

NEUROSPHERE AND ADHERENT CULTURE CONDITIONS  
FOR MALIGNANT GLIOMA STEM CELL LINES

BY

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A THESIS PRESENTED TO THE UNIVERSITY OF FLORIDA  
GRADUATE SCHOOL IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR THE DEGREE OF  
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2010

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## ACKNOWLEDGEMENTS

I thank Dr. William Friedman and Dr. Brent Reynolds who have been tremendous mentors and have been committed to cultivating my research interests and guiding me during this process. I thank Dr. Marian Limacher, Dr. Nabih Asal, and Dr. Jane Douglas who have been indispensable for the APPCI program and who taught me during the early part of my academic career. I also thank the Florida Center for Brain Tumor Research and the Grey Ribbon Crusade for funding my research projects. I thank my research colleagues, specifically Dr. Loic Deleyrolle, Maria Caldeira, Dr. Hassan Azari, and Dr. Vinata Vedam-Mai for their support, guidance and collaboration in the attempt to improve the lives of patients with brain tumors. Finally, I thank the brain tumor patients who donate their clinical information and tumor tissue for the search for a cure. On a personal note, I would like to thank my family – especially my mother and my husband – who have shaped me into who I am today and have supported me unconditionally.

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Abstract of Thesis Presented to the Graduate School  
of the University of Florida in Partial Fulfillment of the  
Requirements for the Degree of Master of Science

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May 2010

Chair: Marian Limacher

Major: Medical Sciences – Clinical and Translational Science

Understanding the cellular and molecular characteristics of malignant glioma that drive tumor growth is important for the development of more effective treatments to improve survival. Malignant glioma tumor cells are grown in culture for detailed study of the cellular mechanisms that drive growth. Although useful, cell culture also has the risk of selecting for certain cellular populations that may not be representative of the tumor from which they originated. These concerns have led to interest in developing glioma cell culture techniques that allow for propagation of cells that drive tumor growth with minimal changes to tumor cellular genetic and phenotypic profiles. Currently, the neurosphere assay (NSA), using serum-free media supplemented with growth factors, is the standard for growing and maintaining glioblastoma multiforme (GBM) tumor-initiating cells (TICs) in culture. Recently, serum free culture conditions supplemented with laminin have been described which grow neural stem cells (NSC) as a monolayer. Some of these data suggest that establishing cell lines from human gliomas is more efficient when the cells are grown as a monolayer and that the NSA is inferior to adherent culture in terms of apoptosis and differentiation of cells.

We report the comparison of the NSA and laminin growth conditions for the growth of GBM cell lines in terms of growth, apoptosis, differentiation, clonal frequency and tumorigenicity in a xenotransplant model. The GBM cell lines did not demonstrate a difference in growth over time in either growth condition. Using multiple assays, the percentage of apoptotic cells was also equivalent in the NSA and laminin growth conditions. Flow cytometry for differentiation markers did not show statistically significant differences between NSA and laminin GBM cells. Clonal frequency, estimated using the limiting dilution assay, of the laminin cells was similar to published clonal frequency of GBM TICs grown using the NSA. Finally, cells grown in both conditions resulted in tumors when transplanted into the flanks of mice, and these mice had similar Kaplan-Meier survival curves.

These data show that GBM cell lines grown in the NSA and laminin growth conditions are not different in terms of growth, apoptosis, differentiation, clonal frequency and tumorigenicity. The development of alternative culture techniques for establishing cell lines is a useful addition to the existing tools for the study of human GBM (hGBM). hGBM cells grown in adherent conditions are not significantly different from cells grown with the traditional NSA.

## CHAPTER 1 INTRODUCTION

### **Overview**

Gliomas represent the most common primary brain tumor in the U.S. with approximately 10,000 new cases of malignant glioma diagnosed each year.<sup>1</sup> Gliomas originate from transformed glial cells (i.e. astrocytes, oligodendrocytes and ependymal cells), and usually result in diffuse infiltration of brain tissue. Understanding the cellular and molecular characteristics of malignant glioma that drive tumor growth is important for the development of more effective treatments to improve survival. Tumor cells are grown in culture to 1) study growth patterns, and 2) provide an endless supply of cells for animal transplantation and other experiments. Although very useful, cell culture also has the risk of selecting for a certain cellular population or changing the genetic or phenotypic expression profile over time.<sup>2,3</sup> Therefore, tumor cells grown in culture may not be truly representative of the tumor from which they originated. These concerns have led to interest in developing glioma cell culture techniques that allow for propagation of cells that drive tumor growth with minimal changes to tumor cellular genetic and phenotypic profiles.

### **Malignant Glioma**

Gliomas are classified by the World Health Organization (WHO) as grade I-IV depending on histologic features.<sup>4</sup> Malignant gliomas are defined as WHO grade III and IV gliomas. The median survival with treatment for WHO grade III gliomas (anaplastic glial tumors) is only 24 months and for WHO grade IV gliomas (glioblastoma multiforme) is a dismal 14 months.<sup>1,5</sup> Treatment for malignant gliomas includes surgical resection, chemotherapy and radiation. However, despite aggressive treatment, all survivors have

tumor recurrence. The median survival after development of recurrence for malignant glioma is 7 months.<sup>6,7</sup> For patients with recurrent glioblastoma multiforme (GBM) the median survival is approximately 2-4 months.<sup>7-9</sup> Management of recurrence can involve supportive care alone—palliative resection, radiation, and chemotherapy—or participation in clinical trials. The poor prognosis of malignant glioma, especially GBM, has been attributed to the limitations of our current treatment regimens. Current treatment includes radiation (XRT) and temozolomide (TMZ). Limited treatment efficacy for malignant glioma has been attributed to tumor cellular and genetic heterogeneity and limited delivery of therapy to the tumor site.<sup>10</sup>

Understanding the cellular make-up of a particular tumor may allow for tailored therapy. An example of this phenomenon is the discovery of the role of O6-methylguanine-DNA methyltransferase (MGMT), a DNA repair gene, in GBM tumor resistance. GBM patients with epigenetic silencing of MGMT, due to methylation of the promoter, have improved survival regardless of treatment.<sup>11</sup> Additionally, GBM patients with silenced MGMT have significantly more improvement in survival after treatment with XRT with concomitant temozolomide (TMZ), now the standard of care for initial treatment of GBM, compared to patients with active MGMT.<sup>12, 13</sup>

The discovery of MGMT status influencing treatment response to TMZ highlights one of the major hurdles in the treatment of malignant glioma – tumor cellular and genetic heterogeneity.<sup>14,15</sup> Cell surface protein expression and molecular defects vary dramatically among and within tumors.<sup>16-22</sup> This heterogeneity represents a significant therapeutic challenge and most likely complements the *de novo* and acquired resistance of these tumors, especially with monotherapy. Blocking proliferation of the

entire tumor population has been the challenge in the development of novel therapies for malignant glioma. This challenge has led to interest in combinatorial therapy approaches as well as tumor cellular assessment to determine which treatments will be most effective.<sup>10</sup> Understanding cellular and molecular malignant glioma defects requires studying these cells in culture and using cultured cells for further experiments.

### **Tumor Stem Cells and Cell Culture**

The origin, maintenance, and resistance of solid tissue malignancies, including malignant gliomas, is attributed to transformed precursors that have the cardinal properties of stem cells.<sup>23,24</sup> These transformed cells with stem cell-like properties are hypothesized to be resistant to conventional therapy based on the notion that conventional therapy targets the heterogeneous body of cancer cells in a relatively non-specific fashion and spares the tumor stem cells due to their unique properties.<sup>25-27</sup> A body of evidence now exists suggesting that brain tumors contain this relatively rare subpopulation of tumor-initiating cells that exhibit stem cell characteristics,<sup>24,28-30</sup> that this population may be responsible for treatment resistance<sup>31</sup> and targeting this population may be an important therapeutic strategy in treating patients with brain tumors.<sup>24,32</sup> Therefore, studying malignant glioma in culture requires the culture conditions to maintain the tumor-initiating cells (TICs) that are hypothesized to drive tumor growth, as well as preserve the genetic and phenotypic properties of these cells. Only with these criteria can cell culture results be relevant for patients with malignant glioma.

### **Neurosphere Assay and Adherent Culture Conditions**

The demonstration of adult neural stem cells (NSC) grown as neurospheres in the 1990s,<sup>33,34</sup> confirmed findings of previous investigators<sup>35-43</sup> and led to widespread

recognition of adult mammalian neurogenesis. Mitotically active stem and progenitor cells were found in discrete regions of the mature brain that could be grown in serum-free culture conditions. The differentiated cells would rapidly die in the neurosphere culture conditions, whereas the NSC would proliferate and generate spheres.

Useful for the maintenance and study of somatic stem cells, the neurosphere assay (NSA) was subsequently used to isolate tumor cells.<sup>44-46</sup> Specifically, the NSA was used to isolate and expand cells from human brain tumors.<sup>28</sup> The NSA is one of the most frequently adopted methods for the enrichment and expansion of both somatic neural stem cells (NSC) and brain TICs.

The NSA is the current standard for identifying and maintaining brain TICs in culture.<sup>47</sup> Brain TICs have been found to be more genetically representative of the original tumor genetically when grown in the NSA compared to serum conditions.<sup>48,49</sup> Furthermore, GBM cells in the NSA bear similarity to stem cells demonstrating potential for indefinite self-renewal, ability for terminal differentiation into glial and neuronal lineages, genetic expression profiles similar to neural stem cells, and genetic stability over serial passage.<sup>48</sup> Since the cells are maintained in serum-free media, their environmental cues are limited to the surrounding cells.<sup>50</sup> The extrinsic cues can be manipulated easily to study the genetic and molecular control of these cells.

However, this assay has notable limitations such as variable composition of cells and overestimation of the proportion of neural stem cells. First, the NSA can produce variable composition of cell types depending on the media,<sup>51</sup> frequency of passaging and whether dissociation is performed before cell differentiation.<sup>52</sup> Moreover, the assay can overestimate the number of stem cells.<sup>53</sup> A 1:1 relationship between stem cell and

neurosphere does not exist and the NSA overestimates the proportion of neural stem cells (usually <5% of the overall NSA population) by an order of magnitude<sup>53</sup>.

