Understanding lithium induced regenerative response in mouse and zebrafish retina and regenerative responses induced in mouse digits on immuno-suppression.

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I think I owe a lot more to the zebrafish and mouse that we experimented on than to any other person. Thank you, little ones, for sacrificing lives for us. I also express my gratitude to IISER Mohali and INSPIRE-DST for facilities and financial support.

CONTENTS

List of figures

List of Tables

Notations

1. **CNS**- central nervous system
2. **MG**- Muller Glia
3. **INL**- inner nuclear layer
4. **ONL**- outer nuclear layer
5. **GCL**-ganglionic cell layer
6. **GC**- ganglionic cell
7. **RGC**- Retinal ganglionic cell
8. **NMDA**- N-methyl-D-aspartate
9. **GSK3B**- Glycogen Synthase Kinase 3 Beta
10. **PCP**- planar cell polarity
11. **RT**- room temperature
12. **BDNF**- Brain-derived neurotrophic factor
13. **BrdU**- Bromodeoxyuridine
14. **PCNA**- Proliferating cell nuclear antigen
15. **PBS**- phosphate buffer saline
16. **PFA**-Paraformaldehyde
17. **DEPC**- Diethyl pyrocarbonate
18. **OCT**- optimum cutting temperature
19. **MCT**- microcentrifuge tube
20. **PCR**- polymerase chain reaction
21. **PBST**- phosphate-buffered saline with Triton
22. **BSA**- Bovine serum albumin
23. **DABCO**- 1,4-diazabicyclo[2.2.2]octane
24. **dpa**- days post-amputation
25. **dpi**- days post-injury
26. **DAMPS**- Damage-associated molecular patterns
27. **PAMPS**- Pathogen-associated molecular pattern molecules
28. **FACS**-Fluorescence-activated cell sorting
29. **SCID**- Severe combined immunodeficiency

Part - 1

Understanding Lithium induced regenerative response in mouse and zebrafish retina

Chapter 1: **ABSTRACT**

The retina is the light-sensitive tissue layer that lines inside of the eye that sends visual signals to the brain through the optic nerve. Eye injuries and certain eye diseases can damage retinal tissue and leads to blindness. Retinal regeneration refers to the recovery of vision in vertebrates that have suffered retinal lesions or retinal degeneration. Lower vertebrates, like zebrafish, shows the extensive regenerative capability of their retina. Mammals like mice have the same genes and pathways, but they lack such extensive regenerative potential. Müller glia is the cells from which all other retinal cell types are regenerated in zebrafish. These cells support the healthy functioning of neighboring neurons and hold the innate regenerative ability. Wnt signaling pathways play indispensable roles in cell fate specification, cellular proliferation, and differentiation. LiCl can inhibit GSK3β activity and stabilize free cytosolic β-catenin efficiently, thereby behaving as an agonist of canonical Wnt signaling. Though lithium potently inhibits GSK-3 beta activity. It is not a general inhibitor of other protein kinases. Lithium shows neuroprotective nature against a wide variety of processes, including anticonvulsants and potassium deprivation. It is also known to promote the neuronal survival and axonal regeneration of retinal ganglionic cells through a Bcl-2-dependent mechanism in the rat model. This study was done to understand the variation in lithium-induced regenerative responses between mouse and zebrafish retina. BrdU staining was used to establish the proliferation status of retina both in terms of the number of cells and their localization. Gene expression patterns in both models were analyzed and compared. Overall this study gives new insights and better comparison of LiCl effect on injury responses between mouse and zebrafish retina models.

Chapter 2: **INTRODUCTION**

Regeneration is a fascinating phenomenon that enables the restoration of lost body parts. All living organisms naturally possess regeneration potential up to some extent, to maintain tissues and organs. Some lower vertebrates like zebrafish, planaria, hydra, and axolotl possess extensive regenerative abilities. More complex animals, like mammals, shows minimal regenerative capacities. These include the formation of scars to promote the healing of injured or amputated body parts, regrowing hair and skin, and the knitting together of fractured bones (Cai, Fu, and Sheng 2007; Tanaka and Reddien 2011; Wan and Goldman 2016). In humans, when the part of the liver is removed or destroyed, it undergoes a phenomenon called compensatory hypertrophy to grow back the remaining tissue to the original size though not the unique shape.

The zebrafish (Danio rerio), a teleost fish can replace a damaged or lost fin, and can significantly repair damaged heart, pancreas, retina, brain, and even spinal cord. Mammals like mice have the same genes and pathways, but they lack such extensive regenerative potential. The retina is the relatively simple and accessible part of the CNS as it lies at the back of the eye. Zebrafish can regenerate retinal layers, thanks to specialized cell types called Muller glia (MG) (Goldman 2014; Wan and Goldman 2016). Even though similar Muller glial cells are present, CNS does not regenerate in mammals, and injuries or disease to the CNS generally result in irreparable damage.

Therefore, unlocking the secrets of MG's reprogramming and comparing these two model organisms will shed light on the possible ways with which we can prompt the mammalian retina to regenerate.

4.2.1 The retinal structural framework and Injury Response

4.2.1.1 The retinal structure

The retina consists of three main nuclear cell layers, namely the inner nuclear layer (INL), outer nuclear layer (ONL), and ganglionic cell layers (GCL). The photoreceptor cells-cones and rods are the most significant components of ONL(Fig:4.2.1). These photoreceptors sense light and transfer the signals to

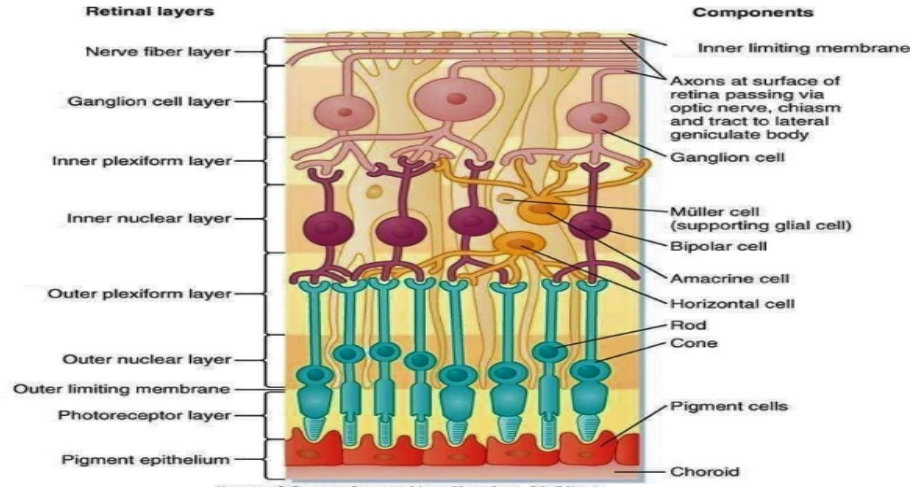


Fig:4.2.1 Different layers of retina and its components (Bruce Koeppen Bruce Stanton 2009). The photoreceptor cells cones and rods sense light and transfer the signals to ganglionic cells (GC) through INL.

ganglionic cells (GC) through INL. INL consist of amacrine cells, bipolar cells, horizontal cells, and Muller Glia (MG).

This kind of anatomical framework and the housing of MG cells in the INL allow MG to monitor any changes in the retinal neuronal homeostasis. Since MG is well-positioned in the retina, it can help in the transfer of various molecules across the layers of the retina. More importantly, such perfect positioning enables muller glia to react to retinal injuries quickly and initiate early injury response like the gathering of various inflammatory molecules and satellite cells. So, this is extremely beneficial considering the significance of Muller glia in retina regeneration.

4.2.1.2 Muller glia: injury response and regeneration

Unlike mammals, zebrafish retina shows extensive regenerative capability. The key to this regenerative response is the major glial component of the retina - Muller glia (MG) cells. They help in maintaining retinal homeostasis and integrity. Injuries can be exacted on the retina by three main ways depending on the type of cells targeted.

i. **Mechanical injury** (simple poke with a needle): damages all retinal layers
ii. **Laser/UV injury**: damages photoreceptor cells
iii. **Chemical injury** (using N-methyl-D-aspartate (NMDA)): damages ganglionic cells (GC).

In Zebrafish, when there is a retinal injury, MG undergoes a reprogramming event that allows them to divide and generate retinal progenitor cells. These progenitor cells are multipotent stem cells and can develop into all major retinal neuron types. Mammals also possess similar MG cells, but its activity in terms of injury response is limited, which can be either protective or detrimental to retinal function(Goldman 2014; Martin and Poché 2019; Wan and Goldman 2016). The process of regeneration of retina moves through 3 main stages:

i. **Dedifferentiation**: cells revert from a specialized function to a simpler state evocative of stem cells.
ii. **Proliferation**: asymmetric cell division, cell multiplication, and active nuclear migration to ONL.
iii. **Redifferentiation:** upon migration, these cells reform into required cell types performing specific functions.

Muller glial cell reprogramming in zebrafish involves the activation of various signaling pathways including, glycogen synthase kinase 3β (Gsk3β)-β-catenin, mitogen-activated protein kinase – extracellular signal-regulated kinase (Mapk-Erk), and Jak-Stat signaling. Wnt expression and β-catenin stabilization play an essential role in MG reprogramming. The inhibitory pathways- let-7 miRNA signaling, Dkk signaling, and Notch signaling are necessary to maintain a quiescent state of MG and to keep a check on cell division (Meyers et al. 2012; Ramachandran, Zhao, and Goldman 2011). Various factors like Ascl1a, Myc-a, and Lin28 also seems to regulate the earliest stages of Müller glial cell reprogramming.

Growth factors and cytokines can arouse the proliferation of MG cells in the damaged mouse retina, but they have a very limited ability to produce new neurons. Forced expression of Ascl1

can direct muller glia to become retinal neurons in the newly born mouse. But, the regions of genetic code that are targetted by Ascl1 are no longer accessible when they reach adulthood.

4.2.2 Wnt-signalling and the Lithium effect

4.2.2.1 Wnt- signalling

Wnt signaling pathways play indispensable roles in cell fate specification, cellular proliferation, differentiation, cell migration, and planar cell polarity (PCP). They are driven by secreted glycoproteins called Wnts and are classified into canonical Wnt/β-catenin or non-canonical (β-catenin-independent) pathways.

