Exploring the Activity of L1CAM and FGFR on Glioma Cell Migration Using Rat and Chick Embryo Models

by

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ACKNOWLEDGMENTS

I would like to dedicate this to Dr. Deni S. Galileo. In my life there are certain people that have made such a significant impact that words alone cannot explain. Dr. Galileo is one of them. I still remember the first time we met in his office. I was very nervous and lacked self-confidence. Here I was just an undergraduate student with one years worth of biology to my credit speaking with an experienced doctoral researcher. Dr. Galileo saw the meeting completely differently. He treated me as not just a student in his lab but a future collaborator. Right away he engaged me by asking me questions and inquiring about my opinions on experimental methods. This would be the start to a productive partnership.

What sets Dr. Galileo apart is his ability to mentor students using a "hands-off" approach. The degree of autonomy and self-responsibility that his methods require truly brings out the best in all of his students. He acts as an overseer, like a captain of a ship, making sure we are all headed in the right direction. In my two years with Dr. Galileo I have learned a great deal about brain tumors and the nervous system, however, I am proud to say I've learned even more about hard work, motivation, and responsibility. Dr. Galileo instills a sense of accountability in his students that lasts not just during the senior thesis process but also for a lifetime.

I could not imagine my collegiate career without Dr. Galileo's guidance. His recommendations have allowed me to attend prestigious conferences such as Experimental Biology in San Diego, C.A. and Cullen Trust MD/PhD Symposium at University of Texas and Baylor College of Medicine. He has also played a large role in my application process to medical school. During my interview at Jefferson Medical College, the faculty interviewer brought up a specific comment by Dr. Galileo. He was impressed with Dr. Galileo's remarks regarding my critical thinking skills and creativity. At that point I took a second to remember my mentor who instilled these qualities within me.

As I wrap up my senior year at University of Delaware, I can't help but think of all the people that have allowed me to accomplish my goals. Without hesitation I can say Dr. G is on the top of that list. I hope every student is fortunate enough to have a supportive mentor like Dr. Galileo in his or her careers. While I may be leaving his lab this spring, I know Dr. G will always remain in my life as my mentor and my friend.

I would also like to sincerely thank all my lab members, past and present: Dr. Murali Temburni, Vishnu Mohanan, Karma Pace, Michelle Pusey, and Ryan Hartman. They have all contributed to making the Galileo lab a supportive environment for academic research.

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LIST OF ABBREVIATIONS

GBM	Glioblastoma multiforme
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
ADAM	A Disintegrin and Metalloproteinase
MAP	Mitogen-activated protein kinase
CAM	Cell adhesion molecule
FN	Fibronectin
Ig	Immunoglobulin
NCAM-L1	Neural cell adhesion molecule L1
CNS	Central nervous system
BM	Brain metastasis
FAK	Focal adhesion kinase
dFGFR	Truncated FGFR
s.e.m	Standard error of the mean
shRNA	Short-hairpin RNA
mRNA	Messenger RNA
RNA	Ribonucleic acid
GFP	Green fluorescent protein
PS	Penicillin/Streptomycin

DMEM	Dulbecco's Modified Eagle's Medium
L1LE	L1 long ectodomain
FACS	Fluorescent-activated cell sorting (flow cytometry)
DNA	Deoxyribonucleic acid
FBS	Fetal bovine serum
PBS	Phosphate buffer saline
PNS	Peripheral nervous system
mAb	Monoclonal antibody
ECM	Extracellular matrix
TX-100	Triton X-100
μm	Micrometer
μg	Microgram
μl	Microliter
M	Molar
mM	Milimolar
μM	Micromolar
EDTA	Ethylenediaminetetraacetic acid

ABSTRACT

Glioblastoma multiforme (GBM), the most lethal of brain tumors, spreads rapidly in the brain. The mechanisms that promote extensive GBM cell migration are not completely known. The Galileo lab has shown that the neural recognition protein L1CAM (L1) is a key factor. It acts by being abnormally expressed by GBM cells, proteolyzed to release a large ectodomain fragment, and autocrine signaling through integrin receptors and fibroblast growth factor receptors. While autocrine stimulation by L1 has been shown to increase glioma cell migration and proliferation, there may be other extrinsic influences at play that promote glioma aggressiveness. Brain cells, or more specifically astrocytes, the most abundant cell in the human brain, also may stimulate GBM cells. In order to determine relative contributions of mixed brain cells or purified astrocytes to glioma cell motility, time-lapse experiments of co-cultured GFP-expressing T98G human glioma cells and chick embryo brain cells were performed. Results showed that chick brain cells may further stimulate glioma cells. Astrocytes also were isolated from primary rat brain tissue and plated as a monolayer for co-cultured glioma cells to interact with. The effect of cell-to-cell contact between astrocytes and glioma cells was inconclusive due to astrocyte sensitivity to the applied methods. L1-FGFR interaction was seen through double immunostaining. Results showed that L1 and FGFR do in fact act as binding partners but also separately to

other molecules and receptors. Furthermore, T98G-dFGFR cells were injected into chick optic tectum to observe the effect of FGFR *in vivo.* Results showed that glioma cells lacking FGFR were minimally invasive compared to the control (T98G-2605) that showed invasive character. In a separate experiment, media was extracted from astrocyte and L1- expressing tumor cell line (U118-L1LE) and placed in cell scratches of cells lacking L1 (T98G-shL1), cells lacking FGFR (T98G-FGFR), and cells expressing L1 (T98G). The cell velocities were recorded using time-lapse microscopy. In addition, cell proliferation rates were determined through cell cycle analysis using propidium iodide staining and FACS. Results showed that paracrine stimulation by astrocytes can stimulate glioma cell motility and proliferation to near autocrine stimulation levels in the absence of L1. This suggests that Astrocytes, via paracrine stimulation, may have other factors or molecules that stimulate glioma cell motility through mechanisms other than FGFR receptors. It was also found that L1 autocrine signaling is the biggest source of stimulation for normal glioma cells.

Chapter 1

INTRODUCTION

1.1 The Central Nervous System

The central nervous system (CNS), while highly complex is mostly made up of two types of cells: neurons and glia [1]. The complexity can be attributed to the diverse subtypes of neurons and glia and how they are connected. Neurons are present in variety of shapes and sizes, each corresponding to their function within the brain. While neurons are most often found in groups known as ganglia in the peripheral nervous system (PNS), they are mostly present as layers (laminae) in the CNS. Glia are the support cells and are responsible for carrying out a variety of functions including structural support, homeostasis, insulation, providing nutrients, and removing pathogens. Together, neurons and glia carry out the main functions of the nervous system: signaling between various parts of the body and performing both voluntary and involuntary actions.

The most abundant glial cells in the brain are astrocytes consisting of about 50% of entire brain mass. When viewed under a microscope, astrocytes appear star shaped, allowing them to be easily identified. They play a major role in providing nutrients to neurons, helping to maintain blood-brain barrier, and in nervous system repair. A tumor that is derived from astrocytes is referred to as astrocytoma. Astrocytomas may be low or high grade (grades I-IV), with low appearing in children and high in adults [2].

1.2 Glioblastoma Multiforme

The most malignant form of cancer associated with the central nervous system is a high-grade astrocytoma called Glioblastoma Multiforme (GBM). GBM can consist of many different cell types including astrocytes and oligodendrocytes [3]. Cancers are rated according to their invasiveness and lethality. Nearly half of all astrocytomas are glioblastomas [4]. GBM is of the highest category, recognized as a grade IV tumor. The end result of GBM is hemorrhaging and necrosis in the brain (Figure 1).

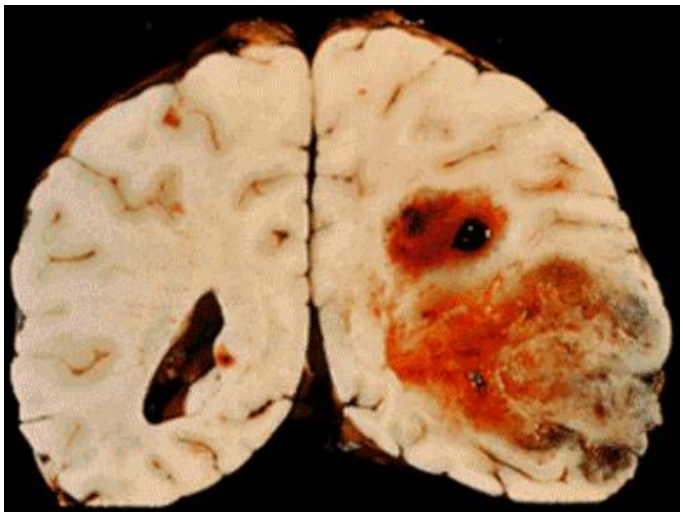

Figure 1. Anatomy of Glioblastoma Multiforme.

