**Acidic pH with coordinated reduction of basic fibroblast growth factor maintains the
glioblastoma stem cell-like phenotype *in vitro***

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Running Title: *Acidic pH and low bFGF maintains GSCs in vitro*

Abstract

Glioblastoma stem cells (GSCs) are a unique subpopulation of cells within glioblastoma multiforme (GBM) brain tumors that possess the ability to self-renew and differentiate into bulk tumor cells. GSCs are resistant to currently available treatments and are the likely culprit behind tumor relapse in GBM patients. However, GSCs are currently inaccessible to the larger scientific community because obtaining a sufficient number of GSCs remains technically challenging and cost-prohibitive. Thus, the objective of this study was to develop a more efficient GSC culture strategy that results in a higher cell yield of GSCs at a lower cost. We observed that the basic fibroblast growth factor (bFGF) is indispensable in allowing GSCs to retain an optimal stem cell-like phenotype *in vitro*, but little change was seen in their stemness when grown with lower concentrations of bFGF than the established protocol. Interestingly, a dynamic fluctuation of GSC protein marker expression was observed that corresponded to the changes in the bFGF concentration during the culture period. This suggested that bFGF alone did not control stem cell-like phenotype; rather, it was linked to the fluctuations of both bFGF and media pH. We demonstrated that a high level of stem cell-like phenotype could be retained even when lowering bFGF to 8 ng/mL when the media pH was simultaneously lowered to 6.8. These results provide the proof-of-concept that GSC expansion costs could be lowered to a more economical level and warrant the use of pH- and bFGF-controlled bioprocessing methodologies to more optimally expand GSCs in the future.

Keywords

Glioblastoma stem cells; cancer stem cells; basic fibroblast growth factor (bFGF); acidic pH; glioblastoma multiforme (GBM)

Introduction

Glioblastoma multiforme (GBM) is the deadliest type of brain cancer. GBM is classified by the World Health Organization as Grade IV, which is reserved for tumors with the highest malignancy. Patients diagnosed with this disease have a median survival rate of only one year (1). Very few effective treatments exist for GBM in part because drug developers have failed to acknowledge the heterogeneity found within the tumor. Recent studies reveal that a small subpopulation of GBM cells called glioblastoma stem cells (GSCs) has stem cell-like characteristics (2, 3). GSCs have been characterized to be resistant to chemotherapy and radiation therapy (2, 4, 5, 6, 7, 8). Furthermore, they have the ability to self-renew, differentiate, and repopulate all the cells found within a tumor. The combined effects of these characteristics of GSCs, also called brain tumor initiating cells, have led many in the scientific community to hypothesize that they are the root-cause behind tumor-initiation, metastasis, and recurrence of tumors after treatment (9).

Therefore, GSCs are an attractive target for novel drug development and are better cell models for preclinical studies. However, GSCs remain largely inaccessible to drug developers and oncology researchers. This is due to their inherent scarcity within tumors, with some estimations being as low as 1% of the GBM cells derived from the surgically resected tumor tissues (10, 11). Efforts are thus being made with small animal models as *in vivo* expansion system. To accomplish GSC expansion, cells are xenografted orthotopically into the brain of immunocompromised mice. Once the tumor is regrown, the mouse brains are collected and dissociated, from which GSCs are isolated and re-implanted into subsequent mice. This process is painstaking, labor-intensive, slow-throughput, and costly. Additional limitation of the approach also includes the necessity to separate the human GSCs from the surrounding mouse brain tissue and the inherent presence of high animal-to-animal variations. Therefore, *in vitro* expansion system of GSCs is urgently needed.

