Understanding the role of Neuregulin during zebrafish retina regeneration

TRIRUPA CHAKRABORTY

Integrated BS-MS dual degree

Department of Biological Sciences

A dissertation submitted for the partial fulfilment of

BS-MS dual degree in Science

Indian Institute of Science Education and Research Mohali

April 2020

CERTIFICATE OF EXAMINATION

This is to certify that the dissertation titled "**Understanding the role of Neuregulin during zebrafish retina regeneration**" submitted by Ms. Trirupa Chakraborty (Reg. No. MS15204) for the partial fulfillment 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 is accepted.

Dr. Santosh B. Satbhai

Dr. Sharvan Sehrawat

Dr. Rajesh Ramachandran
(Supervisor)

Dated : 04/05/2020

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 acknowledgment 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.

T. Chakraborty

Trirupa Chakraborty
(Candidate)

Dated: 04/05/2020

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

First of all, I would like to express my sincere gratitude to my supervisor, Dr. Rajesh Ramachandran, for giving me an opportunity to work in his lab. I am grateful for his generous guidance and support during the entire process of this thesis.

To a great extent, I thank Mansi Chaudhary for the continuous support of my thesis work, for her patience, encouragement, and kindness. My MS thesis work would not have been possible without the assistance and participation of her. I would specially like to thank Ashish for helping me get started with the techniques used in the lab and for being a constant support through my highs and lows. I also thank all the other lab members, Poonam, Anwar, Shivangi, Bindia, Sharanya and Pooja and my friends and batchmates Sapna, Jaskaran, Ajay, Athul, Ziya, Jithin and Pallavi for their helpful suggestions and making it a pleasant journey.

I would also like to thank some of my closest friends, Arindam, Sukanya, Jnanajyoti, Jayashree, Shabduli and Azeez for motivating and cheering me up every time I lost hope. Lastly, I'm grateful to my parents for always being there for me.

Finally, I would like to thank DBS, IISER Mohali and INSPIRE-DST for facilities and financial support

CONTENTS

LIST OF FIGURES

LIST OF TABLES

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. Nrg- Neuregulin protein
13. EGFR- Epidermal growth factor (EGF) receptor
14. Hdac- Histone deacetylase protein
15. IGF- Insulin-like growth factor
16. FGF- Fibroblast growth factor
17. MAPK- Mitogen activated protein kinase
18. BrdU-Bromodeoxyuridine
19. PCNA-Proliferating Cell Nuclear Antigen
20. mpi-minutes post injury
21. hpi- hours post injury

22. dpi-days post injury
22. Ascl1a - Achaete-scute homolog 1 zebrafish protein
23. ascl1a - *Achaete*-scute homolog 1 zebrafish gene/mRNA
24. c-Myc-MYC proto-oncogene protein
25. Yy1- Yin yang 1 protein
26. yy1- Yin yang 1 gene/mRNA
27. RT-PCR-Reverse Transcription PCR
28. RT-qPCR-Quantitative PCR
29. MGPC- Müller glia-derived progenitor cell
30. l-24-60S ribosomal protein l-24 gene
31. Gapdh- Glyceraldehyde 3-phosphate dehydrogenase protein

ABSTRACT

It has long been the goal of vision researchers to find a key to diseases like glaucoma, diabetic retinopathy, etc which cause vision impairment and loss. While the Müller Glial cell comprises the major glial component of the retina and can form progenitors that give rise to all the retinal cell types in *Danio rerio*, mammalian Müller glia is limited by way of regeneration as despite expressing genes required for acquiring stem-cell fate, they fail to act as progenitors *in vivo.* Since retina regeneration is a complex interplay of a number of signaling pathways like Delta-Notch, FGF2-FGFR-MAPK, Insulin-IGF1-PI3K (Goldman, 2014), the key to unraveling ways of coaxing the mammalian Müller glia to act as progenitors for the retinal cell types *in vivo*, might be held in understanding how the different signaling pathways regulate the regeneration associated genes. In our study we explored the nerve-derived factor, Neuregulin (Nrg) and Nrg1-ErbB Signaling in context of zebrafish retina regeneration. The Nrg1-ErbB Signaling has already been shown to have a positive regulation on proliferation of stem cells in regenerating axolotl limb, and in neurogenesis from neural progenitor cells of the developing zebrafish brain as well as for cardiomyocyte proliferation during embryogenesis (Farkas, Freitas, Bryant, Whited, & Monaghan, 2016; Sato et al., 2015; Yaniz-Galende & Hajjar, 2014). Moreover, since the Neuregulins are nerve-derived factors and the ErbB are transmembrane receptors, we hypothesized that the signal of an injury might be mediated to the nucleus of Müller glial cell by Neuregulins via the Nrg1/ErbB signaling cascade, thereby initiating regeneration. From our study we observed that both the Nrg1 and the ErbB receptors showed a similar peak in expression during the proliferative phase. By pharmacological inhibition of the Nrg1-ErbB1 signaling and the Nrg1-Erb2 signaling, we showed a reduction in the number of proliferating cells, thereby suggesting a positive role in proliferation. Additionally, we found that the inhibition of the Nrg1/ErbB1 signaling cascade resulted in a differential expression of ascl1a which required for the proliferation of Müller glial cells. Interestingly however, upon drug inhibition, we found a decrease in the protein expression of Hdac1, whose upregulation during the dedifferentiation phase (0-2dpi) was previously shown to be essential for successful proliferation in zebrafish fin regeneration (Mitra et al., 2018). Overall this study suggests a role of Nrg1 and its ErbB receptors in regulating ~~some~~ known regeneration-associated genes during the proliferative phase of zebrafish retina regeneration.

CHAPTER1: INTRODUCTION

According to WHO, on a global scale, approximately 2.2 billion people are affected by blindness or have impaired vision. Of these 6.9 million have Glaucoma, 3 million have diabetic retinopathy and Age-related Macular degeneration in third in line after cataract and glaucoma, as a cause of blindness (WHO, 2019, n.d.). This makes it imperative for researchers in the field of regenerative medicine to understand and design strategies to regenerate mammalian retina.

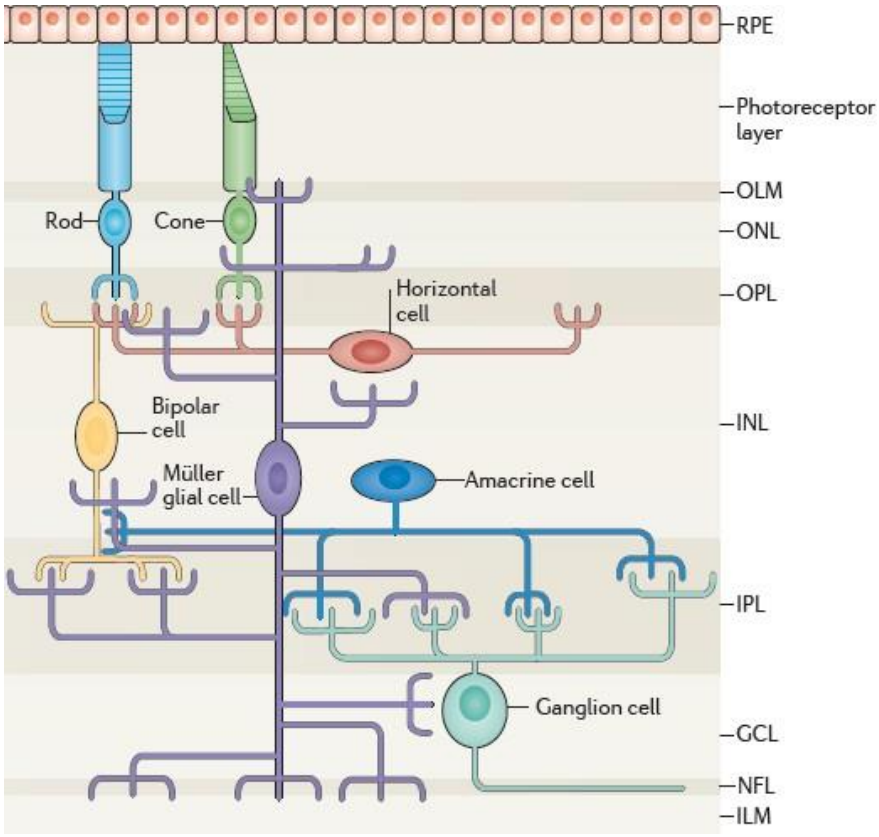
The retina of mammals bears a striking similarity with that of the teleost fish, *Danio rerio* (zebrafish), in terms of retinal cell types and their conserved function. The zebrafish owes its natural regenerative ability to a retinal cell type called Müller glia. While the Müller glia of zebrafish can respond to injury and act as a progenitor for all retinal cell types, the mammalian Müller glia does not act as a progenitor *in vivo*. Yet, the ability of mammalian Müller glia to respond to injury, proliferate and express the regeneration-associated genes warrants an exploration into the molecular pathways which can lead to the Müller glia of mammals adopting a retinal progenitor fate (Goldman, 2014). Besides the ease of rearing, the availability of various genetic and genomic tools such as for creating transgenic lines, various methods of studying effects of injury, screening of drugs, and the simpler progression through the developmental stages, makes zebrafish an ideal model for the study of retina regeneration.

