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Large-Scale Production of Human Glioblastoma-Derived Cancer Stem Cell Tissue in Suspension Bioreactors to Facilitate the Development of Novel Oncolytic Therapeutics

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1. Introduction

Glioblastoma multiforme (GBM, World Health Organization grade IV glioma) is the most common and aggressive form of brain cancer in adults and accounts for 17% of all childhood cancers (Cheng et al., 2010; Dirks, 2008). Even with conventional cancer treatment, the median survival time is between 12-15 months, with a 2-year median survival rate between 8% and 12% (Stupp et al., 2002; Stupp et al., 2005). This grim prognosis is partly due to GBM cells infiltrating the brain and spinal cord thus preventing complete surgical resection. These invasive tumour cells appear to be more resistant to cytotoxic therapy and have a higher proliferative potential (Furnari et al., 2007). Additionally, molecular profiling of GBMs have indicated that there is great heterogeneity within these tumours which may be due to the presence of a sparse population of cancer cells that exhibit stem-cell-like characteristics (Mischel et al., 2003; Parsons et al., 2008; Paugh et al., 2010; Verhaak et al., 2010). Specifically, these cells are able to self-renew and retain the capacity to differentiate but lack proliferative control. These stem-like cancer cells are referred to as either tumour-initiating cells or cancer stem cells (CSCs), and have been identified in a number of hematopoietic malignancies and solid tumours (Hope et al., 2004; C. Li et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004; Stingl & Caldas, 2007). CSCs have also been shown to be more resistant to conventional cancer drugs and have been implicated in the more aggressive nature of tumour drug resistance after remission (Bao et al., 2006; Dean et al., 2005). Hence, by effectively targeting these CSCs the prognostic outcome of GBM patients may be improved. However, the development of novel oncolytic therapeutics to target CSCs is currently hindered by the scarcity of CSCs found within these solid

tumours. Thereby, the development of novel drug treatments may rely on a continuous and reproducible supply of tissue for genomic analysis and high-throughput drug screening.

In order to address this issue, there is a major need for a large-scale cell expansion platform that allows for the generation of a large number of GBM-derived tissue containing cancer stem cells (GBM-CSCs). It has been shown that neurosphere culture can isolate and enrich GBM-CSCs (Singh et al., 2003; Yuan et al., 2004). There has been some controversy with this method of derivation, and recently data was presented by Dirks and colleagues who argued that GBM cell populations expanded in adherent culture, in comparison to neurosphere culture, have uniform access to growth factors and that adherent culture hampers the ability of short-lived progenitor cells to proliferate (Pollard et al., 2009). By comparing expression of differentiation markers on glioma cells grown in both adherent and sphere-forming culture, they found that increased numbers of cells grown as spheres expressed these differentiation markers. However, a source of contention is that the authors only compared one type of serum-free medium for the expansion of these glioma cells as spheres and also grew them in static vessels where the cells did not experience any shear forces. In a contrasting study, De Witt Hammer et al. (2008) showed that the genomic profiles of GBM-derived cells grown in spheroid culture were more representative of the parental tumor profiles, whereas in adherent cultures, genetic changes developed that led to genetic deviations from the parental tumors. Based on our studies with neural precursor cells from multiple regions of the brain and with GBM-derived CSC tissue, we have shown that we can expand these cells reproducibly in suspension culture (Baghbaderani et al., 2008; Baghbaderani et al., 2010; Panchalingam et al., 2010). Additionally, we have shown that the bioreactor-expansion of GBM-derived CSC tissue as spheroids conserves the basic nature of the tissue (Panchalingam et al., 2000). This was achieved by using a serum-free medium, PPRF-h2, which was developed in our laboratory for neural cell isolation and expansion.

Typically, GBM-CSCs are expanded in static culture vessels prior to use in basic cancer research. However, these conventional culture systems for expanding GBM-CSCs are inefficient at generating large numbers of cells and are labour intensive and non-homogenous. Therefore, we looked to develop protocols using suspension bioreactors which can lead to better process control. Shear fields in our suspension bioreactors were controlled with a central impeller that was able to eliminate concentration heterogeneities and lead to homogenous environmental conditions. Moreover, the shear generated by the impeller influenced tissue aggregate size, therefore allowing for the expansion of the generation of homogenous GBM-CSC spheroids. We adapted large-scale suspension bioreactor culture protocols for the expansion of human neural precursor cells to human GBM-derived CSC tissue. Studies were conducted to investigate the role of different bioengineering parameters on the expansion of GBM-derived CSC tissue. Through the refinement of experimental procedures, we were able to expand successfully our cell populations. Additionally, we were able to show that these bioreactor-expanded GBM-derived CSC tissue retained similar genetic characteristics to the initial cell population (Panchalingam et al., 2010). Through the use of a serum-free medium, PPRF-h2 (Baghbaderani et al., 2010), and the development of successful bioprocess cell expansion conditions, we can alleviate the bottleneck in GBM-CSC supply for use in research and potentially for the development of new brain cancer therapies through small molecule screening.

2. Human glioblastoma multiforme (hGBM)

Brain tumours are the leading cause of death resulting from solid cancers in children under the age of 20, and the third leading cause of death in young adults age 20-39 (Jemal et al., 2007). Moreover, brain tumours are diversely defined based on their histological characteristic and tissue origin (Vescovi et al., 2006). In general, brain tumours arise from brain cells (primary brain tumours) or from metastatic tumours originating from other areas of the body (secondary brain tumours) which continue to proliferate in the brain. As the cancer cells proliferate and invade surrounding brain tissue, symptoms may result including hemiparesis, aphasia, and seizures. Additionally, as the tumour grows intracranial pressure also increases due to the space constraints in the skull. The increase in pressure causes secondary complications by damaging or killing healthy brain cells, resulting in symptoms such as headaches, nausea, and changes in mental status (Patkar et al., 2000).

Astrocytic tumours, the most common type of gliomas, are typically classified based on their histological features and fall into two categories: low-grade or high-grade tumours. Low-grade astrocytic tumours (World Health Organization (WHO) Grade I and II) such as pilocytic astrocytomas, have an appearance similar to normal cells and tend to grow slowly. High-grade astrocytic tumours (WHO Grade III and IV), are tumours that tend to grow quickly, invade nearby tissue and the tumour cells have a different appearance than normal cells. GBMs are classified as grade IV astrocytic tumours (Siebzehnrubl et al., 2011). Histopathological features of GBMs usually include cellular anaplasia (undifferentiated cell growth), nuclear atypia, increased mitotic activity, vascular proliferation and extensive necrosis. Additionally, GBMs can arise as primary tumours or from the transformation of lower grade tumours into secondary tumours (Denysenko et al., 2010). Risk factors for gliomas remain elusive, although there have been some factors such as occupation, environmental carcinogens and diet which has been reported to be associated with the development of gliomas (Verma, 2009).

The majority of brain tumours are diagnosed usually when the patient experiences seizures or neurological symptoms (such as cognitive or motor deficits) without a clear cause (DeAngelis, 2001). Currently, there is no method that has been developed for screening or preventing brain cancer. Upon diagnosis, the treatments for brain tumours are multimodal involving surgical resection, radiation and chemotherapy. In children younger than three years of age, it is standard practice to employ chemotherapy instead of radiation due to its negative effect on the developing nervous system (Pollack, 1994). However, in both children and adults, these treatment modalities are not curative and invariably result in relapse and treatment failure. Also, since these treatments are invasive in nature, they usually result in cognitive impairment among other side-effects (Taphoorn & Klein, 2004). Due to the lack of effective therapeutic options, the effective treatment of GBMs represents a major challenge to clinicians.

3. Cancer stem cells

3.1 Cancer stem cell hypothesis

The cancer stem cell hypothesis proposes that tumours arise from stem cell-like tumour-initiating cells (TICs) or cancer stem cells (CSCs), capable of self-renewal but lacking proliferative control, which are the instigators of tumour formation and the precursors of malignancy (Reya et al., 2001; Singh et al., 2004). Under normal physiological conditions,

