Preferential regeneration of photoreceptor from Müller glia after retinal degeneration in adult rat

Jin Wan [a], Hua Zheng [a], Zu-Lin Chen [b], Hong-Lei Xiao [a],
Zhen-Jue Shen [a], Guo-Min Zhou [a,*]

[a] *Department of Anatomy, Histology and Embryology, Shanghai Medical School, Fudan University, 200032 Shanghai, China*
[b] *Laboratory of Neurobiology and Genetics, The Rockefeller University, NY 10021, USA*

Abstract

To determine whether photoreceptor degeneration can stimulate Müller glia to transdifferentiate into neurons in adult mammalian retina, N-methyl-N-nitrosourea (MNU) was injected to induce complete loss of photoreceptors. Following MNU administration, Müller glia underwent reactive gliosis characterized by up-regulation of glial fibrillar acidic protein and nestin, and initiated proliferation through the cyclin D1 and D3 related pathways. Some Müller glia-derived cells were induced to express rhodopsin exclusively. These rhodopsin-positive cells exhibited synaptophysin around them, suggesting possible formation of synapses. After transplanted in to damaged retina, Müller glia migrated, grafted in host retina and produced rhodopsin. These results suggest that degeneration may promote preferential differentiation of Müller glia to photoreceptors and provide a potential therapeutic strategy for retinal degenerative diseases.
© 2007 Elsevier Ltd. All rights reserved.

Keywords: Müller glia; Regeneration; Photoreceptor degeneration; N-Methyl-N-nitrosourea

1. Introduction

As a part of central nervous system, retina has a very limited capacity of regeneration. Injuries or diseases that cause death of retinal neurons would result in permanent blindness. It is known that retinas of low vertebrate species show strong regenerative capacity after lesion, either via retinal pigment epithelium (RPE) transdifferentiation (Ikegami, Mitsuda, & Araki, 2002; Reyer, 1977; Sakaguchi, Janick, & Reh, 1997) or through intrinsic stem cell proliferation and differentiation (Cameron, 2000; Raymond, Reifler, & Rivlin, 1988; Stenkamp & Cameron, 2002). However, in warm-blooded vertebrates, such as birds and mammals, regeneration of retina is very limited (Hitchcock, Ochocinska, Sieh, & Otteson, 2004).

The identification of retinal stem cell in adult rodents has ignited the hope for cell replacement therapies for retinal degenerative diseases, such as retinitis pigmentosa (Tropepe et al., 2000). Recently, significant progresses have been made on stem/progenitor cell transplantation to treat and restore vision loss (Akagi et al., 2003; Chacko, Rogers, Turner, & Ahmad, 2000; Klassen et al., 2004; Young, Ray, Whiteley, Klassen, & Gage, 2000). However, transplantation is difficult as few donor cells can survive, migrate and integrate into the recipient retina (Berson & Jakobiec, 1999; Lu et al., 2002), and those cells tend to be tumorigenic (Arnhold, Klein, Semkova, Addicks, & Schraermeyer, 2004). Therefore, we are interested in whether endogenous progenitors could proliferate and differentiate in response to injuries, and ultimately repair damaged retina. Recent studies have demonstrated the possibility of neural regeneration from glial cells. In developing mammalian central nervous system (CNS), radial glia can differentiate into neurons and glia (Kriegstein & Gotz, 2003; Malatesta, Hartfuss, & Gotz, 2000). Astrocyte-like cells in the subventricular zone and hippocampus from adult brain have the property of neural stem cells (Doetsch, 2003; Doetsch, Caille, Lim, Garcia-Ver-

---

dugo, & Alvarez-Buylla, 1999; Laywell, Rakic, Kukekov, Holland, & Steindler, 2000). With regard to retina, Müller glia can dedifferentiate and mediate regeneration in injured zebrafish retina (Fausett & Goldman, 2006; Yurco & Cameron, 2005). Other studies have shown that cell death stimulates Müller glia to differentiate into retinal neurons, partially restoring lesions in chicken (Fischer & Reh, 2001).

In rodents, Müller glia cells show potential to generate several types of neurons after *N*-methyl-D-aspartate (NMDA) damage to retina (Ooto et al., 2004). These data raise the possibility of Müller glia serving as an injury-induced stem cell. We used MNU injection model to study the effects of photoreceptor degeneration on Müller glia. MNU is known to damage photoreceptor specifically (Kiuchi, Yoshizawa, Shikata, Matsumura, & Tsubura, 2002; Ogino et al., 1993; Yoshizawa et al., 2000; Yuge et al., 1996), while does not cause damage to other retinal cells (Wan et al., 2006). Our results show that injection of MNU in adult rat lead to complete loss of photoreceptors by day 7, and Müller glia were activated to undergo gliosis. Both endogenous and transplanted Müller glia expressed rhodopsin, and were likely to convert into rod photoreceptors. Further examinations revealed that Müller glia did not regenerate into other retinal cell types. These results suggest that retinal degeneration induces Müller glia to preferentially regenerate into photoreceptors.

2. Methods

Adult Sprague–Dawley rats (aged 8–10 weeks) and newborn Z/EG mice (Novak, Guo, Yang, Nagy, & Lobe, 2000) were used in this study. Animals were kept in an air-conditioned room at $22 \pm 2\,°C$ and $60 \pm 10\%$ relative humidity under a 12:12 h light/dark cycle (lights on at 7 am), food and water were available ad libitum. The animals were treated according to the Fudan University Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of rats used and their suffering.

N-Methyl-*N*-nitrosourea (MNU) (Sigma–Aldrich, St. Louis, USA) was kept at $-20\,°C$ in the dark. Immediately before use, MNU powder was dissolved in physiological saline. Experimental rats received a single intraperitoneal injection of 60 mg/kg MNU. Control groups were injected with physiological saline. BrdU was injected into vitreous cavity (30 nmol) and intraperitoneal space (100 mg/kg) 6 h before death at 2 days after MNU. Animals were deeply anesthetized with 10% chloral hydrate, perfused with saline and 4% paraformaldehyde in 0.1 M phosphate buffer. Eyeballs were enucleated, punctured and fixed in 4% paraformaldehyde at $4\,°C$ overnight. After cutting of the cornea and the iris, the lenses were removed and posterior eyecups were dehydrated in 30% sucrose overnight, then embedded in Tissue-Tek OCT compound (TAKARA, Japan). Cryostat sections were cut at 16 μm, and thaw mounted onto Super-Frost Plus slides (Fisher Scientific, Pittsburgh, USA). Sections were air-dried, stored at $-80\,°C$ until use. HE staining process was described as previously (Wan et al., 2006).

Immunostaining was performed as previously (Wan et al., 2006). Briefly, sections were washed in PBS, blocked in 10% normal goat serum at room temperature for 1 h, and then incubated with primary antibodies at $4\,°C$ overnight. For PCNA, cyclin D1 and cyclin D3 immunostaining, sections were incubated in Citrate buffer (pH 6.0) at $95–100\,°C$ 20 min for epitope retrieval. The primary antibodies used in this study include rabbit anti-rat GFAP (1:1000; Dako Corporation, Philadelphia, USA), mouse anti-nestin (1:500; Chemicom, Temecula, USA), rabbit anti-glutamine synthetase (1:5000; Sigma), mouse anti-PCNA (1:200; Lab Vision, Fremont, USA), mouse anti-BrdU (1:400; Roche, Germany), rabbit anti-BrdU (1:200; Lab Vision, USA), mouse anti-rhodopsin (1:5000;

Sigma), mouse anti-protein kinase C α (1:250; BD Bioscience, San Joses, USA), rabbit anti-calbindin D-28k (1:1000; Chemicon), mouse anti-syntaxin (1:1000; Sigma), rabbit anti-synaptophysin (1:250; Abcam, UK), mouse anti-thy1.1 (1:100; Sigma), mouse anti-cyclin D1, cyclin D3 (1:100; Cell Signaling Technology, Danvers, USA), rabbit anti-β-galactosidase (1:1000; MP Biomedicals, Philadelphia, USA). Proper fluorescence-conjugated secondary antibodies were used. The sections were mounted with fluorescent mounting medium (Dako corporation) and examined under a confocal microscope (Zeiss, Germany).

After decapitation, retinas were isolated, nuclear protein extracts were prepared for Western-blot analysis, which was described previously (Kenney & Rowitch, 2000). Briefly, retinas were lysed on ice by sonication in RIPA buffer. The lysate was incubated on ice for 30 min, centrifuged at a speed of $12000g$ for 15 min, and supernatant was collected. Protein concentrations were determined and 30 μg protein of each sample was separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Amersham, Newark, USA). Membranes were blocked for 1 h at room temperature in 5% nonfat dry milk in TBS with 0.01% Tween. The primary antibodies for Western-blotting were mouse anti-cyclin D1 (1:2000), mouse anti-cyclin D3 (1:2000) and mouse anti-β-actin (1:2000, Cell Signaling Technology). Peroxidase-conjugated secondary antibodies were used (Pierce, Rockford, USA). Blots were developed using enhanced chemiluminescence (Pierce). Chemiluminescent immunoreactivity was detected using Kodak X-omat X-ray film. Bands in film were scanned and analyzed by densitometry, using Scionimage software.

