Investigating role of miR-200 family during zebrafish retina regeneration and its interaction with regeneration associated genes

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

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Certificate of Examination

This is to certify that the dissertation titled **"Investigating role of miR-200 family during retina zebrafish regeneration and its interaction with regeneration associated genes"** submitted by **Ms. Sapna Kumari Meena** (Reg. No. MS15154) 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. Rajesh Ramachandran (Supervisor) Dr. Sharvan Sehrawat Dr. Santosh Satbhai

Dated: May 04, 2020

Declaration

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

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

Sapna Kumari Meena

(Candidate)

Dated: May 4, 2020

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

Dr. Rajesh Ramachandran

(Supervisor)

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Sapna Kumari Meena

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NOTATIONS

1. RP-Retinitis pigmentosa
2. MG-Muller Glia
3. MGPC-Muller glia derived progenitor cell
4. R-Rods
5. C-Cones
6. A-Amacrine cells
7. BP-Bipolar cells
8. H-Horizontal cells
9. GC-Ganglion cells
10. ONL-Outer nuclear layer
11. INL-inner nuclear layer
12. GCL-Ganglion cell layer
13. Hdac- Histone deactylase protein
14. Gfap - glial fibrillary acidic protein
15. dpi-days post injury
16. hpi-hours post injury
17. Ascl1a - Achaete-scute homolog 1 zebrafish protein
18. *ascl1a* - Achaete-scute homolog 1 zebrafish mRNA
19. Mmp9-Matrix metallo protease 9 zebrafish protein
20. *mmp9*- Matrix metallo protease 9 zebrafish mRNA
21. ChiP-seq- Chromatin immunoprecipitation- sequencing
22. Co-IP- Co-immunoprecipitation

Abstract

Our eye is one of the most important sensory organs. We perceive 80% of the knowledge through eyes only. Diseases and mechanical insults can lead to vision loss. Although the field of retina regeneration is more than two decade old yet retina regeneration in mammals has still have a long way to go. But unlike mammals, Zebrafish, a teleost fish can regenerate its retina after injury. In zebrafish regenerative ability is attributed to a retinal cell type Muller glia which respond to injury and restore all lost retinal cell types. Muller glia are common to all vertebrates but in zebrafish only these cells undergo nucleus reprogramming upon injury to restore the vision.

miRNA-200 family have been reported to regulate mesenchymal to epithelial transition (MET), cell proliferation, differentiation, cell cycle exit and their role in tumour suppression. Although lots of research studies have elucidated importance of various transcription factors and few micro-RNAs who are known to contribute to muller glia reprogramming, but microRNA-200 role in Zebrafish retina regeneration have not been studied.

In this study, we have explored the role of miR-200 family and their interaction with regeneration associated genes during retina regeneration. We found that knockdown of miR-200a results in increased proliferation of MGPCs. We also explored interaction of miR-200 family with TGF-β signalling. Cyclopia condition was reported upon pharmacological inhibition of TGF-β signalling. We observed higher expression of miR-200a and miR-200b in TGF-β blocked condition in 48hpf embryos. So, may be TGF-β signalling is mediating its regulation through miR-200 family.

Hence, this study sheds some light on miR-200 family role in zebrafish retina regeneration and their interaction with TFG-β signalling in embryo.

Chapter 1: Introduction

About 5% of world's population suffer from vision impairment and retinal blindness. These problems are caused by diseases like, macular degeneration, retinitis pigmentosa, diabetic retinopathies and glaucoma. Regeneration is a process in some organisms which has inherent capability to restore damaged and lost tissues. Unfortunately, mammals cannot regenerate their damaged retina. Tissue regeneration promises a way to restore damaged organs in mammals too. Unlike the mammals, lower vertebrate such as fishes and frogs possess a remarkable regenerating ability(Gemberling et al. 2013). A most widely use model organism is zebrafish, a teleost fish which can regenerate almost all organs(Gemberling et al. 2013). A key goal of researcher working in this field is to unlock the secret of this organism and then enable regeneration in mammals.

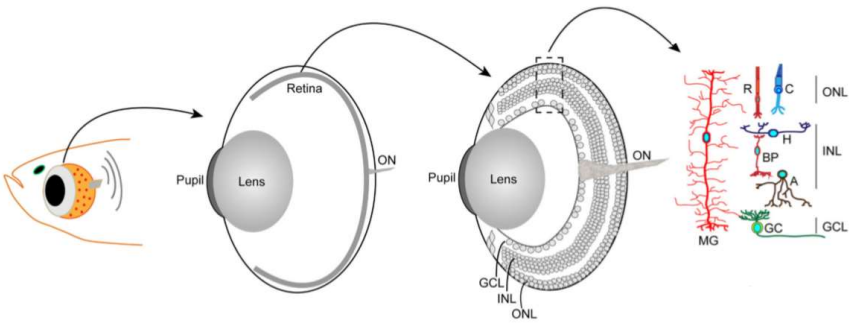
Zebrafish has the Muller glia cell type which undergo cellular reprogramming upon injury, they first dedifferentiate and then proliferate to give rise to Muller glia derived progenitor cells (MGPCs)(Wan and Goldman 2016)(Goldman 2014). These cells then migrate to damaged area and re-differentiate to replaces the damaged retinal cells into new cells(Ramachandran, Fausett, and Goldman 2015)(Wan, Ramachandran, and Goldman 2012).

Despite the field have been studied a lot in years yet a lot of molecular mechanisms are need to unravel. It is hope that this study will provide us first insight of the role of miRNA-200 family in zebrafish retina regeneration and will help in future in designing therapeutic strategies to cure mammalian blindness.

1.1 Zebrafish Retinal Architecture

The zebrafish retina has 6 neural cell types and 1 glial cell types, which resides in three distinct cell layers. Outer nuclear layer (ONL), inner nuclear layer (INL), ganglion cell layer (GCL). The ONL layer consists of photoreceptors rods(R) and cones (C), which sense light and transmit it to the ganglion cells into ganglion cell layer via INL cells namely amacrine cells (A), horizontal cells (H) and bipolar cells (BP). Retina also houses a unique type of cell which named after Heinrich Muller in the respect of the discoverer. The Muller glia cell that extends their processes from ganglion cell layer (GCL) to outer nuclear layer (ONL) and their cell body resides in INL. MG cells also extends its processes in laterally allowing them to interact with neighbouring neurons. These cells are the only glial cells that derived from retinal progenitors(Goldman 2014). (Pic. 1.1)

Such anatomy in zebrafish retina allows, Muller glia cells to keep check on retinal homeostasis, structure and functioning in transfer of wide range of molecules in different cell types and retinal layers(Goldman 2014).



Picture 1.1. A schematic showing the retinal cell types and layers.

Source: Retina regeneration in Zebrafish (2016). Jin Wan and Daniel Goldman, Curr Opin Genet Dev . Published online: 2016 October DOI:10.1016/j.gde.2016.05.009

1.2 Zebrafish Muller glia during injury and regeneration

Several model organisms used to study retina regeneration. But mainly three model organisms rule the field: zebrafish, postnatal chick and mice. While zebrafish naturally regenerate retina upon injury, postnatal chick limited regeneration capacity. However, mice do not regenerate but used as an important mammalian model for testing regenerative strategies for retinal repair in mammals(Goldman 2014).