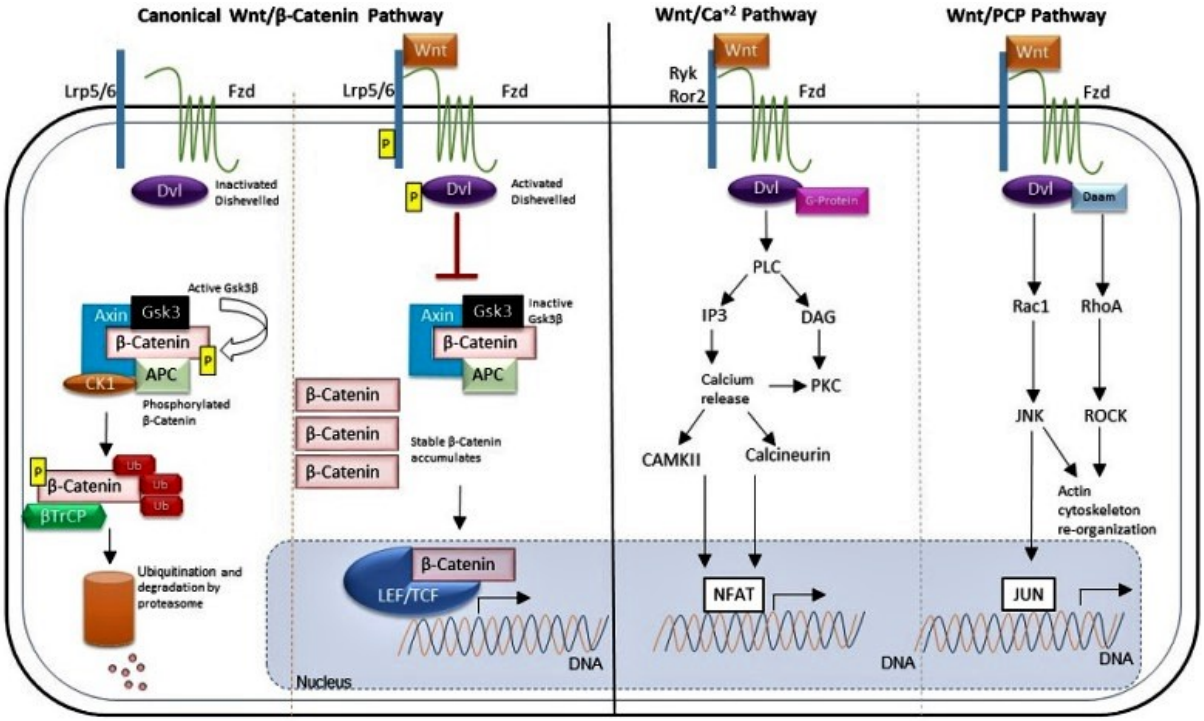


Fig4.2.2 An overview of Wnt signaling pathways (Garcia et al. 2018). Canonical Wnt/β-catenin signaling pathway signals through cytoplasmic stabilization and accumulation of β-catenin in the nucleus to activate gene transcription. In β-catenin-independent Wnt signaling, several alternative signaling mechanisms, including calcium flux, JNK, and heterotrimeric G-proteins, have been involved.

Canonical Wnt/β-catenin signaling is the most studied and is mediated by nuclear translocation of its central effector β-catenin. This pathway signals through cytoplasmic stabilization and accumulation of β-catenin in the nucleus to activate gene transcription. In the absence of Wnt ligands, cytoplasmic β-catenin is prevented from reaching its nuclear targets due to its constitutive degradation by a protein complex containing axin, casein kinase 1 (CK1), glycogen synthase kinase 3 (GSK3β), and adenomatous polyposis coli (APC). Wnt signaling activation is initiated by binding of ligands to Frizzled (Fzd) receptors and the co-receptor low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6). Formation of this receptor complex and recruitment of the scaffolding protein Dishevelled (Dvl) leads to phosphorylation of LRP5/6 and thereby inhibits β-catenin degradation. Stabilized β-catenin then accumulates and translocates to the nucleus, where it forms large protein complexes comprising the T-cell factor/lymphoid enhancer factor (TCF/LEF). Formation of these transcription factors leads to

the induction of Wnt target genes(Meyers et al. 2012; Munnamalai and Fekete 2013; Veien et al. 2008).

In contrast, in β-catenin-independent Wnt signaling, several alternative signaling mechanisms, including calcium flux, JNK, and heterotrimeric G-proteins, have been involved. Non-canonical Wnt signaling is induced by Wnt ligands that bind to a receptor complex of Fzd, Ror1/2 or Ryk. Binding to these receptors induces signaling through the Wnt/planar cell polarity (PCP) and Wnt/Ca2+ pathways. The PCP pathway is initiated when Fzd receptors activate a cascade involving GTPases RAC1, c-Jun N-terminal-kinase (JNK), and the Ras homolog gene family member A (RHOA), and. The other main non-canonical pathway, the Wnt/Ca2+ pathway, is induced when Wnt ligands bind to Fzd receptors, which activate heterotrimeric G proteins. This leads to the activation of phospholipase C and the release of calcium from intracellular stores(Meyers et al. 2012; Munnamalai and Fekete 2013).

4.2.2.2 Lithium Chloride induced Wnt activation and neuroprotection

Lithium chloride has been used as medicine in psychiatric treatments for very long. It is one of the most effective drugs for the treatment of the bipolar disorder, and it also has dramatic effects on morphogenesis in the early development of numerous organisms. LiCl can inhibit GSK3β activity and stabilize free cytosolic β-catenin effectively, thereby acting as an agonist of canonical Wnt signalling (Jorstad et al. 2017; Klein and Melton 1996; Silva et al. 2010). Though lithium potently inhibits GSK-3 beta activity, It is not a general inhibitor of other protein kinases. Though the proper mechanism is not known, there are two existing theories regarding lithium-induced inhibition of GSK-3. According to the first theory, lithium is a competitive inhibitor of GSK-3 with respect to magnesium, but neither competitive to substrate nor ATP. But the second theory state that lithium represses potassium deprivation, thereby inhibiting GSK-3 activity (Kramer, Schmidt, and Lo Monte 2012).

Lithium has explicit *in vitro* and *in vivo* protective effects on various central neurons, which includes the protection of cultured cell lines, including cerebral cortical cells, cerebellar granule cells, hippocampal neurons, and PC12 cells. Lithium shows neuroprotective nature against a wide variety of processes, including anticonvulsants and potassium deprivation (Chuang et al. 2002; Leng et al. 2008). It can also promote the neuronal survival and axonal regeneration of retinal ganglionic cells through a Bcl-2-dependent mechanism (Huang et al. 2003). After an optic nerve injury, lithium can delay the death of axotomized RGCs in a dose-dependent manner within a specified period. Such a beneficial effect of lithium is interrelated with an upregulated level of intraretinal BDNF. BDNF/TrkB pathway plays a crucial role in mediating the neuroprotective nature of lithium (Wu et al. 2014). BDNF binds to the TrkB receptor, and thereby excites certain biological activities in anti-apoptotic pathways such as phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways

Chapter 3: **MATERIALS and METHODS**

4.3.1 Licl administration in mouse and zebrafish

4.3.1.2 In Zebrafish

- Zebrafish were anesthetized using Tricaine methanesulfonate.
- The retina was injured by poking with a 30-gauss needle.
- They were then transferred immediately to tanks with a desired concentration of LiCl.
- LiCl Solution is changed every day until the retina is harvested at four days post-injury.
- For BrdU staining, all the fishes were dipped in 5 mM BrdU solution for 6-8 hours before harvest.

4.3.1.2 In Mouse

- The universally accepted wild type strain-C57BL/6J is used for the experiments.
- Mice were anesthetized by using avertin(222-tribromoethanol).
- LiCl injection was given intraperitoneally every day, starting from one day before retinal injury till the day of harvesting of the retina.
- The retina was injured by first poking with a 30-gauss needle, and then by injecting 2 µl of 100mM NMDA (N-methyl-D-aspartate) intravitreally.
- The retina was then harvested at four days post-injury.
- For BrdU staining, 1 mg/mL BrdU injection was given intravitreally on the second-day post-injury, and 20mM BrdU injection was given intraperitoneally on the second, third, and fourth days post-injury.

4.3.2 Retina dissection for RNA isolation/tissue fixation

- Zebrafish was anesthetized using tricaine methanesulfonate before harvesting the eyes and the retina was dissected using steel forceps and needle. On the other hand, Mice were given Euthanasia with the help of the CO2 chamber, and the harvested eyes were washed in 1xPBS before dissection.
- Dissections were carried out either in 1X PBS (Phosphate Buffered Saline) in DEPEC for harvesting retina for RNA isolation or 4% paraformaldehyde (PFA) for tissue fixation.

- Lens was removed for both isolating RNA/ tissue fixation.

- If the retina is to be 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 and stored at -80ºC.

4.3.3 Tissue fixation and sectioning

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

- Next day give the following serial washings of the fixed tissue at room temperature 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.

- Then remove 500µl of sucrose solution from it and add 500µl of OCT and rotate it for 30 min.

- Then embed them in OCT in small cubes made from aluminium foil.

- The embedded samples are stored at -80ºC until sectioning.

- Section the blocks in cryostats (12µm thickness) and collect the sections on super frost plus slides.

- These slides were dried overnight and then stored in -20ºC.

4.3.4 RNA isolation

- Take the retina stored in trizol from -80ºC and keep on ice to thaw it.

- Homogenize the tissues completely using 200µl pipette till no tissue clumps are visible.

- Then keep at room temperature for 10 mins.
- Add 0.2 volumes (40µl for 200 µl of trizol with tissue) of chloroform and mix it well by inverting the MCT up and down for 20 seconds.
- Then centrifuge it at 12000 rcf for 15mins at 4ºC.
- Using cut tips, collect the aqueous phase slowly without disturbing the phase layer, and transfer it to fresh MCTs.
- Add Isopropanol (double the amount of aqueous phase collected) and keep it at -80ºC for overnight precipitation.
- The next day, centrifuge it at 10000rcf at 4ºC for 20mins.
- Discard the supernatants.
- Wash it with 80% ethanol (200µl per MCT) and centrifuge it at 4ºC for 10 min at 7500 rcf.
- Dry the pellet till alcohol smell is no longer there and dissolve the pellet in DEPC treated water and stores it at -80ºC.
- Check whether there is any contamination of genomic DNA in it by using agarose gel electrophoresis (1%gel). We can also measure the amount of RNA present by measuring the Optical density using a spectrophotometer (nanodrop) and proceed further for cDNA synthesis.

4.3.5 cDNA synthesis (using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit)

The reaction is set for 10µl reaction

- Take a PCR tube and add 5µl Template RNA, 0.5µl Oligo (dT) Primer and 0.5µl Random Hexamer
- Mix it properly and denature it by putting in 650C for 5min, after this, immediately transfer it to ice for about 2 min.
- Add the following to the above mixture:-
  - 5X Reaction Buffer - 2µl
  - RiboLock RNase inhibitor - 0.5µl
  - 10mM dNTP Mix – 1µl
  - RevertAid M-MuLV RT – 0.5µl
- Mix the reaction mixture properly and Spin it down briefly.

- Set the following incubation program in the thermocycler:
    - 25oC – 5 mins
    - 42oC – 60 mins
    - 70oC – 5 mins
    - 4oC – infinite hold

- Store the cDNA in -80o C

4.3.6 Reverse transcription PCR (RT PCR) using Taq Polymerase

- Dilute the synthesized cDNA by 1:4 dilutions

- For 10µl reaction, the following components are used:
    - 20X buffer- 0.5µl
    - dNTP-1µl
    - forward primer and reverse primer(F+R) -0.5µl
    - template cDNA-0.5µl
    - MQ water -7.4µl
    - Taq polymerase -0.1µl

- Amount of template can vary as per standardization of samples.

- Generally, the following cycling conditions are used: -
    - 98ºC - 2min
    - 98ºC - 15secs
    - 60ºC - 30secs
    - 72ºC - depending on the size of the gene (1kb/1min)
    - 72ºC - 5min
    - 4ºC - Infinite hold

- Number cycles can be standardized as per the gel image obtained after loading the PCR product.

- Check the PCR product by agarose gel electrophoresis.

4.3.7 Quantitative PCR (qRT-PCR)

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

- For 5µl reaction, following components are used: -
    - SYBR Master mix- 2.5µl
    - Primers (F+R)-0.5µl
    - Template -0.5µl
    - MQ water-1.5µl

- Analyze the results on an excel sheet and plot the graph.

4.3.8 Immunostaining

- Take the slides stored in -20^{o}C and dried them in 37^{o}C for half an hour.
- Wash the slides three times with 1xPBS, 10 min each by overlaying over the slides.
- Then put the slides in 2N HCL at 37^{o}C for 20min.
- Wash the slides twice with Sodium borate (pH 8.5, 0.1M) for 10 min each.
- Block the tissues using 5% BSA in 1XPBST for four hours.
- Overlay the 1^{o} Antibody diluted in 1%BSA /PBST (1:1000) over the slides after blocking and keep it in 4^{o}C overnight.
- Next day, wash the slides thrice for 10 min each with 1xPBST.
- Overlay 2^{o} antibody diluted in 1%BSA in 1xPBST (1:1000) and keep in RT for 3 hours.
- Wash the slides thrice with PBST for 10 min each.
- Overlay the DAPI solution and keep at RT for 90 seconds.
- Wash the slides thrice with PBST for 10 min each.
- Wash the slides twice with MQ and let it dry for 30mins.
- Then mount the dried slides with DABCO and keep overnight.
- Next day, Take the image of the section under the confocal microscope.

