**Title:**

Müller glia-mediated retinal regeneration in gamma-ray irradiated embryos of zebrafish (*Danio rerio*)

**Short title:**

Retinal regeneration in zebrafish embryo

**Authors:**

Yunsheng Shen 1, Duolin Li 1, Takako Yasuda 1,2, Hiroshi Mitani 1, Shoji Oda 1

**Affiliations:**

1 Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba, 277-8562, Japan.

2 Department of Chemical and Biological Sciences, Japan Women’s University, 2-8-1 Mejirodai, Bunkyo-ku, Tokyo, 112-8681, Japan.

**Correspondence:**

Shoji Oda,

Department of Integrated Biosciences, Graduate School of Frontier Sciences,

The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba, 277-8562, Japan.

Email: [odasho@edu.k.u-tokyo.ac.jp](mailto:odasho@edu.k.u-tokyo.ac.jp)

**Abstract**

The machinery of retinal regeneration in adult and larval zebrafish has been well studied in the recent decades, since retina of zebrafish regenerates throughout life in contrast only in embryonic stage in mammals and birds. It has been believed that zebrafish embryos can conduct retinal regeneration as well as adult and larval zebrafish, however, there is no study to investigate the retinal regeneration in pre-hatching embryos of zebrafish, since it is difficult to injure the retina of the small and rapidly developing embryos. Here, we irradiated pre-hatching embryos (50 hours post-fertilization; hpf) of zebrafish with sub-lethal dose (10 Gy) of gamma-rays and demonstrated that the irradiation induced a large number of apoptotic cell death mainly in the inner nuclear layer (INL) and also in outer nuclear layer (ONL) and ganglion cell layer (GCL). Müller glia were activated and left their original position in the INL to close to the apoptotic cells in the INL and ON. Furthermore, the Müller glia ectopically proliferated, indicating that the Müller glia play the crucial roles in retinal regeneration in pre-hatching embryos as in larval and adult zebrafish. As far as we know, this is the first evidence for retinal regeneration in pre-hatching embryos of zebrafish and will enhance our understanding why adult zebrafish maintain strikingly strong regeneration ability.

(214 words)

**Key words:** Müller glia, retinal regeneration, embryo, zebrafish, irradiation

**1. Introduction**

The structure of the vertebrate retina are highly conserved from fish to human and all vertebrates have three neural layers in retina: it consists of the outer nuclear layer (ONL), which contains rod and cone photoreceptors, the inner nuclear layer (INL), which contains neural cells that process and transmit optic signals, the ganglion cell layer (GCL), which is mainly composed of ganglion cells and responsible for output of visual signals to brain, and the retina pigment epithelium (RPE) in the apical side of retina (Stenkamp, 2007). The RPE is mainly responsible to absorb excessive light, as well as forming the blood-retinal barrier to regulate material transport (Strauss, 2005). In addition, fish retina has the ciliary margin zone (CMZ) in the peripheral side of retina, which contains retinal stem cell (RSCs) and retinal progenitor cell (RPCs) to provide newly generated retinal cells (Centanin *et al.*, 2011).

Six types of neural cells (ganglion cells, bipolar cells, horizontal cells, amacrine cells, rod and cone photoreceptors) are present in the vertebrate retina and Müller glia is the only glial cell in retina which are derived from RPCs (Goldman, 2014). Microglia were also resident in developed retina, although they are not derived from RPC (Ranawat and Masai, 2021). Müller glia distribute through the three nuclear layers and mainly act to support and maintain the neural activities in retina: they release several trophic factors, recycle neurotransmitters, and regulate extracellular ion balance around the neural cells in retina (Schütte and Werner, 1998; Nagelhus et al., 1999; Bringmann et al., 2009; Shen et al., 2012). When retina is injured, Müller glia phagocytize damaged cones to recycle the retinal chromophore in fish, amphibian and mammals (Long *et al.*, 1986; Wang and Kefalov, 2011; Wang *et al.*, 2004; Goldman., 2014). Retina of teleost has been extensively studied in recent decades because of its persistent plasticity and strong ability of regeneration when injured, especially in zebrafish (Stenkamp, 2007). The most impressive function of Müller glia is that they play the central roles to provide progenitor cells at the time of retinal regeneration in adults and larvae.

