Antiviral Drug Ganciclovir Is a Potent Inhibitor of the Proliferation of Müller Glia–Derived Progenitors During Zebrafish Retinal Regeneration

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PURPOSE. The purpose of this study was to investigate the effect of the antiviral drug ganciclovir (GCV) on Müller glia dedifferentiation and proliferation and the underlying cellular and molecular mechanisms in adult zebrafish.

METHODS. A *Tg(1016tuba1a:GFP)* transgenic line was generated to identify injury-induced dedifferentiation of Müller glia. Mechanical retinal damage was induced by a needle-poke injury on the back of the eyes in adult zebrafish. Phosphate-buffered saline or GCV was injected into the vitreous of the eye at the time of injury or through the cornea. The GCV clearance rate from the eye was determined by a reversed-phase HPLC method. Green fluorescent protein (GFP) and bromodeoxyuridine (BrdU) immunofluorescence were used to determine the effect of GCV on retinal regeneration. Cell apoptosis was evaluated by TUNEL staining. Microglia were labeled by vitreous injection of isolectin IB4 conjugates. Quantitative (q)PCR and Western blot analysis were used to determine gene expression in the retina.

RESULTS. Ganciclovir treatment significantly reduced the number of BrdU+ Müller glia–derived progenitor cells (MGPCs) at 4 days post injury. Further analysis showed that GCV had no impact on Müller glia dedifferentiation and the initial formation of MGPCs. Our data indicate that GCV irreversibly inhibited MGPC proliferation likely through a p53-$p21^{cip1}$–dependent pathway. Interestingly, unlike control cells, GCV-treated Müller glia cells were "locked" in a prolonged dedifferentiated state.

CONCLUSIONS. Our study uncovered a novel inhibitory effect of GCV on MGPC proliferation and suggests its potential use as a tool to uncover molecular mechanisms underlying retinal regeneration in zebrafish.

Keywords: ganciclovir, zebrafish, retinal regeneration, Müller glia, cell proliferation

Retinal degenerations following retinal disease or traumatic injury are the leading causes of permanent blindness in mammals.[1] As in most other areas of the central nervous system (CNS), mammalian retinal neurons are not replaced following retinal degeneration.[1,2] In contrast, teleost fish such as zebrafish exhibit a robust regenerative response after retinal injury that repairs the damaged retina and restores vision.[3] Retinal regeneration in zebrafish depends on a type of glia cells—the Müller glia.[4] After retinal injury, resident Müller glia quickly dedifferentiate and proliferate into a cycling population of neurogenic precursor cells that eventually regenerate new neurons and glia.[4–7] Mechanisms underlying Müller glia dedifferentiation and proliferation in zebrafish could therefore be used to design novel therapeutic strategies for improving retinal regeneration in mammals.

Key to successful retinal regeneration is a robust proliferation of Müller glia–derived progenitor cells (MGPCs).[3,8] Tight control over cell proliferation is critical for retina regeneration, as insufficient proliferation will result in incomplete retinal repair, whereas excess proliferation may result in tumor formation. In a mechanical retinal injury model in zebrafish, Müller glia began to divide to produce the first group of MGPCs at 2 days post injury (2 dpi).[4] These MGPCs continue to divide rapidly, and the peak of proliferation in the retina can be observed at 4 dpi before declining and returning to near basal level at 7 dpi.[4] The precise mechanisms responsible for regulating MGPC proliferation in zebrafish are still not completely understood. It has been shown that many genes and signaling pathways are required for Müller glia dedifferentiation and MGPC proliferation in zebrafish, including Hbegf-Mapk, Wnt-β-catenin, Jak-Stat, Fgf/Fgfr, TGFβ, TNFα, insulin, IGF-1, Ascl1a, Lin-28, and Insm1a.[3,9–17] However, whether these genes and pathways are required for both dedifferentiation and proliferation remains unclear. One exception is the transcriptional factors Pax6a and Pax6b, which are reported to be required only for MGPC proliferation.[18]

Ganciclovir (GCV), a synthetic analogue of 2′-deoxyguanosine, was developed in the 1970s as an antiviral drug and is currently used to treat cytomegalovirus (CMV) and herpes simplex virus (HSV) infections.[19–21] Ganciclovir itself is a nontoxic prodrug, which can be phosphorylated into GCV monophosphate by thymidine kinase (TK) from the Herpesviridae family, including CMV, HSV, or Epstein-Barr virus.[22,23] Ganciclovir monophosphate is further converted by normal cellular enzymes into GCV triphosphate, which is a toxic compound that inhibits viral DNA polymerase and DNA chain elongation.[22,23] Besides its canonical antiviral activity, GCV has also been used in research studies to ablate cells genetically modified to express HSV-TK.[24–26] Recently it was reported that GCV itself is a potent inhibitor of microglia activation and proliferation in a mouse model of CNS inflammation, though its underlying mechanism was unclear.[27]

We previously found that intravitreous administration of GCV inhibited retinal regeneration in zebrafish (Xu H, Goldman D, unpublished, 2012). Here we report an unexpected finding that GCV irreversibly inhibited the expansion of MGPCs, but did not inhibit Müller glia dedifferentiation and cell division. Furthermore, we show that GCV treatment resulted in elevated expression of p53 and p21^{cip1}, which may underlie the cell cycle arrest of MGPCs. Overall, our study revealed a novel inhibitory effect of the classic antiviral drug GCV on retinal regeneration in adult zebrafish. Our study also suggests GCV as a tool to uncover the mechanisms controlling the transition of dedifferentiation to rapid progenitor proliferation during retinal regeneration.

MATERIALS AND METHODS

Animals and Eye Injury

The animals used in this study were treated in accordance with the guidelines for animal use and care at Nantong University, as well as the Guide for the Care and Use of Laboratory Animals. Animal treatment was in adherence to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Zebrafish were kept at 28.5°C in a 14-hour/10-hour light/dark cycle. Retinal injuries were performed as described previously.[4] Briefly, fish were anesthetized and the right retina was poked four times, once in each quadrant, with a 30-gauge needle. The needle was inserted through the sclera to the length of the bevel (~0.5 mm).

Generation of the *Tg(1016tuba1a:GFP)* Transgenic Lines

The plasmid for making the *Tg(1016tuba1a:GFP)* transgenic line was generated using the MultiSite Gateway cloning system (Life Technologies, Carlsbad, CA, USA). A 1016-bp goldfish *tuba1a* regulatory element[4] was subcloned into the *p5E-MCS* vector to generate the *p5E-tuba1a* 5′ entry vector. The *p5E-tuba1a* plasmid and the middle-entry plasmid (containing the coding sequence of green fluorescent protein [GFP]) were then cloned into a destination vector (pDestTol2pA2) using the Tol2-based Gateway system. This transgene plasmid DNA (30 pg) and transposase RNA (20 pg) were coinjected into 1-cell stage zebrafish embryos. Injected embryos with GFP expression were selected and raised, and stable transgenic lines with retinal GFP expression at the injury site were generated and validated.