Chapter 4: **RESULTS and DISCUSSIONS**

4.4.1 Lithium induced proliferation status in zebrafish retina

BrdU/PCNA staining is beneficial in understanding the proliferation status of the retinal MG cells. It not only helps in understanding the rate of proliferation but also about the localization of the proliferating cells.

There is a steady uptick in the number of BrdU positive cells as the concentration of lithium increases. On average, the number of BrdU positive cells per section of the retina was found to be about 49, 71, and 121 for injured control, 10mg/Kg LiCl, and 60mg/Kg LiCl, respectively. The bar graph plotted gives a clear comparison of the number of proliferating cells in response to lithium.

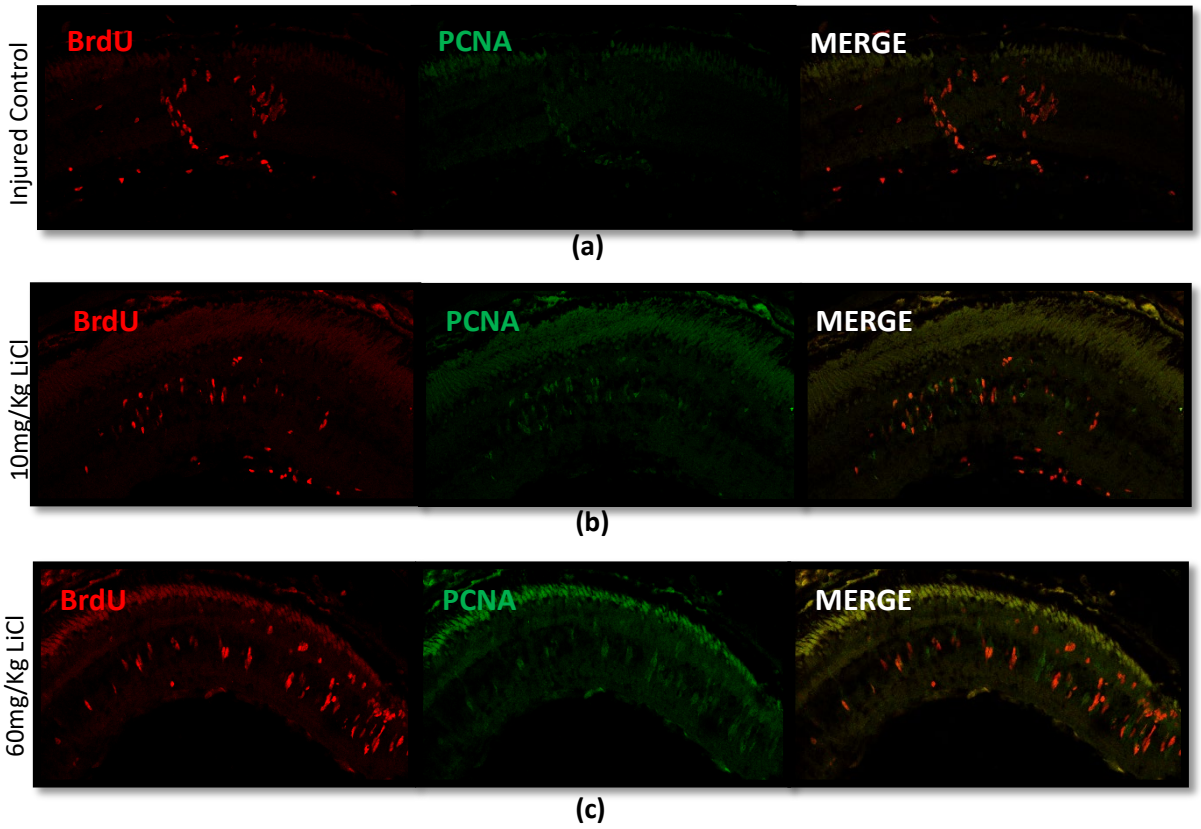
The effect of NMDA and its combined status with LiCl was also assessed. Full-size stitching of retina was done to get a whole retina proliferation status in the image format. It was done by taking individual 20X images of small portions of the retina and then stitching them together with the help of a software called Image Composite Editor. There was a significant increase in the number of BrdU positive cells when LiCl treatment was given along with NMDA injury.

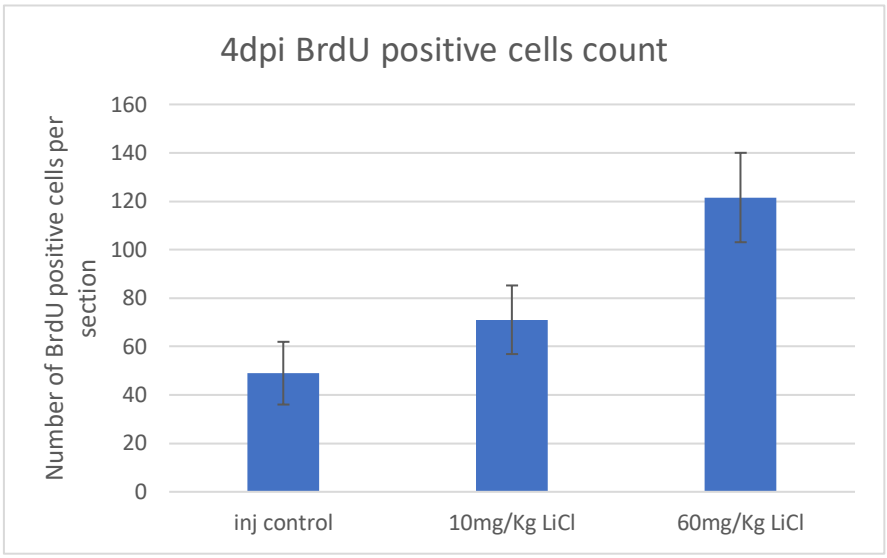
4.4.2 Lithium induced gene regulation in zebrafish retina

Ascl1a is a pro-neural transcription factor that is known to regulate Müller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signaling pathway (Ramachandran, Fausett, and Goldman 2010). The qPCR results show an upregulation of Ascl1a in 4dpi retina on treatment with LiCl, and Ascl1a expression is increasing with increasing concentration of LiCl. RT-PCR result also confirms the same. As expected, complementary to Ascl1a upregulation, Lin28 is also upregulated by LiCl.

HDAC1 is upregulated by LiCl, and especially 60mg/Kg LiCl shows a significant increase as compared to control. Both qPCR and RT-PCR results confirm this. Myc-a is down-regulated by LiCl while Lin 28a is upregulated.

Oct4 and Sox2 are Yamanaka factors, and their overexpression is known to induce pluripotency. An increase in Oct4 and Sox2 expression shows the increase in pluripotent stem cells in response to LiCl treatment.





(d)

Fig 4.4.1: **(a)**, **(b)** and **(c)** are the 4dpi zebrafish retinal images of injured control, 10mg/Kg LiCl, and 60mg/Kg LiCl, respectively, under the confocal microscope showing BrdU positive cells in the red channel and PCNA in the green channel. **(d)** represents the variation in the number of BrdU positive cells at 4dpi.

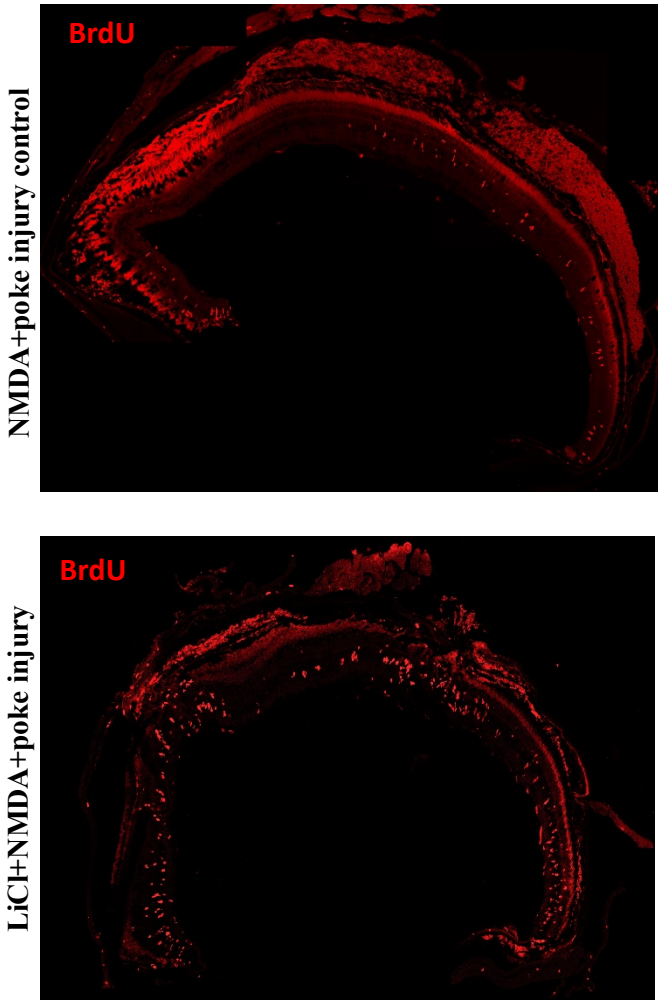
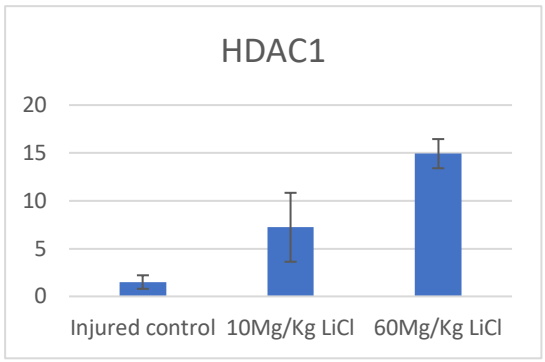
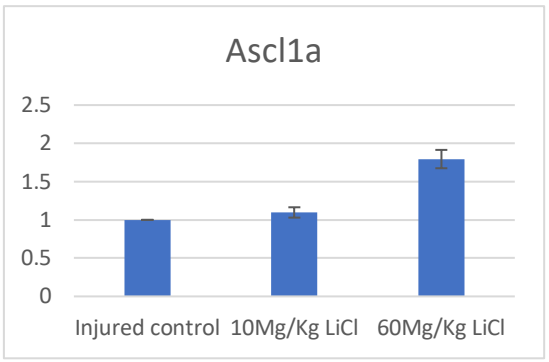


Fig 4.4.2: **(a)** and **(b)** are 4dpi retinal images under confocal microscope for NMDA+poke injury control and LiCl+NMDA+poke injury respectively. **(c)** represents the variation in the number of BrdU positive cells at 4dpi.