To understand the process of retina regeneration several studies have explored various molecular mechanisms and molecular signaling cascades like Wnt Signaling, MAPK/Erk pathways, Jak/Stat pathway, etc along with the involvement of miRNA such as let-7 (Goldman, 2014). From various studies it has been found that unlike mammalian Müller glia which upon an injury, more often undergo fibrosis and glial scarring, Zebrafish Müller glia show a gliotic response followed by the partial reprogramming of the genome. This enables it to attain the retinal stem cell fate, giving rise to differentiated retinal cell types and ultimately repairing it. In order to understand clearly the process of regeneration, an elaboration on the zebrafish retinal architecture is given below.

1.1 ANATOMY OF THE ZEBRAFISH RETINA AND OVERVIEW OF RETINA REGENERATION

1.1.1 Retinal Anatomy

The retina can be broadly divided into three laminar layers: Ganglion cell layer (GCL), Inner Nuclear layer (INL), Outer Nuclear layer (ONL) each separated by a synaptic layer. Six retinal cell types are distributed in these three layers with the photoreceptor cells (Rods and Cones) in the ONL, Horizontal, Bipolar and Amacrine cells in the INL, the Ganglion cell in the GCL and dendritic processes of the Müller glial cells traversing through the three laminal layers with its cyton in the INL. The strategic positioning of the Müller glia across the three layers allows them to interact with the neurons in the vicinity and respond to cell death or damage across the three layers, thus ensuring that a state of homeostasis is maintained throughout the retina (Goldman, 2014; Wan & Goldman, 2016).



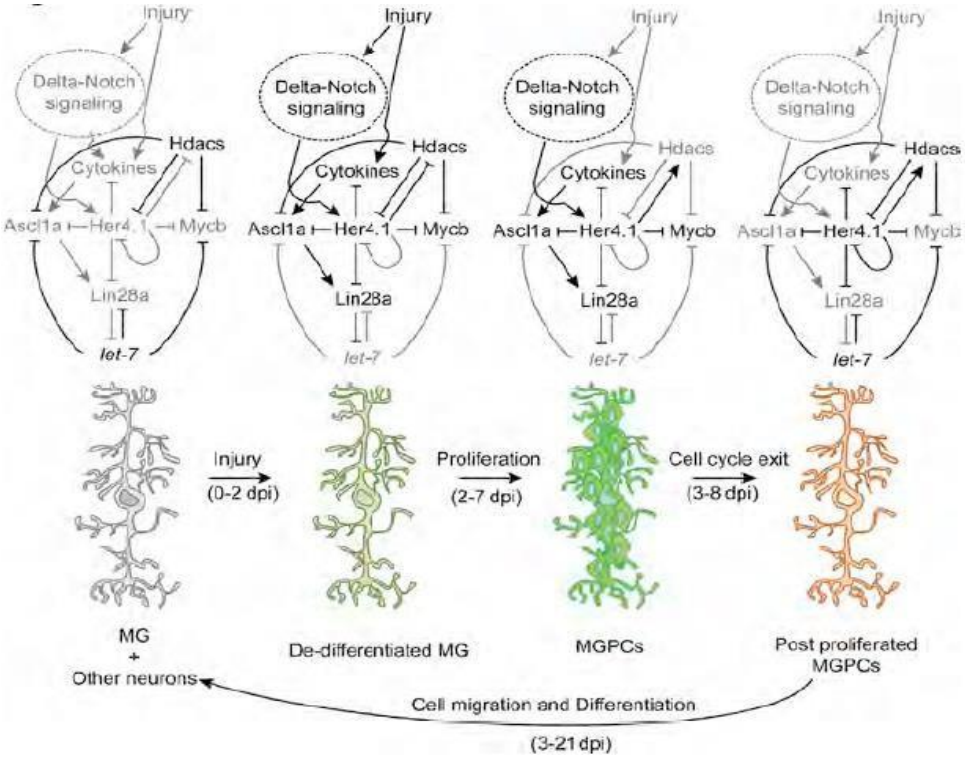
Reference: Goldman, D. *Nat Rev Neurosci* **15,** 431–442 (2014)

Figure 1.1.1: An illustration depicting the retinal anatomy along with the six major retinal cell types and their relative positions in the zebrafish retina. The zebrafish retina comprises 6 retinal cell types, namely, photoreceptor cells (rods and cones), horizontal cells, bipolar cells, amacrine cells and ganglion cells, with the Müller glia (glial cell type) spanning all the layers of the retina.

1.1.2 Process of Retina Regeneration

Some of the widely used injury paradigms are mechanical (eg. stab wound), photobleaching to damage the photoreceptor cells and chemical methods to damage the GCL (Mitra et al., 2019; Sharma & Ramachandran, 2019). The cue to start the process of regeneration is given by the dying cells in the form of secreted factors or through changes in their interactions with the Müller glia. In order to initiate retinal repair broadly three important steps need to occur, namely, a partial reprogramming of the Müller glia to adopt a stem cell fate, formation of Müller glia cell-derived progenitor population by proliferation, differentiation into the neuronal sub-types that had been damaged due to the injury. Further elaborating the steps,

1) Activation and Dedifferentiation- Upon receiving signals from the dying neurons, the Müller glia undergo reprogramming as the result of a pan-retinal induction of Oct4(Sharma et al., 2019), one of the Yamanaka factors. As a result of this reprogramming event, there is an upregulation of pluripotency factors like *lin-28, oct-4, c-myc* and *sox2* (Ramachandran, Fausett, & Goldman, 2010).
2) Proliferation- As a result of the reprogramming, the activated Müller glia begin an interkinetic neuronal migration to the outer nuclear layer and upon reaching the outer limiting membrane these then undergo asymmetrical cell division. The population thus obtained is the multipotent progenitor population which transiently proliferate and , migrate to all cell layers(Goldman, 2014).
3) Redifferentiation- Upon reaching the different retinal layers, the multipotent progenitors exit the cell cycle to regenerate major retinal cell types.



Reference: Mitra et al., iScience 7, 68–84;September 28, 2018

Figure 1.1.2: Regulation of different regeneration-associated genes during regeneration of retina in zebrafish. Retina regeneration is a complex interplay of many developmentally important genes like ascl1a, her4.1, hdacs, lin28 and important signaling like the Delta-Notch pathway. The levels of each component vary differentially over the period of regeneration resulting in the redifferentiation of Müller glia into the retinal cell types

While different injury paradigms result in the ablation of different retinal cell types, the Müller glia-derived progenitor cells (MGPCs) show a skewed proliferation towards those cell-types which have been ablated (Powell, Cornblath, Elsaeidi, Wan, & Goldman, 2016). This hints of an underlying mechanism which might be allowing the MGPCs to sense the cell loss in that layer. Previous studies have shown that secreted factors like Heparin-binding Egf-like growth factor (Hbegf), ADP, Tnfa (tumor necrosis factor-alpha) play a role in inducing Müller glial cell reprogramming and formation of progenitor (Goldman, 2014). In this study we investigated the role of Neuregulins, a family of proteins secreted from dying axons of the peripheral nervous system. A study conducted on the Mexican axolotl (*Ambystoma mexicanum*) showed that Nrg-1/ErbB2 signaling played an important role in proliferation of the blastema and Nrg-1 could

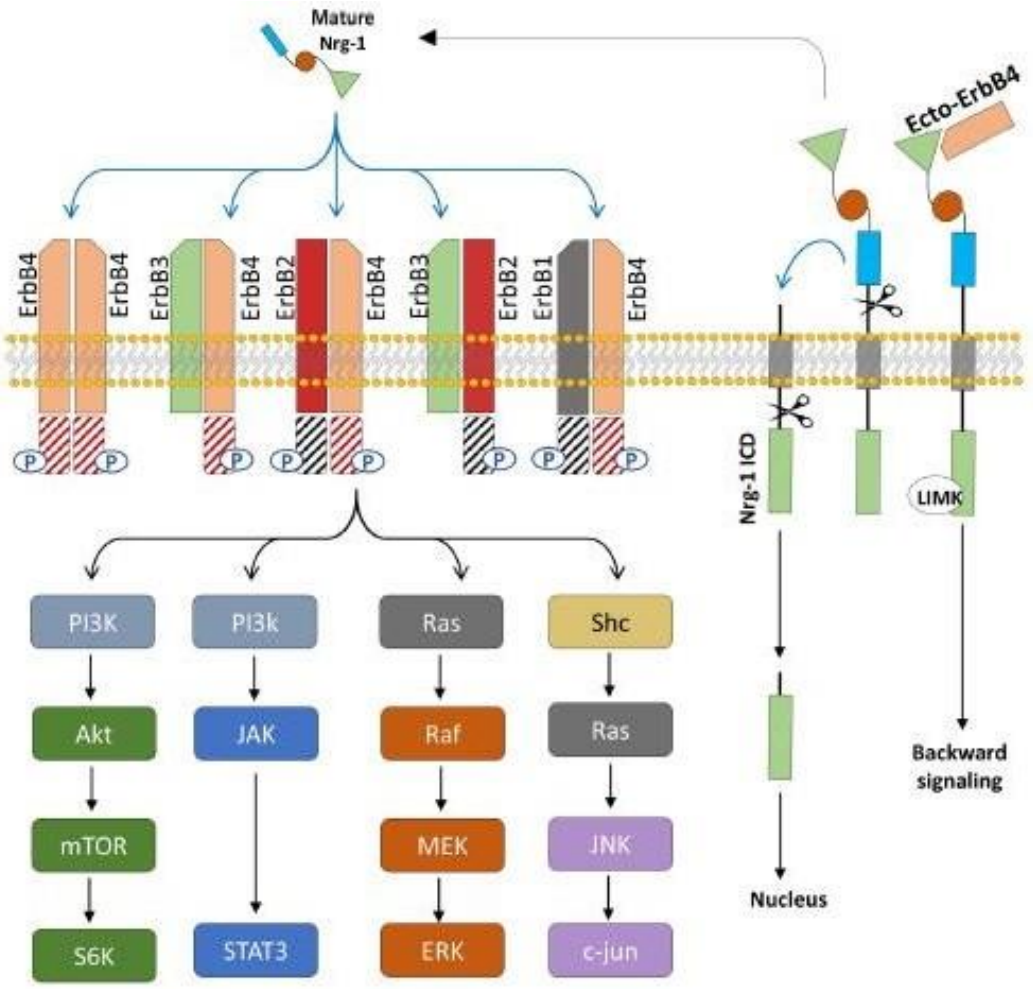
rescue regeneration in limbs that had been denervated (Farkas et al., 2016). Neuregulin 1 type II-ErbB Signaling has also been shown to promote neurogenesis from neural progenitor cells in the developing zebrafish brain (Sato et al., 2015). Moreover, during zebrafish heart regeneration, Nrg1, together with its co-receptor Erbb2 plays a critical role in cardiomyocyte proliferation (Gemberling, Karra, Dickson, & Poss, 2015). Therefore, given the central role that Neuregulins and their corresponding Erbb receptors play in tissue regeneration and a relative dearth of knowledge in context of retina regeneration, in this study we focused on understanding the role of Neuregulins and Nrg/Erbb signaling as factors mediating an injury signal to the Müller Glial cell.

