Understanding the Role of the Hippo-YAP Signaling Pathway in the Process of Retina Regeneration in Zebrafish

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CERTIFICATE OF EXAMINATION

This is to certify that the dissertation titled **"Understanding the Role of the Hippo-YAP Signaling Pathway in the Process of Retina Regeneration in Zebrafish"** submitted by Ms. Pallavi Joshi (Reg. No. MP18031) for the partial fulfillment of the M.S. degree program 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.

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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 form for a degree, diploma or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort has been made to indicate this clearly with due acknowledgement of collaborative research and discussions. This thesis is a bona fide record of the original work done by me, and all sources listed in it have been detailed in the references section.

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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

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Dedicated to my grandfather.

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NOTATIONS

MG- Muller Glia

MGPC- Muller Glia-derived Progenitor Cell

ONL- Outer Nuclear Layer

INL- Inner Nuclear Layer

GCL- Ganglion Cell Layer

hpf- Hours Post Fertilization

hpi- Hours Post Injury

dpi- Days Post Injury

YAP- Yes-associated Protein

TEAD- TEA Domain family member of Transcription Factors

BrdU- 5-bromo-2'-deoxyuridine

EdU- 5-Ethynyl-2’-deoxyuridine

ascl1a- Achaete-scute Homolog 1

hdac1- Histone Deacetylase

her4.1- Hairy-related 4, Tandem Duplicate 1

mmp2- Matrix Metallo Protease 2

oct4- Octamer-binding Transcription Factor 4

sani2- Snail Family Transcriptional Repressor 2

sox2- SRY (Sex Determining Region Y)-box 2

tgfbi- Transforming Growth Factor Beta Induced

tgif1- TGF-β Induced Factor Homeobox 1

GS- Glutamine Synthetase

PKC- Protein Kinase C

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ABSTRACT

Sight is probably one of the most important senses that a number of animals rely on in their daily lives. While the exact anatomy of the eye varies among animals, most vertebrates possess retina as the inner-most layer that helps them with vision. Needless to say, any injury or disease to the retina can cause severe aberrations in vision or even the loss of it. Hence, the body has an intrinsic regenerative capacity to help in such crises. While mammals like humans have an extremely limited potential for regeneration, naturally regenerative organisms like the zebrafish possess excellent reparative mechanisms that can result in full reconstitution of all retinal layers and hence lead to complete restoration of vision. This process of retina regeneration in zebrafish is mainly characterized by the acquisition of a stem cell-like state by the Muller Glial cells which allows them to generate proliferating progenitor populations that can then differentiate into the different cell types. This process requires the interplay of multiple signaling cascades like the TGF-β, Delta-Notch, JAK-Stat and Wnt signaling pathway. However, one such pathway that is not completely understood in this context is the Hippo-YAP signaling pathway. Though recent reports in mice have shown the Hippo pathway to be associated with the quiescence exit of Muller Glial cells and MG cell reprogramming, little is known about its role in a naturally regenerative organism like the zebrafish.

In this study, we try to elucidate the role of the Hippo-YAP signaling pathway by looking at the effects of inhibition of the YAP-TEAD interaction on the proliferative response of the Muller Glial cell-derived Progenitor cells (MGPCs) and on the expression levels of various Regeneration Associated Genes (RAGs) like *ascl1a, hdac1, oct4, sox2* and *tgfbi* in the three phases of retina regeneration, namely, the dedifferentiation phase, the proliferation phase and the redifferentiation phase. Furthermore, this study also looks into the effect of this inhibition on the cell fate by quantifying the redifferentiated cell types like the Muller Glial cells, Amacrine cells and the Bipolar cells. In addition, the study has also looked at how the response to injury is affected when the Hippo-YAP pathway is constitutively kept in the ON state in the homeostatic conditions. Together these experiments show that the Hippo pathway plays an important role in the process of zebrafish retina regeneration and has different functions and underlying mechanisms depending on the phase of retina regeneration.

CHAPTER 1: INTRODUCTION

Vision is one of, if not the most important sense that a number of species in the Animal Kingdom rely on everyday for even the most basic survival activities such as spotting sources of food and nutrition, escaping predators and finding mates. The main organ that facilitates this sense of sight is the eye. Though vision in animals is believed to have been evolved more than 700 million years ago (Scientific American, 2012) even before the Cambrian period, the form and function of the "eye" has evolved hugely over the course of these years. The most pre-historic form included the presence of simple eyespots in some single-celled organisms that simply aided in detection of the presence or absence of light and also its direction and gradient in some cases. Though all types of advanced eyes seen today are able to perform the basic function of image resolution, their specific form and function has evolved in different species to better adapt to the animal's habitat, behavior and other requirements. In humans, the structure and composition of the eye is such that it helps facilitate color vision, motion detection, and depth perception. Any developmental aberration or later damage to this architecture can be detrimental to the eye and the hence to the sense of sight itself.

According to the World Health Organization, about a quarter of the world population suffers from vision impairment, of which around 5% is because of retinal problems that could lead to irreversible blindness. Many common genetic and lifestyle conditions such as glaucoma, diabetic retinopathies, macular degeneration and also traumatic injuries can cause damage to the retina, which in turn can lead to a loss of vision. Many years of research have lead to a significant amount of advancements in this field of medicine. Current treatment options for retinal damage include the use of **visual prosthetics** that contain artificial sensors mostly in the form of photodiode and electrode arrays implanted in the retina; **gene therapy** which involves treating mutations in genes associated with inherited and acquired retinal disorders by making modifications directly at the DNA level; and **cell transplant**, wherein a particular functional form of cell type developed outside the body of the patient is artificially introduced in them. An example of this is the transplantation of the rod-precursor cells. Upon transplantation into the retina, they differentiate into mature photoreceptor cells and can form synaptic connections with the bipolar and horizontal cells in the recipient retina and hence restore scotopic vision (Pearson, 2012). However, most of these options require invasive surgical interventions and the introduction of foreign materials into the body, which can add the risk of an immunological response in the patient body post treatment. Also, many of the therapy options that demonstrate a strong potential are still in the pre-clinical phase and remain to be cleared from clinical phase before application. Combined to the shortcomings in the current therapeutic options is the fact that the mammalian retina has a very limited intrinsic regenerative capacity. Together, this makes the study of the mechanism of retina regeneration an important field in basic sciences in order to find a potentially non-invasive cure for retinal damage and disorders.

1.1. Retinal Anatomy and Architecture

The human eye works on the basic principle of focusing light onto a spot and then translating the image so obtained into electrical impulses that can be relayed to and processed by the brain. The specific tissue that is responsible for sensing, translating and relaying these visual information is the retina of the eye.

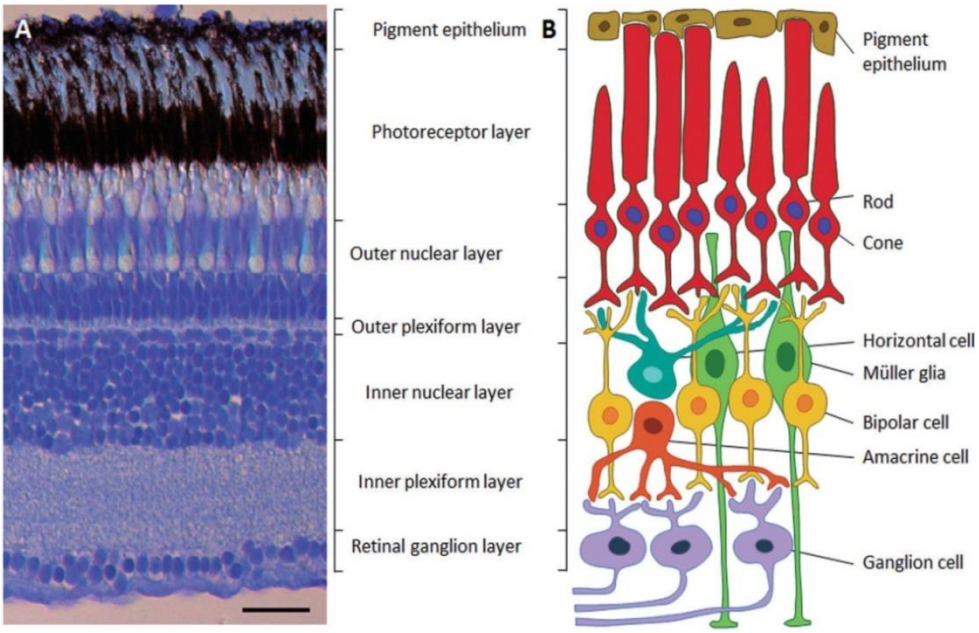


Fig. 1.1: The retinal architecture showing the three retinal layers and the cell types that constitute them.

Source: Gramage, et al. (2014). "The expression and function of midkine in the vertebrate retina". *British Journal of Pharmacology.*

Despite being the inner most layer of the optic cup, the retina is actually a part of the central nervous system. Its outer wall is made up of the melanin-containing retinal pigment epithelium cells which are mainly responsible for photoreceptor maintenance and photopigment renewal. The rest of the human retina is composed of three major layers, namely- the outer nuclear layer (ONL), the inner nuclear layer (INL) and the retinal ganglion cell layer (GCL). Together these are comprised of 6 neuronal cell types and one glial cell. The ONL mainly consists of the light sensing photoreceptor cells- **rods** which facilitates scotopic or night vision, and **cones** that are responsible for photopic or color vision because of their differential sensitivity to a spectral range that encompass blue (short wavelength), green (medium wavelength) and red (large wavelength) light, that is facilitated by the different pigments present in them. The absorption of light by these photopigments is what initiates a change in the membrane potential that causes a release of neurotransmitters and in turn activates the synapsing cells in the inner nuclear layer. The INL consists of three neuronal cell types- the **horizontal cells** which aid in lateral interactions between different photoreceptors and with bipolar

cells, the **bipolar cells** that help to relay the information received from photoreceptors to the ganglion cells, and the **amacrine cells** that help in the communication and integration of information to the ganglion cells. The INL also houses the only glial cell type present in the retina- the **Muller glial cell.** Though the nucleus of the Muller glia lies in the INL, its appendages and extensions span all the three layers and hence allow it to maintain retinal structure and homeostasis (Goldman, 2014). In the GCL, ganglion cells are present whose long axons exit together in the form of the optic nerve and ultimately connect to the visual cortex of the brain, relaying the visual information so that it can be processed in order for an appropriate response to be initiated.

1.2. Zebrafish as a Model Organism for Studying Retina Regeneration

Danio rerio, commonly known as the zebrafish, is a tropical freshwater fish that is native to South Asia. These small, striped fish have been in use as a model organism in the field of medicine from the 1960s. Though being mammals, mice are evolutionarily more similar to humans than zebrafish, these fish have numerous advantages that make it an ideal model system for studying developmental processes, regenerative responses, human diseases and for drug discovery. Though their average lifespan is around 3.5 years, these fish exhibit fast development as they hatch within 48-72 hpf and can grow into adults in about three months. Apart from being cheap and convenient to maintain, they are easy to breed owing to their sexual dimorphism and relatively large egg batches. Since these species exhibit external fertilization and the eggs that they lay are transparent, zebrafish is suitable for studying developmental processes, especially those that occur in the early stages. Being vertebrates, zebrafish share around 70% of the genes with humans and majority of its genome has been completely sequenced. Since zebrafish also have counterparts for about 84% of human disease associated genes, they are frequently used to study human diseases such as cancer, muscular dystrophy, etc. Also, majority of the morphology and physiology of the organ and tissue system present in them are comparable to that in the human body, including the eye. Added to the fact that the retinal architecture in zebrafish and humans are almost identical, zebrafish also have an excellent regenerative capacity, far superior to that exhibited by humans or other mammals.