Drug Delivery, Microglia Labeling, and BrdU Incorporation

Ganciclovir sodium (Santa Cruz Biotechnology, Dallas, TX, USA) was dissolved in PBS at indicated concentrations; 1 μL PBS or GCV was then delivered at the time of injury using the same needle to poke the retina or was injected intravitreally at the indicated time. Intravitreous injection was performed through the front of the eye with a 30-gauge beveled needle attached to a Hamilton syringe (Hamilton Robotics, Ren, NV, USA), and care was taken not to damage the retina or the lens. To label microglia, 1 μL 1 mg/mL isolectin GS-IB4 (isolectin GS-IB4 from *Griffonia simplicifolia*, Alexa Fluor 568 conjugate; Thermo Fisher Scientific, Waltham, MA, USA) was injected intravitreally through the front of the eye 1 day before the fish were killed. For bromodeoxyuridine (BrdU) incorporation, 20 μL 20 mM BrdU solution was injected intraperitoneally into the anesthetized fish.

HPLC Detection of the GCV Levels in the Eye

To determine the clearance rate of injected GCV in the eye, an extraction and reversed-phase high-performance liquid chromatography (HPLC) method was used as previously described.[28] Briefly, eyes were homogenized in 100 μL PBS after PBS or GCV injection, and 100 μL 50% trichloroacetic acid was then added to the homogenate. After shaking for 30 seconds, the deproteinized samples were centrifuged at 2000*g* for 10 minutes. The supernatant was transferred to a new tube and neutralized with 50 μL 2 M NaOH. The tube was vortexed for 10 seconds and then extraction was performed with 5 mL chloroform. Aliquots of the aqueous phase (400 μL) were mixed with 40 μL 1 M NaH_2PO_4 and 0.4 M triethylamine solution, and 30 μL per sample was used for HPLC analysis. High-performance liquid chromatography analyses were performed on a Waters 2695 HPLC system (Milford, MA, USA) equipped with photodiode array detector, auto-sampler, a quaternary pump, online degasser, and column oven. Separation was performed on a Waters Symmetry300 C^{18} column (5.0 μm, 4.6 × 250 mm) maintained at 25 ± 2°C at a flow rate of 1 mL/min and a 10-μL sample injection.The detector wavelength was set at 254 nm. The eluent consisted of 95% (vol/vol) water, and 5% (vol/vol) methanol was used in the isocratic elution program.

RT-PCR and Quantitative PCR

Retinas were dissected and total RNA was extracted using the TRIzol reagent (Invitrogen). RNA (1 μg) was reverse transcribed into cDNA by the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Upper Bavaria, Germany) according to the manufacturer's instructions. Primers for quantitative PCR (qPCR) are listed in the Table. Quantitative PCR was carried out in triplicate using the FastStart Universal SYBR Green Master Mix (Roche Applied Science) on a real-time PCR detection system (CFX96TMReal-Time System; Bio-Rad, Hercules, CA, USA).

Tissue Preparation and Immunofluorescence

Fish were overdosed with tricaine. The eyes were dissected and fixed in 4% paraformaldehyde at 4°C overnight. Fixed samples were prepared for immunofluorescence as previously described.[10] Primary antibodies used for immunofluorescence included rabbit anti-GFP (1:1000, Life Technologies), rat anti-BrdU (1:500; Abcam, Cambridge, MA, USA), and rabbit anti-

TABLE. PCR Primers Used in the Study

Gene Name	Primer Sequence, 5′–3′
RT-ccna2-F	GCGTGCTCCAAGAAAGCACCTTTA
RT-ccna2-R	TTTCCCGCAAATGCGTGTG
RT-ccnb1-F	TGTGATGCAGCATATTGCCAAA
RT-ccnb1-R	GGCAGTGAAGAAATCCGTAAAATAAA
RT-ccnd1-F	CTGGACAGGTTTTTATCTGTGGAGCC
RT-ccnd1-R	GCTTGGAGCTCTGATGTATAGGCAGT
RT-ccne1-F	CACGTTAAGGCTCTCGACATTCAAG
RT-ccne1-R	GCATGGGCTTGTGTAACCTGTGT
RT-cdk1-F	CTGGCAGATTTCGGCTTAGCCCGTGC
RT-cdk1-R	CTTATAGTCTGGCAGAGACTCAACATCTGGC
RT-cdk2-F	CTTAAACCCCAGAATCTCCTCATCAA
RT-cdk2-R	AAACAGTGCCCTCCGAGTAATCAT
RT-p21-F	CCCGCATGAAGTGGAGAAAACC
RT-p21-R	CGGTGTCGTCTCTGGTTCCTGA
RT-p27-F	CGGGAATCACGACTGTAGGGTAAC
RT-p27-R	TGGGCGTTCGGGTCACTTC
RT-p57-F	CTTCAGTCCTCAGAAACAGACGGAAG
RT-p57-R	CATCCGCTCTGCAGATAAACACAGGTG
RT-tp53-F	ACGACCTGAGGGGAGCAAAAAG
RT-tp53-R	TCCCATCACCTTAATCAGAGTCGC
RT-ascl1a-F	GGCGTCCTGTCACCCACCAT
RT-ascl1a-R	ACGCAGTGCTTTGTGTTCTTGGA
RT-hbegfa-F	ATGTCTGACCATCATTGGCCTCC
RT-hbegfa-R	ACCATTCAGCTTGCTGTGCCC
RT-socs3a-F	CAGGGAAGACAAGAGCCGAGAC
RT-socs3a-R	GTCTTGAAGTGGTAAAACGGCAGC
RT-pax6a-F	TGGCTAGCGAAAAGCAACAGATG
RT-pax6a-R	TCGCCATTGGAGCTTATTGAGTTT
RT-pax6b-F	CCCAATGCCCAGCTTCACTATG
RT-pax6b-R	GGACTTGGACAGGGACAGACACTC

PCNA (1: 500; GeneTex, Irvine, CA, USA). For BrdU staining, sections were first treated with 2 N HCl at 37°C for 25 minutes, rinsed in 0.1 M sodium borate (pH 8.5) for 10 minutes, and then processed using standard procedures.

Quantification of the Number of BrdU+ Cells at the Injury Site

Green fluorescent protein and BrdU immunofluorescence were performed on retinal cryosections, and each injury-responsive zone (4 injuries per retina in total) could be clearly distinguished from the others with the help of GFP and BrdU fluorescence. The counting area for each injury site was approximately 400 to 600 μm in width, determined by the actual size of the injury-responsive zone. The number of BrdU+ cells in the inner nuclear layer (INL) per injury was counted by using the Cell Counter plugin for ImageJ software (Plugin/Analyze/Cell Counter [http://rsb.info.nih.gov/ij/plugins/cell-counter.html]).

Immunoblot Analysis

Immunoblot analysis was carried out as described previously.[15] Membranes were incubated with antibodies against zebrafish P53 (1: 500; GeneTex) and β-actin (1:1000; Sigma-Aldrich Corp., St. Louis, MO, USA).

TUNEL Assay

To detect apoptotic cells, the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science) was used according to the manufacturer's protocol. Eyes treated with the neurotoxin ouabain were used as a positive control.

Microscopy and Statistical Analysis

Slides were examined and imaged with a Leica DM4000B upright fluorescent microscope (Wetzlar, Germany) or a Leica SP5 confocal imaging system. For multiple comparisons, a 1-way analysis of variance (ANOVA) followed by Tukey test was used. For single comparison, a 2-tailed unpaired Student's *t*-test was used.

