# Molecular Mechanisms Underlying Retina Regeneration: Inquest of Role of Matrixmetalloproteinase-9

**Sukanya V S**

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# Certificate of Examination

This is to certify that the dissertation titled **"Molecular Mechanisms Underlying Retina Regeneration:Inquest of Role of Matrixmetalloproteinase-9**" submitted by Ms. Sukanya V S (Reg. No. MS10060) 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. Shashi Bhushan Pandit  Dr. Kuljeet Singh  Dr. Rajesh Ramachandran
(Supervisor)

Dated: 24 April 2015

# 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.

Sukanya V S
(Candidate)
Dated: April 24, 2015

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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And thank God! (if exists)

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# Notation

MG - müller glia

INL - inner nuclear layer

ONL – outer nuclear layer

GCL – ganglion cell layer

RPE - retinal pigment epithelium

MMP – matrix metalloproteinase

Hh - hedgehog

ECM - extracellular matrix

$Ca^{2+}$ - calcium ion

$Zn^{2+}$ - zinc ion

cDNA - complementary deoxyribonucleic acid

mRNA – messenger ribonucleic acid

DIG- digoxigenin

UTP – uracil triphosphate

dpi – days post injury

hpi – hours post injury

mpi – minutes post injury

EDTA- ethylenediaminetetraacetic acid

M- molar

μM- micromolar

μl - microliter(s)

mg- milligram(s)

mM- millimolar

ng – nano gram(s)

MO- morpholino oligonucleotide

PCR- polymerase chain reaction

qRT-PCR - quantitative reverse transcription PCR

RT-PCR - reverse transcription PCR

DEPC – diethylpyrocarbonate

SYBRGreen – N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine

NaOH- Sodium hydroxide

SDS – Sodium dodecyl sulfate

IPTG- Isopropyl β-D-1-thiogalactopyranoside

Xgal- 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

PCI – Phenol:Chloroform:Isoamyl Alcohol

EtOH- Ethanol

TEA- triethanolamine

SSC- saline-sodium citrate

AP- Alkaline phosphatase

Nacl- Sodium chloride

NBT-nitroblue tetrazolium

BCIP- 5-Bromo-4-chloro-3-indolyl phosphate

HCl-Hydrochloric acid

$Na_2HPO_4$ – Disodium phosphate

$NaH_2PO_4$ – Monosodium phosphate

OCT- Optimal Cutting Temperature

PCNA - Proliferating cell nuclear antigen

BSA - Bovine serum albumin

# Contents

# Abstract

Mammalian CNS regeneration is highly restrained. While, teleost fish shows remarkable capacity of regeneration in response to damage, restoring vision. The entire retinal regeneration relies on a glial cell, Müller glia. The exact molecular mechanisms involved during the retinal regeneration remains elusive. Here, in this study we looked at the role of Matrix metalloproteinases during retina regeneration. It was found that MMP-9 is expressed in Müller glia (MG) of inner nuclear layer (INL) close to injury site at very early time points. Peak of expression observed during dedifferentiation and pre-proliferative phases and near the end of regeneration, expression returned to the basal values. mRNA *in situ* hybridization and PCNA immunostaining showed that a subset of proliferating and non-proliferating MG cells expresses MMP-9. Also, we observed the regulation of MMP-9 through Notch, Wnt and Hedgehog (Hh) signaling. A reduction in the number of proliferative cells was observed with knockdown mediated by MMP-9 targeting antisense morpholino, these results cumulatively suggests that MMPs plays an important role during retina regeneration.

# Chapter1

# Introduction

Vision is one of the most important senses, connecting us to external world. Eye is the organ which makes power of sight is possible and, retina is the part of eye which generates the pictures (through the cornea and lens) which are via the optic nerve passed on to the brain and perceived as such by it. Vertebrate retina has three distinct layers with six neuronal cell types and one glial cell: the outer nuclear layer (ONL) accommodating cell bodies of photoreceptor cells (rods and cones), the inner nuclear layer (INL) with cell bodies of bipolar, horizontal and amacrine cells, along with the cell bodies of Müller glia, yet glial processes span entire retinal layers and cell bodies of ganglion cells are located in the ganglion cell layer (GCL). Synapses are formed between photoreceptor layer and interneurons called outer plexiform layer (OPL) and synapse between interneuron and and ganglion cells is called inner plexiform layer (IPL). Retinal pigment epithelium (RPE) is the pigmented cell layer overlying retinal visual cells**.(Figure1.1)**

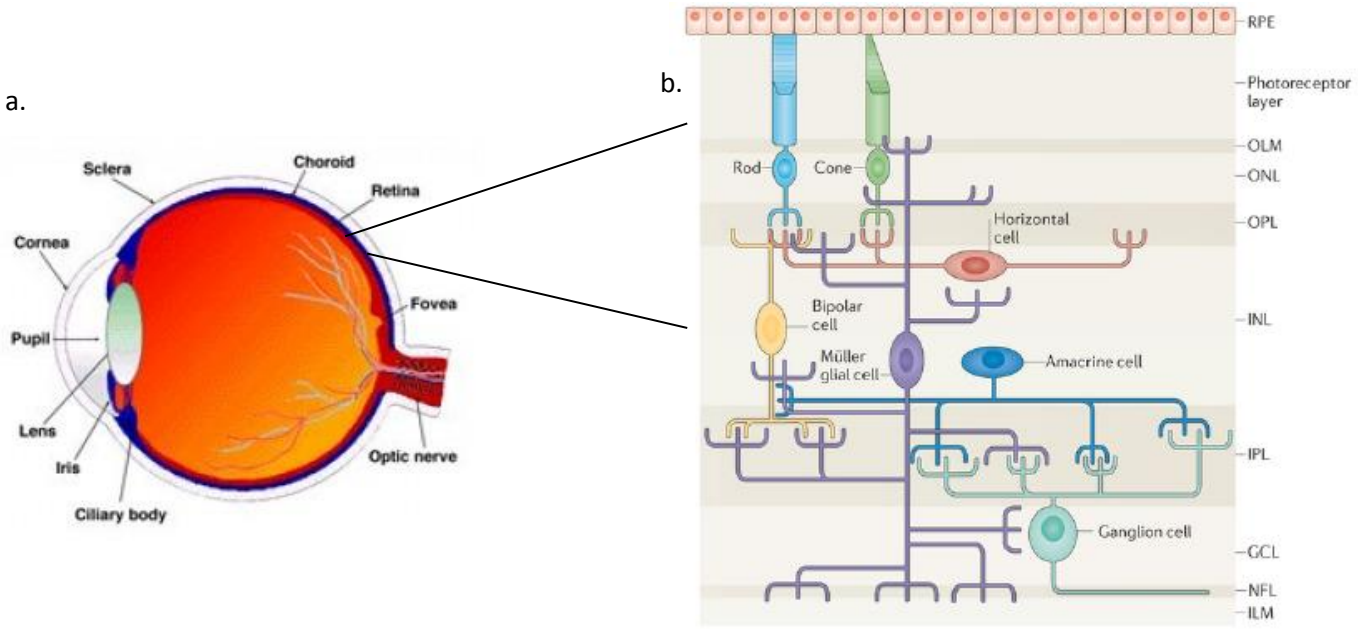



**Figure 1.1**: (a) Sagittal section of the adult human eye[20] (b) Detailed structure of retina showing cellular layers and cell types[3].

Loss of sight can result from traumatic injuries and diseases like macular degeneration diabetic retinopathy, retinoblastoma, etc... Nowadays, various methods of treatment are available like surgical interventions, gene therapy and cell transplants. But, ideal reparative strategy would be self-healing of damage.

