PAX6-Positive Müller Glia Cells Express Cell Cycle Markers but Do Not Proliferate After Photoreceptor Injury in the Mouse Retina

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KEY WORDS
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ABSTRACT
In lower vertebrates, such as fish, Müller glia plays an essential role in the restoration of visual function after retinal degeneration by transdifferentiating into photoreceptors and other retinal neurons. During this process, Müller cells re-enter the cell cycle, proliferate, and migrate from the inner nuclear layer (INL) to the photoreceptor layer where they express photoreceptor-specific markers. This process of Müller cell transdifferentiation is absent in mammals, and the loss of photoreceptors leads to permanent vision deficits. The mechanisms underlying the failure of mammalian Müller cells to behave as stem cells after photoreceptor degeneration are poorly understood. In the present study, we show that photoreceptor injury induces migration of PAX6-positive Müller cell nuclei toward the outer part of the INL and into the inner part of the outer nuclear layer. These cells express markers of the cell cycle, suggesting an attempt to re-enter the cell cycle similarly to lower vertebrates. However, mouse Müller cells do not proliferate in response to photoreceptor injury implying a blockade of the S-phase transition. Our results suggest that a release of the S-phase blockade may be crucial for Müller cells to successfully transdifferentiate and replace injured photoreceptors in mammals. ©2011 Wiley-Liss, Inc.

INTRODUCTION
Inherited retinal degenerations such as retinitis pigmentosa (RP) and Leber congenital amaurosis induce severe and irreversible loss of photoreceptors leading to blindness. As a potential therapeutic approach, cells located in the ciliary marginal zone (CMZ) or Müller glial cells in the inner retina might be used as endogenous retinal stem cells to replace lost photoreceptors (Bhatia et al., 2009; Karl and Reh, 2010; Lawrence et al., 2007). In anamniotes, the CMZ is a highly specialized region situated at the anterior margin of the retina containing retinal progenitors and multipotent cells that proliferate throughout life to generate all types of neurons (Hitchcock et al., 2004; Reh and Fischer, 2001; Reh and Levine, 1998). In the zebrafish retina, the circumferential germinal zone is responsible for persistent neurogenesis where all types of neurons except rod photoreceptors are generated (Hitchcock et al., 2004; Raymond et al., 2006). Here, new rods arise from a second pool of progenitor cells located in the inner nuclear layer (INL) of the differentiated retina (Hitchcock et al., 2004; Raymond and Rivlin, 1987; Vithelick and Hyde, 2000). During vertebrate evolution, a decrease in the size of the CMZ area led to the pars plana localized in the ciliary bodies. As a result, the mammalian retina has an extremely limited intrinsic capacity to induce cell proliferation and to regenerate neuronal cells (Kubota et al., 2002; Nishiguchi et al., 2008; Tropepe et al., 2000). However, a potential source of stem cells may still reside within the Müller glial cell population, which is located in the INL. During histogenesis, retinal neurons are born in a specific temporal order (Cepko et al., 1996; Rapaport et al., 2004). In all vertebrates, retinal ganglion cells (RGCs), horizontal cells, and cones are the first retinal neurons to be born, while rods, bipolar cells, amacrine cells, and Müller glial cells are last to emerge. Interestingly, these six different types of retinal neurons and the Müller glial cells are coming from the same lineage and are differentiated from a common multipotent progenitor (Hatakeyama and Kageyama, 2004; Ohsawa and Kageyama, 2008; Turner and Cepko, 1987). This may explain why Müller cells retain some neuronal stem cell properties and why they can adopt some characteristics of retinal progenitors and transdifferentiate into new neurons. Ganglion and amacrine cell injury induced by N-methyl-D-aspartate (NMDA)-excitotoxicity causes Müller cell transdifferentiation into other retinal cell types in chicken (Fischer and Reh, 2001, 2003) and...
mice (Ooto et al., 2004). During this process, Müller cells can re-enter the cell cycle, switch from a glial to a neuronal phenotype, and express neuronal markers. The mechanisms of neurogenesis involving Müller glial cells have been particularly well studied in the zebrafish retina that grows throughout life and in which rod photoreceptors are continuously added (Bernardos et al., 2007; Otteson and Hitchcock, 2003; Raymond et al., 2006). In the normal retina of fish, the transcription factor paired box gene 6 (PAX6) is expressed by all Müller glial cells at a low level. PAX6-positive Müller cells divide in response to growth factors that regulate the addition of new rod photoreceptors in the growing retina. The new PAX6-positive neural progenitor cells produced in the INL migrate along the radial glial processes to the outer nuclear layer (ONL). During this active migration, PAX6 expression is downregulated and the level of the cone-rod homeobox gene (CRX), another transcription factor essential for the specification into photoreceptors, is upregulated (Bernardos et al., 2007).

