Understanding the role of telomerase in zebrafish retina regeneration

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A dissertation submitted for the partial fulfilment of

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Certificate of Examination

This is to certify that the dissertation titled “understanding the role of telomerase in zebrafish retina regeneration” submitted by Ms. Sapna Meena (Reg. No.MS09114) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr.Kuljeet sandhu Dr. Shashi Bhushan Pandit Dr.RajeshRamachandran

(Supervisor)

Dated: April 25, 2014

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Rajesh Ramachandran at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma,or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Sapna Meena

(Candidate)

Dated: April 25, 2014

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

Dr. Rajesh Ramachandran

(Supervisor)

Acknowledgement

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

Finally, I acclaim my prayer to Almighty for being with me always.

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Abstract

Muller glial cells play the most crucial role in zebrafish retina regeneration. Immediately after injury, they de-differentiate into retinal progenitor cells, proliferate, and re-differentiate into all retinal cell types. Although some of the major singling pathways involved in retina regeneration have been worked out, majority remains unknown. As the involvement of telomerase is evident from earlier research findings in mouse liver and beta cell regeneration, we tried to check whether it plays similar role in zebrafish retina regeneration as well. We found that, one of the telomerase gene transcripts, Tert-004 is expressed in the ganglion cell layer following retinal injury.

Chapter 1

1.1 Introduction

Regeneration is a complete restoration of physiology, morphology and functionality of damaged tissue after injury. Regeneration is different from repair, as in repair mechanism there is scar formation but in regeneration, newly formed tissue is similar to surrounding tissue. Tissues like blood, liver, and skin have maximum regenerative potential whereas Nervous System, kidney, heart etc have lowest regenerative potential. In case of mammalian system, interestingly Peripheral Nervous System can regenerate but Central Nervous System cannot. Achieving mammalian CNS regeneration will be of much importance as CNS injury affects over 5% of the population in developed countries every year and cause irreparable damage. We can get the molecular clues of CNS regeneration from some lower vertebrates like teleost (e.g. zebra fish) and urodeles (e.g. - frog), as they can regenerate their CNS very well. In our lab we used zebra fish (*Danio rerio*) as model system because of its fully sequenced genome and they are easily available, less expensive, easy to handle, and are having short life span. Although zebra fish has ability to regenerate most of its CNS including brain, we used retina as model organ because retina is the only exposed and easily accessible part of CNS and doesn't cause lethality when damaged.

Fig 1 :Zebrafish in aquarium

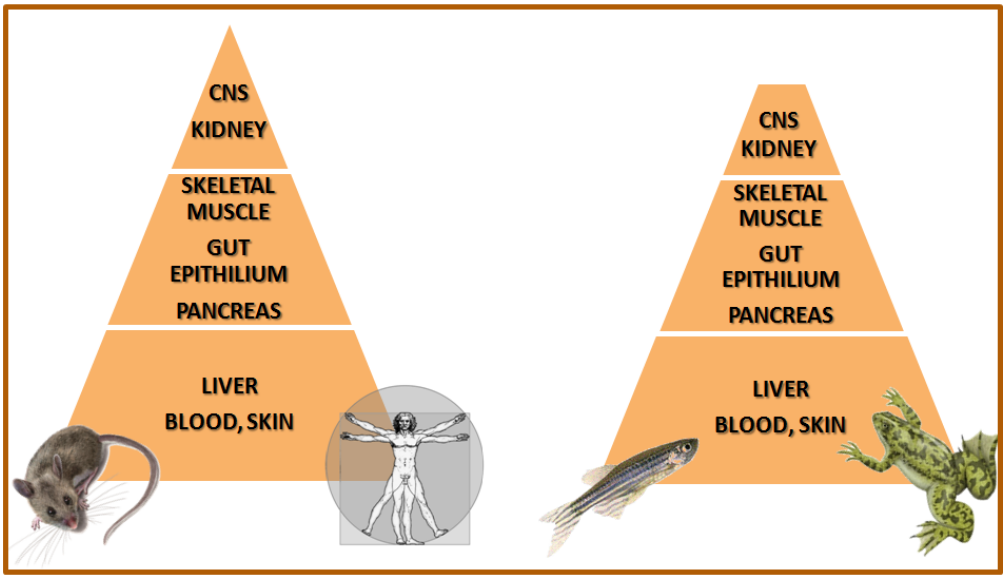

Fig 2: Regenerative potential of different organs

Broadly any vertebrate retina can be divided into 3 distinct layers. From the direction of incoming light, these layers are: Ganglion Cell Layer(GCL), Inner Nuclear Layer(INL) and Outer Nuclear Layer (ONL). GCL contains ganglion cells. The axons of these cells form the optic nerve. Bipolar cells and amacrine cells reside in the GCL. In the ONL rod and cone cells

reside. Muller glial cells which acts as a kind of nourishing cell type and also responsible for retina regeneration, reside in INL. These Muller cells wraps around other cell types and provide nourishment and also, after injury, de-differentiate, proliferate and re-differentiate into all retinal cells.