Bioprocessing strategies hold promise for making GSCs more readily available for novel drug development in a more cost-effective and time-efficient manner (10). An effective bioprocess would need to both enrich the bulk GBM tumor population for GSCs and efficiently expand these cells using a bioreactor. Current *in vitro* enrichment strategy for GSCs involves growing the cells in tumorspheres. Tumorspheres have been shown to be enriched for GSCs and prevent cells from undergoing genetic changes that they would undergo if grown in a traditional adherent culture (3, 6, 12, 13, 14, 15). This lack of genetic mutation helps the cells retain the characteristic of the original tumor which makes them more useful for developing targeted drug therapies. GBM cells are typically cultured as tumorspheres in serum-free Neurobasal-A media (NBE) containing a variety of supplements, including epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (15, 16, 17). GSC media is costly, currently estimated at \$600/L. The most significant portion of this cost comes from the exogenous growth factor supplements, which account for roughly 40% of the media cost (18). Since efficient large-scale expansion of GSCs would require the use of bioreactors in volume scales of several liters, the current cost of the media makes this form of expansion cost-prohibitive.

A number of recent studies have pointed to the necessity of bFGF for GSC maintenance (19, 20, 21, 22, 23). Therefore, it is hypothesized that the optimization of the addition of exogenous bFGF can potentially lead to media cost savings. Additionally, it is widely recognized that cancerous tumors have more acidic microenvironment than benign tissues, and pH has been shown to play a role in stem cell maintenance, tumor progression, and chemotherapy resistance of GBM (24, 25, 26). The pH is an easily manipulated variable in both small- and large-scale culture, making adjustment of pH to increase stemness an inexpensive way to potentially improve GSC media. While bFGF concentration and pH are

relatively simple parameters to adjust, fine-tuning them can lead to significant cost savings making bioprocess expansion of GSCs more economically feasible in the future.

While previous studies have shown the importance of bFGF and acidic pH in GSC culture (15, 16, 19, 27), their combined role and their potential synergy in GSC maintenance have not been explored. Here, we explore the effects of different bFGF concentrations on growth and stemness of GSCs in order to confirm its importance in culture and determine the amount necessary for optimal GSC expansion. Fluctuations of pH during the culture period and its effects on growth and stemness are also analyzed. Finally, expression of various known GSC markers in different culture conditions is compared to observe how bFGF and pH can be altered in concurrence to efficiently provide the greatest yield of GSCs. Our study shows that acidic pH with concurrent low bFGF concentration can synergistically increase the stemness of cultured GSC-like cells.

Materials and Methods

Cell Culture

The three glioblastoma cell lines used in this work were U87 (ATCC, Manassas, VA, USA), SMC448 (a primary GBM cell line generously provided by Dr. Do-Hyun Nam), and JX12 (GBM xenoline generously provided by Dr. G. Yancey Gillespie). The cells were cultured as tumorspheres as previously described (15) in Neurobasal-A medium (pH 7.5) supplemented with 1 mM glutamine, 1% Penicillin/Streptomycin, 0.5x N2, 0.5x B27, 20 ng/mL EGF, 20 ng/mL bFGF, and 8 µg/mL heparin (abbreviated as “NBE”). For cell culture in acidic NBE, pH of the media was adjusted using 10 M hydrochloride (BDH Chemicals, Radnor, PA, USA).

Limiting dilution assay (LDA)

Cells were seeded at six different concentrations in 96 well plates: 20, 10, 5, 4, 2, and 1 cells/well ($n = 20$ per seeding density). Cells were cultured for 2 weeks with supplemental media added every 3 days. On day 14, each well was analyzed for the presence of tumorspheres. Spheres were defined as cell aggregates of at least 50 μm in diameter. Limiting dilution assay (LDA) results were analyzed using Minitab (v17; State College, PA, USA). To test for significant changes, the 95% confidence intervals (CI) of the slopes and intercepts were measured. Treatments with slopes and intercepts with non-overlapping CIs were considered significantly different. Furthermore, clonogenicity index was defined as the slope $\pm$ SE of the LDA linear regression, where higher values indicate higher clonogenicity. Comparison between groups was done either with 2-sample t-test or with ANOVA ($p < 0.05$).

ELISA

ELISA was performed on cell culture supernatants from Days 0, 2, and 6 samples using Human FGF-basic ELISA MAXTM Deluxe Kit (BioLegend, San Diego, CA, USA) and Human EGF ELISA Kit (Thermo Scientific, Waltham, MA, USA) according to manufacturer's instructions. To determine the rate of depletion of bFGF and EGF by the cells, 12-well plates were seeded with 5×10^4 cells/well and cultured for 6 days without media supplementation. Three biological replicates were assayed.

