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The Effect of Natural Extracts on Border Cell and Centripetal Cell Migration in the Developing *Drosophila melanogaster* Egg Chamber

A Thesis

Presented to the Department of Biological Sciences

College of Liberal Arts and Sciences

And

The Honors Program

of

Butler University

In Partial Fulfillment

of the Requirements for Graduation Honors

Kirandeep Kaur

5.8.2020

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Acknowledgements

I would like to acknowledge the following for helping me in the completion of my thesis:

- Dr. Lindsay Lewellyn for allowing me to join her research lab. She has provided generous amount of time, guidance and resources in making this thesis possible.
- The Department of Biological Sciences for their support and resources
- The Butler University Honors Program for their assistance and support

Abstract

Cancer is one of the leading causes of death in the world. Although targeted therapies that specifically inhibit pathways that are activated in cancer cells are becoming more common, often times the specific cancer-causing pathways are not known, or a treatment targeting that pathway has not been developed. In the absence of a targeted therapy, most common cancer treatments target proliferating cells, which can cause many unwanted adverse side effects. Therefore, researchers are testing whether natural extracts or dietary supplements could reduce the growth or metastasis of cancer cells without as many negative side effects. This study uses the *Drosophila melanogaster* egg chamber as a model system to test the effect of two natural extracts (walnut extract and green tea extract) on invasive cell migration. During normal egg formation, two groups of cells - the border cells and the centripetal cells - migrate from the outer epithelial layer into the germ cell cluster. Because cancer cell metastasis involves invasive cell migratory behavior, these normal cellular behaviors can be used as a model for metastasis. To monitor these invasive migratory behaviors, the border cells and centripetal cells were marked with a green fluorescent protein (GFP), and the extent of migration was monitored using fluorescence microscopy. Data collected from these experiments suggest that walnut extract and green tea extract treatment could cause a modest defect in centripetal cell migration, without significantly affecting border cell migration. Future experiments will assess effect of walnut or green tea extract on specific pathways implicated in centripetal cell migration, as well as extend this model to test other natural extracts.

Introduction

Introduction to Cancer

Cancer is one of the leading causes of death in the world, and there are many different types of this heterogenous disease. Each type of cancer affects a different tissue type (such as breast cancer, lung cancer, pancreatic cancer, lymphomas, carcinomas, and leukemia) and will have a different prognosis. Despite the heterogeneity of this disease, the underlying cause of all types of cancer is the accumulation of a set of mutations that change the normal behavior of cells (NCI 2015). These mutations can either be inherited, or they can be acquired. Acquired mutations are due to exposure to mutagens or carcinogens, agents capable of inducing mutation or causing cancer respectively (NCI 2015). Many environmental conditions or lifestyle choices can increase the probability that cancer will develop. For example, excessive exposure UV radiation, exposure to *H. pylori*, and tobacco smoking can all increase the rate of cancer (NCI 2015). In some cases, there is no clear identifiable cause of the mutations; they could even just arise from errors in the DNA replication machinery that replicates the genome in preparation for mitosis.

Cancer causing mutations typically affect pathways that control normal cell behavior. Normal cells, or noncancerous cells, grow and divide in response to specific growth factors and mitogens, ensuring that their rate of proliferation is balanced by rates of cellular death to maintain tissue integrity. Normal cells are subject to the action of checkpoints, which regulate the progression through the cell cycle (NCI 2015). These checkpoints monitor the presence of DNA damage, ensure that the chromosomes have been properly replicated during S phase, assess cell size, and guarantee that the microtubules of the mitotic spindle have been attached appropriately to orient the chromosomes during mitosis (NCI 2015). If DNA damage is detected,

normal cells will attempt to repair that damage; if it is too severe, then the cell will undergo programmed cell death, or apoptosis (NCI 2015). In addition to controlled progression through the cell cycle, normal cells, such as those in an epithelial layer, will maintain their position within the body (NCI 2015). They will form cell-cell junctions with neighboring cells in the tissue layer.

Cancer cells differ from noncancerous cells due to their ability to prolong proliferative mechanisms, degrade growth suppressors, overcome cell death, continuously replicate, create new blood vessels, and initiate invasive behaviors (Hanahan and Weinberg 2011). These abnormal cellular behaviors lead to the formation of tumors, which can either remain in their primary site (benign), or which can metastasize to other parts of the body (malignant). Cancer cells may also have the ability to alter the normal immune response, which can contribute to the complexity of the disease. The immune system, which consists of various specialized cells, tissues and organs, protects the body against foreign bodies and infections (NCI 2015). Cancer cells have the ability to weaken the immune system or disable an immune response, thus inhibiting the body's machinery to eliminate abnormal cells. The central goal of cancer research is to develop therapies or treatments that are able to block one or more of these abnormal behaviors to restore normal cellular functions or induce cancer cell death.

Cancer treatments

Because cancer is such a common, yet devastating disease, many different treatments have been developed to attempt to delay progression or induce remission. The most effective cancer treatments directly target an abnormal signaling pathway that is active in the cancer cells, but which is not active in normal cells (Figure 1). For example, the use of epidermal growth

factor receptor (EGFR) inhibitor therapy can reduce the progression of lung cancer by targeting the cancer cell's ability to sustain proliferative signaling. EGFRs are responsible for controlling cell division and cell survival by inducing tyrosine phosphorylation events. In cancer cells, these receptors can be mutated, leading to the constant activation of EGFR signaling and continuous cell proliferation (Maemondo et al. 2010). Inhibitors of EGFR tyrosine kinases, such as gefitinib, are able to interfere with ligand binding, thus reducing the activation of EGFRs (Maemondo et al 2010). Gefitinib treatment has been shown to be highly effective in turning off the EGFR pathway and thus reducing cancer cell proliferation (Maemondo et al. 2010).

