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The oncolytic herpes simplex virus G47Δ as a therapeutic agent against the glioma stem cell sub-population of glioblastomas

Thesis submitted in accordance with the requirements of the University of Southampton for the degree of Doctor of Medicine by

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ABSTRACT

FACULTY OF MEDICINE

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THE ONCOLYTIC HERPES SIMPLEX VIRUS G47Δ AS A THERAPEUTIC AGENT AGAINST THE GLIOMA STEM CELL SUB-POPULATION OF GLIOBLASTOMAS

By Deva Sanjeeva Jeyaretna

Glioblastoma remains one of the deadliest of human cancers. A sub-population of glioblastoma cells, designated glioma stem cells have been identified as the invasive and adjuvant therapy resistant fraction of the tumour and are presumed to repopulate glioblastomas following surgical resection and adjuvant temozolomide and radiation therapy. To date there is no known cure for glioblastoma. Genetically engineered oncolytic viruses like G47Δ have specific mutations that enable them to preferentially replicate in tumour cells. In this thesis their use against glioma stem cells is examined.

In the first results chapter, the culture and 'stem-like' features of glioma stem cells including their tumour forming capabilities are assessed. The ability of G47Δ to successfully infect, replicate and kill glioma stem cells both in vitro and in vivo is then shown. In the following chapter, co-culture systems between glioma stem cells and endothelial cells are developed to simulate the supportive perivascular niche of glioma stem cells. The G47Δ virus is 'armed' with the antiangiogenic transgenic tumstatin using the Flip-Flop HSV-BAC system. In vitro experiments demonstrate the superior ability of G47Δ-tumstatin to disrupt the co-culture of glioma stem cells and endothelial cells. In vivo testing of the virus demonstrated prolonged survival compared to mock treatments but not the empty vector G47Δ-empty.

In the final results chapter, combined G47Δ and radiation therapy effectively kills glioma stem cells in vitro and prolongs survival of mice bearing glioma stem cell orthotopic xenografts. The two agents show synergistic efficacy against glioma stem cells because G47Δ is able to capitalise on the DNA repair machinery of glioma stem cells following irradiation to enhance its replication abilities and thus its cytotoxic effect. This is the first report of combined efficacy of an oncolytic vector and radiation therapy against glioma stem cells.

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Declaration Of Authorship

I, Deva Sanjeeva Jeyaretna, declare that this thesis " **The oncolytic herpes simplex virus G47Δ as a therapeutic agent against the glioma stem cell sub-population of glioblastomas**" and the work presented in it are my own and has been generated by me as the result of my own original research based upon results from experiments performed as an MD student between January 2007 and August 2009 at the Massachusetts General Hospital, and Harvard Medical School, Boston, USA and associated with the Faculty of Medicine, University of Southampton.

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List of abbreviations:

7-AAD	7-aminoactinomycin D
ABC	ATP-binding cassette
BAC	bacterial artificial chromosome
CI	combination index
CMV	cytomegalovirus
Col IV	type IV collagen
DAPI	4',6-diamidino-2-phenylindole
dNDP	deoxyribonucleioside diphosphate
dNTP	deoxyribonucleotide triphosphate
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
FGF	fibroblast growth factor
Foxn1	forkhead box protein N1
GAG	glycosaminoglycan
GFAP	glial fibrillary acidic protein
HLA	human leukocyte antigen
HSV-1	herpes simplex virus type 1

HUVEC	human umbilical vein endothelial cell
ICP6	infected cell protein 6
ICP47	infected cell protein 47
IDH1	isocitrate dehydrogenase 1
MDR	multidrug resistance
MGMT	O6-methylguanine-DNA methyltransferase
MHC	major histocompatibility complex
MOI	multiplicity of infection
MMP-9	matrix metalloproteinase-9
NC1	non-collagenous domain 1
NDP	ribonucleotide diphosphate
NF1	Neurofibromatosis 1
Olig2	oligodendrocyte transcription factor
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PI3K	phosphoinositol 3 kinase
PKR	RNA-dependent protein kinase pathway
PTEN	phosphatase and tensin homolog
RR	ribonucleotide reductase
SDF1	stromal derived factor 1

STAT3	signal transducer and activator of transcription 3
TAP	transporter associated with antigen presentation
TGF- β	transforming growth factor-β
VEGF	vascular endothelial growth factor

Chapter 1: Introduction

1.1. Overview of the pathology and management of glioblastomas

1.1.1. Glioblastoma epidemiology and outcomes

Gliomas are primary intrinsic tumours of the brain. They encompass a heterogeneous group of tumours with varying clinical and histological features, degrees of malignancy and responsiveness to available treatments. Glioblastomas are the most malignant form of gliomas and also the most prevalent primary brain tumour in adults[1]. The incidence of glioblastoma in the United States is 3.2 per 100,000 person-years[2]. The United Kingdom would be expected to have a proportional number of cases per capita. In recent decades there has been a rise in the incidence of glial tumours, particularly in the elderly and this has partly been attributed to the greater availability of intracranial imaging[3]. There remains a gender bias with glioblastoma being more common in men[4].

Despite the advancements in surgical technique, surgical technology and research into adjuvant therapies, glioblastoma remains an incurable disease. The multitude of treatments available prolong the course of the disease but ultimately never corral it. The most effective strategy at present involves aggressive surgical resection coupled with post-operative radiotherapy with concomitant and adjuvant temozolomide[5, 6]. The evidence for the survival

benefit of radiotherapy for glioblastoma has been well established[7, 8]. Temozolomide is an oral alkylating agent which when used with radiotherapy provides an additional survival benefit[5]. However, the overall survival remains modest and with best treatment the median survival is 14.6 months, with only 27% of patients alive at 2 years and 10% alive at 5 years[5, 9]. For recurrent glioblastoma the prognosis is more severe and the 6-month progression free survival is less than 20%[10, 11].

Surgical techniques have also advanced in the last two decades with the use of microsurgical techniques and intraoperative navigation becoming commonplace in the resection of gliomas. Awake craniotomies, intraoperative mapping of motor tracks with diffusion tensor imaging and cortical and subcortical monitoring have enabled neurosurgeons to aggressively pursue gliomas in areas of close proximity to eloquent cortex, that were previously considered unresectable. Furthermore, a phase III multicenter randomised control trial using intraoperative 5-aminolevulinic acid, which causes fluorescence in tumours cells and guides resection, demonstrated an added 5 month survival benefit for patients receiving a complete macroscopic resection[12, 13]. This evidence along with studies investigating the effect of extent of resection on glioblastoma survival indicate a pivotal role for surgery in glioblastoma[6, 14]. However, given the invasive nature of glioblastomas, surgery alone is not the answer. As we have seen, the addition of temozolomide and radiotherapy while also extending survival still fall far from a cure.

Disappointing as the ultimate clinical outcomes are, the last decade has witnessed great strides in our understanding of this devastating disease. While the definitive cell of origin and precise aetiology of glioblastomas continues to elude us, the investigation into the molecular genetics of glioblastomas has seen much progress.

1.1.2. Recent progress in the management of glioblastoma

Glioblastoma was selected as the first cancer to be comprehensively studied by The Cancer Genome Atlas (TCGA) project. In their study, over 600 cancer-related candidate genes were sequenced in 206 glioblastoma specimens[15]. In addition the investigators examined DNA methylation status, DNA copy number changes and expression of coding and non-coding RNA. TCGA identified mutations and activations in key cellular survival pathways including neurofibromatosis 1 (NF1), epidermal growth factor receptor (EGFR), phophoinositol 3-kinase (PI3K), phosphatase and tensin homolog (PTEN), retinoblastoma and p53. The downstream targets of these pathways fuel the tumour cells and allow for resistance against anti-tumour regulators of apoptosis and senescence[16, 17]. A further study sequencing 20,661 protein coding genes in 22 glioblastoma samples identified further molecular targets, most notably the active site of isocitrate dehydrogenase 1 (IDH1)[18]. This mutation has been linked to improved survival in secondary

glioblastomas, which are glioblastomas that are thought to arise from lower grade glial tumours rather than de novo.

Glioma stem cells have also been identified[19], a sub-population of glioblastomas that mediate the enhanced tumour proliferation and resistance to adjuvant therapy that is the hallmark of glioblastomas. Their exact place in the hierarchy of glioblastomas is unclear, but the study of their biology has provided many leads in the search for a cell of origin for glioblastomas. Glioma stem cells will be discussed in more detail in the following section.

The advances above notwithstanding, glioblastoma remains a universally lethal disease. Its mutations and associated complex molecular pathways shield a tumour that has thus far withstood all conventional modalities of treatment. Further work is required to develop novel methods for treating this deadly disease and many avenues are currently being pursued. One area that has demonstrated some promise is the use of genetically engineered oncolytic herpes viruses as anti-tumour agents[20]. These viruses are able to lyse tumour cells and have a proven safety record. The background to their use and the fundamental principles of oncolytic therapy will be discussed in section 1.3.

1.2. Glioma stem cells

1.2.1. Cancer stem cell hypothesis

In the last decade, the cancer stem cell hypothesis has taken a leading role in the study of a number of tumours including haematopoietic tumours, breast cancer and glioblastoma. While its newfound prominence has been a boon to cancer researchers, the premise of cancer stem cells has in one form or another existed since the 1960s[21]. The fundamental principle is that a small subset of tumour cells, in this case glioma stem cells, represent the origins of the tumour and are a self-sustaining and self-renewing pool of cells that are able to reform the tumour after surgical or adjuvant therapy. Glioma stem cells are also thought to be resistant to the major chemotherapeutic agents and radiotherapy and the evidence for this will be reviewed in the following sections. In addition, they are pleuripotent and the heterogeneous cell types formed by these glioma stem cells include astrocytic cells, oligodendroglial cells and neuronal cells. Recent work showing that glioblastoma cells also contribute to the stroma[22, 23] of the tumour may yet lead to a further increase in the known differentiation products of glioma stem cells.

The groundwork for the discovery of cancer stem cells in solid tumours was laid by research into acute myelogenous leukemia[24, 25], where researchers perfected the appropriate assays, established the use of

surface epitopes particularly those related to normal stem cells to delineate cell lineages and hierarchy, and created lineage maps. Following this, work in solid tumours such as breast and gliomas led to the identification of further cancer stem cell models[26, 27]. In gliomas this began with in vitro studies[27] and culminated with the landmark work by Singh et al[19] who demonstrated that as few as 100 glioma stem cells were able to form tumours in immunodeficient mice, and that these tumours phenotypically resembled their parent tumours. In contrast a million non-glioma stem cells could not reproduce this feat. They also demonstrated the pleuripotent nature of glioma stem cells and their capacity for self renewal. In addition this seminal work introduced CD133, a cell surface antigen, as a marker for glioma stem cells. This marker has since been used in a host of studies on glioma stem cells[28-32], although as elaborated in the next section it is not without its detractors[33, 34].

1.2.2. CD133 – a glioma stem cell marker

Due to its origins in hematopoietic research, the study of cancer stem cells in glioblastomas has largely relied on the use of cell surface markers and growth in suspension. Glioma stem cells are cultured as spheroids and in serum-free media with epidermal growth factor and fibroblast growth factor to enrich for cells bearing the surface marker CD133[19]. CD133 is a transmembrane glycoprotein with an extracellular epitope AC133 that is expressed by a number of cells including glioma stem cells, neural stem cells

and endothelial cell precursors[19, 35, 36]. Following its early prominence as a marker of glioma stem cells, there has been some controversy as to its validity as a stem cell marker. We will first examine the evidence for CD133 as a stem cell marker and then the studies refuting this as well as new reports suggesting a hierarchy in glioma stem cell markers.

Singh et al demonstrated that cell populations enriched for CD133 are highly tumorigenic compared to CD133 negative cells[19]. This was followed by work demonstrating their chemoresistance[37, 38]. Glioma stem cells expressing CD133 demonstrated resistance to a range of chemotherapeutic agents that are and have been used to treat gliomas including etoposide, cisplatin and temozolomide. Interestingly the authors demonstrated that this resistance was not due to decreased cellular uptake of drugs or increased extrusion[37].

In vitro and in vivo studies of glioma stem cells showed an enrichment of CD133 positive cells following irradiation, suggesting a survival advantage for this sub-population[29, 39]. Bao et al demonstrated that CD133 positive cells mediate glioma radioresistance by preferential activation of DNA damage checkpoint kinases allowing for repair of radiation-induced injury[29]. A clinical study of 32 patients with WHO grade III or IV gliomas undergoing radiosurgery and external beam radiotherapy examined the distribution of CD133 positive cells in a subset of patients who underwent surgery for primary and recurrent disease[39]. Examination of recurrent

tumours after gamma knife treatment and external beam radiotherapy revealed an increase in CD133 positive glioma cells, especially in areas of necrosis. In comparison there was a relative paucity of CD133 positive cells in samples of the primary tumour prior to adjuvant treatment. The authors concluded that the CD133 positive cells survived the high dose radiation potentially through the mechanisms delineated by Bao et al[29] and mediated the recurrence of the tumour[39].

Contrary to the early glioma stem cell studies, some investigators have demonstrated that CD133 negative cells are able to form intracranial tumours[33, 40, 41]. Serial orthotopic xenotransplantation caused the CD133 negative tumour cells to then become CD133 positive, clouding the issue further. Work by Jaksch et al has also raised the possibility that the expression of the CD133 epitope may be dependent on stages of the cell cycle[42].

A further theory by Chen et al[43], proposes a cellular hierarchy of self-renewing tumour-initiating cells in glioblastoma. The authors investigated 29 primary glioblastoma specimens and determined that both CD133 positive and negative cells can self-renew in vitro and successfully generate orthotopic tumours in immunocompromised mice. They demonstrate a cellular hierarchy for CD133 negative glioma stem cells suggesting that previous reports may have been studying mixed populations of cells; one group of self-renewing, tumour-initiating cells and another group of more

differentiated CD133 negative cells. This may explain the contrasting results seen in studies of CD133 negative cells. At present no marker has been identified to further subclassify these cells. The data thus far is a long way from providing us with a complete picture and the study of glioma stem cells remains a field in its infancy. Nonetheless, the ability of glioma stem cell models to mimic human glioblastomas phenotypically, demonstrate chemoresistance and radioresistance and actively recruit the vasculature and interact with endothelial cells has provided us with a novel method of studying gliomas that is superior to the primary glioma cell lines used in the past. In the next section we will review the intimate relationship between these cells and the vascular stroma of glioblastomas.

1.2.3. The glioma stem cell perivascular niche

The dependence of tumours on an adequate blood supply for their substantial metabolic needs has been well established[44]. It has been over four decades since the first hypothesis for limiting tumour progression by targeting their vasculature was first published[45]. Since Dr. Folkman's seminal paper a range of antiangiogenic agents have reached clinical trials for a number of tumours including gliomas[46]. Now with the identification of glioma stem cells, new insights are being made into the role of the tumour vasculature in supporting solid tumours.

A number of groups have studied the close proximity of glioma stem cells to the tumour vasculature[47-49]. It has been demonstrated that a supportive perivascular niche exists that nurtures the glioma stem cells[47, 50]. The interaction between glioma stem cells and endothelial cells is thought to function synergistically with endothelial cells promoting self-renewal of glioma stem cells and glioma stem cells secreting growth factors such as vascular endothelial growth factor (VEGF), stromal derived factor 1 (SDF1) and transforming growth factor-β (TGF-β) to recruit and enhance endothelial cell survival[30, 51-53]. The complexity of this relationship is slowly coming to light and there is increasing evidence that in addition to the above factors, there exist paracrine and autocrine pathways that promote the survival of both glioma stem cells and endothelial cells. The main protagonist in this system is nitric oxide, which can be secreted by both endothelial cells and glioma stem cells[49, 54].

A further factor supporting the close proximity of glioma stem cells to endothelial cells and their supportive interactions is the presence of tumour survival pathways triggered by cell surface ligands. The Notch ligands, which are present on the surface of endothelial cells, have been demonstrated to enhance proliferation and stem-like characteristics in glioma stem cells[50, 55, 56]. In addition to the crosstalk between glioma stem cells and endothelial cells, more recent work has demonstrated the ability of glioma stem cells to transdifferentiate into endothelial cells[22, 23]. This marks a paradigm shift from the previous theory of an angiogenic switch, in which gliomas and other cancers were thought to recruit endothelial cells and

mediate the subsequent formation of the vasculature by inducing endothelial cell migration and sprouting of new blood vessels[57].

It has now been shown that glioma stem cells are able to transdifferentiate into endothelial cells[22, 23]. They carry with them the genetic mutations of tumour cells and thus to a certain degree the resistance to therapeutic agents. These include mutations in the EGFR and p53 genes[23]. This transformation highlights the many intricate pathways tumours have developed to shield themselves against conventional therapies. Nonetheless, this supportive perivascular niche of glioma stem cells remains a promising target for a range of therapeutics, particularly anti-angiogenic agents that are currently being employed to treat glioblastoma patients. Pre-clinical work combining antiangiogenic therapy with cytotoxic therapy has been shown to successfully target the glioma stem cell fraction of xenograft tumours in mice[58]. Chapter 4 of this thesis will investigate a similar strategy using an oncolytic herpes simplex virus armed with an anti-angiogenic transgene. Together, the virus and transgene are aimed at targeting glioma stem cells and their supportive perivascular niche. Tumstatin, an endogenous inhibitor of tumour angiogenesis was chosen as the anti-angiogenic transgene. Tumstatin and its effects are discussed in section 4.1.

1.3. Oncolytic herpes simplex viruses

1.3.1. History and fundamental principles

It has been over a hundred years since the first description of a virus as an oncolytic agent[59]. In 1904, Dock, a professor of medicine at the University of Michigan, reported a case of the influenza virus improving the course of a 42 year old woman's myelogenous leukemia[60]. Her symptoms and white cell count improved during a bout of influenza, only to deteriorate afterwards. After this study, many reports using a host of viruses to treat cancers were published, not only in preclinical studies but also in clinical trials[61-68].

The mechanism for accumulation in tumours and tumour-specific replication of wild-type viruses is yet to be clarified. Work in bacteria and vaccinia virus suggests that the porous nature of tumour blood vessels enables the micro-organisms to more easily extravasate from the systemic circulation and infect tumour cells[69]. Moreover, the paradigm of a cell infected by a virus is not that dissimilar to a cell undergoing malignant transformation. They both share a predilection for inactivation of tumour suppressor genes, apoptotic pathways and the interferon response[70]. This makes tumour cells a more receptive environment for viruses, be they wild-type or genetically engineered oncolytic viruses[70].

Oncolytic viral vectors are genetically engineered viruses that take advantage of cancer cell pathways to replicate preferentially in cancer cells while sparing normal cells. In 1991 Martuza et al[71] described the first genetically engineered virus designed for tumor-selective viral replication. The herpes simplex virus type 1 (HSV-1) with a deletion in the thymidine kinase gene was engineered enabling preferential replication in cancer cells where thymidine kinase activity is upregulated. Cellular thymidine kinase is required to catalyze the phosphorylation of thymidine to thymidine monophosphate[72]. Actively dividing cells such as cancer cells upregulate this pathway. The wild type HSV-1 genome carries the gene required to produce thymidine kinase which enables HSV-1 to replicate effectively in quiescent neuronal cells where active DNA synthesis is not occurring. By deleting the thymidine kinase gene the mutant strain is able to preferentially replicate in the actively dividing tumour cells but not the quiescent neuronal cells. As part of the life cycle of the virus, following infection, replication occurs and infected cells are lysed releasing more viral progeny. This enables further cancer cells to be infected and in theory this cycle would continue until all the cancer cells are destroyed. This process is shown schematically in Figure 1.1.

The promise of this early work was met with hurdles as the thymidine kinase mutant strains demonstrated residual neurovirulence[73]. Furthermore, the mutant strain was resistant to the standard antiviral agents acyclovir and ganciclovir leading to safety concerns[73]. This prevented its use in clinical trials and the search for other mutant strains began[73].

Nonetheless, this landmark work renewed the interest of cancer researchers in the use of genetically engineered oncolytic viruses.

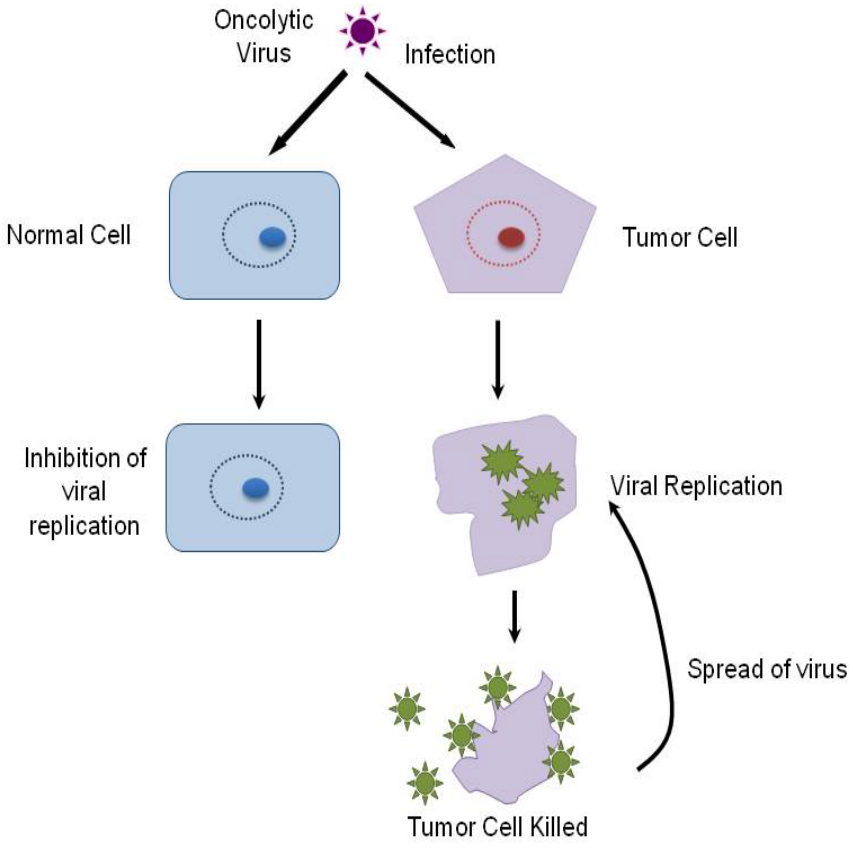

Figure 1.1 Schematic demonstrating viral replication and tumour lysis upon infection of tumour cells by oncolytic HSV-1 viruses. Theoretically viral infection, replication, cell lysis and propagation can continue unimpeded until all tumour cells are killed. Viral cell replication is inhibited in normal cells by the engineered mutations.

Three major strategies have been employed when targeting oncolytic viruses to cancer cells. The first is the deletion or inactivation of viral genes

such as the thymidine kinase mutated strains[71]. Genes responsible for virulence in normal cells but non-essential for replication in tumour cells are typically selected. These include anti-interferon and anti-apoptosis proteins, enzymes involved in nucleotide metabolism and silencing of cellular antiviral defences[70]. The second strategy employed is to use tumour-specific promoters to express essential or crucial viral genes[74]. The final strategy involves engineering viral tropism by developing viral antigens that can bind to specific tumour cell receptors[75].

When selecting a viral agent for oncolytic therapy the first decision involves deciding between a replicating and non-replicating agent. The obvious advantage of a non-replicating vector is the safety afforded by its lack of virulence. Its anti-tumour effect is achieved by the vector expressing transgenes whose proteins act on cancer cells. The most commonly used non-replicating viruses are adenoviruses, retroviruses and the herpes simplex virus[66]. The safety advantages notwithstanding, the main disadvantage of a non-replicating virus is its inability to continue to propagate an anticancer effect beyond the initial treatment dose. Theoretically, replication competent viruses may continue to propagate their anticancer effect as long as there are cancer cells available to be infected.

A number of replication competent viruses, either wild strain or genetically engineered to enable tumour selective replication, have been used in preclinical and clinical studies. These include the herpes simplex virus type-1[71, 76, 77], adenoviruses[78], vesicular stomatitis virus[79], poliovirus[80],

measles[81], Newcastle disease virus[82-84], reovirus[85], West Nile virus[86-88] and mumps virus[89-91].

1.3.2. Herpes simplex virus as a viral vector

The herpes simplex virus type 1 (HSV-1) was selected for this study because of a number of advantages it affords researches using oncolytic viruses. It is a replication competent virus that as part of its life cycle lyses the host cell and thus by virtue of infecting tumour cells and completing its life cycle is able exert an anticancer effect. HSV has been extensively studied and its genome comprehensively examined[92]. It is an enveloped, DNA virus with a 152kb linear double stranded genome[93]. Figure 1.2 demonstrates the two unique segments, one long and one short, that are flanked by repeat sequences. The two repeat sequences are orientated in opposite directions to each other. The genes responsible for virulence in the herpes virus have been clearly identified[92, 94-98]. Furthermore, approximately 30kb of DNA is non-essential for viral replication and can be replaced with transgenes without significant effect on viral replication or titres[99]. This has led to the development of the field of transgene expressing oncolytic HSV and the creation of HSV bacterial artificial chromosome systems which will be discussed later[67].

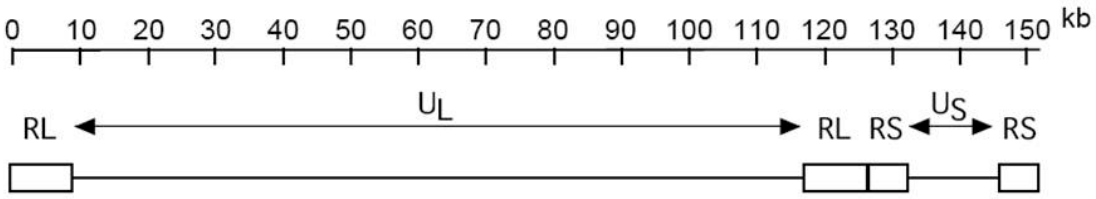

Figure 1.2. Schematic representation of the wild type HSV-1 genome. It has a 152kb linear double stranded genome. Two unique fragments, one long (U_L) and one short (U_S) are flanked by repeat sequences (RL and RS) that are orientated in opposite directions to each other.

For preclinical studies, HSV can be easily generated to high titres (10^{10} viral particles/ml) and infects multiple species[100]. It is able to infect a wide variety of cells including neuronal[101], endothelial[101], epithelial cells[102] and fibroblasts[103]. Its neurotropism is an advantage in neurooncology research as it readily infects neural cells and is an effective method of gene delivery to the nuclei of cells of the central nervous system. HSV also poses no risk of insertional mutagenesis as it does not integrate with host DNA but instead persists as an episome, even during latency[104]. Finally, with appropriately selected mutations, the virus retains its sensitivity to commonly employed antiviral agents, thereby limiting its virulence[73].

Nonetheless, the use of HSV as an oncolytic vector is not without its challenges. Its large genome makes it difficult to manipulate genetically. Host innate immunity to the virus remains a challenge especially in human trials. The majority of human subjects may have a pre-existing immunity to the virus, which would limit its efficacy both as oncolytic vector and as a vessel

for gene delivery[105]. The study of the immune response to oncolytic viruses and virus-infected tumour cells, although beyond the scope of this study, is a major area of glioma research. HSV's neurotropism is also a major concern with the wild type strain, as the resultant untreated encephalitis from a primary infection or latent reactivation is fatal. In addition, HSV's ability to infect replicating and non-replicating cells with life threatening consequences needed to be addressed.

The genetic modification of HSV viral genes to rectify these problems has been studied and Table 1.1 lists the viral genes that have been altered to satisfy safety concerns and to selectively target tumour cells while sparing normal cells[67]. The majority of HSV-1 oncolytic vectors have inactivating mutations in the ICP6 (infected cell protein 6) gene (UL39) or one or both copies of the ICP 34.5 gene (ɣ34.5) or both[73, 106, 107].

The main vector used in this thesis was G47Δ, a triple mutated HSV-1 virus with ICP6, ɣ34.5 and ICP47 mutations[76]. This virus was the result of multiple mutations created to enhance selective tumour cell killing and satisfy safety concerns regarding the intracranial use of the herpes simplex virus. Table 1.2 lists the more common mutant HSV-1 strains that have been tested in clinical trials. G47Δ originated from the mutant virus strain R3616, a mutant derived from the HSV F-strain backbone with a 1kb deletion in both copies of the ɣ34.5 gene[96].

Viral gene	Viral protein	Essential	Function
UL23	TK	No	Thymidine kinase
UL39	ICP6	No	Large subunit of ribonucleotide reductse
ɣ34.5	ICP34.5	No	Neurovirulence, blockade of cell immunity and autophagy
α0	ICP0	No	Silencing interferon induction
US3	US3	No	Anti-apoptotic
US11	US11	No	Blockade of cell immunity
US12/α47	ICP47	No	Inhibits MHC class I antigen presentation
UL2	UD	No	Uracil DNA glycosylase
α4	ICP4	Yes	Viral transcriptional activator

Table 1.1. Viral genes that have been deleted enable selective replication of oncolytic vectors in tumour cells and enhance the safety of normal cells. The protein produced by the gene is listed as is its function. A gene is listed as essential if it is required for viral growth in vitro.

Vector	Mutated virus genes								Parental virus	Backbone wild-type strain	Marker gene	Marker gene promoter	Clinical trial
	ICP6 (UL39)	ICP34.5 (γ34.5)	ICP47 (α47/US12)	Expression of US11	ICP0 (α0)	US3	ICP4 (α4)	Others					
G-207 [107] (Medigene Inc)	LacZ ins (into the 2nd BamHI site)	956 bp del (BstEII-StuI)	+	Late	+	+	+	(Truncation of UL3)*	R3616	F	LacZ	ICP6 (UL39) (endogenous)	Phase I
hrR3 [108] (Medigene Inc)	LacZ ins (into the 2nd BamHI site)	+	+	Late	+	+	+		KOS	KOS	LacZ	ICP6 (UL39) (endogenous)	Phase I
G-47Δ [76] (Medigene Inc)	LacZ ins (into the 2nd BamHI site)	956 bp del (BstEII-StuI)	–	Immediate early	+	+	+	(Truncation of UL3)*	G207	F	LacZ	ICP6 (UL39) (endogenous)	Phase I
HF10 [109] (M's Science Corp/ Nagoya University)	+	+	+	Late	+	+	+	See below **	HF	HF	None	None	Phase I / II
HSV-1716 [110] (Crusade Laboratories Ltd)	+	759 bp del (125213-125972)	+	Late	+	+	+		17+	17+	None	None	Phase II
NV1020 [111] (R7020; Medigene Inc)	+	+/-	+	Late	+/-	+	+/-	See below ***	R7017	F	None	None	Phase II

Table 1.2 Oncolytic HSV-1 vectors undergoing clinical trials. (-) signs represent deletional mutations in the gene described. * These mutations were originally unintended when the vectors were constructed. Truncation of UL3 in G207 and G47Δ is theoretically predicted, but not confirmed. ** Deletion of UL56, duplication of UL53, UL54, and UL55. Syncytial mutant. *** Inactivation of UL24. Deletion of UL56, one copy of ICP0 (a0), LAT, ICP4 (a4). Duplication of UL5, UL6. Insertion of HSV2 glycoproteins G, D and I.

1.3.2.1. ɣ34.5 mutations

ɣ34.5 was originally deleted because of its role in neurovirulence and its mutants were able to retain the ability to replicate in tumours[62, 96, 112]. Its gene product ICP34.5 was later determined to inhibit the RNA-dependent protein kinase pathway (PKR)[113]. Double-stranded RNA produced by HSV activates this protective cellular pathway and allows the host cell to shut down protein synthesis in response to HSV infection[114]. Despite loss of ɣ34.5, these HSV mutants were able to demonstrate preferential replication in tumour cells[115, 116], although the precise mechanisms remain unclear. Early work indicated that tumour cells had a suppressed PKR pathway thereby enabling ɣ34.5 mutants to infect and replicate in these cells[66, 117, 118]. Further studies demonstrated that the PKR pathway was activated rather than suppressed but that viral replication occurred because of a marked increase in the protein synthesis machinery in tumour cells resulting in continued viral replication[115, 119, 120].

The main mutated herpes vector that was trialed in human gliomas was G207[107]. This virus contained the ɣ34.5 mutations discussed above and an inactivating insertion of the E. coli lacZ gene in the UL39 locus[107]. The UL39 locus codes for infected cell protein 6 (ICP6), which is the large subunit of ribonucleotide reductase (RR), an enzyme required for viral DNA synthesis. In herpes simplex viruses this inactivation leads to inefficient viral

DNA replication in a similar manner to the earlier thymidine kinase mutants[106].

1.3.2.2. ICP6 mutation

Ribonucleotide reductase is a heterodimeric enzyme comprising an 88kDa R1 subunit and a 44kDA R2 subunit. Its function is to catalyse the conversion of ribonucleioside diphosphates (NDPs) to deoxyribonucleioside diphosphates (dNDPs). dNDPs are subsequently converted by nucleoside diphosphate kinase to deoxyribonucleotide triphosphate (dNTPs). dNTPs are the building blocks of DNA and an essential substrate during replication of both mammalian cells and herpes simplex viruses. The mutation in the ICP6 coding gene was added to enhance the safety of mutated herpes viruses that ultimately would be injected intracranially. As a result of this mutation the virus preferentially replicates in cells where the mammalian ribonucleotide reductase levels are elevated such as tumours and is replicatively deficient in quiescent cells such as neurons[121].

Furthermore, lacZ is a reporter gene that can be used to monitor the spread of the virus in preclinical studies[121]. Double mutants of ICP6 and ϒ34.5 were chosen for clinical trials as their combined mutations were deemed to be safer for use in humans[107, 122]. This is because single-mutation viruses potentially may select for second site mutations that would enable them to overcome replication restrictions of the first mutation. In addition, short viral replication times, the millions of viral progeny produced

and random viral DNA polymerase mutations may all exert a selection pressure to overcome single mutations[122]. The concern with dual mutations though, is the restrictive effects of the mutations on oncolytic viral efficacy against gliomas.

1.3.3. G47Δ

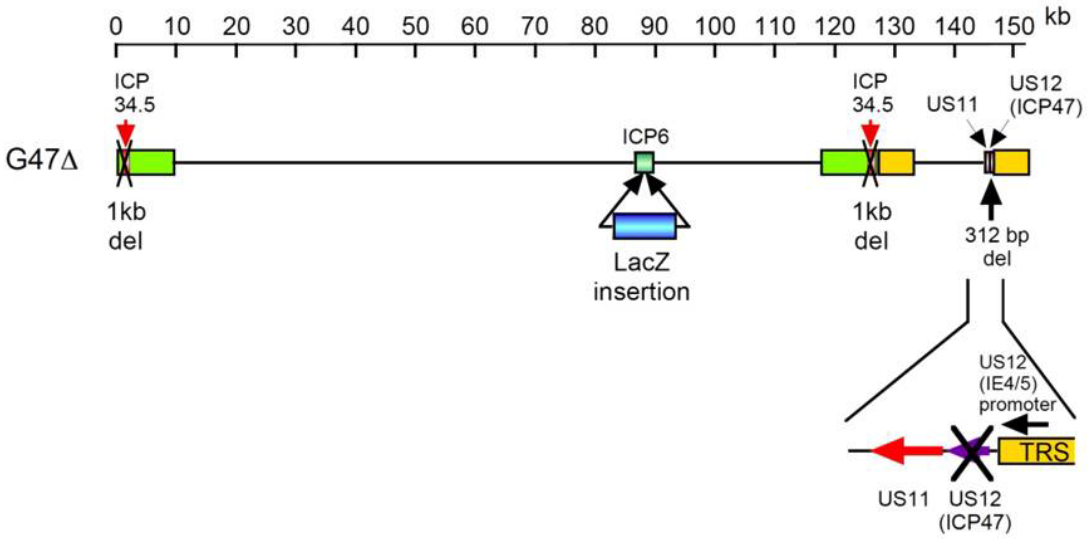

Figure 1.3. Schematic demonstrating the G47Δ oncolytic vector and its engineered mutations. Both ɣ34.5 genes in the inverted repeat sequences, flanking the unique long segment have 1kb deletions. There is also a LacZ insertional inactivation of the ICP6 gene. Finally there is a 312bp deletion in the US12 gene bringing the US11 gene under the control of the immediate-early US12 promoter.

One important strategy with oncolytic herpes viruses has been to employ the virus to trigger or enhance the host immune response to tumour

cells. This was the fundamental idea behind the creation of the next generation oncolytic vector G47Δ[76]. The pathway targeted for this next generation vector was the inhibition of immune presentation by herpes simplex viruses. Viruses, like cancers, are adept at shielding themselves from the host immune system[123, 124].

Following infection by HSV-1 there is a reduction in the expression of major histocompatibility complex (MHC) class I molecules on the surface of host cells[125, 126]. This effect is mediated by the product of the immediate early gene α47 (US12), which binds to the transporter associated with antigen presentation (TAP) and interferes with antigen processing in the endoplasmic smooth reticulum and subsequent antigen presentation by MHC class I molecules to CD8+ T cells[125, 127]. The efficacy of the protein produced by α47, infected cell protein 47 (ICP47), is species specific. It has a predilection for larger mammals and is highly effective in preventing T-cell-mediated cell killing in a range of animal models including pigs, dogs and monkeys[128]. Herpes simplex virus ICP47 is also highly efficacious in humans as a result of its high affinity for human TAP but is significantly weaker in murine models as its binding to murine TAP can be a hundred-fold lower[129, 130]. This is an important consideration when translating work performed in murine models with oncolytic vectors without an α47 deletion to human studies, as α47 expression would be expected to attenuate cytotoxic T-cell responses to virus infected cells in humans but not in mice with an intact immune system.

Based on the above data and using the backbone of the double-mutated G207, a third mutation was added by deleting the non-essential α47 gene[131] and G47Δ was born. The aim was to stimulate the host T-cell response to infected tumour cells via the immune presentation mechanisms discussed above. The deletion of the α47 gene also provided G47Δ with a further advantage over its predecessor G207. α47, also known as US12, is one of the five immediate early genes expressed at the start of HSV infection. Immediate early genes help regulate two further sets of genes that are chronologically expressed in waves and known as early and late genes. Deletion of the α47 gene resulted in the neighbouring US11 gene coming under the control of the US12 immediate early promoter[76]. US11 is normally expressed much later in the infection process and like γ34.5 inhibits the RNA-dependent protein kinase pathway[132]. Thus, expression of US11 at this early stage compensates for the replication deficiencies seen in γ34.5 mutants[133, 134]

The improved replicative ability of α47 mutants like G47Δ is not associated with reacquisition of neurovirulence[76]. To allay safety concerns this has been tested using Patton backbone mutants with either γ34.5 mutations alone, or double mutations in γ34.5 and α47 (SUP1). SUP1 was able to replicate effectively in a glioblastoma cell line that was not permissive for its parental γ34.5 mutant but remained equally attenuated for neurovirulence[122]. The genetic separation in γ34.5 mutants of neurovirulence and PKR suppression and the ability to capitalize on this has enabled the creation of more robust oncolytic vectors like G47Δ. G47Δ would

be expected to replicate more efficiently then its parental strain G207 while maintaining the safety profile of G207.

1.3.4. Safety evaluation of oncolytic viruses

A significant portion of the preclinical data on glioma virotherapy involves the use of G207 and G47Δ. Both these viruses are derived from the wild type HSV strain F. When inoculated into the central nervous system, wild type HSV strain F is one of the least virulent of HSV strains [135]. Safety testing of G207 in mice was carried out at intracerebral and intraventricular doses of 10^7 particle forming units (pfu) which is at or above doses demonstrating efficacy in mouse tumour studies[136]. In this study, the inoculated BALB/c mice survived for over 20 weeks with no ill effects. Two control mice groups were infected with 1.5×10^3 pfu of HSV1 wild type strain KOS or 10^4 pfu of wild type strain F. 80% of mice injected with the KOS strain and 50% of mice injected with strain F died within 10 days.

Safety evaluation of G207 in primates began in the late 1990s with intracerebral injections of G207 in the New World owl monkeys *Aotus nancymae*. This species of primate is highly sensitive to HSV infection[137-139]. In the G207 primate safety study, *Aotus nancymae* were injected with 10^7 to 10^9 pfu of G207 intracerebrally[140]. There were no deaths caused by G207 and post inoculation magnetic resonance imaging did not reveal evidence of encephalitis. Contrastingly, the control primate that received 10^3 pfu of wild type strain F developed severe herpes encephalitis with

symptoms including hemiparesis, meningitis and hemorrhage in the basal ganglia. The primate died quickly thereafter.

A subgroup from the initial cohort were reinoculated a year later with 10^7 pfu of G207 at the same stereotactic coordinates[140]. They demonstrated no sequelae and remained healthy for a further two years after the second dose of G207. Following this study, the biodistribution and shedding of G207 viral particles was investigated. *Aotus nancymae* were inoculated intracerebrally with 10^7 to 10^9 pfu of G207[141]. Post infection, serum anti-HSV antibody titres were raised but neither infectious viral particles nor viral DNA were present in saliva, tears or vaginal secretions[141]. Furthermore, post-mortem analysis demonstrated that G207 DNA was restricted to the brain. The findings from this study suggest that family members living in close proximity to patients receiving oncolytic viral therapy and health care workers treating these patients are at minimal risk of contracting the virus as a result of viral shedding.

The results from the G207 human phase I dose escalation studies of 21 patients with malignant gliomas were published in 2000[62]. G207 was inoculated into human gliomas at doses ranging from 10^6 pfu at one location to 3×10^9 pfu at five intracerebral locations. Clinically none of the patients developed herpes encephalitis and at post mortem examination there were no signs of encephalitis[62]. None of the deaths were attributable to G207 and at the time of reporting, three glioblastoma patients were alive. The γ34.5 mutant HSV 1716 which was developed from the wild type strain 17+

was also evaluated for its safety in two clinical trials. In the first trial, patients with recurrent malignant gliomas were treated with 10^5 pfu of HSV 1716 with no evidence of encephalitis or other ill effects[142]. In the second HSV1716 trial, 12 patients with high grade gliomas underwent surgical resection and HSV1716 was injected into the walls of the tumour cavity[61]. Once again, there was no toxicity associated with viral inoculation.

