THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Characterisation of glioblastoma subtypes: implications for therapy resistance

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BSc (Hons)

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2016

University of Queensland Diamantina Institute

Abstract

Glioblastoma is the most common malignant brain tumour in adults. Categorised by The World Health Organization as a grade IV glioma, it is the most aggressive, invasive form of glioma with a median survival time of 14.6 months. The incidence in the United States is approximately is two to three per 100,000. Glioblastoma is about 40% more common in males than in females and the average age at diagnosis is 64 years. Despite aggressive medical intervention including surgical resection followed by radiotherapy and chemotherapy, treatment increases survival by a median of only 2.5 months. Tumour recurrence occurs in the brain, frequently adjacent to the site of the original tumour. New therapies are needed to combat metastatic disease and tumour regeneration after therapy.

Therapeutic resistance is the major cause of death in patients diagnosed with malignant tumours. Tumour heterogeneity has emerged as a major player in therapeutic resistance. In addition to this, treatments frequently contribute to the evolution of therapy resistance by introducing mutations, which provide the cell with mechanisms to survive further rounds of treatment. The main aim of this thesis was to identify potential targets within glioblastoma that could be exploited to reduce intratumoural heterogeneity and prolong progression free survival.

We used three independent processes to interrogate phenotypic and genetic heterogeneity within populations of primary human glioblastoma cell isolates. In this first experimental chapter, we have investigated whether the cancer stem cell hypothesis may explain the heterogeneity observed within our isolates of human glioblastoma. Cancer stem cells have been a major focus of research since they were implicated in tumour initiation in acute myeloid leukaemia in the 1990s. We tested the cancer stem cell hypothesis using glioblastoma cells. We found that glioblastoma contained “slow” and “fast” cycling populations of malignant cells. Within the slow cycling stem-like population of glioblastoma cells exist a population of cells with superior tumour forming ability. Some of the content of this chapter has been published (Brain, 2011. 134, 1331-1343).

The second chapter explored chromosomal heterogeneity using fluorescence activated cell sorting (FACS) and Flow cytometry-based assays to demonstrate that the “ploidy” of the malignant cells within primary glioblastomas was highly varied and was associated with highly discordant behaviour with respect to *in vitro* growth and proliferation as well as

drug resistance. We also showed that hyperdiploid glioblastoma cells were capable of tumour formation, more metabolically active and sensitive to glycolysis than their euploid counterparts. Some of the content of this chapter has been published (Molecular Biosystems, 2014. 10, 741-758)

In the final experimental chapter, we used another platform to interrogate the heterogeneity of primary glioblastoma cells. Cell surface proteins have been identified as prognostic markers and predictive of response to therapy in a range of human tumours. We analysed the expression of 118 cell surface proteins on primary human glioblastoma cells and identified a small cohort of proteins, which in concert provide a molecular signature of GBM. This data highlights some potential targets for use in the design of anti-tumour specific therapeutics.

GBM is an incurable heterogeneous tumour with a poor response to treatment. The work presented within my thesis identifies a number of potential therapeutic targets that may be used to inform novel treatment strategies and targets. In particular, I provide new data that may be used to reduce the impact of the highly heterogeneous polyploid populations of cells that arise in tumour evolution. Moreover, the targeting of tumour heterogeneity may be used to design more effective therapies to reduce side effects, avoid the induction of further mutational events caused by current therapies and hopefully extend the progression free survival time of patients by slowing tumour growth.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

1. Deleyrolle, L.P. Harding, A. **Cato, K**. Siebzehnrubl, F. A. Rahman, M. Azari, H. Olson, S. Gabrielli, B. Osborne, G. Vescovi, A. Reynolds, B. A. Evidence for label-retaining tumour-initiating cells in human glioblastoma. Brain, 2011. 134, 1331-1343.

2. Donovan, P. **Cato, K**. Legaie, R. Jayalath, R. Olsson, G. Hall, B. Olson, S. Boros, S. Reynolds, B. Harding, A. Hyperdiploid tumour cells increase phenotypic heterogeneity within glioblastoma tumours. Molecular Biosystems. 2014. 10, 741-758.

Publications included in this thesis

No publications included.

Contributions by others to the thesis

This thesis is the product of work performed primarily by myself with contributions from contributors to jointly authored works listed above and collaborators and others listed below. Dr Brent Reynolds and Dr Angus Harding initiated all intellectual reasoning underlying this work. Dr Angus Harding and Associate Professor Nicholas Saunders assisted with the editing of this thesis. Dr Loic Deleyrolle, McKnight Brain Institute, University of Florida performed the imaging of gliomaspheres and animal experiments contained in chapter three. Dr Prudence Donovan, University of Queensland Diamantina Institute, performed the DAPI staining and flow cytometry analysis on passaged cell lines contained in chapter four. Beatrice Large, University of Queensland Diamantina Institute, performed the long term passaging assays contained in chapter four. All other experimental work described in this thesis was performed solely by the candidate. The thesis was written solely by the candidate.

Statement of parts of the thesis submitted to qualify for the award of another degree

None.

Acknowledgements

First, I would like to express my gratitude to Dr Angus Harding for the manner in which you supervised my work as student in your laboratory. You gave your time unreservedly to teach me to be rigorous in experimental design, perform techniques meticulously, and critically evaluate my results. In addition to all this, your energetic and zealous disposition ensured that no day was ever boring.

I am indebted to Associate Professor Nicholas Saunders for agreeing to supervise the completion of my thesis during my final year. Thank you for fitting another student into an already busy schedule. I very much appreciate your time and willingness to assist me. You made what could have been a very difficult time smooth sailing.

I would like to acknowledge the Cancer Council Queensland for the scholarship I received that enabled me to undertake fulltime postgraduate studies. I am also grateful to The Queensland Brain Institute and the University of Queensland Diamantina Institute for providing the facility, resources, and support to staff and students that made my research possible.

I would like to thank our collaborators from the Department of Neurosurgery at the Princess Alexandra Hospital, Brisbane and Dr Sarah Olson and Dr Gemma Olsson in particular, for your strong commitment to supporting research into brain cancer and taking the time to recruit donors of tumour tissue for research. Your support provided us with this wonderful resource for our work. My gratitude also goes to the glioblastoma patients who donated tumour tissue in the hope that one day a cure will be available for this awful disease.

Finally, a very big thank-you to my amazing family. My parents Bob May and Leslie May and my three beautiful children Keshia, Aaron and Cherise. Thank you for always believing in me, supporting and encouraging me throughout my PhD, particularly when I had little time to devote to anything else. I very much appreciate your love, generosity and understanding. Without your support, none of this would have been possible.

Keywords

Glioma, glioblastoma, hyperdiploidy, aneuploidy, heterogeneity, therapy resistance, cell surface proteins.

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 111207, Molecular Targets, 35%

ANZSRC code: 111201, Cancer Cell Biology, 35%

ANZSRC code: 111209, Solid Tumours, 30%

Fields of Research (FoR) Classification

FoR code: 1112, Oncology and Carcinogenesis, 50%

FoR code: 0601, Biochemistry and Cell Biology, 50%

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List of Abbreviations

ABCG2	ATP-binding cassette, sub-family G (white), member 2
AICAR	5-Aminoimidazole-4-carboxamide-1-β-4-ribofuranoside
AML	Acute myeloid leukaemia
AMP kinase	5’ adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BCL-XL	B-cell lymphoma-extra large
BCL-2	B-cell lymphoma 2
BrdU	5-bromo-2’-deoxyuridine
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CD	Human cell differentiation molecules (HCDM)/ cluster of differentiation
CDK1	Cyclin dependent kinase 1
CFDASE	Carboxyfluorescein diacetate succinimidylester
CLL	Chronic lymphoblastic leukaemia
CML	Chronic myeloid leukemia
CNS	Central nervous system
CSC	Cancer stem cells
DDR	DNA-damage checkpoint response activation
DFS	Disease free survival
DMSO	Dimethyl sulfoxide
DXR	Doxorubicin
EdU	5-ethynyl-2-deoxyuridine
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
FITC	Flourescein isothiocyanate

FACS	Fluorescence-activated cell sorting
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GPI	Glycophosphatidylinositol
hGBM	Human GBM
HNSCC	Head and neck squamous cell carcinoma
HSCs	Haematopoietic stem cells
IDH1	Isocitrate dehydrogenase 1
IκB	Inhibitory protein kappa-B
LOH	Loss of heterozygosity
LSC	Leukaemia stem cells
mAbs	Monoclonal antibodies
MAC	Membrane attack complex
MET	Mesenchymal to epithelial transition
MET	Met proto-oncogene tyrosine kinase
MCM	Minichromosome maintenance complex
MG132	Carbobenzoxy-Leu-Leu-leucinal
MGMT	06-methylguanine-DNA-methyltransferase
MM	Multiple myeloma
MRP-1/CD9	Motility-related protein-1
MSH2	MutS homolog 2
MSH6	MutS homolog 6
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
NCAM	Neural cell adhesion molecule
NF1	Neurofibromatosis 1
NF-κB	Nuclear factor kappa-B
NOD/SCID	Nonobese diabetic/severe combined immunodeficiency

Nrf2	Nuclear erythroid-related-factor-2 expression
NSA	Neurosphere assay
NFκ-B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSC	Neural stem cells
PDGFRA	Platelet-derived growth factor receptor alpha
PE	Phycoerythrin
PFS	Progression free survival
phospho-Rb	Phosphorylated Rb
PI	Propidium iodide
PMN	Human neutrophil polymorphonucleocyte
PSMβ5	Proteasome Subunit-Beta Type-5
PTEN	Phosphatase and tensin homolog
p27Kip1	Cyclin-dependent kinase inhibitor B1
Rb	Retinoblastoma
SIRPα	Signal regulatory protein alpha
SOX	Sex-determining region Y protein (Sry)-related high mobility group box -C-phosphate-G- (CpG)
T-ALLs	T cell acute lymphoblastic leukaemias
TGCs	Trophoblast giant cells
TICs	Tumour-initiating cells
TIF	Tumour initiation frequency
TMZ	Temozolomide
tNSCs	Tumour neural stem cells
TP53	Transformation-related protein 53
TSP-1	Thrombospondin-1
VEGF	Vascular endothelial growth factor
WHO	The World Health Organization

YKL40	YKL40/Chitinase-3-like protein-1
2-DG	2-deoxy-D-glucose
5-FU	5-fluoro-uracil
17-AAG	17-allylamino-17-demethoxygeldanamycin

CHAPTER ONE

1 Introduction

1.1 Foreword

Cancer accounts for 22% of deaths in the US, a rate exceeded only by heart disease. While the 5-year survival rate for cancer has risen from 49% in 1977 to 68% in 2008 (American Cancer Society, Cancer Facts and Figures. 2013), the death rate from cancer overall has remained static for the last 50 years (US National Cancer Institute, 2007). Based on previous data, it is estimated that there will be 46,880 deaths from cancer in Australia in 2016. The age-standardised mortalilty rate for cancer for the same year will be 162 deaths per 100,000 persons (Cancer Australia, 2016). Modern medical research has been unsuccessful in its goal to effectively cure most cancers despite the growing number of available techniques, increasing investment and the enormous amount of time that has gone into its study. Target-based study of oncogenic proteins has so far been mostly inadequate resulting in therapies incapable of providing long-term survival benefits for most cancer patients (Butcher, 2005). A greater understanding of a subset of tumours has led to significant improvements in survival demonstrating the benefits of a deeper knowledge of the disease and response of tumour cells to targeted therapies. Promyelocytic leukaemia is an example of this. Predominantly affecting children, the precise chromosomal translocation has been identified and with current therapy the cure rate is 80-90% (Tallman & Altman, 2009). Similarly, the cure rate in children with T cell acute lymphoblastic leukemia's (T-ALLs) is 75%. This is the result of the identification of distinct molecular subgroups with specific gene expression signatures (Van Vlierberghe & Ferrando, 2012). However, the contribution of these statistically high cure rates in overall cancer survival statistics is masked by their relatively low incidence. Despite this, the success of informed targeted therapies in some malignancies highlights the potential for inducing cures in many other cancer types once we have detailed information of their underlying pathobiology. A new experimental approach is required to understand the

underlying mechanisms of therapy resistance in order to develop effective anti-cancer drugs for the majority of tumours that remain incurable today.

Malignant astrocytic gliomas represent 60% of all primary human brain tumours in adults (Furnari et al., 2007; Sakariassen, Immervoll, & Chekenya, 2007). Histologically classified according to The World Health Organization (WHO), glioblastoma multiforme is categorized grade IV, the most aggressive, invasive form of the disease with the poorest prognosis (Network, 2008). Patients with GBM have a median survival time of 14.6 months (Vescovi, Galli, & Reynolds, 2006). The incidence of glioblastoma is 40% more common in men than in women and the average age at diagnosis is 64 years (CBTRUS, 2012). Only 5% of glioma cases present with a family history of glioma and in the majority of these patients there is no known familial genetic abnormality (Wen & Kesari, 2008). Patients with primary GBM present with a grade IV glioma and have no previous history of the disease. Secondary glioma presents initially as a lower grade tumour either grade I, II or III astrocytoma, which subsequently progresses to grade IV. Histopathologically, primary and secondary GBM are indistinguishable (Parsons et al., 2008).

Glioblastoma is refractory to treatment despite aggressive medical intervention (Stupp et al., 2005). This is partly attributable to the blood brain barrier that presents a physical obstruction to the majority of systemically administered chemical compounds, thereby reducing treatment response (Sottoriva et al., 2013). Current treatment of GBM is aggressive and involves surgery to remove the bulk of the tumour followed by chemotherapy and radiotherapy. Surgical debulking is performed to remove pressure on surrounding tissues and must be conservative enough to keep post surgical morbidity to a minimum (Wen & Kesari, 2008). Radiotherapy in conjunction with the chemotherapeutic, temozolomide, is usually administered at 2Gy per day for five days a week over six weeks to an area which encompasses the tumour cavity and a small margin of the surrounding tissues only, again to reduce damage via post-radiation tissue necrosis (Wen & Kesari, 2008). In a study of 573 patients with GBM where 50% received radiotherapy alone and the other 50% received radiotherapy plus temozolomide, the median increase in survival for the radiotherapy plus temozolomide group was only 2.5 months (Stupp et al., 2005). Glioblastoma tumour recurrence usually occurs within tissue that has received intensive radiation and cytotoxic therapy (Garden et al., 1991). This suggests that a subpopulation

of cells is able to evade cytotoxicity and participate in tumour regrowth. Proliferation of therapy resistant cells occurs rapidly with previously resected tumours frequently requiring further surgical debulking (Chaichana et al., 2013).

The genetic abnormalities most commonly found in GBM involve pathways responsible for cell survival, cell cycle control and growth factor signaling (Fomchenko & Holland, 2005). These changes result in loss and inactivation of tumour suppressor genes and genes responsible for DNA repair mechanisms combined with the amplification or over-activation of constitutively activated pathways that function to regulate cell cycle progression and an increase in quantity of growth factor ligands and receptor sensitivity that promote cell proliferation (Fomchenko & Holland, 2005; Wen & Kesari, 2008). Until recently, the common genetic mutations identified in glioma have been used to classify tumours into two broad groups, namely, primary and secondary glioma. A recent genomic study that identified four tumour subtypes within GBM based on common genetic mutations provided a molecular profile that could be used to identify an individual tumour's potential response to therapy and prognosis (Verhaak et al., 2010).

The four subtypes are described in depth elsewhere; key points only will be briefly outlined here (Verhaak et al., 2010). The subtype termed 'classical' was clearly defined by a combination of chromosome 7 amplification with chromosome 10 loss in 100% of cases. There was an average fourfold increase in epidermal growth factor receptor (EGFR) amplification in 97% of tumours categorised as 'classical' with an associated homozygous deletion of cyclin-dependent kinase inhibitor 2A (*CDKN2A*). The 'mesenchymal' subtype was characterized by neurofibromatosis 1 (*NF1)* and phosphatase and tensin homolog (*PTEN)* mutations. This group also exhibited an increase in mesenchymal marker expression, YKL40/Chitinase-3-like protein-1 (*YKL40)* and met proto-oncogene tyrosine kinase (*MET)* as well as that of genes in the nuclear factor kappa-light-chain-enhancer of activated B cells *(NFκ-B)* and tumour necrosis factor pathways although the upregulation of these genes was not exclusively identified in this subtype. The 'proneural' subtype exhibited the highest rate of platelet-derived growth factor receptor alpha (*PDGFRA)* amplifications in association with an increase in gene expression levels. Isocitrate dehydrogenase 1 (*IDH1*) mutations were seen almost exclusively in this group. Transformation-related protein 53 (*TP53*) mutations and loss of heterozygosity (LOH) were

also strong features of this subtype. Proneural development genes including the sex-determining region Y protein (Sry)-related high mobility group box (*SOX)* genes were a feature of this group. The 'neural' subtype was identified based on the expression of neural markers. Synaptotagmin-1 binds calcium at the synapse triggering neurotransmitter release, this along with the neurofilament light polypeptide gene was amongst the genes upregulated in the neural group.

No correlation was identified between CpG methylation status of the 0^6-methylguanine-DNA-methyltransferase (MGMT) promoter and any of the four subtypes. This study found no evidence of transition between subtypes during disease progression. The mesenchymal and classical subtypes derived the greatest benefit from treatment in terms of survival. The purpose of a profile such as this is to develop biomarkers for each glioma subtype that would allow clinicians to tailor treatment to each patient's tumour by predicting points of therapeutic susceptibility and resistance and to aid in prognosis. However, in 2013 Sottoriva et al.. published findings that more than one of the subtypes identified by Verhaak et al.. can be found within a single GBM tumour simply by sampling different regions of the same tumour. This suggests that tumour evolution, from a cell of origin, gives rise to an heterogeneous population of malignant cells with varying fitness levels responding to a variety of selection pressures including therapy and microenvironment resulting in regional differences in genotype and gene expression over time (Sottoriva et al., 2013).

Sturm et al., looked at glioblastoma tumour samples of both children and adults and identified six subgroups based on mutational status, DNA copy-number aberrations and gene expression signatures. They found that mutations in two critical amino acids (K27 and G34) in the H3F3A gene or mutations in IDH1 occur in both adult and childhood glioblastoma and that the two mutant genes were mutually exclusive. In addition to this, they were able to map the six subgroups to specific brain regions. This suggests that glioblastoma tumour treatment could be specifically designed for different epigenetic and biological subgroups from different anatomical brain regions (Sturm et al., 2012).

Therapy resistance is the major problem facing oncologists today. The combination of surgery, radiotherapy and chemotherapy, results in removal of the majority of tumour cells. However, tumour regeneration following treatment suggests that a population of therapy

resistant cells remain to regenerate the tumour. This often leads to the evolution of an aggressive chemoresistant cell population that is refractive to further treatment. Tumour heterogeneity contributes to therapy resistance making this an important research area in the effort to find a cure for cancer.

1.2 Tumour heterogeneity

Tumour heterogeneity is the term used to describe the existence of cells exhibiting different genomic or proteomic profiles or biological activity (Marusyk & Polyak, 2010). Tumours from different organs are genetically diverse. This might be explained in part, by the specific biological properties of the organ combined with the microenvironment of the cells within that tissue. Intertumoural heterogeneity has been partially resolved through subgroup classification, which may predict response to therapy and prognosis (Burrell, McGranahan, Bartek, & Swanton, 2013). Tumours from the same organ within the same individual patient also frequently exhibit genetic differences as well as differences in therapeutic response and aggressiveness of disease. This is called intratumoural heterogeneity (Burrell et al., 2013). Intratumoural heterogeneity presents the biggest obstacle to cancer therapy. It is not simply ‘noise’, that disguises the more frequent deleterious changes that have been the focus of genetic studies to identify oncogenic genes common to certain cancer types (Heng et al., 2009). Intratumoural heterogeneity provides a mechanism of rapid evolution by imparting variation that can overcome a wider range of perturbations. Small subpopulations of cells persist with an elevated expression of a beneficial protein such as an oncogene, or loss of expression of a detrimental protein such as a tumour suppressor gene. These cells exhibit a superior fitness level in a cytotoxic environment increasing their chances of survival. For a trait to contribute to therapeutic resistance it must be heritable. If a trait is not heritable, it does not contribute to the evolvability of a clonal population with the ability to overcome therapy. In some tumour types the stage of the cancer progression can be measured by the genetic mutation load (Khambata-Ford et al., 2007; McFarland, Korolev, Kryukov, Sunyaev, & Mirny, 2013).

GBM is characterized by marked heterogeneity within the cell population of an individual tumour. Cells within a tumour display varying rates of proliferation and migration, a variety of cell-cell interactions, variation in sensitivity to therapy and differences in cell size and cell surface protein profile. The accumulation of this diversity presents a significant

challenge to the development of an effective treatment for the disease. One possible solution to this dilemma is to identify subpopulations of cells within a tumour that promote disease progression and treat these cell populations with individually targeted therapies.

1.3 Tumour evolution

Genomic instability refers to an increase in genetic mutations arising in a cohort of cells (Shen, 2011). It is a potential source of genetic diversity underpinning progressive tumour evolution subject to phenotypic penetrance (Burrell et al., 2013). The presence of a variety of different clones within a tumour is generally considered to be generated stochastically while the expansion of a clonal population within a tumour is determined by selection pressure exerted by external factors, the microenvironment on surrounding cells and cell signalling (Inda, Bonavia, & Seoane, 2014). Mechanisms of genomic instability include mismatch repair, telomere loss, double-strand break repair, deregulated DNA replication and chromosomal missegregation (Burrell et al., 2013; Pikor, Thu, Vucic, & Lam, 2013). These mechanisms are a result of various genetic defects including point mutations, chromosomal deletions (loss of heterozygosity) or insertions, chromosomal rearrangements and aneuploidy (subdiploid and hyperdiploid) (Abdel-Rahman, 2008; Campbell et al., 2010). The phenotypic outcome will vary according to the magnitude of the genetic defect, which could be smaller for a point mutation for example than the loss of an entire chromosome (Burrell et al., 2013).

Treatment including chemotherapy and radiotherapy can initiate exogenous genomic instability introducing new mutations as was demonstrated in patients following treatment for AML (L. Ding et al., 2012). Temozolomide is an alkylating agent used in the treatment of glioblastoma. Alkylating agents work by inducing adducts on DNA causing mispairing of nucleotides. Normally the mispairing is recognised by the mismatch repair system leading to apoptosis (Kondo, Takahashi, Ono, & Ohnishi, 2010). Mispairing of 0^6-methylguanine with thymine in double stranded DNA is normally recognised by the MSH2/MSH6 mismatch repair dimers (Hunter et al., 2006). The results of an analysis of human malignant glioma tissue and blood samples from 30 patients showed that this system was inactivated due to mutations in MSH6 following TMZ therapy (Hunter et al., 2006). Tumours, which responded to the initial round of chemotherapy, were insensitive to

subsequent treatment due to mismatch repair deficiency and with no system in place to prevent cell cycle progression exhibited elevated mutation rates (Hunter et al., 2006).

Environmental conditions can promote tumour progression in individual cohorts of cells. Tumour microvascular proliferation, a key feature of glioma is a disorganised process leading to inconsistency in perfusion levels across the tumour (Junttila & de Sauvage, 2013). Variations in distance from vasculature, affects access to supply of nutrients and signalling proteins. Nitric oxide is produced in the endothelial cells and plays a role in cell signalling (Tredan, Galmarini, Patel, & Tannock, 2007). Variations in nutrient distribution and access to signalling proteins contribute to the regional heterogeneity reported when biopsies are examined from different geographical locations across a tumour (Tredan et al., 2007). Suboptimal perfusion results in poor drug distribution to some regions of the tumour leaving those areas with less than the desired dose (Tredan et al., 2007). Exposure to nitric oxide was shown to be associated with tumour formation and aggression in mouse models of glioma as determined by endothelial nitric oxide synthase protein levels on western blot. Tumourigenesis was reversed when nitric oxide was withdrawn resulting in longer survival time (Charles et al., 2010).

Cellular fitness is context dependent (Junttila & de Sauvage, 2013). A genetic mutation, which is advantageous in one environment, may be deleterious in another. This means that a mutation might be present in a small subpopulation and subsequently undergo clonal expansion in response to a change in the tumour environment such as commencement of cytotoxic therapy (L. Ding et al., 2012; Villanueva et al., 2010). In addition to this, mutations can be silent, known as cryptic genetic variation, and this cryptic variation can be revealed under novel selection pressures and is thought to be responsible for rapid evolution (Whitacre, Lin, & Harding, 2012). A mutation that is deleterious in a normal environment may, in a hostile environment such as hypoxia, improve the likelihood of cell survival, contribute to fitness, undergo positive selection and through clonal expansion become a trait that contributes to a therapeutic resistant population (Loeb & Loeb, 1999; Tian, Olson, Whitacre, & Harding, 2011). Regional microenvironmental differences within a tumour can result in the clonal expansion of different subpopulations across the tumour (Junttila & de Sauvage, 2013).

Another way in which tumour cells can express heterogeneity is through phenotypic plasticity. Tumour cell plasticity is defined as the dedifferentiation of a cell to a different phenotype (Friedmann-Morvinski & Verma, 2014). One example of cellular plasticity is epithelial to mesenchymal transition (EMT). EMT is a process whereby cells display morphological changes including cytoskeletal changes and loss of cell-to-cell signalling facilitating invasion and metastasis formation (Creighton, Chang, & Rosen, 2010). EMT has been reported in tumours in the breast, lung, pancreatic, head and neck and glioblastoma (Ortensi, Setti, Osti, & Pelicci, 2013; Sarkar, Li, Wang, & Kong, 2009; Shrivastava et al., 2015; Q. S. Wang, Kong, Li, Yang, & Feng, 2015; Xiao & He, 2010). Reversible EMT is the process by which cells undergo EMT followed by mesenchymal to epithelial transition (MET) and back again (Luo, Brooks, & Wicha, 2015). This process of transitioning between different states has been reported in breast cancer cells (S. Liu et al., 2014). MET-like breast cancer cells identified at the tumour centre have been recognised as being proliferative while EMT-like cells located at the invasive front were quiescent (Luo et al., 2015). In tumours, cellular plasticity can provide additional mechanisms for subpopulations of cells within a tumour to respond differently to chemotherapy. Cancer metastasis and drug resistance have been shown to be features of EMT (Luo et al., 2015; Saunders et al., 2012). Breast cancer cells that underwent EMT confirmed by changes in protein expression levels, developed insensitivity to paclitaxel (G. Z. Cheng et al., 2007). Similarly, cisplatin resistance correlated with EMT in immunohistochemical analyses of head and neck squamous cell carcinoma (HNSCC) patient tissue samples (Hsu et al., 2010).

GBM cells have been reported to exhibit EMT characteristics in association with increased invasion and therapy resistance following treatment (Piao et al., 2013). Anti-vascular endothelial growth factor (VEGF) therapy slows tumour progression by inhibiting angiogenesis (Batchelor et al., 2007). Bevacizumab (Avastin; Genentech/Roche) is a humanised monoclonal antibody directed against VEGF currently approved for use in the treatment of GBM (Piao et al., 2013). Glioma cells with previous exposure to bevacizumab developed a mesenchymal-like gene expression signature that was associated with an increase in invasion and resistance to further bevacizumab therapy compared with parental cell lines with no previous exposure to bevacizumab (Piao et al., 2013).

There are two proposed models of tumour heterogeneity contributing to tumour evolution, the cancer stem cell model and the clonal evolution model. Both models purport to explain therapy resistance in tumours. This thesis investigates each model in human glioblastoma. Finally, cell surface protein expression has been used by others, to predict prognosis and determine therapeutic response in a variety of tumours. This study analysed cell surface protein expression on human GBM cells to identify a subpopulation of cells of clinical relevance in GBM tumours. Tumour evolution resulting in increased cellular plasticity and heterogeneity is generated by a range of mechanisms leading to a complex set of therapeutic responses. To effectively treat a plastic heterogeneous tumour cell population a combination of therapies will be required that target different subsets of the tumour with unique characteristics.

1.4 The cancer stem cell model

Stem cells exist throughout the body in many human tissues to generate new cells for the dual purposes of ongoing maintenance and repair following injury and disease (Dalerba, Cho, & Clarke, 2007). The three defining properties of stem cells are: extensive self-renewal, generation of a large number of progeny and multi-lineage differentiation potential (Piccirillo et al., 2006). Another key characteristic of stem cells is their relative quiescence, which is essential to prevent premature stem cell depletion over the lifetime of the organism (T. Cheng et al., 2000).

Neural stem cells are generated in the hippocampus, the subventricular zone of the lateral ventricles and the olfactory bulbs (Fomchenko & Holland, 2005; Piccirillo et al., 2006). Their function is to generate new neurons and glia that can migrate to other brain regions to replace damaged and ageing cells throughout the lifetime of the organism (Gage, 2000). The Neurosphere Assay culture system (NSA) is the method of choice to isolate, propagate and culture neural stem cells (NSCs) from embryonic and adult murine and human central nervous system (CNS) (Reynolds & Weiss, 1996; Vescovi et al., 1999). For this reason, it is the method used in the present study to culture brain tumour cells.

Therapy resistance continues to be the greatest obstacle to achieving complete remission in this disease (Ramirez, Weatherbee, Wheelhouse, & Ross, 2013). The cancer stem cell

hypothesis states that tumour initiation and maintenance is driven by a rare subpopulation of tumour cells that phenotypically mimic the cardinal properties of stem cells. This hypothesis was first postulated in the 1990s based on studies of acute myeloid leukaemia (AML) (Bonnet & Dick, 1997; Huntly & Gilliland, 2005; Lapidot et al., 1994) and has since been strengthened by findings in breast, prostate, lung and mesenchymal tumours (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003; Buzzeo, Scott, & Cogle, 2007; Mano et al., 2008). The ability of cells derived from human glioma tissue to generate neurospheres in culture, suggests the presence of tumour cells with neural stem cell properties within CNS tumours (Hemmati et al., 2003; Ignatova et al., 2002; Singh, Clarke, Hide, & Dirks, 2004; Singh et al., 2003), as neurosphere generation is one indicator of the presence of NSCs. Further work (Galli et al., 2004) has demonstrated the isolation, propagation and serial transplantation of tumour neural stem cells (tNSCs) that exhibit very similar functional properties to neural stem cells. Importantly, following implantation of tNSCs, the resulting tumours exhibit the classic *in vivo* features of human glioblastoma multiforme; recapitulating the morphology, genotype and gene expression patterns of primary GBMs, as well as having extensive migratory and infiltrative capacity (Engelman et al., 2007). This indicates that the *in vitro* defined brain tumour stem cells faithfully preserve the key *in vivo* features of human GBM.

Results in chronic myeloid leukemia (CML) patients suggest that a small number of quiescent, malignant stem cells are extremely refractory to treatment and are responsible for patient relapse after therapy (Barnes & Melo, 2006). The short progression free survival (PFS) time of patients with glioblastoma is consistent with the hypothesis that an analogous population of slow-cycling cancer stem cells also exists in human GBM tumours. If this hypothesis is correct, then elimination of slow-cycling, therapy-resistant cancer stem cells will be essential for the long-term survival of glioblastoma patients.

1.5 The clonal evolution model

One of the ways in which tumours exhibit genetic heterogeneity is through variation in ploidy (Craig & Foon, 2008; Zuzana Storchova & Pellman, 2004). Polyploidy is the over duplication of whole chromosome sets. Polyploidy in mammals is defined as greater than two sets of chromosomes (Tovar et al., 2010). An example of this is tetraploidy which

refers to the presence of four sets of chromosomes. This can occur when a cell that has undergone DNA replication subsequently fails mitosis or cytokinesis (Vitale et al., 2011).

Polyploidy exists in normal development. There are at least three known mammalian cell types that utilize polyploidy during development. During placental development, placental trophoblast giant cells utilize polyploidization to reach many times the usual diploid content by preventing mitosis (Davoli & de Lange, 2011; Ullah, Lee, Lilly, & DePamphilis, 2009). Bone marrow megakaryocytes fail to undergo cytokinesis numerous times forming a large nucleus following multiple rounds of mitosis and hepatocytes also fail cytokinesis to form 4N or 8N nuclei (Davoli & de Lange, 2011; Winkelmann, Pfitzer, & Schneider, 1987). Numerous variations of polyploidy also exist.

Aneuploidy refers to the presence of an abnormal number of chromosomes which is not a precise multiple of the haploid chromosome number for that cell (E. M. Torres, Williams, & Amon, 2008). An aneuploid cell can be subdiploid or hyperdiploid, due to the loss or gain of one or more chromosomes (Lacroix & Maddox, 2012). Aneuploidy is a common feature of tumours (Y. Li et al., 2010; Z. Storchova & Kuffer, 2008; Tang, Williams, Siegel, & Amon, 2011) and is sometimes used to classify tumours and predict prognosis (Y. Li et al., 2013; Van Wier et al., 2013). Hyperdiploidy is a clinically relevant feature of tumours including acute lymphoblastic leukaemia (Paulsson et al., 2010). Hyperdiploidy can also contribute to therapeutic resistance by amplification of drug transporters and reducing sensitivity to chemotherapeutics (Anand, Penrhyn-Lowe, & Venkitaraman, 2003; Q. Cheng & Evans, 2005; Gorre et al., 2001). Studies involving yeast revealed that aneuploidy promoted rapid phenotypic evolution and drug resistance to cytotoxic compounds (J. Chen et al., 2012). These findings suggest that aneuploid cells could be contributing to therapy resistance in cancer and may provide an important therapeutic target for future cancer treatments.

1.6 Cell surface proteins

Tumours are also heterogeneous in their expression of cell surface proteins. A vast spectrum of molecules exists on the surface of a single cell and cell types from various organs and tissues exhibit differential cell surface molecule expression. The molecules represented on the cell surface include proteins, glycoproteins and glycolipids, which

function as ligands, receptors, adhesion molecules, and ion channels (Zola et al., 2005). Cell surface proteins may span the cell membrane, binding both extracellular and intracellular ligands, or alternatively through tethering to the external surface of the membrane, trigger protein interactions that promote a cascade of interactions through the membrane and into the cytosol. Proteins that reside on the surface of a cell contribute to the phenotype of that cell and to the range of functions of which it is capable. Processes such as proliferation, apoptosis, invasion, migration, and senescence are initiated through the binding of circulating extracellular ligands to cell surface protein receptors that trigger downstream signaling to intranuclear transcription factors. Immunophenotyping exploits cell surface protein expression to distinguish a subpopulation of cells with a distinct molecular signature (Craig & Foon, 2008; van Dongen et al., 2012).

During the 1980's, the identification of antibodies to leukocyte cell surface molecules by immunologists developed rapidly in various laboratories across the globe utilizing a variety of techniques. This resulted in antibody reactivity that was often variable and confusion with regard to which newly generated antibodies were identical. The human leukocyte Differentiation Antigen (HLDA) workshops were initiated in 1982 to provide consensus on surface protein identification and a universal nomenclature system (Zola, 2001; Zola et al., 2005). Some of the same proteins have since been detected in organs and tissues throughout the body. Additionally, disease biomarkers and molecular targets for therapy have been identified (Zola, 2001). The collective name for these antibodies is now human cell differentiation molecules (HCDM) to encompass the expanding range of human tissue types that exhibit reactivity to these antibodies.

Cell surface proteins have traditionally been widely used in haematologic malignancies for the purposes of diagnosis, monitoring of treatment efficacy and isolation of cancer stem cells (Hofmann et al., 2010). Immunophenotyping of acute leukaemic blast cells using CD markers has facilitated the identification of the cellular lineage allowing clinicians to predict prognosis and response to therapy (Medeiros et al., 2010). Panels of antibodies that detect minimal residual disease in leukaemia following therapy are being investigated to facilitate early detection of relapse and improve treatment outcomes (X. Li et al., 2010).

An increased presence of circulating membranous glycoproteins is thought to be indicative of either apoptosis or protein shedding (Alatrash et al., 2010). The shedding of membrane spanning glycoproteins into the plasma is a characteristic of malignant cells (Komada et al., 1986). Post therapeutic progression free survival and estimation of minimal residual disease may soon be possible by assessment of CD expression levels in circulating plasma. In chronic lymphoblastic leukaemia (CLL), circulating CD20 is understood to reflect a refractory response to therapy (Prevodnik, Lavrencak, Horvat, & Novakovic, 2011).

Cancer stem cells have been identified in AML as the cancer-initiating cell responsible for disease progression and relapse after therapy (Bonnet & Dick, 1997). Immature lymphocytes, also known as blasts, proliferate without fully differentiating to the point that overcrowding inhibits normal proliferation of other cell types (Bonnet & Dick, 1997). One study interrogated leukaemic cells for a cell surface protein expression profile that identified them from amongst other primitive cell types and non-cancerous stem cells (Lapidot et al., 1994). A small proportion of the overall population of AML cells were selected based on their expression of two cell surface proteins, CD34+ and CD38-, which when transplanted into immunocompromised mice were able to proliferate and initiate a disease state identical to that of the donor (Bonnet & Dick, 1997; Lapidot et al., 1994). More recently, monoclonal antibodies targeting CD44, CD47 and CD123 have been shown to independently inhibit disease progression in *in-vivo* models of AML (Majeti & Weissman, 2011).

The identification and isolation of tumour initiating cells in leukaemia using a cell surface protein expression profile (Bonnet & Dick, 1997; Lapidot et al., 1994), has led to a search for a similar phenomenon in solid tissue cancers. Medulloblastoma cells have been shown to include a subpopulation of CD133+ expressing cells that exhibit extensive proliferative ability, ability to differentiate into the cell types specific to the tumour from which they were derived, and in the proportions indicative of that specific tumour type, as well as the ability to form neurospheres in the culture system originally designed for neural stem cells (Singh et al., 2003). In other work, tumour-initiating cells have been isolated from the total population of breast cancer cells using cell surface protein antibodies. A xenograft assay involving transplantation of breast cancer cells into immunocompromised mice showed that as few as 200 CD44+/CD24-/low cells were capable of tumour formation with 100%

efficiency whereas transplantation of 50,000 unsorted breast cancer cells were required to achieve 100% tumour formation (Al-Hajj et al., 2003).

CD protein expression profiles have been recognized as corresponding with tumour progression and prognosis in a range of solid tumours. The increased expression of the tetraspanin protein CD151 has been shown to positively correlate with large invasive tumours and metastases while being negatively correlated with overall survival in hepatocellular carcinoma patients (Ke et al., 2009). Motility-related protein-1 (MRP-1/CD9) is a transmembrane protein found to predict disease free survival (DFS) in patients diagnosed with squamous cell carcinoma of the head and neck. CD9 expression was negatively correlated with disease free survival (DFS) in a study of 153 patients by immunohistochemistry (Mhawech et al., 2004). A small number of cell surface CD proteins have been recognized in GBM as contributing to disease progression and prognosis. Higher expression of CD44 has been correlated with aggressive glioma tumours as determined by immunohistochemistry (Wei et al., 2010).

CD133 expression in glioma has been quite controversial. Initially reported as being a putative cancer stem cell marker (Zeppernick et al., 2008), CD133 positivity now appears to be environmentally induced rather than inherited (L. K. Donovan & Pilkington, 2012; Safa, Saadatzadeh, Cohen-Gadol, Pollok, & Bijangi-Vishehsaraei, 2015). Several labs have shown an increase in stem cell marker expression and CD133 expression, proliferation and migration in cells exposed to a hypoxic environment, suggesting that cells undergoing hypoxia migrate away to regions supplying higher oxygen concentrations where they utilize blood vessels to supply nutrients for tumour growth and progression (Campos & Herold-Mende, 2011; Ohnishi et al., 2013). McCord et al. showed that GBM cells passaged under hypoxic conditions, 7% oxygen, exhibited an increase in CD133 expression in addition to increased proliferation and self-renewal rates in comparison to cells passaged in 20% oxygen (McCord et al., 2009).

1.7 Aims and Hypotheses

The overall aim of my thesis was to interrogate the role of heterogeneity in the development of GBM therapy resistance with a view to designing better treatment paradigms

1.7.1 Hypotheses

1. A rare subpopulation of stem like cells exists within human GBM tumours that are responsible for the initiation and regeneration of tumours following treatment.
2. Cell-surface protein expression can be used to identify cell subpopulations of clinical relevance in GBM tumours.