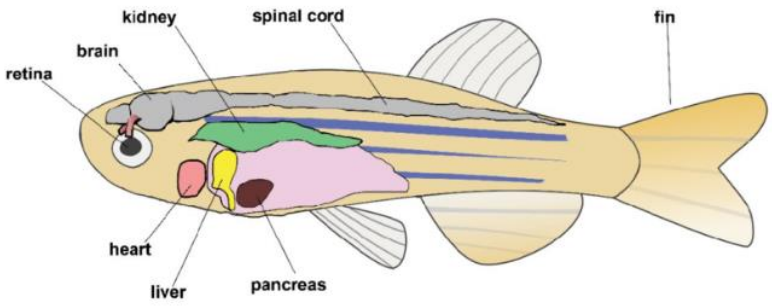


Fig. 1.2: A schematic showing the regenerative potential of zebrafish and the organs that it can regenerate.

Source: Zullo, et al. (2020). "The Diversity of Muscles and their Regenerative Potential Across Animals". *Cells*.

For studying the process of retina regeneration, multiple model systems exist apart from zebrafish, like the postnatal chick that possesses a limited regenerative capacity and the mice which although does not show intrinsic regenerative ability, is frequently used for testing repair strategies in mammals (Goldman, 2014). The zebrafish on the other hand, possesses an excellent regenerative potential, and can regenerate most of its organs such as the kidney, liver, pancreas, spinal cord, fins, heart, brain and the retina. In case of damage to the retina, zebrafish can generate a spontaneous injury response that can ultimately lead to the restoration of all the retinal cell layers and can ultimately also restore vision. Therefore, currently the zebrafish can be described as the best model organism for studying the process of retina regeneration, in the hopes of applying the learnings from their self-reparative strategies to the treatment of human retinal diseases and conditions.

1.3. Regenerative Response in the Zebrafish Retina

Retinal injury can be induced in a number of different ways like introduction of bright light or UV light, toxic chemicals like NMDA or by performing mechanical injury using a needle poke (Goldman, 2014). Though the regenerative mechanism depends on the extent and type of the injury, there are a few characteristic series of events that occur in case of all types of injuries. For the sake of performing experiments in the lab, stab wound by injury is mostly used as it has the advantage of resulting in a uniform injury in all retinal cell layers (Sharma and Ramachandran, 2019).

The response to a mechanical injury is majorly carried out by the Muller glial cells, which are the key players in the process of retina regeneration. This process is mainly divided into the following three phases-

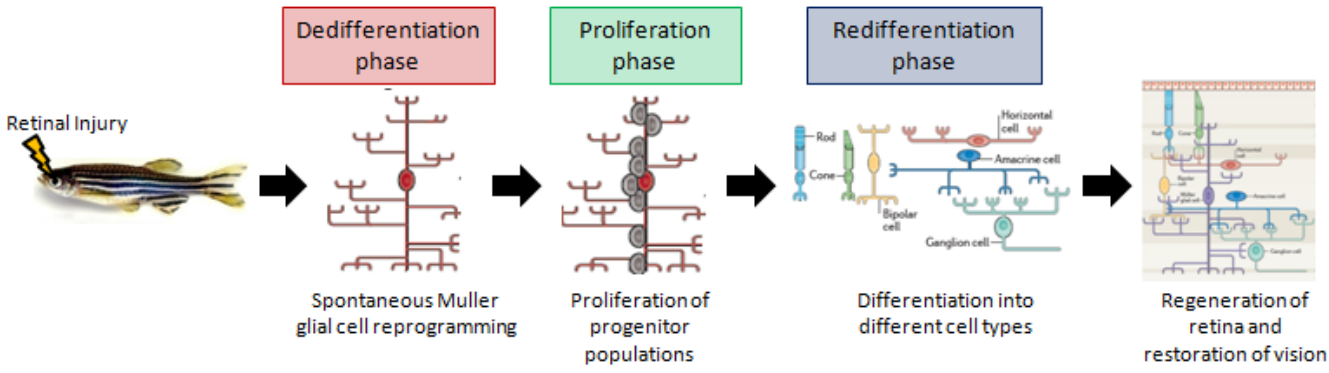


Fig. 1.3: The three phases of retina regeneration, namely, dedifferentiation phase, proliferation phase and the redifferentiation phase in zebrafish in response to injury.

Source: Goldman, (2014). "Müller glial cell reprogramming and retina regeneration". *Nature Reviews Neuroscience*.

1. **Dedifferentiation Phase (0-2 days post injury)-** This phase is characterized by the acquisition of a stem cell like characteristic of the differentiated Muller glial cells near the spot of injury. The initial signals come from the neighboring affected neuronal cells in the form of the release of Tumor Necrotic Factor-α (TNF-α), ADP and Heparin-binding EGF-like growth factor (Hbegf) (Goldman, 2014). This results in the re-entry of these MGs into the cell cycle.

2. **Proliferation Phase (2-4 days post injury)-** In this phase, the Muller glial cell-derived progenitors cells (MGPCs) are formed by interkinetic nuclear migration and asymmetric division. These divide and in turn result in the generation of more MGPCs. This MGPC formation is associated with alteration in the expression of genes such as for Ascl1a and Stat-3 (Goldman, 2014).

3. **Redifferentiation Phase (4-6 days post injury)-** Since MGPCs are multipotent cells, they migrate along the radial fibers to places where retinal cell types are missing and redifferentiate into the different cell types, restore the damaged retinal layers (Belecky-Adams et al., 2013) and ultimately are able to completely restore vision as well.

Multiple signaling cascades are involved in carrying out these three phases of retina regeneration, which include- TGF-β signaling, JAK-Stat signaling, Wnt signaling and Delta-Notch signaling. The TGF-β signaling acts through the Smad pathway to alter gene expression in case of injury. The TGF-β signaling inhibitors like TGFβ-induced factor 1 (Tgif1) has been shown to enhance progenitor proliferation in the injured zebrafish retina (Lenkowski et al., 2013). The JAK-Stat signaling is involved in MG cell reprogramming and this is evident by the fact that the Stat3 expression is observed upon injury in both quiescent MG cells as well as MGPCs, and the knockdown of *stat3* can inhibit the formation of progenitor cells (Kassen et al., 2007; Nelson et al., 2012). Retinal injury in zebrafish also leads to the activation and stabilization of *wnt* and β-catenin in the MGPCs and the inhibition of the Wnt pathway using antagonists like dickkopf (Dkk), leads to the suppression of progenitor formation in the injured retina (Ramachandran et al., 2011; Meyers et al., 2012). On the other hand, the Notch signaling has an inhibitory function since the induction of Notch signaling components like *deltaA, deltaB, deltaC* and *notch1*, and Notch target genes such as *her4* upon injury reduces the number of MG cells that take part in the response to injury (Goldman, 2014; Wan et al., 2012). In addition to this, all four of these pathways directly or indirectly regulate the levels of the RNA-binding protein Lin28 and the microRNA let-7. When the levels of let-7 are high inside the MG cell, it remains in the quiescence state and in the differentiated form. On the other hand, when the levels of Lin28 in the cell are high, the MG cell can undergo a reprogramming event and re-enters cell cycle (Goldman, 2014). Hence, different signaling pathways work together to control the switch of MG cells from quiescent to stem-cell like state, and vice-versa. However one such signaling pathway that is not completely understood in context of the MG cell reprogramming in response to retinal injury is the Hippo-YAP signaling pathway.

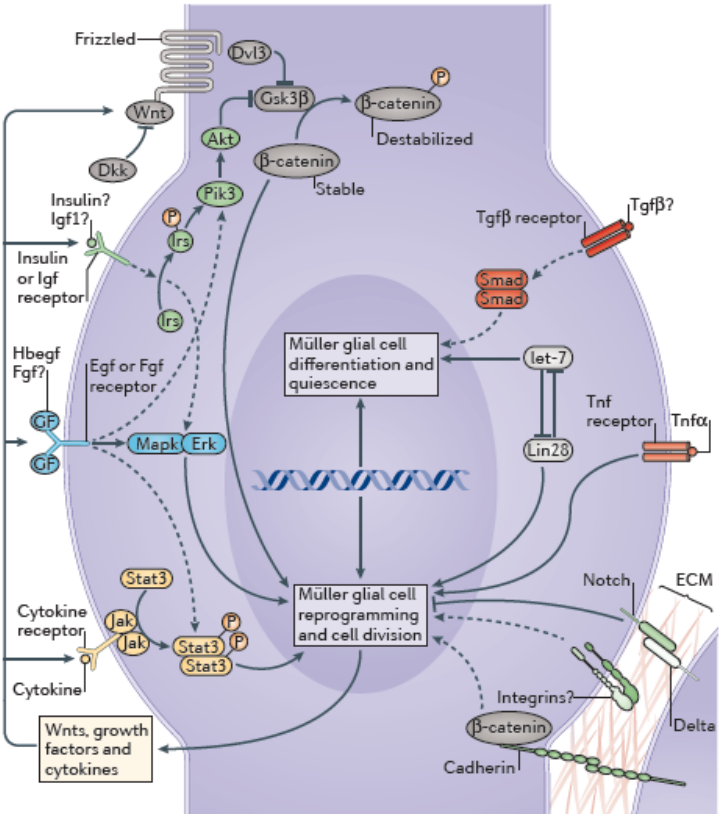


Fig. 1.4: The molecular mechanisms regulating switch between Muller glial cell reprogramming and quiescence.

Source: Goldman, (2014). "Müller glial cell reprogramming and retina regeneration". *Nature Reviews Neuroscience*.

1.4. Hippo-YAP Pathway and its Role in Tissue Regeneration

First discovered in the fruit fly *Drosophila melanogaster* (Harvey et al., 2003; Wu et al., 2003), the Hippo-YAP signaling pathway is an evolutionarily conserved pathway that is mostly involved in organ development and size control (Pan, 2010; Halder and Johnson, 2011). In mammals, its main components include Mst1 and Mst2 (Hippo is *Drosophila),* Salvador (Sav), Lats1 and 2 (Warts in *Drosophila),* Mob (Mob-as-tumor-suppressor in *Drosophila*) and Yes-associated Protein (YAP). In a multi-step phosphorylation cascade, the ultimate target is the transcription factor YAP, whose phosphorylation status determines whether the Hippo-YAP pathway is in the ON state or the OFF state. In the ON state, the YAP is in the phosphorylated form which allows it to be acted upon by the YAP chaperon protein 14-3-3σ which facilitates its proteosomal degradation in the cytoplasm. On the other hand, when the Hippo pathway is in the OFF state, YAP is in the non-phosphorylated form and hence can undergo nuclear localization. Inside the nucleus, it binds to other transcription factors such as the TEA domain family member (TEAD) transcription factors (TEAD1-4) which leads to the expression of the downstream genes which are mostly those associated with cell cycle and

proliferation. Some other transcription factors that YAP binds to are ErbB4 and Smads (binding to which can lead to the activation of the downstream genes), while binding to transcription factors like RunX2, NuRD and p73 can cause a repression in gene expression.

