# Gene Therapy for Malignant Gliomas

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## PREFACE

### Contributions of Authors

Chapter 4: Suicide Gene Therapy contains the partial text of the publication, "Improvement of Antitumour Activity by Gene Amplification with a Replicating but Nondisseminating Adenovirus." Cancer Research 2007; 67(7):3387-3395, where Denis Bourbeau and I are listed as co-first authors. Denis Bourbeau constructed the virus vectors and characterized them *in vitro*. I was responsible for *in vivo* experimentation, and the characterization of the viruses in the GL261 cell line. Although the cytotoxicity graphs presented in Chapter 4, Figure 18 were produced by Denis Bourbeau, I generated similar data independently that is not shown.

The following authors also appear in the publication mentioned above:
Jaime Jairo: virus production
Zafiro Koty: animal technician
Simone P. Zehntner: trained me to isolate immune cells from whole brain tissue
Geneviève Lavoie: generated viruses with Denis Bourbeau
Anne-Marie Mes Masson: provided ovarian cancer cells for the original paper
Josephine Nalbantoglu: co-project supervisor
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**LIST OF ABBREVIATIONS**

5-FC=5-fluorocytosine
5-FdUMP=5-fluoro-2'deoxyuridine 5'monophosphate
5-FU=5-fluorouracil
5-FUMP=5-fluorouridine5'-monophosphate
5-FUTP=5-fluorouridine 5'-triphosphate
AAV=adeno-associated virus
Ad=adenovirus
AdV=adenovirus vector
ARF=alternative reading frame
BBB=blood brain barrier
bFGF=basic fibroblast growth factor
CAR=coxsackie and adenovirus receptor
CD=cytosine deaminase
CDK=cyclin dependent kinase
CKI=cyclin-dependent kinase inhibitor
CNS=central nervous system
CNTF=cilliary neurotrophic factor
CTL=cytotoxic T lymphocyte
CU=UPRT fused with CD
DAG=diacylglycerol
DC=dendritic cells
EGF/R=epidermal growth factor/receptor
EGFRvIII=constitutively active EGFR
ELISA=enzyme-linked immunosorbant assay
ERK=extracellular signal-related kinase
FasL=Fas ligand
FGF2=fibroblast growth factor
FOXO=forkhead box subgroup 'O' transcription factor
GADD45=growth arrest- and deoxyribonucleic acid damage-inducible protein
GAP=GTPase activating protein
GBM=glioblastoma multiforme
GCV=ganciclovir
GDP=guanosine diphosphate
GEF=guanosine exchange factor
GFAP=glial fibrillary acidic protein
GTP=guanosine triphosphate
HER2=human epidermal growth factor type 2
HRP=horse radish peroxidase
HSV-1=herpes simplex virus type 1
IE=immediate early
IFN=interferon
IGF-1=insulin-like growth factor
IL=interleukin

i.p.=intraperitoneal
IP3=inositol triphosphate
IR=inverted repeat
IRES=internal ribosome entry site
IRF-1=interferon regulatory factor-1
ISG=interferon stimulated genes
ISRE=IFN-stimulated response elements
ITR=inverted terminal repeat
JAK=janus kinase
JNK=jun kinase
MAPK=mitogen-activated protein kinase
MLV=murine leukemia retrovirus
MMP=matrix metalloproteinase
MOI=multiplicity of infection
mTOR=mammalian target of rapamycin
NF1=neurofibromatosis type 1
NK=natural killer cells
NSC=neural stem cell
PDGF/R=platelet derived growth factor/receptor
PDK-1=3-phosphoinositide-dependent protein kinase-1
pfu=plaque forming unit
PI3K=phosphoinositide 3-kinase
PIP2=phosphatidylinositol-4,5-bisphosphate
PIP3=phosphatidylinositol-3,4,5-trisphosphate
PKC=protein kinase C
PKR=protein kinase R
PLC-γ=phospholipase-C-gamma
PS=protease gene
PTEN=phosphatase and tensin homologue
rAAV=recombinant adeno-associated virus
Rb=retinoblastoma
RTK=receptor tyrosine kinase
Rv=retrovirus
s.c.=subcutaneous
STAT=signal transducer and activator of transcription
TGF-β=transforming growth factor-β
TK=thymidine kinase
TNF-α=tumour necrosis factor-α
TR=terminal repeats
TRAIL=tumour necrosis factor-related apoptosis-inducing ligand
UPRT=uracil phosphoribosyltransferase
VEGF=vascular endothelial growth factor
VG=vector genomes
VPC=vector producing cell
WHO=World Health organization
XAF1=X-linked inhibitor of apoptosis factor 1

**Abstract**

Gliomas are the most common primary brain tumours found in adults. The median survival of patients diagnosed with the most malignant form, glioblastoma multiforme (GBM), is 9-12 months and has changed little over the years despite advances in medical technology. Gene therapy may offer new solutions to treat this resistant disease. Hence, we tested three different gene therapy strategies.

In our first study, we tested the efficacy of targeted therapy to correct common aberrations found in gliomas including amplification/mutation of receptor tyrosine kinases (RTK) and loss of PTEN, which result in an overactive PI3K/Akt pathway. Without PTEN, FOXO transcription factors are inactivated, and the cell becomes resistant to apoptosis and cell cycle arrest. By using an adenoviral vector (AdV) expressing an activated FOXO1 mutant (AdFOXO1;AAA), we restored apoptosis and cell cycle arrest, reduced tumour volume and prolonged survival in an intracerebral xenograft model.

Secondly, we examined the therapeutic capacity of a novel replicating/non-disseminating AdV expressing the fusion protein of cytosine deaminase and uracil phosphoribosyltransferase (CU). CU can convert the non-toxic pro-drug, 5-fluorocytosine (5-FC) to the tissue diffusible chemotherapeutic drug, 5-fluorouracil (5-FU) to target dividing cells. *In vitro*, the replicating vectors were superior to the non-replicating vectors, but the fully replicating/disseminating vector did not perform considerably better than the replicating/non-disseminating vector suggesting that dissemination may not be advantageous. *In vivo*, the

replicating/non-disseminating vector administered in conjunction with 5-FC prolonged survival in both an athymic and an immunocompetent mouse model. Moreover, an immune bystander effect *in vivo* was mediated by macrophages and T cells.

Lastly, we investigated a method to harness a tool of the immune system, IFN-β; this cytokine is known to have anti-angiogenic, anti-proliferative, and immunomodulatory capabilities. It was tested in clinical trials, but without success due to its short half-life. We generated adeno-associated virus (AAV) vectors that would allow continuous, local supply of IFN-β. AAV-human-IFN-β treatment led to regression and prevented engraftment of tumours and improved survival in an intracerebral xenograft model. Furthermore, AAV-mouse-IFN-β decreased blood vessel density in the tumours of athymic mice (IFN-β is species specific).

These studies point to promising novel therapeutic approaches for malignant gliomas.

**Résumé**

Les gliomes sont des tumeurs primaires de cerveau les plus communes retrouvées dans les adultes. La survie médiane des patients diagnostiqués avec la forme la plus maligne, le glioblastome multiforme (GBM), est de 9 à 12 mois et a peu changé au cours des années en dépit des avances en technologie médicale. La thérapie génique peut offrir de nouvelles solutions pour traiter cette maladie résistante. Durant nos travaux, nous avons examiné trois stratégies différentes de thérapie génique

Dans notre première étude, nous avons examiné l'efficacité de la thérapie visée à corriger des anomalies communes retrouvées dans les gliomes, comprenant l'amplification/mutation de récepteurs de type tyrosine kinase (RTK) et la perte de PTEN, qui mènent en conséquence à une voie activée de PI3K/Akt. Sans PTEN, les facteurs de transcription FOXO sont inactivés, et la cellule devient résistante à l'arrêt du cycle cellulaire et à l'apoptose. En utilisant un vecteur adénoviral (AdV) exprimant une protéine activée du mutant FOXO1 (AdFOXO1;AAA.), nous avons reconstitué les signaux pour l'arrêt du cycle cellulaire et l'apoptose *in vitro* ainsi que *in vivo*.

Deuxièmement, nous avons examiné la capacité thérapeutique d'un nouveau vecteur adénovirale qui a la capacité de se répliquer sans provoquer de lyse cellulaire et qui exprime en plus la protéine de fusion uracile phosphoribosyltransférase/cytosine déaminase (CU). La protéine CU peut convertir le promédicament non-toxique, le 5-fluorocytosine (5-FC) à la drogue

chimiothérapeutique diffusible, le 5-fluorouracile (5-FU) qui a comme cible des cellules en division cellulaire. *In vitro*, les vecteurs à capacité de répliquation étaient meilleurs que ceux qui ne pouvaient pas se répliquer. *In vivo*, le vecteur en présence du 5-FC a prolongé la survie de deux modès animaux (avec et sans sytèmes immunitaires).

Dans un dernier temps, nous avons étudié une méthode pour exprimer l'IFN-β, un cytokine qui est connu pour avoir des capacités anti-angiogeniques, anti-prolifératives, et immunomodulatrices. Nous avons produit des vecteurs virus adeno-associés (AAV) qui ont permis un approvisionnement continu et local de l'IFN-β. Le traitement AAV-humain-IFN-β a produit une regression de tumeurs ainsi qu'avoir inhibé l'implantation de tumeurs, avec une amélioration de la survie dans un modèle intra-crânien de xénogreffes humaines. En outre, l'AAV-souris-IFN-β a diminué la densité de vaisseaux sanguins dans les tumeurs de souris.

Nos études démontrent que ces approches thérapeutiques pourraient être prometteuses pour les patients souffrant de gliomes.

# Chapter 1: Introduction and Review

## RATIONALE AND OBJECTIVE

Considered one of the deadliest cancers, malignant gliomas are the most common primary brain tumour found in adults[200]. They are highly aggressive, invasive, and neurologically destructive. The median survival of patients diagnosed with the most malignant manifestation of glioma, glioblastoma multiforme (GBM), ranges from 9 to 12 months, a statistic that has changed very little over the past two decades despite recent advances in surgery, radiotherapy, and chemotherapy[201,289]. Thus, the search for a cure is a desperate circumstance.

Gene therapy is an exciting novel therapeutic modality that we believe will help to ultimately control and/or cure malignant gliomas. Malignant gliomas are attractive targets for local gene therapy due to their restricted anatomical location and the absence of metastases outside the CNS. Furthermore, gene therapy approaches offer a means to take advantage of the recently discovered molecular properties of gliomas by allowing techniques that can correct rogue signalling pathways, or cause high transient local accumulation of chemotherapeutic drugs, or provide a constant and long term supply of therapeutic agents that stimulate the immune system or have anti-angiogenic effects. Our research objective was to characterize several gene therapy strategies based on targeted therapy, suicide gene therapy, and cytokine gene transfer to ascertain their clinical promise as therapeutic agents by incorporating the use of intracerebral glioma xenograft models. This introductory chapter will provide a comprehensive review of the pathological and molecular characteristics of gliomas, the advantages and disadvantages of vector systems used in gene therapy, and the current gene

therapy strategies for malignant gliomas including the specific research aims of this thesis.

## CHARACTERISTICS OF MALIGNANT GLIOMAS

### Classification and Grading

Glial-like tumours, known as gliomas, are primary brain tumours with morphologies similar to astrocytes (astrocytomas), oligodendrocytes (oligodendrogliomas), mixtures of various glial cells (oligoastrocytomas, and gliosarcomas), ependymal cells (ependymomas) or their precursors[119,289,557]. 60% of all primary brain tumours are astrocytomas, which make them the most common CNS neoplasm and more than half of gliomas are malignant[343,557]. One of the cardinal features of astrocytomas is their propensity to infiltrate throughout the brain, making complete surgical resection impossible; furthermore, these tumours are largely resistant to radiation and chemotherapy[56,119].

The World Health Organization (WHO) classifies gliomas into grades I-IV according to their degree of malignancy as judged by histological features such as high cellularity, cellular pleomorphism, mitotic activity, microvascular proliferation, and necrosis[247]. Grade I gliomas are generally benign and can be surgically cured if they can be resected; grade II tumours are low-grade malignancies that follow a long clinical course and are not curable by surgery; grade III tumours are malignant and lead to death within a few years; grade IV tumours are highly malignant and fatal in less than a year. Oligodendrogliomas and oligoastrocytomas are categorized as grade II and grade III (anaplastic).

Astrocytic tumours are classified accordingly as pilocytic astrocytoma (grade I), astrocytoma (grade II), anaplastic astrocytoma (grade III), and glioblastoma multiforme (GBM; grade IV). The most aggressive and invasive form, GBM, is characterized by intense microvascular proliferation and/or necrosis. The invasive spread results in the formation of multiple secondary foci and frequent recurrent growth, although rarely metastasizing outside the CNS.

GBM can be divided into two subtypes based on clinical characteristics: primary and secondary GBM[289,557]. Primary GBM, typically presents in older patients *de novo*, without any evidence of prior clinical disease and arise rapidly (<3 months). On the other hand, secondary GBM is usually observed in younger patients and develops progressively from low-grade astrocytoma within 5 to 10 years of initial diagnosis, regardless of prior therapy. Although both subtypes are clinically indistinguishable from the time GBM is established, genetic studies of GBMs clearly indicate that distinct genetic mutations are involved in the initiation and progression of these tumours; however, the same genetic pathways appear to be targeted (Figure 1). Genetic lesions that occur with high frequency in low-grade and high-grade gliomas are considered to be involved in the initiation of tumour genesis. Lesions that that are more commonly found only in high-grade gliomas are believed to be involved in the progression of the malignancy. However, due to the rapid clinical course of primary GBM, it is often difficult to delineate initiation from progression.

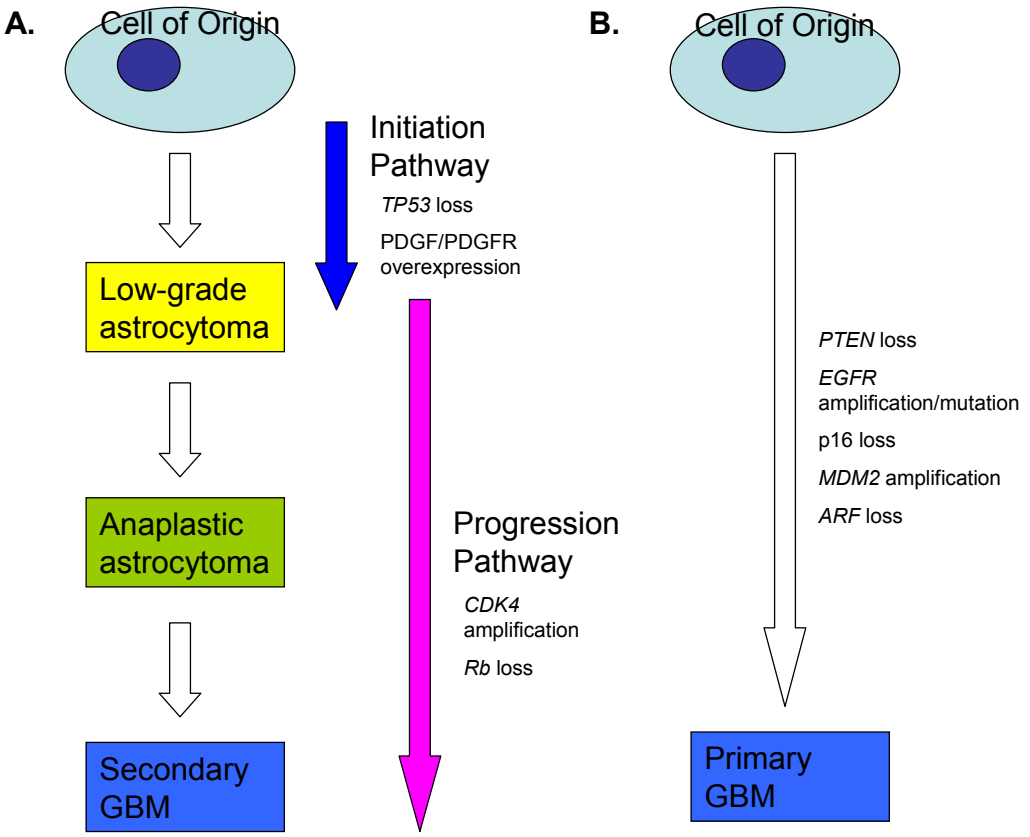



**Figure 1: Genetic pathways in primary and secondary astrocytoma.**

***A***, mutations that are commonly present in low-grade gliomas are associated with the initiation pathway of secondary GBM including loss of *TP53* and the overexpression of PDGF/PDGFR. Low-grade gliomas can progress to higher grade gliomas by acquiring additional mutations such as *Rb* loss and *CDK4* amplification. ***B***, the genetic pathways involved in *de novo* primary GBM are similar to secondary GBM, although the disruptions occur through different mechanisms. For instance, loss of the p53 pathway occurs through the amplification of *MDM2* or the loss of *ARF*. Disruption in the receptor tyrosine pathways occurs more commonly through amplifications or mutation of EGFR. Adapted from Zhu et al[557].

**Cell of Origin**

The cell type that gives rise to gliomas is unknown; however, glioma cells most often resemble immature astrocytes and/or oligodendrocytes in both morphology and gene expression. In fact, many of the same signalling pathways that regulate differentiation and proliferation of glial progenitors are active in gliomas[201]. Recently, self-renewing, multipotent cells expressing the CD133 stem cell-surface marker were isolated from human gliomas[148,443]. When transplanted into adult mouse brains, only the multipotent CD133 positive population was capable of recapitulating the histology of the parent tumour whereas the CD133 negative brain tumour fraction was incapable of forming tumours[148,443]. These findings suggest that gliomas themselves are maintained by a small fraction of cancerous neural stem cells, but the origin of this stem-cell like population is not known. There are two main hypotheses regarding the origin of glioma cells. The first proposes transformation events in terminally differentiated cells result in dedifferentiation of mature astrocytes or oligodendrocytes leading to glioma genesis. The second hypothesis suggests neural stem cells or progenitors to the mature glia that the tumour cell most closely resemble give rise to glioma cells.

Several lines of evidence suggest that mature astrocytes or oligodendrocytes may have dedifferentiated to possess stem cell-like properties resulting from malignant transformation. Studies of explanted embryonic cortical tissue have shown that mature astrocytes in the recipient brain can dedifferentiate into radial glia-like phenotype, indicating that differentiation is not a terminal event[213,446]. Radial glia are neural stem cells that likely come from the same lineage as neuroepithelial

cells and eventually transform into astrocyte-like adult stem cells[306]. Furthermore, dedifferentiation of surrounding astrocytes into radial glia can be induced by a highly selective nontraumatic phototoxic lesion to a limited number of neurons in the adult mouse cortex[271,288]. Oligodendrocyte precursors can also be reprogrammed to become multipotential CNS stem cells by certain extracellular signals such as fetal calf serum or bone morphogenic proteins followed by treatment with basic fibroblast growth factor (bFGF)[250]. Gene transfer of the polyomavirus middle T antigen oncogene into differentiated astrocytes has been demonstrated to result in the production of both oligodendrogliomas and astrocytomas[204]. Although the middle T antigen is not known to be involved in glioma genesis, it is known to activate many of the same growth factor pathways implicated in glioma genesis and can serve as a functional model[204]. Thus, dedifferentiation may result in an astrocyte or oligodendrocyte that is present in a more immature state that can proliferate and migrate like stem cells, which are key properties of astrocytomas.

Another proposed cell of origin for gliomas are neural stem cells (NSCs). NSCs, endowed with the capacity for self-renewal and differentiation, give rise to neuronal and glial progenitors, which consequently give rise to the mature cell types in the brain comprising neurons, oligodendrocytes, and astrocytes [50,146,272,307]. NSCs are localized in the subventricular zone, the lining of the lateral ventricles, the dentate gyrus of the hippocampus, and the subcortical white matter (reviewed in Sanai *et al*[419]). The prevalent germinal region in humans is the subventricular zone, which contains a large population of astrocytes that can

function as neural stem cells[420]. The population of cells that comprise the subventricular zone are as follows[98]: Type A have the ultrastructure of migrating neuronal precursors and are positive for polysialylated neural adhesion molecule, TuJ1, and nestin, but are glial fibrillary acidic protein (GFAP)- and vimentin-negative. Type B are astrocyte-like, proliferate slowly, self-renew, and are characterized by the presence of intermediate filaments; as well, they possess nestin- and GFAP-positive immunoreactivity. Type C cells lack intermediate filaments, proliferate rapidly, and give rise to neuroblasts and oligodendrocyte progenitors. Finally, type E cells are ependymal cells important for regulating cerebral spinal fluid flow. Many gliomas are either periventricular or contiguous with the subventricular zone[419]. In animal models, regions of the brain with stem-cell populations are particularly more sensitive to chemical or viral oncogenesis than other areas of the brain with a low proportion of proliferative cells[419]. Indeed, neural stem cells in mice have been demonstrated to be more susceptible to transformation than differentiated astrocytes [202,203]. The migration of glioma cells may also be explained by progenitor cells. In injured adult brain, nestin-positive progenitor cells migrate from the subventricular zone to the site of injury through mature parenchyma[161,226,357,515]. Gliomas and neural stem cells share this capacity for migration. Insight into the biology of progenitor-cell motility in the CNS may lead to a better understanding of glioma invasion. However, neural stem-cell activity reduces with age[146], such that this hypothesis is not consistent with primary GBMs that arise mainly in patients who are older than 55 years of age[289].

A recent paper by Bachoo *et al*[10] demonstrated that both astrocytes and neural stem cells can serve as the cell of origin for high-grade malignant gliomas. In fact, they show that both neural stem cells and astrocytes have the same propensity towards transformation to a high-grade glioma phenotype indicating that both dedifferentiation of mature astrocytes and neural stem cell transformation may give rise to gliomas. They argue that it is the deregulation of specific genetic pathways, rather than cell-of-origin that dictates the emergence and phenotype of high-grade gliomas[10]. Dai *et al*[78] showed that oligodendrogliomas and oligoastrocytomas can be induced from neural progenitors and astrocytes *in vivo*, although neural progenitors were more susceptible to transformation in their study. Moreover, Dai *et al*[79] demonstrated that oligodendrogliomas can be converted into astrocytomas by altering particular signalling pathways suggesting that a given tumour cell phenotype is achieved by a combination of its signalling pathways and characteristics of the cell of origin. Knowledge of the genetic pathways involved in the establishment and development of GBM will undoubtedly offer novel targets for therapy.

**Commonly Disrupted Pathways and Genetic Lesions in Gliomas**

Most of the genetic alterations in gliomas lead to the disruption of several main cellular systems involving growth factor receptor tyrosine kinase pathways, p53 tumour suppressor pathways and Rb cell cycle regulation pathways. In many cases, forced expression of oncogenes involved in one or more of these pathways, in either NSCs or mature glia in mice, produce tumours similar to human gliomas providing evidence that deregulation of specific genetic pathways, rather than

cell-of-origin dictates the emergence and phenotype of high-grade gliomas. The highest grade of glioma, GBM, invariably demonstrates the greatest number of genetic abnormalities compared to all other grades accompanied by multiple chromosomal losses, structural abnormalities[22,223,395], and double minute chromosomes[19,21,22] indicating amplified genes. Microarray technology has further revealed many new gene alterations and paved a way for gene profiling of glioma subtypes. The most common genetic lesions in gliomas and their pathways will be presented in the following sections.

*Growth Factor Receptor Tyrosine Kinase Pathways (RTKs)*

Gliomas often overproduce growth factors such as epidermal growth factor (EGF)[526], platelet derived growth factor (PDGF)[181], fibroblast growth factor 2 (FGF2)[342,468], cilliary neurotrophic factor (CNTF)[115,516], and/or their tyrosine kinase receptors (RTKs). Mutations or overexpression of RTKs and their ligands are thought to contribute to the formation of malignant gliomas by relaying into the cell growth signals for survival, proliferation, cell scatter, and/or migration[201,289,557]. After binding to a growth factor, RTKs dimerize, autophosphorylate and recruit adaptor proteins that interact and activate various downstream effectors. RTKs signal through several pathways, including Ras-MAPK (mitogen-activated protein kinase), PI3K (phosphoinositide 3-kinase), and PLC-γ (phospholipase-C-gamma) (Figure 2).

Ras is mutated in 30% of human cancers, although no Ras mutations have been found in astrocytomas[33]. Instead, Ras is upregulated indirectly in gliomas by

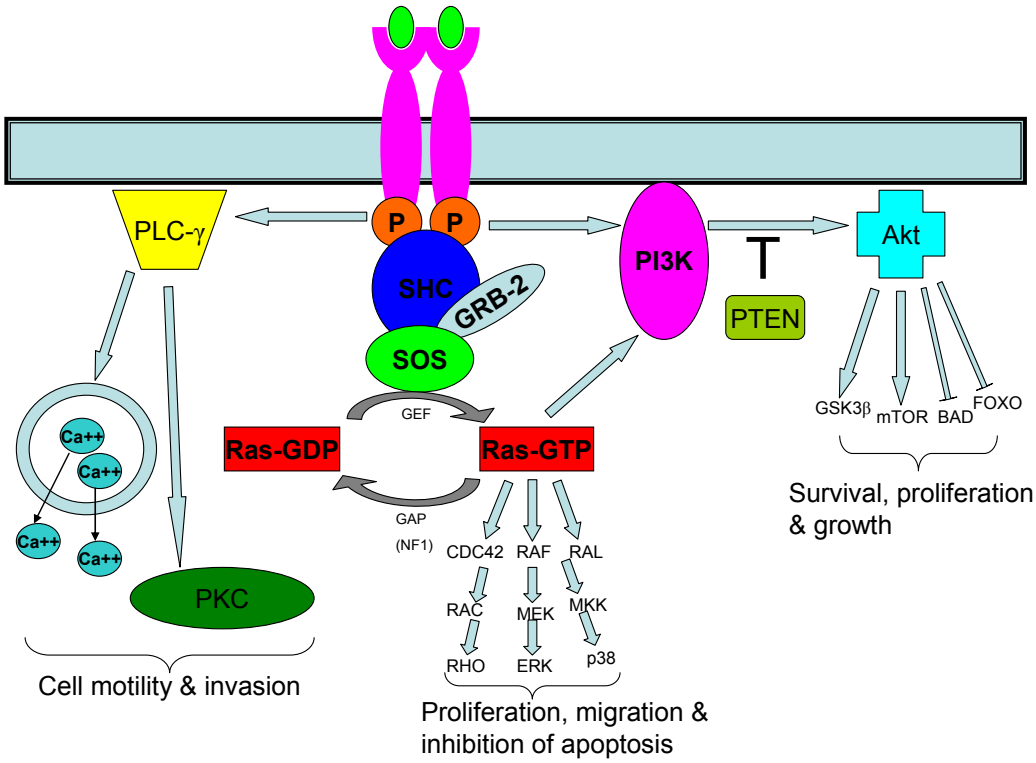



**Figure 2: The signalling pathway for growth-factor-RTK.**

Growth factor binding leads to receptor dimerization which is followed by autophosphorylation. This recruits adaptor proteins such as GRB2 and SHC that activate various downstream effectors such as Ras and PI3K. Ras is activated by guanosine exchange factors (GEFs) such as SOS that convert Ras-GDP to the active form Ras-GTP. GTPase activating proteins (GAPs) such as NF1 negatively regulate Ras-GTP. Ras can activate several different pathways: Rac, Raf, Ral, and PI3K resulting in cell proliferation, survival, and cytoskeletal organization. PI3K is also directly activated by growth factor signalling leading to Akt activation. This leads to the inhibition of FOXO, and BAD, as well as the activation of MDM2 eliciting a cell survival response. Akt activation of mTOR (mammalian target of rapamycin) and glycogen synthase kinase-3β (GSK3β) results in an increase in cell growth and proliferation, respectively. The gene encoding PTEN is frequently lost in gliomas, as well RTKs and their ligands are often overexpressed. Growth-factor-RTK signalling can also contribute to the activation of PLC-γ, which leads to calcium release and activation of PKC resulting in increased cell motility.

overexpression of RTKs and their ligands, or by loss of their negative regulators. Ras is active when bound to guanosine triphosphate (GTP), but inactive when the GTP is hydrolyzed to guanosine diphosphate (GDP). Ras activates many pathways in its GTP-bound form such as the Ral, Rac and Raf pathways, which lead to activation of the MAPKs, ERK, Jun kinase (JNK) and p38[297] (Figure 2). The overall effect of these pathways is to enhance motility, proliferation and progression through the cell cycle and inhibition of apoptosis. Neurofibromatosis type 1 (NF1) protein is a RAS-GTPase that inactivates RAS by hydrolyzing GTP[537]. Loss of both *NF1* alleles has been shown in low-grade astrocytomas[69,556]. Moreover, astrocyte-specific overexpression of *Ras* in transgenic mice leads to the development of astrocytomas[94].

PI3K can be activated by Ras as well as by extra-cellular matrix attachment and by growth factors via their RTKs[53,281] (Figure 2). PI3K phosphorylates its lipid substrate phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3), an important lipid second messenger for downstream proteins such as Akt. Akt interaction with PIP3 through the Akt pleckstrin homology domain leads to a conformational change that permits its phosphorylation by 3-phosphoinositide-dependent protein kinase-1 (PDK-1) and PDK-2, a kinase whose identity remains unclear. This leads to maximum activation of Akt resulting in the many biological effects of the PI3K pathway including cell proliferation, growth, and survival, as well as cellular responses to nutrients[268,281]. PTEN, a lipid phosphatase and tumour suppressor, negatively regulates PI3K by converting PIP3 back to PIP2[322] and blocks inappropriate

activation of the serine threonine kinase Akt. Independently of Akt, the PI3K pathway can also activate cdc42 and Rac which are known for their roles in regulating cytoskeletal movement and cell motility[498]. Overactivation of PI3K is frequently observed in a wide range of human tumour types including gliomas[498]. In fact, overexpression of constitutive *Akt* in conjunction with oncogenic *Ras*, in mouse neural stem cells leads to the development of GBM[202].

Signalling through PLC-γ in GBM is largely implicated in cell motility[517]. Activation of PLC-γ by RTKs leads to the conversion of PIP2 into diacylglygerol (DAG) and inositol-triphosphate (IP3) (Figure 2). This action releases actin binding proteins and activates downstream effectors: protein kinase C and calcium, which have a multitude of effects on adhesion protein turnover and actin-myosin contractility, both of which contribute to motility. Disruption of PLC-γ signalling prevents invasion of glioblastoma cells into normal brain tissue[242].

The most common disruptions in RTK signalling implicated in gliomas are PDGF/PDGFR and EGF/EGFR overexpression[201,289,557] as well as *PTEN* deletion, which will be discussed further. In the adult brain, PDGFR expression is restricted to the ventricular and subventricular zone of the lateral ventricles, whereas PDGF is broadly expressed by neurons and astrocytes[353]. PDGF has been demonstrated in cell culture to cause the oligodendroglial progenitor population to proliferate[302], and in cooperation with bFGF has been shown to prevent further differentiation into mature oligodendrocytes[27,301]. Overexpression of both the PDGF ligand and its receptor are prominent in low-grade astrocytomas[70,193]. Astrocytoma cells can

establish an autocrine stimulatory loop that can potentially mediate a signalling cascade implicated in gliomagenesis[195]. The actual mechanisms leading to PDGF overexpression are not known and gene amplification of the *PDGF-R-α* receptor gene is rarely observed[196]. *In vivo* gene transfer of PDGF alone to both nestin-positive neural progenitors and GFAP-expressing astrocytes induced the formation of oligodendrogliomas and oligoastrocytomas[78]. Interestingly, forced Ras or Akt activity in PDGF-stimulated cells led to a conversion from oligodendroglial to astroglial character presenting a mechanism whereby signalling activity can convert tumour cells from one lineage to another giving rise to gliomas composed of multiple lineages[79]. PDGF mitogenic signals, though, may not be central to the development of astrocytomas[134]. Chronic PDGF stimulation in glial progenitor cells has been shown to actually suppress the activation of both Akt and Ras/MAPK pathways[79]. The most important role of PDGF in tumour initiation may be its capacity to induce tumour cell migration through activation of PI3K and PLC-γ, which are involved in cell migration and scattering[192,525]. Moreover, the overexpression of PDGF follows closely with p53 tumour suppressor loss-of-function mutations[196]. The early loss of p53 may allow the astrocytoma cells to evade apoptosis and the additional overexpression of PDGF could facilitate their capacity to migrate and survive in a microenvironment that would otherwise be unable to provide adequate trophic support. Thus, PDGF overexpression is capable of causing malignant transformation and increasing cell migration but its mitogenic capacity may not play a large role in tumour genesis.

*EGFR*, located on chromosome 7p11-12, is amplified in approximately 50% of all GBMs[526] and may be fundamental in advancing the transformation process towards GBM. This amplification is rarely seen in low-grade gliomas, but the majority of gene amplification events in high-grade astrocytomas involve *EGFR*[20,526]. As with PDGF, an autocrine stimulatory loop with EGF and its receptor is present in GBM[115] and is likewise linked with inducing cell scattering and migration in glioma cells *in vitro*[116]. EGF infusion was found to stimulate the proliferation and migration of transplanted progenitor cells in rats[133]. *In vitro*, EGF, but not PDGF, can stimulate neural stem cell proliferation to undergo self-renewal and generate all three CNS cell types: neurons, oligodendrocytes, and astrocytes[396,397]. Glial progenitors are pushed towards astrocytic and oligodendrocytic differentiation by EGF and CNTF[300,382]. About 40% of the GBMs with *EGFR* amplification also express a constitutively active autophosphorylated variant form (EGFRvIII)[212,527]. Introducing this active form into glioma cells enhances their tumourigenicity *in vivo*[340] by increasing cellular proliferation[323], reducing apoptosis[323], and conferring resistance to chemotherapeutic drugs such as cisplatin[324]. Expression of the constitutively active form of EGFR in neural stem cells and astrocytes, however, does not lead to the formation of gliomas, and requires additional disruptions[10]. For example, Ding *et al*[95] found that oligodendrogliomas and oligoastrocytomas only developed from double transgenic mice expressing astrocyte-specific EGFRvIII and activated Ras. These data are consistent with the supposed role of EGF/EGFR in glioma progression as opposed to initiation.

Furthermore, there is evidence the mutation or amplification of *EGFR* may actually drive the induction of malignant glioma vascularization though a functional link to vascular endothelial growth factor (VEGF). VEGF has been shown to be critical to vascular development by promoting endothelial cell proliferation, differentiation, migration and tubular formation[224]. During embryonic development, the spatial and temporal pattern of VEGF expression correlates tightly with EGFR expression[36]. Studies in cultured astrocytes have also found that VEGF expression is EGF-dependent[163]. In fact, EGFR was found to transcriptionally upregulate VEGF expression in human GBM cells in a manner that involved the PI3K pathway[290]. These observations suggest that *EGFR* amplification may contribute to the high degree of angiogenesis observed in GBM. Nonetheless, the angiogenic process in GBM is far more complicated than that promoted by *EGFR* amplification alone. Tumour hypoxia and ischemia may lead to the induction of angiogenesis that is mediated by VEGF and other angiogenic factors such as fibroblast growth factor, and transforming growth factor-β[62,224].

Loss of the long arm of chromosome 10, which affects 75-90% of high-grade gliomas, is the most frequent genetic alteration in GBM[141,502]. PTEN is a tumour suppressor whose gene is located on chromosome 10q23-24 and is disrupted in 30-44% of high grade gliomas[507]. *PTEN* mutations are found more often in primary GBM (30%) and rarely in secondary GBM (4%)[475] indicating its role in the progression of gliomas. Loss of *PTEN* in tumour cells correlates with an increase in cellular levels of PIP3, and subsequent enhanced activation of

Akt[322,452,532]. Thus, amplification of *EGFR* and loss of *PTEN* result in a hyper-active PI3K-Akt pathway. Consistent with the view that PTEN is involved in glioma progression, rather than initiation, brain or glial cell-specific inactivation of *PTEN* alone does not elicit neoplastic changes[11,178]. Inactivation of *PTEN* in mouse neural stem cells causes increased proliferation, but not transformation, in part by shortening the cell cycle of neural stem cells[178]. In *PTEN* heterozygous mice (*PTEN*+/-), astrocyte inactivation of Rb and its related proteins p107 and p130, however, accelerates the formation of high-grade malignant astrocytomas[534]. *PTEN* deficiency in a background of *Rb* inactivation has also been implicated in increasing astrocytoma cell invasion as well as angiogenesis in a mouse model of spontaneous cancer development[535]. Furthermore, mutations in *EGFR* and *PTEN* contribute to resistance to chemotherapy and radiation which can be attributed to PI3K-Akt over activity[57,59,276,324]. Thus, mutations in tyrosine kinase receptors and *PTEN* contribute greatly to the malignant phenotype of gliomas and can contribute to tumour progression via multiple mechanisms.

*P53 Tumour Suppressor Pathway*

The p53 tumour suppressor is a transcription factor often referred to as the "genome guardian" that regulates cell cycle progression, and apoptosis in response to cellular stress signals such as DNA damage, growth factor withdrawal, and hypoxia[52,187,238,248,337,495]. Stress signals are communicated to the p53 protein in part by a wide range of post-translational modifications. At the same time, stress signals increase the half-life of p53 by degrading the E3 ubiquitin ligase MDM2, which raises the concentration of p53. MDM2 serves a

critical role in the ubiquitination and proteosomal degradation of p53 in the absence of stress signals (Figure 3). Activated p53 acquires the ability to bind specific DNA sequences and enhances the rate of transcription of target genes. Specific stress signals result in different protein modifications of the p53 protein which results in different transcriptional programs and various outcomes in a cell. Some transcriptional targets of p53 include *p21*, involved in cell cycle inhibition, *BAX* and *FAS*, implicated in promoting apoptosis, as well as *GADD45* (*growth arrest- and deoxyribonucleic acid damage-inducible*), concerned with the DNA damage response[495].

Mutations in the *TP53* gene, located on the short arm of chromosome 17p13.1, encoding the p53 tumour suppressor are found in all grades of astrocytoma suggesting that this mutation may be involved in the early phases of secondary GBM formation. Moreover, *TP53* mutations are far less common in primary GBM (~10%) than in secondary GBM (>60%)[265]. In a recent summary, p53 was mutated in 30% (51 of 172) of low-grade fibrillary astrocytomas, 31% (51 of 165) of anaplastic astrocytoma, and 33% (83 of 250) of GBM[26]. Additionally, epigenetic processes may inactivate p53 in a subset of astrocytomas with wild-type p53[38,266,284], suggesting that the frequency of p53 pathway inactivation may be higher than predicted by gene mutation studies alone. In human gliomas, *p53* mutations are primarily point mutations that inactivate one *p53* allele; genomic instability usually leads to the loss or deletion of the second allele[4,67,494]. Furthermore, since p53 functions as a tetramer, the presence of one mutant allele

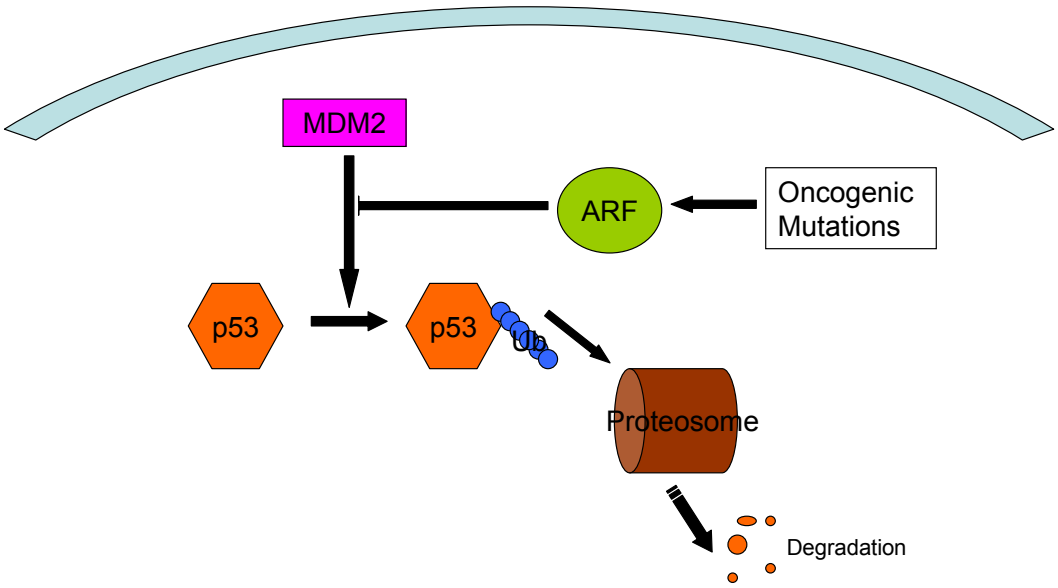



**Figure 3: Regulation of the p53 tumour suppressor.**

MDM2 is an important negative regulator of p53 that promotes the ubiquitination and degradation of p53 in the absence of stress signals. ARF leads to stabilization of the p53 protein by binding directly to MDM2 inhibiting its activity. Loss of p53 or ARF, and amplification of MDM2 are frequent mutations involving this pathway in gliomas.

may have a dominant-negative effect on the other wild-type allele. Li-Fraumeni syndrome is a familial cancer syndrome characterized by the presence of a germ-line mutation in *TP53* that predisposes individuals to the development of various brain tumours, including astrocytoma, providing evidence for the importance of p53 in tumour suppression[291,449]. However, p53 knockout and p53 heterozygous mice do not develop astrocytomas[101,220], although primary p53 null astrocytes do demonstrate increased growth and susceptibility to transformation[25,538]. Furthermore, p53 loss does not synergize with the constitutive activation of EGFR in astrocytes in tumour genesis[10]. Loss of both *NF1* and *p53* in mice, though, leads to the formation of a range of astrocytomas from low-grade to GBM[389]. Loss of p53 alone in neural stem cells of the subventricular zone did provide a proliferative advantage; however, it did not result in transformation and cells still retained the capacity to differentiate along distinct lineages[155]. Transformation of progenitor cells in the subventricular zone to glioblastoma-like tumours does occur when loss of *TP53* is associated with a mutagenic stimulus, such as N-ethyl-N-nitrosourea (ENU) or constitutively active Ras, leading to enhanced self-renewal, increased proliferation and impaired differentiation[155]. Therefore, in both astrocytes and neural stem cells or progenitors, the loss of the gene for p53 alone may be inadequate to initiate astrocytoma, and may necessitate other added genetic or epigenetic events, but loss of p53 does predispose cells towards transformation.

The p53 protein is regulated by a number of proteins including ARF (alternate reading frame; also known as p14 in humans), which is another tumour suppressor

that is commonly deleted in gliomas. ARF blocks p53 degradation and promotes its stabilization by binding directly to MDM2 (Figure 3). The stabilization of p53 enhances apoptosis[233,366,457,553], induces G1 and G2 phase cell cycle arrest[233,378], and blocks oncogenic transformation[366]. Mice that are ARF-null develop spontaneous low-grade gliomas at low penetrance[232] thereby underscoring the importance of this protein in tumour suppression. Primary GBM that lack *TP53* mutations often display amplification or overexpression of *MDM2* or loss of *ARF*[18], whereas secondary GBM often display direct loss of *TP53*[265]. Interestingly, several studies in human gliomas have found that *EGFR* amplification was frequently observed in association with mutation or deletion of *ARF* and *p16* (a cell cycle inhibitor located on the same locus as *ARF*), but not in association with *TP53* mutations[501] indicating mutually exclusive events[89,464].

*Rb Cell Cycle Regulation Pathway*

Another tumour suppressor, Rb, is a major regulator of cell cycle progression at the G1/S checkpoint and is frequently disrupted in high-grade gliomas, but rarely in low-grade astrocytomas[201,289,557], suggesting its importance for tumour progression. Therefore, it is not surprising that high-grade gliomas are characterized primarily by an elevation in mitotic rate. In the normal quiescent cell, hypophosphorylated Rb blocks progression through the G1/S restriction point by binding to E2F, an important cell cycle transcription factor[292,439,454] (Figure 4). Phosphorylation of Rb is effected by the mitogen-induced Ras/MAPK signalling pathway, which induces cyclin D1 transcription. Cyclin D1 then complexes with cyclin-dependent kinases (CDKs) 4 and 6 and initiates the

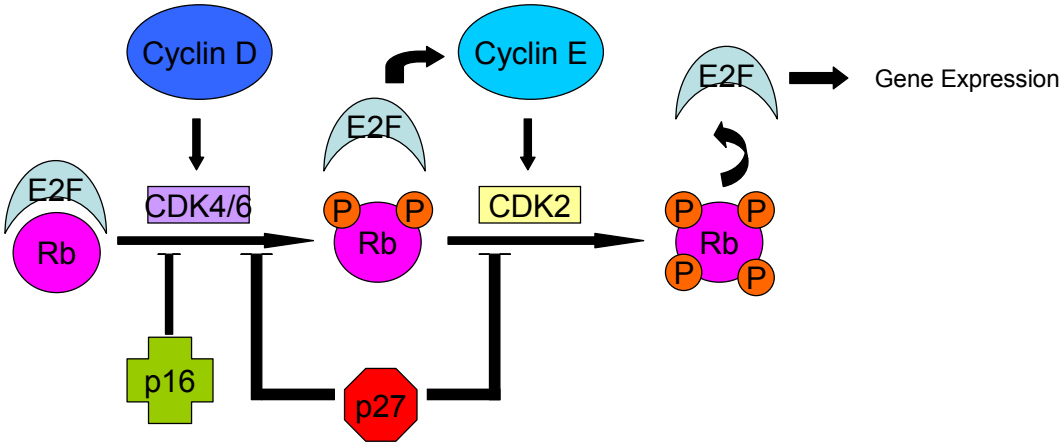



**Figure 4: The Rb cell cycle regulation pathway.**

Hypophosphorylated Rb blocks G1/S transition by binding to E2F, an important transcription factor in cell cycle progression. In response to proliferative signals, Rb is partially phosphorylated by CDK4/6, and completely phosphorylated by CDK2. Hyperphosphorylated Rb leads to the release of E2F, activation of E2F-responsive genes, and the initiation of DNA synthesis. Two families of CKIs regulate this pathway, which include p27 and p16. Loss of Rb, p16, and amplification of CDK4 are regularly detected in gliomas causing increased E2F activity.

phosphorylation of Rb, which is completed by cyclin E/CDK2. Hyperphosphorylated Rb leads to the release of E2F, activation of E2F-responsive genes, and subsequent entry into S phase[292,439,454]. Two families of cyclin-dependent kinase inhibitors (CKIs) negatively regulate this pathway. The INK4 family of CKIs, which includes p16 (also known as INK4A), p15 (also known as INK4B), p18 (also known as INK4C), and p19 (also known as INK4D) specifically inhibit activation of the cyclin D and CDK4/6 complex. The second CKI family is a more general inhibitor of CDKs and includes p21 (also known as WAF1), p27 (also known as KIP1) and p57 (also known as KIP2)[292,439,454].