1.7.2 Aims

To address these hypotheses I will pursue the following three aims:

1. Examine GBM tumours for a subpopulation of stem like cells that exhibit therapeutic resistance.

2. Investigate the relevance of hyperdiploid cells in the evolution of therapeutic resistance in GBM.

3. Identify cell surface proteins of potential clinical relevance in GBM.

CHAPTER TWO

2 Materials and Methods

2.1 Tumour cell line generation from primary tumour tissue

All tumour tissue was obtained from glioblastoma patients during surgical resection following written informed consent complying with guidelines established by the Princess Alexandra Hospital Research Ethics Committee. Tumour tissue was classified by neuropathologists as glioblastoma according to WHO guidelines (D. N. Louis et al., 2007). Patient tumours were washed and mechanically dissociated before being chemically dissociated with trypsin/ethylenediaminetetraacetic acid 0.05% (Gibco) for 10 minutes at 37^0C. Cells were then filtered through a 40µm filter to achieve a single cell suspension. Cells were counted using Trypan Blue to exclude dead cells and cultured in fresh neurosphere media in a 37^0C incubator. Neurosphere media contained DMEM F12 (Sigma), 10% NeuroCult Neural Stem Cell Proliferation Supplement (StemCell Technologies Inc), human epidermal growth factor (hEGF, 20ng/ml, StemCell Technologies Inc.), basic fibroblast growth factor (bFGF, 10ng/ml, StemCell Technologies Inc.) and heparin 2µg/ml (Sigma), Penicillin/Streptomycin 2.8ml in 250ml (Life Technologies) as described in (S. A. Louis et al., 2008). When spheres reached an adequate size ~ 150µm, they were chemically dissociated as described above, counted and reseeded in flasks at a density of 50,000 cells per ml.

2.2 Xenotransplantation assays

All animal work was performed in accordance with the National Health and Medical Research Council Guidelines for the care and use of animals for scientific purposes. Surgical protocols were approved by The University of Queensland Animal Ethics Committee. All surgery was performed on six to ten week old non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice.

2.2.1 Mouse subcutaneous injections of human GBM cells

Cells were chemically dissociated using Trypsin (Gibco) and washed twice in neurosphere media. Cells were resuspended in 5μM CFSE in 5ml cold neurosphere media and incubated for 10 minutes in the dark. CFSE reaction was inhibited with cold neurosphere media containing 10% foetal calf serum. Cells were centrifuged, given two washes in neurosphere media, resuspended in complete neurosphere media and a cell count was performed. Cells were resuspended at 1 x 10^6 cells per 100μl in Matrigel (BD Biosciences) and neurosphere media 1:1 and stored on ice while mice were prepared. Fur was chemically removed from the abdominal region of each mouse using Nair and area cleaned. Mice received subcutaneous injections using a Tuberculin syringe containing 100μl of solution with 1 x 10^6 cells. Animals were monitored daily and tumours measured with a caliper once they became visible. Animals were sacrificed once tumours reached 1cm in diameter.

2.2.2 Mouse intracranial injections of human GBM cells

Cells passaged as neurospheres in serum free media were chemically dissociated with Accutase (Sigma) for 10 minutes at 37^0C and centrifuged into a pellet. Cells were resuspended in neurosphere media to a concentration of 100,000 cells/μl and stored on ice. Mice were anaesthetized with either Ketamine and Xylazine or Isofluorane. Mice were positioned in stereotax. Coordinates for stereotactical injections into adult mice were 2mm laterally to the midline at the bregma and 3mm dorso-ventrally. A small hole was drilled in skull for needle insertion. Mice were injected with 200,000 cells using a 5μl Hamilton Syringe. The wound was closed with vetbond. Post-operatively mice were monitored for signs of neurological and physical deterioration, including ataxia, lethargy, seizures, weight loss, or paralysis. Mice were sacrificed and the tumours removed for further study.

2.3 Flow cytometry

2.3.1 Loading cells with CFSE

GBM cells were aspirated from flask and centrifuged. Cells were then resuspended in warm Trypsin and incubated for three minutes at 37^0C, pipette mixing twice during incubation and centrifuged. Cells were washed in neurosphere media and centrifuged. Staining was performed using 5μM CFSE (Molecular Probes) per 1.0ml cold neurosphere media per 4 x 10^6 cells for 10 minutes protected from light. Adding 5.0ml ice-cold

neurosphere media with 10% foetal calf serum stopped the reaction. Cells were centrifuged for five minutes at 4^0C and rinsed twice in ice-cold neurosphere media, resuspended in fresh media, counted and seeded into tissue culture flasks at 50,000 cells per ml. GBM cells were cultured for five to seven days before analysis.

2.3.2 FACS sorting of CFSE stained cells

Cells were loaded with CFSE as described above. After CFSE was inhibited cells were washed once in PBS and either sorted by fluorescence-activated cell sorting (FACS) or fixed for 10 minutes in 4% PFA and kept at 4^0C until analysis by flow cytometry. Cells were separated into top 5% CFSE stained cells and bottom 85% CFSE stained cells with a 10% gab in between to prevent contamination of populations. Cell sorting was performed on an Influx Cell Sorter. Results were analysed using FLOWJO software.

2.3.3 5-Ethynyl-2'-deoxyuridine incorporation

Cells were chemically dissociated in Trypsin (Gibco) or Accutase (Sigma) and washed twice in PBS. Cells were labeled with EdU (Invitrogen 10μM) for 45 minutes at 37^0C, followed by two washes with neurosphere media and resuspension in complete media. Retention was determined at intervals of 24 hours for five days. In preparation for flow cytometry analysis, cells were treated with Accutase (Sigma), centrifuged, fixed for 10 minutes in 4% PFA on ice, pelleted resuspended in ice-cold methanol and stored at -20^0C. Flow cytometry was performed on a BD LSRII. Analysis was performed with FLOWJO software.

2.3.4 Cancer stem cell marker and cell cycle analysis by flow cytometry

Cells were stained with CFSE in NS media as described above and fixed for 10 minutes in 4% PFA. Cells were washed once in PBS and permeabilized in 100% ice-cold methanol. Cells were either stored at -20^0C or used immediately. For antibody staining, cells were washed once in PBS and blocked in 1% BSA (Sigma) in PBS for 30 minutes at room temperature in the dark. Cells were counted and 1 x 10^6 cells were stained per well. Primary antibodies: CD15 (BD Biosciences 1:100), CD133 (Miltenyl Biotech 1:50), ABCG2 (Abcam 1:200), GFAP (Cell Signaling Technology Technology 1:1,500), Sox2 (Cell Signaling Technology 1:200), Nestin (Merck Millipore 1:400), MCM2 (Cell Signaling Technology 1:200), MCM7 (Cell Signaling Technology 1:100), Cyclin B1 (BD Biosciences

1:100), Phospho-Rb (Cell Signaling 1:200) were diluted in 100ul block and cells incubated for 60 minutes at room temperature in the dark. Cells then underwent two x PBS washes followed by secondary antibody incubation. Alexa Fluor 647 (Life Technologies 1:400) and DAPI (1:500) were applied for 30 minutes at room temperature in the dark. At the completion of secondary antibody incubation, cells were given one PBS wash and stored at 4^0C until analysis by flow cytometry on a BD LSRII. Analysis was performed using FLOWJO software.

2.3.5 Ki67 staining

GBM cells were aspirated from flask, centrifuged, resuspended in warm Trypsin (Gibco) and incubated for three minutes at 37^0C, pipette mixing twice during incubation, Trypsin was then inhibited and cells centrifuged. Cells were washed twice in PBS and fixed for 10 minutes in 4% PFA then washed twice again in PBS and permeabilized in Saponin (Invitrogen) in BSA and PBS for 10 minutes as per manufacturers instructions. Blocking in 3% BSA in PBS was performed at room temperature for 60 minutes. Staining using Ki67 (Novocastra 1:100) was performed at room temperature for 60 minutes. Cells were washed twice in PBS and incubated in secondary antibody and DAPI for 30 minutes at room temperature followed by two PBS washes before analysis by flow cytometry on a BD LSRII.

2.3.6 Isolation of hyperdiploid clones and assessment of proliferative ability

Four GBM tumour cell lines were stained with CFSE and cultured for seven days in neurosphere media. Cells were chemically dissociated with Accutase as described above and sorted by FACS according to CFSE intensity into two groups, the top 5% CFSE bright cells and the bottom 85% CFSE dim cells. Top 5% CFSE bright cells were diluted in media and added to wells of a 384 well plate at one cell per 50µl per well. Developing spheres were monitored daily. Only spheres generated from a single cell in a well were retained for proliferation and further analysis. Clones were amplified into plates from 96 wells through to six wells and then flasks. At this stage, clones were DAPI (Invitrogen 4µg/ml) stained and analysed by flow cytometry using FLOWJO software to determine ploidy status. Three independent hyperdiploid clones were assessed from three primary tumour cell lines for the potential to maintain long-term proliferation. Each cell line was sub-cultured for seven

days over an eleven-week period at 50,000 cells per ml to observe the maintenance of viable, proliferative cultures.

2.3.7 Cell volume measurement

GBM cells were cultured as tumourspheres as described above and chemically dissociated in Accutase (Sigma) and fixed in 4%PFA in PBS, permeabilized in ice-cold methanol and stained with propidium iodide (PI) at a final concentration of 1μM in PBS RNase solution for 30 minutes at room temperature. Cell volume was measured using a Beckman Coulter Quanta SC Flow Cytometer. The instrument software using 10μM latex bead size standards calculated cell volume.

2.3.8 Cell surface protein detection by flow cytometry

Cells were passaged as neurospheres in serum free media as described above for tumour cell line generation. When large spheres had formed, spheres were dissociated via enzymatic reaction using Accutase (Sigma) for 10 minutes, or Trypsin (Gibco) for three minutes at 37^0C. Cells were placed into a flask of media in 37^0C for three hours to recover before staining. After blocking in 2% BSA in neurosphere media for 30 minutes at room temperature, staining was performed in 96 well 'V' bottom plates. Live cells were stained with LIVE/DEAD Fixable Near Infra-red Dead Cell Stain Kit (Invitrogen) according to manufacturers instructions to facilitate isolation of dead cells during flow cytometry analysis. To control for non-specific antibody binding cells were incubated in identical concentrations of isotype control. During analysis of mouse tumours, neural cell adhesion molecule 1 (NCAM-1) (BD Biosciences Alexa Fluor 647 Mouse Anti-Human CD56 1:100) was used to identify human cells and exclude cells of mouse origin. In analyzing primary human tumours, CD45 was used to exclude leukocytes (Violet 450 1:50). CD3 was used as an additional negative control. All CD antibodies and isotype control were purchased from Becton Dickinson unless stated otherwise below in 'Cell surface antibodies'. Cells were incubated for 30 minutes in the dark at 37^0C in CD antibodies diluted in block conjugated to either phycoerythrin (PE) (1:100) or flourescein isothiocyanate (FITC) (1:50). This was followed by fixation in 4% PFA for 10 minutes and two PBS washes. Cells were resuspended in PBS and flow cytometry was performed on either a BD LSRII or a Beckman Coulter Gallios. Results were analysed in FLOWJO software.

2.3.9 Cell surface antibodies

CD antibodies purchased from BD Biosciences unless stated otherwise were conjugated to either PE or FITC (1:100): CD2, CD3, CD4, CD5, CD7, CD8, CD9, CD11a, CD11b, CD11c, CD13, CD14, CD15, CD16, CD16b, CD18, CD19 (BD Horizon), CD20, CD23, CD24, CD25, CD26, CD29, CD30, CD31, CD32, CD33, CD34, CD35, CD36, CD38, CD40, CD44, CD45, CD45RA, CD45RB, CD45RO, CD49a, CD49c, CD49d, CD49f, CD54, CD55, CD56, CD58, CD59, CD61, CD62L, CD62P, CD63, CD69, CD79a, CD79b, CD80, CD81, CD86, CD87, CD88, CD90, CD95, CD103, CD104, CD105 (abcam), CD106, CD110, CD111 (Beckman Coulter), CD117, CD128a, CD128b, CD133 (Miltenyl Biotech), CD134, CD135, CD138, CD140a, CD140b (BD Transduction Laboratories), CD141, CD142, CD146, CD152, CD161, CD163, CD164, CD165, CD166, CD171 (eBioscience), CD183, CD184, CD200, CD206, CD220, CD230 (eBioscience), CD235a, CD243, CD271, CD273, CD274, CD338, HLA-DR (347367), HLA-DR (555561), HLA-DR (555809), HLA-DR (555812), NGFR.

2.4 In vitro assays

2.4.1 Sphere forming assay

To determine sphere-forming frequency, cells were chemically dissociated using Accutase (Sigma) for 10 minutes at 37^0C. Cells were resuspended in media and seeded into a 384 well flat bottom plate usually 10,000 cells per ml, 50µl per well. In the experiment to assess response to temozolomide treatment, cells were seeded at 2,000; 1,000; 500 cells per well and passaged for seven days before counting spheres. Sphere forming frequency was determined by dividing the number of observed gliomaspheres by the number of cells plated initially.

2.4.2 Colony forming assay

To determine colony-forming ability 1000 cells were suspended in 2.0ml of semi-solid media per well of a six well plate. Media consisted of: MegaCult-C 1.7ml (StemCell Technologies Inc.), NeuroCult Neural Stem Cell Proliferation Supplement 330µl (StemCell Technologies Inc.), EGF 6.6µl (human EGF, 10µg/ml, StemCell Technologies Inc.), basic FGF 3.3µl (bFGF, 10µg/ml, StemCell Technologies Inc.) and heparin 3.3µl (0.2% Sigma), penicillin/streptomycin 32µl and Collagen (StemCell Technologies Inc.) 1.3ml. Collagen was kept on ice and added after all other components. The assay continued for three

weeks and colonies were fed fresh media once every seven days. Weekly feeds of 60µl/well consisted of neurosphere media 900µl, EGF 50µl and FGF 25µl and heparin 25µl. Colonies were measured and counted by eye using a grid under a Zeiss Axio Observer.

2.4.3 Cell division rate assay

To identify different cell proliferation rates and isolate a population of slowly dividing cells human glioblastoma cells were loaded with CellTrace CFSE green fluorescent dye (Molecular Probes) and cultured for seven days in neurosphere media. Slow cycling cells were identified by flow cytometry based on their increased fluorescence due to greater dye retention. CFSE is divided equally between daughter cells at cell division resulting in weak fluorescence in cells that have undergone more cell divisions and brighter fluorescent intensity in cells that have undergone few cell divisions. The cells with strong fluorescent intensity, CFSE bright cells (top 5%) dilute the dye at a slower rate than the overall population (bottom 85%). Staining was quantified by flow cytometry (BD LSRII) and FLOWJO software.

2.4.4 2-DG assay

2-Deoxy-$_D$-glucose (2-DG, Sigma) was diluted to a final concentration of 4mM and added to cells plated into 96 well plates at 5000 cells per 100ul per well. Cell viability was measured after seven days. Cells were incubated at 37°C for 2.5 hours in 10µl colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide (MTT) assay (5mg/ml, Sigma) per well. Media was aspirated and 100µl solubilization solution (0.1N HCl, 10% TritonX in isopropanol) added before reading. Colorimetric analysis was performed at 690nm & 570nm using a standard microplate reader. The background absorbance of multiwell plates at 690 nm was subtracted from the 570nm measurements; the data was plotted in Prism and reported as the mean normalized to the vehicle control vials. The drug response assays were performed in triplicate for each cell line per assay with biological replicates per cell line. The data plotted represent the standard error of the mean from three independent experiments.

2.4.5 Temozolomide MTT assay

Cells were plated into 96 well plates at 5000 cells per 100ul media per well, with or without temozolomide (Sigma) to determine resistance to therapy. Temozolomide (TMZ, Sigma) was diluted to final concentration of 60μM in 100μl of media. MTT assay was performed as described above.

2.4.6 L-Lactate metabolic assay

GBM parent and clonal variants passaged under neurosphere conditions were chemically dissociated into a single cell suspension using trypsin and seeded at a density of 1 x 10^6 cells per ml into a flat bottom 96 well plate and incubated for 24 hours at 37^0C. L-Lactate assays were performed according to manufacturers instruction (Eton Bioscience kits). L-Lactate was added to wells at 50μl/well for a 30-minute incubation at 37^0C. Adding 50μl of 0.5M glacial acetic acid per well stopped the reaction. Absorbance was measured at 490nM using a microplate reader.

2.4.7 Immunohistochemistry

Haematoxylin and eosin staining was used to confirm tumour formation in tissue sections from mouse xenograft tumours. Human GBM cells were identified using an anti-human Nestin antibody (Millipore 1:500) alone or in combination with CD133 (Abcam 1:300). A human-specific MCM2 antibody (Santa Cruz 1:200) was used to identify human GBM cells that were competent to divide. Immunocomplexes were visualized in 3,3'-diaminobenzidine using the ABC-Elite peroxidase method (Vector laboratories) or using secondary antibodies conjugated to Alexflour 488 or 568 (Invitrogen 1:500) with DAPI (Invitrogen 1:1000).

CHAPTER THREE

3 Tumour initiation is associated with label retention in human glioblastoma

This work contributed to the following publication:
Deleyrolle, L.P. Harding, A. Cato, K. Siebzehnrubl, F. A. Rahman, M. Azari, H. Olson, S. Gabrielli, B. Osborne, G. Vescovi, A. Reynolds, B. A. Evidence for label-retaining tumour-initiating cells in human glioblastoma. Brain, 2011, 134, 1331-1343.

3.1 Introduction

Cancer stem cells are widely believed to be responsible for the formation and ongoing maintenance of tumours in a number of malignant conditions including CML (Barnes & Melo, 2006; Bonnet & Dick, 1997; Holyoake, Jiang, Eaves, & Eaves, 1999; Lapidot et al., 1994), pancreas (Dembinski & Krauss, 2009; L. Li & Neaves, 2006), head and neck cancers (Prince & Ailles, 2008), melanoma (Roesch et al., 2010) & breast cancer (Al-Hajj et al., 2003). Cancer stem cells have been reported in glioblastoma (Bao et al., 2006; Das, Srikanth, & Kessler, 2008) however controversies exist due to a lack of established functional markers for neural stem cells (Chojnacki, Mak, & Weiss, 2009).

One of the defining characteristics of somatic stem cells is their infrequent cell division due to cell cycle arrest, which is essential to prevent premature stem cell depletion over the lifetime of the organism (T. Cheng et al., 2000). As it is well established that cell cycle arrest protects cells from irradiation (Mohrin et al., 2010) and cytotoxic agents (Dean, Fojo, & Bates, 2005), it follows that the slow-cycling cancer stem cells will be resistant to conventional therapeutic approaches (Reya, Morrison, Clarke, & Weissman, 2001). Several groups have also utilized the propensity of label-retention to identify tumor-initiating cells from solid tumors such as in breast (Krishnamurthy, Wang, Rokhfeld, &

Bieberich, 2008; Pece et al., 2010), skin (Roesch et al., 2010) and pancreatic (Dembinski & Krauss, 2009) cancer.

In light of these observations, I hypothesised that post-therapy tumour regrowth is initiated from a subpopulation of slow cycling chemo/irradiation-resistant cancer stem cells that exhibit the basic stem cell-like property of self-renewal. Cancer stem cells that evade toxicity could then generate a separate population of rapidly dividing progeny that are responsible for repopulating the tumour bulk, much like somatic stem cells generate a rapidly dividing pool of precursor cells to replace damaged tissue. I predict that elimination of slow-cycling, therapy-resistant cancer stem cells is essential for the long-term survival of glioma patients. This subpopulation therefore represents a critical therapeutic target for the treatment of malignant gliomas.

Here, I describe the isolation and characterization of an infrequently cycling (*i.e.* CFSE retaining), tumor-initiating subpopulation in human glioblastoma cells that may represent a target to improve response to therapy.

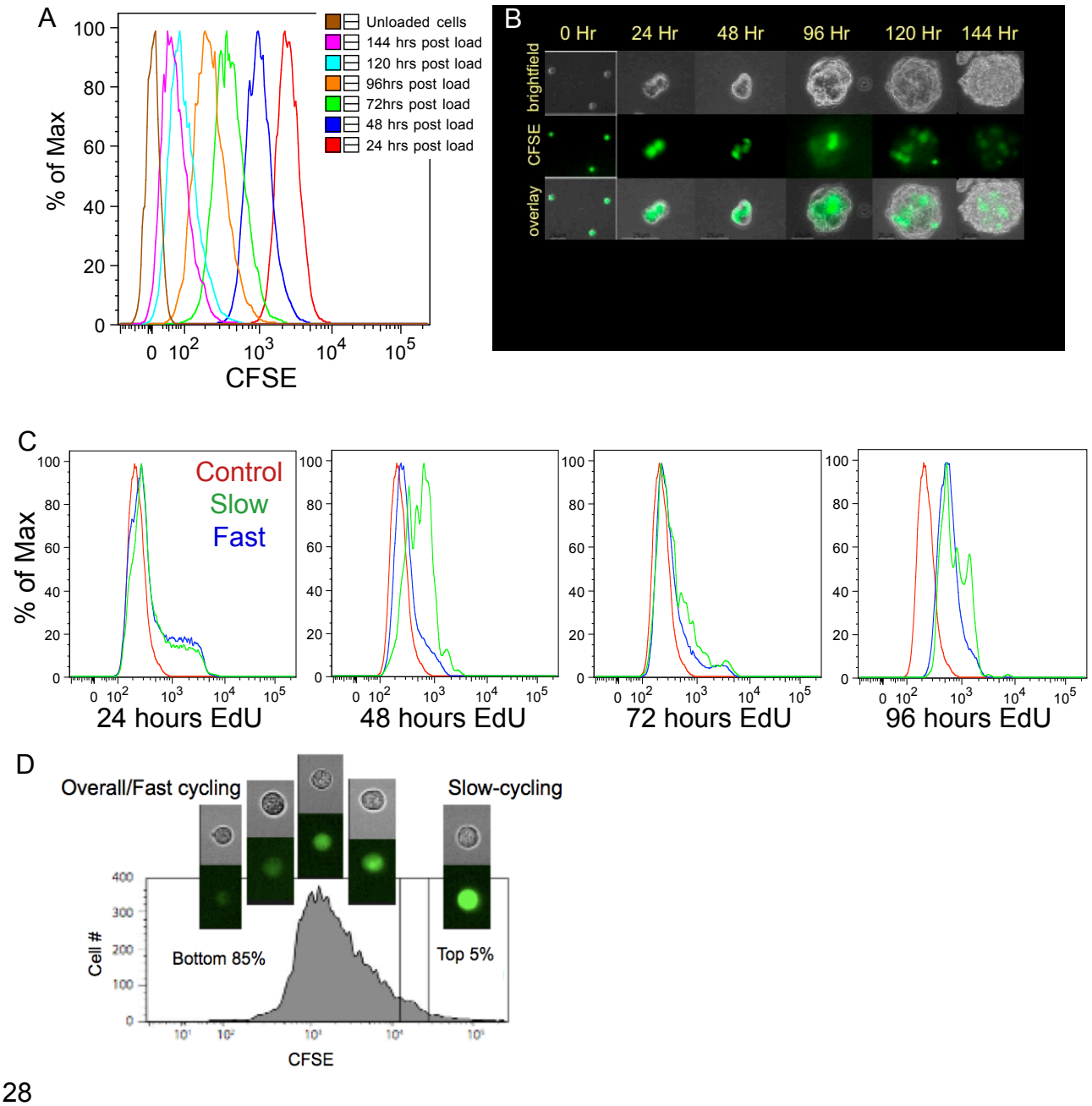
3.2 Results

3.2.1 Slow cycling cells exist *in vitro* in primary human GBM cell cultures

Fluorescent labels are commonly used in the study of cancers to identify quiescent cancer stem cells. The fluorescent label used in this study was the prodrug carboxyfluorescein diacetate succinimidylester (CFSE), which passively diffuses into cells and following cleavage by intracellular esterases, covalently binds to cellular proteins (Quah, Warren, & Parish, 2007). CFSE is equally divided between daughter cells at each cell division, thus providing information about the comparative division rates of all the cells within a population allowing the researcher to quantify the relative frequency of cell division over time based on CFSE staining intensity (De Boer, Ganusov, Milutinovic, Hodgkin, & Perelson, 2006; Hawkins et al., 2007). I chose CFSE because of its extensive use both *in vitro* and *in vivo* as a functional readout of cell division in immunology and cancers (Fei et al., 2010; Hawkins et al., 2007; Quah et al., 2007; Rodel et al., 2005).

All cell lines were generated from primary human GBM tissue, chemically dissociated and seeded as a single cell suspension into serum free neurosphere media (S. A. Louis et al., 2008). Human GBM cells passaged *in vitro* in neurosphere culture conditions maintain the

genotype and phenotype of the original tumour (J. Lee et al., 2006). To test whether staining intensity correlated with cell division in primary human GBM cell culture, I analysed human GBM tumour cell lines established from primary patient tumour tissue. Cells were stained with CFSE and the subsequent reduction in fluorescence was measured by flow cytometry for six consecutive days. The profile of CFSE staining in figure 3.1A shows a progressive decrease in CFSE concentration in human GBM cells from right to left, with unstained cells represented on the far left. This data shows that in human GBM cells, CFSE can be used to track cell divisions over time, and may therefore be a suitable tool to enrich for slow-cycling cancer stem cells in human GBM cell culture systems.



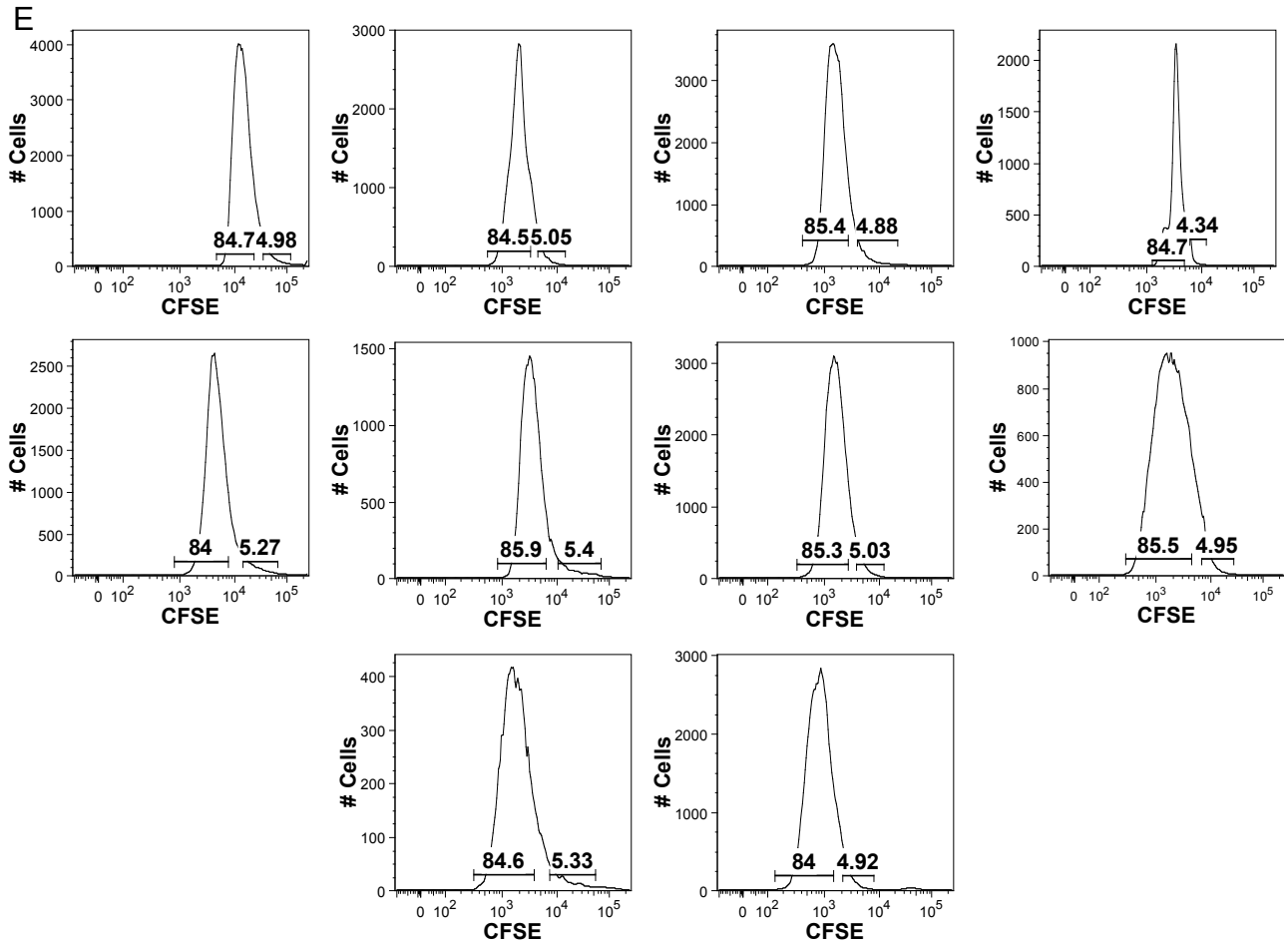


Figure 3-1 Identification of slow cycling human GBM cells *in vitro*. (A) Human GBM cells, established from primary patient GBM tumors were incubated in 5μM CFSE. CFSE is divided equally between daughter cells during cell division. The histogram shows the decreasing CFSE concentration in human GBM cells over six days of proliferation. (B) Tumoursphere formation by human GBM cell lines was observed after cells were stained with 5μM CFSE and allowed to proliferate in neurosphere culture media over 5-10 days. Developing tumourspheres were observed daily under a brightfield microscope. (C) The histograms indicate EdU incorporation and retention by human GBM cells. Cells were stained with CFSE and incubated for 45 minutes in EdU. During flow cytometry analysis, the first gate on CFSE retention established two populations, the top 5% CFSE high population which were called the slow cycling population and the bottom 85% CFSE low cells called the fast cycling population. The histograms show an overlay of the EdU incorporation by the slow cycling cells indicated by the red peak and the fast cycling cells indicated by the blue peak at various times post EdU pulse in hours. The incorporation and retention of EdU into the CFSE bright cells indicates that they are cycling and have undergone fewer cell divisions than the bulk of the population. (D) The figure demonstrates a typical distribution of CFSE levels in a human GBM neurosphere culture that was loaded seven days earlier. Above the flow cytometry histogram are representative cells showing different CFSE staining intensities. A slow-cycling population of cells was identified and isolated based on their capacity to retain CFSE (top 5%). To

the right a 10% gap has been used between populations of cells collected to minimize cross contamination between top 5% and bottom 85%. (E) The histograms show 10 human GBM cell lines. In each line, a slow cycling top 5% and fast cycling bulk 85% was identified based on CFSE retention.

Next, single cells derived in our lab from primary human GBM tumour tissue were loaded with CFSE, and the decrease in CFSE retention was monitored in developing neurospheres over six consecutive days. Neurosphere formation by the CFSE labelled human GBM cells was observed using a combination contrast microscope. In Figure 3.1B slow cycling cells can be seen in developing neurospheres when the majority of surrounding fast cycling cells have diluted out the CFSE marker over a number of cell divisions. These results show that CFSE retaining human GBM cells exist *in vitro* in proliferating cells cultured as neurospheres.

5-bromo-2'-deoxyuridine (BrdU) retention has been used to identify tumour-initiating cancer stem cells from breast tumours (Pece et al., 2010). 5-ethynyl-2-deoxyuridine (EdU) is a thymidine analogue and like BrdU is incorporated into DNA during strand synthesis (Chehrehasa, Meedeniya, Dwyer, Abrahamsen, & Mackay-Sim, 2009). Detection of EdU doesn't require the harsh DNA denaturation necessary with BrdU that can distort cell structure resulting in loss of the target antigen (Lin et al., 2009). To determine whether CFSE label retention correlates with a second, established label retention protocol, I double labelled GBM cells with EdU and CFSE and analysed this by flow cytometry. EdU labelling was performed for 45 minutes and retention was determined at intervals of 24 hours for five days by flow cytometry. The histogram in figure 3.1C shows that the top 5% slow cycling cells, as identified by CFSE-retention, did incorporate EdU and retained more EdU over time than the fast cycling 85% CFSE-low population. Increased EdU retention in the slow cycling cells provides a second, independent confirmation that slow cycling cells have undergone fewer cell divisions, and in combination with Pece et al. verifies the use of CFSE to monitor cell division rates in human GBM cells (Pece et al., 2010).

Utilizing the ability of flow cytometry to sort cell populations based on relative fluorescence, human GBM cells were divided into two populations. A CFSE high top 5% population of slow cycling cells and a bottom 85% CFSE low population of fast cycling cells with a 10% gap between the two to avoid contamination. To the right of the histogram in figure 3.1D a

small percentage of cells exhibit high fluorescent intensity in contrast to the cells to the left of the histogram that show decreasing fluorescence. The cells inserted above the histogram indicate decreasing CFSE concentrations with subsequent cell divisions throughout neurosphere formation. This is representative of the progressive reduction in fluorescent intensity associated with each cell division. We were able to identify a CFSE high, top 5% population in all our human GBM lines. Figure 3.1E shows the CFSE fluorescent intensity in 10 human GBM lines.

3.2.2 Slow cycling cells exist *in vivo* in mouse human GBM tumours

The tumour microenvironment is known to play a key role in tumour development (Hoelzinger, Demuth, & Berens, 2007). In particular, hypoxic regions within the tumour are thought to drive cells into a quiescent state and contribute to therapy resistance (Axelson, Fredlund, Ovenberger, Landberg, & Pahlman, 2005). To test whether slow cycling human GBM cells are quiescent *in vivo*, two xenograft models of GBM tumours were used, an intracranial model, and a subcutaneous model. Human GBM cells were stained with CFSE and immediately injected into the striatum of NOD-SCID mice. Figure 3.2A shows the distortion of the tumour bearing cerebral hemisphere caused by the progressive enlargement of a GBM tumour. Six weeks after human GBM cells were injected into the mouse brain, CFSE bright cells can be seen within the bulk of the tumour in tissue sections using immunofluorescence (Figure 3.2B). This result confirmed the legitimacy of our strategy to use *in vivo* mouse models to study slow cycling cells in GBM and provides further evidence that slow cycling CFSE-retaining cells persist *in vivo*.

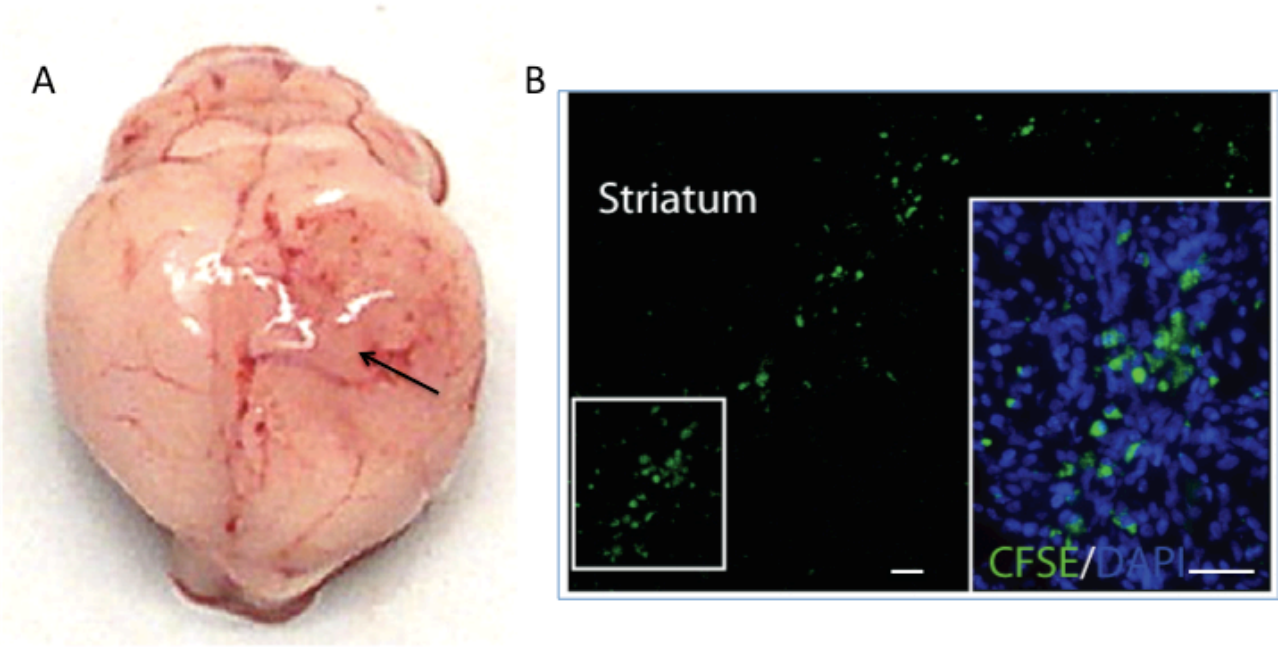


Figure 3-2 Slow cycling cells exist in orthotopic xenograft mouse tumours. (A) Injections of 200,000 human GBM cells were administered into the striatum of anaesthetized mice and animals monitored for signs of deteriorating health during tumour formation, usually three - six months. The arrow indicates the tumour bearing cerebral hemisphere is enlarged and distorted below the site of tumour cell injection. (B) Mouse brain section showing CFSE bright cells**.** CFSE retaining cells can be identified in proliferating mouse brain tumours *in vivo* six weeks post-transplant of 200,000 CFSE stained human GBM tumour cells into the striatum. The scale bar represents 50μm.

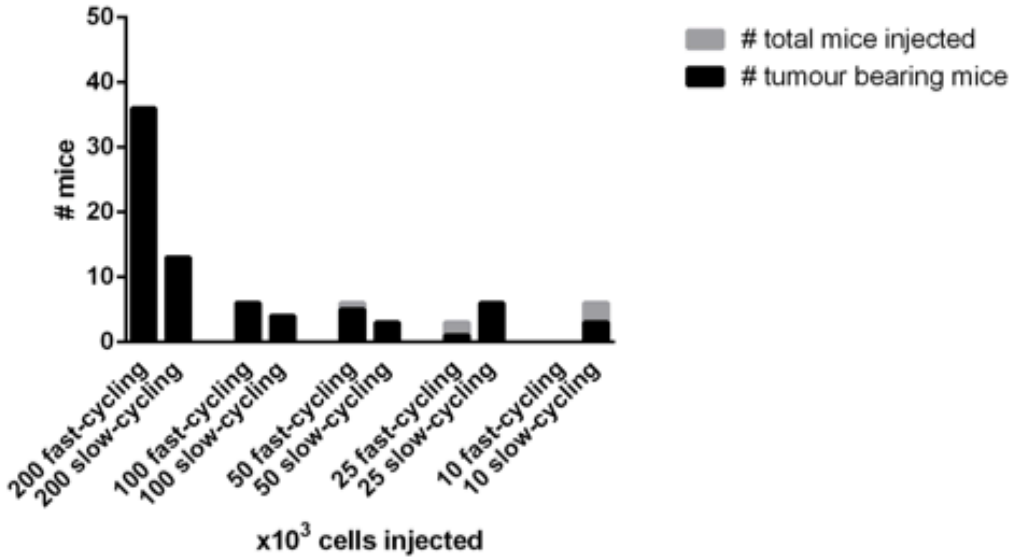
3.2.3 Slow cycling human GBM cells form tumours *in vivo*

Several studies have confirmed that slow cycling cell populations in other tumours are enriched for tumour-initiating cells (TICs). We next sought to confirm whether slow cycling cells are able to form tumours *in vivo*. To determine the capability of human GBM cells to form orthotopic xenograft mouse tumours, 2 x 10^5 human GBM cells were injected into the striatum of SCID mice. This technique resulted in 100% success in generating large tumours that eventually resulted in death.

Next we wanted to compare the tumour forming capabilities of slow cycling cells compared with fast cycling cells. To examine this in human GBM tumours we injected either 2 x 10^5 slow cycling cells or 2 x 10^5 fast cycling cells into the striatum of SCID mice using three cell lines, Line 0, Line 1 and Line 2, and we observed tumour formation in 100% of mice injected. This was followed by limiting dilution assays, where reducing numbers of cells

were injected into mouse brains to determine tumour initiation efficiency. In limiting dilution, only one of three mice injected with 25,000 fast-cycling cells developed a tumour while six out of six mice injected with 25,000 slow-cycling cells developed tumours. When 10,000 slow-cycling cells were injected into mice, tumour initiation was successful in 50% of mice injected (Figure 3.3A and 3.3B). This data shows that tumour initiation is a feature of both the slow cycling and fast cycling cells and not a distinctive feature of slow-cycling human GBM cells. However, slow cycling cells display a small but statistically significant increase in tumour initiation frequency (TIF). These data suggest that slow cycling cells are not quiescent, senescent or terminally differentiated since they are still capable of cycling and tumour initiation and this population is somewhat enriched for cells able to initiate tumours *in vivo*.

