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SUBVENTRICULAR ZONE ISOLATED
NEUROSPHERES

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MIGRATION OF MURINE SUBVENTRICULAR ZONE ISOLATED
NEUROSPHERES

By

Danny C. LeBert

THESIS

Submitted to
Northern Michigan University
In Partial fulfillment of the requirements
For the degree of

Master of Science

Graduate Studies Office

2011

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Danny C. LeBert

January 31st 1984

ABSTRACT

MIGRATION OF MURINE SUBVENTRICULAR ZONE ISOLATED NEUROSPHERES

By

Danny Christopher LeBert

The subventricular zone (SVZ) of the adult brain is a region known to contain a higher concentration of progenitor/stem cells than surrounding tissue. The SVZ progenitor/stem cells can be isolated and purified using neurosphere culturing technique. Neurosphere culturing is an attractive research model in that the lack of serum in culture media keeps progenitor/stem cells from differentiating and has even been shown to maintain genotypic and phenotypic profiles. The migratory capabilities of cancer cells has been explored in detail to elucidate the mechanisms behind recurrence and metastasis. However, the investigation of stem cells' role in recurrence and metastasis has only recently become an area of research. The current study examined the ability of neurospheres to migrate towards glioblastoma (GBM) cells and investigated the interaction of neurospheres with GBM released exosomes as a possible means to subsequent transformation into cancer stem cells (CSCs). Migration assays indicated that neurosphere isolates from the *murine* SVZ did not migrate toward GBM cells but were repelled *in vitro*. Interestingly, neurospheres are also repelled by serum containing media. These findings refute multiple studies that have indicated a chemo-attractive governance of stem cell migration by GBMs.

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DEDICATION PAGE

This thesis is dedicated to my son, Landon.

Hope is the belief that tomorrow will be a brighter day.

That the time and effort will lead to a new or confirmed direction.

Hope is what allows patients, families, and friends
to share and cherish the time they have together.

Hope is the belief that progress and knowledge gained
is one step closer to making a difference.

Hope is the fight for a greater good and the idea that one day an answer will be found.

Why is it important to have hope as a researcher?

One day, we might need to have hope in someone else's work.

That is why,

Hope Starts Here.

ACKNOWLEDGEMENTS

I would like to thank Dr. Robert Winn for his guidance and support, without which this project would not have been possible. I would also like to thank Drs. Richard Rovin and Erich Ottem for their valuable input and patience. A large gratitude also goes out to Vanessa Thibado who kept me motivated and whose help throughout the project was necessary for its' completion. Thanks also to the following members of Upper Michigan Brain Tumor Center for their assistance: Deanna Bougie, and Justin Segula.

This work was financed through generous support from the Upper Michigan Brain Tumor Center, NMU Spooner Grants for Research (D. LeBert), NMU Excellence in Education Grants (D. LeBert), NMU Biology Department Development Funds (D. LeBert), and Dr. Winn's general laboratory funds. Also, thanks to the faculty and staff of the NMU Biology Department for their advice and insight.

This thesis follows the format prescribed by the *MLA Style Manual* and the Department of Biology.

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LIST OF SYMBOLS AND ABBREVIATIONS

Sub-Ventricular Zone (SVZ).....	i
Stromal Derived Factor 1 α (SDF-1 α).....	i
Glioblastoma Multiforme (gbm).....	i
Enzyme Linked Immunosorbant Assay (ELISA).....	i
Passage Number (P#).....	i
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CHAPTER ONE: LITERATURE REVIEW AND BACKGROUND

INTRODUCTION:

The central nervous system (CNS) is composed of several cell types, the most numerous of which are glia and neurons. Glia is further subdivided into astrocytes, microglia and oligodendrocytes. Under normal physiological conditions, glia typically acts as scaffolding upon which neurons build networks and interact. Mutation in glial cells can result in a phenotypic change from a structural role to one characterized by rapid proliferation and invasiveness. (Markert)

Cancer is a class of disease characterized by uncontrolled cell growth, tissue invasiveness and metastasis (Reya et al.). Even after diagnosis and treatment with radiation and chemotherapeutic agents, many cancers prove to be resilient. According to the American Cancer Society, cancers caused approximately 7.6 million deaths in 2007, and the number continues to rise annually. Gliomas result from cancerous processes which induce histological and morphological changes in astrocytes and oligodendrocytes (Markert). The most malignant form of glioma is the grade IV glioblastoma multiforme (GBM) (Vescovi, Galli and Reynolds). Derived from astrocytic precursor cells, grade IV GBMs have a poor patient prognosis.

Gliomas account for 42% of all primary CNS tumors and 77% of all malignant primary CNS tumors (CBTRUS, 2005). Historically, treatment of malignant gliomas has included resection followed by radiotherapy. This form of treatment has achieved a mean survival time of only nine months (Ashby and Ryken). From 1969 to 1991, the

introduction of new chemotherapy agents such as carmustine, lomustine and nimustine (nitrosureas) increased the one year survival rate from 40% to 46% when used concomitantly with radiotherapy (Stewart). The mean survival rate also rose during this time to nearly 12 months. Initially introduced in 1996, temozolomide (TMZ) and the gliadel wafer gained notoriety in 2005 when the New England Journal of Medicine published two papers indicating concomitant use with radiotherapy increased the mean survival to 14.6 months (Hegi et al.; Stupp et al.). Since that time, these treatments have been adopted as the standard of care for malignant glioma treatment. Despite the improvements, current GBM treatment only slightly improve overall survival times, often with the accompaniment of a decreased standard of living due to the treatments. The mechanism for postoperative GBM recurrence remains elusive. Current evidence points toward malignant cell migration into surrounding brain tissue as being the most prominently accepted hypothesis for cancer dispersal (Ehtesham et al.). Surgical resection removes the tumor; distal malignant cells however, are left behind. Subsequent radio- and chemotherapy treatments keep the cells from proliferating initially, but also “select” for cells resistant to the treatments (Holland). It is at this point that the GBM recurs with treatment no longer effective.

BACKGROUND

The unfavorable GBM prognosis is due in part to postoperative tumor recurrence. Studies have shown that recurrence is inevitable for any individual surviving longer than 32-36 weeks (Ammirati et al.). GBM recurrence shows varied presentation of new

lesions which has led to an ambiguous definition. Most commonly, GBM recurrence is defined as a change in tumor growth compared to a previous interval of tumor absence or a loss of prior complete tumor control (Barker et al.).

When reviewing models explaining possible GBM recurrence patterns, it is important to address the processes underlying tumor formation. There are two cancer stem cell related explanations for the initiation of tumor growth: The first hypothesis describes the de-differentiation of cancer cells into cancer stem cells (CSC). The second hypothesis describes the mutation of a stem cell (SC) into a CSC (Civenni and Sommer; Yang and Weinberg). Both hypotheses suggest that tumor progression is due to a subpopulation of tumor cells with stem cell like properties.

Stem cells are not easily identified morphologically or phenotypically, though they are more commonly defined by functional criteria. First, the cells in question must be capable of self-renewal. Second, they must be able to differentiate into all mature cell types. Lastly, the cells must be capable of repopulating *in vivo* (Louis et al.). In 2004, a study by Shiela Singh *et al.*, identified CD133+ (cluster of differentiation 133), brain tumor cells as having stem cell like properties *in vitro*. They proposed CD133 as a stem cell marker, and according to their study, the CD133+ cell populations were the only cells capable of initiating tumors in non-obese diabetic, severe combined immuno-deficient (NOD-SKID) mice (Singh et al.). In recent years CD133 marker has failed to achieve widespread acceptance as an accurate stem cell marker. However the presence of a CSC subpopulation within tumor masses is widely accepted. The growth potential of a tumor appears to be governed by the CSC subpopulation found within its mass. The question

remains whether accumulation of mutations within a cancer cell allows for de-differentiation into a CSC and subsequent tumor formation, or if tumor formation is the result of the cancerous transformation of a stem cell.

The de-differentiation hypothesis is supported by evidence of epithelial-mesenchymal transition (EMT). The process of EMT causes epithelial cells to convert to a less differentiated form allowing them to lose rigid structural constraints, adopt a phenotype more permissive to migration with greater proliferative capabilities (Yang and Weinberg). This process has been definitively linked to neurofibroma formation (Joseph et al.). Neurofibromas are benign nerve sheath tumors found in the peripheral nervous system. Though compelling, there is not enough evidence to confirm that all tumors are caused via this pathway or similar processes. This lack of evidence supports the theory that CSCs arise simply through the mutation of a normal SC. Regardless of the steps leading to their formation, CSCs appear necessary for the formation of a tumor mass. It seems plausible that CSCs may also be responsible for tumor recurrence.

Currently, the accepted model for recurrence is as follows: A cell becomes transformed into a CSC through the mutation of a SC or de-differentiation of a cancer cell. The CSC possesses SC capabilities and are able to proliferate indefinitely to form a tumor. Within the tumor, a sub-population of CSCs accumulates. Over time the CSCs migrate away from the main tumor mass forming satellite cancer sites. Upon tumor resection, the satellite cells remain undetected. If residing in a favorable micro-environment, the CSCs will begin to proliferate and the tumor recurs (Civenni and Sommer).

NEURAL STEM CELLS AND NEUROSPHERES

Embryonic stem cells (ESC) were initially characterized independently by two groups in 1981. Martin Evans and Matthew Kaufman at the University of Cambridge first published a technique for culturing mouse ESCs (Evans and Kaufman). It was not until later that year that Gail R. Martin at the University of California coined the term embryonic stem cell after successfully culturing cells taken from mouse embryos (Martin). Since that time, research has led to the thorough characterization of stem cells and the elucidation of a cellular hierarchy (Ratajczak et al.).

At the top of the hierarchy are totipotent cells that arise during the morula stage of embryonic development. These cells are capable of giving rise to an entire organism, including the extra embryonic tissues required for development. During embryonic development, the cells of the morula begin differentiating after reaching the 16 cell stage (Wilbur). The cells, at this point in development, are considered pluripotent. Including ESCs, the cells at this stage can produce all three germ layers (endoderm, ectoderm and mesoderm), but cannot form the extra embryonic tissues. Below the pluripotent cells in the hierarchy are the multipotent SCs, which include NSCs. These cells can differentiate into multiple cell types and are typically tissue specific.

In 1992 adult NSCs were discovered in mice (Reynolds and Weiss). However, the concept of adult NSCs weren't fully accepted until 2001. It has since been shown that the largest neurogenic region in the adult brain is the subventricular zone (SVZ) (Okano and Sawamoto). Within the SVZ are three cell types: Types B, C and A (Doetsch, Garcia-Verdugo and Alvarez-Buylla). Type B cells divide slowly and have been shown to act as

stem cells (Doetsch et al.). These stem cells produce highly proliferative transit-amplifying or progenitor cells. Progenitor cells are Type C cells. Type C cells generate Type A cells, which are neuroblasts capable of migration as well as differentiation into nerves or glia (Doetsch, Garcia-Verdugo and Alvarez-Buylla).

Stimulation of the SVZ cell types through the addition of growth/neurotrophic factors causes a significant increase in cellular proliferation and migration. Infusion of exogenous epidermal growth factor (EGF) directly into the SVZ has been shown to induce both the migration of cells into the striatum and subsequent differentiation into glia (Craig et al.; Kuhn et al.). Additionally, using SVZ-specific labeling, evidence has indicated the directed migration of SVZ cells towards an area of trauma. In a study by Yamashita *et al.*, middle cerebral artery occlusion (MCAO) was induced to cause ischemia at the striatum. The labeled SVZ cells were tracked and shown to migrate preferentially towards the site of the ischemia (Yamashita et al.). The findings indicate migration was promoted either through ischemia induced disruption of SVZ sequestering or through an alteration of local chemokine expression.

Isolates from the SVZ are commonly cultured as neurospheres. Since the majority of cells isolated from the SVZ are not SCs, neurosphere culture conditions were adopted to aid in the purification of the isolate. The culture media is such that it allows progenitor cells to proliferate without having contact to the culture flask. The result is an overall selection for progenitor (PC) and SCs as these are the cells types capable of contact independent proliferation under the culture conditions (Laks et al.). Once the PCs and SCs begin to proliferate, they form floating clusters of cells known as neurospheres.

At the center of each individual neurosphere is a single PC or SC. The neurospheres are mechanically dissociated at each subsequent passage resulting in an overall increase in PC quantity. The process can be continued indefinitely while keeping the genotypic and phenotypic profile of the cells from changing (Fedoroff et al.). The result is cells that are genotypically identical at passage one and passage 20 with the same ability to differentiate into all of the proper cell types.

The neurosphere assay has become the gold standard for the culturing of primary brain tumors. Cancer cells lack anchorage dependence for proliferation thus making the neurosphere culture system a viable growth system. The neurosphere method is also beneficial in that it allows for a much “cleaner” growth flask meaning that non-cancerous cells are quickly separated from the cells capable of proliferating. Once in culture, the GBM cells respond similarly to the SCs in that CSCs begin forming neurospheres. Like the SCs, the GBM-neurospheres maintain their genetic fingerprint with each subsequent passage. This property allows for *in vitro* testing on cells that are identical to the original tumor sample.

The key to maintaining cellular profiles is linked to the lack of serum within the culture media (Laks et al.). Multiple studies on GBM-neurospheres have supported this finding. Using the GBM-neurosphere method to implant tumors into mice has yielded tumor masses that grow quicker and are more invasive than GBM implants grown with serum (deCarvalho et al.). The neurosphere-induced tumors can be re-isolated and once more cultured using the neurosphere method. The neurospheres formed post-xenograft have been shown to be genetically identical to the cells originally taken from the human

primary brain tumor. The same neurospheres can then be transferred to and cultured in serum-containing media. Within two passages, the cells' genotypic and phenotypic profiles no longer match the original tumor (deCarvalho et al.).

CELL MIGRATION

Cellular migration occurs due to an interaction of secreted factors and surface receptors. The secreted factors include growth factors and chemokines that act as ligands (Ehtesham et al.). These ligands include, but are not limited to neuregulin-1 (Ritch, Carroll and Sontheimer), ephrin-B1 (Nakada et al.), and stromal cell-derived factor-1 alpha (SDF-1 α) (Tiveron and Cremer). When separated from chemo-attractants in the form of a concentration gradient, cells will display polarity in their movement towards the desired ligand (Suzuki and Iwaki). This indicates that cells intrinsically migrate, non-randomly, towards specific signals.

Previous findings have shown that SDF-1 α interacts with the surface receptor CXCR4 to strongly promote cell migration (Ehtesham et al.). The interaction of SDF1- α and CXCR4 is unique in its specificity (Burns et al.). The majority of ligands bind to multiple receptors, however, SDF-1 α has only been found to bind to two, CXCR4 and CXCR7. The interaction between CXCR7 and SDF1- α plays an important role in tumor growth and survival, but no link has yet been made with migration (Burns et al.).

SDF-1 has three isoforms: SDF-1 α , SDF-1 β and SDF-1 γ . SDF-1 α and β proteins are encoded by a single gene at chromosome 10q and are generated by alternative

splicing (Shirozu et al.). SDF-1 α has an additional 4 amino acids at the C-terminal end to total 89 amino acids. Sequence analysis shows that SDF-1 α is highly conserved between species, 92% identical between murine and human according to Entrez-Gene. It is also ubiquitously expressed throughout the body (Shirozu et al.). The third isoform, SDF-1 γ , is only expressed in neurons and Schwann cells.

SDF-1 gene expression is regulated by the transcription factor hypoxia-inducible factor-1 (HIF-1) (Shirozu et al.). HIF-1 is only stable under hypoxic (low oxygen) conditions. HIF-1 binds to HIF-responsive elements (HREs) to promote the expression of glycolysis enzymes, SDF1- α , and vascular endothelial growth factor (VEGF) (Ceradini et al.). These genes promote cell survival and induce angiogenesis. For tumors to grow, they must overcome hypoxic conditions and promote angiogenesis in order to keep nutrients available for the expanding cell population. Endothelial progenitor cells (EPCs) and hematopoietic stem cells (HSCs) are important for these processes. These cells are capable of self-renewal and trans-differentiation (creation of cells outside of a designated differentiation path) and their motility is governed by SDF1- α – CXCR4 interactions (Weidt et al.). Taken together, it is not surprising that SDF-1 α concentrations are abnormally high in glioblastoma tissue (Imitola et al.).

CXCR4 is a 7-transmembrane G protein-coupled receptor expressed in a variety of tissues including the brain and in many cell types (Weidt et al.). Knockout experiments indicate that CXCR4 is involved in several physiological processes. For instance, CXCR4 deficiency experiments in mice yield hematopoietic and cardiac defects (Zou et al.). Another study indicates that CXCR4 is also necessary for vascularization of the

gastrointestinal tract (Tachibana et al.). Subsequent studies using SDF-1 α deficient mice found the same lack of vascularization, thus highlighting the importance of the CXCR4 – SDF-1 α interaction (Weidt et al.).

The migration of neural stem cells has also been linked to the CXCR4 – SDF-1 α interaction. In both the developing and adult brain, CXCR4 and SDF-1 α show a complementary expression pattern (Tiveron and Cremer). Ehtesam et al., (2008) were able to demonstrate that NSCs migrate toward tumor conditioned media in vitro. Migratory capabilities were then neutralized by inhibiting SDF-1 α with an antibody (Ehtesam et al.). Another study, by Imitola et al., (2004) used pre-labeled human NSCs and transplanted them into stroke patients. They were able to track the movement of the NSCs and found their movement was polar in nature towards the sites of damage. These sites were then characterized and found to contain elevated levels of SDF-1 α (Imitola et al.).

Neural stem cells interact with many chemotactic ligands, the result of which is guidance towards a specific location. However, not all ligands act in a chemo-attractive manner. For instance, under normal physiological conditions, NSCs remain sequestered in the SVZ in a quiescence state due to a chemo-repellent interaction with the Slit family of proteins (Wu et al.). Also an important class of CNS chemo-repellents are the semaphorins. The semaphorins consist of more than 20 secreted and membrane bound proteins important for the regulation of axonal guidance (Goshima, Ito et al.). The semaphorins are divided into 8 separate categories based on their domain organization as well as the species in which they are found. A common domain shared by each of the

family members is the presence of a 500 amino acid “Sema” signature found at the amino terminal (Rieger, Wick and Weller). Characterized under the class 3 semaphorins, Sema3A has been shown to be a strong chemo-repellent that effects axonal outgrowth (Goshima, Sasaki et al.). Sema3A is a secreted semaphorin that binds with high affinity to the neuropilin-1 (Nrp-1 receptor).

The neuropilins are a group of 2 transmembrane glycoproteins and are receptors for the class 3 semaphorins. As mentioned previously, Sema3A binds with high affinity to Nrp-1. The other two class 3 semaphorins, Sema3C and Sema3F, bind to both Nrp-1 and Nrp-2 (Nakamura, Kalb and Strittmatter). The neuropilins have extremely short cytoplasmic tails and rely on other proteins, such as the plexins, for signal transduction (Tamagnone et al.). The only other known binding partner for Nrp-1 is the vascular endothelial growth factor isoform VEGF₁₆₅. As a co-receptor, the Nrp-1 interaction increases the binding affinity of VEGF₁₆₅ with the kinase insert domain receptor (KDR), a receptor tyrosine kinase (Goshima, Ito et al.). The presence of Nrp-1 is required for the interaction of VEGF₁₆₅ and KDR (Miao et al.). The VEGF₁₆₅ – KDR interaction is important for cancer cell survival and proliferation (Goshima, Ito et al.). Interestingly, Sema3A and VEGF₁₆₅ compete for the Nrp-1 binding site and have opposing effects on overall cell motility.