Loss of *RB* is detected in 14-33% of GBMs[45,215,489], loss of *p16* is detected in 40-57% of GBMs[45,190,215,431,489], and *CDK4* is amplified by 10-100 fold in 12-14% of GBMs[45,215]. In fact, mutations in the p16-CDK4-Rb pathway have been identified in over 80% of GBMs and in 50% of anaplastic astrocytomas[557]. Overexpression of CDK6[74], cyclin D1[46], and cyclin E[58] have also been reported in some high-grade gliomas. Moreover, the gene that encodes p16 and ARF, called *CDKN2A* on chromosome 9p21[438], is frequently involved in homozygous deletions in 40% of GBMs[215,431] resulting in the disturbance of both Rb and p53 pathways. Methylation of 5' CpG islands of *CDKN2A* was also shown to be associated with lack of expression in GBM[73,137]. Therefore, disruption of both cell cycle control and tumour suppressor pathways may provide an explanation for the intense aggressiveness of GBM. Bachoo *et al*[10] showed that loss of both p16 and ARF, but not of p16, ARF, or p53 alone, results in de-differentiation of neonatal mouse astrocytes into neural stem cells in response to EGF signalling. Furthermore,

transplantation of p16/ARF-/- astrocytes and p16/ARF-/- neural stem cells into brains led to the development of high-grade astrocytomas in response to EGF signalling[10].

Overall, the disruption of particular cell pathways in either neural stem cells or astrocytes leads to the formation of gliomas and targeting frequently disrupted pathways is a potential strategy for developing new therapies.

**New Data from Microarrays**

Microarrays enable researchers to examine the expression of thousands of genes simultaneously from a small quantity of high quality RNA from tumour (or non-tumour) tissue. The RNA is labelled and hybridized on the surface of a chip composed of spotted cDNA clones or probes spotted or synthesized on the surface of the chip (oligonucleotide arrays). Then computational methods are used to find meaningful patterns which might help to objectively define relevant groups of tumours and/or genes. In this way, screening can be achieved for thousands of genes for polymorphism or loss of heterozygosity by single nucleotide polymorphism microarrays, or by comparative genomic-hybridization arrays. Global patterns of epigenetic changes can also be screened. In addition to confirming the genetic aberrations mentioned earlier, microarray has revolutionized clinical medicine by beginning to find ways to objectify diagnosis and prognosis based on expression profiling and bringing to light previously unrecognized genes that may contribute to the initiation or progression of malignant gliomas[313].

Several studies applying expression profiling by microarray have revealed that various grades of glioma have distinct transcriptional patterns. Three distinct molecular subtypes of gliomas were identified by Shai *et al*[436] by gene expression profiling: GBMs, lower grade astrocytomas (includes grades II and III) and oligodendrogliomas. They also found that primary GBMs and secondary GBMs are molecularly distinct and secondary GBMs constitute a highly heterogeneous group likely due to the fact that they arise from lower- grade gliomas. Astrocytomas (grade II, grade III, and GBM) were also found to have a transcriptional profile enriched for genes involved in cellular proliferation, RNA processing, signal transduction and proteosomal function[436]. Oligodendrogliomas, on the other hand, were enriched for proteosomal subunits and genes involved in DNA repair and energy metabolism. These genes may be important for the initiation or progression of gliomas[436]. Tso *et al*[483] also discovered distinct transcription profiles between primary and secondary GBM. They found that primary GBM have a propensity to overexpress extracellular response-associated genes reflecting the process of host-tumour interaction. For example, included in the list of 58 genes found overexpressed only in primary GBM and not secondary GBM are genes associated with inflammation, coagulation, immune/complement responses, angiogenesis, extracellular matrix remodelling, and status of hypoxia/angiogenesis among others[483]. Secondary GBM on the other hand, overexpress primarily mitotic cell components. In fact, all 21 genes that were overexpressed only in secondary GBM are associated with the cell cycle including genes involved with cell cycle control, DNA synthesis and repair, cytokinesis, movements of spindle and chromosomes, DNA bending, kinetochore function,

chromatid separation, and regulation of *TP53*, and mitotic chromosome condensation[483].

Moreover, microarray studies have identified previously unrecognized subsets of gliomas with distinct gene-expression profiles. Mischel *et al*[314] found that primary GBMs, which are morphologically similar, can be further categorized into three groups: those which overexpress EGFR, those with contiguous upregulation of genes on chromosome 12q13-15, and those lacking either change. The EGFR over-expressing GBMs were noted for their upregulation of growth factors, receptors and signal transduction molecules including VEGF, as well as plasma membrane-bound transporters and channels such as the multidrug chemoresistance gene SRI (sorcin) and MLC1, a mutation associated with white matter brain defects. Other growth factors and receptors upregulated in this group include pleiotrophin (PTN), and its receptor (PTRPZ1), endothelin B receptor (ET(B)), as well as the anti-apoptotic protein Bax inhibitor 1 (TEGT) and cyclin D2. Additionally, the EGFR over-expressing tumours had increased expression of extracellular matrix proteins including tenascin C and fibronectin, which have roles in GBM invasion. The upregulation of multiple growth factor-mediated signal transduction pathways in EGFR over-expressing GBMs may promote GBM cell proliferation, survival and angiogenesis. Over-expression of genes on 12q13-15 led to increased expression of CDK4, cyclin D1, and CENTG1, a signal transduction molecule that enhances PI3K and cyclin D1 activity[314]. As well, the 12q13-15 over-expressing group had very high transcript levels for autotoxin, a secreted mobility factor that has been shown to promote tumour cell invasion and

metastasis[330,458], and significantly high transcriptional levels of oligodendroglial genes (*MBP*, *MAG*, *PLP1*, *Nkx2.2*, *Mal*, and *Sox10*)[314]. Thus, transcriptional information may contain more data about individual patients with gliomas than pathological examination giving credence to the development of molecular diagnostics to assign treatments based on genotype rather than phenotype.

Further studies examining the expression profile of the tumour core and white-matter-invading cell populations revealed transcriptional differences. Among many other genes, Hoelzinger *et al*[199] found that autotoxin (ATX), ephrin B3, B-cell lymphoma-w (BCLW), and protein tyrosine kinase 2 beta were more highly expressed in invasive glioma cells. In the tumour core, insulin-like growth factor binding protein 2 (IGFBP2) and vimentin were robustly expressed. Thus new therapies may be designed to optimally to target both cell populations.

In addition to the ability of microarrays to reveal previously undiscovered subsets of gliomas, they can also be used to predict survival outcome[128,345]. Studies have shown that gene expression-based classification of malignant gliomas actually correlated better with survival than histological classification[345]. Poor survival was found to be associated with overexpression of genes involved in matrix structural components, assembly and modification, which may facilitate local invasion and migration[128]. Some of these extracellular growth factors or modulators include VEGF, insulin growth factor binding protein 4 and 6 (IGFBP4 and IGFBP6). As well, poor survival was associated with overexpression of *EGFR*, *AKT1*, and *IGFBP2*. IGFBP2 promotes invasion in GBM by upregulating

a panel of genes involved in invasion, especially *MMP2*[506]. A second group of patients with poor survival was defined by overexpression of genes involved in mitosis such as *Ki-67* and *PCNA*[128]. Longer survival was associated with genes involved in neurogenesis (*BMP2*, *DLL3*, *HDAC4*, *EDNRB*, *HEY2*, and *NTRK*), or genes involved in synaptic transmission[128]. Rich *et al*[399] did not find any prognostic significance associated with *EGF*, *TP53*, *p16*, or *PTEN*, but increased expression of genes that share roles in the regulation of cellular motility, namely *osteonectin* (*SPARC*), *doublecortex*, and *semaphorin*, were also associated with shorter survival. Data from Phillips *et al*[364] found that low expression of PTEN was associated poor prognosis, and high levels of both PTEN and DLL3 expression were associated with the best outcome. Therefore, DNA-microarray analysis can identify clinically relevant subsets of patients with GBM in a robust and reproducible manner.

Besides finding new ways to categorize gliomas, microarrays have identified multiple novel genomic markers and genetic alterations for gliomas. Putative tumour suppressor genes, *TOPORS*, *FANCG*, *RAD51*, *TP53BP1*, and *BIK*, were recently found to be deleted in a subset of tumours[35]. Roversi *et al*[408], confirmed previous genetic anomalies associated with glioma progression using microarray, including +1q32, +7, -10, -22q, *PTEN* and *p16* loss, and disclosed multiple new regions, some correlating with grade malignancy. For instance, only grade I-III gliomas showed losses at 3p26 (53%), 4q13-21 (33%) and 7p15-21 (26%), while GBMs demonstrated 4p16.1 losses (40%) exclusively[408]. By genomic hotspot detection, they found new candidate genes such as *PRDM2* (1p36.21), *LRP1B*

(2q22.3), *ADARB2* (10p15.3), *BCCIP* (10q26.2) and *ING1* (13q34) for losses and *ECT2* (3q26.3), *MDK*, *DDB2*, *IG20* (11p11.2) for gains[408]. Hundreds more genes have been discovered to be differentially expressed between various glioma grades and studies into their functionality in gliomas will provide additional insight into the initiation and progression of gliomas as well as provide novel therapeutic targets.

Therefore, microarrays offer the possibility for improved diagnosis, prognosis, and therapeutic decision making by creating an objective tool to assign the best treatment to patients based on genetic profiles of their tumours for targeted therapy. They also reveal the genetic and cell biological complexities of these tumours.

**VECTORS IN GENE THERAPY**

Increasing knowledge of the genetic lesions present in glioma has contributed greatly to the study of cancer therapeutics that can cater directly towards individual differences in patients with malignant gliomas. Gene therapy, defined as the use of nucleic acids as drugs, has great potential as a novel therapeutic. It offers a means to deliver genes that can have the capacity to restore specific aberrant cell signalling pathways or allow the local accumulation of therapeutic drugs at concentrations that would otherwise be detrimental if delivered systemically. Additionally, particular genes can be delivered that can activate the immune system to specifically aid in tumour clearing or to prevent the progression of disease by blocking angiogenesis. Both viral and non-viral vectors

have been explored in order to deliver therapeutic genes to the tumour mass. For optimal gene delivery a vector must satisfy the following criteria: it must efficiently and safely transduce target tissue, result in gene expression that would be of sufficient duration, and be easily manufactured. The most popular vectors thus far are adenoviruses with 26% of the world's gene therapy clinical trials employing this vector, followed by retroviruses at 24%, and non-viral transfer of naked or plasmid DNA with 17%[112]. Other vectors in clinical trials in order of popularity following non-viral transfer of naked or plasmid DNA are lipofection, poxvirus, vaccinia virus, adeno-associated virus (AAV), herpes simplex virus, and RNA transfer[112]. Viral vectors are useful in gene therapy because of their natural ability to enter and mediate transgene expression in infected host cells; however, their safety in terms of immunogenicity and replicative capacity is a major consideration in vector construction. In contrast, the non-viral transfer of DNA is relatively innocuous; nonetheless, they may be less useful as transfection efficiency is very low. The following sections will review different vector types relevant to cancer gene therapy and address their advantages and disadvantages.

**Retrovirus vectors**

For the past few years until only very recently, retroviruses were the vector of choice among clinical trials for gene therapy[112]. Retroviruses are diploid RNA viruses with a genome around 8-11kb in size, packaged into a virion and enveloped in a lipid coat composed of viral proteins and host cell membrane (reviewed by Buchschacher *et al*[42] and Kurian *et al*[256]; Figure 5A). The genome encodes a packaging signal ($\psi$), structural capsid proteins (*gag*), a viral protease

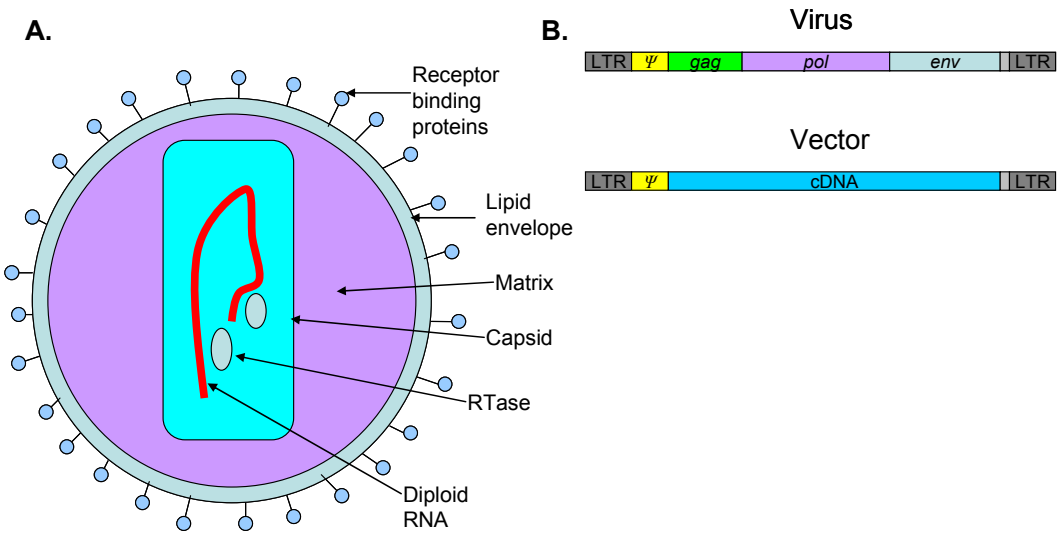



**Figure 5: Retroviruses.**

***A*,** diagram of the retrovirus indicating the location of the lipid envelope, receptor binding proteins, diploid virus RNA, the capsid core, and the viral reverse transcriptase (RTase). ***B***, among other proteins, the retrovirus genome encodes a packaging signal ($\psi$), structural capsid proteins (*gag*), viral reverse transcriptase (*pol*), and envelope glycoproteins (*env*). Flanking the genome are long terminal repeats (LTRs) required for integration. In retroviral vectors *gag*, *pol*, and *env* are replaced by the transgene cDNA leaving $\psi$ and the LTRs intact, but rendering them replication defective.

(*pro*), integrase, viral reverse transcriptase (*pol*) and envelope glycoproteins (*env*) (Figure 5B). Flanking the genome are long terminal repeats (LTRs) that are required for integration and also contain promoter/enhancer regions. Retroviruses can be differentiated by their ability to bind various cellular receptors. Upon binding to cellular receptors, the retroviral envelope fuses with the host cell membrane liberating the virion capsid core into the cytoplasm. The RNA genome must then be reverse transcribed by the viral reverse transcriptase into double-stranded DNA. The resulting complementary DNA sequence forms a complex with the viral integrase protein. This complex is transported to the nuclear membrane where it awaits for the onset of mitosis when the nuclear membrane is disrupted allowing the complex to enter and integrate into the host cell chromosome. Once integrated, the virus utilizes the host cell machinery for viral gene expression.

Retroviral vectors are deleted in the genes *gag*, *pol*, and *env* to render them replication defective for safety purposes, allowing for a level of control over gene expression, but still retaining the ability to integrate into the host genome[42]. The deleted genes are then provided in trans by a viral packaging cell line, helper viruses, or by co-transfection with packaging plasmids during vector production[42]. Thus, the only viral sequences that remain in the retroviral vector are $\psi$ and the LTRs. Stable integration and low immunogenicity due to the removal of genes encoding for viral proteins have contributed to the capacity of retroviral vectors to

induce long-term therapeutic transgene expression. In fact, transgene expression of over two years has been achieved in humans[31].

Nonetheless, retroviral vectors have limited application in cancer gene therapy. Firstly, transgene integration by retroviruses can pose a risk for a secondary malignancy should the transgene integrate within an oncogene. Retroviral integration, in fact, has preferred sites and favours integration near transcription start sites[531] which raises the possibility that retroviruses may particularly induce insertional activation of oncogenes. Three children developed leukemia after treatment with a murine leukemia retrovirus (MLV)-based vector in a gene therapy trial for X-linked severe combined immunodeficiency. The therapeutic MLV vector had integrated in the 5' region of the *LMO2* oncogene, which likely contributed to neoplastic transformation[183,184]. Another disadvantage for retroviruses is their inability to infect non-dividing cells, which is a great disadvantage given that in the majority of brain tumours the internal areas of the tumour mass are not actively dividing. Retroviruses are also inactivated by the complement system, which limits transduction efficiency. Furthermore, retrovirus vectors are difficult to produce to high titers, only moderate titers of $10^6$-$10^7$ plaque-forming units (pfu)/mL are typically achieved using packaging cell lines[296]. Unfortunately, the titers required for treating cancer are much higher than can be produced *in vitro*. To resolve this issue for *in vivo* studies, grafts of vector-producing cells (VPCs) are inserted into the tumour itself, but the probability for graft rejection is a concern. Promising pre-clinical work with VPCs in gliomas led to a phase III clinical trial[379]. However, the results of the clinical trial have been

disappointing and replicating retrovirus are now being explored to increase transduction efficiency[467]. Lentiviruses, a subclass of retroviruses with the added ability to infect both proliferating and non-proliferating cells, are also currently being evaluated for safety as an alternative to MLV. The most widely used lentiviral vector system for CNS gene transfer is based on HIV-1 (human immunodeficiency virus tepe I) and has been tested in pre-clinical glioma models with promising results[249,424]. However, concerns relating to insertional mutagenesis remain as HIV-1 favors active genes for integration[433]; therefore, efforts toward targeted integration in noncoding sequences as well as the development of nonintegrating lentiviral vectors are ongoing[47,539].

**Adenovirus Vectors (AdV)**

Unlike retroviruses, adenoviruses are non-enveloped double-stranded DNA viruses that are capable of infecting both dividing and non-dividing cells (reviewed by Russell[411] and Ghosh *et al*[154]). Adenoviruses are also much more effective for *in vivo* transduction than retroviruses[371]. There are over 50 adenovirus serotypes, and serotypes 2 and 5 have been used extensively in gene therapy. Adenoviruses are 60-90nm in diameter with a 36kb genome that is surrounded by a symmetrical icosahedral protein coat composed of 252 capsomers comprising 240 hexons and 12 pentons projecting from each vertex of the virus[456] (Figure 6A). The penton capsomere is composed of a penton base and a trimeric fibre protein ending in a globular knob protein. Infection is initiated by the binding of this fibre knob protein to the host cell surface molecule, coxsackie and adenovirus receptor (CAR)[16]. Further entry into the cell is mediated by the

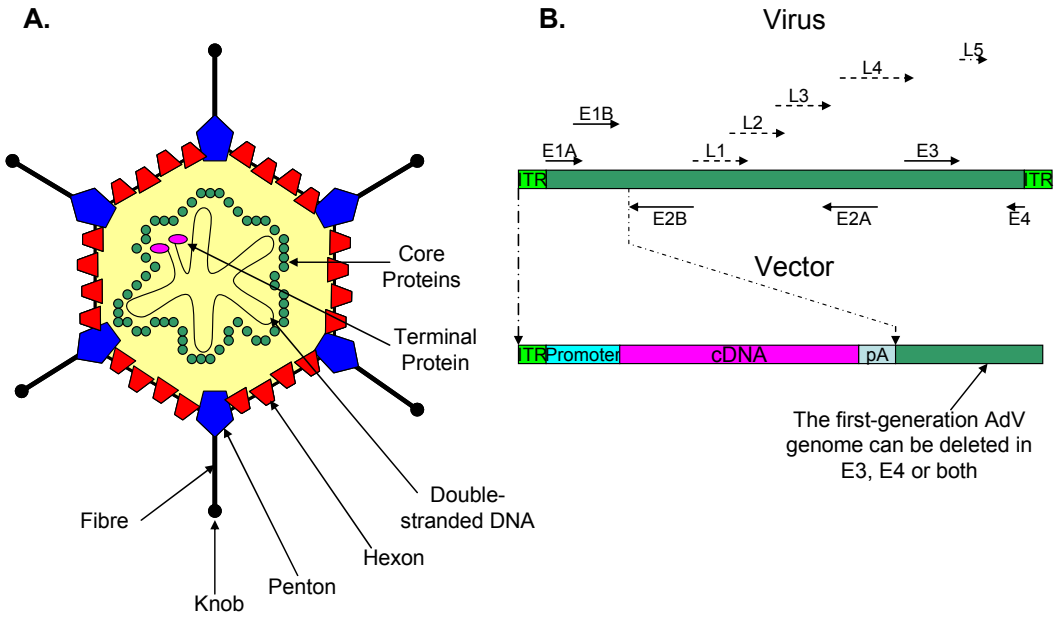



**Figure 6: Adenoviruses.**

***A***, diagram of the adenovirus showing the arrangement of the hexons, the pentons projecting from the vertices of the virus, the location of the trimeric fibre knob protein in the penton capsomere, and the terminal protein attached to the 5' end of the virus genome. A number of other minor proteins also make up the virus capsid termed core proteins. ***B***, the adenovirus genome encodes four distinct early regions (E1-4) and five major late regions flanked by ITRs. Adenovirus vectors are usually deleted in the E1 region and the E3 and/or E4 region with retention of the ITRs allowing for the accommodation of a transgene under a heterologous promoter.

penton base, which binds to the integrins, $\alpha_V\beta_3$ or $\alpha_V\beta_5$, that results in the internalization of the virus into an endosome[305,455,520]. Release of the virus from the endosome into the cytoplasm is caused by conformational changes in viral coat proteins as a result of a decrease in the internal pH. Once released from the endosome, the virus translocates to the nuclear membrane where a dissociation of the viral coat proteins occurs, which leads to the exposure of viral DNA. The viral DNA then enters the nucleus and establishes episomally without the requirement for cell division where it can undergo transcription and replication.

The adenovirus genome is flanked with inverted terminal repeats (ITRs) with a 55kDa terminal protein covalently attached to the 5' end. The genome is divided into two phases according to transcription; four distinct early regions (E1-E4) and five alternatively spliced major late regions (L1-L5) (reviewed by Russell[411] and Ghosh *et al*[154]; Figure 6B). E1A proteins drive the host cell into S phase to provide conditions conducive to viral replication, while E1B binds to the tumour suppressor p53 to block host cell apoptosis. E2A (encodes for DNA binding protein) and E2B (encodes for terminal binding protein and DNA polymerase) provide the machinery for replication of virus DNA and the ensuing transcription of late genes. The E3 region encodes proteins that help to evade the host immune system. E4 proteins facilitate virus messenger RNA metabolism, promote virus DNA replication, shut-off host protein synthesis, and provide resistance to lysis by cytotoxic T lymphocytes (CTLs). The adenovirus structural components which package the virus DNA to produce infectious progeny are encoded by the major

late regions. The nucleus is lysed when these viral particles accumulate leading to virion release.

The choice of recombinant adenovirus vector (AdV) used for a particular application is based on the desired expression level, size of target gene, and the characteristics of target tissues. Initially, AdV were only E1-deleted, which impaired virus replication, however, replication was not completely eliminated as expected because many cells harboured E1-like proteins. Additional deletions in E3 and/or E4 were later created to further reduce the possibility for replication-competent virus contaminants and could also accommodate larger transgenes than E1-only deleted adenoviruses. E1, E3 and/or E4 deleted adenoviruses are now commonly used in gene therapy experiments (also termed first- generation AdV; Figure 6B). Deletion of E1, E2A, E3, and E4 viral genes can generate "gutless" vectors with reduced immunogenicity and even more available space for transgene insertion (28-32kb capacity). Nevertheless, the production of gutless vectors is labour intensive and replication and production requires the presence of a helper virus.

AdV are produced in packaging cell lines that provide the missing early genes in *trans* and are easily produced to a titer of $10^{10}$ pfu/mL, which is an important advantage for large scale production for clinical trials or manufacturing. Additional benefits of AdV are that it can infect a wide range of cell types with high efficiency, has a large insert capacity, no risk for insertional mutagenesis, and is well-characterized and easily manipulated. Furthermore, successful

administration to the brain via the carotid artery has been achieved leading to a large spatial distribution of AdLacZ vector (Nalbantoglu, unpublished). The greatest disadvantage for AdV is its immunogenicity, which has contributed to minor clinical side effects in glioma patients ranging from fevers and chills to headaches[370]. Nonetheless, it has been shown that the immune effect can actually aid tumour clearing by immune cell recruitment to the tumour area[257,258,487]. Furthermore, it can be argued that the transient transfection by AdV may be advantageous in cancer therapy as transgene expression will no longer be required if cure is attained.

Efficient entry of the virus vector into host cells, though, is still dependent on the level of CAR present on the cell surface and CAR is downregulated in many cancers[106]. AdV still manage to enter into cancer cells by binding secondary integrin receptors that are commonly upregulated in cancer, but entry is not as efficient as through CAR. Nonetheless, changing the fibre knob of the AdV has been successful in increasing transduction efficiency and tumour specificity in first generation AdV. For instance, changing the fibre knob to contain an RGD motif can target the AdV to tumour cells that overexpress integrins[486]. Moreover, adding a stretch of lysine residues to the knob can target the AdV to heparan sulfates which are ubiquitous on multiple cell surfaces[97,441]. To further increase transduction efficiency conditionally replicating adenoviruses have been explored and will be discussed in a later section.

**Adeno-Associated Virus Vectors (rAAV)**

Adeno-associated viruses (AAV) are small (20-25nm) single stranded DNA viruses of the *parvovirus* family that, like adenoviruses, are also capable of infecting both dividing and non-dividing cells[367]. They are non-enveloped, icosahedral in shape, and have a broad cell tropism (Figure 7A). Alternate serotypes differ in their tissue tropism and ability to transduce different cell types. AAV-2 was the first and most extensively studied AAV vector system and can efficiently transduce muscle fibres, hepatocytes, and neurons *in vivo*[510]. AAV-2 interacts with the host cell *via* the widely expressed heparan sulphate proteoglycans as its primary receptor[463]. The $\alpha_V\beta_5$ integrins, fibroblast growth factor receptor-1, or hepatocyte growth factor receptor can act as co-receptors for AAV-2 internalization after binding to the cell surface[237,377]. AAV-5 utilizes an α-2,3-N-linked sialic acid for binding to the cell surface and makes use of PDFGR as a co-receptor[88,505]. AAV-4 depends on α-2,3-O-linked sialic acid for cell binding and its co-receptor has not been identified[231]. The required receptors for other AAV serotypes are not known yet. AAV are non-pathogenic in humans and require the presence of a helper virus, such as adenovirus, herpes simplex virus (HSV), or vaccinia virus in order to replicate[429]. In the absence of helper virus, AAV genome integrates specifically into the AAVS1 locus on chromosome 19q in human cells and remains latent[252,283]. The lytic phase is induced upon the presence of helper virus. Their non-replicating life cycle in the absence of helper virus and non-pathogenicity make AAV an attractive vector for gene therapy.

The AAV genome is only 4679bp, and is relatively simple, containing a *rep* and a *cap* gene flanked by inverted terminal repeats (ITRs) of 145bp[448] (Figure 7B).

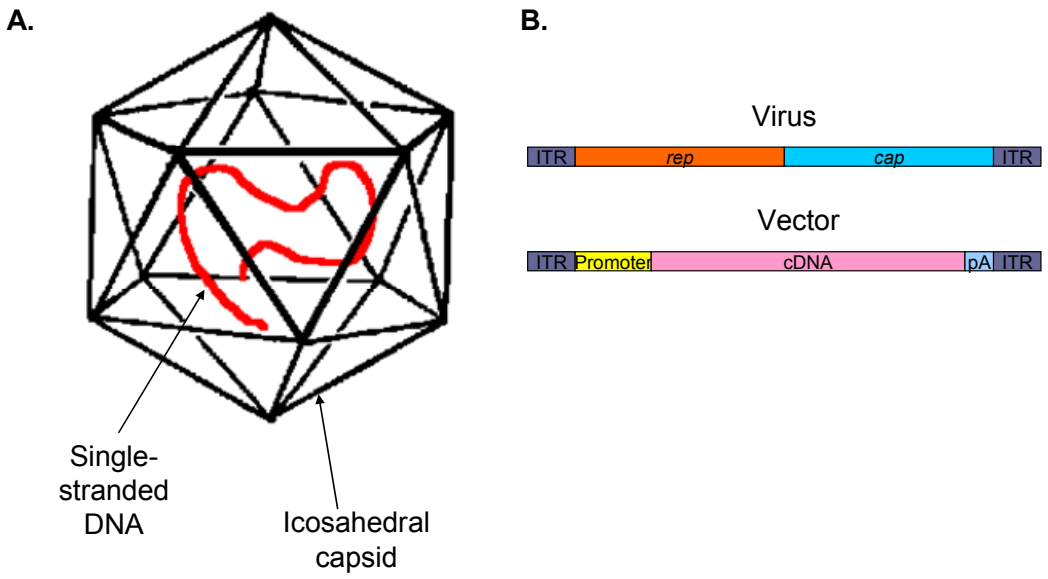



**Figure 7: Adeno-associated virus.**

***A***, the icosahedral capsid houses the single-stranded DNA genome. ***B***, the AAV genome contains the genes *rep* and *cap* bracketed by ITRs. In AAV vectors, the *rep* and *cap* are replaced by a transgene positioned in between the ITRs.

Through a combination of alternative translation start and splicing sites, the small genome is able to express a number of gene products. The *rep* gene encodes four main proteins that control AAV replication, structural gene transcription, and integration into the host genome. Three structural proteins that form the viral capsid are coded by the *cap* gene. The ITRs form a hairpin structure, owing to a palindromic sequence, and enable replication initiation, site specific integration, as well as encapsidation.

The recombinant AAV genome for gene therapy contains the transgene cassette flanked by ITRs and lacks *rep* and *cap* sequences (Figure 7B). The *rep* and *cap* are provided by co-transfection with a plasmid containing *rep* and *cap* during vector production. Helper virus function during vector production is provided by either a helper virus such as adenovirus, or with a plasmid containing the necessary helper virus genes for AAV replication, a mini adenovirus genome with all the structural genes removed.

Since the wild-type genome is only 4.7kb, the amount of foreign DNA incorporated cannot greatly exceed this value, which limits the use of rAAV to small transgenes. Another drawback of rAAV is that integration was found to occur in sites other than AAVS1 and was associated with chromosomal deletions and other rearrangements that have important implications on both the transgene and the host gene expression[312]. However, it is important to mention that the majority of AAV genome remains episomal in infected cells[510].

Their lack of pathogenicity and low immunogenicity along with their capacity for long term gene expression are benefits that rAAV possess over adenoviral vectors. In fact, AAV-2 has been found to be substantially less likely than adenoviral vectors to cause innate and adaptive immune responses to its viral capsid[425]. This can be attributed to the low efficiency of rAAV to transduce antigen presenting cells such as macrophages or dendritic cells *in vivo*[425]. Furthermore, clinical trials with rAAV for severe hemophilia B show that it is well-tolerated and can persist for 3.7 years in skeletal muscle[225,239,293]. Phase I clinical trials for the treatment of Canavan disease, which involved intracerebral delivery of rAAV, showed only minimal signs of inflammation or immune stimulation indicating that use of this vector in the brain is safe and well-tolerated[303]. Although, portal vein infusion of rAAV in a hemophilia clinical trial had induced a transient immune response to the rAAV capsid, which led to the destruction of transduced hepatocytes by cell mediated immunity and declined transgene expression[294]. Still, rAAV shows great promise as a well-tolerated gene therapy vector for the treatment of brain diseases such as glioma.

**Herpes Simplex Virus Type 1 (HSV-1) Vector**

HSV-1 is a common human pathogen that is much larger than both adenovirus and AAV with a genome of ~150kb, encoding approximately 90 genes in a linear double-stranded DNA genome (reviewed by Shen *et al*[437]). The HSV-1 genome is arranged as L (long) and S (short) components that are covalently linked. Each component consists of unique sequences bracketed by inverted repeats (IR) and terminal repeats (TR) (Figure 8B). The virus causes asymptomatic infections of

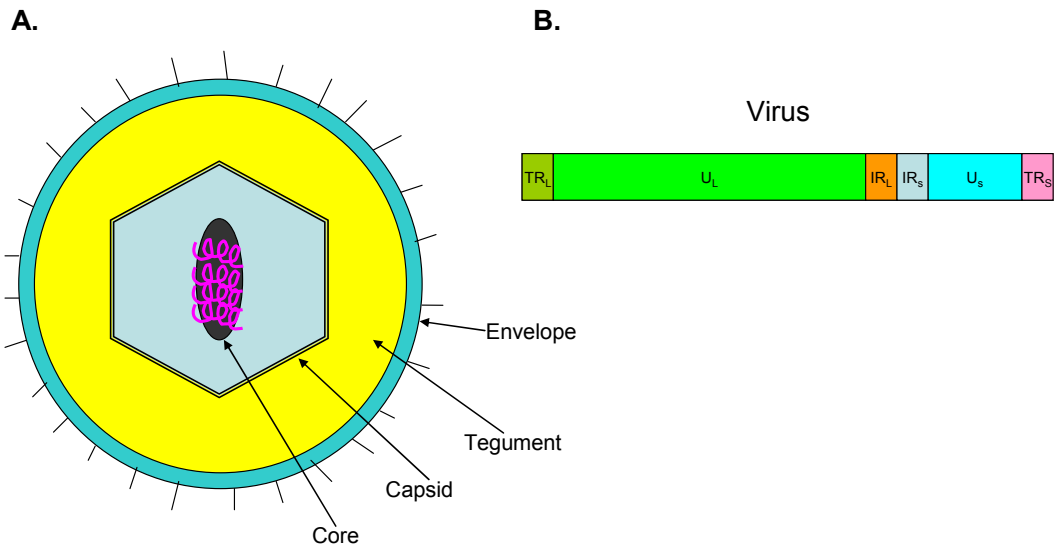



**Figure 8: Herpes Simplex Virus-1.**

***A***, the HSV-1 structure is composed of four main elements: the outer envelope, the core containing the genome wrapped as a spool, the icosadeltahedral capsid, and an amorphous tegument that surrounds the capsid. ***B***, the HSV-1 genome is arranged as L (long) and S (short) components that are covalently linked. Each component consists of unique sequences flanked by inverted repeats (IR) and terminal repeats (TR). The genome encodes for approximately 90 genes.

the nervous system with periodic epidermal manifestations. Infections of non-neuronal cells results in host mRNA degradation and obstruction of protein synthesis[386]. It is an enveloped virus composed of four main components (Figure 8A): (1) the outer envelope is a lipid bilayer with ~13 embedded viral glycoproteins with spikes on its surface. (2) The core contains the genome wrapped as a toroid or spool, and (3) surrounding the core is an icosadeltahedral capsid that is composed of 162 capsomers. The capsid contains channels controlled by tegument proteins that regulate the transport of DNA through the channel. (4) An amorphous tegument surrounds the capsid and contains a matrix of proteins with important roles during HSV-1 infection. Contained within the matrix of the HSV-1 particle is a tegument protein called VP16 that is carried into the cell upon infection. VP16 induces a viral gene cascade beginning with the HSV immediate early (IE) genes, ICP0, -4, -6, -22, and -27. These genes then lead to the expression of the early and late genes required for replication and encapsidation.

As a vector, HSV-1 has a number of advantages; firstly, it has a naturally broad host range due to the capacity for the HSV envelope glycoproteins (gB and gC) to bind to extracellular heparan sulphate moieties of cell surface proteoglycans[533]. In addition, HSV-1 is able to infect both dividing and quiescent cells and can persist for an extended period in a non-integrated state in neurons thereby eliminating concern for insertional mutagenesis[386]. Much of the HSV-1 genome is redundant allowing for large deletions; as much as 50kb of heterologous DNA can be

accommodated without greatly affecting viral growth[508], and up to five transgenes can be inserted into different loci[160].

Replication deficient vectors typically contain deletions of essential, immediate-early genes encoding transcriptional activators to block viral gene expression and reduce cytotoxicity[207]. The first replication deficient HSV-1 vectors were deleted in the IE gene ICP4, but unfortunately, these were toxic as a result of a potent immune response against the other IE genes of the virus. To prevent toxicity, a number of recombinant HSV-1 vectors have been made harbouring a variety of combinations of IE gene deletions including a vector with all five IE genes deleted[254,417,530]. An alternative strategy was to generate HSV amplicons, which are plasmid vectors containing the transgene with only the HSV packaging and cleavage signals along with the viral origin of replication, expressing none of the IE genes[129]. This amplicon can only replicate and be packaged in the presence of a helper virus providing the missing gene products in *trans*. Clinical trials with HSV indicate that HSV vectors in the human brain can be tolerated, but there is great concern about the potential for reactivation of the wild-type virus, which is believed to be latent in the brains of many humans[421]. The biggest challenge that HSV vectors face, however, is the potential for direct toxicity to neurons or persistent cerebral inflammation due to viral protein expression or immune responses[274], which has precluded its use beyond animal models in many cases.

**Non-Viral Gene Transfer**

The non-viral transfer of DNA to target cells circumvents some of the problems associated with viral vectors such as endogenous virus recombination, oncogenic defects, and unexpected immune response. It is also relatively simple to produce on a large scale. Naked DNA is delivered via a number of physical techniques including electroporation, gene gun, ultrasound, and hydrodynamic pressure[339]. Nonetheless, transduction efficiency of naked DNA in the brain remains very low despite the great advantage of avoiding an immune response[434]. Chemical techniques have been much more successful in improving transfection by incorporating gene carrier molecules. Plasmid DNA complexed with cationic lipids to form liposomes have been highly successful and have been used as a vector in phase I clinical gene therapy trials for glioma[221,499]. Levels of transgene expression by the cationic complexes are still several orders of magnitude below viral delivery systems such as AdV[56] despite the delivery of large amounts of DNA to target cells *in vivo*[56]. Improvements to DNA transfer have included the attachment of nuclear localization signals[8,54], viral genome components[234], targeting elements[440] or high mobility group proteins[8] to gene carrier molecules or to DNA directly[339].

## GENE THERAPY STRATEGIES FOR MALIGNANT GLIOMAS

There are a multitude of strategies for gene therapy of malignant gliomas that include the application of targeted therapy, suicide gene therapy, immune system activation, cytokine release, and suppression of angiogenesis. It is doubtful that gene therapy will offer a single agent cure however, as its success will likely be demonstrated from its use in conjunction with traditional cancer treatments such

as surgery, chemotherapy and radiotherapy. The following section will outline the approaches that have generated a great deal of clinical promise.

**Targeted Therapy**

Targeted therapy is the correction of a genetic defect to restore cell cycle or apoptosis defects in cancer cells. Increasing knowledge of the genetic mutations that occur in malignant gliomas has greatly advanced the development of targeted therapy. As mentioned earlier, the most common genetic anomalies found in GBM are defects in p53, ARF, PDGFR and PDGF, EGFR and EGF, members of the Rb pathway (Rb, p16) and PTEN, which result in cell cycle and apoptotic deficiencies. Gene therapy strategies have targeted many of these genetic lesions in pre-clinical studies. For instance, HSV-1 amplicon delivery of siRNA developed against EGFR resulted in *in vivo* growth inhibition and apoptosis[427]. In another example, adenoviral delivery of p16 was able to produce growth arrest in p16 deficient glioma cell lines[273]. PTEN delivered by adenovirus into PTEN null cell lines resulted in inhibition of proliferation and decreased tumourigenicity[61]. As a result of promising pre-clinical data, Adp53 was tested in phase I clinical trials for malignant gliomas and the results show that clinical toxicity was minimal and no signs of viral dissemination were detected[264]. Overall median survival was 10 months, and one patient with GBM continued free of recurrence for more than 3 years. As attestation to the validity and efficacy of targeted gene therapy, Adp53 has recently been approved in China for the treatment of head and neck cancer[361].

Mechanisms of apoptosis are also commonly deregulated in cancer cells. One way to restore apoptosis that was explored is to introduce death receptor ligands such as Fas ligand[6] (Fas-L) or tumour necrosis factor-related apoptosis-inducing ligand[244] (TRAIL or Apo2-ligand) into glioma cells to directly activate caspases. The main advantage of this approach is that it is independent of losses of p53, Rb, or p16. This strategy is also independent of the immunosuppressive effects exerted by transforming growth factor- β (TGF-β), which is overexpressed by gliomas[426] and has been shown to antagonize immune therapeutic approaches[175,218]. Gene therapy approaches using adenoviral delivery of Fas-L increased the mean survival of rats bearing F98 gliomas by 80% compared with AdLacZ or untreated controls[6]. Soluble TRAIL has also been shown to exert strong anti-tumour activity on intracerebral human malignant glioma xenografts[406]; however, the pharmacokinetic profile of TRAIL indicated that the majority of the protein was cleared in 5h[504] necessitating the use of large amounts of protein to inhibit tumour growth. Intratumoural adenoviral delivery of TRAIL was tested as a possible solution in human glioma xenografts in SCID mice and it significantly suppressed tumour growth[244]. A great disadvantage to this approach, however, is the low level of expression of appropriate death receptors on many tumour cells and overt toxicity to the normal brain.

Targeting transcription factors may offer the most direct solution for treating cancer because many oncogenes and tumour suppressors can funnel through the same transcription factors. For example, the amplification of EGFR, and loss of PTEN function can potentially be repaired by overexpression of a single

downstream transcription factor, such as FOXO1, which is of particular interest in this thesis to target defects in tyrosine kinase receptor signalling that results in PI3K-Akt upregulation. We investigated this approach and the following provides a brief background.

FOXO transcription factors are significant downstream effectors of the PI3K-Akt pathway and are major direct substrates of Akt that execute the process of apoptosis and cell cycle arrest through their target genes. Akt negatively regulates FOXO by phosphorylation in the presence of growth factor signalling[37,170]. Akt phosphorylation of FOXO results in the binding of FOXO factors to 14-3-3 proteins, which leads to nuclear export[555]. Once in the cytoplasm, FOXO factors are ubiquitinated in a manner that is dependent on phosphorylation of Akt sites, and are then subjected to degradation by the proteosome[170,211]. FOXO factors belong to the Forkhead family of proteins and are characterized by a conserved DNA binding domain termed the 'forkhead box' (FOX)[230]. The 'O' subgroup of FOX transcriptional regulators includes FOXO1, FOXO3, FOXO4 and FOXO6 and were initially identified in humans, except for FOXO6, at chromosomal translocations in tumours[32,83,147,198,359]. All FOXO factors utilize the same forkhead responsive element and transactivate many of the same genes *in vitro*[145], although recent knockout mice of FOXO1, FOXO3, and FOXO4 indicate that they are functionally diverse[206]. Nuclear localization of FOXO proteins is required for their transcriptional regulatory functions, which include the control of genes involved in apoptosis, such as *Bim*[90,156], and *FasL*[39], and genes involved in cell cycle regulation such as *p27*[91,304], *cyclin D1* and *D2*[383,432]. In cells that carry the

*PTEN* mutation, the PI3K pathway is constitutively active, and this leads to the inactivation of endogenous forkhead transcription factors and the possible initiation and progression of tumours[170]. Several lines of evidence suggest that FOXO factors play a significant role in cancer. Tumourigenicity in nude mice induced by IKKβ or the receptor tyrosine kinase, HER2 oncogene, can be overridden by an active form of FOXO3 or FOXO4, respectively[208]. Cytoplasmic expression of FOXO3 in breast cancer also correlates highly with poor survival of patients[208]. In addition, FOXO factors have been shown to cause apoptosis in prostate carcinoma cells[209] and melanoma cells[165].

Of all the FOXO factors, restoration of FOXO1 activity appears to be the most promising as a cancer therapeutic based on its additional roles in angiogenesis and neovascularization[144,206,368]. Knockouts of FOXO1, FOXO3, FOXO4 have been generated and only FOXO1-null mice are embryonic lethal (E10.5) displaying major defects in normal blood vessel development[144,206]. FOXO3 and FOXO4 mice are grossly indistinguishable from wild-type littermates, but FOXO3-null mice show age-dependent infertility[206]. Additionally, FOXO1 and FOXO3, but not FOXO4 significantly inhibit endothelial cell migration and tube formation[368]. This has important implications for malignant gliomas as they are characterized by intense microvascular proliferation. Tumour capillaries supply hundreds of tumour cells indicating that additional targeting of angiogenesis should increase anti-tumour effects.

In this study, we evaluated the therapeutic potential of adenoviral vector delivery of FOXO1 (AdFOXO1;AAA) in a first generation non-replicating virus to correct the consequences of frequent genetic mutations in EGFR and PTEN in two representative cell lines of GBM, U87 and U251. Both cell lines are PTEN null, but U87 bears a functional wild-type p53 and U251 has an inactive p53 with a missense mutation. Furthermore, EGFR expression in both cell lines has been associated with increased tumourigenicity[235,453,554]. The FOXO1 protein that we tested is mutated in three of its Akt phosphorylation sites; T24, S256, and S319, which have been converted into alanine residues. Thus, FOXO1;AAA is not subject to Akt phosphorylation control and should remain primarily nuclear[329,472]. Our main objective was to characterize the cytotoxic effect of AdFOXO1;AAA by examining its mechanism of cell death, and to analyze its potential as a form of therapy for GBM in an intracerebral mouse model. We sought to determine if AdFOXO1;AAA could successfully restore cell cycle arrest and induce cell-death *in vitro* and prolong survival *in vivo* with xenografts of glioma cells. We also wanted to show if FOXO1;AAA has anti-angiogenic effects and whether it could act as a radio-sensitizer to ascertain its potential as an adjuvant in the clinical setting.

Since the number and type of genetic lesions differ vastly from one patient to the next, it might be more practical in some cases to apply a more generalized therapy that has the goal of locally concentrating chemotherapeutic drugs.

**Enzyme/Pro-Drug Therapy (Suicide Gene Therapy)**

One of the major factors limiting successful cancer chemotherapy is the blood-brain barrier (BBB). The BBB separates the brain from the blood and helps regulate brain function and metabolism by preventing the passage of molecules that do not have the suitable physiochemical features that can penetrate this physical barrier. The BBB makes it difficult to administer chemotherapeutic agents at doses high enough in the brain for a therapeutic effect, as well drug resistance mechanisms in the BBB and the toxicity of chemotherapy complicates matters. An alternative solution to chemotherapy is the use of pro-drugs that are inert over a wide range of doses, but can be converted into pharmacologically active toxic molecules by specific activating enzymes in a targeted population of cells. Gene therapy techniques allow for the targeting of tumour cells and the delivery of non-mammalian activating enzymes that would essentially permit local accumulation of high doses of active anti-cancer drug avoiding systemic toxicity and limiting neural toxicity. Combinations of enzyme/pro-drug that have been tested pre-clinically in a variety of human cancers include nitroreductase/CB1954, cytochrome P450/cyclophosphamide, P450/bioreductives, carboxypeptidase G2/CMDA, and horseradish peroxidase/indole-3-acetic acid among others (reviewed by Dachs *et al*[77]). The most studied enzyme pro-drug combinations are thymidine kinase/ganciclovir (TK/GCV) and cytosine deaminase/5-fluorocytosine (CD/5-FC).

