DEVELOPING CHICK EMBRYO BRAIN SLICE CULTURES TO INVESTIGATE THE ROLE OF N-CADHERIN IN GSC INVASION

by

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ABSTRACT

Glioblastoma (GBM) is the most aggressive form of brain cancer formed from astrocytes, classified as a grade IV astrocytoma by the World Health Organization (WHO). GBM has been associated with poor prognosis and the medium survival time of a patient with GBM is around 14 months. It is theorized that GBM invasion and relapse is connected to the small population of glioblastoma stem cells (GSCs) that is resistant to treatments including radiotherapy and chemotherapy, and how they interact with non-cancer stem cells. A major molecule known to take part in increasing GBM motility and invasion is the L1 cell adhesion molecule (L1CAM). Cleavage of L1CAM ectodomain by the metalloproteinase ADAM10 can induce cell migration and neuronal growth. Along with L1CAM, the transmembrane protein N-cadherin, is also speculated to increase GBM motility by similar processes as L1CAM involving cleavage of the protein's ectodomain by ADAM10. GSC and non-GSC invasion can involve several paths including along neuronal axon bundles, migrating diffusely into brain tissue, or along blood vessels.

My goal was to develop an *ex vivo* brain slice culture system using embryonic chick brain slices to facilitate time-lapse analysis of GBM cell and GSC behavior. *Ex vivo* brain slice cultures survived best in MEM media (with additives) as shown by Sox-2 and laminin stainings which screened for brain viability and blood vessel conditions, respectively. The implantation method used in this study proved to be a successful method to observe live U-118/L1LE cell migration along blood vessels in the live brain slice cultures via time-lapse under the widefield fluorescent microscope. This was

validated with immunostaining and confocal imaging with z-stack analysis. However, less invasion was seen using GSC2016-4, suggesting further investigation with live brain slice cultures and GSC invasion.

A second goal was to determine N-cadherin expression in GSC and GBM cell lines in the Galileo Lab using antibodies against the cytoplasmic domain and ectodomain of N-cadherin. By being able to identify N-cadherin expression in specific cells, further experimentation on how N-cadherin influences GBM motility can be investigated in the future. My second goal was to establish an *ex vivo* organotypic brain slice culture model to visualize live invasion of GSC and GBM cells, particularly along blood vessels.

Western blot analysis, flow cytometry, and coverslip immunostaining with the antibody against the cytoplasmic domain of N-cadherin showed N-cadherin expression in all GSC and GBM cells tested. N-cadherin expression using the antibodies against the cytoplasmic domain and ectodomain was evident in western blot analysis, fixed and live coverslip staining, and immunostained tumors in brain slices.

My results demonstrate that *ex vivo* chick embryo brain slice cultures are a suitable system for analyzing invasiveness of GBM cells and GSCs. Furthermore, N-cadherin appears to be widely expressed in GBM cells and GSCs, although further experiments are needed to determine if it is cleaved and released to facilitate autocrine/paracrine stimulation of motility, invasiveness, and/or proliferation similarly to what occurs with L1CAM.

Chapter 1

INTRODUCTION

1.1 Glioblastoma (GBM)

Glioblastoma (GBM) is the most lethal and aggressive type of brain cancer formed from brain cells. The most invasive and malignant type of glial tumor, GBM is classified as a grade IV astrocytoma by the World Health Organization (WHO), accounting for 77%-81% of all primary malignant tumors of the central nervous system (CNS) (Grech et al., 2020). Although radiotherapy and/or chemotherapy following resection surgery can aid in prolonging the survival of the patient, the high proliferation and motility rate of the GBM cells make them highly resistant to treatment. This results in a median survival of approximately 14 months (Leite et al., 2019), with the 5-year survival rate of around 5% (Ostrom et al., 2014). Despite the medical and surgical advances today, there is still a poor prognosis associated with GBM, encouraging continual research in treatments, and understanding of GBM.

1.2 Cancer stem cell model

Growing evidence from studies have suggested that a small population of cancer cells, termed cancer stem cells (CSCs) or cancer initiating cells (CICs), play a key role in GBM invasiveness and relapse. In GBM, the CSCs which are referred to as glioblastoma stem cells (GSCs), are considered to account for around 1% of the tumor cells, while non-CSCs account for most of the tumor (Sundar et al., 2014). It is believed that the small population of the GSCs is responsible for the unresponsiveness of GBM tumors to

treatment due to self-renewal properties even after removal of the tumor. GSCs are seen to cause the poor prognosis in patients because of the heterogeneity and plasticity in GBM, as well as the interconversion between GSCs and differentiated non-GSCs (Safa et al., 2015).

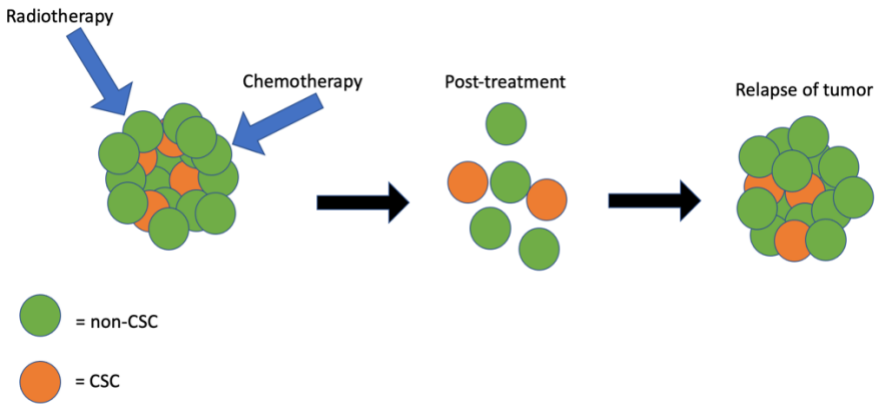

Figure 1. Cancer stem cell model. Most of the tumor is made of non-CSCs, while 1% of the tumor is made of CSCs. CSCs are unresponsive to treatment due to self-renewal properties and the potential interconversion between GSCs and differentiated non-GSCs.

1.3 Perivascular invasion

There are several ways in which GSCs and non-GSCs can exit the tumor, including along neuronal axon bundles, migrating diffusely into brain tissue, or along blood vessels. Here, the focus will be on when the glioma cells migrate at the invasive edge of blood vessels, otherwise known as perivascular invasion (Figure 2). Glioma cells tend to actively seek and migrate along blood vessels (Montana et al., 2011). The perivascular microenvironment consists of endothelial cells, pericytes, microglia, astrocytes, and neurons. This particular manner of invasion contributes to incomplete

surgical resection, leading to the recurrence of the tumor (Hambardzumyan et al., 2015). In contrast to other cancers such as prostate or colorectal cancer, gliomas are rarely seen metastasizing outside of the main organ, with only 0.4-2% of cases metastasizing outside the brain (Beauchesne, 2011). For this reason, gliomas are also thought to be unable to penetrate the basement membrane of blood vessels and enter the vasculature, resulting in the blood vessels being just a migration pathway for the cells (Cuddapah et al., 2014).

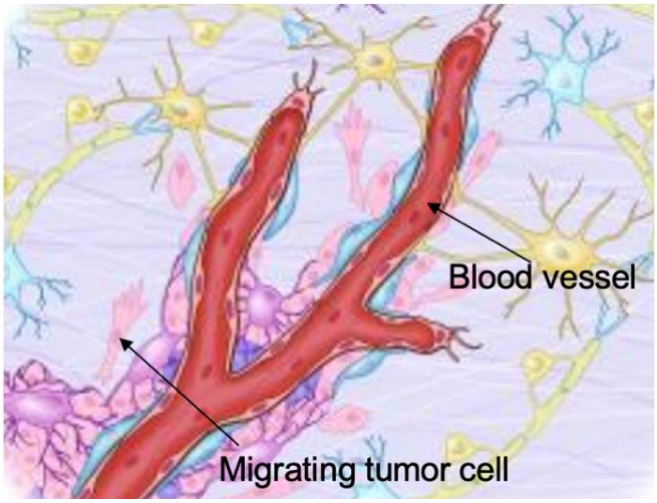

Figure 2. GSCs and tumor cells line migrate along blood vessels as they invade outwards throughout the brain from the initial tumor mass. Image adapted from Hambardzumyan et al., 2015.

1.4 L1 Cell Adhesion Molecule (L1CAM)

1.4.1 Role of L1CAM in GBM

Multiple cell surface proteins likely play a role in GBM cell spread in the brain. The L1 adhesion molecule, or L1CAM has shown to play a major role in GBM motility. L1CAM is a transmembrane protein comprised of an extracellular ectodomain with five fibronectin (FN) domains and six immunoglobin-like (Ig) domains at the amino terminal end. The molecule also has a highly conserved intracellular cytoplasmic tail, with the

full-length molecule weighing at 200-220 kDa (Yang et al., 2011). Being a significant factor in cell-to-cell adhesion, cell signaling, and migration, the molecule is also able to participate in homophilic and heterophilic bonding and binding to other integrins (Linneberg et al., 2018). Normally, L1CAM is expressed during the development of the central and peripheral nervous systems. The molecule has an important role in neuronal migration, axonal growth, guidance and fasciculation, survival of neural cells, and synaptic plasticity (Tonosaki et al., 2014).

L1CAM can be subject to ectodomain cleavage or shedding under tight control during neuronal development or to a greater extent in cancer (Maten et al., 2019). Once the metalloprotease ADAM10 cleaves L1CAM just outside the cell surface, the protein's soluble ectodomain fragment can induce cell migration and neuronal outgrowth. The remaining non-soluble fragment that is embedded in the membrane can also be further cleaved by γ-secretase (Schäfer et al., 2010). L1CAM expression in tumor cells has shown to stimulate cell motility when the ectodomain fragment interacts with integrin receptors (Yang et al., 2011) or FGF receptors (Mohonan et al., 2013). It is thought that the interaction activates the FAK signaling pathways, regulating cell migration, and influencing the invasiveness of the tumor in the brain (Yang et al., 2011). These findings have made L1CAM a potentially important cell surface protein for the spread of GSCs and GBM cell lines.

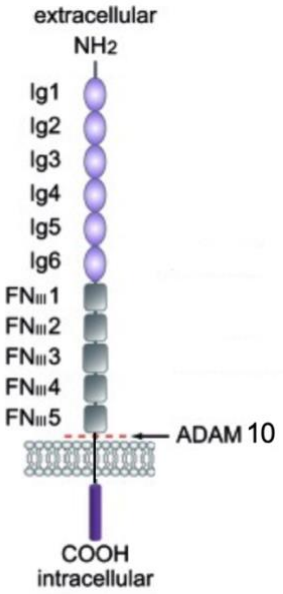

Figure 3. Structure of L1CAM and cleavage site by ADAM10. Adapted from Kiefel et. al., 2012.

1.5 N-cadherin

1.5.1 N-cadherin's role in GBM

Another molecule that is speculated to play a role in GBM motility is N-cadherin. N-cadherin is a 130-135 kDa calcium-dependent transmembrane protein. The protein is a type I transmembrane glycoprotein with five extracellular domains (EC) linked to a functional intracellular domain. The extracellular domain can form adherent junctions between neural cells via strong homophilic adhesion and interacting with other N-cadherin molecules. N-cadherin is known to be correlated to several morphogenetic processes including epithelial cell arrangement, segregation, and association of cells. The protein is highly expressed during embryogenesis, with expressions starting at the gastrulation stage in mesodermal and ectodermal cells. Expression is permanent in glial cells and neurons which are derived from the neural ectoderm (Kohutek et al., 2009).

Past studies have shown that expression of N-cadherin is relatively low in majority of adults, while re-expression of N-cadherin during tumor progression promotes cell migration and metastasis (Cavallaro, 2004). In astrocytomas like GBM, however, associating N-cadherin's expression levels to the invasiveness of the tumor still requires much investigation.

N-cadherin also is a substrate of ADAM10-mediated cleavage. N-cadherin is cleaved proximal to the membrane, yielding a 95 kDa N-terminal fragment and a 40kDa C-terminal fragment (CTF 1). Like L1CAM, a multi-subunit protein complex called the γ-secretase complex will cleave CTF1 to produce a 35 kDa fragment (CTF2) and CTF2 will be released into the cytoplasm (Figure 4). The consequences of these cleavages are still unclear, however, it is suggested that the N-terminal fragment promotes neurite outgrowth and cell migration, while the CTF2 fragment regulates gene transcription (Kohutek et al., 2009; Marambaud et al., 2003).

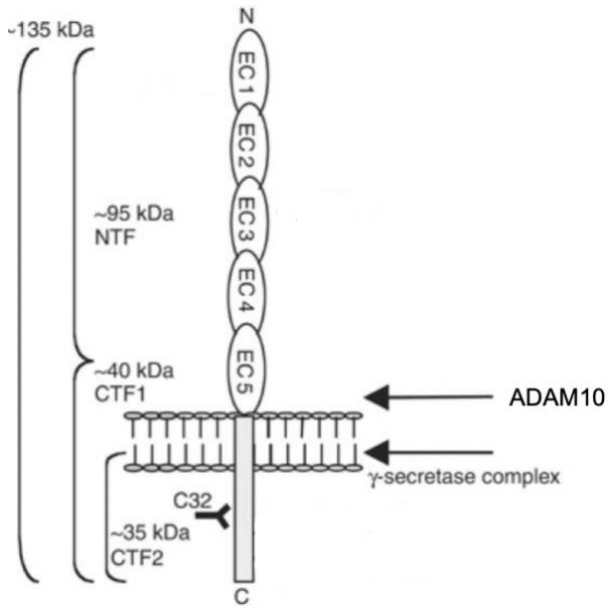

Figure 4. Structure of N-cadherin and cleavage site of ADAM10. Adapted from Reiss et al., 2005.

1.6 Brain slice viability markers

1.6.1 Sox-2

SRY (sex determining region Y)-box 2, or Sox-2, is a transcription factor that is classified as a member of the SoxB1 group among 20 different Sox genes. Sox-2 is notably known for its function during embryonic development, maintaining the pluripotency and directing the neural differentiation of embryonic stem cells (Zhang et al., 2014). The transcription factor is also known for its significance in maintaining stem cell activity in the Central Nervous System (CNS), self-renewal of cells, and an acceptable marker for stemness of neural stem cells (Suh et al., 2007). In GBM, Sox-2 is overexpressed, with its removal shown to prevent GBM growth in past studies with xenograft mouse models (Bradshaw et al., 2016). The expression of Sox-2 is not limited to specific percentage of cells and can be expressed along with other markers

(Berezovsky, 2014). This deems it as an appropriate marker for GSCs (Lathia et al., 2015). Although these properties of Sox-2 have been considered in our lab and past lab members have used Sox-2 as an accurate marker for GSCs, I mainly focused on utilizing Sox-2 to evaluate the viability of brain sections, since the embryonic brain cells appear to be all Sox-2 positive. In the Galileo lab, we have established Sox-2 expression in cells of brain sections as a good indicator for demonstrating the quality and condition of live brain sections, particularly the optic tectum and forebrain of chick embryos. Lack of expression in cells suggest a degenerating condition of the brain sections.

1.6.2 Laminin

The basement membrane is a specialized extracellular matrix that lines the outer basal side of endothelial cells in blood vessels. This structure is essential in the form and function of blood vessels, with cell signaling occurring due to interactions between various proteins. The vascular basement membrane is 20-200 nm thick and made of three-dimensional networks of mainly several different proteins. Type IV collagen isoforms, nidogens, heparan sulfate proteoglycans (HSPG), fibronectins, and laminin are well-known proteins that make up the basic framework of the basement membrane (Hallman et al., 2005; Thomsen et al., 2017). Of the five, laminin expression by immunohistochemistry has shown to be a simple method to visualize the location of blood vessels and other vascular structures. In past mammalian brain models, laminin immunoreactivity was strong in the walls of blood vessels in the frontal cerebral cortex and cerebellum (Erik-dotter-Nilsson et al., 1986). Laminin is a cross-shaped

heterotrimeric glycoprotein and the most abundant non-collagenous basement membrane protein. All 16 different isoforms for laminin have an α-chain, a β-chain, and a γ-chain (Thomsen et al., 2017).

A previous lab member, Amy Lin, evaluated different markers for their ability to stain blood vessels in chick embryo brain sections. She found that the anti-laminin-1 (3H11) monoclonal antibody yielded the best results in specifically staining endothelial cells of blood vessels. The laminin antibody distinctly stained only the outer surface of blood vessels, and the lumen interior was negative (Lin, 2020). These results have encouraged our lab to use the laminin antibody as a specific marker for blood vessel location, and to determine the conditions of the blood vessels while in live chick embryo culture.

1.7 Xenograft model and Organotypic slice culture

Xenograft models are *in vivo* studies on animals that allow for further understanding of results from *in vitro* studies as well as provide evidence for future clinical trials with patients. In the case of GBM, GSCs and GBM cell lines can be injected into animal brains such as a rodent or chick embryos to grasp a better understanding of the development of the tumor in a complete animal environment. However, a disadvantage to this *in vivo* method is that difficulties arise when elements such as cell motility, speed, and the manner in how cells invade (i.e., along blood vessels) in the brain are being assessed. Observing invasion solely on a dish also does not provide

a live tissue culture environment and thus does not give complete insight on how the complex tissue environment influences invasion.

Organotypic slice culture models, however, allow us to assess GSC and GBM motility and invasiveness by blending both *in vitro* and *in vivo* methods (Chokshi et al., 2020). This usually is referred to as an *ex vivo* model. They can especially be useful when tracking the endpoint of the invasion of GSCs or GBM cells after a certain period to see how far the cells moved after implantation. Brain tissue can be maintained with near-normal physiological and morphological characteristics that allow them to survive in culture for a few weeks, making them available for multiple experiments during this time frame. The most common method used in organotypic slice culturing is the method from Stoppini et al (1991) where a rat brain slice is cultured on top of a transparent and porous membrane insert, with media underneath the insert, and the brain slice being exposed to air (Figure 5). There have been minor modifications to this protocol (i.e., use of different media, different inserts, etc.) across different studies (See Table 1). Including xenograft models, many organotypic models have been established in rodents, but not as commonly in chick embryos (Yang et al., 2019). However, in the Galileo lab as well as a few others, chick embryos have proven to demonstrate both human and rat glioma invasion in xenograft models. Invasion patterns are like those observed in rodent models, supporting the use of chick embryos as an *in vivo* model for GBM growth. The use of chick embryos is also an efficient and inexpensive option compared to rodents, making them a favorable system to work with in a short-term study. Injection of the tumor and stem cells also involves simply injecting them directly into the optic tectum (Yang, 2011). For

these reasons, chick embryos are appropriate models to use in both xenograft and organotypic models.

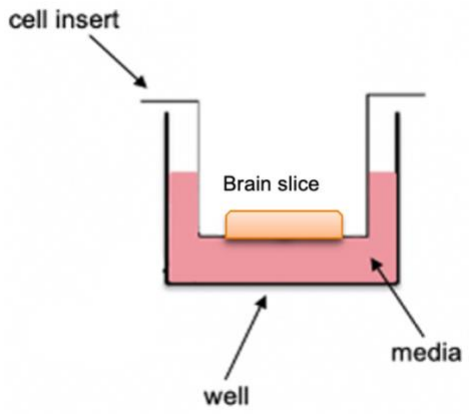

Figure 5. Organotypic brain slice model. Adapted from Ren et al., 2015.

