

A novel double-targeted bacteriophage vector for systemic cancer gene therapy

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Declaration of Own Work

This thesis describes work undertaken in laboratory of Dr. Amin Hajitou at the Imperial College London, in fulfilment of the requirement for the degree of Doctor of Philosophy.

This thesis describes the outcome of my own work. Any work done as a part of collaboration is noted in the text.

No part of this thesis has already been, or is presently being, submitted for any other degree.

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Abstract

A successful cancer gene therapy requires systemic gene delivery vectors capable of efficient and selective gene transfer to tumour sites. Two main approaches have been undertaken for targeted cancer gene therapy: i) restriction of transgene expression in tumour cells by using tumour specific promoters and ii) selective delivery of vectors of gene therapy to tumours by ligand targeting. Amongst many characterised tumour specific promoters, the stress-inducible promoter of the *Glucose-regulated protein 78 (Grp78)* gene holds great potential to improve the practice of cancer gene therapy as its activity is highly induced under conditions often found in the tumour microenvironment such as acidic pH, glucose deprivation and chronic anoxia. However, the clinical application of the *Grp78* promoter in cancer gene therapy has been limited by lack of delivery strategies via the systemic routes. In 2006, an efficient tumour targeted vector, adeno-associated virus/phage termed AAV/Phage or AAVP, was reported. In this AAVP vector, the M13 bacteriophage (phage) was used as a vehicle to target α_v integrin receptors, which are selectively overexpressed in tumours, and to deliver the AAV transgene cassette driving transgene expression from a *cytomegalovirus (CMV)* promoter. Systemic administration of the targeted AAVP viral phage vector has been shown to successfully mediate therapeutic transgene expression in several preclinical tumour models. Thus, the AAVP particle provides a suitable vector candidate for double targeting of cancer after systemic administration. Herein, I combined ligand-directed targeting of AAVP vector with transcriptional targeting using the *Grp78* promoter in one single platform. In the first half of the thesis I show that the double-targeted vector provides sustained and long-term transgene expression in transduced tumour cells *in vitro*. Moreover, compared to the conventional phage carrying the *CMV* promoter, the new hybrid phage provided a significant tumour killing both *in vitro* and *in vivo* using the *HSVtk* gene and ganciclovir suicide therapy. More importantly in chapter 5, I describe a novel mechanism of *Grp78* promoter activation by *HSVtk* and ganciclovir suicide therapy. In chapter 6, in addition to the vector safety, I show the superior tumour transduction *in vivo* after systemic administration of double-targeted AAVP vector over the original AAVP carrying the *CMV* promoter. In Chapter 7, I demonstrate strong dose dependent chemotherapeutic induction of the *Grp78* promoter in the context of AAVP vector. Together, all data presented in this thesis validate the great potential of the *Grp78* promoter in targeted systemic cancer gene therapy and report the efficacy of the double-targeted AAVP vector which proves useful for translation into clinical applications.

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Abbreviations

AAV	Adeno-associated virus
AAVP	Adeno-associated virus/Phage
Amp	Ampicillin
APS	Ammonium persulphate
ATF6	Activating transcription factor 6
Bp	base pair
BSA	Bovine serum albumin
CMV	Cytomegalovirus
Da	Dalton
dH ₂ O	Deionized water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eIF2 α	Eukaryotic initiation factor 2 α
ER	Endoplasmic reticulum
ERSE	Endoplasmic reticulum stress element
EtBr	Ethidium bromide
FBS	Fetal bovine serum
GCV	Ganciclovir
HAT	Histone acetyltransferase
HDACs	Histone deacetylase enzymes
HSVtk	Herpes Simplex Virus thymidine kinase
IRE1	Inositol requiring enzyme 1
ITRs	Inverted terminal repeats
GFP	Green fluorescent protein
Grp78	Glucose-regulated protein 78
Kan	Kanamycin

Kb	kilobase
kDa	kilo Daltons
LTR	Long-terminal repeat
MCS	Multiple cloning site
MFI	Mean fluorescent intensity
Min	minute
mRNA	messenger RNA
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PERK	Protein kinase-like ER kinase
RLU	Relative Luminescence Units
ROI	Region of interest
Puro	Puromycin
rpm	revolutions per minute
SAHA	Suberoylanilide hydroxamic acid
SDS	Sodium dodecyl sulphate
sec	second(s)
TAE	Tris-acetate EDTA
TEMED	Tetramethylethylenediamine
Tet	Tetracycline
TG	Thapsigargin
TNF α	Tumour necrosis factor- α
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
TSA	Trichostatin-A
UPR	Unfolded protein response
VPA	Valporic acid
v/v	volume per unit volume
w/v	weight per unit volume
XBP1	X-box binding protein 1
5-Aza	5-Azacytidine

Chapter 1

Introduction

1.1 Treatment of cancer

Cancer is a large group of diseases that develop by the rapid unregulated growth of cells. Cancer cells can invade neighbouring tissue and/or spread to other organs through the bloodstream or lymphatic system. This process, which is referred to as metastasis, results in the most aggressive types of cancer and therefore has the highest mortality rates. Cancer is a leading cause of death worldwide, with an estimated 7.9 million deaths reported in 2010; it has been predicted to rise to approximately 13.1 million deaths by 2030 (Jemal et al., 2011).

In addition to surgery, conventional cytotoxic therapies, such as chemotherapy and radiation, are currently used for cancer treatment. However, as most cancer cells are resistant to apoptosis and can remove chemotherapeutic drugs from their local environment, conventional therapies have shown limited success, especially against solid, metastatic cancers (Liu, 2009). Moreover both therapies often exhibit high toxicity with a wide variety of severe side effects. In addition, most of the chemotherapeutic drugs circulate around the entire body and in fact the dose that reaches the tumour cells (normalized per gram of tissue) may be as little as 5–10% of the dose that accumulates in normal organs (Bosslet et al., 1998). Thus development of targeted anticancer drugs is arguably one of the most important goals of current anticancer research as this drug release at the tumour tissue will reduce toxicity.

In the past two decades many novel cancer treatments have been developed with the aim to deliver therapeutic drugs directly to tumour tissue and thus to reduce the toxicity for normal

tissues- an objective that conventional therapies have failed to achieve. One promising area with fast-expanding clinical trial data is cancer gene therapy which mostly utilizes vectors derived from viruses.

1.2 Cancer gene therapy

Gene therapy aims to transfer exogenous genes, called transgenes into the diseased cells in order to achieve a therapeutic effect. First developed in 1970s as a gene replacement tool (Friedmann, 1992), gene therapy has been utilised in numerous cancer research projects with the aim of developing efficient vectors that both can be safely administrated and tolerated by the human body and can discriminate between normal and tumour tissues. To date, due to lack of an efficient vector able to target therapeutic transgenes to the tumour tissue after systemic administration, most clinical trials have focused on direct intratumoural administrations of either viral or nonviral vector systems. A local delivery of the transgene is necessary as proof-of-principle, but real clinical benefit can only follow systemic administration in order to reach both metastases and some of the primary tumours that necessitate invasive procedures for local access and delivery.

Indeed, a successful clinical gene therapy will depend on the development of efficient systemic vectors. Such vectors also need to be safe after systemic administration, so that the appropriate genes can be delivered to and be expressed in target cells only. It is well known that the main challenges of clinical gene therapy trials are vector targeting and the high cost of vector production. Therefore development of efficient targeted vectors that mediate efficient and long-term expression of the therapeutic gene in tumour tissues after systemic administration presents an interesting approach to cancer gene therapy.

To date, two main approaches have been used for targeting: i) transcriptional targeting that uses promoters that are active only in the target tumour cells and ii) ligand-targeting of vectors. Each of these two approaches have been attempted individually and showed promising results for vector delivery and transgene expression in preclinical tumour models. Combining these two strategies in one vector system is challenging but would provide a major advance in cancer gene therapy.

1.2.1 Transcriptional targeting

Selective targeting and killing of tumour cells is a major goal of current cancer gene therapy. One targeting approach is to use specific promoters that are active in the tumour tissue only (Sadeghi and Hitt, 2005). The use of such promoters in cancer gene therapy will lead to transcriptional targeting, which can reduce or even eliminate potential toxic side effects of the therapeutic gene in normal tissues. One of the common promoters used in gene therapy vectors is the *cytomegalovirus* (CMV) promoter, which can constitutively express the transgene at high levels. Nonetheless, its non-specific and unregulated activity may become problematic in the context of cancer gene therapy since the expression pattern of the transgene will be constitutive in normal as well as in tumour tissues.

On the other hand, promoters which are active in tumour cells only have been used for targeted gene delivery to tumours in order to deliver transgene expression to the tumour tissue only while sparing the normal organs (Robson and Hirst, 2003).

Transcription targeting was first established by two studies in 1997. Rodriguez *et al.* have restricted transgene expression in prostate-specific antigen (PSA) producing prostate cells by applying the prostate-specific antigen promoter into adenovirus type 5 DNA to drive transgene expression which they referred to as “attenuated replication-competent adenovirus” (Rodriguez *et al.*, 1997). Moreover, using the albumin promoter, Miyatake *et al.* could restrict the replication of the herpes simplex virus type 1 (HSV-1) vector to albumin-expressing liver cells (Miyatake *et al.*, 1997). Since then, numerous types of promoters have been characterised and used in targeted cancer gene therapy, each of which are discussed in the following sections.

1.2.1.1 Tissue-specific promoters

This group of promoters are active, and mediate transgene expression, in specific tissues only. Several tissue-specific promoters that target tumour of a single origin were characterized and used in cancer gene therapy (Robson and Hirst, 2003). Examples include the ovarian-specific promoter to target ovarian cancer (Bao *et al.*, 2002, Selvakumaran *et al.*, 2001), albumin promoter to target hepatocellular carcinoma (Su *et al.*, 1997), and the thyroglobulin promoter for thyroid carcinomas (Zhang *et al.*, 2001). Moreover, the tyrosinase promoter has been used both *in vitro* and *in vivo* to target melanocytes and melanomas (Vile and Hart, 1993, Fecker *et al.*, 2011).

The *PSA* promoter was used in targeting prostate cancer (Wu et al., 2001, Lu and Madu, 2010, Latham et al., 2000). *PSA* is predominantly expressed in prostate cells due to transcriptional activation. The *PSA* promoter has been previously shown to express Herpes Simplex Virus thymidine kinase (*HSVtk*) suicide gene in *PSA*-positive prostate cancer cells and prostate tumours. However, no transgene expression was observed in cells that do not express *PSA* (Gotoh et al., 1998).

Although these promoters proved efficient to induce transgene expression in tumour cells, their activity in both normal and tumour cells is a major drawback.

1.2.1.2 Tumour-specific promoters

Tumour-specific promoters constitute an ideal choice for targeted cancer gene therapy to direct the expression of therapeutic genes. They have been shown to be highly active in tumour cells while having little or no activity in normal cells. Based on their characteristics, tumour-specific promoters have been subdivided into 4 groups (Harrington et al., 2000, Robson and Hirst, 2003): cancer-specific promoters, tumour-type-specific promoters, tumour-vasculature-related promoters, and tumour-microenvironment-related promoters.

1.2.1.2.1 Cancer-specific promoters

Cancer-specific promoters, such as the promoter of the telomerase gene, are specifically active in malignant cells and have great potential in cancer gene therapy to target a wide variety of tumours. According to previous reports, telomerase is highly active in around 85-90% of human cancer cells, while in normal tissues its activity is either low or undetectable (Kim et al., 1994, Broccoli et al., 1995). Telomerase is composed of two subunits- the human telomerase RNA (*hTR*) and a catalytic component called human telomerase reverse transcriptase (*hTERT*)- both of which are required for its activity (Shay and Wright, 2001). The promoters of these two subunits are highly active in cells that are positive for telomerase activity such as tumour and fetal cells. Thus, both of these two promoters can be used in many targeted cancer gene therapy studies to drive therapeutic gene expression. The enhanced killing of telomerase-positive cells upon utilization of either *hTR* or *hTERT* promoters in tumour cells was reported in several studies (Song and Kim, 2004, Plumb et al., 2001). Although the *hTR* or *hTERT* promoters have been broadly utilized for transcriptional regulation of therapeutic genes, they have some limitations for clinical use. For instance, they

possess low activity, and some potential toxicity to certain normal cells have been reported (Xiong et al., 2012).

1.2.1.2.2 Tumour-type-specific promoters

Tumour-type-specific promoters are the promoters of oncofetal genes that are often overexpressed in certain types of tumours and are silent in normal tissues. Examples of the most well-characterised promoters of this group include the promoter of the gene encoding α -fetoprotein, which has been found to be active in fetal liver and hepatocellular carcinomas (Ishikawa et al., 1999), and the promoter of the gene for the carcinoembryonic antigen (CEA), which is active in a proportion of breast, lung, colorectal and pancreatic cancers (Richards et al., 1995). The CEA promoter was extensively used in different vector systems to selectively deliver various therapeutic genes such as the cytosine deaminase (CD) gene or *HSVtk* in CEA- positive cells. The outcome was significant tumour growth suppression or regression with no toxicity to the liver and other normal organs (Brand et al., 1998, Cao et al., 1999, Zhang et al., 2009a, Richards et al., 1995).

Although these promoters mediate transgene expression in tumour tissues and may therefore be good candidates for transcriptional targeting in cancer gene therapy, their application remains limited because they cannot be administered to a variety of tumours.

1.2.1.2.3 Tumour-vasculature-related promoters

Another group of promoters used in targeted cancer gene therapy is tumour-vasculature-related promoters as their genes are overexpressed in proliferating endothelium of the tumour blood vessels. Examples of such promoters that have been used in targeted gene therapy are E-selectin and endothelial-specific kinase insert domain receptor (KDR/flk-1), which are upregulated in tumour endothelium. The promoter of E-selectin and KDR/flk-1 were used to drive the expression of an inflammatory cytokine, tumour necrosis factor- α (TNF- α) and results shown 10- fold increase of transgene expression in endothelioma cells compared to non-endothelioma cells (Jaggar et al., 1997). In addition, the promoters of genes encoding vascular endothelial growth factor receptor (Flt-1), and human preproendothelin-1 have also been used in gene therapy vector systems and proved efficient at driving transgene expression in the vasculature of tumours and metastases (Varda-Bloom et al., 2001, Bauerschmitz et al., 2002). While the usage of these promoters in targeted cancer gene therapy proved efficient to

drive transgene expression in tumour vasculature, their limitation still remains as some of these promoters were shown to be active in normal small vessels and also upregulated in injured vessels (Herz et al., 2012).

1.2.1.2.4 Tumour-microenvironment-related promoters

Tumour-microenvironment-related promoters belong to the genes that are upregulated in response to the tumour microenvironment and physiology. Solid tumours intend to grow faster than their blood vessels leading to an inadequate supply of vital nutrition to the tumour cells. This condition creates a unique tumour microenvironment characterized by hypoxia, acidosis and glucose deprivation which persist in poorly vascularized solid tumours. As part of the cancer cell's adaptation to these conditions, the promoters of some genes, such as hypoxia response elements (HREs) and some of the heat shock family genes, become induced.

Recently, the heat shock genes have been of increasing interest in targeted cancer gene therapy because of their activation in a wide variety of tumours, such as the genes of the *Glucose-regulated protein 78 (Grp78)*, the promoter which responds to glucose starvation and hypoxia. This promoter's activity and its ability to drive transgene expression within areas of tumour hypoxia- which are highly resistant to current forms of treatment- make it even more attractive for use in targeted cancer gene therapy. Indeed, therapeutic transgene expression driven by this promoter is induced in response to insufficient blood supply and tumour necrosis, and reaches high levels leading to complete tumour eradication in preclinical models (Dong et al., 2004, Chen et al., 2000).

For cancer targeting gene therapy, the *Grp78* promoter seems to be an ideal promoter to restrict expression of the therapeutic gene within the tumour tissue and was therefore selected for our studies.

1.2.1.2.4.1 Grp78

The *Grp78* gene encodes a 78-kDa protein – the stress-response chaperone glucose-regulated protein 78 (Grp78). Grp78 gets its name from the discovery in the late 1970s, that upon rapid glucose removal from the culture medium of chick embryo fibroblasts a rapid increase in a protein with a molecular weight of 78-kDa was spotted (Lee, 2001). Grp78, also referred to as BiP (immunoglobulin heavy chain binding protein) (Lee, 2001), is encoded by a single

copy gene in both human and mouse genomes (Ting and Lee, 1988). With similar function and 60% amino acid homology to the 70kDa heat shock protein (HSP70), Grp78 has been categorized within the HSP70 family (Munro and Pelham, 1986). However in contrast to HSP70, Grp78 is not induced by heat stress. Moreover, whereas HSP70 mainly localises in the cytoplasm of cells, the endoplasmic reticulum (ER) retention signal peptide KDEL located in the carboxy-terminus of Grp78, primarily localises this chaperone protein into the ER lumen where it retains its functions as a molecular chaperone in an ATP-dependent manner (Dorner et al., 1990).

Grp78 plays an important role in orchestrating the unfolded protein response (UPR) by binding and assembling unfolded or misfolded membrane and secretory proteins within the ER lumen as well as degrading irreversibly misfolded proteins (Li and Lee, 2006, Zhang and Zhang, 2010, Lee, 2007). Moreover by binding to Ca^{2+} ions in the ER, Grp78 maintains the ER calcium homeostasis and serves as an ER stress signalling regulator. Under normal conditions, Grp78 chaperon binds to three ER transmembrane sensor proteins, protein kinase-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6), maintaining them in an inactive form. However, when mammalian cells are subjected to ER stress, such as ER Ca^{2+} efflux or accumulation of supra-threshold unfolded or misfolded protein load, Grp78 dissociates from these three sensors and binds the unfolded or misfolded proteins to assist their proper folding. Upon dissociation of Grp78 from PERK, IRE1 and ATF6, their downstream pathways are activated, resulting in activation and translocation of some of the transcription factors to the *Grp78* promoter and thus initiation of the UPR (also see introduction of chapter 5) (Ni et al., 2009, Baumeister et al., 2005).

The expression of stress-inducible Grp78 is regulated at the transcription level. Upon blockage of protein glycosylation or depletion of the ER calcium store, the transcriptional activity of the *Grp78* promoter is induced by around 10-25 fold (Lee, 1992, Fernandez et al., 2000, Li et al., 1993). This process, is primarily controlled by multiple stress-inducible elements located upstream of the TATA element of the mammalian *Grp78* promoter. These regulatory elements are composed of: (i) highly conserved copies of the 19 bp sequence CCAATN₉CCACG (N₉ represents conserved 9 bp of the GC-rich sequence) termed ER stress response element (ERSE) (Hong et al., 2005) and (ii) the cAMP response element (CRE), which is located upstream of the ERSE.

The mammalian *Grp78* promoter contains three such ERSEs and one CRE, which collectively serve as a binding site for a variety of transcription factors including YY1, TFII-I and ATF6 in order to activate transcription of the chaperone molecule Grp78.

1.2.1.2.4.1.1 Role of Grp78 in cancer

Compared to normal cells, the rapidly growing tumour cells show high growth rate and increased glucose metabolism. The glucose level of many cancer cells therefor can be as low as 0-2 mM compared with 5mM in normal cells. Moreover, neovascularization and angiogenesis often fail to keep pace with tumour growth leading to hypoxia, acidosis and nutrient deprivation. These intrinsic and extrinsic factors within tumour cells and the tumour microenvironment result in accumulation of unfolded or misfolded ER proteins which subsequently trigger the UPR. Thus, while expression of the Grp78 chaperone protein is maintained at low basal level in major adult organs such as brain, heart and lung, it is highly upregulated in transformed cells (Patierno et al., 1987) as well as in a wide variety of tumours (Lee, 2007, Lee, 2001, Hendershot, 2004, Yu et al., 1999, Li et al., 2006). Interestingly, in addition to solid tumours, Grp78 expression is highly induced in the endothelia of tumour vasculature (Dong et al., 2011).

Overexpression of Grp78 has been shown to correlate with an increased tumour recurrence risk and decreased survival rate in cancer patients (Pfaffenbach and Lee, 2011). Moreover there is a positive correlation between the tumour grade and the level of Grp78 expression in breast, liver, prostate, colon, gastric, and brain tumours (Lee et al., 2008, Wang et al., 2010). Grp78 plays a major role in cancer cell survival by activating the prosurvival pathway. *In vivo* studies with heterozygous Grp78 mice (*Grp78*^{+/-}) demonstrated a marked impediment in tumour progression compared to wild-type Grp78 mice, as tumour size was reduced and apoptosis was promoted (Dong et al., 2008). In addition to primary tumours, overexpression of Grp78 has been shown to assist tumour and lymph node metastasis. Interestingly *Grp78* gene knockout has been shown to inhibit tumour cell invasion *in vitro* as well as growth and metastasis *in vivo* in the *Grp78*^{+/-} xenograft models (Fu and Lee, 2006, Zhang et al., 2006, Dong et al., 2011). It has been demonstrated that during tumour growth and metastasis, Grp78 plays an important role in tumour neovascularisation. Compared to wild type Grp78 mice, the microvessel density (MVD) of the endogenous tumours in the *Grp78*^{+/-} mice was decreased by 70%, while the vasculature of normal organs remained intact (Dong et al., 2011).

