Chapter XX

Cell fate of Müller cells during photoreceptor regeneration in an MNU-induced retinal degeneration model of zebrafish

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Abstract Zebrafish can regenerate several organs such as the tail fin, heart, central nervous system, and photoreceptors. Very recently, a study has demonstrated the photoreceptor regeneration in the alkylating agent *N*-methyl-*N*-nitrosourea (MNU)-induced retinal degeneration (RD) zebrafish model, in which whole photoreceptors are lost within a week after MNU treatment and then regenerated within a month. The research has also shown massive proliferation of Müller cells within a week. To address the question of whether proliferating Müller cells are the source of regenerating photoreceptors, which remains unknown in the MNU-induced zebrafish RD model, we employed a BrdU pulse-chase technique to label the proliferating cells within a week after MNU treatment. As a result of the BrdU pulse-chase technique, a number of $BrdU^+$ cells were observed in the outer nuclear layer as well as the inner nuclear layer. This implies that regenerating photoreceptors are derived from proliferating Müller cells in the zebrafish MNU-induced RD model.

XX.1 Introduction

Retinal degeneration (RD) caused by photoreceptor cell death, including retinitis pigmentosa and age-related macular degeneration, is considered as a major cause for visual loss. It has been reported that at least 50 million individuals are suffering from these diseases (Lund et al. 2003), and the prevalence is increasing with the aging of society (Chakravarthy et al. 2010). In mammals, once photoreceptors are lost, they normally fail to regenerate. In contrast, fish show a tremendous regenerative capacity to offset the loss of photoreceptors (Fischer and Bongini 2010; Nagashima et al. 2013).

Very recently, a study has demonstrated a reproducible and uniform method to create an RD model in zebrafish, in which fish were treated with an alkylating agent, *N*-methyl-*N*-nitrosourea (MNU) (Tappeiner et al. 2013). In this model, a wide and uniform photoreceptor cell loss was observed within a week after MNU treatment followed by photoreceptor regeneration within a month. As with other zebrafish RD models (e.g., intense light injury and stab wound models), a massive proliferation of Müller cells was generally observed to produce new photoreceptors following MNU treatment. In this study, we attempt to show the cell fate of proliferating Müller cells after MNU treatment to address the question "Are proliferating Müller cells indeed the source of regenerating photoreceptors?" using the 5-bromo-2′-deoxyuridine (BrdU) pulse-chase technique with the zebrafish MNU-induced RD model.

XX.2 Materials and Methods

XX.2.1 Animals

All experiments described below were approved by the Committee on Animal Experimentation of Kanazawa University, and all attempts were made to minimize pain and the number of fish used. Adult zebrafish (*Danio rerio*; 3–4 cm in body length, 6–12 months after birth, either sex) were used throughout this study. The fish were kept in water at 28°C unless otherwise stated.

XX.2.2 MNU Treatment

MNU treatment of zebrafish was performed as described previously (Tappeiner et al.

2013). In brief, fish were kept in water containing 10 mM phosphate buffer (pH = 6.3) with a concentration of 150 mg/l of MNU (Toronto Research Chemicals Inc., North York, Canada) for 60 min. After exposure to MNU, fish were washed and kept in fresh water until appropriate time points.

XX.2.3 Intraperitoneal Injection of BrdU

Fish were anesthetized by immersion in 0.033% ethyl 3-aminobenzoate methanesulfonic acid (MS222; Sigma–Aldrich, MO, USA) in PBS, and intraperitoneally injected with 50 μl of 2.5 mg/ml BrdU (Sigma–Aldrich) at 0 (just after MNU treatment), 2, 4, 6, and 8 days post-treatment (dpt), as described previously (Ogai et al. 2012).

XX.2.4 Preparing Retinal Sections

At appropriate time points, fish were euthanized by an overdose (0.1%) of MS-222 for 10 min followed by fixation with 4% paraformaldehyde in PBS overnight at 4°C. The cryosections of the retina were then prepared at 12-μm thickness as described previously (Ogai et al. 2012).

XX.2.5 Hematoxylin–Eosin (HE) Staining

HE staining was performed to observe the structure of the retina. Sections were stained with Mayer's hematoxylin (Wako Pure Chemical Industries, Osaka, Japan) for 2 min followed by washing and counterstaining with 1% eosin-Y (Wako Pure Chemical

Industries) for 2 min. The cleared sections were then observed using a bright-field microscope (DS-Fi1c; Nikon Instech, Tokyo, Japan).