1.2 THE FAMILY OF NEUREGULINS AND THE Nrg/ErbB SIGNALING:

The Neuregulins form a family of growth factors encoded by four genes: NRG1, NRG2, NRG3 and NRG4 of which NRG1 is the most widely studied. Having an EGF-like sequence, the NRG1 has 6 different types (type I-VI) with over 30 different isoforms generated by alternative splicing (Marchionni et al., 1993). NRG1 isoforms have a wide array of functions owing to the tissue-specific expression pattern and diverse biological activities of their isoforms (Britsch, 2007). The Neuregulins help in relaying a signal to the target cell via a receptor-ligand interaction with the ErbB family of receptors. The ErbB family is a group of receptor tyrosine kinase which are transmembrane in nature. The group includes EGF-R (ErbB1), ErbB2, ErbB3, ErbB4, which form heterodimers or homodimers to transmit the signal received upon ligand binding, through various signaling pathways (Kataria, Alizadeh, & Karimi-Abdolrezaee, 2019). Together with their ErbB receptors, the Neuregulins form a complex network of intracellular signaling pathways well-known for playing a central role in the development and repair of the Peripheral nervous system (PNS), during Schwann cell development, survival and myelination. Besides that the Nrg1/ErbB signaling also plays an essential role in neural differentiation, neuronal guidance, synapse formation, neuromuscular junction formation myelination, neural crest cell differentiation and migration (Kataria et al., 2019).

In the zebrafish, the Neuregulins that have been shown to exist are- nrg1, nrg2 (nrg2a, nrg2b, nrg3 (nrg3b) and the erbb receptors present are- erbb1 (egfr), erbb2, erbb3 (erbb3a, erbb3b), erbb4 (erbb4a, erbb4b). In order to explore the role of Nrg1/ErbB signaling in this study we used two drugs- AG1478 (Apex Bio) which blocks the phosphorylation of egfr (erbb1) and

superoxide anion formation, thereby acting as an EGFR kinase inhibitor (D'Anneo et al., 2013); Mubritinib (hereby referred to as Mub)(Sigma-Aldrich) which selectively inhibits erbb2. Using various concentrations of the two drugs we explored the significance of Nrg1/ErbB signaling in context of zebrafish retina regeneration.



Reference: Hardeep Kataria, Arsalan Alizadeh and Soheila Karimi-Abdolrezaee, Progress in Neurobiology, https://doi.org/10.1016/j.pneurobio.2019.101643

Figure 1.2: A schematic of the Nrg1/ErbB Signaling cascade (canonical and non-canonical) and some of their key downstream factors. The Nrg1 is well-studied ligand for the ErbB family of receptors, which through formation of homo- and hetero-dimers and undergo auto-phosphorylation to regulate a number of developmentally important genes.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animal Maintenance

- Zebrafish are raised and housed in an automated water circulation system, and the embryos are kept in tanks in an incubator until 2 weeks post hatching when they are transferred to the water circulation system.
- The zebrafish kept in the water circulation system and the embryos were both kept in 14 hours of light and 10 hours of dark.
- The temperature of both systems was maintained at 27°C.
- pH: Physiological pH
- Embryos used were obtained by breeding of wild-type fish under artificial light.

2.2 Drug injection

- AG1478 (Apex Bio) was reconstituted in DMSO to a stock solution of 16mM and then further diluted to the required concentrations (5µM and 10µM) using milli-Q for performing experiments. The stock was stored at -20°C.
- Mubritinib (Sigma Aldrich) was reconstituted in DMSO to a stock solution of 10.7mM and then further diluted to the required concentrations (1µM and 10µM) using milli-Q for performing experiments. The stock was stored at 4°C.
- The control for experiments involving drug treatment was autoclaved milli-Q.
- The drug injections were performed by Mansi Chaudhary using a Hamilton needle.

2.3 Dissection of retina for RNA isolation/ western blotting

- Zebrafish were anesthetized in Tricaine methanesulfonate.
- A 30 Gauge needle sterilized in 70% ethanol was used to injure the retina at four spots.
- At the desired time points (15hpi, 1dpi, 2dpi, 4dpi, 7dpi), the eyes were dissected from the anesthetized fish. The dissection was performed in a chilled 1XPBS solution prepared in DPEC. The lens was removed and the retina harvested using steel forceps.
- The retinae to be dissected for RNA isolation were kept in 200µl of TRIzol (Invitrogen). For each sample one retina was used.

- The retinae to be used for Western Blotting were stored in 150ul of Laemmli buffer at -80°C. Two retinae per sample were used for used for the experiment.

2.4 Total RNA isolation

- The retina was harvested from uninjured (control) and injured retina at the respective time points (15hpi, 1dpi, 2dpi, 4dpi, 7dpi) and homogenized using the pipette in 200µl of TRIzol in an MCT.
- 0.2volumes of the above (40µl) of Chloroform was added to the MC and slowly inverted.
- The MCT was then kept at room temperature for 5minutes.
- The MCT was then centrifuged at 10,000 rcf for 15minutes at 4°C.
- 35µl of the aqueous layer was collected with a cut tip, in a fresh MCT.
- To this an equal volume (35µl) of isopropanol was added and the tube kept on ice for 20minutes. (To aid in precipitation the tube was flicked in intervals)
- The solution was then centrifuged at 10,000rcf for 10mins at 4°C.
- The supernatant thus obtained was discarded. The pellet was then washed with 80% EtOH by centrifuging at 7600rcf for 10minutes at 4°C.
- After drying away the EtOH the pellet was dissolved in 12µl of DPEC and kept on ice for 30minutes.
- The RNA isolated was then checked on a 1% agarose gel for its quality.

2.5 cDNA Synthesis

(RevertAid First Strand cDNA Synthesis Kit by ThermoFisher was used.)

- The following reagents were added to a sterilized PCR tube kept on ice:

Primer (Oligo(dT)18+ Random hexamer)	0.25µl+0.25µl
Template RNA	5.5µl

- The contents of the tube were mixed gently, centrifuged briefly and then incubated at 65°C for 5minutes. At the end of the duration the tubes were plunged in ice.
- The following components were then added to the tube in the indicated proportion:

5X Reaction Buffer	2µl
RiboLock RNAse Inhibitor	0.5µl
10mM dNTP Mix	1µl

Revert-Aid M-Mμl VRT	0.5μl
Total volume	4μl

- The contents of the tube were mixed gently and centrifuged briefly.
- The tube was then incubated at temperature conditions in the following order:
  -At 25°C for 5minutes
  -At 42°C for 60minutes
  -At 70°C for 5mins
- A required proportion of cDNA thus obtained was diluted in autoclaved Milli-Q in a 1:4 ratio for performing subsequent experiments and the remaining stock stored at -80°C.

2.6 Reverse Transcription-Polymerase chain reaction (RT PCR)

(Primers were diluted to a ratio of 1:10 of 100μM)

- The reaction mixture

20X buffer	0.5μl
2.5mM dNTP	1μl
Primer (Forward+Reverse)	0.2μl
Taq Polymerase	0.2μl
Template DNA	volume as standardized
Autoclaved Milli-Q	7.6μl

- Reaction Conditions

Enzyme activation	95°C for 2minutes
DNA denaturation	95°C for 20seconds
Primer annealing	as standardized for the respective primers
Elongation	68°C for 40seconds
Final Elongation	72°C for 7minutes
Infinite hold	4°C
Number of cycles	25 for Beta-actin gene, 40 for Nrg and ErbB genes

- Primer annealing temperatures

Nrg1-204: 55°C	Egfr-207: 55°C
Nrg1-205: 58°C	ErbB2-201: 55°C
Nrg1-206: 58°C	ErbB3a-203: 60°C

Nrg2a-204: 52°C	ErbB3b-201: 55°C
Nrg2b-202: 58°C	ErbB4a-201: 55°C
Nrg3b-201: 58°C	ErbB4b-203: 55°C

- At the end of the cycle the products were then run on a 1.5% agarose gel.