1.3.5. Clinical trials and future strategies

While the safety record of the oncolytic viruses was demonstrated in the clinical trials discussed above, the maximum tolerated dose was never reached. In addition, the true effect on human tumours has not been clearly established[20]. To date, no phase III trials involving oncolytic herpes simplex viruses have been completed. In theory, the perpetual replication and lysis of tumour cells would result in complete eradication of tumors. Unfortunately, this was not born out in preclinical and clinical study[20, 32]. This failure has been attributed to a number of factors including insufficient viral growth and spread, host immunity, or tumor growth rate outpacing the viral growth rate[143]. Nevertheless, the potential and promise of oncolytic virotherapy is too great to dismiss it.

1.4. Purpose of the proposed studies

The work in this thesis aims to investigate the utility of glioma stem cell models for assessing novel anti-glioma therapeutics and developing new therapeutic options for a disease that is thus far universally fatal. The emphasis is on the use of G47Δ, the latest generation genetically engineered oncolytic herpes simplex virus vector developed by our group. The effects of G47Δ will be tested on glioma stem cells both in vitro and in vivo. In addition, two strategies to augment the anti-tumour effect of G47Δ will be tested. The first will be the addition of the anti-angiogenic protein tumstatin as a transgene expressed by G47Δ. An anti-angiogenic agent was chosen as the transgene to specifically target the supportive perivascular niche of glioma stem cells. The second strategy will be combining radiation treatment with G47Δ to assess their combined efficacy against glioma stem cells. This strategy was selected because radiation therapy is the most commonly employed therapeutic option in human glioblastomas and the combined effect with G47Δ if successful, could inform future clinical trials with G47Δ.

Chapter 2: Materials and Methods

2.1. Cell culture and glioma stem cells

2.1.1. Cell lines and reagents

Human GBM cell line U87 and the African green monkey kidney cell line (Vero cells) used in the studies were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MRC5 human lung fibroblasts were obtained from Dr. Wendy Kallas, Massachusetts General Hospital. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Grand Island NY) supplemented with 10% foetal calf serum (FCS, Invitrogen) at 37°C and in 5% CO_2. Human umbilical vein endothelial cells (HUVECs) and their culture medium EGM-2 were obtained from Cambrex (Walkersville, MD, USA) and cultured as per the vendor protocols. The BT74 cell line, which was originally obtained from Dr. C. David James (University of California, San Francisco, San Francisco, CA, USA, referred to as GBM6 in *Pandita A, Genes Chrom Cancer 2004*[144]), and then was maintained as subcutaneous xenografts in mice at Brigham and Women's Hospital, Boston, Massachusetts.

2.1.2. Isolation and culture of glioma stem cells

Glioblastoma surgical specimens were obtained at Massachusetts General Hospital, Boston, with approval from the Institutional Review Board. Tissues were mechanically minced then digested with 0.1% Trypsin and 10 U/mL of DNaseI at 37°C for 45 min. Digested tissues were washed, triturated and passed through a 100-µm cell strainer. Cells were plated in EF medium which consisted of Neurobasal medium (Invitrogen) supplemented with 3 mmol/L L-Glutamine (Mediatech, Manassas, VA), 1× B27 supplement (Invitrogen), 0.5× N2 supplement (Invitrogen), 2 µg/mL heparin (Sigma, St Louis, MO), 20 ng/mL recombinant human EGF (R & D Systems, Minneapolis, MN), 20 ng/mL recombinant human FGF2 (Peprotech, Rocky Hill, NJ), and 0.5× penicillin G/streptomycin sulfate/amphotericin B complex (Mediatech). In addition, a portion of the digested tissue was grown in DMEM supplemented with 10% FCS to produce standard primary adherent cell cultures. The glioma stem cell cultures were fed once every three days with 1/3 volume of fresh medium. Cell passaging was performed by dissociation of the neurospheres using NeuroCult Chemical Dissociation kit (StemCell Technologies, Vancouver Canada).

2.2. Viral assays

2.2.1. Virus and infection studies.

The wild-type strain F virus of HSV-1 (obtained from Dr. B. Roizman, University of Chicago, IL, USA) and its derivatives G207 and G47Δ, have been described. G207 (*γ34.5⁻, ICP6⁻*) was derived from the R3616 virus by an inactivating insertion of *Escherichia coli lacZ* into *UL39* (ICP6)[107]. G47Δ (*γ34.5⁻, ICP6⁻, ICP47⁻*) was derived from G207 by deleting *α47* and the *US11* promoter (placing the late *US11* gene under control of an immediate-early *α47* promoter)[76]. The ICP6 mutant (FΔ6) is a strain F–derived recombinant with an *ICP6*-inactivating *lacZ* insertion, created by co-transfection of strain F DNA with the XbaI-HindIII fragment of pKX2-βG3[107]. G47ΔBAC virus was generated by homologous recombination between G47Δ DNA and pBAC-ICP6EF, and contains a cytomegalovirus promoter–driven enhanced green fluorescent protein (EGFP) in place of *lacZ* in G47Δ[145]. G47Δ-empty has the BAC and EGFP sequences removed from G47ΔBAC as described by Fukuhara and colleagues[146]. d120BAC virus was generated from d120, which contains a deletion in both copies of *ICP4*, by insertion of EGFP into the *ICP6* locus[147].

2.2.2. Replication assays

Cells were placed in 12-well plates and adherent cells were infected at 70-80% confluency. Glioma stem cells were infected 24 hours after seeding. After 2 hours, cells were washed with PBS and fresh media provided. For replication assays, cells and media were harvested at the indicated times post-infection and processed using three freeze-thaw cycles and sonication.

2.2.3. Virus titration

Monolayer cultures of Vero cells were grown in 6-well plates and infected with serial dilutions of the virus for 45 minutes at 37°C. Following removal of the inoculum, the cells were incubated in DMEM supplemented with 1% FCS and 0.1% pooled human immune globulin (BayGam, Bayer Corporation, Elkhart, IN, USA) at 37°C for 3 to 4 days until visible plaques appeared. The cells were then fixed with methanol for Giemsa staining or with 0.5% glutaraldehyde-2% paraformaldehyde for X-Gal histochemistry. Plaques were counted and the average number of plaques determined from 2 wells. Viral titres were documented as plaque forming units (pfu).

2.2.4. Infection spread assay.

Cells were infected with EGFP-expressing vectors d120BAC or G47ΔBAC. Cells were grown in EF medium and at the indicated timepoints EGFP fluorescence levels were measured using a standard fluorescence plate reader.

2.2.5. Viral cytotoxicity assay.

GBM-SC spheres were dissociated, cells resuspended at 5-10 × 10^6 cells/mL and infected at the indicated multiplicity of infection (MOI) for 45 min at 37°C. After centrifugation to remove unadsorbed virus, cells were seeded in 24-well plates at 2 × 10^4 cells per well in EF medium. The cells were harvested at fixed timepoints, dissociated with trypsin/EDTA, and viability assessed using flow cytometry, the MTS assay (Promega, Madison, WI, USA) or using viable trypan blue-excluding cells counted on a haemocytometer.

2.3. Construction of G47Δ-Tumstatin

The vector pBLAST42 bearing the human Tumstatin gene was obtained from Invivogen (San Diego, CA, USA). The subcloning of the tumstatin gene into the shuttle vector using restriction endonucleases is described in section 4.2.1.

2.3.1. Integration of the human tumstatin shuttle vector with pG47Δ-BAC using Cre recombination

1.5µg of pG47Δ-BAC and 150ng of shuttle vector DNA were mixed with Cre recombinase (Invitrogen, Grand Island, NY, USA) in a total of 10µl and incubated for 1 hour at 37°C. The Cre recombinase is then heat inactivated at 70°C for 10 minutes. The recombined DNA is ethanol precipitated and dissolved in 5µl of distilled water. Electrocompetent DH10B cells (Invitrogen) were transformed with the recombinant DNA using a Gene Pulser II (Bio-Rad, Hercules, CA, USA) and chloramphenicol and kanamycin double resistant clones were isolated.

2.3.2. Production of recombinant virus using FLP recombination

Vero cells (1.5 x 10^5 cells/well) were plated in a 24-well plate. They were co-transfected with 1µg of pG47ΔBACTumstatin DNA and 100ng of FLPe using Lipofectamine PLUS (Invitrogen) as per the vendor's protocols. 5 to 7 days later when viral cytotoxic effects were noted in the cells, the cells were harvested and lysed by three freeze-thaw cycles and sonication. Recombinant virus was purified on Vero cells by limiting dilution. G47Δ-Empty was created using the steps above with an empty shuttle vector.

2.4. Differentiation induction and immunocytochemistry.

To induce differentiation, dissociated cells were plated onto fibronectin/poly-l-ornithine–coated coverslips and grown in 1% FCS-containing medium without EGF or FGF2 for 10 to 14 days. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-X, washed and blocked with 10% goat serum then incubated overnight with primary antibodies at 4°C. Antibodies used were rabbit anti-GFAP (1:200), monoclonal anti-MAP2 (1:150) and rabbit anti-Nestin 130 (1:50)[148]. Cy3-conjugated secondary antibodies (1:400) were incubated at 4°C for 4 h to visualize immunoreactivity before microscopic observation. Antibody manufacturers are listed in Table 2.1.

2.5. Flow cytometric analysis.

Glioma stem cell cultures were dissociated and stained with phycoerythrin (PE)-conjugated anti-CD133/2 according to manufacturer's instructions before analysis with FACScalibur (BD Biosciences, San Jose, CA) or LSRII (BD Biosciences, San Jose, CA). oHSV infection, cell death, and CD133 expression status were examined simultaneously by three-color flow cytometric analysis. G47ΔBAC- or mock-infected cells were collected on days 1 and 3, stained with phycoerythrin-conjugated anti-CD133/2, washed and nonviable cells labeled with 7-AAD (BD Biosciences) before the samples were subjected to flow cytometric analysis. Data were analyzed by FlowJo software (Tree Star, Ashland OR). Antibody manufacturers are listed in Table 2.1. All other flow cytometric antibodies were used as per the vendor’s protocols. Flow cytometric based cell sorting was performed on the FACS Aria (BD Biosciences, San Jose, CA).

2.6. Western blotting

2.6.1. Protein extraction and quantification

Cultured cells were harvested using 200µl of radioimmunoprecipitation assay (RIPA) buffer (Boston Bioproducts) containing an added 2ul of protease inhibitor cocktail (Sigma, St Louis, MO). Cells were sonicated for 30 seconds, before incubating for 30 minutes on ice. Cells were centrifuged 10,000rpm for 15 minutes and protein expression in the supernatant quantified using the bicinchoninic acid assay (BCA, Sigma) by measurement of absorbance at 562nm of each sample relative to a standard curve in a standard fluorescence microplate reader according to manufacturer's instructions.

2.6.2. Gel electrophoresis and Western blotting

20µg of protein from each sample was run on NuPAGE Novex 10% Bis-Tris gels (Invitrogen) under denaturing reducing conditions at room temperature at 150V for 45 minutes. Protein transfer from gel to membrane was carried out at 4°C overnight (approximately 16 hours) at 22V in the transfer blot module (XCell II Blot Module, Invitrogen) according to manufacturer's instructions. Membranes were subsequently blocked with 10ml 2.5% w/v non-fat dry milk for 1 hour at room temperature, washed, then incubated with

primary antibody in PBST for 2 hours at room temperature or overnight at 4°C, in concentrations recommended by the individual antibody manufacturers. Membranes were then washed and incubated with 10ml of diluted secondary antibody to the appropriate species for 1 hour at room temperature in concentrations recommended by the individual antibody manufacturers. Antibody manufacturers are listed in Table 2.1. The membrane was covered with 4ml of enhanced chemiluminescence solution (ECL, GE Healthcare, Piscataway, NJ) and exposed to according to manufacturer's instructions for varying durations depending on the antibody used. β-Actin was used as the housekeeping gene to control for protein loading. The film was scanned using a Canoscan 4200F plate scanner (Canon, Lake Success, NY) and ArcSoft Photostudio V5.5 software (ArcSoft, Fremont, CA).

2.7. Antibodies used for protein expression

Protein	Antibody Manufacturer	Modality
ColIV	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Western blotting
Integrin αVβ3	eBioscience, San Diego CA, USA	Western blotting
pAKT (Ser473)	Cell Signaling Technology, Danvers, MA, USA	Western blotting
PTEN	Cell Signaling Technology, Danvers, MA, USA	Western blotting
Total AKT	Cell Signaling Technology, Danvers, MA, USA	Western blotting
Ribonucleotide reductase M2	Genway Biotech, San Diego, CA, USA	Western blotting
Tumstatin	Dr Raghu Kalluri, Boston, MA, USA	Western blotting
Nestin	Dr. Ron McKay, Bethesda, MD, USA	Immunohistochemistry
GFAP	Sigma, St Louis, MO, USA	Immunohistochemistry
MAP2	Chemicon, Billerica, MA, USA	Immunohistochemistry
CD133, Phycoerythrin conjugated	Miltenyi Biotech, Auburn, CA, USA	Flow cytometry
Olig2, Phycoerythrin conjugated	R&D Systems, Minneapolis, MN, USA	Flow cytometry
CD44 Phycoerythrin conjugated	Invitrogen, Grand Island, NY, USA	Flow cytometry
CD24 Allophycocyanin conjugated	Miltenyi Biotech, Auburn, CA, USA	Flow cytometry
Human HLA-ABC Phycoerythrin conjugated	BD Bisciences, NJ, USA	Flow cytometry

Table 2.1: List of antibodies used for Western blotting, immunohistochemistry and flow cytometry

2.8. Secondary neurosphere formation assay.

Seven days after virus infection, counts of trypan blue–stained cells were determined on a haemocytometer. Viable cells were resuspended in fresh EF medium and seeded into 96-well plates at 1 or 10 cells per well. 14 days later, the number of wells containing neurospheres (diameter, >60 μm) was recorded.

2.9. Angiogenesis and endothelial cell assays

2.9.1. Tumstatin

Human tumstatin protein and antibodies were obtained from Dr Raghu Kalluri, Harvard Medical School, Boston, MA, USA.

2.9.2. Matrigel assay

300µl of Matrigel (BD Biosciences, New Jersey, USA) was plated in wells of a 24-well plate and allowed to set for 30 minutes. 80,000 HUVECs in 500µl of media was added. A further 30 minutes was allowed for endothelial cell branching to take place. For co-culture experiments day 5 glioma stem cell spheres were added in 500µl of media.

2.9.3. Transwell co-culture assay

Transwell co-culture assays were performed in 12-well co-culture plates (BD Biosciences, New Jersey, USA) with transwell inserts with 0.4µm diameter membrane pores that allow the passage of diffusible molecules but not cells. 40,000 endothelial cells were seeded in bottom well in 1ml of media. 24 hours later 80,000 glioma stem cells in a single cell suspension were placed in the top well in 1ml of media. Therapeutic agents were added 24 hours later.

2.9.4. Migration and apoptosis assays

The endothelial cell migration assay was performed using the Calbiochem (Billerica, MA, USA) Innocyte Migration Assay as per the vendor protocols. Apoptosis was measured using an Annexin V apoptosis detection kit (BD Biosciences, New Jersey, USA) and flow cytometric analysis as per the vendor's protocols.

2.9.5. Vascular endothelial growth factor (VEGF) measurement

VEGF levels were measured using the human VEGF Quantikine ELISA Immunoassay from R&D Systems (Minneapolis, MN, USA) as per the vendor protocols.

2.10. Chou-Talalay analysis

Dose response curves and EC50 values (50% effective concentration values) were calculated using the MTS assay (Promega, Madison, WI, USA).

Fixed ratios of virus and radiation treatment were delivered in combination. Combined dose response curves were fitted to Chou-Talalay lines derived from the law of mass action[149, 150]. The equation describing this is:

$$\log(F_a/F_u) = m\log D - m\log D_m,$$

where F_a is the fraction of dead cells, F_u is the fraction of unaffected cells, D is the dose, D_m is the median effect dose and *m* is the coefficient signifying the shape of the dose response curve. Combination index (CI) values were calculated using the equation:

$$CI = (D1/D \times 1) + (D2/D \times 2) + (D1)(D2) / [(D \times 1)(D \times 2)],$$

where D x 1 and D x 2 are radiation and G47Δ doses that are required to achieve a particular F_a; and D1 and D2 are the doses of the 2 agents in combined treatments that are required to achieve the same F_a. A CI value of 1 indicates an additive effect. A value of less than 1 indicates synergism and a value above 1 indicates antagonism.

2.11. In vivo experiments

2.11.1. Acquisition and maintenance of mice

Nude mice (athymic NCr-nu/nu) were obtained from the National Cancer Institute (NCI, Frederick, MD) and maintained under standard conditions. All mice used were aged between 7 and 9 weeks at the time of experimentation. Mice were housed at the animal research facility at the Simches building at Massachusetts General Hospital in full accordance with the rules and regulations of the Centre for Comparative Medicine at Massachusetts General Hospital. All in vivo procedures described below were approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital.

2.11.2. Tumorigenicity studies and immunohistochemistry.

Mice were anaesthetized by intraperitoneal injections of 50mg/kg of pentobarbital in 0.1 ml saline using 1 ml tuberculin syringe with 28G1/2" needle. Sterile technique was observed at all times with alcohol skin prep, the use of sterile instruments and technique and the use of appropriate clothing such as cap, mask and sterile gloves. Surgery was carried out in a

laminar flow hood. Dissociated glioma stem cells were stereotactically implanted into the brains (right striatum, 2.5mm lateral from bregma and 2mm deep) of athymic mice under anesthesia. To do this, the mice were placed on a KOPF stereotactic frame and a skin incision of approximately 1 cm made in the midline of the scalp. A burr hole of approximately 1.5 mm in diameter was made at 1.5 mm to the right to the midline and 1 mm anterior to the coronal suture using an electric high-speed drill with a diamond burr. 50,000 glioma stem cells in a volume of 2µl were injected over 5 minutes at a depth of 2mm from the brain surface using a 10-microliter Hamilton syringe with a needle. The needle was then retracted over 5 minutes. The burrhole was closed with bone wax and the wound closed with a clip applier. Mice were wrapped in 4" x 4" gauze to keep their body temperature warm. Respiration and the colour of their body (especially eyes and the tail) were closely observed. Mice were monitored for signs of discomfort or neurological deterioration and their overall health checked every 2 to 3 days. Mice were sacrificed when they developed significant weight loss or neurological symptoms including hemiparesis. Formalin-fixed, paraffin-embedded sections of brain were stained with Haematoxylin and eosin.

2.11.3. In vivo treatment studies.

2.11.3.1. Intracerebral tumor treatment

At a fixed timepoint after tumor implantation (described in detail in each results section), oncolytic virus was inoculated into the tumours. Preoperative procedures were identical to the implantation methods described above. Mice were placed on the same KOPF stereotactic frame. The wound was reopened, and the same burr hole used. Virus was injected at doses of 2 x 10^6 pfu in a volume of 2µl at the same coordinates in exactly the same manner as tumor cell implantation. The wounds were reclosed with a clip using a clip applicatour. Mice were wrapped in 4" x 4" gauze to keep their body temperature warm. Respiration and the colour of the body (especially eyes and the tail) were closely observed. Animals were followed for survival as described above. Upon sacrifice, the brains were fixed in 4% paraformaldehyde and frozen sections obtained.

2.11.3.2. Radiation therapy

Radiation therapy to glioma stem cells in vitro and in in vivo mice experiments was delivered from a Cesium 137 radioisotope from a sealed source in the Centre for Comparative Medicine at the Simches Building at Massachusetts General Hospital. Radiation therapy was delivered to the mice while they were placed in a radiation shield (provided by Dr. Kamalakannan, Ohio State University) to protect their bodies from receiving a dose of radiation.

2.12. Statistics.

Comparisons of data in cell survival and viral yield assays were performed using a two-tailed Student's *t* test (unpaired) and ANOVA with post-hoc Tukey analysis where appropriate. Survival analysis was conducted by Kaplan-Meier curves, and their comparison was determined by log rank test. *P* values of <0.05 were considered significant. Statistical analysis was performed using Prism (GraphPad Software, La Jolla, CA).

Chapter 3: Establishing glioma stem cell cultures, creating in vivo xenograft models and the efficacy of G47Δ against glioma stem cells

3.1. Introduction

One of the major areas of focus for glioma researchers is the creation of pre-clinical models that mimic human glioblastomas as precisely as possible. Accurate models of glioblastomas are necessary to allow dissection of its intricate survival and proliferation pathways and investigation of novel therapeutics, as well as testing imaging and drug delivery techniques with maximal translational relevance. The preclinical stage of glioblastoma research has largely focused on commercially available glioma cell lines that have been established and propagated in serum containing media (fetal calf serum) for decades. These cell lines, grown as adherent cells, are a poor representation of the genetic and phenotypic diversity seen in human glioblastomas[151, 152].

Another method employed has been the development of transgenic mouse models engineered with mutations in various genes such as NF1, EGFR and platelet-derived growth factor (PDGF), which result in the formation of spontaneous gliomas in mice. While this method has its merits, these models to an extent reflect our current understanding of glioblastomas and are only as good as the mutations they bear. The complexity of the

engineered tumours is therefore partly restricted by our understanding of the triggering mutations in glioblastoma. Furthermore, development of these models requires considerable time, funding and expertise. In addition, current transgenic mouse models are unable to model recurrent glioblastomas satisfactorily[153]. This is a particular weakness as in the clinical setting, recurrent disease is an area of intense study and the focus for development of novel therapeutic agents. Recurrent glioblastoma is also ultimately the cause of death in human patients.

While both these models have certainly aided the study of glioblastomas, the development of glioma stem cell models has provided a further avenue for investigation. As discussed in section 1.2, the tumours formed by these cells resemble their human clinical counterparts in a number of important areas including their pro-angiogenic capabilities and their resistance to chemotherapeutic agents as well as radiation therapy. Furthermore, analysis of their transcriptome and in vivo phenotypes indicates that glioma stem cells grown as spheres most closely resemble their human counterparts compared to conventional cell lines or adherent primary culture cells[151, 152]. The culture of glioma stem cells also allows for clinical correlations to be made with the original tumour's histological features and the patient's clinical course which in the future may help stratify further subtypes of glioblastomas with distinct clinical outcomes. The action of novel therapeutic agents such as oncolytic herpes simplex virus vectors both in vitro and in vivo in this new preclinical model of glioblastoma was still unknown. Thus,

glioma stem cell models were established to test the effect of oncolytic vectors, particularly the latest generation vector G47Δ.

3.2. Glioma stem cells

3.2.1. Establishing glioma stem cell cultures

Glioma stem cell lines were created from primary glioblastoma surgical specimens that were taken immediately from the operating rooms to the pathology department where a sufficient quantity of tissue for histological and molecular analysis was excised for clinical analysis. The remainder of the specimen was taken to the laboratory for dissociation and culture as glioma stem cells. A histological diagnosis of glioblastoma (World Health Organisation Grade IV) in the original patient tissue samples was retrospectively confirmed in all of the specimens used to create glioma stem cell cultures. The processing of the samples in the laboratory began within an hour of the tumour specimens being removed from the patients.

Tissue specimens were mechanically minced and digested with 0.1% trypsin. Dissociated surgical specimens grown in glioma stem cell culture medium typically gave rise to neurosphere formation in 5 to 14 days. The culture medium (EF medium) contains the growth factors epidermal growth factor (EGF) and fibroblast growth factor (FGF) that have previously been shown to promote the growth of glioma stem cells[19]. Once stable cell lines were established they could be chemically dissociated and passaged for more than 3 months. All experiments were conducted on primary cultures of

glioma stem cells between passages 3 and 8 to limit the effects of in vitro selection pressures on the cultures.

3.2.2. In vitro assessment of glioma stem cells

Once the glioma stem cell cultures were established they were tested for their stem-like characteristics including sphere formation assays, self-renewal, multipotency, the presence of putative stem cell markers and in vivo tumorogenicity. Only cell lines that met these criteria were selected for further experiments. Figure 3.1a demonstrates the sphere forming capabilities of these cells when grown in EF media. Not all the surgical specimens robustly formed spheres in EF media. Figure 3.1b demonstrates a cell line that formed very small spheres. Self-renewal of glioma stem cell spheres was tested by plating a single cell suspension into the wells of a 96-well plate containing EF media. 14 days later the wells were examined for spheres and in a mean of 9% (range 5% to 21%) of wells, spheres would form from a single cell suspension demonstrating the ability of these cells to self-renew. These spheres retained the stem-like characteristics of their parent cells.

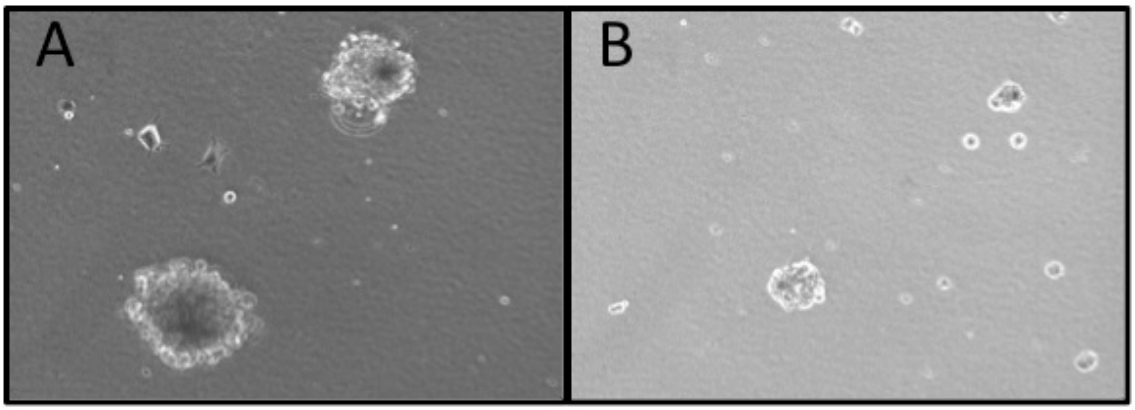

Figure 3.1 Glioma stem cell neurospheres (A,B). Phase microscopy of glioma stem cell neurospheres seen at 10X magnification. Cells are grown in media enriched with FGF and EGF. Day 7 neurospheres are shown.

Next the glioma stem cells were tested for putative stem cell markers, firstly CD133. Flow cytometric analysis of in vitro cultures for the surface epitope AC133 demonstrated varying quantities of the surface protein (2.35% to 97.82%). Figure 3.2 shows the varying quantities of the AC133 surface epitope in the glioma stem cells. Flow cytometric analysis was also used to identify the presence of oligodendrocyte transcription factor (Olig2), a neural stem cell marker which has been implicated in the growth regulation of glioma stem cells[31, 154]. Figure 3.3 demonstrates the Olig2 expressing population of glioma stem cells in one cell line. Immunohistochemistry testing of Nestin, a marker of primitive neuronal stem cells, was positive in these cells as was glial fibrillary acidic protein (GFAP), a marker for astrocytes and neuronal stem cells[155] (Figure 3.4).

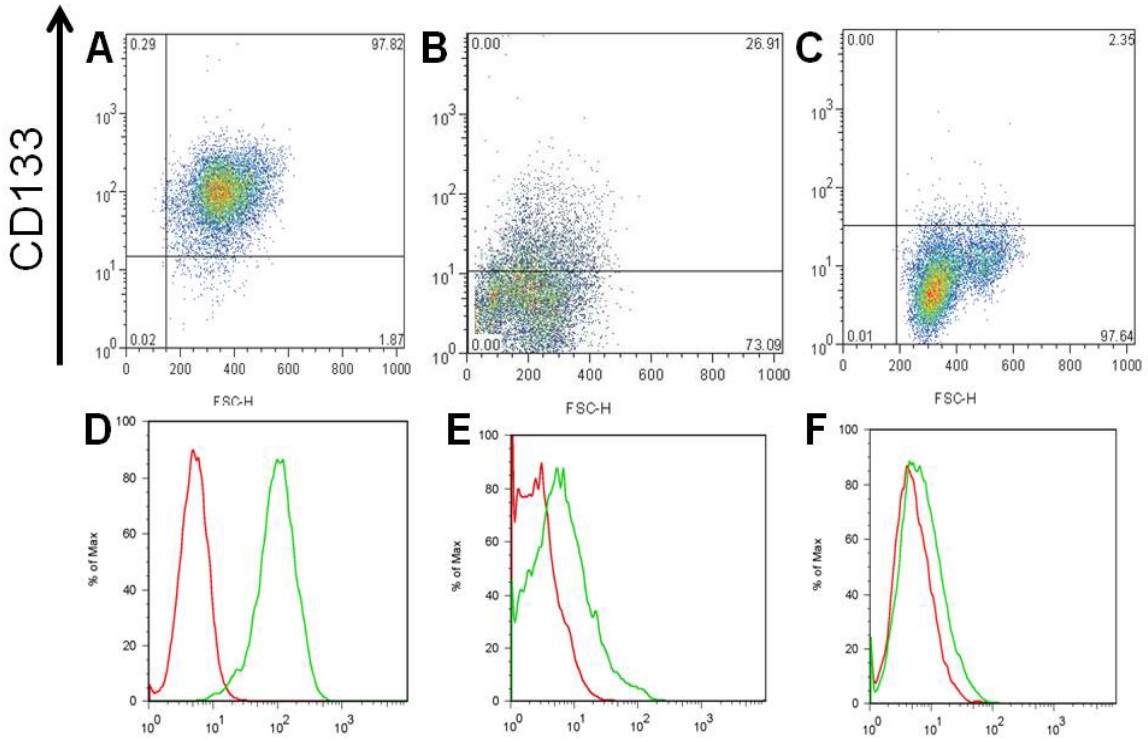

Figure 3.2 Flow cytometric data from three different cell lines demonstrating the varying percentages of CD133 positive cells. A, B and C represent glioma stem cell lines GBM8 (98% CD133 positivity), GBM4 (27%) and GBM18 (2%) respectively, demonstrating the range of CD133 expression by glioma stem cells. D, E and F show histograms of CD133 expression in GBM8, GBM4 and GBM18 respectively. The isotype control is in red and the CD133 positive population is in green.

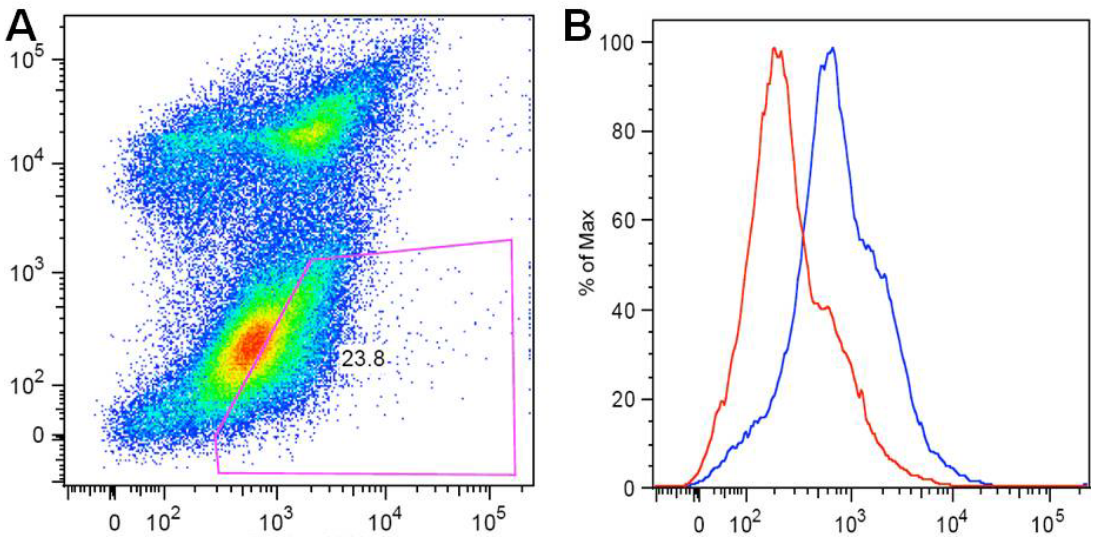

Figure 3.3 Expression of Olig2 marker in glioma stem cells (BT74). (A) demonstrates the 23.8% Olig2 positive population of glioma stem cells in. B demonstrates a histogram of the expression of Olig2 (blue). A histogram for the isotype control (red) is also shown.

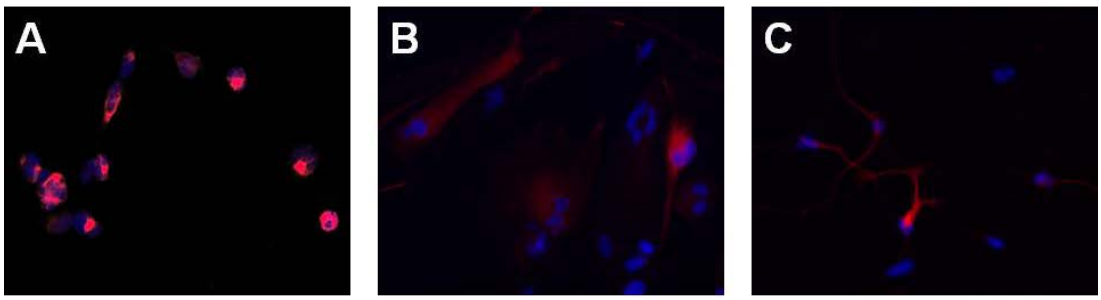

Figure 3.4 Nestin, GFAP and MAP2 expression in glioma stem cells. (A) Immunohistochemical staining in glioma stem cells grown in EF media for the neural/glioma stem cell marker Nestin (red). (B) The astrocytic marker GFAP (red) and (C) neuronal marker MAP2 (red) staining in glioma stem cells grown in serum-containing media to induce differentiation. Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI, blue).

Once the presence of these markers was established, the glioma stem cells were placed in serum-containing media to induce differentiation and produce cells of neuronal or glial lineage. In this media the cell lines consistently changed morphologically and demonstrated the glial marker GFAP and the neuronal marker MAP2 (Figure 3.4). In keeping with their differentiation into neuronal and glial lineages, a concurrent reduction in nestin staining and CD133 positivity was seen. Figure 3.5 shows the reduction in CD133 expression following differentiation of the cells in serum containing media.

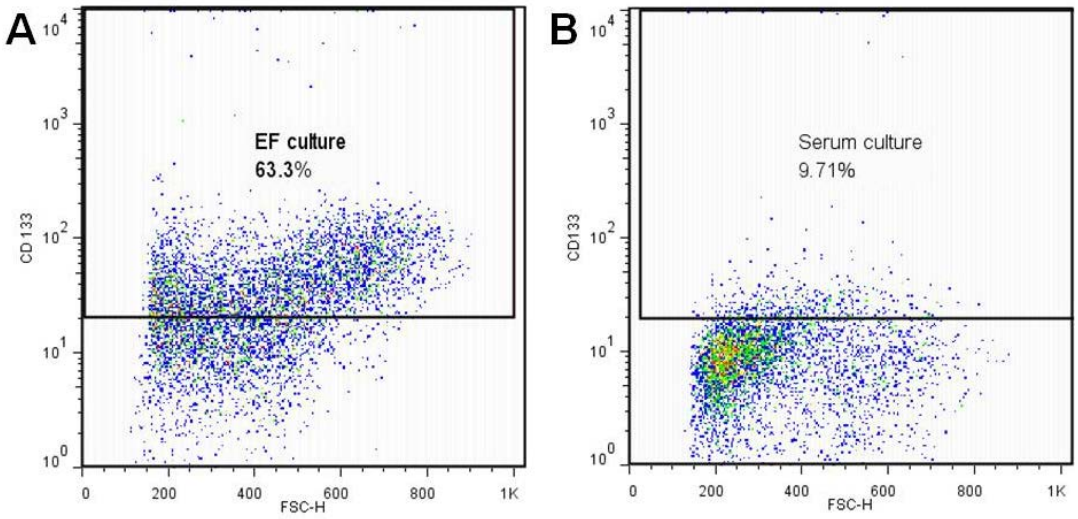

Figure 3.5 CD133 expression in glioma stem cells. GBM4 grown in (A) EF media alone and (B) EF media then subsequently grown in serum-containing media. Expression of CD133 is reduced (63.3% to 9.71%) when glioma stem cells media is changed to serum-containing media.

3.2.3. In vivo assessment of glioma stem cells

The cultured cells were then implanted orthotopically in nude mice to evaluate tumorogenicity. Nude mice, in this case the NCr-*nu/nu* mice, are genetically mutated immunocompromised mice. Their genome contains a mutation in the forkhead box protein N1 (Foxn1) gene making them athymic and thus unable to generate mature T-lymphocytes. This results in a severe restriction of their ability to mount T-cell based immune responses including the rejection of xenografts, thereby making them an ideal host for the human glioma stem cells. The glioma stem cells that were to be implanted were washed and implanted in phosphate buffered saline (PBS). They were not implanted in growth media containing FGF and EGF to prevent any

extraneous growth promoters influencing their ability to form tumours in this orthotopic model.

Once a cell line was demonstrated to form tumours in 100% of mice with 50,000 cells and met the criteria above, it was used for further experiments. Despite the range in CD133 positivity seen in these cells (2.35% to 97.82%), 90% of the cell lines were capable of forming orthotopic tumours in immune compromised mice and this ability was not related to the degree of CD133 expression (personal communication Dr. Hiroaki Wakimoto, Harvard Medical School, Boston, USA). Histopathological analysis of orthotopic tumours formed by the glioma cell lines demonstrated recapitulation of the phenotypic features of astrocytic glial tumours seen in humans.

Figure 3.6 demonstrates some of these features. Two major subtypes of tumours were formed. They were either diffusely infiltrating along white matter tracts or formed more discrete, circumscribed tumours. Figure 3.6A illustrates one tumour that is haemorrhagic, crosses the midline and exerts significant mass effect. Figure 3.6B shows a 10x magnification of a more circumscribed tumour, but even this tumour has cells extending beyond its borders invading into normal brain. Figure 3.6C, also at 10x magnification, demonstrates an invasive tumour infiltrating white matter tracts of the brain. Both figures 3.6 B and C show features of primitive malignant cells with high nuclear to cytoplasm ratios, pleomorphic nuclei and mitotic figures.

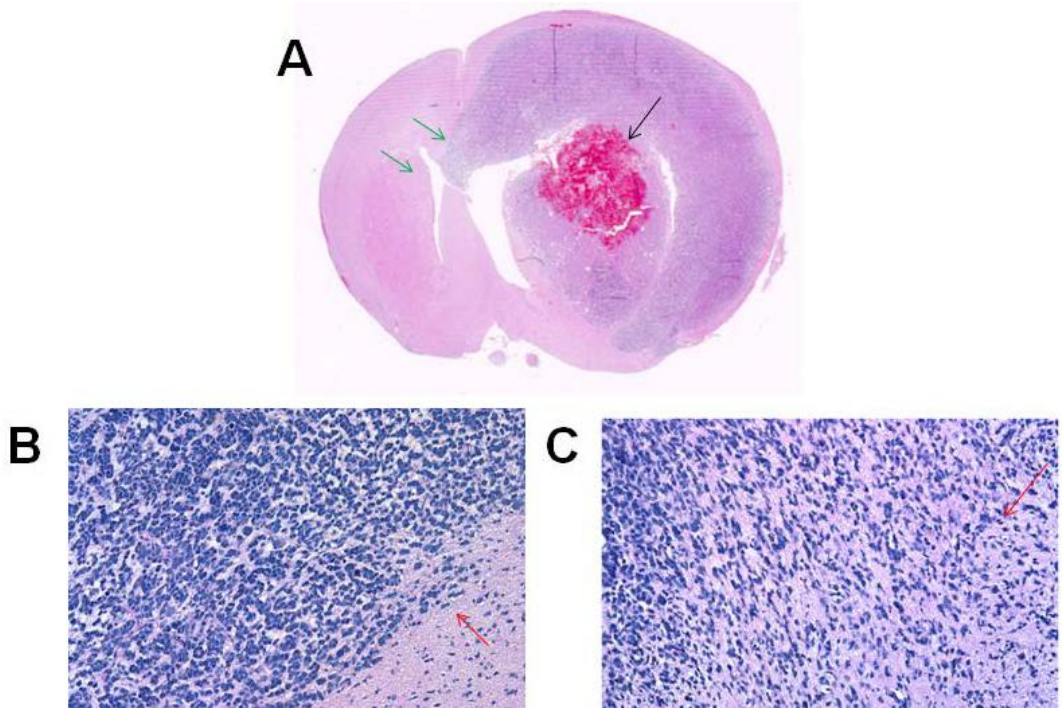

Figure 3.6 In vivo tumours formed by glioma stem cells. (A) GBM18 demonstrating the haemorrhagic tumours that can be formed by glioma stem cells. The black arrow shows the area of haemorrhage and the green arrows indicate the areas where the tumour crosses the midline and the considerable mass effect on the contralateral ventricle. (B) and (C) are 10x magnifications of GBM4 and GBM8 respectively. Figure 3.6A demonstrates the more circumscribed tumours formed by the GBM4 subtype while GBM8 (B) is more invasive and does not have distinct histological borders. In B the red arrow shows glioma cell invading into normal brain and the red arrow in C demonstrates a mitotic figure.

3.3. Effect of oncolytic HSV on glioma stem cells in vitro

3.3.1. Infection and replication of G47Δ in vitro

Having established the glioma stem cell in vitro and in vivo models, the efficacy of the oncolytic vector G47Δ was tested. First, the ability of the vector to infect and replicate in glioma stem cells in vitro was tested. Herpes simplex viruses infect cells by initially binding to glycosaminoglycan (GAG) chains on the surface of cells. The GAG heparan sulphate is the commonly employed binding receptor and is present on a range of cells[156]. Virus glycoproteins gB and gC mediate the binding of the virus to heparin sulphate. Following binding, interaction between the viral glycoprotein gD and entry receptors such as nectin-1, nectin-2 and herpesvirus entry mediator are used to cause fusion of the virus with the cell membrane and enable entry into the cell[157].

