Investigating the role of *miR-143* & *miR-145* in the Course of Zebrafish Retina Regeneration

Ajay Kumar
MS15039

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

Indian Institute of Science Education and Research
Mohali
MAY 2020

Certificate of Examination

This is to certify that the dissertation titled " INVESTIGATING THE ROLE OF ***miR-143*** & ***miR-145*** IN THE COURSE OF ZEBRAFISH RETINA REGENERATION" submitted by Mr. Ajay Kumar(Reg.No. MS15039) 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.Santosh Satbhai **Dr. Sharvan Sehrawat** **Dr. Rajesh Ramachandran (Supervisor)**

Dated: May 4 , 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.

Ajay Kumar

(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)

ACKNOWLEDGEMENT

I duly acknowledge the efforts put in by the faculty members of IISER Mohali, who have guided and helped me in the course of last five years.

I thank my parents, Mrs. Vidhya Devi and Mr. Ram Chander for believing in me, for not losing faith in me and for being constant supporters in the toughest of circumstances. I thank my brother, Narender for being there and encouraging me to 'raise the bar'. I thank my friends, Abhishek, Sapna, Bindia, Ashish and Sharanya for hearing me out and bracing through thick and thin. I also thank Adeeb, Rajesh, Paresh, Dharm and Ravi for being a wonderful company.

I am immensely grateful to Dr. Rajesh Ramachandran for accepting me as his Master's thesis student. Through his courses, his valuable feedbacks and discussions in lab, the anecdotal narrations and his sense of humor, he has inspired me to be a thorough academician, professional and a humble person. I thank him for being a great support. He has been an attentive ear not just through the course of this project, but also during the many dilemmas I have faced in the last 5 years. He truly is a great mentor.

This project would have been impossible without the constant support and encouragement of Rajesh's lab members. I am extremely thankful to Ms. Poonam Sharma for her undying patience, generous encouragement and her guidance for mastering the techniques in the lab.

CONTENTS

LIST OF FIGURES

ABBREVIATIONS

1. MG-Müller Glia
2. MGPC-Müller Glia Derived Progenitor cell
3. R-Rods
4. C-Cones
5. A-Amacrine cells
6. BP-Bipolar cells
7. H-Horizontal cells
8. GC-Ganglion cells
9. ONL-Outer nuclear layer
10. INL-inner nuclear layer
11. GCL-Ganglion cell layer
12. Hdac- Histone deacetylase protein
13. *Hdac*- Histone deacetylase mRNA
14. BrdU-Bromodeoxyuridine
15. PCNA-Proliferating Cell Nuclear Layer
16. MO-Morpholino-modified antisense oligonucleotides
17. dpi-days post injury
18. Ascl1a - Achaete-scute homolog 1 zebrafish protein
19. *ascl1a* - *Achaete*-scute homolog 1 zebrafish mRNA

20. RT-PCR-Reverse Transcription PCR
21. qPCR-Quantitative PCR

Abstract

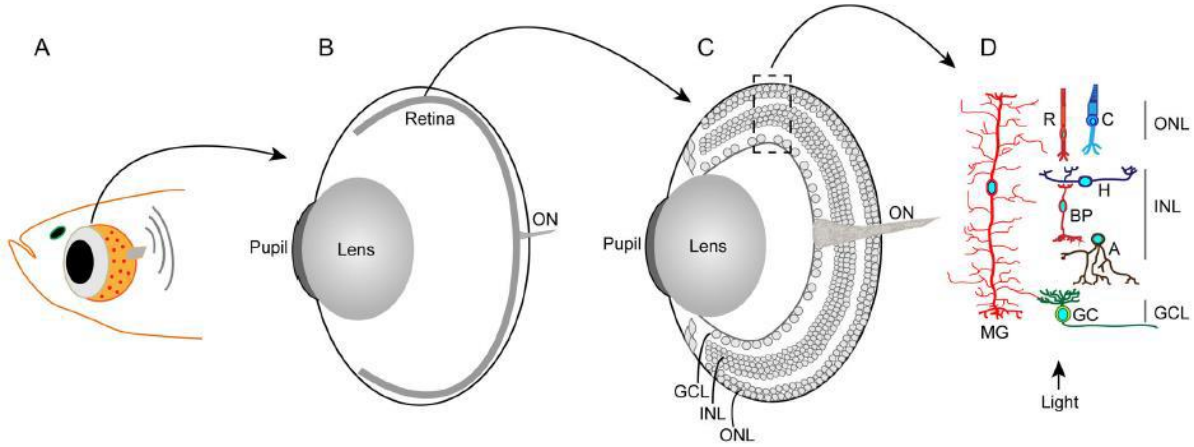
Sight, unarguably the most important of all senses is what allows us to understand the world around us. A human retina if diseased or damaged remains incurable in its ability to restore lost vision regardless of ample scientific advances and breakthroughs. On the contrary, Zebrafish, a teleost fish exhibits extraordinary capacity and ability to regenerate and regain visual function post injury. In Zebrafish, a dominant form of glial cells should be the only type of macroglial cells, Müller Glia are responsible for retina regeneration. These cells respond to injury and undergo reprogramming into a proliferative population. Here in this work, we aim to explore the role of *miR143* and *miR-145* in the retina regeneration process. We looked at the levels of molecular players of regeneration upon *miR-143* knockdown. We observed *miR-145* gene expression in retina, brain and tail region at 24hpf, 48hpf and 6dpf .We observed relatively strong expression in optic rediion and tail rediion.. Even within the eye, its expression is predominantly in retina. We use MO for knocking down the *miR-143* and *miR-145*. We observed decrease in proliferation cells in 1016tuba:gfp in which gfp is marking proliferating Muller glia cells. Also after knocking down *miR-143*, we looked at protein level of some RAG and epigenetic modifier like hdac1 and it doesn't show downregulation. We observed decrease in the significant expression of Ascl1a, lin28 ,Sox2, Lin28a, Myc a/b, H3k27me3, H3k27Ac, ptenb,Oct4, H3k4me3, akt, *p*-Smad3 and β - catenin after knocking down of *miR-143*. Ascl1a, master regulator of retina regeneration, level went down after knocking down *miR-143* which is in accordance with decline in proliferation at 4dpi in immunostaining results. Taken together, our results show the importance of *miR-143/145* in MG reprogramming and proliferation.