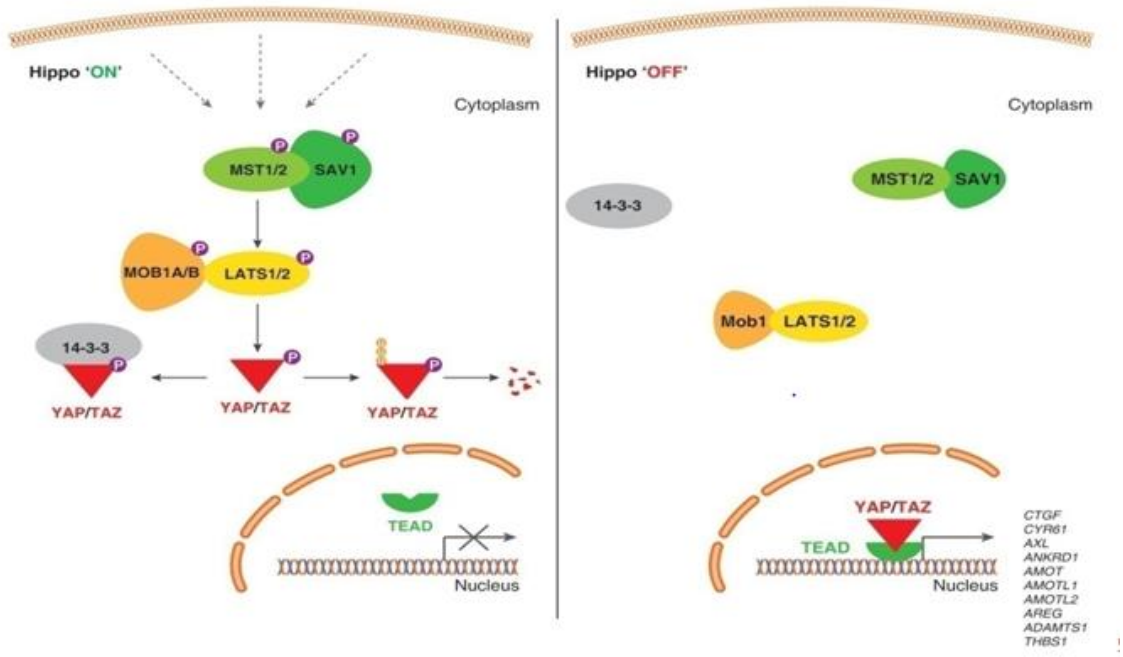


Fig. 1.5: The main components of the Hippo-YAP signaling pathway and their roles in the ON and OFF states.

Source: Boopathy, et al. (2019). "Role of Hippo Pathway-YAP/TAZ Signaling in Angiogenesis". *Frontiers in Cell and Developmental Biology*.

Since, the Hippo-YAP signaling pathway mostly regulates cell proliferation, apoptosis and stem cell renewal; it has been associated with not only developmental processes like organ and tissue formation, but also with regenerative responses; and aberrations in this pathway such as deletions or mutations in the upstream Hippo pathway components such as *Mst1/2, Sav1* and *Lats1/2* or the overexpression of YAP have been reported to result in serious conditions like hyperplasia and tumorigenesis, which can cause various types of cancers in humans such as hepatocellular carcinoma, oral squamous cell carcinoma (OSCC), luminal breast cancer, lung adenocarcinoma and colorectal cancers to name a few (Zheng et al., 2019).

In the developmental process of angiogenesis, the Hippo-YAP pathway affects key events of vascular sprouting, vascular barrier formation and vascular remodeling by regulating endothelial cell proliferation, migration and survival (Boopathy et al., 2019). Moreover, the endothelium-specific deletion of YAP/TAZ can lead to an impairment in the process of vascularization and also

result in embryonic lethality. The YAP/TAZ activation induced by the VEGF-VEGFR2 signaling causes changes in the transcriptional regulation of genes linked to actin cytoskeletal modulation and this cytoskeleton dynamics is what ensures a guided angiogenic response that leads to the formation of a proper vascular system (Wang et al., 2017). Apart from this, the Hippo-YAP pathway is also associated with the genetic regulation of the mammalian heart size. Mice embryos with inactivated Hippo pathway components develop overgrown hearts that have increased cardiomyocyte proliferation. The inhibition of the interaction of YAP with β-catenin on the expression levels of Sox2 and Snai2 reveals that the Hippo pathway negatively regulates a subset of Wnt signaling target genes. Hence, together the Hippo pathway and the Wnt signaling restrict cardiomyocyte proliferation and help in controlling heart size (Heallen et al., 2012). Additionally, the Hippo pathway also plays an important role in cardiac repair and regeneration. It has been shown that the cardiac-specific deletion of *Yap* can impede with the neonatal heart regenerative response. In line with this, a forced expression of the constitutively active form of YAP can stimulate cardiac regeneration in adult hearts (Xin et al., 2013). Another tissue wherein the Hippo pathway helps in regeneration is the intestinal tissue in which it, along with other pathways, leads to the alteration in gene expression in the stem cell compartment of the human intestine upon injury (Hong et al., 2016).

In the context of retina regeneration, recent advancements have been made that have shown the Hippo-YAP signaling pathway to be associated with the Muller glial cell reprogramming and quiescence exit. A 2019 study by Hamon et al. shows that the transcription factor YAP is essential for MG cell reprogramming and its re-entry into the cell cycle and can regulate the basal level of cell cycle genes like Cyclin D1 and Cyclin D3 in quiescent Muller glial cells. They show that a loss of function mutation in YAP results in about a 60% decrease in the proliferative response of the MGs in *Xenopus*. In mice, a YAP CKO condition has no significant effect in the homeostatic condition as the retinal structure remains intact. However, upon performing injury by injecting MNU (methylnitrosourea) to induce photoreceptor death, the cell cycle regulator genes like Cdk6, Ccnd1, Ccnd2 and Ccnd3 exhibit a downregulation in expression levels. Also, the levels of Cyclin D1 and Cyclin D3 that are specifically expressed in the Muller glial cells also decrease. In line with these results, overexpression of YAP5SA, a mutant of YAP that is insensitive to cytoplasmic retention, is sufficient to initiate quiescence exit and facilitate MG cell reprogramming. Along with resulting in an enhanced proliferative response, this gain of function mutation also leads to an increased expression levels in Cyclin D1 and the pro-neural transcription factor Ascl1.

The hypothesis that the Hippo pathway acts as an endogenous blocking mechanism which prevents MGs from adopting a proliferative, stem cell-like state in mammals, and that its repression may be able to stimulate their regenerative capacity was proven in another 2019 study by Rueda et al. This study shows that the bypass of Hippo signaling in the adult MGs by causing MG specific deletion of upstream Hippo components Lats1 and Lat2 and also in gain of

function mutation condition (YAP5SA overexpression) results in elevated Cyclin D1 levels and loss of MG identity and causes spontaneous MG proliferation.

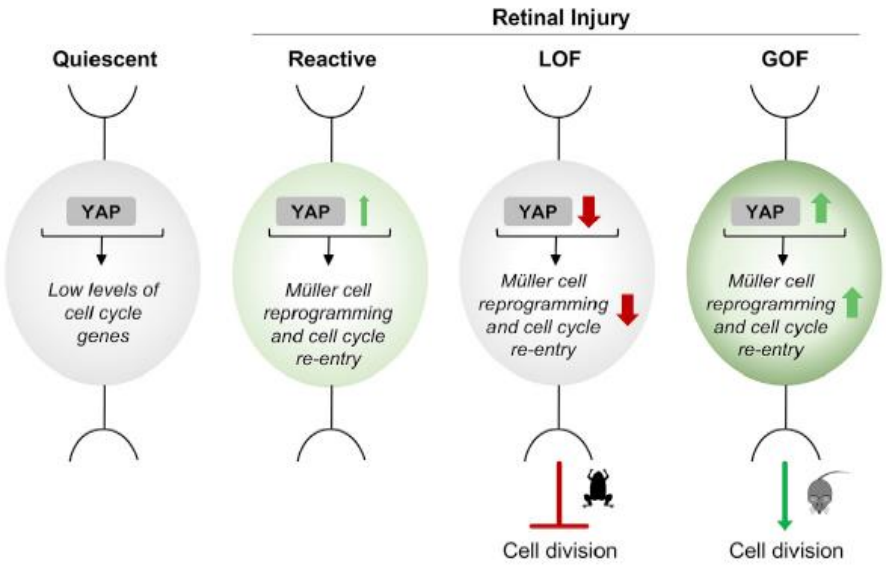


Fig. 1.6: The Hippo-YAP pathway maintains quiescence by maintaining low levels of cell cycle genes. Its upregulation upon injury leads to Muller glial cell reprogramming and results in cell cycle re-entry.

Source: Hamon, A. et al. (2019). "Linking YAP to Muller Glia Quiescence Exit in the Degenerative Retina". *Cell Reports*.

According to previous studies, there is a co-expression of Cyclin D3 and p27KIP1, the CyclinD/CDK complex inhibitor in quiescent adult mice MG cells (Dyer and Cepko, 2000). Due to the presence of this inhibitor, the transition from G1 phase to S phase of the cell cycle is prevented (Dyer and Cepko, 2001a, 2001b). Upon injury, the P27KIP1 expression declines dramatically at 24hpi, while that of Cyclin D3 remains unchanged and this is likely what drives the cell cycle into S phase entry. Though the Cyclin 3 levels do not show any significant changes, there is a drastic increase in Cyclin 1 expression from 12 to 24hpi which declines by 48hpi. In the same 2019 study by Rueda et al, they have shown YAP/TEAD1 complex to be a transcriptional regulator of Cyclin D1 in MG cells, since it is crucial for maintaining high levels of Cyclin D1 expression in all the succeeding MG cell divisions after injury. This was also confirmed by the upregulated Cyclin D1 levels observed in Lats1/2 CKO condition and a forced expression of YAP5SA, both of which also facilitate cell cycle re-entry and proliferation in MG cells.

In zebrafish, the knowledge about the function of this pathway is currently very limited. A 2018 study shows that upon light-induced damage to the zebrafish retina, the expression levels of the key genes associated with the Hippo pathway like *yap1, lats1* and *tead1a* show a rapid increase that returns to basal levels only after 96 hours of continuous light treatment. After 36 hours of constant light treatment, the YAP1 protein in the Muller glia translocates from the cytoplasm to

the nucleus where it can form a complex with TEAD. Disruption of this complex caused by injecting the drug verteporfin can lead to a significant decrease in the number of Muller glia re-entering the cell cycle and generating a regenerative proliferative response (Jia et al., 2018).

Since, the involvement of the Hippo pathway is evident in the process of retina regeneration; it would be interesting to obtain some deeper insights into its functions in a naturally regenerative organism like the zebrafish, which is what this study aims to do.

1.5. Pharmacological Blocking of YAP-TEAD interaction

To keep Hippo-YAP signaling pathway in a constitutively ON state, the YAP-TEAD interaction is inhibited with the use of a drug called Verteporfin. Verteporfin induces the sequestration and proteosomal degradation of YAP by upregulating the levels of the YAP chaperon protein called 14-3-3σ (Wang et al, 2016). As a result, YAP is not able to undergo nuclear localization and hence the expression of the downstream genes is prevented. A benzoporphyrin derivative, verteporfin is light sensitive and is commonly used for photodynamic therapy especially in treating conditions like macular degeneration where the abnormal blood vessels need to be eliminated. Though usually injected, in the experiments part of this study, the drug was mixed in fish system water to make up the required concentrations depending on the condition (Control, 10nM, 100nM and 1µM drug concentration). The fish were dipped in these different concentrations for the set duration depending on the experimental timeline. The fish were kept in dark throughout the duration of the treatment period since any neovascular damage had to be prevented owing to the photosensitive nature of the drug.

OBJECTIVES

The primary aim of this project was to determine the involvement of the Hippo-YAP signaling pathway in the different phases of retina regeneration in the zebrafish. In order to better understand this question, the following were kept as the main objectives of the project-

- To check the effect of the inhibition of the YAP-TEAD interaction on the proliferation response of MGPCs upon retinal injury.
- To determine the effect of this inhibition on the expression levels of the Regeneration Associated Genes.
- To see the effect of this inhibition on the cell fate of the redifferentiated cells formed in the regenerated tissue.
- To determine if pre-injury treatment has any effect on the proliferation response of the MGPCs.