Herpes viruses are known to be able to infect a range of cell types and rather than test for the presence of above receptors, a functional assay was employed. Virus strains expressing the enhanced green fluorescent protein (EGFP) gene driven by the cytomegalovirus (CMV) promoter were used. The CMV promoter was chosen because of its ability for consistent and strong expression of genes in mammalian cells[158]. A replication-competent G47Δ with a bacterial artificial chromosome (BAC) system and EGFP tag (Figure 3.7) was compared to a control virus strain d120BAC.

d120BAC is a replication deficient strain with a similar BAC and EGFP tag. d120BAC is able to infect cells but does not replicate in them. The lack of replication in the d120 strain is a result of a 4.1kb deletion in both copies of the immediate early ICP4 gene[159]. ICP4 is essential for HSV-1 replication but not infection. Therefore, its mutants can infect but not replicate in cells. In addition to testing infection, various MOIs were used including very low concentrations of the virus to allow for detection of multiplication of the virus.

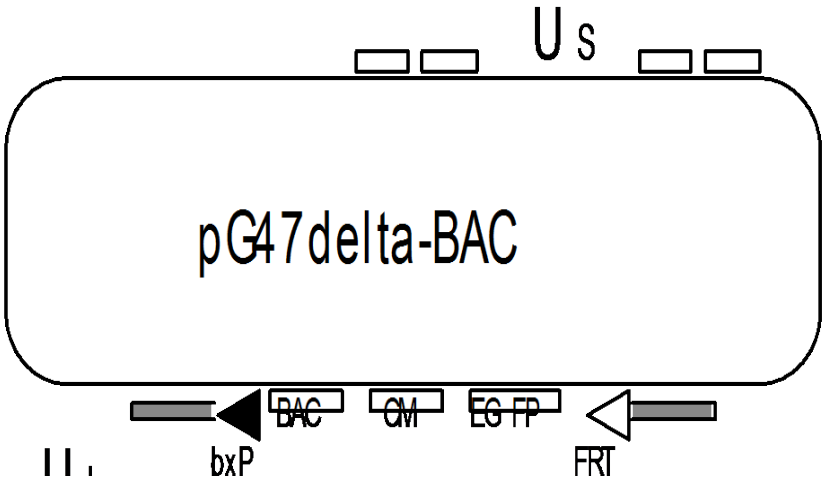

Figure 3.7. Schematic representation of the G47ΔBAC strain. Enhanced green fluorescent protein gene (EGFP) has been inserted in the ICP6 region of the unique long (UL) segment of the genome. The gene is driven by a cytomegalovirus (CMV, denoted CM in the figure) promoter.

Figure 3.8 below shows fluorescent images demonstrating the presence of fluorescent green glioma stem cells infected by G47ΔBAC. Similar findings were seen in cells infected with d120BAC indicating the ability of the virus to successfully infect glioma stem cells. Wells with

G47ΔBAC demonstrated increasing amounts of fluorescence with time. This was not seen in the replication-deficient d120BAC infected cells indicating that the G47Δ strain is able to infect and replicate in glioma stem cells. This was quantified by measuring the level of fluorescence in the infected cells with time.

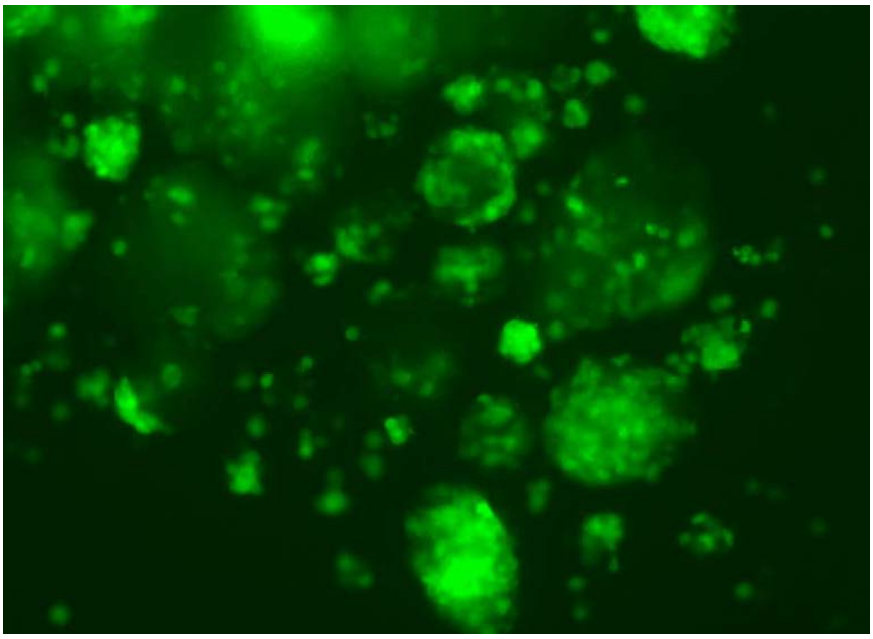

Figure 3.8 Fluorescence microscopy (10x magnification) demonstrating glioma stem cell spheres infected with EGFP expressing G47ΔBAC.

Figure 3.9 demonstrates the increase in fluorescence seen with time following infection by G47ΔBAC. The fall in fluorescence seen at the latter time points particularly with higher multiplicities of infection is due to the virus killing glioma stem cells. The green fluorescent protein expressed by the infected cells only occurs in live infected cells. Figure 3.10 demonstrates the

fluorescence activity of d120BAC infected cells. The fluorescence levels are unchanged over time because d120BAC is able to infect glioma stem cells, but unlike G47ΔBAC it is replication-incompetent. Thus the self-propogation seen with oncolytic vectors, where continued replication and cell lysis continues unabated until all the live tumour cells are lysed, is not seen here.

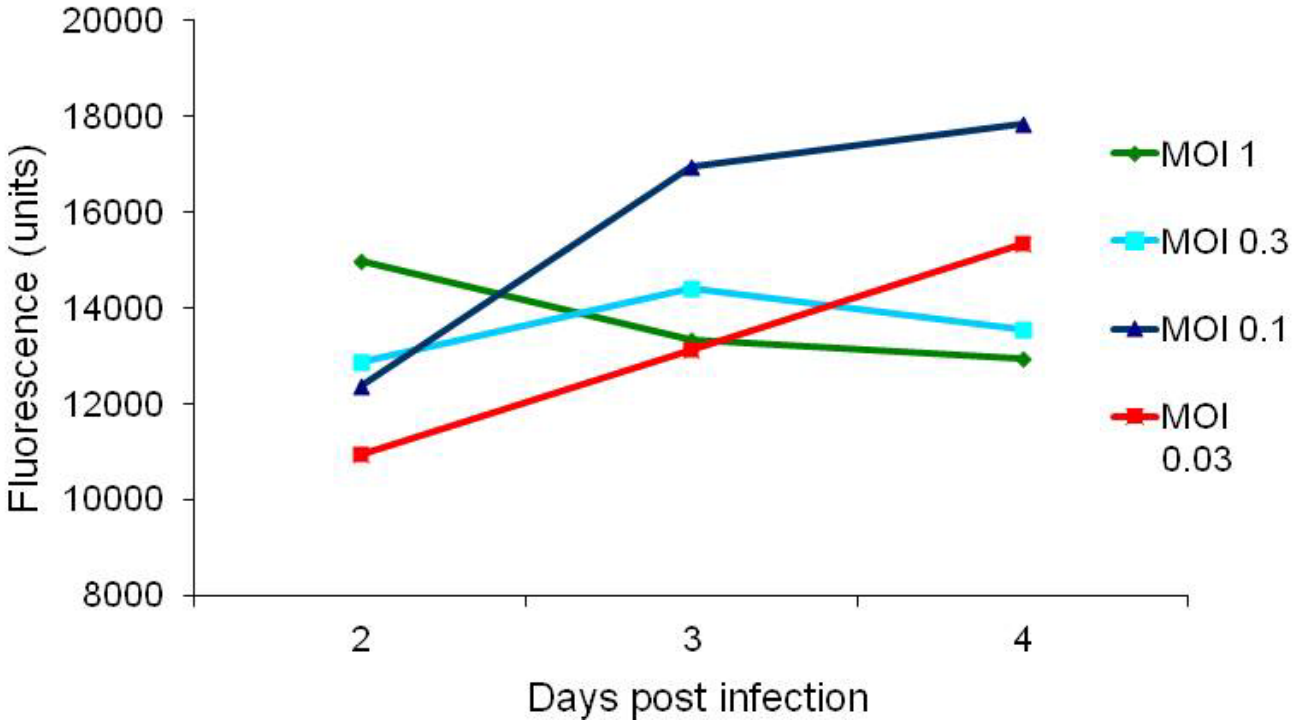

Figure 3.9 Fluorescence measurements in glioma stem cell cultures (GBM8) following infection with G47ΔBAC. Increases in fluorescence are seen with time indicating infection and replication. At the higher multiplicities of infection (MOI) the fluorescence diminishes at later time points because of cell killing.

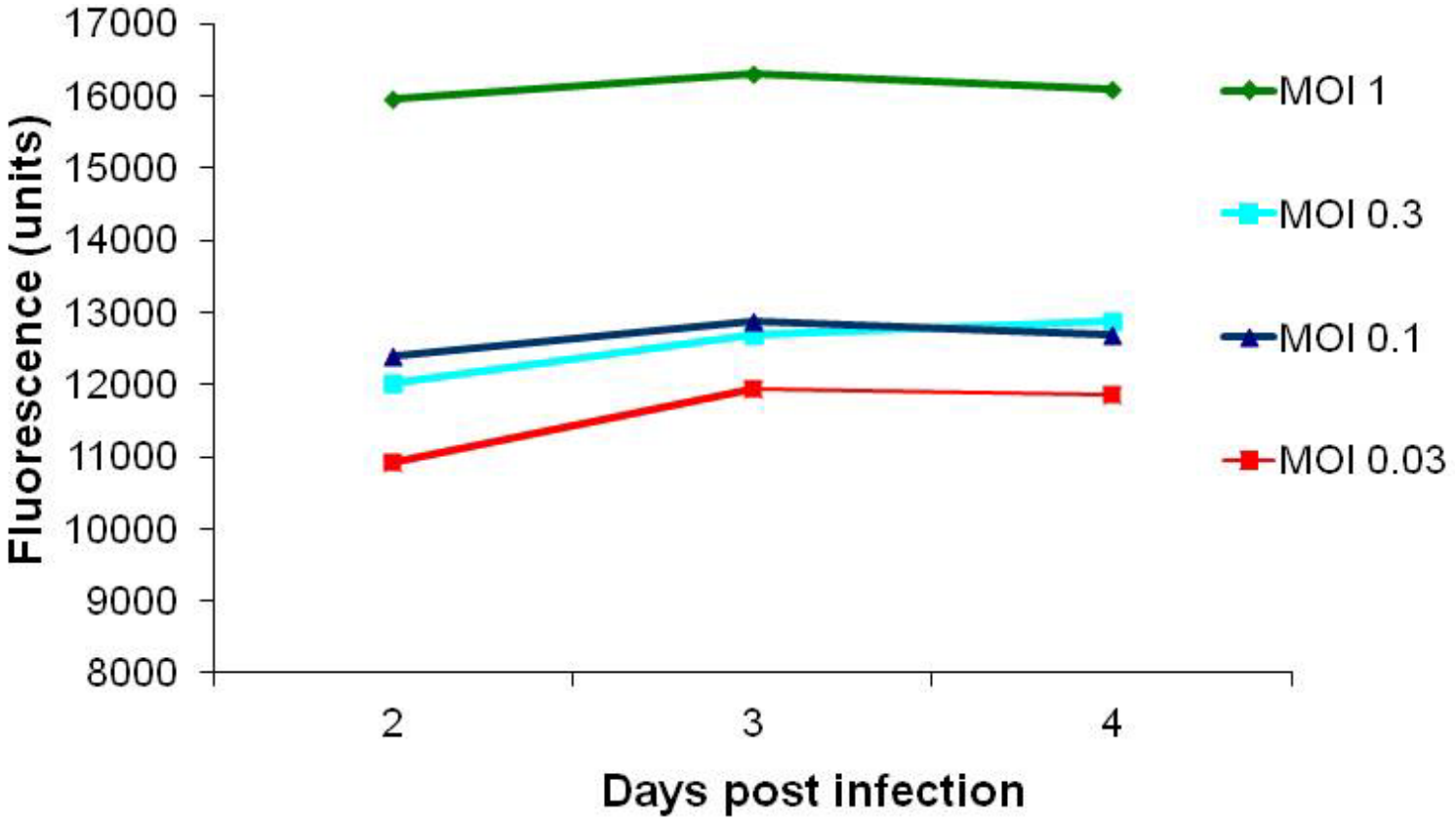

Figure 3.10 Fluorescence measurements in glioma stem cells (GBM8) following infection with the replication incompetent d120BAC. No change in fluorescence is seen in this control experiment because of the inability of d120Bac to replicate.

This experiment demonstrated the ability of the virus to infect gliomas stem cells and indicated that the virus was able to replicate and kill these stem cells. To confirm the replicative abilities of G47Δ and other HSV-1 strains, infection and viral yield assays were performed. Figure 3.11 below demonstrates that wild type HSV-1, the ICP6 mutant virus (FΔ6) and G47Δ are all able to replicate in glioma stem cells in in vitro conditions. All 3 of the viruses demonstrate logarithmic increases in viral yield and this continued self-propagation of the virus is a major advantage of this therapy compared to conventional adjuvant treatments. As expected the wild-type virus, unconstrained by safety mutations, produced the greatest yield at the final timepoint. Nonetheless, the final yield from G47Δ demonstrated its ability to satisfactorily infect and replicate in glioma stem cells. G207 with its deletions in the γ34.5 genes demonstrated poor replicative abilities in the glioma stem cells.

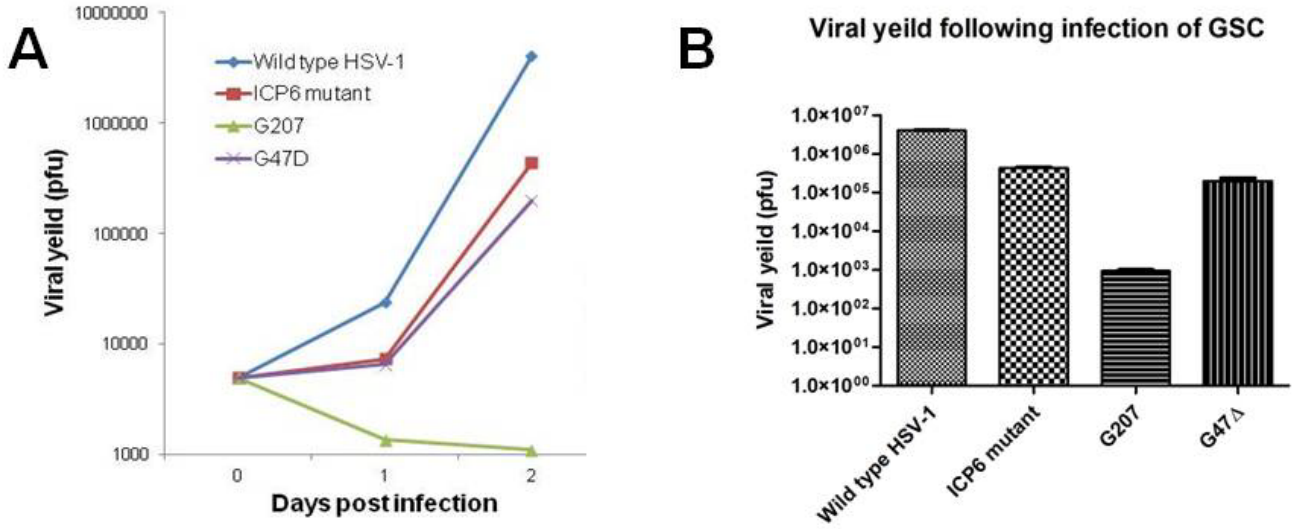

Figure 3.11 Viral yield assays with wild type HSV-1 and mutated oncolytic vectors. (A) All vectors except G207 demonstrated logarithmic increases in viral replication. (B) 48 hour viral yields. Viral yields at 48 hours were statistically different for all 4 viruses (ANOVA with Tukey post-test, $p<0.05$). Wild type HSV-1 and the ICP6 mutant demonstrated higher replication yields than G47Δ, while the yield from G207 was significantly poorer.

3.3.2. Oncolytic HSV abrogates the self-renewal of glioma stem cells in vitro

The above experiments confirmed the ability of G47Δ to infect and replicate within glioma stem cells. Next the effect of G47Δ on the glioma stem cell spheres and their ability to self renew were tested. Figure 3.12 shows microscopy images taken of glioma stem cell spheres after infection with G47Δ. The cells no longer form symmetrical spheres and now aggregate to form distorted clumps of cells. Also visible were single cells that under the microscope did not appear viable, as well as cellular debris. The self-renewal capacity of glioma stem cells that survived infection were tested using the self-renewal assay described earlier in Chapter 2. Post-infection single cell suspensions were plated into wells of a 96 well plate. In contrast to pre-infection glioma stem cells, none of the cells were able to form spheres post-infection.

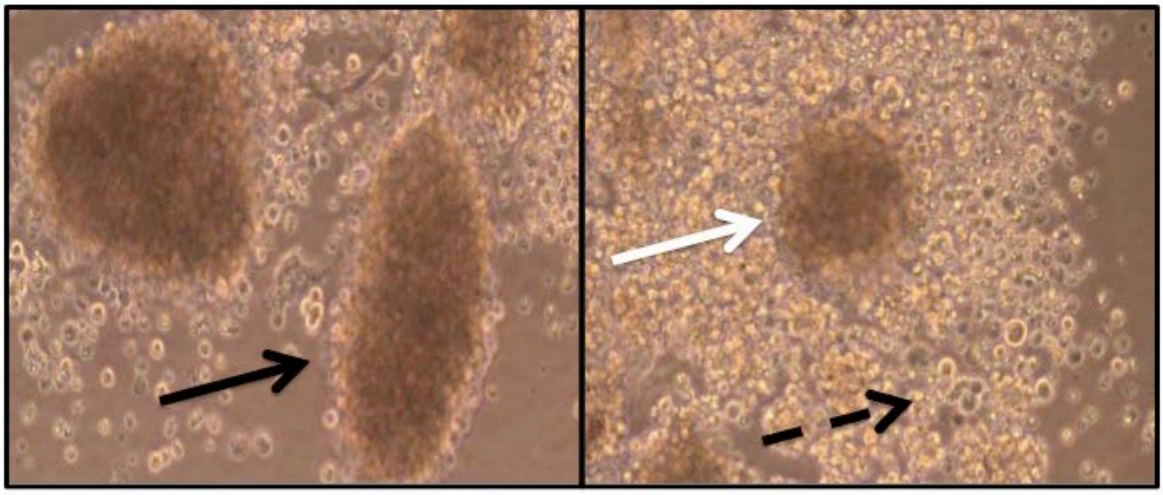

Figure 3.12 Glioma stem cells spheres following infection with G47Δ. Phase microscopy pictures at 10X magnification demonstrating the appearance of glioma stem cell spheres following infection with an oncolytic virus. The morphology of the spheres are altered and with a loss of symmetry (black arrow) and the formation of irregular clumps of cells (white arrow). Cellular debris is present (dashed arrows) and the cells do not appear viable.

To confirm that the dead cells were virus-infected cells, the experiment was repeated using the G47ΔBAC virus and flow cytometric analysis undertaken. Figure 3.13 shows the results of 2-colour flow cytometry demonstrating GFP infected cells and staining for 7AAD, a marker for loss of cell viability. Figure 3.13A shows glioma stem cells expressing GFP following infection with G47ΔBAC. The majority of the cell population is viable. The assay was repeated 5 days later (Figure 3.13B) and flow cytometric analysis demonstrates that the majority of cells are dead and very little GFP expression is seen (12.6%) as only live cells can express GFP.

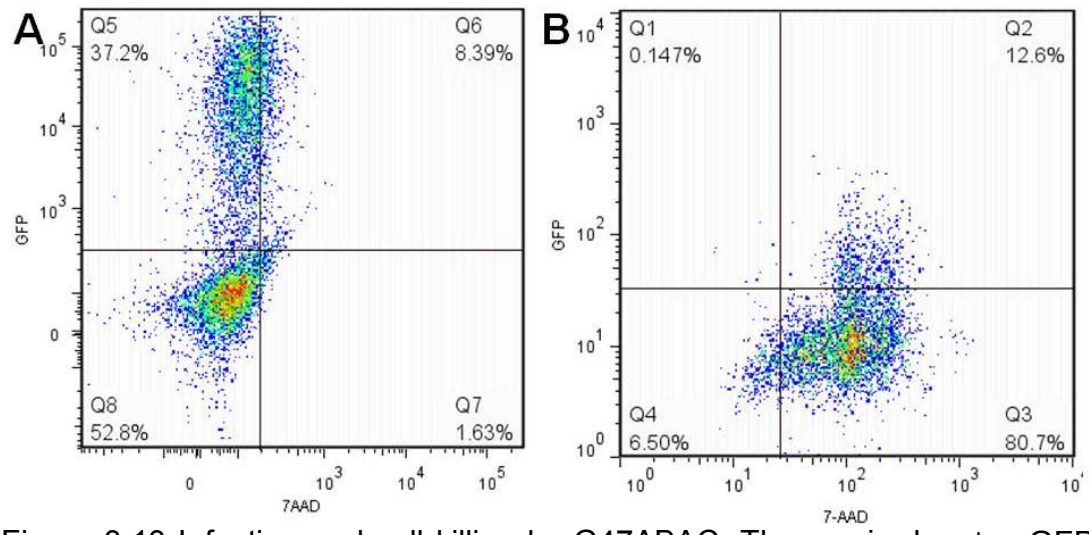

Figure 3.13 Infection and cell killing by G47ΔBAC. The x-axis denotes GFP expression and the y-axis represents the cell death marker 7-AAD. (A) GFP expression following infection with G47ΔBAC. (B) 5 days later the cells are shifted to the right indicating cell death. GFP expression is reduced because only live cells can emit GFP.

3.3.3. Viral cytotoxicity on glioma stem cells

Next we examined the direct cytotoxic efficacy of the viruses using a dose response assay. The virus delivers its cytotoxic effect as part of its life cycle. To complete its replication cycle, herpes simplex viruses lyse the host cell and release further infective particles to restart this cycle. Having demonstrated that the wild type virus and its mutants FΔ6 and G47Δ are able to infect and replicate in glioma stem cells with subsequent flow cytometric

evidence indicating that infected cells are killed, dose response curves were constructed (Figure 3.14-3.15).

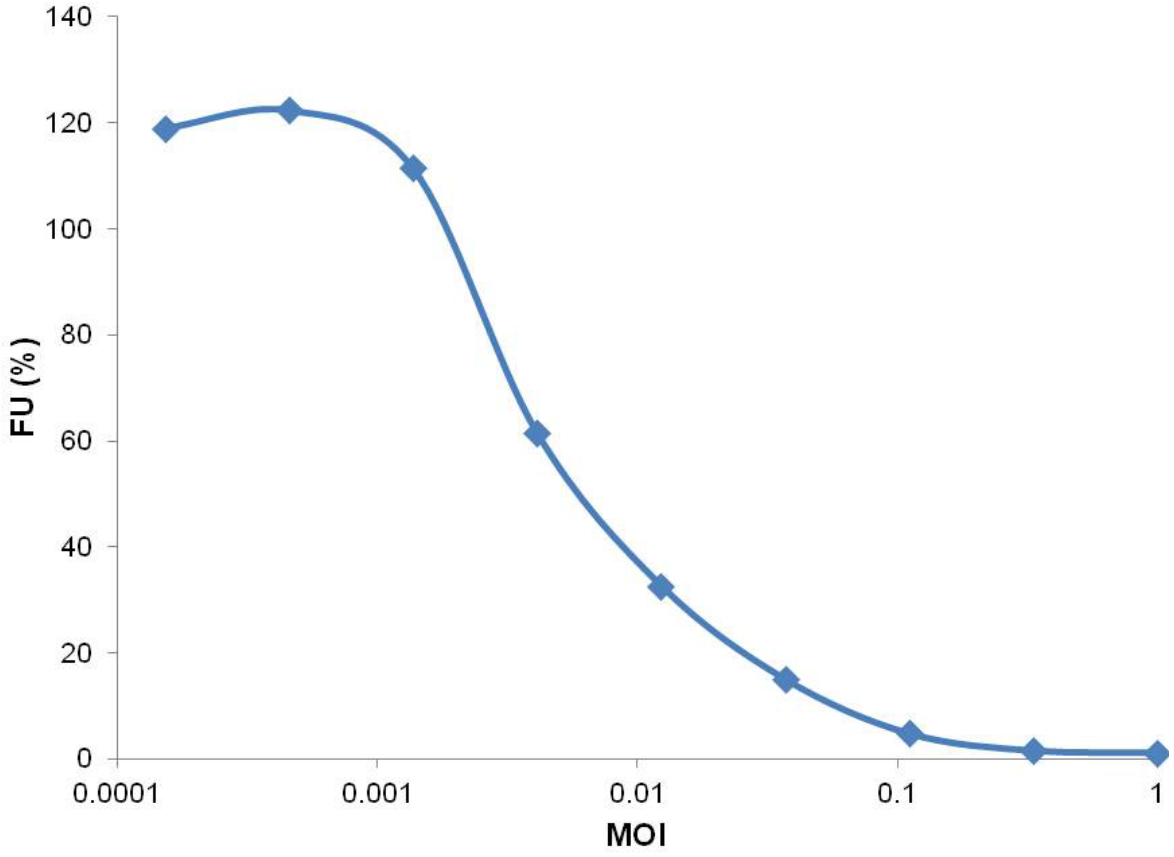

Figure 3.14 Dose response curve for killing of glioma stem cells by G47Δ (FU is the unaffected fraction and equivalent to survival). G47Δ efficiently killed glioma stem cells and the EC50 (Dose at which 50% of the cells are killed) value for G47Δ was a MOI of 0.0057.

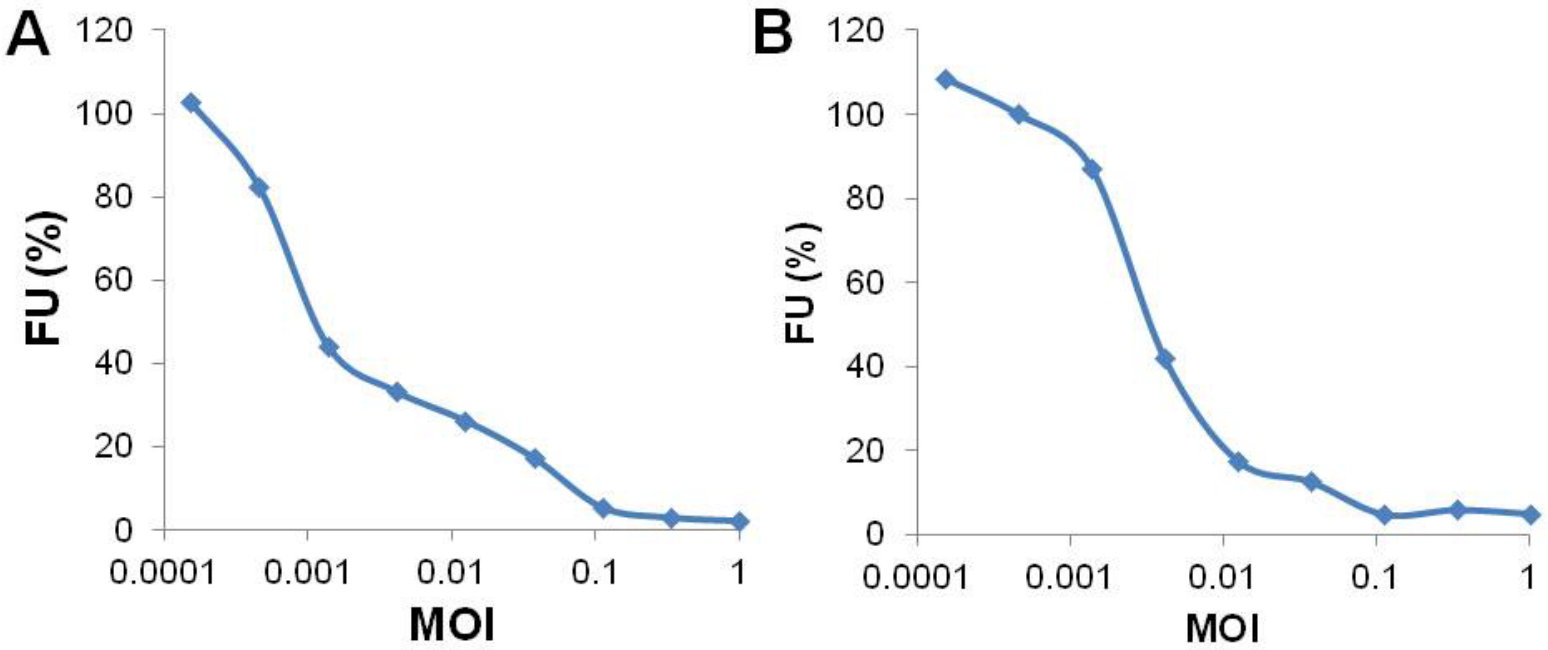

Figure 3.15 Dose response curves for killing of glioma stem cells by (A) wild type HSV-1 and (B) ICP6 mutant (FΔ6). The ICP6 mutant demonstrated similar killing efficiency to G47Δ.

As with the viral yield assays, the wild type virus was most aggressive at killing the glioma stem cells (Figure 3.15A) with an EC50 in a logarithmic range higher than G47Δ (Figure 3.14). The ICP6 mutant and G47Δ both demonstrated efficacy at killing glioma stem cells at a similar range of doses (Figure 3.15B and 3.14). The EC50 for G47Δ which is the dose required to kill 50% of the cells was a multiplicity of infection (MOI) of 0.0057. This relatively low dose of the virus, in cell culture models is able to significantly reduce the number of viable glioma stem cells.

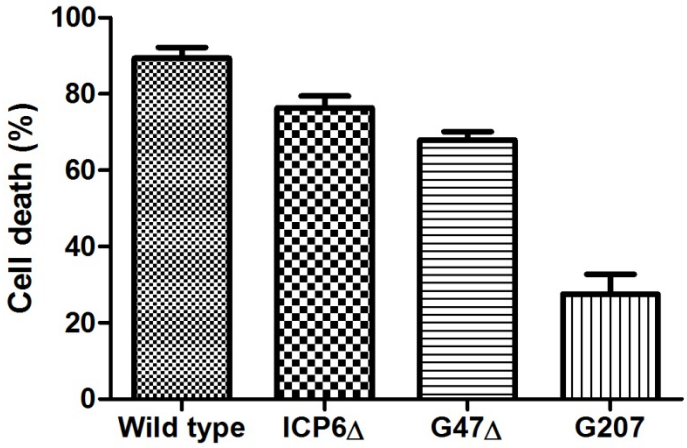

Figure 3.16 Direct comparison of the cytotoxic efficacy of wild type HSV-1 and its derived oncolytic vectors the ICP6 mutant, G47Δ and G207. G47Δ had a similar efficacy to the ICP6 mutant. The wild type strain demonstrated the highest killing with a mean of 89% cell death compared to 68% by G47Δ. G207 was the least effective vector with a mean cell death of 28% which was significantly lower than G47Δ ($p>0.05$, ANOVA with Tukey post-test).

Having established dose response curves, a direct comparison of the cytotoxic effects of oncolytic HSV against glioma stem cells was investigated. Figure 3.16 demonstrates the cell death of glioma stem cells following infection with the herpes viruses. Dissociated glioma stem cells were placed in 24-well plates in EF medium and infected with viruses the following day. A multiplicity of infection of 0.2 was used to allow the virus to demonstrate its ability to infect, replicate and kill by completing its life cycle and then infecting more cells. At seven days post infection, flow cytometric analysis was used to determine the percentage of dead cells in each group. As predicted by the dose response curves, wild type F-strain HSV-1 had the greatest cell killing (Figure 3.16). Its cytotoxic effect was significantly better than G47Δ ($p<0.05$, ANOVA with Tukey post-test). G47Δ had a similar cytotoxic effect as the ICP6 mutant and was markedly more effective than G207 ($p>0.05$ and $p<0.05$ respectively, ANOVA with Tukey post-test). Overall the cytotoxic effect of G207 was poor, in keeping with its weak replicative abilities in glioma stem cells. Having demonstrated effective infection, replication and killing by G47Δ in vitro, an in vivo study using the orthotopic xenograft model of glioma stem cells was performed.

3.4. Effect of G47Δ on an in vivo model of glioma stem cells

Dissociated human glioma stem cell spheres were implanted intracranially in the basal ganglia of athymic immunocompromised Ncr-*nu/nu* mice as described in Chapter 2. Seven days after implantation the tumours were injected with G47Δ or mock-treated. The mock-treated group underwent all the steps of viral treatment including the insertion of a needle into the tumour and injection of a similar volume of phosphate buffered saline. No deaths were seen as a result of tumour implantation or viral or mock treatment.

Figure 3.17 illustrates the Kaplan-Meier survival curves for the two groups of mice. The median survival for the mock-treated group was 66 days (range 63-74 days) compared to 84 days (range 64 to 102 days) for the G47Δ treated mice. Statistical comparison of the two survival curves using the Log-rank analysis demonstrated that the mice treated with G47Δ had a statistically significant survival advantage compared to mock treatment ($p<0.005$). Despite the enhanced survival with G47Δ, no mice were cured and all of the mice in this study died. Upon death their brains were harvested and all of the brains demonstrated increase in size of the gliomas. Therefore, treatment with G47Δ in this model appears to retard the growth of glioma stem cells but was not able to completely eradicate the tumours.

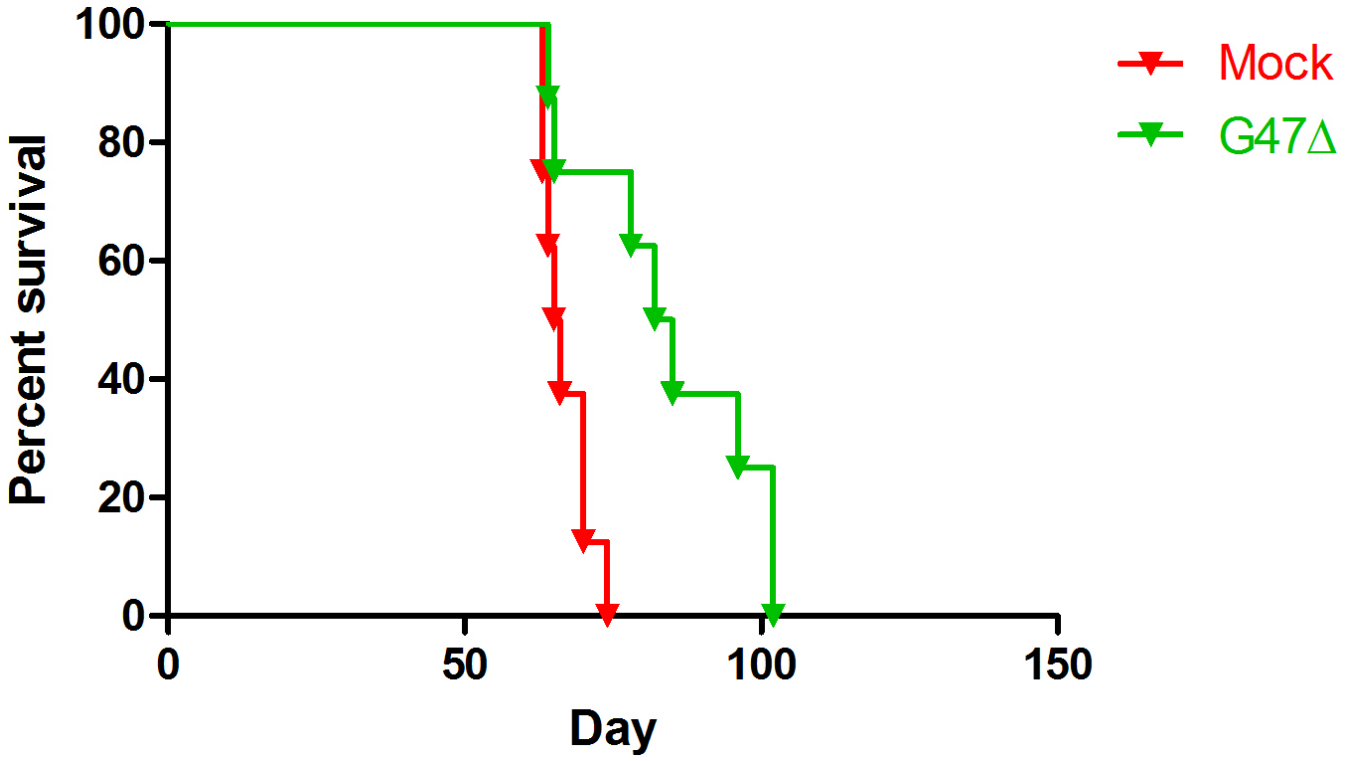

Figure 3.17 Survival following G47Δ treatment in vivo. Kaplan-Meier in vivo survival curves of orthotopic xenografts of glioma stem cells (GBM8) in nude mice (n=8 in each group), treated with either 2 x 10^6 pfu of G47Δ (green) or mock treated (red). G47Δ treated mice demonstrated a significant survival advantage (median survival 84 days compared to 66 days for mock treated mice, $p<0.005$ Log-rank).

3.5. Discussion

The isolation and stable culture of human glioma stem cells provides an excellent model for in vitro and in vivo testing of anti-glioma therapeutic agents as demonstrated here. A number of studies have described the problems with the traditional xenograft model of glioblastomas where cell lines that have been repeatedly passaged in serum culture are used[151, 152, 160]. These tumours do not recapitulate the invasive phenotype of glioblastomas, a feature seen in glioma stem cell models[152, 161]. Transcriptome analysis demonstrated that glioma stem cells were able to maintain the proneural expression signatures of parent tumours[151]. The mesenchymal drift of glioma cells grown in serum-containing media is well established[162, 163]. Interestingly studies of glioma stem cells grown in serum-free conditions but as adherent cultures also display this drift when compared to cells grown as spheres[151]. It is possible that a yet unexplained feature of three dimensional growth of the glioma stem cells (as described in Section 3.2.2) is required to preserve the expression of specific genes. One possible hypothesis is that a feature of anchorage-independent growth which is characteristic of transformed cells, is responsible for maintaining glioma stem cell gene signatures.

More recent work comparing the histological features of some of the glioma stem cell lines described in section 3.2, to their parent patient samples has shown that glioma stem cell xenografts maintain distinctive

cytological and histological features of their parent tumours including primitive neuroectodermal tumour, giant cell and gemistocytic characteristics[161]. The same study demonstrated that genetic mutations harboured by glioma stem cells were a result of mutations in the parent tumour rather than an artifact of in vitro culture. Furthermore, it has been shown that fluid attenuation inversion recovery (FLAIR) MR imaging characteristics of patient tumours correlate with the histological phenotype of glioma stem cell tumours[161].

Glioma stem cell models are not without their weaknesses. The number of in vitro passages that glioma stem cells can sustain is significantly shorter than for traditional glioma cell lines. However, this in vitro propagation may result in accumulation of genetic and phenotypic changes that cause the cells to no longer resemble their parent tumours. For this reason only low passage glioma stem cells were used in all the experiments. Furthermore, large cell numbers (50,000) were used in the xenograft model to form tumours, as this rapidly and predictably generates intracranial tumours. This method is in contrast to the formation of glioblastomas in humans, which like all cancers is postulated to originate from a single cell that has acquired multiple genetic mutations. While this single cell method would be difficult to recapitulate in a xenograft model, the GBM8 cell line is able to form tumours with only 50 cells[32]. Another inherent weakness of glioma stem cell xenografts in mouse models is the requirement for immunocompromised hosts to enable the tumour growth. This weakness of any xenograft model is one all cancer stem cell researchers must accept. The loss of immune

surveillance, and in nude mice the loss of interaction between the tumour and T-cells, is an important factor to consider when interpreting results from xenograft models.

These limitations notwithstanding, the overall evidence points to glioma stem cell models as a more accurate representation of human glioblastomas. This, and the ability to correlate clinical features of the disease in patients to the glioma stem cells, may ultimately lead to the ability to test personalized, novel therapeutics on patients' glioma stem cells and then deliver tailored treatments to glioblastoma patients.

The cell lines used comprise a combination of CD133 positive and negative cells as the cells were not sorted for CD133 positivity prior to experimentation. As discussed previously there exists some controversy as to the pre-eminence of CD133 as a stem cell marker. Moreover, the possibility of stem-like cells in the CD133 negative sub-population continues to be investigated and in this series the 50,000 unsorted cells consistently formed tumours in immunocompromised mice. As stated above, one cell line (GBM8) with greater than 90% CD133 positivity was able to consistently initiate tumours with just 50 cells implanted intracranially[32]. Overall these cell lines have consistently demonstrated tumorogenicity with as few as 50-500 cells[32]. Furthermore, BT74, another glioma stem cell line in this laboratory has been reported to recapitulate the endovascular proliferation and pseudopalisading necrosis seen in human glioblastomas[32]. In addition

to CD133 expression and in vivo tumour formation, a number of stem cell assays were employed to determine the stem-like quality of the cell lines used prior to employing them to test the efficacy of the oncolytic viruses. While the cell cultures used met all these criteria, the precise definition of what constitutes a glioma stem cell continues to evolve and this remains a field that is still in its infancy.

The glioma stem cells are a stable and reproducible model for testing the efficacy of oncolytic vectors derived from the herpes simplex virus. The multi-mutated virus G47Δ was able to successfully infect, replicate and kill glioma stem cells in culture. Moreover, cells surviving the viral infection lost their clonal abilities to reform spheres from single cell suspensions. In vivo G47Δ extended the survival of immunocompromised mice harbouring tumours formed by the glioma stem cells. The wild type strain F virus was used as a positive control in the in vitro experiments and aggressively infected, replicated and lysed the cells. The ICP6 mutant that lacked the large subunit of RR was able to successfully complete its life cycle indicating the presence of an adequate pool of dNTP substrates for the virus in glioma stem cells.