CHAPTER 1: INTRODUCTION

We understand the world around us best using the power of sight. Unfortunately, given the developments, diseased or impaired human retina struggles to recover the lost vision. Fortunately, a teleost fish, Zebrafish (*Danio rerio*), has the capacity and ability to regenerate and regain visual function after injury (Fausett & Goldman, 2006). In mammalian species, this capacity is contrasted by the development of glial scars. Zebrafish shows a strong tendency for Central Nervous System (CNS) regeneration. The retina, a tissue layer that lies at the back of the eye, is a plain, accessible part of the CNS. Consequently, the retina is used to study the regeneration of the CNS. In Zebrafish, retina regeneration mostly depends on Müller Glia, the dominant form of glial cell in the retina. Müller glia responds to retinal injury by re-entering the cell cycle and thereby producing multipotent progenitors that can regenerate cells that are damaged. These progenitors divide asymmetrically to giving rise to Muller glia and neurogenic cluster, which migrate to ablated layers and redifferentiates to restore the damage . Müller glia acquire stem cell-like properties (MG-derived progenitor cells) with the help of various contributing factors and changes in the gene expression.

1.1 Zebrafish retinal architecture

The vertebrate retina consists of three distinct nuclear layers, the outer nuclear layer (ONL), the inner nuclear layer (INL), and the ganglion cell layer (GCL). The ONL consists of photoreceptor cells - rods (R) and the cones (C). The ONL senses light and transmits the information through INL to ganglion cells (GC). INL consists of the interneurons (bipolar, amacrine, horizontal) and Müller cells . Throughout these layers one type of glial cell and six types of neuronal cells occur. Müller Glia is the only type of glial cell which spans all layers of retinal cells. This structure allows them to interact with neighbouring neurons; thus, it helps to move molecules through the different layers of the retina. It also monitors retinal homeostasis and contributes to the structure and function of the retinal system(Goldman, 2014). In regeneration MG play an indispensable role.



Reference: Wan J, Goldman D. (2016) Curr Opin Genet Dev. 40:41-47

Figure 1.1.1 Zebrafish retinal anatomy and structure. The image of zebrafish eye and retinal architecture, showing all its layers and cells, namely outer nuclear layer(ONL) which contains rod(R) and cone(C) photoreceptors, inner nuclear layer(INL) housing amacrine(A) cells, bipolar(BP) cells, horizontal(H) cells and Muller Glia (MG),and ganglion cell layer (GCL)having Ganglion Cells(GC)

1.2 Regeneration mechanism

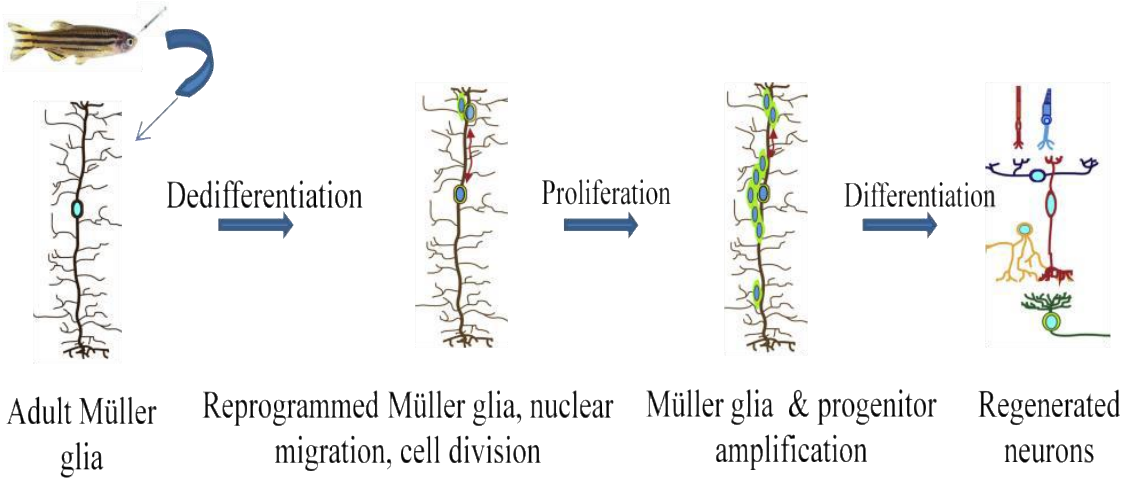
Injury can be induced mechanically (using needle poke)(Fimbel, Montgomery, Burket, & Hyde, 2007),by toxic chemicals or toxic genes(Vihtelic & Hyde, 2000), strong or UV light(Wu et al., 2001), and laser ablation(Yao et al., 2016) MGs evoke a gliotic response (over-expression of tubulin proteins such as glial fibrillary acidic protein (GFAP) and undergo hypertrophy) after detecting damage to the retina. The gliotic response comes with a reprogramming event that enables MGs to have stem cell-like properties.

Retinal regeneration led by Müller Glia requires three major overlapping steps:

i. Dedifferentiation: upon injury, Müller Glia cells attain stem cell-like properties

ii. Proliferation: Interkinetic nuclear migration to the ONL and asymmetric cell division

iii. Re-differentiation: Migration of progenitors to various retinal layers and differentiate into different neuronal cell types



Reference: Wan J, Goldman D. (2016) Curr Opin Genet Dev. 40:41-47

Figure 1.2.1 Injury response by Müller glia. Upon injury, adult Muller glia undergoes reprogramming events to acquire stem cell-like properties. This further proliferates and migrate to different cell layers and form different retinal cell types.

Glycogen synthase kinase 3β (Gsk3β)–β-catenin, Notch, Mapk – Erk and Jak – Stat signalling pathways are some of the early signalling events evoked in zebrafish following a mechanical injury. Wnt signalling is activated on zebrafish retinal injury to cause MG reprogramming and proliferation (Goldman, 2014). Wnt expression and stabilisation of β-catenin occurs in MGPCs. The let-7 miRNA signalling and Dkk signalling are two inhibitory pathways required to maintain an MG quiescent state(Ramachandran, Fausett, & Goldman, 2010). Notch signalling also has an inhibitory function that suppresses the number of MGPCs, which is apparent from an increase in the expression of Notch's target. The pro-neural transcription factor Ascl1a (Achaete-scute homolog 1) is one of the key players which suppress differentiation programs and promote proliferation(Wan, Ramachandran, & Goldman, 2012).

