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Drosophila Tumor Mosaic Models to Study Intercellular Interaction

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Honors Thesis

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Abstract

Drosophila is a powerful genetic model system to study cancer. In patients, a small number of mutations accumulate in cells that change their growth characteristics and eventually lead to the formation of tumors. These tumors are clonal in origin, meaning the cancer arose from the proliferation of a single rogue cell. We have developed similar "clonal" cancer models in the *Drosophila* brain to study how tumor cells interact among each other and with their neighbors. To study such interactions, we need to tag the tumor cells and their neighboring cells. Such differentially marked clone-pairs or 'twin-spots' are ideal for genetic and biochemical analysis. In this proposal, our goal is to develop tools to manipulate either the tumor or the normal neighboring cells or both, and test the effect on tumor growth and progression. These studies will allow deeper analysis of early changes in the tumor that are precursors for the aggressive and invasive characteristics found later. We will use glioma – a lethal brain tumor – as the cancer type of interest, and will use the variety of genetic tools available in flies to generate the twin-spots using different fluorescent tags.

Acknowledgements

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Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Abstract

Glioma are glia-derived brain tumors that grow aggressively and ultimately result in patient death as there is no cure to date despite surgical and therapeutic interventions (radio- and chemotherapy). Many studies have shown that human diseases can be functionally conserved in *Drosophila*, thus, we have come up with experiments to manipulate gene expression in two cell populations that will help us understand more about how cells surrounding a tumor behave. We will use a Drosophila glioma model to study these cell-cell interactions. Establishing our experiment, we expect one population to grow into a glioma tumor and the other population to be comprised of cells that express stress-inducing genes. This will allow us to study the effects of wound-healing related genes to study the effects on the neighboring glioma tumors. **We hypothesize that interclonal interactions promote tumor growth.** We will combine the GAL4-*UAS* system with the *Lex-AOP* system to develop our twin-spot two-clone models. Preliminary results from Cut-lexA, Chimno-lexA, and Ey-lexA crossed with AOP-RFP, and crossing Cut-lexA with Reaper (which induces cell death mediated stress) shows promising results. To further test our hypothesis, we have devised two aims. The **first aim** is to generate a positively marked two-clone interclonal model by generating tumor growth in *Drosophila* Brain and understand the behavior of the glial cell surrounding the dead tumor cell. The **second aim** is to test interclonal tumor interactions using apoptotic and cytotoxic stress by dissecting larvae to determine the effect on tumor size and the intercolonal tumor interactions. By targeting glial cells that grow the closest in proximity to the tumor growth, it will allow us to understand cell behavior of the surrounding cell signals when the tumor cells die.

Chapter 1

Background & Literature Review

1.1 Introductory Description of Glioma

Glioma account for approximately 70% of human malignant primary brain tumors, and glioblastoma multiforme (GBM) is the most commonly diagnosed as adult glioma (World Health Organization grade IV). Current standard of care includes surgery, radiation, and chemotherapy, yet despite these treatments, the median survival time for GBM is only approximately 4.5 months (Yamada and Nakano 2012) with a 72% chance of recurrence in many patients (Milano et al, 2010).

Because human disease linked genes are functionally conserved in *Drosophila* (Chien et al., 2002; Hirth, 2010; Pandey and Nichols, 2011; Read, 2011), it makes *Drosophila melanogaster* a powerful model to study cancer and other human diseases. One advantage of using *Drosophila* genetics is that it can be used to identify new genes and signaling pathways. Another advantage of using *Drosophila* is the cost – it is less expensive and requires less complicated cell manipulation compared to mouse model glioma. In addition, findings from *Drosophila* research e.g., drugs identified in *Drosophila* genetic screens have been proven effective in clinical trials in mammalian models (Dar et al., 2012; Das and Cagan, 2010; Read et al., 2005).

