Understanding the role of Matrix metalloprotease 14 (Mmp-14) and Furin during retina regeneration in zebrafish

KSHITIZ

(MS16045)

A dissertation submitted for the partial fulfilment of

BS-MS Dual Degree in Science

Department of Biological Sciences

Indian Institute of Science Education and Research Mohali

May 2021

Certificate of Examination

This is to certify that the dissertation titled “Understanding the role of Marix metalloprotease 14 (Mmp-14) and Furin during retina regeneration in zebrafish” submitted by Mr. Kshitiz (Reg.No. MS16045) 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. Sharvan Sehrawat Dr. Shashi B. Pandit Dr. Rajesh Ramachandran
(Supervisor)

Dated: 09 May 2021

Declaration

The work presented in this thesis has been carried out by me under the supervision of Dr. Rajesh Ramachandran at the Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali.

This work has not been submitted in part or full for a degree, 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 acknowledgment of collaborative research and discussions. This thesis is a bona fide record of original work done by me and all sources listed within have been detailed in the bibliography.

Date **Place** **Kshitiz**

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

Dr. Rajesh Ramachandran
(Supervisor)

Dedicated to my Mother and Father

And

Late Md Anwar Khursheed

"If there were no regeneration there could be no life.

If everything regenerated there would be no death"

- Richard J. Goss, Principles of Regeneration (1969).

Acknowledgement

I believe I could have written this thesis in a better way, had some thoughts and situations not disturbed my peace of mind, and stolen ideas which otherwise could have very well been used for the betterment of the thesis. Thanks to them and Covid-19 pandemic, those ideas are gone, and so are a few more extra unnecessary pages.

Although I am not sure about whether to be thankful for the various individuals or events who instilled in my mind the idea of pursuing BS-MS, I am definitely deeply in debt to countless marvellous individuals who have helped me in this adventure over 5 years. In this section, it is my desire to thank all of them who have been there over the years, genuinely from the core of my heart, for adding to this wonderful journey, a sense of purpose and a definite shape which also helped in building me, both as a person and a student of science.

First and foremost, thanks go to Dr. Rajesh Ramachandran, my MS Thesis supervisor, who allowed me to work in his lab. I cannot help but wonder at my extremely fantastic luck upon thinking of getting an opportunity to work under him. He always welcomed the new ideas with great appreciation and excitement. He gave constant motivation and complete freedom to work on it. He has always been there for discussions or problems faced, which have also resulted in gaining some fantastic life lessons, definitely along with developing scientific temperament over the years. I sincerely thank him for developing me on personal and professional front.

I consider myself very fortunate to be part of the Fish lab or Tissue Regeneration Lab, mostly known as RR Lab (Rajesh Ramachandran Lab or Retina Regeneration Lab). Being a member of the lab for the past 3 years. I have learnt a lot from all the amazing members of the lab, be it the experiment design or trouble-shootings, and most importantly, time and resource management, along with how to deal with various problems in life. I thank Danio rerio for the sacrifices and being a model organism for the experiments.

I could not have imagined this thesis in this shape without the constant help and support of Sharanya Premraj (aka my GURUMA or Masterni Ji), my PhD mentor. Along with being the most elegant in the lab, she is the person with a very kind soul and calm mind who has been extremely supportive and caring all the time. Apart from teaching me all the techniques and helping in experiments, she has incubated in me the art of generating ideas or experimental design and the presentation skills. She has always considered and critically analysed my ideas with her excellent scientific temperament. She helped and supported me in all my executions, experiments, and problems faced in my personal life. I am thankful for her time-to-time doses of motivation and positive objurgations. If I were to symbolize her with a phrase, that would undoubtedly be "beauty with brains". At last, all I can say is, it was my honour to be her first mentee.

Now, going in the order of seniority in the lab, I would like to start by thanking our Post-Doctoral Fellow, Dr. Poonam Sharma (Poonam Di), for her guidance and suggestions. She has always suggested alternative troubleshooting and has given valuable critiques.

Further, I would like to express my thanks to the go-to daily hacks expert in the earlier days, Dr. Soumitra Mitra (Dada), who has always created a positive environment with his presence along with teaching short-cuts. He was the person who taught various stuff and made me appreciate the beauty of stars and the galaxy (imaginary). I thank Md Anwar Khursheed (Anwar Bhaiya), who had introduced me to the lab when I joined as just a summer intern in 2018. He was always motivated for the various projects, one of which he asked me to pursue. With time and interest for the project, my time in the lab was not just limited to the summer of 2018. I am sometimes surprised by the kindness and calmness he had in him. He always amazed everyone with his theoretical knowledge base of science and experience of share market. Next, I would like to extend my sincere thanks to the couple of RR Lab, Dr. Shivangi Gupta (Shivangi Dii) and my PhD batchmate Mansi Chaudhary (read Komal Bana), for their love and support and the fantastic selfless resource management of the lab (which has helped everyone a lot). They were there to always help in the experiments and listen to the kaands of my life. I am also thankful to Bindia Chawla (Bindia Dii) for all the discussions, whether scientific or related to campus life. I thank Ashish Sethi (Ashish Bhaiya), who has never denied fruitful discussions, help, and support. He is the person who has kept the lab cheered up with his not-so-rich sense of humour. Moving further, I would thank Pooja Shukla (Pooja Jii) for being such a friendly senior and sharing resources and space resembling 'sharing is caring'. She has always kept the lab atmosphere cheered up with her dancing and discussions. My batchmates, Pallavi and Nikhil, have also supported me and critiqued the work, for which I am thankful to them. Coming to the junior-most member and the serious uncle of the lab, I would like to mention the owner of 420, Omkar Desai. He was always there whenever I needed any help, be it scientific or technical. He is the person who helps and cares for others selflessly and has always provided me with mid-night snacks and maggi!

It would be straight away a lie, if I say that I have never been through the dark phases or downs in my life during the past years, but there have always been people (gems) around me to support. I would like to start with my long-distance Libra partner, who has always listened to my non-sense talks and frustrations, Dr. Bhawna Attri. She has been there to give me absurd suggestions to my problems, which never proved to help solve, instead resulting in forgetting them. Priya Bhatt and Shubhangi Jain have constantly supported me, even in all my evil deeds throughout the years of stay at IISER Mohali. Dr. Anushika is also one of my go-to persons on many occasions and has always listened to me. They all have tolerated every bit of me, for which I owe thanks to them all. Next, it has to be the person who has tolerated me for hours in his room when I was going through downs in my thesis year. I thank Vishal Gaur for his support and suggestions on all topics. He has shared lots of maggi, 66mm's and the space of 621. Vishal and Shubhangi deserve a shout-out for the various trips and fantastic views resembling the journey to the moon, which have proved to be stress busters. Mamta has been the person who is present with me from the beginning of the BS-MS journey. She has provided us with lots of delicious food that she made. I acknowledge Celina Meena, Prerna Goel, and Tejendra for all the unforgettable moments spent together and all CoD sessions. Prerna and Tejendra have helped a lot in the formatting and shaping of this thesis.

I am incredibly thankful to the beautiful soul Avantika Kapoor, who had supported me in some tough times and gave me constant support along with some much-needed life lessons.

She always kept me motivated and encouraged me to write this thesis. Thanks to Komal Bali for being such a lovely and senti junior and listening to me at times needed. She is the person with a very friendly and understanding nature. I would like to mention R Awasthi for some fantastic quick hacks and tricks and some really interesting sky gazing sessions.

I am thankful to the IISER Mohali administration and the Department of Biological Sciences for providing me opportunity to pursue BS-MS along with the various facilities. I thank IISERM Library and Computer Centre for granting us access to numerous resources, a great reading environment, and a passable Wi-Fi network. I am thankful to DST-INSPIRE for the fellowship during the degree.

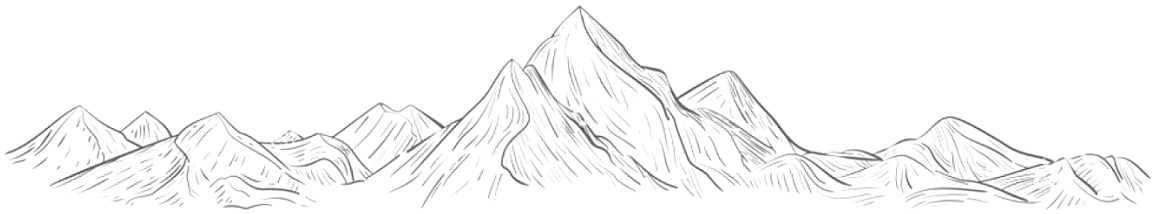
I also thank Gupta Ji (Sushil) for the perfect sugar-optimized cups of tea and for believing in system of delivery on demand.

I extend my thanks to my old friends, my schoolmates Shubham, Disha, Preety Ankit, and Akash, for being there with me for years and constantly checking up on me and for some fantastic call sessions, which proved to be helpful always.

I thank Kartika Goyal for all of her pieces of advice on work as well as in life. The constant motivations and suggestions have always helped. Radhika (Radhu) deserves thanks for such an amazing company, which has kept me cheered up always.

Now that I have successfully (at least I hope so!) completed thanking all the people from the lab, the institute, and friends, I take this opportunity to express my gratitude towards my family. My sincere thanks to my father and my mother for this kind of upbringing and for letting me nurture the desire to be a science student. They have constantly made sure that I do not lack any resources required at any time. I thank my sister, Dr. Varsha, for her constant guidance, scolding, and understanding, and taking a stand for me always. She has always funded my absurd and lavish demands (especially shoes).

I thank the mountains for keeping me alive and active whenever things were falling apart!



******

List of Figures

Contents

Thesis Abstract

Unlike higher vertebrates, where retinal injury leads to scarring, zebrafish exhibits robust regenerative potential to restore the retinal structure and function. Injury to zebrafish retina induces Müller glia (MG) cells to dedifferentiate, proliferate and migrate to the various layers and differentiate into respective cell types to restore the retinal physiology by the help of various factors that assist tissue remodelling. Matrix Metalloproteases (MMPs) are known to be the primary Extracellular Matrix (ECM) remodellers which regulate the collagen and gelatin levels leading to ECM degradation. A class of MMPs, MT-MMP (membrane type MMPs), are known to be inserted in the membrane. We wanted to decipher the role of MMP-14 (MT1-MMP) in the process of zebrafish retina regeneration and understand the significance of its localisation on the membrane, unlike other classes of MMPs which are secreted into extracellular milieu. Upon checking the temporal expression pattern by RT-PCR, we found that *mmp14a* and its activator, *furina*, transcript levels are upregulated during dedifferentiation phase, when Muller glia starts to attain stemness. In-situ hybridisation and immunostaining revealed that *mmp14a* and *furina* are expressed in cells next to proliferating Müller Glia Progenitor Cells (MGPCs). Thus, we hypothesise that Mmp14 is expressed in neighbouring cells which activates pro-Mmp2 secreted into ECM. This activated form of mmp2 then act on MGPCs to aid proliferation. Upon inhibiting various established pro-proliferative signalling pathways such as TGF-beta, Mmp2/Mmp9 activation and prominent oncogenic long non-coding RNA, *malat-1*, the levels of *mmp14a* and *furina* transcripts were upregulated, suggesting that Mmp14a and Furina might be playing the crucial role in regeneration and hence system tries to upregulate them and maintain homeostasis. We also found Mmp14a and Furina to be anti- and pro-proliferative respectively. Furin is observed to be regulating various regeneration associated genes such as *ascl1a*, *mmp9*, *zic2b* and *her4.1*. Further, Furin is also observed to be helping in maintaining proliferation at 8dpi. Hence, to confirm the role of axis in the context of retina regeneration, we would like to overexpress *mmp14a* and then observe if the phenotype is rescued by Furin inhibition. And look at the regulation of various major pathways by the axis.

Abbreviations

AC	Amacrine Cells
ANOVA	Analysis of variance
Ascl1a	Achaete-Scute Complex-Like 1a
BC	Bipolar Cells
BM	Basement Membrane
BMP	Bone Morphogenetic Protein
BrdU	5-Bromo-2'-Deoxyuridine
BSA	Bovine Serum Albumin
CAM	Cell Adhesion Molecules
CD44	Cluster of Differentiation 44
ceRNA	competing endogenous RNA
CNS	Central Nervous System
D.rerio	*Danio rerio*
DIG	Digoxigenin
DPF	Days Post Fertilization
DPI	Days Post Injury
ECM	Extracellular Matrix
Eda	Ectodysplasin A
EdaR	Ectodysplasin A Receptor
EMT	Epithelial to Mesenchymal Transitions
ESC	Embryonic Stem Cell
FGF	Fibroblast Growth Factor
GC	Ganglion Cells
GCL	Ganglion Cell Layer
GFP	Green Fluorescence Protein
GPI	Glycosylphosphatidylinositol
HC	Horizontal Cells
Her4.1	Hairy related 4, tandem duplicate 1
HIF	Hypoxia Inducing Factor
Hpx	Hemopexin
IF	Immuno-Fluorescence

IGF1R	Insulin-derived Growth Factor I Receptor
Il6	Interleukin 6
INL	Inner Nuclear Layer
IPL	Inner Plexiform Layer
Insm1a	Insulinoma-Associated 1a
KO	Knockout
Malat-1	Metastasis-associated lung adenocarcinoma transcript 1
miRNA	micro RNA
MG	Müller Glia
MGPC	Müller Glia derived Progenitor Cell
MMP	Matrix metalloproteinase
MO	Morpholino
MT1-MMP	Membrane Type I - MMP
NGF	Nerve Growth Factor
NMDA	N-methyl-D-aspartate
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
PBS	Phosphate Buffer Saline
PC	Proprotein Convertase
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived Growth Factor
PIF	Pluripotency inducing Factor
qPCR	quantitative Polymerase Chain Reaction
RAG	Regeneration Associated Genes
RNAi	RNA interference
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RTK	Receptor Tyrosine Kinase
shRNA	short hairpin RNA
Stat	Signal transducer and activator of transcription
TEA	Triethylamine
TGF	Transforming Growth Factor
TGN	*trans*- Golgi Network

TIMP	Tissue Inhibitor of Metalloproteases
Tnfa	Tumour Necrosis Factor Alpha
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth Muscle Cells
Zic2b	Zinc finger of cerebellum 2b

Section 1

Introduction and Review of Literature

1.1 Regeneration: the phenomenon and the field of study

Regeneration is primarily renewal or restoration of structure and function of a lost or damaged part of any system achieved by the system itself, without any direct external aids. If we are in a consensus with the definition above, nearly all organisms starting from the primitive protozoon to the complex organism such as human, are capable of regeneration up to a variable extent, *i.e.,* if not for regenerating the complete organism, for specific parts at least, and making it one of the most fascinating as well as most complex but poorly understood field of biology.

The book Principle of Regeneration by Richard J. Goss in 1969 states,

" If there were no regeneration there could be no life. If everything regenerated there would be no death. All organisms exist between these two extremes."

Richard J. Goss states these lines to explain the perfect balance that is maintained by mother nature between the two extremes that would result if all things would regenerate and if none would have regenerated leading to immortality and death in the respective cases. This balance is eventually essential for the existence of life on the planet. Over the course of evolution of life on earth, it has been observed that the balance is being shifted from the one of the extremes of complete regeneration of the organism, in the case of protozoons or the Platyhelminthes such as planaria having capability to regenerate from a part of their body, to the other extreme of very limited regeneration capability as in the case of higher

animals such as humans. At the same time, one can say that for the existence of life on earth, the most important phenomenon is reproduction which allows organisms to have a variability as well as is essential for the stability of a species. Eventually during evolution, regeneration becomes dispensable upon comparing the differences between reproduction and regeneration with reference to the fundamental need, that is, the existence of life.

1.2 Injury and wound healing responses

A tissue is said to be injured or wounded when its physical integrity and/or its functionality is lost. This loss of integrity or functionality can be caused by a variety of chemical, physical, electrical, metabolic or optical destructive stimuli (White LM et al., 2010). Upon an injury, the body's intrinsic machinery gets into action and a variety of intercellular and intracellular pathways are activated in a coordinated manner to repair the damage and restore tissue homeostasis (Gurtner GC et al, 2008). Hence, there is extensive crosstalk of the signalling pathways and communication between the various cell types and the extracellular matrix (ECM) (Eming SA et al, 2014). The initial biological response to a wound is the inflammation of the wounded region. In the case of a physical trauma, the inflammatory response is the formation of haemostatic plug and blood coagulation by the platelets, which also release the various growth factors at the site of injury and help in inducing cell migration to the site (Ryan GB and Majno G, 1977). The next step to this process is tissue formation and remodelling. A series of events like cell proliferation, migration and ECM modulation take place at the wounded tissue to aid this process (Singer AJ and Clark RA, 1999; Darby IA and Hewitson TD, 2007). After inflammation and tissue formation, organisms show a varied response to the healing process. Some organisms or tissues undergo the process of regeneration to restore back the functionality of the tissue while the others undergo fibrosis or the scar formation.

1.2.1 Regeneration

Regeneration can be defined as the complete restoration of the tissue's morphology as well as physiology. In other words, a regenerated tissue would result in homeostasis by achieving the physical integrity as well as the functionality (Birbrair A et al., 2013).

The primitive organisms like hydra and some Phatyhelmenths like planaria along with Arthropods, etc have immense regenerative potential and can regenerate completely. This regenerative potential decrease as we move from these primitive organisms to advanced vertebrates (Gabor MH and Hotchkiss RD, 1979). As we move towards the advanced organisms, we come across vertebrates which vary a lot in their capability to regenerate. Mammals, higher vertebrates, have very poor and limited regenerative capability in adulthood, while aves such as chicks have regenerative capability for once in their lifetime (Purnell LD, 2008). One of the amphibians, *Ambystoma* has immense regenerative capability of its various body parts including limbs (Simon A and Tanaka EM, 2013).

Moving towards the end of the spectrum, we see that lower vertebrates like fishes have immense regenerative potential and can regenerate most of their body parts (Marques IJ et al, 2019), while the higher vertebrates respond to most injuries by formation of scar, with very limited capability to regenerate liver, skin, etc (While LM et al., 2010).

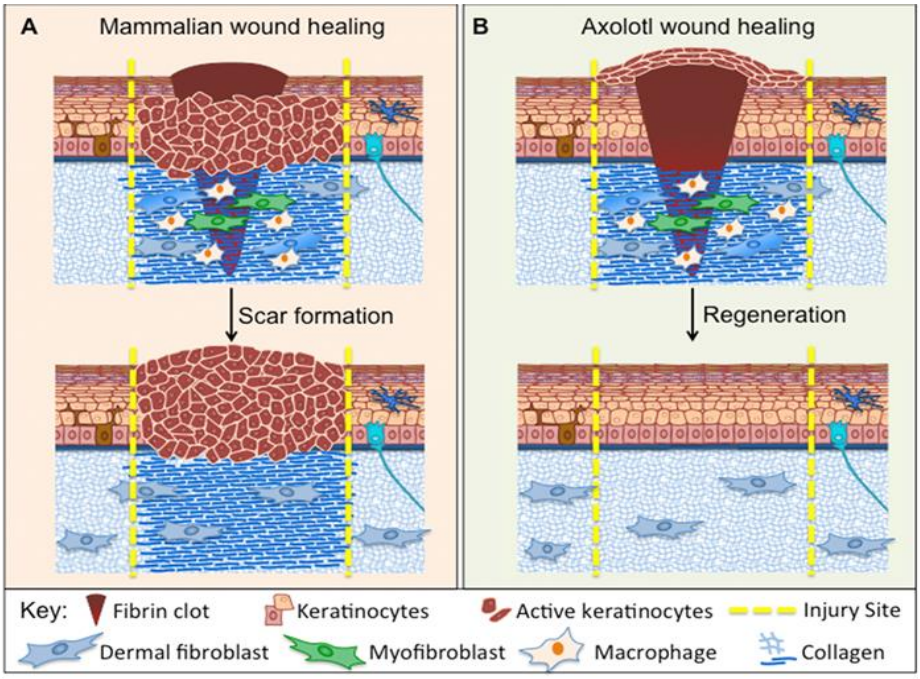


Figure 1.1: Wound healing responses; scarring vs regeneration. (a) In mammalian wound healing, fibrim clot is formed, with proliferation and migration of keratinocytes and fibroblasts under it expressing ECM proteins. Upon formation of provisional matrix, fibroblasts differentiate into myofibroblasts and contract the wound. Process results in scar formation. (b) In axolotl wound healing, keratinocytes migrate over fibrin clot to close the wound, then proliferate to form wound epidermis. Fibroblasts enter the wound bed, proliferate, express ECM proteins and differentiate into myofibroblasts to contract the wound. The cells present in dermis then remodel the ECM and the process results in regeneration. Taken from Erickson JR and Echeverri K, 2018.

After injury, when the tissue undergoes inflammation and is in the phase of tissue formation, it can undergo regeneration to restore tissue homeostasis in presence of secreted components of immune system and upregulated growth factors such as transforming growth factor-beta-1 (TGFb1), basic fibroblast growth factor (FGF-2), angiopoietin, platelet-derived growth factor (PDGF), and VEGF which help to undergo angiogenesis during the stage of tissue formation (Figure 1.1b). In the last stage of this process of regeneration, the tissue undergoes remodelling by retracting and ceasing all the released factors and apoptosis of the macrophages that were in action in the inflammation and tissue formation stages of wound healing. Eventually, tissue is left with cells which undergo Epithelial-mesenchymal transitions in the presence of collagen and other ECM proteins to restore its structural functionality (Darby IA and Hewitson TD, 2007; Gurtner GC et al., 2008; Szabowski, A. et al., 2000).

