Role of DNA Methyl Transferases (Dnmts) during retina regeneration in zebrafish and its interactions with other epigenetic modifiers.

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Certificate of Examination

This is to certify that the dissertation titled “Role of DNA Methyl Transferases (Dnmts) during retina regeneration in zebrafish and its interactions with other epigenetic modifiers” submitted by Mr. Akshai J. Kurup(MS13034) for the partial fulfilment 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.

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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.

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Dated: April 10, 2018

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
- **hpi-** Hours post injury
- **dpi-** Days post injury
- **ONL-** Outer nuclear layer
- **INL-**Inner nuclear layer
- **GCL-** Ganglion cell layer
- **Aza-**Azacytidine
- **BrdU-**Bromodeoxyuridine
- **PCNA**: Proliferating Cell Nuclear Antigen

Abstract

The anatomical structure of zebrafish and mammalian retina are highly similar. However, the reason why mammalian retina fails to regenerate when zebrafish shows a robust regenerative response has been a paradox. The conundrum has been perceived as an opportunity by the scientific community. Endavours form the last two decades have unravelled wealth of information regulating retina regeneration in zebrafish with the hope of restoring mammalian vision following injury.

However, the role of epigenetic factors regulating retina regeneration remains less understood. Here, we have investigated the role of an important epigenetic modifier DNA Methyl Transferases (Dnmts) in zebrafish retina regeneration. Dnmts are known to cause down-regulation of gene expression through methylating promoters. Regulation of *dnmts* during regeneration was explored. It was also revealed that Dnmts have a proliferation-inhibitory role. However, the increased proliferation was not sustained at later time points on *dnmt* inhibition.

Regulation of some of the regeneration associated genes like Ascl1a, Sox2, *mmp9* through Dnmts were also revealed. It was also deciphered that the Dnmts regulate expression of other epigenetic modifiers like Ezh2 and Hdacs. Finally, to assess how global methylation levels impact regenerative programme whole genome bisulfite sequencing was also performed.

Chapter 1

Introduction

The loss of regenerative ability of mammalian retina is baffling especially from an evolutionary perspective. While regenerative potential is coherent among primitive vertebrates like zebrafish, mammalian retina is incapable of self-repair. Recent experiments have, however shown the incomplete regenerative potential of mammalian retina [1]. This could be unleashed through therapeutic intervention if the molecular mechanisms regulating regeneration are fully understood. Zebrafish has been the ray of hope for this problem as it fully restores retinal structure and vision following injury. Its current status as an excellent vertebrate model for developmental biology accentuates the benefits, offering developmental genetics and imaging approaches to dissect out the query [2].

Cellular and anatomical structure of zebrafish and mammalian retina is highly similar [3]. Regenerative ability of zebrafish retina relies upon the glial cells within retina called the Muller glia (MG) cells [4]. Upon injury, they undergo dedifferentiation acquiring stem cell-like/progenitor state. They further proliferate and differentiate to form MG itself as well as other neuronal cell types of retina. Recent studies have identified various genetic and epigenetic factors regulating this response. A number of pluripotency factors are induced during de-differentiation phase which are supressed prior to re-differentiation [3]. The importance of epigenetic modifications in regulating these factors is well known during embryonic development. This particularly acts as a barrier during sexual reproduction, where preparation for next generation require global DNA demethylation to induce totipotency [5].

As retina regeneration involves the reprogramming of Muller glia into multipotent Muller Glial Progenitors, we hypothesised that DNA Methyl transferases may be playing a prominent role in the process. An open accessible chromatin often promotes potency, whereas a closed chromatin is associated with somatic cells. Chromatin structure is greatly impacted by DNA methylation, and promoter demethylation of pluripotency factors are known to cause induced Pluripotency Stem Cell (iPSC) formation [6].

1.1 Retinal Structure

Structure of zebrafish retina is very similar to that of the retina of the other vertebrates. Broadly, they have three different cell layers.

1. The outer nuclear layer (ONL): Rod and Cone photoreceptor cells reside this layer. It is covered on the outer side by the retinal pigmented epithelium.
2. The inner nuclear layer (INL): Mainly comprises of interneurons which help in transmitting information from the ONL to the innermost Ganglion Cell Layer. These include Amacrine cells, bipolar cells, horizontal cells.
3. Ganglion cell layer (GCL): Made up of ganglions. These help in transmitting information to the brain through the axonal endings progressing to form the optic nerve [3].

Spanning all the three cell layers, there is the only glial cell type of the retina-Muller glia. Their main function is to maintain the retinal homeostasis and integrity. The regenerative ability of zebrafish retina is also attributed to Muller glia [3].

1.2 Zebrafish Muller glia during injury and regeneration

Main model systems used for retina regeneration research includes zebrafish, chick and mouse. While zebrafish retina completely regenerates, chick retina shows an incomplete regenerative response. Mammalian model mouse which normally doesn't regenerate is used for testing potential of therapeutic interventions triggering regenerative response. The injury paradigms followed involves chemical, light induced and mechanical methods, out of which mechanical method has the advantage that it damages all the three layers of the retina [6].

The response of Muller glia to injury is initially gliotic. This reactive gliosis can trigger the release of various factors that support survival of neurons. Prolonged gliotic response can be bad for the neurons as it can lead to neurodegeneration and can hinder normal retinal function [3].Muller glia's response to injury involves three phases:

a) Dedifferentiation phase (0-2 Days Post Injury): When the Muller glia reverts into a stem cell like state.

b) Proliferation phase (2-7 Days Post Injury): When the dedifferentiated cells divide to form a population of multipotent Muller Glial Progenitor Cells (MGPCs).
c) Redifferentiation phase (Starts at 7 Days Post Injury): Proliferating MGPCs differentiate into retinal neuronal cell types [7].

1.3 Mechanism of zebrafish retina regeneration

The positioning of the Muller glia in the Inner Nuclear Layer with processes extending to different layers of the retina allows them to protect and monitor the retinal environment [7].

Regenerative response is initiated by the dying cells and the signal comes in the form of secreted factors like tumour necrosis factor-α (Tnfα), which communicates the death of the neurons to Muller glia [8]. However, Tnfα alone has only a very limited effect on cell proliferation. Tnfα in collaboration with an inhibition of notch signalling stimulates Muller glia proliferation [9]. Notch pathway maintains the cell population in a differentiated form. Other secreted factors like Hb-egfa, Insulin, Igf-1, IL11 and Leptin are also necessary for Muller glia reprogramming and progenitor formation. They are produced by the Muller glia and thus, may be acting in an autocrine and paracrine manner [9, 10, 11].

Altered connection of Muller glia with other cells on injury and phagocytosis of injured cells by Muller glia has also reported to be important in the initial phase of regeneration. However the mechanism by which they acts remain less characterised. [3]

Downstream of EGF and cytokine pathways come mitogen-activated protein kinase (MAPK) and Janus kinase (Jak) pathways [13, 14]. PI3K signalling is also induced post injury. These signalling events are required for beta-Catenin and pStat3 expression necessary for injury dependent cell proliferation [14]. Muller glia acquires properties of stem cells by reprogramming its genome, which also suggests that epigenetic modifiers may be regulated by these factors.

Various microRNAs are also involved in the regeneration process, which was brought to light when knockdown of the microRNA processing enzyme DICER triggered Muller glia proliferation. Some of the well characterised microRNAs involved in the process include the *let7* microRNA, *mir-203* etc. [15].

Injury dependent induction of Ascl1a is necessary for the initiation of regenerative response. Ascl1a expression is initiated by TNFa and Hbegfa [8]. Ascl1a stimulates *lin28*, forming an RNA binding protein, which supresses *let7* microRNA. Lin28 and let7 regulate each other and Lin28 is associated with self-renewal and stemness of cells, while the *let7* is well known to promote cellular differentiation by supressing multiple pluripotency factor transcripts [16]. Eventually, Ascl1a supresses expression of *insm1a*. Insm1a is a transcriptional repressor that promotes differentiation [17].

Throughout the process of regeneration, various epigenetic factors have also been reported to be involved. At 4 days post injury condition, promoters of regeneration associated genes including *ascl1a*, *lin28*, *hbegfa* and *insm1a* exhibited high levels of demethylation. Also, there is reported correlation between DNA methylation and chromatin architecture, suggesting that high levels of methylation may be promoting a closed chromatin supressing the expression of different genes [4]

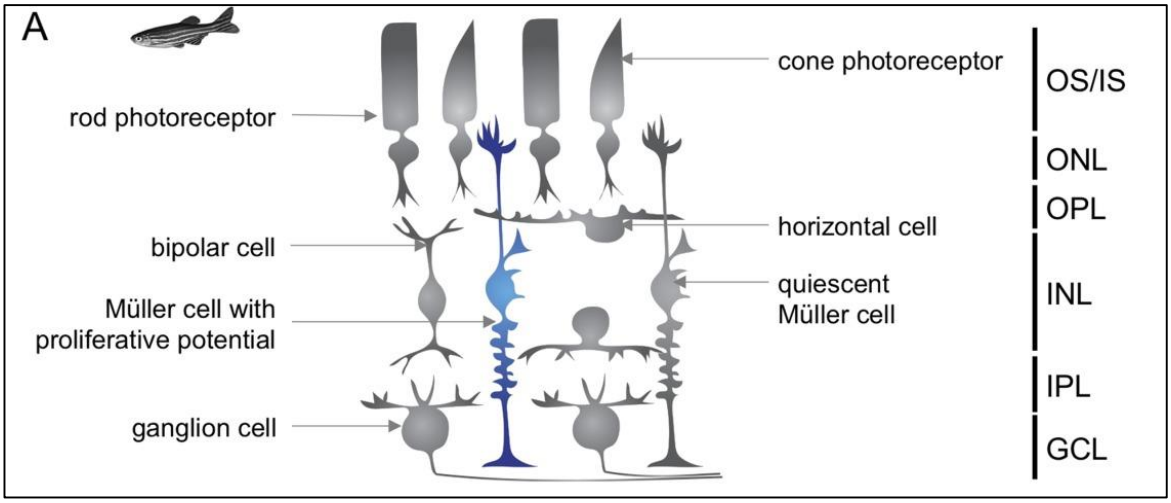


Fig 1.1. A schematic showing the retinal cell types and layers.

Source : Müller glial cell-dependent regeneration of the neural retina: An overview across vertebrate model systems (2016). Hamon A, Roger JE, Yang XJ, Perron M. Dev Dyn published online: 8 JAN 2016 DOI: 10.1002/dvdy.24375.

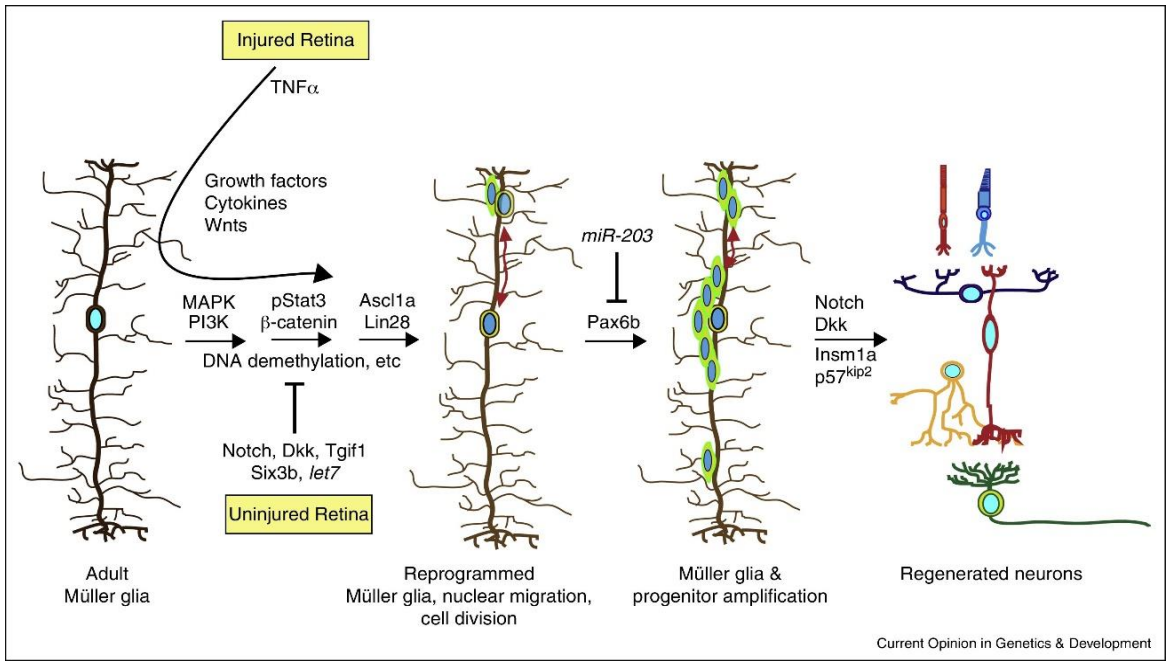


Figure1.2 Different phases of regeneration and the signalling pathways triggering them.