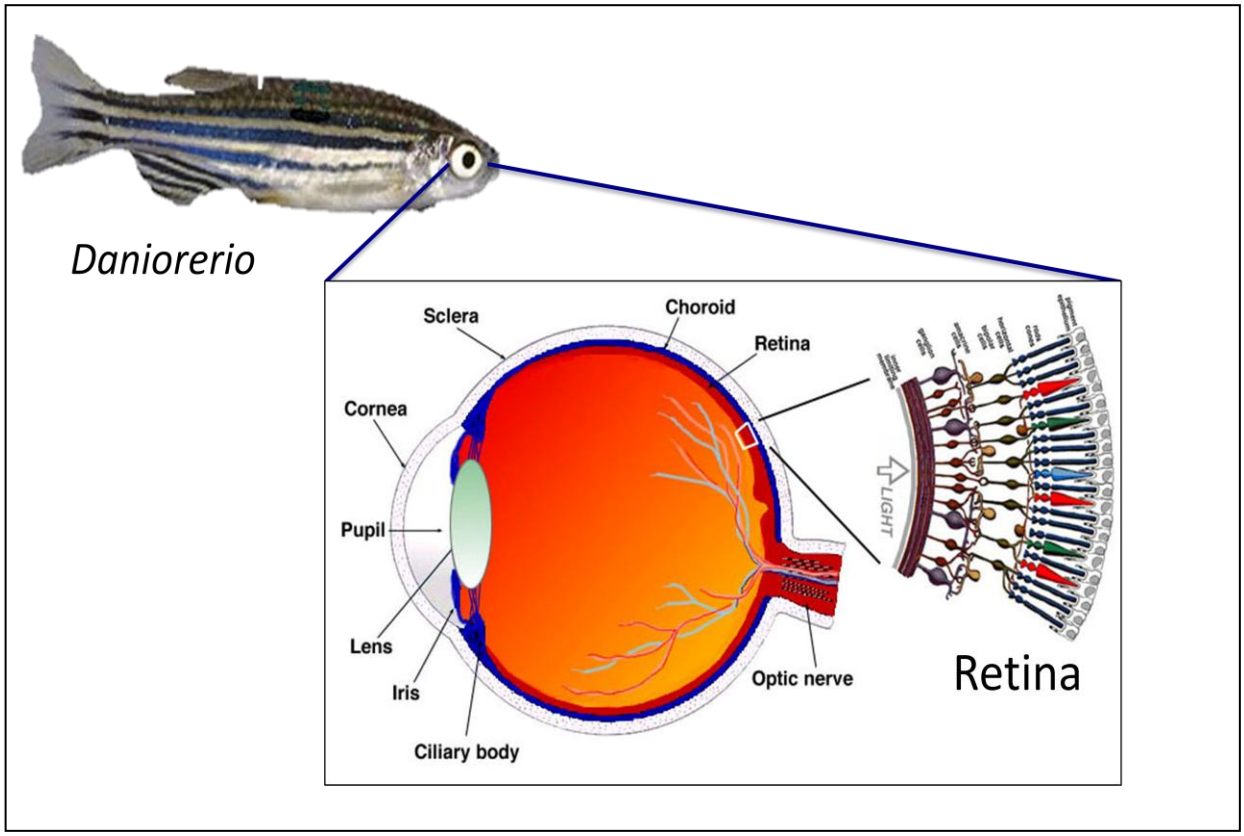

Fig 3: Structure of teleost eye

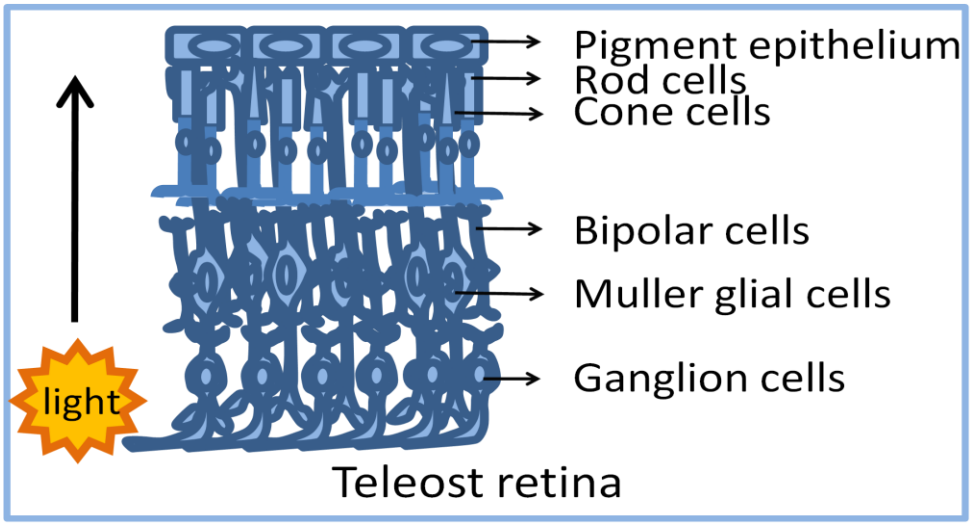

Fig 4: Layers of teleost retina

There are six different types of cells in retina. Rod and Cone photoreceptor cells, Bipolar cells, Amacrine cells, Ganglion cells and Muller glial cells. When muller cells takes up the job of regeneration after injury, they give rise to all cell types present in the retina. In order to achieve that, Muller cells first de-differentiate into retinal progenitor cells, then they proliferate, and again re-differentiate into all the cell types including Muller glia cells.

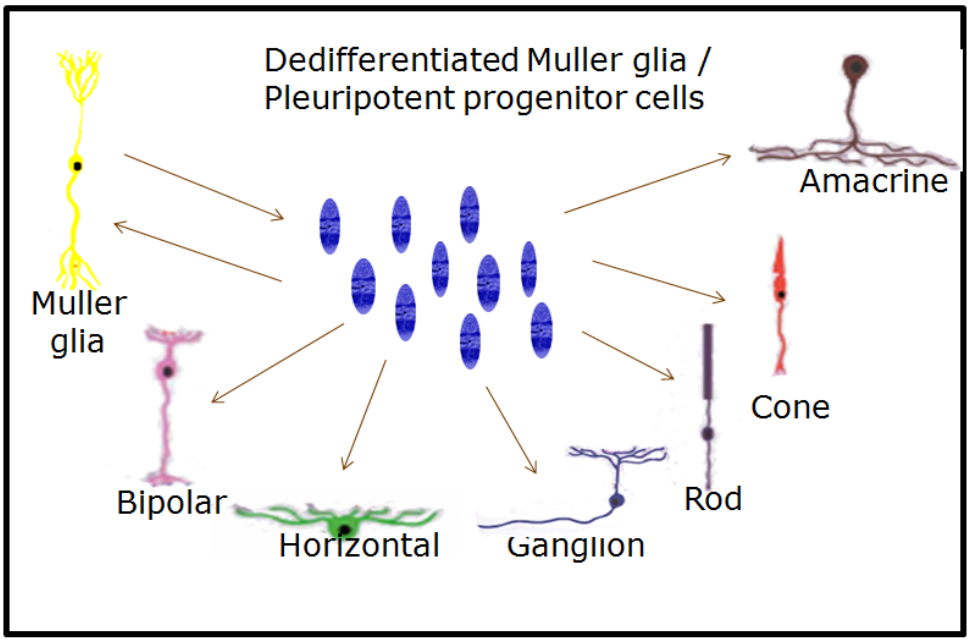

Fig 5: Fate of cells during retina regeneration

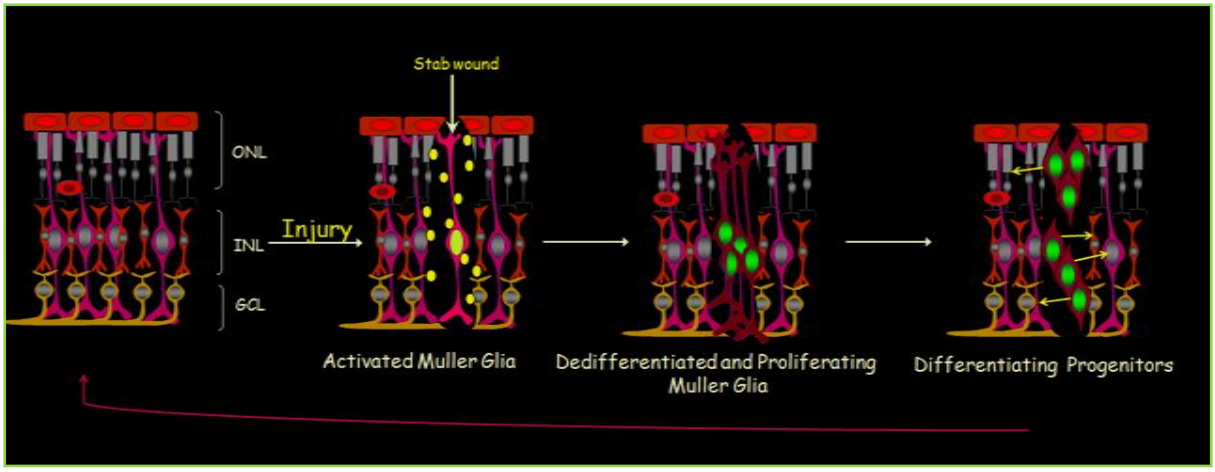

Fig 6: Retina regeneration at a glance

1.2 Review of Literature

1.2.1 Retina Regeneration

Different group around the world have deciphered some of the important molecular clues which are responsible for teleost retina regeneration. Immediately after injury, it is suspected that different cytokines and Fagocytosis are responsible for initiating different signalling cascades. As of now, the molecule known to be induced earliest (1-2 hrs) is Heparin Binding Epidermal Growth Factor (HB-EGF). This molecule, through its receptor (EGFR) in turn activates the MAPK pathway. This pathway is believed to induce the transcription activator Ascl1a, one of the most important hallmark molecules of retina regeneration. This molecule acts via several other pathway to bring about the necessary downstream signalling which ultimately culminates into physiological and functional regeneration of the retina.