1.2.2 Scar formation

The system's self-healing response apart from regeneration is scar formation or fibrosis. Scarring results when tissue fails to transit from the formation phase to the resolving or remodelling phase extension due to altered matrix component deposition. The cells of tissues undergoing the healing process have to be synchronised to result in the proper healing. But, in case of the chronic healing wounds, all the cells of the tissue might not be in sync, that is, in the different stages of healing at a particular time. Due to this asynchrony, the progression to the remodelling phase is not coordinated. This process then results in the deposition of mainly fibroblasts and de-organized extracellular matrix and a failure to regeneration and just forms a scar.

The tissues such as the brain, heart, spinal cord, etc of the advanced adult vertebrate fail to regenerate and form clumps of collagen fibres of small radii to fill the wound with fibroblasts to cover the injury and form a scar (Figure 1.1a). One of the reasons for the scar formation in adults is the complexity of the tissue structure due to which the cells involved in the healing process might not be synchronized and therefore has a de-organized matrix and scar formation instead of regeneration.

The foetus of the advanced vertebrates has an immature immune system, and it is observed that upon injury to the foetus, the inflammation initiated, results eventually in the

regeneration of the tissue. From this, we can safely hypothesise that the immune system might be involved in regulating organisms to switch between the regeneration or scarring response. This would eventually support the statement made in the beginning of this sub-section that the cells involved in the healing process need to be in sync for regeneration otherwise, the cells that would enter the remodelling stage might be killed by the active macrophages and dendritic cells that are present as a component of the earlier stages (Mescher AL and Neff AW, 2005; Gurtner GC et al., 2008).

Scarring in humans also exerts a great deal of negative impact on the health of people in general and also consecutively can display an economic impact (White LM et al., 2010; Gurtner GC et al., 2008).

1.3 Zebrafish as a model system for studying regeneration

The zebrafish scientifically known as *Danio rerio* is a ray finned fish classified in the Chordata phylum of the kingdom Animalia. These are the fresh water fishes which are readily found in the waters of South Asia. *Danio rerio's* establishment as a model organism for scientific study in the lab was pioneered by George Streisinger. He along with his colleagues established the zebrafish facility from 1972 and worked upon production of zebrafish clones and induction of mutation in zebrafish embryos (Streisinger G et al., 1981; Walker C and Streisinger G, 1983). After around 15 years later, zebrafish was well appreciated as a model organism due to the fact that it is easy to maintain and has a short life cycle of nearly 4 months. Later, when the zebrafish genome was published, the scientific community gained even more interest in it by the fact that it shares nearly 70% of its genome with the humans and has at least one ortholog of over 20,000 human proteins (Howe K et al., 2013). Therefore, by studying the zebrafish, we can expect to elucidate the roles and interactions of proteins along with their regulation in an *in vivo* system.

D. rerio seems to be a handy system for studying the developmental biology, oncology, genetics, toxicology, muscular dystrophy, etc and an easy to manipulate system to introduce transgenes, overexpress protein and gene knockdown by microinjection (Xiang J et al., 2009; Hill AJ et al., 2005; Rosen NJ et al., 2009; Plantié E et al., 2015; Veldman MB and Lin S, 2008). Apart from the above mentioned features, these pisces possess an immense regenerative capability for the various organs or parts of their body (Gemberling M et al.,

2013). These lower vertebrates are capable of regenerating heart, brain, liver, pancreas, spinal cord, hair cells of posterior lateral line, kidney, fin and retina (Poss KD *et al.*, 2002; Kizil C et al., 2012; Becker T et al., 1997; Vihtelic TS and Hyde DR, 2000; Nechiporuk A and Keating MT, 2002; Harris JA et al, 2003; Reimschuessel R, 2001; Burkhardt-Holm P et al., 1999; Marques IJ et al., 2019) which makes them the most widely used model organism for studying regeneration. The researchers have been trying to study the interplay of various intercellular and intracellular pathways and elucidate the micro-environment of the regenerating tissue to have a better insight to the non-regenerative capability of humans and other organisms. Also, studying the regeneration in zebrafish would ultimately help us extrapolate and develop novel regenerative therapies for the higher organisms lacking this potential.

1.4 Physiology of eye and retina : Human vs Zebrafish

The basic structure of the human and zebrafish eye is quite similar, as eye of both the organisms have all similar components like lens, cornea, iris, retina and optical nerve which helps in the formation and processing of the image of the object seen by the eye. Upon comparing the two organisms eyes, the minor difference that appears is the shape of the lens which is spheroid in zebrafish which also results in a much lower volume of vitreous as compared to the human eye (Figure 1.2a) (Chhetri J et al., 2013).

Retina is a part of the Central Nervous System (CNS) playing an indispensable role in visualization. When light passes through the lens, it reaches retina which is placed in the innermost part of the eye and is a light sensitive tissue. This light enables the cascade of electrical and chemical impulses to travel in the form of synapse through the neurons and finally reach the optic tectum of the brain by optic nerve, resulting in the perseverance of matter (Thoreson WB and Dacey DM, 2019).

Vertebrate retina consists of six types of retinal neurons and one glial cell type which serve in the whole process of image perseverance (Figure 1.3) (Chhetri J et al., 2013). These cell types are:

- Ganglion cells (GC)
- Horizontal cells (HC)

- Amacrine cells (AC)
- Bipolar cells (BC)
- Rods
- Cones
- Muller glial (MG) cells

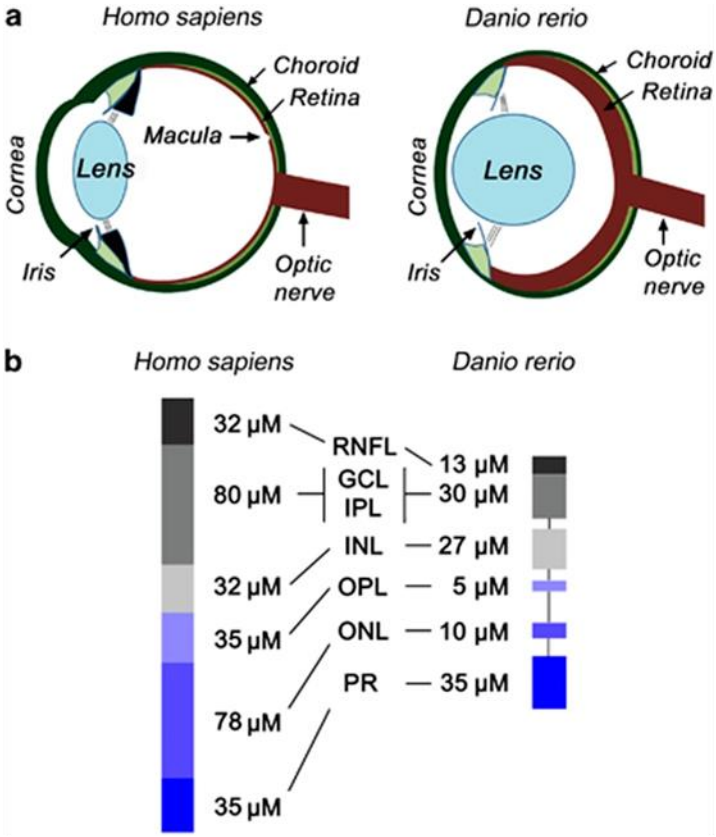


Figure 1.2: Structural comparison of human and zebrafish retina. (a) Overall structure of human and fish eye showing similarity. (b) Comparison between human and zebrafish retinal layers. Taken from Chetri et al., 2014.

The retinal layers are also conserved across the vertebrates consisting of various nuclear and plexiform layers (PL) (Figure 1.2b and Figure 1.3). The layers present and conserved across vertebrates are :

- Outer Nuclear Layer (ONL) – This layer consists the body of rods and cones photoreceptors which sense the light.
- Inner Nuclear Layer (INL) – INL contains the cell bodies of horizontal cells, bipolar cells and amacrine cells.

- Ganglion Cell Layer (GCL) – the ganglion cell bodies reside in the GCL itself with the GC axons are extended into the retinal nerve fibre layer.
- Outer Plexiform Layer (OPL) – This layer observes the synapse between the photoreceptor, cones and rods, and BC (and HC).
- Inner Plexiform Layer (IPL) – The synapse between BC and GC (and AC) occurs at IPL.

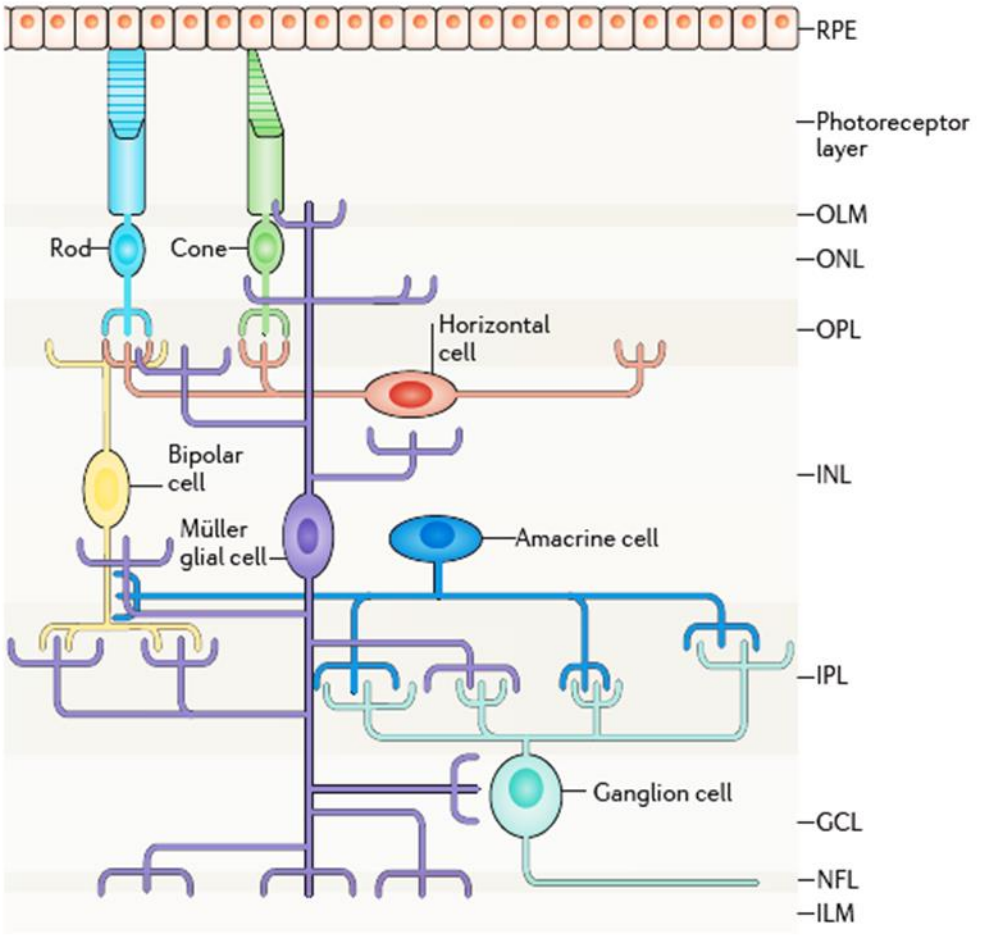


Figure 1.3: Retinal anatomy of zebrafish representing retinal cell types and glial cell type. Taken from Goldman, 2014.

The glial cell type present in the retina, *i.e.*, Muller Glia, whose processes span all the layers of the retina and are capable of giving rise to all the six retinal neuronal types. The glial cells are the differentiated cells which were not known to have the capability to proliferate. But there are various factors discovered, that are upregulated upon injury to induce reprogramming of MG to MGPCs and proliferate and aid migration across layers

and replace the damaged retinal neurons. (Goldman D, 2014; Nelson J et al., 2012, Ramachandran R et al., 2010).

Taken together, the structural similarities of zebrafish and mammalian retina, along with it being the most easily accessible part of CNS, and, above all the capacity to demonstrate a beautiful regenerative response which is absent in higher vertebrates, make zebrafish retina one of the best suitable model system to study retina regeneration aiming towards successful medical intervention of mammalian retinal repair.

1.5 Mechanism of zebrafish retina regeneration

In today's world, diabetes is one of the most common diseases across the globe and it is one of the major causes for blurry vision or loss of eyesight. This happens due to increased pressure in the eye which damages optic nerve or impairs retina causing medical conditions like glaucoma, retinopathy (Jadeja et al., 2020; Gerard Lutty, 2013). Since retina is the most accessible part of the Central Nervous System (CNS), and injuries to it are not lethal, it is one of the best models to study regeneration. Also, studying retinal regeneration in zebrafish would hence help us have understanding of the vertebrate CNS and this knowledge might then be extended to the other parts of CNS and further to the higher vertebrates and mammals and we can develop novel targets for the treatment of various disease conditions and even this would also help in the development of regenerative therapies.

Over the years of research in the field, various models have been developed to injure zebrafish retina (Sharma P and Ramachandran R, 2019) which include:

- Chemical injury by NMDA (N-methyl-D-aspartate) damaging the ganglionic cell layer (GCL).
- Injury by photobleaching which causes damage to the photoreceptor cells.
- Mechanical injury by needle, which injures all the layers and cell types of the retina.

In this study, we have used mechanical injury method with the help of a 30Gauge needle, wherein we puncture all the layers of retina. . It mimics conditions which a Zebrafish can encounter in a normal scenario. Uninjured portions of the same retina can act as control, conferring this model appropriate to study regenerative responses under natural conditions.

As the mechanical injury damages all cell types, it enables us to appreciate the ability of Muller Glia (MG) to give rise to all the retinal cell types. Upon any injury, various factors are released at and near the injury spot including interleukins (Bernardos RL et al., 2007), which initiate inflammatory response and within a few hours after retinal damage, *insm1a* and *stat3* is upregulated in Muller Glia cells (Nelson CM et al., 2012), and with time the

expression of *ascl1a* increases in the reprogramming MG cells (Ramachandran et al., 2010) during dedifferentiation phase to form the multipotent Muller Glia Progenitor Cells (MGPCs). These dedifferentiated MGPCs re-enter cell cycle and actively proliferate asymmetrically to form an MGPC and a differentiated MG cell, and these MGPC give rise to neurogenic cluster which then migrate to the all retinal layers and differentiate into the respective cell types of retina (Goldman d, 2014; Gemberling M et al., 2013).

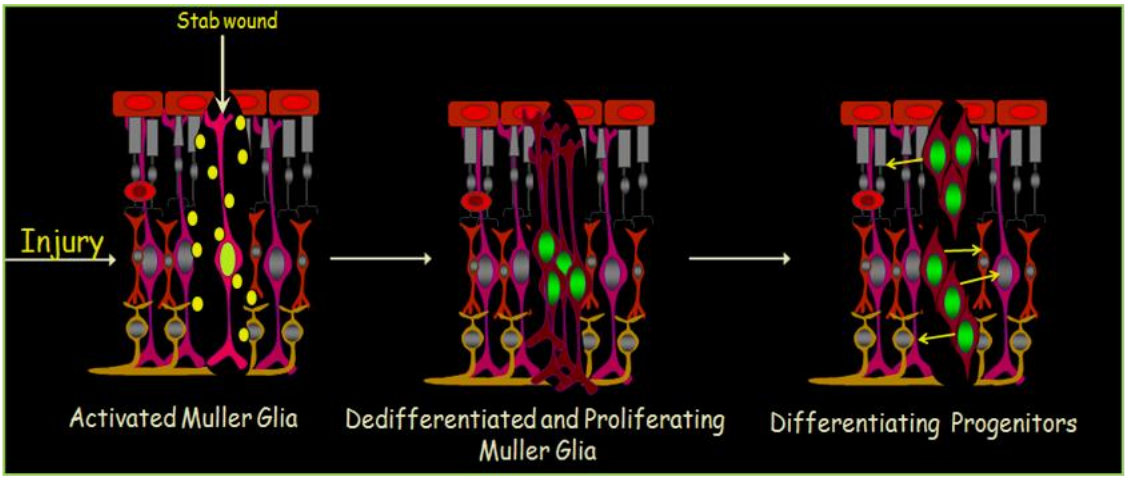


Figure 1.4: Stages of retina regeneration in zebrafish upon stabwound. Taken from Goldman D Lab.

1.6 MMPs and their roles

ECM remodelling is a major event in the wound healing process, when tissue enters the regeneration phase and not the scar formation phase. This is the stage when system retracts the released factors and macrophages, and undergoes the process of ECM modulation and tissue remodelling. The ECM macromolecules play a crucial role in defining the cell and tissue niche at developmental and morphogenesis stages (Visse R and Nagase H, 2003). The major players of ECM and tissue modulation are the Matrix Metalloproteinases (MMPs) or matrixins which play an indispensable role in directing the tissue microenvironment by spatio-temporally defined ECM degradation (Visse R and Nagase H,

2003; Nagase H et al., 2006). If this degradation isn't regulated by the system, it can lead to various diseases and disorders such as arthritis, cancer, atherosclerosis, aneurysms, nephritis, tissue ulcers, and fibrosis (Woessner JF, 1998; Shah PK, 1997). Apart from degrading the ECM proteins such as collagen and fibronectin, these enzymes can also activate a varied number of bioactive molecules, cleave cell surface receptors, aid in release of apoptotic ligands and cytokine inactivation (Lint VP and Libert C, 2007).

1.6.1 Structure of MMPs

A typical MMP is known to have a propeptide of around 80 amino acids, a catalytic metalloproteinase domain of nearly 170 amino acids, a linker peptide of variable length which is also known as hinge region and a hemopexin (Hpx) domain of about 200 amino acids helps in protein-protein interaction (Figure 1.5). Hpx domain also helps in substrate recognition, enzyme activation, protease internalization and degradation. The structure of the pro-peptides of MMPs have some similar and conserved sequences of zinc binding motif HEXXHXXGXXH in catalytic domain and cysteine switch motif PRCGXPD in the propeptide. The coordinate interaction between the Cys-$Zn2^{+}$ from the two motifs keep the MMPs in an autoinhibitory form by preventing the essential interaction of zinc atom binding to the water molecule and activating the proMMP (Nagase H et al., 2006).

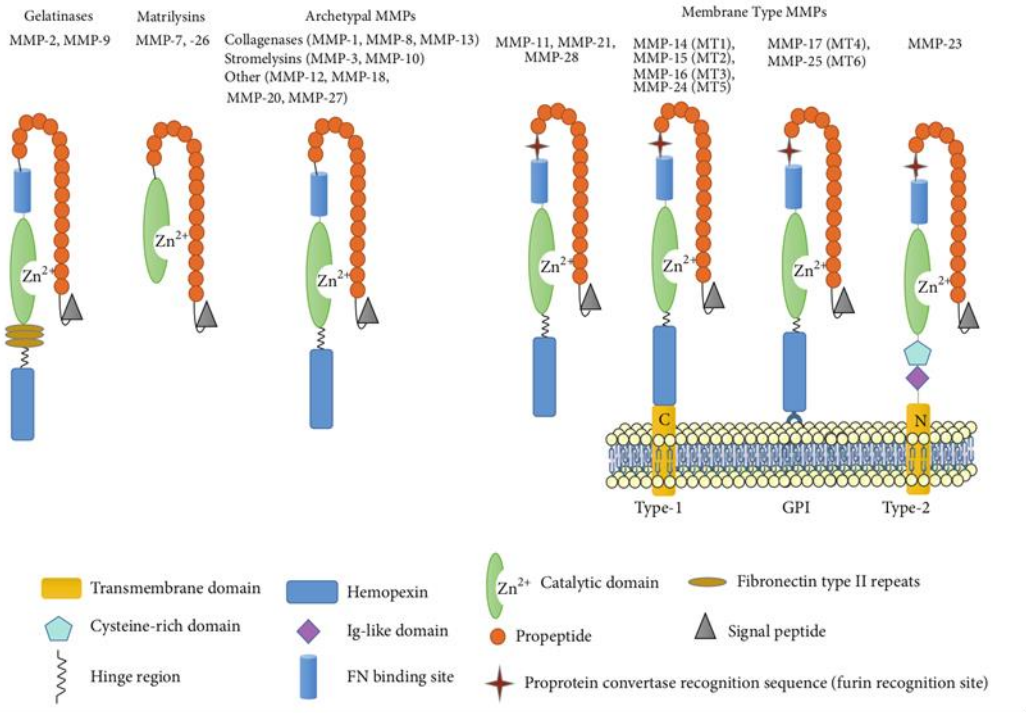


Figure 1.5: Structure and classification of Matrix Metalloproteases (MMPs). Adapted from Brkic M et al., 2015.

