Establishment of Glioblastoma Multiforme Cell Line Bank

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Abstract

Glioblastoma Multiforme (GBM) is a grade IV astrocytoma and is therefore, one of the most aggressive and lethal tumors. Currently, the treatments available are not very successful in maintaining the longevity of a person who receives this diagnosis. However, there is a great deal of research being done on GBM and much of the success is being seen at the molecular level. Due to these positive results, it will be important for further molecular research to continue. The areas that will likely lead to the most successful results involve studying microRNAs, various proteins and enzymes, as well as a plethora of other gene structures. With the importance of GBM research increasing, the collection and analysis of GBM cell lines will be vital. Therefore, through this research the growth rates as well as morphology of different GBM cell lines will be found. Once each of the lines has reached the appropriate amount (>100 million cells) they will then be suspended in TRIzol™ and sent to have an RNA sequence analysis conducted. By the end of the research project the cell lines will have been placed in cryostore for long term preservation to be used by researchers to continue studying GBM.

Keywords: glioblastoma multiforme, cell bank, tumor, growth rates, TRIzol™, RNA sequence

Acknowledgments

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Literature Review

Due to the more recent advances in technology the diagnosis of cancer is not accompanied with as grim a prognosis at it would have been several years ago. Although, even with these advances in technology one malignant glioma tumor, glioblastoma multiforme (GBM), still remains exceedingly difficult to treat (Parsons et al, 2008). This tumor makes up about 60% of the brain tumors found in adults and upon diagnosis, the median survival is about 14 to 15 months (Hanif, Farina et al, 2017).

Malignant gliomas are cancers of the central nervous system and are the most lethal and frequently occurring in this system (Parsons et al, 2008). These types of tumors arise from astrocytes found in the brain which are a type of glial cell ("Astrocytoma Tumors"). Astrocytes give rise to GBM tumors thus giving them the categorization of an astrocytoma (Hanif, Farina et al, 2017). Furthermore, the World Health Organization (WHO) has classified tumors by grade on a scale of I to IV, with grade IV being the most lethal and aggressive (Hanif, Farina et al, 2017). GBM has been given the classification of grade IV (Hanif, Farina et al, 2017).

Upon further examination, GBM has been found to consist of two tumor types, primary and secondary (Ohgaki, Hiroko, and Paul Kleihues. 2007). These two subtypes of GBM have different genetic markers, effect different age groups, and develop differently (Ohgaki, Hiroko, and Paul Kleihues. 2007). Primary GBM tumors constitute the majority of the cases seen, around 90% (Ohgaki, Hiroko, and Paul Kleihues, 2007). These tumors rapidly develop de novo, meaning they arise without the presence of any other cancer or lesion in the body ("NCI Dictionary of Cancer Terms", Ohgaki, Hiroko, and Paul Kleihues, 2007). The genetic markers of a primary GBM tumor are as follows: loss of heterozygosity on 10q, EGFR amplification, $p16^{INK4a}$ deletion, and PTEN mutations (Ohgaki, Hiroko, and Paul Kleihues, 2007). Secondary

GBM tumors only constitute about 10% of the GBM tumors seen but are still aggressive and lethal ("Glioblastoma (GBM)", 2014). This type of GBM develops from the metastases of a tumor that was already present ("NCI Dictionary of Cancer Terms"). The first genetic marker of this tumor type is the mutation seen in the TP53 gene, this is then characterized further by G:C → A:T mutations at CpG sites (Ohgaki & Kleihues, 2007). Secondary tumors also are more likely to be seen in younger patients while primary tumors are more likely to be seen in adult patients (Ohgaki & Kleihues, 2007). While these is a great number of differences between the tumor types similarities exist as well. The most frequently observed genetic alteration, the loss of heterozygosity 10q25-qter, is seen in both tumor types (Ohgaki & Kleihues, 2007).