*TK/GCV*

The most intensely researched enzyme/pro-drug for solid tumours, both pre-clinically and clinically, is the combination of thymidine kinase (TK), isolated

from HSV-1, with the pro-drug, ganciclovir (GCV). In the natural replication cycle of HSV-1, TK is implicated in the production of deoxyribonucleotides to assist viral DNA replication in cells with suboptimal precursor pools[66,113,120]. However, TK also phosphorylates GCV into an intermediate, which is then further phosphorylated by cellular kinases to a di- or tri- phosphate form that can incorporate into DNA as a nucleoside analogue leading to the demise of infected dividing cells, in a manner similar to S-phase chemotherapeutic drugs. GCV-triphosphate is capable of transferring to non-transduced cells via gap-junctions and thus produces a potent bystander effect[127]. Kianmanesh *et al*[243] showed that an additional immune-mediated bystander effect could eliminate not only the main tumour mass, but also anatomically distant metastases when only a fraction of the tumour cells expressed TK.

However, TK/GCV has a number of disadvantages. Firstly, GCV cannot easily cross the blood brain barrier, which complicates the delivery of the pro-drug to tumour tissues. In addition, the requirement for gap junctions to mediate the bystander effect is a weakness as many cancer cells lack gap junction connexins[92]. Still, on the basis of good pre-clinical data and because GCV is already approved as an anti-herpetic agent, numerous clinical trials for various malignancies are utilizing TK/GCV[370].

Clinical trials for malignant glioma using the *HSV-TK* gene have used non-viral, retroviral, and adenoviral vector delivery systems. Phase I trials using cationic liposomes to deliver an HSV-TK plasmid intratumourally followed by GCV

treatment, however, only led to transgene expression in one of the five patients tested[221]. Furthermore, this small trial did not provide reliable information regarding the efficacy of non-viral vector-mediated gene therapy[221].

Phase III clinical trials have been conducted using VPCs generating retroviral vectors carrying the *HSV-TK* gene (Rv.HSV-TK)[379]. This large, randomized, parallel study was conducted by Rainov *et al*[379] and enrolled two-hundred and forty-eight patients with newly diagnosed and previously untreated GBM. The patients in this study were treated using the standard therapy of surgical resection and radiotherapy (50-60Gy), or standard therapy plus adjuvant Rv.HSV-TK. An average volume of 9.1mL of VPCs at a concentration of $10^8$ cells/mL were administered during craniotomy into the margins of the tumour cavity. No differences in median survival, or safety between the patients treated with standard therapy versus gene therapy were found. Interest in the use of retroviral vectors for brain tumours as a result has declined.

In contrast, adenoviral delivery of *TK* (Ad.HSV-TK) may be much more promising than Rv.HSV-TK, although, phase III trials have yet to be completed. In a study by Sandmair *et al*[422] where they compared both Rv.HSV-TK and Ad.HSV-TK in malignant gliomas, the mean survival time in the group treated with Ad.HSV-TK (15 months) was significantly longer ($p<0.012$) than the Rv.HSV-TK (7.4 months) and the control AdLacZ group (8.3 months). Phase IIb studies by Immonen *et al*[217] showed the mean survival of malignant glioma patients treated with Ad.HSV-TK plus GCV (5mg/kg twice daily for 14 days),

was significantly longer (70.6 weeks) than the standard care group (radical tumour excision and radiotherapy; 39.0 weeks). The increased median survival times observed in the Ad.HSV-TK/GCV clinical trials demonstrate the immense potential that suicide gene therapy holds for the treatment of gliomas, although phase III trials will provide a more definitive answer.

*Cytosine deaminase (CD)/5-Fluorocytosine (5-FC)*

A more efficient enzyme/pro-drug combination than HSV-TK/GCV may be the pairing of CD and the approved anti-fungal drug, 5-FC. CD, a bacterial and fungal enzyme that is absent in mammalian cells, is capable of deaminating 5-FC into the highly toxic drug 5-fluorouracil (5-FU), a well-known chemotherapeutic drug used in the treatment of cancer. 5-FU is tissue diffusible and does not require cell-to-cell contact for distribution resulting in a much more effective bystander effect than HSV-TK/GCV[259,481], although this increases the possibility for damage to normal tissues. Also, unlike GCV, 5-FC easily crosses the blood-brain barrier. 5-FU is converted by cellular kinases by phosphorylation into the toxic metabolites 5-fluoro-2'-deoxyuridine 5'-monophosphate (5-FdUMP) and 5-fluorouridine 5'-triphosphate (5-FUTP). 5-FdUMP can block cellular thymidylate synthase resulting in chain termination and cell death in dividing cells, whereas 5-FUTP can disturb RNA processing, which affords 5-FU the capacity to kill both rapidly and slowly dividing tumour cells. Promising pre-clinical data in gliomas show that CD/5-FC is efficacious in causing tumour regression and prolonging survival[102,180,214,260,310] Although CD/5-FC has yet to be tested in clinical trials for

glioma, phase I clinical trials have been conducted for metastatic colon[75] and prostate cancer[131] that showed that this method of therapy is safe.

**Oncolytic Viruses**

One of the greatest barriers to cancer gene therapy is efficient gene transfer[169], limited in part by poor transduction as viral vectors reach only a fraction of the tumour cells. For example in spheroid models *in vitro*, only the superficial cell layers are transduced with AdVs[34,173]. Similarly, a clinical study with an AdV expressing p53 revealed that only cells in the vicinity of the injection site were transduced[264]. Hence, cancer gene therapy strategies are highly dependent on potent bystander effects in order to target all tumour cells[167]. Yet, even with the strong bystander effect provided by the prototypical suicide genes based on *HSV-TK* or *CD*, poor *in vivo* transduction results in high dilution of the toxic product formed by conversion of the prodrugs, explaining in part the poor efficacy obtained in clinical trials[122]. To compensate for the small number of transduced cells, a higher number of infectious viral particles are required (in terms of higher multiplicity of infection –MOI) to improve therapeutic outcome. However, increasing the particle number may not be practical due to increased virus shedding and concomitant systemic toxicity[176,194,255]. As a possible solution, conditional or restricted replicating viruses have been developed known as oncolytic viruses. The goal of these viruses is to selectively infect or replicate in cancer cells, but spare normal cells[63]. Numerous oncolytic viruses are being tested for gliomas including adenoviruses[24,136] HSV-1[240], reovirus[522], vaccinia virus[473], Newcastle disease virus[387], and poliovirus[177]. However, fully replicating viruses

pose safety concerns which include the possibility for spontaneous revertants, the unknown action of many genes, the connection with serious or fatal disease, no clinical trial experience or the current inability to shut-off undesired viral replication[63]. Innate immune responses against viral proteins may also increase toxicity due to complement activation and elevated cytokine levels[55]. Although this may aid in tumour clearing, excessive brain inflammation may cause neurological damage. Nonetheless, phase I clinical trials in malignant glioma were carried out using replicating HSV-1 (G207 and 1716) and adenovirus (ONYX-015)[370].

The 1716 HSV-1 vector has defects in its *γ34.5* gene, whereas the G207 HSV-1 vector has defects in both *UL39* and *γ34.5* genes [240]. The *γ34.5* gene encodes for ICP34.5 which is a protein that inactivates protein kinase R (PKR)[240]. PKR is a cytosolic enzyme that is activated by unusual nucleic acid structures and cytokines such as interferons[85]. It phosphorylates the translation factor E1F2α, inhibiting its function and blocking cellular protein synthesis[523]. It is also associated with the activation of the transcription factor NFκB leading to transcription of proinflammatory genes and enhancing viral clearance in infected cells[85]. Inhibition of PKR is thereby crucial for the HSV-1 life cycle. Activated *Ras* mutants have been shown to inhibit PKR[321]. In addition, many cancer cells have defects in interferon signalling pathways that activate PKR[197,528,536]. Therefore, viruses with mutations in ICP34.5, such as 1716 and G207 can replicate in tumour cells with an overactive Ras or defective interferon signalling

pathways, whereas replication would be limited in normal cells[164]. *UL39* encodes for the viral ICP6 protein that functions as a ribonucleotide reductase[164]. The *UL39* gene product allows wild-type HSV-1 to replicate in quiescent cells, but when it is deleted the virus can only replicate in dividing cells. Viruses with this mutation depend on complementation of ribonucleotide reductase activity by infected cells[164]. Ribonucleotide reductase is tightly regulated by free E2F that is not bound by Rb and is not expressed in quiescent cells, but is upregulated during the G1/S phase of the cell cycle[117]. Therefore, HSV-1 *UL39* mutants replicate specifically in cells with defects in the p16 tumour suppressor pathway. Thus, G207 has the added safety control benefit of replicating not only in cells with defects in Ras but also p16[164] signalling making it even more specific than 1716 for rapidly cycling cells. Replicating HSV-1 vectors can be inhibited by GCV, given that their *TK* gene is still intact, providing an added safety control benefit (TK/GCV does not augment the antitumour efficacy of oncolytic HSV-1 strains)[328,360,372].

Four phase I trials have been performed for the treatment of malignant glioma using replication-competent HSV-1 mutants. Markert *et al*[295] administered G207 by direct intratumoural injection to doses of up to $3x10^9$. It was found to be well tolerated and a maximum tolerated dose was not achieved, indicating that this form of therapy was safe and did not cause encephalitis, which was a pathogenic concern. Rampling *et al*[384] using 1716 also demonstrated that intratumoural injections were well tolerated ($10^5$ pfu was the highest dose tested). Papanastassiou *et al*[356] also tested 1716 using intratumoural injections and no

toxicity was detected. Using a different strategy, Harrow et al[188] injected 1716 into the brain adjacent to the surgical cavity after tumour resection and no clinical evidence of vector related toxicity was detected.

Oncolytic adenoviruses are also being explored in clinical trials for the treatment of malignant gliomas. ONYX-015 is a replicating oncolytic adenovirus that lacks the *E1B* 55kDa gene whose gene product would normally bind to p53 to block apoptosis[24]. This virus has been shown to replicate in and lyse p53 deficient cells, although its p53 specificity is a subject of debate[152,168,186,363,407]. Chiocca *et al*[64] conducted phase I clinical trials in patients with recurrent malignant glioma where doses from $10^7$ to $10^{10}$ pfu were given into a total of 10 sites within the tumour cavity. The vector was shown to be well tolerated as none of the patients experienced vector-related serious adverse events. However, no definitive anti-tumour efficacy was demonstrated. Another example of a replicating adenovirus still in the preclinical phase is delta-24, an *E1A* deleted virus[136]. E1A normally interacts with cellular Rb to drive the cell into S phase. The delta-24 virus takes advantage of the fact that *Rb* mutations are common mutations in glioma and this virus has been shown to selectively lyse *Rb* mutant tumour cells.

To date oncolytic adenoviruses have not resulted in vectors with sufficient anti-tumour activity to sustain complete tumour regression, although a few positive instances were observed[245]. Poor transduction and poor dissemination *in vivo* appear to be largely responsible for this outcome. In terms of functionality, it has been found that the rate of virus replication is slower than the rate of growth of

the tumour reducing its capacity to cure gliomas[173]. At the same time, the inability to control viral replication poses a concern that it may lead to systemic toxicity. Oncolytic vectors carrying suicide transgenes have been developed to improve cytotoxic capacity[72], however safety issues related to the presence of a fully replicating vector still remain. An adenovirus that is capable of replicating its genome, but unable to release virus progeny may prove to be safer and more efficient for enhancing transgene expression[352].

In this study, we have explored such a strategy whereby instead of relying on the dissemination of viral particles as a means of amplification of the therapeutic efficacy (as in oncolytic viruses), we have used genome amplification to increase the number of therapeutic gene copies, thus potentially increasing anti-tumour activity. The replication competent platform we chose is a mutant AdV[352] that is deleted in the *protease* gene (*PS*), which is a late protein responsible for capsid protein maturation and viral assembly[511]. In the absence of *PS*, the adenovirus [Ad(dPS)] replicates its DNA normally since the *E1A* region of the virus is still present, but fails to form infectious particles and is incapable of dissemination. The recombinant AdV we tested also expresses as a fusion protein the suicide gene *CD* with *Uracil Phosphoribosyl Transferase* (*CD::UPRT* )[474]. In the absence of UPRT, the conversion of 5-FU into 5'-fluorouridine 5'-monophosphate (5-FUMP) occurs over a two-step process that only happens when 5-FU is present in high concentrations[7]. UPRT co-expressed or fused with CD bypasses the slow phosphorylation of 5-FU in mammalian cells and has been shown to be superior to CD delivered alone in measures of cytotoxic capacity and bystander effect

[2,34,68,118,315]. Not only is CD::UPRT superior to CD alone, but AdV expressing CD::UPRT (AdCU) followed by 5-FC treatment was shown to be effective even in human primary tumour samples from patients that had developed a resistance to 5-FU[400]. This can be attributed to the ability of AdCU to increase concentrations of 5-FU to levels high enough to successfully affect both the DNA synthetic pathway and the RNA metabolic pathways, thereby affecting both rapidly and slowly replicating cells.

We hypothesized that viral DNA replication would increase the transgene copy number and consequently transgene expression, thereby transforming transduced cells into mega-factories for the production of 5-FU and 5-FUMP. The current work compared the replicating/non-disseminating Ad(dPS)CU-IRES-E1A that expresses CD::UPRT to a non-replicating AdV also expressing CD::UPRT (AdCU) that was previously characterized in glioblastoma cells[34]. Additionally, we tested *in vitro* a replicating and disseminating AdV, the Ad(PS+)CU-IRES-E1A in comparison with its non-disseminating counterpart. Furthermore, we have benchmarked in the spheroid model the efficacy of these AdVs versus oncolysis using wild-type adenovirus (Ad5wt). Finally *in vivo*, we compared the replicating and the non-replicating viruses in intracerebral glioma models using both human xenografts in athymic mice as well as a syngeneic glioma mouse model. We examined the ability of the viruses to cause tumour regression and to improve overall survival.

**Gene Transfer of Cytokines**

Using the tools of the immune system to treat cancer is a very attractive approach. But, due to the presence of the blood-brain barrier, and the scant lymphatic drainage of the CNS, along with the lack of dendritic cells or analogous cells from the brain, anti-tumour immune stimulation in the brain is limited[285]. One therapeutic approach is to use tumour antigens or genetically engineered tumour cells to immunize the host against the tumour. Another strategy to enhance the interaction of immune cells with tumour cells is by using stimulatory cytokines.

Cytokines have multiple functions ranging from the recruitment of different types of leukocytes, to the modulation of angiogenesis and hematopoiesis. They also have the capacity to moderate natural killer (NK) cells and dendritic cells (DC) which can activate antitumour immune responses. Gene therapy techniques have been investigated to provide continuous long-term, local production of cytokine to circumvent problems associated with pharmacokinetic issues, and the blood brain barrier. Interleukins 4 and 12 (IL-4 and IL-12), and human interferon-β (IFN- β) are currently undergoing phase I clinical trials in malignant gliomas[370]. IL-4 is a cytokine that has the capacity to induce a very strong immune response when administered into the brain parenchyma[548], while also having antiproliferative effects on human GBM cells *in vitro* and *in vivo*[477,512,513]. Currently, gene modified autologous glioma cells or fibroblasts expressing IL-4/HSV-TK are being tested in clinical trial[348]. Another study is exploiting a vaccination strategy with irradiated autologous glioma mixed with dendritic cells and fibroblasts transduced with IL-4[347]. An additional tumouricidal cytokine being tested in phase

I clinical trials is IL-12, which will be infused intratumourally with a liposomally encapsulated replication-incompetent Semliki Forest virus vector[390].

Interferon-α (IFN-α) and interferon-β (IFN-β) are type 1 interferons (IFNs) that have great potential as therapeutic anti-cancer agents (reviewed by Borden[28]) and will be explored further in this thesis as a strategy for the treatment of malignant gliomas. IFNs are well-known for their anti-viral, anti-proliferative, anti-angiogenic, and immunomodulatory activities both *in vitro* and *in vivo*. In fact, they were the first cytokines to be applied clinically in cancers such as hairy cell leukemia, chronic myelogenous leukemia, renal cell carcinoma and melanoma[114]. Suppression of the IFN system by malignant cells has been found to be an important contributor to the development of clinical disease. For instance, chromosome 9p, which is the location of the *IFNA* and *IFNB* genes, is often deleted or rearranged in malignant gliomas[23]. Moreover, gene expression profiling and cytogenetic analysis in melanoma, colon cancer, breast cancer, and hematologic malignancies have identified decreases in IFN-stimulated genes (ISGs)[28]. Tumour development has also been associated with epigenetic and genetic silencing of ISG expression, and improved prognosis is often correlated with increases in ISGs[28]. Furthermore, administration of an antibody to IFN has been shown to augment the growth of implanted tumours[172,388]. Thus, restoration of IFN could re-establish signalling and growth regulation in malignant cells as a therapeutic strategy for malignant gliomas, which is of particular interest in this thesis.

Type I IFNs bind to the same receptor, which is composed of two subunits, IFN-αR1 and IFN-αR2, and signal via the activation of receptor-associated tyrosine kinases of the Janus Kinase (JAK) family (reviewed by Doly *et al*[99]). Ligand binding induces *trans*-phosphorylation of the JAKs, TYK2 and JAK1. JAKs in turn phosphorylate and activate latent transcription factors of the STAT (signal transducer and activator of transcription) family, STAT-1α and STAT-2. Together with p48, STAT-1α and STAT-2 form a heterotrimeric complex known as ISGF3, which translocates to the nucleus and binds to IFN-stimulated response elements (ISREs) in the promoters of target genes. Type 1 IFNs also stimulate the MEK/ERK[403] and PI3-K[488,540] pathways, which are critical pathways in growth factor signalling. IFN-β, though, has consistently demonstrated more potency than IFN-α in inducing anti-proliferative effects and apoptosis[29,60,227,404,428]. For instance, although IFN-α and IFN-β have equivalent anti-viral activity per mg of protein, IFN-β is >5 times more effective in apoptosis induction[60]. In addition, gene profiling has identified several hundred genes induced by IFNs where IFN-β was 2-4 fold more potent than IFN-α for almost all genes assessed[76,84,86,151,269]. Therefore, we especially chose to further investigate the anti-glioma potential of IFN-β.

Some of the genes activated by IFN in terms of its anti-cancer apoptotic effects are TRAIL[270], XAF1[270], and Fas[228] with cell death executed via caspase-7 and DNase-γ[415]. In fact, it was shown that IFN-induced apoptosis required the expression of both TRAIL and XAF1, where IFN-β induced TRAIL to a much greater extent than IFN-α[270]. IFN-β can also mediate cell cycle arrest at both the

G1 and S phases in part through the activity of the cell cycle inhibitor p21[471]. Of particular importance, the growth inhibitory effect of IFN-β is restricted to glioma cells whereas normal astrocytes are unresponsive to IFN-β treatment when tested to 500U/mL[150] such that in the clinical situation, normal brain tissue will encounter less toxicity than tumour tissue. There are also several known IFN-inducible proteins with apparent tumour-suppressor activity such as interferon regulatory factor 1 (IRF-1), PKR, 2'-5'-oligoadenylate (2-5A) synthetase, and 2-5A-dependent RNase (RNase L)[30,275]. Furthermore, IFN-β inhibits growth and migration of endothelial cells[5,412] and induces an anti-angiogenic effect by suppressing bFGF[442], MMP-9[287], interleukin (IL)-8[349], and vascular endothelial growth factor[460]. Immunostimulatory effects of IFN-β augment cytotoxic T lymphocyte (CTL) activity by inducing major histocompatibility complex class I expression, enhance the generation of T helper cells, activate NK cells and macrophages, which can aid in tumour eradication[28,478]. Glioma cells transduced by IFN-β have also been shown to produce IL-1β, IL-6, tumour necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1, IFN-γ-inducible protein-10 (IP-10), and heat shock protein (HSP) as well as IFN-β itself, which all exert a strong anti-tumour effect[545]. IFN-β utilizes many facets to eradicate tumours making it a very attractive anti-cancer agent.

Early studies with recombinant IFN-β delivered as a bolus in clinical trials for solid tumours, however, were dissapointing[114]. Pharmacokinetic studies revealed the half-life of IFN-β in serum is relatively short, on the order of five minutes[416],

suggesting insufficient accumulation of biologically active IFN-β in tumours may have limited responses. Furthermore, serum concentrations after a bolus intravenous dose of $6x10^6$ units (U) of IFN-β contained <8 U/mL of IFN-β 1h later, and <2U/mL were detected after an intramuscular or subcutaneous injection[114], which is far below the concentration required to suppress tumour growth and angiogenesis. Moreover, autocrine IFN secretion, rather than exogenous IFN, has been found to regulate growth of glioma cells[518].

Cationic liposome-mediated gene transfer of IFN-β and AdVs expressing IFN-β are currently being tested in clinical trials[111,543]. However, adenoviruses and cationic liposomes are usually capable of only transient transfection. AAV may offer a more stable delivery of IFN-β that could not only cause tumour regression, but aid in preventing tumour recurrence over a longer therapeutic period. Thus, we propose that intratumoural and peritumoural delivery of IFN-β using an AAV vector (AAV-IFN-β) will allow for a constant local supply of this anti-cancer cytokine. This will significantly improve therapeutic outcome by abrogating the issue regarding the short half-life of the protein while also avoiding the side effects caused by systemic delivery. As proof that gene transfer results in higher local concentrations of IFN-β, a study found IFN-β protein, delivered by adenovirus, to be approximately 1500U/mL in the injected tumour area, whereas only 37U/mL of IFN-β were detected in the serum by ELISA[376]. In the present study we generated AAV vectors of both human and mouse IFN-β (due to the species specificity of the cytokine) and characterized their effectiveness both *in vitro* and *in vivo*. In this study we wanted to determine if intratumoural and

peritumoural delivery of AAV-IFN-β could effectively prevent tumour implantation, reduce the volume of established tumours, and significantly prolong survival. At the same time, we wanted to investigate the mechanism of the anti-tumour effect of AAV-IFN-β by examining the anti-angiogenic, anti-proliferative, and immunostimulatory potential of this vector and transgene combination.

Reports from completed clinical trials for malignant glioma gene therapy have thus far concluded uniformly satisfactory tolerability and absence of serious adverse events. However, the greatest shortcoming of gene therapy for malignant gliomas appears to be low efficiency of tumour killing and the limited spatial distribution of transgenes and/or vectors, which fails to eradicate tumours that have disseminated from the main tumour mass. One particular solution could be to use targeted gene therapy to restore aberrant signalling pathways that may deliver more effective apoptotic responses to increase tumour killing efficiency. This strategy will be explored in chapter three through the evaluation of adenoviral delivery of FOXO1, a transcription factor important for promoting apoptosis and cell cycle arrest that can potentially bypass the overexpression of RTKs or the mutation of *PTEN*, a major negative regulator of the PI3K growth-factor signalling pathway. The fourth chapter explores another strategy to improve the efficiency and spatial distribution of transgene product by assessing the potential for a replicating but non-disseminating AdV to deliver CD::UPRT. Finally in chapter five, we considered the efficacy of AAV delivery of IFN-β, to provide a means for long term gene expression of this anti-tumourigenic cytokine to elicit tumour regression and prevent future implantation. It is our hope that

these improvements will prolong survival more than current gene therapy techniques and conventional therapies (*i.e.* radiotherapy, chemotherapy, and surgery) as an adjuvant.

# Chapter 2: Materials and Methods

## CELL LINES AND CELL CULTURE

The human glioblastoma cell lines U87 and U251 and the human embryonic kidney cell line 293A were obtained from ATCC (Manassas, VA). A subclone of the U251 cell line, U251N, was a gift from Dr. PA Forsyth (Calgary, AB). The 293-PS-CymR cell line is a clone derived from the 293 cell line that has been described elsewhere[320]. Human cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. The murine glioma cell line GL261 was obtained from NCI-Frederick (Frederick, MD) and grown in RPMI supplemented with 10% FBS.

The human fetal astrocytes (HFA)[14] and the human brain endothelial cells (HBECs)[369] were a kind gift of the laboratory of Dr. JP Antel (Montreal, QC). HFA were maintained in DMEM supplemented with 10% FBS. HBECs were grown in ECM2 composed of M199 (Gibco, Burlington, ON), human serum (Sigma, Oakville, ON), fetal calf serum, mouse melanoma conditioned media (clone M3; supernatants collected and kept at -20˚C), endothelial cell growth supplement (BD Biosciences, Mississauga, ON), and insulin-transferrin-selenium premix (BD Biosciences).

## VIRUS CONSTRUCTION AND GENERATION

AdFOXO1;AAA was a generous gift from Dr. WR Sellers (Cambridge, MA). It was generated with the pAD-EASY system[191] and was described previously[383]. Linearized shuttle plasmids were cotransfected with pAdEasy-1 into BJ5183 cells. The pAdEasy 1 plasmid contains all Ad5 sequences except nucleotides 1-3,533

(encompassing the E1 genes) and nucleotides 28,130-30,820 (encompassing the E3 genes)[191]. Recombinant adenoviral DNA was restricted with PacI and transfected into 293A cells after isolation. The adenovirus was amplified in 293A cells, released by freeze-thaw extraction followed by CsCl gradient purification. Viral particles were quantified by spectrophotometry at 260nm and 280nm with a BioPhotometer 6131 (Eppendorf, Hamburg, Germany). Viral titers were estimated by cytopathic effect on 293A cells.

AdCU, Ad(dPS)CU, Ad(dPS)CU-IRES-E1A, and Ad(dPS+)CU-IRES-E1A were constructed with the AdenoVator system (Q-Biogene, Irvine, CA) and characterized *in vitro* by Dr. Denis Bourbeau. The suicide gene composed of the fusion of the *CD* and the *UPRT* genes [referred to as *CD::UPRT* or '*CU*'] was obtained from InvivoGen (San Diego, CA). The transgene was first sub-cloned in the shuttle vector pAdenoVator-CMV5(CuO)-IRES-GFP, in which the expression can be silenced in the presence of the cumate repressor (CymR) that binds to the cumate operator (CuO) downstream of the start site[*]. Regulation of the transgene expression was necessary because previous studies had shown that viruses expressing the *CD::UPRT* gene from the strong CMV5 promoter were unstable. The *CD::UPRT* gene was amplified from pGT60-codaupp (InvivoGen), using primers that introduced BglII restriction sites at each end of the fragment. The transgene was sub-cloned into the BglII site of the shuttle vector and was verified by sequencing. Then the E1A gene, including its viral polyA signal, was inserted

[*] D. Bourbeau et al, manuscript in preparation

in place of the GFP downstream of the IRES in order to co-express CD::UPRT and E1A. Using purified DNA from the adenovirus serotype 5 as template, the E1A gene was amplified with the primers that included NheI and MluI restriction sites at the 5' and the 3' ends respectively: 5' CCT AGC TAG CAT GAG ACA TAT TAT CTG 3' and 5' CGA CGC GTC GGA GGT CAG ATG TAA CCA 3'. The junction between the IRES and E1A was designed so that the ATG of E1A would correspond to the 11$^{th}$ ATG of the IRES as it was demonstrated to be the most efficient ATG[374]. This was achieved by replacing the fragment spanning the PmlI to NheI with the PCR product generated with the following primers: 5' CTA GCT AGC CAT GGT TGT GGC AAG CTT ATC 3' and 5' GCC ACG TGT ATA AGA TAC 3'. The final construct was sequenced at the junction of the IRES and E1A to confirm the configuration of the second transgene. The recombinant adenovirus (AdDPS-CuO-CU-IRES-E1A) was generated using the AdEasy system (Q-Biogene) with the Ad(dPS) backbone and following the manufacturer's procedures.

The plasmids containing the human IFN-β gene, pORF-hIFN-β (InvivoGen), and the murine IFN-β gene, pORF-mIFN-β (InvivoGen), were used in the construction of the AAV-hIFN-β vector and the AAV-mIFN-β vector, respectively. Initially, the plasmids containing the IFN-β genes were subcloned into the pcDNA3 plasmid (Invitrogen, Burlington, ON) to attach the CMV promoter and the polyA tail. The pORF-hIFN-β plasmid was digested with AgeI and NheI, while pORF-mIFN-β was digested with SgrAI and NheI. All restriction enzymes were obtained from New England Biolabs (Pickering, ON).

The digestion products corresponding to the IFN-β gene (574bp for human IFN-β; 601bp for murine IFN-β) were ligated with pcDNA3 digested with EcoRV and XbaI. The pcDNA3 plasmids containing the IFN-β gene was then digested with NruI and RsrII to retain only the CMV promoter and the polyA tail. The product of the digestion was then ligated to a plasmid containing inverted terminal repeats (ITRs) of the AAV genome obtained from Dr. J Samulski (Chapel Hill, NC). The final plasmid products were identified by colony hybridization and named pucAAV-hIFN-β and pucAAV-mIFN-β. The hybridization probes were generated from pORF-hIFN-β and pORF-mIFN-β.

The recombinant plasmids were verified by digestion with AhdI for the presence of the ITRs (resulting in bands at 3.6, 1.6, and 1.4 kb) and also with KpnI (bands for mouse: 1.1, 0.5, and 5.1 kb; for human: 1.1 and 5.6 kb) to determine if the gene was in the correct orientation. Plasmids were amplified using *E. coli* DH5α or SURE 2 to prevent recombination (Stratagene, Cedar Creek, TX) grown in LB medium at 30°C supplemented with ampicillin (100μg/mL) and purified using Maxi/Giga plasmid purification kits (Qiagen, Valencia, CA)

The rAAV-2 vectors were propagated in a 3.5 L stirred-tank Chemap type SG bioreactor (Mannedorf, Switzerland) using suspension-growing human embryonic kidney 293 cells in serum-free media. This method has been described in detail[109]. The process was based on triple transfection using 25kDa linear polyethylenimine (PEI) from Polysciences (Warrington, PA) as the transfection agent for the recombinant plasmid, the AAV-2 *rep* and *cap* plasmid, ACG2, and the mini

adenovirus genome plasmid, pXX6 (both plasmids were obtained from Dr. J Samulski). PEI stock solutions (1 mg/mL) were prepared in water, neutralized with HCl, sterilized by filtration (0.22 μm), aliquoted and stored at –80°C. Briefly, bioreactors were seeded at $0.25x10^6$ cells/mL and transfected with the DNA/PEI complexes 24h post-inoculation, when a density of $0.5x10^6$ cells/mL was normally attained. Transfected cells were harvested by centrifugation and then resuspended in harvesting buffer (150mM NaCl, 10mM Hepes pH 7.6). To release the virus, the cells were subjected to three rounds of freeze-thaw (freezing on dry ice for 10 min followed by thawing in a 37˚C water bath). Cellular debris was removed by centrifugation and the supernatants were pooled. Purification was achieved by ion exchange chromatography.

The genome titer for human and mouse AAV-IFN-β was determined using real-time PCR (Cepheid Smart Cycler, Sunnyvale, CA) with the following primers for human-IFN-β: forward, 5'-AATTGAATGGGAGGCTTGAA-3', and reverse, 5'-AGCCAGGAGGTTCTCAACAA-3'; and for mouse-IFN-β: forward, 5'-ATAAGCAGCTCCAGCTCCAA-3', and reverse, 5'-CTGTCTGCTGGTGGAGTTCA-3'. The genome titer for AAV-GFP was determined using primers for the CMV promoter: forward, 5'-CAAGTACGCCCCCTATTGAC-3', and reverse, 5'-AAGTCCCGTTGATTTTGGTG-3'. A standard curve was created from serial dilutions of the corresponding plasmid vector. The DNA was isolated by incubating AAV with 0.3M NaOH at 65°C for 30 min to disrupt the capsid. The

resulting DNA samples were de-salted by passing them through a MicroSpin G-25 column (GE Healthcare, Piscataway, NJ) washed with PBS.

## IN VITRO EXPERIMENTATION

### Virus Infection

Adherent cells were washed with PBS and media was replaced with OPTI-MEM (Invitrogen). AdV or rAAV was added to the OPTI-MEM and incubated with the cells for 3h. OPTI-MEM was replaced with DMEM supplemented with 10% FBS following incubation. Cells were assessed by light microscopy for changes in cell morphology and in some cases photographs were taken using the Leica digital microscope (Richmondhill, ON) and analyzed with OpenLab software (Improvision, Lexington, MA). To evaluate AdFOXO1;AAA or AAV-GFP infection, the green fluorescent protein (GFP) fluorescence was assessed by digital fluorescence microscopy (Leica) and all digital photographs were taken at the same exposure time and examined with OpenLab software (Improvision). Transduction efficiency for AdFOXO1;AAA infected cells was also determined by flow cytometry of unfixed cells to detect GFP fluorescence using a FACSCalibur (Becton Dickenson, Oakville, ON)

Multiplicity of Infection (MOI) for AdVs was based on infectious viral titers estimated by cytopathic effect on 293A cells. On the other hand, MOI for rAAVs was based on the genome titer obtained by real-time PCR.

### Cell Cycle Analysis

DNA content was measured by flow cytometry using a FACSCalibur (Becton Dickenson) to investigate unsynchronized propidium iodide (PI; Sigma)-stained and fixed cells. Cells were initially trypsinized, washed with PBS, centrifuged, and then resuspended in cold PBS. The cold cell suspension was then added dropwise into 100% ice-cold ethanol and vortexed. The suspension was incubated on ice for 15 min then centrifuged. The pellet was resuspended in PBS and 20mg/mL RNase, then incubated for 40 min at 37˚C. PI solution (stock of 1mg/mL diluted to 1:1000 final concentration) and additional PBS was added to the cell mixture following incubation and then samples were analyzed by flow cytometry. At least 10,000 cells were counted for each sample. Analysis for live cells was performed using ModFit LT software (Verity, Topsham, ME). The percent in the sub G1 peak was determined using CellQuest software (Becton Dickenson). The number of live and dead cells was assessed in a separate experiment by flow cytometry of unfixed live cells stained with PI. Membranes of dead and damaged cells are permeable to PI. The percentage of live versus dead cells was ascertained using CellQuest software (Becton Dickenson). All cell cycle and live/dead studies were performed at least twice.

**Annexin-V-PE Staining**

Annexin V-PE (BD Pharmingen, Oakville Ontario) staining was performed as indicated by the manufacturer's instructions and PI was used to distinguish between viable and nonviable cells. Adherent cells were lifted with 0.5mM EDTA, washed twice with cold PBS and resuspended in 1X binding buffer (10mM Hepes/NaOH (pH 7.4), 140mM NaCl, 2.5mM $CaCl_2$) at a concentration

of $1x10^6$ cells/mL. 5μL of Annexin V-PE was then added to 100μL of cell solution. The cells were vortexed and incubated for 15min at room temperature (RT) in the dark. 400μL of binding buffer was added to each tube followed by PI addition. The samples were then analyzed by flow cytometry using FACSCalibur (Becton Dickenson) and the percent undergoing apoptosis was determined using WinMDI software (San Jose, CA).

**Western Blot**

Cells transduced with AdFOXO1;AAA, AdLacZ or mock infected (no virus) at a MOI of 50 were plated the day before in 6 well plates at a density of $1x10^5$ cells. Infection was followed by incubation for 24, 48 or 72 h. Protein lysates were quantified with the BCA protein assay (Pierce, Rockford, IL). Proteins were separated on a 12 % polyacrylamide gel, and transferred onto a Hybond-ECL nitrocellulose membrane (GE Healthcare). Blots were incubated with the following antibodies: rabbit anti-FOXO1 (Cell Signaling, Danvers, MA), rabbit anti-p27 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-cyclin D1 (Neomarker, Fremont, CA), rabbit anti-cyclin D2 (Santa Cruz Biotechnology), mouse anti-FasL (BD Biosciences), rabbit anti-Bim (Affinity BioReagents, Golden, CO), mouse anti-caspase-9 (Oncogene Research Products, Cambridge, MA), or mouse anti-β-actin (Abcam, Cambridge, UK). They were then washed and incubated with the appropriate rabbit, or mouse secondary antibody coupled to horse radish peroxidase (HRP) (Dako, Glostrup, Denmark). Signal was detected in the presence of SuperSignal West Femto substrate (Pierce) and

chemiluminescent images were captured by the CCD camera of GeneGnome (Syngene, Frederick, MD).

In the case of AdCU, Ad(dPS)CU, Ad(dPS)CU-IRES-E1A, and Ad(dPS+)CU-IRES-E1A, cells were plated in 6 well plates at a density of $2.5x10^5$ cells, transduced the next day with the appropriate AdV at a MOI of 50, and incubated for 72 h. Protein lysates were quantified with the DC protein assay (Bio-Rad, Mississauga, Canada) and diluted in Laemmli buffer. Proteins were separated on 10 % polyacrylamide gel (Invitrogen), and transferred onto nitrocellulose membranes. Blots were incubated with the antibody for anti-codA (kindly provided by InvivoGen) or anti-adeno[34], then washed and incubated with secondary antibody coupled to HRP (Amersham, Baie d'Urfe, Canada). Signal detection was performed by ECL (Amersham).

**Immunoprecipitation**

Cells were plated in 10cm dishes at a density of $6x10^5$ and infected the next day with AdV. DMEM supplemented with 10% FBS plus 5μM of trichostatin A (TSA; Sigma) replaced the infection medium (OPTI-MEM) after the 3h incubation with virus. Cells were then incubated for a further 24 or 48h. Whole cell lysates were harvested in the following lysis buffer: 50mM Tris, pH 8.0, 50mM KCl, 10mM EDTA, 1% NP40, 10mM Nicotinamide, 1μM TSA, Roche EDTA free cocktail protease inhibitors, 20mM NaF, and 1mM orthovanadate. Lysates were immunoprecipitated with rabbit anti-acetyl-lysine antibody (Cell Signaling) over-night at 4˚C. Protein A agarose beads (Sigma) were then added to

the samples and incubated for 3h at 4˚C. The samples were washed five times with 50mM Tris (pH 7.5) containing 5mM EDTA, 150mM NaCl and 0.1% Tween 20. The samples were separated by 7% polyacrylamide gel then transferred to an Immobilon-P PVDF membrane (Millipore, Billerica, MA). Blots were incubated with anti-FOXO1 antibody (Cell Signaling) and visualized with SuperSignal West Femto substrate (Pierce). Chemiluminescence was detected using GeneGnome (Syngene).

**Viability Assays (XTT and MTT)**

To determine the viability of HFA and HBECs following AdFOXO1;AAA or AdGFP infection, cells were plated at a density of $1x10^5$ in a 6 well and infected with virus the next day at various MOI. 72 hours later, the cells were washed twice with PBS and the incubated with XTT working solution [1mg/mL XTT (Sigma); permethylsufonate (Sigma)] at 37˚C for one hour. After incubations, optical densities were determined with a Bio-Rad Microplate Reader, model 550 (Mississauga, ON) at 450nm.

The viability of cells directly infected with AAV was also determined with XTT assay. U87 and U251 cells were plated at $5x10^3$ in a 96-well and the next day were incubated with virus for 3 hours in OPTI-MEM at various MOI. This was followed by incubation in DMEM for a further 48 hours. Then the cells were washed twice with PBS, incubated with XTT working solution and optical densities determined at 450nm.

As well, the viability of cells incubated with supernatants from AAV infected 293A cells was determined using an XTT assay and performed as follows: 293A cells were plated at $1x10^5$ in a 6 well and then infected with AAV in OPTI-MEM for 3 h the next day. The media was replaced with DMEM after the 3 h incubation, and then incubated for an additional 48-72h at which time the media was then changed to DMEM for the U87 and U251 cells and ECM2 for the HBECs. The 293A cells were incubated for an additional 48h. U87 and U251 cells were plated at 5000 cells per well in a 96-well plate and were treated the next day with various percentages of supernatant from the infected 293A. HBECs were treated with 100% 293A supernatant. The U87, U251 and HBECs were incubated with supernatant for 48 hours. Then the cells were washed twice with PBS and incubated with XTT working solution, followed by determination of optical density. All XTT assays were performed at least twice in duplicate and statistical significance was determined by one-way ANOVA followed by the Bonferroni post-test.

The potency of AdCU, Ad(dPS)CU, Ad(dPS)CU-IRES-E1A, and Ad(dPS)CU-IRES-E1A was assessed using a doubling dilution assay. Cells were plated at a density of $5x10^3$ cells per well in 96-well plates. The next day, cells were transduced with a doubling dilution of AdVs starting at a MOI of 10 in 50 µl for 5 h, and then 25µM of 5-FC was added for U87 cells and 500µM for U251 cells with 50 µl of media. Each condition had 8 replicates and each experiment was performed twice. Cells were incubated for 6 days and analyzed by MTT assays. Cells were incubated with MTT (Sigma) at a final concentration of 1 mg/ml for 4

h. Then medium was removed, and DMSO was added. Optical density was measured at 490 nm. For the experiments with spheroids, the incubation time was extended to 8 h followed by an overnight incubation with DMSO in the dark.

**Clonogenic Assay**

The clonogenic assay has been described previously[398]. Cells were initially plated at a density of $1x10^5$ in a 6-well and infected the following day at various MOI. The next day, cells were trypsinized and irradiated at various Grey (Gy) inside a Gammacell (Atomic Energy of Canada Ltd, Radiochemical Co., Kanata, ON). Cells were re-plated from 200 to 10,000 cells per well to result in 30-150 colonies per well, with each colony containing >50 cells after 14 days incubation in 5% $CO_2$ at 37˚C. After 14 days incubation, cells were washed with PBS and colonies stained with 0.5% crystal violet (in methanol:$H_2O$ at 1:1) (Sigma). Colonies were counted and plating efficiencies and surviving fractions were calculated using the following formula:

Plating efficiency (PE) = # of colonies without radiation/# of cells plated

Surviving fraction = # of colonies/(# of cell plated x PE)

This assay was performed twice and statistical significance was determined by one-way ANOVA followed by the Bonferroni post-test.

**Bystander Assays**

The bystander assays were performed as previously detailed[34]. Cells were plated at a density of $2x10^5$ cells in 12 well plates. The next day, they were transduced with the appropriate vector at the chosen MOI. After 5 h, transduced cells were

trypsinized and resuspended in 2mL of media. Doubling dilution of cells were generated and spread onto 96 well plates in 50μL. Then, non-transduced cells (bystander cells) were trypsinized, diluted to $1x10^5$ cells/mL, and added to the wells with transduced cells. This resulted in the following ratios of transduced cells: 50 %; 33 %; 20 %; 11 %; 6 %; 3 %; 1.5 %; 0.8 %; 0.4 %; 0.2 %. The next day, 5-FC was added to the cells at the appropriate concentration. The cells were further incubated for 5 days and analyzed by MTT assays. Each condition had 8 replicates and each experiment was performed twice.

**Spheroids**

U87 and U251 spheroids were generated as previously described[34]. Confluent cells were trypsinized, and $5x10^4$ cells were laid per well of 24 well plates covered with 2 % Seaplaque agarose (Cambrex, Rockland, MA) in culture media. Cultures were incubated for a week to allow spheroids to gain a compact and regular spherical shape. Transduction was performed using the appropriate schedule and concentration of AdV expressed in plaque forming units (pfu) per well (one spheroid). The pro-drug 5-FC was added the day following transduction at a concentration of 500μM.

**Enzyme-Linked Immunosorbant Assay (ELISA)**

For examination of IFN-β protein concentrations from infected 293A and U87 supernatants, cells were plated at 5000 cells per well in a 96-well plate in DMEM supplemented with 10% FBS. The next day, the DMEM was removed and cells were infected with AAV in OPTI-MEM for 3 hours. The media was then replaced

with 100μL DMEM. The supernatants were then harvested after a predetermined incubation (2-5 days) and centrifuged to remove cellular debris. 50μL of supernatant (diluted to 100uL by dilution buffer included in the ELISA kit) was applied to an ELISA plate for Human Interferon Beta or Mouse Interferon Beta (both kits from PBL Biomedical Laboratories, Piscataway, NJ) as directed by the manufacturer. Optical Densities were measured with a Bio-Rad Microplate Reader, model 550, at 450nm. The data were analyzed with Microplate Manager (Bio-Rad Laboratories).

**HBEC Migration Assay**

To produce the IFN-β supernatants, 293A cells were plated in a 6 well at $1x10^5$ with ECM2 media. The next day, they were subjected to UV irradiation without media in a Stratalinker (Stratagene) set to 6000μJ/$cm^2$ to increase their susceptibility to AAV transduction[423]. Then 1mL of OPTI-MEM was added per well followed by AAV infection for 3h. OPTI-MEM was replaced with ECM2 after incubation with virus and then cells were incubated for a further 72h. To prepare the HBECs inside a 24 well plate, a cloning ring was positioned in the centre of the well and 100μL of gelatin was placed inside the ring, which was then incubated for 2h. Excess liquid was removed and 5000 HBECs in 100μL were plated in the centre of the ring. U251 cells were plated on the outside of the ring at a density of 10,000 cells in 1mL of ECM2. The ring was removed 48h after plating and the medium was replaced with supernatants from the 293A cells that had been incubated for 72h. Pictures were taken from 0-96h after ring removal

using a light microscope (Leica). The experiment was done twice and measurements were taken from 5 different areas.

## IN VIVO EXPERIMENTATION

### Mouse Models

To prepare tumour cells for intracerebral stereotactic injection into the caudate, subconfluent cells were detached in 0.5mM EDTA, centrifuged, and then resuspended in an appropriate volume of Hanks' buffered saline solution (HBSS). U87LacZ, U251N human glioma cells ($1x10^5$ cells in 3µL HBSS) were implanted intracerebrally into athymic nude mice, and GL261 murine glioma cells ($2x10^5$ cells in 3µL HBSS) were implanted similarly into wild-type C57Bl6 mice as outlined previously[278]. Ten days later mice were injected intratumourally with AdFOXO1;AAA [$1x10^{10}$ pfu/mL], AdGFP [$1x10^{10}$ pfu/mL], Ad(dPS)CU [$5x10^{10}$ pfu/mL] or Ad(dPS)CU-IRES-E1A [$6x10^9$ pfu/mL] in a volume of 3µL. For mice treated with AAV-hIFN-β [$1.1x10^{10}$ vector genomes (VG)/mL], AAV-mIFN-β [$9.49x10^9$VG/mL], or AAV-GFP [$2.9x10^{10}$VG/mL], 6µL the virus was injected either ten days prior or ten days after tumour implantation. The second injection in all cases was performed by re-entering the burr hole created by the initial injection. Some groups of mice receiving Ad(dPS)CU and Ad(dPS)CU-IRES-E1A were given a second injection of virus 21d following the last virus injection. 5-FC for mice was administered intraperitoneally where indicated for 5 days at one or two days following virus injection at a dose of 500mg/kg twice a day.

### Tumour Volume

For tumour volume assessment, mice were sacrificed 35d or 40d post-tumour implantation. The entire brain was removed and frozen in 2-methylbutane cooled with liquid nitrogen. The brain was sectioned on a cryostat (Leica) into 20μm thick coronal sections to reconstruct the β-galactosidase-expressing tumours[278] in order to calculate tumour volumes. The sections were stained histochemically with X-gal, followed by hematoxylin and eosin staining. Tumour volumes were calculated using the following formula: $V=a \times b^2 \times 0.4$ if $a>b$ and $V=a^2 \times b \times 0.4$ if $a<b$, where V=volume; a=(section number at the end of the tumour – section number at the start of the tumour) x section thickness; and b = length of the greatest longitudinal of the tumour. When comparing two groups the two-way *t*-test was used to determine statistical significance. Where three or more groups were being compared, one-way ANOVA followed by the Bonferroni post-test was performed. One-way ANOVA followed by the Kruskal-Wallis post-test or the Mann-Whitney test was used to determine the significance for mice with pre-established U251N due to the non-Gaussian data distribution present in this control group.