1.3 EPIGENETICS AND ITS ARMY

The world around us like the food we consume, the consistency of the air, the contaminants that we are exposed to, and so on, play a key role in shaping our genes. These factors per se exert control over gene expression by factors other than the DNA. Studying changes in a gene's expression due to causes other than changing the genetic code itself is called epigenetics.

Epigenetic modifications can be divided into three categories: methylation of DNA, alteration of the histone and silencing of genes associated with RNA. The process that adds a methyl group to DNA regions called CpG islands (C next to G) is called DNA methylation, mediated by the enzymes called DNA methyltransferases which add methyl groups to these sites causing changes in their structure, thus making the region inaccessible to the transcription machinery and thus silencing of the gene .The silencing of RNA-associated genes can be caused by siRNA, miRNA and other non-coding RNAs. These RNAs bind to and kill the complimentary mRNA transcript, or prevent translation.

1.3.1 Histone modifications

Unless the DNA is spread in each of our cells one could travel 300 times from Earth to the sun and back. Each human diploid cell contains almost 2 m of DNA in length. And how is that packed into a cell sized micro-meter? The solution lies in how the DNA is packaged within the nucleus. DNA wraps around the proteins called histones, 1.65 times to form nucleosomal structures.
The chromatin makes up these nucleosomes. Each nucleosome is wrapped around a 'histone octamer' with DNA.Histone octamer consists of pairs of H2A, H2B, H3 and H4, the fundamental, positively charged histone proteins. The positive charge helps to bind the negatively charged DNA and thus compact it.
Different post-translational covalent modifications such as methylation, acetylation, ubiquitylation, and phosphorylation are made on these histone proteins by epigenetic modification to modify the gene expression with which they are associated. These covalent modifications induce a change in the structure of the chromatin as indicated to control gene expression.

1.3.2 Histone methyl transferases

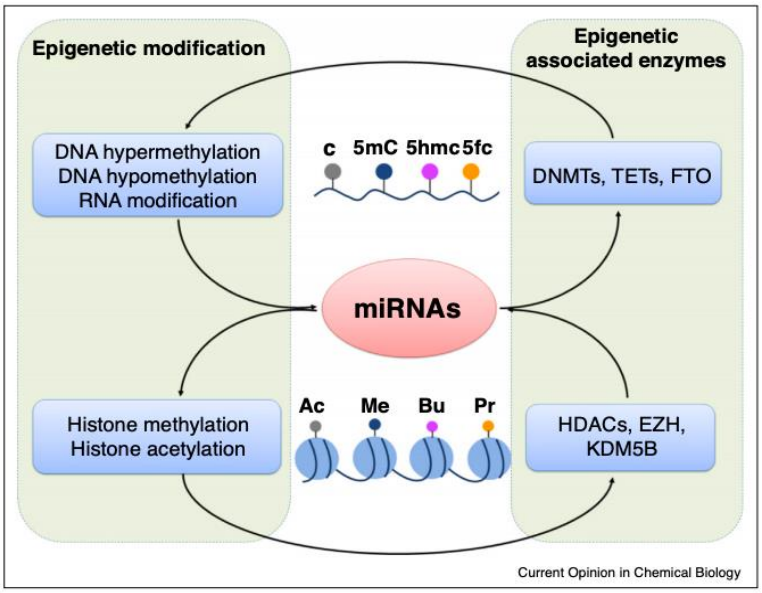
Histone methylation is the method of transfer by histone methyl transferases (HMT) of the methyl groups from S-adenosyl methionine (SAM) substrates to lysine or arginine residues of histone tails. Various HMTs exist to alter specific concentrations of lysine and arginine.

1.3.3 Histone deacetylases (HDACs)

Histone deacetylases (HDACs) are a class of enzymes that extract acetyl groups from the histone lysine amino acids, thereby allowing tighter DNA packing. HDACs make the arrangement of the chromatin a more compact type of heterochromatin, rendering the genes unavailable for transcription. HDACs play an important role in controlling the compaction of chromatine by histone deacetylation. Although many HDACs are widely expressed, little is known about their direct transcription targets and how they control the expression of tissue-specific genes.
HDACs are classified into three main classes – Class I, Class II and Class IV. Class I HDACs (HDAC 1,3 and 8) are present only in the nucleus, while Class II HDACs (HDAC 4, 5, 6, 7, 9 and 10) exist both in the cytoplasm and in the nucleus. Only HDAC11 is class IV and is found in nucleus(Bhalla, 2005)(NCT00140179, 2005).

1.4 Cross talk between microRNAs and epigenetics

By targeting key enzymes responsible for epigenetic reactions such as DNA methyltransferases (DNMTs), histone deacetylases (HDACs), and histone methyltransferases, miRNAs can act as epigenetic modulators(Kwa & Jackson, 2018; Li et al., 2019; Sato et al., 2016).In addition, epigenetic machinery also controls the expression of miRNAs, including DNA methylation, RNA modification, and histone modification. The miRNA-epigenetic feedback loop forms the reciprocity link between miRNAs and epigenetic regulation. microRNA epigenetic feedback loop modulation and its cellular role has emerged as a novel mechanism for regulating the cell cycle, including cell proliferation(Kiga et al., 2014), apoptosis(Matsushima et al., 2011), and differentiation(Chen et al., 2006).

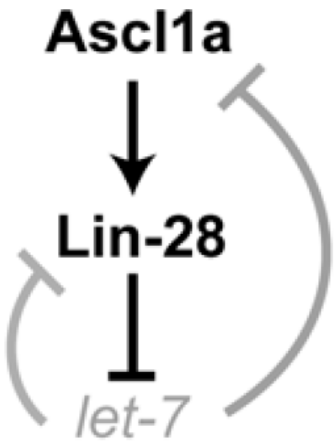


Reference : *Yao Qian et al. Current Opinion in Chemical Biology 2019,51:11–17*

Figure 1.3.1 Schematic illustration of the miRNA-epigenetic feedback loop. miRNAs are regulated by epigenetic regulators, including DNA methylation, RNA modification and histone modification. Epigenetics-associated enzymes are also under the control of miRNA regulation.

1.5 Role of microRNAs in zebrafish retina regeneration

Ascl1a stimulates the expression of lin-28 which suppresses the expression of let-7 miRNA (associated with differentiation).



Reference: Ramachandran et. al Nature Cell Biol. 12:1101–1107

Figure 1.5.1 Gene regulatory loop between Ascl1a,Lin28 and *let7* in MGPCs

.

