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修士論文

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

ゼブラフィッシュ胚における網膜の放射線傷害とミュラーグリアによる網膜組織の再生に関する研究

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1. **Introduction**
   1. **Retina structure in vertebrates**

The cells and structure of the retina are highly conserved in vertebrates (from fish to humans), and all vertebrates have a three-layer structured retina (Stenkamp, 2007). It consists of the outer nuclear layer (ONL), which contains rod and cone photoreceptors, inner nuclear layer (INL), which contains the neural cells that process and transmit optic signals, and the ganglion cell layer (GCL), which is mainly composed of ganglion cells and responsible for output of visual signals to brain. As the other vertebrate animals, the neural retina of fish is composed of the three nuclear layers, ONL, INL, GCL and ciliary margin zone (CMZ) in the peripheral side and retina pigment epithelium (RPE) in the apical side. The CMZ is the center to provide newly generated retinal cells and mainly composed of retinal stem cell (RSC) and retinal progenitor cell (RPC), where RSC is located in the most peripheral area of CMZ, the proliferating RPC is next to RSC, and the post-mitotic progenitor cell is located in the outermost side of CMZ (Centain *et al.*, 2011). The RPE is mainly responsible to absorb excessive light, as well as forming the blood-retinal barrier to restrict material transport (Strauss, 2005).

There are six neural cells in vertebrate retina (ganglion cells, bipolar cells, horizontal cells, amacrine cells, rod and cone photoreceptors) and Müller glia, which is the unique glial cell in vertebrate retina and distribute through the three nuclear layers (Fig. 1.1). The function of rod and cone photoreceptors is to convert photons that enter the eye through the lens into chemical and neural signals (Morris *et al.*, 2005). Bipolar cells connect the ONL and GCL to transmit the signals from photoreceptors to ganglion cells (Stenkamp, 2007). Both horizontal cells and amacrine cells connect INL and ONL to integrate and modify neural signals (Stenkamp, 2007). The ganglion cells are responsible for transmitting visual signals to visual cortex of brain via optic nerve (Goldman, 2014).

Müller glia, as the only glial cells derived from retinal progenitor cell in retina, distributed through the three nuclear layers and mainly act to support and maintain the neural activities by the neural cells in retina (Tout *et al.*, 1993; Shen *et al.*, 2012; Nagelhus *et al.*, 1999). They release several trophic factors, recycle neurotransmitters, and control extracellular ion balance around the neural cells in retina (Bringmann *et al.*, 2009; Pow and Crook, 1996; Schütte and Werner, 1998; Seki *et al.*, 2003). Moreover, it is reported that Müller glia conduct phagocytosis of damaged cones to recycle the retinal chromophore (Long *et al.*, 1986; Wang and Kefalov, 2011; Wang *et al.*, 2004). The retina of teleost fish has been extensively studied in recent decays 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 during the embryonic retinal development and in the retinal regeneration when adult and larval retina is injured (see below).

* 1. **Retina regeneration ability in vertebrates**

In mammals, it is reported that severe damage to the retina mainly triggers reactive proliferation of glial cells and causes reactive cellular hypertrophy. And then Müller glia will transform into fibroblasts and will finally form glial scarring in the retina (Lenkowski and Raymond, 2014). Inhibitory factors secreted by glial scarring can prevent retinal neuron regeneration, which can result in a failure of retina self-repair. Although mammalian Müller glia can respond to injury, proliferating and expressing genes associated with retinal stem cells (Jadhav *et al.*, 2009; Roesch *et al*., 2008), they do not generate retinal progenitor cells in *vivo* (Wilken and Reh, 2016). Müller glia have been observed to give rise to neurons and glial cells in only postnatal rodent and human cell cultures (Das *et al.*, 2006; Lawrence *et al.*, 2007; Löffler *et al.*, 2015).

Adult birds’ retinal neurons could not be regenerated after retina damage (Hayes et al., 2007; Wilken and Reh, 2016). But such regeneration could be observed in postnatal chicks (Fischer *et al.*, 2014). Müller glia proliferation in postnatal chicks is reported to be stimulated by Notch signaling (Ghai *et al.*, 2010; Hayes *et al.*, 2007); and the proliferating cells express progenitor markers, like achaete-scute homologue 1 (ASCL1) (Fischer *et al.*, 2014); and endogenous factors, like fibroblast growth factor 2 (FGF2) (Fischer *et al.,* 2002). However, in previous study, it was observed that Müller glia-mediated regeneration in postnatal chicks were limited because only a little retinal progenitor cells derived from Müller glia differentiated into inner retinal neurons (Raymond and Hitchcock, 1997).

In amphibians, it is widely known that adult newts can regenerate whole eyeball including retina after eye removal. Retinectomy induces RPE to regenerate the entire retina after injury and CMZ also plays a role during regeneration (Araki, 2007). In the frog, Müller glia re-entered the cell cycle and generated new photoreceptors after needle poke injury or neurotoxin-induced photoreceptor damage of retina. And this process is age-dependent which means it is less effective in early tadpole stage and peaks in adults (Langhe *et al.*, 2017).

Zebrafish, a small tropical teleost fish, can regenerate a damaged retina and restore visual behavior both in larval and adult stage (Fausett and Goldman, 2006; Bernadros *et al.*, 2007; Lust and Wittbrodt, 2018). Although the teleost retina has the same structure and function as the mammalian retina, two distinctive features are different from other vertebrate species, which are ciliary margin zone and Müller glia. Ciliary margin zone, in which retinal progenitor cells reside, can add new neurons and glial cells into retina throughout zebrafish lifetime (Johns, 1977) (Fig.1.2). Müller glia can produce multipotent retinal stem cells after retinal injure (Fausett and Goldman, 2006; Bernadros *et al.*, 2007; *Fimbel* et al., 2007; Thummel *et al.*, 2008). These distinctive features may contribute to the strong retinal regeneration ability of zebrafish and have made zebrafish an important model animal to investigate the molecular mechanism underlying retinal regeneration in vertebrates.