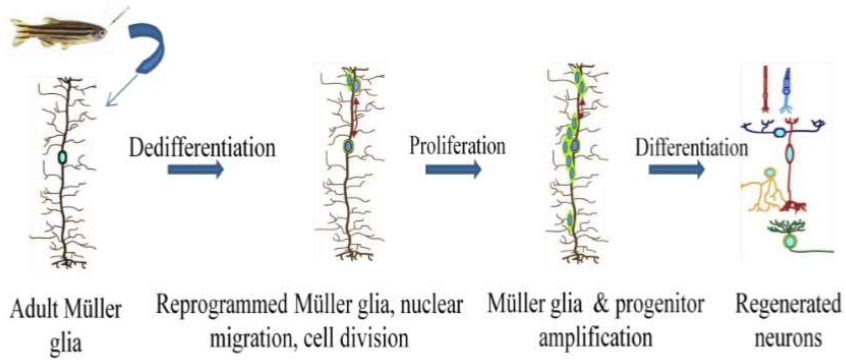
Zebrafish retinal damage can be induced by many ways: bright light, laser ablation, toxic chemicals and mechanical method (using needle poke). In these various injury paradigms mechanical injury by stab wound have advantage of uniform injury in all retinal cell layers(Sharma and Ramachandran 2019).

Muller glia in the immediate vicinity of damage partially and transiently de-differentiate, re-express retinal progenitor and stem cell markers. Their nucleus reprograms, undergo interkinetic nuclear migration and divide once asymmetrically to generate retinal progenitor. These daughter cells proliferate rapidly to generate form a compact neurogenic cluster of surrounding the MG, these multipotent retinal progenitors then migrate along the radial fibres to the damaged place to replace the missing part. (Pic. 1.2)

Zebrafish retina regeneration by Muller glia cells can be summarized in three steps:

1) **Dedifferentiation phase (0-2 days post injury):** This phase is defined by suppression of the pathways that derive MG cells differentiation and quiescence. Also, expression of genes that induces stem cell like characteristics and re-entry in cell cycle in Muller glia cells(Belecky-Adams et al. 2013)(Goldman 2014).

2) **Proliferation phase (2-4 days post injury):** In this phase reprogrammed Muller glia undergo interkinetic nuclear migration and divide asymmetrically to give rise to multipotent progenitor cells named Muller glia derived progenitor cells (MGPCs)(Wan and Goldman 2016).
3) **Re-differentiation phase (4-8 days post injury):** Multipotent Muller glia in the immediate vicinity of damaged cells migrate through along the radial fibres to the appropriate locations to replace missing retinal cell types(Belecky-Adams et al. 2013).



Picture2. A diagram showing phases of retina regeneration.

Source: Retina regeneration in Zebrafish (2016). Jin Wan and Daniel Goldman, Curr Opin Genet Dev . Published online: 2016 October DOI:10.1016/j.gde.2016.05.009

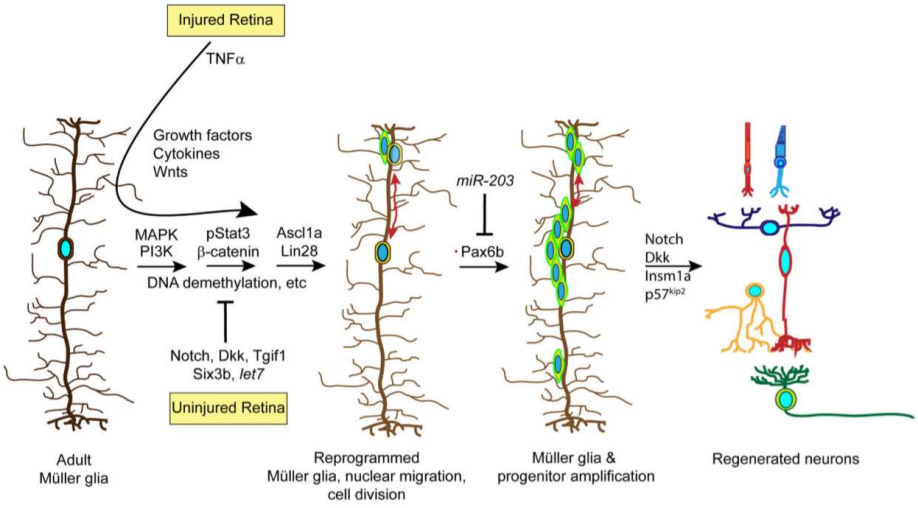
1.3 Mechanisms of zebrafish retina regeneration

When Muller glia senses injury in retina they respond by entering into reactive gliosis state. Reactive gliosis is marked by hypertrophy and activation of cytoskeleton genes like glial fibrillary acidic protein (Gfap) and vimentin(Hippert et al. 2015). However, this reactive gliosis often results in glial scarring and fibrosis in mammals, they rarely divide. In zebrafish also Muller glia respond to injury by hypertrophy, activation of

Gfap and vimentin but this reactive gliosis is transient in zebrafish and often convert into regenerative one by adopting stem cell like properties(Wan and Goldman 2016).

The dying cells initiates regeneration by alteration in their interaction with neighbourhood Muller glia. Damaged cells also secrete growth factors and cytokines, like Hb-egf, TNFα and stimulate Muller glia proliferation(Wan and Goldman 2016). Muller glial reprogramming also includes zebrafish genome changes in DNA methylation and activation of genes associated with multipotency(Powell et al. 2013). Pro-neural gene Ascl1 and pluripotency factor Lin28 expresses as early as 6 hours post injury and are necessary for dedifferentiation of Muller glia. The Lin28 supress expression of Let-7 microRNA. Let-7 microRNA is associated with differentiation and quiescence. It represses expression regeneration associated genes such as, ascl1a, lin-28, oct4, pax6b and c-myc and keep their expression at basal level in uninjured retina(Ramachandran, Fausett, and Goldman 2015).

These injury-responsive reprogrammed Muller glia exhibits interkinetic nuclear migration. Reprogrammed nuclei move from inner nuclear layer (INL) to outer nuclear layer (ONL) and divide asymmetrically, then return to INL. The asymmetric division produces Muller glia derived progenitor cells (MGPCs) and again they undergo interkinetic nuclear migration on pax-6 dependent manner resulting in large number of MGPCs. MicroRNA-203 helps in maintain adequate number of proliferation Muller glia by inhibiting expression of Pax-6b(Rajaram et al. 2014). Notch signalling helps in maintaining Muller glia in differentiated state in uninjured condition and contributes in progenitor differentiation in lost retinal cell types in injured retina(Wan and Goldman 2016). (Pic. 1.3)



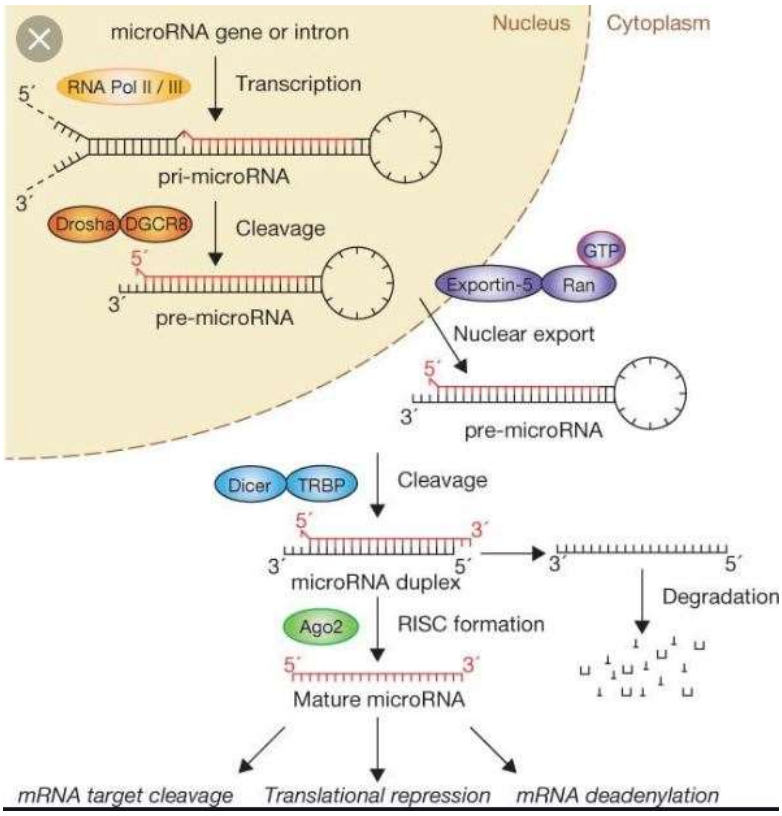
Picture3. A diagram showing phases of retina regeneration and signalling pathways triggering them.