One of the recent papers of our lab(*Sharma et al. Life science alliance ,2019*) showed that Oct4 is very crucial for retina regeneration in Zebrafish and knocking down Oct4 expression induced *miR-143* and *miR-145* expression . This indicates that Oct4 may exert its effect partly by regulating the levels of *miR-143* and *miR-145*.

Induced expression of *miR-143* and *miR-145* in oct4 knockdown, causes *mir-143* and *mir-145* as an interesting molecule as studied in reference to retinal regeneration.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animal maintenance

- Zebrafish(*Danio rerio*) are maintained in an automated water circulation system at 26°C–28°C (14:10 h light/dark cycle).
- The transgenic fish used in this study were previously classified as 1016tuba1a:GFP.
- pH: physiological pH

2.2. Retina dissection for RNA isolation/ western blotting

- Fish had been anesthetized with tricaine methanesulfonate.
- Retina were injured using 30 gauge needle
- The eyes were dissected using steel forceps and a needle at the right time after injury.
- Dissections were carried out in 1X PBS (Phosphate Buffered Saline) for retina harvesting. Lens was detached for RNA separation, and dissection of the retina.
- If the retinae are to be used for RNA isolation, it is placed in Trizol and suspended in Laemmli buffer at -80 ° C if used for western blotting.

2.3. Microscopy

- Bright field microscope (Zeiss) was used for retinal dissection.
- For imaging purposes Confocal microscope (Nikon) was used.

2.4 Tissue fixation and sectioning

1. For tissue fixation, the eyes whose lenses were removed were taken and placed in 4% PFA at 4 ° C overnight.

2. The next day, serial washings of the fixed tissue were performed on a rotor at RT for 45 minutes each:

1mL of 5% sucrose
800µL of 5% sucrose and 400µL of 20% sucrose
500µL of 5% sucrose and 500µL of 20% sucrose
400µL of 5% sucrose and 800µL of 20% sucrose
1mL of 20% sucrose.

3. 500µl of OCT is added and rotated it for 30 min.

4. Within small cubes made of aluminium foil, the tissue is embedded within OCT, and then frozen at-80$_o$C before sectioning.

5. The blocks are cut into cryostats and then the pieces(12µm thickness) are collected on super frost plus slides and they are dried overnight and then stored in - 20$_o$C.

The composition of solutions used are:
a) 4% PFA in 1X Phosphate buffer (made DEPC water) :

- 2g PFA
- 5mL of 10X phosphate buffer
- Make up the volume to 50mL with DEPC water.
- Dissolve it by keeping in 65$_o$C and constant shaking

b) 5% sucrose:

- Dissolve 2.5g sucrose in 50mL of autoclaved water. Store at -20$_o$C.

c) 20% sucrose:

- Dissolve 10g sucrose in 50mL of autoclaved water. Store at -20$_o$C.

2.5 RNA isolation

1. Dissected retinae were collected in MCT with 200µL of Trizol.

2. They were properly homogenised using a pipette.

3. 0.2 volume (40µL) of chloroform was added.

4. The solution was gently shaken for 15–20 seconds.

5. The MCT was kept at room temperature (RT) for 5 minutes.

6. Centrifuged for 10 minutes at 8,000rpm at 4°C.

7. Using a cut tip, 20µL of the aqueous layer was transferred into fresh MCT.

8. Equal amount of Isopropanol was added.

9. Stored at -80°C overnight (or in ice for 20 minutes).

10. Centrifuged at 13,000 rpm, 4°C for 10 minutes.

11. The supernatant was discarded and washed with 80 percent ethanol.

12. At RT, pellet was completely dried and then eluted in DEPC treated water.

13. Eluted RNA was checked on 1% agarose gel by gel electrophoresis and then, stored it in -80°C

2.6 cDNA synthesis

(Kit used – RevertAid First Strand cDNA Synthesis Kit by Thermo Fisher)

1. Following reagents were added into a sterile tube:

   a. Template RNA 2.5 µL

   b. Primer (Oligo (dT)18 +Random Hexamer) 0.25+0.25 µL

2. The content was gently mixed and then incubated for 5 minutes at 65 ° C. The tubes were then transferred back onto the ice.

3. Following components were added in the indicated order:

5X Reaction Buffer	1 µL
RiboLock Rnase Inhibitor	0.25 µL
10mM dNTP Mix	0.5 µL
Revert-Aid M-Mul VRT	0.25 µL
Total volume	5 µL

4. The contents were mixed, briefly centrifuged and incubated at the following temperatures:

- 5 minutes at 25 °C
- 60 minutes at 42°C
- 5 minutes at 70°C

5.The cDNA was then diluted with autoclaved Milli-Q water and stored at -80°C

2.7 Reverse transcription-Polymerase chain reaction (RT-PCR) using Taq Polymerase

1. The reaction mixture (10 µL volume)

10X buffer	1 µL
2.5mM dNTPs	0.5µL
Primers (forward + reverse)	0.2µL
Taq polymerase	0.2µL
Template	(as per standardized volume)
Water	Rest

2. Reaction Parameters

Enzyme activation	95°C for 2 min
DNA denaturation	95°C for 15 sec
Primer annealing	62°C for 30 sec

Elongation	68°C for 30 sec
Final elongation	72°C for 5 min
Infinite hold	4°C

3. PCR products were then checked on 1.5% agarose gel by electrophoresis

2.8 Plasmid isolation

1. 5mL of culture was centrifuged at 13400rpm for 2 min at RT
2. The supernatant was discarded and the pellet was dissolved in 100µL of autoclaved MQ.
3. 100µL of freshly prepared lysis buffer was added and gently tapped. For 1mL of lysis buffer, add 50µL of 20% SDS solution, 20µL of 0.5M EDTA and 10µL of 10N NaOH in 910µL of water.
4. The samples were boiled at 100°C for 2 minutes (until the solution becomes clear).
5. 50µL of 0.5M MgCl2 was added. Tapped and kept on ice for 2 minutes.

