Understanding the role of Wnt signaling in context of zebrafish fin regeneration

Priya Sharma (MP14002)

A dissertation submitted for the partial fulfilment of MS degree in Science

Indian Institute of Science Education and Research Mohali

April 2017

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Certificate of Examination

This is to certify that the dissertation titled "Understanding the role of Wnt signaling in context of zebrafish fin regeneration" submitted by Ms. Priya Sharma(Reg. No. MP14002) for the partial fulfilment of MS 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.

Professor A.K. Bachhawat Dr. Shashi B. Pandit Dr. Rajesh Ramachandran

(Supervisor)

Dated: April 21,2017

Declaration

The work presented in this report has been carried out by me under the guidance of Dr. Rajesh Ramachandran at IISER, Mohali.

This work has not been submitted in part or in full of a degree, a diploma, or a fellowship to 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.

Priya Sharma

(Candidate)

Dated:21April,2017

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

Dr. Rajesh Ramachandran

(Supervisor)

Acknowledgement

I would like to express my sincere gratitude for my project supervisor Dr. Rajesh Ramachandran for his guidance, and suggestions throughout my project work.

I would like to thank Poonam Sharma, Simran Kaur, Anwar, Soumitra Mitra and MS students for their selfless contribution , constant help and support throughout the lab work.

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Above all, I would like to pay my heartiest gratitude and thankfulness to my parents for their lifelong contribution, support and blessings.

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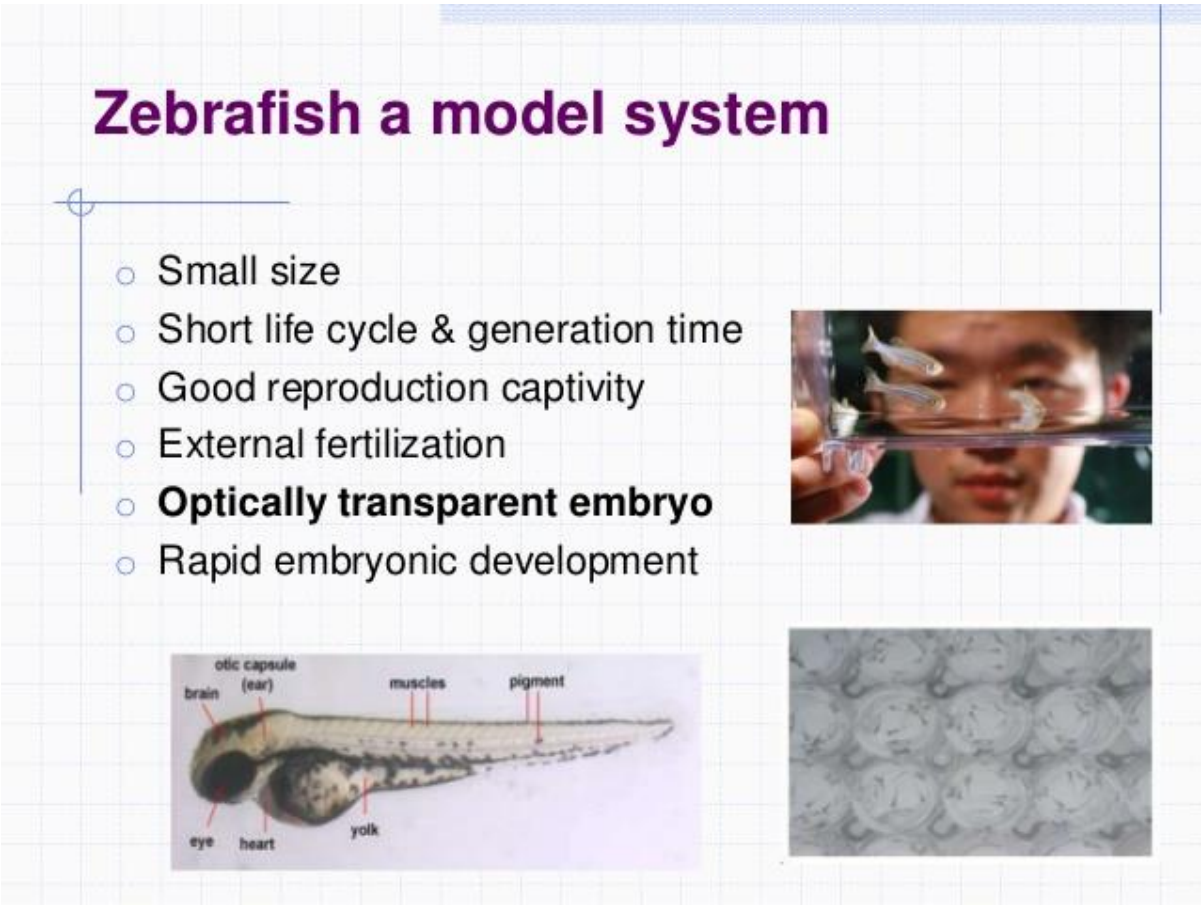
Abstract

Wnt signaling is known to be involved in retina regeneration but its importance in fin regeneration has not been studied yet. LiCl is a positive regulator of Wnt signaling pathway by inhibiting GSK3β. So, we studied the effect of LiCl on zebrafish caudal fin regeneration and we found that LiCl acts a positive regulator of caudal fin regeneration by upregulatingWnt signaling pathway as blastema regeneration has increased from 125µM to 500µM. Along with this, we studied the effect of tumour suppressor gene Ptenb inhibitor and EDTA on fin regeneration and we found that both of these downregulates fin regeneration whereas HDACs inhibitor, LiCl and CaCl2 upregulates fin regeneration. Then, we studied the effect of Lithium ions on embryos which revealed that embryos showed delayed hatching at 8dpf in the presence of Li ions as compared to the control as Lithium upregulates Wnt signaling which further downregulates hyaluronidase enzyme due to which chorion does not break down. Then, we studied about pluripotency factors where there is induction of pluripotency factors during retina regeneration, embryonic development and fin regeneration. mRNA in situ hybridization of pluripotency factors revealed their spatial expression pattern at different time points during embryonic development. Finally, we did immunostaining where it has been shown that notch inhibitor causes induction of sox2 during proliferation of astrocytes in mice retina and DAPT enhances proliferation of cells.

Chapter1

1.1 Introduction

Regeneration is the process of renewal, restoration, and growth that makes genomes, cells, organisms and ecosystems resilient to natural fluctuations or events that cause disturbance or damage. Zebrafish has the ability to regenerate fins, skin, heart and brain during its larval stages. Zebrafish can regenerate photoreceptor cells and retinal neurons, which has been shown to be mediated by the dedifferentiation and proliferation of Müller glia cells. Study of gene expression during regeneration has allowed for the identification of several important signaling pathways involved in the process, such as Wnt signaling, fgf signaling and notch signaling pathways. Regeneration is the renewal through the internal processes of a body or system. It has been known for centuries that salamanders and fishes can regenerate complex tissues much more effectively than mammals. Zebrafish have emerged as a central model system for studying regeneration, due to their ability to regenerate myriad tissues and to the availability of molecular genetic tools. [RZG]



