Understanding the role of DNA methyltransferases (Dnmts) in retina regeneration of zebrafish

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Certificate of Examination

This is to certify that the dissertation titled "Understanding the role of DNA methyltransferases (Dnmts) in retina regeneration of zebrafish" submitted by Ms. Yengkhom Roja Devi (Reg.No.MS12006) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr.Shashi Bhushan Pandit Dr. Mahak Sharma Dr.Rajesh Ramachandran
(Supervisor)

Dated: April 21, 2017

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Rajesh Ramachandran at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Yengkhom Roja Devi
(Candidate)

Dated: April 21, 2017

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr.Rajesh Ramachandran
(Supervisor)

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Notations

- **Dnmt** - DNMT protein in zebrafish
- ***dnmt*** - genes of DNMT in zebrafish
- **Mpf** - Minutes post fertilization
- **Hpf** - Hours post fertilization
- **Dpf** - Days post fertilization
- **Hpf** - Hours post fertilization
- **Dpf** - Days post fertilization
- **ONL** - Outer nuclear layer
- **INL** - Inner nuclear layer
- **GCL** - Ganglion cell layer
- **Aza** - Azacytidine
- **Zeb** - Zebularine
- **Min.** - Minutes
- **BrdU** - Bromodeoxyuridine
- **PCNA** - Proliferating Cell Nuclear Antigen

Contents

Abstract

Lower vertebrates such as fishes and frogs have the ability to regenerate damaged central nervous system. They show a strong regenerative response to retinal injury that can result back in the restoration of visual function, Müller glia, being the key to this regenerative response. If we have a thorough understanding of molecular mechanisms involved in retina regeneration in such lower vertebrates, it can help us design strategies for improving Müller glia dedifferentiation and regeneration of retina in human and help in recovery from retinal damage and vision loss. Many genes are now well established for their important roles in the regeneration of retina in zebrafish (*Danio rerio*); however, roles of epigenetic regulation of genes in this context are not understood clearly. Understanding the epigenetic regulation of a gene is as important as understanding their role in retina regeneration. Here we are investigating some of the roles of epigenetic regulators during zebrafish retina regeneration by studying DNA methyltransferases (Dnmts), a family of enzymes which catalyse DNA methylation. Induction of dnmts and their roles during retina regeneration were examined, and the candidate genes that may be epigenetically regulated by Dnmts during retina regeneration were identified. It was observed that Dnmts are regulated during embryonic development in eyes and are also regulated during the retina regeneration. When Dnmts were blocked using Azacytidine (a pharmacological Dnmts blocker) the increase in proliferation of Müller glial cells was observed, and the increase in proliferation is more significant when we blocked the Dnmts in early de-differentiation phase. Various retina regeneration associated genes are observed to be down regulated or up regulated when Dnmts are blocked during retina regeneration.

Chapter 1

INTRODUCTION

Loss of vision is a major problem affecting the human. Loss of vision may be due to injuries or several diseases. Even though there are various strategies for restoring the sight in vision loss, they have various limitations and low success rate. So if we can develop a reparative strategy such that retina can heal by itself without an introduction of any foreign materials, it would be a great success. We can develop such strategy if we could understand the retina regeneration exhibited in lower vertebrates such as teleost fishes and frogs. Such successful retina regeneration in teleost fishes is because of the Müller glial cells that dedifferentiate, following injury, divide and then give rise to different cell types for retina regeneration(1).

Retina regeneration is widely studied in zebrafish because of its strong regenerative capacity, and also it is a convenient model organism to handle and maintain. Various signaling mechanisms and genes involved in reprogramming of zebrafish Müller glial cells and retina regeneration have been understood; however current understanding is not sufficient enough to explain clearly why Müller glia reprogramming and successful regeneration of retina after injury is not present in mammals. In mammals, after injury, Müller glia responds by reactive gliosis which is often accompanied by hypertrophy (2-3). To some extent, mammalian Müller glial cells can proliferate and also express genes which are present in zebrafish retinal stem cells as a response to injury (4, 5) but they cannot give rise to different cell types in vivo. This observation suggests us that mammalian Müller glia has regenerative potential and at right conditions and circumstances it can repair damaged retina like that in lower vertebrates. Understanding retina regeneration can also give us a clear vision of CNS regeneration.

1.1. Basic Theory

1.1.1. Müller Glial cells and regeneration of retina in zebrafish.

Zebrafish retina like that of other vertebrate's retina is composed of broadly three cellular layers-

- The outer nuclear layer (ONL): This layer is just internal to retinal pigmented epithelium layer. Photoreceptor cells (rods and cons) are present in this layer.
- The inner nuclear layer (INL): Amacrine cells, bipolar cells and horizontal cells which are interneurons reside in this layer.
- The ganglion cell layer (GCL): Ganglion cells are present in Ganglion cells layer.

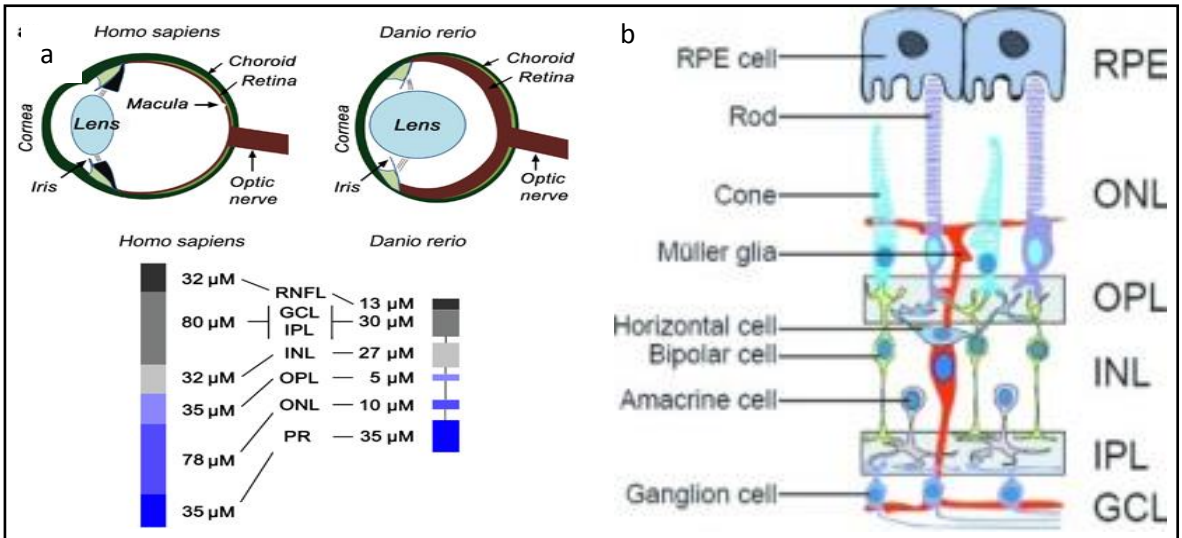


Figure I: a) Comparison of the human eye and zebrafish eye b) Vertebrates retinal cell layers and types of cells. Source: a)Retinal Eye (2014) 28, 367–380; doi:10.1038/eye.2014.19, b) Martinez-De Luna RI, Zuber ME - J Ophthalmic Vis Res (2014).

The photoreceptor cells which are present in ONL absorbed the light falling on the eyes, and the signals are transmitted through the inter-neurons present in the INL and passed to Ganglion cells in GCL. Ganglion cells then collect all the signals and gave it to optic nerves which further transfer the information to the brain to process, allowing us to see. Müller glia is a type of glial cells that are present in the retina, and are common to all the vertebrates. Müller glia's cell bodies are present in INL, but it spans all the retinal layers. It contacts neighboring neurons through its processes. Thus, Müller glia are arranged and positioned in such a way that it can successfully monitor and maintain the retinal homeostasis which can contribute to structure and functions of the retina (6).

1.1.2. The response of zebrafish Müller glia to retinal injury.

To study the regeneration in the zebrafish, various modes of injury is used. Some of them are exposure to intensely bright light for prolonged period, exposure to ultraviolet radiation, injection of toxins into vitreous of eyes (doses of ouabain and NMDA), and mechanical injury, widely done by poking with a needle. Mechanical mode of injury can damage all types of cells in the retina and so is more favored to understand the retina regeneration in wholesome perspective.

Zebrafish Müller glia initiates a transient hypertrophy gliotic response, after injury (7), and is followed by reprogramming event that allows it to acquire retinal stem cells like properties (8). Understanding the mechanisms involved in the transition from gliotic response to regeneration can help us to unlock the potential in mammalian Müller glia to regenerate. There are three essential steps for the retina to regenerate. The First step is dedifferentiation, allowing the Müller glia to acquire stem cell-like characteristics, next is proliferation and forming a population of Müller glia progenitors cells (MGPC) that are multipotent followed by cell cycle exit of MGPC and final step is neuronal differentiation(9). In zebrafish the transition of Müller glia to MPGC takes 2dpi (days post injury), the proliferation of multipotent MGPC happen from 2pi and 7dpi where the peak of proliferation is at 4dpi, the differentiation of MGPC to different retinal cell types starts after 7dpi.

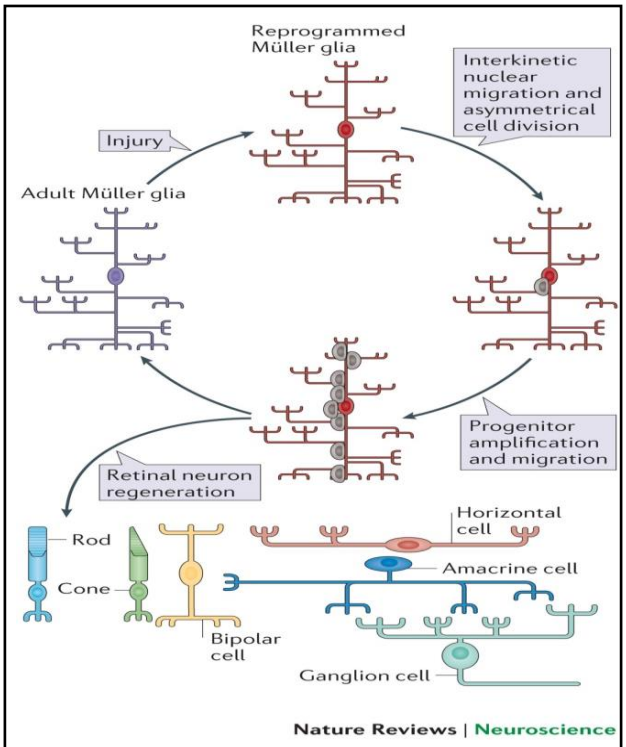


Figure II: Multipotent reprogrammed Muller glia generated in response to injury for retinal repair. Image source: Daniel Goldman, doi:10.1038/nrn3723 | VOLUME 15 |

1.1.3 Signaling mechanisms involved in retina regeneration

Dying cells in damaged retina initiate the retina regeneration by sending information to Müller glia for reprogramming. This information may be through various secreted factors or by their alteration in interaction with processes of the Müller glia. Recent studies have revealed that varieties of growth factors and cytokines are induced in injured retina of zebrafish and can stimulate proliferation of Müller glia when provided with appropriate conditions. These growth factors and cytokines include heparin-binding epidermal-like growth factor a (Hb-egfa), tumour necrosis factor-α (Tnfα), Insulin, Igf-1, IL11 and Leptin(9,10,11). These factors are found to be expressed by MGPC and may be acting in an autocrine and paracrine manner to reprogram Müller glia. Out of these factors, Tnfα is the factor found to be expressed in dying cells, representing a signal that can relate cell death to activation of Müller glia (11). Multiple signaling cascades get activated during retinal regeneration, and some of the signaling pathways which are found to be regulating retina regeneration are glycogen synthase kinase 3β (Gsk3β)–β-catenin, Mapk–Erk, Notch, and Jak–Stat signaling pathways (12-15). Out of these, activation of b-catenin in MGPCs has been confirmed (12, 15). All these pathways are found to be involved in expression of *achaete-scute homologue 1a* (*ascl1a*), after injury (12, 13, 14).