Additionally, neurospheres are not always clonal since they are mobile and can merge with one another.<sup>54</sup>

To overcome these limitations, modifications to the NSA have been proposed. The colony-forming assay is a semi-solid culture with collagen that has been described to prevent the migration and fusion of mouse NSC spheres.<sup>55</sup> Only cells from the large colonies (>2mm) demonstrated stem cell characteristics and the capability for long-term self-renewal (>7 passages).<sup>55</sup> Similarly with human brain TICs, the NSA has been supplemented with methylcellulose in an attempt to decrease sphere motility.<sup>28</sup> Recently, serum free culture conditions supplemented with laminin have been described to grow NSC as a monolayer.<sup>56-59</sup> These techniques were extended to brain TICs with an extracellular matrix<sup>60</sup> and subsequently laminin.<sup>61</sup> Some of these data suggest that establishing cell lines from human gliomas is more efficient when the cells are grown as a monolayer and that the NSA is inferior to adherent culture methods in terms of higher percentages of apoptosis and differentiation of cells.<sup>61</sup>

### **Summary**

Malignant glioma, specifically GBM, has a dismal prognosis despite aggressive treatment with surgery, XRT and chemotherapy. The development of novel therapies requires a comprehensive understanding of GBM TICs, their genetic/phenotypic profiles and what drives their growth. Therefore, developing cell culture techniques that maintain tumor cells that are representative of the original tumor and are relevant for tumor growth is of paramount importance. The NSA had replaced culture media with serum to grow GBM cells since it maintains TICs and preserves characteristics of the original

tumor. Recently, however, the use of serum-free media supplemented with laminin to create adherent cultures has been identified as a possible improvement over the NSA in terms of cellular differentiation and apoptosis. Based on limitations of these data we hypothesized that the NSA and adherent culture condition are not significantly different. We tested this hypothesis by comparing the NSA and adherent culture conditions for the growth of GBM stem cell lines.

## CHAPTER 2 MATERIALS AND METHODS

### **Tumor Samples and Cell Culture**

Fresh brain tumor samples were obtained at the time of surgical excision from patients after obtaining informed consent. GBM tumor samples were dissociated into single cells using trypsin and cultured in the NSA at a cell density of 100,000 to 200,000 cells/mL. These samples were established as cell lines by undergoing long-term propagation (>10 passages) and were subsequently used for NSA vs. adherent culture comparisons (Figure 2-1).

The NSA growth media included: Neural stem cell medium and human proliferation supplement (Stem Cell) with 20ng/mL epidermal growth factor (EGF) (R&D), 10ng/mL basic fibroblast growth factor (bFGF) (R&D) and 2 $\mu$ g/mL heparin (Sigma). Cultures were grown in T25 flasks. The adherent culture required coating of flasks with laminin (Sigma) (diluted 1:10 in neural stem cell medium) for at least 3 hours at 37°C with 5% CO<sup>2</sup>. The same medium was used for the adherent and NSA culture conditions. The NSA cells were grown until formation of 100 micron spheres and the adherent cells were grown until cells were confluent. For passage of cells, the cells were dissociated with 0.05% trypsin-EDTA (Gibco) for 2 minutes at 37°C, counted and re-plated at a density of 50,000 cells/mL. Cells were stained with 0.04% trypan blue at the time of counting to identify dead cells (trypan blue +) (Figure 2-2). These cells were counted and the percentage of dead cells was calculated for each growth condition at the time of each passage. These values were compared using the Student's t-test and a p value of  $\leq 0.05$  was considered significant.

Additionally, the fold expansion at each passage was compared between NSA and the laminin growth conditions. The cells were passaged every 4-10 days depending on the cell line. The NSA and laminin cells were passaged simultaneously for each line for fair comparison (n=4 in each group). The difference was compared to zero using the Student's t-test and p values were calculated. A p value of  $\leq 0.05$  was considered significant.

### **Flow Cytometry**

For apoptosis assays, live cells were evaluated for caspase 3 (1:300) (Calbiochem), annexin V (1:100) (Invitrogen), 1,1',3,3',3',3'-hexamethylindodicarbocyanine iodide (DiIC) (1:1000) (Invitrogen), propidium iodide (PI) (1:1000) (Sigma), and 4',6-diamidino-2-phenylindole (DAPI) (1:2000) (Invitrogen). Cell death was evaluated using PI and DAPI. Subsequently, late, mid and early markers of apoptosis were tested. Annexin V, a marker of late apoptosis, binds phosphatidylserine (PS) which is normally confined to the inner plasma membrane, but becomes exposed with apoptosis. The annexin V cell experiments were followed by testing for activated caspase 3 (activated death protease that catalyzes the cleavage of key cellular proteins<sup>62-64</sup>) expression in live and fixed cells. For fixed cells, analysis with caspase 3 (1:1000) (BD) was performed with cells fixed with 90% methanol at 20°C for 15 minutes. An early measure of cell death is the integrity of the mitochondrial membrane.<sup>65</sup> Cyanine dyes, such as DiIC, penetrate eukaryotic cell membranes and accumulate in the mitochondria with active membrane potentials. When this potential is disrupted, there is less DiIC staining.

The hGBM cell lines were maintained in NSA and adherent culture conditions. To evaluate the purity of the stem cell population in each growth condition, expression of

differentiation markers was tested. Cells were tested for GFAP (astrocytic differentiation), beta tubulin III (neuronal differentiation), and nestin (stem cell marker).<sup>28</sup> Additionally, cells were tested for proliferative potential with Ki67 and MCM2. The cells were fixed and stained with antibodies against the markers. Flow cytometry was performed and percent cell staining and geometric mean were measured. Nestin (1:1000) (Chemicon), glial fibrillary acidic protein (GFAP) (1:2000) (BD), beta tubulin III (1:2000) (Promega), MCM2 (1:500) (Santa Cruz), and Ki67 (1:750) (Invitrogen) were used for analysis of differentiation. Flow cytometry was performed using the LSR II (Becton Dickinson ©). Flow cytometry analysis was performed with Flow Jo (Treestar, Inc.). The percent cells positive and geometric mean values are reported as mean  $\pm$  standard error. The values were compared using the Student's t-test and computing p values. Given the use of multiple assays to test apoptosis, to reduce the risk of an alpha error, significance was determined to be a p value of 0.05 divided by the number of assays (6) resulting in a significant p of 0.008.

### **Limiting Dilution Analysis**

Since reliable molecular or cellular markers of neural or tumor stem cells do not exist, these cells are defined with functional criteria. Included in these criteria is clonogenic capacity, or the ability of one founder cell to create a colony consisting of stem cells and more differentiated cells.<sup>66</sup> The clonal frequency of hGBM cells was tested *in vitro* with the limiting dilution assay<sup>67</sup>. Cells were grown in 96 well plates coated with laminin with dilutions of 1-10 cells/well. The wells that became confluent or close to confluent were considered to have clonogenic cells. For the NSA, cells were

plated and wells were evaluated for number of neurospheres (sphere forming frequency).

A limiting dilution analysis was performed using laminin coated 96 well plates. The wells were coated with laminin for at least 3 hours at 37°C with 5% CO<sup>2</sup>. Medium with GBM cells was serially diluted to plate 1-10 cells/well using the standard medium described above. Cultures were analyzed after 3 weeks. Wells with confluent growth or at least  $\frac{3}{4}$  full by visual inspection were considered full. The percent of wells with less than 75% growth were considered empty and the negative log (-log) of the proportion of negative wells was calculated for each cell density. This result was plotted to determine the clonal frequency of the adherent culture conditions using the extreme limiting dilution analysis (ELDA) software<sup>67</sup> and 95% confidence intervals are reported. For the NSA, sphere forming frequency was used as an estimate of clonal frequency. Similarly, cells were serially diluted and plated in 96 well plates. After 3 weeks of growth, the wells were evaluated for the number of spheres at least 100µm in size. The number of spheres was divided by the number of cells plated and multiplied by 100 to obtain a sphere forming percentage of cells. These data are reported as a mean with standard error.

### **Xenotransplantation**

All animal work was approved by the Institutional Animal Care and Use Committee (IACUC). Non-obese diabetic/severe combined immunodeficient (NOD/SCID) female mice 8-10 weeks of age were anesthetized and received a flank subcutaneous injection of  $1 \times 10^6$  cultured GBM cells in 200 microliters of medium and 100 microliters of Matrigel (BD). The cells had been passaged in either NSA or adherent conditions for at least 5

passages prior to xenotransplantation. The animals were divided into two equal groups based on receipt of cells from NSA (n=5) or those from adherent culture (n=5). The animals were monitored for tumor growth and were euthanized once tumors reached 1.5cm in size. A Kaplan-Meier survival analysis was performed, ratio generated for the NSA and laminin group, and the 95% CI reported to determine the relationship between type of tumor cell culture and length of survival.

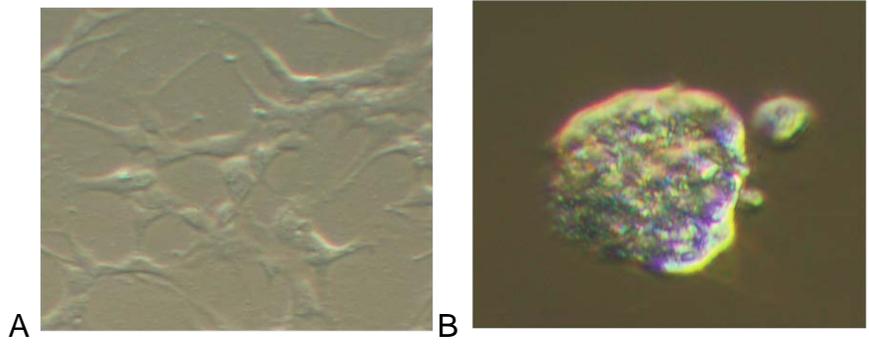


Figure 2-1. hGBM cells in culture. A) Cells grown as a monolayer in laminin. B) Cells grown as spheres in the NSA.

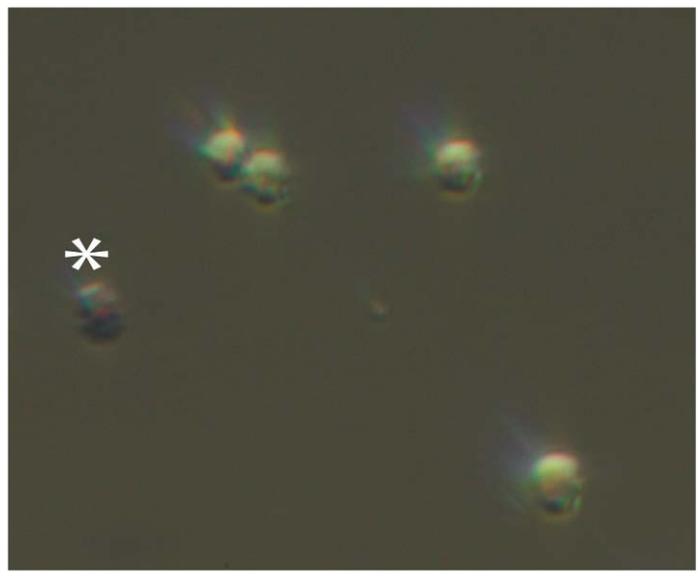


Figure 2-2. hGBM cells stained with trypan blue. Only the dead cells were positive for the dye (asterisk).