Adult avian and mammals cannot spontaneously regenerate retinal neurons damaged by disease or injury. In contrast, teleost fish like zebrafish, retinal damage triggers a spontaneous regenerative response that restores retinal structure and function (Table.1.1). So, zebrafish are widely used as "model organisms" in biological and medical research to study retinal regeneration.

* 1. **Retinal regeneration mechanism in adult zebrafish**

In zebrafish, retinal injury evokes a highly efficient endogenous repair mechanism. When the retina of adult zebrafish is damaged, injured cells secret tumor necrosis factor-α (TNFα) and heparin-binding EGF-like growth factor (Hbegf), and Müller glia contact to the injured cells and then de-differentiated (Ramachandran *et al.*, 2011; Nelson *et al.*, 2013). In the reprogramming process of Müller glia, ASCL1a, a transcription factor, binds directly to the promoter region of the mRNA-binding protein Lin28, an RNA regulatory protein that regulates expression of microRNA let-7, which is one of the factors determining quiescence/activated states of Müller glia. The expression of Lin28 suppresses the expression of miRNA let-7 and induces de-differentiation and cell proliferation of Muller glia (Ramachandran *et al.*, 2010a). In addition, it is reported that several signal pathways play significant roles to regenerate the injured retina, including Wnt and Notch signaling pathways (Ramachandran *et al.*, 2011; Meyers *et al.*, 2012).

The reprogrammed Müller glia lose the characteristics of glial cells and instead show the characteristics of retinal stem cells. They start to proliferate and provide retinal progenitor cells that differentiate to the other retinal cells to replace the damaged retinal cells. The molecular machinery that regulates the differentiation of newly generated progenitor cells in the adult zebrafish retina has been extensively investigated and is going to be elucidated (Qin *et al*., 2009; Goldman, 2014). The process of retinal regeneration in adult zebrafish is briefly summarized in Figure.1.3.

* 1. **Aim of this study**

Zebrafish has been one of ideal models to understand retina regeneration in vertebrates and the regeneration mechanism has been well studied in over decades in larvae and adult zebrafish (Fausset and Ramachandran, 2006; Ramachandran, 2010a, b; Ramachandran, 2011; Thummel *et al.,* 2008). On the other hand, medaka, *Oryzias latipes*, is another model fish and their embryos show similar retinal regeneration ability during development (personal communication by T. Yasuda). There would be no doubt that zebrafish embryos share the regeneration ability with larval and adult zebrafish. However, retinal development and growth are remarkably rapid in zebrafish embryos (Fig. 1.4) and there is almost no research on retinal regeneration in pre-hatching embryos of zebrafish. In this study, zebrafish embryos before hatching were irradiated with gamma-rays and the retinal regeneration process was investigated in detail. Moreover, the behavior of Müller glia was identified during the regeneration process, confirming that the regeneration process in embryos is the same as that of larval and adult zebrafish.

# 2. Material 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 Breeding of Zebrafish

Wild Type Zebrafish strain (a crossbreed between Riken wild type and WIK) was used in this study. Zebrafish were bred and reared in the indoor breeding room with 14 hours light period (9:00-23:00hrs), 10 hours dark period (23:00-9:00hrs) and the water temperature is maintained constantly at 27±1 °C.

Brine shrimp (*Artemia Salina*) which were hatched in 3% saline water at 27 °C from dried dormant eggs, were fed to zebrafish at 10:00, and powdered Tetrafin (Spectrum Brands Japan Inc., Tokyo, Japan) was fed twice a day (11:00 and 17:00). Besides, culturing water exchange was performed twice a week.

## 2.3 Embryo Collection of Zebrafish

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. On the next day, with the stimulus of light, zebrafish started to spawn eggs at 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 0.00001% methylene blue at 28.5 °C. The collected eggs were observed under a stereomicroscope (SZ60, Olympus, Tokyo, Japan) and unfertilized eggs were removed (Kimmel *et al.*, 1995).

## 2.4 Gamma-ray Irradiation

Zebrafish embryos at 29 hpf and 50 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 0.00001% methylene blue. After irradiation, zebrafish embryos were transferred to Petri dish and kept in an incubator at 28.5 °C.

## 2.5 Histological and Immunohistochemical Analysis

### 2.5.1 Fixation

Zebrafish embryos and larvae were anaesthetized in ice water and were fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB) overnight at 4 °C for following embedding and sectioning.

### 2.5.2 Resin Embedding and sectioning

The fixed embryos were washed in phosphate buffered saline (PBS) for 20 minutes, then dehydrated in an ethanol series of 70%, 80%, 90%, 99% and 100% for 30 minutes each and then embedded in plastic resin (Technovit 8100, Heraeus Kulzer, Wehreim, Germany).

The resin samples were sectioned into a complete series of serial sections (8 µm thick) using a microtome (RM2125RT, Leica, Germany). These collected section were placed on the slide glasses (#S9902, MAS-GP, Type A, Matsunami, Japan), for 16 pieces on one slide glass, and dried on the heat plate (65 °C) (PS-53, Sakura Finetek Japan Co., Ltd., Tokyo, Japan) overnight until they were totally attached to slide glasses.

### 2.5.3 Nissl Staining

Sections were firstly washed in water for 1 minute, and then were stained with Nissl solution (add 10 µl 10% acetic acid into cresyl violet solution before staining and stir gently several seconds) for 12 minutes. After staining, section slides were transferred into 70% ethanol twice for several seconds, 80% ethanol for 10 seconds, 90% and 95% ethanol for 1 minute, and 100% ethanol twice for 2 minutes. These slides then were soaked in xylene (#244-00086, FUJIFILM Wako Pure Chemical Co., Osaka, Japan) for 3 times, each for 10 minutes, finally enclosed with softmount (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) and a coverslip (25mm x 60mm, Matsunami Glass Ind., Ltd., Kishiwada, Japan).