In adult mammals, severe damage to retina triggers proliferation of Müller glia and they transform into fibroblasts to form glial scarring in the injured retina, however neural tissues of the injured retina are never regenerated (Lenkowski and Raymond, 2014). Although the mammalian Müller glia can respond to injury to proliferate and express the genes associated with retinal stem cells (Roesch *et al*., 2008; Jadhav *et al.*, 2009), they do not generate retinal progenitor cells in adult (Wilken and Reh, 2016) but do so only in postnatal and *in vitro* (Das *et al.*, 2006; Lawrence *et al.*, 2007; Löffler *et al.*, 2015). Similarly, retinal neurons in adult birds are not regenerated after retinal injury (Hayes et al., 2007; Wilken and Reh, 2016), whereas retinal neurons can be regenerated in postnatal chick when injured (Hayes *et al.*, 2007; Ghai *et al.*, 2010; Fischer *et al.*, 2014). In contrast to mammals and birds, it is widely known that adult newts can regenerate whole eyeball including all retinal tissues after eye removal. Retinectomy induces RPE to regenerate the entire retina and CMZ is also crucial during regeneration (Araki, 2007). In *Xenopus*, Müller glia in injured retina re-entered cell cycle and generate photoreceptors after needle poke or neurotoxin administration and the process of retinal regeneration is age-dependent: it is less effective in early tadpole stage and peaks in adults (Langhe *et al.*, 2017).

Zebrafish, a small tropical cyprinid fish, can regenerate a damaged retina and restore visual behavior both in larval and adult stages, and has been an excellent model to investigate the machinery of retinal regeneration in vertebrates (Fausett and Goldman, 2006; Bernadros *et al.*, 2007; Stenkamp, 2007; Lust and Wittbrodt, 2018). Retina of zebrafish has two distinctive features different from the mammalians: CMZ and Müller glia. RSC localize in CMZ and newly generated neural and glial cells are added to the retina throughout zebrafish lifetime (Johns, 1977). Müller glia of larval and adult zebrafish produce multipotent retinal stem cells after retinal injury (Fausett and Goldman, 2006; Bernardos *et al.*, 2007; Fimbel *et al*., 2007; Thummel *et al.*, 2008). Injured cells secret tumor necrosis factor-α (TNFα) and heparin-binding EGF-like growth factor (HB-EGF), and Müller glia contact to the injured cells and de-differentiated (Ramachandran *et al.*, 2011; Nelson *et al.*, 2013). During the process of Müller glia reprogramming, Achaete-scute homolog 1a (ASCL1a), a transcription factor, directly binds to the promoter region of a mRNA-binding protein Lin28 and suppresses expression of microRNA let-7, which can determine quiescence/activated states of Müller glia, inducing de-differentiation and cell proliferation of Müller glia (Ramachandran *et al.*, 2010a). In addition, several signal pathways, such as Wnt and Notch pathways, play significant roles to regenerate the injured retina in adult zebrafish (Ramachandran *et al.*, 2011; Meyers *et al.*, 2012). The reprogrammed Müller glia lose the characteristics of glial cells and proliferate to provide RPCs that differentiate to all of the retinal cells and replace the damaged retinal cells. The molecular machinery that regulates the differentiation of newly generated progenitor cells in larval and adult zebrafish retina has been extensively investigated and is going to be elucidated (Qin *et al*., 2009; Goldman, 2014).

Zebrafish has been one of excellent models to understand retina regeneration in vertebrates and the regeneration machinery has been extensively studied in over decades in larval and adult zebrafish (Fausett and Goldman, 2006; Ramachandran, 2010a, b; Ramachandran, 2011; Thummel *et al.,* 2008). There might be little doubt that zebrafish embryos share the regeneration ability with larval and adult zebrafish, however, there is almost no research on retinal regeneration in pre-hatching embryos of zebrafish, since retinal development and growth are remarkably rapid in zebrafish embryos so that it is difficult to physically or chemically injure the retina of the small and rapidly developing embryos. In this study, zebrafish embryos before hatching (29 and 50 hours post-fertilization; hpf) were irradiated with gamma-rays and retinal injury was induced to investigate the retinal regeneration process in zebrafish embryos. The behavior of Müller glia was studied during the regeneration process, confirming that the regeneration process in embryos is the same as that reported in larval and adult zebrafish.

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# 2. Materials and methods

## *2.1. Ethics*

All experiments in this study were conducted by the Japanese laws and regulations for the care of experimental animals according to the University of Tokyo Animal Experiment Enforcement Rule. All protocols using in this study were approved by the Animal Care and Use Committee of the University of Tokyo (permit number: C-19-5, C-21-4).

