# Exploring the role of TGF-β signaling and its crosstalk with other pathways during retina regeneration in *Danio rerio.*

Navnoor Kaur Saran

MS14086

Department of Biological Sciences

*A dissertation submitted for the partial fulfilment of BS-MS dual degree in Science.*


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# Certificate of Examination

This is to certify that the dissertation titled "**Exploring the role of TGF- signaling and its crosstalk with other pathways during retina regeneration in *Danio rerio***" submitted by **Ms. Navnoor Kaur Saran** (Reg. No. MS14086) 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.

Prof. Anand Kumar Bacchawat  Dr. Sharvan Sehrawat  Dr. Rajesh Ramachandran
(Supervisor)

Dated: April 25, 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 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.

Navnoor Kaur Saran

(Candidate)

Dated: April 25, 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)

# Acknowledgement

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# Notations

- BrdU: Bromodeoxyuridine
- PCNA: Proliferating Cell Nuclear Antigen
- MGPC: Müller Glia-derived Progenitor cells
- MG: Müller Glia
- ONL: Outer Nuclear Layer
- INL: Inner Nuclear Layer
- GCL: Ganglion Cell Layer
- RT-PCR: Reverse Transcription PCR
- dpi: days post injury
- hpi: hours post injury
- hpf: hours post fertilization
- UC: Uninjured Control
- MO: Morpholino-modified antisense oligonucleotides
- DAPT: N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester
- ALK: Anaplastic lymphoma kinase
- TGFBR: Transforming growth factor-β receptor
- *tgfbi:* transforming growth factor-β induced
- *tgif1:* transforming growth factor-β induced factor homeobox 1

# Abstract

Any injury to retina leads to irreparable damage in mammals. Unlike higher vertebrates, zebrafish possess remarkable regenerative response in retina, driven by Müller Glia (MG), the only glial cell type in retina. Upon retinal injury, the Müller Glia cells undergo de-differentiation and cell division to give rise to a population of MGPCs (Müller Glia derived Progenitor cells), which migrate to damaged layers of retina to differentiate and restore cell population. But the exact mechanism of molecular interplay that orchestrates de-differentiation, proliferation and re-differentiation still remains elusive. Although many signaling cascades and regulatory pathways have been identified to play roles at different stages of retina regeneration, TGF-β signaling pathway remains under-explored. In this study, we analysed the role of a developmentally important signaling pathway *i.e.* TGF-β signaling in the retina regeneration. Also, we explored the communication that TGF-β pathway has with other signaling pathways. We found that TGF-β signaling and Notch signaling interplay is important for retina regeneration. Another signaling that we explored is Shh(Sonic Hedgehog), which is one of the major networks that regulates the key events during developmental processes. We tried to understand how TGF-β signaling is communicating with Notch and Shh signaling to help restore the structural and functional integrity of the retina.

# Chapter 1 – Introduction

**"***If there were no regeneration, there could be no life. If everything regenerated, there would be no death."*

-Richard J. Ross (1969)

Regeneration is one of the most fascinating and complex phenomena in biology. Regeneration propensity lies in all the species, virtually. But, the degree of regeneration varies. While organisms like Echinoderms can regenerate most of their body, the mammals have limited regenerative capacity. Tissue regeneration is a kind of reparative regeneration, a way to restore the function of damaged tissues or for renewal of lost tissues.[1] Mammals cannot regenerate Central Nervous System (CNS).[2] The propensity of the regeneration of the central nervous systems in the fish had been discovered back in the 1950s. This broke the prevalent belief that the central nervous system is not plastic. Teleost fish, such as Zebrafish, have an excellent potential to regenerate most of its body parts which include retina, heart, brain, fin, and liver. Zebrafish can regenerate its nervous tissue and restore the function of the damaged tissue.[3]

One of the most important and accessible part of the CNS is retina. The regenerative property of the retina is attributed to retinal glial cell type - the Müller glia (MG). Upon retinal injury, these Müller glia cells undergo re-programming and attain stem cell-like characteristics which enable them to generate progenitors that replace lost neurons to restore functional and structural integrity of the retina.[4] Since zebrafish retina shares similarities with mammalian retina, understanding the mechanisms underlying retinal repair in zebrafish will provide insights into the regenerative potential of the mammalian retina. Therefore, zebrafish retina may hold the key to regenerating human retina.

## 1.1.A. Microanatomy of Retina

The retina is composed of six neural cell types and one glial cell type in vertebrates. These are organized as three distinct nuclear layers: the outer nuclear layer (ONL), the inner nuclear layer (INL) and the ganglion cell layer (GCL). ONL comprises of the nuclei of photoreceptor cells (rods and cones) which perceive light. INL consists of interneurons (bipolar cells, amacrine cells, and horizontal cells) which transmit information between ONL and GCL. GCL harbours the nuclei of ganglion cells which transfer the incoming information, through the optic nerve, to the brain.[4-6] (Figure1(a)).

Müller glia are the only glial cell type in the retina. Müller glial cell bodies reside in the INL, but they span all retinal layers. They extend processes to outer and inner limiting membranes, also, extend processes laterally to make contact between neighboring neurons. This unique Müller glial architecture allows them to maintain retinal homeostasis and contribute to retinal structure and function[6]

## 1.1.B. Mechanism behind Regeneration

In zebrafish, Müller glia respond to retinal injury by initiating a gliotic response[4] which is characterized by hypertrophy[5] and activation of cytoskeletal genes, like glial fibrillary acidic protein (Gfap)[20]. In mammals, Müller glia rarely divides upon retinal injury, if they do, it results in glial scar formation and fibrosis.

Müller glia-driven retinal regeneration involves three steps:

a) Dedifferentiation: Müller glial cells undergo reprogramming and attain stem cell-like properties after injury;
b) Proliferation: Generation of a proliferating population of multipotent Müller glial cell-derived progenitors (MGPCs);
c) Re-differentiation: Progenitors migrate to different cell layers and differentiate into various neuronal cell types. (Figure1(b)).

Müller glia cells have a property to phagocytose the dead and damaged cells, which results in the release of various cytokines. These cytokines, further, initiate various signaling pathways leading to rapid changes in the gene expression pattern which causes re-programming of Müller glia to MGPCs by activation of multi-potent genes and change in DNA methylation pattern. In zebrafish, the proliferation of these MGPCs occurs from 2dpi to 6dpi, with peak proliferation at 4dpi. After 6dpi, differentiation of MGPCs to different retinal cell types take place , with the peak at 7dpi. (Figure1(c))

## 1.1.C. Signaling mechanisms involved in Müller glia reprogramming

Upon injury, a plethora of genes which encode for growth factors and cytokines are induced and can stimulate Müller glia and progenitor proliferation in an autocrine and paracrine fashion. These include heparin-binding EGF-like growth factor (Hb-egf)[7], tumour necrosis factor-α (Tnfα)[9], Insulin[11], Insulin-like growth factor-1 (IGF-1)[10], Interleukins (IL-11)[7] and Leptin[8] Of various secreted factors, TNFa is known to be expressed by dying cells, representing a signal to communicate cell death to MG[9]. Leptin and IL-6 family cytokines are found to be induced in the injured retina and regulate MG reprogramming and generation of MGPCs via a Jak/Stat signaling pathway. This pathway plays a key role in MG reprogramming as it stimulates expression of ascl1a, lin-28a, hb-egfa and sox3. The cytokine-activated Jak/Stat signaling pathway serves as an early responder to injury as Stat proteins rapidly transduce information to the nucleus via membrane receptors[8].Signaling pathways like Notch[7,12], Mapk-Erk[7], Jak-Stat[8] and Wnt (GSK3β/β- catenin)[13] are known to regulate retina regeneration.

It has been confirmed that β- catenin is activated in MGPCs. Retinal injury in zebrafish results in wnt expression and β- catenin activation in MGPCs. β- catenin, a multifunctional protein, is known to link changes in Wnt and cadherin signaling on the cell surface to gene expression[14]. Inhibition of Wnt signaling in injured retina suppresses progenitor formation. However, pharmacological activation (using lithium or a Gsk3β inhibitor) of the β- catenin signaling in an uninjured retina promotes reprogramming of Müller glial cells and formation of progenitors[15,16].

Notch signaling pathway is known to be regulated during retina regeneration as it defines the zone of injury responsive MG. In the uninjured retina, if Notch is inhibited, it does not stimulate MG dedifferentiation and proliferation which suggests that Notch acts on an injury-induced signaling cascade. Her4.1, the downstream target of Notch signaling, inhibits expression of hb-egf which is necessary and sufficient for MG dedifferentiation and proliferation as it induces various genes, like ascl1a, myc, delta, and pax6b[7].

A range of response genes like ascl1a (achaete-scute homologue 1a)[16,17], insm1a (insulinoma-associated 1a)[18], and mycb (myelocytomatosis oncogene b)[15,16] are known to play a key role in different steps of regeneration. Ascl1a, a proneural basic-helix-loop-helix transcription factor, is induced on retinal injury and is required for MG dedifferentiation. It is necessary for induction of a pluripotency factor lin28a. Lin28a is an RNA-binding protein that is known to act as a post-transcriptional regulator of genes which are involved in developmental timing and self-renewal in embryonic stem cells. It functions via direct interaction with target mRNAs and by disrupting the maturation of specific miRNAs which are involved in embryonic development. Lin28a is known to inhibit let-7 miRNA which is required to keep the cell in the differentiated state. let-7 regulates expression of many regeneration-associated genes, like ascl1a, lin-28,pax6a, pax6b, mps1 and hspd1[17].Ascl1a is necessary for injury-dependent insm1a (a transcriptional repressor) expression, which in turn regulates the Wnt signaling pathway by inhibiting dkk (dickkopf, Wnt inhibitor) expression and activation of expression of Wnt genes, which results in Müller glia reprogramming and progenitor formation[16,18](Figure 1.1.(d)).

The role of epigenetic regulation is still under-explored. Some studies suggest that DNA demethylation contributes to MG reprogramming, whereas DNA methylation might be necessary for MGPCs migration and differentiation[19]. However, epigenetic regulation through acetylation or deacetylation is yet to be investigated. Although some pathways are well established in the zebrafish retina regeneration process, very little is known about pathways important for development in regards to regeneration, like TGF-β signaling pathway.