Shown is post-mortem, section of a cortex of a patient's brain. The red areas indicate hemorrhage while the darker areas indicate necrosis, or collection of dead cells.
Source: http://pathweb.uchc.edu/eATlas/CNS/237.htm

Glioblastoma Multiforme currently has no successful treatment. The primary treatment is surgery along with some form of radiation and chemotherapy [6]. Due to

the tumor cell's migratory properties, it is able to escape resection and more often than not is able to regrow. Even with a combination of these treatment methods the median survival time is no longer than 12 months. Less than 4% of patients diagnosed with GBM survive 5 years. The lethality of this cancer requires more insight into its invasive qualities, which is responsible for this lethality.

1.3 L1 Cell Adhesion Molecule

L1 cell adhesion molecule (L1CAM) is a molecule that is highly expressed in the developing nervous system and tumors of the nervous system [16][17][18][20][31]. L1 is a transmembrane glycoprotein that consists of 6 Ig domains and 5 fibronectin like domains (Figure 2). L1CAM is generally seen at about 220 kDa on western blots but also exists in cleaved (proteolyzed) forms. It is involved in many cell-to-cell interactions. L1 is both homophillic and heterophillic; it can bind to itself and other molecules to provide adhesion between cells. In developing brains, L1 is responsible for neuron migration and can be found generally on the surface of axon growth cones [3]. During brain development, L1 serves as a key factor in guidance of axons to their proper target regions [4]. L1CAM may also be cleaved by proteases such as ADAM10, which allow it to be present in the extra cellular matrix (ECM) in a smaller form [36]. As a cleaved protein, L1 can then serve both an adhesion molecule and signaling factor [44]. In general, L1 is found in three different forms; it can be cell surface, cleaved and present as extracellular matrix protein, or on the surface of released exosome vesicles.

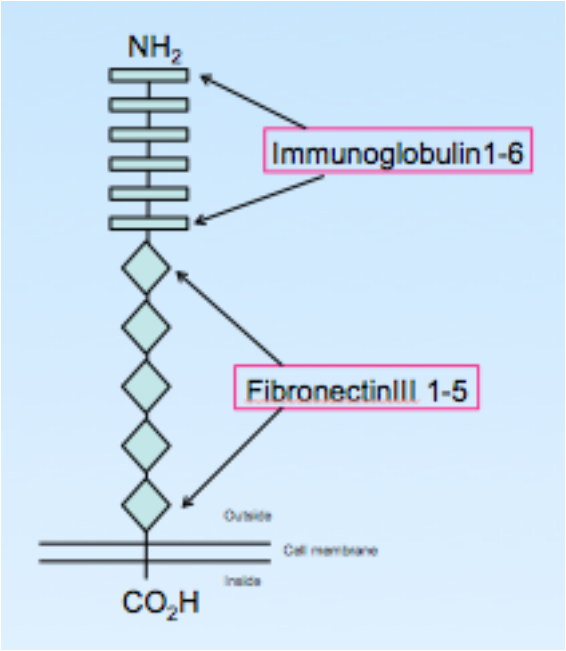

Figure 2. Schematic Diagram of L1CAM. L1 consists of five fibronectin domains and six immunoglobulin domains.

In addition to binding to itself, L1 also can also bind to other receptors including integrins and ECM molecules such as laminin. When bound to these partners, L1 heterodimerizes to an active form. It has been shown that L1 acts through MAP kinase (MAPK) pathways. Inhibition of Focal Adhesion Kinase (FAK) has shown to decrease the effect of L1. Thus, one way L1 promotes its action is through intracellular FAK pathway [46].

1.4 Fibroblast Growth Factor Receptor

An important binding partner for L1 is fibroblast growth factor receptor (FGFR). FGFR is a tyrosine kinase receptor found on the surface of many cell types including GBM cells. It is activated via dimerization and cross phosphorylation. In addition to L1, FGFR has a canonical ligand known as fibroblast growth factor (FGF). It has been found that when FGFR is shut down through a dominant-negative approach, glioma cell migration is decreased [20]. This signals the importance of L1-FGFR binding for glioma cell progression. FGFRs can occur in seven different variants. FGFR1 is found on neurons where as FGFR2 and FGFR3 are normally seen on glia and glioma. FGFR4 is only seen during the early developmental period. When activated, FGFR is involved in signaling for survival, migration, and proliferation.

1.5 Role of L1CAM in Cancer

L1CAM has a prominent role in many cancers of the body. In many cases, tests are conducted to detect the presence of L1 as a diagnostic marker for cancer progression [33]. Over expression of L1 is found in brain, ovarian, renal, breast, and other cancers as well [11][30][31][34][35]. Due to its presence as a cell surface protein, soluble protein, and exosomal surface protein, L1 can signal in a variety of ways that allow for cancer progression. The Galileo lab has shown that shutting down L1 results in significant decrease (~62%) of glioma cell motility [44].

1.6 Autocrine vs. Paracrine Stimulation

As mentioned above, L1 is found in three different forms. It can be present as a transmembrane protein with an extracellular and cytosolic domain. It can be cleaved

by proteases such as ADAM10, which allow it to be present as an extracellular matrix molecule. It can also be expressed on the surface of released extracellular vesicles. All three of these forms of L1 stimulate glioma cell motility. This type of stimulation is technically referenced as autocrine/paracrine signaling. However since the glioma cell produces all forms of L1, this type of stimulatory effect will be referred to as autocrine stimulation in this study (Figure 3). Another potential source of stimulation is from nearby, normal brain cells. In addition to the tumor cells there are other supporting cells, such as astrocytes, that may stimulate glioma cell motility via either released soluble factors or cell to cell contact. This type of stimulatory effect is referred to as paracrine stimulation because the source is a nearby external cell.

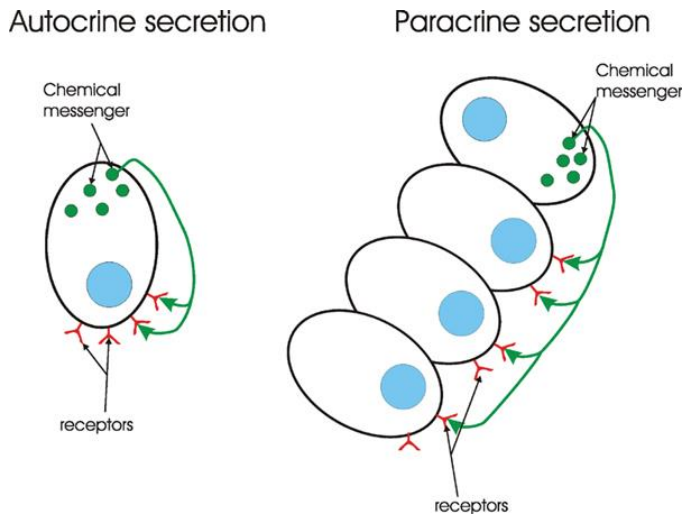

Figure 3. Autocrine vs. Paracrine Stimulation for Glioma Cell Motility

1.7 Research Goals

The overarching goal of this study is to further explore the effects of L1 and FGFR in glioma cell migration by using chick and rat brain models. In particular, the aim is to determine the relative contribution of autocrine vs. paracrine signaling to glioma cell motility. Autocrine stimulation is the intracellular signaling produced by the glioma cells (e.g., by L1 expression and cleavage). Paracrine stimulation is potentially the signaling from surrounding normal cells such as astrocytes. Surprisingly, no work has been done thus far to measure or elucidate the effects that supporting cells may have on glioma progression. If normal brain cells are contributing to glioma cell motility then additional or alternative clinical strategies will be required to combat their stimulation. The molecule or molecules behind the paracrine stimulation will need to be identified so that a strategy can be devised to counteract it.

1.8 Hypothesis and Specific Aims

My hypothesis is that although L1 autocrine stimulation by glioma cells results in greater cell motility and proliferation, there is a significant and measureable paracrine stimulation of glioma cells by normal astrocytes.

- Aim 1: Determine the extent of autocrine vs. paracrine stimulation on glioma cell motility and proliferation

- Aim 2: Determine the difference in stimulation between chick vs. rat animal model for glioma-brain cell co-cultures

- Aim 3: Visualize L1-FGFR cell surface interaction

- Aim 4: Determine the effect of dominant-negative FGFR (dFGFR) expression in glioma cells on tumor migration *in vivo*

Chapter 2

Materials and Methods

2.1 Cell Culture Techniques

The cell lines used in this project include human T98G and U118 MG [20]. Modified cell lines used were T98G-shL1, T98G-pLKO.1, T98G-dFGFR, T98G-2605, U118-L1LE (Table 1). In addition, cultured primary brain cells were isolated from live chick (embryonic day 7) and rat brain tissue separately to grow chick brain monolayers and rat astrocytes. The culture medium for tumor cells and rat astrocytes was DMEM, 10% FBS, 2 mM L-glutamine, and penicillin-streptomycin. For experimentation, the amount of FBS was reduced to 0.5%. Cells with introduced vectors were selected for once a month using small amounts of puromyocin (5 μg/ml).