2.7 Quantitative PCR (qPCR)

- The Master mix used was KOD SYBR (SYBR green containing PCR mix with KOD DNA polymerase from Thermococcus kodakaraensis) qRT-PCR mix (QKD-201; Genetix) and the reaction was carried out in a real-time PCR detection system (MasterCycler RealPlex4; Eppendorf)
- The reaction of a total volume of 5µl had the following components:

Master mix	2.5µl
Primer (Forward+Reverse)	0.2µl
Template	1µl
Autoclaved milli-Q	1.3µl

- The data was then recorded on an excel sheet and the graph plotted.

2.8 Tissue fixation and sectioning

- Before dissection of the eyes at the respective time point, the fish used for the experiments were dipped in 30ml of BrdU for 4hours.
- After the end of the duration and at the respective time point the eye (uninjured or injured) were dissected with the lens removed in freshly prepared 4% PFA.
- The eyes were then stored in the 4% PFA in an MCT and incubated at 4°C overnight for fixation.
- The eyes were then washed in the following serial order, at room temperature at 45minutes each on a rotor at 12rpm:

  1ml of 5 % Sucrose solution

  800µl of 5% Sucrose and 400µl of 20% Sucrose solution 500µl of 5% Sucrose and 500µl of 20% Sucrose solution 400µl of 5% Sucrose and 800µl of 20% Sucrose solution 1ml of 20% Sucrose solution

- 500ml of OCT was then added to the above tube, and it was kept on rotation for 30minutes.
- The tissue was embedded in OCT in small cubes of aluminium foil and flash frozen at -80°C.
- The blocks were then sectioned with a width of 12µM on a Cryostat and transferred onto a super frost plus slides to be dried overnight.
- The slides were then stored at -20°C until further use.
- The solutions used in the above protocol had the following composition:

1) 4% PFA in 1X Phosphate Buffer

   -2g PFA

   -5ml of 10X Phosphate buffer (in DPEC water)

   -volume made up to 50ml using DPEC water

   -dissolved at 65°C with constant shaking until homogenous solution formed

2) 5% sucrose

   - 2.5g sucrose dissolved in 50ml of autoclaved milli-Q water. Stored at -20°C.

3) 20% sucrose

   -10g sucrose dissolved in 50ml of autoclaved milli-Q water. Stored at -20°C.

2.9 Immunostaining

Day 1

- The slides were washed in 1X PBS for 10minutes, 3 times.
- The slides were then dipped in 2N HCl (pre-warmed at 37°C) for 20minutes.
- The slides were then washed twice with 0.1M Sodium Borate solution for 10minutes each.
- The sections on the slides were treated with 3% BSA in 1X PBST (1X PBS+ 0.1% Triton X) at room temperature, for 45minutes.
- The slides were then washed with 1XPBST for 10minutes.

- 500µl of primary antibody (antibody against PCNA, raised in mouse and diluted to 1:1000 in 1% BSA-1XPBST) was laid over the slides, and incubated overnight at 4°C.

Day 2

- The slides were given three washes with 1XPBST for 10minutes each.
- Slides were then incubated in 500µl of Secondary antibody (raised in rabbit and tagged with a fluorophore with excitation at 555nm, diluted to 1:1000 in 1% BSA-1XPBST) at room temperature for 1hour and 30minutes.
- At the end of the duration, slides were washed three times with 1XPBST for 10minutes each.
- This was followed by two washes with autoclaved milli-Q for 10minutes each.
- The slides were then kept at room temperature until dry.
- 80µl of anti-quenching agent, DABCO was added to each of the slides with a sterile cut tip and covered with a coverslip.
- The slide thus prepared was kept to dry overnight at room temperature in the dark. The next day the slides were stored at -20°C until further use.

2.10 Microscopy

- Retinal injury and dissections were both performed under the Bright field microscope (Zeiss).
- For imaging the immunostained slides the Confocal microscope (Nikon) was used.
- Quantification of images (counting PCNA positive cells) was done using software ImageJ.

2.11 Cloning

1) Polymerase Chain Reaction

- The reaction used to add RE digestion sites included the following components:

2XMM Go Taq Polymerase enzyme	5µl
Primers (Forward+Reverse)	0.2µl
Template DNA	0.5µl
Autoclaved milli-Q water	4.3µl

- **The reaction conditions are as follows:**

Enzyme activation	95°C for 5minutes
DNA denaturation	95°C for 30seconds
Primer annealing	as standardized for the respective primers
Elongation	68°C for 2minutes
Final Elongation	72°C for 10minutes
Infinite hold	4°C
Number of cycles	40

- Primer annealing temperatures:

  Nrg1-204: Temperature coμld not be standardized

  Nrg1-205: Temperature could not be standardized

  Nrg1-206: Temperature could not be standardized

  Nrg2a-204: 62°C

  Nrg2b-202: 58°C

  Nrg3b-201: 62°C

- The PCR amplified product was then purified using a kit and eluted in 30μl of Autoclaved water.

2)Plasmid isolation

- Neuregulins digested with Restriction Enzymes XbaI were cloned in PCS2 dam^-.
- Cells with the plasmid cloned, were inoculated from the glycerol stock, in 5ml of sterilized LB media and were allowed to grow for 12-14 hours.
- 1.5ml of the culture was transferred into sterile MCT tubes and centrifuged at 13,400rpm at room temperature.
- After discarding the supernatant, the pellet was dissolved in chilled100μl of autoclaved water by vortexing.
- 100μl of lysis buffer (920ul autoclaved water + 50μl of 20% SDS +20μl EDTA+ 10μl of NaOH) was added to the above tubes and slowly inverted a few times.
- The solution was then incubated at 100°C (drybath) until it became transparent.
- 50μl of MgCl2 was added to the above and gently mixed.
- The tubes were then incubated on ice for 2minutes and centrifuged at 13,400 rpm for 2 minutes at room temperature

- Slowly 50µl of Potassium acetate was added and gently mixed with the supernatant, without disturbing the pellet.
- The tubes were then centrifuged at 13,400 rpm for 2 minutes.
- The supernatant thus obtained was collected into a fresh MCT and after the addition of 600µl of Isopropanol. After shaking it vigorously to assist in precipitation, it was incubated on ice for 10minutes.
- The solution was then centrifuged at 13,400rpm for 2minutes at room temperature.
- After discarding the supernatant, the pellet was washed with 200µl of 70% EtOH and centrifuged at 13,400rpm.
- The supernatant was again discarded and the EtOH dried away by keeping the tube at room temperature for a few minutes. The pellet was then dissolved in 50µl of autoclaved water and run on 1% agarose gel.

3)Digestion Reaction

- The following reactions were set up using the PCR amplified (and purified) insert and the corresponding plasmid DNA.
- **Vector digestion**:

Vector DNA	30µl
Enzyme 1	1.2µl
Enzyme 2	1.2µl
Buffer	6µl
Autoclaved water	21.6µl
Total volume	60µl

Insert digestion:

Insert DNA	29µl
Enzyme 1	1.2µl
Enzyme 2	1.2µl
Buffer	6µl
Autoclaved water	22.6µl
Total volume	60µl

- The tubes were then incubated at 37°C overnight.
- The digested vector was then treated with CIP Phosphatase to reduce the possibility of self

ligation from occurring.

- The buffer used for digestion with XhoI was FD Green and with XbaI was Cut Smart.
- The following RE pairs were used for the corresponding gene :
  - Nrg2a-204: EcoRI/XhoI
  - Nrg2b-202: BamHI/XhoI
  - Nrg3b-201: EcoRI/XbaI

4) Gel Extraction

- After running the PCR amplified product was run on the gel, the bands were cut and put into an MCT.
- Another MCT was stuffed with small bits of Aluminium foil and hole was made at the bottom of it.
- The above stuffed MCT(1) was inserted into another MCT(2). The gel piece was then put inside the MCT(1) and centrifuged at 13500rpm for 10minutes at room temperature.
- To the clear liquid that was collected in MCT(2) an equal volume of PCI and shaken vigorously.
- The MCT was then centrifuged at 13500rpm for 10minutes at room temperature.
- The supernatant was transferred to a fresh MCT and an equal volume of chloroform was added to it and shaken vigorously.
- The tube was again centrifuged at 13500rpm for 10minutes at room temperature.
- The supernatant of volume ‘x’ was transferred to a fresh MCT. To this volume the following were added :
  - ‘y’ volumes of 100% EtOH: $y=7x/3$
  - ‘v’ volumes of Ammonium acetate: $v= 3(x+y)/7$
  - 2.5µl of 10mg/ml glycogen
- The above mixture was then incubated overnight at -80°C.
- The MCT was then centrifuged at 4°C for 30minutes.
- After discarding the supernatant, the pellet was washed with 70% EtOH by centrifuging at 4°C, for 5minutes at 13,500rpm.
- The EtOH was then allowed to dry away after which the pellet was dissolved in 13µl of autoclaved water.