1.6.2 Classification

The MMPs have been categorised into 6 classes based upon their localization and, structural and functional variation (Figure 1.5). These are :

- Collagenases – This class consists of MMP1, MMP8, MMP13, MMP18, which are known to cleave interstitial collagens I, II, and III along with other ECM and non-ECM molecules (Visse R and Nagase H, 2003).
- Gelatinases – These are the enzymes having repeat structures of type II fibronectin in their catalytic domain helping in the binding of these enzymes to gelatin, collagen and leminin and digesting them. Gelatinase A (MMP2) and Gelatinase B (MMP9) belong to this group and are known to have important roles to play in osteogenesis (Allan JA et al., 1995; Patterson ML et al., 2001; Martignetti Jaet al., 2001).
- Stromelysins – MMP3 and MMP10 are the stromelysin 1 and 2 having similar structures but varied efficiency. These function to activate the proMMP1 apart from degrading ECM component (Suzuki K et al., 1990).
- Matrilysinss – These group of MMPs lack the Hpx domain. MMP7 and MMP26 are also known as endometases. MMP-7 processes many cell surface molecules such as pro–α-defensin, Fas-ligand, pro–TNF-α, and E-cadherin, with MMP26 digesting a number of ECM components (Uria JA, López-Otín C, 2000; Park HI et al., 2000).
- Membrane- Type MMPs – These are classified as type I transmembrane proteins (MMP-14, MMP15, MMP16, MMP24) and glycosylphosphatidylinositol (GPI) anchored proteins (MMP-17 and MMP-25). MT1-MMP (MMP-14) has collagenolytic activity and is found to be necessary for angiogenesis (Ohuchi E et al., 1997; Pepper MS 2001).
- Other MMPs – The rest of the MMPs are not classified under any category and have varied functions. MMP12 is necessary for macrophage migration (Shapiro SD et al., 1993). Enamelysin (MMP-20) help in digesting amelogenin, located within newly formed tooth enamel (Li W et al., 2001). The other MMPs that are uncategorised include MMP22, 23, 28 and the expression patterns of MMP28 predict it can be involved in the process of wound healing (Kere U et al., 2002).

1.6.3 Role of MMPs in regeneration

As discussed in Section 1.3, the process of tissue regeneration requires remodelling in the tissue matrix along with inducing progenitor cells to give rise to new cells in the tissue. An essential phenomenon for cell growth and tissue repair is dynamic modulation of physical cell-to-cell contact.

Upon dermal injury, inflammatory process takes over initially, which is then regulated by expression of first collagenase, MMP-8 (Nwomeh BC *et al.*,1998). In due course, MMP-10 is expressed by macrophages and epithelial cells as an injury response. After the inflammation stage, the tissue has to prepare itself for cell proliferation and migration. The initial step taken in this process involves degradation of extra-cellular matrix (ECM) components such as collagen and basement membrane (BM) to decrease ECM stiffness to assist the reprogramming and movement of the progenitor cells and various growth factors to the injured area (Chang C and Werb Z, 2001). Collagenases expressed in the earlier stages initiate degrading the major matrix component, collagen, along with assisting the release of MMP-1 and MMP-13 (Rohini MG *et al.*, 2015), and BM being degraded majorly by MMP-19 (Sadowski T *et al*., 2005). This wound-induced epithelial migration also involves role of MMP7 which helps by cleaving E-cadherin resulting in loosening of cell-cell attachment for migration.

Apart from degrading the matrix, MMPs have a significant role in regulating cell proliferation. It has been shown in Vascular Smooth Muscle Cells (VSMCs) that MMPs regulate the process of cell proliferation majorly by activating various growth factors by their proteolytic cleavage along with regulated receptor cleavage (Xi Wang and Khalil RA, 2018). MMPs also facilitate growth factor or ECM component interaction with the cells to induce proliferation by integrin mediated pathway (Morla AO and Mogford JE, 2000). This is done by releasing growth factors (such as FGF-2) from ECM (Visse R and Nagase H, 2003) or activating growth factors (TGF-h) by cleaving latency-associated peptide (Annes JP *et al.*,2003). It has been shown in retina regeneration that *mmp2/mmp9* also keeps the induction of regeneration associated genes under check in initial phases of cell reprograming (Sharma P *et al.,* 2020).

1.7 Membrane Type 1 – MMP (MMP-14)

While the various class of MMPs are secreted in the extracellular milieu where they can diffuse to the place of action, a class of MMPs, MT-MMPs as described in the section above, are inserted in the membrane as type 1 membrane protein or glycosylphosphatidylinositol (GPI)-anchored protein (Sato et al., 1994; Itoh et al., 1999; Kojima et al., 2000) resulting in a restricted place of action to the pericellular space. This restriction results in their special functional ability over other MMPs to modify the cellular microenvironment. Recently, various studies on MT1-MMP (or MMP-14) have shown its indispensable role during development or regulating the ECM and cell's immediate microenvironment (Ueda J *et al.,* 2003).

1.7.1 MT1-MMP structure and its activation

MT-MMP shares common structure with the other MMPs containing propeptide, catalytic domain, hinge region and hemopexin domain except for having the stalk and transmembrane domain to help with stability and spatial localization and a furin cleavage site (Figure 1.6) (Sato et al., 1994; Brinckerhoff and Matrisian, 2002; Itoh and Nagase, 2002; Seiki, 2003; Zucker et al., 2003).

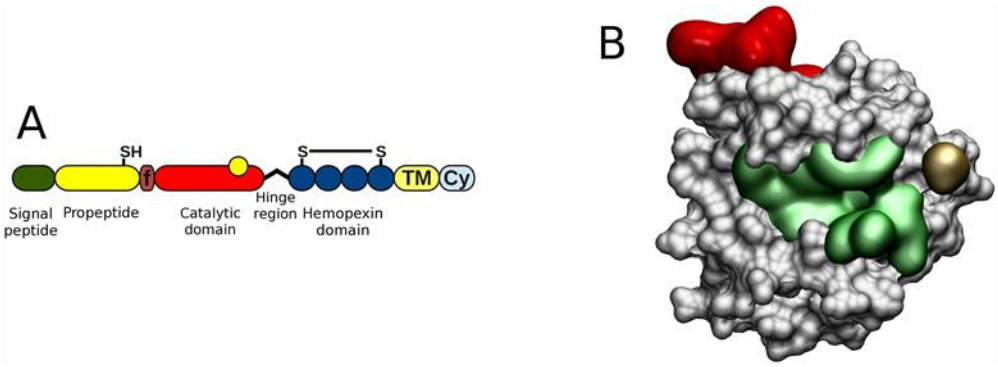


Figure 1.6: Structure of MMP-14 (MT1-MMP). (a) Domain structure of MMP-14, f is furin cleavage site. (b) Surfance representation of MMP-14 catalytic domain. Taken from Pietraszek-Gremplewicz K et al., 2019.
Protein PDB ID: 1BQQ; sites represented: catalytic site (green), MT-LOOP (red), N-glycosylation site Asn229 (yellow)

When this matrixin is released into the cellular space after processing from golgi, the proprotein convertase (PC) Furin (Nagase H et al., 2006), which acts on the Furin cleavage site comprised of basic amino acids RRKR on MMP-14, results in release of the propeptide

domain from the enzyme in the intracellular space. On release of the propeptide, the monomer is then localized onto the membrane for its insertion. Once inserted, it dimerizes with the other cleaved form and forms a complex with TIMP-2 (Gifford V and Iteh Y, 2019). The process of dimerization and forming complex with TIMP-2 results in a fully active trimer MMP-14 which can now act on the ECM components to regulate the cellular microenvironment and also help in activation or increase the accessibiity of various growth factors and other MMPs (Figure 1.7). The activation of MMP-14 requires intermediate TIMP-2 concentration, which when exceeded can cause inhibition of proteins activated by MMP-14 along with its own activity (Nagase H et al., 2006).

1.7.2 Function of MMP-14 in regulating cellular micro-environment

MMP-14 is one of the majorly studied MT-MMP and is well known for its actions on regulating ECM remodelling and angiogenesis. Along with this, it is a well-known zymogen playing significant role in regulating the progression and metastasis of various cancers.

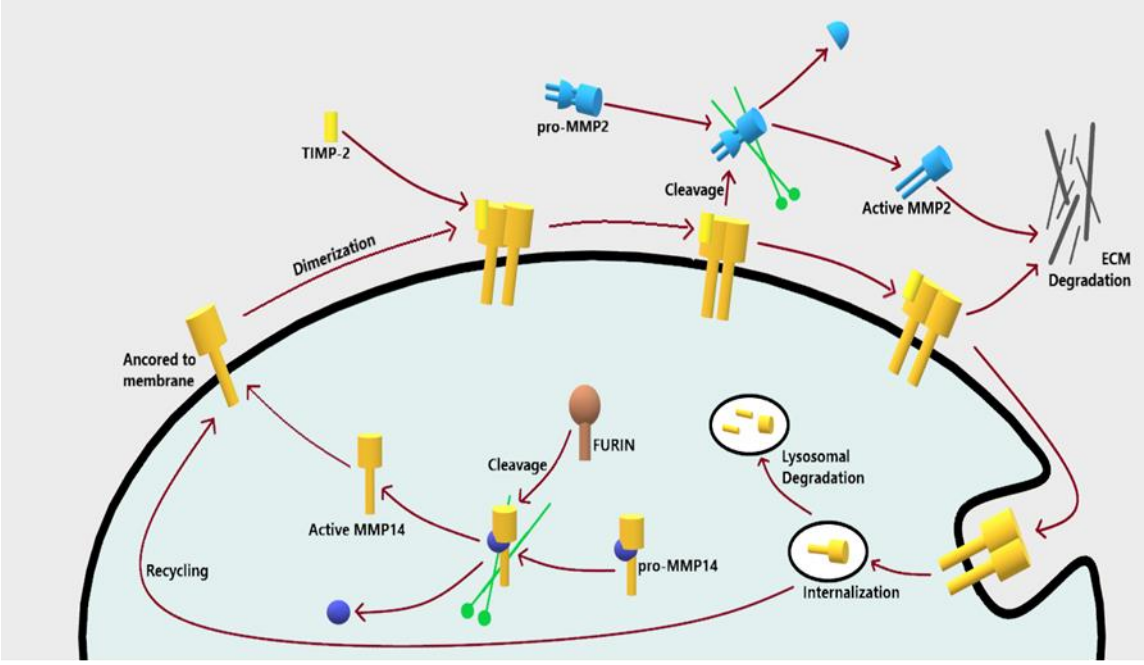


Figure 1.7: Activation and function of MMP-14.

MMP-14 upregulates the levels of angiogenic factors like VEGF, simultaneously cleaving and deactivating the anti-angiogenic factors such as decorin (in cornea) leading to an increase in the formation of new blood vessels from the existing vasculature (Sounni NE *et*

al., 2011; Mimura T *et al.,* 2009; Marc A *et al.,* 2004). MMP-14 mediated collagenolysis of type 1 collagen creates conducive environment for cell mobility, and production of cryptic peptides regulating tissue homeostasis and development (Page-McCaw et al., 2007). MT1-MMP interacts with the molecules on or juxtaposed to cell surface, such as shedding of CD44 adhesion molecules and syndecan-1 (Morishita T *et al.,* 2012; Endo *et al.,* 2003). These interactions regulating the growth factors and cell micro-environment leads to reorganization of actin cytoskeleton, disruption of endothelial tight junctions and BM. This process not only leads to ECM degradation and remodelling, but also assists in cell migration and proliferation (Sounni NE *et al.,* 2011; Marc A *et al.,* 2004).

Apart from directly affecting angiogenesis and collagen degradation mediated remodelling, MMP-14 also activates other MMPs such as gelatinases (pro-MMP-2/9) by forming trimolecular complex with TIMP-2 acting as receptor for pro-MMP2 and another free molecule of MMP-14 cleaves the pro-peptide to activate gelatinase (Al-Raawi D *et al.,* 2011). Activation of gelatinases leads to the degradation of gelatins and type-4 collagen disrupting the BM, a process necessary for achieving invasiveness for cancer cells (Stetler-Stevenson et al., 1993; Seiki, 2003).

The reports and the physiological functions of MMP-14 described signify its importance in development and remodelling. Studies on cancer cells reveal that upon RNAi mediated MMP-14 gene silencing, invasiveness and metastasis of tumours decreases significantly as the matrix is not remodelled due to decreased shedding of adhesion molecules such as CD44, leading to stiff ECM (Ueda J *et al.,* 2003). Also, experiments on mice have shown the physiological significance of MMP-14 by various phenotypic disorders. MMP-14 gene knockout (KO) mice develop significant growth impairment 5 days after birth and undergo severe fibrosis of soft tissue in periskeleton along with delayed bone ossification leading to death at an age of 7 to 12 weeks (Holmbeck K et al., 1999).

Even though its membrane insertion limits its range of action and accessibility to molecules juxtaposed to that cell, MMP-14 has been shown to play an indispensable role during development and angiogenesis. Since, process of development resembles the basic idea of regeneration with cells undergoing proliferation and migration in both, we can hypothesise that MMP-14 might be acting as a major regulator of ECM, due to its unique spatial localization, during the process of retina regeneration in zebrafish.

1.8 Proprotein Convertase Furin

Furin is a calcium dependent proprotein convertase (PC) belonging to family of subtilisin-like serine proteases expressed ubiquitously across organisms (Seidha NG *et al.,* 1998). PCs are involved in proteolytic processing of various propeptides to convert them to biologically active peptides or proteins including neuropeptides, growth factors and their receptors, hormones and cell adhesion proteins (Jaaks P and Bernasconi M, 2017; Seidah NG et al., 2012). The zymogen's canonical cleavage sites are proprotein's paired basic amino acids at RXK or RR (known as Furin cleavage site). This 794 amino acid long PC consists of a conserved catalytic domain having RXK/RR consensus sites and P domain required for the activity of the protein along with pH modulation and calcium demands (Zhou A *et al.,* 1998). Furin achieves its fully functional form upon undergoing two autocatalytic cleavages at its essential prodomain, and plays an indispensable role in protein folding and activation (Thomas G, 2002).

1.8.1 Furin activation and functionality

Furin transcription is driven by cytokine activated or housekeeping gene promotors P1, P1A or P1B, with these promotors harbouring HIF-1 binding sites to induce expression under hypoxic conditions (Ayoubi TA *et al.,* 1994; McMohan S *et al.,* 2005). Translating proprotein then enters in secretory pathways and inserts into ER membrane upon glycosylation (Braun E and Sauter, 2019). First autocatalytic cleavage of propeptide from its N-terminus results in generation and placement of a chaperon for functional folding of catalytic subunit (Shinde U and Inouye M, 1993). Upon enzyme's localization to the *trans*-golgi network (TGN), second and slower autoproteolytic step adds oligosaccharides to N-terminal along with chaperon removal and glycans trimming resulting in enzymatically functional PC (Anderson ED *et al.,* 1997) (Figure 1.8).

Furin accumulated in TGN can then be translocated for its activity to cell surface or early endosomes for cleaving toxins and antigens; or can act on various biosynthetic pathways cleaving pro-beta-NGF and pro-BMP4 by residing at the sorting compartment (TGN) (Thomas G, 2002).

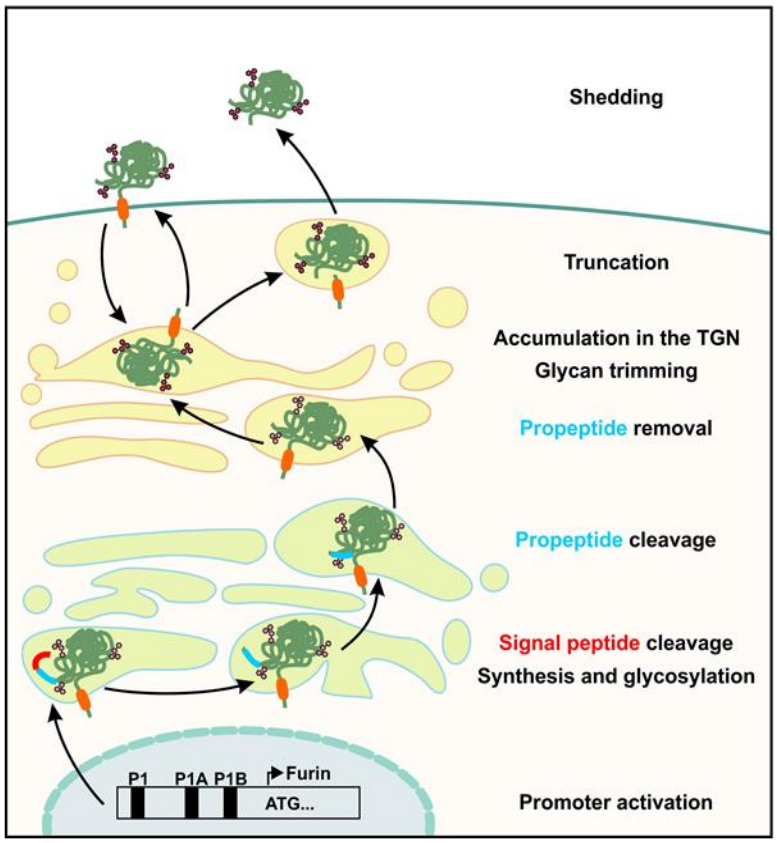


Figure 1.8: Maturation of proprotein convertase Furin. Taken from Braun E and Sauter D, 2019. Domains: transmembrane domain (orange), propeptide (blue), N-terminal signal peptide (red), active domain (green).

1.8.2 Role of Furin in development and homeostasis

Furin is shown to play an important role in regulating cell fate, embryo development and tissue homeostasis apart from a destructive role in activation of toxins and processing of various viral proteins (Braun E and Sauter, 2019; Thomas G, 2012). One of the major cleavage roles of Furin is the cleavage of prototypic target derived neurotrophin pro-beta-NGF to beta-NGF leading to triggering of neurotrophin derived cell fate pathways in innervating neurons. The unprocessed pro-form of beta-NGF having high affinity for receptor of TNF family results in increased apoptosis. In contrast, Furin mediated processed beta-NGF results in its interaction with Trk proto-oncogene RTKs leading to increased cell survival (Lee R *et al.*, 2001) and eventually resulting in homeostasis.

Furin also cleaves transmembrane receptor Notch to assist release of intracellular domain of Notch, activating the development associated genes essential for cell-cell communication , while immature Notch inhibit cell differentiation through a distant unknown signalling pathway (Mumm JS *et al.*, 2000; Bush G *et al.*, 2001). Furin is

hypothesised to be involved in the receptor-ligand spatial uncoupling by switching juxtacrine signalling to paracrine. In tooth enamel, this results in the binding of distantly located TNF family member Eda-1 with its receptor EDAR by cleaving membrane bound Eda-1 to interact with its receptor resulting in proliferation (Chan Y *et al.,* 2001; Tucker AS *et al.,* 2000). Furin promotes yolk sac vasculogenesis in the cardiogenic mesoderm along with playing an important role in heart-looping and maintaining the asymmetry of the embryo by catalysing production of members of TGF-beta family, namely Nodal and Lefty-2 (Constam DB and Robertson ED, 2000).

Various studies on this PC have shown its physiological importance and indispensable role in development. Furin KO in mice has been reported to be hemodynamic insufficient and having cardiac defects resulting in death at 11 embryonic day (Roebroek AJ *et al.,* 1998). KO specific to endothelial cell have also shown cardiac malfunctions resulting in death few days post birth (Kim W *et al.,* 2012).

1.8.3 Furin in tumour progression and invasion

Furin is termed a 'master switch of tumour growth and progression' (Bassi DE *et al.,* 2005; Klein-Szanto AJ and Bassi DE, 2017) for its ability to catalytically activate proteins promoting angiogenesis, cell proliferation and migration and tissue invasion. Furin cleaves various proproliferative inactive growth factors or growth factor receptors such as NGF, PDGF, IGF1R etc. These growth factors mediated signalling pathways promoting tumour growth maintaining the malignancy of the tumour, with Furin and acting as a prognostic marker for various cancers (Jaaks P and Bernasconi M, 2017).

For the tumour to metastasise to other parts of the body, the matrix and the cellular microenvironment is to be modified, which is achieved by the degrading the ECM components resulting in liberation of ECM bound molecules and cell mobility (Jaaks P and Bernasconi M, 2017). As discussed in section 1.7, MMP-14 acts as the potent ECM modifier due to its role in cleaving various molecules and ECM components. Furin cleaves pro-MMP-14 intracllularly at the C terminal of the cleavage site RRKR motif to facilitate the release of propeptide , resulting in an activated matrixin(Yana I and Weiss SJ, 2000).

Another class of molecules mediating the cell-cell and cell-matrix interactions are Cell Adhesion Molecules (CAM), Apart from activating cell survival and cell proliferation

pathways, CAM play a significant role in tumour cell invasion and migration across lymphatic vessels. Furin acts on the integrin alpha-subunits resulting in their activation (Lehmann M *et al.*, 1996). It also processes N- and E-cadherins which promote tumour invasion and migration (Posthaus H *et al.*, 1998; Maret D *et al.*, 2010).

1.8.4 Furin role in zebrafish

Based on sequence homology and functionality, Furin exists as 2 isoforms in zebrafish, namely *furina* and *furinb*, with both the genes being expressed maternally. *furina* zebrafish mutants have been shown to have defects in pharyngeal skeletal domain elements due to hampered Furin activated Edn1 signalling. However, studies show that *furina* loss of function mutant does not result in Edn1 mutant mediated jaw defects. This suggests the compensatory role played by its co-ortholog *furinb* in activating Edn1 signalling (Walker MB *et al.*, 2006). Apart from it, Furin activates VEGF-C inducing Atk phosphorylation during fin regeneration to accompany cell proliferation (Khatib AM *et al.*, 2010).

1.9 MMP-14 interaction with other pathways

MMP-14 is known to be playing a fundamental role in various physiological processes by degrading the ECM. Apart from it, it also interacts with various pathways such as TGF-beta, MMP2/9 mediated cell proliferation, etc to regulate tissue homeostasis and remodelling.