Experimental Cell Line	Control Cell Line
T98G-shL1 (L1 attenuated)	T98G-pLKO.1 (L1 expressing)
T98G-dFGFR (FGFR-, L1 expressing)	T98G-2605 (FGFR+, L1 expressing)
U118-L1LE (L1 expressing)	U118-1879 (L1-)

Table 1. Cell lines created from lentiviral vector infection

2.2 Monolayer Culture Preparation

Embryonic day 7 chick optic tectum (OT) was dissected in sterile conditions (Figure 4). The dissected OTs were placed in small petri dish and minced with scissors. The minced OT were transferred to 15mL Falcon Tube and centrifuged at 800 rpm for 5 minutes. 2 mL of 0.25% trypsin was added and the tube was vortexed. The tube was then incubated at 37°C for 20 minutes. 2 mL of Medium 199 (M199) with soybean trypsin inhibitor and DNaseI was added and the tube was allowed to sit on ice for 5 minutes. The tube was again centrifuged at 800 rpm for 5 minutes. The sample was resuspended in 2mL DNaseI. Cells were counted at this time using hemocytometer. The desired concentration was 1.2 $x10^7$ cells/mL in the stock suspension. The cells were suspended in M199, 10% FBS, and penicillin-streptomycin. They were then plated on tissue culture dishes and incubated in 37°C.

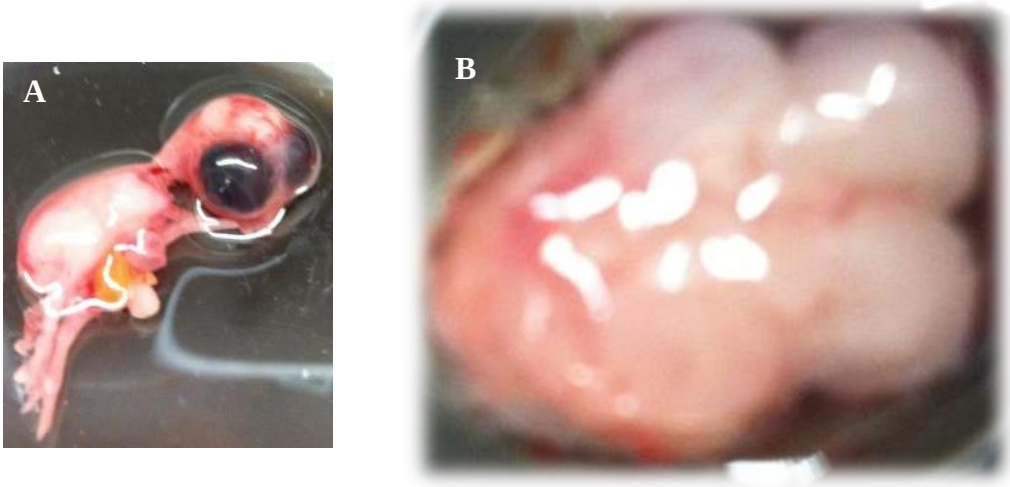

Figure 4. Chick Embryo Dissections. Embryonic day 7 chick is surgically removed from its shell (A). The brain is further dissected to obtain the target OT regions (B).

2.3 Cell Co-Cultures

Both chick brain and rat astrocyte co-culture experiments were set up analogously. In both cases, 6 well dishes were first plated with primary chick brain or rat astrocyte cells to form monolayers. In rat astrocyte experiments, an initial scratch was made on the primary monolayer to allow glioma cells to settle on the plastic and observe the interaction with astrocytes. After several days of growth, the appropriate concentrations of glioma cells were added (~$2.5x10^4$ cells/ml). The glioma cells were allowed to settle on top of the primary monolayers for 1-2 hours then prepared for time-lapse microscopy.

2.4 Antibodies

The following are the antibodies that were used for double immunostaining of L1-FGFR. UJ127 (cat. # GTX72362; Gene Tex), a mouse monoclonal antibody, was used against human L1. It targets the fibronectin repeats present near the surface of the membrane. To bind FGFR1, anti-FGFR (cat. # 13-3100; Invitrogen) was used. It is a mouse monoclonal antibody that binds to human FGFR1.

2.5 Immunostaining

Cells were grown on coverslips in 24 well dishes and fixed using 1% formaldehyde in PBS for 30 minutes at room temperature. After fixation, the coverslips were rinsed three times in PBS. Cells were then incubated with diluted (1/500) primary antibody containing 5% normal goat serum and 0.03% Triton X-100 for an hour at room temperature. Afterwards, the coverslips were rinsed three times with PBS and incubated with the appropriate secondary antibody for 1 hour at room temperature. The dilutions for secondary antibodies were 1/200. For co-staining, L1 primary and secondary antibody staining was conducted first followed by primary and

secondary for FGFR. The coverslips were again rinsed three times with PBS and mounted using 6 μl buffered glycerol on glass slides. The fixed cell slides were examined using Nikon Microphot-FX microscope with Fluorescence objectives.

2.6 Collection of Conditioned medium

L1 ectodomain (L1LE) was collected by using media from the U118-L1LE cell line [20]. These cells actively produce and secrete L1 into the culture medium. At about 2/3 confluence, the media is replaced by 10ml of DMEM with 0.5% FBS in 2mM L-glut, and penicillin-streptomycin. The media is collected and filtered through 0.20 μm filters after 24 hours. Astrocyte conditioned media was collected from a confluent monolayer of rat astrocytes. The same filtering process was used for astrocyte-conditioned medium.

2.7 Cell Cycle Analysis

Cells were first prepared for fixation before analysis. Cells were washed with PBS and treated with 0.05% trypsin for 5 minutes at 37°C. The cells were then transferred to a 15ml tube along with 2ml Soybean trypsin inhibitor/DNAseI. The tubes were centrifuged at 800 rpm for 5 minutes at room temperature. The pellets were resuspended in 0.5 ml PBS and 4.5 ml of 70% ethanol. The tubes were placed in -20°C and stored for a minimum of 3 days. On the day of cell cycle analysis, cells were centrifuged at 800 rpm for 5 minutes. The cells were resuspended in 5ml of PBS and centrifuged at 800 rpm for an additional 5 minutes. Cells were then suspended in 1 ml DNA staining solution. The DNA staining solution was comprised of 200μg/ml of DNAse free RNAse A and 20μg/ml Propidium Iodide in PBS. The cells were then incubated in the dark for 30 minutes and then transferred to filter top FACS tubes.

The cells were then analyzed via Becton Dickinson FACSCalibur Flow Cytometer. ModFit software was used to analyze the specific cell cycle stages of the different cells.

2.8 Time-lapse microscopy for Cell Motility

Cells first were grown to confluence in the appropriate size dish (6-or 12-well dishes). Two horizontal scratches were made near the center of the wells by using 1ml pipette tips. The cells were rinsed three times in PBS to remove the cells that had been lifted. For time-lapse microscopy, DMEM with 0.5% FBS, 2mM L-glutamine, and penicillin-streptomycin was added. After 2-3 hours, the scratches had stabilized and a starting point was established. The dish was then sealed with vaseline to seal the cover and coated on the bottom with rubber cement to keep it from moving within the apparatus. The dish was then transferred to custom culture chamber on ProScan II automated stage (Figure 5). The temperature was maintained at a constant 37^{o}C. The air was maintained at a concentration of 5%CO_2/95% air. MetaMorph Premier software (Molecular Dyanmics Corp.) was used to operate the time-lapse microscope. A CoolSnap ES CCD camera was used to capture images during the experiment at specific intervals over certain duration. A 20x Nikon Plan Fluor ELWD objective was used in this study. Phase contrast images were taken every 10 minutes for 24-hour period.

Figure 5. Features of the automated Time-lapse microscopy system.

1) Fully automated Nikon TE-2000E with epifluorescence, 2) incubator chamber, 3) WPI temperature controller, 4) Tokai Hit stage insert warmer controller, 5) Prior ProScan II flat-top automated stage, 6) Prior stage controller, 7) Photometrics CoolSNAP ES CCD camera, 8) custom 3GHz computer with 2 gigabytes of RAM, 2 hard drives, dual monitors, and MetaMorph Premier software, 9) uninterruptible power supply capable of running entire system. Out of view is CO2 injection system connected to incubator chamber via tubing [21].

2.9 Cell Motility Analysis

The "Track Points" application of the MetaMorph software was used to quantitate the cell motility data obtained from time-lapse microscopy videos. 15 cells per well from three wells were analyzed per condition and tracked using track points. Three different wells were used per condition for a total of 45 cells analyzed. The path of travel of each individual cell was traced by following cell nuclei through the

series of micrographs. The software recorded several variables including velocity, distance, and time. These values were then exported to an Excel spreadsheet to analyze the average velocities. The values were converted from pixels to microns using appropriate conversion factors.

2.10 Embryonic Chick Brain Microinjection

Fertile White Leghorn chicken embryos were obtained from University of Delaware Department of Animal and Food Sciences. 12-20 eggs were placed in a humidified incubator at 37.5 ^{o}C. The eggs were ready for brain microinjections on embryonic day 5 (E5). About 2.5 x 10^4 cells/ml were stained green using the fluorescent vital membrane dye Vybrant DiO (Molecular Probes) and injected directly into the chick optic tectum (midbrain). In order to inject the cells, a drop of Medium 199 is added and a thin membrane is removed to access the chick embryo. The injections were performed using PV830 pneumatic picopump (World Precision Instruments; Sarasota, FL) and a glass micropipet. Once the injection was completed, 1-2 drops of 10mg/ml ampicillin was added and the eggshell was covered with transparent tape. The embryos were kept in the incubator until embryonic day 9 (E9). At this time the embryos were sacrificed so that the brains could be dissected. Once the brain was removed, the optic tectum (the site of the injections) was isolated. The tectum was then fixed in 2% paraformaldehyde in PBS over night.