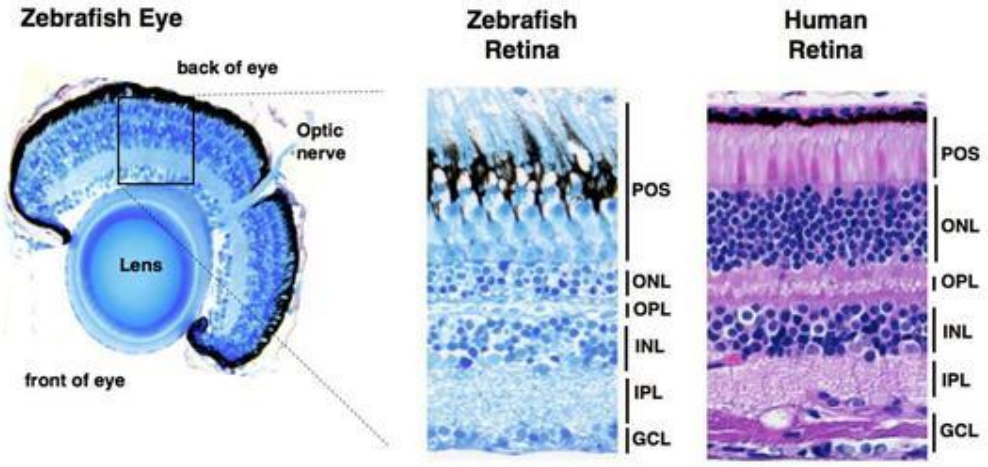
(Wikipedia.org)

Fig 1: Zebrafish as a model organism

Muller glia, are a type of retinal glial cells. They are found in the vertebrate retina, which serve as support cells for the neurons of the retina as all glial cells do. They are the most common type of glial cell found in the retina. They span across the entire thickness of the neural retina. Müller glia have been shown to serve as important mediators of neurotransmitter (acetylcholine and GABA specifically) degradation and maintenance of a favorable retinal microenvironment in turtles. Müller glia have also been shown to be important in the induction of the enzyme glutamine synthetase in chicken embryos, which plays an important role in the regulation of glutamine and ammonia concentrations in the central nervous system. Müller glia have been further identified as fundamental to the transmission of light through the vertebrate retina due

to their unique funnel shape, orientation within the retina and more favorable physical properties.

Any retina of vertebrate can be divided into three distinct layers: Ganglion cell layer(GCL) has ganglion cells, Inner nuclear layer(INL) and Outer nuclear layer(ONL). Axons of these cells forms the optic nerve. Bipolar and amacrine cells reside in the GCL. In the ONL, rods and cone cells are found. Muller glial cells reside in the INL. These muller glial cells wraps around other cell types and provide nourishment also, they have the capability to redifferentiate into other cell types of retina. Basically there are six different kinds of cells in the retina : Rod and cone photoreceptors cells, amacrine cells , ganglion cells and muller glial cells. Among all of these cells, Muller glia are the major glial component of the retina. They are one of the last retinal cell types to be born during development, and they function to maintain retinal homeostasis and integrity. In the case of mammals, Muller glia respond to retinal injury in various ways that can be either protective or detrimental to retinal function. Although these cells can be coaxed to proliferate and generate neurons under special circumstances, these responses are meagre and insufficient for repairing a damaged retina. By contrast, in teleost fish (such as zebrafish), the response of Müller glia to retinal injury involves a reprogramming event that imparts retinal stem cell characteristics and enables them to produce a proliferating population of progenitors that can regenerate all major retinal cell types and restore vision.[JRG]



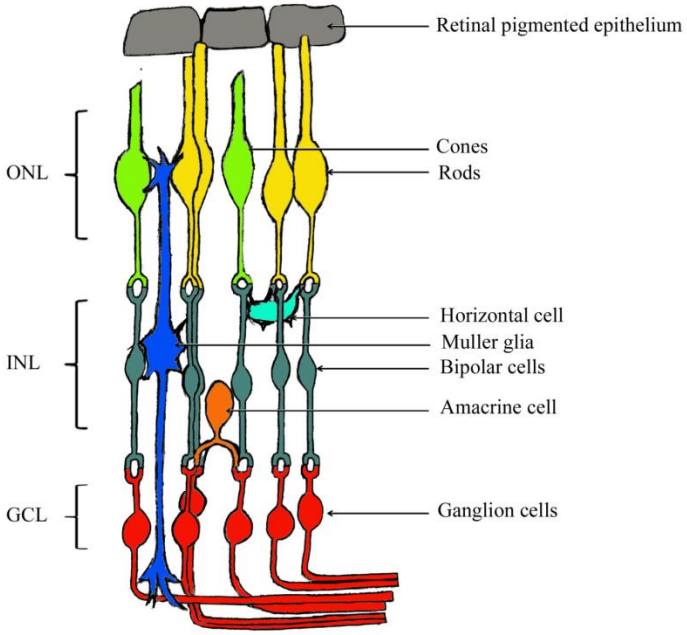


Fig2: Retina of Vertebrate Eye

Pluripotency factors in retina regeneration

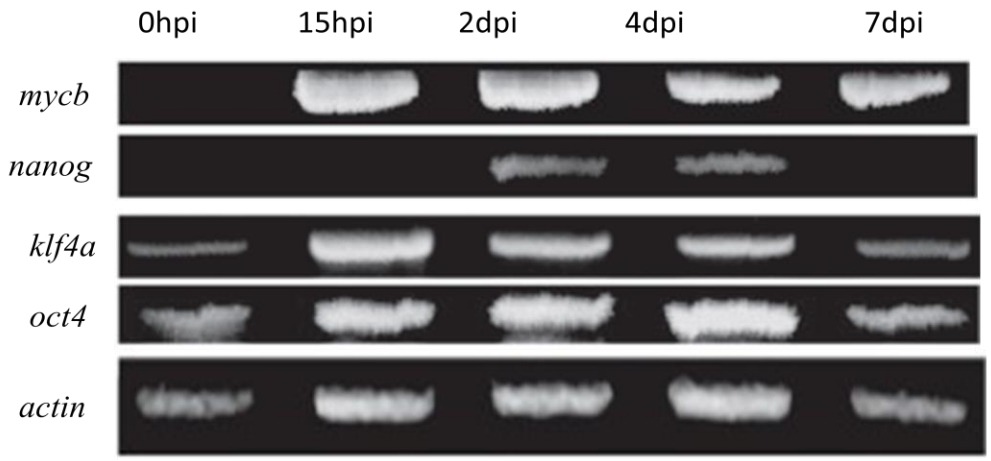


Fig 3: Pluripotency factors in retina regeneration

(Ramachandranet. al 2010)

Here, in the above picture, It can be seen that after post injury to retina, there is induction of pluripotency factors (mycb, nanog, klf4a and oct4) from 15 hpi to 7dpi by Ramachandran et., al 2010.

1.2 Review of literature

A. Pluripotency factors

Pluripotency, the capacity of a cell to give rise to differentiated derivatives that represent each of the three primary germ layers, belongs to the cells that are located within the inner cell mass (ICM) of the developing blastocyst. Functional studies have identified a group of transcription factors, the pluripotency transcription factors that affect the pluripotent capacity. Within this group, the transcription factors Oct4 ,Nanog and Sox2 are crucial for the efficient maintenance of pluripotent cell identity.

Pluripotency transcription factors regulate stem cell pluripotency and differentiation via the colocalization and the cooperation with each other, polycomb repressive complexes (PRC) and microRNAs in the transcriptional and epigenetic regulation of key stem cell genes.Stem cell is a specific cell population with the abilities of self-renewal and multipotent differentiation. According to the origin and potentiality of the cells, mammalian stem cells could be classified into two groups: one is embryonic stem cells (ESCs), which are isolated from ICM of blastocysts; the other is adult stem cells, such as hematopoietic stem cells, neural stem cells and mesenchymal stem cells (MSCs), which are found in adult tissues. In addition, it has been demonstrated that somatic cell can be reprogrammed to pluripotent-like stem cells, otherwise known as induced pluripotent stem-like cells (iPS) by overexpressing specific pluripotency transcription factors, including Oct4, Sox2, c-Myc and Klf4, or Oct4, Nanog, Sox2 and Lin28.[Mak et., al.]

