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Honors Thesis

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Abstract

Glioma are glia-derived brain tumors that have poor prognosis. This thesis project tests Tyrosine Kinase Inhibitor drugs that have shown promise during an ongoing chemical drug screen on an alternate oncogenic pathway. In the ongoing chemical screen, tumors were induced in *Drosophila melanogaster* by expressing $Pten^{RNAi}$ to eliminate the tumor suppressor gene, *Pten*, which negatively regulates the Pi3K pathway. For this thesis project, tumors will be induced through constitutively activating the EGFR (epidermal growth factor receptor) through the Pi3K pathway. Therefore, these two projects individually look at two different types of glioma and aim to determine if certain drugs show promise on more than one type of glioma. A series of crosses over the course of three generations is performed to balance chromosomes of the flies and result in the genotype with the EGFR mutation. Data collection includes exposing these flies to the specific tyrosine kinase inhibitor drugs when added to their food. Larvae is collected, dissected to obtain the brains, washed and mounted on microscope slides after 2 and 3 days of exposure to the drugs. Images are taken of the mounted brains for further data analysis. In order to conclude if these drugs showed similar promise as in the existing chemical screen, images will be examined for enhancement, suppression or no effect in comparison to *Pi3k; Repo* control brains of flies left untreated.

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Introduction

Today, cancer is still one of the leading causes of death and results in 8.2 million people deaths a year (National Cancer Institute, 2018). Of these deaths, about 13,000 of these cancer deaths are from brain cancer, and specifically 9,100 suffered from aggressive form of glioma. Glioma are glia-derived brain tumors with poor prognosis. Current treatments for glioma include surgery, chemotherapy and radiotherapy (John's Hopkins Medicine). While these treatments target tumorous cells, these treatments also effect healthy cells. Therefore, these patients' lives are usually only extended by a few months (Brennan et al., 2013). The lack of life-extending treatments calls for research in treatments that can provide better prognosis.

There are a variety of mutations that can lead to the development of glioblastoma. Some mutations are specific to glioblastoma, while others are common cancer mutations. *TP53* and *PTEN* are common cancer mutations (Liu, 2016). *PTEN* is a tumor suppressor gene and these mutations usually result in the loss of function. In addition, point mutations in EGFR is also common. EGFR mutations result in EGFR amplification. Other common mutations found in glioblastoma include *Pi3KCA* and *Pi3KR1*, *NF1*, *RB1*, and *IDH1* (Liu 2016). Many of these genes regulate cell signaling involving cell proliferation and survival (Liu 2016).

The development of glioma is important in understanding the prognosis of this type of cancer and the need for better treatment. As previously mentioned, glioma are glia-derived brain tumors. Glia cells are products down the line of neuroblast cell division (Figure 1). Neuroblasts undergo asymmetric cell division (Homem et al., 2012). In *Drosophila*, division results in two daughter cells, either a ganglion mother cell or another neuroblast that will undergo further division into two more daughter cells. Ganglion mother cells then divide into a neuron and glia cell. In humans, neuroblast division is more complex, involving the transit amplification of interneurons. Using *Drosophila* as a model organism is beneficial due to their simpler central nervous system and neuroblast division. This division can be important in understanding the development

of glioma depending on the rounds of cell division in development. The amount of neuroblasts can be determined in Miranda staining, whereas the amount of Ganglion Mother cells can be determined through Prospero staining (Brody, 1996). These two stains have been beneficial in analyzing the production of tumors in model organisms.

Drosophila melanogaster is the model organism used in this experiment and has been a pivotal model organism in scientific research. This model organism is beneficial in scientific research due to its accessibility, ease in maintaining, and their short life spans (Figure 2). *Drosophila* has led to many scientific discoveries and has been used in studying many diseases such as cancer, cardiovascular disease, neurological diseases and diseases related to the immune system (Fortini et al., 2000). Another important aspect of *Drosophila* are the similarities their genomes share with humans. 70% of human disease-linked genes are seen in *Drosophila* (Chien et al, 2002; Hirth, 2010; Pandley and Nichols, 2011; Read, 2011), as well as important cellular signaling pathways such as Wnt, Hippo, Notch, Pi3K and EGFR (Read, 2011; Tickoo and Russell, 2002).