RESULTS

GCV Treatment Significantly Decreased the Number of Proliferating MGPCs After Retinal Injury

To evaluate the effects of GCV on zebrafish retinal regeneration, GCV (12–50 μg/eye) or PBS was injected once daily into the vitreous of the eye after retinal injury. Because almost all of the BrdU-labeled cells in the INL after retinal injury are derived from Müller glia,[4] BrdU-positive cells in the INL represent injury-induced MGPCs. Incorporation of BrdU could thus be used as an excellent indicator of retinal regeneration. It has been shown that the proliferation of MGPCs reaches a peak at 4 dpi in this mechanical injury model.[4] We therefore gave the fish a pulse of BrdU 3 hours before they were killed at 4 dpi, and examined the status of regeneration on retinal cryosections. Immunofluorescence showed that GCV treatment significantly reduced the number of BrdU+ cells in the INL after retinal injury in a dose-dependent manner (Figs. 1A, 1B). In the control retina at 4 dpi, clusters of BrdU+ nuclei with an elongated morphology were clearly visible in the INL near the site of injury (Fig. 1A). In contrast, many fewer BrdU+ cells could be seen in the GCV-injected retina (Fig. 1A). Importantly, the number of TUNEL+ cells at the injury site after GCV treatment was comparable to that of the PBS-treated control, suggesting that GCV did not stimulate cell apoptosis at the site of injury (Fig. 1). These results indicate that GCV significantly decreased the number of proliferating MGPCs and therefore inhibited retinal regeneration in adult zebrafish.

GCV Had No Effect on Müller Glia Dedifferentiation

The number of proliferating MGPCs at the injury site observed at 4 dpi could be affected by several cellular events during retinal regeneration, including the activation and dedifferentiation of Müller glia, their asymmetric cell division, and the formation of MGPCs, as well as their later expansion. To investigate whether the observed effect of GCV is due to inhibition of Müller glia activation and dedifferentiation, we generated a transgenic line *Tg(1016tuba1a:GFP)* with the same promoter as described previously to identify these dedifferentiated Müller glia.[4] In this line, a fragment of the α1 tubulin promoter drove GFP expression specifically in dedifferentiated Müller glia and proliferating MGPCs after retinal injury.[4] Consistent with the previous report,[4] characterization of our generated *Tg(1016tuba1a:GFP)* fish showed injury-induced GFP expression exclusively in Müller glia and MGPCs at the injury site (Supplementary Figs. S1C, S1D, Fig. 1A), indicating this line as a valuable tool for analyzing Müller glia dedifferentiation and retinal regeneration.

In the injured retina of the *Tg(1016tuba1a:GFP)* fish, each dedifferentiated Müller glia and its daughter MGPCs displayed a green column-like shape and are referred as a GFP+ column.[29]

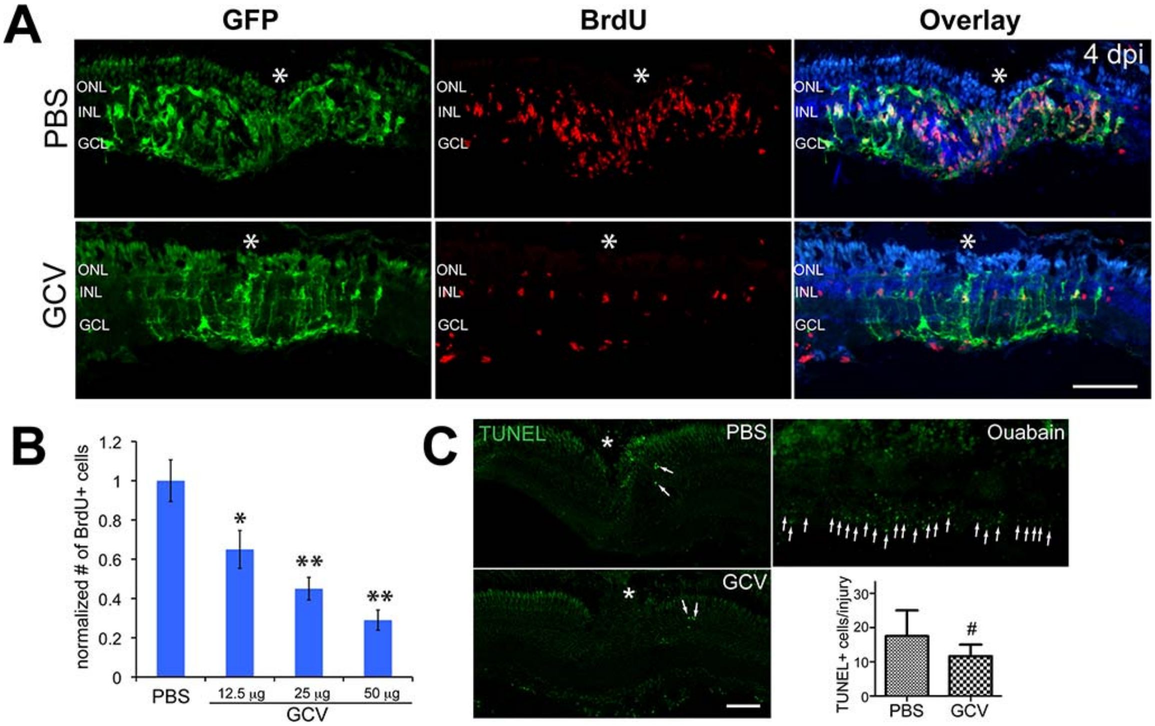

Figure 1. Ganciclovir treatment significantly reduced the number of MGPCs after retinal injury. (**A**) Green fluorescent protein (*green*) and BrdU (*red*) immunofluorescence shows that GCV treatment decreased the number of BrdU+ MGPCs localized to the injury site at 4 dpi. DAPI (4′,6-diamidino-2-phenylindole) channel (*blue*) was added to the overlay images to show retinal layer structures. *Tg(1016tuba1a:GFP)* fish received a pulse of BrdU 3 hours before they were killed at 4 dpi. (**B**) Quantification of BrdU+ MGPCs in (**A**). $^*P < 0.05$; $^{**}P < 0.01$ compared to PBS control, $n = 4$. (**C**) TUNEL staining and quantification of TUNEL+ cell numbers from PBS-, GCV-, or ouabain-treated retina at 3 dpi. *Arrows* mark TUNEL+ cells. #No significant difference, $P > 0.05$, $n = 4$. *Scale bars*: 100 µm. The *asterisks* mark the injury site (needle poke). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; dpi, days post injury; MGPCs, Müller glia–derived progenitor cells.

The GFP transgene expression and the number of GFP+ columns per injury are thus good indicators of Müller glia activation and dedifferentiation. At 4 dpi, confocal microscopy showed that although GCV-treated retina exhibited many fewer BrdU+ MGPCs than PBS-treated control, GFP+ Müller glia identified by their radial morphology were still present at the site of injury (Fig. 2A). Importantly, the number of dedifferentiated Müller glia at the injury sites in GCV-injected eyes determined by the number of GFP+ columns was comparable to that of control (Figs. 2A, 2B; arrows indicate GFP+ columns). We also examined these Müller glia cells at 2 dpi, when they were dedifferentiated and began to go through the first division.[4] Immunofluorescence showed the presence of GFP+ Müller glia at the site of injury in both control and GCV-injected retina (Fig. 2C). Together, these data indicate that GCV treatment had no effect on Müller glia activation and dedifferentiation.