Currently, it is extremely difficult to treat GBM. This difficultly is due to a variety of factors such as the challenge of crossing the blood-brain barrier, age of patient at the onset of the disease, and the lack of understanding of the pathophysiology of the tumor (Carlsson et al, 2014). Treatment currently used is fractionated radiotherapy with temozolomide paired with adjuvant temozolomide (Yi, Yang, et al, 2016). Although, this therapy is often ineffective due to the invasiveness of the tumor. Additionally, GBM has also been seen to be resistant to radiotherapy and chemotherapy (Yi, Yang, et al, 2016). This resistance is thought to come from cells in GBM called glioblastoma stem-like cells (GSCs) (Yi, Yang, et al, 2016). These cells are found to exhibit characteristics similar to those found in stem cells. These characteristics are: self-renewal, drug and radiation resistance, differentiation into different cell lineages, high tumorigenicity, and the pathways similar to normal stem cells (Yi, Yang, et al, 2016). A marker that is frequently found on the surface of normal stem cells is also found on the GSCs. (Yi, Yang, et al, 2016) Transcription factors that deal with the maintenance of stem cells are also found to be expressed in some subpopulations of GSCs, these are c-Myc, SOX2, OCT4,

NANOG, SALL4, STAT3, Bmil, and KLF4 (Yi, Yang, et al, 2016). Each of these transcriptional factors has a different role in giving stem cells the properties they are known to possess (Yi, Yang, et al, 2016). The discovery of these factors shows the importance of continuing the research on the microscopic features of GBM in order to better understand the pathophysiology.

Another factor that contributes to the aggression and threat of GBM is the microRNA (miRNA) alterations found within the tumor tissue. When compared to healthy brain tissue it was seen that there is both more upregulation and downregulation of miRNAs (Shea, Amanda, et al, 2016). The miRNAs seen in cancer have a strong tendency to target the developmental genes, thus making them a strong factor in the regulation of proliferation, differentiation, and apoptosis of cells (Shea, Amanda, et al, 2016). These miRNAs have even been noted to have a role in the evasion of growth suppressors, be key regulators in drug resistance, and induce the replicative immortality of the cells (Shea, Amanda, et al, 2016). Therefore, the understanding of these RNA sequences will be vital to better understanding GBM.

While GBM is not the most prevalent tumor, it is the most lethal (Whiteman, Honor, 2017). Lengthening the median survival of patients with GBM is what fuels the desire to continually learn more about this vicious brain tumor. New research has emerged from scientists from the Massachusetts Institute of Technology dealing with a specific gene, the PRMT5 (Whiteman, Honor, 2017). This gene creates an enzyme that plays a role in gene splicing, removing introns from messenger RNA (mRNA) because they do not code for a protein (Whiteman, Honor, 2017). When these introns remain in the mRNA strand, the strand cannot be exported outside the nucleus and begins to accumulate (Whiteman, Honor, 2017). This process causes genes involved in expression of proliferation to be retained in the nucleus, and thus the cell does not continually divide (Whiteman, Honor, 2017). The group investing hypothesized

that GBM has higher levels of PRMT5 throughout its entire life cycle allowing for the continued proliferation of the cells (Whiteman, Honor, 2017). This theory was proven correct for when they delivered PRMT5 inhibitors to mice models with GBM and human GBM cells in vitro the growth and division of cells was stopped (Whiteman, Honor, 2017). This information as well as other studies conducted demonstrate the importance of continuing to study at the molecular level.

The difficulty that comes in treating this disease is the availably of GBM cell lines available to use for research (NIH R24 NS086554-01). GBM cells can be cultured in the same manner as adult neural stem cells (NSC) through a NeuroSphere Assay (NSA) but the GBM cells are difficult for labs to obtain (NIH R24 NS086554-01). The difficulty in obtaining GBM cells comes from the fact that many institutions cannot get fresh samples because they are not near a hospital performing surgeries on GBM patients (NIH R24 NS086554-01). Although, once the GBM cells are obtained they can often be easier to maintain than NSC cultures because once established they are more forgiving (NIH R24 NS086554-01). That being said the skills required to handle the culture of GBM are still difficult and require the researcher to pay close attention to the culture (NIH R24 NS086554-01).