G207 with its deletion of the γ34.5 genes had markedly restricted replication and hence killing of glioma stem cells. The presumed mechanism for this lack of activity of the virus is the ability of the glioma stem cells to activate the RNA-dependent protein kinase pathway (PKR) and enable host

cell shutdown of protein synthesis in response to HSV infection. G47Δ also contains the γ34.5 deletion but the additional deletion of the α47 gene suppresses the attenuated replicative properties of γ34.5 mutants. The US11 gene, which is normally expressed late in infection and also inhibits the PKR pathway, is expressed earlier in G47Δ as described previously in Chapter 1. This is because as a result of the α47 deletion, the neighbouring US11 gene is under the control of the immediate early US12 promoter resulting in very early expression of US11 following infection. This early expression compensates for the loss of γ34.5 functions.

This deficiency of γ34.5 mutants in glioma stem cells has clinical implications as the majority of oncolytic herpes vectors trialed in humans have been γ34.5 mutants that do not possess the compensating α47 deletion[61, 62, 142, 164, 165]. This data underpins the need for more efficacious vectors like G47Δ to progress to human trials. Nonetheless, despite its increased cytotoxicity against glioma stem cells, G47Δ did not result in any cures in the in vivo trial. The following chapters will investigate two different strategies aimed at targeting this problem and enhancing the anti-glioma effect of G47Δ.

Chapter 4: Developing glioma stem cells - endothelial cells co-cultures and investigation of in vitro and in vivo effects of G47Δ-Tumstatin on glioma stem cells

4.1. Introduction

4.1.1. Antiangiogenic properties of tumstatin

Angiogenesis is a key aspect of tumour behaviour that contributes significantly to the proliferative ability of malignant cells[44]. The supportive perivascular niche of glioma stem cells is therefore an important therapeutic target, and antiangiogenic agents have already been shown to suppress glioma stem cells in murine xenografts[58]. Tumstatin is an endogenous antiangiogenic agent formed from the non-collagenous (NC1) domain of the α3 chain of type IV collagen (Col IV). Tumstatin inhibits tumour angiogenesis by limiting the proliferation capacity of endothelial cells and preventing blood vessel formation in vitro and in vivo[166-168]. Endogenous tumstatin is formed by the cleavage of Col IV in the vascular basement membrane by matrix metalloproteinase-9 (MMP-9). This cleavage is part of a complex regulation of angiogenesis that also sees the release of the potent pro-angiogenic factor VEGF and other anti-angiogenic agents such as endostatin and angiostatin.

Human tumstatin inhibits the proliferation of both human and mouse endothelial cells and induces apoptosis in proliferating endothelial cells[167-169]. It exerts its activity by binding to a family of heterodimeric cellular receptors known as integrins, particularly integrin αVβ3[170]. In addition, studies of the anti-tumour effects of tumstatin, independent of angiogenic pathways have also been reported[171-174]. These include work on glioma cell lines where expression of αVβ3 by tumour cells on the periphery of glioblastomas has also been reported[174, 175].

The last chapter reviewed the effect of G47Δ on glioma stem cells and noted in particular its ability to prolong survival in vivo but its failure to eradicate the tumours. To enhance the efficacy of G47Δ and improve its antiglioma effect, tumstatin was inserted as a transgene to enhance the effect of the virus, particularly on endothelial cells. The aim was to target the supportive perivascular niche of glioma stem cells that was discussed in section 1.2.3. The use of other anti-angiogenic transgenes in oncolytic herpes virus to enhance its anti-tumour effect has been successfully trialled before, but never in a glioma stem cell model[176-178]. Tumstatin was inserted into the virus using the Flip-Flop HSV BAC system[146, 147].

4.1.2. Bacterial Artificial Chromosome (BAC) system and insertion of transgenes

Historically, the creation of a recombinant herpes simplex vector required homologous recombination in mammalian cells with co-transfection of viral DNA and a plasmid containing the appropriate DNA sequence flanking homologous sequences. This process was protracted and required extensive screening of the products to determine if the appropriate recombination had occurred. Advances in BAC technology have markedly expedited the process by enabling the entire herpes virus genome to be cloned as a BAC plasmid and manipulated in *E. coli*[147, 179, 180].

Part of the BAC cloning process involves insertion of replication origin sequences and antibiotic resistance genes. The addition of these extra sequences can inhibit the replicative properties of the final virus produced and thus they are excised as part of the recombination process. In addition to BAC technology, site-specific recombination enzymes were used to precisely control the recombination process. Two distinct and independent site-specific recombination methods were used; digestion with Cre enzymes at loxP sites (Cre/loxP) and FLP at FRT sites (FLP/FRT). These two processes were performed sequentially. Figure 4.1 illustrates the steps involved; and Figure 4.2 shows a detailed view of the transgene.

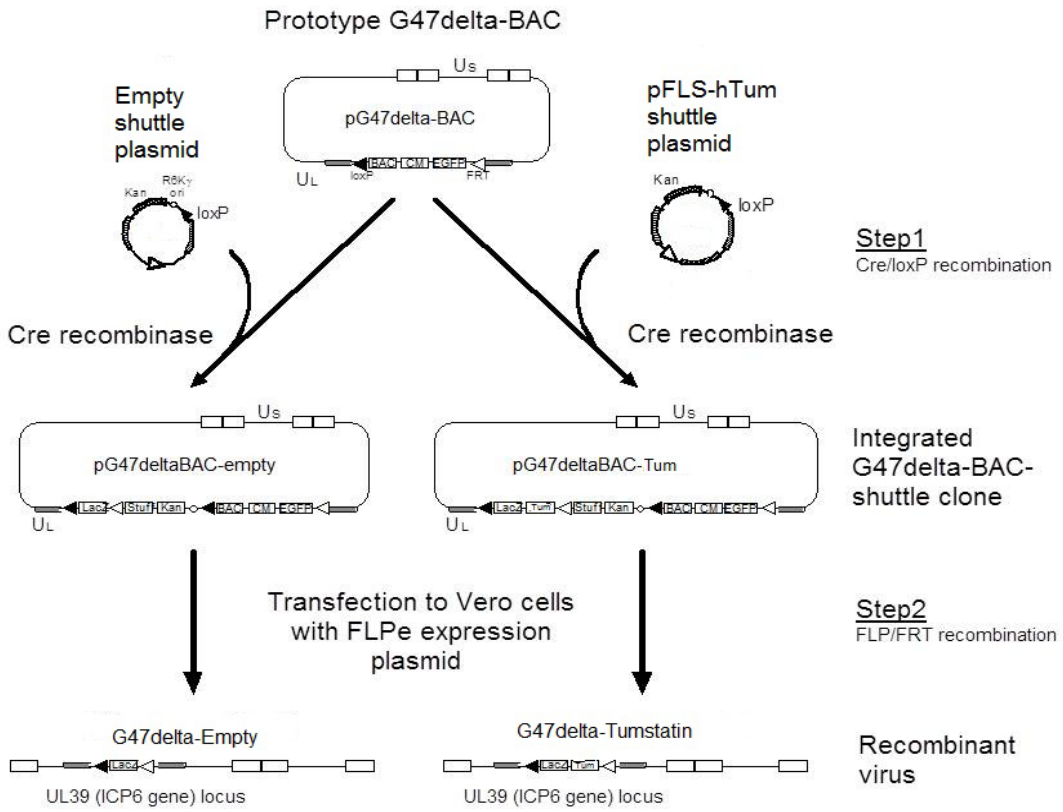

Figure 4.1 Schematic representation of the 2-step recombination processes involved in creating the transgene vector G47Δ-Tumstatin and the control vector G47Δ-Empty. The first step involves Cre recombination at loxP sites. The second step involves the removal of excess sequences using FLP/FRT recombination.

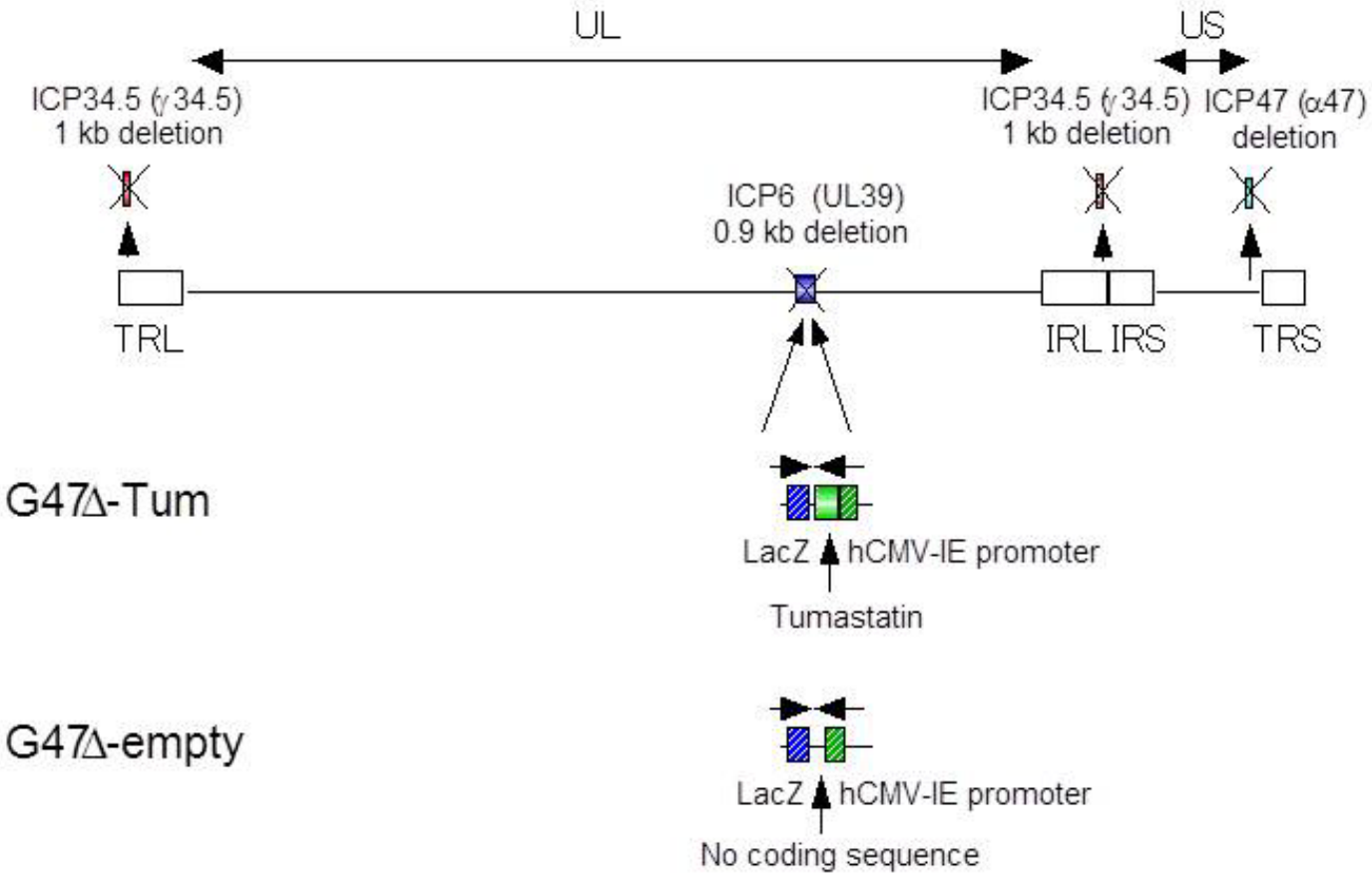

Figure 4.2 Schematic representation of G47Δ, G47Δ-Tumstatin and G47Δ-Empty. The transgene is inserted into the UL39 gene locus.

4.2. Creating G47Δ-Tumstatin and G47Δ-Empty

4.2.1. Constructing the virus

The first step in creating G47Δ-Tumstatin and the control vector G47Δ-Empty, was to construct a shuttle vector with the tumstatin sequence. The standard shuttle vector used was pFLS-Express #4 and the creation of the tumstatin containing shuttle vector is shown in Figure 4.3. The tumstatin DNA sequence was cut out of the plasmid using the restriction endonucleases NheI and SgrAI. Gel purification was used to separate the 818bp band. The pFLS-Express #4 plasmid was digested with the enzymes AvrII and XmaI and the resultant 10634bp product was gel purified. These specific restriction endonucleases were chosen because they produce compatible 5' overhangs with each other, enabling the products to be ligated to form pFLS-hTum (Figure 4.3).

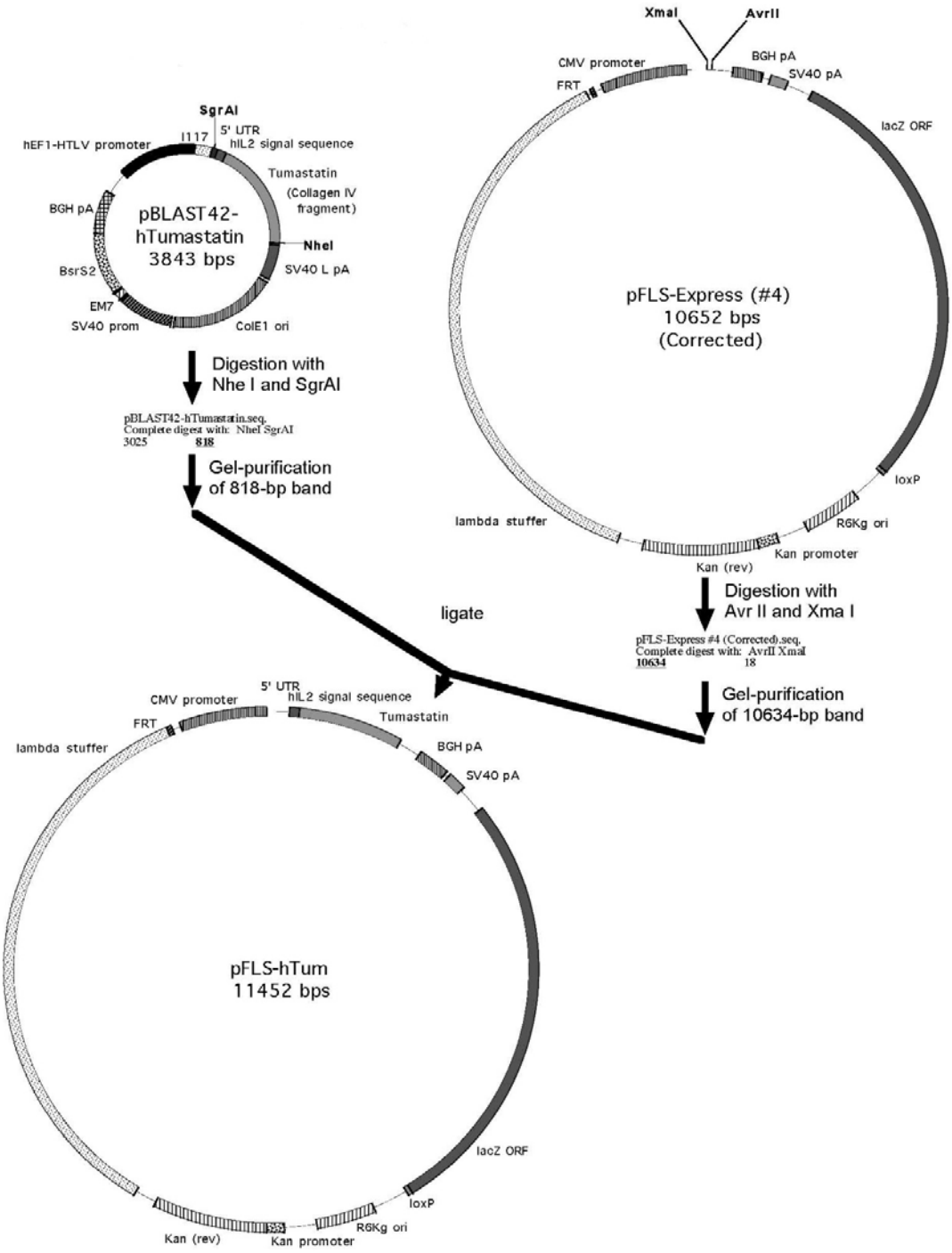

Figure 4.3 Subcloning the human Tumstatin gene (hTum) into the pFLS-Express (#4) shuttle vector using restriction endonucleses with compatible overhangs.

The next step in the creation of G47Δ expressing tumstatin was the in vitro Cre recombination of pFLS-hTum and pG47ΔBAC. The control empty plasmid was subjected to a similar parallel process to create the control vector G47Δempty. The tumstatin and empty vectors have a kanamycin resistance gene and pG47ΔBAC has a chloramphenicol resistance gene. The integrated clone was isolated in *E.coli* using kanamycin and chloramphenicol as selection pressures. The integrated G47ΔBAC-shuttle cone has a genome length of greater than 158kb including the resistance genes and the EGFP gene. Figure 4.4A demonstrates the continued presence of EGFP at this stage. These additional DNA sequences make the virus inefficient when replicating. The subsequent step was FLP recombination in Vero cells. This removes the stuffer sequence, the antibiotic resistance sequences and the EGFP sequence. Figure 4.4B demonstrates the HindIII restriction enzyme digestion of the final product in comparison to G47ΔBAC demonstrating the additional DNA bands in G47Δ-Tumstatin.

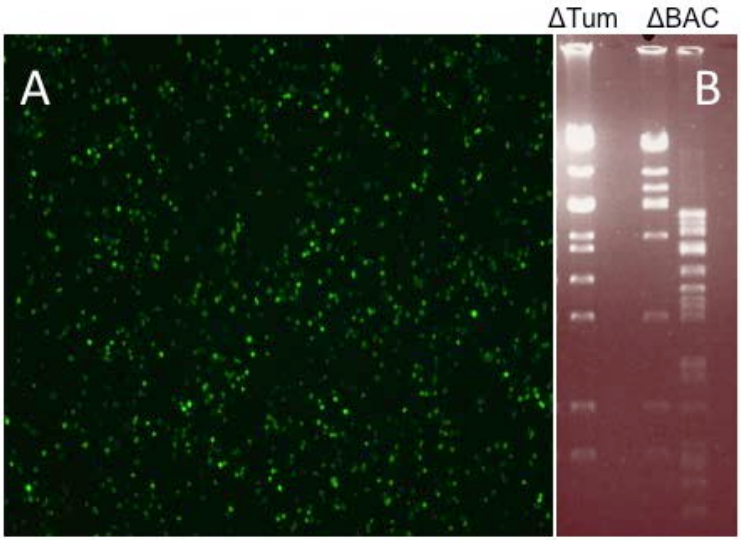

Figure 4.4. (A)EGFP expression of viral clones following Cre recombination and (B)HindIII digestion of G47ΔBAC and G47ΔTumstatin clones demonstrating a change in the restriction pattern. The lane on the far left is a DNA ladder.

4.2.2. Validation of the transgene vector constructs

The final construct for the two vectors is shown in Figure 4.5. The vectors were checked for the presence of EGFP by infecting Vero cells and both were negative. The virus was tested for LacZ staining and both vectors were positive as demonstrated in Figure 4.6. Control cells were not infected with virus and G47Δ-IL12 was used as a positive control.

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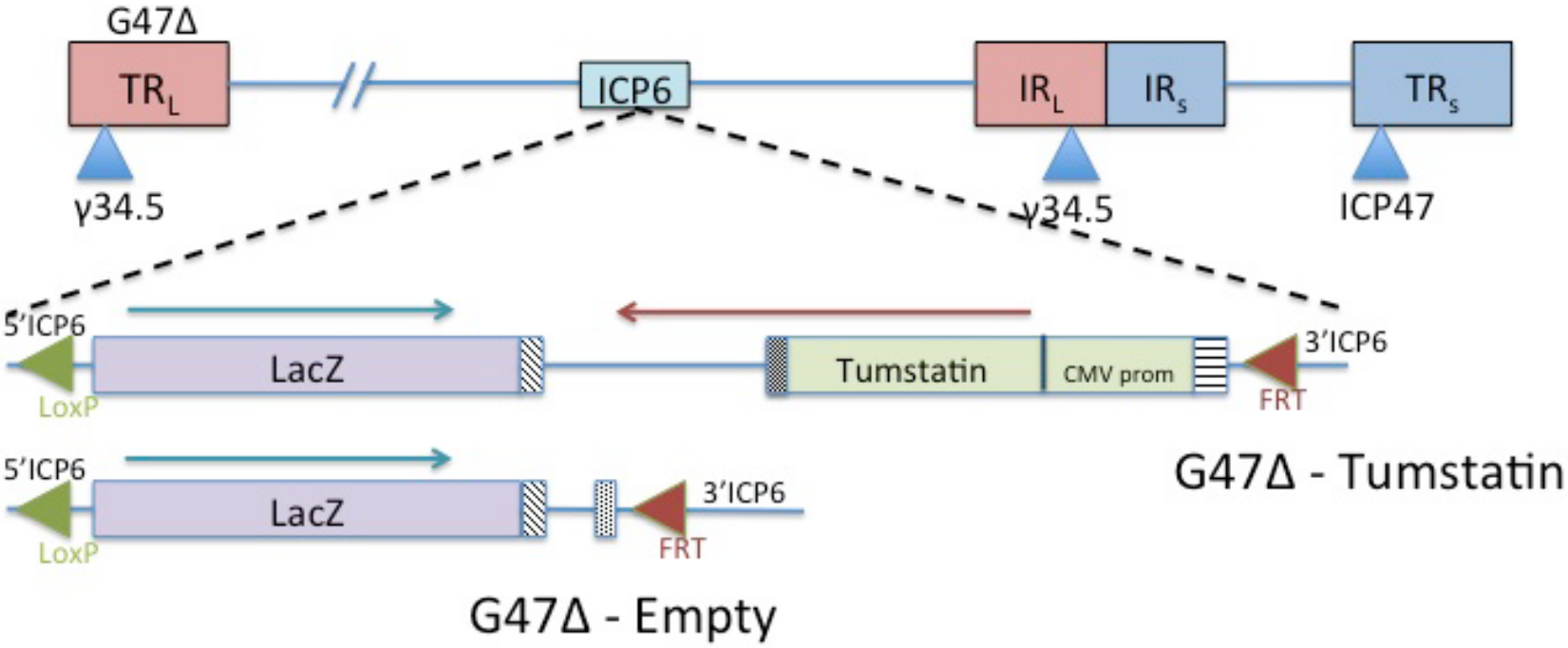

Figure 4.5 Schematic representation of the final construct of G47Δ-Tumstatin and G47Δ-Empty. The tumstatin transgene is located in the UL39 (ICP6) gene locus and driven by a cytomegalovirus promoter (CMV). The LacZ reporter gene is present in both vectors. One loxP site and one FRT site remain at the end of the recombination process.

Figure 4.6 not only demonstrates the expression of LacZ but also the plaque forming capabilities of the virus. Next the expression of tumstatin in infected cells was tested using western blotting. This was done in Vero cells, glioma stem cells and endothelial cells. The Figure 4.7 demonstrates the results in glioma stem cells, the key cell of interest for tumstatin expression

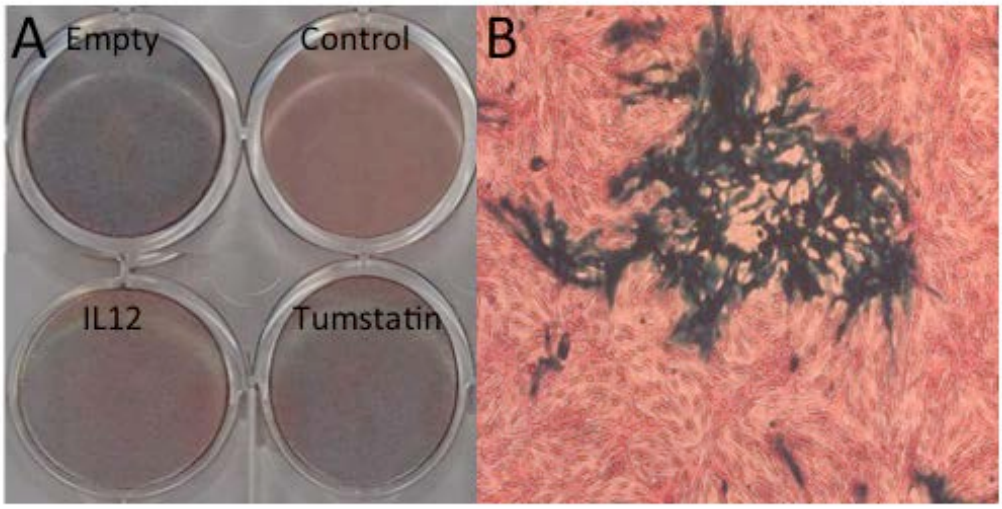

Figure 4.6 LacZ expression (blue) of G47Δ clones. (A) LacZ expression of the final G47Δ clones – Empty, IL12 and Tumstatin. (B) LacZ expression and plaque formation by G47Δ-Tumstatin in Vero cells at 10x microscopy.

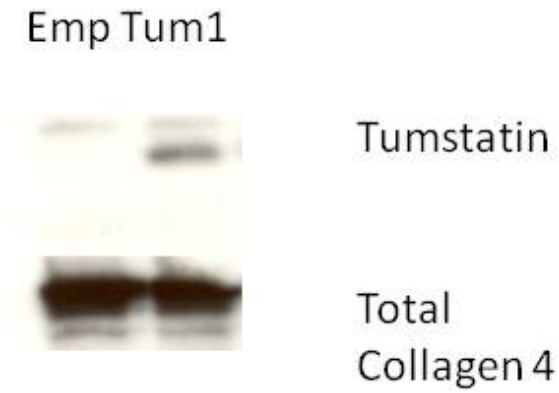

Figure 4.7 Western blot of tumstatin expression by G47Δ-Tumstatin in glioma stem cells (GBM8). Infection with the empty vector was used as a negative control.

Having established the expression of the transgene, LacZ expression, the absence of EGFP and plaque forming capabilities of the vectors, the replicative abilities of the vectors were then compared to G47Δ. Both glioma stem cells and endothelial cells were infected in separate experiments using G47Δ-Tumstatin, G47Δ-Empty and G47Δ as a control. Figure 4.8 demonstrates the viral yield at 24 hours and 48 hours. The growth curves for the 3 viruses are similar suggesting no ill effect from the addition of the transgene. Figures 4.8 C and D demonstrate the viral yields at 48 hours in both glioma stem cells and endothelial cells. No statistically significant differences in the yield of the viruses at 48 hours were seen in either cell line (ANOVA with Tukey post-test, $p>0.05$). In addition to excluding that the generated viruses had not developed any replication restrictive mutations during their creation, this experiment also showed that tumstatin did not have an unknown suppressive or enhancing effect on viral replication.

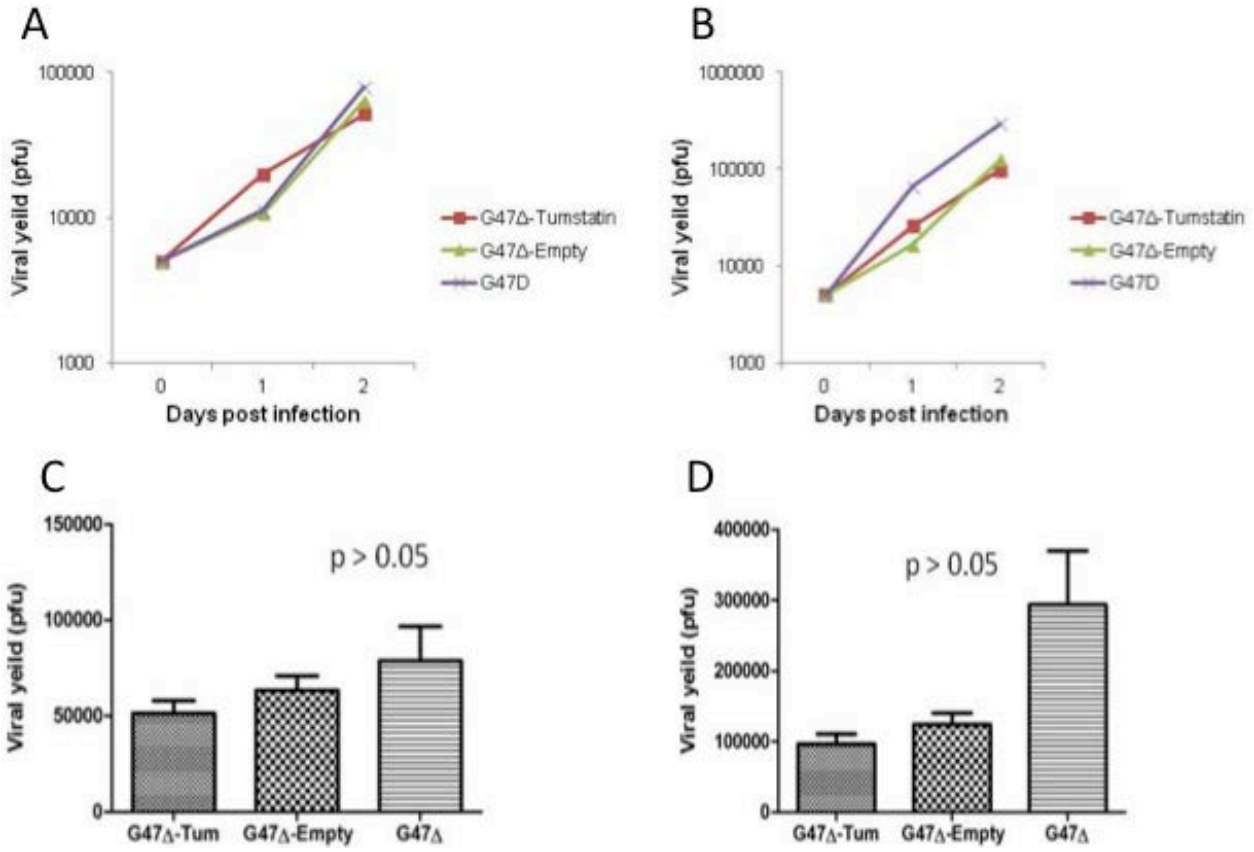

Figure 4.8 (A,B) Viral yield assays in glioma stem cells and endothelial cells respectively. (C,D) Comparison of viral yields from A and B respectively, at the 48 hour time points. No difference was seen between the vectors ($p>0.05$ ANOVA with Tukey post-test).

4.3. In vitro efficacy of viral vectors

4.3.1. Viral cytotoxicity on endothelial cells

The cytotoxicity of the two viruses towards endothelial cells was quantified. A multiplicity of infection of 0.2 (MOI 0.2) was used and cells incubated with the virus for 3 days. Figure 4.9 demonstrates that both viruses are able to effectively kill endothelial cells with an advantage for G47Δ-Tumstatin (Student's t-test, $p<0.03$). The mean survival for G47Δ-Tumstatin was 18% compared to 32% for G47Δ-Empty.

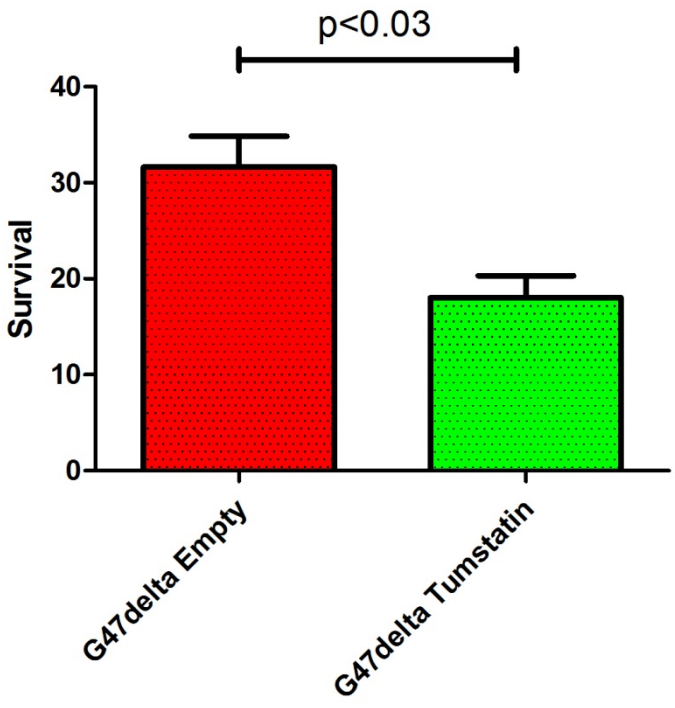

Figure 4.9 Direct comparison of the cytotoxic effect of G47Δ-Empty and G47Δ-Tumstatin using the MTS assay. G47Δ-Tumstatin showed enhanced cytotoxic efficacy (18% vs 32%, $p<0.03$, Student's t-test).

4.3.2. Apoptotic effect of G47Δ-Tumstatin

Following the cytotoxicity assay, the apoptotic function of G47Δ-Tumstatin was measured using flow cytometric analysis. G47Δ-Empty was used as a control virus as the relationship between herpes simplex virus infection and subsequent cellular regulation of apoptosis is complex and oncolytic viruses are known to induce apoptosis[181]. Both vectors induced apoptosis with G47Δ-Tumstatin performing significantly better (39% apoptosis vs 22%, Student's t-test, p=0.04). Cells were infected at a low multiplicity of infection (0.2 MOI) because of the sensitivity of endothelial cells to oncolytic HSV. Cells were harvested at 24 hours to reduce any confounding effect of viral replication.

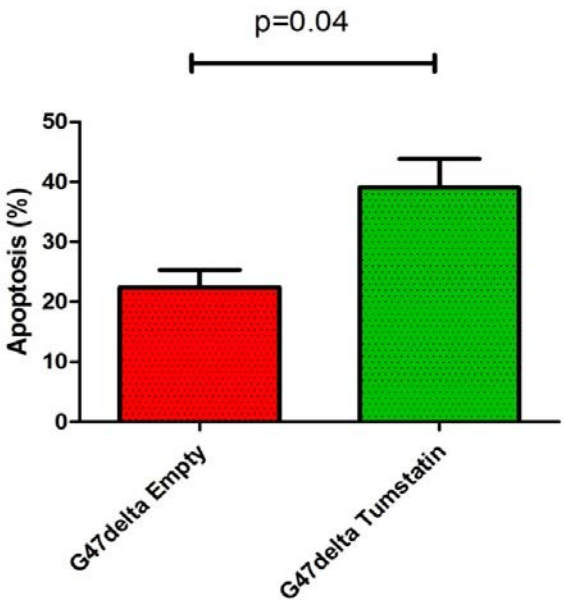

Figure 4.10 Apoptosis assay by flow cytometry (Annexin-V) comparing G47Δ-Tumstatin and G47Δ-Empty. 17% greater apoptosis was noted in the G47Δ-Tumstatin group (p=0.04, Student's t-test).

4.3.3. Effect of G47Δ-Tumstatin on endothelial cell migration and branching

Next, an endothelial cell migration assay was carried out. The percentage of migrated cells was normalized to the number of successfully migrated control cells. Again G47Δ-Tumstatin significantly outperformed the control vector (Figure 4.11, Student's t-test, $p<0.03$).

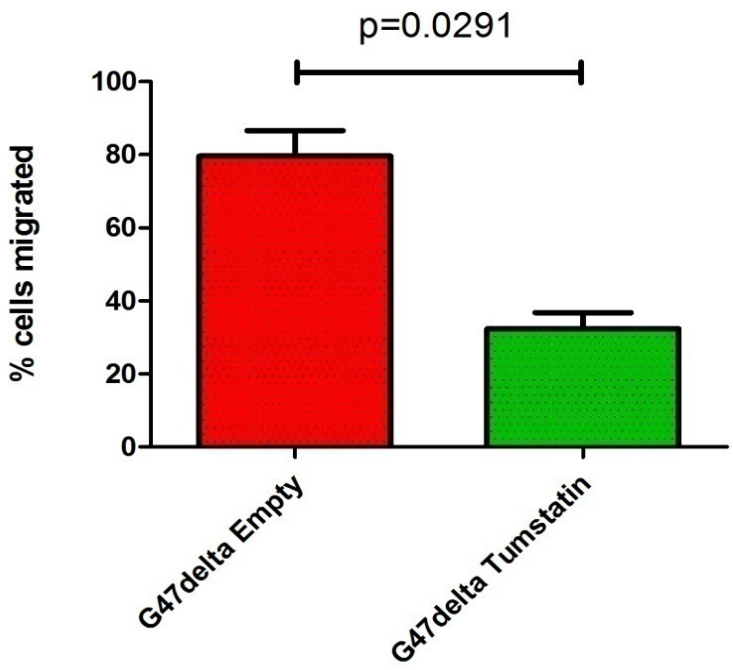

Figure 4.11 Cell migration assay following G47Δ-Tumstatin infection. Reduced migration was observed following infection with G47Δ-Tumstatin compared to empty vector ($p<0.03$, Student's t-test).

Subsequently, an endothelial cell branching assay on matrigel was performed. Endothelial cells plated on matrigel form organised branches with multiple cross links as shown in Figure 4.12a. Figure 4.12b demonstrates the effect of G47Δ-Tumstatin on the endothelial cell branches, with destruction of the branching pattern and a change in the morphology of the cells. The two vectors were compared at low multiplicities of infection (MOI 0.2) for 24 hours to allow for effects of the transgene to manifest but before viral replication destroyed the branches. Again G47Δ-Tumstatin performed significantly better than the empty vector (Figure 4.13, $p<0.02$).

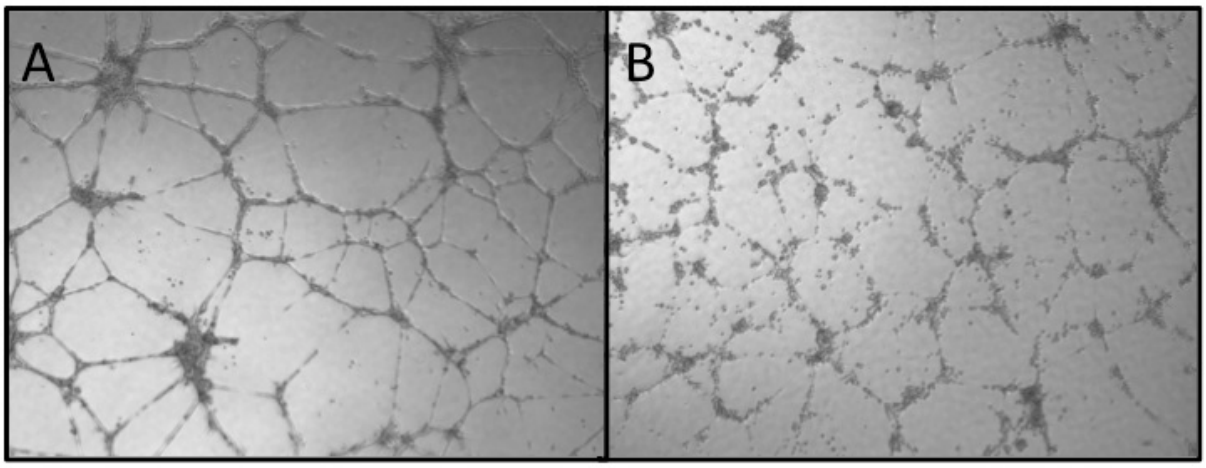

Figure 4.12 Endothelial cell branching assay on matrigel. (A)Normal branching pattern of human umbilical vein endothelial cells (HUVECs) on matrigel. (B)Destruction of the branching pattern by G47Δ-Tumstatin.

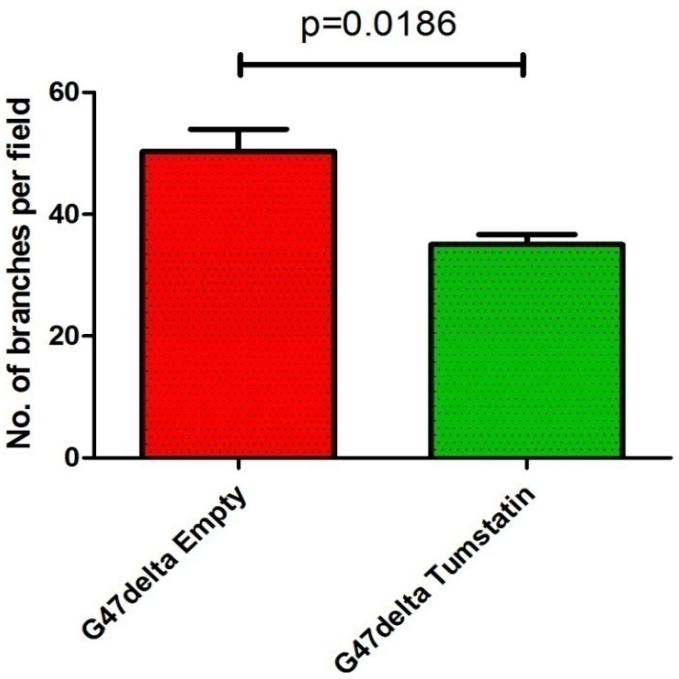

Figure 4.13 Quantitative assessment of the endothelial cell branching assay on matrigel. G47Δ-Tumstatin was more effective at destroying the endothelial cell branches ($p<0.02$, Student's t-test).

4.3.4. Expression of β3 integrin and PI3K pathway components in glioma stem cells

The effects of tumstatin, be they on endothelial cells or tumours cells are mediated via integrin αVβ3. The first step was to determine the presence of β3 integrins on the glioma stem cells. Their presence on endothelial cells is well established and more recently their presence on a number of glioma cell lines including U87 was shown[174]. Their presence on glioma stem cell lines has not previously been studied. Flow cytometric analysis for αVβ3 on glioma stem cell lines, endothelial cells and U87 was undertaken (Figure

4.14). As expected, U87 and endothelial cells demonstrated positivity for αVβ3. One of the two glioma stem cell lines tested also demonstrated the presence of αVβ3. This is the first report of a glioma stem cell line demonstrating the integrin αVβ3.