## *2.2. Fish and embryo collection*

Wild type zebrafish strain (a crossbreed between Riken wild type and WIK) were bred and reared in the indoor breeding room with 14 hours light period (9:00-23:00), 10 hours dark period (23:00-9:00) and the water temperature is maintained constantly at 27±1 ℃. Brine shrimp (*Artemia franciscana*) which were hatched in 3% NaCl water at 27 ℃ from dried dormant eggs, were fed to the zebrafish at 10:00, and powdered Tetrafin (Spectrum Brands Japan Inc., Tokyo, Japan) was fed twice a day (11:00 and 17:00).

Glass marbles were placed at the bottom of tank and two pairs of zebrafish were put into the tank at 18:00 for next day mating (Westfield, 1994). On the next day, zebrafish spawn eggs around 9:00. Eggs, that fell into the gap between the marbles at the bottom of the breeding tank, were collected and transferred into and incubated in a plastic Petri dish with 10-5% methylene blue at 28.5 ℃. The collected eggs were observed under a stereomicroscope (SZ40, Olympus, Tokyo, Japan) and unfertilized eggs were removed (Kimmel *et al.*, 1995).

## *2.3. Gamma-Ray Irradiation*

Zebrafish embryos at 29 and 50 hours post-fertilization (hpf) were irradiated with gamma rays of 10 Gy emitted by 137Cs (Gamma-cell 3000 Elan, MDS Nordion, Ottawa, Canada) at dose rate of 6.67 Gy/min at room temperature in a 15 mL plastic tube containing 2 mL of 10-5% methylene blue. After irradiation, zebrafish embryos were transferred to a Petri dish and kept in an incubator at 28.5 ℃.

## *2.4. Histological and Immunohistochemical Analysis*

Zebrafish embryos and larvae were anaesthetized and fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB) overnight at 4 ℃ for histological examination. The fixed embryos were washed in phosphate buffered saline (PBS) for 20 min, then dehydrated in a series of 70, 80, 90, 99 and 100% ethanol for 30 min each and then embedded in plastic resin (Technovit 8100, Heraeus Kulzer, Wehreim, Germany). Then, the samples were cut into a complete series of serial sections (8 µm thick) using a microtome (RM2125RT, Leica, Germany) and placed on slide glasses and dried on the heat plate (65 ℃) overnight. The sections were washed in water for 1 min, then stained with Nissl solution (add 500 µl of 10% acetic acid into cresyl violet solution and stir gently before staining) for 12 min. The slides with the stained sections were soaked into 70% ethanol twice for several seconds, 80% ethanol for 10 sec, 90% and 95% ethanol for 1 min each, and 100% ethanol twice for 2 min. Then, the slides were soaked in xylene for 10 min three times, finally mounted with Softmount® (FUJIFILM Wako Pure Chemical Co., Osaka, Japan).

For paraffin sectioning, the fixed embryos were dehydrated in the ethanol series for 30 min each and in 100% ethanol for 1 hour. The samples were soaked in xylene for 30 min three times to allow complete penetration. The penetrated samples were immersed in a pre-warmed (65 ℃) 1:1 mixture of xylene and liquid paraffin for 1 hour, and then soaked into liquid paraffin for 30 min three times. Finally, the samples were embedded in paraffin and stored overnight in a refrigerator at 4 ℃. The embedded samples were sectioned into a complete series of serial sections (5 µm thick) using a microtome (RM2125RT, Leica, Germany). These sections were placed on the slide glasses and dried on the heat plate (42 ℃) overnight. The paraffin sections were used for fluorescence immunohistochemistry. The slides with the sections were dried up for 1 day or longer and then heated on a heat plate (65 °C) for 30 sec to melt paraffin. To deparaffinize the sections, the slides were immersed in xylene for 10 min, followed by 2 min soaking in 100%, 95%, 90%, 80% and 70% ethanol, and then 5 min in water. For antigen retrieval, the glass slides with the sections were placed in a heat-resistant (TPX Staining Tray (PMP) Vertical, #2-3029-01, AS ONE, Osaka, Japan) with citric acid buffer (1.4 mM citric acid monohydrate (Fujifilm Wako Pure Chemical Co., Osaka, Japan), 8.2 mM sodium citrate (Nakalai Tesque, Kyoto, Japan), and autoclaved at 120 °C for 20 min, then cooled to room temperature and washed three times with PBS for 3 min each. The sections were incubated in blocking buffer containing 4% normal goat serum in PBS at room temperature, washed in PBS and incubated for 3 hours at room temperature in a humidified chamber with the following primary antibodies: polyclonal anti-cleaved caspase-3 antibody (9661S; Cell Signaling Technology, Danvers, MA, USA) (1:200), monoclonal anti-GS antibody (MAB302; Millipore, CA, USA) (1:500), polyclonal anti-phospho-histone H3 (Ser10) (PH3) antibody (06-570; Millipore, CA, USA) (1:200) and monoclonal ant-PCNA antibody (PC10) (NB500-106, NOVUS Biologicals, CO, USA), which were diluted with primary antibody diluent (0.5% BSA, 0.5% Triton, 0.1% NaN3/PBS). Then, the sections were rinsed three times in PBS for 5 min each. The sections were further incubated with secondary antibodies conjugated with Alexa-488 (A11001, Invitrogen, Carlsbad, CA, USA) (1:500) or Alexa-546 (A11035, Invitrogen, Carlsbad, CA, USA) (1:500) for 2.5 hours at room temperature. Then the slides were rinsed three times in PBS for 5 min each. Some of the slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1:50) and rinsed in PBS three times for 5 min each. Finally, the sections were mounted with fluorescence antifade reagent (AR-6500-01, Flouroshield Mounting Medium, ImmunoBioScience Corp., USA).