stem cells would either self-renew or differentiate into proliferating progenitor cells. These proliferating progenitor cells would then be able to differentiate and give rise to the cell populations of that tissue type (i.e. oligodendrocytes, neurons, or astrocytes) [Figure 1]. According to the cancer stem cell hypothesis, a mutagenic agent (e.g. environmental stresses) would cause the oncogenic transformation of the stem cell or a down-stream progenitor cell. These transformed cancer stem cells would retain some stem cell properties, such as self-renewal, but uncontrollably produce differentiated non-tumourigenic cells that would form the bulk of the tumour. This hypothesis has lead to the identification of CSCs in numerous malignancies, including leukemia, breast cancer, brain cancer, pancreatic cancer, and colon cancer (Hope et al., 2004; C. Li et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004; Stingl & Caldas, 2007).
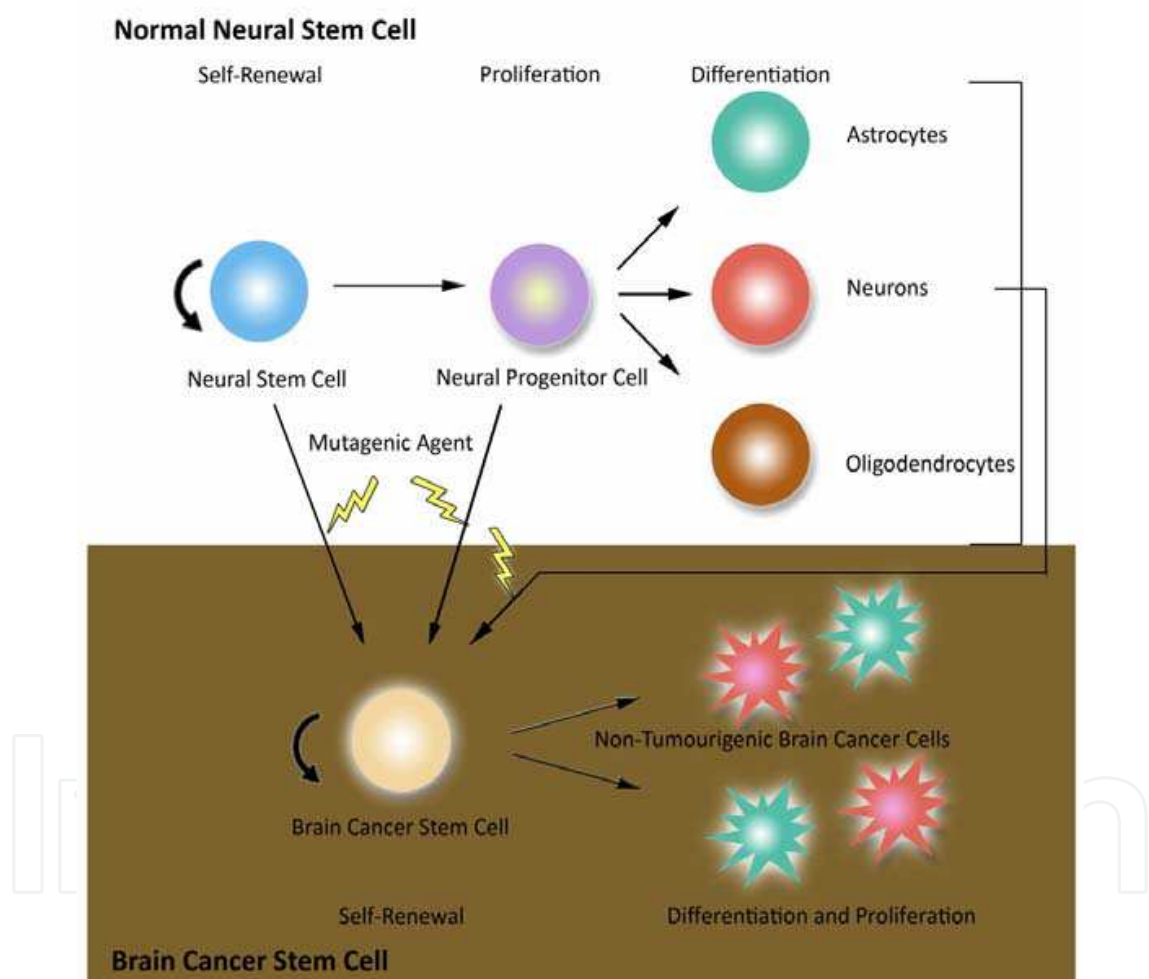

Fig. 1. Proposed mechanism of tumourigenesis through the formation of CSCs. Stem cells are able to self-renew and produce more differentiated progenies. Due to their long-lived, slow-dividing nature, they are exposed to damaging environmental factors over long periods of time, that may lead to the development of oncogenic mutations. The result of these mutations give rise to CSCs (also known as tumour-initiating cells). It has also been hypothesized that CSCs can be formed from the dedifferentiation of committed cells which also acquire oncogenic mutations. These CSCs uncontrollably produce large quantities of non-tumourigenic cells that result in a tumour mass

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3.2 Alternate view of the cancer stem cell hypothesis

Quintana et al. (2008) questioned the existence of CSCs within human melanomas. The authors argued that the original assessment of the scarcity of CSCs in human high-grade melanoma (0.001%), was an artefact of a xenotransplant tumour-initiation assay, caused by residual innate immunity of the recipient mouse. Dissociated melanoma cells were transplanted in non-obese diabetic/severe compromised immunodeficient (NOD/SCID) mice at dilutions of 10^2-10^7, and found that only four of the seven samples generated palpable tumours after 8 weeks. Performing a limiting dilution assay, only 1 in 10^6 cells were observed to be tumour-initiating cells. However, by increasing the observation time to 32 weeks, the CSC frequency increased to 1 in 10^5 cells (Quintana et al., 2008). Because NOD/SCID mice retain some innate immunity, Quintana et al. (2008), utilized a mouse model depleted of natural killer cells (NOD/SCID/*Il2rg-/-*). By utilizing this model along with Matrigel to provide the cells with a more supportive environment, it was found that 15-25% of the tumour cells exhibited CSC activity, thereby creating uncertainty on the existence of a cancer cell hierarchy within human high-grade melanomas (Quintana et al., 2008). The results of this study show that within human melanomas, CSCs might not be as scarce as once thought. However, these results cannot be directly correlated with other solid tumours in the body, since even in mouse models of leukemia there exists wide variations in the frequency of CSCs from higher than 1 in 10^6 cells (*c-myc and bcr/abl/CDKN2*) to 1 in 10^4 cells (*MOZ-TIF and Pten-/-*) (Dick, 2009). From the results presented by Quintana et al. (2008), inferences may be made concerning the population of cancer stem cells within solid tumours. First, there may be human cancers that have a high-frequency of cancer stem cells that do not follow the cell hierarchy model (Eaves, 2008). Second, the optimization of assays, specifically xenotransplant models devoid of immunity, to correctly identify tumour cells that possess any stem cell function need to be further investigated. Third, it is important to develop tumour endpoints to discriminate between CSCs (or progenitors) that have limited self-renewal capacity and CSCs that possess extensive capacity for self-renewal (Dick, 2009).

3.3 Brain cancer stem cells

Brain tumours are phenotypically and morphologically diverse. Additionally, the tumours mass is highly heterogeneous and the CSC hypothesis reasons that this observed heterogeneity is due to the existence of a hierarchical organization, in which CSCs are the parental cell for the other cells present in the tumour (Siebzehnrubl et al., 2011). Consequently, the development of an assay that identifies the cells responsible for maintaining tumour growth may lead to the development of novel effective therapies to treat brain tumours. Also, Singh et al. (2003) showed that human brain cancer cells expressing the surface marker CD133 are capable of tumourigenesis in NOD/SCID mice with as few as 100 cells, whereas the brain cancer cells lacking this expression appear to have no proliferative ability. In addition, they obtained human brain tumours and enzymatically dissociated them to generate a single cell suspension. The cells were inoculated into conditions shown to be conducive for the isolation of neural stem cells. In culture, a minority of the cells formed neurosphere-like clusters (termed tumourspheres), which showed immunoreactivity for Nestin (a marker of neural stem cells) and CD133 (a putative marker of neural stem cells) (Singh et al., 2003). A sphere formation assay was carried out to assess the frequency of the stem cell population which yielded a frequency between 0.3% to 25.1%. They demonstrated that the CD133+ population of cells differentiated in culture into tumour cells that phenotypically resembled the tumour from the patient

(Singh et al., 2003). However, not every CD133+ brain cancer cell was able to form spheres *in vitro*, indicating that the CD133+ brain cancer cells contain a subpopulation that are the 'true' brain cancer stem cells (Singh et al., 2003; Ward & Dirks, 2007).

In 2004, Galli and colleagues (Galli et al., 2004) isolated tumourigenic brain cancer cells from human GBMs that exhibited characteristics consistent with neural stem cells. The cells were found to be unipotent *in vivo* and multipotent *in vitro*, which may have resulted from the influence of the microenvironment on cellular signaling. Also, the cells were capable of generating tumours, through serial transplantations, that had similar histopathological features as the original tumour. As the cells were grown *in vitro*, the growth rate of the cells increased for the later passages, which could be attributed to an increase in the amount of proliferative cells. Interestingly, although all the cell lines studied were identified histopathologically as GBMs, they displayed varying growth kinetics and differentiation profiles. As stated previously, histopathological diagnosis of brain tumours is very subjective and may be unable to identify tumour variability between patients. This study suggests that the variability seen between GBM-cell lines might explain the differential response of tumour patients to treatment modalities (Galli et al., 2004).

Since the seminal paper by Singh et al. (2004), it has been shown by multiple groups, that CD133 is not a definitive CSC marker (Beier et al., 2007; Joo et al., 2008). Not only do CD133+ cells form tumours in an immunodeficient animal model, but also CD133- cells have been shown to form tumours. From this, many new markers for CSCs have been proposed such as stage-specific embryonic antigen 1 (SSEA-1), A2B5, SRY-like high mobility group 2 (Sox2), Sox4 and many others (Fang et al., 2011; Phi et al., 2008; Son et al., 2009; Tchoghandjian et al., 2010).

3.4 Therapeutic implications of the cancer stem cell hypothesis

Ideally, untransformed adult stem cells should remain functional throughout an individual's lifetime and retain the capacity to produce large quantities of undifferentiated progeny. In order to preserve this ability, stem cells have developed mechanisms to protect themselves from external factors that may potentially have an adverse effect on their function. Specifically, one such mechanism is the activation of ATP binding cassette (ABC) transporters, which are integral to DNA repair and inhibit apoptosis (Dean et al., 2005; S. Zhou et al., 2001).

It has been suggested that CSCs can develop from either stem cells or progenitor cells, or dedifferentiate from somatic differentiated cells, upon mutation of cancer-critical genes (B. B. Zhou et al., 2009). If CSCs are derived from stem/progenitor cells, it has been suggested that they would acquire the drug resistance mechanisms found in stem/progenitor cells. In GBMs this may explain the drug resistance observed for these tumours, as well as the high probability of recurrence. Four viewpoints have been developed on the implications of this drug resistance:

- CSCs, through their derivation from normal adult stem cells, acquire natural drug resistance thereby aiding their survival during chemotherapy, and preventing their full elimination from the body (Dean et al., 2005).
- Upon chemotherapy, some cancer cells acquire drug resistance that permits their survival. Following chemotherapy, the surviving cells pass on their drug resistance to their progeny, thereby rendering the original treatment of limited value (Dean et al., 2005).