Growth curves

Cells were grown in 96-well plates at a seeding density of 1000 cells/well and were supplemented with additional media every three days. Cell growth was measured using a water-soluble tetrazolium salt-based proliferation assay at 450 nm according to manufacturer's protocol (Cell Counting Kit-8, Enzo Life Sciences, Farmingdale, NY, USA; Figures 2 and 4). Three biological replicates were assayed.

Western blot

Total cell lysates were collected on Days 0, 3, and 6 of culture. On Days 3 and 6, samples were collected before and after media supplementation. The pH of cell-free media supernatant from culture was measured in triplicate using a pH meter (pHi 570, Beckman-Coulter, Brea, CA, USA). Cell pellets were lysed in NP-40 lysis buffer (Amresco, Solon, OH, USA) with protease, nuclease, phosphatase, and 0.5 M EDTA (G-Biosciences, Saint Louis, MO, USA). Bradford assay was performed on the lysates for protein quantification. Antibodies used were anti- β -actin (BioLegend), anti-SOX2 (Cell Signaling Technology, Danvers, MA, USA), and anti-MET (Cell Signaling Technology), and the blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) in a digital imager (Bio-Rad ChemiDoc MP) and quantified using ImageLab (v4.1; Bio-Rad).

Flow cytometry

Cells were cultured for 5 days in various formulations of NBE. Flow cytometry was performed on BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA) using anti-CD133/1 (Miltenyi Biotec) and FITC secondary antibody (BD Biosciences).

qRT-PCR

SOX2 (NM_003106) and *ACTB* (NM_001101) primers were designed by retrieving nucleotide sequences from NCBI gene database. The primers used were as following: *SOX2* forward: AAG CCC TGA AAG CGC AAG TCC TCA A; *SOX2* reverse: GGC AGT GGT AGT GGT GGC ATT AGC AG; *ACTB* forward: CTC GTC GCC CAC ATA GGA A; and *ACTB* reverse: AGG GCT TCT TGT CCT TTC CTT C. Primers were synthesized by Eurofins Genomics (Huntsville, AL, USA).

RNA isolation was performed using GeneJet RNA Purification Kit (Thermo Scientific) according to manufacturer's protocol for mammalian cultured cells. RNA was quantified using NanoDrop (Thermo Scientific). Complementary DNA (cDNA) was synthesized using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) and Mastercycler Nexus Gradient (Eppendorf, Hauppauge, NY, USA) according to manufacturers' protocols. Quantitative real-time PCR was performed using PerfeCTa SYBR Green FastMix (Quanta Biosciences) according to manufacturer's protocol. Illumina Eco (Illumina, San Diego, CA, USA) was used as the qRT-PCR instrument along with EcoStudy software for the data analysis.

Chemoresistance Assay

To assess chemoresistance under the new media conditions, 1×10^4 U87 or SMC448 cells were seeded to 48 wells of a 96-well plate. Twenty four wells had standard NBE media with 20 ng/mL bFGF, pH 7.5, while the other 24 wells had 8 ng/mL bFGF, pH 6.8. The cells were incubated at 37°C for 24 hours and then treated with either control (DMSO; 0 μ M) or temozolomide (10 μ M and 100 μ M; Enzo Life Sciences) ($n=8$ for each condition). After 48 h of treatment, cell viability was measured using WST-8 as described above according to manufacturer's instructions (Enzo Life Sciences).

Statistical Analyses

All statistical analyses were performed on Minitab or SPSS. For comparison of marker expression and viability, Student's *t*-test or ANOVA with Tukey's HSD post hoc analysis was performed. For LDAs, general linear model was used to generate the linear regressions and to compare the resulting slopes and intercepts. With all these analyses, significance was set at $p < 0.05$.