A



Number of tumours/ Number of mice injected					
Cell population	200,000 cells	100,000 cells	50,000 cells	25,000 cells	10,000 cells
Fast-cycling cells	36/36	6/6	5/6	1/3	N/D
Slow-cycling cells	13/13	4/4	3/3	6/6	3/6

B

Time for tumour formation (weeks) cells per injection					
	200 000	**100 000**	**50 000**	**25 000**	**10 000**
Overall population	11.1 +/- 0.5	12.3.+/- 0.3	13.8 +/- 1	12	ND
Slow cycling cells	19.1 +/- 0.9	15.5 +/- 4.1	19 +/- 1.5	17.3 +/- 0.8	23

Figure 3-3 Slow cycling cells show an increase in orthotopic xenograft tumour initiation frequency. (A) In limiting dilution assays, NOD-SCID mice injected intracranially with 200,000, 100,000, 50,000, 25,000 and 10,000 (slow-cycling only) fast-cycling and slow-cycling primary patient human GBM cells were monitored for tumour formation. (B) The tumour initiating frequency is shown for the overall population and the slow cycling cell population. The time-frame for tumour development is shown as average ± SEM.

3.2.4 Sphere and colony formation are not characteristics exclusive to slow cycling human GBM cells

The neurosphere assay can be used to measure sphere formation and has been shown to be a predictor of GBM disease progression (Laks et al., 2009). To determine the sphere-forming frequency of slow cycling cells, CFSE-labelled cells were sorted according to CFSE intensity into top 5% and bottom 85%, and identical numbers of viable sorted cells were seeded into 384 well plates to assess sphere-forming frequency. Figure 3.4A shows that in two human GBM lines both the slow cycling and fast cycling cells were able to proliferate and form spheres although the slow cycling cells formed fewer spheres and at a slower rate. This experiment was performed in triplicate. This finding suggests that both cell populations are capable of tumour formation and that sphere formation is not a characteristic unique to or enriched in slow cycling cells.

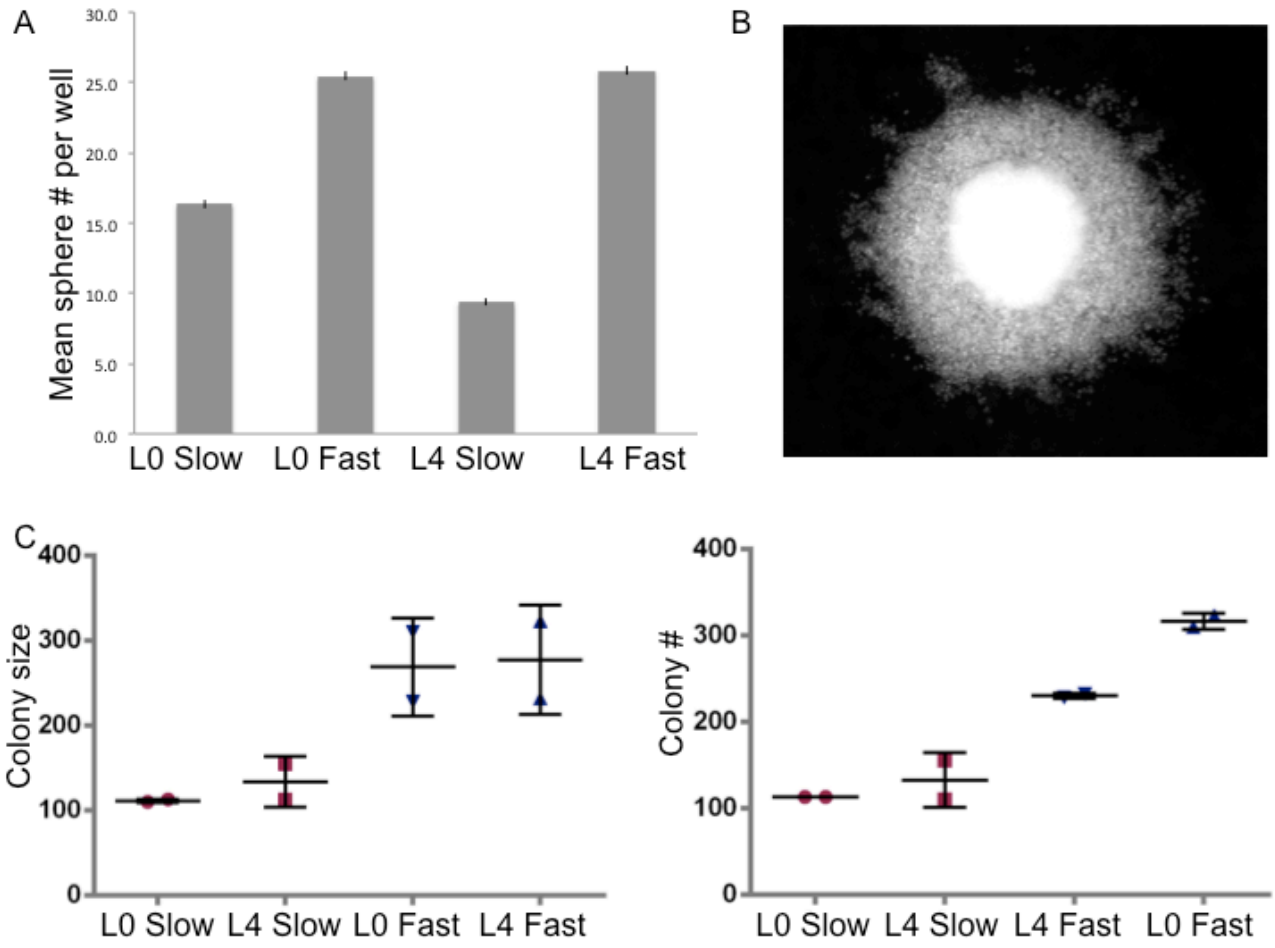


Figure 3-4 Sphere and colony formation are not characteristics exclusive to slow cycling human GBM cells *in vitro*. (A) Two human GBM lines showing sphere formation in slow cycling and fast cycling cells. Cells were seeded into 384 well plates 50µl per well at a density of 10,000 cells per ml. Twenty wells were seeded per row per condition. This was done in triplicate. The mean and SEM were calculated for each, as shown. (B). The image shown is of a typical colony of human GBM cells formed by a cell with extensive self-renewal capabilities in semi-solid media. (C) Colony forming assays representing number and size of colonies generated by the fast cycling cells and slow cycling cells of two human GBM cell lines, Line 0 and Line 4 over three weeks. Cells were seeded in a single cell suspension into six well plates at 1000 cells/ml, two wells per condition. Mean and SD shown.

Similarly, the ability of somatic neural stem cells to form large colonies when grown in solid collagen growth conditions has been established as a robust assay for quantitating the abundance of neural stem cells from culture and primary mouse brain cells (S. A. Louis et al., 2008). To assess the ability of slow cycling cells to proliferate and form large colonies we seeded single cells into a semi-solid collagen media (Figure 3.4B). The formation of large colonies indicates extensive proliferative ability, which is a characteristic of stem

cells. In our colony-forming assay the average colony size was larger in fast cycling cells than slow cycling cells. We also noticed that slow cycling cells formed fewer colonies compared to that of the fast cycling cells (Figure 3.4C). This result shows that fast cycling cells are more proliferative than slow cycling cells and therefore colony formation is not an exclusive characteristic of human GBM slow cycling cells.

3.2.5 Slow cycling human GBM cells are dissimilar to somatic stem cells in their cell cycle progression

Infrequent cell division was used to enrich for cancer stem cell-like cells for two reasons. First, somatic stem cells are predominantly quiescent (that is, in the G0 phase of the cell cycle), and if cancer stem cells (CSC) phenotypically mimic somatic stem cells, a significant proportion of CSCs should also be quiescent and therefore cycle infrequently. Second, identification of a quiescent human GBM CSC subpopulation has important therapeutic implications, as many chemotherapeutic agents target actively cycling cells and quiescent cells are therefore less susceptible to those therapies, as has been observed in CML (T. Cheng et al., 2000; Komarova & Wodarz, 2007). Thus quiescence provides a potential mechanism for the reported resistance to radiotherapy in human GBM cancer stem cells (Bao et al., 2006). By discriminating between the slow and fast cycling cells in human GBM we were able to directly test whether these two distinct populations differed in their sensitivity to radiation treatment.

Quiescent cells pause during the cell cycle; consequently, identifying quiescent cells requires the use of multiple markers, as this subpopulation is defined by the absence of markers. I define quiescence as a reversible state of dormancy during which the cell has temporarily withdrawn from the cell cycle (Cheung & Rando, 2013). Three markers were selected in addition to CFSE to determine if the slow cycling cell population was quiescent. 1) DNA content, which can be used to identify 2n peak, 2) Ki67 which is not expressed by cells in the G0 phase of the cell cycle (Scholzen & Gerdes, 2000), and is used clinically to determine the proliferative potential of patient glioma specimens, to grade tumours and to predict patient prognosis (P. C. Burger, Shibata, & Kleihues, 1986; Karamitopoulou, Perentes, Diamantis, & Maraziotis, 1994) and, 3) EdU which can identify cells actively replicating their genome (Chehrehasa et al., 2009). I define quiescent G0 cells as CFSE-retaining cells that have 2n DNA content and are negative for both Ki67 and EdU.

Initially, somatic mouse adult neural stem cell cultures were examined. Figure 3.5A shows the DNA content and cell cycle distribution of adult mouse somatic neural stem cells and human GBM cells. Incorporation of EdU indicates the presence of actively cycling cells. Ki67 is only expressed by proliferating cells (Scholzen & Gerdes, 2000). Figure 3.5B shows fast cycling mouse adult neural stem cells overlayed with the slow cycling cells and the relative incorporation and expression of EdU and Ki67 as assessed by flow cytometry. The fast cycling mouse neural stem cells expressed Ki67 and incorporated EdU in contrast to the deficit seen in the slow-cycling cells reflecting a rapid proliferation rate. The mouse label-retaining neural stem cells have all the hallmarks of quiescent, G_0 cells, an absence of proteins expressed by cycling cells.

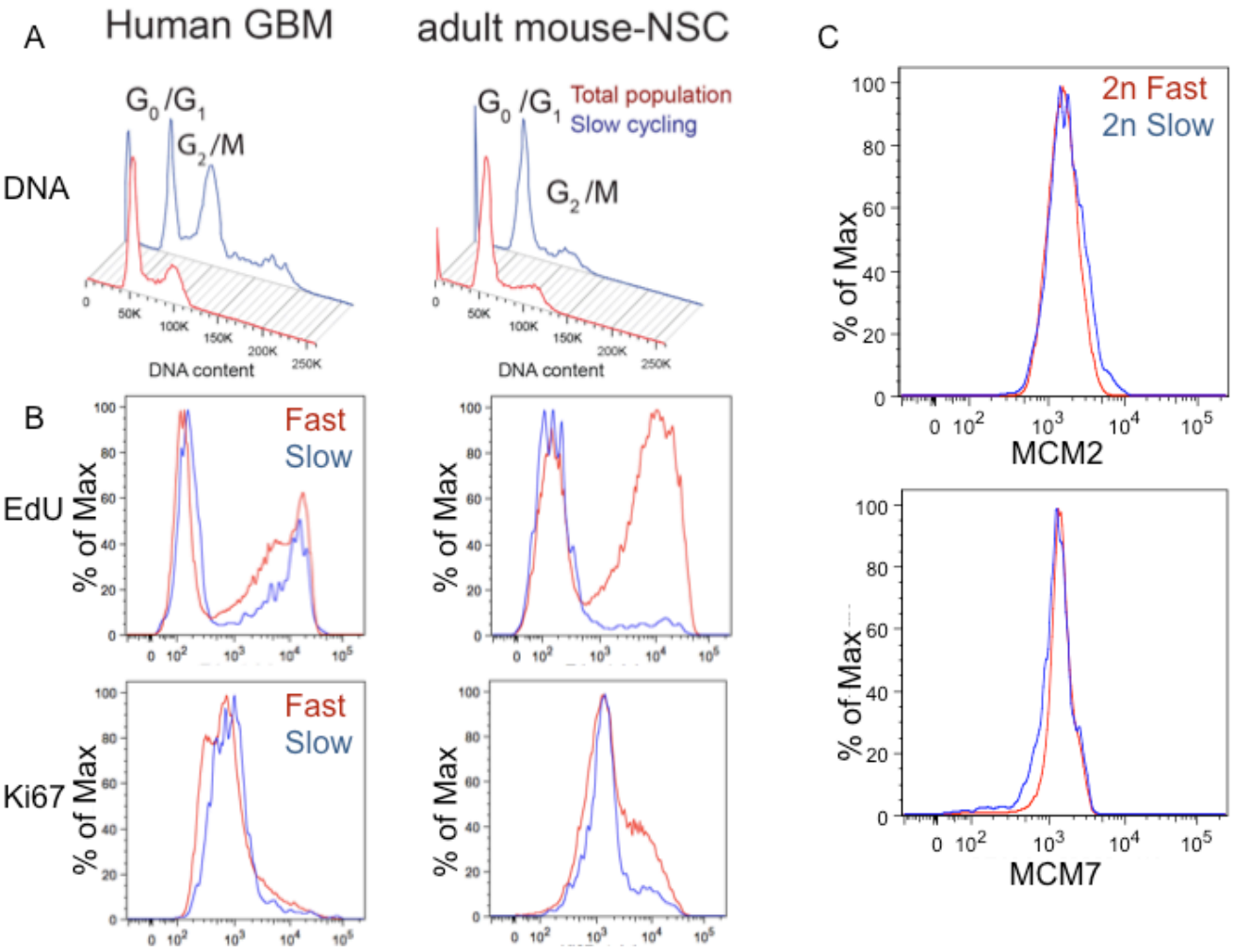


Figure 3-5 Slow cycling human GBM cells are dissimilar to somatic stem cells in their cell cycle progression. (A) DNA staining shows cell cycle profiles for total population and slow cycling mouse adult neural stem cells (mANSC) and human GBM cells. Cells passaged in neurosphere culture conditions were first stained with 5μM CFSE then passaged for five days. Spheres were chemically dissociated and DAPI stained at 4μg/ml with 1 x 10^6 cells per well in a 96 well 'V' bottom plate. During flow cytometry analysis a gate isolating the top 5% CFSE-bright cells was used to identify the slow cycling population. DNA content for each population was determined according to DAPI staining intensity. The red histogram shows the total population, the blue histogram the slow-cycling (top 5% CFSE high) population. There are two peaks in the histograms. The first peak indicates cells in the G0/G1 phase of the cell cycle and 2n DNA content and the second peak indicates the G2/M phase and 4n DNA content. (B) Human GBM and mANSC were incubated for 10 minutes in 5μM CFSE then passaged for five days. CFSE labeled cells were chemically dissociated and incubated for 45 minutes in 10μM 5-ethylnyl-2'-deoxyuridine (EdU), fixed in 4% formaldehyde in PBS and permeabilized in ice-cold methanol. Analysis was performed by flow cytometry. Approximately 250,000 cells were analysed per tube. The red histogram shows the bulk of the population, bottom 85%, CFSE diluting, fast cycling cells. The blue histogram shows the slow cycling, top 5%, CFSE retaining cells. The fast cycling adult mouse NSC retain more EdU and express more Ki67 than the slow cycling adult mouse NSC. Slow and fast cycling human GBM cells incorporate EdU and express Ki67 at approximately the same rate. (C) The figure shows expression of proliferation markers MCM2 and MCM7 in the 2n populations of slow and fast cycling human GBM cells after normalizing for DNA content.

Having confirmed that the CFSE-retaining cell population of our somatic neural stem cells was enriched in cells displaying a quiescent phenotype, we next sought to confirm that the human GBM CFSE-retaining, slow cycling cell population was also enriched in quiescent cells. Surprisingly, and in contrast to our somatic neural stem cell control, human GBM slow cycling cells are not quiescent (G_0), as slow cycling human GBM cells expressed Ki67 and incorporated EdU at rates that approximated that of fast cycling cells. Intriguingly, there was consistently more 4n (G2/M peak) and 4n+ (hyperdiploid) cells in the slow cycling cell population than in the fast cycling population for all lines tested.

Minichromosome maintenance complex (MCM) proteins are only expressed by actively cycling cells. To extend the cell cycle analysis of slow cycling human GBM cells, MCM2 and MCM7 protein expression was assessed for human GBM cells. The MCM2-7 complex activated during S-phase is essential for the separation of the double-stranded DNA at the

replication fork (Vijayraghavan & Schwacha, 2012). After normalizing for DNA content, the MCM protein expression was the same for the fast and slow cycling cells (Figure 5C). This data provides further experimental evidence showing that slow cycling GBM cells are not quiescent but are actively cycling.

In conclusion, the results presented here reveal that CFSE-retention does enrich for quiescent cells in somatic neural stem cell cultures. This confirms that the neurosphere culture system, where long-term proliferation is driven by somatic neural stem cells, does maintain a quiescent subpopulation. In contrast, human GBM cultures grown under identical culture conditions either do not have a quiescent cell population or it is significantly reduced compared to the neural stem cell control.

3.2.6 Hyperdiploid human GBM cells exhibit an increase in stem cell marker expression

During analysis the cell cycle progression of slow and fast cycling cells we noticed that the slow-cycling CFSE-retaining top 5% population of cells contained proportionately more hyperdiploid cells than the human GBM cell population overall (Figure 3.6A and B).

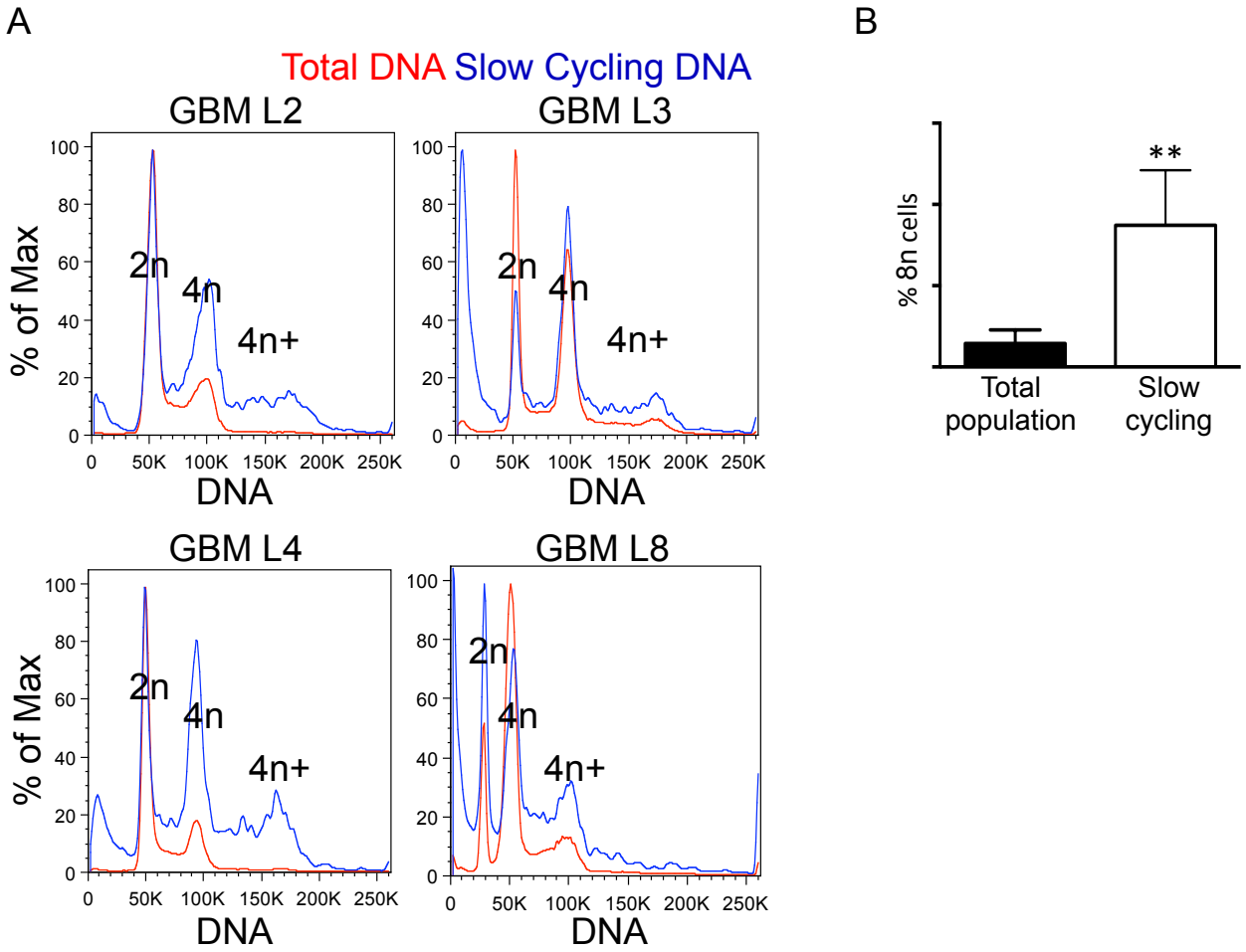


Figure 3-6 Slow cycling human GBM cells do not exhibit stem-like cell characteristics. (A) Cell cycle analysis shows the DNA content of the total cell population in red and the slow cycling population in blue for four human GBM cell lines. (B) The slow cycling human GBM cell population is enriched with a subpopulation of hyperdiploid cells. Human GBM cells were chemically dissociated into a single cell suspension and incubated in DAPI at 4µg/ml before being fixed in 4% formaldehyde and DNA content analysed. The percentage of cells containing 8n DNA was identified for the total population and the slow cycling population in four independent experiments using gating during flow cytometry. An average 2.9% of the total cell population and 17.4% of slow cycling cells fell within the 8n gate (*p* value = 0.0059).

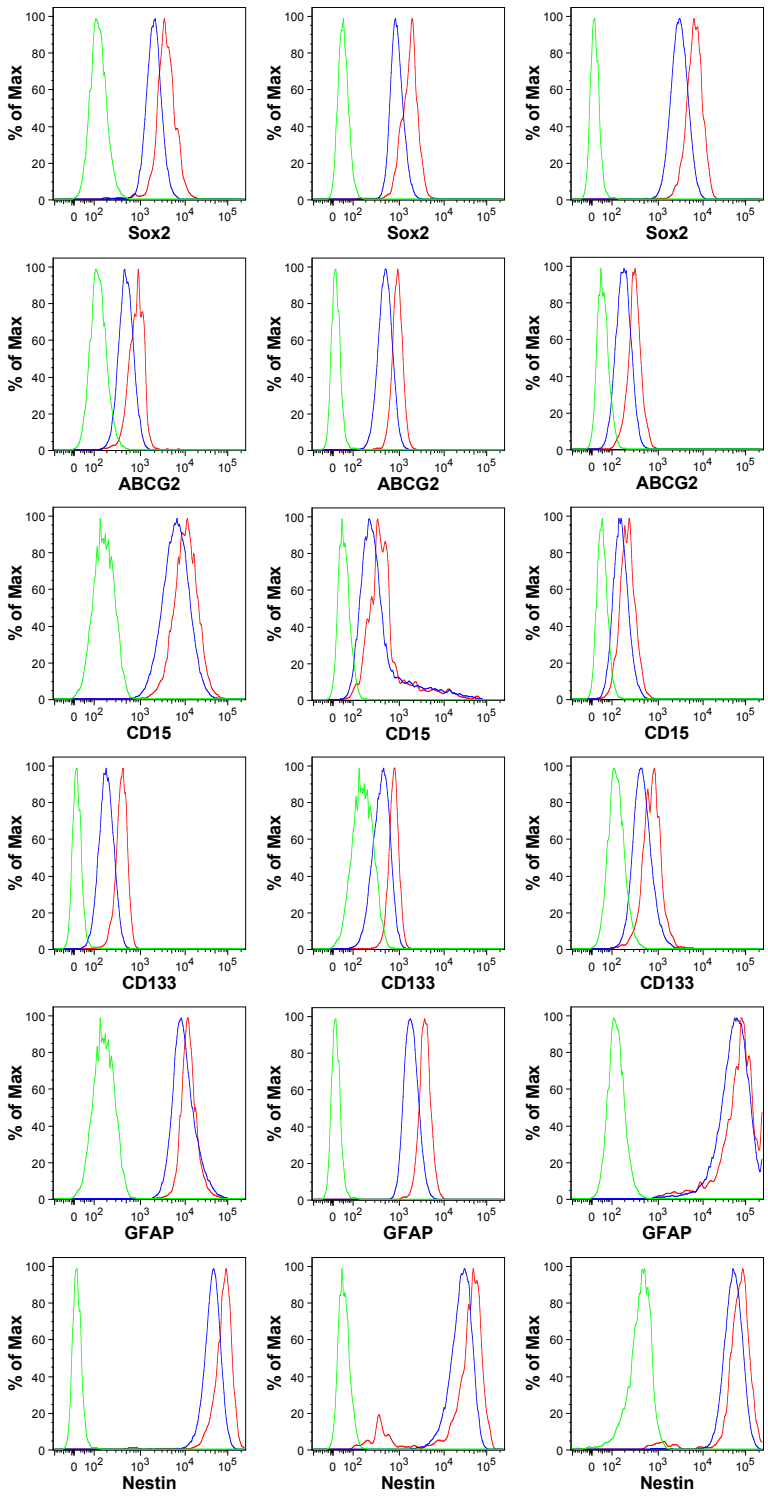
Several studies have reported the use of cell surface protein expression to identify and enrich for brain cancer stem cells (Mao et al., 2009; Ogden et al., 2008). Hyperdiploidy gives rise to cells that display elevated levels of protein expression due to gene amplification and changes in transcriptional programming (Pavelka et al., 2010). Therefore we hypothesised that hyperdiploid GBM cells may display increased expression of cell surface markers commonly used to identify cancer stem cells. If true, enrichment

strategies based on cancer stem cell marker expression would enrich for viable hyperdiploid cells, and these cells could therefore be contributing to the cancer stem cell phenotype reported in these studies. To test this hypothesis, we selected a panel of six stem cell markers (Table 3.1) and compared the cancer stem cell marker expression in the 4n+ hyperdiploid population to the bulk population of three passaged human GBM lines.

Table 3-1 Known stem cell functions. Table describes the known functions of stem cell markers in the analysis.

Marker	Protein	Function	References
ABCG2	ATP binding cassette membrane transporter/ membrane pump	An efflux pump that utilizes ATP to move small substances, including toxins across the cell membrane providing protection. Particularly active in brain endothelial cells contributing to the blood brain barrier and in neoplastic cells.	Bleau, A. M. 2009.
CD15	Stage-specific embryonic antigen 1 (SSEA-1)	Cell-cell adhesion particularly astrocytes.	Mao, X. G. 2009. Pruszac, J, 2009.
CD133	Prominin-like 1	Located in the cytoplasm and on the cell surface in human neural stem cells. Function unknown. Possibly associated with migration and polarity. CD133 antigen with the glycosylated epitope AC133 is only expressed by stem cells.	Bao, S. 2006. Dell 'Albani. J. 2008.
GFAP	Glial fibrillary protein	The most specific protein used to identify astrocytic cells. An astrocytic intermediate filament protein. Phosphorylation of GFAP regulates polymerization and depolymerization of filaments in mitosis.	Das, S. 2008. Imura, T. 2006.
Nestin	A cytoplasmic intermediate filament protein	Expressed in undifferentiated cells and radial glia during development. Nestin phosphorylation in mitosis facilitates dissociation of intermediate filaments. It is down regulated at differentiation. Reexpression has been seen in reactive astrocytes associated with injury and ischaemia.	Zhang, M. 2008. Park, C. H. 1971. Kornblum, H. I. 2001
Sox2	SRY (sex determining region Y box 2)	A transcription factor that maintains neural stem cells, it's lost at neural differentiation. Sox2 is overexpressed in glioma.	Brazel, C. Y. 2005. Pevny, L. H. 2010.

The markers selected were CD15, CD133, ATP-Binding Cassette, sub-family G (white), member 2 (ABCG2), Glial fibrillary acidic protein (GFAP), Nestin and Sox2. The ABCG2 drug transporter is widely understood to export chemotherapeutic substances from stem cells and cancer stem cells and is reportedly expressed at high levels in numerous cancers including glioma (Bleau, Huse, & Holland, 2009; X. Li et al., 2010). CD133 is reputed to be a marker for both stem cells and brain cancer stem cells and to confer radioresistance to glioma cells that express it on the cell surface (Bao et al., 2006; Dell'Albani, 2008). CD15 is a known stem cell marker and more recently purported to be a marker of brain tumour stem cells (Mao et al., 2009; Pruszak, Ludwig, Blak, Alavian, & Isacson, 2009). The intermediate filament protein GFAP is expressed on stem cells in the nervous system (Das et al., 2008; Imura, Nakano, Kornblum, & Sofroniew, 2006). Nestin is recognized as an intermediate filament protein and commonly used stem cell marker because of its expression by cells in the developing neural tube (Kornblum & Geschwind, 2001; Park, Bergsagel, & McCulloch, 1971; M. Zhang et al., 2008). Sox2 is a well-recognized marker of adult neural stem cells and has been identified both *in vitro* and *in vivo* (Brazel et al., 2005; Pevny & Nicolis, 2010). We analysed the expression of these six potential cancer stem cell markers in human GBM cells by flow cytometry. Interestingly when the hyperdiploid cells were compared with the diploid cell population, an increase in stem cell markers was apparent in the hyperdiploid population (Figure 3.7). Stem cell markers are used in other cancers to identify the tumour initiating cell population. As the 4n+ population of human GBM cells lies within the label retaining group, previous studies may have overlooked this population, attributing the traits of a hyperdiploid minority of cells to a cancer stem cell population.



Secondary Only Control

Diploid

Hyperdiploid

Figure 3-7 Hyperdiploid primary human tumour cells express higher levels of stem cell markers. Cells from three primary human GBM tumours were stained with antibodies for six recognized stem cell markers: Sox2, ABCG2, CD15, CD133, GFAP and Nestin. During flow

cytometry analysis, gating isolated the hyperdiploid cells in each cell population so that antibody labelling could be directly compared.

Strikingly, in all lines tested and for all cancer stem cell markers, the hyperdiploid subpopulation displayed elevated expression of cancer stem cell markers relative to the bulk population. These results indicate that hyperdiploid tumour cells will be enriched in strategies designed to isolate putative cancer stem cells, and suggests the intriguing hypothesis that many of the clinically relevant phenotypes linked with cancer stem cells (such as tumour initiation and therapy resistance) could in fact also be displayed by hyperdiploid GBM tumour cells.

3.3 Discussion

The notion that a self-renewing, infrequently cycling, cancer stem-like cell population is responsible for tumor initiation is well established in leukemias (Graham et al., 2002; Holyoake et al., 1999). While an infrequently cycling compartment has been described in solid tumors such as breast cancer (Krishnamurthy et al., 2008; Pece et al., 2010), pancreas adenocarcinoma (Dembinski & Krauss, 2009) and melanoma (Roesch et al., 2010) a similar population has yet to be identified in brain tumors.

3.3.1 CFSE can be used to identify slow cycling cells

Here we identified a subpopulation of label-retaining cells within human GBM that exhibited slower progression through the cell cycle, compared to the bulk of the tumor cells as well as an enhanced ability to form tumors *in vivo*; features that are consistent with a tumor-initiating cell. To track cell divisions and identify slower cycling cells, we used functional labeling with the lipophilic, non-fluorescent precursor, CFDASE. The probe is activated by intracellular esterase activity converting it to fluorescent CFSE while covalently coupling it to amino groups where it becomes cell permanent and is diluted in half at each cell division (Lyons, 2000). Cells exhibiting higher CFSE epifluorescence over time corresponded to slow dividing cells, which was confirmed using nucleoside analog incorporation of EdU followed by a chase period, correlating label-retaining cells with CFSE intensity (Golmohammadi et al., 2008). This shows that CFSE retention directly correlates with EdU retention (Figure 2B), providing direct confirmation for the hypothesis that a subset of human GBM cells cultured as tumorspheres retain CFSE due to reduced

cell division. In sum, these results, together with the extensive literature using CFSE to track cell division (Graham et al., 2002; Lyons, 2000; Moore, Houghton, & Lyle, 2012), demonstrate CFSE labeling as a valid approach to identify and isolate sub-fractions of cells based on frequency of cell division.

3.3.2 CFSE retaining cells are actively cycling

Although the primary aim of this study was the enrichment of quiescent cancer stem cells, our data provided a series of unexpected findings that are not consistent with the classical cancer stem cell model. A basic cell cycle analysis yielded the surprising result that in GBM tumoursphere cultures and *in vivo* GBM tumours, slow cycling cells were not enriched in quiescent cells but in fact were enriched in cells harboring a 4n DNA content that were positive for EdU and Ki67, and therefore actively cycling, although at a slower rate. This is unlikely to be due to experimental artifact, as CFSE has been extensively characterized *in vitro* and *in vivo* as a marker for cell division (De Boer et al., 2006; Hawkins et al., 2007). In addition, in our hands and using identical culture conditions, somatic adult neurosphere cultures revealed that CFSE retention does enrich for cells displaying all the hallmarks of cellular quiescence (i.e. 2n DNA content, Ki67 negative and EdU negative). These results confirm that in cultures containing quiescent cells, CFSE-retention does enrich for the G_0 sub-population. To provide additional experimental evidence that the slow cycling human GBM cells were not quiescent, we tested them for expression of the replication licensing proteins MCM2 and MCM7, which are absent from quiescent cells and expressed only in cycling cells that have been licensed to replicate their genome (Blow & Gillespie, 2008). CFSE retaining GBM cells expressed both MCM2 and MCM7, confirming that the majority of CFSE-retaining cells are licensed to replicate their genome and therefore not in a quiescent state. Altogether these data suggest that unlike CML, where a quiescent cancer stem cell population has been identified using CFSE retention, the CFSE retaining human GBM cell population does not contain a significant number of quiescent cells.

3.3.3 Slow cycling human GBM cells do not exhibit superior sphere or colony forming ability

To extend this unexpected outcome, we then used functional assays to assess the enrichment of cancer stem cells from GBM using CFSE retention. The frequency of sphere

formation under neurosphere culture conditions correlates with the presence of somatic neural stem cells and progenitor cells (Azari, Rahman, Sharififar, & Reynolds, 2010). If CFSE retention does enrich for cancer stem cells, then one prediction would be a commensurate increase in the sphere forming frequency due to an increase in the number of cancer stem cells and progenitor cells within the CFSE retaining population. The slow cycling human GBM population displayed a significantly reduced sphere forming capacity to the tumour bulk. To extend these findings we used a second functional readout for stem cell function, the colony-forming assay. In this system, only somatic stem cells can generate large colonies during culture due to the stem cell property of self-renewal that is absent from the progenitor and differentiated subpopulation. In our hands, human GBM cultures were incapable of generating the large colonies found in somatic neural stem cell cultures invalidating this assay system for the identification of GBM cancer stem cells. However, when comparing the slow cycling population with the tumour bulk, the slow cycling population always generated significantly fewer colonies than the tumour bulk. This is the opposite of what would be expected if the slow cycling population were enriched in cancer stem cells. However, this result could be due to a number of factors. Within a heterogeneous cell population cell cycle times may vary resulting in a greater contribution from some cells than others. Some cell divisions may be asymmetrical giving rise to a subpopulation of cells incapable of proliferation.

3.3.4 Slow cycling cells were more proficient at tumour formation and harbor a hyperdiploid subpopulation with increased stem cell marker expression

In contrast to the predictions of the stem cell hypothesis, when we injected 50,000 of either slow or fast-cycling cells into the striatum of NOD-SCID mice, tumours formed in all mice. However, the slow-cycling cells were more proficient at tumour formation, requiring fewer injected cells than fast-cycling cells. Upon injection of 25,000 cells, slow-cycling cells formed tumours 100% (n=6) of the time while the total cell population formed tumours at a rate of 1:3 (n=3) at this dilution. With the intracranial injection of 10,000 cells, the slow-cycling cell population was capable of tumour formation in 50% of cases (3/6). In isolation, this finding might suggest support for the cancer stem cell hypothesis. When taken in combination with our data showing that increased levels of stem cell markers are expressed in the population of label retaining cells that exhibited a DNA content of 4n+, we hypothesize that the hyperdiploid cells within the top 5% CFSE retaining cells are the tumour initiating cells.

3.3.5 Slow cycling cells have an advantage during tumour regeneration.

The finding that slow cycling cells are more proficient at tumour formation than fast cycling cells is counter intuitive. It seems logical that a population of fast cycling cells would normally generate a tumour faster than a slow cycling cell population and as a result, be the cohort that were more proficient at tumour formation. However, tumour regeneration following surgery, radiotherapy and chemotherapy is believed to be the result of a small number of therapy resistant cells that are capable of replication and go on to form another tumour, often adjacent to the site of the original tumour. Our findings that fewer slow cycling cells were required for tumour formation than fast cycling cells suggest that the slow cycling cells have an advantage during tumour regeneration.

3.3.6 Hyperdiploid cells may have superior survival capabilities.

Our data revealed a feature of slow cycling cells. Within the slow cycling cell population there was a cohort of cells with an increased DNA content. The finding that slow cycling cells were more proficient at tumour formation suggests that extra copies of genes could provide a cell with the ability to withstand a harsh environment by supplying nutrients or overcome cell cycle inhibition mechanisms to evade cell death. We wanted to investigate further the potential role and advantageous characteristics of slow cycling 4n+ DNA cells in GBM.

3.4 Conclusions

The distribution of CFSE staining within human GBM cultures reveals that cells within the human GBM culture have varied rates of proliferation. This finding suggests that CFSE-retention can be used to isolate infrequently cycling cancer cells based on a functional marker (dye retention). The slow-cycling cell population in GBM do not exhibit the typical stem cell characteristics of quiescence and recognised stem cell marker presentation. They do not form larger colonies than their fast-cycling counterparts or exhibit superior sphere formation.

However, the slow-cycling cell population exhibited superior tumour forming ability *in vivo.* The greatest challenge facing cancer researchers, clinicians and patients today is tumour regeneration following therapy. These findings suggest that fewer slow cycling cells are

required at the completion of therapy for efficient tumour regeneration. Within the slow cycling cell population we identified a cohort of cells with a hyperdiploid DNA content that displayed elevated expression of cancer stem cell markers relative to the bulk population implicating hyperdiploid cells within the slow cycling fraction as a population of interest for further study. A deeper understanding of the potential points of fragility of this cohort is required to be able to begin to identify therapeutic candidates.

CHAPTER FOUR

4 Hyperdiploid glioblastoma cells are capable of tumour formation and show increased sensitivity to metabolic inhibition

This work contributed to the following publication:

Donovan, P. Cato, K. Legaie, R. Jayalath, R. Olsson, G. Hall, B. Olson, S. Boros, S. Reynolds, B. Harding, A. Hyperdiploid tumour cells increase phenotypic heterogeneity within glioblastoma tumours. Molecular Biosystems, 2014. 10, 741-758.

4.1 Introduction

Therapeutic resistance is one of the most challenging obstacles thwarting the efforts of researchers involved in the development of anticancer drugs today. The development of therapeutic resistance mechanisms is widespread across cancer types and progressive throughout the course of the disease (Garraway & Janne, 2012; Szakács, Paterson, Ludwig, Booth-Genthe, & Gottesman, 2006). The potential mechanisms underpinning GBM resistance to conventional therapy are diverse. Therapy resistance can be mediated by microRNA expression (Besse, Sana, Fadrus, & Slaby, 2013), receptor tyrosine kinase amplification and activation (Hatanpaa, Burma, Zhao, & Habib, 2010; Khalil, Jameson, Broaddus, Lin, & Chung, 2013; Osuka et al., 2012), activation of signal transduction pathways (Chautard et al., 2010; Golding et al., 2009; Kim et al., 2012; H. F. Li, Kim, & Waldman, 2009; J. Wang et al., 2010), nuclear erythroid-related-factor-2 expression (Nrf2) (P. K. Sharma & Varshney, 2012), enhanced DNA repair and homologous recombination (Bao et al., 2006; Lim et al., 2012; Naidu, Mason, Pica, Fung, & Pena, 2010), hypoxia and tumour microenvironments (Hardee et al., 2012; Jamal, Rath, Tsang, Camphausen, & Tofilon, 2012; Yang, Lin, Wang, Guo, & Wang, 2012), expression of heat shock proteins (Brondani Da Rocha et al., 2004; Fedrigo et al., 2011), and generation of a cancer stem cell phenotype (Eyler & Rich, 2008).