CHAPTER 2: MATERIALS AND METHODS

2.1. Zebrafish Maintenance

1. Fish were maintained in 14/12 light-dark cycle.
2. The temperature of the system was kept at 27°C and pH maintained at 7.4
3. Fish were fed twice a day

2.2. Retina Dissection for RNA Isolation and Western Blotting

1. Fish was anesthized using Tricaine methanesulfonate.
2. Each retina was injured using a 30 gauge needle in 4 different orientations.
3. At the set time of injury, eyes were dissected using steel forceps and needle. Dissections were carried out either in 1X PBS (Phosphate Buffered Saline) for harvesting retina. The dissected retina was taken and stored in Trizol and Laemmli buffer for RNA and Western samples respectively.

   NOTE: For retina dissections, the fish were kept for dark adaptation at least 4 hours prior to the time of dissection. This makes it easier to remove the pigment epithelium from the retina since it turns black.

2.3. Tissue Fixation and Sectioning

1. The lens were removed and the rest of the intact eye tissue was put into 4% PFA (Paraformaldehyde) and kept overnight at 4°C.
2. The next day, serial sucrose washings were given to the fixed tissue as a method for cryoprotection for 45 minutes each on a rotor in the following order-

   1ml of 5% sucrose
   800µl of 5% and 400µl of 20% sucrose
   500µl of 5% and 500µl of 20% sucrose
   400µl of 5% and 800µl of 20% sucrose
   1ml of 20% sucrose

3. Then 500µl of OCT was added and the MCTs were put on the rotor for 30 minutes.

4. These tissues were then aligned and embedded in small cube-shaped OCT containing aluminium foil containers and the samples were stored at -80°C until sectioning.
5. The blocks were then sectioned into slices of 12µm thickness in Cryostat and the sections were collected on Super frost plus slides. The slides were stored at -20°C after overnight drying.

Composition of solutions used-

4% PFA: 2g PFA

5ml 10X Phosphate buffer

Volume made up to 50ml using DEPC water.

Solution kept at 65°C in a water bath with constant stirring to dissolve mixture.

5% Sucrose: 2.5 g Sucrose

50ml Autoclaved water

Mixture dissolved and stored in -20°C

20% Sucrose: 10 g Sucrose

50ml Autoclaved water

Mixture dissolved and stored in -20°C

2.4. RNA Isolation

1. The dissected retinas stored in Trizol were taken out from -80°C and allowed to thaw.
2. The tissue was homogenized using a 200µl pipette or a homogenizer until no clumps were visible.
3. After an incubation of 5 minutes at RT, 40µl (0.2 volume) of chloroform was added and the solution was mixed gently by inverting the MCTs up and down for 15-20 seconds.
4. After an incubation of 5 minutes at RT, the solution was centrifuged at 12,000 RCF for 15 minutes at 4°C.
5. The upper phase so obtained was collected using cut tips and put into fresh MCTs without disturbing the interphase.
6. Double volume of isopropanol was added. After mixing properly, the MCTs were kept overnight in -80°C for better yield.

7. Next day after thawing, samples were centrifuged at 13,000 RPM for 20 minutes at 4°C.
8. After discarding the supernatant, the pellets were washed with 200µl of 80% ethanol (made in DEPC water) and samples were centrifuged at 13,000 RPM for 5 minutes at 4°C.
9. The supernatant so obtained was discarded and this step was repeated.
10. After discarding the supernatant, the pellets were allowed to dry for 15-20 minutes till ethanol evaporated completely.
11. The dried pellets were dissolved in 15-20µl of DEPC treated water and the MCTs were kept on ice for 30 minutes.
12. The concentrations of these samples were then checked by measuring the OD on a Nanodrop and running the samples on 0.8% gel.

2.5. cDNA Synthesis

For 10µl reaction-

1. The following components were added in a PCR tube:-

   Template RNA- 2.5µl

   Oligo (dt) Primer- 0. 5µl

   Random Hexamer- 0. 5µl

   Nuclease Free Water- 2.5µl

2. After a short spin, the mixture was put for denaturation at 65°C for 5 minutes, after which the tubes were put immediately on ice for 2-3 minutes.
3. The following components were added to the mixture:-

   5X Reaction Buffer- 2µl
   RiboLock RNase Inhibitor- 0.5µl
   RevertAid M-MuL V RT- 0.5µl
   10mM dNTP Mix- 1µl

4. After a short spin, the tubes were put in a PCR machine with the following conditions:-

   25°C- 5 min
   42°C- 60 min
   70°C- 5 min
   4°C- Infinite hold

5. The cDNA so obtained was stored in -80°C.

2.6. Reverse Transcription Polymerase Chain Reaction (RT PCR)

1. The synthesized cDNA was diluted using Milli-Q water (1:4 dilutions) and the following components were put for a 10µl reaction-

    20X Buffer- 0.5µl
    dNTP- 1µl
    Primer (F+R)- 0.5µl
    Template cDNA- 0.5µl
    Taq Enzyme- 0.1µl
    MQ water- 7.4µl

2. After giving a short spin, the PCR tubes were put in the PCR machine with the following cycling conditions over 30 cycles-

    95°C- 2 min
    95°C- 20 secs
    60°C- 30 secs
    72°C- 7 min
    4°C- Infinite hold

3. The PCR product so obtained was checked on 1.2% Agarose gel.

2.7. Quantitative PCR (qPCR)

1. qPCR was carried out using the KOD SYBR qPCR Master Mix (pure gene) and the following components were added for 5µl reactions:-

    Master Mix- 2.5µl
    Primer (F+R)- 0.5µl
    Template cDNA- 0.5µl
    MQ water- 1.5µl

2. After a short spin, the plate was put in the qPCR machine.
3. The data so obtained was analyzed and graphs for the genes were plotted.

2.8. Western Blotting

1. Sample Preparation:

- Retina samples stored in 2X Laemmli buffer were taken out from -80°C and allowed to thaw.
- Tissue was homogenized using a homogenizer or a 200µl tip until the solution was translucent.
- The MCTs were vortexed briefly and kept immediately on ice. This step was repeated 10 times for each sample.
- The samples were boiled at 95°C for 10 minutes on a heat block.
- These were then centrifuged at 6000 RPM for 6 minutes at 4°C.
- After tapping, the samples were given a short spin and were stored in -80°C.

2. The western glass plates were cleaned and set. Leakage was checked by pouring water in the cast.
3. 12% Resolving gel was prepared with the following composition and poured in the cast-
    - Resolving Buffer-2.5ml
    - 30% Acrylamide-4ml
    - MQ Water- 3.3ml
    - 10% SDS- 100µl
    - 10% APS- 100µl
    - TEMED- 6µl
4. After solidification, stacking gel with the following composition was prepared and poured-
    - Stacking Buffer-625µl
    - 30% Acrylamide-667µl
    - MQ Water- 3.603ml
    - 10% SDS- 50µl
    - 10% APS- 50µl
    - TEMED- 5µl
5. Combs were inserted and the gel was allowed to solidify.
6. Samples were taken out from -80°C and allowed to thaw.
7. After a short spin, samples were loaded on the gel, along with protein ladder for reference.
8. The gel was run at 91V for approximately 3 hours.
9. Prior to setting up the transfer, PVDF membrane was charged with methanol for 5 minutes, which was followed by two washes with water and one with transfer buffer.
10. Transfer was set up in the transfer buffer by making a sandwich of the cut gel, the charged membrane and the blotting papers.
11. The transfer was put for 90 minutes at 71V.

12. After removing the transfer, the blot was put for blocking in 10% Skimmed milk for 1 hour on a rotor shaker at low speed (~35)
13. Three washes with 0.05% 1X PBST were given at high speeds (~60), each for 10 minutes.
14. The blot was incubated in 1° Antibody for 3 hours at RT or overnight at 4°C.
15. The antibody was collected and three 10-minute washes with 0.05% 1X PBST were given.
16. The blot was incubated in 2° Antibody for 2 hours at RT.
17. Antibody was collected and three 10-minute washes with 0.3% 1X PBST were given.
18. The blot was developed in ImageQuant LAS4000 using ECL or stored in 4°C in 0.05% 1X PBST.

2.9. Immunostaining

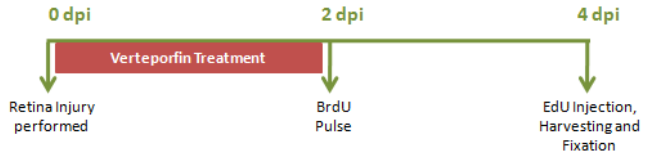
1. Slides were taken out from -80°C and allowed to dry in 37°C for half an hour.
2. The slides were then given three 1ml 1XPBS washes, each with 10 minutes of incubation.
3. Meanwhile, 50ml of 2N HCl was prepared and prewarmed at 37°C.
4. The slides were put in 2N HCl at 37°C for exactly 20 minutes.
5. The slides were then given two successive Sodium Borate (0.1M, pH 8.5) washes for 10 minutes each.
6. 1ml of 6% BSA prepared in 1X PBST was used for blocking with an incubation time of 2-3 hours at RT.
7. After removing the BSA, 250µl of 1° Antibody (1:1000 dilution in 1% BSA/PBST) was put in each slide and slides were incubated at RT for 3 hours.
8. 1° Antibody was collected and three 1X PBST washes were given, each of 10 minutes.
9. 250µl of 2° Antibody (1:1000 dilution in 1% BSA/PBST) was added in each slide and slides were incubated at RT for 3 hours.
10. After three 10-minute 1X PBST washes, signal was checked under fluorescence microscope.
11. If signal was strong, slides were allowed to dry for 15-20 minutes before mounting with 60µl of DABCO.
12. After overnight drying, images were taken under the confocal microscope or the slides were stored in -20°C.

2.10. Microscopy

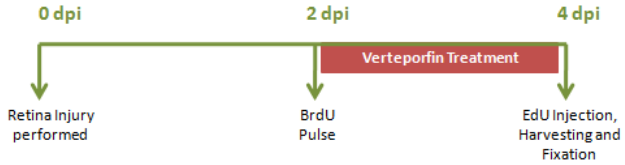
1. Bright field microscope from Zeiss was used for performing retina injuries and dissections.
2. Confocal Microscope from Nikon was used for cell counting and fluorescence imaging.

2.11. Experimental Timelines

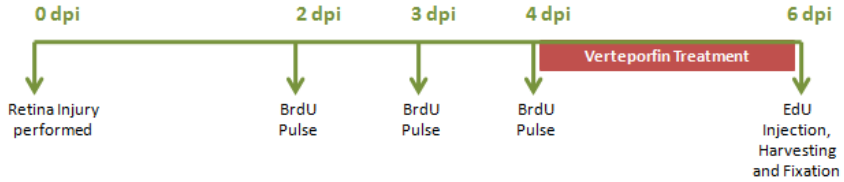
1. For Inhibition of YAP-TEAD interaction in Dedifferentiation phase



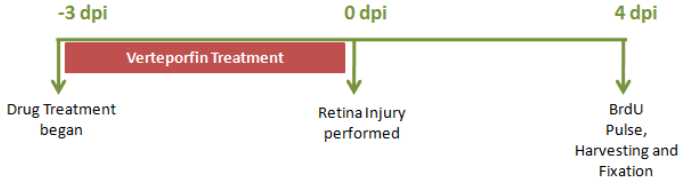
2. For Inhibition of YAP-TEAD interaction in Proliferation phase



3. For Inhibition of YAP-TEAD interaction in Redifferentiation phase



4. For Inhibition of YAP-TEAD interaction prior to Injury



CHAPTER 3: RESULTS AND DISCUSSION

3.1. Inhibition of YAP-TEAD interaction in dedifferentiation phase reduces proliferation in MGPCs.

To check for the proliferative response of the MGPCs on inhibiting the YAP-TEAD interaction in the dedifferentiation phase from 0-2dpi, the number of BrdU labeled cells were counted in each condition. It was observed that the proliferative response of the MGPCs at 4dpi decreases with an increase in the concentration of the verteporfin drug. The number of EdU +ve cells and collocalization also exhibited the same trend, proving that the YAP-TEAD interaction is essential for proper MGPC proliferation response.