1.9.1 MMP-14 and TGF-beta signalling

The pleiotropic factor Transforming Growth Factor-beta (TGF-beta) generally promotes EMT and is known to play paradoxical role by repressing early tumorogenesis, but stimulating advanced tumour growth and metastasis (Bierie B and Moses HL,2006; Padua D and Massague M, 2009). TGF-beta is also known to play biphasic role in retina regeneration performing proproliferative functions and activation of RAGs, and then causing the cell cycle exit of MGPCs to migrate to the various layers (Sharma P *et al.*, 2020). The Tgf-beta protein is sequestered in the extra-cellular matrix in the form of inactivated small latent complex formed by propeptide homodimers (Jenkins G, 2008). This latent Tgf-beta can be catalytically activated by proteolytic activity of MMP-14 and gelatinases. The transmembrane matrixin MMP-14 mediates its effect by its membrane

colocalization with alpha-v-beta-8, which acts as a cell surface shuttle to present latency associated Tgf-beta to MMP-14 for proteolytic activation and release during vascular and brain development (Jenkins G, 2008; Proctor JM *et al.*, 2005; Cambier S *et al.*, 2005). Based on the studies, we hypothesise that MMP-14 is required for the release of Tgf-beta sequestered in ECM and its activation.

1.9.2 MMP-14 and MALAT-1

Apart from the protein expressions and their regulations by various Transcription Factors (Tfs), cellular and physiological processes are also regulated by various ubiquitous or tissue specific non-coding RNAs such as miRNAs, shRNAs or long non-coding RNAs (lncRNA) which act at the level of transcription, post transcription or translation (Castro-Oropeza R *et al.*, 2018). One of the lncRNAs, metastasis-associated lung adenocarcenoma transcript 1 (MALAT-1) is known to be overexpressed in cancer stem cells (CSCs) of various tumours such as pancreas and breast (Jiao F *et al.*, 2015; Zeng L *et al.*, 2017). MALAT-1 has a role in regulating proliferation and migration of cancer stem cells (CSCs) by regulating Snails, Slugs and Cadherins mediated EMT triggering (Jiao F *et al.*, 2014). MALAT-1 can affect

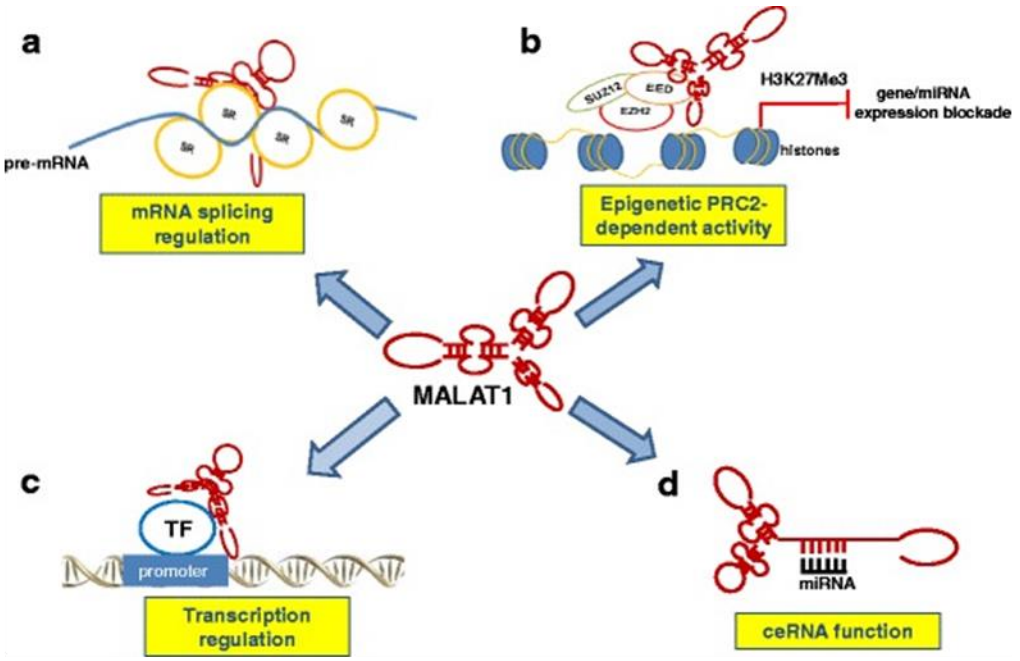


Figure 1.9: MALAT-1 functions in various tumors. (a) Affect on mRNA transcription by regulating splicing. (b) MALAT-1 interaction with PRC2 components such as EZH2, EED resulting in epigenetic regulation. (c) Fascilitating of TFs binding to promotor by MALAT-1. (d) MALAT-1 sequesters levels of miRNA, acting as ceRNA. Taken from Braun E and Sauter D, 2019.

levels and activity of various genes by direct transcription or epigenetic regulations, along with regulating the splicing events and facilitating promoter binding of various genes involved in human cancers (Figure 1.9) (Amodio N *et al.*, 2018). MALAT-1 is known to act as competing endoenous RNA (ceRNA) for miR200c, miR145, miR22, etc. MALAT-1 acting as ceRNA regulates the levels of MMP-14 by sequestering the levels of miR22, having MMP-14 and Snails as its functional targets (Luan W *et al.*, 2016).

******

Section 2

Materials and methods

2.1 Animal maintenance

Zebrafish were maintained at the temperature between 25-28-degree C with light: dark cycle of 14hours:10hours. These fish were fed with prawn feed and live artemia twice a day. Fish embryos were obtained by crossing wild-type fish and embryos were further maintained at 28-degree C and were fed crushed prawn feed dissolved in water.

2.2 Retinal injury and drugs delivery

Zebrafish were anesthetized using tricaine followed by retinal injury using a 30- gauge needle. To injure the retina, the eye ball was slightly pressed from one corner of the eye with the help of a tweezer resulting in the back of the opposite corner of the eye to come up. A 30-gauge needle was inserted through the back of the eye resulting in injuring all retinal layers.

For treatments with various inhibitors of different molecules and signalling pathways, either fish were dipped in micromolar solutions of the drugs or the drugs were injected into the vitreous humour using a Hamilton syringe following injury.

2.3 BrdU pulsing and eye or retina dissection

Injured control or drug treated fish were given BrdU pulsing on a day suitable and advantageous for the experiments. If not specified, most of the times, the eyes were harvested from 4dpi retinae with or without any treatment as that day has the peak of proliferation and hence, maximum number of BrdU positive cells. For BrdU pulsing, fish were either dipped in 5mM solutions of BrdU for 4 to 5 hours or they received IP injection of 20mM BrdU 4 to 5 hours before anaesthesia.

Following BrdU pulsing, the eye was pulled out completely from the eye socket of an anesthetized fish using a tweezer and was used for further procedures like tissue fixation. For RNA isolation, eyes were pulled out without BrdU pulsing and the retinae were dissected out using sharp surgical tweezers and needles. Retina dissection for RNA isolation was carried out by placing the eye in a solution of chilled 1X PBS.

2.4 RNA isolation

1. After harvesting the eyes, retinal dissections were performed in the 1X PBS solution.
2. Tissues were suspended in 200 µL (X) of Trizol and homogenized with a piston or a pipette.
3. Following homogenization, 0.2X volume of chloroform was added, and was mixed 10 to 15 times by inverting the MCTs upside down and the MCTs were kelp at room temperature for 5 to 10 minutes.
4. MCTs were centrifuged at 12,000 rcf for 15 minutes at 4-degree C. The upper most layer containing the RNA (~40uL) was transferred into new MCTs using cut tips.
5. Equal volume of isopropanol was added to the tubes and the MCTs were kept on ice for 20 minutes or in -80 freezer for overnight to precipitate the RNA.
6. After precipitation, MCTs were centrifuged at 13,000 rpm for 30 minutes at 4-degree C.
7. Followed by centrifugation, pellets were washed with 80% ethanol in DEPC water.
8. Washed pellets were centrifuged at 7600 rcf for 10 minutes at 4-degree C, followed by air drying and dissolution in 10 µL (one retina) or 13uL (two retinae) of DEPC water.

Following reagents were used in the process of extracting the whole RNA:

1. 10X Phosphate buffer saline (PBS) solution composition:
    2.76g $NaH_2PO_4 \times H_2O$ (monobasic)
    11.36g Na_2HPO_4 (dibasic)
    87.6g NaCl
    1.87g KCl
    DEPC up to 1 litre
2. Trizol (Sigma)
3. Chloroform (Sigma)
4. Isopropanol (Sigma)

5. Ethanol (Sigma)
6. DEPC water

2.5 Preparation of cDNA.

Following reagents were used for cDNA synthesis:
1. mRNA from retinal tissue/embryonic tissue
2. Thermo scientific RevertAid RT First-strand cDNA synthesis kit
3. PCR Thermocycler

After thawing, the mRNA and the components of the cDNA synthesis kit, they were placed on ice. Further, following steps were performed:

1. Following components were added into a sterile PCR tube in the indicated order (for 20uL reaction):

Components	**Amount**
Template mRNA	upto 5µg of RNA
Oligo (dT)Primers	1µL
Random Hexamer primers	1µL
Nuclease-free water	upto 12µL

2. PCR tube was incubated at 65 °C for 5 minutes and was immediately transferred onto ice.
3. After incubating the PCR tube on ice for 2 minutes, following components were added into the mix in the indicated order:

Components	**Amount**
5X reaction buffer	4 µL
RiboLock RNase inhibitor	1 µL
dNTPs Mix	2 µL
RevertAid M-MuLV RT	1µL

4. Components were gently mixed and centrifuged, and the following program was run on the thermocycler.

Temperature	Time
25 °C	5 minutes
42 °C	60 minutes
70 °C	5 minutes

5. The cDNA was then diluted as desired and used for performing RT-PCR (Reverse Transcription-PCR) and qPCR.

Working dilution was stored at 4-degree C for short time and at -20-degree C for longer time and neat cDNA was stored at -80-degree C.

2.6 Genomic DNA isolation

Amplification of the promoter elements was done using zebrafish genomic DNA. Reagents required for isolating the genomic DNA were as follows:

1. TEN buffer

Components	Final concentration
1M Tris-HCl (pH 8)	100mM
0.5M EDTA	1mM

2. 10% SDS solution
3. Proteinase K (10mg/ml)
4. Phenol: Chloroform: Isoamyl alcohol (PCI)
5. Chloroform
6. Ethanol
7. Ammonium acetate Buffer
8. TE buffer

Components	Final concentration
1M Tris-HCl (pH 8)	100mM
0.5M EDTA	1mM
5M NaCl	150mM

Steps taken for isolating the genomic DNA were as follows:

1. Zebrafish fin was clipped and suspended in 500 µL of TEN buffer.
2. After adding 1% v/v SDS, the fin was homogenized.

3. For degrading proteins that are present in the cell, proteinase K was added to a final concentration of 200 µg/ml and incubated for 4 hours at 37-degree C.
4. Following proteinase K treatment, an equal volume of PCI was added into it and mixed gently by inverting the MCT upside down.
5. The aqueous phase was transferred into a fresh MCT after centrifuging the above MCT at 6000rpm for 5 min at room temperature.
6. An equal volume of chloroform was mixed into the aqueous phase, which was further centrifuged at 12000 rpm for 5 min at room temperature for collecting the upper phase.
7. The upper phase was transferred into the fresh MCT, and 1/10th volume of ammonium acetate buffer was added into it.
8. Further, 2 volume of isopropanol was added and mixed into it by inverting the MCT gently.
9. With the help of needle or tip, the mesh was taken out in a fresh MCT, and washed with 70% ethanol at 12000 rpm for 10 min.
10. Following 70% ethanol wash, the pellet was air dried and dissolved in TE buffer.

2.7 PCR (Polymerase chain reaction) Amplification.

Following reagents were added to a PCR tube:

Components	**Amount**
20X reaction buffer	0.5µL
2.5mM dNTPs	1µL
2.5nM Forward primer	0.1µL
2.5nM Reverse primers	0.1µL
Template	10ng
Taq polymerase	0.1µL
MQ Water	upto10µL

PCR tube containing these reagents were subjected to the following thermo-cycler cycling conditions:

Steps	Temperature	Time
Initial denaturation	95°C	2min
Cycling	95°C	20sec
(24-35cycles)	58°C (variable)	30sec
	68°C /72°C	1min/kb
Final extension	72°C	5min
Storage	4°C	Infinite hold

Further, the final PCR product was run on agarose gel electrophoresis for amplification.

2.8 Quantitative PCR (qPCR).

The qPCR was performed in ABI QuantStudio4 machine. Following components were added to each PCR plate well:

Components	Amount
KOD SYBR qPCR Master Mix (Pure gene)	2.5µL
10pM Forward primer	0.1µL
10pM Reverse primer	0.1µL
Template	0.25µL (5ng)
MQ Water	Final volume to 5µL

A protocol was run on ABI QuantStudio4 machine, which was followed by analysing the data on excel sheet using the ΔΔCT method.

2.9 Cloning of full length cDNAs into plasmid.

The cDNA which was prepared from the total RNA isolated from 24hpf embryo was used to amplify several cDNAs needed for the study. These cDNAs were cloned in in pET22B+ plasmid for their future use for protein expression or in pCS2+ plasmid by using restriction enzymes for future use for *in vitro* transcription reaction for making RNA probes under T3 promotor which was further to be used in mRNA *in situ* hybridization or under SP6 promotor for *in vitro* mRNA synthesis.

2.10 Plasmid isolation.

1. 1mL of culture was centrifuged at 13,500rpm for 2 min at RT.
2. Supernatant was then discarded and pellet was dissolved by vortexing in 100µL of chilled autoclaved MQ.
3. 100µL of freshly prepared lysis buffer was added and gently tapped.

    1mL of Lysis Buffer was prepared freshly by adding 50µL of 20% SDS solution, 20µL of 0.5M EDTA and 20µL of 10N NaOH in 910µL of MilliQ-water.

4. The samples were boiled at 100°C for 5 minutes (till the solution becomes clear).
5. 50µL of 0.5M MgCl2 was added. Tapped and kept on ice for 1 minute and centrifuged at 13,000 rpm for 2 min at RT.
6. 50µL of 3M Potassium Acetate buffer was added, mixed gently and centrifuged at 10,000rpm for 2 min at RT.

    60mL of 5M Potassium Acetate, 11.5mL glacial acetic acid and 28.5mL H_2O were mixed to get 100ml of Potassium Acetate Buffer. It was Stored in 4°C.

7. Supernatant was transferred into another MCT containing 600µL of Isopropanol and kept on ice for 5 min and then was centrifuged at 13,000rpm for 2min at RT.
8. 70% ethanol wash was given and then the pellet was dried completely.
9. Pellet was dissolved in 30µL of autoclaved MQ.
10. 1uL plasmid was run on agarose gel to check the quality and the concentration was determined by the spectrophotometer.
11. Stored at -20-degree C.

2.11 Ultra-competent cells preparation.

1. 5mL primary culture of *E.coli* DH5α strain was incubated at 37°C overnight
2. Secondary culture (1% of primary culture) was incubated at 18°C till OD_{600} reached the value of 0.6-0.8.
3. Culture was kept on ice for 10-15 minutes and then centrifuged at 2500g for 10 minutes at 4°C.
4. Pellet was re-suspended in 80 mL of TB buffer. [TB Buffer: 10mM of PIPES + 15mM of $CaCl_2.2H_2O$ + 250mM of KCl + 55mM of $MnCl_2.4H_2O$, pH = 6.8 is set using KOH.].

5. Culture was kept on ice for 10 minutes and then centrifuged at 2500g for 10 minutes at 4°C.

6. Pellet re-suspended in 20mL of TB buffer.

7. DMSO is added to a final concentration of 7% (1.4mL+18.6mL of TB buffer).

8. Kept it on ice for 10 minutes.

9. 100µL volumes were aliquoted and stored at -80°C.

2.12 Restriction digestion.

1. Following reagents were added to an MCT:

Components	**Amount**
DNA	5µg
Restriction digestion buffer (10X)	3µL
Restriction digestion Enzyme	1µL
MQ Water	Final volume to 30µL

2. The reaction was briefly centrifuged and incubated at 37 °C for 6-8 hours or overnight depending on the need.

3. The digested product was run on an agarose gel and isolated using a gel extraction kit or by manual gel extraction method.

2.13 The *in vitro* transcription reaction of making RNA probe.

1. Following reagents were added to an MCT:

Components	**Amount**
DNA	0.5-1µg
Transcription buffer (10X)	1µL
DIG/Fluorescein RNA labelling mix	0.5µL
RNA polymerase (SP6/T7/T3)	0.5µL
DEPC Water	up to 10µL

2. The reaction was mixed and briefly centrifuged.

3. The reaction was further incubated at 37 °C for 4 hours.

4. The reaction was stopped by adding 1ul of 0.5M Tris EDTA.

5. Precipitation was performed by adding 1µL of 5M LiCl, 0.5µL of 10mg/ml glycogen and 18µL of 100% ethanol. Further this reaction was mixed and kept overnight at -80 °C.
6. Next day, the reaction mix was centrifuged for 30 minutes at 14,000rpm.
7. Pellet was washed twice with 80% ethanol at 14,000rpm for 15minutes.
8. Further, RNA pellet was air dried and dissolved in 20µL of DEPC water.

2.14 Manual gel extraction.

1. Desired band was cut from agarose gel and collected in a MCT containing small pieces of aluminium foil. A small hole was made at the base of MCT using a 30G needle.
2. This MCT was then placed inside another MCT, both of them taped together.
3. This setup was then centrifuged at 10,000rpm for 10 minutes at RT.
4. Equal volume of lower layer of PCI (Phenol: Chloroform: Isoamyl alcohol) was added into the flow through and mixed properly.
5. Centrifuged at 10,000rpm for 10 minutes at RT.
6. Centrifuged sample was kept on ice for 1 minute to get a differentiable interface and then, the aqueous layer was carefully pipetted out without disturbing the lower phase and collected in another MCT.
7. Equal amount of Chloroform was added and mixed thoroughly.
8. Centrifuged at 10,000rpm for 10 minutes at RT.
9. Upper aqueous layer was carefully taken out and collected in another MCT.
10. Half volume of 7N Ammonium Acetate and twice the volume of Isopropanol was added and mixed properly.
11. The sample was kept at -80°C overnight.
12. Centrifuged at 13,000rpm for 30 minutes at 4°C.
13. 70% ethanol wash (500µL) was given twice in MQ water(in DEPC if to be used for probe or mRNA synthesis) and pellet was dried completely.
14. The pellet was then eluted in MQ (DEPC for probe or mRNA) and checked on 1% agarose gel.
15. Stored at -20°C.

2.15 Morpholino electroporation, mRNA transfection, and knockdown-rescue.

Lissamine-tagged MOs (Gene Tools) of 0.5 µl (0.5 to 1.0 mM) volume were injected at the time of injury by a Hamilton syringe of 5 µl capacity. MO delivery was accomplished through electroporation by placing the positive electrode on the fish's eye that received the MO and then giving 5 electric pulses of 70mV with duration of 500 mSec each.

Morpholino used in this study is *malat1* MO (against *malat1* splice site).

Transfection mixture contained two solutions constituted in equal volumes. (A) 4-5 µg of mRNA mixed with HBSS (Hanks balanced salt solution), (B) Lipofectamine messenger max reagent (Invitrogen, Cat number LMRNA001) mixed with HBSS. Both the solutions were kept at room temperature for 10 minutes and then mixed dropwise followed by 30 minutes incubation at room temperature. The 0.5uL of this resultant solution was then injected in zebrafish's injured eye using a Hamilton syringe.

2.16 Tissue fixation and cryo-sectioning.

1. The zebrafish eyes were dissected in 4% paraformaldehyde solution for removing the lens.
2. Eyes were then fixed overnight at 4 °C in 4% paraformaldehyde solution on a a shaker at slow speed.
3. Fixed eyes were dehydrated by a series of sucrose solutions of increasing concentrations for 45 minutes. Series of sucrose solutions used were as follows:

    5% Sucrose (1000µL)
    5% Sucrose (800µL) + 20% Sucrose (400µL)
    5% Sucrose (500µL) + 20% Sucrose (500µL)
    5% Sucrose (400µL) + 20% Sucrose (800µL)
    20% Sucrose (1000µL)

4. Eyes were further washed with the mixture of 1000µL of 20% Sucrose and 500 µL of OCT compound (Optimum Cutting Temerature compound) (2:1) for 30 minutes.
5. Eyes were then embedded into the blocks of OCT and were frozen and stored in -80 °C.

6. Frozen blocks were taken out from the freezer and using a Lyca cryosectioning machine, 8-12microns retinal sections were taken on poly-lysine coated slides.

2.17 mRNA *in situ* hybridization.