Regeneration of retina has been observed in a variety of organisms during either their development or for some, even during their adult life. Among those with the ability to regenerate during adulthood are fish, birds and amphibians. The story is changed for mammals where regeneration is not observed, rather mammalian retina responds to injury through reactive gliosis causing more damage than cure. Previous studies demonstrated that the successful regeneration process relies on a glial cell, Müller glia (MG), which normally does not generate neurons during development, but can regenerate neurons after retinal injury. Müller glia was generally assumed that they were responsible for maintaining tissue structure and homeostasis. However, researchers have reported that these cells acquire stem cell characteristics in response to injury, generating proliferating population of multipotent progenitor cells. But, until now the exact molecular mechanism involved in the retinal regeneration is a mystery. Understanding mechanisms could suggest novel strategies for stimulating retina regeneration in mammals.

Zebrafish **(Figure1.2)** shows remarkable capacity to regenerate their central nervous system following damage. Zebrafish (*Danio rerio*) is a small tropical fresh-water fish, which gained popularity due to their short generation time, ability to produce large number of offspring and the transparency of the embryos – making them an ideal model organism to study the development of vertebrates using a genetic approach and also utilized in exploring pathophysiology of diseases.

**Figure1.2*:*** Adult zebrafish fish

Traditionally, the zebrafish has been used as a model for studying the regeneration of the fins. Since retina is the most easily ‘approachable part of the brain’, and morphology and function of the retina are basically conserved among vertebrate species, zebrafish have also been used for studying the regeneration of the retina and optic nerve.

Extracellular matrix (ECM) is a network of proteins and carbohydrates providing mainly mechanical support and intracellular communication. It is known that, ECM remodelling is crucial for development, homeostasis, tissue repair and cancer regulating cell migration, differentiation, morphogenesis, proliferation, adhesion and apoptosis. Primarily, dynamic nature of ECM is affected by degradation of its components by various proteases as serine proteases, threonine proteases, and matrix metalloproteinases. Matrix metalloproteinases (MMPs) family is one of the most abundant classes of proteases which collectively can degrade all components of extracellular matrix. MMPs are mostly excreted proteins with several conserved domains. Basically, there are three common domains pre domain, pro domain and catalytic domain. The gelatinases (MMP-2 and -9) further contain a series of three fibronectin type II inserts in the catalytic domain, which facilitate binding of gelatine and collagen. All MMPs require $Zn^{2+}$ and $Ca^{2+}$ for catalytic activity. MMPs show differences in their substrate specificity, membrane binding and regulation that make this a very versatile family of

| Subfamily | Name(s) | ECM Substrate(s) |
|---|---|---|
| Collagenase | MMP-1/Collagenase-1 | Collagen I, II, III, VII, VIII, X, XI; gelatin, entactin/nidogen, fibronectin, laminin, vitronectin, aggrecan |
| | MMP-8/Collagenase-2 | Collagen I, II, III; gelatin, entactin, aggrecan, tenascin |
| | MMP-13/Collagenase-3 | Collagen I, II, III, VI, VIII, IX, X, XIV; gelatin, fibronectin, vitronectin, aggrecan, osteonectin |
| | MMP-18/Collagenase-4 | Collagen IV |
| Gelatinase | MMP-2/Gelatinase-A | Collagen I, III, IV, V, VII, X, XI; gelatin, fibronectin, fibrillin, elastin, laminin-5, vitronectin, aggrecan, osteonectin, tenascin |
| | MMP-9/Gelatinase-B | Collagen IV, V, XI, XIV; gelatin, decorin, fibrillin, elastin, laminins, vitronectin, aggrecan |
| Stromelysin | MMP-3/Stromelysin-1 | Collagen III, IV, V, VII, IX, X, XI; gelatin, decorin, elastin, entactin/nidogen, fibronectin, fibrillin, laminin, vitronectin, aggrecan, osteonectin |
| | MMP-10/Stromelysin-2 | Collagen III, IV, V; gelatin, elastin, fibronectin, aggrecan |
| | MMP-11/Stromelysin-3 | Fibronectin, laminin, aggrecan |
| Matrilysin | MMP-7/Matrilysin-1 | Collagen I, IV; decorin, elastin, fibrillin, fibronectin, laminin, vitronectin, aggrecan, osteonectin |
| | MMP-26/Matrilysin-2 | Collagen IV, gelatin, fibronectin, fibrin/fibrinogen |
| Membrane-type | MMP-14/MT1-MMP | Collagen I, II, III; gelatin, fibronectin, vitronectin, aggrecan |
| | MMP-15/MT2-MMP | Fibronectin, laminin, entactin, aggrecan, proteoglycans |
| | MMP-16/MT3-MMP | Collagen III, fibronectin |
| | MMP-17/MT4-MMP | Gelatin, fibrin/fibrinogen |
| | MMP-24/MT5-MMP | Fibronectin, gelatin, proteoglycans |
| | MMP-25/MT6-MMP | Collagen IV, gelatin, laminin-1, fibronectin, proteoglycans, fibrin/fibrinogen |
| Other MMPs | MMP-12/Macrophage elastase | Fibronectin, elastin, laminin, proteoglycans, fibrin/fibrinogen |
| | MMP-19/RASI-1 | Collagen IV, gelatin, laminin, fibronectin, fibrin/fibrinogen |
| | MMP-20/Enamelysin | Amelogenin, aggrecan |
| | MMP-21/XMMP | Gelatin |
| | MMP-23/CA-MMP | Gelatin |
| | MMP-27/CMMP | Gelatin |
| | MMP-28/Epilysin | Not determined |

**Table-1**: Classification of Matrix metalloproteinases and ECM substrate specificity[7].

enzymes with a multitude of physiological functions, many of which are still not fully understood. Main subdivisions include: collagenases, gelatinases, stromelysins and matrilysins, membrane- type and sixth group different from others **(Table-1).** In recent past, researchers are interested in MMPs because of their vital role in number of physiological and pathological processes. In zebrafish 83 zinc proteases have been identified, out of which 26 are MMP orthologs (In humans 24) and only three MMPs: MMP-2,MMP-9 and two isoforms of membrane type MMP(MT-MMP α and β) has been characterized.The purpose this study is to determine the role of MMP-9 during zebrafish retina regeneration. Although, there are a plethora of research papers on the role of MMP-2 and -9 in diverse malignant processes owing to role in tumor metastasis and angiogenesis.In regeneration context there is one study, indicating that gelatinases MMP-2 and MMP-9, expressed in osteoclasts, plays a pivotal role in the regeneration of zebrafish scales. In a microarray analysis (Ramachandran R. *et al.*), MMPs RNA level induction was obseerved, where MMP-2 showed 1.5 fold change and MMP-9 showed 38.89 fold change in 4 day post injured retina compared to uninjured retina. We think that MMP-9 can be potential candidate supporting remodelling for progenitor cell migration and differentiation of regenerated neurons. Here, we looked at spatio-temporal expression of MMP-9 and its actual role during process of retinal regeneration. Also, we tried to find potential regulation of MMP expression via different signaling pathways.

# Chapter2

# Materials

## 2.1 Care and maintenance of zebrafish

Zebrafish were kept in AQUATIC ECO-SYSTEMS (Pentair system) that filters and aerates the water required for a healthy environment. The tank temperature is generally maintained between 26-28.5 °C and the lighting conditions are 14:10 hr (light: dark). The pH of the water is maintained between 6.8 and 7.5. Fishes are fed on *Artemia salina* given 2 or 3 times a day.

## 2.2 Retinal Injury and Dissection

Tricaine methanesulfonate(0.2%), 30G needle, Stainless steel forceps-5F, 70% Alcohol, Sterile plastic Petri dish, Sponge bed for anesthetized fish.