Primary culture of retinal Müller cells were prepared as described previously (Das et al., 2006). In brief, retinas from postnatal 7 days Z/EG mice were carefully dissected; avoid possible contamination of retinal pigmented epithelium and ciliary epithelium. Then retinas were digested with 0.25% trypsin and 0.1% type I collagenase (Sigma). Dissociated retinal cells were seeded in culture dishes containing DMEM/F12 supplemented with N2 (GIBCO), 2 mM glutamine, 0.1% penicillin-streptomycin and 10% FBS. Cultures were incubated at $37\,°C$ in 5% CO_2. After 7 days, retinal aggregates and debris were removed by forcibly pipetting. This procedure was repeated every 3 days. A further purified flat cell population was obtained after three passages.

Total RNA from cultured Müller cells and adult retina (positive control) were isolated using Trizol reagent method. The procedure was performed following RT-PCR kit instructions (Invitrogen, San Diego, USA). PCRs were performed as described previously (Das et al., 2006). The primers for opsin were 5′-CATGCAGTGTTCATGTGGGA-3′ (forward) and 5′-AGCAGAGGCTGGTGAGCATG-3′ (reverse); for mGluR6 were 5′-CACAGCGTGATTGACTACGAG-3′ (forward) and 5′-CTCAGGCTCAGTGACACAGTTAG-3′ (reverse); for HPC1 were 5′-A AGAGCATCGAGCAGCAGAGCATC-3′ (forward) and 5′-CATGGCCATGTCCATGAACAT-3′ (reverse); for Brn3b were 5′-GGCTGG AGGAAGCAGAGAAATC-3′ (forward) and 5′-TTGGCTGGATGGC GAAGTAG-3′ (reverse); for β-actin were 5′-CTGTGGCATCCACGA AACTAC-3′ (forward) and 5′-CGGACTCGTCATACTCCTGCT-3′ (reverse). PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide.

Within 6 h after receiving MNU injection, intravitreal transplantation of Müller cells was performed. Processes have been described previously (Young et al., 2000), briefly, rats were anesthetized and their pupils were dilated by 1% topical tropicamide. Under a surgical microscope, a glass micropipette connected to a 10-μl Hamilton microsyringe was inserted to the point vitread to the corneoscleral junction, avoid damaging lens and ciliary body. A total of $5–10 \times 10^4$ cells in 2 μl volume were delivered into vitreous cavity. Animals were sacrificed 2, 5, 7, 15 days after transplantation. Animals with eye inflammation or cataract were excluded for further analysis.

3. Results

3.1. Photoreceptor degeneration was induced by MNU

Seven days after MNU intraperitoneal injection, photoreceptors in outer nuclear layer (ONL) were completely lost

(Fig. 1b), while all three nuclear layers were intact in control retina (Fig. 1a), consistent with previous reports (Kiuchi et al., 2002; Yoshizawa et al., 2000). The loss of photoreceptor cells was confirmed by immunostaining of rhodopsin. Photoreceptors in control retina were positive for rhodopsin, with a characteristic honeycomb appearance in ONL (Fig. 1c). Five days after MNU, with the progressive death of photoreceptors, only 2–3 layers of cells were left. The remainder photoreceptors still expressed rhodopsin and appeared disorganized in ONL (Fig. 1d). Seven days after MNU injection, expression of rhodopsin was not detected (Fig. 1e). These results suggest that a dose of 60 mg/kg MNU can lead to complete degeneration of photoreceptors in adult rat retina by day 7. After a longer period (28 days) of observation, rhodopsin was no longer re-expressed at damaged sites (Fig. 1f).

3.2. Müller glia underwent gliosis after photoreceptor degeneration

Following acute retinal injury, Müller glial cells undergo gliosis to help maintain a homeostatic environment for protection of neurons. Gliosis is manifested by changes in
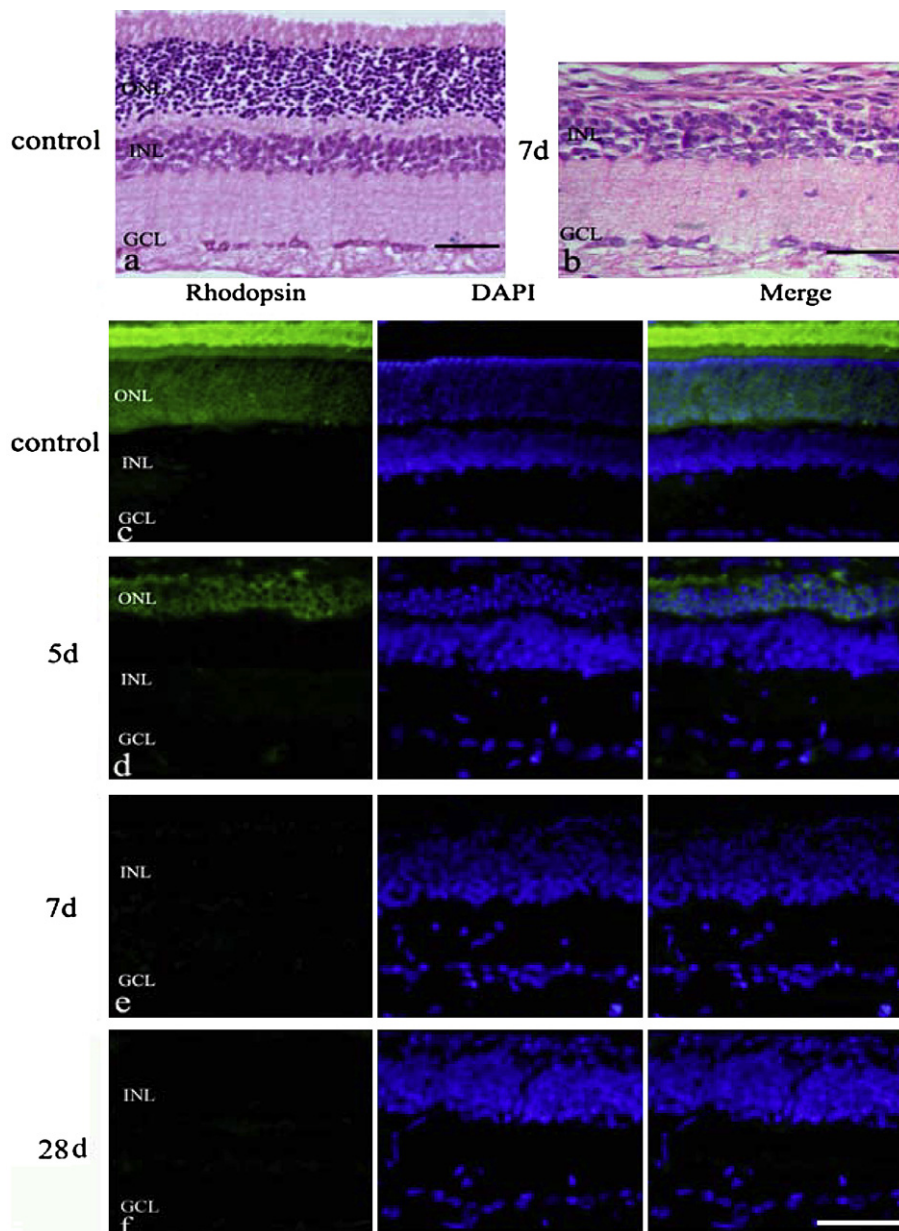

Fig. 1. Photoreceptor degeneration induced by MNU injection. (a) Section of saline-treated retina for H&E staining. (b) Complete photoreceptor loss was induced 7 days after MNU injection. (c) Rhodopsin was expressed in photoreceptors, with a characteristic appearance of honeycomb ONL. (d) With the progression of photoreceptor degeneration, expression of rhodopsin was decreased 5 days after MNU. (e) Immunoreactivity was not detected at day 7 and (f) in a longer period. ONL, outer nuclear layer. Scale bar, 50 μm.
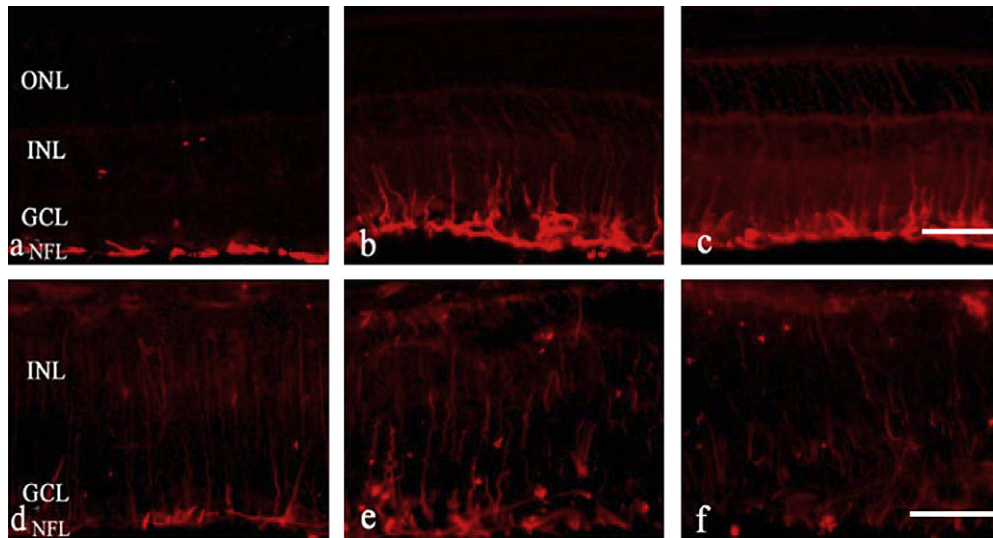
Fig. 2. Up-regulation of GFAP in MNU-treated retina. (a) GFAP was expressed in astrocytes and end-feet of Müller cells in control retinas. (b) GFAP was induced greatly in processes 2 days after MNU injection. These GFAP-positive processes were twisted and extended from NFL to INL. (c) GFAP-labeling was evident at day 5. (d) At day 7, GFAP immunoreactivity in NFL was decreased. However, positive processes extended the entire thickness of retina. At day 15 (e) and 28 (f), expression of GFAP was less intense and discontinuous. INL, inner nuclear layer; NFL, nerve fiber layer. Scale bar (50 μm) in (c) applies to (a–c). Scale bar (50 μm) in (f) applies to (d–f).