2.11 Vibratome Sectioning

A Vibratome Series 1000 Sectioning system was used for sectioning midbrains embedded in 3.5% agar and 8% sucrose in PBS. The embedded midbrains were glued to a stable base with super glue and then placed into the PBS bath on the vibratome for

sectioning. Regular dual edge razor blades were used to cut 200um thin slices. These slices were then mounted on slides in glycerol and coverslipped and examined under fluorescence microscope.

2.12 Statistical Methods

For conditioned media experiments, the average of three repeats is reported with +/- SEM. In order to compare two different conditions, Student's t-test was conducted to determine significance. A difference of * or #, $P<0.05$ was considered as significant.

Chapter 3

Results

3.1 Conditioned Media Time-Lapse Experiments

3.1.1 Paracrine stimulation by astrocytes results in increased glioma cell migration when L1 is not expressed

Glioma cells with L1 attenuated (T98G-shL1) were plated and grown to confluency. Three different types of media conditions were used: L1LE (from U118-L1LE grown in DMEM with 0.5% FBS); Astrocyte (from primary rat astrocytes grown in DMEM, 0.5% FBS); Control (DMEM, 0.5% FBS). The average velocities of the three spots analyzed per condition (N=45 cells) were calculated and used to plot the motility graph. As shown in Figure 6, T98G-shL1 cells treated with L1LE media had an average velocity of 0.115 microns/second; Astrocyte conditioned media had an average velocity of 0.113 microns/second; the control media had an average velocity of 0.017 microns/second. Both autocrine (via L1LE) and paracrine (via astrocytes) signaling resulted in increased glioma cell motility compared to the control (*,$p << 0.001$ in comparison with the velocity of control cell).

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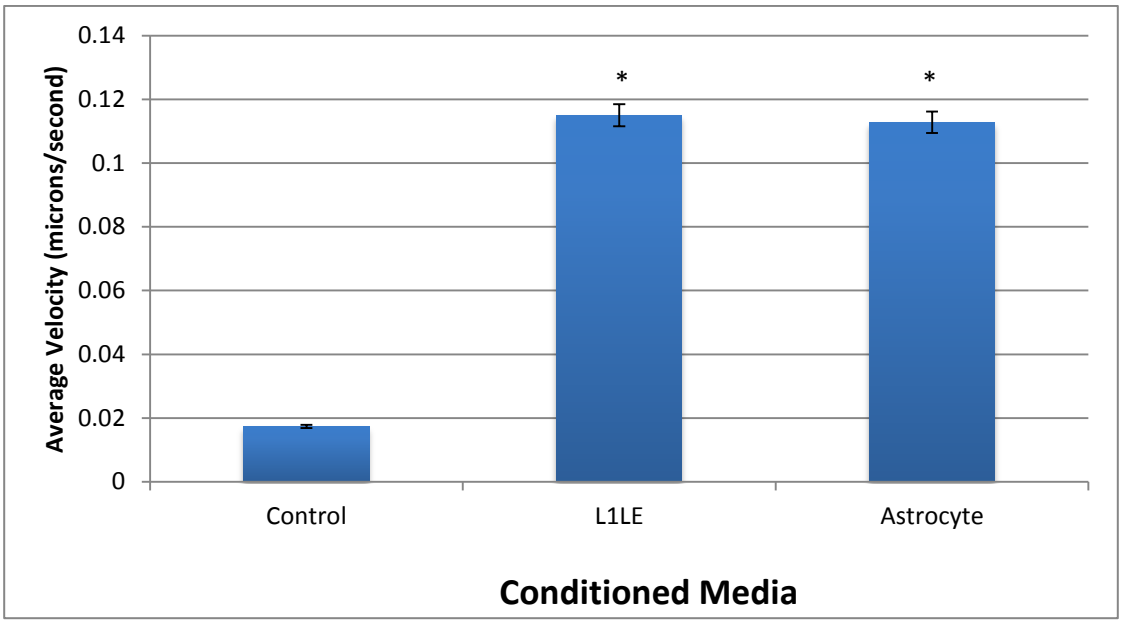


Figure 6. Conditioned Media results of T98G-shL1 glioma cells treated with control, L1LE, and Astrocyte Conditioned Media.

3.1.2 In the presence of L1 autocrine stimulation, neither excess autocrine stimulation nor paracrine stimulation results in increased glioma cell motility

Glioma cells expressing L1 (T98G) were plated and grown to confluency. Three different types of media conditions were used: L1LE (from U118-L1LE grown in DMEM, 0.5% FBS); Astrocyte (from primary rat astrocytes grown in DMEM, 0.5% FBS); Control (DMEM, 0.5% FBS). The average velocities of the three spots (N=45 cells) analyzed per condition were calculated and used to plot the motility graph. As shown in Figure 7, T98G cells treated with L1LE media had an average velocity of 0.161 microns/second; Astrocyte conditioned media had an average velocity of 0.160 microns/second; the control media had an average velocity of 0.162 microns/second. Both autocrine (via L1LE) and paracrine (via astrocytes) signaling did not result in increased glioma cell motility compared to the control. Compared to Figure 6, Figure

7 shows the effects of adding L1LE and astrocyte conditioned medium to a cell line that already produces and secretes its own L1. There was no additional stimulation observed.

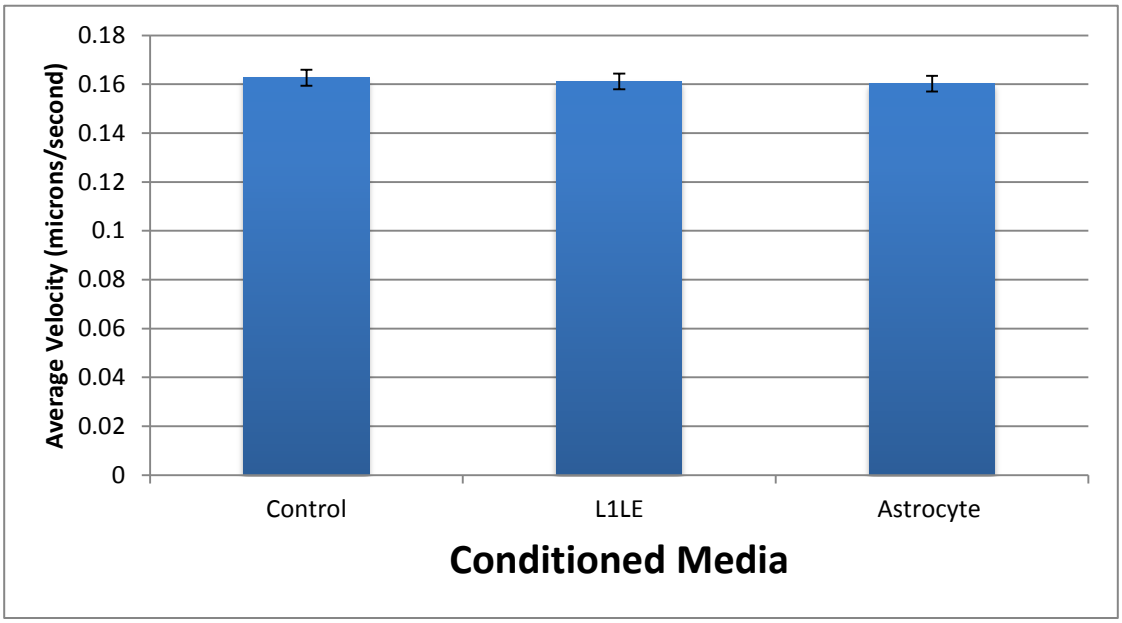


Figure 7. Conditioned Media results of T98G glioma cells treated with Control, L1LE, and Astrocyte Conditioned Media.

3.1.3 Paracrine stimulation from astrocytes results in an increase in glioma cell motility

Glioma cells lacking FGFR function (T98G-dFGFR) were plated and grown to confluency. Three different types of media conditions were used: L1LE (from U118-L1LE grown in DMEM, 0.5% FBS); Astrocyte (from primary rat astrocytes grown in DMEM, 0.5% FBS); Control (DMEM, 0.5% FBS). The average velocities of the three spots analyzed (N=45 cells) per condition were calculated and used to plot

the motility graph. As shown in Figure 8, T98G-dFGFR cells treated with L1LE media had an average velocity of 0.065 microns/second; Astrocyte conditioned media had an average velocity of 0.086 microns/second; the control media had an average velocity of 0.056 microns/second. Both autocrine (via L1LE) and paracrine (via astrocytes) signaling resulted in increased glioma cell motility compared to the control (*,p<<0.001 in comparison with the velocity of control cell), however the paracrine signaling effect by astrocytes was greater.