The stress-inducible Grp78 is also required for tumour cell proliferation. The direct correlation between Grp78 level and proliferation rate of a range of glioblastoma cells has been previously demonstrated. Grp78 knockdown in glioblastoma cells leads to decreased tumour cell proliferation (Pyrko et al., 2007). In addition to tumour cell survival and proliferation, Grp78 plays an important role in blocking apoptosis in stressed cells.

Although Grp78 primarily resides in the ER lumen, upon ER stress, a fraction of the overproduced Grp78 can be distributed to regions outside the ER such as mitochondria, cytoplasm (Ni et al., 2009), plasma membrane and can be released even into the culture medium. Within the cytoplasm, this molecular chaperone binds to caspase-7 and caspase-12 and inhibits their activation, thus blocking apoptosis. Moreover, the mitochondrial Grp78 fraction is capable of forming complexes with pro-apoptotic proteins and prevents cytochrome *c* release from the mitochondria, blocking apoptosis (Lee, 2007, Zhang and Zhang, 2010).

Interestingly, Grp78 in the tumour cell surface has been used as a reliable receptor for tumour targeting. Indeed, systemic administration of synthetic chimeric peptides composed of Grp78 binding motifs fused to a proapoptotic sequence could efficiently target tumours and inhibit their growth without affecting normal tissues (Arap et al., 2004). Moreover in a variety of tumours, cell surface Grp78 has been shown to promote cell proliferation, survival and metastasis by binding to plasma proteinase inhibitor α 2-macroglobulin and activation of its downstream signalling pathway (Misra et al., 2006, Shu et al., 2008, Zhang and Zhang, 2010).

1.2.1.2.4.1.2 Role of Grp78 in drug resistance

Previous reports showed that increased expression of Grp78 in a variety of cancers such as prostate, breast and lung tumours in addition to glioma is correlated with cancer progression, metastasis and drug resistance (Wang et al., 2009, Fu and Lee, 2006, Zhang et al., 2009c, Racek et al., 2008). Although development of drug resistance in tumour cells is rather complex and involves various mechanisms, induction of Grp78 is arguably a major contributing factor.

Glioblastoma multiforme (GBM; grade IV), the most aggressive form of gliomas, is highly resistant to chemotherapy. Interestingly, the level of Grp78 chaperone molecule is markedly induced in human glioblastoma cell lines and malignant glioma specimens, while Grp78 expression is maintained at a low level in normal adult brain. As a proof of principle,

knockdown of Grp78 resulted in sensitization of glioblastoma cells to chemotherapeutic drugs such as temozolomide (Pyrko et al., 2007). In addition to glioblastoma cells, significant induction of Grp78 has been reported in the vasculature derived from human glioma specimens both *in vitro* in cell culture and *in situ* in tissue. Interestingly, similar to glioblastoma cells, human brain endothelial cells derived from blood vessels of malignant glioma (TuBECs) show resistance to various chemotherapeutic drugs such as temozolomide, etoposide and CPT-11. Indeed, down regulation of *Grp78* in TuBECs using small interfering RNA has been shown to significantly reverse resistance to chemotherapy. By suppressing the Grp78, previous studies could successfully sensitize human breast cancer cells to etoposide-induced apoptosis (Reddy et al., 2003). Thus tumour drug resistance resulting from high levels of Grp78, might be due to the prosurvival property of this chaperone molecule.

1.2.1.2.4.1.3 The *Grp78* promoter and targeted gene therapy

The use of the *Grp78* promoter in cancer gene therapy was first reported by Dr Lee's group (Little et al., 1994). The team had fully characterised the efficacy of this promoter in several tumour systems. They used the *Grp78* promoter in the context of a retroviral system to drive the expression of the *HSVtk* gene in tumours followed by ganciclovir (GCV) administration. GCV, a nontoxic prodrug, phosphorylated by the *HSVtk* enzyme, and then converted by cellular kinases into triphosphate GCV, which incorporates into DNA leading to inhibition of DNA polymerase and resulting in cell death. Studies of *HSVtk* driven by the *Grp78* promoter demonstrated complete eradication of murine tumours and human xenografts in mice with no recurrence of tumour growth (Dong et al., 2004, Chen et al., 2000).

Previous work demonstrated that the *Grp78* promoter is highly induced in human breast carcinomas and that its activity is not affected by either p53 or estrogen receptor status (Dong et al., 2004). Suppression and eradication of human breast xenografts such as MCF7, MDA-MB-435 and T47D in nude mice were observed after using the *Grp78* promoter to drive *HSVtk* in the context of a retroviral vector (Dong et al., 2004). Similar results were reported in a murine mammary adenocarcinoma cell line (TSA) in immune-competent mice (Chen et al., 2000). Moreover, another group assessed the efficacy of *HSVtk* under the control of the *Grp78* promoter in gastroesophageal junction and gastric adenocarcinoma cells, and found significant cell death *in vitro* and tumour regression *in vivo* following GCV treatment (Azatian et al., 2009).

Induction of the *Grp78* promoter in response to photodynamic therapy (PDT) has also been reported (Luna et al., 2002). PDT generates cytotoxic singlet oxygen species, which are potent activators of the *Grp78* promoter. Inducible expression of *HSVtk* under the control of the *Grp78* promoter was also observed after PDT of stably retroviral-transduced mouse mammary carcinoma cells.

Interestingly, transgenic mice in which a *LacZ* transgene was driven by the rat *Grp78* promoter showed high transgene expression in cancer cells, while no promoter activity was detected in major adult organs (Dong et al., 2004). Thus, transcriptional control mediated by *Grp78* promoter offers a novel approach for human cancer gene therapy. In addition to the vector safety, this can increase gene transcription because, unlike viral promoters such as *CMV*, mammalian promoters are not silenced in eukaryotic cells (Brooks et al., 2004). This can lead to stronger and longer-term transgene expression from the vector.

Despite these potential advantages of the *Grp78* promoter in cancer gene therapy, transcriptional targeting alone is not sufficient to ensure gene expression in the target cell, which also requires efficient introduction of the therapeutic transgene cassette in the correct cells. A real clinical application and benefit of the *Grp78* promoter will depend on the generation of vectors that can specifically and efficiently deliver a *Grp78*-driven transgene expression cassette to the tumour tissue after systemic administration. Thus, further optimization of vectors, especially ligand targeting of vectors, may facilitate application of the *Grp78* promoter for efficient gene therapy.

1.2.2 Ligand-directed targeting

Cancer gene therapy is limited by side effects that result from non-specific delivery of therapeutic genes. To circumvent this limitation, ligand-directed targeting of gene delivery vectors has been attempted. The ligand-targeting strategy using animal viruses involves manipulation of ligands on the outer viral coat proteins to mediate binding to the cells of interest. This process often necessitates ablation of the virus natural tropism for mammalian cells and displaying specific peptide ligands on their surface to retarget them to specific cell type receptors within the host. A number of such ligands have been identified by phage display technology, an *in vivo* screening method in which peptides homing to tissue-specific receptors are selected after intravenous administration of a phage display random peptide library (Pasqualini and Ruoslahti, 1996). Through this process several tumour-specific and tumour-associated endothelial cell-specific receptors, which hold great potential for ligand-

directed targeting in cancer gene therapy, have been characterised and some have been assessed in preclinical tumour models (Hajitou et al., 2006a) (Table 1.1). In recent years, in addition to primary tumours, targeting the tumour vasculature has been of great interest in cancer gene therapy as vascular cells are readily accessible through the systemic circulation of the vector and are also known to express several different surface markers that are absent or barely detectable in normal quiescent blood vessels. Furthermore given the estimates that up to 100 tumour cells are sustained by a single endothelial cell (Folkman, 1997), gene targeting of tumour vasculature might mediate a pronounced anti-cancer effect. However, a successful tumour-vasculature-targeted therapy will depend on the development of efficient vectors that can deliver and express therapeutic genes specifically in the tumour vasculature of the primary and metastatic tumours.

Table 1.1. List of receptors being targeted in cancer therapy. (Hajitou et al., 2006a)

<i>Receptor</i>	<i>Function/class</i>	<i>Vehicle</i>	<i>Localization</i>	<i>Homing motif</i>
GRP 78	Stress response	Yes	Tumor cells	WIFPWIQL
α_v Integrins	Cell adhesion	Yes	EC, tumor cells	RGD4C
CD13	Protease	Yes	EC, pericytes	CNGRC
APA	Protease	N/D	Pericytes, stromal cell	CPRECES
NG2/HMWMAA	Proteoglycan	N/D	Pericytes, tumor cells	GSL
MMP-2/MMP-9	Protease	Yes	EC, tumor cells	CTTHWGFTLC
IL-11-R	Cytokine receptor	Yes	EC, tumor cells	CGRRAGGSC
Prohibitin	Membrane chaperone	Yes	EC	CKGGRAKDC
HSP90	Heat shock	Yes	EC, tumor cells	CVPELGHEC

EC, endothelial cells; N/D, not determined.

1.2.2.1 Ligand targeting of viral gene therapy vectors

In the past decade, several techniques have been developed with the aim of facilitating vector targeting to specific cells after systemic administration. However, for systemic targeted cancer gene therapy, the choice of an efficient vector system capable of mediating an appropriate expression level of the therapeutic gene to cancer cells is the most crucial step.

Nonviral vectors, such as gene gun, magnetofection, lipoplexes, inorganic nanoparticles and injection of naked DNA, hold some potential due to their mass production, safety and low host immunogenicity. Nevertheless their low level of transfection and transgene expression still represent a great challenge (Li and Huang, 2006).

In contrast to nonviral vectors, viral vectors have arguably proved to be the vector of choice as they naturally evolved to infect mammalian cells and can mediate high level of transduction. Although in 2007, viral vectors were used in 70% of clinical trials involving gene therapy (Waehler et al., 2007), many groups are focused on improving viral vectors features for gene therapy purpose. Most progress in viral gene therapy has involved Lentiviruses, Adenoviruses (Ad) and Adeno-associated viruses (AAV) (Bouard et al., 2009). In particular, Ads are attractive for cancer gene therapy and are the most frequently used vectors in clinical trials (Kay, 2011). Ads contain a 36 kb double-stranded DNA genome which provides plenty of space for inserting large sequence fragments. In addition to the ease of vector manipulation and the capability to produce high titers, Ad vectors are known to mediate efficient transgene expression in both dividing and non-dividing cells. Despite all the advantages of Ad vectors, they can induce severe toxicity by stimulating host immune response after systemic delivery of the required dose of vector. Thus for selective killing of tumour cells, many studies have focused on modifying the adenoviral genome in such a way to achieve cancer specificity at different stages through the adenoviral life cycle (Green and Seymour, 2002). One main approach has involved a specific deletion in the Ad genome and generation of replication-incompetent Ad vectors which attenuate the viral amplification and thus inhibit the infection. These engineered vectors can be further modified to selectively replicate in tumour cells and mediate their lysis. The ONYX-015 vector is one such example of a replication-incompetent Ad vector. This vector cannot defend itself against the products of the p53 tumour suppressor gene and thus is destroyed in healthy cells. In contrast, as the p53 gene is defective in most tumour cells, ONYX-015 can proliferate within these cells and mediate their lysis (McCormick, 2000). Moreover, if the tumour lysis mediated by these vectors is inefficient, researchers can insert therapeutic genes into the Ad vector genome in order to enhance its antitumour efficacy (Oosterhoff et al., 2005).

AAVs, which are members of the parvovirus family, are also promising vectors and have been used in a variety of gene-therapy applications as they confer low immunogenicity within the host. Moreover, AAVs have been shown to mediate stable and long-term gene expression *in vivo* (Duan et al., 1998). Additionally, AAV vectors are purified to high titers and, like Ads, can transduce both dividing and non-dividing cells. However, one of the severe

limitations of AAV vectors for gene therapy is their limited capacity for gene cloning which is due to their small genome (Kay, 2011).

AAVs contain a 4.7 kb single-stranded DNA genome encoding for two genes: *rep* which is responsible for viral DNA replication and *cap*, which directs packaging of the viral genome. *Rep* and *cap* open reading frames (ORFs) are flanked by two inverted terminal repeats (ITRs) (Buning et al., 2008). Upon entry into the host cell, the AAV single-stranded DNA is converted to transcriptionally active double-stranded DNA and remains episomal (Duan et al., 1998). To generate recombinant AAV, *rep* and *cap* are replaced by transgene cassettes, leaving the ITR cis elements which serve as an origin of replication (Buning et al., 2008, Samulski et al., 1987).

Despite the fact that animal viruses such as Ad and AAVs hold potential for targeted cancer gene therapy, their native tropism for mammalian cells must be eliminated prior to re-targeting them to specific receptors. This process often makes the vectors quite unstable and also leads to reduced cells transduction resulting from entry via non-natural receptors (Kay, 2011). Moreover, addition of targeting peptides derived from phage display screenings to eukaryotic viruses has not been successful, as this process seems to alter the structure of the viral capsid and diminish the targeting properties of the peptide ligands themselves (Hatefi and Canine, 2009). Indeed, peptides isolated from phage libraries are selected in the protein structural context of the pIII capsid protein of the filamentous M13 bacteriophage (phage) and are then translated into different protein structures of a viral capsid protein. Due to these limitations, bacteriophage itself has been proposed as a safe vector for targeted gene therapy (Piersanti et al., 2004, Ghaemi et al., 2010, Di Giovine et al., 2001).

1.2.2.2 Bacteriophage gene therapy

Another approach in ligand-directed gene therapy uses targeted bacteriophage directly for gene delivery to mammalian cells without the need to transfer peptides from phage display to a eukaryotic vector. Bacteriophage, which is a prokaryotic virus, has no tropism for mammalian cells, but can be engineered to deliver genes to such cells. They are safe and can be targeted by a ligand displayed on their capsid, to a specific mammalian receptor after systemic administration. This can be achieved by DNA sequence insertion into the phage Gene III which encodes the pIII minor coat protein. Moreover, phage-based vectors have some potential advantages over animal viruses for targeted delivery of transgenes into mammalian cells. First, there are no known natural receptors for phage on mammalian cells.

Additionally, bacteriophage have been used for antibiotic therapy before the discovery of antibiotics and are safe even in children after systemic administration (Barrow and Soothill, 1997). In 2006, the US Food and Drug Administration (FDA) announced that it had approved the use of some bacteriophage preparations on ready-to-eat (RTE) meat and poultry products as antibacterial food additives (Lang, 2006). Moreover, bacteriophage are cheap to produce in bacteria and have very high titers (Hajitou et al., 2007). Most importantly, unlike eukaryotic viruses, bacteriophage do not require further context modification of their capsid; the targeting peptides are actually selected and isolated directly to target specific cell-surface receptors after screening of a phage display peptide library. Despite all these advantages, however, phage-based vectors are not ideal as bacteriophage only infect bacteria and cannot efficiently express transgenes upon entry into eukaryotic cells.

To circumvent this limitation, Hajitou *et al.* created a new generation of targeted hybrid prokaryotic-eukaryotic vectors. These vectors are chimeras between two single-stranded DNA viruses: (i) an adeno-associated virus (AAV2) and (ii) a targeted filamentous M13 bacteriophage termed AAV/phage (AAVP) (Hajitou et al., 2006b).

1.2.2.3 Adeno-Associated Virus/Phage (AAVP)

In the AAVP vector, devoid of any sequence encoding the AAV capsid, the AAV transgene cassette containing a *CMV* promoter flanked by inverted terminal repeats (ITRs) from AAV serotype 2 (Fig. 1.1a) was inserted into an intergenomic region of the phage genome and packaged with the phage DNA into the bacteriophage capsid (Fig. 1.1b). This AAVP vector was genetically modified to display a double-cyclic Arginine-Glycine-Aspartate (CDCRGDCFC, RGD4C) motif, on the minor pIII coat protein of the bacteriophage (Hajitou et al., 2006b). The RGD4C motif has been shown to specifically target α_v integrin receptors which are overexpressed in tumour blood vessels and tumour cells but are absent or are barely expressed in normal vascular endothelial cells (Pasqualini et al., 1997, Ruoslahti, 1996). The use of AAV ITRs in the RGD4C AAVP results in improved transduction efficiency which is associated with an improved fate of the delivered transgene, through maintenance of the entire mammalian transgene cassette, better persistence of episomal DNA and formation of concatamers of the transgene cassette (Hajitou et al., 2006b).

AAVP-mediated reporter transgene expression was observed in mammalian cells treated with targeted RGD4C AAVP only, while control non-targeted RGD4C AAVP or AAVP bearing mutated/ scrambled ligand RGD4C motifs were unable to bind or transduce mammalian cells

in vitro and *in vivo* (Hajitou et al., 2006b). This ligand-directed AAVP was systemically delivered to tumour-bearing mice and proved efficient in expressing both imaging and therapeutic genes into tumours specifically. Indeed, the *in vivo* biodistribution of the RGD4C AAVP vector after systemic administration to mice bearing human prostate xenografts or rats bearing human soft tissue sarcoma was evaluated using *in vivo* Positron Emission Tomography (PET) imaging of HSVtk and [¹⁸F]-FEAU or bioluminescence imaging of the luciferase reporter gene. Interestingly, the results demonstrated specific tumour mediated transgene expression (Hajitou et al., 2008, Hajitou et al., 2006b).

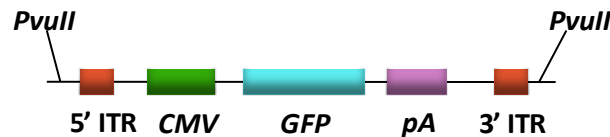
More interestingly, despite the nonspecific hepatic clearance of phage particles (Geier et al., 1973), systemic administration of targeted RGD4C AAVP resulted in no gene transduction of the liver or other control organs (Tandle et al., 2009, Paoloni et al., 2009, Hajitou et al., 2006b, Hajitou et al., 2008). These findings make phage a more advantageous vector for systemic delivery than eukaryotic viral vectors which widely transduce the normal organs after systemic administration (Donsante et al., 2007).

Therapeutic efficiency of the targeted RGD4C AAVP vector was evaluated using vector carrying the *HSV1-tk* gene in combination with the prodrug GCV. Interestingly, a single intravenous administration of the RGD4C AAVP-*HSVtk* vector in immunodeficient nude mice bearing Kaposi's sarcoma KS1767 tumours, resulted in marked tumour suppression compared with mice treated with vehicle or with non-targeted AAVP (Hajitou et al., 2006b). Similar tumour growth suppressive effects were reported in DU145-derived prostate carcinomas and UC3-derived bladder carcinomas in nude mice (Hajitou et al., 2006b). The RGD4C AAVP-*HSVtk* vector was also systemically administered to athymic rats bearing an orthotopic model of SKLMS human soft tissue sarcoma and the results at day seven post GCV treatment demonstrated an extensive tumour destruction and tumour blood vessel apoptosis which resulted in tumour growth suppression (Hajitou et al., 2008).

Further to these experiments, targeted RGD4C AAVP carrying *Tumour Necrosis Factor α* (*TNF α*) was systemically administered to mice bearing human melanoma (Tandle et al., 2009). This systemic administration of targeted RGD4C AAVP/*TNF α* resulted in both tumour growth inhibition, and induction of apoptosis in tumour blood vessels. In a study carried out under the direction of the National Cancer Institute (NCI)- USA, targeted RGD4C AAVP was used to deliver *TNF α* to naturally occurring cancers diagnosed in pet dogs (Paoloni et al., 2009). In this study, single and repeated dosing using targeted AAVP-*TNF α* vector proved safe in treated animals. Remarkably, repeated therapy resulted in complete

tumour eradication in a few dogs with aggressive cancers such as fibrosarcoma (Paoloni et al., 2009).

a)



b)

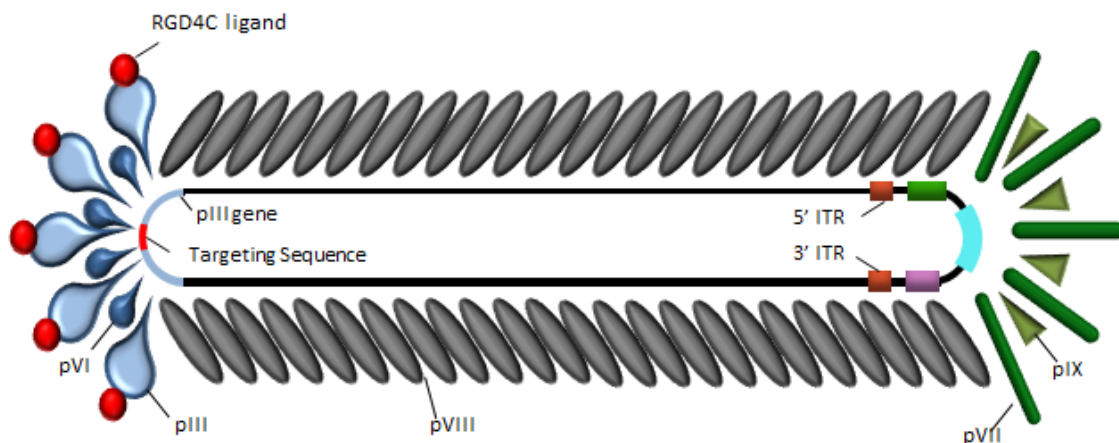


Figure 1.1. Schematic diagram of targeted AAVP vector. a) A mammalian transgene cassette flanked by ITRs from AAV2. b) AAVP particle bearing the targeting double-cyclic RGD4C ligand on the pIII minor coat protein. The virus structure consists of 2,700 - 3,000 copies of the major coat protein pVIII with approximately 5 copies of the four minor capsid proteins pIII, pVI, pVII and pIX which are located at the ends of the filamentous particle. A mammalian transgene cassette flanked by ITRs from AAV is inserted into the intergenomic region of an RGD4C-FUSE5 bacteriophage. (ITR: inverted terminal repeat, pA: polyadenylation site).