### 2.5.4 Paraffin Embedding and Sectioning

The fixed embryos were washed in phosphate buffered saline (PBS) for 20 minutes, then dehydrated in an ethanol series of 70%, 80%, 90%, 99% and 100% for 30 minutes each and in 100% ethanol for 1hour. Then samples were soaked in xylene (#244-00086, FUJIFILM Wako Pure Chemical Co., Osaka, Japan) for 30 minutes three times to allow full penetration. The penetrated samples were immersed in a pre-warmed (65 °C) 1:1 mixture of xylene and liquid paraffin for 1 hour, and then soaked into liquid paraffin three times for 30 minutes. Finally, the samples were embedded into paraffin and stored overnight in a refrigerator at 4 °C.

The paraffin 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 (#S9902, MAS-GP, Type A, Matsunami, Japan), for 5-10 pieces on one slide glass, and dried on the heat plate (42 °C) (PS-53, Sakura Finetek Japan Co., Ltd., Tokyo, Japan) overnight until they were totally attached to slide glasses.

### 2.5.5 Fluorescence Immunohistochemistry

The paraffin sections were used for fluorescence immunohistochemistry. For staining, the slides with the sections were dried up for 1 day or longer and then heated on a heat plate (65 °C) for 30 seconds to melt paraffin. To deparaffinize the sections, the slides were immersed in xylene for 10 minutes each, followed by 2 minutes soaking in 100%, 95%, 90%, 80% and 70% ethanol, and 5 minutes 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) which was added with 150 mL of citric acid buffer (2.7 mL Citric Acid (FUJIFILM Wako Pure Chemical Co., Osaka, Japan), 2.1g/100 mL DW, 12.3 mL Sodium Citrate (Nakalai Tesque, Kyoto, Japan), 14.7 g/100 mL DW, 135 mL DDW, pH 6.0), and autoclaved at 120 °C for 20 minutes. The heat-resistant container was then cooled to room temperature and the slides were washed 3 times with PBS for 3 minutes each time.

These sections were incubated in blocking bugger containing 4% normal goat serum in PBS at room temperature, washed in PBS and incubated 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) (anti-PH3) antibody (06-570; Merck, Darmstadt, Germany) (1:200) and monoclonal ant-PCNA antibody (PC10) (NB500-106, NOVUS Biologicals, CO, USA) was diluted with primary antibody diluent (0.5% BSA, 0.5% Triton, 0.1% NaN3/PBS) for 3 hours at room temperature in a humidified chamber. These sections were rinsed 3 times in 150 mL of PBS for 5 minutes each time.

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 150 mL of PBS for 5 minutes each time. Some of the slides were counterstained with DAPI (1:20) and rinsed in PBS three times for 5minutes. At last, sections were enclosed by the fluorescence antifade reagent (AR-6500-01, Flouroshield Mounting Medium, ImmunoBioScience Corp., USA).

### 2.5.6 Nissl Staining and Immunohistochemistry Imaging

All immunohistochemistry images were acquired using a microscope (BX-50, Olympus, Tokyo, Japan) with 20x and 40x objective and photographed with a digital camera (DS-Ri1, Nikon, Tokyo, Japan) equipped on the microscope.

## 2.6 Quantification of Width of Zebrafish Embryo Retina and Inner Nuclear Layer

In the measurement of embryo retina size and inner nuclear layer width, 3 adjacent sections, which with the largest diameter of the lens, were chosen and intervals among the chosen sections was 8 µm. The distance between the exterior top of lens and the apical side of the retina, which represents the width of retina, was measured on the photos using ImageJ v1.53a, indicated by the solid yellow line in Fig.2.1 A. Similarly, the width measurement of INL was conducted according to the distance between the basal and apical side (Fig.2.1 B).

## 2.7 Photoreceptor Cell Density Quantification

To quantify the photoreceptor cell density after irradiation, the number of photoreceptor cells in the central part of the retina was counted in 3 Nissl-staining sections which presented the largest diameter of the lens. The corresponding regions were examined in the control embryos.

## 2.8 Image Processing and Statistical Analysis

The images were processed by ImageJ (Version 1.53a) image processing software. Statistical analysis and graphical representation of the data were conducted by Microsoft Excel. Error bars represents Standard Deviation. Two tailed Student’s *t* test and one-way ANOVA followed by Tukey’s test were used for comparison to determine the statistical significances. The *p* value less than 0.05 was considered significant.

# 3. Results

## 3.1 Early development of retina in zebrafish embryos

A previous investigation in medaka embryo has reported that medaka embryo at 72 hours post-fertilization (hpf) is most sensitive to gamma-ray exposure (Yasuda *et al.*, 2018). Due to a lack of research on the effects of irradiation on embryonic development of zebrafish, it is difficult to specify at which developmental stage zebrafish embryo is most sensitive to irradiation of gamma-rays. Firstly in this research, I investigated the early development of retina of zebrafish embryos and determined the developmental stage of zebrafish embryo at which the retina of zebrafish embryo develops to the same degree as the retina of the 72 hpf medaka embryo.

Based on the morphological characteristics, pigmentation of eye in zebrafish and medaka embryos occurred in a similar manner, however, the timing of the eye pigmentation was 29 and 72 hpf, respectively, and largely different between them (Fig. 3.1). In zebrafish embryo, the characteristic layered structure in retina had not developed at 39 hpf (Fig. 3.2 A) and outer nuclear layer (ONL) began to be formed at 49 hpf (Fig. 3.2 B); three cellular layers, ONL, inner nuclear layer (INL) and ganglion cell layer (GCL), were clearly recognizable at 60 hpf (Fig. 3.2 C). These observations indicate that the structure of the retina of zebrafish embryo at 49 hpf are close to those of medaka embryo at 72 hpf, of which retina shows roughly differentiated ONL, INL and GCL and photoreceptors begin to be differentiated in the ONL (Fig.3.2 D).

Müller glia first differentiated in the central part of the retina apart from the CMZ area in 72 hpf zebrafish embryos not in 53 hpf (Fig. 3.3 A, E). On the other hand, Müller glia first appeared in the central part of the retina in 96 hpf medaka embryo (Fig.3.3 C).