An emerging tumour subpopulation that has been shown to contribute to therapy resistance in a variety of solid tumours are tumour cells that contain elevated levels of genomic DNA (i.e. hyperdiploid cells) (Balsas, Galan-Malo, Marzo, & Naval, 2012; Castedo et al., 2006; Lagadec, Vlashi, Della Donna, Dekmezian, & Pajonk, 2012; S. Sharma et al., 2013; Q. Wang et al., 2012). Based on results where we identified that hyperdiploid cells were enriched in the CSFE-retaining, slow cycling population, we sought to determine whether GBM-hyperdiploid cells represent a therapy-resistant subpopulation within adult brain tumours. This data identified a slow cycling, tumourigenic hyperdiploid tumour cell population present within GBM patients that can initiate and maintain tumour growth *in vivo*, and are resistant to conventional therapy. These results revealed a GBM hyperdiploid tumour cell subpopulation that has the phenotypic potential to contribute to the evolution of therapy resistance in patients. In this chapter, I show that GBM hyperdiploid tumour cells are larger and more metabolically active than euploid tumour cells, and that hyperdiploid tumour cells are vulnerable to therapies targeting tumour metabolism. Together, these results support the hypothesis that hyperdiploid tumour cells are a clinically relevant GBM tumour cell subpopulation that contributes to the phenotypic heterogeneity present within GBM patient tumours. Recent work is beginning to identify hyperdiploid specific drugs (A. J. Lee et al., 2012; Marxer et al., 2012; Rello-Varona et al., 2009; Roh, van der Meer, & Abdulkadir, 2012; Vitale et al., 2007), which may provide a therapeutic opportunity to deplete hyperdiploid cell subpopulations from solid tumours.

4.2 Results

4.2.1 Slow cycling human GBM cells are not delayed in G2/M

In multiple lines *in vitro*, CFSE-retaining, slow cycling cells display a significant increase in 4n DNA content. Figure 4.1A shows the 4n DNA content in passaged primary human GBM cells, orthotopic xenograft mouse models, and fresh primary human GBM cells from patient tumours. Passaged cells amplified from primary patient tumour tissue exhibited levels of 4n+ DNA varying between patients from 1.5% -12% of the total population (Figure 4.1B). This is consistent with our hypothesis that a proportion of slow cycling cells are delayed or arrested during G2 and/or mitosis.

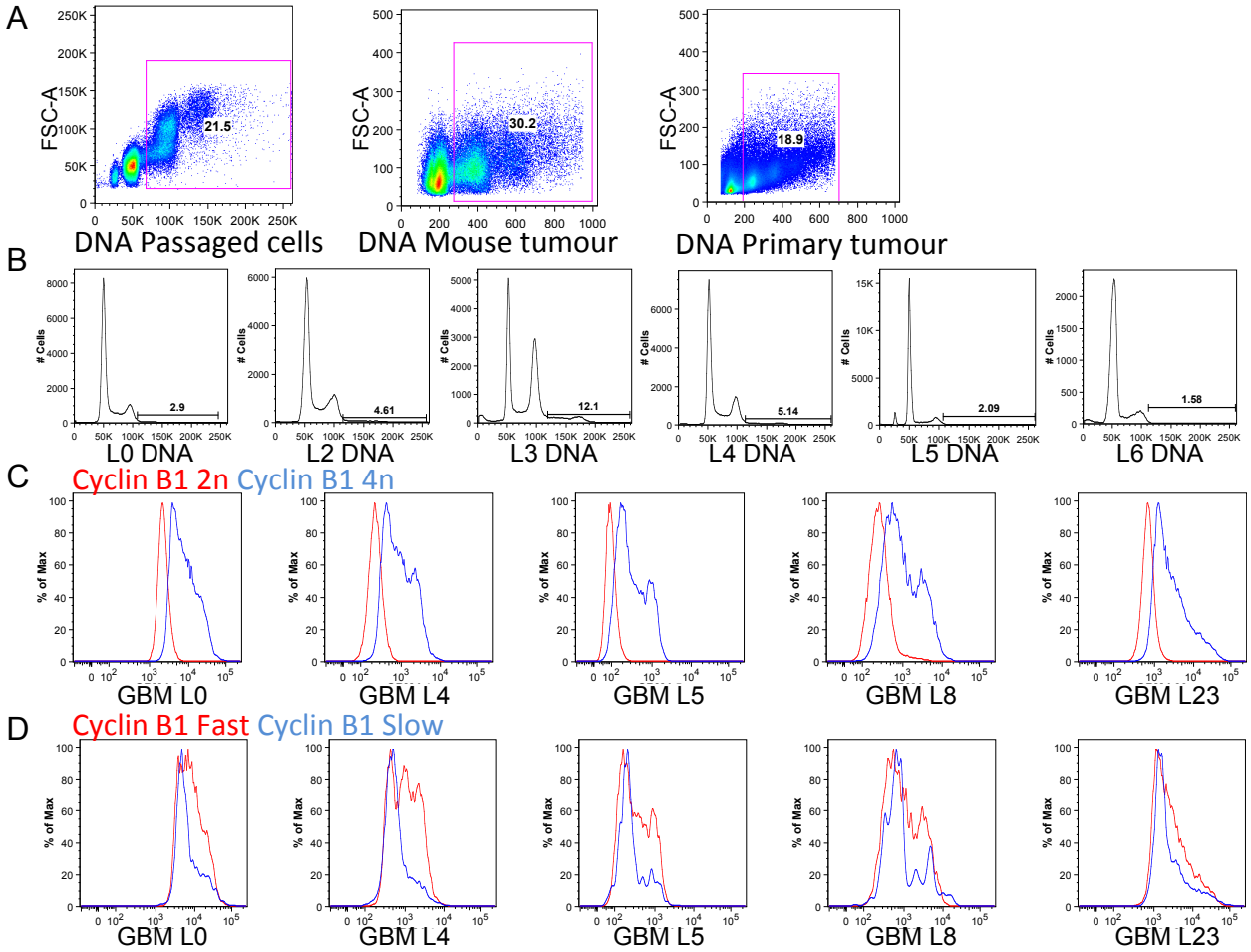


Figure 4-1 Hyperdiploid cells exist in primary patient tumour tissue, passaged human GBM cells, and xenograft tumours. DNA content was determined using DAPI. (A). The panel on the left shows the DNA content of GBM tumour cells cultured as tumourspheres. The gate isolates the cells with a 4n DNA content. The 4n DNA population shown in the panel to the left represents 21.5% of the total cell population in cells cultured from a patient tumour. The centre panel shows the DNA content of a mouse xenograft tumour where the 4n cell population makes up 30.2% of the total tumour cell population. The panel on the right shows the DNA content of fresh primary human glioblastoma tumour cells. The subpopulation of 4n cells shown is 18.9% of the total tumour cell population. (B). Cells cultured as tumourspheres from primary patient tumour tissue exhibited levels of hyperdiploidy varying between 1.5% and 12%. Six patient lines are shown. (C). The histograms show cyclin B1 expression in cells from five human GBM tumours passaged *in vitro*. The 2n population was negative for cyclin B1 and the 4n population was positive, confirming that the 4n population contained cells in G2 phase while the 2n population did not. Cyclin B1 expression was normal for all five tumours assessed validating the use of cyclin B1 levels for identification of a delay in G2M progression. (D). The 4N population of the fast cycling cells expressed higher levels of cyclin B1 than the 4n population of the slow cycling cells which is inconsistent with a G2 checkpoint arrest in the slow cycling cells.

To test this hypothesis, the slow cycling population was screened for increased expression of cyclin B1. Cyclin B1 is required for mitotic regulation. Expression accumulates throughout G2 phase and reaches a peak during mitosis. Cyclin B1 binds to cyclin dependent kinase 1 (CDK1) the primary driver of mitosis (Boxem, 2006). Analysis of a diploid cell population would usually reveal an initial absence of cyclin B1 and following DNA synthesis, cyclin B1 should be present in the 4n population. A delay in G2M progression would be recognized by overexpression of cyclin B1 in the cells undergoing DNA replication in G2, as they accumulate before mitosis. To first verify the staining profile of our antibody, cyclin B1 expression in the total tumour cell population was compared with the 2n (G0/G1 sub-population) and the 4n (G2/M sub-population) (Figure 4.1B). These results for five primary human GBM cell lines indicate that while the 2n population is negative for Cyclin B1, the 4n population is positive, confirming that cyclin B1 levels increased during G2/M as expected and validating the cyclin B1 antibody under the experimental conditions routinely used in our lab for flow cytometry analyses.

Next, cyclin B1 expression was compared between the slow cycling cells and the fast cycling cells. Cyclin B1 expression was absent in the 2n population of the slow cycling and fast cycling cells. Strikingly, and in the same experiment, when we compared the cyclin B1 staining of the slow cycling 4n cells with the 4n fast cycling cell population, the slow cycling cell population expressed less cyclin B1 than the fast cycling population (Figure 4.1C & D). This is the opposite of what would be expected for a G2/M accumulation and is inconsistent with a G2 or mitotic checkpoint arrest. A population arrested in G2/M would be expected to express higher than usual levels of cyclin B1. This suggests that the 4n population of the slow cycling cells were actively replicating their genome and not in G2 arrest.

Hyperdiploidy in tumour cells can lead to cellular senescence initiated by DNA-damage checkpoint response activation (DDR) (Di Micco et al., 2006). The DDR once initiated, slows progression through the cell cycle while DNA replication is performed correctly and completely and DNA strand repair is carried out. If the DDR is unable to achieve this the cell normally enters replicative senescence or is redirected to the apoptosis pathway (B. B. Zhou & Elledge, 2000). For a cell to contribute to tumour progression it must be capable of

DNA replication and be actively cycling. It is therefore important to determine whether hyperdiploid tumour cells are actively cycling and capable of contributing to tumour growth.

The finding that slow cycling cells contain an increase in the 4n and 4n+ cells that are not in G2/M phase suggests two possibilities. First, these are tumour cells that have failed cytokinesis but did not die, are senescent, and gradually accumulate in the slow cycling subpopulation over time. Second, these cells are a viable, hyperdiploid, tumour cell population that cycle less frequently but nevertheless are viable and therefore may play a non-trivial role in GBM disease. We next sought to determine whether the 4n population of the slow cycling cells were proliferating and therefore capable of contributing to disease progression.

4.2.2 Proliferating hyperdiploid cells exist in tumoursphere lines derived from patient tumours

To ascertain whether the hyperdiploid cells were actively cycling or senescent, Ki67 expression levels were analysed in cells cultured as tumourspheres. Ki67 is expressed exclusively by cycling cells and absent in senescent cells (Scholzen & Gerdes, 2000). Ki67 labeled cells were stained with DAPI and the 4n+ cells were analysed to determine Ki67 expression. Gating on the 4n+ population revealed that a proportion of GBM hyperdiploid tumour cells expressed Ki67, indicating they were actively replicating their genome. Figure 4.2 shows the expression of Ki67 in the 2n, 4n, and 4n+ populations of human GBM cells indicating that all three populations are actively proliferating.

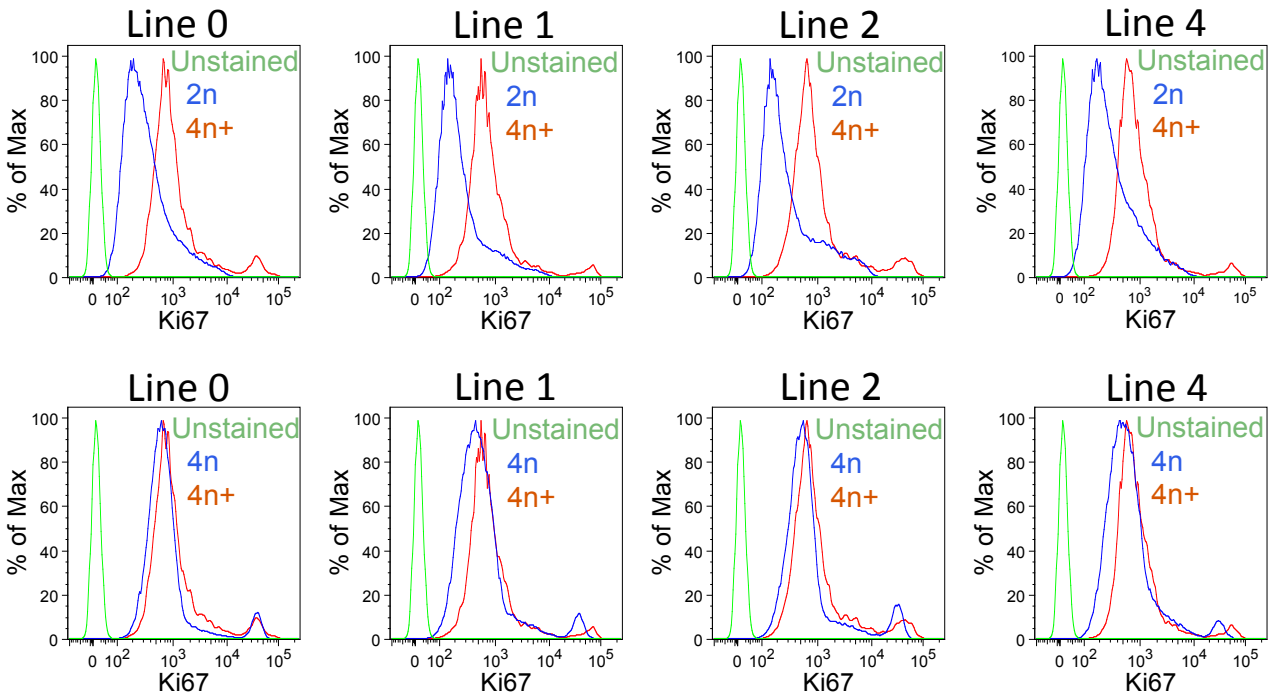


Figure 4-2 Proliferating hyperdiploid cells exist in tumoursphere lines derived from primary patient tumours. Ki67 labeled cells were stained with DAPI. Gates isolated the 4n+ population to assess Ki67 expression in the hyperdiploid subpopulation of cultured human GBM cells. The figure shows Ki67 protein expression for four human GBM cell lines. The upper panels show cells containing 2n DNA, overlaid with the hyperdiploid population (4n+), as assessed by DAPI staining. The lower four panels show an overlay of cells containing 4n DNA with hyperdiploid cells.

The proportion of 4n+ GBM cells expressing Ki67 indicates that this sub-population was clearly not senescent, however it is possible that these cells routinely fail cytokinesis due to defects in G2/M progression and are therefore not a viable sub-population. Cytokinesis failure has a number of potential outcomes. Activation of the tumour suppressor p53 induces a checkpoint arrest in G_1 resulting in cells that either undergo apoptosis or are simply unable to divide and contribute to tumour progression (Lacroix & Maddox, 2012). Another possible outcome of failed cytokinesis is the formation of binucleate cells and aneuploidy (Sagona & Stenmark, 2010).

4.2.3 Hyperdiploid clones can maintain long–term growth under tumoursphere culture conditions

To confirm that GBM hyperdiploid cells are capable of long term proliferation CFSE-retaining cells were sorted from the bulk population by flow cytometry and seeded 96 well

dishes at a density of one single cell per well. Nine days post-seed, wells containing a single sphere were identified (i.e. clonal population) and these clones were amplified for cell cycle analysis. Over 100 clones were screened from four separate GBM parent lines. Hyperdiploid clones were isolated from three of the patient lines and amplified as tumourspheres. Only clones with a hyperdiploid DNA profile, as assessed by DAPI concentration, were maintained in culture. Figure 4.3A compares the DNA content of three hyperdiploid clones with the diploid parent cell line.

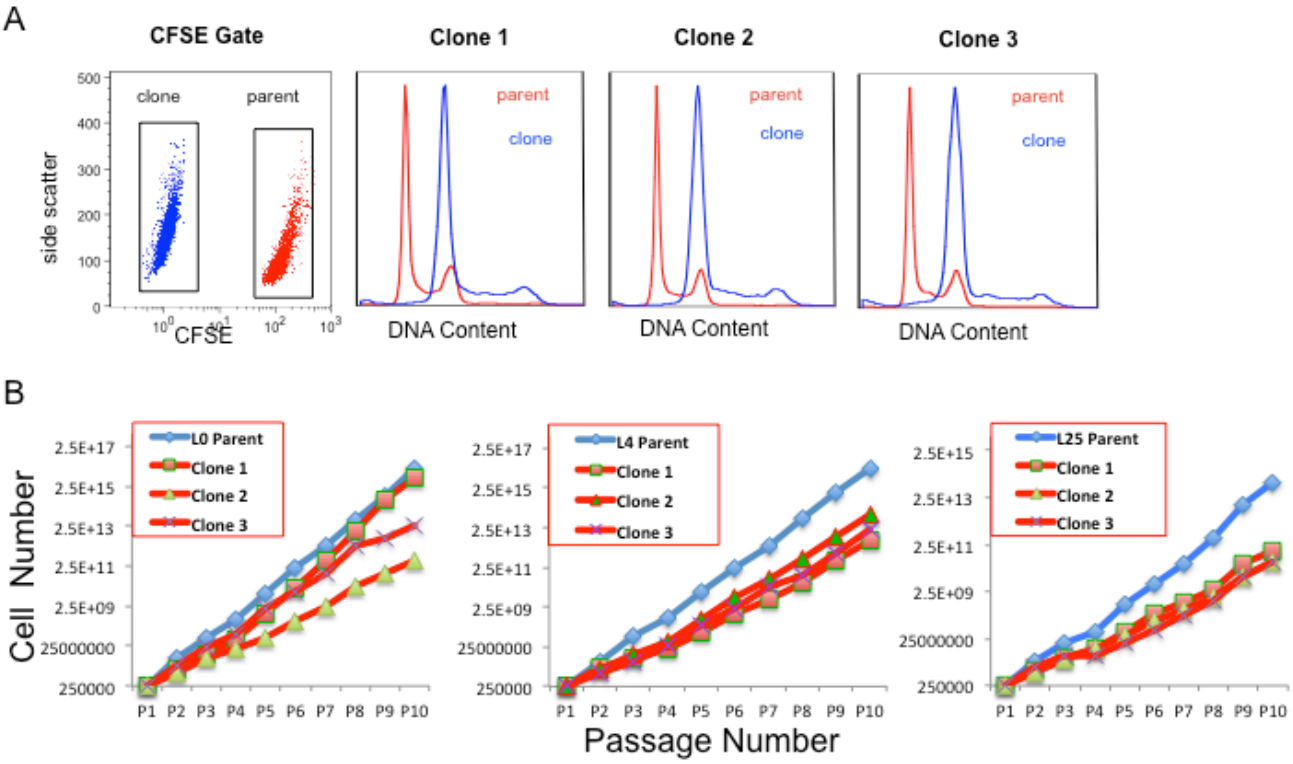


Figure 4-3 Hyperdiploid clones exhibit an increase in DNA content. (A). Clonal cell lines were generated from three primary human GBM tumours. The parent GBM tumour cell line and clonal cell lines were dissociated into single cells, the parent population was stained with CFSE for easy identification, and all cells were fixed and permeabilized. Each clonal cell line was spiked with CFSE labeled parent cells as a control and all the cells were stained with DAPI to assess DNA content. The parent cell line was isolated from the clonal lines during flow cytometry based on CFSE fluorescence. All the clonal lines shown in blue, exhibited hyperdiploidy as indicated by the increase in DNA content over that of the parental line, shown in red. (B). Hyperdiploid cells are viable and capable of long-term proliferation *in vitro*. The charts show three parent lines (L0, L4, L25) in blue and three clones of each parent line shown in red over 10 passages.

To formally assess the ability of hyperdiploid clonal lines to proliferate long term in culture conditions, three clones from each line were compared to their parental control populations with respect to their ability to maintain long-term growth under tumoursphere culture conditions. Parental and clonal lines were passaged under tumoursphere conditions for seven days when spheres were dissociated and cell counts performed, this continued for 10 passages. All of the hyperdiploid clones isolated were able to maintain long-term growth, and therefore expressed one of the key hallmarks of cancer, unbridled proliferation (Hanahan & Weinberg, 2000). While the hyperdiploid cell populations exhibited long-term proliferative capacity, they did not proliferate with the same efficiency as the parent cell lines (Figure 4.3B). These results confirm that a proportion of 4n and 4n+ GBM cells are viable as evidenced by the ability to maintain a tumoursphere culture *in vitro*, and suggest the hypothesis that these cells may play an active role in GBM disease.

4.2.4 Viable hyperdiploid cells are present in mouse xenograft tumours and primary patient tumours

It was crucial to confirm that the presence of hyperdiploid cells was not an artifact of tissue culture. To do this we attempted to identify a subpopulation of hyperdiploid cells in xenograft mouse brain tumours. NOD SCID mice were anaesthetized and 200,000 hyperdiploid cells amplified in sphere cultures and dissociated into a single cell suspension were injected into the striatum of each mouse. When large brain tumours had formed, they were surgically removed and chemically dissociated. Near infrared live-dead stain was used to exclude dead cells from our analysis. Human specific CD monoclonal antibody CD56, which recognized human glioblastoma cells but not mouse cells, was used to isolate human cells from surrounding and infiltrating mouse cells in xenograft experiments.

Figure 4.4A shows the gating strategy used to isolate human GBM cells within orthotopic xenograft tumours. Human GBM cells were isolated from freshly resected primary human brain tumours using a similar gating strategy. CFSE staining intensity was used to isolate control cells, near infrared live/dead stain was used to exclude dead cells, and DAPI was used to analyze the DNA profile (Figure 4.4B).

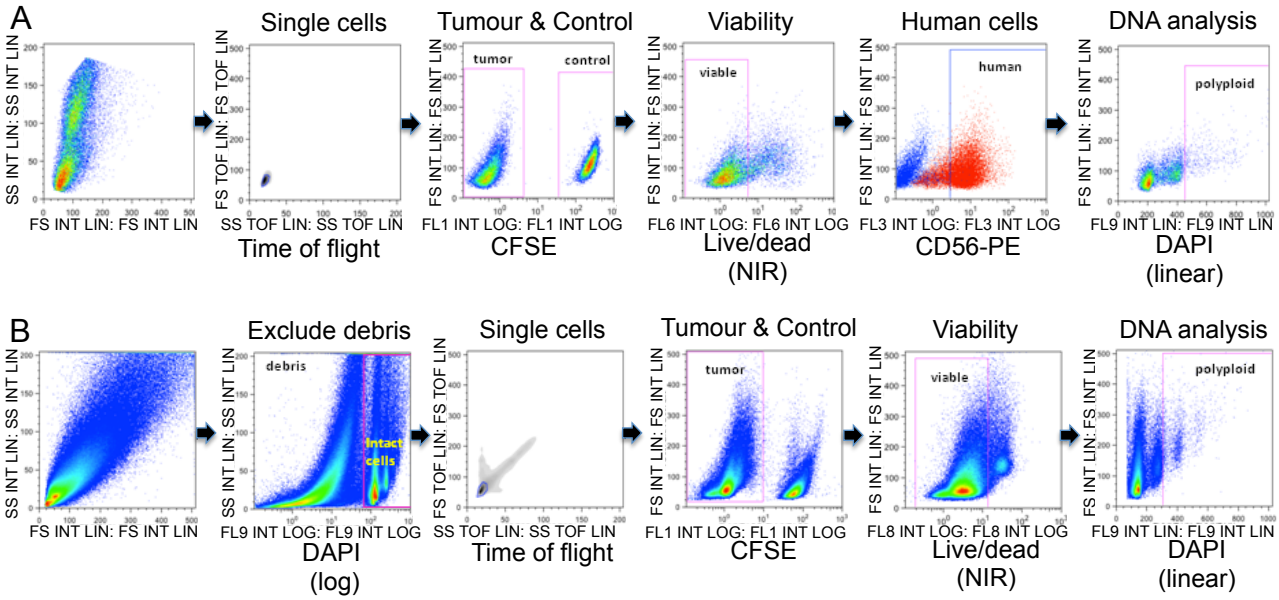


Figure 4-4 Gating strategy used to isolate human GBM cells in orthotopic xenograft tumours and primary human brain tissue. (A) The figure shows the gating strategy used for mouse xenograft tumour cells. CFSE staining was used to isolate control cells from tumour cells. To isolate the live cells, near infrared live–dead stain was used to exclude dead cells that take up the fluorescent label. CD56 labels neural cell adhesion molecule 1 (NCAM-1) which is present on the surface of human neural and glial cells. It was used to isolate human tumour cells from surrounding and infiltrating mouse brain cells. The DNA content of live human tumour cells was detected according to DAPI staining intensity. (B) Shows the gating strategy used for fresh primary human tumour cells. CFSE staining was used to isolate control cells from tumour cells. To isolate the live cells, near infrared live–dead stain was used to exclude dead fluorescently labeled cells. The DNA content of live human tumour cells was detected according to DAPI staining intensity.

Figure 4.5A shows the DNA content of primary patient GBM cells that have been passaged as tumourspheres. The gate isolates the 4n+ population. In this tumoursphere culture, 5.88% of cells fall within this gate. Figure 4.5B shows the DNA content of an orthotopic xenograft tumour analysed by flow cytometry using DAPI. The 4n+ cells constituted 7.88% of the tumour cell population in this mouse.

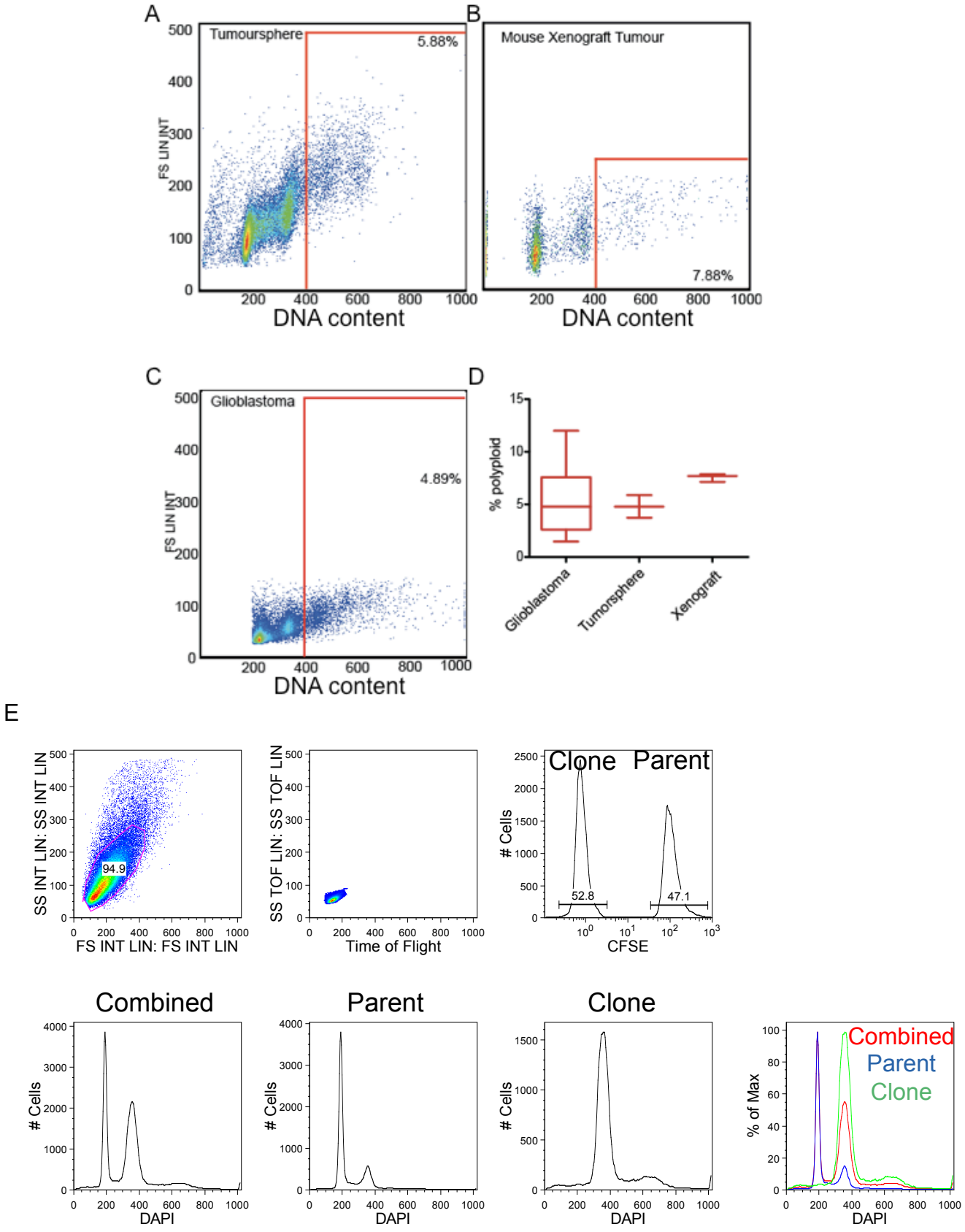


Figure 4-5 Hyperdiploid cells exist in primary patient tumour tissue and passaged cell lines and xenograft tumours. The DNA content was determined using DAPI. (A) Shows the DNA content of a GBM tumour cell line passaged as tumourspheres. The gate isolates the aneuploid

cell population, which represents 5.88% of the total cell population. (B) Shows the DNA content of a mouse xenograft tumour where the aneuploid cell population makes up 7.88% of the total tumour cell population. (C) The DNA content of primary human glioblastoma tumour cells is shown where a subpopulation of aneuploid cells shown is 4.89% of the total tumour cell population. Cell cycle analyses of all primary GBM tumours identified a viable subpopulation with an elevated DNA content. (D) The box and whisker plot indicates the aneuploid cell distribution in a single cell suspension of human glioblastoma tumours, cultured tumoursheres, and xenograft mouse tumours. (E) The figure shows the gating strategy used for comparing DAPI staining in parents and clones. GBM Line 4 is shown with one of the clones derived from it. The clone was stained with CFSE to enable both populations to be analysed in the same tube. The figure shows the DAPI content of each separately and then as an overlay. The clone, shown in green, clearly consists mostly of 4n and 8n DNA.

Finally, we analysed the ploidy status of fresh primary patient tumour tissue using DAPI by flow cytometry. Primary glioma tissue contained a subpopulation of cells with an increased DNA content as assessed by DAPI concentration shown in figure 4.5C. All the human GBM tumours we analysed harbored a hyperdiploid subpopulation. In the tumour shown the hyperdiploid cell population was 4.89% of the total cell population. The box and whisker plot in figure 4.5D shows the percentage of hyperdiploid cells in passaged tumourspheres, primary patient tumours, and orthotopic xenograft tumours. Combined, these results confirm the presence of viable, proliferative hyperdiploid cells in passaged tumourspheres, xenograft tumours, and primary patient glioma tissue.

Donovan et al., contains a CGH analysis of three hyperdiploid clonal cell lines and the corresponding parent line generated in our lab. All three clones displayed chromosomal gains and losses compared to the parental bulk population. The clones exhibited almost double the DNA content of the euploid parent. CGH analysis revealed that all three clones were hyperdiploid with near-tetraploid chromosomal content. Overall, 1297 genes with copy number variations were identified, however, only 16 genes were affected in all of the three clones. A subsequent analysis of the functional networks over-represented in the cohort of 16 genes affected across all three clones identified that “Cellular Growth and Proliferation”, “Cancer”, “Cell Cycle”, “Cell Death and Survival” and “DNA Replication, Recombination and Repair” were the networks affected (P. Donovan et al., 2014).

4.2.5 Hyperdiploid cells are capable of forming tumours *in vivo* which have the hallmarks of high grade glioma

The gold standard assay to assess tumourigenic potential of cells for brain cancer is the intracranial xenograft disease model (Morton & Houghton, 2007). Therefore, we used a xenograft tumour model to determine whether hyperdiploid cells were capable of driving disease *in vivo*. Figure 4.5E shows the gating strategy used to assess the DNA content of our hyperdiploid clones. We injected 200,000 hyperdiploid cells passaged as tumourspheres into the striatum of NOD SCID mice exactly as described in (Deleyrolle et al., 2011). Figure 4.6A is a schematic of the GBM mouse models we generated *in vivo*. We injected a total of 29 mice consisting of L0 Parent: N=8, L0 Clone: N=5, L4 Parent: N=4, L4 Clone: N=6, L25 Parent: N=3, L25 Clone: N=3. Mice were monitored daily for signs of disease and were sacrificed when they began to lose weight and/or displayed symptoms of disease in line with our animal ethics protocol. The survival data generated was used to generate a Kaplan-Meir survival curve. Figure 4.6B shows the survival data for mouse orthotopic xenograft tumours for three parent cell lines and a corresponding clonal variant of each. The median survival time was longer for mice injected with hyperdiploid clonal lines than parental lines, however, the difference was shown to be insignificant in all cases using the log-rank test. These data show that hyperdiploid clones are capable of driving disease progression similar to that of the bulk tumor population.

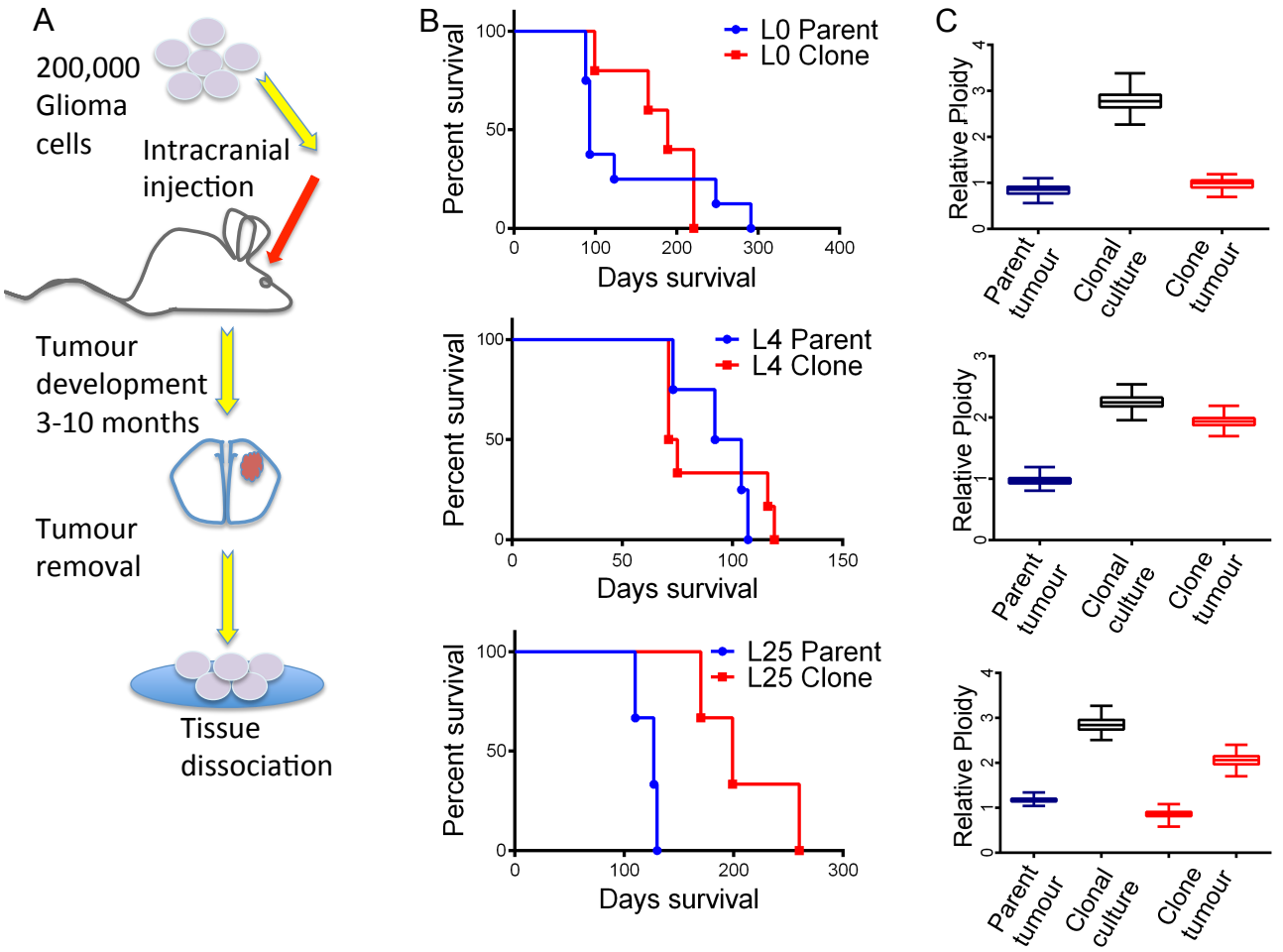


Figure 4-6 Hyperdiploid human GBM cells can initiate tumour formation *in vivo* and exhibit variations in genomic stability in xenograft tumours. (A) Schematic of xenograft tumour initiation in mice. Cells from three hyperdiploid clonal variants and the three-matched parent diploid controls were dissociated from tumoursphere cultures into single cell suspensions and injected into the striatum of SCID mice (2 x 10^5 cells per mouse). When large tumours formed, they were surgically removed and mechanically and chemically dissociated. (B) Kaplan-Meier survival curve showing mouse survival following striatal tumour cell injection of hyperdiploid clonal cell lines and matched parental controls. We injected a total of 29 mice consisting of L0 Parent: N=8, L0 Clone: N=5; L4 Parent: N=4, L4 Clone: N=6; L25 Parent: N=3, L25 Clone: N=3. (C) Stability of hyperdiploidy in orthotopic xenograft tumour cells. The DNA content of human GBM diploid parent tumour cells, hyperdiploid cultured clones and hyperdiploid xenograft tumour clones was determined according to DAPI staining intensity using flow cytometry analysis as described in the Materials and Methods. The clone derived from patient Line 0 (upper panel) showed a marked loss of DNA content *in vivo*. The tumour generated from the hyperdiploid clone generated from patient Line 4 (centre panel), maintained an approximate two-fold DNA content *in vivo* despite some genetic drift. Two separate populations evolved from the tumour generated from the hyperdiploid clone derived from patient Line 25 (lower panel), one of which displayed a marked loss in DNA content and a second separate population that maintained a two-fold DNA content *in vivo*.

To determine the ploidy of the parent and hyperdiploid clonal orthotopic xenograft tumors we analysed the DNA content by flow cytometry. Mouse brain tumours were diced and chemically dissociated before fixation; DAPI staining intensity was assessed to determine DNA content. In both parent and hyperdiploid clonal lines there was 100% tumour formation *in vivo*. Tumours generated from parental cell lines maintained a near diploid DNA content and a similar proportion of hyperdiploid DNA content to their corresponding tumoursphere cultures. In contrast, the ability of clones to maintain a hyperdiploid DNA profile after *in vivo* tumour formation varied from cell line to cell line. Hyperdiploidy was lost *in vivo* in the tumour that developed from the clone of patient Line 0 resulting in a subdiploid DNA content evident during flow cytometry analysis. The tumour generated from the clone of patient Line 4 maintained a relatively stable hyperdiploid state *in vivo* despite some genetic drift. The tumour generated by the clone from patient Line 25 developed two populations *in vivo*, one hyperdiploid and the other subdiploid. (Figure 4.6C). This data is illustrative that maintenance of the hyperdiploid state *in vivo* is variable across different patient tumours being stably maintained in some and lost in others.

Next, we examined the histopathological features of the xenograft tumors following haematoxylin and eosin staining. Figure 4.7 shows histological sections of xenograft tumours from parental cell lines and hyperdiploid clonal lines that are consistent with the histopathological features of high-grade human glioma. Parental and hyperdiploid clonal variants both exhibited pleomorphic (variable shape and size of cell/nucleus) astrocytic tumour cells with marked nuclear atypia and brisk mitotic activity (more than 20 mitoses/ten high power fields). This data confirms the use of orthotopic xenograft models of glioblastoma in immunocompromised mice with regard to the histopathology of the tumours generated using this method.