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- Chemotherapy selects for drug-resistant CSC variants that then repopulate the tumour with progenies that are also drug-resistant after chemotherapy (Dean et al., 2005).
- CSCs and their resulting progenies are inherently drug-resistant and therefore therapies have little or no effect on the tumour (Dean et al., 2005).

Presently, it is not clear which viewpoint represents the correct tumour model. However with the discovery of CSCs and their role in the formation of tumour drug-resistance, it is clear that the development of effective cancer therapeutics must be focused on this cell population. The use of surgery, radiation therapy and chemotherapy are not only invasive and ineffective, but are also standardized for the histopathology of the tumour without regards to its molecular basis. Hence, a systems biology approach to analyze *in vitro* expanded CSCs within hGBMs can lead to the development of oncolytic drugs that specifically target the molecular pathways within the GBM-CSC population (Foltz et al., 2006; Foltz et al., 2009; Shah et al., 2011; Yan et al., 2011). In order to generate a clinically-relevant number of patient-specific GBM-CSCs, it is necessary to optimize the *in vitro* culture environment, such that the basic genetic nature of the GBM-CSCs does not significantly change.

4. Culture conditions

Initially, GBM-derived CSCs were isolated and expanded in neurosphere culture based on protocols developed for the isolation and expansion of murine and human neural precursor cells (NPCs). However, a recent publication evaluated the derivation and expansion of GBM-derived CSC-lines in adherent culture. In this section both neurosphere and adherent culture will be discussed and evaluated.

4.1 Neurosphere cultures

In a landmark paper, Reynolds and Weiss (Reynolds & Weiss, 1992) were the first researchers to isolate neural stem cells (herein referred to as neural precursor cells (NPCs)) from the adult mouse brain using the neurosphere formation assay. They generated a single cell suspension of cells from the striatial tissue (including the subventricular zone), and inoculated this cell suspension into culture dishes void of attachment substrates. In these conditions, free floating spheres (termed neurospheres) formed from NPCs present in the murine brain. This was confirmed by analyzing the cells for defining stem cell characteristics, namely (1) self-renewal, and (2) differentiation into astrocytes and neurons (unipotent). Their methods were adapted over the years by key research groups for the isolation and expansion of human NPCs and GBM-derived CSC lines (Baghbaderani et al., 2008; Baghbaderani et al., 2010; Galli et al., 2004; Kelly et al., 2009; Panchalingam et al., 2010; Singh et al., 2003; Svendsen et al., 1998; Vescovi et al., 1999).

Conventionally, GBM-derived CSC lines are derived from a single cell suspension of dissociated GBM-derived cells inoculated into suspension conditions (either ultra-low attachment vessels or uncoated tissue culture vessels), and allowed to expand as neurospheres in serum-free culture medium. GBM-derived neurospheres are heterogeneous by nature and contain both stem and progenitor cells. However, it has been shown that the genomic changes in GBM cells expanded as neurospheres resemble the same changes detected in the primary tumours of the corresponding patients (Ernst et al., 2009). Additionally, in contrast to adherent culture, cells in neurosphere cultures maintain these genomics over the culture period (De Witt Hamer et al., 2008).

4.2 Adherent cultures

Recently, Dirks and colleagues (2009) reported on work which involved modifying the original protocol by Reynolds and Weiss (1992), by adding laminin as an attachment factor and culturing the cells as a monolayer of adherent cells. The authors expanded GBM-derived CSC lines in adherent and neurosphere culture and showed the cells in neurospheres expressed higher levels of differentiation markers. Also, higher levels of cell death were found within the neurospheres (Pollard et al., 2009). Using copy-number gains on the chromosomes and differentiation propensity, Pollard et al. (2009) showed that differentiated adherent cells were similar to parental tumours and that the adherent cells exhibited genomic characteristics commonly associated with glioblastomas. However, as mentioned in the previous section, there have been many groups that have shown that cells expanded in neurosphere culture exhibit similar genomics as seen in the parental tumour. Pollard et al. (2009) also did not mention the size of spheres that were analyzed. It is critical to consider the size of spheres in culture since mass transfer limitations in large spheres can result in the formation of necrotic centers (due to an inadequate supply of oxygen and nutrients to cells in the center of the spheres). This could have biased their comparison of neurospheres and adherent cells [for a comprehensive review please refer to Reynolds and Vescovi (2009)]. Based on our extensive work with neurosphere culture, and the work that has been done by numerous other groups, we chose to isolate and expand our GBM-derived CSC lines as neurospheres.

5. Development of PPRF-h2 medium

PPRF-h2 medium was developed to support the long-term expansion of human NPCs (hNPCs) in culture while retaining the multipotency of the cells (Baghbaderani et al., 2010). This medium was developed from an existing medium utilized for the expansion of murine NPCs (mNPCs) called PPRF-m4 (Sen et al., 2001). Modifications to this pre-existing medium focused on the dose-dependent effect of basic fibroblast growth factor (bFGF) and human leukemia inhibitory factor (hLIF) on the expansion and differentiation of hNPCs in culture. Additionally, the effect of dehydroepiandrosterone (DHEA) on the proliferation and differentiation of hNPCs was investigated. The different medium modifications studied are summarized in Table 1.

Growth Medium	Base Medium	Growth supplements added to the base medium
Control	PPRF-m4	10 μg/L bFGF
PPRF-h1	PPRF-m4	20 μg/L bFGF + 10 μg/L hLIF
PPRF-h2	PPRF-m4	20 μg/L bFGF + 10 μg/L hLIF + 1.0 μM DHEA

Table 1. The formulations of three different growth media that were developed by adding growth supplements (i.e. bFGF, hLIF and DHEA) to an existing serum-free medium (PPRF-m4) (Baghbaderani et al., 2010).

Fetal cortical-derived hNPCs were expanded in static suspension culture in the three different culture modifications outlined in Table 1. Cells expanded in PPRF-h2 resulted in a higher viable cells density over five passages. The cumulative cell-fold expansion over five passages in PPRF-h2 medium was $2.17\pm0.5\times10^4$, which was significantly higher than cells expanded in PPRF-h1 ($0.59\pm0.1\times10^4$) or control medium ($0.07\pm0.03\times10^4$) [Figure 2A and 2B]

(Baghbaderani et al., 2010). Cells in PPRF-h2 and PPRF-h1 medium also formed larger aggregates (approximately 500 μm). The aggregates were morphologically different compared to aggregates expanded in PPRF-m4 medium [Figure 2C] (Baghbaderani et al., 2010) which adhered to the surface of the culture flask. This altered the phenotype of the cells as differentiation analysis showed a significantly higher number of neurons were derived from hNPCs expanded in PPRF-h2 medium (43.6±2%) compared to the control - PPRF-m4 medium (22.9±2%) (Baghbaderani et al., 2010).
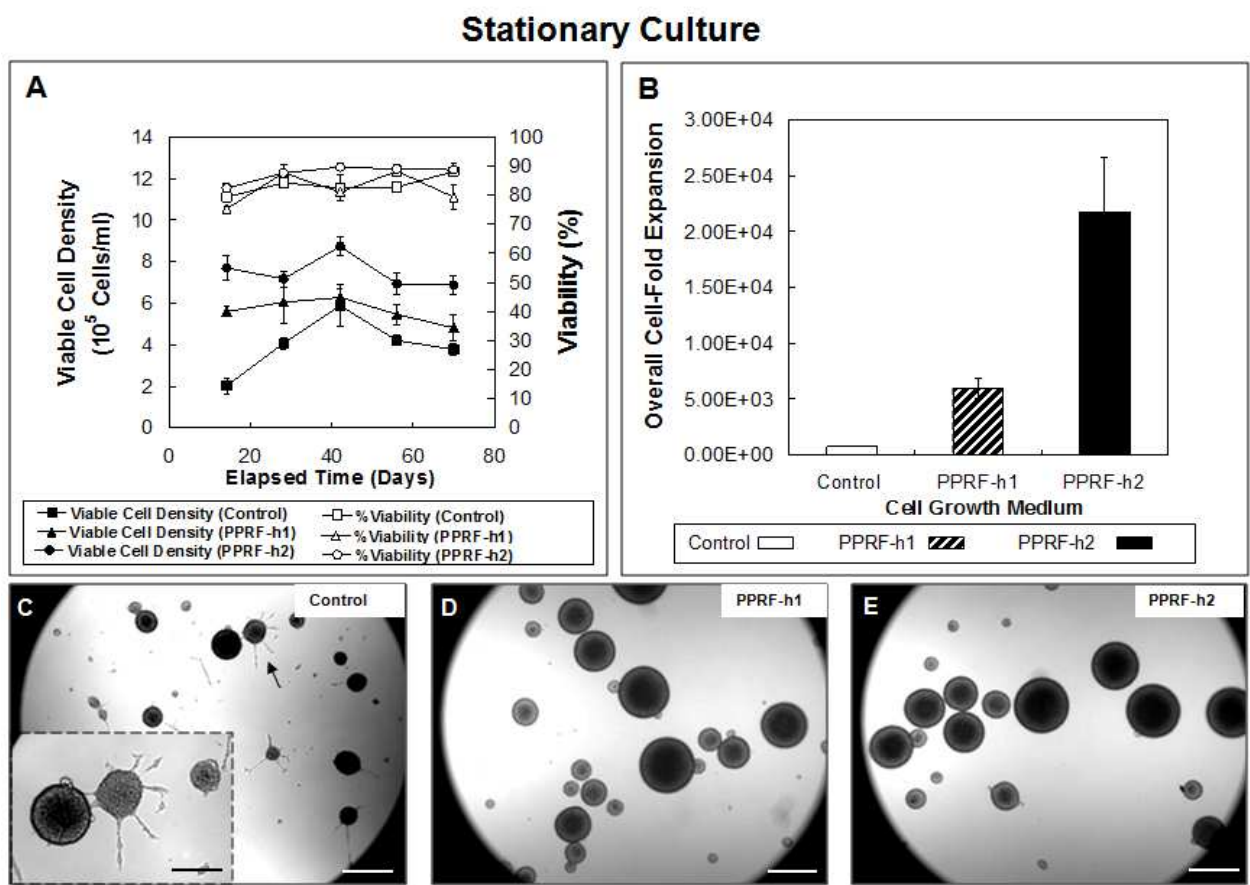