## 3.2 Irradiation of zebrafish embryo with gamma-rays

Based on the morphological similarities of the developing retina between zebrafish and medaka embryos at 72 hpf described above, I irradiated the zebrafish embryos at 29 and 50 hpf with 10 Gy gamma-rays and investigated the effects on the retinal development. The irradiated samples were chemically fixed, sectioned and stained at 24 hours post-irradiation (hpi) to detect induction of apoptosis (Fig. 3.4). When the zebrafish embryos irradiated at 50 hpf, more apoptotic cell deaths were induced in the retina than the embryos irradiated at 29 hpf, 24 after the irradiation. I also found that clusters of apoptotic cell debris were formed not only in the retina but also in the other parts of the brain of the irradiated embryos (Fig. 3.5 A-B). The amount of the induced apoptosis in the zebrafish embryos irradiated at 50 hpf was similar to those induced in the medaka embryos which was irradiated at 96 hpf and cultured for 24 hours (Fig. 3.5 C). Based on these observations, I selected 50 hpf zebrafish embryos to irradiate gamma-rays and investigate the effects induced.

## 3.3 Induction of apoptosis in irradiated retina of zebrafish embryo

In the Nissl-stained sections of non-irradiated embryos at 60 to 96 hpf, no apoptosis occurred (Fig. 3.6 A-C). In contrast, apoptosis began to appear at 10 hpi when 50 hpf embryo was irradiated (magenta arrows in Fig. 3.6 D) and large masses of apoptotic cell debris was obvious at 22 hpi (magenta arrows in Fig. 3.6 E). The most of apoptosis were induced in the INL, whereas a few numbers of apoptosis were induced in the ONL (magenta arrows in Fig. 3.6 E). These results demonstrate that gamma-ray irradiation causes severe damage in the embryonic retina after 22 hpi in zebrafish. In the retina of the 48 hpi embryos (96 hpf), no apoptosis cell debris was present (Fig. 3.6 F), suggesting that apoptotic cell death is induced within 22 hours after the irradiation and not after 48 hours.

The eyeball of the irradiated embryos at 22 hpi and 48 hpi were smaller than those of the non-irradiated embryos (Fig. 3.7 A). Although there is no statistical significance of the difference between the thickness of the INL of the irradiated and control embryos at 22 hpi, presumably because of the small number of the sample (n = 3 for each) (Fig. 3.7 B), the smaller size of the eyeballs of the irradiated embryos could be attributable to the cell deaths and the cessation of cell proliferation induced after the irradiation in the retinal cells of the irradiated embryos.

## 3.4 Müller glia in INL ectopically proliferate in irradiated retina

Anti-glutamine synthetase (GS) antibody, anti-cleaved caspase-3 antibody and anti-PCNA antibody, were used to localize Müller glia, apoptotic cells and proliferating cells, respectively, in the irradiated retina of zebrafish embryo at 22 and 48 hpi when the embryos were irradiated 10 Gy of gamma-rays at 50 hpf (Fig. 3.8).

In the retina of the non-irradiated embryo at 72 hpf, GS-positive Müller glia were present in the INL (white arrowheads in Fig. 3.9 A) and there were no apoptotic cells (Fig. 3.9 B). In the retina of the 22 hpi irradiated embryos, Müller glia located in the central part of the retina changed their cell shape to be round or amoeboid (white arrows in Fig. 3.3 B and sky-blue arrows in Fig. 3.9 D) and some of the Müller glia looked to surround cleaved caspase-3-positive cells, those were apoptotic (yellow arrows in Fig. 3.9 F, F’). Furthermore, some of the Müller glia left their original location in INL and migrated into the ONL, close to the cleaved caspase-3-positive (apoptotic) cells in the ONL at 22 hours after the irradiation (yellow arrows in Fig. 3.10 A’).

PCNA-positive cells, those were proliferating, were only present in Ciliary Marginal Zone (CMZ) (not-filled arrows in Fig. 3.11 A, C) and no cleaved caspase-3-positive cells were found in the non-irradiated 72 hpf embryos (Fig. 3.11 B). In the 22 hpi embryos, cleaved caspase-3-positive cells were present both in the INL and ONL (yellow arrows in Fig. 3.11 E), suggesting that gamma-ray irradiation induced apoptotic cell death in those cells. I also found that PCNA-positive cells were also presented near cleaved caspase-3-positive (apoptotic) cells (sky-blue arrows in Fig. 3.11 F, Fig. 3.12 A’-C’), in addition to those in CMZ (areas enclosed with dashed-dotted line in Fig. 3.11 F, Fig. 3.12 B, C). These ectopic proliferating cells in the INL and ONL were not found in the retinas of the non-irradiated embryos (Fig. 3.11 A).

Interestingly, the shape of the cleaved caspase-3 positive cells in Fig. 3.9 E are obviously different from those shown in Fig. 3.11 E: the cleaved caspase-3 positive cells in Fig. 3.9 E are round and small, whereas the cleaved caspase-3 positive cells in Fig. 3.11 E are amorphous and larger. Apoptosis processes are divided into 3 main steps: cell shrinkage, plasma membrane blebbing and formation of apoptotic dobies, those are phagocytosed by phagocytes. The cleaved caspase-3 positive cells in Fig. 3.9 E might be the cells in early apoptotic process of cell shrinkage and those in Fig. 3.11 E in a form of green dots cluster might be apoptotic cells phagocytosed by glial cells.

In the not-irradiated embryos at 72 hpf, PH3-positive proliferating cells were only in CMZ (Fig. 3.13 B) and this observation was consistent with the result with anti-PCNA antibody (Fig. 3.11 A). In the retina of the irradiated embryos at 22 hpi, GS-positive Müller glia in the INL changed their cell shape to be amoeboid (sky-blue arrow in Fig. 3.13 D) and some of them were present in ONL (green arrows in Fig. 3.13 D), as show in Fig. 9 and 10. Especially, I found that some of the Müller glia were PH3-positive in the irradiated retina (Fig. 3. 13 D, E, F), indicating that some of the Müller glia were proliferating at 22 hpi in the irradiated embryonic retina (yellow arrows in Fig. 3.13 D, E, F and Fig. 3.14).