The signaling pathways mentioned are important in cellular mechanisms such as growth, development and cell proliferation (Figure 3). When these signaling pathways are mutated or do not operate as they should, cancer can result. Glioblastomas are associated with many different mutations in these oncogenic pathways (Phillips et al, 2006; Verhaak et al., 2002). Many glioblastomas include mutation that leads to overexpression of receptors of tyrosine kinases like EGFR (Read, 2013). Tyrosine kinases are important in signaling cascades in cells that activate specific genes (Paul et al., 2004). In normal functioning cells, tyrosine kinases like EGFR are important for cellular functions. For example, cell proliferation is promoted (Huang et al., 2007). However, in mutant EGFR, the kinase is constitutively active and therefore, constantly stimulates RAS that promotes migration and blocks apoptosis leading to tumor development (Boockvar et al., 2003). However, glioma requires activation of two of these genetic pathways to result in cancer. In humans, the most common oncogenic pathways activated are the co-activation of EGFR and Pi3K (Raizer, 2011). Modeling this co-activation in *Drosophila* to induce glioblastoma is the critical first step in this thesis project. The effectiveness of specific tyrosine kinase inhibitor drugs that had showed

interesting results on glioma induced by a *Pten* mutation through the RAS pathway are tested on a different mutation for this thesis project. The previous drug screen focused on glioma induced through a negative mutation, while this project focuses on an EGFR mutation that is constitutively active.

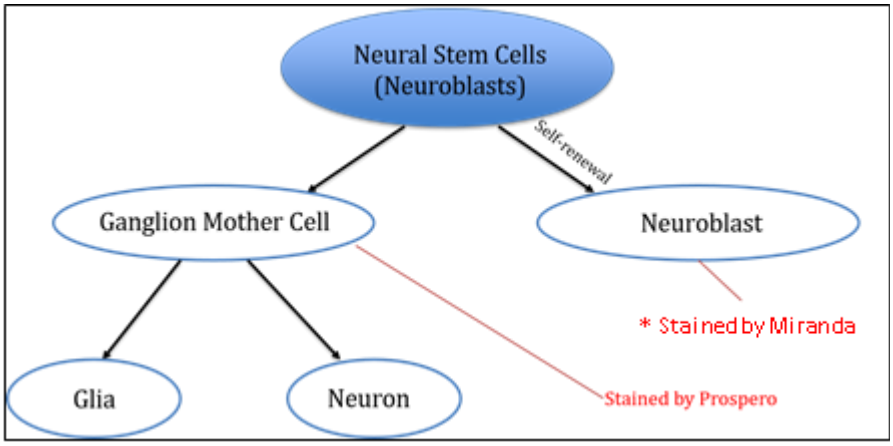


Figure 1

Neuroblast stem cells undergo asymmetric cell division. They can divide to self-renew into another neuroblast or into ganglion mother cells (GMC) that further divide into a glia cell or neuron. Each cell type can be identified based on biomarkers like Miranda for Neuroblasts and Prospero for GMC.

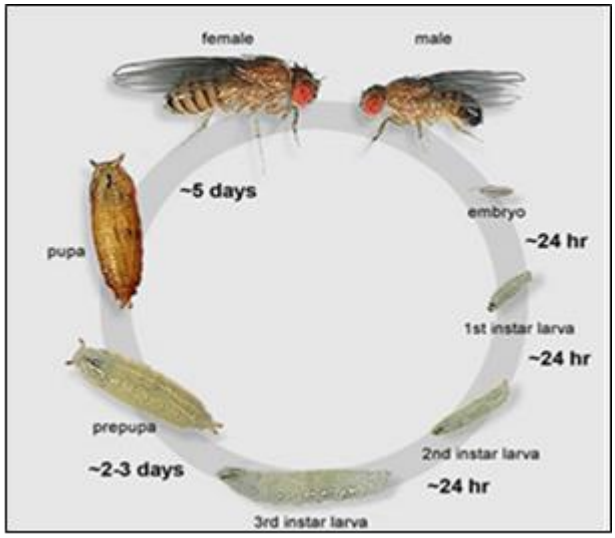


Figure 2 (Maynard, 2009)

In this figure, the life cycle of *Drosophila* is shown, along with the timeframe of each stage. The short lifespan from embryo, larvae and adult provides advantages in the research field to complete many trials or experiments in a short amount of time.