To date, the reports continue to show that AAVP can be used to target gene therapy to distant tumours after systemic administration, a route that is applicable for localized and metastatic disease. The ligand-directed RGD4C AAVP particle provides a suitable vector system candidate to target a *Grp78*-driven transgene expression cassette to the tumour tissue after systemic administration. Combining ligand and transcriptional targeting in one single vector

would lead to a safer system with high specificity and strong transgene expression at the transcription level. Such a system would provide a novel double-targeted vector that would enhance the clinical applicability of the *Grp78* promoter in cancer gene therapy.

1.3 Therapeutic genes for cancer gene therapy

In addition to generation of an efficient tumour-targeted vector for cancer gene therapy, the choice of the right therapeutic gene to eliminate tumour cells is important. Many different therapeutic genes with numerous different functions have been characterised and used in cancer gene therapy including antiangiogenesis genes, cellular stasis genes and suicide genes, which mediate cell death when expressed.

As tumour growth and invasion are highly dependent on tumour vasculature, anti-angiogenesis genes are emerging as promising therapeutic genes for many cancers. However for successful tumour treatment, continuous expression of anti-angiogenesis genes such as human Angiostatin (hANG), human Troponin I (hTnI) or soluble receptor (sFlt) for vascular endothelial growth factor (VEGF) is needed (Ohlfest et al., 2005).

Other groups of therapeutic genes such as Rexin-G (which is known to interfere with the cyclin G1 gene) and p53 (an important cell-cycle regulator which is defective in cancerous cells) have also been used in cancer gene therapy in clinical trials and showed promising results against several different tumours such as pancreatic cancer, colon cancer (Gordon et al., 2004), head and neck squamous cancers, prostate cancer, glioma and bladder cancer (Merritt et al., 2001, Swisher et al., 2003, Cross and Burmester, 2006) respectively.

In addition to the above, TNF α has been shown to have antivascular and antitumour activity (ten Hagen and Eggermont, 2003) and has been utilised in cancer gene therapy against several different tumours such as soft tissue sarcoma, pancreatic and rectal cancers and melanoma. However due to severe systemic toxicity of TNF α at therapeutic doses, there has been limited clinical utility of this gene (Burton and Libutti, 2009). Thus many studies attempted intratumoural injection of this gene and/or used inducible promoters to restrict its expression within the tumour cells (Rasmussen et al., 2002, Senzer et al., 2004).

Suicide genes which necessitate the presence of the prodrug for their function as a therapeutic gene, have been proposed by Moolten (Moolten, 1986). *HSVtk* from the virus Herpes Simplex, the most widely used suicide gene in cancer gene therapy (Miller, 1992), has been

considered as an ideal therapeutic gene for cancer therapy. GCV, the prodrug of the HSVtk, is not toxic and can easily penetrate into tumour tissues and can be further taken up by individual cells after systemic injection. Moreover upon reaching the tumour cells, the protein product of *HSVtk* suicide gene which has 1000 times more affinity for the GCV than the mammalian tk, phosphorylates the GCV (Elion et al., 1977). Upon generation of monophosphate GCV, host cell kinases will further phosphorylate the GCV monophosphate and generate the highly toxic GCV triphosphate. As a guanosine analogue, the triphosphate form of GCV can incorporate into replicating DNA and inhibit DNA polymerase and further initiate the apoptotic pathway. Interestingly, GCV monophosphate, is also believed to induce cell death by being incorporated into the entire genome (Mesnil and Yamasaki, 2000). Although the exact mechanism of cell death with *HSVtk*/GCV is not fully understood, it has been clearly shown that cell apoptosis is due to GCV-induced cell-cycle arrest but not due to a direct chemical effect (Wei et al., 1998).

The converted toxic GCV, of about 500 Da molecular weight, can be transported from transduced cells to neighbouring non-transduced cells through the Gap junctions and induce apoptosis of cells which do not contain *HSVtk* (Mesnil and Yamasaki, 2000). This recent unique phenomenon referred to as the bystander effect can augment the effect of toxic drugs by several fold and reduce the need for transduction of a high number of cells in order to mediate an efficient therapeutic effect.

Despite the fact that *HSVtk*/GCV holds a great potential for cancer gene therapy, its toxicity for the dividing healthy tissue has limited its clinical usefulness. Thus, to circumvent the above problem, either direct intratumoural injection or using tumour-type-specific promoters such as α -fetoprotein to drive *HSVtk* expression have been attempted (Su et al., 1997).

However, intratumoural perfusion of the vector is not a practical option for treatment of many tumour types and metastases. Thus development of a tumour targeted *HSVtk* delivery system should overcome the above limitations. Indeed as discussed earlier, targeted RGD4C AAVP vector was previously used to systemically deliver *HSVtk* under the control of *CMV* promoter to tumour-bearing mice and the results demonstrated significant tumour size reduction or marked tumour growth suppression compared to controls, with no evidence of systemic toxicity (Hajitou et al., 2006b).

Moreover by transducing the tumour endothelial cells, recently targeted RGD4C AAVP/*CMV*-*HSVtk* has been shown to induce cell death in tumour vasculature and more importantly in non-transduced tumour cells both *in vitro* and *in vivo* (Trepel et al., 2009). These data suggest the presence of a bystander effect between the endothelial cells and

tumour cells, implying that the therapeutic efficacy of RGD4C AAVP/*HSVtk* will not be completely limited by transduction efficiency. Thus with its ability to specifically target and infect tumour vasculature, avoid normal tissues and mediate substantial tumour cell death via the *HSVtk*/GCV treatment, AAVP holds considerable promise to achieve *HSVtk* clinical applicability.

On the other hand previous reports demonstrated strong expression of the *HSVtk* under the control of the *Grp78* promoter in a variety of human tumours and its ability to eradicate murine tumours and human xenografts in mice. Thus, as a potential therapeutic gene I aimed to utilise the *HSVtk* suicide gene in the context of a double-targeted RGD4C AAVP/*Grp78* vector for systemic cancer gene therapy.

Work presented in this thesis

This work centres around construction and further evaluation of a double-targeted RGD4C AAVP/*Grp78* vector with the aim of combining ligand-directed chimera and transcriptional targeting to safely deliver transgene expression to tumour cells after systemic administration of the vector.

- Chapter 3: constructing a double-targeted vector carrying either *GFP* or *Luc* reporter genes, or the *HSVtk* therapeutic gene. The generated double-targeted vector was further fully characterised *in vitro* using breast cancer and glioblastoma models.
- Chapter 4: Focusing on the effect of *CMV* promoter silencing in the context of AAVP while showing long-term transgene expression mediated by the AAVP/*Grp78* vector *in vitro* using human and rat glioblastoma models.
- Chapter 5: Showing a novel mechanism of *Grp78* promoter induction by *HSVtk* and GCV suicide gene therapy.
- Chapter 6: Presenting *in vivo* biodistribution of transgene expression as well as therapeutic effects of the final ligand-directed and transcriptionally targeted AAVP vector in subcutaneous models of glioblastomas in mice.
- Chapter 7: Describing the effects of chemotherapeutic drugs on *Grp78* promoter induction.

Chapter 2

Materials and Methods

2.1 General material sources

Unless otherwise stated, all chemicals were purchased from Sigma Aldrich. All restriction enzymes were purchased from New England Biolabs.

2.2 Plasmid

Plasmid	Source	Description
pDrive-rGRP78	InvivoGen	pDRIVE bearing the rat GRP78 promoter.
pBluescript II SK(+/-)	Stratagene	Phagemid vector contains <i>Amp^R</i> sequence for bacterial maintenance. T7 and T3 promoters flank the multiple cloning site (MCS), which is situated in the <i>lacZ</i> gene.
pGL4.20	Promega	Containing the luciferase reporter gene <i>Luc2</i> (<i>Photinus pyralis</i>) and mammalian selectable marker <i>puro^R</i> gene under the control of SV40 early enhancer promoter. multiple cloning site (MCS) located upstream of <i>Luc2</i> for the cloning of a promoter of choice.
pPUR	Clontech	Selection vector that has the <i>puro^R</i> gene under the control of SV40 early promoter for mammalian selection and <i>Amp^R</i> gene for bacterial maintenance.

2.3 Oligos

Oligo	Sequence (5'-3')
Rat-XBP-F	TTACGAGAGAAAACTCATGGGC
Rat-XBP-R	GGGTCCAACCTTGTCCAGAATGC
Hu-XBP-F	TTACGAGAGAAAACTCATGGCC
Hu-XBP-R	GGGTCCAAGTTGTCCAGAATGC
GAPDH-F	CCTGTACGCCAACACAGTGC
GAPDH-R	ATACTCCTGCTTGCTGATCC
Grp78-R	TCACAGAGTCCAGCAACAGG
PIII-F	TAATACGACTCACTATAGGGCAAG CTGATAAACCGATAACAATT
PIII-R	CCCTCATAGTTAGCGTAACGATCT

All oligos were made by Invitrogen. The stock of 100 pmol/ μ l was stored at -20°C and 8 pmol/ μ l stock was kept at 4°C. Forward primers (5'-3') are named ending in (F) and reverse ending in (R).

2.4 DNA cloning

2.4.1 Agarose gel electrophoresis

Electrophoresis of DNA was carried out as previously described (Sambrook, 1989). Agarose gels (between 0.5% to 2.5%) were prepared in 1x TAE (40mM Tris-acetate, 1mM EDTA) containing 0.5 μ g/ml ethidium bromide (EtBr) for DNA visualisation. Samples were mixed with loading buffer, then loaded onto the gel alongside a DNA ladder and run at 80 Volts until the dyes had reached the desired position on gel. The bands were visualised under ultraviolet light using the UVP Bio Doc-It imaging system. The concentration of DNA was evaluated by visual comparisons of samples with known standards on agarose gels under UV transillumination. If DNA fragments were to be purified, a slice of agarose gel containing the DNA band of interest was cut out and isolated using the Qiaquick Gel Extraction kit (Qiagen), eluted in EB buffer.

2.4.2 Standard Polymerase Chain Reaction (PCR)

Standard PCR was set up as follows: 1x GoTaq reaction buffer, 250µM dNTPs, 1.25 units GoTaqTM DNA Polymerase (Promega), 20 pmol of each primer, 10% v/v of DMSO, 1mM MgCl₂ in a final volume of 50µl. PCR reactions were carried out in the Biorad PCR machine. PCR conditions for GoTaqTM DNA Polymerase were as follows: denaturing at 94°C for 5 minutes (1 cycle) followed by further denaturing at 94°C for 30 seconds, annealing at 55 °C-60°C for 1 minute and elongation at 72°C (35 cycles; elongation time was determined on the basis of the processivity of the polymerase being 1 minute per kb of DNA). A final elongation step at 72°C for 5 minutes (1 cycle) was carried out. PCR products were analysed by agarose gel electrophoresis

2.4.3 Endonuclease restriction digestion

Restriction digests were performed using 3 units of restriction enzyme per 1µg of DNA in 1x reaction buffer, 1% Bovine Serum Albumin (BSA) and appropriate amount of H₂O. The mixture was incubated for 2-3 hours at the supplier recommended temperature. When necessary, restriction enzymes were heat-inactivated for 20 minutes at 65°C. Digested DNA was analysed by agarose gel electrophoresis.

2.4.4 Dephosphorylation of DNA fragments

For DNA dephosphorylation, 1pmol of digested DNA from the digestion reaction was added to 1 unit of Alkaline Phosphatase (AP) (Roche) and 1× AP reaction buffer. The reaction mixture was incubated at 37°C for 1 hour, and AP was irreversibly inactivated by heating at 65°C for 30 minutes. The dephosphorylation process was necessary to prevent the self-ligation of cloning vectors.

2.4.5 DNA ligation

Each ligation reaction was performed in a final volume of 20-30µl, containing vector/insert at the ratio of 1:6, 1x ligase buffer and 30 units of T4 DNA ligase (Fermentas). A tube of vector without insert was also included in order to estimate the background generated by vector alone. The mixture was incubated at 22°C for 4 hours, followed by overnight incubation at

18°C. The mixture was next transformed into *E.coli* Competent Cells. The next day, colonies were picked and grown in 5ml of Luria-Bertani (LB) broth containing appropriate antibiotics. After overnight incubation at 37°C in a shaker incubator, shaking at 250 revolutions per minute (rpm), plasmids were extracted and analysed by restriction digestion.

2.4.6 Ethanol precipitation of DNA

Final concentration of 10% v/v 3M Sodium acetate (NaAc) plus 200-250 v/v of ice-cold absolute ethanol were added to the DNA then mixed and incubated at -20°C overnight. After centrifugation, the DNA pellet was washed with 1 ml of 70% Ethanol and centrifuged again for 15 minutes. Next, the ethanol was discarded and the pellet of DNA was air-dried before re-suspension in 10-20 µl of EB buffer.

2.4.7 Generation of DNA blunt ends

To make DNA blunt ends, DNA polymerase I large fragment (Klenow) (1unit/ µg DNA) and dNTP mix (33µM) (Invitrogen) were added to DNA. After 15 minutes incubation at 25°C, the reaction was stopped using 10mM EDTA, followed by further incubation at 75°C for 20 minutes.

Blunt ended DNA was further purified by ethanol precipitation and pellet was re-suspension EB buffer.

2.4.8 DNA sequence analysis

DNA sequence analysis of the *Grp78* promoter was carried out by the DNA Core Laboratory in the Automated sequencing facility of Imperial College London using the Grp78-R primer (see section 2.3).

2.5 Protein analysis methods

2.5.1 Protein extraction from tissue cultured cells

To extract whole cell protein from cultured cells, first the medium was removed and cells were washed twice with cold 1x PBS, then RIPA lysis buffer (Thermo Scientific) supplemented with a protease-inhibitor cocktail (Roche) and phosphatase inhibitor sodium orthovanadate (2 mM) were added directly to the cells. A total of 150 µl of lysis buffer was used per well for cells grown on a 24-well plate. Using a pipette tip, the bottom of the wells was scraped to remove all cell residues. The entire lysate was collected and instantly placed on ice prior to centrifugation at 13,000 rpm for 10 minutes at 4 °C. The supernatant was harvested and stored at -80°C.

2.5.2 Quantification of protein

Protein concentration was quantified by using the Bradford method (Bradford, 1976). 315µl of Bradford reagent was mixed with 34µl dH₂O and added to 1µl of lysate. Then, 100 µl of samples were added to a clear 96-well plate in triplicate and the absorbance measured at 595nm using a Versamax microplate reader. The protein concentration of the samples was derived from a standard BSA curve.

2.5.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gels were prepared as follows for the BioRad mini-protean gel apparatus. The 8% resolving gel consisted of 2.4 ml lower buffer (1.5 M Tris-HCl, 0.4% SDS pH 8.8) and 2.6 ml of 30% acrylamide, 4.9 ml water, 32 µl of 15% ammonium persulphate (APS) and 5 µl of TEMED. The mixture was well mixed and poured immediately between the glass plates. The resolving gel was poured to about $\frac{3}{4}$ the height of the gel and overlaid with 300 µl of water. Once polymerised, the water was removed and the stacking gel (0.75 ml of 30% acrylamide, 1.87 ml of upper buffer (0.5 M Tris-HCl, 0.4% SDS pH 6.8), 4.87 ml water and 7.5 µl TEMED, 75 µl 15% APS) was poured on top of the stacking gel. The comb was immediately inserted and the stacking gel was allowed to polymerise. A total of 50- 70 µg of sample proteins were mixed with sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 20% Glycerol, 0.01% bromophenol blue and 100 mM DTT) and heated at 100°C for 5 minutes

before loaded onto the gel. For all gels 8 μ l of full-range rainbow molecular weight marker (GE Healthcare) was also loaded. Gels were run in 1x running buffer (192 mM glycine, 25 mM Tris-base, 1% SDS) at 80 volts until the bromophenol blue present in the samples reached the resolving gel, then the voltage was increased to 140 volts until the dye reached the bottom of the resolving gel.

2.5.4 Western Blot analysis

Following separation by SDS-PAGE electrophoresis, proteins were transferred in 1x transfer buffer (25 mM Tris, 192 mM glycine, 0.02% SDS, 20% (v/v) methanol) onto immobullin-P PVDF transfer membrane using the BioRad Protean II mini trans-blot electrophoresis apparatus. PVDF membranes were first pre-soaked in 100% pure methanol for 1 minute, then placed in transfer buffer for a few minutes prior to transferring at 400mA for 1 hour. Once transferred, non-specific protein binding sites on the membrane were blocked by incubation with blocking buffer (1x TBS-T and 5% (w/v) milk [Marvel]) for 1 hour at room temperature. Subsequently the membrane was incubated with primary antibody for 2-3 hours at room temperature or overnight at 4°C according to the manufacturer's instructions. Primary antibodies were goat anti-Grp78 (C-20, 1:400), mouse anti-GAPDH (6C5, 1:1000) (Santa Cruz Biotechnology), rabbit anti-HSVtk (1:100) (Dr. William Summers, Yale university, USA), mouse anti-ATF6 (IMG-273, 5 μ g/ml), (Imgenex, USA) and rabbit-anti phosphor-eIF2 α (1:1000), (Cell Signaling). Apart from the anti phosphor-eIF2 α which was diluted in 1x TBS-T and 5% (w/v) BSA, the rest of primary antibodies were diluted in 1x TBS-T and 5% (w/v) milk. After incubation with primary antibody, membranes were washed for 3x 5 minutes in blocking buffer. Next, membranes were incubated with a horse radish peroxidase (HRP)-conjugated secondary antibody for 1 hour. Membranes were washed 3x 10 minutes in 1x TBS-T and secondary antibodies were detected with Super Signal West Pico chemiluminescence (ECL) (Thermo Scientific) according to the manufacturer's instructions and exposed to X-ray films ((Biomax MS, Kodak). The density of each band was further quantified using ImageJ software and results were normalized against the density of the corresponding GAPDH band for each condition.

2.6 Bacterial work

2.6.1 Bacterial strains

Competent *E.Coli* DH5 α cells (ATCC) were used for routine cloning.

Kanamycin-resistant *Escherichia coli* (K91 Kan^R) was kindly provided by Professor Pasqualini & Arap, USA. This host bacterial strain was used for propagating filamentous phage (Smith and Scott, 1993).

2.6.2 Media

Autoclaved LB media or LB-agar plates (LB with 15g/l agar) were used for general bacterial growth. To select for a plasmid or AAVP vector, either one or a combination of antibiotics as shown below, were added to the medium.

Antibiotics	Final concentration
Kanamycin (Kan)	100 μ g/ml
Tetracycline (Tet)	40 μ g/ml
Ampicillin (Amp)	50 μ g/ml

2.6.3 Transformation

2.6.3.1 Preparation of competent cells

A single colony of *E.coli* DH5 α cells was used to inoculate 5 ml of LB broth and grown overnight at 37°C, in a shaker incubator shaking at 250 rpm. This culture was used to inoculate a 300ml to 1 litre LB medium and grown at 37°C until the OD₆₀₀ = 0.4-0.6. The cells were harvested by centrifugation (15 minutes, 4000 rpm) and the cell pellet was resuspended in ¼ of the initial volume of solution A (10 mM MnCl₂, 50 mM CaCl₂, 10 mM MES (from MES stock of 50 mM pH 6.3)) and incubated on ice for 30 minutes. The cells were harvested by centrifugation (15 minutes, 4000 rpm) using pre-chilled centrifuge bottles and rotors. The supernatant was discarded and the pellet was resuspended in 1/40th the volume of solution A containing 15% glycerol. 100- 200 μ l aliquots in pre chilled eppendorf tubes were quick frozen on a dry ice/ ethanol bath and then stored at -80°C until required.