A previous lab member, Jackson Doerr, was able to demonstrate invasion of GSCs along blood vessels after the chick embryo was dissected, and fixed sections were immunostained for laminin (Doerr, 2021). My main interest is to visualize live movement of GSCs and/or GBM cells along blood vessels with the organotypic slice culture model, while making changes to the various protocols shown in Table 1 to successfully implant GSCs and GBM cells into the live brain slice. Organotypic slice cultures can also be potentially beneficial when testing treatments or inhibitors on the motility of GSC or GBM cells in the brain. Inhibitors cannot be tested, nor can the effects be visualized in a chick embryo in the egg (xenograft model), but the live brain slice culture can allow us to directly supply inhibitors into the media, which will infiltrate into the brain slice, to observe the effects.

Table 1. Different references and their methods for organotypic slice culturing

Reference	Membrane inserts used	Media used
Stoppini et al., 1991	30-mm diameter, porous (0.4um) membrane insert (Millicell-CM, Millipore) in a 35mm petri dish	Mixture of MEM + Hepes (50%; Gibco No. 079-01012), horse serum (25%) and Hank's solution (25%) Glucose was added to reach a final concentration of 6.5 mg/ml
Ohnishi et al., 2006	Millicell cell culture inserts (0.4um) for 6-well plates (Millipore)	50% Eagle's MEM (Earle salt with L-glutamine, 25μM HEPES, and NaHCO3), 25% HBSS, 25% heat-inactivated horse serum, 6.5mg/ml D-glucose, 100U/ml penicillin, 100μg/ml streptomycin, and 2.5μg/ml amphotericin B
Aaberg-Jessen et al., 2013	Millicell cell culture inserts (0.4um) for 6-well plates (Millipore)	50% Optimem 1 (GIBCO), 25% horse serum (GIBCO), 25% Hanks Balanced Salt Solution (HBSS, GIBCO), supplemented with 25 mM D-glucose, 1% penicillin/streptomycin (Cambrex), 3% non-essential amino acids (Cambrex) and 2% glutamine (Cambrex)
Croft et al., 2018	Millicell cell culture inserts for 6-well plates (Millipore (UK) Ltd.)	Basal medium eagle (BME), 26.6 mM HEPES, pH 7.1, 19.3 mM NaCl, 5 mM NaHCO3, 511 μM ascorbic acid, 40 mM glucose, 2.7 mM CaCl2, 2.5 mM MgSO4, 1% (v/v) GlutaMAX (Life Technologies, Paisley, UK), 0.033% (v/v) insulin, 0.5% (v/v)

		penicillin/streptomycin (Life Technologies), in ultrapure H2O, sterile filtered (0.2 μm), plus 25% (v/v) heat inactivated horse serum
Humpel., 2015	Millicell cell culture inserts (0.4um) for 6-well plates (Millipore)	50% MEM/HEPES (Gibco), 25% heat-inactivated horse serum (Gibco/Lifetech, Austria), 25% Hanks' solution (Gibco), 2 mM NaHCO3 (Merck, Austria), 6.5 mg/ml glucose (Merck, Germany), 2 mM glutamine (Merck, Germany), pH 7.2
Ren et al., 2015	Millicell cell culture inserts (0.4um) for 6-well plates (Millipore)	50% Eagle's MEM with HEPES, 25% HBSS, 25% heat-inactivated horse serum, 6.5 mg/mL glucose, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin.
Moser et al., 2003	30-mm diameter, porous (0.4um) membrane insert (Millicell-CM, Millipore) in a 35mm petri dish	50% MEM/HEPES (Gibco), 25% heat-inactivated horse serum (Gibco/Lifetech, Austria), 25% Hanks' solution (Gibco), 2 mM NaHCO3 (Merck, Austria), 6.5 mg/ml glucose (Merck, Germany), 2 mM glutamine (Merck, Germany), pH 7.2

1.8 Research goals

My main research goal was to develop a live *ex vivo* brain slice culture model that allows time-lapse observation of GSC and GBM cell invasion and experimental treatments. Live culture experiments were performed to test what media maintained the most viable brain slices based on blood vessel integrity and brain tissue conditions, and a new technique of implanting GSC and GBM cells into brain slices was conducted. Time-lapse also was run to track live invasion with both the widefield and confocal microscopes to determine which type of fluorescent microscope would provide the best quality in imaging the invasion process. My second goal was to assess N-cadherin expression and possible cleavage of N-cadherin in GSC and GBM cell lines in our lab. Ultimately, these experiments will help us understand if N-cadherin plays a potential role as L1CAM does in stimulating cell motility and invasiveness in GBM cell and GSC lines.

Chapter 2
MATERIALS AND METHODS

2.1 Cells and cell culture

A human glioblastoma cell line, U-118 MG, was obtained from the American Type Culture Collection (ATCC, Manassas, VA). U-118/L1LE and U-118/1879 were two variants of the U-118 cell line that were used across different experiments and are described in Li et al. (2010). U-118 cells were transduced with a K1879/L1LE lentiviral vector that encodes for the L1 long ectodomain (L1LE), creating U-118/L1LE cells that express and secrete the L1 ectodomain. U-118 cells were also transduced with an empty K1879 lentiviral vector to create L1-negative U-118/1879 control cells. Both variants were further infected twice with a lentiviral vector that encodes the mCherry fluorescent protein (p.Ultra-hot; Addgene plasmid #24130). Cells were cultured in "Complete media" which consists of Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, #D5671) supplemented with 10% fetal bovine serum (Gemini, #900-108), 2mM L-glutamine (Sigma Aldrich, #G7513), and 100 ug/mL penicillin-streptomycin (Cellgro, #30-003-CI).

Patient-derived glioblastoma stem cells (GSCs) were created from GBM specimens received from the Tissue Procurement Center of the Helen F. Graham Cancer Center and Research Institute in Newark, Delaware. "GSC 2016-4" and "2015-2" were two main GSC lines used across different experiments. Naming of the cell lines are represented as "the year the samples were obtained- the tumor sample number from that year". Both GSC lines were transduced with a lentiviral vector that encodes for green

fluorescent protein (GFP, K72; Mohanan et al., 2013). Cells were dissociated from tissue specimens and maintained in "Failsafe" media which consists of a 1:1 mixture of DMEM/F12 (Sigma-Aldrich, #D8437), 1% fetal bovine serum, 15mM HEPES buffer (Sigma Aldrich, #H0887), 2mM L-glutamine, 100 ug/mL penicillin-streptomycin, 2% B27 supplement without vitamin A (Gem21 NeuroPlex, Gemini Bio-Products, Catalog #400-161), and 2.5ug/mL Heparin (Thermo Fisher Scientific, Catalog #9041-08-1) (Murrell et al., 2013). Every three days, cells were supplemented with 10ng/mL of basic fibroblastic growth factor (bFGF; Bio Vision, Catalog #4037-1000) and 20ng/mL transforming growth factor-alpha (TGF-α; Bio Vision Catalog #4339-1000) by adding these two growth factors directly to the culture dish.

All cells were primarily passaged in 10 cm tissue culture dishes. Cells were passaged using 0.05% or 0.25% trypsin/EDTA solution (Sigma Aldrich, #T3924) for 2 minutes and centrifuged in their respective media. All cells were incubated at 37˚C and 5% CO_2 conditions.

Table 2. Different cell lines and their media

Cell line	Media
U-118/L1LE, U-118/1879	DMEM, 10% fetal bovine serum, 2mM L-glutamine, 100 ug/mL penicillin-streptomycin
GSC2016-4, GSC2015-2	1:1 mixture of DMEM/F12, 1% fetal bovine serum, 15mM HEPES buffer, 2mM L-glutamine, 100ug.mL penicillin-streptomycin, 2% B27 supplement without

	vitamin A, 2.5ug/mL Heparin, bFGF, TGF-a

2.2 N-cadherin expression in GSC and GBM cell lines

2.2.1 Western blot analysis

Cell lysates for western blots were obtained from confluent 60 mm dishes. Media was aspirated from the dish and rinsed twice with 1X PBS. While setting the dish on ice, 500 uL of 1X RIPA buffer with protease inhibitor was put into the dish for 10 minutes. Cells were scraped off the dish with a cell scraper and transferred into a 1.5 mL centrifuge tube. The cells in the buffer were then sonicated on ice for 2-5 minutes with a Biologics ultrasonic homogenizer. Lysates were kept in the freezer at -20˚C until usage. Protein concentrations were measured using a Bradford assay (ThermoScientific). Samples for gel electrophoresis were prepared by combining NuPAGE™ LDS Sample Buffer 4X (Invitrogen), NuPAGE™ 10X Reducing Agent (Invitrogen), cell lysate, and distilled water to reach a volume of 40uL and adjust protein concentrations. Samples were denatured for 5 minutes at 95˚C before loading. A 4-20% tris-glycine gel (Invitrogen) was put into an electrophoresis chamber that was filled with NuPAGE™ SDS Running buffer (Novex NP0001) and 1 mL of NuPAGE antioxidant (NP0005) for every 400 mL of running buffer. In addition to 5 uL of PageRuler™ Plus Pre-stained Protein Ladder (10 to 250 kDa; Thermofisher 26619) mixed with 5 uL of Magic Mark XP (Invitrogen; LC5602) in the first and last lanes, samples were loaded into the wells of

the gel. The gel was run at 100 VDC for approximately two hours until the sample dye reached the bottom of the gel. Protein samples were then transferred from the gel onto a PVDF membrane overnight at 25 volts in transfer buffer. Following transfer, blots were briefly rinsed in tris buffered saline with 0.1% Tween-20 (TBST). The blots were then blocked in 5% nonfat dry milk in TBST for 1 hour at room temperature. Blots were rinsed 3 times for ten minutes each in TBST before incubating them in a solution with a 1:1000 dilution of primary antibody and 1% nonfat dry milk in TBST. Blots were incubated in the primary antibody solution for 1 hour at room temperature in constant agitation. After primary antibody incubation, the blot was rinsed 3 times for ten minutes each in TBST. The blot was then incubated in HRP-conjugated secondary antibody solution at a 1:20000 dilution of secondary antibody and 1% nonfat dry milk in TBST for 1 hour at room temperature in constant agitation. The blot was finally rinsed 3 times for ten minutes each in TBST. For imaging, the blot was developed using a 1:1 mixture of Lumigen ECL Ultra reagents A and B for detecting protein bands via chemiluminescence for 1 minute. The blot was imaged using the Bio-Rad ChemiDoc imager.

Table 3. Lysates used in western blot analysis

Cell lysate names	Description of lysate
U-118/L1LE	GBM cell line, U-118 cells transduced with a K1879/L1LE lentiviral vector that encodes for the L1 long ectodomain (L1LE)
U-118/1879	GBM cell line, U-118 cells transduced with an empty K1879 lentiviral vector to create L1-negative control cell line

GSC2016-4 (P22)	GSC line, 4th tumor sample obtained in 2016 and passaged 22 times
GSC2015-2 (P17)	GSC line, 2nd tumor sample obtained in 2015 and passaged 17 times
GSC2018-1A (P27)	GSC line, 1st tumor sample obtained in 2018, "A" refers to the line made from single dissociated cells which is different from "B" which refers to cells from tumor chunks left over from the sample

2.2.2 Immunostaining on coverslips

Fixed and permeabilized immunostaining

12 mm diameter round glass coverslips were sterilized with 70% ethanol, and each placed in a well of a 24-well plate. Once the coverslips dried, they were rinsed 2-3 times with sterile water or 1X PBS and treated with 0.5 mL of 0.2mg/mL poly-L-ornithine for 30 minutes at room temperature. After 30 minutes, the coverslips were rinsed 2-3 times with sterile water before plating the cells. From a 60-70% confluent 10 cm dish of cells, approximately 1/40th of the cells were placed into each coverslip in the 24-well plate. Up to1 mL of fresh media was added to each well. Cells were left in the incubator overnight at 37˚C and 5% CO_2 to allow them to growth and adhere onto the coverslips. The following day, media in the wells were discarded using a transfer pipette. Cells were fixed onto the coverslips by adding 1 mL of 1% paraformaldehyde (PFA, Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer per well for 30 minutes at room temperature. After fixing, the fixative was discarded and cells were rinsed with twice with 1 mL of PBSTG (1X PBS, 5% normal goat serum, and 0.1% Triton X-100). Cells were then incubated in primary antibody solution with 2 ug/mL of

primary antibody and PBSTG for 1 hour at room temperature with 1 mL of this solution per well. Negative controls were incubated in PBSTG. After incubation in primary antibody solution, cells were rinsed twice with PBSTG and incubated in a 1:200 dilution of fluorescent secondary antibody and PBSTG in the dark for 1 hour at room temperature. Cells were then rinsed in 1X PBS, and then rinsed in 3 ug/mL of bisbenzimide for 5 minutes to counterstain nuclei. A final rinse of cells in 1 X PBS was done after treatment with bisbenzimide, and the cells on coverslips were left in 1 X PBS until mounting onto slides. Each coverslip was placed on 6 uL of mounting media (N-Propyl Gallate + 80% glycerol) and the edges were sealed with nail polish. The slides were kept at 4˚C until imaging under a Plan Apo 20X/0.75 objective lens on a Nikon Eclipse E800 fluorescent microscope. Photos were captured using a SPOT Insight 2.0 Megapixel color camera and the same exposure levels for each image was adjusted via the SPOT Advanced software.

Live immunostaining

A day after cells adhered to the coverslips, media was discarded from the wells. The entire procedure for live immunostaining on coverslips occurred on ice. The cells were rinsed with 1X PBS and incubated in primary antibody solution with 2 ug/mL of primary antibody and culture media (DMEM + heat-inactivated 10% FBS + P/S + L-Glut) for 1 hour on ice with 1 mL of this solution per well. After primary antibody incubation, cells were rinsed twice with culture media and incubated in a 1:200 dilution of fluorescent secondary antibody and culture media for 1 hour on ice. Cells were rinsed twice with culture media and fixed with 1% PFA in 0.1 M cacodylate buffer for 20

minutes, with 1 mL of fixative per well. The cells were rinsed with 1 X PBS twice and left in 1X PBS until mounting onto slides. Each coverslip was placed on 6 uL of mounting media (N-Propyl Gallate + 80% glycerol) and the edges were sealed with nail polish. The slides were kept at 4˚C until imaging with the Nikon C2si+ confocal system.

Table 4. Cell lines used in fixed and live coverslip staining

Fixed cells on coverslips	Live cells on coverslips
GSC2016-4/K72 (P26), U-118/1879 mCherry 2x, U-118/L1LE mCherry 2x	GSC2016-4/K72 (P40), U-118/1879 mCherry 2x, U-118/L1LE mCherry 2x

2.2.3 Flow cytometry (FACS)

Fixed and permeabilized FACS

Using 0.05% trypsin/EDTA, cells were trypsinized from a 60 mm dish in complete media for 2 minutes. Cells were resuspended in complete media and centrifuged in a 15 mL tube at 800 rpm for 5 minutes. Media was aspirated, leaving only the cell pellet in the tube. The cell pellet was resuspended in fixative (0.1 M cacodylate buffer in 1% PFA) and put into microfuge tubes with 1 mL of cell solution per tube. Cells were left to fix for 30 minutes in room temperature. After fixing, cell solutions were spun down in the microfuge at 2000 rpm for 5 minutes. The fixative was discarded, and the cells were rinsed twice with PBSTG (1X PBS, 5% normal goat serum, and 0.1% Triton X-100). After rinsing, cells were resuspended in 2 ug/mL of primary antibody in PBSTG for 1 hour at room temperature. Negative controls were incubated in PBSTG. After primary antibody incubation, cells were spun down and rinsed twice in PBSTG.

Cells were then resuspended in a 1:200 dilution of fluorescent secondary antibody in PBSTG for 1 hour at room temperature in the dark. Cells were rinsed for a final time in PBSTG and each tube of cells was resuspended in 0.5 mL of PBSTG. Cell solutions were filtered into a FACs tube with a blue filter cap (Falcon #0877123) before taken to be analyzed. If not analyzed on the same day, cells were fixed again with 1% formaldehyde in PBS with 0.1% BSA and stored at 4˚C.

Live FACS

For cells analyzed with antibodies against the ectodomain, live immunostaining was conducted. Cells from a 60 mm dish in complete media were trypsinized for 2 minutes with 0.05% trypsin/EDTA. Cells were resuspended in complete media and centrifuged in a 15 mL tube at 800 rpm for 5 minutes. Media was aspirated, leaving only the cell pellet in the tube. While on ice, the cell pellet was resuspended in 1 mL of culture media containing heat-inactivated (HI) serum (DMEM and 5% heat-inactivated fetal bovine serum) and 2 ug/mL of primary antibody. The cells were incubated in primary antibody on ice for 1 hour. Negative controls were incubated in the HI serum media. Cells were then spun down and the media was aspirated. Cells were rinsed with 10 mL of HI serum media using the cold centrifuge, and the media was aspirated. The cell pellet was resuspended in a 1:200 dilution of fluorescent secondary antibody and 1 mL of HI serum media. Incubation in secondary antibody occurred for 1 hour on ice. Cells were spun down again, and media was aspirated before the final rinse in HI serum media. The media was aspirated, and cells were resuspended in 1 mL of cold HI serum

media. The cell solution was filtered through a blue filter cap into a FACs tube and analyzed.

Cell lines used in fixed and live flow cytometry:

- GSC2016-4 ITGA6 (+) P15
- U118/L1LE

2.3 Injection and dissection experiments

Fertilized White Leghorn chicken eggs were obtained from the Department of Animal and Food Sciences at the University of Delaware and incubated at 37.5˚C in a Humidaire incubator. Embryonic ages of the chick eggs were kept track of by how long they were incubated. Embryonic day 0 or E0 eggs refer to the first day eggs were received and placed in the incubator. Eggs were taken out of the incubator at E5 to be injected with different GSC and GBM cell mixtures. GSC lines were transfected with a lentiviral vector that encodes for green fluorescent protein, while GBM cell lines were transfected with fluorescent marker, mCherry. Prior to injecting, the eggs were sterilized with 70% ethanol. Once sterilized, a small hole outlining the air pocket on the blunt side of the egg was cut. The hole was covered with two pieces of tape, forming an x over the hole. After cutting a small hole in the tape, drops of serum-free DMEM was used to coat the air space membrane. The membrane was then removed with forceps and around 50,000 cells in 5uL were injected into the optic tectum using a glass micropipette and PV830 pneumatic picopump (World Precision Instruments). Before injection, cells were trypsinized, resuspended in Failsafe media, and counted using a hemocytometer. Cells were mixed with a small amount of 1% Fast Green FCF dye. Once injection was

complete, drops of 50 mg/mL ampicillin (Sigma Aldrich, Catalog #A9518) was put on top of the embryo. The hole in the egg was sealed again with tape, and all injected eggs were placed back into the incubator until E15. At E15, eggs were taken out of the incubator for dissection of the embryos. Embryonic brains were dissected, and regions of the brain (optic tectum, forebrain, and hindbrain) were fixed in a 24-well plate in 2% paraformaldehyde (PFA) in 0.1 M sodium cacodylate buffer (Electron Microscopy Science, Catalog #11652) and stored overnight at 4˚C. The next day, brain regions were rinsed in 1X PBS three times, each time for one hour. Brain regions were left in 1X PBS until sectioning.