The genetic tools available in *Drosophila melanogaster* enable us to mimic tumor growth that happens in humans into a fruit fly model. This genetic toolkit allows manipulation of gene expression using genetic mosaics to create somatic clones (Bier 2005; St Johnston, 2009, Lee and Luo, 2001). Somatic clones are groups of cells that are genetically different from the surrounding normal cells, and usually show behaviors that are different from normal cells. For example, somatic clones can show cancer like effects by dividing rapidly, forming benign masses, and eventually showing changes akin to metastasis. The ability to generate and study the growth and progression of such tumor-forming somatic

mosaic clones one of the distinct advantages of the *Drosophila* model. These somatic mosaic clones can be identifies by cellular biomarkers (e.g., Green fluorescent protein, GFP), which allows tracking and analysis of tumor cells, and their surrounding normal cells.

Somatic clones can be induced in many different *Drosophila* tissues to mimic different disease conditions, for example, the epithelial imaginal discs, the brain, the ovaries/testes of developing flies, and the intestinal cells of larvae or adults. Thus, many different disease models can be studies using the mosaic model in flies. In all cells, initially development happens through a strict growth control process where a pool of uncommitted cells is generated. As development proceeds, these cells become specialized through a process called differentiation into key cell types like neurons, epithelial cells, etc. In addition, stem cells participate in controlling growth and producing cells with specialized functions.

1.2 Glioma Development in *D. melanogaster*

Drosophila brains are bilaterally symmetrical structures with a ventral nerve cord and two dorsal lobes (Spindler and Hartenstein, 2010) that contain neurons and glia (Apitz and Salecker, 2014). Therefore, we can use *Drosophila* glioma model to study the intercellular interaction between the surviving tumor cells as well as the dying cell to examine the molecular signals that promote glioma. Currently we induce glioma by activating two genes, PI3K and Ras, in *Drosophila* glial cells. These are fly counterparts of human genes commonly activated in glioma patients. Because there are ample genetic tools to study *Drosophila*, it provides an ideal model to study cell-to-cell signaling events and also provides the ability to dissect multi-gene interactions, identify new gene functions, and cell-type specific manipulation of gene expression in vivo (Kango-Singh, 2014).

Both in flies and humans, a pool of uncommitted cells generates stem cells in the brain that are referred to as Neuroblasts. These cells self-renew and produce a group of differentiated cells by undergoing two to three rounds of cell division to form Neurons and Glia, two major cell types in the brain. In flies, two types of neuroblasts are described. Type I neuroblasts divide to produce a Ganglion Mother Cell, which further divides to form 2 cells – 2 Neurons or 2 Glia or a neuron and a glia each – to form a small cluster of cells (Figure 1.1). Type II neuroblast cells are more clustered because they divide to form Interneuron cells which through additional rounds of cell division give rise to a larger cluster of cells. Both these neuoblast types and their descendants are found in the human brain as well. Hence, studying defects in growth regulation in flies can provide insights on human diseases like glioma.

