Investigating the Role and Regulation of Ezh2 in Zebrafish Retina Regeneration

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Certificate of Examination

This is to certify that the dissertation titled “Investigating the role and regulation of Ezh2 in zebrafish retina regeneration” submitted by Ms. Priyanka Sudheendra Jamadagni (Reg.No.MS11050) 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.

Dr. Shashi Bhushan Pandit Dr. Kuljeet Singh Sandhu Dr. Rajesh Ramachandran
(Supervisor)

Dated: April 22, 2016

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.

Priyanka Sudheendra Jamadagni
(Candidate)

Dated: April 22, 2016

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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Abstract.

In contrast to mammals, a teleost Zebrafish shows complete retinal regeneration in response to injury. Studying it would provide insights into the mechanisms that don't occur in the mammalian retina causing various ophthalmologic defects. It has been established that retina regeneration proceeds through three stages as a result of Muller glia cell reprogramming - dedifferentiation, proliferation, and re-differentiation/cell migration. There are wide range of factors that trigger and sustain the Muller glial cell reprogramming that is primarily responsible for regeneration to manifest. Of them, I have tried to elucidate the roles of epigenome modifiers, like Enhancer of Zeste homologue 2 (Ezh2) which is the catalytic domain of the polycomb repressive complex 2 (PRC2), involved in H3 lys27 methylation (H3K27me3).

Chapter 1

Introduction

1.1 Basic Theory

Regeneration is the complete or partial restoration of morphology and function of a tissue, after a damage or injury. Possession of this attribute makes an organism resilient to the environmental and physiological stresses that cause damage or injury.

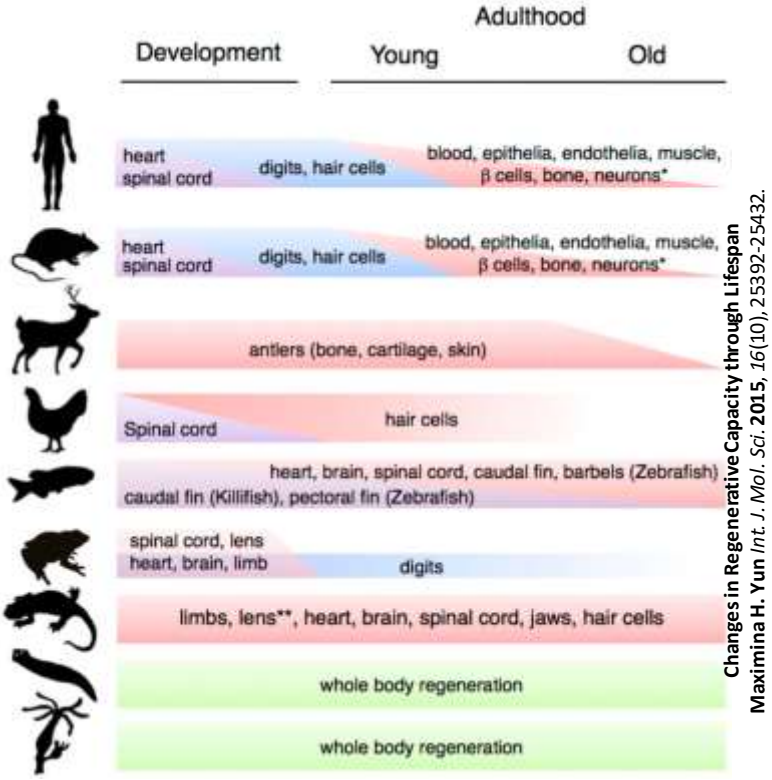


Fig.1 Regeneration potential across species

Teleosts exhibit an enormous potential for regeneration. They are capable of producing new neurons in the adult central nervous system and of replacing damaged neurons by those newly generated. One such telost fish is the zebrafish. It can regenerate a vast array of its organs - brain, spinal cord, hear, retina, liver, pancreas and fins, making it apt for studying regeneration. It is a convenient model organism for forward as well as reverse genetic approaches, easy in breeding and maintenance and its transparent embryos are very useful in developmental studies. [1]

Retina being the most accessible part of the Central nervous system that regenerates in zebrafish, is very useful in studying neuronal regeneration. It is observed that upon injury, same cell types respond in zebrafish and humans, only, in the zebrafish retina the Muller glia cells turn into a neuronal progenitor and regenerate the damaged neurons. In the human retina they cause scarring in human retina. [2]

Zebrafish retina is divided into three sections: Outer nuclear layer (ONL), inner nuclear layer(INL) and ganglion cell layer (GCL). ONL consists of photoreceptor cells – rods and cones. INL consists of the horizontal, amacrine, bipolar and Muller glial cells. Horizontal and bipolar cells are interconnecting neurons between the ONL and INL, and INL and GCL respectively. They regulate the input from the photoreceptor cells. Ganglion cells receive visual information from Rods and cones and their axons for the optic nerve. Muller Gliais a type of glial cells. They span all the retinal layers and have their nucleus in the INL. Their major function is to maintain retinal extracellular environment.

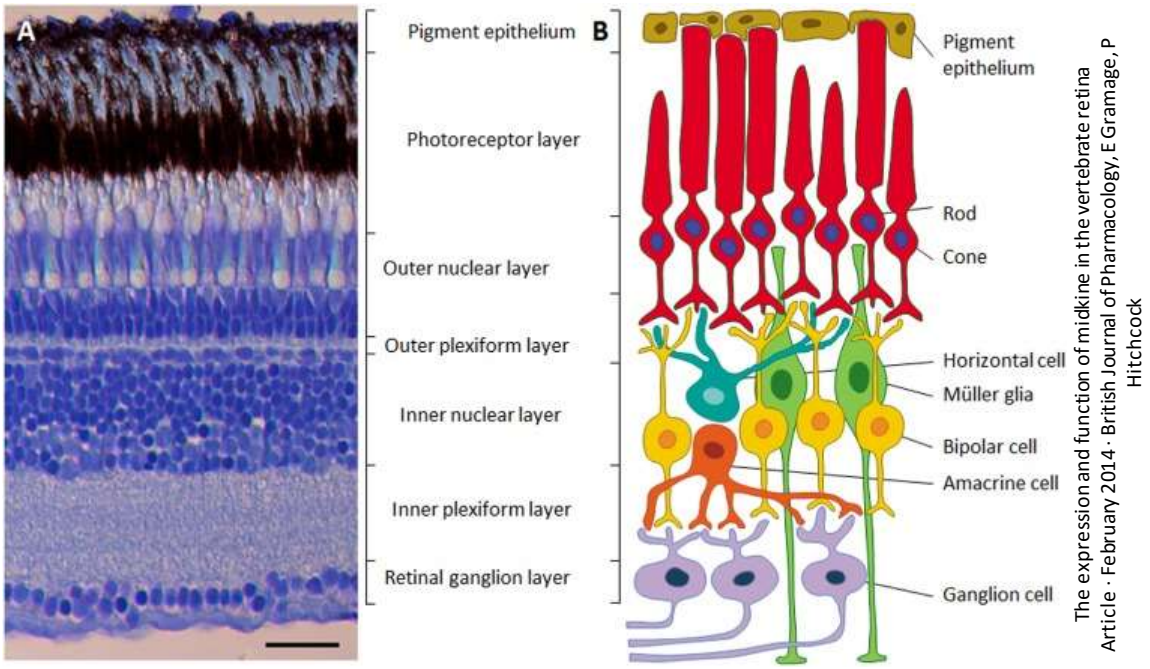


Fig.2 Zebrafish retinal cell layers

In order to experimentally study regeneration in the zebrafish retina, various injury paradigms have been used. These include: prolonged exposure to intense bright light and short exposure to ultraviolet (UV) light; intra-vitreal injection of toxins (such as ouabain and NMDA); expression of a toxic gene (such as bacterial nitroreductase, which, in combination with a pro-drug, generates a cytotoxic product); and mechanical injury (such

as that resulting from a needle poke). Of these, mechanical injury generally destroys all retinal cell types in a circumscribed region of the retina thus giving a wholesome perspective.

After injury the Muller glia cells are triggered to dedifferentiate and form neuronal progenitor cells that proliferate and re-differentiate into specific neurons that replace the injured neurons across the retinal cell layers.

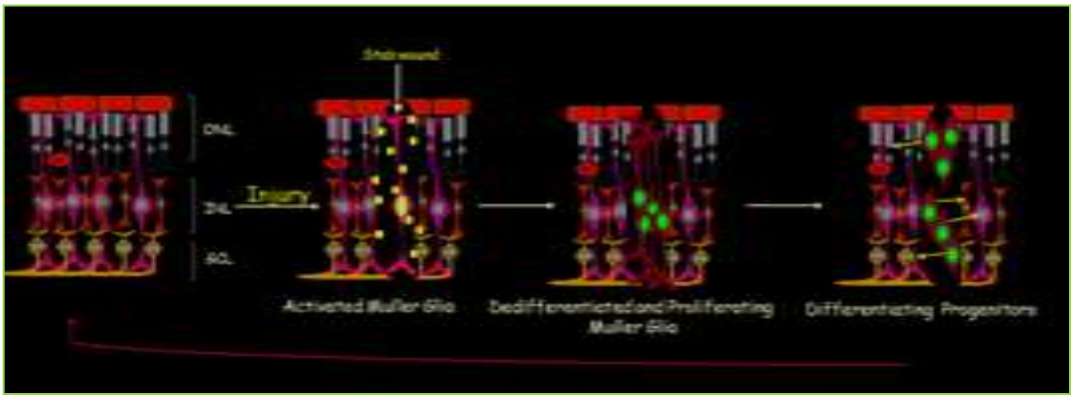
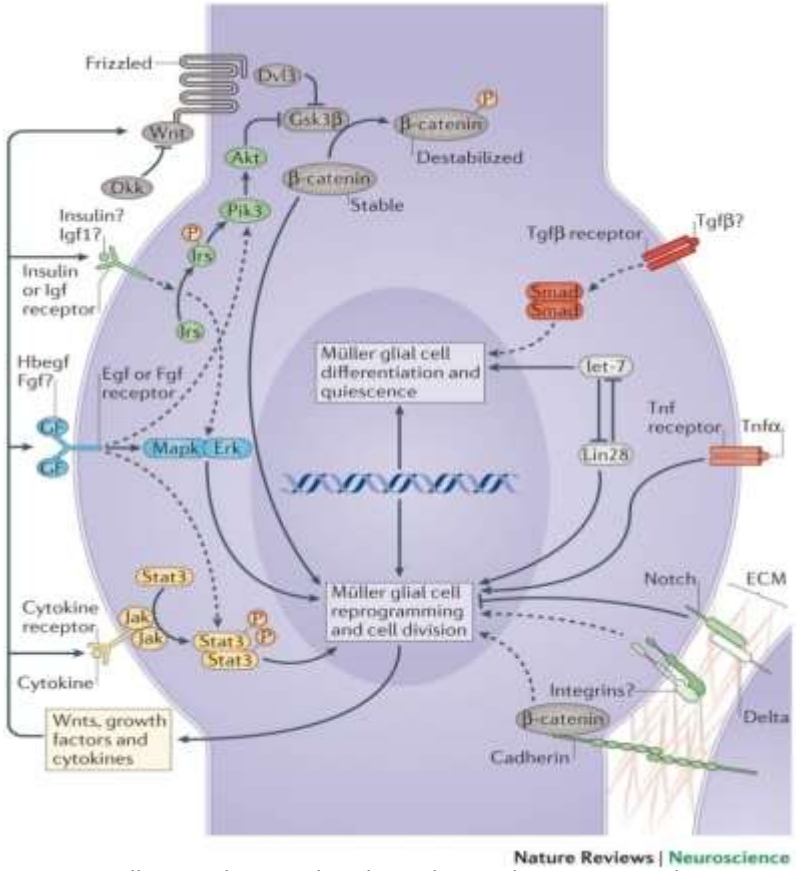