Source: Retina regeneration in Zebrafish (2016). Jin Wan and Daniel Goldman, Curr Opin Genet Dev . Published online: 2016 October DOI:10.1016/j.gde.2016.05.009

1.4 MicroRNA-200 family

MicroRNAs are a class of small noncoding RNAs (18-24 bp) that are not translated into any proteins and polypeptides. MicroRNAs are small single stranded RNAs that are either encoded in genome as a gene or as a part of intron of another gene. The primary transcripts of miRNA(pri-miRNA) is processed in two steps by endoribonucleolytic steps. First, nuclear RNAse III enzyme DROSHA cleaves primary transcripts and generates nearly a 70-nucleotides long hairpin precursor miRNA (pre-miRNA). This pre-miRNA comes in the cytoplasm for next cleaving step. Here cytoplasmic RNAse III enzyme DICER cleaves pre-miRNA into 18-24 bp long miRNA/miRNA duplex. One of the less stable miRNA strand from this duplex gets incorporated in miRNA-induced silencing complex (miRISC) and another strand is degraded. This mature single stranded miRNA sequence specially “seed sequence” (7-8 bp long) at the 5’ end of the miRNA hybridizes to the 3’ untranslated region (3’-

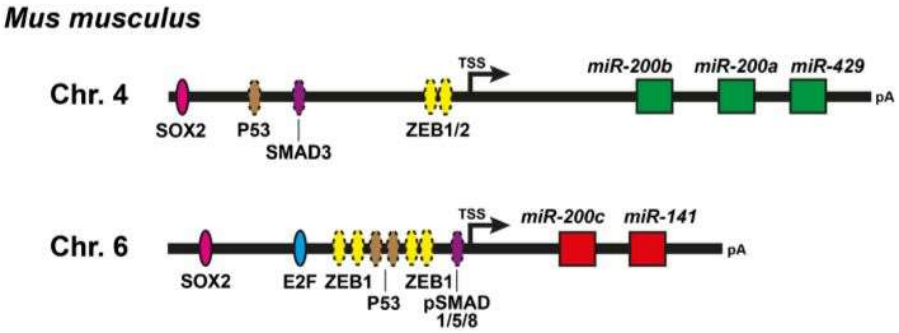
UTR) of target mRNA. This partially or fully hybridization between miRNA and target mRNA induces posttranscriptional gene silencing. The miRNA mediate gene silencing by direct degradation of mRNA, destabilization of mRNA, and translational inhibition of mRNA transcripts(Trümbach and Prakash 2015). (Pic. 1.4)



Picture4. A diagram showing microRNA biogenesis

Source: Many roads to maturity: microRNA biogenesis pathways and their regulation (2009). Julia Winter, Stephanie Jung, Sarina Keller, Richard I. Gregory, Sven Diederichs Nature Cell Biology. Published online: March 2009 DOI: 10.1038/ncb0309-228

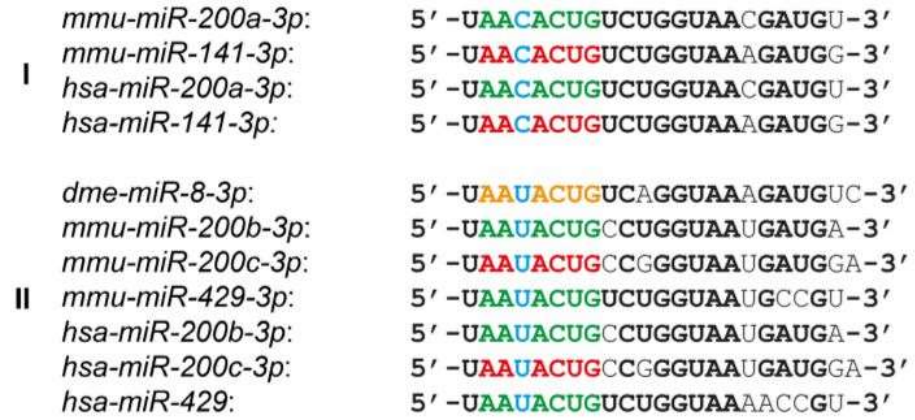
There are five members in miR-200 family: *miR-200a*, *miR-200b*, *miR-200c*, *miR-141* and *miR-429*. These are organised in two gene clusters. The tricistronic *miR200a/b/429* cluster is transcribed from common promoter and is located at chromosome no. 4 in mouse. The bicistromic *miR200c/141* also expresses from a common promoter and located at chromosome no. 6 in mouse(Trümbach and Prakash 2015). (Pic. 1.5)



Picture5. A schematic showing structure of miR-200 family gene cluster in mouse.

Source: The conserved miR-8/miR-200 microRNA family and their role in invertebrate and vertebrate neurogenesis (2015). Dietrich Trümbach, Nilima Prakash Cell tissue research. Published online: 30 May 2014 DOI 10.1007/s00441-014-1911-z

These miRNAs are further divided into two groups based on their seed sequence. *miR200a* and *miR141* has seed sequence AACACUG and other group *miR200b*, *miR200c* and *miR429* only differs by one nucleotide and have seed sequence AAUACUG(Trümbach and Prakash 2015). (Pic. 1.6)



Picture6. A schematic showing seed sequence of miR-200 family.

Source: The conserved miR-8/miR-200 microRNA family and their role in invertebrate and vertebrate neurogenesis (2015). Dietrich Trümbach, Nilima Prakash Cell tissue research. Published online: 30 May 2014 DOI 10.1007/s00441-014-1911-z