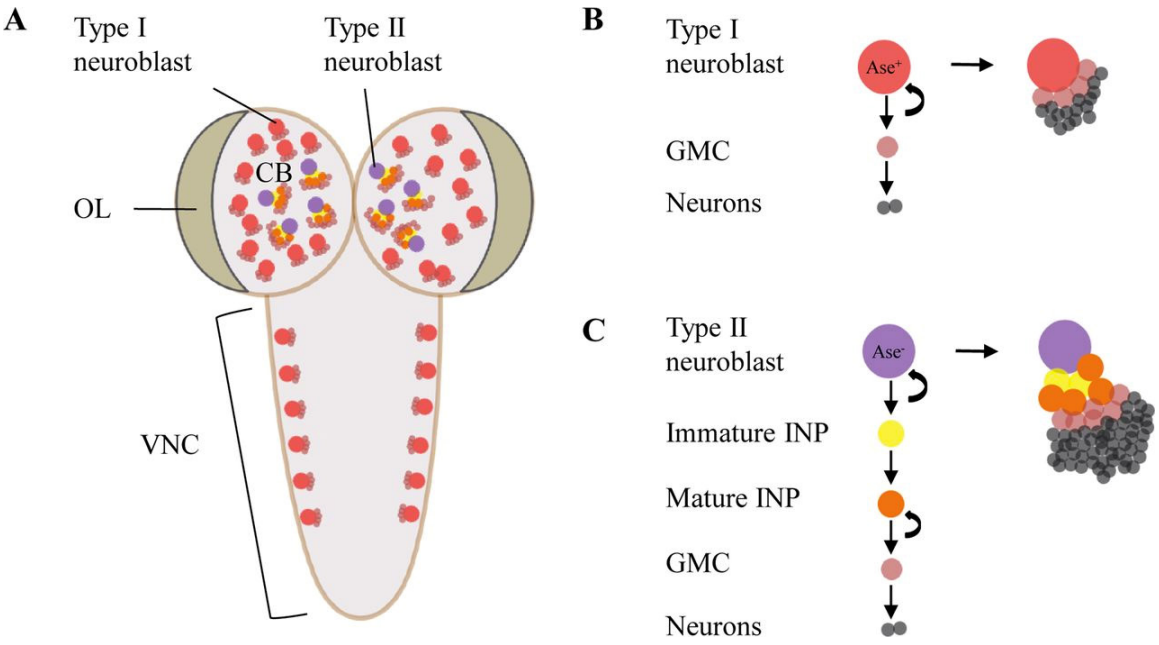


Figure 1.1 Displays how the two different types of neuroblasts differentiate in *Drosophila.* Imagine obtained from:

https://www.google.com/search?biw=1280&bih=728&tbm=isch&sa=1&ei=bL24XI3LEYiKtQWtuaCQDw&q=anatomy+of+3rd+instar+larvae+brain+lobe&oq=anatomy+of+3rd+instar+larvae+brain+lobe&gs_l=img.3...10836.12388..12534...0.0..0.166.1137.7j4.....1....1..gws-wiz-img.wY5P42FUNzk#imgrc=DGYp0JHlmwEuUM

1.3 GAL4-*UAS* and LexA-*lexAOP* System

The Gal4 and UAS (upstream activating sequence) system is a potent tool for targeting gene expression. In *Drosophila*, these components are carried in separate parental lines (Busson, 2007). When combined it into one system via crossing two parental lines together, offspring bear both systems that can be used to activate the sequence of the gene of interest. In such a case, the Gal4 transcription factor that activates the transcription of its target genes by binding to the UAS cis-regulatory site; which controls the sequence for the targeted gene's sequences (Busson, 2007). This system has been well established in our lab to produce tumor growth with *RNAi* and $RasV^{12}$.

Similar to the GAL4-*UAS* system, the LexA-*LexAOP* works the same way. Both are in individual parental genes. But once crossed, the offspring will carry the LexA-*LexAOP* line that enables LexA to act as a driver and bind to LexAOP to activate the transcription of the targeting gene (del Valle Rodriguez, Alberto et al, 2011). In this experiment, we used LexA-*LexAOP* to express Reaper expression in fruit flies.

Creating a binary system with both Gal4-*UAS* and LexA-*LexAOP* into one fly will allow researchers to simultaneously perform two manipulations of gene expression in vivo (del Valle Rodriguez, Alberto et al, 2011). Researchers can "check whether two reporters are expressed in the same or different cells" by using "GAL4 to report the expression of one gene by driving Green Fluorescent Protein (GFP) expression and LexA to report expression of another gene by driving RFP expression" (del Valle Rodriguez, Alberto et al, 2011). This system has been done in the past, but had not been established using *RNAi* and $RasV^{12}$ in combination with Reaper yet, which is one of our goals in this experiment.

1.4 Hypothesis and Specific Aims

We plan to establish a method to manipulate gene expression in two cell populations. One population is expected to grow into a glioma tumor while the second is expected to comprise of cells that express stress – or wound – healing related genes to study effects on the neighboring glioma tumors. This will allow generation of two genetically distinct mosaic populations in the developing the *Drosophila* brain, and allow us to test our **hypothesis that interclonal interactions promote tumor growth**. We have devised two aims for this project:

Aim 1 Generate a positively marked 2-clone interclonal model.