**Survival Assessment**

During long term survival studies, animals were monitored daily regarding nutrition, hydration, agility etc. Animals were euthanized if they showed any signs of lethargy, significant neurological morbidity, or severe weight loss. All experiments were carried out according to the guidelines of the institutional Animal Care Committee. Survival was assessed with Kaplan-Meier analysis.

**CD31 Immunostaining**

Fluorescent immunostaining for the presence of CD31 positive blood vessels was performed on frozen brain sections from mice that were sacrificed 35 days post-tumour implantation. Sections from the longest longitudinal section of tumour were air-dried for 30 min and then fixed with 4% PFA for 10min. They were washed twice with PBS, treated with 0.1% Triton X-100 for 5min, washed again, then blocked with 5% normal goat serum and 0.1% Triton X-100 for 10 min. Rat anti-mouse CD31 antibody (BD PharMingen) diluted to 1:500 in blocking solution was incubated with the sections for 2h at RT. After three washes, the sections were incubated in the dark for 30 min with Alexa 555 conjugated anti-rat antibody (Molecular Probes, Eugene, OR) diluted to 1:200. Nuclei were stained with Hoechst (Molecular Probes) for 10 min at a 1:1000 dilution. The sections were washed and mounted using Immu-mount (Shandon, Lipshaw, PA). Sections were visualized by fluorescence microscopy using the Leica digital microscope. Photographs were analyzed using OpenLab (Improvision). Quantification was performed by photographing five different areas of the tumour in a 200X field followed by a count of the number of CD31 positive blood vessels in the photographs. For experiments with AdFOXO1;AAA statistical significance was determined by two-way *t*-test. Since more than two groups were being compared in experiments with AAV-IFN-β significance was determined by one-way ANOVA followed by the Bonferroni post-test.

**Analysis of Immune Infiltrates by Flow Cytometry**

Tumour bearing mice were anaesthetized with 10mL/kg of somnitol and perfused transcardially with PBS containing 6000U heparin/L. The forebrain was dissected and placed in HBSS. Samples were mechanically dissociated, passed though a 70μm filter, and then centrifuged. The cell pellets were resuspended and blocked at room temperature for 20 min. with 24G2 hybridoma supernatant (anti Fc receptor) containing 100μg/mL rat Ig, 2% FBS and 0.01% sodium azide. FACS buffer (HBSS, 2% FBS and 0.01% sodium azide) was added to the samples after incubation and then centrifuged. Cell pellets were resuspended and incubated with the following anti-mouse monoclonal antibodies for 1h at 4°C: fluorescein isothiocyanate (FITC)-conjugated anti-NK-1.1; R-phycoerythrin (R-PE)-conjugated anti-CD45 (30-F11); PerCP-Cy5.5-conjugated anti-CD11b; and allophycocyanin (APC)-conjugated anti-TCR-beta chain (H57-597). All antibodies were obtained from BD Pharmingen. Following incubation, samples were washed, cell pellets were resuspended with PBS and analyzed by flow cytometry using a FACSCalibur (Becton Dickenson). Statistical significance was determined by *t*-test.

**Quantification of *In Vivo* Human IFN-β Expression**

Animals were implanted with tumour and then infected with either AAV-hIFN-β, AAV-GFP, or left untreated 10 days later. Mice were sacrificed 3 and 5 days post virus infection. Brain tissue was homogenized and the RNA collected using the Qiagen RNAeasy kit following the manufacturer's instructions. RT-PCR was performed using M-MLV-RT (Invitrogen) in the following manner: total RNA was added to a mixture of oligo$(dT)_{12-18}$ (Invitrogen), and 20mM dNTP mix

(Invitrogen), and then heated to 65˚C for 5 min followed by a quick chill on ice. 5X first strand buffer (Invitrogen), and 0.1M DTT (Invitrogen) was added to the mixture and incubated at 37˚C for 2 min. 200 units of M-MLV-RT was added and the mixture was incubated for 50 min. at 37˚C. The reaction was inactivated by heating at 70˚C for 15 min. Quantitative Real-time PCR was then performed using primers for human-IFN-β described above in the Cepheid Smart Cycler. Serial dilutions of pucAAV-hIFN-β were used for the standard curve.

**TUNEL Staining**

TUNEL staining was performed on the sections with the longest tumour longitudinal. Sections were fixed with 4% PFA for 10 min, washed with PBS, treated with 0.1% Triton X-100 for 5min, and then washed again. A positive control was created by incubating a section with 1μg/mL DNAse I in Gibco React 4 buffer (added 0.1mM DTT) for 10 min, which was then washed extensively with $H_2O$. TUNEL solution, containing 30 units of Terminal Transferase (Promega, Madison, WI), Biotin-16-2”-deoxy-uridine-5’-triphosphate (Roche, Basel, Switzerland), Terminal Transferase buffer (final concentration: 100mM cacodylate buffer, pH 6.8; 1mM $CoCl_2$, 0.1mM DTT), and $H_2O$ (to 100μL total), was then added to the brain sections and incubated for 1h at 37˚C. The sections were washed with PBS then blocked with 10% goat serum in PBS for 10 min at RT. Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:1000) was added and incubated for 30 min at RT. Nuclei were stained with Hoechst (Molecular Probes) (1:1000) for 10 min. After washing, sections were mounted with Immu-mount (Shandon). Fluorescence microscopy

was performed with Leica digital microscope and photographs were analyzed with OpenLab software (Improvision). Quantification was performed by photographing five different areas of the tumour in a 400X field followed by a count of the number of CD31 positive blood vessels in the photographs. Significance was determined by two-way *t*-test.

**Statistical Analyses**

All statistical analyses were performed using GraphPad Prism software (San Diego, CA) with the critical significance level (*P*) set to 0.05.

# Chapter 3: Targeted Therapy

# The Potential for the FOXO1 Transcription Factor for Gene Therapy for Malignant Glioma

**PREAMBLE**

In this chapter, we exploit the capacity of a member of the FOXO family of transcription factors, FOXO1, to induce apoptosis in glioma cells as a potential form of therapy. Forkhead transcription factors lie downstream of the PI3K-Akt pathway and are known to regulate genes involved in apoptosis, cell cycle arrest, nutrient availability, DNA repair, stress, and angiogenesis. Akt inactivates FOXO1 by phosphorylation, which results in its localization from the nucleus to the cytoplasm and subsequent proteosomal degradation. PTEN is an important negative regulator of the PI3K-Akt pathway and is commonly mutated in glioma. In cells that carry this mutation, Akt is constitutively active leading to phosphorylation of endogenous forkhead transcription factors and facilitating their nuclear export. The cell, as a result, becomes resistant to apoptosis and cell cycle arrest. Using adenovirus-mediated delivery of a form of FOXO1 that cannot be phosphorylated by Akt (AdFOXO1;AAA), we determined whether AdFOXO1;AAA could restore cell cycle arrest and apoptosis in human GBM cell lines that are PTEN-null but differ in their p53 status. As well, we examined if AdFOXO1;AAA could prolong survival in an intracerebral xenograft model and determined whether AdFOXO1;AAA could act as a significant radiosensitizer to examine its potential as an adjuvant to radiotherapy.

## RESULTS

### Evaluation of Cytotoxic Activity

We first compared efficiency of AdV-mediated gene transfer between U87 (p53 wild-type) and U251 (p53 mutant) human GBM cells. Since the *FOXO1;AAA* transgene was fused with the gene for *green fluorescent protein* (*GFP*) to facilitate FOXO1;AAA localization, transduction efficiency was analyzed by flow cytometry for the percentage of GFP-positive cells after AdFOXO1;AAA infection at various MOI. Both U87 and U251 exhibited high transduction efficiencies of 80-100% at similar MOI. To examine if AdFOXO1;AAA causes morphological changes consistent with apoptosis in U87 and U251 cells, we examined them by light microscopy 24h after infection with AdV. U87 and U251 human glioma cells typically display long cellular processes characteristic of astrocytes. AdFOXO1;AAA infection of U251 cells at a MOI of 50 caused gross morphological changes consistent with early apoptosis such as cytoplasmic shrinkage (Figure 9A). In contrast, infection of U87 cells with AdFOXO1;AAA did not lead to changes in cell morphology at 24 hours. Infection with AdLacZ vector control also did not change the cellular morphology of either cell type (Figure 9A). Since susceptibility to AdV is similar between U87 and U251 cells, it is unlikely that the morphological differences observed between the two cell types is related to variations in AdFOXO1;AAA transduction efficiency.

We then compared the subcellular localization of FOXO1;AAA with AdFOXO1;AAA at 24 and 48h after infection. At both time points FOXO1;AAA expression in U251 cells remained primarily nuclear. Apoptotic U251 cells,

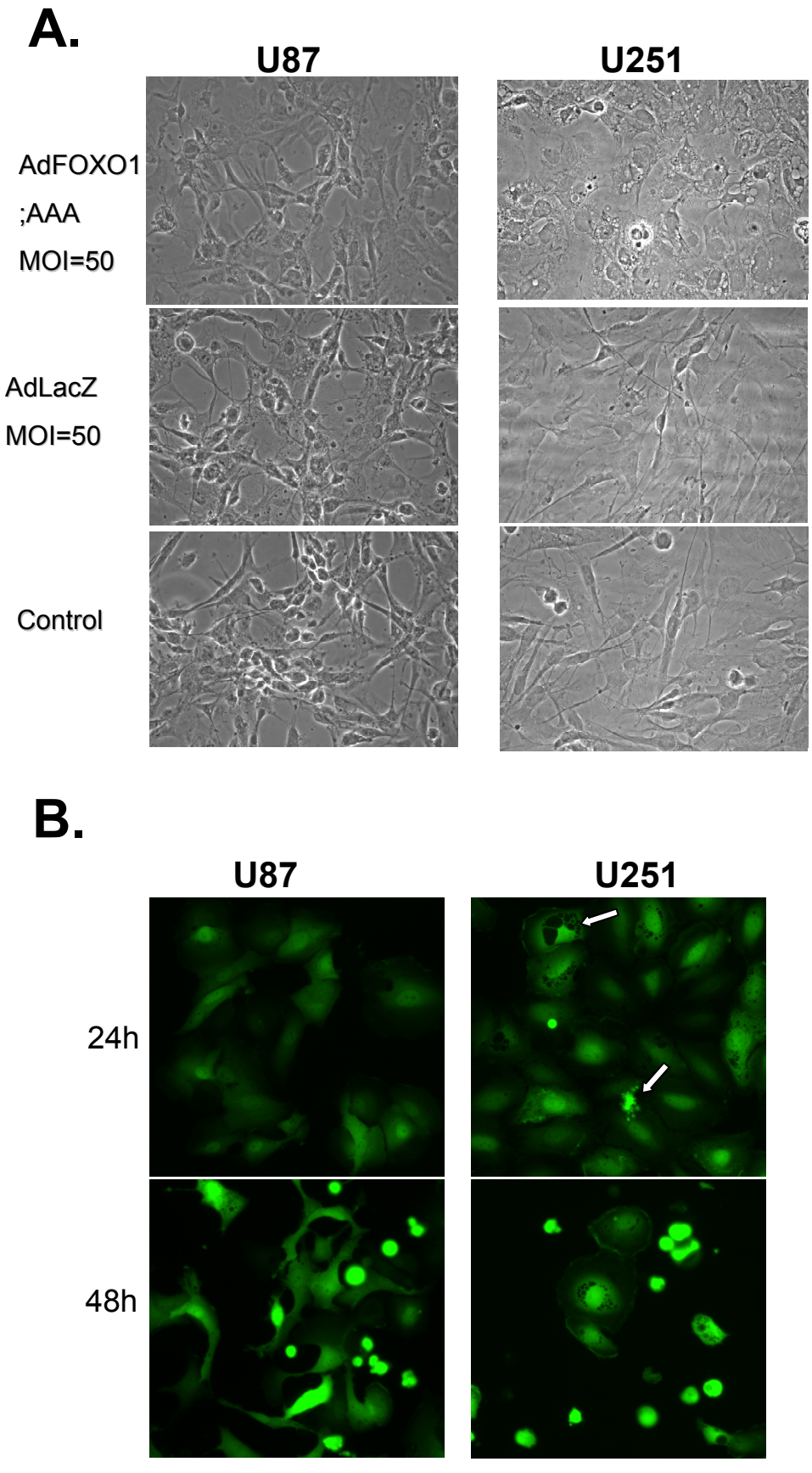

A.
U87
U251
AdFOXO1
;AAA
MOI=50
AdLacZ
MOI=50
Control
B.
U87
U251
24h
48h

**Figure 9: Comparison of morphological changes.**

***A***, light microscopy of U87 and U251 cells 24h following infection with AdFOXO1;AAA, or AdLacZ at a MOI of 50 or mock-infected (control). 200X magnification. ***B***, fluorescence microscopy of AdFOXO1;AAA infected cells at 24 and 48h post-infection. Apoptotic U251 cells (white arrows) showing nuclear condensation and membrane blebs were readily observed along with rounded dead cells. *FOXO1;AAA* is fused with *GFP* to allow for localization of the FOXO1;AAA protein. 200X magnification.

indicated by nuclear condensation, and appearance of membrane blebs, were also readily observed along with rounded dead cells (Figure 9B). In contrast, although FOXO1;AAA was primarily nuclear in U87 cells at 24h, at 48h FOXO1;AAA became evenly distributed between the cytoplasm and the nucleus (Figure 9B). Some cell death was also evident (Figure 9B). The cytoplasmic localization of FOXO1;AAA suggests that in U87 cells, FOXO1;AAA may be regulated in an Akt phosphorylation independent manner. This possible attenuation of its protein activity may explain why cell morphology was not altered as significantly in U87 cells as it was in U251 cells.

To investigate the degree of cell death triggered by AdFOXO1;AAA infection, we examined the sub-G1 peak by flow cytometric analysis of fixed propidium iodide (PI)- stained cells. For fixed cells, this method provides quantitative data regarding DNA content per cell through PI intercalation with the DNA. The sub-G1 peak represents DNA fragments that could indicate necrosis and/or apoptosis[350,373]. There was a dose-dependent increase in the sub-G1 peak for both U87 and U251 cells infected with AdFOXO1;AAA over a period of 72 hours; the higher the MOI used, the more cell death was observed in both cell lines (Figure 10A). On the other hand, no difference was observed between uninfected cells or AdLacZ infected cells. The AdFOXO1;AAA-induced death, however, was much more pronounced in U251 cells where the percentage of cells in the sub-G1 peak reached 90% or more in cells infected with a MOI of 30 or above. In U87 cells, a peak of only ~40% cell death was reached with a MOI of 100 even when 90% of the cells were transduced at this MOI. Similar data were obtained with flow

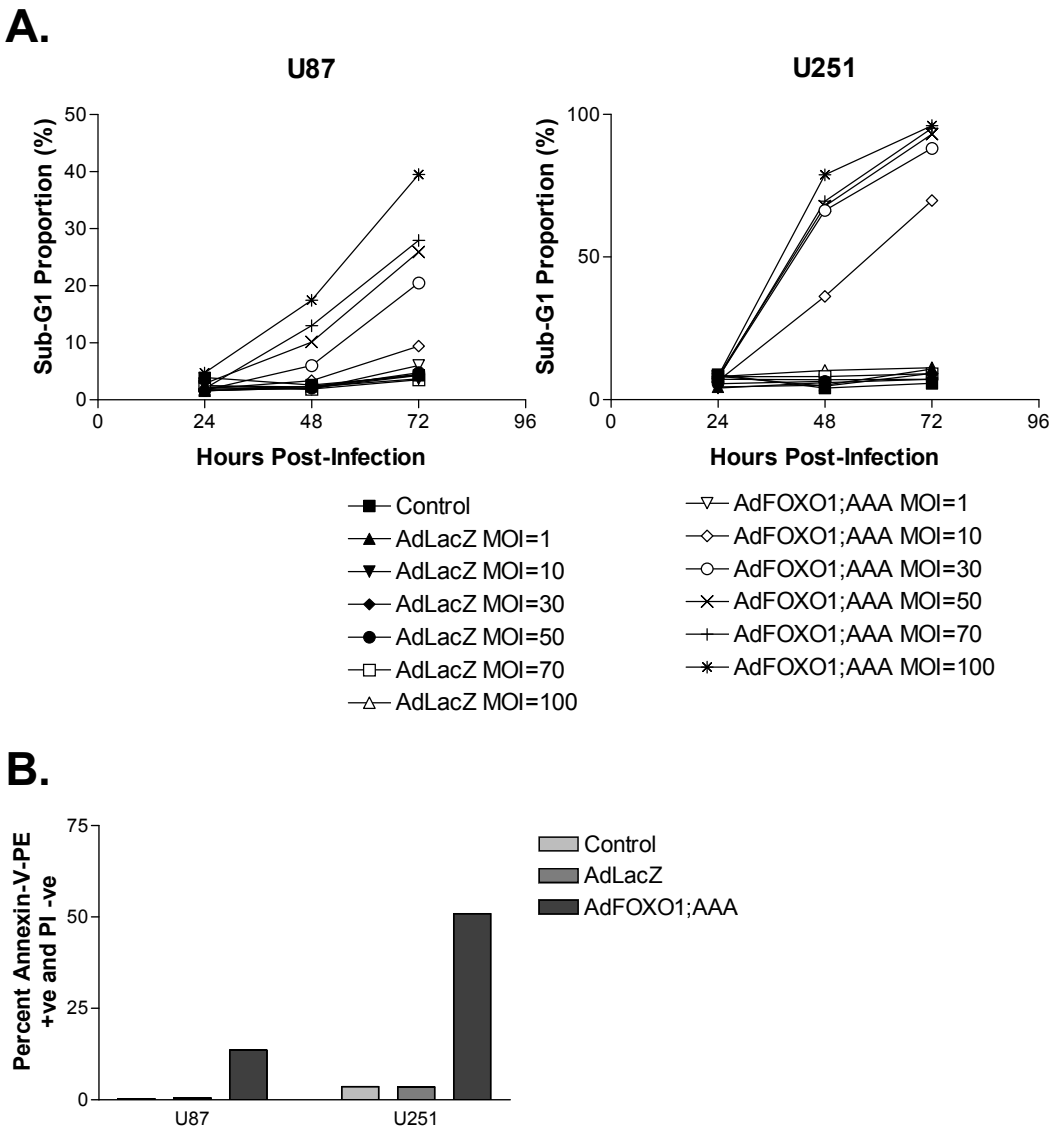



**Figure 10: Cytotoxic activity *in vitro*.**

***A***, flow cytometric analysis of the sub G-1 portion of cells infected with AdV or left uninfected over a 72h period. ***B***, cells stained with both Annexin-V-PE and PI and examined by flow cytometry 48h after adenovirus infection at MOI of 50. Shown is the percentage of only live cells undergoing apoptosis, which are Annexin-V-PE-positive and PI-negative. Gates were used to remove dead and damaged PI-positive cells. These experiments were repeated twice and representative data are shown. Histograms of these data are located in Supplementary Figure 2.

cytometry of unfixed PI stained cells, which provided a count of live and dead cells (Supplementary Figure 1). [For unfixed cells, PI only stains cells whose membranes are compromised.] AdFOXO1;AAA infection, therefore, led to a dose dependent increase in the sub-G1 fraction and cell death, but U87 cells were less sensitive than U251 cells.

In order to confirm whether the dose-dependent cell death was attributable to apoptosis, flow cytometric analysis of Annexin-V-PE stained cells was performed at 48 hours post-infection at a MOI of 50. Annexin-V labels phosphatidylserine residues that become externalized in early apoptosis whereas the sub-G1 peak indicates cells that are in late apoptosis or already dead. The proportion of live cells (PI negative) infected with AdFOXO1;AAA and actively undergoing apoptosis (staining positively for Annexin-V-PE, but negatively for PI) was greater than AdLacZ-infected or control cells and was also greater in U251 cells than in U87 cells (50.9% vs. 13.6%; Figure 10B and Supplementary Figure 2).

Taken together, these data indicate that AdFOXO1;AAA-mediated dose-dependent increase in cell death is a result of its capacity to induce apoptosis. Furthermore, AdFOXO1;AAA causes apoptosis to a greater extent in U251 cells than in U87 cells.

**Cell Cycle Analysis**

FOXO1 can induce cell cycle arrest by regulating the expression of cell cycle inhibitors and promoters[43,44]. We analyzed the cell cycle profile of

AdFOXO1;AAA infected glioma cells by flow cytometry of fixed PI-stained cells. For U87 cells at 24h post-infection, there was no difference in the proportion of cells in G1-phase compared to control; however, they exhibited a dose-dependent decrease in the S-phase proportion (Figure 11). At the same time the proportion of U87 cells in the G2/M-phase was greater than control, suggesting cell cycle arrest. Interestingly, the S-phase decrease was transient in the U87 cells as the S-phase proportion increased at 72h and this was accompanied by a drop in the G1 phase proportion without a further increase in the G2/M fraction (compared to 24h) suggesting the cells were proceeding through the cell cycle. Similar to U87 cells, U251 cells at 24h post-infection with AdFOXO1;AAA also had a higher proportion of cells in the G2/M phase compared to uninfected control, indicating arrest at this phase (Figure 11). U251 cells also demonstrated a decrease in the S-phase proportion compared to control. However, unlike U87 cells, after 48h the majority of U251 cells infected with AdFOXO1;AAA were sub-G1 and were not plotted. These data demonstrate that AdFOXO1;AAA is capable of inducing cell cycle arrest in both U87 and U251 cells, and that arrest is followed by extensive apoptotic death in U251 cells.

**Mechanisms of AdFOXO1;AAA Cell Cycle Arrest and Apoptosis**

Our data show that U87 and U251 cells respond differently to AdFOXO1;AAA infection, where U251 cells experience apoptosis to a greater extent than U87 cells. Additionally, U87 cells undergo transient cell cycle arrest whereas in U251 cells, the arrest is followed mainly by apoptosis. In order to determine what may

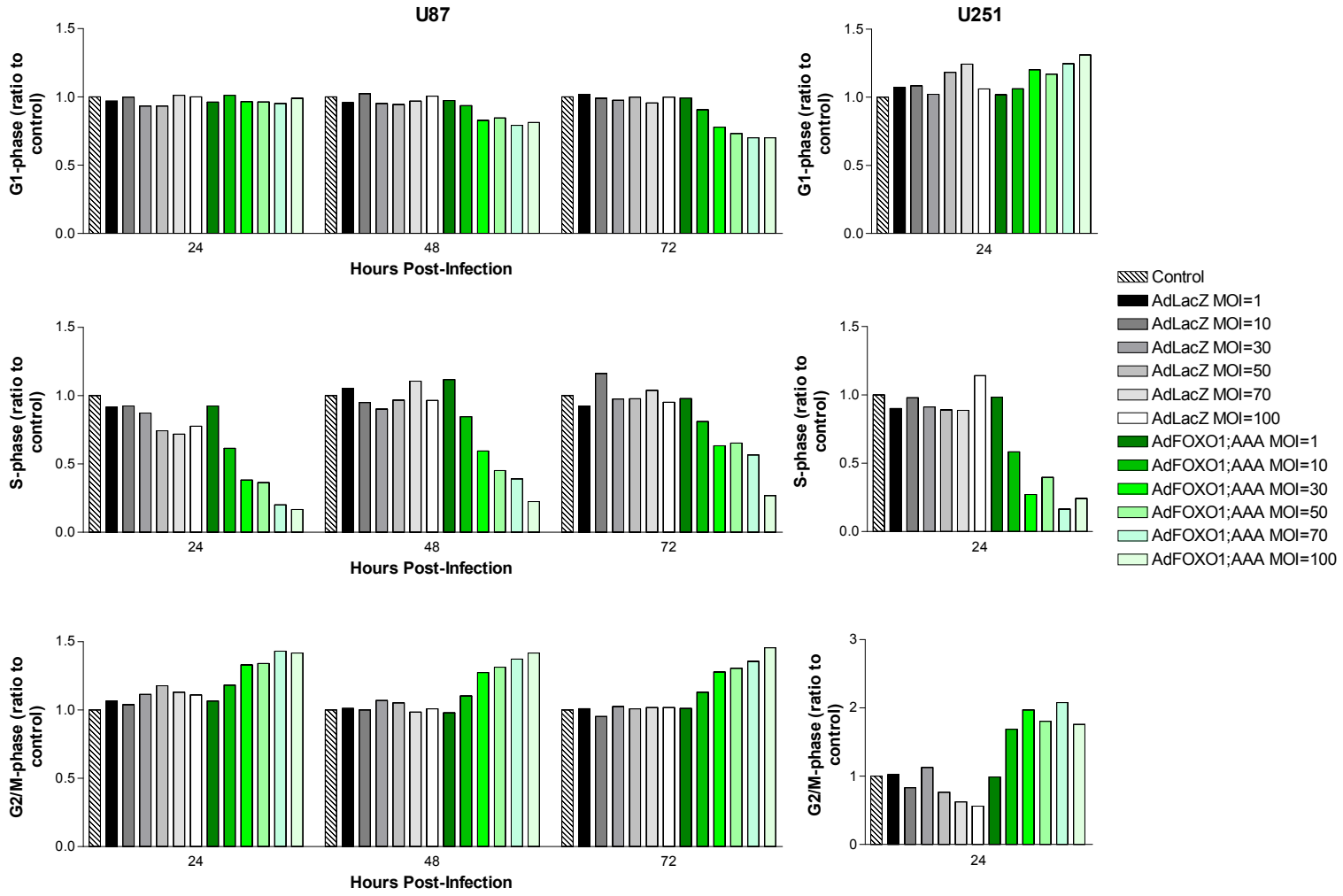

U87
U251
G1-phase (ratio to control)
S-phase (ratio to control)
G2/M-phase (ratio to control)
Hours Post-Infection
24
48
72
0.0
0.5
1.0
1.5
2
3
Control
AdLacZ MOI=1
AdLacZ MOI=10
AdLacZ MOI=30
AdLacZ MOI=50
AdLacZ MOI=70
AdLacZ MOI=100
AdFOXO1;AAA MOI=1
AdFOXO1;AAA MOI=10
AdFOXO1;AAA MOI=30
AdFOXO1;AAA MOI=50
AdFOXO1;AAA MOI=70
AdFOXO1;AAA MOI=100

**Figure 11: Cell cycle analysis by flow cytometry after infection with AdV.**
U87 and U251 cells were examined over a 72h time period; however, U251 cells infected with AdFOXO1;AAA at 48h and 72h were mostly sub-G1 and were not plotted. The percentage of cells in each cell cycle phase was evaluated by ModFit LT and plotted relative to uninfected control cells. These are representative data for an experiment repeated twice. These data are also represented as percentages of the total population in Supplementary Figure 3.

be contributing to these differential responses, immunoblot analyses were performed of FOXO1 and of the protein products of its target genes.

Infection with AdFOXO1;AAA at a MOI of 50 led to expression of FOXO1 at ~80kDa and ~50kDa (possible degradation product) in both U87 and U251 cells 24 hours post-infection (Figure 12). There were undetectable levels of endogenous FOXO1 expression in both U87 and U251 glioma cell lines, and also undetectable FOXO1 protein levels in AdLacZ infected cells. Noticeably, in U87 cells, there was a decline in FOXO1 expression over a period of 72 hours, which may only be partly related to increased cell death because only ~26% are dead at this time point (Figure 2) and 70% of the population is still transduced (fluoresced green) at 72h at a MOI of 50. Of interest in the U87 cells is the appearance of an extra band at ~55kDa at 48 and 72h that is not observed in U251 cells, which may indicate additional protein degradation bands or a cleavage product. In U251 cells, maximum expression was seen at 48 hours, and most cells were dead by 72 hours, where FOXO protein was no longer detected. Thus, the decline in FOXO1 expression in U87 cells may be related to cell death and protein degradation or cleavage, whereas in U251 cells, the decreased expression is due mostly to cell death.

To determine the molecular mechanism contributing to the observed cell cycle arrest, we examined the expression profile of FOXO target genes: p27, cyclin D1 and D2. The p27 protein is a cyclin dependent kinase inhibitor that is transcriptionally regulated by FOXO factors[304] and can cause both G1 and G2

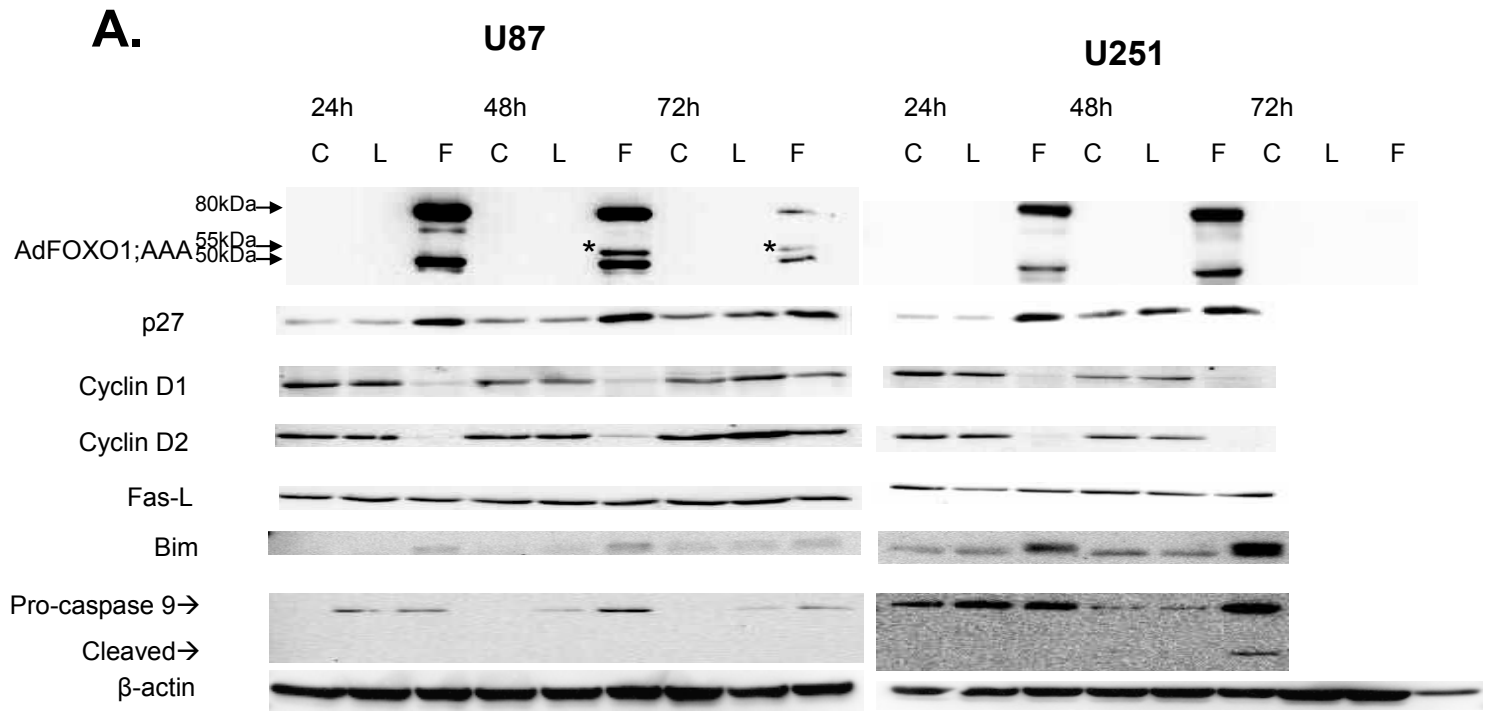

A.
U87
U251
24h
48h
72h
C L F C L F C L F
C L F C L F C L F
80kDa
55kDa
50kDa
AdFOXO1;AAA
*
*
p27
Cyclin D1
Cyclin D2
Fas-L
Bim
Pro-caspase 9→
Cleaved→
β-actin
B.
+TSA
24 48
IP: acetyl-lysine
IB: FOXO1
U87
U251

**Figure 12: Mechanism of cell cycle arrest and apoptosis.**

***A***, protein expression of FOXO1 and its target genes over time following AdV infection by immunoblotting with anti-FOXO1, -p27, -cyclin D1, -cyclin D2, -FasL, -Bim, -caspase-9, and -β-actin. The blots for U87 and U251 cells for Bim expression were processed at the same time. *, indicates bands that are only seen in AdFOXO1;AAA infected U87 cells. For U251 cells, 72h immunoblots were only performed for FOXO1 protein expression. C=mock infected; L=AdLacZ infected; F=AdFOXO1;AAA infected. ***B***, immunoprecipitation of cells infected with AdV in the presence of TSA with anti-acetyl lysine for acetylated residues on the FOXO1;AAA protein followed by immunoblotting with anti-FOXO1.

arrest[124]. D-type cyclins in complexes with cyclin dependent kinases 4 or 6 promote cell cycle progression from G1 to S, and are down regulated by FOXO factors in a manner that does not require binding to DNA[383]. At 24h post-infection in both U87 and U251 cells, p27 was upregulated in response to AdFOXO1;AAA, whereas cyclin D1 and D2 were downregulated (Figure 12A), which was consistent with results in the literature[383]. Interestingly, the expression profile of cell cycle proteins in U87 cells changed during the course of the 72h infection. Cyclin D1 and D2 expression levels in response to AdFOXO1;AAA returned after an initial down-regulation at 24h and the expression of p27 also declined slightly. In contrast, expression of neither p27 nor cyclins D1 and D2 in U251 cells changed from 24 to 48h. Therefore, the cell cycle arrest seen at 24h post-infection with AdFOXO1;AAA may be mediated by a down-regulation of D-type cyclins and an increase in p27. The transient arrest in U87 cells may be the result of declining FOXO1;AAA expression along with a return of D-type cyclins and a fall in p27 levels.

FOXO1 is also known to transcriptionally upregulate pro-apoptotic protein levels of Bim[90,156] and FasL[39]. Fas is a death receptor protein that transmits a suicide signal to the cell upon FasL binding that leads to caspase 8-dependent cell death[229,462]. Bim is a pro-apoptotic member of the Bcl-2 family that binds to pro-survival Bcl-2 molecules, neutralizing their function[346], thereby promoting apoptosis[156]. Caspase-9 is an initiator caspase of the mitochondrial apoptotic signalling pathway, as opposed to the death receptor pathway (such as Fas), that cleaves cellular substrates and leads to the biochemical and morphological

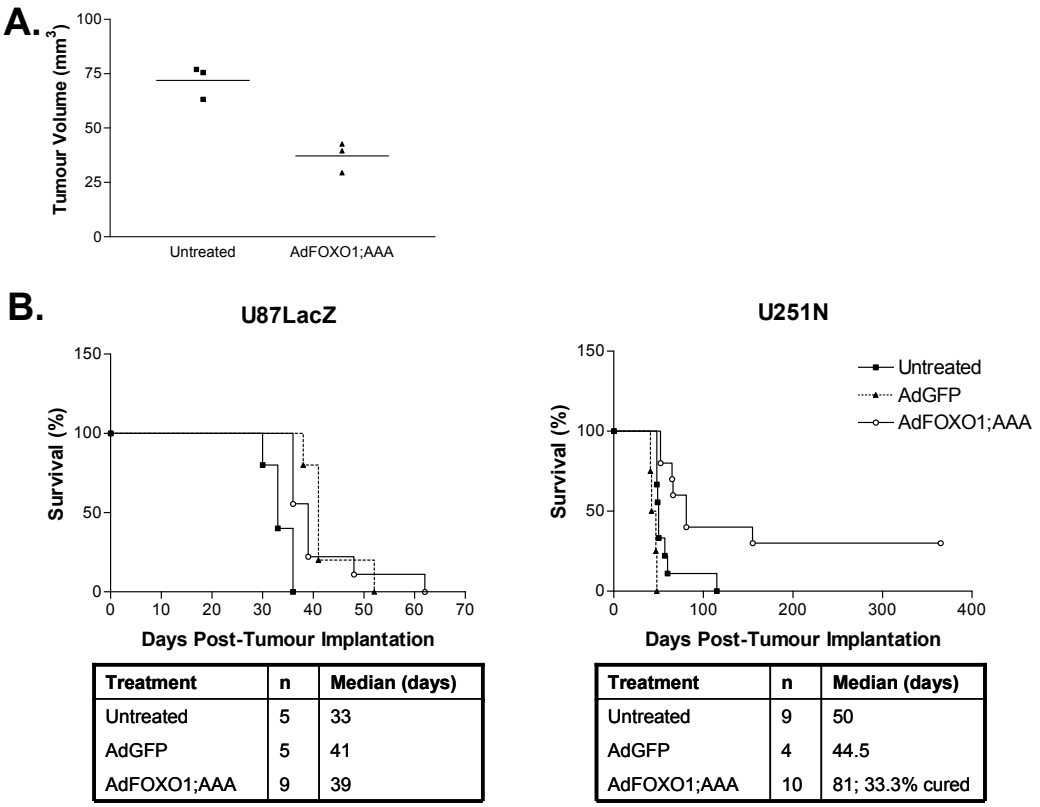
changes that characterize apoptosis[216]. Pro-caspase-9 must be cleaved in order to be active[216]. In U87 cells at 48h, Bim was modestly upregulated, but not FasL, nor cleaved caspase-9, even though pro-caspase-9 was increased (Figure 12A). At 72h, pro-caspase-9 and Bim levels fell, consistent with our previous observations that FOXO1;AAA expression is transient in U87 cells. On the other hand, in U251 cells, the expression of Bim and cleaved caspase-9 increased, with no change in FasL expression, suggesting that susceptibility to apoptotic death may be attributed to Bim activation and consequent death mediated via caspase-9 that does not involve FasL upregulation.

The observation that U87 cells appear less sensitive to AdFOXO1;AAA raises the possibility that the FOXO1;AAA proteins may be inhibited by other regulatory controls that are independent of phosphorylation. In particular, FOXO1 proteins are also regulated via acetylation. Several studies have demonstrated that acetylation of FOXO1 represses its activity by decreasing its affinity with target DNA[80,138,298]. Since FOXO1 acetylation is a transient event, the abundance of acetylated FOXO1 may be less detectable in the absence of deacetylase inhibitors such as trichostatin A (TSA)[362]. Therefore, to enrich for acetylated FOXO1 protein, cell samples were treated in the presence of TSA. Immunoprecipitation (IP) of whole cell lysates with anti-acetyl-lysine followed by immunoblotting with anti-FOXO1 antibody showed that at 48h post-infection in U87 cells, FOXO1 protein was acetylated (Figure 12B). Acetylated FOXO1 was not detected in U251 cells or at 24h in U87 cells. Taken together, these data suggest that the transient effect of AdFOXO1;AAA on the expression of cell cycle and apoptotic

proteins in U87 cells could be related to alterations in FOXO1 acetylation status. These data also indicate that FOXO1;AAA is not regulated by acetylation in U251 cells, which may contribute to the vulnerability of U251 cells to AdFOXO1;AAA induced apoptosis.

### *In Vivo* Cytotoxic Capacity

To assess the potential of AdFOXO1;AAA as a therapeutic agent, athymic mice were implanted intracerebrally with human U87LacZ or U251N glioma tumours. The stable transfection of the *LacZ* gene into U87 cells allowed easy identification of tumour versus non-tumour tissue for calculation of tumour size. U251N are a subclone of the U251 cell line with a higher tumour take. Ten days following tumour implantation, mice were given intratumoural injections of AdFOXO1;AAA, AdGFP, or were left untreated. AdFOXO1;AAA successfully caused tumour regression in the U87LacZ athymic mouse model at 35 days post-tumour implantation (Figure 13A). However, in long term survival studies, there was no significant difference between mice implanted with U87LacZ and treated with AdFOXO1;AAA, or AdGFP vector control (Figure 13B). This suggests that the apoptotic effect of AdFOXO1;AAA on U87LacZ is not robust enough to lead to prolonged survival of treated animals bearing U87LacZ tumours. In contrast mice implanted with U251N and treated with AdFOXO1;AAA did have a significantly longer median survival than AdGFP treated and untreated animals (Figure 13B). In fact, AdFOXO1;AAA treated animals survived 1.8X longer than AdGFP treated mice and a cure rate of 33.3% was achieved (as assessed by the proportion of animals which survived one year, the end point of the experiment).




| Treatment | n | Median (days) |
|---|---|---|
| Untreated | 5 | 33 |
| AdGFP | 5 | 41 |
| AdFOXO1;AAA | 9 | 39 |

| Treatment | n | Median (days) |
|---|---|---|
| Untreated | 9 | 50 |
| AdGFP | 4 | 44.5 |
| AdFOXO1;AAA | 10 | 81; 33.3% cured |

**Figure 13: *In vivo* tumour volume and Kaplan Meier survival curves.**

***A***, Tumour volume was measured 35 days after intracerebral implantation of athymic mice with U87LacZ cells and treated with AdFOXO1;AAA or left untreated ($P$=0.042 by two-tailed $t$-test). ***B***, survival of mice implanted with U87LacZ or U251N cells followed 10 days later by intratumoural injection with AdFOXO1;AAA ($3x10^7$pfu) or AdGFP ($3x10^7$pfu) or left untreated. Mice bearing U251N tumours and treated with AdFOXO1;AAA and AdGFP have significantly different median survivals by Kaplan Meier analysis ($P$<0.0001). Cure rate is defined as the percent of mice alive at 365 days post-tumour implantation when the experiment was terminated.

This is consistent with the *in vitro* data which demonstrated that U251 cells were more sensitive to AdFOXO1;AAA induced apoptosis than U87 cells.

**Assessment of Anti-Angiogenic Effects**

Given that FOXO1 has been shown to have anti-angiogenic activity[368], we tested whether AdFOXO1;AAA could reduce the viability of human brain endothelial cells (HBECs) by XTT assay. The XTT assay utilizes the capacity of metabolically active cells to reduce the tetrazolium salt, XTT, to orange coloured compounds of formazan where the dye intensity is proportional to the number of viable, metabolically active cells. We found that AdFOXO1;AAA significantly decreased HBEC viability in comparison to AdGFP vector control (Figure 14A). In contrast, increasing the MOI of AdGFP did not decrease HBEC viability.

Subsequently, we examined the capacity of AdFOXO1;AAA to decrease blood vessel density *in vivo* by staining the tumours of U87LacZ implanted mice for CD31, a marker for angiogenic vessels. Infection *in vivo* with AdFOXO1;AAA led to a significant reduction in the number of angiogenic vessels compared to untreated mice 3 and 5 days post-virus implantation (Figure 14B). The anti-angiogenic effect, though, was short-lived as 10 days following virus implantation, the blood vessel density was no longer significantly different than in untreated mice (Figure 14B). Thus, AdFOXO1;AAA has an anti-angiogenic effect that may be the result of its capacity to reduce viability of endothelial cells, which may contribute to tumour regression.

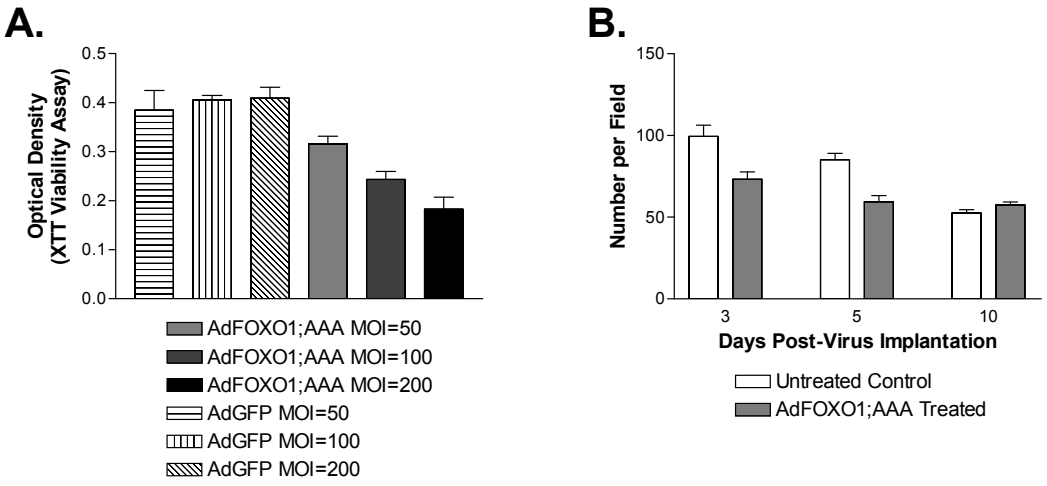



**Figure 14: Anti-angiogenic effect of AdFOXO1;AAA infection.**

***A***, HBEC viability expressed as optical density determined by XTT assay 72h following infection with AdFOXO1;AAA or AdGFP at various MOI. This experiment was repeated twice. AdFOXO1;AAA significantly decreased HBEC viability in comparison to AdGFP vector control ($P<0.01$ for MOI 100 and $P<0.001$ for MOI 200 by one-way ANOVA followed by the Bonferroni post-test). ***B***, quantification of CD31-positive blood vessels within the tumours of athymic mice implanted with U87LacZ and treated with AdFOXO1;AAA or left untreated. Sections with the greatest longitudinal of the tumour were stained and 5 pictures were taken at 200X with the number CD31-positive vessels recorded. N=2 mice per group at each time point. Infection *in vivo* with AdFOXO1;AAA led to a significant reduction in the number of angiogenic vessels compared to untreated mice 3 and 5 days post-virus implantation ($P=0.0126$ and $P=0.0015$, respectively by two-way *t*-test).

**Response of normal human neural cells**

To assess potential collateral damage caused by AdFOXO1;AAA infection in the brain, we investigated the effect of this virus on human fetal astrocytes (HFA) by XTT assay. Infection with AdFOXO1;AAA decreased the viability of HFA, whereas AdGFP did not affect viability compared to untreated control (Figure 15). These data indicate that consideration must be taken to minimize AdFOXO1;AAA damage to normal tissues if it is used as a therapeutic agent.