GCV Inhibited the Proliferation of MGPCs

The above results suggest that GCV may inhibit retinal regeneration at a later stage, such as the division of Müller glia that generates the first group of MGPCs, or subsequent MGPC expansion. We therefore first investigated whether GCV inhibited the initial formation of MGPCs by examining the number of BrdU+ cells in the INL at 2 dpi when MPGC formation was just beginning.[4] Surprisingly, GCV treatment had no effect on the number of BrdU+ cells in the INL at 2 dpi (Figs. 2C, 2D), suggesting that GCV did not inhibit Müller glia division and the initial MGPC formation.

We next investigated the effect of GCV on MGPC proliferation. For this purpose, we labeled a population of proliferating MGPCs with a pulse of BrdU at 45 to 48 hours post injury (hpi) in wild-type zebrafish. Fish then received intravitreous PBS or GCV injection once daily until 4 dpi. To trace the progeny of these labeled MGPCs, retinas were collected and examined at 2, 3, and 4 dpi (Fig. 3A). In the control retina, a rapid expansion of the BrdU-labeled MGPCs could be observed from 2 to 4 dpi (Figs. 3B, 3C). In contrast, the expansion of MGPCs in the GCV-treated group occurred at a significantly lower rate (Figs. 3B, 3C). These data indicate that GCV blocked retinal regeneration by inhibiting MGPC proliferation.

The Inhibitory Effect of GCV on MGPC Proliferation Was Irreversible

As we observed a strong inhibitory effect of GCV on MGPC proliferation at 4 dpi, we asked whether MGPCs could recover from this inhibition when GCV was removed from the eye. To determine the clearance rate of GCV from the eye of adult zebrafish, 1 µL 50 µg/µL GCV was injected into the vitreous. At 0, 1, and 2 hours after the injection, injected eyes were collected and homogenized in PBS. Ganciclovir was then extracted and its concentration was determined by HPLC as described previously.[28] Our results showed that GCV was rapidly removed from the eye after vitreous injection (Supplementary Figs. S2A–D), and its half-life in the eye is between 1 and 2 hours (Supplementary Fig. S2E).

We next performed a washout experiment to determine if MGPCs could recover from GCV treatment. Fish received intravitreous GCV or PBS injection once daily for 4 days, and then received PBS injection once daily to further wash out GCV for another 4 days (Fig. 4A). Because the half-life of GCV in the

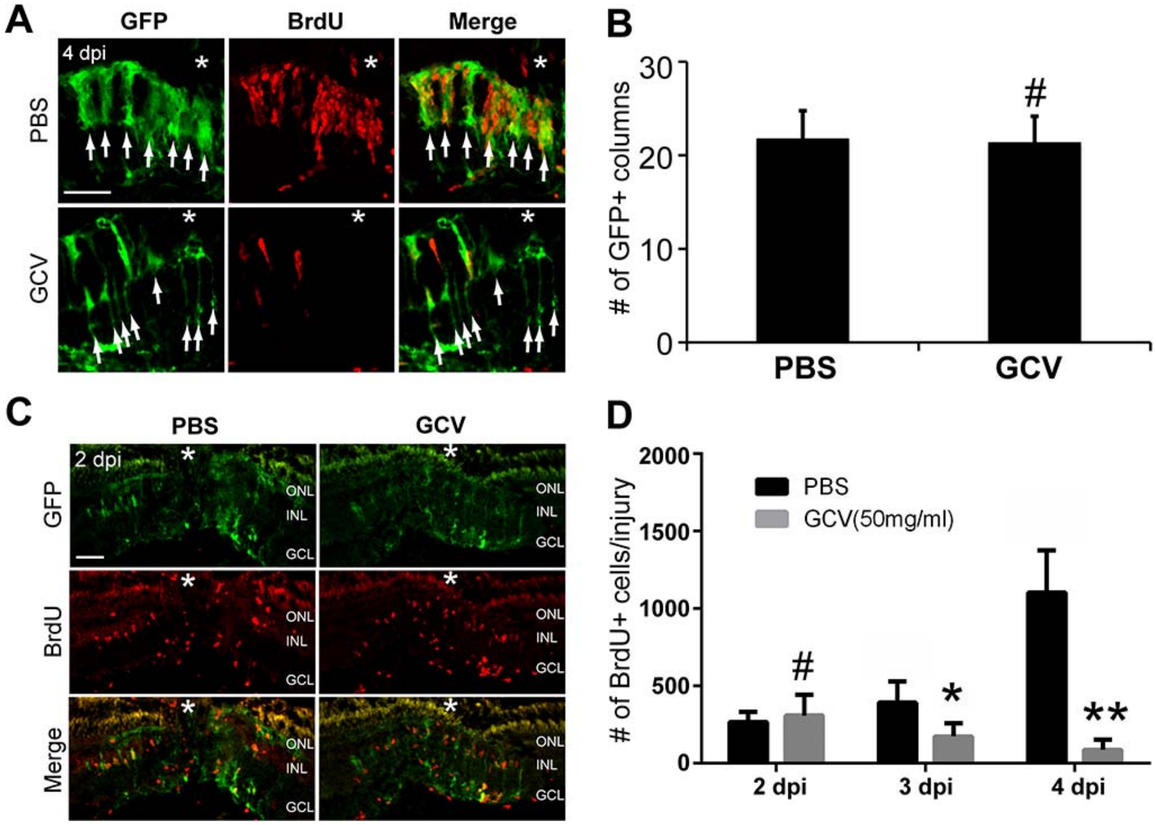

Figure 2. Ganciclovir had no effect on Müller glia dedifferentiation and the initial MGPC formation. (**A**) Confocal microscopy of retinal sections after GFP and BrdU immunofluorescence shows the presence of GFP+ Müller glia near injury site in PBS- or GCV-treated retina at 4 dpi. *Tg(1016tuba1a:GFP)* fish were given a pulse of BrdU 3 hours before they were killed at 4 dpi. Note that there are many fewer BrdU+ MGPCs in GCV-treated retina than in control. *Arrows* indicate GFP+ columns. Each GFP+ column represents an activated Müller glia and its daughter progenitor cells. (**B**) Quantification of the number of GFP+ columns at the injury site at 4 dpi. #No significant difference, $P > 0.05$, $n = 4$. (**C**) Immunofluorescence shows the initial formation of MGPCs in the INL in PBS- or GCV-treated retina at 2 dpi. *Tg(1016tuba1a:GFP)* fish received a pulse of BrdU 3 hours before they were killed at 2 dpi. (**D**) Quantification of the number of BrdU+ MGPCs per injury at 2, 3, and 4 dpi in PBS- or GCV-treated retina. Fish received a pulse of BrdU 3 hours prior to killing at 2, 3, or 4 dpi. #No significant difference, $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$. $n = 4$ for each group. *Scale bars*: 50 μm. The *asterisks* mark the injury site (needle poke).

eye is 1 to 2 hours, the 4-day washout experiment is sufficient to eliminate the drug from the eye and also provides enough time for MGPCs to recover. The number of proliferating MGPCs was determined by BrdU incorporation at 4, 6, and 8 dpi. Immunofluorescence of BrdU showed that the number of BrdU-positive cells in the INL at 6 and 8 dpi was not significantly different from that at 4 dpi, suggesting that the inhibitory effect of GCV on MGPC proliferation was irreversible (Fig. 4A). Indeed, a single intravitreous GCV injection at 1 or 2 dpi was sufficient to inhibit retinal regeneration, determined by the number of BrdU+ cells in the INL at 4 dpi (Fig. 4B). Together, these data indicate that the impact of GCV on MGPC proliferation was strong and irreversible.