Using knowledge from the preliminary data, we will make additional crosses with Cut-lex-A and other stocks such as UAS Gal4 and LexAOP Reaper based system to generate tumor growth in the *Drosophila* brains. We will also make crosses of Cut-lexA with $RasV^{12}$ that will introduce big tumor growth to *Drosophila* to try to understand the behavior of the glia cells surrounding the dead tumor cells. The final goal is to make: *UASGFP LexAOPRFP/ UASPtenRNAi; RasV12/cutlexA; repoGal4/+* larvae. In these the *repoGal4* will drive GFP, and activate Ras and PI3K (through *UASPtenRNAi*), whereas Cut-LexA will drive RFP and LexAOP-Reaper.

Aim 2 Test interclonal tumor interactions using apoptotic and cytotoxic stress.

Using antibodies as markers, we will dissect larvae of the type generated in Aim 1 to test (1) effect on tumor size, and (ii) interclonal tumor interactions. We hope to target those glia cells that grow as closest in proximity to the tumor growth that will allow us to further examine and understand behavior of the surrounding cells signals when the tumor cells die.

Chapter 2

Materials and Methods

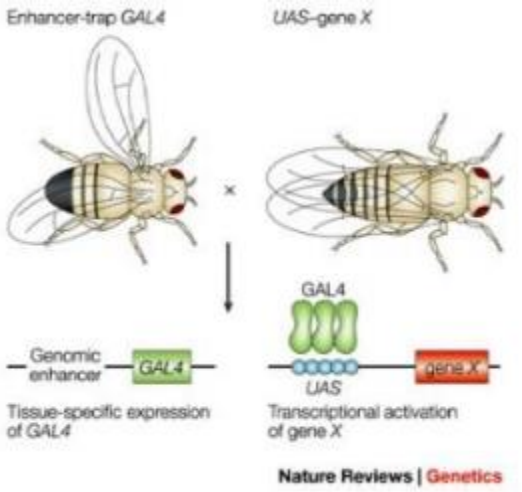
2.1 *Drosophila melanogaster* as Model Organism

The cost, availability, and ease of use makes *Drosophila melanogaster* an ideal organism of interest for genetic studies. Compare to other organisms such as mice, *D. melanogaster* are more cost efficient to maintain and perform experiments on. They have a quick generation turnover rate; metamorphoses from egg to larvae to pupa to adult within the span of 12 days. They also have a high fecundity rate that makes it more readily available. In addition, *D. melanogaster* has a small number of chromosomes, which makes it less complicated than organisms with a larger number of chromosomes to manipulate. *D. melanogaster* has many basic biological, physiological, and neurological properties that are conserved from human diseases, with approximately 75% of known human disease genes have recognizable matches in the fly genome; making them a powerful tool to understand disease mechanics and develop novel therapeutics (Pandey et al, 2011).

Fruit flies used in this experiment were obtained from colleagues as well as from Bloomington, Indiana. They were maintained at 24°C room temperature and placed in 29°C temperature when necessary. Rearing flies at this temperature increase the generation turnover rate compare to room temperature.

2.2 Tissues Specific Gene Expression Using Gal4-*UAS* System and LexA-*LexAOP* System

D. melanogaster underwent many crossing schemes in order to create two lines with various components of Gal4-*UAS* system and LexA-*LexAOP* system that later would be used to combine into one line. The Gal4-*UAS* system, was used to co-activate the glioma-causing gene using expressions of *UAS* $RasV^{12}$ and *UAS PteniRNAi* with the Repo Gal4 promoter/driver (Figure 2.1). The Repo Gal4 will drive the GFP and activate the $RasV^{12}$ and *PI3K* pathway through *UAS PteniRNAi*, which introduces tumor growth in the brain. The LexA flies underwent multiple crossing schemes conducive to creating a line with LexAOP Reaper that would be expressed using the LexA as a driver (Figure 2.2). The LexA driver created a protein that allowed it to bind to the transcriptional activator of the LexAOP Reaper. Once bound, the transcriptional activator is activated and thus the targeted gene is expressed. The targeted gene in this line is Reaper.



2.1 Diagram from Nature Reviews depicting the mechanism of how Gal 4-*UAS* system operates within the genome of an organism.