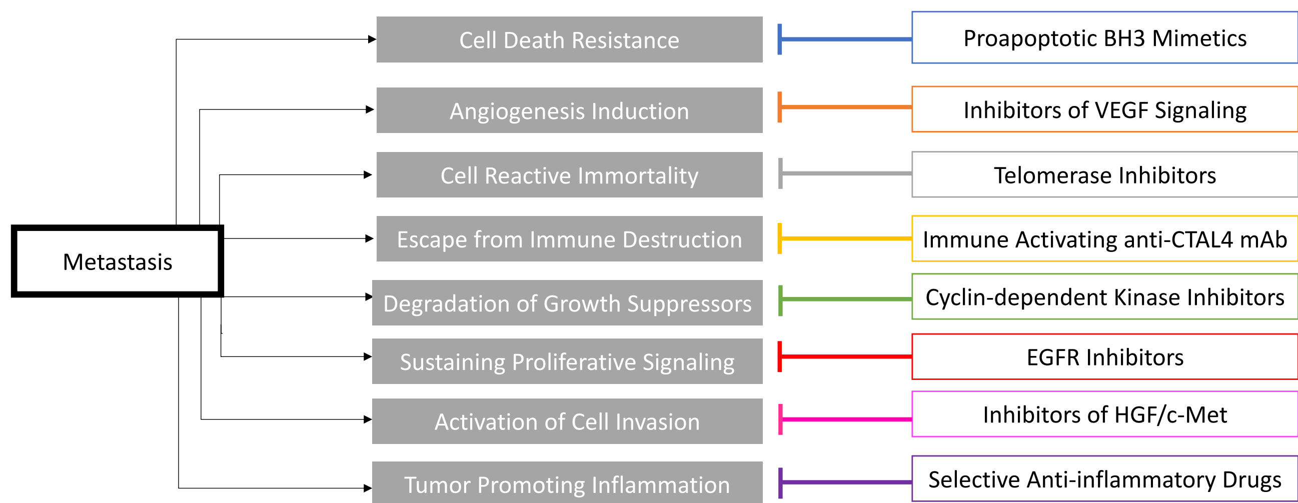

Figure 1: Current therapeutic treatments for cancer. Development of therapies and preventative treatments (color) target the different hallmarks of cancer (gray) (Hanahan and Weinberg 2011).

Other therapies have been able to delay cancer progression or induce remission by altering the body's immune response. Normally, the immune system is able to help eliminate abnormal cells, such as cancer cells, but often times cancer cells will dampen or weaken the immune response to allow their survival. In immune therapy, the patient's immune system is enhanced so that it can more effectively target and eliminate cancer cells. A specific drug used to

treat melanoma, Keytruda, works in this way. Keytruda is a monoclonal antibody (mAb) that binds to programmed-death 1 receptor (PD-1) and prevents binding to immune-suppressing ligands, which are often expressed on the surface of cancer cells (Raedler 2015). By blocking the interaction between PD-1 and the cancer suppressing ligand, this can allow the activation of the T-cell adaptive immunity response causing a destruction of the cancer cells. Keytruda has been shown to prolong survival of patients with metastatic melanoma (Raedler 2015).

In the absence of a targeted therapy, other standard cancer treatments attempt to target dividing cells. Because cancer cells proliferate at a higher rate than normal cells, this can lead to a somewhat selective method to kill cancer cells in the body. Site specific radiation or whole-body radiation can be used to induce mutation and DNA damage in replicating cells, ultimately resulting in cell death (NCI 2019). Other treatments include chemotherapy, which again targets dividing cells. For example, some chemotherapy drugs disrupt the microtubules, which are used to build the mitotic spindle (NCI 2015). By disrupting the microtubules, this can block the ability of the cell to progress through mitosis, which can ultimately lead to apoptosis. One major limitation to this type of therapy is that not all replicating cells are cancerous. Therefore, radiation and chemotherapy cause many short term and long term side effects due to the damage to normal dividing cells. These can include hair loss, shortness of breath, and dry mouth. More long-term effects, called "late effects," include congestive heart failure, coronary artery disease, hypertension and osteoporosis (ASCO 2019; NCI 2015). Therefore, in the absence of a targeted therapy, another cancer treatment with fewer side effects would be a significant improvement.

Natural Extracts as Therapeutic Agents

Many researchers have turned to more natural cancer remedies, such as extracts from curcumin, theaflavins, and others (Desai 2008). Many of these natural treatments are high in antioxidants, which can prevent cancer growth by inducing toxicity and ultimately inducing cancer cell death (Abraham et al. 2017). Antioxidants can target pathways that regulate cell proliferation or other processes involved in tumor progression. These pathways include the NFkB, Akt/p13 and VEGF pathways (Abraham et al. 2017). For example, NFkB is a transcription factor that is responsible for cell growth and the autoimmune and inflammatory responses (Xia et al. 2014). In cancer cells, there is an increase or constitutive activation of NFkB signaling, causing an increase in cell growth and proliferation (Xia et al. 2014). Clinically, treatment of oral squamous cell carcinoma with alpha-tomatine, a saponin in tomato, was able to induce a downregulation of the NFkB pathway (Abraham et al. 2017), suggesting that this could be a viable option for targeting this pathway in cancer cells.

Walnut extracts contain high levels of antioxidants and phytosterols, specifically alpha-linolenic acid and beta-sitosterol, which may be able to slow tumor growth by inhibiting cell proliferation and inducing apoptotic death (Hardman et al. 2019). These components of the extract are able to inhibit the activity of many different kinases, one being the phosphatidylinositol 3-kinase/serine/threonine kinase 1 (P13K/Akt) which normally promotes cancer cell survival in breast cancer patients (Hardman et al, 2019). In a clinical trial, breast biopsies were taken from diagnosed breast cancer patients before and after 2 to 3 weeks of consuming 2 ounces of walnuts per day. The results showed a significant suppression of P13K/Akt activity, which correlated with reduced cancer cell survival (Hardman et al. 2019).

These findings suggest that even fairly modest changes to the diet could have a significant impact on cancer progression.

Green tea extract contains antioxidants and molecules such as epigallocatechin-3-gallate (EGCG) that prevent tumor progression by inhibiting the interaction of specific tumor promoting ligands (okadaic acid, TPA, and teleocidin) with their cellular receptors (Fujiki et al. 2018). In a clinical study, it was shown that green tea consumption led to a significant decrease in stage I breast cancer recurrence in patients who consumed more than 5 cups of green tea daily as compared to those who consumed less than 4 cups of green tea daily (Nakachi et al. 1998). Additionally, studies have suggested that drinking 10 Japanese-size cups of green tea daily can reduce the recurrence of colorectal tumors (Fujiki et al. 2018). These studies again suggest that dietary changes could have a significant impact on cancer prevention, progression, and recurrence.

Drosophila melanogaster **as a Model**

The fruit fly, *Drosophila melanogaster,* is an excellent model system that can be used to study genetics, developmental biology, cell signaling, and cell migration (Hudson and Cooley 2014). Since the biology of fruit flies has been well studied, and many genetic and molecular tools are available, targeted modifications can be made to generate invertebrate models that mimic human diseases. For example, Pagliarini and Xu (2003) performed a screen in *Drosophila* to identify combinations of mutations that could induce the formation of metastatic tumors, which were also labeled with green fluorescent protein, or GFP. Once a model system has been developed, it could then be easily and quickly screened for small molecules or other treatments that would alter the disease phenotype. For example, the previously developed model (Pagliarini and Xu 2003) or others could be screened to identify compounds that prevented metastasis of the

GFP-labeled cancer cells (Gladstone & Su 2011). Because many of the mutations that induce tumor growth in flies are similar to those seen in humans, this type of work provides a useful starting point for the development of novel therapies.