   For 100mL, 60mL of potassium acetate, 11.5mL glacial acetic acid, and 28.5mL H_2O were mixed. Stored at 4°C.
6. Immediately tapped and centrifuged at 13400rpm for 2 min, RT.
7. The supernatant was transferred into another MCT containing 600µL of Isopropanol.
8. Kept on ice for 5 min.
9. Centrifuged at 13400rpm for 2min, RT.
10. 70% ethanol wash and the pellet was dried completely
11. Pellet was dissolved in 50µL of autoclaved MQ.
12. Stored at -20°C

2.9 Plasmids and restriction digestion for probe making

1. Following components were added in a reaction mixture:

Plasmid	5 µL
MQ water	3.5µL
10X buffer	1µL

(Depending on the restriction enzymes used)

Restriction enzymes - 0.5µl

1. Incubate at 37°C for 1 hour
2. On an agarose gel it is tested to confirm the size of their digested fragments and to determine the insert's orientation.
3. Based on the orientation, large-scale digestion is performed with sufficient restriction enzymes to linearize the plasmid using the **above** enzymes.
4. The following components are added in an MCTs:

Plasmid	35 µL
10X buffer	4µL
Enzymes	1µL

5. Incubated at 37^{o}C for 3 hours or overnight.
6. Loaded the digested products on an agarose gel and excised the linearized DNA fragments from the agarose gel.
7. The gel is extracted and purified.

2.10 RNA probe reaction

1 .Following reaction was set up:

RNA Polymerase buffer	1 µL
Template DNA (linearised plasmid)	500ng to 1µg (7µL)
Dig /Fl	0.5µL
RNA polymerase	0.5µL
Nuclease-free water	1 µL
Total	10µL

2 .Incubated at 37°C for 4 hours.

3 .Added following:

0.5M Tris-EDTA	1µL
5M LiCl	1µL
10mg/mL glycogen	0.5µl
Absolute ethanol	17.5µL

4. Tapped to mix well, kept at -80°C overnight for precipitation.

5. Centrifuged at 4°C for 30 min at13,000 rpm.

6. The supernatant is discarded and it is washed with 70% ethanol (200µL) and then centrifuged for 10 min at 13,000rpm, 4°C.

7. Dried and dissolved the pellet in 10µL of DEPC treated water.

8. Validated the probe on an agarose gel and Stored at -80°C.

9. Tapped to mix well, kept at -80°C overnight for precipitation

10. Centrifuged at 4°C for 30 min at13,000 rpm.

11. The supernatant is discarded and it is washed with 70% ethanol (200µL) and then centrifuged for 10 min at 13,000rpm, 4°C.

12. Dried and dissolved the pellet in 10µL of DEPC treated water.

13. Validated the probe on an agarose gel and Stored at -80°C.

2.11 Western blot

Sample preparation

- Retina were collected in 2X Laemelle Buffer and homogenized properly. (2X Lamelle Buffer – 4mL of 10% SDS + 2mL of Glycerol + 1.2mL of 1M Tris-HCl (pH 6.8) + 2.8mL of MilliQ-water + 0.02% of Bromophenol blue. Store at 4°C).
- Brief vortexing and ice incubation was given for 10 times .
- Centrifuged at 6000 RPM for 10 minutes.
- Transferred supernatant to a fresh MCT.
- Heated the sample at 100°C FOR 10 minutes.
- Stored in -80°C

Day 1

1.Resolving gel was casted.

(12% Resolving gel –2.5mL Resolving Buffer + 4mL 30% Acrylamide 3.3mL MilliQ-water + 100µL 10% SDS + 100µL 10% Ammonium Persulfate + 6µL

TEMED)

2.Stacking gel was casted

(Stacking gel – 625µL Stacking Buffer + 667µL 30% Acrylamide + 3603µL MilliQwater + 50µL 10% SDS + 50µL 10% Ammonium Persulfate + 5µL TEMED)

3.Samples were taken out from -80°C and thawed.

4.Samples were loaded on the gel along with Protein Ladder.

5. The gel was run for 3 hours at 80V.

6. The transfer was set up using PVDF membrane for 70 minutes at 70V. (PVDF membrane was charged with methanol for 1 minute and then washed with MilliQ- water).

7. Blot was then blocked in 10% skim milk for 1 hour.

8. 0.01% PBST (For 200mL of 1X PBST, add 200µL of TWEEN20) washes were given for 15minutes, four times.

9. Incubated blot in the primary antibody of choice overnight at 4°C.

Day2

1. 0.01% PBST washes were given for 15 minutes each, four times.
2. Incubated blot in secondary antibody for 1 hour at RT.
3. 0.01% PBST washes were given for 15 minutes each, four times.

Blot was then developed in ImageQuant LAS4000.

2.12 Immunostaining

Day 1

- Slides were taken out from -20°C and dried at 37°C for 1 hour.
- 1XPBS washes for 10minutes, twice.

- Fixed tissue with 4% PFA solution for 20minutes (This step is done only in the case ofMO slides).
- Treated the slides with 2N HCl (pre heated to 37°C) for 20 min. (*Use Sodium Citrate for epitope retrieval while performing beta-catenin immunostaining).
- Washed the slides with 0.1M Sodium borate solution twice for 10 minutes each.
- Blocked the sections using 6% BSA in 1X PBST (1XPBS + 0.1% Triton X) for atleast 30minutes.
- Overlaid the slides with primary anitbody of choice (PCNA, BrdU, β-catenin or Hdac1), 500µL per slide (Antibody is diluted in 1:500 ratio in 1% BSA in 1XPBST).
- Incubated the slides at 4°C overnight.

* To perform immuno-histochemistry using beta-catenin antibody, heat mediated antigen retrieval is done. For this, 10mM of Sodium Citrate (2mL in 200mL of MilliQ-water) is preheated for about 20 minutes till it starts boiling. And then, slides are kept in this boiling Sodium Citrate for 20 minutes.

Day 2

- Washed slides with 1X PBST, 3 times for 10 minutes each.
- Overlaid the slides with desired secondary antibody, 500µL per slide (Antibody is diluted in 1:1000ratio in 1% BSA-1X PBST solution).
- Incubated slides for 3 hours at RT.
- Washed slides with 1X PBST 3 times for 10 min each.
- Washed slides with autoclaved MilliQ-water 3 times for 10 min each.
- Dried slides for 1 hour at RT.
- Mounted slides in DABCO and left at RT in dark overnight.
- Stored slides at -20°C.

2.13 Whole-mount *in situ* hybridization

DAY 1

After rinsing and refixation, the embryos were prehybridized for 2h at 56°C and then incubated in hybridization buffer (TEN solution, 50% Formamide, 5% Dextran Sulphate, 1% RMB blocker and 500ng-1µg probe) overnight.