So far, very little is known about the ability of Müller cells to divide in the mouse retina after massive photoreceptor cell death. Although Del Debbio and colleagues (2010) recently reported that the activation of Notch and Wnt signaling stimulates Müller cell proliferation and improves visual performance in a rat model of RP, the molecular mechanisms involved in transdifferentiation of Müller cells are still poorly understood. Here, we tried to increase our understanding on why Müller cells fail to replace dead photoreceptors in the injured mouse retina. To do so, we used the model of light-induced photoreceptor degeneration and a genetic model of RP, the rd10 mouse. Our observations indicate that photoreceptor injury activates PAX6-positive Müller cell nuclei to translocate from their normal location in the middle of the INL toward the outer INL and even into the inner part of the ONL (inner ONL). The expression of several molecular markers suggests that these Müller cells attempt to re-enter the cell cycle but fail to do so, resulting in a nonproliferative gliosis. We hypothesize that a partial blockade before the S-phase may explain, at least in part, why Müller cells cannot complete their transdifferentiation into photoreceptors in the mammalian retina.

**MATERIALS AND METHODS**

**Animals and Light Exposure**

All procedures were conducted in accordance with the ARVO statement for the use of Animals in Ophthalmic and Vision Research and with the regulations of the Veterinary Authority of Zurich. Wild-type 129S6/SvEvTac mice (Taconic, Eiby, Denmark), rd10 mice (Jackson lab, Bar Harbor, USA), and C57BL/6 control mice (Jackson lab, Bar Harbor, USA) were raised in cyclic light (12-h light–dark; 60 lux at cage level) and maintained at the University Hospital Zürich. For light exposure, adult (8–10 weeks of age) 129S6/SvEvTac mice were dark adapted overnight. Pupils were dilated under red light with 1% cyclopentolate (Cyclogyl, Alcon, Cham, Switzerland) and 5% phenylephrine (Ciba Vision, Niederwangen, Switzerland) 45 min before exposure. Mice were then exposed to 13,000 lux of white light for 2 h. After exposure, mice were kept in darkness for 12 additional hours before they were returned to cyclic light. Light-exposed mice were sacrificed at different time points after light offset (n = 3 for each group), while rd10 mice, along with their C57BL/6 wild-type controls, were sacrificed at different ages ranging from postnatal day 10 (PND10) to PND168 (n = 3 for each time point).

**Injections**

To test for proliferating cells, wild-type mice received daily intraperitoneal injections of bromodeoxyuridine (BrdU, 50 mg/kg of body weight; Sigma, Buchs, Switzerland) starting immediately after light exposure for four consecutive days. For intravitreal injections, mice were anesthetized with an intramuscular injection of ketamine (75 mg/kg) and xylazine (23 mg/kg). Intravitreal injections were done as described earlier (Pernet et al., 2005) with 2 l of a mixture of insulin (400 ng per injection) and fibroblast growth factor (FGF2, 130 ng per injection) (Peprotech, London, UK, #AF-100-18B), of the vehicle Phosphate Buffer Saline/Bovine Serum Albumin (PBS/BSA), or of 20 mM NMDA (Sigma).

**RNA Preparation, cDNA Synthesis,**

**and Semiquantitative Real Time-PCR**

RNA isolation from retinal tissue and reverse transcription were performed as described (Joly et al., 2009). Specific primer pairs, designed to span large intronic sequences or to cover exon-intron boundaries, were used to amplify cDNA: Actb (β-actin) (forward, 5′-CAAC GGCTCCGGCATGTCG; reverse, 5′-CTTTGCTCGGG CCTCG), Pax6 (forward, 5′-AGTTCTTCGCAACCTGG CTA; reverse, 5′-CTCTGAGCTCCTACCCAGT). cDNA amplification and quantification were performed as described in the Supporting Information.

**Immunofluorescence and Western Blot**

The primary antibodies used are summarized in Table 1. See the Supporting Information detailed protocols.