expression of intermediate filament protein, secretion of signaling molecules and increased proliferation.

Expression changes of intermediate filament protein GFAP and nestin were the early signs of gliosis. Immunofluorescent analysis showed that GFAP was confined in astrocytes and some end-feet of Müller cells located in nerve fiber layer (NFL) and ganglion cell layer (GCL) (Fig. 2a). Two days after MNU injection, the expression of GFAP protein was increased greatly in glial processes which twisted and extended into inner nuclear layer (INL) (Fig. 2b). At day 5, GFAP-immunoreactive processes were also detected in ONL (Fig. 2c). At day 7, GFAP-positive processes extended the entire thickness of retina. However, immunoreactivity of GFAP in NFL was decreased compared with its expression at day 2 (Fig. 2d). At 15 days (Fig. 2e) and 28 days (Fig. 2f), GFAP expression in the processes became discontinuous, and appeared less intense than at early stages. Therefore, GFAP protein expression was rapidly induced in Müller glia after photoreceptor degeneration.

Nestin expression was also up-regulated in Müller cells after retinal injury. In control retina, anti-nestin antibody labeling was located in blood vessels close to outer margin of INL and some astrocytes in GCL (Fig. 3a–c). Expression of nestin was greatly increased 2 days after MNU treatment, and overlapped with glutamine synthetase (GS), a Müller glia-specific marker (Fig. 3d–f). Intensively stained nestin-positive processes extended across the entire thickness of retina, from NFL to photoreceptor layer.

Proliferation of Müller glia was assessed by double labeling with anti-GS and anti-PCNA antibodies. Retinal neurons or glia did not show signs of proliferation in control retina (Fig. 4a–c). Two days after MNU injection, many PCNA-positive nuclei were found in INL (Fig. 4d–

f), 93.87 ± 2% PCNA-labeled nuclei were immunoreactive for GS (4428 cells in 9 sections, total 3 animals examined). Double staining retina for PCNA and DAPI showed that PCNA-labeled nuclei were located through the center of INL (Fig. 4g–i), suggesting that they were nuclei of Müller glia.

Up-regulation of GFAP, nestin and PCNA expression suggest Müller glia were stimulated into active gliosis after retinal damage at early stage. To further analyze the identity of cells that are derived from the Müller glia in a later period, we used BrdU as a lineage tracer. Previous study reported Müller glia entered cell cycle at day 2 after retinal damage and underwent a single division (Ooto et al., 2004), we injected rats with BrdU at day 2 after MNU treatment and analyzed at 2, 7, 15 days.

BrdU-labeling first appeared in retina 6 h after BrdU injection, labeled nuclei scattered through the center of INL (Fig. 5b). Quantification analysis showed that 547 ± 8.6 cells per section were BrdU-positive at day 2, after that day, the number of BrdU-labeled cells gradually decreased. At day seven, 354 ± 10.55 cells per section were found positive (Fig. 5c). While at day 15, only 233 ± 5.4 cells per section remained BrdU-labeled (Fig. 5d). The decline of BrdU-positive cells was likely due to cell death. Double staining for BrdU and cell-specific markers were performed to identify which cell type incorporated BrdU. As Müller cells have been detected to divide by PCNA immunoreactivity, we used anti-GS and anti-BrdU antibodies to further confirm their proliferation. We found nearly all BrdU-positive nuclei co-expressed GS in INL at day 2 (Fig. 5f–h), with a similar distribution pattern to PCNA immunostaining. Microglia has also been reported to be activated in damaged retina (Roque, Imperial, & Caldwell, 1996). In our study,
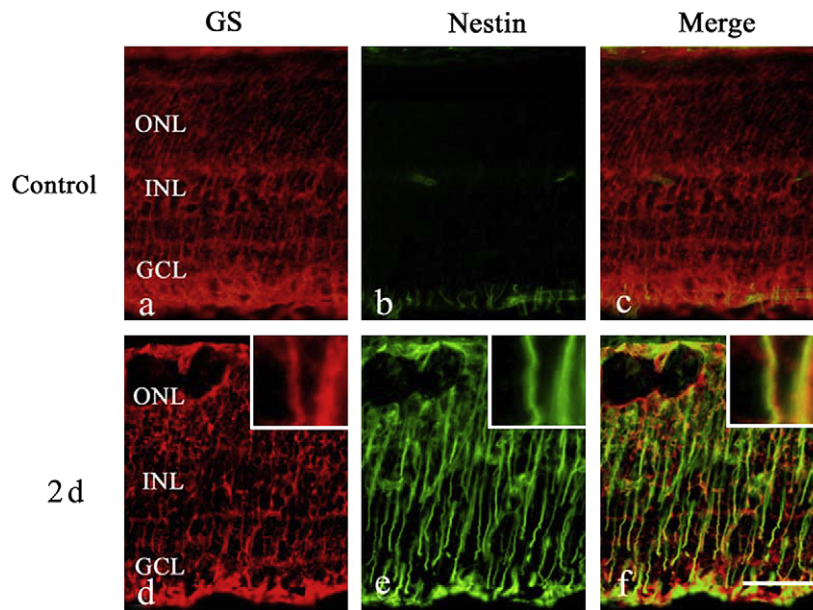
Fig. 3. Induction of nestin in Müller cells after retinal injury. (a–c) In control retinas, nestin was labeled in blood vessels near INL and some astrocytes in GCL. (d–f) Two days after MNU, nestin expression was dramatically increased, and co-expressed with GS, indicating Müller cells were stimulated to express nestin after photoreceptor degeneration. Higher magnifications of the images were shown in the boxes. INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 50 μm.
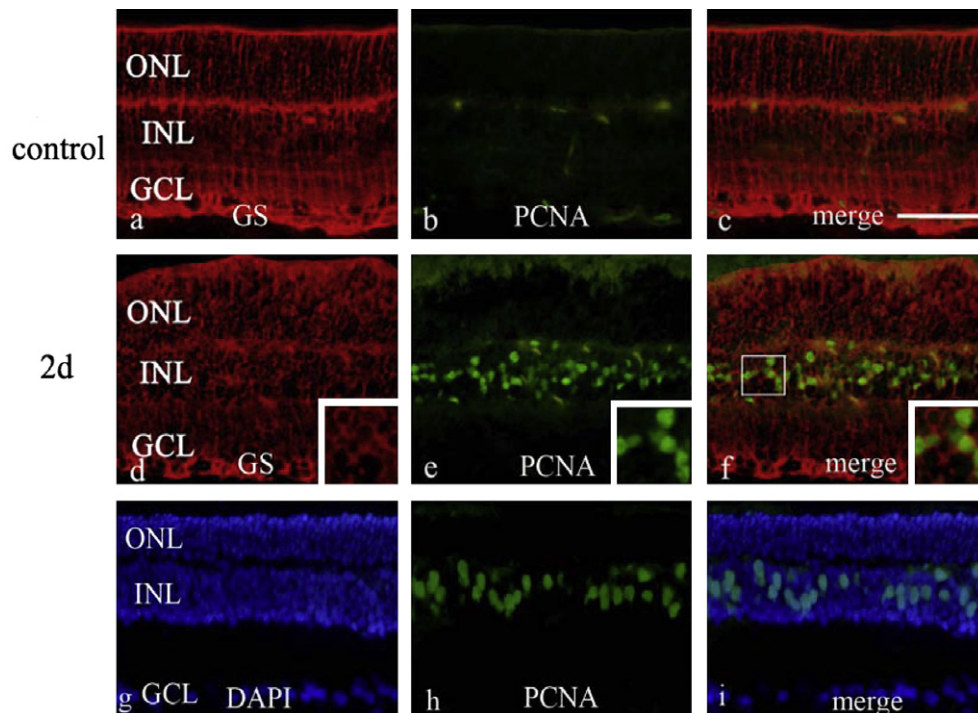