DAY 2

After incubation in hybridization buffer, the embryos were washed twice in Solution I (50% formamide, 2XSSC) at 65°C and thrice in Solution II (2x SSC) at 37°C. Following this embryos were RNase A treated, washed twice with 2x SSC at 37°C and with 1x PBST at RT. Then embryos were kept in blocking solution (1X Maleate,0.05% Triton and 1% RMB blocker) for 2 hr at RT and incubated overnight at RT with anti-DIG-AP antibody at a 1/2500 dilution in blocking solution.

DAY 3

After washing embryos twice with 1X Maleate and twice with Genius 3 buffer(100mM Tris-HCl,100mM NaCl and 50mM $MgCl_2$) containing 0.1% Tween, staining was done using conventional nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) precipitation by alkaline phosphatase.

CHAPTER 3: RESULT AND DISCUSSION

Result 1. Spatial expression pattern of *miR-145* in zebrafish embryos at 24 hpf.

Whole mount in-situ hybridisation of *miR-145* showed that it is expressed at 24 hpf. At 24hpf, it's expression was clearly observed in the eyes, spinal cord and tail. During this period, *miR-145* expression in the optic capsule and tail region was relatively strong. Even within the eye, its expression is predominantly in retina.

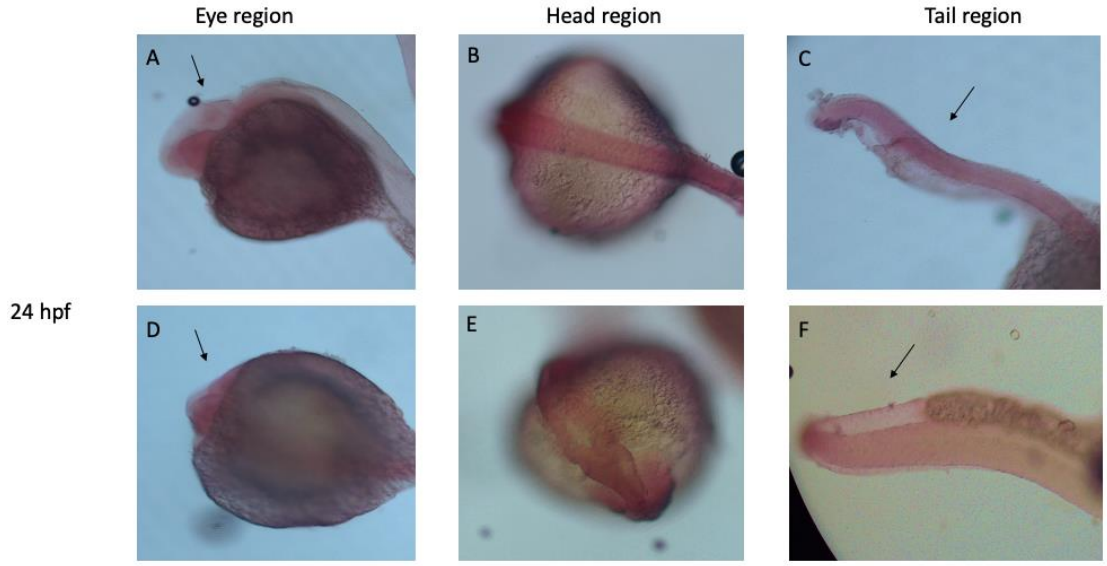


Figure 3.1 mRNA *in situ* hybridization shows expression of pre/pri *miR-145* in eye region(A,D),head region(B,E) and tail region(C,F) of 24 hpf Zebrafish embryos.

Result 2. Spatial expression pattern of *miR-145* in zebrafish embryos at 48 hpf.

At 48 hpf, *miR-145* expression was clearly observed in the eyes, spinal cord and tail. During this period, *miR-145* expression in the optic capsule and tail rediion was relatively strong. Even within the eye, its expression is predominantly in retina.

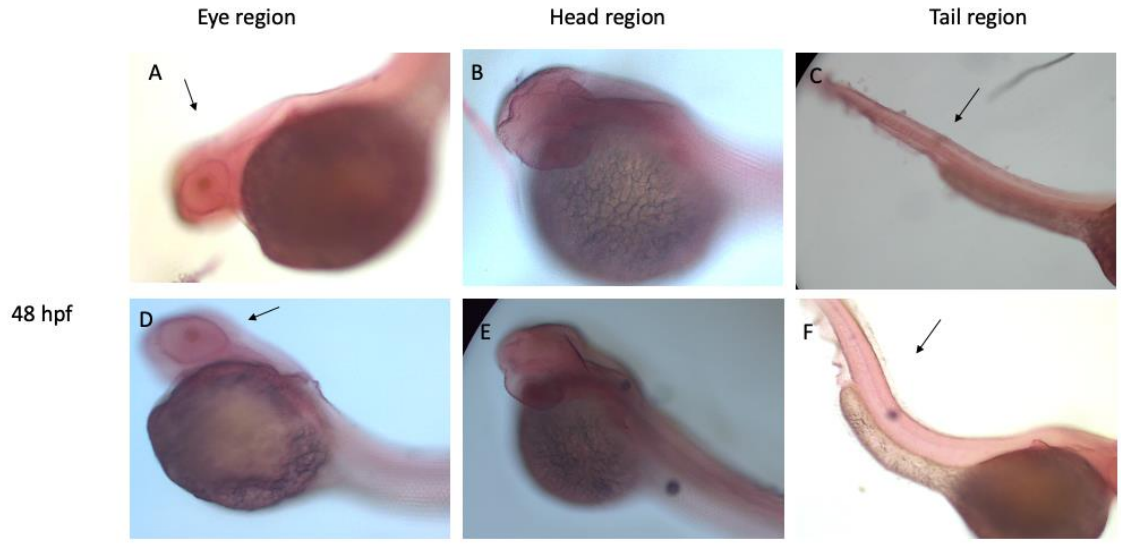


Figure 3.2 mRNA *in situ* hybridization shows expression of pre/pri *miR-145* in eye region(A,D),head region(B,E) and tail region(C,F) of 48 hpf Zebrafish embryos.

Result 3. Spatial expression pattern of *miR-145* in zebrafish embryos at 6dpf.

