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Cryopreservation of cancer-initiating cells derived from glioblastoma

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1. ABSTRACT

Glioblastoma multiforme (GBM) represents the most devastating adult brain tumor. GBM follows a hierarchical development in oncogenesis, with a sub-population of cells - brain tumor stem cells (BTSCs), exhibiting tumor-initiating potential. BTSCs possess extensive self-renewal capability and can repopulate the entire tumor mass. They are resistant to conventional therapies, suggesting that they are the likely candidates of tumor recurrence. Their eradication is thus important for an effective cure. Previous works showed that human-derived BTSCs could be stably maintained for 10-15 passages in serum-free condition, and gene expression and karyotypic hallmarks similar to the primary tumors were preserved. However, primary cells have been shown to sustain additional karyotypic aberrations owing to the harsh conditions of extended *in vitro* serial passage. Several investigators have proposed passaging these cells in xenograft models. A limitation of such an approach is the inability to return to identical passages for experimental repetitions, or the unavailability of suitably-aged mice for implantation. We have devised a method to cryopreserve BTSCs and that important characteristics were maintained, establishing a repository for drug screening endeavors.

2. INTRODUCTION

Several cancers of the hematopoietic system, brain, breast and colon have been shown to originate from a sub-population of cells commonly called cancer stem cells, or tumor-initiating cells (1-7). The definition of a cancer stem cell relies on functional characteristics such as the ability to serially engraft and form tumors that recapitulate the human disease pathophysiology, and does not always point to the origin of the cancer cell being a *bona fide* stem cell. A variety of transgenic and xenografted animal models have been used to identify the BTSC, here interchangeably termed the GBM-initiating cell. Elegant transgenic mouse experiments revealed that mutational deletions in common tumor suppressors such as p53 and Pten, or p53 and Nf1 led to astrocytomas with 100 percent penetrance, only if the mutational deletions occurred in the neural stem cell compartment (8, 9). In glioma xenograft models, tumors have been shown to arise from implantations of flow-sorted cells expressing various markers such as CD133 and SSEA-1 (10, 11). While these data strongly suggest that BTSCs exist, the xenograft model is not without limitations. Factors such as the strain of mouse, time to formation of tumor and co-injection with an extracellular matrix have been shown to alter the tumor-initiating cell

frequency, thus questioning the rarity, identification and definition of such cells (12). Nevertheless, the xenograft model remains important for several reasons: Implantations of BTSCs grown under serum-free condition form tumors that recapitulate the gene expression, phenotypic and karyotypic profiles of their primary tumors (13), these xenografts are thus important "replicas" of human tumors that can be prospectively tested with new candidate compounds, yet have retrospective clinical history, gene expression, and paraffin tissue blocks for mining prognostic indicators. This collection of human xenograft "replicas" will also be amenable to molecular characterization and clustering according to the four subtypes defined in The Cancer Genome Atlas (14). Efforts are therefore important in preserving the integrity of such human-derived BTSCs.

Our previous work established the essential components of a rapid snap-freezing technique - vitrification, for cryopreserving BTSCs (15). We will discuss how reduction of serum is important to maintain cells in the undifferentiated state, with rapid solidification of the cell-liquid mixture using liquid nitrogen to minimize water crystal formation. We discuss our evidence in the context of current BTSC literature, explaining why vitrification is a superior method for preserving essential traits of BTSCs. Finally, we utilized a small molecule screen interrogating common oncologic pathways to show that BTSCs from a repository preserved in this manner retained essential signaling mechanisms. This establishes the feasibility of a BTSC repository for drug screening efforts.

3. VITRIFICATION: A CRYOPRESERVATION TECHNIQUE FOR GBM-INITIATING CELLS

BTSCs are enriched in both spherical structures called neurospheres, and adherent layers cultured on laminin, in the presence of serum-free media supplemented with growth factors (3, 6, 7, 16). A comparison of the transcriptional profiles using various parametric statistical tests revealed that major signaling pathways were preserved among published BTSC models (17). Our work on vitrification of BTSCs as spherical structures remains important for the following reason: While major signaling pathways were preserved, the lack of genetic drift in transcriptome profiles did not imply that *bona fide* stem cell properties remained unaltered. Indeed, we had shown previously that BTSCs passaged extensively *in vitro* sustained changes in tumor stem cell frequency, karyotype and surface marker expression, yet generated transcriptome profiles that clustered with the original low passage BTSCs in a principal component analysis map (15). As such, a detailed side-by-side comparison of clonal characteristics from these two culture conditions remains to be performed. We recognize that BTSCs as neurospheres are heterogeneous (18-20), and we attempted to address the importance of maintaining clonal properties in our choice of cryopreservation method.