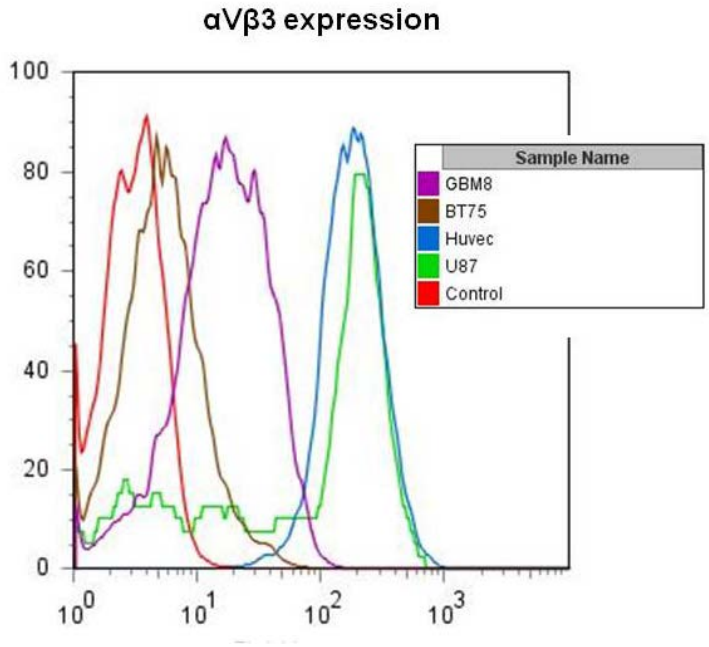

Figure 4.14 Flow cytometric analysis of the surface integrin αVβ3 in endothelial cells, glioma stem cells and a glioma cell line. GBM8 and BT75 are glioma stem cell lines. HUVEC – Human umbilical vein endothelial cells. U87 – serum cultured glioma cell line. Control – Isotype control.

Following its binding to αVβ3, tumstatin acts via the PI3K/PTEN/AKT/mTOR pathway, detailed in section 4.6. Factors that can prevent the successful activation of apoptosis by tumstatin include the lack of expression of αVβ3 integrins on the cell, PTEN deficiency and the constitutive activation of the Akt/mTOR pathway. To determine if glioma cell

lines would be susceptible to tumstatin the above were tested. Endothelial cells, which are susceptible to tumstatin, demonstrated the expected profile of expressing the integrin and PTEN without high levels of pAKT. None of the glioma cell lines nor U87 expressed this profile, suggesting resistance to tumstatin (Figure 4.15).

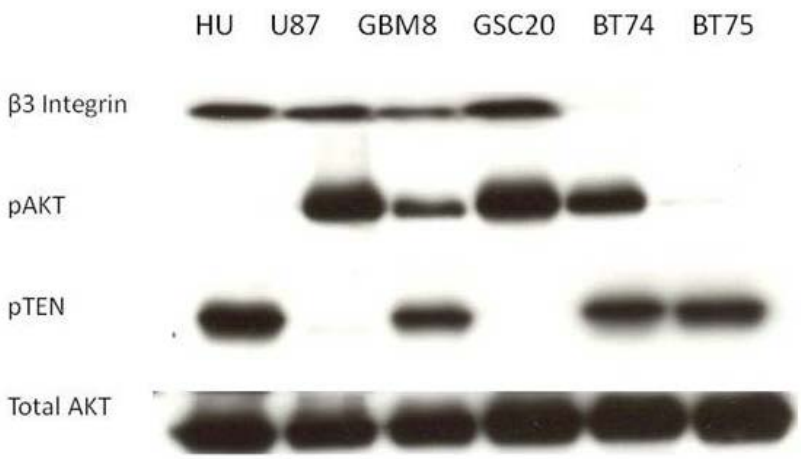

Figure 4.15 Western blot analysis for the β3 integrin, pAKT (Ser473), PTEN and total AKT. HU – HUVECs, U87 – serum cultured glioma cell line, GBM8, GSC20, BT74, BT75 – glioma stem cell lines.

To confirm the hypothesis that tumstatin would have no effect on the glioma stem cell lines, the cell lines were treated with the tumstatin protein. As a positive control endothelial cells were also treated. As expected, tumstatin significantly reduced the survival of endothelial cells but had no effect on the survival of glioma stem cells (Figure 4.16).

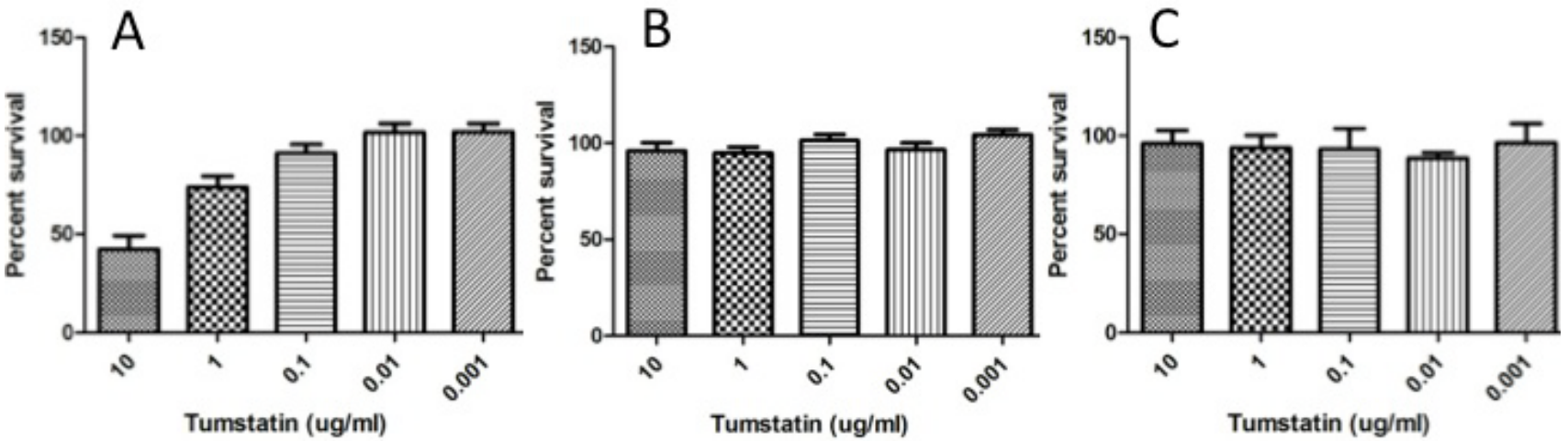

Figure 4.16 Cytotoxic effect of the tumstatin protein on (A) endothelial cells and 2 glioma stem cell lines (B) BT75 and (C) GBM8. Survival was measured using the MTS assay.

4.4. Co-culture experiments with glioma stem cells and endothelial cells

4.4.1. Establishing the transwell co-culture model

To better understand the relationships between glioma stem cells and endothelial cells, in vitro experiments were designed to try and simulate the in vivo interaction between glioma stem cells and endothelial cells. The first experiment involved the development of a transwell co-culture system. Figure 4.17 demonstrates the co-culture system with a porous membrane separating the glioma stem cells above and the endothelial cells below. Although there is no direct cell-to-cell contact, secreted factors can be transported across the membrane. Once the culture system was in place, the effect of co-culture on the glioma stem cells was tested. As a control, glioma stem cells were co-cultured with fibroblasts.

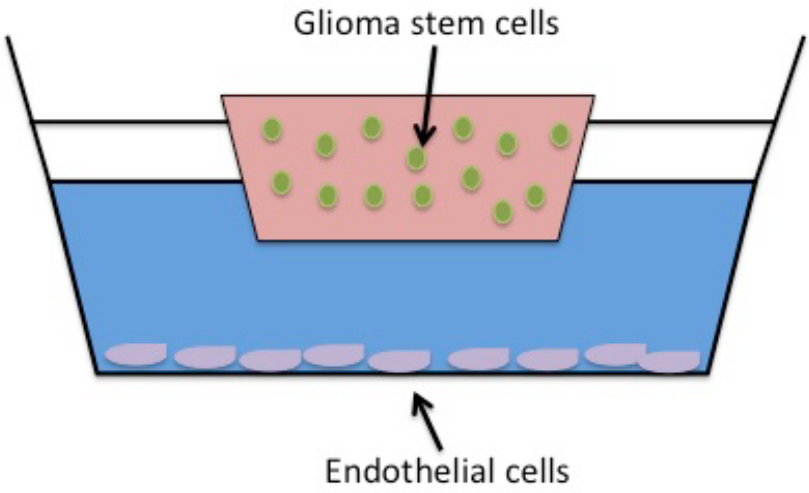

Figure 4.17 A schematic of the transwell co-culture system where endothelial cells are co-cultured with glioma stem cells. A porous membrane separates the two components and secreted factors flow freely between them.

Co-cultured glioma stem cells spheres were dissociated and cell death measured using flow cytometry analysis. Figure 4.18 demonstrates the improved survival of glioma stem cells co-cultured with endothelial cells compared to control fibroblasts (Student's t-test, $p<0.02$). The dissociated spheres were also plated as single cell solutions into 96-well plates and a clonal assay performed to assess the number of cells that were able to form secondary spheres. Again co-cultured glioma stem cells performed better (22% vs 13%, Student's t-test, $p<0.03$)

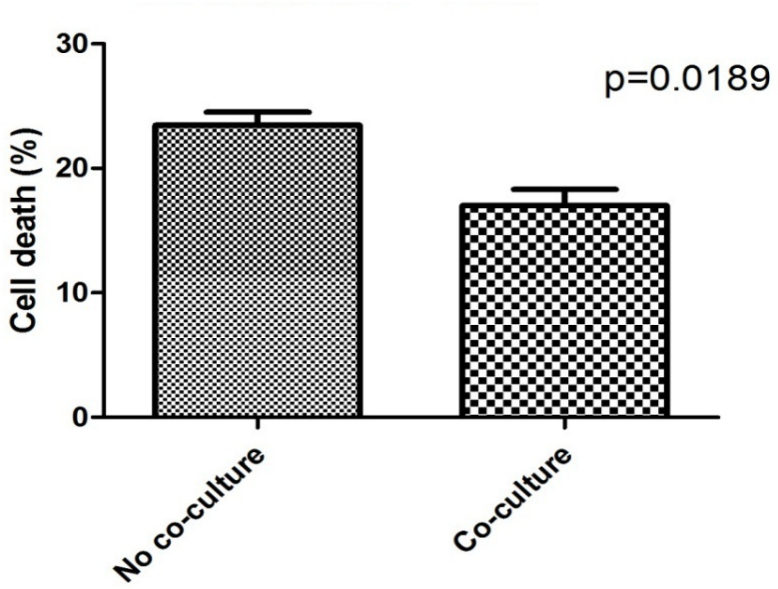

Figure 4.18 Comparison of differences in the rate of cell death in co-cultured and control glioma stem cells. Glioma stem cells co-cultured with endothelial cells demonstrated reduced cell death (Student's t-test, $p<0.02$)

The dissociated spheres were also tested for CD133 levels after co-culture to determine if the levels of this glioma stem cell marker would increase in response to simulation of the glioma stem cell perivascular niche. Co-culture with endothelial cells did not significantly affect CD133 expression by glioma stem cells. Data from a low CD133 expressing cell line is shown in Figure 4.19. A low CD133 expressing cell line was chosen to enable detection of even small changes in CD133 expression.

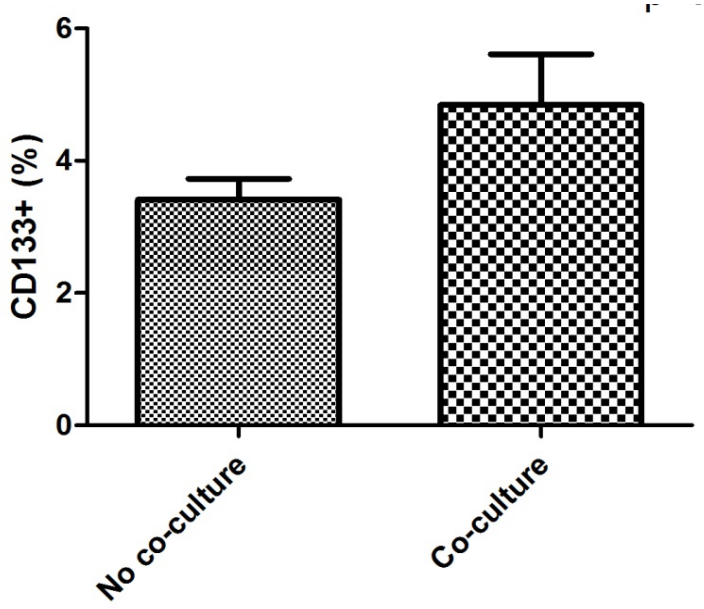

Figure 4.19 Comparison of CD133 expression in glioma stem cells following co-culture or control conditions. No significant difference was seen ($p>0.05$, Student's t-test).

4.4.2. Viral efficacy in transwell co-culture

The ability of co-culture to affect the infectivity of G47Δ was tested using G47ΔBAC expressing EGFP and again no difference was seen between the groups (Figure 4.20, Student's t-test, $p>0.05$). The mechanisms for cellular suppression of herpes viruses including the RNA-dependent protein kinase pathway discussed in Chapters 1 and 3 would not be expected to change in co-culture. Although the possible increased proliferation from co-culture with endothelial cells that has been reported previously[47] could affect replication, this was not seen here. Viral titres were also not changed by co-culture (Student's t-test, $p>0.05$).

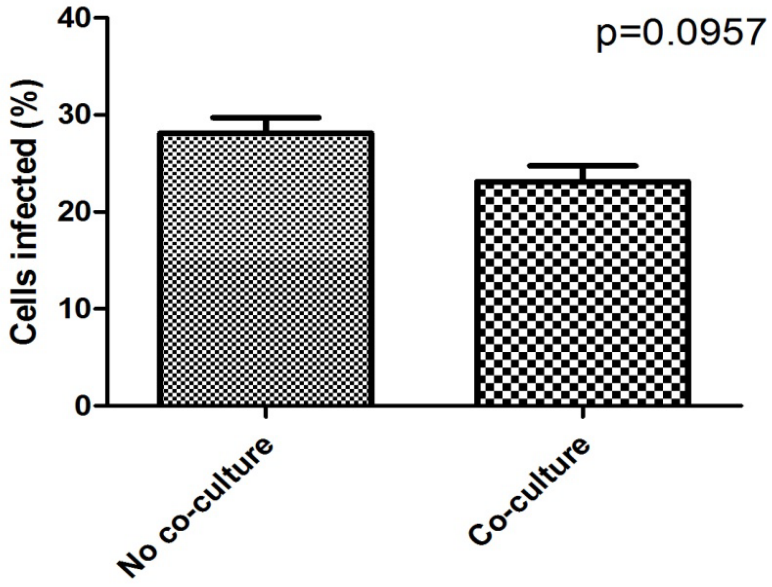

Figure 4.20 Ability of the EGFP expressing G47ΔBAC to infect cell under co-culture or control conditions. No difference in infectivity was seen ($p>0.05$, Student's t-test).

The transwell co-culture system was also used to test the effects of infection of glioma stem cells by the two vectors, on endothelial cells. Glioma stem cells were infected at MOI 0.5 with either G47Δ-Tumstatin or G47Δ-Empty for 24 hours. The cells were then washed and co-cultured with endothelial cells in fresh media. After a further 24 hours endothelial cells were harvested and the migration assay performed as a means of assessing endothelial cell function. 44% of endothelial cells in the G47Δ-Empty group migrated compared to 14% in the G47Δ-Tumstatin group (Figure 4.21, $p=0.001$) demonstrating that tumstatin is effective at disrupting endothelial cell function even when expressed by infected glioma stem cells. The addition of tumstatin offers a significant advantage in the disruption of

endothelial cells - glioma stem cells interaction compared to infection by the control vector.

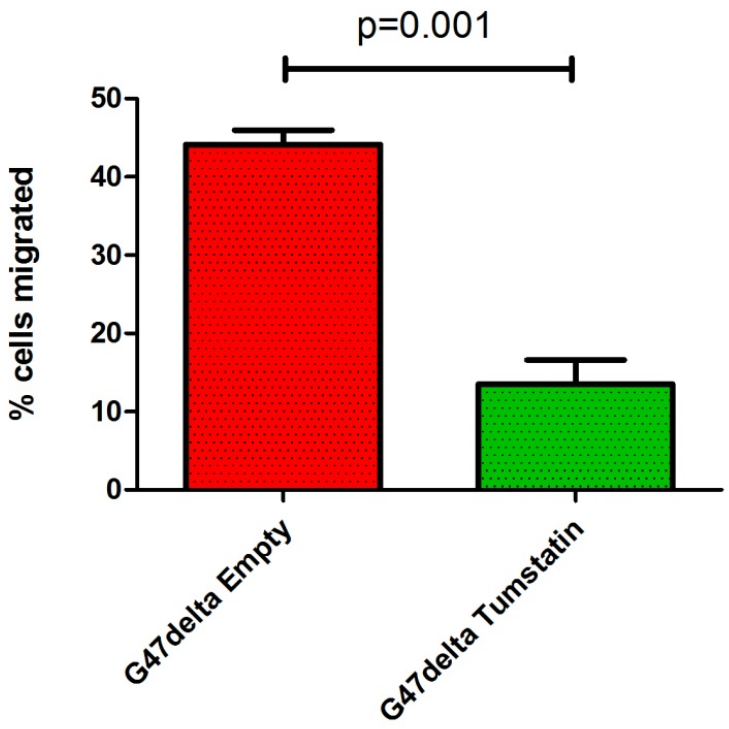

Figure 4.21 Comparison of the efficacy of the two vectors against endothelial cells in co-culture conditions. Infected glioma stem cells (MOI 0.5) were co-cultured with endothelial cells in the transwell system. After 24 hours in co-culture, endothelial cells were harvested and a migration assay performed. G47Δ-Tumstatin treated cells demonstrated inferior migration capabilities compared to the empty vector (p=0.001, Student's t-test).

4.4.3. VEGF production by glioma stem cells

Previous reports have demonstrated that glioma stem cells secrete vascular endothelial growth factor (VEGF), a very potent stimulator of endothelial cell growth and function[30, 182]. Furthermore, under hypoxic conditions the secretion of VEGF under the control of hypoxia inducible factor 2α is known to rise[182]. The secretion of VEGF by glioma stem cells grown in the laboratory was measured using a commercially available ELISA detection kit. Figure 4.22 demonstrates the level of VEGF secretion at day 2 and day 9 under normoxia and at day 2 at hypoxic conditions. As previously noted, glioma stem cells actively secrete VEGF and dramatically increase the level of secretion in hypoxic conditions.

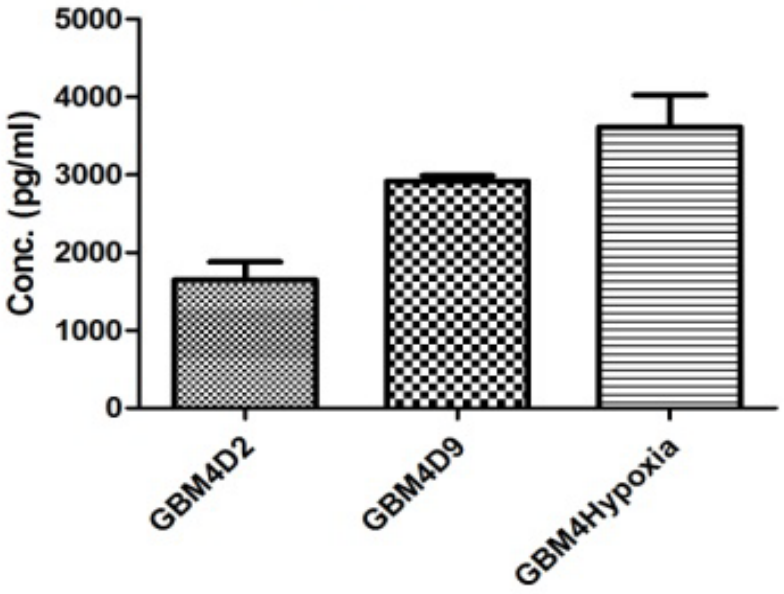

Figure 4.22. VEGF secretion by glioma stem cells and the effect of hypoxia on VEGF secretion. VEGF secretion by a glioma stem cell line at day 2 and day 9 under normoxia and at day 2 under hypoxic conditions is shown. VEGF levels in the media rise with time and in hypoxic conditions.

Having established that glioma stem cells secrete VEGF, a functional assay was used to test the effect of the secreted VEGF on endothelial cells. Endothelial cells were plated on Matrigel in 12-well plates. As expected the endothelial cells formed branching tubes on the matrigel surface. Once the branches formed, the endothelial cell media was removed and replaced with conditioned media (media harvested from day 2 glioma stem cell cultures) or control media. Figure 4.23 demonstrates that conditioned media was able to sustain the endothelial branches for a further 48 hours but control media was not. The addition of Bevacizumab, a monoclonal antibody to VEGF was able

to abrogate the effect of conditioned media (Figure 4.23D), further evidence for the role of VEGF in the conditioned media.

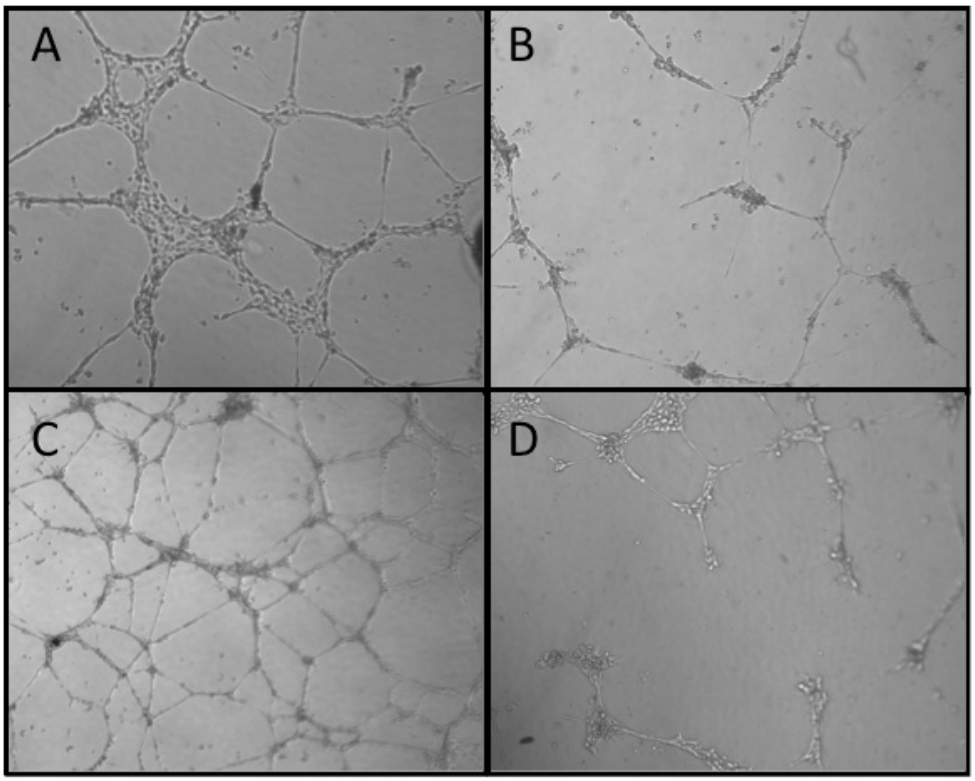

Figure 4.23 Endothelial cell branching assay on matrigel (A-D). Media conditioned by glioma stem cells (A) contains VEGF and prolongs survival of endothelial cell branches on matrigel compared to control media (B). Bevacizumab (D) effectively destroys the endothelial branching pattern despite the presence of VEGF in the conditioned media compared to control wells containing glioma stem cell conditioned media(C).

A similar effect was seen when glioma stem cell spheres were added to the matrigel culture of endothelial cells. The stem cell spheres also

appeared to attach to the endothelial cell branches rather than to the matrigel itself although no quantitative analysis of the phenomenon was be performed (Figure 4.24). Interestingly, the addition of bevacizumab to the mixed glioma stem cells and endothelial cells co-cultures on matrigel was not as effective at destroying the branches as it was with conditioned media. Even at doses that reduced VEGF levels to below 20pg/ml, co-culture preserved the branching pattern. There are a number of possible mechanisms for the resistance to Bevacizumab seen in this co-culture system, one being that other secreted factors are in play in addition to VEGF. This is corroborated by the fact that previous studies have identified other endothelial cell pro-survival factors[51, 52].

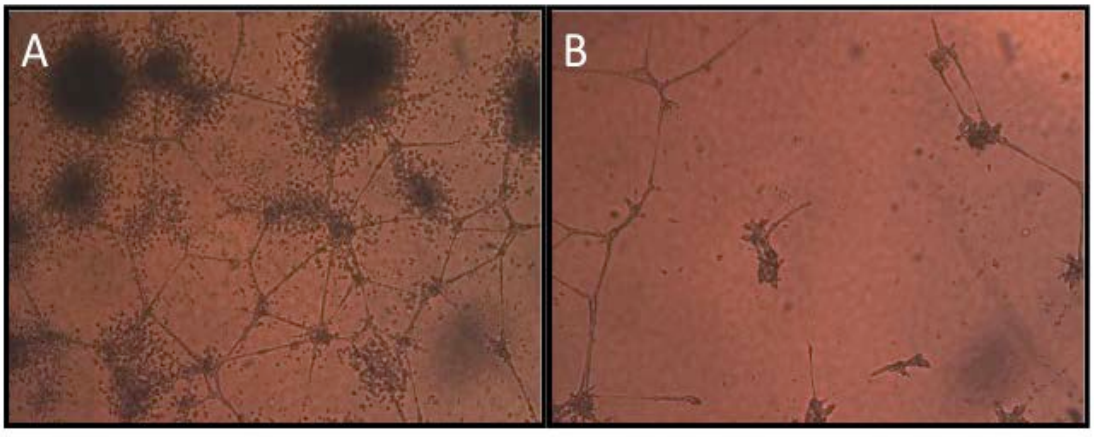

Figure 4.24 Glioma stem cell spheres attach to endothelial cell branches and prolong the survival of the branching pattern beyond 48 hours (A) compared to control wells with no glioma stem cells(B).

The experiment was repeated using the transwell system described above and this time the addition of bevacizumab easily overcame the co-culture with glioma stem cells and killed the endothelial cells. The remaining possibilities include a pro-survival mechanism requiring cell-to-cell contact and a ligand such as the Notch signalling pathway. The survival mechanism responsible for the survival of treated endothelial cells in contact co-culture was not investigated further as the focus of the study was to develop a model to test oncolytic viruses. However, more recent work by other laboratories has raised the possibility that CD133 positive glioma stem cells may be able to transdifferentiate into endothelial cells. This results in the endothelial cells inheriting mutations from their parent cells which may cause them to be more resistant to antiangiogenic therapies[22, 23].

4.4.4. G47Δ-Tumstatin disrupts the glioma stem cells - endothelial cells interaction in matrigel co-culture

As with transwell co-culture, infected glioma stem cells were introduced into the co-culture system. Again, this was to replicate the injection of virus into the tumour parenchyma, where the majority of infected cells would be tumour cells. Infection of endothelial cells led to rapid killing of the endothelial cell component of co-culture. Figure 4.25 demonstrates the effect of viral infection on the co-culture system. Both vectors were able to disrupt the endothelial cell branches but G47Δ-Tumstatin was more effective than the empty vector ($p=0.001$).

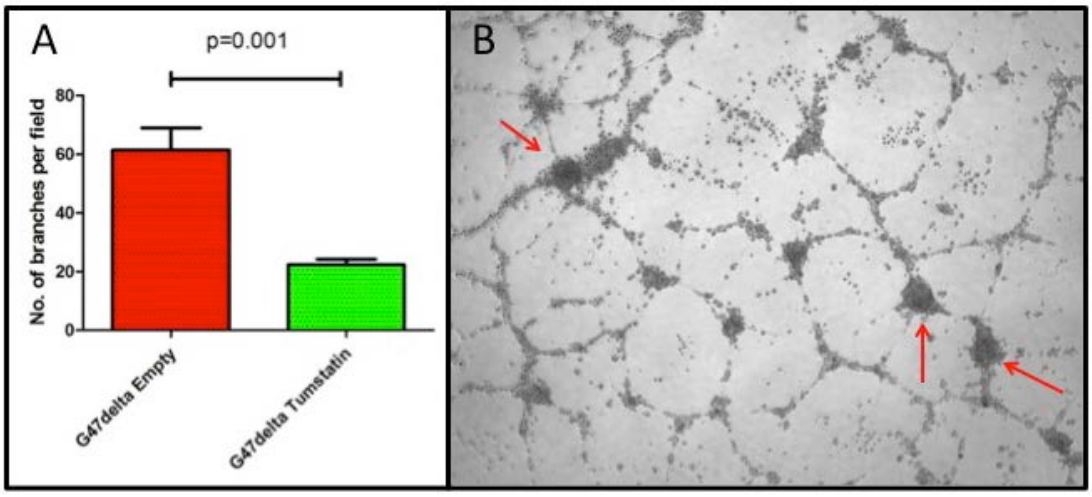

Figure 4.25 A. Quantitative assessment of the ability of both vectors to destroy endothelial cell branches in co-culture on matrigel. Infected glioma stem cell spheres were co-cultured with endothelial cells on matrigel. G47Δ-Tumstatin was more effective at destroying the branches (p=0.001, Student's t-test). B. Destruction of the endothelial branches in the co-culture model by G47Δ-Tumstatin (red arrows – infected glioma stem cell spheres).

4.5 In vivo efficacy of G47Δ-Tumstatin

Having established the in vitro efficacy of G47Δ-Tumstatin at killing endothelial cells and disrupting glioma stem cells – endothelial cells co-culture systems, the orthotopic xenograft model discussed in section 3.4 was used to assess the in vivo efficacy. G47Δ-Tumstatin was compared to the empty vector and to mock treatment. As described in section 3.4, mock-treated mice were treated just as virus-treated mice, but received intracranial injections of phosphate buffered saline.

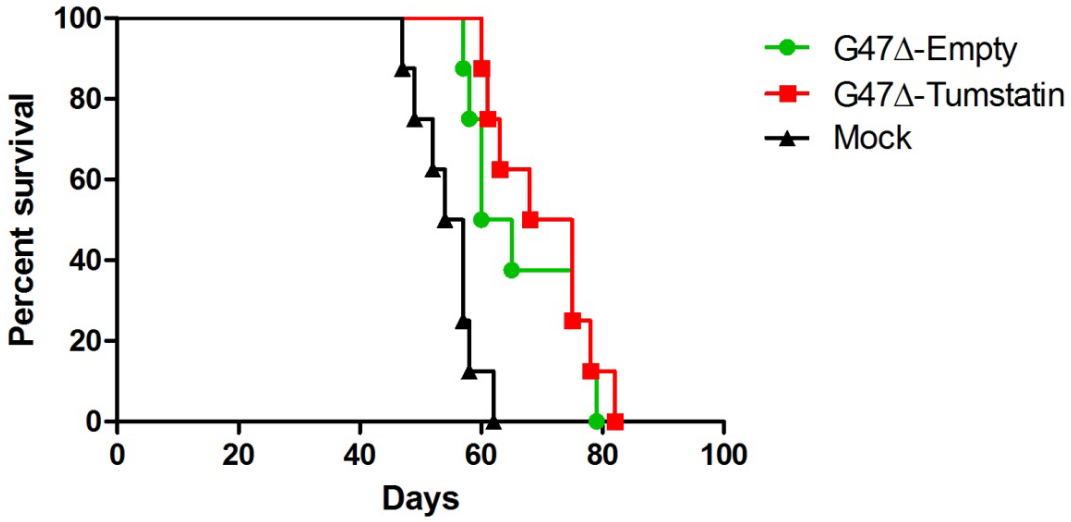

Figure 4.26 Survival analysis of orthotopic xenotransplants of glioma stem cells (GBM8) treated by either mock injections, or 2 x 10^6 pfu of either G47Δ-Tumstatin or G47Δ-Empty. Both vectors performed better than mock treatment (Log rank, $p<0.005$). G47ΔTumstatin did not demonstrate a survival advantage over G47Δ-Empty (Log rank, $p=0.44$).

There were no deaths related to tumour implantation or virus treatment. Mice brains were harvested at death and all the mice died from progression of the brain tumours. The median survival in the mock treated group was 56 days (range 47 - 62 days) and for mice treated with G47Δ-Empty and G47Δ-Tumstatin it was 63 days (range 57 -75 days) and 72 days (range 60 – 82 days) respectively (Figure 4.26). The survival advantage of treatment by either virus was statistically significant compared to mock treatment (Log rank, $p<0.005$). However, neither of the virus treatments were curative. No clear advantage was demonstrated for the vector expressing tumstatin compared to the empty vector (Log rank, $p=0.44$).

4.6 Discussion

The traditional method of creating recombinant HSV vectors involved homologous recombination techniques using purified HSV DNA and recombination plasmids being co-transfected in cells. This technique was labour intensive with multiple attempts at viral selection and structure confirmation until the appropriate clone was created. The inefficiency of this method has been a major hurdle in the creation of new oncolytic HSV vectors. The use of BAC technology has enabled the manipulation of the entire HSV genome in *E coli* bacteria. The technique described in this chapter where BAC technology is combined with two site specific recombination steps (Cre/loxP and FLP/FRT) has been designated the Flip-Flop (FLP/FRT and Cre/loxP) HSV-BAC system[147].

Using standard sub-cloning techniques of restriction enzyme digestion and ligation to manipulate the HSV-BAC DNA and insert transgenes would be unfeasible as the DNA sequence is typically more than 150 kilobases. Thus the use of site-specific recombination systems are particularly beneficial for precise and reproducible recombination. Another technique employed to aid accurate reconstruction is the inefficiency of HSV virions in handling and packaging DNA fragments larger than 156 kilobases[183, 184]. The addition of a stuffer sequence, antibiotic resistance genes, and the EGFP gene increases the HSV DNA size to greater than 156 kilobases. This prevents the efficient replication of viruses that have not undergone the

second recombination step involving FLP/FRT which excises these additional sequences. Furthermore, by removing the EGFP gene, viral clones can be screened for their lack of EGFP. These advances in cloning technology and our improved understanding of the HSV genome has enabled the creation of pure, appropriate recombined clones in greater than 99% of attempts[147].

The role of extracellular matrix proteins in the perivascular niche of glioma stem cells is gaining prominence and their activity on tumour cell proliferation and migration is slowly being elucidated[53]. Furthermore, laminins have been demonstrated to be essential in the culture of adherent glioma stem cells[185, 186]. More recently integrin α6 has been reported to be a marker of the glioblastoma perivascular niche and that gliomas stem cells express high levels of this surface protein[48]. Integrins αVβ3 and αVβ5 are known to be expressed by glioma bulk tumour cells, their vasculature and correlate with the grade of tumour[175]. αVβ3 positive tumour cells are located at the invasive borders of gliomas[175], an area that may be associated with invading glioma stem cells. The flow cytometric results and protein expression analysis in section 4.3.2 are the first report of the presence of integrin αVβ3 on glioma stem cells. However, their presence was not universal with only certain cell lines expressing the surface protein.

Tumstatin has a proven record of efficacy in preclinical studies of angiogenesis[166-169, 187, 188]. In addition, anti-tumour effects

independent of anti-angiogenic actions have also been reported[174]. The activity of tumstatin is via integrin αVβ3 and the PI3K/PTEN/AKT/mTOR. For successful apoptosis by tumstatin, cells must express the integrin αVβ3. In addition, intracellular pathways that inhibit the Akt-mTOR pathway, an intact PTEN and factors that prevent constitutive activation of Akt potentiate the activity of tumstatin[174]. Proliferating endothelial cells meet the criteria outlined above for tumstatin susceptibility but glioma stem cells do not and thus the anti-tumour activity of tumstatin in glioma stem cell models is purely restricted to the endothelial cell component.

The effect of tumstatin on endothelial cell migration in the published literature is mixed. One report suggests that tumstatin's activity does not extend to endothelial cell migration, but purely to reduce protein synthesis and induce apoptosis[170]. In contrast extracellular matrix bound VEGF is known to promote endothelial cell migration via αVβ3 and this binding is competitively inhibited by tumstatin[189]. Furthermore, more recent work has confirmed an anti-migratory effect of tumstatin on endothelial cells[190]. The experimental results in this chapter demonstrate a clear reduction in migration following G47Δ-Tumstatin infection. This may in part reflect a higher cytotoxic effect of the virus, as only viable cells would be able to complete the experiment.

Both the empty vector and G47Δ-Tumstatin are capable of killing glioma stem cells and endothelial cells. This ability to affect both components

of the perivascular niche is an advantage in any anti-glioma therapy. G47Δ-Tumstatin demonstrated an added advantage in disrupting endothelial cell function in the in vitro models of glioma stem cells - endothelial cells co-culture. This advantage was seen even in a contact co-culture model where testing with bevacizumab, a potent monoclonal antibody to VEGF appeared ineffective. One potential hypothesis of the resistance in the presence of bevacizumab may be the transdifferentiation of glioma stem cells into endothelial cells[22, 23]. Wang et al[23] noted that using a γ-secretase inhibitor to target the Notch pathway rather than targeting VEGF with bevacizumab, prevented the transition of CD133 positive tumour cells to endothelial cell progenitors. The advantage for G47Δ-Tumstatin may be its ability to kill glioma stem cells as well, thus disrupting any protective effect the glioma stem cells have on endothelial cells. The prevention of neo-angiogenesis and the role of the glioma stem cells in this process is certainly an area worthy of further study.

The in vivo failure of G47Δ-Tumstatin to increase survival compared to G47Δ-Empty may reflect a number of possibilities. Endothelial cells are sensitive to G47Δ viruses and while in vitro there was enhanced efficacy with the tumstatin transgene, this effect was not translated to the in vivo model. Glioma stem cells in human glioblastomas would have to regulate endogenous anti-angiogenic peptides like tumstatin during tumour development. It is possible that in vivo factors not seen in the in vitro model enable the tumour to successfully resist tumstatin's activity. The viruses were only tested on human endothelial cells but human tumstatin is known to work

on mouse endothelial cells. Glioma stem cells in orthotopic tumours which represent a more comparative model to their state in human gliomas are adept at adapting to anti-angiogenic agents. A number of possible adaptive mechanisms may be at play.

In addition to the example of transductive adaptation where glioma stem cells are able to transdifferentiate into endothelial cells in response to antiangiogenic agents[22, 23], there have been reports of gliomas changing their phenotype to a more invasive model in response to anti-angiogenic agents[191]. Other anti-angiogenic studies have suggested that a metronomic model of delivery of antiangiogenic agents is preferred to a single dose model[192] and this is one more consideration for further work in this area. The use of anti-angiogenic agents in glioblastoma remains a promising area of study but more work is required to delineate the resistance seen with their use.

Chapter 5: Therapeutic synergy between G47Δ and radiation therapy in glioma stem cells

5.1. Introduction

5.1.1. Radiotherapy for glioblastoma

The evidence for the use of radiotherapy for malignant gliomas has long been established with a study published over three decades ago demonstrating a survival advantage for patients treated with radiation compared to those receiving best supportive care[193]. This survival advantage persists even in modern series of older patients with glioblastoma[194]. Glioblastoma patients today receive approximately six weeks of irradiation to clearly defined tumour-involved volumes of the brain. The infiltrative nature of the disease requires the radiotherapy field to have a wide margin and commonly 2cm is added to the enhancing tumour or tumour bed borders. The total dose over the six-week period is typically 60Gy in 2Gy fractions.

Despite the survival benefit of radiotherapy, glioblastomas demonstrate significant radioresistance with almost universal recurrence of the tumours, some within areas receiving high doses of radiation[195]. Dose

escalation investigations have not demonstrated a curative dose even at 90Gy[196]. A number of strategies have been employed to circumvent the radioresistance of these tumours including modulating the fractionation regimes, stereotactic radiosurgery and the use of radiation sensitizers. All three strategies have had limited success.

5.1.2. Radiation sensitizers in glioblastoma

The current standard of practice as discussed in Chapter 1, is the use of concomitant and adjuvant temozolomide with radiotherapy[5]. Temozolomide, an alkylating agent, has an anti-tumour effect of its own but has also been shown in vitro to sensitize glioma stem cells to radiation[197]. Thus part of the benefit seen in their concomitant use may be due to radiosensitization of the tumour.

Other radiosensitizers have been trialled including misonidazole and bromodeoxyuridine. Misonidazole induces the formation of free radicals and depletes radioprotective thiols. It was intended to act as a radiosensitizer of hypoxic cells, but in a randomised trial it failed to confer a survival benefit[198]. Bromodeoxyuridine is a synthetic analogue of thymidine that radiosensitizes cells by incorporating into DNA. As with misonidazole, a phase III randomised trial in anaplastic astrocytomas demonstrated no survival advantage for bromodeoxyuridine[199].

5.1.3. Biology of radiation resistance and glioma stem cells

Preclinical models of glioblastomas have elucidated some of the mechanisms behind the radioresistance seen in this tumour. Two common mutations seen in glioblastomas are loss of PTEN and amplification or mutation of EGFR[15]. Both these mutations lead to activation of the PI3K pathway. Studies of glioma cells with mutated, constitutively active EGFR have demonstrated its role in radioresistance, mainly via the PI3K pathway[200]. The discovery of glioma stem cells has led to further discoveries on the mechanisms of radioresistance. Following irradiation, CD133 positive glioma stem cells have been shown to preferentially activate DNA damage checkpoint responses and repair radiation induced DNA damage[29]. The same study proposed that CD133 positive glioma stem cells are responsible for the radioresistance of glioblastomas, and that these cells subsequently repopulate the tumour leading to tumour recurrence.