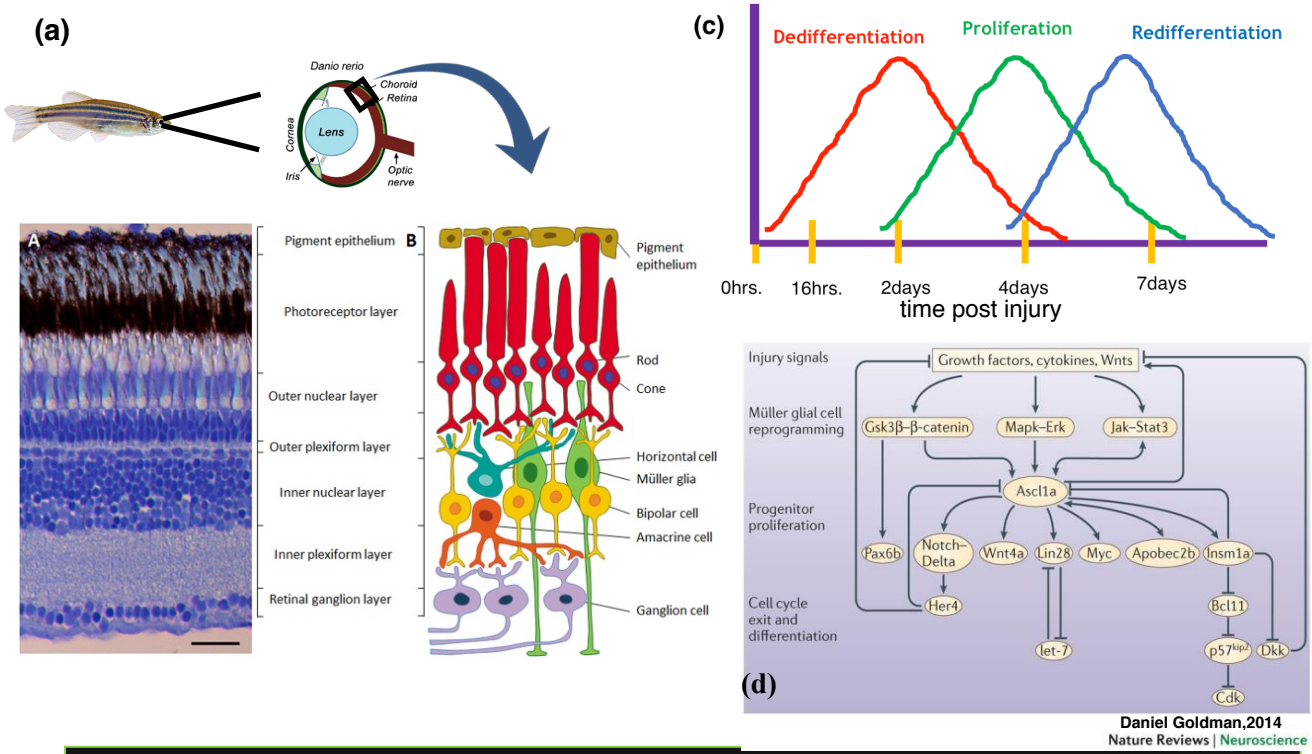


**Figure1.1. Retina regeneration at a glance.** (**a**) Micro-anatomy of Retina (Gramage *et al,* 2014). - OPL- Outer plexiform layer(synapses between cells of ONL and INL), IPL- Inner plexiform layer (synapses between cells of INL and ganglion cells), OLM- Outer Limiting Membrane, ILM- Inner Limiting Membrane, RPE- Retinal Pigment Layer (absorbs light), NFL-Nerve Fibre Layer (Axonal process of ganglion cells); (**b**) Regeneration Mechanism (Daniel Goldman) - 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 migration of injury-responsive MG to ONL for division and then back to INL; (**c**)Different stages in retina regeneration and (**d**) Signaling Cascades that regulate retina regeneration. Growth factors and cytokines are released as injury signals, initiating MG-reprogramming and proliferation. (Daniel Goldman, 2014)

## 1.2. Transforming Gowth factor-β (TGF-β) signaling pathway

TGF-β signaling plays essential roles in pattern formation during embryonic development, cell growth and proliferation, cellular differentiation, extracellular matrix production, angiogenesis, tissue repair and apoptosis throughout development and adulthood. The effects of TGF-β are different, even opposite, depending on the cell type and the conditions. The cellular context dictates the nature of the response. TGF-β can inhibit cell proliferation but also promote cell growth. Not only can it enhance stem cell pluripotency, it can also promote differentiation. [21,22]

Upon ligand binding, two type I receptor components and two type II components assemble into a complex. These both components are Serine/Threonine protein kinases. Type I gets phosphorylated by type II receptors.The signal is further propagated by type I receptors.[23] TGF-β has exclusive affinity for type I receptor TGFBR1( known as ALK5) and type II receptor TGFBR2.[24] The canonical pathway is the SMAD pathway which is activated directly by TGF-β cytokines. The transcriptional output is regulated by receptor-mediated activation of certain transcription factors i.e. SMADs. SMAD proteins help in context-dependent modulation of the signaling. SMAD proteins which undergo receptor-mediated phosphorylation are known as receptor-regulated SMAD proteins or R-SMAD proteins (Smad1, Smad2, Smad3, Smad5, Smad9). Following this, there is a formation of a heteromeric complex with the Common mediator Smad (Co-Smad) *i.e*. Smad4.[25,26] Even in absence of ligands, R-Smads shuttle between cytoplasm and nucleus. Upon their phosphorylation and formation of heteromer with Smad4, R-Smads are retained in nucleus as transcriptional regulators.[27,28]Every Smad complex recruits particular co-repressors or co-activators and targets particular set of genes. Depending on cell type, SMAD cofactors differ.[29]Also, SMADs can recruit histone acetyltransferases.[30]Apart from SMAD mediated signaling, there is activation of other pathways by TGF-β as well. These include various branches of MAP kinase pathways, Rho-like GTPase signaling pathways, and phosphatidylinositol-3-kinase/AKT pathways.[31]

TGF-β family ligands are initially synthesized as pre-pro-TGF-β, a monomer and have a latency associated peptide(LAP). Further, it undergoes proteolysis and dimerisation of monomers. Thus, pro-TGF-β homodimer is created. Upon further proteolysis, a small latent TGF-β complex (SLC)

is created in which two LAP chains are cleaved out but maintain connection due to non-covalent bonds. This helps in maintaining TGF-β in its inactive state. [32,33] This is further connect ed with latent TGF-β binding protein (LTBP) , to form the resultant protein known as large latent TGF-β complex (LLC).[34,35] After secretion from the cell, the LLC interacts with extracellular matrix components. Mainly, the release of TGF-β from LCC is dependent on numerous molecules, including proteases like plasmin,matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 9 (MMP9) etc.[36,37]

It has been reported that TGF-β is influenced by several other signaling pathways like RAS, WNT, Hedgehog, Notch, tumour necrosis factor (TNF) *etc*.[38] Sonic Hedgehog (Shh) signaling governs the precisely regulated developmental processes of multicellular organisms. It has a vital role in establishing the patterns of cellular differentiation and post-embryonic tissue regeneration and repair processes. The TGF-β and Sonic hedgehog (Shh) pathways both mediate cell proliferation. In a study, it was shown that exogenous TGF-β increased Shh expression, and inhibition of TGF-β signaling abrogated the cyclosporine-induced up-regulation of Shh expression.[39] Another signaling cascade, the Notch pathway plays a crucial rule in regulation of cell proliferation, cell fate, differentiation and apoptosis in all metazoans. It has already been shown that the two major signaling pathways function both independently and synergistically during development. The NICD(intracellular domain of Notch1) and Smad3 have been shown to interact directly, both in vitro and in cells. This indicates that Notch and TGF-β pathways communicate by direct protein-protein interactions.[40,41]

Pharmacological inhibiton of these signaling pathways is a great method to understand the roles of these individually as well as their crosstalk. SB-431542 is a specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7.[42]. DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester) is a γ-secretase inhibitor which fully blocks Notch signaling [43]. Cyclopamine appears to interfere with these signaling events by influencing Smo function, as it antagonizes Hh pathway activity in a Ptch-independent manner and exhibits attenuated potency toward an oncogenic, constitutively active form of Smo[44].