5)Ligation reaction

- The digested product obtained above after gel extraction is incubated at 40°C for 5minutes to remove all secondary structures.
- The OD value was then measured using nanodrop and the ligation reaction was set up according to the following relationship:

  Mass of vector added= 50ng

  Mass of insert added= (4 x size of insert (bp) x mass of vector)/ size of vector (bp)

- The reaction :

Vector	0.7µl
Insert	2.7µl
Buffer	2µl
Ligase	1µl
Autoclaved water	13.6µl

- The reaction was then incubated at around 18°C overnight.

6)Transformation Reaction

- 5µl of the ligated product was added to 100µl of ultra-competent cells in an MCT and tapped gently to mix.
- The MCT was then kept on ice for 30minutes.
- The cells were then given a heat shock by incubating the tubes at 42°C for 75seconds.
- Immediately after, the MCT was plunged in ice for 5minutes.
- 1ml of LB media was added to the MCT and incubated at 37°C on rotation for 1hour.
- After the incubation, the MCT was centrifuged for 4minutes at room temperature.
- The pellet obtained was re-suspended in 100µl of the media and plated on an Ampicillin plate.
- The plates were then incubated overnight at 37°C for 12-16 hours.
- The positive colonies were screened by the following method:

  -Each colony was lysed in 20µl of autoclaved water for 25seconds.

  -An equal volume (20µl) of Phenol-Chloroform-Isoamyl alchohol was added and vortexed for a minute.

- The mixture was then spun at maximum speed for 5minutes and the supernatant then run on 1% agarose gel.

-The relative shift compared to an undigested vector DNA was observed for positive colonies.

- These colonies were again digested with their corresponding enzymes as a further check.

2.12 Western Blotting

- Sample Preparation

-Drug treated retinae were dissected at the 2dpi time point along with the uninjured retinae and transferred into 150µl of Laemmli buffer.

-The samples were then homogenized with a pestle.

-Each of these were vortexed in cycles for 30seconds with incubation on ice for 1minute. This was done for a total of 20minutes.

-The samples were then incubated at 95°C for 10minutes and can then be stored at -80°C until further use.

- Gel run and transfer

-The resolving gel (10% for GAPDH and 12% for the other genes) and the stacking gel were casted in the respective order and with the following composition:

Resolving gel (12%):

2.5ml Resolving buffer (1.5M Tris, pH8.8)+ 4ml 30% Acrylamide+ 3.3ml Autoclaved Milli-Q water+ 100µl 10% SDS+ 100µl 10% Ammonium persulphate+ 6µl TEMED

Resolving gel (10%): 2.5ml Resolving buffer (1.5M Tris, pH8.8)+ 3.33ml 30% Acrylamide+ 3.97ml of Autoclaved Milli-Q+ 100µl of 10% SDS+ 100µl of 10% APS+ 6µl of TEMED

Stacking gel: 625µl Stacking buffer + 667µl 30% Acrylamide+ 3603µl of Autoclaved Milli-Q water +50µl 10% SDS+ 50µl 10% Ammonium persulphate +5µl TEMED

-Samples kept in -80°C were thawed and then loaded onto the gel along with a 3-colour pre-stained protein ladder.

-The gel was then run at 25A.

-After the run, the required portion of the gel was cut and the PVDF membrane was cut and dipped in Methanol for 5minutes, to charge.

-The membrane and the gel were washed with milliQ water twice.

-The membrane was washed with transfer buffer and then a wet transfer was set up at 70V for 90 minutes.

-The blot was then incubated in 10% skimmed milk, overnight at 4°C on a shaker for probing Oct4 and HdAc and for one hour at room temperature on shaker for GAPDH, MYC, ASCL1a, YY1.

-The blot was washed with 0.01% PBST (200µl of TWEEN was added for every 200ml of 1X PBST), three times for 10minutes each.

-The blot was then incubated in the respective primary antibody, for 1 hour for GAPDH and overnight for the other proteins.

-After the incubation the blot was washed with 0.01% 1X PBST, three times for 10minutes each and then incubated in the respective secondary antibody (1hour for GAPDH and 2 hours for the remaining proteins).

-The blot was then washed in 0.3% 1X PBST three times, for 10minutes each.

-The blot was then developed in Image Quant LAS4000 using ECL (Biorad).

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Isoform nrg1-206 is upregulated during the proliferative phase of zebrafish retina regeneration

While several studies have highlighted the pro-proliferative function of nrg1 in regions of the CNS like the developing brain of zebrafish (Sato et al., 2015) and in rat CNS clonal cell line (Edwards & Bottenstein, 2006), we wanted to study the temporal expression pattern of Neuregulin in the adult zebrafish retina upon injury by focal stab method. As the dedifferentiation phase lasts between 0-2dpi, the proliferative phase between 2-7dpi with its peak on 4dpi, and the re-differentiation phase starts from 4-6dpi onwards, we followed the expression relative to the uninjured retina (control) on 15hpi, 1dpi, 2dpi, 4dpi and 7dpi. Upon performing RT-PCR and quantifying the mRNA expression through RT-qPCR we observed a rise in the levels of a particular isoform nrg1-206 starting from 15hpi upto 2dpi followed by a fall in its level of expression. A similar trend was observed even in the RT-PCR data though the expression appeared to be highest during 4dpi. However, both RT-PCR and RT-qPCR results suggest that the transcript nrg1-206 might be playing a role during the initiation of the proliferative phase of zebrafish retina regeneration.

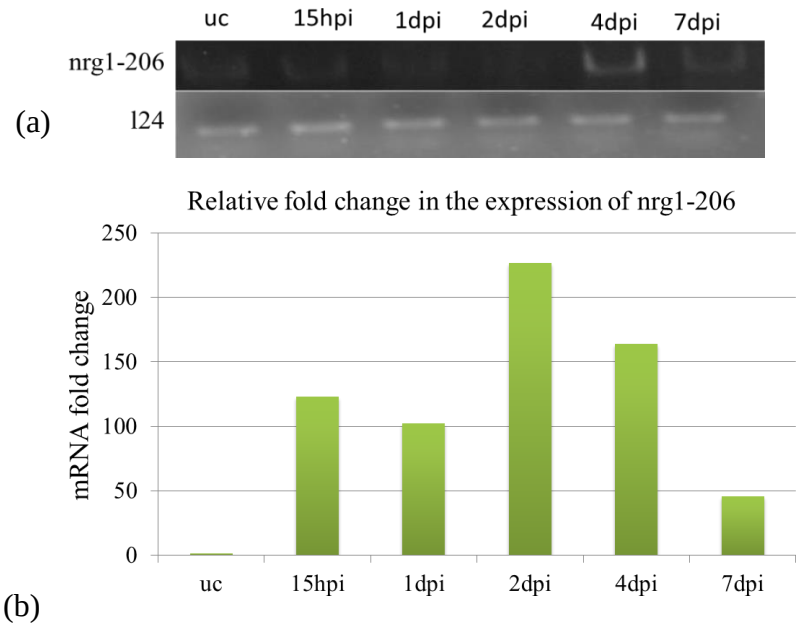


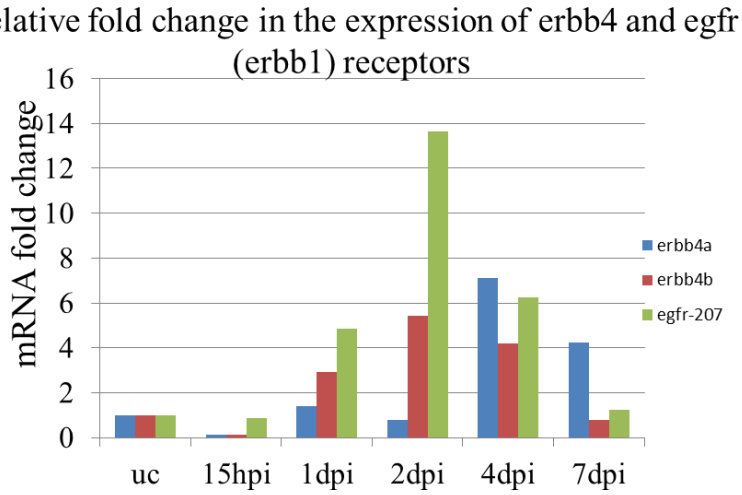
Figure 3.1: Temporal expression pattern of nrg1 during zebrafish retina regeneration.

(a)RT-PCR data showing a rise in expression of nrg1-206 during the peak of proliferation on 4dpi. (b) RT-qPCR quantifying the fold change of nrg1-206 reaffirming the spike in its expression during the proliferative phase of regeneration.