The Sema3A – Nrp-1 signal cascade has yet to be fully characterized. What is known is the first step involves the lipid raft-dependent endocytosis of Nrp-1 upon binding to Sema3A (Salikhova et al.). The specific mechanisms underlying this process remain largely unknown. Upon localization to the cytoplasm, Nrp-1 interacts with

synectin. Synectin (also commonly known as GIPC) is a postsynaptic density 95, disk large, zona occludens-1 (PDZ) adaptor protein. Synectin is required for the coupling of the Nrp-1 containing lipid raft to motor myosin VI (Naccache, Hasson and Horowitz). The synectin – Nrp-1 interaction is required for developmental angiogenesis in zebra fish as well as endothelial cell migration (Wang, Mukhopadhyay and Xu). Semaphorin 3A induces cell migration through the activation of the Rac1 GTPase (Kolodkin et al.). Rac1 moderates the signaling cascade controlling F-actin dynamics, the end result yields increased motility away from the source of Semaphorin 3A.

Historically, the role of chemokines in the brain was thought to be limited to mediating normal neural development. Evidence of neurogenesis in the adult brain and subsequent identification of adult neural stem cells led to a broadening of these roles with a distinct link to cancer. As discussed previously, SDF1- α plays an important role in promoting angiogenesis through the attraction of EPCs to a growing tumor mass. The use of proteomic screens of GBM surface proteins demonstrated that another chemokine was affecting GBM progression. The proteomic screen indicated Nrp-1 to be nearly ubiquitously expressed in cells of the most malignant cases (Eustace et al.). A study of 12 malignant glioma cell lines showed Semaphorin 3A to be highly expressed indicating a possible autocrine signaling pathway (Rieger, Wick and Weller). The result of such a pathway could result in dispersal of malignant cells through the brain.

EXOSOMES AND CELL TRANSFORMATIONS

Exosomes are membrane vesicles secreted by multiple cell types including tumor cells (Keller et al.). An exosome is formed when an early endosome invaginates forming an internal luminal vesicle (ILV). The ILV can go down either of two pathways, lysosomal degradation or fusion with the plasma membrane and subsequent release as exosomes (Johnstone). Exosomes have been found to vary greatly in their morphologic features and content (Iero et al.). Once in the extracellular matrix, exosomes can be taken up by neighboring cells through endocytosis (Johnstone).

Exosomal release begins with the initial invagination of vesicles at the limiting membrane of the cell (Fevrier and Raposo). This invagination keeps proper orientation of extracellular proteins, while cytosolic proteins are engulfed within the 50-90 nm ILVs. The ILVs accumulate and form multivesicular bodies (MVBs) within the cell (Fevrier and Raposo). At this stage, the exosome is considered to be each of the ILVs contained within a single MVB. The whole process creates MVBs, and more specifically, ILVs with a common set of molecules, but also with cell-type specific components. It is therefore possible to determine the cellular origin of an exosome by looking at the specific protein/mRNA components (van Niel et al.).

Analysis of exosome containing serum using Western-blotting and immunocytochemistry is used to identify cellular origin of exosomes using proteomics. These methods have identified the following exosomes based on cell-type specific mRNAs; MHC classes I and II (antigen presenting cells), CD-3 (T cells), GluR2/3 (neurons) and EGFRvIII (cancer cells) (van Niel et al.; Al-Nedawi et al.). As mentioned

before, exosomes contain common components including but not limited to: chaperone protein mRNA (Hsc70 and Hsp90), sub-units of trimeric G-proteins, endosomal sorting complex required for transport (ESCRT) proteins (TSG101) and proteins involved in transport and fusion (Annexins, Rab2, Rab7) (van Niel et al.). Other studies have shown that certain molecules such as caspase mRNAs are found in higher concentrations within the exosomes than are found within the cell they originated from (Cocucci, Racchetti and Meldolesi). This led to research investigating the specific molecular packaging into exosomes.

Studies on protein sorting into ILVs indicate that mono-ubiquitination of targets as well as ESCRT machinery may play a role in preferential sorting. The sorting begins with target molecule mono-ubiquitination recognition by the endosomal-associated protein, Hrs, which forms a complex with signal transducing adaptor molecules (STAM), epidermal growth factor receptor substrate 15 (Eps15) and clathrin at the limiting membrane. Tsg101 of ESCRTI is recruited to the complex, which in turn recruits ESCRTII and ESCRTIII. The machinery is then broken down and recycled by Vps4 an AAA-ATPase (Babst et al.). However, not every mRNA within the ILV is ubiquitinated thus implying an alternative ESCRT – independent mechanism. An example is the sorting of the pre-melanosomal protein (Pmel17) which has been observed within ILVs, while lacking the ubiquitin tag (Theos et al.).

Once packaged and released from the host cell, exosomes can be taken-up by other cells. This may enable cells to communicate or “cross-talk” with one another. Communication by mRNA shuttling may provide cells a means of adapting to changes in

their immediate environment. This process may also be important in cell survival. Such is the case with complement activation of the membrane attack complex (MAC) in K562 erythroleukemia cells (Pilzer and Fishelson). Activation induces accumulation of MACs within the cell until reaching lytic concentrations. However, these cells preferentially sort MACs into ILVs and release them from the cell (Pilzer and Fishelson). Not all ILV interactions end favorably for the cell. Macrophages have been observed receiving CCR5 receptors from ILVs. This accumulation of CCR5, a co-receptor for the human immune deficiency virus (HIV) induces an increased risk of disease progression for individuals with the HIV infection (Mack et al.). Other studies have shown T lymphocytes receiving the death receptor ligand, Fas, which in turn leads to increased frequency of apoptosis (Kim et al.). Also affected by the release of ILVs is the immune system.

The immune system is typically activated during initial tumor formation but becomes blunted by tumor induced activation of suppressive pathways. Immunosuppression ranges from dysfunctional dendritic cells to apoptosis of antitumor effector T cells in cancer patients (Iero et al.). The exact mode of action has yet to be discovered but a broad array of soluble factors and cell to cell contact mediated interactions have been correlated with immune dysfunction. A model is beginning to take shape in which tumor cells evade the immune system through the secretion of ILVs. The general idea is that ILVs can present antigens which stimulate an immune response (Iero et al.). The immune system essentially ignores the growing tumor body as it deals with the antigen presenting ILVs. Immune-response eliciting ILV antigens include gp100, CEA and HER2-Neu (Andre et al.).

Evidence behind ILV-exosome release from tumor cells and their subsequent role in cell survival and immune avoidance has been steadily growing. Exosomes can be taken into cells and have their contents expressed such as the case discussed earlier in which macrophages took in exosomes containing CCR5. It therefore seemed plausible that cells could take in cancer released exosomes and express tumor-specific proteins. To investigate this theory, tumor-released exosomes containing the oncogenic epidermal growth factor receptor EGFRvIII were isolated from serum of tumor-bearing mice. These exosomes were introduced to cells not expressing the oncogenic factor. The exosomes were not only taken up by the cells, but the EGFRvIII was expressed. The expression was then linked to the activation of MAPK and Akt pathways as well as morphological transformation. (Al-Nedawi et al.)

PROJECT OBJECTIVES

Cell motility regulates tumor invasiveness and is an integral portion of all recurrence and metastasis models. The interactions of the ligands SDF-1 α with CXCR4 and Sema3A with Nrp-1 have a role in the cellular migrations observed in both the developing and adult CNS. These ligand-receptor interactions are an interesting link in tumor recurrence in that they are normal physiological cues. This project investigates an alternative recurrence model. The model suggests that NSCs migrate towards a tumor mass through the attractive interactions of SDF-1 α and CXCR4. The NSCs interact with an increasing gradient of exosomes as they near the tumor. These exosomes contain

tumor-specific mRNA and may cause a subsequent transformation into a CSC. The end result of this process would be the formation of a secondary or recurrent tumor.

The alternative recurrence model predicts that neurospheres will migrate in a directed manner towards a tumor. To test, GBM-containing and GBM-conditioned media was used in a transwell assay. Characterization of overall migration patterns was achieved by comparison to the migration of neurospheres towards unmodified media. Migration towards the unmodified media was considered intrinsic cellular motility and set as a baseline. The results indicated that the hypothesized migration of neurospheres towards tumor cells was not supported by the data. An overall repulsive effect upon the neurospheres was observed, which led to an investigation of the exosomes contained within the GBM-conditioned media. Migratory governance of exosome-free, GBM-conditioned media was compared to GBM-conditioned media from which it was derived. Finally, SDF-1 α was added to GBM-conditioned media in hope of returning neurosphere migration capabilities.

In order to support the alternative model, neurospheres must be shown to migrate towards SDF-1 α containing media. Neurosphere migration characteristics were also investigated in response to FBS conditioned media. Commercially obtained FBS has been shown to attract tumor cells *in vitro*, due to a wide assortment of proteins contained in it. Exosomes are also found in FBS. To test whether these exosomes play a role in the governing of neurosphere motility, three experimental conditions were tested using the transwell assay; normal FBS, exosome free FBS and MEME containing isolated FBS

exosomes. To my knowledge, this is the first study to investigate the effects of FBS on neurosphere motility.

CHAPTER TWO: ISOLATION, PROLIFERATION AND DIFFERENTIATION OF MURINE NEURAL STEM CELLS:

BACKGROUND

Neural stem cells can be isolated from the adult brain. The region known as the sub-ventricular zone contains a high concentration of NSCs and is a common target for isolation procedures. Not all cells within the SVZ are NSCs however. To confirm the presence of NSCs, the SVZ isolate must be passed in culture a minimum of seven times using the neurosphere culturing method. Finally, the cultured cells must be differentiated into the three common cells of the CNS; astrocytes, oligodendrocytes and neurons.

MATERIALS AND METHODS

SUBVENTRICULAR ZONE ISOLATION

Mice, strain C57BL/6, obtained from Harlan Laboratories were euthanized through the approved IACUC protocol # 060. The mice were saturated with 70% ethanol to reduce the risk of contamination. The dissections occurred under sterile conditions and began with the decapitation of the mouse. The optic tract was separated from the eyes by making a small incision behind the orbital bones. The scissors were inserted on the posterior – dorsal portion of the head and between the scalp and calvaria. An anterior slice to the snout allowed the scalp to be peeled aside exposing the occipital bone. A shallow cut beginning at the occipital bone, up through the sagittal suture and through the frontal bone gave access to the brain. Once the cranial bones were peeled aside, the brain

(no longer attached to the spinal column and optic tracts) was gently teased out of the cranium.

The extracted brain was placed into a petri dish containing phosphate buffered saline (PBS) with 2% glucose on its dorsal side exposing the ventral region and the clearly visible optic chiasm. A sterile blade was used to slice down through the optic chiasm resulting in anterior and posterior sections. A clean vertical slice in the previous step was important to minimize the damage to the SVZ. The posterior section is discarded and the anterior portion of the brain was positioned caudal side up. The now visible SVZ was removed using forceps and placed into a centrifuge tube containing PBS supplemented with 2% glucose.

NEUROSPHERE CULTURING

Once the SVZ isolates have settled at the bottom of the centrifuge tube (Biologix Research Co.), the glucose solution is removed and the cells are re-suspended in 2.0 mL of “complete” Neurocult NSC Proliferation Medium (mouse) (Stem Cell Technologies, Vancouver, BC.). Complete media is prepared as follows: 1.0 mL of Neurocult NSC Proliferation supplements (mouse) is added to every 9.0 mL of “Basal” Neurocult NSC Proliferation Medium (mouse). Next, 1.0 μ L of 10 μ g/mL rh-FGF-b (fibroblast growth factor) is added to every 1.0 mL complete media, followed by the addition of 20 ng/mL rh-EGF (2.0 μ L for every 1.0 mL complete proliferation media) and 2.0 μ g/mL Heparin

(1.0 μ l for every 1.0 mL complete media). All reagents were obtained from Stem Cell Technologies.

Cells were re-suspended through mechanical trituration using a 1000 μ L pipette. After approximately 50 triturations being careful not to introduce air bubbles, much of the SVZ will have dissociated into single cells. The suspension was removed and filtered through an 80 μ m filter (BD Falcon, Sparks MD) into a new centrifuge tube to ensure a single cell suspension. Any tissue remaining in the original sample was re-suspended in 2.0 mL complete media, and the procedure was repeated as necessary. The resulting suspension was pelleted by centrifugation at 150 x g for 5 minutes (Eppendorf, Hamburg, Germany). Again the supernatant was removed and the cells are gently re-suspended in 2.0 mL of complete media. The cells were counted using 0.4% trypan blue stain (Gibco-Invitrogen, Carlsbad, CA.) and a hemocytometer (American Optical, Southbridge, MA.) and plated at 2×10^6 cells / 10.0 mL complete media in a T-25 cm^2 flask (Nunc, Roskilde, Denmark). Neurospheres begin forming within 24 hours and detach from the plate within 48 hours. The cultures are passed when the neurosphere colonies become visible to the naked eye but before the medium becomes acidic, indicated by a color change of the media indicator.

When neurospheres are ready for passage, the entire culture suspension is collected and centrifuged at 150 x g for 7 minutes. The supernatant is discarded and the pellet is re-suspended in 2.0 mL of complete proliferation media. Gentle trituration separates the neurospheres into a single cell suspension. At this point, the cells are again counted using trypan blue and a hemacytometer. The cells are seeded at a density of 5 x

10^5 / 10.0 mL for a T-25 cm² flask at each subsequent passage. Between passages, neurospheres were cultured at 37^oC and 5.0% CO₂ (Revco, Twinsburg, OH.).

DIFFERENTIATION ASSAY

Once neurospheres have been passed greater than seven times, they must be differentiated to definitively claim the presence of NSCs. To differentiate, neurospheres are pelleted by centrifugation at 90 x g for 5 minutes. The complete proliferation media is aspirated and the pellet is re-suspended in 10.0 mL of Complete Neurocult NSC Differentiation Medium (mouse). A solution of nine parts Neurocult NSC Differentiation Basal Media is added to one part Neurocult NSC Differentiation Supplements to create the Complete medium. The suspension is again centrifuged, (90 x g for 5 minutes) after which the supernatant is removed. Pelleted neurospheres are re-suspended in 2.0 mL of Complete Differentiation Media and a single cell suspension is obtained using gentle mechanical dissociation. A cell count is performed as described previously and cells are plated at a density of 5×10^4 cells/well in 24 well plates. A poly-l-ornithine coverslip (Becton-Dickinson, Franklin Lakes, NJ.) is placed in each well prior to the addition of cells. After 6-8 days, the plates are checked using an inverted light microscope (Microscoptics Incorporated, Holly, MI.) to establish the viability of the cells. If viable, differentiation status can be determined looking at cell morphology. Differentiated cells will spread out on the cover slips and heterogeneous cell populations will be observed. All of the differentiation media and supplements were purchased from Stem Cell Technologies.

IMMUNOLABELING PROCEDURE

For wells with differentiated cells, the cover slips are transferred to new 24 well plates containing 1.0 mL of 4% paraformaldehyde in PBS pH 7.4 (Gibco-Invitrogen). After a 30 minute incubation the paraformaldehyde is aspirated and the cells are permeabilized by the addition of 1.0 mL of PBS containing 0.3% Triton X-100 (Fisher Scientific, Hampton, NH). Following a five minute incubation, the Triton X-100 solution is removed and the cover slips are washed three times using PBS. The cells are now ready to be antibody (Ab) labeled. The primary antibodies used were: Mouse IgG anti-microtubule associated protein 2 (MAP2, neurons), rabbit polyclonal glial fibrillary acidic protein (GFAP, astrocytes) and mouse oligodendrocyte IgM marker O4. The working dilutions for the primary antibodies were as follows; MAP2 – 1:200, GFAP – 1:100 and O4 – 1:50. Each primary antibody was diluted to a working concentration with PBS enriched with 10% goat serum. The antibodies were then added to the 24-well plate at a volume of 200 μ L. The plates were incubated for 2 hours at 37°C before washing 3 times (5 minutes each) with PBS.

To the appropriate wells, 250 μ L of secondary antibody was added. The secondary antibodies were added to each well at a volume of 200 μ L. The secondary antibodies used were FITC-conjugated Affini-Pure goat anti-mouse IgM, μ chain specific, FITC-conjugated Affini-Pure goat anti-rabbit IgG (H + L) and Texas Red conjugated goat anti-mouse IgG. The working dilutions were 1:100 for the FITC-conjugated Abs, and 1:75 for the Texas Red conjugated Ab. After a 30 minute incubation at 37°C unbound Ab was washed off using three PBS washes (five minutes

each). After the final wash, 200 μ L of distilled water (dH₂O) was added to each well (all primary and secondary Abs were obtained from Stem Cell Technologies). Cover slips were transferred from the dH₂O to a microscope slide and were mounted using a single drop of Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Incorporated, Burlingame, CA.). The slips were visualized using fluorescent microscopy. The filters used to visualize each flourochrome are shown in Table 1.

Table 1: Immunostaining Fluorochromes. The following fluorochromes were used for the immunostaining of neurospheres during the differentiation assay.

Fluorochrome	Absorption peak (nm)	Emission peak (nm)
Fluorescein, FITC	492	520
Texas Red, TR	596	620
DAPI	350	460

RESULTS

The cells isolated from the mouse SVZ were placed into culture and began forming neurospheres within 24 hours (Figure 1). By day four, the media became acidic and the cells were passed. This procedure was continued and the cells were passed a total of 27 times. The cells were typically passed every six days; with an average growth

range of 5-7 days. During each passage, the cells were counted (Figure 2). Even at P27, the neurospheres were able to repopulate the flask within seven days.