XX.2.6 Immunohistochemistry

The localization of proliferating cells was visualized by immunohistochemistry (Ogai et al. 2012). In brief, antigen retrieval was performed in 10 mM citrate buffer (pH = 6.0) for 5 min at 121°C or 2 M HCl for 30 min at 37°C for proliferating cell nuclear antigen (PCNA) or BrdU immunohistochemistry, respectively. Following washing and blocking, the sections were incubated with anti-PCNA (1:500; Sigma–Aldrich) or anti-BrdU (1:500; Sigma–Aldrich) antibody at 4°C overnight. Visualization was performed with Alexa Fluor 488-conjugated secondary antibody (1:500; Thermo Fisher Scientific, MA, USA) for 60 min at 23°C. Nuclear staining was performed by 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Wako Pure Chemical Industries). The sections were then observed using a fluorescent microscope (DS-Fi1c).

XX.2.7 Statistical analyses

The data were presented as means ± standard error of the mean. The thickness of ONL and the number of $PCNA^+$ cells were analyzed by one-way analysis of variance followed by Tukey's *post hoc* test using SigmaPlot (version 12; Systat Software, Inc., CA, USA). A p value of <0.05 was considered statistically significant.

XX.3 Results

XX.3.1 MNU treatment selectively depleted outer nuclear layer (ONL) in zebrafish retina

First, to confirm the effect of MNU on the zebrafish retina, we obtained retinal sections at 0 (control), 3, 5, 8, 16, 24, and 32 dpt and stained using the HE method. As a result of MNU treatment, the collapse of ONL was observed from 3 to 8 dpt followed by a regeneration of ONL by 32 dpt (Fig. XX.1) in the same manner as previously reported (Tappeiner et al. 2013).

XX.3.2 MNU treatment induced cell proliferation in the inner nuclear layer (INL) in zebrafish retina

Next, to show cell proliferation after MNU treatment, we stained the sections with PCNA at the same time points as in HE staining. As a result of PCNA staining after the MNU treatment, we observed a massive cell proliferation in INL, putatively Müller cells, within a week that peaked at 5 dpt (Fig. XX.2).

XX.3.3 Regenerating photoreceptors were derived from proliferating Müller cells after MNU treatment

Given that a massive cell proliferation was observed within a week after MNU treatment and photoreceptor regeneration took place from 10 to 30 dpt, we theorized that regenerating photoreceptors may originate from proliferating Müller cells. To test this theory, we injected BrdU at 0, 2, 4, 6, and 8 dpt (*pulse*) to label proliferating cells within 8 days after MNU treatment when the massive Müller cell proliferation was

observed (Fig. XX.2). This was followed by BrdU detection at 32 dpt (*chase*; Fig. XX.3a) when ONL was reconstructed (Fig. XX.1). As a result of the BrdU pulse-chase experiment, we could see a number of $BrdU^+$ cells in ONL, as well as INL at 32 dpt (Figs. XX.3b, c).

XX.4 Discussion

Whether genetic or non-genetic (i.e., hereditary or acquired), RD can result in a serious visual loss, which damages the quality of life (Mitchell and Bradley 2006). By using a zebrafish RD model that is capable of regenerating lost photoreceptors, it may be possible to understand not only the degenerative but also the regenerative mechanisms underlying RD. Very recently, Tappeiner et al. (2013) proposed a novel RD model utilizing MNU in zebrafish. MNU has been widely used in RD research in rodents for more than a couple of decades (Smith et al. 1988; Koriyama et al. 2014). MNU can selectively, uniformly, reproducibly, and at any time kill photoreceptors in the retina, which makes it simpler to produce RD models than genetic and/or light-injury RD models (Fausett and Goldman 2006; Pennesi et al. 2012).

In this study, we showed that MNU could kill photoreceptors characterized by the reduction of ONL thickness and that a massive proliferation of Müller cells was observed after MNU treatment, which is consistent with the previous findings (Tappeiner et al. 2013), with a few exceptions. In a previous study, the cell count of ONL significantly dropped only at 8 dpt, whereas in this study we observed a significant thinning of ONL from 3 to 15 dpt. This discrepancy may be explained by the

difference in the method of ONL quantification. The previous study used the cell count of ONL, whereas in this study we used the thickness of ONL. Thinning of ONL occurs not only by cell loss, but also by the reduction of cell size and cell-to-cell distance. However, the cell size and the distance between cells after MNU treatment appeared comparable to that of the control retina (Figs. XX.1a–c). Therefore, we can assume that photoreceptors were indeed lost in this study.

Another difference with the previous study is in time course of cell proliferation. Tappeiner et al. (2013) reported that cell proliferation in INL peaked at 8 dpt, whereas in this study cell proliferation was most active at 5 dpt (Figs. XX.2b, c). At this point, it is difficult to explain the lag in cell proliferation. It is however certain that considerable and rapid proliferation of Müller cells occurs within a week after MNU treatment.

This study further showed that proliferating Müller cells may be a source of regenerating photoreceptors, as a number of $BrdU^+$ cells were observed in ONL as well as INL (Fig. XX.3). This implies that some of the proliferating Müller cells may migrate and differentiate into new photoreceptors (Nagashima et al. 2013), whereas others may remain Müller cells to maintain retinal stem-cell burden and retinal structure.