At 6 dpf, *miR-145* expression was clearly observed in the eyes and brain. During this period, *miR-145* expression in the optic capsule was relatively strong. Even within the eye, its expression is predominantly in retina. This result suggests *miR-145* may play a crucial role in the early embryonic development of zebrafish.

Figure 3.3 mRNA in situ hybridization shows expression of pre/pri miR145.

Result 4. Morpholino mediated knock down of *miR-143/145* in regenerating retina and its impact on MG reprogramming.

To investigate the role of *miR-145* and *miR-143* in retina regeneration, we knockdown its expression using specific lissamine tagged morpholino against mature miRNA sequence in zebrafish(1016tuba:GFP transgenic) retina. We observed that there is significant decrease in number of GFP+ve cells, which are proliferating cells, on knocking down *mir-145* and *miR-143* expression at 4dpi. This signifies that *miR-145* and *miR-143* plays role in proliferation of MGPC.

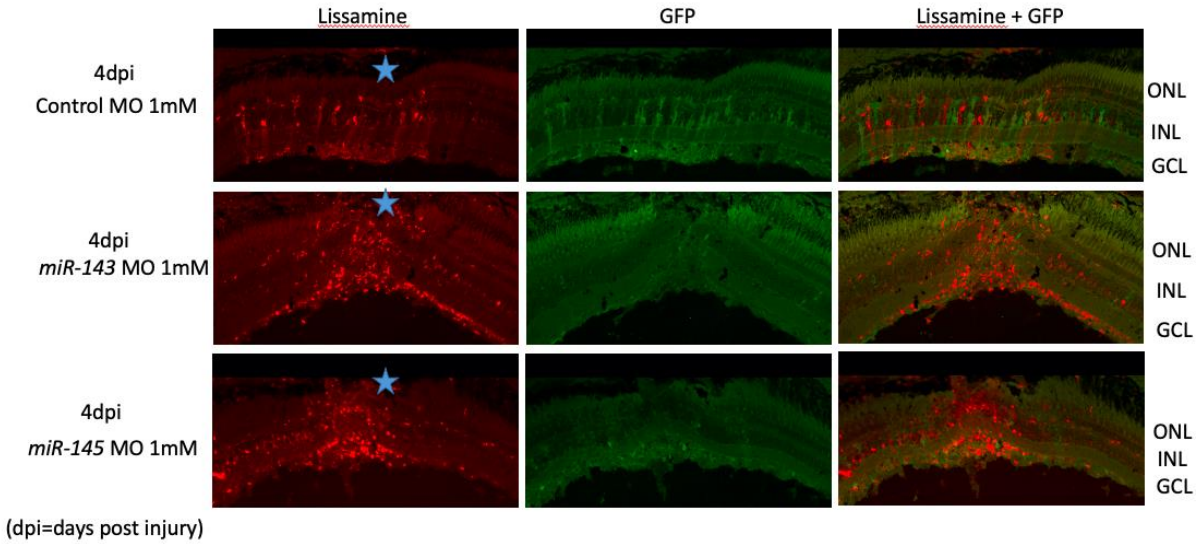


Figure.3.4 Effect if miR-143 and miR-145 knockdown on proliferation of MGPC

Result 5. Protein Expression pattern of regeneration associated molecules in retina treated with MO against *miR-143*

To impute the function of *miR-143* in retina regeneration of zebrafish, we looked at the levels of molecular players of regeneration upon mir-143 knockdown. We observed that the levels of Lin28a, Myc a/b, H3k27me3, H3K27Ac, Ptenb, Oct4, H3K4me3, Sox2, Akt, *p*-Smad3 and β - catenin decreased in a concentration dependent manner on knocking down *mir-143*. This may partly explain the decrease in proliferation of MGPC we observed in retina. We also looked at epigenetic modifiers and found that its levels also significantly decreased. Contrary to our expectations, Ascl1a, master regulator of retina regeneration, levels went down on knocking down *miR-143.*

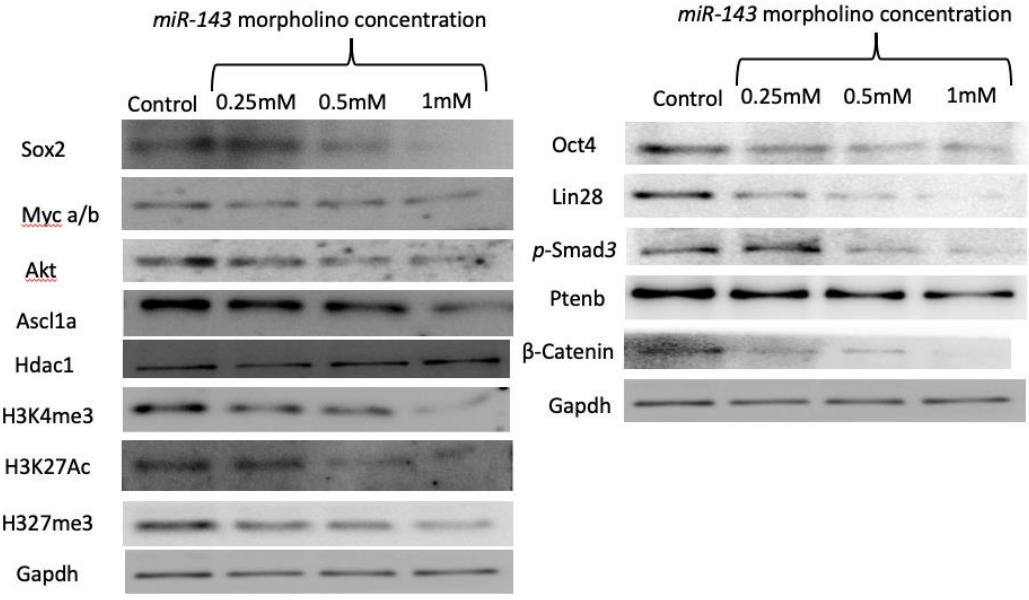


Figure 3.5 Western blot analysis of regeneration associated proteins on *miR-143* knockdown