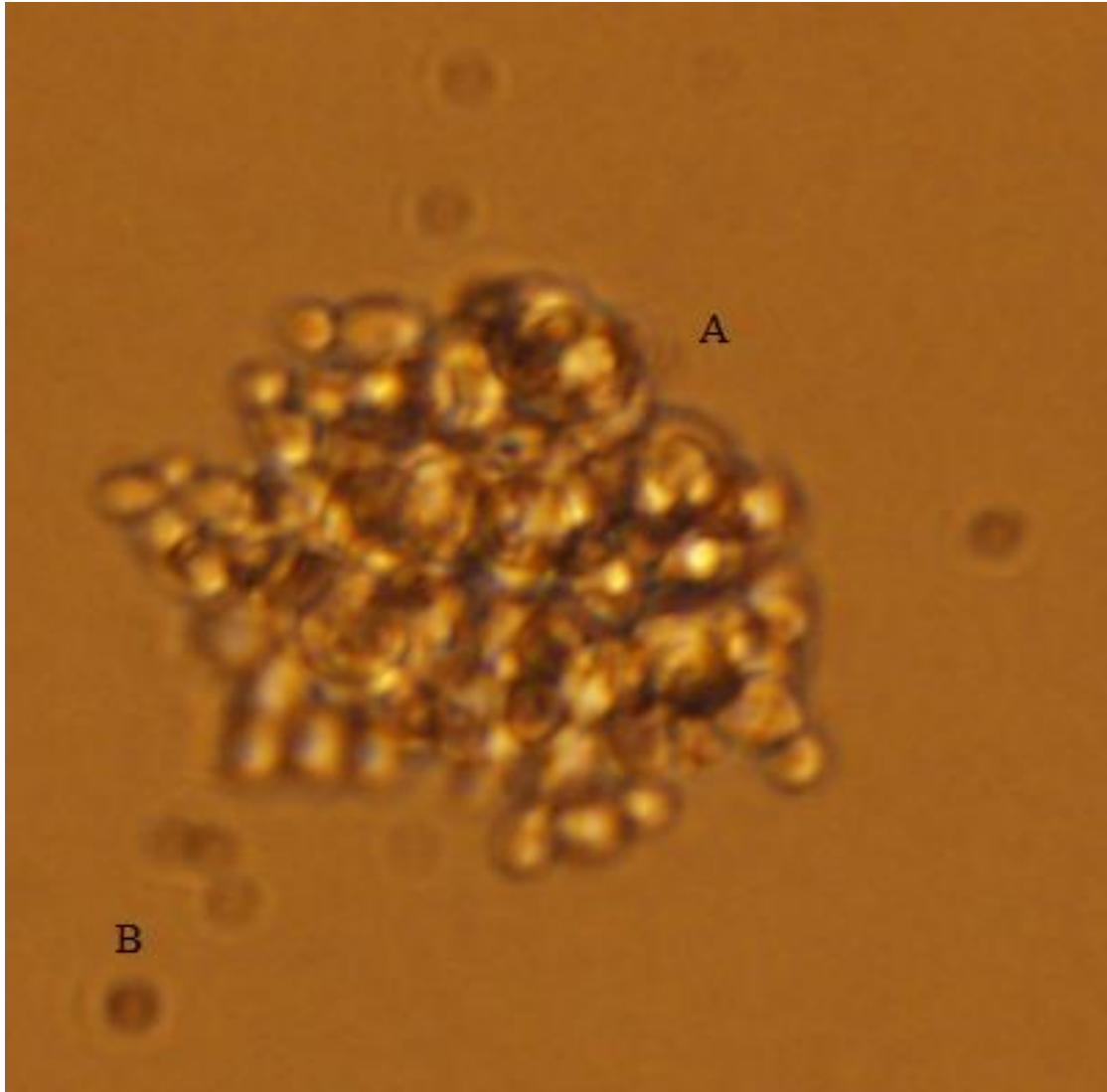


Figure 1: Image taken of P3 cells isolated from the mouse SVZ. (A) A Floating neurosphere in the signature spheroid profile. (B) Early passages contained cells not derived in the central nervous system such as red blood cells (RBCs). These cells were typically smaller, did not form neurospheres, and stayed attached to the base of the flask.

Cell Count Through P10

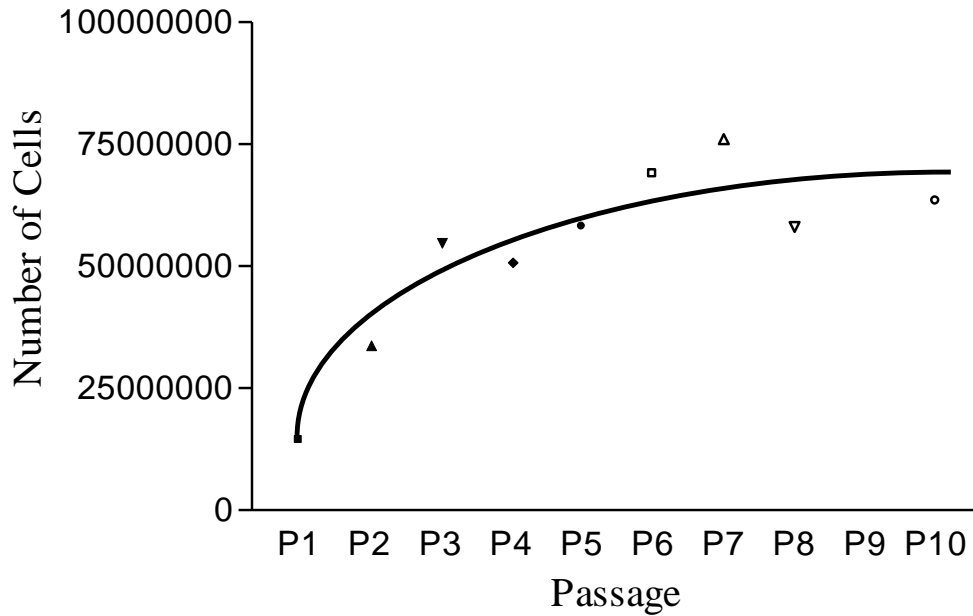


Figure 2: Continued proliferation of cells isolated from the mouse SVZ. Cells were passed every 5-7 days at a concentration of 5×10^5 cells / 10.0 mL of complete proliferation media. During each passage, the total number of cells was recorded.

After passing the neurospheres to the P7 stage, differentiation into the three CNS cell types was necessary to confirm the presence of stem cells. The differentiation assay was performed using the Stem Cell Technologies Differentiation Assay protocol described previously. The only change to the protocol was the addition of the nucleus stain, DAPI, during the mounting step. Positive staining was observed with DAPI as well as the oligodendrocyte marker, O4-FITC conjugated. The Texas-Red and MAP2 samples (astrocyte and neuron markers respectively) were negative. The Texas-Red conjugated secondary antibody was negative in the sample containing all three primary antibodies (Data not shown). FITC labeling was positive in the sample containing the three primary

antibodies, but the presence could not be definitively linked to the presence of neurons since FITC was used in the secondary antibody for both neurons and oligodendrocytes. These findings indicate that the neurospheres were un-able to differentiate into the three common cells of the CNS.

DISCUSSION

The stem cell isolation was conducted on two separate occasions. For the first attempt, the SVZ of the mice brains were isolated and dissociated as the protocol describes. The cells were placed into culture without performing cell counts. These cells were then incubated for 48 hours before microscopic observation was performed. At the 48 hour point, the media had turned yellow indicating a decrease in pH. The culture media was cloudy, a possible indication of bacterial contamination. The media was replaced and streptomycin was added at a concentration of 100 $\mu\text{g} / \text{L}$. After 48 hours, the media displayed the previous characteristics, and were deemed contaminated and discarded.

A second isolation was performed as before, this time however, the cells were counted prior to plating. The cells were plated at a density of 2×10^6 cells / 10 mL of media per plate. The cells were observed regularly and neurospheres formed quickly. Even at this density the media became acidic and the plates filled rapidly with cells. The initial observance of neurospheres gave confidence during the first few cell passings and by P4 the culture media appeared uncontaminated. Because the initial cell propagation

was rapid, it is possible that the “contamination” observed during the initial isolation was caused by an overabundance of cells. It is even more likely since the initial cultures were not of a known cell density and thus an extremely large number of cells could have been plated.

The neurospheres obtained during the second isolation were eventually passed to P27. It was a decision of the investigator to stop passage of the neurospheres, not due to contamination or an inability propagate. The cells were observed microscopically during and between each passage and the cells forming the neurospheres were much smaller than expected. Initially it was thought the cells were RBCs that had clumped together. However the ability of the cells to repopulate the cell plates indicated the cells were proliferating ruling out RBCs. It was also suggested that the proliferating cells were possibly prokaryotic in origin. However, this hypothesis was rejected by the positive nucleus staining with DAPI. Informal consultations with stem cell experts also confirmed the relatively small physical appearance of stem cells in culture.

The ability to repopulate the cell plates along with the positive DAPI staining was compelling evidence that the cells forming the neurospheres were eukaryotic. The formation of neurospheres at P27 was also suggestive to the presence of stem/progenitor cells since the formation of neurospheres post P7 is typically sufficient to claim SC existence. The final test in the determination of the presence of SCs was the differentiation assay. In its most basic form, the differentiation assay is simply a change in media that triggers cells to undergo progression down their innate lineage.

The first step of the differentiation assay was plating the cells, forming the neurospheres, on poly-L-ornithine coated slides in differentiation media. During this time cells should stop proliferating and begin differentiating. The majority of cells continued proliferating with only a small number attaching to the cover slips. The result of which were very small sample sizes on each slip. The washing steps caused additional removal of cells compounding the sample size issue. The poor results of the differentiation assay can be attributed, at least in part, to the small number of cells on each slide. The observation of FITC labeling of the O4 marker was encouraging. The labeling indicates that the cells were in-fact differentiating. A differentiation assay with clear positive results for each of the cell markers is necessary before confirmation of stem/progenitor cell presence can be made. For this reason, the cells isolated from the SVZ will be referred to from this point forward simply as neurospheres.

CHAPTER THREE: STROMAL DERIVED FACTOR 1α (SDF- 1α) AND FETAL BOVINE SERUM (FBS) INDUCED MIGRATION OF SVZ DERIVED NEUROSPHERES:

BACKGROUND

When separated from a chemokine source, NSCs display polarity in their movement either towards or away from the source depending on the ligand. Chemokines play an important role in the body's normal functions as well as cancer formation and progression. To form a tumor mass, cancer cells must have a constant supply of nutrients. Through the process of angiogenesis, GBMs are able to overcome hypoxia and continue to proliferate. Angiogenesis is induced, in part, through the release of the hypoxia-related chemokine SDF- 1α . Previous research has indicated that SDF- 1α interacts with the cell surface receptor CXCR4 to promote cell migration (Ehtesham et al.). It was therefore of interest to determine whether the neurospheres isolated from the SVZ would respond in a similar manner. Also of interest was the effect FBS would have on neurosphere migration. Cancer cell migration assays from our own laboratory have indicated that FBS acts as a strong chemo-attractant (data not shown). It was hypothesized to also induce migration of neurospheres in an attractive manner. Both studies were performed in order to gain a better understanding of the neurosphere migration capabilities.

MATERIALS AND METHODS

TRANSWELL ASSAY

The QCM™ 24-Well Colorimetric Cell Migration Assay was commercially obtained from Chemicon International. A general diagram of the transwell assay is shown in figure 3. The first step was to prepare the media placed into the outer chamber. To test the effect of FBS, 9.0 mL of minimum essential medium eagle (MEME), (BioWhittaker Incorporated, Walkersville, MD.) was mixed with 1.0 mL of FBS (PAA Laboratories, Pasching, Austria) yielding a 10% FBS solution. A total of 500 µL was taken out of the 10% FBS solution and placed into each of the outer chambers. Into the inner chamber, 300 µL of neurospheres at a density of 7×10^5 cells / 1.0 mL were seeded. The inner chamber was then placed into the outer chamber and incubated at 37⁰C and 5% CO₂ for 20 hours. The procedure was repeated for the experiments involving SDF-1 α . The SDF-1 α was tested at concentrations of 25.0, 50.0, 100.0 and 200.0 ng/mg in MEME. Each condition was repeated at least three times (three wells) for each of the migration experiments. An example of the SDF-1 α assay is depicted in figure 4.

During the incubation, cells are free to migrate. The inner chamber contained pores 8.0 µm in diameter. These pores are small enough to inhibit the passive passage due to gravity, but large enough to allow a cell to actively pass through. Once through the pores, the neurospheres remain suspended in the media or settle to the base of the outer chamber. These cells were then physically scraped off the floor of the outer chamber, and quantified using the Cell Titer-Glo assay (Promega, Fitchburg, WI.). Cells that had detached from the underside of the inner chamber were not collected.

In addition to each experimental sample, a group of five control samples were assayed using the transwell assay. As a negative control, unmodified MEME was used in the outer chamber of the assay. The control indicated the total random migration of the neurospheres during each assay. To measure a relative quantity of migratory neurospheres, the Cell Titer-Glo assay was used.

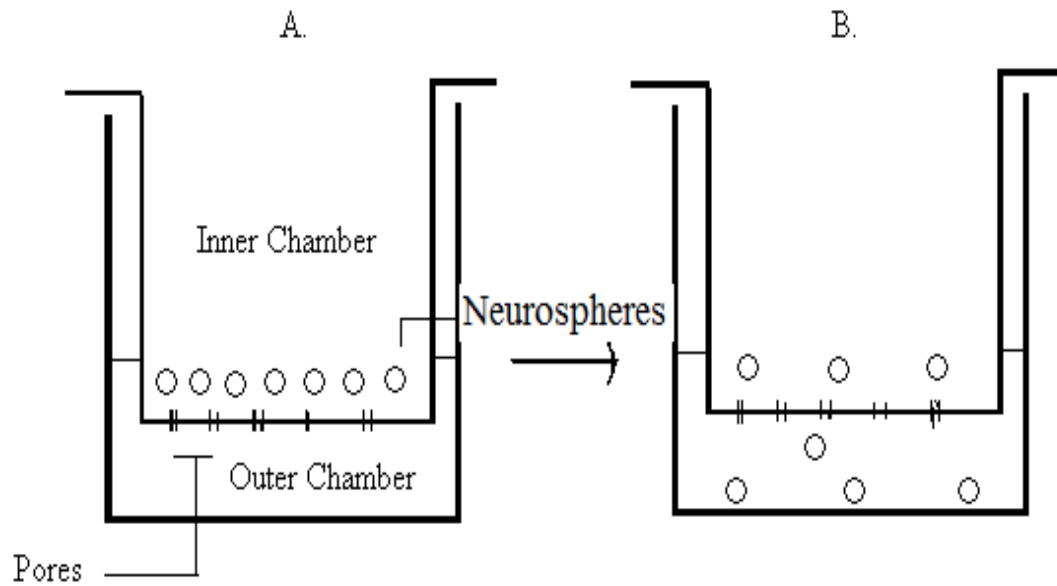


Figure 3: Transwell Assay. (A) 300 μL of Neurospheres are placed into the inner chamber at a density of 7×10^5 cells / 1.0 mL. The inner chamber is inserted into the outer chamber containing the experimental solution. The assay is then incubated to allow the cells to migrate through the pores. (B) Cells that have migrated from the inner chamber into the outer chamber were quantified using the Cell Titer Glo assay.

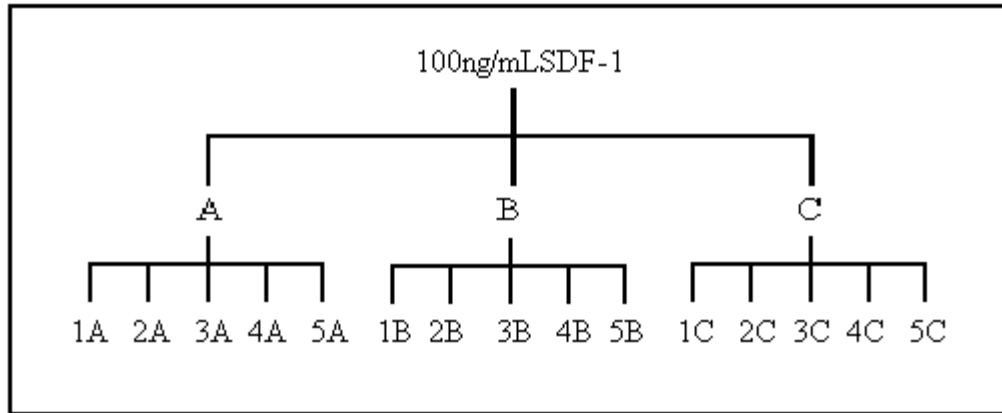


Figure 4: Transwell assay sample preparation. First, the experimental solution was made (100 ng/mL SDF-1 α). The solution was then used in three transwell assay chambers (A,B,C). After the incubation period, the cell suspension in each of the three chambers was subdivided into five separate wells for quantification using the Cell Titer Glo assay.

CELL TITER- GLO ASSAY

After the 20 hour incubation, the inner chamber was removed from the outer chamber and discarded. Each outer chamber was manually scraped to re-suspend the migratory neurospheres. From each suspension, five - 100 μ L samples were transferred to five individual wells of an opaque bottom 96-well plate (Becton-Dickson - Falcon). To each sample, 100 μ L of the complete Cell Titer Glo reagent was added. The complete Cell Titer Glo reagent was prepared by adding 10.0 mL of the Cell Titer Glo solvent to the Cell Titer Glo substrate. The plate was incubated at room temperature and rocked gently using a random orbital shaker (Stovall – Thermoscientific, Waltham, MA) for three minutes. The plates were then removed from the shaker and incubated for another 10 minutes at room temperature. Relative Light Units (RLUs) were obtained for each

well by measuring luminescence using the Modulus plate reader (Promega). For each transwell assay, background luminescence was determined by adding 100 μ L of complete reagent to 100 μ L of un-modified MEME.

RESULTS

The SDF-1 α enriched MEME induced an increase in neurosphere migration as expected. At concentrations of 100.0 ng/mL and 50.0 ng/mL, the SDF-1 α induced a statistically significant increase in neurosphere migration compared to the control (Figure 5). The 25.0 ng/mL sample was unable to induce any change in neurosphere motility (Figure 5). The 25.0 ng/mL sample was tested during three migration assays, each with a similar outcome. The final concentration of 200.0 ng/mL also generated significant change in migration, albeit acting as a chemo-repellent. This finding correlates with prior research that has found SDF-1 α to act as a chemo-repellent in high concentrations (Poznansky et al.). Taken together, the data indicates that the chemo-attractive effect of SDF-1 α on neurosphere migration is concentration dependent.

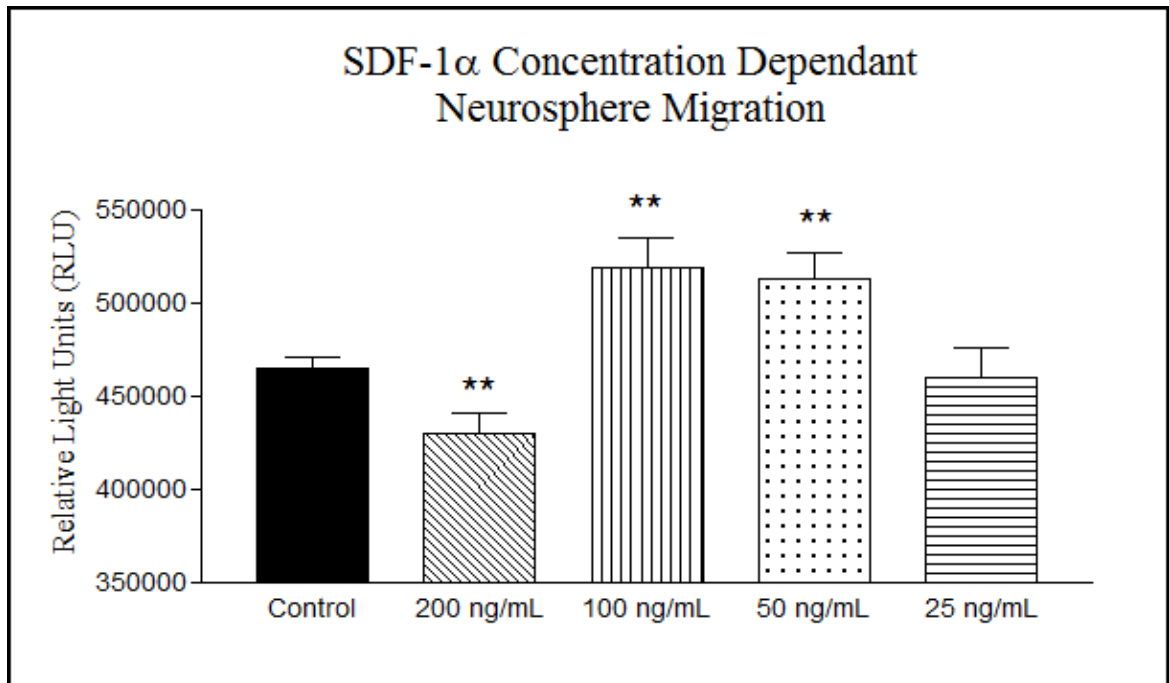


Figure 5: SDF-1 α induced migration of neurospheres is concentration dependent. (1) Control – unmodified MEME, (2) 200.0 ng/mL SDF-1 α (3) 100.0 ng/mL SDF-1 α (4) 50.0 ng/mL SDF-1 α (5) 25.0 ng/mL SDF-1 α . Bars 3 and 4 induced a statistically significant increase in neurosphere migration. Bars 2 also reached statistical significance, but acted as a chemo-repellent. P value ≤ 0.01 . The 25.0 ng/mL SDF-1 α did not cause a change in neurosphere migration. All samples were analyzed using a student's t-test.