(a)



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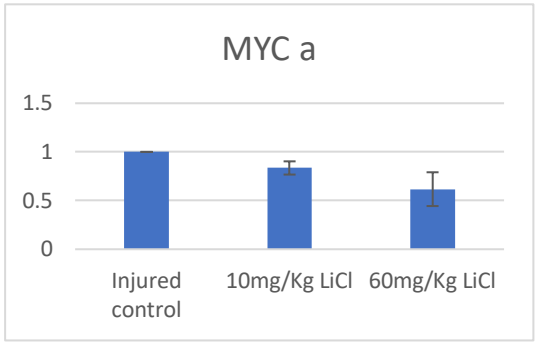

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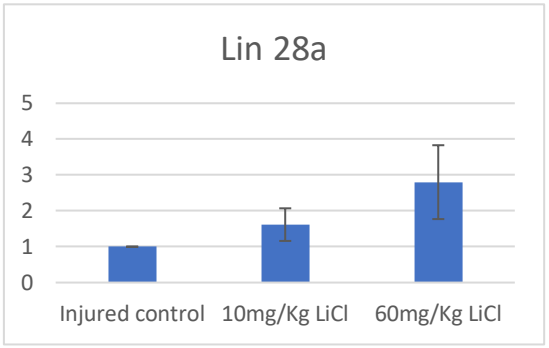

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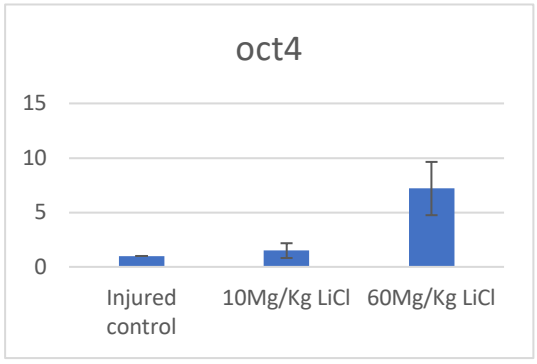

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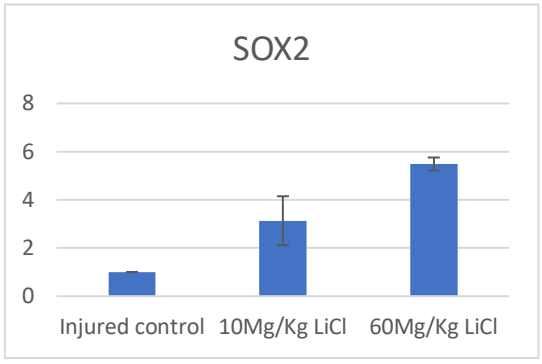

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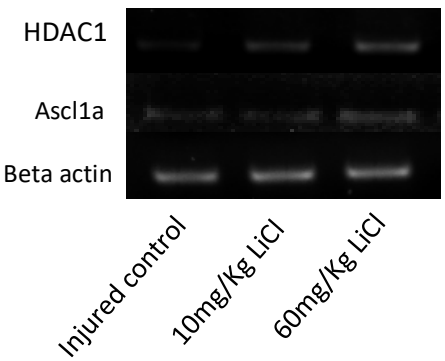


(g)

Fig 4.4.3: **(a)**, **(b)**, **(c)**, **(d)**, **(e)**, **(f)** represent qPCR results of zebrafish samples for the genes HDAC1, Ascl1a, Myc-a, Lin28a, oct4 and sox2 respectively. **(g)** represent RT PCR results for the genes HDAC1 and Ascl1a. The first sample is injured control, followed by 10mg/Kg LiCl and 60mg/Kg LiCl.

4.4.3 Lithium induced proliferation status in mouse retina

Unlike its effect in the zebrafish retina, lithium induces the proliferation of retinal ganglionic cells (RGC) rather than MG cells in the mouse retina. The number of BrdU positive cells is very high in LiCl treated mouse retina, and it extends throughout the whole ganglionic cell layer. The number of proliferating cells in the NMDA injured control were very few to nil while in LiCl treated retina, it was about 15-20 times more.

There is a significantly high increase in the number of proliferating cells in the LiCl treated retina with poke injury alone as compared to that with NMDA+poke injury. This may be because of the ablation of some RGCs by the action of NMDA.

The proliferation status of mouse retina on NaCl treatment under similar conditions was also considered to check if it is a salt effect. The number of proliferating cells is very few in NaCl treated mouse retina. This helps in proving that such a high proliferation of RGCs is the effect of LiCl, and it cannot be replicated by just any salts.

4.4.4 Gene expression pattern in mouse retina in response to LiCl treatment

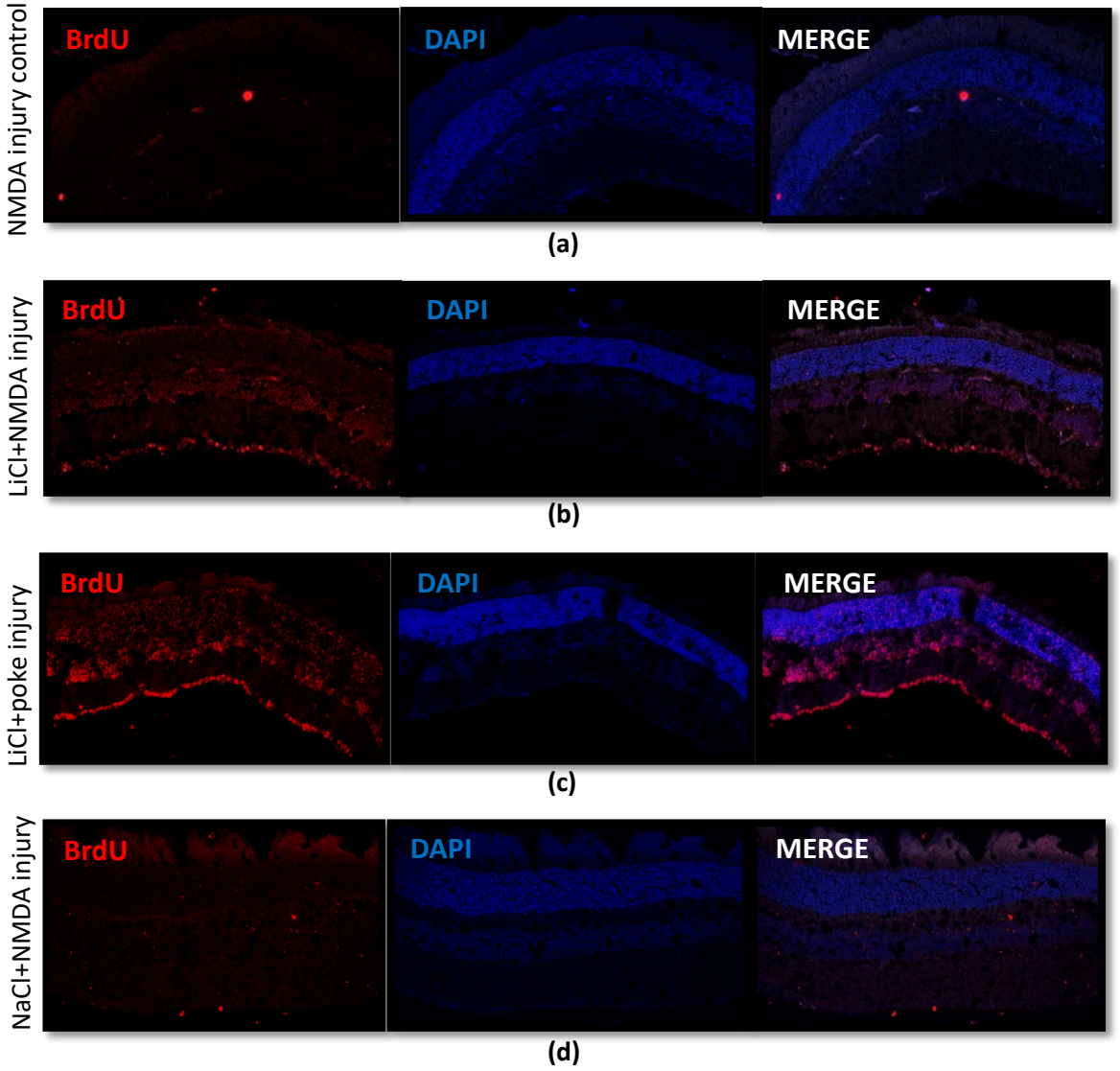
For both the samples used for doing qPCR, injury is made by using NMDA as well as by poking with 30 gauss needle.

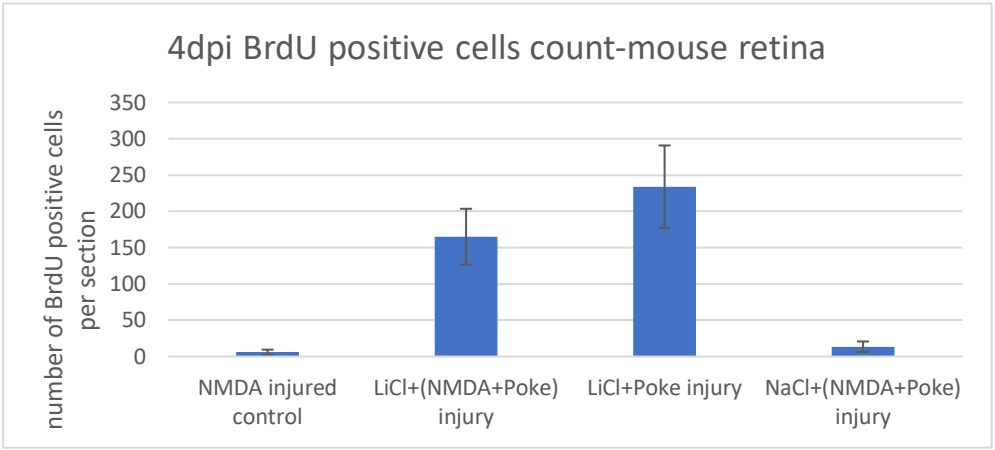
Lithium is known to show neuroprotective nature towards ganglionic cells through BDNF(Brain-derived neurotrophic factor) regulation. The upregulation of intraretinal BDNF in the LiCl treated retinal sample as per the qPCR data compliments well with this.

CTNNB1 is crucial for beta-catenin formation. So it can be considered as an indicator of beta-catenin level. As shown by the qPCR results, there is not much of a difference in the regulation of CTNNB1. This points out that It may not be canonical Wnt signaling that is responsible for the effect of LiCl in the mouse retina.

qPCR results for c-Myc and Lin28a shows an interesting anomaly. The trend in their regulation is opposite to that in zebrafish. On 60mg/Kg LiCl treatment, c-Myc is upregulated in mouse retina while Lin28a is down-regulated.

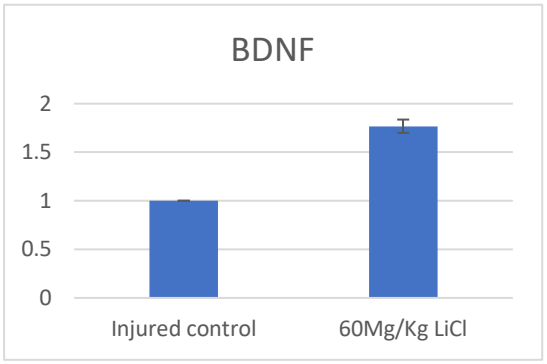
Ascl1a, which is a proneural transcription factor, is upregulated as suggested by qPCR results when the mouse is treated with LiCl. Oct4, which is one of the Yamanaka factors, is also upregulated, and this suggests the increase in the number of pluripotency stemcells on treatment with LiCl.





(e)

Fig4.4.4: **(a)**, **(b)**, **(c)** and **(d)** are the 4dpi mouse retinal images of injured control, 60mg/Kg LiCl with NMDA injury,60mg/Kg LiCl without NMDA injury, and 60mg/Kg NaCl with NMDA injury respectively, under the confocal microscope. **(e)** represents the variation in the number of BrdU positive cells at 4dpi.