Source : Müller glial cell-dependent regeneration of the neural retina: An overview across vertebrate model systems (2016). Hamon A, Roger JE, Yang XJ, Perron M. Dev Dyn published online: 8 JAN 2016 DOI: 10.1002/dvdy.24375.

1.4 DNA Methylation, Histone Acetylation and Histone Methylation

DNA Methylation is a very important and most studied epigenetic mark. This is known to be a repressive epigenetic mark. The binding of transcription factors to the promoter region is hindered by DNA methylation, thereby preventing expression of the particular gene. These marks are written on the genome by a set of enzymes-DNA Methyl Transferases (Dnmts). The mechanism of DNA methylation is conserved in mammals and zebrafish. In zebrafish, the methylation machinery involves 8 Dnmts (Dnmt1, Dnmt2, Dnmt3, Dnmt4, Dnmt5, Dnmt6, Dnmt7, Dnmt8) [18].

While the role of Dnmt2 in methylating DNA remains elusive, it has shown to be involved in tRNA methylation. This is one of the robust mechanisms preventing degradation of these RNAs under stress. Rest of the Dnmts are involved in DNA methylation. Also known as maintenance methylation, Dnmt1 photocopies methylation pattern from one DNA strand to the other during DNA replication. Other Dnmts, like mammalian homologs DNMT3A and DNMT3B, establish DNA methylation from the scratch (*de novo* methylation). Even though the specific targets of particular Dnmts remain unidentified in zebrafish, all of them are shown to be involved in eye development. The methylation reaction occurs when the Dnmts transfer a methyl group from S-adenosyl methionine (SAM) to the CpG islands. Non-CpG methylation is also present in zebrafish, however, at basal levels without any reported functions [18].

Induced pluripotent cell formation involves genome wide demethylation. This is known to induce the expression of genes necessary for potency, and methylation of these targets occur during differentiation. This process is also well documented during germ cell formation, fertilization and cell fate determination. Exploring the precise promoter targets of Dnmts along with other chromatin modifications like histone acetylation, histone methylation, 3 dimensional organisation would help us understand the gene regulatory network better. This may direct us to unravel the roles of novel genes involved in retina regeneration as well [3].

It is thus important to understand how DNA Methyl transferases collaborate with Histone Deacetylases (Hdacs) and PRC complexes. Hdacs assist chromatin remodelling

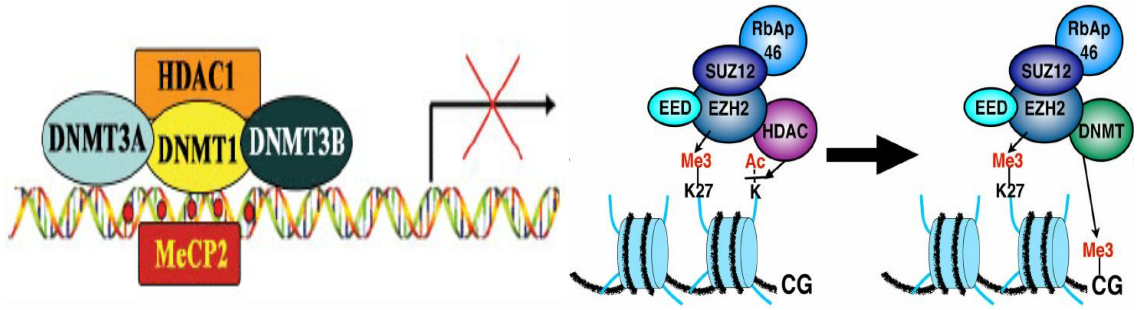
by acting as a catalyst for acetyl-L-lysine side chains of histone proteins. This supresses the expression of the particular gene [19]. PRC2 contributes to chromatin compression through methylation of histone H3 at lysine 27. Its activity is known to regulate cell identity and differentiation. PRC2 also contributes to chromatin compaction, and catalyses the methylation of histone H3 at lysine. Interplay of these factors and the modifications that they make is known in various models. There is a reported direct interaction between PRC2 enzymatic component EZH1 and DNMT1 in various systems [20]. The H3K27me3 marks and DNA methylation show strong antagonism as reported in zebrafish during development and human embryonic stem cells. However, DNA methylation shows correlation with histone acetylation and H3K9me3. [21]

1.5 Bisulfite sequencing

Bisulfite sequencing is the current gold standard for assessing genomic regions modified by DNA methylation. It involves bisulfite treatment, where unmethylated cytosines are converted to uracils. Methylated cytosines remain unchanged. Next generation sequencing enable measurement of DNA methylation at base-pair resolution for the whole genome [22].

1.6 Pharmacological inhibition

5-Azacytidine is a cytosine analogue that is metabolized and incorporated into the DNA. Dnmts are tricked to irreversibly bind the incorporated form of Azacytidine, thereby preventing the further activity.



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Kundakovic, Marija & Chen, Ying & Guidotti, Alessandro & Grayson, Dennis. (2008). The Reelin and GAD67 Promoters Are Activated by Epigenetic Drugs That Facilitate the Disruption of Local Repressor Complexes. Molecular pharmacology. 75. 342-54. 10.1124/mol.108.051763.

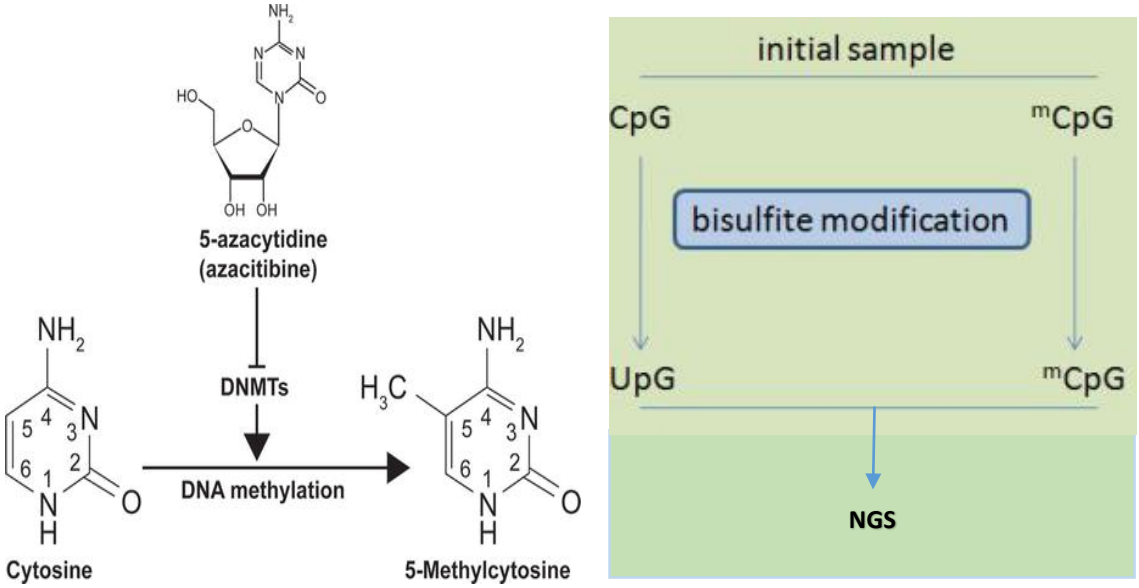


Figure 1.4 a) Mechanism of action of Dnmts and azacytidine. b) Bisulfite sequencing

DNA Hypermethylation in Breast Cancer *By Le Huyen Ai Thuy, Lao Duc Thuan and Truong Kim Phuong DOI: 10.5772/66900*

Chapter2
Results and observations

2.1 Temporal regulation of *dnmts* in zebrafish retina

Post injury, whole retina RNA was isolated at desired time points and RT-PCRs were performed.

dnmt1 mRNA levels decreased 12 hours post injury (hpi) after injury. It again increased, peaking at 2 and 4 days post injury (dpi), finally went down at 8dpi. It was present in uninjured retina also. *dnmt4* expression went down at 4hpi and again came to the normal levels at 12hpi. It completely turned off by 8dpi. *dnmt5* is present in uninjured retina and the expression levels remained unchanged till 12hpi. It increased at 1dpi, subsequently went down at 2dpi and completely vanished by 4dpi. *dnmt6* was not present in uninjured retina. However, it's expression started in injured retina at 12hpi, peaking at 1dpi. Further, it's expression went down gradually and completely turned off by 8dpi. *Dnmt8* expression remained unchanged till 4 dpi. However it turns off by 8dpi (**Figure 2.1**).

Time course data clearly shows that *dnmts* are regulated post injury. Pattern of regulation, eventhough not strictly shared between all *dnmts*, also shows commonalities. Expression of all *dnmts* comes to basal levels or turns off by 8dpi. Except *dnmt6*, others show expression in uninjured retina also hinting at other housekeeping functions.

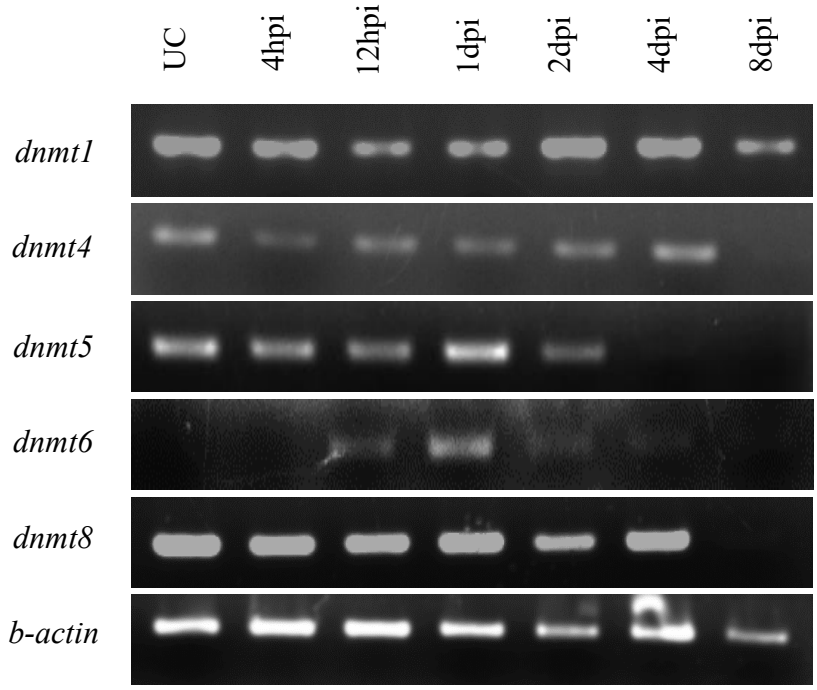


Figure 2.1 RT-PCR showing mRNA levels of *dnmt*s in regenerating retina at different time points post injury.

2.2 Effect of blocking Dnmts on retina regeneration.

To check the role of Dnmts in cell proliferation, immunostaining against PCNA was performed on retinal cryosections in Dnmt inhibited conditions. 5-Azacytidine (Aza) was used as a pharmacological inhibitor of Dnmt activity. Drug treatment was carried out by dipping injured fish in 1μM concentration of Azacytidine.

There was an increase in PCNA positive cells at 2.5dpi as well as 4dpi on Dnmt inhibition (**Figure 2.2**).