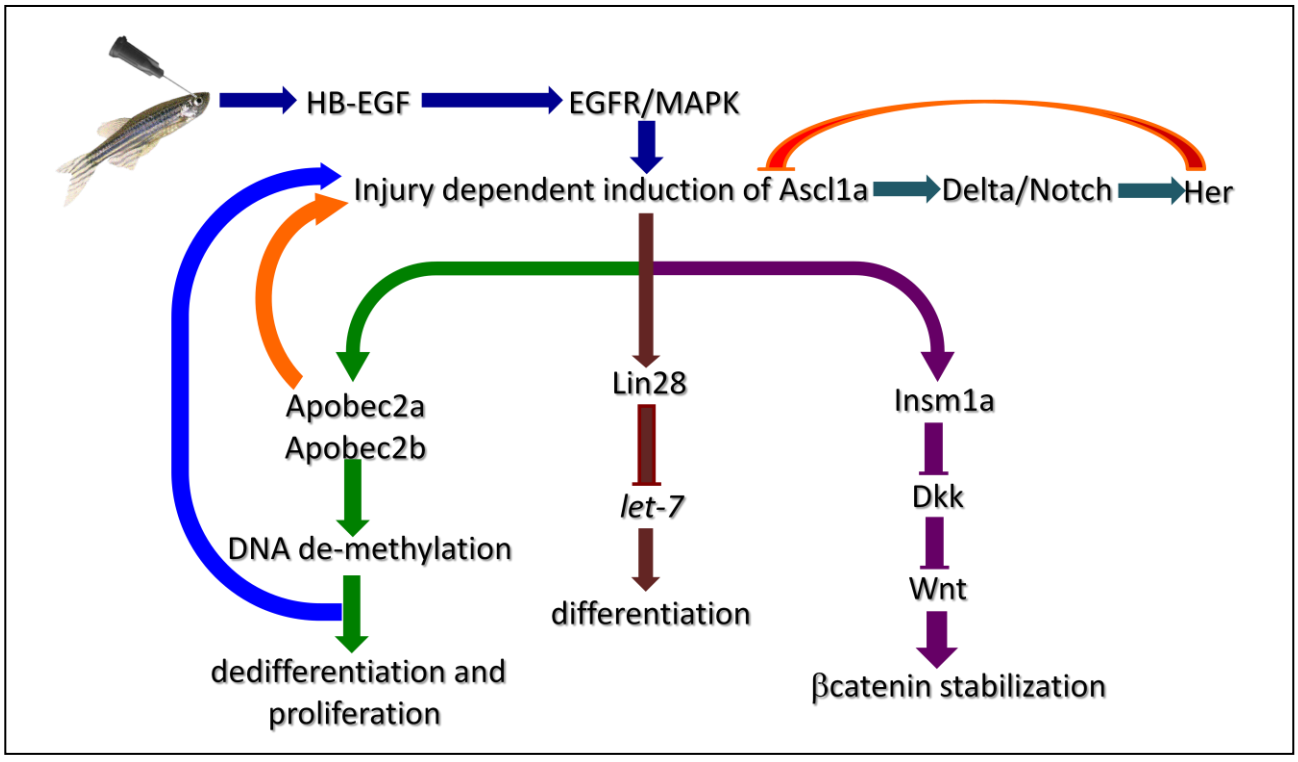

Fig 7: different signaling pathways in retina regeneration

In one pathway, it was reported that Ascl1a activates Apobec2b and Apobec2b along with

Apobec2a (another Apobec family protein, which is also upregulated during retina regeneration) bring about some changes in DNA de-methylation pattern which is believed to be responsible for De-differentiation and proliferation of Muller glial cells into Retinal progenitor cells. Through a second pathway, Ascl1a downregulates *let7* miRNA by activating Lin28, which is a microRNA binding protein. *let*7 miRNA helps in maintaining the differentiated state of the cells. So, in absence of this, cells tend to start de-differentiating. The third signalling cascade started by Ascl1a acts via activation of a transcription repressor Insm1a which down-regulates Dkk, a Wnt inhibitor. Once activated, canonical Wnt pathway helps in de-differentiation of Muller glia into retinal progenitor cells and proliferation of the same. In a positive feedback loop, Apobec2a/2b feeds back to further upregulation of Ascl1a. Through another negative feedback loop, via Delta/Notch pathway, expression of Ascl1a as well as the MAPK pathway get down-regulated.

1.2.2 Telomere

A telomere is a specialized chromatin structure of repetitive nucleotide (TTAGGG) sequences at each end of a chromatid, which protects the distal ends of eukaryotic chromosome and prevents from degradation or recombination. Telomeres continuously lose TTAGGG repeats after each cell division because of end replication problems. Besides, there are a few more reasons for telomere to get shortened, like, UV radiation, oxidative stress which leads to genomic instability, replicative senescence and apoptosis.

1.2.3 Telomerase

Telomerase is a ribonucleoprotein complex with specific reverse transcriptase activity and adds DNA sequence repeats to the 3'end of DNA strands in the telomere regions based on its own RNA which acts as templates.

As a result, every time the chromosome is copied, only 100–200 nucleotides are lost , which causes no damage to the organism's DNA.

Telomerase consist of two component- Telomerase reverse transcriptase (*TERT*) and Telomerase RNA template (*TERC*).By using *TERC, TERT* adds repetitive sequences to compensate for lost ends and thus maintains telomeric length. Loss of telomerase activity leads to aging and also prevents cancer.

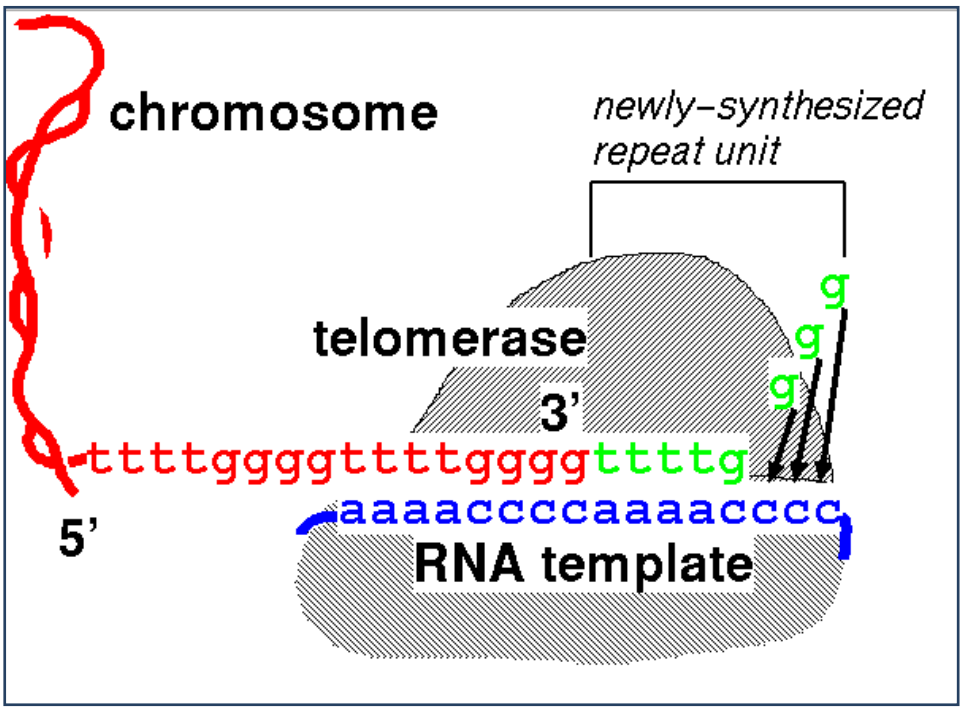

Fig 8: telomere and telomerase

Site	Expression
Germ cells & Embryonic Stem Cells	+
Somatic cells	-

Table 1: expression of Telomerase in different cell type

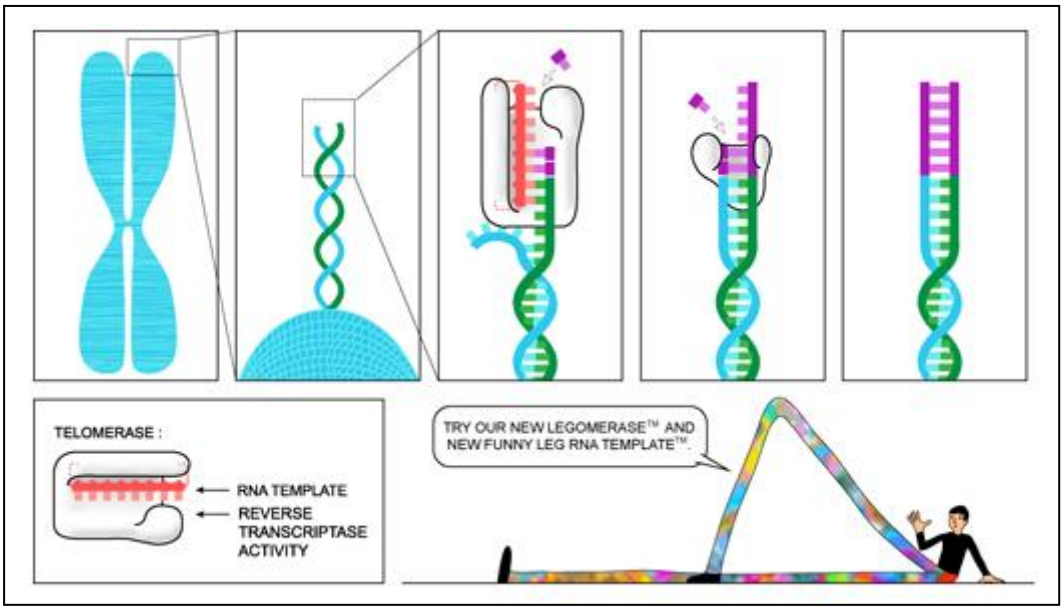

Fig 9: function of telomerase

1.3 Materials and Methods

1.3.1 Retinal injury

Table 2 : materials required for retinal injury

s.no.	Materials
1	Tricaine methanesulfonate
2	30G needle
3	Forceps
4	70% alcohol
5	Petri dish
6	Sponge bed for anesthetized fish
7	Stemi DV4 Zeiss dissecting microscope

Take out zebra fishes from tank and put it in a beaker containing tricianemethanesulfonate for anesthetize fish and then take out anesthetized fish from beaker and put it in sponge bed under dissecting microscope. Then gently pull one eye from its socket with the help of forceps and exposed back side of the eye then injured with 30G needle four times at each retinal quadrant once. After giving injury fish were put back in water.