Although various cryopreservation techniques have been developed for a range of cells such as human/mouse embryonic stem cells and mouse neural precursor cells, these studies have largely relied on gross morphological appearances and have ignored examining the genetic profiles and quantitative analysis of cell types (both stem and differentiated forms) of samples (21-25). Vitrification has been commonly used in the cryopreservation of cells involved in reproduction, human embryonic stem cell bodies, and cell-containing constructs used in tissue engineering (24, 26-28). Vitrification works on the principle of rapid glass induction with liquid nitrogen instead of ice crystal formation with slow-cooling methods, and results in significantly better viability upon thawing. For validation of vitrification as a method of cryopreservation for BTSCs, the cellular heterogeneity of tumor cells and their ability to recapitulate glioma pathophysiology would have to be taken into consideration.

3.1. Preservation of essential properties

To validate our vitrification method, we sought to analyze several parameters associated with the BTSC: Tumor stem cell frequency, preservation of surface marker expression, gene expression profile, karyotypic hallmarks, and the ability to recapitulate the original tumor morphology when engrafted in an immune-compromised mouse (29). Standard freezing techniques with high serum have been used in many cellular systems because of their less complex preparatory steps. Mao *et al.* have discussed the efficacy of freezing single cells from dissociated neurospheres in either serum-free medium or 90 percent fetal bovine serum in the presence of 10 percent dimethyl sulfoxide (30). Previous work showed that slow-freezing with high serum content resulted in differentiative outgrowths of human embryonic stem cells upon thawing (21). Similarly, we observed that although freezing with 90 percent fetal bovine serum yielded the best viability of tumor spheres, it also resulted in differentiative outgrowths (15), with concomitant changes in expression of various genes (Figure 1A). The maintenance of BTSCs in their undifferentiated state is important because these cells have been shown to generate tumors whereas their lineage-committed progenitors and differentiated cells do not. Indeed, a therapeutic approach aimed at differentiating BTSCs to eliminate tumor-initiating potential has been suggested (31).

Investigators in the BTSC field have relied on modifications of the neurosphere assay to approximate tumor stem cell frequency, and this assay has been shown to reliably translate into survival outcome of engrafted mice (32-34). In particular, the efficacy of drug treatments is assayed for a prolonged period following drug withdrawal, to allow for remnant BTSCs with self-renewal potential to recover. We have shown that vitrification did not alter the BTSC frequency *in vitro* over 3-4 passages, although the gold standard for this assay would be a limiting dilution analysis carried out in immune-compromised mice (Figure 1B) (10). The neurosphere assay is not without limitations as transiently amplifying progenitors and cellular aggregations can confound data interpretation (18-20). It is thus crucial to carry out such assays at clonal densities over an extended length of time and several generations to measure *bona fide* BTSC frequency.