To analyse the increase in cell proliferation quantitatively, exact number of PCNA positive cells were counted in control and Aza treated sections at 2.5 and 4dpi and graphs were plotted. At 2.5dpi, PCNA +ve cell number went up from 38 to 65 on Dnmt inhibition. While at 4 dpi, PCNA positive cell number went up from 45 to 87 **(Figure 2.2), (Graph 2.1)**.

This makes the anti-proliferative effects of Dnmts evident during zebrafish retina regeneration.

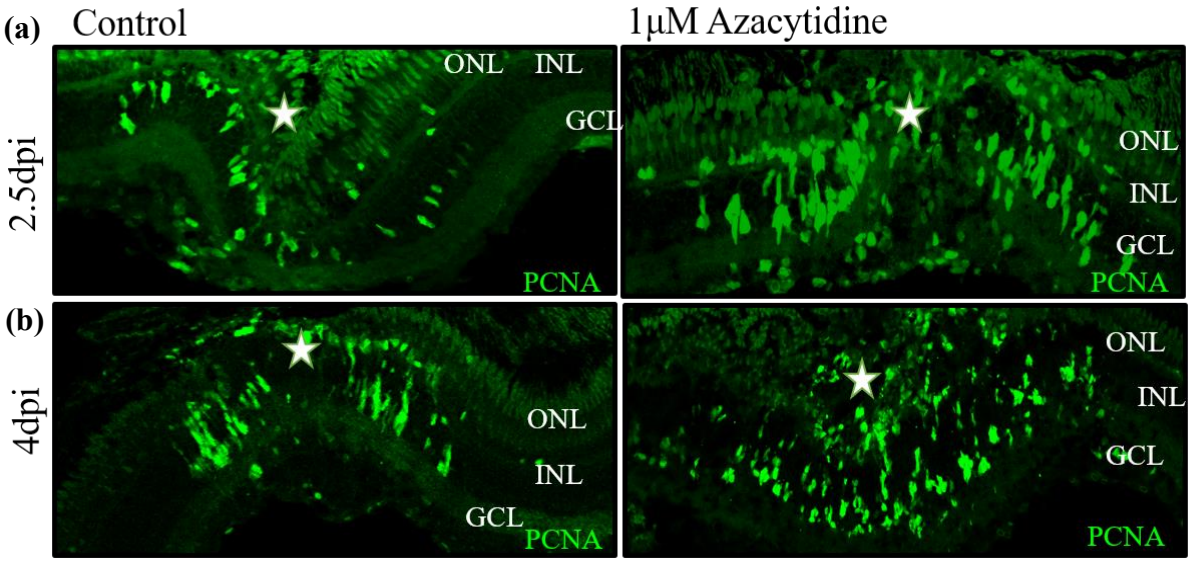
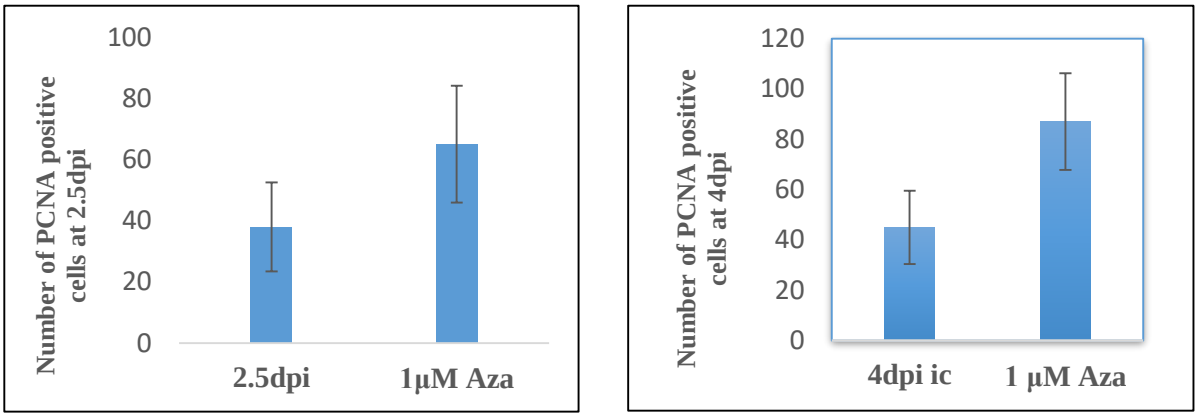


Figure 2.2 (a) Immunohistochemistry and Immunofluorescence (IF) microscopy showed an increased span and number of proliferating cells marked by PCNA, at 2.5 dpi on control and Dnmt inhibited (using 1µM Azacytidine) retinal sections. (b) IF microscopy showed an increase in the cell number marked by PCNA, in Aza treated sections as compared to the 4dpi control.



Graph 2.1 a) Number of PCNA positive cells at 2.5dpi in control and 1µM Aza treated retinas. The cell number increased from 38 to 65 on Aza treatment.

b) Number of PCNA positive cells at 4dpi in both control and 1µM Aza treated retinas. Dnmt blockade increased the number of PCNA positive cells from around 45 to 87.

2.3 Effect of Dnmt inhibition in different phases of regeneration.

In order to assess whether Dnmt inhibition has a permanent effect on regeneration, three sets of experiments were carried out. These experiments would also answer in which phase of regeneration Dnmts are having an influential role.

1. **Control**. In this set fish were continuously put in water and eyes were harvested at 6dpi, 10dpi, 14dpi and 30dpi. BrdU pulses were given on 3dpi, 4dpi and 5dpi to mark the proliferating cells. Duration of each BrdU pulse was 5 hours.
2. **Experiment set-1 (Water to Aza).** In this case fish were put in water till 4dpi. From 4dpi to 8dpi they were put in Dnmt inhibitor Aza. Eyes were harvested at 6dpi, 10dpi, 14dpi and 30dpi with Brdu labelling on 3dpi, 4dpi and 5dpi.
3. **Experiment set-2 (Aza to Water).** In this case fish were put in Aza till 4dpi. From 4dpi to 8dpi they were put in water. Eyes were harvested at 6dpi, 10dpi, 14dpi and 30dpi with Brdu labelling on 3dpi, 4dpi and 5dpi. **(Figure 2.3)**

Observations

At 6dpi, Aza to water showed an increase in proliferation compared to control and Aza to Water. Cell proliferation in Water to Aza and control remained almost the same.

At 10 dpi, Aza to water showed a depletion in BrdU positive cells indicating that the increased proliferation that this set displayed at 6dpi is not sustained till 10dpi. BrdU +ve cell numbers remained almost the same in control and Water to Aza set.

At 14dpi, Aza to water maintained fewer BrdU +ve cells as compared to Water control and Water to Aza. The number of cells in the latter two again remained nearly the same.

At 30 dpi, the number of BrdU +ve cells decreased drastically compared to its status at 14dpi. This number was less in comparison with the BrdU +ve cells of the control and Water to Aza case. **(Figure 2.3), (Graph 2.2)**

It was thus concluded that the Dnmt inhibition in the initial phase of regeneration (0-4dpi) leads to an increased proliferation initially (4dpi and 6dpi). However, this increase is not sustained at later time points (10dpi, 14dpi, 30dpi). Blocking Dnmts in the second phase of regeneration (4-8dpi) does not have any effect **(Graph 2.2)**.

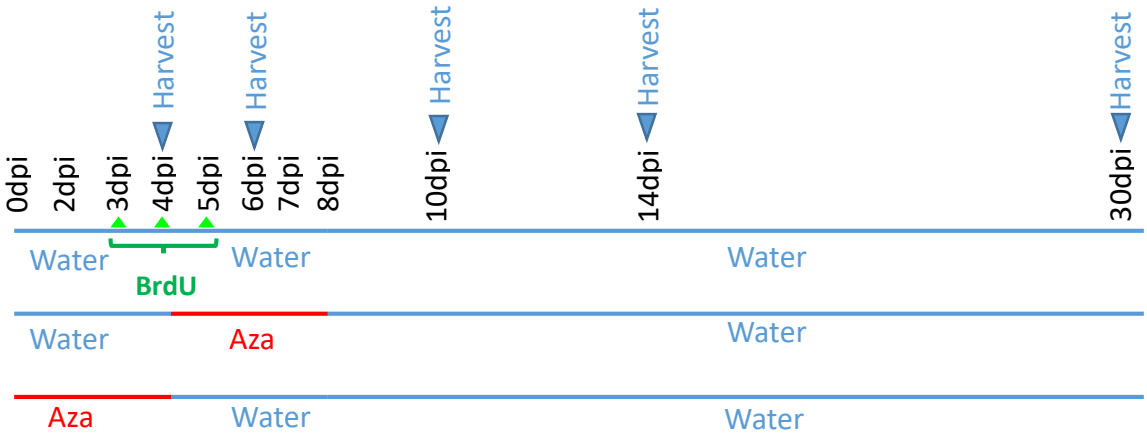


Figure 2.3 Schematic showing the experimental regime.

In the first case which is the control, fish were put in water continuously. In the second case, Azacytidine treatment was given for the duration 4-8dpi. Whereas in the third set Azacytidine treatment is limited to 0-4dpi. Eyes were harvested at 6dpi, 10dpi, 14dpi and 30dpi. BrdU, which marks the cells in the S-phase was given as pulses of 5 hours on 3dpi, 4dpi and 5dpi. Immunostaining for BrdU and counting of BrdU positive cells were performed for assessing cell proliferation.

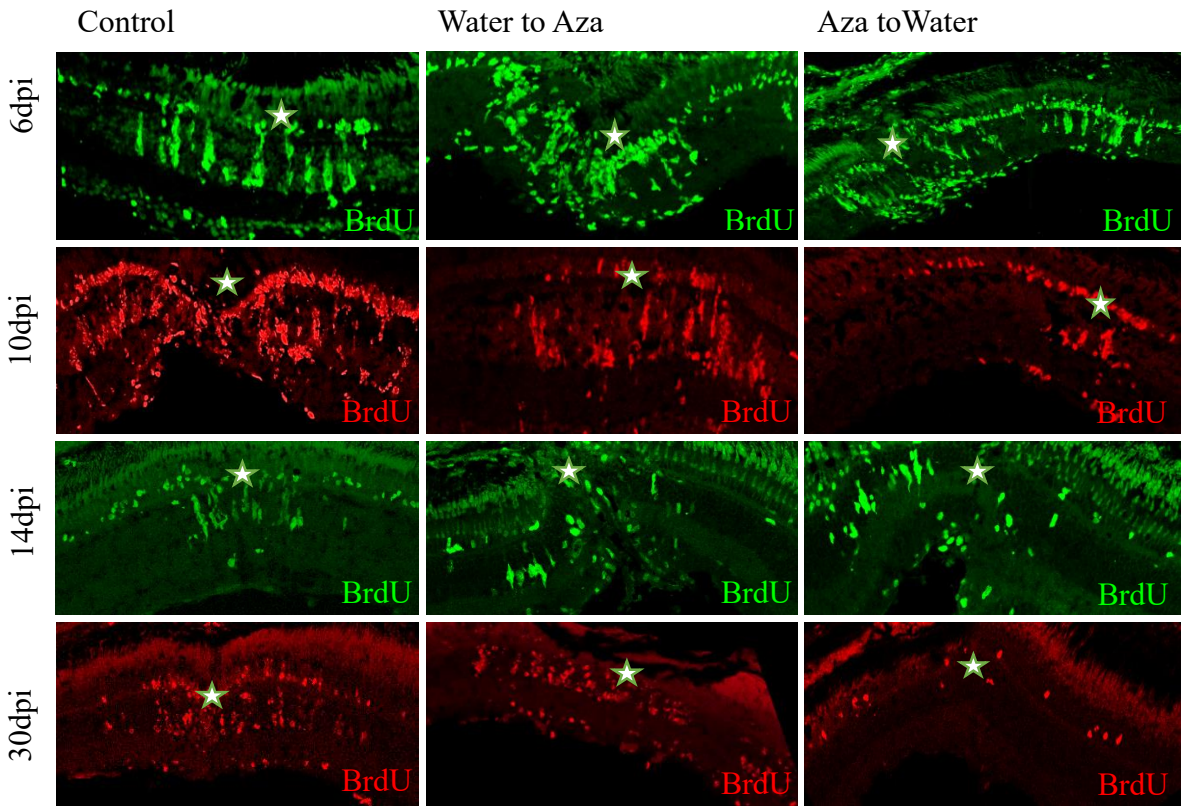
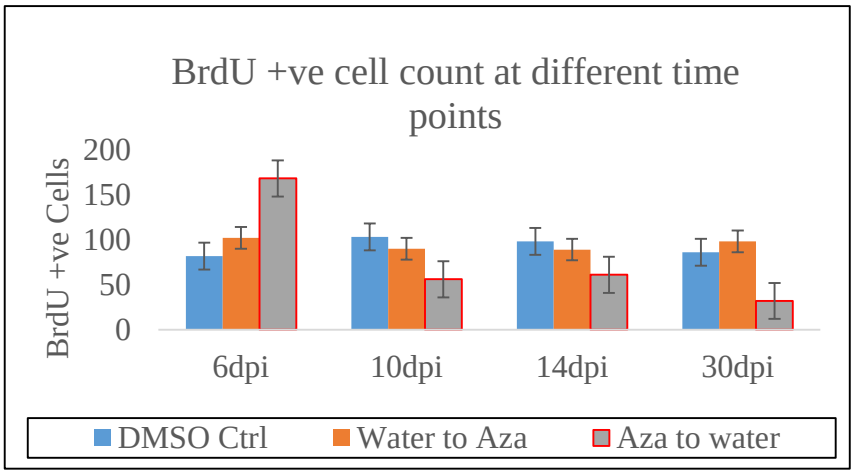