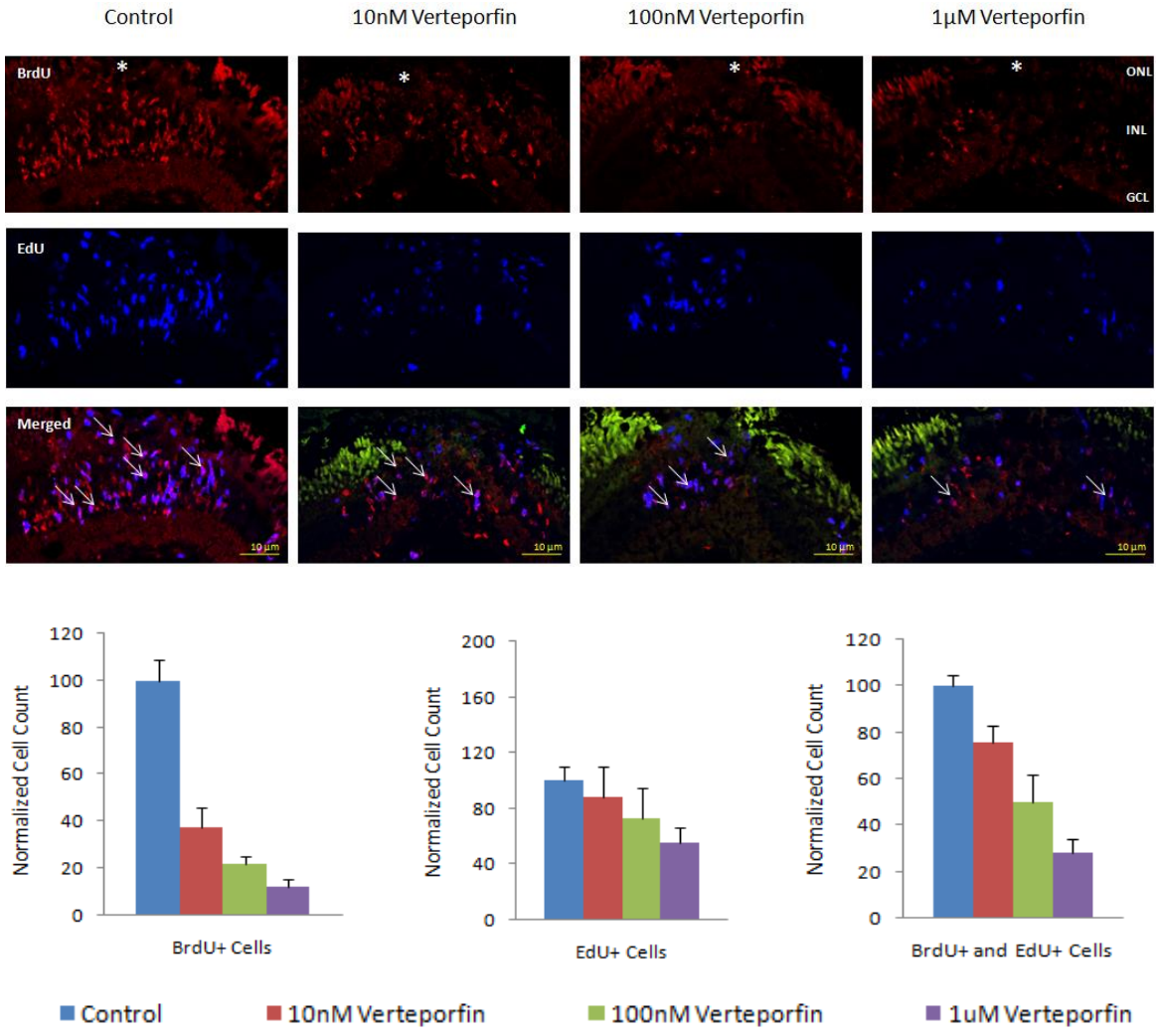


Fig. 3.1: The immunostaining images and cell count plots showing a decrease in the proliferation of BrdU labeled MGPCs with an increase in verteporfin concentration in the dedifferentiation phase.

3.2. Effect of YAP-TEAD interaction inhibition during dedifferentiation phase on RAGs.

The qPCR and Western Blotting data show that the levels of Regeneration Associated Genes like *ascl1a, hdac1, her4.1, mmp2, tgfbi, tgif1, snai2* and *oct4* increase, while that of *sox2* decreases at 4dpi with an increase in the concentration of the verteporfin drug when the YAP-TEAD interaction is inhibited in the dedifferentiation phase from 0-2dpi.

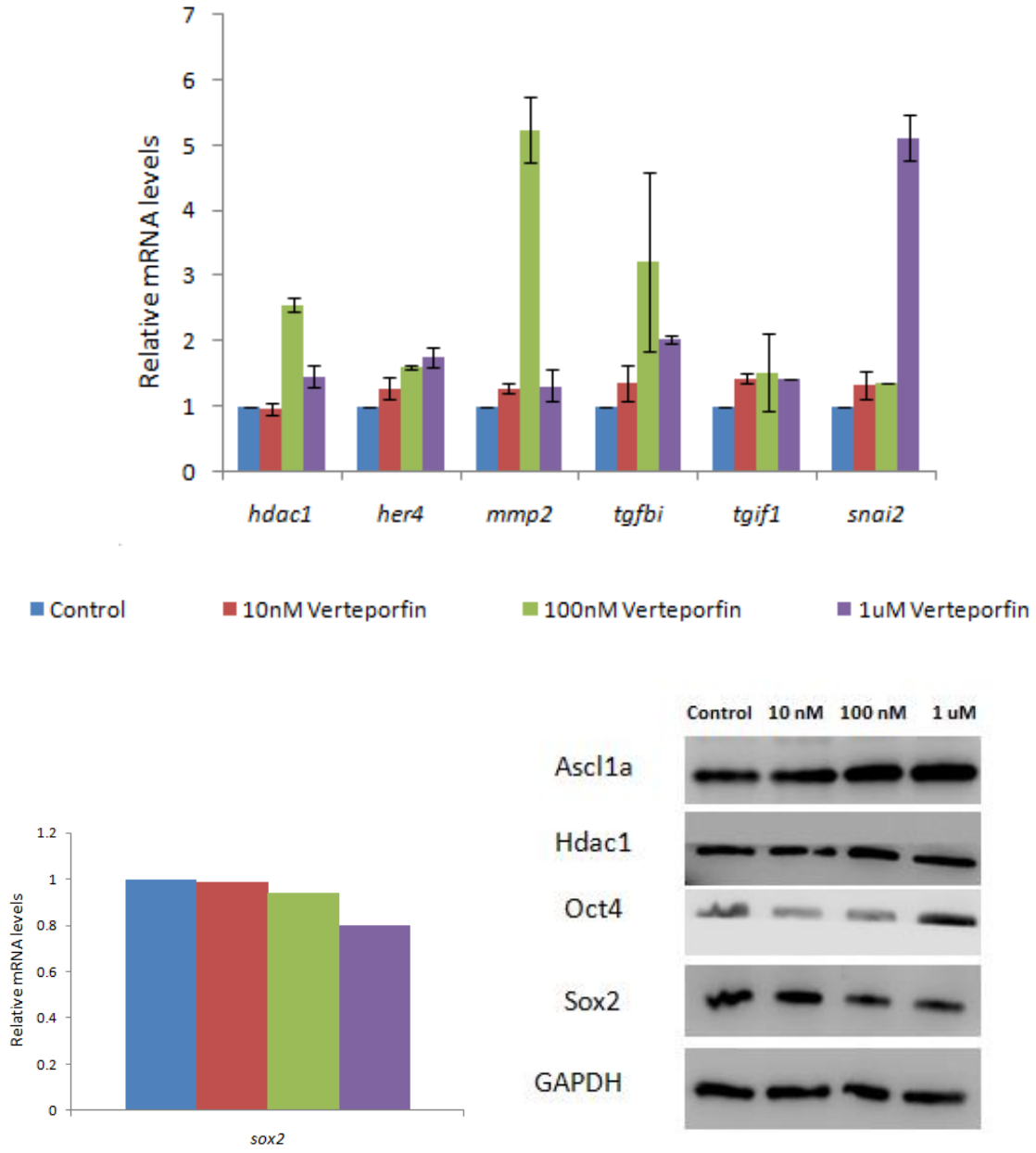


Fig. 3.2: The qPCR and western blotting data showing the effect on the expression levels of Regeneration Associated Genes on inhibition of the YAP-TEAD interaction in the dedifferentiation phase (0-2dpi).

3.3. Inhibition of YAP-TEAD interaction in proliferation phase enhances proliferation and this increase is due to the lack of cell cycle exit.

To check for the proliferative response of the MGPCs on inhibiting the YAP-TEAD interaction in the proliferation phase from 2-4dpi, the number of BrdU labeled cells were counted in each condition and it was observed that the proliferative response of the MGPCs at 4dpi increases with an increase in the concentration of the drug. The increase in collocalization of BrdU +ve and Edu +ve cells also increases, and since there are almost no BrdU +ve and EdU –ve cells, it can be concluded that the increase in proliferation is not due to the recruitment of new cells in the process. This could suggest that the same cells are undergoing more number of cell divisions due to a lack of cell cycle exit.