Methodology

Passage of Primary Tumor

We received primary GBM tumor samples fresh in a vial promptly following their removal from the patient. The tissue was then transferred to the lab on ice and placed in the hood. We then transferred the tumor piece onto a sterile plastic surface on which the blood vessels were removed with a scalpel. We diced the sample with the scalpel and once the pieces became small enough we added 500 µL of 0.5% trypsin and the dicing continued. We moved the

solution of tissue and trypsin into a 15mL conical tube and another added 500 µL of 0.5% of trypsin. The conical tube was then placed in a water bath at 37°C for 10 minutes. After the 10 minutes passed we added 1 mL of trypsin inhibitor and DNAse and then titrated the mixture five times.

We then passed the solution through a 40 µm strainer into a 50 mL conical tube. The liquid collected was transferred to a 15 mL conical tube and spun down in a centrifuge at 800 rpm for five minutes. The pellet formed from this centrifugation was red. We removed the supernatant liquid, added 1 mL of red blood cell (RBC) lysis buffer, and titrated the solution. The tube was again placed in a water bath for 10 minutes. After 10 minutes we added 2 mL of complete media to the conical tube and then centrifuged the tube for five minutes at 800 rpm. This pellet created was no longer red. We removed the supernatant liquid and added 1 mL of complete media. All of the liquid was then placed in a T25 flask with 5 mL of complete media and placed in an incubator.

Passaging of Cell Lines

Twice a week the T25 flaks containing the cell lines were checked to see if the cells had become confluent and should be passaged. To passage the cell lines, we brought the T25 flaks to a sterile hood. The cell solution was moved to a 15 mL conical tube labeled with that lines identification number. If the cells were attached to the bottom of the T25 flask we hit the flask gently to detach the cells, or we added a small amount of trypsin to facilitate the movement of the cells. The cell solution was then spun down at 800 rpm for five minutes. We removed the supernatant, added 1 mL of trypsin to the pellet, and titrated the solution five times. We then placed the conical tube in a water bath at 37ºC for two minutes. After two minutes in the water

bath we brought the conical tube back to the hood and added 1 mL of trypsin inhibitor, titrated the solution five times, and then it was spun down for five minutes at 800 rpm. Once a pellet was observed we removed the supernatant. Then we added 1 mL of phosphate buffer solution (PBS) and titrated five times.

Once the cells were suspended in PBS we counted the number of cells present using a hemocytometer. We brought an Eppendorf tube containing 90 µL of trypan blue to the hood and 10 µL of the cell suspension was added and mixed in well. We loaded the hemocytometer with 10 µL of the trypan blue cell suspension and then took it to the microscope to be counted. We counted each of the four quadrants in the hemocytometer. To find the total number of cells present in the solution, we divided the number counted on the hemocytometer by four. Next, we multiplied by the dilution factor of 10 and then by the constant for the hemocytometer which is 10,000. Next, we set up a proportion to solve for the amount of cell solution we put into the flask for incubation:

$$\frac{Number\ of\ cells\ present\ in\ solution\ (calculated)}{1000\mu L\ (1mL)} = \frac{Number\ of\ cells\ to\ be\ plated}{X\ (volume\ of\ cells\ to\ be\ plated\ from\ the\ 1000uL)}$$

The number of cells to be plated depends on the size of the flask being used. A T25 flask is plated with 250,000 cells, a T80 is plated with 1,000,000 cells, and a T175 is plated with 2,000,000 cells.

Once X has been found the appropriate flask has the appropriate amount of media added. A T25 gets 5 mL of complete media, a T80 gets 20 mL of complete media, and a T175 gets 40 mL of complete media. Once the correct amount of media was added then we then added the

correct amount of the cell solution (X). Afterwards, we looked at the flask under the microscope to ensure that cells were added to the flask. The flask was then placed in the incubator.

Banking the Cell Lines

Banking cells requires that 100 Million cells are present in the flasks in the incubator. The 100 million cells should be present in ten T175 flasks with 10 million cells per flask. Once the cells in the ten T175 flasks reached confluence, we took 2 mL from one flask to count the cells present. The number of cells we obtained from the 2 mL was then multiplied by the total number of milliliters from each T175 flask to determine the total number of cells from all the flasks. The total number of milliliters in each T175 flask was found when the cell suspension was moved into 50 mL conical tubes. The 2 mL removed and counted previously was then plated in a T25 flask and labeled with the cell line identification number, "Banked", and the date.