Results

Epidermal growth factor and basic fibroblast growth factor contribute to stemness of GBM cells in vitro

In order to confirm that EGF and bFGF are important for GSC growth, GBM cells were grown with both growth factors, bFGF only, EGF only, and without either growth factors. These cells were analyzed by limiting dilution assay, a hallmark *in vitro* functional assessment of stemness (28, 29, 30, 31). The assay results elucidated that both growth factors contributed to enhanced sphere formation capability, thus signifying the importance of bFGF and EGF in GSC maintenance (Figure 1A). Sphere formation ability was highest when both growth factors were present, and EGF was seen to have a more significant impact on sphere formation than bFGF. The degradation rates of bFGF and EGF in cell culture were analyzed using ELISA on media samples collected throughout the cell culture period. Results showed that bFGF concentration in media decreased approximately 50% when cells were grown without any additional media supplementation during the culture period (Figure 1B). To rule out non-specific degradation of the growth factors, NBE was incubated for the same period of time without cells at 37°C, and the concentration of bFGF remained constant (data not shown). These results indicated that the GBM cells actively utilize bFGF during culture. In contrast, EGF concentration either remained at its baseline level or increased (Figure 1B). This increase can likely be attributed to production and excretion of EGF into the extracellular environment by the cells at a rate higher than cell consumption of the growth factor. Because the cells more readily utilized bFGF and because bFGF is a much more significant portion of the overall media cost than EGF (35% vs. 3%) (18), subsequent studies focused on studying the effects of bFGF in GSC culture.

Basic FGF concentration has a minimal effect on growth and stemness

In order to determine how bFGF concentration could be affecting GSC growth and stemness, cells grown with 0, 8, 14, and 20 ng/mL exogenous bFGF were analyzed. In the U87 cells, there was no significant difference in growth rate at any concentration of bFGF (Figure 2A). SMC448 cells did not grow at 0 ng/mL bFGF, but the growth rates at all other bFGF concentrations were comparable (Figure 2A). To determine the effect of bFGF concentration on stemness, LDA was performed on cells grown at the different concentrations of bFGF. For both cell lines, sphere formation capability was lower at 0 ng/mL bFGF, but little difference was seen between the cells grown at 8, 14, and 20 ng/mL bFGF (Figure 2B and 2C). This indicated that bFGF concentration as low as 8 ng/mL can be used without negatively affecting GSC growth and stemness.

GSC marker expression in in vitro culture varies over time

Ideally, GSC marker expression should remain constant throughout the cell culture period. This is important because establishing consistent culture conditions that promote GSC growth, rather than dilution by non-stem cells, is an important step towards implementing efficient large-scale GSC production. To test, Western blot analysis was performed on cell lysates collected before and immediately after the addition (i.e., within 5 minutes) of supplemental media throughout the culture period. Surprisingly, the expression of GSC markers SOX2 and MET (17, 32, 33, 34, 35) changed dynamically throughout the culture period (Figure 3). The marker expressions typically increased as the media became more acidic, especially for the patient-derived SMC448 and JX12 cells. Interestingly, the MET and SOX2 expression changes varied depending on the cell line, perhaps due to the differences in their GBM molecular subtype (36). Overall, these results were unexpected and indicated that other factors besides bFGF were affecting stemness.

Acidic pH slows growth but promotes clonogenic tumorsphere formation

We analyzed a number of different factors in GSC media that could account for the fluctuations in GSC marker expression. As previously mentioned, EGF concentration did not decrease throughout culture (Figure 1B), and the glucose concentration also remained in excess (data not shown). However, we observed that the culture pH fluctuated between media supplementations (Figure 3). There was a significant decrease in the pH between supplementations, but it increased slightly after supplementation. Considering that even a small change in pH can have a substantial impact on biological systems, further investigation of the potential role of pH in GSC marker expression was warranted. The trends in pH expression were compared with the trends in GSC marker expression. Generally, pH and GSC marker expression were inversely proportional (Figure 3).