The second arm of the experimental set determined whether the presence of FBS stimulated a change in neurosphere migration. When MEME was supplemented with 10% un-modified FBS, a significant change ($P \leq 0.001$) in neurosphere motility was observed (Figure 6). The addition of FBS caused a significant decrease in neurosphere migration when compared to the control. To test the validity of the initial findings, the experiment was repeated twice more, each with the same outcome. These findings, along with the other two trials (data not shown), indicate that FBS influences neurospheres in a chemo-repellent manner.

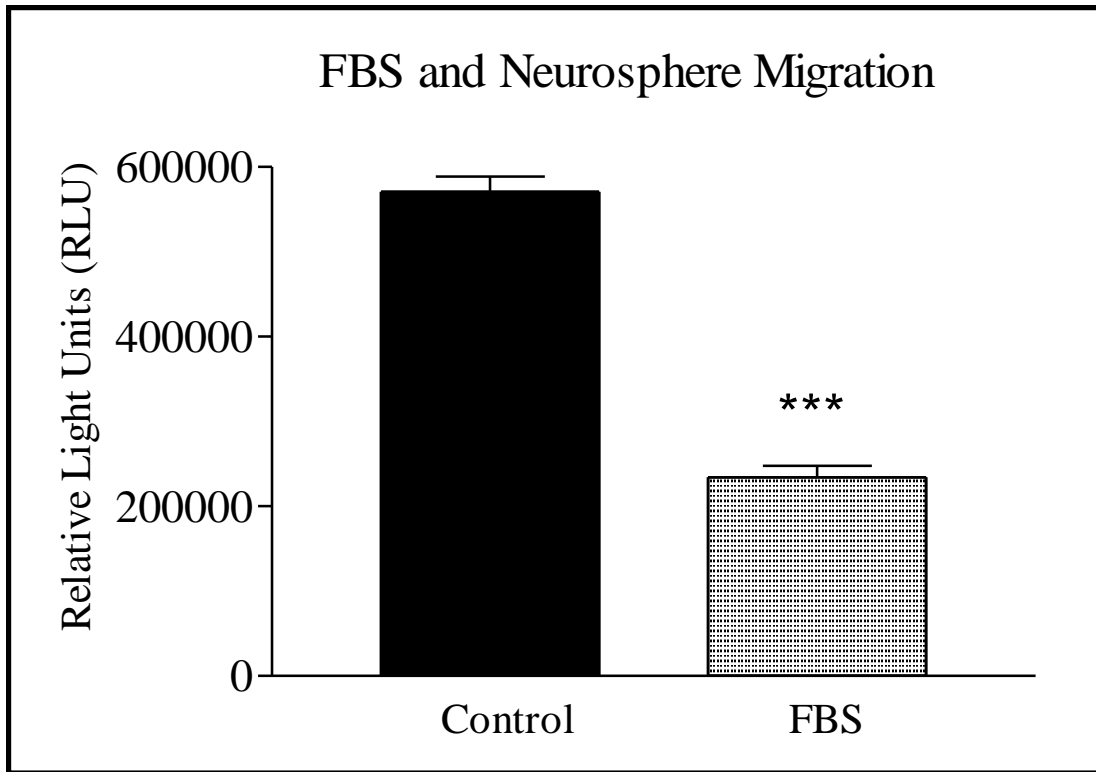


Figure 6: Effect of MEME + 10% FBS on neurosphere migration. The addition of 10% un-modified FBS caused a statistically significant reduction in neurosphere migration ($P \leq 0.001$).

DISCUSSION

An extensive literature review indicated that SDF-1 α was a chemo-attractant capable of justifying the alternative recurrence model. The study performed here was able to achieve a statistically significant increase in neurosphere migration toward SDF-1 α (50.0 and 100.0 ng/mL concentrations), in a concentration dependent manner. Three separate transwell assays were performed using SDF-1 α at a concentration of 25.0 ng/mL, none of which caused a significant change in overall migration rates. The

concentration of SDF-1 α necessary to induce migration was larger than expected but fell within the recommended concentrations.

One possible explanation lies in the different species of origin of the neurospheres and the SDF-1 α . The neurospheres were isolated from a mouse, while the SDF-1 α was of human origin. The SDF-1 α was of human origin since the GBM cell lines to be utilized during the later portions of the experiments were also of human origin. To determine if the species differences were of importance, the protein sequences were analyzed for homology. The differences between the human and *murine* SDF-1 α are quite slim with roughly 92% homology (Entrez-Gene). The receptor, CXCR4, is also similar between species with a 91% homology between *murine* and human (Entrez-Gene). The highly conserved nature of both the ligand and receptor indicates both the importance of the interaction between species, as well as the likelihood that human SDF-1 α can interact with the *murine* CXCR4. It seems likely then that the high concentration threshold of SDF-1 α is not the result of a sequence difference between species. The mechanisms behind the concentration threshold may require future research to further elucidate the mechanism(s). However, the high threshold may not be of real importance but rather a curiosity to the investigator.

The other interesting observation was the chemo-repellent effect of the 200.0 ng/mL SDF-1 α . Recent studies have indicated that T-cell migration is controlled, at least in part, by the SDF-1 α – CXCR4 interaction (Poznansky et al.). It was found that at concentrations greater than 100 nM, SDF-1 α repels T cells in a CXCR4 dependant

manner. The findings within this study support the concept that SDF-1 α acts as both an attractant and repellent in a concentration dependant manner.

The most surprising outcome from the set of experiments was the chemo-repellent nature of FBS on neurosphere migration. Proteomic screens have indicated that FBS is rich in chemo-attractive proteins, a finding supported by findings within our own lab, that GBM cells will migrate towards FBS enriched media. The data presented within this study clearly demonstrates that neurospheres were repulsed by the MEME + 10% FBS. The cause of the repulsion has yet to be clearly elucidated, but a few causes are possible.

For the same reason that neurospheres were un-responsive to SDF-1 α at concentrations of 25.0 ng/mL, it is likely the chemo-attractants within the un-modified FBS (including SDF-1 α), were at concentrations below the threshold necessary induce neurosphere migration. The low levels of chemo-attractants would thus explain the lack of attractiveness towards the FBS enriched media. Furthermore FBS contains a wide array of proteins including chemo-repellents. A possible sensitivity to chemo-repellents with a resistance to moderate levels of SDF-1 α would explain the repulsive effect of FBS on the neurospheres.

The repulsive effects of the MEME + 10% FBs upon the neurospheres could also be an innate response based on the cell composition of the neurospheres. Research has indicated that CSC cultures from primary brain tumors grown as neurospheres exhibit characteristics opposite those of cells of the same origin grown in the presence of serum. For instance, primary cultures grown in serum begin differentiating within days while cells of the same origin grown as neurospheres remain undifferentiated for over a year

(deCarvalho et al.). Even after a year the neurospheres will differentiate into the cells of the CNS when transferred to serum containing media. Primary cultures grown in serum have also been compared neurosphere cultures after implantation into mice (Li et al.). The invasiveness of each culture was analyzed and the results indicated that neurospheres were much more invasive than the differentiated cells of the serum containing cultures. A second experiment determined that the passage number of the serum containing cultures also played a role in invasiveness with later passages being more invasive (deCarvalho et al.). One important commonality between these studies was that while primary cultures in serum and primary cultures in stem cell media can be of the same origin, the cells quickly diverge in terms of phenotypic characteristics and even genomic makeup. The end result of the divergence are two distinct cell types with separate characteristics.

The same general concept can be applied to the results observed in this study. It is possible that the neurospheres begin differentiating in the presence of FBS into the different cell lineages during the 20 hour migration incubation. If true, these newly differentiated cells could be less invasive (migrate less) than the cells of the control group in serum free media (ie: those that still have the neurosphere phenotype). Testing this hypothesis may be difficult after an incubation of only 20 hours. One possibility would be to test the levels of SOX2 in the neurospheres before and after the transwell assay. The undifferentiated stem cells should have a much higher expression of SOX2 than would cells beginning to undergo differentiation (Masui et al.). This test would strengthen the hypothesis, as SOX2 expression is an indicator of differentiation status.

CHAPTER FOUR: THE INFLUENCE OF GBM CELL LINES ON THE MIGRATION OF NEUROSPHERES ISOLATED FROM THE SVZ:

BACKGROUND

Glioblastomas are known to release chemokines under hypoxic and high stress conditions *in vivo*. Stress can be applied to GBM cell cultures *in vitro* to induce a similar release of chemokines simulating an *in vivo* response. The alternative recurrence model predicts the hypoxia-induced release of SDF-1 α by GBMs *in vivo* induces the migration of neurospheres towards the tumor. To test, GBM cell lines were stressed using nutrient and oxygen deprivation. Under these conditions, the cells were expected to release chemokines which in turn would cause a change in neurosphere migration. The transwell assay was performed to determine if the nutrient deprivation of GBM cell lines did in fact cause a change in neurosphere migration.

MATERIALS AND METHODS

CELL LINES

Three human glioma cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cell lines were T98 G, U-138 MG and the U-87 MG. The T98G cell line (ATCC# CRL-1690) was derived from a 61-year-10 month old caucasian male in 1979. This particular cell line is classified as a glioblastoma multiforme with fibroblast morphology and is grown in Minimum Essential Medium – Eagle's (MEME; ATCC), with 10% FBS supplement. The U-138 MG cell line (ATCC#

HTB-16) was derived from a 47-year-old caucasian male. Pathologically, U-138 MG cell line is a GBM with polygonal morphology, and is cultured in MEME, with 10% FBS. The U-87 MG cell line (ATCC# HTB-14) was derived from a 44-year-old caucasian female. The U-87 MG cell line is classified as a grade III astrocytoma and displays epithelial morphology and was cultured in MEME with 10% FBS. All cell lines were cultured at 37°C and a 5% CO₂ atmosphere. Cells were passed on an as-needed basis which was typically every three to four days, or when the monolayer of cell growth covered more than 70% of the surface area of the flask. The cells were scraped from the base of the flask to disrupt adhesion, and then aspirated with the medium into a collection tube and centrifuged. The expended growth medium was aspirated and the cells were re-suspended into fresh growth medium. The re-suspended cells were then plated at a density approximately 25% of their previous density.

TRANSWELL ASSAY

The transwell assay was performed as described previously with the exception of the following changes; For the first set of experiments GBM cell lines were plated into the outer transwell chamber at a density of $5.0 - 7.5 \times 10^5$ cells / mL in un-supplemented MEME. The cells were incubated for 24 hours before inserting the inner transwell chamber. The remaining steps were carried out as previously described. For the second set of experiments, GBM lines were cultured with un-supplemented MEME in sealed culture flasks. The cultures were sealed to inhibit gas exchange and thus induce a

hypoxic growth environment. After 24 hours the media was removed from the flask and centrifuged (150 x g for 20 min) to remove suspended cells. The supernatant was collected and centrifuged once more to further remove cellular debris from the conditioned media. The collected media was termed GBM-conditioned media. Complete cell removal was determined through the examination of the conditioned media with an inverted light microscope. Cell-free, GBM-conditioned media was placed into the outer transwell chamber and the migration assay was conducted. As a final experiment, 100 ng/mL SDF-1 α was added to the GBM-conditioned media.

RESULTS

The effect of GBM cells on neurosphere migration was investigated *in vitro* using the transwell assay. Three GBM cell lines; U87, U138 and T98 were used during the first experiment. Each cell line was plated into the outer transwell chamber in serum-free, unmodified MEME and deprived of oxygen. After 24 hours dissociated neurospheres were plated into the inner transwell chamber. The results indicate that neurosphere migration in the experimental groups differed from the control groups. There was a significant reduction ($P \leq 0.001$) in neurosphere migration when influenced by both the T98 (Figure 7) and U87 cell lines (Figure 8). Migration of neurospheres also appears to be reduced when separated from the U138 cell line (Figure 8) however the reduction in migration failed to reach statistical significance. Similar results were observed in two subsequent trials (data not shown). Taken together, GBM cell lines govern neurosphere

migration in a chemo-repellent manner as demonstrated using the transwell migration assay.

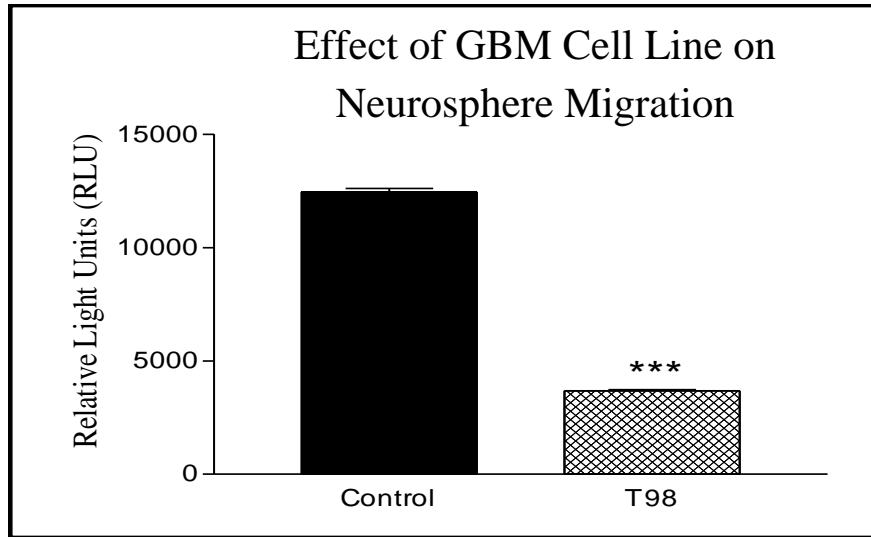


Figure 7: The chemotactic effect of the T98 GBM cell line on SVZ isolated neurospheres. When compared to the control group, the T98s induced a significant reduction in neurosphere migration ($P \leq 0.001$).

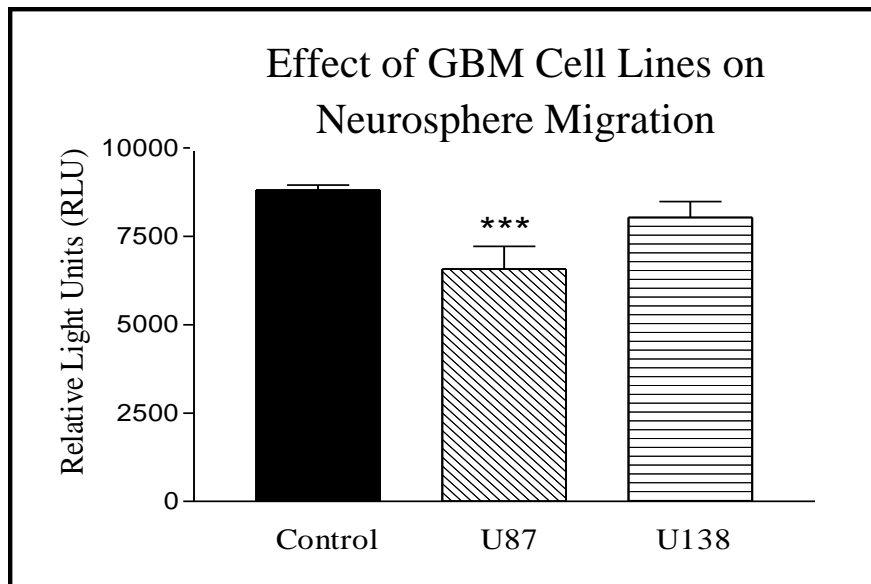


Figure 8: The effect of the U87 and U138 GBM cell lines on the migration of SVZ neurospheres. Both GBM cell lines caused a reduction in neurosphere migration, however, only the U87 cell line reached statistical significance ($P \leq 0.001$).

The change in neurosphere migration was thought to be due to the release of soluble chemokines from the GBM cell lines. To test the validity of the assumption, GBM-conditioned media was utilized in the second series of assays. The GBM-conditioned media from each of the three cell lines was found to alter neurosphere migration in a chemo-repellent fashion. Conditioned media from the three cell lines; U87, U138 and T98 caused a statistically significant reduction in neurosphere migration (Figure 9). These findings provide evidence that GBMs act in a chemo-repellant manner upon neurospheres through the release of chemokines or soluble factors in vitro.

The final experiment investigated the effect of an exogenous addition of 100.0 ng/mL SDF-1 α to the GBM-conditioned media samples. It was hypothesized the addition would negate the repulsive effect of the GBMs. This hypothesis was supported, as SDF-1 α addition caused a significant change in neurosphere migration resulting in an overall increase in neurosphere migration (Figure 10). It should be noted the SDF-1 α addition did not bring the neurosphere migration back to the levels observed with the control groups.

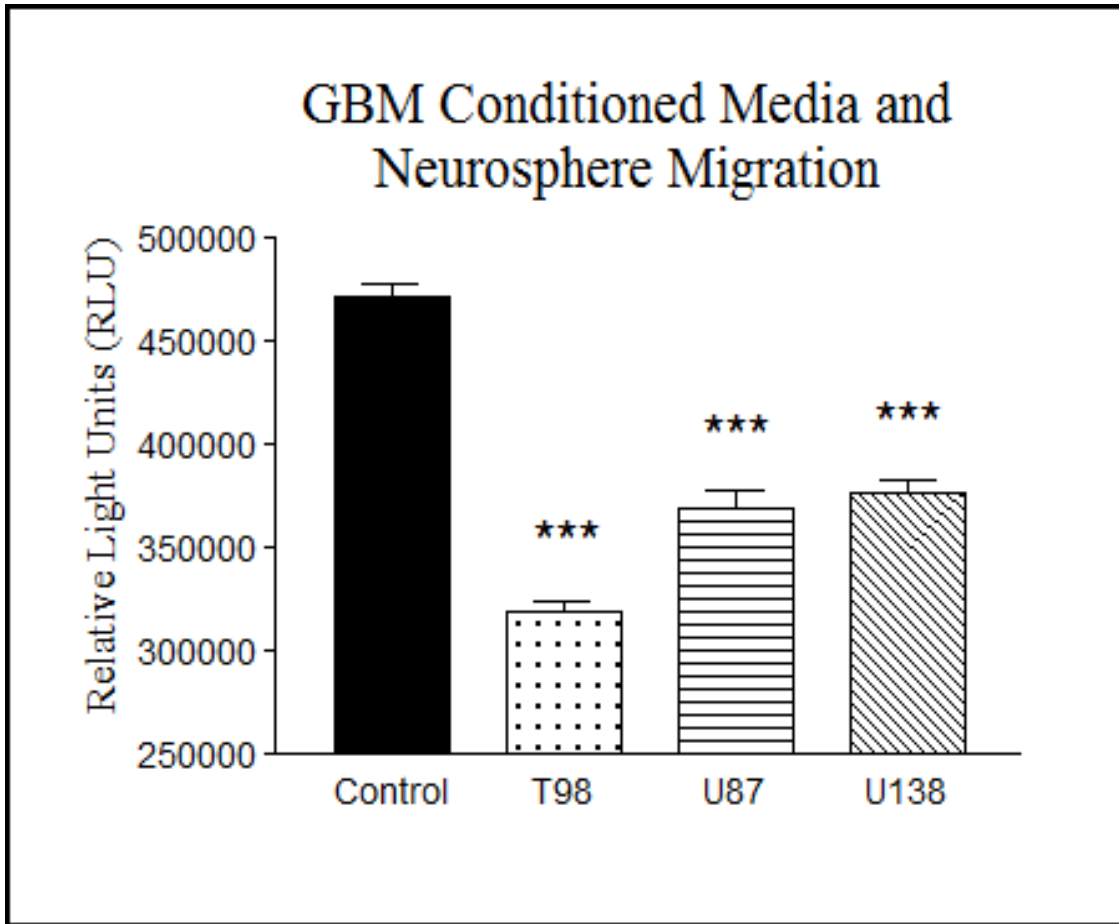


Figure 9: Effect of GBM-conditioned media from the T98, U87 and U138 cell lines on neurosphere migration. All three cell lines caused a statistically significant ($P \leq 0.001$) reduction in neurosphere migration when compared to the control. These findings support the GBM induced neurosphere repulsion found previously, indicating the observed effects are due to chemokine release.