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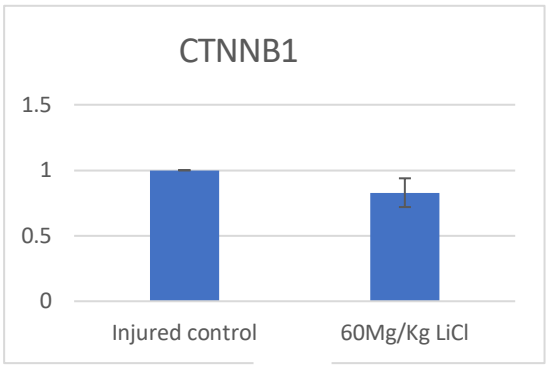

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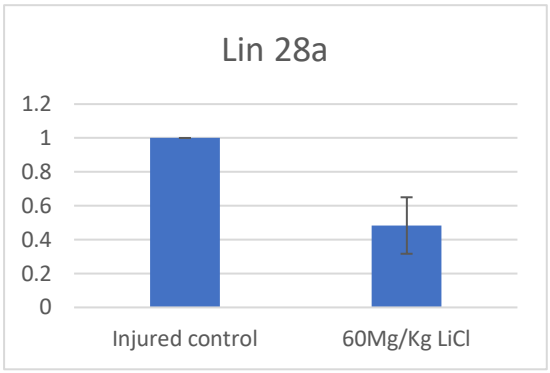

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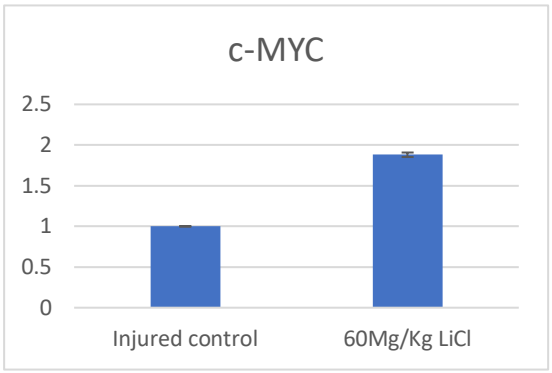

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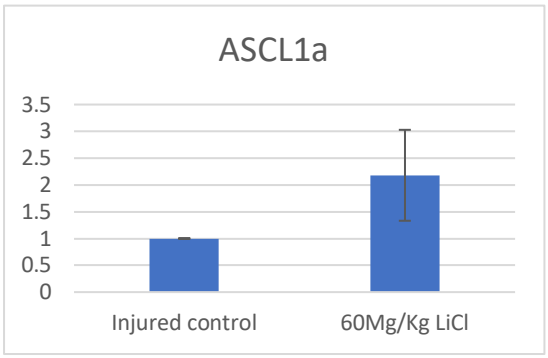

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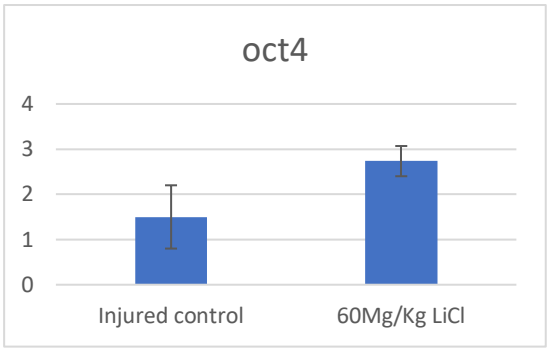


(f)

Fig 4.4.5: **(a)**, **(b)**, **(c)**, **(d)**, **(e)**, **(f)** represent qPCR results of 4dpi mouse retina samples for the genes BDNF, CTNNB1, Lin28a, c-Myc, Ascl1a and oct4 respectively. Injured control represents NMDA+Poke injury control and 60mg/Kg LiCl represent NMDA+Poke injury with 60mg/Kg LiCl treatment.

Chapter 5: **CONCLUSION**

This study gives a comparison in lithium-induced regenerative responses in both mouse and zebrafish models. Though lithium-induced proliferation in the retina of both model organisms, unlike in zebrafish retina, proliferation was seen in the ganglionic cell layers in the mouse retina. There is a decrease in proliferation observed when mouse retina was given NMDA along with NMDA compared to the one with LiCl treatment alone. But, unlike in mouse retina, there is an increase in proliferation in zebrafish retina given NMDA injection along with LiCl treatment. This may be due to the ablation of some retinal ganglionic cells by the effect of NMDA. The proneural transcription factor, Ascl1a, was also upregulated in both model organisms in response to lithium, implying induction of initial regenerative responses in both. Upregulation pluripotency factors Oct4 and Sox2 suggest an increase in the number of pluripotent stem cells in both mouse and zebrafish retina. The level of HDAC1, which is a pro-proliferative gene, is also increasing with increasing concentration of LiCl in the zebrafish retina. Its also observed that Myc and Lin28a are regulated oppositely in these two models. qPCR data suggest no considerable change in the beta-catenin level of LiCl treated mouse with respect to control. CTNNB1 give intructions for the formation of beta-catenin protein.It may not be the activation of Wnt signaling that is responsible for these effects in mouse retina as there is no significant change in the beta-catenin level. So, in a nutshell, we can conclude that, while lithium is activating Wnt signaling and thereby promoting regeneration in zebrafish, it may be showing a neuroprotective nature towards retinal ganglionic cells through a Wnt independent pathway.

Chapter 6: **FUTURE PERSPECTIVE**

The retina is the relatively simple and accessible part of the CNS as it lies at the back of the eye. So, trying to elucidate mechanisms involved in retina regeneration and injury response is highly significant. This study gives an overview of the difference in regenerative response induced by LiCl in mouse and zebrafish retina. As a next step, GSK3b inhibitor can be used, and its effect on both models can be analyzed. It will also help to give better clarity on the lack of significant variation in beta-catenin for LiCl treated mouse retina. Insitu hybridization and Western blotting can also be done to provide better stability to the PCR results. Since there is an opposite trend in the regulation of c-Myc and Lin28a between the two models, working more on these genes may hold the key to increase regenerative potential in mammals. Morpholino based knockdown of these genes can be done, and its effect on both the models can be compared. A study can be done using different concentrations of LiCl to understand the change in the response pattern with varying concentrations. Gene expression pattern at various time points can also be analyzed and the result compared between two models. This study also gives scope for works to be done on other parts of the body like the fin, heart, and brain in zebrafish and digits in the mouse.

Part-2

Understanding regenerative responses induced in mouse digits on immuno-suppression

Chapter 1: **ABSTRACT**

The mouse digit is an excellent mammalian model because its regeneration process closely resembles that in humans. The extreme distal tip can regenerate via the formation of a bud of undifferentiated cells (a blastema). But amputations and injuries in the more proximal level of the digits and limbs usually result in scar wounding. In these clinically more critical situations, the inability of blastema formation is the most significant barrier for successful regeneration. The immune system, known as the first line of defence against invading pathogens, is integral to tissue development, homeostasis and wound repair. The inflammation following injury significantly contributes to tissue repair and scar formation. In contrast, excessive inflammation led by immune cells causes pathological fibrosis that debilitates tissue function and may lead to organ failure. In contrast to adults, neonates do not mount a robust fibrotic but a more angiogenic response that facilitates tissue regeneration after injury. Therefore, since immune cells regulate both fibrosis and angiogenesis during tissue healing, targeting the immune system to promote neoangiogenesis with minimal fibrosis would be an exciting approach to stimulate regeneration. The function of RAG1 is to exert variable, diverse, and joining (V[D]J) recombination of T-cell receptors (TCRs) and immunoglobulins in developing lymphocytes. RAG1 deficient mice have small lymphoid organs that do not contain mature Band T lymphocytes. So, this strain of mouse will be a great model in understanding the regulation of regenerative response at an immunosuppressive state. Mouse digits were amputated proximally by completely taking the nail bed off. As a preliminary result, the number of circulating lymphocytes in the bloodstream for wild type and RAG1-/- mouse was compared using FACS analysis. Oct4 (a pluripotency factor) expression was checked using qRT-PCR to understand the status of pluripotent stem cells in RAG1-/- mouse digits at 12 days post-amputation(dpa)compared to normal Wildtype. Regulation of insm1a and mmp9 in RAG1-/- mouse digits were also analyzed. Hence, this study sheds some light on the impact of immunosuppression on digit regeneration.

Chapter 2: **INTRODUCTION**

Regeneration is a fascinating phenomenon in which lost tissues are functionally re-established. In a typical regeneration model observed in the limbs of salamanders, the regenerative process includes bone, muscle, tendons, joints, nerves, blood vessels, mesenchyme, and epidermis. After amputation, lineage-restricted stem cells move to the central distal-most region of the limb and form a structure called the blastema, a heterogeneous population of cells that can re-enter the cell cycle and reutilize developmental mechanisms to restore lost structures. The salamander blastema can reconstitute the limb with identical form and function to an un-amputated limb, as many times as needed (Mescher 1976; Namenwirth 1974).

Regeneration and wound repair are two separate biological processes by which organisms heal wounds. While amphibians can spontaneously regenerate lost appendages, mammals generally form scars over the injury site through wound repair. There are numerous examples of regeneration in mammals, including the seasonal growth of deer antlers (Li 2012), ear hole closure in rabbits and mice (Gawriluk et al. 2016; ten Koppel 2001), and digit tip regeneration in humans, monkeys, and mice (Borgens 1982; Illingworth 1974). Epimorphic regeneration, which is blastema-mediated, is considered to be distinct from the regeneration of individual damaged tissues, such as skin and bone. In general, mammals display tissue-specific regenerative abilities (e.g., healing bone fractures) but a limited capacity to coordinate a multi-tissue regenerative response.

Though Mammals are generally considered to have lost the capacity to regenerate their limbs after amputation, both humans and mice are able to restore the extreme tip of their digits through a regenerative response similar to that witnessed in amphibians. This regenerative response establishes the foundation on which we can explore methods to stimulate more extensive regeneration in humans.

5.2.1 Digit tip regeneration

Mouse digit tip is one of the well-characterized tissue regeneration models in mammals. Digit tip regeneration has been recorded in mammals, including mice and juvenile humans. Digit tip regeneration viewed in both mice and humans includes the coordinated regrowth of the nail organ, including nail epithelial cells, and the terminal phalanx (Lehoczky, Robert, and Tabin 2011; Rinkevich et al. 2011; Takeo et al. 2013).Upon regrowth of the nail after amputation of the digit tip, undifferentiated mesenchymal cells, including fate-restricted progenitor cells, collect under the wound epithelium and develop the so-called blastema (Neufeld 1980). Growth and differentiation of these mesenchymal cells lead to digit regeneration. However, neither nail nor digit regenerates when amputated proximally to the nail, and it is undiscovered why this limitation exists.

Amputations that remove up to 50% of the terminal phalanx are considered distal amputations, and this results in the recovery of the general morphology after about a month, resembling non-amputated digits. Unlike salamanders, distal amputation in mice affects only the distal portion of the bone, nail, and the skin without impairing muscles or tendons. When amputations occur

more proximally, removing over 60% of the mouse digit tip, the wound heals, but the digit tip does not grow back(Han et al. 2008; Lehoczky et al. 2011; Sensiate and Marques-Souza 2019).

