Understanding The Role of *zebs* and *cadherins* in Zebrafish Retina Regeneration

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Certificate of Examination

This is to certify that the dissertation titled “Understanding the role of zebs and cadherins in Zebrafish Retina Regeneration” submitted by Ms. Komal Meena (Reg.No.MS14095) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report is accepted.

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(Supervisor)

Dated: April 26, 2019

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 acknowledgment of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within detailed in the bibliography.

Komal Meena

(Candidate)

Dated: April 26, 2019

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)

Acknowledgment

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Contents

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Notation

- BrdU: Bromodeoxyuridine
- PCNA: Proliferating Cell Nuclear Antigen
- TSA: Trichostatin A
- VPA: Valproic acid
- MG: Müller Glia
- ONL: Outer Nuclear Layer
- INL: Inner Nuclear Layer
- GCL: Ganglion Cell Layer
- RT-PCR: Reverse Transcription PCR
- RT: Room temperature
- dpi: Days post injury
- hpi: Hours post injury
- UC: Uninjured Control
- MO: Morpholino-modified antisense oligonucleotides
- hdac: Histone deacetylase gene or transcript in zebrafish
- Hdac: Histone deacetylase protein in zebrafish
- HDAC: Histone deacetylase protein in mammals
- Ascl1a : Achaete-scute homolog 1 zebrafish protein
- ascl1a : Achaete-scute homolog 1 zebrafish mRNA
- Mmp9: Matrix metalloprotease 9 zebrafish protein
- mmp9: Matrix metalloprotease 9 zebrafish mRNA
- YAP: Yes-associated protein
- EMT: epithelial-to-mesenchymal transition
- ZEB: Zinc finger E-box-binding homeobox protein in human
- zeb: Zinc finger E-box-binding homeobox protein encoded by zeb

Abstract

Retinal cells degenerate in a variety of different diseases like diabetic retinopathy, glaucoma, age-related macular degeneration. Unfortunately, mammals are incapable of regenerating retinal cell types. Mammals exhibit a limited propensity for regeneration in tissues and organs like skin, skeletal muscle and liver but CNS (central nervous system) do not regenerate in mammals. The retina is a part of CNS and zebrafish (*Danio rerio*) can regenerate most of its organs like heart, liver, fin, including retina which makes zebrafish a suitable model for studying tissue regeneration. Zebrafish act as a unique model system to understand the molecular mechanism involved in retina regeneration. The fish and mammalian retina structure are similar and composed of identical cell types with conserved function. Müller glial cells have an essential role in zebrafish retina regeneration. Upon injury, Müller glial cells undergo reprogramming and divide asymmetrically into multipotent progenitor cells, and these progenitor cells give rise to all retinal cell types. Retina regeneration requires many growth factors and signaling cascades like transforming growth factor(TGF-β), Sonic Hedgehog(SHH), and WNT signaling pathways. These pathways are involved in EMT(epithelial-to-mesenchymal transition). EMT is a critical step in development and regeneration. The crosstalk of these signaling pathways in EMT are sophisticated and remain explored. In this project, we have investigated the effect of YAP nuclear inhibition on *zebs* and *cadherins* and crosstalk of *zebs* and *cadherins* with HDACs and klf4 in early dedifferentiation phase, and we showed that *zebs* and *cadherins* could have an essential role in zebrafish retina regeneration since EMT can be closely similar to dedifferentiation and proliferation.

Chapter 1. Introduction

Retina regeneration is the restoration of vision invertebrates that have a retinal injury or degenerative diseases. Degenerative diseases often lead to visual impairment. Retinal cells degenerate in a variety of different diseases like diabetic retinopathy, glaucoma, age-related macular degeneration[1]. Unfortunately, mammals are incapable of regenerating retinal cell types. Mammals exhibit a limited propensity for regeneration in tissues and organs like skin, skeletal muscle and liver but CNS (central nervous system) do not regenerate in mammals. The Retina is a part of CNS and zebrafish has a capacity to regenerate most of its organs like heart, liver, fin, including retina which makes zebrafish a suitable model for studying tissue regeneration. The fish and mammalian retina structure are similar and composed of identical cell types with conserved function. In telofish, upon injury, Müller glial cells undergo reprogramming and divide asymmetrically into multipotent progenitor cells, and these multipotent progenitor cells give rise to all retinal cell types[2].

Therefore, understanding of the molecular mechanism involving in Müller glial cell reprogramming and retina regeneration in zebrafish may provide the basis, which will be helpful for the patients with retinal diseases[1, 2].

1.1 Retinal Anatomy:

Vertebrate's retina situated at the back of the eye and is a complex structure which helps in vision. Vertebrate's retina is divided into three layers mainly: ONL (outer nuclear layer), INL (inner nuclear layer), and GCL (ganglion cell layer). ONL contains photoreceptor cells (rods and cones) which sense the light and transduce the signals to ganglion cell in GCL through bipolar, amacrine, and horizontal cell which are types of interneurons, in INL[3].

1.2 Müller glia and their response to retinal injury:

Although the Cell bodies of Müller glia are lie in INL, they are the only cell types which span through all retinal cell layers. Depending upon the severity of the injury, Müller glia responds to retinal injury by reprogramming. Reprogramming of Müller glial cell mainly divided into three part:

Dedifferentiation-After injury, MG acquire stem-cell-like properties and called MGPCs

(Müller Glia Progenitor Cell)

Proliferation- MGPCs increases in number.

Redifferentiation-MGPCs migrate to the injury place and give rise to the different retinal cell types.

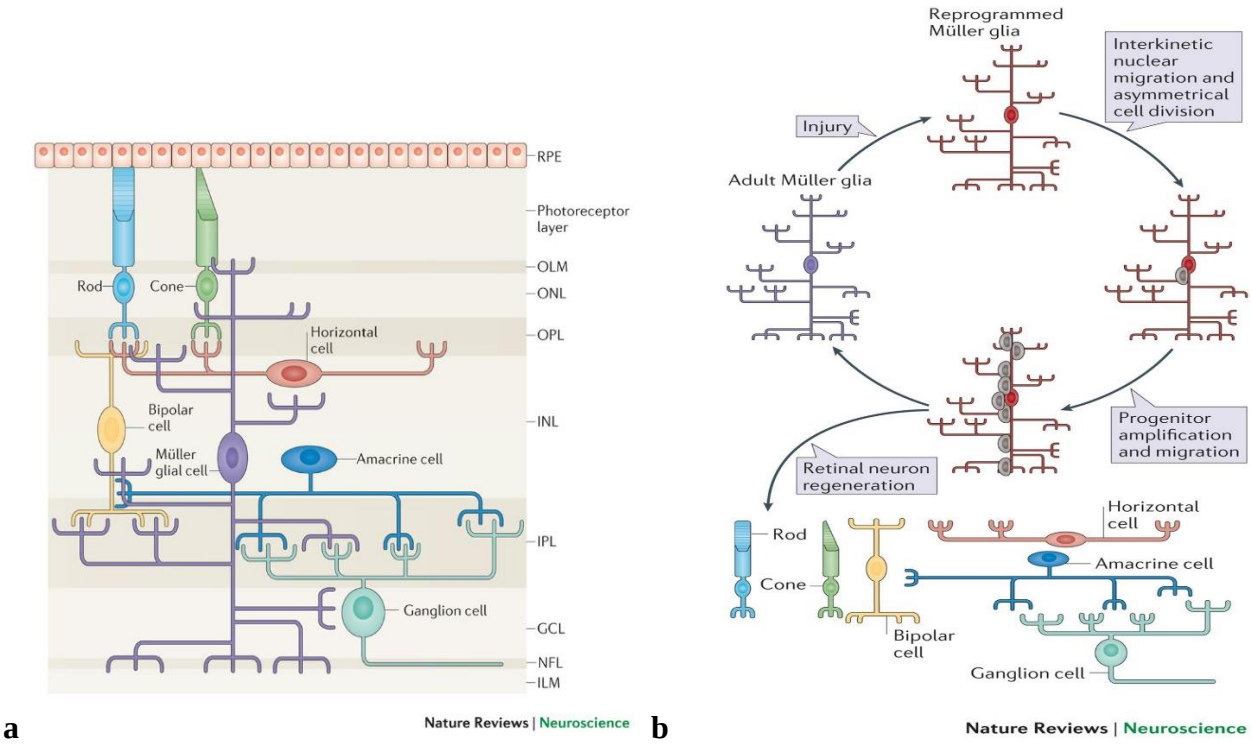

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Figure 1.1. Retina regeneration at a glance[3]: **(a)** Retinal Anatomy. OPL- Outer plexiform layer (synapses between cells of ONL and INL), IPL- Inner plexiform layer (synapses between cells of INL and ganglion cells), OLM- Outer Limiting Membrane, ILM- Inner Limiting Membrane, RPE- Retinal Pigment Layer (absorbs light), NFL-Nerve Fibre Layer (Axonal process of ganglion cells); **(b)** Regeneration Mechanism. After reprogramming, MG cells migrate to ONL and undergo asymmetrical cell division at OLM generating multipotent progenitors. These progenitors migrate to all retinal layers, exit cell cycle and form different cell types, regenerating retina. Interkinetic nuclear migration is the migration of injury-responsive MG to ONL for the division and then back to INL.