## 2.3 RNA Isolation, RT-PCR and Semi-qRT PCR

1. RNA Isolation by TRIzol, chloroform, Isopropanol, 70% Alcohol, DEPC water.
2. cDNA preparation (Roche cDNA synthesis kit)
3. PCR reactions use Taq polymerase and gene-specific primers (Table 2.1)
4. Quantitative PCR (qPCR) was carried out with Lightcycler SYBR Green Fluorescein Master Mix (Roche) on Realplex thermal cycler Eppendorf.

| Gene | Accession no. | Sequence (5′→3′) |
|---|---|---|
| L24 | NM_173235.3 | Fwd: CAAGAAGGGCCAGTCTGAGG<br>Rev: GCTGATTTAGCAGGAGCCTTG |
| MMP-9 | NM_213123.1 | Fwd:TCAAAATGAGACTTGGAGTCCTGGCG<br>Rev:GGGTGCTTTGTTCACAACAAATCGGC |
| MMP-9 RT | NM_213123.1 | Fwd: TGACAAAACAACCACTGCTTCCACC<br>Rev: AACCCAGAACTGTCTCTCTGAGAAG |

**Table-2:** Sequences of the primers used to develop the *In-situ* hybridisation probe and to quantify relative gene expression levels.

## 2.4 Cloning, Transformation and Plasmid Isolation

Cloning:- Primers were designed based on the zebrafish MMP-9 sequence (Table-2). The TOPO-TA Cloning Kits (Invitrogen) are specifically designed to clone Taq polymerase-generated PCR products for sequencing.

Transformation: IPTG (0.1 M), Xgal (20mg/ml), Ampicillin-100mg/ml, PCI (Himedia)

Plasmid Isolation:

1. Lysis Buffer (1ml prepration) : 920µl water, 50µl 20% SDS, 20µl 0.5M EDTA (pH – 8.0 ), 10µl 10N NaOH
2. Potassium acetate buffer(100ml) : 60 ml of potassium acetate (5M autoclaved ), 11.5 ml glacial acetic acid and 28.5ml Water

Plasmid Digestion: CutSmart Digestion buffer, Not I HF

## *2*.5 *In vitro* transcription (Probe Making)

Transcription buffer-10x, SP6 RNA polymerase 1000 U, DIG RNA labelling mix-10x, Template (plasmid)- 100-500ng, 0.5M Tris-EDTA, 5M $LiCl_2$, Glycogen-10mg/ml,100% EtOH.

## 2.6 mRNA *in situ* hybridization

**Day1:** 20x SSC, Proteinase K buffer, TEA solution, Hybridization solution,
TEN solution.

**Day2**: 5X Maleate buffer, 1X Maleate/0.05% Triton X-100/1% RMB blocker Solution, Antibody: Blocker solution α-DIG-AP 1:5000, RNase Buffer: 0.5M Nacl, 10mM Tris-HCl, 0.5M EDTA.

**Day 3**: NBT/BCIP, Genius 3 buffer

## 2.7 Cryo-Sectioning

1. 1 M phosphate buffer (10x PB):weigh 21.97g of $Na_2HPO_4$ and 5.42 g of $NaH_2PO_4$ dissolve in 150 ml of deionized water. Adjust pH 7.4 by adding 2N HCl (accordingly).Then, make up the final volume up to 200ml by deionized water.
2. 10x Phosphate buffered saline (10xPBS):weigh 75.97g of NaCl, 9.93g of $Na_2HPO_4$ and 3.59g of $NaH_2PO_4$ dissolve in 800ml of deionized water. Adjust pH 7.4 by adding 2N HCl (accordingly).Then, make up the final volume up to 1000ml by deionized water.

3. 4% Paraformaldehyde (PFA): weigh 2g of PFA, add 1x PB and make up volume to 50 ml by adding deionized water and allow to get completely dissolve heating at 65$^{o}$C for approximately 1 hour
4. 5% Sucrose: dissolve 2.5g in 1x PB and make up volume to 50 ml by adding deionized water
5. 20% Sucrose: dissolve 10g in 1x PB and make up volume to 50 ml by adding deionized water
6. OCT cryostat embedding medium ( Tissue Tek)
7. Superfrost slides (Fisher Scientific)

## 2.8 Immunohistochemistry

1. 2 N HCl: 42 ml of 37% HCl and make volume upto 50 ml
2. Sodium borate: 0.1 M , pH-8.5 adjusted by NaOH
3. Blocking solution: 3% BSA with 0.1% Triton X-100 in 1× PBS
4. Primary and secondary antibodies: PCNA Antibody (F-2) mouse monoclonal $IgG_{2a}$, goat anti-mouse IgG (H+L) secondary antibody Alexa Fluor 555 conjugate, Secondary antibody Alexa Fluor 488 (H+L) goat anti-rat.

## 2.9 Notch, Wnt, Hedgehog signaling inhibitors

1. DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) a γ-secretase inhibitor, used to inhibit the Notch signaling pathway, used at final concentration of 40μM.
2. XAV939 (3,5,7,8-Tetrahydro-2-[4-(trifluoromethyl)phenyl]-4H thiopyrano[4,3d]pyrimidin-4-one) is a Tankyrase (TNKS) inhibitor antagonizing Wnt signaling, used final concentrations of 100μM and 10μM.
3. Cyclopamine ((3β,23R)-17,23-Epoxyveratraman-3-ol) inhibitory effect is mediated by direct binding of drug to the seven membrane spanning receptor Smoothened (Smo).

## 2.10 Morpholino-Modified Antisense Oligonucleotide-Mediated Gene Knockdown

Lissamine-tagged MMP-9 targeting Morpholino (Gene Tools) used is 5'-GCTGCATATCCCACTGGCATCGAGAC-3'.

## 2.11 Immunofluorescence microscopy

Slides were examined with Nikon $A1^{+}$ confocal microscope system.

### Chapter 3

# Methods

## 3.1 Retinal Injury and Drug Injection

Selected healthy adult fishes. Anesthetized the fish in 0.2% Tricaine methanesulfonate in a glass beaker until it gets temporarily immobilized. Placed anesthetized fish on a moist sponge bed under the dissecting scope. By using forceps, exposed the back of right eye and poke four times (in different retinal quadrant) through the sclera with needle, up to the length of the bevel. Drugs were delivered intravitreally through the front of the eye using a Hamilton syringe equipped with a 30 gauge needle.

## 3.2 Whole eye retina fixation

Fishes were anesthetized with 0.2% tricaine methanesulfonate, collected eyes over a course of different time points. Pulled out the intact eye out of its socket using forceps. Placed the eyes in 4% PFA and without disturbing retina take out the lens. Transfer the eye into 1.5 ml micro-centrifuge tube containing 4% PFA at 4°C.Kept the eyes in 4% PFA overnight for fixation. For cryoprotection of eyes used sucrose solutions of 5% and 20% sucrose .First, removed the PFA buffer and added 5% sucrose, allowing gentle shaking on rotator. Then, added increasing concentration of sucrose by mixing 5 and 20% sucrose at a ratio 2:1, 1:1, and 1:2 for 45 min each at room temperature. Finally add 20% sucrose keeping overnight at 4°C. Eyes are then mixed with a 2:1 solution of 20% sucrose and OCT for 30 min. Placed the eye in aluminium moulds made from wrapping the foil around electroporation cuvette with OCT embedded in it. Freeze the sample at -$80^{0}$C.Section tissue at 10-12 μm and collected on superfrost slides. Kept slides overnight at room temperature and store at −20°C.

## 3.3 RNA Isolation

Total RNA was isolated from control and injured retinae using TRIzol (mixture of guanidine thioacyanate and phenol).

Over treated fishes with 0.2% tricaine methanesulfonate before dissecting the eye. Harvested four retinae in 1X PBS put it into a1.5- ml microcentrifuge tube containing 200µl of TRI reagent (Sigma). Homogenized tissue samples by using syringe. Added 0.2 volume (40µl) of chloroform. Shake for 15 sec and centrifuge at 1000 rpm at $4^0$C for 10-20 min. The mixture separates into 3 phases with the upper clear aqueous phase containing the RNA, transfered upper phase into new tube and add 40-50µl of Isopropanol. Incubated at $4^0$C for10-20 min. Centrifuged at max.speed 13000 rpm at $4^0$C for 20 min and discarded supernatant. Washed pellet with 70% EtOH. Gave max rpm spin, 10 min, 4ºC and air-dry pellet for 5-10 min. Dissolve pellet in 15-20µl DEPC water.