In the non-irradiated embryos at 96 hpf, PCNA-positive proliferating cells were present only in CMZ (areas enclosed with dashed-dotted line in Fig. 3.15 C, F, I). In contrast, PCNA-positive cells were present not only in CMZ but also appeared in the INL at 48 hpi in the irradiated embryos (Fig. 3.15 D, F, G, I). A fraction of the ectopic PCNA-positive cells in the INL of the irradiated embryos (yellow arrows in Fig 3.15 F, I) were not round but spindle-shaped, strongly suggesting that they were Müller glia. The other ectopic proliferating cells in the INL were smaller and round-shaped, suggesting that they might be amacrine cells and horizontal cells. These results strongly suggest that Müller glia and the other retinal cells (amacrine cells and horizontal cells) in the INL ectopically proliferated in the retina at 2 days after the irradiation in the irradiated zebrafish embryo, presumably to repair the damaged retina.

## 3.5 Photoreceptors regeneration in irradiated zebrafish embryos

In the retina of the irradiated zebrafish embryo at 22 hpi, a major body of the apoptotic cell death was induced in the INL and a few was induced in the ONL (magenta arrows in Fig. 3.16 D). The apoptotic cells in the ONL in the immunofluorescence staining experiments described above (green arrow in Fig. 3.9 F’, Fig. 3.10 A’, yellow arrows in Fig. 3.11 E, F, sky-blue arrows in Fig. 3.12 A’, B’, C’) were confirmed. These results strongly suggest that photoreceptors were damaged by irradiation and conducted apoptotic cells death in zebrafish embryos, in contrast that the photoreceptors of medaka embryo at 96 hpf do not conduct apoptotic cell death after gamma-ray irradiation (personal communication by T. Yasuda).

The photoreceptors first appear in at 49~50 hpf in embryonic retina of zebrafish and well developed after 72 hpf (Morris and Fadool, 2005; Stenkamp, 2007). The typical morphology of normal ONL is a dense arrangement of slender conical photoreceptors as shown in Fig. 3.16 A-C’. At 22 hours after the irradiation, a large number of apoptotic cells were present in the INL and ONL of the irradiated retina and the characteristic layered structure of the retina was severely disturbed (Fig. 3.16 D). At 48 hpi, no apoptotic cell death was found in the retina of the irradiated embryo, however, cells with the almost same morphology as the round cells in INL were partially present in the ONL (yellow arrows in Fig. 3. 16 E’) with the normally layered photoreceptors (orange arrows in Fig. 3. 16 E’). Then, at 72 hpi, in a half of the irradiated embryos (3 embryos among examined 6 in total), the photoreceptors in the ONL showed the normal appearance (orange arrows in Fig. 3. 16 F’), however, the ONL structure with photoreceptors in the remaining 3 embryos were still severely disturbed and the ONL and INL were not divided (Fig. 3.16 G, G’). It would be noteworthy that there were individual differences in the repair ability among zebrafish embryos after gamma-ray irradiation.

Furthermore, in the retina of the irradiated embryo at 48 hpi, PCNA-positive (proliferating) cells appeared not only in CMZ (areas enclosed with dashed-doted line) and the INL (yellow arrows and magenta arrows in Fig. 3.17 C, D, D’) but also in the ONL (green arrows in Fig. 3.17 C, D, D’). The cell shape of the PCNA-positive cells in the ONL were not typical of normal cone or rod photoreceptors, instead, they showed a round and smaller form, suggesting that they were progenitor cells of photoreceptor. Compared with the irradiated embryos, there were no proliferating cells in the ONL of the not-irradiated embryos at 96 hpf (Fig. 3.17 A, B, B’). It might be noteworthy that the spindle-shaped PCNA-positive (proliferating) cells were present in the INL of the irradiated embryo (magenta arrows in Fig. 3.17 C, D, D’), those might be Müller glia, and the PCNA-positive cells in the ONL might be photoreceptor progenitor cells (green arrows in Fig. 3.17 C, D, D’). These results strongly suggest that Müller glia dedifferentiate to the progenitor cells and play an essential role in photoreceptor regeneration and differentiation after irradiation in zebrafish embryos as in adult and larval zebrafish (Fimbel *et al.*, 2007; Bernardos *et al.*, 2007).

The retina size and INL width in the unrepaired irradiated embryos at 72 hpi were significantly smaller and thinner than those of the non-irradiated embryos (Fig. 3.18 A, B), suggesting that 72 hours are not enough for the complete restoration of the damaged retina since retina of zebrafish embryos are developing rapidly even without irradiation.

In normal development of zebrafish, the photoreceptors in ONL of 120 hpf embryos were packedly arranged (Fig. 3.19 A, A’) and the photoreceptors in ONL showed almost normal arrangement in the irradiated and repaired embryos, however, they were more loosely arranged than the not-irradiated embryos (Fig. 3.19 B, B’). Moreover, the density of photoreceptors in the ONL (labelled area enclosed with red dashed line in Fig. 3.19) indicated that less photoreceptors were distributed in the ONL of the irradiated embryos at 72 hpi than the non-irradiated embryos (Fig. 3.19 C). Since the photoreceptor progenitor cells appeared in the ONL at 48 hpi (Fig. 3.17 C, D, D’), these loosely arranged cells in the ONL could be newly formed photoreceptors.

In the irradiated embryos at 48 hpi, apoptotic cells had disappeared and the eye size and INL width of the irradiated embryos were still smaller and thinner than non-irradiated embryos. The cell arrangement in the INL and ONL of the irradiated embryos were still clearly disturbed. Some of the cells in the ONL showed round cell shape, strongly suggesting that they were not differentiated photoreceptors (Fig. 3.20 A, A’). The results presented in this study suggest that the Müller glia in the INL moved to the ONL and de-differentiated to progenitor cells of photoreceptors.

In the irradiated embryos at 72 hpi, the abnormal round cells were still present in the ONL and some of the adjacent cells showed normal morphology of photoreceptors in a half of the irradiated embryos (Fig. 3.20 B, B’), suggesting that the progenitor cells in the ONL differentiated to photoreceptors and the ONL began to be restored to be the normal photoreceptor layer.