## *2.5. Image Processing and Statistical Analysis*

## All immunohistochemistry images were acquired using a microscope (BX-50, Olympus, Tokyo, Japan) equipped with a digital still camera (DS-Ri1, Nikon, Tokyo, Japan). The images were processed by ImageJ (Version 1.53a) software. Statistical analysis was conducted with Microsoft Excel. Two tailed Student’s *t* test and one-way ANOVA followed by Tukey’s test were conducted to determine the statistical significances and *p* values less than 0.05 were considered significant.

# 3. Results

## *3.1. Effects of gamma-ray irradiation on retinal development of zebrafish embryos*

The characteristic layered structure of retina was not formed in 39 hpf embryos and outer nuclear layer (ONL) was formed in embryonic retina only at 49 hpf during development of zebrafish embryos. Then, the three nuclear layers, ONL, inner nuclear layer (INL) and ganglion cell layer (GCL), are clearly recognized at 60 hpf (Stenkamp, 2007; Fig. 1, S1). In this study, we irradiated zebrafish embryos at 29 and 50 hpf with 10 Gy of gamma-rays and then chemically fixed the embryos at 24 hours post-irradiation (hpi) and histologically examined them. In the embryos irradiated at 50 hpf, more clusters of apoptotic cells appeared in their brain and retina 24 hours after the irradiation than those irradiated at 29 hpf (Fig. S2). We further investigated the radiation response in the zebrafish embryos irradiated at 50 hpf, since apoptotic cell death was induced in the zebrafish embryos irradiated at 50 hpf in similar numbers as in the medaka embryos irradiated at 96 hpf, which are in the most susceptible stage to irradiation in the embryonic development of medaka (Yasuda et al., 2018; Fig. S2)

Apoptotic cells first appeared 10 hours after the irradiation when 50 hpf embryos were irradiated (Fig. 1B) and large clusters of apoptotic cell debris were present 22 hours after the irradiation of gamma-rays (10 Gy) (Fig. 1D). The induced apoptotic cells were mainly present in the INL, whereas a few of them were induced in the ONL and GCL (Fig. 1D). Since no apoptotic cell death was present in the retina of non-irradiated embryos of 60 hpf (Fig. 1A) and 72 hpf (Fig. 1C), it is indicated that irradiation of 10 Gy of gamma-rays induces apoptotic cell death in retina of zebrafish 50 hpf embryo. In the retina of the 48 hpi embryo (98 hpf), the apoptotic cell debris was absent (Fig. 1F), indicating that apoptotic cell death was induced within 22 hours after the irradiation and then removed before 48 hpi. The INL thickness of the irradiated embryos at 48 hpi were significantly thinner than those of the non-irradiated embryos (Fig. 1G); the thinner INL of the irradiated embryos might be attributable to the cell loss because of the apoptosis of retinal cells.

## *3.2. Müller glia ectopically proliferate in INL of irradiated embryos*

Glutamine synthetase (GS)-positive Müller glia were absent in 53 hpf embryos (Fig. S3) and first differentiated in the central part of the INL of 72 hpf embryos (Fig. 2A) and they were all Sox2-positive (Fig. 2B). Irradiation of gamma-rays (10 Gy) onto 50 hpf embryos induced a large number of apoptotic cell death in the INL 22 hours after the irradiation (Fig. 2D), whereas apoptotic cells were absent in the retina of 72 hpf embryos without the irradiation (Fig. 2C). In the retina of the 22 hpi irradiated embryos, Müller glia in the INL changed their cell morphology to be hypertrophied or amoeboid (Fig. 2E). Some of the Müller glia left their normal location in the INL and migrated into the ONL and looked to surround cleaved caspase-3-positive apoptotic cells in the ONL (Fig. 2E, F), strongly suggesting that Müller glia in the INL play a crucial role to phagocytize apoptotic cell debris and repair the injured retina, as with in larval and adult zebrafish.