## CHAPTER 3 RESULTS

Cells were counted at every passage and expansion curves for the NSA and adherent culture conditions were not different by comparing difference in fold expansion with zero using the Student's t-test ( $p=0.24$ ) (Figure 3-1). Similarly, cell death was measured at the time of passage by counting the number of cells positive for trypan blue during the cell counts. Cell death between the two groups was the same by Student's t-test ( $p=0.7$ ) ( $n=9$ ) (Figure 3-2).

Cells were also analyzed for markers of death, late phases of dying and early markers of dying. Initially dead cells were identified using either PI or DAPI to stain cells with compromised cell membranes.<sup>68-70</sup> Similar to the trypan blue data, no statistical difference was found between NSA or laminin cells in PI ( $p=0.27$ ) (Figures 3-3, 3-4) or DAPI staining ( $p=0.93$ ) (Figures 3-5, 3-6). These experiments are summarized in Figure 3-7.

Using a marker of late apoptosis, annexin V, was evaluated in live cells<sup>71</sup>. Percentage of cells expressing annexin V in laminin cells was  $7.6\% \pm 1.8$  (Figure 3-8) compared to  $10.1\% \pm 1.5$  in NSA cells (Figure 3-9) ( $p=0.32$ ). These experiments are summarized in Figure 3-10.

Using a caspase 3 inhibitor conjugated to a flurochrome (PE), activated caspase 3 was quantified in live cells. The staining was equivalent between NSA ( $5.9\% \pm 1.0$ ) and laminin cells ( $7.2\% \pm 1.2$ ) ( $p=0.41$ ) (Figure 3-11, 3-12). Conversely, measuring the amount of antibody staining activated caspase 3 in fixed cells, the laminin cultured cells demonstrated a trend for increased immunoreactivity ( $4.4\% \pm 0.6$ ) when compared to NSA culture ( $2.2\% \pm 0.6$ ) ( $p=0.026$ ) (Figure 3-13, 3-14).

Live cells were tested for DiIC, identifying apoptotic cells as those without DiIC staining. No statistically significant difference between the NSA and laminin cells was found ( $p=0.55$ ) (Figure 3-15, 3-16). Based on multiple markers of apoptosis, no difference exists in terms of apoptosis between GBM cells grown in NSA or laminin culture conditions (Figure 3-17).

The NSA and adherent cells had no significant differences in GFAP, Nestin, beta tubulin III, Ki67 and MCM2 staining using the Student's t-test, although there was a trend for more GFAP and beta tubulin III staining and less Ki67 and MCM2 staining in the cells grown with laminin (Figure 3-18). The data shows the laminin cell staining as a percent of the NSA cell staining. Based on our data, assuming a difference in staining of 50% between cells grown in the NSA and laminin conditions, with an alpha error of 0.05 and power of 80%, we would require at least 50 experiments with both culture conditions to show statistical significance in the trends we found. Given the strong evidence of no differences in the culture conditions, we did not pursue this.

Using a standard calculation for clonogenic frequency<sup>67</sup> (by taking the negative log of the proportion of empty wells) the clonal frequency of the cell lines grown in laminin was determined to be 5.5% or 1 cell out of 18.1 (95% CI 15.9 - 20.6) (Figure 3-19). This value was similar to the mean clonal frequency of hGBM cells grown in the NSA of  $7.1\% \pm 0.4$  (data not shown). These results are similar to the reported clonal frequency of hGBM cell lines grown in the NSA.<sup>29, 72</sup>

The growth of the tumors and survival analysis was the same for hGBM cells grown in the NSA (median survival 78 days) and those grown with laminin (median survival 71 days) (ratio 1.099, 95% CI 0.8 to 1.4) (Figure 3-20, 3-21).

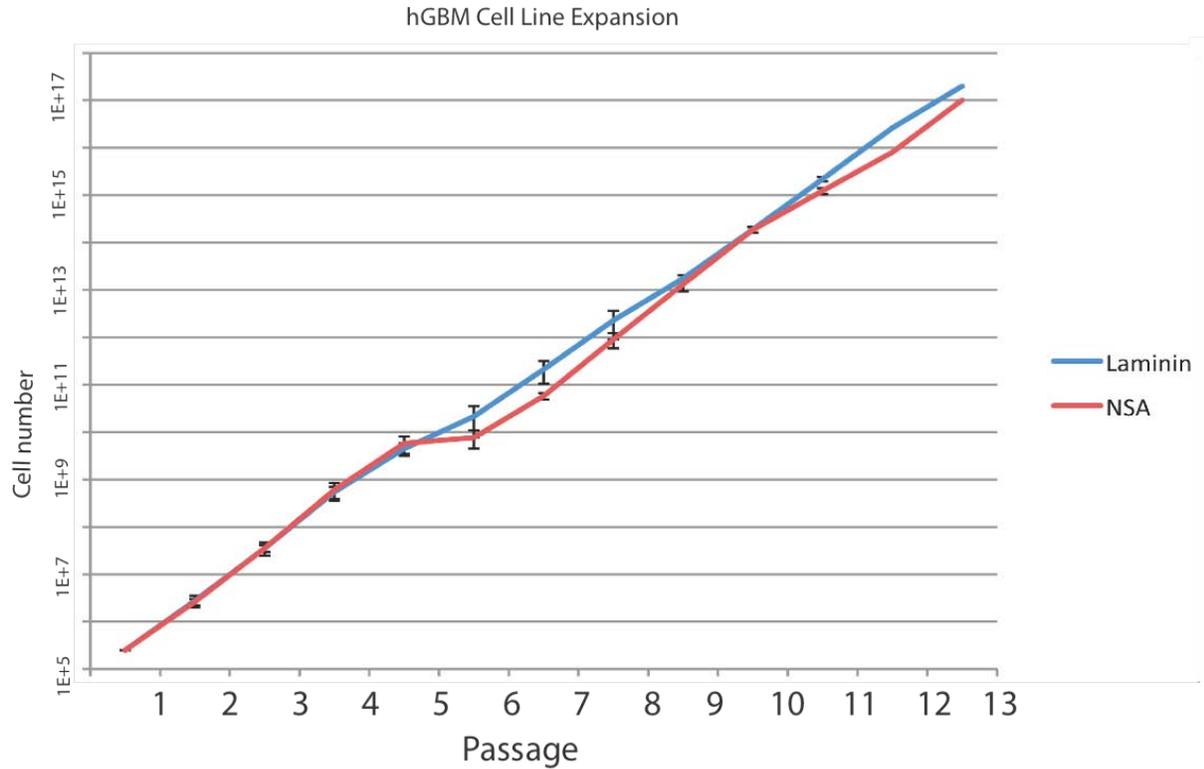


Figure 3-1. Number of cells obtained in vitro over multiple passages of GBM cell lines grown in NSA and adherent culture conditions. Standard error is shown with the error bars. The fold expansion over time in the two different conditions was not statistically different ( $p=0.24$ ).

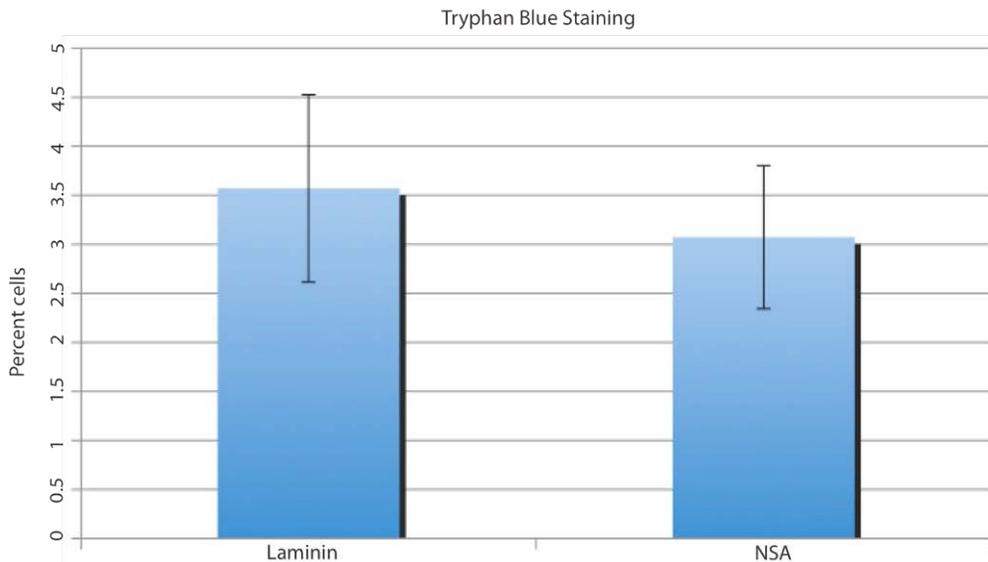


Figure 3-2. Percent of cells positive for trypan blue denoting cell death at the time of trypsinization of neurospheres or cells grown on laminin. Standard error is shown with the error bars. No statistically significant difference was found ( $p=0.7$ ).

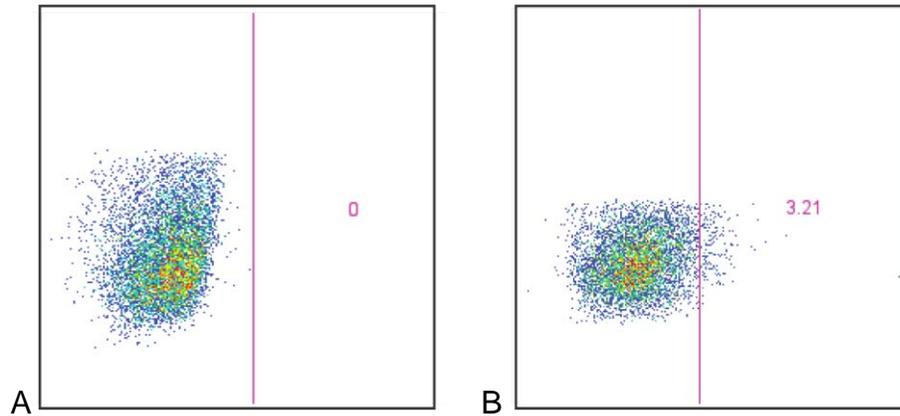


Figure 3-3. Flow cytometry plots of laminin PI experiment. A) Laminin cells with no PI. Using the PI autofluorescence of the cells, a gate was identified (pink line) to separate PI positive and PI negative cells. B) Applying this gate to the cells stained with PI, 3.21% of the cells were found to be PI positive/dead.