**Radiosensitization by AdFOXO1;AAA**

The use of radiation in the brain is known to cause serious tissue damage. An agent that would sensitize cells to radiation could reduce the amount that would need to be delivered for a therapeutic effect, or could enhance the effect of radiation. As gliomas are known for being radio-resistant, it was important to investigate the ability of AdFOXO1;AAA to sensitize gliomas to radiation given its role in DNA damage induced cell death[210]. Using a clonogenic assay, we found that AdFOXO1;AAA plus radiation had a greater effect than either agent alone in decreasing clonogenicity in both U87 and U251 cells compared to uninfected control (Figure 16). At 10Gy in U87 cells, the radiosensitizing effects of AdFOXO1;AAA infection at an MOI of 50 was significant compared to untreated control and AdLacZ infected cells (Figure 16A). Moreover, the combined effect of AdFOXO1;AAA and radiation in U87 cells led to a 10-fold decrease in clonogenicity compared to control (Figure 16B). The U251 cells were even more sensitive than U87 cells as both AdLacZ and AdFOXO1;AAA decreased the surviving fraction to the same extent that AdFOXO1;AAA had in U87 cells with

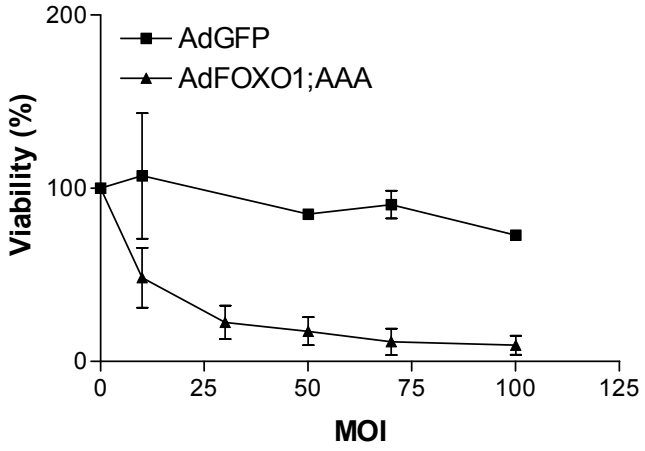



**Figure 15: HFA viability measured by XTT assay.**

Cells were infected at various MOI with AdFOXO1;AAA or AdGFP and incubated for 72h. This experiment was performed twice in duplicate and expressed as the percentage of control untreated cells.

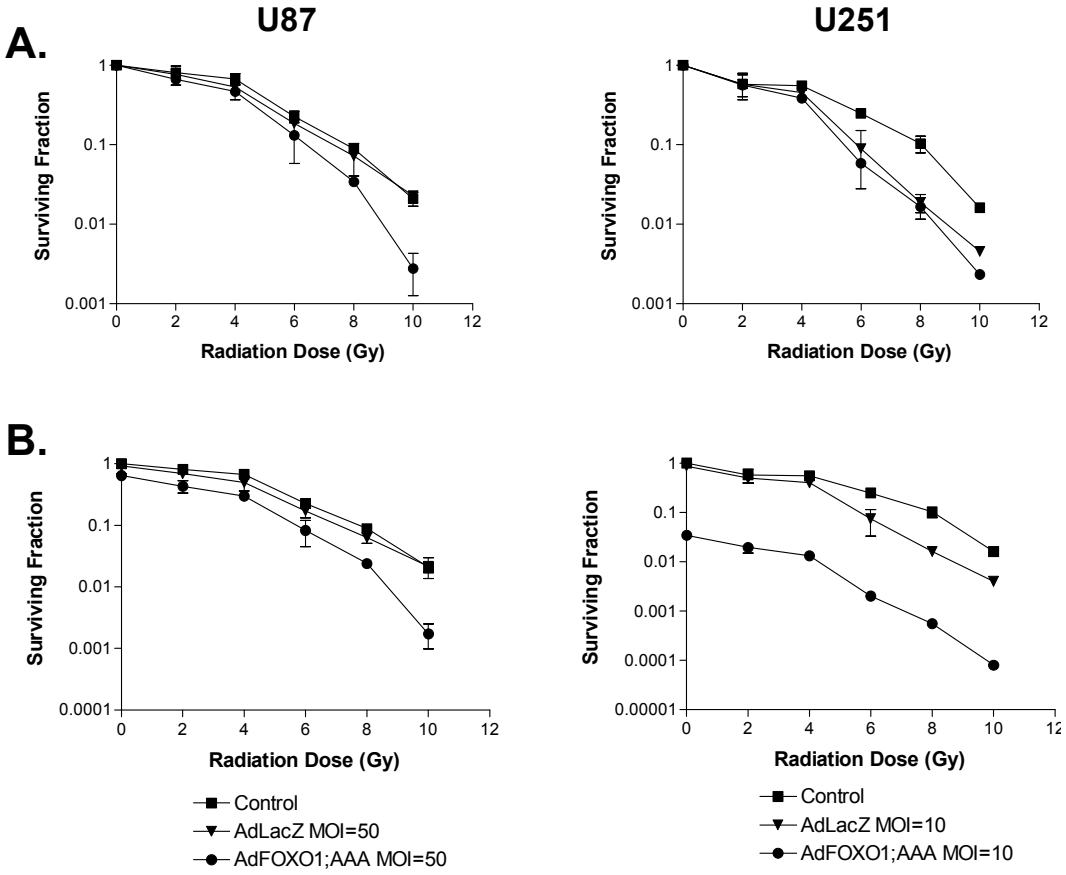
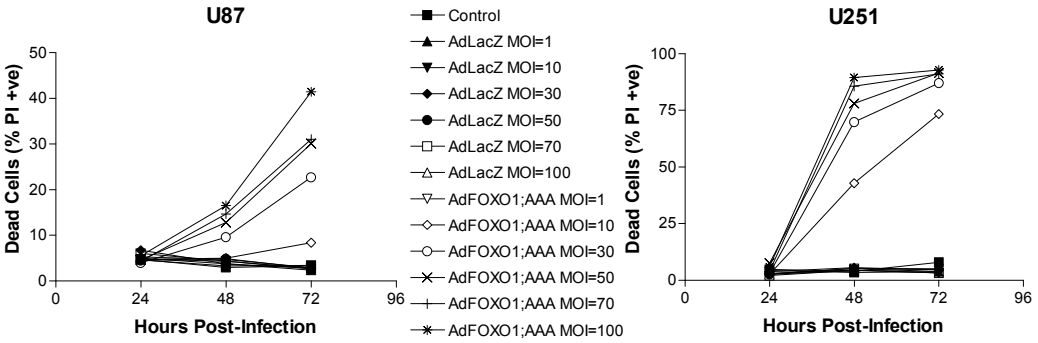



**Figure 16: Clonogenic assay for radiosensitivity.**

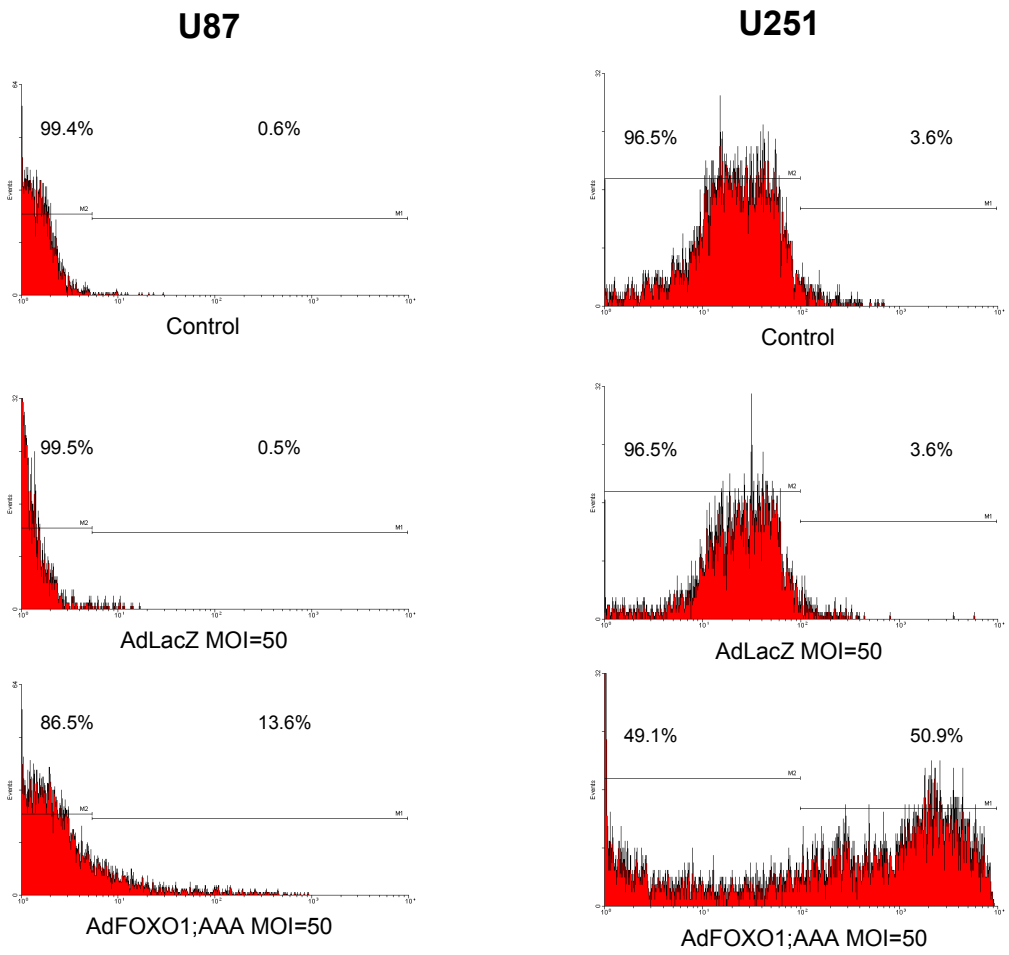
Cells were infected with virus, irradiated 24h later and then re-plated. 14 days after irradiation the number of colonies that formed was counted. The top panels (***A***) illustrate the effect of radiation alone—the surviving fraction was normalized such that all treatment groups would begin with a surviving fraction of 1.0 at 0Gy even if survival was not 100% at 0Gy. The bottom panels (***B***) show the effect of radiation and the virus combined. These experiments were done twice and the surviving fraction was calculated as described in the Materials and Methods. At 10Gy for U87 cells in (A), AdFOXO1;AAA infection was significant compared to untreated control and AdLacZ infected cells ($p<0.05$). AdFOXO1;AAA infection of U251 cells in (A) was also significant beginning at 6Gy ($p<0.05$) compared to control. At 8Gy for U251 cells in (A), radiosensitivity with AdLacZ infection was detectable ($p<0.05$), but AdLacZ had no effect on U87 cells. One-way ANOVA followed by the Bonferroni post-test were used to determine significance.

an even lower MOI of 10 (Figure 16A). The radiosensitizing effect of AdFOXO1;AAA infection of U251 cells was also significant at a lower radiation dose than U87 cells beginning at 6Gy as opposed to 10Gy (Figure 16A). In fact, AdFOXO1;AAA infection and radiation together in U251 cells led to an overall 100-fold decrease in clonogenicity compared to control (Figure 16B). Surprisingly, radiosensitivity with AdLacZ infection was detectable beginning at 8Gy for U251 cells (Figure 16A), but AdLacZ had no effect on U87 cells (Figure 16A). Thus, AdFOXO1;AAA has potential as an adjuvant to radiotherapy, and in the case for U251 cells, adenovirus infection itself has radiosensitizing capabilities.



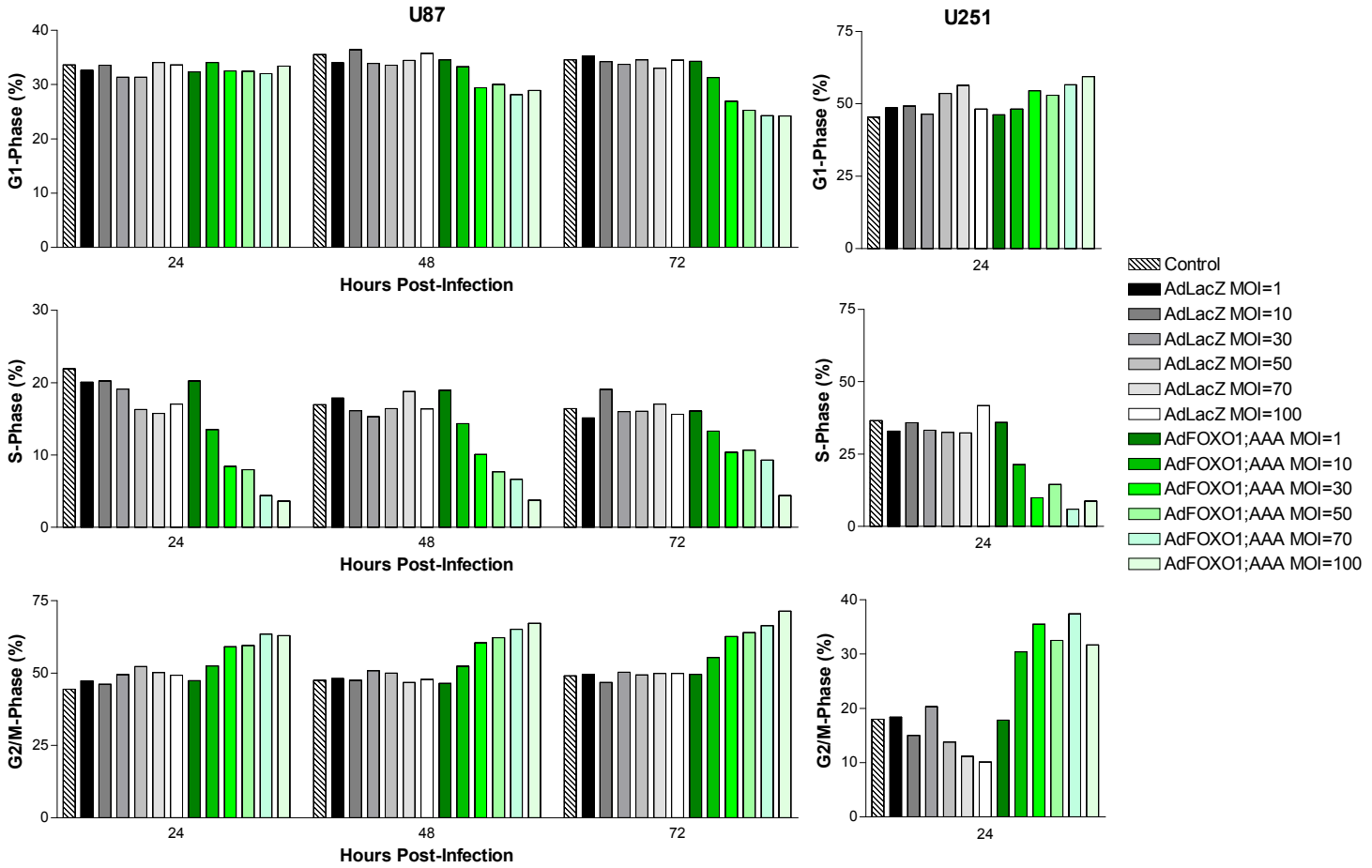

**Supplementary Figure 1: PI staining of unfixed cells to determine the live/dead proportion.**

Cells that stain positively for PI are dead and are represented as a percentage of the total population. These are representative data of an experiment done twice.




**Supplementary Figure 2: Annexin-V flow cytometry histograms.**

The region on the right (M1) represents cells that are actively undergoing apoptosis (Annexin-V-positive and PI-negative). The region on the left (M2) are live cells that are neither Annexin-V- nor PI-positive. Cells were infected at MOI of 50 and analyzed by flow cytometry 48h post-infection. Only adherent cells were analyzed and a gate was used to exclude dead and damaged cells (PI-positive cells).


U87
U251
G1-Phase (%)
S-Phase (%)
G2/M-Phase (%)
Hours Post-Infection
24
48
72
Control
AdLacZ MOI=1
AdLacZ MOI=10
AdLacZ MOI=30
AdLacZ MOI=50
AdLacZ MOI=70
AdLacZ MOI=100
AdFOXO1;AAA MOI=1
AdFOXO1;AAA MOI=10
AdFOXO1;AAA MOI=30
AdFOXO1;AAA MOI=50
AdFOXO1;AAA MOI=70
AdFOXO1;AAA MOI=100

**Supplementary Figure 3: Cell cycle analysis.**

These data are the same as in Figure 11, but expressed as percents taken directly from ModFit LT analysis.

# Chapter 4: Suicide Gene Therapy

## Improvement of Antitumour Activity by Gene Amplification with a Replicating but Nondisseminating Adenovirus

**PREAMBLE**

Gene therapy is a promising approach for cancer treatment; however efficacy of current vectors remains insufficient. To improve the success of suicide gene therapy, we constructed an adenoviral replication competent vector that has its protease gene deleted, and expresses bacterial cytosine deaminase fused with bacterial uracil phosphoribosyltransferase (CD::UPRT). The pro-drug, 5-FC is transformed into the highly toxic and tissue-diffusible 5-FU by CD::UPRT in infected cells. This vector is incapable of producing infectious particles, but is able to undergo a single round of replication, thereby increasing transgene copy number and expression. In both the presence or absence of 5-FC, we determined whether the replication competent vector [Ad(dPS)CU-IRES-E1A] was significantly more efficacious for *in vitro* tumour cell killing and bystander effects compared to first generation non-replicating vectors. For *in vivo* experiments, in which virus was injected into preestablished intracerebral glioma xenografts and followed by 5-FC treatment, we investigated whether Ad(dPS)CU-IRES-E1A could reduce tumour volume and extend survival more than first generation vectors.

## RESULTS

### Description of the Ad Genomes

The structures of the AdVs used in this study are presented in Figure 17A. The two non-replicating AdVs [AdCU and Ad(dPS)CU], consist of the same backbone and differ only in the presence of the protease gene [deleted in (delta) PS]. These are first generation AdV, deleted in E1 and E3 regions, and expressing the CD::UPRT fusion gene in a dicistronic cassette with the GFP. The two replicating AdVs [Ad(dPS)CU-IRES-E1A and Ad(PS+)CU-IRES-E1A] express the same CD::UPRT fusion gene, but the GFP gene is replaced with the E1A gene, allowing DNA replication. The AdV containing the PS gene [Ad(PS+)CU-IRES-E1A] can package and disseminate after replication, while the deltaPS (dPS) AdV can only replicate but cannot disseminate. Wild-type adenovirus serotype 5 (Ad5wt) was chosen to compare directly the suicide gene approach with oncolytic approaches, as Ad5wt should be as potent if not more so than most oncolytic AdVs described in the literature since it is not impaired by any mutations.

All the AdVs were validated by PCR-based assays for the structure of the CD::UPRT expression cassette; the deletion of the protease gene in Ad(dPS)CU and Ad(dPS)CU-IRES-E1A; and the presence of E1A genes in both replicating AdVs (Supplementary Figure 4A). We also tested whether the presence of the prodrug 5-FC would affect the characteristics of the replicative AdVs. The addition of 5-FC reduced viral protein expression (Supplementary Figure 4B) and yield of viral particles produced from Ad(PS+)CU-IRES-E1A by about 4-fold

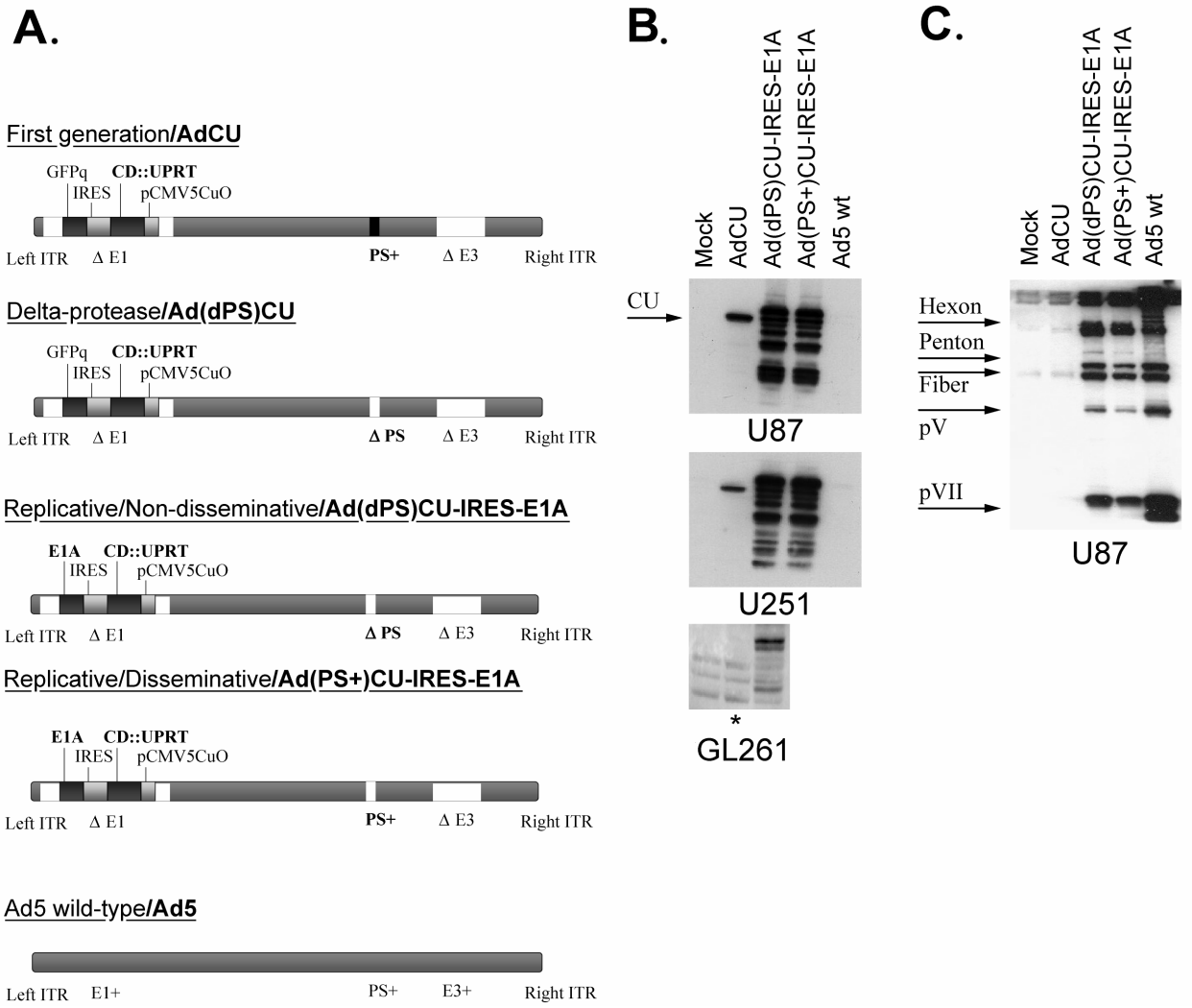

A.
First generation/AdCU
GFPq
IRES
CD::UPRT
pCMV5CuO
Left ITR
Δ E1
PS+
Δ E3
Right ITR
Delta-protease/Ad(dPS)CU
GFPq
IRES
CD::UPRT
pCMV5CuO
Left ITR
Δ E1
Δ PS
Δ E3
Right ITR
Replicative/Non-disseminative/Ad(dPS)CU-IRES-E1A
E1A
IRES
CD::UPRT
pCMV5CuO
Left ITR
Δ E1
Δ PS
Δ E3
Right ITR
Replicative/Disseminative/Ad(PS+)CU-IRES-E1A
E1A
IRES
CD::UPRT
pCMV5CuO
Left ITR
Δ E1
PS+
Δ E3
Right ITR
Ad5 wild-type/Ad5
Left ITR
E1+
PS+
E3+
Right ITR
B.
Mock
AdCU
Ad(dPS)CU-IRES-E1A
Ad(PS+)CU-IRES-E1A
Ad5 wt
CU
U87
U251
*
GL261
C.
Mock
AdCU
Ad(dPS)CU-IRES-E1A
Ad(PS+)CU-IRES-E1A
Ad5 wt
Hexon
Penton
Fiber
pV
pVII
U87

**Figure 17: Characterization of adenovirus vectors used in this study.**

***A***, schematic representation. *AdCU* vector is an adenovirus deleted in the E1 and E3 regions, the expression cassette is under the control of the CMV5(CuO) promoter. This promoter allows the repression of transcription during AdV production in 293 cells expressing the CymR repressor. This was needed for efficient AdV production [D. Bourbeau *et al*, manuscript in preparation]. The expression cassette expresses the fusion gene *CD::UPRT* and the *GFP* gene, the latter being under the translational control of an IRES. *Ad(dPS)CU* is a variant of the *AdCU* that bears the additional deletion of its *PS* gene. *Ad(dPS)CU-IRES-E1A* has the same structure as the *Ad(dPS)CU*, but the *GFP* reporter gene was replaced with the *E1A* gene rendering this AdV replicative. *Ad(PS+)CU-IRES-E1A* differs from the replicating/non-disseminating virus by having the *PS* gene thus being both replicating and disseminating. Ad5wt was used in this study to benchmark the suicide gene approach to an oncolysis approach. ***B*,** suicide gene expression. 'CU' indicates CD::UPRT protein expression. ***C*,** viral protein expression in tumour cells lines infected with the AdVs. Tumour cells were plated in 6 well plates at a density of $2.5x10^5$ cells, transduced the next day with AdVs at a MOI of 50, and incubated for 48 h. Blots were incubated with either anti-codA (B), or anti-Ad5 (C). In B, in the lane marked with *, GL261 cells were transduced with Ad(dPS)CU.

(Supplementary Figure 4C). This suggested that in this backbone, oncolysis in combination with suicide gene expression may not be optimal.

**Transgene Expression and Viral protein Expression**

The effect of the various AdV was evaluated in the human U87 and U251 and the murine GL261 glioma cell lines. The human glioma cells were efficiently transduced at low MOIs, while the murine glioma cells required higher MOIs. GL261 cell lines displayed low transduction even at MOI as high as 200 to 500 (data not shown and Supplementary Table 1). We evaluated by Western blot analysis the expression of the *CD::UPRT* fusion gene produced after transduction of these cell lines with the first generation AdV and the two replicating AdVs (Figure 17B). The AdCU-transduced cells expressed CD::UPRT as a single 78 kDa protein, whereas in cells transduced with the replicating AdVs, the expression of the CD::UPRT gene was increased and secondary degradation products appeared. When the expression of the viral proteins was analyzed, only trace amounts of proteins were detectable in cells transduced with the first generation AdCU (Figure 17C). In contrast, with both replicating AdVs, high levels of viral proteins were detected, albeit to a lesser extent than with Ad5wt. Furthermore, the level of viral protein expressed was similar between cells transduced with either Ad(dPS)CU-IRES-E1A or Ad(PS+)CU-IRES-E1A, suggesting that the PS deletion did not affect the extent of AdV genome replication and transgene expression.

**Comparison of Cytotoxic Activities *In Vitro***

To evaluate whether Ad(dPS)CU-IRES-E1A can provide improved cytotoxic activity as compared with AdCU, we used doubling dilutions of AdV to determine the MOI that would reduce cell viability to 50% at a constant concentration of 5-FC (Figure 18). In the U87 cells, the replicating/non-disseminating Ad(dPS)CU-IRES-E1A was 45 times more potent than its first generation counterpart; infection at a MOI as low as 0.2 could inhibit 50% cell viability, whereas the AdCU had to be used at a MOI of 9 to obtain the same result. In the absence of 5-FC, AdCU was not toxic at any of the MOIs tested, whereas in the absence of 5-FC, the replicating/non-disseminating AdV was as good as the AdCU + 5-FC (compare Figure 18A to 18B). The MOI required to reduce cell viability to 50% was compared for all the AdVs in the two cell lines (Figure 18C). The U87 cells were the most responsive, while U251 were less responsive due to moderate sensitivity to 5-FU (Supplementary Table 1). No differences were observed between Ad(dPS)CU and AdCU confirming that the deletion of the *PS* gene had no effect on the cytotoxic activity of the vector. Moreover, when the replicating/non-disseminating Ad(dPS)CU-IRES-E1A was compared to its PS+ counterpart, Ad(PS+)CU-IRES-E1A, the cytotoxic activity showed a modest improvement of 2-fold (Figure 18C), demonstrating only poor oncolytic activity by the Ad(PS+)CU-IRES-E1A. Additionally, GL261 murine glioma cells infected with Ad(dPS)CU at MOI of 10 required 250μM of 5-FC to reduce viability to 50%, whereas Ad(dPS)CU-IRES-E1A only required 10.7μM to achieve the same killing at the same MOI demonstrating that GL261 cells were also more sensitive to Ad(dPS)CU-IRES-E1A despite their low transduction efficiency (Figure 18D).

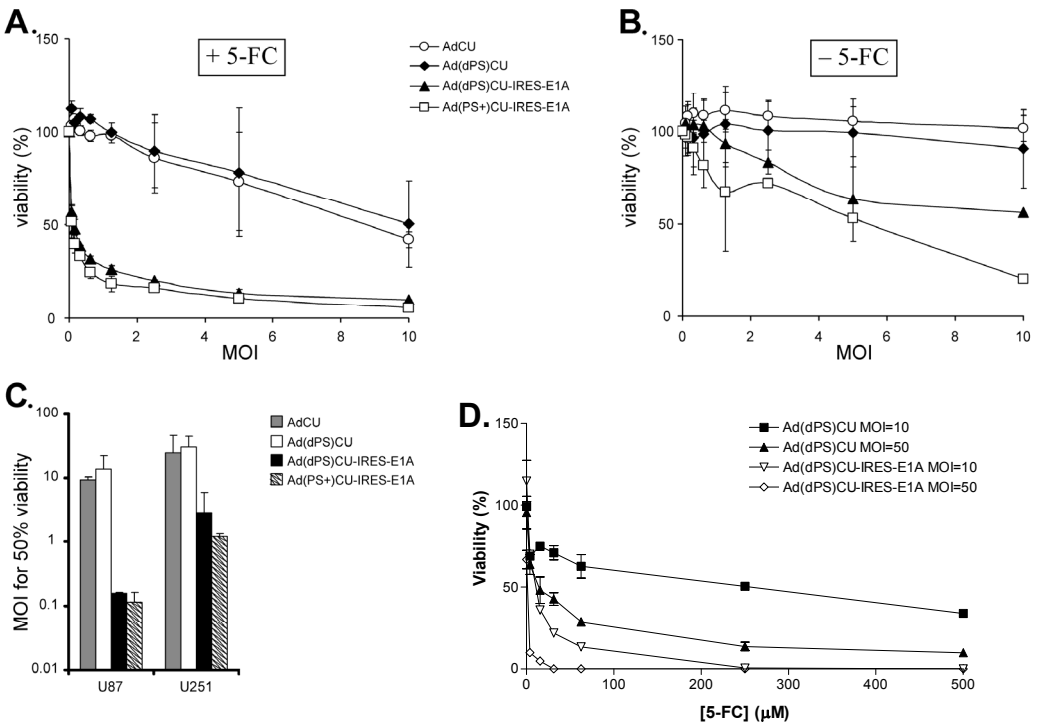



**Figure 18: Cytotoxicity assays.**

Cells were plated at a density of $5x10^3$ cells per well in 96-well plates. The next day, cells were transduced with doubling dilutions of AdVs starting at a MOI of 10 for 5 h, and then 5-FC (25µM for U87 and 500µM for U251) was added. Cells were incubated for 6 days and analyzed by MTT assays. Curves obtained with the U87 cells: ***A***, with 5-FC; ***B***, without 5-FC. ***C***, evaluation of the initial MOI required to produce 50% viability. Data were obtained from curves displayed as in (A) and generated for each cell line. Each dilution had 8 replicates and each experiment was performed twice. ***D***, evaluation of the amount of 5-FC required to reduce viability of GL261 murine glioma cells at a MOI of 10 and 50 analyzed by XTT. This experiment was performed twice.

**Bystander Activity of the Replicating/Non-Disseminating AdV**

To achieve clinical success, a strong bystander activity is of paramount importance. Therefore, we evaluated whether the benefits observed at low titers of virus with the Ad(dPS)CU-IRES-E1A would also translate into an improvement in a bystander assay. U87 cells transduced with either MOI of 10 or 100 were mixed at different ratios with non-transduced (bystander) cells. Cells transduced with MOI of 10 of Ad(dPS)CU-IRES-E1A were more potent at reducing cell viability of the entire population than cells transduced with MOI of 100 of AdCU (Figure 19A). When a MOI of 10 was used for both AdVs, the replicating/non-disseminating virus was 14 times better than AdCU (3% cells versus 42%) for killing 50% of the U87 cell population (Figure 19B). Furthermore, similar results were obtained with the U251 cells (11% versus >100%; Figure 19B). Although at MOI of 10, the first generation AdCU did not display any bystander activity in U251 cells, with MOI of 100 bystander activity was observed at 11% in U251 (data not shown).

**Comparison of Cytotoxic Activities in Spheroid Models**

Monolayer cell cultures do not reproduce the three-dimensional structure of tumours. In cancer gene therapy, accessibility of cells to the viral vector is very important and specifically for AdV, transduction efficiency varies greatly between cells in monolayers and in spheroids (three-dimensional structures). It was previously demonstrated that in spheroid cultures, only superficial cell layers are transduced with the first generation AdCU, which co-expresses the GFP protein[34]. Therefore, we tested the anti-tumoural activities of the various AdVs in U87

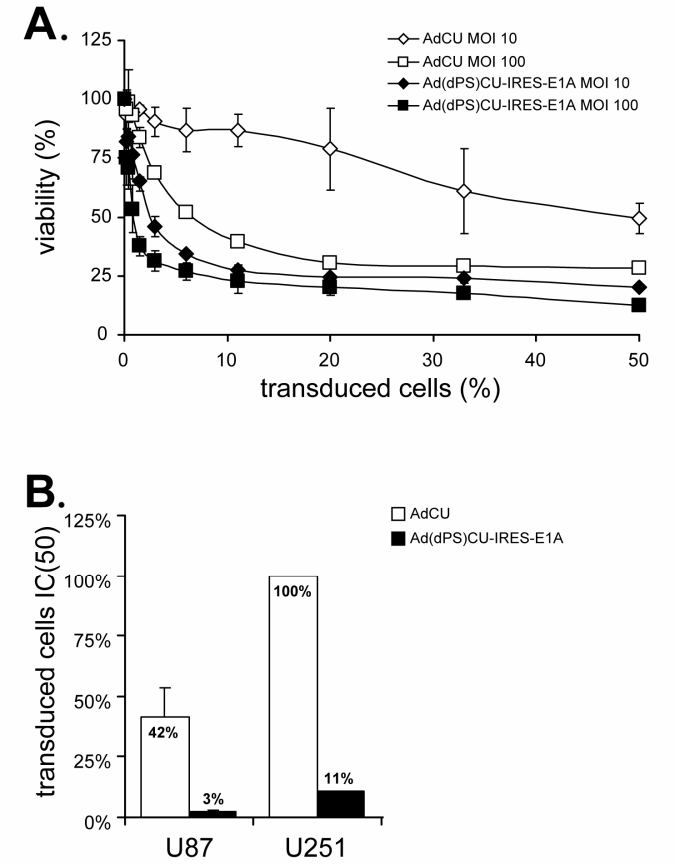



**Figure 19: Analysis of bystander effect.**

Cells were plated at a density of $2x10^5$ in 12 well plates. The next day, they were transduced with AdVs at MOIs of 10 and 100. After 4 h, transduced cells were trypsinized and resuspended in 2mL of media. Doubling dilutions of cells were generated and plated into 96 well plates in 50µL. Then, non-transduced cells (bystander cells) were trypsinized, diluted to $1x10^5$ cells/mL, and 50µL were added to the wells containing the transduced cells. These dilutions were done with 5-FC (25µM for U87 and 500µM for U251). This resulted in the following ratios of transduced cells: 50%, 33%, 20%, 11%, 6%, 3%, 1.5%, 0.8%, 0.4%, and 0.2%. The cells were incubated for 6 days and analyzed by MTT assays. ***A***, curves obtained with the U87 cells. ***B***, evaluation of the $IC_{50}$ of transduced cells. Values for U87 and U251 cells were obtained from curves using MOI of 10 as displayed in (A). Each condition had 8 replicates and the experiment was done twice.

spheroids. Both the replicating/non-disseminating and the replicating/disseminating virus reduced viability close to or below 50%, respectively, at a lower viral dose (particle number) than the first generation AdCU (Figure 20A). Furthermore, viability was still significantly reduced to levels around 50% even when the number of infectious particles of the replicating AdVs was decreased by 5-fold, suggesting that the number of viral particles of replicating AdVs can be reduced by 25-fold, while still matching the performance of first generation AdCU (Figure 20A). However, none of the AdVs tested were successful at significantly reducing the viability of U251 spheroids (Figure 20B), most probably due to the resistance of these p53 mutant cells to 5-FU[34].

Nevertheless, we found that the suicide gene therapy approach was far more potent than oncolysis in these spheroids when we benchmarked these viruses against the oncolytic activity of Ad5wt. Indeed, oncolysis with Ad5wt only reduced viability to 70% with either $1x10^6$ pfu or even $5x10^6$ pfu, whereas the Ad(dPS)CU-IRES-E1A achieved 69% with only $2x10^5$ pfu and 47% with $5x10^6$pfu (Figure 20A). Overall, these data suggest that the higher efficacy of the replicative platform can compensate for poor transduction.

**Comparison of Cytotoxic Activities in Established Xenograft Models**

As a first step to examining if Ad(dPS)CU-IRES-E1A is capable of causing tumour regression *in vivo*, we assessed tumour volume in athymic nude mice implanted intracerebrally with U87LacZ human glioma cells and injected intratumourally 10 days later with virus, and treated with 5-FC (500mg/kg twice

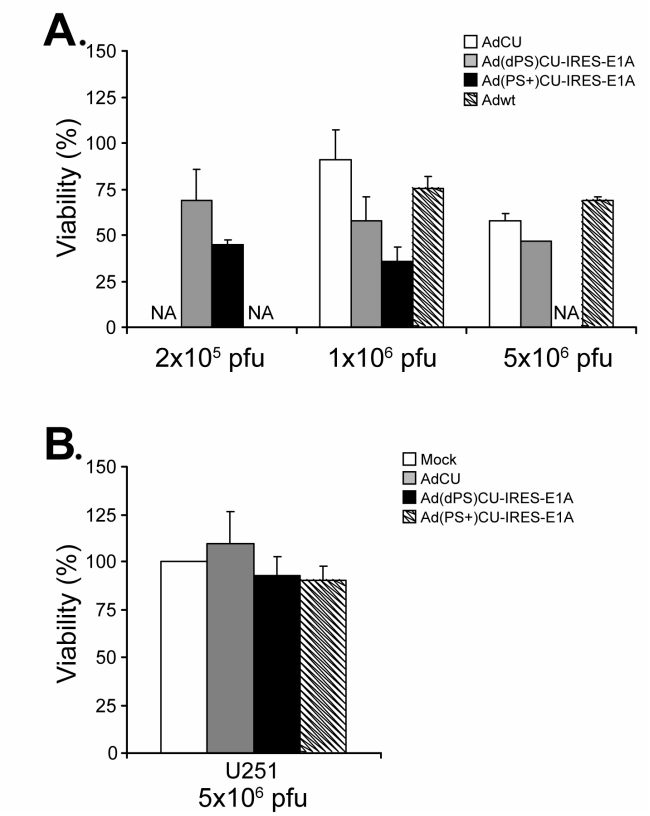



**Figure 20: Cytotoxic activity in spheroids.**

**A**, U87 spheroids were transduced with $1x10^6$ pfu, $5x10^6$ pfu, or $2x10^5$ pfu and treated with 500μM of 5-FC. At each dose, the shown viruses were used. The diameters of the U87 spheroids were ~1mm. Spheroids were incubated for 10 days after the treatment and analyzed by MTT assay. **B**, U251 spheroids were treated with $5x10^6$pfu and 500μM of 5-FC on day 0, 3, and 6. Viability was assessed on day 10. The diameters of these spheroids were ~0.5mm.

daily given i.p.). At 35 days post-tumour implantation, Ad(dPS)CU-IRES-E1A treated mice had significantly smaller tumours (19.8±18.8mm$^3$) than animals that received only 5-FC (159.6±52.30mm$^3$ ; Figure 21).

To determine whether these results would translate into an extended life span for tumour-bearing mice, we performed long-term survival studies. We first evaluated the best dosing regimen for 5-FC given the cytotoxic capacity of the replicating/non-disseminating virus alone (Figure 18A). To allow the transduced cells to convert as much 5-FC as possible to accumulate the toxic 5-FU, before the virus itself eliminates the tumour cells, 5-FC was administered at either 1 or 2 days post virus injection. These studies revealed that survival of mice treated with 5-FC 1 day after virus injection with Ad(dPS)CU-IRES-E1A resulted in a significant increase in median survival as compared with the cohort that was treated 2 days after virus injection ($p=0.0005$).

In survival studies we tested the effect of providing two injections of virus spaced 21 days apart, where the first injection is administered 10 days following tumour implantation. 5-FC was given the day after virus injection in both injection periods. Mice treated with Ad(dPS)CU-IRES-E1A and 5-FC had a much greater median survival of 117 days than any group tested (Figure 22A). Ad(dPS)CU plus 5-FC treated animals only had a median survival of 76 days, which is similar to the median survival obtained after a single virus injection (data not shown). Treatment with Ad(dPS)CU-IRES-E1A alone without 5-FC led to a small improvement in survival (median survival of 60.5 days) as compared with

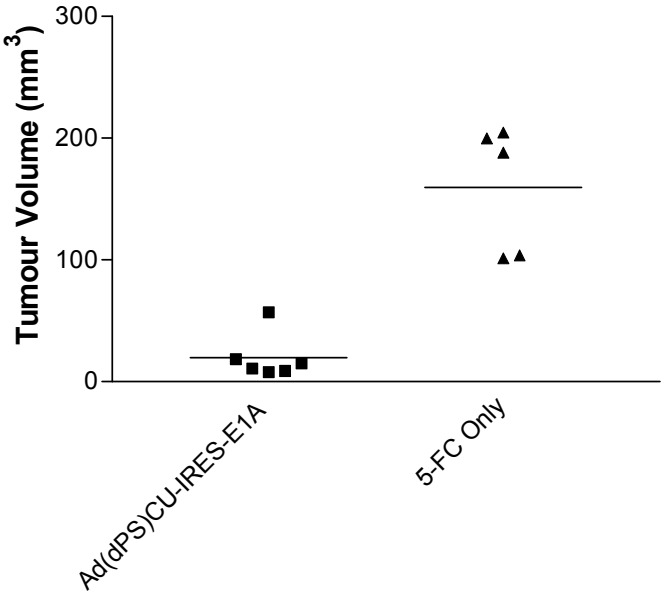
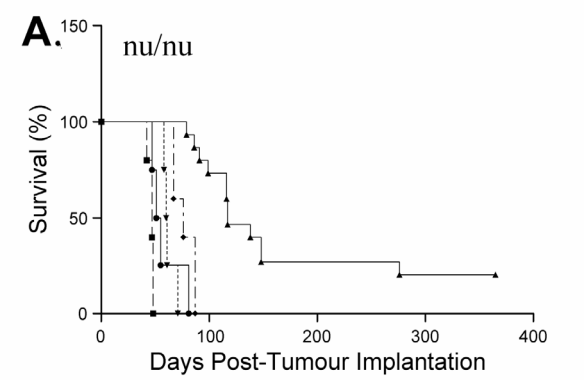
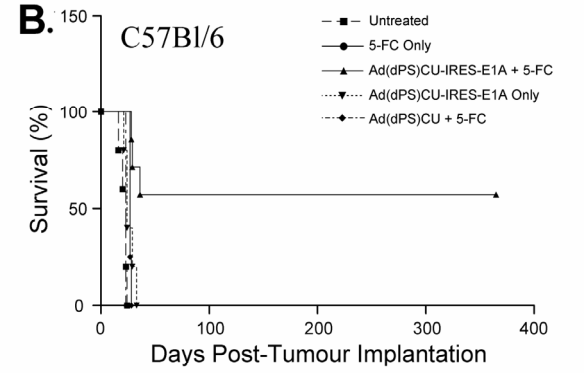



**Figure 21: Tumour volume of athymic mice.**

U87LacZ xenografts were implanted in athymic mice and 10 days later they were injected with Ad(dPS)CU-IRES-E1A or left untreated. All mice were provided with 5-FC treatment the next day as described in Materials and Methods. Mice were sacrificed 35 days after tumour implantation and tumour volume was calculated from sequential brain sections as described in Materials and Methods. $P$=0.0002 by two-tailed $t$-test.




| Treatment | n | Median (days) |
|---|---|---|
| Untreated | 5 | 47 |
| 5-FC Only | 5 | 53 |
| Ad(dPS)CU-IRES-E1A +5-FC | 15 | 117 |
| Ad(dPS)CU-IRES-E1A Only | 4 | 60.5 |
| Ad(dPS)CU+ 5-FC | 5 | 76 |




| Treatment | n | Median (days) |
|---|---|---|
| Untreated | 5 | 23 |
| 5-FC Only | 4 | 27 |
| Ad(dPS)CU-IRES-E1A +5-FC | 7 | >365 |
| Ad(dPS)CU-IRES-E1A Only | 5 | 24 |
| Ad(dPS)CU+ 5-FC | 5 | 23 |

**Figure 22: Kaplan-Meier survival curves.**

U87LacZ xenografts in athymic mice (***A***) and GL261 murine glioma cells in C57Bl/6 mice (***B***). Animals were implanted with tumour cells and 10 days later were injected with the various viral recombinants, followed the next day by 5-FC treatment as described in Materials and Methods. Mice were monitored daily and were euthanized if they showed any signs of lethargy, significant neurological morbidity, or severe weight loss. The experiment was terminated at 365 days after tumour implantation.

untreated controls and mice treated with 5-FC only (median survivals of 47 and 53 days, respectively). Due to the capacity of Ad(dPS)CU-IRES-E1A to further prolong survival after a second injection compared to Ad(dPS)CU, the results support the idea that Ad(dPS)CU-IRES-E1A is likely more capable of penetrating a larger tumour mass.

To assess if the immune system can be a contributor to the effect observed with Ad(dPS)CU-IRES-E1A, a syngeneic glioma model was used by implantation of GL261 murine glioma cells in wild type C57Bl/6 mice. AdV mediated gene transfer is quite poor in these cells, with less than 1% of cells being transduced with an AdLacZ recombinant virus at a MOI of 500 (data not shown; Supplementary Table 1). Nevertheless, infection at a MOI of 50 with Ad(dPS)CU-IRES-E1A decreased cell viability to 50% in the presence of 1.4μM of 5-FC (figure 18D), indicating that GL261 can be sensitized to killing with 5-FC and suggesting that the majority of the cell killing that occurs after addition of 5-FC is achieved through a bystander effect.

The same protocol as described above was used to evaluate the effect of Ad(PS)CU and Ad(dPS)CU-IRES-E1A (Figure 22B). In this model, untreated mice have a median survival of 23 days, whereas mice treated with 5-FC alone have a median survival of 27 days. Ad(dPS)CU-IRES-E1A injected animals treated with 5-FC had a dramatically higher survival rate. In fact, 365 days after tumour implantation, when the experiment was terminated, this treatment group had a survival rate of 57.1%. In contrast, animals treated with Ad(dPS)CU-IRES-

E1A alone and mice treated with Ad(dPS)CU with 5-FC had median survivals of only 24 and 27 days, respectively, with survival curves very similar to control groups. We compared the extent of immune cell infiltration by flow cytometry by analyzing and quantifying T cells, macrophages and natural killer (NK) cells in the brains of mice treated with the AdV and 5-FC at different times after AdV injection (4 and 10 days). As shown in Figure 23, for the 10-day time point, Ad(dPS)CU-IRES-E1A elicited greater macrophage and T-cell infiltration, which may be a major reason for its superior efficacy in this immunocompetent model.