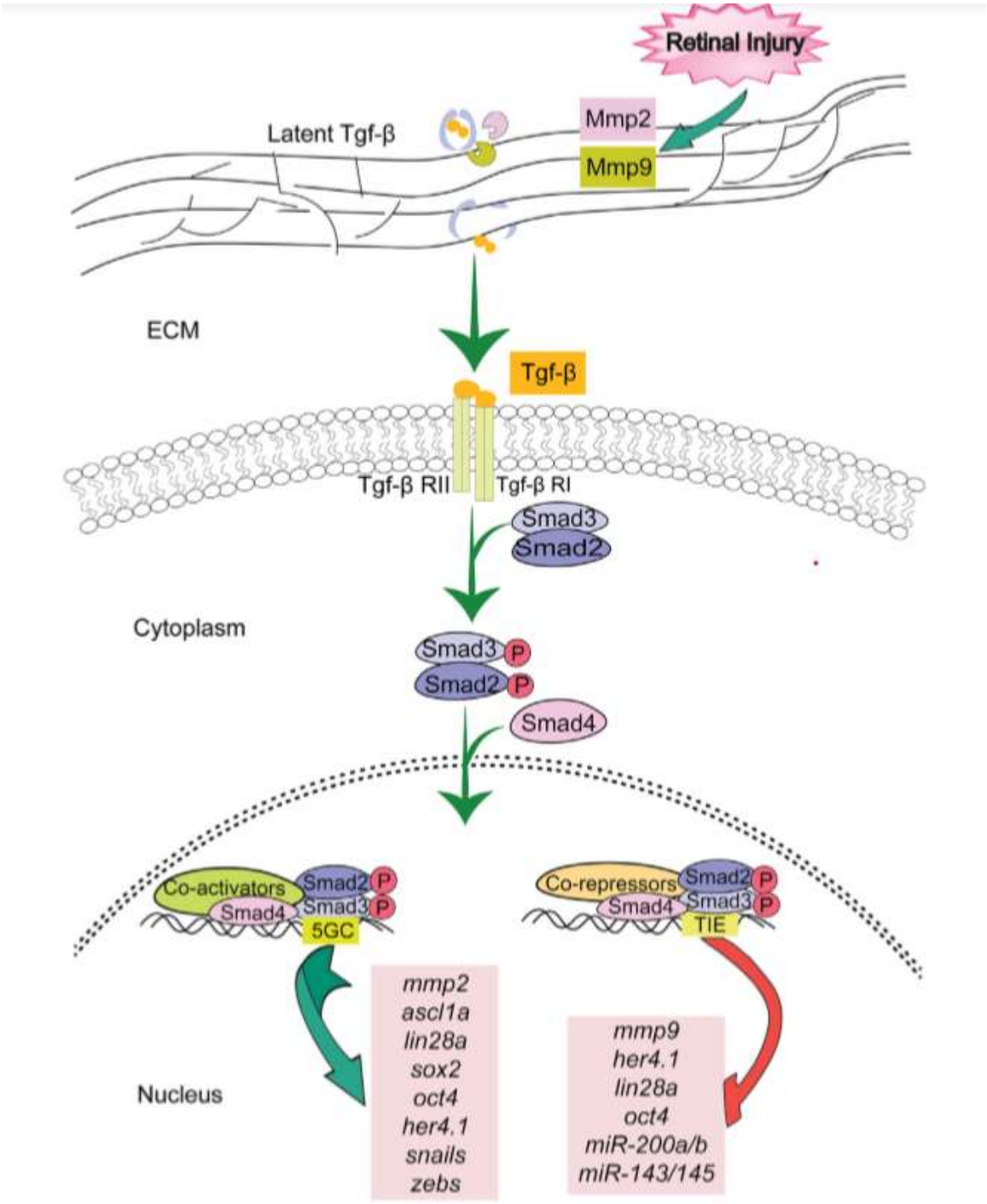
miR-200 family in mammals reported to regulate epithelial to mesenchymal transition (EMT), stemness and somatic cell reprogramming into induced pluripotent stem cells(iPSC)(Gill et al. 2011)(Bracken et al. 2014)(Wang et al. 2013). It is also reported for its role as tumour suppressor in various human cancers(Peter 2009)(Rajaram et al. 2014)(Feng et al. 2014).

ZEBs and miR200 family regulate each other's expression in reciprocal feedback loop. MicroRNA200 regulate EMT by inhibition of zinc finger-enhancer binding (ZEB1 and ZEB2) transcription factors. ZEBs also regulate MET by repressing expression of miR200 family(Brabletz and Brabletz 2010).

1.5 Interaction of MicroRNA-200 family and TGF-β signalling

The transforming growth factor beta (TGF-β) superfamily regulates many functions in developing embryo including cell growth, differentiation, adhesion, migration and apoptosis(Miyazawa et al. 2002)(Morikawa, Derynck, and Miyazono 2016). It also functions in tumour suppression and promotion at different stages(Akhurst and Derynck 2001).

miR-200 family and TGF-β signalling regulate each other in negative feedback loop to regulate EMT and MET. The miR-200 family shown downregulates components: TGF-β, TβR-1 and Smad2 to prevent EMT and also TGF-β signalling represses miR-200 family expression to prevent MET. The TGF-β signalling repress miR-200 family activation of transcriptional repressor ZEB1 and ZEB2(Gregory et al. 2011). miR-200 family downregulates TGF-β signalling to provide resistance from cancers(Truong et al. 2014)(Izumchenko et al. 2014). Blocking of TGF-β signalling from inhibitors results in elevated expression of miR-200a and miR-200b in zebrafish retina regeneration. They also reported that protein injection of TGF-β1 in injured retina results in dose-dependent downregulation of miR-200 family(Sharma et al. 2020). (Pic. 1.7)


Retinal Injury
Mmp2
Mmp9
Latent Tgf-β
ECM
Tgf-β
Tgf-β RII
Tgf-β RI
Smad3
Smad2
Cytoplasm
Smad3 P
Smad2 P
Smad4
Co-activators
Smad2 P
Smad4
Smad3 P
5GC
Co-repressors
Smad2 P
Smad4
Smad3 P
TIE
mmp2
ascl1a
lin28a
sox2
oct4
her4.1
snails
zebs
mmp9
her4.1
lin28a
oct4
miR-200a/b
miR-143/145
Nucleus

Picture7. A diagram showing TGF-β signalling in zebrafish retina regeneration.

Source: Biphasic role of TGF-β signalling during Muller glia reprogramming and retina regeneration in zebrafish. Poonam Sharma, Rajesh Ramachandran (2020) iScience. Published online: 212 February 2020 DOI: 10.1016/j.isci.2019.100817

1.6 Pharmacological inhibition of TGF-β signalling

SB431542 is an inhibitor of TGF-β type 1 receptor (Tβ-R1). SB431542 inhibits Tβ-R1 kinase activity by ATP-mimetic inhibition and blocks phosphorylation of downstream components SMADs and inhibits their nuclear localization resulting in repressed signalling pathway(Hjelmeland et al. 2004)(Halder, Beauchamp, and Datta 2005).

Chapter 2: Materials and Methods

2.1 Zebrafish maintenance

1. Fish are maintained in 14 hours of light and 10 hours of dark cycle
2. Temperature: 27°C
3. pH: 7.4

2.2 Retina dissection for RNA isolation/ western blotting

1. Zebrafish was anesthetize using Tricaine methanesulfonate.
2. Retina were injured using 30 Gauge needle.
3. At the time of harvesting, eyes were dissected using steel forceps and needle. Dissection were carried out in 1XPBS (Phosphate buffered saline) for harvesting retina, but for tissue fixation dissected in fresh 4% PFA. Lens removed for both RNA isolation and tissue fixation but not for western blotting sample preparation. Retina were submerged into 200uL Trizol for RNA isolation (Store at -20°C). For western blotting retina were suspended in 200uL Laemmli buffer and stored at -80°C.

   Note: For RNA isolation keep the environment free from RNAse. Wipe workbench and equipment with 70% ethanol and talk less while dissection.

2.3 Tissue fixation and sectioning

1. Eyes whose lenses are removed kept in 4% PFA overnight for fixation.
2. Next day, following washes of sucrose given at RT on rotor for 45 minutes each-

a. 5% sucrose 1mL
b. 5% sucrose 800uL and 20% sucrose 400µL
c. 5% sucrose 600uL and 20% sucrose 600µL
d. 5% sucrose 400µL and 20% sucrose 800µL
e. 20% sucrose 1mL

3. Remove 500uL of 20% sucrose and add approximately 500uL of OCT. Rotate for 45 minutes at RT on rotor.
4. Meanwhile make a small cube with aluminium foil.
5. After 45 minutes, fill the cube with OCT and submerged the tissue in it.
6. Store the block at -80°C until sectioning.
7. Blocks are sectioned in Leica cryostat and superimposed on super frost glass slide. Dry the sections overnight at RT and then store at -20°C.

❖ 4% PFA preparation:

- Add 2gm PFA
- Add 5mL of 10X Phosphate buffer
- Make up the volume with Milli-Q
- Keep the falcon on hot water bath at 65°C and shake in between until solution becomes colourless.
- Store at -20°C.