We spun down each of the 50 mL conical tubes for 5 minutes at 800 rpm. The supernatant was then removed. Using the total number of cells calculated we added the appropriate amount of cryostore to each 50 mL concial to make it so each milliliter of cryostore had one million cells. One milliliter of each cell suspension was placed in a cryovial and the cryovials were placed in a Mr. Frosty. The Mr. Frosty allows the cell suspension to freeze slowly over 24 hours in the -80°C freezer. Once the 24 hours had passed we took the cryovials out of the Mr. Frosty and placed them in a container for long term storage in the -80°C freezer.

Cell Line Creation

Once the GBM cells were expanded to 100 million cells and banked in the -80°C freezer they were said to be a cell line.

Defrosting Cell Lines

Before removing the vial from the -80°C freezer a T25 flask with 5 mL of complete media needed to be equilibrated for one hour. Once the hour passed, we removed the cryovial containing the cryostore cell suspension from the freezer and placed it in a 37°C water bath for no more than two minutes. Next, we moved the cryostore cell suspension to a 15 mL conical tube in the hood using a pipette. Dropwise, we slowly added 10 mL of warm complete media. The conical tube was then spun down for five minutes at 800 rpm. Once the five minutes had passed we brought the conical tube back to the hood, removed the supernatant, and suspended the pellet in 1 mL of complete media from the T25 flask that was equilibrated. We then added the 1 mL back to the T25 and placed it in the incubator.

TRIzol™ Protocol

The cells being suspended in TRIzol™ were removed from the freezer in small groups because the defrosting of the cells needed to occur in a time sensitive manner. Once we had removed the vials from the -80°C we brought them into the hood to twist the cap just enough to the reduce any pressure that could have accumulated. With the cap retightened the vials were then placed in a water bath at 37°C for no more than two minutes. Under no circumstance should the cell suspension be vortexed. After two minutes the vials were removed, wiped with ethanol, and brought into the hood. We titrated the mixture to homogenize the cells in the cryostore. The solution was then transferred to a 15 mL conical tube. Next, dropwise we added 5 mL of warm media to the cell suspension to dilute the cryostore. The 15 mL conical tube was then centrifuged at 800 rpm for five minutes. After the five minutes, the conical tube was brought back to the hood and the supernatant was removed. We added the TRIzol™ reagent at a ratio of 0.75 mL of

TRIzol™ to 5-10x10^6 cells (ratio for an animal subject). The solution was pipetted five times to homogenize the solution. Once the cells were suspended in the TRIzol™ we transferred the solution to an Eppendorf tube. The Eppendorf tubes can be stored in the -80°C for up to one year.

Results

Growth Rate

The numbers used in the calculation of the growth rates were taken after 4 to 6 passages in order for the cells to reach a complex equilibrium (Deleyrolle, Loic P., et al., 2011). Within the culture of the neural cells there exist two types of cells long-term proliferating (LTP) cells and short-term proliferating (STP) cells (Deleyrolle, Loic P., et al., 2011). LTP's are the cells being measured, they are defined as infinite in their replication, and assumed to be stem cell like (Deleyrolle, Loic P., et al., 2011). STP's have a finite life and are thought to be more similar to progenitor cells (Deleyrolle, Loic P., et al., 2011).

The equation used to calculate the rate of LTP cell symmetric division (LTPCSD) is:

$$K_{ll} = \frac{\ln(F)}{t_f}$$

The *F* present in the equation is the fold expansion of the cell line. It is taken by dividing the cells present at the start of the passage, T_i, by the cells counted at the end of the passage, T_f.

$$F = \frac{T_f}{T_i}$$

The t_f present in the equation represents the amount of time between the beginning of the passage and the end of the passage. The unit for the t_f variable is days. When calculating the LTP

cell symmetric division rate the *F* and t_f were the average numbers calculated from five rounds of passaging (Deleyrolle, Loic P., et al., 2011).