Notably, in mammalian MNU-induced RD models, Müller cell proliferation took place from 3 to 7 days after MNU treatment but showed no signs of photoreceptor regeneration (Taomoto et al. 1998). It is possible that the major difference in the ability of photoreceptor regeneration between fish and mammals may be laid in the ability of migration and/or differentiation into photoreceptors rather than Müller cell proliferation.

Therefore, we hope that investigating such differences in the migration/differentiation abilities of Müller cells may add new insight into therapeutic advances in the treatment of RD in the future.

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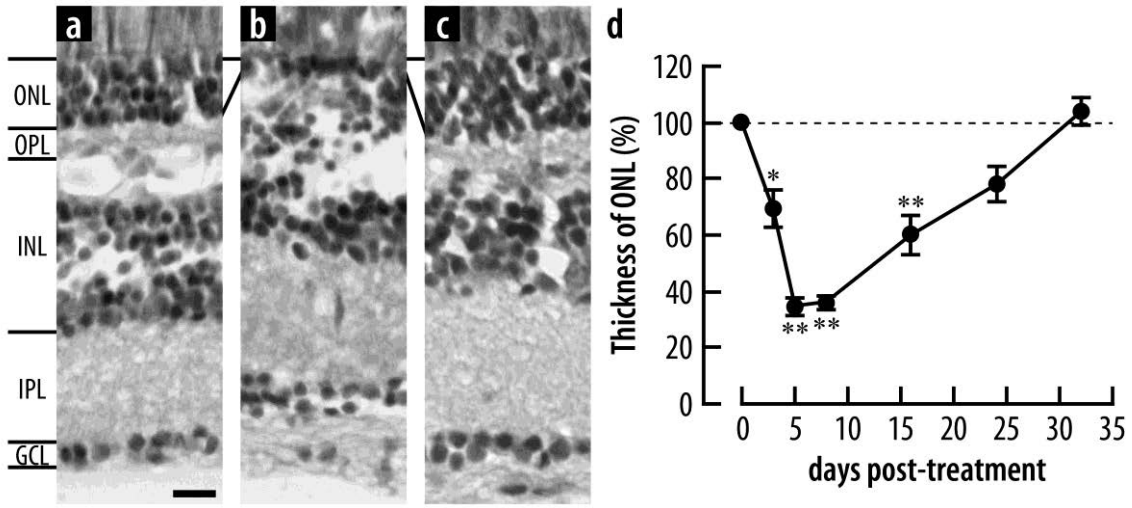
Figure Captions

Fig. XX.1 Changes in ONL thickness after MNU treatment in adult zebrafish. (**a**–**c**) Representative images of HE staining following MNU treatment. At 8 dpt, the thickness of ONL was significantly thinner (**b**) than control retina (**a**). The retinal structure at 32 dpt (**c**) was comparable to control retina (**a**). (**d**) Quantification of ONL thickness; $n = 3$ each, $*p < 0.01$, $**p < 0.001$. OPL: outer plexiform layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bar in (**a**), 10 μm.

Fig. XX.2 Cell proliferation in zebrafish retina following MNU treatment. (**a**, **b**) Representative images of PCNA immunohistochemistry. In control retina, few $PCNA^+$ cells were observed in INL (**a**), whereas a number of $PCNA^+$ cells were observed at 5 dpt (**b**). (**c**) Quantification of $PCNA^+$ cells after MNU treatment; $n = 3$ each, $*p < 0.01$, $**p < 0.001$. Scale bar in (**a**), 10 μm.

Fig. XX.3 Fate of proliferating cells following MNU treatment. (**a**) Experimental setup. At 0, 2, 4, 6, and 8 dpt, BrdU was intraperitoneally injected to label proliferating cells during this period. At 32 dpt when the retinal structure was restored (Fig. XX.1), immunohistochemistry was performed against BrdU. (**b**, **c**) Representative images of BrdU immunohistochemistry. A number of $BrdU^+$ cells were observed in ONL as well as INL. Note that the retinal structure was comparable to control retina (Fig. XX.1a). Scale bar in (**b**), 10 μm.


a
b
c
d
ONL
OPL
INL
IPL
GCL
Thickness of ONL (%)
120
100
80
60
40
20
0
0 5 10 15 20 25 30 35
days post-treatment
*
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**
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Fig. 1