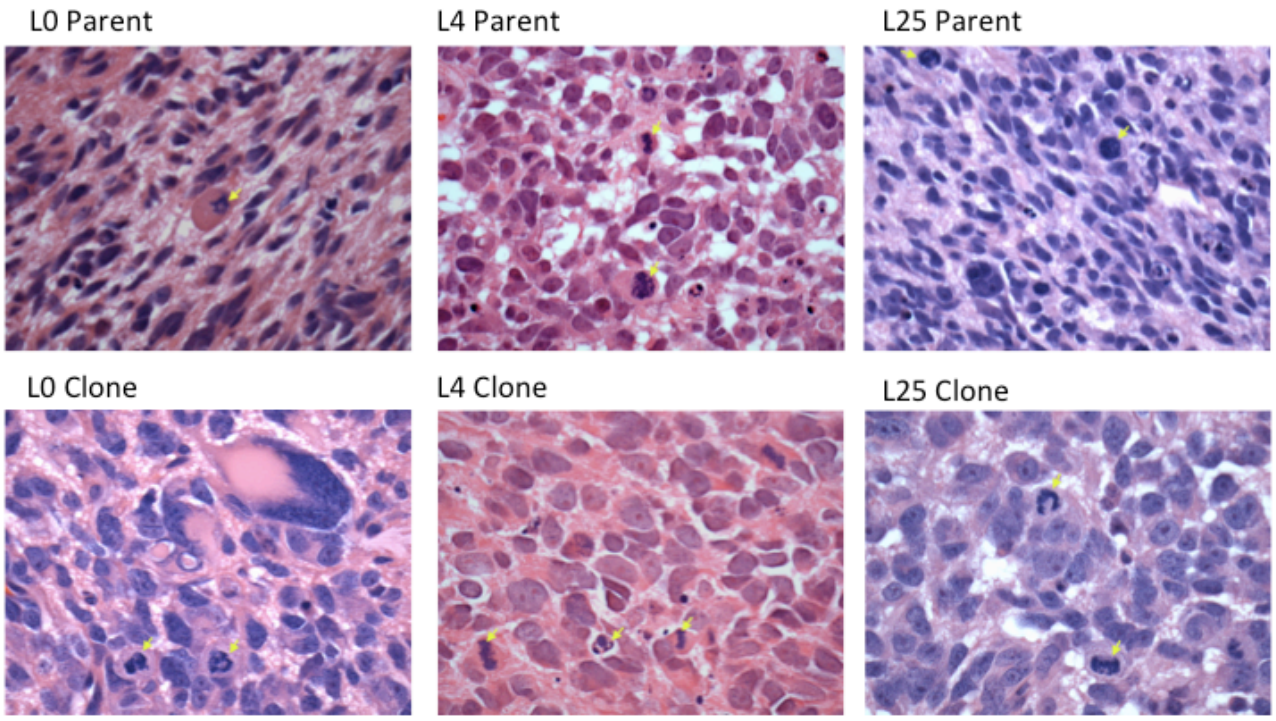


Figure 4-7 Haematoxylin and eosin (H&E) staining of xenograft tumours generated from diploid parent cells and hyperdiploid clonal cells. Histopathological features of high-grade glioma were identified by a pathologist in H & E stained sections of paraffin embedded tumour tissue. Pleomorphic (variation in cell/nuclear size, shape, staining) astrocytic tumour cells can be seen with marked nuclear atypia and brisk mitotic activity (more than 20 mitoses/ten high power fields). Representative atypical mitotic figures are indicated by yellow arrows.

From this data I concluded that the xenograft tumour model I established shows that hyperdiploid cells are capable of tumour formation *in vivo* and that mouse brain tumours that develop from hyperdiploid glioma cells exhibit features consistent with primary patient GBM tumours. In one patient line, the hyperdiploid cell is a stable cell state, maintained during both serial passaging and growth *in vivo*. In two other patient lines, the hyperdiploid state is a pseudo-stable cell fate, with hyperdiploidy being maintained during long-term passaging *in vitro* but lost during *in vivo* disease.

4.2.6 Hyperdiploid cells show resistance to conventional therapy

Hyperdiploid cells present in other solid tumors including colon cancer, breast cancer and ovarian cancer (Kusumbe & Bapat, 2009; Paulsson et al., 2013) display resistance to cytotoxic therapies (Castedo et al., 2006; Moore et al., 2012; Senovilla et al., 2012; Vitale

et al., 2011). We therefore predicted that GBM hyperdiploid cells might be resistant to the conventional cytotoxic treatments.

To this end, cells were treated with the chemotherapeutic currently used in first line management of GBM to determine whether hyperdiploid cells exhibited resistance and were therefore capable of contributing to disease recurrence after therapy. Temozolomide (TMZ) is a chemotherapeutic DNA methylation agent currently in use to treat GBM. To assess the response of hyperdiploid cells to TMZ, hyperdiploid clones were seeded into media containing TMZ. The colourimetric MTT assay was used to determine the response to chemotherapy. The response was compared to that of the bulk of the overall tumour population. Hyperdiploid cells showed a reduced sensitivity to TMZ when passaged in the drug for five consecutive days in comparison to the overall tumour cell population (Figure 4.8). This data shows that the hyperdiploid cell population exhibited relative therapeutic resistance when compared to the parent population in response to one of the current first line treatments in clinical use for glioma.

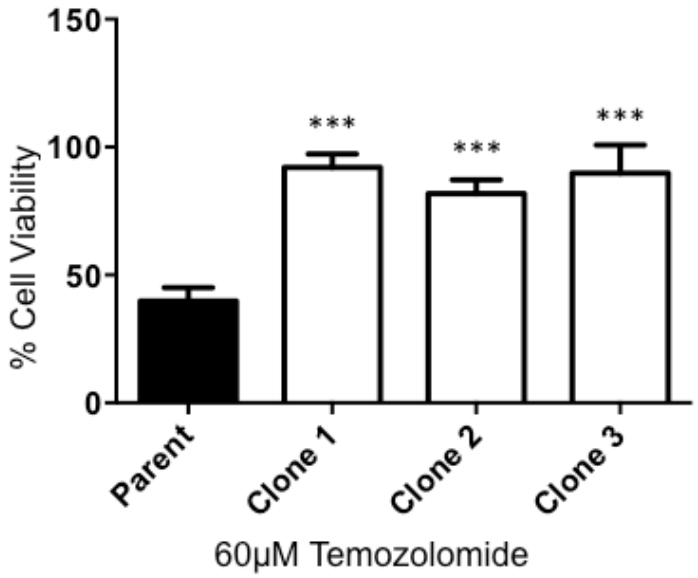
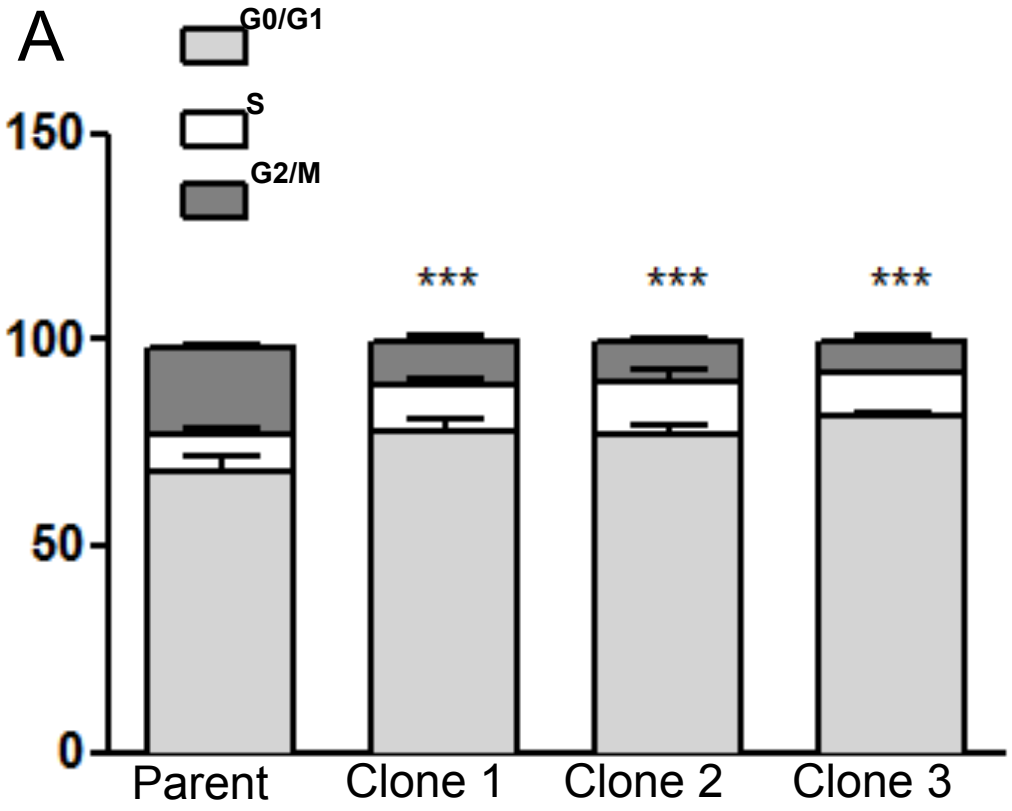


Figure 4-8 Hyperdiploid cells show therapeutic resistance. Cell viability was assessed using the MTT assay following treatment with temozolomide. Tumoursphere cultures from Line 0 (N=3) and 3 aneuploid clonal lines derived from Line 0 (N=3 per clone) were treated with 60μM temozolomide and assessed for viability seven days after treatment. The three hyperdiploid clonal lines were significantly more resistant to temozolomide than the parental control line. * Denotes significance as determined by ANOVA with Tukey post-test; **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

4.2.7 Hyperdiploid tumour cells cycle less frequently than euploid tumour cells

Experimental data from yeast (Rancati et al., 2008; Eduardo M Torres et al., 2007), mammalian cell culture (Williams et al., 2008) and tissue (Gentric, Celton-Morizur, & Desdouets, 2012) all indicate that polyploidy reduces cellular proliferation. Consistent with a reduced proliferative phenotype, we have shown that GBM hyperdiploid cells represent a subpopulation in primary tumor culture, mouse xenograft tumors, and primary patient tumors. To formally address the replicative phenotype of GBM polyploid tumor cells, we analysed the cell cycle distribution of the parent and tetraploid cells grown under tumorsphere culture conditions. Consistent with a reduced frequency of entering the cell cycle, the hyperdiploid clonal tumorsphere cultures had a reduction in cells in the G_2/M phase of the cell cycle with a commensurate increase in the G_0/G_1 peaks (Figure 4.9A).



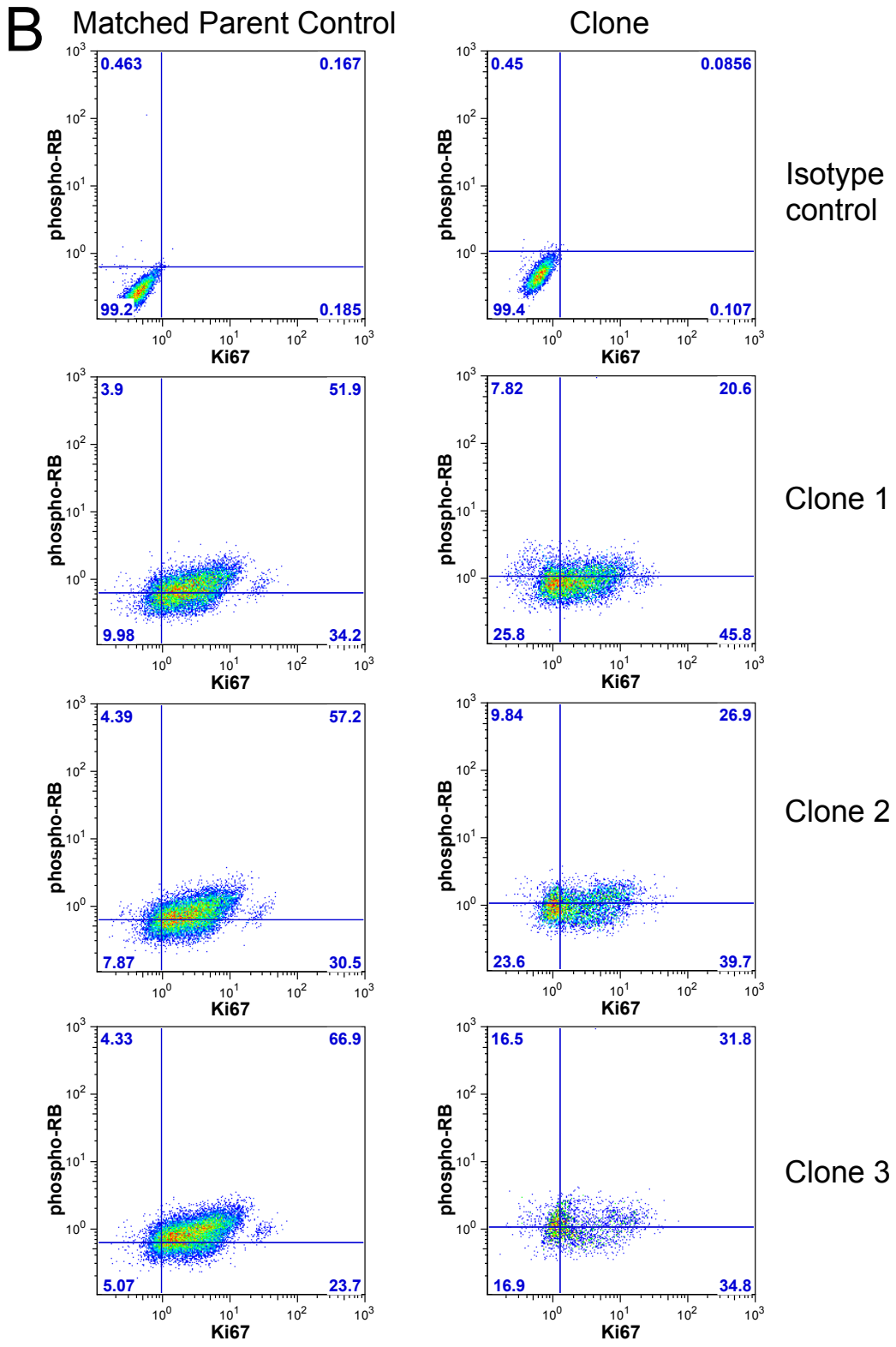


Figure 4-9 Slow cycling cells exist in *in vitro* GBM cell culture and exhibit a higher DNA content than the bulk of the population. (A) Hyperdiploid clonal tumoursphere cultures exhibited

a pronounced reduction in cells in the G_2/M phases of the cell cycle with a commensurate increase in the G_0/G_1 phases. (B) The expression levels of two proliferation markers, phosphorylated Retinoblastoma (phospho-Rb) protein and Ki67 were analysed in hyperdiploid clones and the matched parental control. Parental cells and hyperdiploid clones were grown under tumorsphere conditions for five days, then dissociated into a single cell suspension and the parental population was incubated in CFSE for identification during flow cytometry analysis. All cells were fixed, permeabilized, and blocked and each clonal population was mixed in with the corresponding CFSE labeled parent control. Then the mixed populations were stained in the same tube to achieve identical staining conditions for phosphorylated Rb (*y* axis) and Ki67 (*x* axis). Phosopho-Rb (1:50 Cell Signalling). Ki67 (1:100 Novocastra). In the clonal populations, an increase in double negative non-cycling cells (phospho-RB-/Ki67-, lower left quadrant) corresponded with a decrease in double positive proliferating cells (phospho-Rb+/Ki67+, upper right quadrant), relative to the matched parent control population.

The retinoblastoma (Rb) tumour suppressor gene produces a protein which blocks entry into S phase, initiating the G_1 checkpoint (Harbour & Dean, 2000). Rb binds to E2F preventing transition from G_1 to S phase through repression of gene transcription (Giacinti & Giordano, 2006). Three Rb states have been identified, namely, unphosphorylated, hypophosphorylated and hyperphosphorylated. Rb is unphosphorylated in early G_1 (Narasimha et al., 2014). In the hyperphosphorylated state the protein is inactive. Rb proteins are phosphorylated by cyclin D- and cyclin E-dependent kinases allowing progression into S phase (Nevins, 2001). The hyperphosphorylated form of Rb is expressed in late G_1, S, G_2 and M phases of the cell cycle (Giacinti & Giordano, 2006). We chose phosphorylated Rb (phospho-Rb) and Ki67 to determine the relative proliferation rates of parental and hyperdiploid clonal cultures. Ki67 protein is highly expressed in cycling cells and rapidly degraded once cells exit the cell cycle (Scholzen & Gerdes, 2000). A comparison of the hyperdiploid clones with the parental controls revealed a 2.97 fold increase in non-cycling cells in the hyperdiploid clones. Cells that were negative for both Ki67 and phospho-Rb (*p* value = 0.0008) were deemed non-cycling (Figure 4.9B). This data indicates that the hyperdiploid cells cycle less frequently due in part, to the presence of a G_0/G_1 checkpoint delay in hyperdiploid human GBM cells.

4.2.8 Hyperdiploid cells are larger and exhibit increased metabolic activity and glycolysis inhibition sensitivity compared to euploid tumour cells

Increased DNA content is associated with an increase in overall cell size, and a direct correlation exists between ploidy status and increases in cell size (Williams et al., 2008). We hypothesized that increased cell size is a conserved phenotype across the entire hyperdiploid cell population and that this attribute could be exploited in the development of anti-aneuploid therapeutics.

The total cell volume of hyperdiploid clones was compared with the parental cell line to determine the relative cell size differences between the euploid bulk population and the hyperdiploid clones. The results confirmed those of previous work by others (Cavalier-Smith, 2005; Chan & Marshall, 2010; Gregory, 2001; Jorgensen et al., 2007; Neumann & Nurse, 2007; Petrov, 2002), our hyperdiploid cells were approximately double the size of the parent euploid cells (Fig. 4.10).

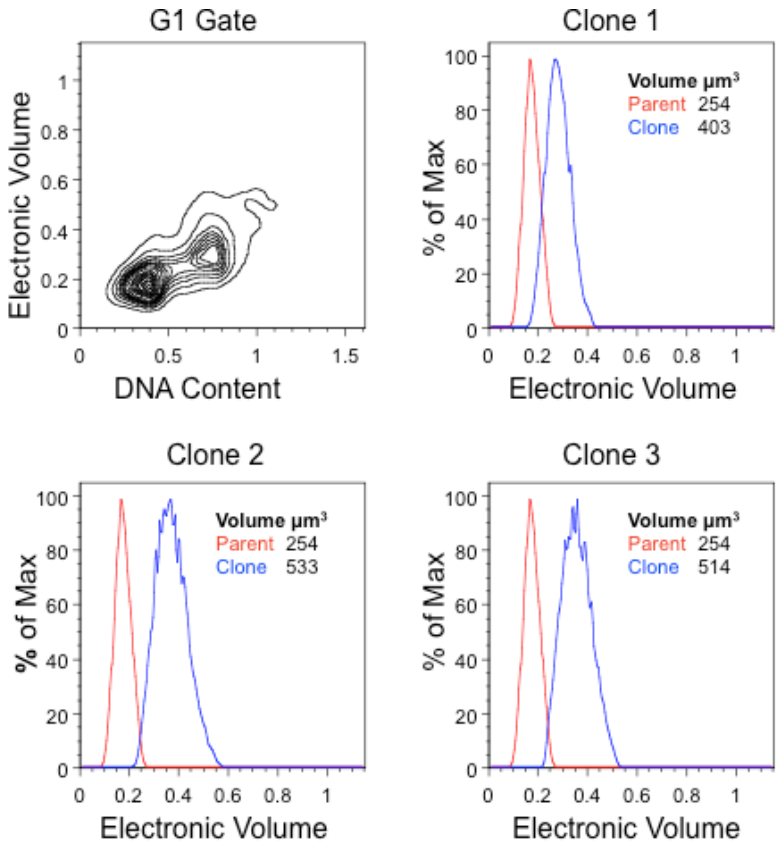


Figure 4-10 Hyperdiploid cells are larger than euploid tumour cells. Aneuploid clones and parent cell lines were passaged as tumourspheres. DNA content was analysed by DAPI concentration and cell volume using BD Quanta flow cytometer. DNA content was used to identify

cells in G0/G1. Electronic volume of parent and clonal line in G0/G1 was calculated from 10,000 cells. In the histograms, the electronic volume distribution shown in red represents the parent population and the hyperdiploid clones are shown in blue. The mean cell volume was calculated using 10,000 cells based on 10µm diameter beads.

There are a number of possible cellular adaptations required to compensate for an increase in DNA content including a corresponding increase in metabolic demand associated with an increase in organelle activity and protein production. An increase in these activities would place an increased demand on the glycolytic pathway. To assess whether this was occurring in hyperdiploid cells L-Lactate production was measured, which is an established method for assessment of metabolic pathway activity in tumour cells (Jain et al., 2012). L-lactate production is the result of an intracellular accumulation of pyruvate as a consequence of glycolysis, increased lactate dehydrogenase activity, or disturbances in mitochondrial function (Williams et al., 2008). As predicted, the increase in size of the hyperdiploid tumour cells corresponded with an increased level of L-Lactate production when compared to the parental euploid population (Figure 4.11A).

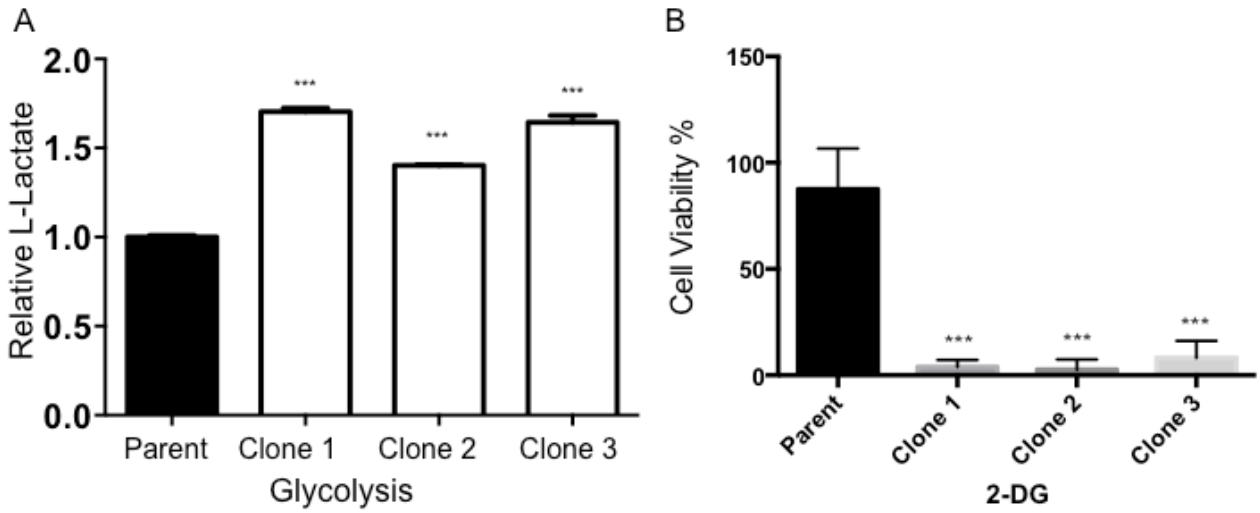


Figure 4-11 Hyperdiploid cells exhibit increased metabolic activity and glycolysis inhibition sensitivity compared to euploid tumour cells. (A). Parent and hyperdiploid clonal cell lines were seeded at 1 x 10^6 cells/ml and passaged as tumourspheres for 24 hours. The relative L-Lactate production shown in the figure represents the results of three independent cultures per line. Significance was determined using ANOVA with Tukey post-test, *** p≤ 0.001. (B). Parental tumourspheres (N=3) and hyperdiploid clonal cultures (N=3 per clone) were treated with 4mM 2-Deoxy-D-glucose (2-DG). Viability was assessed seven days after treatment. The three aneuploid

cultures were significantly more sensitive to 2-DG than the parental control line. *Denotes significance as determined by ANOVA with Tukey post-test; ****p≤ 0.0001, *** p≤ 0.001.

To determine whether the demand for an increase in metabolism in hyperdiploid cells was a potential therapeutic target, cells were treated with an inhibitor of glycolysis. Treatment with 2-deoxy-D-glucose (2-DG), an established inhibitor of glycolysis (S. Gupta, Farooque, Adhikari, Singh, & Dwarakanath, 2009; Kalia, Prabhakara, & Narayanan, 2009; F. Zhang & Aft, 2009) had a significantly greater effect on cell viability in the hyperdiploid clonal lines than on the euploid parental population (Figure 4.11B). The results of this assay showed that administration of the optimal dose of 2-DG reduced sphere formation in the euploid parental cells while at the same time eliciting a more dramatic response from the hyperdiploid clones. This data supports our hypothesis that hyperdiploid GBM cells are larger in size and exhibit an increased metabolic demand compared to their euploid counterparts. Additionally, the finding that hyperdiploid GBM cells are more sensitive to glycolysis inhibition than euploid tumour cells suggests that this is a possible therapeutic target with the advantage of cellular specificity.

4.3 Discussion

Genomic instability and aneuploidy are common characteristics of tumours (Wodarz & Gonzalez, 2006) (Korzeniewski, Spardy, Duensing, & Duensing, 2011; E. M. Torres et al., 2010). In the process of monitoring patients diagnosed with Barrett's oesophagus for progression to oesophageal adenocarcinoma, surgeons regularly perform endoscopic examinations and tissue biopsies. This enables oncologists to observe disease progression at a cellular and subcellular level. Galipeau et al., identified an unstable 4n intermediate cell population in premalignant oesophageal adenocarcinoma tissues *in vivo* that progressed to aneuploidy as a result of continued genomic instability ((Galipeau et al., 1996). Vitale et al., studied the effects of nocodazole or cytochalasin D (a cytokinesis inhibitor) on p53-/- human colon carcinoma HCT116 cells *in vitro*. The initial response was an increase in the number of polypolid cells and a reduction in cell death compared to the control p53 competent cells. Cells were FACS-purified to establish a tetraploid population and amplified in vitro. Notably, p53-/- tetraploid cultures accumulated a population of cells with an ~2n DNA content. Videomicroscopy revealed that p53-/- underwent multipolar cell

divisions with a higher frequency than p53+/+ control cells and that the resulting sub tetraploid cells were capable of normal bipolar cell divisions thereafter (Vitale et al., 2010).

Despite the observation that almost all cancers have degrees of aneuploidy, the contribution of genetic imbalance to the pathophysiology of cancer remains an ongoing question in cancer research. Direct experimental evidence supporting a pivotal role for aneuploidy in tumor initiation has been provided in a series of seminal studies (Castillo, Morse, Godfrey, Naeem, & Justice, 2007; Davoli & de Lange, 2012; Fujiwara et al., 2005; Zheng et al., 2012), however the role of aneuploidy in advanced disease is less well understood. It was first hypothesized by Boveri (Boveri, 1902) that aneuploidy may cause the uncontrolled proliferation of cancer cells. Here we have identified a hyperdiploid tumor cell subpopulation of potential clinical significance that is present in adult brain GBM tumors. These cells are viable, stably hyperdiploid, able to maintain long-term proliferation and drive tumor growth *in vivo*, and are resistant to conventional therapy.

This hypothesis has been countered with experimental observations showing that hyperdiploidy, in particular polyploidy, reduces cellular proliferation. For example, studies of hyperdiploid yeast strains have widely reported that aneuploid strains have a proliferative disadvantage (Rancati et al., 2008; Eduardo M Torres et al., 2007). The growth disadvantage of hyperdiploidy has also been demonstrated in mammalian cells derived from aneuploid mice, where hyperdiploid cells showed proliferation defects under standard tissue culture conditions (Williams et al., 2008). In tissues, polyploidy is associated with a markedly decreased replicative capacity (reviewed in (Gentric et al., 2012)). Consistent with the idea of polyploidy providing a growth disadvantage in adult brain cancer, GBM hyperdiploid clonal cultures were less proliferative than the parental tumor cell population. Cell cycle analyses confirmed that hyperdiploid tumor cells cycle less frequently than the euploid tumor bulk, which is caused (at least in part) by a delay in G_0/G_1. Altogether, our data and historic studies all support the hypothesis that hyperdiploidy comes with the fitness cost of decreased replicative capacity.

The reduced proliferative capacity of aneuploid tumor cells would be predicted to provide a selective disadvantage in the competitive tumor environment; a prediction borne out by the observation that aneuploid cells represent a relatively small cell subpopulation within patient and xenograft tumours as well as during growth under tumorsphere cell culture

conditions. However, this situation can change during the therapeutic selection pressures that occur as a result of treatment. We have shown here that GBM hyperdiploid cells are resistant to the DNA damaging therapy commonly in use to treat GBM, temozolomide. These results are consistent with tetraploid models of colon carcinoma, in which tetraploidy (a specific form of polyploidy) was shown to provide resistance to DNA-damaging agents (Castedo et al., 2006).

Infrequent cell cycle is a well-established drug resistance mechanism, and provides a plausible explanation as to why GBM aneuploid tumor cells are resistant to temozolomide. Quiescent (G_0) haematopoietic stem cells (HSCs) are resistant to the anti-proliferative chemo-therapeutic agent 5-fluoro-uracil (5-FU) (Randall & Weissman, 1997; Wilson et al., 2008), and become sensitive to 5-FU treatment when they are forced from G_0 into a proliferative state by treatment with IFNα (Essers et al., 2009). Further, HSCs can be protected from the effects of irradiation by increasing the proportion of HSCs in G_0 through a variety of treatments *in vivo* (Davis, Mungunsukh, Zins, Day, & Landauer, 2008; Forristal et al., 2013; Johnson et al., 2010). In cancer, the chemo-protective effect of cell cycle-mediated drug resistance is well established (Shah & Schwartz, 2001). For example, Schmidt and colleagues demonstrated that colon adenocarcinoma cells arrested in G_1 by over-expression of $p27^{Kip1}$ are significantly more resistant to a variety of chemotherapeutic agents, including temozolomide (Schmidt et al., 2001). Using a mouse xenograft model, Naumov et al. showed that the DNA intercalating compound doxorubicin (DXR) effectively reduced the metastatic tumor burden but spared non-cycling tumor cells, which persisted during therapy and subsequently developed into metastases after DXR therapy was discontinued (Naumov et al., 2003).

More recently, label-retention has been used to phenotypically identify infrequently dividing cells that are resistant to chemotherapy from a variety of tumor types (Dembinski & Krauss, 2009; Fillmore & Kuperwasser, 2008; Kusumbe & Bapat, 2009; Moore et al., 2012). Studies examining the cancer stem cell phenotype have also shown that quiescence provides protection against cell death induced by DNA-damage agents (G. Chen, Bradford, Seidel, & Li, 2012; Y. Chen et al., 2012) and chemotherapy (Kobayashi et al., 2012). Recently, a landmark study by Kreso et al. revealed how chemotherapy selects for minor, infrequently cycling subpopulations using lineage tracking in mouse models of cancer evolution (Kreso et al., 2013). Collectively these studies provide strong support for

the hypothesis that infrequent cell cycle contributes to the evolution of therapy resistance in cancer, and here we identify hyperdiploidy as an additional mechanism to generate slow-cycling cell subpopulations within solid tumors.

Why do aneuploid tumor cells cycle less frequently? One intriguing possibility is that the difference in cell size between aneuploid and diploid cells determines the frequency of cell division. This study found that GBM hyperdiploid tumor cells have a two-fold larger cell volume compared to their diploid counterparts. Cell growth, cell size and cell division are co-regulated to ensure cells are large enough to divide at mitosis (Turner, Ewald, & Skotheim, 2012). Studies in yeast revealed a size requirement for G_1-S transition, with smaller cells delaying in G_1 until a sufficient size was reached to maintain viable progeny after cell division (Johnston, Pringle, & Hartwell, 1977; Martin-Castellanos, Blanco, de Prada, & Moreno, 2000). Complementary studies in animal cells show that mammalian cells also delay in G_1 to allow an appropriate cell size to be achieved (Dolznig, Grebien, Sauer, Beug, & Mullner, 2004; Kafri et al., 2013). Thus, one plausible hypothesis is that the larger aneuploid tumor cells arrest during G_0/G_1 to allow sufficient growth to occur before committing to division.

In human development, aneuploidy is a normal mechanism contributing to organ development. Trophoblast giant cells (TGCs) are required for placental development following embryo implantation. Endoreplication in TGCs produces polyploid nuclei thought to facilitate an increased capacity for synthesis of secreted proteins including hormones, cytokines, adhesion molecules and extracellular matrix proteins in addition to promotion of angiogenesis and invasion during placental development (D. Hu & Cross, 2010). Polyploidy is maintained in TGCs through endoreplication by preventing M phase after completion of S phase (Elledge, 1996). Unhavaithaya et al. studied glial cells in normal development of the model organism *Drosophila melanogaster*. Polyploidy of glial cells is required to maintain a functioning blood brain barrier during larval brain development in *Drosophila* (Unhavaithaya & Orr-Weaver, 2012). Polyploid neurons have been found throughout the mammalian brain during normal brain development. While their functions have not been fully elucidated, it is postulated that chromosome gains could contribute to developmental processes (Kingsbury et al., 2005). It is conceivable, given that hyperdiploidy is required for normal organ development in insects and mammals that the

contribution to tumour progression is the adaptation of an evolutionarily conserved mechanism to maintain organ function.

Hyperdiploidy may contribute to therapy resistance through additional mechanisms other than cell cycle effects. Both temozolomide and ionizing radiation can inhibit the proliferative capacity of tumor cells by inducing senescence (Hirose, Berger, & Pieper, 2001; Jinno-Oue et al., 2010; Knizhnik et al., 2013). A recent study has shown that aneuploid cells preferentially escape from therapy-induced senescence, providing another mechanism for aneuploid tumor cells to resist the effects of conventional therapy (Q. Wang et al., 2012). Aneuploidy also generates phenotypic changes through changes in gene expression (Galitski, Saldanha, Styles, Lander, & Fink, 1999; Pavelka et al., 2010), and there is a growing body of evidence suggesting that aneuploid-dependent phenotypic changes provide adaptive advantages during therapy in a wide range of clinical settings. In fungal pathogens, chromosomal gains are thought to be responsible for anti-fungal drug resistance and immune-evasion (reviewed in (Kronstad et al., 2011; Selmecki, Forche, & Berman, 2010)). In experimental yeast models of therapy resistance, chromosomal gains provide a selective advantage under chemotherapeutic, cytotoxic, and anti-fungal drugs (G. Chen et al., 2012; Pavelka et al., 2010). Bortezomib (Velcrade) is an important drug for the treatment of multiple myeloma (MM). In a myeloma cell line model of cancer therapy resistance, polyploid myeloma cells displayed a five-fold resistance to the proteasome inhibitor bortezomib (Velcade) that is associated with over-expression of the proteasome subunit PSMβ5, the cellular target of bortezomib (Balsas et al., 2012). It is also possible that aneuploid cells are more resistant to genomic toxins due to simple gene duplication, which could preserve gene function through redundancy mediated genetic buffering (Elena, Carrasco, Daros, & Sanjuan, 2006).

It must also be noted that genotoxins such as TMZ and γ-radiation directly cause the generation of hyperdiploidy within GBM patients, based on the classic study by Yung and colleagues (Yung, Shapiro, & Shapiro, 1982) and more recent results obtained in breast cancer (Lagadec et al., 2012). Lagadec and colleagues increased the prevalence of polyploidy using radiation or pharmacological induction and showed that the resulting polyploid tumor cells display a pluripotent, tumor-initiating phenotype (Lagadec et al., 2012). These data support the provocative hypothesis that radiotherapy increases the aggressiveness of a patient tumour by elevating the frequency of hyperdiploid cells within

the surviving tumor cell population (Lagadec et al., 2012). Altogether there is a growing body of literature that provides a strong precedent for the ability of aneuploid cells to contribute to the evolution of therapy resistance and disease recurrence in a variety of solid tumors, and this study provides support for the idea that this hypothesis also holds true for adult brain cancer.

A number of lines of evidence suggest that aneuploid tumour cells can be specifically targeted with drugs that have little or no impact on normal euploid cells. Tumour cells exhibited heightened sensitivity when treated *in vitro* with 5-Aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR) compared with non-cancerous cells (Jose et al., 2011). AICAR is an AMP kinase activator that regulates cellular responses to metabolic stress (Rattan, Giri, Singh, & Singh, 2005). AICAR treatment resulted in a dose dependent reduction in tumour cell viability and proliferation, and an increase in apoptosis in contrast with little effect on non-cancer cells (epithelial cells and fibroblasts) (Jose et al., 2011). Jose et al. showed that AICAR sensitivity was increased in cells with a low steady-state ATP and a rapid proliferation rate. In another study, single chromosome gains in mouse embryonic fibroblasts were used to model the response of aneuploid cells to chemical compounds inducing genotoxicity, proteotoxicity, and energy stress. Aneuploid cells treated with AICAR and 17-allylamino-17-demethoxygeldanamycin (17-AAG) underwent apoptosis at doses that had minimal toxic effects on euploid cells (Tang et al., 2011). 17-AAG interferes with protein folding through its inhibition of HSP90 (Krishnamoorthy et al., 2013). These data show that drugs targeting already disrupted cellular functions have the potential to selectively inhibit aneuploid cell proliferation and tumour progression.

One strategy being actively pursued to overcome tumor heterogeneity is the identification of tumor subpopulations most likely to contribute to therapy resistance, followed by the development of new drug regimes that specifically target these subpopulations. This approach aims to decrease the adaptive capacity of the tumor to maintain the efficacy of the primary targeted or chemotherapeutic regimes for longer periods of time. Several independent groups have made inroads into the development of therapeutic strategies that specifically target aneuploid tumor cells (A. J. Lee et al., 2012; Marxer et al., 2012; Rello-Varona et al., 2009; Roh et al., 2012; Vitale et al., 2007). Extensive genetic heterogeneity could impede the development of general therapeutic strategies targeting the aneuploid

subpopulation of cells. We have shown that hyperdiploid tumor cells have a two-fold increase in cell volume that is proportional to their doubled genome. As the positive relationship between genome size and cell volume has been evolutionarily conserved throughout eukaryotes (Cavalier-Smith, 2005; Chan & Marshall, 2010; Gregory, 2001; Petrov, 2002), a larger cell volume may be a common phenotype within the hyperdiploid tumor cell subpopulation, which may increase the sensitivity of hyperdiploid tumors cells to inhibitors of tumor metabolism. Consistent with this hypothesis, hyperdiploid clonal cultures were more metabolically active than the diploid lines, and showed an increased sensitivity to inhibition of glycolysis.

Cancer cells rely on the glycolytic pathway to maintain their metabolic function resulting in high glucose requirements, known as the Warburg Effect (Warburg, 1956a). The increased glucose metabolism in tumour cells is facilitated by greater expression of the Glut1 transporter than normal cells (Birnbaum, Haspel, & Rosen, 1987). 2-DG is an inhibitor of glucose metabolism (Aft, Zhang, & Gius, 2002). Breast cancer cells exhibit a higher glucose uptake when treated with 2-DG thereby increasing 2-DG uptake after which they undergo apoptosis (Aft et al., 2002). When hyperdiploid GBM cells with 2-DG in this study, sphere formation inhibition was inversely proportionate to 2-DG dose. While euploid GBM counterparts were also inhibited by 2-DG, the effect on the hyperdiploid cells was far greater. This suggests that careful optimization of drug dose has the potential to maximize the effect of 2-DG on hyperdiploid GBM cellular metabolism while having a minimal impact on the metabolism of normal healthy cells.

4.4 Conclusions

Interestingly, hyperdiploidy appears to confer inhibition to proliferation, hindering tumour progression and gene amplification (Rexer & Arteaga, 2012), facilitating therapy resistance (Corcoran et al., 2010). Hyperdiploid cells cycle more slowly than their euploid counterparts and therefore exhibit slower tumour development. Conversely, chromosome duplication can potentially contribute to drug resistance by facilitating the emergence phenotypic diversity leading to a selective advantage during cellular stress. These results suggest that tumor metabolism may be a point of fragility within hyperdiploid tumor cells that can be therapeutically exploited to target this subpopulation within patient tumors. Our hope is that this insight will contribute to the development of anti-hyperdiploid treatments,

which can be used to reduce cellular heterogeneity in solid tumors and improve the efficacy of frontline therapies.

CHAPTER FIVE

5 A small cohort of cell surface proteins emerge as viable therapeutic targets in glioblastoma

5.1 Introduction

Two studies published approximately two decades ago, identified a small cohort of cancer-initiating cells from the total cancer cell population based on their cell surface marker expression (Bonnet & Dick, 1997; Lapidot et al., 1994). Using mouse models of acute myeloid leukaemia (AML), limiting dilution assays revealed that the majority of AML cells exhibited limited proliferative ability *in vivo* (Bonnet & Dick, 1997; Lapidot et al., 1994). When cell surface marker expression of tumor-initiating cells was analysed, both groups found that only CD34+/CD38- AML cells possessed extensive proliferative ability capable of reproducing the original cancer in mouse models (Bonnet & Dick, 1997; Lapidot et al., 1994). Mice intravenously injected with CD34+/CD38- leukaemic cells developed leukaemia in contrast to mice injected with CD34- or CD34+/CD38+ cells that did not (Lapidot et al., 1994). Bonnet et al., compared normal and leukaemic cells in AML to identify the cells capable of producing leukaemic clones. Primitive normal stem cells were identified as being the targets of leukaemic transformation rather than progenitors because consistent with the results of the study performed by Lapidot et al. only CD34+/CD38- cells were capable of extensive proliferation and tumour initiation in immunocompromised mice (Bonnet & Dick, 1997; Lapidot et al., 1994).