In addition to developing fly models that mimic human diseases, we can also utilize developmentally controlled morphogenetic events as a model system to study cancer. Often times, cancer cells display behaviors that are reminiscent of those seen during development. For example, cancer cells can display epithelial-to-mesenchymal transition, where they lose the apical basal polarity and junctional structures seen in epithelial cells and become migratory (Pagliarini and Xu 2003). This is a normal transition seen during embryonic development in many organisms therefore, by studying how this process occurs normally during development, we can understand how it may be initiated and how it could be prevented in cancer cells.

The developing egg provides an excellent system to study invasive cell behaviors. Each fruit fly egg develops from a multicellular structure called an egg chamber. Each egg chamber goes through 14 developmental stages before forming a mature egg. The egg chamber starts its development within the germanium where a germline stem cell divides to produce a cystoblast cell that undergoes 4 rounds of mitosis to generate a cluster of 16 germ cells; these cells are surrounded

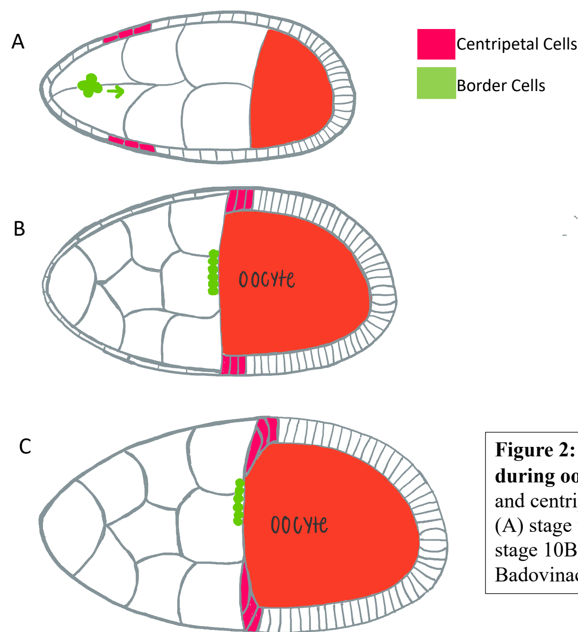

Figure 2: Follicle cell behaviors during oogenesis. Border Cell (green) and centripetal cell (pink) migration at (A) stage 9, (B) stage 10a, and (C) stage 10B. (Adapted from Horne-Badovinac & Bilder 2004).

by a layer of somatic follicle cells. Follicle cells, themselves, exhibit a number of stereotypical morphogenetic behaviors during oogenesis, including mitotic divisions, cell shape changes, and collective cell migrations (Figure 2; Horne-Badovinac & Bilder 2004). These cell movements are essential for normal egg formation.

Two of these stereotypical follicle cell movements involve an invasive form of cell migration in which the follicle cells leave their normal position on the outside of the egg chamber and migrate into the germline. At the beginning of stage 9, six to ten of the most anterior follicle cells are specified as border cells; the border cells break contacts with the rest of the follicular epithelium and start migrating through the germ cell cluster toward the oocyte, which is located at the posterior of the egg chamber (Figure 2). This process is similar to the epithelial-to-mesenchymal transition seen during cancer cell metastasis. Coincident with border cell migration, the main body follicle cells change shape from cuboidal to columnar and undergo a shift or migration to cover the oocyte at the posterior by stage 10a (Figure 2; Montell 2003; Horne-Badovinac & Bilder 2004).

During stage 10b, the centripetal cells, which are near the boundary between the oocyte and the nurse cells, invade into the germline to separate these two compartments (Fig. 3; Montell 2003). These invasive cell behaviors - border cell migration during stage 9, and centripetal cell migration in stage 10b - can be used as a model to study cancer metastasis.

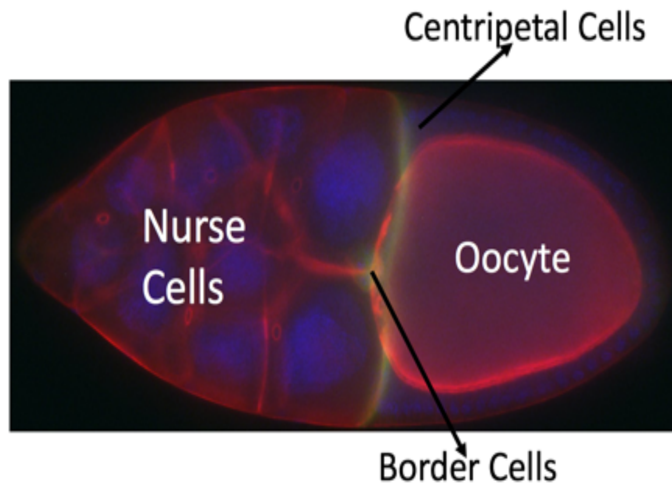

Figure 3: **Position of the border cells and the centripetal cells at stage 10 of oogenesis.** *Slbo-GAL4; UAS-GFP* stage 10b egg chamber stained with phalloidin (red) and DAPI (blue). Green indicates the GFP that is expressed in the border cells and centripetal cells.

Border cell migration requires the activation of specific signaling pathways as well as changes in cell adhesion in order to allow this cluster of cells to migrate from the anterior to the posterior of the egg chamber. One of the important signaling pathways is the JAK-STAT signaling pathway. The JAK-STAT pathway involves the binding of an extracellular ligand to a receptor at the cell surface (Prasad et al. 2015). This leads to the activation of the JAK intracellular kinases, and ultimately to activation of the STATs, which are transcription factors that can activate expression of genes that promote cell growth and survival and regulate the immune response (Prasad et al. 2015). This pathway is necessary to specify the border cells as well as to promote their collective migration during stage 9 (Montell 2003). The PDGF and VEGF signaling pathways also contribute to the migratory behavior of border cells. PVF1, also known as VEGF related factor 1, binds to PVR, a receptor tyrosine kinase that functions with various epidermal growth factor receptors (EGFRs) to promote cell migration (Prasad et al. 2015). Ligand binding to the EGFRs on the oocyte helps the border cells to migrate to the oocyte at stage 10 (Prasad et al. 2015). EGFRs are responsible for controlling cell division and cell survival signals by inducing tyrosine phosphorylation events. Mutations that constitutively activate the EGFR can lead to unregulated cellular proliferation (Maemondo et al. 2010). Thus, there are multiple pathways that promote border cell migration, and which can also be mis-regulated in cancer cells.

The migration of the centripetal cells is a less understood. However, it is known that it is regulated by multiple signaling pathways, including the decapentaplegic (Dpp), Notch, EGFR, and the JAK-STAT pathways (Wu et al. 2008). Dpp is a critical component in centripetal cell migration because of its ability to downregulate the expression of *bun,* a gene that is important

for regulating the expression of Notch, a critical signaling pathway that promotes centripetal cell migration (Wu et al. 2008).