Figure 2.4 IF Microscopy at 6dpi, 10dpi, 14dpi and 30dpi for the experiments involving Water control, Aza to Water, Water to Aza. At 6dpi, the proliferation was highest for Aza to water as marked by BrdU. However, at later time points Aza to water maintained the lowest number of BrdU +ve cells.



Graph 2.2 Graphical representation of BrdU+ve cells at different time points for Water control, Water to Aza, Aza to Water at different time points. Aza to water showed an increase in BrdU+ve cell number in 6dpi as compared to control. At later time points (10dpi, 14dpi, 30dpi) Brdu+ve cell number was less than control for Aza to Water set. Compared to controls, Water to Aza set did not show any considerable difference in BrdU+ve cell number.

2.4 Effect of Dnmt inhibition on apoptosis rate.

From the previous experiments, it was noted that Dnmt inhibition in 0-4dpi leads to an increase in BrdU +ve cells at 4dpi and 6dpi. However at 10dpi, 14dpi and 30dpi, BrdU +ve cell number was less than that of controls for this set.

We hypothesised that the drastic decline in BrdU +ve cells in Aza to Water set at 10dpi is because of apoptosis. To check this, TUNEL assay was performed on 10dpi cryosections for control, Water to Aza and Aza to Water sets.

TUNEL assay showed the apoptosis rate to be highest in Aza to Water set. As compared to controls, Water to Aza set showed slightly higher apoptosis rates. **(Figure 2.5, Graph2.3).**

This validates the hypothesis that the initial increase in BrdU +ve cells declines at the later time points because of apoptosis. This is particularly evident in Aza to Water set.

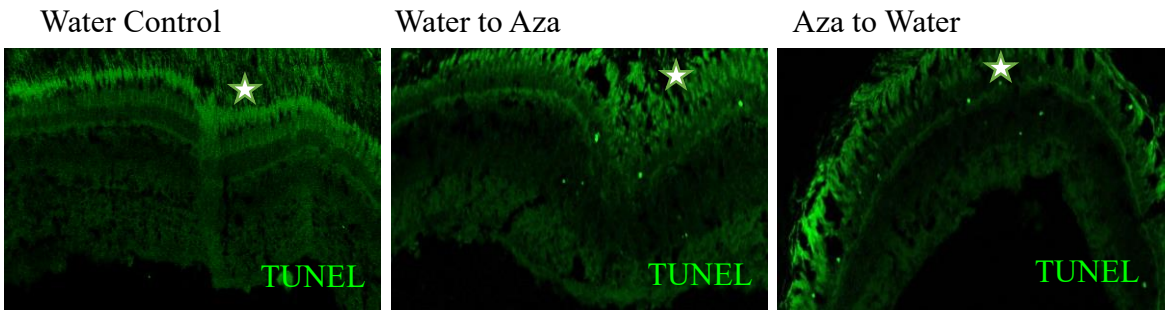
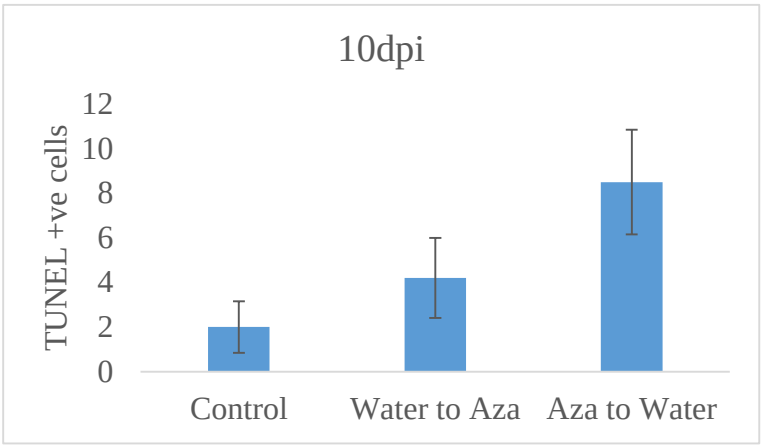


Figure 2.5 TUNEL assay at 10dpi. Water to Aza and Aza to Water experimental sets show higher apoptosis than control, out of which the number of apoptotic cells is however highest for Aza to Water.



Graph 2.3 Shows the number of TUNEL +ve cells in Control, Water to Aza, and Aza to Water sets. Aza to Water shows the highest value with 8.5 TUNEL +ve cells. Number of TUNEL +ve cells in Water to Aza is 4.2, while in control this is just 2.

2.5 Effect of Dnmt inhibition on redifferentiation phase of regeneration.

As the Dnmt inhibition from 0-4dpi (Aza to Water) takes its toll on cell survival, next the cell migration and redifferentiation levels were investigated. 0-4dpi involves de-differentiation and chances were high that these genes are involved in re-differentiation. Also, Dnmts have been shown to be a key factor determining cell fate determination and ageing [11].

14dpi retinal sections were co-immunostained for differentiated Muller glia marker Glutamine synthetase (GS) and BrdU. Correspondingly, co-immunostaining for HuD (Amacrine cell marker) and BrdU was also performed. The co-localization of BrdU and cell-type specific markers indicates that the MG cells marked by BrdU during the proliferation are able to get re-differentiated and take up the fates of Amacrine cells and MG cells itself **(Figure 2.6)**.

Despite the higher MGPCs death rate, as suffered by the Aza to water set, BrdU+ve cells showed colocalization with GS and HuD cell markers. In Water to Aza set also, redifferentiation was observed to occur efficiently.

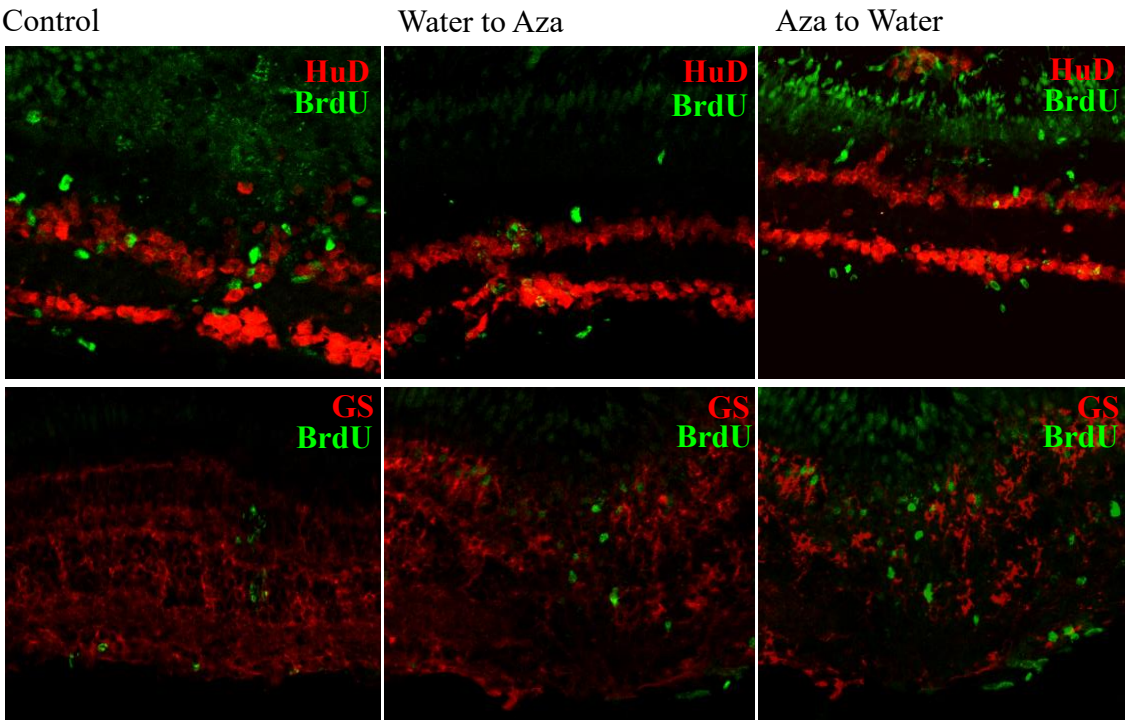


Figure 2.6 IF microscopy showed the cell-type specific staining in 14dpi retinal sections following the experimental regime described in Figure 2.3 for BrdU and HuD, where HuD is an amacrine cell specific marker, and for BrdU and GS, where GS is a differentiated Muller glia marker. Co-localization of BrdU with GS and HuD was evident in all the cases indicating differentiation of MGPCs into differentiated Muller glia and amacrine cells respectively.

2.6 Effect of Dnmt inhibition on regeneration associated genes.

With the blockade of Dnmts from 0-4dpi, showing an effect on the regenerative programme, further made us to check if the levels of regeneration associated genes also get effected upon such inhibition **(Figure 2.7, 2.8)**. This helped us in understanding the molecular basis behind the effect of Dnmt inhibition.

The expression of certain regeneration associated genes in Dnmt depleted condition at 2.5dpi was checked using RT-PCR and qPCR. The mRNA *insitu* hybridisation was also performed to check the effect of Dnmt blockade on the levels of *mmp9* and *zic2b* mRNA.

It was found that upon Dnmt blockade using 1μM Aza at 2.5 dpi, mRNA expression levels of *her4.1*, *ascl1a*, *miR34a* increased. However, *mmp9* and *zic2b* levels went down (**Figure 2.7 a**). The results were further validated using qPCR, which showed an upregulation of *sox2* and *insm1a* as well **(Figure 2.7 b)**. The mRNA *insitu* hybridisation showed that *zic2b* and *mmp9* expression levels go down upon Dnmt inhibition **(Figure 2.8).**

The binding sites of *miR34a* on *ascl1a*, *sox2* and *mmp9* mRNA were located and the effect of this binding was studied. As *miR34a* was up-regulated upon Dnmt inhibition, we checked its effect on the protein levels of Sox2 and Ascl1a. Contrary to their mRNA expression levels, Ascl1a and Sox2 protein levels showed a decrease, might be because of binding of *miR34a* on *ascl1a* and *sox2*, thereby leading to their cleavage and degradation, and preventing their translation **(Figure 2.9)**.

Thus, Dnmt blockade leads to downregulation of *mmp9* and *zic2b,* which are necessary for regeneration. Reduced expression of these factors may be the reason for non-survival of MGPCs beyond 6dpi. *miR34a,* which is suppressed by Dnmts could be targeting *ascl1a*, *mmp9* and *sox2* transcripts.