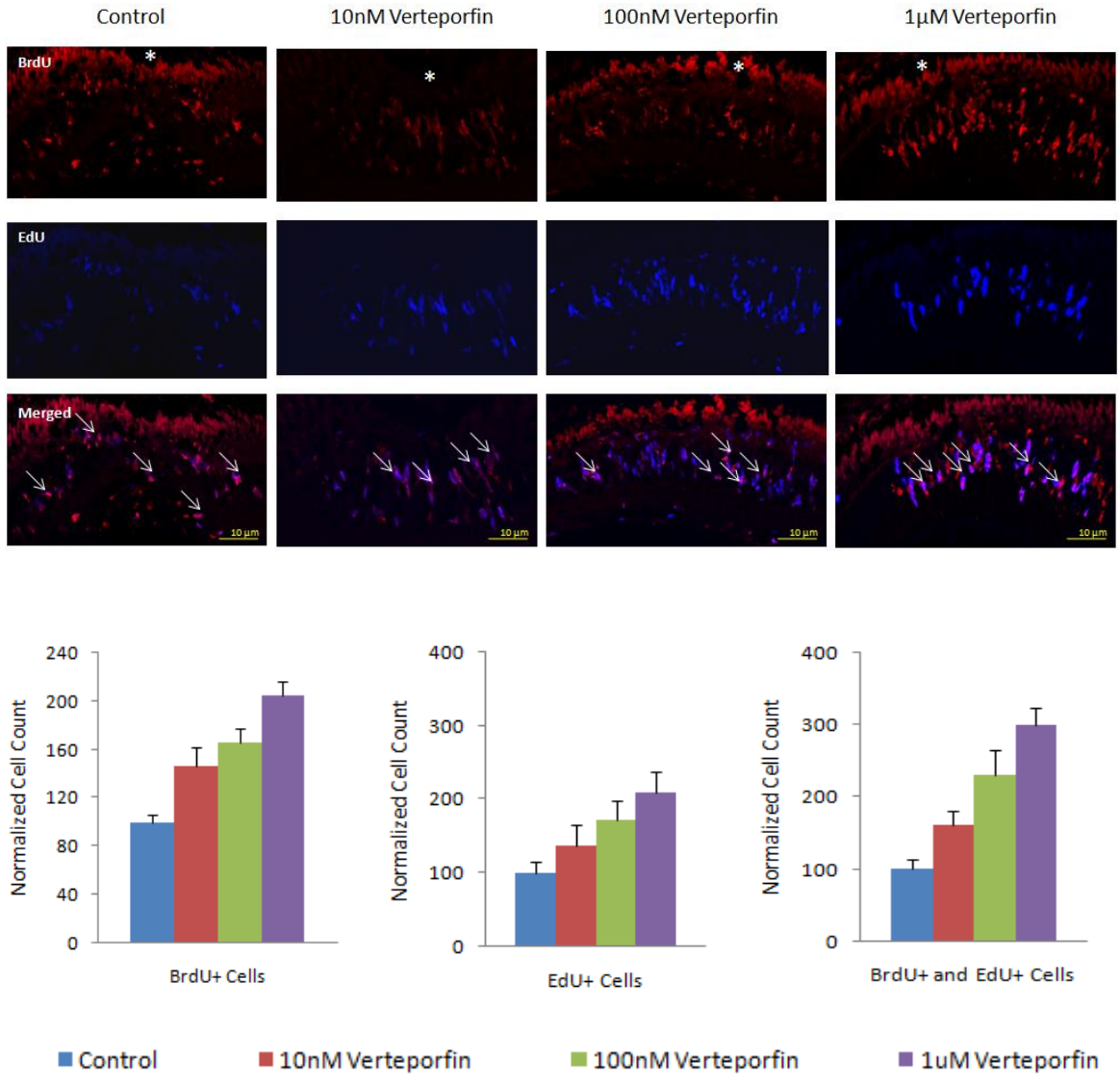


Fig. 3.3: The immunostaining images and cell count plots showing an increase in the proliferation of BrdU labeled (2dpi) and EdU labeled (4dpi) MGPCs with an increase in verteporfin concentration in the proliferation phase (2-4dpi).

3.4. Effect of YAP-TEAD interaction inhibition during proliferation phase on RAGs.

The qPCR and Western Blotting data show that the levels of Regeneration Associated Genes like *ascl1a, sox2* and *oct4* decrease, while that of *tfgbi, mmp2* and *hdac1* increase at 4dpi with an increase in the concentration of the verteporfin drug when the YAP-TEAD interaction is inhibited in the proliferation phase from 2-4dpi.

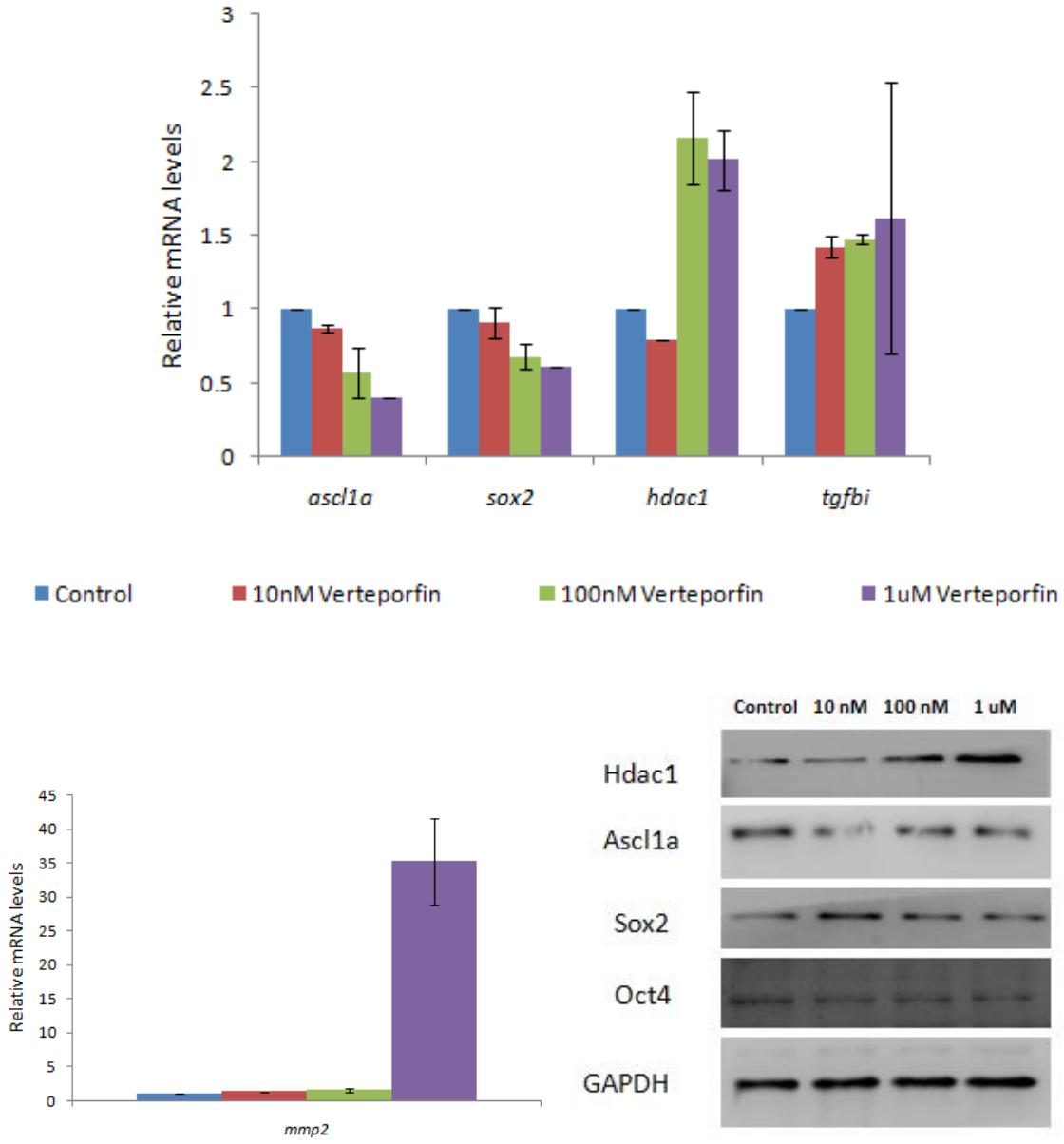


Fig. 3.4: The qPCR and western blotting data showing the effect on the expression levels of Regeneration Associated Genes on inhibition of the YAP-TEAD interaction in the proliferation phase (2-4dpi).

3.5. Inhibition of YAP-TEAD interaction in redifferentiation phase enhances proliferation and this increase is also due to the lack of cell cycle exit.

To check for the proliferative response of the MGPCs on inhibiting the YAP-TEAD interaction in the redifferentiation phase from 4-6dpi, the number of BrdU labeled cells were counted in each condition and it was observed that the proliferative response of the MGPCs at 6dpi increases with an increase in the concentration of the drug. The increase in collocalization of BrdU +ve and Edu +ve cells also increases, and since there are almost no BrdU +ve and EdU –ve cells, it can be concluded that the increase in proliferation in this phase also is not due to the recruitment of new cells in the process. This could again suggest that the same cells are undergoing more number of cell divisions due to a lack of cell cycle exit.

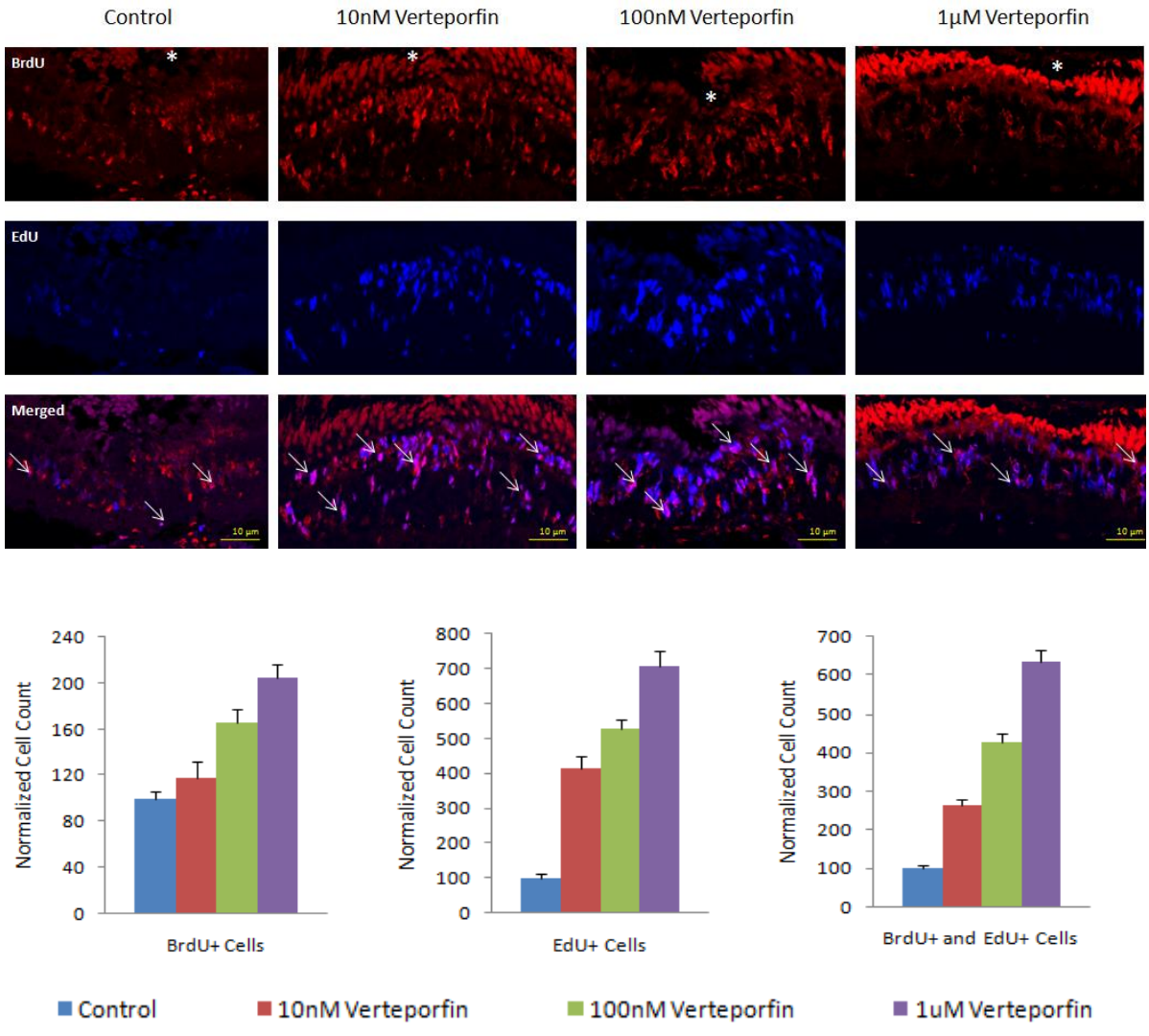


Fig. 3.5: The immunostaining images and cell count plots showing an increase in the proliferation of BrdU labeled (2, 3 and 4dpi) and EdU labeled (6dpi) MGPCs with an increase in the verteporfin concentration in the redifferentiation phase (4-6dpi).

3.6. Effect of YAP-TEAD interaction inhibition during redifferentiation phase on RAGs.

The qPCR and Western Blotting data show that the levels of Regeneration Associated Genes like *ascl1a, tgfbi, her4.1* and *oct4* decrease, while that of *sox2* and *hdac1* increase at 6dpi with an increase in the concentration of the verteporfin drug when the YAP-TEAD interaction is inhibited in the redifferentiation phase from 4-6dpi.