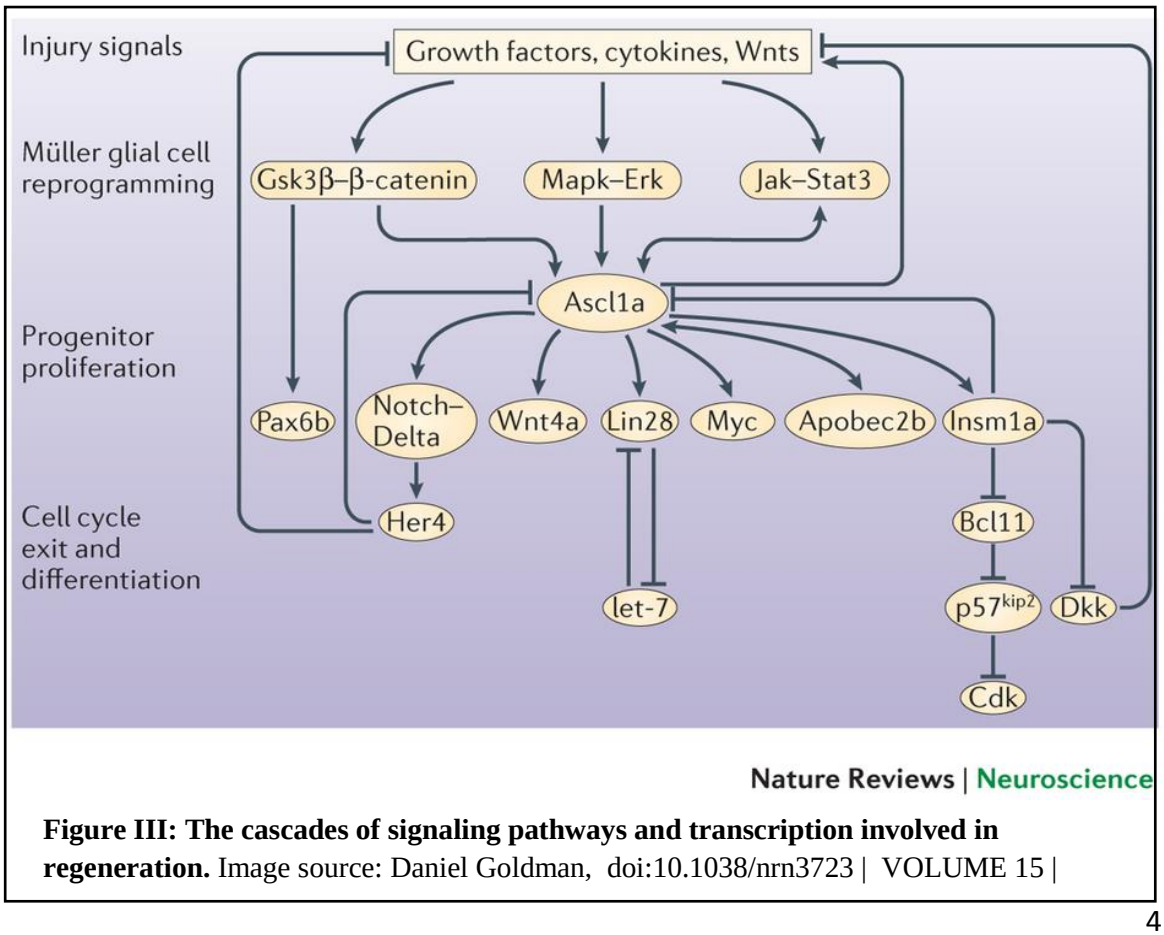


Figure III: The cascades of signaling pathways and transcription involved in regeneration. Image source: Daniel Goldman, doi:10.1038/nrn3723 | VOLUME 15 |

The expression of *ascl1a* results in genetic activation of Müller glia for proliferation and suppressing of cellular differentiation. Ascl1a can regulate the expression of *Wnts, Lin 28, Myc, Apobec , insm1a* and *stat* which plays a major role in early induction in retina regeneration. Retina regeneration after the early reprogramming event is progressed by the suppression of *let-7* micro RNA, contributed by Lin28, and induction of Wnt signaling which are essential for the proliferation of MGPCs. Then at later time point, the regenerative response is restricted by the reappearance of *let-7*miRNA, delta-notch signaling and Insm1a mediated transcription repressive events that are very important for prevention of tumor formation and restoration of retinal homeostasis (16).

1.1.4. DNA methylation and Dnmts

DNA methylation is an epigenetic mark which involves transferring of a methyl group to the C-5 of cytosine covalently by DNA methyl transferases (DNMT) (19). In the methylation reaction S-Adenosylmethionine (SAM) a modified amino acid donates the methyl group. Methylation of DNA Methylation occurs generally in CpG dinucleotides in somatic cells, while methylation in non-CpG context is also observed to present in high amount in embryonic stem cells in case of mammals (20). Non-CpG methylation is also reported in case of zebrafish (21). DNA methylation represses expression of gene by recruiting proteins that can repress gene or by inhibiting the binding of transcription factors to DNA (Figure IV)

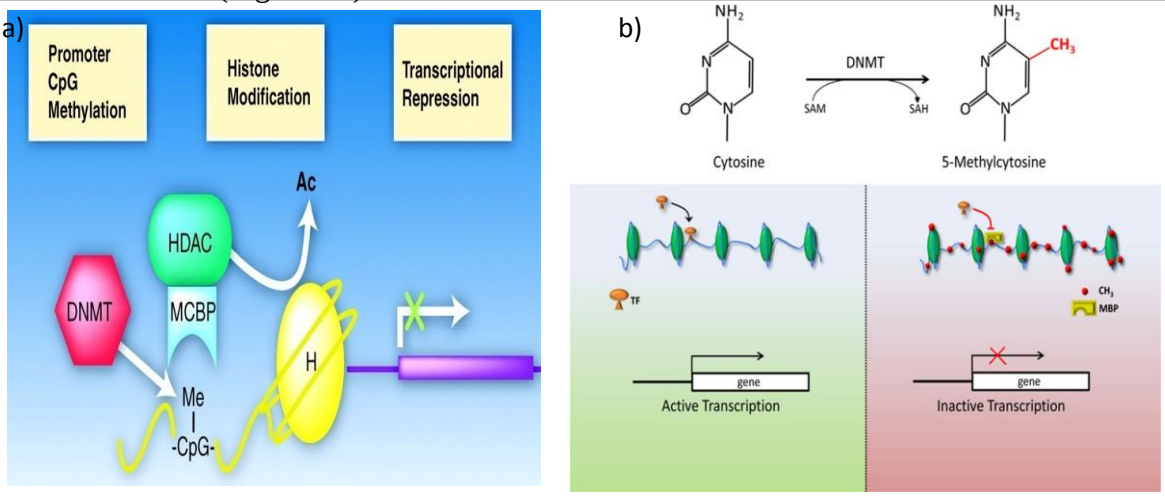


Figure IV: Repression of gene expression by: - a) recruitment of methylated cytosine binding protein b) Inhibiting binding of transcription factors. Image source:Norman J. Lacayo, Jorge F. DiMartino, Michael C. Wei and Gary V. Dahl,DOI: 10.1158/1078-0432.CCR-06-0651

Family of DNMTs consists of DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L (22). DNMT1 is known as maintenance methyl transferase because it preferentially methylate semi-methylated DNA, and so copy methylation patterns to daughter strands during DNA replication (22). DNMT3A and DNMT3B performed *de novo* methylation and have more preference for unmethylated CpG dinucleotides (23). Even though DNMT3L doesn't have catalytic activity by itself, it helps DNMT3A and DNMT3B by increasing their ability to bind to SAM (24). Methylation of some of the regions of the genome, especially repetitive elements require Cooperation among different Dnmts (25).

There are eight Dnmts present in zebra fish from Dnmt1 to Dnmt8 and their mechanism of DNA methylation in zebrafish similar to that of mammals.

Pharmacological blockers of DNMTS

Azacytidine – Azacytidine is an analog of cytosine nucleoside and after they get metabolized to 5-aza-2′-deoxycytidine-triphosphate, cytosine get replaced with azacytosine. DNMTs can recognize the azacytosine - guanine dinucleotide and can methylate it by nucleophilic attack which leads to formation of a covalent bond between carbon-6 atom of the cytosine ring and DNMTs. Because of this covalent bond the DNMTS will get trapped and will not be able to perform the methyl transferase function and further degrade the trapped DNMTS (26).

Why understanding the roles of Dnmts in retina regeneration is important?

Even though various genes are now well established for their important roles in retina regeneration, roles of epigenetic are not understood clearly. Examining the epigenetic regulations of genes becomes very important for having a complete understanding on retina regeneration. Reprogramming of Müller glia to form MGPCs has similar characters to that of somatic cell reprogramming to generate induced Pluripotent stem cells (iPSCs). During reprogramming for formation of iPSC, it is observed that de-methylation event is exhibited by pluripotency genes, and this enables their chromatin to become more open and accessible for gene expression (17, 18). So, demethylation events might also be exhibited by genes regulating reprogramming of Müller glial cell to form MGPC during retina regeneration.