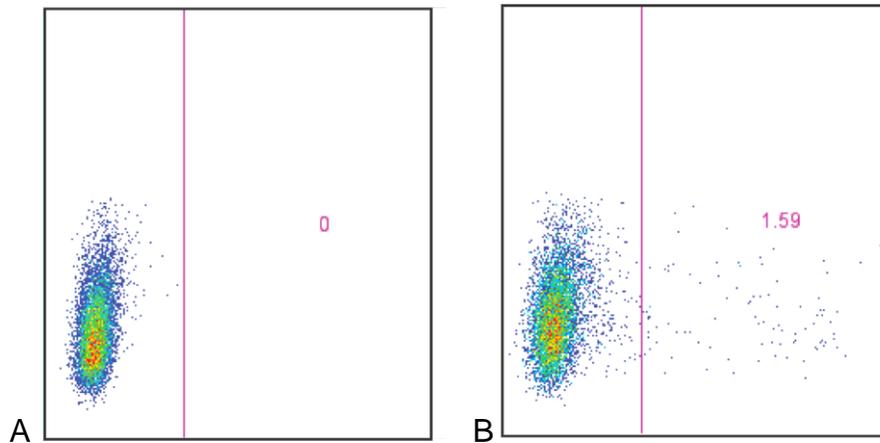


Figure 3-4. Flow cytometry plots of NSA PI experiment. A) NSA cells with no PI. Using the PI autofluorescence of the cells, a gate was identified (pink line) to separate PI positive and PI negative cells. B) Applying this gate to the cells stained with PI, 1.59% of the cells were found to be PI positive/dead.

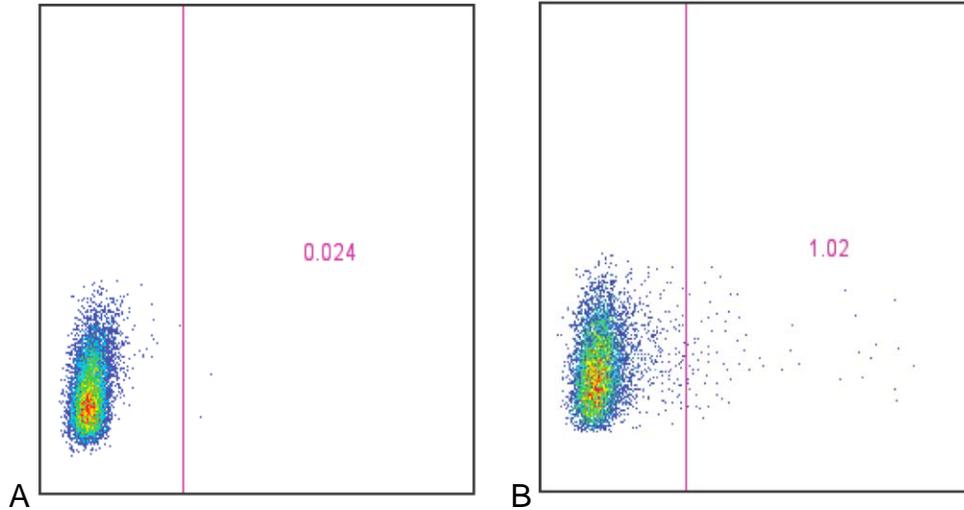


Figure 3-5. A) Flow cytometry plots of laminin DAPI experiment. Laminin cells with no DAPI are shown on the left. Using the DAPI autofluorescence of the cells, a gate was identified (pink line) to separate DAPI positive and DAPI negative cells. B) Applying this gate to the cells stained with DAPI, 1.02% of the cells were found to be DAPI positive/dead.

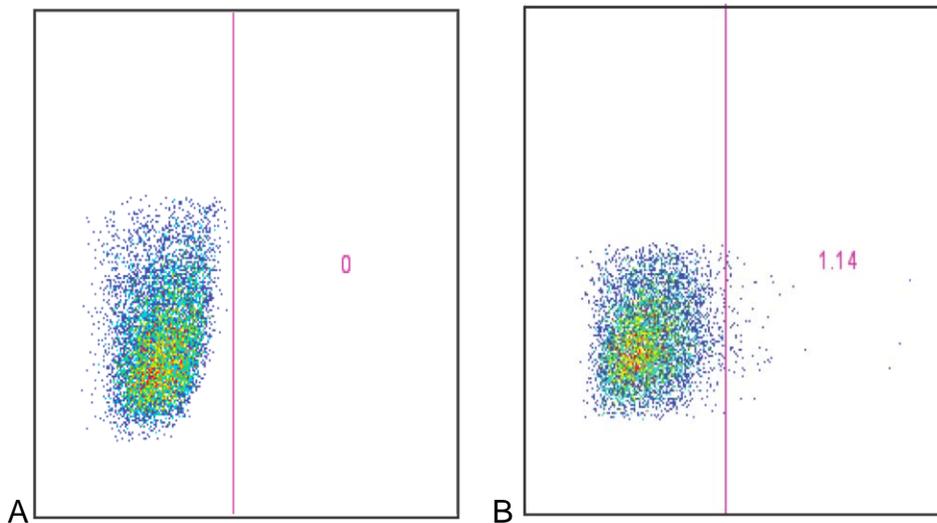


Figure 3-6. Flow cytometry plots of NSA DAPI experiment. A) NSA cells with no DAPI. Using the DAPI autofluorescence of the cells, a gate was identified (pink line) to separate DAPI positive and DAPI negative cells. B) Applying this gate to the cells stained with DAPI, 1.14% of the cells were found to be DAPI positive/dead.

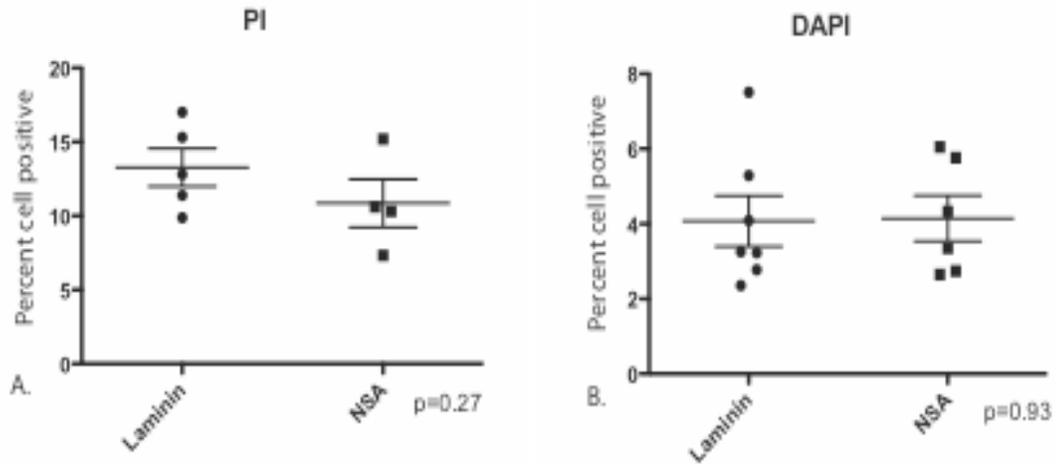


Figure 3-7. Summary of PI and DAPI experiments. A) Percent cells positive for PI. B) Percent cells positive for DAPI. The mean and standard error are shown in the graphs. No statistical differences were found between cells grown in NSA and laminin culture conditions.

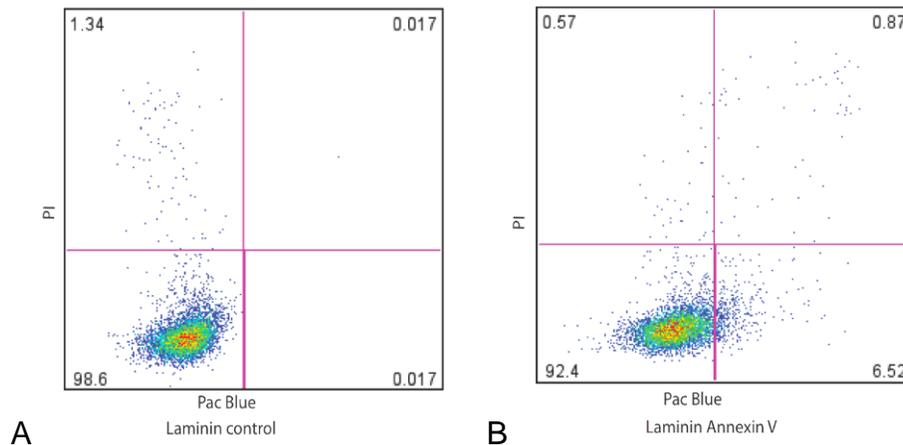


Figure 3-8. Flow cytometry plots demonstrating annexin V and PI staining in cells grown in laminin conditions. A) Cells stained with PI only to identify dead cells. Using the autofluorescence of pacific blue (the fluorochrome conjugated to the annexin V antibody) of these cells, a gate was identified (pink lines) to separate live/dead cells (PI-/+) and annexin V -/+ cells (pacific blue -/+). B) This gate was applied to cells stained with PI and an antibody to annexin V conjugated to pacific blue. 6.52% of the cells were positive for annexin V and apoptotic. 0.87% of the cells were positive for annexin V but also positive for PI and therefore already dead.

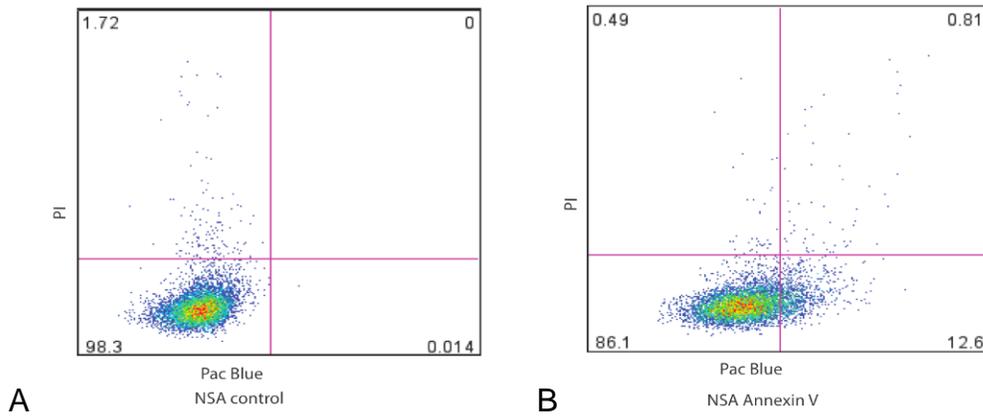


Figure 3-9. Flow cytometry plots demonstrating annexin V and PI staining in cells grown in NSA conditions. A) Cells stained with PI only to identify dead cells. Using the autofluorescence of pacific blue (the fluorochrome conjugated to the annexin V antibody) of these cells, a gate was identified (pink lines) to separate live/dead cells (PI-/+) and annexin V -/+ cells (pacific blue -/+). B) This gate was applied to cells stained with PI and an antibody to annexin V conjugated to pacific blue. 12.6% of the cells were positive for annexin V and apoptotic. 0.81% of the cells were positive for annexin V but also positive for PI and therefore already dead.