Cell mixtures used in injection experiment:

- GSC2015-2/K72 (P33) + U118/L1LE mCherry 2x
- GSC2016-4/K72 (P20) + U118/L1LE mCherry 2x
- GSC2016-4/K72 (P24) + U118/1879 mCherry 2x
- GSC2016-4/K72 (P24) + U118/L1LE mCherry 2x

2.4 Vibratome sectioning

Brain regions from the injection experiment were screened for visible tumor formation based on GFP or mCherry positive cells with the Nikon SMZ stereo dissecting microscope equipped with epifluorescence. Regions with tumors were placed in aluminum foil molds and embedded in an agar solution (3.5% agar, 8% sucrose in 1X PBS) until the agar solidified. Excess agar from the block of agar containing the section was trimmed off and the remaining block was secured onto a stainless-steel square on a

dish using superglue. The dish was then filled with 1X PBS, submerging the agar block with the section. Using a Vibratome 3000, the brain region was cut into 350 um thick sections using half of a thin double-edge razor blade attached to the system. Brain sections were then screened for tumors again and placed in 10 cm dishes filled with 1X PBS to allow for easy handling when immunostaining brain sections. Dishes with brain sections were kept at 4˚C until immunostaining.

2.5 Live dissection and vibratome sectioning experiment

In preparation for handling live brain slices, all equipment was sterilized with 70% ethanol. 6-well plates with Transwell polyester membrane inserts (Corning, 0.4 um, 24mm diameter) in each well were also prepared with 1 mL of media in each well under the insert. Initial experimentation in testing for the most optimal media to culture live brain slices in consisted of three different types of media: 1) 50% Medium 199 (Gibco, #11150-059) + 25% Hank's Balanced Salt Solution (Corning, #21-020-CV) BSS + 25% horse serum (Gibco, #2025-03) + penicillin-streptomycin + L-glutamine + 15mM HEPES buffer, 2) 50% MEM (Corning, #10-010-CV) + 25% HBSS + 25% horse serum + penicillin-streptomycin + L-glutamine + 15mM HEPES buffer, and 3) 50% Opti-MEM (Gibco, #31985-047) + 25% HBSS + 25% horse serum (Gibco) + penicillin-streptomycin + L-glutamine + 15mM HEPES buffer. The plates with media were placed on ice until brain slices were ready to place onto the inserts. E15 embryos were dissected for optic tectum and forebrain regions. Once dissected, brain regions remained in a 60 mm dish on ice for the remainder of the process. Prior to dissecting, a 4% low melting point agarose

solution was prepared in 1X HBSS and kept in a 40˚C water bath to maintain liquid form (Yang et al. 2019). Dissected brain regions were placed into aluminum foil molds and embedded in the agarose solution. Embedded brain regions were placed on a 10 cm dish on ice until sectioning on the Vibratome. Excess agarose from the block of agarose and section was trimmed off and the remaining block was secured onto a stainless-steel square on a 10cm dish using superglue. Ice was constantly refilled in the reservoir space underneath the dish in the Vibratome 3000 to keep the brain slices chilled and alive while slicing. The dish with the agarose block glued to the SS square was then filled with Medium 199 (50% Med199 + 25% HBSS + 25% horse serum + penicillin-streptomycin + L-glutamine + 15mM HEPES buffer), submerging the block with the section. Sections were sliced into 350um slices in this media using a sapphire knife (Delaware Diamond Knives) attached to the Vibratome 3000 system. 2-3 slices were then carefully placed onto the membrane inserts of the pre-prepared 6-well dish while on ice as according to the Stoppini protocol (Stoppini et al. 1991). Any excess media on the brain slices or membrane insert was removed using a micropipette, and the brain slices on inserts in the 6-well plate were incubated at 37˚C in 5% CO_2 for 24 hours. After 24 hours, media in the wells under the inserts was replaced with fresh media and replenished once every 2 days (Yang et al. 2019). Brain slices were observed until flattening of the brain slices and attachment to the membrane. Flattening of the brain slices on the membrane inserts is an indication of healthy slices (Humpel et al., 2015).

After confirmation of the best media in the initial testing experiment, out of the three media, MEM media (#2 above) was the only media used for live brain slice culturing in following experiments.

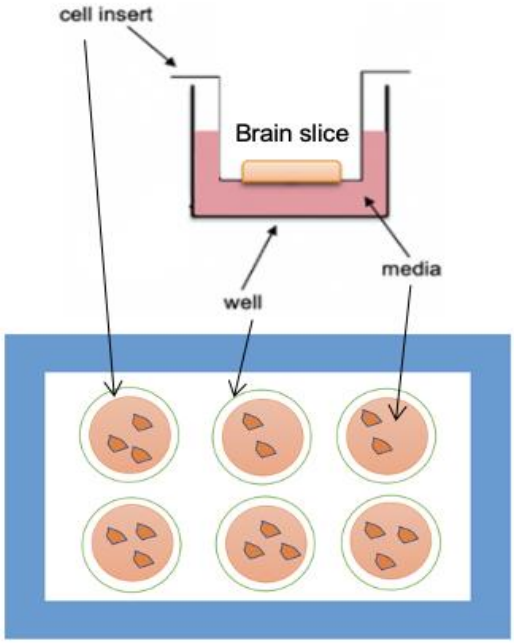

Figure 6. 6-well insert plate diagram.

2.6 Implanting cells into brain slices

Once brain slices have attached and flattened out onto the polyester membrane insert in a 6-well plate (approximately 5 days after placing the brain slices into the membrane inserts), cells were ready to be implanted into the center of the brain slices. Connecting a sterile 1mm biopsy punch pen (Robbins Instruments, #20335) to the aspirator tube, the pen was directly set down on the center of a brain slice to allow a 1mm hole to be cut out with aspiration of the tissue. The biopsy pen should not be pushed down too hard as this will puncture a hole into the membrane insert. After the holes were

created in each brain slice, a 60%-70% confluent 10 cm dish of cells was trypsinized and resuspended in complete media. The cell solution was centrifuged, and the media was aspirated. The cells were resuspended and mixed thoroughly in 10 mL of complete media, of which 1 mL was used and mixed with 100 uL of Matrigel (Corning, Reference #354234) to make a 10% Matrigel mixture containing the cells. Matrigel was thawed on ice prior to mixture with the cell solution. Taking a micropipette, 1uL of the 10% Matrigel mixture was placed into the holes of the brain slices (Figure 7). After each placement, the brain slice culture should be returned to the incubator to allow the Matrigel to solidify after a few minutes.

This cell injection method was not compatible with the 35 mm glass-bottom dish (FluoroDish, World Precision Instruments, FD35-100) and Millicell cell culture inserts made of polytetrafluoroethylene or PTFE (Sigma-Aldrich, 0.4um, 30mm Diameter). Brain slices did not attach to those membrane inserts in a reasonable time, causing the biopsy pen to aspirate the whole brain slice instead of puncturing a hole as seen with the brain slices on the 6-well insert membrane.

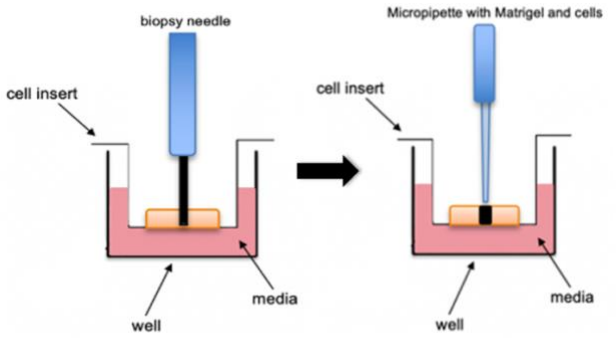

Figure 7. Implanting cells into brain slices. After a hole is created in the center of the brain slice using a biopsy pen (left), 1uL of the 10% Matrigel mixture was placed into the hole.

2.7 Organotypic slice culture using 35 mm glass-bottom dishes with inserts

Live brain slices were also cultured in FluoroDish 35 mm glass-bottom dishes (World Precision Instruments) with or without an additional PTFE membrane insert. 10 days after culturing the live brain slices on the polyester inserts in 6-well insert plates, and 5 days after cells were injected into the brain slices (11 days total), brain slices were cut out from the large polyester membrane insert, but the piece of membrane underlying the brain slice was left attached to it. When cutting out the brain slices, the membrane was kept as much as possible to allow enough weight and area to secure the brain slices down on the glass bottom dish. In the first condition with the 35 mm glass-bottom dish, injected brain slices were cut out from the polyester membrane insert and placed directly onto the glass-bottom dish. Around 1 mL of MEM media covered the top of the brain slice, and the dish was sealed with Vaseline around the rim of the lid to retain moisture in the dish. In the second condition, injected brain slices were cut out from the polyester membrane insert and placed on top of the PTFE membrane insert for the 35 mm glass-bottom dish. This insert, with the brain slice and polyester membrane, was then placed in the 35 mm glass bottom-dish, creating a condition where the brain slice was on two different membrane inserts. 1.6 mL of MEM media filled the glass-bottom dish under the PTFE insert. The dish was sealed with Vaseline around the rim of the lid to retain moisture in the dish. Both dishes were incubated at 37˚C in 5% CO_2 until used for time lapse.

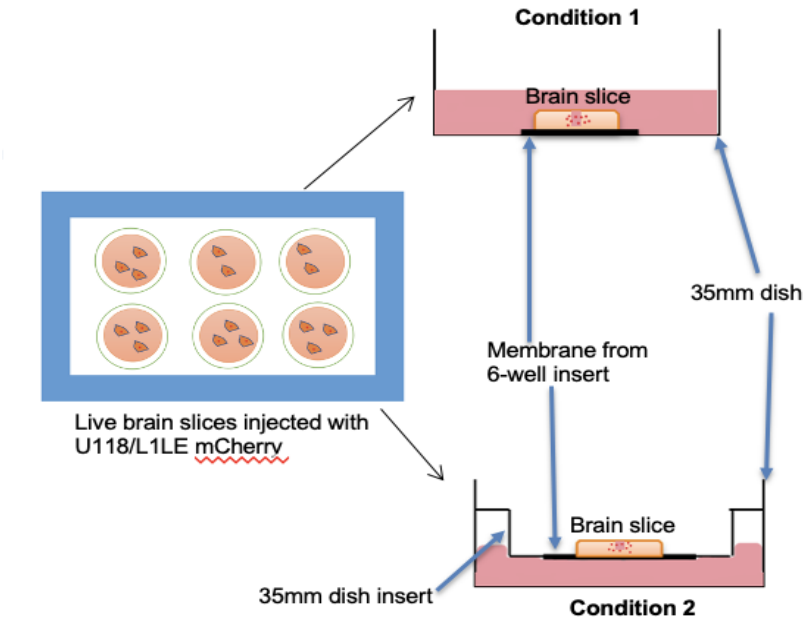

Figure 8. Two different conditions in 35mm dish. Condition 1 submerges the brain slice on membrane in the media (top). Condition 2 placed the brain slice on the membrane on the insert of the 35mm dish, with media under the insert (bottom).

2.8 Immunostaining brain sections

The 350 um slices from fixed brains and from post-time-lapse analysis of live sections were immunostained using antibodies against specific proteins of interest. Depending on the primary antibody used, immunostaining took anywhere from 2 to 4 days. On Day 1, the agar or membrane surrounding the brain slice was first trimmed to allow each brain slice to fit in a well of a 24-well plate. To facilitate the process of putting brain slices into each well, 1X PBS was added to fill up each well prior to placing the fixed brain slices into the wells. After placing the brain slices into the wells, the 1X PBS was removed and replaced with 350 uL of primary antibody solution per well, which consisted of 2 ug/mL of primary antibody in PBSTG (1X PBS, 0.1% triton X, and 5% normal goat serum). Brain slices were left to incubate in primary antibody solution for

24 hours at 4˚C in constant gentle agitation. Negative controls were incubated in PBSTG. On Day 2, slices were rinsed three times in PBSTG, for 1 hour each time. Slices were then incubated in 350 uL of a 1:200 dilution of secondary antibody and PBSTG for 20 hours at 4˚C in constant agitation. Depending on whether the primary antibody was directly conjugated, or the staining protocol only called for primary and secondary antibodies, the slices would be mounted onto slides on Day 2 or 3 respectively. If mounted on Day 2, slices would be rinsed 2 times in PBSTG for 1 hour each time, and the last rinse would be in 1X PBS for 1 hour. All rinses are also in 4˚C in constant agitation. On Day 3, brain slices were rinsed three times, 1 hour each, with PBSTG. After rinsing, they were left to be incubated in 350 uL of a 1:250 dilution of tertiary antibody/streptavidin in PBSTG. If brain slices were to be mounted on Day 3, slices would be rinsed 2 times in PBSTG, for 1 hour each time, and the last rinse would be in 1X PBS for 1 hour. On Day 4, slices were rinsed 2 times in PBSTG, for 1 hour each time, and the last rinse was in 1X PBS. Slices were mounted onto slides in wells created by layering two pieces of electrical tape (adding up to approximately 254 um in thickness) on top of each other and punching a square hole in the center. The layered tape was then placed on top of the slide and the brain slice was wicked of excess PBS with filter paper and placed into the center of the square hole. Mounting media (N-Propyl Gallate + 80% glycerol) was added on top of the brain slice and in the square hole. The brain slice was covered with a 22 x 40 mm no. 1.5 coverslip, which was then sealed with nail polish around the periphery. Slides with brain slices were kept at 4˚C until imaged.

Bisbenzimide was added on the day prior to mounting to stain for total cell nuclei. In addition to the antibody the brain slices were incubated in on that day, brain slices were incubated in 10 ug/mL of bisbenzimide solution.

Table 5. Staining protocols for different antibodies

Day	Staining for Sox-2	Staining for laminin	Staining for L1CAM, N-cadherin, and ADAM10
Day 1	2 ug/mL anti-Sox-2 10 ug/mL Bisbenzimide	2 ug/mL anti-laminin	2 ug/mL anti-L1CAM 2 ug/mL anti-N-cadherin against ectodomain and cytoplasmic domains 2 ug/mL anti-ADAM10
Day 2	Mount	1:200 AlexaFluor647-conjugated GAM IgG 10 ug/mL Bisbenzimide	1:200 Biotin-SP-conjugated AffinitiPure GAM IgG
Day 3		Mount	1:250 AlexaFluor647-conjugated Streptavidin 10 ug/mL Bisbenzimide
Day 4			Mount

2.9 Confocal imaging

Brain slice sections and live cells immunostained on coverslips were analyzed via a confocal microscope system. This consisted of a Nikon C2si+ system with an Eclipse Ti2-E inverted microscope with Plan Apo objectives, and Nikon's acquisition and analysis software, NIS-elements (Nikon Instruments, Version 5.20.02). This software was used to detect 4 colors with specific wavelengths. Acquisition of z-stacks, or a compilation of images over a range of focal distances, allowed production of a composite image with the software. Images could also be displayed as a movie of the entire z-stack or a 3-D volume render of the imaged tumor. For live cells immunostained on coverslips, only 2-D images were taken at a specific z-plane. Imaging brain slice sections involved the 2-D images along with the acquisition of z-stacks using the confocal system.

One time-lapse experiment was performed using the confocal microscope to collect z-stacks over time. For this, a new environmental chamber system was designed and fabricated to ensure brain slices were kept at 37˚C and 5% CO_2 over the course of the experiment. Brain slices were kept in a glass bottom 35 mm dish on PTFE membranes and submerged in media. Images were acquired in z-stacks at 2.40 um intervals approximately every 10 minutes for 20 hours using a Nikon S Plan Fluor ELWD 20x/0.45 DIC objective to accommodate the needed increased working distance.

2.10 Time-lapse with the wide-field microscope

To visualize the movement of cells in live brain slices for both the 6-well insert plate and the two conditions of the 35 mm glass-bottom dish, the time-lapse imaging

system was used as originally described by Fotos et al. (2006). Time-lapse imaging of migrating cells in the live brain slice was performed using a customized incubator chamber. Stage movement was controlled by an adjustable ProScan II automated stage (Prior Scientific, Rockland, MA). Time-lapse takes between 20 to 24 hours, and during this time, the brain slice cultures were maintained at atmospheric conditions of 5% CO_2 / 95% air and 37˚C using a gas injection controller (Forma Scientific, Marietta, OH), a warm air temperature controller (Air Therm, World Precision Instruments), and a temperature-controlled stage insert (Tokai Hit, Shizuoka-ken, Japan). Cell images were captured via a Retiga CoolSnap ES CCD camera (Photometrics, Tucson, AZ). The time-lapse system was controlled by MetaMorph software (version 7.8.12.0; Molecular Devices Corporation, Downingtown, PA). Cells were tracked using the "Track Points" application of MetaMorph. After time-lapse, brain slices were immunostained.

Table 6. Antibody information

Antibody	**Company**	**Catalog #**	**Type of antibody**	**Experiment**
L1CAM (UJ127) mouse monoclonal IgG_1	Santa Cruz	sc-53386	Primary	Immunostaining brain slices
N-cadherin (8C11) ectodomain mouse monoclonal IgG_1	Santa Cruz	sc-53488	Primary	Western blot, immunostaining cells on fixed and live coverslips, immunostaining on brain slices, fixed and live flow cytometry
N-cadherin (H-2) cytoplasmic mouse monoclonal IgG_1	Santa Cruz	sc-393933	Primary	Western blot, immunostaining cells on fixed coverslips,

				immunostaining on brain slices, fixed flow cytometry
ADAM10 (B-3) mouse monoclonal IgG_1	Santa Cruz	sc-28358	Primary	immunostaining on brain slices
Laminin-1 (3H11-s, IgG_1)	DSHB	F1NM47	Primary	Immunostaining on live brain slices
Sox-2 (E-4) AlexaFluor 546 direct conjugated, IgG_1	Santa Cruz	sc-365823	Primary	Immunostaining on live brain slices
Peroxidase-conjugated AffiniPure GAM IgG	Jackson ImmunoResearch	115-035-062	Secondary	Western blot
AlexaFluor 488 conjugated AffiniPure GAM IgG	Jackson ImmunoResearch	115-545-146	Secondary	Immunostaining on brain slices for laminin, fixed and live flow cytometry, immunostaining cells on fixed and live coverslips
Cy3-conjugated AffiniPure GAM IgG	Jackson ImmunoResearch	115-165-166	Secondary	Immunostaining cells on fixed and live coverslips
AlexaFluor647-conjugated AffiniPure GAM IgG	Jackson ImmunoResearch	115-605-146	Secondary	Immunostaining on brain slices for laminin
Biotin SP-conjugated AffiniPure GAM IgG	Jackson ImmunoResearch	115-065-146	Secondary	Immunostaining on brain slices for L1CAM, N-cadherin, and ADAM10
AlexaFluor647-conjugated Streptavidin	Jackson ImmunoResearch	016-600-084	Tertiary	Immunostaining on brain slices for L1CAM, N-cadherin, and ADAM10

Chapter 3

RESULTS

3.1 N-cadherin expression in GSC and GBM cells

3.1.1 N-cadherin was expressed in GSC and GBM cell lines using western blot analysis

Western blot analysis was used to detect N-cadherin expression in GSC and GBM cell lines with antibodies against the cytoplasmic and ectodomains. Results showed that N-cadherin was expressed using antibodies against both domains for the GSC and GBM cell lines tested. Cell lines used for N-cadherin detection included: U-118/L1LE, GSC2016-4 (P22), GSC2015-2 (P17), GSC2018-1A (P27), U-118/1879.