Fig. 2. Stationary culture of fetal cortex-derived hNPCs in three different growth medium: control medium, PPRF-h1 and PPRF-h2. Shown are (A) viable cell density and viability and, (B) overall cell-fold expansion of hNPCs from P10 to P14 over 70 days in the three different growth media. Also shown are photomicrographs of cells grown in (C) control medium, (D) PPRF-h1 medium and (E) PPRF-h2 medium at 14 days post-inoculation (P14). The inset shows a higher magnification of the aggregates (shown by arrow) adhered to the surface of the culture flask in the control medium. Scale bars represent 500 μm (scale bar in the inset is 250 μm) (Baghbaderani et al., 2010)

Since the presence of bFGF and hLIF in the growth medium exhibited a potent effect on the expansion and differentiation of hNPCs, the dose-dependent effect of bFGF and hLIF on the expansion of hNPCs was also investigated. Levels of bFGF were varied between 0-40 μg/L in PPRF-h2 medium. While there was a dose-dependent effect of bFGF on the proliferation of hNPCs (i.e. cell-fold expansion of hNPCs increased with increasing levels of bFGF), the addition of bFGF above 20 μg/L did not provide any significant beneficial effect on the

proliferation of hNPCs (Baghbaderani et al., 2010). Additionally, hLIF was varied between 0-20 µg/L. We observed that above 10 µg/L that there was no added beneficial effect on the growth of the hNPCs (Baghbaderani et al., 2010). Based on this study, a final concentration of bFGF and hLIF in PPRF-h2 medium was maintained at 20 µg/L and 10 µg/L, respectively (Baghbaderani et al., 2010).

6. Large-scale production of human glioblastoma-derived cancer stem cell tissue

The development of therapies to target CSCs is hindered by the scarcity of the CSCs within solid tumours. In brain cancers, the frequency of cells expressing CD133 varied with pathological subtype and grade from 1% to as high as 45% (Joo et al., 2008). As discussed previously, not every CD133+ cell is capable of forming a sphere *in vitro* (an assay for CSC identification), and therefore a subpopulation within the CD133+ exists that contains the scarce 'true' CSCs or TICs (Ward & Dirks, 2007). It is this lack of pure populations of CSCs for research that hinders the development of effective cancer therapies. In developing effective cancer therapeutics, it will be necessary to observe and quantify the interactions within brain tumours which require drug regimes and therapeutics to be individually tailored. This would require a large number of cells obtained from each individual, so that numerous treatment modalities could be tested on it. The availability of CSCs *in vivo* limits the type of analyses that can be performed, restricts the knowledge that can be gained about the basic cancer stem cell biology and hinders the development of effective, individualized cancer therapeutics. Thus it is necessary to develop a bioprocess that is capable of producing a reproducible, clinically-relevant number of cells.

Conventionally, the *in vitro* expansion of stem/progenitor cells has been performed in stationary tissue culture flasks. Although large cell numbers could be produced using this method of culture, it becomes increasingly laborious as the number of tissue culture flasks increases. Additionally, with handling a large number of tissue culture flasks, the probability of introducing cell culture variability between flasks becomes high, thereby reducing reproducibility between cultures. Such issues would be detrimental to the development of therapeutics since this culture variability would lead to increased phenotypic variability in cells expanded in different flasks, thereby impairing subsequent analysis to determine therapeutic targets. In order to generate large numbers of stem cells reproducibly, suspension bioreactors have been used for the expansion of a number of adult stem cell/progenitor lines. Figure 3 shows a comparison of the large-scale production of CSCs using tissue culture flasks and suspension bioreactors. A 1.0 L suspension bioreactor, for example, is equivalent to 200 T-25 tissue culture flasks using a 5.0 mL volume. The use of suspension bioreactors can generate a reproducible, large number of cells with well defined characteristics, while enabling control and maintenance of a homogenous environment.

Suspension bioreactors to expand a population of adult and embryonic precursor cells have been discussed in detail in literature. We have expanded human and murine neural precursor cells, murine mammary epithelial stem cells and breast cancer stem cells as aggregates in suspension culture (Baghbaderani et al., 2008; Baghbaderani et al., 2010; Youn et al., 2006; Youn et al., 2005). We have shown that protocols developed for these cell types can be adapted to other cell types, such as hGBM-derived CSC tissue. hNPCs bioreactor expansion protocols were adapted for the expansion of hGBM-derived CSC tissue (Panchalingam et al., 2010). Baghbaderani et al. (2008) found that inoculating hNPCs at

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1.0×10^5 cells/mL was conducive for cell growth. Operating in a semi-fed-batch mode resulted in a maximum cell density of 3.0×10^6 cells/mL over 18 days, corresponding to a 36-fold expansion (Baghbaderani et al., 2008). From these protocols, we investigated the effect of key culture parameters on the proliferation and phenotype of hGBM-derived CSC tissue expanded in suspension bioreactors. These key bioengineering parameters were: (1) liquid shear, (2) inoculation density, (3) feeding strategy, (4) medium composition and, (5) hypoxia.
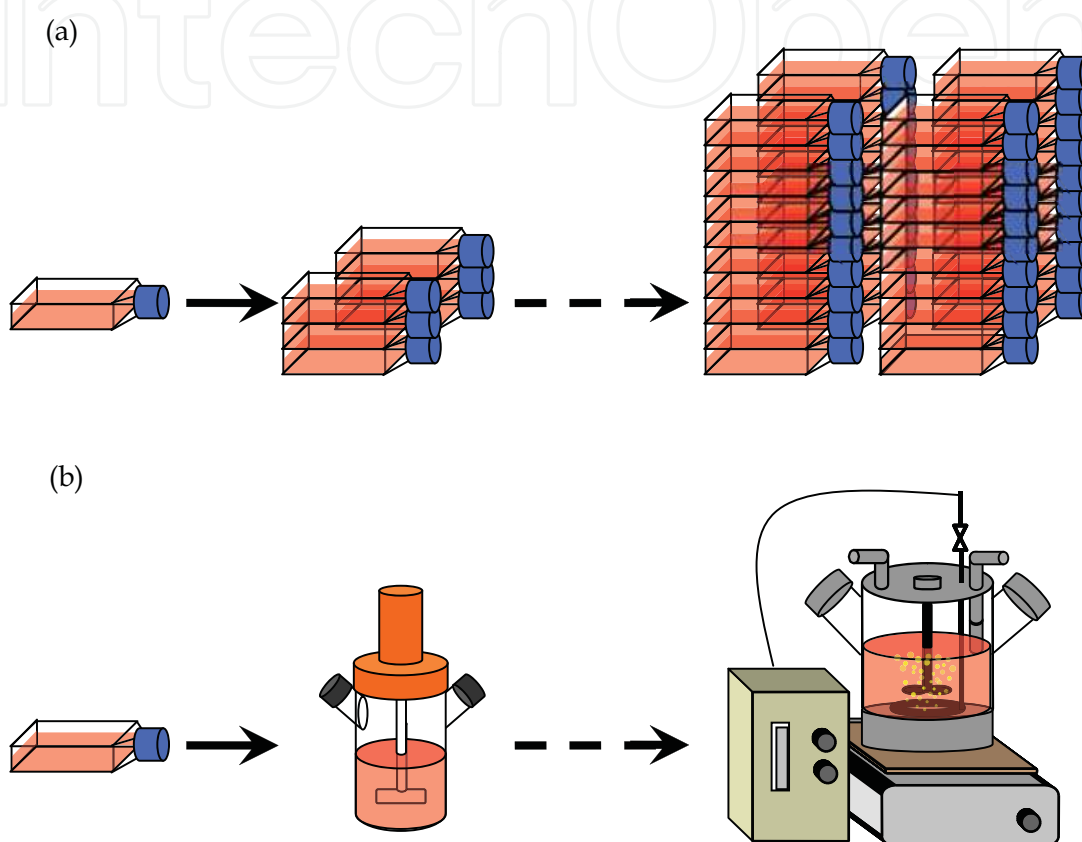

Fig. 3. Comparison of large-scale CSC cultures using tissue culture flasks and bioreactors. Two methods of scale-up to larger volumes are available: (a) large number of T-flasks, and (b) a single bioreactor. Bioreactors are a significantly better alternative for anchorage independent cells due to their reproducible, homogeneous, controllable environment

6.1 Bioengineering issues
6.1.1 Bioreactor liquid shear
Within suspension bioreactors, liquid shear control ensures adequate oxygen mass transfer and bulk fluid mixing. However, it is also important to make sure that hydrodynamic shear associated with the level of turbulence is within acceptable limits. The shear that is seen in stirred suspension bioreactors can have a negative impact on shear sensitive mammalian cells, but can also maintain aggregate size within acceptable limits (Kallos & Behie, 1999; Kallos et al., 1999; Sen et al., 2001). Also, the maximum shear stress occurs near the tip of the impeller, where turbulence is at its greatest (Cherry & Kwon, 1990). Briefly, Cherry and Kwon (1990) developed a relationship for maximum shear stress that is experienced by a cell on the surface of an aggregate. This relationship is represented by:

$$\tau_{\max} = 5.33\rho(\varepsilon\nu)^{1/2} \tag{1}$$

where $\tau_{\max}$ is the maximum shear stress (Pa), ρ is the medium density (kg/m³), and ν is the kinematic viscosity (m²/s). The power dissipation per unit mass, ε, can be calculated using the vessel working volume (V, m³) and density:

$$\varepsilon = \frac{P}{V\rho} \tag{2}$$

where P is the power input to the system (W), which is estimated by the Nagata (1975) correlation using the power number (N_p). Since the power number is correlated with Reynolds number (and therefore with agitation rate), shear stress can be controlled by adjusting the agitation rate of the culture vessel. For hNPCs, we found that a maximum shear stress between 0.35 to 0.80 Pa may sustain the expansion of these cells in small-scale suspension culture (Baghbaderani et al., 2008). Within our 125 mL suspension bioreactors this corresponds to an agitation speed of 70 - 130 rpm.