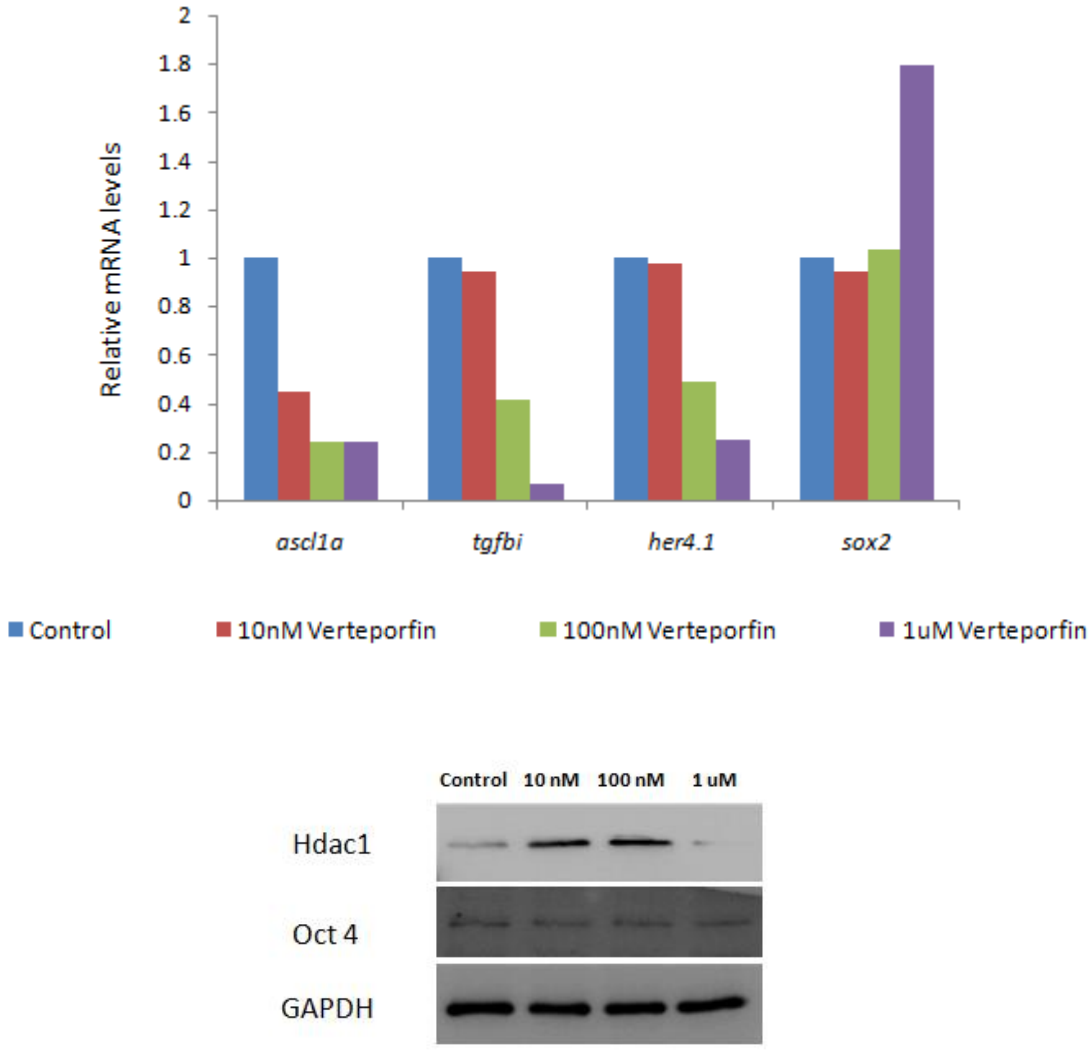


Fig. 3.6: The qPCR and western blotting data showing the effect on the expression levels of Regeneration Associated Genes on inhibition of the YAP-TEAD interaction in the redifferentiation phase (4-6dpi).

3.7. Inhibition of YAP-TEAD interaction decreases Muller Glial cell fate of progenitor population.

To check for the effect of the YAP-TEAD interaction on the formation of the differentiated Muller Glial cells (seen as slender appendages spanning multiple layers in the images below) in the regenerated tissue, immunostaining was performed using BrdU to label the proliferating cells and the antibody for the Muller Glial cell marker Glutamine Synthetase (GS), and the collocalization was measured to indicate the MGPCs that got differentiated into Muller Glial cells. It was observed that the number of BrdU +ve and GS +ve cells (i.e., collocalization) was decreased in the 1µM Verteporfin condition as compared to the control at 6dpi.

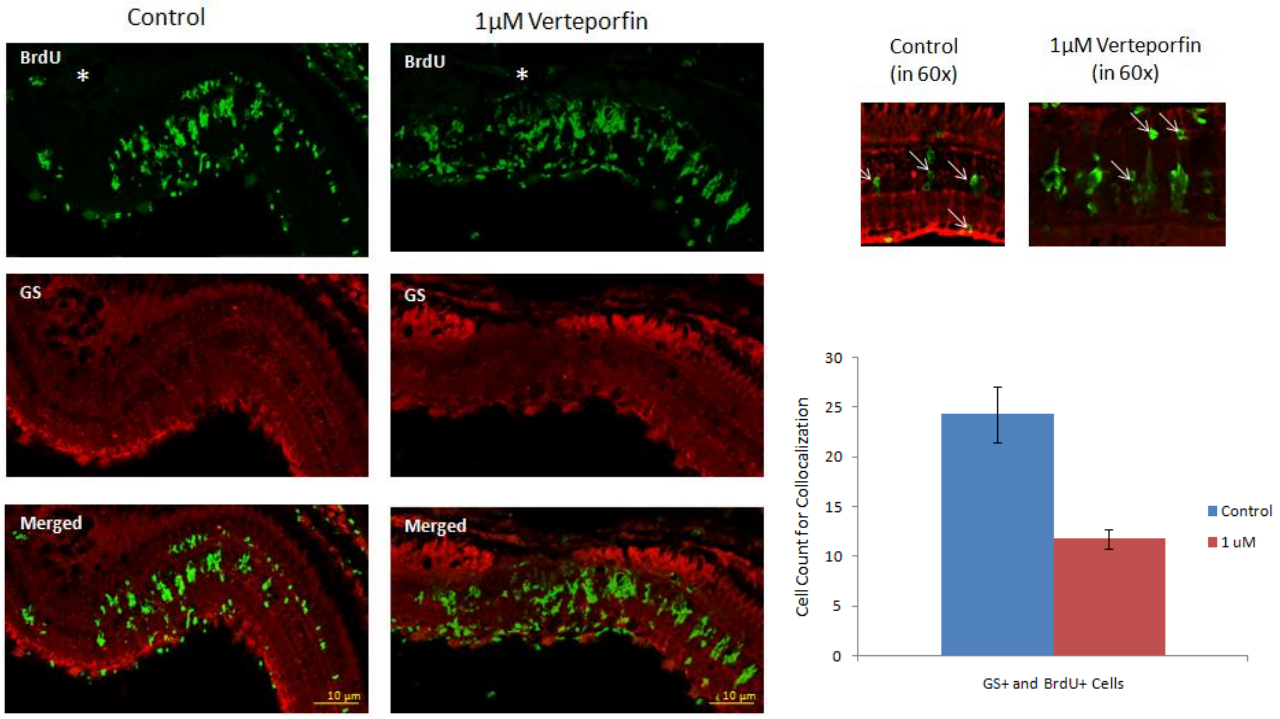


Fig. 3.7: The immunostaining images and the cell count plot showing a decrease in the number of Muller Glial cells formed on inhibition of YAP-TEAD interaction from 4-6dpi.

3.8. Inhibition of YAP-TEAD interaction increases Amacrine cell fate of progenitor population.

To check for the effect of the YAP-TEAD interaction on the formation of the Amacrine cells (seen as globular cells present as two uniform layers in the images below) in the regenerated tissue, immunostaining was performed using BrdU to label the proliferating cells and the antibody for the Amacrine cell marker HuC/D, and the collocalization was measured to indicate the MGPCs that got differentiated into amacrine cells. It was observed that the number of BrdU +ve and HuC/D +ve cells (i.e., collocalization) was increased in the 1µM Verteporfin condition as compared to the control at 6dpi.

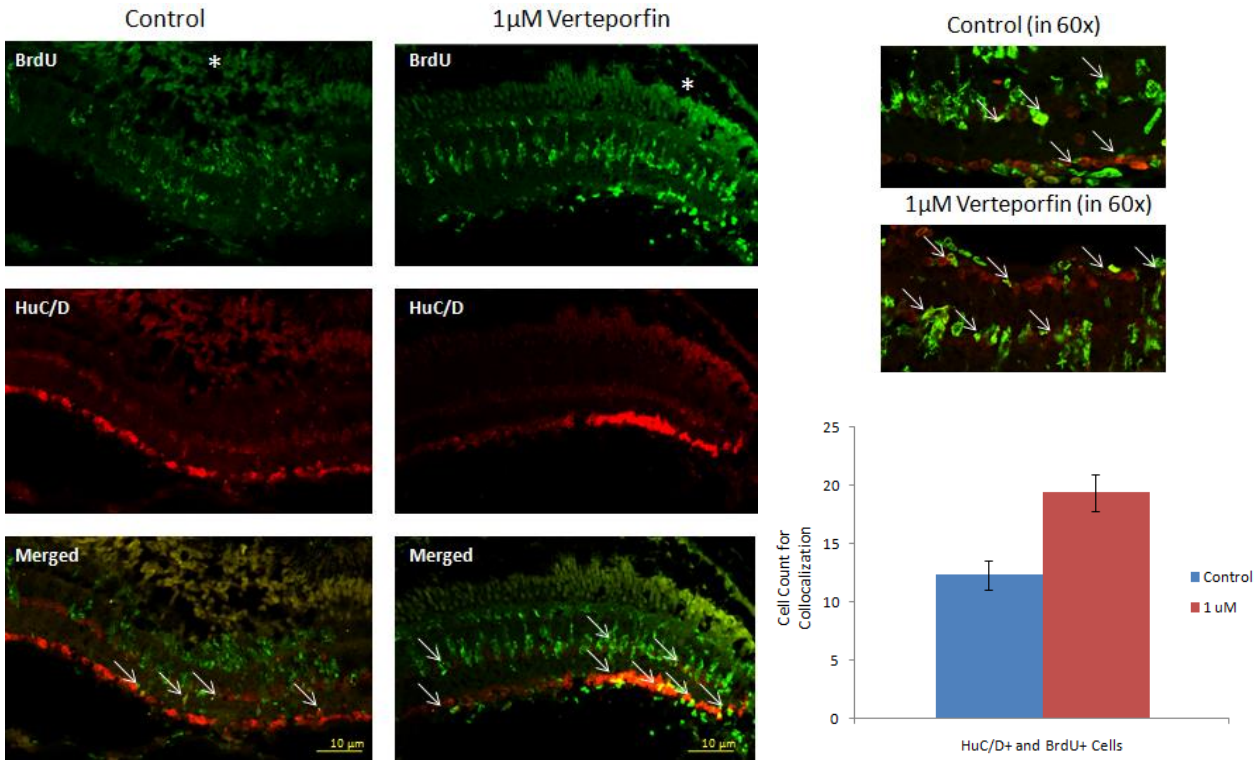


Fig. 3.8: The immunostaining images and cell count plots showing an increase in the number of Amacrine cells formed on inhibition of YAP-TEAD interaction from 4-6dpi.