Line = R24-37					
Start Date	End Date	*tf* (days)	Beginning Cell Count	End Cell Count	Expansion
11-Jul-17	1-Aug-17	15	50,000	950,000	19
1-Aug-17	17-Aug-17	16	50,000	950,000	19
17-Aug-17	18-Sep-17	13	50,000	1.150Mil	23
18-Sep-17	2-Nov-17	22	50,000	300,000	6
2-Nov-17	28-Nov-17	26	50,000	150,000	3
		Mean *tf*:18.4		Mean Expansion (F): 14	LTP: 0.143
Line = R24-63					
Start Date	End Date	*tf*(days)	Beginning Cell Count	End Cell Count	Expansion (F)
31-May-17	27-Jun-17	27	250,000	1.6Mil	6.4
27-Jun-17	7-Aug-17	41	50,000	375,000	7.5
7-Aug-17	7-Sep-17	31	50,000	2.05Mil	41
7-Sep-17	25-Oct-17	48	50,000	700,000	14
25-Oct-17	13-Dec-17	49	50,000	375,000	7.5
		Mean *tf*: 39.2		Mean Expansion (F): 15.3	LTP: 0.070
Line = L2					
Start Date	End Date	*tf*(days)	Beginning Cell Count	Cell Count	Expansion
10-Aug-17	23-Aug-17	13	50,000	3.075Mil	61.5
23-Aug-17	5-Sep-17	13	50,000	1.05Mil	21
5-Sep-17	18-Sep-17	13	50,000	3.225Mil	64.5
18-Sep-17	3-Oct-17	15	50,000	7.6Mil	152
3-Oct-17	17-Oct-17	14	50,000	2.725Mil	54.5
		Mean *tf*: 13.6		Mean Expansion (F): 70.7	LTP: 0.313
Line = CA7					
Start Date	End Date	*tf* (days)	Beginning Cell Count	End Cell Count	Expansion (F)
3-Oct-17	23-Oct-17	21	50,000	2.375Mil	47.5
23-Oct-17	31-Oct-17	8	50,000	166,666	3.33
31-Oct-17	16-Nov-17	16	50,000	2Mil	40
16-Nov-17	30-Nov-17	14	50,000	1.8Mil	36
30-Nov-17	13-Dec-17	13	50,000	8.5Mil	170
		Mean *tf*: 14.4		Mean Expansion (F): 59.4	LTP: 0.2835
Line = CA9					
Start Date	End Date	*tf* (days)	Beginning Cell Count	End Cell Count	Expansion
6-Jul-17	27-Jul-17	21	50,000	425,000	8.5
27-Jul-17	7-Aug-17	11	50,000	675,000	13.5
7-Aug-17	23-Aug-17	16	50,000	400,000	8
23-Aug-17	7-Sep-17	15	50,000	400,000	8
7-Sep-17	22-Sep-17	15	50,000	650,000	13
		Mean *tf*: 15.6		Mean Expansion (F): 10.2	LTP: 0.148

Photographs

Line R24-37

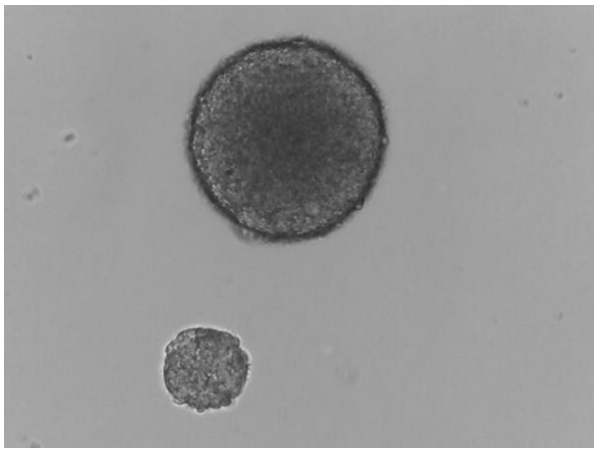

Figure 1. Photo of cells from Line R24-37. Magnification 10x. Shows neurospheres. The darkened center of the larger neurosphere marks cell death.