We inoculated hGBM-derived CSC tissue at 7.5x10⁴ cells/mL and operated at either low shear (60 rpm) or high shear (100 rpm) in 125 mL suspension bioreactors under batch conditions and grown as neurospheres. hGBM-derived CSC tissue inoculated in high-shear conditions reached a maximum cell density of 9.30x10⁵ cells/mL compared to cells inoculated in low-shear conditions which reached a maximum cell density of 6.15x10⁵ cells/mL (p-value = 0.046). This corresponded with a cell-fold expansion of 14.4 and 7.0 for the high- and low-shear condition, respectively. Moreover, we observed that the mean neurosphere diameter on day 22 was around 300 μm for the low-shear condition. Whereas, in the high-shear condition the mean neurosphere diameter did not exceed 200 μm. Over the entire course of the study, the average neurosphere size in the low-shear condition was higher than for the cells expanded in the high-shear condition. Additionally, the standard deviation of the diameters of neurospheres expanded in the high-shear condition was lower than the neurospheres expanded in the high-shear condition. This implies our high-shear expansion of hGBM-derived CSC tissue can lead to more homogenous culture morphology.

6.1.2 Inoculation density

Inoculation density is an important parameter in cell culture. Inoculating at too low a cell density may not be sufficient to support cell growth or cause a considerably prolonged lag phase. Such a low cell density will lead to a lack of cell-to-cell contact, which has been shown to be crucial to initiate cell expansion. Moreover, the release of autocrine factors released by the cells inoculated at a low density may not be sufficient for cell growth. In contrast, inoculating the cells at too high a cell density can result in (1) a lower cell-fold expansion and, (2) the need to sparge oxygen into the culture in order to maintain adequate oxygen transfer. To successfully grow hNPCs a sufficiently high inoculation density was required for their expansion (1.0x10⁵ cells/mL) (Baghbaderani et al., 2008). Additionally, preliminary experiments on the expansion of hGBM-derived CSC tissue in static suspension culture experiments found that inoculating hGBM-derived CSC tissue at 5.0x10⁴ cells/mL resulted in the highest cell-fold expansion (Baghbaderani et al., 2008). We further investigated the influence of inoculation density on the expansion of hGBM-derived CSC tissue in our 125 mL stirred suspension bioreactors. Cells were inoculated at a density of 5.0x10⁴ cells/mL and 1.0x10⁵ cells/mL. An inoculation density of 5.0x10⁴ cells/mL resulted

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in the highest cell fold expansion (19.9) with the cells attaining a maximum viable cell density of 1.12×10^6 cells/mL. Additionally, we observed that at the higher cell density, that the consumption of key nutrients, namely glutamine and glucose, was higher compared to the cells inoculated at the lower cell density. Additionally, the nutrient uptake of bioreactor-expanded hGBM-derived CSC tissue to hNPCs, was higher, even when comparing hNPCs inoculated at 1.0×10^5 cells/mL (compared to 5.0×10^4 cells/mL for hGBM-derived CSC tissue). The rapid loss of nutrients from the culture medium may be mitigated by using a semi-fed-batch approach.

6.1.3 Feeding strategy

As cells proliferate in culture, they metabolize key nutrients in the medium (i.e. glutamine and glucose) and produce toxic by-products (i.e. ammonium and lactate) (Butler, 2004). Within tumours, the rate of glycolysis and glutaminolysis are higher than their untransformed counterparts, and nutrient limitations have been shown to be an important factor on cancer tissue expansion (DeBerardinis et al., 2008; Elstrom et al., 2004; Youn et al., 2006). The 'Warbug' effect states that in tumour cells the increased rate of glycolysis is accompanied with an increase in the lactate production of the tumour cells (Kim & Dang, 2006; Vander Heiden et al., 2009). This contrasts with their untransformed counterparts which - (1) have comparatively low rates of glycolysis and, (2) metabolize glucose to pyruvate (part of aerobic respiration) within the mitochondria. This 'effect' is a metabolic hallmark of aggressive tumours, as the cells utilize anaerobic respiration in order to maintain their need for large amounts of energy. Additionally, within cell culture glutamine serves as not only an energy source for the cells, but also as a source of reduced nitrogen for maintenance of nucleotide biosynthesis and non-essential amino acids (Newsholme et al., 2003). From this information and our previously discussed studies, it was surmised that periodic medium replacement within our stirred suspension bioreactor hGBM-derived CSC tissue cultures may be beneficial. Baghbaderani et al. (2008) replaced 40% of the culture medium every 6 days for the expansion of hNPCs in suspension bioreactors. However, hGBM-derived CSC tissues utilized their nutrients at a higher rate than hNPCs and also produce the associated metabolic by-products at a higher rate. Therefore, in our work we examined the effect that a 40% medium replacement every 6 days as well as every 2 days had on the expansion and phenotype of bioreactor-expanded cells. Total medium replacement was not considered, since it has been suggested that cells in culture secrete endogenous factors that support cell proliferation (Zandstra & Nagy, 2001). It was found that hGBM-derived CSC tissue expanded in 125 mL suspension bioreactors in a 2-day fed-batch mode (40% medium replacement every 2 days) resulted in the highest expansion (90 cell-fold expansion) [Figure 4] (Panchalingam et al., 2010). Additionally, it was seen that as the cultures progressed, significantly larger aggregates were seen in the 2-day fed-batch conditions, resulting in average aggregate sizes around 600 μm compared with 350 and 450 μm in the batch and 6-day fed-batch conditions, respectively, on day 24. Baghbaderani et al. (2008) found that average aggregate diameters of < 600 μm would not compromise cell viability and would maintain the phenotypic characteristics of the hNPCs. Nutrient and metabolite analysis was performed for all the culture conditions, and it was observed that in the fed-batch conditions there was an increase in the specific consumption rate of glucose and glutamine. This resulted in an increase in the specific production rate of lactate and ammonium in the fed-batch culture conditions compared to the batch conditions. The level

of lactate and ammonium however did not rise above 2.0 g/L and 2.0 mM, which is generally considered detrimental for mammalian cell growth (Butler, 2004). The difference in nutrient uptake between batch and fed-batch conditions illustrates the activation of different metabolic pathways that may affect the phenotype bioreactor-expanded hGBM-derived CSC tissue.
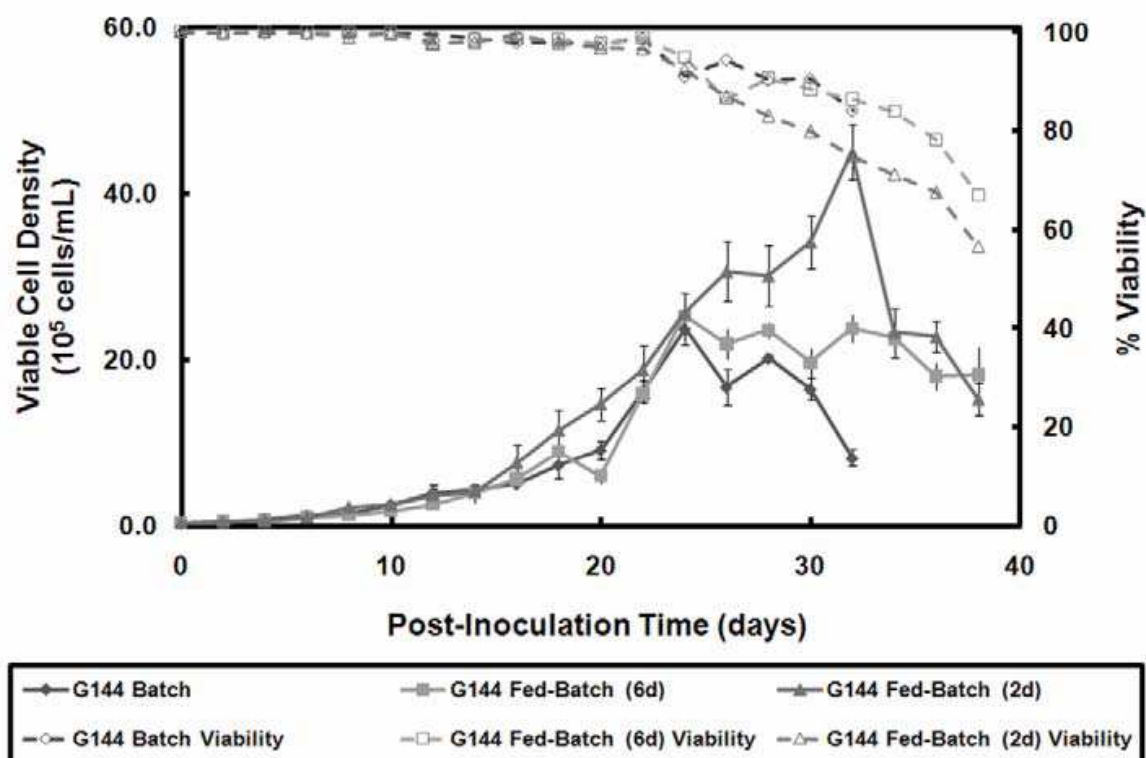