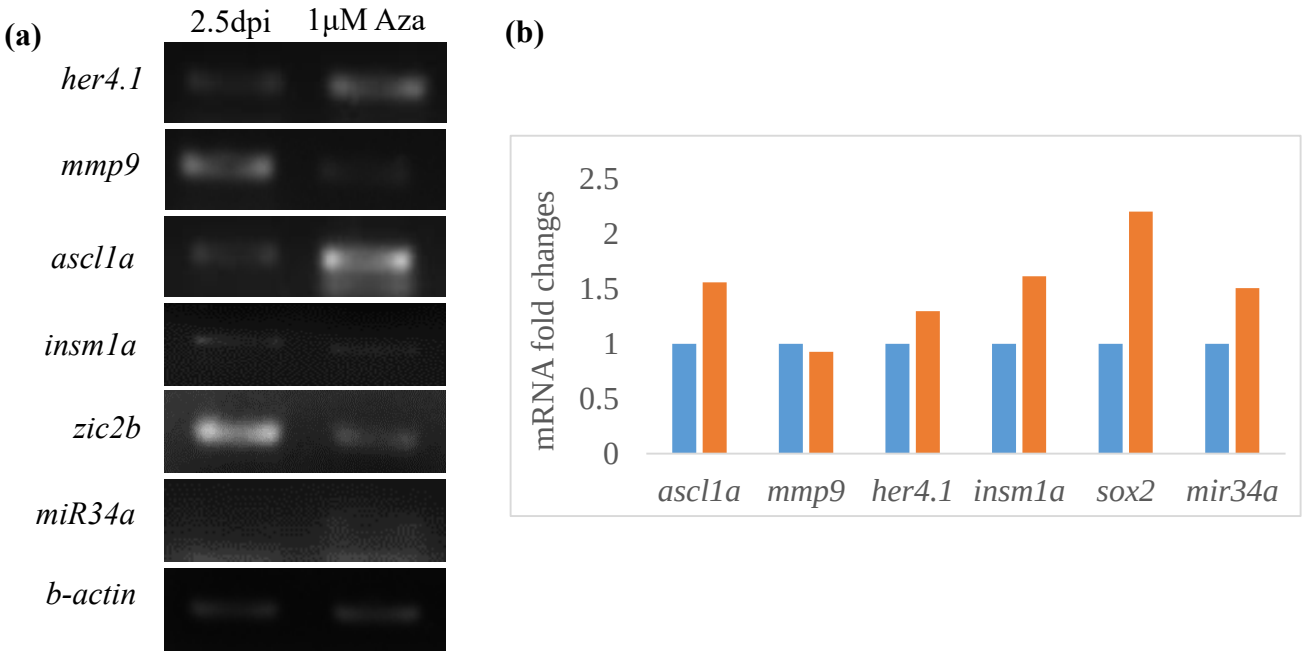


Figure 2.7 (a, b) RT-PCR (n=3) and qPCR(n=1) analysis showed the regulation of *her4.1, mmp9, ascl1a, insm1a, zic2b, sox2, miR34a*.

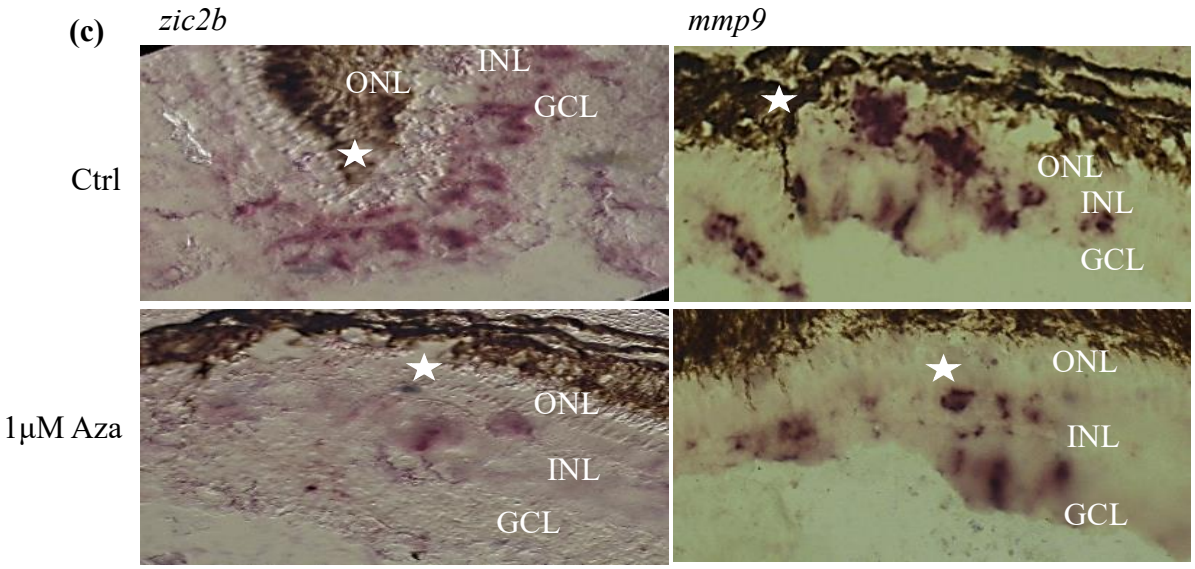


Figure 2.8 mRNA *insitu* hybridisation of *zic2b* and *mmp9* in control and 1μM Aza treated retinas.

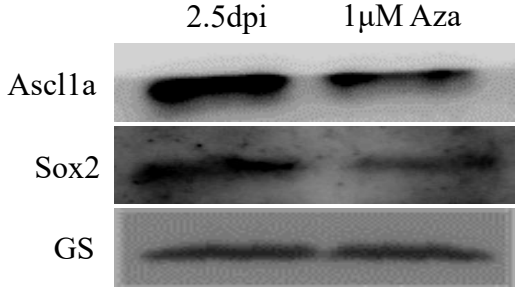


Figure 2.9. Western blotting analysis showed the protein expression levels of Ascl1a and Sox2 upon Aza treatment. GS served as the loading controls.

2.7 Effect of Dnmt inhibition on other epigenetic modifiers.

As a primer to understand the cross-talks between different epigenetic modifiers, the regulation of expression levels of other epigenetic modifiers by Dnmts were explored.

2.7.1 Effect of Dnmt inhibition on Ezh2 expression levels.

To envisage the mechanism of regulation of Ezh2 by Dnmt, RT-PCR, qPCR and mRNA *insitu* hybridisation of *ezh2* were carried out in 1μM Aza treated and control retinas.

RT-PCR showed an upregulation of *ezh2* under Dnmt inhibited condition. This result was confirmed using qPCR and mRNA *insitu* hybridisation **(Figure 2.10 a, b, c)**.

To check if the increase in transcript levels are reflected at translational levels also, immunohistochemical analysis of Ezh2 was performed in control and 1μM Aza treated eye sections, which confirmed an increase in the protein levels of Ezh2 in Dnmt inhibited background **(Figure 2.11)**.

2.7.2 Effect of Dnmt inhibition on Hdac expression levels.

Another important class of transcriptional repressor whose expression levels under Dnmt inhibition were also investigated were Hdacs.

RT-PCR showed an increase in the expression levels of all *hdacs* except *hdac1*. This was further confirmed in the case of *hdac1, hdac3, hdac5* and *hdac6* by qPCR analysis also **(Figure 2.12 a, b)**.

The *hdac1* mRNA expression did not change as evident by qPCR, the protein expression was also analysed, since there is *miR34a* binding site on *hdac1* mRNA. However, protein expression also did not show any difference upon Aza treatment, as confirmed by immunostaining and western blotting analysis **(Figure 2.13 a, b)**.

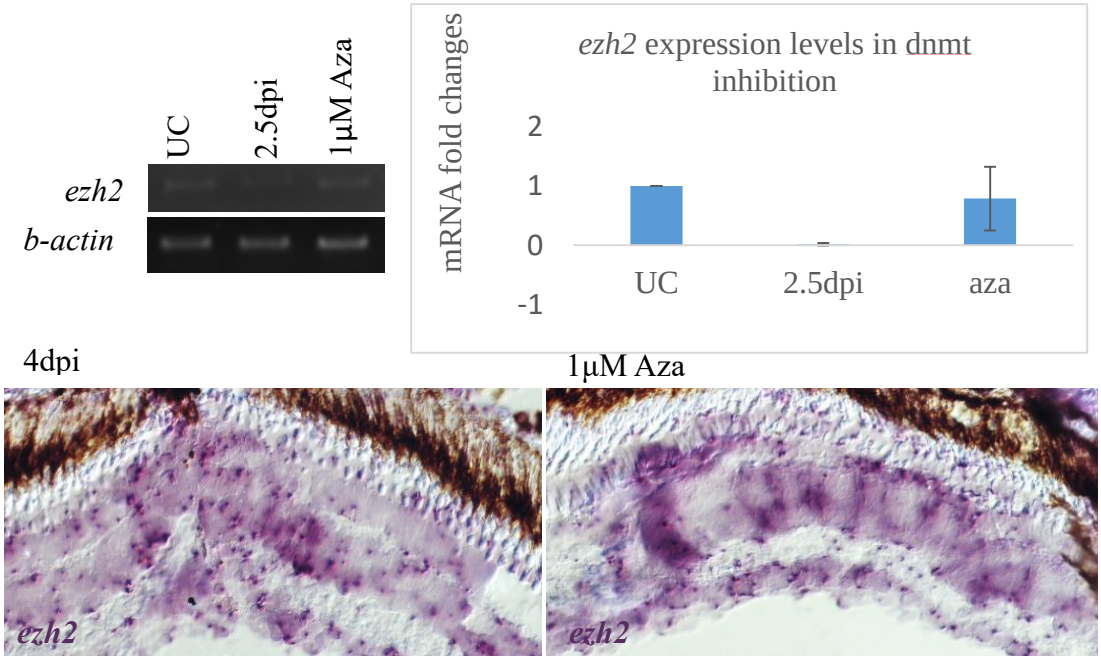


Figure 2.10 (a, b) RT-PCR and qPCR comparing mRNA levels of *ezh2* in Uninjured Control (UC), 2.5dpi control and 1uM Aza treated retinas at 2.5dpi, showed an increase in *ezh2* mRNA levels (n=3).

c) mRNA *insitu* hybridisation of *ezh2* showed an increase in its levels upon Aza treatment as compared to the control, at 4dpi.

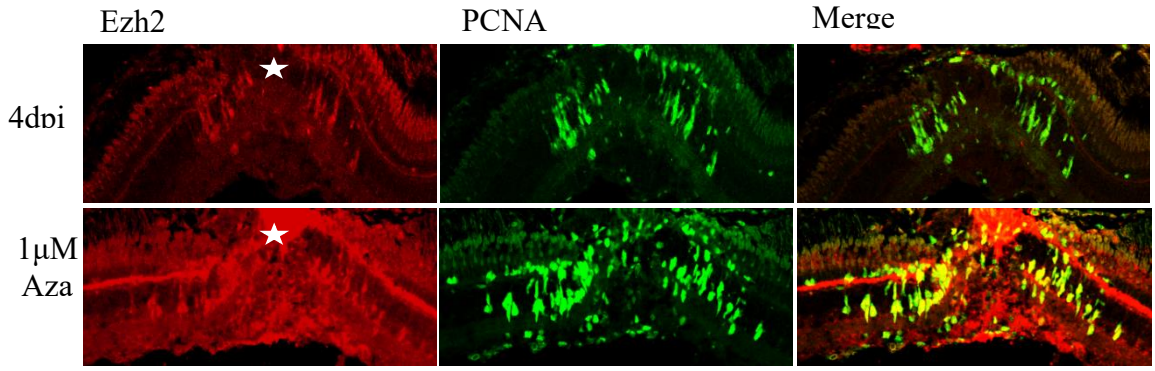


Figure 2.11 IF microscopy showed an increase in the expression of Ezh2 protein and PCNA in Aza treated condition as compared to the control, at 4dpi.