The initial identification and isolation of tumour initiating cells in leukaemia, has led to a search for a similar phenomenon in solid tissue cancers. A molecular signature identifying cells capable of tumour initiation could be used to identify a population of clinical interest when designing targeted treatments for therapeutically resistant cell populations (Ke et al., 2009; McClelland, Zhao, Carskadon, & Arenberg, 2009; Rorive et al., 2010; Shim, Oishi, & Fujii, 2009; Taran, Kobos, Sitkiewicz, & Sporny, 2007; Teicher & Fricker, 2010). In breast

cancer studies, tumour-initiating cells have been isolated from the total population of breast cancer cells using CD antibodies. A xenograft assay involving transplantation of breast cancer cells into immunocompromised mice showed that as few as 200 $CD44^{+}/CD24^{-/low}$ cells were capable of tumour formation with 100% efficiency whereas transplantation of 50,000 unsorted breast cancer cells were required to achieve 100% tumour formation (Al-Hajj et al., 2003). This study suggests that cell surface proteins can be used to identify cells within a tumour that possess tumour-initiating capabilities in solid tumours (Al-Hajj et al., 2003). Another more recent study in 2013 also identified $CD44^{+}/CD24^{low+}$ breast cancer cells as exhibiting superior tumour initiation ability compared to CD44+/CD24-/low cells in limiting dilution assays in immunocompromised mice (Azzam et al., 2013).

The identification of stem cells in the adult mammalian brain in 1992 (Reynolds & Weiss, 1992) has led to the hypothesis that a population of brain tumour stem cells could be responsible for tumour initiation and progression in the central nervous system (Reynolds & Weiss, 1992; Vescovi et al., 2006). Over the past decade, CD133 has been widely reported as a putative cancer stem cell marker (Jamal et al., 2012; Singh et al., 2003). CD133 positivity now appears to be environmentally induced rather than inherited. A recent study found that a hypoxic environment increased CD133 expression in glioblastoma cells *in vitro* resulting in a more stem-like tumour cell phenotype (Kolenda et al., 2011). This suggests that stem-like properties may not be fixed but may depend upon environmental cues. This is consistent with a recent study showing that breast cancer cells possess considerable plasticity *in vitro* in which the cancer cells can transition between stem-like states and various differentiation states (P. B. Gupta et al., 2011). Digoxin has been shown to inhibit hypoxia inducible factors in vitro and in vivo. HIF1α and CD133 were inhibited in mice with flank GBM xenografts treated with intraperitoneal injections of digoxin. Digoxin inhibited HIF1α (63%) and CD133 (72%) as analyzed by western blot of biopsy tissue (Bar, Lin, Mahairaki, Matsui, & Eberhart, 2010).

In addition to identifying tumour-initiating cells, CD proteins are aberrantly expressed in a wide range of cancers and have been recognized as useful prognostic markers in predicting disease progression and selecting between treatment options. For example, the increased expression of the tetraspanin protein CD151 has been shown to positively

correlate with large invasive tumours and metastases while being negatively correlated with overall survival in hepatocellular carcinoma patients (Ke et al., 2009). Motility-related protein-1 (MRP-1/CD9) is a transmembrane protein found to predict disease free survival (DFS) in patients diagnosed with squamous cell carcinoma of the head and neck. CD9 expression was negatively correlated with DFS in a study of 153 patients using immunohistochemistry on formalin fixed paraffin embedded tissue (Mhawech et al., 2004). CD44 expression levels positively correlated with disease recurrence and poor prognosis in head and neck squamous cell carcinoma (HNSCC) in a study analyzing cells extracted from mouse xenografts of human tumour cells by flow cytometry (Joshua et al., 2012). Additionally, CD44 expression was found to be involved in metastasis formation in osteosarcoma through *in vivo* experiments using CD44 knockout mice (Weber et al., 2002). These studies indicate that cell surface proteins are useful markers of disease progression in a range of human solid cancers.

There are only a few published studies examining CD expression in various brain cancer types. These studies have found similar relationships between select CD protein expression and disease outcome. Moreover, these studies have also shown that the expression of various CD proteins may also contribute to disease development and progression. Read et al., found that approximately 35% of medulloblastomas contained CD15+ cells analysed by immunohistochemistry and flow cytometry. They identified CD15 as a marker for tumour-propagating cells in a mouse model of medulloblastoma (Read et al., 2009). There are currently only a small number of CD proteins with known correlations to glioma disease progression and prognosis. Higher expression of CD44 has been correlated with aggressive glioma tumours as determined by immunohistochemistry (Wei et al., 2010). CD63 is a tetraspanin protein that complexes with integrins to mediate binding although little is currently known about the effects of this on tumour progression (Pols & Klumperman, 2009). It is a known independent prognostic marker of disease progression and reduced survival times in astrocytoma (Rorive et al., 2010). In glial tumours, CD63 is more highly expressed in higher-grade tumours and correlated with shorter survival times in GBM (Rorive et al., 2010). Interestingly, a study using tissue microarrays revealed that CD90 expression was higher in human GBM tissue than in healthy brain (He et al., 2012). Subsequent neurosphere analysis *in vitro* showed that CD90+ cells possessed superior sphere forming ability when compared with CD90- cells.

Additionally, CD90+ cells accumulated in regions of vascularity in GBM tissues (He et al., 2012). These results suggest the possible involvement of CD90+ cells in tumour growth and progression. Collectively, these studies support the hypothesis that cell surface proteins that are upregulated in glioma tumour cells could be contributing to disease progression.

5.2 Hypothesis

Cell-surface protein expression can be used to identify cell subpopulations of clinical relevance in GBM tumours.

5.3 Aims

The aim of this study was to identify a panel of glioma-specific CD proteins that can be used to identify tumour cell populations of clinical interest. Identification of specific tumour cell populations is a prerequisite for further studies on the relative contribution of specific CD proteins to the phenotypic properties (e.g. proliferation, apoptosis, drug resistance) of glioma cells in patients.

5.4 Results

5.4.1 Development of a robust flow cytometry screening platform to quantitate CD expression in GBM cells

In the first instance, a robust screen was developed that was capable of analyzing a large number of live cells by flow cytometry, while at the same time excluding false positives. As the population of interest may be attributable to a small subpopulation of cells, it was important to be able to assay large numbers of cells and then progressively i) exclude dead cells, ii) exclude cell clumps iii) determine the level of non-specific background staining, iv) exclude cells from other tissues (non-tumour cells).

Live tumour cells were isolated using a gating strategy that excluded dead cells based on fixable live-dead staining (Svendsen et al., 2011). Here, live cells with intact plasma membranes exclude the fluorescent dye, whereas dead cells have lost plasma membrane

integrity and take up the dye (Life Technologies LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit). Traditionally propidium iodide has been used for this purpose (Crissman & Steinkamp, 1973) but to facilitate the identification of multiple cell populations in a single experiment it was necessary to use a fluorophore that would not overlap with any others in use and would be retained following fixation (Crissman & Steinkamp, 1973; Hudson, Upholt, Devinny, & Vinograd, 1969). This was achieved by using a near infra-red fluorescent dye that fluoresces upon binding to free amines released when cell membranes break down producing intensely fluorescent cells with compromised membranes and minimal fluorescence in intact cells, resulting in a 50 fold difference in fluorescence intensity between live and dead cells (Perfetto, Ambrozak, Nguyen, Chattopadhyay, & Roederer, 2006) (Figure 5.1A). The live-dead stain had to be far enough away from other fluorophores that no overlap in excitation/emission spectra would occur. The excitation spectra for near infrared is 633/635nm and the emission is ~780nm. This will not overlap with fluorescein isothiocyanate FITC (Ex: 495– Em: 530nm), PE (Ex: 496 - Em: 578) or violet (Ex: 405nm - Em: 450nm) fluorophores (Figure 5.1B).

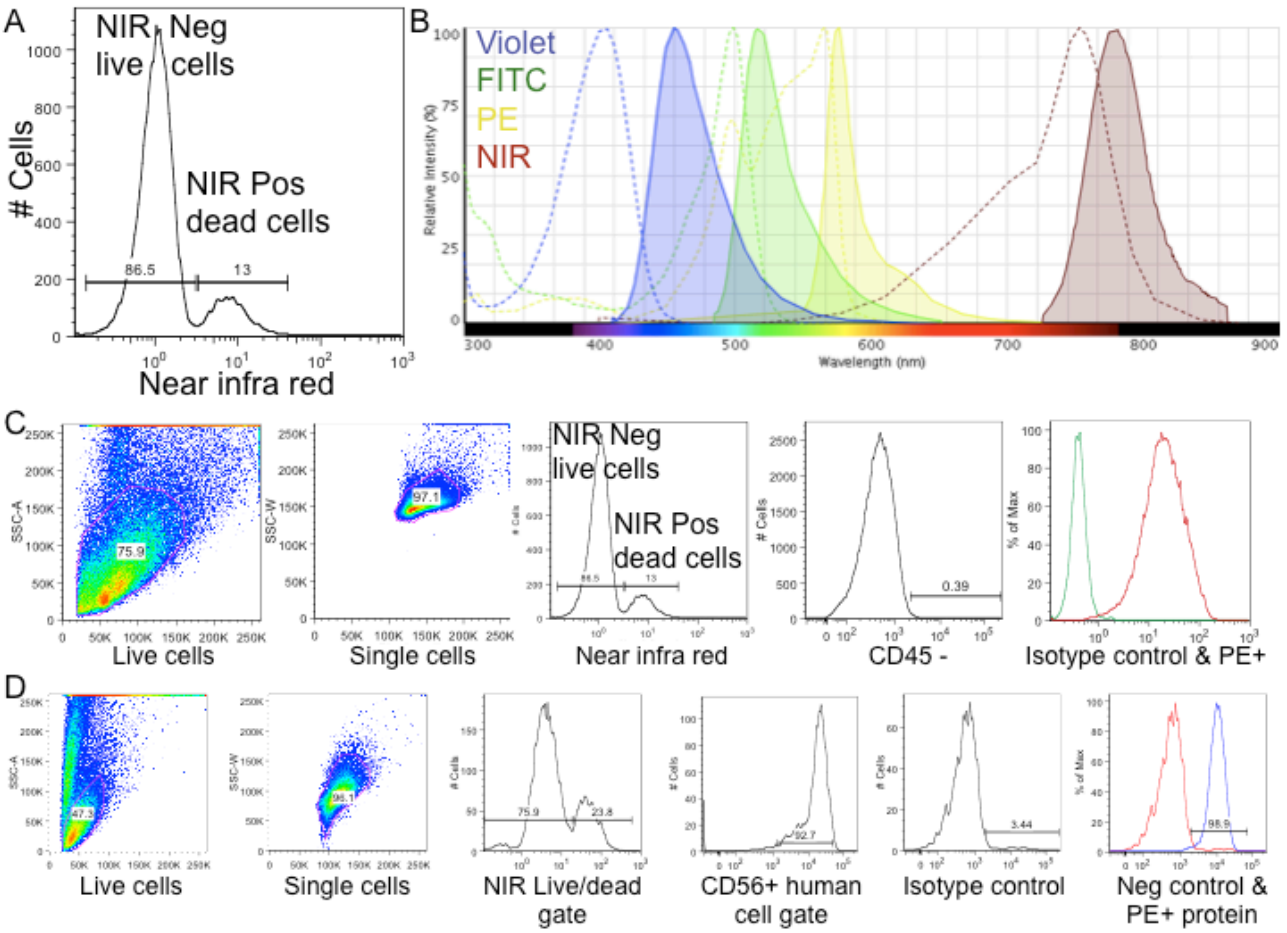


Figure 5-1 Cell surface markers were used to identify populations of interest. (A) Gating strategy used to exclude dead cells. The histogram shows near infrared (NIR) Live/dead fixable cell stain (Invitrogen). Dead cells exhibit strong fluorescence, live cells mild fluorescence. (B) Spectral viewer (generated using the Invitrogen web site) showing absorption and fluorescence emission spectra of fluorophores selected to achieve minimal overlap. Violet (450/40) used to exclude lymphocytes, Near IR (760/780) to exclude dead cells, PE (575/26) and FITC (530/30) were used to identify populations of interest. (C) Shows the gating strategy used in flow cytometry analysis of primary human GBM cells. Forward scatter and side scatter on the Becton Dickinson LSRII were used to isolate the live, single cells. The first panel on the left shows exclusion of dead cells and debris. The second panel shows the single cell gate, which was used to exclude doublets. The third panel shows the fixable Live/dead Near infrared stain, which is taken up by dead cells that have compromised membranes and is excluded by cells with intact membranes. Panel 4 shows CD45 used to exclude lymphocytes. The last panel shows the isotype control, in green, used at identical concentrations to the antibodies to exclude non-specific staining and an example of the PE positive expression of a protein of interest, shown in red. (D) Gating strategy used for mouse xenograft tumours. Forward scatter and side scatter shown were used to exclude dead cells and debris and isolate the live cell population shown in the first panel on the left. The second panel shows the single cell gate that excludes doublets. The third panel shows the fixable Live/dead Near infrared stain that is excluded by live cells. Panel 4 shows the human cell gate. CD56, which is only expressed on human cells, was used to exclude mouse cells from xenograft tumour cell populations. The isotype control shown in panel 5 was used at identical concentrations to the antibodies of interest to determine the level of non-specific staining for exclusion. The last panel shows an example of the isotype control and the PE positive expression of a protein of interest.

Clumps were excluded using Time of Flight on the Beckman Coulter Gallios or forward and side scatter on the Becton Dickinson, LSRII. Time of flight is a function of cell size and is determined by the duration of the passage of the cell through the point where the laser beam intersects with the stream of cells in the flow cytometer, whereby cells with a larger diameter take longer to pass through the beam than smaller cells. Using this method, clumps of cells can be excluded based on size using the Beckman Coulter Gallios (Sleeman, Kendrick, Ashworth, Isacke, & Smalley, 2006). Forward scattered light is used to detect cell surface area. Side scattered light is used to detect granularity. These parameters allowed detection and exclusion of debris and clumps and isolation of live, single cells using the Becton Dickinson LSRII (Loken & Herzenber, 1975; Tzur, Moore, Jorgensen, Shapiro, & Kirschner, 2011) (Figures 5.1C and D).

Antibodies can bind non-specifically to cells due to protein-protein interactions, as well as binding Fc receptors on the cell surface (Hulspas, O'Gorman, Wood, Gratama, & Sutherland, 2009; Krutzik & Nolan, 2003). To quantify the level of non-specific background staining, cells were also incubated in identical concentrations of an isotype control (Figures 5.1C and D). This was used to determine the lower limits of positive staining. The majority of the proteins analysed were directly conjugated to either PE or FITC. The gating strategy used excluded cells that fell outside of our population of interest so that only the human brain tumour cells remained for analysis. To identify tumour and non-tumour cells *in vivo* and in primary patient tumours, cell surface markers were used to identify proteins expressed on cells we wanted to include or exclude. CD56, known as (NCAM) is a marker of human cells and is not expressed by murine cells (Figure 5.1D). CD56 was used to identify human cells in murine subcutaneous and intracranial tumours excluding cells that did not express CD56. All fresh primary human cells were stained with CD45 to gate out and therefore exclude lymphocytes from further study (Figure 5.1C).

5.4.2 Identification of a preliminary CD panel based on the screening of glioma tumoursphere cell lines for protein expression levels of a large cohort of cell surface proteins

Using the gating strategy described above, the expression levels of 118 cell surface proteins in primary tumoursphere cell lines derived from ten patient tumours were analysed by flow cytometry (Figure 5.2). Tumour cell lines were passaged from primary human tumours obtained directly from surgery at the Princess Alexandra Hospital Brisbane with patient consent and human ethics approval. Cells were cultured as tumourspheres, as this has been shown to more closely preserve the original genotype and phenotype of the cell than cells cultured in serum (J. Lee et al., 2006). Maintaining cell lines in a state as close as possible to the cells of the original tumour was important as the initial results would determine which proteins would be selected for further study.

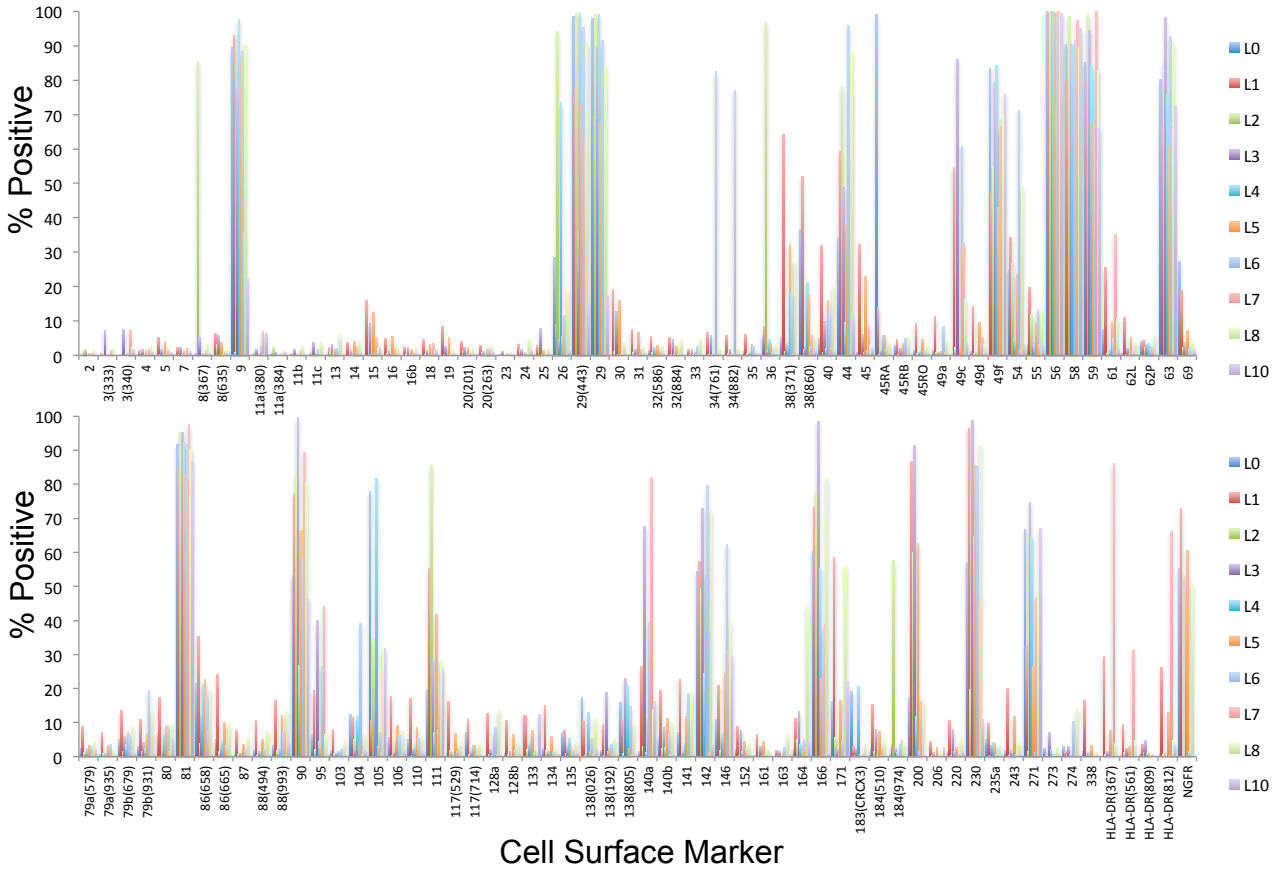


Figure 5-2 A panel of 118 cell surface proteins was analysed using ten human GBM passaged cell lines to select a subset for further study. Human GBM cell lines were passaged as tumourspheres, chemically dissociated into single cells, incubated in cell surface protein antibodies and fixed prior to flow cytometry analysis. From this initial screen a subset of proteins were selected for further study. The X-axis represents the cell surface proteins and their names expressed as a CD. The Y-axis represents the percentage of cells that were positive for the protein.

There are two methods for measuring protein expression. The level of protein expression can be calculated based on the overall fluorescence intensity of a population. Alternatively, protein expression can be defined based on the threshold of fluorescence intensity determined by the isotype control. We used the latter method. The advantage of using this method was that the result is not affected by experimental variation such as relative fluorescence. Staining intensity is significantly affected by variables such as cell number. The inclusion of an unstained control for each experiment each time was designed to improve accuracy and consistency when determining the percentage positive. This

provides a quantitative number that can be used across experiments. Using an isotype control, a threshold value for non-specific staining was determined by drawing a gate around the cells positively expressing the isotype control. Based on this threshold, any cell with staining fluorescence above this was deemed positive. Within the 118-CD screen, the proportion of positive cells ranged from 0-100% within each cell line (Fig 5.2). In addition, there was significant variation of CD protein expression within a subset of CD proteins across cell lines, while others were highly expressed across all lines studied. Some CD proteins were not expressed in any of the human GBM patient lines.

Some potentially useful patterns were suggested from this initial screen. First, CD markers exist that may be uniformly expressed in all GBM tumour cells and are therefore constant across GBM patients. These CD proteins can be used as a positive control for CD staining in experiments and potentially in assessing clinical samples. Second, a subset of CD proteins exists whose expression is highly variable. This subset of CD markers may be differentially expressed between tumour cell sub-populations, and may therefore be used to identify specific tumour cell sub-populations of clinical interest.

5.4.3 Selection of a small panel of cell surface markers for validation of results

From the initial panel of 118 cell surface proteins screened, 26 were selected to study further to determine the level of experimental variation with two additional proteins that served as negative and positive controls. The selection of each of the 26 proteins to continue studying was based on positivity for one or more of the following three factors.

1. Significant expression across all lines analysed. Significant was defined as 75 % of cells in a tumour expressed the protein. Within this 75% of positive cells, variation in fluorescent intensity from individual cells reflecting more or less protein on one cell than another was not used to include or exclude cells. A small number of proteins were consistently highly expressed across all lines tested in each experiment.

2. Some proteins were highly expressed in some lines but not all lines. Because one characteristic of human GBM is the existence of a very heterogeneous cell population it was important to include proteins that were strongly expressed in some lines even though they may have been only weakly expressed in other lines, as this may still be representative of an important cell population in different subsets of glioma patients. This

is consistent with the findings by Verhaak et al. that a number of subtypes exist within GBM exhibiting different genetic and molecular profiles (Verhaak et al., 2010).

3. Proteins that exhibited less than 10% expression in all lines were excluded from the study unless they were previously identified as prognostic markers or as being involved in tumour progression in glioma or other cancers by other research groups. The proteins we selected to continue studying based on published relevance to tumours are listed in table 4.1. We included these in our analysis particularly as the tumoursphere culture system, although the best *in vitro* tumour model available to us may differ from *in vivo* tumours and from primary tissues due to important tumour microenvironment conditions that are not easily replicated *in vitro*. Therefore, the inclusion of CD's identified as potential drivers of disease in other cancers increases the probability of identifying a prognostic panel of CD markers. CD133 is one example of a protein that was rarely expressed in the patient lines used in the initial screen, but which was included for further analyses because a number of research groups had reported links between CD133 expression and stem like cancer cells in GBM and other tumours (G. Liu et al., 2006; Singh et al., 2003; Zeppernick et al., 2008).

Table 5-1 CD proteins selected based on identification by others. Some CD proteins had previously been identified by other researchers as prognostic markers in cancer. These proteins were included in the study specifically because they were reported to be clinically relevant in glioma or other cancers in work by other research groups.

CD	Tumour Association	Authors
9	Increased expression was seen in more advanced gastric cancer	Souyer, S. 2009.
	Expression was an independent prognostic factor in SCC of head & neck	Mhawech, P. 2004.
44	Upregulated in more aggressive head & neck tumours	Benzion, J. 2010.
	High serum levels were associated with poor response to treatment in non-Hodgkin's lymphoma	Ristamaki, R. 1977.
47	Increased expression was associated with reduced overall survival in AML.	Majeti, R. 2009.
	Upregulated in human & murine myeloid leukaemia	Jaiswal, S. 2009.
49c	An indicator of poor prognosis in colon cancer	Hashida, H. 2002
49f	Detected in ALL cells but absent in control cells. Expresssion increased following treatment.	DiGuiseppe, J. A. 2009.
59	Strongly expressed in adenocarcinoma of the prostate & associated with disease progression & poor prognosis.	Xu, C. 2005.
63	Strong expression correlated to shorter survival times in GBM & lower levels in lower grade glioma.	Rorive, S. 2010.
90	Higher expression levels correlated to shorter survival times in AML	Buccisano, F. 2004.
105	Higher levels in patients with disease recurrence & shorter progression free survival in patients with laryngeal carcinoma.	Marioni, M. 2010.
133	Higher expression levels were associated with shorter survival times & an increase in tumour grade in glioma.	Zeppernick, F. 2008.
	Increased levels corresponded with an increase in tumour grade & shorter survival times.	Zhao, P. 2010.
146	A marker of poor prognosis in epithelial ovarian cancer.	Aldovini, D. 2006.
	Higher levels associated with shorter survival times in prostate cancer	Fritsche, F. R. 2008.
151	Over-expression seen in pancreatic andenocarcinoma.	Zhu, G. H. 2010.
	Over-expression was correlated with metastasis & invasion in intrahepatic cholangiocarcinoma.	Huang, X. Y. 2010.
166	Expression was a prognostic marker of relapse in prostate cancer.	Kristiansen, G. 2005.
171	Upregulation seen between the stages of benign nevi & malignant melanoma.	Fogel, M. 2002.
184	High levels of CD184 in the nucleus was an independent predictor of poor survival in colorectal cancer.	Speetjens, F. M. 2009.
200	Found to promote metastasis in SCC.	Stumfova, M. 2010.

To increase the statistical confidence of the initial analysis 12 patient human GBM lines were screened twice each for expression of the 26 cell surface proteins selected for the smaller panel (Figure 5.3). This data led to the conclusion that the constant CD's were constant across all GBM lines, supporting the hypothesis that these CD proteins were universally expressed.

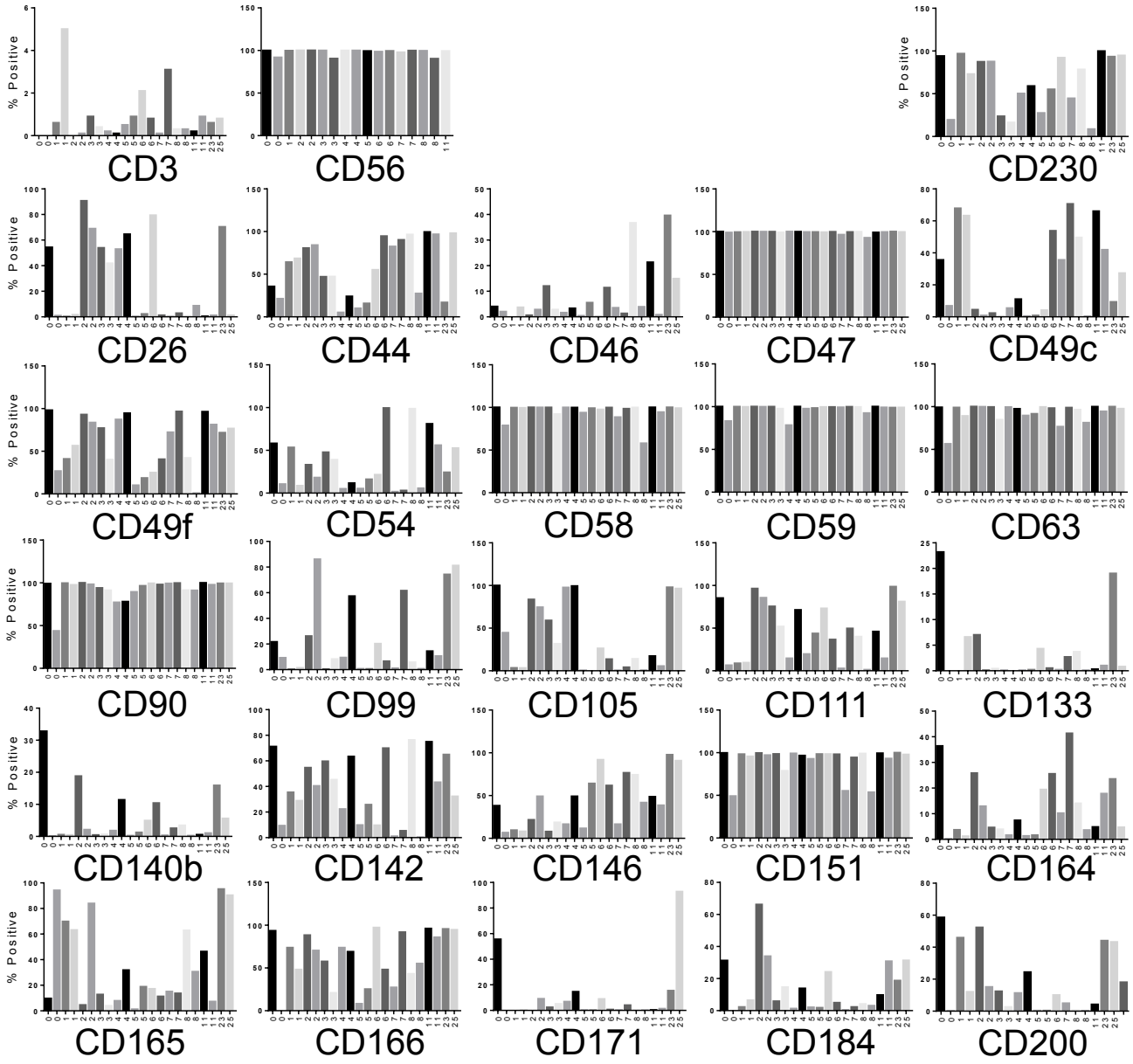


Figure 5-3 Twenty-six cell surface proteins were selected for further study. The x-axis titles are the cell surface protein names. The numbers below each column on the X-axis represent the cell line names. Protein expression, shown as the percentage positive appears on the Y-axis. The figure represents 12 passaged cell lines and 22 experiments as some cell lines were analysed in

duplicate (L0x2, L1x2, L2x2, L3x2, L4x2, L5x2, L6x2, L7x2, L8x2, L11x2, L23x1, L25x1). CD3 was used as a negative control and CD56 as a positive control.

Two of the proteins that were invariant were selected to serve as internal controls to highlight experimental noise and assess staining efficiency when replicating experiments. CD3 was either not expressed at all or expressed at very low levels so this was selected as a negative control. CD56 was used as a positive control for passaged cell lines to determine the level of experimental variability and was consistently strongly expressed in all cell lines analysed with around 99% of cells in all lines expressing the protein. A small cohort of proteins was consistently strongly expressed in all cell lines tested. The remaining CD's were variable across all lines with no obvious patterns suggesting the possible involvement of other factors such as environmental conditions.

This data confirmed the original selection of 26 proteins for further study as the majority of proteins selected were moderately or strongly expressed in at least some of the lines analysed. One exception to this was CD133 that was weakly expressed but had been selected originally because of its reported expression in glioma studies undertaken by other researchers. We chose to retain it despite its poor expression levels because of the large quantity of literature on GBM referring to its relevance (He et al., 2011; Jamal et al., 2012).

The majority of cell surface proteins examined were highly varied across all cell lines. This was assessed by comparing the average number of percentage positive cells in each cell line for a set of proteins (Figure 5.3). In some cases, the variability was very high however, five proteins analysed, consistently exhibited very little variability, these were CD47, CD58, CD59, CD63, and CD90.

Most cell surface proteins analysed expressed great variability even within a single cell line. This was noticeable when two separate experiments compared the cell surface protein expression of a single cell line. To confirm this result, four human GBM lines were selected for study in triplicate to ascertain whether consistent CD protein expression could be seen when the same line was analysed on three separate occasions. Consistent with all previous results, cell surface protein expression was variable and the standard deviations ranged between small and large. These results were consistent with the results

seen between different lines (Figure 5.4). The standard deviation was very large in some of the proteins examined. This could be due to environmental effects. Culturing cells for different lengths of time in different sized flasks could affect oxygen and nutrient availability to cells within spheres. Cells on the outer edge of a large sphere may be able to access greater quantities of oxygen and nutrients in media compared to cells located at the centre of a sphere. Environmental variation could potentially change the expression levels of some proteins in cells within a flask. It was therefore important to determine and compare the cell surface protein expression of proliferating cells *in vivo*.

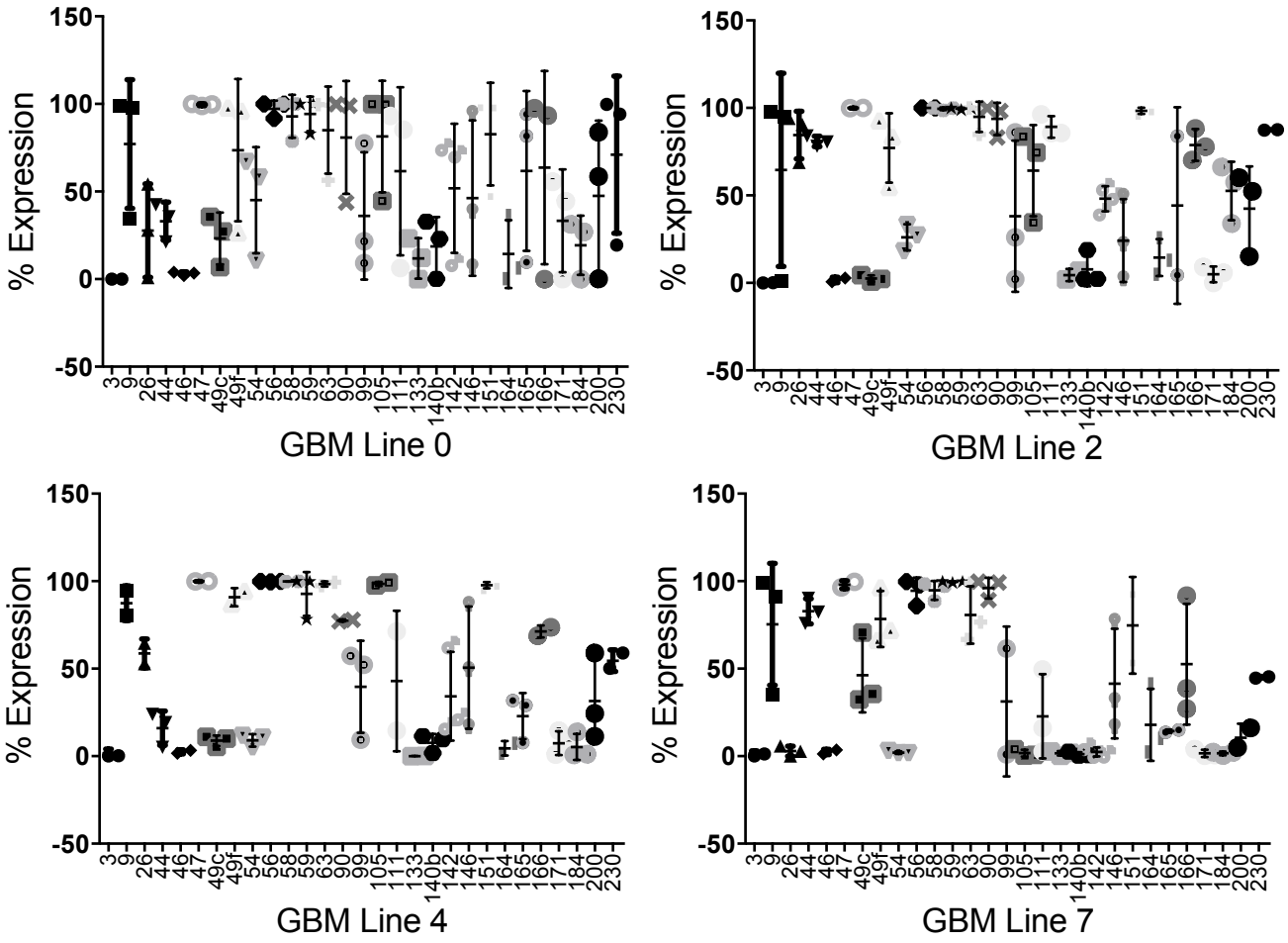


Figure 5-4 Cell surface protein expression varies within a cell line. The figure shows the percentage of cells that were positive for the 26 proteins in the small panel across four human GBM lines all performed in triplicate. The standard deviation for each protein is shown.

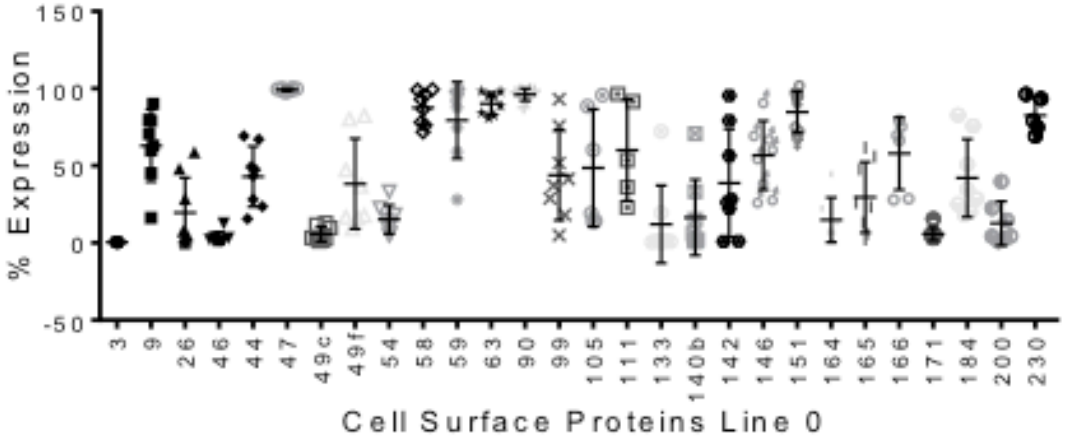
5.4.4 Potential CD markers identified *in vitro* are also expressed *in vivo*

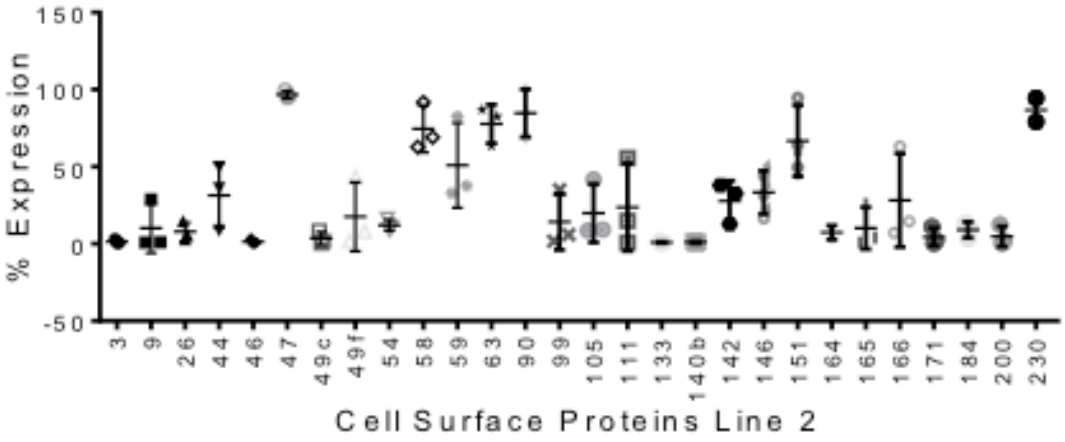
The tumour micro-environment is a critical player in tumour initiation and disease progression (M. Hu & Polyak, 2008), and therefore may regulate the expression of cell surface proteins, either through epigenetic, transcriptional or post-translational effects (these combined effects are here-after referred to as plasticity), or through natural selection increasing the frequency of clonal subpopulations that display elevated fitness within *in vivo* selection conditions (Greaves & Maley, 2012). To confirm the protein expression levels seen *in vitro*, *in vivo* surface protein expression was analysed using established NOD-SCID xenograft GBM tumour models (Deleyrolle et al., 2011).