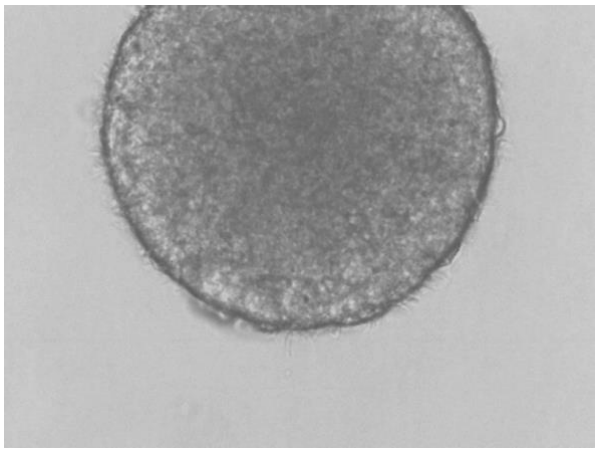

Figure 2. Photo of cell from Line R24-37. Magnification 20x. Picture of larger neurosphere.

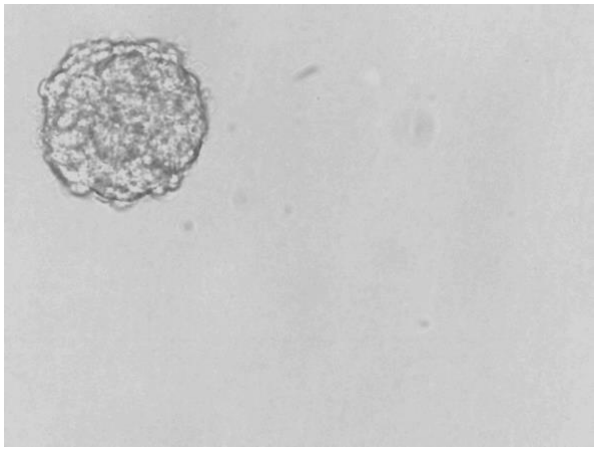

Figure 3. Photo of cells from Line R24-37. Picture of smaller neurosphere. Magnification 20x. This is a conglomeration of multiple cells.

Line R24-63

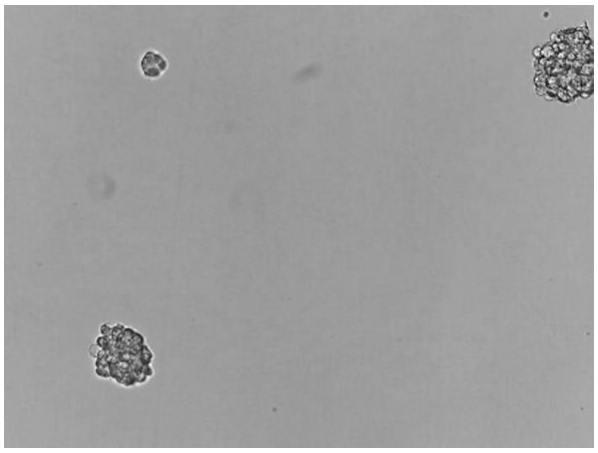

Figure 4. Photo of cells from Line R24-63. Magnification 10x. Neurosphere present. Neural progenitor cells can be seen in the neurospheres.

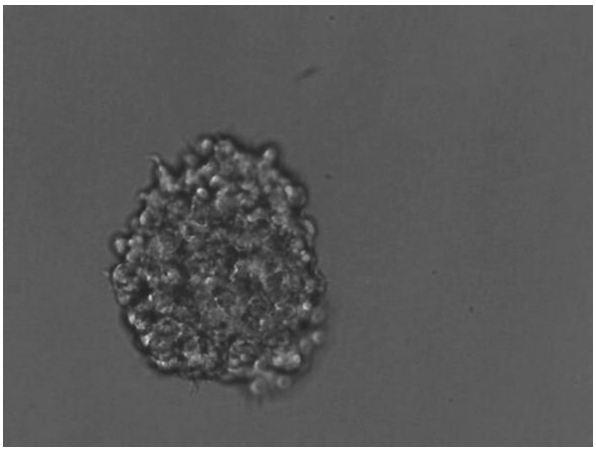

Figure 5. Photo of cells from Line R24-63. Magnification 20x. Small neurosphere.