5.1.4. Combining oncolytic herpes viruses with radiotherapy and cytotoxic therapies

The ubiquity of radiation treatment in the management of cancers is a testament to its utility. However, the ability of radiotherapy to cure cancers is rare. The use of radiation sensitizers was previously discussed in section

5.1.2; in this chapter, an alternative strategy where radiation therapy is used as a sensitizer for viral oncolytic therapy will be investigated. A number of oncolytic herpes viruses have been used in conjunction with radiotherapy to treat tumours. R3616, a γ34.5 mutant, has been trialled in preclinical models of glioblastoma. The tumours were inoculated with the virus and then irradiated. In xenograft flank models tumour regression was greater with this dual treatment[201]. Similarly in an orthotopic model, median survival was significantly greater in the combined radiation therapy and R3616 group compared to single treatment groups[202]. The effect of combined oncolytic viral therapy and radiation treatment on glioma stem cells has not been previously studied. Recent work in combining cytotoxic agents with oncolytic viral therapy to treat glioma stem cells has demonstrated promising results when the chemotherapeutic agents temozolomide and etoposide were used[203, 204].

In this chapter the effect of radiotherapy on glioma stem cells and the efficacy of combining G47Δ with radiotherapy will be examined. In addition, the ability of the two agents to act synergistically will be tested and the mechanism of their interaction delineated. Furthermore, the changing patterns of cellular markers in glioma stem cells in response to radiotherapy will be investigated. Finally, the in vivo action of radiotherapy and G47Δ in combination will be studied in an orthotopic, xenograft mouse model of glioma stem cells.

5.2. Radiosensitization of glioma stem cells in vitro

5.2.1. In vitro treatment of glioma stem cells with radiotherapy

Cultured glioma stem cells were treated with radiotherapy at varying doses to establish dose response curves. Interestingly, the previously reported radioresistance of glioma stem cells is not a universal phenomenon. Dose response curves from 2 glioma stem cell lines with varying resistance to radiotherapy are shown below. Like all the cell lines used they have a mixed population of CD133 positive and negative cells. GBM8 has greater than 90% CD133 positivity but in contrast to previous data[29] discussed in section 5.1, GBM8 was exquisitely sensitive to radiotherapy with an EC50 (dose at which 50% of cells are killed) of 2Gy (Figure 5.1). By comparison, BT74 a glioma stem cell line with only a third of its cells expressing the surface marker CD133, was extremely radioresistant with an EC50 of 22Gy when treated in vitro (Figure 5.2). Thus after treatment with 22Gy delivered as a single dose, half the BT74 glioma stem cells were still alive. To put this degree of resistance to radiation therapy in perspective, a 22Gy dose when delivered to humans is given over 15 days in 2Gy fractions with breaks after every 5 days.

In Chapter 3 the use of G47Δ to treat glioma stem cells and its ability to increase survival in xenograft models of glioma stem cells was noted. Nonetheless, no cures were achieved. This, combined with the marked

radioresistance of glioma stem cells and previous studies suggesting enhanced combined efficacy, led to the investigation of the efficacy of the two agents used in conjunction.

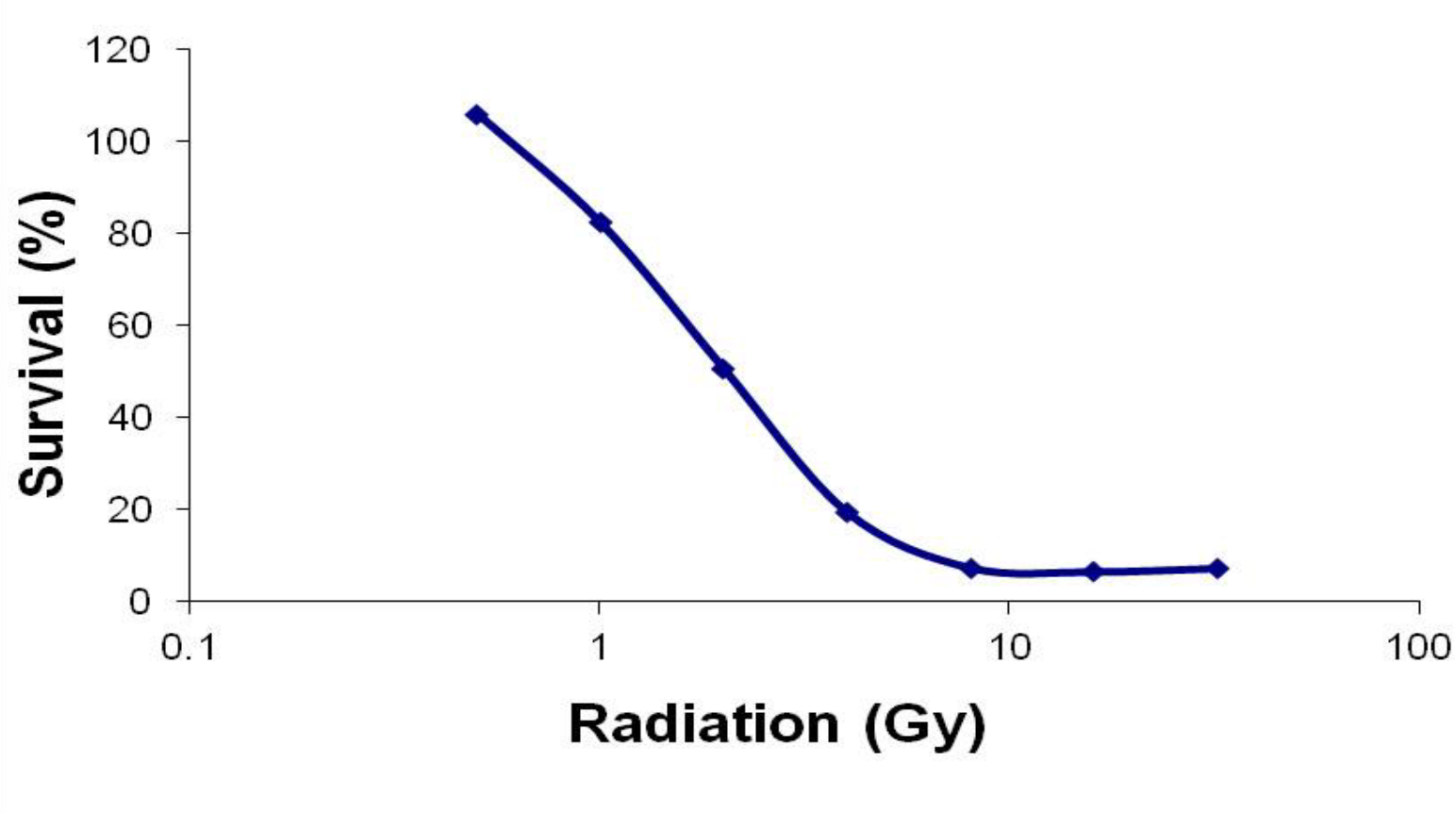

Figure 5.1 Dose response curve measuring the survival of GBM8 glioma stem cells following irradiation. The EC50 value for radiation treatment in this glioma stem cell line is 2Gy.

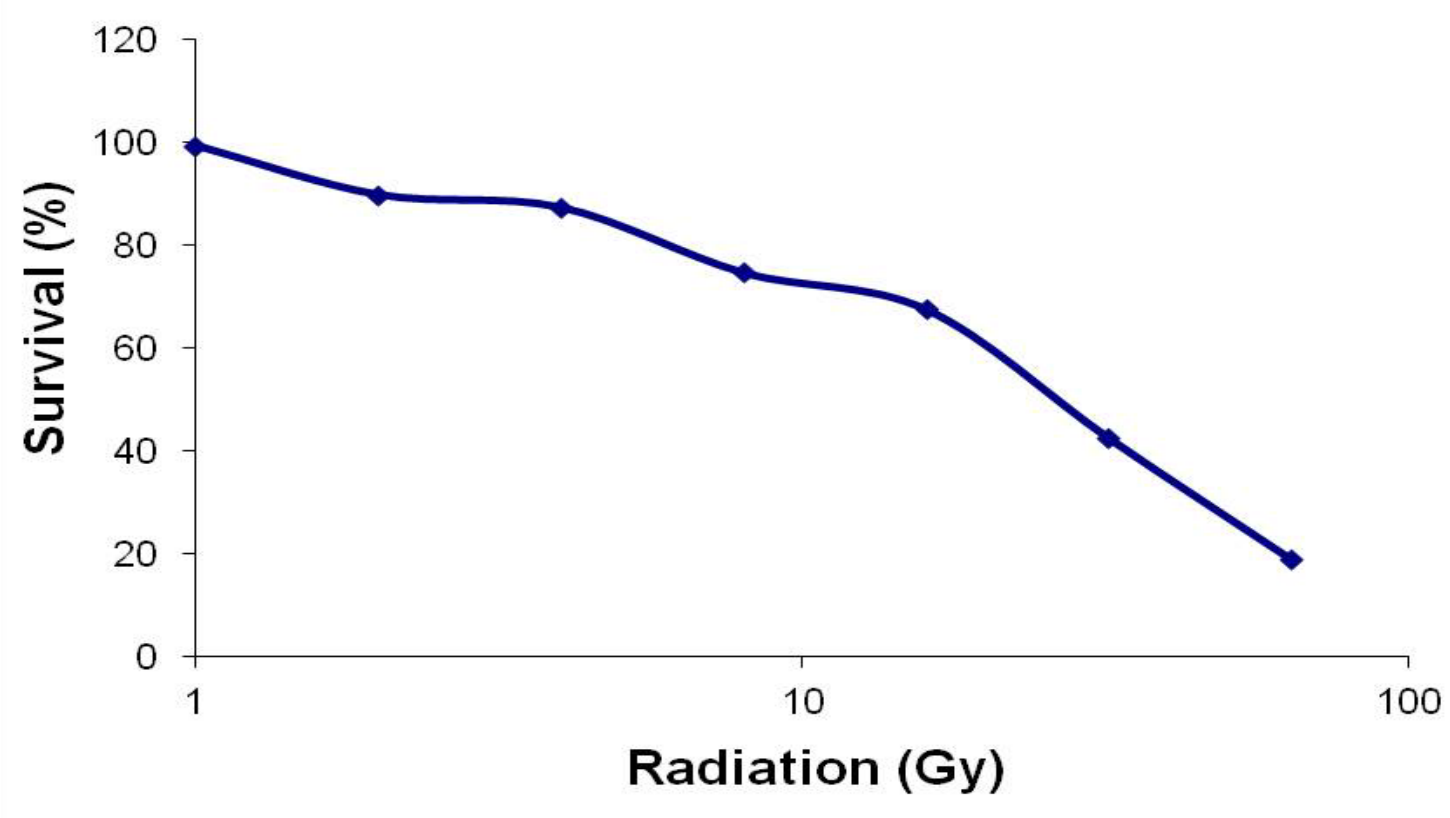

Figure 5.2 Dose response curve measuring the survival of BT74 glioma stem cells following irradiation. The EC50 value for radiation treatment in this glioma stem cell line is 22Gy.

5.2.2. Combined efficacy of G47Δ and radiotherapy on glioma stem cells

In section 5.1 the role of radiosensitizers in increasing the efficacy of radiotherapy was discussed. Radiosensitizers may be able to exert an additive or synergistic effect when given in combination, hence in the following experiments the ability of radiotherapy and G47Δ to synergize was studied. Radioresistant glioma stem cells were treated with G47Δ and radiotherapy in 5 separate regimes as outlined below:

- Simultaneous treatment with radiotherapy and G47Δ
- Pre-treatment with G47Δ and then treatment with radiotherapy
- Pre-treatment with radiotherapy followed by infection with G47Δ
- G47Δ alone
- Radiotherapy alone

Treatments were separated by 24 hours. Based on previous work on irradiation of glioma stem cells where doses between 3 to 5Gy were sufficient to produce changes in the DNA damage response of glioma stem cells[29], a dose of 4Gy was chosen for the preliminary studies. Figure 5.3 demonstrates the cytotoxic effects of the 5 treatment regimens. The most efficacious combination was the pre-treatment of glioma stem cells with radiotherapy prior to infection with G47Δ ($p<0.0001$, ANOVA with Tukey post-test). Mean survival for the group pre-treated with radiotherapy was

47% compared to 63% in the simultaneous treatment group and 66% in the group where radiation treatment was delayed.

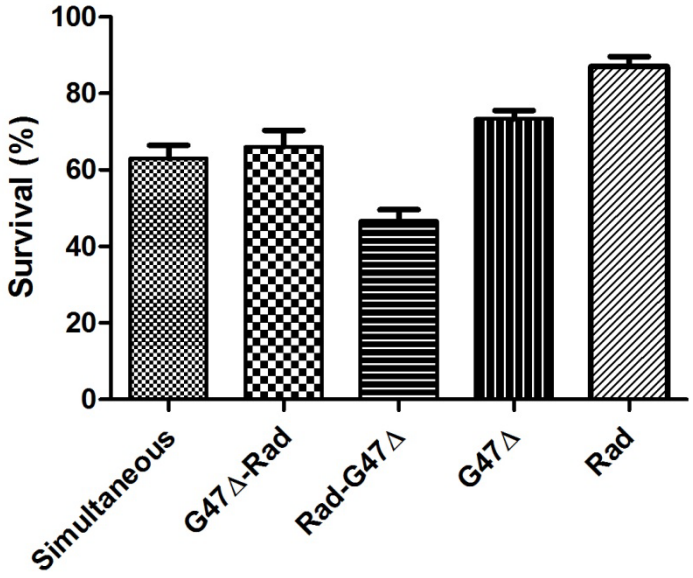

Figure 5.3 Dosing regimens tested to determine the optimum schedule for the combined treatment of G47Δ and radiotherapy (4Gy). Treatment was delivered individually, simultaneously or at 24h intervals. Survival was analyzed using an MTS assay. Pre-treating radioresistant glioma stem cells (BT74) with 4Gy of radiotherapy enhanced the killing of the cells by G47Δ ($p<0.0001$, ANOVA with Tukey post-test).

The results from this experiment suggest that pre-treatment of glioma stem cells with irradiation prior to infection with G47Δ is the most efficacious strategy. Therefore, radiotherapy must be sensitizing the glioma stem cells to G47Δ. This is a reversal of the typical approach to improving outcomes with radiotherapy in glioblastoma patients where radiation sensitizers are

used[198, 199]. To determine if the enhanced activity was a result of increased viral replication, measurement of viral titres post radiotherapy was undertaken.

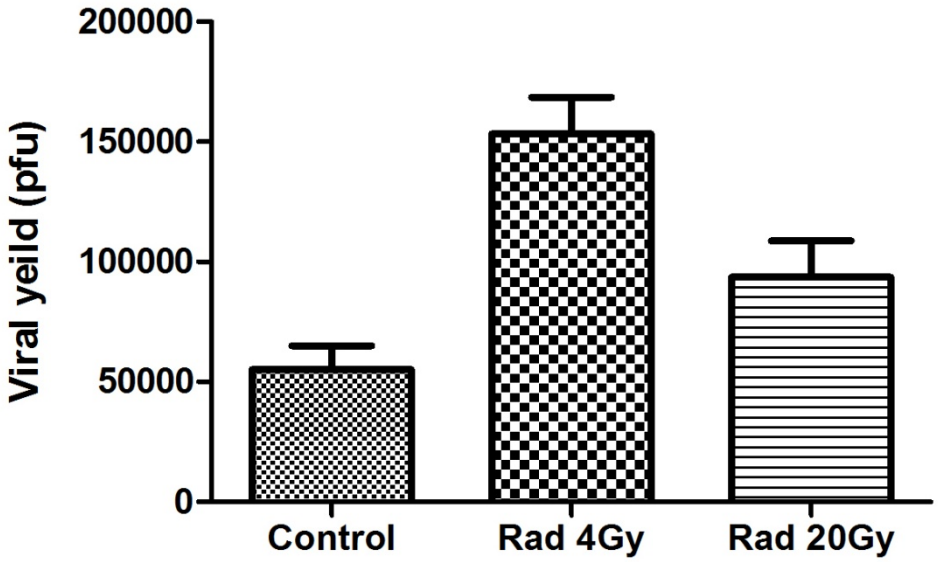

Figure 5.4 Replication of G47Δ in control glioma stem cells and following pre-treatment with 4Gy or 20Gy irradiation. Treatment with 4Gy of radiation therapy significantly enhanced replication of G47Δ compared to no radiation therapy or high dose radiation at 20Gy in BT74 glioma stem cells ($p<0.01$, ANOVA with Tukey post-test).

Glioma stem cells were infected with G47Δ following irradiation with 4Gy or 20Gy. Control cells were not irradiated and all cells were infected 24 hours after the treatment groups were irradiated. Viral titres were measured 36 hours post-infection. Pre-treatment of glioma stem cells with 4Gy of

radiotherapy significantly enhanced the viral titres produced ($p<0.01$, ANOVA with Tukey post-test). Treatment with high dose radiotherapy did not produce the same effect ($p>0.05$, ANOVA with Tukey post-test). This may have been a result of additional cell killing at the high dose suppressing viral bursts or initiation of a more quiescent stage in glioma stem cells which would suppress viral replication. The 4Gy dose was chosen for all future experiments.

The effect of pre-treatment with radiation was tested on the HSV-1 wild type strain and other mutant viruses. Figure 5.5 demonstrates the results. Irradiating the glioma stem cells had no effect on the viral yield of HSV-1 wild type strain ($p>0.05$, Student's t-test) indicating that the enhanced effect seen with G47Δ may be a compensatory phenomenon for one of the engineered mutations of G47Δ. Infection of irradiated glioma stem cells resulted in a significant growth advantage for the ICP6 mutant (FΔ6) ($p=0.01$, Student's t-test) indicating a possible role for ribonucleotide reductase activity in the irradiated cells. Interestingly, radiation did not seem to significantly affect the poor replicative activity of G207 ($p>0.05$, Student's t-test), seen in Chapter 3.

Having established that both the ICP6 mutant and G47Δ demonstrate enhanced replication following pre-treatment of the glioma stem cells with irradiation and that the cytotoxic effect of radiotherapy and G47Δ is better if

given sequentially with the virus added after radiotherapy, an analysis of the combination effect was carried out.

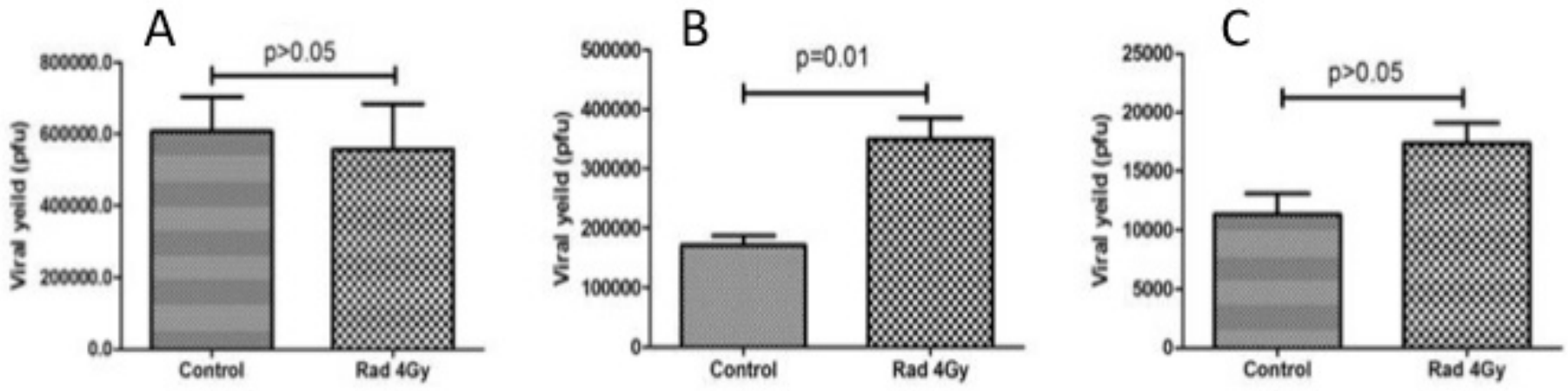

Figure 5.5 Viral yields of (A) wild type HSV-1, (B) the ICP6 mutant and (C) G207 in BT74 glioma stem cells pre and post irradiation with 4Gy. Neither wild type HSV-1 nor G207 demonstrated significant changes in replicative abilities following radiation treatment ($p>0.05$, Student's t-test). The ICP6 mutant FΔ6 demonstrated a significant increase in replication following irradiation ($p=0.01$, Student's t-test).

5.2.3. Combination index

Determining the combined efficacy of two agents is an intricate process dependent on a number of variables. The cytotoxic effect of two agents needs to be defined more than just as agent A + agent B being greater than agent A or B. This simplistic definition becomes unusable in a situation where one agent may have 65% cytotoxicity and the other 85%, where the combined efficacy cannot be 150% cell death.

One mathematical model for calculating the combined efficacy of two agents is the Chou-Talalay method, which is based on the law of mass action and thus independent of the knowledge of the mechanisms of action of the agents involved[149, 150]. Having established dose response curves for radiotherapy and for G47Δ, the EC50 values for each agent was calculated (Figure 5.1, Figure 5.2, Figure 3.14). Based on the EC50 values of both agents, a constant ratio drug combination analysis is carried out to determine the combination index which is a measure of the interaction between the two agents. The result combination index values are plotted against the cytotoxic effect which is termed the fraction affected (f_a). f_a ranges are plotted between 0.2 to 0.8. Figure 5.6 demonstrates the results of the Chou-Talalay analysis for a radioresistant glioma stem cell line (BT74) and Figure 5.7 shows the analysis for a more radiosensitive glioma stem cell line (GBM8).

A combination index (CI) of 1 denotes an additive effect between the two agents. A CI of greater than 1 is defined as an antagonistic reaction and a value below 1 is defined as synergistic. In the radioresistant glioma stem cell line (BT74), the CI value at a f_a of 0.2 is >1 but at all other f_a the CI is <1 indicating synergism (Figure 5.6). The radiosensitive glioma cell line (GBM8) demonstrated a CI <1 throughout indicating a synergistic relationship (Figure 5.7).

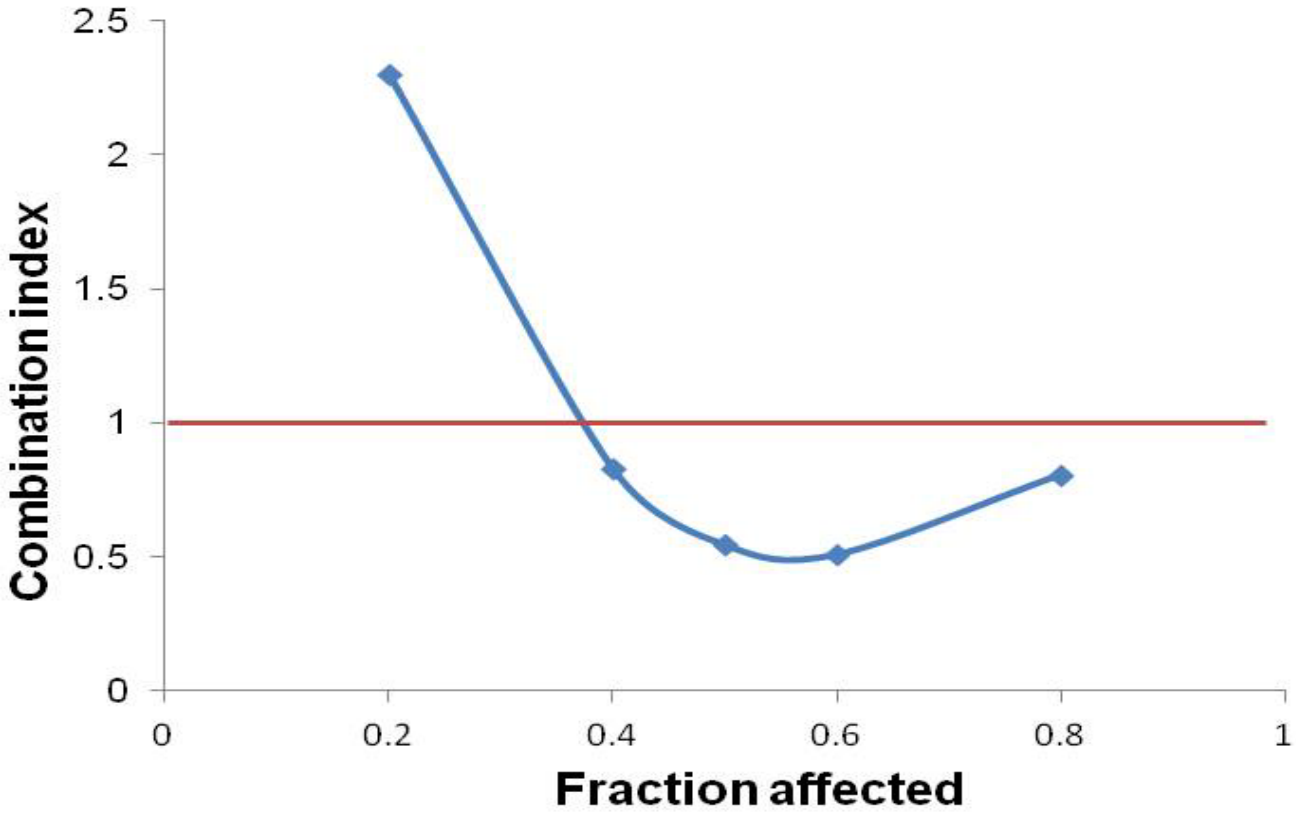

Figure 5.6 Chou-Talalay analysis in a radioresistant cell line (BT74). The red line marks a combination index (CI) of 1 which is the level for an additive effect. At a f_a of 0.2 there is an antagonistic relationship between radiotherapy and G47Δ but at all other fa the relationship is synergistic.

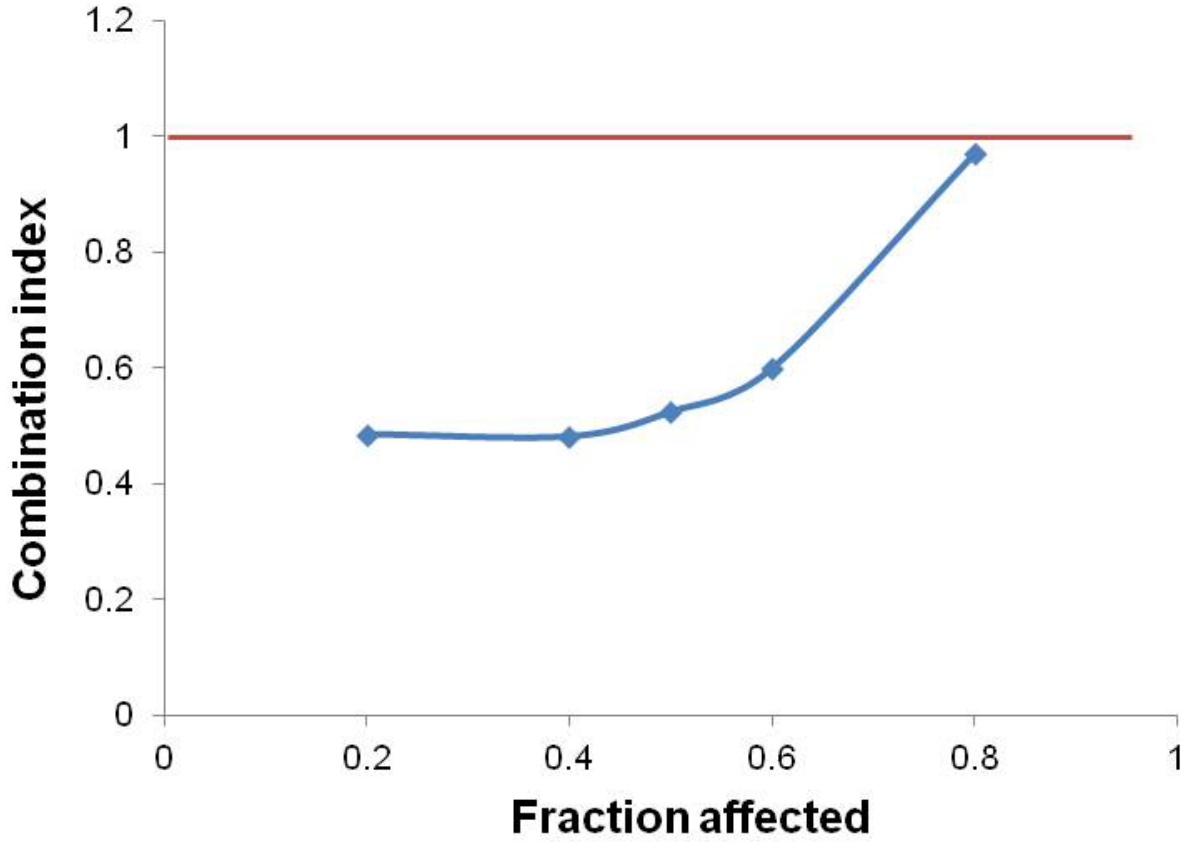

Figure 5.7 Chou Talalay analysis in a radiosensitive cell line (GBM8). The red line marks a combination index (CI) of 1 which is the level for an additive effect. In this experiment all the CI values were below 1 indicating a synergistic effect.

5.2.4. Mechanism of synergy between radiotherapy and G47Δ on glioma stem cells

The increase in replication by both the ICP6 mutant and G47Δ, but not wild-type HSV-1, suggests that the glioma stem cells may be responding to the cytotoxic stress of radiotherapy by upregulating the expression of ribonucleotide reductase (RR). This is an established survival mechanism for cancers undergoing radiotherapy[205-208]. As both G47Δ and the ICP6 mutant have insertional mutations at the UL39 gene, they are unable to produce the RR enzyme which is crucial for the synthesis of DNA. Thus, their replication is restricted to cells with an adequate level of the protein to sustain viral replication. In cells where there is an upregulation of RR their replication is enhanced. Protein analysis of glioma stem cells pre- and post-radiation was carried out to test this hypothesis. In the three glioma stem cell lines tested, all demonstrated an increase in the R2 subunit of RR following irradiation (Figure 5.8).

This increase in RR following radiotherapy would explain the increase in replication and killing seen in glioma stem cells by G47Δ and the ICP6 mutant, but not wild type HSV-1. G207 would also be expected to demonstrate increased replication, however the benefits of raised RR levels do not appear to compensate for the suppression of γ34.5 mutants in glioma stem cells.

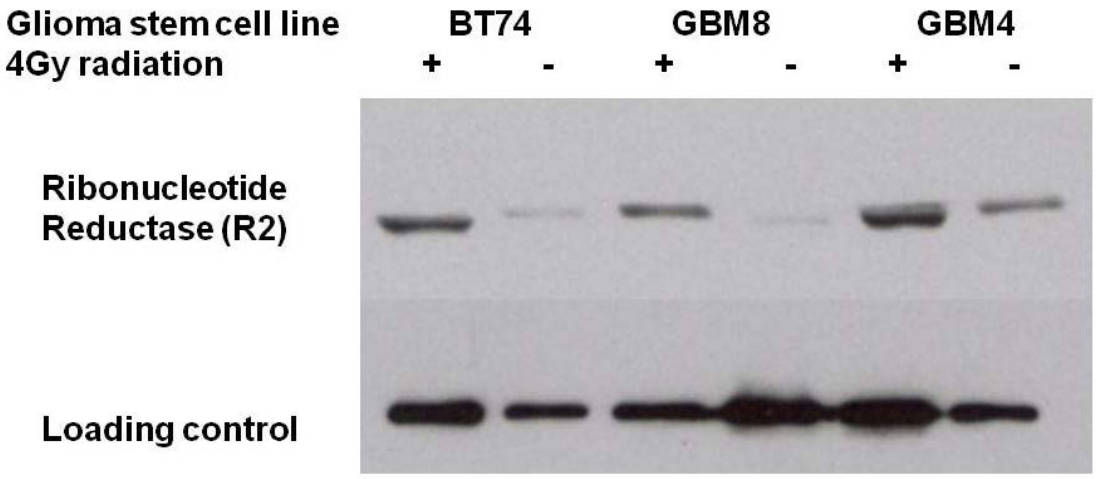

Figure 5.8 Western blot analysis of the expression of the R2 subunit of ribonucleotide reductase in 3 glioma stem cell lines, 24 hours after irradiation with 4Gy. All 3 glioma stem cell lines showed increasing quantities of R2 protein following 4Gy of irradiation.

5.2.5. Changing biomarkers following irradiation in glioma stem cells

The resistance of glioma stem cells to radiotherapy, particularly by CD133 positive cells has previously been shown[29]. The glioma stem cells used in this study are a mixed population of CD133 positive and negative cells. Nonetheless, the level of CD133 expression did not correlate with radioresistance with the highest CD133 expressing glioma stem cell line demonstrating relative radio-sensitivity. To investigate this further, 2 new biomarkers CD44 and CD24 were tested. CD44/24 double positive glioma

stem cells have been shown to be radioresistant (unpublished data, personal communication from Dr. Kamalakannan, Ohio State University, USA). Both the glioma cell lines tested demonstrated a subpopulation of double positive cells (Figure 5.9). Following irradiation with 4Gy the subpopulation in BT74 increased from 17% to 66%. In GBM8 the rise was more dramatic from 7% to 56%. However, this was on a background of far greater cell death in GBM8 as the 4Gy of irradiation is double the EC50 for this cell line. Nonetheless a radioresistant population survives and theoretically in humans these cells could repopulate the tumour following radiotherapy. Not all the surviving cells were CD44/24 double positive suggesting that these were by no means the only markers involved.

The ability of G47Δ and radiation therapy to synergistically kill glioma stem cells has been demonstrated (Figure 5.6 and 5.7). To determine the ability of the virus to infect the radioresistant sub-population of glioma stem cells, 2 colour flow cytometry was used to investigate the capability of G47ΔBAC to infect CD44 positive cells. Figure 5.13A and 5.13B below demonstrate the results. G47ΔBAC is able to infect the CD44 sub-population of glioma stem cells both pre and post radiation. In Chapter 3 it was shown that infection of glioma stem cells by G47ΔBAC resulted in death of the cells. The CD44 surface marker was chosen to represent the CD44/24 positive population as this marker, as will be discussed in section 5.4, has been linked to cellular pathways of radiation resistance.

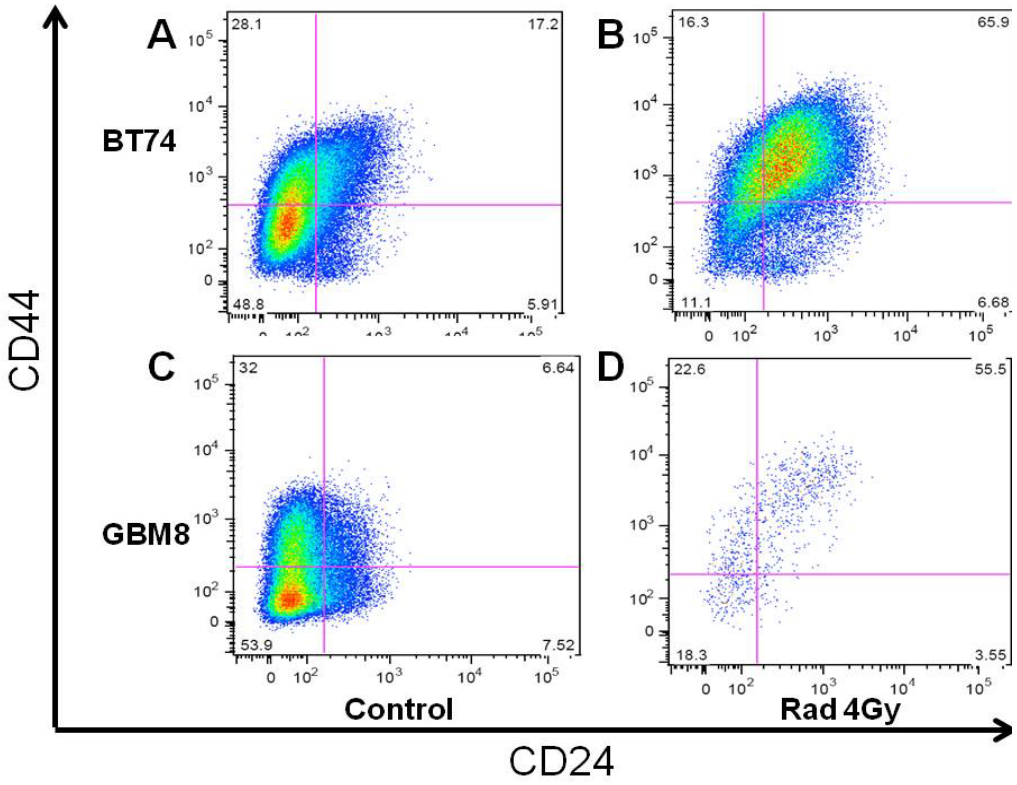

Figure 5.9. Flow cytometric analysis of the markers CD44 and CD24 in 2 glioma stem cell lines pre and post irradiation with 4Gy. Figure A and B show the radioresistant cell line BT74 pre and post radiation respectively. The CD44/CD24 double positive cells represent the radioresistant population and post irradiation there is an increase in the sub-population from 17% to 66%. Similarly in the more radiosensitive GBM8 cells (C and D) there is an increase in same population from 7% to 56%. However, in GBM8 there has been a marked reduction in the total number of cells demonstrated by the reduced cell density seen in D.

5.3. In vivo results

5.3.1. Survival analyses

Having established the in vitro efficacy of combining G47Δ with radiation therapy, the glioma stem cell, orthotopic, xenograft model was used to test the combined efficacy in vivo. The radioresistant glioma stem cell line BT74 which in vitro had an EC50 of 22Gy and a CI<1 in f_a 0.4-0.8 was used. 6 mice were assigned to each group at random and treated with mock infection, 4Gy of radiotherapy, G47Δ or both G47Δ and 4Gy of radiotherapy. Mice were irradiated in 2GY fractions on day 7 and day 8 post-tumour implantation and G47Δ injected on day 9. Both the radiotherapy alone group and mock treated group received mock injections of phosphate buffered saline. The results are shown in Figure 5.10

Treatment with G47Δ and 4Gy irradiation significantly improved survival ($p<0.001$, Log rank) compared to either G47Δ or radiation therapy alone. The median survival for the radiation group was 76 days, compared to 84 days and 96 days respectively for the G47Δ group and the double treated group. In this radiation therapy resistant glioma stem cell line, G47Δ treatment performed better than radiation alone. It should be noted that the 4Gy dose is below the in vitro EC50 value for this cell line but at a dose known to increase ribonucleotide reductase levels and enhance viral

replication. All the mice died from tumour progression and again no cures were seen in any of the groups.

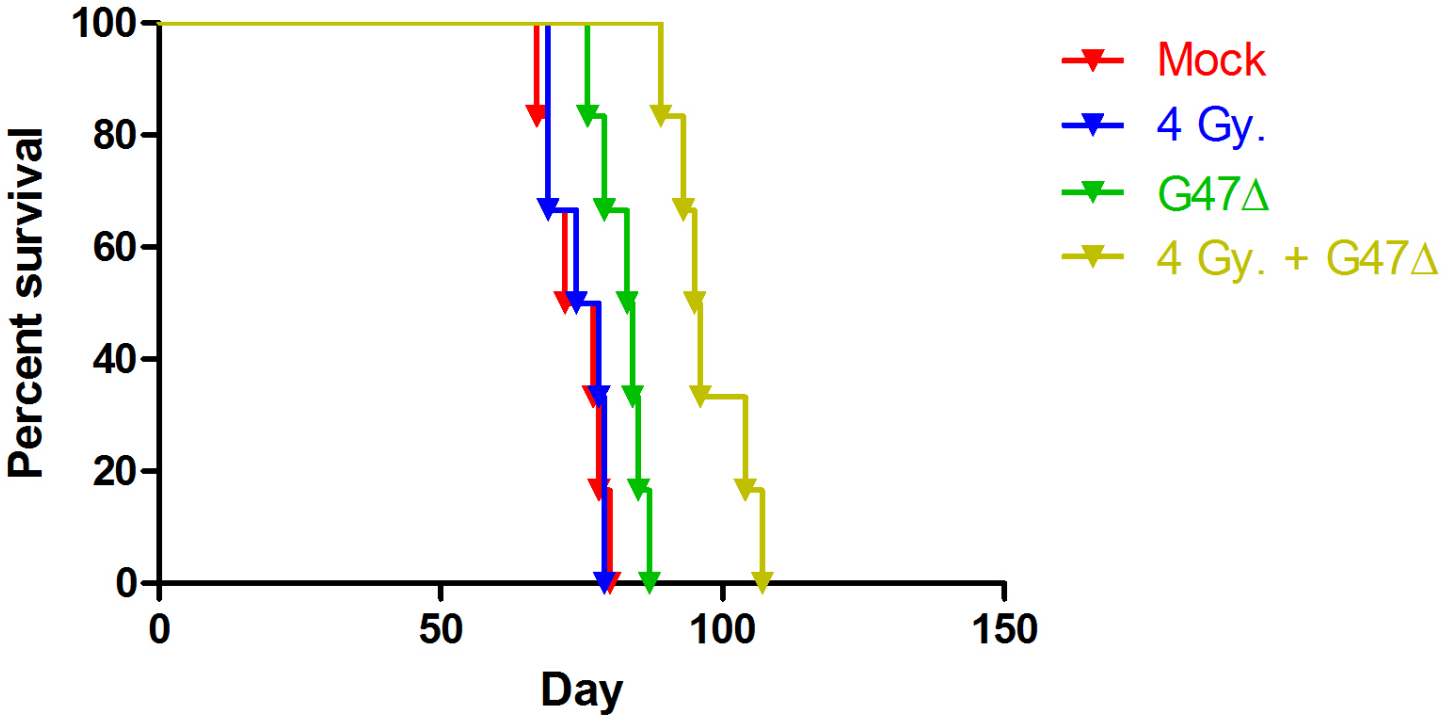

Figure 5.10: Survival post radiotherapy and /or G47Δ . Mice were randomised to be treated with either mock infection, 4Gy of radiotherapy, G47Δ or both G47Δ and 4Gy of radiotherapy (n=6 per group). Mice were irradiated in 2GY fractions on day 7 and day 8 post-tumour implantation and G47Δ injected (2 x 10^6 pfu) on day 9. The dual treatment group survived for significantly longer than any other group ($p<0.001$, Log rank).