To test the effect of acidic culture conditions on growth rate, U87, SMC448, and JX12 cells were grown at pH 6.8 and pH 7.5. Consistent with previous report (26), a slower growth rate was observed in the cells grown at pH 6.8, but the cells still exhibited an ability to proliferate (Figure 4A). Seeing that the cells display more stem cell-like characteristics when grown at acidic pH, the slight decrease in growth rate could be acceptable if overall GSC yield is increased by the practice. Furthermore, LDA was performed on cells cultured at pH 6.8 and 7.5 (control, the pH of fresh NBE). In acidic media, sphere formation capability of U87 cells and JX12 cells increased, suggesting an increase in the presence of GSCs (Figure 4B). This is consistent with previous report that showed a relationship between acidic pH and GSC phenotype (24).

Stem cell-like phenotype can be maintained by lowering both bFGF concentration and pH

Based on the above results with bFGF and pH, it was hypothesized that lowering both the bFGF concentration and the pH of the media would result in increased growth of GSCs at a lower cost than current practices allow. To test this hypothesis, flow cytometry was performed to compare the expression of CD133, a putative GSC marker, in cells grown in four different NBE compositions: pH 7.5 and 20 ng/mL bFGF (current standard); pH 7.5 and 8 ng/mL bFGF; pH 6.8 and 20 ng/mL bFGF; and pH 6.8 and 8 ng/mL bFGF (Figure 5A). In U87 cells, CD133 expression was lower when cells were grown at pH 7.5 and 8 ng/mL bFGF, but its expression in all other experimental groups was comparable. In SMC448 and JX12 cells, the pH 6.8 experimental groups had higher CD133 expression than the pH 7.5 experimental groups. There was no statistical difference between the cells grown at 8 ng/mL bFGF versus 20 ng/mL bFGF in pH 6.8.

In order to further elucidate the relationship between pH and bFGF, qRT-PCR and limiting dilution assays were performed. Quantitative RT-PCR was used to quantify the difference in SOX2 expression between cells grown in pH 7.5 and 20 ng/mL bFGF and cells grown in pH 6.8 and 8 ng/mL bFGF. In all the tested cell lines, those grown in the pH 6.8/8 ng/mL bFGF had significantly higher SOX2 expression than those grown in the conventional pH 7.5/20 ng/mL bFGF media (Figure 5B). This corroborated the flow cytometry data in Figure 5A, further indicating that culture conditions of low pH and low bFGF concentration retain or even increase the GSC marker expressing population.

Finally, the clonogenicity of cells grown in pH 7.5 and 20 ng/mL FGF and cells grown in pH 6.8 and 8 ng/mL FGF were compared using LDA (Figure 5C). There was no statistical difference in the clonogenicity between the cells grown in these different culture conditions. This finding indicates that as long as the media pH is simultaneously lowered, the clonogenicity of the cells is not significantly decreased by decreasing the concentration of bFGF. In other words, we can lower the bFGF concentration in the media by over 50% and

lower the pH of the media to decrease the cost of GSC culture, but retain a high yield of GSCs.

Lowered bFGF concentration and acidic pH retains chemoresistance

Chemoresistance is one of the functional characteristics of GSCs (4, 37). To provide further evidence that stem-like characteristics have been retained in the new media condition with lowered bFGF and acidic pH, we treated U87 and SMC448 cells with 0, 10, and 100 μ M temozolomide (TMZ) for 48 h to test whether their resistance to the drug was affected by the media. In all tested conditions, post-treatment cell viability remained high and was not significantly different to the standard NBE culture condition (Figure 6). For SMC448 cells treated with 100 μ M TMZ, we observed an even greater resistance to TMZ than in standard NBE culture conditions. These results suggest that the lowered bFGF and acidic pH helps retain or even increase the chemoresistance, further providing support for the use of the new culture condition to enhance or enrich GSC-like population *in vitro*.

Discussion

Our findings confirmed that bFGF has a direct impact on the functional stemness of GBM cells. The cells had very similar responses to concentrations of bFGF between 8 and 20 ng/mL, suggesting that we can decrease the concentration of bFGF used in the media by over 50% and still maintain the GSC-like phenotype. This is significant because bFGF contributes the largest portion of the cost of GSC media (18), and decreasing its usage will allow for more cost-effective culture of GSCs in the future. We also showed that bFGF is not solely responsible for the maintenance of GSC marker expression and stemness *in vitro*. The pH of the culture media was found to also have a profound impact on GSC marker expression. By

decreasing the pH of the media to 6.8, we can further enhanced the GSC-like phenotype of the cells.