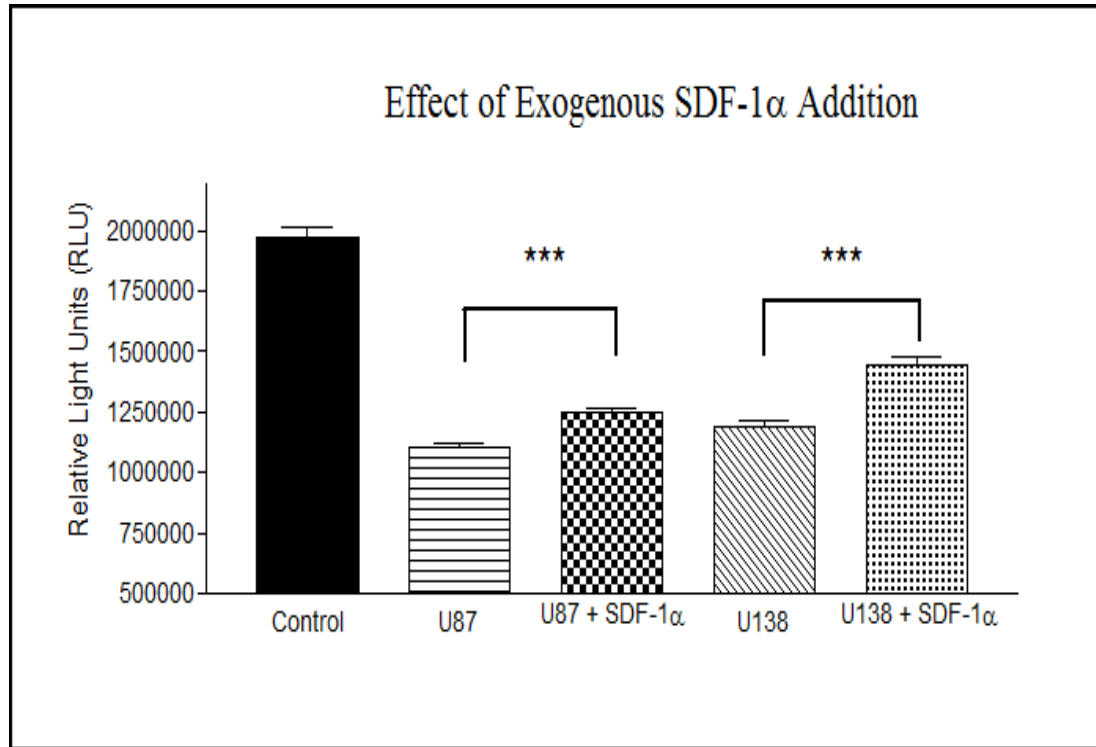


Figure 10: Effect of exogenous addition of 100.0 ng/mL SDF-1 α to GBM-conditioned media. The U87 and U138 cell lines caused significant reduction in neurosphere migration compared to the control. The addition of SDF-1 α to the conditioned media caused a significant increase in migration for both cell lines. ($P \leq 0.001$)

DISCUSSION

To overcome the growth limitations of hypoxia, gliomas release SDF-1 α and VEGF. Together, these proteins promote the recruitment of endothelial progenitor cells and subsequently the formation of new blood vessels. While the role in angiogenesis is quite clear, side effects of SDF-1 α release have yet to be fully elucidated. Hypothesized here is a possible role in tumor recurrence and metastasis. The same attractive properties exerted on the EPCs could extend to other cell types including stem cells / neurospheres.

Attraction of these cells could be the first step towards the formation of a secondary tumor, as hypothesized by the alternative recurrence model.

The attractive nature of exogenous SDF-1 α as observed in the previous experiments was the basis for determining whether GBM cell line released factors (including SDF-1 α) would be sufficient to induce a similar effect on neurosphere migration. Glioma cell lines were deprived of nutrients, including oxygen, for 24 hours prior to being used in the assay. The nutrient deprivation was thought to induce the release of hypoxia related proteins. It was hypothesized that the SDF-1 α release under these conditions would thus increase. This SDF-1 α increase would then attract neurospheres, an effect measurable using a transwell assay. Under these conditions however, the GBM cell lines did not cause an increase in neurosphere migration but rather a significant decrease when compared to control groups.

Glioma-conditioned media was utilized in the second series of experiments and yielded many benefits over culturing the GBM cells within the outer transwell chamber. First, the exclusion of GBM cells from the transwell assay would confirm the repulsive effects were due to the release of soluble factors and not the physical presence of the cells. This in-turn would eliminate the possibility of a physical GBM - neurosphere interaction causing the repulsion. Secondly, by removing the GBM cells from the outer chamber, cells collected post-migration from the outer chamber would be exclusively neurospheres. This allowed for an accurate migration quantification. The final advantage of using conditioned media was that gas exchange could be prevented during the

incubation stage. This yielded a better simulation of hypoxia and a hypothesized increase in SDF-1 α release.

The results from the second set of experiments clearly confirmed the initial findings. The GBM-conditioned media caused a significant reduction in neurosphere migration when compared to the control groups. Further, the repulsive effects of the GBM cell lines could be conclusively linked to the release of proteins into the culture media and not due to a physical interaction between the GBM cells and the neurospheres. While the data appears to refute the hypothesized alternative recurrence model, it does indicate that GBMs govern neurosphere migration behaviors in the transwell assay.

The second set of experiments also aimed to determine whether the repulsive nature of GBM-conditioned media could be reversed with the addition of exogenous SDF-1 α . The addition of 100.0 ng / mL SDF-1 α did cause a significant attraction of neurospheres when compared to GBM-conditioned media not enriched with SDF-1 α . Interestingly, the SDF-1 α addition did not bring the neurosphere migration rates back to the baseline levels observed with the control groups. Taken together, the results suggest the neurospheres used in the study are either less sensitive to SDF-1 α or highly sensitive to chemo-repulsive proteins being released by the GBM cell lines.

The findings also indicated a need to determine the physiological relevance of the hypoxia simulation used in the study. A determination of GBM released protein levels before and after the 24 hour nutrient deprivation would answer whether the hypoxia simulation had any effect on the GBM cell lines. Testing the protein levels after longer

nutrient deprivation periods would also determine if an increase in nutrient deprivation should be used in further studies.

CHAPTER FIVE: SDF-1 α CONCENTRATION IN HYPOXIA INDUCED GBM CELL LINES

BACKGROUND

To overcome hypoxic conditions *in vivo*, GBMs release elevated levels of VEGF and SDF-1 α . Hypoxia can be simulated for cells cultured *in vivo*, as was performed within this study. To determine whether the hypoxia induction caused the desired increase in SDF-1 α release, a sandwich enzyme linked immuno-sorbant assay (ELISA) was performed. Levels of SDF-1 α were measured at four time points, to evaluate the effect of the hypoxia induction. The times points tested were nutrient deprivation periods of 24, 48 and 72 hours. The SDF-1 α level at each of the experimental time points was then compared to the control group. The control group was GBM-cells grown in un-supplemented MEME with gas exchange allowed. A student's t-test was performed to determine statistical significance between samples. The result of which was an evaluation of the effectiveness of the hypoxia simulation performed in the study.

MATERIALS AND METHODS

EXPERIMENTAL SAMPLES

The sandwich ELISA was utilized to determine SDF-1 α concentrations released by four common GBM cell lines. The GBM cell lines included the U87MG, U138MG, T98G and SF-767s. The SF-767s were cultured in MEM + Glutamax (Gibco) and supplemented with 10% FBS. The other GBM cell lines were cultured as described

previously. Each cell line was plated at a density of 2×10^6 cells / 10.0 mL media supplemented with 10% FBS. The cells were incubated for 24 hours under normal culture conditions to allow the cells to attach to the floor of the culture dishes. After the 24 hour period, the FBS containing media was exchanged for un-supplemented media and the cells were again incubated for 24 hours. To quantify the amount of SDF-1 α released by cells cultured in un-supplemented media with proper gas exchange, supernatant samples were collected. Cells and cellular debris were then removed by the centrifugation steps as described previously. The samples were then compared to a standard curve prepared for each trial to quantify of SDF-1 α levels for each cell line and used as the control data.

Following the determination of baseline SDF-1 α levels for each cell line, the sandwich ELISA was used to determine the effect of the 24 hour nutrient deprivation. Each cell line was plated at a concentration of 2×10^6 cells / 10.0 mL media supplemented with 10% FBS. After 24 hours, the media was exchanged for 10.0 mL of serum free media as described. At this time, the flasks were sealed to prevent gas exchange. The cell lines were then incubated for 24 hours before samples of the media were taken. The same procedure was repeated changing the final incubation to 48 hours and then to 72 hours.

SANDWICH ELISA

All reagent preparation was performed as specified by R and D Systems (Minneapolis, MN.). After bringing the reagents to room temperature, 50 μ L of assay diluent RD1-55 was added to each well. Next, 50 μ L of Standard, Control or cell supernatant was added to the diluent containing wells. The plates were then incubated for two hours at room temperature on an orbital shaker set to 500 rpm. Once the incubation period was completed, the media was aspirated and each well was washed with 400 μ L of wash buffer four times. When the wells were completely dry, 100 μ L of mouse SDF-1 α conjugate was added. Again, the plate was incubated for 2 hours at room temperature on an orbital shaker. Incubation was followed by the aspiration/washing step described previously and the subsequent addition of 200 μ L of substrate solution to each well. The plate was then incubated for 30 minutes in darkness before adding 50 μ L of stop solution to each well, the result of which was a color change based on the amount of SDF-1 α . Lastly, the optical density was recorded at 450 nm.

To prepare the experimental samples, the four cell lines were cultured as described above. The hypoxic time points were arranged in a manner that allowed all samples to be taken on the same day. From each culture plate, 7.0 mL of media was removed, transferred to 15 mL conical tubes and centrifuged for 20 minutes at 150 x g. The supernatant was removed after the centrifugation and the procedure was repeated, the result of which was GBM-conditioned media devoid of both living and dead cells. From each experimental sample, 25 μ L of conditioned media was diluted with 225 μ L of Calibrator Diluent RD6Q as recommended by R and D Systems.

RESULTS

To determine the SDF-1 α concentrations in GBM-conditioned media, the SDF-1 α standard curve was constructed as recommended by R and D Systems (Figure 11). From the standard curve, the line of best fit was calculated yielding the equation $y = 0.000121x + 0.004221$. The SDF-1 α levels were determined using the line of best fit and the corrected absorbance values from each experimental sample. The corrected absorbance values were determined by subtracting the average absorbance of the 0 ng/mL SDF-1 α standard sample from the average absorbance value for each experimental sample.

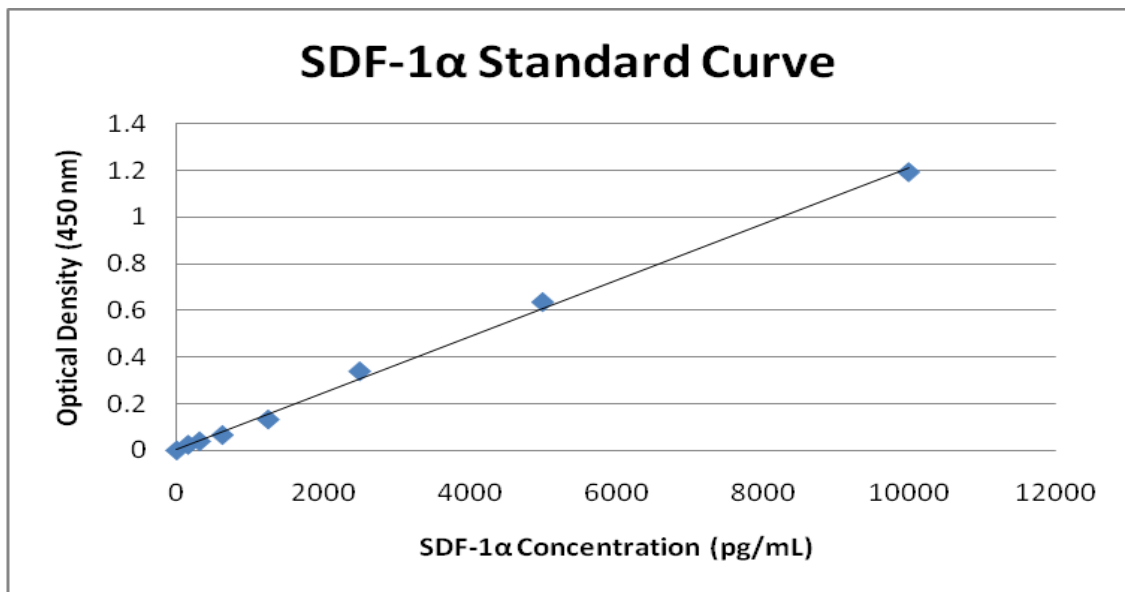


Figure 11: SDF-1 α standard curve. The standard curve was constructed as recommended by the R and D Systems protocol. The equation obtained through the construction of the line of best fit was used to determine the SDF-1 α concentrations in the GBM-conditioned media samples. The equation for the line of best fit was found to be $y = 0.000121x + 0.004221$ with an $R^2 = 0.997609$.

The sandwich ELISA was initially performed to determine the SDF-1 α concentration in GBM-conditioned media at a cell density of 2×10^6 cells / 10.0 mL media (control groups). Each of the experimental samples yielded an average absorbance between 0.11 and 0.12 at 450 nm (Figure 12). A student's t-test was performed on the average absorbance values, the data from which indicated that none of the experimental samples were statistically different. Next, the average absorbance, and equation for the line of best fit were used to calculate SDF-1 α concentrations. The GBM samples were initially diluted 10-fold for the sandwich ELISA so the SDF-1 α concentrations were multiplied by 10 to find the actual concentrations shown in table 2. The SDF-1 α concentrations for each cell line were as follows: 1190 pg / mL for the T98s, 525 pg / mL for the SF767s and the U138 cell line released a negligible amount of SDF-1 α (0.333 pg/mL). All three concentrations were below the exogenous levels necessary to induce neurosphere migration. Due to the 10-fold dilutions of the initial GBM samples the U87 reading fell below the baseline level which meant that calculations could not be performed on the sample.

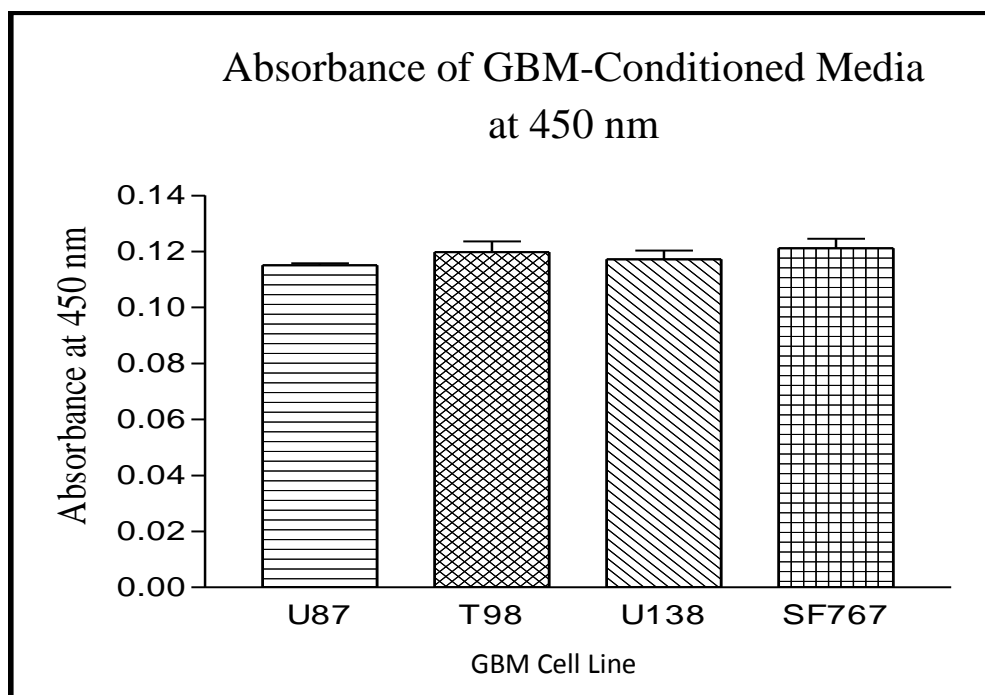


Figure 12: Average absorbance at 450 nm for the four cell lines tested using the SDF-1 α sandwich ELISA. None of the cell lines displayed an absorbance value that was significantly different than any of the other cell lines. It is also important to note that the samples taken from each cell line were diluted 10 fold as recommended by the manufacturer.

Table 2: Results from the sandwich ELISA on the four GBM samples (U87, T98, U138 and SF767). The samples were tested for SDF-1 α release. For each sample, the standard deviation between samples is shown along with the average SDF-1 α concentration. The corrected absorbance was found by taking the average absorbance and subtracting the “blank” recording. This calculation caused the U87 sample to fall below the threshold for calculations.

Sample	Average Absorbance at 450 nm	Corrected Absorbance	SDF-1 α Concentration (pg/mL)
U87	0.1157	0.002625	-
T98	0.1316	0.018525	1190
U138	0.1173	0.004225	0.333
SF767	0.1236	0.010525	525

A second trial was conducted in which the experimental samples were not diluted in order to obtain SDF-1 α levels for the U87 cell line. The precision of the initial trial was also determined by performing a second trial. By not using the recommended 10-fold dilution, each of the four GBM cell lines yielded SDF-1 α concentrations. The U87, T98, U138 and SF767 cell lines released SDF-1 α concentrations of 107.5 pg / mL, 151.7 pg / mL, 152.5 pg / mL and 45.03 pg / mL respectively (Table 3). The data from the second sandwich ELISA indicates GBM-conditioned SDF-1 α concentrations are magnitudes of order lower than the exogenous concentrations found to induce neurosphere migration. After determining the concentrations for the four GBM cell lines, a follow-up experiment was performed to determine the effect of longer nutrient deprivation on the release of SDF-1 α .