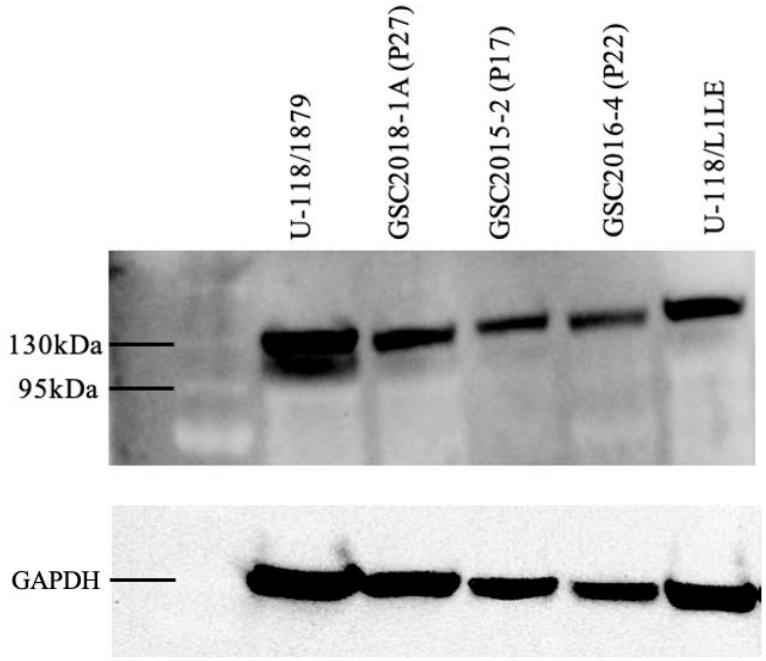

Figure 9. Western blot of GSC and GBM cell lines for N-cadherin detection for the antibody against the ectodomain. Protein ladder is in the first lane. GAPDH was used as a control.

All cell lines expressed full-length N-cadherin for the antibody against the ectodomain, showing a band between 130kDa and 135kDa (Figure 9). The U-118/1879 lysate appeared to have another band at around 120kDa, which is most likely due to processing of the protein via glycosylation or phosphorylation of the protein. This can be seen faintly in the other cell lines. The U-118 cell lines also appear to express more N-cadherin than any of the GSC lines as seen by comparing density of the bands at 130kDa.

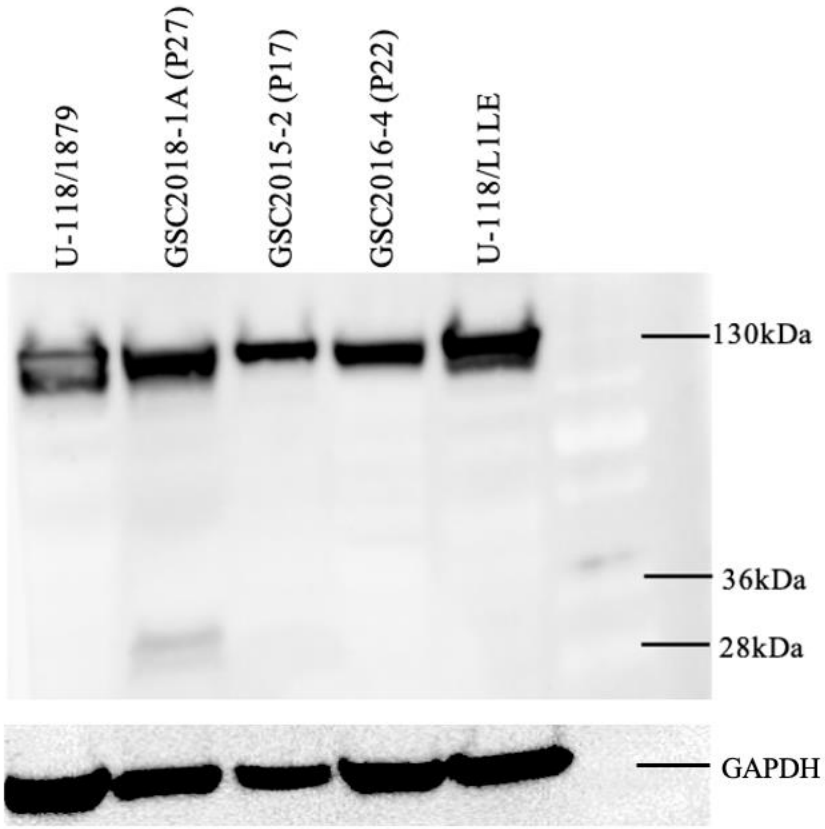

Figure 10. Western blot of GSC and GBM cell lines for N-cadherin detection for the antibody against the cytoplasmic domain. Protein ladder is in the final lane. GAPDH was used as a control.

Full-length N-cadherin was once again detected in all cell lines for the antibody against the cytoplasmic domain (Figure 10). Processing bands at around 120kDa were observed again in U-118/1879, GSC2018-1A (P27), and U-118/L1LE cell lines. This is most likely due to glycosylation or phosphorylation of the protein. Additionally, another band was seen at around 30kDa - 35kDa for GSC2018-1A cells. This may indicate cleavage by the γ secretase complex, which cleaves the CTF1 fragment of N-cadherin and releases the CTF2 fragment (Approximately 35kDa) into the cytoplasm.

3.1.2 N-cadherin was expressed in GSC and GBM cell lines in fixed/permeabilized cell coverslip staining

Cells grown and immunostained on coverslips were analyzed using a Plan Apo 20X/0.75 objective lens on a Nikon Eclipse E800 epifluorescent microscope. All images shown with U-118 cells are merged images where cells nuclei are shown in red, while protein immunostaining is shown in green. Images with GSC2016-4 cells are merged where cells nuclei are shown in green, while protein immunostaining are shown in red. All images were zoomed in by 3X to show cells in detail. The three cell types stained for N-cadherin with antibodies against the cytoplasm and ectodomains of N-cadherin were: U-118/L1LE mCherry 2x, U-118/1879 mCherry 2x, and GSC 2016-4/K72 (P26).

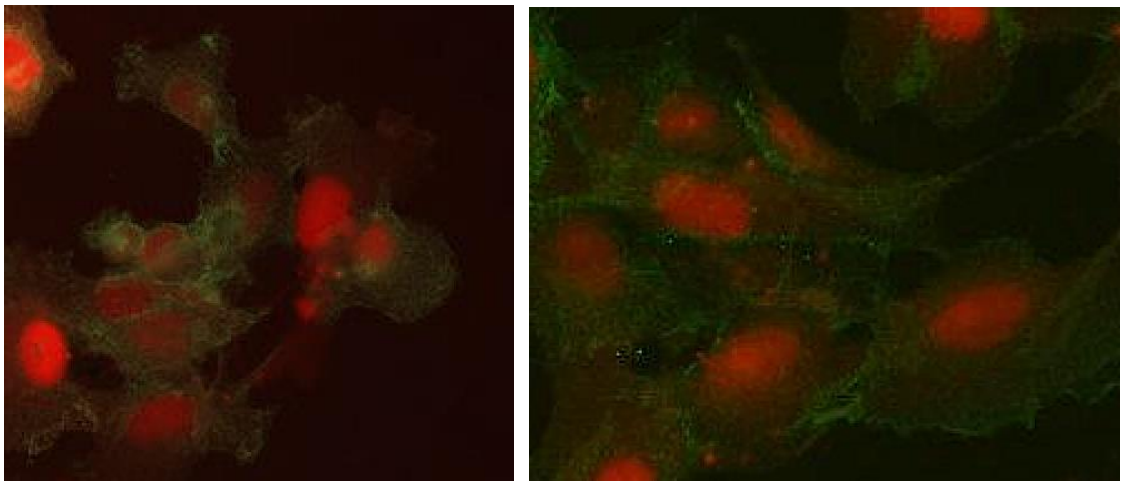

Figure 11. Fixed and permeabilized coverslip staining of U-118/L1LE mCherry 2x cells for N-cadherin expression. Antibodies were against the cytoplasmic (left) and ectodomains (right) of N-cadherin in U-118/L1LE mCherry 2x cells.

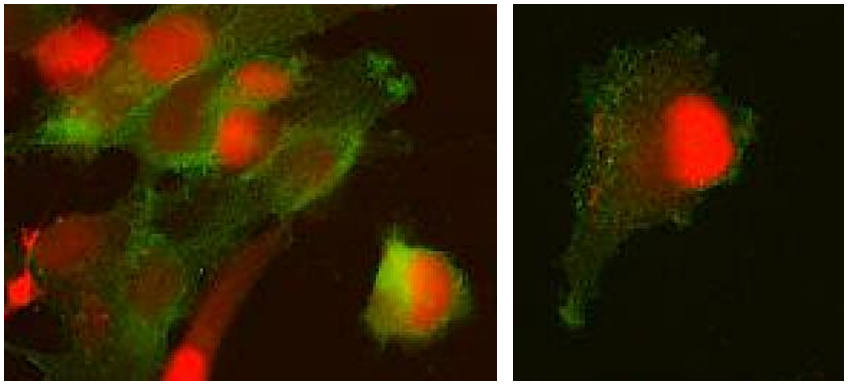

Figure 12. Fixed and permeabilized coverslip staining of U-118/1879 mCherry 2x cells for N-cadherin expression. Antibodies were against the cytoplasmic (left) and ectodomains (right) of N-cadherin in U-118/1879 mCherry 2x cells.

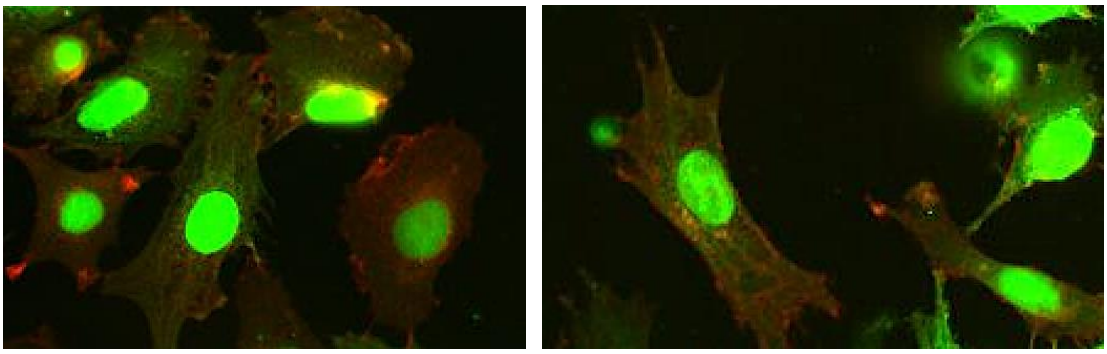

Figure 13. Fixed and permeabilized coverslip staining of GSC2016-4/K72 (P26) cells for N-cadherin expression. Antibodies were against the cytoplasmic (left) and ectodomains (right) of N-cadherin in GSC 2016-4/K72 (P26) cells.

N-cadherin immunoreactivity was clearly seen in all three cell lines for antibodies against the cytoplasmic and ectodomains (Figures 11-13). This supports expression seen in the western blot analysis for these three cell lines.

3.1.3 N-cadherin was expressed in GSC and GBM cell lines in live cell coverslip staining

N-cadherin was analyzed as a potential marker protein on the cell surface in three cell lines. Live cell staining for N-cadherin was conducted on ice for GSC2016-4/K72 (P40), U-118/L1LE mCherry 2x, and U-118/1879 mCherry 2x. All images with U-118 cells are merged images where cell nuclei are shown in red, while the immunostaining of N-cadherin is shown in green. Images with GSC2016-4 cells are merged images where cell nuclei are shown in green, while the immunostaining of N-cadherin is shown in red. The GSC2016-4 cells are shown both merged and without the fluorescent nuclei for easy comparison and visualization of N-cadherin protein expression.

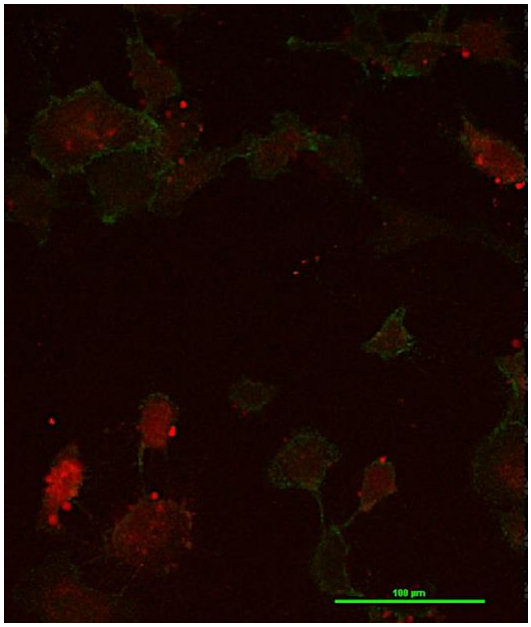

Figure 14. Live coverslip staining for N-cadherin expression in U-118/L1LE mCherry 2x cell line. File (11-5-21 N-cadherin 8C11 Alexa488 20x).

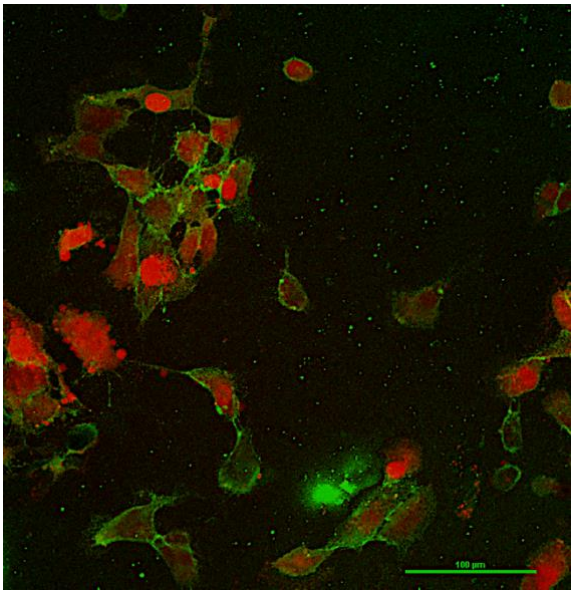

Figure 15. Live coverslip staining for N-cadherin expression in U-118/1879 mCherry 2x cell line. File (10-28-21 N-cadherin 8C11 Alexa488 20x).

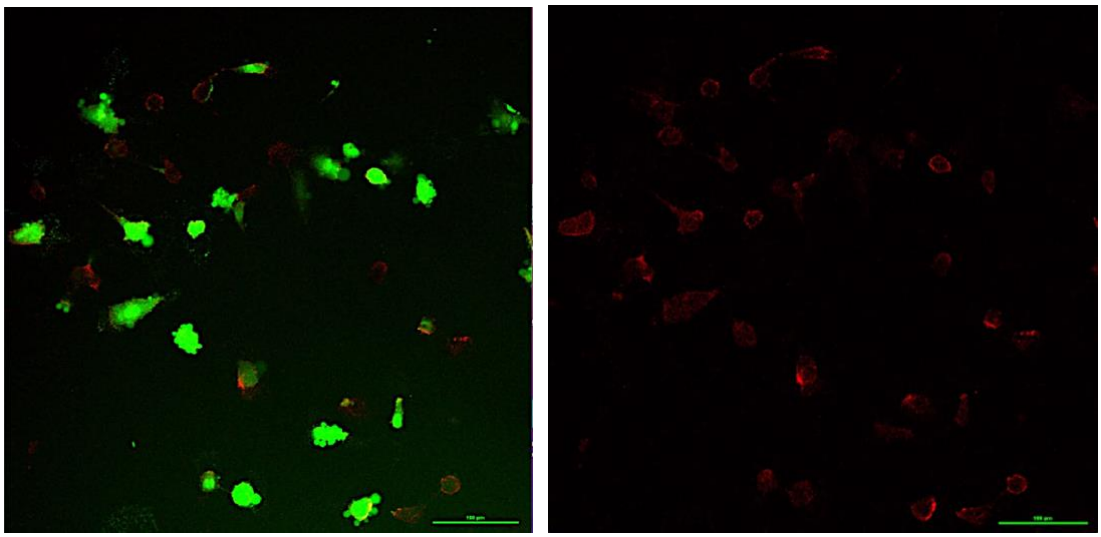

Figure 16. Live coverslip staining for N-cadherin expression in GSC2016-4/K72 (P40) line. Merged image (left) and image with only protein expression (right) are shown. File (11-5-21 N-cadherin 8C11 Cy-3 20x).

All cells showed N-cadherin expression after using for the antibody against the ectodomain for the three live GSC and GBM cell lines immunostained on ice (Figures 14-16). Despite the different passage number of the GSC 2016-4/K72 line vs. the fixed coverslip staining, no apparent difference in protein expression level was seen. The live cell staining also supported N-cadherin expression data analyzed in the western blot for these three cell lines.

3.1.4 U-118/L1LE and GSC2016-4 ITGA6 (+) P15 cells were positive for N-cadherin in fixed FACS analysis, but not live FACS analysis

Flow cytometry analysis of both fixed and live cells measured the expression level of N-cadherin in U-118/L1LE cells and GSC2016-4 ITGA6 (+) P15 cells using antibodies against the cytoplasmic and ectodomains of N-cadherin. Flow cytometry analysis of live cells only measured N-cadherin expression in the two cell lines for the

antibody against the ectodomain. Flow cytometry of fixed cells for N-cadherin expression with the antibody against the cytoplasmic domain in U-118/L1LE cells showed that 96.3% of the cell population was positive (Figure 17). In fixed cell flow cytometry using the antibody against the ectodomain, 6.8% of the cell population was positive for N-cadherin (Figure 18).