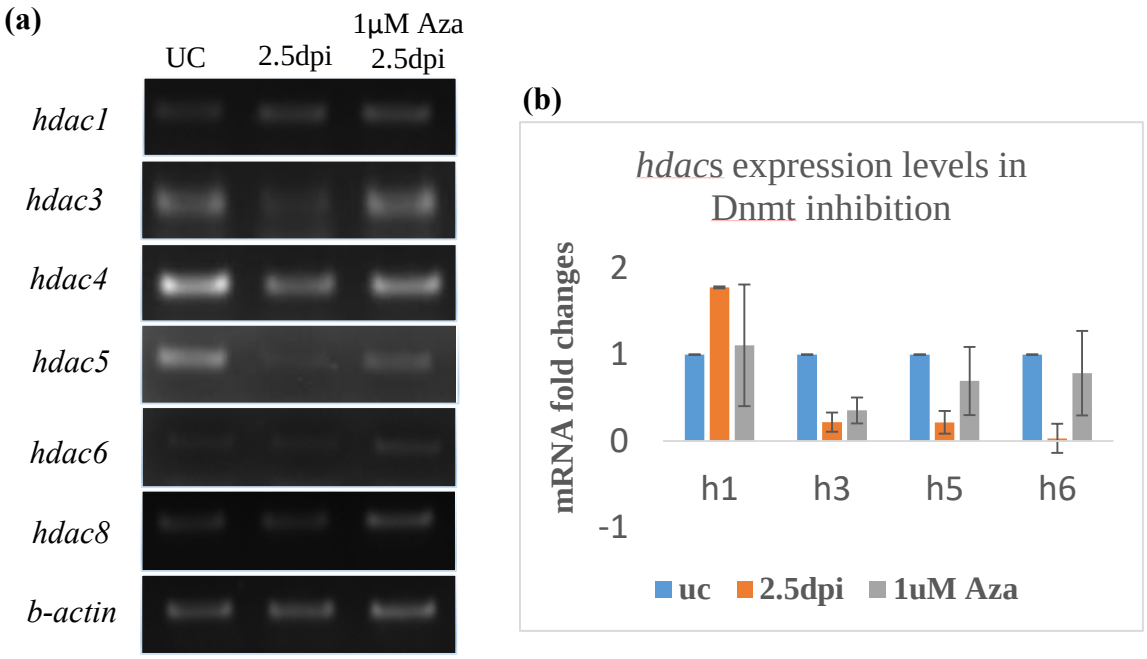


Figure 2.12 (a, b) RT-PCR and qPCR analysis showed the regulation of various *hdac*s upon Dnmt inhibition, at 2.5dpi (n=3).

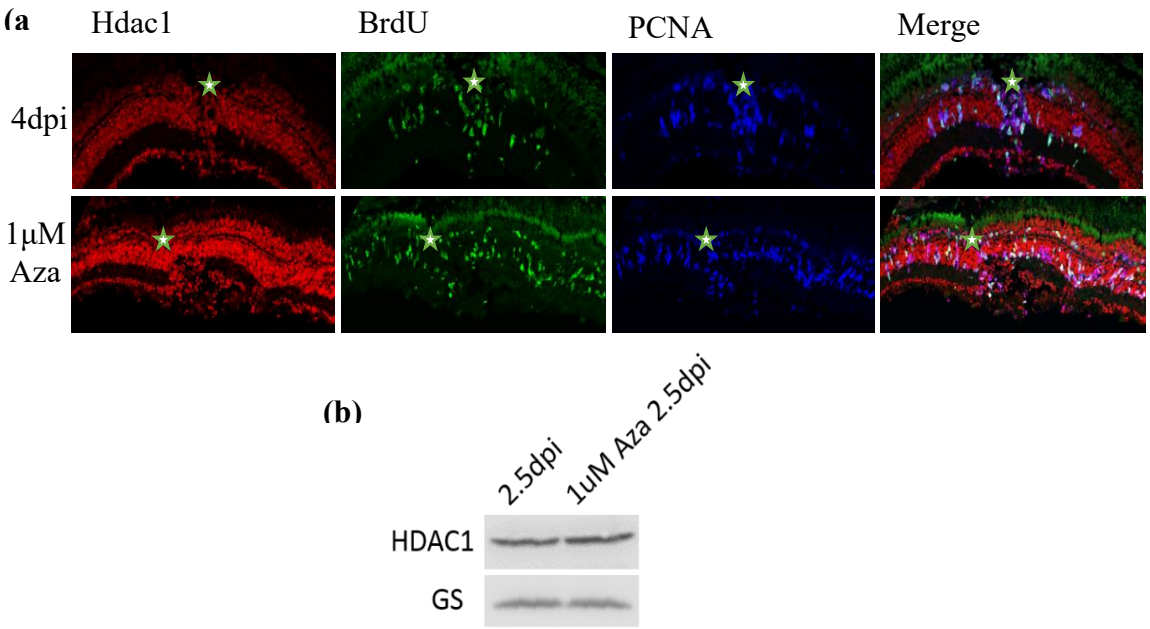


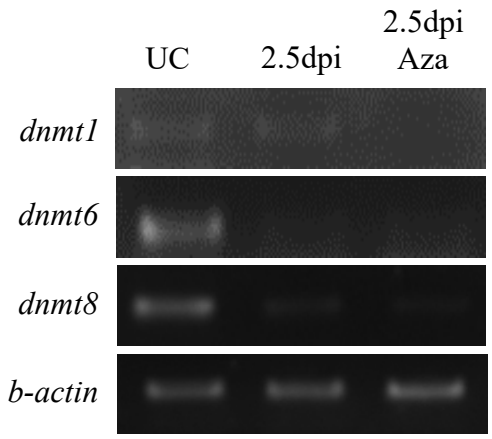
Figure 2.13 (a) Immunohistochemistry & IF microscopy in 4dpi and (b) Western Blotting analysis in 2.5dpi, done for Hdac1, BrdU and PCNA, showed that Hdac1 levels remained unchanged, irrespective of the proliferation in 1µM Aza treated retinas as compared to the control.

2.7.3 Effect of Dnmt inhibition on Dnmt expression levels.

To analyse if Dnmts autoregualte, RT-PCRs of Dnmts were performed in Dnmt depleted condition.

RT-qPCRs showed a decrease in mRNA expression levels of *dnmt1*, *dnmt6* and *dnmt8* on 1μM Aza treatment. This was confirmed in the case of Dnmt1 using mRNA-*insitu* hybridisation. It was thus shown that Dnmts autoregulate **(Figure 2.14)**.

It was thus concluded that the inhibition of Dnmts lead to an increase in the expression of ezh2 and all hdacs except hdac1. Dnmts showed a decrease in expression on Dnmt inhibition showing autoregulation.



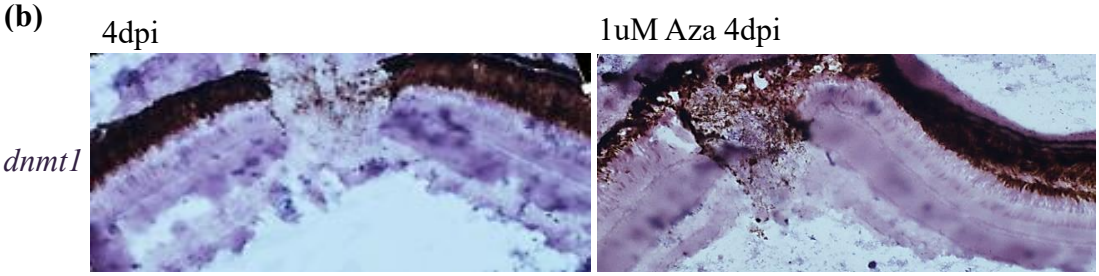


Figure 2.14 (a) RT-PCR for *dnmt1*, *dnmt6* and *dnmt8* in UC, 2.5dpi control and 1µM Aza treated retinas at 2.5dpi, showed a decrease in the levels of these *dnmt*s. (b) mRNA *insitu* hybridisation for *dnmt1* in 1µM Aza treated sections showed the decrease in *dnmt1* , as compared 4dpi control. .

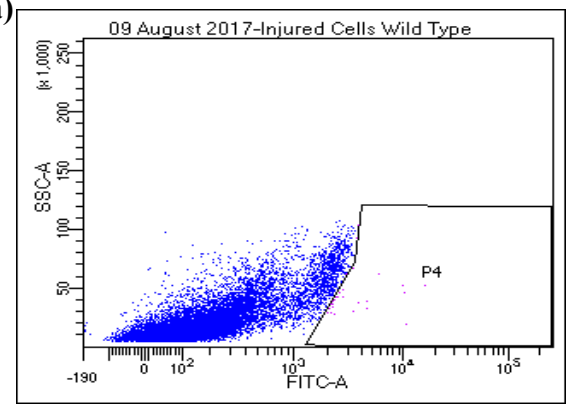
2.8 Sorting of Muller glia and whole genome bisufite sequencing.

In order to assess how the global methylation levels change during retina regeneration at different phases, whole genome bisulfite sequencing was performed. This would help in understanding how the methylation level of promoters of regeneration associated genes change during regeneration.

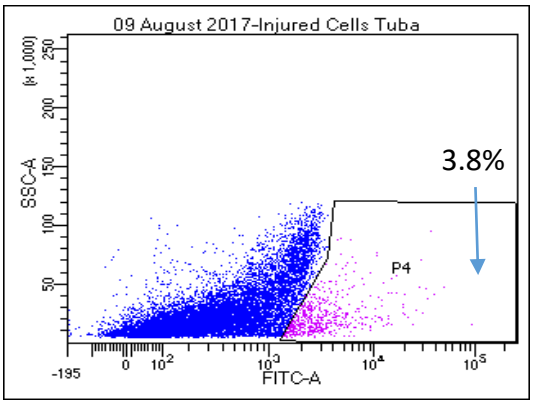
This is being done at whole retina levels for UC, 12hpi, 2dpi, 4dpi and 8dpi and the sequencing is going on. To do it at the level of Muller glia alone, Fluorescence Activated Cell Sorting (FACS) for Muller glia was standardized. Zebrafish carrying transgene *1016tuba:GFP* was used for this purpose. These fish express GFP in MGPCs and the expression of GFP is maximum at 4dpi [25].

On FACS, GFP +ve MGPCs formed a separate population (**Figure 2.15 a**), this constituted to around 3.8% of total cell population of retina. Doublets were gated out to prevent false postives (**Figure 2.15 b**). Ungated plot consisting of all cells of retina formed a very heterogenous population (**Figure 2.15 c**).

(a)

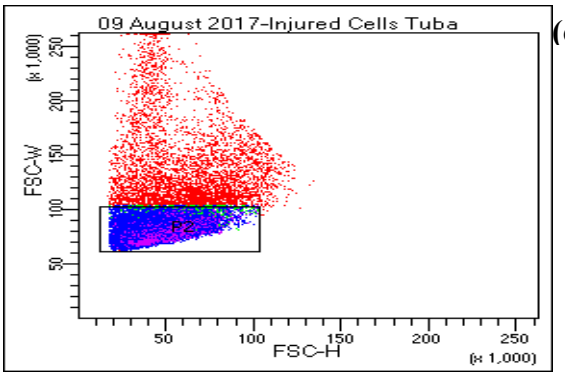


Bivariate WT



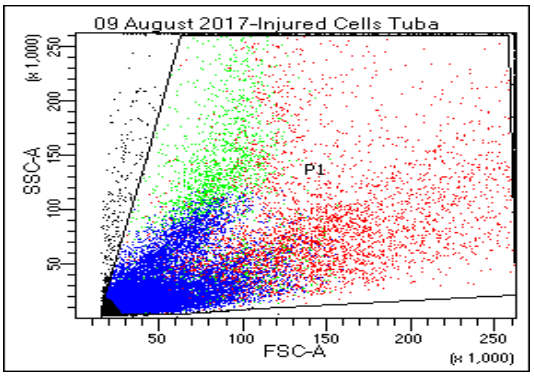
Bivariate *1016tuba*:GFP

(b)



Doublet Avoidance

(c)



Ungated

Figure 2.15 (a) Bivariate FACS plots for WT and *1016tuba:GFP* transgenic fish. (b) Doublet plots to gate out doublets of GFP+ve and GFP-ve cells. Lower values of FSC-W are constituted by singlets. (c) Ungated plot showing all cells of retina.