Table 3: Results from the second trial of the sandwich ELISA on the four GBM cell lines. The conditioned media was not diluted prior to testing. Each sample resulted in a corrected absorbance value above threshold for calculations. The resulting SDF-1 α concentrations were once again below the exogenous levels found to induce neurosphere migration.

Sample	Average Absorbance at 450 nm	Corrected Absorbance	SDF-1 α Concentration (pg / mL)
U87	0.1302	0.017125	107.5
T98	0.1355	0.022425	151.7
U138	0.1356	0.022525	152.5
SF767	0.1227	0.009625	45.03

To determine whether a longer nutrient deprivation would have caused a change in concentrations, each sample was tested at four time points. SDF-1 α concentrations found in the conditioned media of nutrient deprived (including oxygen) GBM cultures of 24, 48, and 72 hours were compared to the control groups (Figure 13). A student's t-test was performed to compare the average absorbance of each experimental sample. Testing of the four GBM cell lines found only the comparison of the T98 control to the T98 72-hour deprivation sample reach statistical significance (Figure 13b). The average absorbance of each sample was then used to determine the corrected absorbance values.

The corrected absorbance was subsequently drawn on to procure the SDF-1 α concentrations for each experimental sample (Table 4). For the U87 cell line, only the corrected absorbance value for the 72-hour sample was large enough to calculate SDF-1 α concentrations (142 pg / mL). The four time points of the T98 cell line resulted in a wide range of SDF-1 α concentrations (209 pg / mL – 1950 pg / mL). Importantly, a positive correlation was observed between the duration of nutrient deprivation and SDF-1 α release for the T98 samples. The corrected absorbance in the control group for the U138 cell line was too low to calculate the SDF-1 α concentration. However, the same correlation of nutrient deprivation and SDF-1 α release was observed for the three time points tested (0.33 pg / mL – 125 pg / mL). The SF767 cell line again followed the positive correlation observed in the other cell lines (325 pg / mL – 1492 pg / mL).

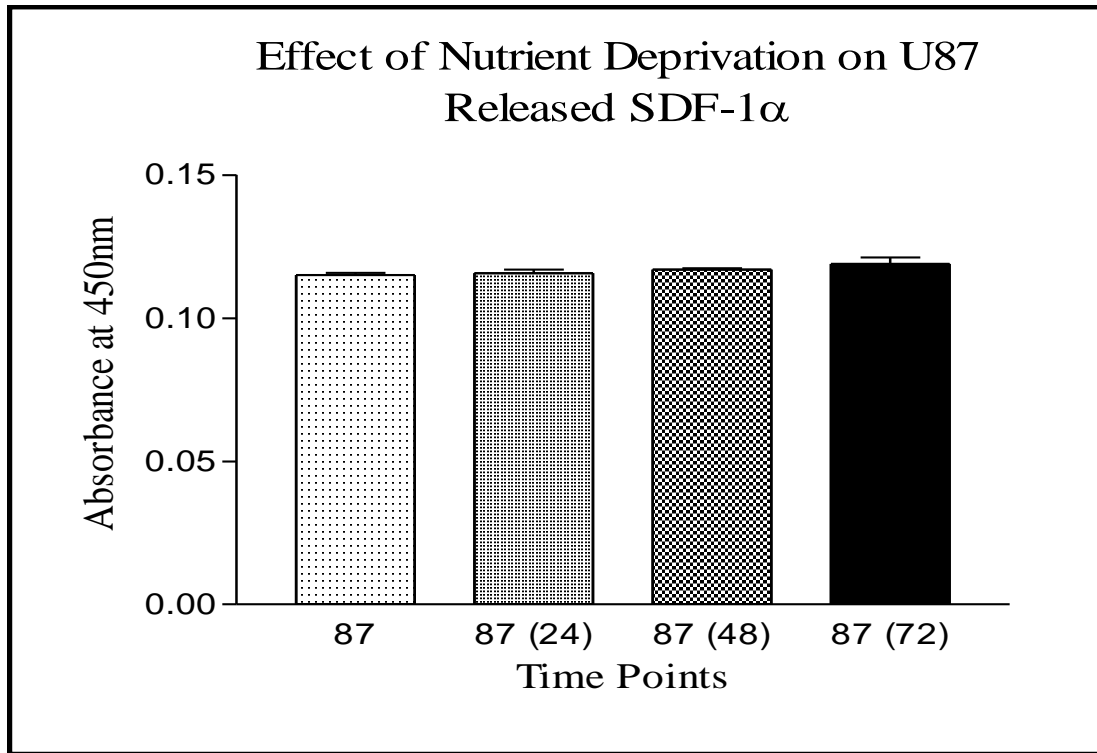


Figure 13a: Average absorbance at 450 nm at differing time points for the U87 cell line using the SDF-1 α sandwich ELISA during the in vivo nutrient deprivation study. The first column represents media taken from cells not deprived of nutrients. No significant difference in SDF-1 α release was obtained at any of the time points tested.

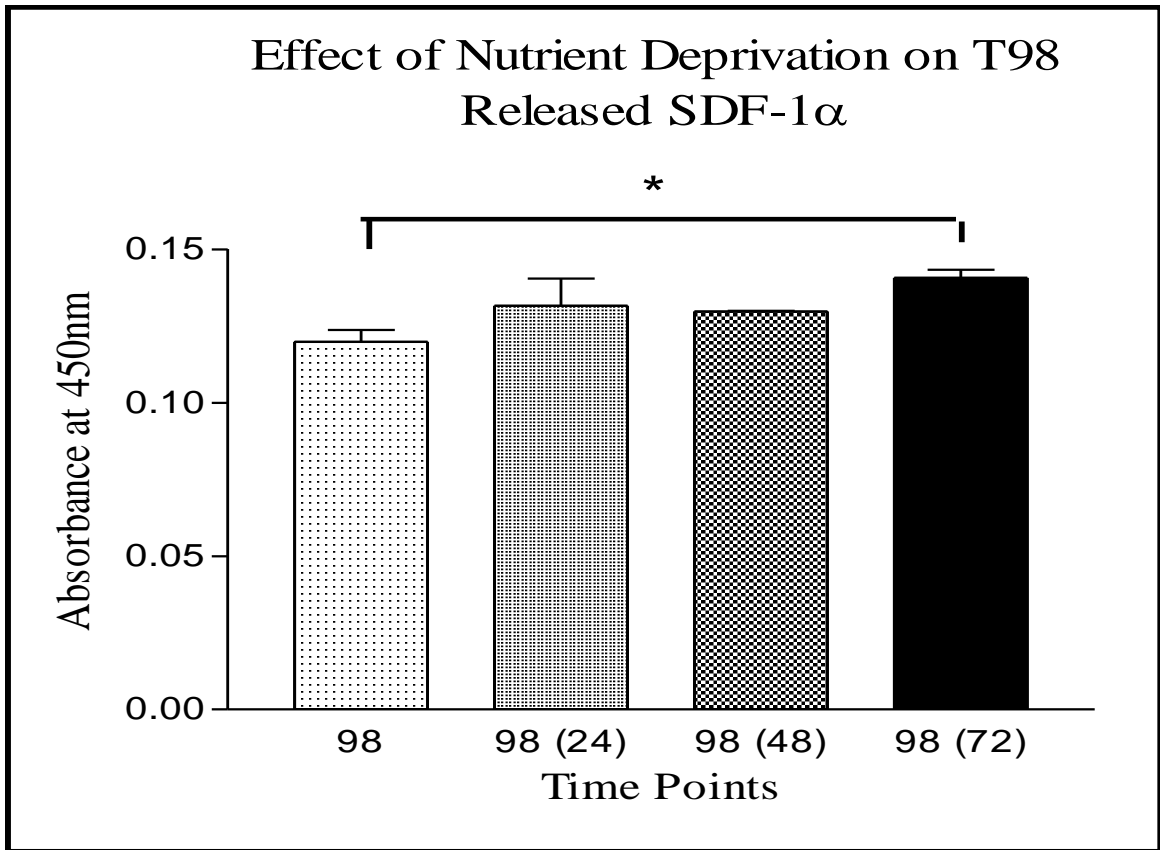


Figure 13b: Average absorbance at 450 nm at differing time points for the T98 cell line using the SDF-1 α sandwich ELISA during the in vivo nutrient deprivation study. The first column indicates media taken from T98 control group (cells not nutrient deprived). A significant increase in SDF-1 α release was only observed at the 72 hour point.

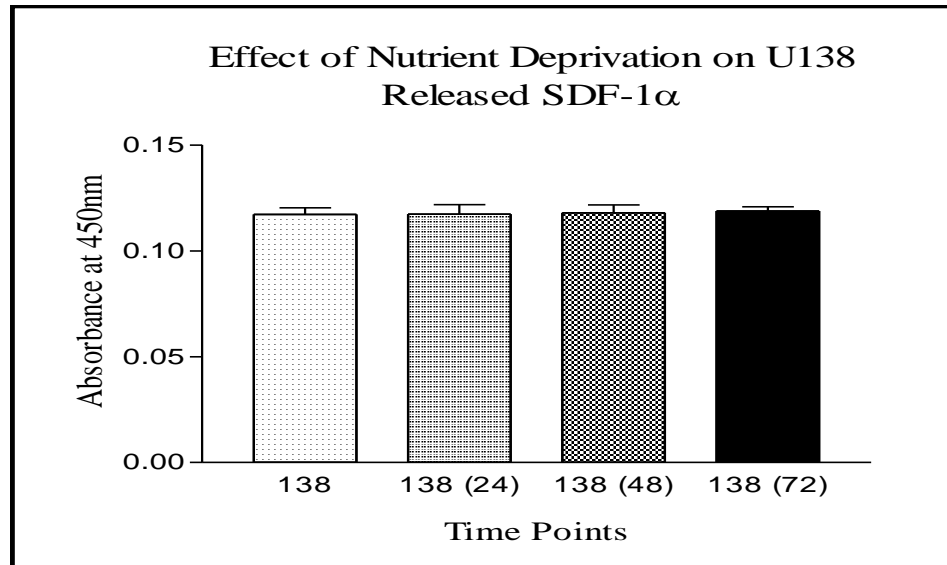


Figure 13c: Average absorbance at 450 nm at differing time points for the U138 cell line using the SDF-1 α sandwich ELISA during the in vivo nutrient deprivation study. The first column indicates media taken from the U138 control (not nutrient deprived). No significant increase in SDF-1 α was observed at any of the time points tested.

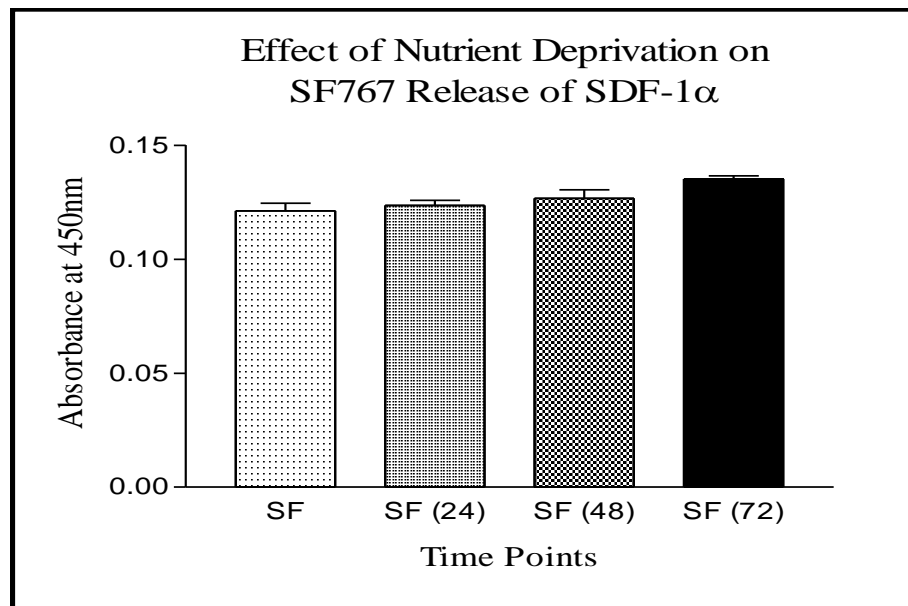


Figure 13d: Average absorbance at 450 nm at differing time points for the SF767 cell line using the SDF-1 α sandwich ELISA during the in vivo nutrient deprivation study. The first column indicates media taken from SF767 control (not nutrient deprived). No significant increase in SDF-1 α was observed at any of the time points tested.

Table 4: Time course sandwich ELISA. The four cell lines (U87, T98, U138 and SF767) were tested using the sandwich ELISA procedure at increasing time points to determine the effect nutrient deprivation had on overall SDF-1 α release. A positive correlation between SDF-1 α release and increased nutrient deprivation is observed.

Sample	Average Absorbance at 450 nm	Corrected Absorbance	SDF-1 α Concentration (pg / mL)
U87	0.1151	.002025	-
U87 (24)	0.1157	.002625	-
U87 (48)	0.1169	.003825	-
U87 (72)	0.119	.005925	142
T98	0.1198	.006725	209
T98 (24)	0.1316	.018525	1190
T98 (48)	0.1297	.016625	1034
T98 (72)	0.1407	.027625	1950
U138	0.1172	.004125	-
U138 (24)	0.1173	.004225	0.333
U138 (48)	0.118	.004925	58.7
U138 (72)	0.1188	.005725	125
SF767	0.1212	.008125	325
SF767 (24)	0.1236	.010525	525
SF767 (48)	0.1267	.013625	784
SF767 (72)	0.1352	.022125	1492

DISCUSSION

At the time of writing, no data has been published on GBM released SDF-1 α concentrations. The one published piece of data on SDF-1 α quantification looked at release by human dental pulp cells. The study found that SDF-1 α was released at a concentration of 45133 ng / L (45 ng / mL) (Gong et al.). Based on the limited information available, it is hypothesized that exogenous SDF-1 α levels necessary to induce neurosphere migration in this study were far greater than levels released by the GBM cell lines. Even so, it was of interest to the investigator to determine the SDF-1 α concentrations released by the GBM cell lines used in the transwell assay.

The GBM cell lines were cultured at a density of 2×10^6 cells / culture. At this density, SDF-1 α concentrations were expected to be below the 45 ng / mL found in the prior study. The transwell assays performed previously found migration to be concentration dependant, with a necessary SDF-1 α concentration of 50 ng / mL. In order for GBM cell lines to induce similar neurosphere migration, the SDF-1 α levels in the conditioned media would be expected to be near the 50 ng / mL threshold. Any concentration below the threshold would explain the inability of conditioned media to attract the neurospheres. To test the validity of this theory, the 24-hour GBM - conditioned media (the same time point used in the previous transwell assays) was tested to determine SDF-1 α concentrations. The concentrations varied between cell lines (0.33 pg/mL for the U138 cell line – 1190 pg/mL in the T98 cell line), however, none of the samples approached the exogenous 50 ng/mL threshold. The findings confirm that 24-hour nutrient deprivation of GBM cell lines does not induce the release of SDF-1 α at

concentrations possible of attracting the isolated SVZ neurospheres using the transwell assay.

The findings of the 24-hour deprivation were analyzed to determine the efficacy of the nutrient deprivation. The transwell assays used 24-hour nutrient deprivation to induce the release of SDF-1 α . The sandwich ELISA was used again to determine if a longer nutrient deprivation period would increase SDF-1 α release. Time points of 48 and 72 hours were compared to the 24 hour time point and a control of MEME containing FBS. The data indicated the 24-hour deprivation did not cause a significant increase in SDF-1 α concentrations compared to the control. The data did indicate a positive correlation between an increase in the duration of nutrient deprivation and SDF-1 α release. However, only the 72-hour time point in the SF767 cell line reached statistical significance.

CHAPTER SIX: INVOLVEMENT OF THE NEUROPILIN-1 – SEMA3A INTERACTION AS AN EXPLANATION FOR NEUROSPHERE REPULSION:

BACKGROUND

Due to the chemo-repulsive influence of GBM cell lines on neurosphere migration, a literature review was performed to find a candidate responsible for the effect. The review led to the hypothesis that GBM cell lines may release an elevated level of Sema3A, a member of the semaphorin family of proteins (Goshima, Ito et al.). If the neurospheres were expressing the receptor to Sema3A, Nrp-1, the interaction would cause chemotaxis in a repulsive manner. To determine whether the hypothesis was possible, real time PCR was performed on neurosphere lysate in hopes of finding Nrp-1 mRNA.

RNA extraction was used to obtain intact Nrp-1 mRNA strands. A blast search was used in order to build complementary forward and reverse mRNA primers specific to the Nrp-1 mRNA (Integrated DNA Technologies, Coralville, IA.).

The forward primer used the sequence:

5' – TGG GAA GAT TGC ACC TTC TCC TGT – 3'

The reverse primer used the sequence:

5' – TTC TGC CCA CAA TAA CGC CCA ATG – 3'

Used together, the primers resulted in an overall amplification of a 381 BP sequence of the Nrp-1 mRNA. The calculation for the amplified sequence length would be important for determining whether an amplified sequence was from the Nrp-1 mRNA.

MATERIALS AND METHODS

RNA EXTRACTION

RNA was extracted from the isolated neurospheres by using the spin column protocol for animal cells in the manufacturer's published protocols for the RNeasy Kit (Qiagen, Hilden, Germany). The buffer RLT, which contains guanidine thiocyanate, was used to lyse the cells in highly denaturing conditions. Under these conditions, RNases become inactive allowing for the purification of intact RNA. Homogenization of the cells was then performed by passing the cells through a QIAshredder homogenizer (Qiagen). The addition of 70% ethanol to the lysate promotes selective binding of RNA to the spin column membrane. The spin column was then washed to remove contaminants, and the RNA was eluted in RNase-free water. The samples were used within 1 hour of RNA extraction.

REAL TIME - POLYMERASE CHAIN REACTION (RT - PCR)

Dilution of the primers was performed to prepare the master mix. For example, an 8 sample experiment would dilute 8 μL of primer into 72 μL of nuclease free water. Once each primer was diluted, the experimental tubes were set up as follows:

1.0 μL experimental sample
18.5 μL nuclease free water
1.0 μL of forward primer
1.0 μL of reverse primer
0.5 μL of AMV reverse transcriptase
3.0 μL of Acces Quick™ Master Mix
<hr/>
25.0 μL Total

The experimental samples were prepared along with positive controls, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta actin. Amplification of the samples was carried using the following parameters:

40 PCR cycles:

Denaturation: 94°C for 15 min

Annealing: 55-60°C for 1 min

Extension: 72°C for 30 sec

Final Extension: 72°C for 3 min

RESULTS

Three RT-PCRs were performed on separate mRNA extractions. None of the three reactions were positive for Nrp-1 mRNA in the neurosphere cell line (Figure 14). There was also no amplification of GAPDH indicating an un-successful extraction of mRNA. A second cell line, the T98 glioma cell line was also tested. The T98 cell line was shown to contain both beta actin and GAPDH mRNA (Figure 14). Taken together, the mRNA extraction protocol was effective but the reason behind the lack of mRNA amplification from the neurosphere line remains to be answered.