This entire regeneration process requires multiple signaling pathways and growth factors like transforming growth factor (TGF-β), Sonic Hedgehog (SHH), and WNT signaling pathways. These pathways are involved in EMT (epithelial-to-mesenchymal transition). EMT is a crucial

step in development and regeneration. Signaling molecule like transforming growth factor (TGF-β) superfamily, the WNT family and fibroblast growth factor (FGF) family, SNAIL family (SNAIL1 and SNAIL2) are essential to regulate the primary EMT during early embryonic development. SNAIL and ZEBs are essential for repressing E-cadherin which leads to weakening of the cell-cell junction[4]. E-cadherin is one amongst the first vital molecules in cell-cell adhesion in animal tissue tissues. It is localized on the surfaces of animal tissue cells in regions of cell-cell contact referred to as adherens junctions. Calcium-dependent cell adhesion molecules (CAMs), the cadherin glycoproteins are expressed by a variety of tissues, mediating adhesion through homotypic binding. Classical cadherins – E- and N-cadherins being the simplest characterized – play vital roles within the formation of tissues throughout the biological process, neurulation, and organogenesis. The suppression of E-cadherin expression is thought to be one amongst the most molecular events accountable for dysfunction in cell-cell adhesion. Most tumors have an abnormal cellular structure, and loss of tissue integrity will result in a native invasion. Loss of E-cadherin-mediated-adhesion characterizes the transition from benign lesions to invasive, metastatic cancer[5].

1.3 E-cadherin and its crosstalk with ZEB:

Multiple signaling pathways regulate the expression of ZEBs such as WNT, TGF-β, signaling, and microRNAs. ZEB1 belongs to the ZEB family of transcription factors characterized by the presence of 2 zinc finger clusters, which are responsible for DNA binding, and a centrally located homeodomain. Expression ZEB1, a nuclear factor induces an epithelial-to-mesenchymal transition and by repressing the E-cadherin gene at the transcriptional level confers a metastatic phenotype on carcinomas. Through zinc finger clusters, ZEB1 can bind to specific DNA sequences named E-boxes. By recruiting co-suppressors or co-activators, ZEB1 can either down-regulate or up-regulate the expression of its target genes. ZEB1 directly links to the E-box located in the promoter of CDH1, the gene encoding E-cadherin and recruit transcriptional co-repressor leading to repression of CDH1 transcription and induction of EMT[6]. ZEB1, to regulate E-cadherin independently of CtBP interacts with the SWI/SNF chromatin-remodeling protein BRG1 and its traditional co-repressor. Blocking of the ZEB1 and BRG1 complex induces the expression of E-cadherin and down-regulation of the mesenchymal marker vimentin. By recruiting the CtBP co-repressor to its CtBP-interacting domain (CID), ZEB1 represses transcription. BRG1 is a vital

molecule required for the regulation E-cadherin and the induction of EMT by ZEB1. ZEB1/BRG1 as a new transcriptional mechanism regulating E-cadherin expression and epithelial-to-mesenchymal transdifferentiation. This makes ZEBs significant to study in the context of retina regeneration[7].

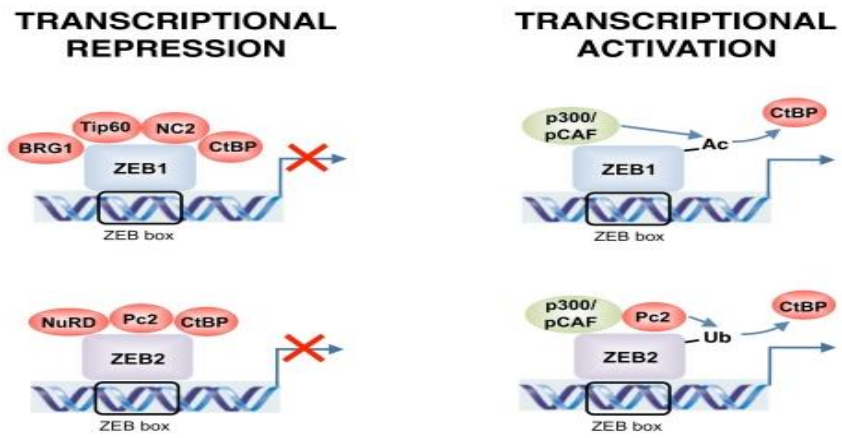

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Figure 1.3: ZEB1 and ZEB2 as transcriptional activator or repressor. ZEB1 and ZEB2 act as transcriptional activator or repressor depending upon the targeted gene and tissue. Post-transcriptional modifications of ZEB1 and ZEB2 alter the set of co-activators and co-repressors bound and switch both proteins from transcriptional repressors to activators[8].

1.4 E-cadherin and its crosstalk with hippo signaling pathway:

Contact inhibition of cell growth is crucial for embryonic development and maintenance of tissue structure in adult organisms, and loss of contact inhibition of proliferation is the main characteristic of the growth of a tumor. The Hippo signaling pathway controls organ size and contact inhibition of proliferation by inhibiting cell proliferation and promoting programmed cell death[8]. The protein kinase cascade of the Hippo signaling pathway stimulates the nuclear exclusion and inactivation of transcriptional co-activator Yes-associated protein (YAP) and its paralog TAZ (transcriptional activator with PDZ binding motif). YAP, TAZ (transcription co-activators) and TEAD

(transcription factor) are the downstream transcriptional machinery and effectors of the pathway. Formation of the YAP/TAZ-TEAD advanced ends up in transcription of growth-promoting genes.

Conversely, disrupting the interactions of the advanced decreases cell proliferation. Hippo signaling pathway components needed for E-cadherin–dependent contact inhibition of proliferation. Knockdown of the Hippo signaling pathway component or overexpression of YAP inhibits the decrease in cell proliferation caused by E-cadherin homophilic binding at the cell surface, freelance of alternative cell-cell interactions. E-cadherin-mediated cell-cell contact directly regulates the Hippo signaling pathway to regulate cell proliferation, in addition to its role in cell-cell adhesion[9, 10].

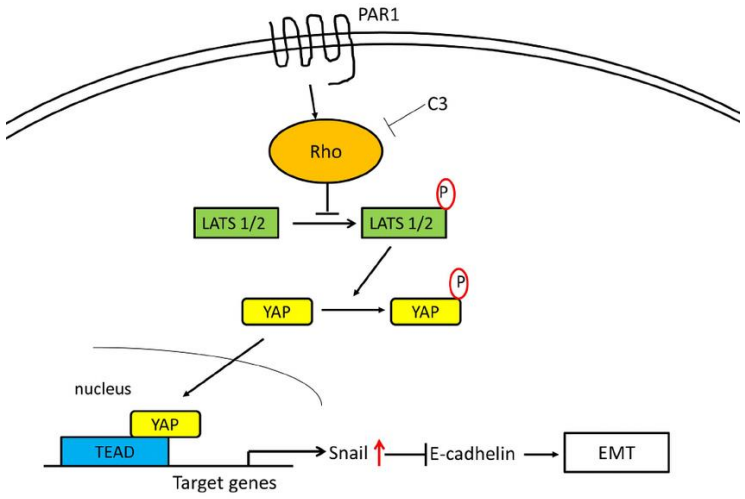

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Figure 1.4: Inhibition of the Hippo-YAP pathway and EMT[11]. Inhibition of yap nuclear translocalization leads to down-regulation of snails which further lead to EMT.