3.9. Inhibition of YAP-TEAD interaction increases Bipolar cell fate of progenitor population.

To check for the effect of the YAP-TEAD interaction on the formation of the Bipolar cells (seen as oblong vertical cells in the images below) in the regenerated tissue, immunostaining was performed using BrdU to label the proliferating cells and the antibody for the Bipolar cell marker Protein Kinase C (PKC), and the collocalization was measured to indicate the MGPCs that got differentiated into bipolar cells. It was observed that the number of BrdU +ve and PKC +ve cells (i.e., collocalization) was increased in the 1µM Verteporfin condition as compared to the control at 6dpi.

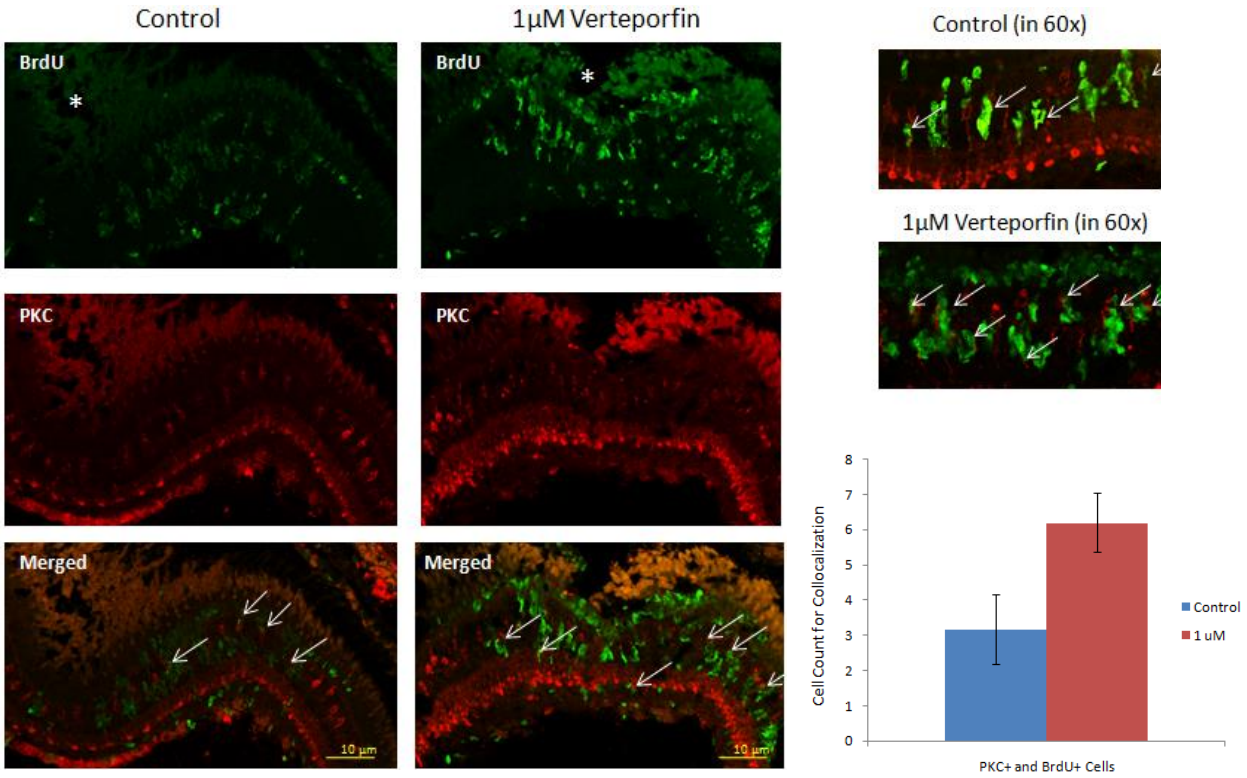


Fig. 3.9: The immunostaining images and cell count plots showing an increase in the number of Bipolar cells formed on inhibition of YAP-TEAD interaction from 4-6dpi.

3.10. Inhibition of YAP-TEAD interaction prior to injury reduces proliferation.

To check for the proliferative response of the MGPCs on inhibiting the YAP-TEAD interaction in the homeostatic conditions i.e., prior to any injury given, the number of BrdU labeled cells were counted in each condition and it was observed that the proliferative response of the MGPCs at 4dpi decreases with an increase in the concentration of the verteporfin drug.

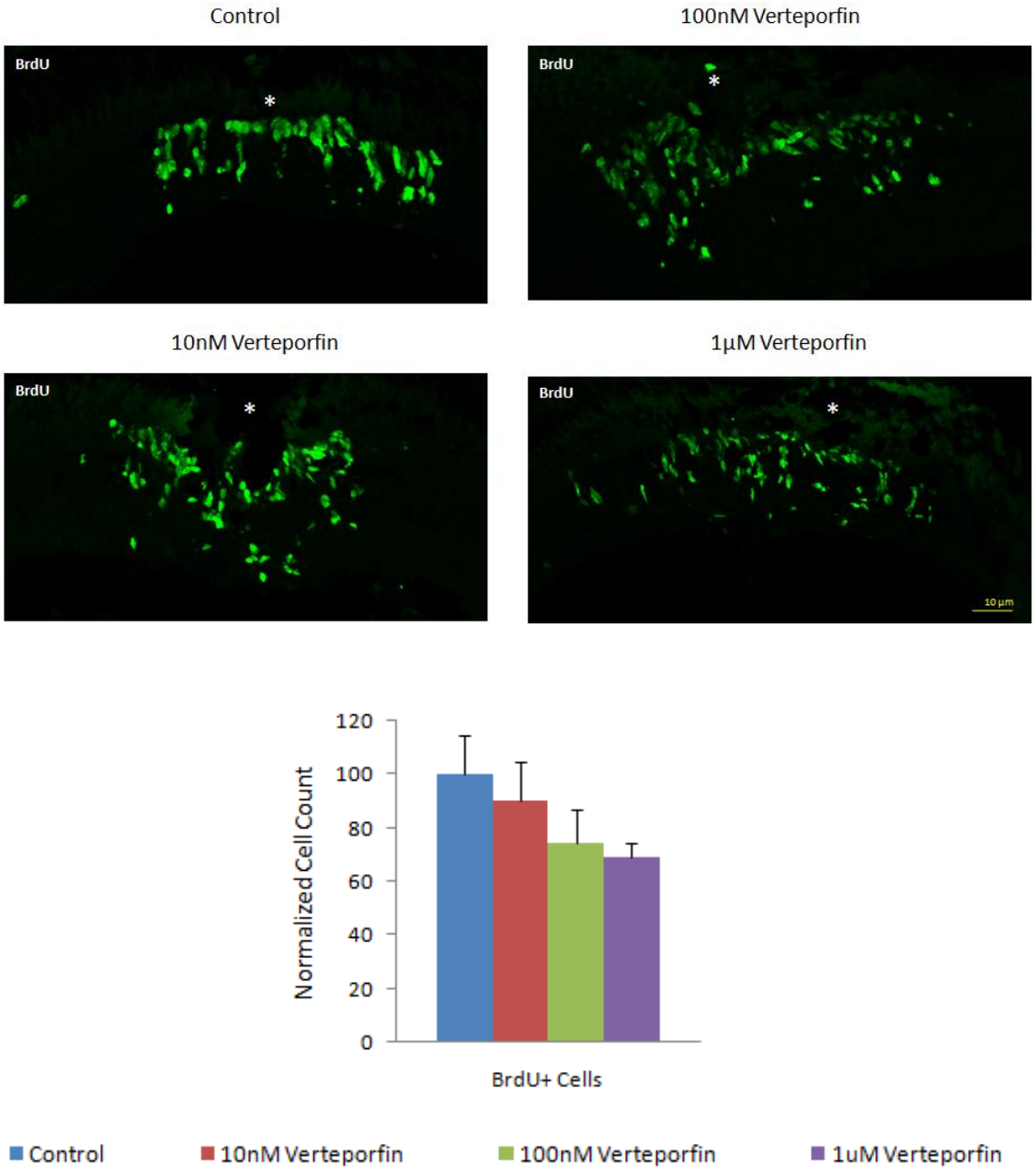


Fig. 3.10: The immunostaining images and cell count plot showing a decrease in the proliferation of BrdU labeled MGPCs with an increase in verteporfin concentration on inhibition of the YAP-TEAD interaction in the homeostatic condition.

CONCLUSION

This study to understand the involvement of the Hippo-YAP signaling pathway in the process of retina regeneration in zebrafish shows that the inhibition of the YAP-TEAD interaction in the dedifferentiation phase of zebrafish retina regeneration causes a decrease in the proliferative response of the Muller Glial cell-derived Progenitor cells (MGPCs). This verteporfin drug treatment also has an effect on the levels of Regeneration Associated Genes as it leads to an increase in the expression levels of *ascl1a, hdac1, her4.1, mmp2, tgfbi, tgif1, snai2* and *oct4* and leads to a decrease in the levels of *sox2*. Both the cell count and the trend in the RAG levels show concentration dependency. Inhibition of the YAP-TEAD interaction in the proliferation phase leads to an increase in the proliferation response of the MGPCs and causes a decrease in the expression levels of *ascl1a, sox2* and *oct4*, while causing an increase in the levels of *hdac1, tgfbi* and *mmp2* with an increase in the concentration of the drug. Inhibition of the YAP-TEAD interaction in the redifferentiation phase also leads to an increase in the proliferative response of the MGPCs and causes a decrease in the levels of *ascl1a, her4.1, tgfbi* and *oct4* and an increase in the levels of *hdac1* and *sox2*. The increase in the proliferative response of the MGPCs in both the proliferation and redifferentiation phase is due to the lack of these cells to exit cell cycle, and not because new cells are recruited in this response to injury. The inhibition of the YAP-TEAD interaction also has an effect on the cell fate of the regenerated cells. With an increasing drug concentration, there is an increase in the number of Bipolar and Amacrine cells formed. However, there is a decrease in the number of Muller Glial cells formed and this could be explained by the increase in the number of MGs that remain undifferentiated and partake in the increased proliferative response. Also, inhibition of the YAP-TEAD interaction in the homeostatic condition, i.e., keeping the Hippo pathway in a constitutively ON state prior to any injury leads to a decrease in the proliferative response of MGPCs upon injury.

Looking at the above observations together, one can conclusively state that the Hippo-YAP signaling pathway plays an important function in retina regeneration and has different roles and mechanisms of action in the different phases of regeneration.

FUTURE PROSPECTS

Though this study gives some insight into what happens when the Hippo-YAP signaling pathway is constitutively kept ON by inhibiting the YAP-TEAD interaction, it would also be interesting to know how exactly does this happen. Since, YAP can interact with a number of transcription factors like TEAD, those of the Smad family, Runx2 and NuRD to name a few, depending on which it leads to the activation or repression of the expression of the downstream genes, looking at the binding partners of YAP in these three phases by performing co-immunoprecipitation and mass spectrometry would give a better insight into the underlying mechanisms of the altered expression of the Regeneration Associated Genes. Also, performing mRNA *in situ* hybridization to check for and confirm the expression levels of RAGs can help to further validate the qPCR results reported in this study.

In addition to this, the decrease in the MGPCs' proliferation response in the homeostatic condition can be further studied by looking at the change in the mRNA and protein expression levels of RAGs, binding partners of YAP and the effect on cell fate on inhibiting the YAP-TEAD interaction prior to injury.

Since, the increase in the proliferative response of the MGPCs is due to lack of cell cycle exit, the effect on the levels of Cyclins such as Cyclin D1 and Cyclin D3, and Cyclin Dependepnt Kinases (CDKs) such ad CDK6 can also be looked at to get a better insight into how there is continued cell cycle and division and no differentiation process in these cells.

Ultimately to compare these results and better understand the role of this pathway in the context of mammalian retina regeneration, these studies can be repeated and performed on mice.

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