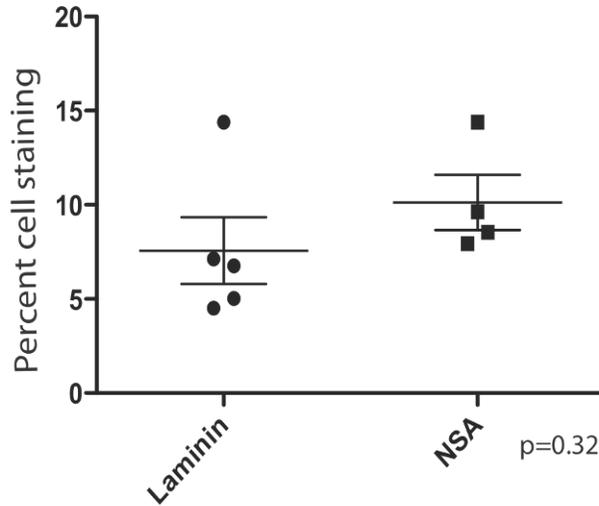


Figure 3-10. Summary of annexin V experiments. The percentage of cells positive for annexin V are shown for both growth conditions. The mean and standard error are shown. No statistically significant differences were found.

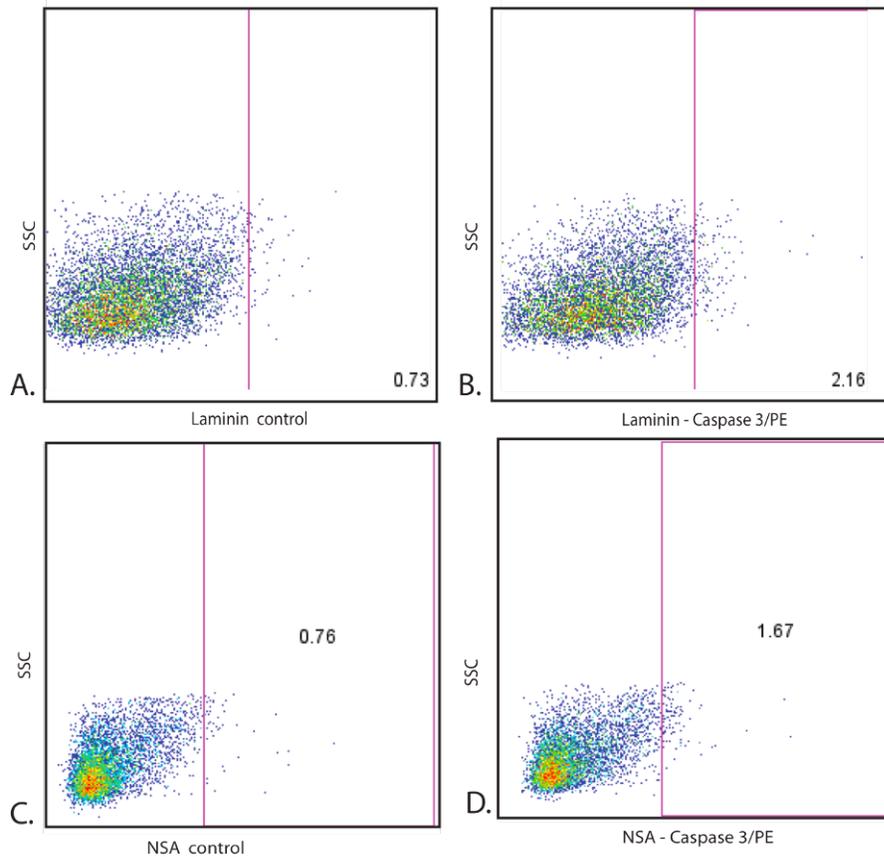


Figure 3-11. Flow cytometry plots demonstrating caspase staining in live cells grown in NSA and laminin culture conditions. A) Cells without caspase 3 inhibitor. Using the PE (fluorochrome conjugated to the caspase 3 inhibitor) autofluorescence of cells, a gate was identified (pink line) to separate PE +/- cells. B) Applying this gate to cells with caspase 3 inhibitor, 2.16% of laminin cells were found to express activated caspase 3. C) Similarly, a gate was chosen using NSA control cells. D) Applying this gate to cells with caspase 3 inhibitor, 1.67% of cells were found to express activated caspase 3.

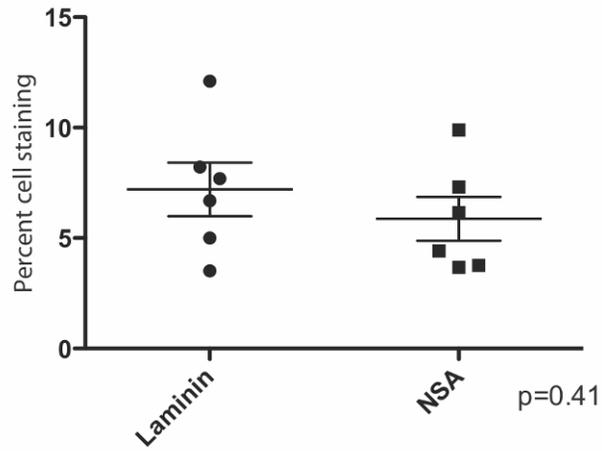


Figure 3-12. Summary of activated caspase 3 experiments in live cells grown in laminin or NSA. Mean and standard error of percent cells positive are shown. No statistically significant differences were found.

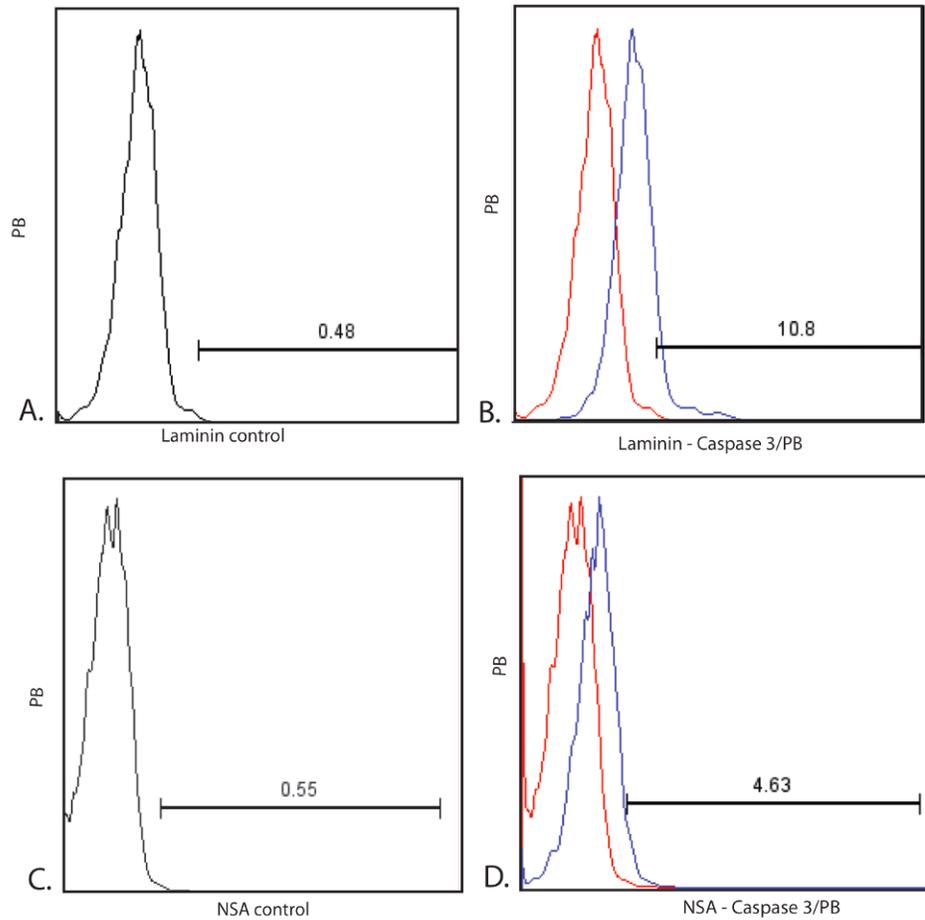


Figure 3-13. Flow cytometry histograms of cells grown in laminin or NSA conditions and subsequently fixed and stained with an antibody to activated caspase 3. A,C) Using the autofluorescence of pacific blue (fluorochrome conjugated to the caspase 3 antibody) of the cells with no antibody, a gate was identified to separate pacific blue +/- cells. B,D) Applying these gates to cells with the antibody to activated caspase 3, 10.8% of laminin and 4.63% of NSA cells were found to express activated caspase 3.

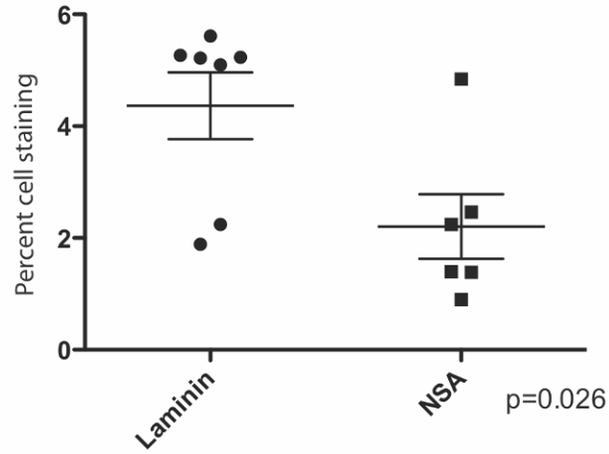


Figure 3-14. Summary of caspase 3 experiments using fixed cells. The percent cell staining is shown with mean and standard error. Although there was a trend for increased activated caspase 3 expression in cells grown in laminin, this did not reach statistical significance.