In the embryos irradiated at 50 hpf, PCNA-positive cells in CMZ were still present 22 hours after the irradiation, indicating that the stem cells and progenitor cells in the CMZ did not cease cell proliferation even after the gamma-ray irradiation (Fig. 3B). In contrast that PCNA-positive cells were present only in CMZ of the not-irradiated 72 and 96 hpf embryos (Fig. 3A, C), we found ectopic proliferating cells in the INL of the 48 hpi irradiated embryos (arrowheads in Fig. 3D). We also conducted immunostaining with anti-phospho-histone H3 antibody and confirmed the presence of the ectopically proliferating cells outside the CMZ of the irradiated embryos 22 hours after the irradiation (Fig. S4). These results strongly suggest that Müller glia ectopically proliferated to repair the injured retina in the irradiated early embryo, as with in larval and adult of zebrafish.

## *3.3. Photoreceptors regeneration in irradiated zebrafish embryos*

Photoreceptors first differentiated in retina of 49 - 50 hpf zebrafish embryos and were well developed in the embryos later than 72 hpf (Morris and Fadool, 2005; Stenkamp, 2007; Fig. 1, S1). The typical histology of the normal ONL during embryonic development is the dense arrangement of slender conical photoreceptors as shown in Fig. 4A, C, E, H. Apoptotic cell death was also induced in the ONL of the irradiated embryos (Fig. 2D, F) and the three-layered structure of the retina was severely disturbed 22 hours after the irradiation (Fig. 4B). Apoptotic cells disappeared in the retina of the irradiated embryos within 46 hours after the irradiation (Fig. 1F), although the ONL with photoreceptors was only formed partially but round cells similar to those in the INL were present instead of the slender conical photoreceptors, obviously those were not differentiated photoreceptors (Fig. 4D).

In contrast that the photoreceptors of the normally developed retina of zebrafish embryos were densely packed in the ONL (Fig. 4H), those in the restored retina of the irradiated embryos were more sparsely arranged 5 days after the irradiation (Fig. 4I, K), although the size of eyeballs and INL thickness were not significantly different from those of the not-irradiated embryos (Fig. S5). In this study, the histological appearance of the ONL was restored to be normal in a half of the irradiated embryos (3 of 6 embryos in total) (Fig. 4F, I) and still severely disturbed in the other half of the irradiated embryos (3 of 6 embryos in total) 3 days after the irradiation (Fig. 4G, J), so the densities of photoreceptors in the embryos with restored retina were measured and compared with the controls in Fig 4K.

In all of the irradiated embryos, the injured retina was almost completely restored within 10 days after the irradiation: size of eyeball and INL thickness were not significantly different between the non-irradiated control embryos and the irradiated embryos (Fig. 5A, B), however, we found that the density of photoreceptors in the ONL of the irradiated embryos were still smaller than the control embryos (Fig. 5C).

1. **Discussion**

In this study, we investigated the irradiation-induced retinal injury in pre-hatching embryos of zebrafish and found that apoptotic cell deaths in the brain and retina were induced most prominently when the embryos were irradiated just before the differentiation of the retinal three layers (ONL, INL, and GCL) and Müller glia (50 hpf). The apoptotic cell deaths were mainly induced in the INL, but also in the ONL and GCL, and development of the three layers was not delayed by irradiation. GS-positive Müller glia changed their morphology to surround the apoptotic cells in the INL and some migrated into the ONL in the irradiation-injured retina. On the other hand, stem cells and progenitor cells in the CMZ did not stop proliferation after the irradiation, while the Müller glia ectopically proliferated in the irradiated retina. The dead cells in the irradiated retina disappeared within 48 hpi and the laminar structure and ocular atrophy were restored in the 5 dpf larvae, but photoreceptors were still more sparsely distributed in the ONL of irradiated retina even in 10 dpf larvae, suggesting that there was an overall lack of cell number in the irradiated retina. These findings strongly suggest that Müller glia in pre-hatching embryo were activated after the irradiation-induced injury in developing embryonic retina and phagocytose the dead cell debris, and proliferate to provide cells for retinal regeneration, as in adult and larval zebrafish.