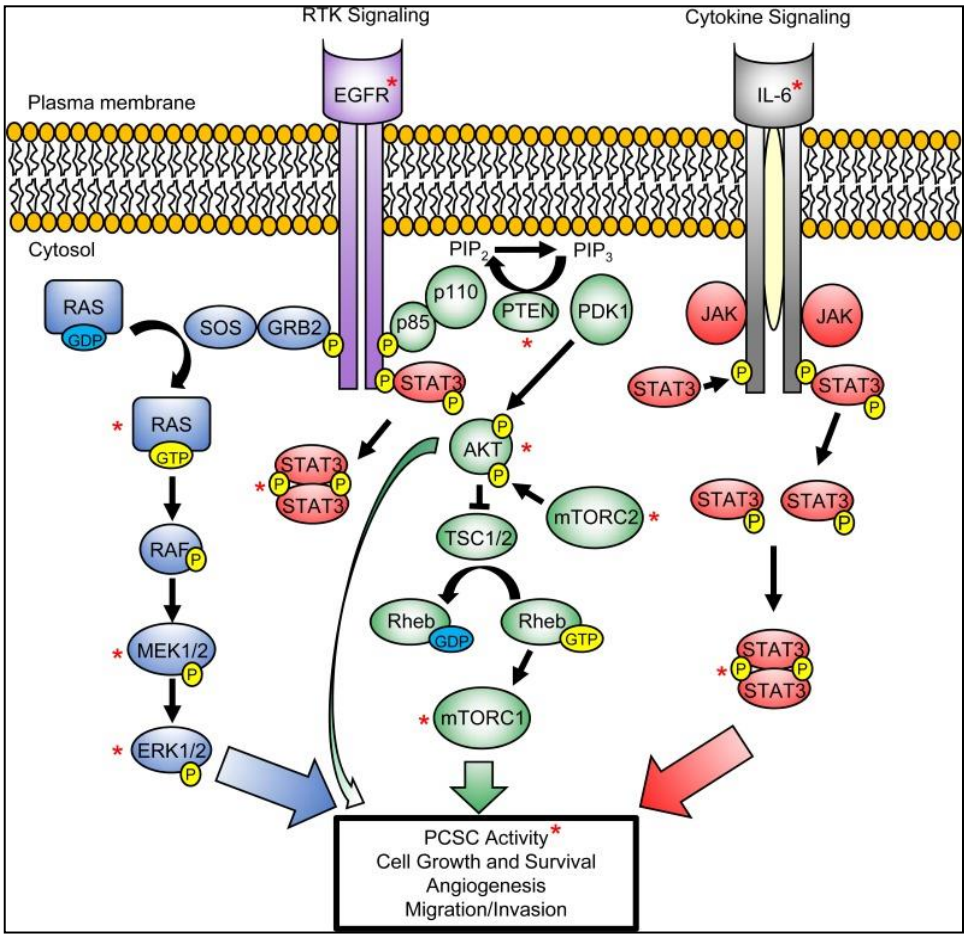


Figure 3 (Rybak, 2014)

These signaling pathways including Ras, Pi3K, and STAT3 converge and act directly or cross actively to mediate tumorigenesis. If mutations in these pathways or present receptors are found, tumors such as GBM can result.

Methods

Drosophila crosses to generate *Pi3K EGFR$^{\lambda top}$* glioma:

The overall drug screen consists of seven steps and the drugs tested throughout this project were P1A9 and P1G10. As mentioned, modeling the co-activation of the EGFR and Pi3k pathway is the critical first step in inducing glioblastoma in the model organism. The co-activation of these pathways required a series of steps over the course of three generations of *Drosophila*. This is accomplished with balancer chromosomes. Balancer chromosomes are specific stocks in *Drosophila* that are rearranged chromosomes that can be used to maintain a mutant genotype or help in complex genotype construction (Miller et al., 2018). However, lethality is a possible issue with these oncogenic mutations and can cause issues determining crossing schemes to obtain specific genotypes. For this project, three generations of crosses were completed to obtain the final genotype for the *Pi3k; Repo* stock (Figure 4). In each generation, female virgins are collected and used in the next step. Recombination, or genetic exchange between chromosomes, occurs in female flies (John et al., 2016). Using female virgins eliminates the risk of recombination when trying to obtain a specific genotype. Once this genotype was obtained, the stock was crossed with *EGFR$^{\lambda top}$* resulting in the final cross with induced glioma. Once glioma was successfully induced in *Drosophila*, the final cross and final balanced stocks were maintained with a 2:1 female to male ratio to result in maximal egg production for data collection.

Drug Screen:

Larvae from control stocks and the *Pi3K; Repo X EGFR$^{\lambda top}$* cross are collected on day 3 of their life span when they are in their early third instar stage (Figure 2). Third instar larvae are added to food that has been infused with the specific tyrosine kinase inhibitor chemicals. The larvae are exposed to the inhibitor drugs for an additional 3 days and are collected again at day 5 and 6 of age. When collected after exposure, the larvae are dissected to obtain the brain and ventral cord. Before imaging, the dissection is

successfully prepared. Following dissection, larvae samples are fixed for 20 minutes in 150 microliters of PBS and 50 microliters of 16% PFA. The larvae samples are then washed for 10 minutes in 1mL of PBS-T. The sample is then mounted onto microscope slides for imaging and are protected and secured by Vectashield (Vector Labs, USA). Imaging is completed using fluorescent microscopy for both the control stocks and main cross.