B. Fin Regeneration:

Urodele amphibians and teleost fish possess remarkable capabilities for epimorphic regeneration, that is, the regrowth of complex tissues such as an amputated limb or fin. In contrast, mammalian regenerative abilities are extremely limited. The molecular mechanisms of epimorphic regeneration require further definition, and it is not known why mammals cannot regenerate.

It has been seen that using the zebrafish caudal fin regeneration model, it has been examined the hypothesis that fibroblast growth factors (Fgfs) initiate blastema formation from fin mesenchyme. *,fibroblast growth factor receptor 1* (*fgfr1*) is expressed in mesenchymal cells underlying the wound epidermis during blastema formation and in distal blastemal tissue during regenerative outgrowth. *fgfr1*transcriptscolocalize with those of *msxb* and *msxc,* putative markers for undifferentiated, proliferating cells. A zebrafish Fgf member, designated *wfgf,* is expressed in the regeneration epidermis during outgrowth. Inhibition of Fgf signaling during ongoing fin regeneration prevents further outgrowth while downregulating the established expression of blastemal *msx* genes and epidermal *sonic hedgehog.*It has been indicated that zebrafish fin blastema formation and regenerative outgrowth require Fgf signaling.

Epimorphic regeneration requires the presence or creation of pluripotent cells capable of reproducing lost organs. Zebrafish fin regeneration is mediated by the creation of blastema cells. Fgf has regeneration specific requirement, initiating fin regeneration, and controlling blastema formation. [Cristi et al., Development 2007]

- Activation of Wnt/beta-catenin signaling by overexpression of *wnt8 increases proliferation of progenitor cells in the regenerating fin.*
- Overexpression of *wnt5b reduces expression of Wnt/beta-catenin target genes, impairs proliferation of progenitors and* inhibits fin regeneration.

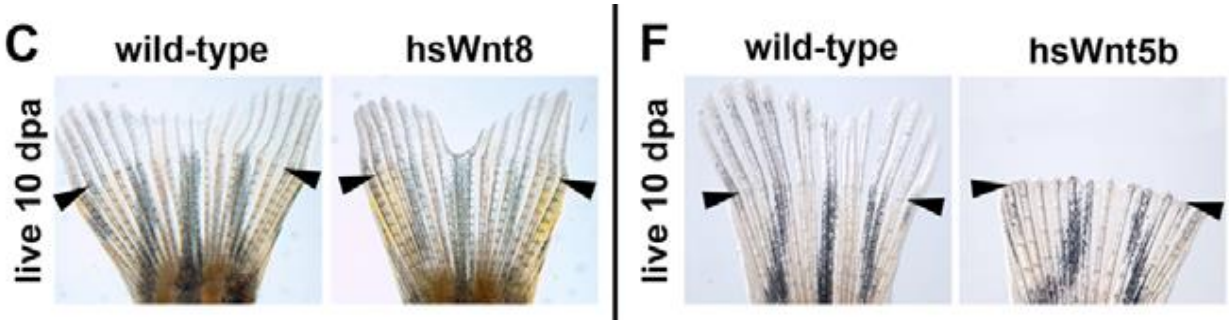


Fig4:Overexpression of Wnt8 and reduced expression of Wnt5

(Cristi et al, Development 2007)

C. Wnt signaling pathway

Wnt signaling pathways are a group of signal transduction pathways made of proteins that pass signals into a cell through cell surface receptors.

- Canonical Wnt pathway,
- Noncanonical planar cell polarity pathway,
- NoncanonicalWnt/calcium pathway.

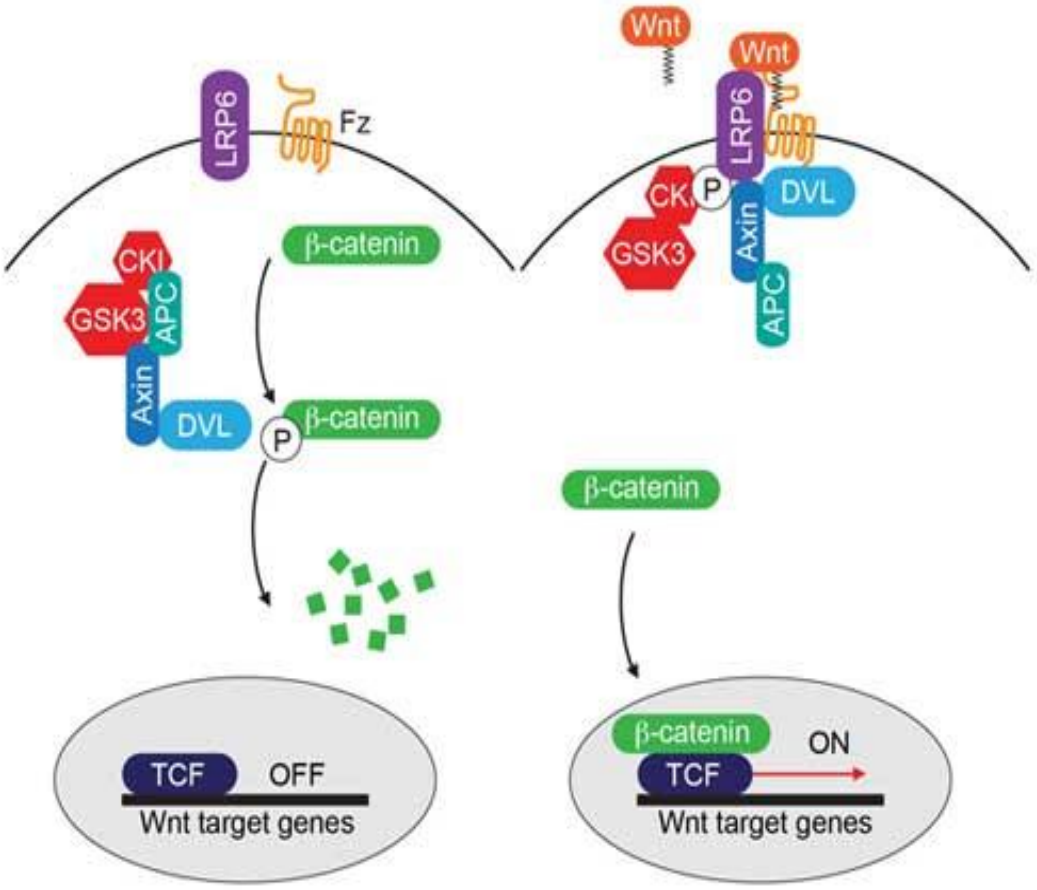


Fig5:Wnt signaling

(Wormbook.org)

All three pathways are activated by binding a Wnt-protein ligand to a Frizzled family receptor, which passes the biological signal to the Dishevelled protein inside the cell. The canonical Wnt pathway leads to regulation of gene transcription. The noncanonical planar cell polarity pathway regulates the cytoskeleton that is responsible for the shape of the cell. The noncanonical

Wnt/calcium pathway regulates calcium inside the cell. Wnt signaling pathways use either nearby cell-cell communication (paracrine) or same-cell communication (autocrine). They are highly evolutionarily conserved in animals, which means they are similar across animal species from fruit flies to humans.

Wnt signaling was first identified for its role in carcinogenesis, then for its function in embryonic development. The embryonic processes it controls include body axis patterning, cell fate specification, cell proliferation and cell migration. These processes are necessary for proper formation of important tissues including bone, heart and muscle. Its role in embryonic development was discovered when genetic mutations in Wnt pathway proteins produced abnormal fruit fly embryos. Wnt signaling also controls tissue regeneration in adult bone marrow, skin and intestine. Later research found that the genes responsible for these abnormalities also influenced breast cancer development in mice.[Meyer et., al.]