Fig.3 Retina regeneration at a glance

This trigger could be because of secretion of signalling molecules from damaged cells, Müller glia or infiltrating microglia; altered contact between damaged cells and Müller glia; and phagocytosis of injured cells by Müller glia.

Recent studies have suggested that growth factors, such as heparin-binding epidermal growth factor (EGF)-like growth factor a (Hb-egfa), and cytokines, such as tumour necrosis factor-α (Tnfα), are necessary for Müller glial cell reprogramming and progenitor formation in the injured retina. These factors are produced in Müller glia at the injury site and therefore may act in an autocrine and paracrine manner. TNFα and ADP are also released from injured retinal neurons. For regeneration to occur successfully, after triggering Muller glia, various signalling cascades also need to be activated. [3]



Muller Glial cell reprogramming and retina regeneration Dan Goldman, 432 JULY 2014 VOLUME 15, Nature reviews neuroscience

Fig.4 Signalling pathways that have been shown to regulate retina regeneration are indicated by solid lines, whereas those indirectly implicated or hypothesized to be involved are indicated by dashed lines. Secreted factors that regulate the proliferation of Müller glia are indicated outside the cell (those that affect the proliferation of Müller glia in birds and mammals but which have not yet been tested in zebrafish are annotated with a question mark).

1.2 Enhancer of Zeste Homologue:

Timely activation and repression of genes and inturn signalling pathways requires efficient epigenetic regulation. The Polycomb group proteins (PRC1 and PRC2) are one such group of epigenetic regulators. They have been known to be critical to maintaining gene repression during development. Enhancer of zeste homologue 2 (Ezh2) is the catalytic subunit of the PRC2 complex that catalyzes di- and trimethylation of lysine27 on histone H3 (H3K37me2/3). This marks repressive transcription. This transcriptionally repressive state is thought to be due to PRC2/EZH2-EED-mediated H3K27 methylation and subsequent recruitment of PRC1 which facilitates condensation of chromatin and formation of heterochromatin. [5]

There are two mammalian homologs- Ezh1 and Ezh2 that form similar PRC2 complexes but exhibit contrasting repressive roles.PRC-Ezh2 catalyzes H3K27me2/3 and its

knockdown affects global H3K27me2/3 levels, PRC2-Ezh1 performs this function weakly. Ezh1 is more abundant in non-proliferative adult tissues while Ezh2 expression is tightly associated with proliferation. [4]

EZH2 is a member of the SET domain family of lysine methyltransferase which function to add methyl groups to lysine side chains of substrate proteins. It depends on a cofactor SAM (S-Adenosyl methionine**)** to act as a methyl donor. Unlike other SAM-dependent methyl transferase, SET domain proteins bind their substrate and SAM cofactor on opposite sides of the active site of the enzyme. This orientation of substrate and cofactor allows SAM to dissociate without disrupting substrate binding and can lead to multiple rounds of lysine methylation without substrate dissociation. [6]

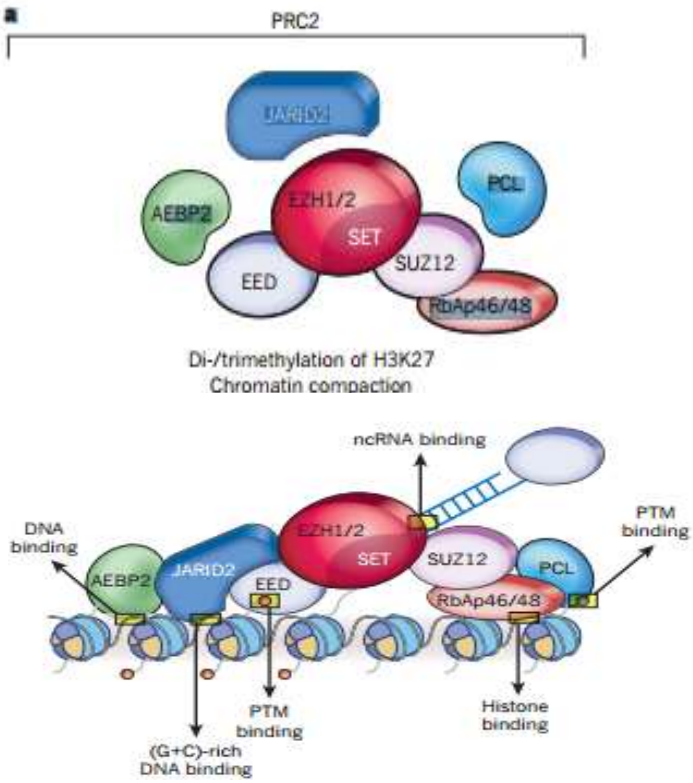


Fig.5 Subunits of Ezh2 and their binding domains on the DNA

The minimum components required for methyltransferase activity of the PRC2/EED-EZH2 complex are EED, EZH2 and SUZ12. The PRC2/EED-EZH2 complex may also serve as a recruiting platform for DNA methyltransferases DNMT1, DNMT3A, DNMT3B, thereby linking two epigenetic repression systems. The PRC2 complex may also interact with HDAC1 and HDAC2.

1.3 Literature Review:

The methylated H3-K27 chromatin mark is commonly associated with silencing of differentiation genes in organisms ranging from plants to flies to humans. PRC2 target genes are highly enriched for transcription factors and signalling components that control cell differentiation. Since there is normally little EZH2 in differentiated adult tissues, EZH2 overabundance could shift expression profiles to promote a return to or reinforcement of a stem cell-like state

Studies on human tumors show that EZH2 is frequently over-expressed in a wide variety of cancerous tissue types, including prostate and breast. The highest EZH2 levels correlate with advanced stages of disease and poor prognosis. Although the mechanistic contributions of EZH2 to cancer progression are not yet determined, functional links between EZH2-mediated histone methylation and DNA methylation suggest partnership with the gene silencing machinery implicated in tumor suppressor loss. [7]

Ezh2 has also been suggested to play a role in cell division, where heterochromatin formation is required for proper chromosome segregation.[8]

During X-inactivation, it is thought that EZH2 is involved in initiating heterochromatin formation by trimethylating H3K27 and that other histone methyltransferases and histone marks may be involved in maintaining the silenced state.[9]

EZH2 may also play a role in activation of transcription, independently of PRC2[10]

With such strong implications about the role of Ezh2 in development and cancer, but not much yet about its roles in regeneration, it poses as an interesting molecule to be studied in reference to retinal regeneration.

Chapter2

Experimental methods and techniques:

2.1Materials:

2.2 Experimental methods:

(i) Retinal injury and harvesting:

1. Zebrafish is anesthetized using Tricane methanesulfonate.
2. Then fish is kept on a sponge bed. Retina is injured using 30 gauge needle and stainless steel forcep.
3. At a desired time after injury, dissect out eyes using a steel forceps and either dip in 1X PBS for harvesting retina or 4% paraformaldehyde for tissue fixing. .

(ii) Microscopy:

1. Bright field microscope (Zeiss) is used for dissecting retina
2. Confocal microscope (Nikon) is used for imaging.

(iii) RNA Isolation:

1. Dissect two retinal tissues dissected is stored in TRI agent (200ul)either at -80^{o}C or used immediately.
2. The tissues are homogenized using either a pestle or a pipette.
3. Add 0.2 volume of Chloroform - 40µl
4. Mix it by inverting the tube 5-6 times
5. Place stationary on the bench for 5mins.
6. Centrifuge at 12000 rcf at room temperature for 10mins.
7. Carefully remove only the aqueous phase – 50ul (to avoid DNA contamination, avoid the lower layer).
8. Add equal of isopropanol and keep at -80^{o}C overnight for at least 1hr for precipitation.
9. Centrifuge at 12000 rcf at 4^{o}C for 10 mins.
10. Discard the supernatant and wash pellet with 70% ethanol – 200ul.
11. Centrifuged at 7500rcf at 4^{o}C for 10 mins.
12. Dry pellet and dissolve in millique H_2O and confirm on a 2% agarose gel by gel electrophoresis.
13. Store at -80^{o}C.