Fig. 4. Viable cell density and percent viability of hGBM-derived CSC tissue grown in batch, 6-day fed-batch, and 2-day fed-batch culture modes. All data points represent the average of duplicate bioreactors ± S.D. (Panchalingam et al., 2010)

Cell phenotype was assessed by (1) using flow cytometry to quantify the expression of the surface antigen CD133, (2) differentiation analysis to observe the propensity of cells to differentiate into the three neural lineages and, (3) microarray analysis to identify whether the bioreactor expansion process changed the basic nature of the hGBM-derived CSC tissue. The hGBM-derived CSC tissue in the fed-batch conditions maintained their CD133 expression greater than 90% for the entire course of the experiment. In comparison, cells expanded in the batch conditions had a CD133 expression of 95% on day 24 which decreased rapidly as cell viability decreased (Panchalingam et al., 2010). Immunocytochemical analysis of undifferentiated cells revealed that the hGBM-derived CSC tissue were positive for GFAP (GFAP+, a marker of astrocytes) and negative for Nestin (Nestin-, a marker of neural stem cells) (Panchalingam et al., 2010). It has been shown that GFAP+/Nestin- cells are a subset of cells derived from glial progenitor cells (Miyaguchi, 1997). Upon differentiation, the cells spontaneously differentiated into primarily GFAP+/β-tub+ (a marker of neurons) cells. Genomic analysis of the hGBM-derived bioreactor-expanded CSC tissue revealed that there were more genomic changes within the 2-day fed-batch condition compared to the batch condition. However, Gene Ontology (GO)

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enrichment analysis revealed that key genomic constructs were less affected than in the other culture conditions [Figure 5]. These findings show that the bioreactor-expansion of our hGBM-derived CSC tissue using a 2-day fed-batch methodology results in cells maintaining their phenotypic and genomic characteristics.
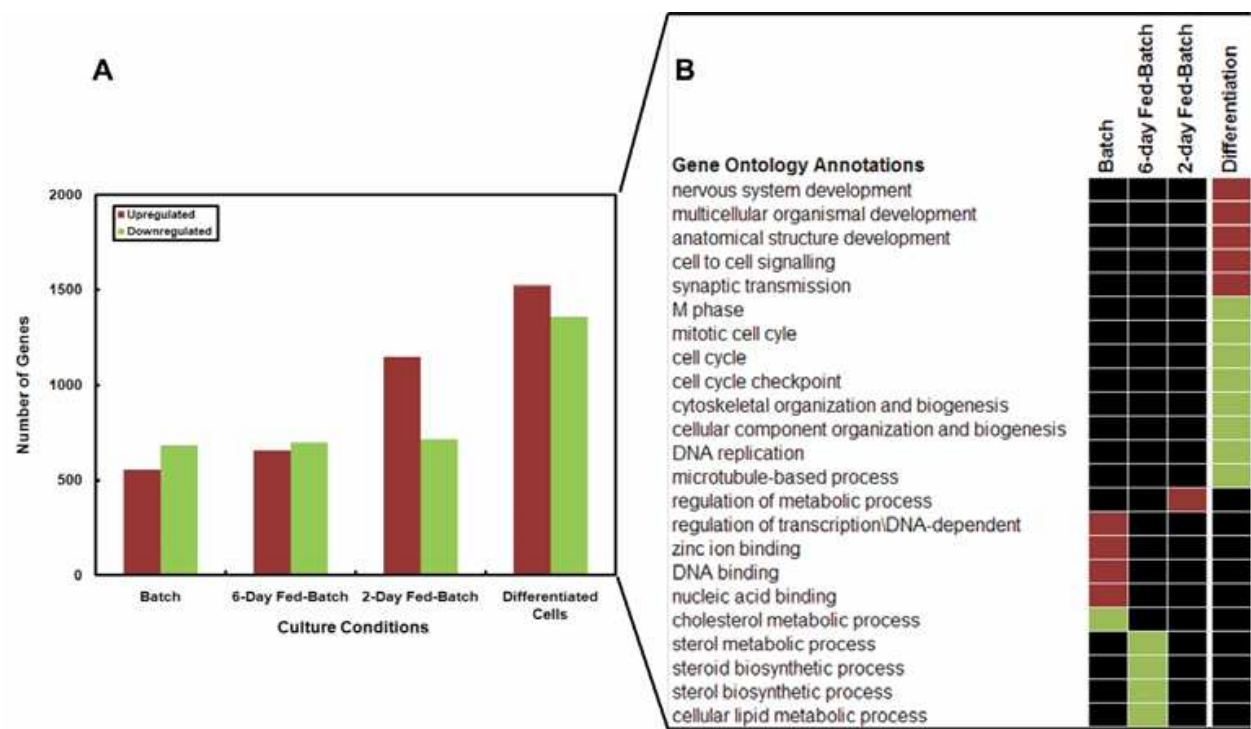

Fig. 5. Differentially-expressed genes of bioreactor-expanded GBM-derived CSC tissue grown in batch, 6-day-fed-batch and 2-day fed-batch culture modes. Shown are (A) the number of genes that showed a fold changed greater than 2 with a p-value < 0.007 and, (B) gene ontology (GO) annotations for the differentially-expressed genes with a p-value < 0.007 for the three different culture conditions on day 24 compared to day 0. Red represents above-average expression level, green represents below-average expression level, and black represents no change in the gene expression with cells before inoculation into the bioreactors (Panchalingam et al., 2010)

6.1.3 Culture medium
Within our laboratory, the influence of different culture components on the growth kinetics and phenotype of different stem/progenitor lines has been evaluated (Baghbaderani et al., 2010; Jung et al., 2010). As was mentioned in previous sections, studies were done to elucidate the influence of bFGF, hLIF and DHEA on the proliferation and phenotype of hNPCs (Baghbaderani et al., 2010). We observed that these chemical mitogens had a significant effect on the growth kinetics and phenotype of hNPCs. Moreover, we evaluated the effect our growth medium, PPRF-h2, had on the growth kinetics of hGBM-derived CSC tissue in suspension culture compared to another growth medium used extensively by other research groups, Neurocult NSA (STEMCELL Technologies, Vancouver, Canada). Two glioma cell lines (SN184-grade II glioma-derived and SN143-hGBM-derived) were inoculated at 5.0×10^4 cells/mL into 125 mL stirred suspension bioreactors in either PPRF-h2 or NSA medium. Here, the SN184 and SN143 cells in PPRF-h2 medium reached a maximum

viable cell density of 2.7×10^6 cells/mL and 3.4×10^6 cells/mL (80.6 and 73.2 cell-fold expansion), respectively. In NSA medium, the SN143 and SN184 cells reached a maximum viable cell density of 2.2×10^6 cells/mL and 3.1×10^6 cells/mL (data not shown). Also, we saw that the cells in both media had similar aggregate size distributions. Although the growth kinetics of the cells were similar in both media, it will be important to evaluate the genomics of the cells as they may have been altered through the culture period. This is currently being evaluated within our laboratory.

6.1.4 Hypoxia

The expansion of hNPC and GBM-derived CSC tissue *in vitro* has often been done at atmospheric oxygen levels of 21% (Baghbaderani et al., 2010; Panchalingam et al., 2010). However, the physiological levels of O_2 in the brain vary between 2.5% to 12.5%, and in solid tumours it has been reported that the oxygen tension can be below 0.1% in necrotic regions (Bar, 2011; Panchision, 2009). hGBM tumours were shown to experience mild to moderate/severe hypoxia, with oxygen concentrations between 0.5% to 2.5% for mild hypoxia and 0.5% to 0.1% for moderate/severe hypoxia (Bar, 2011). This agrees with the observations by many researchers who note that hypoxia tends to correlate with increased tumour aggressiveness (Evans et al., 2004; Helczynska et al., 2003; Jogi et al., 2002; Kunz & Ibrahim, 2003). This may be due to the different genes that are upregulated in hypoxic conditions, which are primarily controlled by hypoxia-inducible factors 1α, 2α, and 3α (HIF-1-3α) (Bar, 2011). HIF-1α has been shown to repress p53, which is a major effector of mitotic arrest and apoptosis and can also block normal CNS precursor differentiation (Gustafsson et al., 2005; Hammond & Giaccia, 2005; Panchision, 2009). Also, it has been shown that HIF-1α and HIF-2α can result in an increase in the expression of CD133 (Z. Li et al., 2009; Soeda et al., 2009). Expanding hGBM-derived CSC tissue *in vitro* under physiological oxygen concentrations (1-7%), results in the tumour stem cell signature genes being overexpressed and also enhances the stem-cell like phenotype of the expanded GBM cells (McCord et al., 2009; Seidel et al., 2010). Additionally, Pistollato et al. (2009), cultured Grade III and IV (GBM)-derived precursor cells (referred to as high-grade gliomas - HGG) which proliferated to a higher extent in a hypoxic environment (Pistollato et al., 2009). In their study, they compared the expansion of these HGG cells at 2%, 5% and 20% O_2 tension and found that HGG cell expansion was significantly higher at 2% and 5% O_2 tension than at 20% O_2 tension (Pistollato et al., 2009). At these lower oxygen tensions, there was also a selective suppression of mitotic arrest and apoptosis. Also, at these low oxygen tensions, HIF1α expression repressed BMP signalling (BMP under high oxygen tension promotes differentiation) thereby conferring resistance to differentiation and death (Pistollato et al., 2009). In using *in vitro* expanded GBM-CSCs for the development of novel oncolytic therapeutics it will be necessary to be able to control the tumour microenvironment *in vitro* within set limits. The use of a large-scale bioprocess with control capabilities, which is also able to effectively control oxygen levels in vitro, will aid in generating clinically-relevant numbers of GBM-CSCs.