1.2. RESULTS AND OBSERVATIONS

1.2.1. Expression of *dnmts* in developing embryos

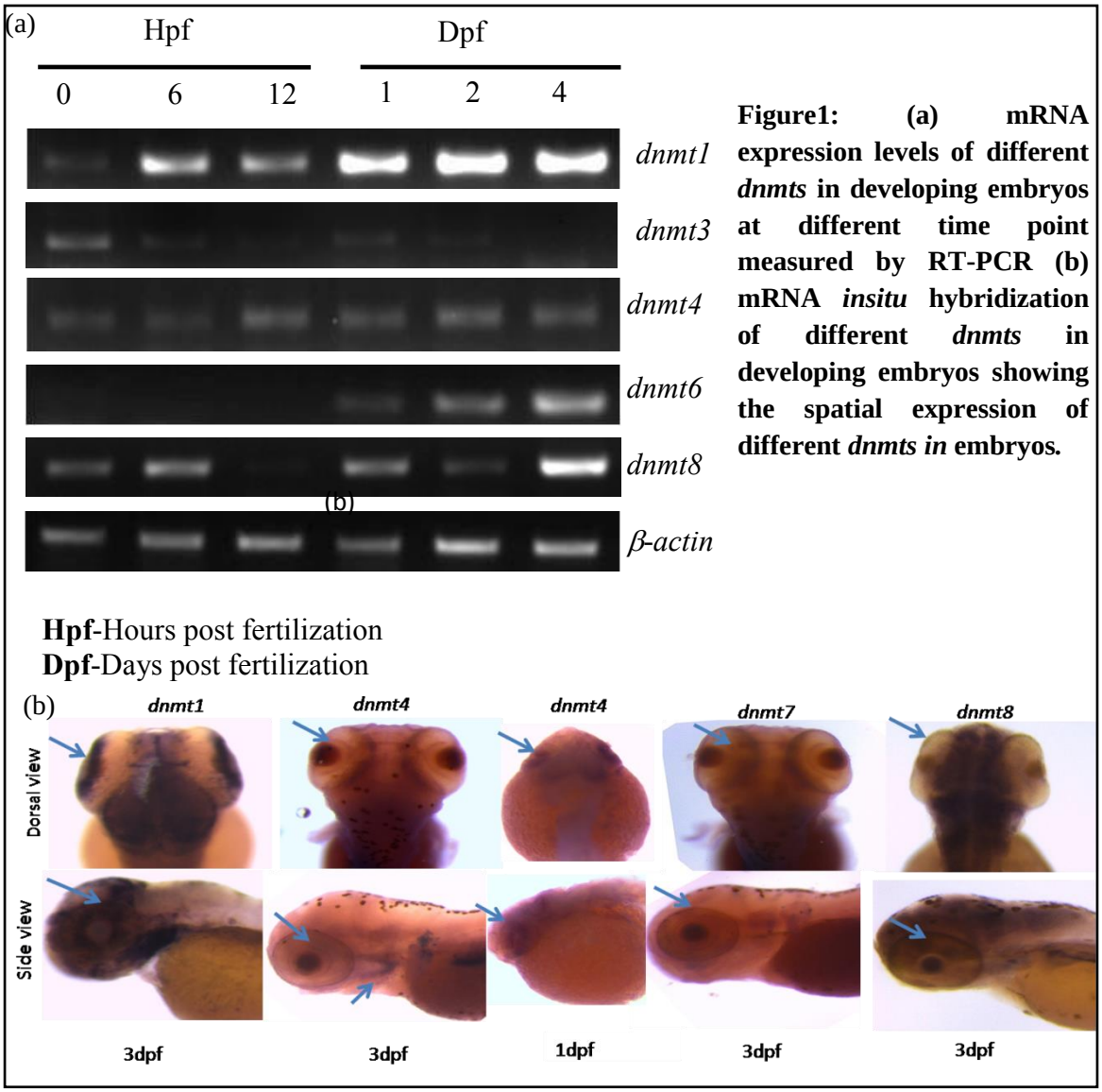


Figure1: (a) mRNA expression levels of different *dnmts* in developing embryos at different time point measured by RT-PCR (b) mRNA *insitu* hybridization of different *dnmts* in developing embryos showing the spatial expression of different *dnmts in* embryos.

Observations: *dnmt1* mRNA expression increases gradually from 6hpf. *dnmt3* mRNA expression level shows wobble trends of expression where it's level decreases till 12hpf, increases its expression in 1dpf and again decreases after 1dpf. *dnmt4* mRNA level increases in 12hpf and remain almost the same in the later time point. *dnmt6* started it's

expression from 1dpf and increases gradually. *dnmt8* mRNA level also shows wobble trend where it's level decreases in 12hpf, increases again in 1dpf, then decreases in 2dpf and again increases. Spatial expression of *dnmts: dnmt1* and *dnmt8* are expressed in highly in eyes and brain in 3dpf. *dnmt4*and *dnmt7* are expressed in eyes in minor level in 3dpf, and *dnmt4* level is high in 1dpf as compare to 3dpf.

1.2.2. Expression of *dnmts* in regenerating retina of zebrafish

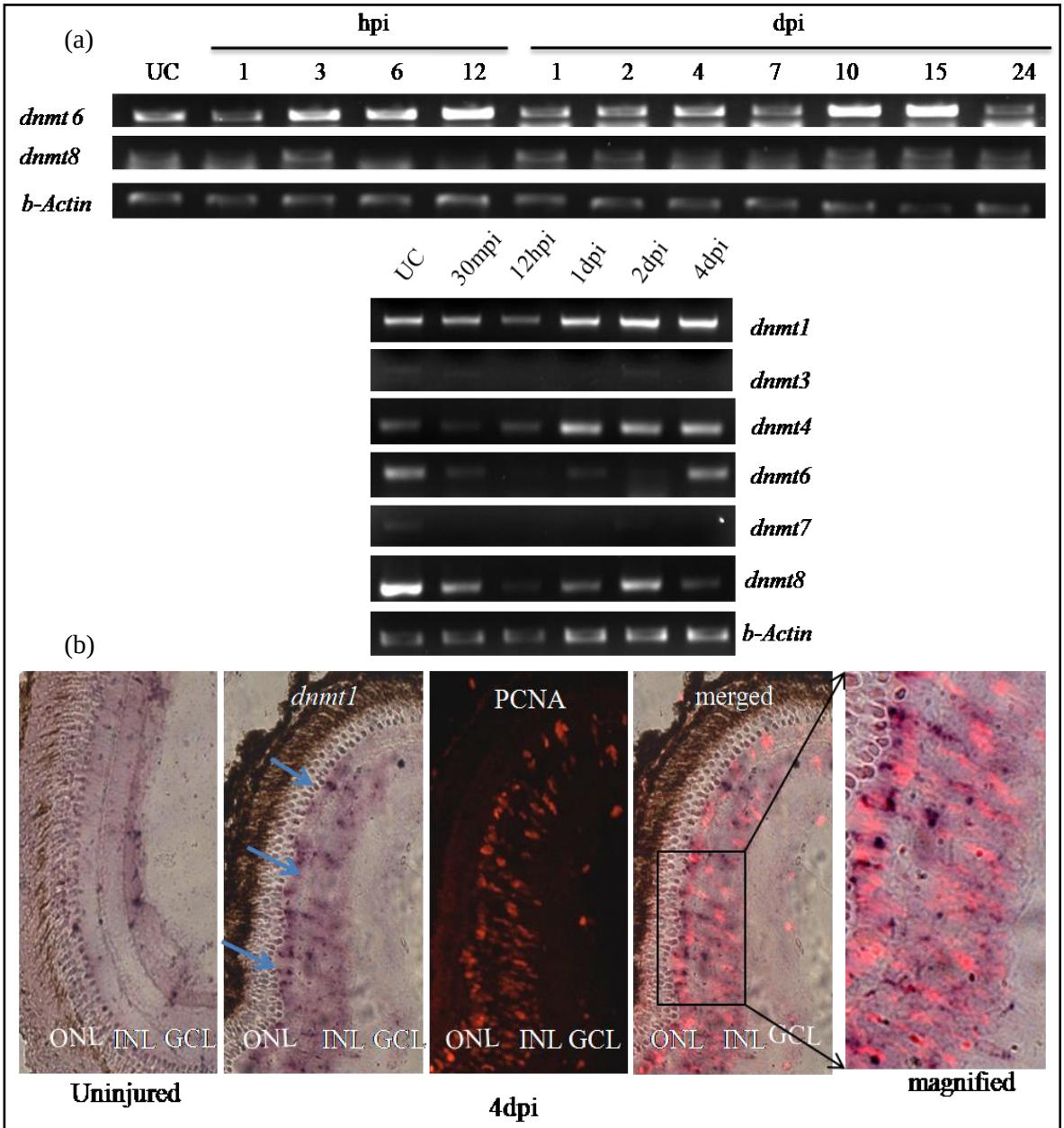


Figure2: (a) mRNA expression levels different *dnmts* in regenerating retinas at different time points, before and after retina injury. (b) mRNA *in situ* hybridization of dnmt1 on uninjured and 4dpi retina section, immunostaining of PCNA is done on the same section.

Observations: *dnmt1*mRNA is present in uninjured control and it decreases at 12hpi, and increases from 1 dpi. *dnmt3* is present in UC and decreases and vanished till 1dpf, and it

reappears again at 2dpi. *dnmt4* is present in UC, and the level remains constant till 12hpi and its expression level increases from 1dpi. *dnmt6*and *dnmt8* mRNA expression level shows a wobble trend and this trend are observed even at the late phase of regeneration. *dnmt7* is present in UC, and is found to be expressed only at 2dpi.

mRNA *in situ* hybridization of *dnmt1* is done on 4dpi retina section because expression of *dnmt1* is observed to be maximum at 4dpi from RT PCR results. At 4dpi *dnmt1* mRNA is found to be present and is restricted to the injury spot. In UC no such restricted expression of *dnmt1* is observed. When mRNA *in situ* hybridization signals of *dnmt1* is co-labelled with PCNA immunostaining signals it is observed that not all PCNA positive cells are having *dnmt1* expression and not all cells which are showing *dnmt1*expressions are PCNA positive.

1.2.3. Effects on retina regeneration when Dnmts are blocked using Pharmacological blockers.

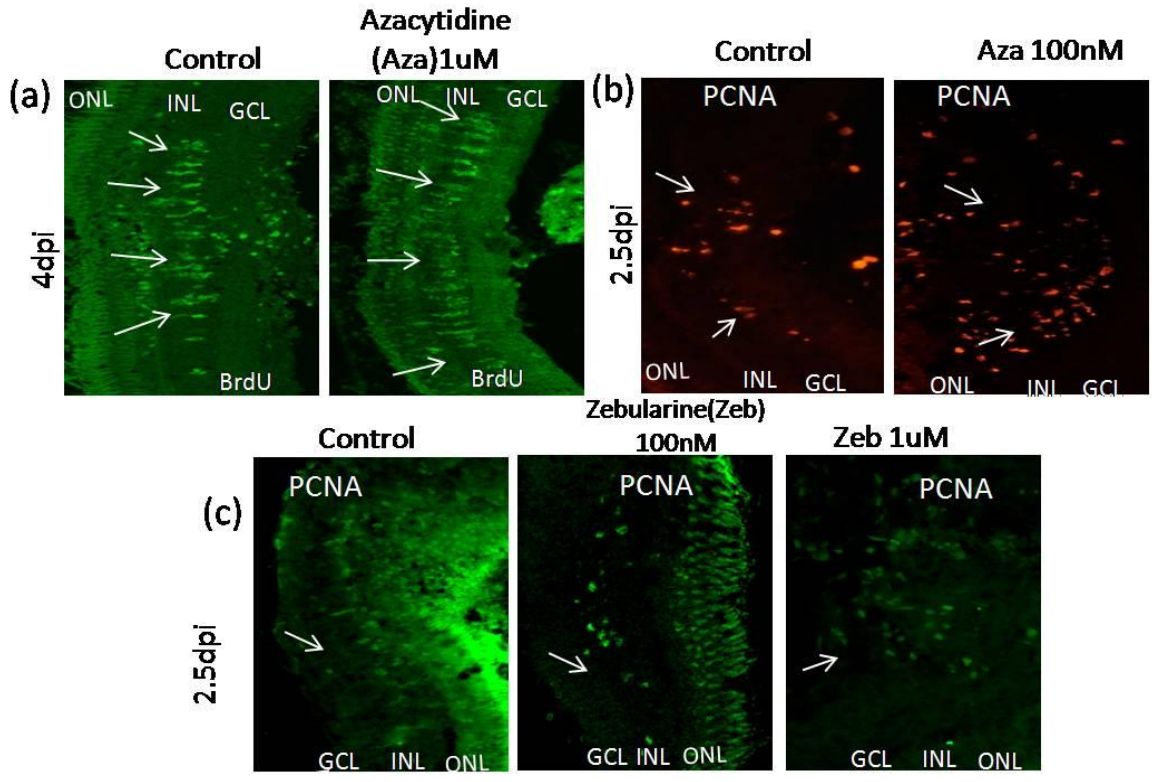
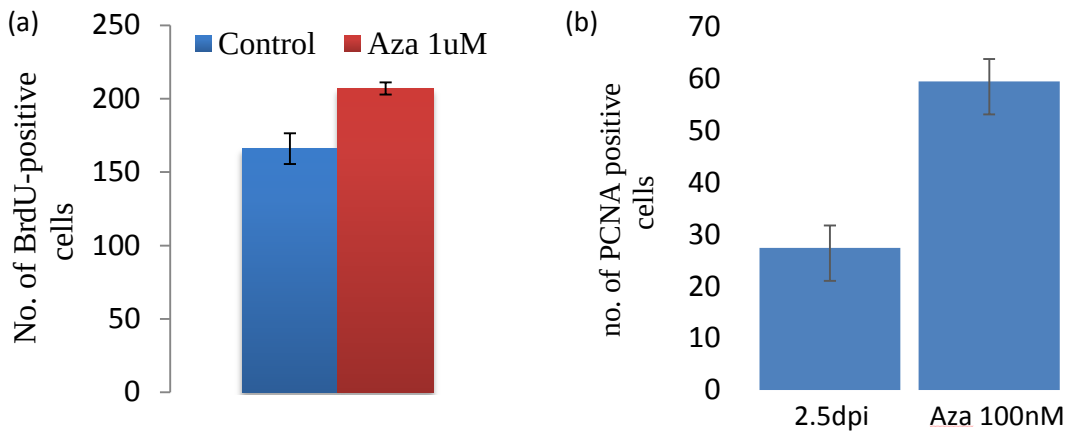