(vi) cDNA preparation (kit used – Biorad):

1. Master Mix for the reaction (for 20ul reaction):

5X iScript Reaction Mix	4ul
iScript Reverse Transcriptase	1ul
RNA	upto 4ug
Water	Make up the volume to 20ul

2. Reaction parameters:

(v)Polymerase chain reaction:

1. Reaction mixture (20ul volume):

20X buffer	1ul
dNTPs	2ul
Primers (forward + reverse)	0.4ul
Taq polymerase	0.4ul
Template	(as per standardized volume)
Water	16.2ul

2. Reaction Parameters:

   Enzyme activation - 95^{o}C for 2mins

   DNA denaturation - 95^{o}C for 15secs

   Primer annealing - 62^{o}C for 30secs

   Elongation - 68^{o}C for 30secs

   72^{o}C – 7 min

   Infinite hold - 4^{o} C

(vi)Semi quantitative real- time polymerase chain reaction:

1. Reaction mixture(for a 5ul reaction):

Master Mix	2.5ul
Primer	0.2ul
Template	(as per standardized volume)
Water	(make up the volume to 5ul)

2. Reaction parameters same as the general PCR.

(vii) RNA probe systhesis:

1. Single digest the plasmid with CDS of the gene of interest at the forward end of the CDS.
2. Validate the digested product on an agarose gel.
3. Gel-elute/ Precipitate the digested product.
4. Set up the probe reaction (20ul):

SP6 Buffer	2ul
Template(digested DNA)	4ul (1.1ng)
Dig-UTP	1ul
SP6 RNA polymerase	1ul
H_2O	12ul

5. Incubate at 37^{o}C for 4 hours.
6. Validate the probe prepared on an agrose gel
7. Stop the reaction and precipitate the probe at -80^{o}C overnight or one hour

0.5M Tris EDTA	2ul.
5M LiCl	2ul
10mg/mL glycogen	1ul
Absolute EtOH	35ul

8. Centrifuge at 4^{o}C for 15 min max speed.
9. Wash with 70% EtOH (200µL) and centrifuged for 10 min.
10. Dry and dissolve the pellete in 50µL of DEPEC water.
11. Validate the probe prepared on an agrose gel
12. Store at -80^{o}C after aliquoting.

(viii) Cryo-protection and Sectioning

1. Incubate lens removed eye in 400ul 4% PFA in 1X phosphate buffer overnight fot tissue fixing.
2. Serial washings of the fixed tissue for 45mins at RT each on a rotor:

    1ml of 5% sucrose

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

    500ul of 5% and 500ul of 20% sucrose

    400uL of 5% and 800ul of 20% sucrose

    1ml of 20% sucrose.
3. Add 500ul of OCT in the existing 20% sucrose and mixed on rotator for 30 min.
4. Make a block with OCT in aluminium foil box and keep at -80^{o}C for freezing.

5. Section the tissue block using cryo-section machine and store slides at -20^{o}C

Composition of solutions used for tissue fixation and cryoprotection:

1. 4% PFA in 1X Phosphate buffer:

   2g PFA + 5mL of 10X phosphate buffer

   Makeup the volume to 50mL with autoclaved water. Store at -20^{o}C.
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.

(ix) Immuno-fluorescence staining:

Day 1

1. Incubate slides stored at -20^{o}C at 37^{o}C for 30 min.
2. Wash the slides using 1X PBS (twice- 10 min each).
3. Treat the slides with 2N HCL (pre heated to 37^{o}C) for 20 min.
4. Wash the slides with 0.1M sodium borate solution twice for10 min each.
5. Block the sections using 3% BSA PBST (1X PBS with 0.1% Triton) for at least 1hr.
6. Overlay the slides with 1^{o} Ab of choice, 300ul per slide (Ab is diluted in 500µL of 1X PBST+1% BSA).
7. Incubate the slides at 4^{o}C overnight.

Day 2

1. Wash slides with PBST (0.1% Triton) 3 times for 10 min each.
2. Overlay the slides with desired 2^{o} Ab, 300ul per slide (Ab is diluted in 500µL of1X PBST+1% BSA).
3. Incubate slides for 3 hours at RT or at 4^{o}C overnight.

Day 3

1. Wash slides with PBST 3 times for 10 min each.
2. Wash slides with autoclaved water 3 times for 10 min each.
3. Dry slides for 30mins at RT.
4. Mount slides in DABCO and leave at RT in dark overnight.
5. Store slides at -20^{o}C.

Composition of solutions:

1. 1X PBS

5mL PBS (from 10X stock)+ 45mL autoclaved MQ

2. 1X PBST

   5mL PBS (from 10X stock)+ 45mL autoclaved MQ+ 0.1% Triton(50µL)

3. 1X PBST + 3% BSA

   5mL PBS (from 10X stock)+ 45mL autoclaved MQ+ 0.1% Triton(50µL) + 1.5gm BSA

4. 1X PBST+ 1%BSA

   5mL PBS (from 10X stock)+ 45mL autoclaved MQ + 0.1% Trit(50µL) + 0.5gm BSA

5. 0.1M Sodium Borate

   9.53g Sodium Borate in 500mL of deionized H_2O

(x)In-situ hybridization:

Day 1

1. Hydrate the slides in following sequence for 1 min each :
   a. 100% EtOH
   b. 95% EtOH
   c. 70% EtOH
   d. 50% EtOH
   e. 2XSSC
2. Incubate slides in Proteinase K solution for 1-5 min at 37^{o}C
   a. Prewarmed Proteinase K buffer to 37^{o}C
   b. Added 250µL of 10mg/mL proteinase K
3. Rinse slides briefly in room temp DEPEC water. (2-3mins)
4. Rinsed slides in 0.1M TEA pH 8.0 for 3 min.
5. Rinse in Acetic anhydride/TEA for 10 min
   a. Add 130µL of acetic anhydride to dry dish.
   b. Add 50mL of TEA
6. Dehydrate the slides in SSC and EtOH series for 1 min each:
   a. 2X SSC
   b. 50% EtOH
   c. 70% EtOH
   d. 95% EtOH
   e. 100% EtOH

7. Air dry the slides for at least 1 hour at RT.
8. Pre warm the hybridization solution at 56^{o}C (150ul per slide).
9. Probe preparation:
    a. Add probe to hybridization solution and mixed (as per concentration of the probe).
    b. Boil probe and hybridization solution mix at 100^{o}C for 10 min.
    c. Plunge immediately on ice for 2-3mins
10. Added 150ul of Hyb/probe solution to each slide and coverslip with siliconized hybri slips
11. Place slides in humid chamber dampened with 50% formamide/5X SSC and incubate at 56^{o}C overnight.

Day 2

1. Preheat 50% formamide/2X SSC solution to 65^{o}C.
2. Preheat two 50mL RNase buffer washes, one to 37^{o}C and the other to 65^{o}C.
3. Preheat two 2X SSC washes to 37^{o}C.
4. Soak slides with cover slips in 2X SSC for 30 min at RT on shaker table
5. If the slides do not come off, gently teased them apart from slide with forceps.
6. Rinse slides in 50% formamide/2X SSC solution for 30 min at 65^{o}C.
7. Gently agitate for the first 5 min.
8. Rinse slides in 2X SSC for 10 min at 37^{o}C (twice).
9. Add 100µL of RNase (10mg/mL) to the 37^{o}C RNase buffer: Incubated slides for 30 min.
10. Wash slides in 65^{o}C RNase buffer for 30 min.
11. Wash slides for 2-3 hours in 1X Maleate buffer/0.05% Triton X-100/1% RMB blocker solution at RT
12. Wash slides in 1X Maleate buffer for 5 min (twice).
13. Incubate slides with 300ul antibody (of choice) diluted 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 in Genius buffer twice for 5 minutes each.
3. Added NBT/BCIP, incubated at room temperature in dark for colour reaction.
4. Colour detection: In bright field microscope.

Composition of solutions used for In-Situ 1st day:

1) 20X SSC

   Dissolved 87.6g of NaCl in350mL of DEPEC H_2O

   Added 44.12g sodium citrate

   Bring final volume to 500mL with DEPEC H_2O.

2) TEA solution

   9.3g Triethanolamine (TEA)

   Bring upto 490mL with DEPEC H_2O

   Add 12-14 NaOH pellets

   pH to 8.0, bring up to 500mL

3) Proteinase K buffer

   25mL 1.0 M Tris-HCL

   25mL 0.5M EDTA

   Bring up to 250mL with DEPEC H_2O

4) Hybridization solution (50mL)

   3.6mL TEN solution

   25mL 100% formamide

   10mL 50% Dextran sulphate

   5mL 10% RMB blocker

   6.4mL DEPEC H_2O

   Stored at -20^oC

1) TEN solution

   5mL of 1.0M Tris-HCL, pH 7.5

   30mL of 5M NaCl

   1mL of 0.5M EDTA

Composition of solutions used for In-situ 2nd day:

1) RNase buffer:0.5M NaCl, 10mM Tris-HCL,1mM EDTA

   29.23g NaCl

   10mL 1.0M Tris- HCl, pH 7.5

   2mL 0.5M EDTA

   Brought up to 1L with deionized H_2O

2) 1X Maleate/0.05% Triton X-100/1% RMB blocker solution

   2mL of 5X Maleate stock

5µL Triton X-100

1mL of 10% RMB blocker

Make 3mL aliquots and freezed at -20^{o}C

3) 5X Maleate buffer

8g Maleic acid in 850mL deionized H_2O

pH to 7.5 using lots of NaOH pellets

Added 43.8g NaCl

Bring up to 1L with deionized H_2O

Chapter 3

Results

3.1 Temporal regulation of *ezh2* after injury.

Semi-quantitative PCR

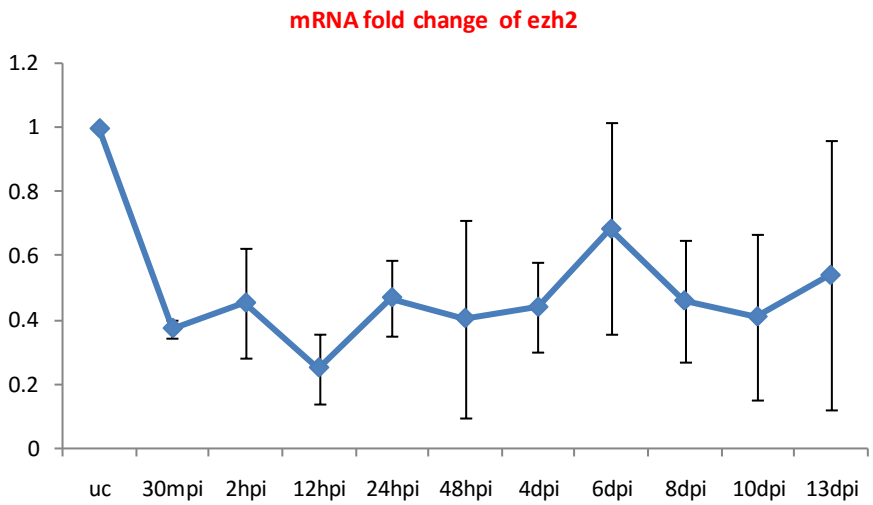
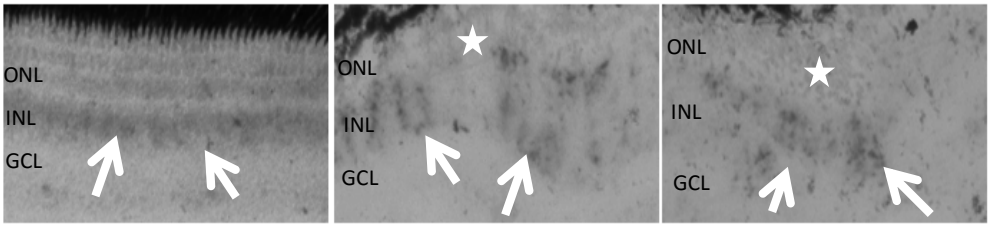