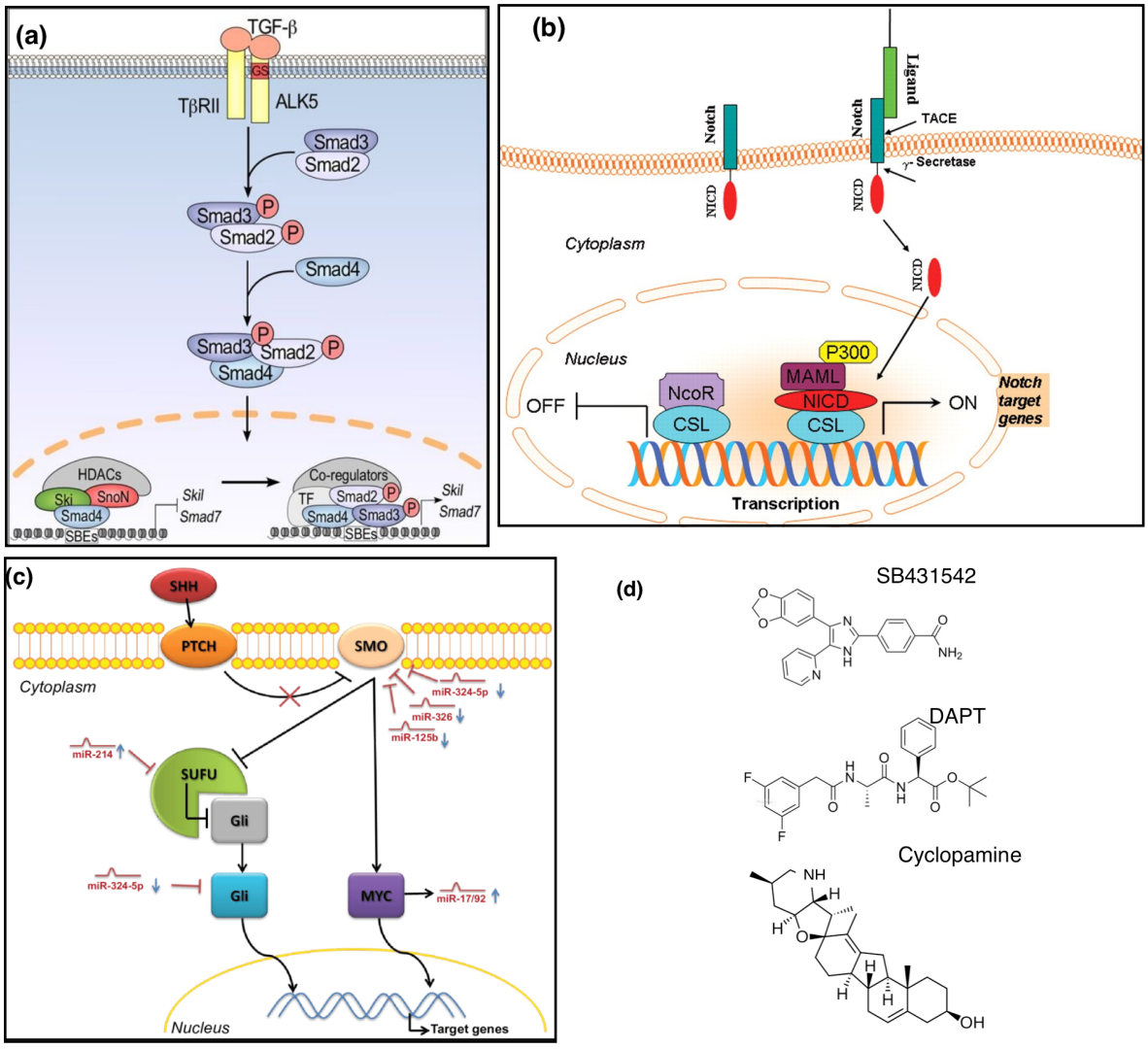


**Figure 1.2. Signaling pathways and their inhibitors.** (**a**) TGF-β signaling pathway (Angeles C. *et al*, Signal Transduction and Targeted Therapy, 2018) - TGF-β induces the phosphorylation of Smad2/3 proteins to form the R-Smad/Smad4 complex, which associates with specific transcription factors and cofactors to modulate the expression of its target genes. (**b**) Notch pathway (Brendan D'souza *et al*, Current Topics in Developmental Biology, 2010) - Notch signaling is initiated when the ligands bind to the Notch receptor. It results in proteolytic cleavage by γ-secretase, resulting in release of Notch Intra-cellular Domain(NICD), which is transported to nucleus and interacts with DNA binding proteins like CSL and activates expression of target genes. (**c**) Shh signaling (Visani *et al*, International Journal of Brain Disorder Treatment, 2015) - In the absence of Shh ligand, PTCH1 inhibits SMO resulting in GLI1 sequestration in the cytoplasm by SUFU. In the presence of Shh, PTCH1 suppression of SMO is stopped resulting in the nuclear accumulation of GLI1 and activation of target genes. (**d**) Inhibitors - SB-431542 is a specific inhibitor of kinase activity of TGF-β receptors .DAPT is a γ-secretase inhibitor which fully blocks Notch signaling. Cyclopamine appears to interfere with signaling events by influencing Smo function.

# Chapter 2. Materials and Methods

## 2.1. Zebrafish maintenance

Zebrafish are maintained in automated water circulation system that continuously filters and aerates the system water to maintain the requisite water quality. The circulating system also helps to filter excess food and fish excreta. The room temperature is maintained between 26-28.5 °C, with the lighting conditions are set at 14 hour light with 10 hour dark period. The pH of the system water is maintained between 6.8 and 7.5.

## 2.2. Retinal injury and harvesting

- The zebrafish is anaesthetized using 0.02% Tricaine methanesulfonate.
- Then, it is kept on a moist sponge bed under dissecting scope. Injury is given to retina using 30 gauge needle and stainless steel forceps.
- Dissection of the eyes is done using steel forceps and needle. If the retina is to be harvested, dissections are carried out either in 1X PBS (Phosphate Buffered Saline),,otherwise if the tissue has to be fixed, eyes are kept in 4% paraformaldehyde (PFA).

## 2.3. Microscopy

- Bright field microscope (Zeiss) was used for harvesting whole eye or retina.
- Confocal microscope (Nikon) was used for imaging.

## 2.4. RNA Isolation

- Dissected retinae are collected in 200µL of Trizol reagent in MCT.
- Homogenized the tissue properly using a pipette.

- 0.2 volume (40µL) of chloroform was added.
- The solution was shaken gently for 15-20 seconds.
- The MCT was kept still for 5 minutes at room temperature (RT).
- Centrifuged for 10 minutes at 8,000rpm at 4°C.
- Using a cut tip, 20µL of the aqueous layer was transferred into fresh MCT.
- Equal of Isopropanol was added.
- Stored at -80°C overnight (or in ice for 20 minutes).
- Centrifuged at 13,000 rpm, 4°C for 10 minutes.
- The supernatant was discarded, and 80% ethanol wash was given.
- Pellet was dried completely at RT and then, eluted in DEPC treated water.
- Eluted RNA was checked on 1% agarose gel by gel electrophoresis and then, stored in -80°C.

## 2.5. cDNA Preparation

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

- Following reagents were added to a sterile tube:
    1. Template RNA 2.5µL
    2. Oligo $(dT)_{18}$ Primer 0.25 µL
    3. Random Hexamer Primer 0.25 µL
- The contents were mixed gently and were incubated at 65°C for 5 minutes. Then, the tubes were transferred back on ice and incubated for 5 minutes.
- Following components were added in indicated 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

- The contents were mixed, centrifuged briefly and were incubated at following temperatures:
    1. 5 minutes at 25°C
    2. 60 minutes at 42°C
    3. 5 minutes at 70°C
- The cDNA was then diluted using autoclaved Milli-Q water and stored at -80°C.

## 2.6. 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 | 0.2µL |
| Template | (as per standardized volume) |
| MilliQ-Water | Rest |

- Reaction Parameters:

| | |
|---|---|
| Enzyme activation | 95°C for 2 min |
| DNA denaturation | 95°C for 20 sec |
| Primer annealing | 62°C for 15 sec |
| Extension | 72°C for 30 sec |
| Final extension | 72°C for 7 min |
| Infinite hold | 4°C |

- PCR products were then checked on 1% agarose gel by gel electrophoresis.

*The annealing temperature varies depending upon the primers used.

## 2.7. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

(Kit used – KOD SYBR® qPCR Mix)

- Reaction mixture (5µL volume):

| | |
|---|---|
| Master Mix (KOD) | 2.5µL |
| Forward+Reverse Primer | 0.5µL |
| Template | 0.5µL |
| MilliQ-Water | 1.5µL |

- Reaction Parameters:

| | |
|---|---|
| Enzyme activation | 95°C for 2 min |
| DNA denaturation | 95°C for 20 sec |
| Primer annealing | 62°C for 15 sec |
| Extension | 72°C for 30 sec |
| Final extension | 72°C for 7 min |
| Infinite hold | 4°C |

- Data was then analyzed and plotted in Excel.

## 2.8. TOPO TA Cloning

(Kit used – TOPO TA Cloning Kit by Invitrogen)

- Desired gene was amplified by PCR (same protocol as above except extension time was adjusted depending on the size of amplicon) and was run on 1% agarose gel.
- The gene band was cut under UV light and purified using manual gel extraction protocol.
- The following reaction was set up in a MCT:

| | |
|---|---|
| Salt solution | 0.5µL |
| pCR 2.1-TOPO Vector | 0.3µL |
| Insert | 1µL |
| Autoclaved MQ | 1.2µL |
| Total | 3µL |

- The MCT was incubated at RT for 1 hour.
- The reaction mixture was then transformed into ultra competent cells.

## 2.9. Manual Gel Extraction

- Desired gene band was cut from agarose gel and collected in a MCT containing little pieces of aluminium foil. A small hole was made at the base of MCT using a needle.
- This MCT was then placed inside another MCT and both of them are taped together.
- The entire setup was then centrifuged at 10,000rpm for 10 minutes at RT.
- Equal volume of PCI (Phenol:Chloroform:Isoamyl alcohol) was added into the flow through and mixed properly.
- Centrifuged at 10,000rpm for 10 minutes at RT.
- Aqueous layer was carefully taken out and collected in another MCT.
- Equal amount of Chloroform was added and mixed thoroughly.
- Centrifuged at 10,000rpm for 10 minutes at RT.
- Upper aqueous layer was carefully pipetted out and collected in another MCT.
- Half volume of 7N Ammonium Acetate and twice the volume of Isopropanol were added and mixed properly.
- The sample was kept at -80°C overnight.
- Centrifuged at 15,000rpm for 10 minutes at 4°C.
- 70% ethanol wash (500µL) was given and pellet was dried completely.
- The pellet was then eluted in DEPC MQ and checked on 1% agarose gel.
- Stored at -20°C.

## 2.10. Ultra competent cells preparation

- 5mL primary culture of *E.coli* strain DH5α was incubated at 37°C overnightSecondary culture (1% of primary culture) was incubated at 18°C till $OD_{600}$ reached the value of 0.6-0.8.
- Culture was kept on ice for 10-15 minutes.
- Centrifuged at 2500 x g for 10 minutes at 4°C.
- Pellet was re-suspended in 80 mL of TB buffer. [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.]
- Then kept on ice for 10 minutes.
- Centrifuged at 2500 x g for 10 minutes at 4°C.
- Pellet re-suspended in 20mL of TB buffer
- DMSO is added to a final concentration of 7% (1.4mL+18.6mL of TB buffer)
- Kept it on ice for 10 minutes.
- 100µL volumes were aliquoted and stored at -80°C.

## 2.11. Bacterial Transformation

- Competent cells stored at -80°C were thawed on ice.
- 3µL of TOPO cloned product was added into 100µL of competent cells.
- Incubated on ice for 30 minutes.
- Heat shock was given at 42°C for 50 sec.
- Kept on ice for 5 minutes.
- 1mL of LB media was added into it and the vial was incubated at 37°C for 30 minutes.
- Centrifuged at 4,000rpm for 5 minutes.

- Supernatant was discarded and remaining sample was plated on ampicillin added LB-agar plates. Plates were incubated at 37°C overnight.

## 2.12. Plasmid Isolation

- 5mL of culture was centrifuged at 13,500rpm for 2 min at RT.
- The supernatant was discarded and pellet was dissolved in 100µL of autoclaved MQ.
- 100µL of freshly prepared lysis buffer was added and gently tapped.
  (1mL of Lysis Buffer = add 50µL of 20% SDS solution, 20µL of 0.5M EDTA and 20µL of 10N NaOH in 910µL of MilliQ-water).
- The samples were boiled at 100°C for 2 minutes (till the solution becomes clear).
- 50µL of 1M MgCl2 was added. Tapped and kept on ice for 2 minutes.
- Centrifuge at 13,00rpm for 2 min at RT.
- 50µL of 3M Potassium Acetate buffer was added and tapped immediately.
  (100mL of Potassium Acetate Buffer = 60mL of 5M Potassium Acetate, 11.5mL glacial acetic acid and 28.5mL $H_2O$ were mixed. Stored at 4°C).
- Centrifuged at 10,000rpm for 2 min at RT.
- Supernatant was transferred into another MCT containing 600µL of Isopropanol.
- Kept on ice for 5 min.
- Centrifuged at 13,000rpm for 2min at RT.
- 70% ethanol wash was given and pellet was dried completely.
- Pellet was dissolved in 50µL of autoclaved MQ.
- Stored at -20°C.