1.5 Regulation of EMT by KLF4:

Krüppel-like factor 4 (KLF4), belonging to the Krüppel-like factor family, is a zinc finger-type transcription factor highly expressed in various human tissues, including differentiated, post-mitotic epithelial cells of the gastrointestinal tract. KLF4 acts as a tumor suppressor in Gastrointestinal cancer, and it negatively regulates cell proliferation and promotes tissue

differentiation, and loss of KLF4 expression is a predictor of poor survival. Recent studies indicated that KLF4 was a critical negative regulator of EMT and during the EMT process expression of klf4 has reduced. Studies showed that down-regulation of E-cadherin is associated with the invasive and undifferentiated phenotype in many GI cancers, including esophageal cancer, gastric cancer, colorectal cancer, gallbladder cancer, liver cancer, and pancreatic cancer. Another adhesion molecule N-cadherin, which typically expressed on mesenchymal cell surface, is correlated with the motility and invasive potential of cancer cells. Reduced E-cadherin expression and up-regulated N-cadherin expression were associated with the EMT, invasion and metastasis potentials of pancreatic cancer cells. Studies are showing that the cadherin-switch from E-cadherin to N-cadherin has a critical function in cancer progression and is essential for increased motility and migration. Many transcription factors could repressed E-cadherins, such as ZEB (ZEB1 and ZEB2), the Snail (Snail1 and Snail2) and Twist. These transcription factors are downstream of signaling pathways, including transforming growth factor-β (TGF-β), Wnt/β-catenin, Notch, fibroblast growth factor (FGF), signal transducer and activator of transcription-3 (STAT-3), epidermal growth factor (EGF) and nuclear factor (NF)-κB. Loss expression of E-cadherin leads to the release of its intracellular partner β-catenin which after that translocates into the nucleus and transcriptionally modulates several genes, such as cyclin D1, CD44, c-Myc, and vascular endothelial growth factor (VEGF), and promotes cancer development and progression. Through interference with TGF-β, Notch, and Wnt signaling pathways, KLF4 negatively regulates EMT of GI cancers[12].

1.6 HDACs and their role in regeneration:

Histone deacetylases or HDACs are members of one of the classes of genome epigenetic regulators. They are a class of enzymes which remove an acetyl group from the ε-amino group of a lysine on a histone protein. This property of Hdacs allows tighter wrapping of DNA around histones, ultimately leading to transcriptional repression. HDACs have divided into four classes and two families: the classical and silent information regulator 2 (Sir2)-related protein (sirtuin) families. In humans, members of the classical family include HDAC1, -2, -3 and -8 (class I); HDAC4, -5, -6, -7, -9 and -10 (class II); and HDAC11 (class IV). They share sequence similarity and require Zn^{2+} for deacetylase activity. The sirtuin family contains seven members (SIRT1–7,

class III), which show no sequence resemblance to members of the classical family and require NAD+ as the cofactor[13].

The levels of histone acetylation play a crucial role in chromatin remodeling and the regulation of gene transcription. HDACs are key enzymes regulating important cell processes such as cell-cycle progression and apoptosis. Another way by which HDACs are recruited to DNA independently of DNA methylation involves the interaction with transcription factors and nuclear receptors. Interaction with transcription factors, HDAC1 and HDAC2, are included in transcriptional repression regulated by the retinoblastoma protein Rb. Treatment with TSA, a classical HDAC inhibitor, prevents the Rb-mediated repression of gene transcription. By overexpression and aberrant recruitment of HDACs to their promoter region transcriptional repression of tumor-suppressor genes could be a common phenomenon in tumor growth. Treatment with HDAC inhibitors leads to the inhibition of tumor cell growth and an increase in both the acetylation of the promoter and gene expression. Transcription factors like Snail recruit HDAC1, HDAC2, and the co-repressor complex mSin3A to the E-cadherin promoter to repress its expression and studies are showing that the acquisition of invasive potential by carcinomas implicated in down-regulation or loss of function of E-cadherin. These characteristics make HDACs another important molecule to study in the context of retina regeneration[13].

Chapter 2. Materials and Methods

2.1 Retinal Injury and harvesting the retina for RNA isolation

1. Zebrafish anesthetized using tricaine methanesulfonate.
2. Then it was kept on a wet sponge bed. Retina was injured using 30 gauge needle and forceps.
3. At a desired time after injury, eyes were dissected using steel forceps and needle. Dissections were carried out either in 1X PBS (Phosphate Buffered Saline) for harvesting retina or 4% paraformaldehyde (PFA) for tissue fixation.
4. Lens removed for both isolating RNA/ tissue fixation.
5. If the retina used for RNA isolation they should be stored in trizol and if to be used for western blotting they should be suspended in laemmli buffer in -80◦C.

2.2 Microscopy

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

2.3 RNA Isolation

1. Take the stored embryos/dissected retinas from -80oC and thaw it.
2. Homogenize the tissues (use 200µl pipette for retinas and homogenizer in case of embryos) entirely so that no tissue clumps are visible. Allow it to settle for 5 min.
3. Add 0.2 volumes (40µl) of chloroform and mix it gently by inverting the MCT up and down ten times.
4. Then centrifuge it at 12000 rcf for 15min at 4oC.
5. Using cut tips collect aqueous phase slowly without disturbing the middle phase layer and put it in fresh MCTs.

6. Add an identical volume of Isopropanol and keep it on ice for 10-15 mins or incubate overnight at -80oC.
7. Then centrifuge it at 13000rcf at 4oC for 30min.
8. Discard the supernatants.
9. Wash it with 75% alcohol (200µl) and centrifuged it at 4oC for 5 min at 12000 rcf.
10. Dry and dissolve the pellet in DEPC treated water and stores it at -80oC.
11. Check whether there is any contamination of genomic DNA in it by using agarose gel electrophoresis (1%gel) and if the isolated RNA is pure and free from any genomic DNA contamination we measure the amount of RNA present by measuring the Optical density using spectrophotometer (Nanodrop) and proceed further for cDNA synthesis.

2.4 cDNA Preparation

(Kit used – RevertAid First Strand cDNA Synthesis Kit by Thermo Fisher)

1. Following reagents were added into a sterile tube:

   - Template RNA 2.5µL
   - Primer (Oligo (dT)18 + Random Hexamer) 0.25 + 0.25 µL
   - The contents mixed gently and incubated at 65°C for 5 minutes. Then, the tubes were transferred back on the ice.

2. Following components added in the indicated order:

   - 5X Reaction Buffer 1µL
   - RiboLock RNase Inhibitor (20U/µL) 0.25µL
   - 10mM dNTP Mix 0.5µL
   - Revert-Aid M-Mul V RT (200U/µL) 0.25µL
   - Total Volume 5µL

3. The contents were mixed, centrifuged briefly and incubated at following temperatures:

   - 5 minutes at 25°C
   - 60 minutes at 42°C

- 5 minutes at 70°C

4. The cDNA was then diluted with nuclease-free water and stored at -80°C.

2.5 Reverse transcription-Polymerase chain reaction (RT PCR) using Taq Polymerase

1. Dilute the synthesized cDNA by 1:3 dilutions (1µl cDNA+ 3µl Milli-Q water) and set reactions for 10µl.

- 20X buffer- 0.5µl
- dNTP-1µl
- forward primer and reverse primer(F+R) -0.4µl
- template cDNA-0.5µl
- MQ water -7.7µl
- Taq polymerase -0.1µl

2. Put cycling conditions as:-

- 95oC - 2min
- 95oC - 15secs
- 62oC - 30secs
- 72oC - depending on size of gene (1kb/1min),
- 72oC - 5 min
- 4oC - Infinite hold
- Put 30 cycles.

3. PCR product checked by Agarose gel electrophoresis.

2.6 Quantitative PCR (qRT-PCR)

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

1. We set 5µl reaction:-

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

2. Analyze the data on an excel sheet and plot the graph.

2.7 Tissue fixation and sectioning

1. Take the eyes whose lenses were removed and put them into 4% PFA in 4oC overnight.

2. Next day give washings of the fixed tissue at RT for 45mins each on a rotor:

- 1ml of 5% sucrose
- 800µl of 5% and 400µl of 20% sucrose
- 500µl of 5% and 500µl of 20% sucrose
- 400µl of 5% and 800µl of 20% sucrose
- 1ml of 20% sucrose.