**Quantification of Translocated**

**PAX6-Positive Cells**

To quantify translocated Müller cell nuclei, PAX6-positive nuclei were counted in the outer INL (at the border of the INL to the outer plexiform layer, OPL) and also in the inner ONL (cells which crossed the OPL). The most transversal sections passing through the optic nerve were selected, and quantification was performed in three sections per animal. An average of three to four mice
was used per treatment except for the groups unexposed to light and treated with PBS/BSA or insulin/FGF2 where two mice were used. Results were expressed as cells per millimeter retinal length. The direction of this translocation will be referred as an apical movement in the rest of the work.

RESULTS

Light-Induced Photoreceptor Injury Causes an Apical Translocation of PAX6-Positive Müller Cell Nuclei to the Outer INL and the Inner ONL

The transcription factor PAX6 is involved in both embryonic and adult neurogenesis (Osumi et al., 2008) and in response to brain injury PAX6 expression is strongly induced in proliferating progenitor cells of the hippocampus (Nakatomi et al., 2002). To localize PAX6 in response to photoreceptor injury in the mouse retina, we performed immunofluorescence at different time points after exposure to bright visible light (Fig. 1A–D). In non-exposed control mice, PAX6-positive Müller cell nuclei were mainly detected in the outer INL (arrows) and few localized to the inner part of the photoreceptor layer (Fig. 1A, arrowheads). Double labeling revealed that most of these cells also expressed GS indicating that they were of Müller cell origin. The largest number of PAX6-positive Müller cell nuclei in the outer INL was detected in the region with the most severe photoreceptor injury close to the optic nerve head. However, some translocated cells were also detected in the retinal periphery (Fig. 1E). Western blot analysis did not show a significant increase of PAX6 protein levels in total extracts from retinae with light-induced photoreceptor injury (Fig. 1F). Similarly, Pax6 mRNA levels remained at basal levels after light exposure (data not shown). Our data suggest that light-induced photoreceptor injury caused the apical migration of some PAX6-positive Müller cell nuclei toward or even into the damaged ONL.

Mutation-Induced Photoreceptor Injury Causes Translocation of PAX6-Positive Müller Cell Nuclei to the Outer INL and Inner ONL in the rd10 Mouse, a Genetic Model for RP

Next, we analyzed whether the observed translocation of PAX6-positive Müller cell nuclei was specific for the light-damaged retina or whether it might be a general mechanism induced upon photoreceptor injury. The rd10 mouse carries a mutation in exon 13 of the β subunit of the cGMP phosphodiesterase and is a well-established genetic model for RP (Chang et al., 2007; Gargini et al., 2007). Photoreceptor degeneration starts at PND18 and peaks around PND25 (Barhoum et al., 2008; Gargini et al., 2007). PAX6 immunofluorescence was performed on retinae from wild type and rd10 mice at PND28 (Fig. 2A). In the rd10 mouse, many PAX6-positive Müller cell nuclei (as indicated by the colocalization with GS) were detected in the outer INL (arrows) and few localized...
Fig. 1. Light-induced photoreceptor injury causes the appearance of PAX6-positive Müller cell nuclei in the outer INL and inner ONL. Wild-type 129S6 mice were exposed to bright light for 2 h and analyzed at indicated time points after light exposure. A: Time series after light exposure. Arrows and arrowheads point to PAX6-positive Müller cell nuclei in the outer INL and the inner ONL, respectively. B–D: Identification of PAX6-expressing cells in control retinae and at 4 days after light exposure. Blue: nuclei stained with DAPI. Red: PAX6. Green: Calbindin (B), Calretinin (C), or glutamine synthase, GS (D) as indicated. E: Regional differences in the translocation of PAX6-positive Müller cell nuclei (red) in the inferior retina at 4 days after light exposure. Control retina was not exposed to light. Note the decreased ONL thickness in the light-exposed retina (DAPI). F: PAX6 protein levels in total retinal extracts. PAX6 signals were quantified, normalized to ACTB, and expressed relatively to nonexposed controls. Shown are mean values ± SEM of n = 3. Abbreviations: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer; ONH, optic nerve head; CB, ciliary body; C, control; h, hours; d, days. Scale bars: (A–D) 50 μm; (E) 100 μm.
even in the inner ONL (arrowheads). In wild-type mice of the same age, however, only very few PAX6-positive cells localized to the outer INL (stars). These cells were GS negative and most likely corresponded to the PAX6-positive horizontal cells already described in Fig. 1B. Note that almost no PAX6/GS double positive cells remained in