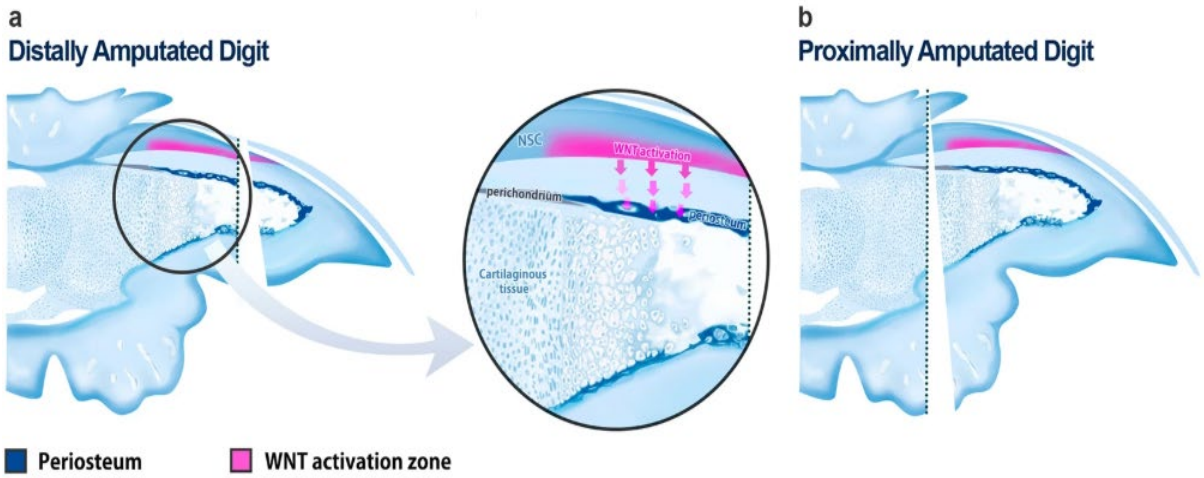


Fig 5.2.1: **(a)**Distal amputations preserve the Wnt activation zone (pink) and bone progenitor periosteal cells (darkest blue). **(b)** Proximal amputation removes the Wnt activation zone and the periosteum of the digit tip. Without Wnt activation and periosteum, no distal appositional bone regrowth is expected to occur. If present, these structures will promote distal appositional bone regrowth, nail bed extension (Sensiate and Marques-Souza 2019).

Terminal phalangeal bone is the main structure shaping the mouse digit tip in size and form. Unlike all long bones, additional ossification centre located at the distal tip of the bone helps in further increasing the length of the distal phalanx, through intramembranous ossification. Although distal amputation eliminates part of the terminal phalanx formed by endochondral ossification, bone regrowth after amputation is exclusively due to distal intramembranous ossification. Similar to that of bone formation by intramembranous ossification during development, bone regrowth after distal amputation of the mouse digit tip also depends on Wnt signalling(Sensiate and Marques-Souza 2019; Takeo et al. 2013).

5.2.2 Immune system, Lymphocytes and Regeneration

5.2.2.1 Immune system and lymphocytes

The overall function of our immune system is to prevent or restrict infection. The immune system can distinguish between healthy and unhealthy cells by identifying a variety of danger cues called danger-associated molecular patterns (DAMPs). Infectious microbes like bacteria and viruses release a different set of signals recognized by the immune system called pathogen-associated molecular patterns (PAMPs). If an immune response can't be activated when needed, problems like infections will arise. But, when an immune response is activated without the presence of a real threat, different problems like allergic responses and autoimmune disease may occur (National Institute of Allergy and Infectious Diseases (NIAID) 2013).

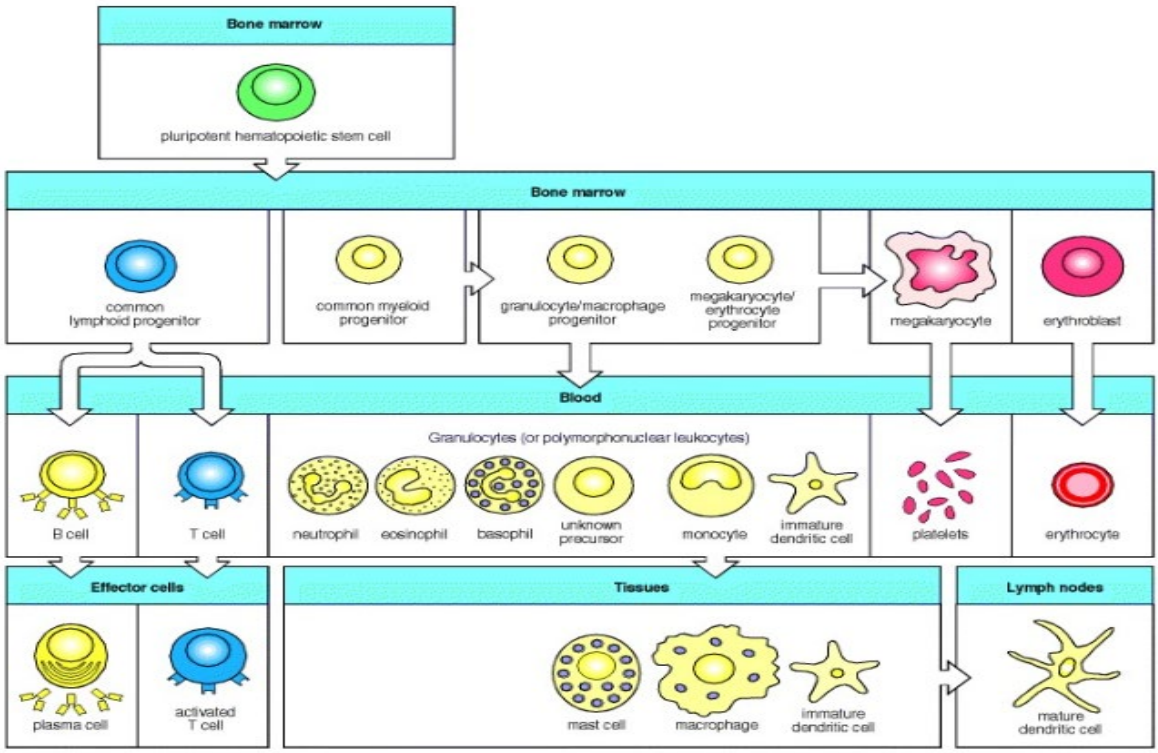


Fig 5.2.2: The cells of immune system and progenitor cells they are derived from. All the cellular elements of blood arise from hematopoietic stem cells in the bone marrow. The pluripotent cells divide to form a common lymphoid progenitor that gives rise to the T and B lymphocytes, and a common myeloid progenitor that gives rise to different types of leukocytes, erythrocytes, and the megakaryocytes that produce platelets (Janeway CA Jr, Travers P, Walport M 2001).

White blood cells (WBCs) are an essential part of our immune system. Lymphocytes are white blood cells that are the main types of immune cells in the body. They are formed in the bone marrow and circulated in the blood and found lymph tissue. They help our body fight against bacteria, viruses, and other toxins that make you sick (King, Toler, and Woodell-May 2018).

There are two categories of lymphocytes - B lymphocytes and T lymphocytes, and they are commonly referred to as B cells and T cells. Both types originate from hematopoietic stem cells in the bone marrow. Some of these cells travel from there to the thymus and become T cells. Other cells remain in the bone marrow and later become B cells. B cells make antibodies, which are proteins generated by the immune system to fight against antigens. Each B cell is set to make only one specific antibody. Each antibody matches a distinct type of antigen and thereby marking the antigen for destruction. T cells help the body control the immune response to foreign substances and kill cancer cells(Janeway CA Jr, Travers P, Walport M 2001; Stacy R. Sampson 2017).

There is one more type of lymphocyte known as the natural killer (NK) cells. It originates from the same place as B and T cells. NK cells are specialized in killing cancer cells and virus-infected cells and respond quickly to several foreign substances (Lowry and Zehring 2017).

5.2.2.2 Immune modulation in wound healing and regeneration

During embryonic and postnatal development, the immune system monitors processes such as ductal formation, branching morphogenesis, and angiogenesis. Similar functions are sustained in some adult tissues to maintain normal homeostasis. Injury or disease evokes an inflammatory response that can either promote functional restoration of the tissue regeneration or a rapid healing response that may protect the organism at the expense of maintaining structure and function(Aurora and Olson 2014).

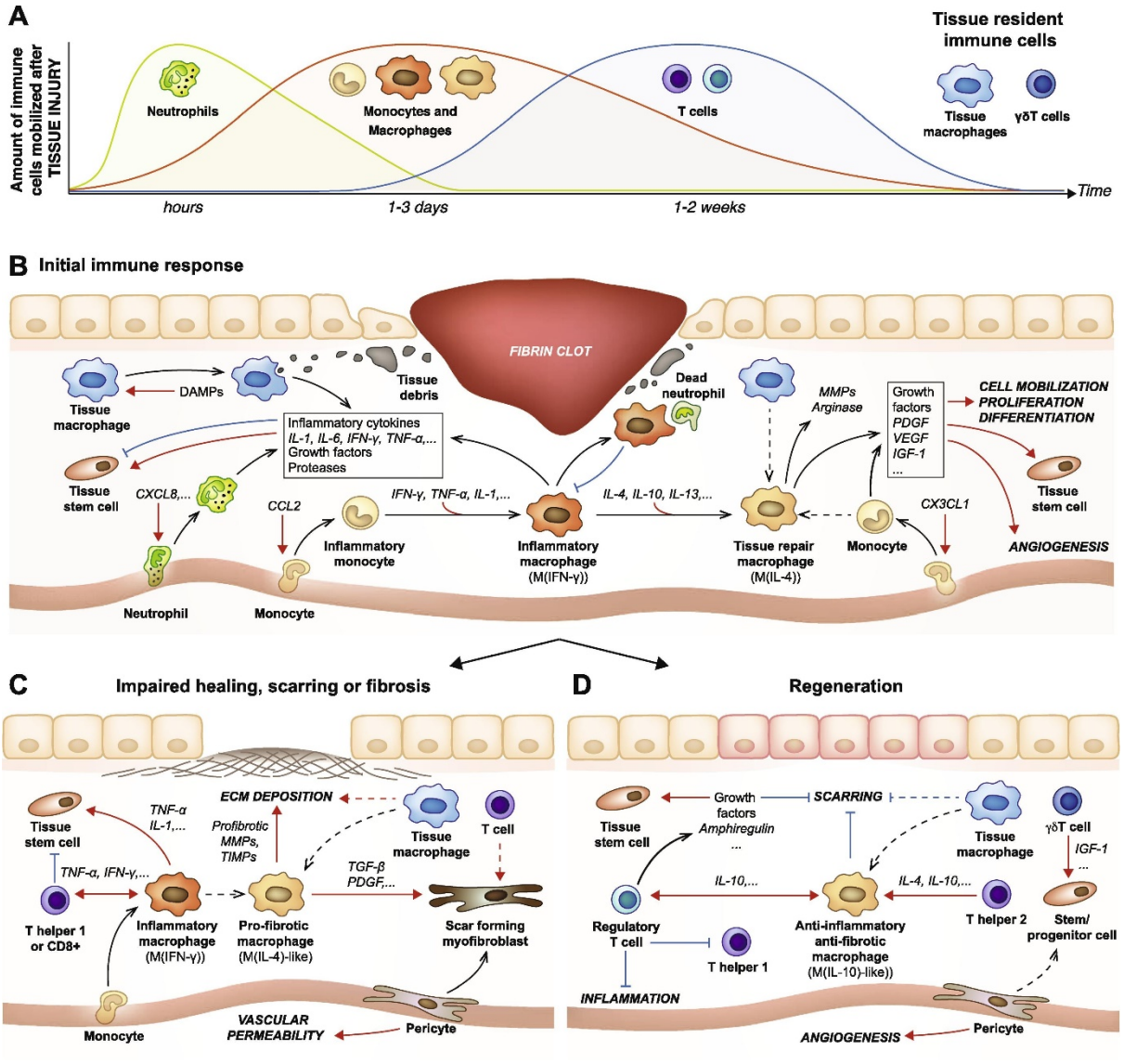


Fig 5.2.3: The main actors of the immune response following tissue injury (Julier et al. 2017). (A) Dynamics of immune cell mobilization after tissue injury, (B) Overview of the initial inflammatory phase following tissue injury, C) Overview of the immune mechanisms that can impair tissue healing or drive to scarring and fibrosis, (D) Overview of the pro-regenerative immune mechanisms. Black arrows indicate a differentiation path or secretion of immune modulators/morphogens. Black dashed arrows indicate a possible differentiation path. Red arrows indicate induction. Blue arrows indicate inhibition.