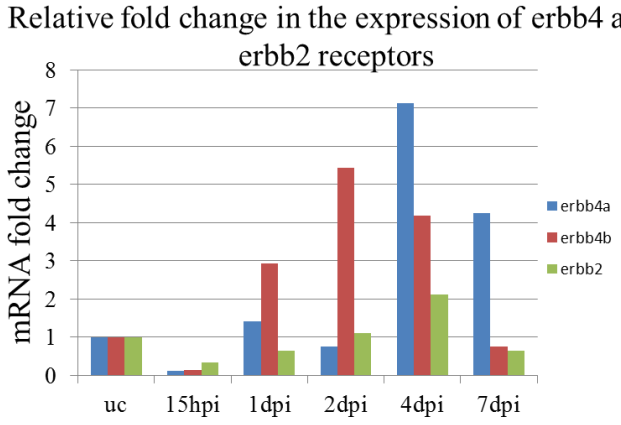
3.2 erbb receptors show a rise in expression during the proliferative phase of zebrafish retina regeneration

Since Nrg1 is a well-known ligand for the erbb receptors and the binding of Nrg1 induces a conformational change in the receptors resulting in the formation of homo- and heterodimers. While the conventional receptors for Nrg1 are ErbB2, ErbB3 and ErbB4, EGF-R doesn't act as a receptor. However, EGF-R heterodimerizes with ErbB4 to take part in the Nrg1/ErbB signaling cascade (Iwakura & Nawa, 2013). Also, since ErbB2 and ErbB3 forms heterodimers with ErbB4, we studied the temporal expression of these genes in relation with their respective heterodimer counterpart (Kataria et al., 2019). On performing a RT-qPCR with one set of samples, we observed that the heterodimers EGF-R-ErbB4, ErbB2-ErbB4, and ErbB3-ErbB4 show a rise in their expression during 2-4dpi which is the proliferative phase. This is similar to the pattern of expression of nrg1 as seen in Figure 3.1. This suggests that Nrg1-ErbB ligand-receptor pair might be playing a role in retina regeneration in the zebrafish model. Since it has been shown that YY1, responsible for tethering chromatin to the nuclear periphery, positively regulates the

expression of ERBB2 by interacting with AP-2 in Mammary Cancer cell lines (Begon, Delacroix, Vernimmen, Jackers, & Winkler, 2005), we compared the temporal kinetics of the two in context of our regeneration model. We observed that indeed, even in context of retina regeneration in zebrafish, both yy1 and erbb2 (comparing figure 3.2 b and d) seemed to show a peak in their expressions on 4dpi, suggesting a link between the two with respect to retina regeneration.

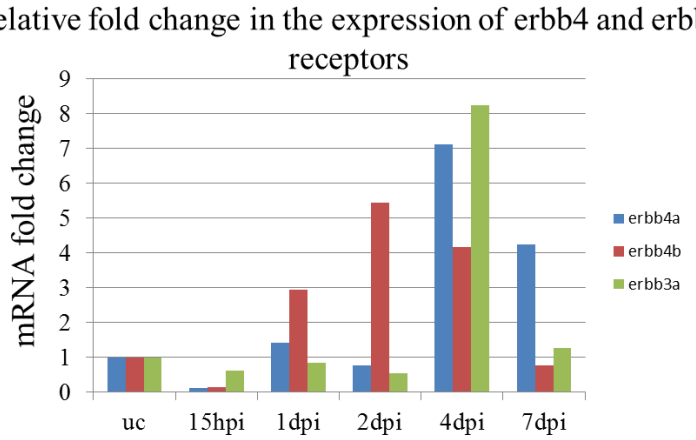


(a)



(b)

(c)



(d)

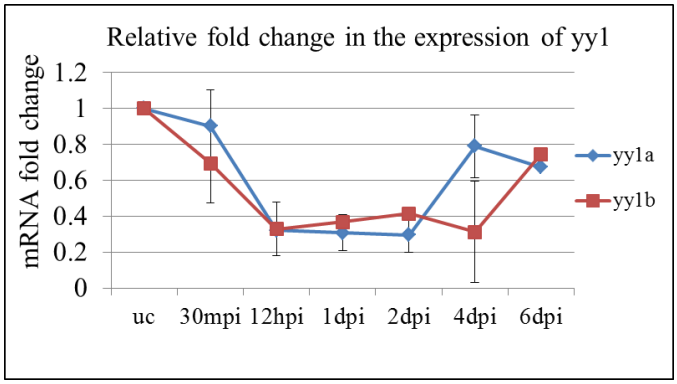
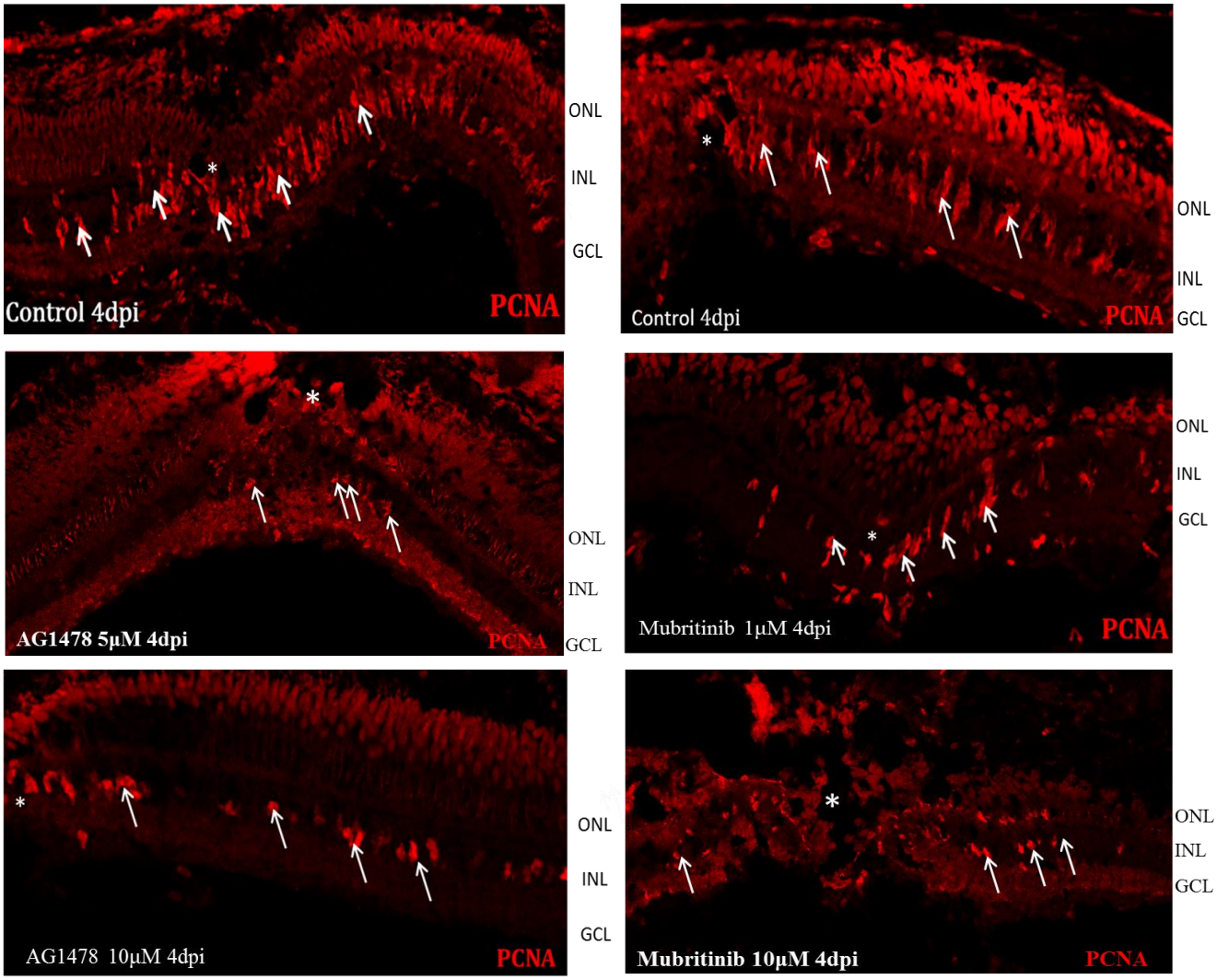


Figure 3.2: mRNA expression pattern of the erbB receptors shows a rise in the levels during the proliferative phase of retina regeneration. Quantification of mRNA levels of (a)erbb4 with respect to egfr (b)erbb2 with respect to erbb4 and (c)erbb3 with respect to erbb4 reveals similar trends of the respective heterodimers suggesting nrg1- mediated regulation during retina regeneration. (d)RT-qPCR showing the temporal kinetics of yy1 in different injury time points (Courtesy of Mansi Chaudhary).

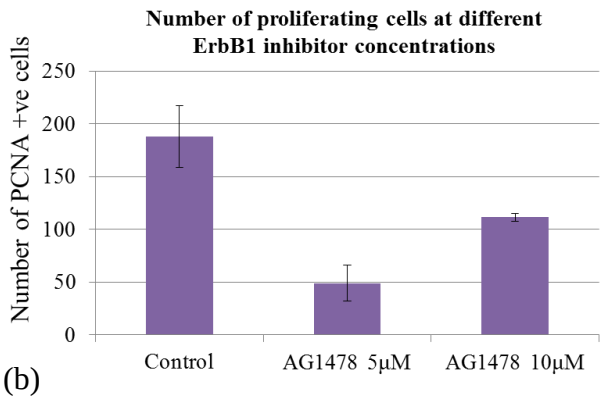
3.3 Pharmacological inhibition of EGF-R and ErbB2 result in reduction in the number of proliferating Müller glial progenitor cells upon injury

The Nrg1/ErbB signaling has been shown to regulate neural development in the CNS by promoting neurogenic cell division in the sub-ventricular zone of the developing zebrafish brain (Sato et al., 2015). In the PNS Farkas *et al.* showed the importance of the Nrg1/ErbB2 signaling in axolotl limb regeneration. So based on our inference from the RT-PCR and RT- qPCR data (done on one set of samples) in figure 3.1 and figure 3.2, we wanted to investigate whether the Nrg1/ErbB signaling cascade was involved in Nrg1 mediated retina regeneration.