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**Fig. 2.** PAX6-positive Müller cell nuclei appear in the outer INL and inner ONL of rd10 mice. **A:** PAX6 (red) and GS (green) localizations were analyzed by immunofluorescence in retinae of wild type and rd10 mice at PND28. Nuclei were stained with DAPI (blue). Arrows and arrowheads point to PAX6/GS double-positive Müller cell nuclei in the outer INL and the inner ONL, respectively. (*) label PAX6-positive, GS-negative cell nuclei (presumably horizontal cells, see Fig. 1) in the outer INL. **B:** Determination of PAX6 protein levels in retinae of wild type and rd10 mice at postnatal days as indicated. ACTB served as loading control. The bar diagram shows the average of three independent experiments (mean ± SEM). Statistical analysis was performed using a Student’s t-test between wild type and rd10 mice for each time point (*p < 0.05). C: Pax6 mRNA expression in retinae of wild type (black) and rd10 (red) mice at postnatal days as indicated (n = 3; mean ± SEM). Expression levels were normalized to Actb and expressed relatively to wild type at PND10, which was set to 1. Statistical analysis was performed using a two-way ANOVA followed by a Tukey post hoc test (*p < 0.05, ***p < 0.001). Abbreviations: PND, postnatal day; IPL, inner plexiform layer; other abbreviations as in Fig. 1. Scale bar: 50 μm.
the middle part of the INL in the rd10 retina suggesting that most of these nuclei had shifted their position toward the region with injured photoreceptors.

Western blot analysis of protein extracts from wild type and rd10 retinas showed that PAX6 levels declined in the ageing wild-type retina from PND10 to PND168 (Fig. 2B). After an initial similar decrease from PND10 to PND21, PAX6 protein levels in rd10, however, slightly increased (Fig. 2B). A similar expression pattern was detected for Pax6 mRNA using qRT-PCR (Fig. 2C). Wild-type mice displayed a 69% increase of Pax6 mRNA levels from PND10 to PND21 before expression reached steady-state levels (Fig. 2C, black line). Rd10 mice, however, presented a constant increase of their Pax6 mRNA expression from PND10 to PND168 (Fig. 2C, red line). It is interesting to note that Pax6 mRNA levels increased from PND10 to PND21, whereas PAX6 protein levels decreased in the same time window in both wild-type and rd10 mice. This points to a post-transcriptional regulation of PAX6 protein during aging and suggests that the rd10 mutation does not influence the normal regulation of PAX6 expression in the young retina. Overall, our data show that the appearance of PAX6-positive Müller cell nuclei in the outer INL/inner ONL is a general response to photoreceptor injury, independent of the damage-inducing stimulus (light, gene mutation) and supports our earlier conclusion that PAX6-positive Müller cells are attracted apically toward the layer of injured photoreceptors (ONL).

Translocation of PAX6-Positive Müller Cell Nuclei to the Outer INL Is Specific for Photoreceptor Injury

To determine whether the translocation of PAX6-positive Müller cell nuclei toward the ONL was a phenomenon occurring generally in a damaged retina or was caused specifically by the injury of photoreceptors, we injected NMDA to induce excitotoxic death of ganglion cells and amacrine cells (Siliprandi et al., 1992). Already 3 days after NMDA treatment, PAX6 staining almost completely disappeared in the GCL due to the severe loss of RGCs and of the displaced amacrine cells normally present in this layer (see Fig. 3). The loss of amacrine cells also caused a pronounced thinning of the INL. Importantly, however, PAX6-labeled Müller cell nuclei did not translocate and remained in the center of the INL at 3 and 15 days post-NMDA-injection (see Fig. 3). The complete absence of such a migration after NMDA treatment was confirmed by immunofluorescence stainings of SOX9, a protein which specifically localizes to the nuclei of Müller cells (Supp. Info. Fig. 1). Thus, the appearance of Müller cell nuclei in the outer INL and inner ONL after light exposure or in the rd10 mouse was specific for photoreceptor injury. This suggests that dying photoreceptors may induce a signaling cascade to activate an oriented migration of PAX6-positive Müller cell nuclei toward the injured photoreceptor layer. Such a cellular response may be induced by the retina in an attempt to minimize or repair the damage.