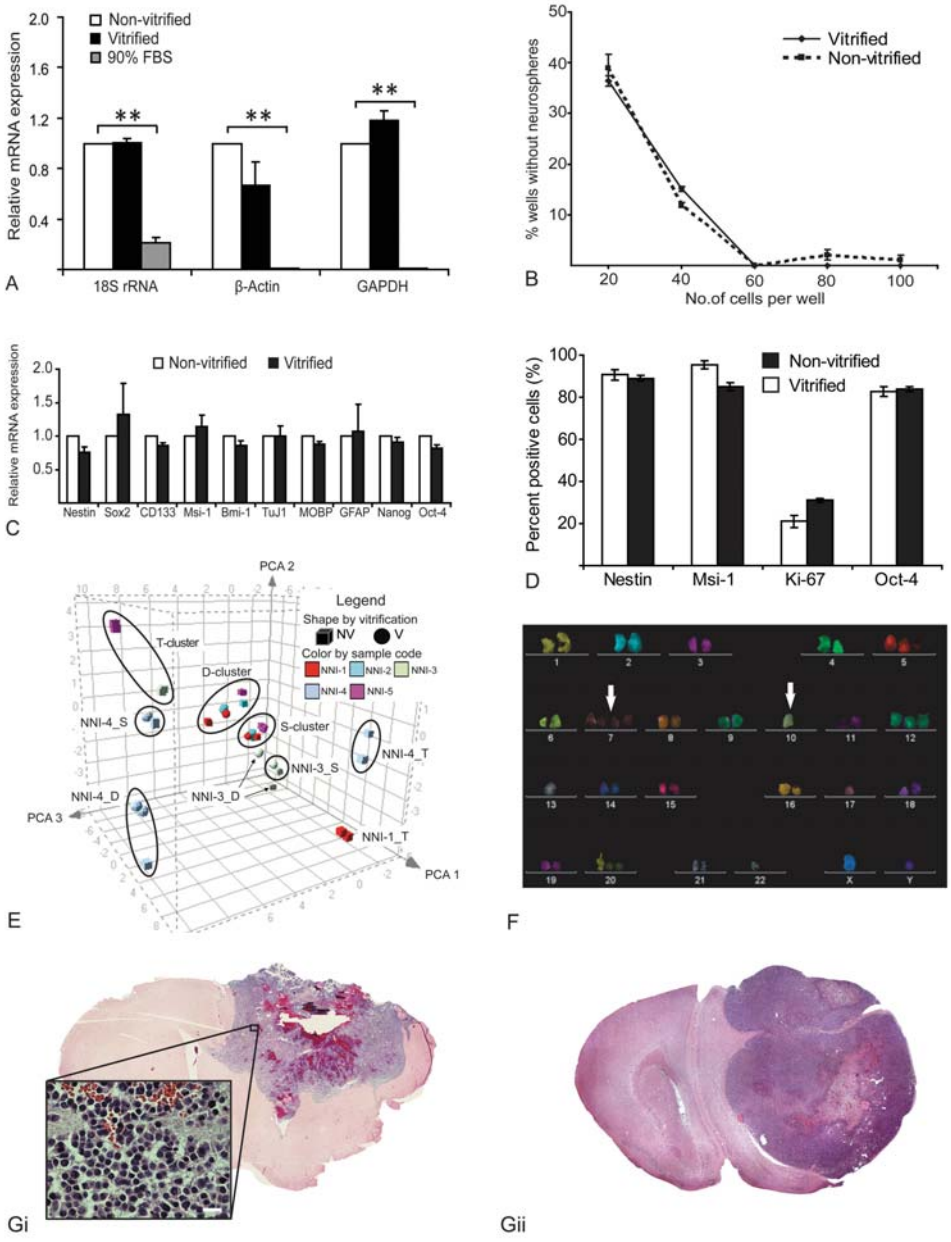

Figure 1. Vitrified GBM-initiating cells using low serum content preserved their BTSC frequency, gene expression, surface marker expression, karyotypic profile and created xenografts that recapitulated the original disease pathophysiology. (A) Cryopreservation with high serum content led to altered levels of several constitutively expressed genes upon thawing and re-culturing. Data were normalized against the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene. ** denotes *p* less than 0.01. (B) Vitrified cells preserved their BTSC frequency. (C) Vitrified BTSCs preserved mRNA levels of key stemness and differentiation genes. (D) Vitrified BTSCs preserved cell numbers expressing common stemness markers. (E) Principal component analysis map of BTSCs showed that vitrified and non-vitrified cells in each of stemness and differentiated states clustered together. BTSCs were genetically distinct from the primary tumor. Two BTSC lines (NNI-1_S in S cluster and NNI-4_S) exhibited distinct transcriptomic profiles even though they showed identical GBM histology of their primary tumors. BTSC lines from a previous manuscript (15) are re-named here for simplicity: NNI1-1 replaces S0305, NNI-2 replaces S0405, NNI-3 replaces S0805, NNI-4 replaces S0306, NNI-5 replaces S0807. (F) BTSCs preserved karyotypic hallmarks of GBM, notably amplification of chromosome 7 and loss of chromosome 10. (Gi) Anaplastic oligoastrocytoma BTSC intracranial xenograft exhibited extensive infiltration, hemorrhaging and displayed the typical "fried egg" morphology and "chicken wire" patterning of stroma. Scale bar represents 20 microns. (Gii) Intracranial xenograft established from serum-grown U87MG displayed spatially constrained, well-lineated tumor margins. Figures C, D and F were reproduced with permission from, (15). Figures B and E were adapted from data obtained in (15).

Several surface markers have been associated with tumor neural stem cells such as Oct-4, Nanog, Bmi-1, Nestin, CD133, aldehyde dehydrogenase, SSEA-1, and the ability to efflux the Hoechst 33342 dye (10, 11, 32, 35, 36). Although some of these markers have been associated with a worse prognosis in GBM tumors, they are by no means the perfect markers for BTSCs as they also mark normal neural stem cells (37, 38). In our vitrification technique, we determined the preservation of such BTSC markers by qRT-PCR (Figure 1C), immunofluorescence (Figure 1D) as well as quantitatively by flow cytometry. We observed that vitrification preserved BTSC marker expression. Of note, we and others observed a high frequency of cells staining for both TuJ1 and GFAP upon induction of BTSC differentiation. This may indicate an aberrant differentiation pathway in BTSCs (15, 39).

Recent work utilizing gene expression data from The Cancer Genome Atlas categorized GBM tumors into four classes: Proneural, Neural, Classical and Mesenchymal (14, 40). These classes coincided with unique chromosomal aberrations, strongly suggesting that karyotypic profiles drive GBM gene expression and disease progression. We observed that our vitrified BTSCs maintained gene expression profiles similar to the original low passage BTSCs (Figure 1E) (15). Two morphological and genetic subtypes of BTSCs could be identified even though the primary tumors from which these BTSCs were derived showed similar histology. It thus remains a key question as to whether BTSC subtypes contribute to clinical heterogeneity in treatment response. We also observed that BTSCs formed a unique cluster on the principal component analysis map distinct from primary tumors (denoted by T-suffix), while BTSCs induced to differentiate in the presence of serum (denoted by D-suffix) clustered more closely with the primary tumors. These data imply that BTSCs are a genetically distinct set of cells from primary tumors, thus, therapeutic strategies based on molecular targeting using conventional serum-grown cancer cells may have to be revisited. Indeed, the common choice of drug, temozolomide, has recently been shown to be ineffective against CD133-expressing BTSCs located at the core of tumors which exhibit low oxygen tension promoting MGMT expression, while progenitors and differentiated cells at the periphery were arrested (41). Designing effective therapeutic strategies against GBMs thus represents a paradigm shift in understanding the cells-of-origin and their contribution to clinical heterogeneity in treatment response.