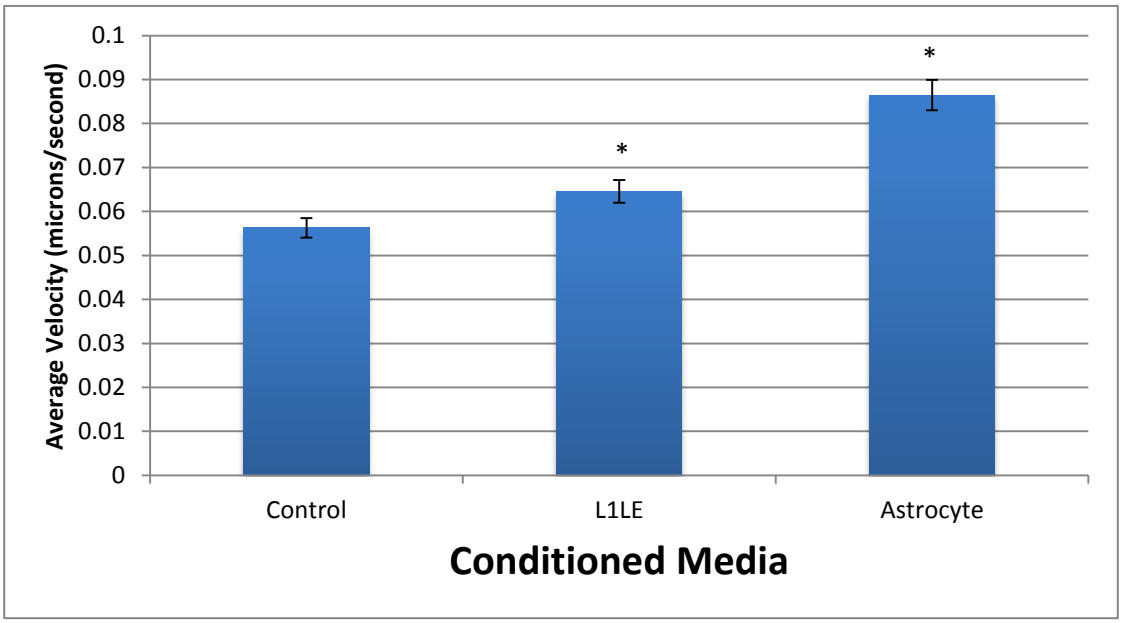
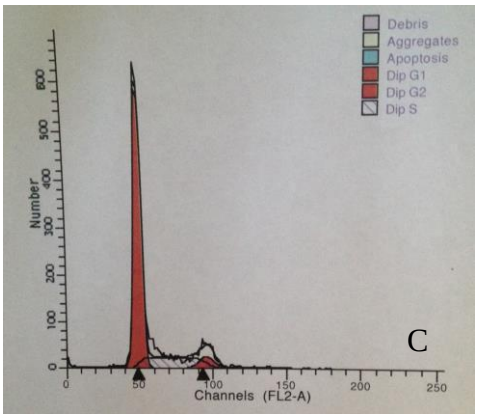


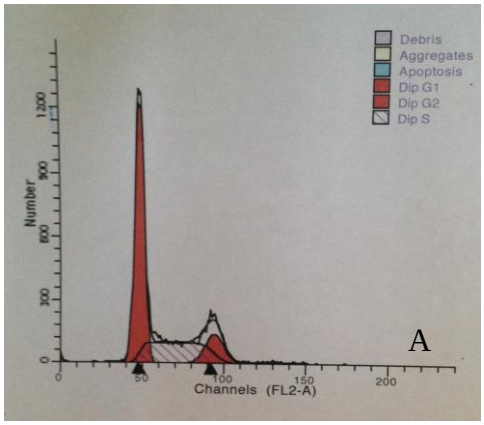
Figure 8. Conditioned Media results of T98G-dFGFR glioma cells treated with Control, L1LE, and Astrocyte Conditioned Media.

3.2 Conditioned Media Cell Cycle Experiments

3.2.1 FACS analysis to determine the cell cycle of T98G-shL1 cells treated with L1LE, Astrocyte, and Control Media

Cells were grown in DMEM with 0.5% FBS and later trypsinized, fixed, then stained with propidium iodide. Three samples (~50,000 cells each) per condition were analyzed using FACS to determine the cell DNA content. The data obtained was then analyzed using ModFit software that converts DNA content into distinct cell cycle phases (Figure 9). The percentage of cells in S phase was used for determining the extent of proliferation. T98G-shL1 with L1LE media had an S phase percentage of 29.0± 0.86% (Figure 9A); with astrocyte media had an S phase percentage of 28.12± 0.18% (Figure 9B); and with control media had an S phase percentage of 21.6± 0.65% (Figure 9C, Tabulated results for all phases of the cell cycle are shown in Table 2). Thus, both L1LE- and Astrocyte-conditioned media resulted in increased proliferation of T98G cells that lacked L1 expression (*,$p << 0.001$ in comparison with the S% of control cell).





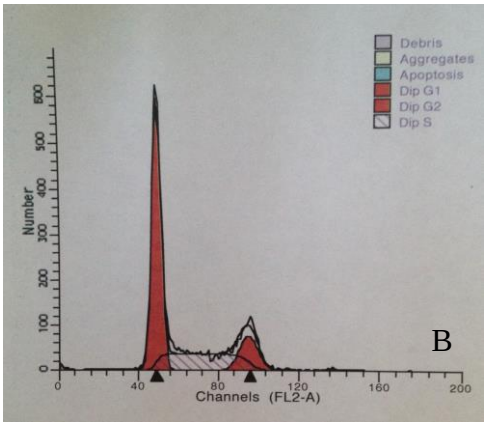


Figure 9. Cell Cycle Analysis results of T98G-shL1 cells treated with Control (top), L1LE (bottom, right), and Astrocyte (bottom, left) Conditioned Media.

Media Condition	G0 G1%	S%	G2 + M%
Control Media	72.18 ± 0.57	21.6 ± 0.65	6.22 ± 0.07
L1LE Media	58.02 ± 0.83	*29.0 ± 0.86	12.98 ± 0.24
Astrocyte Media	56.7 ± 0.05	*28.12 ± 0.18	15.18 ± 0.24

Table 2. Percentage of Cells at Different Cell Cycle Stages of T98G-shL1 glioma cells treated with control, L1LE, and astrocyte conditioned media.

(*,$p << 0.001$ in comparison with the S% of control cell).

3.3 Chick Brain vs. Rat Brain Monolayer Co-Cultures

3.3.1 L1 expression by glioma cells increases cell motility on E7 chick OT monolayers

Primary chick optic tectum cells were isolated and cultured from day 7 embryos. Confluent monolayers were established in about a week. T98G-pLKO.1 and T98G-shL1 tumor cell lines labeled with DiO and added on top of the monolayer separately at a density of $2.5x10^4$ cells/ml in a 35 mm well of a 6-well plate (Figure 10 and 11). The tumor cells were allowed to settle and attach for two hours before the onset of time-lapse microscopy. Two types of cell interactions were observed. There were instances where the GFP labeled glioma cells gently rested on top of the monolayer and moved at random. These cells were not tracked because they had not attached to the monolayer of brain cells. In other instances, it was noticed that the glioma cells would attach, spread, and start to actively move on the monolayer. These were the cells that were tracked. T98G-pLKO.1 cells had an average velocity of 0.336 microns/minute, which is quite rapid (Figure 12). T98G-shL1 cells had a reduced average velocity of 0.109 microns/minute. There was a significant 62% decrease in the cell motility of T98G cells that lacked L1 (*,$p<<0.001$ in comparison with the velocity of control cells). In addition, there was no observable detrimental effect of blue light illumination on chick brain or T98G cells.

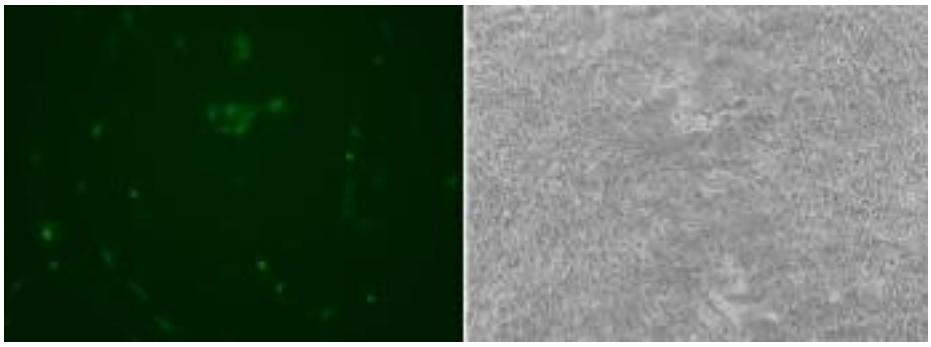


Figure 10. Fluorescent and Phase images of T98G-pLKO.1 and chick brain monolayer co-cultures.