On the other hand, in the remained half embryos, I found that photoreceptors in the ONL have restored to the normal structure, although they were arranged still loosely (Fig. 3.20 C, C’) and it can be concluded that photoreceptors were regenerated within 2 days.

1. **Discussion**

The self-repair ability is enhanced after retinal damage in some non-mammalian vertebrates such as fish and frogs and the most representative species is zebrafish (Fimbel *et al.*, 2007; Fischer and Reh, 2001; Langhe e*t al.*, 2017). The mechanism of retinal regeneration in adult zebrafish has been well studied in recent decades but the regeneration process in embryonic stage of zebrafish remains to be addressed. In this research, before-hatching embryos of zebrafish were irradiated with gamma-rays, and the retina regeneration process was investigated. My study firstly demonstrated an irradiation-induced retinal injures and regeneration in zebrafish embryos and that Müller glia play the central roles during retina regeneration in zebrafish embryos as in larval and adult zebrafish.

## 4.1 Retina regeneration in zebrafish embryos is mediated by Müller glia in a similar process in larval and adult zebrafish.

Most of the previous studies on retinal regeneration were conducted on adult zebrafish retina. Many injury methods were employed to destroy retinal neurons or all retina, such as high-intensity light damage which causes photoreceptors degeneration (Bernardos *et al.*, 2007; Bailey *et al.*, 2010; Raymond *et al.*, 2006; khan *et al.*, 2020), neurotoxins administration leading to retinal cell death (Fimbel *et al.*, 2007), physical surgery like needle impalement into retina (Yurco and Cameron, 2005). Many researches showed that Müller glia firstly respond to the cell damage after retinal injury, and then Müller glia migrate to the damaged cells and conduct phagocytosis of the damaged retinal cells (Goldman, 2014; Morris *et al*., 2005; Bailey *et al.*, 2010). It is also suggested that microglia in retina also conduct phagocytosis of the injured cells in adult zebrafish (Craig *et al.*, 2008; Fischer *et al.*, 2014). Then, Müller glia start to de-differentiate and enter cell cycle to proliferate. The Müller glia are reprogrammed to be retinal stem cells and conduct self-renewal and symmetrical cell division to generate neural precursor cells in the injured retina (Nagashima *et al.*, 2013). These precursor cells differentiate into all of the retinal cells to restore the structure of retina injured (Fausett and Goldman*.*, 2006; Ramachandran *et al.*, 2010b).In the process of Müller glia-dependent regeneration, Secretion of tumor necrosis factor-α (TNFα), Wnts, and heparin-binding EGF-like growth factor (Hbegf) has been reported to promote Müller glia reprogramming and progenitor formation (Ramachandran *et al.*, 2011; Wan *et al.*, 2012; Nelson *et al.*, 2013).

In this research, I found that some Müller glia surrounded the apoptotic cells in the irradiated INL (yellow arrows in Fig. 3.9 F) or Müller glia migrated to apoptotic cells in the ONL (yellow arrows in Fig. 3.10 A’) after the irradiation. These observations were consistent with those in the injured retina of adult zebrafish (Yurco and Cameron, 2005; Morris *et al*., 2005; Fausett and Goldman, 2006; Bailey *et al.*, 2010). There are two possibilities to explain this interesting behavior of Müller glia in the irradiated retina. The one might be that Müller glia conducted phagocytosis of these apoptotic cells in embryonic retina. Microglia also would be involved in the phagocytosis of the injured retinal cells, since it is reported that microglia settle into the embryonic retina at 48 hpf in zebrafish embryos (Renawat and Masai, 2021). The other one might be that Müller glia migrate close to apoptotic cells to provide progenitor cells for the restoration of the damaged retinal structure.

I also found that one spindle-like proliferating cell appeared in the INL of the irradiated retina after the irradiation (yellow arrow in Fig. 3.12 C’). Based on the cell morphology, this finding strongly suggests that Müller glia proliferation was induced after the irradiation. In addition, Müller glia obviously proliferated in the ONL of the irradiated retina (yellow arrows in Fig. 3.13 F, Fig. 3.14 C’). These results indicate that Müller glia re-entered the cell cycle after the retinal injury. Interestingly, my finding demonstrates that Müller glia located close to the proliferating cell (sky-blue arrows in Fig. 3.14 F’). Based on the reprogramming process of Müller glia in adult zebrafish (Goldman, 2014), this Müller glia might just started to proliferate and provided the progenitor cells there. Anti-Sox2 (an indicator of reprogrammed progenitor cells) and anti-GS double immune-staining will confirm the reprogramming and dedifferentiation of Müller glia in irradiated embryonic retina.

Proliferating cells were still present in the INL at 48 hpi (96 hpf) (yellow and green arrows in Fig. 3.15 F, I), whereas no proliferating cells were present in the INL of the non-irradiated embryos. These spindle-like signals (yellow arrowhs in Fig. 3.15 F; magenta arrows in Fig. 3.17 C, D, D’) might represent the cell body of Müller glia and the round cells (green arrows in Fig. 3.15 I; yellow arrows in Fig. 3.17 C, D, D’) might be newly generated retinal neural cells in INL, such as amacrine cells, horizon cells and bipolar cells. It may be conclusive that these proliferating neural cells in the irradiated INL might differentiated from the de-differentiated Müller glia, as reported in adult zebrafish (Fausett and Goldman, 2006; Bernardos *et al.*, 2007; Fimbel *et al.*, 2007; Bailey *et al.*, 2010).

Taken together, it can be concluded that retina regeneration in zebrafish embryos is a process mediated by Müller glia (Fig.4.1; Fig. 4.2), which is the same as in larval and adult zebrafish (Bernardos *et al.*, 2007; Lust and Wittbrodt, 2018).