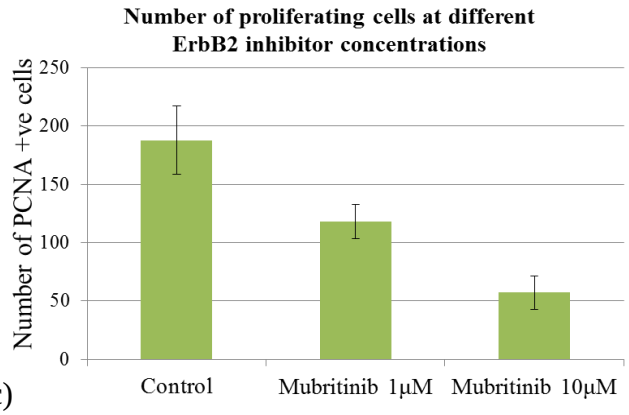
We therefore used AG1478, an EGF-R inhibitor and Mubritinib, an ErbB2 inhibitor to study the effect of blocking the Nrg1/ErbB signaling after an injury. We chose the 4dpi time point for performing an immunostaining after drug treatment on the day 0, since proliferation reaches its peak on 4dpi. Since the Proliferating Cell Nuclear Antigen (PCNA) acts as the processivity factor of DNA Polymerase, we immune-stained for PCNA to visualize the actively proliferating cells upon drug treatment. After quantifying the images observed under the Confocal microscope, we observed that with an increasing concentration of Mubritinib the number of proliferating cells reduced. However, AG1478 yielded a higher suppression of proliferation at 5µM than at 10µM. Since the sample size for 5µM treatment was only one, the ambiguity might be resolved upon increasing the sample size. Notwithstanding the internal discrepancy, however, we still observe a reduction in proliferation in both scenarios when compared to the control. This result is in concurrence with our previous observations that the Nrg1/ErbB signaling plays a role in mediating retina regeneration.



(a)



(b)



(c)

Figure 3.3: Blockade of ErbB1 (Egfr) and ErbB2 using AG1478 and Mubritinib respectively, reduces proliferation when compared to the control. (a)Immunostaining of

PCNA shows the number of actively proliferating cells (AG1478 treatment in the first panel vertically and Mubritinib treatment in the second panel vertically), with their corresponding quantifications done in (b) and (c)

3.4 Blockade of Nrg1/ErbB signaling results in the increased expression of Yy1 and also regulates the expression of regeneration associated genes like Ascl1a, hdac1 when compared to controls

Since our previous data suggests that the Nrg1/ErbB signaling is active during the proliferative phase we performed western blotting to study the expression of regeneration associated genes like Ascl1, Hdac1 and c-Myc. We found that while in AG1478 treated retina the protein levels of Ascl1a reduced with the increase in the drug concentration which correlates well with our observation that the proliferation of cells reduced when compared to the controls, the opposite trend was observed in case of Mubritinib. This could suggest that the Nrg1-Egfr signaling and the Nrg1/ErbB2 signaling regulates Ascl1 differently. We also observed that in case of both drugs, there was a decrease in the levels of Hdac1 with increasing drug concentrations. Since it has been shown previously that during the dedifferentiation phase which lasts up to 2dpi, Hdac1 acts as a pro-proliferative gene (Mitra et al., 2018) and so its decrease during the blockade suggests a possible explanation for the reduced proliferation seen in figure 3.3. Despite being a downstream player in the Nrg1/ErbB signaling we did not observe any change in the expression pattern of c-Myc. From a previous study done in Mammary Cancer cells, it was found that YY1 interacted with endogenous AP-2 to induce the activation of ERBB2 (Begon et al., 2005). The western blotting, however very interestingly showed an increase in the expression of Yy1 upon blocking the Nrg1/ErbB signaling. Juxtaposing this with the temporal kinetics of yy1 obtained from the RT-qPCR (courtesy of Mansi Chaudhary), we believe it could hint at a possible feedback loop involving Yy1 and ErbB (Egfr / ErbB2) wherein ErbB negatively regulates Yy1.

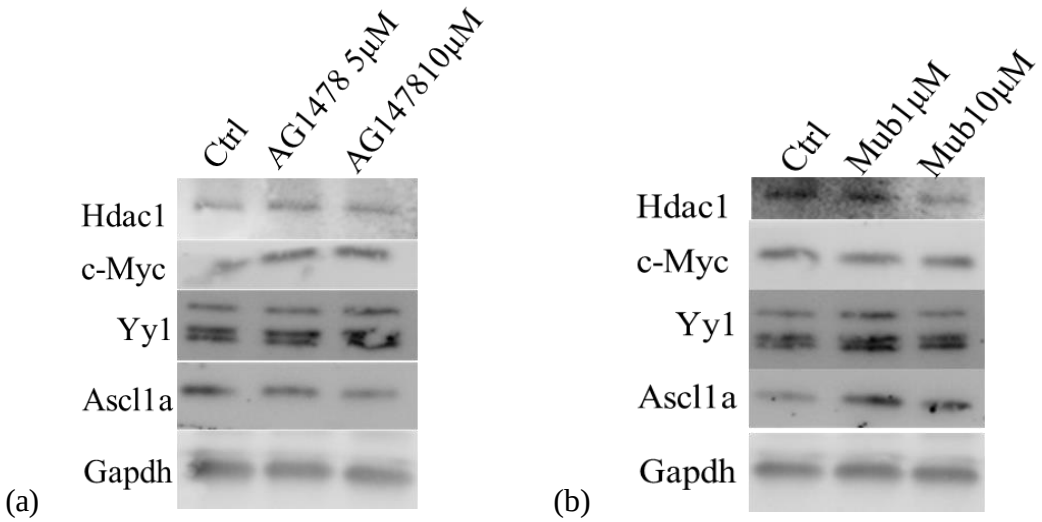


Figure 3.4: Western blotting to show the effect of Nrg1/ErbB blockade on Hdac1, Yy1 and two of the regeneration-associated genes, c-Myc and Ascl1a. (a)effect of Egfr blocking by increasing concentrations (5μM,10μM) of AG1478 and (b)effect of ErbB2 blocking by increasing concentrations (1μM, 10μM) of Mubritinib on Hdac1, Yy1, c-Myc and Ascl1a with Gapdh as the loading control.

CONCLUSION

In this study we explore the role of Nrg1/ErbB signaling in context of retina regeneration in the zebrafish model. We provide preliminary evidence to show that upon injury, the levels of a particular transcript of Nrg1 (Nrg1-206) are upregulated right from the dedifferentiation phase (0-2dpi) to and reach its peak in the proliferative phase (2-4dpi). A similar kinetics is shown by the membrane-spanning tyrosine kinase receptor of Nrg1, ErbB, notably the heterodimer pairs, ErbB1-ErbB4, ErbB2-ErbB4, ErbB3-ErbB4 which also show a peak in expression at the transcript level during the proliferative phase (2-4dpi). To further probe the role of the signaling cascade, we used an ErbB1 (EGF-R) inhibitor AG1478 and an ErbB2 inhibitor, Mubritinib. The blockade of both these receptors showed a decreased proliferation when compared to the control. On tracking the expression of some of the regeneration associated genes like c-Myc and Ascl1a, we find that while c-Myc shows no change in its protein expression, Ascl1a shows dissimilar trends in case of the two drugs which might be owing to different regulatory roles of the two signaling pathways on Ascl1a. The study also provides an exciting insight to the role of Yy1 in retina regeneration. The pharmacological inhibition reveals that the Nrg1/ErbB signaling negatively regulate expression of yy1. Correlating recent studies done by Mansi Chaudhary (unpublished data) with the observations in this study suggest a link between Nrg1-mediated retina regeneration and Yy1, in zebrafish.

FUTURE PERSPECTIVES

My study shows evidence suggestive of an underlying role of the Nrg1/ErbB signaling cascade as a mediator of an injury signal to the Müller glial cell nucleus. While these preliminary results show that Nrg1 has a positive regulation on proliferation, further experiments like *in-situ* hybridization to see spatial localization of Nrg1, western blotting to see the pattern of expression of Nrg1 at the protein level, and analyzing the effect of knockdown of Nrg1 using Morpholino based RNAi interference would be needed to confirm the inferences of the present study. We would also need to investigate the effect of the knockdown and drug-induced blockade on the regeneration associated genes like insma1, oct4, lin-28, etc over a longer range of time points. Besides, since the pharmacological inhibition of the Nrg1/ErbB signaling showed an increase in the levels of Yy1, and due to previous reports linking it to ErbB2 expression in Mammary cancer cell lines, it would be interesting to investigate the link between the signaling and Yy1 and the regulation of the regeneration-associated genes involved in zebrafish retina regeneration.