D. Effect of LiCl on fin regeneration:

Comparison of normal and LiCl treated catfish clearly shows that regeneration of amputated caudal fins was inhibited or delayed after lithium treatment. After caudal fin amputation, the fish were exposed 3 h daily to 35 mM lithium chloride for 9 days. The effects of lithium chloride treatment were evaluated by analyzing the caudal fin structure at 3, 6 and9 days after amputation. Comparison of normal and LiCl treated fish clearly shows that regeneration of amputated caudal fins was inhibited or delayed after lithium treatment. By the third day after amputation (dpa) either no epidermal cap or blastema ever formed or the epidermal cap had an abnormal morphology in lithium treated fish. By the 3 and 6 dpa no lepidotrichial matrix deposition was observed in the lithium treated fish compared to control fish. Unlike the control fish that completely regenerate their caudal fins after 9 dpa and have fully mineralized lepidotrichia, lithium treated fish have small blastema.

In some treated fish, small amounts of new lepidotrichial matrix were observed at this time, in somefin rays. Ultrastructural observations have shown differences between control and lithium treated fish. Thus, in the lithium treated fish we observed expanded intercellular spaces between epidermal cells and many apoptotic cells.[Otilia et., al. Micron 2013]

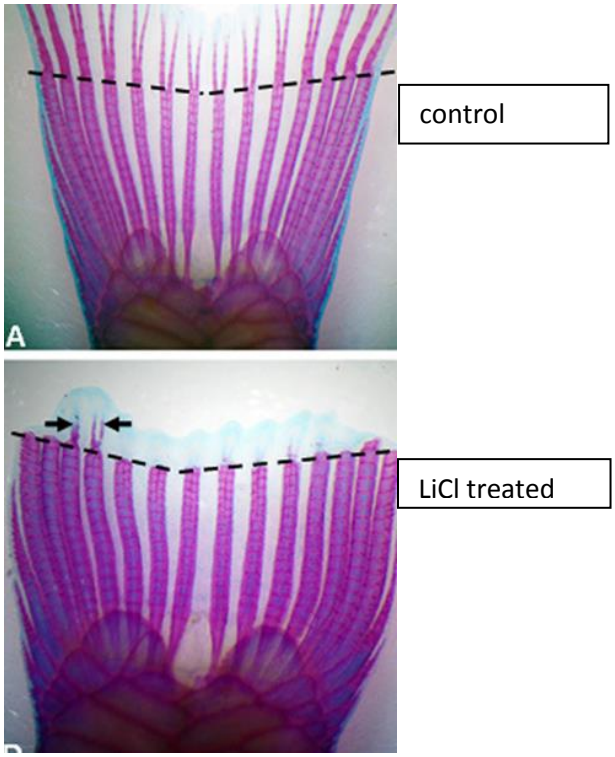


Fig6:Effect of LiCl on fin regeneration

(Otiliaetal., Micron 2013)

Generally, It has been seen that LiCl enhances Wnt signaling pathway by inhibiting GSK3 and beta catenin goes to nucleus and helps in regeneration.

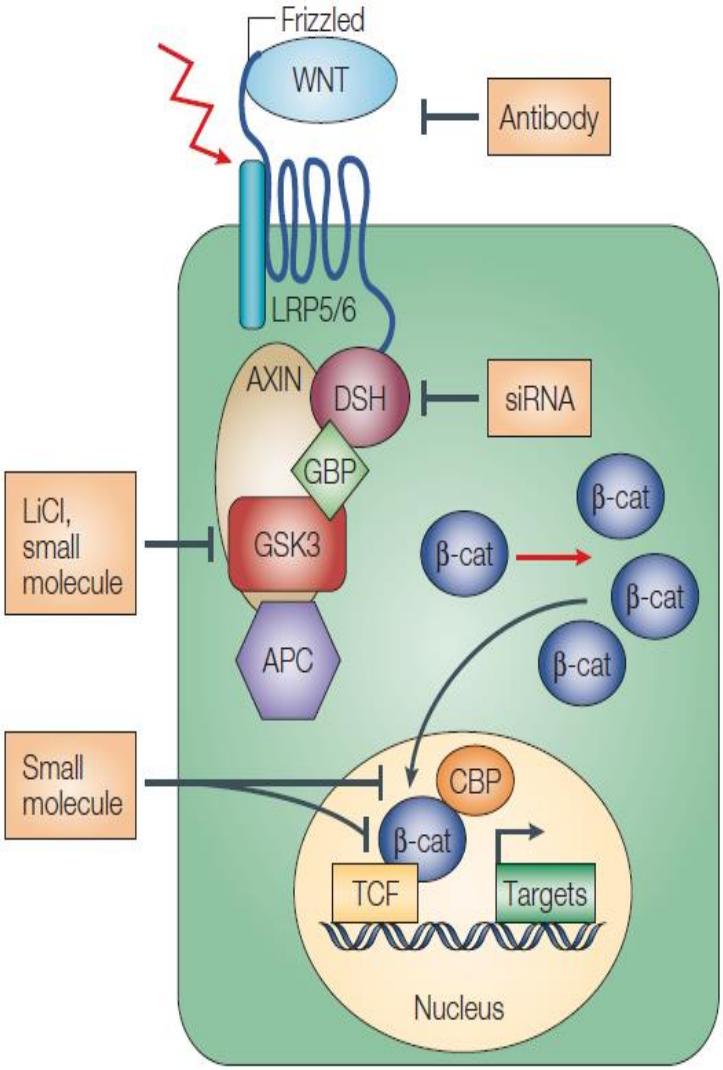


Fig7:LiCl inhibits GSK3

(Galliet.,al2012)

1.3 Materials and Methods

Experiment 1

Methodology:

Three different concentrations of LiCl were prepared as 125uM, 250uM and 500uM and add 50 ml of water to each with a blank control . Then, the caudal fins of fishes were cut and add one fish per concentration. After 4,6 and 8 dpa, observe the results.

Experiment 2

Methodology:

The caudal fins of zebrafish were cut and then were dipped in different concentrations of drugs as mentioned below, then the effect of regeneration was checked.

Drugs	Concentration	dpa
VPA	0.65mM	6
EDTA	0.5mM	6
CONTROL	Water	6
PTEN INHIBITOR	5uM	6
CACL2	0.5mM	6
LICl	0.5mM	6

Table 1: Different concentrations of drugs

Exeriment3:

Methodology:

Here, the effect of lithium chloride on zebrafish embryos is being checked out. So, take 10 zebrafish embryos in water and add lithium chloride on them. Keep a control along with it in which no lithium chloride is added. Observe effect of lithium chloride on zebrafish embryos at 8dpf.