	Gal 4/UAS System	Lex A/ Lex AOP System
Transcriptional Activator	Gal 4	Lex A
Effector	UAS	Lex AOP

2.2 Table illustrating how GAL 4/UAS and Lex A/Lex AOP works as transcriptional activator and effector.

2.3 Experimental Design

To generate this model, we will take advantage of techniques that allow us to manipulate genes under different regulatory mechanisms. For example, using the Gal4-*UAS* system, we will co-activate the glioma causing genes. In our system, expression of *UASRasV*^{12} and *UASPtenRNAi* with the repo GAL4 driver results in induction of glioma brain tumors in the developing larva.

For this experiment, we plan to combine this system with the LexA-*LexAOP* system, which also facilitates the misexpression of genes in flies. We have tested Cut-lexA, Chimno-lexA, and Ey-lexA with AOP-RFP to track which cells in the fly's brain will be affected. Secondly, we tested the effect of a stressed inducing gene Reaper in this system by crossing Cut-LexA to LexAOP Reaper. The preliminary data have shown promising results of detecting the Red Fluorescent Protein (RFP) biomarker in Reaper cells. Antibody staining revealed that out of the three LexA lines tested, Cut-lex-A had cells growing closest in proximity to the glial cells in the brain lobes. From there, Cut-lex-A was further crossed and amplified to aid in future studies. This mutant line was put through a specific crossing scheme with the correct phenotypic F1 flies used to continue in the next crosses.

2.4 Using Immunohistochemistry as a Tool to Study Protein Expression and Localization in 3rd instar *D. melanogaster* larvae

After achieving the desired phenotypes, either stocks or more crosses were made using flies from the Cut-LexA lines. Once those lines produce offspring with the phenotypes we seek, 3rd instar larvae were dissected, mounted, and photographed. D. *melanogaster* brain lobes are dissected and stained using standard protocols for immunohistochemistry. Generally, immunohistochemistry assays are used to show protein expression and localization.

For the dissection process, we prepared a silicon agar plate with Phosphate Buffer Saline (PBS) that has a pH of 7.4 to dissect 3rd instar larvae on. The PBS provides the sample an isotonic solution to dissect in and it also prevents the fly from sticking to the silicon plate. After separating the brain lobes into 100μL of PBS and discarding the rest, the samples were fixed in 100μL of 8% parafamaldehyde (PFA) for 20 minutes. Then, the samples were transferred to an eppendorf tube with 1ml of PBST detergent (Triton). The eppendorf tubes were placed on the nutator for 10 minutes. PBST detergent was then removed using a vacuum and this washing process was repeated 2 more times. Once the washing is completed, if using antibodies, the primary bodies were added into the eppendorf tube and incubated at 4°C overnight. Antibodies were used to identify molecular markers staining on the brain lobes. The washing process using PBST was repeated 3 more times the following day. Secondary antibodies were added following the wash. The primary antibodies expression were detected by secondary antibodies containing fluorescence tagged, which shows up under the microscope. The eppendorf

tube were wrapped in foil and placed on the nutator for an additional 2 hours. The samples were then washed 3 more times before they was mounted.

The mounting occurred on a slide under a stereomicroscope. After the samples were mounted, Vectashield were placed on top of the samples and topped by a cover slip. The cover slips were glued in place using nail polish around the perimeter of the slip to seal the slip in place. Images of the slides were taken at 20X using the electron microscope (Olympus Fluoview 100). A Z-stack image was generated by manually setting the focus of the starting and ending points. The Z-stack image generated by the camera took picture in slices, and then can be stacked/combined into one whole image. The samples were then taken at 40X. At least five different samples of the same genotype were taken per magnification.

Chapter 3

Results

3.1 Introduction

Both Gal4-*UAS* and LexA-*LexAOP* systems play a crucial part in this experiment by providing us a means to manipulating the gene expression of the targeted gene. When both systems work in conjunction, we expect to have a positively marked two-clone interclonal model in *Drosophila* illustrated by cellular biomarkers such as GFP and RFP. Our goal was to generate such a model (*UASGFP LexAOPRFP/ UASPtenRNAi; RasV12/cutlexA; repoGal4/+*) to test interclonal tumor interaction using apoptotic and cytotoxic stress to determine the effect on tumor size and the intercolonal tumor interactions.