Microglia Activation and Accumulation at the Injury Site Were Not Affected by GCV

Previous studies have shown that after retinal injury, phagocytic microglia accumulate at the site of injury in adult zebrafish.[7,15] Interestingly, a recent study reported that GCV itself is a potent inhibitor of microglia proliferation and activation in a mouse model of CNS neuroinflammation, although the underlying mechanism remains unknown.[27] As microglia accumulation and neuroinflammation may play a role in retinal regeneration in zebrafish, we investigated the effect of GCV on microglia accumulation and proliferation after retinal injury at 2 to 3 dpi. Consistent with the previous study,[15] no proliferating microglia were observed in the retina (Figs. 5A–D). Importantly, the number of microglia at the injury site in GCV-treated retina was comparable to that of control (Figs. 5A–E), suggesting that the inhibitory effect of GCV on retinal regeneration was independent of microglia.

Induction of p53 and $p21^{cip1}$ by GCV

The inhibitory effect of GCV on MGPC proliferation prompted us to examine the expression of cell cycle–related genes after GCV injection. Quantitative PCR analysis showed that GCV significantly decreased the expression of cyclin A2, cyclin B1, and cyclin D1 but had no effect on cyclin E1 at 3 dpi (Fig. 6A). Ganciclovir also significantly decreased the expression of Cdk1 and Cdk2 (Fig. 6A). Strikingly, high induction of a Cdk-dependent inhibitor $p21^{cip1}$ (gene *cdkn1a*, Fig. 6B) was found after GCV treatment at 3 dpi. Ganciclovir had no effect on the mRNA level of $p27^{kip1}$ and $p57^{kip2}$ (Supplementary Fig. S3A). Since it is well known that $p21^{cip1}$ can be directly regulated by the tumor suppressor p53,[30,31] we next examined the expression of p53 by qPCR and Western blot analysis. Indeed, GCV induced a small but significant increase of p53 at both the mRNA and protein level at 3 dpi (Figs. 6C, 6D). We also examined the expression of cell cycle genes at 2 dpi, when

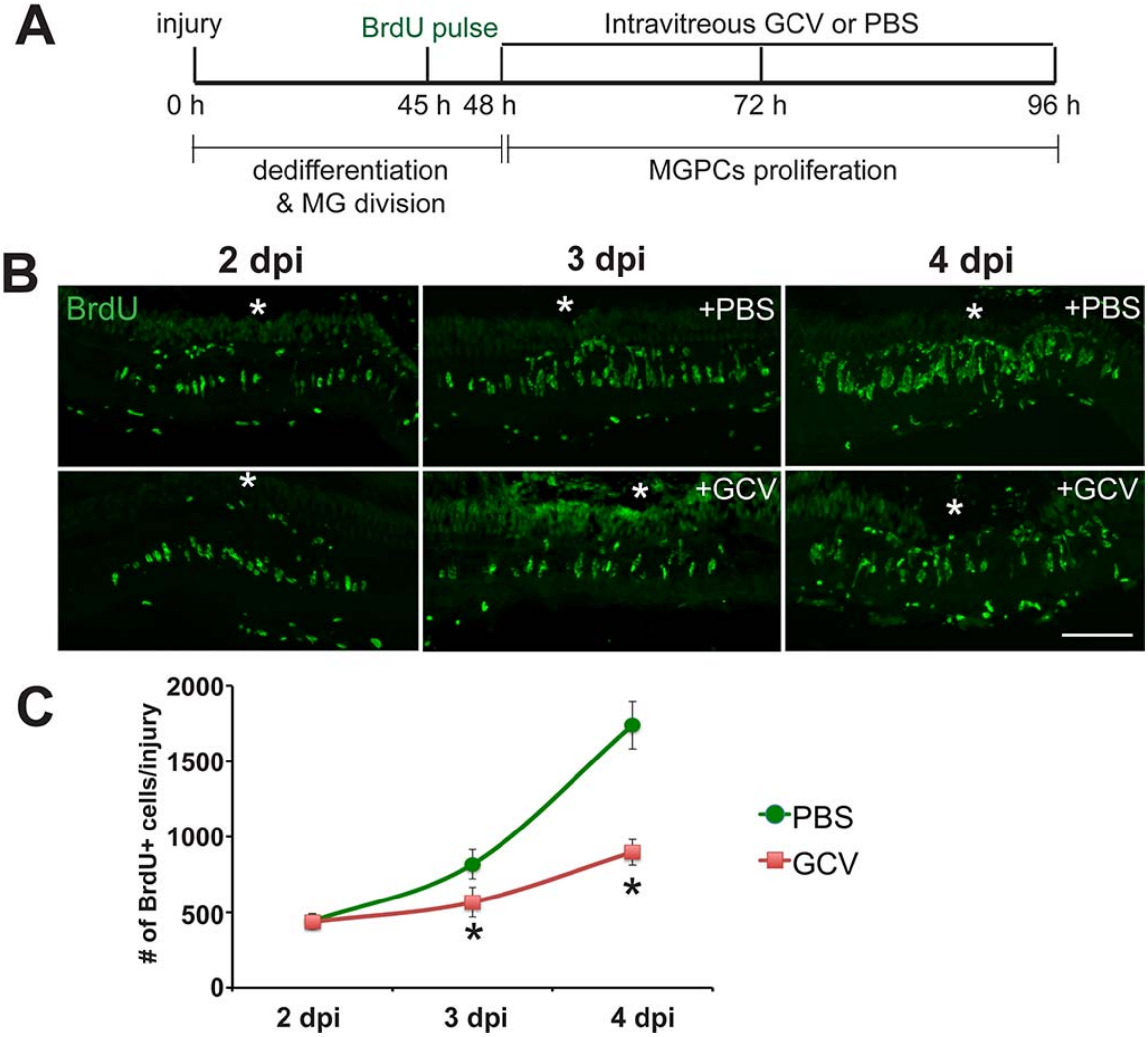

Figure 3. Ganciclovir inhibits MGPC proliferation. (**A**) Schematic illustration showing the experimental timeline. Retinas of wild-type zebrafish were injured, and all fish received a pulse of BrdU at 45 hpi. Fish then received intravitreous PBS or GCV injection once daily until 4 dpi. Retinas were collected at indicated time points (48, 72, and 96 hpi). (**B**) Bromodeoxyuridine immunofluorescence showing that GCV inhibits the expansion of MGPCs in the retina. Bromodeoxyuridine signals are shown in *green*. (C) Quantification of (**B**). $^*P < 0.05$ compared to PBS-treated control. $n = 3$ for each group. *Scale bars*: 100 µm. The *asterisks* mark the injury site (needle poke).