## 2.13. Plasmid Digestion

The cloned gene (TOPO clone) was linearized by digesting it with restriction enzyme. Following components were added in the reaction mixture:

| | |
|---|---|
| 1. 10X Reaction Buffer | 5µL |
| 2. Template (Manually isolated) | 20µL |
| 3. Enzyme | 3µL |
| 4. Autoclaved MQ | 22µL |
| Total | 50µL |

- Incubated at 37°C overnight.
- Ran on agarose gel, extracted and purified.

  Following enzymes were used:

| | |
|---|---|
| *ascl1a* | XhoI |

## 2.14. RNA Probe Synthesis

- Following reaction was set up:

| | |
|---|---|
| 1. Buffer (10X) | 1µL |
| 2. Digested Template | 1µL (100-200ng) |
| 3. UTP (DIG-labelled or Fluroscein-labelled) | 0.5µL |
| 4. Enzyme (T7 or SP6) | 2µL |
| 5. DEPC treated MQ | 5.5µL |
| Total | 10µL |

- Incubated at 37°C for 4 hours.
- Added following to precipitate:
  1. 0.5M Tris-EDTA 1µL
  2. 5M LiCl 1µL
  3. 10mg/mL glycogen 0.5µL
  4. Absolute ethanol 70µL
- Tapped to mix properly and kept in -80°C overnight for precipitation.
- Centrifuged at 4°C for 30 min at 13,500 rpm.

- Washed with 80% ethanol (200µL) and centrifuged for 10 min at 13,500rpm, 4°C. Dried and dissolved the pellet in 20µL of DEPC treated water.
- Check the probe on agarose gel.
- Stored at -80°C.

Following enzymes were used:

| | |
|---|---|
| *ascl1a* | SP6 Polymerase |

## 2.15. Tissue Fixation

- Lens was removed from eye in 4%PFA (4%PFA in 1X Phosphate Buffer). The eye was incubated in 4%PFA solution at 4°C overnight.
- Fixed tissue was washed by following sucrose solutions in mentioned order for 45 minutes at RT on a rotor:

1. 5% Sucrose in 1X Phosphate Buffer 1mL
2. 5% Sucrose + 20% Sucrose 800µL + 400µL
3. 5% Sucrose + 20% Sucrose 500µL + 500µL
4. 5% Sucrose + 20% Sucrose 400µL + 800µL
5. 20% Sucrose in 1X Phosphate Buffer 1mL

- 500µL of OCT was added into the existing solution and mixed on rotor for 30 min.
- Block was prepared with OCT in aluminium foil with eye at the center and kept in -80°C for freezing.
- Fixed tissue was sectioned using cryo-sectioning machine.
- The slides were dried (overnight RT) and stored at -20°C.

## 2.16. Immunostaining

Day 1

- Slides were taken out from -20°C and dried at 37°C for 1 hour.
- 1X PBS washes for 10minutes, twice.
- Fixed tissue with 4% PFA solution for 20minutes (This step is done only in the case of MO slides).
- Treated the slides with 2N HCl (pre heated to 37°C) for 20 min. (*Use Sodium Citrate for epitope retrieval while performing beta-catenin immunostaining).
- Washed the slides with 0.1M Sodium borate solution twice for 10 minutes each.
- Blocked the sections using 6% BSA in 1X PBST (1XPBS + 0.1% Triton X) for atleast 30minutes.
- Overlaid the slides with primary anitbody of choice (PCNA, BrdU, β-catenin or Hdac1), 500µL per slide (Antibody is diluted in 1:500 ratio in 1% BSA in 1XPBST).
- Incubated the slides at 4°C overnight.

Day 2

- Washed slides with 1X PBST, 3 times for 10 minutes each.
- Overlaid the slides with desired secondary antibody, 500µL per slide (Antibody is diluted in 1:1000ratio in 1% BSA-1X PBST solution).
- Incubated slides for 3 hours at RT.
- Washed slides with 1X PBST 3 times for 10 min each.
- Washed slides with autoclaved MilliQ-water 3 times for 10 min each.
- Dried slides for 1 hour at RT.
- Mounted slides in DABCO and left at RT in dark overnight.
- Stored slides at -20°C.

## 2.17. TUNEL Assay

- Slides were taken out from -20° and dried by keeping them at 37°C for 1hour.
- 1X PBS washes were given for 10 minutes, 2 times.
- Permeabilized the retinal sections in 1mL Trypsin at 37°C for 15 minutes.
- (Pre-warmed Trypsin at 37°C for 20 minutes).
- Overlaid the 45µL label solution and 5µL enzyme solution on each slide.
- Incubated for 1 hour at 37°C in a humified chamber in dark.
- 1X PBS washes were given for 10 minutes, 2 times.
- Slides were dried and mounted slides in DABCO and left at RT in dark overnight.

## 2.18. mRNA *in situ* Hybridization

Day 1

- The slides were taken out from -20°C and dried by keeping them at 37°C for 2 hours.
- Hydrated the slides in following sequence for 1 minutes each :
    1. 100% EtOH
    2. 95% EtOH
    3. 70% EtOH
    4. 50% EtOH
    5. 2XSSC
- Incubated slides in Proteinase K solution for 5 minutes at 37°C .
  (Proteinase K Buffer: 5mL of 1M Tris-HCl (pH = 8) + 5mL 0.5M EDTA + 40mL of DEPC water)
    1. Pre warmed Proteinase K buffer to 37°C.
    2. Added 160µL of 10mg/mL proteinase K.
- Rinsed slides briefly in room temp DEPC water (2-3minutes).
- Rinsed slides in 0.1M TEA pH 8.0 for 3 minutes.
  (0.1M TEA: 0.93g of Triethanolamine (TEA) + 50mL of DEPC H2O + 173µL of 10N NaOH (for adjusting pH = 8)
- Rinsed in Acetic anhydride/TEA for 10 minutes.

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

- Dehydrated the slides in 2XSSC and EtOH series for 1 minutes each:
    1. 2X SSC
    2. 50% EtOH
    3. 70% EtOH
    4. 95% EtOH
    5. 100% EtOH
- Air dried the slides for at least 1 hour at RT.
- 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:
    1. Added probe to hybridization solution and mixed (as per concentration of the probe).
    2. Boiled probe and hybridization solution mix at 100°C for 10 minutes.
    3. Kept immediately on ice for 2-3minutes.
- Added 300µL of Hyb/probe solution to each slide and coverslip with siliconized hybrid slips.
- Placed slides in humid chamber dampened with 50% Formamide/5X SSC and incubate 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 final volume to 500mL.
TEN Solution (3.6mL): 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

- Preheated 50% formamide/2X SSC solution to 65°C.
- Preheated two 50mL RNase buffer washes, one to 37°C and the other to 65°C.
- Preheated two 2X SSC washes to 37°C.
- Soaked slides with cover slips in 2X SSC for 30 minutes at RT on shaker table.
- If the coverslips do not come off, gently teased them apart from slide with forceps.
- Rinsed slides in 50% Formamide/2X SSC solution for 30 minutes at 65°C.
- Gently agitated for the first 5 minutes.
- Rinsed slides in 2X SSC for 10 min at 37°C (twice).
- Added 100µL of RNase (10mg/mL) to the 37°C RNase buffer, incubated slides for 30 minutes.
- Washed slides in 65°C RNase buffer for 30 minutes.
- Washed slides for 2-3 hours in 1X Maleate buffer/0.05% Triton X-100/1% RMB blocker solution at RT
- Washed slides in 1X Maleate buffer for 5 minutes (twice).
- Incubated slides with 500µL antibody (Anti-DIG/Anti-FL) diluted in 1X Maleate/0.05% Triton X-100/1% RMB blocker solution (1:2500 dilution) overnight at RT.

  (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.)

Day 3

- Washed slides twice with 1X Maleate buffer for 5 minutes.
- Washed 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 $MgCl_2$)
- Added 500µL of NBT/BCIP dissolved in Genius buffer (1:50 dilution), incubatedat room temperature in dark for color reaction.
- Color detection: In bright field microscope.

## Primer and MO List

Mentioned sequences are in 5'- 3' direction.

| | |
|---|---|
| control MO | CCTCTTACCTCAGTTACAATTTATA |
| gli1a MO | TCGAGTCATCCAAGAAGTCTCATCATGGAGAAG |

Table1. Sequence of MO.

| | |
|---|---|
| ascl1a FL Fwd | ATGGACATCACCGCCAAGATGGAAATAAGCG |
| ascl1a FL Rev | TCAAAACCAGTTGGTGAAGTCCAGGAGCTC |
| tgfbi FL Fwd | ACTGACCGGATCCAGATGCTGGTCTTAATGATCACCTGGTC |
| tgfbi FL Rev | CAGCACTCTCGAGTGGTCACTCACAATTTTAGGAGGCAGTG |
| her4.1 FL Fwd | GAAACTCTACTGACAAACAAGCTG |
| her4.1 FL Rev | GATGTTGTCCATCTTCGTTTAGTGC |
| ascl1a RT Fwd | ATCTCCCAAAACTACTCTAATGACATGAACTCTAT |
| ascl1a RT Rev | CAAGCGAGTGCTGATATTTTTAAGTTTCCTTTTAC |
| her4.1 RT Fwd | GCTGATATCCTGGAGATGACG |
| her4.1 RT Rev | GACTGTGGGCTGGAGTGTGTT |
| β-actin RT Fwd | GCAGAAGGAGATCACATCCCTGGC |
| β-actin RT Rev | CATTGCCGTCACCTTCACCGTTC |
| mmp2 RT Fwd | CTCAGATCCGTGAGATCT |
| mmp2 RT Rev | CTTTGGTTCTCCAGCTTCAGG |
| mmp9 RT Fwd | GGAGAAAACTTCTGGAGACTTG |
| mmp9 RT Rev | CACTGAAGAGAAACGGTTTCC |
| tgfbi RT Fwd | CGCTGACCTCAACAAACTCATGAGAG |
| tgfbi RT Rev | TGGTCACTCACAATTTTAGGAGGCAG |

| | |
|---|---|
| tgfbr2a RT Fwd | TGAGACTCGACAGCTGCCTGAGTC |
| tgfbr2a RT Rev | AGGCTCATAATCCCGCACATCTCC |
| tgfbr2b RT Fwd | AGATGTAACGCCATCGGAGAGG |
| tgfbr2b RT Rev | AAACTGCTGCCACTCCCTGG |
| tgfbr3 RT Fwd | GTTGTCATAGAGAACATCTGTCCCAAG |
| tgfbr3 RT Rev | AGGCTTCATCCGGTAACATACACTC |
| tgif1 RT Fwd | TATTTCTGCCTGTCATGAAGGTG |
| tgif1 RT Rev | ATTTATAAACCAGTTGCAAACCTG |
| snail1a RT Fwd | AGCTGGAATGTCAGAACGACACTTC |
| snail1a RT Rev | GTCTGACGTCCGTCCTTCATCTTC |
| snail2 RT Fwd | AGCACGTATTCGGGACTCATGAAGC |
| snail2 RT Rev | CAGGAAAACGGTTTCTCACCCGTG |
| deltaB RT Fwd | AAGAATGGCGGCAGTTGTAATGATTTG |
| deltaB RT Rev | AGATCCACACATTCACCACCGTTG |
| deltaD RT Fwd | AAATGGAGGAAGTTGCACTGATC |
| deltaD RT Rev | AAGATCGAGACACTGAGCATCATTC |
| notch1a RT Fwd | ACGGATTCACTCCACTGATGATCGCATC |
| notch1a RT Rev | TCGGTTCCGAATGAGGATCTGGAAG |