## 3.4 RT-PCR

RNA isolated from retinae at different time points, used to make cDNA using Roche Reverse Transcriptase.Using Taq polymerase and gene-specific primers, PCR reaction followed described cycling conditions(x 35 cycles) ,Veriti 96 well Thermal cycler,

(Table 3.1).

| Steps | Time | Temperature in $^o$C |
|---|---|---|
| Initial denaturation | 15'' | 95.0 |
| Denaturation | 15'' | 95.0 |
| Annealing | 1' | 60.0 |
| Extension | 2' | 72.0 |
| Final extension | 7' | 72.0 |
| Hold | ∞ | 4.0 |

**Table 3.1:** Cycling parameters

## 3.4 Agarose gel electrophoresis

- We make 1% agarose solution: Weighed 1g agarose in 100ml 1 X TAE, carefully boiled the solution to dissolve the agarose.
- Cooled down solution to room temperature. (Stir or swirl the solution while cooling)
- Added ethidium bromide stock(10mg/ml) in the gel solution.(Be very careful when handling the concentrated stock)
- Stirred the solution to disperse the ethidium bromide
- Inserted the comb at one side of the gel tray, and then pour solution into it.
- When the gel is cooled and become solid, carefully removed the comb. The holes that remain in the gel are the wells.
- Together with the rack, we put gel into a tank with TAE. The gel must be completely covered with TAE, with the wells at the end electrode that will have the negative Using a micropipette loaded mixture of sample (5µl) and loading dye (2µl) into the wells.
- Applied electric current of 90-120 V
- The gel is placed under UV-transilluminator, all observations are recorded.

## 3.5 Cloning, Transformation and Plasmid Isolation

PCR product extraction from agarose gel: After PCR amplification, we cut out the band from agarose gel and put it into 0.5ml microcentrifuge tube (MCT) containing some cut pieces of aluminium foil and small hole at bottom made by needle piercing. Placed this 0.5ml MCT into an empty 1.5 ml MCT and centrifuged this at 7000rpm for 5 minutes at room temperature.

TOPO-TA cloning:-

| Reagent | Volume |
|---|---|
| PCR product | 1µl |
| Salt solution | 0.5µl |
| TOPO vector | 0.3µl |
| Water | 1.2µl |

**Table 3.2**

After setting up TOPO cloning reaction kept for 1hour incubation at room temperature. Then, we proceeded to transform TOPO construct into competent E.coli DH5-α strain.

Transformation:-

- Took out the competent cells from -80$^0$C and thawed the cells by keeping them on ice.

  NOTE: Competent cells should be kept cool, they are sensitive to being warm and in that condition we may not get result we wanted!
- Once cells get thawed, we added 3µl of plasmid and mixed gently.
- Kept for incubation in ice for half an hour.
- Gave heat shock at 42$^0$C for 45 seconds without shaking and immediately after kept on ice for 5 minutes.
- Added 1ml of LB media and incubated for 30-35 minutes at 37degree.
- Cells were spread on selective medium (antibiotic –ampicillin) containing LB agar plates.
- Incubate at 37$^0$C overnight/16-18hours. Pick the single colonies to culture.
- Next day, spread the culture on IPTG (40µl) + X gal (40µl) containing agar plate and incubate the plates at 37°C for 24 hours.
- Blue and white colonies appeared on the agar surface.
- Selected the transformed white colonies and add into 1.5ml micro centrifuge tube containing 20µl of deionized water.
- Kept for five minutes to dissolve and gently mix it.
- Added PCI solution to it and centrifuge at 13,000 rpm for 10 minutes, room temperature. And check on 1% agarose gel.

Plasmid Isolation:-

- Centrifuged 1.5ml culture at 1000rpm for 1-2 minutes at 4 degree, twice.
- Added 100µl of pre-chilled water to the pellet and re-suspend it by gentle vortex.
- Added freshly prepared Lysis Buffer (100µl) and mix by tapping. (no vortex)
- Kept for incubation at 100$^0$C for about 2 minutes.
- Added 50µl Mgcl2 (1M) and kept in ice for 2 minutes.
- At room temperature, centrifuged sample at 12,000 rpm for 2 minutes.

- Then, added 50µl potassium acetate buffer (3M) {sodium acetate also can be used} and mix immediately and gently.
- Centrifuged at room temperature for 2 minutes at 1000rpm.
- Took 60µl of isopropanol in a fresh tube.
- Cleared the supernatant into these isopropanol containing tubes and keep on ice.
- Again, centrifuged at maximum speed (13,000-14,000 rpm) at room temperature for 2 minutes.
- Gave a 70% ethanol (200µl) wash, dry the pellet and re-suspended in TE buffer/ deionized water
- Run it on gel or stored for later usage.
- Running plasmid on gel: - Mixed plasmid solution (5µl) with loading dye (2µl) and load the sample into wells of 1 % agarose gel. Give an electric supply of 80 - 120 volts.

## 3.6 Plasmid Digestion

| Reagent | Volume for single reaction |
|---|---|
| Plasmid | 2.5 µl |
| HF- Not I enzyme | 1 µl |
| Buffer | 2 µl |
| Water | 14.5 µl |

**Table 3.3**

- Allowed for incubation at $37^0$C at 1 hour and check on 1% agarose gel.
- Added equal volume (20µl) chloroform and vortex.
- Then, centrifuged at 13,000 rpm at room temperature for 10 minutes. And took out the aqueous phase carefully transfered into another MCT.

## 3.7 *In vitro* transcription (Probe making)

| Reagent | Volume for single reaction |
|---|---|
| Transcription buffer (10X) | 4µl |
| Template (Digested Plasmid) | 7 µl |
| DIG- UTP | 2 µl |
| SP6 RNA Polymerase | 2 µl |

| | |
|---|---|
| water | 25 µl |

**Table 3.4**

- Incubated the reaction at $37^0$C at 4 hours.
- Added 4µl TE, 4µl $LiCl_2$, 2µl glycogen and tap it gently.
- Then, add 70µl of 100% EtOH and again tap it. Kept in $-80^0$C overnight.
- Centrifuged at $4^0$C for 15 minutes at 13,000 rpm and give 100% EtOH wash (200µl).
- Dry the pellet and dissolved in 50µl of deionized water and store in $-80^0$C.

## 3.8 mRNA *in situ* hybridization

In situ hybridization (ISH) was done on retinal sections with digoxigenin-labelled complementary RNA probes.

**Day1: Hybridization**

1. Hydrated slides by following washes 1minute each:
   a.100% EtOH
   b.100% EtOH
   c.95% EtOH
   d.70% EtOH
   e.50% EtOH
   f. 2X SSC
2. Prewarmed Proteinase K buffer to $37^0$C and incubate slides for 1-5 minutes (250 µl of 10mg/ml) and wash slides with DEPC water.
3. Rinsed slides in 0.1M TEA pH 8.0 for 3 minutes.
4. Rinsed in Acetic anhydride/TEA for 10 minutes.
5. Rehydrated the slides by following washes for 1 minute each:
   a. 2X SSC
   b.50% EtOH
   c.70% EtOH
   d.95% EtOH
   e.100% EtOH
   f.100% EtOH
6. Air dried the slides for at least an hour at room temperature.
7. Prewarmed hybridization solution to $56^0$ C

8. Boiled probes with water at $100^0$C for 5 minutes (64µl-probe volume = volume of DEPC water).Immediately cooled on ice and added to Hybridization solution and mix.