Experiment 4:

Methodology:

First of all, RNA isolation was done from zebrafish embryos and fins and then cDNA was prepared from RNA and then set the PCRs for given pluripotency factors.

i. RNA Isolation protocol

Embryos and fins were taken at the mentioned time points , then grind these embryos using a pestle until embryos extract become clear by taking 200ul of trizol. Add 0.2 volume of chloroform- 40µl and mix for 15-20 minutes. Centrifuge at 4000-7000 rpm at RT for 5 min. Separate upper aqueous layer using a cut tip and transfer it to other MCT. Add, 0.6 volume of isopropanol then keep it at -80^{o}C for overnight or for minimum one hour. Then, On second day, after 20 min. centrifuge at 4000rpm at 4^{o}C for 10 minutes. Discard superenatant and then, give 80% ethanol wash , centrifuge at maximum speed for 10 minutes. Pellet was air dried and dissolved in 30ul of MQ and store it at -80^{o}C and check on gel.

ii. cDNA Preparation(kit used-biorad)

1. Master mix for the reaction(for 20ul reaction)

5X iScript Reaction mix	4ul
iScript Reverse transcriptase	1ul
RNA	4ug
Water	Make up the volume to 20

2. Reaction Parameters-

   5 minutes-25^{O}C

   30 minutes-42^{O}C

   5 minutes-85^{O}C

   Hold-4^{O}C

3. Polymerase chain Reaction

1. Reaction mixture(20ul volume)

20x buffer	1ul
dNTPs	2ul
Primers (Forward+Reverse)	0.4ul
Taq polymerase	0.4ul
Template	as per standardized volume
Water	16.2ul

2. Reaction Parameters

   Enzyme activation-95^{O}C for 2 minutes

   DNA Denaturation-95^{O}C for 15 seconds

   Primer annealing-62^{O}C for 30 seconds

   Elongation- 68^{O}C for 30 seconds

   72^{O}C-7minutes

   Infinite hold-4^{O}C

Experiment 5:

Methodology:

Here, mRNA insitu hybridization was done and dig labelled probes were prepared. Then, spatial expression of pluripotency factors at the eyes, notochord and dorsal region of zebrafish embryos in different pluripotency factors(klf4a,nanog,oct4 and mycb) as mentioned were prepared.(klf4a,nanog,oct4 and mycb).

1. **Protocol for Whole Mount in situ Hybridization**

   Day 1

Prehybridization:RNase Free

- Rinse in 100% MetOH for 5 min, RT
- Rinse in 50:50 MetOH and Xylene for 5 min, RT
- Rinse in 100% Xylene for 30 min, RT
- Rinse in 100% MetOH for 30 min, RT
- Rehydrate tissue at RT

  A. 90% MetOH for 5 min

  B. 70% MetOH for 5min

  C. 50% MetOH for 5 min

- Rinse in 1X PBS /0.1% Tween 2 times, 15 min each at RT
- Make Proteinase K buffer and prewarm to 37^{o}C
- Add 25ul Proteinase K to 37^{o}C buffer, add to tissue for 15 min in 37^{o}C water bath
- Rinse embryos for 10-15 sec in 1X PBS/0.1%Tween at RT

Re-fixation of tissue:

a. Remove 1X PBS/0.1% Tween and add 4% paraformaldehyde in PBS for 20 min at RT

B. Remove fixative and wash with PBS/Tween for 5 min 2 times

- Rinse in 0.1 M TEA for 3 min at RT
- Incubate tissue in acetic anhydride/ TEA solution for 10 min at RT
- Rinse for 30 min in 1X PBS/0.1% Tween
- Prewarm prehybridization and hybridization solutions to 56^{o}C
- Add 500ul Prehybridization solution to each tube, incubate for 1-2 hrs at 56^{o}C
- Prepare DIG/FL probes(2ug/2ul each)

  a. Add calculated amount of DEPC water to tubes containing probes and heat at 65^{o}C for 10 min

  b. Place immediately on ice

- Remove prehybridization solution from tubes with tissue, add 436ul of hybridization solution, add DEPC/ probe

  17. Hybridize overnight at 56^{o}C

DAY 2

Post hybridization

- Prewarm the following solutions:

  10ml 50% formamide/ 2X SSC to 65^{o}C

  10 ml 2XSSC AT 37'C

  10 ml RNase buffer/ 0.1%Tween solution to 37^{o}C

  10ml RNase buffer/0.1% Tween solution to 65^{o}C

- Rinse in 2X SSC, RT
- Wash 1 hour in 50% formamide/ 2X SSC in 65ºC water bath
- Wash in 37'C 2X SSC 3 times for 10 min each in 37ºC water bath
- RNase A:

  a. Add 20ul of RNase A to 10 ml of 37ºC RNase buffer/ 0.1 % Tween

  b. Incubate tissue with RNase buffer/ 0.1% Tween solution for 1 hr at 37ºC

- Wash in 65'C RNase buffer/ 0.1% Tween without Rnase A for 30 min
- Wash 2 times for 15 min each in 2X SSC at 37ºC
- Wash in 1X PBS/0.1% Triton for 15 min at RT
- Block with Maleate/ Triton/ RMB blocker solution for 2 hours
- Incubate in alpha -DIG/alpha -FL antibodies in 1X Maleate / 0.05% Triton/ 1% RMB blocker solution overnight at RT.

Day 3

- Wash with 1X Maleate buffer twice for 5-10 min
- Incubate 2 times/5 min each in Genius 3 and 0.1% Tween
- Transfer embryos to a 24 well plate
- Prepare NBT/BCIP per manufacturer's instructions , incubate 0.5 to 2 hrs at RT with NO agitation in dark.
- Wash in Alk. Phosphate for 30 min at RT
- Wash in 1X phosphate for 5 min at RT.
- Fix in 4% para and phosphate for 30 min at RT
- Rinse 5 min in 1X phosphate or PBS
- Transfer tissue to container, store in glycerol/PBS(2:1)or 100% glycerol at 4ºC in dark

RNA Probe Synthesis:

1. Single digest the TOPO cloned construct of the CDS of the gene of the interest.
2. Validate the digested product on the agarose gel.
3. Gel elute/Precipitate the digested product.
4. Set up the probe reaction(40ul):

Buffer(10X)NEB 3.1	10ul
Template(Digested DNA)	100-500ng
Dig-UTP	2ul
SP6 RNA polymerase	2ul
water	25ul

Table 2: Set up for Probe reaction

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

0.5M Tris EDTA	4ul
5M LiCl	4ul
10mg/ml glycogen	2ul
Absolute ethanol	70ul

Table 3: Precipitation of the probe

8. Centrifuge at 4^{O}C for 15 minutes.
9. Wash with 70% ethanol(200ul) and centrifuge for 10 minutes.
10. Dry and dissolve the pellet in 50ul of DEPEC water.
11. Validate the probe prepared on agarose gel.
12. Store at -80^{O}C for aliquoting.

Experiment6:

Methodology:

Immunostaining

Day1

If slides are stored in -20^{o}C,take them out and keep in 37^{o}C for 2-3 hrs.Wash with 1XPBS for 10 minutes twice each. Keep slides in coupling jars containing 2N HCl at 37^{o}C for 20 minutes. Wash slides with sodium borate (0.1M) to neutralize and incubate at RT for 10 minutes twice. Add 1X PBS+3%BSA+0.1% Triton and incubate for 30 minutes at RT. Overlay slides with primary antibody and keep the container overnight at 4^{o}C.

Day2

Wash the slides using 1XPBST for 10 minutes twice then overlay the slides using secondary antibody(2:1000ul) then wash thrice with 1XPBST for 10 minutes each and then wash it with MQ twice for 10 minutes each then dry the slides for an hour, then put coverslip using dabco.

Microscopy: Confocal microscope(Nikon) is used for imaging.