Fig. 4. Expression of PCNA in Müller glia. (a–c) PCNA protein was hardly detected in normal retinal cells. (d–f) However, the number of PCNA-positive nuclei increased dramatically in INL 2 days after MNU, 93.87 ± 2% PCNA-labeled nuclei co-expressed GS in Müller glia. (g–h) Counterstained by DAPI, PCNA labeling was located in the center of INL. Higher magnifications of the boxed areas were shown in downright corners. Data were expressed as mean ± SEM%. INL, inner nuclear layer. Scale bar, 50 μm.

expression of ED-1 (a marker for activated phagocytes) was distributed in GCL, IPL and OPL (Fig. 5i), however, BrdU-labeled cells did not co-express ED-1 in INL (Fig. 5j–k). Thus, Müller glia is a dominant cell type to re-enter cell cycle after photoreceptor degeneration.
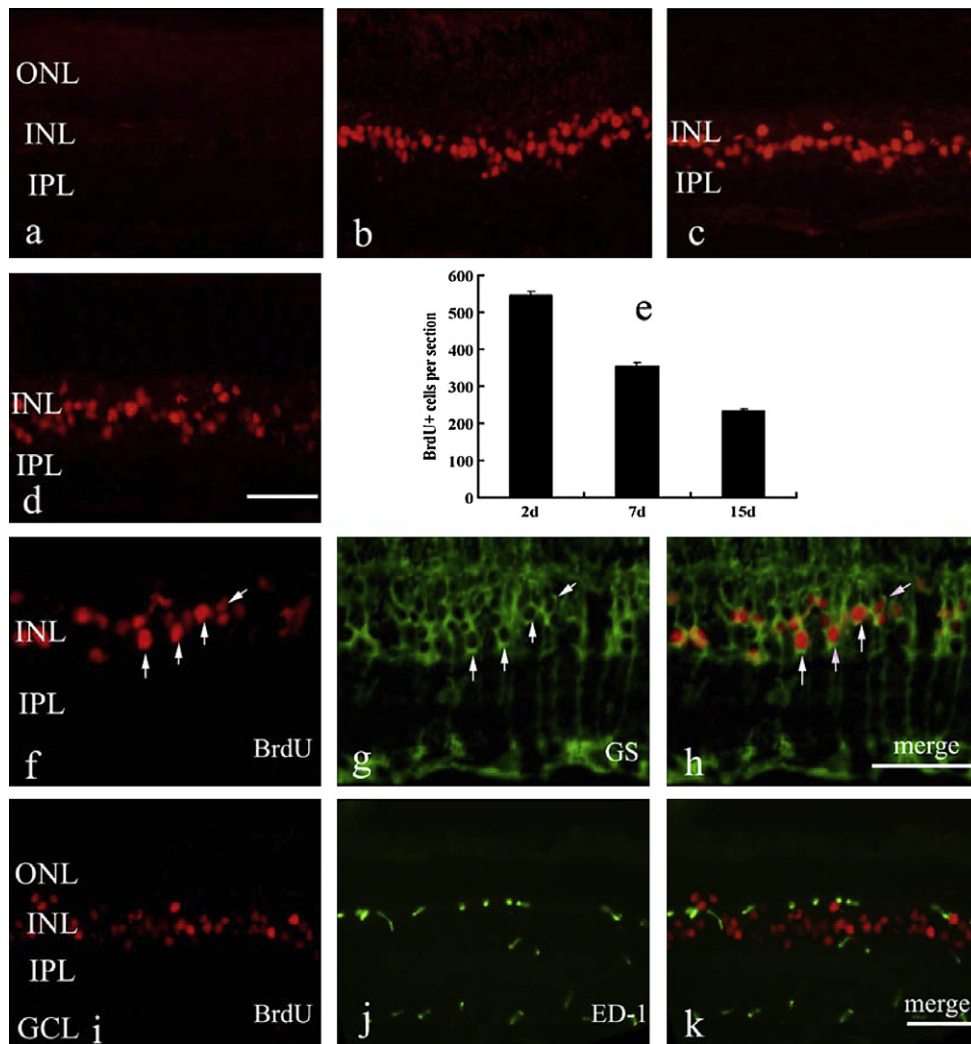
Fig. 5. Photoreceptor degeneration stimulated Müller glia re-entry into cell cycle. (a) Vertical section of saline-treated retina was labeled for BrdU immunofluorescence. BrdU was administrated at day 2 after MNU, and (b) retinas were harvested at 6 h, (c) 7 and (d) 15 days. BrdU-labeled nuclei were found in INL at different time points. (e) Quantification showed that the number of BrdU-labeled nuclei increased evidently at day 2, and then decreased with increasing time after MNU. Cell counts were made from 10 sections of 3 animals at each time point. Data were expressed as a mean ± SEM. (f–h) BrdU-labeled cells (red) co-expressed with GS (green). Whereas (i–k) BrdU-positive cells (red) did not overlap with ED-1 (green) in INL. INL, inner nuclear layer. Scale bar (50 μm) in (d) applies to (a–d). Scale bar (50 μm) in (h) applies to (f–h). Scale bar (50 μm) in (k) applies to (i–k). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

3.3. The proliferation of Müller glia was through cyclin D1 and D3 related pathways

After the onset of acute photoreceptor apoptosis, Müller cells re-entered cell cycle, activated from quiescent G0 state, passed through G1/S checkpoint and undergo proliferation. Cyclin D–CDK complexes are believed to play a role during G1 phase. Cyclin D3 is known to be expressed in Müller glia (Dyer & Cepko, 2000) and cyclin D1 is involved in the proliferation of Müller glia (Kohno, Sakai, & Kitahara, 2006; Yoshida et al., 2004). Double labeling with anti-GS and anti-cyclin D1 or cyclin D3 were performed. Cyclin D1 was not expressed in normal retinal cells (Fig. 6a), consistent with previous reports (Kohno et al. 2006; Yoshida et al., 2004). Two days after MNU, cyclin D1-positive cells were distributed in INL and co-expressed with GS (Fig. 6b), cyclin D1 was induced during prolifera-

tion of Müller glia. Later, immunoreactivity of cyclin D1 was not evident at day 7 (Fig. 6c). Cyclin D3-labeled nuclei overlapped with GS in control retina (Fig. 6d), indicating that cyclin D3 was expressed in normal Müller glia. At day 2 (Fig. 6e) and 7 after MNU (Fig. 6f), cyclin D3 was still expressed in Müller cells.