3.2 Results

Preliminary results (Figure 3.1) reveal the perspective LexA line growth in the brain lobe. We determined that Ey-LexA and Chimno-LexA lines did not have cell growth in the closest proximity to glial growth compared to Cut-LexA, and thus, Cut-LexA was selected to be the line we focused our study on. Illustrated in Figure 3.2 is an image of the Cut-LexA and its cell growth in perspective to glial cell growth.

From there, we generated the following lines shown on List 3.4. Line #9 (Figure 3.3) that was generated shows successful differentially marked clone-pairs or “twin-spots” using the Gal4 and LexAOP binary system.

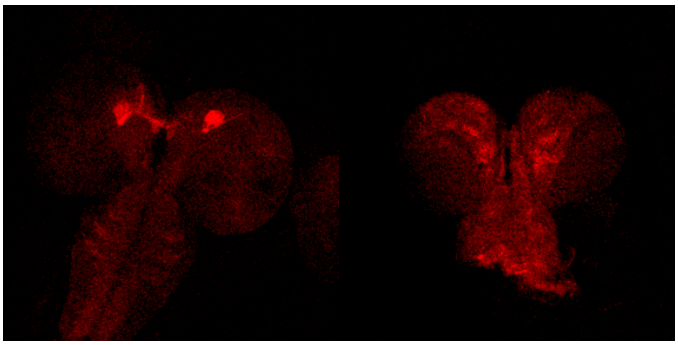


Figure 3.1 Left: *Ey LexA x LexAOP Reaper*; Right: *Chimno-LexA x Lex AOP Reaper*. Images obtained by: Minh Ho and Kirti Snidgha

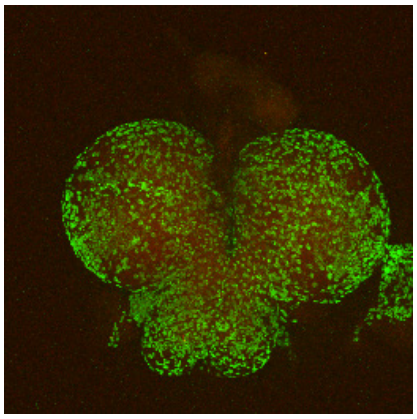


Figure 3.2 *Cut LexA x LexAOP Reaper*. This displays the close proximity of the Cut-LexA line with the glial cells. The GFP represents the glial cells and the RFP represents the Cut-LexA cells. Image obtained by: Minh Ho and Kirti Snidgha

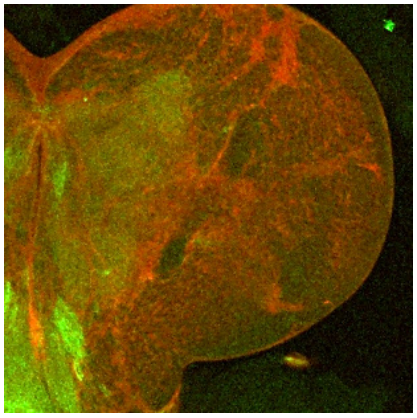


Figure 3.3 *UAS RFP Lex AOP GFP; Cut-LexA/Cyo; Repo Gal 4/TM6B x Ywhsflip; Sp/Cyo; LexAOP Reaper/TM6B*

1. Repo Gal4 UAS GFP
2. Pteni; $RasV^{12}$ x Repo GFP.
3. YwhsFlp; Sp/Cyo; LexAOP Reaper/TM6B
4. YwhsFlp; Cut-lexA/Cyo; TM3/TM6B*
5. YwhsFlp; Cut-lexA/Cyo; Lex AOP Reaper/TM6B*
6. YwhsFlp; Cut-lexA/Cyo; Repo Gal4/TM6B*
7. UAS Pteni; UAS Ras V12/ RasV12; Lex AOP Reaper/TM6B
8. UAS RFP lex AOP GFP; Cut lexA/Cyo; RepoGal4/TM6B.
9. *UAS RFP Lex AOP GFP; Cut Lex A/Cyo; Repo Gal 4/TM6B x Ywhsflip; Sp/Cyo; LexAOP Reaper/TM6B*

Table 3.4 Display stocks and crosses we generated over the span of the experiment.