Cell-cell adhesion must also be tightly regulated to promote migration of both the border cells and the centripetal cells. DE-cadherin is a key regulator of cell-cell adhesion; it is a trans-membrane protein that interacts with another DE-cadherin on a neighboring cell and then connects to the cytoskeleton to form an adherens junction. Increasing or decreasing DE-cadherin in either the border cells or nurse cells can lead to defects in border cell migration (Niewiadomska et al. 1999). Proper regulation of DE-cadherin is also required for centripetal cell migration. Reducing DE-cadherin levels in the follicle cells disrupts the structure of the columnar epithelium, which alters the position of the centripetal cells during their migration (Wu et al. 2008). Cancer cells also show changes in cadherin levels, especially during the process of metastasis (Wu et al. 2008). Therefore, the border cells and centripetal cells share many similar characteristics with cancer cells, which make them a good model for testing the effect of different natural treatments.

Thesis Description

The goal of this study is to use the developing fruit fly egg as a model system to study the effect of natural extracts on two developmentally controlled forms of invasive cell migration, border cell and centripetal cell migration. Both walnut and green tea extract are high in antioxidants, and they have been shown to be useful in the prevention of various cancer types, including lung and colon cancer. However, because metastasis, or the migration of cancer cells from the primary tumor site to other places in the body, is the leading cause of cancer-related deaths, identifying natural extracts that are able to inhibit invasive cell migration in the fruit fly can provide avenues for the development of novel cancer treatments. Therefore, I propose to test the hypothesis that walnut and green tea extract will affect the migration of the border cells and centripetal cells in the developing egg chamber. The treatment of adult female flies with walnut and green tea extract in their diet may block or reduce the degree of invasive cell migration.

Methods and Materials

Genetics

Flies expressing Slbo-GAL4 were crossed with flies expressing UAS-GFP. The female offspring from this cross will express the GAL4 protein in the border cells and centripetal cells. In these cells, GAL4 protein binds to the UAS sequence of DNA and promotes expression of transgene that is downstream of this sequence, in this case, GFP, or green fluorescent protein. Therefore, the developing egg chambers produced by these female offspring will express GFP in the border cells and centripetal cells.

Extracts

Walnut juice was obtained from a dozen walnut fruits. The fruits were placed in a 2L beaker and covered with distilled water. After boiling for 1 hour on a hot plate, walnut juice was filtered through a sieve. It was then filtered through Mira-cloth for purified extraction of walnut juice. After refrigeration of the walnut juice, three different walnut treatments were made (Table 1). Green tea powder was obtained from Bigelow Green Tea Classic packets. Three different green tea treatments were made (Table 1). One control treatment was also made.

Name	Compound	Water	Yeast
Control	N/A	12.0 mL	6.64 g
W1	6.0 mL Walnut Extract	0.0 mL	3.32 g
W2	3.0 mL Walnut Extract	3.0 mL	3.32 g
W3	1.0 mL Walnut Extract	5 mL	3.32 g

G1	0.32 g Green Tea Extract	6 mL	3.32 g
G2	0.16 g Green Tea Extract	6 mL	3.32 g
G3	0.08 g Green Tea Extract	6 mL	3.32 g

Table 1: Composition of the experimental yeast paste treatments.

Ovary Dissection and Staining

Slbo-GAL4/+; UAS-GFP/+ female flies were kept in vials that contained cornmeal molasses fly food (which included cornmeal, agar yeast, molasses, tegosept, and propionic acid). A total of 8-10 female flies and 3-5 male flies were placed into a new treatment vial. Each treatment vial consisted of a spoonful of either control, W1, W2, W3, G1, G2, or G3 yeast paste on top of the normal fly food. Flies were incubated for 40-55 hours at 25°C prior to dissection.

Ovaries were dissected in Schneider's media and placed in 1.7 mL Eppendorf tube. Ovaries were fixed in 4% formaldehyde + PBS solution for 15 minutes. The ovaries were washed in PBS + 0.1% Triton solution and were then rocked for 5 minutes. This washing and rocking step was repeated for a total of 3 washes in PBT.

After washing, ovaries were stained in a 1:500 dilution of phalloidin (TRITC) and 1:500 dilution of DAPI in PBS + 0.1% Triton. DAPI was used for the visualization of DNA and phalloidin was used for the visualization of the actin cytoskeleton. Upon removal of staining solution after 20 minutes in the dark, 2 washes in PBS + 0.1% Triton solution were performed. Ovaries were mechanically disrupted and settled for 10 minutes prior to being mounted in Slowfade Antifade and transferred onto glass slides.

Imaging and Analysis

Images of egg chambers were obtained using a Leica DM5500 compound fluoresce microscope and Leica DFC7000 T camera. Leica Application Suite X Software was used to take z-stacks of each egg chamber with the 20x objective. Each egg chamber imaged was classified into either stage 9, 10a or 10b of development. The analysis tools in the Fiji/ Image J software were used to take measurements of relative size and distances of different structural features in the egg chamber, such as the total length and width of the egg chamber and the distance traveled during border cell migration (Figure 4). The distance that the border cells migrated was measured relative to the position of the main body follicle cells as a readout for the progress of this behavior. Centripetal cell migration was analyzed through visual identification on the confluency of the centripetal cell at the anterior of the oocyte (Figure 2E).

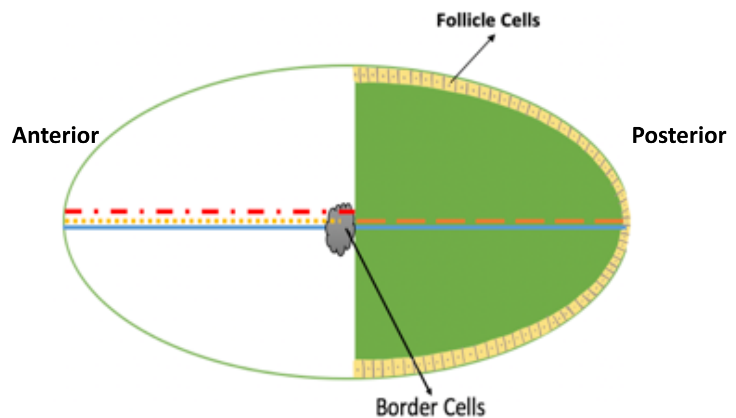

Figure 4: Technique used to monitor border cell migration and oocyte size. The length of the egg chamber (blue solid line) and the oocyte length (orange dashed line) were measured using the line tool in Fiji software. Yellow dashed line measured length from anterior of egg chamber to border cells and red dashed line measured the distance between the anterior of egg chamber to anterior of oocyte.