Immunochemistry:

In addition to images following drug exposure, Miranda staining is also completed in attempts to explain tumor progression. Larvae was dissected and prepared similarly to the samples prepared after drug exposure. Following fixing, Miranda antibody 1:200 dilution is added to the sample and incubated overnight at $4^{\circ C}$. The primary antibody is removed, and the samples washed with PBST, then incubated with the appropriate secondary antibody fused to a fluorophore (e.g. anti-mouse Cy3). After 2h the samples are washed for a series of 3 rounds of 10 minute washes. The stained brains are mounted and protected with Vectashield (Vector Labs, USA). The stained slides are imaged using confocal microscopy (Olympus Fluoview 3000).

Quantification of data:

Quantification of data was completed by analyzing the central brain region, or the CBR. The CBR is located between the two occipital disks and ventral cord (Figure 5). The width of the CBR was measured in pixels. These widths were then compared between the crosses and controls exposed to drug treatment to determine the effects. Suppression was seen if the size of the CBR decreased in comparison to the cross untreated and is more similar to the size of the control. Likewise, enhancement was observed in the width of the CBR is greater than the cross untreated and larger than the control.

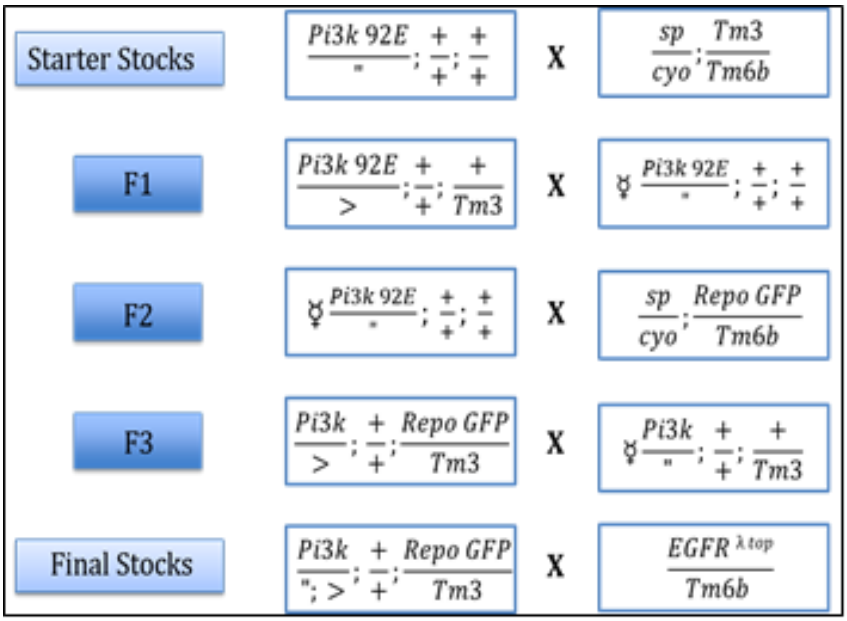


Figure 4

A series of crosses were designed with balancer chromosomes to obtain the wanted genotype for *Pi3k; Repo* to induce tumor when crossed with $EGFR^{\lambda top}$. This was accomplished over three generations.

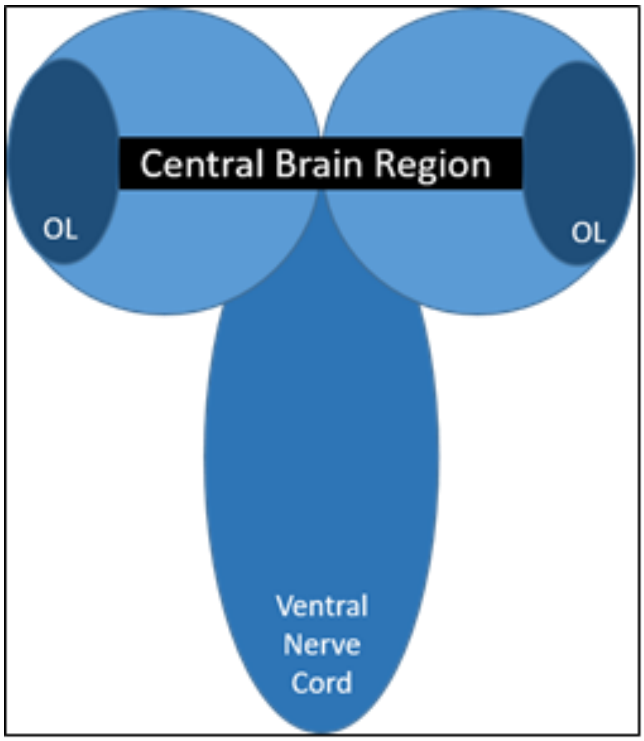


Figure 5

The CBR is located in between the two occipital lobes of the brain. The ventral nerve cord is the posterior part of the *Drosophila* brain.