REFERENCES

Begon, D. Y., Delacroix, L., Vernimmen, D., Jackers, P., & Winkler, R. (2005). Yin Yang 1 Cooperates with Activator Protein 2 to Stimulate *ERBB2* Gene Expression in Mammary Cancer Cells. *Journal of Biological Chemistry*, *280*(26), 24428–24434. https://doi.org/10.1074/jbc.M503790200

Britsch, S. (2007). The neuregulin-I/ErbB signaling system in development and disease. *Advances in Anatomy, Embryology, and Cell Biology*, *190*, 1–65.

D'Anneo, A., Carlisi, D., Emanuele, S., Buttitta, G., Di Fiore, R., Vento, R., … Lauricella, M. (2013). Parthenolide induces superoxide anion production by stimulating EGF receptor in MDA-MB-231 breast cancer cells. *International Journal of Oncology*, *43*(6), 1895–1900. https://doi.org/10.3892/ijo.2013.2137

Edwards, J. M., & Bottenstein, J. E. (2006). Neuregulin 1 growth factors regulate proliferation but not apoptosis of a CNS neuronal progenitor cell line. *Brain Research*, *1108*(1), 63–75. https://doi.org/10.1016/j.brainres.2006.06.025

Farkas, J. E., Freitas, P. D., Bryant, D. M., Whited, J. L., & Monaghan, J. R. (2016). Neuregulin-1 signaling is essential for nerve-dependent axolotl limb regeneration. *Development*, *143*(15), 2724–2731. https://doi.org/10.1242/dev.133363

Gemberling, M., Karra, R., Dickson, A. L., & Poss, K. D. (2015). Nrg1 is an injury-induced cardiomyocyte mitogen for the endogenous heart regeneration program in zebrafish. *ELife*, *4*, e05871. https://doi.org/10.7554/eLife.05871

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

Iwakura, Y., & Nawa, H. (2013). ErbB1-4-dependent EGF/neuregulin signals and their cross talk in the central nervous system: Pathological implications in schizophrenia and Parkinson's disease. *Frontiers in Cellular Neuroscience, 7*. https://doi.org/10.3389/fncel.2013.00004

Kataria, H., Alizadeh, A., & Karimi-Abdolrezaee, S. (2019). Neuregulin-1/ErbB network: An emerging modulator of nervous system injury and repair. *Progress in Neurobiology, 180*, 101643. https://doi.org/10.1016/j.pneurobio.2019.101643

Marchionni, M. A., Goodearl, A. D. J., Su Chen, M., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., … Gwynne, D. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature*, *362*(6418), 312–318. https://doi.org/10.1038/362312a0

Mitra, S., Sharma, P., Kaur, S., Khursheed, M. A., Gupta, S., Ahuja, R., … Ramachandran, R. (2018). Histone Deacetylase-Mediated Müller Glia Reprogramming through Her4.1-Lin28a Axis Is Essential for Retina Regeneration in Zebrafish. *IScience*, *7*, 68–84. https://doi.org/10.1016/j.isci.2018.08.008

Mitra, S., Sharma, P., Kaur, S., Khursheed, M. A., Gupta, S., Chaudhary, M., … Ramachandran, R. (2019). Dual regulation of lin28a by Myc is necessary during zebrafish retina regeneration. *Journal of Cell Biology*, *218*(2), 489–507. https://doi.org/10.1083/jcb.201802113

Powell, C., Cornblath, E., Elsaeidi, F., Wan, J., & Goldman, D. (2016). Zebrafish Müller glia-derived progenitors are multipotent, exhibit proliferative biases and regenerate excess neurons. *Scientific Reports*, *6*(1), 24851. https://doi.org/10.1038/srep24851

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, T., Sato, F., Kamezaki, A., Sakaguchi, K., Tanigome, R., Kawakami, K., & Sehara-Fujisawa, A. (2015). Neuregulin 1 Type II-ErbB Signaling Promotes Cell Divisions Generating Neurons from Neural Progenitor Cells in the Developing Zebrafish Brain. *PLOS ONE*, *10*(5), e0127360. https://doi.org/10.1371/journal.pone.0127360

Sharma, P., Gupta, S., Chaudhary, M., Mitra, S., Chawla, B., Khursheed, M. A., & Ramachandran, R. (2019). Oct4 mediates Müller glia reprogramming and cell cycle exit during retina regeneration in zebrafish. *Life Science Alliance*, *2*(5), e201900548. https://doi.org/10.26508/lsa.201900548

Sharma, P., & Ramachandran, R. (2019). Retina Injury and Retina Tissue Preparation to Study Regeneration in Zebrafish. *BIO-PROTOCOL*, *9*(24). https://doi.org/10.21769/BioProtoc.3466

Wan, J., & Goldman, D. (2016). Retina regeneration in zebrafish. *Current Opinion in Genetics & Development*, *40*, 41–47. https://doi.org/10.1016/j.gde.2016.05.009

WHO. (2019, October 8). Blindness and vision impairment [Fact sheet]. Retrieved April 2, 2020, from World Health Organization website: https://www.who.int/en/news-room/fact-sheets/detail/blindness-and-visual-impairment

WHO. (n.d.). Blindness and vision impairment prevention. Retrieved April 2, 2020, from World Health Organization website: https://www.who.int/blindness/causes/priority/en/index7.html

Yaniz-Galende, E., & Hajjar, R. J. (2014). Stem cell and gene therapy for cardiac regeneration. In *Cardiac Regeneration and Repair* (pp. 347–379). https://doi.org/10.1533/9780857096708.4.347

PRIMER LIST

Mentioned sequences are in 5'-3'.

Table2: List of RT and FL primer used. RT-Reverse transcription; FL- Full Length

nrg1-204 FL Fwd	ATGCTAGCGAATTCACCATGGCGATTCTGCCAGGACG
nrg1-204 FL Rev	ATGCTAGCCTCGAGTCACACAGCTATAGGATCCTGGTTGGC
nrg1-205 FL Fwd	ATGCTAGCGAATTCACCATGGCTGAGGTGAAAGCAGG
nrg1-205 FL Rev	ATGCTAGCCTCGAGTCACACAGCTATAGGATCCTGGTTGG
nrg1-206 FL Fwd	ATGCTAGCGAATTCACCATGAAGTCGGAGGCGGCGGAGG
nrg1-206 FL Rev	ATGCTAGCTCTAGATCACACAGCTATAGGATCCTGGTTGGC
nrg2a-204 FL Fwd	ATGCTAGCGAATTCACCATGAAGAATCCAGTGTTGGCAGACG
nrg2a-204 FL Rev	ATGCTAGCCTCGAGCTAGTGGGGTGCATTGTCCGG
nrg2b-202 FL Fwd	ATGCTAGCGGATCCACCATGAGGAGGCTTGATCCCGTGC
nrg2b-202 FL Rev	ATGCTAGCCTCGAGTTAAAGGGGTGCATTGTCCGGTCTG
nrg3b-201 FL Fwd	ATGCTAGCGAATTCACCATGAGTGAGAGAACAGCACTAGGGG
nrg3b-201 FL Rev	ATGCTAGCTCTAGATCAGCTCTTGGGATGGTCGAGG
nrg1-203 RT Fwd	GCGAGTCGTAGACCACAAACAAGCTG
nrg1-203 RT Rev	GCTTTGCCATTACTCCAGCATTG
nrg1-205 RT Fwd	CCTGCAGCTGGTCAATCATTAC
nrg1-205 RT Rev	GTAGTCATGGCTGAAACATACCTCTCG
nrg1-206 RT Fwd	CCTGCAGCTGGTCAATCATTAC
nrg1-206 RT Rev	CATGGCTGAAACATACCTCTCG
nrg2a-204 RT Fwd	GGGGATTGAATTTATGGAGGCAG
nrg2a-204 RT Rev	CGTTCTCCCTTCATGTCTGTG
nrg2b-202 RT Fwd	ACTTCCAGCCACCGGCACGAG

nrg2b-202 RT Rev	CAGAGCAGACACGTACCTGTCGC
nrg3b-201 RT Fwd	GCTGGGCATTGAGTTTATGGAGAGTG
nrg3b-201 RT Rev	GGAGAACGAGCAGCTTCTGCAG
egfr-207 RT Fwd	CCACATTCAGAGGTGACCC
egfr-207 RT Rev	CTGTTCTGATTGGCGCAGG
erbb2-201 RT Fwd	GATGGAGGAAAGGTGCCG
erbb2-201 RT Rev	GACTCATCTGATCTTCGTTCTGG
erbb3a-203 RT Fwd	GACACCGACTGCTTTGCCTGC
erbb3a-203 RT Rev	CGGTGCCAACACAGGCTTTAG
erbb3b-201 RT Fwd	AACCTCTACTCCGGAGAGCC
erbb3b-201 RT Rev	CAGGGCCTGTACATCCTCG
erbb4a-201 RT Fwd	GGCCTCAAGAACCTGACAGAAATCC
erbb4a-201 RT Rev	GTTGGTGCAAGCGAAGCAG
erbb4b-203 RT Fwd	CACAGACTGCTTCGCTTGC
erbb4b-203 RT Rev	CCGATCCCATCACATGCTTTGG
l-24 RT Fwd	CGACCCAGAGCAGCAAGG
l-24 RT Rev	AGCACATCAGAGTTTAGC
beta-actin RT Fwd	GCAGAAGGAGATCACATCCCTGGC
beta-actin RT Rev	CATTGCCGTCACCTTCACCGTTC