Statistical Analysis

For each treatment, the percent ratio was calculated of the average length of egg chamber to border cells over the average length of the egg chamber to the anterior oocyte. Standard deviations were calculated for each percent ratio. Then the ratio of the oocyte size relative to egg chamber size was calculated for all treatments. Standard deviation was again calculated for each

percent ratio. The 2-tailed t-test was used to determine statistical significance; significance was determined at p-values < 0.05.

Results

Effect on Border Cell Migration

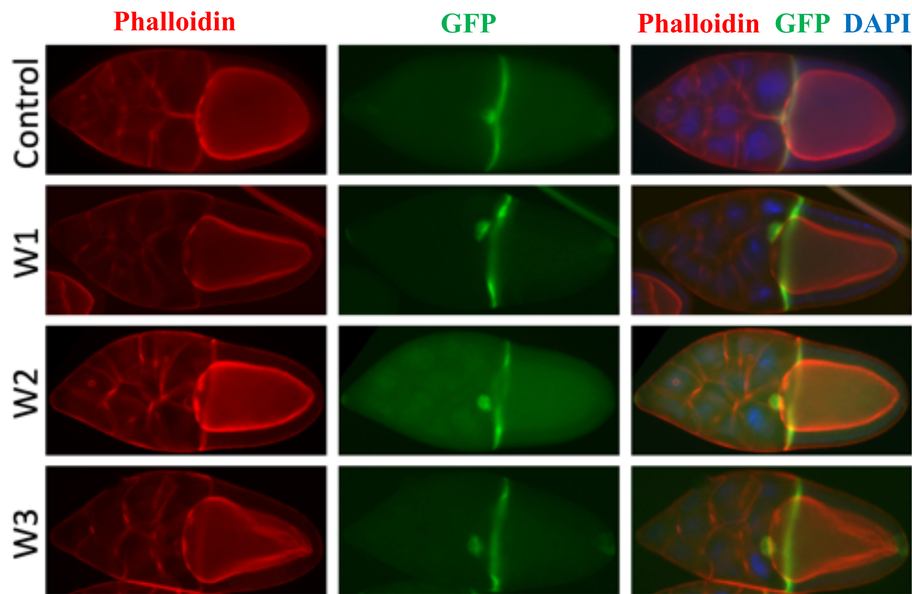

Figure 5: Treatment with walnut extract alters centripetal cell position without affecting border cell migration. Ovaries from Slbo-GAL4; UAS-GFP flies treated with control or walnut extract yeast paste. GFP is expressed in the border cells and centripetal cells, and the tissue was stained with phalloidin (*red*) and DAPI *(blue*). W1 treatment contained 6mL walnut extract, yeast food, and no water. W2 treatment contained 3mL walnut extract, yeast food, and 3 mL water. W3 treatment contained 1mL walnut extract, yeast food, and 5mL water. All fluorescence images shown are of egg chamber in stage 10 of oogenesis.

Initial observations suggested that treating female flies with walnut extract did not have a significant effect on border cell migration when compared to controls (Fig. 5), as the border cells did always reach the oocyte by stage 10 of oogenesis. Additionally, there was no dose-dependent

effect observed from the various walnut extract solutions; increasing the concentration of walnut extract in the yeast paste did not alter the phenotype. This experiment was repeated, and the effect of green tea extract was also assessed (Fig. 6). Again, there was no significant difference in border cell migration when compared to control and increasing the concentration of either treatment did not enhance the phenotype.

To confirm this result, I quantified the distance traveled by the border cells to the total distance from the anterior of the egg chamber to the anterior of the oocyte; this ratio was then converted to a percent, with 100% indicating complete migration (Fig. 7A, B). In the controls, the border cells always migrated 100% of the distance, whereas the border cells in both treatment groups showed migration slightly over 100%. All green tea treatments were significantly different from the control treatment (p-value < 0.05; Fig. 7B). In contrast, the average percent ratios of the walnut treatments were not significantly different from the control treatment (Fig. 7B). The observation that the migration was >100% suggests that the border cell cluster traveled all the way to the oocyte, but that the structure of the oocyte was slightly altered relative to the control. Instead of forming a straight vertical border at the anterior end, in line with the columnar follicle cells, the oocyte was more convex at the anterior end, which made it appear that the border cells migrated past 100%.