2.6.3.2 Heat shock

DH5α competent cells, were thawed on ice and mixed with 10 to 100 ng of plasmid DNA. The cells were then incubated on ice for 20 minutes. The cells were heat-shocked at 42°C for 40-45 seconds and immediately returned back on ice for 2 minutes. A volume of 250 µl of super optimal broth (SOC) medium (Invitrogen) was added to each reaction and incubated for 30-60 minutes at 37°C with 250 rpm of shaking. Dilutions of the transformation culture were plated on LB-agar plates containing the appropriate selective antibiotic and incubated overnight at 37°C.

2.6.4 Recovery of plasmid DNA- Qiagen miniprep

A single transformed bacterial colony was used to inoculate 5ml of LB medium containing the appropriate selective antibiotic. The culture was incubated overnight, shaking at 37°C. Plasmid DNA was extracted using a Miniprep kit (Qiagen) according to the manufacturer's instructions. The culture of bacteria was centrifuged at 10,000×g for 10 minutes, then the bacterial pellet was thoroughly resuspended in 250µl P1 Resuspension Solution (50mM Tris.HCl, 10mM EDTA, and 100µg/ml RNase A) and lysed in 250µl P2 Cell Lysis Solution (0.2M NaOH and 1% SDS). The mixture was neutralized by adding 350µl of N3 Neutralization Solution (4.2M guanidine hydrochloride, 0.9M potassium acetate, pH 4.8) and centrifuged at 17,900×g for 10 minutes at room temperature. The clear lysate was applied to a QIAprep spin column in collection tubes, centrifuged for 1 minute and the flow-through was discarded. The column was washed with PB buffer (5M guanidine hydrochloride 30% ethanol) and spun for 1 minute. The QIAprep spin column was washed with PE buffer (10 mM Tris-HCL60%, 80% ethanol) by centrifugation at 17,000×g for 1 minute. The plasmid DNA was eluted with 30µl EB buffer by centrifugation for 1 minute at 17,000×g. DNA was stored at -20°C.

2.7 Phage work

2.7.1 Phage production

production and purification of AAVP protocol has been carried out as reported by Hajitou *et al.* (Hajitou et al., 2007).

Kanamycin-resistant *E.coli* (K91) was grown in 7ml Terrific Broth (TB) at 37°C, in a shaking incubator (250rpm) until the cells reached log-phase growth. This was achieved by growing cells to the optical density (OD) of between 1.6 and 2.0 (OD₆₀₀). Next, AAVP was incubated with 1ml of K91 for 1hour at room temperature, then transferred to 450 ml LB broth containing tetracycline and kanamycin and incubated for 14-16 hours at 37°C, with shaking (250rpm).

The culture was then centrifuged at 6000×g for 30 minutes at 4°C, the supernatant was collected and the procedure was repeated again under the same conditions in order to remove bacterial debris. To precipitate the phage particles, the supernatant was incubated with Polyethylene-glycol/Sodium chloride (PEG/NaCl) (15% supernatant volume), for 2-3 hours on ice and then centrifuged at 10,000×g for 30 minutes at 4°C. The resulting viral pellet was resuspended in 10ml PBS with agitation for 30 minutes at 37°C. The precipitation with PEG/NaCl was repeated for a second time for 30 minutes on ice, followed by centrifugation at 14,000×g for 30minutes, at 4°C. The pellet was resuspended in sterile PBS, then filtered through a 0.45µm filter before titration.

2.7.2 Titration

Serial dilutions of the AAVP were prepared in PBS in order to achieve final dilutions of 10⁸ and 10⁹. Then 5µl of the above dilutions were used to infect 1ml K91 cells (OD₆₀₀ =1.6-2) for 20 minutes at room temperature. Volumes of 20 and 200µl of infected cells were plated onto LB-Agar plates containing 40ug/ml Tetracycline and 100µg/ml Kanamycin. After overnight incubation at 37°C, the colonies were counted and titers were expressed as bacterial Transducing Units per µl (TU/µl).

2.7.3 PCR to verify the correct display of RGD4C

The purity of the phage was checked by PCR. 5µl of the 1:1000 diluted phage in molecular biology grade water was mixed with standard PCR mixture (2.4.2). In order to amplify and sequence the targeting ligand inserted into the pIII phage DNA, the PIII-F and PIII-R primers were used (2-3). The PCR products from both targeted RGD4C AAVP and control insertless non-targeted vectors were run on an agarose gel and visualized using a transilluminator. RGD4C AAVP which display a targeting peptide within the site of the gene of the pIII coat protein display a shift on the gel compared to parental insertless non-targeted AAVP.

2.7.4 Transduction of eukaryotic cells by AAVP

Cells were seeded into 48–well culture plates. The cells were transduced when they reached approximately 80% confluency. Prior to transduction, cells were counted in order to calculate the number of vector TU/cell to add to each well. The medium was removed and replaced with 100µl serum-free medium containing either targeted RGD4C or non-targeted insertless AAVP phage encoding *luciferase (Luc)* or *Green Fluorescent Protein (GFP)* reporter genes or the *HSVtk* therapeutic gene at varying TU/ cell. Incubation of cells with vectors was carried out for 4 hours by manually mixing every 30 minutes in order to maintain homogeneous distribution of phage before adding 400µl of complete medium to the cells. GFP expression was monitored using a Nikon Eclipse TE2000-S fluorescence microscope fitted with a Nikon digital camera (DXM1200F); luciferase expression was quantified using a Turner Biosystems microplate luminometer and therapy was induced by addition of the GCV prodrug. Each variable experiment was set up in triplicate.

2.8 Cell Culture

2.8.1 Cell lines

The Human Embryonic Kidney (HEK-293) cell line was obtained from the American Type Culture Collection (ATCC), Human breast cancer cells from Michigan Cancer Foundation-7 (MCF-7) and human glioblastoma U87 cells (gift from Cancer Research UK) were also used. The rat glioblastoma 9L cell line was kindly provided by Dr Hrvoje Miletic, Norway. All the above cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing

4.5 mg/ml glucose sodium pyrovalate, and supplemented with 10% foetal bovine serum (FBS), 100 units/mL penicillin, 100µg/ml streptomycin, and 2mM L-Glutamine (Sigma-Aldrich) at 37°C, in a humidified CO₂ (5%) incubator.

When cells reached 80-90% confluence, the medium was discarded and cells were washed once with PBS in order to remove residual serum. The cells were incubated at 37 °C with pre-warmed trypsin- ethylenediaminetetraacetic acid (Trypsin-EDTA) for about 1-2 minute(s) until they were detached from the flask. Pre-warmed complete medium was added to the cells in order to inactivate the trypsin. HEK293 and 9L cells were regularly split 1:6, while MCF-7 and U87 were split 1:4.

Cells were tested for *Mycoplasma* contamination regularly by using the MycoAlert *Mycoplasma* detection kit (Lonza).

2.8.2 Freezing and thawing of the cells

Cells were harvested where they were 70- 90% confluence and frozen. After trypsinization and centrifugation at 170×g for 5 minutes, the cell pellet was re-suspended in fresh cooled freezing medium (5% DMSO and 95% FBS). The cells were aliquoted into cryogenic vials, then placed into an isopropanol containing freezing container and frozen overnight at –70°C. Frozen cells were transferred to a liquid nitrogen container the following day.

To thaw cells, complete medium was added to the frozen cells. Then, thawed cells were transferred to 9ml complete medium and centrifuged at 170×g for 5 minutes. The supernatant containing DMSO was discarded and the cell pellet was re-suspended in 5ml of complete medium and incubated in a 25cm² tissue culture flask.

2.8.3 Stress treatment

Thapsigargin (TG) was purchased from Sigma-Aldrich. TG was prepared as a 1.92 mM stock solution in DMSO and added to cell cultures at 300 nM final concentration for 16 hours. A23187 was dissolved in DMSO and added to cell culture at 0.5µM and 2µM final concentrations for 5 hours.

2.8.4 Cell proliferation assay

For the proliferation assay, the parental 9L, U87 and MCF-7 cell lines were seeded at a density of 10^4 cells/ well of a 24-well plate in triplicate. Cells grown in 10% FBS-containing medium as well as 0.5% FBS-containing medium, were trypsinized on days 2, 3, 4, 5 and 6 and counted using trypan blue. Viable cells were counted by two observers and the experiment was performed twice (Correa et al., 2006).

2.8.5 Transfection of Cells

All plasmid transfections were carried out by using FuGene 6 transfection reagent (Roche) according to manufacturer's instructions. FuGene 6 transfection reagent was mixed with serum free medium and incubated at room temperature for 5 minutes. Then the DNA plasmid was added to diluted FuGene 6 reagent (1:3 w/v ratio of DNA to FuGENE 6) and incubated for 45 minutes at room temperature. The transfection mixture was added dropwise to 70-80% confluent cells, which were then placed at 37°C for a further incubation of 48 hours. In order to assess transfection efficacy, vectors carrying the reporter genes *GFP* or *Luc* were used.

2.8.6 Generation of cells stably expressing the *Luc* reporter gene

A pGL4-20 plasmid (Promega) containing a *Luc* reporter gene as well as a puromycin resistance gene was used for this purpose. This plasmid was transfected into 70-80% confluent cells in a 6-well plate. After 48 hours, cells of each were split and stably selected using appropriate puromycin concentration for approximately 2 weeks. Resistant cell clones were pooled, cultured in a 24 cm² plate, then screened for luciferase activity by using the Luc-Screen® System (Applied Biosystems). The cells were expanded and continuously grown under selection.

Prior to the start of the above experiment, the minimal concentration of puromycin required to kill cells that do not have the resistance gene was determined. Briefly, 5×10^4 cells were plated per well of a 48-well plate, and when 70-80% confluent, cells were treated with different concentration of puromycin (0-10 µg/ml). Each concentration was applied in triplicate. Antibiotic containing medium was replaced every 2 days and cell killing was daily monitored under the microscope. The minimal puromycin concentration for 9L cells was 7µg/ml and for U87 cells was 1µg/ml.

2.9 Luciferase enzymatic assay (in vitro)

The Luc-Screen® System (Applied Biosystems) was used to determine luciferase reporter expression in cells transduced with AAVP carrying the *Luc* reporter transgene. Luc-Screen® reagents include two buffers which are added to culture medium in a 2:1:1 (culture medium: Buffer 1: Buffer 2) ratio.

At indicated time-points, the old culture medium was replaced with 200µl of serum free DMEM. Lysis buffer (buffer 1, 100µl) was added to the cells and incubated at room temperature for 15minutes. Before addition of buffer 2 (100µl), 1µl of lysate was removed and used for Bradford assay.

After 10 minutes incubation at room temperature, luciferase expression was quantified. Luciferase assays were performed in triplicate and normalised to 1µg cell protein.

2.10 Fluorescence Activated Cell Sorting (FACS)

The percentage of cells expressing GFP following stable cell transduction with either RGD4C AAVP/*Grp78-GFP-puro^R* or RGD4C AAVP/*CMV-GFP-puro^R* was determined using Fluorescence Activated Cell Sorting (FACS). U87 and 9L cells were seeded in 6-well plates at a density of 3×10^5 cells/well in 6-well plates using complete DMEM. After 48 hours, cells were washed with PBS and trypsinised. Trypsin was neutralised with complete DMEM and cells centrifuged at 2000 rpm for 5minutes. The medium was removed and the cell pellet was resuspended in PBS containing 0.5% FBS and kept on ice until individual cells were analyzed by FACS. FACS analysis was performed using a FACScalibur instrument. GFP fluorescence signals were detected using a blue argon laser and standard FITC detector settings. To eliminate debris and dead cells, cells were manually gated on forward- and side-scatter characteristics. A total of 20,000 individual cells were amounted within a manual gate in each condition.

To validate reactivation of the *CMV* promoter activity, stably transduced U87 and 9L cells for *GFP* reporter gene as well as U87 and 9L parental cells, were seeded in 6-well plates. 24 hours later, cells were treated with DNA methyltransferase inhibitor and HDACs inhibitor drugs for different time intervals, ranging from 1 to 3 days. Cells were monitored by fluorescent microscope during the time intervals. Afterwards, cells were washed, trypsinized and resuspended in PBS containing 0.5% FBS and analyzed by FACS.

The median fluorescence intensity (MFI) was used to define the fluorescence intensity of each cell population analyzed and % of GFP positive cells were calculated by normalizing to the control (parental cells grown in the similar condition). Data were analyzed using FlowJo software.

2.11 In vitro cell killing using vectors carrying the HSVtk

Each cell line, 9L and U87, was plated in triplicate at 3×10^4 cells/well of a 48-well plate. After 24 hours, cells were transduced with either RGD4C targeted or insertless AAVP vector containing either *Grp78* or *CMV* promoter driving the *HSVtk* suicide gene at the dose of 2×10^6 TU vector particles/cell. Three days post-vector transduction, 9L and U87 cells were treated with 300 nM TG for 16 hours. Then, cells were incubated with medium containing 20 μ mol/L GCV. Fresh GCV was added daily to the cells which were monitored under a microscope on a daily basis. Surviving cells were counted using the trypan blue dye exclusion method.

2.12 Generation of stably transduced cells

70% confluent 9L or U87 cells were transduced with either targeted RGD4C AAVP/*Grp78-puro*^R or targeted RGD4C AAVP/*CMV-puro*^R AAVP phage encoding *Luc* or *GFP* reporter genes, or the *HSVtk* therapeutic gene at 2×10^6 TU/ cell. At day 3 post-vector transduction, cells were trypsinized and suspended in medium containing an appropriate dose of puromycin (7 μ g/ml for 9L cells and 1 μ g/ml for U87 cells) in which only few cells could be seen per field of view of the microscope. Parental non-transduced cells were used as a control.

The medium was removed and replaced with fresh medium containing puromycin every 2-3 days. After around 2 weeks, all the control cells were killed by and puromycin- resistant single cell clones, which were derived from single cells, were monitored under the microscope. When the puromycin- resistant single cell clones were visible, either a population of clones or single cell clones were collected and expanded. Single cell clones were isolated and picked by autoclaved filter paper (Whatman). 4-5 mm² paper was at first soaked with trypsin, then by using sterile forceps the paper was brought in contact with the cell clone and then transferred into a well of a 24-well plate and grown until 60-70% confluent. Subsequently the paper was removed and the cells were trypsinized and expanded.

At this point, stable selected clones were frozen and stored at -80°C until needed. Note that all the stable selected cells were always maintained in medium containing puromycin.

Stable selected *GFP* clones or population were imaged and monitored under a fluorescent microscope every 2-3 days and further analysed by FACS.

Luciferase assay was performed to determine luciferase reporter expression in stable selected *Luc* clones or cell population.

2.13 *In vitro* chemotherapy

9L parental cells as well as 9L cells stably transduced with either RGD4C AAVP/*CMV-HSVtk-puro^R* or RGD4C AAVP/*Grp78-HSVtk-puro^R* were plated at 7×10^4 cells/ well of a 24-well plates. After 24 hours, cells were incubated with increasing concentrations of cisplatin (0-100 μ M) (kind gift from Dr. Paul Mintz, Imperial College London) or temozolomide (0-250 μ M) for 12 hours. Chemotherapeutic drug concentrations were obtained from relevant literature (Mandic et al., 2003, Gaddameedhi and Chatterjee, 2009, Jiang et al., 2009, Pyrko et al., 2007).

2.14 RNA Methods

2.14.1 RNA extraction

Using an RNeasy Mini Kit (Qiagen), total RNA was extracted from untreated or 9L and U87 cells treated with GCV for various times or with Thapsigargin for 16 hours. Briefly, cells (around 10^6) were washed with cold 1x PBS and then 600 μ l of buffer containing 10 μ l/ mL β -mercaptoethanol was added to each well of 6-well plate. Cells were scraped using a rubber policeman and then an equal volume of 70% ethanol was added to the lysates and mixed by pipetting. Samples were applied to RNeasy spin columns in collection tubes, centrifuged for 15 seconds at 15,000 rpm, and the flow-through was discarded. The RNeasy column was washed once with 700 μ l buffer RW1 (15 seconds at 15,000 rpm centrifugation), and twice with 500 μ l buffer RPE (15 seconds and 2 minutes respectively at 15,000 rpm centrifugation). The column was placed in a new 1.5ml collection tube and total RNA, bound to the membrane, was eluted with 30 μ l RNase-free water by centrifugation for 1 minute at 15,000 rpm. RNA was stored at -80°C until used. Total RNA concentration and purity were

determined by measuring the absorption at 260nm using the Nanodrop ND-1000 spectrophotometer (Agilent Technologies, DE, USA) and analysed on a 2% agarose gel.

2.14.2 Reverse transcription

1µg of total RNA, extracted from either U87 or 9L cells, was reverse-transcribed for cDNA synthesis using a “Super Script III First-Strand Synthesis SuperMix” (Invitrogen). According to the manufacturer’s instructions, 1µg of total RNA was mixed with 1µl Annealing Buffer and 50 ng random hexamers in a total volume of 8µl (made up with RNase-free water) and incubated for 5 minutes at 65°C. The reaction was placed back on ice and added to 10µl of First-Strand Reaction Mix and 2µl of SuperScript III Enzyme Mix. The reverse-transcription reaction was incubated for 7 minutes at 25°C, followed by 50 minutes at 50°C and terminated at 85°C for 5 minutes. cDNA was stored at –20°C until used.

2.14.3 Semiquantitative RT-PCR

Semiquantitative RT-PCR was carried out by following the standard PCR condition (2.4.2) and using 1µl of synthesised cDNA (around 500 ng) to determine both unspliced and spliced isoforms of *XBP1*. The 5’ to 3’ sequences of primers used to detect unspliced and spliced *XBP1* mRNA are indicated in 2.3 (Samali et al., 2010).

The size of PCR products for both human and rat species are as follows; unspliced *XBP1* = 289 bp, spliced *XBP1* = 263 bp. The PCR products were checked on a 3.5% agarose gel.

As an internal standard control, a PCR using both primers for GAPDH (2.3) was performed on each cDNA sample and the products were checked on a 2% agarose gel (Wang et al., 2005).

2.15 In vivo work

Cells (80-90% confluent) were trypsinized, counted, washed twice with PBS by centrifugation and resuspended in serum-free DMEM medium. To establish tumours in mice, a total of 10⁶ cells from 9L rat glioblastoma or 10⁷ cells from U87 human glioblastoma were subcutaneously implanted into the right flank of 5-7 weeks old female immunodeficient nude mice (20-25gram). Tumour growth was monitored and once tumours reached the desired volume, tumour-bearing mice were anesthetized by 2% isoflurane and 98% oxygen

inhalation and received a single intravenous dose of targeted or insertless non-targeted AAVP (5×10^{10} TU /mouse resuspended in 200µl PBS) through the tail vein. To compare the growth of tumours in mice between the different groups and to establish tumour growth curves, tumour size was monitored by caliper measurement and tumour volume was calculated from the below formula and expressed as the mean tumoural volume $\pm$ standard error of the mean (s.e.m), (Hajitou et al., 2006b, Hajitou et al., 2008, Arap et al., 2004):

$$\text{Tumour volume (mm}^3\text{)} = (\text{width})^2 \times (\text{length}) \times 0.4$$

2.15.1 Bioluminescence imaging

Vectors carrying the reporter gene *Luc* encoding the firefly luciferase were used to evaluate gene delivery to tumours as well as vector biodistribution. Luciferase expression was monitored over a time course in order to determine initiation and maximal expression after administration of vector. To monitor the luciferase expression, mice were anesthetized and administered with 100 mg/kg of D-luciferin (Gold Biotechnology) by subcutaneous injection into the loose skin over the neck. Five minutes post D-luciferin injection, photonic emission was imaged using the *In Vivo* Imaging System (IVIS 100) (Caliper life Sciences). To avoid image saturation various imaging times (from 1 second to 5 minutes) were applied depending on signal strength. A region of interest (ROI) was defined manually either over the tumours or whole body to measure signal intensities. Within experiments the area of the ROI was kept constant and the intensity was recorded as total photon counts per second per cm² per steradian (photons/sec/cm²/sr) within a ROI. Negative control, insertless AAVP vectors were used in these experiments to validate specificity. For all the representative images the same colour scale bar was applied.

2.15.2 In vivo gene therapy using vectors carrying the HSVtk

To assess the therapeutic efficacy of the *HSVtk* suicide gene *in vivo*, tumour-bearing mice were randomly sorted and RGD4C targeted or insertless AAVP vector carrying the therapeutic gene *HSVtk* was delivered intravenously through the tail vein. At day 3 post vector injection, mice received intraperitoneal injections of the GCV prodrug at a dosage of 75 mg/kg/day until the experiment had ended or mice were sacrificed.

Growth of tumours in mice between the different groups was measured by caliper and tumour viability was monitored by Bioluminescence imaging of tumour cells stably expressing the *Luc* reporter gene.

2.16 Histology

Mice were culled and tumours surgically removed. Harvested tumours were embedded in OCT and snap frozen in liquid nitrogen, then stored at -80° C. Tumour sections were made at 7µm thickness with a Cryostat microtome and placed on glass slides. The sections were air dried for 30-60 minutes and either stained immediately or stored at -80° C.