Müller glia firstly respond to the retinal injury and they phagocytose damaged retinal cells with microglia in larval and adult zebrafish (Goldman, 2014; Morris *et al*., 2005; Bailey *et al.*, 2010; Ranawat and Masai, 2021). Some of Müller glia are reprogrammed and conduct symmetrical cell division to generate neural precursor cells in the injured retina, renewing themselves (Nagashima *et al.*, 2013; Fausett and Goldman*.*, 2006; Ramachandran *et al.*, 2010b).As summarized above, we obtained the evidence in this study, that Müller glia play the central roles in retinal regeneration in zebrafish embryos as in the injured retina of larval and adult zebrafish (Yurco and Cameron, 2005; Morris *et al*., 2005; Fausett and Goldman, 2006; Bailey *et al.*, 2010). Also in embryos, Müller glia might re-enter the cell cycle after the retinal injury and act as retinal stem cells as in larval and adult zebrafish. The finding that all of the Müller glia in the INL of the 72 hpf embryos were Sox2-positive (Fig. 2) supports this idea, since Sox2 is the well known transcription factor involved in the self-renewal of neural stem cells and its maintained expression is critical for the regenerative activity of Müller glia in larval zebrafish (Lust and Wittbrodt, 2018). It has been reported that a similar molecular machinery is employed in embryonic and postembryonic retinogenesis in zebrafish (Harris and Perron, 1998; Perron *et al.*, 1998) and the recent single-cell RNA-seq analysis has provided the evidence that embryonic and postembryonic retinogenesis share a similar developmental program (Xu et al., 2020). The results obtained in this study strongly support the conclusion that retina regeneration in zebrafish pre-hatching embryos is a process mediated mainly by Müller glia, which is the same as in larval and adult zebrafish retinal regeneration (Bernardos *et al.*, 2007; Lust and Wittbrodt, 2018).

There have been numerous studies on the retinal regeneration in adult and larval zebrafish and most studies used neurotoxins or mechanical surgery (such as needle impalement) to injure retina in adult and larval zebrafish (Yurco and Cameron, 2005; Fimbel *et al.*, 2007; Fausett and Goldman, 2006; Lust and Wittbrodt, 2018) and some studies employed heater strip or high-intensity light (or laser) (Bernardos *et al.*, 2007; Bailey *et al.*, 2010; Raymond *et al.*, 2006; khan *et al.*, 2020). Up to now, there have been very limited studies on the repair of injured retina during embryonic development of zebrafish, probably due to the difficulty to precisely injure the small retina of early embryos. Furthermore, some damage methods require a long time for operation but the embryonic development of zebrafish is very rapid: the embryo hatch within 4 days after fertilization. For example, high-intensity light illumination for 24 hours was used to destroy photoreceptors in adult zebrafish retina (Khan *et al.*, 2020). The rapid development of zebrafish embryo during the light illumination might make the experiment so complicated and so difficult. Recent studies have shown that irradiation can induce neurotoxicity in fish embryos (Geiger *et al.*, 2006) and developing central nervous system in fish embryos is highly sensitive to irradiation (Yasuda *et al.*, 2018). At the same time, it takes a very short time to irradiate and induce retinal injury in embryos, making it possible to injure the embryonic retina at the specific development stage during the embryonic development with the high reproducibility. In this study, the irradiation-induced injury model allows us to investigate the damage induction and repair process reproducibly and precisely even in the rapid developing zebrafish embryos.

Since a previous study reported that apoptotic cell death in embryonic brain is most effectively induced by gamma-ray irradiation at 72 hpf medaka embryo (Yasuda *et al.*, 2018), we examined 29 hpf and 50 hpf zebrafish embryos in the present study and found that more apoptotic cell deaths were induced in the brain and retina of 50 hpf zebrafish embryos than in 29 hpf zebrafish embryos. In the previous studies, earlier stage embryos at segmentation period (24 hpf), gastrula period (8 hpf) or very early stage embryos (2 - 6 hpf) were irradiated to investigate the effects of irradiation on zebrafish embryonic development and some of them reported the induction of apoptotic cell death in embryonic eye after developed (Geiger *et al.*, 2006; Bladen et al., 2007; Zhou *et al.*, 2014; Szabó *et al.*, 2018). We found only one report that apoptotic cell deaths were induced in zebrafish embryos irradiated at 48 hpf (Barrett *et al.*, 2018) and the results in the present study are consistent with it.