3. Then add 500µl of OCT and rotate it for 30 min.

4. Then embed the in OCT in small cubes made from aluminum foil, and the embedded samples are kept frozen at -80oC until sectioning.

5. Then section the blocks in cryostats (12µm thickness) and collect the sections on super frost plus slides and dried overnight and then stored in -20oC.

The composition of solutions used:

- 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

➢ 5% sucrose:

- Dissolve 2.5g sucrose in 50mL of autoclaved water. Store at -20oC.

➢ 20% sucrose:

- Dissolve 10g sucrose in 50mL of autoclaved water. Store at -20oC.

2.8 Immunostaining

Day 1

Slides were taken out from -20°C and dried at 37°C for 30minutes.

1. 1X PBS wash for 10min, twice.
2. Fix tissue with 4% PFA solution for 20min (This step is done only in the case of MO slides).
3. Treat the slides with 2N HCl (preheated to 37°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 in 1X PBST (1XPBS + 0.1% Triton X) for 3 hours at RT.
6. Overlay the slides with 1° Ab of choice (PCNA or BrdU), 500µL per slide (Ab has diluted in 1:1000 ratio in 1% BSA in 1XPBST).
7. Incubate the slides at 4°C overnight.

Day 2

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

2.9 Plasmid Isolation

1. 1.5mL of culture centrifuged at 10,000rpm for 2 min, RT.
2. The supernatant form the above step discarded, and the pellet dissolved in 100µL of autoclaved prechilled MQ.
3. 100µL of freshly prepared lysis buffer was added and gently tapped.
   - For 1mL of lysis buffer:
     - 50µL of 20% SDS solution
     - 20µL of 0.5M EDTA
     - 10µL of 10N NaOH 920µL of water.
4. The samples boiled at 100°C for 2 minutes (until the solution becomes clear).
5. 50µL of 1M MgCl2 added. Tapped and kept on ice for 2 minutes.
6. Centrifuge at 10,000rpm for 2 min, RT.
7. 50µL of 3M potassium acetate buffer added.
   - For 100mL of potassium acetate(Stored at 4°C)
     - 60mL of potassium acetate
     - 11.5mL glacial acetic acid
     - 28.5mL H2O
8. Tapped immediately and centrifuged at 10,000rpm for 2 min, RT.
9. The supernatant transferred into another MCT containing 600µL of Isopropanol.
10. Kept on ice for 5 min.
11. Centrifuged at 13,000rpm for 2min, RT.
12. 70% ethanol wash and the pellet dried completely.
13. Pellet dissolved in 50µL of autoclaved MQ.

14. Stored at -20°C.

2.10 mRNA in situ Hybridization

Day1

1. The slides were taken out from -20°C and dried by keeping them at 37°C for 1 hour.
2. Hydrated the slides in the following sequence for 1 min each :
   - 100% EtOH
   - 95% EtOH
   - 70% EtOH
   - 50% EtOH
   - 2XSSC
3. Incubated slides in Proteinase K solution for 5 min at 37°C
   - Proteinase K Buffer:
     - 5mL of 1M Tris-HCl (pH = 8)
     - 5mL 0.5M EDTA
     - 40mL of DEPC H2O.

i. Pre-warmed Proteinase K buffer to 37°C.

ii. Added 160µL of 10mg/mL proteinase K.

4. Rinsed slides briefly in room temp DEPC water (2-3mins).
5. Rinsed slides in 0.1M TEA pH 8.0 for 3 min.
   - 0.1M TEA:
     - 0.93g of Triethanolamine (TEA)
     - 50mL of DEPC H2O

- 173µL of 10N NaOH (for adjusting pH = 8)

6. Rinsed in Acetic anhydride/TEA for 10 min.

7. Added 130µL of acetic anhydride to 50mL of 0.1M TEA

8. Dehydrated the slides in 2XSSC and EtOH series for 1 min each:

- 2X SSC
- 50% EtOH
- 70% EtOH
- 95% EtOH
- 100% EtOH

9. Air dried the slides for at least 1 hour at RT.

10. Pre-warmed the hybridization solution at 56°C (300µL per slide).

- Hybridization Solution (50mL):
  - 3.6mL TEN solution
  - 25mL 100% Formamide
  - 10mL 50% Dextran Sulphate
  - 5mL 10% RMB blocker
  - 6.4mL of DEPC H2O.
- Probe preparation:
  - Add probe to hybridization solution and mixed (as per concentration of the probe).
  - Boil probe and hybridization solution mix at 100°C for 10 min.
  - Keep immediately on ice for 2-3mins.

11. Added 300µL of Hyb/probe solution to each slide and coverslip with siliconized hybrid slips.

12. Place slides in humid chamber dampened with 50% Formamide/5X SSC and incubated at 56°C overnight.

- 20X SSC Stock (500mL):
  - 87.6g of NaCl in 350mL of DEPC H2O
  - 44.12g of sodium citrate
  - Rest DEPC water to bring the final volume to 500mL.
- TEN Solution (3.6mL):
  - the 500µL of 1M Tris-HCl (pH-7.5)
  - 3mL of 5M NaCl
  - 1mL 0.5M EDTA.
- 10% RMB Blocker (50ml):
  - 10% RMB blocker added to Maleic acid (100mM maleic acid + 250mM NaCl), heated and pH set to 7.5 to dissolve.

Day 2

1. Preheat 50% formamide/2X SSC solution to 65°C.
2. Preheat two 50mL RNase buffer washes, one to 37°C and the other to 65°C.

- RNase buffer (50mL):
  - 5mL of 5M NaCl
  - 500µL of Tris-HCl (pH 7.5)
  - 100µL of 0.5M EDTA
  - Rest H2O.

3. Preheat two 2X SSC washes to 37°C.
4. Soak slides with coverslips in 2X SSC for 30 min at RT on the shaker table.

1. If the coverslips do not come off, gently teased them apart from the slide with forceps.
2. Rinse the slides in 50% Formamide/2X SSC solution for 30 min at 65°C.
3. Gently agitate for the first 5 min.
4. Rinse slides in 2X SSC for 10 min at 37°C (twice).
5. Add 100µL of RNase (10mg/mL) to the 37°C RNase buffer, Incubate slides for 30 min.
6. Wash slides in 65°C RNase buffer for 30 min.
7. Wash slides for 2-3 hours in 1X Maleate buffer/0.05% Triton X-100/1% RMB blocker solution at RT
8. Wash slides in 1X Maleate buffer for 5 min (twice).
9. Incubate slides with 500µL antibody (Anti-DIG) diluted in 1X Maleate/0.05% Triton X-100/1% RMB blocker solution (1:2500 dilution) 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.

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

3. Added 500µL of NBT/BCIP dissolved in Genius buffer (1:50 dilution), incubated at room temperature in the dark for color reaction.

4. Colour detection: In a bright field microscope.

- 5X Maleate Buffer (1L):
  - 58g of Maleic acid in 850 mL of H2O (pH to 7.5 using NaOH, 43.8g of NaCl)
  - Rest water to bring volume up to 1L.

Chapter 3. Experiments and Results

3.1 *zebs* and *cadherins* temporally regulated during retina regeneration.