Table2. List of RT and FL primers used. RT – Reverse Transcription; FL- Full Length

# Chapter 3. Experiments and Results

## 3.1. Components of TGF-β signaling pathway are regulated during retina regeneration.

To investigate the temporal expression of the components of TGF-β signaling pathway, the zebrafish eyes were injured. The retinae were dissected out at different time points and mRNA was isolated. Then, cDNA was synthesized and RT-PCR were set for TGF-β signaling components. It was observed that these components were expressed soon after injury, as soon as 6hpi (hours post injury) . Being a house-keeping gene, *β-actin* is used as control. The TGF-β signaling pathway components had a distinct pattern. *tgif1* (TGF-β induced factor homeobox 1) interacts with SMADs to modulate gene expression. It is induced soon after injury, with its maximum expression at 7dpi (days post injury), which is the peak of re-differentiation. The receptors, *tgfbr2b* and *tgfbr3* are induced after injury. They show a similar profile, with increased expression at 15hpi(start of de-differentiation), 4dpi (peak of proliferation) and 7dpi (peak of re-differentiation), with dramatic reduction at 2dpi (peak of de-differentiation). However, *tgfbr2a* expression is already present in uninjured control, it undergoes slight increase from 2dpi to 7dpi. This implies that the receptors have a crucial role at 4dpi *i.e.* at peak of proliferation. Another important component, *tgfbi* (transforming growth factor β induced*)* is a downstream gene of the TGF-β signaling pathway and it also sees up regulation after injury, with maximum induction at 4dpi and 7dpi. Matrix metalloproteases, *mmp2* and *mmp9* are known for activation of TGF-β ligand. They are also up-regulated post injury. This implies TGF-β signaling pathway has an important role to play during retina regeneration. (Figure3.1).

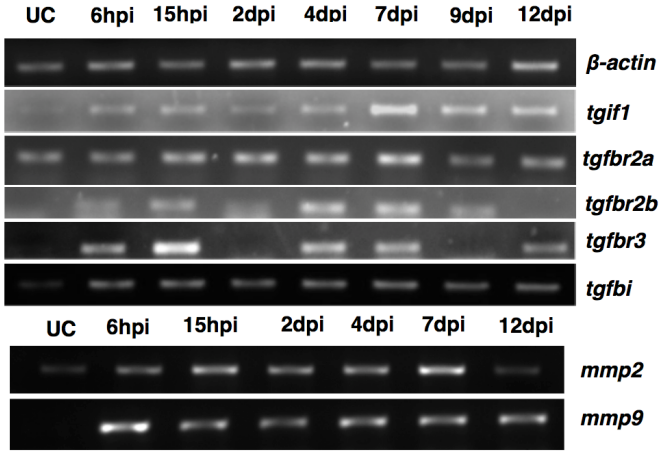


**Figure 3.1. TGF-β signaling pathway associated genes are regulated during retina regeneration.** mRNA expression levels of various TGF-β signaling pathway components at different time points after retinal injury. Method: RT-PCR.

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## 3.2. Inhibition of TGF-β signaling causes reduction in MG proliferation at the site of injury, whereas its activation causes increased MG proliferation.

In the next set of experiments, we injected the pharmacological inhibitor of TGF-β signaling pathway i.e. SB431542 in the retina soon after injury. SB431542 prevents the kinase activity of the TGF-β receptors. The fish line used for this experiment is 1016 *tuba1a*:GFP transgenic line . The tuba1a gene encodes a tubulin isoform, which is only expressed in developing or regenerating nervous system. During retina regeneration, this is exclusively expressed in the Müller Glia cells which have entered into cell cycle. Therefore, GFP is tagged with *tuba1a* promotor to mark MG cells. The eyes were harvested at 4dpi, fixed, sectioned and stained for BrdU (bromodeoxyuridine, a thymidine analog) and PCNA (Proliferating Cell Nuclear Antigen), which are the markers for cell proliferation. It is observed that significantly less number of MG cells enter the cell cycle after the treatment and, the number of proliferating cells has also

decreased as compared to the control. (Proliferation has been used as assay for regeneration in these experiments).

In yet another experiment in the 1016 *tuba1a*:GFP transgenic line, activated TGF-β1 protein was injected soon after the injury and the eyes are harvested at 4dpi. This induction of TGF-β signaling shows an increased number in cells expressing GFP and cells stained for BrdU and PCNA. (Figure3.2.b).

These both experiments show that **TGF-β signaling pathway has a pro-proliferative role when it comes to retina regeneration.**

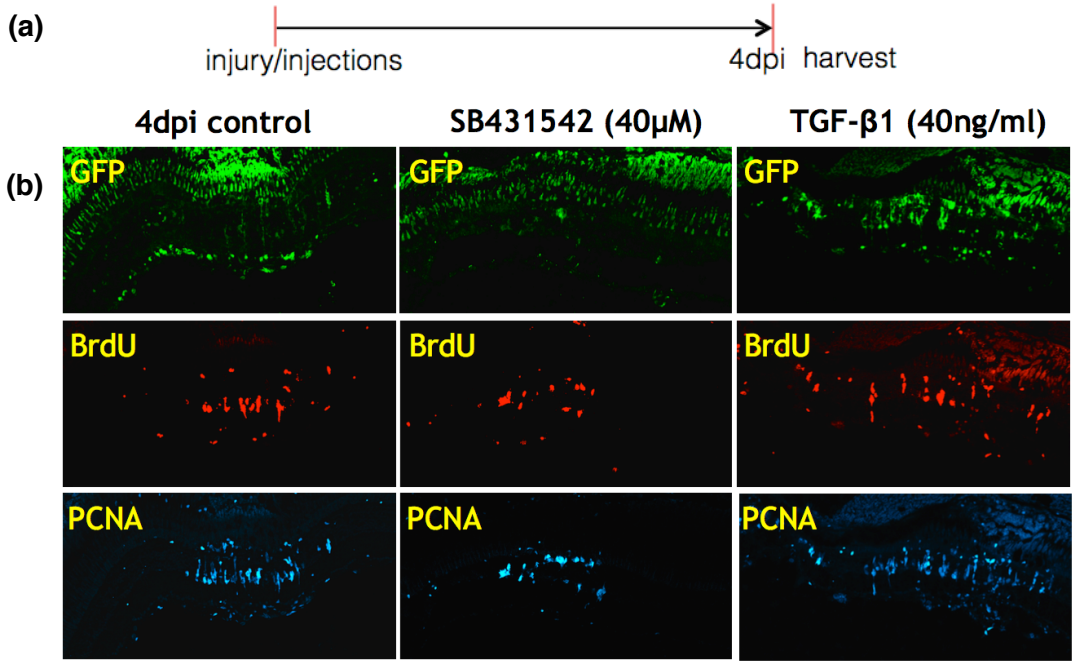


**Figure 3.2. TGF-β signaling pathway has a pro-proliferative role when it comes to retina regeneration.** (**a**)Experimental schematic : the eyes are harvested at 4dpi after giving BrdU pulse for 4 hours; (**b**) Co-immunostaining of BrdU (red) and PCNA (blue). Green channel shows expression of GFP , which is exclusively expressed in proliferating MG .Method: Immunostaining

## 3.3 Crosstalk between TGF-β signaling pathway and Notch signaling.

In the next set of experiments, we tried to explore the crosstalk between TGF-β and Notch signaling. We started with injecting the pharmacological inhibitors of both pathways separately as well as in a combination, into wild-type zebrafish eyes. SB431542 inhibits kinase activity of TGF-β receptors. DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester) inhibits γ-secretase, causing a blockade for Notch signaling. The eyes were harvested at 4dpi, fixed, sectioned and stained for PCNA. It was observed that inhibition of TGF-β pathway caused attenuated cell proliferation. However, Notch pathway inhibition caused a huge expansion of proliferation zone. around the site of the injury. To much interest, the combined inhibition of both the pathways had an overall increase in proliferating area, even showing an increase in a dose-dependent combined inhibition.The effect of TGF-β pathway inhibition has been masked by the effect of the Notch pathway inhibition.This data suggests that TGF-β signaling has a pro-proliferative role, but the **Notch signaling has an anti-proliferative role in reina regeneration.** Therefore, Notch signaling must have a role in keeping the cells in differentiated state. In combined inhibition, it can be inferred that Notch signaling blockade masks the effect of TGF-β signaling blockade. This was suggestive of the idea that these both pathways must have some interacting factors.(Figure 3.3.1)

To check the effect at mRNA levels, we injected a combined dosage of both the inhibitors. The retinae were extracted at 2dpi. The mRNA was extracted, cDNA was synthesized and RT-PCR and qRT-PCR were run for some components of both the signaling pathways. *β-actin* is used as control. Upon combined blockade, the expression of the ligands (d*eltaB, deltaD*) and the receptors (*notch1a*) is increases as compared to control, but decreases in a dose-dependent manner. The same happened to the expression of its downstream gene, *her4.1*. However, the receptor of TGF-*β* signaling *i.e. tgfbr3* and its downstream genes *i.e. tgfbi, snail1a* and *snail2* are down-regulated.(Figure 3.3.2.a & b)

*her4.1* is known to have anti-proliferative role. It is a downstream gene of the Notch signaling. Upon the combined blockade *her4.1* shows an increase as compared to injured control. The expression, however , decreases in a concentration-dependent manner This indicates that TGF-*β* has some role to play in this up-regulation. Next, we checked the spatial expression of *her4.1* by *mRNA in situ* hybridization. The eyes were harvested at 4dpi, fixed and sectioned. As expected, *her4.1* mRNA expression is almost negligible in retina with Notch pathway blockade. , since *her4.1* is downstream gene of the Notch pathway. However, in TGF-*β* signaling blockade the expression of *her4.1* is increased. The combined inhibition also shows the same effect, *i.e.*. increased expression as compared to the control. This shows that TGF-*β* pathway has a negative effect on *her4.1* expression. ***her4.1* is upregulated by Notch signaling activation, but down-regulated by TGF-*β* pathway blockade.**(Figure 3.3.2.c)