Two mouse models were used to look at the *in vivo* expression of these same 26 proteins. NOD/SCID mice were chosen because they have no adaptive immune response and would therefore tolerate the implantation of foreign cells. The next experiment was designed to assess the variation in protein expression of xenograft tumours generated from a single cell line on a single day. This was done to exclude variations in protein expression due to differences in passage number, days in culture media and flask size. Injections of 1,000,000 cells of a single GBM cell line, line 0, were performed subcutaneously on the same day, into the flank of eight mice. In addition to this, three mice were injected with cells from L2 and L4. The cells were harvested on different days because tumours were removed according to their size so that there were sufficient cells for analysis by flow cytometry. Not all tumours reached a sufficient size at the same time due to differing tumour growth rates.

Tumours were measured regularly to determine size. When a tumour of 1cm diameter had developed, the mouse was euthanized. The tumour was removed, diced and cells chemically dissociated for analysis by flow cytometry in the same manner as the passaged cell lines. All cells were incubated in a human-specific CD monoclonal antibody (CD56) that recognizes 100% of human GBM cells and does not recognize mouse cells in addition to the panel of CD proteins. Only CD56 positive cells were included in the analyses to exclude invading or surrounding mouse cells from the analysis. The cell surface protein expression pattern of the subcutaneous mouse tumours from a single cell line was remarkably consistent with the results obtained using many passaged cell lines. Protein expression overall was highly variable, except for CD47, which remained invariant; and

CD59 and CD90, which were strongly expressed by each mouse tumour. Figure 5.5 shows the results of protein analyses of eight subcutaneous tumours generated from the same passaged human tumour cell line injected into the mice on the same day and two additional lines with three tumors each generated from Line 2 and Line 4. Overall, the standard deviations are indicative of very variable CD protein expression within a cell line in the subcutaneous mouse tumours. This data suggests that variability in protein expression was not a direct result of *in vitro* passaging techniques, as it remained a feature of *in vivo* xenograft tumours.





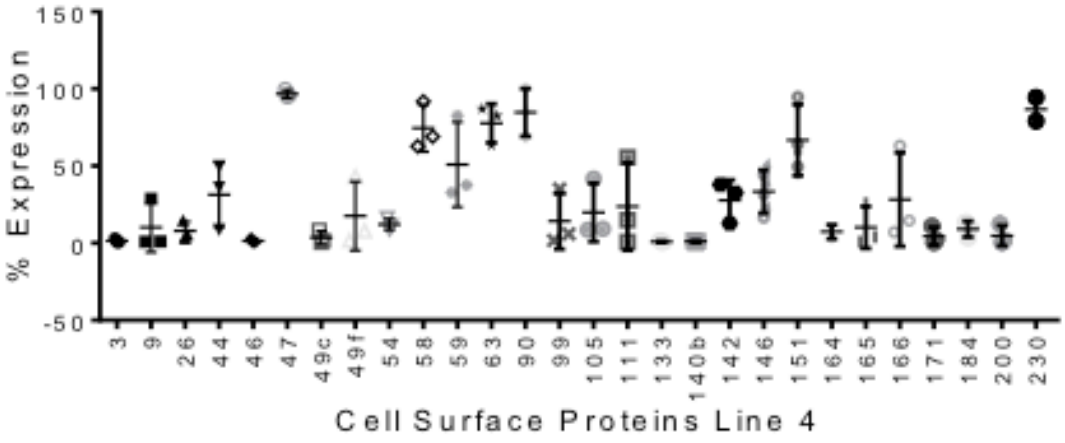


Figure 5-5 Cell surface protein expression is very variable in xenograft tumours. Due to differences between *in vitro* assays and in the *in vivo* microenvironment, cell surface protein expression was analysed on cells from subcutaneous tumours generated on the flank of mice. Subcutaneous injections delivered 1,000,000 human GBM cells from three cell lines of the same passage number on the same day into NOD/SCID mice and large tumours were allowed to form. Line 0 n=8 tumours, Line 2 n=3 and Line 4 n= 3. Tumours were harvested independently when each tumour had reached sufficient size for analysis of all or most proteins in the panel. In order to exclude cells of mouse origin, only CD56+ cells were included in the analysis.

The cell surface proteins in the previous experiment were differentially expressed in subcutaneous tumours even though they all originated from the same cell line, injected into the mice on the same day. However, tumours were harvested at different time points due to variation in growth. Tumours were only resected when they reached sufficient size to be assessed by flow cytometry, which requires a large number of cells for analysis. To confirm that variable cell surface protein expression was not due to the differing periods of time individual tumours took to reach sufficient size, we injected two mice subcutaneously using line 0 on the same day. The resulting tumours were harvested on the same day, 165 days later, cells counted and stained with the same aliquot of antibody. Analysis of these results, shown in Figure 5.6 confirmed that a high degree of variation in CD expression exists even under these stringent experimental conditions. This data suggests the hypothesis that CD protein expression is highly plastic, and dynamically regulated depending on a wide range of factors including environmental conditions and/or stochastic effects (Heddleston et al., 2011).

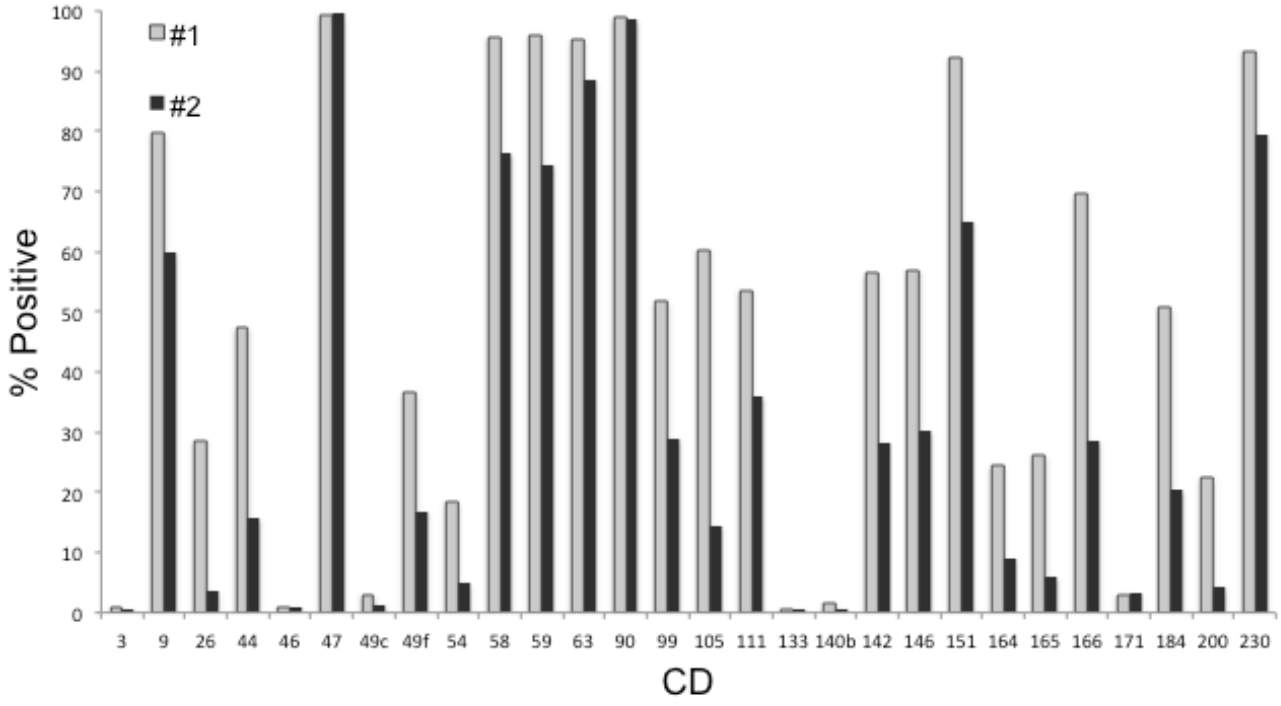


Figure 5-6 Cell surface protein expression is variable in xenograft tumours generated from the same cell line at the same time. To minimize the effects of variation caused by differences in cell line, passage number, and length of time to tumour formation, cells were injected into two mice on the same day and tumours harvested and analysed 165 days later.

5.4.5 *In vivo* confirmation of *in vitro* assays using intracranially injected glioma cells

Although the subcutaneous model is a widely used approach to study tumour biology *in vivo*, GBM rarely metastasizes from the brain, and the brain microenvironment is different to *in vitro* tumoursphere and subcutaneous environments. As discussed above, differences in microenvironments may significantly alter CD expression. To ascertain the influence of the brain environment on cell surface protein expression during tumour development 200,000 passaged human tumour cells were injected intracranially into the right striatum of anaesthetized mice positioned in a stereotax. When the mice showed signs of physical deterioration as a result of large tumour formation, (weight loss, tenting of skin due to dehydration, scruffy fur, changes in posture or gait, fits or behavioural changes) they were euthanized and the brain was removed. Fifteen tumours generated from 10 human GBM lines were analysed (Figure 5.7). Protein expression was analysed by flow cytometry. Upon analysis, expression of the selected cell surface proteins in intracranial xenograft tumours was highly variable with consistently large standard

deviations. CD56 was used to exclude cells of mouse origin from the analysis and ensure that only cells of human origin were analysed (Deleyrolle et al., 2011). Consistent with the results of the subcutaneous tumours, cell surface protein expression in intracranial xenograft tumours was highly variable and CD47 was the most consistently highly expressed protein in orthotopic xenograft tumours. This shows that CD47 is strongly expressed by human GBM cells in the brain microenvironment and that our earlier results were not simply an artifact of tissue culture.

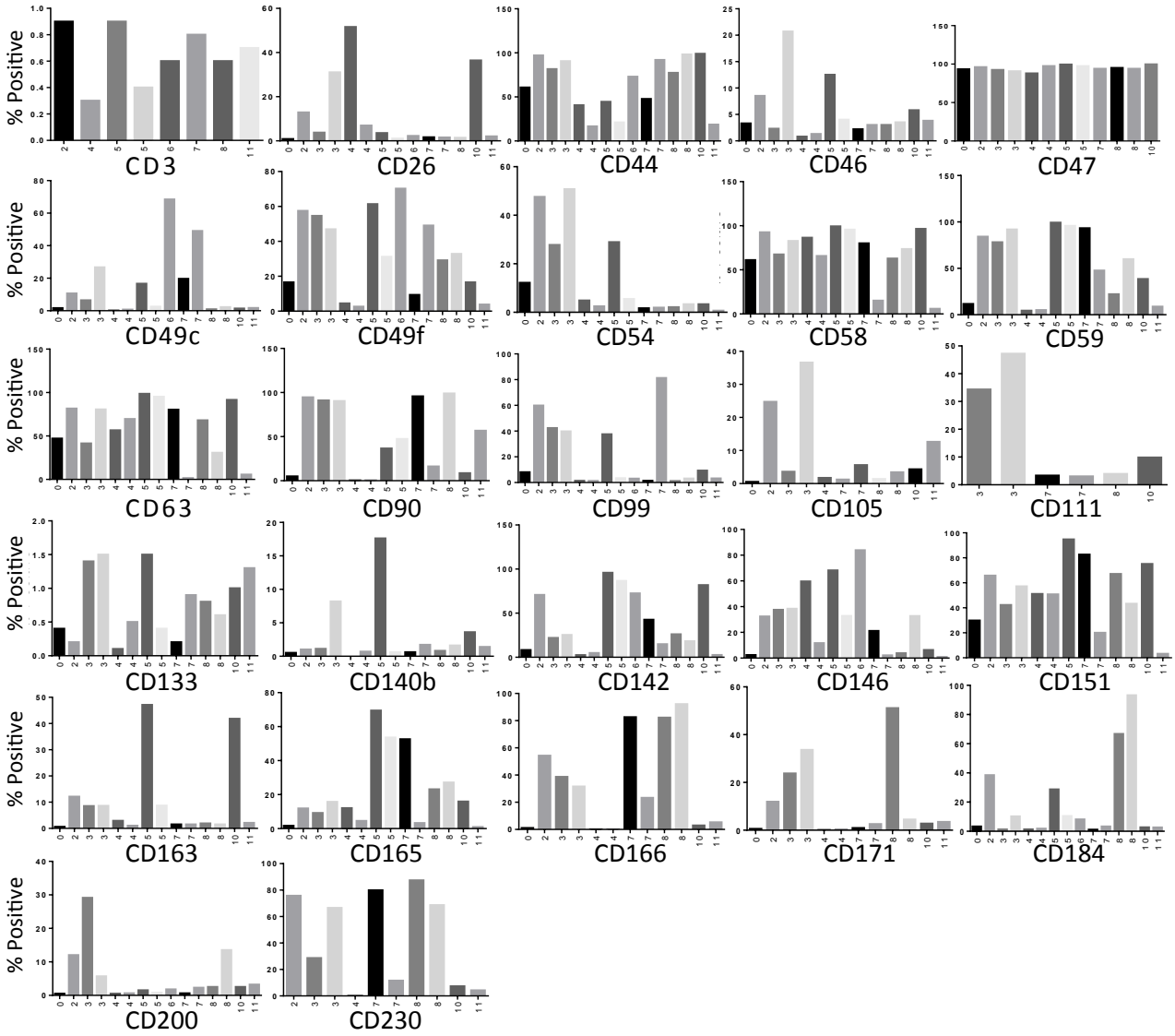


Figure 5-7 Expression of most cell surface proteins is very variable in mouse intracranial tumours. To determine the effects of the brain microenvironment on cell surface protein expression. Ten human GBM cell lines were injected intracranially into 15 mice (five lines were performed in duplicate). On some occasions, due to small cell numbers, less than the full CD panel of 26 proteins was analysed after tumour harvest.

5.4.6 Confirmation of cell surface protein expression profiles using primary glioma tumour tissue

Finally, to confirm that the panel of CD proteins was also expressed in patient tumours, fresh primary brain tumour cells straight from surgery at the Princess Alexandra Hospital were analysed. Cells were immediately dissociated and red blood cells were lysed using a red cell lysis buffer. CD45 that recognizes white blood cells was used to exclude these from the analysis. CD56 was used as a positive control. Six primary human tumours were used for this analysis. CD47 expression remained consistently high between patients, as predicted from the *in vitro* and *in vivo* tumour models described above. CD expression profiles overall varied significantly between patients (Figure 5.8). Average protein expression between patients tended to have a large standard deviation with the notable exception of CD47.

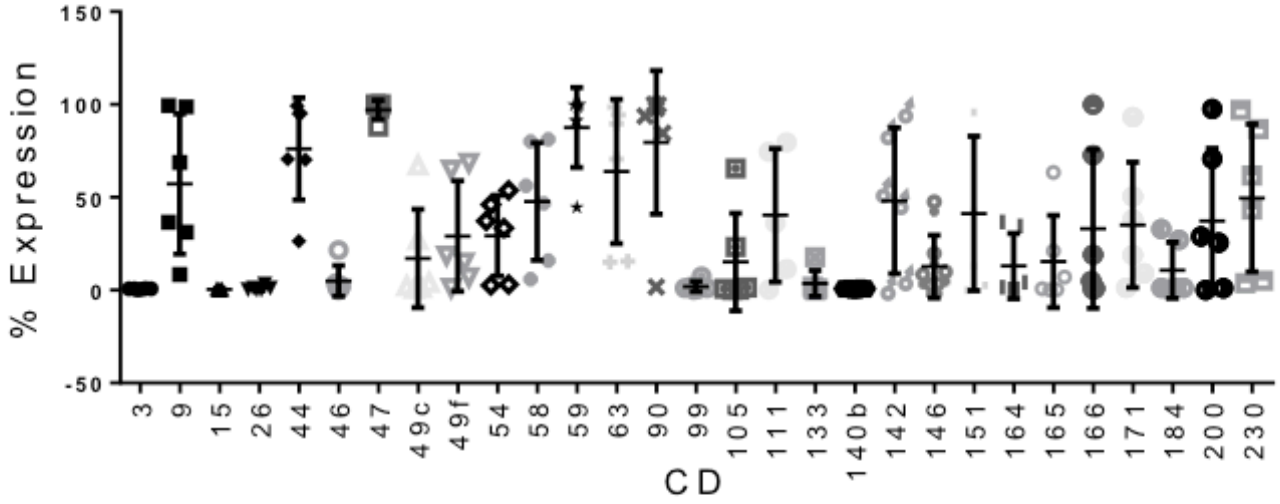


Figure 5-8 Expression of some cell surface proteins is very variable in primary human tumour tissue. The figure shows the percentage of cells that exhibited positive cell surface protein expression. Six primary patient tumours were analysed immediately following surgical removal. Red blood cells were lysed in red cell lysis buffer. Cells were diced and chemically dissociated into a single cell suspension prior to incubation in antibodies and fixation for flow cytometry analysis. CD45 was used to exclude lymphocytes.

Regardless of whether the cells were cultured *in vitro* or proliferated as part of a xenograft tumour or primary human tumour, cell surface protein expression overall was variable. These results suggest that with regard to the majority of proteins studied here, rather than being associated with a specific cell type, cell surface protein expression is very plastic.

Interestingly though, three proteins were consistently highly expressed in all tumour models we analysed. CD59 is also known as compliment regulatory protein. It controls cell lysis by inhibiting the formation of the membrane attack complex (Madjd et al., 2003). CD90 is also known as Thy-1 cell surface antigen and is an adhesion molecule used as a marker for stem cells and axonal processes on mature neurons (Buccisano et al., 2004). In a recent publication, CD90 exhibited increased expression levels in high-grade gliomas when compared to low-grade glioma and normal brain tissue and was associated with increased sphere forming ability leading to the conclusion that it was a potential marker for high-grade glioma (He et al., 2011).

CD47 is known as integrin associated protein and interacts with thrombospondin-1 (TSP-1) and signal regulatory protein alpha (SIRPα). SIRPα is expressed on macrophages and upon activation capable of inhibition of phagocytosis. For this reason CD47 has been dubbed the 'don't eat me' signal (Willingham et al., 2012). In addition, TSP-1 anti-angiogenic activity is reportedly inhibited in cancer cells by the presence of high levels of CD47 (Kalas et al., 2013).

5.4.7 CD47 is expressed in GBM.

Blocking CD47 binding to it's ligand SIRPα has the potential to expose tumour cells to circulating immune cells with the ability to remove the tumour cells using the body's natural defense system (Willingham et al., 2012). When we looked at the distribution profile of CD47, we noticed that in addition to consistently high expression levels, in all tumour models analysed CD47 displayed a tight distribution. Figure 5.9 shows the percentage of cells expressing CD47 in eight passaged cell lines and the distributions in each. Expression levels are all above 98% and distributions are small.

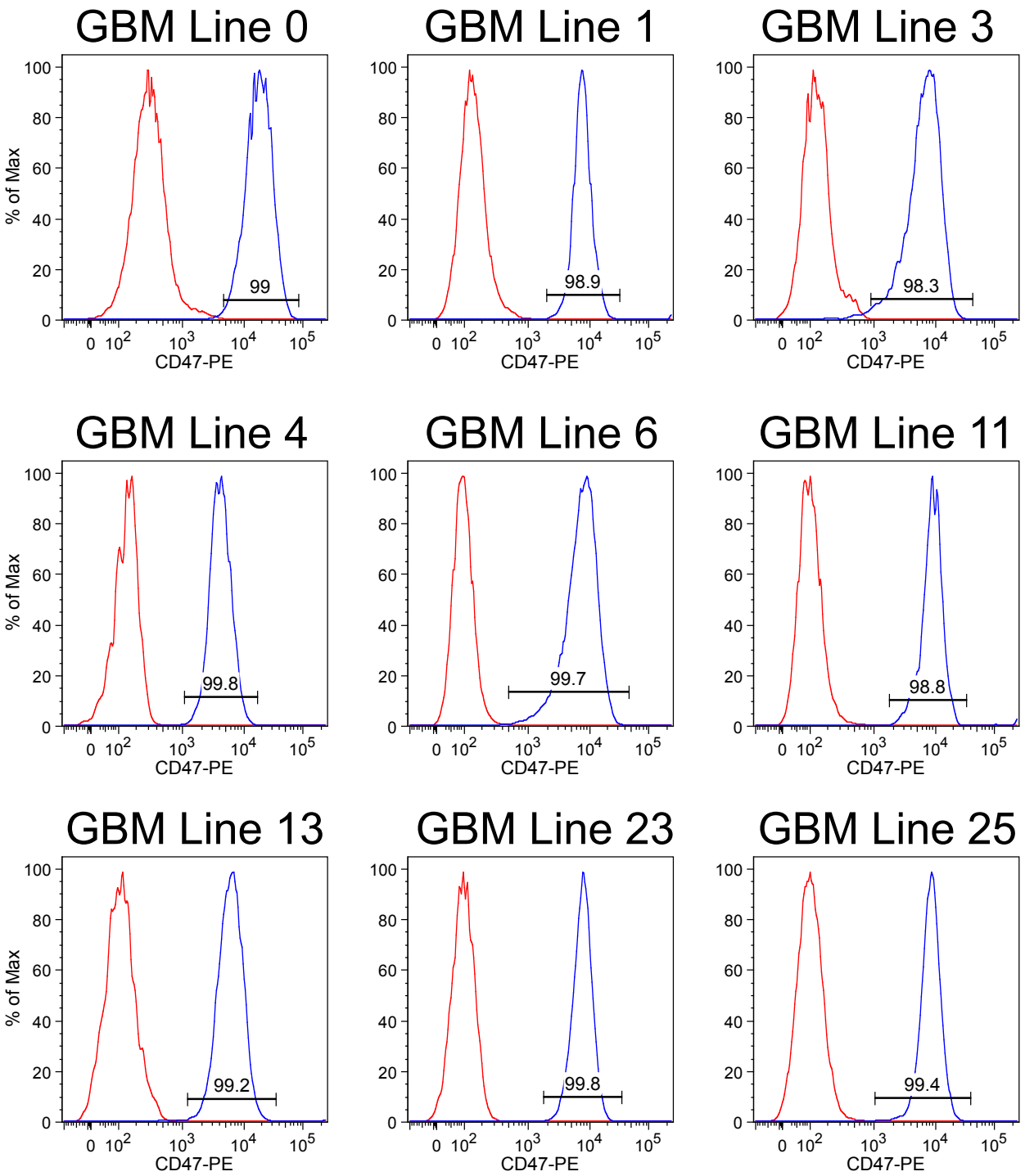


Figure 5-9 CD47 is strongly expressed in human GBM cell lines. Expression is shown for passaged human GBM cell lines from nine different patient tumours. CD47 was consistently expressed in above 98% of cells.

We confirmed these results in xenograft subcutaneous mouse tumours shown in Figure 5.10. Here we have used two replicates of three human cell lines injected to form

subcutaneous tumours in mice. CD47 expression levels shown are all above 96% with narrow distributions.

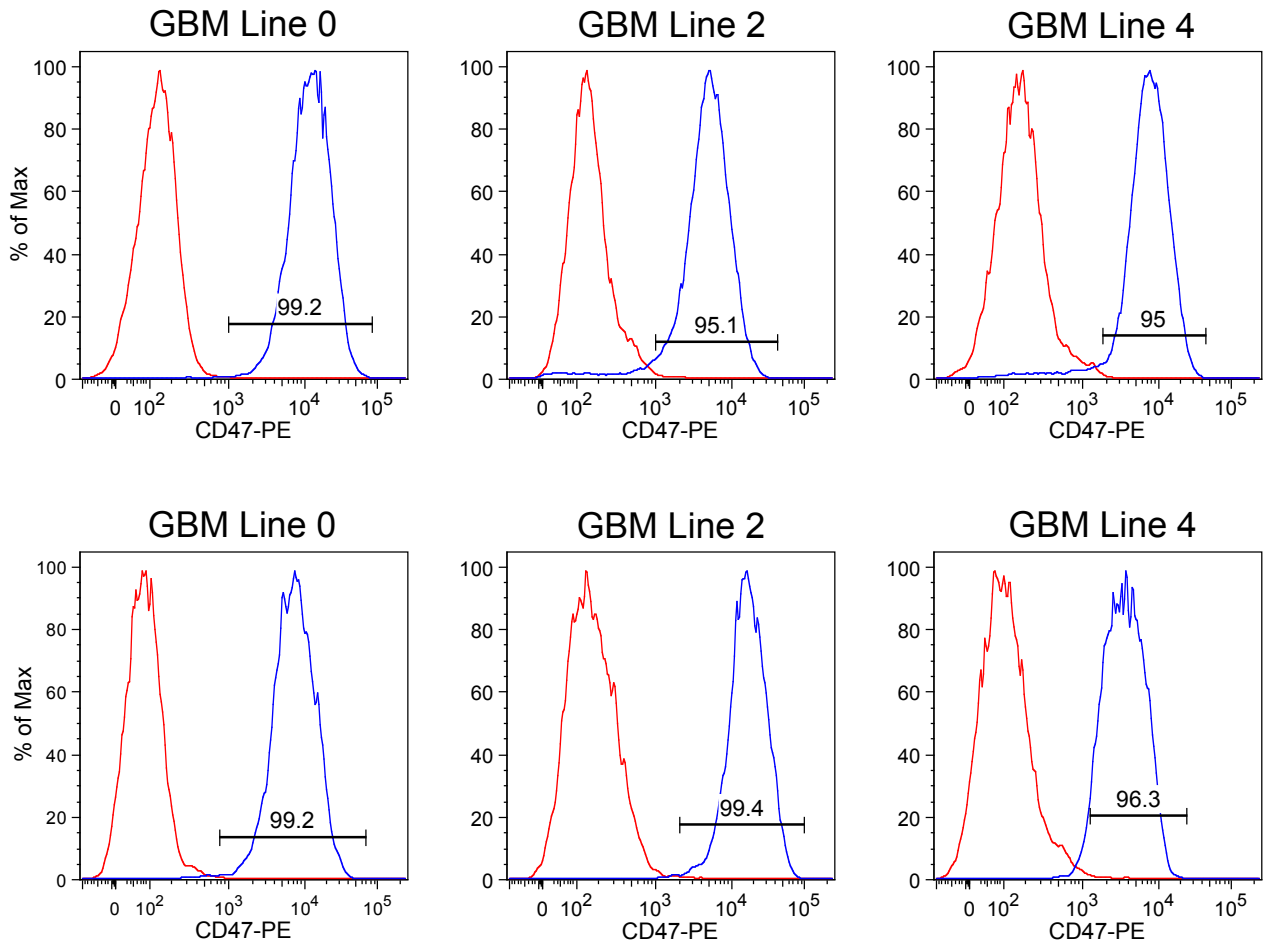


Figure 5-10 CD47 is strongly expressed in subcutaneous xenograft tumours. One million human GBM cells from three patient tumours were injected into two mice each and allowed to proliferate until large tumours had formed. CD47 in subcutaneous mouse tumours was consistently expressed in over 95% of cells.

The same analysis applied to eight orthotopic xenograft tumours is shown in Figure 5.11 where CD47 expression was consistently above 86% and distributions remained small.

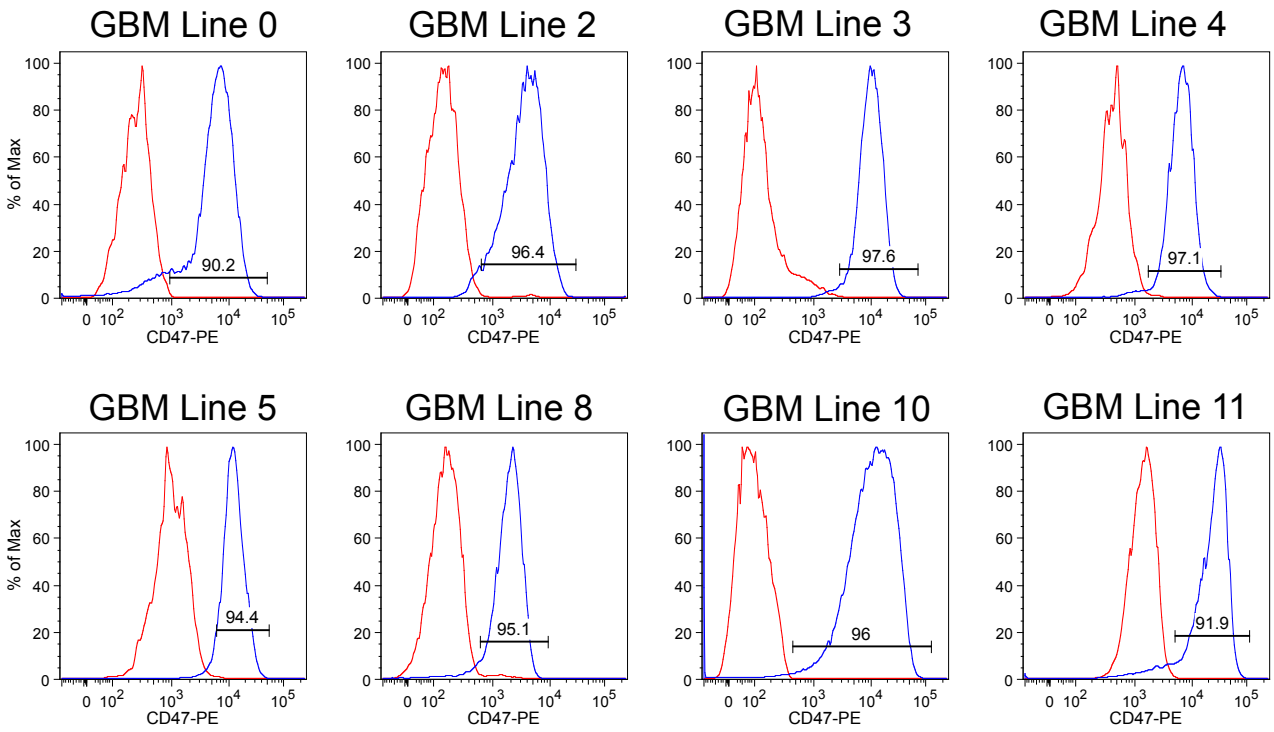


Figure 5-11 CD47 is strongly expressed in orthotopic xenograft tumours. NOD/SCID mice received intracranial injections of 200,000 human GBM cells into the right striatum under general anaesthetic and were monitored until large brain tumours formed. The x-axis represents the human GBM cell line injected into the mouse brain. The figure shows the percentage of cells expressing CD47 in eight mouse brain tumours. CD47 was expressed on above 86% of tumour cells.

Finally, fresh brain tumour tissue removed from patients during surgery was analysed to determine whether these results were consistent with that of patient tumours or simply an artefact of cell culture conditions. Figure 5.12 shows that CD47 expression across six patient tumour lines was above 90% and the size of distributions were inversely correlated with the percentage of overall CD47 positivity. Normal human brain tissue is very difficult to obtain as it is rarely surgically removed and therefore our lab was not able to use normal healthy brain cells as a control in our study. Work by others suggests that CD47 is variably expressed in normal tissue and could provide a viable target for anti-cancer therapeutics that would enable glioma cells to be recognized and removed by the immune system with minimal impact on healthy tissue (Willingham et al., 2012).

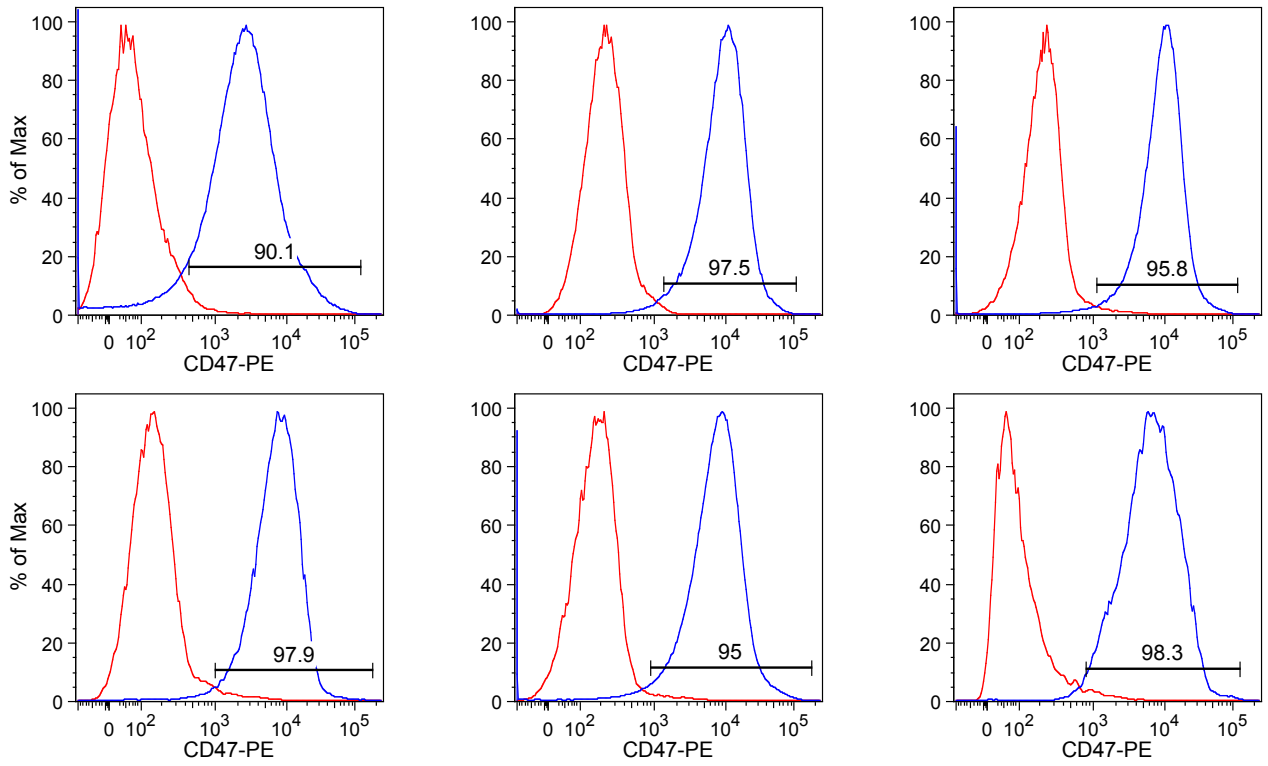


Figure 5-12 CD47 is strongly expressed in primary patient human GBM tumours. The figure shows CD47 expression in six primary human tumours.

5.4.8 Gene expression levels of five cell surface proteins are higher in GBM tumours than normal brain tissue

As we had been unable to obtain normal human brain cells to compare to the primary human brain tumour cells we studied *in vitro* and *in vivo*, we used publically available gene expression data to determine differences in gene expression levels between normal brain tissue and GBM tumours. We analysed the gene expression data of the 26 cell surface proteins we had previously selected for further study using the TCGA database. Ten normal brain tissue samples were compared to 524 GBM tumours. The GBM tumours have been divided between four subtypes identified by Verhaak et al. based on the TCGA data (Verhaak et al., 2010). The four subtypes were proneural (n=137), mesenchymal (n=159), neural (n=87) and classical (n=146). Five of the 26 proteins were significantly more highly expressed in all four GBM subtypes compared to normal human brain tissue. The five proteins were CD9, CD44, CD63, CD164, and CD184 (Figure 5.13). The proteins identified are all receptors known to be involved in functions including cell adhesion, migration, and activation. Of the five proteins, four are known to functionally interact with

another protein within the group. The functions and interactions are briefly outlined in Table 5.2.

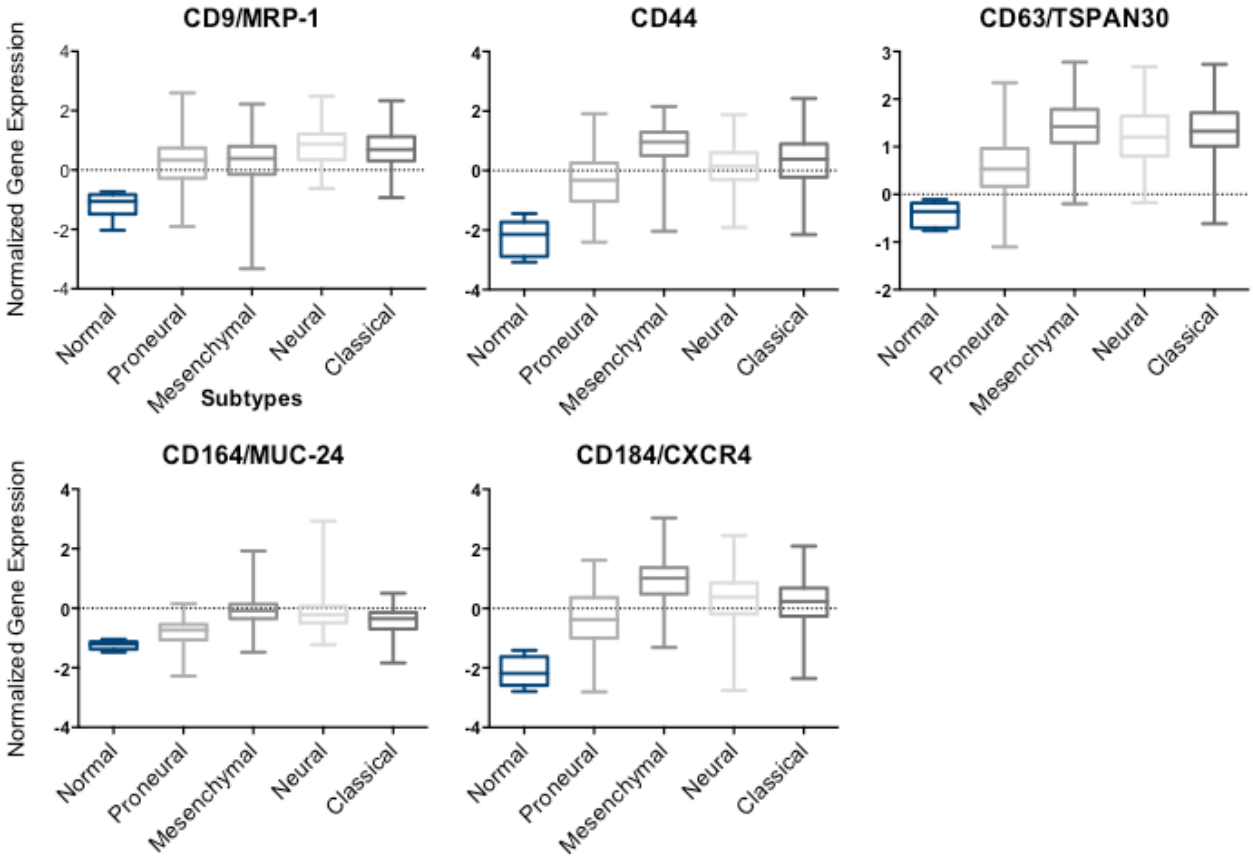


Figure 5-13 Comparison of CD gene expression data from the publically available TCGA database. The TCGA database analysed normal human brain tissue (n=10) and tumour tissue (n=524) which was divided into four subtypes by Verhaak et al. Gene expression of five proteins from the subset of 26 that I selected for further study was significantly higher in all GBM subtypes than in normal brain tissue.

Table 5-2 Table lists known functions of five cell surface proteins upregulated in GBM. The table briefly lists known functions of five cell surface proteins that are significantly upregulated in all four GBM subtypes compared to normal brain tissue. The analysis was performed on publically available TCGA gene expression data for GBM. Functions were obtained from the references within the table and The Human Protein Atlas, UniProt and the Atlas of Genetics and cytogenetics in Oncology and Haematology.

Protein	Known Functions
CD9/MRP-1	Receptor. Tetraspanin. Expressed on haematopoietic epithelial cells and during nervous system development. Interacts with many other proteins including tetraspanins, integrins and glycoproteins. Involved in cellular differentiation, adhesion, signal transduction.
CD44	Receptor. Glycoprotein. Involved in cell-cell interactions, cell adhesion, cell migration. Acts as a receptor for hyaluronic acid.
CD63/TSPAN30	Receptor. Tetraspanin. Regulation of cell development, activation, growth and motility. Interacts with many proteins including integrins, other tetraspanins (including CD9 and CD151), kinases, adaptor proteins and other proteins.
CD164/MUC-24	Receptor. A transmembrane sialomucin. An adhesion receptor. Promotes tumourigenicity through the CXCR4/SDF-1 α axis in *in vivo* ovarian cancer models Huang, A.F. 2013. Mol Cancer, 12:115.
CD184/Fusin/CXCR4	Receptor. Chemokine receptor specific for stromal cell-derived factor-1 (SDF-1). Involved in cerebellar development. May mediate hippocampal-neuron survival. Involved in cell migration and brain development.