For checking the temporal expression of *zebs* and *cadherins*, we injured the retina of the fish and harvested at different time points (6hpi, 15hpi, 2dpi, 4dpi, 7dpi, 9dpi, 12dpi) and uninjured control. We performed RT-PCR and qPCR and found that **expression of *zebs* and *cadherins* is increasing during the early dedifferentiation and late redifferentiation phases of Zebrafish retina regeneration.**

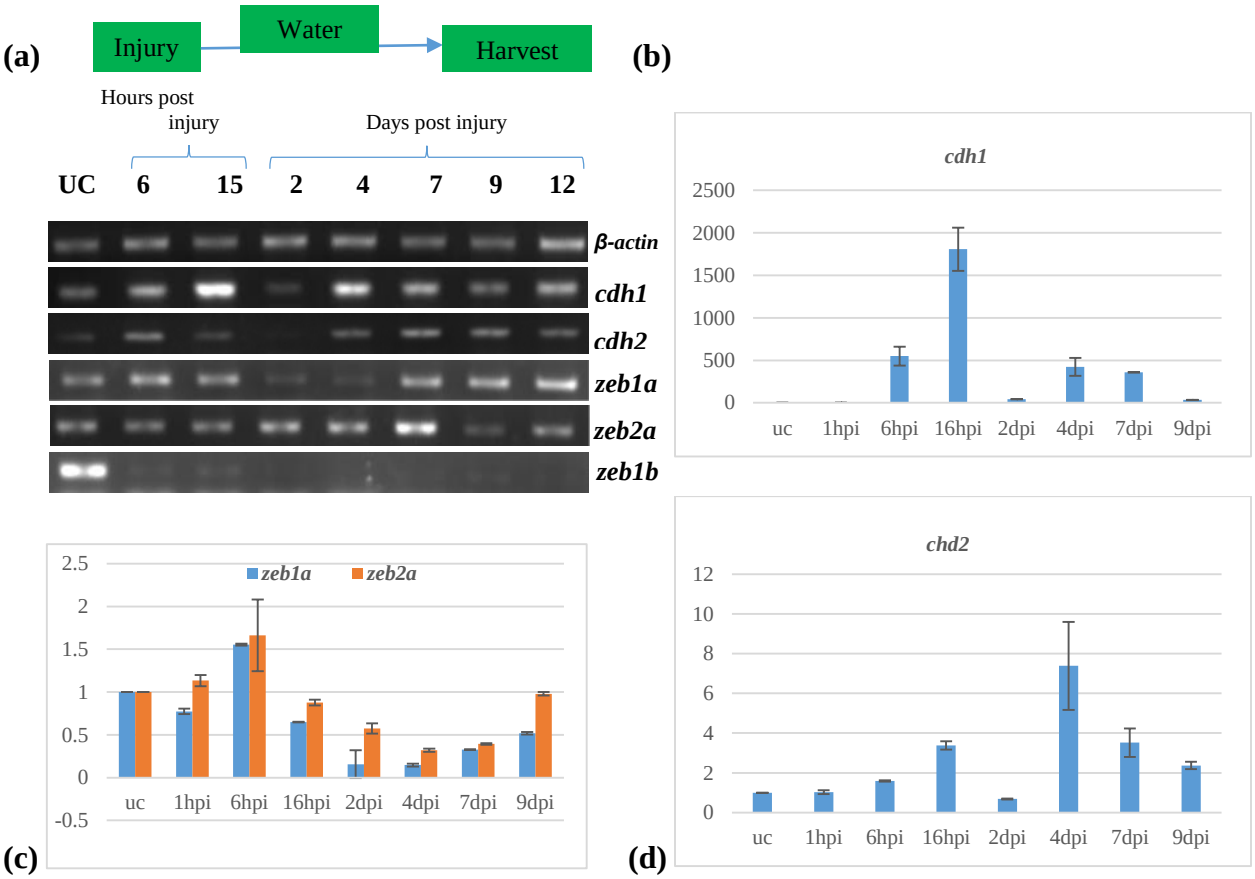


Figure 3.1: Temporal expression of *zebs* and *cadherins* during retina regeneration at different time points **(a)**RT-PCR showing the increasing expression of zebs and cadherins in early dedifferentiation and late differentiation phase. **(b)** qPCR data of *cdh1*gene in different injury time points reveals dual peaks of their expression at 16 hpi and 4dpi. **(c)** qPCR data of *zeb1a* and *zeb2a* gene in different injury time points

reveals dual peaks of their expression at 6 hpi and 9dpi. **(d)** qPCR data of *cdh1*gene in different injury time points reveals dual peaks of their expression at 16 hpi and 4dpi.

3.2 YAP nuclear translocalization inhibition during early dedifferentiation phase reduces proliferation.

Verteporfin is a photosensitizing drug(is a benzoporphyrin derivative monoacid ring A). Verteporfin is an inhibitor of the interaction of YAP with TEAD, which, in turn, blocks transcriptional activation of targets downstream of YAP. Yes-associated protein (YAP) is a central mediator of the Hippo pathway. Overexpression of YAP stimulates cell proliferation whereas knocking down YAP or treating cells with Verteporfin inhibited cell proliferation, even in the presence of growth factors. YAP overexpressed in many types of human cancers with its expression level associated with patient outcomes. Thus, inhibiting YAP function might give a unique therapeutic approach.

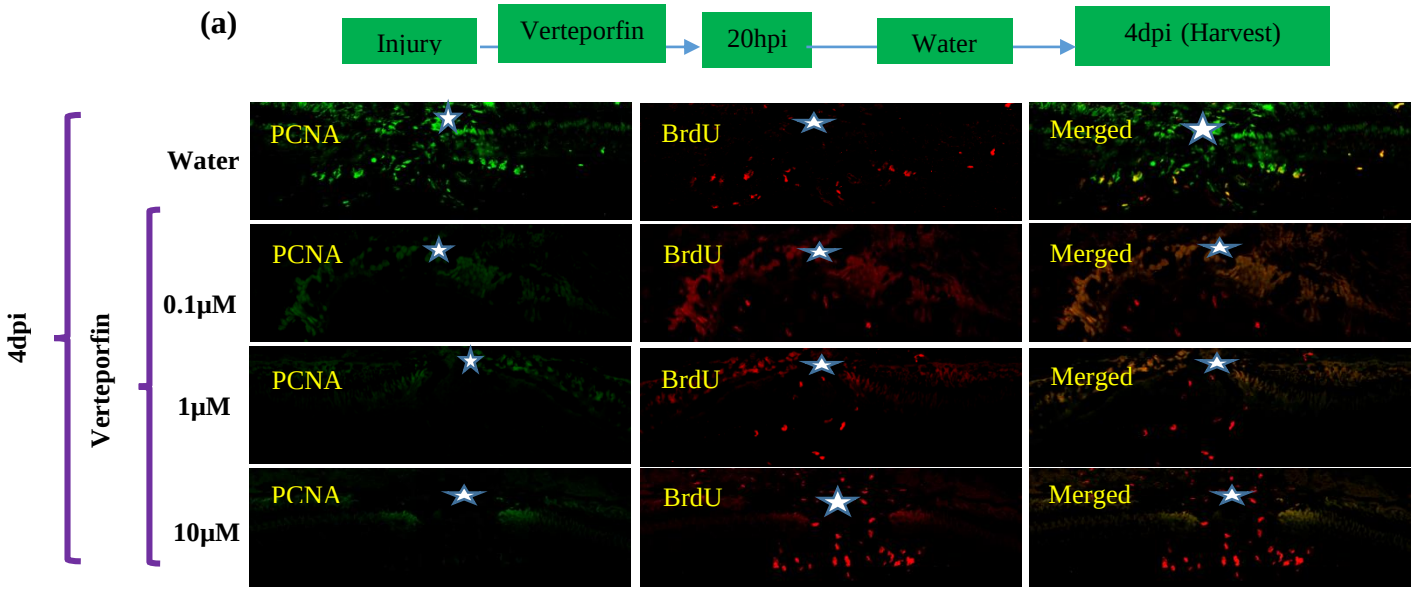
So for this experiment, we inhibited the YAP nuclear localization using pharmacological inhibitor verteporfin. Fish after injury dipped in different concentration of verteporfin which was 0.1µM, 1 µM, 10µM and water (control). Fish in drug were transferred back to water after 20 hpi, and then eyes were harvested (at 4 dpi), fixed and sectioned. The sections were then immuno-stained against PCNA and BrdU to mark the proliferating cells. Finally, the number of PCNA and BrdU positive cells were counted and analyzed. Significant reduction observed in the number of proliferating MG cells as compared to control at injury spot on inhibition of YAP nuclear translocalization, but this proliferation increased in a dose-dependent manner. This results indicated **YAP nuclear translocalization inhibition during early dedifferentiation phase reduces proliferation**, but we found dose-dependent increases in proliferation, may be due to transferring the fish from drug to water after 20hpi causes the withdrawn effect of the drug (verteporfin).