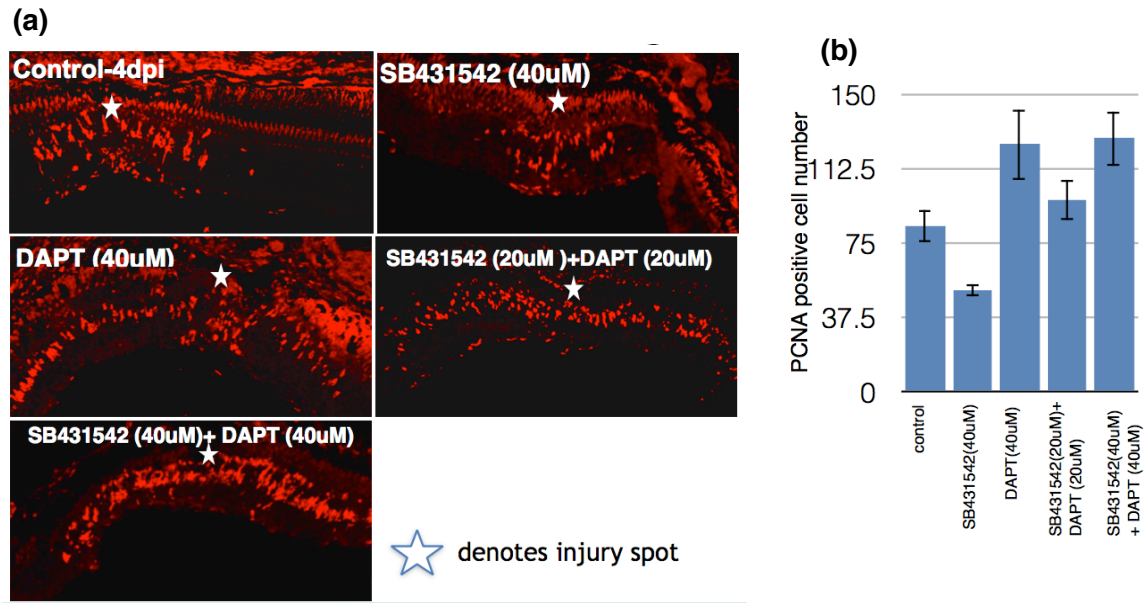


**Figure 3.3.1. Effect of blockade of TGF-β signaling pathway and Notch signaling on retina regeneration.** (**a**) SB431542 treatment causes reduced proliferation, however DAPT treatment causes huge augmentation, which is also seen in combined blockade. This suggests that both these signaling pathways are antagonists. Method:PCNA immunostaining (**b**) PCNA positive cell count data for each treatment.

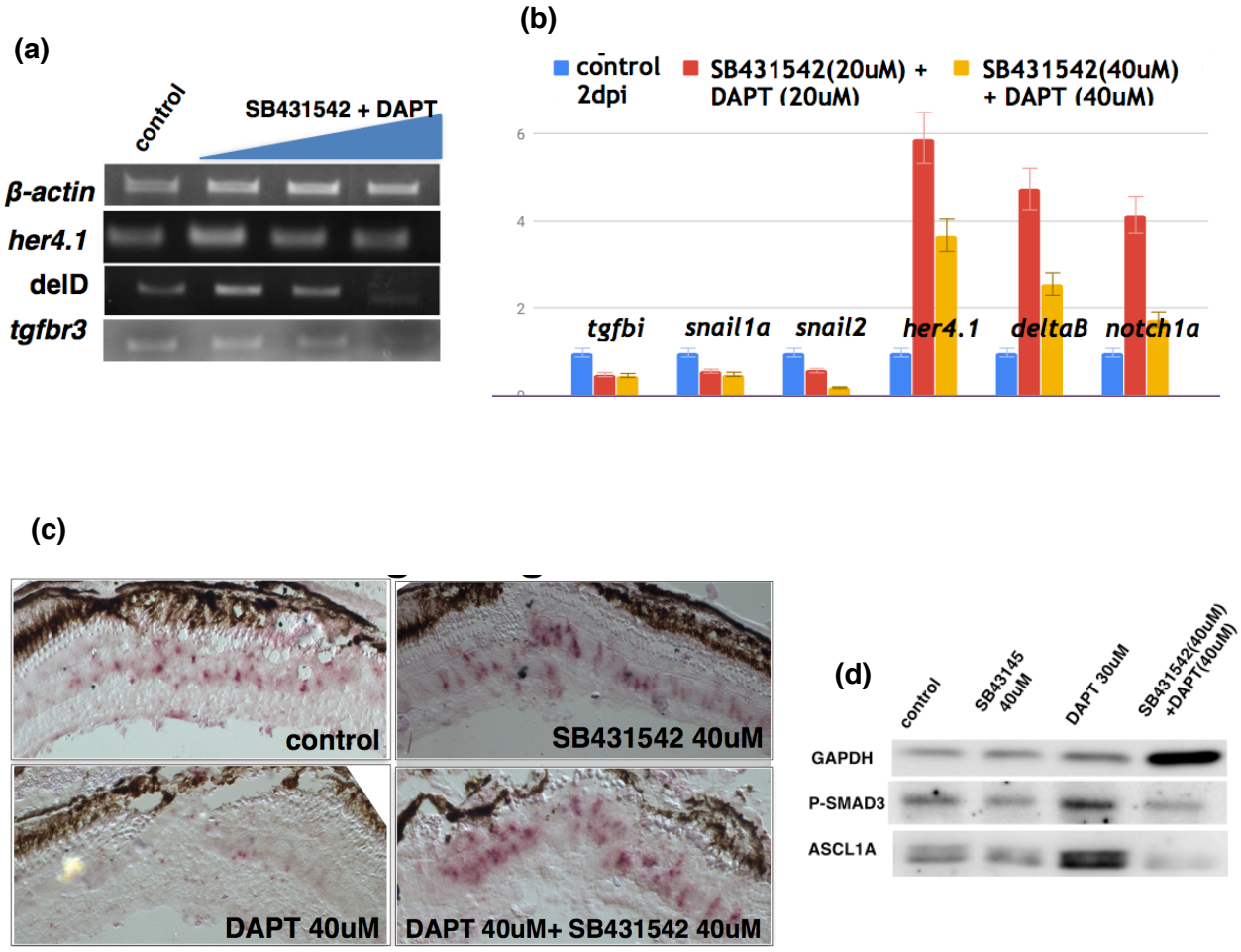


**Figure 3.3.2. Crosstalk between TGF-β signaling pathway and Notch signaling during retina regeneration.** (**a & b**). Gene expression of components of both signaling pathways on different concentrations of combined blockade. Method: RT- PCR and qRT-PCR; (**c**)Effect of the pharmacological inhibition of both the signaling pathways on the expression of *her4.1*. DAPT -treated retina has next to negligible expression, while SB431542-treated retina has higher expression as compared to control Method:mRNA *in situ* hybridization ; and (**d)** Western Blot analysis shows up-regulation of p-Smad3 and Ascl1a in Notch pathway inhibition, but down-regulation in TGF-β signaling pathway blockade.

The western blot analysis suggests that p-Smad3 and Ascl1a is down regulated upon TGF-$\beta$ blockade, but is up-regulated in Notch signaling blockade. Ascl1a is a pro-proliferative gene and p-Smad3 is a component of TGF-$\beta$ pathway. The protein data that **Notch signaling has an inhibitory effect on p-Smad3 and Ascl1a.**(Figure 3.3.2.d).(Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) is considered a house-keeping gene because of its ubiquitous expression. Therefore, it is used as control for loading.)

In another set of experiments, we tried to see the effect of both of the signaling in zebrafish development. The embryos ere collected and dipped in SB431542 or DAPT or a combination of the both. The embryos were harvested at 48hpf (hours post fertilization). A BrdU pulse was given for 4 hours before harvesting the embryos. SB431542-treated embryos developed shorter tails and had less pigmentation. DAPT- treated embryos were smaller with curved tails and less pigmentation. In combined inhibition, the embryos had severe deformities. The survival rate of the embryos was decreased drastically upon inhibition. In control, 26 out of 27 embryos developed; in SB41542 treatment, 9 out of 60 survived; in DAPT treatment 35 out of 43 survived; in combined treatment 1 out of 60 survived till 48hpf.

In another set, PTU (1-phenyl 2-thiourea) was added 12 hours after fertilization to prevent pigmentation and generate transparent embryos for mRNA *in situ* hybridization. mRNA *in situ* hybridization was done for *her4.1* and *tgfbi.* In embryos, *her4.1* is expressed in parts of the nervous system and has decreased expression in both the treatments. This result is not along the lines of the observation in retina, where *her4.1* expression on TGF-$\beta$ pathway inhibition increases. *tgfbi* (a downstream gene of TGF-$\beta$ pathway) expression is seen in yolk sac cells and various parts of nervous system. The expression is decreased in SB431542-treated embryos; but in DAPT- treated embryos, it is increased. This suggests that Notch and TGF-$\beta$ pathways have a different equation in the developing organism as compared to retina regeneration.