❖ 5% sucrose preparation

- Dissolve 2.5 gm of sucrose in Milli-Q water and mix well. Store at -20°C.

❖ 20% sucrose preparation

- Dissolve 10 gm of sucrose in Milli-Q water and mix well. Store at -20°C.

2.4 RNA Isolation

1. Take the dissected retinae collected in 200uL Trizol in MCT/ stored retina from -80°C and thaw it.
2. Homogenise the retina with 200uL tip/ using homogeniser if sample is embryos until no clumps are visible. Allow it to settle down for 5 minutes at RT.
3. Add 0.2 volume (40uL) of chloroform and mix gently by inverting MCT upside down for 5-6 times.
4. Incubate at RT for 5-10 minutes.
5. Centrifuge for 20 minutes at 10000 RCF at 4°C.
6. Take the aqueous phase slowly using small cut tip in fresh MCT without disturbing organic phase.
7. Add equal volume of Isopropanol and shake vigorously for 1 minutes.
8. Store at -80°C for overnight or incubate in ice for 20 minutes.
9. Centrifuge at 10000 RCF at 4 °C for 10 minutes.
10. Discard the supernatant and wash pellet with 80% alcohol (200uL) and centrifuge at 7600 RCF for 10 minutes.
11. Discard the alcohol and dry the pellet.
12. Dissolve RNA in DEPC treated water.
13. To check if RNA is successfully isolated and is free from genomic DNA contamination run it on 1% agarose gel. Check the RNA concentration using nano drop and proceed with cDNA synthesis.

2.5 cDNA synthesis

(RevertAid First Strand cDNA Synthesis Kit by Thermo Fisher)

Total reaction volume – 10 uL

1. Add the following components in PCR tube:

   Template RNA – 3 µl

   Oligo (dT) Primer - 0.5µl

   Random Hexamer - 0.5µl

Nuclease free water - 2µl

2. Mix it properly and then give a short spin. Put the PCR tube at 65°C for 5 minutes and then immediately place the tube on ice for 2 minutes.
3. Add the following to the tube:

   5X Reaction Buffer - 2µl

   RiboLock RNase inhibitor - 0.5µl

   10mM dNTP Mix – 1µl

   RevertAid M-MuLV RT – 0.5µl
4. Mix it and give short spin. Then incubate in following conditions:

   25°C - 5min

   42°C - 60min

   70°C - 5min

   4°C - infinite hold
5. Dilute (1:4) the cDNA with Milli-Q water and store at -80°C.

2.6 Reverse Transcription-Polymerase chain reaction (RT PCR)

Total reaction volume -10uL

1. Mix well and spin briefly following components before adding to the PCR tubes-

   20X buffer- 0.5µl

   dNTP-1µl

   forward and reverse primer (F+R) -0.4µl

   template cDNA-0.5µl

   MQ water -7.1µl

   Taq polymerase -0.5µl
2. PCR conditions-

   95°C - 2min

   95°C - 20secs

   60°C - 30secs

   68°C - depending on size of gene (1kb/1min),

72°C - 5 min

4°C - Infinite hold

3. Check the PCR product in agarose gel electrophoresis.

2.7 Quantitative PCR (qRT-PCR)

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

1. Add following components after mixing well and short spin Total-

   Master mix - 2.5µL

   Primers(F+R) - 0.25µL

   Template - 0.25µL

   MQ water - 2µL

2. Analyse the data on an excel sheet and plot the graph.

2.8 PCR reaction using GoTaq

1. Add the following components-

   Gotaq master mix – 50uL

   Primers (F+R) – 4uL

   Template – 1uL

   Water – 45uL

2. Set up following cycling conditions in PCR machine -

   95°C - 2min

   95°C - 20secs

   60°C - 30secs

   68°C - depending on size of gene (1kb/1min),

   72°C - 5 min

   4°C - Infinite hold

3. Load 5uL on gel to check. Extract PCR products from manual gel extraction method.

2.9 Manual Gel Extraction

1. Make a small hole using a needle in MCT and then add tiny pieces of aluminium foil in it.
2. Cut the band and collect in above prepared MCT.
3. Place this MCT inside another MCT and tape them together. Then centrifuge at 10000 RPM for 10 minutes at RT.
4. Remove the above MCT and add equal volume of PCI (Phenol : Chloroform : Isoamyl alcohol) in solution.
5. Centrifuge at 10000 RPM for 10 minutes at RT.
6. Take out the aqueous layer in new MCT.
7. Add 1/10th volume of 10M Ammonium acetate and twice volume of Isopropanol. Mix properly.
8. Keep the sample at -80°C for overnight.
9. Next day, centrifuge at 13500 RPM for 30 minutes at 4°C.
10. Wash the pellet with 70% ethanol and centrifuge at 13500 RPM.
11. Dry the pellet and elute in 20uL MQ water. Check on agarose gel.
12. Store at -20°C.

2.10 Ligation reaction

1. Add the following components-

    Salt solution – 0.5uL

    2.1 TOPO Vector – 0.3uL

    Insert – 1.5uL

    MQ water- 0.7uL
2. Incubate it at 25°C for at least 3 hours.
3. Check on gel.

2.11 Bacterial Transformation

1. Take out competent cells from -80°C and thaw them on ice.
2. Add ligated product into 100uL of competent cells.
3. Incubate on ice for 30 minutes and tap in between.
4. Give heat shock at 42°C for 45 seconds.
5. Keep immediately on ice for 5 minutes.
6. Add 800uL of LB media into it.
7. Keep the MCT into 37°C bacterial incubator for 1-2 hours.
8. Centrifuge at 4000 RPM for 5 minutes to pellet down the cells.
9. Discard 700uL of supernatant and then dissolve the pellet into remaining supernatant.
10. Plate the cells on resistance containing LB agar plate.
11. Incubate plate at 37°C overnight.

2.12 Checking for transformed positive clones

1. Take a new Lb agar plate. Make small boxes into it using marker.
2. Pick individual colonies and patch it on small boxes in new LB agar plate.
3. Grow the colonies for overnight.
4. Next day, pick the colonies from each patch and perform PCI method to look for positive clones.
5. Pick the colonies from patch using toothpicks and mix them in 20uL MQ water in PCR tubes.
6. Add 20uL PCI to it and vortex it briefly. Spin down cells.
7. Take the aqueous layer and load it in agarose gel.
8. Mark the patches which show positive shift in their plasmid size.
9. Confirm the positive clone again using restriction digestion reaction.
10. After confirming take positive clone and grow it overnight in LB media containing resistance at 37°C.
11. Make glycerol stock of it.

2.13 Ultracompetent cells preparation

1. Incubate 5mL primary culture of *E.*coli strain DH5α at 37°C overnight.
2. Incubate secondary culture (1% of primary culture) at 18°C till OD600 reach the value of 0.6-0.8.
3. Keep the culture on ice for 10-15 minutes.
4. Centrifuge at 2500x g for 10 minutes at 4°C.
5. Resuspend the pellet in 80mL of TB buffer.
6. Place on ice for 10 minutes.
7. Centrifuge at 2500x g for 10 minutes at 4°C.
8. Re-suspend the pellet in 20mL of TB buffer.
9. Add DMSO to a final concentration of 7%. (1.4 mL DMSO+ 18.6mL of TB buffer)
10. Place it on ice for 10 minutes.
11. Aliquot in 100uL volumes in MCTs and store at -80°C.