1.3.2 RNA isolation

From different time points post injury (0hr, 6hr, 12hr, 1dpi, 2 dpi, 3 dpi, 4 dpi, 5 dpi, 6 dpi, 7 dpi, 8 dpi, 9 dpi, 10 dpi, 11 dpi, 12 dpi, 13 dpi, 14 dpi, 16 dpi, 21 dpi)

Eye was taken out in petri dish containing 0.85M NaCl then then extract 4-6 retina and put it in eppendorf containing 200µl trizol. Then we did homogenization no tissue clumps should be visible and then add 0.2 volume chloroform and mix for 15-20 minute in rotator at speed 25 rpm. Centrifuge at 4000-7000rpm at room temperature for 5minute.Cut tips at ends and slowly take RNA from upper layer. Then add 0.6 volume of isopropanol and keep it in -80°C for overnight or in ice for 20 minute. Next day or after 20 minute centrifuge at 4000rpm at 4°c (fast cool) for 10 minute. Then discard supernatant and give 75% - 80% alcohol wash, centrifuge at maximum speed for 10 min. Pellet was air dried and dissolved it in 30µl MQ and store it in -80°C and check on a gel.

- Take nanodrop reading of all time course RNA

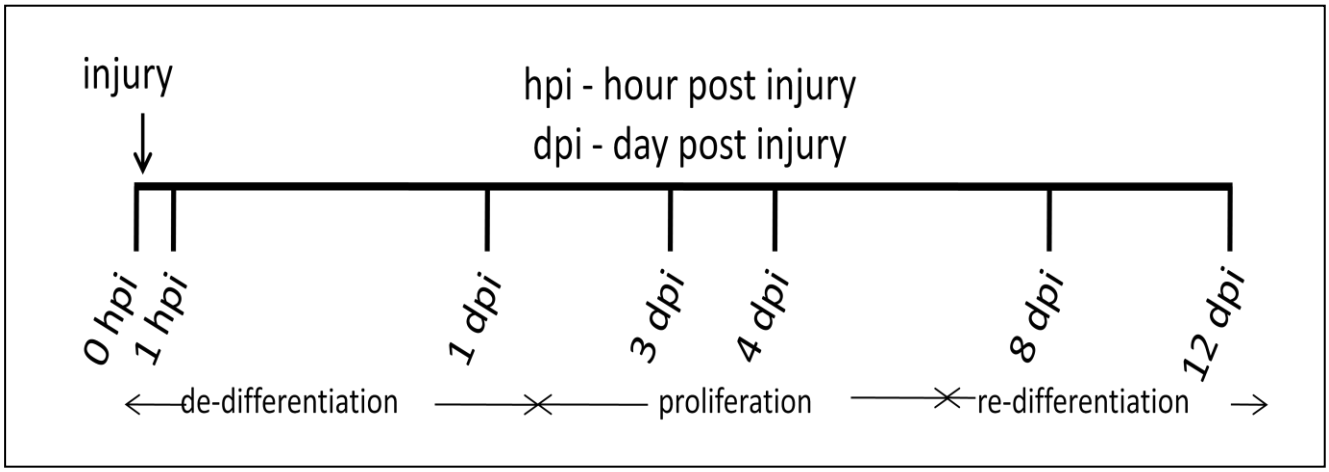

Fig 10: schsematic of the course of experiment

1.3.3 cDNA preparation

We used transcriptor first strand cDNA synthesis kit from Roche to prepare cDNA from total RNA.

We took 10µl RNA, 0.5µL random primer, 0.5µl oligo dT and 2µl of DNTPs in PCR tubes. Denature at 65°C for 5 minute.Take out PCR tubes and keep them on ice immediately. Add 0.5µl RNAse inhibitor, 4µl buffer and 1.5 µl H2O and then add 1µl reverse transcriptase. Run reverse transcriptase (RT) protocol.Take out PCR tubes and store it in -20°C. Add 60µl MQ in 20µl cDNA(4 folds) before putting PCR reaction.

Then we put RT PCR reaction by using these cDNA as template.

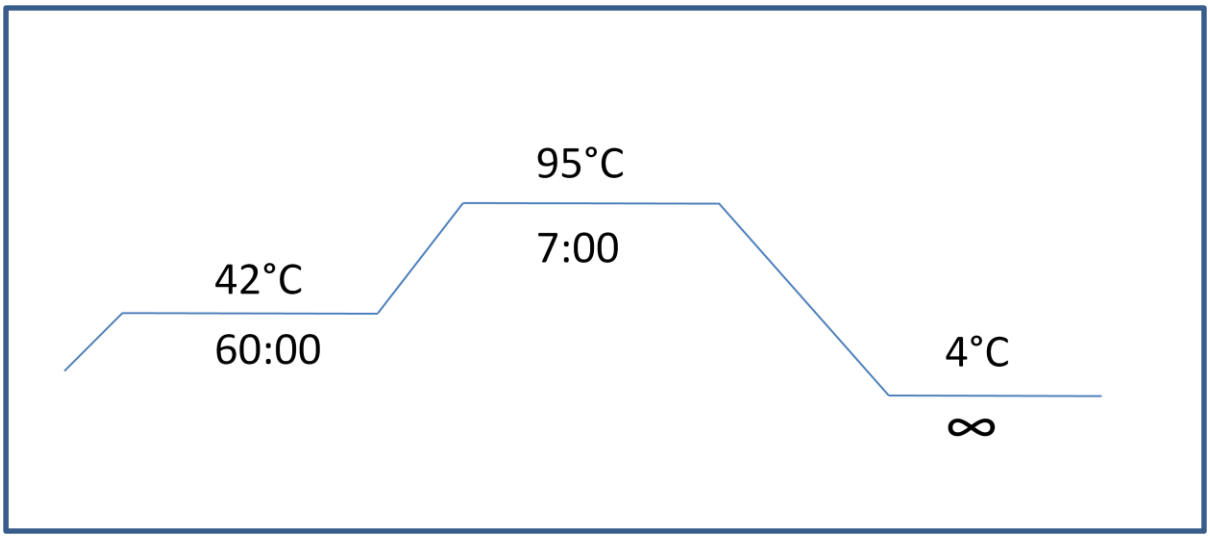

Fig 11 : protocol of RT PCR

1.3.4 Polymerase Chain Reaction(PCR)

We used actin as control so we put pcr reaction to check whether these cDNA were prepared correctly or not

Table 3 : materials required for PCR

s.no.	Ingradient	Volume(25 µl)
1	Buffer	2.5µl
2	DNTPs	2.5µl
3	Forward primer	1µl
4	Reverse primer	1 µl
5	Template	1 µl
6	MQ	15 µl
7	Taq polymerase	2 µl

Put reaction and run actin protocol.Then take out pcr tubes and check on gel.