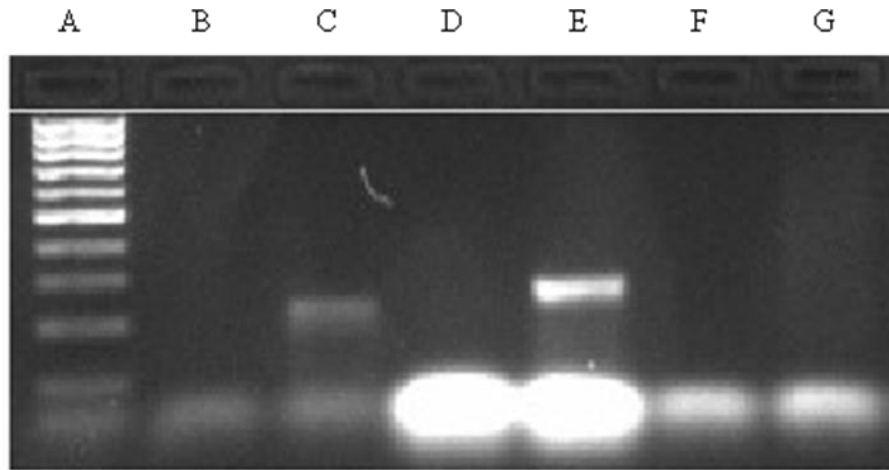


Figure 14: Electrophoresis of PCR products. Lane assignments are as follows: (A) Fisher™ exACT Gene 1000 bp PCR DNA Ladder (B) Neurosphere, beta actin (C) T98 beta actin (D) Neurosphere GAPDH (E) T98 GAPDH (F) Neurosphere, Nrp-1 (G) T98, Nrp-1, The T98 glioma cell line was positive for the controls, GAPDH and beta actin as indicated by the appropriate banding in lanes C and E. The neurosphere cell line did not display amplification of any of the PCR amplified sequences.

DISCUSSION

Sema3A was recently shown to be expressed nearly ubiquitously by GBMs. The same cells were also shown to express the receptor Nrp-1 and migrate through autocrine signaling (Bagci et al.). It seemed likely the SVZ isolated neurospheres would also express Nrp-1. The results of the RT-PCR indicate the neurospheres do not transcribe Nrp-1 mRNA as no amplification of the mRNA was achieved. Also discouraging was the lack of GAPDH and beta actin amplification within the neurosphere lysates. This would suggest a failed protocol, however, amplification in the control cell lines was achieved. The lack of amplification is discouraging, and efforts to investigate the findings have continued. Of note however, a lack of mRNA does not necessarily mean Nrp-1 is not already expressed on the cells. In order to determine if Nrp-1 is in fact present, a western blot needs to be performed. At the same time, a western blot should be performed to determine if Sema3a is being released by the GBM cell lines. At this point it seems unlikely the Sema3a – Nrp-1 interaction is the reason for the GBM induced repulsion of neurospheres. Further studies will be needed to determine the factor(s) responsible for the findings.

CHAPTER SEVEN: EFFECT OF EXOSOMES ON NEUROSPHERE MIGRATION:

BACKGROUND

The role of exosomes in cancer progression has yet to be fully determined. Exosomes play many roles in normal cellular physiology making it likely that exosomes play multiple roles in cancer. Exosomes are known to contain cancer-specific mRNAs that when taken into normal cells are translated and expressed (Baj-Krzyworzeka et al.). The cancer specific mRNAs code for proteins important in cancer progression including those necessary for cellular migration. The repulsive nature of GBM cells on neurosphere migration has led to the hypothesis that exosomes may carry mRNA coding for receptors to chemo-repellent ligands. To test, exosome-free GBM-conditioned media was used in the transwell assay and compared to exosome containing media. The comparison of exosome-free to exosome-containing media of the same origin would give a generalized assessment of exosome involvement in neurosphere migration.

MATERIALS AND METHODS

EXOSOME FREE MEDIA

The creation of GBM-conditioned media was performed as described in chapter 4. The conditioned media was centrifuged (Beckman – Coulter, Brea, CA.) for 10 minutes at 300 x g to remove living cells. The supernatant was collected and centrifuged at 2000 x g for 20 minutes which pellets dead cells. To remove cellular debris the supernatant

was centrifuged at 10000 x g for 30 minutes. Exosomes were pelleted by centrifuging at 100000 x g for 70 minutes twice. The centrifugation steps are thought to pellet the exosomes without removing proteins found in the conditioned media. The exosome-free conditioned-media was collected and subsequently used in the transwell assays as the exosome free (-) sample. The procedure was used to create exosome-free media for the U87MG, U138MG and T98G cell lines.

After analysis of the samples, exosome-free media was used in the transwell assay. The GBM-conditioned media from the T98, U138 and U87 cell lines were split into two groups, A and B. From group A the exosomes were removed creating an exosome free sample. The splitting into two separate groups was essential in that each group was derived from the same original sample. The migration induced by GBM-conditioned media (B) was compared to the migration induced by the exosome-free GBM-conditioned media (A) using an unpaired student's t-test to determine statistical significance.

SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Ultracentrifugation at 110,000 x g is considered sufficient for the specific separation of exosomes from conditioned media. To confirm, GBM-conditioned media and exosome-free GBM-conditioned media assayed by SDS-PAGE. First, 1,000 mL of 1X NuPAGE SDS running buffer is prepared by combining 50 mL of 20X NuPAGE SDS running buffer with 950 mL of dH₂O. The 1X running buffer is separated into volumes

of 800 mL and 200 mL. To the 200 mL sample, 500 μ L of NuPAGE antioxidant is added. The sample containing the antioxidant was added to the inner chamber, and the other to the outer chamber. The gels were run at 250 V for 40 minutes (Bio-Rad, Hercules, CA.), and visualized using Simply Blue Safestain (Invitrogen). (Figure 15).

PREPARATION OF FBS SAMPLES

Three experimental FBS samples were used during the experiments: un-modified FBS, exosome-free FBS (-) and exosome-enriched FBS (+). The FBS (-) was produced by centrifuging un-modified FBS at 110,000 x g for 24 hours. The end result of the centrifugation step was an exosome-free supernatant and pellet of exosomes. The supernatant was collected and used as the FBS (-) sample. The exosome pellet was re-suspended in 1.0 mL of MEME and used as the FBS (+) sample. As with the exosome-free GBM-conditioned media, SDS-PAGE was performed to ensure the centrifugation was exosome specific (Figure 16).

RESULTS

The specificity of the exosome removal process was tested using SDS-PAGE. The procedure gave evidence to the presence or absence of bands between samples. The GBM-conditioned media was tested first. Two cell lines were tested (T98 vs. T98 exosome-free and U87 vs. U87 exosome-free) for similarities and differences in protein banding patterns. The SDS-PAGE does not give a protein quantification, but the results

show similar banding patterns between the exosome-containing and exosome-free samples (Figure 15). The finding indicates the ultracentrifugation step did not drastically change protein levels found in the samples.

The FBS samples were also tested using SDS-PAGE. The banding patterns and intensities were once again observed in each of the four FBS samples (un-modified FBS, FBS (-), and FBS (+)). The gel indicates that each of the samples have similar banding patterns but there is a large disparity in banding intensity (Figure 16). The strong protein smears observed in the exosome-enriched samples coupled with the faint smears of the exosome-free samples indicates the ultracentrifugation procedure may not have been exosome specific.

After analysis of the samples, exosome-free media was used in the transwell assay. The results of the study support our previous findings indicating a chemo-repulsive effect of GBM-conditioned media on neurosphere migration (Figure 17). When compared to the control group, each cell line (T98, U87 and U138) caused a significant reduction in neurosphere migration. Interestingly, the data fails to show a reduction in neurosphere migration when comparing the GBM-conditioned media to the exosome-free GBM-conditioned media (Figure 17). The original hypothesis was that removal of exosomes would cause a decrease in neurosphere migration. However, the transwell assay did not show a decrease in migration. In fact, the T98 exosome-free media caused an increase in migration as compared to the exosome-containing T98 media.

To test the validity of the preliminary findings, a second trial was performed. The second assay tested the effect of exosome removal from GBM-conditioned media in the

T98 and U87 cell lines. This time, the removal of exosomes caused a statistically significant reduction in neurosphere migration in each cell line (Figure 18). Both the T98 and U87 cell lines showed a significant reduction in neurosphere migration when exosomes were removed from the media. These findings support the initial hypothesis. More trials are needed however to determine the actual effect of exosome removal.

The previous migration assays uncovered the intriguing chemo-repulsive effect of FBS on neurosphere migration. Due to the similar chemo-repulsive nature observed using GBM-conditioned media, it was hypothesized that exosomes found within the FBS may play a similar role. The data from the FBS transwell assays indicate that all three FBS samples caused a reduction in neurosphere migration compared to control (Figure 19). These findings support the previous chemo-repellent effect of FBS on neurosphere migration. The main purpose for the experiment, however, was to investigate the effect of exosome removal. The removal of exosomes from FBS caused no change in neurosphere migration compared to un-modified FBS. This contradicts the initial hypothesis, as it indicates exosome removal does not affect migration of neurospheres. Interestingly however, a comparison of the FBS (+) and un-modified FBS samples saw a significant change in migration. The exosome enriched sample caused an increase in neurosphere migration as compared to the exosome free sample (Figure 19).

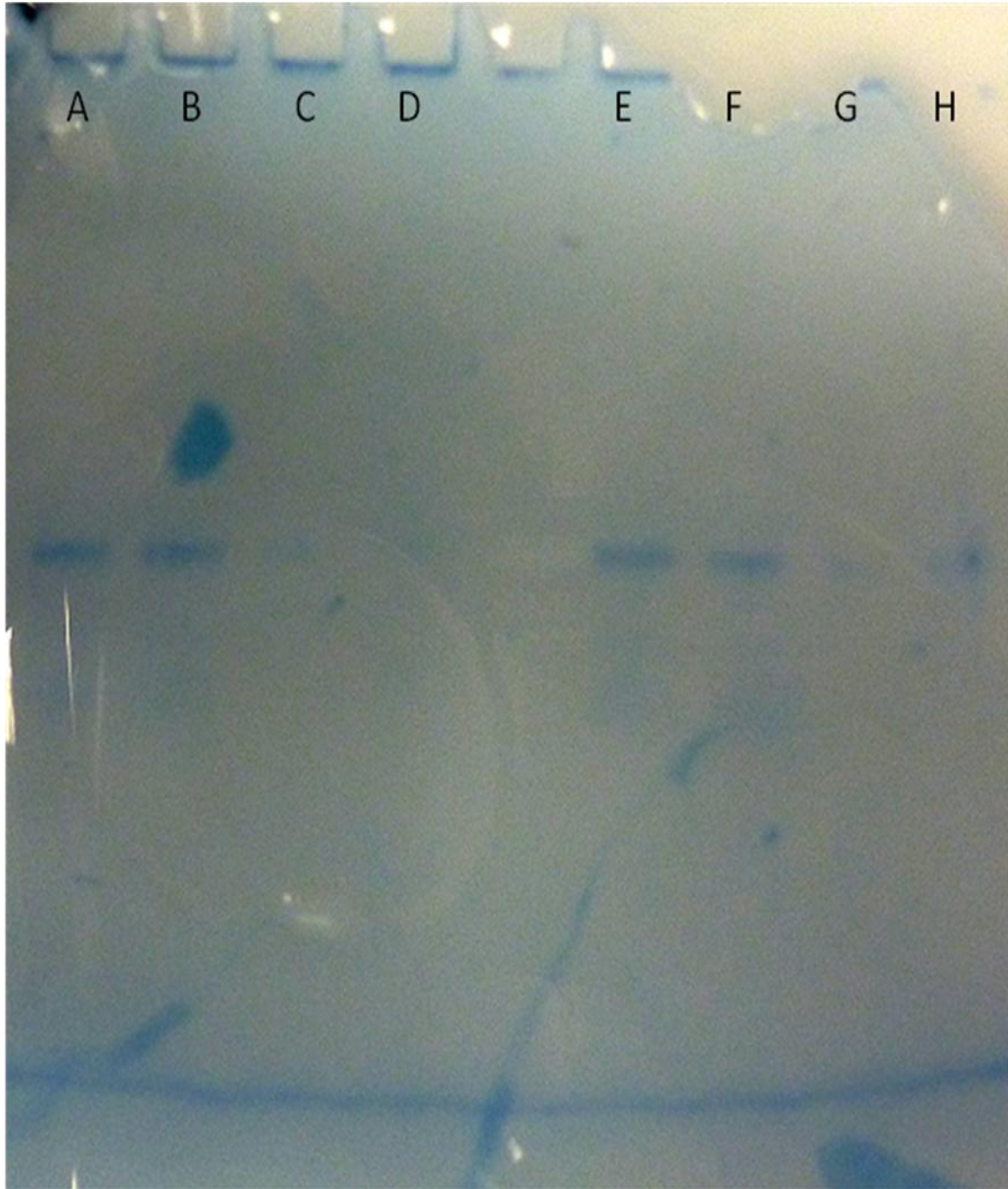


Figure 15: SDS-PAGE performed on GBM-conditioned media in vitro. The samples were run in duplicate. Lane assignments were as follows: (A,B) exosome free T98 supernatant (C,D) exosome free U87 supernatant (E,F) T98 supernatant (G,H) U87 supernatant. The procedure does not quantify protein levels, but does show similar banding characteristics between the GBM-conditioned media and exosome free media for both cell lines. This indicates that the exosome removal step was not removing soluble proteins.

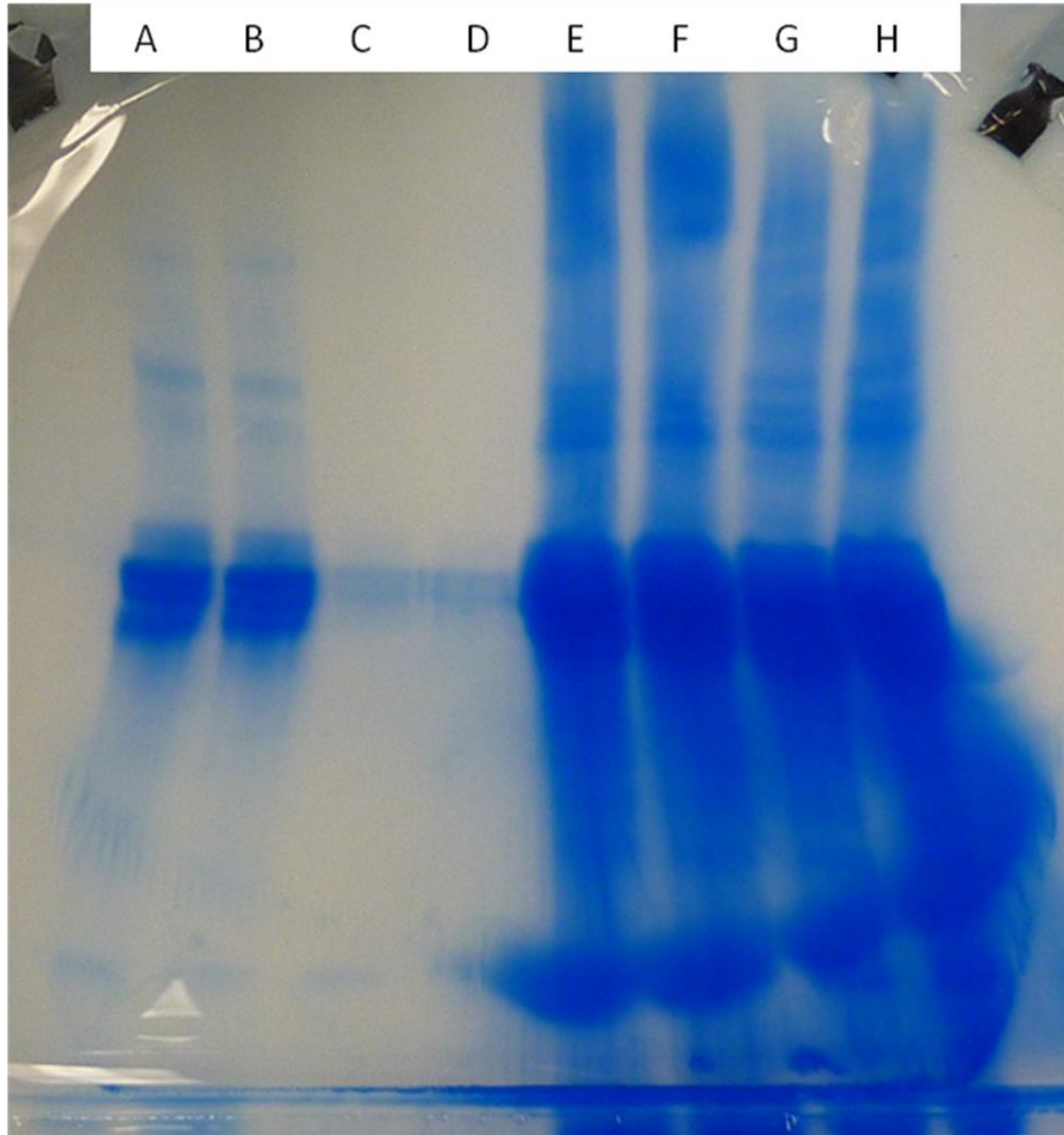


Figure 16: SDS-PAGE performed on un-modified FBS samples to determine the effectiveness of the exosome removal process. The samples are (A,B) un-modified FBS (C,D) FBS (-) (E,F) exosome enriched (+) FBS pellet (G,H) FBS (+). The procedure does not quantify protein levels but indicates the exosome removal procedure is only marginally specific. The protein smears observed in the un-modified FBS samples are much fainter in the FBS (-) samples. Conversely, the protein smears in the FBS (+) samples appear much more concentrated than in the un-modified FBS samples. Together, these findings indicate that the observed changes in neurosphere migration may be exosome independent.

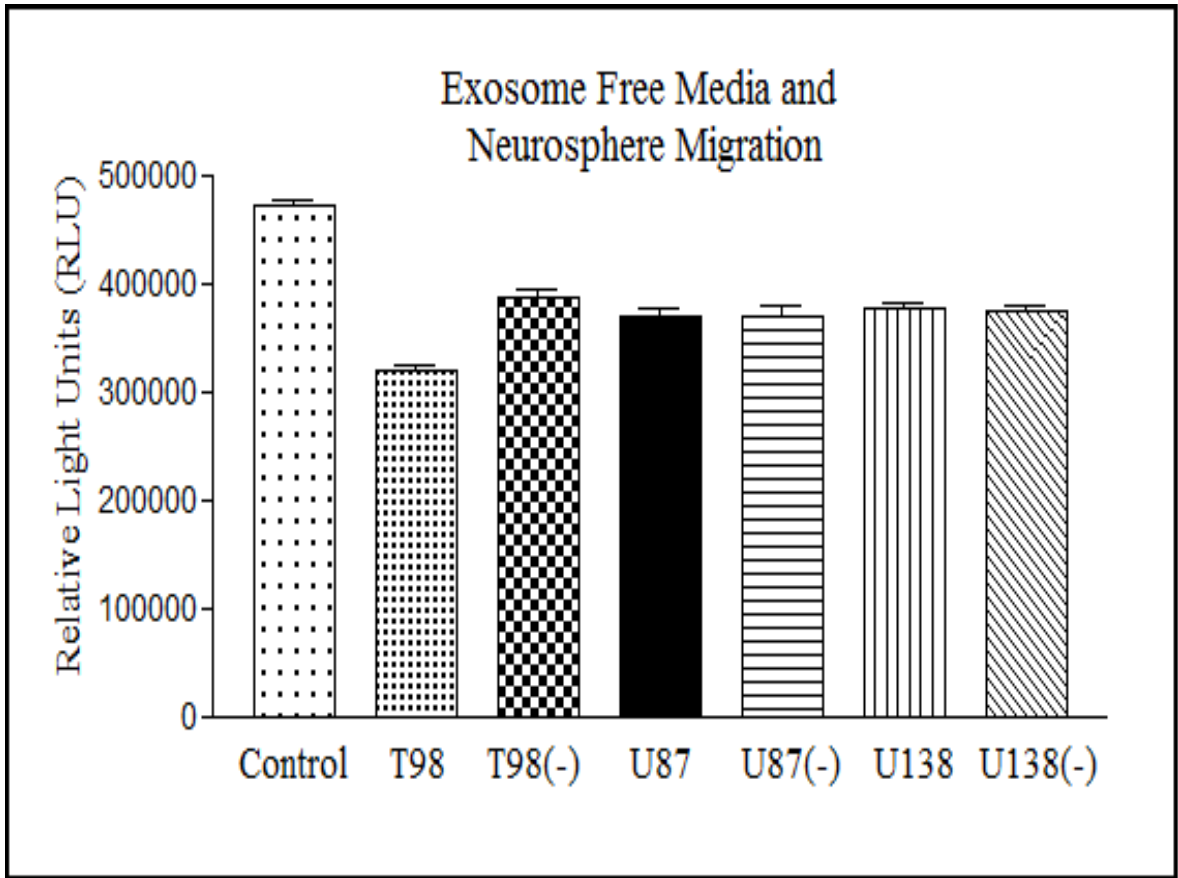


Figure 17: Effect of exosome removal on neurosphere migration. The initial findings indicated that neurosphere migration may be increased with exosome removal from conditioned media as observed with the T98 and T98(-) groups.