2.16.1 Haematoxylin and Eosin (H&E) staining

Sections of snap-frozen tumours were air dried for a few minutes to remove moisture, then immersed in filtered 0.1% haematoxylin for 10 minutes. Next, the sections were washed in running tap water for 5 minutes. This was followed by immersing the sections in 1% eosin for 2 minutes and then they were briefly dipped in water until the eosin stop streaking. Stained tumour sections were next dehydrated in 70%, 95% and 100% ethanol for 10 minutes, 30 and 60 seconds, respectively. The slides were cleared by immersing them in xylene for several times and mounted in DPX mounting media before adding the coverslips.

2.17 Statistics

Statistical analyses were performed by using GraphPad Prism software (version 5.0). Error bars represent standard error of the mean (s.e.m). P values were generated by ANOVA and denoted as follows: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Chapter 3

Generation and functional characterization of the dual tumour-targeting phage

3.1 Introduction

One of the major challenges in cancer gene therapy is generating a systemic gene delivery vector that can selectively target malignant tissues and mediate efficient level of transgene expression solely within tumour cells. In order to strictly ensure transgene expression in target cells, one main strategy is to introduce one or multiple checkpoints which act as barriers to cell entry and gene expression within the host.

Transcriptional targeting has been widely introduced in gene therapy vectors and has been considered an important checkpoint barrier. In cancer gene therapy, many tumour specific promoters have been identified and characterized; however, the promoter encoding *Grp78* has gained an increasing interest because of its high activity in a variety of primary tumours and metastases.

Indeed, previous reports have shown induced therapeutic gene expression, driven by the *Grp78* promoter in various tumours leading to complete tumour eradication while no therapeutic gene expression was detected in normal tissues (Dong et al., 2004, Chen et al., 2000, Azatian et al., 2009).

In addition to transcription targeting, ligand-directed targeting of vectors has been considered as another major checkpoint in systemic targeted gene therapy. In 2006, Hajitou *et al.* have reported an efficient systemic vector able to specifically target α_v -integrins overexpressed on tumour vasculature and tumour cells (Hajitou et al., 2006c).

This targeted vector, which is a chimera between two single stranded DNA, AAV and a targeted filamentous M13 bacteriophage (AAVP), was genetically modified to express the RGD4C motif on the minor pIII coat protein of the filamentous phage thereby specifically targeting AAVP to the α_v -integrins. The RGD4C AAVP vector proved efficient in targeting tumour vasculatures and tumour cells, and drive transgene expression within those cells after systemic administration.

Moreover the targeted RGD4C AAVP vector carrying the *HSVtk* gene under the control of the *CMV* promoter demonstrated significant tumour growth suppression following GCV administration (Hajitou et al., 2006b, Hajitou et al., 2008).

Although the *CMV* promoter has been shown to drive high level of transgene expression within cells, its non-specific activity and its susceptibility to silencing is problematic in the context of cancer gene therapy. *Grp78* is therefore an attractive candidate as an inducible promoter to tightly control and mediate high levels of transgene expression within primary tumour, metastasis and tumour vasculature.

Thus, for the first time we aimed to combine transcription targeting and ligand-directed targeting in one vector system in order to generate a double-targeted vector.

The results of this chapter mainly describe the generation of a double-targeted AAVP vector displaying the RGD4C ligand and carrying the *Grp78* inducible promoter. The next aim was to compare gene expression levels by the double-targeted vector to those obtained by using the original AAVP vector carrying the standard *CMV* promoter in cancer cells *in vitro*. Moreover, the therapeutic efficacy of the double-targeted AAVP vector carrying therapeutic genes was assessed *in vitro* against glioblastoma cells.

3.2 Results

3.2.1 Generation of double-targeted phage vector

3.2.1.1 Construction of RGD4C AAVP/Grp78-GFP and RGD4C AAVP/Grp78-HSVtk vector

To generate the double-targeted vector displaying the RGD4C ligand and carrying the *Grp78* promoter, the *CMV* promoter was removed from the RGD4C AAVP vector and replaced by the *Grp78* promoter. I used the *Grp78* promoter of rat as a proof of principle because the promoter sequences of *Grp78* are highly conserved among rodents and human, with 86% sequence identity between humans and rats (Ting and Lee, 1988). Moreover, it has been shown that this promoter is active in a wide range of species including human, rat and murine cells (Dong et al., 2004, Chen et al., 2000). Thus, a pDrive-rGRP78 plasmid containing the rat rGrp78 promoter was purchased from Invivogen. In the original RGD4C AAVP vector, the *CMV* promoter is flanked by *NheI* and *NotI* restriction sites. Thus, the 768-bp region containing the *CMV* promoter was removed from AAVP by *NheI* and *NotI* double restriction digestion. The strategy of cloning aimed at first flanking the rat *Grp78* promoter by *NheI* and *NotI* restriction sites followed by ligation into the AAVP plasmid vector. This cloning procedure was carried out in two steps:

Step-1. Subcloning of the *Grp78* fragment into the *XbaI* site of the MCS of pBluescript II in order to flank the *Grp78* fragment by *SpeI* and *NotI* (*SpeI* can ligate to *NheI*).

Step-2. The plasmid generated in step-1 was used to release the *Grp78* promoter flanked by *SpeI* and *NotI* followed by ligation into the AAVP plasmid.

Step-1. To release the 689bp fragment containing the rat *Grp78* promoter, the pDrive-rGRP78 was double digested with *PstI* and *NcoI*. The purified *Grp78* fragment from the agarose gel was blunted with DNA polymerase then ligated to *XbaI* linkers. After restriction digestion with *XbaI*, the *Grp78* was ligated into the *XbaI* site of the pBluescript II plasmid (Fig. 3.1a). The unique *XbaI* site in the pBluescript is located between the *NotI* and *SpeI* restriction sites. The positive recombinants were identified by *XbaI* digestion and agarose electrophoresis. In order to determine the correct orientation of *Grp78* among the positive

clones, pBluescript-*Grp78* was digested with *Sma*I, a unique restriction site located in the *Grp78* promoter as well as in pBluescript adjacent to the *Spe*I site (Fig. 3.1a).

The recombinant plasmid showing the right direction for the *Grp78* promoter was digested with the restriction enzymes *Not*I and *Spe*I in order to release the *Grp78* promoter. The *Grp78* promoter fragment was then ready to replace the *CMV* promoter in the AAVP plasmid.

Step-2. The *Spe*I/*Not*I fragment containing the *Grp78* promoter was ligated into the AAVP plasmid digested with *Nhe*I and *Not*I (Fig. 3.1b). Double digestion with *Not*I and *Eco*RI were performed to release the *Grp78* promoter and to detect the positive colonies (Fig. 3.1b).

Targeted RGD4C AAVP/*Grp78*-*GFP* and RGD4C AAVP/*Grp78*-*HSVtk* as well as control insertless vectors driving *GFP* and the *HSVtk* transgenes under the control of the *Grp78* promoter were generated following the above cloning procedure.

3.2.1.2 Construction of the RGD4C AAVP/*Grp78*-*Luc* vector

A different cloning strategy was followed in order to construct vectors carrying the *firefly luciferase* (*Luc*) reporter gene as the *Nhe*I restriction site is also located within the *Luc* region of the AAVP/*CMV*-*Luc* (Fig. 3.2). Briefly, AAVP/*Grp78*-*GFP* plasmids were digested with *Not*I and *Sph*I in order to remove a fragment containing *GFP* and replaced by a *Not*I-*Sph*I *Luc*-containing fragment from the AAVP/*CMV*-*Luc* (Fig. 3.2).

Targeted and control vectors were amplified, isolated and purified from the culture supernatant of host bacteria (*E. coli* K91) as described in material and methods.

Finally, the *Grp78*-R primer (Section 2.3) was designed to confirm the *Grp78* promoter sequence in all the vectors generated above.

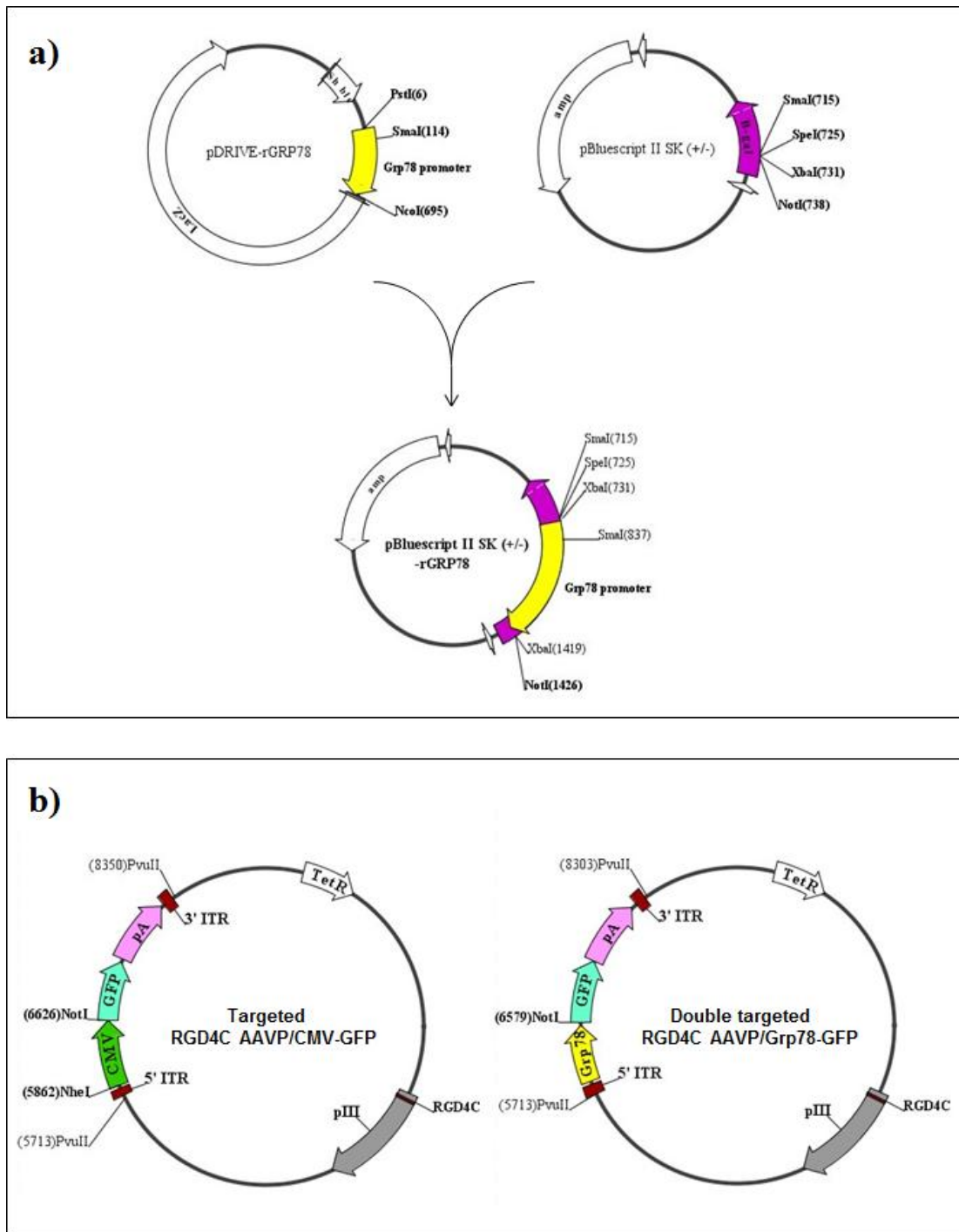


Figure 3.1. Schematic chart describing the construction of the double-targeted RGD4C AAVP/*Grp78* vector. **a)** Plasmid maps of the pDrive-rGRP78 and pBluescript II SK (+/-). Restriction sites used for the subcloning of *Grp78* promoter into pBluescript plasmid are shown. The *Grp78* promoter was released by *Pst*I and *Nco*I, ligated to *Xba*I linkers, then inserted into the *Xba*I site of the pBluescript. **b)** the *Grp78* promoter flanked by *Spe*I and *Not*I was released from pBluescript and ligated into the AAVP plasmid digested with *Nhe*I and *Not*I. (Shble: zeocin-resistance gene, LacZ: β -galactosidase gene, amp: ampicillin resistance gene, TetR: tetracycline resistance gene, ITR: inverted terminal repeat, pA: polyadenylation site).

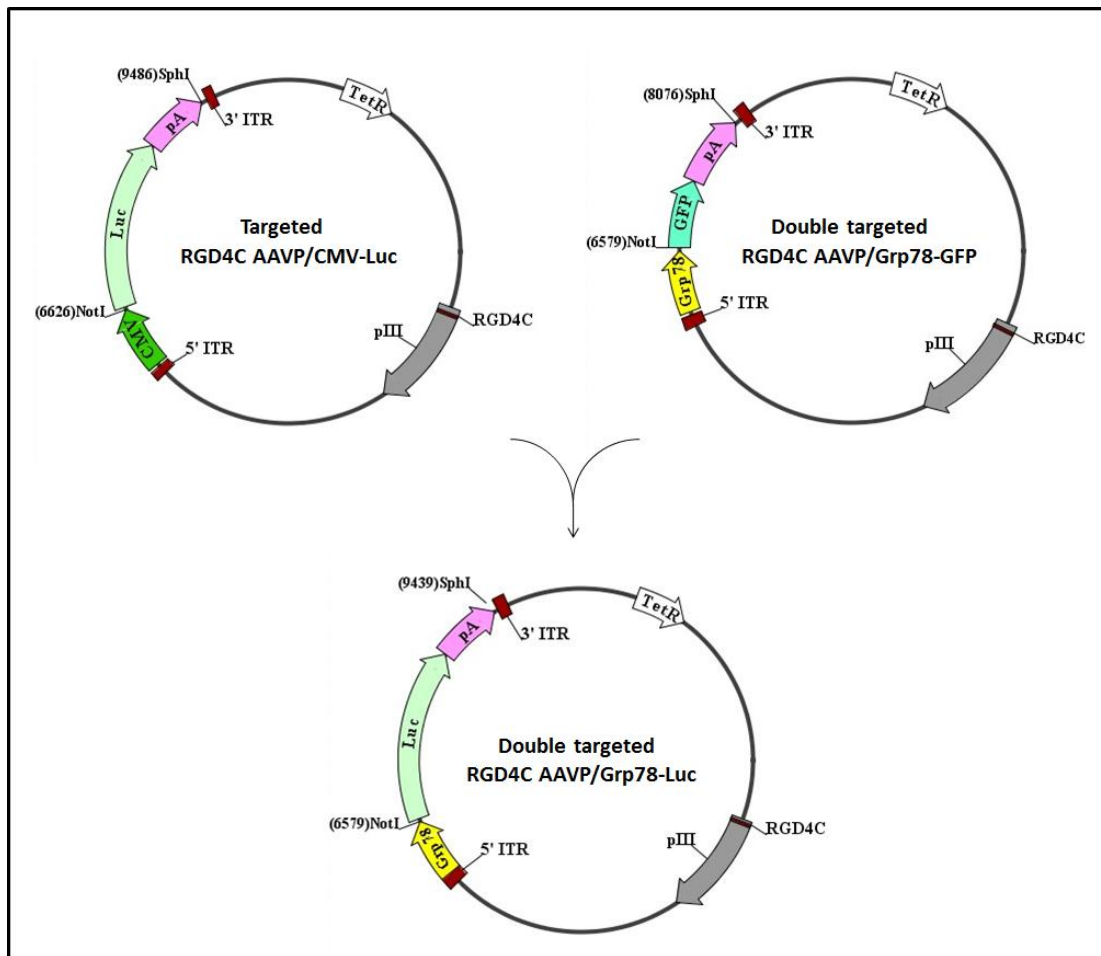


Figure 3.2. Schematic chart describing the construction of the RGD4C AAVP/*Grp78-Luc*. The *Luc* gene flanked by *Sph*I and *Not*I was released from the AAVP/*CMV-Luc* plasmid and ligated to the RGD4C AAVP/*Grp78-GFP* plasmid digested with *Sph*I and *Not*I to replace *GFP*. (*Luc*: luiferase gene, *Tet*^R: tetracycline resistance gene, ITR: inverted terminal repeat, pA: polyadenylation site).

3.2.2 Characterization of a double-targeted vector using the HEK293 standard cellular model

3.2.2.1 Transfection and induction of Grp78 in the context of the AAVP plasmid

Before starting production of the phage viruses we aimed to test whether the *Grp78* promoter retains its inducible function in the AAVP plasmid constructs generated above. Thus, HEK293 cells were transfected with the AAVP/*Grp78*-GFP naked plasmid. After 4 days, detectable GFP expression could be seen in a few cells transfected with this plasmid. Then, the HEK293 cells were treated with thapsigargin (TG), a standard inducer of the *Grp78* promoter. TG acts through blockage of the endoplasmic reticulum calcium adenosine triphosphatase (ATPase) pump and depletes the endoplasmic reticulum Ca^{2+} store (Denmeade and Isaacs, 2005, Li et al., 1993) which is required for normal protein folding, glycosylation and subunit association. At day 4 post plasmid transfection, cells were treated with 300nM TG for 16, 24 and 48 hours (Fig. 3.3). The TG concentration was chosen based on previously published data (Li et al., 1993, Harding et al., 2000). GFP expressing cells were monitored under a fluorescent microscope and at day 8 post-TG treatment, higher levels of GFP expression was observed from the AAVP/*Grp78* plasmid construct treated with TG compared to untreated cells.

These results confirm the inducible function of the *Grp78* promoter in the plasmid constructs generated. Therefore, these plasmids were used to produce the AAVP phage viral particles.

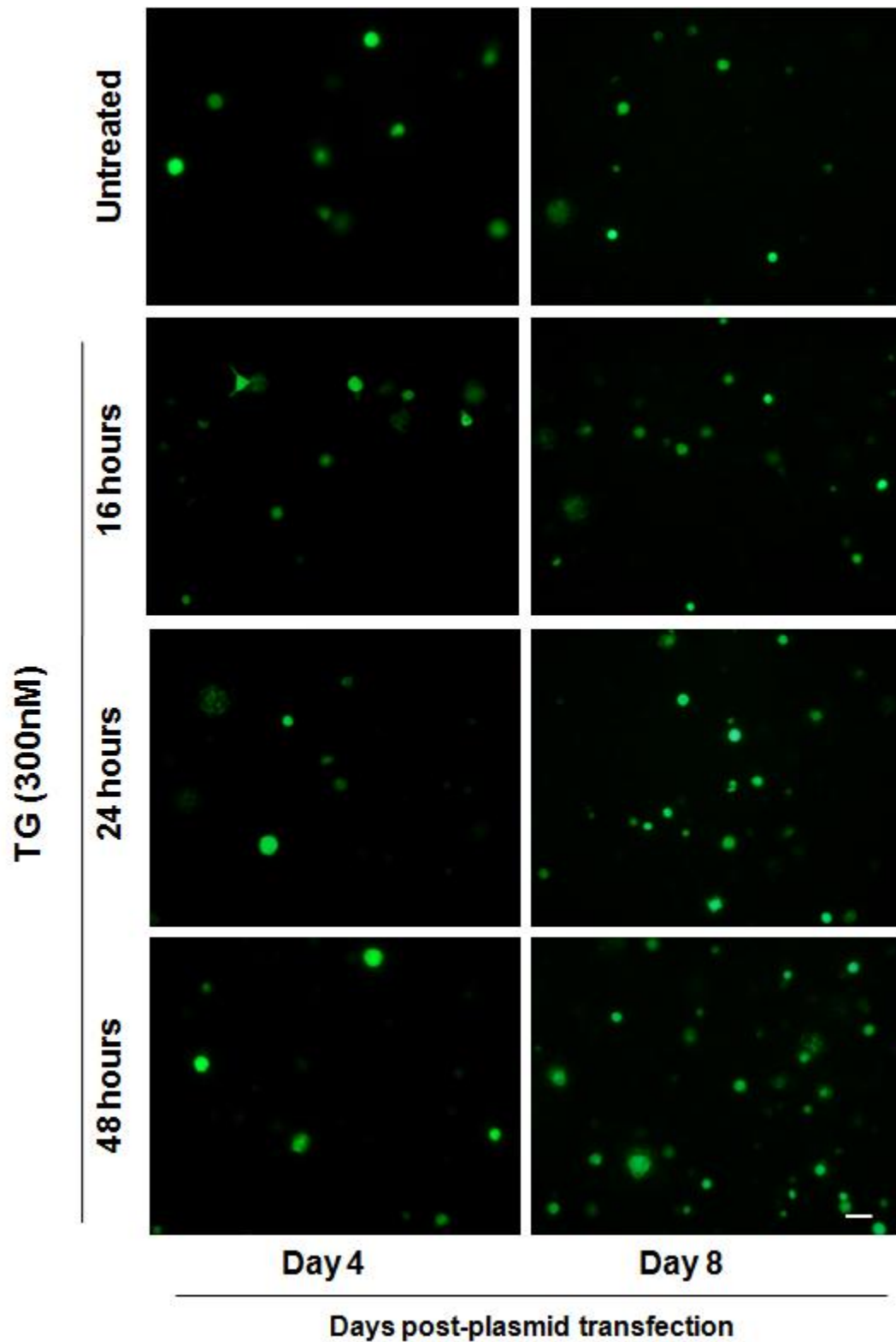


Figure 3.3. ER stress induces *GFP* transgene expression driven by the *Grp78* promoter in HEK293 cells. Cells were plated in 48-well plates to reach 70-80% confluence, then transfected with the AAVP/*Grp78-GFP* plasmid. At day 4 post-transfection, cells were incubated with 300nM TG for 16, 24 and 48 hours. As control, untreated cells were also included for comparison. GFP expression was observed under fluorescence microscope using a 20x objective and photographed at the indicated days. Scale bar = 45 μ m.