Light-Induced Photoreceptor Injury Causes a Nonproliferative Gliosis of Müller Cells

Several reports demonstrate that Müller cells can proliferate in mammals and birds after NMDA-induced excitotoxicity (Fischer and Reh, 2001; Karl et al., 2008; Ooto et al., 2004). In addition, a recent report suggests rare but detectable proliferation and dedifferentiation of Müller cells in the rat retina during genetically induced photoreceptor degeneration (Del Debbio et al., 2010). To determine whether photoreceptor injury not only caused a translocation of PAX6-positive Müller cell nuclei but also a proliferation of Müller cells as a sign of neuronal regeneration, we injected BrdU intraperitoneally into...
light-exposed mice (see Fig. 4). BrdU is commonly used to specifically label neural progenitors in the S-phase of the cell cycle. As expected, BrdU incorporation was not detected in dark control retinae but strongly stained proliferating cells in the thymus which was used as positive control (Fig. 4A, inset). Six hours after light exposure, few cells in the ONL were BrdU-positive (Fig. 4A). Incorporation of BrdU peaked at 1 day after exposure. BrdU-
specific labeling became weaker at 2 days and almost had disappeared at 3 days after exposure, probably due to ongoing photoreceptor degeneration and clearance of cellular debris from the subretinal space. Incorporation of BrdU was restricted at all times to the ONL, the cell layer where light exposure induced photoreceptor injury and degeneration. Colocalization of BrdU and DNA Ligase IV (LiG4), a marker for DNA repair, suggested that BrdU was incorporated in photoreceptor cells most likely because of ongoing DNA repair (Fig. 4B; Taupin, 2007) which has been previously reported for light-damaged photoreceptor cells (Gordon et al., 2002) as well as in the rd1 mouse (Menu dit Huart et al., 2004). Since we did not detect any cells positive for Ki-67 (Fig. 4C), another proliferation marker, we conclude that retinal cells (including Müller cells) did not proliferate in response to light-induced photoreceptor injury.

Müller cell gliosis was evaluated by analyzing expression of glial fibrillar acidic protein (GFAP) by immunofluorescence (Fig. 4D) and levels of Gfap mRNA by qRT-PCR (data not shown). In control retinæ, GFAP expression was restricted to astrocytes and to the endfeet of Müller cells in the nerve fiber layer. Already at 1 day, but mostly at 2 and 3 days after light exposure, GFAP was dramatically up-regulated in the radial processes of Müller cells throughout the retina (Fig. 4D), as previously reported (Ekström et al., 1988). At the mRNA level, Gfap expression was increased 10-fold after 6 hours and peaked after 2 days with a 12.5-fold increase (data not shown). We also investigated the expression of Kir4.1 whose expression was reported to decrease during proliferative gliosis (Bringmann et al., 2000). However, such a decrease of Kir4.1 expression was not detected after light exposure and at 10 days, Kir4.1 labeling was even stronger compared to the control retina (Supp. Info. Fig. 2) as previously reported (Iandiev et al., 2008). Taken together, these data suggest that photoreceptor injury caused by bright light leads to a non-proliferative gliosis of Müller cells.

**Müller Cells Activated by Photoreceptor Injury Express Markers of the Cell Cycle**

Even though our data did not provide any evidence of BrdU incorporation in Müller cells after light-induced photoreceptor injury, we analyzed the expression of phospho-histone 3 (PH3), an intrinsic mitotic marker regulating the transition from the G2- to the M-phase (Gurley et al., 1978; Hendzel et al., 1997; Juan et al., 1998). Mice were sacrificed at 6 hours and at 1 day after light exposure and double immunofluorescence combining PH3 and GS detection was performed (Fig. 5A). In the intact adult retina, PH3 labeling was undetectable. Six hours after light stress, however, PH3 was strongly and specifically detected in Müller cell nuclei (Fig. 5A, inset, arrowheads). This expression was transient as PH3 was detected only at low intensity in very few translocated Müller cell nuclei at 1 day after light exposure (Fig. 5A, inset). In addition to be associated with cell cycle progression, histone H3 phosphorylation has been shown to occur concomitantly with the transcriptional activation of some immediate early-response genes such as Jun and Fos (Nowak and Corces, 2004). Thus, we followed the expression of FOS in control and light-exposed retinas by immunohistochemistry (Fig. 5B). In control adult retinæ, FOS was expressed in RGCs (Fig. 5B, arrowheads) while Müller cell nuclei (Fig. 5B, arrows and inset) became FOS-positive at 6 hours after light. After 1 day, FOS expression was no longer detectable in Müller cells but only subsisted in RGCs (data not shown). Very similar observations were made by in situ hybridizations detecting Fos transcripts (Hafezi et al., 1997). The simultaneous and transient up-regulation of PH3 and FOS suggests that PH3 may be phosphorylated to facilitate expression of early-response genes and may thus not indicate an active G2-phase.