Finally, we found the difference in the retinal regeneration among the irradiated embryos. Embryonic development of zebrafish is very rapid (Westerfield, 2000) and it could be possible that we failed to irradiate all of the embryos at exactly the same developmental stage in this study. Zebrafish embryos restored the injured retina in their histological appearance, eyeball size, INL thickness within 8 days after the irradiation, however, the photoreceptors in the ONL were not recovered. Presumably it might be adaptive and/or crucial for zebrafish embryos to develop visual system as rapidly as possible to escape from predators. Cone photoreceptors are formed from 48 to 60 hpf during normal embryonic development of zebrafish (Stenkamp, 2007) and rod photoreceptors develop around 120 hpf (Morris and Fadool, 2005). In addition to this, our another finding that cell proliferation in the CMZ did not cease (Fig. 3B) and retinal development and regeneration were both on going even after the irradiation, strongly suggest that zebrafish embryos might prioritize retinal development over repairing injured retina.

# 5. Conclusions

It has been natural to speculated that zebrafish embryos can conduct retinal regeneration as well as adult and larval zebrafish, however, there is few studies to investigate the retinal regeneration in pre-hatching embryos of zebrafish, since it is difficult to selectively injure only the retina of the small and rapidly developing embryos. In the present study, we established the irradiation-induced retina injury model in zebrafish pre-hatching embryos with gamma-rays and investigated the process of retinal regeneration in zebrafish pre-hatching embryos. The findings of this study indicate that Müller glia play the crucial roles in retinal regeneration in pre-hatching embryos as in larval and adult zebrafish. It was also demonstrated that regeneration of the developing retina from irradiated damage might involve a process of rapid removal of dead cells and replenishment of retinal various cells from the CMZ over time after hatching after juvenile growth. These findings might enhance our understanding why the retina regenerates throughout life in zebrafish and also why adult zebrafish maintain strikingly strong regeneration ability.

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**Authors contributions**

Conceptualization: Takako Yasuda, Hiroshi Mitani, Shoji Oda.

Data curation: Yunsheng Shen, Duolin Li, Takako Yasuda, Hiroshi Mitani, Shoji Oda.

Investigation: Yunsheng Shen, Duolin Li, Takako Yasuda.

Project administration: Takako Yasuda, Hiroshi Mitani, Shoji Oda

Writing – original draft: Yunsheng Shen, Duolin Li, Shoji Oda.

Writing – review & editing: Takako Yasuda, Hiroshi Mitani, Shoji Oda.

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**Conflict of interests**

The authors declare that there is no conflict of interests.

**Data availability statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**Figure legends**

**Fig. 1.**

**Effects of gamma-ray irradiation on retinal developmental in zebrafish embryo.**

There was no apoptotic cells in non-irradiated 60 hpf (A), 72 hpf (C) and 96 hpf (E) zebrafish embryos. Embryos at 50 hpf were irradiated with gamma-rays (10 Gy) and Nissl-stained histological sections were prepared at 10 hpi (B), 22 hpi (D) and 48 hpi (F). Clusters of apoptotic cells were present mainly in the INL of the retina of the 10 and 22 hpi embryos and absent in the 48 hpi embryos (Arrowheads in B and D). Size of retina and thickness of INL were measured in the 72 hpf and 4 dpf (96 or 98 hpf) not-irradiated embryos and in the embryos irradiated at 50 hpf and 22 hours and 46 hours after the irradiation (n=3 for each). The data are presented by means ± SD. p values less than 0.05 were considered to be significant by Student's t-test. \*, p < 0.05; \*\*, p < 0.01. Scale bars = 50 µm.

**Fig. 2.**

**Induction of apoptotic cell death in irradiated embryonic retina and morphology changes of Müller glia.**

Glutamine synthetase (GS)-positive Müller glia aligned in the central part of the INL (A), those were all Sox2-positive (B), and no apoptotic cell death was observed (C) in the non-irradiated 72 hpf embryos. In the embryos irradiated with 10 Gy of gamma-rays at 50 hpf, a large number of cleaved caspase 3-positive cells (green) was induced mainly in the INL 22 hours after the irradiation (D) and GS-positive Müller glia (red) changed their cell morphology and left their normal location (arrowheads in E). Enlarged and marged image of dotted box in D and E is shown in F. Some of the Müller glia (red) looked to migrate close to the apoptotic cells (arrowheads in E, F). Apoptotic cells (green) were also present in the ONL (F). Scale bars = 20 µm.

**Fig. 3.**

**No cessation of cell proliferation in CMZ and ectopic cell proliferation in retina of gamma-ray irradiated embryo.**

PCNA-positive proliferating cells were present only in CMZ in the non-irradiated 72 hpf (A) and 96 hpf (C) embryos, whereas proliferating cells appeared ectopically in the INL and ONL 48 hours after the irradiation in the embryos irradiated at 50 hpf (arrow heads in D). In the irradiated embryos at 22 hours after the irradiation, a few PCNA-positive mass were found in the INL in addition to the proliferative cells in the CMZ but they might be clusters of pseudo-positive apoptotic cell debris (arrow heads in B). Scale bars = 20 µm.