Line L2

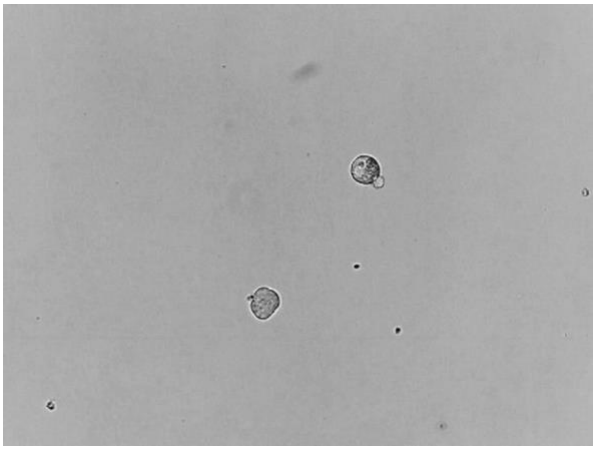

Figure 6. Photo of cells from Line L2. Magnification 10x. These are neural progenitor cells and they will come together to form a neurosphere.

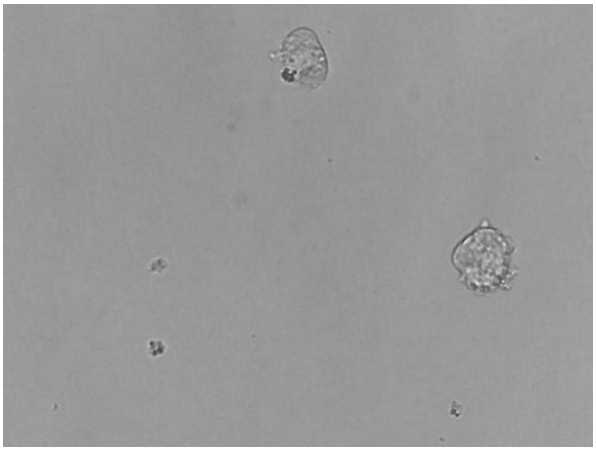

Figure 7. Photo of cells from Line L2. Magnification 20x. These are neural progenitor cells.

Line CA7

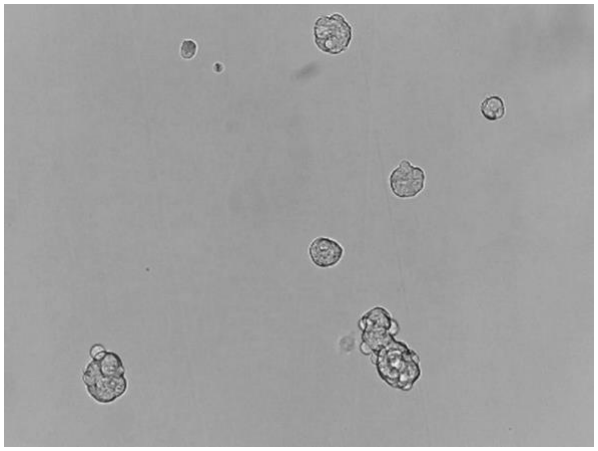

Figure 8. Photo of cells from Line CA7. Magnification 10x. Beginning of the neural progenitor cells forming the neurosphere.

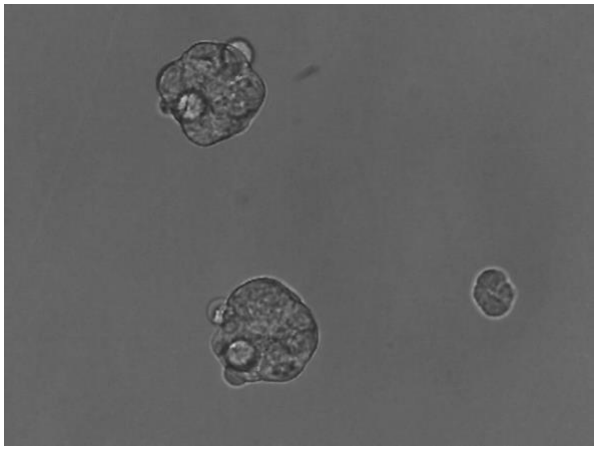

Figure 9. Photo of cells from Line CA7. Magnification 20x. Beginning of the neurosphere formation.