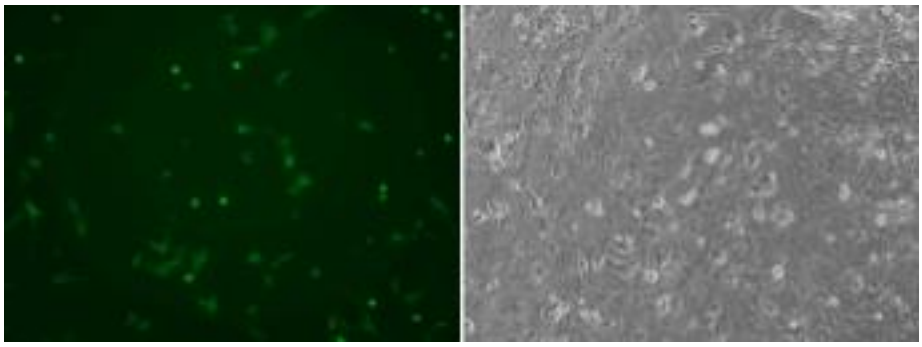


Figure 11. Fluorescent and Phase images of T98G-shL1 and chick brain monolayer co-cultures

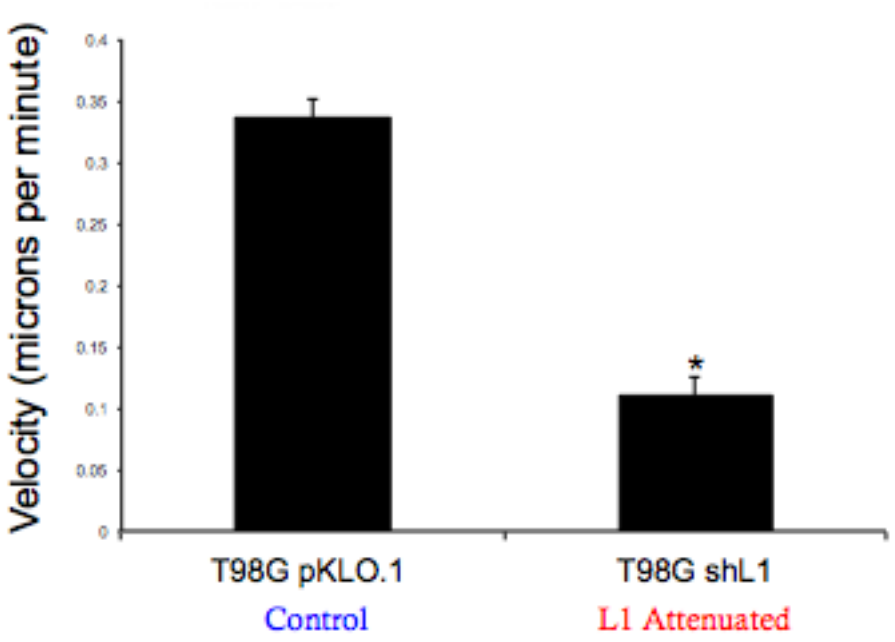


Figure 12. Average Velocity of Glioma Cells on E7 Chick OT Monolayers

3.3.2 Rat Astrocytes are sensitive to blue light irradiation

Rat brains were obtained and further dissected to isolate the cerebrum. After further dissociation and trypsin treatment, the single rat brain cells were plated in a tissue culture dish. The brain cells were then trypsinized and replated on a glass dish. Rat astrocytes adhered faster to the glass substratum than other rat brain cells. The astrocytes were allowed to settle overnight. The media containing the other rat brain cells was removed and fresh media was added. These rat astrocytes were then plated in 6 well dishes along with T98G-pLKO.1 and T98G-shL1 cells separately at a density of 2.5×10^4 cells/ml. Time-lapse microscopy showed images of rat astrocytes deteriorating and retracting from their original positions. After examining just

astrocyte controls illuminated with blue light (necessary to track to DiO labeled T98G cells), the same result was observed. Thus, the rat astrocytes appeared to be sensitive to the repeated blue light illumination that is necessary to view the labeled T98G cells (Figure 13).

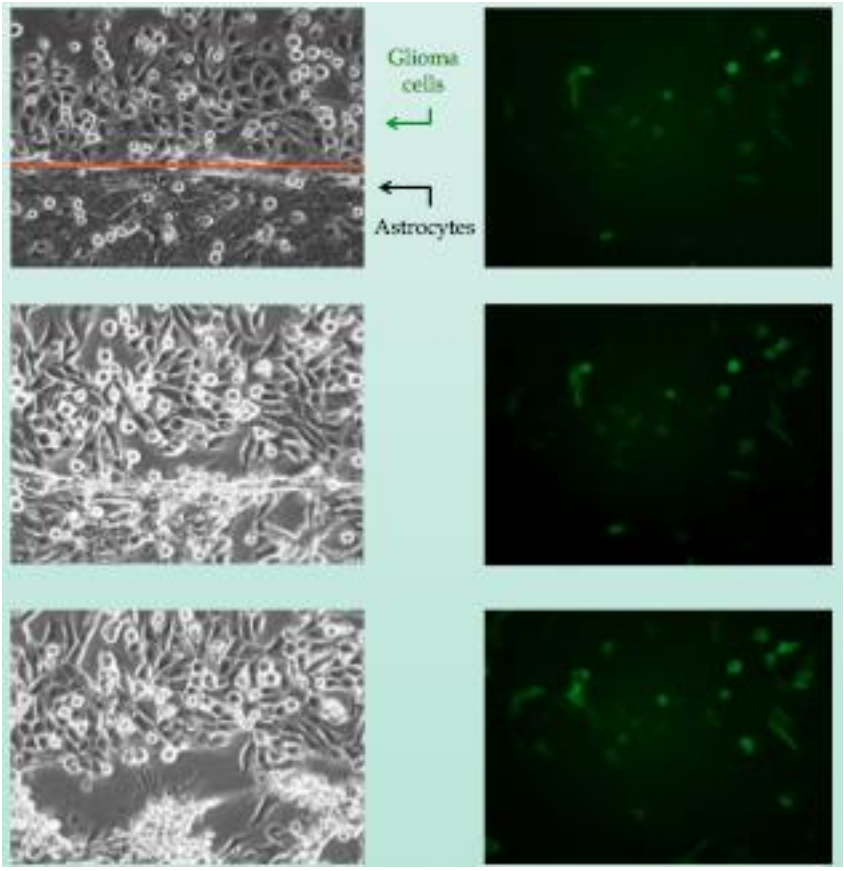


Figure 13. Phase and Fluorescent images of Rat Astrocytes sensitivity to blue light irradiation.

3.3.3 Glioma cells treated with vybrant diI result in less cytotoxicity of rat astrocytes

Because of the phototoxicity of the blue light illumination that was required for visualization of DiO labeled T98G cells, I alternatively labeled T98G cells with Vybrant DiI, which can be illuminated with longer wavelength green light (Figure 14). This should be less phototoxic to the astrocytes. T98G-shL1 cells were trypsinised and collected in a 15ml falcon tube. The cells were centrifuged and washed with PBS. 5 microliter/ml of vybrant dye I stock was added to the glioma cells and thoroughly mixed. The glioma cells were then allowed to incubate at 37°C for an hour. The tumor cells were then rinsed with PBS twice and plated on top of rat astrocyte monolayers. Time-lapse microscopy was conducted to measure glioma cell motility when co-cultured on rat astrocytes. Although the rat astrocytes were unharmed when exposed to green light, the tumor cells displayed no movement from their original start point. Due to this observation, glioma cell motility could not be recorded.

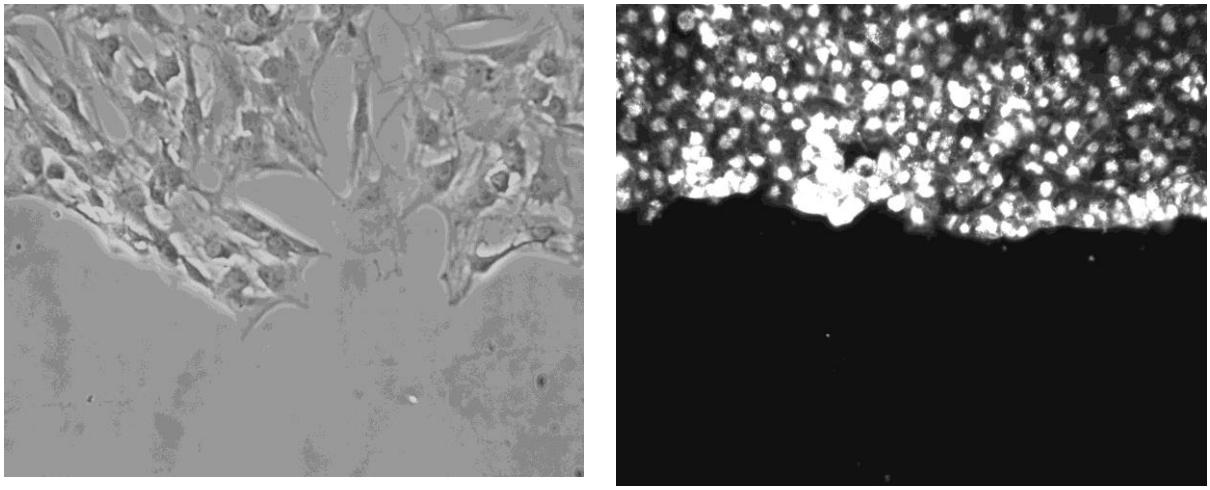


Figure 14. Phase and Fluorescent images of Astrocytes and Glioma cells treated with Vybrant DiI. Phase image (left) of astrocytes surviving the 24 hour period exposure to green light. Fluorescent image (right) of tumor cells treated with Vybrant DiI.