DAY 1

Reagents used:

1. 100% Ethanol
2. 95% Ethanol
3. 70% Ethanol
4. 50% Ethanol
5. 2X SSC solution (made from 20X SSC solution)

   The 20X SSC solution was prepared by dissolving 87.7g of NaCl in 350ml of DEPC water. Further 44.12g of sodium citrate was dissolved in it and volume was made up to 500ml.
6. Proteinase K buffer

   25ml Tris-HCl

   25ml 0.5M EDTA

   Bring the volume up to 250ml with DEPC water
7. Proteinase K enzyme (10mg/ml)
8. TEA Solution

   Add 9.3g Triethanolamine (TEA) to 490ml water. Add 173µL of 10N NaOH solution to bring the pH to 8.0. Finally, bring the volume up to 500ml with DEPC water.
9. TEN solution

   5ml 1.0M Tris-HCl (pH 7.5)

   30ml 5M NaCl

   1ml 0.5M EDTA
10. Hybridization Solution

    3.6ml TEN solution

    25ml 100% Formamide

    10ml 50% Dextran sulphate

    5ml 10% RMB blocker

6.4ml DEPC water

Store at -20 °C

Steps:

1. Slides were dried for 3-4hours at 37-degree C.
2. Slides were hydrated by serial ethanol solution for 1 minute each:

   100% Ethanol

   100% Ethanol

   95% Ethanol

   70% Ethanol

   50% Ethanol
3. Ethanol was washed off by keeping the slides in 2X SSC solution for 1 minute.
4. The slides were then incubated in pre-warmed Proteinase K Solution (10mg/ml Proteinase K) for 6minutes for 10micron sections.
5. Slides were rinsed in DEPC water at room temperature for 1-2 minutes.
6. Slides were kept in 0.1M TEA solution for 3 minutes.
7. Slides were then treated with TEA solution with acetic anhydride for 10 minutes.
8. Slides were dehydrated with a series of 1 minute each SSC and Ethanol washes:

   2X SSC solution

   50% Ethanol

   70% Ethanol

   95% Ethanol

   100% Ethanol

   100% Ethanol
9. Slides were then air dried for at least 1 hour.
10. Hybridization solution was pre-warmed at 56 °C.
11. For preparing the probe mix, the probe was boiled for 10 minutes at 100 °C to open up the secondary structures with 64µL of water. The mixture was immediately transfered onto ice and hybridization solution was added to it (with a total volume of 300µL for each slide).
12. 300µL of probe solution was added to each of the slides and it was coverslipped using Hybrislips.

13. Slides were kept overnight at 56 °C in a humidified chamber, which was damped using 50% Formamide/5X SSC.

DAY 2

Reagents used:

1. 2X SSC solution
2. 50% Formamide/2X SSC solution
3. RNase buffer

RNase Buffer solution was prepared by mixing 5ml of 5M NaCl, 500µL of 1M Tris (pH7.5) and 100µL of 0.5M EDTA in DEPC water to bring the final volume to 50ml.

4. 5X Maleate buffer

Maleate buffer was made by dissolving 58g of Maleic acid in 850ml of MQ water, and then pH of this solution was adjusted to 7.5 using NaOH pellets. Further 43.8g of NaCl was dissolved into the solution and volume was made up to 1L using MQ water.

5. 1X Maleate/0.05% Triton/ 1% RMB blocker solution

This solution was made by mixing 2ml of 5X Maleate stock solution, 5µL of Triton X-100 and 1ml of 10% RMB blocker. 3ml aliquots were made and frozen at -20 °C.

Steps taken:

1. Slides along with cover slips were soaked in 2X SSC solution for 20 min at room temperature on a shaker.
2. Hybrislips were gently removed, and slides were soaked in pre-warmed 50% Formamide/2X SSC solution for 30 min at 65 °C. The Coplin jar was gently agitated at an interval of first 5min twice.
3. Slides were then rinsed twice, using 2X SSC solution at 37 °C for 10 min each.
4. RNase A (100ul of 10mg/ml) was added into RNase buffer and slides were incubated in this solution for 30 min at 37 °C.
5. Slides were washed in RNase buffer for 30 min at 65 °C.
6. Slides were incubated with 1X Maleate/0.05% Triton/ 1% RMB blocker solution for 2-3 hours at RT.
7. Slides were then washed twice with 1X Maleate buffer for 5 minutes each at RT.

8. After washing, slides were incubated overnight with anti-DIG/anti-FL antibody having 1:2500 dilution in 1X Maleate/0.05% Triton/1% RMB blocker solution.

DAY 3:

Reagents used:

1. 1X Maleate buffer
2. Genius buffer

   The genius buffer was prepared by dissolving 5mL of 1M Tris-HCl (pH9.5), 1mL of 5M NaCl and 5mL of MgCl2 in MQ water to make the volume up to 50mL.
3. NBT/BCIP solution, which was mixed with Genius buffer at 1:50 dilution.

Steps taken:

1. Slides were washed twice with 1x Maleate buffer for 5 min each.
2. Slides were then washed twice with Genius buffer for 5 min each.
3. Slides were incubated with NBT/BCIP solution in the dark for colour reaction until the signals developed.
4. Signals were detected under the microscope.

Fluorescence mRNA *in situ* hybridization was also performed in the same manner as above; except, on the 2nd day, a tyramide reaction was performed instead of NBT/BCIP color reaction.

2.18 Immuno-fluorescence study.

DAY 1

Reagents used:

1. 1X PBS (diluted for 10X PBS)
2. 4% Paraformaldehyde (Sigma) solution (made in 1X Phosphate buffer)
3. 2N HCl
4. 0.1M Sodium borate solution (pH8.5)
5. 3% Bovine serum albumin (BSA) in 1X PBST (PBS with 0.1% Triton X 100)
6. 1% BSA in 1X PBST (1X PBS with 0.1% Triton X 100)
7. A primary antibody of choice

Steps taken:

1. Slides were taken from-20-degree C and dried at 37-degree C for 30 minutes.
2. Slides were washed thrice using 1X PBS, 10 min each.
3. Slides were treated with 2N HCl for epitope retrival (pre-heated at 37 °C) for 20 minutes.
4. HCl solution was neutralized using two 0.1M Sodium borate wash for 10 minutes each.
5. Blocking of the sections was carried out by incubating the slides with 3% BSA-PBST solution for 1-2 hours at room temperature.
6. Following blockade, slides were overlaid with primary antibody (antibody dilution was 1:500, i.e., 1 µL of antibody in 500 µL of 1% BSA-PBST solution), for overnight at 4 °C.

DAY 2

Reagents used:

1. 1X PBST
2. Secondary antibody
3. DABCO

Steps taken:

1. The primary antibody was recovered from the slides and the slides were washed thrice with 1X PBST solution for 10 min each.
2. Slides were overlaid with 500uL of the secondary antibody of choice (secondary antibody dilution was 1:1000) for 3 hours at room temperature.
3. Following incubation, the slides were washed three times with 1X PBST for 10 min each and signals were checked under the nikon fluorescence microscope..
4. The slides were washed with MQ water for 5 min and then dried in dark for 20-30 minutes.
5. Further the slides were coversliped with 70uL DABCO and stored at -20 °C.

2.19 Western blotting.

Sample preparation:

1. Retina was dissected out and immersed in 2X Laemmli buffer.

Components	Amount
10% SDS	4ml
Glycerol	2ml
1M Tris-HCl (pH6.8)	1.2ml
Bromophenol blue	0.002gm
MQ water	up to 10ml

2. Samples were then homogenized using a piston.
3. Following homogenizing samples were vortexed briefly for 10-15 seconds and kept on ice for 2-3 minutes intermittently alternatively for 20 min.
4. Samples were boiled at 100 °C for 10 min and stored at -80 °C.

Reagents and tools required:

1. Western blotting gel apparatus
2. Resolving buffer (4X)

   For preparing this, 18.7g of Tris base was dissolved in 85ml of water, and pH was adjusted to 8.8 with HCl. The final volume was made up to 100ml using MQ water.

3.`The composition of the 12% resolving gel

Components	Amount
Resolving buffer	2.5ml
30% Acrylamide	4ml
MQ water	3.3ml
10% SDS	100µL
Ammonium persulfate	100µL
TEMED	6µL

4. Stacking Buffer (1X)

   For preparing this, 12.08g of Tris base was dissolved in 85ml of water, and pH was adjusted to 6.8 with HCl. The final volume was made up to 100ml using MQ water.

5. The composition of stacking gel

Components	Amount
Stacking buffer	625uL
30% Acrylamide	667uL
MQ water	3603uL
10% SDS	50µL
Ammonium persulfate	50µL
TEMED	5µL

6. Composition of running buffer (10X) (pH 8.3)

Components	Amount
Tris base	30g
Glycine	144g
SDS	10g
MQ water	up to 1000ml

7. PVDF membrane
8. Skimmed milk
9. PBST (PBS with 0.1% TWEEN20)

Steps taken:

1. The resolving gel was made between the glass plates of the assembly and 50% isopropanol was overlaid to avoid bubbles.
2. Once resolving gel was solidified, the gel was washed with MQ water to remove isopropanol, and stacking gel was made over it and a gel comb was placed for forming the wells.
3. Once stacking gel got solidified, the comb was removed, and wells were washed using MQ water. The gel assembly was then placed in a tank carrying 1X running buffer.
4. Samples were loaded into the wells, and SDS gel electrophoresis was performed for 3 hours at 91V.
5. Protein was transferred from the SDS-Gels to PVDF membranes in the presence of transfer buffer for 1 hour.
6. Once the protein is fully transferred, blots were blocked with 5% skimmed milk-PBST for 1 hour.

7. After blocking, the blots were washed three times using 0.05%PBST for 10 min each.
8. Blots were incubated with primary antibody at 4 °C overnight or for 2-3 hours at RT.
9. The blots were then washed thrice with 0.05%PBST for 10 min each.
10. Blots were incubated with secondary antibody for 1-2 hour at room temperature.
11. After antibody incubation, blots were washed thrice with 0.3%PBST for 10 min each.
12. Blots were developed using ImageQuant LAS4000.

2.20 Microscopy, cell counting, and statistical analysis.

Nikon N*i*-E fluorescence microscope assembled with fluorescence optics and Nikon A1 confocal imaging system was used for all the retinal imaging purposes. The $PCNA^+$ and $BrdU^+$ cells were visualized and counted by directly looking at the fluorescence present in the retinal sections. The ISH^+ cells were visualized, imaged and counted using the same microscope by looking though the bright field. Every section of the retinal tissue was visualized and counted for fluorescence and ISH signals, and more than three retinae were used for each experiment. The statistical analysis of the data for all the experiments was done using a two-tailed unpaired students' *t*-test. Comparison based studies were analysed using analysis of variance test (ANOVA), and further, a Bonferroni/Dunn *post hoc t-test* was performed using Stat View software. Error bars in all the histograms represent the standard deviation in between the different datasets.

******

Section 3

Results

3.1 *mmp14a* and *furina* are induced post retinal injury

As discussed earlier about the major role of MMP-14 in extra-cellular matrix (ECM) remodelling, alongside the fact that matrix remodelling plays a crucial role in the process of regeneration motivated us to check the role of *matrix metalloprotease 14 (mmp14)* during retina regeneration in zebrafish.

In zebrafish, two isoforms of *mmp14* exist; namely *mmp14a* and *mmp14b*. The two isoforms have been shown to have a significant similarity of 63% in their protein sequences (Figure 3.1a), with the latter one having an additional stretch of Arg and Glu towards protein's C-terminal. We were encouraged to check for the temporal expression of *mmp14a* and *mmp14b* transcripts, along with the levels of Mmp14 activating PC, Furin isoforms: *furina* and *furinb*.

The fish retinae were subjected to mechanical injury by a 30 gauge needle and were then harvested at various time points for RNA isolation during the phases of dedifferentiation (15hpi or hours post injury), proliferation (4dpi or days post injury) and differentiation (8dpi or days post injury) along with Uninjured Control (UC) state. First strand cDNA was synthesised from isolated mRNA and subsequently Reverse Transcription Polymerase Chain Reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) were used to determine the transcript levels by the help of gene specific primers. Both the *mmp14* isoforms *mmp14a* and *mmp14b* showed upregulated expression upon injury with their expression peaking at 15hpi and 2dpi (Figure 3.1b, 3.1c), the phase where majority of Muller Glia (MG) dedifferentiate. The upregulation in the early phase of injury might imply that *mmp14* could be involved in the remodelling of the ECM by degradation of various ECM components. Further, the transcript levels of the its activator PC isoform *furina* were

increasing and overlapping with the expression pattern of *mmp14a* and *mmp14b*, with the levels of *furinb* being unaltered upon retinal injury suggesting it might not be involved in the process of retina regeneration (Figure 3.1b, 3.1c).

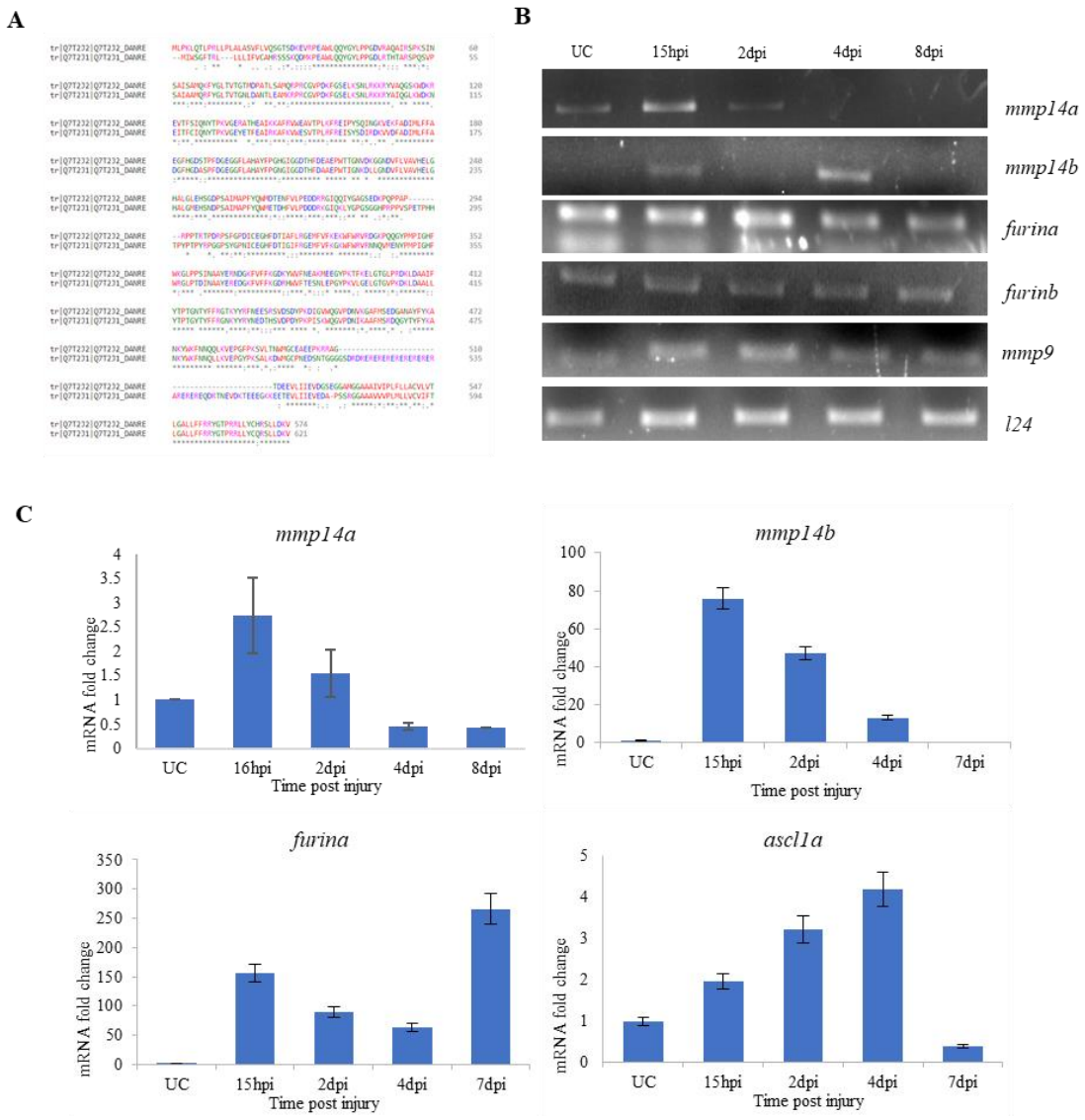


Figure 3.1: Upregulation of *mmp14* and *furina* after retinal injury. (a) Clustal Omega alignment of *mmp14a* and *mmp14b* bearing 63% amino acid similarity. (b) RT-PCR of mRNA levels and (c) qRT-PCR of mRNA fold change of *mmp14* and *furin* at various time points post injury showing upregulation of *mmp14* and *furina* at 15hpi and 2dpi. (hpi: hours post injury, dpi: days post injury, *l24* is internal control, *mmp9* and *ascl1a* are positive controls).

Further, to check for the localization of cells expressing *mmp14a* and *furina*, mRNA *in-situ* hybridization (ISH) was performed on retinal sections at 4dpi time point. Before harvesting eyes for Paraformaldehyde (PFA) fixation, fishes were also subjected to Bromodeoxyuridine (BrdU) pulsing for 4 hours to mark all the proliferating cells during

that time period in the tissue. BrdU being the analogue of thymidine, is incorporated in the DNA of cells in the S phase of cell cycle, marking the proliferating cell during the pulsing. ISH showed that *mmp14a* and *furina* are expressed across retinal layers near the injury point at 4dpi, the proliferation phase during regeneration. Immuno-fluorescence was performed on the ISH sections to mark association/localization of *mmp14a*$^{+}$ and *furina*$^{+}$ cells with respect to the actively proliferating BrdU^{+} cells. Both, *mmp14a* and *furina* were found to be excluded from the BrdU labelled MGPCs. Interestingly, both the transcripts were expressed by the cells present next to MGPCs (Figure 3.2) suggesting their role in regulating the micro-environment around proliferating MGPCs.

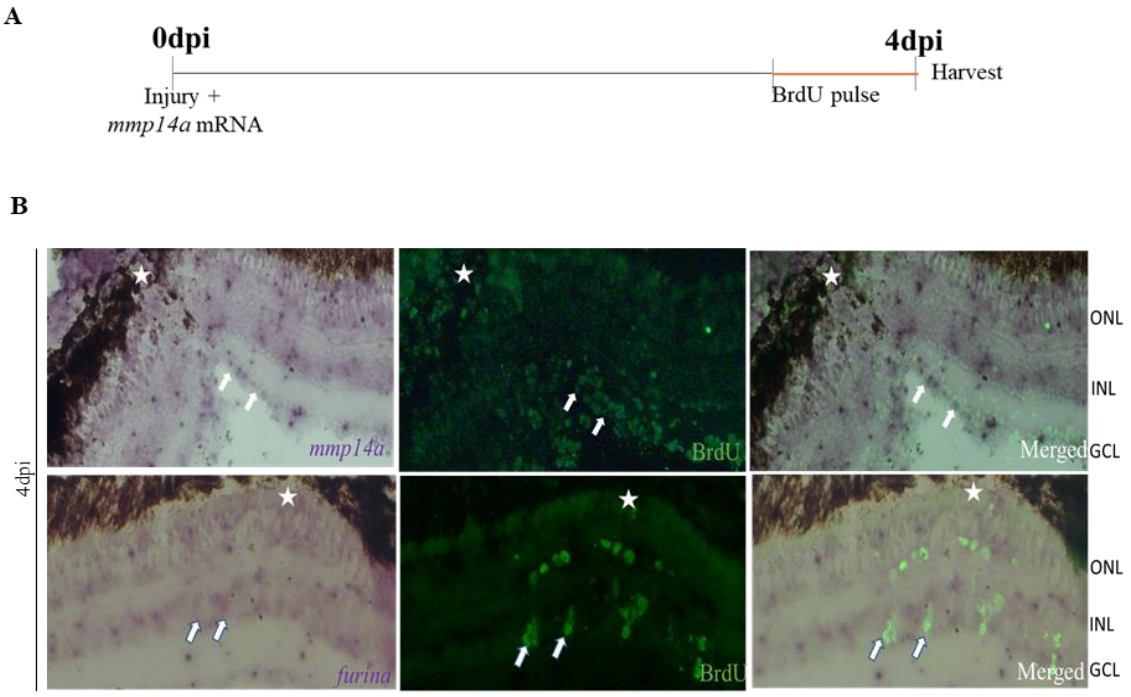


Figure 3.2: Expression of *mmp14* and *furina* upon retinal injury. (a) An experimental timeline that describes injury, BrdU pulsing and retina harvest at 4dpi. (b) ISH along with BrdU Immuno Fluorescence shows the expression of *mmp14a* and *furina* in injured retina at 4dpi. *Mmp14a* and *furina* are excluded from actively proliferating MGPCs (BrdU^{+}).
(BrdU: Bromodeoxyuridine; ONL: Outer Nuclear Layer, INL: Inner Nuclear Layer, GCL: Ganglion Cell Layer).

3.2 *mmp14* and *furina* levels are regulated by various pathways

Further, we looked for *mmp14a* and *furina* levels by inhibiting or activating various pathways/ effectors to understand the effect of pathways which are established to be crucial in retina regeneration.

3.2.1 Tgf-beta inhibition induces transcription of *mmp14a* and *furina*

Studies show that Transforming growth factor - beta (Tgf-beta) is sequestered in the ECM, and activation of Tgf-beta signalling requires degradation of matrix for the release of Tgf-beta. This necessitates the activation of Mmp-14/2/9. Tgf-beta is a prominent signalling involved in the retina regeneration. Upon SB431542 mediated Tgf-beta signalling inhibition we observe a decline in actively proliferating MGPCs (Sharma P *et al.,* 2020). To check for its effect on *mmp14a* and *furina* levels, we injected SB431542 at various concentrations in zebrafish eye upon injury and the retinae were harvested at 2dpi, the phase of *mmp14a* and *furina* maximum expression, for mRNA isolation followed by RT-PCR. *mmp14a* transcript levels were found to be increasing on inhibiting TGF beta signalling, in a concentration dependent manner. *furina* levels also showed a significant increase as compared to 2dpi Injured control (IC) (Figure 3.3a).