9. On each slide Hyb/probe solution (60µl) and coverslip by HybridSlips.

10. Incubated slides at $56^0$ C overnight in humid chamber moistened with 50% Formamide/5X SSC.

**Day 2: Post Hybridization**

1. Prewarmed 50% Formamide/2X SSC solution to $65^0$C.
2. Prewarmed two RNase buffers, one to $37^0$C and other to $65^0$C.
3. Prewarmed two 2X SSC to $37^0$C.
4. Immersed the slides in 2X SSC for 30 minutes on shaker table.
5. Washed the slides with 50% Formamide/2X SSC solution at $65^0$C for 30 minutes.
6. Washed slides with 2X SSC at $37^0$C 10 minutes, twice.
7. Added 200µl of RNase A (10mg/ml) to $37^0$C RNase buffer and incubate slides for 30 minutes. And, then washed slides in $65^0$C RNase buffer for 30 minutes.
8. Rinsed slides with 1X Maleate/0.05% Triton X-100/1%RMB blocker solution at room temperature for 2-3 hours.
9. Washed slides twice with 1X Maleate buffer for 5 minutes.
10. Incubated slides with antibody diluted in 1X Maleate/0.05% Triton X-100/1%RMB blocker solution overnight at room temperature.

**Day 3: Detection- NBT/BCIP**

1. Washed slides twice with 1X Maleate buffer for 5 minutes.
2. Incubated twice/5 minutes each in Genius 3
3. Added NBT/BCIP, incubate overnight at room temperature in dark.

## 3.9 Semi- qRT PCR

| Reagent | Volume for single reaction |
|---|---|
| qPCR SYBR Green Mix | 5 µl |
| Forward primer | 0.4 µl |
| Reverse primer | 0.4 µl |
| Formamide | 0.4 µl |
| Template(cDNA) | 0.5-1 µl |
| Water | 3.3 µl |

**Table 3.5**

## 3.10 Immunohistochemistry

Kept the slides at $37^0$C for 1 hour for drying. Gave three washes of 1x PBS (1 ml per slide). Rinsed the slides with Sodium borate three times,10 minutes each. Added 3% BSA in 1x PBST on sections incubated for 30 minutes at room temperature. Diluted primary antibody (1:500) in 1% BSA in 1x PBST. Placed slides in a moist air tight chamber keep it over overnight at $4^0$C.Removed the primary antibody and wash slides with 1x PBST, three times 10 minutes each. Incubated the slides with secondary antibody (diluted 1:1000 in 1% BSA in 1x PBST) for 3 hours at room temperature in moist chamber. Washed in 1x PBST 3 times, 10 minutes each. And then, washed in deionized water three times, 10 minutes each. Air dried the slides for about an hour at room temperature before putting coverslip.

## 3.11 Morpholino electroporation

Anesthetized zebrafish in Tricaine.Kept them on wet sponge. Wraped zebrafish in a moistened piece of paper , covering the gills, but leaving the eye exposed. Made injury on eye by using 30 guage needle.Injected lissamine-tagged morpholino using Hamilton syringe during time of injury(~1μl), into the vitreous of the adult zebrafish eye. Put them back into water to recover. We usually only inject the right eye, using the left eye as an uninjected control. Lissamine-tag morpholinos are positively-charged and electrodes drive electroporated morpholinos towards the negative electrode. Electroporation parameters were kept as five consecutive 50-msec pulses, at 72 V. After electroporation returned fish immediately into water.

## 3.12 Immunofluorescence microscopy

Cell counts were determined by counting fluorescently labelled cells in retinal sections visualized using fluorescent microscopy. Statistical analysis done using GraphPad prism.

## Chapter 4

# Results and Discussion

## 4.1 Injury dependent induction of MMP-9

First, we checked the temporal expression pattern of MMP-9 during the process of retina regeneration **(Figure4.1a,b)**.Compared to uninjured control retina, peak expression was observed at 6 hours post injury (hpi) and 2days post injury (dpi),indicating that it is an early induced gene. This expression suggests that MMP-9 might play multiple roles during the retina regeneration.

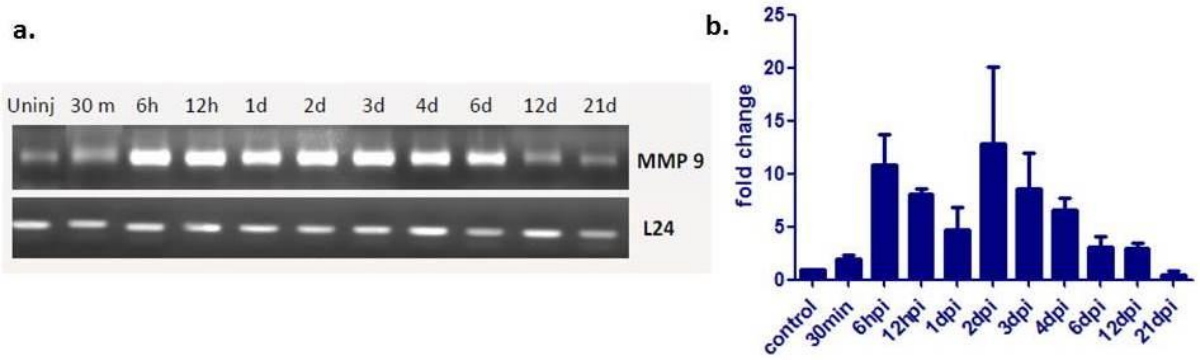



**Figure 4.1: (**a) RT-PCR and (b) semi-quantitative RT-PCR showing time course of injury-dependent gene expression. (Experiment repeated three times)

## 4.2 Spatial expression of MMP-9

mRNA *In situ* hybridization and immunofluorescence microscopy showed that MMP-9 is expressed in Muller glia (MG) cells (Fig.4.2,a).Further, some of these MMP-9+ cells incorporated PCNA(Fig.4.2,c).MMP-9 localisation in MG observed in 2 dpi and 4dpi data is shown in (Fi. 4.2,a-f ).Quantification showed increase in MMP-$9^{+}$ cells in 2dpi and decrease at 4dpi,consistent to RT- PCR data. Also, the number $MMP^{+}$ cells which were PCNA+ is less in both 2dpi and 4dpi (Fig.4.2 c,f). We could say that, a subset of proliferating MG cells and non-proliferating MG cells expresses MMP-9.