Results

The *Pi3K EGFR$^{\lambda top}$* model of glioma growth:

The *Pi3k EGFR$^{\lambda top}$* model of glioma growth showed similar results when compared to the *Pten^{i}; Ras^{V12}* model. In both models, there was a significant increase in size of the CBR when the cross was compared to the control. In Figure 6, this significant difference is observed. In both studies, the controls showed a CBR of 145-170 pixels in width. In both mutations, the crosses showed an enhanced CBR of 230 pixels in width.

Following the completion of the cross scheme for co-activation of Pi3K and EGFR and prior to drug exposure, control images were completed and analyzed for both the control stock and the cross (Figure 7C). The area examined for analysis was the central brain region (CBR). This region was analyzed by determining the average width of the central brain region in pixels for both the control stock and co-activated cross (Figure 7B). On average, the control stock showed CBR of 455.50 pixels, whereas the co-activated cross showed a larger CBR of 707.25 pixels (Figure 7A).

Miranda staining was completed and analyzed to compare *Repo* control to the *Pi3k; Repo X EGFR* cross to explain glia overgrowth. Miranda staining can be seen in the red stained cells showing successful staining of neuroblast stem cells (Figure 8). Distribution of neuroblast cells, size and number were analyzed n the comparison. Miranda staining, when compared to control and cross, revealed the cells in the cross are not clearly or evenly distributed and vary in size. In the control, they are widely spaced and similar in size across the CBR.

Drug screening in Pi3K EGFR model:

As mentioned, P1A9 and P1G10 had previously shown interesting effects on tumor progression when examined with the *Pten* mutation via the RAS pathway. The drug screen steps were the same between both mutations examined. These specific tyrosine kinase inhibitors were tested on the *Pi3K; Repo X EGFR* mutation to determine if the effects were similar for a different mutation. The size of the CBR in the *Pten^{i};*

Ras^{V12} mutation after drug exposure for three days showed enhancement from P1A9 and suppression for P1G10 when compared to the *Repo* control (Figure 9).

P1A9 and P1G10 showed interesting effects on with the *Pi3K; Repo X EGFR* mutation as well. The control when exposed to P1A9 for three days had CBR on average of 460 pixels in width After 3 days of drug exposure, the cross on average exhibited a CBR width of 928 pixels (Figure 10). In comparison, after three days of exposure to P1G10, no larvae surfaced for the control drug vials. In addition, very few (3/20) larvae rose across the 3 drug vials. Of the larvae surfaced and dissected, extremely fragile brain lobes were observed in addition reduction in the CBR (Figure 11).