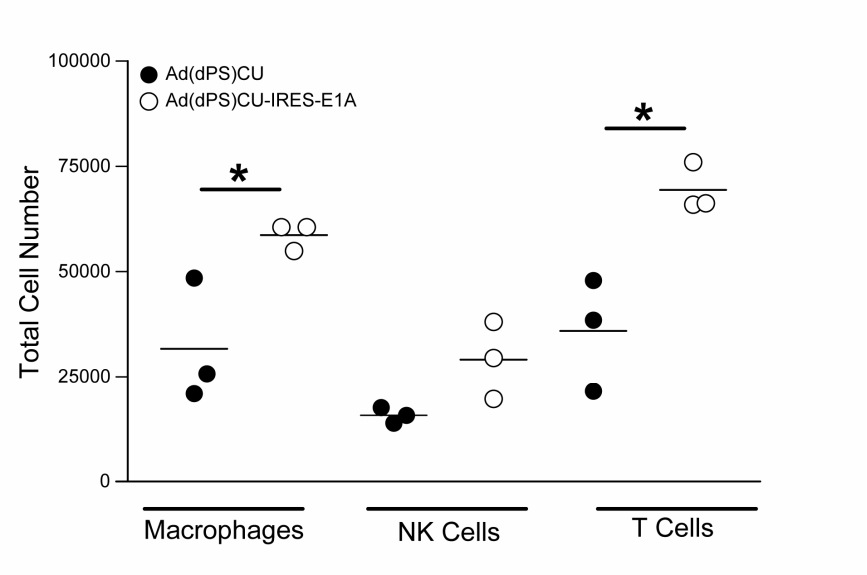


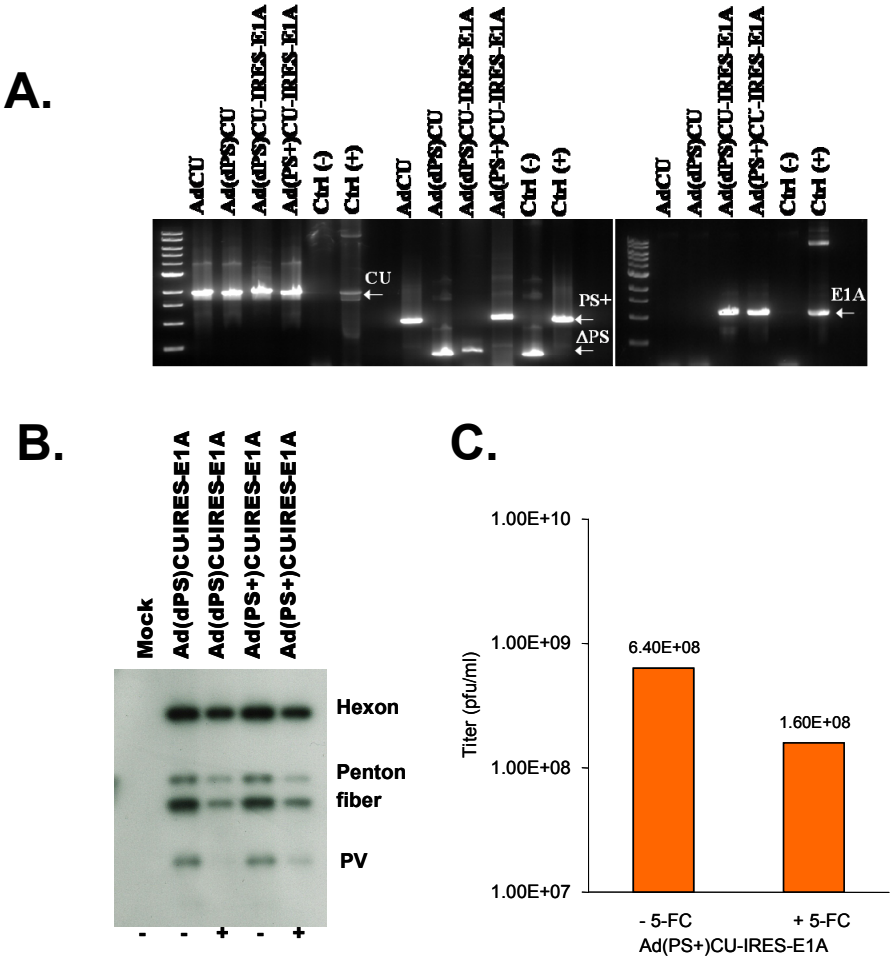

**Figure 23: Quantification of immune cells in the brain of tumour-bearing animals by flow cytometry analysis.**

C57Bl/6 mice were implanted with GL261 cells and 10 days later were injected with either Ad(dPS)CU or Ad(dPS)CU-IRES-E1A, followed the next day by 5-FC treatment. Ten days after viral recombinant injection, animals were euthanized and brain tissue was dissociated for immunostaining as described in Materials and Methods. Populations of immune cells (macrophages, NK cells, and T cells) were identified and quantified by flow cytometry. Statistical significance was determined by *t*-test (two-tailed); *, $P<0.05$.

| | $IC_{50}$ for 5-FU | MOI for 50% transduction | Intercellular transport with calcein* |
|---|---|---|---|
| U87 | 3 μM | 15 | good |
| U251 | 13 μM | 10 | good |
| GL261 | NA | >500 (500=1%) | NA |

*The procedure for intercellular transport analysis was described elsewhere (Bourbeau et al, 2004).

**Supplementary Table 1: Characteristics of the cell lines used in this study.**




**Supplementary Figure 4: AdV genotypic and phenotypic characterization.**

***A***, genomes were tested by PCR for the presence of the *CD::UPRT* gene, the *PS* gene deletion, and the *E1A* gene re-insertion. ***B***, viral protein expression was visualized in the U87 glioblastoma cells after infection with the replicating AdVs in the presence and absence of the pro-drug by Western blot analysis. ***C*,** titers of Ad(PS+)CU-IRES-E1A were evaluated in the presence and absence of 5-FC in 293 cells.

# Chapter 5: Gene Transfer of Cytokines

## AAV-Delivered IFN-β in Causes Regression and Prevents Implantation of Tumours in an Intracerebral Glioma Model

**PREAMBLE**

Interferon-β (IFN-β) has enormous potential as a therapeutic for cancer treatment. It has been shown to have anti-proliferative, immunomodulatory, and anti-angiogenic properties. Specifically, it has been shown to suppress MMP-9, bFGF, IL-8, and VEGF, which are factors involved in angiogenesis. Furthermore, it can promote apoptosis via TRAIL, Fas, and XAF-1. By augmenting CTLs, NKs, and DCs, IFN-β can also cause immunomodulation. Early clinical studies with recombinant interferon-beta delivered as a bolus in clinical trials have, however, resulted in disappointing results. The poor outcome is likely due to the relatively short half-life of the protein. Thus, we propose that intratumoural and peritumoural delivery of IFN-β using adeno-associated virus vector (rAAV) will allow for a constant local supply of this cytokine, which could significantly improve therapeutic outcome and possibly prevent tumour recurrence in patients that have already undergone surgical resection. To achieve our objective, we generated AAV vectors which effectively deliver human and murine interferon beta (AAV-hIFN-β and AAV-mIFN-β, respectively) and characterized their anti-tumour capacity. By examining whether AAV delivery of IFN-β could decrease glioma cell viability, reduce angiogenesis, augment the immune system, prevent tumour engraftment as well as prolong survival in an intracerebral xenograft model we ascertained the therapeutic potential of this form of gene therapy.

## RESULTS

### Description of AAV-IFN-β genome

The structures of the AAV vectors used in this study are presented in Figure 24A. The human- and the murine-IFN-β AAV vectors (AAV-hIFN-β and AAV-mIFN-β) consist of the same backbone. The rAAV-2 genome was deleted in its viral *rep* and *cap* genes and in place is the human- or murine-*IFN-β* with a CMV promoter and the SV40 polyA. Flanking the recombinant AAV genome are the inverted terminal repeats (ITRs).

Colony hybridization was used to detect ampicillin positive clones, and the structure of the genome was confirmed by restriction enzyme digestion as outlined in the Methods and Materials. Furthermore, real-time PCR was used to titer the AAV vectors with primers for the human- or murine-*IFN-β* transgene or for the *CMV* promoter in the case of the AAV-GFP vector control. Titers were determined from a standard curve set by serial dilutions of the plasmid vector containing the respective IFN-β transgene and are expressed as vector genomes (VG)/mL.

### Evaluation of IFN-β Production from AAV-IFN-β

To confirm that the AAV-IFN-β vectors lead to the production of IFN-β, ELISAs were performed using supernatants of AAV infected 293A cells. AAV-hIFN-β infection of 293A cells led to increased production of human IFN-β over a period of 5 days to 20300±467pg/mL (~2000U/mL) (Figure 24B). AAV-mIFN-β also led

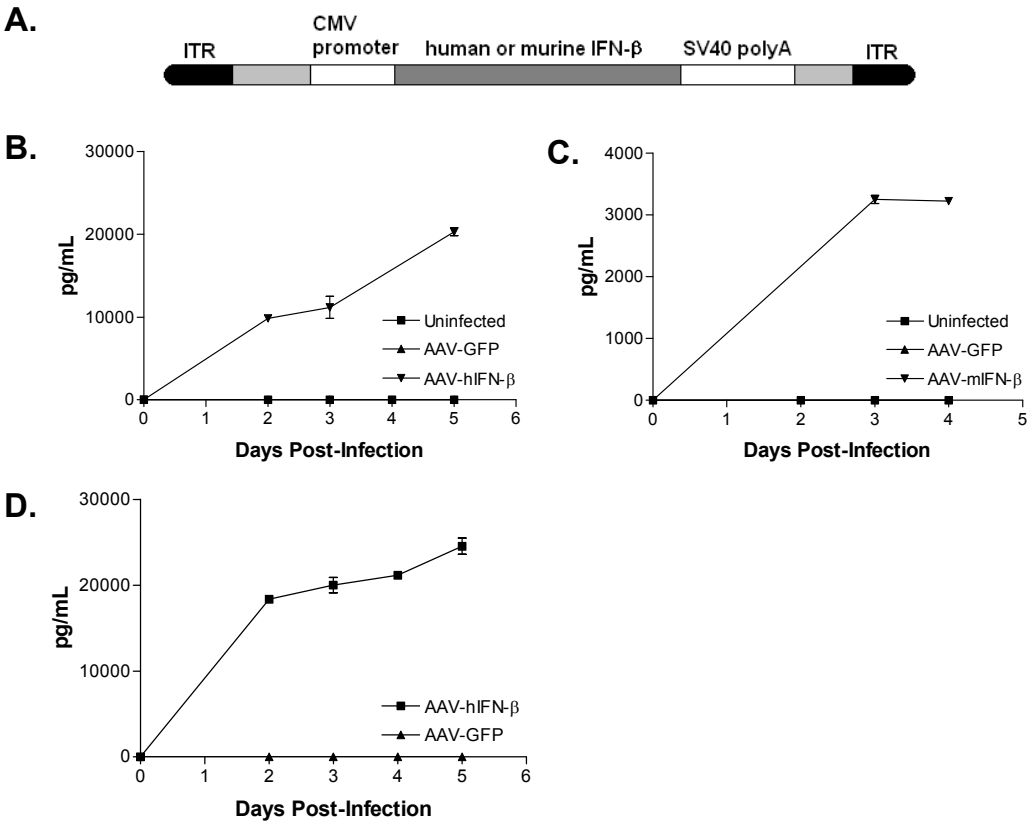



**Figure 24: AAV construct carrying the IFN-β transgene.**

***A***, depiction of the AAV-hIFN-β and AAV-mIFN-β vector genomes. The therapeutic transgene is driven by a CMV promoter, completed by an SV40 polyA, and flanked by AAV ITRs. ***B-D***, IFN-β protein production after infection with AAV virus at a MOI of 1000 or mock infected as measured by ELISA of the following: ***B***, human IFN-β from 293A supernatants; ***C***, murine IFN-β from 293A supernatants; ***D***, human IFN-β from U87 supernatants. ELISAs were performed twice. Note that the sensitivity of the assay differs between the human and murine IFN-β.

to the production of murine IFN-β that increased to 3224± 26pg/mL over a period of 4 days (Figure 24C). Uninfected cells and AAV-GFP infected cells did not yield any measurable levels of IFN-β (Figure 24A and B). Therefore, both AAV-mIFN-β and AAV-hIFN-β infection of 293A cells results in the production of murine and human IFN-β, respectively.

Next we determined if AAV-hIFN-β infection of U87 human glioma cells could produce human IFN-β given that *in* vivo intratumoural injections rely on the capacity of tumour cells to efficiently express the therapeutic transgene. Supernatants from AAV-hIFN-β infected U87 cells *in vitro* demonstrated that they are readily infected by AAV and infection over 5 days led to the production of 24548±927pg/mL of human IFN-β (Figure 24D), levels equivalent to those produced by the 293A cells. Mock-infected and AAV-GFP infected U87 cells did not produce any measurable levels of human IFN-β (Figure 24D). Subsequently, we wished to determine if intratumoural injection of AAV-hIFN-β into pre-established U87LacZ tumours in nude mice would result in an increase in human IFN-β mRNA production. Real-time-PCR performed on mRNA collected from homogenized brain samples 10 days post-infection demonstrated that AAV-hIFN-β successfully resulted in transcript levels of human IFN-β *in vivo* (2.63±1.13fg of hIFN-β mRNA/μg of total RNA), whereas levels were undetectable in untreated and AAV-GFP treated mice (n=2 per group). Taken together, the AAV vectors designed to deliver IFN-β increase expression and transcript levels of IFN-β in infected cells *in vitro* and *in vivo*.

**Cytotoxic Activity *In Vitro***

Since human IFN-β is known to induce apoptosis and cell cycle arrest by regulating a myriad of genes[28], we examined whether AAV-hIFN-β would result in a decrease in glioma cell viability. XTT assays were performed to assess this characteristic in two human GBM cell lines, U87 (p53 wild-type) and U251 (p53 mutant). Direct infection of U87 cells with AAV-hIFN-β led to a substantial decrease in cell viability compared to untreated control at both MOI of 100 and 1000 (Figure 25A) without any dose-dependent effects. The lack of dose-dependency with AAV-hIFN-β infection can be attributed to the known resistance of these cell types to IFN-β cytotoxic effects[316]. On the other hand, AAV-GFP led to a dose-dependent decline in viability, although AAV-hIFN-β still outperformed AAV-GFP at similar MOI. This indicates that AAV may have intrinsic cytotoxic properties.

In addition, there was a clear difference in sensitivity to AAV-hIFN-β infection between U87 and U251 cells. Direct infection with AAV-hIFN-β was not as effective in reducing viability in U251 cells as it was in U87 cells as viability only decreased to 47.3±2.2% at MOI of 1000 (Figure 25A, right) with no dose-dependent effects; whereas viability was decreased to 10.4±0.8% in U87 cells at MOI of 1000 (Figure 25A, left). AAV-GFP was also only mildly effective in reducing U251 cell viability. The reduced efficacy of direct infection in U251 compared to U87 may be related to transduction efficiency (determined by fluorescence microscopy of AAV-GFP infected cells), which is lower in U251

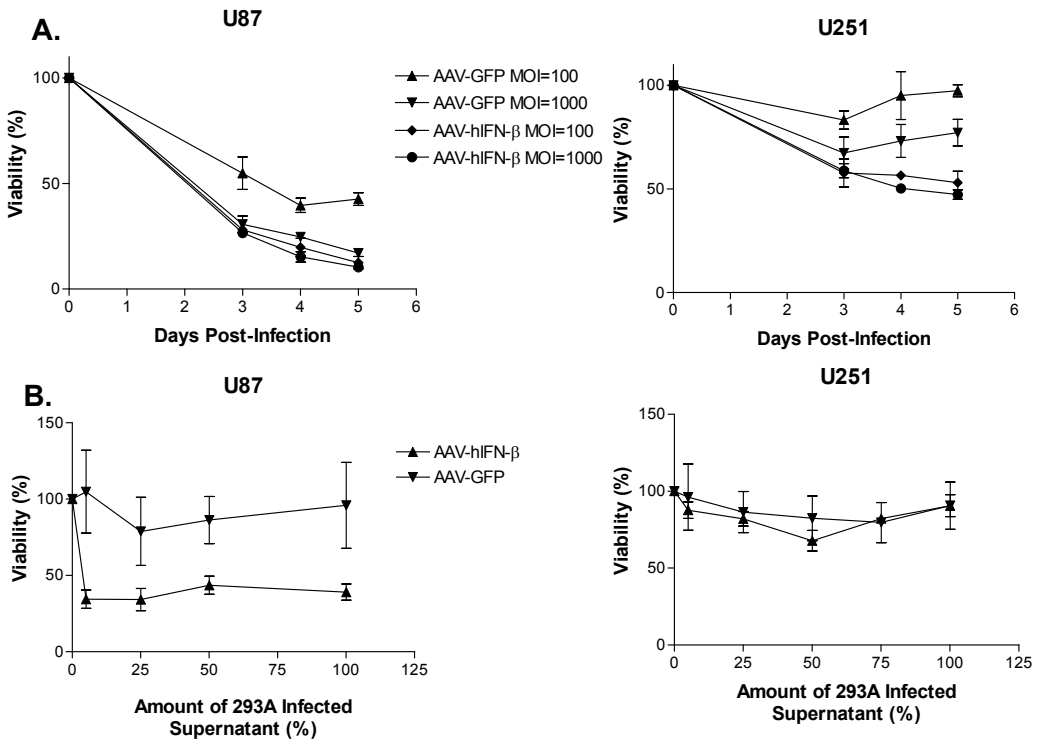



**Figure 25: Cytotoxic activity *in vitro.***

***A***, U87 and U251 human glioma cells were infected with either AAV-GFP or AAV-hIFN-β at different MOIs and viability was measured by XTT assay. Results are reported as a percentage of the viability of mock-infected control cells and were completed three times. For U87 cells, AAV-GFP vs. AAV-hIFN-β at MOI=100, $P<0.001$ and at MOI=1000, $P<0.05$. MOI=100 vs. MOI=1000 for U87 cells infected with AAV-GFP, $P<0.001$, and for AAV-hIFN-β infected, $P>0.05$. For U251 cells, AAV-GFP vs. AAV-hIFN-β at MOI=100, $P<0.001$ and at MOI=1000, $P<0.001$. MOI=100 vs. MOI=1000 for U251 cells infected with AAV-GFP, $P<0.01$, and for AAV-hIFN-β infected, $P>0.05$. Statistical significance determined on day 5 post-infection by one-way ANOVA followed by the Bonferroni post-test. ***B***, cell viability, as measured by XTT, of U87 and U251 cells after treatment with 293A supernatants for 48h. The supernatants were obtained previously from 293A cells infected for 48h with AAV-GFP or AAV-hIFN-β at an MOI of 1000 or mock-infected (performed three times).

cells compared to U87 cells (data not shown). Thus, direct infection by AAV can reduce viability *in vitro*, but the presence of IFN-β offers an advantage as AAV-hIFN-β delivered a greater cytotoxic effect than AAV-GFP in both U87 and U251 cells at similar MOI (Figure 25A).

IFN-β is a secreted cytokine which leads to paracrine or autocrine activation of signalling cascades. Thus, we investigated whether supernatants from infected 293A cells could also have an effect on glioma cell viability. Similar to the decreased viability obtained by direct infection, treatment with 100% supernatant from 293A cells infected with AAV-hIFN-β also lowered viability to 39.1±5.3% in U87 cells; in contrast, incubation with supernatants from AAV-GFP infected 293A cells did not lead to a similar decrease in viability. However, analogous to the curves obtained with the application of recombinant IFN-β[150,471], there was no dose-dependent effect with increasing amounts of supernatant. Moreover, supernatants from AAV-hIFN-β infected 293A cells did not decrease viability of the U251 cell line (Figure 25B, right).

Therefore, while there are differences in sensitivity between glioma cell lines, AAV-hIFN-β can decrease viability of human glioma cells through both direct infection of glioma cells and the application of its supernatants from infected 293A cells, but direct infection is much more effective than the application of supernatants. Additionally, the AAV-2 vector itself has cytotoxic effects.

**Effects of AAV-hIFN-β On Endothelial Cell Migration**

IFN-β has been shown to inhibit growth and migration of endothelial cells and suppress factors involved in angiogenesis[28]. To determine whether AAV-hIFN-β was capable of decreasing human brain endothelial cell (HBEC) migration we assayed migration of HBECs in response to chemotactic cues secreted by glioma cells. Briefly, this assay consisted of plating HBECs in the center of a cloning ring, which was surrounded by U251 glioma cells. The extracellular matrix secreted by U251 served as a chemotactic signal for the migration of HBECs. When the cloning ring was removed 48h later, a gap of ~100μm was created between the U251 cells and the HBECs. Cells were photographed and the distance between the U251 cells and HBECs was measured over time. Over a 96 hour period, the supernatant from AAV-hIFN-β infected 293A cells prevented migration of HBEC as the gap distance did not decrease, remaining the same distance as it was at time 0 (Figure 26A). On the other hand, the gap decreased when 293A supernatants from AAV-GFP and untreated cells were applied (Figure 26A). XTT studies indicated that viability was similar whether HBEC had been treated with supernatants obtained from 293A cells infected with AAV-hIFN-β or vector control (AAV-LacZ; Figure 26B). Therefore, AAV delivered hIFN-β in 293A supernatants decreased the migratory capacity of HBEC towards glioma cell matrix without inducing an anti-proliferative effect.

### *In Vivo* Assessment of AAV-hIFN-β Anti-tumour Capacity

Next, we investigated if AAV-hIFN-β had an effect *in vivo*. We used two protocols in our athymic mouse models. In the first model, athymic mice were injected intracerebrally with human glioma cells and were given a single

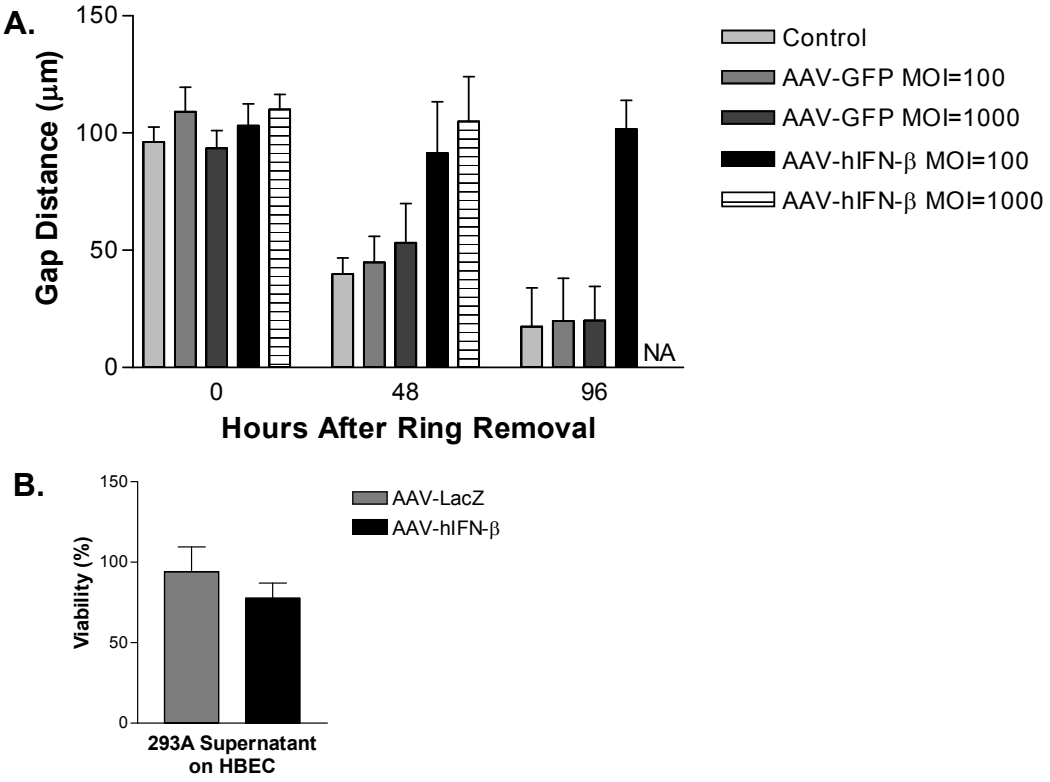



**Figure 26: HBEC migration and viability assays.**

***A***, HBECs were plated in the centre of a 24-well dish separated by a 100µm circular barrier from surrounding U251 cells. 48h after plating the barrier was removed and 293A supernatants from cells infected with AAV-GFP, AAV-hIFN-β at various MOI, or mock-infected control cells were applied to the HBEC and U251 co-culture. The gap distance is the space between U251 cells and HBECs. This experiment was repeated twice. ***B***, viability of HBECs (measured by XTT assay) 48h after the application of 293A supernatants from cells infected with AAV-LacZ or AAV-hIFN-β and repeated twice; $P$=0.3740 by two-tailed $t$-test.

intratumoural injection of AAV-hIFN-β ten days later to reproduce the clinical treatment of a pre-established tumour. In the second paradigm, athymic mice were injected with AAV-hIFN-β ten days prior to tumour implantation to institute IFN-β expression peritumourally. The second paradigm serves to mimic a treatment which could serve to prevent tumour recurrence following post-surgical resection. To determine if AAV treatment was capable of causing tumour regression, mice were sacrificed 40 days after U87LacZ tumour implantation and the volume calculated from sequential tumour sections. In both *in vivo* models, mice that were treated with AAV-hIFN-β had much smaller tumours than untreated or AAV-GFP treated animals (Figure 27A and B) indicating that the recombinant AAV-hIFN-β was capable of causing tumour regression (Figure 27A) and preventing tumour engraftment (Figure 27B). In fact, in some cases in both *in vivo* models, no tumour was visible at 40 days (Figure 27A and B).

Implantation with U251N produced similar results (Figure 27C); control untreated animals had significantly larger tumours than mice implanted with U251N followed by AAV-hIFN-β infection. In mice with pre-established U251N tumours, AAV-hIFN-β treatment yielded significantly smaller tumours than AAV-GFP treatment. AAV-hIFN-β pre-treatment, however, did not significantly decrease tumour volume in comparison to control, although two out of the five mice did not have a visible tumour. Nonetheless, these results are consistent with *in vitro* cell viability data that show that in U87 cells both AAV-hIFN-β infection of tumour cells and secreted IFN-β from infected cells decreased U87 cell viability. Also coherent with *in vitro* results in U251N cells, only direct infection,

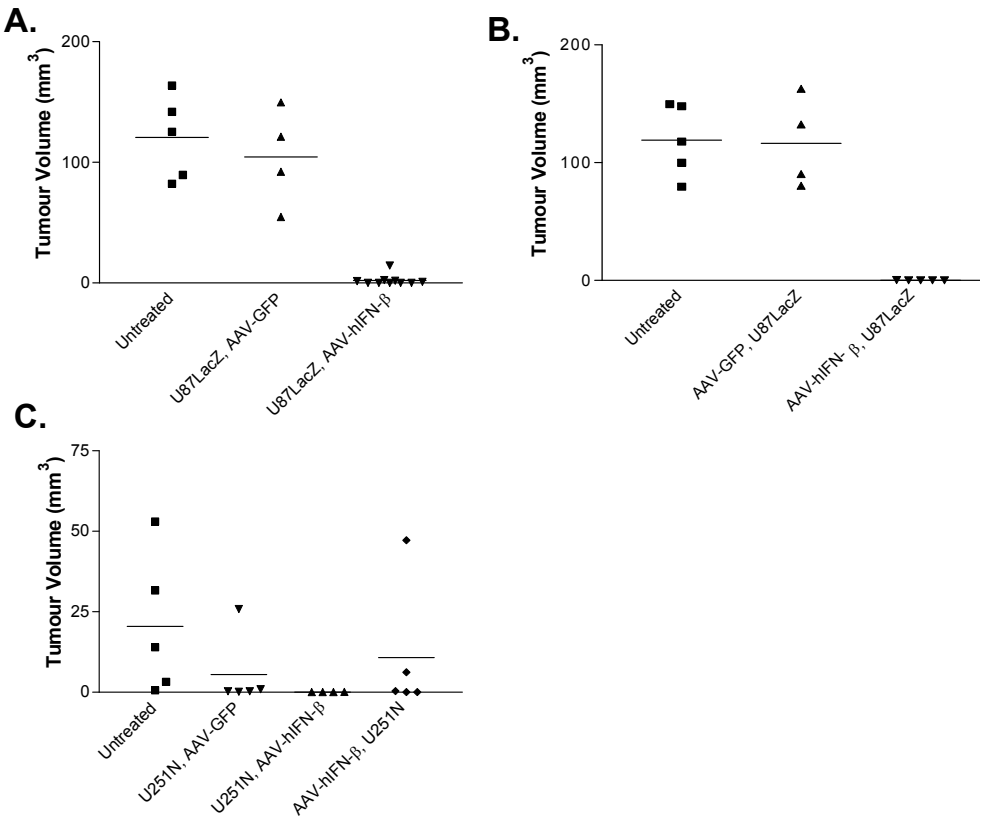
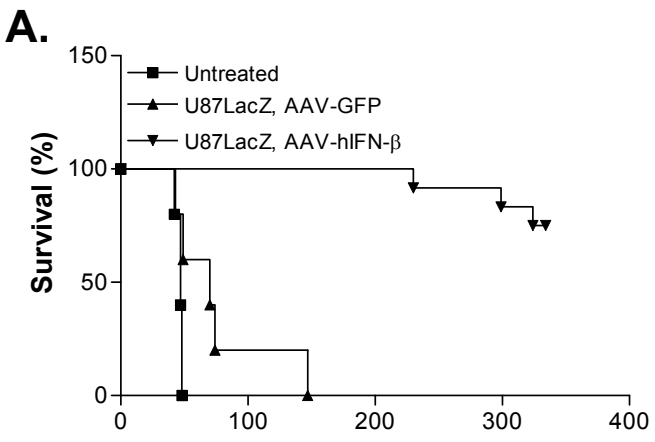



**Figure 27: *In vivo* tumour volume.**

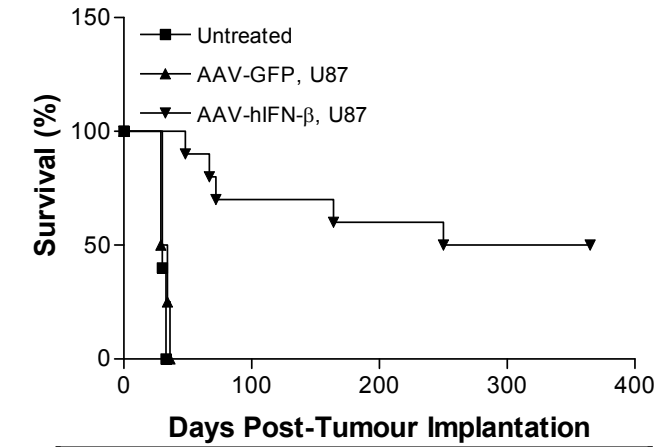
Intracerebral tumour volume of mice (calculated using sequential brain sections) treated with AAV-hIFN-β ($6.6x10^7$VG), AAV-GFP ($1.74x10^8$VG) or left untreated was determined 40 days post-tumour implantation for mice treated either (***A***) 10 days following implantation of U87LacZ tumour cells, or (***B***) 10 days prior to implantation of U87LacZ cells. In both (A) and (B), $P<0.001$ for all comparisons with AAV-hIFN-β treated mice vs. AAV-GFP or untreated mice. Statistical significance was determined by one-way ANOVA followed by the Bonferroni post-test. ***C***, shows the intracerebral tumour volume of mice 35 days after U251N tumour implantation. Whether AAV or U251N cells were given first with a 10-day interval is indicated. For mice with pre-established U251N tumours, AAV-hIFN-β treated animals had significantly smaller tumours than control ($P<0.05$ by one-way ANOVA followed by the Kruskal-Wallis post- test). Mice with pre-established U251N tumours and treated with AAV-hIFN-β also had smaller tumours than mice treated with AAV-GFP ($P=0.0159$ by the Mann-Whitney test).

or intratumoural injection of virus could decrease cell viability or tumour volume. On the other hand, AAV-GFP was unable to significantly decrease tumour volume in either xenograft model, although it was able to decrease cell viability *in vitro*.

To assess whether the capacity for AAV-hIFN-β to reduce tumour volume translated into improved survival we performed one-year-long survival analyses. Mice that were injected intratumourally with AAV-hIFN-β after tumour implantation had a much longer survival than mice left untreated or injected with AAV-GFP (Figure 28A). In fact, 75% of the animals treated with AAV-hIFN-β were cured, with cure rate defined as the survival proportion at 365 days post-tumour implantation when the experiment was terminated, whereas untreated mice had a median survival of 47 days, and AAV-GFP treated mice had a median survival of 70 days (Figure 28A). Mice that were injected with virus prior to tumour implantation also showed an improvement in survival compared to uninfected and AAV-GFP treated animals (Figure 28B). Untreated animals and AAV-GFP treated animals had median survivals of 30d and 31.5d, respectively, whereas AAV-hIFN-β treated animals had a median survival of 307.5 days and 50% of the animals were tumour-free at 365 days post-tumour implantation. Mice that died had large tumours and those that survived to 365 days were healthy exhibiting normal grooming and feeding behaviour. These results are in agreement with the outcome obtained in the tumour volume study and suggest that AAV-hIFN-β has robust anti-tumoural properties.




| Treatment | n | Median (days) |
|---|---|---|
| Untreated | 5 | 47 |
| AAV-GFP | 5 | 70 |
| AAV-hIFN-β | 12 | >365 (75% cure) |




| Treatment | n | Median (days) |
|---|---|---|
| Untreated | 5 | 30 |
| AAV-GFP | 4 | 31.5 |
| AAV-hIFN-β | 10 | 307.5 (50% cure) |

**Figure 28: Kaplan Meier survival curves.**

Survival of mice treated with AAV-hIFN-β, AAV-GFP or left untreated was assessed by Kaplan Meier survival curves for mice injected either (***A***) 10 days following U87LacZ tumour cell implantation, or (***B***) 10 days before U87 tumour implantation. Cure rate is defined as the survival percentage at 365 days post-tumour implantation when the experiment was terminated.

**Mechanisms of the *In Vivo* Anti-Tumour Outcome**

Since AAV-hIFN-β decreased glioma cell viability and prevented HBEC migration *in vitro*, we determined whether these mechanisms could have contributed to the tumour regression and extended survival observed *in vivo*. To examine apoptosis *in vivo,* mice were sacrificed 40 days post-tumour implantation and brain sections were stained by terminal transferase dUTP nick end labelling (TUNEL). Mice with pre-established tumours treated with AAV-hIFN-β had a higher number of TUNEL positive cells per 400X field compared to untreated animals (Figure 29A). Therefore, these data suggest that one mechanism whereby AAV-hIFN-β causes tumour regression *in vivo* is by inducing apoptosis in human glioma cells.

To investigate the anti-angiogenic effects of IFN-β, we examined blood vessel density by staining for CD31 in brain sections with the greatest cross-section of tumour. AAV-hIFN-β did not induce any significant change in blood vessel density compared to control indicating that the apoptotic capacity of AAV-hIFN-β is much more important in its ability to induce tumour regression in the mouse xenograft model. In contrast, when mice were treated before tumour implantation with AAV-mIFN-β, they had a lower density of CD31-positive blood vessels compared to untreated animals (Figure 29B) and compared to animals treated intratumourally with AAV-hIFN-β (Figure 29B). Thus, AAV-mIFN-β has a species selective anti-angiogenic effect on mouse brain blood vessels suggesting that AAV-hIFN-β might also have an effect on HBECs in human patients.

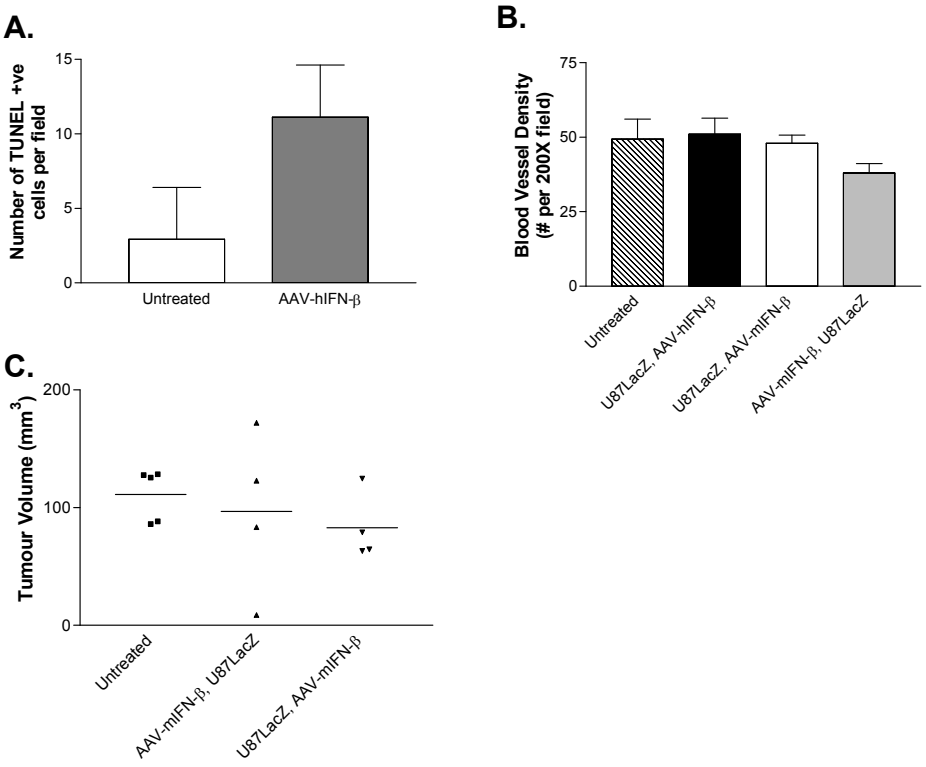



**Figure 29: *In vivo* anti-tumour mechanism.**

***A***, quantification of TUNEL staining of mouse brains with pre-established tumours treated with AAV-hIFN-β or untreated; $P$=0.0004 by two-tailed $t$-test. Pictures were taken at 400X and the number of TUNEL positive nuclei per field in the section with the greatest tumour longitudinal was recorded. Untreated mice, n=9; AAV-hIFN-β treated mice, n=7. ***B***, quantification of CD31-positive blood vessels within the tumours of mice implanted with U87LacZ either before or after treatment with AAV-hIFN-β, AAV-mIFN-β, AAV-GFP, or without treatment. Pictures were taken at 200X and the number of CD31 positive blood vessels documented. Untreated, n=7; U87LacZ, AAV-hIFN-β, n=2; U87LacZ, AAV-mIFN-β, n=4; AAV-mIFN-β, U87LacZ, n=4. Mice treated with AAV-mIFN-β prior to U87LacZ implantation had less CD31 positive blood vessels compared to untreated animals ($P$<0.01) and animals treated intratumourally with AAV-hIFN-β ($P$<0.05). Significance determined by one-way ANOVA followed by the Bonferroni post-test. **C**, tumour volume of mice treated with AAV-mIFN-β, or untreated, either before of after U87LacZ tumour implantation. Mice were sacrificed 40d post-tumour implantation.

In order to evaluate the strength of the anti-angiogenic and anti-proliferative effect of AAV-mIFN-β on human glioma xenografts, the tumour volume at 40d post-tumour implantation was examined in mice that were treated either before or after tumour implantation with AAV-mIFN-β. Treatment with AAV-mIFN-β in either scenario did not lead to a significant difference in tumour volume compared to untreated controls (Figure 29C). This indicates that anti-angiogenesis alone is not sufficient to induce tumour regression in this model; nonetheless, anti-angiogenesis may still contribute to the anti-tumour properties of AAV-hIFN-β in human trials.

**Immunostimulatory Effects of AAV-IFN-β**

IFN-β is a well known immunomodulatory cytokine, and it was important to determine if the recombinant AAV-IFN-β could also stimulate the immune system to aid in tumour clearing. We used a syngeneic intracerebral glioma model in which C57Bl/6 wild-type mice were pre-treated with AAV and implanted 10 days later with GL261 murine glioma cells to assess for immune infiltrates one week following tumour implantation. Total cell numbers of microglia, macrophages, T cells and NK cells were collected by flow cytometry from homogenized whole brain. AAV-mIFN-β led to a moderate increase in NK cells compared to animals with untreated tumours (Figure 30). In contrast, AAV-hIFN-β did not increase any of the immune infiltrates tested compared to animals with untreated tumours suggesting the mouse immune system may not respond to human IFN-β, neither as a cytokine nor as a foreign protein; at the same time, this data demonstrates that AAV itself does not mount a substantial immune response.

However, mice treated with AAV-GFP had increased levels of macrophages and NK cells compared to animals with untreated tumours suggesting the foreign GFP protein elicits an immune response. Thus, the immune system may have a modest role in aiding tumour clearing in the brain when treated with AAV-IFN-β in a manner that is species specific.

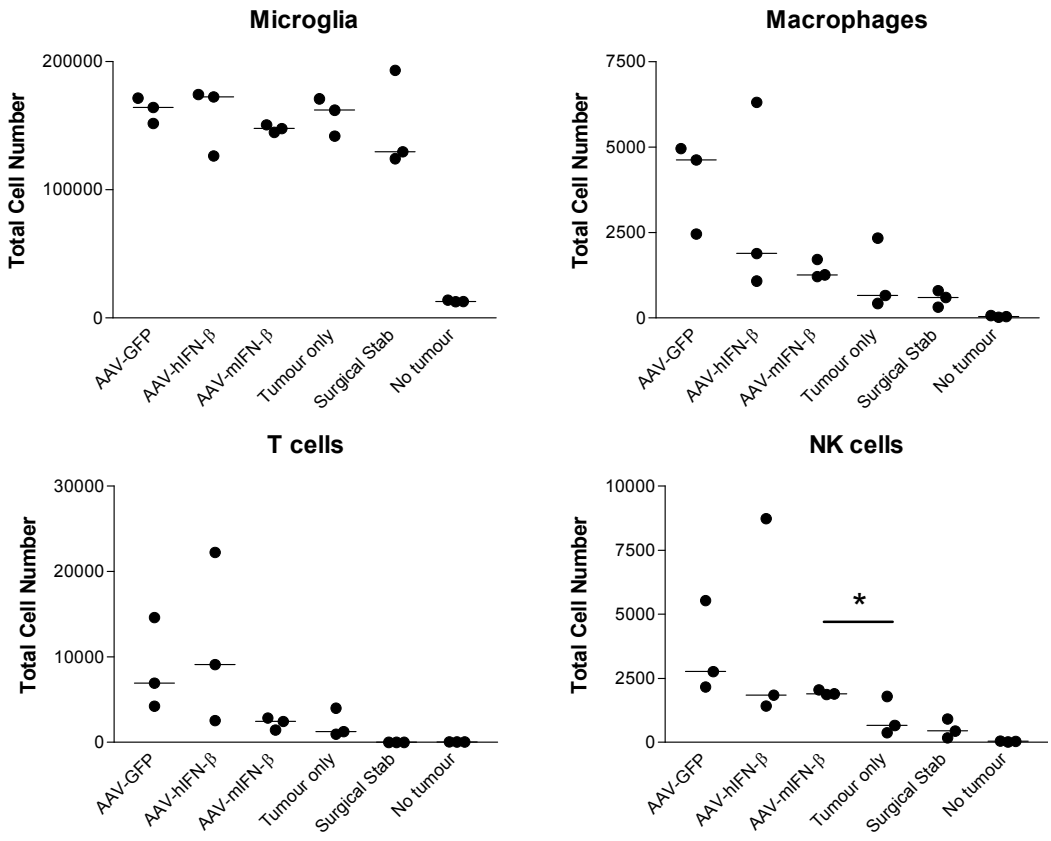



**Figure 30: Quantification of immune cells in the brain of tumour-bearing animals by flow cytometry analysis.**

Using a syngeneic mouse model, levels of immune infiltrates were determined in homogenized brain tissue 7 days post-tumour implantation by flow cytometry. Wild-type C57Bl/6 mice were treated 10 days prior to GL261 murine glioma implantation with AAV-GFP, AAV-hIFN-β, AAV-mIFN-β, or left untreated. Mice that were administered surgical stabs received them at the same time interval had they been injected with virus and implanted with tumour, although, no agents were delivered. Mice listed as having "no tumour" were also not treated with virus. AAV-mIFN-β lead to a moderate increase in NK cells compared to mice with untreated tumours (*, $P$=0.0471). AAV-GFP increased levels of macrophages ($P$=0.0220) and NK cells ($P$=0.0433) compared to mice with untreated tumours. Statistical significance was determined by one-tailed $t$-test.

# Chapter 6: Discussion

GBM continues to elude curative solutions with conventional therapy. Malignant gliomas are difficult to resect due to the lack of a defined tumour edge that invades into normal brain tissue and growth near critical areas of the brain. Radiation treatment is often performed following surgical resection to kill residual tumour cells, or if the tumour is in a non-operable region of the brain, to relieve symptoms associated with the disease. However, the radiation dose that can be provided is limited due to the sensitivity of normal brain tissue and the given dose is often insufficient to kill GBM cells; furthermore, GBM are commonly radio-resistant. Chemotherapy can be used as a primary therapy or used as an adjuvant following surgery and radiation. As well, it can be used as palliative treatment for patients who have failed prior therapy, or used in combination with radiation as a sensitizer. However, GBM tend to be chemo-resistant or gradually develop resistance in spite of new chemotherapeutic agents, new drug combinations, and new delivery methods. Moreover, systemic and CNS toxicity causes serious long term side effects for patients[107].

Here, we explored several new therapeutic modalities based on gene therapy to address some of the difficulties associated with conventional therapy while also investigating ways to improve upon current gene therapy strategies. Issues regarding resistance to radiation and apoptosis could be confronted with AdFOXO1;AAA to target disrupted signalling pathways in gliomas implicated in radioresistance and defects in apoptosis. Moreover, chemoresistance, systemic toxicity, low bioavailability due to the BBB, and poor transduction efficiency could be alleviated through the use of the replicating/non-disseminating vector,

Ad(dPS)CU-IRES-E1A, plus 5-FC. At the same time, this enzyme/pro-durg combination also provides a more generalized killing mechanism than targeted therapy with AdFOXO1;AAA. Finally, we show that AAV-IFN-β could be used as a possible adjuvant to surgical resection to prevent tumour recurrence and could also be used to regress non-operable tumours. These three gene therapy strategies and their capacity to improve survival in murine tumour models will be discussed.