Tissue resident cells including tissue-resident macrophages and γδT cells sense tissue damage and trigger the mobilization of other immune cells. Neutrophils are followed by monocytes/macrophages and T cells. Tissue damage is sensed by tissue-resident macrophages via DAMPs. Neutrophils are the first circulating immune cells supplied to the site of injury, promoting inflammation and monocyte/macrophage recruitment (Prame Kumar, Nicholls, and Wong 2018). The inflammation is initially sustained by pro-inflammatory M(IFN-γ) macrophages, before being eventually resolved with the help of M(IL-4) macrophages. M(IFN-γ) macrophages stimulate effector T cells in a positive-feedback loop. Effector T cells may also inhibit the regenerative capacity of tissue resident stem/progenitor cells via inflammatory cytokines. M(IL-4)-like macrophages with a pro-fibrotic activity encourage ECM protein deposition and consequent fibrosis (scarring), preventing full regeneration of the original tissue (Braga, Agudelo, and Camara 2015). Pericytes increase immune cell mobilization and differentiate into scar forming myofibroblast via growth factors such as TGF-β1. A critical number of macrophages displaying an anti-inflammatory/anti-fibrotic phenotype contribute to regeneration through a crosstalk with Tregs, which in turn help sustain the anti-inflammatory/anti-fibrotic phenotype via secretion of anti-inflammatory cytokines such as IL-10 (Aurora and Olson 2014, 2014; Chen et al. 2018; Julier et al. 2017; Prame Kumar et al. 2018). Tregs may also enhance the regenerative capacity of endogenous stem/progenitor cells through secretion of growth factors.

5.2.3 Rag1$^{-/-}$ Mouse Strain

V(D)J recombination is the novel mechanism of genetic recombination that happens only in developing lymphocytes during the initial stages of T and B cell maturation. V(D)J recombination convenes a diverse collection of immunoglobulin and T-cell receptor genes in the developing B and T-lymphocytes by rearrangement of various V (variable), D (diversity), and J (joining) gene segments (Jung and Alt 2004). Recombination-activating gene 1 (RAG1) Catalytic component of the RAG complex is a multiprotein complex that mediates the DNA cleavage activities by adding a double-strand break between the recombinant signal sequence and the adjacent coding segment. At the time of V(D)J recombination. Though RAG2 is not a catalytic component, it is needed for all known catalytic activities (Fugmann et al. 2000). The RAG complex also performs a role in pre-B cell allelic exclusion.

Recombination-activating gene 1 (RAG1) deficiency is an autosomal recessive disorder that results in severe combined immunodeficiency (SCID) because of the complete lack of mature T and B lymphocytes. RAG1 deficient mice have small lymphoid organs that do not contain mature Band T lymphocytes. The thymus of the RAG-1 mutant mouse is small and holds immature, large, CD8-CD4 double-negative thymocytes expressing the IL-2 receptor. Besides, the bone marrow and the spleen of the RAG-1 mutant mouse carry a population of dull B220-positive cells, a fraction of which may depict pro-B cells (Ménoret et al. 2013; Mombaerts et al. 1992; van Til et al. 2014). The suspension of B and T cell differentiation happens at an early stage and associates with the incapability to perform V(D)J recombination.

Chapter 3: **MATERIALS and METHODS**

5.3.1 Fluorescence-activated cell sorting (FACS)

- Blood samples were collected at 6dpa and 12dpa timepoints.
- Take six microcentrifuge tubes and add 25µl of blood in each MCT.
- Add the mix of CD8, CD4, CD3, and CD19 antibodies in one tube. Also, add these antibodies separately in 4 different MCTs and keep an MCT with the blood sample without any antibody. The MCT having the blood sample with no added antibodies will be used as unstained control. The sample with a combined mix of all antibodies will be used for final analyses. In contrast, the rest of the tubes with individual antibodies and unstained will be used for fluorescence compensation. 5µl of antibodies were added to each tube by keeping their concentration at 1:300 in each MCT.
- Incubate the samples at 40^0C by keeping them in ice for 45 mins.
- Add 20X the volume(600µl) of RBC lysis buffer to the samples and keep at room temperature for 10 mins.
- Add 600µl of 1X PBS and spin at 3000rpm and at room temperature for 5 mins.
- Discard the supernatant, add 500µl of 1X PBS and spin again at 3000rpm and at room temperature for 5 mins.
- A white pellet will be visible at the bottom after discarding the supernatant. Dissolve this pellet in 200µl PBS. Use this prepared samples for cell sorting and analysis with the help of the FACS machine/Flow cytometer.

RBC lysis buffer composition:

- NH_4Cl – 155Mm
- $NaHCO_3$ – 12Mm
- EDTA (pH-8) – 0.1mM

5.3.2 RNA isolation

- Take the 12 dpa digit sample stored in trizol from -80ºC and keep on ice to thaw it.
- Homogenize the tissues completely by first using homogenizer and then with 200µl pipette till no tissue clumps are visible.

- Then keep at room temperature for 10 mins.
- Add 0.2 volumes (40µl for 200 µl of trizol with tissue) of chloroform and mix it well by inverting the MCT up and down for 20 seconds.
- Then centrifuge it at 12000 rcf for 15mins at 4ºC.
- Using cut tips, collect the aqueous phase slowly without disturbing the phase layer, and transfer it to fresh MCTs.
- Add Isopropanol (double the amount of aqueous phase collected) and keep it at -80ºC for overnight precipitation.
- The next day, centrifuge it at 10000rcf at 4ºC for 20mins.
- Discard the supernatants.
- Wash it with 80% ethanol (200µl per MCT) and centrifuge it at 4ºC for 10 min at 7500 rcf.
- Dry the pellet till alcohol smell is no longer there and dissolve the pellet in DEPC treated water and stores it at -80ºC.
- Check whether there is any contamination of genomic DNA in it by using agarose gel electrophoresis (1%gel). We can also measure the amount of RNA present by measuring the Optical density using a spectrophotometer (nanodrop) and proceed further for cDNA synthesis.

5.3.3 Reverse transcription PCR (RT PCR) using Taq Polymerase

- Dilute the synthesized cDNA by 1:3 dilutions
- For 10µl reaction, the following components are used:
    - 20X buffer- 0.5µl
    - dNTP-1µl
    - forward primer and reverse primer(F+R) -0.5µl
    - template cDNA-0.5µl
    - MQ water -7.4µl
    - Taq polymerase -0.1µl
- Amount of template can vary as per GAPDH standardization of samples.
- Generally, the following cycling conditions are used: -
    - 98ºC - 2min
    - 98ºC - 15secs
    - 60ºC - 30secs

- 72°C - depending on the size of the gene (1kb/1min)
- 72°C - 7min
- 4°C - Infinite hold

- Number cycles can be standardized as per the gel image obtained after loading the PCR product.

- Check the PCR product by agarose gel electrophoresis.

5.3.4 Quantitative PCR (qRT-PCR)

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

- For 5µl reaction, following components are used: -
  - SYBR Master mix- 2.5µl
  - Primers (F+R)-0.5µl
  - Template -0.5µl
  - MQ water-1.5µl

- Analyze the results on an excel sheet and plot the graph.

Chapter 4: **RESULTS and DISCUSSIONS**

5.4.1 Number of lymphocytes are negligibly low in the case of RAG1-/- mouse.

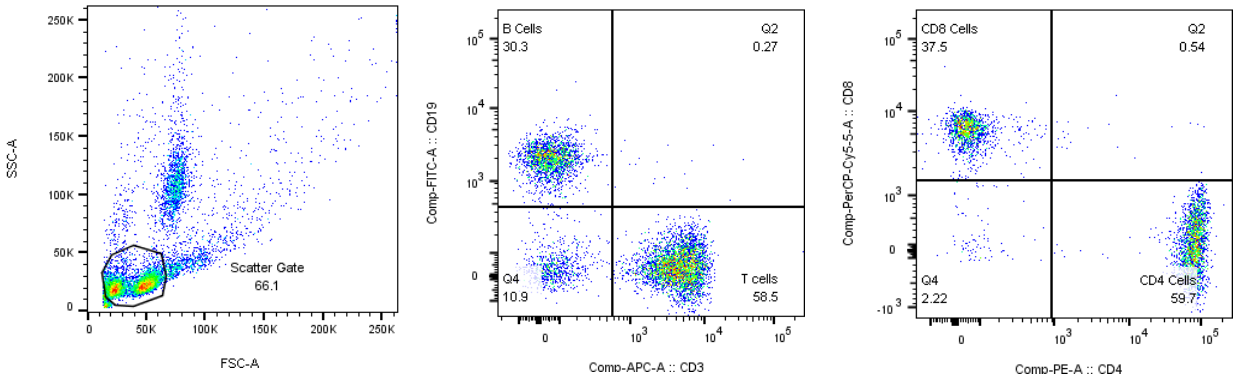
FACS analysis helps in understanding the amount of circulating T cells and B cells in the bloodstream. The mouse is anesthetized using avertin(2,2,2-tribromoethanol). Blood is collected from the venous sinus by Retro-orbital bleeding with the help of capillary tubes. APC-CD3, FITC- CD19, PE-CD4, and PerCP CY5.5-CD8 were the antibodies used for marking T-cells, B-cells, T-helper cells and cytotoxic T-cells respectively. The use of four different fluorophores attached antibodies helps in their efficient comparison. Individual antibody added samples along with unstained were used for fluorescence compensation. From the FACS data and as depicted by the graphical representation of the analysis, the number of T-cells and B-cells is negligibly low in the case of RAG1-/- mouse strain. This confirms the absence of mature T-cells and B-cells as a result of RAG1 deficiency. Since lymphocytes can inhibit the regenerative capacity of tissue-resident stem/progenitor cells via inflammatory cytokines, this mouse strain proposes the presence of a better regenerative potential.

5.4.2 Increased pluripotency in rag1-/- mouse digits at 12dpa

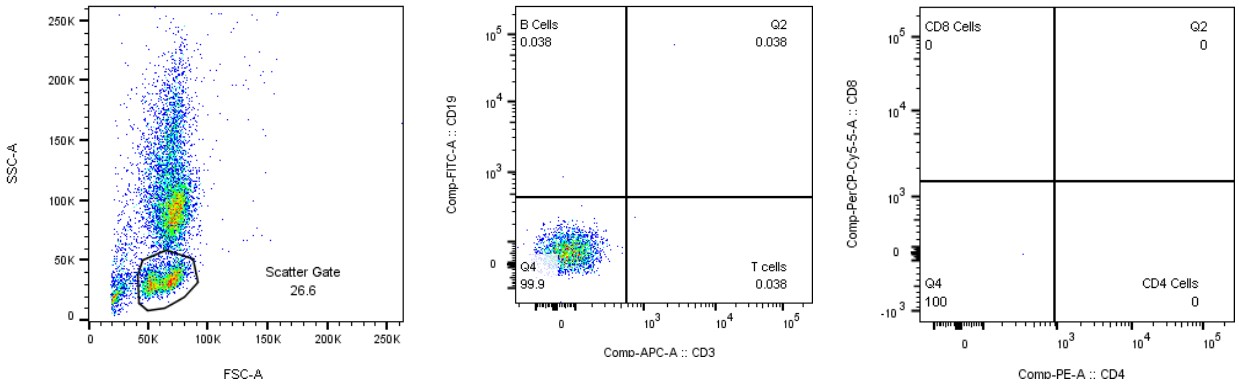
Pluripotency is described as the ability of a cell to produce all of the cell types of an organism. It is a characteristic of cells in the inner cell mass of the mammalian preimplantation blastocyst as well as of embryonic stem cells. A group of transcription factors is crucial for the establishment and maintenance of the pluripotent state. Among these factors, Oct4 is central to the machinery governing pluripotency (Shi and Jin 2010). Mouse digits were amputated proximally by completely taking the nail bed off. The Oct4 level is significantly very high in 12dpa digits of RAG1-/- mouse compared to control. This suggests a considerable increase in the number of pluripotent stem cells in RAG1-/- mouse digits with respect to control at 12 DPA.