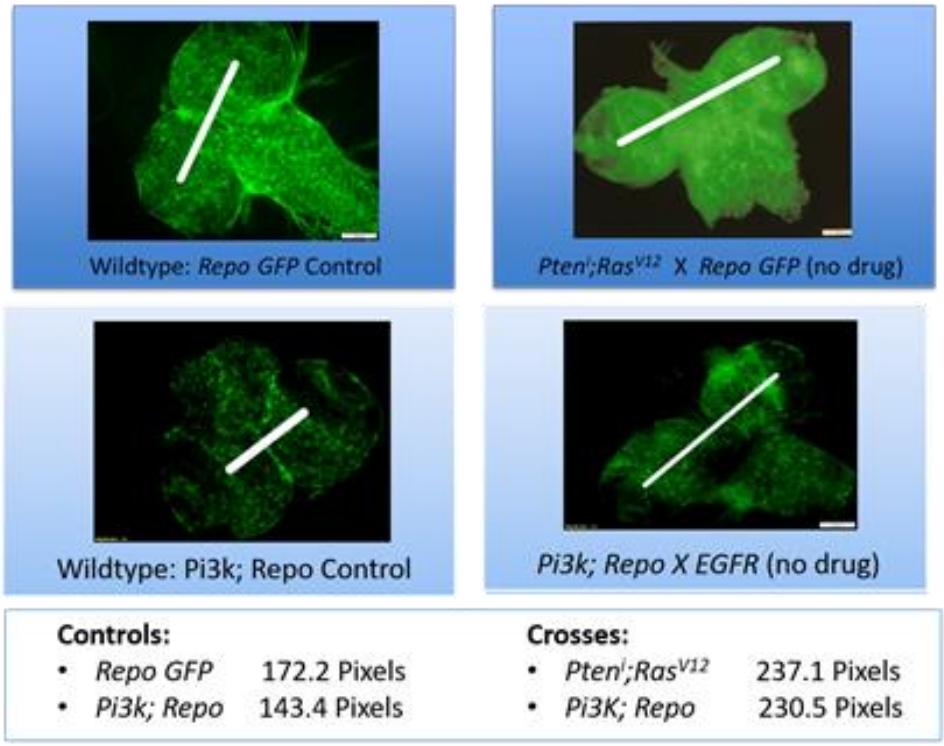
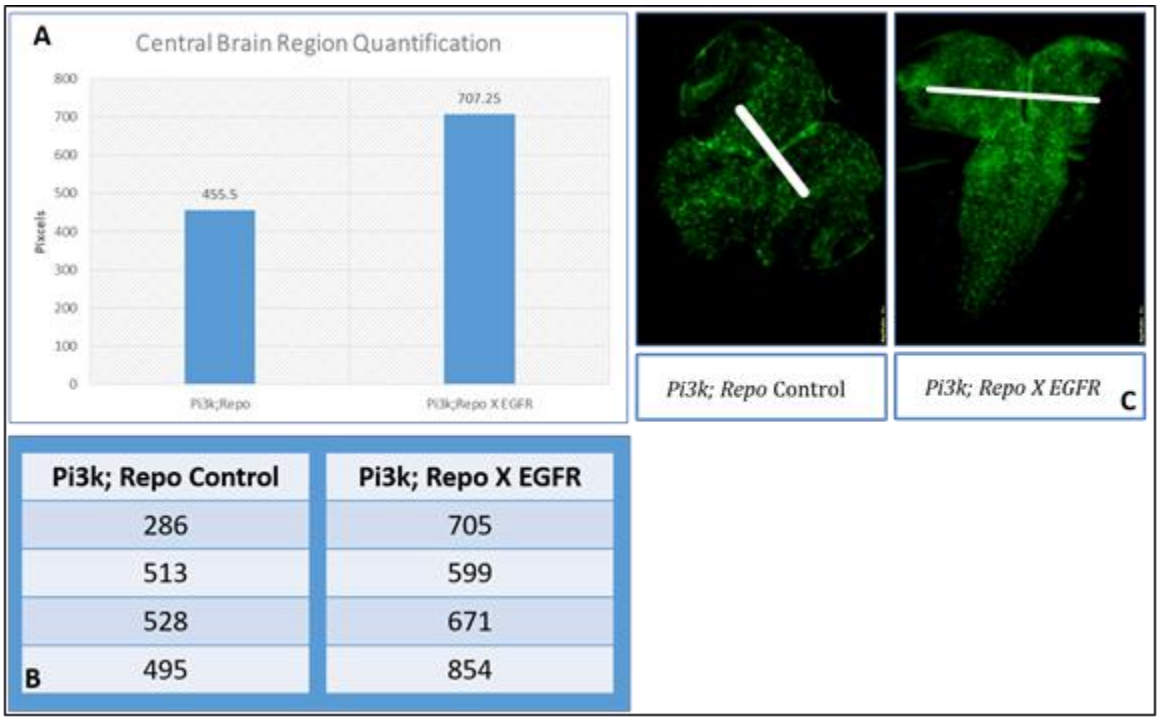


Figure 6

This figure shows the comparison between the studies of the two different mutations. In both studies, the controls show a CBR of 140-170 pixels. In addition, in both studies the activated mutations show a CBR 230 pixels.



Pi3k; Repo Control	Pi3k; Repo X EGFR
286	705
513	599
528	671
495	854

B

Figure 7

On average, the CBR was larger in the co-activated cross (A). This was analyzed from 4 brains in both genotypes (B). The images were completed using fluorescent microscopy (C).