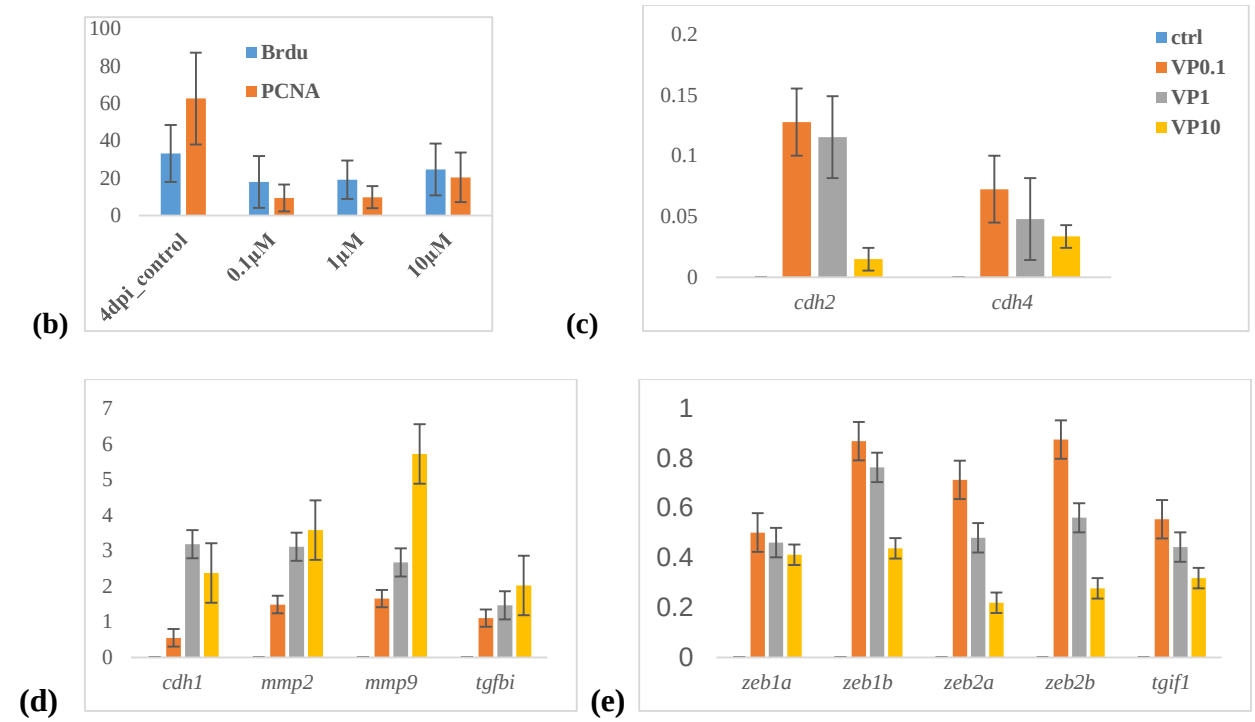
Further to validate the effect of YAP nuclear translocalization inhibition during early dedifferentiation phase, we checked the temporal expression pattern of *zebs* and *cadherins* and other regeneration associated genes. For performing this fish after injury, were dipped in different concentration of verteporfin which 0.1µM, 1 µM, 10µM, and water. Fish in drug were transferred

back to water after 20 hpi, and then eyes were harvested (at 4 dpi). The retina dissected and mRNA isolated for performing RT-PCR and qPCR.

We analyzed the data from RT-PCR and qPCR and found that the expression of genes like cdh1, *mmp2*, *mmp9*, *tgfbi* is increasing and *cdh2*, *cdh4*, *zebs*, and *tgif1* is decreasing. **Increased expression of cdh1 is due to the downregulation of *zebs*, and *snails***. Disrupting the YAP/TEZ/TEAD complex prevent YAP to enter into the nucleus which in turn down-regulate the *zebs*, and *snails*.

Further, to understand this reduction in proliferation upon inhibition of YAP nuclear translocalization mRNA level of the regeneration-associated gene like *ascl1a* and *mmp9* were analyzed by mRNA in situ hybridization. Here, we injured the fish and dipped them in different concentration of verteporfin, 0.1µM, 1 µM, 10µM and water (control) for 20 hpi and then fish which were in verteporfin, transferred back to water and harvested at 4dpi. The eyes were then fixed and sectioned. *ascl1a* and *mmp9* mRNA in-situ hybridization showed the increased proliferation upon inhibition of YAP nuclear translocalization. These findings indicate that **ascl1a and mmp9 have a vital role to play during zebrafish retina regeneration. The decreases in cell proliferation and mRNA level is due to the YAP nuclear translocalization inhibition. Verteporfin drug phosphorylate the YAP protein. Phosphorylated YAP does not bind to the TAZ/TEAD complex, and the complex helps in transcription.**





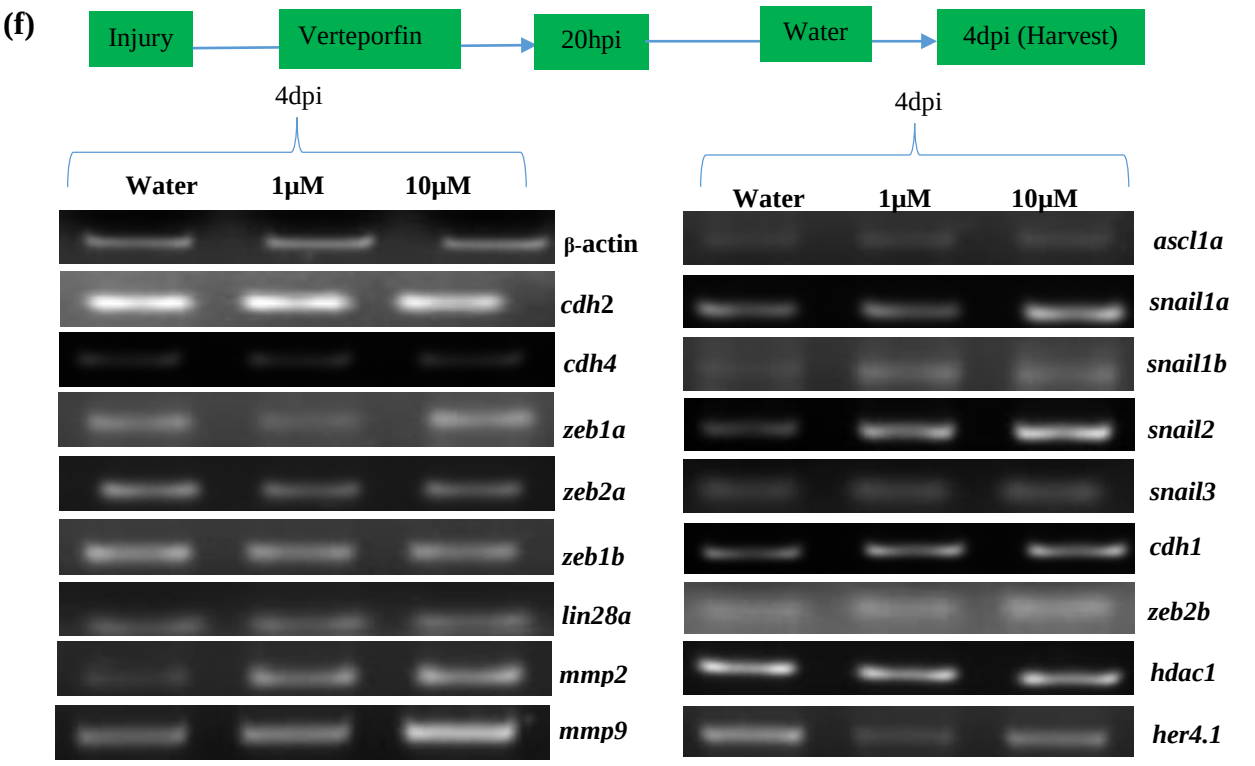


Figure: 3.2.1 YAP nuclear translocalization inhibition during early dedifferentiation phase reduces proliferation. **(a)** Immunostaining against BrdU and PCNA showing proliferative cell in the retina. The

proliferation is decreasing, compared to control but increasing in a dose-dependent manner in o.1µM, 1 µM, 10 µM drug treatment. **(b)** Graphical representation of cell count data of PCNA and BrdU positive cells (Method-manual cell counting and plotting) **(c, e)** qPCR data of *cdh2*, *cdh4* and *zeb1a*, *zeb1b*, *zeb2a*, *zeb2b*, and *tgif1* gene shows a decrease in their expression upon YAP nuclear translocalization inhibition. **(d)** qPCR data of *cdh1*, *mmp2*, *mmp9* and *tgfbi* shows an increase in their expression upon YAP nuclear translocalization inhibition. **(f)** RT-PCR of *zebs*, *cadherins* and other regeneration associated genes shows that the expression of *zebs*, *cdh2*, and *cdh4* is decreasing and the expression of *cdh1*, *mmp2*, *mmp9* is increasing. (qPCR data of these genes also shows the same results)