(TB Buffer- 10mM PIPES + 15mM $CaCl_2.2H_2O$ + 250mM KCl + 55mM $MnCl_2.4H_2O$, pH-6.8 is set using KOH)

2.14 Plasmid isolation

1. Take 1.5mL of overnight grown culture in 1.5mL MCT and centrifuge it at 13500 RPM for 2 minutes.

2. Discard the supernatant and add remaining culture to the MCT and centrifuge again.

3. Discard again and elute pellet in 100uL Milli-Q water. Vortex to mix pellet until no clumps are visible.

4. Add 100uL freshly lysis buffer, mix and boil at 100°C for 2 minutes.

Composition of lysis buffer is following:

20% SDS - 50µl

0.5M EDTA - 20µl

10N NaOH - 10µl

MQ water - 920µl

5. Add 50uL of 0.5M MgCl2, mix properly and incubate on ice for 2 minutes.
6. Centrifuge for 2 minutes at 13500 RPM and add 50uL of 3M potassium acetate buffer. Mix gently by inverting MCT upside down.
7. Centrifuge again at 13500 RPM for 2 minutes.
8. Take the supernatant in new MCT and add 600uL of Isopropanol, mix well and incubate in ice for 5 minutes.
9. Spin at 13500 RPM for 2 minutes and then discard the supernatant.
10. Wash the pellet with 70% ethanol and centrifuge again at 13500 RPM for 2 minutes.
11. Dry the pellet and elute it in 50uL of Milli-Q water.

2.15 Restriction digestion reaction for probe making

1. Add the following components in the MCTs-

Plasmid - 5uL

MQ water - 3.5uL

10X buffer - 1uL

Restriction enzyme - 0.5uL

2. Incubate the MCTs at 37°C for 1 hour.
3. To check the size fragments of digested run on the agarose gel electrophoresis and determine the orientation of the insert.
4. Put the large-scale digestion reaction using suitable enzymes.
5. Add following components in MCT-

Plasmid - 35µl

10X buffer - 4µl

Enzymes – 1µl

6. Incubate at 37°C for 3 hours or overnight.
7. Run the product on agarose gel and cut the linearized band size from gel and store in new MCT.
8. For fragments purification use gel extraction kits.

2.16 RNA probe reaction

1. Add following components in MCT-

   Template DNA (linearized plasmid) – 500ng-1µg (7µl)

   RNA Polymerase buffer - 1µl

   Dig/Fl - 0.5µl

   RNA polymerase - 0.5µl

   Nuclease free water - 1µl

   Total - 10µl
2. Incubated at 37°C for 4 hours.
3. Add 1uL of 0.5M Tris-EDTA for stopping the reaction.
4. Add 1uL of 5M LiCl for precipitation.
5. Add 0.5uL of glycogen and tap it.
6. Add 18uL of 100% ethanol, mix properly and keep at -80°C.
7. Next day/after one-hour centrifuge at 13500 RPM for 15 minutes.
8. Discard the supernatant and wash the pellet with 100% ethanol.
9. Centrifuge at 13500 RPM for 10 minutes.
10. Dry the pellet and elute in 15uL DEPC water. Store at -80°C.

2.17 Immunostaining

1. Take out the slides from -20°C and keep for drying at 37°C for 30 minutes.
2. Wash the slides with 1XPBS 3 times for 10 minutes each.
3. Meanwhile, prepare 2N HCL (50mL) and prewarm at 37°C.
4. Put the slides in 2N HCL at 37°C.
5. Wash the slides 2 times with Sodium borate (0.1M, pH 8.5) for 10 minutes.
6. Blocking of tissue using 4% BSA in 1XPBST at RT for 2-3 hours.
7. Remove BSA and then add 1° antibody diluted in 1% BSA /PBST (1:1000) and incubate it at RT for 3 hours or overnight at 4°C.
8. Collect 1° antibody and wash the slides with 1XPBST 2 times for 10 minutes each.
9. Add 2° antibody diluted in 1% BSA /PBST (1:1000) and incubate it at RT for 3 hours.
10. Wash the slides with 1XPBST 3 times for 10 minutes each.
11. Dry the slides and mount with DABCO.
12. Take images under confocal microscope.

2.18 Western blotting

❖ Sample preparation-

- Put retina in 2X Laemmli buffer and homogenise properly.
  (2X Laemmli buffer – 4mL of 10% SDS, 2mL of Glycerol, 1.2mL of 1M, pH6.8 Tris-HCL, 2.8mL of MQ, 0.02% Bromophenol blue. Store at 4°C.)
- Vortex briefly and then keep on ice. Repeat it for 10 times.
- Boil the sample at 100°C for 10 minutes.
- Store at -80°C.

❖ Day1-

1. Clean the glass slides.
2. Cast the resolving gel.

(12% Resolving gel – 2.5mL Resolving Buffer, 4mL 30% Acrylamide, 3.3mL MQ Water, 100uL 10% SDS, 100uL 10% Ammonium Persulfate, 6uL TEMED)

3. Let it solidify. Then cast the stacking gel.
   (Stacking gel – 625uL Stacking Buffer, 667uL 30% Acrylamide, 3603uL MQ-Water, 50uL 10% SDS, 50uL 10 % Ammonium Persulfate, 5uL TEMED)
4. Let the stacking gel dry. Take out the samples from -80°C and thaw.
5. Load the samples on gel. Load protein ladder for reference.
6. Run the gel at 25A for approximately 3 hours.
7. Charge membrane with methanol and then wash with water twice. Again, wash with transfer buffer.
8. Make sandwich of gel, membrane and blotting paper in transfer buffer.
9. Set up the transfer using PVDF membrane for 90 minutes at 71V.
10. Remove transfer and block the blot in 10% skim milk for 3 hours at RT or overnight at 4°C. Keep the blots on the rotor at low speed for incubations and on high speed for washes.
11. Wash the blot with 0.05% 1XPBST three times for 10 minutes each.
12. Incubate in 1° antibody for 3 hours at RT or overnight at 4°C.

❖ Day 2

1. Collect the 1° antibody.
2. Wash 3 times with 0.05% 1XPBST for 10 minutes each.
3. Incubate with 2° antibody at RT for 2 hours.
4. Collect 2° antibody.
5. Wash 3 times with 0.3% 1XPBST for 10 minutes each.
6. Keep the blots in 0.05% 1XPBST.
7. Develop blots in ImageQuant LAS4000.

2.19 mRNA *in situ* hybridization on embryos and cryosections

Embryos - Adapted from (Thisse and Thisse 2014)

Retina cryosections- Adapted from (Barthel and Raymond 2000)

Chapter 3: Results and Discussion

3.1 miR-200 family is expressed in zebrafish retina

As a first step of understanding miR-200 family regulation in retina regeneration and its interaction with regeneration associated genes, expression of miR-200 family in zebrafish retina is checked. MicroRNA-200 family expression is analysed using in situ hybridization experiment, which revealed that the microRNAs expresses in zebrafish retina(6dpf). (Fig. 3.1) Probes were made for Pre-microRNA sequence (80-90 bp) because DIG labelled probes can only detect longer sequences. After ISH experiment, it is noted that all three probes (miR-200a, miR-200b and miR-200c) are giving same signal, which means that probes are hybridizing to the same region. LNA probes are not used due high costs.

miR-200a miR-200b miR-200c

Figure 3.1 Mir-200 family expression in zebrafish retina (6dpf)

3.2 Promoter analysis using Bioinformatics approach to find transcription factors binding sites of miR-200 family

For finding potential transcription factors binding sites at promoters of these microRNA sequence, first sequence of these microRNAs with 3000 bp upstream and downstream is obtain from miRbase software and copied in Snapgene software. Then sequence of several transcription factors is searched from previous literature and their known binding motif srquence is checked on the promoter of these microRNAs using copy paste in Snapgene. Below is the table of transcription factors with the number of binding sites. *Sox2* found to have the greatest number of binding sites. (Fig. 3.2)