D-type cyclins such as D1 and D3 mediate progression through the G1/S-phase of the cell cycle (Donjerkovic and Scott, 2000; Sherr, 1995). Expression of both cyclin D1 and cyclin D3 was transiently induced in retinæ of light-exposed mice as shown by Western blotting (Fig. 6A). Detection of phospho-STAT3 and STAT3 served as controls for retinal degeneration (Samardzija et al., 2006). The retinal localization of both cyclins was determined by immunofluorescence (Fig. 6B–D). Colocalization with CRALBP, a marker for Müller cells, revealed a basal expression of cyclin D3 in Müller cell nuclei (Fig. 6B) as previously reported (Dyer and Cepko, 2000). Cyclin D1, however, was not detected in control retinae (Fig. 6C). Two days after light-induced photoreceptor injury, both cyclins were detected in Müller cell nuclei with more cells positive for cyclin D3 than for cyclin D1. In addition, the spatial distribution of cyclin D3-positive Müller cell nuclei indicated that expression of cyclin D3 might colocalize with PAX6 in those Müller cell nuclei which were translocated after photoreceptor injury. This was confirmed by the double labeling with anti-PAX6 and anti-cyclin D3 antibodies (Fig. 6D). Our results show that light-induced photoreceptor injury did not cause proliferation of Müller cells as evidenced by the lack of BrdU incorporation (see Fig. 4), but nevertheless induced expression of markers involved in cell cycle progression.

**FGF2 and Insulin Are Not Sufficient to Release the Cell Cycle Blockade and to Trigger Müller Cell Proliferation After Light-Induced Photoreceptor Injury**

The work by Fisher and colleagues showed that the intravitreal application of a combination of insulin and fibroblast growth factor 2 (FGF2) in chicken causes Müller cells to express transcription factors specific for retinal progenitors, to proliferate, and, for a fraction of them, to transdifferentiate into neurons (Fischer et al., 2002). Our results described earlier suggest that Müller cells in the mouse retina attempt but do not succeed to re-enter the cell cycle and thus fail to proliferate in response to photoreceptor injury. With the aim to
support Müller cells in their attempt to dedifferentiate and proliferate, wild-type mice were injected with a combination of insulin and FGF2 into the vitreous 1 day after light exposure. Mice were sacrificed 3 days after injections, and retinal immunofluorescence was performed to visualize localization of PAX6-expressing Müller cell nuclei (Fig. 7A). Mice not exposed to light displayed the same pattern of PAX6 localization, irrespective of whether they received injections of vehicle or of insulin/FGP2, or whether they received no injection at all (Fig. 7A, top row). This suggests that the application of insulin/FGP2 and/or of vehicle did not influence the localization of PAX6-positive Müller cell nuclei in the absence of photoreceptor injury. Mice with

Fig. 5. Phosphohistone 3 (PH3) is transiently expressed by Müller cells during photoreceptor degeneration. A: PH3 (red) and GS (green) were detected by immunofluorescence in retinas before (control) and at 6 h and 1 day after light exposure of wild-type mice. Insets show magnifications of boxed areas. B: Localization of FOS (red) and Müller cells (GS-positive, green) in control retinas and at 6 h after light exposure. In nonexposed mice, FOS was detected in ganglion cells (arrowheads) while light-exposed retinae showed additional FOS staining in Müller cell nuclei (arrows, inset). Nuclei were stained with DAPI (blue). Abbreviations as in Figs. 1 and 2. Scale bar: 100 µm.
injured photoreceptors after light exposure showed a similarly increased number of PAX6-positive Müller cell nuclei in the outer INL and inner ONL (Fig. 7A, bottom row) as observed earlier (see Fig. 1). We have also performed quantification of these cells (Fig. 7B). Injections of vehicle and/or insulin/FGF2 increased these numbers slightly (Fig. 7A, bottom row and Fig. 7B). However, no difference was detected between the vehicle group and the insulin/FGF2 group (Fig 7B). This suggests that injections per se and not the application of the trophic factors might have a slight effect on the translocation of PAX6-positive Müller cell nuclei in response to photoreceptor injury. Furthermore, translocated PAX6-positive Müller cell nuclei did not show any signs of BrdU incorporation, even after vehicle or insulin/FGF2 injections (data not shown), suggesting that this treatment did not enhance proliferation of Müller cells in the mouse retina with injured photoreceptors.