Figure 3: (a) Immunostaining of BrdU on 4dpi control retinas and 4dpi Dnmt blocking drug (Aza 1µM) treated retinas, showing the increase in the number of BrdU-positive cells in drug treated condition. BrdU pulse is given on 4dpi for 4 hours. (b) Immunostaining of PCNA on 2.5dpi control and 2.5dpi Aza 100nM treated retinas, showing increased in the number of PCNA positive cells in drug treated condition. (c) Immunostaining of PCNA on 2.5dpi control, 2.5dpi Zebularine 100nM, and Zebularine 1µM treated retinas, showing increased in number of PCNA positive cells in drug treated in higher concentration.



Graph1: (a) Injured fishes were treated with Aza (1µM) and retina harvested at 4dpi after 4 hours BrdU pulsing after immunostaining of BrdU number of BrdU-positive cells were counted in both control and drug treated condition and compared between the two. (b) Injured fishes were treated with Aza (100nM) and retina harvested at2.5dpi, after immunostaining of PCNA number of PCNA positive cells were counted in both control and drug treated condition and compared between the two

Observations: When fishes are treated with Azacytidine 1µM, the number of BrdU -positive cells increases as compared with control at 4dpi. When fishes are treated with Aza 100 nM, the increase in the number of proliferating cells (PCNA positive cells) are much higher as compared with control and the level of increase is more in 100nM Aza than 1µM Aza. This observation may be because of cytotoxicity of Azacytidine at higher concentration. When the injured fishes are treated with another Dnmts blocker, Zebularine, the number of proliferating cells increases in treatment with 1µM Zeb, but there is no significant increased in number of proliferating cells when treated with 100nM Zeb.

<u>Effects on Müller glia cells proliferation when Dnmts are blocked at the de-differentiation phase of regeneration.</u>

Fishes eyes were injured, and four sets of regeneration conditions were made:

1. Fishes after the injury put to water and allowed to regenerate in normal condition.
2. Fishes after the injury put in Aza (100nm) continuously till harvesting of eyes.
3. Fishes after the injury put in Aza (100nM) till 2dpi and allowed it to regenerate normally from 2dpi till harvesting of eyes.
4. Fishes after the injury put in water, allow regenerating in normal condition up to 2dpi and then transfer to Aza (100nM) till harvesting of eyes. Eyes were harvested at 4dpi and immunostaining of PCNA was done.

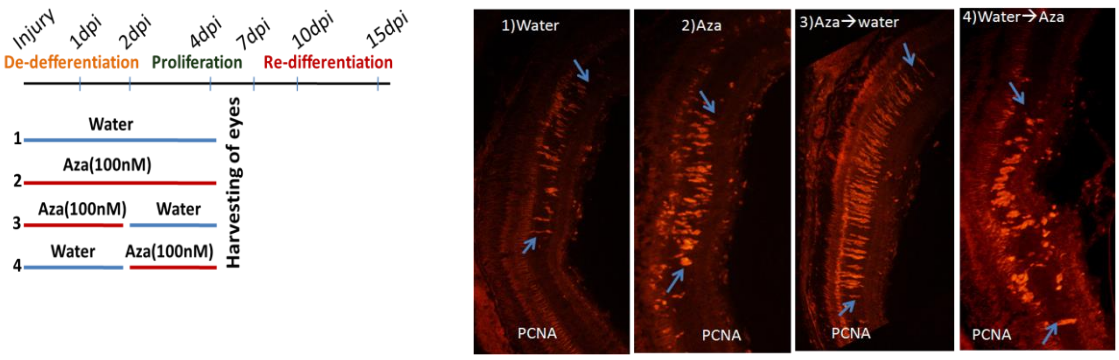
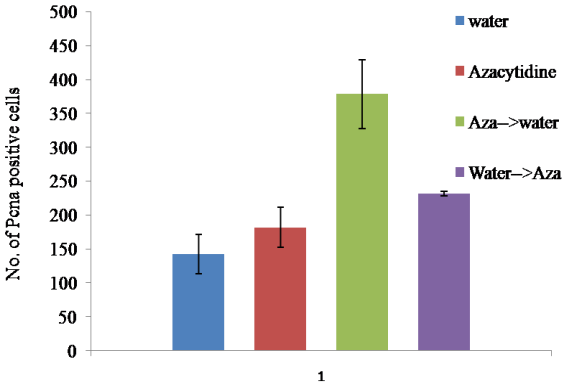


Figure4: Immunostaining of PCNA on 4dpi retinas where after injury fishes were dipped in 1) water till 4dpi, 2) Dnmts blocker, Aza (100nM) till 4dpi, 3)First two days in Aza (100nM) then transfer to water till 4dpi, 4) First two days in water and transfer to Aza till 4dpi.

Graph2: Comparing number of PCNA positive cells on 4dpi retinas where after injury fishes were dipped in 1) water till 4dpi, 2) Dnmts blocker, Aza (100nM) till 4dpi, 3)First two days in Aza (100nM) then transfer to water till 4dpi, 4)First two days in water and transfer to water till 4dpi. Fishes whose Dnmts are blocked at early phase up to 2dpi has the highest



Observations: Fishes treated with Dnmt blocker after injury continuously till 4dpi has more number of proliferating cells as compared with control 4dpi.Fishes that are treated with Dnmts blocking drug during the initial phase of regeneration; treated with Aza (100nM) immediately after injury till 2dpi has the highest number of proliferating cells at 4dpi as compared with control 4dpi and other conditions. Fishes treated with Aza from 2dpi till 4dpi has more number of proliferating cells as compared with control 4dpi and fishes treated with Aza continuously for 4dpi, but the number is less as compared with the fishes whose Dnmts are blocked only up to 2dpi. These observations suggested that the roles of Dnmts are more crucial during the early phase that is the de-differentiation phase of regeneration.

Effects on Müller glia cells proliferation and redifferentiation when Dnmts are blocked at re-different phases of regeneration.

Fishes eyes were injured, and four sets of regeneration conditions were made:

1. Fishes after the injury put to water and allowed to regenerate in normal condition till harvesting of eyes.

2. Fishes after the injury put in Aza (100nM) till 4dpi and transfer to waterfrom 4dpi till harvesting of eyes.

3. Fishes after injury put in water, allow regenerating in normal condition up to 4dpi and then transfer to Aza (100nM) till harvesting of eyes.

4. BrdU pulses were given at 4dpi and 5dpi for 4 hours each. Eyes were harvested at 4dpi. Immunostaining of BrdU was done to compare the number of proliferating cells. Co-immunostaining of BrdU and cells specific proteins was done to check the effect of Dnmts in re-differentiation.

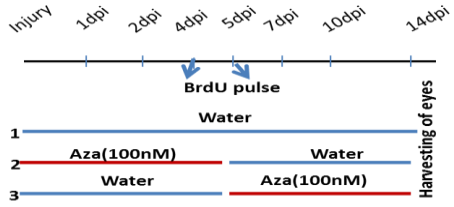
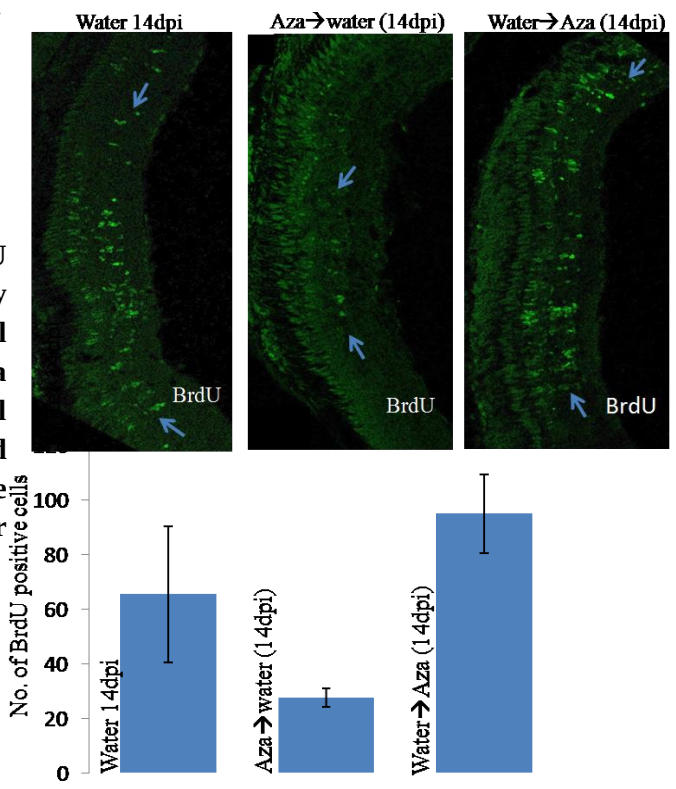


Figure5: Immunostaining of BrdU on 14dpi retinas where after injury fishes were put in: 1) water till 14dpi, 2) First four days in Aza (100nM) then transfer to water till 14dpi, 4) First 4days in water and transfer to Aza till 14dpi.BrdU pulse was given on 4th and 5th day for four hours each.



Graph3: Comparing the number of BrdU-positive cells on 14dpi retinas where after injury fishes were put in: 1) water till 14dpi, 2) First four days in Aza (100nM) then transfer to water till 14dpi, 3) First 4days in water and transfer to Aza till water till 14dpi. Fishes whose Dnmts are blocked at early phase up to 4dpi has the least number of proliferation at 14 dpi.