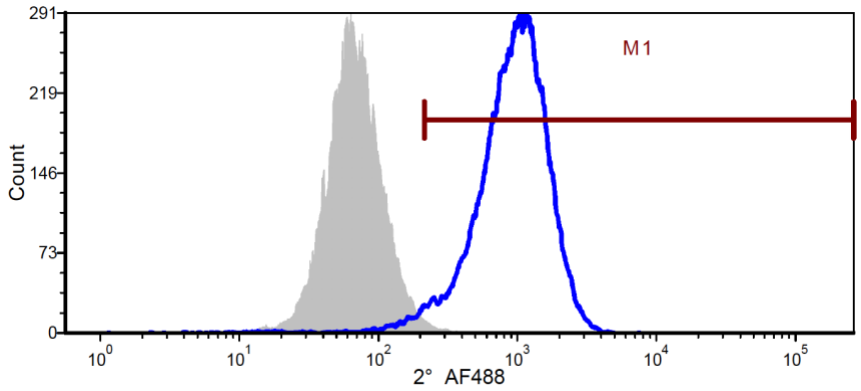

Figure 17. Fixed cell flow cytometry of U-118/L1LE cells for N-cadherin expression. Shaded curve shows cells incubated in secondary-control condition. Antibody was against the cytoplasmic domain of N-cadherin in U-118/L1LE cells. The M1 region denotes positive staining levels.

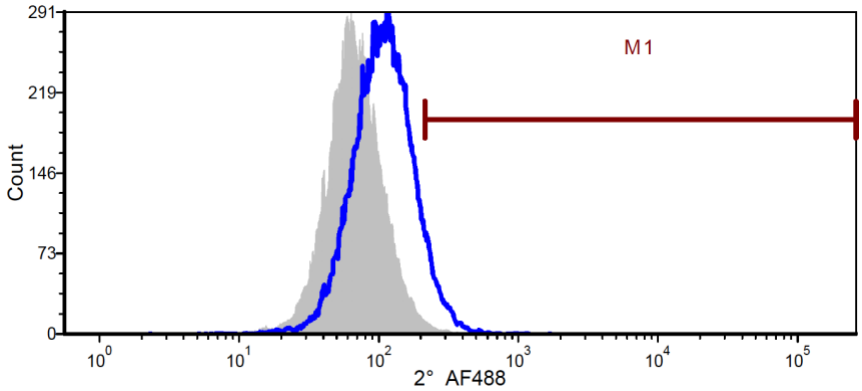

Figure 18. Fixed cell flow cytometry of U-118/L1LE cells for N-cadherin expression. Shaded curve shows cells incubated in secondary-control condition. Antibody was against the ectodomain of N-cadherin in U-118/L1LE cells. The M1 region denotes positive staining levels.

Fixed cell flow cytometry for N-cadherin expression using the antibody against the cytoplasmic domain of N-cadherin in GSC2016-4 ITGA6 (+) P15 cells showed that 92.5% of the cell population was positive for N-cadherin (Figure 19). In fixed cell flow cytometry using the antibody against the ectodomain of N-cadherin, 23.1% of the cell population was positive for N-cadherin (Figure 20).

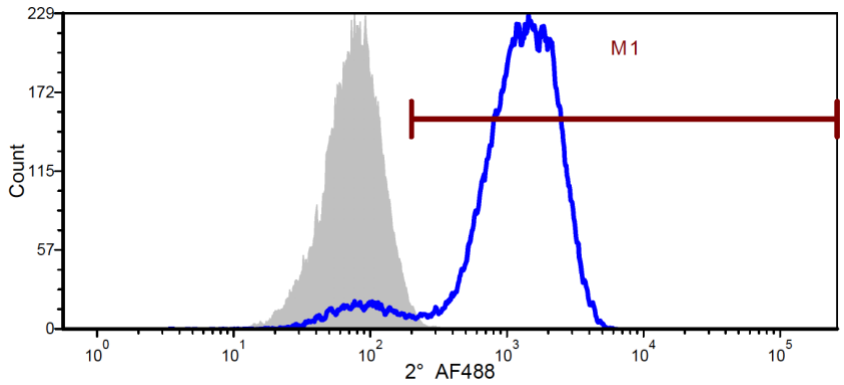

Figure 19. Fixed cell flow cytometry of GSC2016-4 ITGA6 (+) P15 cells for N-cadherin expression. Shaded curve shows cells incubated in secondary-control condition. Antibody was against the cytoplasmic domain of N-cadherin in GSC2016-4 ITGA6 (+) P15. The M1 region denotes positive staining levels.

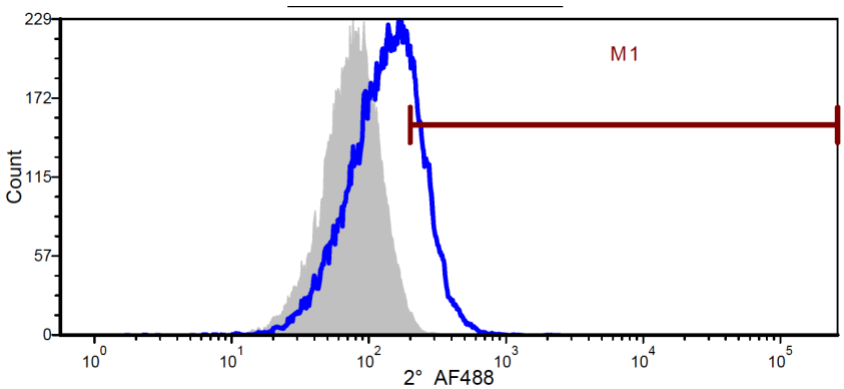

Figure 20. Fixed cell flow cytometry of GSC2016-4 ITGA (+) P15 cells for N-cadherin expression. Shaded curve shows cells incubated in secondary-control condition. Antibody was against the ectodomain of N-cadherin in GSC2016-4 ITGA6 (+) P15. The M1 region denotes positive staining levels.

Live cell flow cytometry for N-cadherin expression using the antibody against the ectodomain of N-cadherin in U-118/L1LE cells showed that 0.6% of the cell population was positive for N-cadherin (Figure 21). In live cell flow cytometry for the antibody against the ectodomain of N-cadherin for GSC2016-4 ITGA6 (+) P15, 0.6% of the cell population was positive (Figure 22).

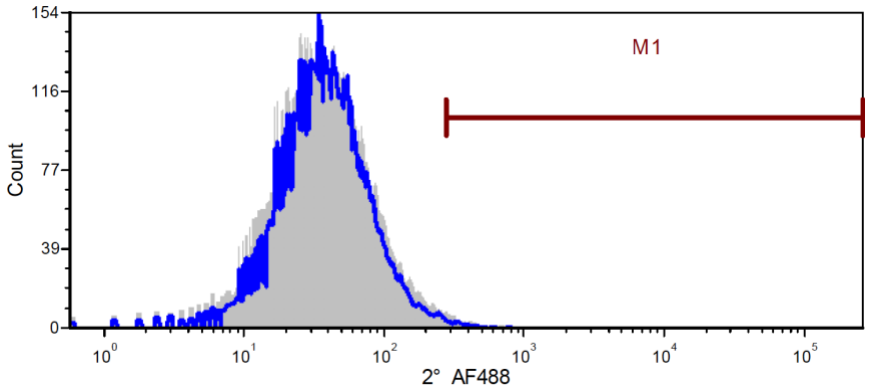

Figure 21. Live cell flow cytometry of U-118/L1LE cells for N-cadherin expression. Shaded curve shows cells incubated in secondary-control condition. Antibody was against the ectodomain of N-cadherin in U-118/L1LE cells. The M1 region denotes positive staining levels.

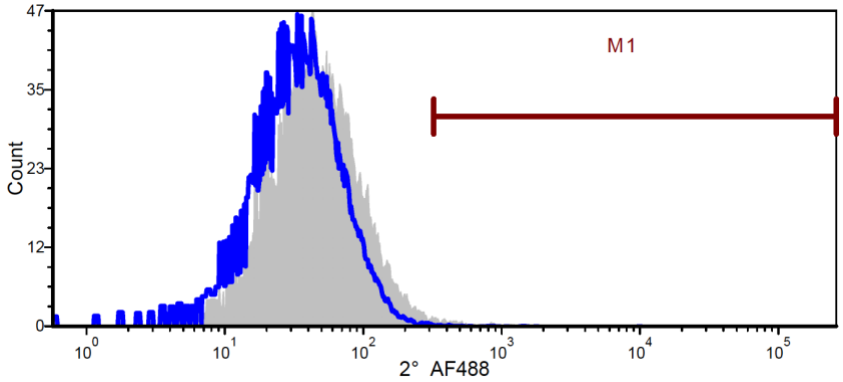

Figure 22. Live cell flow cytometry of GSC2016-4 ITGA6 (+) P15 for N-cadherin expression. Shaded curve shows cells incubated in secondary-control condition. Antibody was against the ectodomain of N-cadherin in GSC2016-4 ITGA6 (+) P15. The M1 region denotes positive staining levels.

N-cadherin expression levels using antibodies against the ectodomain of N-cadherin in both cell lines were low in both fixed and live flow cytometry analyses compared to the expression levels seen in western blot and coverslip analyses. From visual analysis of western blots and coverslip staining, expression of both cytoplasmic and ectodomains of N-cadherin appeared approximately equal. However, this was not the case for quantitative analysis by flow cytometry. N-cadherin expression using the ectodomain antibody was consistently lower than that when using the cytoplasmic domain antibody in fixed cell flow cytometry. In live cell flow cytometry, expression using both cytoplasmic and ectodomain antibodies were equally low.

3.2 Xenograft model experiments

Cell mixtures consisting of GSCs and GBM cells lines were injected into E5 embryo midbrains, and different protein expression in the cells of the resulting tumors were analyzed via immunostaining and confocal microscopy of vibratome sections. In a cell mixture, the GSC was either green GSC2016-4/K72 or green GSC2015-2/K72 while the GBM cell was either red U-118/L1LE mCherry 2x or red U-118/1879s mCherry 2x. The cell mixtures created and injected into embryos across different experiments were: GSC2015-2/K72 (P33) + U118/L1LE mCherry 2x, GSC2015-2/K72 (P33) + U118/1879 mCherry 2x, GSC2016-4/K72 (P20) + U118/L1LE mCherry 2x, GSC2016-4/K72 (P24) + U118/1879 mCherry 2x, GSC2016-4/K72 (P24) + U118/L1LE mCherry 2x. Of the two GSC2015-2 cell mixtures, all embryos from the GSC2015-2/K72 (P33) + U118/1879 mCherry 2x mixture did not survive for unknown reasons. GSC2015-2 cells also did not growth at as high a rate as did GSC2016-4 cells, which resulted in increased use of the

GSC2016-4 cells across experiments. The two GSC2016-4/K72 + U118/L1LE mCherry 2x cell mixtures had different passage numbers for GSC2016-4/K72, with one at passage 20 and the other at passage 24, which should be inconsequential. The two cell lines are the same but were used in different injection experiments to make the same GSC2016-4/K72 + U118/L1LE mCherry 2x mixture.

U-118/L1LE and U-118/1879 cells were visualized by the red channel (mCherry), while the GSCs are visualized by the green channel (GFP). The blue channel represents immunostaining for the protein of interest (L1CAM, N-cadherin, or ADAM10).

L1CAM immunostaining was analyzed in tumors from cell mixtures of GSC2015-2/K72 (P33) + U118/L1LE mCherry 2x cells, GSC2016-4/K72 (P20) + U118/L1LE mCherry 2x cells, and GSC2016-4/K72 (P24) + U118/1879 mCherry 2x cells. L1CAM expression was evident in tumors from the first two mixtures that contained U-118/L1LE cells (Figures 23 and 24), while no expression was seen in tumors from the GSC2016-4/K72 (P24) + U118/1879 mCherry 2x mixture (Figure 25). This was observed as no staining in the blue channel for this tumor mixture and only red and green cells appeared in the merged image.

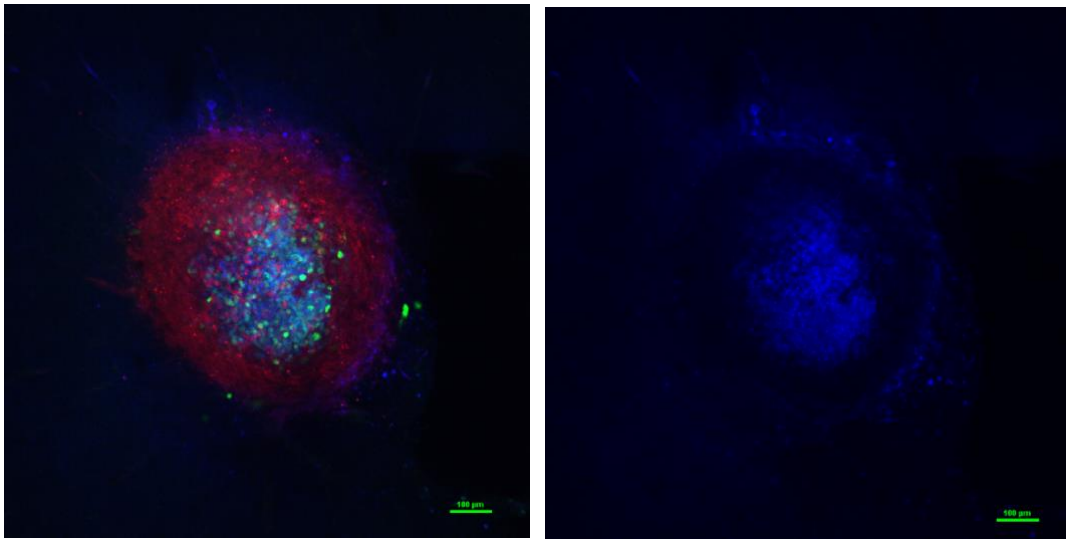

Figure 23. Confocal images of L1CAM immunostaining (in blue) in an optic tectum tumor formed from a mixture of GSC2015-2/K72 (P33) + U118/L1LE mCherry 2x cells. The merged image is on the left, while L1CAM staining only is on the right. File (6-29-21 inj #5b 4 color L1CAM 20x).

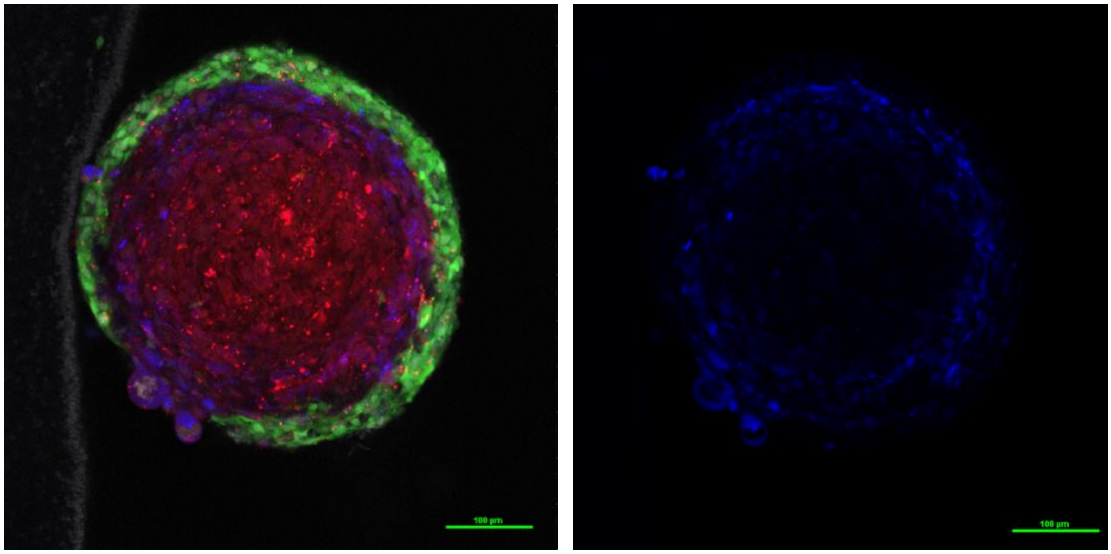

Figure 24. Confocal images of L1CAM immunostaining (blue) in an optic tectum tumor formed from a mixture of GSC2016-4/K72 (P20) + U118/L1LE mCherry 2x cells. The merged image is on the left, while L1CAM staining (blue) is on the right. File (6-29-21 inj #19a 4 color L1CAM 20x).

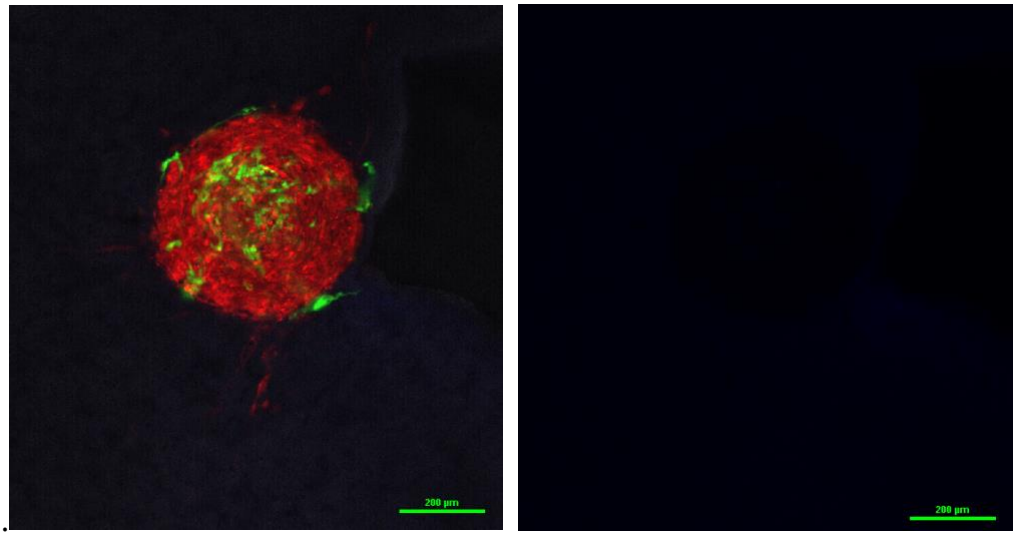

Figure 25. Confocal images of L1CAM immunostaining in an optic tectum tumor resulting from a mixture of GSC2016-4/K72 (P24) + U118/1879 mCherry 2x cells. The merged image is on the left, while L1CAM staining is not seen in the blue-only channel (right). File (11-18-21 inj #17a 4 color L1CAM 10x).

N-cadherin expression using the antibody against the ectodomain was analyzed in tumors resulting from mixtures of GSC2015-2/K72 (P33) + U118/L1LE mCherry 2x, GSC2016-4/K72 (P24) + U118/L1LE mCherry 2x, and GSC2016-4/K72 (P24) + U118/1879 mCherry 2x tumor cells. For all three mixtures, weak expression levels of N-cadherin were seen as the expression was only clear when looking at the blue (N-cadherin) channel and not merging the other channels (Figures 26 - 28).