3.2.2.2 Transduction and induction in the context of RGD4C AAVP/Grp78 phage viral particle

Next, it was determined whether the stress-inducible *Grp78* promoter is functional *in vitro* in the context of the AAVP phage viral particle. As an initial *in vitro* model, the normal immortalised HEK293 cell line was used. These cells had previously been used as a standard *in vitro* model for transduction by RGD4C AAVP as they express high levels of the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors of the RGD4C ligand (Hajitou et al., 2007, Hajitou et al., 2006b).

To date, in most published reports, cells transduced with *Grp78* expression cassettes have been selected and the promoter activity was thus analysed in vector-transduced and selected cells. Herein, no selection during transduction was applied. Hence, HEK293 cells were incubated with RGD4C AAVP/*Grp78*-GFP or control insertless AAVP/*Grp78*-GFP at 2×10^6 TU/cell. After 5 days, no GFP expression was observed in cells transduced with RGD4C AAVP/*Grp78*-GFP. However, when HEK293 cells transduced with targeted RGD4C AAVP/*Grp78*-GFP were treated with 300 nM or 400nM TG for 16hours at day 5 post-vector transduction, GFP expression was induced compared to untreated cells (Fig. 3.4). Since the GFP expression level was similar with both TG concentrations used above, it was decided to use the low 300 nM TG in future experiments. Moreover, induction of the *Grp78* promoter was confirmed by using the calcium ionophore A23187 that also depletes calcium in the endoplasmic reticulum (Fig. 3.4). Transduced HEK293 cells with targeted RGD4C AAVP/*Grp78*-GFP were treated with either 0.5 or 2 μ M of A23187 for 4 hours (Resendez et al., 1988). The data showed that induction of the *Grp78* promoter was achieved in both conditions (Fig. 3.4).

Also no GFP expression was detected in cells that received control insertless AAVP/*Grp78*-GFP vector lacking the RGD4C and either TG or A23187.

These results show that the *Grp78* promoter is inducible and functional in the context of the AAVP phage viral particle. Importantly, the RGD4C AAVP/*Grp78* can function as a double-targeted vector at the transcriptional level with the *Grp78* promoter as well as at the capsid level with the RGD4C ligand to target α_v integrin receptors on HEK293 cells.

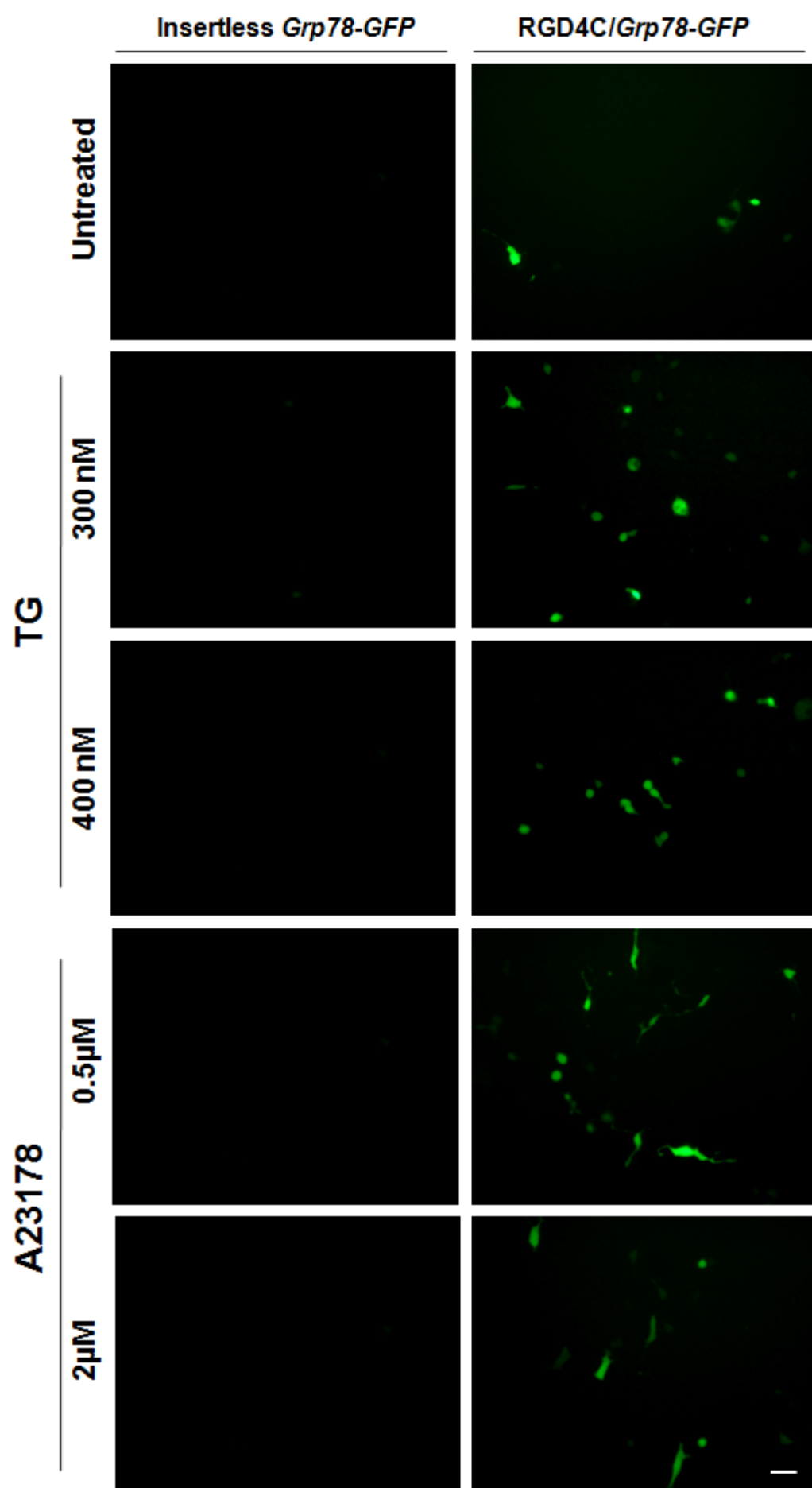


Figure 3.4. Double-targeted RGD4C AAVP/Grp78 phage viral particles mediate GFP transgene expression in HEK293 cells upon TG treatment. Human HEK293 cells (70-80% confluent in a 48 -well plate) were incubated with targeted RGD4C AAVP/Grp78-GFP or control insertless AAVP/Grp78-GFP (2×10^6 TU/cell). Five days later, cells were incubated with either TG (300 or 400 nM) for 48 hours or A23178 (0.5 or 2 μ M) for 5 hours. A control condition of untreated cells was also included for comparison. GFP expression was observed under fluorescence microscope using a 20x objective and photographed at the indicated days. Scale bar = 45 μ m.

3.2.3 *In vitro* characterization of the double-targeted vector in tumour cell models

3.2.3.1 Targeted RGD4C AAVP/CMV vector- mediated transgene expression in tumour cells *in vitro*

Next I aimed to evaluate the efficacy of the double-targeted RGD4C AAVP-Grp78 vector to transduce cancer cells *in vitro* by using the 9L rat and U87 human glioblastoma, as well as the MCF-7 human breast cancer cell lines. Prior to evaluation of the double-targeted vector, it was first sought to confirm and assess the ability of the conventional RGD4C AAVP/CMV vector to transduce these cells. Tumour cells 9L, U87 and MCF-7 were incubated with the targeted RGD4C AAVP/CMV-GFP vector. Insertless AAVP/CMV-GFP was used as a negative control. GFP expression was detected in all these cell lines incubated with the targeted RGD4C AAVP/CMV vector while no transgene expression was observed in cells treated with the control vector (Fig. 3.5). These results indicate that the RGD4C AAVP vector can successfully transduce 9L, U87 and MCF-7 tumour cells in a ligand-dependent manner.

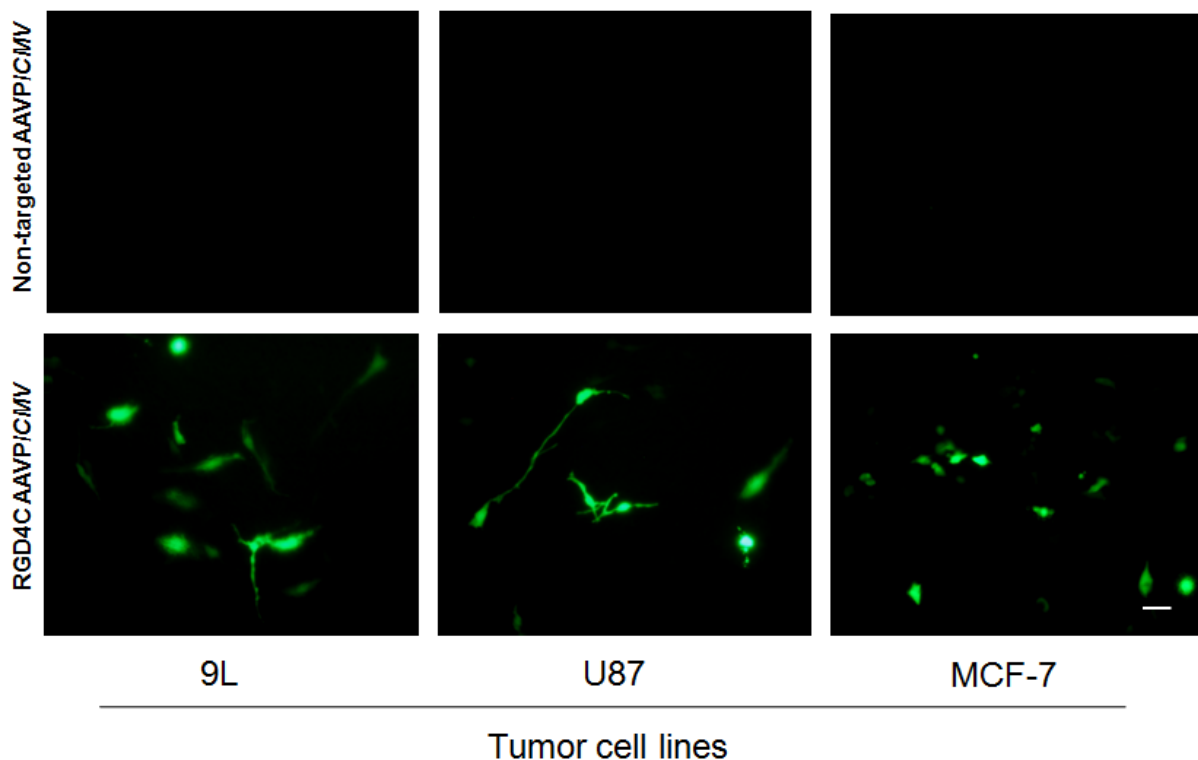


Figure 3.5. Targeted RGD4C AAVP/CMV particles mediate GFP transgene expression in tumour cells. 9L, U87 and MCF-7 tumour cells (70-80% confluent in a 48 well-plate) were incubated with targeted RGD4C AAVP/CMV-GFP along with control insertless AAVP/CMV-GFP (1×10^6 TU/cell). GFP expression was analysed under a Nikon Eclipse fluorescence microscope using a 20x objective and photographed. Scale bar = 45 μ m.

3.2.3.1.1 Optimal dose of AAVP vector for tumour cell transduction

Previous reports have demonstrated that between 8-11% of HEK293 cells transduced with 1×10^6 TU/cell of RGD4C AAVP/CMV-GFP show transgene expressions (Hajitou et al., 2007). Thus, prior to assessing the stress-inducible function of the *Grp78* promoter in the context of the AAVP vector in tumour cells, I first aimed to determine the optimal AAVP dose for transduction of tumour cells. For this purpose, 9L, U87 and MCF-7 cells were transduced with increased doses of targeted RGD4C and the insertless control; AAVP/CMV-*Luc*, at 0.25×10^6 , 0.5×10^6 , 1×10^6 , 1.5×10^6 and 2×10^6 TU/cell. Previous studies have shown that transduction with RGD4C AAVP/CMV results in transgene expression within 48-72 hours post-vector transduction and reaches a maximum level by one week (Hajitou et al., 2007, Hajitou et al., 2006b). At day 7 post-vector transduction, cells treated with either targeted or control AAVP-*Luc* were lysed, and *Luc* transgene expression was analysed by

luciferase assay. The results were then normalised to protein level (Fig. 3.6). The results confirmed successful transduction of both tumour cell lines with targeted RGD4C AAVP/*CMV* and also showed that transgene expression was dose-dependent with the highest expression obtained at 2×10^6 TU/cell. Most importantly, a very negligible transgene expression was observed in MCF-7 cells incubated with a high dose of control insertless AAVP (Fig. 3.6a) while no *luciferase* transgene expression was detected in 9L cells transduced with control AAVP (Fig. 3.6b). These results demonstrate that the specificity of ligand-targeted AAVP vector is intact even with high phage particle/cell doses. Therefore, it was decided to transduce all tumour cells at 2×10^6 TU/cell to achieve the highest transgene expression.

3.2.3.2 Characterisation of AAVP vector-mediated transgene expression

Subsequently, I assessed the efficacy of the double-targeted vector to transduce 9L, U87 and MCF-7 tumour cells by using the RGD4C AAVP/*Grp78-Luc* vector. Transduction efficiency was determined and compared to that of the targeted RGD4C AAVP/*CMV-Luc*. Insertless AAVP/*Grp78-Luc* and AAVP/*CMV-Luc* vectors were used as negative controls. Luciferase quantification was performed on transduced cells at the indicated days (Fig. 3.7). Luciferase quantification by RGD4C AAVP/*Grp78-Luc* and RGD4C AAVP/*CMV-Luc* vectors was normalised to control insertless AAVP/*Grp78-Luc* and AAVP/*CMV-Luc* vectors, respectively (Fig. 3.7).

At day 5 post-vector transduction, the basal *CMV* promoter activity in 9L glioblastoma cells was approximately two fold higher than that of the *Grp78* promoter (Fig. 3.7a). However, at day 7 post-vector transduction the *CMV* promoter activity decreased while the *Grp78* promoter activity increased by 2- fold to become 1.5- fold stronger than the *CMV* activity (Fig. 3.7a). Treatment with TG resulted in a 1.2- fold increase of *Grp78* promoter activity, while transgene expression from the *CMV* promoter remained unchanged (Fig.3.7a).

In U87 cells, the basal *CMV* promoter activity was 11- fold higher than that of the *Grp78* promoter at day 3 post-vector transduction (Fig. 3.7b). Interestingly, at day 7 the *Grp78* promoter activity increased by ~3- fold while the *CMV* promoter activity dramatically decreased to levels similar to those of the *Grp78* promoter. TG treatment of cells resulted in a

1.7- fold increase of the *Grp78* promoter activity at day 7 post-vector transduction, while only a slight increase of the *CMV* promoter activity was detected (Fig. 3.7b).

For MCF-7 cells, the basal *CMV* promoter activity was 15- fold higher than that of the *Grp78* promoter at day 5 post-vector transduction. A dramatic decrease of the *CMV* promoter activity was observed overtime at day 10 post-vector transduction, but was still stronger (6.3-fold) than that of the *Grp78* promoter. In contrast, the basal *Grp78* promoter activity increased by 1.2- fold at day 10 post-vector transduction. TG treatment of cells resulted in a 2.2- fold increase of the *Grp78* promoter activity while a slight increase in the *CMV* promoter activity (1.2) was detected (Fig. 3.7c).

In summary, these results show that: (i) the basic activity of the *Grp78* promoter in the context of the RGD4C AAVP vector varied from one tumour cell line to another with the highest level in the 9L glioblastoma cells, and (ii) depletion of ER calcium, caused by TG, stimulated the *Grp78* promoter activity in all three cell lines, indicating that the *Grp78* promoter is active in a stress inducible manner in the context of AAVP viral phage particles.

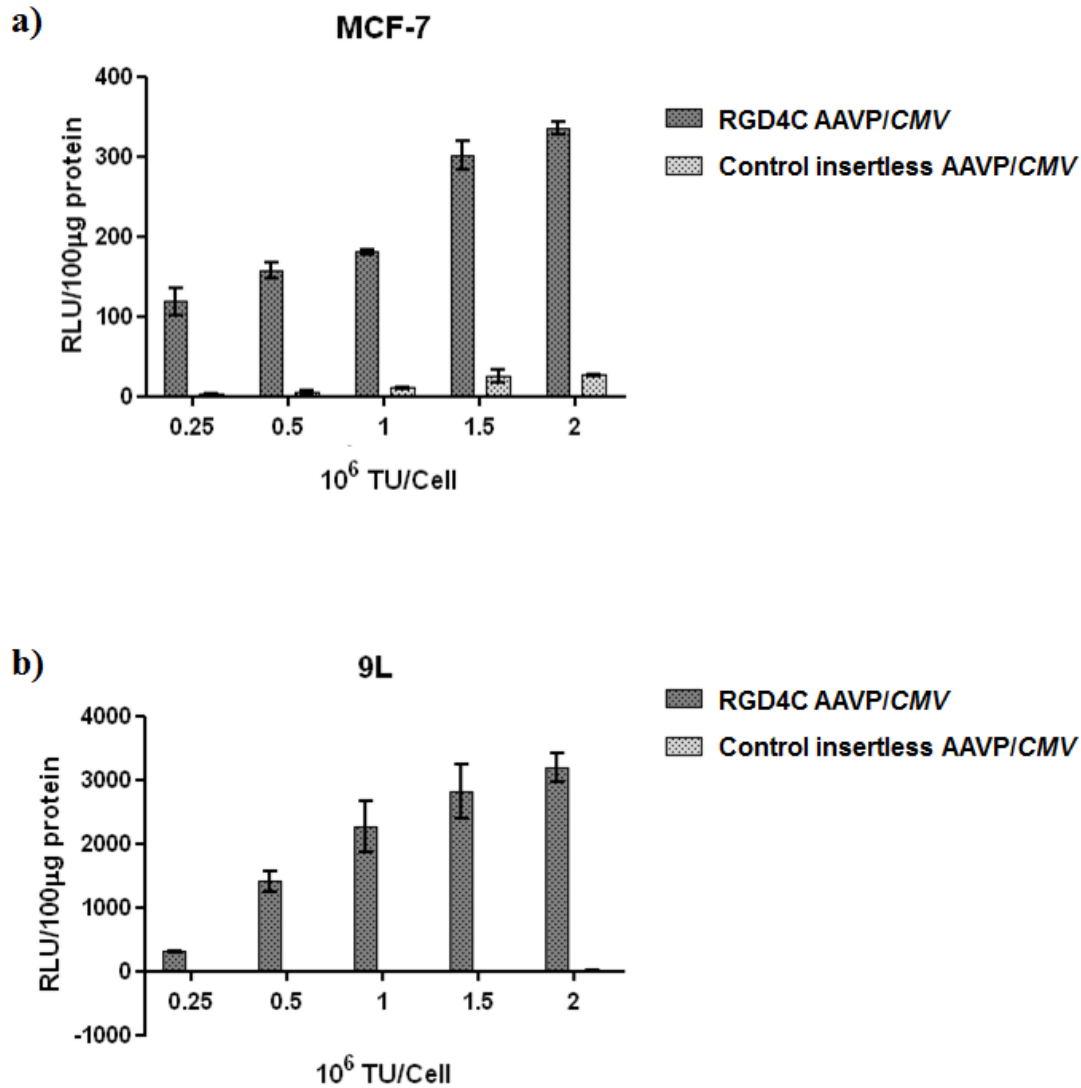


Figure 3.6. RGD4C AAVP mediated dose-dependent transgene expression in MCF-7 and 9L cells. Cells were seeded in 48-well plates and grown until 70-80% confluent before transduction for 4 hours with control AAVP/CMV or targeted RGD4C AAVP/CMV carrying the *Luc* reporter transgene. Each experiment was performed twice with results of one representative experiment shown here. Luciferase expression in **a)** MCF-7 cells and **b)** 9L cells, following transduction with different AAVP TU/cell. The luciferase assay was performed at day7 post-transduction and normalised to protein concentration. Results represent the average Relative Luminescence Units/100 µg (RLU/100µg) protein of triplicate wells +/- the standard error of the mean (s.e.m).