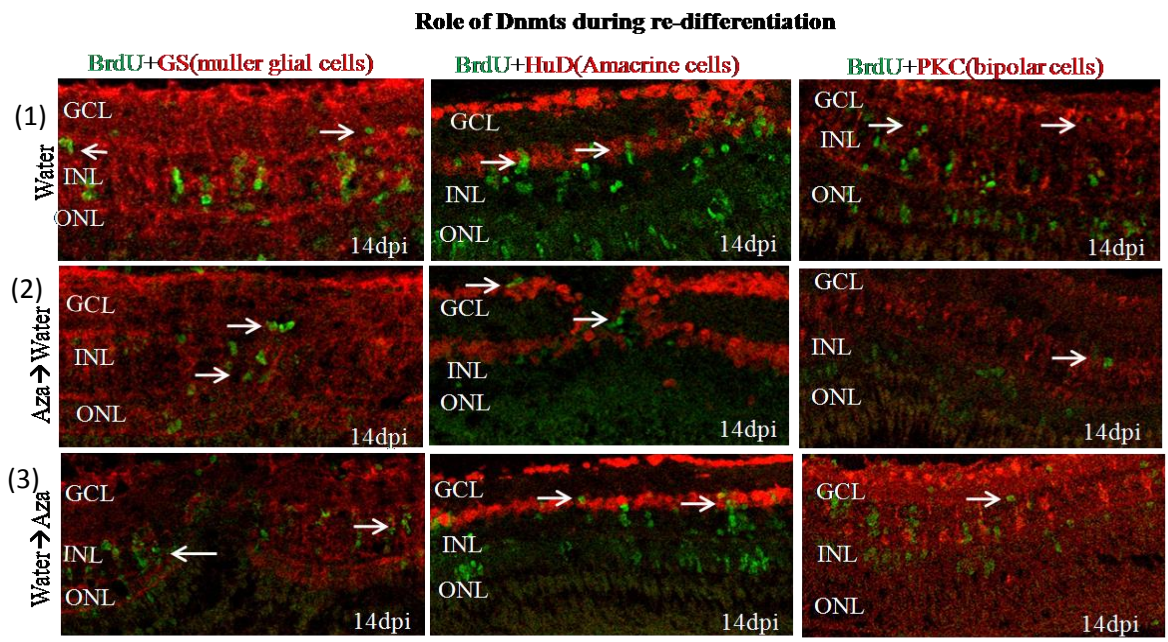


Figure6: Co-immunostaining of BrdUand cell specific staining on 14dpi retinas. GS stains for Müller glial cells, HuD stains the Amcrine cells and PKC will stain the bipolar cells. Fishes after injury fishes were put in: 1) water till 14dpi, 2) First four days in Aza (100nM) then transfer to water till 14dpi, 4) First 4days in water and transfer to Aza till 14dpi.BrdU pulse was given on 4th and 5th day for four hours each. BrdU-positive cells are stained in green, cells specific staining stained in red.

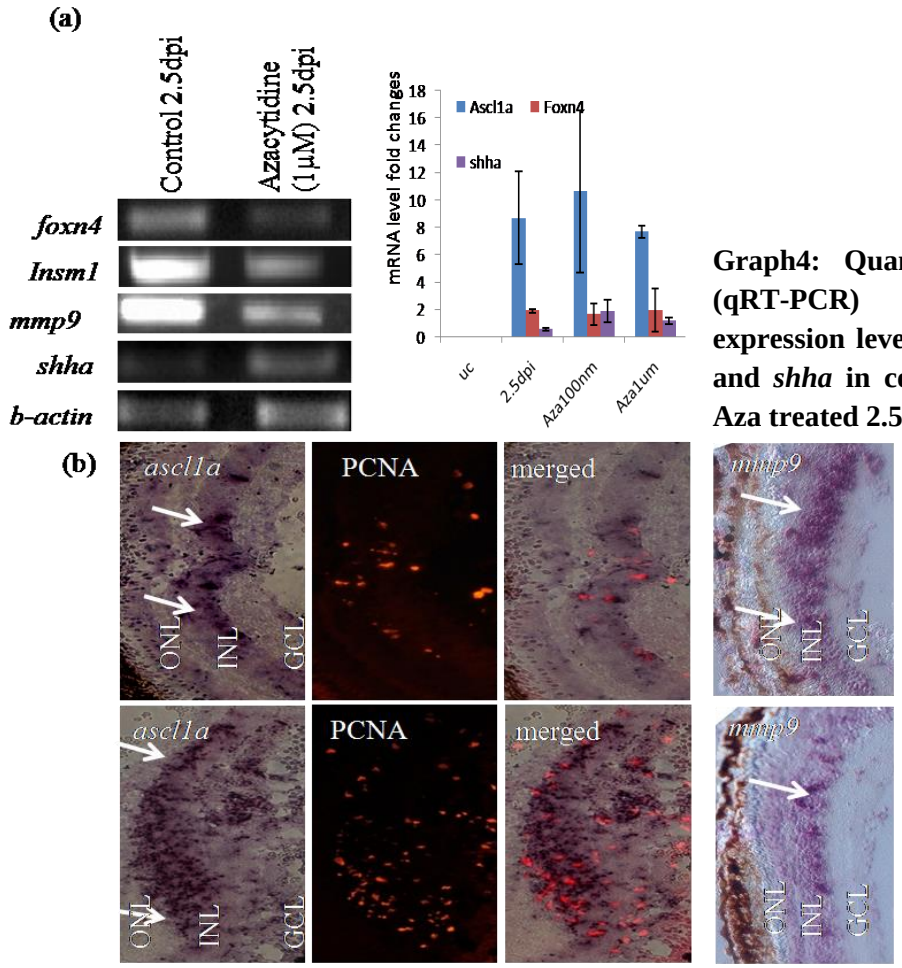
Observation: It is observed that when fishes are dipped in Aza till 4dpi then allowed to regenerate in normal condition (water), the number BrdU-positive cells at 14dpi is significantly less as compared with control 14dpi. Fishes that were allowed to regenerate normally in water during early phase till 4dpi and Dnmts are blocked from 4dpi till 14 dpi has the higher number of BrdU-positive cells as compared with control 14dpi.

When co-immunostaining of BrdU and cells specific staining were done, GS stains for Müller glial cells, HuD stains for amacrine cells and PKC will stain for bipolar cells, it is observed that in all the three conditions mentioned above the BrdU-positive Müller glial cells are able to form into specific cell types(amacrine cells and bipolar cells) at 14dpi.

This observation suggested that proliferating cells that have their Dnmts blocked at early phase till 4dpi are not able to survive at the later phase of regeneration at 14dpi whereas those proliferating cells which have their Dnmts blocked from 4dpi to 14dpi can survive and the number is even more than the control 14dpi.

1.2.4. Regulations of retina regeneration associated genes by Dnmts.

Looking at the expression level of various retina regeneration associated genes when Dnmts are blocked during retina regeneration.



Graph4: Quantitative analysis (qRT-PCR) of mRNA expression level of *ascl1a, foxn4* and *shha* in control 2.5dpi and Aza treated 2.5dpi

Figure7: (a) Comparing the mRNA level of *foxn4, Insm1a, mmp9* and *shha* (retina regeneration associated genes) in control 2.5dpi and Aza treated 2.5dpi by RT-PCR. b) Comparing the mRNA level of *ascl1a*and *mmp9*in control 2.5dpi and Aza treated 2.5dpi by mRNA *in situ* hybridization.

Observations: - It is observed that when Dnmts are blocked some of the retina regeneration associated genes get regulated during retina regeneration. mRNA expression level of *foxn4, Insm1a* and *mmp9* at 2.5dpi are observed to be decreased when Dnmts are blocked and mRNA level of *shha* are seen to be increased. Quantitative analysis confirms this observation by real time PCR (qRT-PCR). mRNA *in situ* hybridization of *ascl1a*and *mmp9* also showed that Dnmts regulate their expression. *ascl1a* mRNA level is more than control at2.5 dpi when Dnmts are blocked while *mmp9* mRNA level is less than the control at 2.5dpi.

Confirmation of regulation of regeneration associated genes by Dnmts in developing embryos.

Figure10: - Comparing the mRNA level of *ascl1a, foxn4* and *shha* in control 3dpf and Aza(100nM) treated 3dpf embryos by whole mount mRNA *insitu* hybridization.

Observations: mRNA level of ascl1a is observed to be increased in Aza treated 3dpf embryos as compared with control 3dpf, which similar to what we observed in retina regeneration. *foxn4* mRNA level is observed to be increased and *shha* mRNA level decreased in Aza treated 3dpf embryos as compared with control 3dpf which is opposite to what we observed in retina regeneration.

1.3. METHODS AND PROTOCOLS

METHODS

1.3.1. To check expression of *dnmts*in developing embryos

a) Checking expressions of *dnmts* in developing embryos temporally:-

Fishes were put for breeding, and fertilized eggs were collected, and allowed it to develop giving them optimum conditions of light and temperature. The developing embryos were collected at different time points in MCT having trizol (200µl) and stored in -80^{o}C. RNA was isolated from these stored embryos. cDNA was synthesized from the RNA we isolate, and RT-PCR was done to look at the expression of various *dnmts* during embryonic development.

b) Checking expressions of *dnmts* in developing embryos spatially:-

dnmt8, *dnmt4* and *dnmt7* were amplified using Gotaq and then inserted it in Topo vector and were transformed in Dh5alpha E.coli strand. Individual colonies from transformed plates were picked and patched on a new plate. Then positive clones were screened by using Phenol Chloroform Isoamyl Alcohol (PCI). Those colonies which are having plasmid with shifted size were allowed to amplify in LB media. Plasmids were isolated from these cultures. Clones were confirmed by doing restriction digestion. Orientations of the insert were checked by restriction digestions, using SnapGene. Then the plasmids were linearized by the desired restriction enzymes. Probe reactions labeling with digoxigenin (dig) were set using either T7 RNA polymerase or SP6 RNA polymerase depending on the orientation of the insert.

Embryos were treated with PTU (Phenyl thiourea) from 24 hours post fertilization (hpf) to prevent from melanin development. 3 days post fertilization (dpf) embryos were collected and fixed in 100% methanol stored in -20^{o}C. Whole mount mRNA *in situ* hybridizations of the prepared RNA probes

were performed in these fixed embryos to look at the spatial expression of *dnmts* in the developing embryos.

1.3.2. To check expression of *dnmts*in regenerating retina of zebrafish

a) Checking expressions of *dnmts* in regenerating retinas temporally:-

Fishes eyes were injured and allowed their eyes to regenerate. The regenerating retinas were collected at different time points after injury in MCT having trizol (200µl) and stored in -80^{o}C. RNA was isolated from these stored retinas or immediately after dissection. cDNA was synthesized from the isolated RNA and RT PCR was done to look at the expression of various *dnmts* during the course of retina regeneration.

b) Checking expressions of *dnmts* in regenerating retinas spatially:-

Fishes eyes were injured and allowed their eyes to regenerate. The regenerating eyes were harvested at 4days post injury and their lenses were removed. Eyes were then fixed in 4% Paraformaldehyde and sucrose washes were performed. Blocks which have fixed eyes embedded in it have been made using OCT for cryosectioning. Then eyes were sectioned in cryostat and the slides were dried and stored it in -20^{o}C and mRNA *in situ* hybridization of *dnmts* were done on this slides.