1.4 RESULTS

Experiment 1: **Effect of Lithium Chloride on fin regeneration**

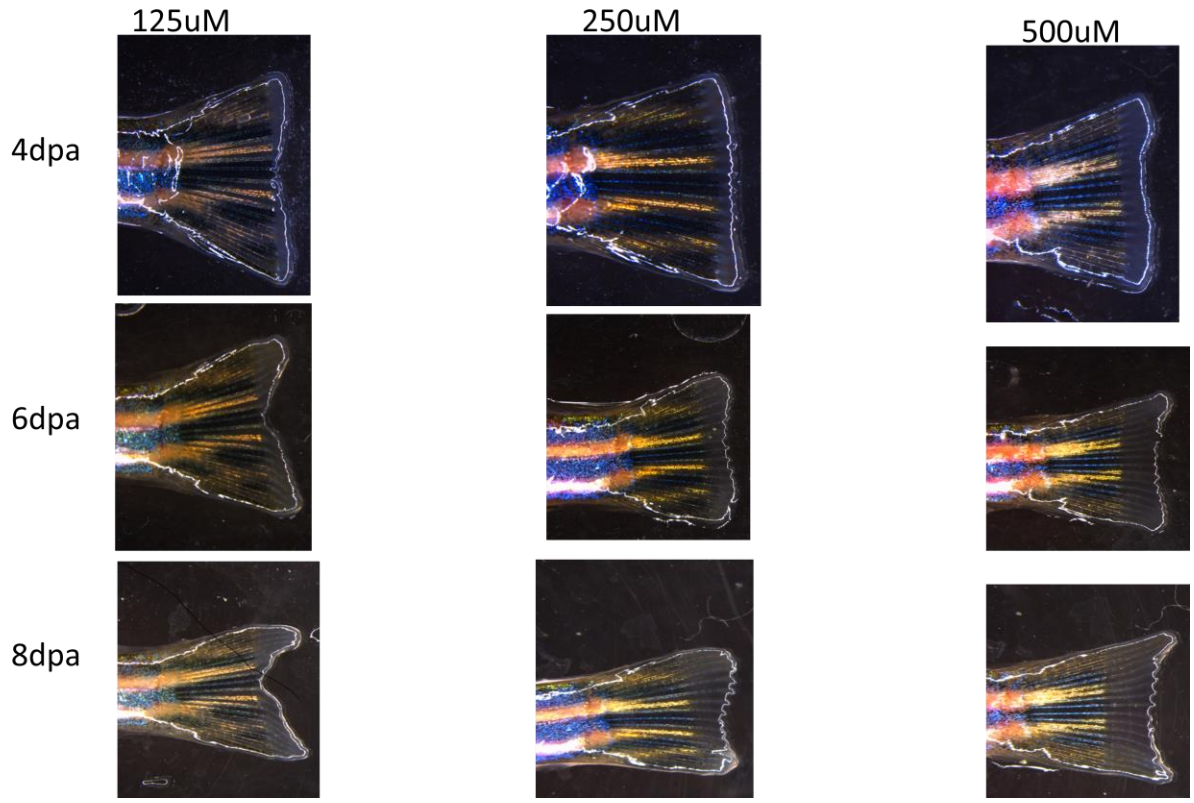
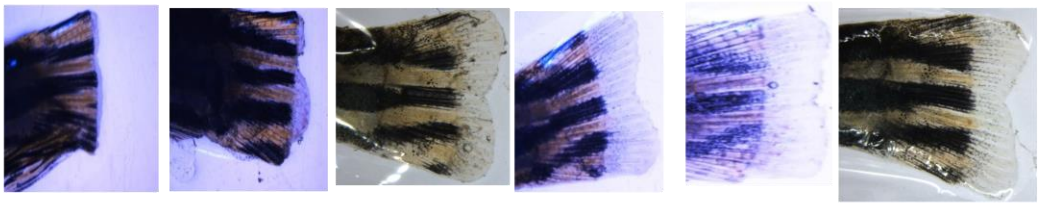


Fig8: Effect of lithium Chloride on fin regeneration

Here, it can be seen that the rate of blastema regeneration has increased from 125µM to 500µM starting from 4dpa to 8dpa.It implies that LiCl acts as a positive regulator of caudal fin regeneration by enhancing Wnt signaling pathway.

Experiment2:

HDACs play essential role during fin regeneration whereas CaCl2 and LiCl enhance proliferation



CHEM	VPA	EDTA	CONT	PTEN INHIBITOR	CaCl2	LiCl
CONC	650µM	500 µM	WATER	5µM	500 µM	500 µM
dpa	6	6	6	6	6	6

Fig9: HDAC play essential role during fin regeneration whereas CaCl2 and LiCl enhance proliferation

Zebrafish caudal fin regeneration was checked here in the above chemicals as mentioned and it can be seen that pten inhibitor,CaCl2 and LiCl enhances blastema formation whereas in contrast to this Valproic acid and EDTA inhibits caudal fin regeneration. VPA basically blocks HDACS, in the presence of VPA , fin regeneration is inhibited, means HDACS play an important role in fin regeneration whereas EDTA is achelating agent, it chelates Calcium ions stating that Calcium ions also play an essential role in fin regeneration.

Experiment3: **Embryos showed delayed hatching at 8dpf**

Fig10: Embryos showed delayed hatching at 8dpf

Here, the effect of LiCl in zebrafish embryos was checked out, So, it can be seen that there is delayed hatching of zebrafish embryos in the presence of LiCl and it acts as a inhibitor of hatching at 8 dpf of zebrafish embryos as compared with a control. Lithium ions activate Wnt signaling which further downregulates hyaluronidase enzyme and in the absence of this enzyme chorion does not break down, that's why embryos showed delayed hatching at 8dpf as compared to normal scenario where embryos hatch at 3dpf.

Experiment4:

Expression pattern of pluripotecy factors during zebrafish embryonicdevelopment

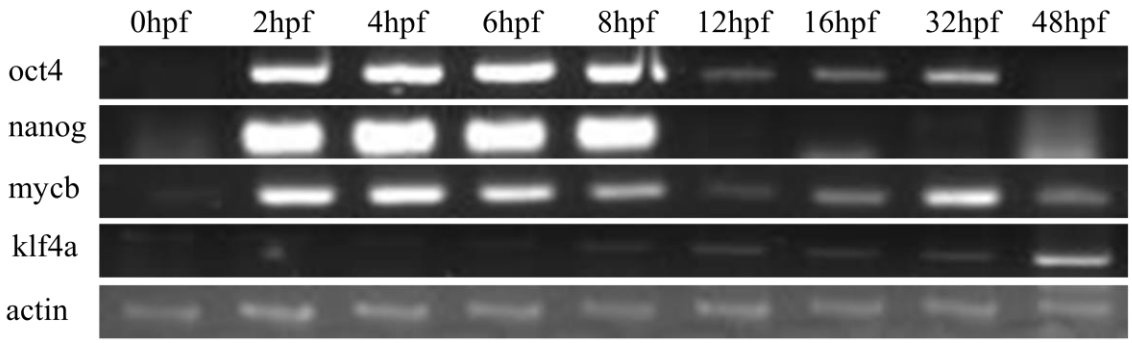


Fig11: Expression pattern of pluripotency factors during embryonic development

During zebrafish embryonic development, oct4, nanog and mycb, mRNA levels has been induced at 2 hours post fertilisation. In contrary to this, klf4a is induced at 2days post fertilization.