*Justification for the use of xenograft models*

Without a doubt, murine tumour models have their limitations and the ultimate test relies on human clinical trials, but the application of a suitable animal model is necessary for the development of new therapeutic approaches. The ideal glioma mouse model should have the following characteristics: (1) predictable and reproducible *in vitro* and *in vivo* growth patterns; (2) infiltrating but non-metastatic progression; (3) and poor immunogenicity. U87 and U251N intracerebral xenograft models were chosen in these experiments for multiple reasons. Firstly, xenograft models allow for the assessment of *in vitro* parameters *in vivo*. Secondly, U87 and U251N have a high degree of tumour take (near 100%) and grow well unlike the T98G, SF-767, and SF-126 xenograft models that have low tumour takes and grow very slowly[354,409]. Furthermore, the use of an athymic mouse reduces the possibility for spontaneous regression as a result of immune reaction, which is observed with C6 rat glioma models[13]. However, U87 and U251 mouse models do have their restrictions; for instance, U87 xenografts do not exhibit the invasiveness of high-grade gliomas, although U251N

xenografts do. Furthermore, while U251N and U87LacZ originated from human malignant glioma tumours and retain many of the original characteristics, it cannot be ruled out that the multiple passaging of these cells may have altered their genetic profile[93].

Our use of an intracerebral implantation model versus a flank implantation model also adds to the stringency of our experiments. Gene expression profiles of glioma tumours grown under s.c. flank xenografts, intracerebral xenografts, and cells grown *in vitro* were shown to be all different [51], clearly indicating that the brain provides a unique environment that cannot be recapitulated s.c.. Reports have also found that glioma tumours implanted in the flank exhibit decidedly different growth parameters[355]. Furthermore, intracerebral models can mimic the actual environment of a glioma tumour more closely[253] because issues important to drug delivery and adenoviral infection such as the BBB and the unique immune environment of the brain can be assessed. Therefore, the tumour models used in this study are a fair indicator of the ability of various gene therapy strategies to eradicate tumours and extend survival in the clinical setting.

**Targeted Gene Therapy: AdFOXO1;AAA**

AdFOXO1;AAA holds potential as a therapeutic adjuvant for the treatment of one of the deadliest cancers, by targeting the frequently disrupted growth-factor-RTK-PI3K-Akt pathway. Hyperactivity in this pathway leads to excessive cell proliferation, migration, and resistance to apoptosis and cell cycle arrest. By using an adenoviral vector to express a FOXO1 mutant transcription factor that cannot

be negatively regulated by Akt phosphorylation, we were able to restore apoptosis and cell cycle arrest *in vitro* in glioma cells lines in a dose-dependent manner, reduce *in vivo* tumour volume and prolong survival in an intracerebral mouse model with a pre-established glioma xenograft tumour.

*Mechanism of AdFOXO1;AAA-mediated apoptosis and cell cycle arrest*

AdFOXO1;AAA mediated apoptosis was related to an increase in Bim expression followed by caspase-9 activation, but did not appear to involve an upregulation of FasL (Figure 12A). Correspondingly, cell cycle arrest was associated with a downregulation of cyclin D1 and D2 along with an increase p27 (Figure 12A). Both mechanisms of cell cycle arrest and apoptosis are consistent with the literature and support the direct transactivation of the *Bim*[90,156] and *p27*[451] promoter by FOXO1;AAA, and the DNA-binding-independent repressor activity of FOXO1;AAA on cyclin D1 and D2 expression[383].

The effects of AdFOXO1;AAA on U87 and U251 cell cycle arrest and apoptosis follow a course of action that is similar to the mitogen starvation response seen in mouse embryonic fibroblasts lacking the complete retinoblastoma gene family (*Rb*-, *p107*-, and *p130*-null; *TKO* MEFs)[125]. FOXO1 protein is normally activated in starved cells as a part of its role as a downstream effector of the insulin signalling pathway[325]; therefore, it is not unusual that over-expression of FOXO1;AAA would result in a response that is comparable to starvation. Moreover, U87 and U251 cells are similar to the *TKO* MEFs in the sense that the Rb signalling pathway in these cells is also defective due to deletions in p15, p16,

and p19 cell cycle inhibitors. Starved *TKO* MEFs cells can complete S phase, but are blocked from completing the cell cycle by two mechanisms: (1) the majority undergo apoptosis[81,124,125,414]; while (2) surviving cells arrest in the G2 phase of the cell cycle[125]. *TKO* MEFs fail to undergo G1 arrest due to mitogen-deprivation because this action is critically dependent on the Rb tumour suppressor family (Rb, p107, and p130)[81,414]. Mitogen-starvation-induced G2 arrest is mediated by upregulation of the cell cycle inhibitors p21 and p27 (high levels of p27 are sufficient) that act as potent inhibitors[124] of cyclin A- and cyclin B1-associated kinase activity, which are essential for entry into mitosis[338]. FOXO1;AAA overexpression as a result of AdFOXO1;AAA infection also leads to high levels of p27, resulting in cell cycle arrest in the G2 phase as opposed to the G1 phase (Figure 11). The apoptosis observed in the *TKO* MEFs could also be traced to Bim; *TKO*-Bcl2 MEFs that overexpress Bcl2, a Bim binding partner, are resistant to mitogen-induced apoptosis[125]. Therefore, the absence of a functional Rb pathway along with p27 expression offers an explanation for the G2 arrest observed in AdFOXO1;AAA infected cells and the expression of Bim likely contributed to apoptosis by sequestering Bcl2.

*Differences in sensitivity to AdFOXO1;AAA-induced apoptosis*

However, we found that not all PTEN null human glioma cells are equally sensitive to AdFOXO1;AAA-induced apoptosis. U251 human glioma cells are much more sensitive to the dose-dependent apoptotic effects of FOXO1;AAA than U87 cells (Figure 10A). This is also reflected in Figure 12A, where in U87 cells at 72h, cyclin D1 and D2 expression returned and Bim expression was not

greatly upregulated; in contrast, D-type cyclin expression did not return in U251 cells, and there was an increase in Bim and cleaved caspase-9 expression. Likewise, infection with AdPTEN results in anoikis (apoptosis of cells following loss of contact with the extracellular matrix) in U251 cells[82] and G1 cell cycle arrest in U87 cells[142,277].

The effects of AdFOXO1;AAA were far more apparent *in vivo*. For instance, the relatively lower capacity for AdFOXO1;AAA to induce apoptosis in U87 cells compared to U251 cells, resulted in an inability to significantly improve survival in U87LacZ xenografts (Figure 13B), although short-term tumour regression was observed (Figure 13A). In contrast, tumour eradication and prolongation of survival with a cure rate of 33.3% was achieved in the U251N xenografts (Figure 13B), which can be attributed to the greater capacity of AdFOXO1;AAA to induce cell death in U251 cells. More than 90% of U251 cells were killed *in vitro* with AdFOXO1;AAA treatment using a MOI as low as 30 (Figure 10A) whereas only ~40% of U87 cells infected at MOI of 100 underwent cell death. This indicates that the successful induction of apoptosis is paramount for prolongation of survival. Thus, in certain tumour types, AdFOXO1;AAA could be successful as a therapeutic agent.

*Why is there a difference in sensitivity?*

Several discernible differences in the regulation of FOXO1 protein between U87 and U251 cells may account for the increased capacity of FOXO1;AAA to induce

apoptosis in U251 cells and the resistance of U87 cells; these will be considered below.

FOXO1 protein is mainly regulated via changes in protein-interactions, subcellular localization, protein stability, and transcriptional activity[491,500]. At a genetic level, the two cell lines tested differ in their status of a very important tumour suppressor, p53; as mentioned earlier, U87 cells are p53 wild-type, whereas U251 are p53 mutant. Wild-type FOXO3 protein has been shown to interact with the p53 protein, and increase the half-life of p53 in conditions that result in nuclear localization of FOXO protein such as stress or nutritional deprivation[336]. It can be argued that the interaction of p53 and FOXO1;AAA may sequester FOXO1;AAA proteins in U87 cells preventing their full activation, or they may co-operate to induce cell cycle arrest in this particular cell type by boosting both p21 and p27 expression. In another instance, the p53 tumour suppressor has been shown to indirectly inhibit FOXO function by inducing the protein kinase SGK, which results in FOXO3 phosphorylation and subsequent relocalization from the nucleus to the cytoplasm[546]. However, the effects of SGK on FOXO3 function is dependent on Thr-32 phosphorylation[546] and the equivalent residue in FOXO1, Thr-24, is altered in our phosphosite mutant[491] negating the possibility for SGK regulation of FOXO1. Nonetheless, consistent with our results, adenoviral delivery of a triple phosphosite mutant of FOXO3 has been shown to induce apoptosis to a higher extent in a p53 mutant melanoma cell line compared to a p53 wild-type melanoma cell line[165].

However, there is evidence to suggest that the p53 status of the cell line may not be an indicator of susceptibility to FOXO factor induced death. For instance, in a study that compared the effect of FOXO3 in p53 mutant and p53 wild-type MEF cells[547], both cell lines underwent apoptosis in response to FOXO3 induction, and the wild-type p53 cell line actually had a greater proportion of apoptotic cells than the p53 mutant cell line. But, this study also showed that the presence of p53 protein, regardless if it was wild-type or mutant, was required for FOXO3 mediated death as p53-null MEFs were resistant[547]. These findings offer an explanation for why FOXO1;AAA had induced some degree of apoptosis in both U87 and U251 cells, but do not indicate why one cell type may be more susceptible to FOXO1;AAA-induced apoptosis. Future studies involving a larger panel of glioma cell types including primary human glioma samples may provide a more definitive answer regarding the importance of p53 status in determining susceptibility to AdFOXO1;AAA-induced apoptosis. Furthermore, until more FOXO1 protein partners are discovered, the significance of p53 in FOXO1 mediated cell death will be largely unknown underscoring the complexity of FOXO1 regulation.

*Differences in subcellular localization*

The subcellular location of FOXO1;AAA protein may offer insight into the varying response to AdFOXO1;AAA infection. In order for FOXO1 to perform its transcriptional regulatory functions, the protein must be present in the nucleus. Transduction efficiencies in U87 and U251 cells were relatively similar, but the subcellular localization of the FOXO1 protein was very different between the two

cell lines. In U251 cells, the FOXO1;AAA protein was found almost exclusively in the nucleus (Figure 9B). On the other hand, the FOXO1;AAA protein was found in both the nucleus and the cytoplasm in U87 cells at 48h post-infection indicating that less FOXO1;AAA proteins were engaged in transcriptional activity than in U251 cells. Based on the observation that higher MOIs are associated with higher levels of protein and higher levels of apoptosis (Figure 10), if less protein is available to transactivate genes in U87, this may provide one explanation for the attenuated capacity of AdFOXO1;AAA to induce apoptosis in U87 cells. Thus, high levels of FOXO1;AAA in the nucleus may signal towards apoptosis and lower levels towards cell cycle arrest.

However, it is difficult to determine why FOXO1;AAA was found in the cytoplasm of U87 cells as this mutant is considered by many to be constitutively nuclear[39,130,329,383]. Subcellular localization of wild-type FOXO1 is controlled primarily by phosphorylation that is associated with nuclear export into the cytoplasm and subsequent repression of transcriptional activity[170,491,555]. Akt phosphorylation in the conserved forkhead DNA binding domain (S256) leads to disruption of the nuclear localization signal and DNA binding, as well it exposes T24 and S319 to Akt phosphorylation that facilitates its nuclear export. In addition to Akt, wild-type FOXO1 is also phosphorylated by other protein kinases, such as SGK[40], CK1[123], and DYRK1A[393,529] (Figure 31). Activation of Akt, and/or SGK, CK1 and DYRK1A leads to phosphorylation of a stretch of four serine residues in FOXO1 which form an acidic patch inducing high affinity binding of 14-3-3 proteins, thereby assisting nuclear export[394,491]. FOXO1;AAA,

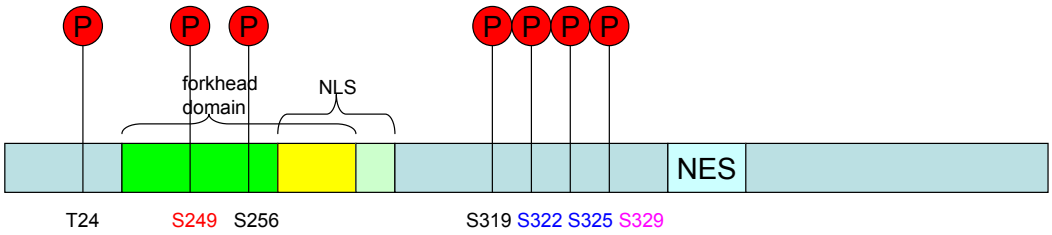



**Figure 31: FOXO1 phosphorylation sites.**

The residues that are phosphorylated by the following kinases are indicated. Black=Akt and SGK sites, blue=CK1, magenta=DYRK, and red=CDK2. The Akt sites are altered to alanine residues in FOXO1;AAA and cannot be phosphorylated.

however, cannot be phosphorylated by Akt, so its regulation differs from that of wild-type FOXO1. First of all, SGK cannot phosphorylate the FOXO1;AAA mutant protein because SGK phosphorylates the same substrate motifs sites as Akt[40]. Secondly, CK1 requires the presence of a motif that is already phosphorylated by Akt, namely the third C-terminal site, in order for CK1 to phosphorylate its two other sites[393]. The DYRK1A site appears to be constitutively phosphorylated and regulation of this site is independent of Akt and CK1 activity[393]. Mutation of the DYRK1A site from serine to alanine does result in increased nuclear localization[529], but since this site is unaltered in FOXO1;AAA the phosphorylation state at this site should not be different between the wild-type and phosphosite mutant forms of FOXO1. Therefore, subcellular localization of FOXO1;AAA may be controlled in a fashion that is Akt phosphorylation site independent or phosphorylation independent altogether.

A recent study showed that CDK2 can regulate FOXO1 by phosphorylation on Ser 249, which is unaltered in our phosphosite mutant[210]. Phosphorylation on S249 leads to cytoplasmic localization and decreased activity in both wild type and FOXO1;AAA in a p53-independent fashion, as measured by target gene expression[210]. CDK2 may be more active in U87 cells leading to higher levels of cytoplasmic FOXO1;AAA protein. Given that increased EGFR activity in glioma cells results in higher levels of cell cycle proteins such as CDK2[331] and that EGFR mRNA expression is much higher in U87 than in U251 cells[341], this suggests that the autocrine stimulatory loop may be much greater in U87 cells leading to higher levels of cell cycle proteins. However, other studies have shown that EGFR

protein expression is greater in U251 cells than in U87 cells[453], thereby opposing this suggestion. Alternatively, FOXO1;AAA has been shown using microarray to strongly downregulate cyclin E2 expression[383], which is a regulator of CDK2 activity and is often deregulated in cancer[447]. If less FOXO1;AAA is present in the nucleus in U87 than in U251 cells, then it is probable that levels of cyclin E are higher in U87 cells contributing to increased CDK2 activity resulting in further sequestration of FOXO1;AAA into the cytoplasm and resistance to apoptosis. Still, it is not known if cyclin E levels are higher in U87 than in U251 cells. Future experiments will address levels of CDK2 and cyclin E in AdFOXO1;AAA infected cells to determine their significance in altering FOXO1;AAA subcellular localization.

On the other hand, the increased cytoplasmic FOXO1;AAA protein in U87 cells may be phosphorylation independent. The nuclear export of FOXO proteins is dramatically accelerated by phosphorylation[222,391,392], but the binding of the export receptor, Crm1 to FOXO proteins is not phosphorylation-state dependent[37]. It is unknown whether Crm1 is more active in U87 cells which would also result in higher levels of cytoplasmic protein. All in all, it is difficult to determine the reason for the increased cytoplasmic localization in U87 cells compared to U251 cells as the regulation of FOXO1;AAA protein is still being elucidated.

*Variations in FOXO1;AAA degradation and/or proteolytic cleavage bands*

Importantly, though, is that inhibition of FOXO1 transcription is not solely due to nuclear exclusion[482]. FOXO1 is also controlled through alterations in protein

stability by proteolysis and proteosomal degradation. FOXO1;AAA, however, is unable to associate with the ubiquitin ligase, Skp2, and displays a loss in ubiquitination and proteosomal degradation[211,299]. FOXO ubiquitination and consequent degradation is dependent on the phosphorylation of S256[211], which is altered in the mutant used in this study. Thus, proteolysis, rather than proteosomal degradation, is a post-translational control that could possibly contribute to the attenuated FOXO1;AAA-induced apoptosis, and the observed fall in FOXO1;AAA expression in U87 cells (Figure 12A).

Western blot analysis of AdFOXO1;AAA infected cells showed multiple bands below the main ~80kDa FOXO1 band (Figure 12A). In particular, there was a ~55kDa band present at 48h and 72h in U87 AdFOXO1;AAA infected cells that was absent in U251 infected cells (Figure 12A). Previously, androgens were found to regulate FOXO1 through a proteolytic mechanism in prostate cancer cells in a manner that is independent of Akt phosphorylation and the proteosome but that involved lysosomal acidic cysteine proteases[209], although a particular protease was not pinpointed. The breakdown product of the FOXO1 protein in that study was missing ~120 amino acids and continued to bind DNA, yet had no transcriptional activity[209]. Since forkhead factors bind as monomers to DNA[145], the effect of this truncated protein may reduce the number of DNA binding sites available to transactivation competent FOXO1 or FOXO1;AAA proteins. The ~55kDa band detected in the samples of AdFOXO1;AAA infected U87 cells (Figure 12A) may also be transactivation incompetent and bind DNA reducing the capacity of full-length FOXO1;AAA to transactivate apoptotic genes. Although

the cell lines tested in our study were not treated with androgens, it is likely that U87 cells may have an activated pathway that leads to proteolytic cleavage involving cysteine proteases as in androgen-induced proteolysis. Cathepsins L and B are cysteine proteases that are upregulated in gliomas; in fact, their expression levels were found to correlate with invasion and malignancy[461]. Furthermore, enhanced cathepsin L expression was demonstrated to be mediated by Ras, which is upregulated in multiple tumours[71]. Thus, it remains to be seen in future studies if U87 cells have higher levels of cathepsins L and B than U251 cells, which may explain the appearance of the extra band at ~55kDa and the degradation of the FOXO1;AAA protein over 72h in the U87 cells (Figure 12A).

*Differences in FOXO1 acetylation status*

FOXO1 transcriptional control is also carried out by modulation of DNA binding affinity, acetylation/deacetylation, and association with co-factors[491]. Phosphorylation as well as acetylation can affect DNA binding activity[298,552]; however, the main phosphorylation site in the DNA binding domain of FOXO1 is altered to an alanine residue in FOXO1;AAA rendering this mutant protein unresponsive to DNA-binding regulation by phosphorylation. Thus, acetylation status may have a larger role than phosphorylation in affecting FOXO1;AAA DNA binding activity.

FOXO factors bind to the transcriptional coactivators CBP (CREB-binding protein) and to p300, thereby connecting these transcriptional regulators to the transcriptional machinery[140,493]. CBP and p300 directly acetylate FOXO factors at

several conserved lysine residues[41,139,319,362,493] and are shown to have dual functions: (1) they are required components for FOXO-mediated transcriptional activity; and (2) they attenuate FOXO activity on target genes. It was speculated that formation of the FOXO-CBP complexes led to histone acetylation and disruption of the tight nucleosomal DNA configuration for transcriptional activation where afterwards the acetylation of FOXO factors would attenuate the specific transcriptional activity[492]. A recent study elegantly demonstrated that acetylation of FOXO1 reduces its DNA-binding ability[298]. Using insulin response sequence (IRS)-containing luciferase reporter constructs, Matsuzaki *et al*[298] found that mutants mimicking constitutively acetylated states had lower reporter construct activity than wild-type or mutants resembling the non-acetylated state. In our study we found that FOXO1 was acetylated at 48h in U87 cells, but was not acetylated in U251 cells (Figure 12B). It is possible that FOXO1;AAA in U251 may have a deficit in acetylation that increases its transactivation potential and susceptibility to apoptosis by AdFOXO1;AAA. Consistent with this possibility is the finding that a combined acetylation and phosphorylation mutant FOXO1 protein prominently potentiated promoter activity by 3.6-fold over wild-type FOXO1 using an IRS-containing luciferase construct[298]. Separate acetylation and phosphorylation mutants were also tested and only increased promoter activity by 1.4- and 2.5-fold, respectively over wild-type FOXO1[298]. Thus, acetylation may partially repress FOXO1;AAA in U87 cells resulting in their blunted response to AdFOXO1;AAA-induced apoptosis, whereas U251 cells may be more susceptible due to the deacetylated state of FOXO1;AAA.

However, data regarding the actual effects of acetylation/deacetylation on FOXO1 activity are conflicting. TSA is not the only deacetylase inhibitor that is capable of increasing FOXO1 acetylation. Nicotinamide, an inhibitor of SIRT1 deacetylases is able to enhance FOXO acetylation by TSA and a number of independent studies have also shown that SIRT1 mediated deacetylation alters FOXO activity[41,319,444]. Motta *et al*[319] demonstrated that SIRT1 directly deacetylated FOXO3 resulting in the downregulation of p27 and Bim. Brunet *et al*[41], on the other hand, showed that SIRT1 increased the ability of FOXO3 to induce p27 and resistance to oxidative stress but inhibited its ability to induce cell death. Nonetheless, Brunet *et al*[41] utilizing a triple mutant FOXO3 found that it did not interact with SIRT1 in the absence of stress stimuli suggesting that the presence of FOXO factors in the nuclei is not sufficient to promote the interaction between SIRT1 and FOXO3. Thus, it is most probable that the FOXO1;AAA used in this study also does not interact with SIRT1 since the glioma cells are grown in full medium and not subject to nutritional deprivation, UV or $H_2O_2$, which are known activators of SIRT1[319].

*AdFOXO1;AAA has anti-angiogenic effects*

Importantly, the clinical potential of FOXO1 is supported not only by its capacity to induce apoptosis, and cell cycle arrest, but also by its ability to regulate neovascularization and angiogenesis[144,206]. FOXO1 regulates post-natal vessel formation and maturation via angiopoietin 2 and eNOS[368]. We demonstrate in this study that AdFOXO1;AAA not only has the capacity to decrease viability of HBECs *in vitro* (Figure 14A), but *in vivo* infection also leads to a decrease in

blood vessel density in the tumours of athymic mice when compared to untreated controls (Figure 14B). Consistent with our observations, a FOXO3 phosphorylation mutant has been found to promote apoptosis of endothelial cells by reducing FLIP, a caspase-8 inhibitor important for protecting endothelial cells from apoptosis[444]. Moreover, it has been found that a phosphorylation site mutant FOXO3 can block VEGF induced endothelial cell survival, proliferation, and cell cycle progression[1]. Clinically, this has important implications as malignant gliomas are highly vascular tumours that produce high levels of VEGF. Thus, the increase in survival observed in U251N and the tumour regression observed in U87LacZ implanted mice could be partially due to the anti-angiogenic effects of FOXO1;AAA thereby adding to the anti-tumour outcome.

One of the most important determinants in endothelial differentiation is the local environment and during embryonic development they acquire organ-specific properties; thus, endothelials cells are very heterogeneous with regard to cell surface molecules and permeability[149,401]. For these reasons HBECs were chosen for our *in vitro* model to investigate the impact of AdFOXO1;AAA on the brain endothelium. However, it is important to note that the endothelium of malignant gliomas differs widely from the resting endothelium of normal human brain[49,162]. For instance, the integrin $\alpha_V\beta_3$ is strongly expressed on glioma blood vessels and additional adhesion molecules also differ compared to the resting endothelium[157,158]. Moreover, brain tumour endothelial cells may comprise a perivascular niche that maintains cancer stem cells in a stem cell-like state[49]. Consequently, the isolation of intratumoural endothelial cells is of particular

importance. However, no immortalized cell line for human glioma endothelial cells exist and methods to purify these cells are extremely difficult as tumour vasculature consists of a complex mix of immature vessels, sprouts, or non-functional glomeruloids surrounded by glioma cells, pericytes and activated immune cells[308]. Still, AdFOXO1;AAA may affect glioma endothelial cells differently than normal endothelial cells and future studies to address the effects of AdFOXO1;AAA in glioma endothelial cells are warranted.

*Ways to improve AdFOXO1;AAA tumour specificity*

Unfortunately, the AdFOXO1;AAA was found to be cytotoxic to human fetal astrocytes (Figure 15), which indicates that a cautionary approach must be taken if this virus is to be used as a therapeutic agent. One possible suggestion for future development would be to make the vector more tumour specific by changing, for instance, the promoter region of the transgene to one that is activated by proteins that are commonly upregulated in tumours, such as E2F[358,485]. Using this strategy, only rapidly cycling tumour cells expressing high levels of E2F will be subject to the apoptotic effects of AdFOXO1;AAA. Altering the fibre knob to change AdV tropism to target tumour-associated antigens would also greatly improve tumour specificity. Some examples of cell receptors that are upregulated in gliomas and can be targeted include integrins[519], EGFR[282], EGFRvIII[126], FGFR[108], and urokinase plasminogen activator receptor (UPAR)[318]. So far, adenoviruses altered to target integrins and EGFR have been tested in gliomas and have resulted in enhanced infectivity[135,174,309,490]. Targeting to $\alpha_v$ integrins was achieved by insertion of an integrin-binding sequence, RGD-4C, into the HI-loop of the AdV

fibre knob protein, which allows the virus to anchor directly to integrins[135,174,521]. EGFR targeting was accomplished by using a single-chain bispecific antibody directed against the human EGFR and against the fibre knob of the AdV[174,309,490]. Nevertheless, in vivo intratumoural injections of AdFOXO1;AAA in our study did not reveal any animals that died due to vector toxicity and cure was achieved suggesting that AdFOXO1;AAA does not cause widespread damage when delivered locally and that the dose provided ($3x10^7$pfu) was sufficient for prolongation of survival.

*AdFOXO1;AAA radiosensitization*

Despite the success of AdFOXO1;AAA in extending the survival of mice in our U251N xenograft model, it was not intended to be utilized as a single agent cure. Consequently, we exploited the role that FOXO1 has in DNA damage to examine the potential that AdFOXO1;AAA would have as an adjuvant to radiotherapy by investigating its ability to increase the sensitivity of glioma cells to radiation. UV- and γ-radiation lead to the expression of Gadd45, which is directly activated by FOXO protein binding to the *Gadd45* promoter[143,479]. Gadd45 is important for DNA repair, and cell cycle arrest at G2/M[509,550], as well it has been shown to induce apoptosis in cancer cells[470,476]. In our study, we found that infection with AdFOXO1;AAA in both U87 and U251 cells made them more radiosensitive (Figure 16). Interestingly, in U251 cells, adenovirus infection itself sensitized the cells to radiation possibly because the adenovirus E4orf6 protein has been shown to inhibit DNA double strand break repair in this cell line[189]. Nevertheless, AdFOXO1;AAA outperformed AdGFP in decreasing the surviving fraction

(Figure 16B, right). The increase in radiosensitivity in response to AdFOXO1;AAA infection has important clinical implications as radiation in the brain can lead to damage to the white matter, which is associated with cognitive decline. Normal CNS tissue can tolerate up to 60Gy of radiation, but this may be far below the threshold required to kill malignant glioma cells, and the risk of residual tumour is high. The administration of AdFOXO1;AAA could help reduce the level of radiation delivered to preserve normal tissue and/or to deliver a much more effective local tumouricidal effect. Furthermore, stereotactic delivery of AdFOXO1;AAA in tumour masses considered inoperable, followed by radiation treatment may also improve the rate of success.

*Comparison with Adp53 targeted therapy*

Many other genes have been investigated for targeted therapy against gliomas such as p53, p16, Rb, PTEN, and Fas Ligand among others[56,261]. Use of p53 is currently being clinically investigated in human glioblastoma[264]. Adp53, in fact, has been approved for clinical use for the treatment of head and neck cancer in China[361]. Thus, Adp53 pre-clinical data can provide perspective regarding the possible use of AdFOXO1;AAA as a therapeutic strategy to restore apoptosis. In an earlier study performed in the same laboratory with similar techniques to this current study, Li *et al*[279] using Adp53 in a U87LacZ intracerebral model, reported a cure rate of approximately 20-40%, which outperforms AdFOXO1;AAA in U87LacZ (Figure 13B). On the other hand, Lang *et al*[267] who conducted pre-clinical studies with Adp53 in athymic mice on established intracerebral U251 tumours, reported no cures, only a meagre 11 day increase in median survival in

comparison to control, and 6.5 day increase in comparison to vector control with an initial tumour size of $1x10^6$ and vector treatment 3d post-tumour implantation. Our data demonstrated a 1.8 times increase in survival in the U251N intracerebral model and a 33.3% cure rate (Figure 13B). Furthermore, U251N xenografts are invasive suggesting that AdFOXO1;AAA treatment can lead to a substantial bystander effect that may be partly related to its anti-angiogenic abilities. These pre-clinical data suggest that FOXO1 may offer an alternative form of therapy for tumours that are resistant to p53 gene therapy.

Therefore, based on its capacity to extend survival, decrease tumour volume, and sensitize cells to radiation, adenoviral delivery of FOXO1;AAA has great potential as a form of adjuvant therapy for patients with malignant gliomas. However, the use of AdFOXO1;AAA may be limited to a specific population of patients due to genetic heterogeneity in tumour cells and varying sensitivities to its apoptotic effects. To examine a gene therapy approach that would be more comprehensive targeting mainly S-phase cells, we tested a replicating suicide gene therapy vector that could potentially concentrate chemotherapeutic agents in the tumour mass and transduce with higher efficiency than first generation non-replicating vectors.

**Suicide Gene Therapy: Ad(dPS)CU-IRES-E1A and 5-FC**

Research into new chemotherapies is complicated by the presence of the blood-brain barrier, which makes it difficult to systemically administer chemotherapeutic agents at adequate doses. The short half-life of

chemotherapeutic agents, such as 5-FU, systemic toxicity, and drug resistance mechanisms further complicate matters. Gene therapy strategies were investigated to counter these problems to allow for sustained local release of high concentrations of chemotherapeutic agent. However, current approaches with non-replicating, first generation adenoviruses were hampered by poor transduction efficiency. Oncolytic viruses that replicate and lyse cancer cells were explored as a possible solution[435], but these fully replicating viruses pose many safety concerns[63]. Therefore, it was our goal to improve suicide gene activity using adenoviral replication and not dissemination since it satisfies many bio-safety issues that oncolytic viruses do not. In this study, we evaluated the anti-tumour activity of an adenovirus that not only expresses the suicide fusion protein CD::UPRT, but is also capable of one round of replication without producing disseminated virus particles[352] (Ad(dPS)CU-IRES-E1A). The virus in this study is unique in that the protease gene has been removed, but the E1A region of the virus is intact. This results in a virus that can amplify its transgene copy number in infected cells through viral genome replication but cannot package new virus particles since the protease gene is required for the maturation, assembly, and release of virions[352]. There is also a lack of viral shedding (as shown by plaque assays), and replication is controlled since it only occurs once, reducing the probability for spontaneous revertants[352]. Our data demonstrates that Ad(dPS)CU-IRES-E1A is more efficacious than first generation non-replicating vectors and the oncolytic Ad5wt in both cytotoxic and bystander capacity; as well, it performs better than non-replicating viruses in *in vivo* survival studies.

*Increased capacity for Ad(dPS)CU-IRES-E1A to increase CD::UPRT expression*

Western blot analysis revealed that the replicating/non-disseminating virus, Ad(dPS)CU-IRES-E1A, was indeed able to induce higher levels of CD::UPRT expression in all cell lines tested, including the murine glioma cell line, GL261, compared to the first generation non-replicating vector (Figure 17B). Murine cells are only semipermissive to adenovirus infection due to several blocks in the late phase of the lytic cycle, but early genes are still expressed in murine cells, including those of the E2 region encoding proteins required for genome amplification. Therefore, the increased transgene expression observed in the GL261 cells and the human glioma cells can be attributed to virus replication. Another striking aspect of the increased expression levels observed in the murine GL261 cells was that this cell line was relatively resistant to AdLacZ infection. At a MOI of 500, less than 5% of GL261 cells were transduced, suggesting that viral replication from the protease deleted virus greatly amplifies CD::UPRT expression as CD::UPRT is not detected in GL261 cells infected with the non-replicating Ad(dPS)CU (Figure 17B). Therefore, deletion of the protease gene results in higher levels of genome amplification and protein expression in all glioma cell lines tested.

*The effect of Ad(dPS)CU-IRES-E1A on viability*

Viability assays demonstrate that fewer particles of Ad(dPS)CU-IRES-E1A were required to produce a cytotoxic effect compared to AdCU (Figure 18) likely due to its capacity to augment CD:UPRT protein expression. The replicating/non-disseminating virus, Ad(dPS)CU-IRES-E1A, achieved a 50% reduction in

viability with a MOI as low as 0.2 in U87 cells with only 25μM of 5-FC, whereas AdCU required an MOI of 9, making Ad(dPS)CU-IRES-E1A 45-times more potent than its first generation counterpart. The fact that fewer particles of Ad(dPS)CU-IRES-E1A could produce a cytotoxic effect is crucial because adenoviral particles at high concentrations can be toxic; injection of greater than $10^{10}$ pfu of adenoviral vector is toxic in the brain and is accompanied by confusion, hyponatremia, and seizures[480]. Reducing the number of particles required could reduce side effects commonly associated with high titers.

In the absence of 5-FC, in fact, Ad(dPS)CU-IRES-E1A was as potent as AdCU in the presence of 25μM of 5-FC in cytotoxic capacity (Figure 18B). This can be attributed to the presence of the E1A cassette in the replicating virus, which is known to have natural anti-tumoural properties[411]. E1A is capable of inducing cells into S-phase to allow for effective viral propagation. In doing so, it binds to Rb which releases E2F. E1A-released E2F induces host expression of the *ARF* gene leading to the accumulation of p53. High levels of p53 in the nucleus leads to growth arrest or apoptosis that is presumed to contribute to the anti-tumourigenic effect[410]. A disseminating AdV expressing a dicistron composed of the CD and E1A was studied by Akbulut *et al*[3], but the modest efficacy they reported differs dramatically with our results. These differences are likely due to the superiority of our strong CMV5 promoter versus their L-plastin promoter, and also to the higher activity of the CD::UPRT versus the CD alone, as our lab and others have shown that the fusion of the CD with the UPRT improves its anti-tumour activity[34,118,474]. Consistent with the *in vitro* cytotoxicity data,

Ad(dPS)CU-IRES-E1A without 5-FC in athymic mice marginally improved survival in comparison to untreated controls (median survival: 60.5 days vs. 47 days; p=0.0062; Figure 22A). However, the data in our syngeneic model did not yield similar results with Ad(dPS)CU-IRES-E1A in the absence of 5-FC compared to untreated control (median survival: 24 days vs. 23 days; Figure 22B). This may be due to the increased tumour burden in the syngeneic mice, which received a tumour load of $2x10^5$ as opposed to $1x10^5$ for the athymic mice. Alternatively, the genetic profile of the GL261 cell line may not permit E1A apoptotic effects. For example, one study by Rao *et al*[385] found that only a small proportion of A549 lung cancer cells (17%) were subject to E1A-induced apoptotic effects while the majority of cells permitted viral replication and were resistant to E1A-induced apoptosis. Thus, the effect of the E1A gene is weak and may contribute only a small part to the cytotoxic effect of the replicating/non-disseminating virus. Therefore, for the most part, the increase in survival and cytotoxicity is contingent on the supply of the pro-drug, 5-FC.

Another important observation in the cytoxicity assays show that Ad(PS+)CU-IRES-E1A, the fully replicating/disseminating vector, does not significantly outperform Ad(dPS)CU-IRES-E1A, indicating poor oncolytic activity. For example, in U87 cells, both replicating vectors required an MOI of ~0.2 to achieve 50% decrease in viability in the presence of 5-FC (Figure 18C). These results are consistent with a study by Lambright *et al*[263] that showed that replicating adenoviruses combined with the suicide gene, *HSV-TK,* had not increased anti-tumour activity in the presence of GCV despite high level

production of the suicide transgene by the replicating vector in the absence of GCV. Moreover, when tumour cells were transduced with Ad(PS+)CU-IRES-E1A or Ad(dPS)CU-IRES-E1A the level of viral protein expressed was reduced in the presence of the pro-drug (Supplementary Figure 3B). Titers of the Ad(PS+)CU-IRES-E1A were also reduced by 4-fold in the presence of the pro-drug (Supplementary Figure 3C). Thus, the combination of a suicide gene and oncolysis may not result in much improvement. In contrast with this observation, Rogulski *et al*[402] observed a significant improvement with a combination of HSV-TK in a replicating vector in the C33A tumour model; however, the same vector did not benefit from the pro-drugs in the DU145 tumour model[132].

The anti-tumour effect of the transgene and replicating vector combination, though, may be dependent on the nature of the suicide gene. For example, Nakamura *et al*[327] using a replicating HSV-1 in combination with suicide genes, found that HSV-1 mediated oncolysis combined with CD/5-FC, but not TK/GCV, had more pronounced anti-tumourigenic effects compared with oncolysis alone. They attributed this discrepancy to mechanistic differences in the effects of GCV versus 5-FC. Phosphorylated GCV can affect both viral and genomic DNA synthesis; however, the 5-FU metabolite 5-fluorodeoxyuridylate affects cellular DNA synthesis more than viral DNA synthesis[171]. Additionally, 5-FU within a broad range of concentrations enhances replication of adenovirus vectors[17]. Thus, it is possible that the combination of an oncolytic vector carrying the transgene for the conversion of 5-FC to 5-FU, but not for the phosphorylation of GCV may have more tumour killing capacity. Then again, according to our observations

"armed oncolytic viruses" may not offer a significant advantage over Ad(dPS)CU-IRES-E1A based on cytotoxic capacity and observations of viral titer.

*Bystander assays*

Nonetheless, a potent capacity to incite cytotoxic death in untransduced cells (bystander effect) is vital for efficient gene transfer as not all cells can realistically be transduced in a solid tumour mass with current gene transfer technology. The *in vitro* bystander assay demonstrated that as few as 3% of cells transduced with Ad(dPS)CU-IRES-E1A at MOI of 10 can reduce the viability of U87 cells to 50% in the presence of 25μM 5-FC (Figure 19). This indicates a considerable bystander effect compared to AdCU, which required an addition of 42% transduced cells (almost a 1:1 ratio). Only at MOI of 100 could AdCU achieve a bystander effect by reducing U87 cell viability to 50% with ~8% transduced cells. Similarly, in U251 cells only 11% of Ad(dPS)CU-IRES-E1A infected cells at MOI of 10 were required to reduce viability by 50% whereas AdCU required 100% transduction to produce 50% killing (Figure 19B). Our data firmly demonstrate that very few particles of the replicating/non-disseminating AdV can kill large numbers of glioma tumour cells. These data imply that a higher concentration of 5-FU is produced by the replicating virus, due to increased transduction efficiency, which can diffuse and kill a greater number of neighbouring uninfected cells.

The *in vitro* bystander effect is most evident in the three-dimensional spheroid assays, which most closely resemble the structure of an actual tumour. At a pfu of $1x10^6$, the viability of U87 spheroids was reduced with Ad(dPS)CU-IRES-E1A, whereas AdCU was unable to reduce viability (Figure 20A) demonstrating that the replicating/non-disseminating virus is also more capable than AdCU in penetrating a three-dimensional mass. We also found that the replicating/non-disseminating virus not only outperformed first generation adenoviral vectors, but oncolysis as well in the spheroid assay. In a 10 day period Ad5wt was not able to reduce the viability of U87 below 70%, even with $5x10^6$ pfu, whereas at that same pfu, Ad(dPS)CU-IRES-E1A reduced viability well below 50% (fig 20A). The fact that oncolysis was not unable to reduce viability below 70% neither with $1x10^6$pfu nor $5x10^6$pfu suggests that oncolysis was limited by the time for each cycle of replication, cell lysis, and re-infection rather than by particle number. A similar observation was reported by Grill *et al*[173] who found that the speed at which oncolysis penetrates a spheroid was slower than the growth of the latter. These results may explain why in clinical trials with ONYX-015 anti-tumour activity was only seen if viral treatment was combined with chemotherapeutic drugs, such as 5-FU, as oncolysis alone was not sufficient[245].

In the spheroid model the "armed oncolytic virus," Ad(PS+)CU-IRES-E1A, fared slightly better than the non-replicating/non-disseminating, Ad(dPS)CU-IRES-E1A, indicating that it may have an enhanced capacity to penetrate past the superficial layers of the spheroid. However, at increasing pfu from $2x10^5$ to $1x10^6$, both Ad(PS+)CU-IRES-E1A and Ad(dPS)CU-IRES-E1A did not increase

their killing capacity, presumably because only the superficial layers were transduced and 5-FC/5-FU diffused poorly through the spheroid (Figure 20A).

Moreover, Ad(dPS)CU-IRES-E1A did not alter the viability of the U251 spheroids (Figure 20B). Our group has previously shown U251 spheroids to be highly resistant to the AdCU/5-FC treatment, although collagen invasion was prevented following the treatment[34]. This suggested that mitotic cells at the surface of the spheroid were killed, but not the non-mitotic core of the spheroid. With the hope of achieving a reduction in viability with the replicating vector, we attempted three rounds of treatment spread over 6 days (days 0, 3, and 6) but without success. We must conclude that some tumours will not be sensitive to our treatment. Whether this insensitivity correlates with the p53 status of the tumour, as our previous work suggested[34] needs to be confirmed in a larger population of primary tumour cells. In this case, co-infection with Adp53 and Ad(dPS)CU-IRES-E1A may yield more favourable results with U251 spheroids. Nevertheless, the U87 spheroids responded favourably to treatment and prompted an *in vivo* evaluation for vector efficacy.

*In vivo comparison*

*In vivo* studies with pre-established intracerebral human glioma xenografts in athymic mice validated the *in vitro* results showing that the replicating/non-disseminating vector was capable of penetrating a three-dimensional tumour mass providing better suicide gene activity than the non-replicating vector. Control athymic mice (5-FC only; no virus) after 35 days had tumours 8-times larger than

Ad(dPS)CU-IRES-E1A plus 5-FC treated mice (Figure 21). Furthermore, the median survival for athymic mice treated with Ad(dPS)CU-IRES-E1A and 5-FC was 1.5-fold more than the median survival for Ad(dPS)CU and 5-FC treated mice (117 vs. 76 days), and was 2.5-fold longer than untreated control animals (47 days) (Figure 22A). A study by Miller *et al*[311] using a first generation adenoviral vector to deliver cytosine deaminase (AdCMVCD) found that mice implanted with U87 and treated with AdCMVCD and 5-FC resulted in only a 1.4-fold increase in median survival compared to control (67 days vs. 49 days). Our median survivals also compare favourably to a study by Conrad *et al*[72] that also used a U87 xenograft mouse model but injected 3 doses of conditionally replicating virus. Their "armed" replicating oncolytic adenovirus containing the transgene for cytosine deaminase (delta-24 CD) demonstrated a 2-fold increase in survival (74 days) compared to PBS control (34 days) and showed a 1.3-fold increase compared to the "unarmed" oncolytic delta-24 virus (55 days)[72]. In comparison, we demonstrated a median survival of 117 days with the replicating/non-disseminating Ad(dPS)CU-IRES-E1A.

In another study, Tai *et al*[467] utilized a single injection of replication competent retrovirus expressing CD followed by multiple cycles of 5-FC administration for 8 consecutive days at 3-week intervals in athymic mice. In their study they showed a survival of 100% for more than 100 days. However, toward the end of each three-week rest interval, the animals reportedly became moribund regaining normal behaviour only with the commencement of the next cycle of 5-FC administration. These results can be explained by the possibility that the

concentration of 5-FU achieved with each cycle of 5-FC administration was not high enough to completely eradicate the tumour and in a sense created a chronic disease condition. On the other hand, a cure rate of 20% in athymic mice was demonstrated in our study with only two treatment cycles. Moreover, the use of retroviral vectors in cancer therapy should be approached carefully as they can pose a risk for insertional mutagenesis that can contribute to the development of novel malignancies[524]; this is not a concern with the use of an adenoviral vector. Most importantly, retrovirus can only infect dividing cells[42,256], which is a disadvantage for GBM because the internal areas of the tumour mass are not actively dividing. Adenovirus, on the other hand, is capable of infecting both dividing and non-dividing cells.

Thus, our *in vivo* results substantiate the *in vitro* results, suggesting that the increased survival of Ad(dPS)CU-IRES-E1A treated animals is due to its replicative properties namely increased transgene production, cytotoxic capacity, and bystander effect, since Ad(dPS)CU, which is unable to replicate, could not achieve the same survival proportions. At the same time, the replicating/non-disseminating virus compares favourably to the oncolytic virus delta-24 and to the "armed" replicating retroviruses.

*Immune bystander in vivo*

The ability of Ad(dPS)CU-IRES-E1A to replicate, though, may not be the only factor contributing to its ability to prolong survival as the immune system is known to be a major factor in the *in vivo* bystander effect[182,241,365]. To elucidate

the importance of the immune system in tumour clearing and the bystander effect, we sought to evaluate the suicide gene vector in a syngeneic mouse model. We chose to use the GL261 murine glioma tumour model in C57Bl/6 mice for several reasons other than the fact that it provides an immunocompetent model. The GL261 tumour was originally generated by intracerebral injection of 3-methylcholantrene into C57Bl/6 mice and was sustained by serial intracerebral and subcutaneous transplantations of tumour into the syngeneic mouse strain[9]. It is a well-characterized and predictable model where the tumours have a rapid growth rate, high degree of tumour take and do not give metastases[465]. In fact, their aggressiveness has been compared to rat glioma models such as 9L, F98, and CNS-1 that also have a high tumour take and short median survival time[465], unlike the C6 rat glioma[280,418] and the 4C8[514] mouse glioma models that are much less aggressive. Importantly, GL261 tumours exhibit an invasive property, which is not observed in the U87 xenograft models. Nonetheless they are moderately immunogenic and this must be taken into consideration when evaluating experimental data. Other models do exist that are considered non- or weakly immunogenic such as F98, CNS-1 and RG2, but since no evidence of spontaneous regression was observed in our experiments as 100% of the control mice succumbed to their tumours (Figure 22B) immunogenicity likely did not greatly affect our outcomes.

Our results in the GL261 immunocompetent mouse model were even more pronounced than in the athymic mouse model. The presence of Ad(dPS)CU-IRES-E1A plus 5-FC dramatically increased survival in relation to control and

Ad(dPS)CU plus 5-FC groups (Figure 22B). In fact a cure rate of 57.1% was achieved with Ad(dPS)CU-IRES-E1A and 5-FC, whereas in the athymic mice, the same treatment only cured 20%. Using a similar syngeneic model, Lumniczky *et al*[286] transduced GL261 *ex vivo* with an adenoviral vector encoding both UPRT and TK and implanted these transduced cells into C57Bl/6 mice. Then they treated the mice with GCV and 5-FU. With this combination using 100% transduced cells, they reported comparable cure rates to our study, but with a shorter observational period of time (100 days post-tumour implantation). However, their use of *ex vivo* transduced cells is not as clinically relevant as intratumoural delivery of vector where the three-dimensional structure of the tumour mass prevents every cell from being transduced. In fact, in that study, when the ratio of transduced versus untransduced cells was lowered to 1:4 and 1:9, the median survival dropped to approximately 28 and 32 days, with survival rates improving upon irradiation. Additionally, our use of the relatively non-toxic pro-drug 5-FC versus their use of 5-FU also serves as a safety advantage, where we can increase 5-FC concentrations to augment local 5-FU concentrations to a much larger degree, and unlike 5-FU, 5-FC can cross the blood brain barrier. This work by Lumniczky *et al*[286] provides perspective to our work suggesting that the replicating/non-disseminating virus is able to lead to widespread tumour cell killing in a manner that is as effective as though every cell were transduced, which is currently clinically impossible. Our vector may also have radiosensitizing capabilities as suggested by the results of Lumniczky *et al*[286]. The *in vivo* data from our syngeneic model indicate strongly that the replicating/non-disseminating virus is able to persist in the presence of a competent immune

system, and is effective in eliciting an anti-tumour response that is greater than that seen in the athymic mouse model, suggesting that immune involvement augments the anti-tumour response.