1.3.3. To check effects on retina regeneration when Dnmts are blocked using Pharmacological blockers

a) Checking the amount of Müller glial proliferation after injuries when Dnmts are inhibited by Pharmacological blockers.

Fishes were pre-dipped in Azacytidine for 1 hour and then injured their eyes with 30 gauge needles and after injury again dipped it in Azacytidine. Fishes which were not pre dipped in Aza, injured and put back in water were taken as control. Fishes were given BrDU pulse (dipped in BrDU) for four hours at 4dpi and their eyes were harvested and lenses were removed and fixed in 4% Paraformaldehyde and sucrose washes were done. Blocks which have fixed eyes embedded in it have been made using OCT for cryosectioning. Then eyes were sectioned in cryostat and the slides were dried and stored it in -20^{o}C. Then immunostaining for BrdU or PCNA was performed on the tissue sections. Immunostained slides were observed under fluorescence microscope, images were captured and numbers of BrdU-positive cells or PCNA positive cells were counted.

Experiments were repeated for Azacytidine taking different concentrations and for another Dnmt blocking drug Zebularine.

b) Looking at effect on Müller glia cells proliferation when Dnmts are blocked at De-different phase of regeneration.

Fishes eyes were injured and four sets of regeneration conditions were made as explained in section 1.2.3.Eyes were harvested at 4dpi. Same steps were followed as above (5.1.3a) to fix the tissues, sucrose washes, making of OCT blocks, sectioning and immunostaining of PCNA to compare the number of proliferating cells.

c) Looking at effect on Müller glia cells proliferation and re-differentiation when Dnmts are blocked at re-different phases of regeneration.

Fishes eyes were injured and four sets of regeneration conditions were made s given in section4.3.3.BrdU pulses were given at 4dpi and 5dpi for 4 hours each. Eyes were harvested at 4dpi.

Same steps were followed as above (5.1.3a) to fix the tissues, sucrose washes, making of OCT blocks, sectioning and immunustaining of BrdU to compare the number of proliferating cells. Co-immunostaining of BrdU and cells specific proteins was done to check the effect of Dnmts in re-differentiation.

1.3.4. To look at the regulation of retina regeneration associated genes by Dnmts.

a) Looking at the expression level of various retina regeneration associated genes when Dnmts are blocked during retina regeneration.

Fishes were pre-dipped in Aza for 1 hour and then injured and after injury again dipped them in Aza. Fishes which are not drug treated are also injured and put back in water. 2.5 days after injury the eyes of the fishes (both control and drug treated) were harvested and retinas were dissected. RNA was isolated from these dissected retinas, cDNA synthesized from the isolated RNA. Then PCR reactions using taq polymerase for certain genes specific RT primers were put. Expression of β-actin mRNA was used as internal control. Band intensity of the amplified PCR products were compared between the control and drug treated conditions. Then Quantitative PCR (qPCR) is carried out in duplicate using KOD SYBR qPCR Master Mix (pure gene). The levels of expression of retina

regeneration associated genes were also checked by doing mRNA *in situ* hybridization on drug treated regenerating retinas tissues and compare it with the normal regenerating retinas.

b) Confirming the regulation of regeneration associated genes by Dnmts in developing embryos.

Fertilised eggs were allowed to develop in Dnmt blocking conditions (treated with Aza), whole mount mRNA *in situ* hybridization of some of the genes which are observed to be regulated by Dnmts in retina regeneration were done on 3dpf embryos and the expression levels are compared with the control (develop in normal conditions). Embryos were treated with PTU to prevent melanin development.

PROTOCOLS:-

1.3.5. RNA isolation

1. Take the stored embryos/dissected retinas from -80^{o}C and thaw it.
2. Homogenize the tissues (use 200µl pipette for retinas and homogenizer in case of embryos) completely so that no tissue clumps are visible. Allow it to settle for 5 min.
3. Add 0.2 volumes (40µl) of chloroform and mix it gently by inverting the MCT up and down ten times.
4. Then centrifuge it at 10000 rcf for 20min at 4^{o}C.
5. Using cut tips collect aqueous phase slowly without disturbing the middle phase layer and put it in fresh MCTs.
6. Add equal volume of Isopropanol and keep it in -80 for 10-20min.
7. Then centrifuge it at 10000rcf at 4^{o}C for 10min.
8. Discard the supernatants.
9. Wash it with 70% alcohol (200µl) and centrifuged it at 4^{o}C for 10 min at 7600 rcf.
10. Dry and dissolve the pellet in DEPC treated water and stores it at -80^{o}C.
11. Check whether there is any contamination of genomic DNA in it by using agarose gel electrophoresis(1%gel) and if the isolated RNA is pure and free from any genomic DNA contamination we measure the amount of RNA present by measuring the Optical density using spectrophotometer (Nano drop) and proceed further for cDNA synthesis.

1.3.6. cDNA synthesis (Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit)

Set reaction for 5µl reaction

1. Add the following components in a PCR tube:-
   - Template RNA – 1.5µl
   - Oligo (dT) Primer - 0.25µl
   - Random Hexamer - 0.25µl
   - Nuclease free water -1µl
2. Mix it properly and put in 65^0C for 5min, after this immediately put it in ice for about 2 min.
3. Add the following to the above mixture:-
   - 5X Reaction Buffer - 1µl
   - RiboLock RNase inhibitor - 0.25µl
   - 10mM dNTP Mix – 0.5µl
   - RevertAid M-MuLV RT – 0.25µl
4. Mix the reaction mixture properly. Spin it down briefly and give the following incubation:-
   - 25^oC - 5min
   - 42^oC - 60min
   - 70 - 5min
   - 4^0C - infinite hold
5. Store the cDNA in -80^o C.

1.3.7. Reverse transcription Polymerase chain reaction (RT PCR) using Taq Polymerase

1. Dilute the synthesisedcDNA by 1:4 dilutions (1µl cDNA+ 4µl Milli-Q water) and set reactions for 10µl.
   - 20X buffer- 0.5µl
   - dNTP-1µl
   - Primers (F+R) -0.4µl
   - template cDNA-0.5µl
   - MQ water -7.1µl
   - Taq polymerase -0.5µl

2. Put cycling conditions as:-

- 95°C - 2min
- 95°C - 30secs
- 60°C - 30secs
- 68°C – depends on the size (1kb/1min),
- 72°C - 7 min
- 4°C - Infinite hold
- Put 35 cycles.

3. Check the PCR product by Agarose gel electrophoresis.

1.3.8. Qantitative PCR (qRT-PCR)

qPCR is carried out using KOD SYBR qPCR Master Mix (pure gene)

We set 5µl reaction:-

- Master mix- 2.5µl
- Primers (F+R)-0.25 µl
- Template -0.25 µl
- MQ water-2 µl

Analyse the data on excel sheet and plot the graph.

1.3.9. PCR reaction using Gotaq

1. Add the following components:-

- Gotaq mastermix - 50 µl
- Forward + Reverse primers – 4 µl
- Template - 1 µl
- Water – 45 µl
- Total- 100 µl

2. Put cycling conditions as:-

95°C - 2min
95°C - 30secs
60°C – 1 min
68°C -2min
72°C - 7 min
4°C - Infinite hold

Put 35 cycles.

3. Check the PCR product in agarose gel electrophoresis by loading 5µl of it.
4. If it shows a single clean band purify the PCR product using PCR purification kit. If multiple bands were there then load the whole PCR in gel electrophoresis and cut the band of desired specific size and collect it in 1.5ml MCT.

1.3.10. TOPO TA Cloning

1. Perform manual gel extraction from the collected specific band.

Put the cut aluminium foil pieces in 1.5ml MCTs and transfer the cut gel into it. Make a very small hole at the bottom of the MCT using a 30G needle. Put this MCT inside another MCT and taped it. Centrifuge it at 14000rpm for 5 min and collect the flow through. Add equal volume of PCI to the flow through, mix it properly and centrifuge it again at 14,000rpm for 10 min. Take the aqueous layer and add again equal volume of chloroform, mix it properly and centrifuge it at 14,000 rpm for 10 min. Transfer the aqueous layer into a new MCT and add ethanol (final concentration should be 70%), 7N Ammonium acetate (make final as 3N), glycogen (1µl in 100µl). Mix it properly and put it in -80^{o}C for at least 30 min. Then centrifuge this sample at 14,000rpm, 4^{o}C for 20-30 min. Discard the supernatant and wash the pellet with 70% ethanol twice, spin at 14,000 rpm, 4^{o}C for 5 min. Then dry the pellet and elute it in 10µl of nuclease free water.

2. Add the following components for ligation:-

- Salt solution – 0.5µl
- Vector – 0.3 µl
- Insert – 1.5 µl
- H_2O - 0.7 µl

Incubate this mixture for at least without disturbing it.

3. Transform this ligated product into competent cells.

- Thaw competent cells stored in -80^{o}C on ice.
- Take 3µl of ligated plasmid and add in 100µl of the thawed competent cells and mixed it gently.
- Incubate it in ice for 30 min.
- Give a heat shock in water bath at 42^{o}C for 80secs.

- Add 1ml of LB media into it and incubate at 37^{o} C for 1 hour.
- Then centrifuge it at 4,000 rpm for 4min.
- Discard some of the supernatant, dissolve the pellet in the remaining supernatant and plate it on Ampicillin resistant LB agar plates and incubate it in 37^{o}C for overnight.

Checking for transformed positive clones

1. Next day pick the individual colonies and patch it on new Amp resistant LB agar plates and allow the bacteria to grow.
2. Pick the individual patched colonies and perform PCI method to look for positive clones.

Pick the colonies from each patch using tips or toothpicks and dissolve it in 20µl of MQ water. Add 20µl of PCI to it (shake the PCI properly before using). Vortex it briefly and spin it down. Take the aqueous layer and load it in agarose gel for gel electrophoresis. Mark the patches which are showing positive shift in their plasmid size (size of vector +insert). Positive clones are taken and mix it in 5ml LB media having Amp and incubate in 37^{o}C overnight for growth.

1.3.11. Plasmid isolation

2. Take 1.5ml of the overnight incubated cultures in MCTs and centrifuge it at 14,000rpm for 2 min.
3. Discard the supernatant and elute it in 100µl of MQ water and vortex it.
4. Add 100µl of freshly prepared lysis buffer, mix it properly and boil it in 100^{o}C for 2 min.

Compositions of lysis buffer are:-

- MQ water - 920µl
- 20% SDS - 50µl
- 0.5M EDTA – 20µl
- 10N NaOH - 10µl

5. Add 50µl of 0.5M $MgCl_{2,}$ mix properly and put it in ice for 2 min.

6. Then spin for 2 min at 14,000rpm and add 50µl of 3M potassium acetate buffer in it and mix it properly by inverting the MCT up and down.
7. Spin it again at 14,000 rpm for 2 min.
8. Take the supernatant in a new MCT and add 600µl of Isopropanol to it, mix it properly and put it in ice for 5 min.
9. Spin at 14,000rpm for 2 min.
10. Discard the supernatant and wash the pellet with 70% ethanol.
11. Dry the ethanol and elute it in 50µl of Nuclease free water.