6.2 Large-scale production of hGBM-derived CSC tissue in computer-controlled suspension bioreactors

6.2.1 Scale-up using suspension bioreactors

In order to generate a large-number of clinically-relevant CSCs, it is necessary to scale-up our current process to larger volume bioreactors. Large volume bioreactors have been

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developed and used for the last 50 years for the culture of different organisms such as bacteria, yeast and mammalian cells for pharmaceutical applications (Eibl, 2009). These applications include protein production [i.e. production of tissue plasminogen activator (tPA) and recombinant erythropoietin (rEPO)] and viral vaccine production (i.e. Hepatitis A vaccine) from recombinant cells (Demain & Vaishnav, 2009). However, in comparison to the more robust cells currently used in industry, stem cells are fragile and are metabolically slower. Therefore, in order to scale-up production of stem/progenitor cells (or in our case CSC tissue), it is necessary to consider key variables which may have a more significant affect when moving from smaller volumes to larger volumes. Two of these key variables are (1) oxygen supply, and (2) hydrodynamic shear in the liquid medium.

The specific oxygen consumption rates of mammalian cells have been reported to be between 1.7×10^{-17} and 17×10^{-17} mol O_2/cell•s (Butler, 2004). In our laboratory, we have found that single cell suspensions of hNPCs and GBM-derived CSC tissue have specific oxygen consumption rates are 5.87×10^{-17} and 2.99×10^{-17} mol O_2/cell•s respectively (Baghbaderani et al., 2008). Typically for small bioreactors (less than 1.0 L) the oxygen demands of cells can be satisfied by gas diffusion from the headspace through the culture surface. However, this may not be adequate in larger bioreactors. As the working volume of the bioreactor increases, the surface area-to-volume (SAV) ratio (also referred to as the aspect ratio) decreases. If we were to consider that we have two bioreactors, one 125 mL and one 500 mL, the SAV ratio would decrease from 33.0 m^{-1} to 15.6 m^{-1}. Therefore, as we scale-up to larger volume bioreactors, the area available for oxygen transfer through the interfacial surface of the medium within the bioreactor, decreases. There are three parameters which could be modified in order to maintain adequate oxygen supply in order to meet the oxygen demands of the expanding cells (1) sparging oxygen into the culture, (2) increasing the agitation rate of the impeller, or (3) increasing the oxygen concentration in the headspace. Spargers have been implemented in bacterial fermentation, where high cell densities require high oxygen supply. However, since sparging is associated with the production of gas bubbles that can burst at the surface of the culture, it can cause cell damage which is not recommended for the cultivation of stem cells (Shuler & Kargi, 2002). Increasing the agitation rate is another option, since this would increase the dissolution of oxygen into the medium. However, as the agitation rate increases, the hydrodynamic shear increases which could be detrimental if it causes excessive cell damage. Increasing the oxygen concentration in the headspace is also a viable option for small bioreactors, however, this may not be sufficient to maintain adequate oxygen supply as the vessel volume increases. Thus far, we have evaluated the use of 500 mL bioreactors for the expansion of GBM-derived CSC tissue. From our work we have found that increasing the oxygen level in the headspace has been sufficient to maintain adequate oxygen supply within our bioreactor cultures.

Hydrodynamic shear is an important characteristic in the culture of cells. The Kolmogoroff theory of turbulent eddies (Cherry & Kwon, 1990) and the Nagata correlation (Nagata, 1975) can be used to determine the agitation rate to scale-up the cultures.

6.2.2 Process control

In order to have a reproducible supply of CSCs in a bioreactor culture process, it is necessary to control key culture process parameters within a strict limit. These parameters are typically temperature, dissolved oxygen concentration and pH. In order to control these

process variables (PV) we use standard process control techniques. Typically, the PV is measured by a sensor or probe that is connected to a computer control system (controller) [Figure 6]. The controller receives the measurement from the probe, called the control variable (CV), and compares it with a predetermined set point (SP) value (this difference is called the error or offset (ϵ)). If there is no difference between the CV and SP (ϵ=0), then the controller does not take any action. However, if there is a difference between the CV and the SP (ϵ≠0), then the controller initiates a corrective feedback in order to shift the process to the SP. The controller output is applied through a final control element (FCE) which manipulates the input values into the bioreactor in order to apply the corrective feedback necessitated by the controller. The manipulated input value is called the manipulated variable (MV). The method by which a controller determines the corrective feedback necessary is through the combination of proportional, integral and derivative control.

Proportional control (P-only) is the simplest control strategy. The controller output to the FCE is proportional to the error (ϵ) and is given by the equation:

$$MV = K_c \epsilon + bias \tag{3}$$

where K_c is the controller gain which is the ratio of the change in the controller output to the change in the error. The bias term is the output of the controller when the error is zero. There are a few shortcomings of using just a proportional control including its relatively slow response to a disturbance in a system, and often the system does not reach the predetermined SP.

Integral controllers (I-only) are used to minimize the error by continually changing the FCE. The governing equation for this controller is:

$$MV = \frac{1}{T_i} \int \epsilon dt + bias \tag{4}$$

where T_i is the integral time constant. However, the integral controller by itself has a response period that is grossly larger than the response period of the proportional controller. Typically, integral control is combined with proportional control (PI) which has a response period that is 1.5 times the response period of the P-only controller. The equation for a PI controller is:

$$MV = K_c \left(\epsilon + \frac{1}{T_i} \int \epsilon dt \right) \tag{5}$$

A PI controller provides better dynamic control than either a P-only or I-only controller. One disadvantage of a PI controller is that a sustaining error could cause the integral term to increase and saturate the controller output (known as reset windup) (Seborg, 2011).

Derivative (D-only) controllers anticipate the behaviour of a process by calculating the rate of the change of the error. The equation for derivative action is:

$$MV = T_d \frac{d\epsilon}{dt} \tag{6}$$

Where Td is the derivative time constant. Using derivative time decreases lags in the process response. In combination with the PI controller we obtain a PID controller which would have a response similar to P-only control. The overall process control equation is:

$$MV = K_c \left(\epsilon + \frac{1}{T_i} \int \epsilon dt + T_d \frac{d\epsilon}{dt} \right) \tag{7}$$

The growth and proliferation of hGBM-derived CSC tissue was therefore investigated in computer-controlled suspension bioreactors, using a PID controller.
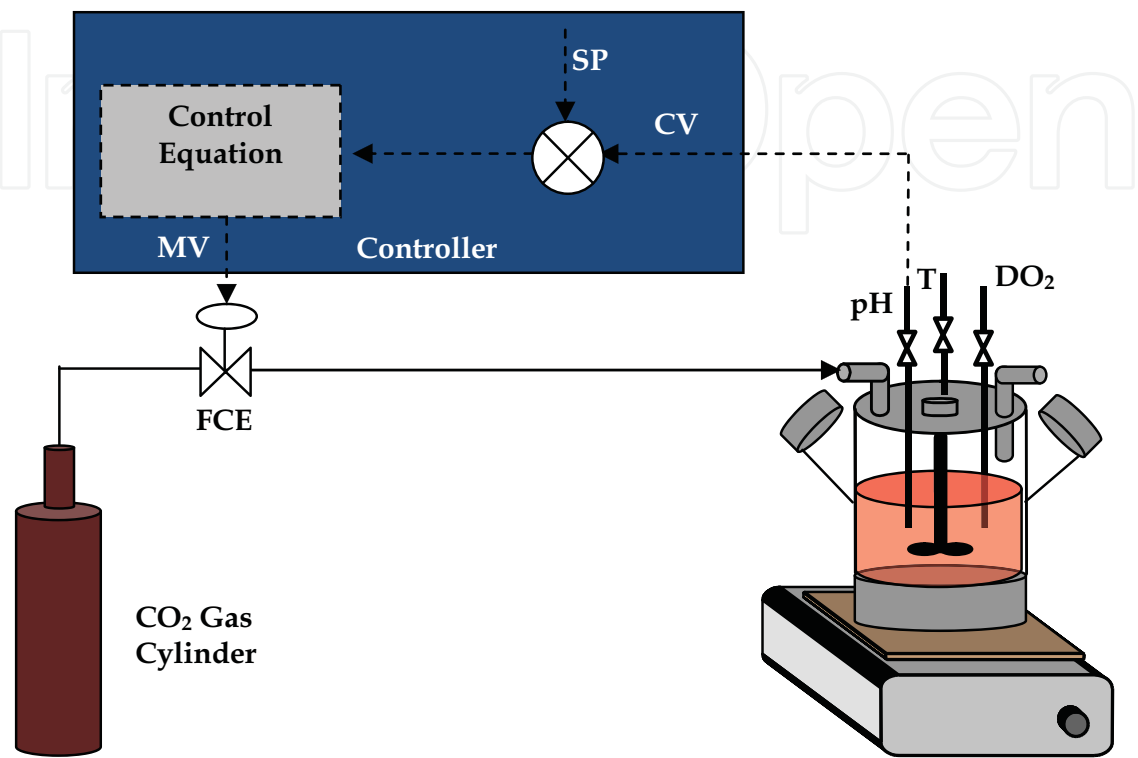