Chapter 3

Materials and Methods

3.1. Retinal injury and harvesting

- Anesthetize zebrafish using Tricaine methanesulfonate.
- Injure retina using 30 gauge needle.
- At a desired time after injury, dissect out eye using steel forceps and needle.
- Use 1X PBS (Phosphate Buffered Saline) for harvesting retina or 4% paraformaldehyde(PFA) for tissue fixing

3.2. Microscopy

- For retina dissection- Bright field microscope (Zeiss).
- For Imaging- confocal microscope (Nikon).

3.3. RNA Isolation

- Suspend dissect retina in 200µL of TRI reagent in MCT.
- Homogenize using a pipette.
- Add 0.2 volume (40µL) of chloroform.
- Shake the solution gently for 15-20 seconds.
- Incubate for 5 minutes at room temperature (RT).
- Centrifuge for 20 minutes at 10000rcf at 4°C.
- Using a cut tip, transfer 30µL of the aqueous layer into fresh MCT.
- Add□2 volumes (60µL) of Isopropanol.
- Incubate on ice for 10 minutes.
- Centrifuge at 10,000 rcf, 4°C for 10 minutes.
- Discard the supernatant, and give 80% ethanol wash at 7600rcf.
- Allow the pellet to dry completely at RT and then, elute in DEPC treated water.
- After Elution, Check RNA on 1% agarose gel by gel electrophoresis and then, store in -80°C

3.4. cDNA Preparation

RevertAid First Strand cDNA Synthesis Kit by Thermo Fisher was used,

- Add the following components into a PCR tube:

1. Template RNA	2.5µL
2. Primer (Oligo dT + Random Hexamer)	0.25 + 0.25 µL

Mix the components and incubate at 65°C for 5 minutes. Transfer the tubes back on ice.

Again add the following components in order

1. 5X reaction buffer	1µl
2. RiboLock RNase Inhibitor (20U/µL)	0.25µL
3. 10mM dNTP Mix	0.5µL
4. Revert-Aid M-Mul V RT (200U/µL)	0.25µL
Total Volume	5µL

- Mix the contents, centrifuge briefly and were incubate at following temperatures:

1. 5 minutes at 25°C
2. 60 minutes at 42°C
3. 5 minutes at 70°C

- Dilute the cDNA with nuclease free water and store at -80°C.
- Dilute the cDNA 1:8 before PCRs

3.5. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

- Reaction mixture (10µL volume):

10X buffer	1µL
2.5mM dNTPs	0.5µL
Primers (forward + reverse)	0.2µL
Taq polymerase	0.1µL
Template	Standardised by b-actin
Water	rest

- PCR parameters

	Reaction Parameters:	
Enzyme activation	95°C for 2 min	
DNA denaturation	95°C for 20 sec	
Primer annealing	60°C for 30 sec	35 cycles
Elongation	68°C for 30 sec	
Final elongation	72°C for 7 min	
Infinite hold	4°C	

- Check PCR products on 1.5% gel.

3.6. Qantitative PCR (qRT-PCR)

For qPCR use KOD SYBR qPCR Master Mix (pure gene)

We set 5µl reaction:-

1. Master mix - 2.50 µl
2. Primers (F+R) - 0.25 µl
3. Template - 0.25 µl
4. MQ water - 2.00 µl

Analyse the data on excel sheet and plot the graph.

3.7 PCR reaction using Gotaq

Add the following components:-

1. Gotaq mastermix	-	50 µl
2. Primers (F+R)	–	4 µl
3. Template	-	1 µl
4. Water	–	45 µl
Total	-	100 µl

Cycling conditions as:-

95^0C	-	2min
95^0C	-	30secs
60^0C	–	1 min
68^0C	-	2min
72^0C	-	7 min
4^0C	-	Infinite hold

x 35 cycles.

After checking 5ul on gel, manual gel purification was performed.

3.8 Manual Gel Extraction

- Cut band and collect in an MCT containing little pieces of aluminium foil. Make a small hole using a needle.
- Place this MCT inside another MCT and tape them together.

- Centrifuge the setup at 10,000rpm for 10 minutes, RT.
- Add Equal volume of PCI (Phenol : Chloroform : Isoamyl alcohol) into the flow-through and mix properly.
- Centrifuge at 10,000rpm for 10 minutes, RT.
- Take out the aqueous layer and collect in another MCT.
- Add equal amount of chloroform and mix thoroughly.
- Centrifuge at 10,000rpm for 10 minutes, RT.
- Take out the aqueous layer collect it in another MCT.
- Add 1/10th volume of 10M ammonium acetate and twice the volume of Isopropanol and mix properly.
- Keep the samples at -80°C overnight.
- Centrifuge at 15,000rpm for 30 minutes, 4°C
- Give 70% ethanol wash at 13400rpm and leave for complete drying
- Do Elution in autoclaved MQ and check on 1% agarose gel.
- Store at -20°C.

3.9 Ligation

Add the following components for ligation:-

1. Salt solution – 0.5µl
2. pCR 2.1 TOPO Vector – 0.3 µl
3. Insert – 1.5 µl
4. H_2O - 0.7 µl

Incubate this mixture for at least without disturbing it.

3.10 Bacterial Transformation

- Thaw competent cells stored at -80°C ice.
- Add 3µL of TOPO cloned product into 100µL of competent cells.
- Incubate on ice for 30 minutes.
- Heat shock at 42°C for 50 sec.
- Keep on ice for 5 minutes.
- Add 1mL of LB media into it and the vial and incubate at 37°C for 30 minutes.

- Centrifuge at 4,000rpm for 5 minutes.
- Discard the supernatant and plate the remaining sample on ampicillin containing LB-agar plates.
- Incubate plates at 37°C overnight.

Checking for transformed positive clones

- Next day pick the individual colonies and patch it on new Amp resistant LB agar plates and allow the bacteria to grow.
- 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 37^0C overnight for growth.

3.11. Ultracompetent cells preparation

- Incubate 5mL primary culture of *E.*coli strain DH5α at 37°C overnight.
- Incubate Secondary culture (1% of primary culture) at 18°C till OD600 reach the value of 0.6-0.8.
- Keep the culture on ice for 10-15 minutes.
- Centrifuge at 2500 x g for 10 minutes at 4°C.
- Resuspend the pellet in 80 mL of TB buffer.
- Place on ice for 10 minutes.
- Centrifuge at 2500 x g for 10 minutes at 4°C.
- Re-suspend the pellet in 20mL of TB buffer
- Add DMSO to a final concentration of 7% (1.4mL+18.6mL of TB buffer)

- Place it on ice for 10 minutes.
- Aliquot 100µL volumes and store at -80°C.

TB Buffer: 10mM of PIPES + 15mM of $CaCl_2.2H_2O$ + 250mM of KCl + 55mM of $MnCl_2.4H_2O$, pH = 6.8 is set using KOH.

3.12. Plasmid isolation

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

Compositions of lysis buffer are:-

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

- Add 50µl of 0.5M $MgCl_2$, mix properly and put it in ice for 2 min.
- 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.
- Spin it again at 14,000 rpm for 2 min.
- 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.
- Spin at 14,000rpm for 2 min.
- Discard the supernatant and wash the pellet with 70% ethanol.
- Dry the ethanol and elute it in 50µl of Nuclease free water.

3. 13. Plasmid Digestion

Linearize the cloned gene (TOPO clone) by digesting it with restriction enzyme.

- Add the following components in the reaction mixture:

1. 10X Reaction Buffer	5µL
2. Template (Manually isolated)	30µL
3. Enzyme	3µL
4. Autoclaved MQ	12µL

Enzymes

1. Mmp9	-	Not I
2. Zic2b	-	Xho I
3. Ezh2	-	Xho I
4. Dnmt1	-	Stu I

3.14. RNA probe reaction

- 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*,sp6 for *Zic2b,Ezh2*)
Nuclease free water	- 0µl
Total	– 10µl

- Incubate it in 37^{o}C for 4 hours.
- Add 1µl of 0.5M Tris EDTA for stopping the reaction
- Add 1µl of 5M LiCl2 for precipitation
- Add 0.5µl of Glycogen and tap it
- Add 18µl of 100% ethanol, tap it and mix properly and keep it in -80oC
- Next day or after one hour centrifuge at 14,000rpm for 15 min
- Discard the supernatant and wash the pellet with 100% ethanol, centrifuge it at 14,000 rpm for 10 min.
- Dry the pellet and dissolve it in 15µl of DEPC water and store at -80oC.

3.15 Tissue fixation and sectioning

- Take the eyes after removing lenses and put them into 4% PFA in 4^0C overnight.
- 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.

- Add 500µl of OCT and rotate it for 30 min.
- Embed the in OCT in small cubes made from aluminium foil and keep the embedded samples frozen at -80oC until sectioning.
- Section the blocks in cryostats (12µm thickness) and collect the sections on super frost plus slides and dry overnight and then store in -20oC.

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 65oC and constant shaking

2. 5% sucrose:

   Dissolve 2.5g sucrose in 50mL 1X phosphate buffer. Store at -20oC.

3. 20% sucrose:

   Dissolve 10g sucrose in 50mL of 1X phosphate buffer. Store at -20oC.

3.16. mRNA *in situ* Hybridization

Day 1

- Take out slides from -20°C and dry them by keeping them at 37°C for 1 hour.
- Hydrate the slides in following sequence for 1 min each:
    1. 100% EtOH
    2. 95% EtOH
    3. 70% EtOH
    4. 50% EtOH
    5. 2XSSC
- Incubate slides in Proteinase K solution for 5 min at 37°C
    1. Pre warm Proteinase K buffer to 37°C.
    2. Add 160µL of 10mg/mL proteinase K.
- Rinse slides briefly in room temp DEPC water (2-3mins).
- Rinse slides in 0.1M TEA pH 8.0 for 3 min.
- Rinse in Acetic anhydride/TEA for 10 min
    1. Add 130µL of acetic anhydride to 50mL of 0.1M TEA
- Dehydrate the slides in 2XSSC and EtOH series for 1 min each:
    1. 2X SSC
    2. 50% EtOH
    3. 70% EtOH
    4. 95% EtOH
    5. 100% EtOH
- Air dry the slides for at least 1 hour at RT.
- Pre warm the hybridization solution at 56°C (300µL per slide).
- Probe preparation:
    1. Add probe to hybridization solution and mix (as per concentration of the probe).
    2. Boil probe and hybridization solution mix at 100°C for 10 min.
    3. Keep immediately on ice for 2-3mins.
- Add 300µL of Hyb/probe solution to each slide and coverslip with siliconized hybri slips.
- Place slides in humid chamber dampened with 50% Formamide/5X SSC and incubate at 56°C overnight.

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 upto 50ml with DEPC water.

b) TEA (50ml) :

Triethanolamine	–	0.93 g
DEPC water	–	50ml
10N NaOH	-	170µl

c) Proteinase K solution (50ml):

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

d) Hybridisation solution (50ml):

TEN solutions	–	3.6ml
100% Formamide	–	25ml
50% Dextran sulphate	–	10ml
DEPC water	–	6.4ml DEPC water

Store this in - 20oC

e) TEN solution (50ml)

0.1M Tris HCl (pH7.5)	–	5ml
5M NaCl	–	30ml
0.5M EDTA	–	1ml

Day2

- Preheat 50% formamide/2X SSC solution to 65°C.
- Preheat two 50mL RNase buffer washes, one to 37°C and the other to 65°C.
- Preheat two 2X SSC washes to 37°C.
- Soak slides with cover slips in 2X SSC for 30 min at RT on shaker table
- If the coverslips do not come off, gently teased them apart from slide with forceps.
- Rinse slides in 50% Formamide/2X SSC solution for 30 min at 65°C.
- Gently agitate for the first 5 min.
- Rinse slides in 2X SSC for 10 min at 37°C (twice).
- Add 100µL of RNase (10mg/mL) to the 37°C RNase buffer, Incubate slides for 30 min.