1.3.12. Plasmid confirmation and restriction digestions

2. Add the following components in an MCTs:

- Plasmid - 5µl
- MQ water- 3.5µl
- 10X buffer - 1µl
- Restriction enzymes – 0.5µl

Enzymes used:

dnmt4 –StuI+BamHI and StuI+XbaI

dnmt7 –StuI+XbaI and StuI+BamHI

Confirmed *dnmt1* clone was taken from Evelyn Abraham (Last year MS student).

3. Incubate at 37^{o}C for 1 hour
4. Check on agarose gel and confirm their digested fragments size and determine the orientation of the insert.
5. Put large scale digestion ,with suitable restriction enzymes depending on the orientation, to linearise the plasmid

dnmt8 –BamHI(inserted in forward orientation)

dnmt4 –BamHI (inserted in forward orientation)

dnmt7 –XhoI (inserted in reverse orientation)

6. Add the following components in an MCTs:

- Plasmid - 35µl
- 10X buffer - 4µl
- Enzymes – 1µl

7. Incubate at 37^{o}C for 3 hours or overnight.

8. Load the digested products on agarose gel and excise the linearised DNA fragments from agarose gel.
9. Follow the manual gel extraction method as given earlier in TOPO TA cloning protocol.

1.3.13. RNA probe reaction

2. Add the following component in MCTs.

- Template DNA (linearised plasmid) – 500ng to 1µg (7µl)
- Buffer (10X) NEB 3.1 - 1µl
- Dig/Fl – 0.5µl
- RNA polymerase – 0.5µl(T7 RNA polymerase
- for *dnmt1, dnmt8* and *dnmt4,* SP6 RNA polymerase for *dnmt7*)
- Nuclease free water- 0µl
- Total – 10µl

3. Incubate it in 37^{o}C for 4 hours.
4. Add 1µl of 0.5M Tris EDTA for stopping the reaction
5. Add 1µl of 5M $LiCl_2$ for precipitation
6. Add 0.5µl of Glycogen and tap it
7. Add 18µl of 100% ethanol, tap it and mix properly and keep it in -80^{o}C
8. Next day or after one hour centrifuge at 14,000rpm for 15 min
9. Discard the supernatant and wash the pellet with 100% ethanol, centrifuge it at 14,000 rpm for 10 min.
10. Dry the pellet and dissolve it in 15µl of DEPC water and store at -80^{o}C.

1.3.14. Whole mount mRNA*insitu*hybridization on fixed embryos.

Day 1: Prehybridization, RNase free

1. Rinse the embryos in 100% methanol for 5min in room temperature (RT).
2. Rinse it in 50:50 methanol and Xylene for 5 min in room temperature.
3. Rinse it in 100% Xlylene for 30 min in room temperature.
4. Rinse it in 100% Methanol for 30 min in room temperature.
5. Then rehydrate the embryos at room temperature by following:
    a) 90% methanol for 5 min
    b) 70% methanol for 5 min
    c) 50% methanol for 5 min

6. Then rinse the embryos twice in 1XPBS/0.1% Tween for 15 min each at RT.
7. Prepare Proteinase K solution and prewarm it in 37^{o}C.
8. Add 25µl of Proteinase K to the solution and add this solution to the embryos for 15 min and keep it at 37^{o}C
9. Then add 1XPBS/0.1% Tween for 10-15 secs at RT.
10. Fix the tissue again with 4% Paraformaldehyde (PFA) made in phosphate buffer. Remove 1XPBS/0.1% Tween and add 4% Paraformaldehyde at RT for 20 min.
11. Remove the PFA and wash with 1XPBS/0.1% Tween twice for 5 min.
12. Then rinse the embryos with 0.1M TEA for 3 min at RT.
13. Incubate the embryos in Acetic anhydride/TEA solution (add 130µl of acetic anhydride to 50ml of TEA solution) for 10min at RT.
14. Then rinse the embryos in 1XPBS/0.1% Tween for 30 min.
15. Prepare the prehybridization and hybridization solution and prewarm them at 56^{o}C.
16. Add 500µl of prewarmedprehybridization solution to each MCT having embryos and incubate them at 56^{o}C for 1-2 hours.
17. Take 100µl of Prewarmedhybridization solution and add 1µl of prepared RNA probes of *dnmt1*, *dnmt4*, *dnmt7* and *dnmt8*, mix it properly and boil it at 100^{o}C for 10 min.
18. Immediately put it in ice for 5min.
19. Remove the prehybridization solution from the tubes having embryos and add the hybridization solution having the RNA probe.
20. Allow it to hybridize overnight at 56^{o}C.

Compositions of solutions used for 1st day *Insituhybridization:*

a) 20X SSC(50ml)

- Dissolve 8.76g of NaCl in 35ml of DEPC water
- Add 4.412g of sodium citrate to it bring the volume up to 50ml with DEPC water.

b) TEA (50ml) :

- Triethanolamine – 0.93 g
- DEPC water – 50ml
- 10N Sodium hydroxide (NaOH) - 170µ

c) Proteinase K solution (50ml):

- 1M Tris (pH8) – 2.5ml
- 0.5M EDTA – 2.5ml
- DEPC water – 20ml

d) Prehybridization (50ml) and hybridization solutions (50ml):

- TEN solutions – 3.6ml
- 100% Formamide – 25ml
- 50% Dextran sulphate – 10ml
- DEPC water – 6.4ml for Prehybridization solution, no DEPC water for hybridization solution.
- Store this in -20^{o}C

e) TEN solution

- 0.1M TrisHCl (pH7.5) – 5ml
- 5M NaCl – 30ml
- 0.5M EDTA – 1ml

Day 2: Post hybridization protocols

1. Prepare and prewarm the following solutions:
   a) 10 ml 50% formamide/ 2X SSC to 65^{o}C.
   b) 10 ml of 2X SSC at 37^{o}C
   c) 10ml RNase buffer/ 0.1% Tween solution to 37^{o}C
   d) 10ml RNase buffer/ 0.1% Tween solution to 65^{o}C
2. Rinse briefly in 2X SSC at RT for 5min.
3. Add 50% formamide/ 2X SSC and put in 65^{o}C for 30min.
4. Wash with 2X SSC at 37^{o}C thrice for 10 min.
5. Add 20µl of RNase A to 10ml RNase buffer/ 0.1% Tween solution kept in 37^{o}C, and add it to the embryos and keep it in 37^{o}C for 1 hour.
6. Wash the embryos with 10ml of 65^{o}C RNase buffer/ 0.1% Tween solution without RNase A; keep it in 65^{o}C fo 30 min.
7. Wash in twice with 2X SSC at 37^{o}C for 15 min each.
8. Wash it with 1X PBS/ 0.1%Triton for 15 min at RT.
9. Block the tissues with 1X Maleate/Triton/RMB blocker solutions for two hours.

10. Add anti-Dig antibody in 1X Maleate/Triton/RMB blocker solutions and incubate the tissues in it for overnight at RT (1:2500 dilution of antibody)

Compositions of solutions used for 2nd day *Insituhybridization:*

a) RNase buffer (10ml)
   - NaCl – 1ml
   - 1M Tris (pH7.5) - 500µl
   - 5M EDTA - 100µl
   - MQ Water – bring up to 10ml

b) 5X Maleate buffer
   - Maleic acid - 8g
   - MQ water- 850ml
   - Adjust the pH to 7.5 using NaOH pellets
   - Add 43.8g of NaCl

   Brought up to 1L with MQ water

c) 1X Maleate/0.05% Triton X/1% RMB blocker solution
   - 5X Maleate stock – 2ml
   - TritonX-100 - 5µl
   - 10% RMB blocker – 1ml
   - Make 3ml aliquots and freeze at -20^{o}C.

Day3:

1) Wash the embryos twice with 1X Maleate buffer for 5min.
2) Incubate it in Genius buffer twice for 5min.
3) Add NBT/BCIP in Genius buffer (1:50 dilution) and incubate it in RT in dark.
4) Check the development of signal in bright field microscope; check the development of signals at time intervals of about 30 min.
5) When enough signals are developed stops the reaction by removing the substrate and adds 1X PBS.
6) Take the image of the embryos.

1.3.15. Retina injury and harvesting

1. Anesthetize the fish in 0.02% TricaineMethanesulfonate in a glass beaker until the fish loses its balance.
2. Place the anesthetized fish on a moist fish tissue under the bright field microscope. Poke the eyes of the fish using 30 gauge needle and with help of forceps
3. After desire time post injury, dissect the retinas in 1XPBS for RNA isolation or remove the lenses in 4% PFA for tissue fixation.

1.3.16. Tissue fixation and sectioning

1. Take the eyes whose lenses were removed and put them into 4% PFA in 4^{o}C overnight.
2. Next day give serial washings of the fixed tissue at RT for 45mins each on a rotor:

   1ml of 5% sucrose

   800µl of 5% and 400µl of 20% sucrose

   500µl of 5% and 500µl of 20% sucrose

   400µl of 5% and 800µl of 20% sucrose

   1ml of 20% sucrose.
3. Then add 500µl of OCT and rotate it for 30 min.
4. Then embed the in OCT in small cubes made from aluminium foil and the embedded samples are kept frozen at -80^{o}C until sectioning.
5. Then section the blocks in cryostats (12µm thickness) and collect the sections on super frost plus slides and dried overnight and then stored in -20^{o}C.

Composition of solutions used:

1. 4% PFA in 1X Phosphate buffer(made DEPC water) :

   2g PFA

   5mL of 10X phosphate buffer

   Make up the volume to 50mL with DEPC water.

   Dissolve it by keeping in 65^{o}C and constant shaking
2. 5% sucrose:

   Dissolve 2.5g sucrose in 50mL of autoclaved water. Store at -20^{o}C.
3. 20% sucrose:

   Dissolve 10g sucrose in 50mL of autoclaved water. Store at -20^{o}C.

1.3.17. Immunostaining

2. Take the slides out from -20^{o}C and dried them in 37^{o}C for half an hour.
3. Wash the slides three times with 1xPBS, 10 min each by overlaying over the slides.
4. Then put the slides in 2N HCL for 20min.
5. Wash the slides twice with Sodium borate (ph 8.5, 0.1M) for 10 min each.
6. Block the tissues using 3%BSA in 1XPBST for one hour.
7. Overlay the 1^{o} Antibody diluted in 1%BSA /PBST (1:1000) over the slides after blocking and keep it in 4^{o}C overnight.
8. Next day, we then wash the slides twice for 10 min each with 1xPBST.
9. Overlay 2^{0} antibody diluted in 1%BSA in 1xPBST(1:1000) and keep in RT for 3 hours.
10. Wash the slides thrice with PBST for 10 min each.
11. Wash the slides twice with water and let it dry for 1 hour.
12. Then mount the dried slides with DABCO.
13. Take the image of the section under fluorescence microscope

1.3.17. *In situ* hybridization on cryosections.

Day 1: Hybridization.

1. Hydrate the slides in following sequence in coupling jar for 1 min each :

    100% EtOH

    95% EtOH

    70% EtOH

    50% EtOH

    2XSSC

2. Incubate slides in Proteinase K solution for 6 min at 37^{o}C

-Prewarmed Proteinase K buffer to 37^{o}C and add 250µL of 10mg/mL proteinase K

3. Rinse slides briefly in room temp DEPEC water for 2 min.
4. Rinse them in 0.1M TEA pH 8.0 for 3 min.
5. Rinse in Acetic anhydride/TEA for 10 min.