To further quantify changes of cyclin D1 and D3 during proliferation, we did Western-blot for these two molecules. Our results showed that cyclin D1 protein increased at day 2 (1.65 ± 0.11-fold to the control), then down-regulated to the level of control at day 7 (1.01 ± 0.05-fold), and decreased at day 15 (0.85 ± 0.04-fold to the control) (Fig. 6g and h). Cyclin D1 was not detectable by immunohistochemistry, but was detected weakly by Western-blot in control retina. Since cyclin D1 has been shown to be expressed in lymphocytes (Kaplan, Meyerson, Husel, Lewandowska, & MacLennan, 2005; Palmero, Holder, Sinclair,
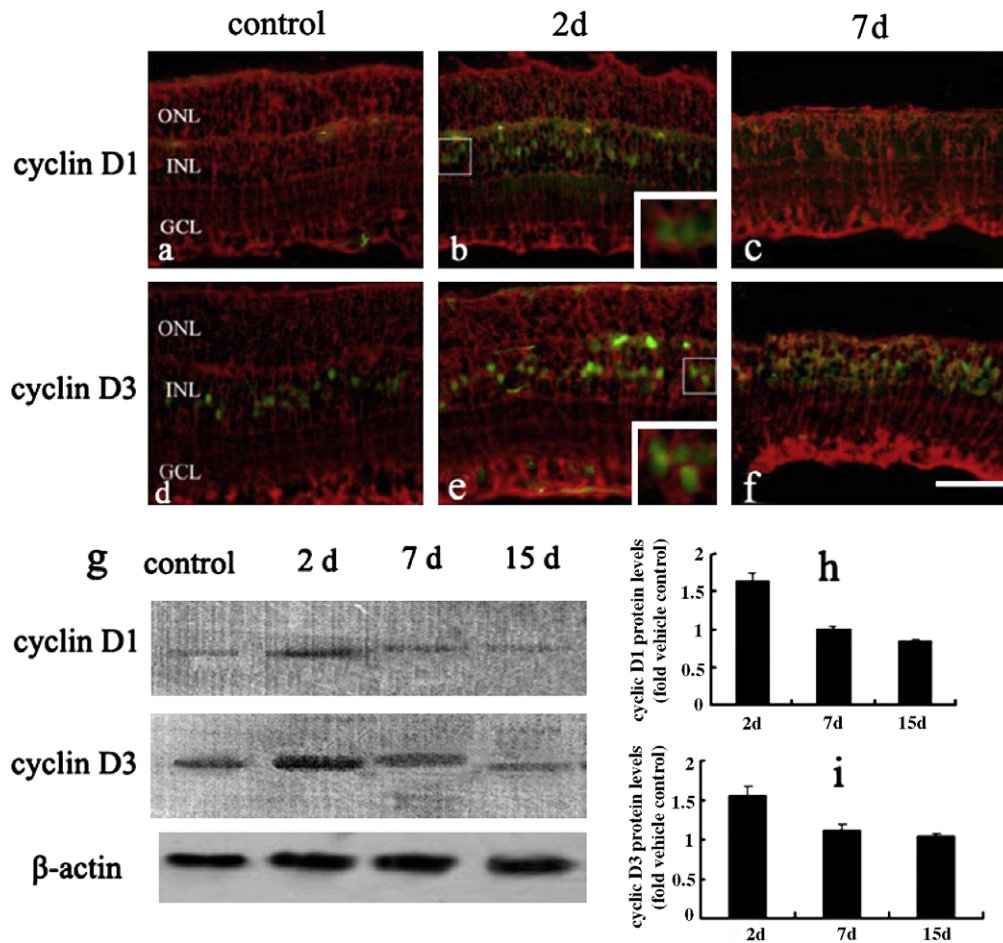
Fig. 6. Expression of cyclin D1 and D3 changed during the proliferation of Müller cells. Retina sections from the control, 2 and 7 days were double stained with GS (red) and cyclin D1 (green) or cyclin D3 (green). (a) Cyclin D1 was not expressed in control retinas. (b) Expression of cyclin D1 was induced at day 2, and cyclin D1-positive cells co-expressed GS in INL. (c) At day 7, immunoreactivity of cyclin D1 was not obvious. (d) Expression of cyclin D3 was located in Müller cells, and the number of Müller cells expressing cyclin D3 increased 2 (e) and 7 (f) days after retinal injury. Higher magnifications of the boxed areas in (b) and (e) were shown on the downright corner. (g) Western-blot analysis revealed changes of cyclin D1 and D3. (h and i) The quantification of Western-blot for cyclin D1 and D3 were expressed as a mean ± SEM, in fold changes compared with the control. Scale bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Dickson, & Peters, 1993), the weak positive band could be derived from lymphocytes in blood vessels of the retina. Cyclin D3 protein was visibly increased on day 2 (1.55 ± 0.12-fold), day 5 (1.63 ± 0.15-fold) and day 7 (1.12 ± 0.01-fold) compared to the control, but decreased on day 15 (1.05 ± 0.01-fold) (Fig. 6g and i). Changes of cyclin D1 and cyclin D3 levels correlated to BrdU uptake in Müller glia, indicating that they were involved in the proliferation of Müller cells.

3.4. Müller glia-derived cells expressed rhodopsin and GS

Müller glia cells have been reported to differentiate into bipolar cell and photoreceptor in rat retina treated by NMDA (Ooto et al., 2004). To identify the cells derived from Müller glia in photoreceptor damaged retina, we double labeled BrdU with retinal specific markers. Before staining, each section was pre-stained with DAPI to exclude any remaining photoreceptors with characteristic round, small

and condensed nuclei. As shown in Fig. 7, some BrdU-labeled cells were immunoreactive for rhodopsin at day 15 after MNU (Fig. 7a–c). Cells co-expressed BrdU and rhodopsin had small and round nuclei, similar to those of photoreceptors. These results suggest that rhodopsin-positive rods are regenerated from Müller cells after damage. We also labeled other neuron markers in MNU-treated retinas with antibodies specific to bipolar (protein kinase C α), horizontal cell (calbindin D-28k), amacrine cell (syntaxin) and ganglion cell (thy1.1). However, there is no BrdU-labeling in these cell types (data not shown). Müller glia-derived cells also expressed GS (Fig. 7d–f). Statistic analysis showed that 7.65 ± 0.2% Müller glia-derived cells expressed rhodopsin, adopting the phenotype of rod photoreceptor (2390 cells, 12 sections examined, $n = 4$ animals), whereas 58.13 ± 3% BrdU-labeled cells expressed GS (1785 cells, 9 sections examined, $n = 3$ animals). Rod photoreceptors and Müller glia may be produced from proliferating Müller glial cells in photoreceptor degenerating retina.
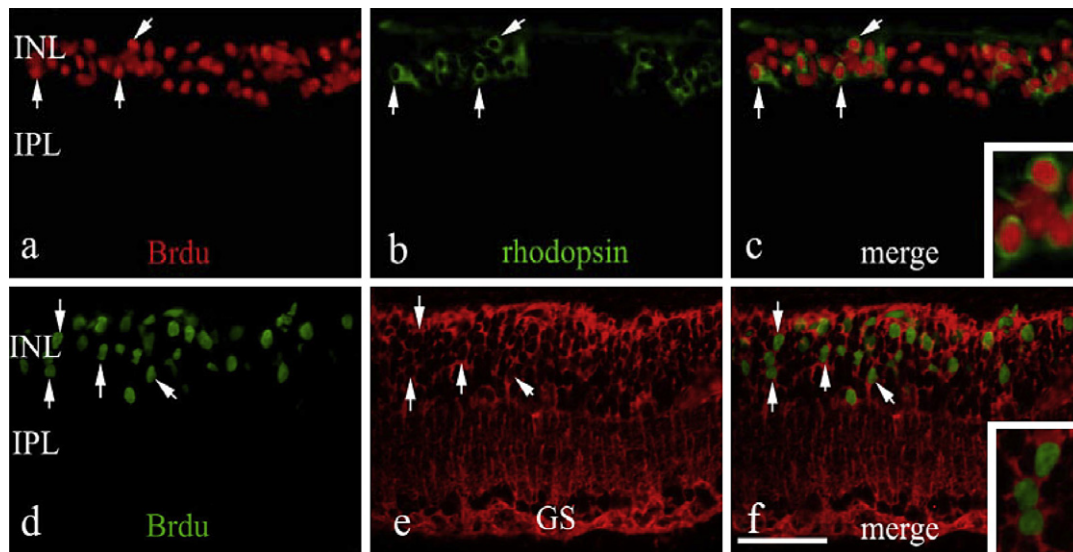
Fig. 7. Müller glia-derived cells expressed rhodopsin and GS. Sections obtained from MNU injection were double stained with BrdU and rhodopsin or GS. (a–c) 7.65 ± 0.2% BrdU-labeled Müller cells (red) co-expressed with rhodopsin (green), a specific marker of photoreceptor. (d–f) 58.13 ± 3% BrdU-labeled cells (green) expressed GS (red). Overlapped cells were shown by arrows. Higher magnifications of the images were shown in boxes. Scale bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

3.5. Expression of synaptophysin around regenerated photoreceptors

We then assayed whether these regenerated rhodopsin-positive cells formed connections with neighboring cells, we used immunohistochemistry for synaptophysin, which was a presynaptic protein labeled only rod terminals (spherules) and cone terminals (pedicles) with bipolar cells (Brandstatter, Lohrke, Morgans, & Wassle, 1996). In control retina, expression of synaptophysin appeared multi-layered at terminal of photoreceptors in OPL and was distributed throughout the IPL (Fig. 8a), consistent with the
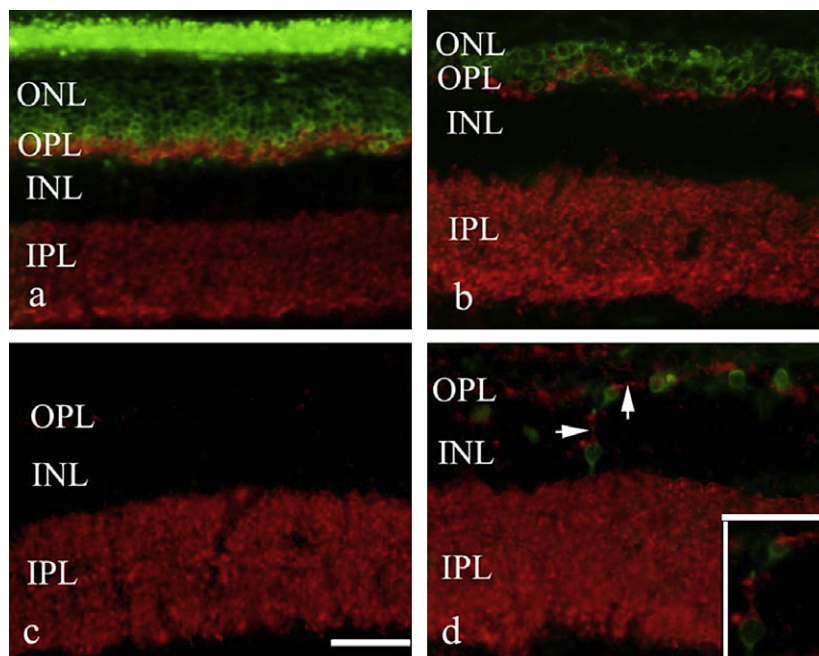

Fig. 8. Re-expression of synaptophysin after photoreceptor degeneration. (a) In control retinas, synaptophysin (red) was expressed in OPL and throughout IPL. Expression of synaptophysin in OPL was partly overlapped with rhodopsin (green) at terminals of photoreceptor. (b) Immunoreactivity of synaptophysin in OPL appeared single-layered due to loss of photoreceptor at day 5. (c) With the complete loss of photoreceptor, synaptophysin was not detected in OPL at day 7. (b and c) Synaptophysin in IPL was not affected obviously. (d) Synaptophysin (red) was detected again around regenerated rhodopsin-positive cells (green) as indicated by arrows. Higher magnification of the image was shown in box. OPL, outer plexiform layer; IPL, inner plexiform layer. Scale bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

previous report (Brandstatter et al., 1996). At 5 days after MNU, synaptophysin immunoreactivity in OPL became single-layered due to loss of photoreceptors (Fig. 8b). At day 7, synaptophysin were not detected in OPL anymore (Fig. 8c). However, its expression in IPL at different time points remained the same as that in control retina (Fig. 8a–c). We found synaptophysin was distributed around regenerated rhodopsin-positive cells, with a discontinuous and dotted appearance (Fig. 8d). Re-expression of synaptic vesicle proteins by regenerative rods suggests that these cells have the potential to grow neurites and form synapses with neighboring cells.