- Wash slides in 65°C RNase buffer for 30 min.
- Wash slides for 2-3 hours in 1X Maleate buffer/0.05% Triton X-100/1% RMB blocker solution at RT
- Wash slides in 1X Maleate buffer for 5 min (twice).
- Incubate slides with 500µL antibody (Anti-DIG) diluted in 1X Maleate/0.05% Triton X-100/1% RMB blocker solution (1:2500 dilution) overnight a

a) RNase buffer (10ml)

NaCl – 1ml
1M Tris (pH7.5) - 500µl
5M EDTA - 100µl
MQ Water – bring upto 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 upto 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 -20oC.

Day 3

- Wash slides twice with 1X Maleate buffer for 5 minutes.
- Wash in Genius buffer twice for 5 minutes each.
- Add 500µL of NBT/BCIP dissolved in Genius buffer (1:50 dilution), incubated at room temperature in dark for colour reaction.
- Colour detection using bright field microscope.

Genius Buffer (50mL):
5mL of 1M Tris-HCl (pH = 9.5)
1mL of 5M NaCl
5mL of MgCl2

3.17. Immunostaining

Day 1

- Take out slides from -20°C and dry at 37°C for 30minutes.
- Give 1X PBS wash for 10min, twice.
- Fix tissue with 4% PFA solution for 20min (This step is done only in the case of MO slides).
- Treat the slides with 2N HCl (pre heated to 37°C) for 20 min.
- Wash the slides with 0.1M Sodium borate solution twice for10 min each.
- Block the sections using 3% BSA in 1X PBST (1XPBS + 0.1% Triton X) for at least 30min.
- Overlay the slides with 1° Ab of choice, 500µL per slide (Ab is diluted in 1:500 ratio in 1% BSA in 1X PBST).
- Incubate the slides at 4°C overnight.

Day 2

- Wash slides with 1X PBST, 3 times for 10 min each.
- Overlay the slides with desired 2° Ab, 500µL per slide (Ab is diluted in 1:1000 ratio in 1% BSA-1X PBST solution).
- Incubate slides for 3 hours at RT or at 4°C overnight.
- Wash slides with 1X PBST 3 times for 10 min each.
- Wash slides with autoclaved water 3 times for 10 min each.
- Dry slides for 1 hour at RT.
- Mount slides in DABCO and leave at RT in dark overnight.
- Store slides at -20°C.

3.18 Western Blotting

Sample preparation

- Dissect out and put retina in 2X Lamelle Buffer and homogenize properly.
  (2X Lamelle Buffer – 4mL of 10% SDS + 2mL of Glycerol + 1.2mL of 1M Tris-HCl (pH 6.8) + 2.8mL of MilliQ-water + 0.02% of Bromophenol blue. Store at 4°).
- Vortex briefly and keep on ice intermittently for 10 minutes
- Boil the sample at 100°C for 10 minutes.
- Store in -80°C

Day1

- Cast the resolving gel.
  (12% Resolving gel – 2.5mL Resolving Buffer + 4mL 30% Acrylamide + 3.3mL MilliQ-water + 100µL 10% SDS + 100µL 10% Ammonium Persulfate + 6µL TEMED)
- Cast the stacking gel.
  (Stacking gel – 625µL Stacking Buffer + 667µL 30% Acrylamide + 3603µL MilliQ-water + 50µL 10% SDS + 50µL 10% Ammonium Persulfate + 5µL TEMED)
- Take out samples from -80 and thaw.
- Load the samples on the gel along with Protein Ladder.
- Run the gel for 3 hours at 80V.
- Set up the transfer using PVDF membrane for 70 minutes. Charge the membrane using
  methanol for 1 minute and wash in transfer buffer.
- Block the blot in 10% skim milk for 1 hour.
- 0.01% PBST (For 200mL of 1X PBST, add 200µL of TWEEN20) washes were given for 10 minutes, three times.
- 0.01% PBST (For 200mL of 1X PBST, add 200µL of TWEEN20) washes were given for 15minutes, four times.
- Incubated blot in primary antibody of choice overnight at 4°

Day2

- Give 0.01% PBST wash for 10 minutes each, three times.
- Incubate blot in secondary antibody for 1 hour at RT.
- Give 0.01% PBST wash for 15 minutes each, four times
- Incubated blot in secondary antibody for 1 hour at RT.
- 0.01% PBST washes were given for 15 minutes each, four times.
- Blot was then developed in ImageQuant LAS4000.

3.19 TUNEL Assay

- Take out the slides from -20° and dry them by keeping them at 37°C for 1hour.
- Give 1X PBS wash for 10 minutes, 2 times.
- Permeablise the retinal sections in 1mL Trypsin at 37°C for 15 minutes. (Pre-warmeTrypsin at 37°C for 20 minutes).
- Overlay the 45µL label solution + 5µL enzyme solution on each slide.
- Incubate for 1 hour at 37°C in a humified chamber in dark.
- Give 1x PBS wash for 10minutes, 2 times.

3.20 Genomic DNA Isolation

- Suspend 20 dissected retina in 500ul of TEN Buffer .
- Add 1% V/V SDS and homogenize it for 1 minute.
- Add proteinase k to a final concentration of 200ug/ml, Tap it gently and Incubate for 4 hours at 37^0C.
- Add equal volume of PCI, Mix by inverting the tube (No vortexing or tapping).
- Spin at room temperature, 6000 RPM, 5 minutes, Collect the aqueous phase using a cut tip.
- Add equal volume of chloroform, invert the MCT gently, spin down at 12000rpm.
- Collect the upper interface and repeat the chloroform wash again.
- Add 1/10th volume of CH3COONa buffer and mix by gently inverting the tube.
- Add 2 volume of isopropanol and Mix gently by inverting.
- A needle like mesh will form, take it out using a tip.
- Put it in 70% alcohol.
- Spin it down at 12000 RPM, 10 minutes.
- Remove supernatant.
- Dissolve the pellet in TE buffer.

TEN Buffer.	TE Buffer
100mM Tris, pH=8.	10 mM Tris, pH=8.
1mM EDTA.	1mM EDTA.
150mM NaCl.	

3.21 FACS

- Suspend the dissected retina(6) in 500ul of L15 media.
- Add 1mg/ml of hyaluronidase and incubate at room temperature for 15 minutes.
- Wash off the hyaluronidase using L15 media twice. Do the spinning at 1000rpm, 1 minute during washing.
- Resuspend the retinas in 500ul of L15 media.
- Add 0.01% of trypsin, Incubate at room temperature for 15minutes with intermittent pipetting for separating cells.
- BD FACS ARIA instrument was used for doing the sorting.

Chapter 4.
Conclusions and discussions

It was observed that Dnmts are regulated during retina regeneration. The temporal expression patterns showed some commonalities like coming to basal levels by 8dpi. However it was not exactly the shared.

The anti-proliferative role of Dnmts was evident from the blocking expreiments. Blocking at 2.5 and 4 days post injury led to an increase in cell proliferation. Inhibiting Dnmts at different phases of regeneration made it clear that inhibition in pre-proliferative phase (0-4 dpi) is only making an impact on cell proliferation. Eventhough, the cell proliferation went up at initial time points, these cells were not able to survive till 30 dpi and were undergoing apoptosis. Surviving cells were still able to migrate and form other retinal cell types like Amacrine cells and differentiated Muller glia.

While analysing the regeneration associated gene expression in the pre-proliferative phase under Dnmt inhibition, it was noted that the protein expression levels of pro-proliferative genes Ascl1a and Sox2 are going down. This is despite the fact that the mRNA transcript levels of these genes increases. This may be because of the involvement of micro-RNAs that target these genes. *miR34a* remains as a candidate micro-RNA whose expression goes up under Dnmt depletion. It has it's binding site present on *ascl1a* and *sox2* transcripts also. *mmp9,* which is important for cell migration was also going down. Anti-proliferative genes like insm1a levels showed an increase also.

Reduction in expression of pro-proliferative genes and increase in expression of anti-proliferative genes may be the reason for non-survival of MGPCs at later stages.

While analysing the expression of other epigenetic modifiers in Dnmt inhibited condition, it was noted that the expression of Ezh2 and all *hdacs* except Hdac1 was going up. Dnmt expression was going down in Dnmt inhibited condition showing auto regulation.

Finally, MGPCs were forming a separate population on FACS while using 4dpi retinal cells from *1016tuba:GFP* fish. It is also important to understand how DNA methylation changes at a global level during regenerative program. This would help understand how different regeneration associated genes are regulated by DNA methylation and can reveal novel players also.

Chapter 5
Future outlook

- Morpholino knockdown of individual *dnmts* and studying the effect on regeneration.
- Analysing whole genome bisulfite sequencing to unravel how global methylation levels changes during retina regeneration.
- Comparing the whole genome bisulfite sequencing results with microarray and RNA-seq data to figure out correlations of DNA methylation levels with gene expression.
- Comparison of whole genome bisulfite sequencing results with Hi-C results. Similar comparison can be made between ChIP-seq results of chromatin modifiers (histone methylations and acetylations) and bisulfite sequencing result. This would decipher how chromatin-structure is affected by DNA methylation and how different epigenetic modifiers collaborate.
- Blocking of different signalling pathways and analysing expression levels of different *dnmts.*

Index

List of primers used

dnmt1-RT-Fwd	5'ACCTTTGGTGTGCTGCAGGCTGGAC3'
dnmt1-Rt-Rev	5'AACCAGGGCACTCATGTCCTTGCAG3'
dnmt3-RT-Fwd	5'GACGGACGGTGGTGGTAATG3'
dnmt3-RT-Rev	5'CTGACAAAAAGCAGCACCTGAGC3'
dnmt4-RT-Fwd	5'CAAGATGACTGCCACGGCTG3'
dnmt4-RT- Rev	5'CTGTTCACACTCTCATCTGCGG3'
dnmt6-RT-Fwd	5'TGATGGGATCGCAACAGGGC5'
dnmt6-RT-Rev	5'CGACCGGTGCCCCTCGTAG5'
dnmt7-RT-Fwd	5'GGAGCAATGTCGTTCAGGTGC5'
dnmt7-RT-Rev	5'TCGTTCACAGGAACTGGCTCTG3'
dnmt8-RT-Fwd	5'CAACCATGACCAGGACTTTGAGC3'
dnmt8-RT-Rev	5'GAAGTGTCCTGTGGTTGAAGGTC3'
hdac1 RT- Fwd	GACAGCACCATTCCTAATGAGCTCC
hdac1 RT Rev	TATCGTGAGCACGAATGGAGATGCG
hdac3 RT Fwd	CCCAGGAACTGGTGACATGTATGAAG
hdac3 RT Rev	ACAAACTCCACACATTCTCCATGTCC
hdac4 RT Fwd	CAAGCTCACGGCCAAATGTTTTGGC
hdac4 RT Rev	GCAGTGAACGCCAGTATTTACTCTGG
hdac6 RT Fwd	TGATTCATCAGCCGTGAAGGATCAGG
hdac6 RT Rev	ATAACGCCCGCAAAGCACCTTATAGC
beta actin RT Fwd	GCAGAAGGAGATCACATCCCTGGC
beta actin RT Rev	CATTGCCGTCACCTTCACCGTTC

ezh2 RT Fwd	CTGCCAGTGTAGCTCAGAATGTCAG
ezh2 RT Rev	CTTTGATGAAGATTCCCCACCCGGCC
ascl1a RT Fwd	ATCTCCCAAAACTACTCTAATGACATGAACTCTAT
Ascl1a RT Rev	CAAGCGAGTGCTGATATTTTTAAGTTTCCTTTTAC
her4.1 RT fwd	GCTGATATCCTGGAGATGACG
her4.1 RT rev	GACTGTGGGCTGGAGTGTGTT
insm1a RT Fwd	CCAAGAAAGCCAAAGCCATGCGGAAGC
insm1a RT Rev	TTATTGCTTTCCGCGCTCTGCTTGGGTTTG
mmp9_RT_Fwd	GGAGAAAACTTCTGGAGACTTG
mmp9_RT_Rev	CACTGAAGAGAAACGGTTTCC

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