GCV has not yet had any observable effect on MGPC proliferation. At this time point, we observed a significant induction of p21^{cip1} and p53 (Figs. 6B, 6C), whereas the expression of most other cell cycle–related genes was comparable to that of control except for cyclin A2 (Supplementary Fig. S3B). Therefore, induction of p53 and p21^{cip1} was an early event after GCV treatment. Since p21^{cip1} is a well-known cell cycle inhibitor, our data are consistent with the idea that the induction of p53 and p21^{cip1} by GCV caused a cell cycle arrest and thus inhibited MGPC proliferation.

It has been shown that the transcription factors Pax6a and Pax6b are required only for MGPC proliferation, and the impact of GCV on MGPC proliferation is similar to that of Pax6a/6b knockdown.[18] We therefore examined the expression level of Pax6a/6b mRNA in PBS- and GCV-treated retina at 2 and 3 dpi. Quantitative PCR analysis showed that the expression of Pax6a and Pax6b in GCV-treated retina was comparable to that of control at 2 dpi (Supplementary Fig. S3C). A small but significant decrease of Pax6b expression in GCV-treated retina was found at 3 dpi, whereas no significant difference in Pax6a expression was observed at this time point (Supplementary Fig. S3C). Since Pax6b was reported to specifically regulate the first cell division of MGPCs,[18] it is possible that in addition to the induction of P53 and P21^{cip1}, GCV may also downregulate Pax6b expression to inhibit MGPC proliferation.

Postmitotic Müller Glia Remained in a Dedifferentiated State After GCV Treatment

After the proliferation of MGPCs reaches its highest level at 4 dpi, dedifferentiated Müller glia downregulate the expression of regeneration-associated genes (RAGs) and gradually return to their preinjury status after 7 dpi.[4,10] However, the exact mechanism regulating this transition remains unknown. Examining PBS- or GCV-treated retina at 8 and 12 dpi, we had an unexpected finding—that in contrast to control Müller glia, which had almost completely turned off GFP transgene expression, Müller glia cells in GCV-treated retina still exhibited strong GFP fluorescence at these late time points (Fig. 7A). Immunofluorescence of BrdU showed that these GFP+ Müller glia were not proliferating (Fig. 7A). Because the GFP transgene is a marker for dedifferentiated Müller glia, this

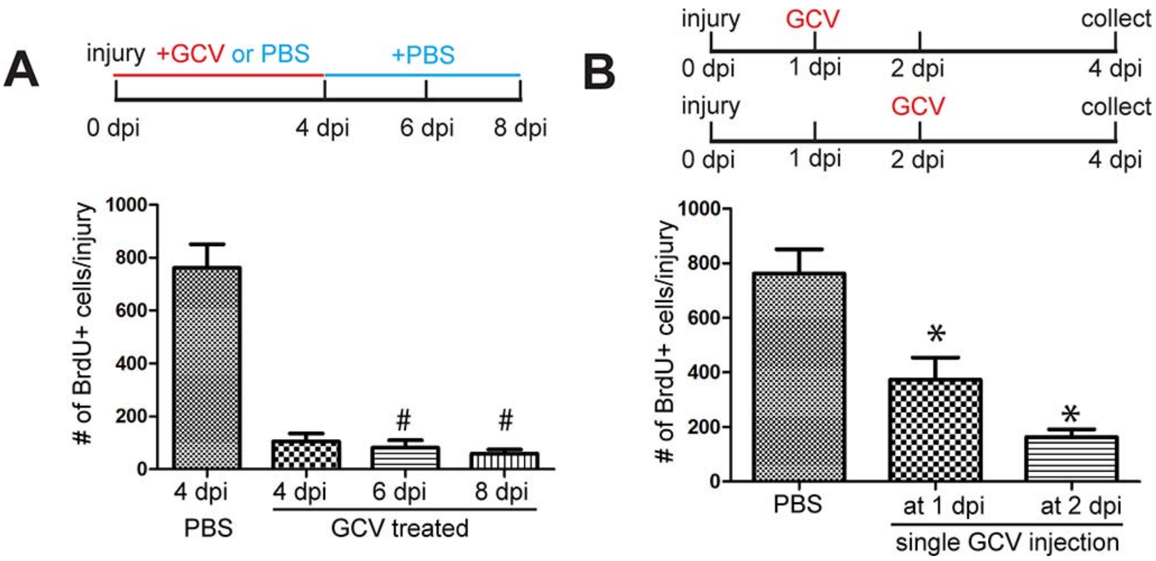

Figure 4. Irreversible effect of GCV on MGPC proliferation. (**A**) Experimental timeline and quantification of the number of BrdU+ MGPCs at indicated time points after recovery. Fish received intravitreous GCV or PBS injection once daily from 0 to 4 dpi, and then received PBS injection once daily from 4 to 8 dpi. A pulse of BrdU was given 3 hours prior to killing at 4, 6, and 8 dpi. (**B**) A single GCV injection is sufficient to inhibit MGPC proliferation at 4 dpi. Fish received a single intravitreous PBS or GCV injection at 1 or 2 dpi. Bromodeoxyuridine was given 3 hours prior to killing at 4 dpi. The quantification shows the number of BrdU+ MGPCs per injury at 4 dpi. #$P > 0.05$ compared to GCV-treated group at 4 dpi; *$P < 0.05$ compared to PBS control. $n = 3$ for each group.

suggests that Müller glia in GCV-treated retina at 8 and 12 dpi were still dedifferentiated. Consistent with this notion, qPCR analysis showed that the expression of *gfp* and several RAGs in GCV-treated retina was significantly higher than that of PBS-treated control at 12 dpi (Fig. 7B). Importantly, Müller glia in GCV-treated retina maintained a relatively stable and high level of expression of *gfp* and several RAGs from 4 to 12 dpi (Fig. 7C). These data indicate that instead of returning to a preinjury status, Müller glia in GCV-treated retina were "locked" in a prolonged dedifferentiated state.

DISCUSSION

Ganciclovir, an antiviral nucleoside analogue, was traditionally used in therapies against CMV and HSV infections. In this study, we investigated the novel effect of GCV on retinal regeneration