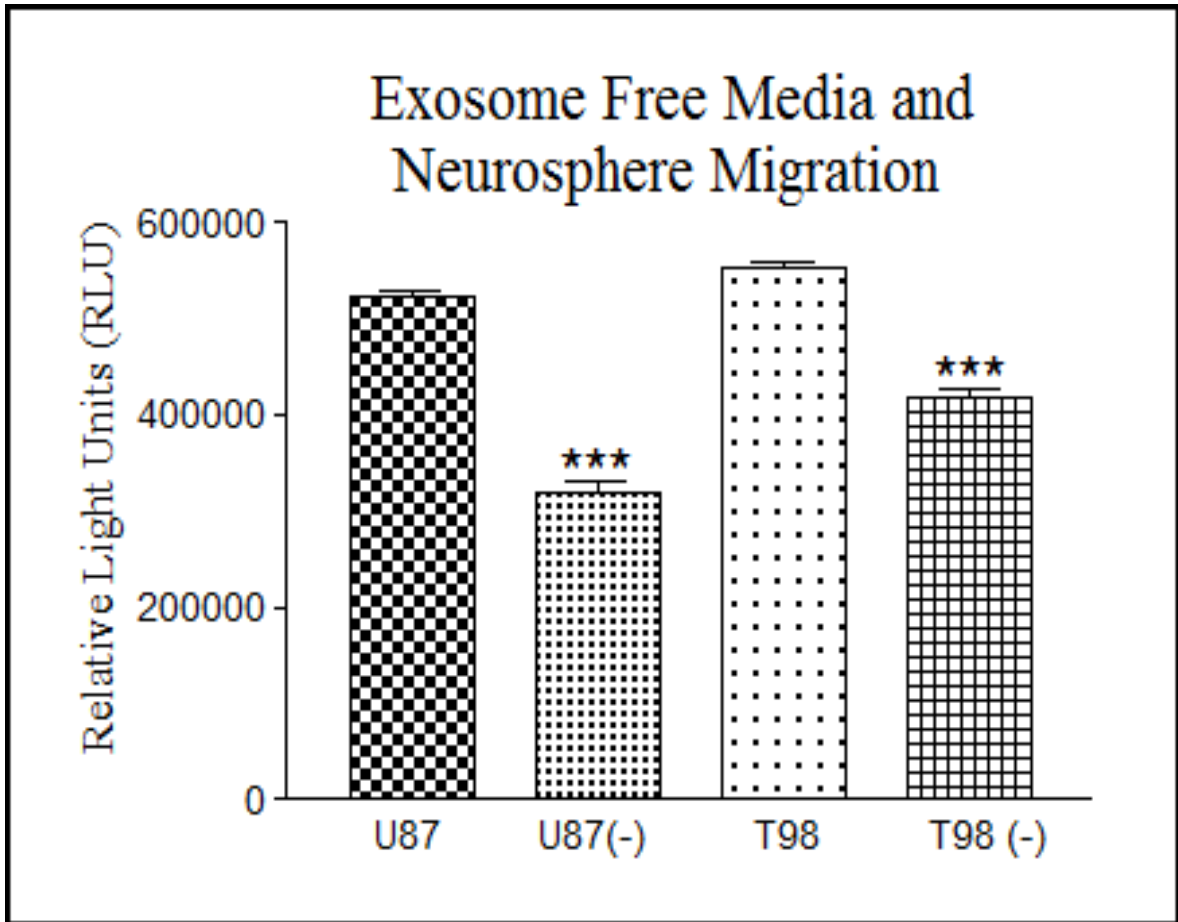


Figure 18: Effect of exosome removal on neurosphere migration. Tumor conditioned media from the U87 and T98 cell lines were compared to exosome free GBM-conditioned media of the same origin. In both cases, the overall neurosphere migration was significantly reduced with exosome removal.

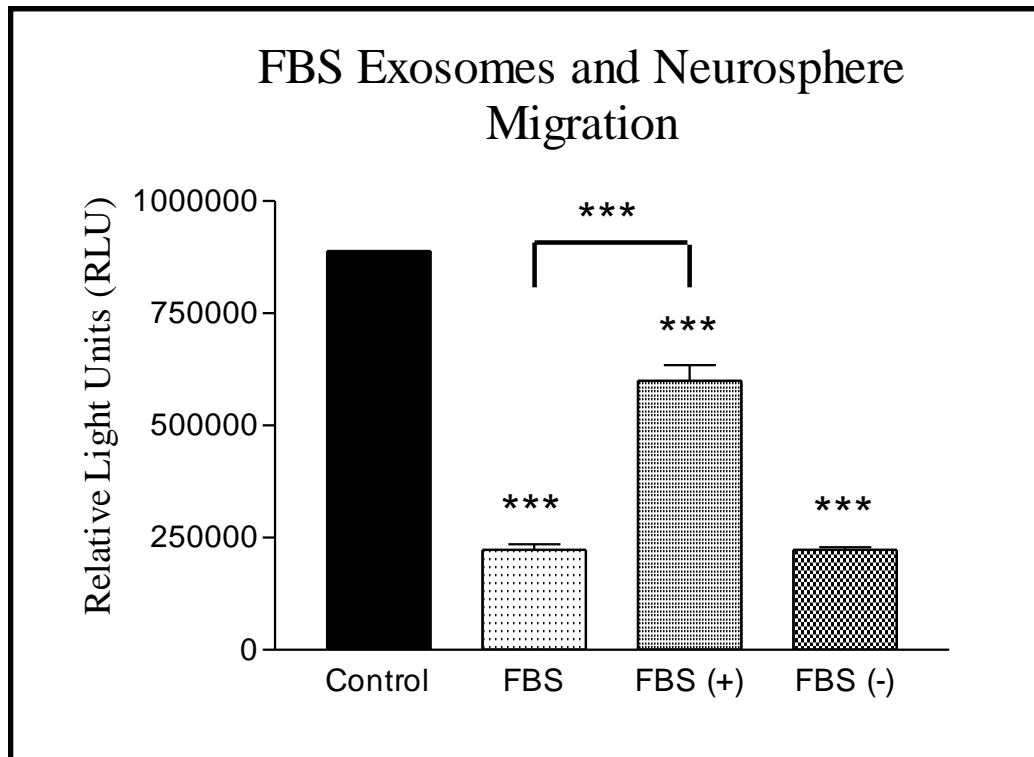


Figure 19: Effect of un-modified, exosome free and exosome enriched FBS on neurosphere migration. All three samples caused a statistically significant reduction in neurosphere migration compared to the control sample. The FBS free sample did not cause a change in migration as compared to the un-modified FBS; however, the FBS enriched sample caused a significant increase in neurosphere migration compared to the exosome free sample.

DISCUSSION

The purpose of the study was to determine if exosomes play a role in neurosphere migration. To obtain exosomes, samples were spun at 110,000 x g. The high speed centrifugation was thought to be sufficient for the separation of exosomes from solution. Furthermore, the spin was thought specific to the isolation of exosomes meaning that exosomes would be the only factor removed from the conditioned media. To test,

exosome-free samples were compared to un-modified samples of the same origin using SDS-PAGE.

The first samples analyzed were GBM-conditioned media from the T98, U87 and U138 cell lines. The protein banding varied between cell lines but was similar in samples taken from the same cell type, including a comparison of exosome-free and un-modified samples. It should be noted that SDS-PAGE does not quantify or positively identify proteins in a sample but it can be used to make general observations as to changes in banding intensities between samples such as between exosome-containing and exosome-free media. The samples tested using SDS-PAGE were also used in the initial transwell assay. Only the T98 cell line showed any change in overall neurosphere migration. Surprisingly, the removal of exosomes from the T98 conditioned media did not cause an overall reduction in migration, but rather a net increase. A second trial was run using the same cell lines to verify the initial findings. Results of the second trial did not support the initial findings. This time the T98 and U87 cell lines saw a reduction in neurosphere migration when exosomes were removed.

At the same time the GBM-conditioned media was tested, a parallel study was conducted using FBS samples. As previously discussed, the FBS samples included un-modified FBS, exosome-free FBS and exosome-enriched FBS. Just as in the GBM-conditioned media study, protein levels were observed using SDS-PAGE. The gel shown in figure 16 indicates the exosome removal process may not be exosome specific. The un-modified FBS sample contains prominent protein smears, while the exosome-free sample is nearly devoid of banding. This observation can be quickly associated with a

poor exosome removal process. However, the protein smears could be due to the presence of exosome bound proteins. The centrifugation steps would increase the exosome density and the protein banding could be interpreted instead as an indicator of exosome presence. Regardless, the findings from the SDS-PAGE illuminate the need for a more conclusive test to be performed to identify the presence or absence of exosomes. Therefore, findings from this study cannot be conclusively correlated to the presence or absence of exosomes. However, the series of migration assays performed do appear to be influenced by the removal or enrichment with GBM derived exosomes. It is the opinion of the author that future exosome studies will support the initial findings reported in this study, and definitively link exosomes to a change in cellular motility.

CHAPTER 8: SYNOPSIS AND CONCLUSIONS

BACKGROUND

The basis of this project was built upon five initial hypotheses; 1) Neurospheres could be isolated from the subventricular zone (SVZ) of the adult mouse brain; 2) Neurospheres would display directed migratory capabilities when separated from chemo-attractants; 3) Neurospheres would preferentially migrate towards glioblastoma cell lines; 4) Migration would be due in large part to CXCR4 receptor interaction with GBM-released SDF-1 α and would be independent of the physical presence of the GBM cells; 5) Motility would be reduced when exosomes were removed from tumor-conditioned media. These hypotheses were tested and several conclusions could be drawn.

RESULTS

ISOLATION, PROLIFERATION AND DIFFERENTIATION OF MURINE NEURAL STEM CELLS:

The initial hope was to isolate cells from the SVZ of the adult mouse brain. This was achieved and the isolates were maintained using the neurosphere culturing technique. The neurospheres were cultured to passage 27 and were able to continuously re-populate the flask, a clear stem-cell indicator. A positive confirmation of stem cells also includes testing the differentiation capabilities of the isolates. Neural stem cells should have the ability to differentiate into oligodendrocytes, astrocytes and neurons. Because of issues

discussed previously, the SVZ isolates were only differentiated into oligodendrocytes as confirmed through a positive identification of the oligodendrocyte marker O4. A lack of positive markings for neurons and astrocytes means the isolates could not be positively identified as neural stem cells. Although the cells were able to form neurospheres for 25 passages, a quality possessed by stem cells, lack of complete differentiation did not allow for complete confirmation of stem cells.

STROMAL DERIVED FACTOR 1α (SDF- 1α) AND FETAL BOVINE SERUM (FBS) INDUCED MIGRATION OF SVZ DERIVED NEUROSPHERES:

In order to determine the effect of GBMs on neurosphere motility, the neurospheres needed to be capable of inducible migration. To test, neurospheres were separated from known chemo-attractant SDF- 1α . Exogenous, incremental additions of SDF- 1α to un-modified media allowed for a measurement of motility in a model of escalating concentrations. The neurospheres responded to the addition of exogenous SDF- 1α , albeit in a dose-dependent manner. SDF- 1α concentrations between 50 and 100 mg/mL acted as statistically significant chemo-attractants. The 200 mg/mL SDF- 1α samples caused the opposite to occur, a net decrease in neurosphere migration. This confirms the findings of other groups, in that high SDF- 1α may also act as a chemo-repellent when found at high concentrations. Surprisingly, SDF- 1α levels as high as 25 mg/mL had no effect on migration. This indicates an SDF- 1α concentration dependence in the governance of neurosphere migration.

A lack of information from the literature led to the investigation of the influence of FBS on neurosphere migration. FBS had previously been shown to induce the migration of the GBM cell lines used within the study. The same effect was expected to occur with the use of the SVZ isolated neurospheres. Again, the neurospheres acted opposite to the expectations as overall migration was significantly reduced when exposed to FBS. The repulsive effect of the FBS is both intriguing and novel and identifies an area that needs future studies to elucidate the mechanism(s).

THE INFLUENCE OF GBM CELL LINES ON THE MIGRATION OF NEUROSPHERES:

The SVZ isolated neurospheres were induced to migrate using exogenous additions of SDF-1 α . Doing so indicated the net movement of the neurospheres could be influenced by external stimuli. Since the addition of SDF-1 α was sufficient to induce migration, it was hypothesized the addition of nutrient deprived GBM cells would cause a similar response. The GBM nutrient deprivation was performed to induce the release of tumor derived SDF-1 α . The results of the transwell migration assays indicate that nutrient deprivation of GBM cell lines is not sufficient to cause an increase in neurosphere migration. Furthermore, GBM cell lines consistently caused an overall decrease in neurosphere migration through the release of soluble factors. Within the context of the project, GBM cell lines appear to act as a chemo-repellent to SVZ isolated neurospheres. These findings are very surprising in that other labs have indicated GBMs act as strong chemo-attractant to neural stem cells. A plausible cause for the findings

could be a disconnect between the protein-protein interactions of *murine* neurosphere receptors and human derived brain tumors. The highly conserved nature of the proteins between species leaves this hypothesis in doubt however. To truly test, a complete proteomic screen should be performed on the receptors found on the neurospheres as well as the tumor-derived proteins within the conditioned media.

SDF-1 α CONCENTRATIONS IN HYPOXIA INDUCED GBM CELL LINES:

The concentration dependent, SDF-1 α induced migration coupled with the chemo-repulsive nature of the GBM cell lines led to an investigation of the GBM derived SDF-1 α concentrations. As expected, the 50 ng/mL concentration threshold of exogenous SDF-1 α was found to be greater than the concentrations released by the GBM cell lines. A comprehensive literature review indicated limited information on normal concentrations of SDF-1 α . The study performed here was novel in that it determined SDF-1 α concentrations for the U138, T98 and SF767 GBM cell lines.

The validity of the first sandwich ELISA was tested by performing a second trial. Each of the four cell lines tested were found to release SDF-1 α into the conditioned media. The concentrations varied less between cell lines and it is the opinion of the investigator that the second trial yielded more accurate results mainly because the samples were not initially diluted. The efficacy of the nutrient deprivation on SDF-1 α release was also tested. The results show that as the duration of the nutrient deprivation increased, so did the release of SDF-1 α . Statistical analysis could not be performed since

only one ELISA was performed for each time point. Future ELISAs will be performed and are expected to show the increase to be statistically significant.

NEUROSPHERE PRODUCTION OF NEUROPILIN-1:

Once the attraction of neurospheres to GBM cell lines was shown to be incorrect, we began initial investigations into elucidating the mechanism behind the findings. The sandwich ELISA experiments demonstrated that GBM-released SDF-1 α levels were below the threshold concentrations determined during the exogenous additions necessary to induce migration. The first thought was to check the neurospheres for the expression of CXCR4, the receptor to SDF-1 α . A hypothesized low level expression of CXCR4 would help explain the lack of chemo-attraction to the GBM cell lines, but would not address the chemo-repulsive effects observed. The investigation turned instead to GBM-released chemo-repellents. A semaphorin family member, Sema3A became the likely answer to the search as it has shown to be nearly ubiquitously released by GBMs. The RT-PCR was performed to test for the presence of Nrp-1 mRNA. None of the samples displayed an amplification of the mRNA target however. While the lack of mRNA decreases the likelihood of protein expression it cannot be ruled out. A western blot assay would definitively determine whether or not Nrp-1 is present on the neurospheres.

ROLE OF EXOSOMES IN NEUROSPHERE MIGRATION:

The results of the exosome-free transwell assays were quite interesting. The issue with the study however, was with the exosome removal process. As mentioned previously, SDS-PAGE was performed on the exosome-free and exosome-containing media samples of the same origin. The image shown in figure 16, the end result of the SDS-PAGE, illustrates striking differences in banding intensities. The differences in protein banding can be interpreted in two ways. The changes in banding intensity could indicate the exosome removal process was also removing soluble proteins. This scenario could cause changes in migration rates due to the presence or absence of soluble proteins, thus making a definitive link to exosomes impossible. However, the SDS-PAGE results could also be interpreted in a positive light as well. The ultracentrifugation was used to specifically isolate, and condense exosomes into a dense pellet. The exosome pellet would be rich in membrane bound proteins. Because of this, one may expect to see a difference in protein banding and in turn the differences in protein intensities could be interpreted as a positive identification of exosome presence. Regardless of which explanation is correct, it is believed that a more definitive exosome removal process is needed for further studies. It is the opinion of the author that such a process would yield similar findings and support the hypothesized chemo-attractive nature of exosomes based on the contents within exosomes as indicated in the literature.

CONCLUSIONS:

- Cells capable of continual self-renewal were successfully isolated from the SVZ of the mouse brain and subsequently cultured as neurospheres through passage 25.
- Both SDF-1 α and FBS were able to induce neurosphere migration. The SDF-1 α acted as a chemo-attractant in a concentration dependant manner, however, FBS acted unexpectedly as a chemo-repellent.
- *Murine* neurospheres isolated from the SVZ do not migrate preferentially towards GBM cell lines. The data indicates that neurospheres are repulsed by GBM cells. This suggests that glioblastoma cell lines excrete chemo-repellents which act to “outcompete” the attractants.
- Neurosphere repulsion is tumor-conditioned media dependent since removal of the GBM cells did not cause a change in the Neurosphere motility.
- Quantification of SDF-1 α from tumor-conditioned media using Sandwich-ELISA indicated levels far below the concentrations needed exogenously to induce neurosphere migration.
- Neuropilin-1 mRNA was not found in the neurosphere lysate.
- Preliminary data indicates that exosomes influence migration rates of SVZ isolated neurospheres. The SDS-PAGE assay clearly indicates a need for an adjustment in the exosome isolation procedure for use in future studies.

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