## 4.2 Müller glia might play an important role in photoreceptor regeneration and differentiation after irradiation

The findings in this study also demonstrate that apoptotic cells were induced in the ONL (yellow arrows in Fig.3.11 E, sky-blue arrows in Fig. 3.12 A’), which is consistent with the result in the Nissl staining observation (magenta arrows in Fig. 3.16 D). At the same time, Müller glia migrated close to the apoptotic cells in the ONL (green arrows in Fig. 3.9 F’, Fig. 3.10 A’), suggesting that Müller glia scavenged the damaged photoreceptors. On the other hand, proliferating cells were present close to the apoptotic photoreceptors in the ONL (sky-blue arrowheads in Fig. 3.12 A’, B’) and proliferating Müller Glia were also identified in the ONL (yellow arrowheads in Fig. 3.13 F, Fig. 3.14 C’). In larval and adult zebrafish, it has been reported that Müller glia proliferate and migrate to the ONL after photoreceptor damage by high-intensity light (Bernardos *et al*., 2007; Fausett and Goldman, 2006). These findings strongly suggest that Müller glia participated in the phagocytosis of the damaged photoreceptors after the irradiation in zebrafish embryos.

In addition, one proliferating cell (green arrows in Fig. 3.17 C) and a cluster of small proliferating cells were found in the ONL of the irradiated retina (green arrows in Fig. 3.17 D-D’). Cone photoreceptors are formed from 48 to 60 hpf during normal embryonic development of zebrafish (Stenkamp, 2007) and rod photoreceptors develop before 120 hpf (Morris and Fadool, 2005). Although it is surprising to find the cell proliferation in the CMZ did not cease even after the irradiation (Fig. 3.17 C, D, D’), the proliferating cells located in the ONL (green arrows in Fig. 3.17 D, D’) clearly demonstrate that regeneration of photoreceptors was going in the ONL after irradiation. Another point which should be mentioned is that these distinctive characteristic cells (green arrows in Fig. 3.17 D, D’) were present in the same position where the abnormal round cells were present in the ONL in Nissl staining (Fig. 3.16 E, E’). It can be possible that these abnormal round cells appeared in Nissl staining (Fig. 3.16 E, E’) might be the progenitor cells for photoreceptor. The best way to test this possibility is double-labelling immunostaining with zpr-1, a cell marker of photoreceptor, and atoh7, an indicator of neurogenesis, which will be conducted in the future.

Interestingly, I found that one PCNA-positive Müller glia located in the INL (magenta arrow in Fig. 3.17 C) and that the PCNA-positive proliferating cells (green arrows in Fig. 3.17 C) located in the ONL just beneath the PCNA-positive Müller glia (magenta arrow in Fig. 3.17 C). This proliferating cell in the ONL might be a progenitor cell derived from de-differentiated and proliferating Müller glia in the INL (magenta arrow in Fig.3.17 C). Linage-trace experiments of Müller glia would confirm this interpretation.

## 4.3 Cell shape of apoptotic cells in irradiated zebrafish embryos

Process of apoptotic cell death can be commonly divided into 3 main steps: cell shrinkage and chromatin condensation, nucleus and organelle collapsing and membrane blebbing, and apoptotic bodies formation and phagocytosis by macrophages or the other immune cells (Wyllie, 1997; Elmore, 2007; Abou-Ghali *et al.*, 2015). Apoptosis is an energy-consuming process that involves the activation of a group of cysteine proteases called caspases. The molecular pathway of apoptosis is initiated by the cleavage and activation of caspase-3. Apoptotic cells sequentially conduct DNA fragmentation, degradation of proteins in nucleus and cytoplasm, apoptotic body formation and release “eat-me” signals for phagocytes (Elmore, 2007).

The cleaved caspase-3 positive (apoptotic) cells in Fig. 3.9 E and Fig. 3.11 E (yellow arrows) showed different cell shapes: the cleaved caspase-3 positive (apoptotic) cells in Fig. 3.9 E was present in a form of green dots, whereas cleaved caspase-3 positive cells in Fig. 3.11 E (yellow arrows) were in a form of a cluster of green dots, although there is a possibility that the difference of cell shape could be an artifact during chemical fixation. Generally, “apoptotic bodies” are much smaller than the large green spots shown in Fig. 3.9 E which might represent intact cells before conducting the process of apoptosis. The small green spots in Fig 3.9 E might be “apoptotic bodies”, however, “apoptotic bodies” usually appear in mass as shown in Fig. 3.12 A' and B' (sky-blue arrows). The green cell clusters in Fig. 3.11 E might represent a microglia which phagocytosed and gather the apoptotic bodies.

* 1. **Establishment of retina injury model by irradiation in zebrafish embryo**

There have been numerous studies on the retinal regeneration in adult and larval zebrafish for the recent decades and most studies employed neurotoxins or mechanical surgery (such as needle impalement) to establish retinal regeneration model in adult and larval zebrafish (Yurco and Cameron, 2005; Fimbel *et al.*, 2007; Fausett and Goldman, 2006; Lust and Wittbrodt, 2018). Some studies employed heater strip or high-intensity light (or laser) to damage photoreceptors in adult and larval zebrafish (Bernardos *et al.*, 2007; Bailey *et al.*, 2010; Raymond *et al.*, 2006; khan *et al.*, 2020). However, 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. Similarly, the size of zebrafish larvae is too small to operate a retina surgery. Neurotoxins should be administrated by injection into the eye socket or eyeball of adult zebrafish. Mechanical damage should operate a surgery on the eyes, but zebrafish embryos are too small to perform such surgical manipulations on them. Furthermore, some damage methods require a long period of the operation but the embryonic development of zebrafish is very rapid: the embryo hatch within 4 days after fertilization, which is extensively rapid than medaka (Fig. 4.3). 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 difficult.

Recent studies have shown that irradiation can induce neurotoxicity in fish embryos (Geiger *et al.*, 2006) and developing central nervous system in vertebrate 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 induce pinpoint damage during the embryonic development at the specific development stage. In this study, the irradiation-induced injury model allows me to observe the damage induction and repair process precisely and reproducibly even in the rapid developing zebrafish embryos.