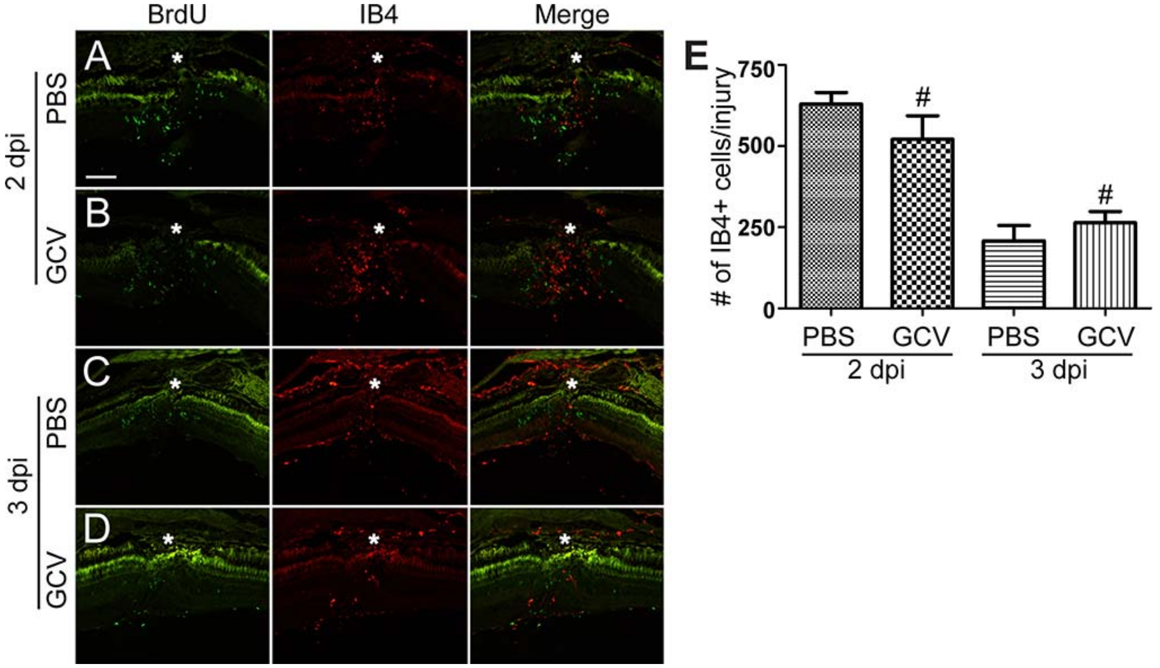

Figure 5. Ganciclovir treatment had no effect on microglia accumulation at the injury site. (**A–D**) Immunofluorescence shows the BrdU+ MGPCs (*green*) and IB4+ microglia (*red*) at the injury site at 2 and 3 dpi. Note that all the IB4+ microglia were BrdU negative. (**E**) Quantification of the number of microglia at the injury site at 2 and 3 dpi. The *asterisks* mark the injury site (needle poke). #$P > 0.05$ compared to PBS-treated control. $n = 3$ for each group. *Scale bars*: 100 μm.

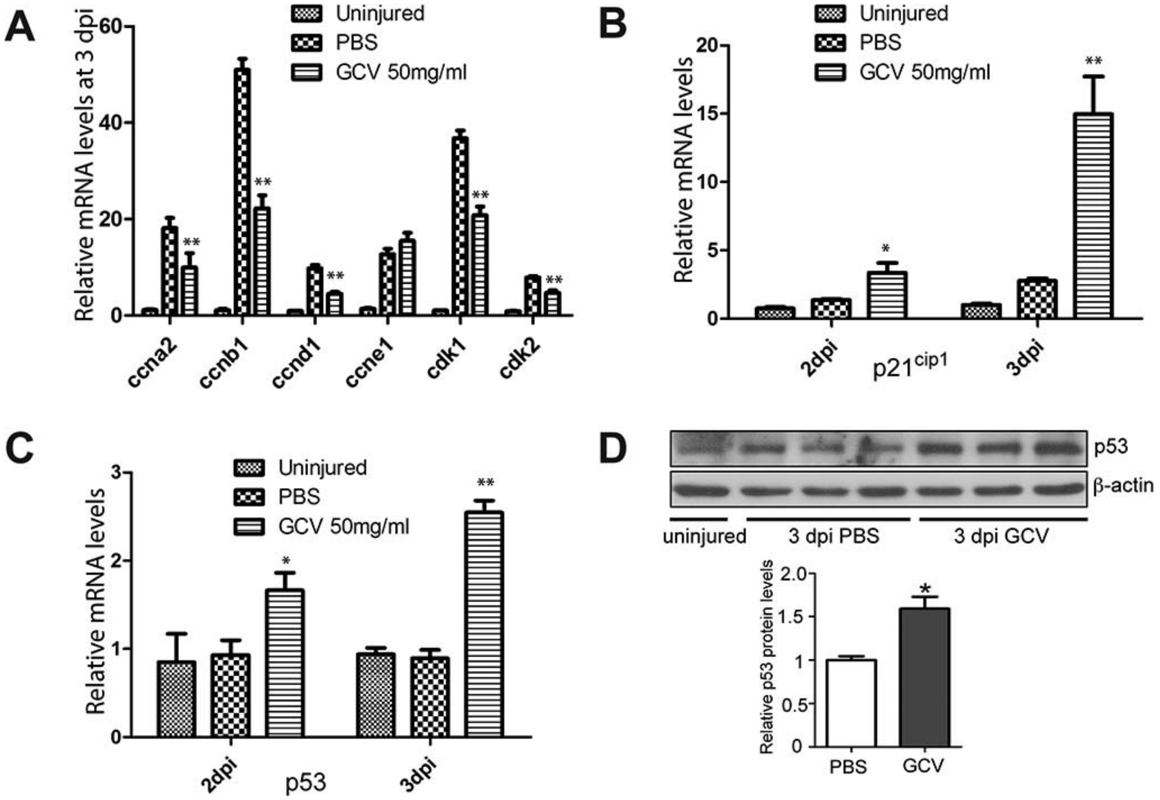

Figure 6. Induction of p53 and p21^{cip1} after GCV administration. (**A**) qPCR shows that GCV treatment inhibits the expression of *ccna2*, *ccnb1*, *ccnd1*, *cdk1*, and *cdk2* significantly at 3 dpi. (**B**) Ganciclovir treatment increases the expression of p21^{cip1} at 2 and 3 dpi significantly. (**C**) Ganciclovir significantly increases the expression level of p53 mRNA at 2 and 3 dpi. (**D**) Western blot analysis shows elevated expression level of the p53 protein in GCV-treated retina compared to that of control at 3 dpi. $n = 3$ for each group. $^{*}P < 0.05$ compared to PBS control; $^{**}P < 0.01$ compared to PBS control.