3.4 L1-FGFR Interaction

3.4.1 L1 and FGFR co-staining revealed areas of high interactions as well as areas of no interaction

Previous work from our lab showed that L1 ectodomain (L1LE) signaled through FGFRs [20], but it was never shown that L1LE co-localized with FGFRs on the cell surface. Therefore, I attempted to double-label T98G-shL1 cells for L1LE binding and FGFRs. T98G-shL1 cells were plated on coverslips in a 24 well plate. L1LE was added into the culture as a source of soluble extracellular L1. First, L1 was stained by mcAb UJ127 and then FGFR was co-stained with anti-FGFR antibody. Proper primary and secondary controls were used to make sure there was no non-specific staining. Fluorescence microscopy was used to examine the co-staining. Individual images were taken of the L1 and FGFR staining and then merged in Photoshop to examine the final result. There were areas of L1-FGFR co-localization around the tumor cell surface and thereby presumably interacting but there also were areas where L1 and FGFR were not co-localized and therefore not interacting. Thus from these experiments I can conclude that L1 and FGFR are binding and interacting on the surface of glioma cells, however there are other proteins and receptors that they may bind as well. These include integrins for L1 and FGF for FGFR.

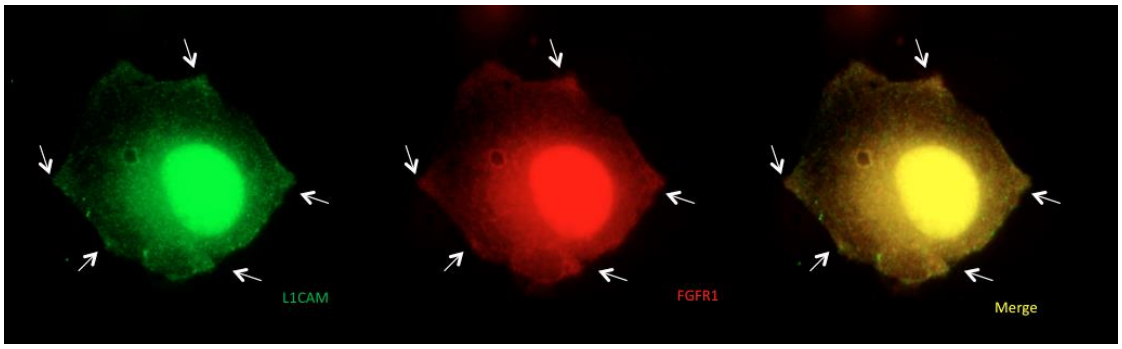


Figure 15. L1-FGFR Interaction. Co-staining of L1 (green), FGFR (red), and their merged image (yellow).

3.5 *In Vivo* Chick Microinjections of T98G-2605 and T98G-dFGFR Glioma Cells

3.5.1 *In vivo* chick microinjections of T98G-2605 showed some invasive behavior

Although our lab previously showed that FGFRs were necessary for full L1LE stimulation of motility *in vitro* [20], no experiments were done *in* vivo and, so, I performed injections into chick embryo brains. T98G-2605 glioma cells were selected for with 10 micrograms/ml of puromycin. Once they reached confluency, T98G-2605 cells (expressing GFP) were trypsinised and collected for *in vivo* injections. Chick eggs were incubated until embryonic day 5. They then were removed and opened for injection. $2.5x10^4$ cells/microliter were directly injected into the chick optic tectum ventricle. The injected eggs were then incubated till embryonic day 9, removed, dissected, fixed, vibratome sectioned, and examined through both phase and fluorescent microscopy. In general, there was about a 50-60% survival rate of the eggs after being injected. Several eggs (~12 per condition) were injected at a time to counteract this effect. Upon qualitative examination, the T98G-2605 cells showed some invasive characteristics moving from the ventricle where they were injected

towards the embryonic brain tissue regions. Based on prior studies it is reasonable to predict that the glioma cells are moving towards the embryonic brain regions.

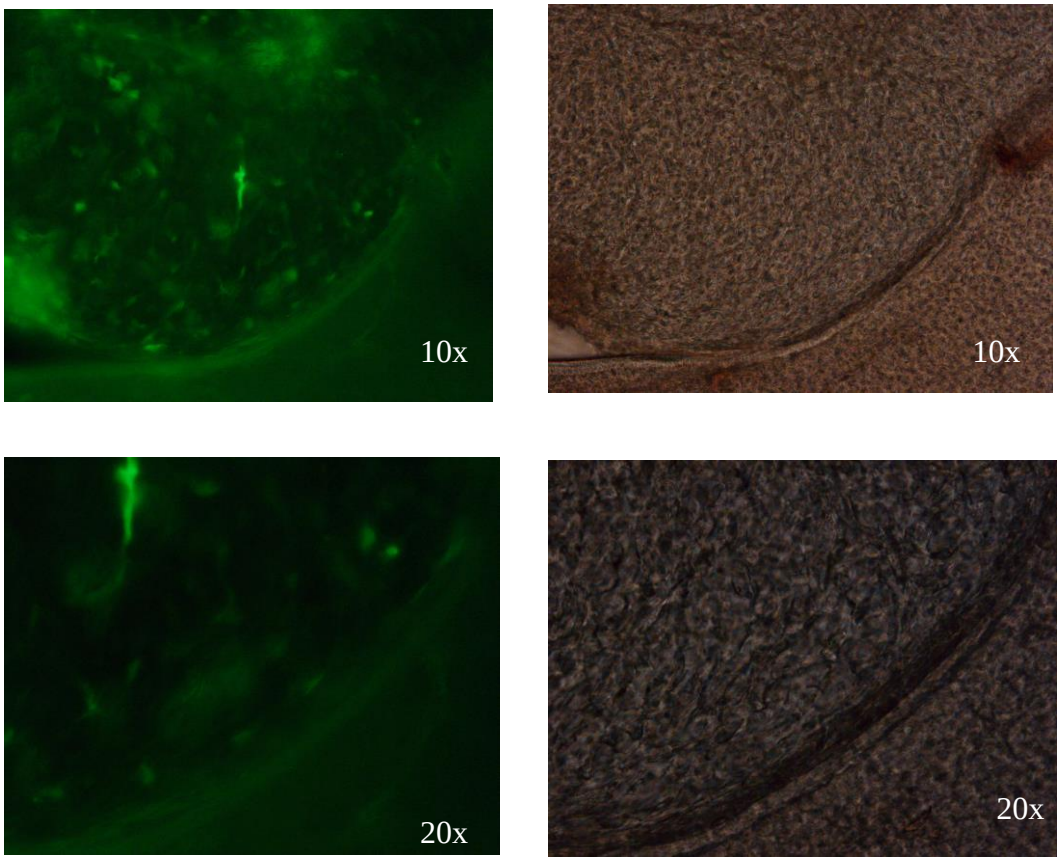


Figure 16. Fluorescent (left) and phase (right) images of injected T98G-2605

3.5.2 *In vivo* chick microjections of T98G-dFGFR showed minimal invasive behavior

T98G-dFGFR glioma cells were selected for with 10 micrograms/ml of puromycin. Once they reached confluency, T98G-dFGFR cells (expressing GFP) were trypsinised and collected for *in vivo* injections. Chick eggs were incubated until embryonic day 5. They then were removed and opened for injection. $2.5x10^4$ cells/microliter were injected directly into the chick optic tectum ventricle. The injected eggs were then incubated till embryonic day 9, removed, dissected, fixed, sliced via vibratome sectioning, and examined through both phase and fluorescent microscopy. In general there was about a 50-60% survival rate of the eggs after being injected. Several eggs (~12 per condition) were injected at a time to counteract this effect. Upon qualitative examination, the T98G-dFGFR cells showed minimal invasive characteristics residing mainly in their original point of injection in the ventricle and not invading towards the embryonic brain regions. Thus, from these experiments I can conclude that FGFR plays a role in glioma progression.