Line CA9

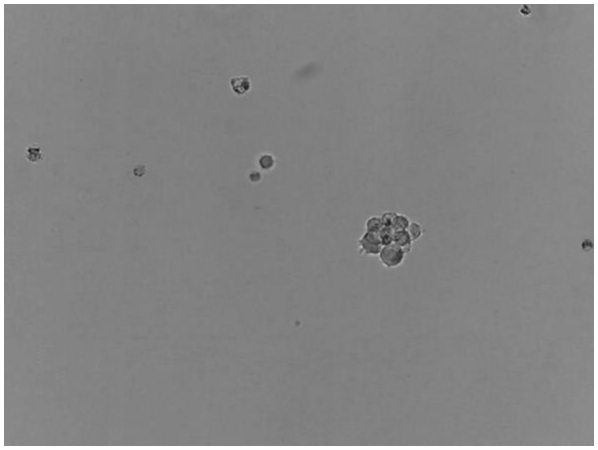

Figure 10. Photo of cells from Line CA9. Magnification 10x. Beginning of neural progenitor cells forming the neurosphere.

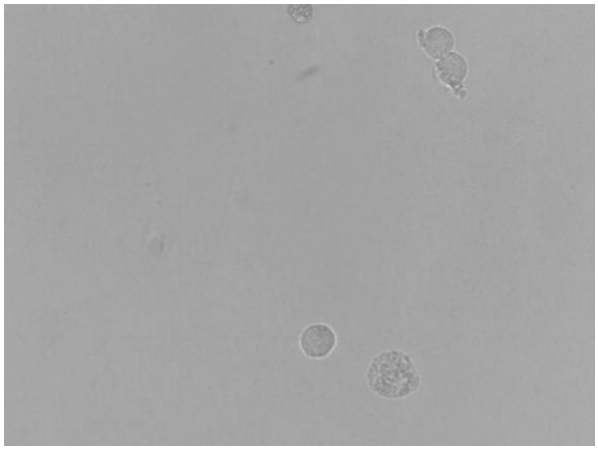

Figure 11. Photo of cells from Line CA9. Magnification 20x. Individual neural progenitor cells.

Discussion

From the study conducted it can be seen that Glioblastoma Multiforme cells lines can be established in culture when obtained from a human subject. It was also shown that it is possible for the GBM cells lines to expand enough to obtain the high volume of cells (>100 million) needed to create a cell line bank. The time required to bank each of the lines differed depending on the growth rate. The growth rates, which were based on the Long-Term Proliferating (LTP) cell symmetric division, were decent for each of the cell lines. The growth rates found will allow for researchers to predict the amount of time required to grow the cells. This will allow researchers to create a more accurate time line for their experiment.

The differences seen in the growth rates most likely occur because the of the genetic variation between GBM tumor cells. The GBM could also differ because the samples came from patients at different stages in their life and the tumors could have been at a different stage in their life cycle. These seemingly small differences can lead to significant changes in morphology and growth rate.

Furthermore, after the cryopreservation process, some of the cell lines were defrosted, plated, and have been successfully grown. Thus, demonstrating the cryopreservation process does not destroy the cells. All of this information together will allow for researchers to make an educated decision when deciding what GBM cell line to use in their experiment. This research will also allow for potential further classification of GBM tumors.

Limitations

The success of this experiment and data collection relied heavily on the relationship our lab has with the Brain Bank at the University of Florida. If any other researchers wish to establish a bank such this one, they would have a difficult time without such a relationship.

Therefore, the labs hoping to replicate this would need to create a relationship such a this before beginning.

Future Research

The creation of the cell bank opens the doors for a plethora of future research. The first piece of research that could be found is the RNA sequence of each of the cell lines. This information will determine if any specific genes are over or under expressed in the GBM line. It could also lead to the potential determination of what genetic markers signal a primary or secondary GBM tumor. Furthermore, researchers could obtain different lines of the GBM cells from our bank to conduct research on them. Such projects that could be performed are testing the effectiveness of a new treatment, implanting cells in vivo to test treatments, or conducting further genetic analysis. The research that could be done on these cells is endless, making this bank vital in furthering research on this cancer.

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