The role of both bFGF supplementation and low pH in GSC culture have been demonstrated previously (15, 16, 19, 27), but, to the best of our knowledge, this is the first time that their combined roles have been explored. Consistent with previous results, our data show that acidic pH can lead to increased stem cell marker expression (Figure 5). Furthermore, we found that acidic pH and lowered bFGF concentration can have synergistic impact in increasing the stemness characteristics of cultured GSC-like cells, such as the expression of stemness markers (Figure 5A), tumorsphere formation capability (Figure 5B), and resistance to chemotherapeutic (Figure 6).

Future studies will need to more specifically elucidate the roles of acidic pH and bFGF in GSC maintenance as well as the means of precisely manipulating them to yield the greatest number of GSCs. In a recent study, Ihling et al. comprehensively identified significant changes in the proteome of a prostate cancer cell line that are induced by acidic conditions (38). They found seven proteins that were either upregulated or downregulated in response to acidosis, all of which are recognized as relevant for tumor growth. A similar study in GBM cell lines would help elucidate the role of acidic pH in GSC maintenance. In addition, a comprehensive look at the effects of bFGF on the GBM proteome is another area for future research. A greater understanding of the effects of pH and bFGF will lead to the possibility of an optimized media composition and, ultimately, allow for a more economical scale-up to bioreactor processing of GSCs to provide a greater number of cells for future research applications.

Bioprocessing has great potential for the future of GBM research. Static batch culture typical in cancer cell culture is poorly controlled, labor intensive, non-homogeneous, and inefficient for large scale expansion (39). Bioprocessing strategies not only increase the scale

of culture, but also allow for better control of the culture conditions. Even bench-scale stirred-tank bioreactors afford the ability to control agitation rate and to monitor for changes in pH and dissolved oxygen concentration in order to ensure homogeneity of the populations within the reactor (40). Throughout the process of static culture, the pH of the media fluctuates due to the release of acidic byproducts into the environment by the cells as they grow and the addition of supplemental media (Figure 3). We are currently making efforts to apply the results described here to a 4-L stirred-tank bioreactor operated as pH-stat to simultaneously control pH and bFGF via optimized feed rates.

Large-scale bioprocessing of GSCs can be used in the future to not only make cells available for basic research purposes, but also for applications like high throughput drug screenings. These bioprocessing strategies will make future oncology research easier and more consistent. However, in order to make these strategies feasible, the cost of the media must first be drastically reduced. Using the combined pH and bFGF control strategy proposed here, the effective cost of media could be reduced by more than 30%.

As the amount of evidence supporting the cancer stem cell hypothesis continues to expand, it is becoming increasingly clear that these largely uncharacterized, treatment-resistant cells need to be a primary focus in future cancer studies. Treatments specifically targeting this small subpopulation of cells have the potential to increase overall patient survival and decrease the occurrence of metastasis for a variety of cancers, including glioblastoma. One of the biggest obstacles that must be overcome to achieve this goal is the difficulty of obtaining a large number of these rare cells for *in vitro* study. The data presented here suggest culture parameters that can be referred to for future work on optimization of media, as well as in scaling up culture bioprocesses to increase the overall *in vitro* yield of GSC-like cells.

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Figure Captions

Figure 1: Exogenous supplementation of growth factors is necessary to retain stemness. (A) Limiting dilution assay was performed on U87 and SMC448 GBM cell lines grown in Neurobasal media supplemented with both epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), with EGF only, with bFGF only, and without either growth factors. Results indicate that both bFGF and EGF promote sphere formation. Sphere formation capability was highest in the presence of both bFGF and EGF (i.e., had smallest x-intercept), and the presence of either growth factor enhanced sphere formation. (B) The amount of EGF and bFGF in the culture media was measured using ELISA and was found that there was no significant decrease in their levels over a six-day period ($n=3$, mean $\pm$ SE). There was a significant increase in the amount of EGF on Day 6 ($p < 0.05$), likely due to autocrine production of the growth factor by cells. Negative control media containing no cells over the same time period also showed no significant change in EGF and bFGF levels (data not shown), indicating that there is an insignificant amount of non-specific degradation of either growth factor during the culture period.