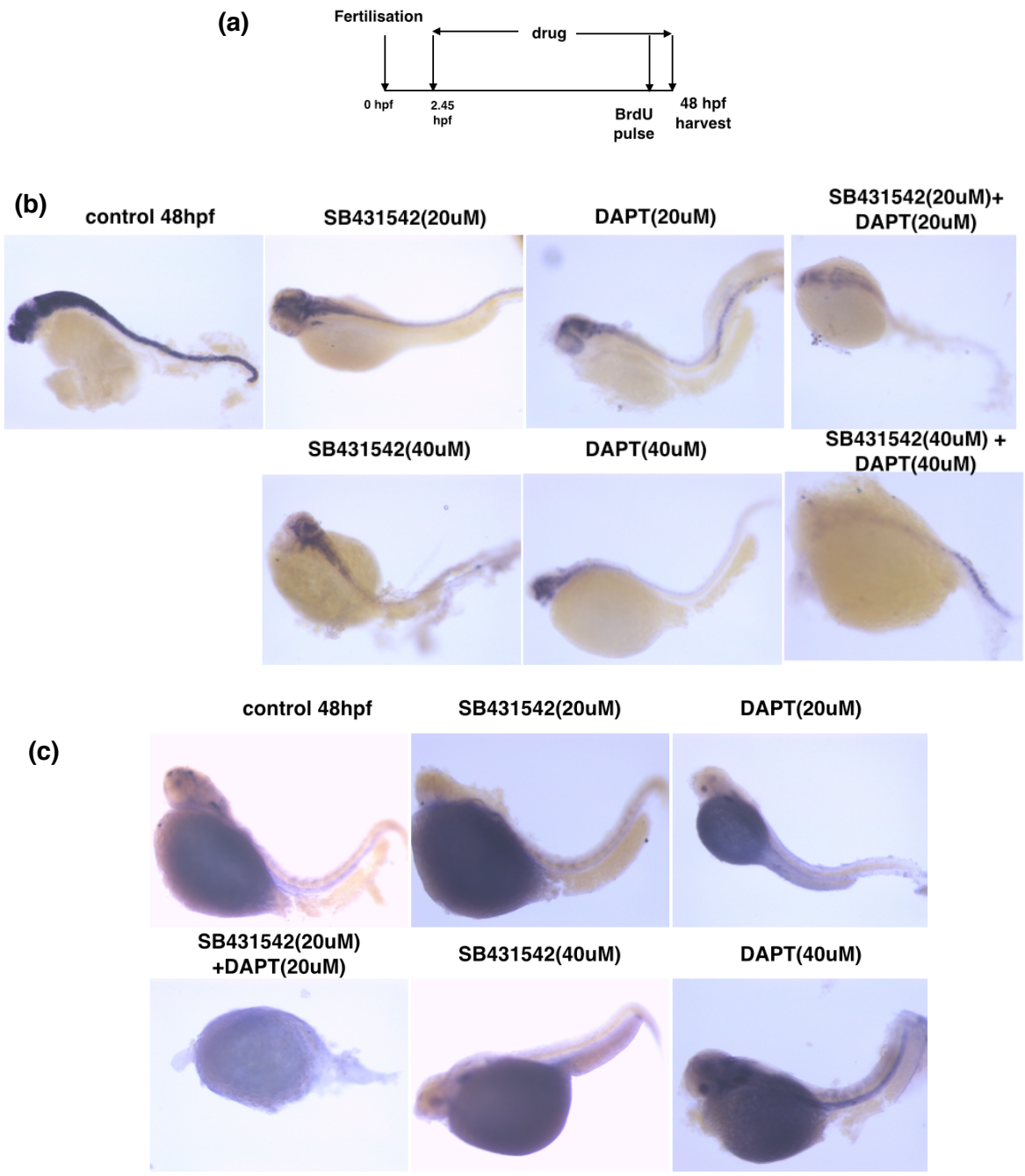


**Figure 3.3.3. Regulation of *her4.1* and *tgfbi* expression by TGF-β pathway and Notch signaling during zebrafish development (a)** Experimental schematic: Embryos are dipped in inhibitors at 2.45hpf (hours post-fertilization) and harvested at 48hpf (**b**) *her4.1* expression profile upon inhibition of TGF-β pathway and Notch signaling. Its expression reduces in both individual treatments as well as in combined boockade. (**c**) *tgfbi* expression profile upon blockade of TGF-β pathway and Notch signaling.

## 3.4 Crosstalk between TGF-β signaling pathway and Shh signaling.

Upon inhibition of the TGF-*β* pathway and Shh signaling in the embryos, it was seen that the embryos developed cyclopia, *i.e.* eye fusion. This suggested that Shh signaling and TGF-*β* pathway must have a common pathway that through which they play a role in the nervous system development of the organism.

For exploring the roles in retina regeneration, we started with the individual as well as combined inhibition of both the pathways. Cyclopamine was used for Shh signaling inhibition. The eyes were injured and injected with drugs and were extracted at 4dpi. Later, they were fixed, sectioned and stained with PCNA. PCNA positive cells were counted and the data was analysed. It was seen that in all the cases, the proliferation decreased. (FIgure 3.3a) Therefore, **TGF-*β* pathway and Shh signaling have a pro-proliferative role in retina regeneration.** In another set of experiment, we injected activated TGF-*β* protein along with cyclopamine. It was observed that activated TGF-*β* protein was able to partially rescue the reduced proliferation.(FIgure 3.3b)This was an indication that **TGF-*β* pathway and Shh signaling play a synergistic role when it comes to retina regeneration.**

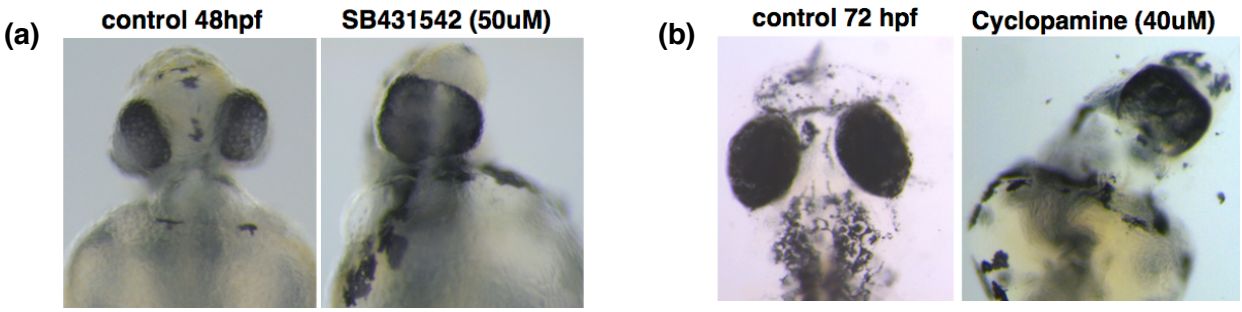


**Figure 3.4.1. Inhibition of the TGF-*β* pathway and Shh signaling in the embryos :** (**a**) In SB431542- treated embryos, the inhibition of TGF-*β* pathway causes cyclopia. (**b**) Blockade of Shh signaling by the treatment of embryos by cyclopamine also causes cyclopia.

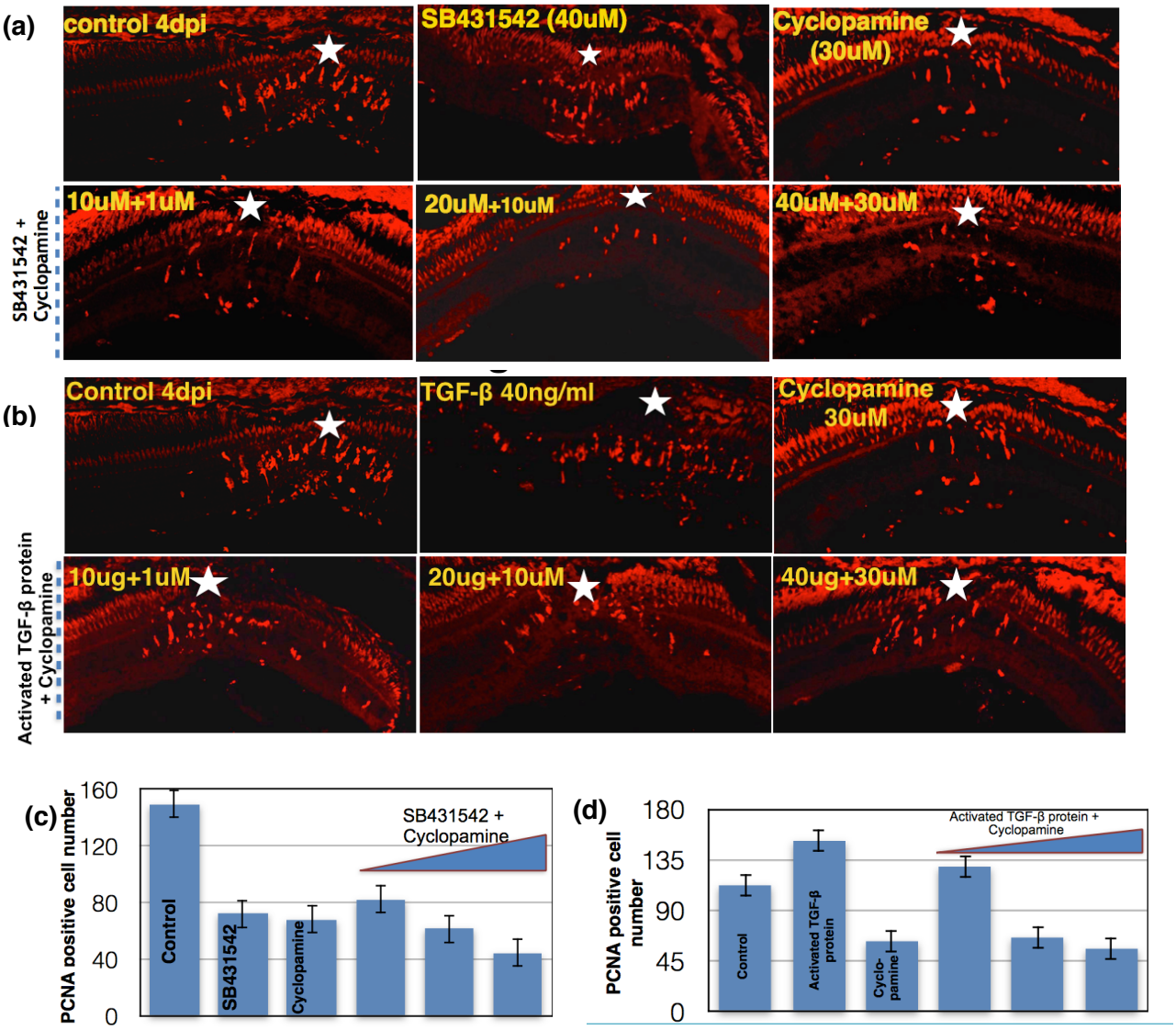


**Figure 3.4.2. Functional synergism between TGF-β signaling pathway and Shh signaling during retina regeneration.** (**a**) Inhibition of both TGF-β signaling pathway and Shh signaling decreases the proliferation at the site of injury. The result is additive when both the blockades are done simultaneously. Method: PCNA immunostaining;(**b**) Activation of TGF-β signaling pathway causes an increased proliferation. It also partially rescues proliferation in retina with Shh pathway inhibition Method: PCNA immunostaining; (**c**). Proliferating cell count data for (a); (**d**) Proliferating cell count data for (b)

a*scl1a* has well characterized pro-proliferative role in retina regeneration. Our next step was to see whether these pro-proliferative pathways, *i.e.* TGF-*β* pathway and Shh signaling have any role to play in *ascl1a* regulation. Hence, we did an mRNA *in situ* hybridization for *ascl1a* in retinae with individual blockade of TGF-*β* pathway and Shh signaling; combined blockade and; in a set where TGF-*β* pathway was activated but Shh signaling was blocked. It was seen that *ascl1a* expression reduced in both the treatments. The effect was even more in combined treatment. However, in activated TGF-*β* protein and cyclopamine injected retinae, the expression is still less than the control, but more than the inhibitor treatments. (Figure 3.4.3.)This clearly suggests that **TGF-β pathway and Shh signaling have a role in up-regulation of *ascl1a*, which is pro-proliferative gene.**

For further exploring the roles of TGF-*β* pathway and Shh signaling in retina regeneration, we injected the retina with *gli1a* morpholino. Gli1a is a putative transcriptional mediator of Shh signaling. Knockdown of *gli1a* caused decreased proliferation. This effect is further elevated by TGF-*β* blockade. This indicates that ***gli1a*** **has a role in up-regulating the proliferation associated genes**. In combination with TGF-*β* blockade, proliferation is nearly negligible. (Figure 3.4.4.)