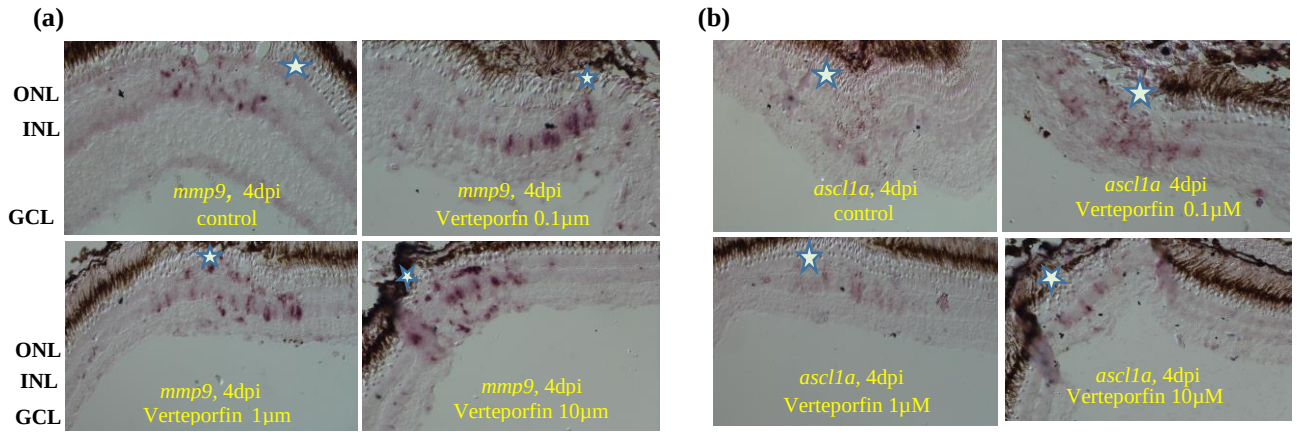


Figure: 3.2.2 mRNA *in situ* hybridization of regeneration-associated genes. **(a)** mRNA in situ hybridization shows the increased level of *mmp9* upon inhibition of YAP nuclear translocalization. **(b)** mRNA in situ hybridization shows the increased level of *ascl1a* upon inhibition of YAP nuclear translocalization.

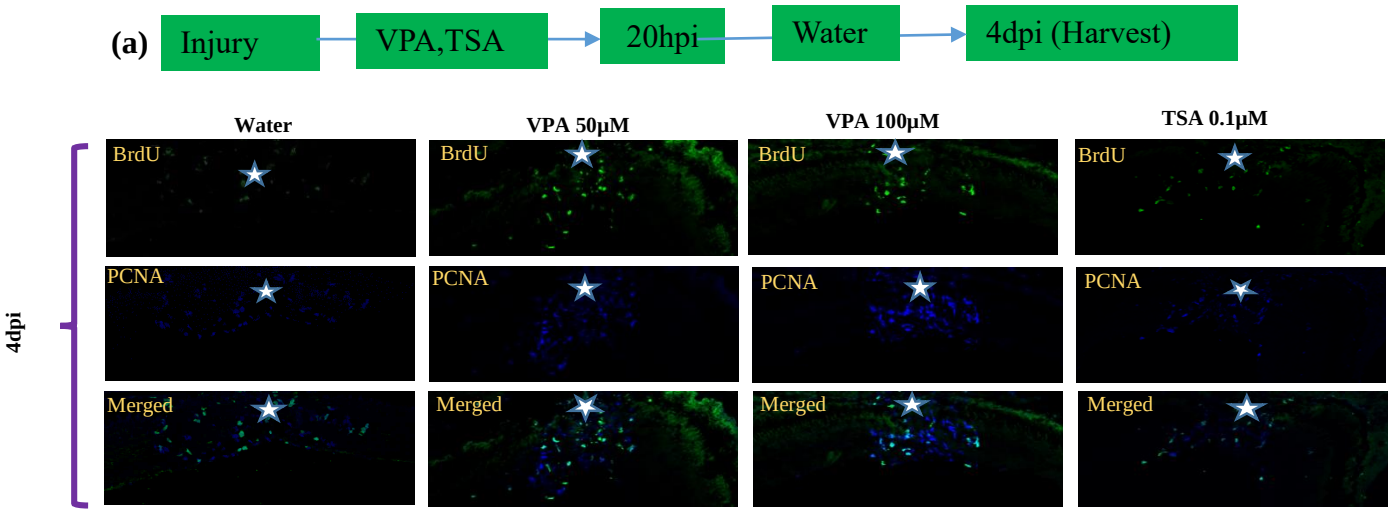
3.3 Inhibiting Hdacs by Trichostatin A (TSA) and Valproic acid (VPA) causes reduction in MG proliferation at the site of injury.

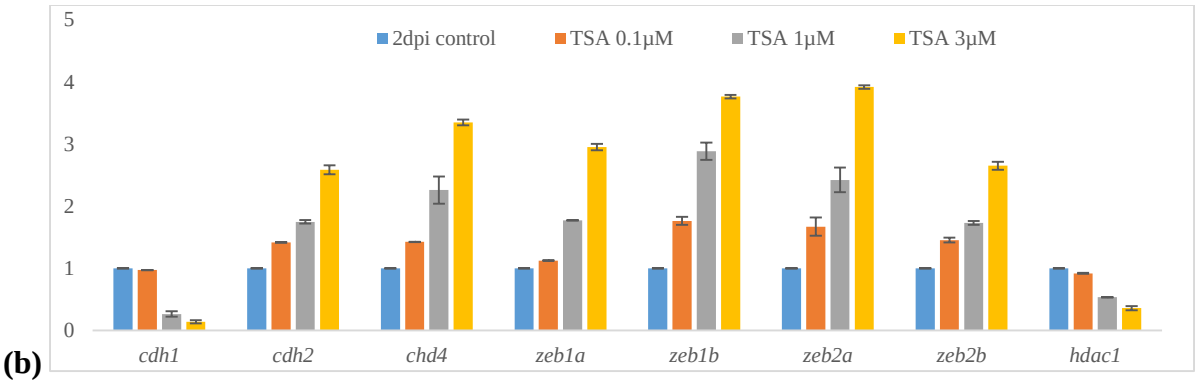
Trichostatin A is usually known as TSA and valproic acid as VPA used as a pharmacological inhibitor of Hdac activity. For this experiment, fish after injury dipped in two concentrations of VPA solutions, 50µM and 100µM and one concentration of TSA solution, 0.1µM and water(control) for 20hpi, after that fish transferred back to water. Eyes were harvested at 4 dpi, fixed and sectioned. The sections were then immuno-stained against PCNA and BrdU to mark the proliferating cells. Finally, the number of PCNA and BrdU positive cells were counted and analyzed. A notable reduction in the number of proliferating MG cells observed at injury spot on

inhibition of Hdacs with TSA and VPA as compare to control. This reduction in number was also concentration dependent. **This indicates that Hdacs helps to maintain the cells in normal state during retina regeneration.**

Further to validate the inhibition effect of Hdac on *zebs* and *cadherins* using TSA, we performed qPCR and analyzed the data. For that, fish after injury dipped in three concentrations of TSA solutions 0.1µM, 1µM, 3µM and water (control) for 20hpi. Then fish were transferred back to water. Eyes harvested at 4dpi, the retina dissected and mRNA isolated for performing RT-PCR and qPCR. Upon inhibition of Hdacs during early dedifferentiation phase using TSA, an associated decreases in E-cadherins (*cdh1*) mRNAs while increases in *cdh2*, *cdh4,* and *zebs* mRNA seen.