Expression pattern of pluripotency factors during fin regeneration

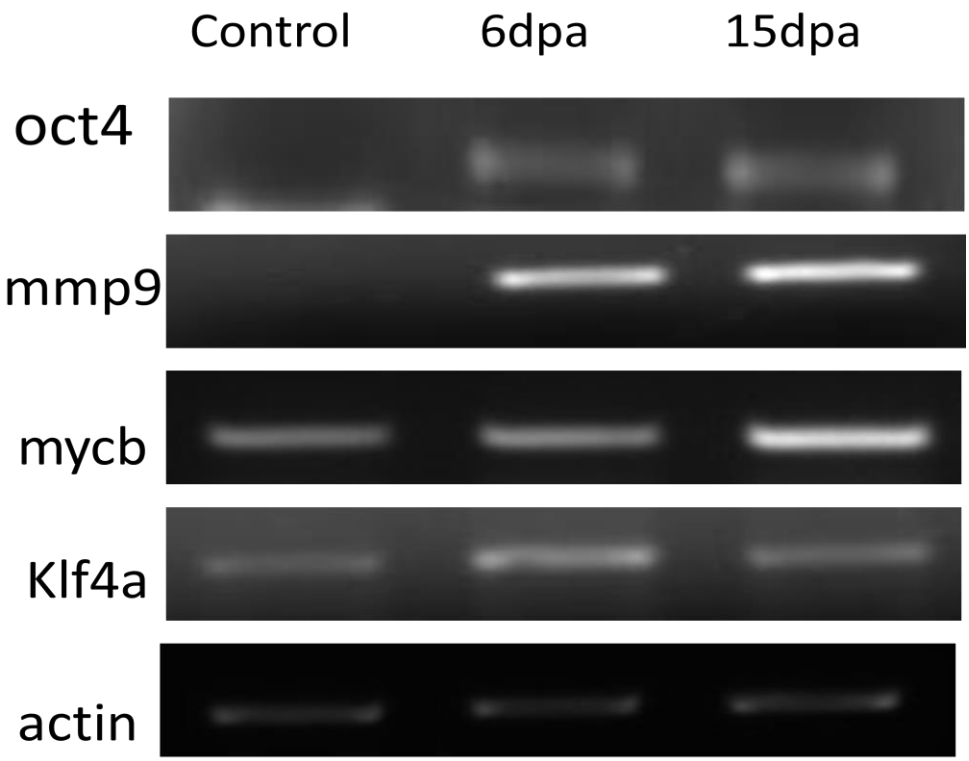


Fig12:Expression pattern of pluripotency factors during fin regeneration

During the course of zebrafish fin regeneration pluripotency factors (klf4a, mycb and oct4) are induced at 6 days post amputation (klf4a), or at 15 dpa (mycb) or at both 6dpa and 15dpa (oct4). Alongside this matrix metalloproteinase (mmp9) expression is also increased at 6dpa as well as 15dpa.

Experiment5:

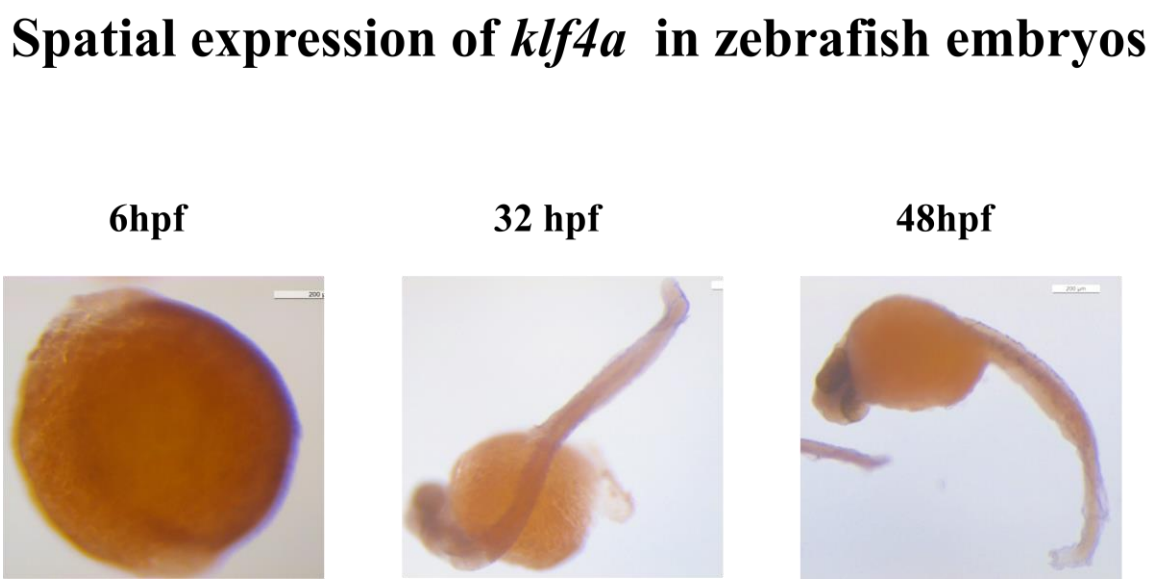


Fig 13: Spatial expression of Klf4a in zebrafish embryos

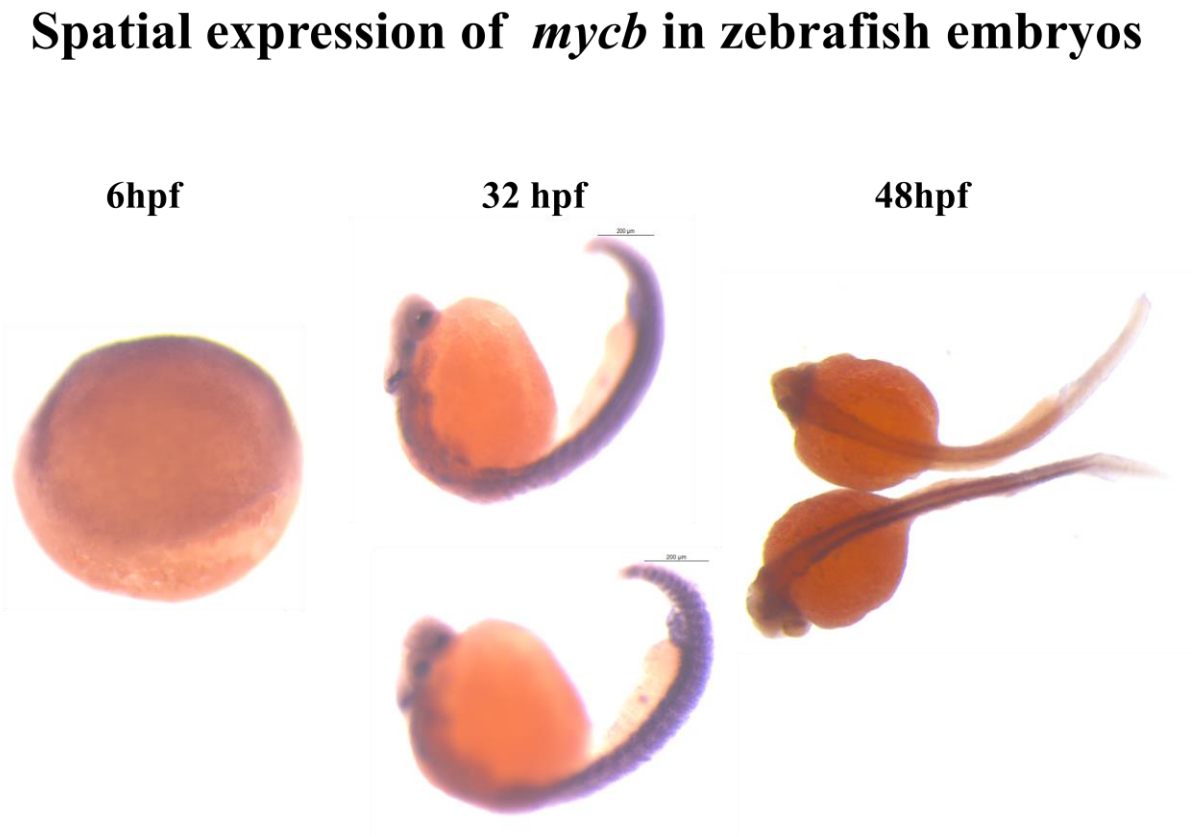


Fig 14: Spatial expression of mycb in zebrafish embryos

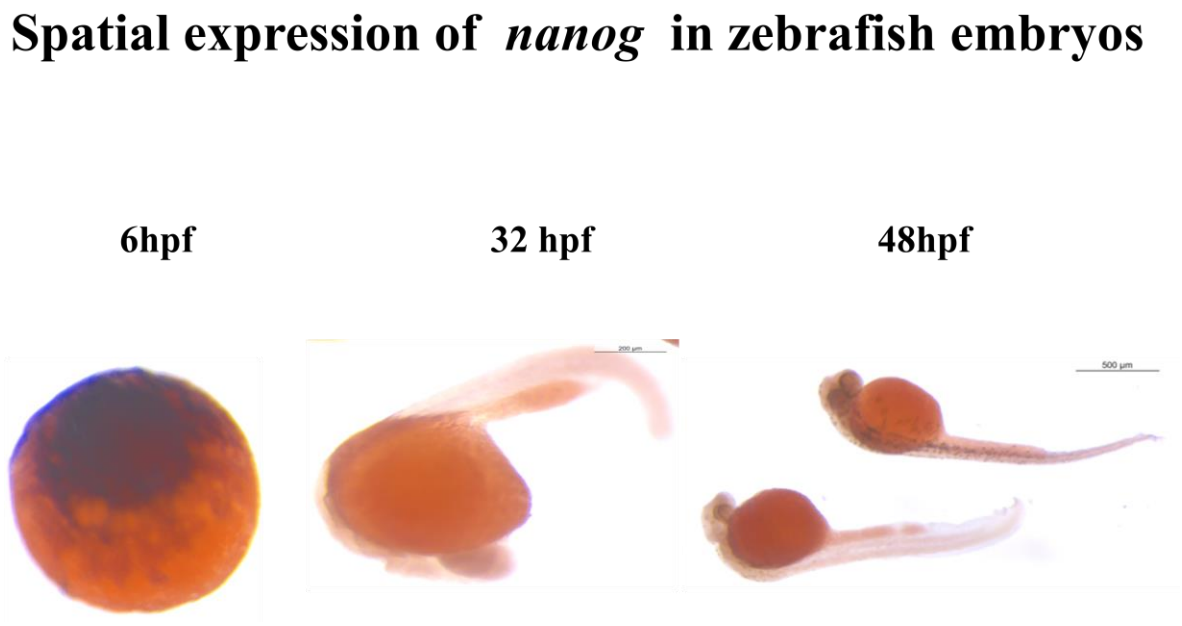


Fig 15:Spatial expression of nanog in zebrafish embryos

Fig 16: Spatial expression of oct4 in zebrafish embryos.

The spatial expression of klf4a can be seen in eyes at 48hpf. The expression of mycb can be seen in notochord region at 32hpf and 48hpf prominently. The spatial expression of nanog is seen in dorsal region at 6hpf whereas spatial expression of oct4 is seen at 6hpf dorsal region and eyes at 48hpf.

Experiment6:

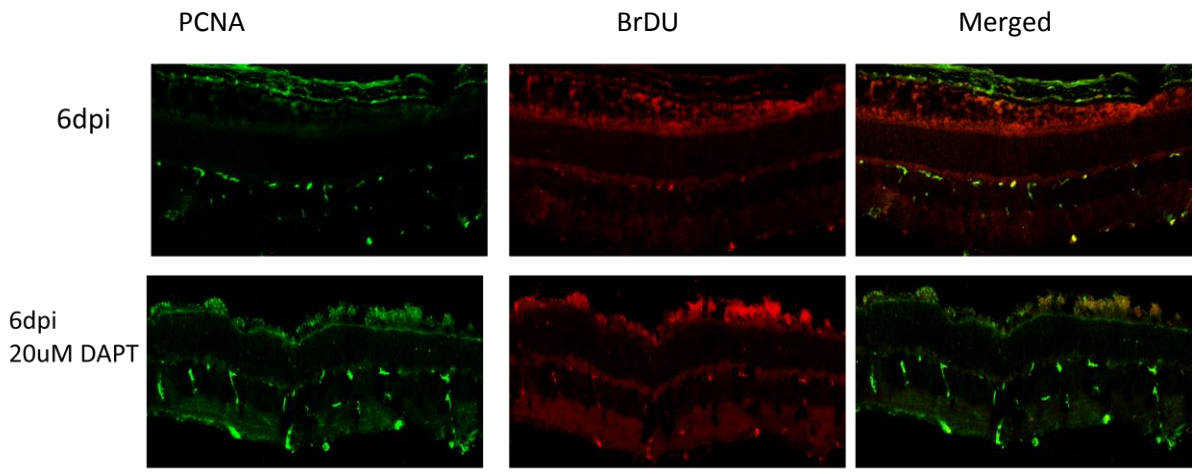


Fig 17:DAPT enhances proliferation

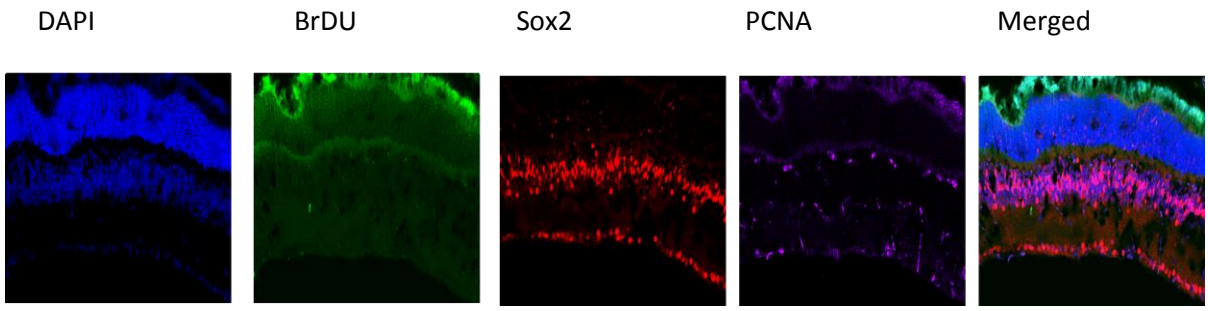


Fig 18:Notch inhibition induces sox2 during proliferation of astrocytes in mice retina

Here, in the above first picture, 20uM DAPT is being used and PCNA and BrDU staining is done, cells at 6dpi with DAPT shows proliferation as compared to the control.

In the second image, it can be seen that notch inhibition induces sox2 during proliferation of astrocytes in mice retina.

2. Summary and Conclusions

2.1 Concluding Remarks

1. LiCl acts as a positive regulator of caudal fin regeneration by enhancing Wnt signaling pathway as the blastema regeneration increased with increasing concentrations of LiCl.
2. Ptenb inhibitor and EDTA acts as a negative regulator of fin regeneration whereas HDACs, LiCl and CaCl2 plays an important role in fin regeneration.
3. Embryos showed delayed hatching at 8dpf in the presence of Li ions as compared to the control at 3dpf. As Li ions activate Wnt signaling pathway which further downregulates hyaluronidase enzyme and in the absence of this enzyme chorion does not break.
4. Pluripotency factors has been induced during retina regeneration, embryonic development and fin regeneration.
5. During mRNA in situ hybridisation, spatial and temporal expression of pluripotency factors has been obtained.
6. In mammalian retina, notch inhibition induces sox2 during proliferation of astrocytes and DAPT enhances proliferation.

2.2 Future Outlook

1.To check effect of LiCl on pluripotency factors in fin regeneration.

2. To check mechanism of delayed hatching in zebrafish embryos.

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