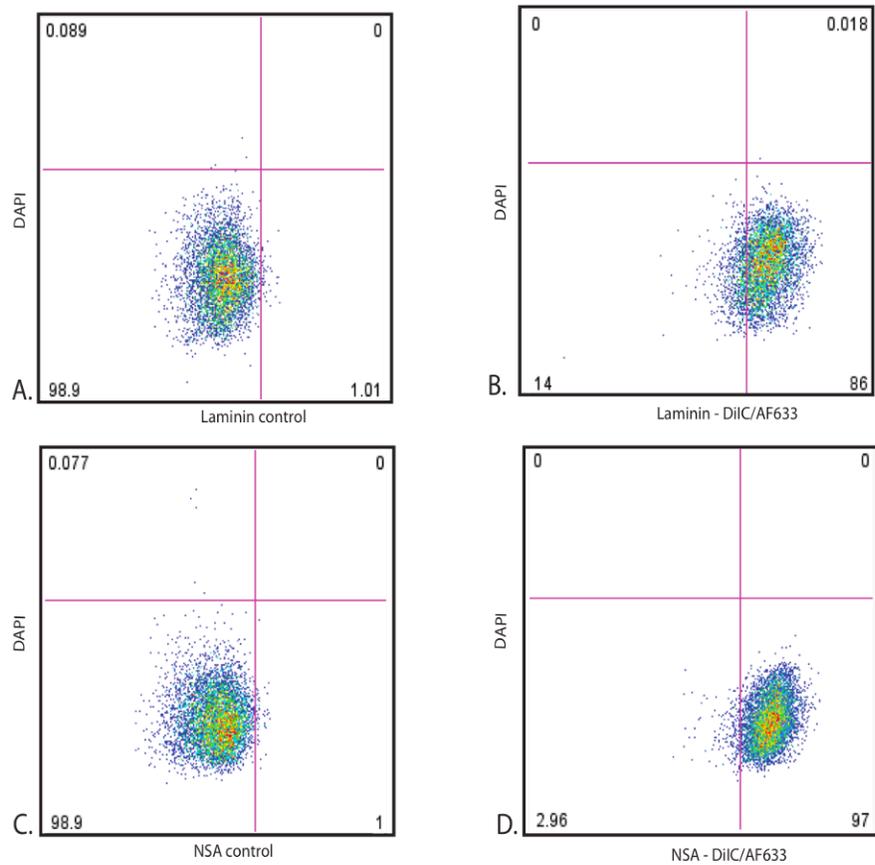


Figure 3-15. Flow cytometry plots showing DiIC staining in NSA and laminin grown cells. A, C) Cells stained with DAPI only were used to chose a gate (pink lines) to separate live/dead cells (DAPI-/+) and DiIC +/- (AF633 fluorochrome +/-). B, D) These gates were applied to cells stained with DAPI and DiIC conjugated to AF633. 86% of laminin grown cells and 97% of NSA grown cells were positive for DiIC (non-apoptotic).

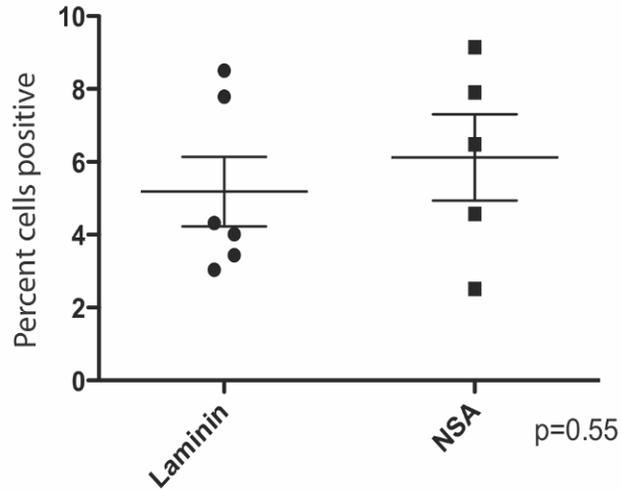


Figure 3-16. Summary of DiIC experiments. The percent cells positive for each experiment, mean and standard error are shown. No significant differences were found between laminin and NSA grown hGBM cells.

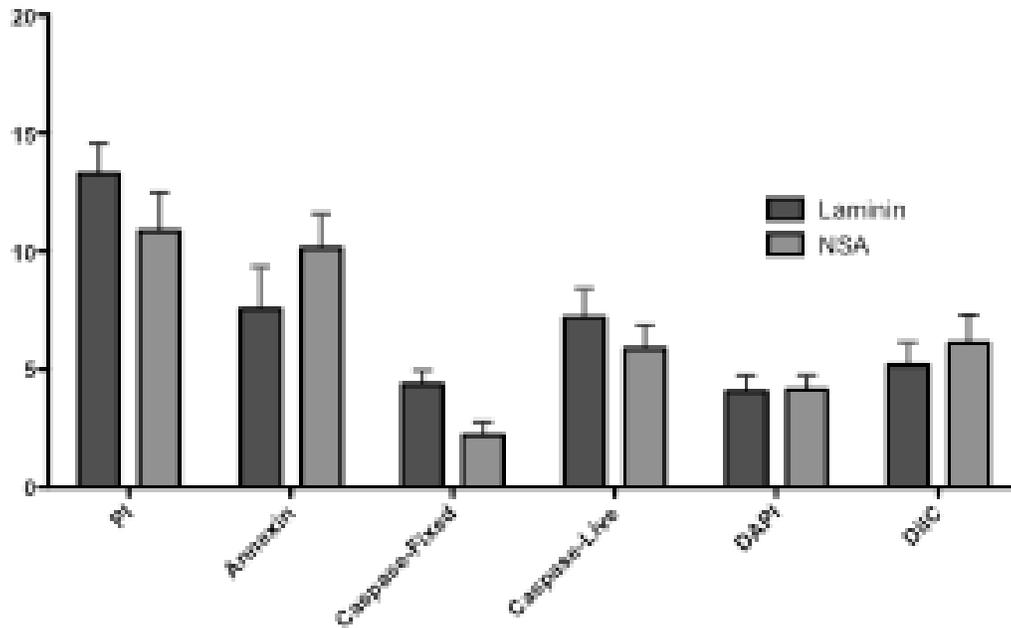


Figure 3-17. Summary of apoptosis markers by flow cytometry percent cells positive in NSA and laminin grown GBM cells.  $p = NS$  for all comparisons. The mean and standard error are shown in the graph.

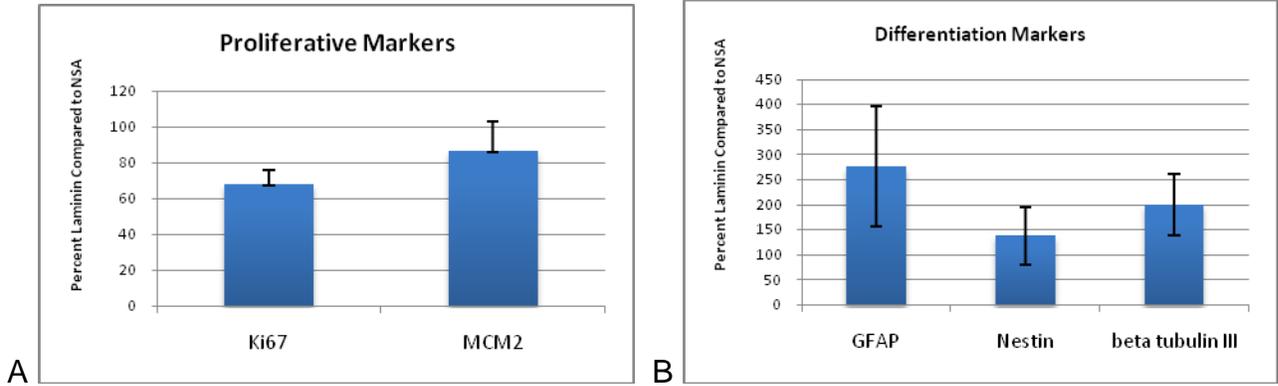


Figure 3-18. The geometric mean of laminin cells as a percent of NSA cell geometric mean from flow cytometry. A) Proliferative markers Ki67 ( $p=0.22$ ) and MCM2 ( $p=0.51$ ). B) Differentiation markers GFAP ( $p=0.44$ ), Nestin ( $p=0.52$ ), and beta tubulin III ( $p=0.18$ ). The mean and standard error are shown in the graphs.

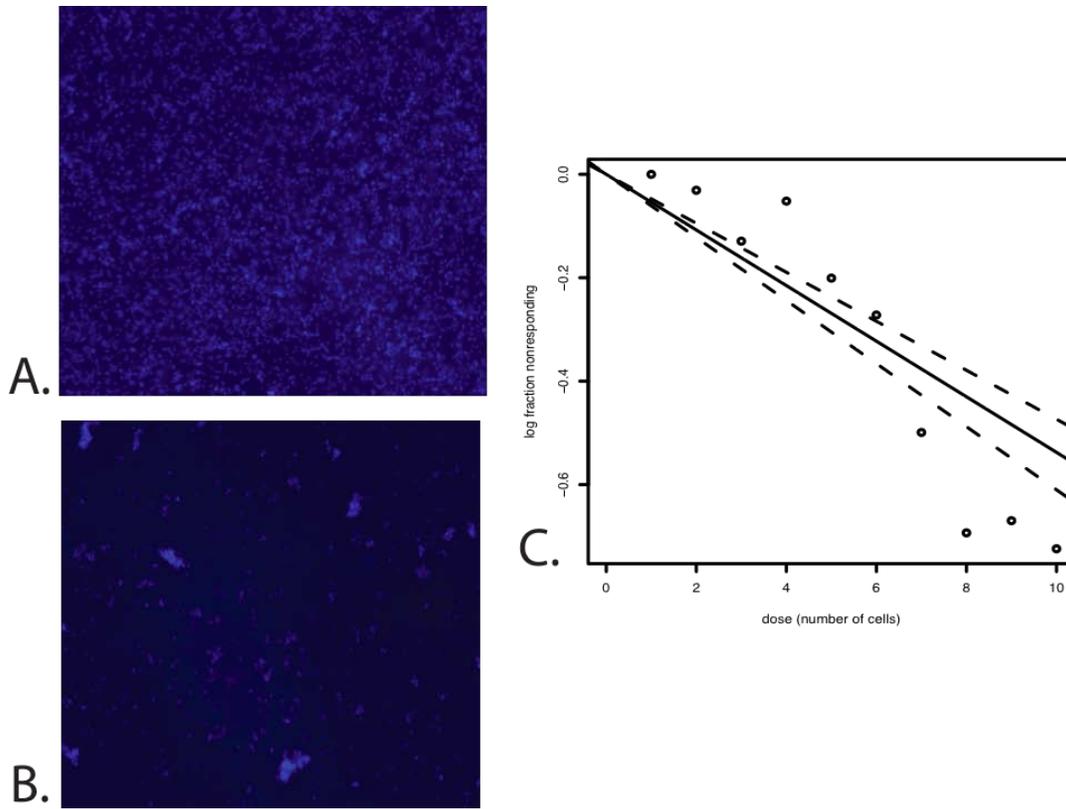


Figure 3-19. Limiting dilution assay of cells grown using laminin. Determination of clonogenic capacity was based on ability of cell grown on laminin to fill a well. A) Full well after growth of cells on laminin. B) Example of a well considered not full after growth of cells on laminin. C) Graph of the log of the number of non-full wells and the dose of the cells plated.



Figure 3-20. Mouse after implantation of GBM cells. Example of a subcutaneous (SQ) tumor growing in the flank of a mouse.