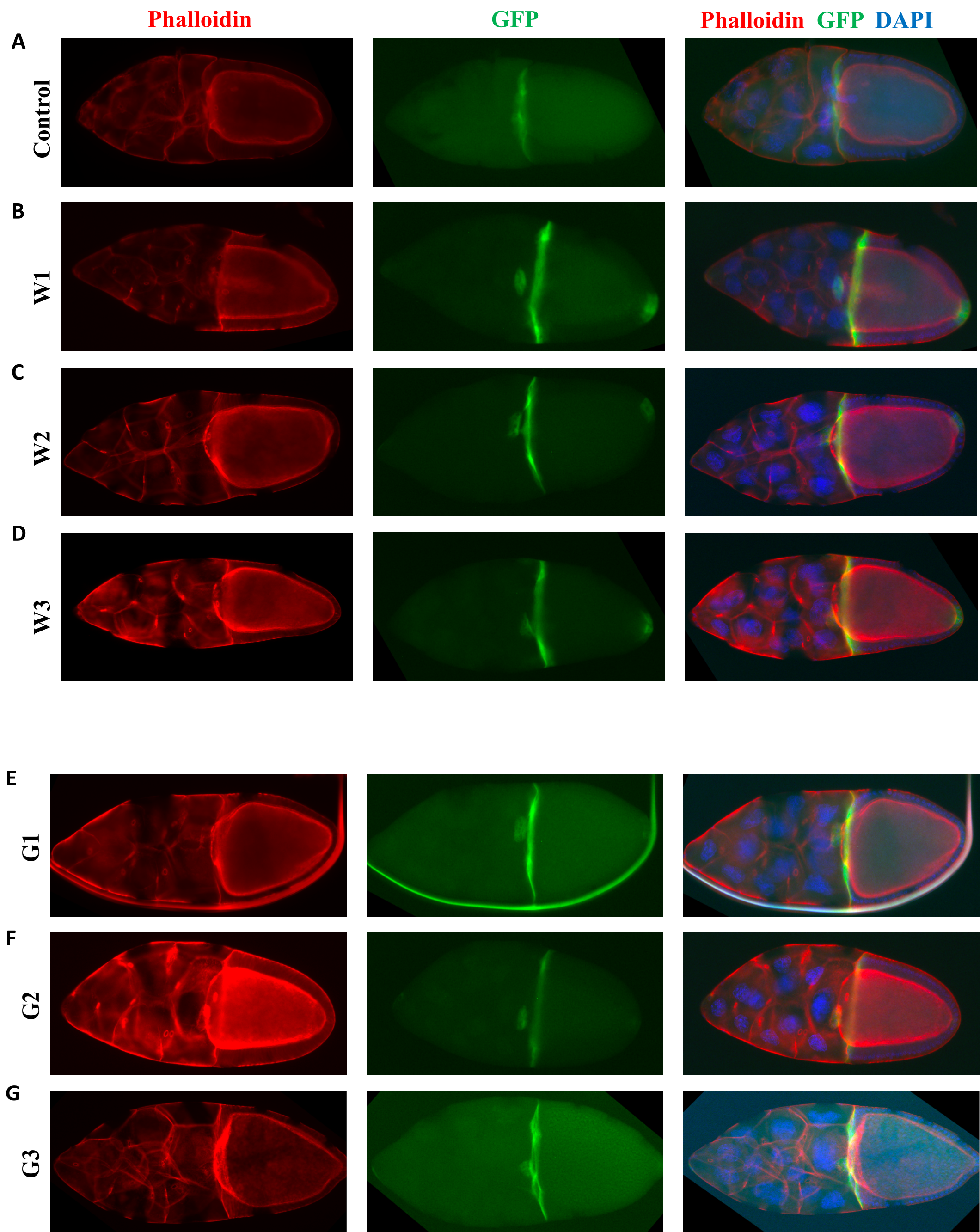

Figure 6: Treatment with walnut extract or green tea alters centripetal cell position without affecting border cell migration. Ovaries from Slbo-GAL4; UAS-GFP flies treated with control, walnut extract, or green tea yeast paste. GFP is expressed in the border cells and centripetal cells, and the tissue was stained with phalloidin (*red*) and DAPI *(blue*). W1 treatment contained 6mL walnut extract, yeast food, and no water. W2 treatment contained 3mL walnut extract, yeast food, and 3 mL water. W3 treatment contained 1mL walnut extract, yeast food, and 5mL water. All fluorescence images shown are of egg chamber in stage 10 of oogenesis. (E) G1 treatment contained 0.32 green tea extract, yeast food and 6mL water. (F)G2 treatment contained 0.16g green tea extract, yeast food and 6mLwater. (G)G3 treatment contained 0.08g green tea extract, yeast food and 6mL water. All fluorescence images shown are of egg chamber in stage 10 of oogenesis.

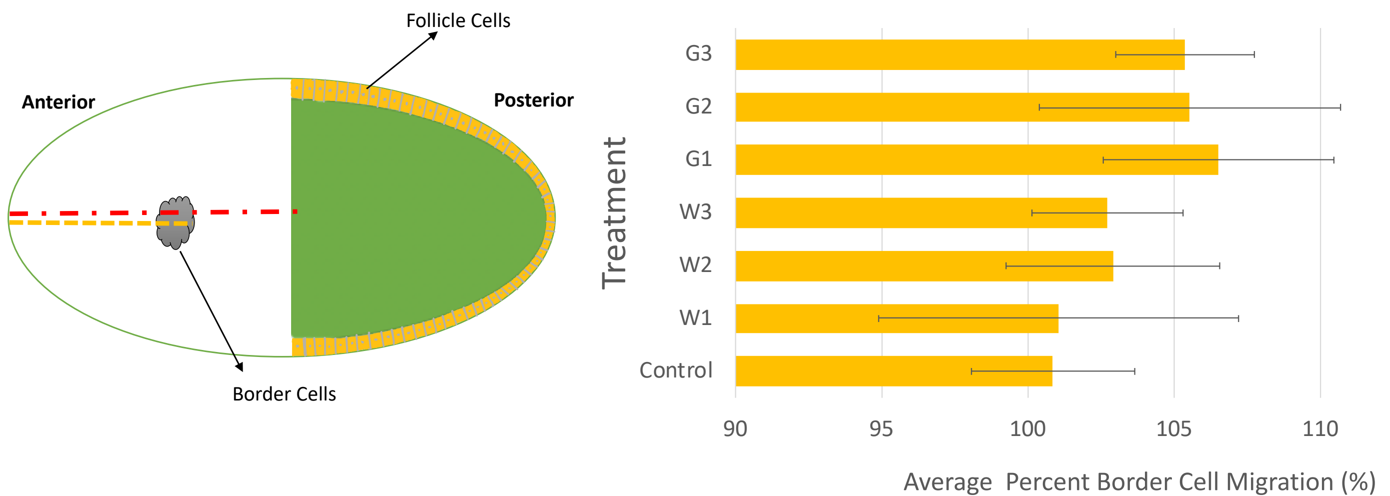

Figure 7: Average percent border cell migration. Border cell migration was determined by dividing the distance from the anterior of the egg chamber to the border cell cluster (yellow line) by the total distance from the anterior of the egg chamber to the anterior edge of the oocyte (red line). Sample size is 12 for each condition. Asterisk indicates significant difference compared to control; 2-tailed t-test performed; ($p < 0.05$). Distance was measured in units of µm. Error bars reflect standard deviation.

Effect on Centripetal Cell Migration

Although the two treatments tested did not reduce border cell migration, I also wanted to test whether there was an effect on centripetal cell migration. During the initial treatment with walnut extract, I observed that there was a change in the localization of the GFP-expressing centripetal cells compared to the controls. Normally, the centripetal cells created a vertical border between the oocyte and the nurse cell cluster. This localization was disrupted in the egg chambers derived from the walnut-treated flies (Fig. 8). By focusing on the area near the oocyte-nurse cell boundary, I observed some curvature defects in which the centripetal cells localize posterior to the oocyte boundary, and the oocyte boundary is more convex than in controls, which typically show a straight vertical boundary (Fig. 8). Increasing the concentration of walnut extract did not significantly alter this phenotype. This alteration in the centripetal cells suggests that walnut extract could be affecting centripetal cell migration.