To determine whether the five genes that were significantly more highly expressed in GBM were similarly upregulated in other solid tumour types, we analysed the TCGA microarray data of three solid tumour types. Mircoarray data with organ specific controls was only available for breast cancer, rectal adenocarcinoma, and colon adenocarcinoma. We found that CD44 was the only protein of the five that was upregulated in other tumour types and it was upregulated in just two of the three solid tumours we analysed; colon adenocarcinoma (normal n=19, tumour n=155) and rectal adenocarcinoma (normal n=3, tumour n=69) (Figure 5.14).

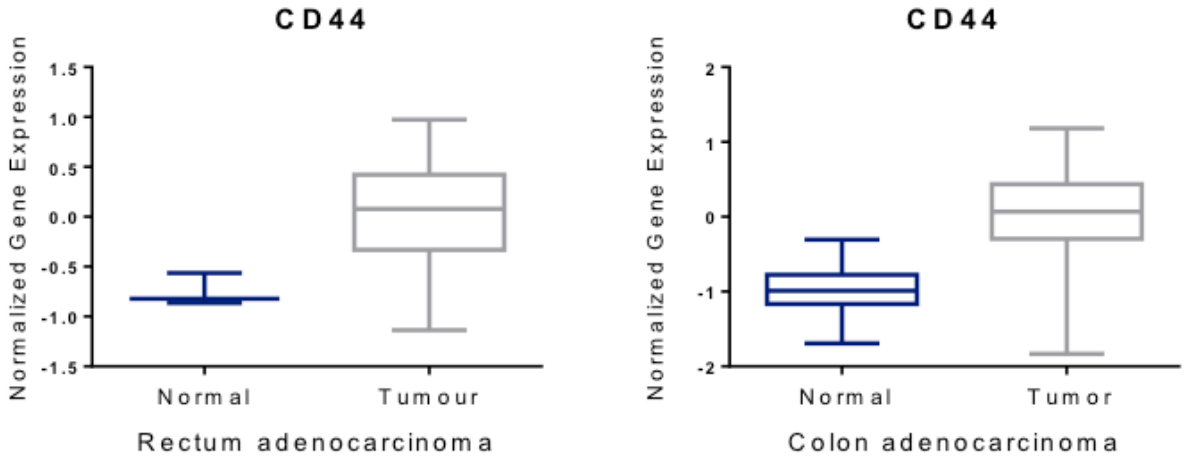


Figure 5-14 CD44 is significantly upregulated in two other solid tumour types. The micro array data on the TCGA database revealed that CD44 was significantly more highly expressed in two other solid tumour types, rectum adenocarcinoma (normal n=3, tumor n=69) and colon adenocarcinoma (normal n= 19, tumour n=155).

5.5 Discussion

Cell surface markers have been identified in a variety of tumour types as contributing to disease progression (Joshua et al., 2012) and as prognostic indicators (Ke et al., 2009). We analysed glioma cells to determine whether CD proteins could be used to identify a subpopulation of cells of clinical relevance in GBM. We screened ten human GBM lines to determine the expression levels of 118 CD proteins by flow cytometry. The purpose of this study was to identify subpopulations of cells of clinical relevance in GBM as potential future therapeutic targets. Of the 118 cell surface markers screened, we selected 26 to study in more depth based on several features. Some were selected due to strong expression across multiple cell lines interrogated. Other proteins were interesting because they were highly expressed in some lines although not in all lines. Some proteins were chosen because they had been identified as proteins of interest in other tumour types by other investigators.

5.5.1 Three cell surface proteins were consistently highly expressed *in vitro* and *in vivo*

The cell surface expression of the majority of the proteins analysed was variable whether the cells were obtained from passaged cell lines, xenograft tumours or unpassaged fresh primary tumour tissue straight from the human brain. A prognostic marker must be

expressed consistently at some stage or in a particular subtype to be useful in diagnosis or prognosis. CD56 was consistently highly expressed and used as a positive control for identification of human cells in xenograft models. Throughout this analysis, three proteins of interest were consistently highly expressed in passaged cell lines, xenograft tumours and fresh primary human glioma cells. CD47, CD59, and CD90 have all been reported to provide benefits to glioma cells promoting tumour development and therapeutic resistance (He et al., 2012; Maenpaa, Junnikkala, Hakulinen, Timonen, & Meri, 1996; Willingham et al., 2012).

5.5.2 CD59 provides protection against complement lysis

CD59 is a known regulator of complement (Cai et al., 2014). Complement system proteins are produced in the liver and circulate in the serum component of blood, and reside on plasma membranes as cell surface receptors in an inactivated form as pro-proteins (Carroll, 2004). Cleavage of the inactivated proteins leads to formation of the membrane attack complex (MAC). The complement system/innate immune system does not adapt like the adaptive immune system but can be recruited by it (Kopp, Hebecker, Svobodova, & Jozsi, 2012). The complement system acts in a few ways: boosting phagocytosis of antigens, recruiting macrophages and neutrophils, promoting lysis of foreign cells. The cell surface protein CD59 also known as protectin or membrane inhibitor of reactive lysis inhibits formation of the membrane attack complex (Cashman, Ramo, & Kumar-Singh, 2011).

Immunohistochemical staining of normal human nervous system tissue revealed that CD59 was present on endothelial cells as well as neurons and glia in the CNS (Vedeler et al., 1994). Increased CD59 expression in the human brain is associated with the normal process of myelination and in reactive astrocytes in disease states in the CNS (Zajicek, Wing, Skepper, & Compston, 1995). These data suggest that CD59 has a role in regulation of complement during normal brain development. It is expressed on all major neuronal cell types providing protection during crucial developmental processes. In addition to this CD59 is activated during reactive astrocytosis, occurring as a result of injury and disease (Pekny, Wilhelmsson, Bogestal, & Pekna, 2007).

Tumours have been shown to exhibit increased levels of complement-regulatory proteins including CD59 enabling them to withstand attack by complement (Durrant & Spendlove, 2001). CD59 is strongly expressed in glioma cell lines and primary glioma tumours providing remarkable protection against complement lysis in comparison to other cell lines (Maenpaa et al., 1996). Antibody therapy that targets membrane complement regulatory proteins including CD59 *in vivo* significantly enhanced the cytotoxic effects of Herceptin on lung cancer cells (W. P. Zhao, Zhu, Duan, & Chen, 2009). Administration of a human monoclonal antibody, which neutralizes CD59 in vivo, enhanced the therapeutic effect of rituximab in B-lymphoma (Macor et al., 2007). In combination with other strategies, the ability to disable CD59 and therefore resistance to complement, could slow aggressive tumour development following initial treatment by rendering cancer cells sensitive to complement cell lysis.

5.5.3 CD90: a potential cancer biomarker

CD90 is a glycophosphatidylinositol (GPI) anchored cell surface antigen. GPI proteins are synthesized as precursor proteins that insert into the extracellular aspect of the membrane (Mayor & Riezman, 2004). Also known as Thy-1, CD90 is expressed on human thymocytes, neurons, endothelial cells, fibroblasts, and T-cells. It is involved in a range of functions including cell adhesion, migration and death, neurite outgrowth, and tumour development (Rege & Hagood, 2006).

A number of lines of evidence suggest a contribution of CD90 protein expression to both solid and liquid tumour progression. Strong expression of CD90 correlated with invasion and metastases in hepatocellular carcinoma (B. Q. Cheng, Jiang, Li, Fan, & Ma, 2012). Studies by True et al. found that while benign prostate tissue showed limited CD90 protein expression, protein expression was upregulated in prostate cancer stromal cells (True et al., 2010). They concluded that in contrast to other proteins that showed no increase in expression in tumour tissue, CD90 was a potential cancer biomarker. Other work that focused on identifying prognostic biomarkers in 148 elderly patients with AML by flow cytometry revealed that increased CD90 expression predicted shorter survival times and correlated with unfavourable cytogenetics or unfavourable drug resistance profiles (Buccisano et al., 2004).

Recently CD90 was reported by He et al., to be a prognostic marker for aggressive gliomas. Detected on a variety of cells but predominantly on endothelial cells on tissue microarrays it was referred to as a cancer stem cell marker. Exhibiting strong expression on glioma tissues with increased expression surrounding vessels it was very weakly expressed in normal brain tissue on tissue microarrays. Additionally, CD90+ cells exhibited a superior sphere forming ability when compared with CD90- cells *in vitro* (He et al., 2012). These findings are consistent with our results, which showed that the expression of CD90 on the surface of glioma cells was usually above 80% ranging between 60% and 100%.

5.5.4 CD47 is integral to normal nervous system development

In the brain CD47 resides on the surface of neurons, microglia, myelin, oligodendrocytes and schwann cells while the SIRPα receptor is situated on the surface of microglia. Microglia are derived from macrophages that integrate into the central nervous system during development and like microglia express both the CD47 ligand and SIRPα receptor (Gitik, Liraz-Zaltsman, Oldenborg, Reichert, & Rotshenker, 2011). Microglia typically contain a small soma and long processes, which extend and retract to scan the local environment. The role of microglia is in leading a reconnaissance mission in search of pathogens, damaged tissue or other indicators of changes to environmental homeostasis (Kierdorf & Prinz, 2013). After detection of a disruption to the normal environment by receptor activation, microglia commence phagocytosis and initiate recruitment of other immune cells, to assist with clearance of dying cells, pathogens etc. Damage to the integrity of the vasculature where injury or tissue distortion occurs facilitates the entry of circulating macrophages that further assist with phagocytosis (Kierdorf & Prinz, 2013).

5.5.5 The “Don't eat me” signal

CD47 is known as the “don't eat me” signal because one of its functions is cellular protection from phagocytic cells by self-recognition, which is established through receptor interaction (Azuma, Nakagawa, Dote, Higai, & Matsumoto, 2011). CD47 is expressed on the surface of both cancerous and noncancerous cells (Barclay & van den Berg, 2013; Y. Liu et al., 2002). However, higher CD47 expression was seen on cancer cells than on normal healthy cells (Majeti et al., 2009; Tseng et al., 2013). CD47 interacts with a number

of receptors expressed on the cell surface including integrins, signal regulatory protein-α (SIRPα) and thrombospondin-1. SIRPα is expressed on macrophages and dendritic cells (Legrand et al., 2011). The binding of CD47 with SIRPα inhibits phagocytosis by initiating a signaling cascade following phosphorylation of the cytoplasmic tail of the SIRPα receptor (Tseng et al., 2013; X. W. Zhao et al., 2011). This action is utilized in normal cellular function and tissue maintenance. Immature haematopoietic cells express CD47 to protect themselves as they travel to their final destination in the bone marrow (Willingham et al., 2012). Human neutrophil polymorphonucleocyte (PMN) cell migration is regulated in part by CD47 binding to SIRPα (Y. Liu et al., 2002).

Cancer cells expressing the ‘don't eat me signal’ can evade normal immunosurveillance systems enabling them to continue to grow and divide (Willingham et al., 2012). The development of anti-CD47 antibodies has led to the identification of the phagocytic effect of the CD47-SIRPα interaction in AML *in vitro* and *in vivo*. The anti-CD47 antibody used *in vitro* preferentially facilitated the phagocytosis of AML stem cells without any increase in the phagocytosis of their normal counterparts. The author advocated the use of monoclonal anti-CD47 therapy as part of a targeted combination therapy in the elimination of leukaemia cells (Majeti et al., 2009).

Willingham et al., found that CD47 expression levels correlated negatively with survival times in a range of solid tumours with cancer cells expressing an average of 3.3 fold more CD47 than normal, adjacent, noncancerous cells, as assessed by quantitative flow cytometry (Willingham et al., 2012). The normal adjacent tissues used in this study were sarcoma, ovarian, breast, bladder, colon, liver, pancreatic, lung, kidney, and head and neck squamous cell carcinoma. Normal human brain tissue is notably absent from this list. The administration of CD47 antibody has been shown to slow tumor progression, prolong life and in small tumors antibody therapy halted disease progression completely in *in vivo* xenograft studies. High CD47 mRNA expression levels in patient samples of glioma and GBM tumours correlated with shorter progression-free and overall survival times and could potentially be a clinically relevant prognostic factor in some tumours (Willingham et al., 2012).

5.5.6 CD47 blocking antibodies prevent the SIRPα interaction and inhibit tumour growth

The blood brain barrier (BBB) provides an effective obstruction to deleterious molecules under normal circumstances and significant difficulties in designing therapies capable of crossing the BBB to researchers and oncologists. To determine the efficacy of antibody therapy on brain cancer, glioblastoma cells were transplanted into the brain of NOD/SCID/γ mice, which lack B, T, and NK cells but produce macrophages capable of phagocytosis. Purified blocking anti-CD47 monoclonal antibodies (mAbs) were injected intraperitoneally resulting in significant reduction in tumor growth (Willingham et al., 2012). The response to treatment shows that the CD47 blocking antibody crossed the BBB and inhibited tumor growth in orthotopic xenograft glioblastoma tumors when administered and absorbed systemically. A recent study into the adaptive immune response of T-cells to macrophage antigen presentation that followed anti-CD47 antibody mediated phagocytosis of tumor cells revealed a cytotoxic T-cell immune response was launched which could be utilized in treating tumours by exploiting adaptive and innate immune responses in concert (Tseng et al., 2013).

As a result of the findings from the study by Willingham et al., the anti-CD47 B6H12 monoclonal antibody is currently in phase-1 clinical trials at Stanford Medical School. The study analysed the response to the anti-CD47 antibody in ovarian, breast, colon, and GBM cells in vitro and to breast, colon, bladder, ovarian and glioblastoma cells *in vivo* in xenograft models of immune deficient mice resulting in significant inhibition of tumour growth. Engraftment of breast cancer cells into fully immune competent mice was undertaken to determine the safety and efficacy of antibody therapy administered systemically resulting in significant inhibition of tumour growth with no unacceptable toxicity (Willingham et al., 2012). The outcome of the clinical trials may be different for glioblastoma tumours as the blood brain barrier poses an additional obstacle, however, a breach to the integrity of the BBB during surgery or as a result of tumour progression has the potential to allow larger than usual molecules past the barrier at least temporarily.

5.5.7 The interaction of CD47 with TSP-1 blocks nitric oxide signaling and promotes radiation damage

Thrombospondin-1 (TSP-1) is a matricellular protein ligand that also binds to CD47 (Osz, Ross, & Petrik, 2014). Inhibition of the interaction of CD47 with TSP-1 has been shown to reduce radiation damage *in vivo* due to the ability of TSP-1 to block nitric oxide signaling. Nitric oxide has a cytoprotective effect on hypoxic soft tissue preventing ischaemic damage (Isenberg et al., 2008; Maxhimer, Shih, Isenberg, Miller, & Roberts, 2009). The ability to impart radiation resistance could be of benefit to radiotherapy patients by protecting surrounding healthy tissue from ischemia while radiosensitivity is required for effective therapeutic eradication of tumour cells. For this strategy to be effective, targeted interference with the CD47, TSP-1 interaction would be required on healthy non-tumour cells while at the same time promoting the interaction between CD47 and TSP-1 on tumour cells.

5.5.8 Increased gene expression in cell surface proteins suggests potential therapeutic targets in GBM

We compared the gene expression data of the cohort of 26 cell surface proteins selected for further study in GBM tumours above with normal brain tissue using the publically available data from The Cancer Genome Atlas Data Portal. We identified five cell surface proteins with significantly higher gene expression in all four GBM subtypes, identified by Verhaaak et al. when compared to normal brain tissue (Verhaak et al., 2010). All five proteins identified were cell surface receptors with roles in cellular processes including cell adhesion, activation, and migration.

CD9, also known as motility related protein 1 (MRP-1) a member of the tetraspanin family interacts with a number of other proteins including integrins, growth factors and other tetraspanins (Hwang et al., 2012; Murayama, Oritani, & Tsutsui, 2015). Through these interactions, CD9 is involved in cellular adhesion, migration proliferation and survival (Murayama et al., 2015). CD9 was found to be upregulated in *in vitro* and in *in vivo* models of ovarian cancer acting as an oncogene with an anti-apoptotic action through the constitutive activation of NF-κB (Hwang et al., 2012). In this study, the CD9-specific monoclonal antibody ALB6 injected into the mouse peritoneum resulted in a reduction in

tumour weight compared to that of control mice injected with IgG_1 in *in vivo* models of ovarian cancer (Hwang et al., 2012).

Our analysis of GBM showed, like the ovarian cancer model examined by Hwang et al., showed that CD9 expression was significantly higher in tumours compared to normal tissue. The role of CD9 in cell adhesion, migration, proliferation and survival (Murayama et al., 2015) combined with evidence of significantly higher expression in tumour tissue suggests that this protein is likely to be contributing to tumour progression. A CD9-specific inhibitor such as ALB6, which reduced tumour burden in *in vivo* mouse models of ovarian cancer, could potentially slow tumour cell proliferation in GBM and in combination with other treatments slow disease progression (Hwang et al., 2012).

CD44 is an adhesion molecule that acts as a receptor for a number of molecules including hyaluronic acid (Louderbough & Schroeder, 2011). Its function is to mediate cell-cell communication and communication between cells and the extracellular matrix thereby promoting cell survival, migration, invasion and angiogenesis (Louderbough, Brown, Nagle, & Schroeder, 2011). After finding increased CD44 expression in human GBM microarray data from the TCGA, we looked for a similar pattern in other solid tumours. Of the five genes upregulated in human GBM compared to normal brain tissue, only CD44 was upregulated in colon adenocarcinoma and rectal adenocarcinoma with respect to normal control tissues. The remaining four genes were expressed either at similar or lower levels compared to the corresponding control tissue.

Yoshida et al. showed that CD44 expression levels increased with histopathological tumour grade in glioma and that treatment with a CD44 monoclonal inhibitor reduced migration in GBM cells *in vitro* whereas normal brain tissue expressed little or no CD44 (Yoshida, Matsuda, Naito, & Ishiwata, 2012). This evidence suggests that specific inhibition of CD44 positive cells could potentially target cancer cells with little or no effects on normal cells. A low molecular weight inhibitor developed from fungus known as F-19848 A was shown to inhibit the CD44-hyaluronic interaction *in vitro* in HEK293 cells (Hirota-Takahata et al., 2007). More recently, a humanized monoclonal antibody (RG7356) specific for CD44 showed specificity and cytotoxicity for leukaemia B cells in immune deficient mice engrafted with CLL while having little or no effect on normal B cells (S. Zhang et al., 2013). Findings by Birzele et al. indicate that the CD44 inhibitor RG7356 was effective in blocking the interaction between CD44 and hyaluronic acid *in vivo* (Birzele et

al., 2015). The results of a phase 1 clinical trial of RG7356 in patients with solid tumours have not yet been posted (U.S National Institutes of Health NCT01358903).

CD44 cell surface protein expression has been shown to be higher in multiple types of solid tumours than in the corresponding normal tissue contributing to cellular communication, survival, migration and invasion ((Louderbough et al., 2011) which all promote tumour progression. The development of a humanized monoclonal antibody that was both cytotoxic and specific for leukaemia cells while sparing normal cells is exciting for two reasons (Birzele et al., 2015; Y. Zhang et al., 2013). Specificity of a therapy reduces the potential side effects for the patient making therapy more tolerable, improving quality of life and reducing patient stress. Secondly, clinical trials already underway in the U.S if successful, could lead to approval for a CD44 inhibitor in other cancers resulting in the potential for the same therapy to be used to slow tumour progression in GBM patients.

CD63, also known as TSPAN30 is a tetraspanin receptor that interacts with many proteins including integrins, other tetraspanins (including CD9 and CD151), kinases, adaptor proteins and other proteins (Kang et al., 2014; Radford, Thorne, & Hersey, 1996). CD63 is involved in regulation of cell development, activation, growth, motility, and survival (Rorive et al., 2010). Rorive et al., reported high CD63 expression in glioblastoma, that this correlates with a poor prognosis in GBM and that CD63 appears to be involved in cellular proliferation (Rorive et al., 2010). A phosphatidylinositol 3-kinase (PI3K) inhibitor, IPI-145 was introduced in early phase clinical trials and found to be well tolerated by haematological patients and healthy control subjects at doses that effectively inhibited CD63 (Akinleye, Avvaru, Furqan, Song, & Liu, 2013). Trials with this inhibitor are ongoing.

Our findings that CD63 was more highly expressed in GBM than in normal tissue confirms the findings of Rorive et al., that CD63 expression was higher in GBM cells and increased with poor prognosis (Rorive et al., 2010). Studies showing that CD63 contributes to cell activation, growth, motility and survival suggest that this protein could contribute to a range of processes necessary for tumour progression. An effective PI3K inhibitor already being trialed in human patients could potentially become a future treatment for GBM patients.

CD164 also known as MUC-24 is a transmembrane sialomucin that acts as an adhesion receptor (Havens et al., 2006). Doyonnas et al., showed that CD164 functions are regulated through cell type-specific post-translational modifications (Doyonnas et al., 2000). In prostate cancer, CD164 was found to promote metastasis through the chemokine receptor type 4 (CXCR4) / stromal cell-derived factor-1 (SDF-1) interaction (Havens et al., 2006). A recent study showed that CD164 promoted tumourigenicity through the CXCR4/SDF-1 α axis in an *in vivo* ovarian cancer model (Huang et al., 2013). Upregulation of CXCR4/SDF-1 interaction through CD164 induces cellular proliferation, migration, and invasion through activation of the PI3 kinase/Akt signaling pathway (Huang et al., 2013). PI3K signaling has been shown to be disrupted in a variety of cancers including GBM (Lino & Merlo, 2011) Interestingly, CXCR4 was significantly upregulated in all GBM subtypes analysed by the TCGA compared to normal brain tissue, as discussed below. This suggests that a relationship could exist between CXCR4 and CD164 in GBM. Forde et al. showed that knockdown of CD164 inhibited cell migration mediated by the CD184 ligand, SDF-1 (Forde et al., 2007). I speculate that inhibiting CD164 might lead to a reduction in CD184/SDF-1 mediated cell migration and downregulation of the PI3K/Akt signaling pathway thereby reducing tumour cell proliferation and metastasis in GBM.

During normal brain development, CD184 binding to the ligand SDF-1 has been shown to mediate hippocampal-neuron survival and cell migration in brain development (Bagri et al., 2002). CD184 also known as CXCR4 is a chemokine receptor specific for SDF-1 (also known as CXCL12). Studies show that CD184 protein contributes to functions that support tumour progression. Small-cell lung cancer cells were shown to express high levels of CD184, which upon binding the ligand SDF-1 elicited firm adhesion to stromal cells (J. A. Burger, Spoo, Dwenger, Burger, & Behringer, 2003). Burger et al. found that disruption of CD184 binding to SDF-1 through the introduction of T140, a CD184-specific chemokine receptor antagonist sensitized CLL cells *in vitro* to drug-induced apoptosis increasing the efficacy of current therapy (M. Burger et al., 2005). When we analysed the expression of CD184 in GBM using the TCGA data we noticed that CD184 expression was significantly higher in all four GBM subtypes than in normal brain tissue. Strong CD184 expression in human GBM tumours analysed by the TCGA suggests that binding of the ligand SDF-1 could contribute to tumour cell migration and invasion in human GBM. The receptor

antagonist T140 is a potential candidate for *in vitro* studies to test the effects of CD184 inhibition in GBM tumour cells.

5.6 Conclusions

Cell surface proteins contribute to a wide range of cellular functions that can be exploited by cancer cells in tumour progression and invasion. One particularly interesting mechanism is the ability to evade phagocytosis by the innate immune system. Before terminal differentiation, normal circulating cells use this same 'recognition of self' marker to remain in the circulation during normal development and for the purpose of cell turnover for healthy organ maintenance (Willingham et al., 2012). Exploitation of this property equips the cancer cell with an advantage, which allows it to circumvent mechanisms, which would otherwise have eliminated it from the population.

The survival advantage that CD47 confers upon a cell through its binding with SIRPα emitting the 'don't eat me' signal contrasts with the survival disadvantage it imparts when it interacts with thrombospondin-1 leading to an increase in radiosensitivity. The ability to capitalize on the diversity of receptors with which CD47 interacts by permitting TSP-1 binding to increase radiosensitivity or inhibiting binding to SIRPα to abrogate the don't eat me signal suggests CD47 could be a useful tool in future combination therapies to eradicate an aggressive tumour cell population. These opposing actions could be further exploited if treatment were to involve inhibition of the SIRPα/CD47 interaction combined with permission of the TSP-1/CD47 interaction to radiosensitise tumour cells while in normal healthy adjacent tissue blocking the TSP-1/CD47 interaction to impart radioresistance. Radiation damage to healthy tissue is recognized as a long-term complication for healthy tissue that has historically been unavoidably negatively impacted by localized radiotherapy treatment (Brandes et al., 2008).

Evidence suggests that the majority of GBM cells exhibit strong CD59 and CD47 expression and that these proteins are viable therapeutic targets in the treatment of tumor progression and disease recurrence. CD59 antibody therapy could potentially enhance the effects of CD47 antibody therapy by exposing protected cells to complement cell lysis in combination with existing surgical and chemotherapeutic treatments.

This study found that five cell surface proteins exhibited a significant increase in gene expression in comparison to normal human brain tissue based on the TCGA microarray data. Recent literature has reported that the ratio of glial cells to neurons in the normal human brain is approximately 1:1 in contrast to historical estimations of a glial to neuron ratio of 10:1 (Hilgetag & Barbas, 2009) (Azevedo et al., 2009). Based on this data we consider normal brain tissue to be a most suitable tissue for comparison of cell surface protein expression in GBM The genes we identified are all involved in functions that can potentially increase tumour aggression, invasion, and metastasis suggesting that they are potential targets for anti tumour therapies. It is of particular interest that for some of these proteins, inhibitors are available which could be deployed in neurosphere and mouse models to determine their ability reduce intratumoural heterogeneity and/or slow disease progression. Overall, my work has identified a cohort of viable targets, some with existing inhibitors that have the potential to reduce subpopulations of tumour cells that may contribute to disease progression and at the same time increase the cytotoxic response to commonly used chemotherapeutics. As the tenure of my PhD candidature has expired, it was not possible to test these inhibitors myself. Instead, in the final discussion I have outlined my recommendations for future directions arising from this work.

CHAPTER SIX

6 General discussion and conclusions

The aim of this thesis was to identify new therapeutic targets in human GBM using “close to patient” models of human GBM. Using these models, we have identified a population of cells within human GBM capable of contributing to therapeutic resistance and tumour relapse after therapy. Furthermore, this work identified a small cohort of cell surface proteins of potential clinical value in GBM that could be exploited in targeted approaches to GBM treatment. The key findings from this work are summarized in Figure 6.1 and its accompanying table.

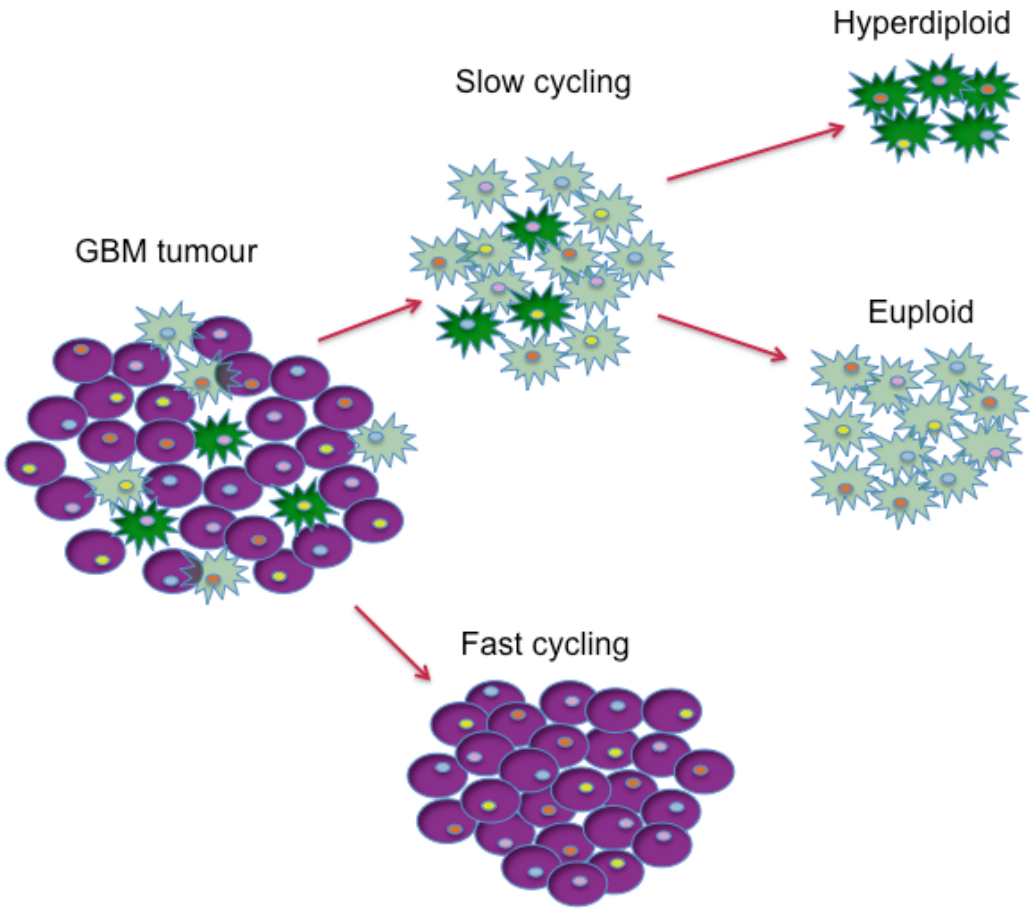


Figure 6-1 GBM is a heterogeneous tumour. A simple schematic summarizes our data showing that GBM tumours are heterogeneous and consist mostly of fast cycling cells and a small population of slow cycling cells. Intratumoural heterogeneity in cell surface protein expression is indicated by different coloured spots in cells. The slow cycling cell population consisted mostly of euploid cells and a small fraction of hyperdiploid cells.

Table 6-1 Table of summary of findings. Table briefly outlines findings including characteristics identified for each cell population of interest.

Cohort of cells	Characteristics identified
Total GBM cell population	Cell surface protein expression was highly variable A small number of proteins were highly expressed *in vitro* and *in vivo* The highly expressed proteins have known roles in cellular proliferation, adhesion, migration, invasion, metastasis, drug resistance and immune system evasion Inhibitors have been developed for some of the identified proteins Comprised of fast cycling and slow cycling cells
Slow cycling cells	Not quiescent but actively cycling More proficient at tumour formation than fast cycling cells Comprised of euploid and hyperdiploid cells
Hyperdiploid cells	Capable of tumour initiation Resistant to conventional therapy Sensitive to metabolic inhibition – a potential point of fragility

6.1 Tumour initiating hyperdiploid cells are present in GBM

A major finding of this thesis was that a hyperdiploid population of tumour initiating cells existed in primary cultures of human GBM. Tumour initiating hyperdiploid cells have been identified in other cancers. One recent study showed that diploid mouse ovarian cancer cells failed cytokinesis (Lv et al., 2012). The product of the failed cytokinesis was tetraploidy which resulted in the generation of aneuploid cells following chromosomal mis-segregation. The subsequent intraperitoneal injection of mouse ovarian surface epithelial cells revealed that only late passage aneuploid cells were able to form tumours in contrast

to diploid cells, which did not form tumours (Lv et al., 2012). Thus, ploidy status was a selection pressure that generated aneuploid tumour initiating cells. Our search for therapeutic targets in GBM led us to the identification of a tumour initiating hyperdiploid subpopulation of GBM cells that possess a potential point of fragility which could be exploited to slow disease progression.

6.2 Cancer cells are sensitive to metabolic inhibition

An important finding from this work was the novel observation that specific inhibitors of metabolism could deplete hyperdiploid GBM cells. This suggests that the dependence of hyperdiploid cells on the glycolytic pathway may be a viable therapeutic target in GBM. We hypothesize, based on our work with slow cycling cells, that hyperdiploid cells may be endowed with superior tumour initiating ability. Therefore, I hypothesize that a low carbohydrate diet could have maximum impact on hyperdiploid human GBM cells while also impeding metabolism in euploid cancer cells and increasing tumour cell sensitivity to other therapies without damaging normal cell metabolism.

It is noteworthy that, the Ketogenic diet that is currently in use to treat children with refractory epilepsy was given to mice with brain tumours. The results showed that when the diet was administered in restricted amounts, tumour growth and vascularity were significantly reduced and progression free survival was significantly increased (W. Zhou et al., 2007). As this diet is already in clinical use to treat another pathological condition, it is conceivable that it could be implemented to compliment current treatment regimes and has the potential to improve therapeutic response and progression free survival (Schwartz et al., 2015). The following patents have also been generated: Ketogenic diet composition for the treatment of chemo therapy and/or radiation therapy. WO2012113572 A1, Ketogenic diet composition for the treatment of chemo therapy and/or radiation therapy patients. US20140011749 A1, Regulation of cancer using natural compounds and/or diet. WO2014159500 A1, Targeting cancer with metabolic therapy and hyperbaric oxygen. WO2014085652 A1. In addition to this, clinical trials are currently underway into the ketogenic diet in adults with GBM. As an example: Calorie-restricted, ketogenic diet and transient fasting during reirradiation for patients with recurrent glioblastoma (ERGO2). NCT01754350.

Normal cells use oxygen and glucose to generate adenosine triphosphate (ATP) to store energy in a process known as oxidative phosphorylation (Garber, 2006). Cancer cells have an increased reliance on glucose for ATP production even in the presence of oxygen (anaerobic glycolysis). This is known as the "Warburg effect" (Warburg, 1956b). The increased glucose requirements of cancer cells present a potential therapeutic target that could specifically inhibit cancer cell metabolism without negatively impacting normal cell metabolism. Ketones are produced in the liver as an energy source when carbohydrate (glucose) is in short supply. They are produced from fatty acids and oxidized in the mitochondria bypassing glycolysis (W. Zhou et al., 2007).

The Ketogenic diet is low in carbohydrates and high in fats providing energy for normal cells that can utilize ketones for energy production and starves cells that cannot metabolize ketones (W. Zhou et al., 2007). Results from studies utilizing high fat, low carbohydrate diets suggest that cancer cells could be specifically targeted with therapies that reduce glucose availability leading to cell death and tumour regression while normal cells continue to generate ATP through oxidative phosphorylation. A study of tumour progression in mice on a carbohydrate restricted diet showed a reduction in tumour progression and an increase in tumour cell sensitivity to antitumor therapies (Ho et al., 2011). The finding that human GBM tumours contain a tumour initiating subpopulation of hyperdiploid cells that rely on increased energy requirements above those of both euploid cancer cells and healthy non cancerous cells suggests that carbohydrate restriction might improve response to treatment in patients with GBM.

6.3 Hyperdiploid human GBM cells are sensitive to proteasome inhibition

Previous studies have shown that proteasome activity in aneuploid cells can be targeted specifically without negatively impacting euploid cell populations (Tang et al., 2011). MG132 is a peptide aldehyde that inhibits proteasome activity by binding to the active site of the beta subunits in the 20S core of the 26S proteasome complex (Guo & Peng, 2013). MG132 promotes cell cycle arrest and induces apoptosis through the regulation of reactive oxygen species and subsequent defects in mitochondrial function as a result of proteasome inhibition (J. J. Chen, Huang, & Chen, 2005; Guo & Peng, 2013; Han, Moon, You, & Park, 2009). Nuclear factor kappa-B (NF-κB) regulates the expression of the

antiapoptotic genes BCL-2 and BCL-XL (Ortiz-Lazareno et al., 2014). Treatment with MG132 interferes with NF-κB function (Ortiz-Lazareno et al., 2008). Inhibitory protein kappa-B (IκB) inhibits NF-κB function through its association with the NF-κB dimer in the cytoplasm. This association maintains NF-κB in an inactive form (Gilmore & Herscovitch, 2006). The degradation of the NF-κB inhibitor, IκB is prevented by treatment with MG132 (Nakajima, Kato, Takahashi, Johno, & Kitamura, 2011). Colon cancer cells underwent apoptosis following treatment with MG132 as a result of mitochondrial induction of proapoptotic factors (W. X. Ding et al., 2007). Similarly, leukaemia cells entered apoptosis at the highest frequency when MG132 treatment was combined with another cytotoxic agent, either paclitaxel or Doxorubicin (Bravo-Cuellar et al., 2013; Ortiz-Lazareno et al., 2014). These data suggest that administering MG132 in combination with other treatments could produce a targeted effect on therapy resistant hyperdiploid tumour cells.

6.4 Limitations to *in vitro* and *in vivo* studies

It was not possible to obtain normal human brain tissue as a comparator for *in vitro* and *in vivo* assays therefore we used publically available data on normal human brain cells to determine normal protein expression levels where possible. Despite this limitation, we were able to show significant differences in gene expression between human GBM tumours and normal human brain tissue for a small cohort of candidates.

Xenotransplantation assays are an established method used to examine tumour progression and complexity *in vivo*. However, due to the necessity for animals to be immunodeficient to some degree, transplanted cells are not within the environment normally experienced by primary tumour cells in a human patient. Immunodeficiency is necessary to ensure that transplanted cells survive but also masks any potential contribution to tumour cell survival that may have been otherwise present with an intact immune system. It should be noted that even “immunodeficient” mice retain remnants of immunity (albeit unbalanced) resulting in the possibility that relevant human tumourigenic cells are eliminated by the residual xenogenic eradication system (Meacham & Morrison, 2013). These limitations are unavoidable and common to the vast majority of cancer studies. Despite these limitations, we were able to generate xenograft tumours that resembled primary human GBM tumours histopathologically.

Orthotopic xenograft models of human GBM are labour intensive, time-consuming experiments. Murine brain tumours in our mouse model took up to ten months to fully develop and required constant monitoring. Time constraints prevented limiting dilution injections of hyperdiploid clonal cell populations into mice to determine the tumour initiation efficiency of hyperdiploid tumour cells. It is conceivable that superior tumour initiation shown in slow cycling cells in chapter two was due to an enrichment of hyperdiploid cells within the slow cycling fraction rather than the total population of slow cycling cells. We hypothesize that fewer hyperdiploid human GBM cells than euploid human GBM cells are required for *in vivo* tumour initiation. The majority of tumour cells are eradicated at the completion of therapy in patients however, human GBM tumours invariably regenerate. A population of cells with the capacity to regenerate tumours from very small numbers would pose a serious threat to complete remission of the disease. Superior tumour initiating ability would establish hyperdiploid cells as a target for treatment with the potential to increase progression free survival or inhibit tumour regrowth completely.

6.5 Future directions

To extend the findings discussed in this thesis, glucose deprivation in *in vivo* hyperdiploid tumour models could potentially reveal changes in therapeutic response to current treatment protocols. Following intrastriatal injection of hyperdiploid cells mice would be placed on either a normal diet or a low glucose diet and monitored for signs of tumour development. At the end of the experiment tumour size for the two groups and survival curves would be compared to assess differences in time for diploid and hyperdiploid cells to form large tumours. Future work should also include other anti-hyperdiploid therapies and the impact they have on the response to TMZ treatment. Tang et al. showed that 5-aminoimidazole-4-carboxamide riboside (AICAR) and 17-allylamino-17demethoxy-geldanamycin (17-AAG) were able to inhibit aneuploid tumour cell proliferation while sparing euploid cells *in vitro* and *in vivo* (Tang et al., 2011). I would like to test these therapies on human GBM hyperdiploid cells *in vitro* and orthotopic xenograft tumour models generated from euploid and hyperdiploid cells.

Cell surface proteins can serve as specific therapeutic targets when they are differentially expressed on normal cells and tumour cells. The CD9-specific monoclonal antibody ALB6

effectively reduced proliferation in tumours generated in mice from an ovarian cell line (Hwang et al., 2012). RG7356 inhibited the interaction between CD44 and hyaluronic acid in *in vivo* tumours generated from eight cancer cell lines (Birzele et al., 2015) and was approved for use in a phase 1 clinical trial in the U.S. A PI3K inhibitor, IPI-145 was effective in inhibiting CD63 activity in phase 1 clinical trials in haematological patients (Akinleye et al., 2013). The T140 CD184 receptor antagonist sensitized CLL cells to existing therapy (M. Burger et al., 2005). To extend the results discussed in this thesis and the work of others I would like to test these inhibitors *in vitro* using human GBM cells and then *in vivo* using orthotopic xenograft tumour models of GBM. The ultimate goal of this research was to provide new insight into therapeutic resistance in GBM. These findings have revealed a subpopulation of GBM cells and a small cohort of proteins that represent potential treatment targets. My hope is that this work leads to the validation of new treatment regimes that will improve therapeutic outcomes in this aggressive and incurable disease.

CHAPTER SEVEN

7 References

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