3.6. Transplanted Müller glia produced rhodopsin in MNU-damaged retina

The above results showed that photoreceptor degeneration could induce endogenous Müller glia to differentiate into rods. To test further whether exogenous transplanted Müller cells have potential for neural regeneration in toxin-damaged retinas, we injected cultured Müller glia into adult retina treated by MNU. Cultured Müller cells were isolated from Z/EG transgenic animal, and they were easily distinguished from endogenous Müller glia by anti-Lac Z immunostaining. Before transplantation, the purity of cultured Müller cells was analyzed by immunocytochemistry and RT-PCR. Double labeling of Lac Z and GS showed that almost all Lac Z-labeled cells were also detected GS-positive (Fig. 9a–c). RT-PCR analysis showed that cultured Müller cells did not express transcripts of neurons, including rhodopsin (for rods), Brn3b (for ganglion cells), HPC1 (for amacrine cells) and mGluR6 (for bipolar cells), which indicated retinal neurons did not contaminate cultured Müller cells (Fig. 9d). We chose to transplant at 6 h after MNU injection since MNU has a half-life of less than 1 h at physiological pH 7 (Severs, Barnes, Wright, & Hicks, 1982), then most MNU was cleared from the blood after 6 h. After transplantation, Müller cells survived, maintained Lac Z expression in both normal and damaged retinas. When transplanted into normal rats, most donor cells tended to aggregate in vitreous cavity and rarely migrate in recipient retina (Fig. 9e). In contrast,
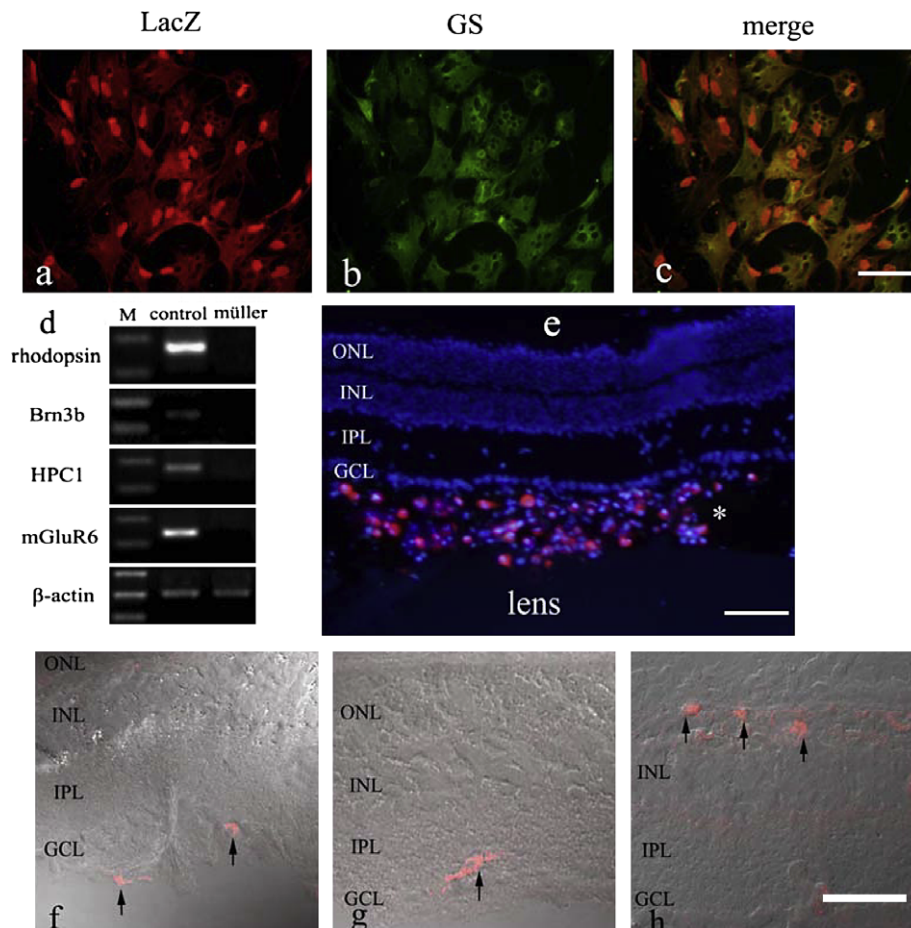

Fig. 9. Transplantation of Müller cells into degenerating retina. Cultured Müller cells from Z/EG mice were immunostained positive with Lac Z (a) and GS (b). (d) RT-PCR showed that cultured Müller cells did not express transcripts of retinal neurons: rhodopsin, Brn3b, HPC1 and mGluR6 mRNA. (e) Müller cells transplanted into normal retina were immunostained Lac Z positive (red) and congregated in vitreous cavity (∗). In contrast, Müller glia transplanted in MNU-damaged retina migrated from vitreous chamber to GCL (arrows in f), IPL (arrow in g) and to outer margin of INL (arrows in h). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer. Scale bar (50 μm) in (c) applies to (a–c). Scale bar (50 μm) in (h) applies to (f–h). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)
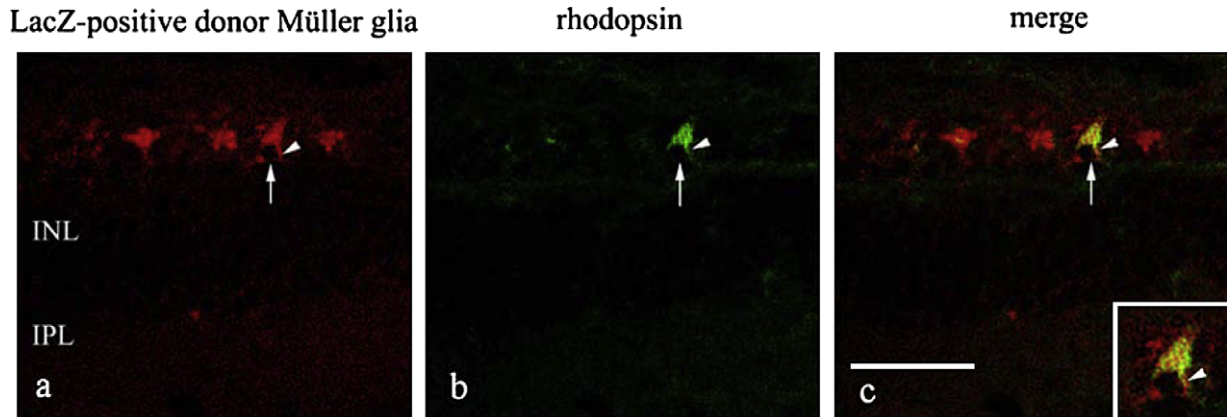
Fig. 10. Transplanted Müller glia produced rhodopsin in photoreceptor degeneration retina. Transplanted Müller cells were double stained with Lac Z and specific markers for retinal neurons. (a) Grafted cells maintained Lac Z expression, and rhodopsin was also detected (arrow in b) 15 days after transplantation. (c) Lac Z+/rhodopsin+ cell (arrow) extended process (arrow head) toward INL. Higher magnification of the merge image in (c) was shown in the box. Scale bar, 50 μm.

Lac Z-labeled Müller glial cells were observed to migrate into damaged retinas, from GCL (Fig. 9f), IPL (Fig. 9g) and to reach outer margin of INL (Fig. 9h). Quantification data showed that $1.48 \pm 0.1\%$ donor cells (50 sections, 5 animals examined) were detected to migrate.

Grafted Muller glia cells maintained Lac Z expression (Fig. 10a) and produced rhodopsin at damaged site after transplantation (Fig. 10b). Cell double-positive for Lac Z and rhodopsin extended process toward INL (Fig. 10c). Quantification showed $4.25 \pm 0.1\%$ Lac Z-labeled cells produced rhodopsin (612 cells, 12 sections examined, $n = 5$ animals). Molecular markers specific for other retinal neurons were not detectable in Lac Z-labeled donor cells (date not shown). These results suggest that photoreceptor degeneration can also induce exogenous transplanted Müller cells to differentiate into rhodopsin-positive photoreceptors. They further support the hypothesis that Müller glia have the potential for regeneration of specific neurons after retinal degeneration.