3.2.2 Gelatinases inhibition induces *mmp14a/b*

Gelatinases (Mmp2/9) have been shown to be necessary for regeneration of zebrafish retina (Kaur S et al., 2018). We wanted to check if the Mmp14 levels are upregulated upon SB3CT mediated Mmp2/Mmp9 inhibition. At 15hpi, we saw a dose dependent increase in the levels of both, *mmp14a* and *mmp14b,* as was anticipated (Figure 3.3b). This signifies the importance of *mmp14* mediated activation of gelatinases, which are further required for the activation of Tgf-beta along with Mmp14.

3.2.3 *malat*-1 knockdown results in decreased *mmp14a*

metastasis associated lung adenocarcenoma transcript – 1 (*malat-1*) is a major lncRNA which has been shown to be necessary for growth of tumour cells across various cancers. To check for the effect of *malat-1* on *mmp14*, we injected lissamine tagged *malat-1* morpholino (MO) at the time of injury to the retina, followed by electroporation to ensure

the entry of MO into the retina. Upon qRT-PCR we found a drastic decrease in levels of *mmp14a* on knocking down malat1 in a concentration dependent manner (Figure 3.3c).

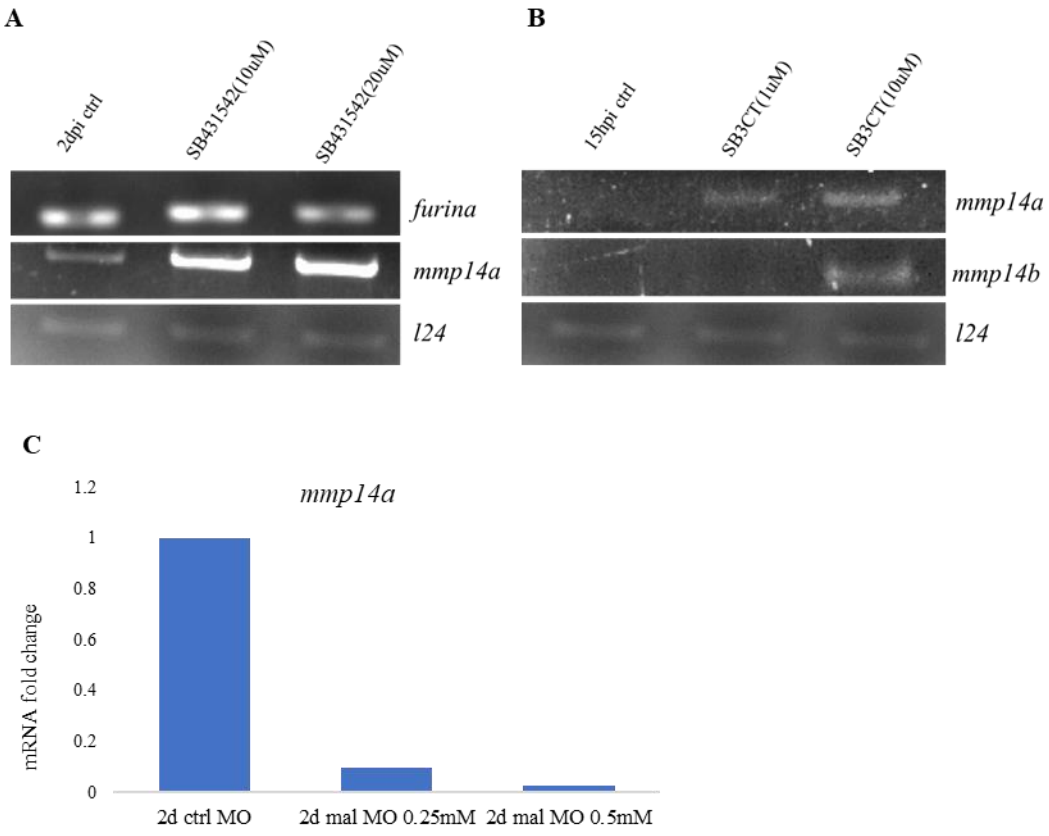


Figure 3.3: Regulation of *mmp14* and *furina*. RT-PCR shows (a) upregulation of *furina* and *mmp14a* levels upon SB431542 mediated Tgf-beta inhibition. (b) upregulation of *mmp14a* and *mmp14b* upon Mmp2/Mmp9 inhibition caused by SB3CT. (c)) shows a concentration dependent downregulation of *mmp14a* in 2dpi retinae electroporated with increasing concentration of *malat-1* targeting MO

3.3 *mmp14a* is required for MGPCs proliferation

We wanted to check for the role of *mmp14a* on the actively proliferating MGPCs. To see the effect of *mmp14a* overexpression, we cloned and in-vitro transcribed *mmp14a* mRNA, which was then along with eGFP mRNA, acting as a control, was injected in the injured retina by the help of Hamilton syringe. The fishes were then subjected to 4 hours BrdU pulsing before harvesting. Immunofluorescence performed at 4dpi shows that *mmp14a* overexpression resulted in decreased number of actively proliferating $BrdU^{+}$ cells and

PCNA^{+} cells with respect to 4dpi control (Figure 3.4). We would check for involvement of various genes and proteins resulting in the phenotype observed.

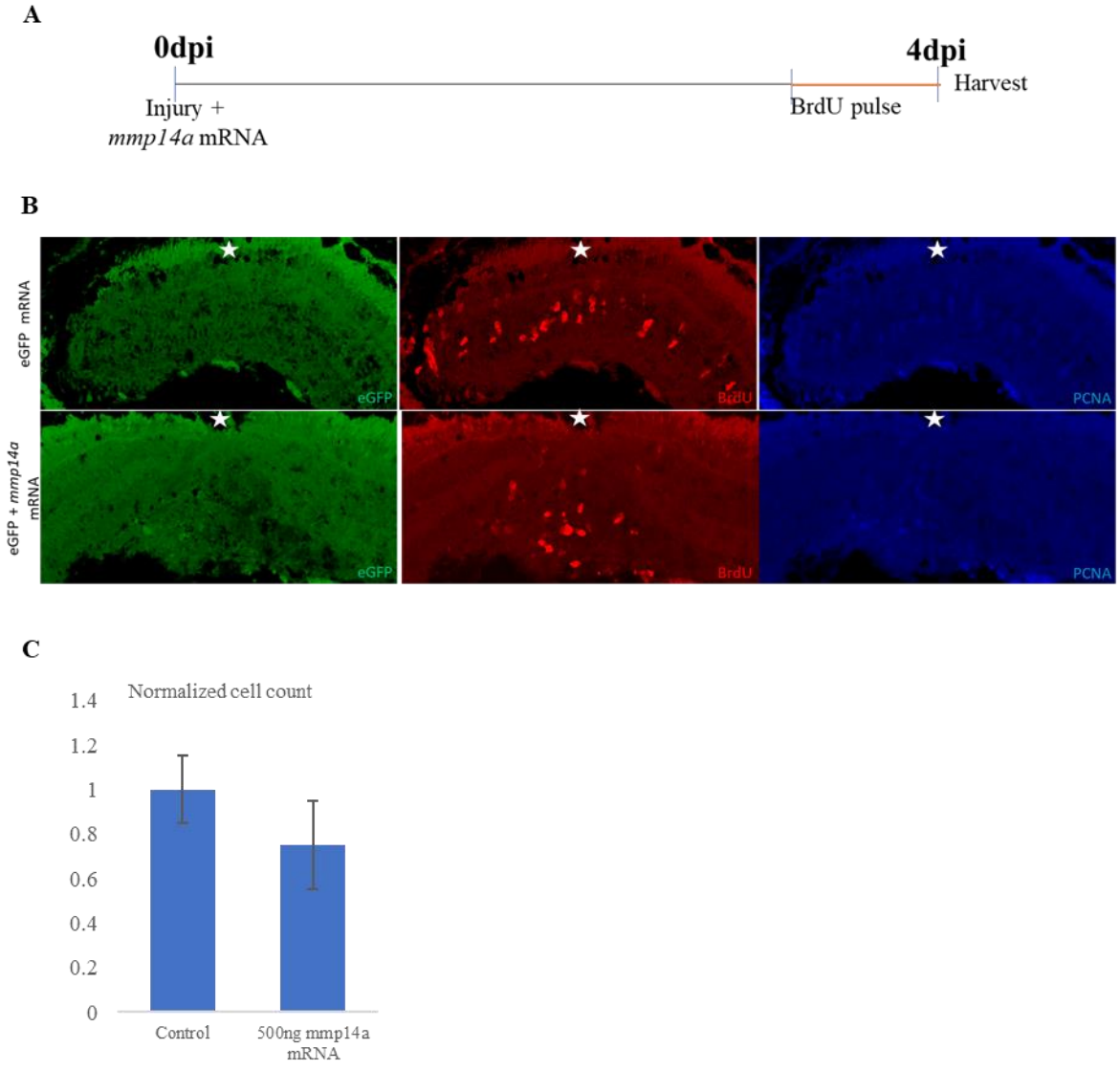


Figure 3.4: mmp14a overexpression reduces MGPCs proliferation. (a) (a) An experimental timeline that describes injury, *mmp14a* mRNA transfection, BrdU pulsing and retina harvest at 4dpi.. (b) BrdU and PCNA ImmunoFluorescenec shows a decrease in the number of actively proliferating MGPCs (BrdU^{+}) and decrease in proliferation (PCNA^{+}) at 4dpi as an effect of *mmp14a* overexpression. (c) quantification by cell count of the same.

3.4 Furin is necessary for MGPCs proliferation

Further, we sought to check for the role of Furin Mmp14 axis in the context of retina regeneration. We blocked Furin, which mediates its effect by activating Mmp14 and various other pathways, by Furin Inhibitor II (FI) drug which inhibits the activity of Furin and other PCs. To ensure inhibition of Furin, the concentrations taken were significantly lower than the inhibition constant (Ki) for Furin. Furin Inhibitor drug was delivered to the retina, at the time of injury using Hamilton syringe. For immunofluorescence experiments, fishes were later subjected to 4 hours BrdU pulsing at 4dpi prior to harvesting the eye. IF performed at 4dpi revealed that FI mediated inhibition significantly decreased number of actively proliferating $BrdU^{+}$ and Proliferating Cell Nuclear Antigen (PCNA) labelled MGPCs on inhibiting Furin activity, in a concentration dependent manner (Figure 3.5).

Additionally, we wanted to observe the involvement and regulation of various genes, which maybe mediating the phenotype obtained at 4dpi on inhibiting furin. mRNA extraction and RT-PCR showed a decrease in levels of *zic2b*, a positive regulator of proliferation. Surprisingly, we obtained an increase in the levels of *mmp9* and *ascl1a*, genes known to be expressed during retina regeneration (Figure 3.6). Since, *ascl1a* and *mmp9* are necessary but not sufficient, we assume that inhibition of Furin resulted in a decreased activity of Mmp14, due to which the ECM remodelling failed. Checking various regeneration associated genes and pluripotency factors would help us better understand the cause for the phenotype obtained.

FI mediated inhibition showed an unexpected increase in the levels of *mmp14a* and *furina* transcripts which might be a way the system is trying to compensate for decrease in active mmp14 levels, by increasing the transcription of mmp14 and its activator.

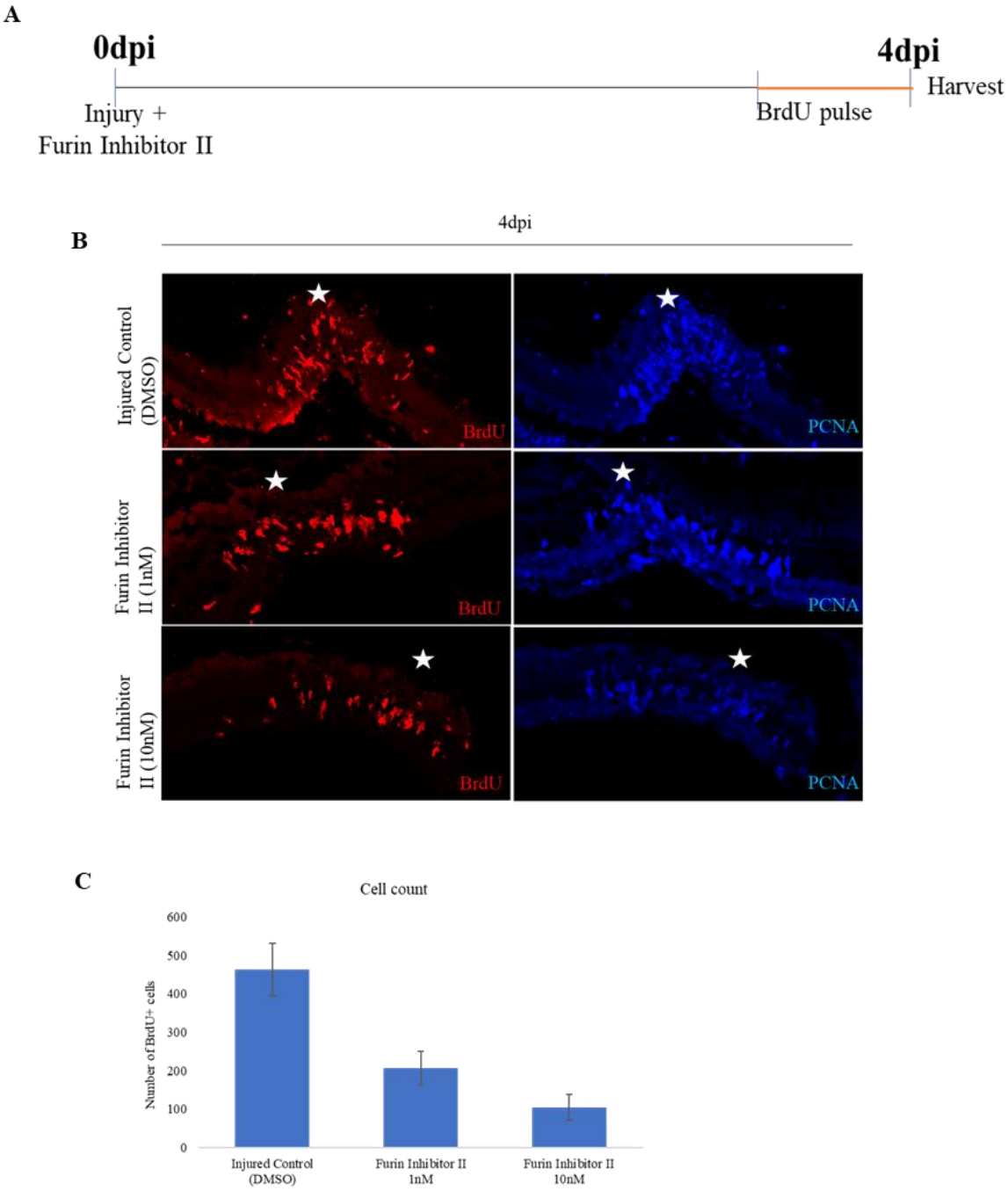


Figure 3.5: Inhibition of Furin activity causes reduction in MGPCs proliferation. (a) An experimental timeline that describes injury, Furin Inhibitor II treatment, BrdU pulsing and retina harvest at 4dpi. (b) BrdU and PCNA ImmunoFluorescenec shows a concentration dependent decrease in the number of actively proliferating MGPCs ($BrdU^{+}$) and decrease in proliferation ($PCNA^{+}$) at 4dpi as an effect of Furin Inhibitor II. (c) quantification by cell count of the same.

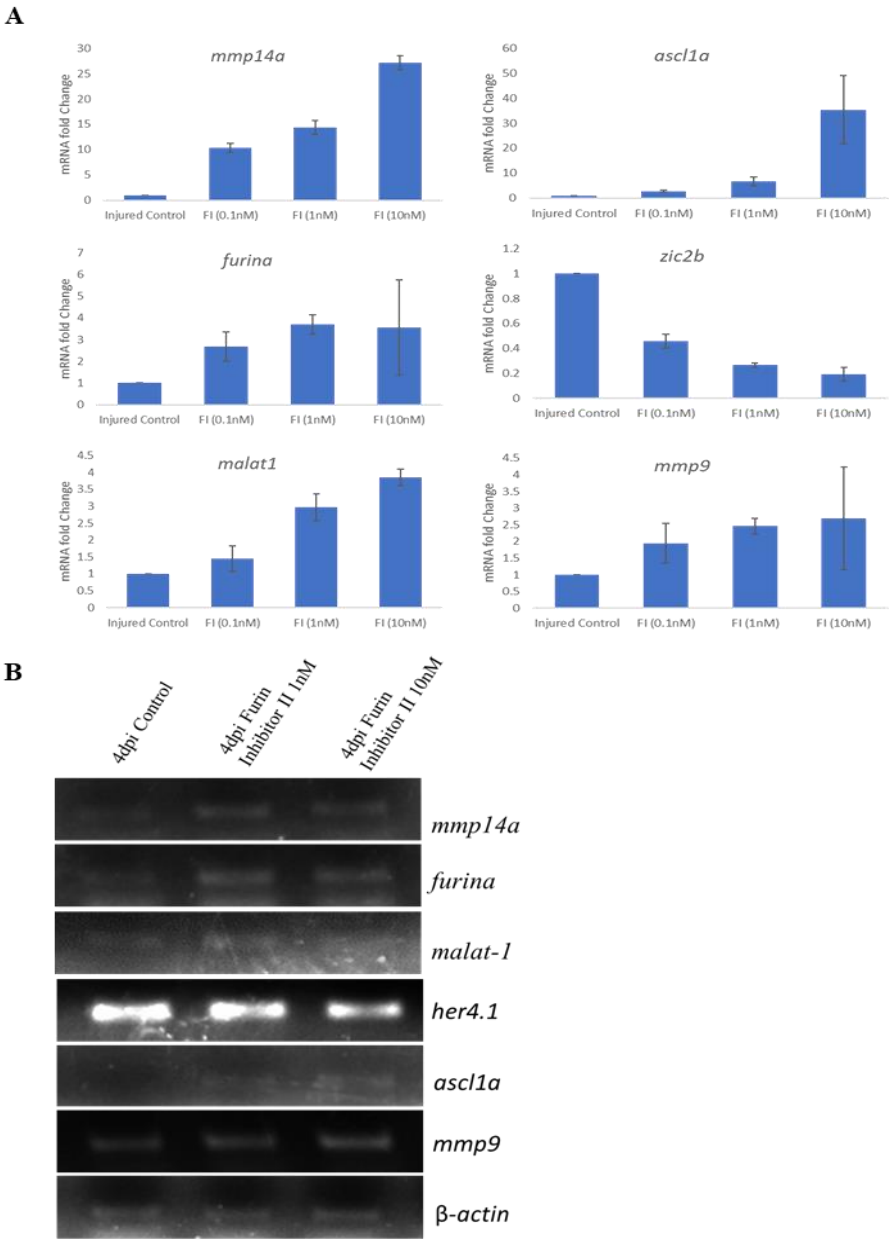


Figure 3.6: Inhibition by Furin Inhibitor II results in regulation of regeneration associated genes. (a) qRT-PCR and (b) RT-PCR shows the regulation of regeneration associated genes *her4.1*, *ascl1a*, *mmp9*, and *zic2b* along with *furina* and *mmp14a* levels as an effect of compromised activity of Furin by Furin Inhibitor II.

3.5 Furin is required for maintaining MGPCs proliferation

Further, we wanted to check for the role of Furin in cell cycle exit or maintaining MGPCs proliferation at 8dpi. Furin activity was blocked by Furin Inhibitor II drug from 4dpi to 8dpi phase by delivering the drug through cornea at 4dpi. Actively proliferating cells were marked at 4dpi and 8dpi by BrdU and Ethylene diUridine (EdU), respectively. Immunofluorescence on retinal sections at 8dpi revealed that number of actively

proliferating EdU$^+$ cells decreased upon late inhibition of Furin. Also, inhibition resulted in decreased BrdU$^+$ EdU$^+$ cells representing the cell population that is maintained in the proliferation phase. This suggests that Furin might be involved in inducing proliferation of MGPCs and maintaining it.