Next, we demonstrated that vitrification preserved the karyotypic aberrations of BTSCs similar to their original low passage cells (15). We noted the preservation of signature hallmarks of GBMs: Amplification of chromosome 7 (where *EGFR* is located) and loss of chromosome 10 (where *PTEN* is located) in all four of our BTSC lines tested (Figure 1F). Lee *et al.* demonstrated that BTSCs cultivated under serum-free condition maintained the karyotypic profiles of the primary tumors (13). In contrast, conventional serum-grown cells contained chromosomal aberrations not reflective of the primary tumor (42). These findings underscore the importance of studying BTSCs and we now have a method to cryopreserve these cells. Interestingly, we were able to detect additional karyotypic changes in one of our lines that was extensively passaged *in vitro* (more than 50 passages), which correlated with altered BTSC frequency and surface marker gene expression, even though these altered BTSCs generated similar transcriptomic profile to the original low passage tumor spheres. We believe this highlights the importance of our vitrification method in being able to freeze down low passage cells, and thaw them only when needed for further experiments. Continued passaging *in vitro* to maintain the cells would be deleterious.

The ability of BTSCs to serially transplant and reform gliomas that recapitulate the original human disease morphology provides unequivocal evidence for the definition of a cancer stem cell (29). We were able to recapitulate glioma disease patterns when we implanted our vitrified cells in immune-compromised mice. In particular, when we implanted NNI-8 anaplastic oligoastrocytoma tumor spheres, we obtained glioma xenografts that were highly infiltrative and displayed the typical "fried egg" histology of oligodendroglial cells with "chicken wire" patterning of the stroma, and extensive hemorrhaging (Figure 1Gi). In contrast, gliomas formed from conventional serum-grown cells were spatially constrained, had a well-delineated margin and were seldom hemorrhagic (Figure 1Gii) (13). BTSCs grown under serum-free condition are therefore important because they generate gliomas that recapitulate the original disease profile. Although many investigators have utilized the more simplified version of cryopreservation with high serum content or serum-free medium containing 10 percent dimethyl sulfoxide, we believe further assays to quantitatively measure the preservation of BTSC characteristics would be important to compare different methods side-by-side (16, 30, 43). Our work describes a comprehensive approach to assess these data; specifically, we highlight the preservation of karyotypic hallmarks using vitrification over an extended period of 3 years. We are currently engaged in experiments to demonstrate the preservation of BTSC frequency *in vivo* through limiting dilution analyses to validate our vitrification method. This is analogous to limiting dilution assays performed in hematological diseases (2).

3.2. Model for maintenance of GBM-initiating cells

Although BTSCs can be enriched in tumor spheres under serum-free condition, in many instances, clinical material is limited, compounded by a lack of methods to preserve such cells at convenient time points. In addition, the field of stem cell biology is complicated by the difficulty of returning to identical passages of cells for replication of experiments. We have now devised a novel technique for cryopreserving BTSCs - vitrification that maintains the integrity of these cells and circumvents the problems previously described. We propose that a combination of vitrification and *in vivo* serial passaging in immune-compromised mice will provide a convenient means of preserving the GBM-initiating population (Figure 2). Our method may also be applicable to other neoplastic