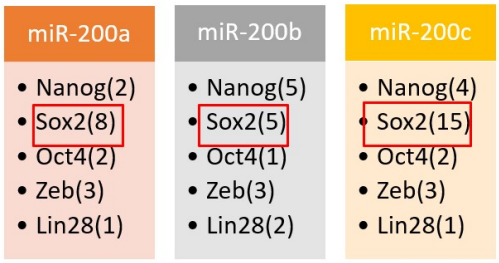


Figure 3.2 Transcription factors on microRNAs promoter

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3.3 miR-200a/b levels elevate upon knockdown of *Sox2*

From promoter analysis, Sox2 found to have highest number of binding sites. To see regulation between *Sox2* and the microRNAs, miR-200a and miR-200b mRNA expression was analysed upon *Sox2* knockdown using qPCR experiment. For knockdown *Sox2,* first retina is injured using 30-gauge needle and morpholino against *Sox2* is injected in retina. Retinae harvested at 2dpi. It is found that *Sox2* knockdown results in increased expression of miR-200a and miR-200b, which suggest that *Sox2* transcription factor is negatively regulating the microRNAs. (Fig. 3.3)

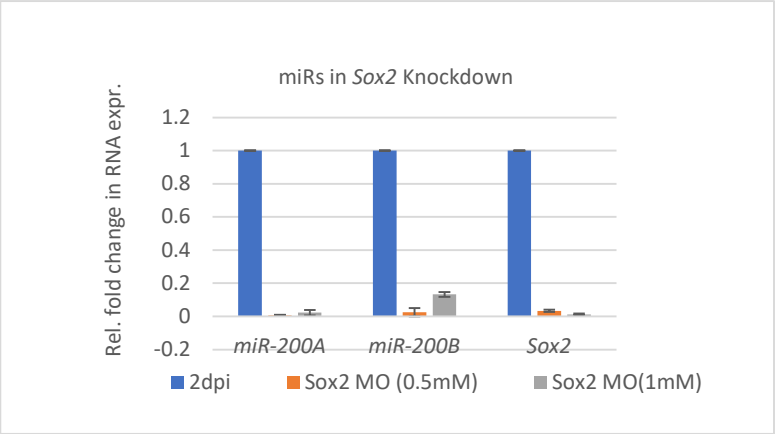
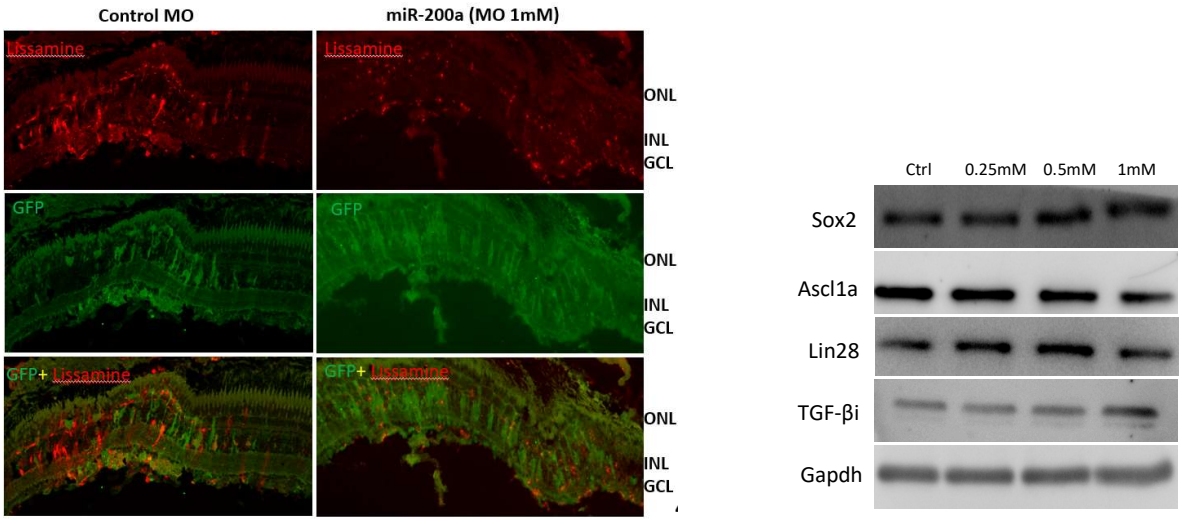


Figure 3.3 Sox2 knockdown results in increased expression of miR-200a and miR-200b.

3.4 miR-200a knockdown results in increased proliferation

Next, we knockdown microRNA-200a to see what happens to the proliferation status. Retinae from *1016tuba1a:GFP* transgenic line were harvested at 4dpi peak proliferation time point for immunostaining experiment. We observed increased proliferation. (Fig. 3.4 a) To see what are the background players of increased proliferation, western blotting experiments were done at 2dpi at the start of proliferation. Sox2, lin28 pluripotency factors were found to be upregulated. TGF-βi was also found to be upregulated. TGF-β signalling is pro-proliferative during zebrafish retina regeneration(Sharma et al. 2020). These factors may explain the increased proliferation. Ascl1a is downregulated, which is surprising because it is reported to be necessary for zebrafish retina regeneration. It expresses as early as 6 hpi and facilitates de-differentiation of Muller glia. (Fig. 3.4 b)



(a) (b)

Figure 3.4 Effect of microRNA-200a knockdown. (a) Immunostaining (b) Western blotting

3.5 Target analysis using Bioinformatics approach to find targets

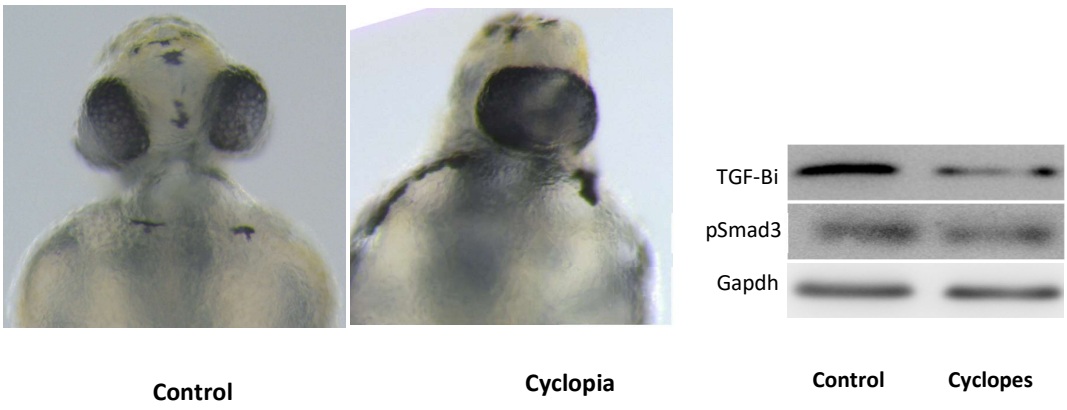
Target were found out from Targetscan bioinformatic tool and then data was fed into Gene profiler tool to sort in associated signalling pathways. We found below target pathways. (Table 3.5.1)

Target pathways of miR-200a	Target pathways of miR-200b and miR-200c
Wnt signaling pathway	cell population proliferation
ErbB signaling pathway	regulation of cell population proliferation
Hedgehog signaling pathway	ErbB signaling pathway
Notch signaling pathway	Wnt signaling pathway
Cell cycle	Cell adhesion molecules (CAMs)
regulation of cell population proliferation	Hedgehog signaling pathway
cell population proliferation	Cell cycle
cell-cell adhesion	Signaling by TGF-beta Receptor Complex
Signaling by TGF-beta family members	