5.3.2. Analysis of in vivo tumours

A further group of mice (n=3) were implanted with glioma stem cells and treated with 4Gy radiation therapy. A control group of mice (n=3) were also implanted with glioma stem cells. The mice were killed on day 45 and their brains and associated tumours removed. The tumours were processed using the protocol described in Chapter 2 and 3 as for human brain tumour samples. Cell were separated using flow cytometry to sort for cells expressing human leukocyte antigens (HLAs). Interestingly the tumours formed in mice were very sensitive to trypsin dissociation. Similarly digestion with collagenases also rendered the samples unsuitable for analysis. Figure 5.11 demonstrates the resultant flow cytometric analysis of the digested mouse brains. The protocol was subsequently modified to remove chemical digestion of the tumours and the brains were dissociated mechanically. Figure 5.12 demonstrates the results following mechanical dissociation. Viable cells were obtained, selected for by using a human antigen and grown in glioma stem cell cultures.

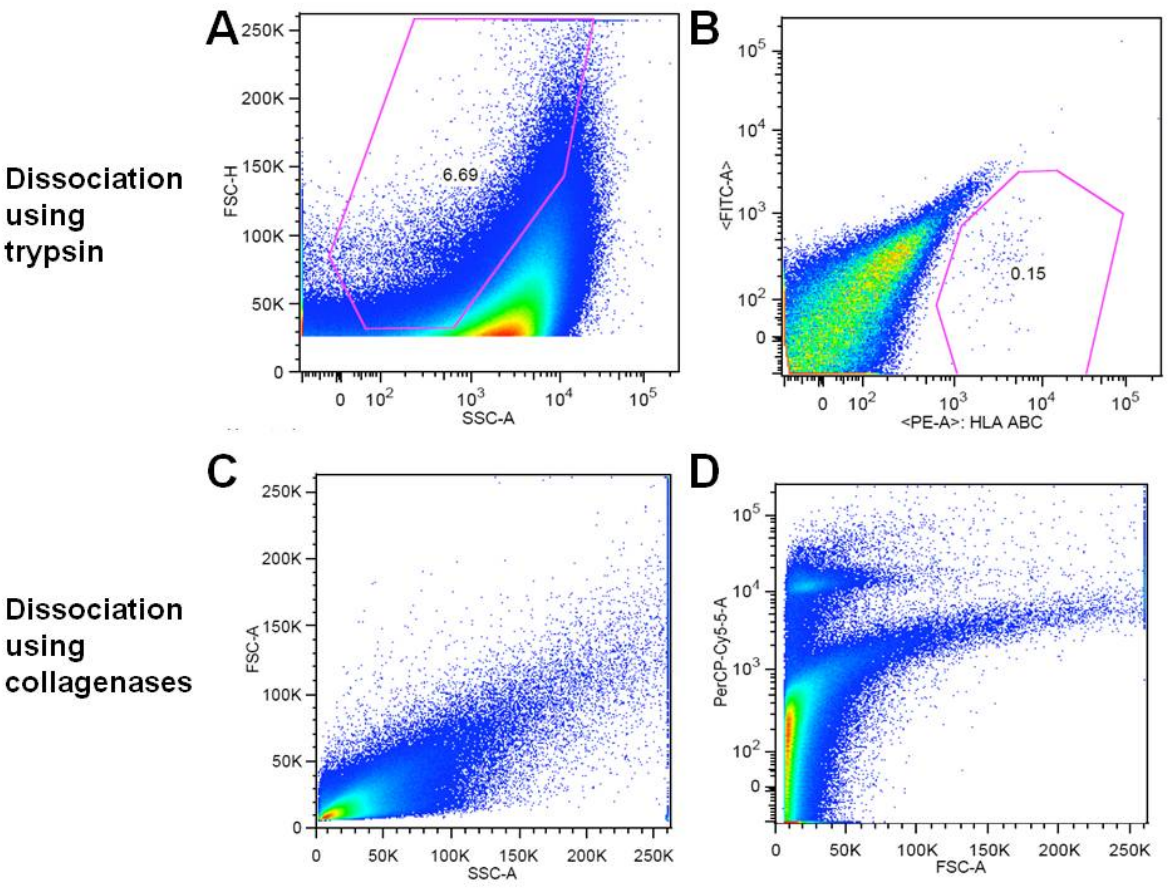

Figure 5.11 Flow cytometric data demonstrating that dissociation of glioma stem cell xenografts with trypsin (A and B) or collagenases (C and D) results in unusable cellular debris. In (B) only 0.15% of cells were viable and stained for the human HLA antigen.

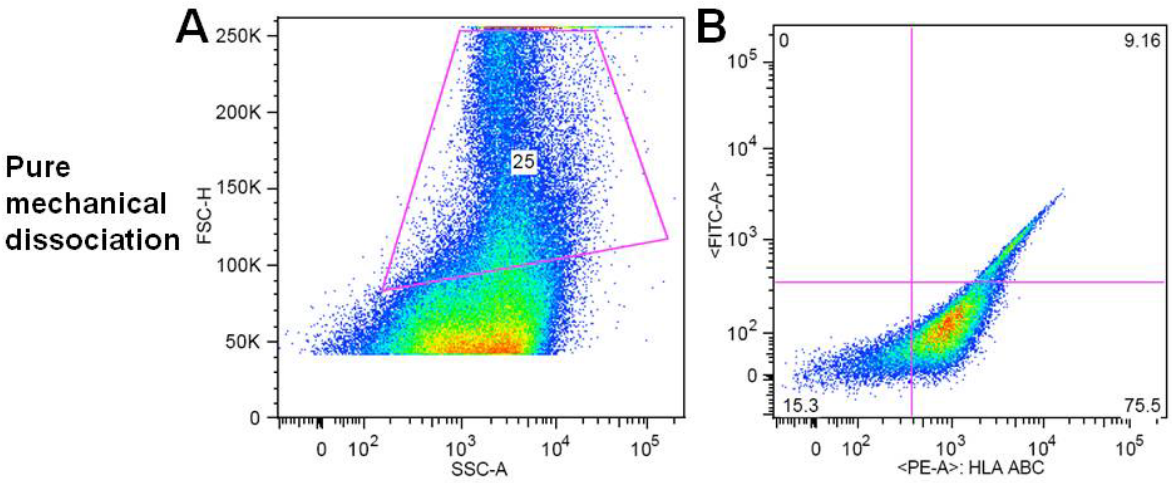

Figure 5.12 Flow cytometric data of glioma stem cell xenografts dissociated using mechanical dissociation. (A) demonstrates that 25% of the cells were viable and (B) demonstrates the cells positive for the human antigen (HLA-ABC) prior to sorting.

Once the cells were dissociated and sorted for the human antigen they were grown in EF media. The cells from tumours surviving in vivo radiation were tested for their CD44 positivity and the ability of the G47ΔBAC EGFP expressing virus to infect this CD44 positive subset of cells. The glioma stem cells surviving in vivo radiation possesed higher levels of CD44 expression compared to pre-irradiation glioma stem cells (Figure 5.13 A and C). Subjecting the glioma stem cells that survived in vivo radiation therapy to a further 4Gy of in vitro radiation, increased the size of the CD44 sub-population (Figure 5.13 C and D). G47ΔBAC was able to infect the CD44 sub-population of glioma stem cells that had survived radiation therapy in vivo (Figure 5.13 C and D) and as demonstrated in Chapter 3, the natural history following infection, is glioma stem cell death.

Figure 5.14 quantifies the change in the percentage of the CD44 subpopulation following radiation therapy in vitro and in vivo. Radiation therapy significantly increased the size of the CD44 sub-population Irrespective of whether treatment was delivered in cell cultures models or in mice tumours ($p<0.0001$, ANOVA with Tukey post-test), indicating a role for CD44 in glioma stem cell radioresistance.

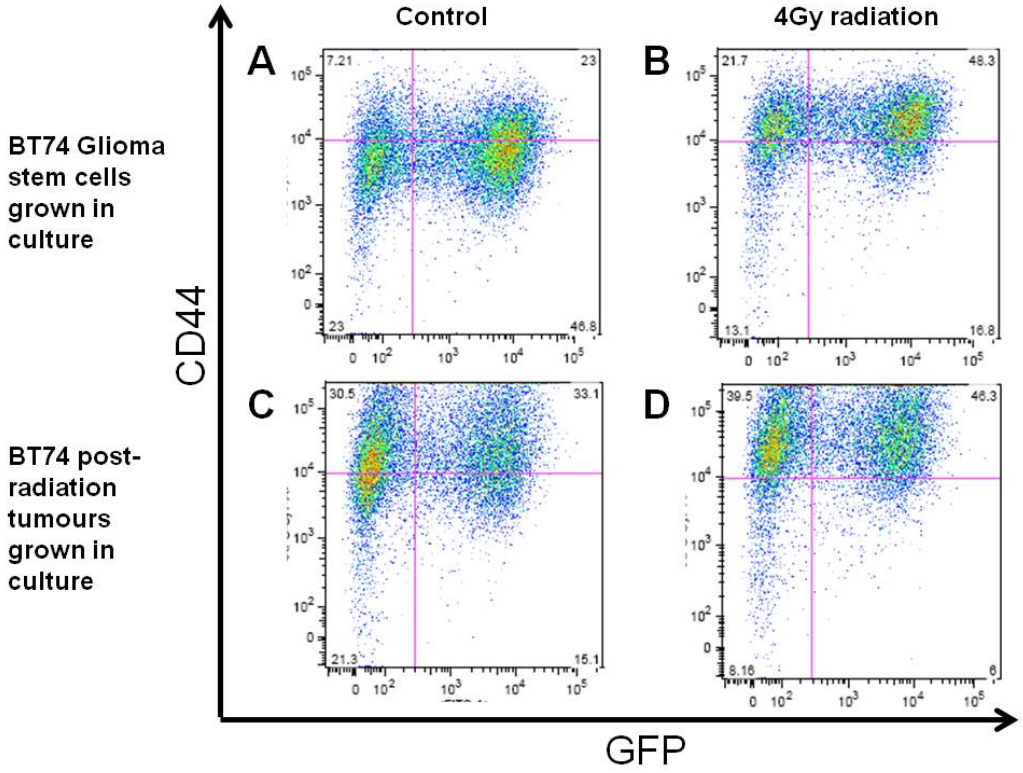

Figure 5.13 CD44 expression of BT74 glioma stem cells and the ability of G47ΔBAC to infect this sub-population of cells. (A) Control cells, (B) Cells following 4Gy of in vitro radiation, (C) Cell culture of tumours surviving 4Gy of in vivo radiation and (D) Cells from C following a further 4Gy of in vitro radiation. G47ΔBAC is able to infect CD44 positive cells in all 4 groups.

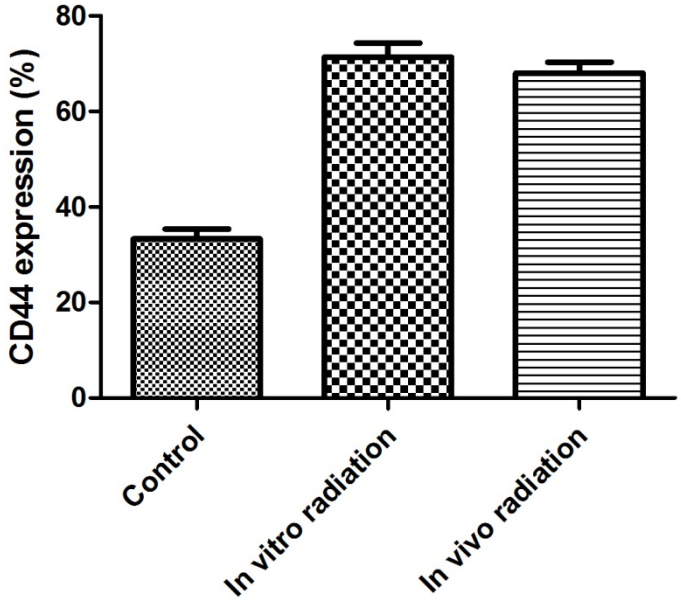

Figure 5.14 Quantitative assessment of CD44 expression in BT74 following radiation treatment using flow cytometric analysis as demonstrated in Figure 5.13. Both in vitro and in vivo radiation significantly increases the percentage of the CD44 expressing sub-population ($p<0.0001$, ANOVA with Tukey post-test).

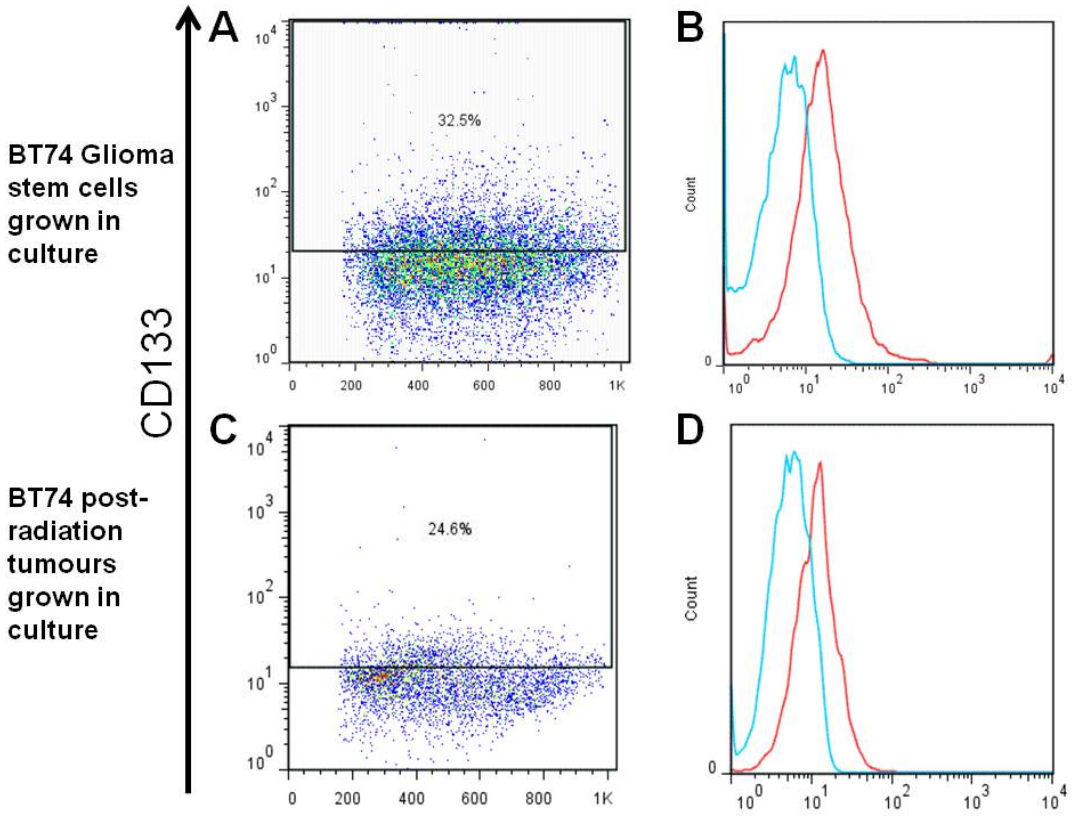

Figure 5.15 CD133 expression in BT74 glioma stem cells in cell culture and following in vivo radiation treatment. (A) CD133 positive glioma stem cells in culture(32.5%) and (C) CD133 positive glioma stem cells following 4Gy in vivo radiation. (B) and (D) are the histrograms representing the CD133 positive populations (red) and the isotype controls (blue) of A and C respectively.

CD133 expression in BT74 glioma stem cells did not demonstrate significant changes following in vivo radiation with 4Gy ($p>0.05$, ANOVA with Tukey post-test). This data is demonstrated in Figures 5.15 and 5.16 suggesting that in this glioma stem cell line (BT74), there was no survival advantage among CD133 positive cells during radiation therapy.

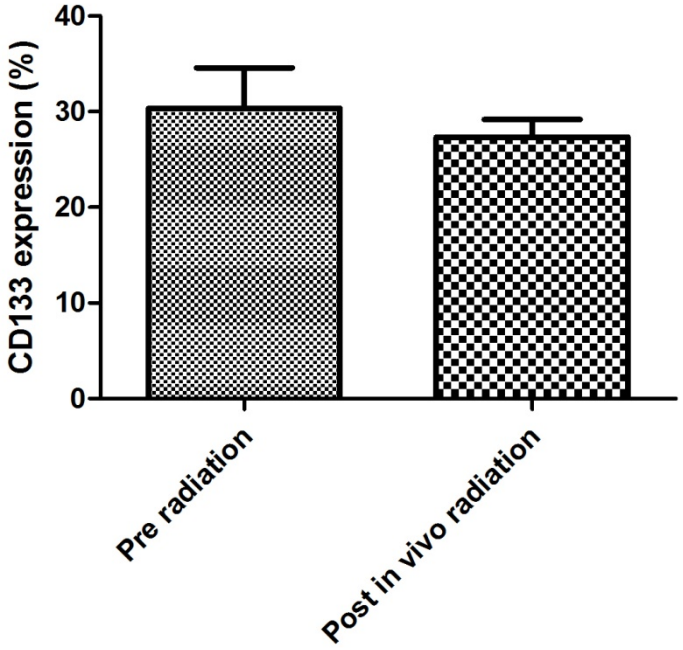

Figure 5.16 Quantitative assessment of CD133 expression in BT74 following radiation treatment using flow cytometric analysis as demonstrated in Figure 5.15. Following in vivo radiation, BT74 glioma stem cells showed no change in the size of the CD133 positive sub-population. ($p>0.05$, ANOVA with Tukey post-test).

5.4. Discussion

Treating glioma stem cell cultures both in vitro and in vivo with radiotherapy has provided interesting insights into their biology. The first point to note is that their resistance to irradiation in vitro is not universal. While even the most radio-sensitive glioma stem cell line harbours a radio-resistant CD44/24 double positive sub-population, the cultured cells overall are sensitive to irradiation. All of the cell lines tested upregulate RR as part of their DNA repair machinery and do this independent of their p53 status. BT74 which was the most resistant has a mutated p53[144], GBM4 is heterozygous for p53 mutation and GBM8 which was relatively sensitive to radiation therapy has wild-type p53 (unpublished data, personal communication from Dr. Hiroaki Wakimoto, Harvard Medical School, Boston, MA, USA).

RR as discussed in Chapter 1, converts ribonucleoside diphosphates to deoxyribonucleotide diphosphates. It is a rate limiting enzyme in the process of creating deoxyribonucleotide triphosphates, the fundamental substrate for DNA repair and synthesis. The precise manner in which RR and the DNA damage machinery respond to ionizing radiation remains unclear. Radiation therapy kills tumour cells by causing double stranded DNA breaks either by directly damaging DNA or by the production of free radicals. The ability of tumour cells to withstand this DNA damage is dependent on a number of factors including its ability to resist pro-apoptotic

pathways and to repair its damaged DNA. The latter process is dependent on RR activity.

Human RR is a heterotetramic enzyme composed of two homodimeric subunits, the large R1 and smaller R2. R1 has a half life of greater than 20 hours and sufficient quantities exist throughout the cell cycle[209]. There are at least two isoforms of the small subunit of RR designated R2 and p53R2[210-213]. The R2 subunit is regulated by the cell cycle and in keeping with its role, its levels are highest during S phase and it is degraded during mitosis.

Two different mechanisms have been postulated for the role of RR in the tumour response to radiotherapy. The first is the upregulation of the R2 subunit and therefore an increase in deoxyribonucleotide diphosphates available for use in the process of DNA repair. A number of preclinical studies have confirmed this hypothesis, including work in cancer cells over-expressing R2 resulting in reduced DNA double-strand breaks following radiotherapy and thus increased resistance[205, 214]. The de novo synthesis of RR following exposure to ionizing radiation requires 18 to 24 hours and levels remain raised for 48 hours[210, 214, 215]. This mechanism is in keeping with the findings of this study where R2 levels were raised 24 hours after exposure to radiotherapy. However, earlier time points were not assayed to monitor for more a rapid change in RR levels. Increases in RR

levels have also been demonstrated as a result of post-transcriptional or DNA damage dependent stabilization[216, 217].

The second mechanism employs p53R2, a p53 inducible form of R2 that requires the wild type form of p53[211]. This mechanism may play a role in the GBM8 cell line, but is unlikely to have a major role in glioblastomas where 35% of tumours have a p53 mutation or homozygous deletion and the p53 signalling pathway is dysfunctional in 87% of tumours[15]. The therapeutic research into RR and radiotherapy has focused primarily on the use of RR inhibitors such as hydroxyurea and triapine to sensitive cancers to the cytotoxic effects of radiotherapy[218, 219]. In the case of hydroxyurea, the therapeutic effect has been questionable and significant adverse effects reported[220, 221].

The use of oncolytic viruses with RR (ICP6) mutations following radiotherapy is an effective strategy for capitalizing on the cellular DNA damage responses of glioma stem cells that are surviving the cytotoxic stress of radiotherapy by repairing their DNA. Interestingly G207 despite containing this mutation was unable to capitalize on the upregulation of RR post-radiotherapy. This may reflect the inability of G207 to overcome the restrictions on its replicative abilities due to its ɣ34.5 deletions. As discussed in Chapter 1 and 3, G47Δ is not encumbered with these difficulties because of its additional α47 mutation which has restored some of the replicative defects suffered from the ɣ34.5 deletions. The synergistic response seen on

the Chou-Talalay analysis of the efficacy of combined G47Δ and radiotherapy is an indicator of the strength of this approach and the advantage of usurping a tumour-protective cellular response, to enhance therapeutic cytotoxicity.

The Chou-Talalay analysis demonstrated a synergistic relationship between G47Δ and radiation therapy in both radiosensitive and radioresistant glioma stem cell lines. The one exception was the CI value for BT74, at a f_a of 0.2, where it was >1, indicating an antagonistic relationship. This phenomenon is not uncommon and the synergy between agents tends to be stronger at higher doses[149]. In addition, in cancer therapeutic studies, the effects at higher doses are more clinically relevant than at low doses[149]. Furthermore, this f_a level is below 4Gy and the ability of radiotherapy to trigger RR upregulation at lower doses in BT74 was not tested.

From a clinical perspective, the sensitization of the tumour to G47Δ by radiation therapy is more likely to produce an effect than vice versa. This is because radiation therapy affects the entire tumour whereas viral therapy, certainly in the initial stages would only affect a small fraction of the tumour. At early timepoints, viral therapy would act mainly along the needle tract and in the vicinity of the injection site. With time, the volume of tumour cells infected will increase and having pre-sensitized the tumour with radiation, the replication of the virus would be expected to be enhanced. Nonetheless, only

with a well planned clinical trial can these questions be answered in human glioblastomas.

In this chapter, the level of expression of CD133 did not correlate with the degree of radioresistance in the small number of cell lines tested. Previous work in this field, which was discussed in section 1.2.2 and 5.1.3, has shown that CD133 positive cells have more efficient DNA repair following irradiation and thus are more radioresistant[29]. In section 1.2.2 the hierarchy of CD133 expression was also discussed and it is possible that classification purely on CD133 expression is insufficient to designate a radioresistant group. Furthermore, no clear pathway linked to the glycoprotein CD133 has been established to provide a mechanism of radioresistance based on CD133 expression. There remains the possibility that a yet undiscovered link exists between CD133 and a cellular pathway of radioresistance. It may be that lessons can be learned from the study of temozolomide resistance in glioma stem cells.

Recent work has suggested that CD133 negative cells may be more able to withstand temozolomide cytotoxicity due to increased activity of the enzyme O6-methylguanine-DNA methyltransferase (MGMT)[222]. MGMT is a DNA repair enzyme and increased levels of MGMT activity in tumour cells mediates resistance to DNA-alkylating agents such as temozolomide[223]. Thus it is MGMT activity that confers chemo-resistance. Similarly for radioresistance, the importance of markers notwithstanding; there remains a

need for the underlying links to the mechanisms of resistance to be defined more clearly.

In section 5.2.6 the CD44/24 double positive sub-population of glioma stem cells demonstrated enhanced survival when in vitro cultures were treated with radiation therapy. The CD44 and CD24 markers of this sub-population of cells are currently being investigated and data thus far has shown that these cells have established pathways of radioresistance (unpublished data, personal communication from Dr. P. Kamalakannan, Ohio State University, USA). CD44 positive glioma stem cells alter their metabolism to produce low levels of reactive oxygen species (unpublished data, personal communication from Dr. P. Kamalakannan, Ohio State University, USA). This is a well-established method of tumour survival during radiation therapy[224]. CD44 expressing glioma stem cells also have higher expression of multidrug resistance (MDR) genes and ABC transporters. The double positive cells also mediate self renewal and tumour initiation via signal transducer and activator of transcription 3 (STAT3) and NANOG pathways (unpublished data, personal communication from Dr. P. Kamalakannan, Ohio State University, USA). This is an area of ongoing work which aims to shed new light on the complex survival pathways of glioma stem cells following radiation therapy. The ability of the virus to infect and kill the radioresistant sub-population of glioma stem cells is a very promising result.

While the in vitro and in vivo efficacy remains promising and the double treated mice group had significant increases in survival, no cures were seen. It is possible that dose escalation studies may yield more successful results, although the more fruitful approach may be multi-model therapy with the addition of further agents that are able to enhance the cytotoxic effects of the G47Δ and radiation therapy.

Chapter 6: Conclusions

Glioblastoma remains one of the most malignant cancers in humans[225]. Despite the best available treatments including radical surgical resection and combined radiotherapy and temozolomide, median survival is less than two years[5]. This is a devastating disease to patients and their families.The invasive nature of this disease precludes any surgical cure. Before any successful treatments of glioblastomas can be developed, we require representative models of this invasive tumour to test therapeutic agents capable of matching the tumour's aggressiveness. In an attempt to meet these needs, the work in this thesis has focused on developing glioma stem cell models and employing oncolytic herpes simplex vectors as anti-glioma agents. The aims were to mimic the human tumour as best as possible in pre-clinical models and then test an agent that if successful could be taken to human clinical trials. It was important that the agent, in this case G47Δ, would fit into existing treatment paradigms which at present are surgical resection followed by radiation and temozolomide therapy. G47Δ was also tested with a transgene expressing an anti-angiogenic agent as anti-angiogenesis is currently an area of intense clinical study in glioblastomas. Furthermore, G47Δ's precursor G207 has undergone extensive preclinical animal model testing and phase I human testing to demonstrate its safety for use in human glioblastoma patients.

In this thesis, glioma stem cell cultures were successfully established and propagated. These cultures were then used as glioblastoma models both in vitro and in vivo to study the use of genetically engineered herpes simplex viruses as anti-glioma agents. Glioma stem cells are able to mimic their parent tumours and produce invasive in vivo tumour models[32, 161]. Glioma stem cell cultures also enabled the study of the interaction between tumours and their microenvironment, specifically endothelial cells, as described in Chapter 4. This led to the testing of a tumstatin transgene expressing vector in in vitro models of glioma stem cells and endothelial cells and in vivo. The glioma stem cells were also able to mimic the radioresistance seen in human tumours and allow the study of novel approaches to overcoming this resistance. All of this work was carried out using a mixed population of CD133 positive and negative cells. This model of using a mixed population of cells worked well and consistently formed tumours in vivo. In addition, this model is in keeping with the evolution of our understanding of the role of CD133 expression in glioma stem cells as discussed in Chapter 1.

The results in Chapter 5 demonstrated the need for further study into new markers for identifying a radioresistant subpopulation of glioma stem cells. This work is currently in progress by another group investigating the mechanisms of radioresistance by CD44/24 double positive cells. In addition, further study is warranted into the DNA repair mechanisms of glioma stem cells following radiotherapy to better understand these pathways and target them with new therapeutic agents. In Chapter 5, one strategy using a

ribonucleotide reductase deficient herpes virus was shown, but other methods including using ribonucleotide reductase inhibitors to disrupt the DNA repair machinery of glioma stem cells may also be of clinical utility.

Developing a pre-clinical model that accurately resembles human glioblastomas is of major importance if we are to efficiently test novel therapeutics and predict success in clinical trials. In Chapter 3 we reviewed the strengths and weaknesses of glioma stem cell models and other preclinical models of glioblastomas. It is likely that no one model will truly establish itself as the best and most accurate representation of human glioblastoma. What is more probable is that each model has its strengths and weaknesses, and each contributes a part to our understanding of this complex and intricate cancer. To that end the development of glioma stem cell models is another important and useful addition in the search for new treatments to corral one of the most malignant of human cancers.

All three of the results chapters in this thesis investigated the use of oncolytic herpes viruses against glioma stem cells. Overall the in vitro results demonstrated excellent efficacy but the in vivo results were mixed. In Chapter 3 G47Δ was able to extend survival in mice with xenograft tumours formed by glioma stem cells. Bearing in mind the previously reported radioresistance and chemoresistance of glioma stem cells[29, 37], the use of oncolytic herpes viruses represents a valuable addition to our armamentarium of anti-glioma therapies. In Chapter 4, the efficacy of

tumstatin expressing G47Δ was tested against G47Δ-Empty. The addition of the transgene was efficacious in vitro, but did not appear to confer an in vivo killing advantage against the glioma stem cells. The possible reasons for this were discussed in Chapter 4. Part of the failure of the in vivo experiment could possibly be due to the innate ability of G47Δ to target endothelial cells without the tumstatin transgene. G47Δ did effectively infect and kill glioma stem cells and endothelial cells, the two primary components of the glioma stem cell perivascular niche. This is an important advantage when considering G47Δ for use in clinical trials.

In Chapter 5, G47Δ demonstrated synergy with radiation therapy, the most common treatment administered to glioblastoma patients. The survival of mice was extended considerably by dual treatment compared to even G47Δ alone. While these results are very promising, no cures were seen in any of mice with xenograft tumours, a feature that unfortunately mirrors the clinical outcome of human glioblastomas.

To truly understand why the treatments studied did not result in complete cures and why this outcome eludes us in clinical practice, it may first be necessary to understand the origins of glioma stem cells. Two possibilities have been hypothesized. The first is that glioma stem cells are normal neural stem cells that have acquired mutations resulting in their transformation into glioma stem cells. The second is that glioma stem cells are differentiated glial cells that have transformed and de-differentiated into

glioma stem cells. At present there is no clear evidence for either aetiology and this question is thus difficult to answer[226-230]. If glioma stem cells do originate from normal neural stem cells, it would therefore follow that they would possess all the DNA repair mechanisms and cellular protective mechanisms of neural stem cells[231]. These defence mechanisms would have evolved over millions of generations to protect us from toxic agents that we may ingest, background ionizing radiation and even viruses. Normal neural stem cells are also known to secrete pro-angiogenic agents and transdifferentiate into endothelial cells, features that have been adapted by glioma stem cells[232, 233].

To combat such a formidable enemy we have enlisted the use of viruses that have also evolved with humans, to infect our cells and in the case of herpes simplex viruses as part of their life cycle, lyse human cells. The reports of viruses killing tumour cells extend back for over a century[59, 60]. The first report of a clinical trial of viral therapy for malignant disease dates back to 1949, where patients with Hodgkin's lymphoma were administered with infectious hepatitis virus[234]. 7 of the 22 patients demonstrated improvements. However, this form of a clinical trial is almost unrecognizable today and the side effects suffered by trial participants included death and the development of hepatitis. Over the last two decades we have ushered in the modern era of viral oncolytic therapy by successfully harnessing the potential of viruses by genetically engineering them to target human tumours selectively. While G47Δ demonstrated efficacy against glioma stem cells in all three major investigations in this thesis, more study in

this area is required. To reach our goal of successfully treating human glioblastomas with oncolytic viruses, we need to examine further the specific obstacles that are hindering oncolytic virotherapy. Tumour specific characteristics and the barriers in the microenvironment surrounding the tumour are areas of current study. By delivering the virus directly into glioblastomas or their tumour bed following surgical resection, we are circumventing the problems with systemic delivery and the difficulties with penetrating the blood-brain barrier.

One major consideration for viral propagation is the role of the immune system in modulating virus and tumour activity. Thus far, the effect of the immune system is one feature that cannot be studied in mouse models of glioma stem cells. This is an inherent weakness of any xenograft model. The host immune response to an oncolytic virus is a double-edged sword that can result in viral clearance or enhance T-cell mediated anti-tumour pathways[235, 236]. The presence of anti-HSV antibodies in humans is another hurdle not seen in murine models[237, 238]. Pre-immunization of rat models to HSV-1 lowers the efficacy of HSV-1 based oncolytic vectors[239]. Various immune cells are thought to limit the propagation of herpes simplex viral oncolytic therapy including neutrophils, microglia and natural killer cells[240, 241]. Preclinical work has demonstrated that administration of cyclophosphamide to inhibit this immune response enhances the effect of a mutant ICP6 oncolytic HSV-1 vector[240, 242]. These studies highlight the importance of considering the use of adjuvant immunosuppressants in any trial of oncolytic herpes vectors.

Another consideration is the ability of the virus to spread within tumours, particularly highly invasive tumours such as glioblastoma. Recent work by our group has shown that after only 24 hours post-injection of G47Δ in a highly invasive glioma stem cell model, G47Δ had spread a significant distance and was infecting invasive glioma stem cells[32]. Glioblastomas also protect themselves by maintaining areas of high interstitial pressures and hypoxic regions which protect tumour cells from standard treatments. The effect of tumour interstitial pressures and virus delivery requires more study. In contrast to standard anti-glioma therapeutics, hypoxia enhances the replication of oncolytic herpes simplex viruses, a significant advantage when targeting glioma stem cells[243].

The real strength of oncolytic herpes viruses is their ability to synergise with existing treatment paradigms. Radiation therapy, like viruses, has been used to treat cancers for over a century[244]. The studies in Chapter 5 examined for the first time the combined benefit of these agents against glioma stem cells. The results were very promising with enhanced efficacy seen in vitro and in vivo. Furthermore, oncolytic viruses were able to kill a subset of CD44/24 double positive radioresistant cells. Similar cytotoxic efficacy has also been shown by G47Δ in combination with temozolomide and etoposide in glioma stem cell models[203, 204]. Radiation therapy and temozolomide are the current standard of care following surgical resection. Given the pre-clinical evidence for the use of G47Δ, not only as a single

agent but also in combination therapy, there is a need for a clinical trial of G47Δ in conjunction with standard adjuvant therapy. As radiation therapy is mainly used in glioblastoma at first presentation and not in recurrent disease, a G47Δ trial in primary disease should be considered. There may also be a role for the use of anti-angiogenic agents. Blood vessels formed by glioblastomas are structurally and functionally abnormal and play a protective role by raising interstitial fluid pressures and creating hypoxic areas within the tumour[245]. The effects of vessel normalization by anti-angiogenic agents, particularly anti-VEGF agents, not only enhances the cytotoxicity of radiation therapy by paradoxically improving oxygenation, but also reduces interstitial fluid pressures in glioblastomas[245]. Both these mechanisms may further potentiate the action of G47Δ.

The use of oncolytic viruses is expanding and with our increased understanding of their use in combination with currently employed chemotherapeutic and radiation regimes, this trend will continue. Their self-replication and propagation combined with the genetically engineered attenuation in normal tissues makes them an ideal agent to combat tumour cells that have themselves lost self-regulation of cellular proliferation. Further study into the interplay between viruses and their human hosts especially the role of innate immunity, is required to enhance the anti-tumour effect of oncolytic viruses while maintaining their attenuation in normal tissues. There is mounting pre-clinical evidence suggesting that the time is ripe for clinical trials of oncolytic viruses such as G47Δ. Only with well planned clinical trials which take into account the strengths and weaknesses of G47Δ and from the

outset plan to quantify the effects of G47Δ therapy on human glioma stem cells in vivo, will we start to evaluate the true potential of oncolytic virotherapy and the utility of testing therapeutics in pre-clinical glioma stem cell models.

Appendix 1: **Presentations arising from this work**

The role of combined radiotherapy and oncolytic herpes simplex virus in treating radio-resistant glioma stem cells

Jeyaretna DS, Palanichamy K, Barnard Z, Wakimoto H, Kesari S, Curry WT, Chakravarti A, Rabkin S, Martuza RL.

World Federation of Neurosurgeons World Congress

August 30th-September 4th 2009. Boston, Massachusetts.

Glioma therapy using an oncolytic herpes simplex virus armed with tumstatin

Jeyaretna DS, Wakimoto H, Buhrman J, Kuroda T, Rabkin S, Martuza R.

American Association of Neurosurgeons Annual Meeting

2nd-6th May 2009. San Diego, California

Modelling the Glioma Stem Cell - Endothelial Cell Interaction and its Susceptibility to Oncolytic Herpes Simplex Virus

Jeyaretna DS, Wakimoto H, Rabkin S, Martuza R.

American Society of Gene Therapy 11th Annual Meeting

May 28th-June 1st 2008. Boston, Massachusetts

References

1. Wen, P.Y. and S. Kesari, *Malignant gliomas in adults.* N Engl J Med, 2008. **359**(5): p. 492-507.
2. *CBTRUS 2012 statistical report: Primary Brain and Central Nervous System Tumors Diagnosed in the United States in 2004-2008.*, Central Brain Tumor Registry of the United States.
3. Fisher, J.L., et al., *Epidemiology of brain tumors.* Neurol Clin, 2007. **25**(4): p. 867-90, vii.
4. Porter, K.R., et al., *Prevalence estimates for primary brain tumors in the United States by age, gender, behavior, and histology.* Neuro Oncol. **12**(6): p. 520-7.
5. Stupp, R., et al., *Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma.* N Engl J Med, 2005. **352**(10): p. 987-96.
6. Sanai, N., et al., *An extent of resection threshold for newly diagnosed glioblastomas.* J Neurosurg. **115**(1): p. 3-8.
7. Laperriere, N., L. Zuraw, and G. Cairncross, *Radiotherapy for newly diagnosed malignant glioma in adults: a systematic review.* Radiother Oncol, 2002. **64**(3): p. 259-73.
8. Shapiro, W.R., et al., *Randomized trial of three chemotherapy regimens and two radiotherapy regimens and two radiotherapy regimens in postoperative treatment of malignant glioma. Brain Tumor Cooperative Group Trial 8001.* J Neurosurg, 1989. **71**(1): p. 1-9.
9. Stupp, R., et al., *Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial.* Lancet Oncol, 2009. **10**(5): p. 459-66.
10. Lamborn, K.R., et al., *Progression-free survival: an important end point in evaluating therapy for recurrent high-grade gliomas.* Neuro Oncol, 2008. **10**(2): p. 162-70.
11. Wong, E.T., et al., *Outcomes and prognostic factors in recurrent glioma patients enrolled onto phase II clinical trials.* J Clin Oncol, 1999. **17**(8): p. 2572-8.
12. Stummer, W., et al., *Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial.* Lancet Oncol, 2006. **7**(5): p. 392-401.
13. Stummer, W., et al., *Extent of resection and survival in glioblastoma multiforme: identification of and adjustment for bias.* Neurosurgery, 2008. **62**(3): p. 564-76; discussion 564-76.
14. Lacroix, M., et al., *A multivariate analysis of 416 patients with glioblastoma multiforme: prognosis, extent of resection, and survival.* J Neurosurg, 2001. **95**(2): p. 190-8.
15. *Comprehensive genomic characterization defines human glioblastoma genes and core pathways.* Nature, 2008. **455**(7216): p. 1061-8.

16. Villalonga-Planells, R., et al., *Activation of p53 by nutlin-3a induces apoptosis and cellular senescence in human glioblastoma multiforme.* PLoS One. **6**(4): p. e18588.
17. Lee, J.J., et al., *PTEN status switches cell fate between premature senescence and apoptosis in glioma exposed to ionizing radiation.* Cell Death Differ. **18**(4): p. 666-77.
18. Parsons, D.W., et al., *An integrated genomic analysis of human glioblastoma multiforme.* Science, 2008. **321**(5897): p. 1807-12.
19. Singh, S.K., et al., *Identification of human brain tumour initiating cells.* Nature, 2004. **432**(7015): p. 396-401.
20. Liu, T.C., E. Galanis, and D. Kirn, *Clinical trial results with oncolytic virotherapy: a century of promise, a decade of progress.* Nat Clin Pract Oncol, 2007. **4**(2): p. 101-17.
21. Clarke, M.F., et al., *Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells.* Cancer Res, 2006. **66**(19): p. 9339-44.
22. Ricci-Vitiani, L., et al., *Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells.* Nature. **468**(7325): p. 824-8.
23. Wang, R., et al., *Glioblastoma stem-like cells give rise to tumour endothelium.* Nature. **468**(7325): p. 829-33.
24. Lapidot, T., et al., *A cell initiating human acute myeloid leukaemia after transplantation into SCID mice.* Nature, 1994. **367**(6464): p. 645-8.
25. Bonnet, D. and J.E. Dick, *Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell.* Nat Med, 1997. **3**(7): p. 730-7.
26. Al-Hajj, M., et al., *Prospective identification of tumorigenic breast cancer cells.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 3983-8.
27. Singh, S.K., et al., *Identification of a cancer stem cell in human brain tumors.* Cancer Res, 2003. **63**(18): p. 5821-8.
28. Facchino, S., et al., *BMI1 confers radioresistance to normal and cancerous neural stem cells through recruitment of the DNA damage response machinery.* J Neurosci. **30**(30): p. 10096-111.
29. Bao, S., et al., *Glioma stem cells promote radioresistance by preferential activation of the DNA damage response.* Nature, 2006. **444**(7120): p. 756-60.
30. Bao, S., et al., *Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor.* Cancer Res, 2006. **66**(16): p. 7843-8.
31. Bao, S., et al., *Targeting cancer stem cells through L1CAM suppresses glioma growth.* Cancer Res, 2008. **68**(15): p. 6043-8.
32. Wakimoto, H., et al., *Human glioblastoma-derived cancer stem cells: establishment of invasive glioma models and treatment with oncolytic herpes simplex virus vectors.* Cancer Res, 2009. **69**(8): p. 3472-81.
33. Wang, J., et al., *CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells.* Int J Cancer, 2008. **122**(4): p. 761-8.
34. Son, M.J., et al., *SSEA-1 is an enrichment marker for tumor-initiating cells in human glioblastoma.* Cell Stem Cell, 2009. **4**(5): p. 440-52.