    -Add 130µL of acetic anhydride 0.1M TEA.

6. Dehydrate the slides in SSC and ethanol series for 1 min each:

2X SSC

50% EtOH

70% EtOH

95% EtOH

100% EtOH

7. Dry the slides for at least 1 hour at RT.
8. Pre warm the hybridization solution at 56^{o}C.
9. Probe preparation:

-Add probe to hybridization solution and mixed properly and boil probe and hybridization solution mi at 100^{o}C for 10 min.Plunge immediately on ice for 2mins

10. Add the hybridization/ probe mix solution to each slide and coverslip with siliconized hybrid slips
11. Place slides in humid chamber dampened with 50% formamide/5X SSC and incubate at 56^{o}C overnight.

Day 2: Post hybridization.

1. Preheat the following solutions:
    - 50% formamide/2X SSC solution to 65^{o}C.
    - Two 50mL RNase buffer washes, one to 37^{o}C and the other to 65^{o}C.
    - Two 2X SSC washes to 37^{o}C.
2. Soak slides with cover slips in 2X SSC for 30 min at RT on shaker table
3. Gently remove the hybrid slip apart from slide with forceps.
4. Rinse slides in 50% formamide/2X SSC solution for 30 min at 65^{o}C, gently agitate for the first 5 min.
5. Rinse slides twice in 2X SSC for 10 min at 37^{o}C.
6. Incubate slides in RNase buffer having 100µl of RNaseA (10mg/mL) in 37^{o}C for 30 min.
7. Then wash slides in 65^{o}C RNase buffer for 30 min.
8. Wash slides for 3 hours in 1X Maleate buffer/0.05% Triton X-100/1% RMB blocker solution at RT
9. Wash slides twice in 1X Maleate buffer for 5 min.
10. Incubate slides with 300ul antibody (anti dig) diluted (1:2500 dilution) in 1X Maleate/0.05% Triton X-100/1% RMB blocker solution overnight at RT.

Day 3

1. Wash slides twice with 1X Maleate buffer for 5 minutes.
2. Wash twice with Genius buffer for 5 minutes each.

3. Add NBT/BCIP,diluted in genius buffer(1:50) and incubate it at room temperature in dark for colour reaction.
4. Detect colour in bright field microscope.

Solution compositions are same with that of whole mount *insitu*hybridization.

CHAPTER2

SUMMARY AND CONCLUSION

2.1 Concluding Remarks

It is observed that Dnmts are regulated during retina regeneration and their level of regulation is different for different Dnmts. Interestingly the expression pattern of *Dnmt1, Dnmt4 and Dnmt8* are found to be similar in retina during retina regeneration and embryonic development. *Dnmt1*. *Dnmt4*, *Dnmt7* and *Dnmt8* are expressed in retina and brain during embryonic development. During retina re generation Dnmt1 are found to be induced at the injury site at 4dpi. These observations gave us an idea that Dnmts may be playing a role during retina regeneration as they are clearly being regulated during after injury.

When Dnmts are blocked with pharmacological inhibitor, we observed the number of proliferating Müller glial cells increases at 4dpi. By blocking Dnmts at different phases of regeneration it is observed that, the number of proliferating Müller glial cells is much more at 4dpi when they are blocked at early phase of regeneration. This suggested us that Dnmts may be having an important role during the early phase of regeneration, reprogramming to form MGPC from Müller glia. When Dnmts are blocked till peak proliferation and allowed them to regenerate. We observed that the cells which have their Dnmts blocked are not able to survive till later phase; however, if we blocked the Dnmts from 4dpi till 14dpi the number of proliferating cells is more as compared with control. So, this tells us that Dnmts are somehow maintaining the proliferation of MGPCs. It is possible that different type of Dnmts are having their important roles at different time points, as the blocker we are using blocked all the types of Dnmts, the results we are observing may be the combinatorial effects of blocking different Dnmts.

We observed that different regeneration associated genes are either down regulated or up regulated when Dnmts are blocked during retina regeneration. Level of expression of

*ascl1a*and *shha*are increased; while level of expression of *mmp9, foxn4* and *insm1a* are observed to be decreased as compare with the control. Those genes which are showing increase in level of expression when Dnmts are blocked may be having methylation sites on their promotor and so their expression is directly regulated by Dnmts. The other possible way is by regulating the expression of its upstream activator genes. Those genes which are down regulated by blocking of Dnmts may be because of the activation of their inhibitor which was repressed by methylation.

2.2.Future Outlook

In order to identify the role and exact function of different Dnmts, MO knockdown of different Dnmts can be done. By doing this experiment, we can acquire clear idea on the way Dnmts are regulating retina regeneration.

We can look whether the increased or decreased in level of expression of the genes in blocking of Dnmts reflect in the protein level of such genes

Dnmts are also known to be interacting with other epigenetic modifier like Histone de-acetylase (HDAC), and Ezh2 (polycomb group protein). So, we can check whether epigenetic regulations of genes during retina regeneration are done co-operatively with other epigenetic modifiers.

Comparing the methylation status of uninjured fishes DNA and DNA from regenerating retina by doing bi-sulphite sequencing can tell us the putative target genes which might be regulated by Dnmts. We can compare it with the result we observed from the experiments done by blocking of Drug using Pharmacological method

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List of primers used

dnmt1-RT-Fwd	5'ACCTTTGGTGTGCTGCAGGCTGGAC3'
dnmt1-Rt-Rev	5'AACCAGGGCACTCATGTCCTTGCAG3'
dnmt1-FL-Fwd	5'CAAAATCGAACTTGAAATGCCTACC3'
dnmt1-FL-Rev	5'ATAAAACATCACATGAATGGCACTGC3'
dnmt3-RT-Fwd	5'GACGGACGGTGGTGGTAATG3'
dnmt3-RT-Rev	5'CTGACAAAAAGCAGCACCTGAGC3'
dnmt4-RT-Fwd	5'CAAGATGACTGCCACGGCTG3'
dnmt4-RT- Rev	5'CTGTTCACACTCTCATCTGCGG3'
dnmt4-FL-Fwd	5'GCCCTTGAGGTGGCTAGTTTACGTGC3'
dnmt4-FL-Rev	5'GCAGCCGACACCTCTATGGCGTCAA3'
dnmt6-RT-Fwd	5'TGATGGGATCGCAACAGGGC5'
dnmt6-RT-Rev	5'CGACCGGTGCCCCTCGTAG5'
dnmt7-RT-Fwd	5'GGAGCAATGTCGTTCAGGTGC5'
dnmt7-RT-Rev	5'TCGTTCACAGGAACTGGCTCTG3'
dnmt7 -FL-Fwd	5'GTGCCATTCTGTGATTGGTTCTTTGTC3'
dnmt7-FL-Rev	5'GAGTTTGGTGCAATTTATTTTTCATC3'
dnmt8-FL-Fwd	5'CAACCATGACCAGGACTTTGAGC3'
dnmt8-FL-Rev	5'GAAGTGTCCTGTGGTTGAAGGTC3'

BIBLIOGRAPHY

1. Fausett, B.V, &Goldman, D. A role forα1 tubulin-expressing Müller glia in regeneration of the injured zebrafish retina. J. Neurosci.26,6303-6313
2. Ooto, S. Potential for neural regeneration in the adult mammalian retina]. Nippon GankaGakkaiZasshi110, 864–871 (2006).
3. Wan, J. et al. Preferential regeneration of photoreceptor from Müller glia after retinal degeneration in adult rat. Vision Res. 48, 223–234 (2008).
4. Jadhav, A. P., Roesch, K. &Cepko, C. L. Development and neurogenic potential of Müller glial cells in the vertebrate retina. Prog. Retin. Eye Res. 28, 249–262 (2009).
5. Roesch, K. et al. The transcriptome of retinal Müller glial cells. J. Comp. Neurol. 509, 225–238 (2008).
6. Reichenbach, A. &Bringmann, A. New functions of Müller cells. Glia 61, 651–678 (2013).
7. Thomas JL, Ranski AH, Morgan GW, Thummel R: Reactive gliosis in the adult zebrafish retina. Exp Eye Res 2016, 143:98-109.
8. Ramachandran R, Fausett BV, Goldman D: Ascl1a regulates Müller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signaling pathway. Nat Cell Biol 2010, 12:1101-1107.
9. Wan J, Ramachandran R, Goldman D: HB-EGF is necessary and sufficient for Müller glia dedifferentiation and retina regeneration. Dev Cell 2012, 22:334-347.
10. Zhao XF et al.: Leptin and IL-6 family cytokines synergize to stimulate Müller Glia reprogramming and retina regeneration. Cell Rep 2014, 9:272-284.
11. Nelson CM et al.: Tumor necrosis factor-alpha is produced by dying retinal neurons and is required for Müller glia proliferation during zebrafish retinal regeneration. J Neurosci 2013, 33:6524-6539.

12. Ramachandran, R., Zhao, X. F. & Goldman, D. Ascl1a/ Dkk/β-catenin signaling pathway is necessary and glycogen synthase kinase-3β inhibition is sufficient for zebrafish retina regeneration. Proc. Natl Acad. Sci. USA 108, 15858–15863 (2011).
13. Wan, J., Ramachandran, R. & Goldman, D. HB-EGF is necessary and sufficient for Müller glia dedifferentiation and retina regeneration. Dev. Cell 22, 334–347 (2012).
14. Nelson, C. M. et al. Stat3 defines three populations of Müller glia and is required for initiating maximal Müller glia proliferation in the regenerating zebrafish retina. J. Comp. Neurol. 520, 4294–4311 (2012).
15. Meyers, J. R. et al. β-catenin/Wnt signaling controls progenitor fate in the developing and regenerating zebrafish retina. Neural Dev. 7, 30 (2012).
16. Ramachandran, R., Fausett, B. V. & Goldman, D. Ascl1a regulates M・ler glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signaling pathway. Nature Cell Biol. 12, 1101–1107 (2010).
17. Mikkelsen, T. S. et al. Dissecting direct reprogramming through integrative genomic analysis. Nature 454, 49–55 (2008).
18. Polo, J. M. et al. A molecular roadmap of reprogramming somatic cells into iPS cells. Cell 151, 1617–1632 (2012).
19. Robertson KD. DNA methylation and human disease. Nat Rev Genet. 2005;6(8):597-610
20. Lister R, Pelizzola M, Dowen RH, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. Nature. 2009; 462(7271):315-22
21. Shimoda N, Yamakoshi K, Miyake A, Takeda H. Identification of a gene required for de novo DNA methylation of the zebrafish no tail gene. DevDyn. 2005;233:1509–16.
22. Probst AV, Dunleavy E, Almouzni G. Epigenetic inheritance during the cell cycle. Nat Rev Mol Cell Biol. 2009;10(3):192-206.
23. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 1999;99(3):247-57

24. Kareta MS, Botello ZM, Ennis JJ, Chou C, Chedin F, et al. Reconstitution and mechanism of the stimulation of de novo methylation by human DNMT3L. J Biol Chem. 2006;281(36):25893-902
25. Bourc'his D, Xu GL, Lin CS, Bollman B, Bestor TH, et al. Dnmt3L and the establishment of maternal genomic imprints. Science. 2001;294(5551):2536-9
26. Stresemann, C.; Lyko, F. (2008). "Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine". International Journal of Cancer. 123 (1): 8–13.