A more detailed investigation of the immune infiltrates in the syngeneic mouse model by examining brain tissue using flow cytometry provided data that suggest the immune system of the C57Bl/6 mice may play a role in prolonging survival by contributing a bystander effect. Brains of mice that had been infected with Ad(dPS)CU-IRES-E1A and treated with 5-FC had a higher number of T cells and macrophages ten days after vector administration than mice infected with Ad(dPS)CU and treated with 5-FC, although the number of NK cells were the same in both groups. Immunofluorescent staining of T-cells in the tumour area was consistent with the flow cytometry data (data not shown). The fact that treatment with Ad(dPS)CU-IRES-E1A produced a higher immune response than Ad(dPS)CU may be due to the ability of the replicating virus to increase response towards tumour antigen(s). Since Ad(dPS)CU-IRES-E1A has been shown *in vitro* to have a much greater cytotoxic capacity, more tumour antigens as well as viral proteins may be released when GL261 cells are killed as compared to tumours treated with Ad(dPS)CU. The ensuing debris left by Ad(dPS)CU-IRES-E1A would need to be cleared by a larger population of phagocytic cells, such as macrophages, which in turn would engage more T cell effectors. Consistent with our results, Uckert *et al*[487] showed that when murine mammary adenocarcinoma cells were transduced with both CD and HSV-TK genes and inoculated s.c. into immunocompetent syngeneic mice followed by 5-FC and GCV, tumour

development was inhibited. Athymic mice in the same study, on the other hand, developed tumours indicating that T-cell-mediated immune mechanisms played an important role in tumour eradication. Kuriyama *et al*[257,258] found that retrovirally transduced CD followed by 5-FC gene therapy in an immunocompetent mouse model of hepatocellular carcinoma could not only induce anti-tumour effects, but could also lead to protective immunity through induction of CD4+ and CD8+ T lymphocytes and macrophages. Studies have also shown that tumours treated with the suicide gene *HSV-TK* also contain macrophages as well as lymphocytes[243,497]. This bystander immune response could offer an explanation for the much higher survival rate of Ad(dPS)CU-IRES-E1A plus 5-FC treated immunocompetent mice in comparison to the athymic mice. Thus, the replicating properties of Ad(dPS)CU-IRES-E1A may serve to increase the immune bystander effect more than the non-replicating vector, Ad(dPS)CU.

Still, there is a possibility that the immune response may be vector induced, which would indicate that the immune response is unfortunately not tumour-specific. Since Ad(dPS)CU-IRES-E1A is capable of producing more viral proteins as shown by Western blot analysis (Figure 17C), it is possible that it may lead to greater adenoviral antigen expression and viral vector clearance. Cartmell *et al*[55] showed that adenovirus injection into the brain stimulates the secretion of interleukin (IL)-1, IL-6 and tumour necrosis factor-α resulting in a very early inflammatory response. However, this process does not appear to impair Ad(dPS)CU-IRES-E1A mediated anti-tumour effects since it was successful in

inducing tumour regression and extending survival. Nevertheless, it is not inconceivable that even if the vector is cleared (i.e. no more transgene expression) tumour immunogenicity could still be increased such that remaining cells are killed by the immune system.

The immune response caused by the vector could also lead to chronic inflammation, which was a concern brought about in a study by Dewey *et al*[87]. This study noted chronic brain inflammation with damage to normal surrounding tissue, and along the needle track following suicide gene therapy with adenovirus vectors expressing HSV-TK followed by GCV treatment. In contrast, chronic brain inflammation has not been observed in several other experimental models involving the administration of adenovirus encoding HSV-TK, instead only short-term, dose-dependent inflammatory responses have been described[48,55,445]. Interestingly, the observations of Dewey *et al* appear very similar to those seen in a rat with an intracerebral stab wound[153,344], indicating that their results could be due to poor surgical technique rather than the effects of adenoviral infection. Furthermore, in a phase I clinical trial conducted by Trask *et al*[480] using an adenoviral vector carrying the transgene for HSV-TK in combination with GCV, they did not observe severe or widespread inflammatory changes in normal brain tissue as shown in the rats in the study by Dewey *et al*. In addition, the syngeneic mice that survived to 365 days in our study did not show any overt side effects following vector administration and showed no indications of illness (were feeding normally and active) at 365 days when the experiment was terminated. Observations of the brain tissue of mice that were not cured showed large

tumours, indicating these mice died from their tumours and not from any vector-related death. Nonetheless, characteristically human traits such as changes in memory or personality due to potential neurological damage caused by vectors cannot be easily assessed in animal models. The observation that the mice did not show any obvious signs of neurological damage at 365 days suggests that the inflammation present in the brain is transient and not chronic.

*Ways to improve vector efficacy*

In addition to the immune bystander effects, evidence from our studies indicate that Ad5-based vectors have a very poor transduction efficiency in GL261, which presupposes that the majority of the suicide gene effectiveness of Ad(dPS)CU-IRES-E1A plus 5-FC comes from its potent bystander effects. We predict that our current results could be further improved using an adenoviral serotype that infects this murine cell line better than Ad5. Studies are currently underway to test the efficiency of Ad(dPS)CU-IRES-E1A using an adenoviral vector of serotype 35 (Ad35) instead of serotype 5 (as used in this study) since preliminary studies show that Ad35 can achieve a much higher transduction efficiency than Ad5 in many cell types including GL261, U87 and U251 (data not shown). Tumour specificity and transduction efficiency can be further improved by altering the fibre knob of the adenovirus to change its tropism[159,166] or placing the gene under a tumour specific promoter[358] as mentioned earlier with AdFOXO1;AAA. Vector spread could also be improved by intravascular delivery through the intracarotid artery, which has been shown to be effective in targeting tumour foci distant from the main tumour mass using adenoviral and herpes simplex virus vectors as well as

cationic liposomes[12,380]. The BBB could be made more permeable temporarily by an agent such as RMP-7, a bradykinin analog[12], to allow virus entry. In fact, intracarotid delivery is currently being explored in our laboratory as an alternative to intratumoural delivery. Thus, it is advantageous that adenovirus is well-characterized such that changes in its structure and genome can be performed with ease. All these potential alterations could further improve the cytotoxic and bystander capacity of the replicating adenovirus vectors evaluated in this study.

*Clinical potential*

Ad(dPS)CU-IRES-E1A in combination with 5-FC treatment holds a great deal of clinical promise for the treatment of gliomas and possibly other malignancies (as evidenced by our results in ovarian cancer cell lines; data not shown). For one, the 5-FC prodrug has many advantages as it has already been approved for CNS fungal chemotherapy, and unlike 5-FU is freely permeable through the blood-brain barrier. 5-FC kinetics show that it is greater than 80% bioavailable[77], and unlike GCV (used in suicide gene therapy approaches with HSV-TK), it can accumulate after multiple dosing[77]. It also has excellent oral bioavailability, which would make administration much simpler. For fungal infections, a dose of 4g/day is usually used, which results in peak serum levels of 88-94μg/mL or 680-728μM, and a serum concentration of 770μM is considered toxic[496]. Our *in vitro* studies only required 25μM of 5-FC to achieve tumour cell killing with the replicating/non-disseminating virus. One possible concern is that the high efficiency of Ad(dPS)CU-IRES-E1A in converting 5-FC to 5-FU may mean that the dose of 5-FC will need to be adjusted from the dose normally provided for

fungal infections to prevent neurotoxicity due to high 5-FU production. Overall, 5-FC is an attractive alternative to systemic infusion of the highly toxic 5-FU.

Phase I clinical trials using a combination of CD and 5-FC in non-replicating vectors have not yet been conducted in glioma patients; however, this form of gene therapy has been tested in colon carcinoma[75] and prostate cancer[131], where it has been shown to be well-tolerated. It is highly unlikely, however, that the combination of CD::UPRT/5-FC will be used as a single treatment modality in patients. 5-FC has been shown to act as a radiosensitizer, and the combination of suicide gene therapy and radiotherapy has proven to be advantageous *in vivo*[262,286,450]. Future experiments will explore the use of the replicating/non-disseminating virus with 5-FC as an adjuvant to radiotherapy.

In conclusion, we show here that adenoviral replication of a protease deleted vector substantially improves suicide gene expression and activity and is better than first generation AdVs and oncolysis. As well, our observations suggest that the immune competence of a patient with cancer may be a critical factor in achieving successful gene therapy using this combination of a replicating/non-disseminating vector and 5-FC administration. This may be an important consideration given that various studies have demonstrated that patients with GBM are immunosuppressed[96,405]. Finally, our *in vivo* data underscore the potential that this form of therapy may have in a clinical setting in offering a new form of therapy for patients suffering with incurable gliomas.

Recurrent disease, however, is a great challenge in the treatment of malignant gliomas. A gene therapy approach that could offer long term treatment to prevent recurrence rather than the transient therapeutic transduction of adenoviruses may be more successful in the treatment of malignant gliomas. Harnessing the tools of the immune system for long term treatment was therefore investigated.

**Gene Transfer of Cytokines: AAV-IFN-β**

Recombinant AAV is a vector that is capable of long term gene transfer[225,239,293] and is also non-pathogenic in humans[510]. We show here that rAAV delivery of IFN-β, a pluripotent cytokine, is extremely efficacious in extending survival in an intracerebral mouse model of glioma producing cure rates of 75% at one year post-tumour implantation with a single intratumoural administration (Figure 28A), surpassing the targeted and suicide gene therapies investigated earlier using the same xenograft model. IFN-β protein was explored as a therapeutic agent in gliomas in the past due to its well known anti-proliferative, anti-angiogenic, and immunomodulatory capacities[28], but its short half-life resulted in its failure in clinical trials[114,416]. The success of AAV-IFN-β can be attributed to its ability to locally and continuously deliver IFN-β eliminating problems associated with the low stability of the protein.

*Effects of AAV-IFN-β on cell viability*

U87 and U251 glioma cells are generally categorized as being resistant to IFN-β treatment[316]. Gene transfer by direct infection of AAV-hIFN-β in U87 and U251 human glioma cells, however, resulted in a significant decline in viability

compared to control (Figure 25A) although, a dose dependent effect of AAV-hIFN-β was not observed likely due to intrinsic resitance to IFN-β. U87 cells experienced a much greater decline in viability than U251 cells in response to direct infection, which can be explained by the fact that AAV has higher transduction efficiency in U87 cells than U251 cells as observed by AAV-GFP infection (data not shown). Several lines of evidence also indicate that IFN-β gene transfer is much more effective in delivering a cytocidal response than exogenous IFN-β protein. Treatment with 100-10,000 U/mL of recombinant human-IFN-β on U87 and U251 cells led mainly to growth inhibition mediated through cell cycle arrest in the S-phase and only to a lesser extent, apoptosis at higher concentrations[205,459,544]. However, when AAV-hIFN-β at an MOI of 30,000 vector genomes per cell was applied to U251, it led to morphological changes along with Annexin-V staining consistent with apoptosis[544]. These results suggest that perhaps only direct gene transfer of glioma cells or very high concentrations of IFN-β will elicit an apoptotic response in cells considered to be resistant to exogenously applied IFN-β. Studies have also shown that the presence of high IFN-β mRNA levels produced from the viral vector may make the infected cells more susceptible to IFN-β treatment[185,351]. The presence of IFN-β mRNA may result in prolonged phosphorylation of proteins involved in the signal transduction pathway of IFN-β, such as JAK1, Tyk2, and STAT-1[545]. Our *in vtiro* results correlated with our *in vivo* results whereby AAV-hIFN-β injected intratumourally led to tumour regression and prolongation of survival.

Interestingly, AAV-GFP direct infection also had an effect on cell viability, although supernatants from 293A cells infected with AAV-GFP did not have any effect on viability in U87 or U251 cells. This suggested that AAV itself may have inherent anti-oncogenic properties. Raj *et al*[381] showed that AAV could mediate the killing of cells in an ATM-dependent manner. The hairpin structures present in the inverted terminal repeats in the AAV genome are thought to trigger a DNA damage response that leads to apoptosis in cells that do not have an active p53[105]. Although we did observe AAV-mediated killing, in contrast to their results we found that AAV-GFP lead to a greater viability decrease in U87 cells even though they have a wild-type p53 (Figure 25); however, U87 cells do transduce better than U251 cells with AAV, which may have decreased the capacity of AAV-GFP to induce killing in U251 cells. Still, the effects of AAV mediated killing may be cell-type specific and p53 status may not reliably predict susceptibility. In any case, AAV-GFP was ineffective in reducing tumour volume (Figure 27A, B and C) or extending survival to same extent as AAV-hIFN-β (Figure 28A and B) *in vivo* indicating a weak anti-tumour effect. This is possibly related to an inability of AAV-GFP to extend its anti-tumour capacities to untransduced cells, in other words, a lack of bystander effect. In contrast, a strong bystander effect has been demonstrated for human IFN-β. Adenoviral delivery of IFN-β into a small percentage (1-10%) of cells was sufficient to block tumour formation and led to regression of established tumours in athymic mice[376]. Thus, the concomitant delivery of IFN-β and AAV results in the production of a secreted cytokine that diffuses to untransduced tissues with the added benefit of the inherent anti-oncogenic properties of AAV.

The application of AAV-hIFN-β infected 293A supernatants also had a significant effect on the viability of U87 cells. Although the AAV-hIFN-β infected 293A supernatants were less effective than direct infection (Figure 25B), these results indicate that the secreted hIFN-β is functional as an anti-tumour agent. The cytoxicity curve observed with the 293A supernatants on the U87 cells resembles the profile of recombinant IFN-β on other glioma cell types where the curves are characterized by the greatest decline in viability at lower concentrations, but plateau with increasing concentrations[150,471]. On the other hand, 293A supernatants had no effect on U251 viability (Figure 25B). Several possibilities may account for the difference in sensitivity between U87 and U251 cells to AAV-hIFN-β infected 293A supernatants such as the presence or absence of the genes encoding IFN-β and –α (*IFNB* and *IFNA*). U87 cells are deleted in both *IFNA* and *IFNB* genes[316], and U251 is presumed to have an intact and functional *IFNB* gene as expression of IFN-β can be induced from U251 but not U87 by polyI:polyC[316,335]. However, it is unlikely that the presence or absence of these genes confers resistance or sensitivity. A study by Miyakoshi *et al*[316] on 19 malignant glioma cell lines differing in their presence or absence of *IFNB* and *IFNA* was unable to find a correlation with cellular sensitivity to IFNs. Moreover, although variable density or affinity of type 1 IFN receptors in different cell lines has been described[179,404], several studies do not support that receptor numbers determine anti-proliferative efficacy[179,541] nor do copy numbers of the interferon receptor gene on chromosome 21q[251]. The most likely explanation for the differences in sensitivity may be related to the p53 status of glioma cell lines.

Takaoka *et al*[469] demonstrated that IFN-β leads to an increase in p53 protein levels, which can contribute to tumour suppression in the face of stress signals. These data are consistent with our observations that U87 cells, which are p53 wild-type, were more sensitive to AAV-hIFN-β treatment than U251, which are p53 mutant. Therefore, it was not likely that the number of IFN type-1 receptors, or the presence or absence of IFN genes led to increased susceptibility to IFN-β-induced decrease in cell viability in U87 cells, but was more likely related to the presence of a functional p53. As was predicted *in vitro*, *in vivo* AAV-hIFN-β injected peritumourally (*i.e.* injected before tumour implantation) was capable of preventing tumour implantation of U87LacZ tumours, but was not able to significantly prevent implantation of U251N tumours (although two out five mice did not exhibit any U251N tumour). Overall, the cytotoxicity data from direct infection and the application of supernatants underscore the *in vivo* feasibility of both intratumoural infection and peritumoural infection for effective tumour cell killing.

*In vivo apoptosis by AAV-IFN-β*

Since AAV-hIFN-β had reduced glioma cell viability *in vitro* (Figure 24), we determined whether it could do the same *in vivo*. Indeed, AAV-hIFN-β intratumoural injection was found to decrease tumour volume in both U87LacZ and U251N xenografts (Figure 27A and C) and increased the number of cells undergoing apoptosis in the tumour area (Figure 29A). Other studies support this observation, for instance, Hong *et al*[205] observed higher numbers of TUNEL positive cells in mice implanted intracerebrally with U87 cells and treated with

recombinant human IFN-β protein. Adenoviral delivery of human IFN-β in human bladder cancer also showed an increase in TUNEL positive cells[219]. Hence the capacity of AAV-hIFN-β to reduce tumour volume and prolong survival *in vivo* is associated with its capacity to induce apoptosis *in vivo*, which is consistent with the *in vitro* data that show that it is capable of decreasing glioma cell viability.

*Anti-angiogenic properties of AAV-IFN-β*

Moreover, *in vitro*, AAV-IFN-β had decreased migration of HBECs, therefore, we also investigated if it could have an effect on the mouse vasculature *in vivo*. Although AAV-hIFN-β was unable to decrease blood vessel density in tumours, AAV-mIFN-β was able to do so (Figure 29B), which underscores the importance of species specificity in evaluating the therapeutic potential of IFN-β. Consistent with our studies, murine IFN-β was shown to decrease blood vessel density in a mouse model of human prostate cancer cells engineered to produce murine IFN-β[103]. Although, some other studies were not able to see an effect of murine IFN-β on mouse blood vessel density in the presence of a human breast carcinoma[376], or a mouse colorectal cancer liver metastases model[466]. On the other hand, studies have also noted effects of human IFN-β on the mouse vasculature. A significant decrease in mean intratumoural blood vessel density was observed in an immunocompetent model of melanoma treated with AAV-hIFN-β[459]. Mice implanted intracerebrally with U87 cells and treated with recombinant human IFN-β also had decreased blood vessel density, but no change in the level of VEGF or bFGF[205]. Adenoviral delivery of both human and murine IFN-β resulted in decreased microvessel density in an athymic mouse model of human bladder

cancer and was associated with decreases in bFGF and MMP-9[219]. Therefore, it is possible that the AAV-hIFN-β in our study may have aided to keep the tumour small by preventing further blood vessel migration or growth without a detectable change in blood vessel density. Alternatively, the differences between these studies and ours may be related to the mode of delivery, the tissue type, and/or vector type.

Interestingly, only when AAV-mIFN-β is applied before tumour implantation is there an anti-angiogenic effect in our athymic mouse model (Figure 29B). This may be due to the fact that mouse brain endothelial cells may be transduced and subject to the anti-proliferative effects of IFN-β, although *in vitro* analysis of AAV-GFP infection of HBECs shows that they are not readily infectable (data not shown). It is more likely that transfection of other non-tumour tissues such as neurons, which transduce easily by AAV-2[236], are secreting IFN-β, preventing endothelial cell migration towards the tumour. Nonetheless, given that mouse IFN-β has been shown not to have any anti-proliferative effects on human tumours *in vitro*[375], the observation that murine IFN-β was unable to decrease tumour volume in human glioma xenografts (Figure 29C) suggests that the anti-angiogenic effect of mouse IFN-β may be fairly weak. Without a human patient population, though, it is difficult to assess if human IFN-β may have stronger anti-angiogenic effects on human glioma endothelial cells than murine IFN-β has on the mouse brain endothelial cells.

*Immunostimulatory capacity of AAV-IFN-β*

Another anti-tumour property of IFN-β that we investigated with AAV-IFN-β is the known immunostimulatory capabilities of IFN-β. Although AAV-hIFN-β had not increased the number of immune infiltrates compared to untreated tumour control, likely due to reasons relating to species specificity and the low immunogenicity of rAAV, mouse NK cell numbers were increased by AAV-mIFN-β as evidenced by flow cytometric analysis of the GL261 immunocompetent syngeneic mouse model of glioma (Figure 30). IFN-β enhances the maturation of NK cells from a pre-NK cell pool and stimulates the secretion of pore-forming protein, which may contribute to tumour cell killing.

In a human clinical trial of melanoma using recombinant human IFN-β, increases in NK activity were also observed for the first 96h, but fell below pre-treatment levels after 4 weeks[121]. Other studies in immunocompetent mice also support our observations regarding the capacity for mouse IFN-β to induce NK cells. Ryuke *et al*[413] in an immunocompetent melanoma mouse model, noted NK cell induction by liposome-mediated interferon-β gene therapy without any significant difference in the number of T cells or macrophages compared to control. Systemic delivery of adenovirus expressing IFN-β led to tumour regression of colorectal liver metastases in a mouse model that was also attributed to NK cell activity and not T cells or macrophages[466]. We also did not observe an upregulation of macrophages or T cells in our study; however, other studies have reported an upregulation in response to IFN-β[246,333,551] likely due to variations in tissue type, or mode of IFN-β delivery. The capacity of mouse IFN-β to induce NK cells may also be applied to the athymic mouse model. When athymic mice bearing human

breast carcinoma tumours were treated with anti-asialo GM1, a substance that depletes NK cells, it severely reduced the anti-tumour effect of adenoviral delivered mouse-IFN-β[375]. In an intracerebral study in athymic mice by Mizuno *et al*[317], cationic liposomal delivery of human IFN-β also led to an induction of NK cells. Thus, our study and others substantiate the claim that IFN-β induces NK cells which may contribute to the anti-tumour mechanism in both immunocompetent and athymic mice.

Although our study did not reveal a difference in T cell numbers between AAV-mIFN-β treated animals and tumour only control animals (Figure 30), Natsume *et al*[332] using murine IFN-β gene transfer with cationic liposomes reported increased infiltration of cytotoxic T-lymphocytes after immunocytochemical analysis in a GL261 mouse model. They further demonstrated that *in vivo* depletion of CD8+, but not CD4+ cells, decreased the efficacy of liposomally delivered murine IFN-β [lip(psV2muIFN-β)][334]. In addition, by characterizing blastic cell lines generated from tumour infiltrating lymphocytes and spleen cells co-cultured with irradiated murine GL261 glioma cells, blastic cell lines of lip(psV2muIFN-β) treated animals were found to have specific cytotoxic activity against GL261 while blastic cell lines could not be induced from untreated mice [334]. When surviving animals at 50 days post-IFN-β treatment were re-challenged with either subcutaneous or intracerebral injections of GL261 no tumour developed for a further 50 days[334]. There are several reasons for why we may not have observed the same increase in T cells. Firstly, our method of analysis may not be as sensitive, for instance, it is possible that IFN-β may lead to high local

concentrations of T cells, but at numbers not high enough to detect by whole brain flow cytometry analysis. Alternatively, the T cells generated in our model of mice treated with AAV-mIFN-β may have higher tumour specificity than the T cells generated by untreated mice that also have tumours, but our methods are unable to detect this likelihood. Other reasons may be that the upregulation of T cell infiltrates may have occurred earlier or later than the time of sampling, or the increase may be a function of the combination of vector type and IFN-β. On the other hand, it might be advantageous that a large immune response is not elicited by AAV-IFN-β as one of our goals was to establish long term expression of IFN-β to prevent tumour recurrence. The mild immune response to AAV-IFN-β is in line with the recognized low immunogenicity of AAV. Although, AAV-GFP elicited an immune response by increased macrophages and NK infiltrates, this was likely due to the presence of the GFP protein as mouse and human AAV-IFN-β did not produce the same response (Figure 30). Given the prolonged survival of athymic mice treated with AAV-IFN-β in our study, which do not have a significant number of T cells, but have NK cells and macrophages, it can be speculated that in immunocompetent animals, T cells may not contribute to a large extent to the anti-tumour capacity of IFN-β.

*Comparison of AAV-IFN-β in other studies*

The use of AAV to deliver IFN-β intratumourally has been explored in other tumour models that consistently substantiate its clinical use. In one study, Yoshida *et al*[544] also used an intracerebral athymic mouse model, but with U251-SP (a subclone of U251) human glioma cells. In this model, $2x10^5$ tumour cells were

implanted followed seven days later with a regimen of intratumoural injection of AAV every other day. The mice were injected once or six times with virus. Similar to our study with U251N, mice treated intratumourally with AAV-hIFN-β in their study also had lower tumour volumes than mice treated with vector control, substantiating our results. One-time injection of AAV-hIFN-β into U251-SP tumours in their study led to a median survival of 63.7±15.3, whereas when it was injected six times, the median survival increased to >120 days. The lower survival for their mice injected once compared to our study, where 75% of animals were cured and no median was reached (Figure 28A), is likely due to the fact that they used U251-SP cells. Its parental cell line, U251, was shown *in vitro* in our study to be less sensitive than U87 cells to AAV-hIFN-β infection and to supernatants from AAV-hIFN-β infected 293A cells (Figure 25). Their mice also received a larger initial tumour burden, but the treatment was started earlier at 7 days post-tumour implantation instead of 10 days, as in our study. Still, this study by Yoshida *et al*[544] is consistent with our results in terms of the significant efficacy of AAV-humanIFN-β. Taken together, the pre-clinical data strongly support the value of AAV-hIFN-β for malignant glioma therapy and suggest that multiple injections may be more effective than a single injection.

Our study is the first to explore an intracerebral pre-treatment protocol with AAV injection first followed by implantation of U87 cells 10 days later. This was to examine the capacity of AAV-IFN-β to prevent tumour engraftment in the brain, which is important clinically to assess whether it could prevent tumour recurrence

in cases where patients have already undergone surgical removal. Furthermore, it was important to examine this paradigm intracerebrally because of the unique environment of the brain which would take into account the BBB and the immunologically distinct milieu. Indeed, AAV-hIFN-β in our study was fully capable of preventing U87LacZ tumour implantation in the brain leading to a cure rate of 50% at 365 days (Figure 28B). In contrast, U251N xenografts were not prevented from implanting (Figure 27C) likely due to their resistance to secreted IFN-β as observed *in vitro* (Figure 25A). Nonetheless, coherent with our U87LacZ xenograft results, pre-treatment studies with AAV-hIFN-β performed in non-intracerebral tumour models such as renal cell carcinoma, melanoma, and neuroblastoma, also prevented tumour engraftment[459,460]. In a study by Streck *et al*[459], subcutaneous U87 tumours failed to engraft when the hosts were treated with intravenous AAV-hIFN-β. However, subcutaneous U87 cells have a vastly different genetic profile and behaviour compared to intracerebral tumours[51,355] and cannot properly predict the activity of the virus in the brain. Furthermore, the tail vein injections of AAV explored by Streck *et al*[459] would not be efficacious in the case of a brain tumour. Instead, to increase vector spread vascular injections via the intracarotid artery would allow better proximity to the tumour mass to circumvent pharmacokinetic issues regarding the short half-life of this cytokine and would increase bioavailability. Nonetheless, the fact that Streck *et al*[459] was able to deliver AAV via tail vein injection provides evidence that intracarotid injection of AAV-hIFN-β would be able to persist and target disseminated tumour cells. AAV-hIFN-β, thereby, clearly has an ability to prevent tumour implantation

for multiple tumour types. Future studies will also explore a vascular route of delivery to increase vector spread.

*Other vectors utilizing the IFN-β transgene*

AAV was not the only vector tested to deliver IFN-β. For example, retrovirus has been used to deliver IFN-β[104], but due to its risk for insertional mutagenesis and formation of novel malignancies, it is not ideal as a cancer therapeutic. On the other hand, adenoviral and non-viral vectors are currently being tested for the delivery of IFN-β to tumours in clinical trials. It is important to keep in mind, however, the clinical trials for adenoviral and non-viral cationic liposomal delivery of IFN-β are delineated for intratumoural delivery[111,543], which is not our sole objective. One of our main goals is to create a peritumoural site for IFN-β production to prevent future implantation after surgical resection.

To provide perspective regarding the relative efficacy of our AAV-hIFN-β vector we examined the pre-clinical work done by Qin *et al*[375,376] using recombinant adenovirus expressing human IFN-β that eventually led to a phase I clinical trial protocol by Eck et al[110]. Qin *et al*[375,376] demonstrated 100% survival at 100 days post virus treatment in a subcutaneous human glioma xenograft model. At 100 days, our much more stringent intracerebral tumour model also exhibited 100% survival with a single injection of virus and we carried our survival studies to one year. At one year, our surviving mice were healthy and active suggesting that the mice did not have any remaining tumour and did not suffer long lasting side effects from the vector. Furthermore, it is important to mention that adenovirus

was not our choice of vector to achieve peritumoural production of IFN-β. In order for peritumoural production to take place, normal non-tumour tissue would need to be infected with virus and employed to produce IFN-β protein. Since adenovirus is known to elicit an immune response, inducing inflammation in non-tumour tissue is not a favourable side effect. AAV also has the potential to offer much longer term expression than adenovirus due to its low immunogenicity. Therefore, AAV-hIFN-β is comparable, if not, possibly more effective, than adenoviral delivery of IFN-β by offering many more advantages including decreased inflammation and longer gene expression.

Cationic liposomes, which are thought to produce less inflammation than viruses, have also been used in a preliminary clinical trial to deliver IFN-β intratumourally to malignant gliomas with some success[543]. In this study, transgene expression and antitumour activity were detected in four of five patients. Two patients had stable disease 10 weeks following commencement of therapy, and two other patients showed partial response with >50% tumour reduction. Side effects were limited to mild headache, and brain edema. These results are promising as they suggest the feasibility and safety of IFN-β therapy in the brain, but considering the higher transfection efficiency and expression levels of viral vectors, it is possible that AAV delivery may be able to further improve results. For instance, when cells were treated *in vitro* with the IFN-β cationic liposomes used in the clinical trial mentioned above, liposomes were seen in the cytoplasm of all cultured human glioma cells, but transduction efficiency was limited to approximately 10 to 20% of the cells over 36 to 48h[543]. On the other hand, AAV

can achieve close to 100% transduction efficiency at a MOI of 1000 vector genomes in U87 cells (data not shown). Persistent gene expression is also not possible with cationic liposomes. In the liposome clinical study mentioned above, expression of IFN-β reached a maximum at approximately four days in most patients, and the liposomes had to be injected stereotactically every week, which was impractical[543]. On the other hand, long term gene expression of a single administration of AAV encoding interferon-alpha of at least one year was reported in the serum of immunocompetent mice[100] and we have shown that a single injection of AAV-hIFN-β can prolong survival in our human xenograft model.

*Clinical potential of AAV-IFN-β*

Clinically, AAV-IFN-β treatment holds the greatest potential as an adjuvant to neurosurgical techniques following resection as it has proven to prevent tumour implantation and cause tumour regression in an *in vivo* athymic mouse model. Tumours that are otherwise inaccessible by surgery could also be treated with intratumoural injections of AAV-IFN-β. Nevertheless, neurotoxicity from increased IFN-β concentrations is an important concern. In a phase I/II clinical trial that involved intravenous delivery of recombinant IFN-β (Betaseron) in patients with recurrent malignant gliomas, side effects included fever, chills, headache, fatigue, dementia, disorientation, personality change, myaglia, and arthralgia where severe malaise tended to have a cumulative effect and was the most common cause of dose reduction[549]. In fact, two patients are believed to have died due to treatment related complications. The maximum tolerated dose

for Betaseron was found to be 180-360x$10^6$ U with neurotoxicity being more problematic at higher doses. Unfortunately, no complete responses were reported and stabilization was short-lived; the median time to progression for all responders was 24 weeks. It is important to consider that these toxicities are related to systemic delivery of IFN-β where large doses of recombinant IFN-β protein were required to overcome the pharmacokinetic issues associated with the short serum half-life of IFN-β.

Gene delivery of IFN-β, on the other hand, would allow for concentrated local production of cytokine that would also help to decrease side effects related to systemic delivery and increase the local therapeutic dose. Indeed, a prospective phase I/II clinical trial using intratumoural cationic liposome delivery of IFN-β demonstrated no toxicity attributable to the liposomes (mild side effects such as headache and brain edema were observed)[543]. Toxicity in non-human primates using adenoviral delivery of IFN-β showed that it produced localized, transient cerebral edema, and minor inflammation was observed in the contralateral brain where a vector control was administered[110], but overall the adenovirus vector was well tolerated. The favourable tolerance to intratumoural gene delivery of IFN-β indicates that it may be safer than systemic delivery of IFN-β protein. Furthermore, the highest concentration of IFN-β protein detected in the tumour bed after cationic liposome delivery in the study mentioned earlier was 24 U/mL[543], suggesting that much lower doses of locally produced IFN-β protein can lead to a therapeutic effect when compared to the 180-360x$10^6$ U Betaseron that was delivered systemically that led to unfavourable side-effects. Thus,

intratumoural and peritumoural delivery of AAV-hIFN-β may offer a much better alternative to recombinant IFN-β protein, adenoviral vectors, and cationic liposomes. AAV gene transfer could decrease side effects related to high doses of systemic recombinant IFN-β protein, lessen the inflammation posed by adenovirus, and could be administered by a single virus injection without requiring frequent, repeated injections as cationic liposomes require.

Moreover, the anti-cancer effects of IFN-β can combine with several conventional chemotherapeutic agents, such as nitrosoureas, and with radiotherapy[542]. Although, an *in vitro* study of the combination of IFN-β and radiosensitization reported mainly infra-additive effects in human glioblastoma cell lines[430], suggesting that IFN-β and radiotherapy may not offer a large benefit. Nevertheless, 5-FU combined with adenoviral delivery of IFN-β resulted in long term survival in a mouse model of colorectal liver metastases[65]. In a phase II study, IFN-β was combined with nimustine hydrochloride (ACNU) and radiotherapy after hyperbaric oxygenation with 50% response rate in the glioblastoma group[15]. Interferon-β plus ranimustine (MCNU) and radiotherapy was also tested in clinical trials for gliomas[503]. However, all patients in these clinical trials eventually succumbed to their tumours[15,503]. Prospective applications of AAV-IFN-β could include its use with conventional therapies to further increase the likelihood of a favourable therapeutic outcome.

Other strategies to boost tumour cell killing by IFNs have resorted to administering IFN-β or -α in combination with dendritic cells (DCs) into gliomas

to augment CTL specific tumour killing[326,484]. This resulted in increased antigen presentation of glioma-derived antigens to specific T cells by the DCs and enhanced CTL specific tumour cell lysis[326,484]. Future studies will involve a combination of AAV-hIFN-β and chemotherapeutic agents such as 5-FU, or DCs to augment immunotherapy.

We show here that AAV-hIFN-β may prove to be a powerful clinical adjuvant aiding in a cure for malignant gliomas. It is our hope that the ability of AAV-IFN-β to induce tumour regression, prevent tumour engraftment, prolong survival, and cure animals in our mouse model can be translated into reliable human clinical responses.

**Final Conclusion and Summary**

The search for alternative forms of therapy for malignant gliomas is a desperate circumstance as current therapies only marginally improve survival. In this thesis, we have explored several exciting new gene therapy strategies: targeted therapy, suicide gene therapy and cytokine therapy. AdFOXO1;AAA has demonstrated that it can restore apoptosis and cell cycle arrest; Ad(dPS)CU-IRES-E1A has shown that it is more efficacious than non-replicating first generation adenoviruses and possesses an incredible bystander effect; and AAV-IFN-β treatment has shown that it not only causes tumour regression, but can also prevent tumour engraftment. Most importantly, each has proven to be effective in prolonging survival in our mouse models. Some key considerations to further improve the success of these gene therapy strategies include increasing vector

tumour specificity by changing the promoter region to an E2F responsive element; enhancing vector spread by intracarotid delivery; and augmenting transduction efficiency by altering the virus tropism, for instance, by using Ad35 instead of Ad5. The ultimate test, however, are human clinical trials. In conjunction with standard therapies, such as chemotherapy, radiotherapy, and surgery these gene therapy strategies considered here have enormous potential to provide new and hopefully curative treatment options for patients with malignant gliomas.

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# Appendix: Research Compliance Certificates

U Aug 9'06
APPROVED

www.mcgill.ca/research/compliance/animal/forms/

# McGill University Animal Care Committee
# RENEWAL of Animal Use Protocol

For: Research ☒ Teaching ☐ project

For Office Use Only:
Protocol #: 4971
Approval end date: Dec. 31, 2006
Facility Committee: MNI
Renewal#: 1st 2nd

| | | | |
|---|---|---|---|
| **Principal Investigator:** | Josephine Nalbantoglu, PhD | **Protocol #** | 4971 |
| **Protocol Title:** | Gene therapy of experimental brain tumours | **Phone:** | 514-398-5920 |
| **Unit, Dept. & Address:** | MNI, 3801 University Street, Montreal | **Fax:** | 514-398-7371 |
| **Email:** | josephine.nalbantoglu@mcgill.ca | **Level:** C | **Funding source:** CIHR 20244 20511 20549 |
| **Start of Funding:** | 10/2004 | **End of Funding:** | 03/2008 |
| **Emergency contact #1 + work AND home phone #s:** | Zafiro Koty - 221-0742 (pager) | | |
| **Emergency contact #2 + work AND home phone #s:** | Josephine Nalbantoglu - 398-5920; 341-2784 | | |

## 1. Personnel and Qualifications

List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/graduate student, fellow). If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to *www.ahlm/care.mcgill.ca* for details. Each person listed in this section must sign. *(Space will expand as needed)*

| Name | Classification | Animal-Related Training Information | Occupational Health Program* | Signature *"Has read the original full protocol"* |
|---|---|---|---|---|
| Josephine Nalbantoglu, PhD | PI | Theory course 2005 (won't handle animals) | | |
| Zafiro Koty, BSc | Technician | Mouse and Rat workshops 2005 | | |
| Cara Lau, BSc | Grad student | Mouse workshop 2005 (won't handle rats) | | |
| Vinit Srivastava, MSc | Grad student | Mouse workshop 2005 (won't handle rats) | | |
| David Huang, MSc | Grad student | Mouse workshop 2005 (won't handle rats) | | |

* Indicate for each person, if participating in the local Occupational Health Program, see http://www.mcgill.ca/research/compliance/animal/occupational/ for details.

*Approved by:*

## 2. Approval Signatures

| | | |
|---|---|---|
| **Principal Investigator/ Course Director** | | **Date:** 16 JAN. 2006 |
| **Chair, Facility Animal Care Committee** | | **Date:** Jan. 16, 2006 |
| **UACC Veterinarian** | | **Date:** 11 JAN 2006 |
| **Chairperson, Ethics Subcommittee** *(D level or Teaching Protocols Only)* | — | **Date:** — |
| **Approved Animal Use Period** | **Start:** Jan 1, 2006 | **End:** Dec 31, 2006 |

## 3. Summary (in language that will be understood by members of the general public)

**AIMS AND BENEFITS:** Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge *(was section 5a in main protocol)*.

Malignant brain tumours represent a relatively common fatal disease. Despite advances in neurosurgery and radiotherapy, life expectancy of patients suffering from brain tumours has not increased over the past 30 years. Newly developed gene therapy approaches combined with conventional therapy promise to be a major advance in

McGill University

# APPLICATION TO USE BIOHAZARDOUS MATERIALS*

Projects involving potentially biohazardous materials should not be commenced without approval from Environmental Health & Safety. Submit applications before 1) starting new projects, 2) renewing existing projects, or 3) changing the nature of the biohazardous materials within existing projects.

1. PRINCIPAL INVESTIGATOR: Josephine Nalbantoglu PHONE: 398-5920
DEPARTMENT: Neurology & Neurosurgery FAX: 398-7371
ADDRESS: Montreal Neurological Institute, 3801 University E-MAIL: josephine.nalbantoglu@mcgill.ca
PROJECT TITLE: Gene therapy of experimental brain tumours

2. EMERGENCY: Person(s) designated to handle emergencies
Name: Josephine Nalbantoglu Phone No: work: 398-5920 home: 341-2784
Name: Zivart Yasruel Phone No: work: 398-8534 home: 336-8979

3. FUNDING SOURCE OR AGENCY (specify): CIHR
Grant No.: Beginning date: 01.04/2005 End date: 31.03/2008

4. Indicate if this is
☒ Renewal: procedures previously approved without alterations.
Approval End Date: 30.09.2005
☐ New funding source: project previously reviewed and approved under an application to another agency.
Agency: Approval End Date:
☐ New project: project not previously reviewed.
☐ Approved project: change in biohazardous materials or procedures.
☐ Work/project involving biohazardous materials in teaching/diagnostics.

CERTIFICATION STATEMENT: Environmental Health & Safety approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in Health Canada's "Laboratory Biosafety Guidelines" and in the "McGill Laboratory Biosafety Manual".

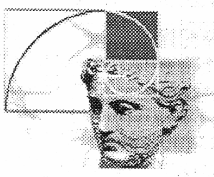
Containment Level (select one): ☐ 1 ☒ 2 ☐ 2 with additional precautions ☐ 3

Principal Investigator or course director: SIGNATURE date: 14 (day) 12 (month) 2005 (year)

*Approved by Environmental Health & Safety:* SIGNATURE date: 20 (day) 12 (month) 05 (year)

Expiry: 31 (day) 03 (month) 08 (year)

*as defined in the "McGill Laboratory Biosafety Manual"



# Montreal Neurological Institute and Hospital

A Teaching and Research Institute at McGill University

# Internal Radioisotope User Permit

Issued by:

**The Radiation Safety Committee of the Montreal Neurological Institute and Hospital**

**Authorized by the Canadian Nuclear Safety Commission**

**CNSC Radioisotope Licence Number: 01187-2-08.5**

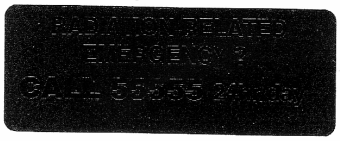
| | | | | |
|---|---|---|---|---|
| 1. | Radioisotope User Permit Number | : | **MNI_001 (Rev.2)** | RSO Office: 398-8927 |
| | **Classification** | : | **Basic** | RSO Pager: 406-3069 |
| | Date of Issue | : | May 1, 2003 | Locating: 8888-53333 |
| | Revision Date | : | August 1, 2006 | Home: 525-0220 |
| | Expiry Date | : | April 30, 2008 | |
| 2. | Name of Principal Investigator | : | **Antel, Jack** | |
| 3. | **Department** | : | **Neuroimmunology** | |
| 4. | **Location(s) approved by this permit** | : | **WB010 e** | |
| 5. | Radioisotopes approved by this permit | : | See non-shaded cells in table below. | |

**Note: The permit holder needs written authorization by the CNSC for projects requiring more than 10,000 exemption quantities (E. Q.) of a radioactive substance.**

| approved for use of: | $^{3}H$ | $^{11}C$ | $^{14}C$ | $^{18}F$ | $^{32}P$ | $^{35}S$ | $^{45}Ca$ | $^{51}Cr$ | $^{125}I$ |
|---|---|---|---|---|---|---|---|---|---|
| 10'000 exemption quantities (E. Q) | 10 TBq or 270 Ci | 1 GBq or 27m Ci | 1 TBq or 27 Ci | 0.1 GBq or 2.7 mCi | 100 MBq or 2.7 mCi | 1 TBq or 27 Ci | 10 GBq or 270 mCi | 10 GBq or 270 mCi | 10 GBq or 270 mCi |
| **your possession limit is:** | **1 GBq or 27mCi** | 370 MBq or 10 mCi | 370 MBq or 10 mCi | 370 MBq or 10 mCi | | 370 MBq or 10 mCi | | **370 MBq or 10 mCi** | 370 MBq or 10 mCi |

6. Personal Dosimeters Required :YES

7. Method of Disposal




: All radioactive waste (solid and liquid) must be disposed of through the containers provided by the McIntyre Waste Management Facility (McGill) and brought to the central waste storage cage in the basement of the MNH/I (room 045 B). RTS procedures have to be followed.

8. Special Conditions:

| |
|---|
| Gloves and lab coats mandatory. |
| Weekly wipe tests required in areas where radioisotopes are used. |
| Ring badges required for staff using > 50MBq (~1.5mCi) of $^{32}P$ |
| Monitoring of all work surfaces where $^{32}P$ is used at the end of the work day. |
| Radio-iodinations: Must be carried out in a working fume hood. Schedule thyroid monitoring. Use of proper survey equipment during radio-iodine manipulations. |

The Radiation Safety Officer, MNH/I: E. Meyer (E.Meyer, RSO, ext. 8927)

Montreal Neurological Institute and Hospital

A Teaching and Research Institute at McGill University

# Internal Radioisotope User Permit

Issued by:

**The Radiation Safety Committee of the Montreal Neurological Institute and Hospital**

**Authorized by the Canadian Nuclear Safety Commission**

**CNSC Radioisotope Licence Number: 01187-2-08.4** (or any revisions thereof)

1. Radioisotope User Permit Number : **MNI_014 (Rev.2)**
   **Classification** : Basic
   Date of Issue : May 1, 2003
   Revision Date : October 27, 2005
   Expiry Date : April 30, 2008

2. Name of Principal Investigator : **Nalbantoglu, Josephine**

3. **Department** : **Neuroimmunology**

4. **Location(s) approved by this permit** : **WB 010 (d, e, f )**

5. Radioisotopes approved by this permit : See non-shaded cells in table below.

**Note: The permit holder needs written authorization by the CNSC for projects requiring more than 10,000 exemption quantities (E. Q.) of a radioactive substance.**

| approved for use of: | $^{3}H$ | $^{11}C$ | $^{14}C$ | $^{18}F$ | $^{32}P$ | $^{35}S$ | $^{45}Ca$ | $^{51}Cr$ | $^{125}I$ |
|---|---|---|---|---|---|---|---|---|---|
| 10'000 exemption quantities (E. Q) | 10 TBq or 270 Ci | 1 GBq or 27mCi | 1 TBq or 27 Ci | 0.1 GBq or 2.7 mCi | 100 MBq or 2.7 mCi | 1 TBq or 27 Ci | 10 GBq or 270 mCi | 10 GBq or 270 mCi | 10 GBq or 270 mCi |
| your possession limit is: | 1 GBq or 27mCi | 370 MBq or 10 mCi | 370 MBq or 10 mCi | 370 MBq or 10 mCi | **370 MBq or 10 mCi** | 370 MBq or 10 mCi | 370 MBq or 10 mCi | 370 MBq or 10 mCi | 370 MBq or 10 mCi |

6. Personal Dosimeters Required : YES

7. Method of Disposal : **All radioactive waste** (solid and liquid) must be disposed of in compliance with RTS rules using the containers provided by the McIntyre Waste Management Facility (McGill) and brought to the central waste storage cage in the basement of the MNH/I (room 045 B).

RSO Office: 398-8927
RSO Pager: 406-3069
Locating: 8888-53333
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