Fig. 6 Variation in *ezh2* in time after retinal injury

There is a dip in the mRNA content of *ezh2* immediately after injury and it seems to peak after at 6dpi.

3.2 Spatial expression of *ezh2*around the site of injury in the zebrafish retina.

In-situ hybridization of *ezh2* at 4dpi and 6dpi compared with uninjured control retina.



Uninjured control 4dpi 6dpi

Fig.7 In-situ *ezh2* expression in uninjured retina and around the site of injury at 4dpi and 6dpi

Co-localization of *ezh2*In-situ signal with proliferating cells at the site of injury.

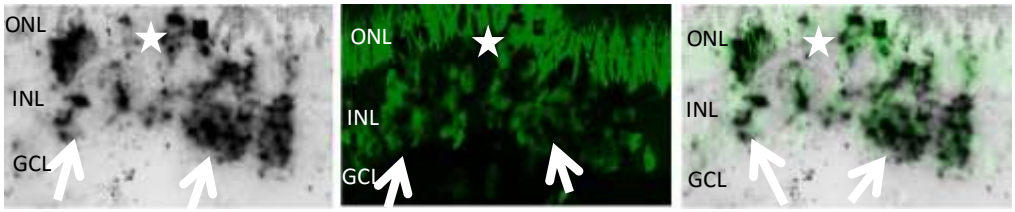


Fig.8 In-situ *ezh2* expression co-localizatized with BrdU positive cells in the INL

Ezh2 expression is concentrated around the spot of injury as compared to its pan retinal expression in the uninjured control. This expression co-localizes with the BrdU positive Muller glia cells as shown in Fig.8.

3.3 Affect of Ezh2 inhibition on Muller glial cell proliferation at the site of injury.

Ezh2 is inhibited using two pharmacological inhibitors GSK343 and UNC1999 (both act as competitors to the binding of co-factor SAM to the SET domain of Ezh2 in the PRC2 complex, inhibiting the methyl transfer to the peptide substrate)

Fig.9 Affect of Ezh2 inhibition on cell proliferation as compared to cell proliferation at 3dpi

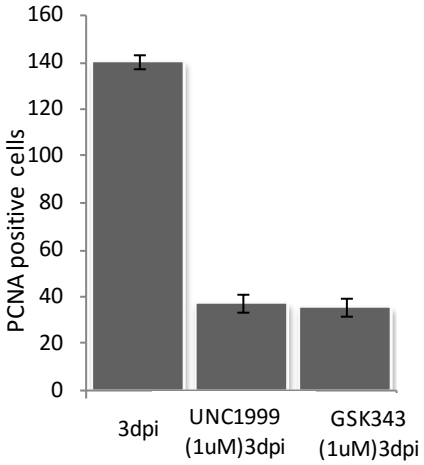


Fig.10 Cell count for PCNA positive cells

There is a decrease in the number of proliferating cells in around the site of injury on inhibition of Ezh2.

Concentration dependence in GSK343

There is continuous decrease in the number of proliferating cells with increase in concentration of Ezh2 inhibitor GSK343

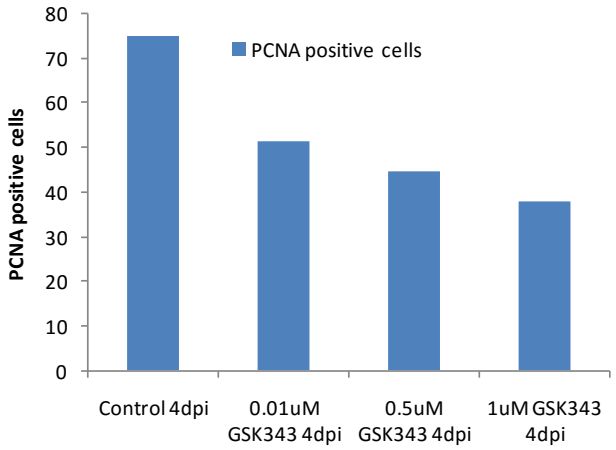


Fig.11 PCNA positive cells in increasing concentration of pharmacological inhibitor.

3.4 Affect of Ezh2 inhibition in different phases of zebrafish retinal regeneration.

Inhibiting Ezh2 in the Muller glia dedifferentiation phase.

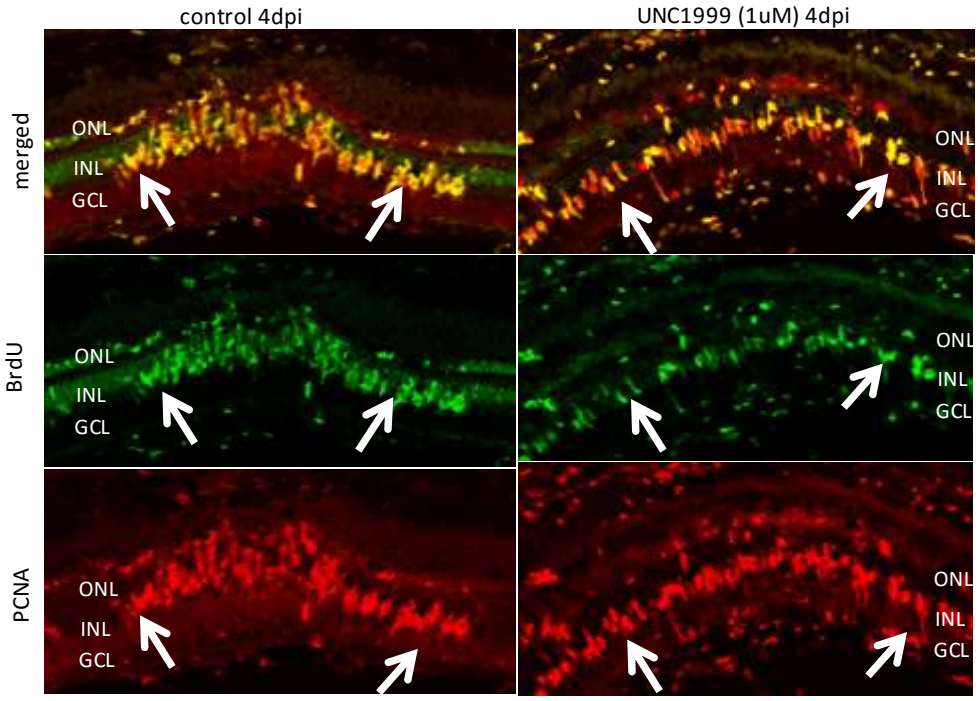


Fig.12 Affect of inhibiting Ezh2 in the dedifferentiation phase

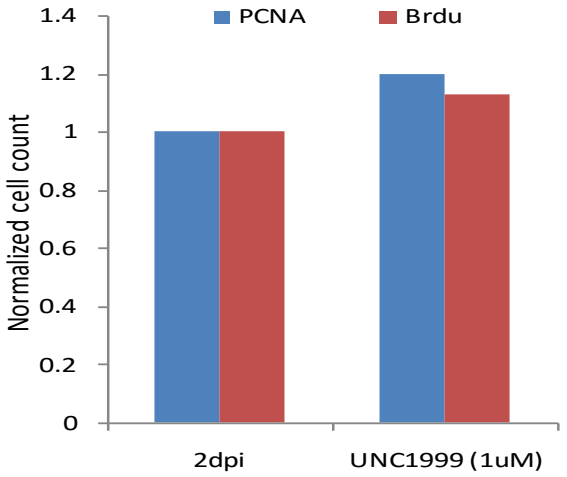


Fig.13 Quantitative analysis of the affect of inhibiting Ezh2 in the dedifferentiation phase

There is an increase in the span of proliferating cells around the site of injury with not a substantial change in their number as compared to the 4dpi control.

Inhibiting Ezh2 in the early proliferation phase.