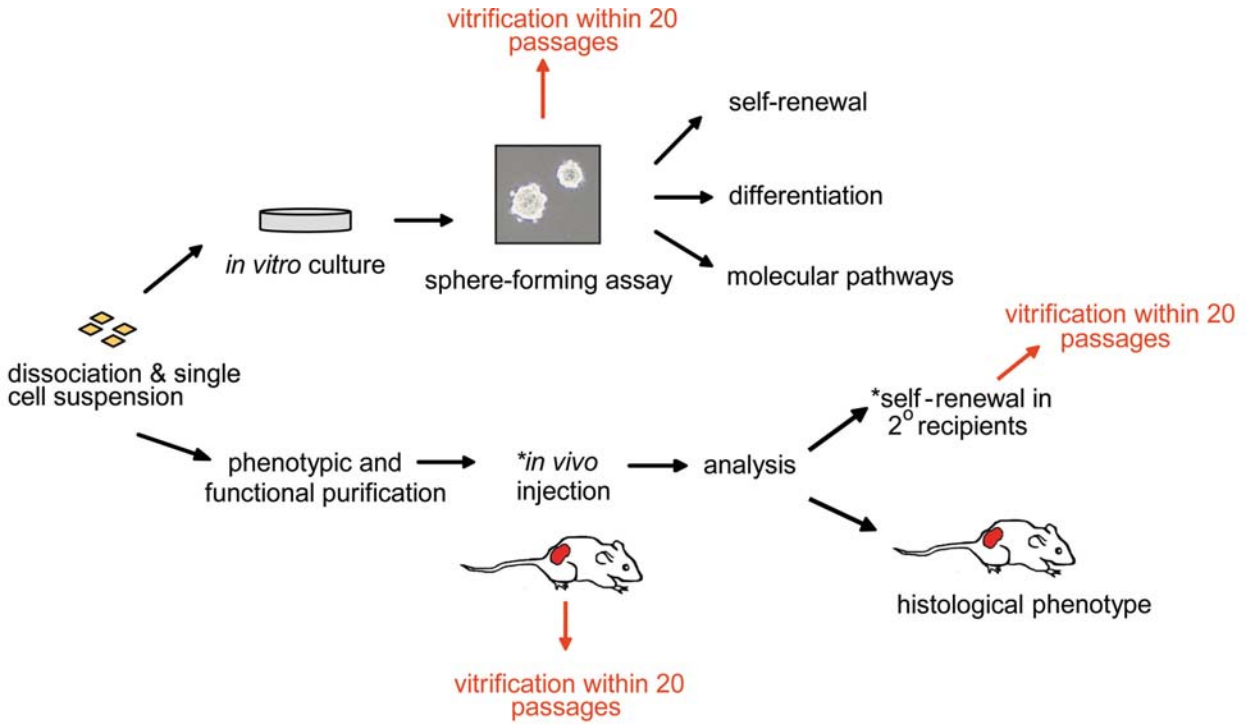

Figure 2. Vitrification can be combined with *in vivo* serial passaging to maintain a repository of GBM-initiating cells. We propose that the vitrification method can be utilized at the indicated stages (red) to cryopreserve BTSCs. These cells can subsequently be thawed and expanded at a later stage for biological assays, such as drug screening, or subjected to serial passaging in immune-compromised mice. Vitrification permits long-term storage of such cells without changes to their genotypic and functional properties. Orthotopic implantation is used for glioma xenograft rather than the typical flank model; diagram is illustrative. Reproduced with permission from, (15).

stem-like cells grown in spheroid manner, such as those isolated from breast (mammospheres), prostate (prostaspheres) and colon (colonospheres) (44-49).

4. GBM-INITIATING CELL SUBTYPES AND THEIR RELATION TO PUBLISHED CELL COLLECTIONS

Recent literature implicated BTSC subtypes that initiated tumor growth irrespective of CD133 status (50-52). Beier and colleagues demonstrated the utility of a 24-gene signature derived from BTSCs that segregated these tumor-initiating cells into type I (proneural) and type II (mesenchymal) classes (17). We sought to define our BTSC collection by performing an unbiased hierarchical clustering (Figure 3A), as well as clustering based on the 24-gene signature (Figure 3B). Our data indicated that all our BTSCs were proneural, and published BTSC lines (both grown as spheres and as adherent layers with laminin) segregated according to their classes as previously defined (Figure 3C) (16, 17, 53). Importantly, we noted that most proneural BTSCs were upregulated in key genes ASCL1 and DLL3, previously documented as proneural genes in GBM tumor molecular classification by The Cancer Genome Atlas (14). In contrast, proneural BTSCs were downregulated in the TGFbeta pathway. The mesenchymal BTSCs demonstrated strong TGFbeta response genes such as TGFbeta-induced and COL1A2 (17, 54). These data suggest that our vitrified BTSC collection contains biological and signaling patterns consistent with published literature, and that cell morphology may not be an ideal criterion for classifying BTSCs.

5. APPLICATION: DRUG SCREENING AND COMMON ONCOLOGIC PATHWAYS

Our initial principal component analysis indicated that BTSCs (Figure 1E, S cluster) possessed a different transcriptome from the tumor mass (Figure 1E, T cluster) which was composed of a mix of relatively undifferentiated and more committed lineages (Figure 1E). Serum is one of the factors that induces differentiation of BTSCs, thus, traditional drug screening efforts using commercially procured cell lines grown in serum-containing media may not be ideal. Work by others has shown that BTSCs contribute to tumor initiation and propagation due to a halt in the differentiation potential. Indeed, tumor involution resulted from exposing BTSCs to the differentiative effects of bone morphogenetic proteins (BMPs) (31). In addition, a newly discovered proto-oncogene Pleiomorphic Adenoma Like 2 (PLAGL2) has been found to promote tumor growth by blocking differentiation, and sustaining BTSC self-renewal via the Wnt signaling pathway (55). These data strongly implicate BTSCs as the culprits responsible for the perpetuating trait of GBM tumors, and underscore the need to design therapeutics aimed at eradicating this population.