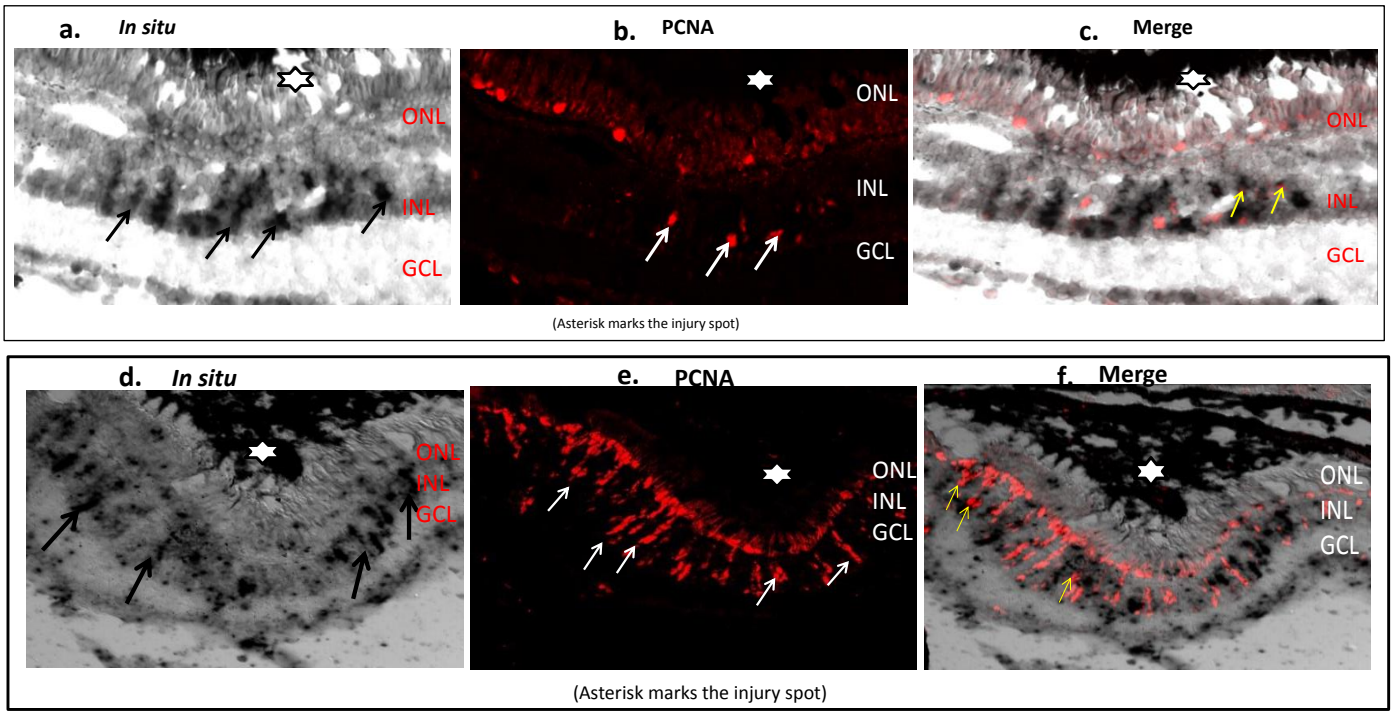



**Figure 4.2**: ISH and immunofluorescence microscopy shows localization of MMP-9 expressing cells with PCNA+ progenitors at 2dpi (a-c) and at 4 dpi (d-f).

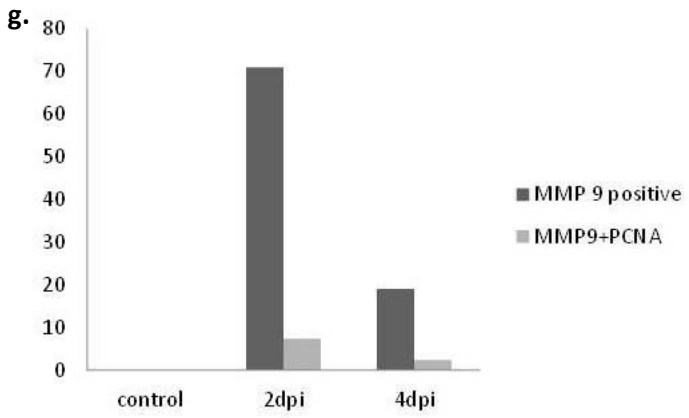
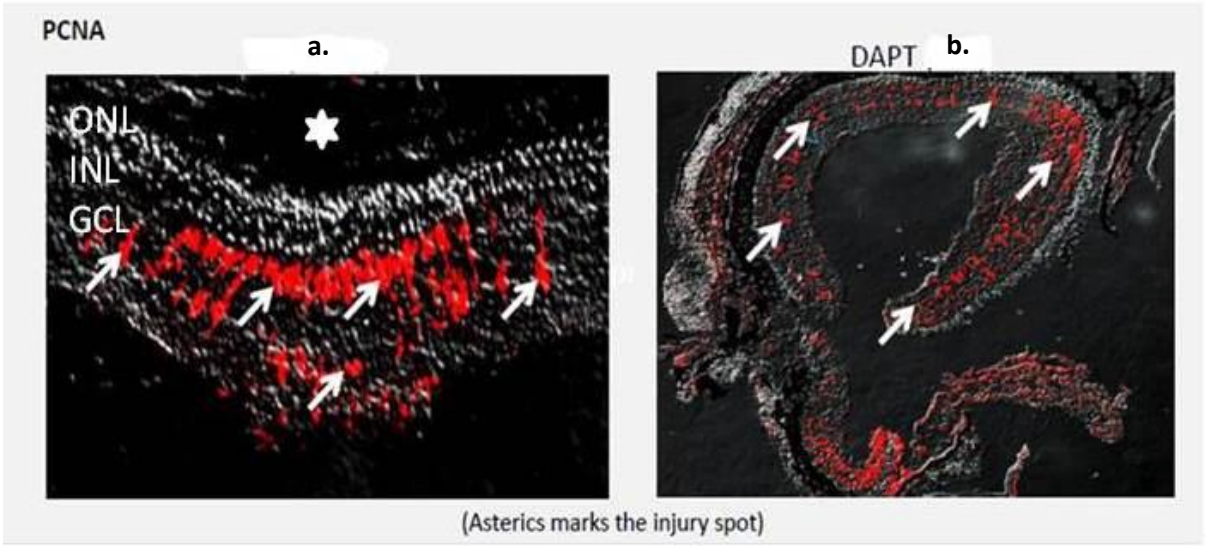
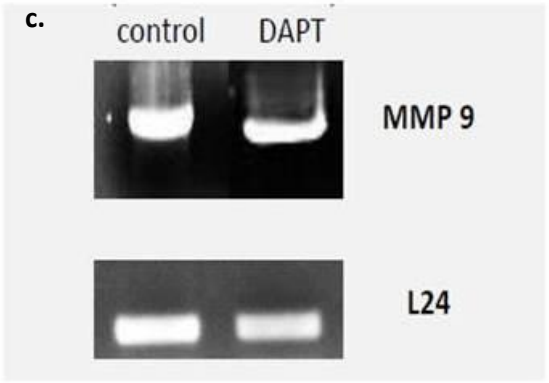


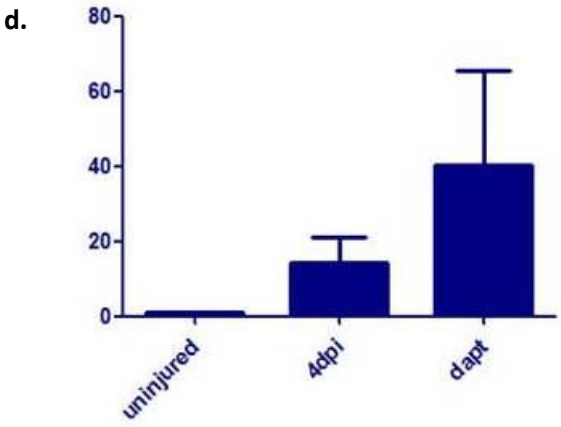

**Figure 4.2**: g. Quantification of MMP $9^{+}$ and $PCNA^{+}$ cells shown

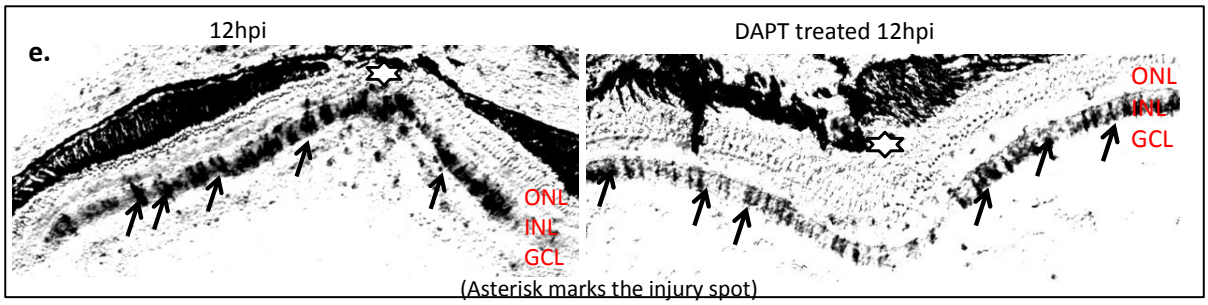
## 4.3 MMP-9 upregulation on inhibiting Notch signaling

We next investigated the significance of MMP-9 expression around 12hpi-4 dpi when MG undergoes dedifferentiation and MG-derived progenitors shows peak proliferation, respectively. Compared with previous studies, DAPT inhibition results in expansion of 1qzone of injury dependent progenitors (Fig. 4.3 a).It was observed that in DAPT treated retina MMP-9 expression was upregulated (Fig 4.3 b,c). ISH at 12hpi also shows reminiscent data(Fig 4.3 d), suggesting that MMP-9 may play role in expanding zone of proliferation in absence of Notch.