35. Salven, P., et al., *VEGFR-3 and CD133 identify a population of CD34+ lymphatic/vascular endothelial precursor cells.* Blood, 2003. **101**(1): p. 168-72.
36. Uchida, N., et al., *Direct isolation of human central nervous system stem cells.* Proc Natl Acad Sci U S A, 2000. **97**(26): p. 14720-5.
37. Eramo, A., et al., *Chemotherapy resistance of glioblastoma stem cells.* Cell Death Differ, 2006. **13**(7): p. 1238-41.
38. Kang, M.K. and S.K. Kang, *Tumorigenesis of chemotherapeutic drug-resistant cancer stem-like cells in brain glioma.* Stem Cells Dev, 2007. **16**(5): p. 837-47.
39. Tamura, K., et al., *Accumulation of CD133-positive glioma cells after high-dose irradiation by Gamma Knife surgery plus external beam radiation.* J Neurosurg. **113**(2): p. 310-8.
40. Beier, D., et al., *CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles.* Cancer Res, 2007. **67**(9): p. 4010-5.
41. Joo, K.M., et al., *Clinical and biological implications of CD133-positive and CD133-negative cells in glioblastomas.* Lab Invest, 2008. **88**(8): p. 808-15.
42. Jaksch, M., et al., *Cell cycle-dependent variation of a CD133 epitope in human embryonic stem cell, colon cancer, and melanoma cell lines.* Cancer Res, 2008. **68**(19): p. 7882-6.
43. Chen, R., et al., *A hierarchy of self-renewing tumor-initiating cell types in glioblastoma.* Cancer Cell. **17**(4): p. 362-75.
44. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
45. Folkman, J., *Tumor angiogenesis: therapeutic implications.* N Engl J Med, 1971. **285**(21): p. 1182-6.
46. Goel, S., et al., *Normalization of the vasculature for treatment of cancer and other diseases.* Physiol Rev. **91**(3): p. 1071-121.
47. Calabrese, C., et al., *A perivascular niche for brain tumor stem cells.* Cancer Cell, 2007. **11**(1): p. 69-82.
48. Lathia, J.D., et al., *Integrin alpha 6 regulates glioblastoma stem cells.* Cell Stem Cell. **6**(5): p. 421-32.
49. Charles, N., et al., *Perivascular nitric oxide activates notch signaling and promotes stem-like character in PDGF-induced glioma cells.* Cell Stem Cell. **6**(2): p. 141-52.
50. Hovinga, K.E., et al., *Inhibition of notch signaling in glioblastoma targets cancer stem cells via an endothelial cell intermediate.* Stem Cells. **28**(6): p. 1019-29.
51. Folkins, C., et al., *Glioma tumor stem-like cells promote tumor angiogenesis and vasculogenesis via vascular endothelial growth factor and stromal-derived factor 1.* Cancer Res, 2009. **69**(18): p. 7243-51.
52. Ikushima, H., et al., *Autocrine TGF-beta signaling maintains tumorigenicity of glioma-initiating cells through Sry-related HMG-box factors.* Cell Stem Cell, 2009. **5**(5): p. 504-14.
53. Gilbertson, R.J. and J.N. Rich, *Making a tumour's bed: glioblastoma stem cells and the vascular niche.* Nat Rev Cancer, 2007. **7**(10): p. 733-6.

54. Eyler, C.E., et al., *Glioma stem cell proliferation and tumor growth are promoted by nitric oxide synthase-2.* Cell. **146**(1): p. 53-66.
55. Shih, A.H. and E.C. Holland, *Notch signaling enhances nestin expression in gliomas.* Neoplasia, 2006. **8**(12): p. 1072-82.
56. Zhang, X.P., et al., *Notch activation promotes cell proliferation and the formation of neural stem cell-like colonies in human glioma cells.* Mol Cell Biochem, 2008. **307**(1-2): p. 101-8.
57. Bergers, G. and L.E. Benjamin, *Tumorigenesis and the angiogenic switch.* Nat Rev Cancer, 2003. **3**(6): p. 401-10.
58. Folkins, C., et al., *Anticancer therapies combining antiangiogenic and tumor cell cytotoxic effects reduce the tumor stem-like cell fraction in glioma xenograft tumors.* Cancer Res, 2007. **67**(8): p. 3560-4.
59. Kelly, E. and S.J. Russell, *History of oncolytic viruses: genesis to genetic engineering.* Mol Ther, 2007. **15**(4): p. 651-9.
60. Dock, G., *The influence of complicating diseases upon leukemia.* Am J Med Sci, 1904. **127**: p. 563-592.
61. Harrow, S., et al., *HSV1716 injection into the brain adjacent to tumour following surgical resection of high-grade glioma: safety data and long-term survival.* Gene Ther, 2004. **11**(22): p. 1648-58.
62. Markert, J.M., et al., *Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial.* Gene Ther, 2000. **7**(10): p. 867-74.
63. Sandmair, A.M., et al., *Thymidine kinase gene therapy for human malignant glioma, using replication-deficient retroviruses or adenoviruses.* Hum Gene Ther, 2000. **11**(16): p. 2197-205.
64. Immonen, A., et al., *AdvHSV-tk gene therapy with intravenous ganciclovir improves survival in human malignant glioma: a randomised, controlled study.* Mol Ther, 2004. **10**(5): p. 967-72.
65. Shand, N., et al., *A phase 1-2 clinical trial of gene therapy for recurrent glioblastoma multiforme by tumor transduction with the herpes simplex thymidine kinase gene followed by ganciclovir. GLI328 European-Canadian Study Group.* Hum Gene Ther, 1999. **10**(14): p. 2325-35.
66. Aghi, M. and S. Rabkin, *Viral vectors as therapeutic agents for glioblastoma.* Curr Opin Mol Ther, 2005. **7**(5): p. 419-30.
67. Jeyaretna, D.S. and T. Kuroda, *Recent advances in the development of oncolytic HSV-1 vectors: 'arming' of HSV-1 vectors and application of bacterial artificial chromosome technology for their construction.* Curr Opin Mol Ther, 2007. **9**(5): p. 447-66.
68. Jeyaretna, D.S., S.D. Rabkin, and R.L. Martuza, *Oncolytic herpes simplex virus therapy for peripheral nerve tumors.* Neurosurg Focus, 2007. **22**(6): p. E4.
69. Yu, Y.A., et al., *Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins.* Nat Biotechnol, 2004. **22**(3): p. 313-20.
70. Kirn, D., R.L. Martuza, and J. Zwiebel, *Replication-selective virotherapy for cancer: Biological principles, risk management and future directions.* Nat Med, 2001. **7**(7): p. 781-7.

71. Martuza, R.L., et al., *Experimental therapy of human glioma by means of a genetically engineered virus mutant.* Science, 1991. **252**(5007): p. 854-6.
72. Evans, J.S., et al., *Herpesviral thymidine kinases: laxity and resistance by design.* J Gen Virol, 1998. **79 (Pt 9)**: p. 2083-92.
73. Markert, J.M., et al., *Reduction and elimination of encephalitis in an experimental glioma therapy model with attenuated herpes simplex mutants that retain susceptibility to acyclovir.* Neurosurgery, 1993. **32**(4): p. 597-603.
74. Kambara, H., et al., *An oncolytic HSV-1 mutant expressing ICP34.5 under control of a nestin promoter increases survival of animals even when symptomatic from a brain tumor.* Cancer Res, 2005. **65**(7): p. 2832-9.
75. Frampton, A.R., Jr., et al., *HSV trafficking and development of gene therapy vectors with applications in the nervous system.* Gene Ther, 2005. **12**(11): p. 891-901.
76. Todo, T., et al., *Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing.* Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6396-401.
77. Kemeny, N., et al., *Phase I, open-label, dose-escalating study of a genetically engineered herpes simplex virus, NV1020, in subjects with metastatic colorectal carcinoma to the liver.* Hum Gene Ther, 2006. **17**(12): p. 1214-24.
78. Kirn, D., *Clinical research results with dl1520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned?* Gene Ther, 2001. **8**(2): p. 89-98.
79. Hadaschik, B.A., et al., *Oncolytic vesicular stomatitis viruses are potent agents for intravesical treatment of high-risk bladder cancer.* Cancer Res, 2008. **68**(12): p. 4506-10.
80. Toyoda, H., et al., *Oncolytic treatment and cure of neuroblastoma by a novel attenuated poliovirus in a novel poliovirus-susceptible animal model.* Cancer Res, 2007. **67**(6): p. 2857-64.
81. Nakamura, T. and S.J. Russell, *Oncolytic measles viruses for cancer therapy.* Expert Opin Biol Ther, 2004. **4**(10): p. 1685-92.
82. Pecora, A.L., et al., *Phase I trial of intravenous administration of PV701, an oncolytic virus, in patients with advanced solid cancers.* J Clin Oncol, 2002. **20**(9): p. 2251-66.
83. Lorence, R.M., et al., *Overview of phase I studies of intravenous administration of PV701, an oncolytic virus.* Curr Opin Mol Ther, 2003. **5**(6): p. 618-24.
84. Freeman, A.I., et al., *Phase I/II trial of intravenous NDV-HUJ oncolytic virus in recurrent glioblastoma multiforme.* Mol Ther, 2006. **13**(1): p. 221-8.
85. Kelly, K., et al., *Reovirus-based therapy for cancer.* Expert Opin Biol Ther, 2009. **9**(7): p. 817-30.
86. Southam, C.M. and A.E. Moore, *Clinical studies of viruses as antineoplastic agents with particular reference to Egypt 101 virus.* Cancer, 1952. **5**(5): p. 1025-34.
87. Southam, C.M. and A.E. Moore, *West Nile, Ilheus, and Bunyamwera virus infections in man.* Am J Trop Med Hyg, 1951. **31**(6): p. 724-41.

88. Southam, C.M., B. Bronstein, and L.F. Webber, *Effect of West Nile and Ilheus viruses on mouse leukemias.* Cancer Res, 1951. **11**(9): p. 669-75.
89. Okuno, Y., et al., *Studies on the use of mumps virus for treatment of human cancer.* Biken J, 1978. **21**(2): p. 37-49.
90. Asada, T., *Treatment of human cancer with mumps virus.* Cancer, 1974. **34**(6): p. 1907-28.
91. Shimizu, Y., et al., *Immunotherapy of advanced gynecologic cancer patients utilizing mumps virus.* Cancer Detect Prev, 1988. **12**(1-6): p. 487-95.
92. Ward, P.L. and B. Roizman, *Herpes simplex genes: the blueprint of a successful human pathogen.* Trends Genet, 1994. **10**(8): p. 267-74.
93. Grandi, P., et al., *Design and application of oncolytic HSV vectors for glioblastoma therapy.* Expert Rev Neurother, 2009. **9**(4): p. 505-17.
94. Efstathiou, S., et al., *The role of herpes simplex virus type 1 thymidine kinase in pathogenesis.* J Gen Virol, 1989. **70 (Pt 4)**: p. 869-79.
95. Meignier, B., et al., *Virulence of and establishment of latency by genetically engineered deletion mutants of herpes simplex virus 1.* Virology, 1988. **162**(1): p. 251-4.
96. Chou, J., et al., *Mapping of herpes simplex virus-1 neurovirulence to gamma 134.5, a gene nonessential for growth in culture.* Science, 1990. **250**(4985): p. 1262-6.
97. Cameron, J.M., et al., *Ribonucleotide reductase encoded by herpes simplex virus is a determinant of the pathogenicity of the virus in mice and a valid antiviral target.* J Gen Virol, 1988. **69 (Pt 10)**: p. 2607-12.
98. Sedarati, F. and J.G. Stevens, *Biological basis for virulence of three strains of herpes simplex virus type 1.* J Gen Virol, 1987. **68 (Pt 9)**: p. 2389-95.
99. Martuza, R.L., *Conditionally replicating herpes vectors for cancer therapy.* J Clin Invest, 2000. **105**(7): p. 841-6.
100. Spear, P.G. and R. Longnecker, *Herpesvirus entry: an update.* J Virol, 2003. **77**(19): p. 10179-85.
101. Guzman, G., et al., *Expression of entry receptor nectin-1 of herpes simplex virus 1 and/or herpes simplex virus 2 in normal and neoplastic human nervous system tissues.* Acta Virol, 2006. **50**(1): p. 59-66.
102. Shah, A., et al., *HSV-1 infection of human corneal epithelial cells: receptor-mediated entry and trends of re-infection.* Mol Vis. **16**: p. 2476-86.
103. Tiwari, V., et al., *Herpes simplex virus type 2 entry into cultured human corneal fibroblasts is mediated by herpesvirus entry mediator.* J Gen Virol, 2007. **88**(Pt 8): p. 2106-10.
104. Heldwein, E.E. and C. Krummenacher, *Entry of herpesviruses into mammalian cells.* Cell Mol Life Sci, 2008. **65**(11): p. 1653-68.
105. Wald A, C.L., in *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis.*, C.-F.G. Arvin A, Mocarski E, et al., Editor. 2007, Cambridge University Press. Cambridge.
106. Mineta, T., S.D. Rabkin, and R.L. Martuza, *Treatment of malignant gliomas using ganciclovir-hypersensitive, ribonucleotide reductase-deficient herpes simplex viral mutant.* Cancer Res, 1994. **54**(15): p. 3963-6.

107. Mineta, T., et al., *Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas.* Nat Med, 1995. **1**(9): p. 938-43.
108. Goldstein, D.J. and S.K. Weller, *Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 lacZ insertion mutant.* J Virol, 1988. **62**(1): p. 196-205.
109. Nakao, A., et al., *Clinical experiment of mutant herpes simplex virus HF10 therapy for cancer.* Curr Cancer Drug Targets, 2007. **7**(2): p. 169-74.
110. MacLean, A.R., et al., *Herpes simplex virus type 1 deletion variants 1714 and 1716 pinpoint neurovirulence-related sequences in Glasgow strain 17+ between immediate early gene 1 and the 'a' sequence.* J Gen Virol, 1991. **72 (Pt 3)**: p. 631-9.
111. Meignier, B., et al., *In vivo behavior of genetically engineered herpes simplex viruses R7017 and R7020. II. Studies in immunocompetent and immunosuppressed owl monkeys (Aotus trivirgatus).* J Infect Dis, 1990. **162**(2): p. 313-21.
112. Chambers, R., et al., *Comparison of genetically engineered herpes simplex viruses for the treatment of brain tumors in a scid mouse model of human malignant glioma.* Proc Natl Acad Sci U S A, 1995. **92**(5): p. 1411-5.
113. He, B., M. Gross, and B. Roizman, *The gamma(1)34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1alpha to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase.* Proc Natl Acad Sci U S A, 1997. **94**(3): p. 843-8.
114. Chou, J. and B. Roizman, *The gamma 1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programed cell death in neuronal cells.* Proc Natl Acad Sci U S A, 1992. **89**(8): p. 3266-70.
115. Sarinella, F., et al., *Oncolysis of pancreatic tumour cells by a gamma34.5-deleted HSV-1 does not rely upon Ras-activation, but on the PI 3-kinase pathway.* Gene Ther, 2006. **13**(14): p. 1080-7.
116. Andreansky, S., et al., *Evaluation of genetically engineered herpes simplex viruses as oncolytic agents for human malignant brain tumors.* Cancer Res, 1997. **57**(8): p. 1502-9.
117. Smith, K.D., et al., *Activated MEK suppresses activation of PKR and enables efficient replication and in vivo oncolysis by Deltagamma(1)34.5 mutants of herpes simplex virus 1.* J Virol, 2006. **80**(3): p. 1110-20.
118. Farassati, F., A.D. Yang, and P.W. Lee, *Oncogenes in Ras signalling pathway dictate host-cell permissiveness to herpes simplex virus 1.* Nat Cell Biol, 2001. **3**(8): p. 745-50.
119. Kim, S.H., et al., *Human breast cancer cells contain elevated levels and activity of the protein kinase, PKR.* Oncogene, 2000. **19**(27): p. 3086-94.
120. Kim, S.H., et al., *Neoplastic progression in melanoma and colon cancer is associated with increased expression and activity of the*

interferon-inducible protein kinase, PKR. Oncogene, 2002. **21**(57): p. 8741-8.
121. Boviatsis, E.J., et al., *Antitumor activity and reporter gene transfer into rat brain neoplasms inoculated with herpes simplex virus vectors defective in thymidine kinase or ribonucleotide reductase.* Gene Ther, 1994. **1**(5): p. 323-31.
122. Mohr, I., et al., *A herpes simplex virus type 1 gamma34.5 second-site suppressor mutant that exhibits enhanced growth in cultured glioblastoma cells is severely attenuated in animals.* J Virol, 2001. **75**(11): p. 5189-96.
123. Vossen, M.T., et al., *Viral immune evasion: a masterpiece of evolution.* Immunogenetics, 2002. **54**(8): p. 527-42.
124. Dunn, G.P., et al., *Cancer immunoediting: from immunosurveillance to tumor escape.* Nat Immunol, 2002. **3**(11): p. 991-8.
125. Hill, A., et al., *Herpes simplex virus turns off the TAP to evade host immunity.* Nature, 1995. **375**(6530): p. 411-5.
126. Jennings, S.R., et al., *Effect of herpes simplex virus types 1 and 2 on surface expression of class I major histocompatibility complex antigens on infected cells.* J Virol, 1985. **56**(3): p. 757-66.
127. York, I.A., et al., *A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes.* Cell, 1994. **77**(4): p. 525-35.
128. Jugovic, P., et al., *Inhibition of major histocompatibility complex class I antigen presentation in pig and primate cells by herpes simplex virus type 1 and 2 ICP47.* J Virol, 1998. **72**(6): p. 5076-84.
129. Tomazin, R., et al., *Herpes simplex virus type 2 ICP47 inhibits human TAP but not mouse TAP.* J Virol, 1998. **72**(3): p. 2560-3.
130. Ahn, K., et al., *Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47.* Embo J, 1996. **15**(13): p. 3247-55.
131. Mavromara-Nazos, P., M. Ackermann, and B. Roizman, *Construction and properties of a viable herpes simplex virus 1 recombinant lacking coding sequences of the alpha 47 gene.* J Virol, 1986. **60**(2): p. 807-12.
132. Mulvey, M., et al., *Regulation of eIF2alpha phosphorylation by different functions that act during discrete phases in the herpes simplex virus type 1 life cycle.* J Virol, 2003. **77**(20): p. 10917-28.
133. Cassady, K.A., M. Gross, and B. Roizman, *The herpes simplex virus US11 protein effectively compensates for the gamma1(34.5) gene if present before activation of protein kinase R by precluding its phosphorylation and that of the alpha subunit of eukaryotic translation initiation factor 2.* J Virol, 1998. **72**(11): p. 8620-6.
134. Cassady, K.A., M. Gross, and B. Roizman, *The second-site mutation in the herpes simplex virus recombinants lacking the gamma134.5 genes precludes shutoff of protein synthesis by blocking the phosphorylation of eIF-2alpha.* J Virol, 1998. **72**(9): p. 7005-11.
135. Dix, R.D., R.R. McKendall, and J.R. Baringer, *Comparative neurovirulence of herpes simplex virus type 1 strains after peripheral or intracerebral inoculation of BALB/c mice.* Infect Immun, 1983. **40**(1): p. 103-12.

136. Sundaresan, P., et al., *Attenuated, replication-competent herpes simplex virus type 1 mutant G207: safety evaluation in mice.* J Virol, 2000. **74**(8): p. 3832-41.
137. Katzin, D.S., et al., *Experimental herpes simplex infection in the owl monkey.* Proc Soc Exp Biol Med, 1967. **125**(2): p. 391-8.
138. Hunt, R.D. and L.V. Melendez, *Herpes virus infections of non-human primates: a review.* Lab Anim Care, 1969. **19**(2): p. 221-34.
139. Melendez, L.V., et al., *Natural herpes simplex infection in the owl monkey (Aotus trivirgatus).* Lab Anim Care, 1969. **19**(1): p. 38-45.
140. Hunter, W.D., et al., *Attenuated, replication-competent herpes simplex virus type 1 mutant G207: safety evaluation of intracerebral injection in nonhuman primates.* J Virol, 1999. **73**(8): p. 6319-26.
141. Todo, T., et al., *Viral shedding and biodistribution of G207, a multimutated, conditionally replicating herpes simplex virus type 1, after intracerebral inoculation in aotus.* Mol Ther, 2000. **2**(6): p. 588-95.
142. Rampling, R., et al., *Toxicity evaluation of replication-competent herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma.* Gene Ther, 2000. **7**(10): p. 859-66.
143. Wong, H.H., N.R. Lemoine, and Y. Wang, *Oncolytic Viruses for Cancer Therapy: Overcoming the Obstacles.* Viruses. **2**(1): p. 78-106.
144. Pandita, A., et al., *Contrasting in vivo and in vitro fates of glioblastoma cell subpopulations with amplified EGFR.* Genes Chromosomes Cancer, 2004. **39**(1): p. 29-36.
145. Todo, T., et al., *In situ expression of soluble B7-1 in the context of oncolytic herpes simplex virus induces potent antitumor immunity.* Cancer Res, 2001. **61**(1): p. 153-61.
146. Fukuhara, H., et al., *Triple gene-deleted oncolytic herpes simplex virus vector double-armed with interleukin 18 and soluble B7-1 constructed by bacterial artificial chromosome-mediated system.* Cancer Res, 2005. **65**(23): p. 10663-8.
147. Kuroda, T., et al., *Flip-Flop HSV-BAC: bacterial artificial chromosome based system for rapid generation of recombinant herpes simplex virus vectors using two independent site-specific recombinases.* BMC Biotechnol, 2006. **6**: p. 40.
148. Tohyama, T., et al., *Nestin expression in embryonic human neuroepithelium and in human neuroepithelial tumor cells.* Lab Invest, 1992. **66**(3): p. 303-13.
149. Chou, T.C., *Drug combination studies and their synergy quantification using the Chou-Talalay method.* Cancer Res. **70**(2): p. 440-6.
150. Chou, T.C., *Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies.* Pharmacol Rev, 2006. **58**(3): p. 621-81.
151. Schulte, A., et al., *A distinct subset of glioma cell lines with stem cell-like properties reflects the transcriptional phenotype of glioblastomas and overexpresses CXCR4 as therapeutic target.* Glia. **59**(4): p. 590-602.
152. Lee, J., et al., *Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of*

primary tumors than do serum-cultured cell lines. Cancer Cell, 2006. **9**(5): p. 391-403.
153. Hambardzumyan, D., et al., *Genetic modeling of gliomas in mice: new tools to tackle old problems.* Glia. **59**(8): p. 1155-68.
154. Ligon, K.L., et al., *Olig2-regulated lineage-restricted pathway controls replication competence in neural stem cells and malignant glioma.* Neuron, 2007. **53**(4): p. 503-17.
155. Doetsch, F., et al., *Subventricular zone astrocytes are neural stem cells in the adult mammalian brain.* Cell, 1999. **97**(6): p. 703-16.
156. Spear, P.G., *Herpes simplex virus: receptors and ligands for cell entry.* Cell Microbiol, 2004. **6**(5): p. 401-10.
157. Spear, P.G., R.J. Eisenberg, and G.H. Cohen, *Three classes of cell surface receptors for alphaherpesvirus entry.* Virology, 2000. **275**(1): p. 1-8.
158. Davis, M.G. and E.S. Huang, *Transfer and expression of plasmids containing human cytomegalovirus immediate-early gene 1 promoter-enhancer sequences in eukaryotic and prokaryotic cells.* Biotechnol Appl Biochem, 1988. **10**(1): p. 6-12.
159. DeLuca, N.A., A.M. McCarthy, and P.A. Schaffer, *Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4.* J Virol, 1985. **56**(2): p. 558-70.
160. Li, A., et al., *Genomic changes and gene expression profiles reveal that established glioma cell lines are poorly representative of primary human gliomas.* Mol Cancer Res, 2008. **6**(1): p. 21-30.
161. Wakimoto, H., et al., *Maintenance of primary tumor phenotype and genotype in glioblastoma stem cells.* Neuro Oncol. **14**(2): p. 132-44.
162. McKeever, P.E., R.D. Davenport, and P. Shakui, *Patterns of antigenic expression of human glioma cells.* Crit Rev Neurobiol, 1991. **6**(2): p. 119-47.
163. Paulus, W., C. Huettner, and J.C. Tonn, *Collagens, integrins and the mesenchymal drift in glioblastomas: a comparison of biopsy specimens, spheroid and early monolayer cultures.* Int J Cancer, 1994. **58**(6): p. 841-6.
164. Papanastassiou, V., et al., *The potential for efficacy of the modified (ICP 34.5(-)) herpes simplex virus HSV1716 following intratumoural injection into human malignant glioma: a proof of principle study.* Gene Ther, 2002. **9**(6): p. 398-406.
165. Markert, J.M., et al., *Phase Ib trial of mutant herpes simplex virus G207 inoculated pre-and post-tumor resection for recurrent GBM.* Mol Ther, 2009. **17**(1): p. 199-207.
166. Maeshima, Y., et al., *Identification of the anti-angiogenic site within vascular basement membrane-derived tumstatin.* J Biol Chem, 2001. **276**(18): p. 15240-8.
167. Maeshima, Y., et al., *Tumstatin, an endothelial cell-specific inhibitor of protein synthesis.* Science, 2002. **295**(5552): p. 140-3.
168. Maeshima, Y., et al., *Extracellular matrix-derived peptide binds to alpha(v)beta(3) integrin and inhibits angiogenesis.* J Biol Chem, 2001. **276**(34): p. 31959-68.

169. Hamano, Y. and R. Kalluri, *Tumstatin, the NC1 domain of alpha3 chain of type IV collagen, is an endogenous inhibitor of pathological angiogenesis and suppresses tumor growth.* Biochem Biophys Res Commun, 2005. **333**(2): p. 292-8.
170. Sudhakar, A., et al., *Human tumstatin and human endostatin exhibit distinct antiangiogenic activities mediated by alpha v beta 3 and alpha 5 beta 1 integrins.* Proc Natl Acad Sci U S A, 2003. **100**(8): p. 4766-71.
171. Han, J., et al., *A cell binding domain from the alpha3 chain of type IV collagen inhibits proliferation of melanoma cells.* J Biol Chem, 1997. **272**(33): p. 20395-401.
172. Shahan, T.A., et al., *Inhibition of tumor cell proliferation by type IV collagen requires increased levels of cAMP.* Connect Tissue Res, 1999. **40**(3): p. 221-32.
173. Shahan, T.A., et al., *Identification of CD47/integrin-associated protein and alpha(v)beta3 as two receptors for the alpha3(IV) chain of type IV collagen on tumor cells.* Cancer Res, 1999. **59**(18): p. 4584-90.
174. Kawaguchi, T., et al., *The PTEN/Akt pathway dictates the direct alphaVbeta3-dependent growth-inhibitory action of an active fragment of tumstatin in glioma cells in vitro and in vivo.* Cancer Res, 2006. **66**(23): p. 11331-40.
175. Bello, L., et al., *Alpha(v)beta3 and alpha(v)beta5 integrin expression in glioma periphery.* Neurosurgery, 2001. **49**(2): p. 380-9; discussion 390.
176. Parker, J.N., et al., *Engineered herpes simplex virus expressing IL-12 in the treatment of experimental murine brain tumors.* Proc Natl Acad Sci U S A, 2000. **97**(5): p. 2208-13.
177. Liu, T.C., et al., *Dominant-negative fibroblast growth factor receptor expression enhances antitumoral potency of oncolytic herpes simplex virus in neural tumors.* Clin Cancer Res, 2006. **12**(22): p. 6791-9.
178. Liu, T.C., et al., *Oncolytic HSV armed with platelet factor 4, an antiangiogenic agent, shows enhanced efficacy.* Mol Ther, 2006. **14**(6): p. 789-97.
179. Saeki, Y., et al., *Herpes simplex virus type 1 DNA amplified as bacterial artificial chromosome in Escherichia coli: rescue of replication-competent virus progeny and packaging of amplicon vectors.* Hum Gene Ther, 1998. **9**(18): p. 2787-94.
180. Tanaka, M., et al., *Construction of an excisable bacterial artificial chromosome containing a full-length infectious clone of herpes simplex virus type 1: viruses reconstituted from the clone exhibit wild-type properties in vitro and in vivo.* J Virol, 2003. **77**(2): p. 1382-91.
181. Stanziale, S.F., et al., *Infection with oncolytic herpes simplex virus-1 induces apoptosis in neighboring human cancer cells: a potential target to increase anticancer activity.* Clin Cancer Res, 2004. **10**(9): p. 3225-32.
182. Li, Z., et al., *Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells.* Cancer Cell, 2009. **15**(6): p. 501-13.
183. Wade-Martins, R., Y. Saeki, and E.A. Chiocca, *Infectious delivery of a 135-kb LDLR genomic locus leads to regulated complementation of*

low-density lipoprotein receptor deficiency in human cells. Mol Ther, 2003. **7**(5 Pt 1): p. 604-12.
184. Wade-Martins, R., et al., *An infectious transfer and expression system for genomic DNA loci in human and mouse cells.* Nat Biotechnol, 2001. **19**(11): p. 1067-70.
185. Pollard, S.M., et al., *Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens.* Cell Stem Cell, 2009. **4**(6): p. 568-80.
186. Fael Al-Mayhani, T.M., et al., *An efficient method for derivation and propagation of glioblastoma cell lines that conserves the molecular profile of their original tumours.* J Neurosci Methods, 2009. **176**(2): p. 192-9.
187. Hamano, Y., et al., *Physiological levels of tumstatin, a fragment of collagen IV alpha3 chain, are generated by MMP-9 proteolysis and suppress angiogenesis via alphaV beta3 integrin.* Cancer Cell, 2003. **3**(6): p. 589-601.
188. Sudhakar, A. and C.S. Boosani, *Inhibition of tumor angiogenesis by tumstatin: insights into signaling mechanisms and implications in cancer regression.* Pharm Res, 2008. **25**(12): p. 2731-9.
189. Hutchings, H., N. Ortega, and J. Plouet, *Extracellular matrix-bound vascular endothelial growth factor promotes endothelial cell adhesion, migration, and survival through integrin ligation.* Faseb J, 2003. **17**(11): p. 1520-2.
190. Boosani, C.S., A.K. Varma, and A. Sudhakar, *Validation of Different Systems for Tumstatin Expression and its in-vitro and iv-vivo Activities.* J Cancer Sci Ther. **2009**: p. 8-18.
191. Rose, S.D. and M.K. Aghi, *Mechanisms of evasion to antiangiogenic therapy in glioblastoma.* Clin Neurosurg. **57**: p. 123-8.
192. Bergers, G. and D. Hanahan, *Modes of resistance to anti-angiogenic therapy.* Nat Rev Cancer, 2008. **8**(8): p. 592-603.
193. Walker, M.D., T.A. Strike, and G.E. Sheline, *An analysis of dose-effect relationship in the radiotherapy of malignant gliomas.* Int J Radiat Oncol Biol Phys, 1979. **5**(10): p. 1725-31.
194. Keime-Guibert, F., et al., *Radiotherapy for glioblastoma in the elderly.* N Engl J Med, 2007. **356**(15): p. 1527-35.
195. Chan, J.L., et al., *Survival and failure patterns of high-grade gliomas after three-dimensional conformal radiotherapy.* J Clin Oncol, 2002. **20**(6): p. 1635-42.
196. Fitzek, M.M., et al., *Accelerated fractionated proton/photon irradiation to 90 cobalt gray equivalent for glioblastoma multiforme: results of a phase II prospective trial.* J Neurosurg, 1999. **91**(2): p. 251-60.
197. van Nifterik, K.A., et al., *Differential radiosensitizing potential of temozolomide in MGMT promoter methylated glioblastoma multiforme cell lines.* Int J Radiat Oncol Biol Phys, 2007. **69**(4): p. 1246-53.
198. Nelson, D.F., et al., *A randomized comparison of misonidazole sensitized radiotherapy plus BCNU and radiotherapy plus BCNU for treatment of malignant glioma after surgery: final report of an RTOG study.* Int J Radiat Oncol Biol Phys, 1986. **12**(10): p. 1793-800.
199. Prados, M.D., et al., *Phase III randomized study of radiotherapy plus procarbazine, lomustine, and vincristine with or without BUdR for*

treatment of anaplastic astrocytoma: final report of RTOG 9404. Int J Radiat Oncol Biol Phys, 2004. **58**(4): p. 1147-52.
200. Li, B., et al., *Mutant epidermal growth factor receptor displays increased signaling through the phosphatidylinositol-3 kinase/AKT pathway and promotes radioresistance in cells of astrocytic origin.* Oncogene, 2004. **23**(26): p. 4594-602.
201. Advani, S.J., et al., *Enhancement of replication of genetically engineered herpes simplex viruses by ionizing radiation: a new paradigm for destruction of therapeutically intractable tumors.* Gene Ther, 1998. **5**(2): p. 160-5.
202. Bradley, J.D., et al., *Ionizing radiation improves survival in mice bearing intracranial high-grade gliomas injected with genetically modified herpes simplex virus.* Clin Cancer Res, 1999. **5**(6): p. 1517-22.
203. Cheema, T.A., et al., *Enhanced antitumor efficacy of low-dose Etoposide with oncolytic herpes simplex virus in human glioblastoma stem cell xenografts.* Clin Cancer Res. **17**(23): p. 7383-93.
204. Kanai, R., et al., *Oncolytic virus-mediated manipulation of DNA damage responses: synergy with chemotherapy in killing glioblastoma stem cells.* J Natl Cancer Inst. **104**(1): p. 42-55.
205. Stanziale, S.F., et al., *Ionizing radiation potentiates the antitumor efficacy of oncolytic herpes simplex virus G207 by upregulating ribonucleotide reductase.* Surgery, 2002. **132**(2): p. 353-9.
206. Hreshchyshyn, M.M., et al., *Hydroxyurea or placebo combined with radiation to treat stages IIIB and IV cervical cancer confined to the pelvis.* Int J Radiat Oncol Biol Phys, 1979. **5**(3): p. 317-22.
207. Vokes, E.E., et al., *Hydroxyurea, fluorouracil, and concomitant radiotherapy in poor-prognosis head and neck cancer: a phase I-II study.* J Clin Oncol, 1989. **7**(6): p. 761-8.
208. Barker, C.A., et al., *In vitro and in vivo radiosensitization induced by the ribonucleotide reductase inhibitor Triapine (3-aminopyridine-2-carboxaldehyde-thiosemicarbazone).* Clin Cancer Res, 2006. **12**(9): p. 2912-8.
209. Kolberg, M., et al., *Structure, function, and mechanism of ribonucleotide reductases.* Biochim Biophys Acta, 2004. **1699**(1-2): p. 1-34.
210. Kunos, C.A., et al., *Modulating radiation resistance by inhibiting ribonucleotide reductase in cancers with virally or mutationally silenced p53 protein.* Radiat Res, 2009. **172**(6): p. 666-76.
211. Tanaka, H., et al., *A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage.* Nature, 2000. **404**(6773): p. 42-9.
212. Xue, L., et al., *Wild-type p53 regulates human ribonucleotide reductase by protein-protein interaction with p53R2 as well as hRRM2 subunits.* Cancer Res, 2003. **63**(5): p. 980-6.
213. Zhou, B., et al., *The human ribonucleotide reductase subunit hRRM2 complements p53R2 in response to UV-induced DNA repair in cells with mutant p53.* Cancer Res, 2003. **63**(20): p. 6583-94.

214. Kuo, M.L., et al., *Overexpression of the R2 subunit of ribonucleotide reductase in human nasopharyngeal cancer cells reduces radiosensitivity.* Cancer J, 2003. **9**(4): p. 277-85.
215. Eaton, J.S., et al., *Ataxia-telangiectasia mutated kinase regulates ribonucleotide reductase and mitochondrial homeostasis.* J Clin Invest, 2007. **117**(9): p. 2723-34.
216. Kuo, M.L. and T.J. Kinsella, *Expression of ribonucleotide reductase after ionizing radiation in human cervical carcinoma cells.* Cancer Res, 1998. **58**(10): p. 2245-52.
217. Chabes, A. and L. Thelander, *Controlled protein degradation regulates ribonucleotide reductase activity in proliferating mammalian cells during the normal cell cycle and in response to DNA damage and replication blocks.* J Biol Chem, 2000. **275**(23): p. 17747-53.
218. Kunos, C.A., et al., *Phase I trial of pelvic radiation, weekly cisplatin, and 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, NSC #663249) for locally advanced cervical cancer.* Clin Cancer Res. **16**(4): p. 1298-306.
219. Rose, P.G., et al., *Long-term follow-up of a randomized trial comparing concurrent single agent cisplatin, cisplatin-based combination chemotherapy, or hydroxyurea during pelvic irradiation for locally advanced cervical cancer: a Gynecologic Oncology Group Study.* J Clin Oncol, 2007. **25**(19): p. 2804-10.
220. Whitney, C.W., et al., *Randomized comparison of fluorouracil plus cisplatin versus hydroxyurea as an adjunct to radiation therapy in stage IIB-IVA carcinoma of the cervix with negative para-aortic lymph nodes: a Gynecologic Oncology Group and Southwest Oncology Group study.* J Clin Oncol, 1999. **17**(5): p. 1339-48.
221. Symonds, R.P., et al., *Concomitant hydroxyurea plus radiotherapy versus radiotherapy for carcinoma of the uterine cervix: a systematic review.* Cancer Treat Rev, 2004. **30**(5): p. 405-14.
222. Beier, D., et al., *Temozolomide preferentially depletes cancer stem cells in glioblastoma.* Cancer Res, 2008. **68**(14): p. 5706-15.
223. Hegi, M.E., et al., *MGMT gene silencing and benefit from temozolomide in glioblastoma.* N Engl J Med, 2005. **352**(10): p. 997-1003.
224. Diehn, M., et al., *Association of reactive oxygen species levels and radioresistance in cancer stem cells.* Nature, 2009. **458**(7239): p. 780-3.
225. Tran, B. and M.A. Rosenthal, *Survival comparison between glioblastoma multiforme and other incurable cancers.* J Clin Neurosci. **17**(4): p. 417-21.
226. Bachoo, R.M., et al., *Epidermal growth factor receptor and Ink4a/Arf: convergent mechanisms governing terminal differentiation and transformation along the neural stem cell to astrocyte axis.* Cancer Cell, 2002. **1**(3): p. 269-77.
227. Sakariassen, P.O., H. Immervoll, and M. Chekenya, *Cancer stem cells as mediators of treatment resistance in brain tumors: status and controversies.* Neoplasia, 2007. **9**(11): p. 882-92.
228. Liu, C., et al., *Mosaic analysis with double markers reveals tumor cell of origin in glioma.* Cell. **146**(2): p. 209-21.

229. Persson, A.I., et al., *Non-stem cell origin for oligodendroglioma.* Cancer Cell. **18**(6): p. 669-82.
230. Lindberg, N., et al., *Oligodendrocyte progenitor cells can act as cell of origin for experimental glioma.* Oncogene, 2009. **28**(23): p. 2266-75.
231. Frosina, G., *The bright and the dark sides of DNA repair in stem cells.* J Biomed Biotechnol. **2010**: p. 845396.
232. Li, Q., et al., *Modeling the neurovascular niche: VEGF- and BDNF-mediated cross-talk between neural stem cells and endothelial cells: an in vitro study.* J Neurosci Res, 2006. **84**(8): p. 1656-68.
233. Wurmser, A.E., et al., *Cell fusion-independent differentiation of neural stem cells to the endothelial lineage.* Nature, 2004. **430**(6997): p. 350-6.
234. Hoster, H.A., R.P. Zanes, Jr., and E. Von Haam, *Studies in Hodgkin's syndrome; the association of viral hepatitis and Hodgkin's disease; a preliminary report.* Cancer Res, 1949. **9**(8): p. 473-80.
235. Wakimoto, H., et al., *Effects of innate immunity on herpes simplex virus and its ability to kill tumor cells.* Gene Ther, 2003. **10**(11): p. 983-90.
236. Zhu, H., et al., *Immune analysis on mtHSV mediated tumor therapy in HSV-1 seropositive mice.* Cancer Biol Ther, 2007. **6**(5): p. 724-31.
237. Corey, L. and P.G. Spear, *Infections with herpes simplex viruses (2).* N Engl J Med, 1986. **314**(12): p. 749-57.
238. Corey, L. and P.G. Spear, *Infections with herpes simplex viruses (1).* N Engl J Med, 1986. **314**(11): p. 686-91.
239. Herrlinger, U., et al., *Pre-existing herpes simplex virus 1 (HSV-1) immunity decreases, but does not abolish, gene transfer to experimental brain tumors by a HSV-1 vector.* Gene Ther, 1998. **5**(6): p. 809-19.
240. Fulci, G., et al., *Depletion of peripheral macrophages and brain microglia increases brain tumor titers of oncolytic viruses.* Cancer Res, 2007. **67**(19): p. 9398-406.
241. Breitbach, C.J., et al., *Targeted inflammation during oncolytic virus therapy severely compromises tumor blood flow.* Mol Ther, 2007. **15**(9): p. 1686-93.
242. Fulci, G., et al., *Cyclophosphamide enhances glioma virotherapy by inhibiting innate immune responses.* Proc Natl Acad Sci U S A, 2006. **103**(34): p. 12873-8.
243. Aghi, M.K., et al., *Hypoxia enhances the replication of oncolytic herpes simplex virus.* Mol Ther, 2009. **17**(1): p. 51-6.
244. Bernier, J., E.J. Hall, and A. Giaccia, *Radiation oncology: a century of achievements.* Nat Rev Cancer, 2004. **4**(9): p. 737-47.
245. Jain, R.K., et al., *Angiogenesis in brain tumours.* Nat Rev Neurosci, 2007. **8**(8): p. 610-22.