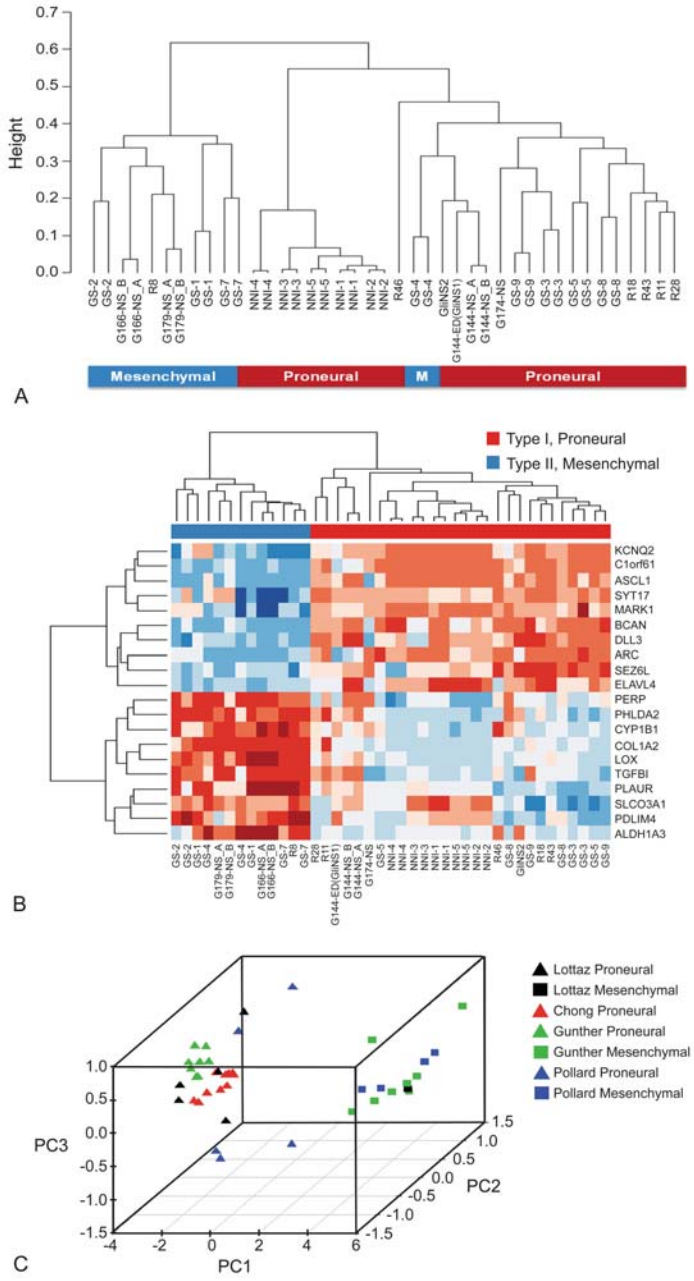

Figure 3. Vitrified GBM-initiating cells could be grouped into two subtypes and were differentially regulated by Proneural genes and TGFbeta signaling pathways. An integrated non-parametric analysis of gene expression in 4 glioma stem cell datasets is shown: (A) An unbiased whole transcriptome (all commonly expressed genes between Illumina & Affymetrix platforms) hierarchical clustering based on a non-parametric rank-sorted binning (500 quantile bins) of each expression dataset, using a spearman rank correlation distance metric and complete linkage. Grouping of samples into Type I (Proneural) represented as red bar and Type II (Mesenchymal) represented as blue bar, based on classification by Lottaz *et al.* (17). (B) Heatmap clustering of 4 BTSC datasets based on a signature of 20/24 genes (4 genes: NGC, GLUR2, SOX11 and unknown 213484_at were not adequately represented on the Illumina Human ref8v2 gene array) published by Lottaz *et al.* (17). Clustering was based on a spearman rank correlation distance of a non-parametric rank sorted binning (500 bins/sample) of gene level averaged expression data for each dataset. (C) 3D principal component analysis (PCA) of spearman rank correlation distance between the models in each of the BTSC datasets. The first 3 principal components are shown capturing more than 90 percent of the total variance in the dataset. Plotting symbols demarcate Type I (Proneural,▲) and Type II (Mesenchymal, ■) BTSC subtypes, while colors demarcate the different sources of BTSCs: Red, Chong *et al.*, (15); Green, Gunther *et al.*, (53); Black, Lottaz *et al.*, (17) and Blue, Pollard *et al.* (16).