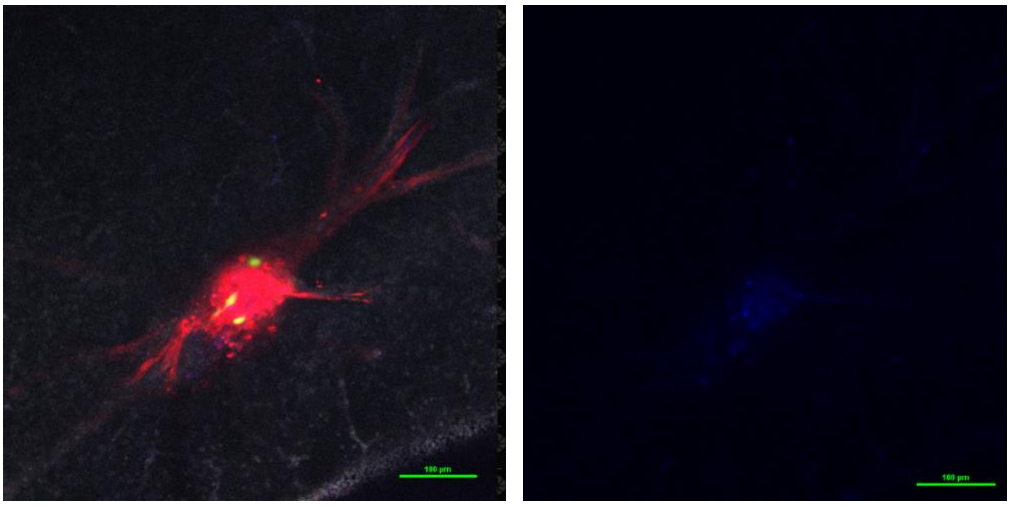

Figure 26. Confocal images of N-cadherin immunostaining in an optic tectum tumor resulting from a mixture of GSC2015-2/K72 (P33) + U118/L1LE mCherry 2x cells. The antibody was against the N-cadherin ectodomain. The merged image is on the left, while N-cadherin-only staining (blue) is on the right. File (6-29-21 inj #5d 4 color N-cadherin 20x).

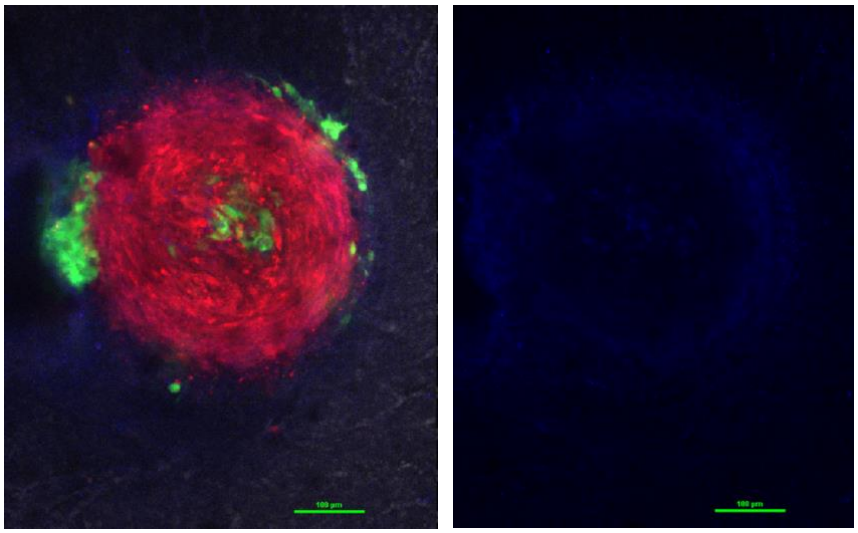

Figure 27. Confocal images of N-cadherin immunostaining in an optic tectum tumor resulting from a mixture of GSC2016-4/K72 (P24) + U118/L1LE mCherry 2x cells. The antibody was against the N-cadherin ectodomain. The merged image is on the left, while N-cadherin-only staining (blue) is on the right. File (11-18-21 inj #5b 4 color N-cadherin 10x).

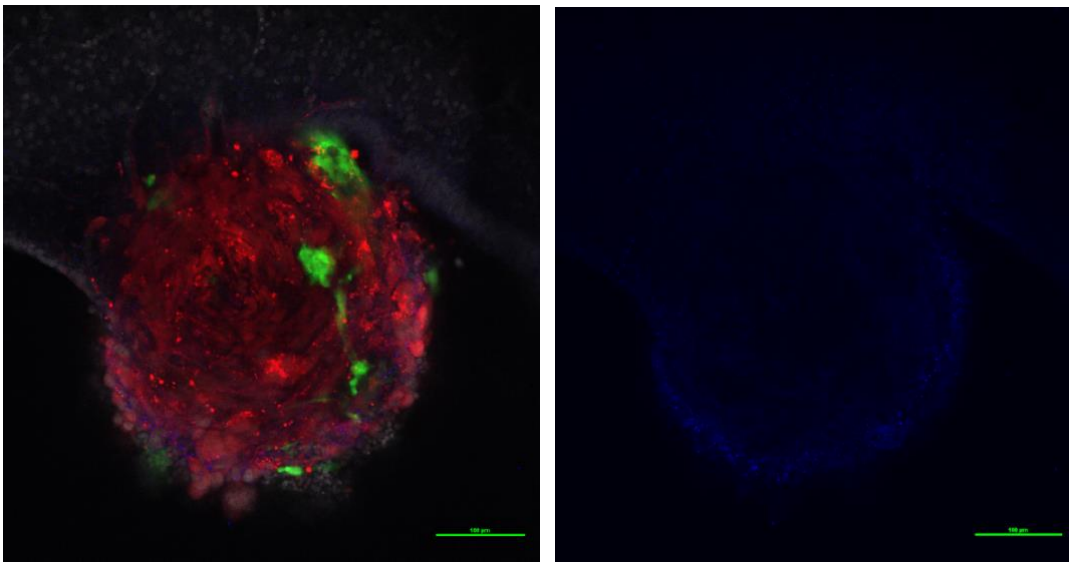

Figure 28. Confocal images of N-cadherin immunostaining in an optic tectum tumor resulting from a mixture of GSC2016-4/K72 (P24) + U118/1879 mCherry 2x cells. The antibody was against the N-cadherin ectodomain. The merged image is on the left, while N-cadherin-only staining (blue) is on the right. File (11-18-21 inj #17f 4 color N-cadherin 20x).

ADAM10 expression appeared only in tumors from GSC2015-2/K72 (P33) + U118/L1LE mCherry 2x cells (Figure 29). Specifically, ADAM10 expression was seen in the center of the tumor where GFP expression from GSC2015-2/K72 was highest. No ADAM10 expression was seen in the other two GSC2016-4 tumor mixtures as nothing was seen in the blue-only channel (Figures 30 and 31).

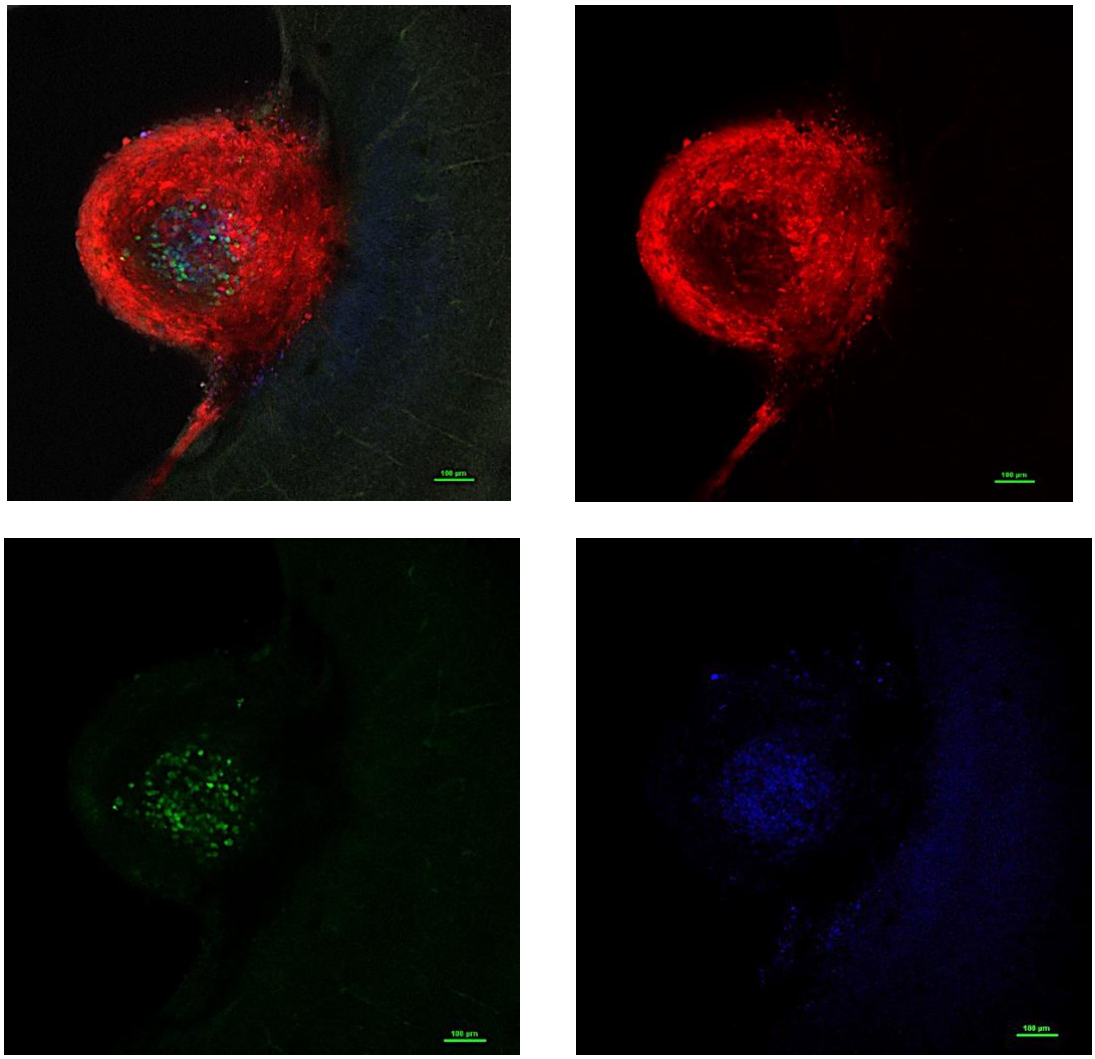

Figure 29. Confocal images of ADAM10 immunostaining in an optic tectum tumor resulting from a mixture of GSC2015-2/K72 (P33) + U-118/L1LE mCherry 2x cells. Top left shows the merged image with all channels. Top right shows mCherry expression in U-118 cells in the red channel. Bottom left shows GFP expression in GSCs staining in the green channel, Bottom right shows ADAM10 immunostaining in the blue channel. File (6-29-21 inj #6a 4 color ADAM10 10x).

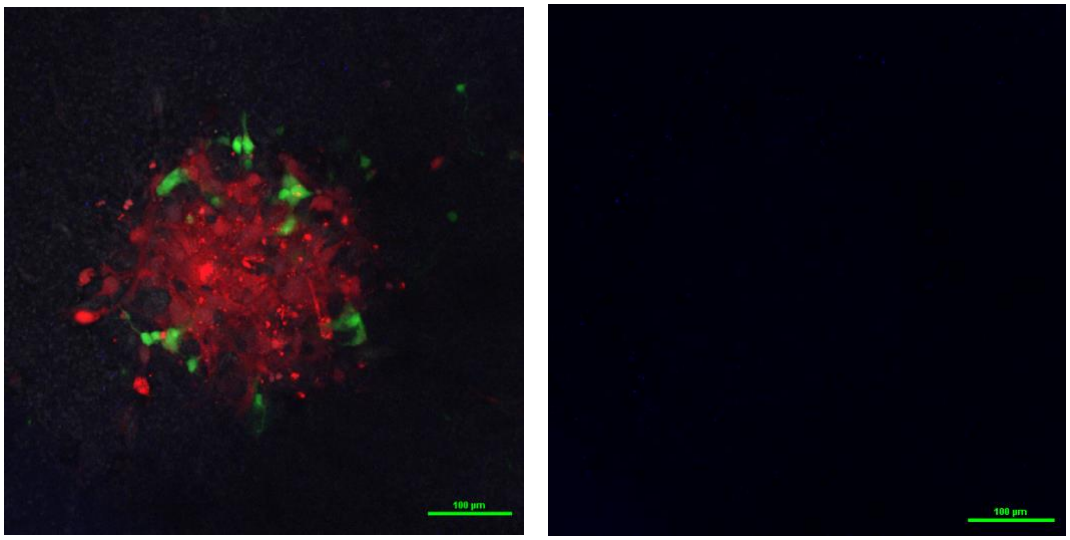

Figure 30. Confocal images of ADAM10 staining in an optic tectum tumor resulting from a mixture of GSC2016-4/K72 (P24) + U118/1879 mCherry 2x cells. The merged image is on the left, while ADAM10-only staining is not seen in the blue channel (right). File (11-18-21 inj #13c 4 color ADAM10 20x).

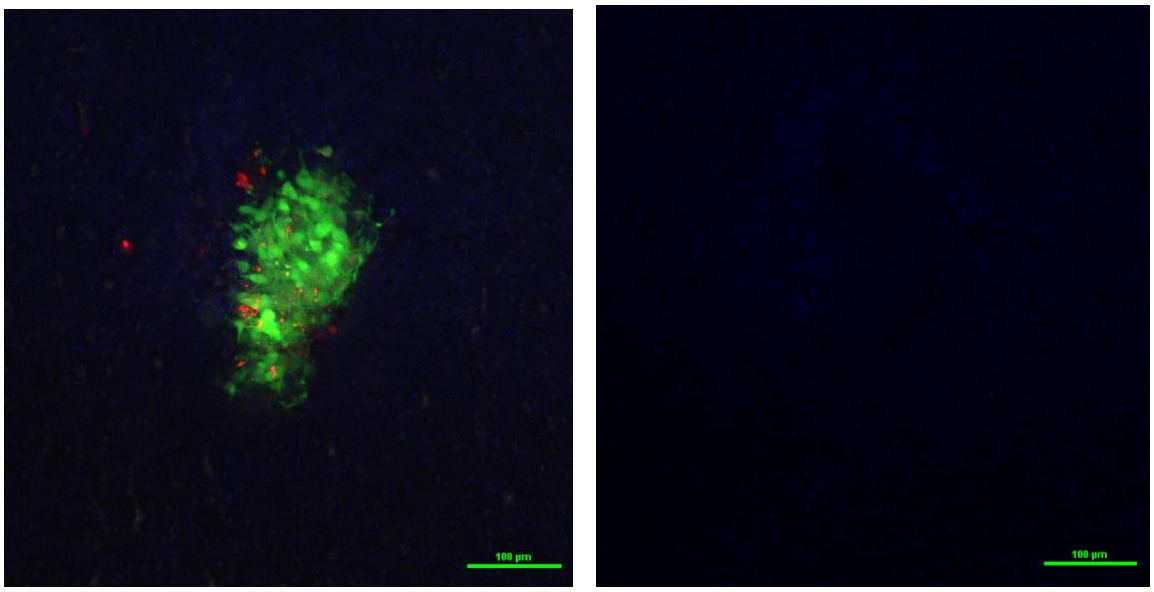

Figure 31. Confocal images of ADAM10 staining in an optic tectum tumor resulting from a mixture of GSC2016-4/K72 (P24) + U118/L1LE mCherry 2x cells. The merged image is on the left, while ADAM10 staining is not seen in the blue channel (right). File (11-18-21 inj #5c 4 color ADAM10 20x).

In a separate injection experiment, N-cadherin expression with the antibody against the cytoplasmic domain was only tested for in the GSC2016-4/K72 (P28) + U-

118/L1LE mCherry 2x mixture. The GSC2015-2/K72 and U-118/1879 mCherry 2x cell lines were not prepared due to time constraints and not having a plate of the cells prior to injection. No N-cadherin expression was seen with the cytoplasmic antibody in the cell mixture as shown by the absence of color in the blue-only channel (Figure 32).

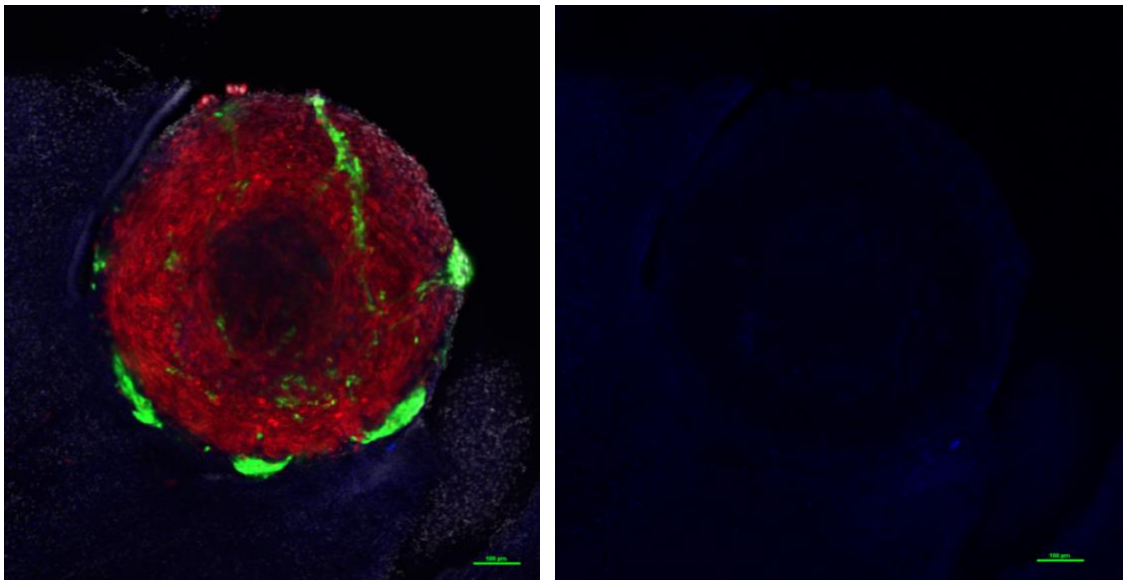

Figure 32. Confocal images of N-cadherin immunostaining in an optic tectum tumor resulting from a mixture of GSC2016-4/K72 (P28) + U118/L1LE mCherry 2x cells. The antibody was against the N-cadherin cytoplasm. The merged image is on the left, while N-cadherin staining is not seen in the blue channel (right). File (1-13-22 inj #14 OT 4 color N-cadherin 20x)

3.3 Immunostaining of live brain slice cultures

3.3.1 MEM resulted in the healthiest brain slices

In the initial experiment with live brain slice cultures, it was necessary to determine what media maintained the best condition of the brain slices. Brain slices were cultured in 6-well insert plates with either Medium 199, Opti-MEM, or MEM medium with various additives for 9 days. On the 10th day, brain regions were analyzed for blood vessel and brain viability via immunostaining for laminin and Sox-2 respectively and

confocal microscope imaging. Laminin expression was observed in the blue channel, while Sox-2 expression was observed using the red channel, with individual cells expressing Sox-2 appearing red. Total nuclei were also stained with bisbenzimide and imaged in the white channel. In Figure 33 below, blood vessels in brain regions that were cultured in Opti-MEM or Medium 199 appeared segmented and clumped rather than continuous. Sox-2 expression also appeared low in both types of media, especially in the brain slice cultured in Opti-MEM (Figure 34). Fewer cells are expressed Sox-2, shown by the greater number of black spaces or non-existent red nuclei. Out of all three types of media, MEM medium showed blood vessels that were continuous and intact with uniform expression of Sox-2 across all cells in the brain slice. With the other two types of media, it appeared as if the blood vessels had broken up and formed balls of endothelial cells that still expressed laminin. This suggested that culturing brain slices in MEM media (with additives) provided the best viability of the brain slice and the blood vessels during the 9 days of culturing compared to the other two media.