**Fig. 4.**

**Repair process of embryonic retina after gamma-ray irradiation.**

The three-layered structure of the retina was obvious in 72 hpf (A), 96 hpf (C) and 5 dpf (E) zebrafish embryos, and the slender conical photoreceptors were densely arranged in the ONL of the 5 dpf embryo (H). In the embryos irradiated at 50 hpf with gamma-rays (10 Gy), apoptotic cell death was induced in the ONL in addition to the INL and the layered structure of the retina was severely disturbed 22 hours after the irradiation (B). Although apoptotic cells were removed in the retina 46 hours after the irradiation, the ONL with photoreceptors was only partially developed (D). The damaged ONL was restored within 3 day after the irradiation in a half of the irradiated embryos (F, n =3), whereas not restored in the other half of the irradiated embryos (G, n = 3). Even in the restored retina, the photoreceptors in the ONL were less and arranged more sparsely than the ONL of the not-irradiated embryos (E, F, K). All histological sections were Nissl stained. The density of photoreceptors were measured as describe in Materials and Methods. The data are presented by means ± SD. Rectangle areas in E, F, G are presented in E’, F’, G’. Scale bars =20 µm.

**Fig. 5.**

**Comparison of eyeball size, INL thickness and density of photoreceptors between not-irradiated and irradiated embryos.**

Size of eyeball, INL thickness and density of photoreceptors of the not-irradiated embryos and the embryos irradiated at 50 hpf (n=3 for each) were compared at 10 dpf as described in Materials and Methods. The data are presented by means ± SD. Student’s t test was employed and p values less than 0.05 were considered to be significant. \*, p < 0.05; \*\*, p < 0.01.

**Supplementary Fig. 1.**

**Embryonic development of retina in zebrafish.**

Histological sections of developing retina of pre-hatching zebrafish embryos at 33 (A), 39 (B), 49 (C), 60 (D), 72 (E) and hatched larva at 96 (F) hpf were presented. Scale bars = 20 µm.

**Supplementary Fig. 2.**

**Apoptotic cell death induced 24 hours after irradiation of gamma-rays in zebrafish and medaka embryos.**

Zebrafish embryos at 29 hpf (A) and 50 hpf (B) were irradiated with 10 Gy of gamma-rays and Nissl-stained histological sections were prepared 22 - 24 hours after the irradiation. Apoptotic cell death were induced mainly in the INL of the retinas. Medaka embryos at 72 hpf were irradiated with 10 Gy of gamma-rays and histological sections were prepared 24 hours after the irradiation (C). Scale bars = 100 µm.

**Supplementary Fig. 3.**

**Absence of glutamine synthetase (GS)-positive cells in 53 hpf zebrafish embryo.**

Zebrafish embryos of 53 hpf were immuno-stained with anti-Glutamine synthetase (GS) antibody, which is widely used to localize Müller glia. It was demonstrated that GS-positive Müller glia had not yet developed at 53 hpf in zebrafish embryo. Scale bars = 50 µm.

**Supplementary Fig. 4.**

**Ectopic cell proliferation in INL of irradiated embryonic retina.**

Glutamine synthetase (GS)-positive (red) Müller glia were located in the INL and phospho-Histone 3 (PH3)-positive proliferating cells (green) were present only in the CMZ (dotted circle) in the 72 hpf not-irradiated embryonic retina (A). In the ONL of the irradiated retina, PH3-positive ectopically proliferative cells were found (arrowheads in B, C). Merged images of immunostaining with anti-GS (red) and anti-PH3 (green) are shown in A to D and enlarged image of dotted box in C is shown in D. Immunostaining image with anti-GS (red) and anti-PH3 (green), which become D when merged, are separately presented in E and F, respectively. An ectopically proliferative cell in the irradiated ONL was GS-positive (yellow arrowheads in D-F). Scale bars = 20 µm.

**Supplementary Fig. 5.**

**Comparison of eyeball size and INL thickness between not-irradiated and irradiated embryos.**

Size of eyeball and INL thickness of the not-irradiated embryos and the embryos irradiated at 50 hpf (n=3 for each) were compared at 5 dpf as described in Materials and Methods. The data are presented by means ± SD. One-way ANOVA followed by Tukey’s test was employed and p values less than 0.05 were considered to be significant. \*, p < 0.05; \*\*, p < 0.01.