Conclusion

microRNAs play important roles during development and tissue regeneration. In the present study, we found *miR-145* to be expressed in different regions of the CNS, including retina. That is indicative of its importance during development. Interestingly, during retina regeneration, much of the developmental signalling mechanisms are recapitulated such as expression of pluripotency factors (Mycs and Lin28). In this light, it was interesting to investigate if this microRNA impacts retina regeneration as well in adult fish. Along with *miR-145*, we also set to investigate the impact of the closely related *miR-143* in retina regeneration. By MO mediated gene knock down, we found both of these microRNAs positivity impacted MG towards proliferation. This indicates their possible roles in upregulation of regeneration associated genes which assist MG in reprogramming and proliferation. To further verify this idea, we performed WB analysis for a few of the regeneration associated molecules such as Ascl1a, Lin28a, Myc a/b, H3K27me3, H3K27Ac, Ptenb, Oct4, H3K4me3, Sox2, Akt, *p*-Smad3 and β - catenin. Interestingly, we found expression of some of these proteins to be positively influenced by *miR-143/145*. This could explain the reduction in the number of gfp+ MG in 1016tuba:GFP retina treated with MO against *miR-143/145*. Taken together, our results suggests *miR-143/145* to be important regulator of MG reprogramming and proliferation which they achieve by regulating key regeneration associated genes, epigenetic modifier and epigenetic modification.

Future perspective

For future studies, we need to check for microRNA binding sites in the genes that we have found to be impacted by them. Then we can use expression vectors with GFP fused with those genes, mutated or unmutated microRNA binding sites, to look for GFP expression. We can also look for novel targets based in microRNA databases and RNAseq analysis in injured retina that has already been published. We can do *in situ* for matured microRNA in regenerating retina to know their exact site of action. We can also write about generating knockout fish to knock out the *miR-143/145* cluster and then studying regeneration in those fish. To effect of over-expression of *mir-143* and *mir-145* on zebrafish retina regeneration can also be studied.

MO Sequence list

1. miR-143 MO, 59-GAGCTACAGTGCTTCATCTCA-39 (Lagendijk et al, 2011)
2. miR-145 MO, 59-GGGATTCCTGGGAAAACTGGAC-39 (Lagendijk et al, 2011)

Reference

Bhalla, K. N. (2005). Epigenetic and Chromatin Modifiers As Targeted Therapy of Hematologic Malignancies. *Journal of Clinical Oncology*, *23*(17), 3971–3993. https://doi.org/10.1200/JCO.2005.16.600

Chen, J.-F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., … Wang, D.-Z. (2006). The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nature Genetics*, *38*(2), 228–233. https://doi.org/10.1038/ng1725

Fausett, B. V, & Goldman, D. (2006). A Role for α1 Tubulin-Expressing Müller Glia in Regeneration of the Injured Zebrafish Retina. *The Journal of Neuroscience*, *26*(23), 6303 LP – 6313. https://doi.org/10.1523/JNEUROSCI.0332-06.2006

Fimbel, S. M., Montgomery, J. E., Burket, C. T., & Hyde, D. R. (2007). Regeneration of Inner Retinal Neurons after Intravitreal Injection of Ouabain in Zebrafish. *The Journal of Neuroscience*, *27*(7), 1712 LP – 1724. https://doi.org/10.1523/JNEUROSCI.5317-06.2007

Goldman, D. (2014). Müller glial cell reprogramming and retina regeneration. *Nature Reviews Neuroscience*, *15*(7), 431–442. https://doi.org/10.1038/nrn3723

Kiga, K., Mimuro, H., Suzuki, M., Shinozaki-Ushiku, A., Kobayashi, T., Sanada, T., … Sasakawa, C. (2014). Epigenetic silencing of miR-210 increases the proliferation of gastric epithelium during chronic Helicobacter pylori infection. *Nature Communications*, *5*(1), 4497. https://doi.org/10.1038/ncomms5497

Kwa, F. A. A., & Jackson, D. E. (2018). Manipulating the epigenome for the treatment of disorders with thrombotic complications. *Drug Discovery Today*, *23*(3), 719–726. https://doi.org/https://doi.org/10.1016/j.drudis.2018.01.004

Li, Y., He, Q., Wen, X., Hong, X., Yang, X., Tang, X., … Liu, N. (2019). EZH2-DNMT1-mediated epigenetic silencing of miR-142-3p promotes metastasis through targeting ZEB2 in nasopharyngeal carcinoma. *Cell Death & Differentiation*, *26*(6), 1089–1106. https://doi.org/10.1038/s41418-018-0208-2

Matsushima, K., Isomoto, H., Yamaguchi, N., Inoue, N., Machida, H., Nakayama, T., … Nakao, K. (2011). MiRNA-205 modulates cellular invasion and migration via regulating zinc finger E-box binding homeobox 2 expression in esophageal squamous cell

carcinoma cells. *Journal of Translational Medicine*, *9*(1), 30. https://doi.org/10.1186/1479-5876-9-30

NCT00140179. (2005). Valnoctamide in Mania. *Https://Clinicaltrials.Gov/Show/NCT00140179*.

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. https://doi.org/10.1038/ncb2115

Sato, S., Katsushima, K., Shinjo, K., Hatanaka, A., Ohka, F., Suzuki, S., … Kondo, Y. (2016). Histone Deacetylase Inhibition in Prostate Cancer Triggers miR-320–Mediated Suppression of the Androgen Receptor. *Cancer Research*, *76*(14), 4192 LP – 4204. https://doi.org/10.1158/0008-5472.CAN-15-3339

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. https://doi.org/10.1002/1097-4695(20000905)44:3<289::AID-NEU1>3.0.CO;2-H

Wan, J., Ramachandran, R., & Goldman, D. (2012). HB-EGF Is Necessary and Sufficient for Müller Glia Dedifferentiation and Retina Regeneration. *Developmental Cell*, *22*(2), 334–347. https://doi.org/https://doi.org/10.1016/j.devcel.2011.11.020

Wu, D. M., Schneiderman, T., Burgett, J., Gokhale, P., Barthel, L., & Raymond, P. A. (2001). Cones Regenerate from Retinal Stem Cells Sequestered in the Inner Nuclear Layer of Adult Goldfish Retina. *Investigative Ophthalmology & Visual Science*, *42*(9), 2115–2124.

Yao, K., Qiu, S., Tian, L., Snider, W. D., Flannery, J. G., Schaffer, D. V., & Chen, B. (2016). Wnt Regulates Proliferation and Neurogenic Potential of Müller Glial Cells via a Lin28/let-7 miRNA-Dependent Pathway in Adult Mammalian Retinas. *Cell Reports*, *17*(1), 165–178. https://doi.org/https://doi.org/10.1016/j.celrep.2016.08.078