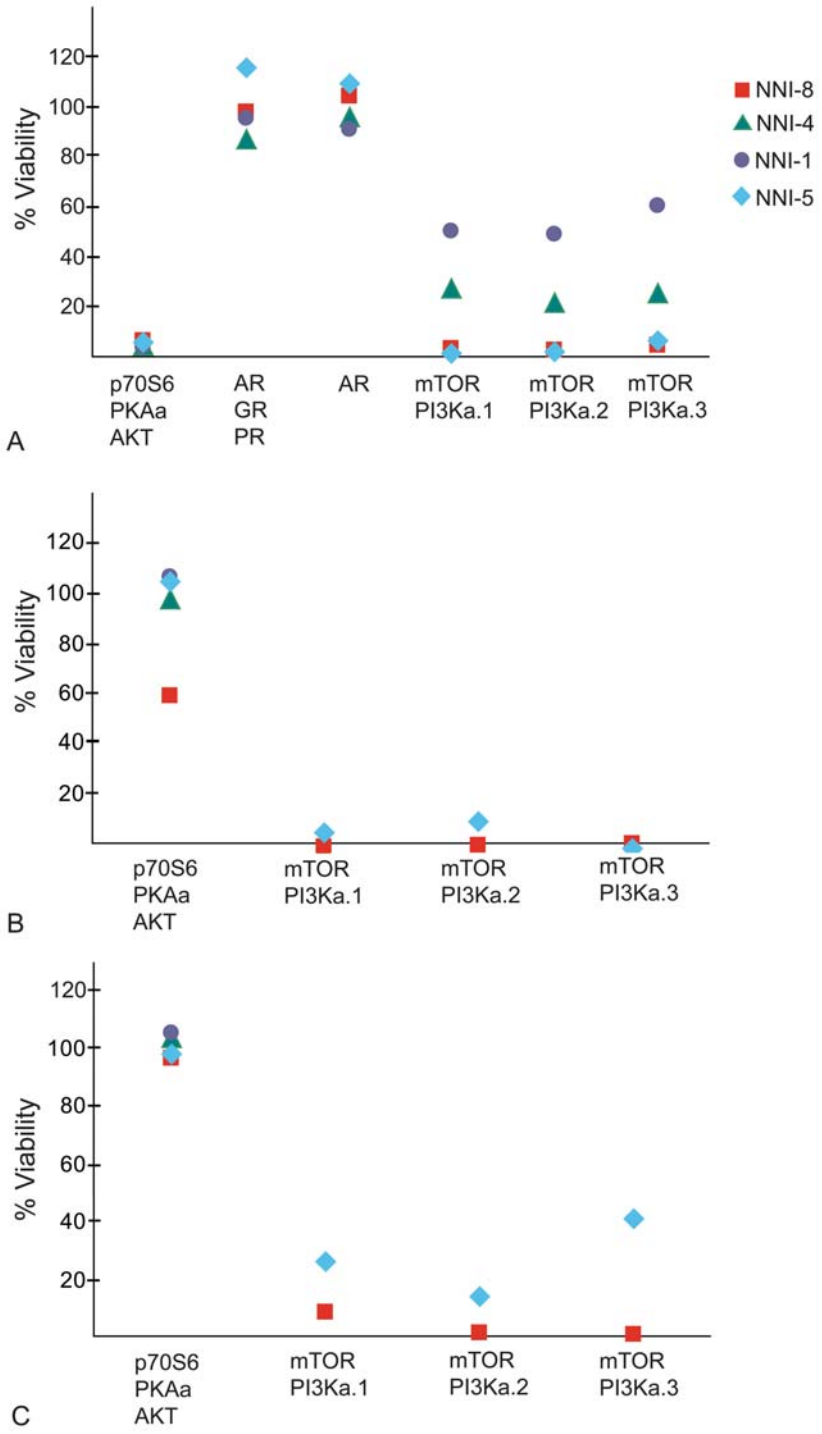

Figure 4. GBM-initiating cell viabilities after treatment with small molecule inhibitors of key signaling pathways. We designed the viability-based screen using tumor spheres treated with candidate compounds by adapting a previously published protocol (33). Briefly, BTSCs were dissociated with Accutase (eBioscience) and seeded in 96-well plate format at 10,000 cells per well in serum-free media containing growth factors (15). A recovery period of 3-4 days was allowed before compounds were added, along with replenishment of growth factors. Viabilities were measured at day 7 following drug addition using the standard alamarBlue® assay according to the manufacturer's instructions (61). Twenty microliters of alamarBlue® dye were added to each well and further incubated for 16 hours before absorbances at 570 and 600 nanometers were measured. At 10 micromolar (A), BTSC lines which demonstrated less than 20 percent viability were re-screened in similar manner at 1 micromolar (B) and 0.1 micromolar (C) to approximate IC_{50} doses.