RT-PCR results also suggest the upregulation of both Insm1a and MMP9 in 12dpa RAG1-/- mouse digits.

(a) Wild Type (C57BL/6J) mouse



(b) *RAG1-/-* mouse strain



(c)

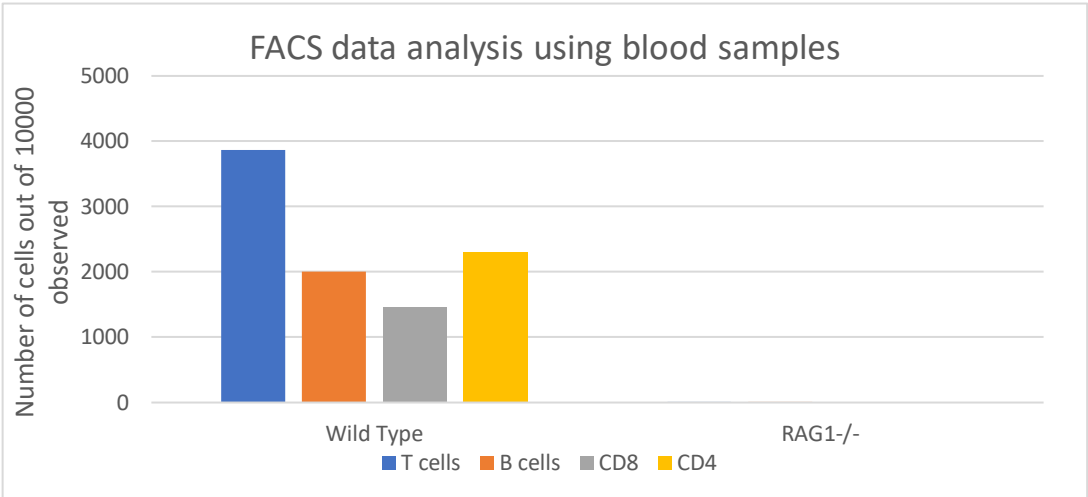
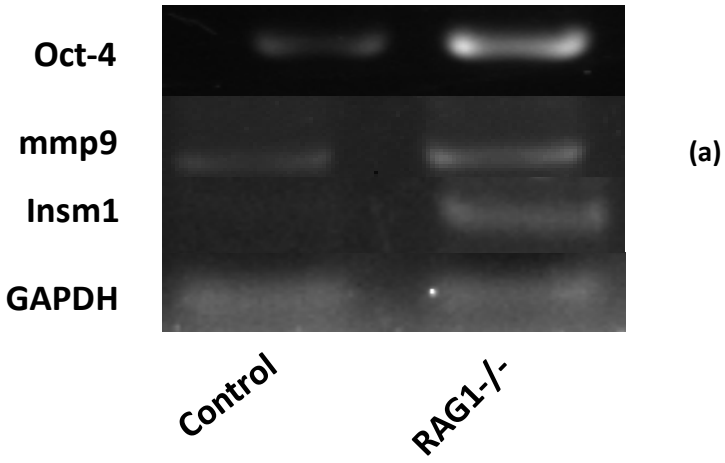
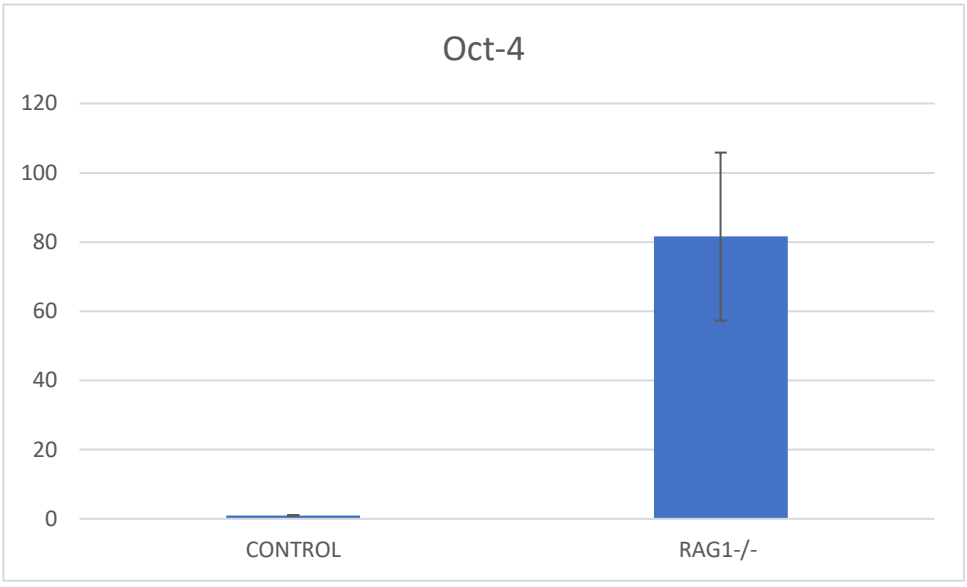


Fig 5.4.1: (**a**) FACS analysis data of control wild type mouse blood sample. (**b**) FACS analysis data of *RAG1-/-* mouse blood sample. (**c**) graphical representation of the number of lymphocytes in the circulating blood of wild type and RAG*1-/- mouse.*



(a)



(b)

Fig 5.4.2: (**a**) RT PCR result for the genes Oct-4, mmp9, and Insm1 using mouse digits samples at 12dpa. (**b**) qPCR result for the gene Oct-4 using mouse digit samples at 12dpa.

Chapter 5: **CONCLUSION**

From this study of understanding regenerative responses in mouse digits on immunosuppression, we can conclude that the reduction in the number of lymphocytes in the circulating bloodstream increases the pluripotency state of mouse digit tissues. Lymphocytes can also inhibit the regenerative capacity of tissue resident stem/progenitor cells via inflammatory cytokines. The use of RAG1-/- mouse strain is beneficial in this study as the RAG1 deficient mouse has no mature T or B cells. This is confirmed from the FACS analysis result, where the number of lymphocytes in the bloodstream of RAG1-/- mouse is negligibly low compared to normal wildtype. The use of four different fluorophores attached antibodies also helps in their efficient comparison.

A group of transcription factors is crucial for the establishment and maintenance of the pluripotent state. Among these factors, Oct4 is central to the machinery governing pluripotency. The upregulation of pluripotency factor Oct4, as confirmed by both RT-PCR and qPCR results, suggest an increase in the number of pluripotent stem cells in the 12dpa mouse digits. Insulinoma-associated1a (insm1a) is a zinc-finger transcription factor performing a range of functions in cell formation and differentiation (Gong et al. 2017). Matrix metalloproteinase 9 (MMP-9) plays a critical role in wound healing and inflammation (Yang et al. 1999). Both Insm1a and MMP9 were upregulated in RAG1-/- mouse digits. The expression of MMP9 suggests an early regenerative response, while the expression of Insm1a suggests new motor neuron development.

In a nutshell, the absence of matured T and B cells RAG1 deficient mouse is responsible for the increase in the pluripotency status of digit tissues, thereby showing better regenerative potential. This study is a small footstep towards the induction of proper regenerative potential in wild type mice. Wild type mice may be given immunosuppressor drugs in the later stages of the projects to see if they are showing the same gene regulation trend as in RAG1-/- mouse.

Chapter 6: **FUTURE PERSPECTIVE**

Regeneration is an intriguing phenomenon of the restoration of lost body parts. Lower vertebrates like zebrafish possess extensive regenerative potential, whereas it's very limited in the case of mammals. The reason for this variation is not entirely known yet. In this study, it's been shown that genetically modified RAG1-/- mouse shows increased pluripotency at 12 days post-amputation. As the next step in this project, some immunosuppressive drugs like FTY720 or Rapamycin can be given to normal Wild type mice to check if they show the same gene regulation trend as RAG1-/- mouse. In-situ hybridization and western blotting can also be done to confirm these results. Expression of genes that are known to be essential for amphibian limb regeneration can also be checked with these digit samples to see if the pattern of gene regulation is similar under immunosuppression. Digits samples at multiple time points can be taken to see if there is any variation in this trend over time. With the help of the lymphocyte isolation kit, lymphocyte can be isolated from wild type mouse and injected on to RAG-/- mouse to see if there is any change in pluripotency status. Live imaging of digits needs to be taken at different time intervals to see if there is any growth as seen externally. Calcein and Alizarin red staining can be used to understand relative bone growth.EdU/BrdU staining of mouse digits can also be done to visualize proliferation status in the tissue.

T1: List of Primers Used

Zebrafish Primer used

RT_bactin_fwd	GCAGAAGGAGATCACATCCCTGGC
RT_bactin_rev	CATTGCCGTCACCTTCACCGTTC
RT_HDAC1_fwd	GACAGCACCATTCCTAATGAGCTCC
RT_HDAC1_rev	TATCGTGAGCACGAATGGAGATGCG
RT_Ascl1a_fwd	ATCTCCCAAAACTACTCTAATGACATGAACTCTAT
RT_Ascl1a_rev	CAAGCGAGTGCTGATATTTTTAAGTTTCCTTTTAC
RT_Myc-a_fwd	AGCAGCAGTGGCAGCGATTCAGAAGATG
RT_Myc-a_rev	TGGAGACGTGACAGCGCTTCAAAACTAGG
RT_Lin28a_fwd	TAACGTGCGGATGGGCTTCGGATTTCTGTC
RT_Lin28a_rev	ATTGGGTCCTCCACAGTTGAAGCATCGATC
RT_Oct4_fwd	AGATAACGCACATATCCGATGATCTAGGCCTG
RT_Oct4_rev	TGCGGGTGAGCATGCATGAATTGAGACATTG
RT_Sox2_fwd	GAAAAACAGCCCGGACCGCATCAAGAGACC
RT_Sox2_rev	GTCTTGGTTTTCCTCCGGGGTCTGTATTTG

Mouse Primers used

RT_GAPDH_fwd	CATTGTGGAAGGGCTCATGACCAC
RT_GAPDH_rev	CTTGATGTCATCATACTTGGCAGG
RT_Insm1a_fwd	GCCGAGGACATCCTGGCTTTCTAC
RT_Insm1a_rev	GCACCTGAAGGAGGATCACCTGTC
RT_Oct4_fwd	TCAGCTTGGGCTAGAGAAGGATGTGG
RT_Oct4_rev	TTCCATAGCCTGGGGTGCCAAAGTG
RT_BDNF_fwd	GGAGCCTCCTCTACTCTTTCTGC
RT_BDNF_rev	CCTTTTGTCTATGCCCCTGCAGC
RT_CTNNB1_fwd	CAGATGCAGCGACTAAGCAGGAAG
RT_CTNNB1_rev	TGGTCAGATGACGAAGAGCACAGATG
RT_Lin28a_fwd	GAAGATCCAAAGGAGACAGGTGC
RT_Lin28a_rev	CAATTCTGGGCTTCTGGGAGC
RT_c-Myc_fwd	CAAAGACAGCACCAGCCTGAG
RT_c-Myc_rev	CCTCATCTTCTTGCTCTTCTTCAGAG
RT_Ascl1a_fwd	AGCAGCTGCTGGACGAGCACGAC
RT_Ascl1a_rev	AGATGCAGGATCTGCTGCCATCCTGC

T2: Bio legend FACS Antibody list

Cat. No.	Description	Size
100312	APC anti-mouse CD3ε Antibody	100 µg
152404	FITC anti-mouse CD19 Antibody	100 µg
100732	PerCP anti-mouse CD8a Antibody	100 µg
100408	PE anti-mouse CD4 Antibody	200 µg

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