## 4.4 MMP-9 downregulation on blocking Wnt signalling

Now, we wanted to look MMP-9 regulation by Wnt signalling at 4dpi and 12hpi.Previous studies, shows that genetic and pharmacological inhibition of Wnt signaling suppresses injury-dependent proliferation of MG-derived progenitors. Here also, we could see that with Wnt inhibitor XAV 939, a tankyrase inhibitor, there is a reduction in number of proliferating cells at 4dpi in a concentration dependent manner (Fig 4.4 a). PCR with reverse transcription (RT-PCR) indicate that MMP-9 expression is suppressed in Wnt inhibited retina (Fig 4.4 b,c).Consistently, at 12hpi we could not

detect any MMP-9+ cells in XAV 939 treated retinae. This concludes a Wnt-dependant MMP-9 induction.

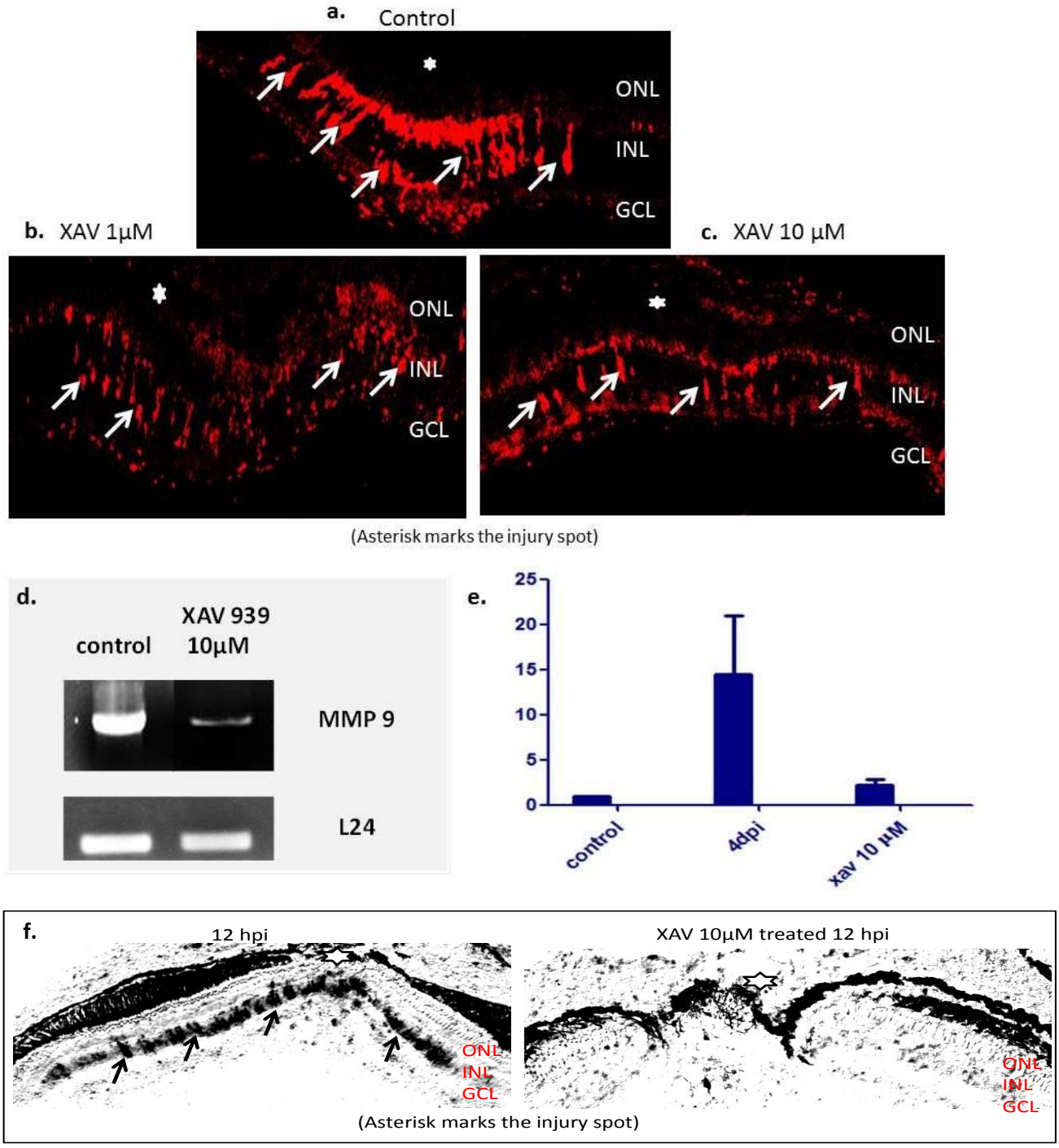



**Figure 4.4** At 4dpi upon treatment with XAV 939 (a-c) PCNA immunofluorescence microscopy on retinal sections shows reduction in proliferation with 1µM and 10µM treatment compared to untreated retina.Also, (d) RT- PCR and (e) Semi qRT-PCR shows decrease in MMP-9 expression (f) ISH shows no detectable MMP+ cells at 12hpi in Wnt inhibited retinae.

## 4.5 MMP-9 expression on blocking Hedgehog signaling

The role of Hedgehog (Hh) during retina regeneration is debatable, with some reports emphasizing increased proliferation and others pointing to a role in cell cycle exit. Also, they are known to induce cell migration and invasion in cancer. So, we next wanted to determine whether Hh pathway-induced cell migration can be correlated with the expression and/or activity of MMP-9.To block the Hh pathway we use Cyclopamine, inhibitory effect is mediated by direct binding to Smoothened (Smo).Our result show that at 4dpi, proliferation ($PCNA^{+}$) is reduced in cyclopamine treated retina(Fig 4.5a).RT-PCR shows that MMP-9 expression got diminished(Fig. 4.5b).Further, mRNA ISH indicates that the $MMP\text{-}9^{+}$ is reduced drastically with cyclopamine treatment. These results suggest that hedgehog induced MMP-9 might play role in cell migration during retina regeneration.