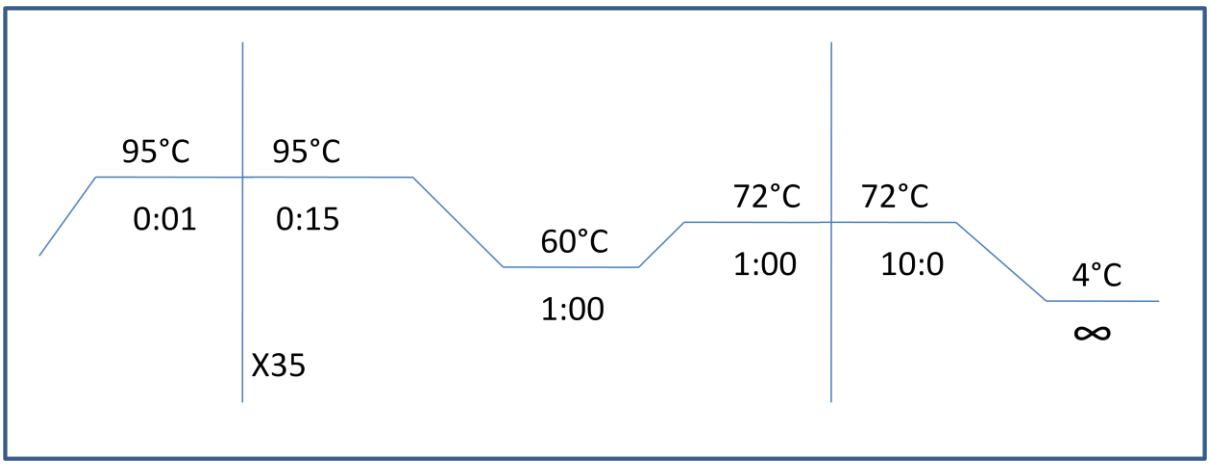

Fig 12 : protocol of actin

There are four telomerase transcripts - Tert 001, Tert 201, Tert 003, Tert 004.

Primers

Reverse primer – CAGTCAGCCATCAAGTGTTTATGAGC

Reverse primer is same for all the four Tert transcript

Tert 001 : forward primer - GAAGCGGGAAATAGAACTCCCCAAG

Tert 201 : forward primer – TTCTGCACCAAAGCTGAGGTCGGAG

Tert 003 : forward primer – CTGCTGCAGGCGCTCAGGGAAGCGTG

Tert 004 : forward primer – GTCAGAGTGTGAACAGAAACCCTTCG

We did pcr at different temperature (56°, 58°, 60°, 62°, 64°, 66°c) to see amplification whether these four transcript of Tert gene are expressed or not in retina during regeneration.

Except Tert 001, all three are amplified.

Tert 201, Tert 003, Tert 004 are amplified at 66°C

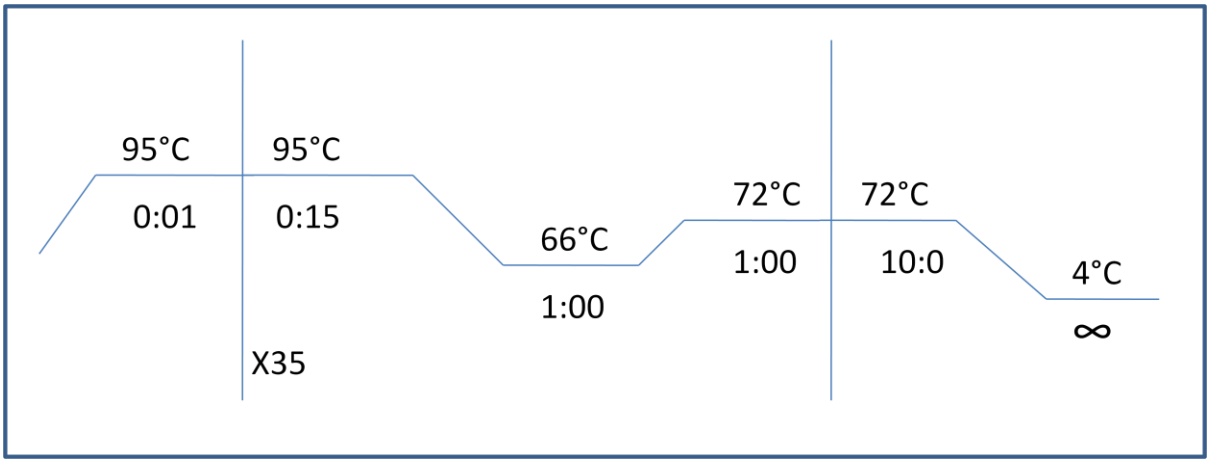

Fig 13 : protocol of Tert

1.3.5 Topo TA cloning

We cloned Tert 004 in TOPO TA cloning vector. The TERT 004 transcript was of 450bp, which was cut and put in a tube before purifying it using gel extraction kit. We did PCR purification in which we add 100µl binding buffer(BB) and boil it for 10 minute so that BB and pcr product mixed then we load on column and centrifuge at 13000rpm for 1 minute then discard flowthrough then add 100µl binding buffer to column then again centrifuge at 13000rpm for 1 minute then discard flowthrough and then wash with 500µl wash buffer then centrifuge at 13000 rpm for 1 minute then discard flowthrough and again add wash buffer , centrifuge and discard then we did dry spin then elute in 30µl prewarmed nucleus free water (55°C) then incubate for 3 minute then centrifuge at 10000 rpm for 5 minute at room temperature then run on gel.

Then we put ligation reaction in which we add 0.5µl salt solution, 0.3µl vector and 1.0µl insert (pcr purified) and then incubate for 1 hr. Then transform it into competent cells by putting 5µl plasmid in *E.coli* cells then leave it in ice for 30 minute then put it in water bath for 42 second at 42°C for heatshock then put 1 ml LB (Luria Broth) in *E.coli* cells and put it in incubator for 20-30 minute then centrifuge at 3000 rpm for 5 minute then discard flowthrough and add 200ml LB and mix it then spread it in ampicillin plate and leave for 15 min for dry then put it in incubator for overnight growth. Then in morning there were some colonies in plates, put those plates at 4°C

Take 3µl MQ in PCR tubes and then put single colonies on those 6-8 pcr tubes and patch on ampicilin plate. Leave for overnight growth. Then on next day take 5µl MQ in pcr tubes and take patched colonies from patched plate and put it in pcr tube then boil at 100°C for 2 minute in PCR Machine then we did pcr.

1.3.6 Making antisense DIG labelled RNA probe

We did Topo-TAII plasmid digestion from fast digest kit. Firstly we add 14.5µl H_2O, 2µl fast digest buffer, 2.5µL DNA, 1.0µl NOTI restriction enzyme(for tert003) and BamHI for tert004 then incubate for 30 minute at 37°C . Then we did gel extraction. Then we took 4µl transcription buffer (10X), 10µl template, 2µl DIG-UTP, 2µl SP6 RNA polymerase and 28µl H_2O. Then incubate at 37°C for 4 hr. after 4 hr. to stop the reaction we add 4 µl 0.5M Tris EDTA then we add 4µl 5M $Licl_2$ then 2µl 10mg/ml glycogen then tab gently and then add 70µl 100% ethanol then tab and keep it in -80°C for overnight or 1 hr. Then centrifuge at 10000rpm at 4°C for 15 minute then one more washing with absolute alcohol then dry and dissolve it in 50µl MQ or hybridisation buffer then store in -80°C.

1.3.7 Tissue fixation and cryoprotection

4% PFA (Paraformaldehyde)

Take 2gm PFA in 50ml falcon tube and then take 50 ml 1X phosphate buffer. Dissolve it.

5% Sucrose – take 2.5gm sucrose in 50ml falcon tube then add 5ml 10X PBS(Phosphate buffer saline) and volume makeup with MQ then mix it.

20%Sucrose – take 10gm sucrose in 50ml falcon tube then add 5ml 10X PBS(Phosphate buffer saline) and volume make up with MQ then mix it.

We have to fix eye, we did with control, 4dpi (day post injury).

Firstly we take out eye from fish in 4% PFA then we gently remove lens by torn cornea then put in MCT containing 4% PFA buffer for fixation at room temperature (RT) for 2 hr. at rotator, then remove buffer and add 600ml 5% sucrose and put it in rotator for 45 minute at

RT, then remove 5% sucrose and add 400ml 5% sucrose and 200ml 20% sucrose and put it in rotator for 45 minute at RT, then remove solution and add 300ml 5% sucrose and 300ml 20% sucrose and put it in rotator for 45 minute at RT, then remove solution and add 200ml 5% sucrose and 500ml 20% sucrose and put it in rotator for 45 minute at RT, then remove solution and add 600ml 20% sucrose and incubate at 4°C for 45 minute or overnight then add 400ml OCT and rotate for 30 minute in rotator then make a cube with aluminium foil and put OCT and then put eye in that block and put it in -80°C.

Blocks were ready for sectioning. We take 4-5 slides and set block on cryosectioning machine then take section on each slide. Store those slide at room temperature for dry then store it in -20°C.