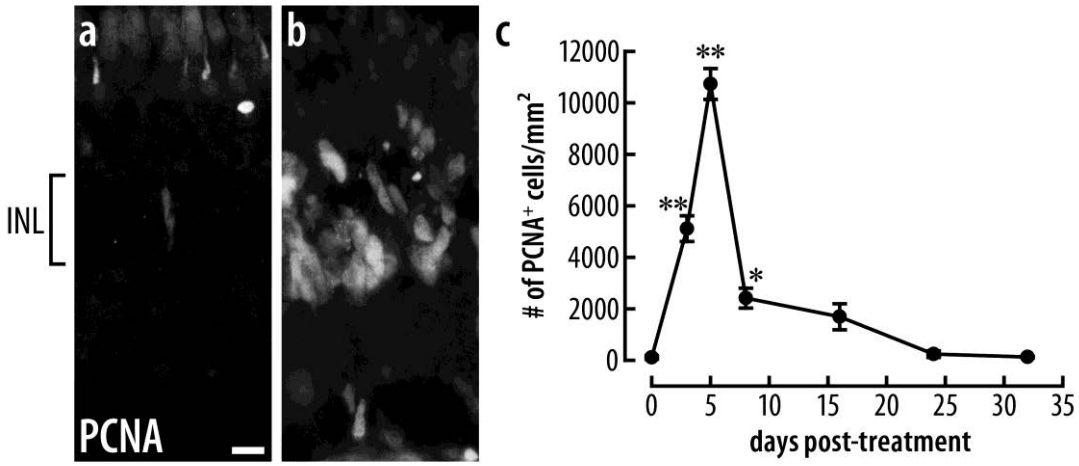
a
b
c
INL
PCNA
# of PCNA+ cells/mm2
12000
10000
8000
6000
4000
2000
0
0 5 10 15 20 25 30 35
days post-treatment
**
**
*

Fig. 2

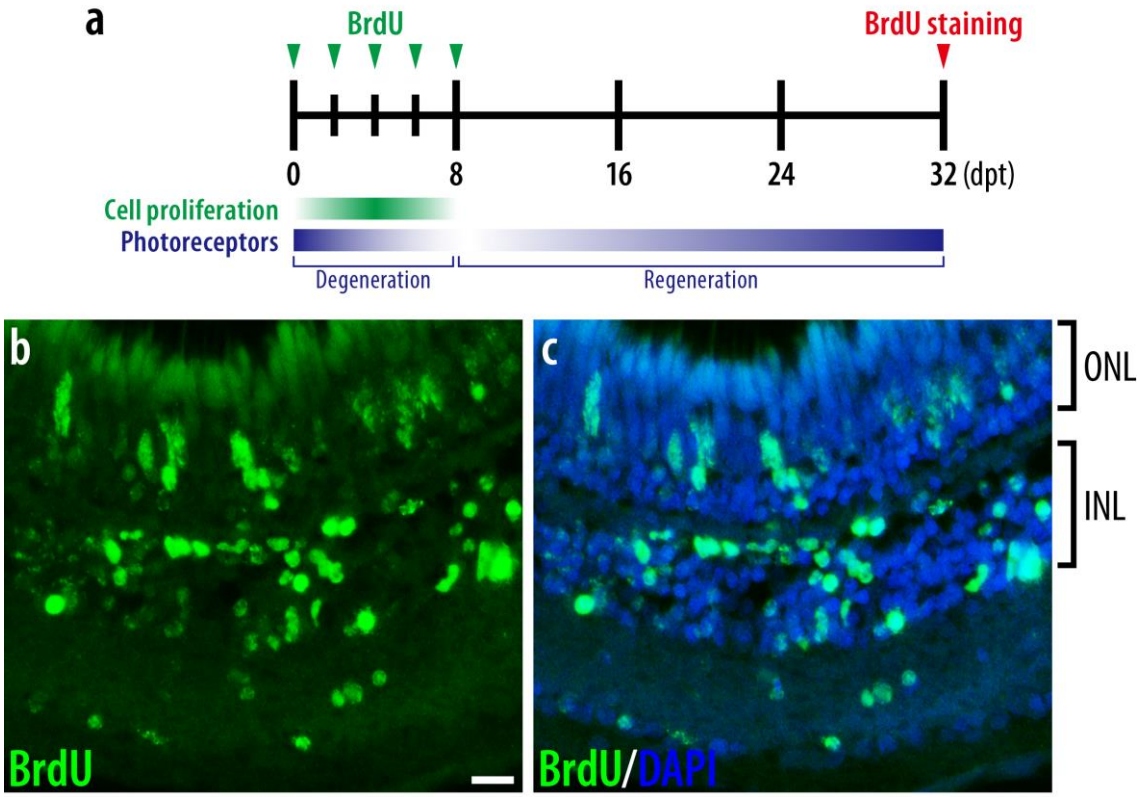
a
BrdU
BrdU staining
0
8
16
24
32 (dpt)
Cell proliferation
Photoreceptors
Degeneration
Regeneration
b
BrdU
c
BrdU/DAPI
ONL
INL

Fig. 3