Table 1. Small molecule inhibitor targets and *in vitro* biochemical assay IC_{50} values

Target	IC_{50} value / micromolar	Reference
p70S6, PKAalpha, Akt	PKAalpha, 0.0026; AKT1, 0.0235; p70S6K, 0.00612	This study
mTOR, PI3Kalpha.1	mTOR, 0.00783; PI3Kalpha, 0.00864	This study
mTOR, PI3Kalpha.2	mTOR, 0.0316; PI3Kalpha, 0.0184	This study
mTOR, PI3Kalpha.3	mTOR, 0.0069; PI3Kalpha, 0.0051	This study
Akt III/SH-6	25 (cell-based)	59, Calbiochem
PI3K	50 (cell-based)	59, LY294002, Calbiochem
Rapamycin	0.1 (cell-based)	59, Calbiochem
Androgen receptor	0.00149	This study
Androgen, glucocorticoid, progesterone receptors	AR, 0.0127; GR, 0.000813; PR, 0.00285	This study

In an effort to understand the signaling mechanisms regulating BTSCs, we treated our vitrified cells with small molecules targeting common oncologic pathways. We observed the following: Compounds targeted against recently published BTSC signaling pathways reduced the viability of our cells significantly, indicating that our vitrified cells maintained common signaling pathways, and not all BTSC lines behaved similarly even though the primary tumors from which they were derived exhibited identical morphologies upon histological analyses (ie. a gradation of response could be seen).

Compounds that were effective against our BTSCs at a range of 0.1 to 10 micromolar and for which published data is available included small molecules against mTOR, PI3K and Akt (Figure 4, Table 1). We noted that effective concentrations for our BTSC lines corresponded to the low *in vitro* biochemical IC_{50} values of the small molecule candidates, consistent with death by specific pathway targeting. Hyperactive PI3K-Akt signaling promotes tumorigenic cell behavior in gliomas by increasing cell survival, proliferation, invasion and angiogenesis (56-58). Eyler *et al.* demonstrated that inhibition of the PI3K-Akt pathway using small molecule inhibitors against PI3K, Akt or its downstream target mTOR (at approximately IC_{50} doses, Table 1) preferentially targeted the CD133-expressing BTSC compartment, with concomitant apoptosis and reduction in BTSC frequency and invasiveness, ultimately translating to prolonged survival of pretreated BTSC-intracranial xenografted animals (59). These data are consistent with an activated PI3K-Akt signaling in our BTSCs. We observed that not all BTSC lines were uniformly inhibited, suggesting the exciting notion that BTSC subtypes may contribute to clinical heterogeneity in treatment response. This remains to be evaluated.

In contrast, we observed no such reduction in viability (up to 10 micromolar) using small molecules targeting the androgen receptor (AR), glucocorticoid receptor (GR) and progesterone receptor (PR), common therapeutic targets in breast and prostate cancers. Indeed, these receptor types are present in a small collection of astrocytic neoplasms (60). However, no effective clinical therapy has borne out to-date. In agreement, the IC_{50} values for these compounds indicate that BTSCs are not likely to be targeted via this pathway.

6. SUMMARY

Work with clinical specimens is often limited by the amount of material available, as well as by the method by which investigators can generate more material of similar integrity to conduct further studies. Although BTSCs can be reliably maintained by serial transplantations in immune-compromised mice, practically, it is not always possible to have suitably-aged mice for implantations. Moreover, the very nature of serial passaging renders it impossible to return to identical passages for experimental repetitions. We have established vitrification as a method for preserving the integrity of BTSCs. We demonstrate that essential properties such as surface marker expression, BTSC frequency and karyotypic patterns are preserved. Importantly, vitrified cells form serially transplantable gliomas that recapitulate the actual human disease morphology. Finally, we show evidence that our vitrified cells can be utilized for drug screening efforts and possess signaling mechanisms consistent with published literature. We believe that vitrification as a means to cryopreserve BTSCs will allow studies to be conducted with the ability to duplicate experimental conditions and cell line parameters.

7. ACKNOWLEDGEMENTS

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Abbreviations: GBM: glioblastoma multiforme; BTSC: brain tumor stem cell; p53: tumor protein 53; PTEN: phosphatase and tensin homolog; Nf1: ; neurofibromatosis type 1; CD133: complementarity determinant 133; SSEA-1: stage-specific embryonic antigen ; qRT-PCR: quantitative real-time polymerase chain reaction; TuJ1: neuron-specific class III beta-tubulin; GFAP: glial fibrillary acidic protein; PCA: principal component analysis; MGMT: methyl-guanine methyl transferase; EGFR: epidermal growth factor receptor; ASCL1: Achaete-scute homolog 1; DLL3: Delta-like 3; TGFbeta: transforming growth factor-beta; TGFbeta-induced: transforming growth factor, beta-induced; COL1A2: collagen alpha-2(I) chain; BMP: bone morphogenetic protein; PLAGL2: Pleiomorphic Adenoma Like 2; mTOR: mammalian target of rapamycin; PI3K: phosphoinositide 3-kinase; NGC: neuroglycan C; GLUR2: glutamate receptor 2; SOX11: Sry-related HMG-box 11; IC_{50}: half maximal inhibitory concentration.

Key Words: Glioblastoma Multiforme, Brain Tumor Stem Cells, Vitrification, Review

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