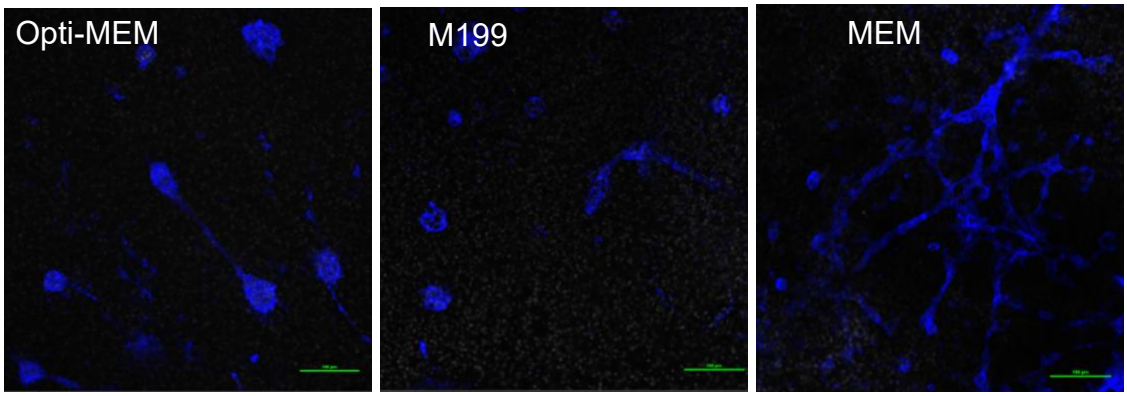

Figure 33. Confocal images of Laminin expression in the same brain regions after 9 days of culturing in the three different types of media. (8-27-21 dissection, laminin, 20x).

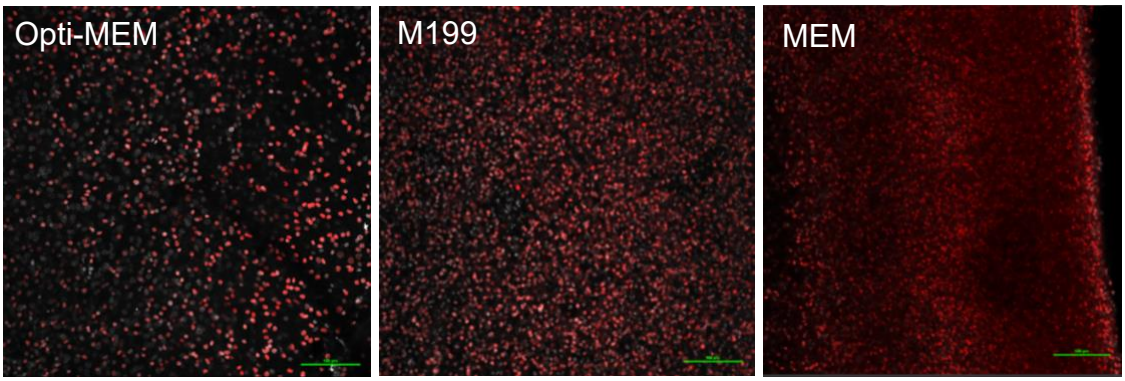

Figure 34. Confocal images of Sox2 expression in the same brain section as Fig. 33 after 9 days of culturing in the three different media. (8-27-21 dissection, Sox2, 20x).

3.3.2 MEM was optimal for live brain slice culturing

After observing that MEM was the best option in brain slice culturing out of the three media tested, a second dissection experiment was conducted to culture brain slices in MEM. E15 chick embryos were dissected and optic tectum and forebrain slices were cultured on a 6-well insert plate following the protocol mentioned. 6 days after culturing, several of the brain regions were stained for laminin and Sox2 to assess the conditions of the blood vessels and brain slice. The remaining brain slices were kept in culture until further use. As seen in Figure 35 and 36 below, the blood vessels in the brain region were in good condition as they were not balled up or segmented as seen previously in brain regions that were cultured in Opti-MEM or Medium 199. The brain slice condition was also good as determined by Sox2 expression seen in the majority of cells in the brain slice. Once again, MEM was considered as an appropriate option of medium for culturing brain slices.

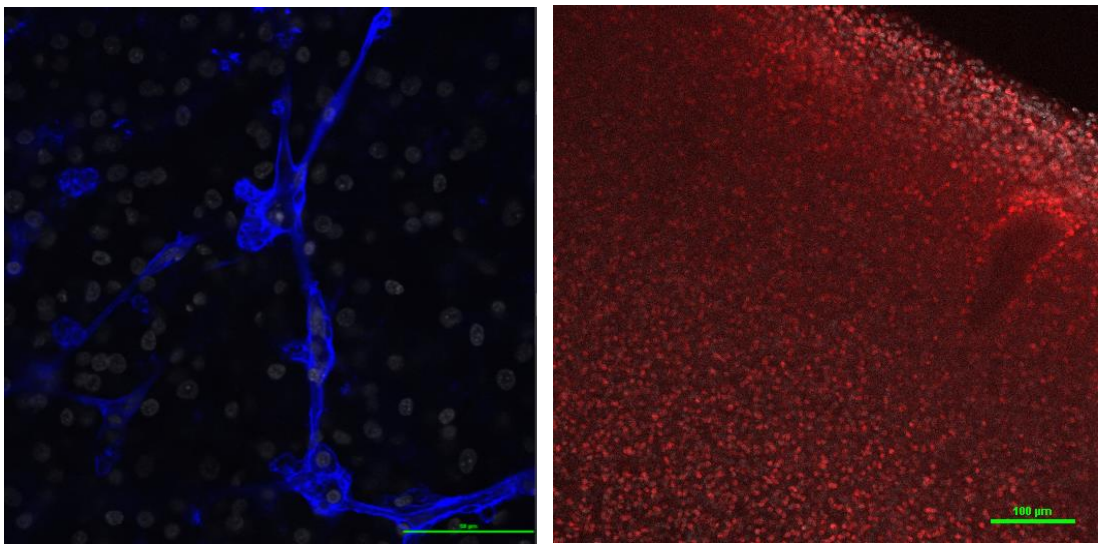

Figure 35. Confocal images of laminin and Sox-2 expression. Left panel, laminin expression in blood vessels of brain region after 6 days of culturing in MEM media (left, 9-15-21 dissection, laminin, 60x oil). Right panel, Sox-2 expression of same brain section after 6 days of culturing in MEM media (right, 9-15-21 dissection, Sox-2, 20x)

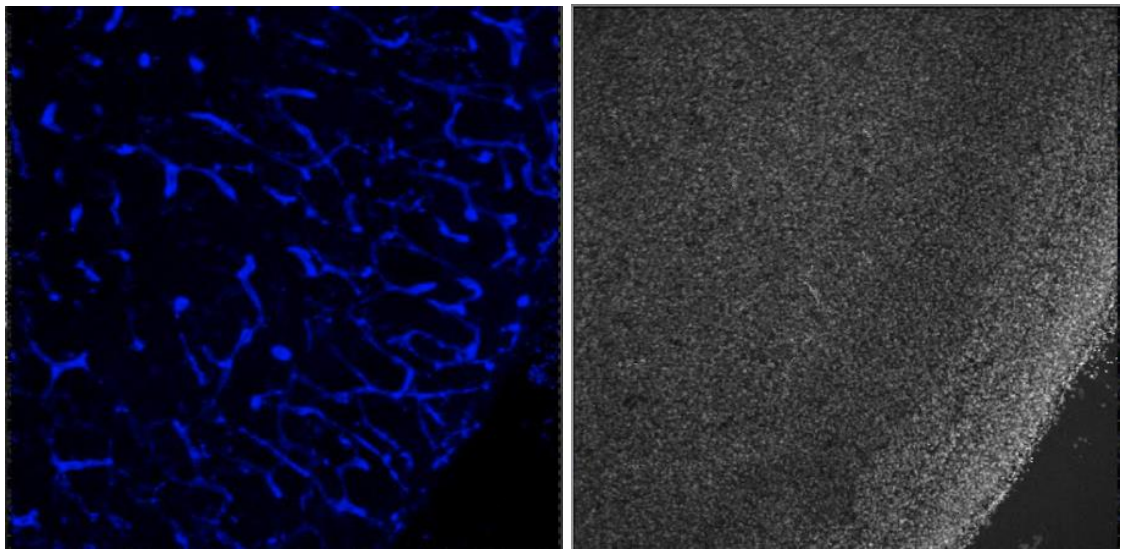

Figure 36. Confocal images of laminin and total nuclei. Left panel, laminin expression in blood vessels of brain region after 6 days of culturing in MEM media (left, 9-5-21 dissection, laminin, 10x). Right panel, nuclei expression of same brain region after 6 days of culturing in MEM media (right, 9-15-21 dissection, bisbenzimide, 10x)

3.3.3 U-118/L1LE cells align along blood vessels in live brain slices

After establishing the best medium for brain slice culturing, it was necessary to test if tumor cells would invade the brain slice along blood vessels and/or by other means. Using the remaining brain slices that were cultured in the previous experiment (14 days in culture), U-118/L1LE mCherry 2x cells were implanted into the center of each brain slice following the method described previously. Cells were cultured in the brain slice for another 5 days, fixing and staining them on the 6th day for laminin and to observe blood vessel configuration (19 days of brain slice culture, and 5 days of implanted cell culture). Staining results indicated that some implanted cells were potentially lining blood vessels as they invaded outwards from the initial 1mm implantation cavity hole as seen in Figure 37 below. Blood vessels in this experiment appeared slightly more segmented and not as continuous as previously seen in the two other brain slice culture experiments. This was most likely due to the 3-week timeframe the brains were cultured in. 3 weeks in culture may be too long of a timeframe to maintain live brain slices before they start deteriorating.

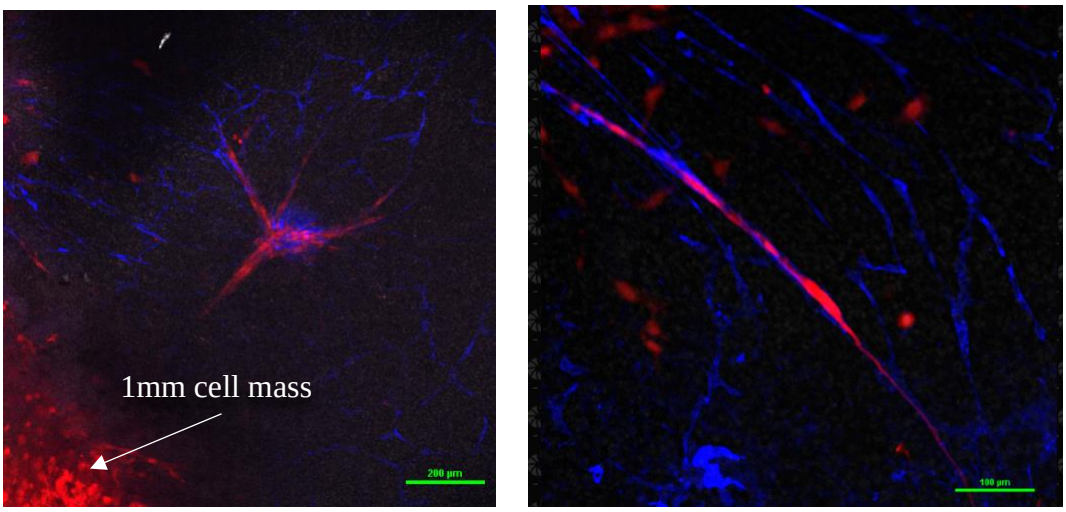

Figure 37. Confocal images of U-118/L1LE cells invading along apparent blood vessels in brain slices, beginning at the 1mm cavity where they were first implanted (left, 9-29-21 injection OT #4, laminin-647, 10x). U-118/L1LE cells invading along apparent blood vessel in another brain region (right, 9-29-21 injection OT #3, laminin-647, 20x).

With suggestions that some U-118/L1LE cells potentially invaded outwards from the cell mass of a brain slice along blood vessels, another experiment was conducted to implant cells into live brain slice cultures and analyze them at a shorter time frame. Previous analysis of staining showed that brain slices begin to deteriorate if cultured for 3 weeks, encouraging analysis of the live culture to occur within a 2-week time frame. E15 chick embryos were dissected, and optic tectum sections were sliced and put into the 6-well insert plate. One day after plating the brain slices into the inserts, they had not attached yet to the membrane and could still be removed. However, by day 2, brain slices had begun to attach to the membrane insert, making it difficult to remove them. By day 5, brain slices were completely attached to the membrane insert, allowing an optimal condition to aspirate a hole in the center of the brain slice with a biopsy pen and insert 1uL cells. 4 days after implantation or 9 days after brain slice culturing (10 days total), a 20-hour time-lapse was run using the widefield microscope with the slices on inserts in

the 6-well culture plate. Cell movement along potential blood vessel structures from the 1 mm cell mass (starting point) was seen in the video created by the time-lapse images.

After a time-lapse video was created, and movement of U-118/L1LE cells along apparent blood vessels was confirmed, slices were fixed to prepare for staining. Staining results showed that cells were migrating along the blood vessel from the initial 1mm cell mass (Figure 37). This was further elucidated because the blood vessel and cells were in the same focal plane based on z-stack images (Figure 38). Nuclei from the brain slice (white channel) were also found above and below the blood vessel and cells, indicating that the cells were invading within the brain slice.

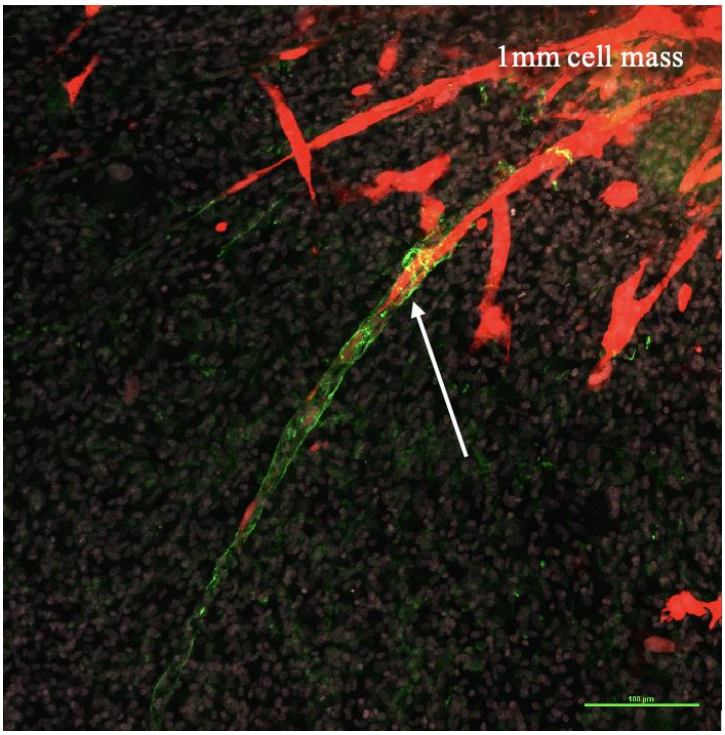

Figure 38. Confocal images of U-118/L1LE mCherry 2x cells (red) invading along blood vessel (green). (1-28-22 implantation, live brain slice slide #4, laminin-488, bis 20x).

3.3.4 U-118/L1LE cells align along blood vessels in 35mm glass-bottom dish brain slice cultures

Having run a time-lapse with only the plastic 6-well plate using the widefield fluorescence microscope, it was predicted that better quality results would show if time-lapse was run with the confocal microscope. To ensure the best possible image resolution with the confocal microscope, coverslip-bottom dishes were necessary. A new S Plan 20x objective lens was purchased to give the increased working distance and optical correction for light traveling through the Tokai Hit stage insert and coverslip-bottom dish, since the standard 20x Plan Apo lens was not adequate. The following day after time-lapse of the live brain slices in the 6-well insert plate, 2-3 brain slices from two inserts were cut out following the above-mentioned method to create two conditions using 35 mm coverslip-bottom dishes. At this point, the brain slices were cultured for 10 days, and cells had been cultured in the brain slices for 5 days (11 days total). In condition 1, brain slices attached to the membrane were directly placed on the coverslip-bottom dish, submerged in MEM media. In condition 2, the brain slices attached to the membrane were placed on the insert of the 35 mm dish to create a double layer of membrane and placed in the coverslip-bottom dish. MEM filled the bottom of the dish under the insert. Both conditions were time-lapsed for 20 hours with the confocal microscope and another 20 hours with the widefield microscope. They were then stained for laminin immediately after.

Widefield microscope video results from compiled time-lapse images for both conditions showed that U-118/L1LE cells invaded outwards from the initial 1mm cell

mass potentially along blood vessel structures. This was also further confirmed in laminin staining results (see Figure 39 below), especially for condition 1. A 3-D render video created by compiling z-stack images showed that the blood vessel (green channel) and the U-118/L1LE cells (red channel) were in the same focal plane within the brain slice (shown by white nuclei), further suggesting that the cells invaded along blood vessels in the brain slice. This was not as evident in condition 2, particularly because brain slices from condition 2 deteriorated quickly after time-lapse. They were unable to be stained because of this. Thus condition 1 appeared to be a more favorable brain slice culture method than condition 2.

It was also expected that the confocal time-lapse would provide better image quality than that of the widefield microscope, especially with the two conditions in the glass-bottom dish. However, results showed that the widefield microscope yielded quality images of cell invasion in brain slice cultures in the 6-well insert plate and the glass-bottom dishes. Although the confocal time-lapse did give the advantage of having individual z-planes that could be analyzed if desired, this was not deemed sufficiently advantageous to offset the simplicity of performing time-lapse of brain slices in the plastic 6-well plates.