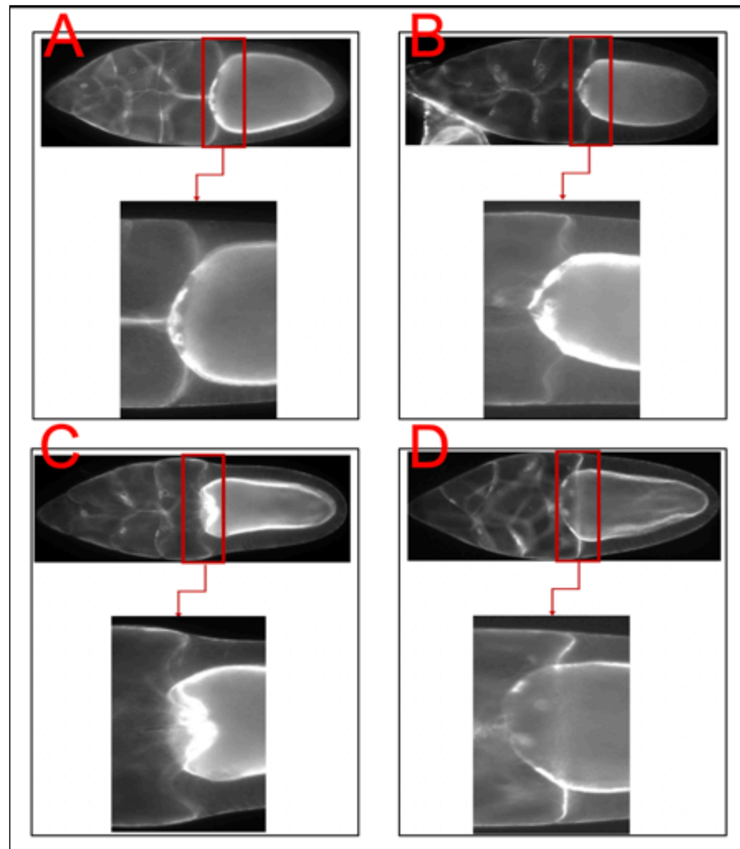

Figure 8: Preliminary treatment on how walnut extract affects centripetal cell migration. Stage 10 egg chambers from the control treatment (A), W1 treatment (B), W2 treatment (C), and W3 treatment (D) are shown. Egg chambers are stained with GFP, DAPI, and phalloidin. Zoomed-in panels show a specific area where the position of centripetal cells are altered. Centripetal cell layout in treatments W1, W2, and W3 differs from control treatment.

We repeated this walnut treatment and also tested whether the green tea extract would affect centripetal cell migration. We again saw a defect in the curvature and positioning of the centripetal cells at the nurse cell-oocyte boundary compared to the controls (Fig. 9). These findings suggest that the extracts could be causing a change in the migratory pattern or path of the centripetal cells during stage 10b of oogenesis, or a change in oocyte shape or structure that inhibits normal centripetal cell movement.

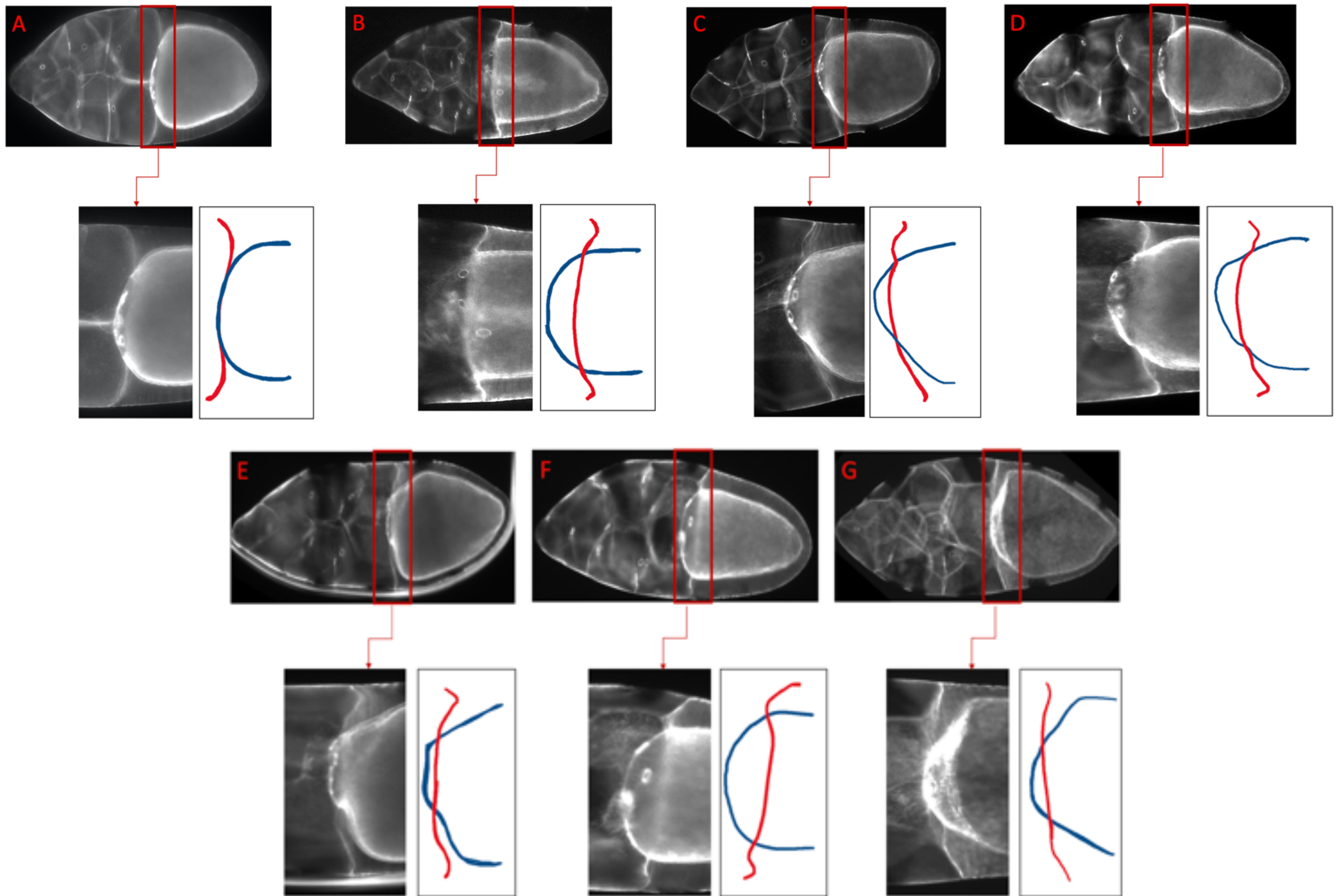

Figure 9: Walnut and green tea extract affects centripetal cell migration. Grayscale images of phalloidin staining of stage 10 egg chambers from the control (A), W1 (B), W2 (C), W3 (D), G1 (E), G2 (F), and G3 (G) treatment. Magnified view shows the region of the egg chamber where the centripetal cells migrate between the nurse cells and oocyte. Red and blue tracing images show centripetal cell boundary (red line) in reference to the oocyte (blue line).