**DISCUSSION**

Photoreceptor injury caused the appearance of PAX6-positive Müller cell nuclei in the outer INL and inner ONL indicating an apical translocation of these cells toward the site of damage. The increased expression of cyclins suggests that these Müller cells attempt to re-enter the cell cycle but lack of BrdU incorporation implies a blockade of DNA synthesis (S-phase) preventing cell proliferation. This blockade might be part of the
explanation why mammalian Müller cells fail to regenerate lost photoreceptors in the degenerating retina.

PAX6 is a key regulator of eye development (Callaerts et al., 1997), and misexpression of PAX6 can induce fully differentiated ectopic eyes in flies and frogs (Chow et al., 1999; Halder et al., 1995). PAX6 is strongly expressed during retinal development to retain multipotency and proliferation capacity of retinal progenitor cells (Ashery-Padan and Gruss, 2001; Walther and Gruss, 1991). Moreover, PAX6 is also detected in Müller glia and other

Fig. 7. Intravitreal injections after light-induced photoreceptor injury slightly increase translocation of PAX6-positive Müller cells toward the outer INL and the inner ONL. A: Wild-type mice were or were not exposed to light as indicated. One day after light exposure, mice received an intravitreal injection of PBS/BSA vehicle or of insulin/FGF2 or were not injected. Three days after injection, the distribution of PAX6-positive (red) and GS-positive (green) Müller cell nuclei was analyzed. Nuclei were stained with DAPI (blue). Arrowheads point to PAX6-positive Müller cell nuclei in the inner ONL. Insets: Higher magnification of boxed areas. B: Quantification of PAX6-positive Müller cells in the outer INL (red bar) and in the inner ONL (green bar), expressed per millimeter retina. The number of mice used for each condition is indicated in the bar graph. The right panel shows an example of quantification where cells marked with red arrowheads were counted in the group “outer INL” and cells marked with white arrowheads were counted in the “inner ONL” group. Statistical analysis was performed using a one-way ANOVA followed by Tukey post hoc test (*p < 0.05). The red star shows the significance for the “INL/OPL group,” whereas the green star shows the significance for the “OPL/ONL group” between noninjured and light-injured mice, respectively. Scale bar: 100 μm. Abbreviations as in Fig. 1.
cell types of the adult mouse retina (see Fig. 1) where it might be involved in the molecular response to retinal injury (Bernardos et al., 2007; Jones et al., 1998; Roesch et al., 2008).

In two models of induced and inherited photoreceptor degeneration, PAX6-positive Müller cell nuclei translocated apically to the outer INL and inner ONL. This might be a specific response to photoreceptor injury, because we did not observe a similar translocation after NMDA-induced excitotoxic damage of ganglion cells and amacrine cells. It is unclear how photoreceptor injury activates this response, but findings in fish show that only Müller cells whose apical processes were adjacent to damaged photoreceptors migrated toward the ONL (Bernardos et al., 2007) proposing that a local injury signal mediates this reaction. Our observation that the number of translocated Müller cell nuclei was largest in the retinal area of strongest light-induced photoreceptor degeneration (Fig. 1E) may support this conclusion.

In posthatched chickens, it was proposed that the migration of Müller cell nuclei might be attributable to the interkinetic nuclear migration process (IKNM) (Fischer and Reh, 2003), which corresponds to oscillations of the nucleus from the apical to the basal cell surface concomitantly with phases of the cell cycle. During development, nuclei in the M-phase stay confined to the apical surface of the neuroepithelium, while nuclei in the S-phase occupy more basal locations (Baye and Link, 2007, 2008). In zebrafish, Müller glia nuclei reenter the cell cycle in response to photoreceptor apoptosis and migrate along the radial fiber through a mechanism reminiscent of the IKNM (Bernardos et al., 2007). The observed transient expression of cell cycle markers in Müller cells (Figs. 5 and 6) may be supportive of an activation of such a mechanism during photoreceptor apoptosis in the mouse retina. Evidence implicates the Notch pathway in the regulation of this process (Del Bene et al., 2008; Ghai et al., 2010) as well as in the regulation of Pax6 gene expression (Onuma et al., 2002), but the precise molecular mechanisms still need to be investigated.