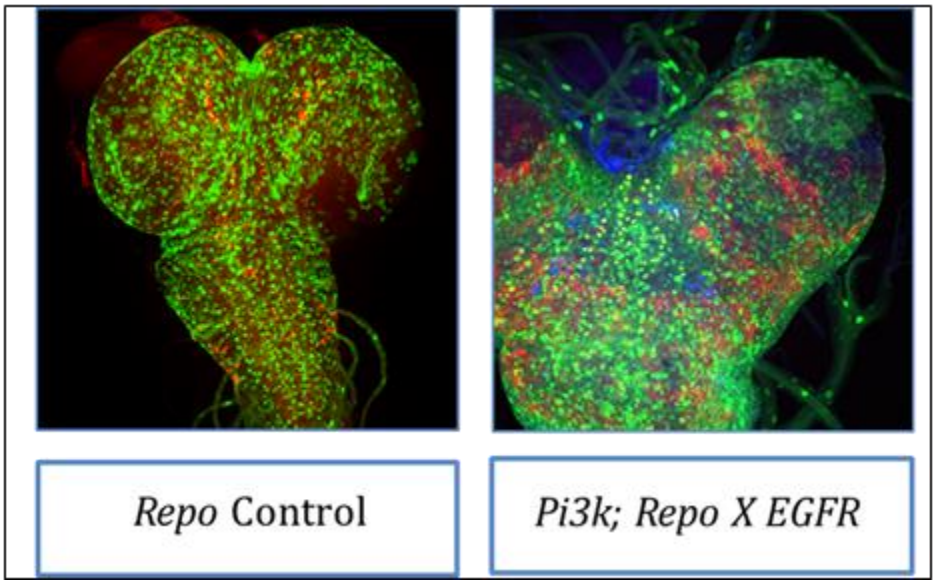


Figure 8

Miranda staining is visible in the red stained neuroblast cells. The cross (right) shows inconsistency in size, number and distribution of neurbolast cells when compared to the *Repo* control.

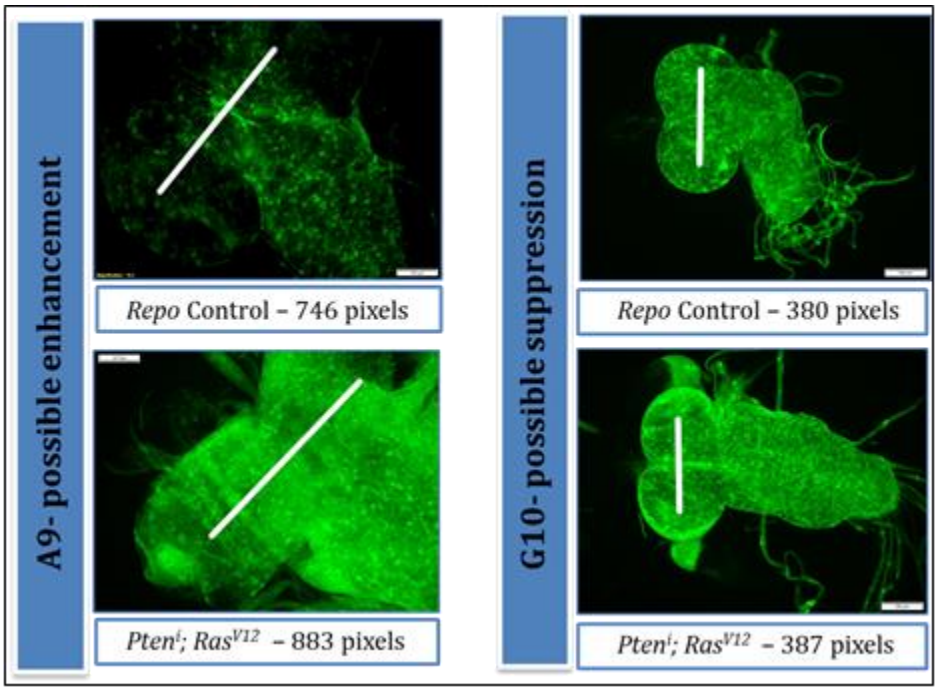


Figure 9

P1A9 showed enhancement of CBR through the *Pten*^{i}*; Ras*^{V12} mutation, whereas P1G10 showed suppression of the tumor.

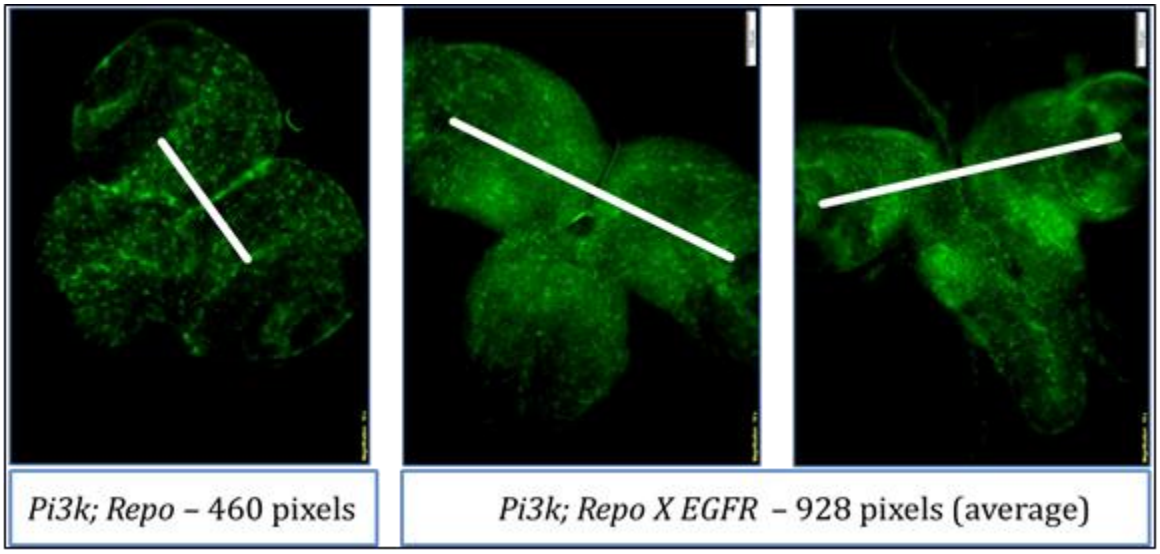


Figure 10

P1A9 showed an increase in the width of the CBR in the cross when compared to the control.