One possible explanation for the altered centripetal cell position could be that these treatments change the oocyte size. To test this possibility, the ratio of the oocyte size was measured relative to the egg chamber size (expressed as a percentage). For the control treatment, the average percent ratio was about 45% and the percent ratio for the each of the extract treatments were within +/- 1% (Fig. 10). No significant difference was found between any of the treatments and the control (p-value > 0.5; Fig.10). These findings suggested the walnut or green tea treatments were not changing the size of the oocyte relative to the egg chamber

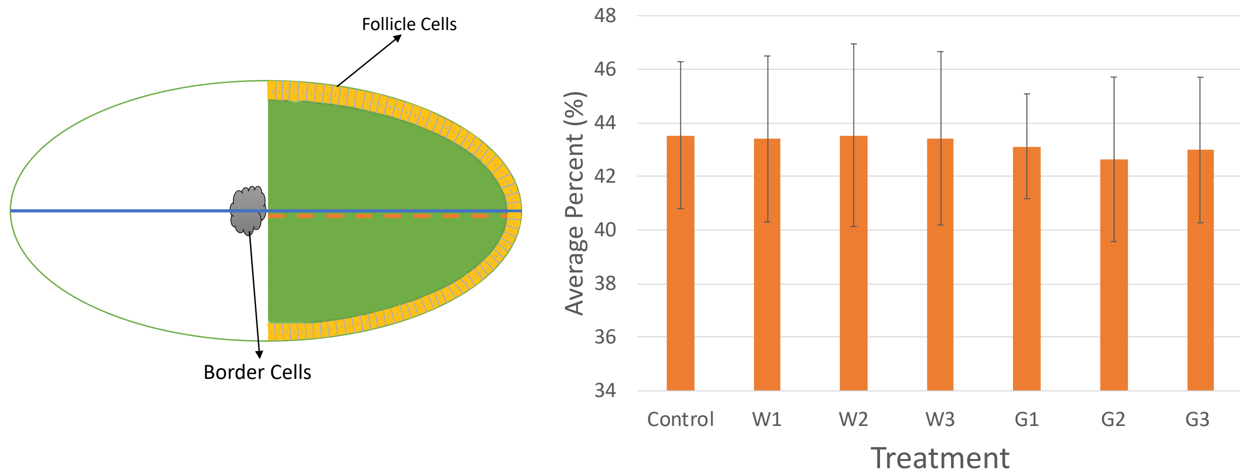

Figure 10: Average size of oocyte relative to total egg chamber length in stage 10 *Drosophila melanogaster* egg chambers. For a particular egg chamber, oocyte length (orange line) was divided by total egg chamber length (blue line) and then multiplied by 100. Sample size is 12 for each condition. Error bars are standard deviation. No significant difference was found between the treatments (2-tailed t-test).

Discussion

Our data partially supported our hypothesis that walnut and green tea extract would alter the invasive migratory processes in the developing egg chamber. There was no significant effect of either treatment on the process of border cell migration. However, we did see a modest effect on the alignment of the centripetal cells at the anterior end of the oocyte in both treatment conditions. While we did observe some changes in the migratory behavior of the centripetal cells, these differences were subtle. Additional experiments will be required in order to confirm these results.

Because we did not see an impact of either treatment on border cell migration, it suggests that green tea extract and walnut extract do not significantly impact any of the signaling pathways required for this behavior, at least at the concentrations used. We always saw that the border cells reached the oocyte by stage 10, but we did not do any analysis of the progression of border cell migration during stage 9. Therefore, it is possible that we could have missed a more subtle effect on the speed or migratory path taken by the border cells.

Our data did suggest that both treatments had a subtle yet consistent effect on centripetal cell migration or positioning. Although less it known about centripetal cell migration, Dpp, Notch, and JAK-STAT signaling pathways have been implicated in this behavior. Based on the results, we can propose multiple different mechanisms to explain the change in centripetal cell migration that we observe. (1) There is a defect in Dpp signaling that is causing the centripetal cells to not migrate correctly to separate the nurse cells from the oocyte. Specifically, Dpp signaling maintains *bun*'s gene expression, which is crucial for Notch, a signaling pathway that promotes the separation of centripetal cells during stage 10b of oogenesis (Wu et al. 2008). (2) There is a change in the Notch signaling pathway. During stage 10b, there is a buildup of Notch

in centripetal cells which promotes changes in the centripetal cells (Xu and Gridley 2012). It is possible that walnut or green tea extracts could impact the activity of this pathway. (3) There could be a defect in the binding of the VEGF related factor-1 to PVR which would be affecting the initiation of the JAK-STAT pathway and hence affecting the promotion of cell migratory behavior. (4) There could be a chance in DE-cadherin levels or stability. DE-cadherin mediates cell-cell adhesion and altered DE-cadherin levels has been shown to alter centripetal cell migration (Wu et al. 2008). (5) Finally, we could also imagine that this effect is caused indirectly by altering the structure of the oocyte. In both treatments, we found that the anterior boundary was not straight, but had a convex curvature. If both treatments alter the oocyte structure, then it is possible that this change could disrupt the ability of the centripetal cells to follow their normal migratory path.

As discussed earlier, anti-cancer drugs target the seven hallmarks of cancer which are displayed by cancer cells (Hanahan and Weinberg 2011). One of these abnormal behaviors is the ability of cancer cells to migrate and metastasize. Our results suggest that at least one type of invasive cell migration, centripetal cell migration, is reduced or altered following treatment with walnut or green tea extract. Although we did not assay for these effects, walnut or green tea could reduce cancer cell proliferation or induce apoptosis. Previous studies have shown that EGCG, an antioxidant found in green tea, has tumor suppressing properties (Hardman et al. 2019), and alpha-linolenic acid and beta-sitosterol, antioxidants from walnut extract, may also be able to reduce P13K/Akt kinase activity to decrease cancer cell survival (Fujiki et al. 2018). Therefore, it is possible that altering the diet to include these could target multiple aspects of cancer cell behavior.

Future Directions

To our knowledge, this is the first study that has looked at the effect of these extracts on border cell migration and centripetal cell migration. Therefore, many additional experiments must be performed in order to confirm these results and gain more insight into the specific pathways that could be altered. In addition, we did not have a straightforward way to quantify the changes in centripetal cell migration. Therefore, additional methods will need to be developed to strengthen these observations.

To further explore the centripetal cell phenotype, we could try to see how walnut extract or green tea extract affect pathways know to control migration. For example, future studies will focus on monitoring the activity of the Dpp, Notch, and JAK/STAT signaling pathways in control and treated egg chambers. We could also monitor DE-cadherin levels and distribution in both conditions compared to control. As mentioned previously, we could monitor border cell migration during stage 9 (to see if the progression of the border cell cluster aligns with the movement of the follicle cells towards the anterior, as it does in control). We could also test much higher concentrations of each of the extracts to see if we see a stronger phenotype or any block to cell proliferation or induction of apoptosis.

Once we have established a set of molecular tools (such as readouts for the indicated pathways) and analysis methods, we would like to test other natural extracts, such as black tea or papaya extract. We would like to see whether these extracts would have a stronger effect on these invasive cell behaviors.

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