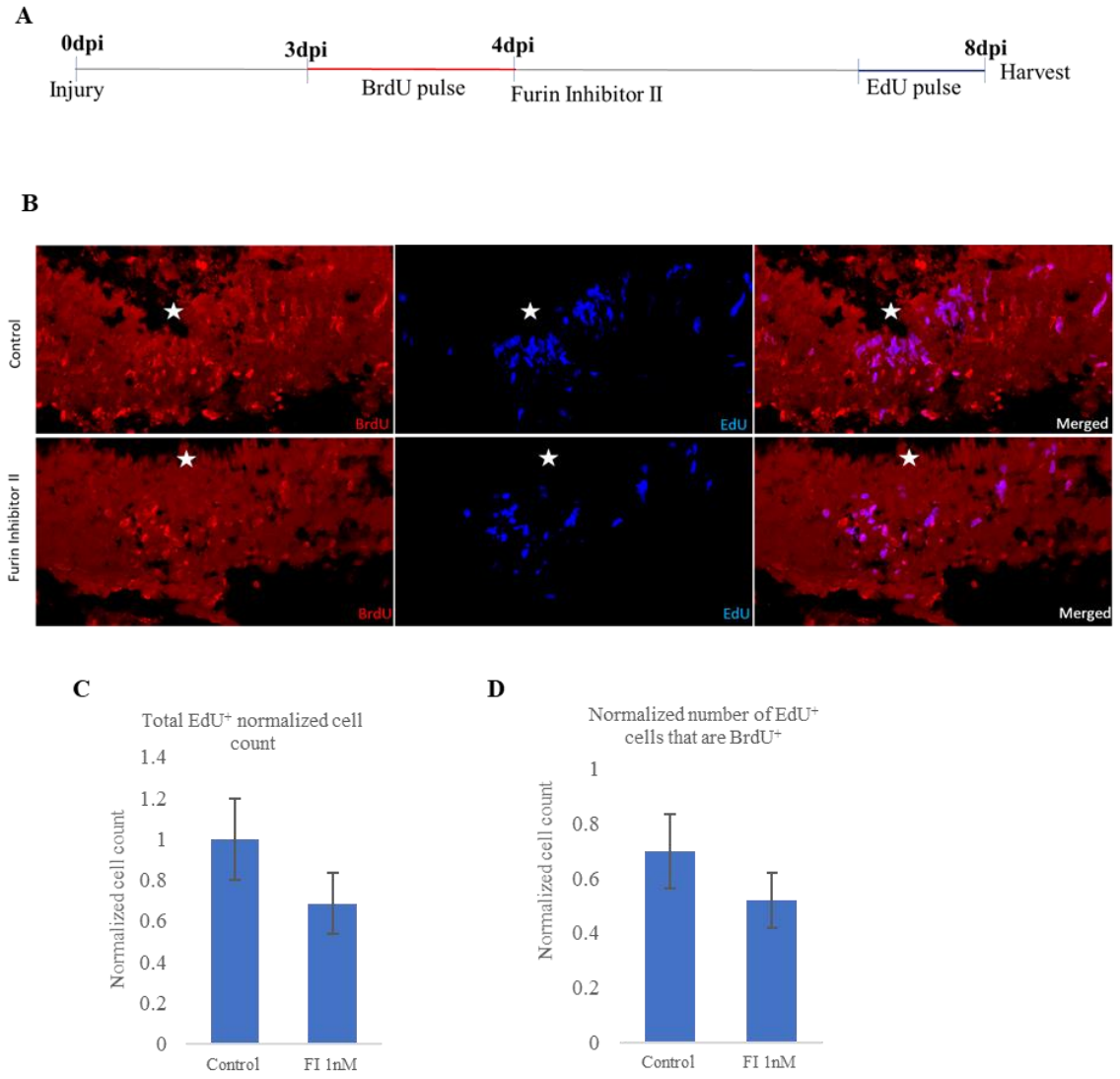


Figure 3.7: Late inhibition of Furin activity reduces MGPCs proliferation at 8dpi. (a) An experimental timeline that describes injury, BrdU pulsing at 3dpi and 4dpi followed by Furin Inhibitor II treatment at 4dpi, EdU treatment and retina harvest at 8dpi. (b) BrdU and EdU Immuno Fluorescenec shows a decrease in the number of actively proliferating MGPCs (EdU$^+$) at 8dpi as an effect of Furin Inhibitor II. It also shows a decrease in the EdU cells that are also (BrdU$^+$). (c) quantification by normalized cell count of EdU$^+$ cells. (d) quantification by normalized cell count of EdU$^+$ BrdU$^+$ cells.

******

Section 4
Discussions & Conclusions

4.1 Discussions

Over the years, the field of zebrafish retina regeneration has progressed and studies have shown the involvement of various transcription factors (TFs) and signalling pathways/molecules during different phases of regeneration: de-differentiation, proliferation and differentiation. Despite having knowledge on the role of TFs like Ascl1a and Insm1a, in regulating de-differentiation and proliferation of MGPCs, very less is known about the involvement of extra-cellular matrix (ECM) remodelers such as MMPs.

Apart from genetic regulations, reprograming of Muller glial cells to MGPCs also requires ECM degradation, failing to which the retina doesn't regenerate. Degradation of ECM is majorly facilitated by matrix metalloproteases (MMPs). Role and advantages of spatial localization of membrane inserted MMP-14 still needs to be explored.

The present study shows that Mmp14 and Furin are upregulated during the initial days following retinal injury. This upregulation corresponds to the peak of dedifferentiation and initial phase of proliferation of reprogramed MGPCs. Process of proliferation requires loosened ECM with degraded components which we suggest is an effect of Mmps activity. *mmp14a* and *furina* are localized in the cells next to the MGPCs, suggesting the two to be expressed in the same cell and Furin activity results in activation of Mmp-14.

Also, the involvement and regulation of *mmp14a* and *furina* upon inhibiting Tgf-beta, or gelatinases shows that the genes might be involved in regulating these pathways or proteins during regeneration. Upon *malat-1* knockdown, the decrease in *mmp14* levels suggest that either *malat-1* might be playing a role in transcription promotion of *mmp14a* or acting as a ceRNA for *mmp14a* targeting miRNA.

We have seen that *mmp14a* overexpression results in decrease in the number of proliferating MGPCs. It might be either dissolving the whole ECM by degrading all the components resulting in a non-optimized environment for proliferation. Also, mRNA transfection might be resulting in substrate (Mmp-14) dependent enzyme (Furin) inhibition. Hence, we need to further check for the activity of Furin and levels of effector molecules.

Further, the study shows that Furin plays an important role during regeneration as disruption of its activity by Furin Inhibitor II results in the decreased proliferation. This might be an effect meditated by inactive Mmp14, or a commulative effect of Furin activity inhibition resulting in less degradation of ECM. Further, transcription levels of various proproliferative genes such as *ascl1a, mmp9* shows an increment, unlike what was anticipated. This data suggests a need for the analysis of levels of various pluripotency factors and protein levels of RAGs. At 8dpi, it has been observed that Furin is required for maintaining the cells in proliferation phase, as upon Furin inhibition, cells are seen to be exiting cell cycle. We can further check for the effect of Furin Inhibitor II on regulating cell cycle exit. This suggests that we can also check for the proteins involved in the process of cell cycle exit.

4.2 Conclusions

Our data, taken together sheds some light on the role of Mmp-14 and Furin during zebrafish retina regeneration. The genes are upregulated during process of regeneration with the expression peaking at 15hpi and 2dpi suggesting involvement in dedifferentiation and proliferation. Further, the regulation of *mmp14a/b* and *furina* levels upon SB431542 or SB3CT mediated inhibition suggests that roles of genes might be fundamental during regeneration and hence the system tries to alter its levels to compensate for the inhibition. The decrease in proliferation upon Furin inhibition shows the necessary role of Furin acting as proprotein convertase and as a result alters the levels of regeneration associated genes and *mmp14a* and *furina*.

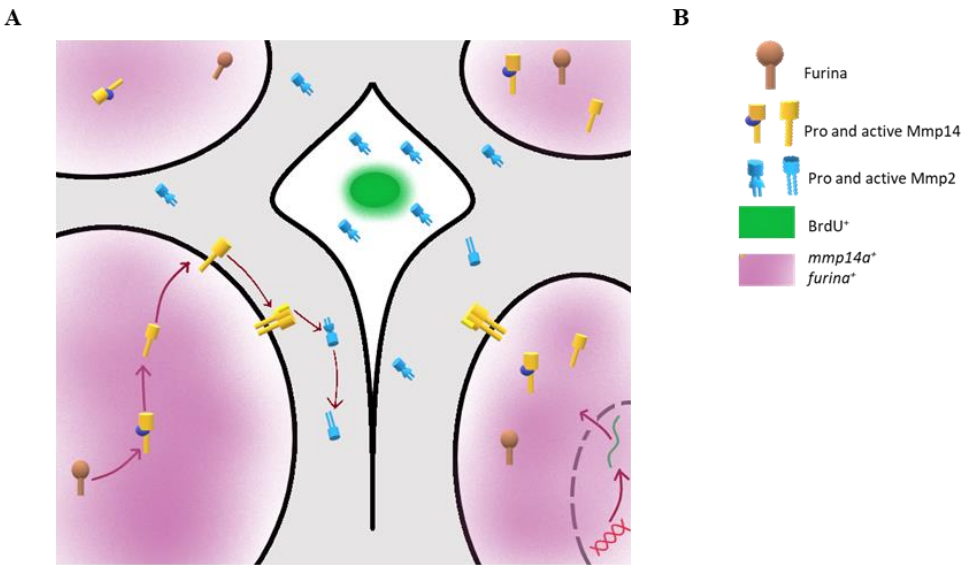


Figure 4.1: Schematic showing hypothesis of pro-Mmp-2 activation by Mmp-14. (a) Schematic showing activation of proliferating MGPCs secreted pro-Mmp-2 by Mmp-14 synthesized and matured in neighbouring cells. (b) Illustrations used for (a).

We suggest that Mmp-14 and Furin might be expressed in the cells neighbouring MGPCs and the spatially localised active Mmp-14 inserted into the membrane could be involved in activation of pro-Mmp2 secreted by proliferating MGPCs to act back on the MGPCs

(Figure 4.1a). Also, Mmp-14 present on the membrane would be degrading the ECM and resulting in setting free the signalling molecules such as Tgf-beta, essential for regeneration. Hence, upon inhibition of the two pathways, the feedback loop might be acting to increase levels of Mmp14 and Furin to facilitate cleavage of pro-Mmp2 and ECM components (Figure 4.2). Further, we suggest that Furin might be involved in maintaining the proliferation during late phases upon injury.

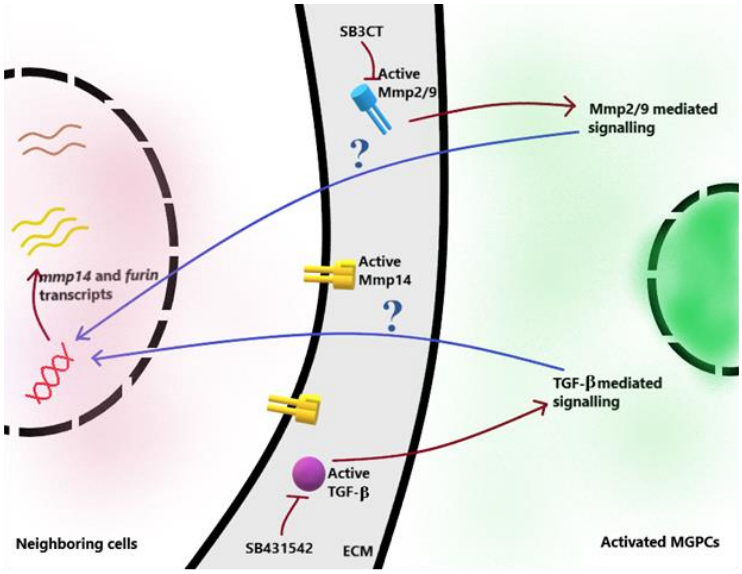


Figure 4.2: Schematic showing hypothesis of *mmp-14* and *furin* regulation. Schematic showing possible molecular mechanisms for regulation of *mmp14* and *furin* by Tgf-beta signaling and Mmp-2/9. (Illustrations from Figure 4.1 (b)).

******

Section 5

Bibliography

Books

1. Principles of Regeneration, Richard J. Goss, 1969, Academic Press.
2. Principles of Regenerative Biology, Bruce M. Carlson, 2007, Academic Press.

Articles

1. Allan, J. A., Docherty, A. J., Barker, P. J., Huskisson, N. S., Reynolds, J. J., & Murphy, G. (1995). Binding of gelatinases A and B to type-I collagen and other matrix components. *The Biochemical journal*, *309 (Pt 1)*(Pt 1), 299–306.
2. Al-Raawi, D., Abu-El-Zahab, H., El-Shinawi, M., & Mohamed, M. M. (2011). Membrane type-1 matrix metalloproteinase (MT1-MMP) correlates with the expression and activation of matrix metalloproteinase-2 (MMP-2) in inflammatory breast cancer. *International journal of clinical and experimental medicine*, *4*(4), 265–275.
3. Amodio, N., Raimondi, L., Juli, G., Stamato, M. A., Caracciolo, D., Tagliaferri, P., & Tassone, P. (2018). MALAT1: a druggable long non-coding RNA for targeted anti-cancer approaches. *Journal of hematology & oncology*, *11*(1), 63.
4. Anderson, E. D., VanSlyke, J. K., Thulin, C. D., Jean, F., & Thomas, G. (1997). Activation of the furin endoprotease is a multiple-step process: requirements for acidification and internal propeptide cleavage. *The EMBO journal*, *16*(7), 1508–1518.
5. Annes, J. P., Munger, J. S., & Rifkin, D. B. (2003). Making sense of latent TGFbeta activation. *Journal of cell science*, *116*(Pt 2), 217–224.

6. Ayoubi, T. A., Creemers, J. W., Roebroek, A. J., & Van de Ven, W. J. (1994). Expression of the dibasic proprotein processing enzyme furin is directed by multiple promoters. *The Journal of biological chemistry*, *269*(12), 9298–9303.
7. Bassi, D. E., Fu, J., Lopez de Cicco, R., & Klein-Szanto, A. J. (2005). Proprotein convertases: "master switches" in the regulation of tumor growth and progression. *Molecular carcinogenesis*, *44*(3), 151–161.
8. Becker, T., Wullimann, M. F., Becker, C. G., Bernhardt, R. R., & Schachner, M. (1997). Axonal regrowth after spinal cord transection in adult zebrafish. *The Journal of comparative neurology*, *377*(4), 577–595.
9. Bernardos, R. L., Barthel, L. K., Meyers, J. R., & Raymond, P. A. (2007). Late-stage neuronal progenitors in the retina are radial Müller glia that function as retinal stem cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *27*(26), 7028–7040.
10. Bierie B, Moses HL. Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. Nat Rev Cancer. 2006 Jul;6(7):506-20. doi: 10.1038/nrc1926. PMID: 16794634.
11. Birbrair, A., Zhang, T., Wang, Z. M., Messi, M. L., Enikolopov, G. N., Mintz, A., & Delbono, O. (2013). Role of pericytes in skeletal muscle regeneration and fat accumulation. *Stem cells and development*, *22*(16), 2298–2314.
12. Braun, E., & Sauter, D. (2019). Furin-mediated protein processing in infectious diseases and cancer. *Clinical & translational immunology*, *8*(8), e1073.
13. Braun, E., & Sauter, D. (2019). Furin-mediated protein processing in infectious diseases and cancer. *Clinical & translational immunology*, *8*(8), e1073.
14. Brinckerhoff, C. E., & Matrisian, L. M. (2002). Matrix metalloproteinases: a tail of a frog that became a prince. *Nature reviews. Molecular cell biology*, *3*(3), 207–214.
15. Brkic, M., Balusu, S., Libert, C., & Vandenbroucke, R. E. (2015). Friends or Foes: Matrix Metalloproteinases and Their Multifaceted Roles in Neurodegenerative Diseases. *Mediators of inflammation*, *2015*, 620581.
16. Burkhardt-Holm, P., Oulmi, Y., Schroeder, A., Storch, V., & Braunbeck, T. (1999). Toxicity of 4-chloroaniline in early life stages of zebrafish (Danio rerio): II. Cytopathology and regeneration of liver and gills after prolonged exposure to waterborne 4-chloroaniline. *Archives of environmental contamination and toxicology*, *37*(1), 85–102.

17. Bush, G., diSibio, G., Miyamoto, A., Denault, J. B., Leduc, R., & Weinmaster, G. (2001). Ligand-induced signaling in the absence of furin processing of Notch1. *Developmental biology*, *229*(2), 494–502.
18. Cambier, S., Gline, S., Mu, D., Collins, R., Araya, J., Dolganov, G., Einheber, S., Boudreau, N., & Nishimura, S. L. (2005). Integrin alpha(v)beta8-mediated activation of transforming growth factor-beta by perivascular astrocytes: an angiogenic control switch. *The American journal of pathology*, *166*(6), 1883–1894.
19. Castro-Oropeza, R., Melendez-Zajgla, J., Maldonado, V., & Vazquez-Santillan, K. (2018). The emerging role of lncRNAs in the regulation of cancer stem cells. *Cellular oncology (Dordrecht)*, *41*(6), 585–603.
20. Chang, C., & Werb, Z. (2001). The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. *Trends in cell biology*, *11*(11), S37–S43.
21. Chhetri, J., Jacobson, G., & Gueven, N. (2014). Zebrafish--on the move towards ophthalmological research. *Eye (London, England)*, *28*(4), 367–380.
22. Constam, D. B., & Robertson, E. J. (2000). Tissue-specific requirements for the proprotein convertase furin/SPC1 during embryonic turning and heart looping. *Development (Cambridge, England)*, *127*(2), 245–254.
23. Darby, I. A., & Hewitson, T. D. (2007). Fibroblast differentiation in wound healing and fibrosis. *International review of cytology*, *257*, 143–179.
24. Eming, S. A., Martin, P., & Tomic-Canic, M. (2014). Wound repair and regeneration: mechanisms, signaling, and translation. *Science translational medicine*, *6*(265), 265sr6.
25. Endo, K., Takino, T., Miyamori, H., Kinsen, H., Yoshizaki, T., Furukawa, M., & Sato, H. (2003). Cleavage of syndecan-1 by membrane type matrix metalloproteinase-1 stimulates cell migration. *The Journal of biological chemistry*, *278*(42), 40764–40770.
26. Erickson, J. R., & Echeverri, K. (2018). Learning from regeneration research organisms: The circuitous road to scar free wound healing. *Developmental biology*, *433*(2), 144–154.
27. Gabor, M. H., & Hotchkiss, R. D. (1979). Parameters governing bacterial regeneration and genetic recombination after fusion of Bacillus subtilis protoplasts. *Journal of bacteriology*, *137*(3), 1346–1353.

28. Gemberling, M., Bailey, T. J., Hyde, D. R., & Poss, K. D. (2013). The zebrafish as a model for complex tissue regeneration. *Trends in genetics : TIG*, *29*(11), 611–620.
29. Gifford, V., & Itoh, Y. (2019). MT1-MMP-dependent cell migration: proteolytic and non-proteolytic mechanisms. *Biochemical Society transactions*, *47*(3), 811–826.
30. Goldman D. (2014). Müller glial cell reprogramming and retina regeneration. *Nature reviews. Neuroscience*, *15*(7), 431–442.
31. Gurtner, G. C., Werner, S., Barrandon, Y., & Longaker, M. T. (2008). Wound repair and regeneration. *Nature*, *453*(7193), 314–321.
32. Harris, J. A., Cheng, A. G., Cunningham, L. L., MacDonald, G., Raible, D. W., & Rubel, E. W. (2003). Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (Danio rerio). *Journal of the Association for Research in Otolaryngology : JARO*, *4*(2), 219–234.
33. Hill, A. J., Teraoka, H., Heideman, W., & Peterson, R. E. (2005). Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicological sciences : an official journal of the Society of Toxicology*, *86*(1), 6–19.
34. Holmbeck, K., Bianco, P., Caterina, J., Yamada, S., Kromer, M., Kuznetsov, S. A., Mankani, M., Robey, P. G., Poole, A. R., Pidoux, I., Ward, J. M., & Birkedal-Hansen, H. (1999). MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell*, *99*(1), 81–92.
35. Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J. C., Koch, R., Rauch, G. J., White, S., Chow, W., … Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, *496*(7446), 498–503.
36. Itoh, Y., Kajita, M., Kinoh, H., Mori, H., Okada, A., & Seiki, M. (1999). Membrane type 4 matrix metalloproteinase (MT4-MMP, MMP-17) is a glycosylphosphatidylinositol-anchored proteinase. *The Journal of biological chemistry*, *274*(48), 34260–34266.
37. Jaaks, P., & Bernasconi, M. (2017). The proprotein convertase furin in tumour progression. *International journal of cancer*, *141*(4), 654–663.

38. Jadeja, R. N., Thounaojam, M. C., Bartoli, M., & Martin, P. M. (2020). Implications of NAD^+ Metabolism in the Aging Retina and Retinal Degeneration. *Oxidative medicine and cellular longevity*, *2020*, 2692794.
39. Jenkins G. (2008). The role of proteases in transforming growth factor-beta activation. *The international journal of biochemistry & cell biology*, *40*(6-7), 1068–1078.
40. Jiao, F., Hu, H., Han, T., Yuan, C., Wang, L., Jin, Z., Guo, Z., & Wang, L. (2015). Long noncoding RNA MALAT-1 enhances stem cell-like phenotypes in pancreatic cancer cells. *International journal of molecular sciences*, *16*(4), 6677–6693.
41. Jiao, F., Hu, H., Yuan, C., Wang, L., Jiang, W., Jin, Z., Guo, Z., & Wang, L. (2014). Elevated expression level of long noncoding RNA MALAT-1 facilitates cell growth, migration and invasion in pancreatic cancer.
42. Kaur, S., Gupta, S., Chaudhary, M., Khursheed, M. A., Mitra, S., Kurup, A. J., & Ramachandran, R. (2018). let-7 MicroRNA-Mediated Regulation of Shh Signaling and the Gene Regulatory Network Is Essential for Retina Regeneration. *Cell reports*, *23*(5), 1409–1423.
43. Khatib, A. M., Lahlil, R., Scamuffa, N., Akimenko, M. A., Ernest, S., Lomri, A., Lalou, C., Seidah, N. G., Villoutreix, B. O., Calvo, F., & Siegfried, G. (2010). Zebrafish ProVEGF-C expression, proteolytic processing and inhibitory effect of unprocessed ProVEGF-C during fin regeneration. *PloS one*, *5*(7), e11438.
44. Kim, W., Essalmani, R., Szumska, D., Creemers, J. W., Roebroek, A. J., D'Orleans-Juste, P., Bhattacharya, S., Seidah, N. G., & Prat, A. (2012). Loss of endothelial furin leads to cardiac malformation and early postnatal death. *Molecular and cellular biology*, *32*(17), 3382–3391.
45. Kizil, C., Kaslin, J., Kroehne, V., & Brand, M. (2012). Adult neurogenesis and brain regeneration in zebrafish. *Developmental neurobiology*, *72*(3), 429–461.
46. Lee, R., Kermani, P., Teng, K. K., & Hempstead, B. L. (2001). Regulation of cell survival by secreted proneurotrophins. *Science (New York, N.Y.)*, *294*(5548), 1945–1948.
47. Lehmann, M., El Battari, A., Abadie, B., Martin, J. M., & Marvaldi, J. (1996). Role of alpha v beta 5 and alpha v beta 6 integrin glycosylation in the adhesion of a colonic adenocarcinoma cell line (HT29-D4). *Journal of cellular biochemistry*, *61*(2), 266–277.