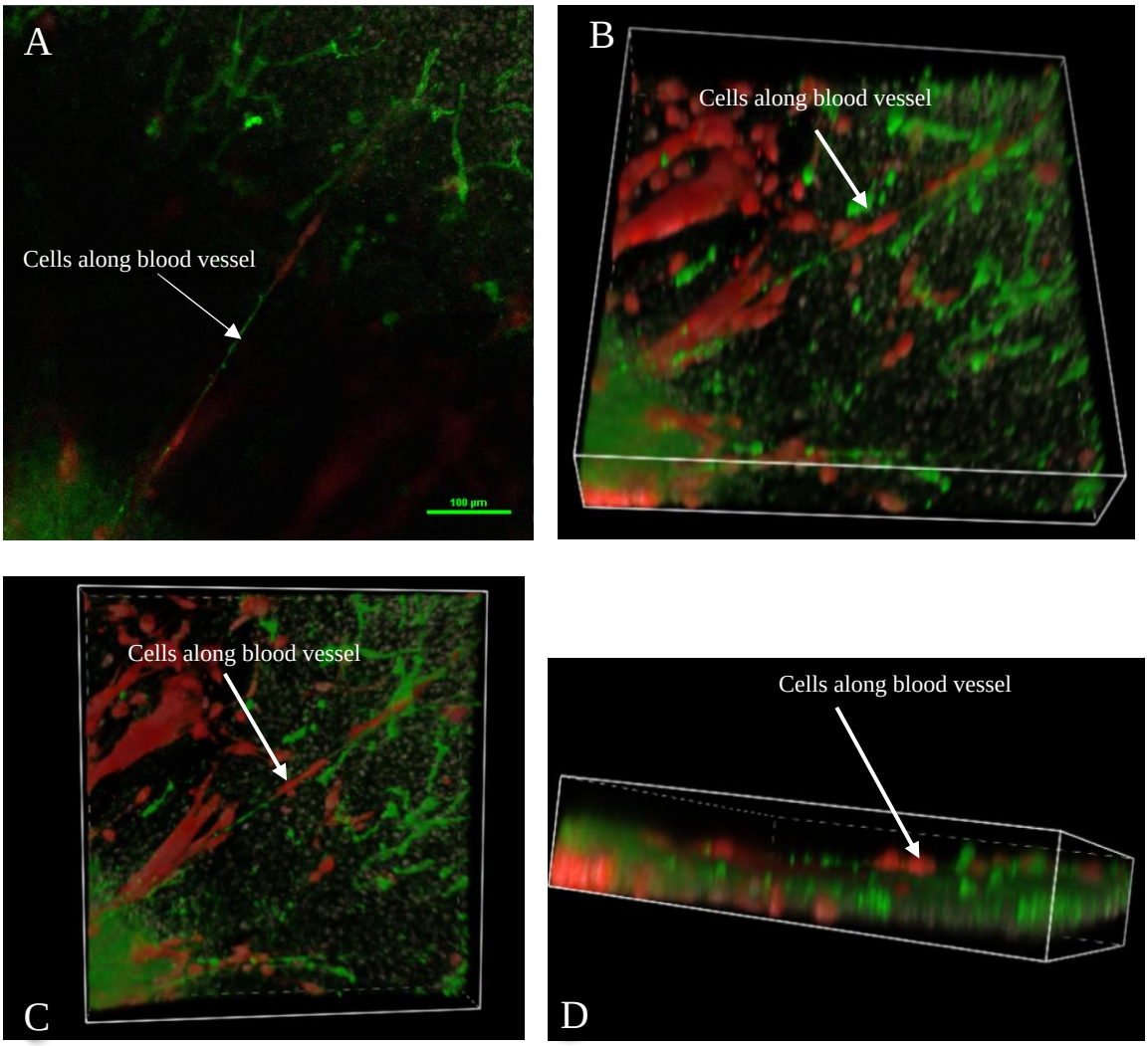

Figure 39. Confocal images of lamini and U-118/L1LE mCherry 2x cells in brain slices. Condition 1: brain slice on membrane directly on glass-bottom dish, laminin—Alexa488, 20x. A) Image shows U-118/L1LE mCherry 2x cells (red) lining a blood vessel (green). B, C, D) Images show 3-D volume render using multiple z-planes. Cell nuclei are shown in white. The tumor cells and blood vessel appear in the same focal plane in the 2-D image and through multiple z-planes.

3.3.5 GSC2016-4/K72 (P29) cells do not align along blood vessels in live brain slices

In a separate experiment, green GSC2016-4 (P29) cells were implanted into the center of each brain slice in a 6-well plate following the method described previously. After 6 days of observing the cell movement in the brain slice, they were time-lapsed with the widefield fluorescent microscope to observe the GSC invasion patterns in brain

slices as done for U-118/L1LE mCherry 2x cells. Based on time-lapse results, GSCs showed significantly less outward invasion and specifically movement along blood vessels compared to that of the U-118/L1LE cells.

3.3.6 Main conclusions from experimental results

From my results, I am able to make the following conclusions about the development of methods for ex vivo slice culturing, implanted cell behavior in those ex vivo slices, and expression of N-cadherin in GSCs and GBM cell lines:

Ex vivo culturing

- Out of the three different brain slice culture media tested, MEM media (with additives) provided the best viability of the brain slice (Sox-2) and the blood vessels (laminin).
- U-118/L1LE mCherry 2x cells use blood vessels as a path for invading outwards from the cell mass created in a live brain slice culture.
- In brain slices attached to the membrane and directly placed on the coverslip-bottom dish while submerged in media, U-118/L1LE mCherry 2x cells also invade along blood vessels from the cell mass.
- Time-lapse of the live brain slice culture in a 6-well insert plate by widefield fluorescent microscopy is equally effective as that of confocal microscopy.

N-cadherin expression

- According to western blots, U-118/L1LE, U-118/1879, and GSC2018-1A (P27), GSC2016-2 (P17), and GSC2016-4 (P22) cell lysates expressed full-length N-cadherin for the antibodies against the ectodomain and cytoplasmic domain.

- According to live and fixed coverslip staining results, U-118/L1LE mCherry 2x, U-118/1897 mCherry 2x, and GSC2016-4/K72 cells expressed N-cadherin for antibodies against the ectodomain and cytoplasmic domain.
- Fixed cell flow cytometry for N-cadherin expression using the antibody against the cytoplasmic domain in GSC2016-4 ITGA6 (+) P15 and U-118/L1LE cells showed that a high percentage of the cell populations were positive for N-cadherin. This was not the case for fixed and live flow cytometry for N-cadherin expression using the antibody against the ectodomain in the two cell lines.
- Weak N-cadherin expression was seen using the antibody against the ectodomain in GSC2015-2/K72 (P33) + U118/L1LE mCherry 2x, GSC2016-4/K72 (P24) + U118/L1LE mCherry 2x, and GSC2016-4/K72 (P24) + U118/1879 mCherry 2x tumor cells. No N-cadherin expression was seen using the antibody against the cytoplasmic domain in of GSC2016-4/K72 (P28) + U118/L1LE mCherry 2x tumor cells.

Chapter 4

DISCUSSION

4.1 N-cadherin expression in different GSC and GBM cell lines

Several different GSC and GBM cell lines were chosen for analysis of their N-cadherin expression. N-cadherin expression was identified in various GSC and GBM cell lines with antibodies against the cytoplasmic and ectodomains. The expression of N-cadherin across the GSCs and two GBM cell lines in the western blot and live and fixed coverslip stainings was consistent with previous research that showed that N-cadherin was expressed in all glioblastoma cases in the study, particularly in the cell membranes (Reszsec et al., 2015). In contrast to the western blot and coverslip staining results, flow cytometry indicated low N-cadherin expression using the antibody against the ectodomain of GSC2016-4 ITGA6 (+) P15 and U-118/L1LE cell lines for both live and fixed protocols (See Figures 18 and 20-22). This discrepancy in N-cadherin expression, however, may be because of trypsin with EDTA removes the calcium available to cells during trypsinization. ETDA is a chelating agent that binds to certain metal ions such as calcium, while N-cadherin is dependent on calcium for homophilic binding to occur between cells. Removal of the calcium will interfere with N-cadherin's binding ability and leaves the protein to be vulnerable against proteases (Shapiro et al., 2009). In the protocols for both fixed and live flow cytometry, cells are initially trypsinized with trypsin/EDTA to remove them from the dish. An alternative method of removing cells would need to be considered to maintain the structure of the N-cadherin proteins on the cell surface for detection by the antibody against the ectodomain. To do this,

trypsinization could be performed in media containing calcium and magnesium followed by immunostaining for N-cadherin and FACS analysis. Positively identifying and quantifying cell surface expression of N-cadherin in GSCs and GBM cell lines is important as N-cadherin has been suggested to increase cell motility and tumor invasion (Reszsec et al., 2015).

Another interesting finding was that staining fixed cells for N-cadherin was highest using the anti-cytoplasmic domain during flow cytometry (potentially for the abovementioned reason), but highest using the anti-ectodomain in stained vibratome sections containing mixed tumors visualized with the confocal microscope. Cells in those in vivo tumors would not have been subjected to trypsin treatment, which might explain why the anti-ectodomain antibody worked to visualize N-cadherin expression in the tumors. This notion is also consistent with good staining using the anti-ectodomain antibody with cells grown on coverslips, where they similarly would not have been subjected to trypsin treatment before antibody incubation.

4.2 Organotypic slice culture model

Creating a live brain slice culture model that maintains the *in vivo* experiment was important if it was to be used as an alternative to actual *in vivo* experiments. Examples of the need for an alternative to *in vivo* experiments would be to directly observe GMB cell invasion along blood vessels and attempts to inhibit invasion using drugs, neither of which can be done in the chick embryo brain tumor model *in vivo*. Based on previous protocols (see Table 1), I used Transwell membrane inserts and a 6-

well plate to construct a brain slice culture system. It was standard practice to expose the brain slices to air, as this allowed for better maintenance of the brain slice throughout incubation (Stoppini et al., 1991). Different types of media have been successfully utilized throughout various past protocols such as MEM and Opti-MEM (See Table 1), but in my experiments, MEM media appeared to produce the best results in maintaining viable brain slices as indicated by Sox-2 and laminin stainings (Figures 33-36). A rather surprising standard component added to the media was 25% horse serum, which provides survival promoting effects on neurons, astroglia or microglia and aids in the flattening process of the brain slice. Flattening of the brain slices is also crucial to confirming the viability of the brain slice and ensuring proper attachment to the membrane insert (Humpel et al., 2015). Brain slices generally took 5 days to flatten and securely attach onto the membrane before implantation of GSC or GBM cells was performed. On the fifth day, cells were implanted via the biopsy pen method in a 10% Matrigel mixture. Matrigel, which solidifies at 37˚C, allowed me to have a starting point for the implanted cell mass to eventually invade outwards into the brain slice. By knowing the starting location of the cell mass, it was easier to observe any cells that extended out from the cell mass presumably invading along blood vessels. Initial live invasion of U-118/L1LE mCherry 2x cells along blood vessels were observed in optic tectum slices via time-lapse with the widefield fluorescent microscope. Blood vessels were not visible with the time-lapse, but certain patterns of cell movement across the 20-hour period suggested that cells were lining a blood vessel structure as they invaded out from the cell mass. After this observation with the time-lapse, brain slices were fixed and immunostained for laminin

and confocal images were taken to confirm that cells were lining blood vessels. Z-stack images and 3D movies (Figure 38) of blood vessels and red U-118 cells in the same focal plane with brain cell nuclei above and below further indicated that U-118/L1LE cells can use blood vessels as a path to invade out into the brain slice. This also suggested that our live brain slice model and cell implantation are successful methods in observing GBM cell motility and invasion along blood vessels. This is important for a good *ex vivo* model because GBM cell invasion in patients with GBM frequently occur along blood vessels.

However, the same amount of invasion could not be seen with GSC2016-4 cells implanted into live brain slice cultures. GSC2016-4 cells were implanted into brain slices and were observed for any movement for 6 days. Brain slice cultures were then time-lapsed under the widefield microscope to confirm any invasion along blood vessels. Based on the series of time-lapse images capture in a 20-hour period, no apparent movement could be seen as with U-118/L1LE cells. This may be because GSCs potentially invade out of the cell mass at a slower rate than U-118/L1LE cells. Another possible reason may be because after a hole was cut out with the biopsy pen in the brain slices, the rim of the hole was lifted, allowing space between the membrane surface and the brain slice. This resulted in implantation of some of the GSCs under the brain slice, decreasing the opportunity of the cells to invade into the brain slice and ultimately along blood vessels. Despite all brain sections being sliced into 350 um slices, there will be discrepancies in the thickness and shape of the slices. This should be considered for future preparations with the biopsy pen and can be resolved by allowing another day for

the rim of the hole of the brain slices to reattach to the membrane before implanting cells into the hole. All in all, further investigation of understanding the invasion patterns of GSC2016-4 in live brain slice cultures is necessary.

4.3 Time-lapse using widefield vs. confocal microscopy

It was hypothesized that noticeable differences would be observed in time-lapse images of the brain slice cultures using the widefield fluorescent microscope vs. the confocal microscope. However, overall, there were no large differences and the time-lapse with the widefield microscope was just as effective as that of the confocal microscope to visualize fluorescent cell invasion into the brain slices. Preparations were also generally easier with the widefield microscope as live cultures could remain in the 6-well insert plate, while cultures had to be transferred to the 35mm insert glass-bottom dish when running a time-lapse with the confocal microscope. This was because the distance between the membrane and the bottom of the glass-bottom dish was less than the distance between the membrane and the bottom of the well of a 6-well plate, facilitating imaging with the confocal when brain slices were in the 35mm glass-bottom dish. However, even after transferring brain slices over to the 35mm dishes (as done in Conditions 1 and 2), no differences were seen in image quality using the confocal vs. the widefield microscope. Time-lapse using the widefield microscope was consistently effective in both individual 35mm dish conditions and the 6-well insert plate. The only disadvantage of the widefield fluorescence was that a time-lapse experiment could only be run for one color of fluorescence at a time unless one wanted to illuminate each cell

type with both the relevant excitation light as well as the irrelevant illumination light and capturing of resultant black images. This was a limitation of the MetaMorph software. Thus, brain slices implanted with green GSC2016-4 (P30) were time-lapsed on a separate day from brain slices implanted with U-118/1879 mCherry 2x or U-118/L1LE mCherry 2x to avoid illuminating cells many times with the irrelevant light that might contribute to phototoxicity. No phototoxicity was observed using the stated method.

4.4 *In vivo* Experiments

In previous *in vivo* experiments by our lab, it was found that L1CAM plays a stimulatory role GSC and GBM cell behavior and how GSC and GBM cells organize themselves into a mixed cell tumor. Past lab member Kyle Plusch used green GSC2015-2 and GSC2016-4 cells that were each mixed with either red L1-positive U-118/L1LE GBM cells or L1-negative U-118/1879 GBM cells. In the tumor mixture with GSC2015-2 and U-118/L1LE cells, GSC2015-2 cells were found inside of the tumor mass while U-118/L1LE cells were on the outside (Plusch 2018). This agreed with results found from my green GSC2015-2/K72 (P33) and red U-118/L1LE mCherry 2x mixed cell experiment (Figure 23). In addition to this, I found consistent tumor organization as Plusch had identified when I mixed green GSC2016-4 with red U-118/L1LE cells (Figure 24). The green GSC line organized itself outside of the tumor mass and the red U-118/L1LE cells inside. The organization was opposite for a tumor mixture of GSC2016-4 and U-118/1879 cells. As concluded before by Plusch, I was able to confirm that L1CAM influenced tumor formation as cells of the tumor mixture organized themselves

differently based on the presence or absence of L1CAM. L1CAM expression from the U-118/L1LE cells in the two tumor mixtures was denoted by staining in the blue channel. GSC and GBM cells were shown by the green and red channels, respectively.

With the same three tumor mixtures (GSC2015-2 + U-118/L1LE, GSC2016-4 + U-118/L1LE, and GSC2016-4 + U-118/1879), each tumor was immunostained for N-cadherin and ADAM10 expression. The purpose of assessing the expressions of these two proteins was to understand if expression levels were different *in vivo* compared to *in vitro.* Antibodies against the cytoplasmic domain and ectodomain of N-cadherin were used to determine N-cadherin expression in all tumor mixtures. In western blot results, it was shown that full-length N-cadherin was expressed in GSC2015-2 (P17), GSC2016-4 (P22), U-118/L1LE, and U-118/1879 cell lysates using the antibodies against the cytoplasmic and ectodomain. Live and fixed cell staining on coverslips further confirmed that the GSC2016-4 line, U-118/L1LE, and U-118/1879 cells expressed N-cadherin with antibodies against the two domains. Flow cytometry results, particularly for the fixed condition with the antibody against the cytoplasmic domain, also indicated high expression in U-118/L1LE cells with 96.3% of the cell population positive for N-cadherin. These results suggested that I would expect similar outcomes for *in vivo* tumor mixtures. However, there was no evidence of N-cadherin expression for the antibody against the cytoplasmic domain in the GSC2016-4 and U-118/L1LE mixture (Figure 32). In contrast to this, N-cadherin expression was evident using the antibody against the ectodomain for all three tumor mixtures, with expression mostly seen on the periphery of the tumor mass (Figures 26-28). Differences in N-cadherin expression levels noted *in*

vitro and *in vivo* may be greatly impacted by the discrepancies in environmental conditions and how the GSCs and GBM cells interact with one another within the tumor. Ultimately, these inconsistencies call for further investigation of N-cadherin's role in mixed tumors.

ADAM10 is a membrane-bound protein that plays a role in GBM cell migration by cleaving L1CAM and N-cadherin (Kohutek et al., 2009). Knowing the significant role of ADAM10 in cell motility, determining its expression levels in tumor mixtures was of particular interest. Of all three tumor mixtures, ADAM10 expression was seen only in tumors formed from the GSC2015-2 and U-118/L1LE cell mixture. Moreover, expression of ADAM10 was mostly apparent in the center of the tumor mass where GSC2015-2 cells were most concentrated and not in the periphery where U-118/L1LE cells were concentrated (Figure 29). The other two tumor mixtures with GSC2016-4 did not appear to express any ADAM10 (Figures 30 and 31), which may suggest that GSC2015-2 expresses significantly more ADAM10 than do GSC2016-4 cells.

4.5 Conclusions and future directions

Overall, I was able to develop a working live brain slice culture model using chick embryos where GSC and GBM cells could be implanted in the brain slice to observe live invasion along blood vessels and throughout the section in general. My results have demonstrated that this brain slice culture model is an appropriate method to maintain viable sections that allow visual tracking of live invasion by time-lapse with

both the widefield fluorescent and confocal microscopes. The brain slice culture model can be eventually utilized in drug treatments, beginning with cell signaling inhibitors to understand the effects of the inhibitors in an *ex vivo* model as seen in previous studies with cell cultures. (Anderson et al., 2016. I was also able to determine that N-cadherin expression can be seen *in vitro* in GSC2016-4, GSC2015-2, U-118/L1LE, and U-118/1879 using both antibodies against the cytoplasmic and ectodomains, but results were inconclusive for N-cadherin expression in mixed tumors using the antibody against the ectodomain. These findings for N-cadherin expression leave more to be investigated in understanding N-cadherin's role in *in vivo* tumor formation and how this influences GSC motility. To grasp a better understanding of N-cadherin's role, one should conduct additional *in vitro* and *in vivo* experiments to further characterize of N-cadherin expression and potential cleavage via ADAM10. N-cadherin's potential role in GSC and GBM motility can also be studied by using a short hairpin lentiviral vector to attenuate N-cadherin expression in certain GSC or GBM cell lines that express it. This was previously done for L1CAM when determining L1CAM's role in GSC motility (Yang et al. 2011; Mohanan et al., 2012). The motility of N-cadherin attenuated and control cell lines could be tracked and compared with each other to conclude whether the attenuated cells have reduced velocity, and the ability of attenuated cells to invade brain tissue and to form mixed tumors could be assessed.

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