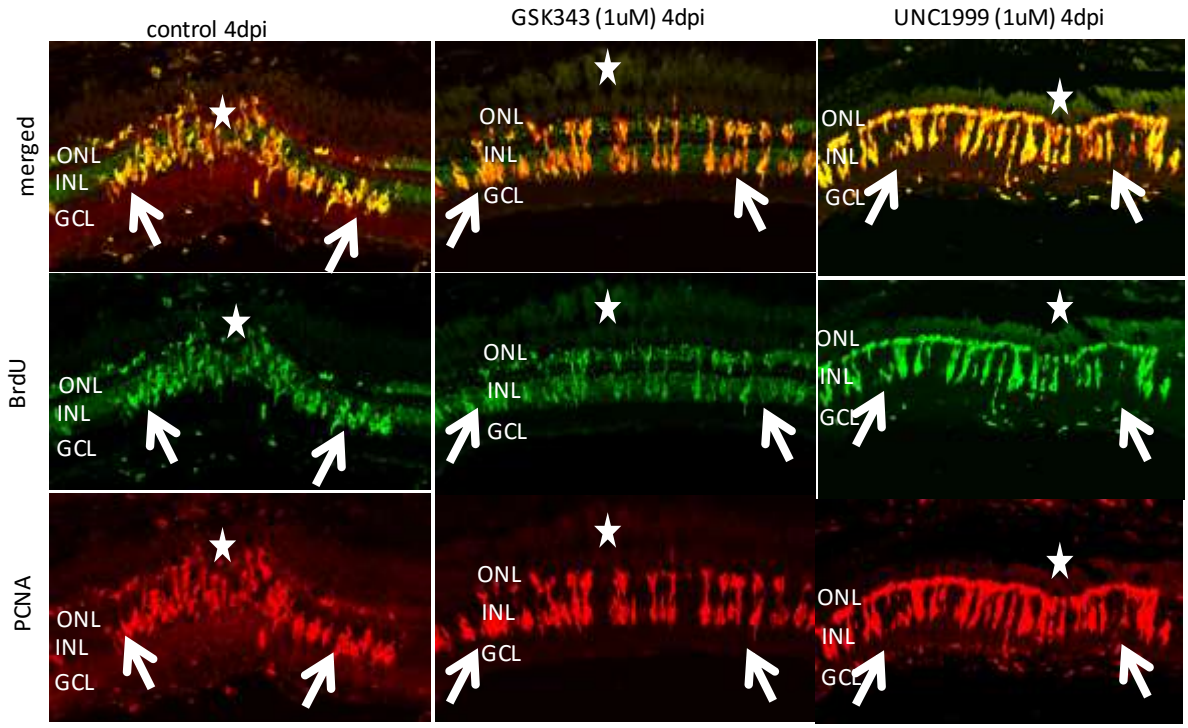


Fig.14 Affect of Ezh2 inhibition in easrly phase of proliferation on cell proliferation

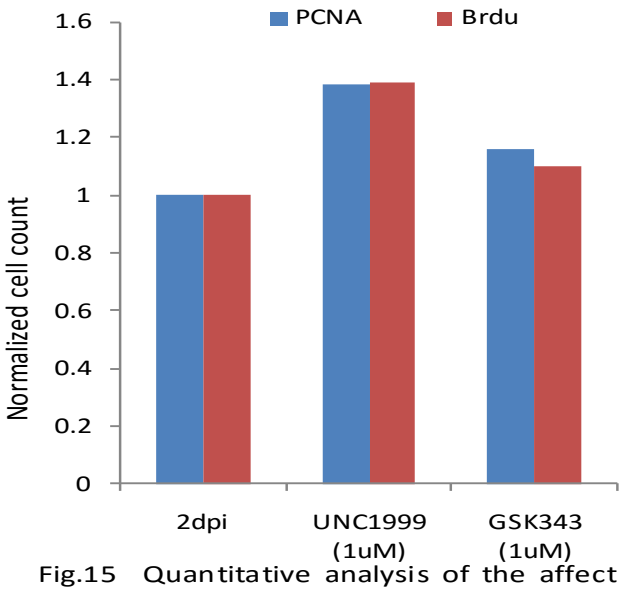


Fig.15 Quantitative analysis of the affect of Ezh2 inhibition in early phase of proliferation on cell proliferation

There is an increase in the span of proliferating cells around the site of injury with not a substantial change in their number as compared to the 4dpi control.

Inhibiting Ezh2 in the re-differentiation/migration phase:

1. Inhibiting Ezh2 after the peak of proliferation (4dpi-10dpi)

   There is decrease in the number of cells migrated to the ONL and GCL; the cells retained in the INL seem to retain Muller glial cell like morphology.

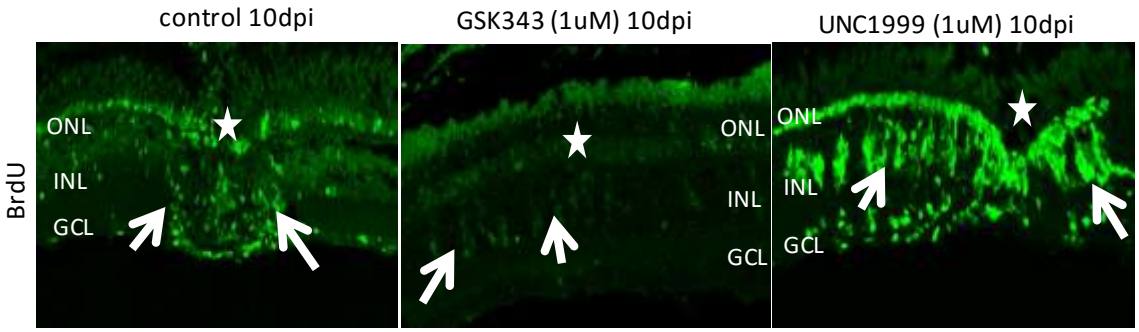


Fig.16 Affect of Ezh2 inhibition from 4dpi to 10dpi on cell migration/ re-differentiation

There is decrease in the number of cells migrated to the ONL and GCL; the cells retained in the INL seem to retain Muller glial cell like morphology.

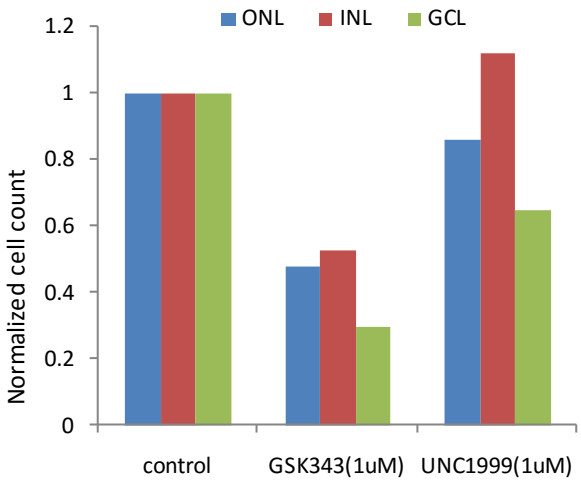


Fig.17 Qualitative analysis of the affect of Ezh2 inhibition from 4dpi to 10dpi on cell migration/ re-differentiation

2. Inhibiting Ezh2 until peak of proliferation (4dpi) and taking off the inhibition till 15dpi.

   There is decrease in the number of cells migrated to the ONL and GCL; the cells retained in the INL seem to retain Muller glial cell like morphology.

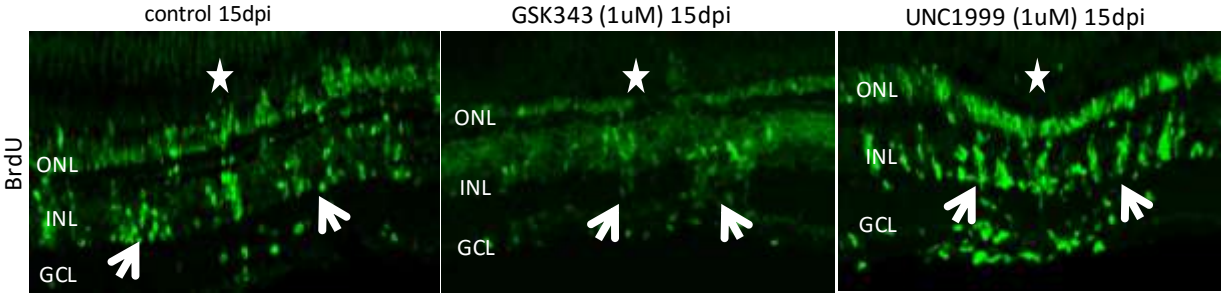


Fig.18 Affect of Ezh2 inhibition until 4dpi on cell migration/re-differentiation

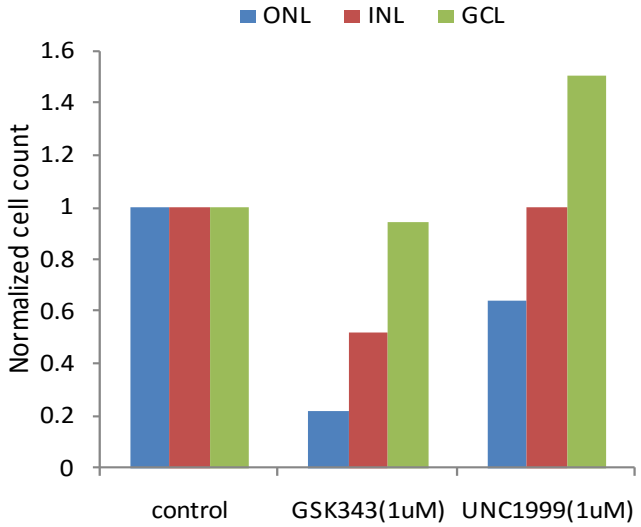


Fig.19 Qualitative analysis of the affect of Ezh2 inhibition until 4dpi on cell migration/ re-differentiation

3.5 Affect of inhibiting Ezh2 on genes known to play a role in zebrafish retina regeneration.

The following genes' regulation was checked in pharmacological inhibition of Ezh2:

Sonic Hedgehog (Shh) signalling pathway: shha, pax6b, patch. Inhibiting Shh signalling pathway drastically reduces the number of proliferating cells, thus hindering regeneration.

Notch signalling pathway: Her4.1. Her4.1 restricts the span of regeneration around the site of injury.

Other genes: Mycb, Sox, Ascl, Lin28, Zic, mmp9.