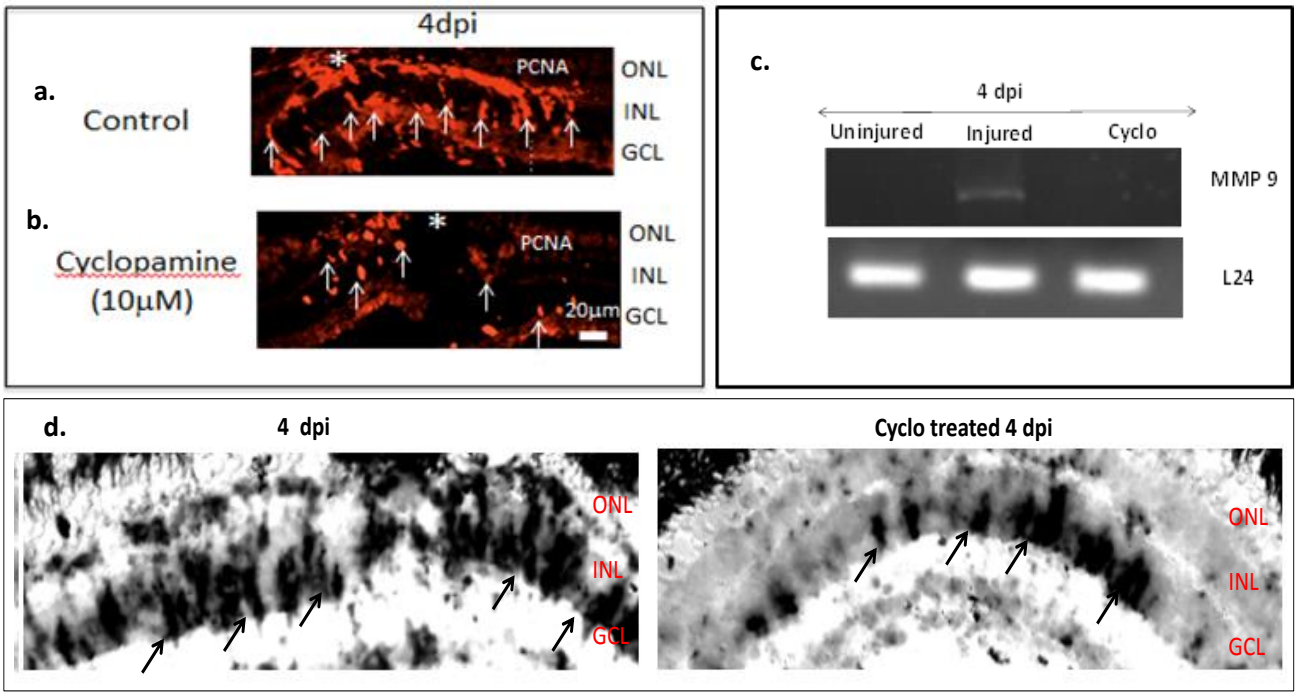



**Figure 4.5** At 4pi on Hh blocking (a) PCNA immunofluorescence microscopy on retinal sections shows reduction in proliferation (b) RT PCR and (c) Semi qRT-PCR shows decrease in MMP-9 expression (d) ISH also indicates reduction in MMP+ cells.

## 4.6 Ascl1a-dependent suppression of MMP-9 gene expression in injured retina

Ascl1a (achaete-scute homologue 1a) is a basic helix-loop-helix transcription factor that is involved in almost all aspects of retina regeneration. It regulates genes that are responsible for generating Müller glial cell-derived progenitors, such as those encoding Wnts, growth factors

and also controls (directly or indirectly) the expression of proteins and microRNAs that inhibit progenitor formation and proliferation, such as Notch. Since, Ascl1a is also an early induced we check it's effect on MMP-9 induction. To test this, we knocked down Ascl1a with previously validated ascl1a-targeting morpholino-modified antisense oligonucleotides (MOs), which is a well-characterized approach for suppressing mRNA translation in zebrafish(Fig. 4.6a) and assayed MMP-9 expression at 2dpi.We could see suppression of mmp9 level in ascl1a knockdown reina (Fig.4.6 b,c).

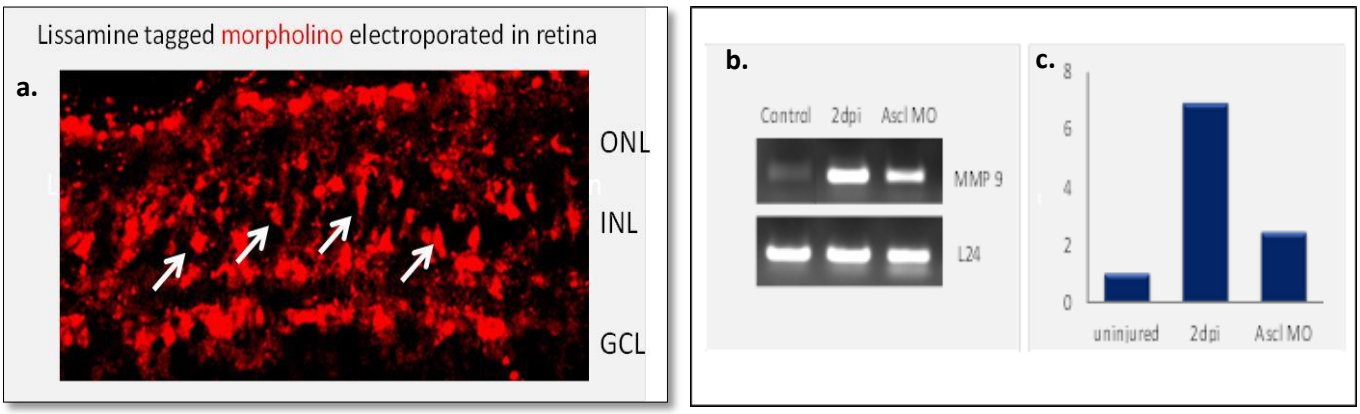



**Figure4.6** (a) Presence of electroporated MO in retina (b) RT-PCR and (c) Semi qRT-PCR shows Ascl1a knockdown suppresses MMP-9 gene.

## 4.7 Reduction in proliferation on knocking down MMP-9

We were interested in determining if MMP-9 expression was necessary for process of retina regeneration. For this analysis the expression of MMP-9 was knocked down in the adult retina using electroporated morpholino-modified antisense oligonucleotides (Fig4.7a). We could clearly see that with MMP-9 knockdown, the number of MG proliferating progenitors got reduced (Fig 4.7 a-c). Quantitatively we confirmed that there is a drastic decrease in number of proliferating cells in MMP-9 knockdown retinae, even though there were a few proliferating cells also (which might depend on efficacy of knockdown).

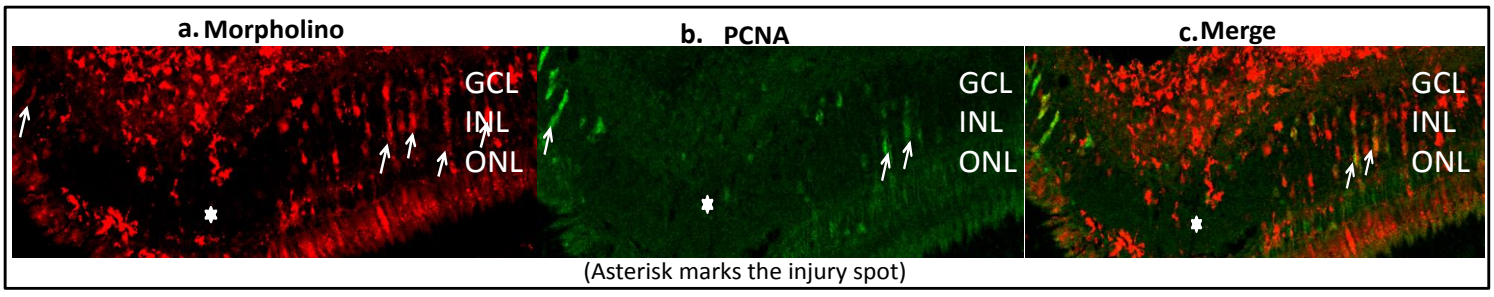



**Figure 4.7** (a-c) Immunofluorescence microscopy shows effect of MMP-9 knockdown on MG proliferation.

## Chapter 5

# Conclusion

Here, we report first ever expression pattern and regulation analysis of zebrafish MMP-9 (gelatinaseB) during retinal regeneration**.** The data shows the spatiotemporal expression profiles of MMP-9 at different time points- post injury during course of retina regeneration, implies that it's an early induced gene with various potential function during the process. mRNA *in situ* analysis showed mmp 9 expression in a subset of proliferating and non-proliferating Muller glia cells of retina, which we have to further analyse significance of this expression pattern. Targeted gene knockdown reduced the number of proliferating cells. In future we have to check at late time points to look the effect of MMP-9 on differentiation stage. We also checked MMP-9 regulation via Wnt signaling, Notch signaling and Hedgehog signalling, indicating potential correlation and multitudinous role it has. But, still we are not clear about hierarchical regulation or interdependency of MMP-9 with these aforementioned and other signalling cascades. This study strongly indicates that MMP-9 plays a vital role during retina regeneration and advance analysis of MMP could help in resolving the enigma of self- healing mechanism for retina damage, a step ahead.

## Chapter 6

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