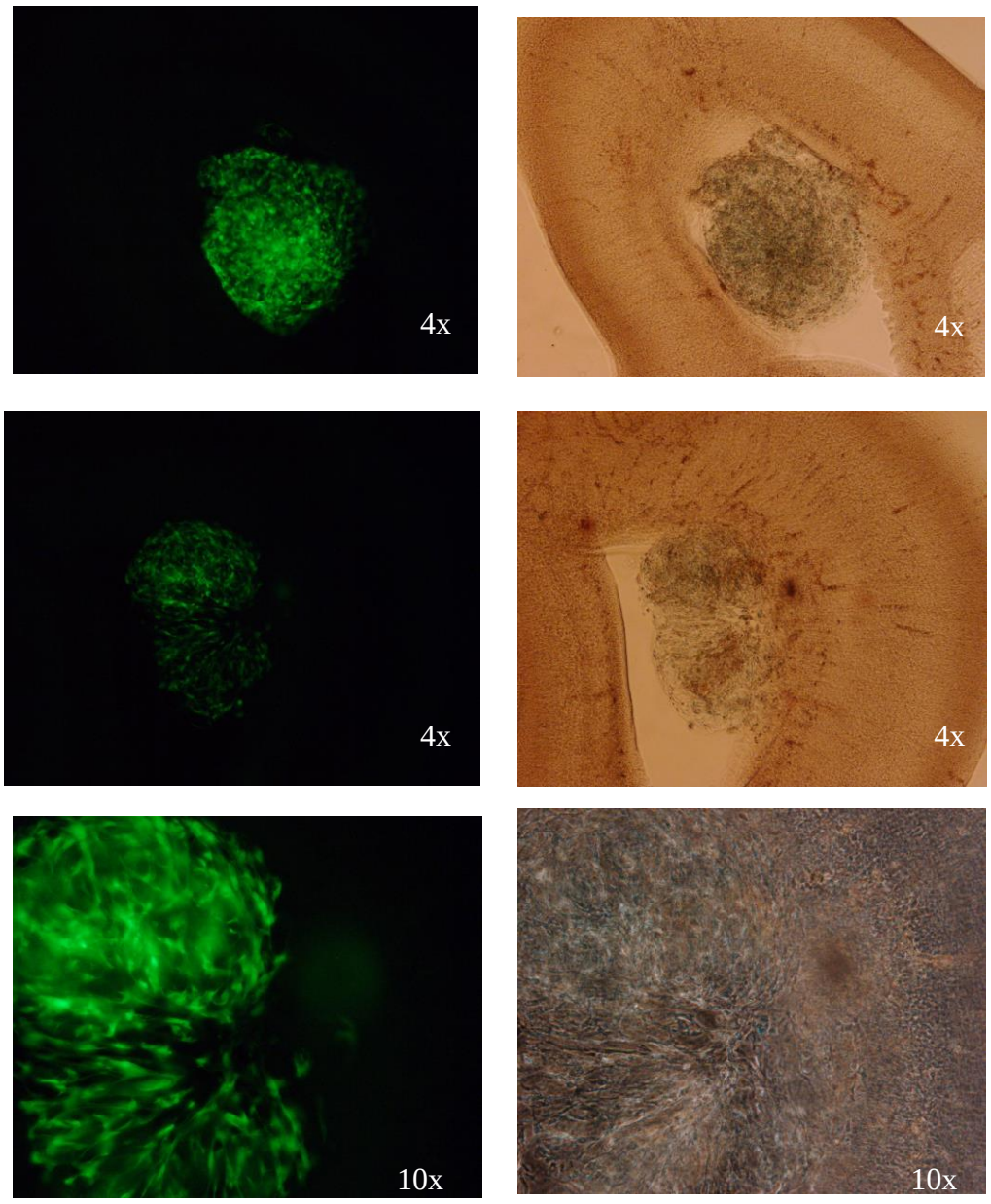


Figure 17. Fluorescent (left) and phase (right) images of injected T98G-dFGFR cells

Chapter 4

Discussion

4.1 New Role of Astrocytes in Glioma Progression

Prior to this study there was minimal to no reported findings on the effects of brains cells, such as astrocytes, on glioma cell migration. After a thorough review of the literature, one article suggested the possibility of glia progenitor cell recruitment driving aggressive glioma growth [47]. Massey et al. suggest that platelet-derived growth factor (PDGF) may have a role in the tumor's development and progression. The data from my experiments signals towards a factor produced by astrocytes that is involved in glioma stimulation. Our work did not investigate a specific target molecule or factor for the observed stimulation. L1 is largely expressed in the CNS by neurons and it is not expected that astrocytes produce enough L1 to cause a significant increase in stimulation, as was observed. However, the answer could lie in the process of cleaving L1. Through proteomic analysis of astrocytic secretions, there have been more than 30 proteins identified that include proteases [48]. As mentioned in the background, ADAM10 is a protease that is involved in cleaving L1 to allow glioma cells to migrate faster. There is a possibility that astrocytes express proteases that may cleave L1. Since astrocyte induced increase in glioma velocity is a new finding there is not enough literature to rule out or rule in a certain molecule or factor. Instead, experiments testing the astrocyte conditioned medium for potential factors will need to be conducted to narrow the source of stimulation.

4.2 Primary Brain and Glioma Cell Co-cultures Provide New Insight

In general most cancer biologists interested in glioma migration have been content to study tumor cell velocities on culture dishes. This method largely neglects the potential impact of their interactions with brain cells. When a tumor forms, develops, and progresses in the brain it does so through its interactions with other brain cells. While it may be difficult to quantify the average cell velocities *in vivo*, this study introduces a novel technique of measuring glioma cell velocity on cultured primary brain cell monolayers. In the literature, one recently published study in 2013 has already begun to investigate GBM migration using co-cultures [49]. Romao et al. have shown that co-culturing GBM cells with neonatal neurons can impact migration rate. I have demonstrated a similar result with glioma cells on chick brain monolayers (see results section 3.3). My findings together with current trends in glioma migration studies point toward a new approach for measuring glioma cell velocities via co-cultures with primary brain cells.

4.3 L1-FGFR Co-localization

Evidence of L1-FGFR binding was searched in the literature for co-staining images. There was mention of L1's fibronectin type III modules interacting with FGFR; however, these results were obtained through surface plasmon resonance analysis and not through immunostaining [50]. Although several references were made to L1-FGFR signaling, no adequate immunohistochemistry had been performed according to our search. Thus our co-staining of L1-FGFR is one of the first of its kind. We show that L1 and FGFR bind on the cell surface of glioma cells. In the areas of no binding, it can be concluded that L1 and FGFR are interacting with other known-binding partners (L1 with integrins, and FGFR with FGF).

4.4 Results Directly Advance Prior Findings in DSG Laboratory

Each aim and consequent result in my study was built on prior work done in our lab. Thus each new result provides greater insight into a past problem but also requires further studies. The following are major conclusions that address our past lab results.

4.4.1 Paracrine stimulation by astrocytes can stimulate glioma cell motility and proliferation to near autocrine stimulation level in the absence of L1

In the past our lab has shown the significance of L1 for glioma cell migration. Its role in stimulating glioma velocity is well documented. In this study, I suggest an alternate source of stimulation. Through examination of cell velocities of glioma cells treated with astrocyte-conditioned media, it is seen that their velocities can be boosted to the same high level as is the case by introducing L1. This indicates a need to further investigate the role of astrocytes and normal brain cells and their interactions with glioma cells.

4.4.2 L1 autocrine signaling provides the most stimulation for normal glioma cells

When examining glioma cells that normally produce L1 (T98G), it was seen that no additional source of stimulation (L1LE or Astrocyte conditioned medium) resulted in an increase in cell velocity. Our lab has mainly investigated ways to counter the effects of L1 stimulation. However, until now, it was not known whether the autocrine/paracrine stimulation from just the glioma cells or the paracrine stimulation from the normal brain cells resulted in greater stimulation. From this study, it can be concluded that L1 signaling does in deed provide the most stimulation for glioma cells.

4.5 Future Work

4.5.1 Optimize methods for determining effect of astrocyte cell-to-cell contact on glioma cell motility

Through this study it was determined that astrocytes were sensitive to blue light irradiation. Thus Vybrant diI was used to combat this issue. This solved the issue of astrocyte cytotoxicity by allowing them to live when exposed to green light, however the dye treatment methods on glioma cells resulted in lack of movement. The glioma cells displayed no movement after being treated with Vybrant diI for the recommended concentration and exposure time. In order to obtain velocity measurements, either optimal condition for dye treatment will need to be developed or an alternate staining technique will need to be used. Knowing the effect of astrocyte cell-to-cell contact will compliment the results of astrocyte conditioned media.

4.5.2 Live cell staining of L1-FGFR interaction

In our method of observing L1-FGFR binding we fixed the glioma cells from the beginning before the addition of the antibodies. An alternate method would be to live stain the glioma cells and then fix them afterwards. The glioma cells could be treated with antibodies on ice and kept alive while treating with antibodies and then later fixed and observed. Images also need to be observed with a higher magnification to be sure that co-localization exists on the cell surface.

4.5.3 Delayed dissection of chick brains microinjected with glioma cells

It was difficult to firmly determine the effects of glioma cell *in vivo*. Embryos injected with T98G-2605 and dissected at E9 began to show the previously determined invasive character of migrating into the brain ventricles. An alternate method would be to dissect embryos at E12 and allow for further glioma cell progression. Yang et al.

also used 30% Matrigel to get invasion into the brain. It is a possibility that we did not see invasion due to our omission of Matrigel in our injection mix.

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