Qualitative analysis

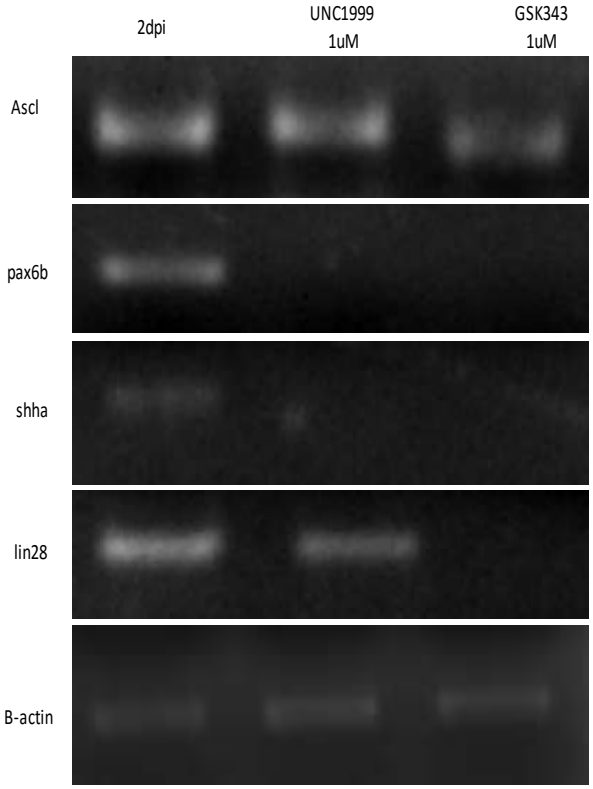


Fig.20 (a) Qualitative analysis of regeneration linked genes in Ezh2 inhibition

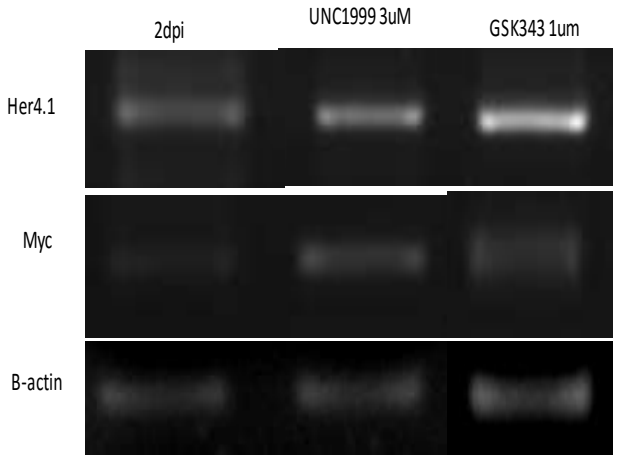


Fig.20 (b) Qualitative analysis of regeneration linked genes in Ezh2 inhibition

There is a decrease in the expression of pax6b, shha, lin28, and a slight decrease in Ascl expression, and an increase in the expression of her4.1 and mycb as compared to the expression of b-actin- the housekeeping control. This is next validated with RT-PCR.

Semi-quantitative PCR

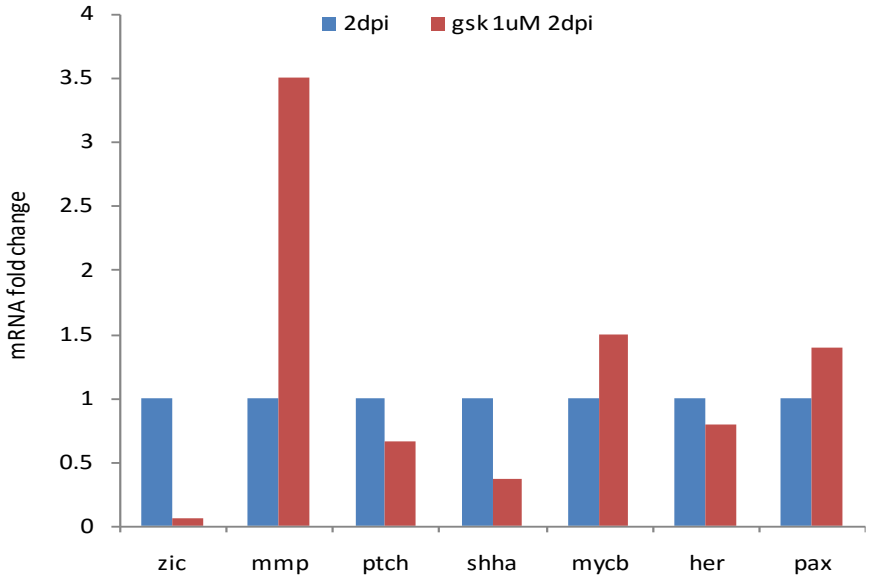


Fig.21 Semi quantitative real time analysis of regeneration linked genes in Ezh2 inhibition

To visualize the expression of these genes at the spot of injury, In-situ and immuno fluorescence staining of the genes was performed.

In-situ hybridization

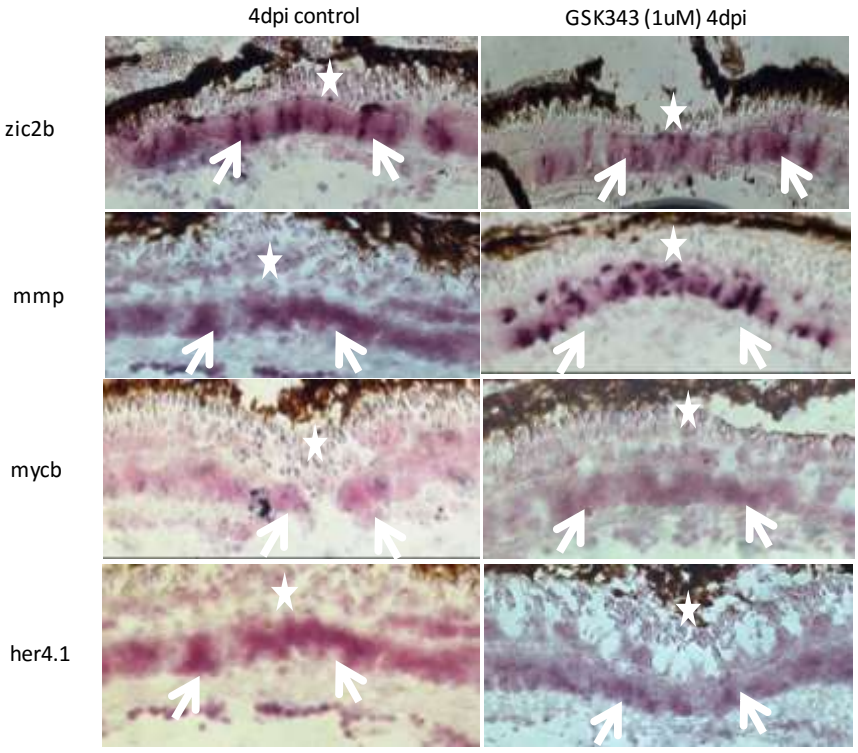


Fig.22 In-situ of genes playing a role in regeneration in Ezh2 inhibition

Immuno-fluorescence staining:

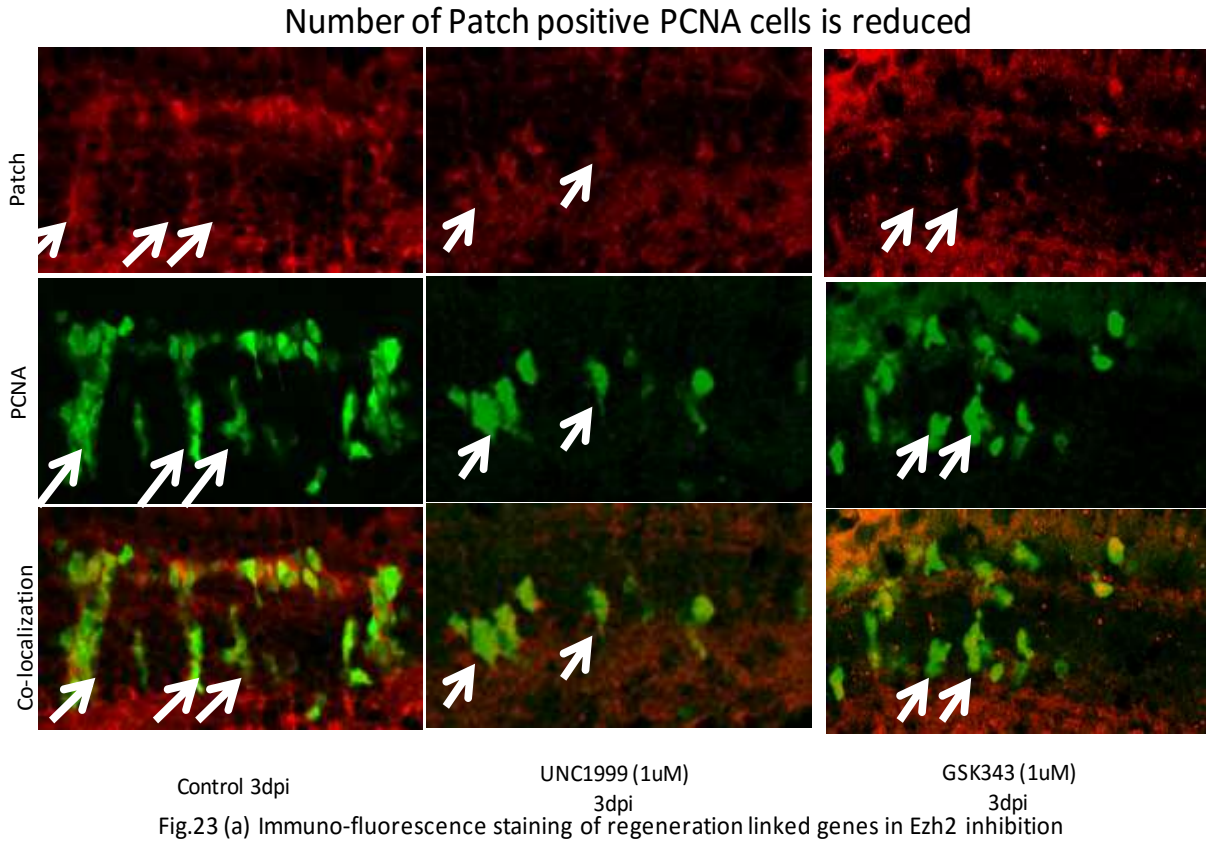


Fig.23 (a) Immuno-fluorescence staining of regeneration linked genes in Ezh2 inhibition

There is decrease in the percentage of Patch positive proliferating cells in the inhibition of Ezh2, as depictd in the graph below.