1.3.8 Immunostaining

1XPBS + 3%BSA +0.1% triton and 1XPBS + 1%BSA +0.1% triton

First day

we incubate the slides at 37°C for an hr. because they were kept in -20°C for dry then washed slides with 1XPBS for 10 minute twice then block the sample using 1XPBST (3%BSA) for 30 minute or an hr. then overlay the slide using primary antibody(anti-mouse) and antibody should be diluted in (2:500)µl ratio-2µl antibody and 500µl 1XPBST(1%BSA) then keep the container in 4°C for overnight.

Second day

wash the slides using 1XPBST for 10 minute twice then overlay the slide using secondary antibody (2:1000µl) then wash thrice with 1XPBST for 10 minute each and then wash it with MQ twice for 10 minute each then dry the slide for an hr. then put coverslip using dabco.

1.3.9 mRNA *in situ*-hybridization

Proteinase K buffer (37°C)

Add 5ml 1.0M Tris-Hcl, 5ml 0.5M EDTA and make up with MQ to make 50ml and put it in incubator

20X SSC

Dissolve 87.6g og NaCl in 350ml of DEPC water and add 44.12g of sodium citrate and bring to final volume of 500ml with DEPC water

For 2X SSC – add 5ml 20X SSC and make up with MQ to make 50ml MQ

TEA

Add 0.9g triethanolamine hydrochloride and add 173µl NaOH and make up MQ

50% Formamide/2XSSC

Take 25ml formamide and 5ml 20XSSC and make up with MQ

RNAase Buffer

Take 5ml 5M NaCl, add 500µl 1M Tris HCl and 100µl EDTA then makeup with MQ

Genius buffer

100ml 0f 10X Tris-HCl/NaCl stock in 800 ml MQ and check pH, pH should be 9.5 and while mixing add 100ml of $MgCl_2$stock (no need to adjust pH at thid point)

Daily Procedures

Day1

Put the slide in EtOH series 100%, 100%, 95%, 70%, 50% and 2XSSC for hydration for 1 minute each then incubate slides in prewarmed Proteinase K buffer solution for 1-5 minute and then rinse the slide with MQ and then rinse it with 0.1M TEA for 3 minute again rinse it

with TEA for 10 minute and then dehydrate the slide in series of 2XSSC, 50%EtOH, 70%, 95%, 100%, 100% ethanol for 1miute each then dry slide for 1 hr. Then take hybridization solution 200µl/slide in MCT and add probe 3µl/slide to hybridization buffer and then put it in 100°C in thermoblock for 10 minute then put hybridization/probe solution to each slide and put coverslip with siliconized hybrid slips then placed it in humid chamber dampened with formamide/5XSSC and incubate at 56°C overnight.

Day2

Preheat 50% Formamide / 2 X SSC solutions to 65°C. Preheat two 50ml RNase buffer washes, one to 37°C and other to 65°C. Preheat two 2X SSC washes to 37°C. Soak slides with cover slip at room temperature 2X SSC for 30 minute on shaker table then gently remove cover slip. Rinse slides at 37°C 2X SSC solution for 10 minute, twice. Add 100µl of RNase A (10mg/ml) to the 37°C RNase buffer, incubate slides for 30 minute. Wash slides in the 65°C RNase buffer for 30 minute. Wash slides for 2-3 hr.in 1X Maleate/ 0.05% Triton X-100/ 1% RMB blocker solution at room temperature (thaw 3ml aliquot and add 7ml of MQ). Wash slides in 1X Maleate buffer for 5 minute, twice. Incubate slides with 205µl with antibody of choice diluted in 1X Maleate /0.05% Triton X-100/ 1% RMB blocker solution overnight at room temperature and add antibody solution to cover well water-tight chamber slip and add lower slides over cover well and place slide cover slip-up in humid chamber.

Day 3

Washed with genius buffer thrice for 10 minute each and put NBT/BCIP reagent to cover slides and then incubate for overnight then did detection.

1.3.10 Phylogenetic tree

Gene sequences of all organisms (human, mouse, zebrafish) were taken from ensemble and then run clustal omega to see the evolutionary relationship between organism then make a tree.

1.4 Results

1.4.1 Cloning

Tert-001 not expressed in uninjured and not even in injured retina whereas Tert -201, Tert-003, Tert-004 are expressed.

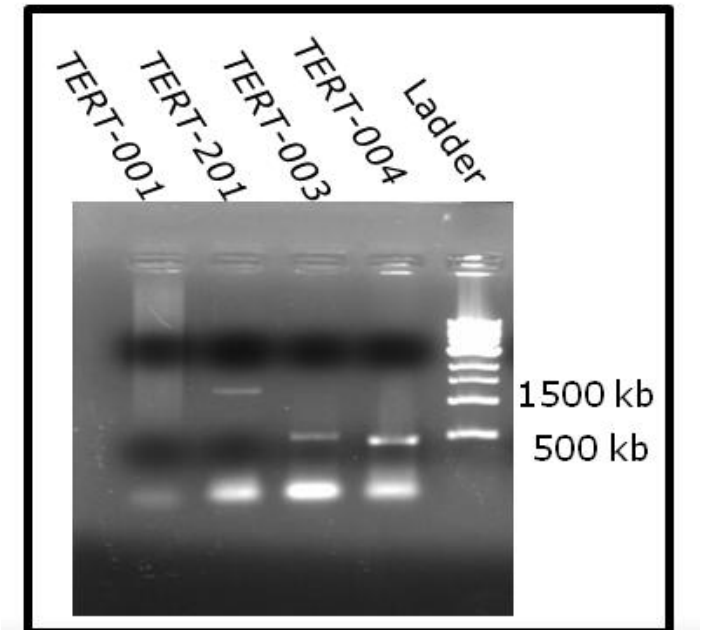

Fig 14 : RT PCR of different Tert transcripts

Cloned telomerase transcript in Topo-TA plasmid.