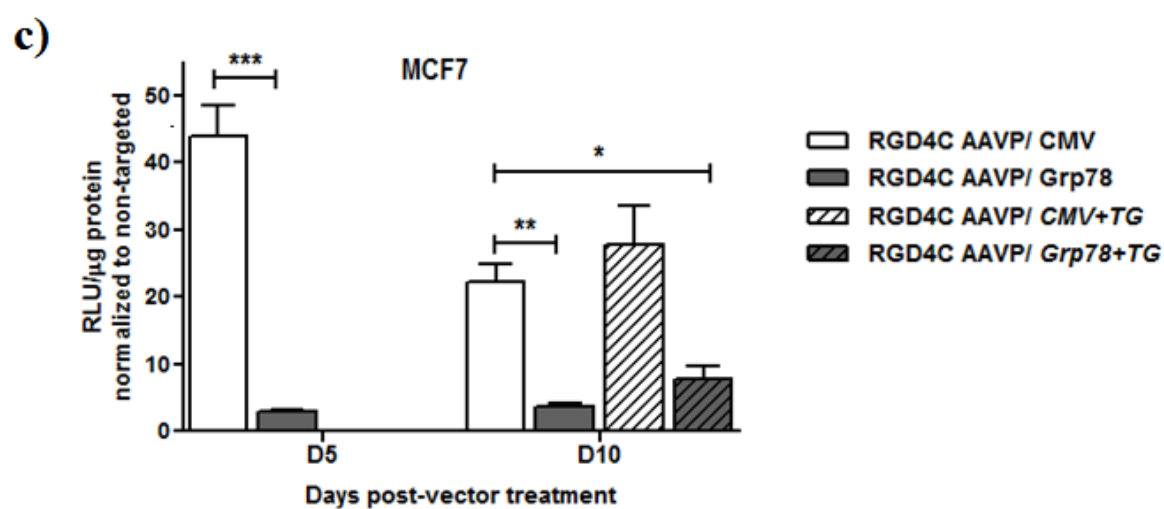
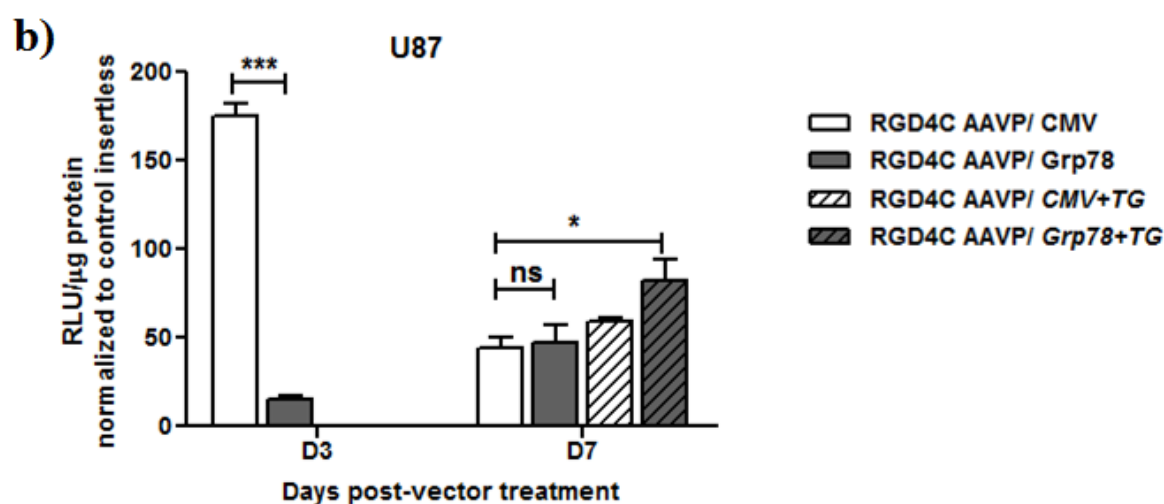
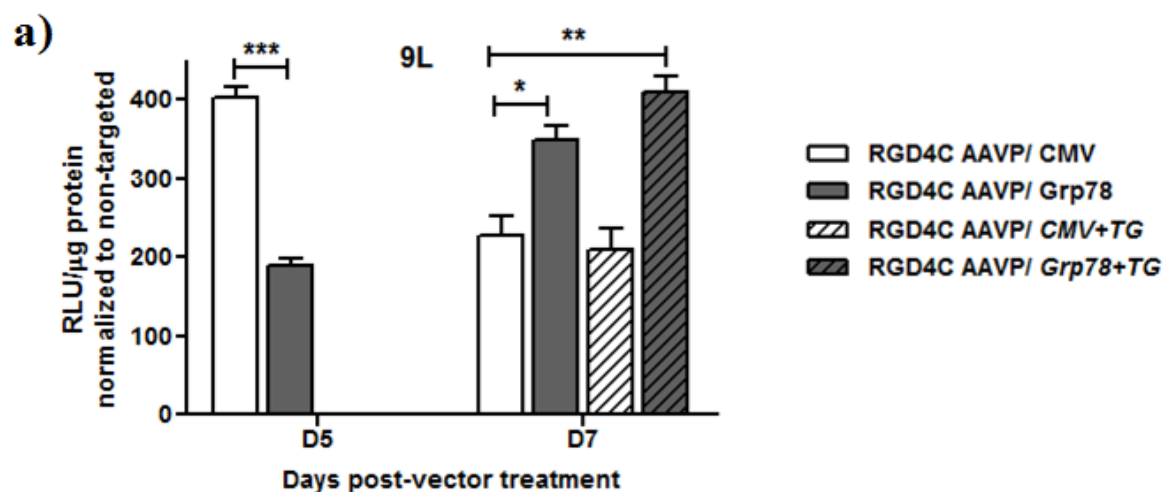


Figure 3.7. Double-targeted RGD4C AAVP/*Grp78* mediated transgene expression in tumour cells. Breast adenocarcinoma and glioblastoma cells (70-80% confluent in a 48-well plate) were incubated with either double-targeted RGD4C AAVP/*Grp78-Luc* or RGD4C AAVP/*CMV-Luc*. Control AAVP containing either *Grp78* or *CMV* promoter were used as negative controls. Luciferase assay was performed at the indicated days and normalised to protein concentration using the Bradford assay. Results represent the average Relative Luminescence Units/ μg protein (RLU/ μg) of triplicate wells. Luciferase quantification of targeted RGD4C AAVP/*Grp78-Luc* and RGD4C AAVP/*CMV-Luc* have been normalised to control insertless AAVP/*Grp78-Luc* and AAVP/*CMV-Luc* respectively. Error bars represent standard error of the mean (s.e.m). **a)** 9L cells were treated with TG (300nM-16hr) at day 5 post- vector transduction. **b)** U87cells were treated with TG at day 3 post-vector transduction. **c)** MCF-7 cells were treated with TG at day 5 post-vector transduction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ns: non-significant.

3.2.3.2.1 Evaluation of endogenous *Grp78* level in different tumour cell lines

Luciferase transgene expression mediated by the double-targeted RGD4C AAVP/*Grp78* vector demonstrated different levels of *Grp78* promoter activity in 9L, U87 and MCF-7 cells. To further evaluate the *Grp78* activity within our selected tumour cell lines, I examined the level of endogenous *Grp78* by western blot analysis (Fig. 3.8a). Interestingly, similar to the results above, the Western blot results normalized to loading control GAPDH demonstrated different levels of *Grp78*; with 9L cells expressing the highest level, U87 exhibiting intermediate level, while MCF-7 had the lowest level of *Grp78* expression (Fig. 3.8b).

Over-activity of the endogenous *Grp78* promoter has been previously reported in malignant tissues, including tumour-associated vasculature and tumour cells themselves (Pfaffenbach and Lee, 2011, Virrey et al., 2008, Dong et al., 2011, Lee, 2007). Moreover, up-regulation of *Grp78* level in the majority of malignant glioma cells with varying levels from one cell line to another had previously been shown (Pyrko et al., 2007). It has been reported that *Grp78* protein level is required for glioma cell growth; thus, cells with the fastest proliferation rate exhibit the highest level of *Grp78* and down regulation of *Grp78* level will markedly slow down cell growth (Pyrko et al., 2007).

To address the correlation between *Grp78* activity and cell proliferation rate, the growth curves of all three cell lines were examined. 9L, U87 and MCF-7 cells grown in complete DMEM medium were plated in 6- well plates, then the cells were trypsinized, counted daily and numbers displayed graphically to depict proliferation rates over 5 days (Fig. 3.8c).

Interestingly, 9L cells with the highest Grp78 protein level showed the highest proliferation rate relative to the other two cell lines. U87 and MCF-7 cells showed intermediate and low proliferation rates, respectively. These results are in agreement with previous studies showing the positive correlation between Grp78 protein level and cell proliferation rate.

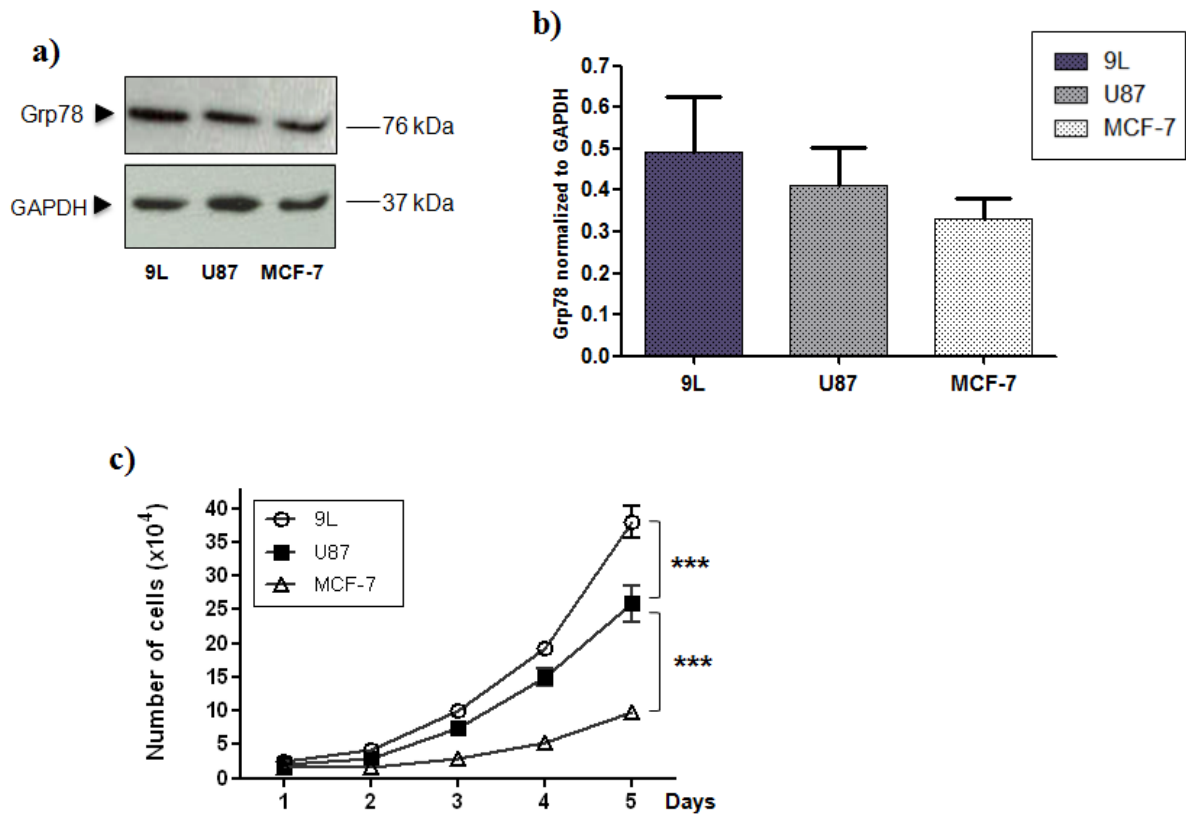


Figure 3.8. Characterisation of the endogenous Grp78 level in tumour cells. **a)** 9L, U87 and MCF7 cells were grown under standard conditions until 70-80% confluent, then lysed and their endogenous Grp78 protein levels determined by western blot. **b)** The intensity of Grp78 protein bands obtained by three independent Western blots (3.8b) were measured and normalised to GAPDH intensity. **c)** Proliferation rates of 9L, U87 glioblastoma and MCF7 breast adenocarcinoma were quantified by counting cells over time. For growth curves 9L, U87 and MCF-7 cells (1×10^4) were plated in 24-wells plates and grown under standard conditions. Triplicates were collected on days 1, 2, 3, 4 and 5 with trypsin- EDTA, and counted using trypan blue. This experiment was performed twice. Results represent the average of 3 independent western blot analyses. Error bars represent standard error of the mean (s.e.m). *** $p < 0.001$.

3.2.3.2.2 Induction of Grp78 level by TG in tumour cells

To verify the effect of TG on the endogenous Grp78 protein level, the tumour cells 9L, U87 and MCF-7 were incubated with 300nM TG for 16 hours. Then equal amount of sample proteins extracted from treated or untreated cells with TG were subjected to western blot analysis. Endogenous Grp78 level was normalized to GAPDH and the results confirmed induction of Grp78 level by 1.4-, 3.4- and 2.2 -fold upon TG treatment in 9L, U87 and MCF-7 cells, respectively (Fig. 3.9).

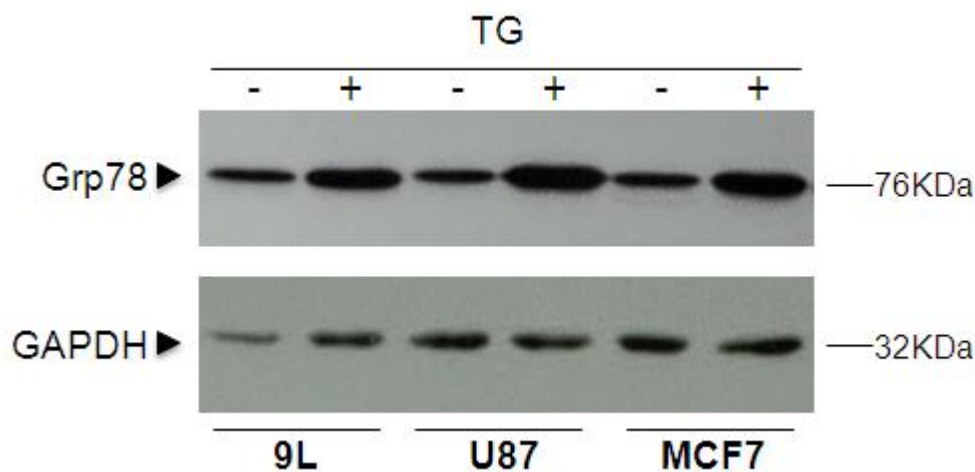


Figure 3.9. Thapsigargin induces endogenous Grp78 in tumour cells. 9L, U87 glioblastoma and MCF7 breast adenocarcinoma cells were grown in the presence or absence of 300nM TG for 16 hours, then lysed, harvested and analysed by Western blot with antibodies against Grp78 and also GAPDH as a control.

3.2.3.3 Tumour cell killing efficacy of a double-targeted vector carrying the HSVtk suicide gene

3.2.3.3.1 The double-targeted RGD4C AAVP/Grp78-HSVtk vector mediated strong cell death in combination with GCV in 9L glioblastoma cells in vitro

Next, I determined the therapeutic efficacy of the double-targeted RGD4C AAVP/Grp78 in 9L cancer cells. This was achieved by two different experiments. First, 9L cells were

transduced with either double-targeted RGD4C AAVP/*Grp78* or targeted RGD4C AAVP/*CMV* carrying the *HSVtk* suicide gene at 2×10^6 TU/cell. Insertless AAVP vectors were used as negative controls. At day 3 post-vector transduction, cells were stressed using TG in order to induce the *Grp78* promoter prior to GCV treatment. After 5 days of GCV treatment, live cells were counted using trypan blue method. The results are shown as the number of surviving cells at day 5 post GCV treatment normalized to the number of cells grown in the absence of GCV (Fig. 3.10a and 3.10b). Addition of GCV resulted in cell death by > 90% of the 9L tumour cells transduced with the double-targeted RGD4C AAVP/*Grp78*-*HSVtk* (Fig. 3.10b). The *Grp78* promoter driving *HSVtk* expression proved to be 10 times more efficient than the *CMV* promoter after GCV treatment *in vitro* in TG-stressed 9L cells (Figs. 3.10a and 3.10b). Representative phase contrast images also depict relatively more cell death in cells transduced with RGD4C AAVP/*Grp78*-*HSVtk* particles (Fig. 3.12a).

To further confirm the above results I applied another strategy in which I could quantify the percentage of live cells by luciferase assay. For this purpose, stably luciferase-labelled 9L cells were transduced with either double-targeted RGD4C AAVP/*Grp78* or targeted RGD4C AAVP/*CMV* carrying the *HSVtk* gene at 2×10^6 TU/cell. As negative controls, insertless AAVP vectors were used. Cells were incubated with TG prior to GCV treatment as described above. After 5 days of GCV treatment, surviving cells were calculated by luciferase assay. The results were further normalised to protein concentration. Consistent with previous experiments, the data were calculated by dividing the percent conversion under GCV by those under non-GCV conditions. The results indicated > 74% cell death upon GCV treatment in 9L tumour cells transduced with the double-targeted RGD4C AAVP/*Grp78*-*HSVtk* (Fig. 3.10d). However, interestingly, upon TG stress treatment, cell death was increased to 90% which was 3.2 times more efficient than the *CMV* promoter (Fig. 3.10c and 3.10d).

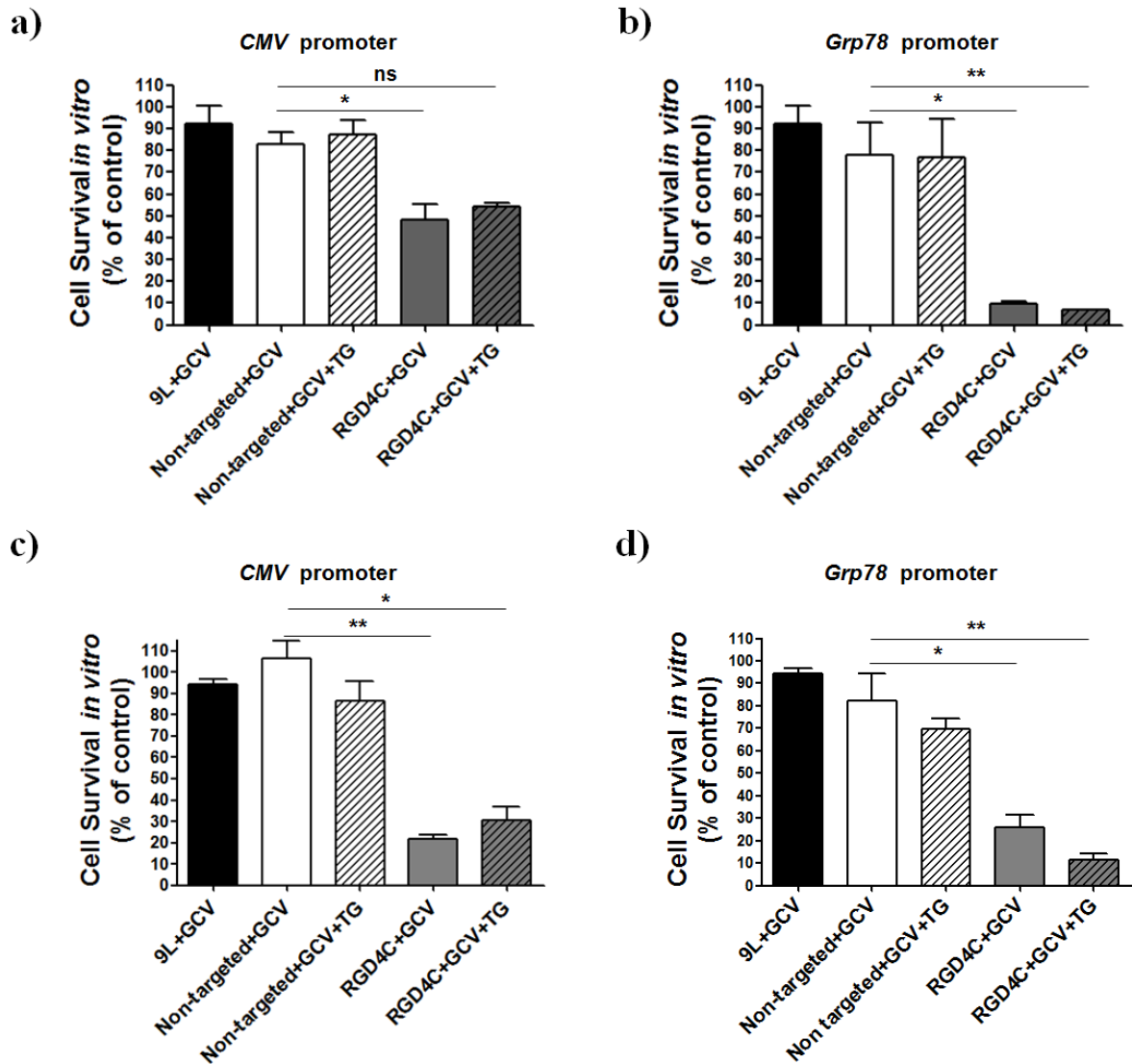


Figure 3.10. Therapeutic efficacy *in vitro* of the double-targeted RGD4CAAVP/Grp78 driving *HSVtk* expression in 9L rat glioblastoma cells. 9L cells (70-80% confluent in a 48-well plate) were incubated with either double-targeted RGD4C AAVP/Grp78-*HSVtk* or targeted RGD4C AAVP/CMV-*HSVtk*. Insertless AAVP containing either the Grp78 or CMV promoters were used as negative controls. At day 3 post- vector transduction, cells were treated with TG (300nM for 16hr), followed by GCV treatment for 5 days. Viable cells were counted using the trypan blue method. *HSVtk*/GCV mediated **a)** by double-targeted RGD4C AAVP/Grp78 and **b)** by targeted RGD4C AAVP/CMV in 9L cells. **c, d)** the same as above; however, instead of 9L parental cells, 9L-luciferase stably selected cells were incubated with either targeted RGD4C AAVP/CMV-*HSVtk* or double-targeted RGD4C AAVP/Grp78-*HSVtk*, respectively. After 5 days of GCV treatment, cells were lysed and the luciferase assay was performed. The results were normalised to protein concentration using the Bradford assay. Data were calculated by dividing the percent conversion under GCV by that under non-GCV conditions. Data represent the mean s.e.m obtained from triplicate wells and are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$ and ns: non-significant.