4. Discussion

Our results show that Müller glia in adult rat retina undergo gliosis and proliferation in response to photoreceptor degeneration, and some of the progeny cells express rod-specific marker. Newly regenerated cells express presynaptic protein, thus may integrate into retinal circuits. Transplanted Müller glia also show regenerative capacity by migrating into the retina and expressing rod-specific marker in photoreceptor degenerating retina. These results further demonstrated regenerative potential of Müller glia in adult mammalian retina.

There have been some reports that indicate Müller glia generate several types of new neurons in NMDA treated retina (Fischer & Reh, 2001; Ooto et al., 2004). However, rods are preferentially regenerated from Müller glia-derived cells in this study, and we failed to find BrdU-labeled Müller glia cells express specific markers for other retinal neurons. The differences of the results between the previous and the current studies might be due to different microenvironment present for cell fate determination. First, different signals may be released from damaged neurons, and these signals may initiate different regenerative response. Previous studies report that photoreceptor must be destroyed to trigger the regeneration of retina in goldfish (Braisted, Essman, & Raymond, 1994; Braisted & Raymond, 1992), which suggest that Müller cells sense change of neuron-derived signaling molecules and react to them. In current study, MNU causes photoreceptor degeneration, an average of 547 Müller cells per section are triggered to proliferate at early stages. Whereas NMDA damages ganglion cells in mice and amacrines in chicken, much fewer cells (20 cells per section) in INL proliferate in respond of NMDA (Ooto et al., 2004). However, such signals initiating response of Müller glia are poorly understood. Until recent study proves that endothelin-2 act as a stress signal derived from injured photoreceptors, and contributes to activation of Müller cells (Rattner & Nathans, 2005). Second, both extrinsic and intrinsic cell signaling pathways are involved in promoting neuronal regeneration from Müller glia in retina. For example, exogenous insulin and FGF2 stimulate Müller cells into undifferentiated stage (Fischer, McGuire, Dierks, & Reh, 2002; Fischer & Reh, 2002). The progenitor properties of Müller cells are also mediated by Notch and Wnt pathway (Das et al., 2006). Furthermore, Wnt (Osakada et al., 2007) and Shh (Wan, Zheng, Xiao, She, & Zhou, 2007) pathways play role in photoreceptor regeneration from Müller cells. Therefore, it would be interesting to further investigate what signals are responsible for direction of cell differentiation from the Müller glia. This line of study may provide clues to regulate regenerative capacity of these progenitor-like cells for future therapy.

We suppose proliferation of Müller glia cells is closely associated with differentiation. Müller cells are detected as positive for BrdU-labeling in MNU-exposed retinas,

demonstrating re-entry of cell cycle from quiescent G0 state, passing through G1/S checkpoint. Cyclin D–CDK complexes are believed to act during early the G1 phase and trigger cell cycle progression. Expression changes of cyclin D1 and cyclin D3 correlate with BrdU-labeling, consistent with the previous study that cyclin D1 and cyclin D3 are involved in proliferation of Müller glia. Rapid up- and down-regulation of cyclin D1 and cyclin D3 levels not only prevent Müller cells from massive gliosis and the formation of glial scar but also result in exiting from cell cycle and initiate differentiation. During up-regulation of cyclin D1 and D3, retinoblastoma protein (Rb) is activated and accumulated. Rb can bind to more than 110 different proteins, and several of which are tissue-specific transcription factors (Morris & Dyson, 2001). Then Rb plays an important role in cell fate determination. Regarding to retina, Rb regulates efficient cell cycle exit of progenitors and the maturation of rod photoreceptor. In the absence of Rb, rhodopsin is not expressed and developing rods fail to undergo chromatin condensation. Furthermore, Rb acts upstream of Nrl in rod development (Zhang et al., 2004). Therefore, Rb is likely associated closely with regeneration of rod photoreceptor in current study.

Whether the regenerated photoreceptors have integrated into retinal functional circuit is still under investigation. Expression of synaptophysin, a synaptic vesicle membrane protein, was used to indicate establishment of potential functional synapses. We show that synaptophysin immunoreactivity completely disappeared by day 7 after NMU, but reappeared later in OPL and around rhodopsin-labeled cells. It has been reported that synaptophysin is present on the regenerative photoreceptors at all stages and synaptophysin can rapidly localize to regenerated terminals in amphibians at early stage (Yang, Standifer, & Sherry, 2002). We will further identify the function of synapses and cell type with which the regenerated rods might be in contact with.

Microenvironment from host retina may influence the fate of grafted cells. We found grafted Lac Z-labeled Müller glia migrated from the vitreous and dispersed throughout the MNU-treated recipient retina, while Müller glia transplanted in normal retina tended to attach closely to vitreous lamina. Previous reports also demonstrate that neural progenitors do not incorporate into normal retina (Takahashi, Palmer, Takahashi, & Gage, 1998), suggesting that intact mature retinas do not provide an attractive environment for integration and migration of exogenous cells. Even in neonatal retina, damages to host retina would help facilitate incorporation of grafted cells (Das et al., 2006). Therefore, injury cues might guide cell migration and promote incorporation of the donor cells. Both transplanted retinal progenitor cells (Chacko et al., 2000; Qiu et al., 2005) and Müller glia (Kubota, Nishida, Nakashima, & Tano, 2006) can differentiate into photoreceptors upon injection into the subretinal space, which may offer an environment conducive for differentiation of photoreceptors and expression of rhodopsin by the engrafted cells. How-

ever, we used intravitreal injection since barrier is easily formed by Müller glia processes in subretinal space, which may inhibit migration and differentiation of donor cells. Furthermore, intravitreal injection makes it easy to observe survival and migration of donor cells.

In summary, the adult mammalian retina has the potential for regeneration. Both endogenous and exogenous Müller glia produce photoreceptors after injury in the adult retina. Our results raise the possibility that Müller glia may become a source of photoreceptor for future cell replacement therapy. Our further investigation will focus on efficient generation of photoreceptors from Müller glia-derived cells, thus enhancing potential of replacing damaged neurons.

Acknowledgments

References

Akagi, T., Haruta, M., Akita, J., Nishida, A., Honda, Y., & Takahashi, M. (2003). Different characteristics of rat retinal progenitor cells from different culture periods. *Neuroscience Letters, 341*, 213–216.

Arnhold, S., Klein, H., Semkova, I., Addicks, K., & Schraermeyer, U. (2004). Neurally selected embryonic stem cells induce tumor formation after long-term survival following engraftment into the subretinal space. *Investigative Ophthalmology and Visual Science, 45*, 4251–4255.

Berson, E. L., & Jakobiec, F. A. (1999). Neural retinal cell transplantation: Ideal versus reality. *Ophthalmology, 106*, 445–446.

Brandstatter, J. H., Lohrke, S., Morgans, C. W., & Wassle, H. (1996). Distributions of two homologous synaptic vesicle proteins, synaptoporin and synaptophysin, in the mammalian retina. *Journal of Comparative Neurology, 370*, 1–10.

Braisted, J. E., & Raymond, P. A. (1992). Regeneration of dopaminergic neurons in goldfish retina. *Development, 114*, 913–919.

Braisted, J. E., Essman, T. F., & Raymond, P. A. (1994). Selective regeneration of photoreceptors in goldfish retina. *Development, 120*, 2409–2419.

Cameron, D. A. (2000). Cellular proliferation and neurogenesis in the injured retina of adult zebrafish. *Visual Neuroscience, 17*, 789–797.

Chacko, D. M., Rogers, J. A., Turner, J. E., & Ahmad, I. (2000). Survival and differentiation of cultured retinal progenitors transplanted in the subretinal space of the rat. *Biochemical and Biophysical Research Communications, 268*, 842–846.

Das, A. V., Mallya, K. B., Zhao, X., Ahmad, F., Bhattacharya, S., Thoreson, W. B., et al. (2006). Neural stem cell properties of Muller glia in the mammalian retina: Regulation by Notch and Wnt signaling. *Developmental Biology, 299*, 283–302.

Doetsch, F. (2003). The glial identity of neural stem cells. *Nature Neuroscience, 6*, 1127–1134.

Doetsch, F., Caille, I., Lim, D., Garcia-Verdugo, J. M., & Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell, 97*, 703–716.

Dyer, M. A., & Cepko, C. L. (2000). Control of Muller glial cell proliferation and activation following retinal injury. *Nature Neuroscience, 3*, 873–880.

Fausett, B. V., & Goldman, D. (2006). A role for alpha1 tubulin-expressing Muller glia in regeneration of the injured zebrafish retina. *Journal of Neuroscience, 26*, 6303–6313.