1.4.2 Time course of gene expression

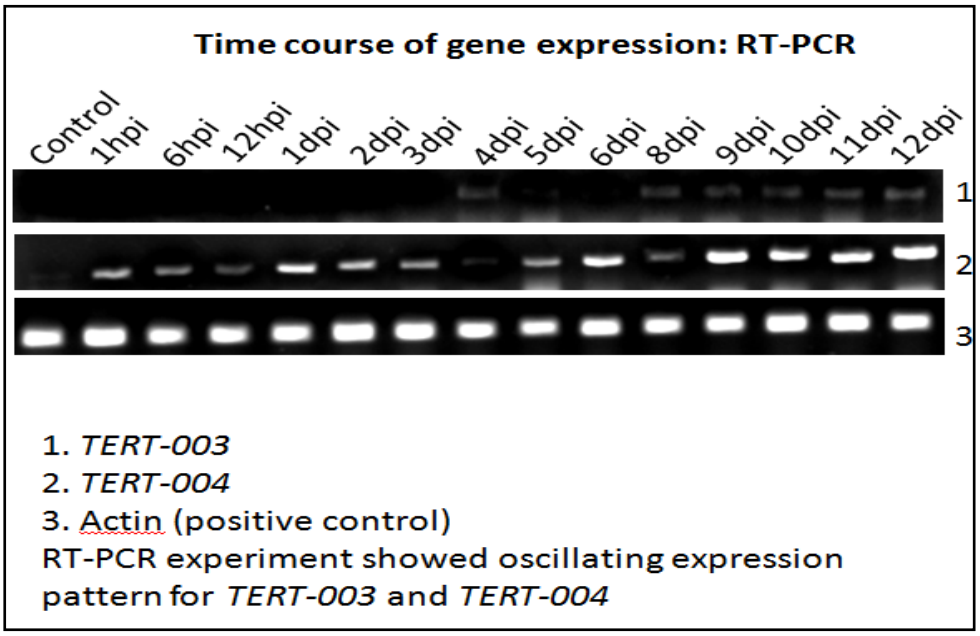

Fig 15: time course of TERT 003/004

1.4.3 mRNA In-situ hybridization of Tert -004

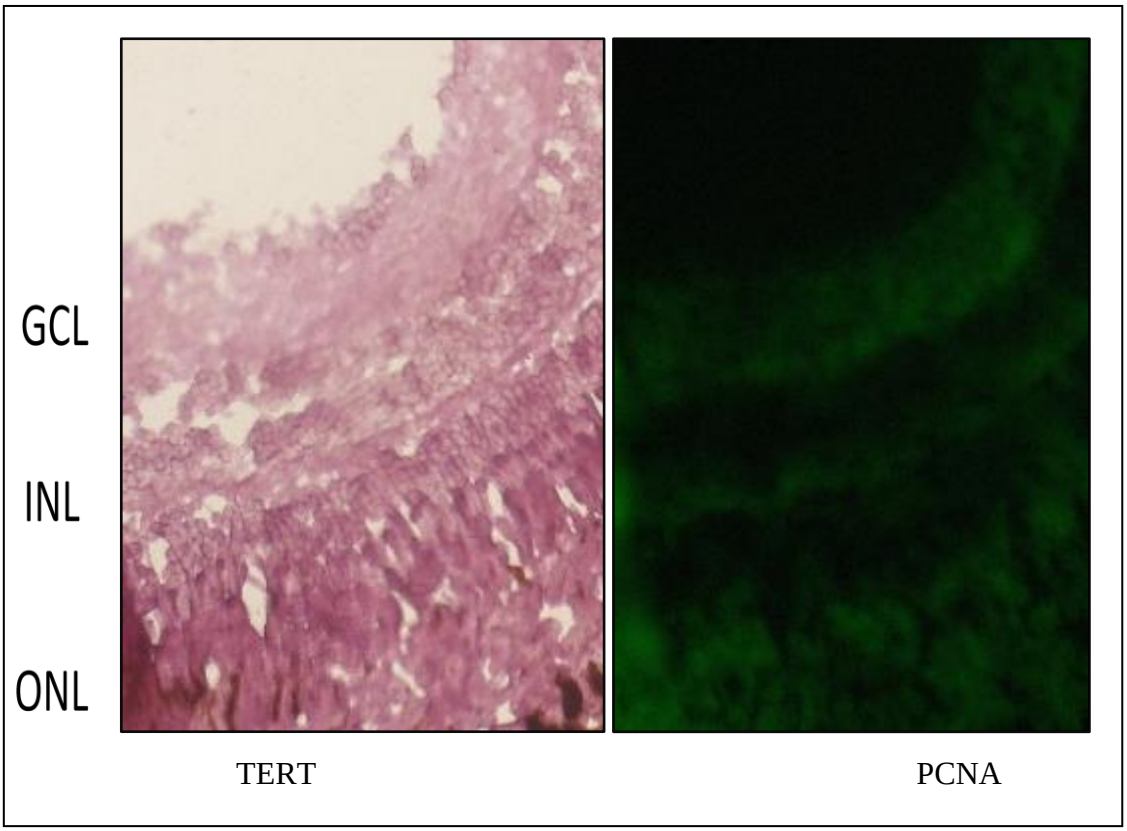

Fig.16a :tert 004 is not expressed in uninjured condition

Eyes were injured using needle poke method and were harvested on 4 dpi. 3 hours before harvesting intraperitonialBrdU injection was given.

Following this *In-situ* was done using *TERT-004* specific Digoxigenin labeled antisense RNA probes. Expression was predominantly in ganglion cell layer.

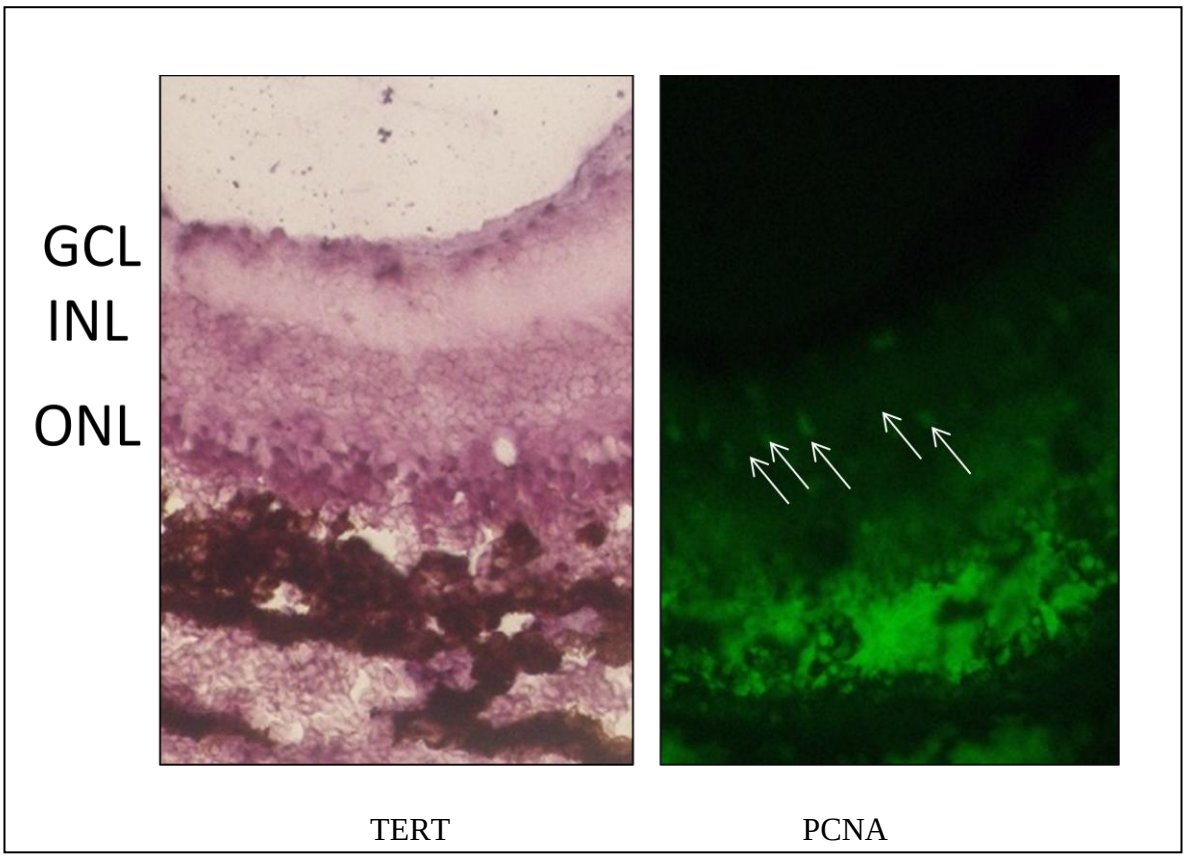

Fig.16b: mRNA *in situ* at 4dpi

At 4 dpi, *TERT 004*is expressed in ganglion cell layer. Cells expressing *TERT 004* are also positive for PCNA.