Fig. 6. A schematic diagram of a process control system for controlling pH, temperature (T) and dissolved oxygen (DO_2) within a suspension bioreactor system. Using pH control as an example: the pH probe measures the pH and transmits a signal carrying that pH value (CV) to the controller where it is compared with a pre-determined SP value. The deviation between the CV and SP is sent to the control equation, where if there is a deviation present, a corrective feedback is provided to the FCE to shift the culture PV to the SP. For example, if the controller recognizes that there is a pH increase in the medium, it will output a corrective feedback (MV) to the FCE, which in this case increases the CO_2 flow to the headspace, which in turn decreases the pH of the culture

6.2.3 Proliferation of hGBM-derived CSC tissue in computer-controlled suspension bioreactors

We inoculated two 500 mL computer-controlled suspension bioreactors at a density of 5.0×10^4 cells/mL in PPRF-h2 medium operating in batch mode. Using the Kolmogoroff theory of turbulent eddies (Cherry & Kwon, 1990) and the Nagata correlation (Nagata, 1975) we determined that an agitation rate of 85 rpm would provide the same maximum shear as experienced by a 125 mL suspension bioreactor operating at 100 rpm. Our computer-controlled suspension bioreactor setup is illustrated in Figure 7. We controlled the dissolved oxygen concentration at 70% air saturation (15% atmospheric oxygen concentration), the pH at 7.4 and the temperature of the vessel at 37°C. Throughout the culture period we observed that the growth kinetics of hGBM-derived CSC tissue grown in the computer-controlled

suspension bioreactors were similar to hGBM-derived CSC tissue expanded in 125 mL suspension bioreactors operated in an incubator maintained at 5% CO_2 and 37°C. After 450 hours in culture, the hGBM-derived CSC tissue attained a maximum viable cell density of 1.12×10^6 and 1.26×10^6 cells/mL in the 125 mL and 500 mL bioreactors, respectively. Neurospheres generated in the 500 mL bioreactors had similar morphologies with the neurospheres expanded in the 125 mL bioreactors. However, we saw that the neurosphere size distribution in the 500 mL bioreactors had an observable shift towards larger neurosphere diameters and hypothesized that scaling-up our bioreactor process using maximum shear, would allow us to generate similar sized neurospheres in the 500 mL and 125 mL bioreactors. However, our observations here suggest that maximum shear may not be the only factor impacting neurosphere size within our bioreactors. Additionally, we found that oxygen supply through the headspace was sufficient in order to meet the oxygen demands of the cells. Further analysis of their differentiation characteristics and CD133 expression, showed that these cells retained similar phenotypic characteristics as neurospheres expanded in the 125 mL suspension bioreactors. The conclusion of this study was that a computer-controlled suspension bioreactor process would be amenable for the large-scale expansion of clinically-relevant numbers of GBM-derived CSC tissue. Through the use of a computer-controlled suspension bioreactor process, we can control key bioprocess parameters in order to generate high numbers of GBM-CSCs for the development of novel oncolytic therapeutics.
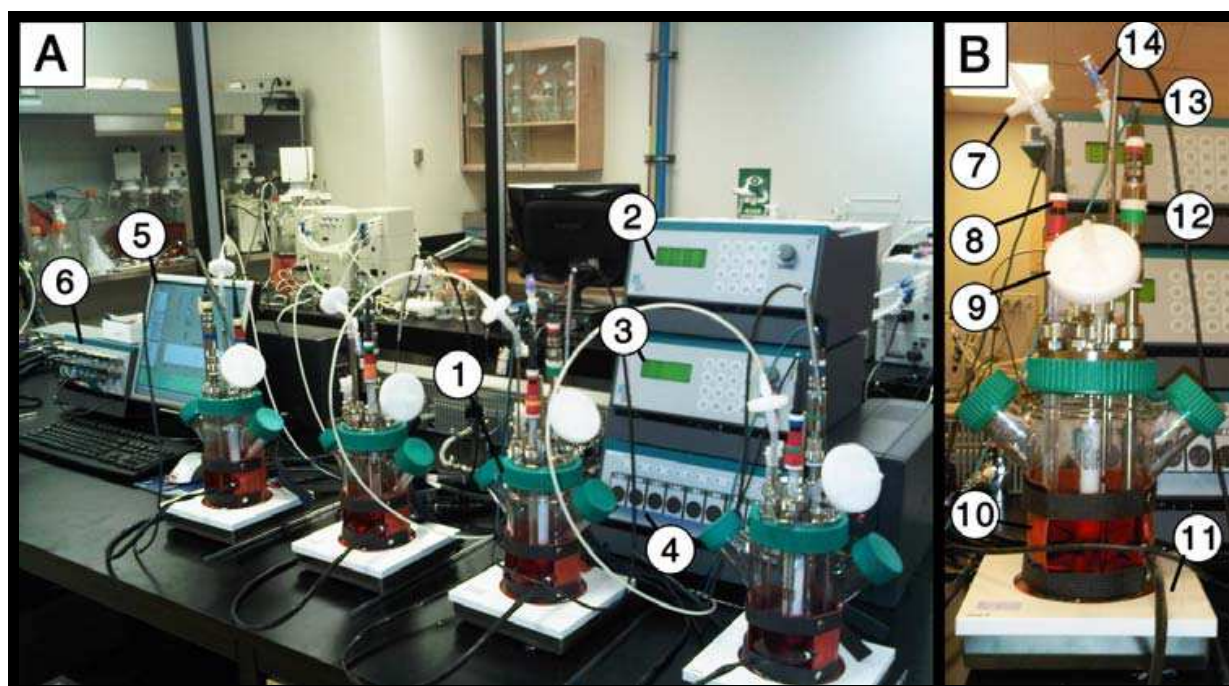

Fig. 7. Photographs of 500 mL DASGIP computer-controlled suspension bioreactors. (A) Experimental setup for the 500 mL suspension bioreactor system showing: (1) 500 mL suspension bioreactor, (2) temperature and agitation module TC4SC4, (3) gas mixing station MX 4/4, (4) multipump modules MP8, (5) DASGIP control and data acquisition computer, and (6) DASGIP sensor module PH8PO8. (B) A closer view of the 500 mL DASGIP suspension bioreactor, showing: (7) gas inlet to the bioreactor connected to O_2, N_2, CO_2 and air gas cylinders via gas mixing station MX 4/4, (8) pH probe, (9) gas outlet from bioreactor, (10) heating jacket, (11) stirring plate, (12) dissolved O_2 probe, (13) temperature probe and, (14) bioreactor sampling port

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7. Conclusion and perspective

The highly aggressive brain cancer, hGBM, is thought to arise from a sparse population of cells within these tumours, with stem cell-like characteristics, called CSCs. Hence, the development of effective cancer therapies may rely on developing methods that specifically target these cells. However, the scarcity of CSCs *in vivo* represents a major impediment to such research, as there is an insufficient supply for basic biochemical and genetic analyses. It is therefore necessary to develop a bioprocess to expand CSCs *in vitro* in a controlled environment. Our objective was to produce a large amount of hGBM-derived brain CSC tissue in computer-controlled suspension bioreactors in order to facilitate the development of novel oncolytic therapeutics. In this chapter we focused on two areas of our research: - (1) the development of a culture medium to successfully grow the GBM-derived CSC tissue and, (2) the development of bioprocess conditions to grow GBM-derived CSC tissue in computer-controlled suspension culture. We employed successfully a serum-free medium (PPRF-h2), developed in our laboratory (Baghbaderani et al., 2010) for the expansion of human neural precursor cells to expand GBM-derived CSC tissue. Using this medium we were able to isolate and support the expansion of GBM-derived CSC tissue from primary tumours (Panchalingam et al., 2010). We also investigated a number of key bioengineering parameters on the expansion of GBM-derived CSC tissue within well-mixed suspension bioreactors. Through the refinement of these bioprocess parameters, we successfully expanded GBM-derived CSC tissue to large cell numbers in suspension bioreactors, while showing that this expanded GBM-derived tissue retained similar genomic characteristics to the initial cell population (Panchalingam et al., 2010). The bioreactor protocols we developed were applied to the expansion of hGBM-derived CSC tissue in computer-controlled suspension bioreactors, which we have shown are able to control our process within very strict limits. The use of a computer-controlled bioreactor process to expand hGBM-derived CSC tissue can play an important role in the development of novel drug treatments by ensuring a continuous homogenous supply of tissue for genomic analysis and high-throughput drug screening.

8. Acknowledgements

9. References

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Cancer Stem Cells - The Cutting Edge

Edited by Prof. Stanley Shostak

Over the last thirty years, the foremost inspiration for research on metastasis, cancer recurrence, and increased resistance to chemo- and radiotherapy has been the notion of cancer stem cells.The twenty-eight chapters assembled in Cancer Stem Cells - The Cutting Edge summarize the work of cancer researchers and oncologists at leading universities and hospitals around the world on every aspect of cancer stem cells, from theory and models to specific applications (glioma), from laboratory research on signal pathways to clinical trials of bio-therapies using a host of devices, from solutions to laboratory problems to speculation on cancersâ€™ stem cellsâ€™ evolution. Cancer stem cells may or may not be a subset of slowly dividing cancer cells that both disseminate cancers and defy oncotoxic drugs and radiation directed at rapidly dividing bulk cancer cells, but research on cancer stem cells has paid dividends for cancer prevention, detection, targeted treatment, and improved prognosis.

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