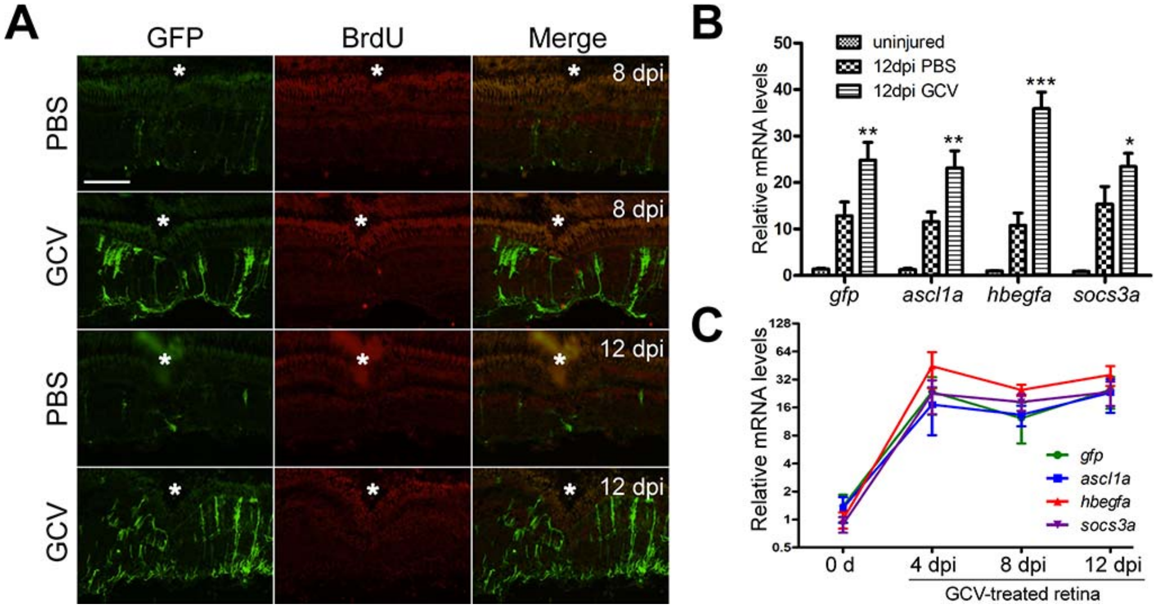

Figure 7. Müller glia remained in a dedifferentiated state after GCV treatment. (**A**) Green fluorescent protein and BrdU immunofluorescence showing the presence of strong GFP+ Müller glia at the injury site in GCV-treated retina at 8 and 12 dpi. Only very weakly GFP-expressing cells were found in the PBS-treated control. *Tg(1016tuba1a:GFP)* fish were given a pulse of BrdU 3 hours before they were killed at 8 or 12 dpi. (**B**) qPCR analysis of the expression of *gfp*, *ascl1a*, *hbegfa*, and *socs3a* in PBS- or GCV-treated retinas at 12 dpi. (**C**) qPCR analysis of the same genes in GCV-treated retinas at 0, 4, 8, and 12 dpi. $n = 4$ for each group. $^{**}P < 0.01$; $^{***}P < 0.001$ compared to PBS control. *Scale bars*: 100 µm. The *asterisks* mark the injury site (needle poke).

in zebrafish. We obtained the unexpected finding that GCV itself is a potent inhibitor of MGPC proliferation. Interestingly, GCV did not appear to inhibit early regeneration events, such as Müller glia dedifferentiation and the formation of MGPCs. Instead, it inhibited MGPC proliferation, likely by causing cell cycle arrest through a p53-p21^{cip1} pathway. In addition, we found that Müller glia remained in a prolonged dedifferentiated state after GCV treatment.

Cell cycle control is important for normal growth and development, and deregulation of cell cycle often leads to growth defects and carcinogenesis.[32,33] The close cooperation between cyclins and cyclin-dependent kinases (CDKs) is necessary for cell cycle progression through different phases. Most cyclins promote CDK activity, whereas CDK inhibitors (CKIs) restrain CDK activity.[34] Cyclin-dependent kinase inhibitors are negative regulators of cell cycle progression, and are divided into the CIP/KIP (p21^{cip1}, p27^{kip1}, and p57^{kip2}) and the INK4 subfamily (p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, p19^{INK4d}).[35] Despite their crucial role in cell proliferation and differentiation, few studies have explored the role of cyclins/CDKs/CKIs in retinal regeneration in zebrafish. One recent study showed that p57^{kip2} was induced in MGPCs after retinal injury, and it promoted cell cycle exit of the MGPCs after 4 dpi.[12] Given that cyclins/CDKs/CKIs have been shown to have broad functions besides cell cycle control,[34] it will be interesting to further investigate their roles in retinal regeneration in zebrafish.

The CKI p21^{cip1} interacts with and inhibits cyclin E/Cdk2 complexes and PCNA,[36] and therefore mediates cell cycle arrest and apoptosis. Expression of p21^{cip1} is tightly controlled by a variety of factors, including the growth factor/MAPK pathway and the tumor suppressor p53.[37] Induction of p21^{cip1} could also be regulated in a p53-independent manner.[38] Interestingly, it has been previously shown that cell cycle arrest and a p53-mediated p21^{cip1} induction were observed in GCV-treated tumor cells genetically modified to express HSV-TK in vitro.[39,40] These results are similar to our findings in this study, although only GCV itself was used here. In our study, we observed an early and significant induction of p53 and p21^{cip1} after GCV treatment. It is likely that the induction of p21^{cip1} was regulated by p53 in the MGPCs after GCV administration. However, we cannot exclude additional pathways promoting p21^{cip1} expression in the presence of GCV.

The novel finding that GCV itself is a potent inhibitor of MGPC proliferation is intriguing. To date, there are only a limited number of studies reporting that GCV alone could strongly inhibit cell proliferation. In one study, it was shown that GCV itself could inhibit human lymphocyte proliferation in vitro.[41] It was also recently reported that GCV is a strong inhibitor of CNS microglia activation and proliferation in a mouse model of neuroinflammation,[27] though the underlying molecular mechanism was unknown. In our study, there is no evidence showing that the fish used here were infected with HSV or CMV. Importantly, we reproduced our results on several lines of wild-type and transgenic zebrafish obtained from different labs (not depicted). In addition, no viral TK sequence was found in the zebrafish genome. Therefore, the action of GCV on Müller glia seems to be independent of TK activity. However, we cannot rule out the possibility that GCV may act on an unknown virus that has infected our zebrafish. The kinetics of GCV metabolism and the active form of the compound have not been studied per se in zebrafish. Therefore it is also possible that a GCV metabolic product different from that in mammals is responsible for the effect of GCV in fish.

A very interesting phenomenon after GCV administration is the prolonged dedifferentiation of postmitotic Müller glia cells. To our knowledge, this is the first report showing that a single drug could "lock" activated Müller glia cells in a prolonged dedifferentiated state. The mechanism underlying this phenomenon is still unknown and requires further investigation. It has been shown that GCV treatment of HSV-TK–expressing tumor cells resulted in the activation of MAP kinase (MAPK).[42] Since MAPK activation is essential for the dedifferentiation of Müller glia and RAG expression after mechanical retinal injury,[13,14] it is possible that GCV activated MAPK in Müller glia through an unknown pathway, which in turn led to the high expression of RAGs and subsequent prolonged activation. Another possibility is that there are unknown communicating signals between the dedifferentiated Müller glia and the proliferating MGPCs that function to downregulate RAG expression in Müller glia and promote their transition to a preinjury status. Ganciclovir might thus activate Müller glia in an indirect manner, by inhibiting the proliferation and expansion of the MGPC population.

In conclusion, our study uncovered a novel effect of the antiviral drug GCV on retinal regeneration in a mechanical retinal injury model in adult zebrafish. Ganciclovir strongly inhibited the proliferation of MGPCs in the injured retina, likely by causing a cell cycle arrest through a p53-p21^{cip1}–dependent pathway. Ganciclovir also prevented the downregulation of RAGs at late stages and thus kept Müller glia in a dedifferentiated state. Because of the strong and irreversible inhibitory effect of GCV on MGPC proliferation, GCV may be used as an effective tool to selectively limit the expansion of MGPCs in future studies. Possibilities for GCV application include, but are not limited to, (1) investigating the impact of MGPC cell number on their migration and differentiation; (2) comparing the transcriptome of postmitotic Müller glia with MGPCs; and (3) instances in which there is too much MGPC proliferation and a need to control their cell number. Furthermore, GCV could be useful in future studies to investigate the molecular mechanism regulating the transition of dedifferentiation to rapid progenitor proliferation during retinal regeneration.

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