There are few studies on the effects of irradiation on zebrafish during the embryonic period before hatching. Since a previous study reported that medaka embryo is most sensitive to gamma-ray irradiation at 72 hpf (Yasuda *et al.*, 2018), I selected 29 hpf and 50 hpf zebrafish embryos for irradiation and examined which stage is more sensitive to gamma-ray irradiation. In 29 hpf zebrafish embryos, eye pigmentation starts, which might be consistent with the retina pigmentation in 72 hpf medaka embryos (Fig. 3.1 A, B). On the other hand, the three-layered retina structure developed in 50 hpf zebrafish embryos was very similar to that in 72 hpf medaka embryos (Fig. 3.2 B, D). I found that more apoptotic cell death was induced in 50 hpf zebrafish embryos than in 29 hpf zebrafish embryos, in which only small-mass of apoptotic cell debris was observed (Fig. 3.5 A, B) and concluded that 50 hpf zebrafish embryos is more sensitive to gamma-ray irradiation. The previous studies on the effects of irradiation on zebrafish embryonic development were conducted on embryos at the gastrula period (5.25~10 hpf) and segmentation period (10~24 hpf) and the impacts of irradiation on the following development were investigated (Zhou *et al.*, 2014; Szabó *et al.*, 2018; Geiger *et al.*, 2006). There is only one report that high levels of apoptosis are induced in the embryos irradiated at 48 hpf (Barrett *et al.*, 2018). This finding is consistent with the present result, and 50 hpf zebrafish embryo was selected to be irradiated in this study.

* 1. **The hypothesis of strong retina regeneration ability in zebrafish**

In zebrafish, even adults are able to regenerate their fins, skin, heart, hair cells in the lateral line of the body, and central nervous system and are often used to investigate the mechanisms of tissue regeneration (Akimento *et al.*, 1995; Rieger and Sagasti*.*, 2011; Poss *et al.*, 2002; Ma *et al.*, 2008; Harris *et al.*, 2003). I think the reason why zebrafish have such a strong regenerative capacity is an important biological question that deserves to be focused on. Here, I would like to propose my working hypotheses.

Generally, fish, amphibians, and reptiles grow throughout their lifetime, unlike birds, and mammals, that reach their maximum adult size after the growth period (Williams, 2017). Zebrafish belong to the family of carp, Cyprinidae. In the natural state, adult carp are about 50-80 cm in length and their lifetime are about 20-years. In contrast, adult zebrafish are only about 3 cm in length and with about 2 or 3-year lifetime. It is reported that the retina still grows in adult zebrafish (Van Houcle *et al.*, 2019). In the other words, zebrafish could be juvenile through its lifetime, growing up until their death.

Neoteny is a phenomenon in which a species retains juvenile or even embryonic characteristics into post-juvenile or even adult life. It is a popular belief that one of the possible mechanisms of appearance of many specific features is developmental retardation, or neoteny (Somel *et al.*, 2009). If neoteny might occur in zebrafish, the strong regenerative capacity through embryonic, larval and adult periods can be explained. For example, fibroblast growth factor 5 (FGF5) is widely expressed in embryonic tissues of Asian icefish (*Protosalanx chinensis*) during embryonic development but not in adult (Zhang *et al.*, 2020). It is reported that FGF5 was involved in the neural development in zebrafish during the transition from larvae to adult (Leerberg *et al.*, 2019). It is also reported that FGF signaling, which FGF5 is involved, still works in photoreceptor maintenance in the adult zebrafish retina (Hochmann *et al.*, 2012). Although FGF5 plays a role in hair growth in mice (Ozawa *et al*., 1998), this gene expression was at a low level in central nervous system in adult mice (Haub *et al.*, 1991). FGF5, a factor that is still expressed in nervous system of adult zebrafish but not in adult mice, could be a feature of the neotenic nervous system of adult zebrafish. It has been reported that a similar molecular machinery is used 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 extensively strong capacity of retinal regeneration in adult zebrafish might be endowed as a result of neoteny or accelerated sexual maturation in ancient zebrafish.

# 5. Conclusions and perspectives

The regeneration mechanism in adult and larval zebrafish has been well studied in recent decades. It has been believed that zebrafish embryos can conduct retinal regeneration as we as adult and larval zebrafish, however, there is no study to investigate the retinal regeneration in before hatching embryos of zebrafish, since. it is difficult to injure the small and rapidly developing retina of the embryos. In the present study, I employed gamma-ray irradiation to injure zebrafish embryos and established irradiation-induced retina injury model in zebrafish embryos. Using this model, I investigated the process of retinal regeneration after irradiation in zebrafish embryo.

The findings of this study demonstrated that gamma-ray irradiation induced a large number of apoptotic cell death mainly in the INL and some in the ONL after 22 hours irradiation in zebrafish embryos irradiated at 50 hpf. Müller glia change their cell shape and left their original position in the INL, then migrated to surround the apoptotic cells in the INL and ONL. The Müller glia ectopically proliferate there and produce newly generated retinal cells, suggesting that the Müller glia de-differentiated to be retinal stem cells and generated retinal cells including photoreceptors to restore the injured retina after irradiation. It can be concluded that the retina regeneration process in zebrafish embryos shares the same process in the larval and adult zebrafish.

These findings enhance our understanding of retina regeneration in zebrafish throughout lifetime, providing a whole new sight to figure out why zebrafish possesses strikingly strong regeneration ability.

Finally, a number of limitations should be considered. Firstly, whether Müller glia reprogrammed after injury response should be confirmed because reprogramming is an initial step for Müller glia proliferation and progenitor cells production. Secondly, I should confirm whether the abnormal round-shape cells in the ONL at 48 hpi are photoreceptor progenitor cells by double immunofluorescence staining in the future. Similarly, the identity of proliferating cells in the INL also need to be figure out in order to make the retina regeneration process more clearly. Thirdly, the reason why some embryos repair their retina but others do not repair also need to be discussed in the future plan.

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# Table and Figures

**Table 1.1: Retina regeneration ability in vertebrates**

|  |  |  |  |
| --- | --- | --- | --- |
| Animals | Embryo | Larvae | Adult |
| Fish | ? | O | O |
| Amphibian | O | O | O |
| Avian | O  (Limited) | N.D | X |
| Mammal | O  (Limited, only in cell culture) | N.D | X |

O: possess retina regeneration ability

X: possess limited or no retina regeneration ability

N.D: no data