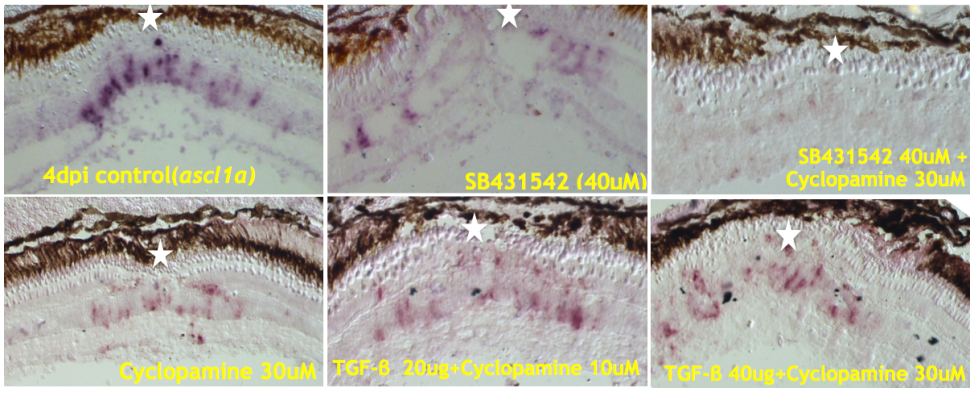


**Figure 3.4.3. Regulation of *ascl1a* expression by TGF-β pathway and Shh signaling during retina regeneration :** *ascl1a* expression is reduced in individual as well as combined blockade of TGF-β pathway and Shh signaling. However, on injecting activated TGF-β protein with cyclopamine, the expression is partially rescued. This suggests that *ascl1a* is up-regulated by both pathways.

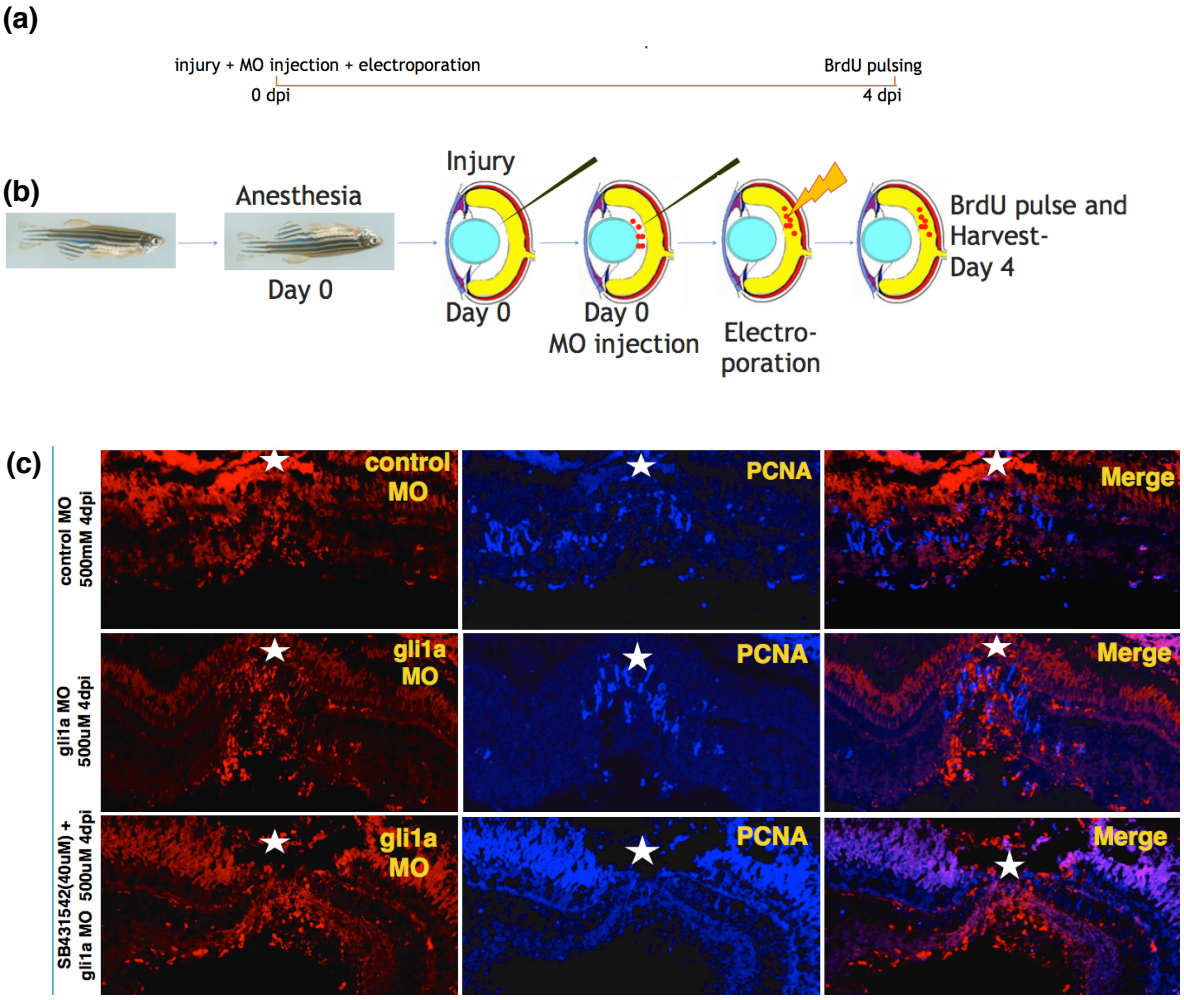


**Figure 3.4.4. Synergistic role of TGF-β signaling and Shh signaling component on retina regeneration.** (**a** & **b**) Experimental Regime : MO is injected and electroporated at the time of injury. The eyes are harvested at 4dpi. (**c**) Immunostaining of PCNA (blue) : *gli1a* Knockdown causes decreased proliferation as compared to control. Upon combination with SB431542, this effect is further elevated, suggesting functional synergism. MO are tagged with a fluorescent molecule, lissamine (red) to track successful electroporation.

# Chapter 4. Conclusion

TGF-β pathway associated genes have a distinct pattern post-retinal injury. This suggests its role in retina regeneration. Induction of TGF-β causes an increased proliferation, while its inhibition leads to an attenuated effect. Notch inhibition causes huge augmentation in number of cells undergoing proliferation. In combined inhibition, it masks the effect of TGF-β pathway inhibition, giving an overall increase. This tells us that TGF-β pathway has a pro-proliferative role and Notch signaling has an anti-proliferative role. Both are antagonistic to each other. Increase in *her4.1* (downstream genes of the Notch signaling) expression is seen upon combined blockade of both Notch signaling and TGF-β pathway. *her4.1,* therefore, becomes a link in connecting the two pathways. Increased levels of p-Smad3 and Ascl1a are increased in Notch inhibition suggests that Ascl1a expression is modulated by the Notch signaling through p-Smad3.

Interruption of TGF- β during embryonic development in zebrafish leads to cyclopia, which is a characteristic feature of insufficient Shh signaling. This suggests that TGF-β signaling and Shh pathway act through common pathway for eye development. Shh signaling inhibition also shows alleviation in the cell proliferation. Combined with TGF-β pathway inhibition, it shows a synergistic effect. This suggests that both TGF-β signaling and Shh signaling are pro-proliferative. Reduced proliferation with Shh pathway blockade is partially rescued when activated TGF-β protein is also injected. Knockdown of *gli1a* (a mediator of Shh signaling) cause decreased proliferation. This effect is further elevated when combined with TGF-β signaling blockade. This indicates that *gli1a* has a key role in up-regulating proliferation-associated genes. In combination with TGF-β pathway inactivation, proliferation is nearly negligible. However, all these are preliminary results. More experiments need to be done to unravel the network that is the regulating regeneration process.

The deviation in levels of TGF-β pathway associated genes upon Notch/Shh inhibition (and vice versa) suggests there is crosstalk which occurs to restore the structural and functional integrity of the retina. While TGF-β and Shh apparently might have a synergistic relation, TGF-β and Notch have antithetical roles to play when it comes to retina regeneration.

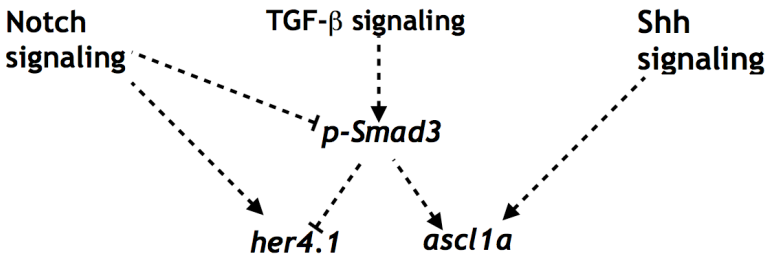


Figure 4.1. Schematic representation of the signaling pathways determined from this study.

# Future Outlook

This study gives insights about the preliminary pathway involved in the process of zebrafish retina regeneration. Further experiments need to be performed to validate current results and decipher the interplay between various signaling pathways and molecules. To get a better understanding about how the TGF-β Signaling pathway is involved in retina regeneration, we need to know how all the components of this pathway are regulated both temporally and spatially. Since TGF-β Signaling pathway is known to behave differently depending on its cellular context, it would be interesting to perform blockades at different time points and see the effects on the proliferation. This could be done for three of the signaling pathways. And also, we need to unravel in which phase of regeneration they are regulated and regulate other signaling molecules. Since most of this study focuses on the transcripts and their transcriptional regulation but any signaling pathway becomes meaningful only when it is dealt at translational level. We plan to further validate the data by Western blotting analysis and mRNA *in situ* hybridization of the candidate genes. We plan to do Western Blotting assay at different time points post injury and in relation with various regeneration-associated genes and in various blockade conditions. We need to validate the interaction of target proteins with the DNA and other proteins to ascertain the probable interactions. Assays like ChiP-seq and mass- spectrometry could be used to pin-point the intersections between the signaling pathways

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