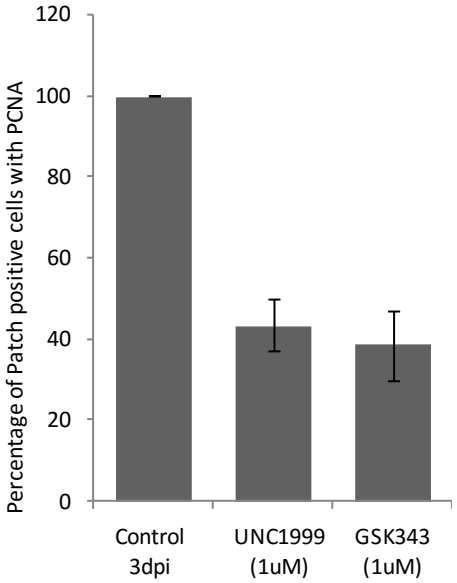


Fig.24 (a) Cell count analysis of Immuno-fluorescence staining of regeneration linked genes in Ezh2 inhibition

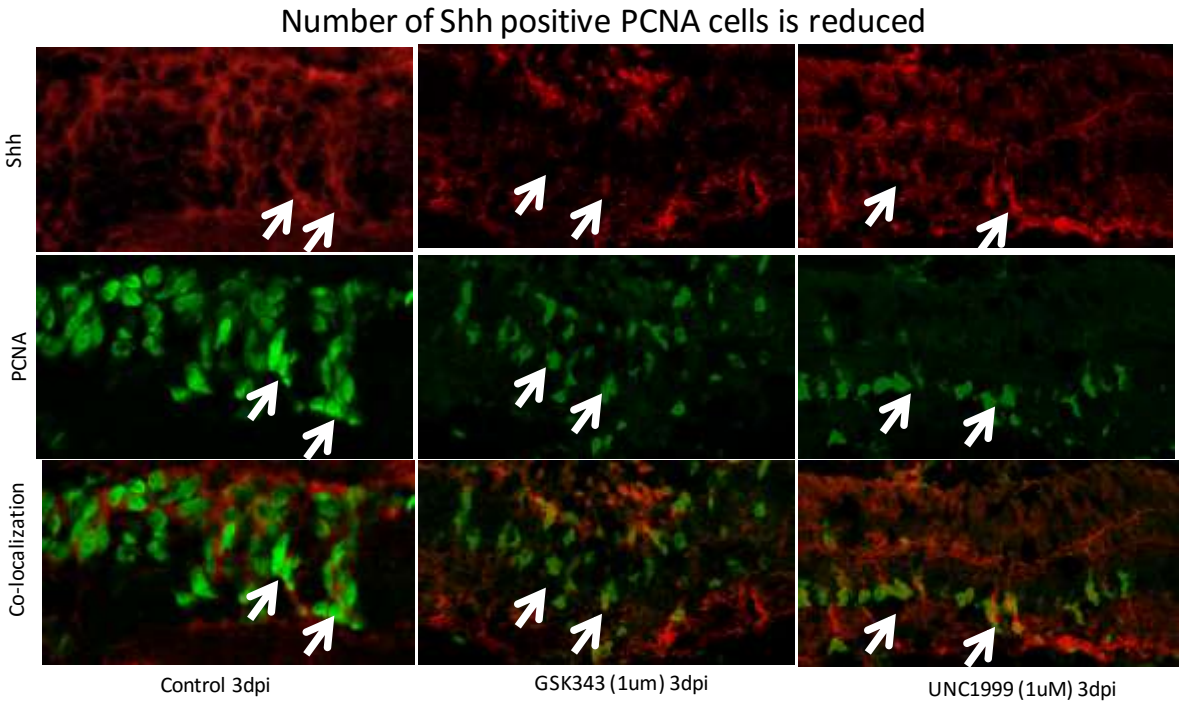


Fig.23(b) Immuno-fluorescence staining of regeneration linked genes in Ezh2 inhibition

There is a decrease in the number of shh positive proliferating cells in the inhibition of Ezh2.

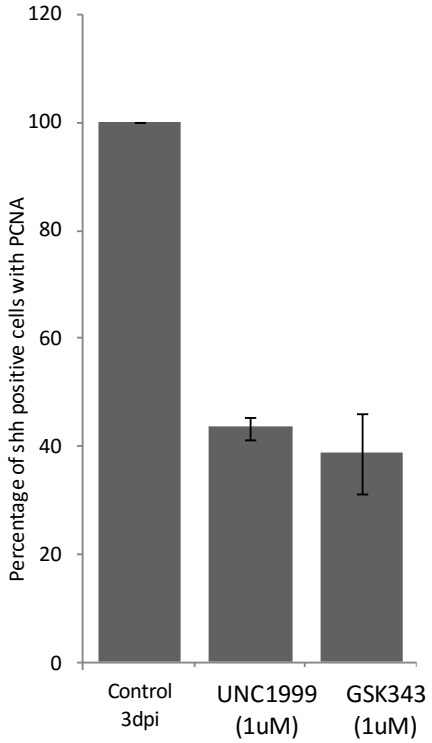


Fig.24 (b) Cell count analysis of Immuno-fluorescence staining of regeneration linked genes in Ezh2 inhibition

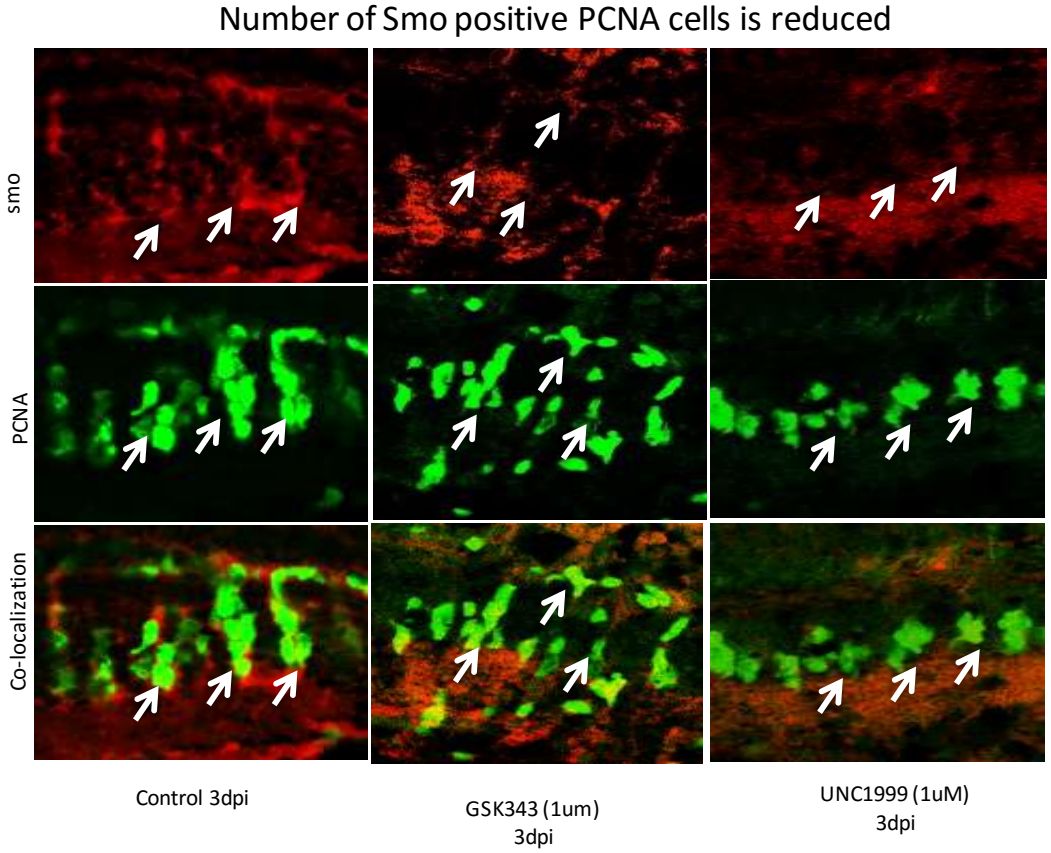


Fig.23 (c) Immuno-fluorescence staining of regeneration linked genes in Ezh2 inhibition

There is decrease in the number of smo positive proliferating in inhibition of Ezh2 as compared to the control.

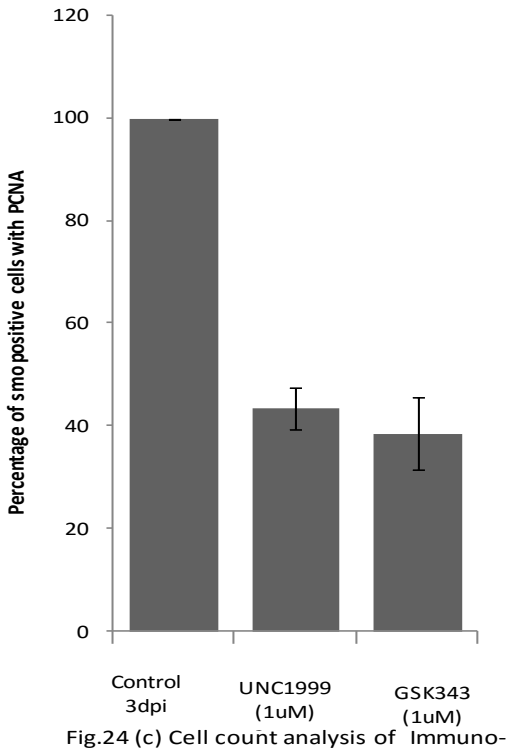


Fig.24 (c) Cell count analysis of Immuno-fluorescence staining of regeneration linked genes in Ezh2 inhibition