Figure 2: Growth and stemness of GBM cells can be maintained even at minimal concentration of bFGF. (A) Growth of U87 and SMC448 GBM cells with varying concentrations of bFGF media supplementation were assayed using WST-8 proliferation assay ($A_{450\text{ nm}}$). The growth was not significantly different when cells were supplemented with media supplemented with bFGF concentration between 8 and 20 ng/mL. Presence of bFGF was necessary for growth, especially for SMC448, as seen in the 0 ng/mL growth curves ($n=3$, mean $\pm$ SE; Note: Some error bars are smaller than the symbols). (B) Sphere formation capability was assayed using limiting dilution assay. For both U87 and SMC448,

sphere formation was enhanced by the presence of bFGF, but this capability was not significantly different among the tested bFGF concentration. (C) Representative microscope images of U87 and SMC448 grown at 0, 8, and 20 ng/mL bFGF concentration are shown (scale bars = 100 μ m). Absence of bFGF severely hindered tumorsphere formation.

Figure 3: GSC protein marker expression changes dynamically in culture. (A) Representative Western blot images of cell lysates of U87 and SMC448 cultured in standard NBE with 20 ng/mL bFGF. (B) MET and SOX2 GSC protein marker expressions were quantified from Western blots with respect to time (normalized to β -actin expression). Corresponding pH profile was also measured ($n=3$, mean $\pm$ SE; Note: Some error bars are smaller than the symbols). Marker expression changes were found to be a function of the changes in the media pH.

Figure 4: Acidic pH slows growth but increases tumorsphere formation capability. (A) Growth of U87, SMC448, and JX12 GBM cells cultured at pH 7.5 and pH 6.8 for 6 days were assayed using WST-8 proliferation assay (A_{450nm}) ($n=3$, mean $\pm$ SE; Note: Some error bars are smaller than the symbols). Cells grown in pH 6.8 had slower growth rate. (B) Limiting dilution assay results showed that the tumorsphere formation capability was either similar or greater in GBM cell lines grown at pH 6.8 compared to control (pH 7.5), suggesting equal or greater stemness.

Figure 5: Stemness can be maintained by simultaneously lowering both bFGF concentration and pH. (A) Flow cytometry analysis of CD133 GBM stem cell marker was performed for U87, SMC448 and JX12 GBM cells. Cells were cultured in pH 7.5 and pH 6.8 with bFGF concentrations of 8 ng/mL and 20 ng/mL. (B) GBM stem cell marker SOX2 was analyzed

using qRT-PCR. SOX2 expression was significantly higher in the pH 6.8, 8 ng/mL bFGF experimental group than the pH 7.5, 20 ng/mL bFGF experimental group in all three tested GBM cell lines. (C) Limiting dilution assay (LDA) was performed to compare the clonogenicity of the pH 6.8 and 8 ng/mL bFGF experimental group against the control group (pH 7.5, 20 ng/mL bFGF experimental group). Clonogenicity index was defined as the slope $\pm$ SE of the linear regression of the LDAs. There was no statistically significant difference in clonogenicity between the two experimental groups in any of the cell lines tested ($p > 0.05$).

Figure 6: Chemoresistance to temozolomide is retained when simultaneously lowering both bFGF concentration and pH. (A) WST-8 assay was performed on U87 and SMC448 cells grown with either 20 ng/mL bFGF, pH 7.5 or 8 ng/mL bFGF, pH of 6.8. Cells were treated with 0, 10, or 100 μ M of temozolomide for 48 hours. Cells grown under new culture conditions retained chemoresistance as indicated by high viability (***) $p < 0.001$. (B) Representative images of cells from A. Pictures confirm chemoresistance and show morphology retention in the presence of temozolomide (scale bars = 100 μ m).

(A)

(B)

(A)

(B)

(C)

(A)

(B)

(A)

(B)

(A)

(B)