Table 3.5 Target pathways of miR-200 family

3.6 Blocked of TGF-β signalling in embryo gave cyclopia phenotype

From all above pathways, TGF-β pathways chose to proceed further. Recently, in one of our lab paper TGF-β signalling found to be downregulate miR-200a and miR-200b. Upon blocking TGF-β signalling using SB431542 drug, miR-200a and miR-200b levels decreases. Upon injecting TGF-β protein, levels of microRNAs downregulated(Sharma et al. 2020). So, to investigate whether the same kind of interaction exist between TGF-β signalling and microRNAs, we blocked TGF-β signalling using SB431542 drug in the embryo. Freshly fertilized embryos were kept in 40uM SB431542 drug for 2 days. Everyday water was changed and drug was added. Embryos were harvested at 48hpf. Cyclopic embryos with one single big eye were observed at 48hpf. (Fig. 3.6 a) Inhibition of TGF-β is checked using western blotting analysis. TGF-βi and pSmad3 protein levels were downregulated showing that our drug is blocking TGF-β signalling. (Fig. 3.6 b)



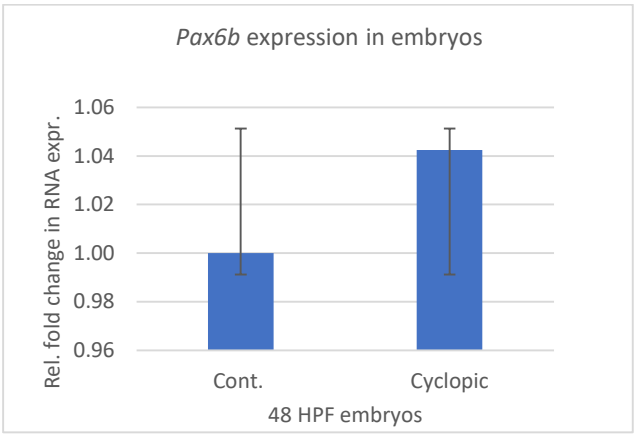
(a) (b)

Figure 3.6 a Cyclopia phenotype in TGF-β blocked condition

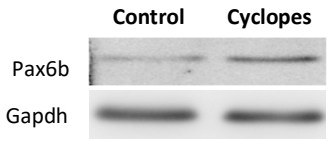
Figure 3.6 b SB431542 drug is blocking TGF-β signalling

3.7 TGF-β signalling regulates miR-200a/b expression

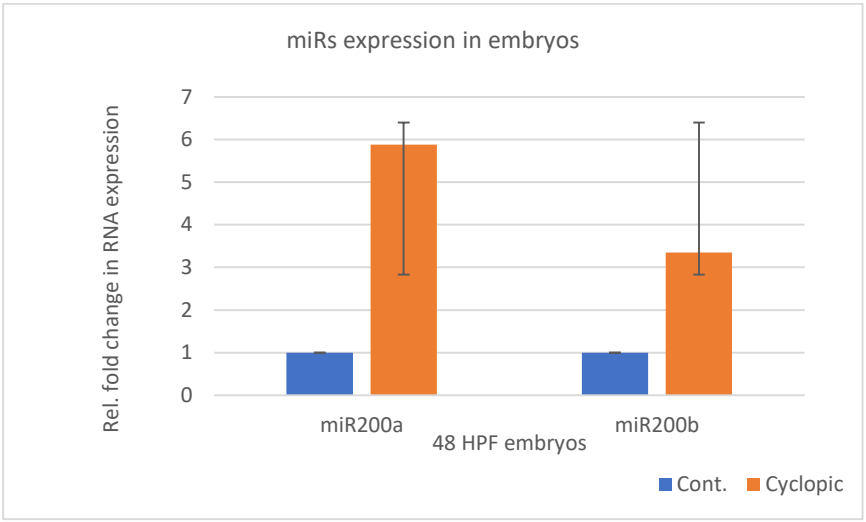
Next, we checked for expression of Pax6b because it has been shown to cause cyclopia in the embryo. Higher levels of Pax6b prevents single eye field's separation in two eyes in the embryo. qPCR experiment showed higher levels of Pax6b. (Fig. 3.7 a) Western blotting analysis also showed higher protein levels of Pax6b in cyclopic embryos. (Fig. 3.7 b) To checked what happens to miR-200a and miR-200b in the TGF-β blocked condition qPCR experiment was done. We found upregulation of miR-200a and miR-200b in TGF-β blocked embryos. (Fig. 3.7 c)



(a)



(b)



(C)

Figure 3.7 a Pax6b qPCR experiment

Figure 3.7 b Pax6b western blotting

Figure 3.7 c miR-200a and miR-200b RNA levels using qPCR

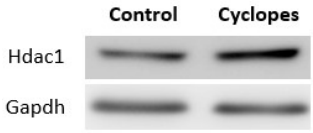
3.8 HDAC1 overexpression also induces cyclopia phenotype

Western blotting analysis also revealed upregulation of HDAC1 protein in cyclopia embryos. (Fig 3.8 a) So, next we assessed whether *hdac1* can also induce cyclopia in embryos. *hdac1* mRNA injected at the single cell stage embryo. After, 72 hpf cyclopes were observed. Here, 250ng, 500ng, 750ng and 1000ng mRNA injected but only 250ng and 1000ng injected survived. Therefore, we images are shown of those survived embryos. (Fig. 3.8 b)

Control 72 HPF HDAC1 mRNA 250ng HDAC1 mRNA 1000ng

(a)

Figure 3.8 a HDAC1 protein levels revealed in cyclopes



(b)

Figure 3.8 b HDAC1 mRNA injected embryos shows cyclopia.

Conclusion

From this study of Investigating role of miR-200 family during zebrafish retina regeneration, we could conclude that microRNA-200 family plays an important role in inducing adequate number of progenitors Muller glia during zebrafish retina regeneration as knockdown of these miR-200a results in increased proliferation. We found that it interacts with Sox2, lin28 pluripotency factors and TGF-β signalling which is a pro-proliferative pathway during retina regeneration in zebrafish. May be Sox2 acts as transcription factor on the promoter of miR-200 family as we observed knockdown of Sox2 showed elevated levels of miR-200a and miR-200b. Also, Sox2 has highest number of binding sites on the promoter of miR-200 family. When TGF-β signalling blocked in the embryo, they showed cyclopia phenotype. In the TGF-β blocked condition miR-200a and miR-200b levels were upregulated, also western blotting analysis of miR-200a knockdown showed increase in the protein levels of TGF-β, suggesting they negatively regulate each other's expression.

In nutshell, it can be concluded that miR-200 family induces adequate number of MGPCs cells by interacting with regeneration associated genes.

Future Perspective

My study gives some preliminary insights of miR-200 family's role during zebrafish retina regeneration. My thesis mainly focused on miR-200a family knockdown. A lot more experiments are need to be done to get a complete picture of miR-200 family. Following key experiments could be done to take this work forward. Immunostaining experiments upon knockdown of miR-200b and miR-200c knockdown to assess proliferation status is remaining. Western blotting analysis to know interaction of miR-200b and miR-200c with regeneration associated genes. In situ hybridization experiments with BrdU and PCNA staining to know whether they are expressing in proliferating or non-proliferating cells is needs to be done for miR-200 family using LNA probes.

Predicted transcription factors on the promoters of miR-200 family are required to confirm using ChIP experiments. Predicted targets of these miR-200 family also need to be confirm using cell culture-based assay. Also, expression of this miR-200 family could be assessed at various stages for example de-differentiation, proliferation and re-differentiation to know what kind of role they may be playing at various time points.

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