Fischer, A. J., McGuire, C. R., Dierks, B. D., & Reh, T. A. (2002). Insulin and fibroblast growth factor 2 activate a neurogenic program in Muller glia of the chicken retina. *Journal of Neuroscience, 22*, 9387–9398.

Fischer, A. J., & Reh, T. A. (2001). Müller glia are a potential source of neural regeneration in the postnatal chicken retina. *Nature Neuroscience, 4*, 247–252.

Fischer, A. J., & Reh, T. A. (2002). Exogenous growth factors stimulate the regeneration of ganglion cells in the retina. *Developmental Biology, 251*, 367–379.

Hitchcock, P., Ochocinska, M., Sieh, A., & Otteson, D. (2004). Persistent and injury-induced neurogenesis in the vertebrate retina. *Progress in Retinal and Eye Research, 23*, 183–194.

Ikegami, Y., Mitsuda, S., & Araki, M. (2002). Neural cell differentiation from retinal pigment epithelial cells of the newt: An organ culture model for the urodele retinal regeneration. *Journal of Neurobiology, 50*, 209–220.

Kaplan, D., Meyerson, H., Husel, W., Lewandowska, K., & MacLennan, G. (2005). D cyclins in lymphocytes. *Cytometry Part A, 63*, 1–9.

Kenney, A. M., & Rowitch, D. H. (2000). Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. *Molecular and Cellular Biology, 20*, 9055–9067.

Kiuchi, K., Yoshizawa, K., Shikata, N., Matsumura, M., & Tsubura, A. (2002). Nicotinamide prevents *N*-methyl-*N*-nitrosourea-induced photoreceptor cell apoptosis in Sprague–Dawley rats and C57BL mice. *Experimental Eye Research, 74*, 383–392.

Klassen, H. J., Ng, T. F., Kurimoto, Y., Kirov, I., Shatos, M., Coffey, P., et al. (2004). Multipotent retinal progenitors express developmental markers, differentiate into retinal neurons, and preserve light-mediated behavior. *Investigative Ophthalmology and Visual Science, 45*, 4167–4173.

Kohno, H., Sakai, T., & Kitahara, K. (2006). Induction of nestin, Ki-67, and cyclin D1 expression in Müller cells after laser injury in adult rat retina. *Graefe's Archive for Clinical and Experimental Ophthalmology, 244*, 90–95.

Kriegstein, A. R., & Gotz, M. (2003). Radial glia diversity: A matter of cell fate. *Glia, 43*, 37–43.

Kubota, A., Nishida, K., Nakashima, K., & Tano, Y. (2006). Conversion of mammalian Muller glial cells into a neuronal lineage by in vitro aggregate-culture. *Biochemical and Biophysical Research Communications, 351*, 514–520.

Laywell, E. D., Rakic, P., Kukekov, V. G., Holland, E. C., & Steindler, D. A. (2000). Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *Proceedings of the National Academy of Sciences of the United States of America, 97*, 13883–13888.

Lu, B., Kwan, T., Kurimoto, Y., Shatos, M., Lund, R. D., & Young, M. J. (2002). Transplantation of EGF-responsive neurospheres from GFP transgenic mice into the eyes of rd mice. *Brain Research, 943*, 292–300.

Malatesta, P., Hartfuss, E., & Gotz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development, 127*, 5253–5263.

Morris, E. J., & Dyson, N. J. (2001). Retinoblastoma protein partners. *Advances in Cancer Research, 82*, 1–54.

Novak, A., Guo, C., Yang, W., Nagy, A., & Lobe, C. G. (2000). Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis, 28*, 147–155.

Ogino, H., Ito, M., Matsumoto, K., Yagyu, S., Tsuda, H., Hirono, I., et al. (1993). Retinal degeneration induced by *N*-methyl-*N*-nitrosourea and detection of 7-methyldeoxyguanosine in the rat retina. *Toxicologic Pathology, 21*, 21–25.

Ooto, S., Akagi, T., Kageyama, R., Akita, J., Mandai, M., Honda, Y., et al. (2004). Potential for neural regeneration after neurotoxic injury in the adult mammalian retina. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 13654–13659.

Osakada, F., Ooto, S., Akagi, T., Mandai, M., Akaike, A., & Takahashi, M. (2007). Wnt signaling promotes regeneration in the retina of adult mammals. *Journal of Neuroscience, 27*, 4210–4219.

Palmero, I., Holder, A., Sinclair, A. J., Dickson, C., & Peters, G. (1993). Cyclins D1 and D2 are differentially expressed in human B-lymphoid cell lines. *Oncogene, 8*, 1049–1054.

Qiu, G., Seiler, M. J., Mui, C., Arai, S., Aramant, R. B., de Juan, E., et al. (2005). Photoreceptor differentiation and integration of retinal progenitor cells transplanted into transgenic rats. *Experimental Eye Research, 80*, 515–525.

Rattner, A., & Nathans, J. (2005). The genomic response to retinal disease and injury: Evidence for endothelin signaling from photoreceptors to glia. *Journal of Neuroscience, 25*, 4540–4549.

Raymond, P. A., Reifler, M. J., & Rivlin, P. K. (1988). Regeneration of goldfish retina: Rod precursors are a likely source of regenerated cells. *Journal of Neurobiology, 19*, 431–463.

Reyer, R. W. (1977). The amphibian eye: Development and regeneration. In F. Crescitelli (Ed.). *The Visual System in Vertebrates. Handbook of Sensory Physiology* (Vol. 7(5), pp. 309–390). New York: Springer.

Roque, R. S., Imperial, C. J., & Caldwell, R. B. (1996). Microglial cells invade the outer retina as photoreceptors degenerate in Royal College of Surgeons rats. *Investigative Ophthalmology and Visual Science, 37*, 196–203.

Sakaguchi, D. S., Janick, L. M., & Reh, T. A. (1997). Basic fibroblast growth factor (FGF-2) induced transdifferentiation of retinal pigment epithelium: Generation of retinal neurons and glia. *Developmental Dynamics, 209*, 387–398.

Severs, N. J., Barnes, S. H., Wright, R., & Hicks, R. M. (1982). Induction of bladder cancer in rats by fractionated intravesicular doses of *N*-methyl-*N*-nitrosourea. *British Journal of Cancer, 45*, 337–351.

Stenkamp, D. L., & Cameron, D. A. (2002). Cellular pattern formation in the retina: Retinal regeneration as a model system. *Molecular Vision, 8*, 280–293.

Takahashi, M., Palmer, T. D., Takahashi, J., & Gage, F. H. (1998). Widespread integration and survival of adult-derived neural progenitor cells in the developing optic retina. *Molecular and Cellular Neuroscience, 12*, 340–348.

Tropepe, V., Coles, B. L., Chiasson, B. J., Horsford, D. J., Elia, A. J., McInnes, R. R., et al. (2000). Retinal stem cells in the adult mammalian eye. *Science, 287*, 2032–2036.

Wan, J., Zheng, H., Hu, B. Y., Xiao, H. L., She, Z. J., Chen, Z. L., et al. (2006). Acute photoreceptor degeneration down-regulates melanopsin expression in adult rat retina. *Neuroscience Letters, 400*, 48–52.

Wan, J., Zheng, H., Xiao, H. L., She, Z. J., & Zhou, G. M. (2007). Sonic hedgehog promotes stem-cell potential of Müller glia in the mammalian retina. *Biochemical and Biophysical Research Communications, 363*, 347–354.

Yang, H., Standifer, K. M., & Sherry, D. M. (2002). Synaptic protein expression by regenerating adult photoreceptors. *Journal of Comparative Neurology, 443*, 275–288.

Yoshida, K., Kase, S., Nakayama, K., Nagahama, H., Harada, T., Ikeda, H., et al. (2004). Distribution of p27(KIP1), cyclin D1, and proliferating cell nuclear antigen after retinal detachment. *Graefe's Archive for Clinical and Experimental Ophthalmology, 242*, 437–441.

Yoshizawa, K., Yang, J., Senzaki, H., Uemura, Y., Kiyozuka, Y., Shikata, N., et al. (2000). Caspase-3 inhibitor rescues *N*-methyl-*N*-nitrosourea-induced retinal degeneration in Sprague–Dawley rats. *Experimental Eye Research, 71*, 629–635.

Young, M. J., Ray, J., Whiteley, S. J., Klassen, H., & Gage, F. H. (2000). Neuronal differentiation and morphological integration of hippocampal progenitor cells transplanted to the retina of immature and mature dystrophic rats. *Molecular and Cellular Neuroscience, 16*, 197–205.

Yuge, K., Nambu, H., Senzaki, H., Nakao, I., Miki, H., Uyama, M., et al. (1996). *N*-Methyl-*N*-nitrosourea-induced photoreceptor apoptosis in the mouse retina. *In Vivo, 10*, 483–488.

Yurco, P., & Cameron, D. A. (2005). Responses of Müller glia to retinal injury in adult zebrafish. *Vision Research, 45*, 991–1002.

Zhang, J., Gray, J., Wu, L., Leone, G., Rowan, S., Cepko, C. L., et al. (2004). Rb regulates proliferation and rod photoreceptor development in the mouse retina. *Nature Genetics, 36*, 351–360.