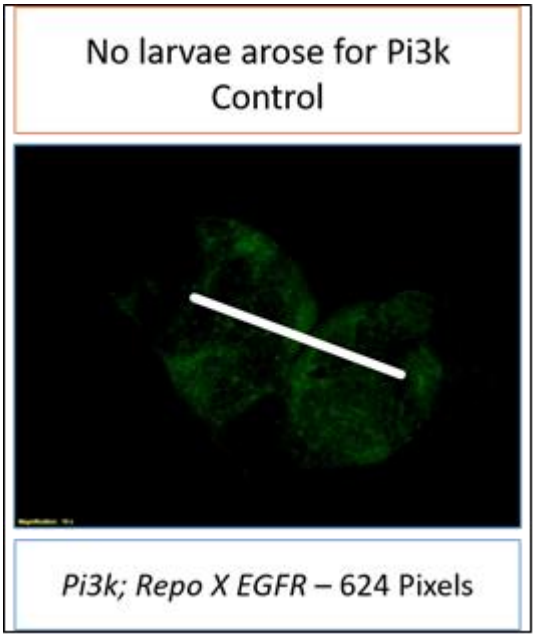


Figure 11

P1G10 had reduction in CBR in comparison to the untreated control. The treated control had death of larvae after exposure.

Discussion

The increase in the width of the CBR in *Pi3K; Repo X EGFR* cross in comparison to the control stock seen in Figure 6 in conjunction with results from Miranda staining supported successful co-activation of the Pi3K pathway and EGFR pathway for tumorigenesis. Further, the unequal distribution, increase in size and number of neuroblast cells in the cross compared to the Repo control explains how tumor progression may occur. An increase in number of neuroblast stem cells and unequal distribution can result in greater number and unequal distribution of glia seen in glioma tumors due to the asymmetric cell division these stem cells undergo. Determining successful co-activation of these oncogenic pathways and successful tumor progression was important before the tyrosine kinase inhibitor drugs were tested.

P1A9 and P1G10 showed interesting effects on tumor progression for the $Pten^{i}$*;* Ras^{V12} mutation. P1A9 showed enhancement across the CBR, while P1G10 showed suppression across the CBR. When these two drugs were examined with the *Pi3K; Repo X EGFR* mutation, P1A9 showed similar results. Enhancement was seen across the CBR after three days of exposure when compared to the control. However, P1G10 showed an interesting response after drug exposure for this constitutively active mutation. P1G10 indicated possible suppression, as well as possible lethality. Larvae images were unable to be obtained for the control, as larvae did not survive to day three following the addition to the drug vials. In addition, the larvae dissected for the cross that arose in small numbers had extremely fragile brains. The reduction in size of the CBR compared to the untreated control images indicated possible suppression; however, the fragility and lack of surviving larvae indicated possible lethality.

Conclusion

In conclusion, this thesis project tested specific tyrosine kinase inhibitor drugs that had shown interesting effects on a negative mutation, *Pten*, through the RAS pathway on a constitutively active mutation of *EGFR* through the Pi3K pathway. The critical first step of co-activating the EGFR and Pi3K pathway was successfully accomplished through a series of crosses across three generations. The successful genotype completion was seen through control images and Miranda staining. The two specific tyrosine kinase inhibitor drugs tested were P1A9 and P1G10. P1A9 had shown enhancement when tested on $Pten^{i}; Ras^{V12}$ mutation. Enhancement was also observed when P1A9 was tested on *Pi3K; Repo X EGFR* mutation. P1G10 had shown promising suppression when tested on the $Pten^{i}; Ras^{V12}$ mutation. P1G10 indicated possible suppression when compared to the untreated control. Most importantly, P1G10 also indicated possible lethality on this specific mutation. Future directions include retesting P1G10 to examine this possible lethality and determine if suppression or lethality is observed. In addition, other tyrosine kinase inhibitor drugs that had shown promising effects on the original drug screen should be tested to further examine the *Pi3K; Repo X EGFR* mutation. These specific drugs include P1A4, P1D2, P1D3, and P1D10.

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