The nature of the postulated Müller cell-activating signal in the degenerating retina (see above) may depend on the injured cell type and/or on the nature of the death-inducing stimulus. This may also influence the response induced in Müller cells. Excitotoxic damage to ganglion cells and amacrine cells after NMDA application leads to Müller cell dedifferentiation and proliferation (Fischer and Reh, 2001; Fischer et al., 2004; Karl et al., 2008; Ooto et al., 2004), whereas damage to photoreceptors by light exposure did not (see Fig. 4). Instead, Müller glia cells strongly upregulated expression of GFAP, which may negatively influence the re-entry into the cell cycle (Dyer and Cepko, 2000; Fischer and Reh, 2003). In spite of the lack of proliferation, the transient expression of PH3 and of the cyclins D1 and D3 argues that Müller glia nevertheless attempt to re-enter the cell cycle in response to photoreceptor injury. D-type cyclins and their corresponding cyclin-dependent kinases are reported to control entry into the S-phase through the regulation of the phosphorylation status of retinoblastoma protein (Giacinti and Giordano, 2006; Sherr, 1995). Despite the increased expression of cyclins, however, the lack of BrdU incorporation suggests that the S-phase cannot be initiated and that the cell cycle is blocked by an unknown mechanism. A candidate protein in this respect might be the cyclin kinase inhibitor p27Kip1. In the retina, a fast drop of the p27Kip1 levels preceded re-entry of Müller cells into the cell cycle after neurotoxin-induced retinal injury (Dyer and Cepko, 2000). The controlled release of the S-phase blockade may be a key to drive mouse Müller cells into dedifferentiation and proliferation, eventually leading to the regeneration of photoreceptors in the injured retina. The lack of BrdU incorporation and the detection of PH3, a supposedly endogenous marker of the G2-phase, are in apparent discrepancy. However, PH3 activation was recently detected in all phases of the cell cycle in newborn mouse retinae (Barton and Levine, 2008). This suggests that H3 phosphorylation may not reflect accurately the status of the cell cycle. Instead, the high level of PH3 may be involved in the transcriptional activity related to Müller cell gliosis (Nakazawa et al., 2007).

Injections of insulin and FGF2 have been shown to activate the neurogenic program in Müller glia of chicken (Fischer et al., 2002). Similarly, injections of epidermal growth factor or FGF1 alone or in combination with insulin promoted Müller cell proliferation and dedifferentiation after NMDA-induced injury of cells in the inner retina of mice (Karl et al., 2008). After photoreceptor injury in the outer retina, however, injections did not induce proliferation but only slightly increased the apical translocation of Müller cell nuclei to the outer INL and inner ONL (see Fig. 7). Furthermore, this effect was not specific for the factors injected and was rather caused by the mechanical damage inflicted by the injections per se as reported before (Cao et al., 2001). The discrepancy in the proliferative capacities of the Müller cells after white light treatment or NMDA injections is not clear but might involve the possibility that NMDA treatment can directly alter the physiology of Müller cells by activating their NMDA receptors (Lebrun-Julien et al., 2009; Takeda et al., 2008). Light exposure, however, targets photoreceptor cells through photon absorption by the visual pigment rhodopsin (Grimm et al., 2000) and may not directly target Müller glia cells. This may explain why Müller cells are more reactive after NMDA treatment than after light exposure.

Although Müller cell nuclei translocation occurred in both models of photoreceptor degeneration, total PAX6 protein expression was only upregulated in the retina of the rd10 mouse (Fig. 2B) and not after light-induced degeneration (Fig. 1F). This difference might be based on the fact that the mutation in the rd10 mouse is permanently present and causes a chronic stress with slow photoreceptor degeneration, whereas light exposure induces an acute degeneration with the toxic stress (light) present only for 2 h. Because PAX6 levels in the rd10 mouse tended to increase only around PND28 (see Fig. 2), roughly 10 days after photoreceptors started to
degenerate, a long-lasting presence of the toxic stimulus seems to be required for Pax6 up-regulation. A very similar observation was made in the rd1 mouse where degeneration starts around PND12 but increased gene expression of Pax6 was only detected around PND21 (Jones et al., 1998). Our observation that during normal postnatal development, Pax6 protein levels declined, whereas Pax6 mRNA levels increased until PND21 suggests a complex regulation of gene and protein expression, which may also contribute to the control of Pax6 levels in response to photoreceptor injury.

In conclusion, our study shows two different models of photoreceptor degeneration that some Pax6-positive Müller cell nuclei translocate in response to photoreceptor injury toward the site of damage. These migrating cells express markers of the cell cycle but do not replicate their DNA. This suggests that these cells attempt to re-enter the cell cycle but fail to overcome an endogenous blockade and thus do not proliferate and transdifferentiate. As a consequence, photoreceptors are not regenerated in the mouse retina. A controlled transdifferentiation might be essential to unleash the neurogenic potential of Müller cells in the mammalian retina.

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