1.4.4 Optic nerve lesion

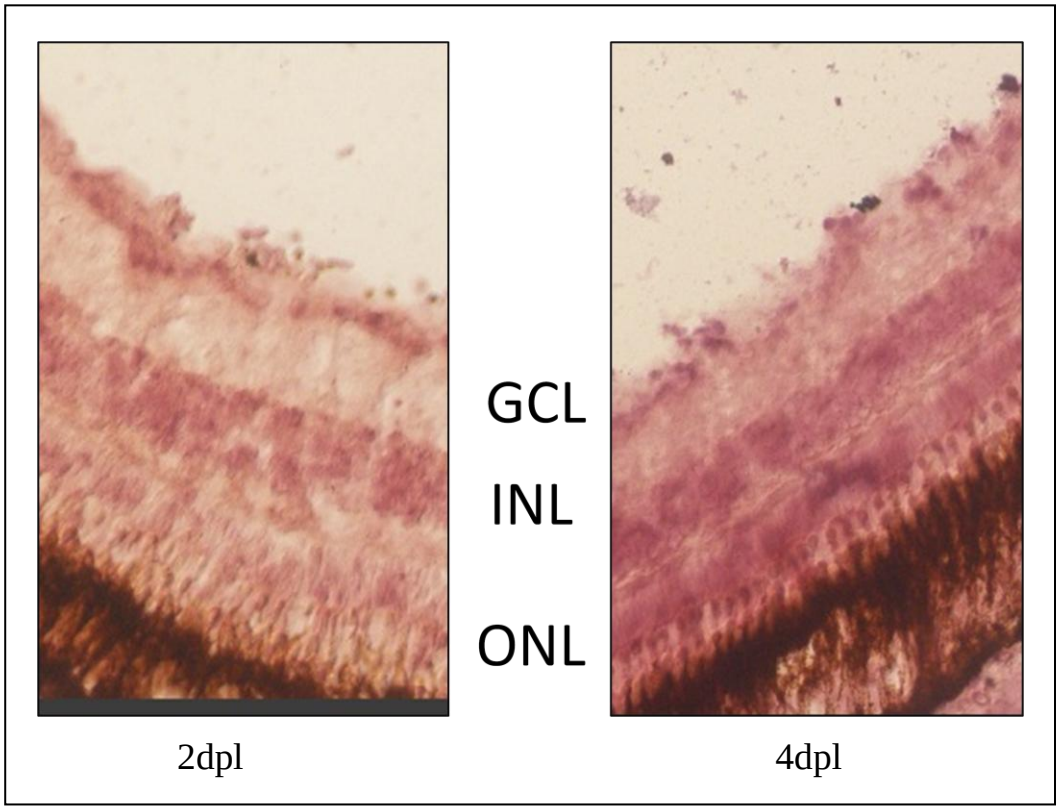

Fig.17 : mRNA *insitu*of optic nerve lesion at 2dpl and 4dpl

Where dpl is day post lesion

To confirm whether *TERT*expression was due to retinal injury or its exclusively a response from ganglion cell layer, we decided to perform optic nerve lesion which disturb largely the optic nerve and ganglion cell layer, retaining rest of the retina intact, Interestingly we found the optic nerve crush did not cause induction of TERT 004 in the GCL as found in injured condition.

1.4.5 TERT Hedgehog signalling and Notch signalling

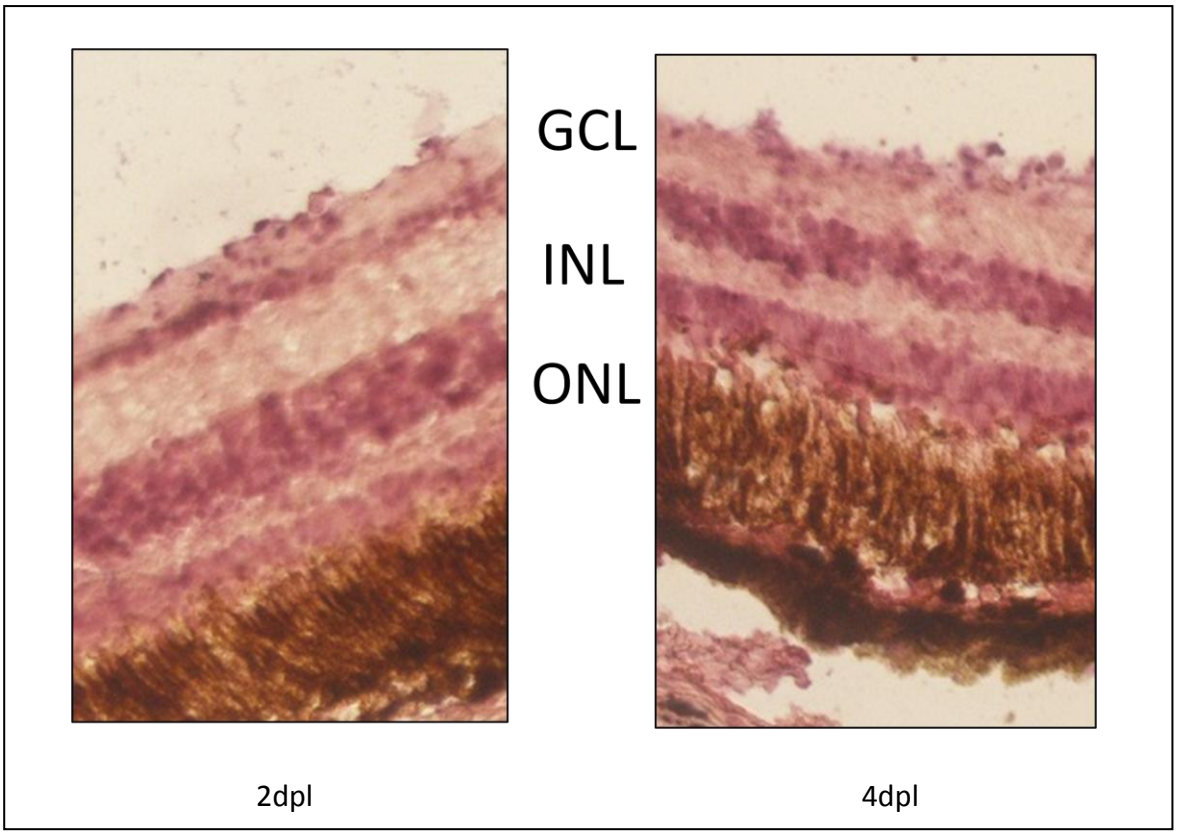

Fig.18: Expression of TERT 004 in blocked bedgehog and notch signalling background

To check if hedgehog or notch signaling takes part in telomerase activity during regeneration, we blocked hedgehog using cyclopamine drug and notch signaling using DAPT drug. There is no prominent effect on the expression of *TERT*.

1.4.6 Phylogenetic tree

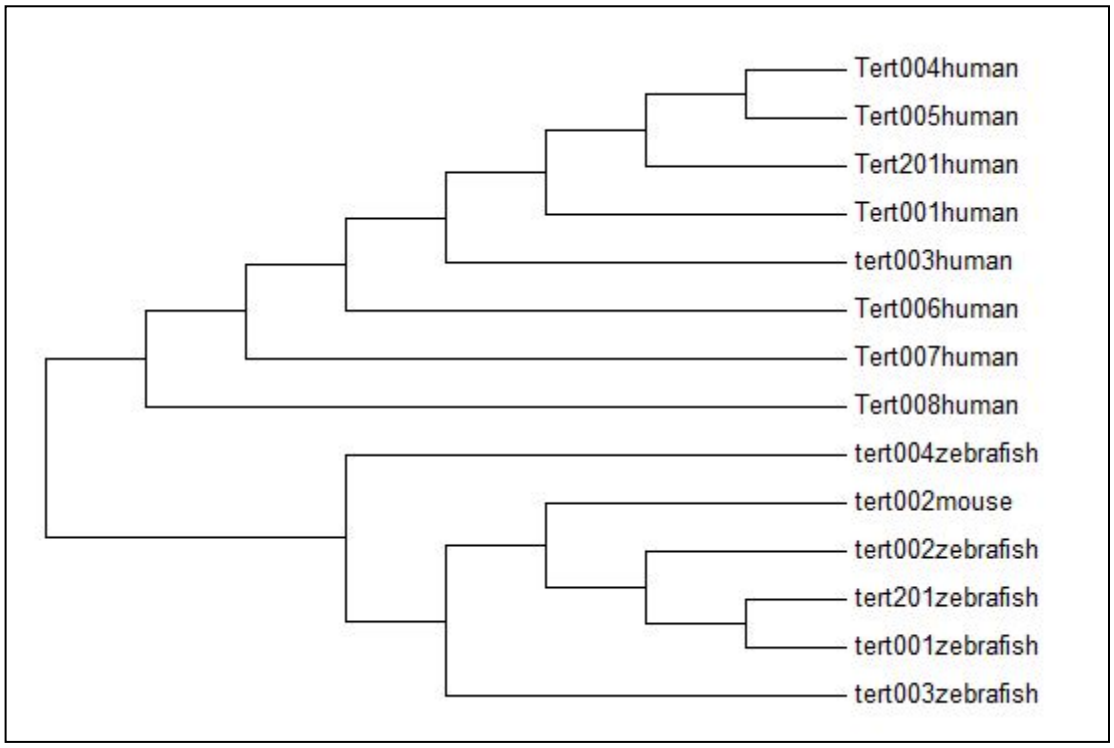

Fig 19 : cladogram of telomerase transcript from zebrafish, mouse, human

Chapter 2

2.1 Summary & Conclusions

TERT-004 induced in ganglion cell layer following injury. This expression might be required for dedifferentiation and Muller Glial derived retinal regeneration. It is already reported to have a role in mouse liver regeneration. Whether *TERT* functions in retina in a similar way needs to be further investigated. It would be ideal to look for the regeneration of *TERT* knockdown zebrafish retina using morphilono based antisense oligo nucleotide. Evaluation of other *TERT* transcripts apart from *TERT-004* also could shed more light into mechanistic understanding of *TERT* gene function in retina in both uninjured and post injured conditions.

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