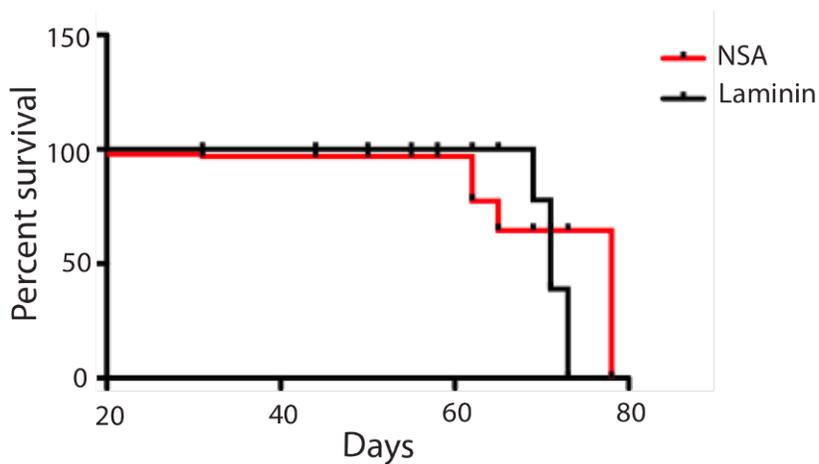


Figure 3-21. Kaplan Meier graph demonstrating similar survival of NOD/SCID mice after implantation of hGBM cells grown in either NSA and laminin culture conditions. Mice were euthanized once tumors reached 1.5cm in any dimension.

## CHAPTER 4 DISCUSSION

### **Summary and Significance of Results**

The NSA is the current standard for identifying and maintaining brain TICs in culture.<sup>47</sup> However, this assay has notable limitations including variable composition of cells<sup>51,52</sup> and overestimation of proportion of neural stem cells.<sup>53</sup> To overcome these limitations, modifications to the NSA have been proposed, including the addition of laminin to grow cells flat. Some advantages of adherent culture conditions suggested by these reports include a more pure stem cell population, decreased differentiation, increased tumorigenicity, and decreased cellular apoptosis.<sup>61</sup>

Our experiments are the first to directly compare the NSA and laminin culture conditions for hGBM cell lines using multiple *in vitro* and *in vivo* assays. To test potential differences in the proportion of CSCs, we used cell culture techniques, as stem cells are defined by functional criteria. Growing our hGBM cell lines with the two different culture conditions demonstrated equivalent growth curves with long-term expansion. These growth data were supported by the finding that NSA and laminin cells had no differences in expression of proliferation markers (Ki67 and MCM2). Furthermore, cells grown in both conditions had similar clonal frequency (ability of a single cell to generate a large number of progeny). These results in sum show that cells grown in the NSA and laminin culture conditions have no statistically significant difference in the proportion of stem cells and progenitors.<sup>67,73</sup>

Recent reports claim that cells from NSA spheroids have increased differentiation although this finding was not statistically analyzed.<sup>61</sup> As CSCs differentiate, they lose their stem cell characteristics such as self-renewal, generation of a large number of

progeny and multipotency.<sup>74</sup> Consequently, CSC culture should maintain relatively undifferentiated CSCs that are multipotent (retain the ability to differentiate when exposed to a differentiation agent). In our study, the NSA and laminin cells were not statistically different with regard to staining for nestin (relatively undifferentiated cells), GFAP (astrocyte differentiation) or beta tubulin III (neuronal differentiation). The differences between our results and other reports may be due to overgrowth of the neurospheres in other studies, resulting in increased cellular differentiation.<sup>75</sup>

In addition to the purity of the CSC population, increased tumorigenicity was described as an advantage of laminin-cultured cells. Laminin has also been implicated in hGBM invasion and migration based on *in vitro* migration analyses.<sup>76,77</sup> However, in our xenograft model, hGBM cells grown in laminin did not show statistically significant increased tumorigenicity or invasion. The animals implanted with NSA and laminin-cultured cells had similar tumor growth and survival times. The growth of the cells *in vivo* may have been affected by the use of matrigel.<sup>78</sup> However, since both groups of cells grew *in vivo* equally with the same growth conditions, the likelihood of a difference between the cells is low.

Moreover, our hGBM cells grown in NSA and adherent culture conditions did not show statistically significant differences in cell death or dying as quantified by apoptotic markers. The previous culture studies demonstrated increased apoptosis in cells grown with NSA compared to cells grown in adherent conditions.<sup>60,61</sup> This literature demonstrated increased apoptosis in NSA cells using annexin V and the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (detecting fragmented DNA) assays<sup>56,79</sup> The decreased apoptosis with laminin was hypothesized to be due to

laminin preventing cellular apoptosis that is secondary to detachment from the extracellular membrane.<sup>56</sup> However, this difference was not quantified statistically nor confirmed with other techniques. Cell death is a heterogeneous process involving multiple and sometimes overlapping pathways.<sup>80</sup> Therefore, we used multiple apoptosis assays to quantify the difference in cell dying and death between cells in the NSA and laminin growth conditions. Immunofluorescence with a flow cytometer was used for several reasons.<sup>80</sup> First, fluorescence has higher sensitivity due to improved signal-to-noise ratio compared to chromogenic techniques. Since this detection method does not involve an enzymatic reaction, variables that can change results such as buffer and pH are not relevant. Secondly, flow cytometry has the distinct advantage of providing both single cell and entire population analyses. Lastly, using fluorescent antibodies to the targets of interest allows for the use of several fluochromes with different absorption/emission spectra and the testing of three to four apoptotic assays simultaneously.

Given the heterogeneity of apoptotic pathways and the numerous assays available to measure apoptosis, using multiple tests to quantify apoptosis is prudent. We used a robust analysis of early and late apoptosis as well as cell death using multiple assays and demonstrated no significant difference between NSA and laminin cultured cells. The previously reported differences in apoptosis are limited by testing of only one or two apoptosis measures.

Based on functional criteria such as our long-term proliferation data, cells grown in the NSA and laminin culture conditions have no differences in the proportion of stem cells and progenitors. Laminin grown cells also do not show increased clonal frequency

*in vitro* or increased tumorigenicity in a xenograft model compared to the NSA. Furthermore, cells grown in the NSA and laminin have no differences in cell death or dying. These data show no advantage of the laminin culture method to the NSA.

### **Study Limitations**

The goal of this study was to study the NSA and laminin growth conditions for differences in cellular growth, apoptosis, clonal frequency and tumorigenicity. Limitations of the study include lack of genomic comparison between GBM TICs grown in the two different growth conditions.

This study demonstrated that the clonal frequency of GBM TICs grown in laminin is similar to those grown in NSA. These data should be confirmed with a limiting dilution analysis *in vivo* with a xenotransplantation model. The *in vivo* SQ flank model would allow for evaluation of tumor-initiating capacity, not just clonal frequency. Additionally, the *in vivo* limiting dilution assay could be repeated with an intracranial model. This model would evaluate tumor-initiating capacity in a model more representative of GBM tumor growth and avoid the possible confounding factor of matrigel.

### **Future Plans**

These data need to be supplemented with genomic comparisons. Genomic data would reveal if the two different growth conditions result in selection for cells with differing genotypes. These types of experiments are part of our future plans. Also, further analysis of the adherent, serum-free growth conditions should be completed using other types of tumor cells *in vitro* before its benefits can be determined.

Developing novel and effective therapy for hGBM is challenging due to tumor heterogeneity, robustness and imperfect tumor models. The development of alternative culture techniques for establishing cell lines is a useful addition to the existing tools for

the study of hGBM. Although potentially more convenient for certain applications, such as migration analyses, hGBM cells grown in adherent conditions are not significantly different from cells grown with the traditional NSA.

## REFERENCES

1. Statistical Report: Primary Brain Tumors in the United States, 2000–2004. Central Brain Tumor Registry of the United States; 2008. Accessed 2/2/2009.
2. Colombatti M, Dipasquale B, Del-l'Arciprete L, Gerosa M, Tridente G. Heterogeneity and modulation of tumor-associated antigens in human glioblastoma cell lines. *J Neurosurg.* Sep 1989;71(3):388-397.
3. Clavreul A, Jean I, Preisser L, et al. Human glioma cell culture: two FCS-free media could be recommended for clinical use in immunotherapy. *In vitro Cell Dev Biol Anim.* Oct 2009;45(9):500-511.
4. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK. Pathology and genetics of tumours of the nervous system. *World Health Organization Classification of Tumours of the Nervous System, Editorial and Consensus Conference Working Group.* Lyon, France: IARC Press; 2007.
5. Brandes AA, Tosoni A, Franceschi E, Reni M, Gatta G, Vecht C. Glioblastoma in adults. *Crit Rev Oncol Hematol.* Apr 2 2008.
6. Wong ET, Hess KR, Gleason MJ, et al. Outcomes and prognostic factors in recurrent glioma patients enrolled onto phase II clinical trials. *J Clin Oncol.* Aug 1999;17(8):2572-2578.
7. Carson KA, Grossman SA, Fisher JD, Shaw EG. Prognostic factors for survival in adult patients with recurrent glioma enrolled onto the new approaches to brain tumor therapy CNS consortium phase I and II clinical trials. *J Clin Oncol.* Jun 20 2007;25(18):2601-2606.
8. Huncharek M, Muscat J. Treatment of recurrent high grade astrocytoma; results of a systematic review of 1,415 patients. *Anticancer Res.* Mar-Apr 1998;18(2B):1303-1311.
9. Colombo F, Barzon L, Franchin E, et al. Combined HSV-TK/IL-2 gene therapy in patients with recurrent glioblastoma multiforme: biological and clinical results. *Cancer Gene Ther.* Oct 2005;12(10):835-848.
10. Reardon DA, Rich JN, Friedman HS, Bigner DD. Recent advances in the treatment of malignant astrocytoma. *J Clin Oncol.* Mar 10 2006;24(8):1253-1265.
11. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med.* Mar 10 2005;352(10):997-1003.
12. Stupp R, Hegi ME, Mason WP, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol.* May 2009;10(5):459-466.

13. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. Mar 10 2005;352(10):987-996.
14. Louis DN, Ohgaki H, Wiestler OD, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol*. Aug 2007;114(2):97-109.
15. Burger PC, Vogel FS, Green SB, Strike TA. Glioblastoma multiforme and anaplastic astrocytoma. Pathologic criteria and prognostic implications. *Cancer*. Sep 1 1985;56(5):1106-1111.
16. Kurpad SN, Zhao XG, Wikstrand CJ, Batra SK, McLendon RE, Bigner DD. Tumor antigens in astrocytic gliomas. *Glia*. Nov 1995;15(3):244-256.
17. Vick WW, Wikstrand CJ, Bullard DE, et al. The use of a panel of monoclonal antibodies in the evaluation of cytologic specimens from the central nervous system. *Acta Cytol*. Nov-Dec 1987;31(6):815-824.
18. Choe G, Horvath S, Cloughesy TF, et al. Analysis of the phosphatidylinositol 3'-kinase signaling pathway in glioblastoma patients *in vivo*. *Cancer Res*. Jun 1 2003;63(11):2742-2746.
19. Chakravarti A, Zhai G, Suzuki Y, et al. The prognostic significance of phosphatidylinositol 3-kinase pathway activation in human gliomas. *J Clin Oncol*. May 15 2004;22(10):1926-1933.
20. Hermanson M, Funa K, Hartman M, et al. Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. *Cancer Res*. Jun 1 1992;52(11):3213-3219.
21. Chaffanet M, Chauvin C, Laine M, et al. EGF receptor amplification and expression in human brain tumours. *Eur J Cancer*. 1992;28(1):11-17.
22. Fleming TP, Saxena A, Clark WC, et al. Amplification and/or overexpression of platelet-derived growth factor receptors and epidermal growth factor receptor in human glial tumors. *Cancer Res*. Aug 15 1992;52(16):4550-4553.
23. Huntly BJ, Gilliland DG. Leukaemia stem cells and the evolution of cancer-stem-cell research. *Nat Rev Cancer*. Apr 2005;5(4):311-321.
24. Vescovi A, Galli R, Reynolds B. Brain tumour stem cells. *Nat Rev Cancer*. Jun 1 2006;6(6):425-436.
25. Huntly BJ, Gilliland DG. Cancer biology: summing up cancer stem cells. *Nature*. Jun 30 2005;435(7046):1169-1170.

26. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature*. Nov 1 2001;414(6859):105-111.
27. Jordan CT, Guzman ML, Noble M. Cancer stem cells. *N Engl J Med*. Sep 21 2006;355(12):1253-1261.
28. Ignatova TN, Kukekov VG, Laywell ED, Suslov ON, Vrionis FD, Steindler DA. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers *in vitro*. *Glia*. Sep 2002;39(3):193-206.
29. Galli R, Binda E, Orfanelli U, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res*. Oct 1 2004;64(19):7011-7021.
30. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature*. Nov 18 2004;432(7015):396-401.
31. Bao S, Wu Q, McLendon R, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature*. Dec 7 2006;444(7120):756-760.
32. Rich JN. Cancer stem cells in radiation resistance. *Cancer Res*. Oct 1 2007;67(19):8980-8984.
33. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*. Mar 27 1992;255(5052):1707-1710.
34. Gritti A, Frölichsthal-Schoeller P, Galli R, et al. Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain. *J Neurosci*. May 1 1999;19(9):3287-3297.
35. Hamilton A. The division of the differentiated cells in the central nervous system of the white rat. *J. Comp. Neurol*. 1901;11:297-320.
36. Kershman J. The medulloblast and the medulloblastoma. *Arch. Neurol. Psychiat.* 1938;40:937-967.
37. Bryans WA. Mitotic activity in the brain of the adult white rat. *Anat. Rec*. 1959;133:65-71.
38. Altman J, Das GD. Autoradiographic and histological studies of postnatal neurogenesis. I. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions. *J Comp Neurol*. 1966;126(3):337-389.

39. Dacey ML, Wallace RB. Postnatal neurogenesis in the feline cerebellum: a structural-functional investigation. *Acta Neurobiol Exp (Wars)*. 1974;34(2):253-263.
40. Kaplan MS, Hinds JW. Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. *Science*. Sep 9 1977;197(4308):1092-1094.
41. Messier B, Leblond CP, Smart I. Presence of DNA synthesis and mitosis in the brain of young adult mice. *Exp Cell Res*. Feb 1958;14(1):224-226.
42. Paton JA, Nottebohm FN. Neurons generated in the adult brain are recruited into functional circuits. *Science*. Sep 7 1984;225(4666):1046-1048.
43. Goldman SA, Nottebohm F. Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. *Proc Natl Acad Sci U S A*. Apr 1983;80(8):2390-2394.
44. Fang D, Nguyen TK, Leishear K, et al. A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer Res*. Oct 15 2005;65(20):9328-9337.
45. Ricci-Vitiani L, Lombardi D, Pilozzi E, et al. Identification and expansion of human colon-cancer-initiating cells. *Nature*. Jan 4 2007;445(7123):111-115.
46. Eramo A, Lotti F, Sette G, et al. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ*. Mar 2008;15(3):504-514.
47. Chaichana K, Zamora-Berridi G, Camara-Quintana J, Quinones-Hinojosa A. Neurosphere assays: growth factors and hormone differences in tumor and nontumor studies. *Stem Cells*. Dec 2006;24(12):2851-2857.
48. Lee J, Kotliarova S, Kotliarov Y, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer Cell*. May 2006;9(5):391-403.
49. De Witt Hamer PC, Van Tilborg AA, Eijk PP, et al. The genomic profile of human malignant glioma is altered early in primary cell culture and preserved in spheroids. *Oncogene*. Mar 27 2008;27(14):2091-2096.
50. Jensen JB, Parmar M. Strengths and limitations of the neurosphere culture system. *Mol Neurobiol*. Dec 2006;34(3):153-161.
51. Arsenijevic Y, Weiss S, Schneider B, Aebischer P. Insulin-like growth factor-I is necessary for neural stem cell proliferation and demonstrates distinct actions of epidermal growth factor and fibroblast growth factor-2. *J Neurosci*. 2001;21(18):7194-7202.

52. Caldwell MA, He X, Wilkie N, et al. Growth factors regulate the survival and fate of cells derived from human neurospheres. *Nat Biotechnol.* May 2001;19(5):475-479.
53. Reynolds BA, Rietze RL. Neural stem cells and neurospheres--re-evaluating the relationship. *Nat Methods.* May 2005;2(5):333-336.
54. Singec I, Knoth R, Meyer RP, et al. Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. *Nat Methods.* Oct 2006;3(10):801-806.
55. Louis SA, Rietze RL, Deleyrolle L, et al. Enumeration of neural stem and progenitor cells in the neural colony-forming cell assay. *Stem Cells.* Apr 2008;26(4):988-996.
56. Hall PE, Lathia JD, Caldwell MA, French-Constant C. Laminin enhances the growth of human neural stem cells in defined culture media. *BMC Neurosci.* Jan 1 2008;9:71.
57. Pollard SM, Conti L, Sun Y, Goffredo D, Smith A. Adherent neural stem (NS) cells from fetal and adult forebrain. *Cereb Cortex.* Jul 1 2006;16 Suppl 1:i112-120.
58. Conti L, Pollard SM, Gorba T, et al. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol.* Sep 1 2005;3(9):e283.
59. Sun Y, Pollard S, Conti L, et al. Long-term tripotent differentiation capacity of human neural stem (NS) cells in adherent culture. *Mol Cell Neurosci.* Jun 1 2008;38(2):245-258.
60. Fael Al-Mayhany TM, Ball SL, Zhao JW, et al. An efficient method for derivation and propagation of glioblastoma cell lines that conserves the molecular profile of their original tumours. *J Neurosci Methods.* Jan 30 2009;176(2):192-199.
61. Pollard SM, Yoshikawa K, Clarke I, et al. Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. *Cell Stem Cell.* Jun 5 2009;4(6):568-580.
62. Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ.* Feb 1999;6(2):99-104.
63. Janicke RU, Ng P, Sprengart ML, Porter AG. Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. *J Biol Chem.* Jun 19 1998;273(25):15540-15545.
64. Janicke RU, Sprengart ML, Wati MR, Porter AG. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem.* Apr 17 1998;273(16):9357-9360.

65. Cossarizza A, Kalashnikova G, Grassilli E, et al. Mitochondrial modifications during rat thymocyte apoptosis: a study at the single cell level. *Exp Cell Res.* Sep 1994;214(1):323-330.
66. Gritti A, Galli R, Vescovi AL. Clonal analyses and cryopreservation of neural stem cell cultures. *Methods Mol Biol.* 2008;438:173-184.
67. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *J Immunol Methods.* Aug 15 2009;347(1-2):70-78.
68. Troiano L, Ferraresi R, Lugli E, et al. Multiparametric analysis of cells with different mitochondrial membrane potential during apoptosis by polychromatic flow cytometry. *Nat Protoc.* 2007;2(11):2719-2727.
69. Mazzini G, Ferrari C, Erba E. Dual excitation multi- fluorescence flow cytometry for detailed analyses of viability and apoptotic cell transition. *Eur J Histochem.* 2003;47(4):289-298.
70. Li F, Ackermann EJ, Bennett CF, et al. Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nat Cell Biol.* Dec 1999;1(8):461-466.
71. Martin SJ, Reutelingsperger CP, McGahon AJ, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med.* Nov 1 1995;182(5):1545-1556.
72. Inagaki A, Soeda A, Oka N, et al. Long-term maintenance of brain tumor stem cell properties under at non-adherent and adherent culture conditions. *Biochem Biophys Res Commun.* Sep 28 2007;361(3):586-592.
73. Breivik H. Haematopoietic stem cell content of murine bone marrow, spleen, and blood. Limiting dilution analysis of diffusion chamber cultures. *J Cell Physiol.* Aug 1971;78(1):73-78.
74. Potten CS, Loeffler M. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development.* Dec 1990;110(4):1001-1020.
75. Gritti A, Galli R, Vescovi A. Cultures of stem cells of the central nervous system. In: Federoff S, ed. *Protocols for Neural Cell Culture*: Humana Press; 2001:173-197.
76. Ziu M, Schmidt NO, Cargioli TG, Aboody KS, Black PM, Carroll RS. Glioma-produced extracellular matrix influences brain tumor tropism of human neural stem cells. *J Neurooncol.* Sep 2006;79(2):125-133.

77. Mahesparan R, Tysnes BB, Read TA, Enger PO, Bjerkvig R, Lund-Johansen M. Extracellular matrix-induced cell migration from glioblastoma biopsy specimens *in vitro*. *Acta Neuropathol.* Mar 1999;97(3):231-239.
78. Hughes CS, Postovit LM, Lajoie GA. Matrigel: A Complex Protein Mixture Required for Optimal Growth of Cell Culture. *Proteomics.* Feb 16.
79. Pollard SM, Yoshikawa K, Clarke ID, et al. Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. *Cell Stem Cell.* Jun 5 2009;4(6):568-580.
80. Galluzzi L, Aaronson SA, Abrams J, et al. Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. *Cell Death Differ.* Aug 2009;16(8):1093-1107.

## BIOGRAPHICAL SKETCH

Maryam Rahman, MD, was born and raised in Florida. She obtained her BA in chemistry from Johns Hopkins University, where she was a member of the varsity women's fencing team. She earned her MD from the University of Florida in 2005 with membership in AOA and the Chapman Humanism Society. She completed her general surgery internship at the University of Florida in 2006 and is scheduled to finish her neurosurgical residency in 2013. She is currently a member of the American Association of Neurological Surgery (AANS), Congress of Neurological Surgeons (CNS) and the Shands Cancer Committee. She was awarded the Chuck Shank award in 2009 for Excellence in Neurosurgery. Dr. Rahman is also the co-director of the neuroanatomy course for the first year medical students.

She is pursuing her research interest in neuro-oncology with a 2-year fellowship in the laboratory of Dr. Brent Reynolds studying tumor stem cells and their role in developing novel therapy for glioblastoma multiforme. Additionally, earned her Master of Science in Medical Sciences with a concentration in clinical and translational research through the Advanced Postgraduate Program in Clinical Investigation (APPCI), an NIH-sponsored program. She has been awarded three research grants to support her work and was awarded the Department of Surgery's Resident Research Award in 2009.