3.3 Challenges

Generating complex genotypes with an extensive number of steps came with multiple challenges. Whether it was due to contamination or unexpected results that differ from our expectations, we had to analyze, troubleshoot, and restart when needed. After generating and obtaining data for the lines mentioned above, we tried to create the final targeted line with the genotype of *UASGFP LexAOPRFP/ UASPtenRNAi; RasV12/Cut-LexA; repoGal4/+* larvae. However, we had a contamination in Line #6 and lost Lines #4 and #5 in the process, which forces us to remake those stocks. We successfully remade Line #6, although that cost us some time. However, when we tried to use Line #6 and cross it with the balancer *YwhsFlp; Sp/Cyo; TM3/TM6B* in a three-step process to regenerate Line #4 – before using Line #4 to recreate Line #5 – we encountered flies that had inconsistent phenotypes that require us to troubleshoot.

For this cross, we had acquired the correct phenotype for the parental stocks. After crossing the parental stocks with each other, offspring (F1) with phenotypes of male, red eyes, curly wings, stubbles, and TM3 were to be collected in order to cross it with *YwhsFlp; Sp/Cyo; TM3/TM6B* to yield an F2 (genotype of *YwshFlp; Cut- LexA/cyo; TM3/TM6B*) that can be used to replace the lost stock of Line #4 (Table 3.4). However, the F1 males collected had every phenotype we sought except eye color. We kept seeing a mixture of red-eyed males and white-eyed males, instead of the consistent red-eyed males that were targeted. This step was repeated four more times, yet the F1 yield had a consistent mixture of red and white-eyed males. Thus, we were unable to replace Line #4 and therefore, could not recover Line #5, preventing us from achieving the end goal: *UASGFP LexAOPRFP/ UASPtenRNAi; RasV12/cutlexA; repoGal4/+*.

The generation time for *Drosophila* is approximately 12 days long, and thus, each time we repeated this experiment, it took a month to compete, with a total of four month spent regenerating this stock. We were only able to repeat this experiment four times within the time span allotted for this thesis.

Chapter 4

Discussion

4.1 Significant results

From the preliminary results, the Cut-LexA illustrates that it grows closest in proximity to the glial cells. We made Repo Gal4 UAS GFP as a control to see the amount of normal glial cells presented in the Gal4-*UAS* system. We also made *Pteni; $RasV^{12}$ x Repo GFP* to visualize the amount of glial cells when tumor cells are present in the brain to use for the control for our second aim. While there were many crosses and stocks made from the experiment, the genotype that we wanted was not successfully made. However, the twin-spots generated using the binary systems of Gal4 and LexAOP was successfully modeled, although the expression appeared to be weak. Thus, we were able to complete a part of Aim 1. Because we were unable to obtain that correct genotype, we couldn't advance on to Aim 2.

4.2 Expected Outcomes

With more time to possibility generate the desired stock from the first aim that contains an overexpression of *$RasV^{12}$* and *PtenRNAi* to stimulate tumor growth in one population, and stimulate Reaper to cause cell death in another population of the cell, it is expected that the genotype modeled will allow us to study how the interclonal interaction influences the growth of tumors. This two-marked population can be quantified in the brain and we can test this interclonal interaction by controlling the tumor, surrounding cells, or both in the population. With the binary system, we can see where glioma is in the human brain and try to drive that expression.

4.3 Future Research

For the future research, we wish to generate the second aim and examine the interclonal interaction between tumors and neighboring cells. We also will try a different strategy and test it from a different LexA line such as using Chimno-LexA and Diachete-LexA. For this experiment, we targeted every glial cell growth in the brain by choosing Cut-LexA. However, if we try another strategy and target the glial cells that only grow in the frontal area – since the expression of glioblastoma grows mainly in the frontal area of the brain in humans – we can use Chimno-LexA and Dichaete-LexA line instead. The experimental plan will be the same but we will use a different type of LexA line and hopefully that will allow us to successfully understand tumor growth interactions better in *Drosophila* and thus, can better understand human glioma.

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