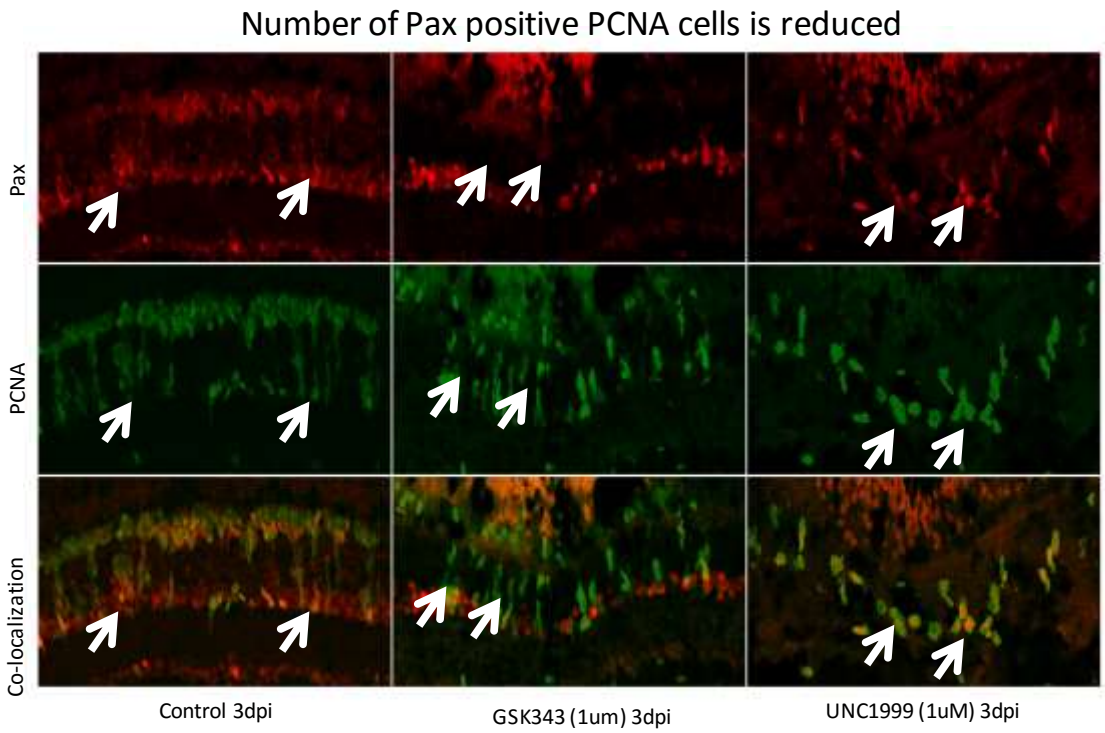


Fig.23 (d) Immuno-fluorescence staining of regeneration linked genes in Ezh2 inhibition

There is decrease in the number of pax positive proliferating in inhibition of Ezh2 as compared to the control.

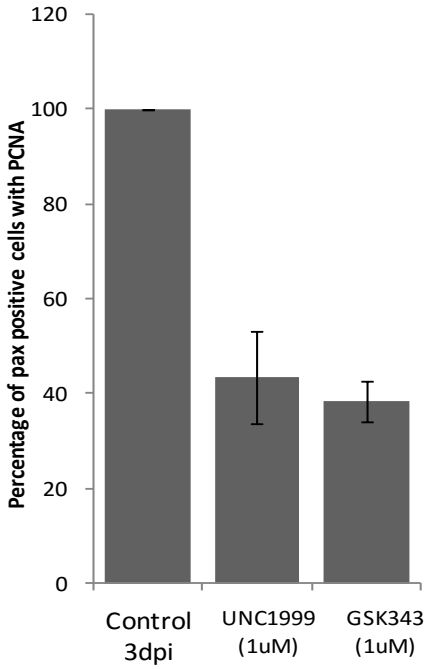


Fig.24 (d) Cell count analysis of Immuno-fluorescence staining of regeneration linked genes in Ezh2 inhibition

3.5 Affect of inhibiting regeneration linked signalling pathways and genes on *ezh2* expression

Semi-quantitative PCR analysis

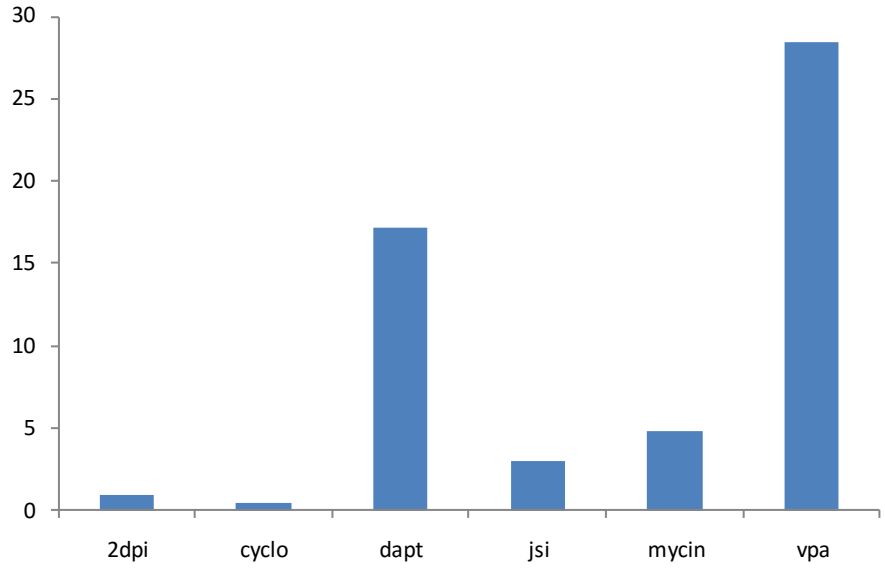


Fig.25 Semi quantitative real time analysis of Ezh2 in inhibition of regeneration linked pathways

Cyclopamine (cyclo) is Sonic Hedgehog signalling inhibitor, DAPT is notch signalling inhibitor, JSI is Jak-stat signalling inhibitor, mycin is Mycb inhibitor, VPA is HDAC inhibitor.

3.6 Potential specific binding sites of PRC2 complex on the genome.

Stretches of GC-rich DNA, termed CpG islands, can initiate recruitment of PRC2 in embryonic stem cells when they are transcriptionally-inactive. Surprisingly, we find that GC-rich DNA from bacterial genomes can also initiate recruitment of PRC2 in embryonic stem cells. [11]

ChIP-Seq maps for the PRC2-components Ezh2 and Suz12 reveal >3000 sites in the mouse genome significantly enriched for one or both factors. Roughly three-quarters of these PRC2 bound sites correspond to known gene promoters: Ezh2 occupies 2461 promoters, while Suz12 occupies 1944 promoters. [12]

3.7 Affect of Ezh2 inhibition on embryonic development.

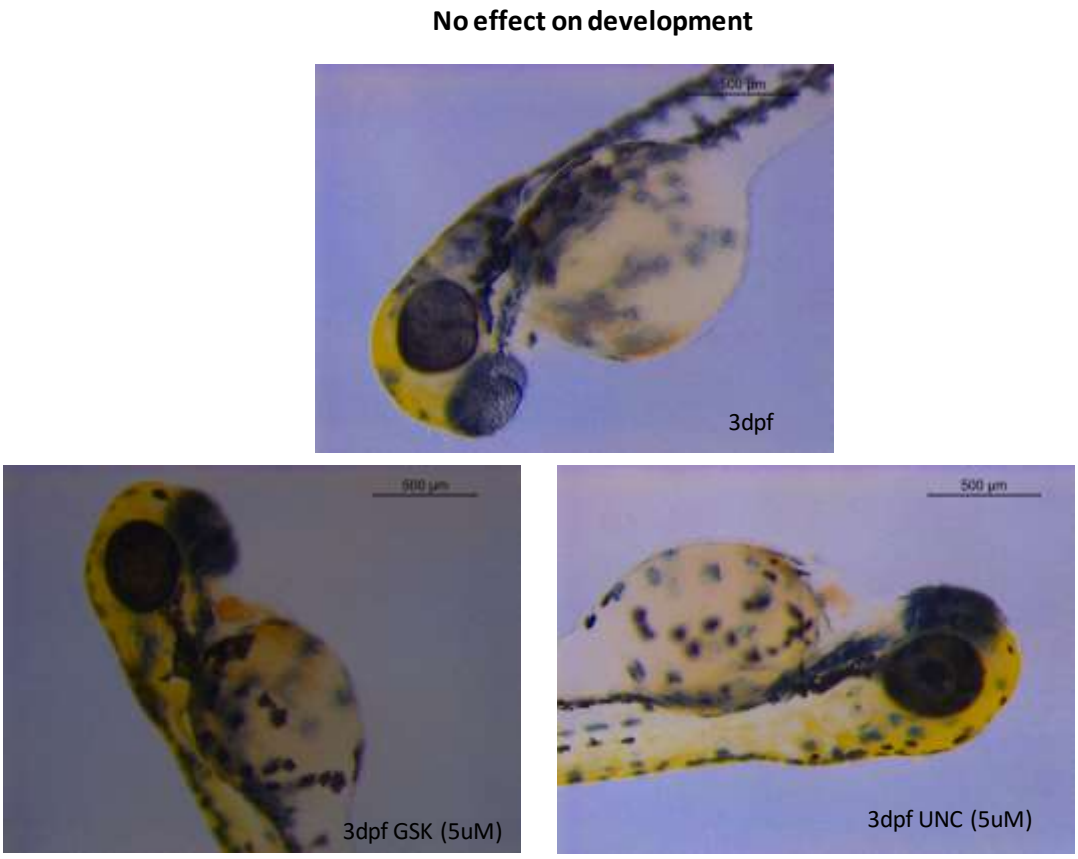


Fig. 26 Effect of Ezh2 inhibition on development.

There is no phenotypic difference in the development of the embryos in pharmacological inhibition of Ezh2 as compared to the control.

Chapter 4

Conclusions and Discussion

Ezh2, a histone methyl transferase and the catalytic subunit of the PRC2 complex is known to be a gene repressor. The expression pattern studies suggest that it is concentrated around the injury spot in proliferating Muller glial cells and its transcription varies as regeneration progresses.

The inhibition studies suggest that Ezh2 is necessary for efficient retinal regeneration. Its inhibition in early phases increases the span of proliferating cells suggesting that it is important in restricting the zone of proliferation.

Late phase inhibition causes lesser number of cells to migrate to the ONL and GCL with Muller glial cells in intact morphology retained in the INL. This suggests Ezh2 activity might be necessary for complete re-differentiation or migration of proliferating cells in the area of injury.

Ezh2 is linked in some way to the other signaling pathways like the Sonic hedgehog-signaling pathway, Notch signaling pathway that are known to play an important role in regeneration.

There also seems to be an inverse link between them as the inhibition of Ezh2 affects expression of genes in these pathways and other regeneration linked genes.

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