48. Li, W., Gibson, C. W., Abrams, W. R., Andrews, D. W., & DenBesten, P. K. (2001). Reduced hydrolysis of amelogenin may result in X-linked amelogenesis imperfecta. *Matrix biology : journal of the International Society for Matrix Biology*, *19*(8), 755–760.
49. Luan, W., Li, L., Shi, Y., Bu, X., Xia, Y., Wang, J., Djangmah, H. S., Liu, X., You, Y., & Xu, B. (2016). Long non-coding RNA MALAT1 acts as a competing endogenous RNA to promote malignant melanoma growth and metastasis by sponging miR-22. *Oncotarget*, *7*(39), 63901–63912.
50. Lutty G. A. (2013). Effects of diabetes on the eye. *Investigative ophthalmology & visual science*, *54*(14), ORSF81–ORSF87.
51. Maret, D., Gruzglin, E., Sadr, M. S., Siu, V., Shan, W., Koch, A. W., Seidah, N. G., Del Maestro, R. F., & Colman, D. R. (2010). Surface expression of precursor N-cadherin promotes tumor cell invasion. *Neoplasia (New York, N.Y.)*, *12*(12), 1066–1080.
52. Marques, I. J., Lupi, E., & Mercader, N. (2019). Model systems for regeneration: zebrafish. *Development (Cambridge, England)*, *146*(18), dev167692.
53. Marques, I. J., Lupi, E., & Mercader, N. (2019). Model systems for regeneration: zebrafish. *Development (Cambridge, England)*, *146*(18), dev167692.
54. Martignetti, J. A., Aqeel, A. A., Sewairi, W. A., Boumah, C. E., Kambouris, M., Mayouf, S. A., Sheth, K. V., Eid, W. A., Dowling, O., Harris, J., Glucksman, M. J., Bahabri, S., Meyer, B. F., & Desnick, R. J. (2001). Mutation of the matrix metalloproteinase 2 gene (MMP2) causes a multicentric osteolysis and arthritis syndrome. *Nature genetics*, *28*(3), 261–265.
55. McMahon, S., Grondin, F., McDonald, P. P., Richard, D. E., & Dubois, C. M. (2005). Hypoxia-enhanced expression of the proprotein convertase furin is mediated by hypoxia-inducible factor-1: impact on the bioactivation of proproteins. *The Journal of biological chemistry*, *280*(8), 6561–6569.
56. Mescher, A. L., & Neff, A. W. (2005). Regenerative capacity and the developing immune system. *Advances in biochemical engineering/biotechnology*, *93*, 39–66.
57. Mimura, T., Han, K. Y., Onguchi, T., Chang, J. H., Kim, T. I., Kojima, T., Zhou, Z., & Azar, D. T. (2009). MT1-MMP-mediated cleavage of decorin in corneal angiogenesis. *Journal of vascular research*, *46*(6), 541–550.

58. Morishita, T., Uzui, H., Nakano, A., Mitsuke, Y., Geshi, T., Ueda, T., & Lee, J. D. (2012). Number of endothelial progenitor cells in peripheral artery disease as a marker of severity and association with pentraxin-3, malondialdehyde-modified low-density lipoprotein and membrane type-1 matrix metalloproteinase. *Journal of atherosclerosis and thrombosis*, *19*(2), 149–158.
59. Morla, A. O., & Mogford, J. E. (2000). Control of smooth muscle cell proliferation and phenotype by integrin signaling through focal adhesion kinase. *Biochemical and biophysical research communications*, *272*(1), 298–302.
60. Mumm, J. S., Schroeter, E. H., Saxena, M. T., Griesemer, A., Tian, X., Pan, D. J., Ray, W. J., & Kopan, R. (2000). A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. *Molecular cell*, *5*(2), 197–206.
61. Nagase, H., Visse, R., & Murphy, G. (2006). Structure and function of matrix metalloproteinases and TIMPs. *Cardiovascular research*, *69*(3), 562–573.
62. Nechiporuk, A., & Keating, M. T. (2002). A proliferation gradient between proximal and msxb-expressing distal blastema directs zebrafish fin regeneration. *Development (Cambridge, England)*, *129*(11), 2607–2617.
63. Nelson, C. M., & Hyde, D. R. (2012). Müller glia as a source of neuronal progenitor cells to regenerate the damaged zebrafish retina. *Advances in experimental medicine and biology*, *723*, 425–430.
64. Nelson, C. M., Gorsuch, R. A., Bailey, T. J., Ackerman, K. M., Kassen, S. C., & Hyde, D. R. (2012). Stat3 defines three populations of Müller glia and is required for initiating maximal müller glia proliferation in the regenerating zebrafish retina. *The Journal of comparative neurology*, *520*(18), 4294–4311.
65. Nwomeh, B. C., Liang, H. X., Diegelmann, R. F., Cohen, I. K., & Yager, D. R. (1998). Dynamics of the matrix metalloproteinases MMP-1 and MMP-8 in acute open human dermal wounds. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society*, *6*(2), 127–134.
66. Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M., & Okada, Y. (1997). Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. *The Journal of biological chemistry*, *272*(4), 2446–2451.

67. Padua, D., & Massagué, J. (2009). Roles of TGFbeta in metastasis. *Cell research*, *19*(1), 89–102.
68. Page-McCaw, A., Ewald, A. J., & Werb, Z. (2007). Matrix metalloproteinases and the regulation of tissue remodelling. *Nature reviews. Molecular cell biology*, *8*(3), 221–233.
69. Park, H. I., Ni, J., Gerkema, F. E., Liu, D., Belozerov, V. E., & Sang, Q. X. (2000). Identification and characterization of human endometase (Matrix metalloproteinase-26) from endometrial tumor. *The Journal of biological chemistry*, *275*(27), 20540–20544.
70. Patterson, M. L., Atkinson, S. J., Knäuper, V., & Murphy, G. (2001). Specific collagenolysis by gelatinase A, MMP-2, is determined by the hemopexin domain and not the fibronectin-like domain. *FEBS letters*, *503*(2-3), 158–162.
71. Pepper M. S. (2001). Extracellular proteolysis and angiogenesis. *Thrombosis and haemostasis*, *86*(1), 346–355.
72. Pietraszek-Gremplewicz, K., Karamanou, K., Niang, A., Dauchez, M., Belloy, N., Maquart, F. X., Baud, S., & Brézillon, S. (2019). Small leucine-rich proteoglycans and matrix metalloproteinase-14: Key partners?. *Matrix biology : journal of the International Society for Matrix Biology*, *75-76*, 271–285.
73. Plantié, E., Migocka-Patrzałek, M., Daczewska, M., & Jagla, K. (2015). Model organisms in the fight against muscular dystrophy: lessons from drosophila and Zebrafish. Molecules (Basel, Switzerland), 20(4), 6237–6253.
74. Poss, K. D., Wilson, L. G., & Keating, M. T. (2002). Heart regeneration in zebrafish. *Science (New York, N.Y.)*, *298*(5601), 2188–2190.
75. Posthaus, H., Dubois, C. M., Laprise, M. H., Grondin, F., Suter, M. M., & Müller, E. (1998). Proprotein cleavage of E-cadherin by furin in baculovirus over-expression system: potential role of other convertases in mammalian cells. *FEBS letters*, *438*(3), 306–310.
76. Purnell L. D. (2008). Traditional Vietnamese health and healing. *Urologic nursing*, *28*(1), 63–67.
77. Ramachandran, R., Fausett, B. V., & Goldman, D. (2010). Ascl1a regulates Müller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. *Nature cell biology*, *12*(11), 1101–1107.

78. Reimschuessel R. (2001). A fish model of renal regeneration and development. *ILAR journal*, *42*(4), 285–291.
79. Roebroek, A. J., Umans, L., Pauli, I. G., Robertson, E. J., van Leuven, F., Van de Ven, W. J., & Constam, D. B. (1998). Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase Furin. *Development (Cambridge, England)*, *125*(24), 4863–4876.
80. Rohani, M. G., & Parks, W. C. (2015). Matrix remodeling by MMPs during wound repair. *Matrix biology : journal of the International Society for Matrix Biology*, *44-46*, 113–121.
81. Rosen, J. N., Sweeney, M. F., & Mably, J. D. (2009). Microinjection of zebrafish embryos to analyze gene function. *Journal of visualized experiments : JoVE*, (25), 1115.
82. Ryan, G. B., & Majno, G. (1977). Acute inflammation. A review. *The American journal of pathology*, *86*(1), 183–276.
83. Saarialho-Kere, U., Kerkelä, E., Jahkola, T., Suomela, S., Keski-Oja, J., & Lohi, J. (2002). Epilysin (MMP-28) expression is associated with cell proliferation during epithelial repair. *The Journal of investigative dermatology*, *119*(1), 14–21.
84. Sadowski, T., Dietrich, S., Koschinsky, F., Ludwig, A., Proksch, E., Titz, B., & Sedlacek, R. (2005). Matrix metalloproteinase 19 processes the laminin 5 gamma 2 chain and induces epithelial cell migration. *Cellular and molecular life sciences : CMLS*, *62*(7-8), 870–880.
85. Sato, H., Kinoshita, T., Takino, T., Nakayama, K., & Seiki, M. (1996). Activation of a recombinant membrane type 1-matrix metalloproteinase (MT1-MMP) by furin and its interaction with tissue inhibitor of metalloproteinases (TIMP)-2. *FEBS letters*, *393*(1), 101–104.
86. Sato, H., Kinoshita, T., Takino, T., Nakayama, K., & Seiki, M. (1996). Activation of a recombinant membrane type 1-matrix metalloproteinase (MT1-MMP) by furin and its interaction with tissue inhibitor of metalloproteinases (TIMP)-2. *FEBS letters*, *393*(1), 101–104.
87. Seidah, N. G., & Prat, A. (2012). The biology and therapeutic targeting of the proprotein convertases. *Nature reviews. Drug discovery*, *11*(5), 367–383.
88. Seidah, N. G., Day, R., Marcinkiewicz, M., & Chrétien, M. (1998). Precursor convertases: an evolutionary ancient, cell-specific, combinatorial mechanism

yielding diverse bioactive peptides and proteins. *Annals of the New York Academy of Sciences*, *839*, 9–24.

89. Seifert, A. W., Kiama, S. G., Seifert, M. G., Goheen, J. R., Palmer, T. M., & Maden, M. (2012). Skin shedding and tissue regeneration in African spiny mice (Acomys). *Nature*, *489*(7417), 561–565.
90. Seiki M. (2003). Membrane-type 1 matrix metalloproteinase: a key enzyme for tumor invasion. *Cancer letters*, *194*(1), 1–11.
91. Shah P. K. (1997). Inflammation, metalloproteinases, and increased proteolysis: an emerging pathophysiological paradigm in aortic aneurysm. *Circulation*, *96*(7), 2115–2117.
92. Shapiro, S. D., Kobayashi, D. K., Pentland, A. P., & Welgus, H. G. (1993). Induction of macrophage metalloproteinases by extracellular matrix. Evidence for enzyme- and substrate-specific responses involving prostaglandin-dependent mechanisms. *The Journal of biological chemistry*, *268*(11), 8170–8175.
93. Sharma, P., & Ramachandran, R. (2019). Retina Injury and Retina Tissue Preparation to Study Regeneration in Zebrafish. *Bio-protocol*, *9*(24), e3466.
94. Sharma, P., Gupta, S., Chaudhary, M., Mitra, S., Chawla, B., Khursheed, M. A., Saran, N. K., & Ramachandran, R. (2020). Biphasic Role of Tgf-β Signaling during Müller Glia Reprogramming and Retinal Regeneration in Zebrafish. *iScience*, *23*(2), 100817.
95. Shinde, U., & Inouye, M. (1993). Intramolecular chaperones and protein folding. *Trends in biochemical sciences*, *18*(11), 442–446.
96. Simon, A., & Tanaka, E. M. (2013). Limb regeneration. *Wiley interdisciplinary reviews. Developmental biology*, *2*(2), 291–300.
97. Singer, A. J., & Clark, R. A. (1999). Cutaneous wound healing. *The New England journal of medicine*, *341*(10), 738–746.
98. Sounni, N. E., Paye, A., Host, L., & Noël, A. (2011). MT-MMPS as Regulators of Vessel Stability Associated with Angiogenesis. *Frontiers in pharmacology*, *2*, 111.
99. Sounni, N. E., Paye, A., Host, L., & Noël, A. (2011). MT-MMPS as Regulators of Vessel Stability Associated with Angiogenesis. *Frontiers in pharmacology*, *2*, 111.
100. Stetler-Stevenson, W. G., Liotta, L. A., & Kleiner, D. E., Jr (1993). Extracellular matrix 6: role of matrix metalloproteinases in tumor invasion and

metastasis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *7*(15), 1434–1441.

101. Streisinger, G., Walker, C., Dower, N., Knauber, D., & Singer, F. (1981). Production of clones of homozygous diploid zebra fish (Brachydanio rerio). *Nature*, *291*(5813), 293–296.

102. Suzuki, K., Enghild, J. J., Morodomi, T., Salvesen, G., & Nagase, H. (1990). Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin). *Biochemistry*, *29*(44), 10261–10270.

103. Szabowski, A., Maas-Szabowski, N., Andrecht, S., Kolbus, A., Schorpp-Kistner, M., Fusenig, N. E., & Angel, P. (2000). c-Jun and JunB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin. Cell, 103(5), 745–755.

104. Thomas G. (2002). Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nature reviews. Molecular cell biology*, *3*(10), 753–766.

105. Thoreson, W. B., & Dacey, D. M. (2019). Diverse Cell Types, Circuits, and Mechanisms for Color Vision in the Vertebrate Retina. *Physiological reviews*, *99*(3), 1527–1573.

106. Tucker, A. S., Headon, D. J., Schneider, P., Ferguson, B. M., Overbeek, P., Tschopp, J., & Sharpe, P. T. (2000). Edar/Eda interactions regulate enamel knot formation in tooth morphogenesis. *Development (Cambridge, England)*, *127*(21), 4691–4700.

107. Ueda, J., Kajita, M., Suenaga, N., Fujii, K., & Seiki, M. (2003). Sequence-specific silencing of MT1-MMP expression suppresses tumor cell migration and invasion: importance of MT1-MMP as a therapeutic target for invasive tumors. *Oncogene*, *22*(54), 8716–8722.

108. Uría, J. A., & López-Otín, C. (2000). Matrilysin-2, a new matrix metalloproteinase expressed in human tumors and showing the minimal domain organization required for secretion, latency, and activity. *Cancer research*, *60*(17), 4745–4751.

109. Van Lint, P., & Libert, C. (2007). Chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation. *Journal of leukocyte biology*, *82*(6), 1375–1381.

110. Veldman, M. B., & Lin, S. (2008). Zebrafish as a developmental model organism for pediatric research. *Pediatric research*, *64*(5), 470–476.

111. Vihtelic, T. S., & Hyde, D. R. (2000). Light-induced rod and cone cell death and regeneration in the adult albino zebrafish (Danio rerio) retina. *Journal of neurobiology*, *44*(3), 289–307.

112. Visse, R., & Nagase, H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circulation research*, *92*(8), 827–839.

113. Walker, C., & Streisinger, G. (1983). Induction of Mutations by gamma-Rays in Pregonial Germ Cells of Zebrafish Embryos. *Genetics*, *103*(1), 125–136.

114. Walker, M. B., Miller, C. T., Coffin Talbot, J., Stock, D. W., & Kimmel, C. B. (2006). Zebrafish furin mutants reveal intricacies in regulating Endothelin1 signaling in craniofacial patterning. Developmental biology, 295(1), 194–205.

115. Wan, J., & Goldman, D. (2016). Retina regeneration in zebrafish. *Current opinion in genetics & development*, *40*, 41–47.

116. Wang, X., & Khalil, R. A. (2018). Matrix Metalloproteinases, Vascular Remodeling, and Vascular Disease. *Advances in pharmacology (San Diego, Calif.)*, *81*, 241–330.

117. White L.M., Roy S., Gordillo G.M., Kalliainen L.K., Melvin W.S., Ellison E.C., and Sen C.K. Wound healing and regeneration. 2010, Physiology and Medicine, 1:193.

118. Woessner J. F., Jr (1998). Role of matrix proteases in processing enamel proteins. *Connective tissue research*, *39*(1-3), 69–149.

119. Xiang, J., Yang, H., Che, C., Zou, H., Yang, H., Wei, Y., Quan, J., Zhang, H., Yang, Z., & Lin, S. (2009). Identifying tumor cell growth inhibitors by combinatorial chemistry and zebrafish assays. *PloS one*, *4*(2), e4361.

120. Yana, I., & Weiss, S. J. (2000). Regulation of membrane type-1 matrix metalloproteinase activation by proprotein convertases. *Molecular biology of the cell*, *11*(7), 2387–2401.

121. Zeng, L., Cen, Y., & Chen, J. (2018). Long non-coding RNA MALAT-1 contributes to maintenance of stem cell-like phenotypes in breast cancer cells. *Oncology letters*, *15*(2), 2117–2122.

122. Zhou, A., Martin, S., Lipkind, G., LaMendola, J., & Steiner, D. F. (1998). Regulatory roles of the P domain of the subtilisin-like prohormone convertases. *The Journal of biological chemistry*, *273*(18), 11107–11114.

123. Zucker, S., Pei, D., Cao, J., & Lopez-Otin, C. (2003). Membrane type-matrix metalloproteinases (MT-MMP). *Current topics in developmental biology*, *54*.

******

Appendix 1:

List of primers

Primer	Ensembl ID	Sequence (5'-3')
qPCR and RT Primers		
RT_furina_Fwd	ENSDARG00000062909	CACACTGCTTCCTCCCAGACCTC
RT_furina_Rev		GAAGCCGGGGTTACACTCTGTG
RT_furinb_Fwd	ENSDARG00000070971	GTGGCTGGACTTAATGACTACGGCGT
RT_furinb_Rev		CTCTGCACCTGGTTCTGGTGCAAGC
RT_mmp14a_Fwd	ENSDARG00000002235	CATGAGTGAGGATGGAGCCAAC
RT_mmp14a_Rev		TTAAACCTTATCGAGCAGAGAGCGG
RT_mmp14b_Fwd	ENSDARG00000008388	GCATTCATGAGTCGAGATCAAGGTTATAC
RT_mmp14b_Rev		TTAAACCTTGTCCAGTAGGGAGCGT
RT_ascl1a_Fwd	ENSDARG00000038386	ATCTCCCAAAACTACTCTAATGACATGAACTCTA
RT_ascl1a_Rev		CAAGCGAGTGCTGATATTTTTAAGTTTCCTTTTAC
RT_b-actin_Fwd	ENSDARG00000037746	GCAGAAGGAGATCACATCCC TGGC
RT_b-actin_Rev		CATTGCCGTCACCTTCACCGTTC
RT_her4.1_Fwd	ENSDARG00000056732	GCTGATATCCTGGAGATGACG
RT_her4.1_Rev		GACTGTGGGCTGGAGTGTGTT
RT_l24_Fwd	ENSDARG00000099104	CGACCCAGAGCAGCAAGG
RT_l24_Rev		AGCACATCAGAGTTTAGC

RT_malat1_Fwd	ENSDARG00000251562	AAACCCATCCACTCGCTCTG
RT_malat1_Rev		CTTATCTCCAGGTAGCGGCG
RT_mmp9_Fwd	ENSDART00000062845.5	GGAGAAAACTTCTGGAGACT TG
RT_mmp9_Rev		CACTGAAGAGAAACGGTTTC C
RT_zic2b_Fwd	ENSDARG00000037178	CGCGGGTGTAGTGTCTTTACGCATTC
RT_zic2b_Rev		GGGCACTTAAGGATCCCCGAAAATAC
Cloning Primers		
*mmp14a*_Fwd_FL_EcoRI	ENSDARG00000002235	ATGCTAGCGAATTCACCATGTTACCGAAACTGCAGACGTTACCTC
*mmp14a*_Rev_FL_XhoI		ATGCTAGCCTCGAGTTAAACCTTATCGAGCAGAGAGCGGTG
*mmp14a*_Fwd_UTR_BamHI	ENSDARG00000002235	ATGCTAGCAGGCCTCTGCTCTATAGGTGCATTTATC
*mmp14a*_Rev_UTR		ATGCTAGCGGATCCGATGGAAAAGTGAAGGATGTGT
*furina*_Fwd_FL_BamHI	ENSDARG00000062909	ATGCTAGCGGATCCACCATGGATCTCAGGCTTGCCTCATTGAC
furina-_Rev_FL_XhoI		ATGCTAGCTCGAGAAGAGCACTTTGTGTTTTGATAAGG

******

“The end of one chapter is just the beginning of another,

Read on… the best part is always yet to come.”

-Susan Gale