Further, we also checked for the protein level of Hdac1 and Ascl1a upon inhibition of Hdacs by western blot. For this experiment, fish after injury dipped in three concentrations of VPA solutions, 10µM, 50µM and 100µM and water (control) and three concentrations of TSA solution, 0.1µM, 1µM, 3µM and water (control) for 20hpi. Then fish were transferred back to water. Eyes harvested at 4dpi, the retina dissected and protein samples prepared using in a laemmli buffer. We perform the western blot, analyzed the data, and we found that **the protein level of Hdac1 and Ascl1a is decreasing in a dose-dependent manner.**





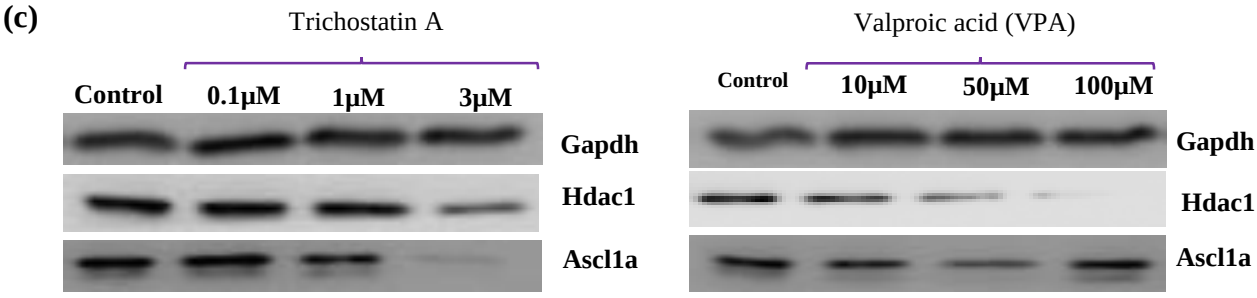


Figure: 3.3 Inhibiting Hdacs by TSA and VPA causes a reduction in MG proliferation at the site of injury. (a) Immunostaining against PCNA and BrdU showing a decrease in a number of proliferating cells upon inhibiting Hdacs by TSA and VPA. (b) qPCR data shows decreased expression of cdh1 and increased expression of zebs upon inhibition of Hdacs by TSA. (c) Western blot shows that the protein level of Ascl1a and Hdac1 decreases in Hdacs inhibition.

3.4 *klf4a* might be regulating EMT retina regeneration in zebrafish.

Klf4 is an anti-tumorigenesis factor. KLF4 in mice and human share high amino acid similarities with zebrafish klf4a. Klf4 acts as a tumor suppressor in gastrointestinal cancer in mice. For this experiment, we used the klf4a morpholino. Morpholino helps in achieving gene knockdown by preventing cells from making a specific protein. Knocking down the expression of the gene helps in learning about the function of a particular gene or protein. So we injected the klf4a morpholino in fish eyes in different concentrations which are 0.25mM,

0.5mM, 1mM and control (water). We harvested the eyes at 2dpi, the retina dissected and mRNA isolated. We performed RT-PCR and analyzed the data. We found that the Knockdown of *klf4a* results in a decrease in mRNA levels of E-cadherin, *zeb1a*, *zeb1b* while an increase in that of N-cadherin (*cdh2*), *zeb2b* and *miRNA-200* family. Klf4 negatively regulates EMT which down-regulate E-cadherins and up-regulate N-cadherins[15].

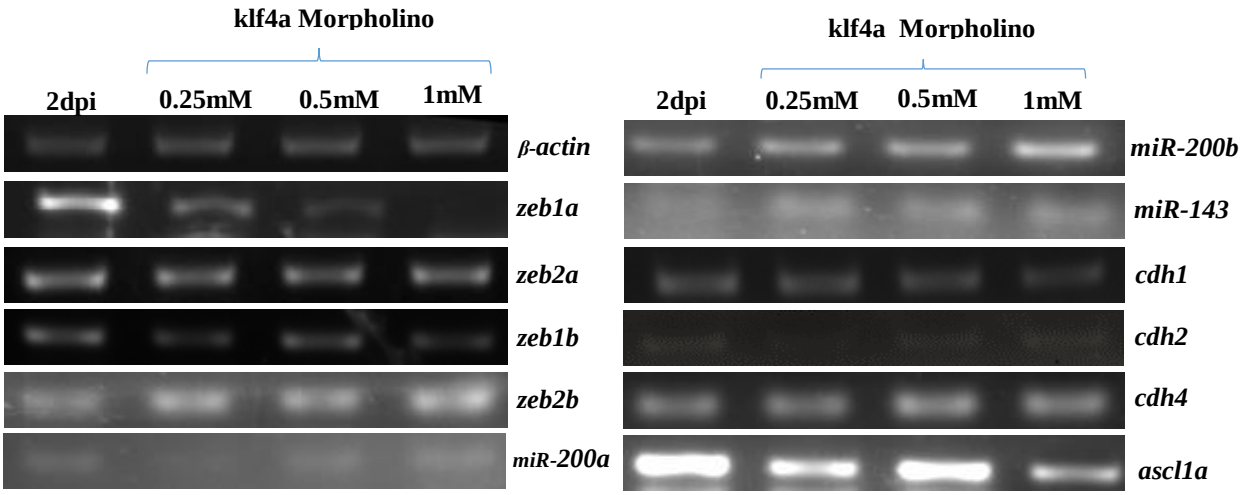


Figure: 3.4 Knockdown of *klf4a* shows reduced expression of E-cadherin. RT-PCR shows the decreased expression of *cdh1* gene and increased expression of *cdh4* gene**.**

Chapter 4. Discussion and conclusion

Temporal expression of *zebs* and *cadherins* in different time points of post-retinal injury suggests their role in retina regeneration in zebrafish. *zebs* and *cadherins* are crucial factors in proliferation and redifferentiation phases of retina regeneration as we found that the temporal expression of *zebs* and *cadherins* reveals dual peak of their expression during early dedifferentiation and late differentiation phases of zebrafish retina regeneration

It is shown by previous studies that *zebs* are repressor of E-cadherins(*cdh1*) which are essential in maintenance of normal cell architecture and disrupting the YAP/TAZ/TEAD complex (which helps in EMT) by inhibiting the YAP to enter into the nucleus we found that YAP nuclear translocalization inhibition during early dedifferentiation phase reduces the cell proliferation. At mRNA level, inhibition of YAP nuclear translocalization increases the levels of regeneration-associated genes like *mmp9* and *ascl1a*, and *cadherins* while reduces the levels of *zebs* mRNAs.

According to literature, HDACs are key enzymes regulating critical cell processes such as cell-cycle progression and apoptosis. Treatment with HDAC inhibitors leads to the inhibition of tumor cell growth and an increase in both the acetylation of the promoter and gene expression[13]. By inhibiting Hdacs with its pharmacological inhibition TSA and VPA we found that inhibition of HDACs during early dedifferentiation phase reduces cell proliferation with an associated decrease in E-cadherin (*chd1*) while an increase in *zebs, chd2,* and *cdh4* mRNAs. This indicates that Hdacs helps to maintain the cells in the normal state because by overexpression and aberrant recruitment of HDACs to their promoter region transcriptional repression of tumor-suppressor genes could be a common phenomenon in tumor growth[13]. The protein level of Ascl1a and Hdac1 decreases in HDACs inhibition.

Further, we also studied the role of klf4a in EMT, since klf4 have shown an essential part in organogenesis and plays an important role in differentiation[14]. Klf4 regulates EMT by down-regulating E-cadherin whereas up-regulating zebs and snails[15]. In this study knockdown of *klf4a* shows a decrease in mRNA levels of E-cadherin, *zeb1a, zeb1b* while an increase in that of N-cadherin (*cdh2*), *zeb2b* and *miRNA-200* family.

Chapter 5. Future Outlook

This study gives insights into a significant pathway involved in retina regeneration. It requires many more experiments for its validation and fine-tuning. YAP nuclear translocalization inhibition shows that crosstalk of *zebs* with *cadherins* is important for retina regeneration in zebrafish and EMT plays a crucial role in the regeneration. This need to be verified by checking the spatial expression pattern of *zebs* and *cadherins* and the temporal and spatial expression pattern of *zebs* and *cadherins* during zebrafish embryonic development. Also, we need to validate the interaction of YAP with *cadherins* by chip assay. To understand the role of klf4a we need to check the temporal and spatial expression pattern of *zebs* and *cadherins* upon knockdown of *klfa*.

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