3.2.3.3.2 The double-targeted RGD4C AAVP/Grp78-HSVtk vector mediated strong cell death in combination with GCV in U87 glioblastoma cells in vitro

Therapeutic efficacy of the double-targeted vector was assessed in 9L cells through two different methods: i) counting live cells using trypan blue and ii) quantifying the percentage of live cells by luciferase assay of stably luciferase-labelled cells. Since similar data were obtained with both methods, I decided to validate therapeutic efficacy of the double-targeted AAVP/Grp78-HSVtk in U87 cells by simply counting the live cells. All the steps followed were similar to the experiment in 9L cells. Transduction of the U87 human glioblastoma cells with the double-targeted RGD4C AAVP/Grp78-HSVtk vector followed by GCV treatment resulted in ~75% cell death (Fig. 3.11b). However, upon TG stress treatment the cell death increased to 87% (Fig. 3.11b). Interestingly, the *Grp78* promoter driving *HSVtk* expression was approximately 3 times more efficient compared to the *CMV* promoter after GCV treatment *in vitro* in TG-stressed U87 cells (Fig. 3.11a, 3.11b and 3.12b).

Taken together, these results demonstrate that *HSVtk*/GCV suicide gene therapy by the double-targeted vector RGD4C AAVP/Grp78-HSVtk was more efficient than the RGD4C AAVP/CMV-HSVtk in both the 9L and U87 cell lines (Fig.3.10, 3.11 and 3.12).

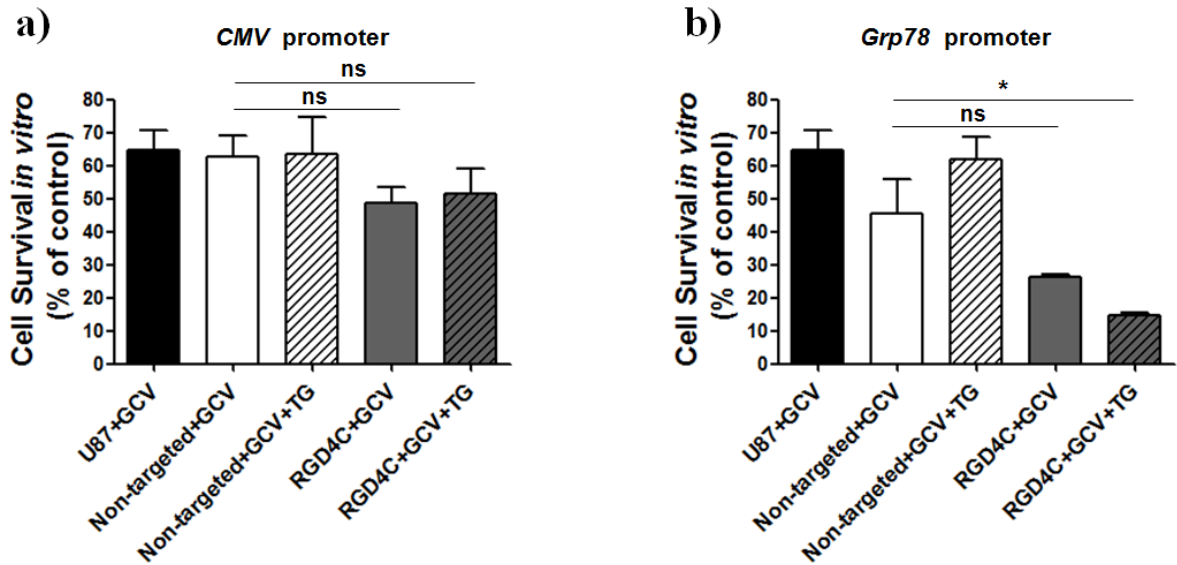
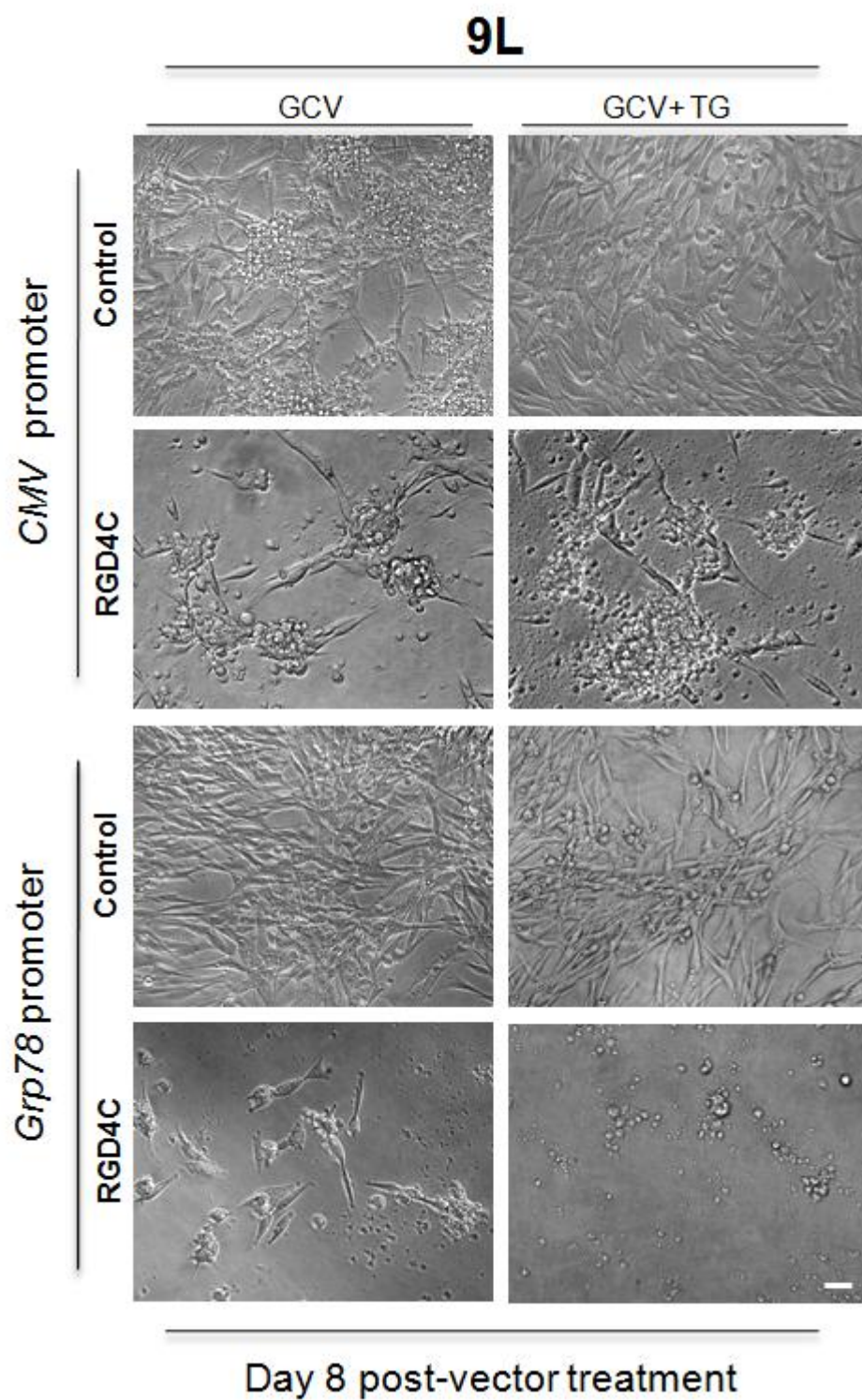


Figure 3.11. Therapeutic efficacy *in vitro* of the double-targeted RGD4CAAVP/*Grp78* driven *HSVtk* expression in U87 human glioblastoma cells. U87 cells (70-80% confluent in 48-well plate) were incubated with either double-targeted RGD4C AAVP/*Grp78*-*HSVtk* or targeted RGD4C AAVP/*CMV*-*HSVtk*. Insertless AAVP containing either *Grp78* or *CMV* promoter were used as negative controls. At day 3 post- vector transduction, cells were treated with TG (300nM for 16hr), followed by GCV treatment for 5 days. Viable cells were counted using trypan blue method. Data were calculated by dividing the percent conversion under GCV condition by that under non-GCV condition. Data represent the mean s.e.m obtained from triplicate wells. *HSVtk*/GCV mediated **a)** by the double-targeted RGD4C AAVP/*Grp78* and **b)** by the targeted RGD4C AAVP/*CMV*. These data are representative of two independent experiments. * $p < 0.05$ and ns: non-significant.

a)



b)

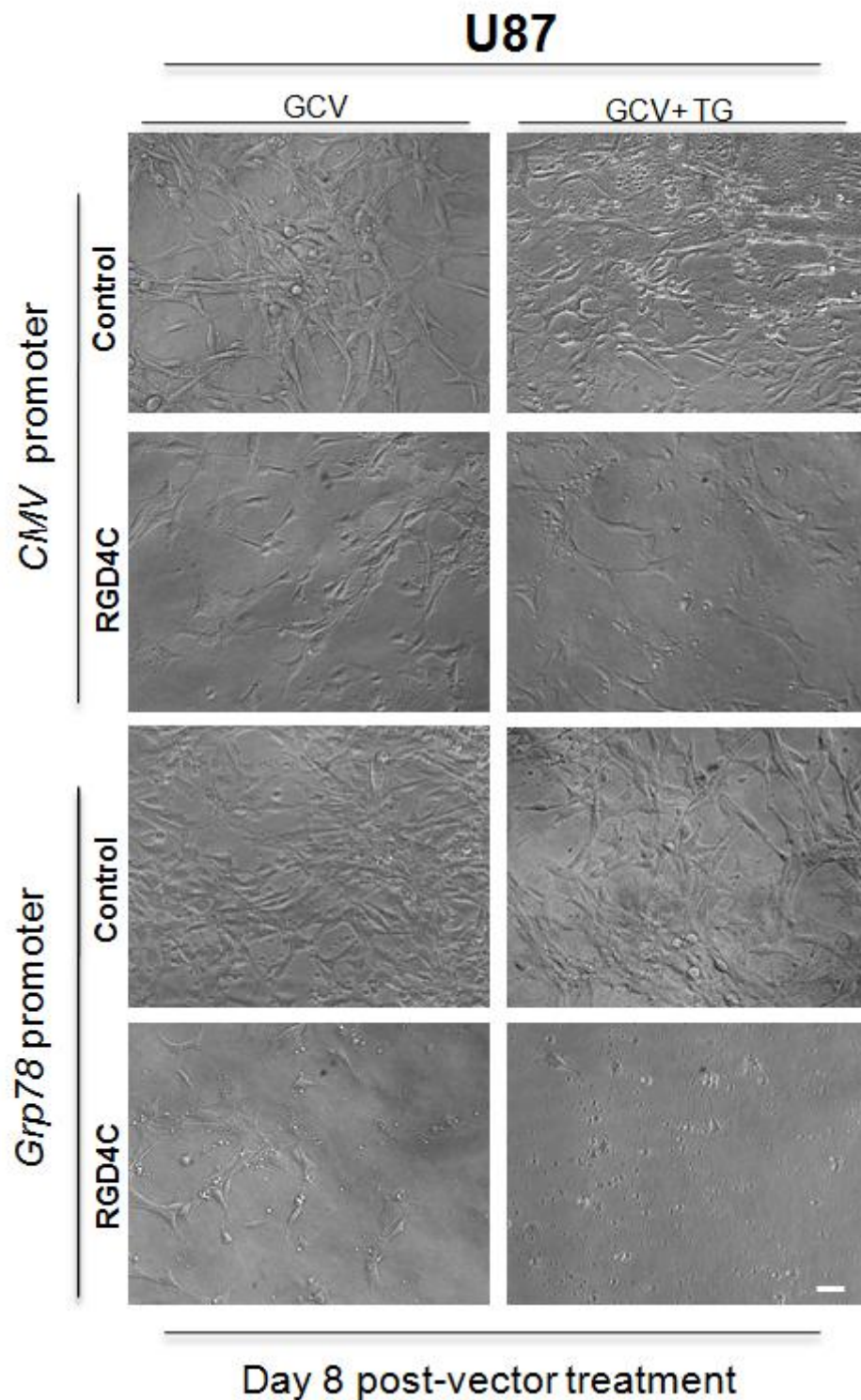


Figure 3.12. Phase contrast images showing tumour cell killing efficacy of the double-targeted RGD4C AAVP/*Grp78*-HSVtk in glioblastoma cells *in vitro*. a) The rat 9L and b) U87 human tumour cells (70-80% confluent in 48-well plate) were incubated with either double-targeted RGD4C AAVP/*Grp78*-HSVtk or targeted RGD4C AAVP/*CMV*-HSVtk. Insertless AAVP containing either *Grp78* or *CMV* promoter were used as negative controls. Cells were treated with TG (300nM for 16hr) at day 3 post- vector transduction, followed by

GCV treatment for 5 days. Cells were photographed using a 20x objective at day 5 post GCV treatment. Scale bar = 45µm.

3.3 Discussion

Transcriptional and ligand-targeting of vectors have been attempted to yield targeted systemic vectors for selective cancer gene therapy. Each of these two approaches have been used individually and showed promising results in preclinical tumour models.

Recently a novel prokaryotic-eukaryotic hybrid viral vector termed as AAV/Phage or AAVP was reported in which the AAV transgene cassette containing a *CMV* promoter was inserted into an intergenomic region of a targeted M13 filamentous bacteriophage genome (Hajitou et al., 2006b). Tumour targeting of imaging and therapeutic transgenes after systemic delivery of targeted AAVP has been established. Herein, I aimed at further increasing the tumour targeting and efficacy of gene delivery by AAVP by driving transgene expression with a mammalian tumour specific promoter, the stress-inducible promoter of the glucose regulated protein *Grp78* gene. The *Grp78* gene is overexpressed under conditions often found in tumour microenvironment such as acidic pH, glucose deprivation and chronic anoxia. Previous studies reported the selective activation of the *Grp78* promoter in tumour cells, but not in normal organs (Li and Lee, 2006, Lee, 2007). More interestingly high therapeutic transgene expression driven by this promoter in a variety of tumours *in vivo* resulted in complete eradication of tumours with no recurrence of tumour growth (Dong et al., 2004, Chen et al., 2000). To date, targeted gene therapy has been attempted by either using ligand-directed vectors or by transcriptional targeting. Herein, I used the targeted AAVP vector in order to combine these two targeting approaches into one vector system, and generated a novel double-targeted particle for systemic gene delivery to cancer cells or tumours.

First I showed that the rat *Grp78* promoter, in the context of the double-targeted AAVP vector, retains its ability to respond to ER stress by TG leading to up-regulation of transgene expression of *GFP* and *Luc* by AAVP. These results were obtained *in vitro* by using the normal immortalized human embryonic kidney HEK293 cells as well as tumour cells such as 9L rat glioblastoma, U87 human glioblastoma and MCF-7 human breast cancer. These data are in agreement with previous studies showing that the *Grp78* promoter activity is independent of the p53 or phosphatase and tensin homolog (PTEN) status in glioma and breast cancer cells. Indeed, the p53 and PTEN status is variable in 9L cells (Lilge, 2002)

(mutated p53 gene- wild type PTEN), U87 (wild type p53- mutated PTEN) (Lu et al., 2004) (Senatus et al., 2006) and MCF-7 cells (wild type p53 protein)(Lim et al., 2009). Interestingly, transgene expression driven by the double-targeted RGD4C AAVP/*Grp78-Luc* showed better persistence overtime than the targeted RGD4C AAVP/*CMV-Luc* in all tested cell lines. Initially the basal *CMV* promoter activity proved higher than that of the *Grp78* in all cell lines; however, luciferase transgene expression from the *CMV* promoter decreased dramatically overtime, while a steady increase in *Grp78* promoter activity was observed. Although, viral promoters express high levels of the transgene upon entry into mammalian cells, it is well known that their long-term activity becomes silenced in cells. Our results are in agreement with other studies reporting transgene silencing with *CMV* promoters. Although the *CMV* promoter is active in mammalian cells, it is susceptible to transcriptional inactivation by DNA methylation and histone deacetylation (Mehta et al., 2009). In contrast eukaryotic promoters such as *Grp78* can sustain long-term transgene expression in mammalian cells.

Luciferase quantification showed stronger basal activity of the *Grp78* promoter compared to *CMV* at day 7 post-vector transduction of 9L and U87 cells, and increased further after TG treatment. These data are consistent with previous studies reporting elevated *Grp78* protein level in malignant glioma specimens, human malignant glioma cells as well as in vasculature derived from human glioma specimens, both *in situ* in tissue and *in vitro* in primary cell cultures (Pyrko et al., 2007, Virrey et al., 2008). The *Grp78* overexpression in glioma cells correlated with the rate of cell proliferation (Pyrko et al., 2007). Interestingly, transgene expression by the double-targeted AAVP/*Grp78* reached the highest level in the 9L cells that exhibit the highest rate of proliferation compared to U87. The hormone-dependent MCF-7 breast cancer cells with the least proliferation rate showed the lowest luciferase expression driven by the *Grp78* promoter. Consistently, analysis of *Grp78* gene expression in human breast tumours from patients showed overexpression of the *Grp78* in 100% of the aggressive hormone-independent breast tumours compared to hormone-dependent tumours (Fernandez et al., 2000).

Treatment with TG resulted in 2.2- fold increase of *Grp78* activity in MCF-7 cells. Induction of the *Grp78* promoter activity in MCF-7 by TG treatment was previously reported in the context of retroviral vectors (Dong et al., 2004). The authors reported an 8- fold increase of transgene expression driven by *Grp78* in a retroviral vector upon TG treatment of MCF-7.

These differences can be explained by the fact that the measurements of transgene expression in other studies were carried out on vector-selected cells stably expressing the transgene under the control of the *Grp78* promoter. In our work, I did not apply any selection force to select for vector-transduced cells, as compared to previous reports in which the cells were selected after transduction with vectors carrying a *Grp78* transgene expression cassette. Indeed, AAVP has been designed for systemic gene therapy; thus, this part of our work aimed at mimicking more accurately the *in vivo* situation where no selection can be applied to select for vector-transduced cells after systemic administration.

After confirming the efficacy of the double-targeted vector to deliver reporter transgene expression in tumour cells, I subsequently evaluated the therapeutic effect of *HSVtk* as a therapeutic gene combined with the GCV prodrug. The advantage of *HSVtk*/GCV therapeutic approach is that the converted cytotoxic triphosphate GCV can spread from transduced cells to non-transduced cells via gap junction intercellular communication. This “bystander effect” may overcome the low transduction of tumours and the need for all cells to be transduced in order to achieve a therapeutic effect. Expression of *HSVtk* in 10 to 20% of the tumour cells can result in complete tumour cell death *in vitro* and tumour eradication *in vivo* upon cell implantation in mice (Freeman et al., 1993). The *HSVtk*/GCV bystander effect has already been reported in 9L and U87 cells (Trepel et al., 2009, Zhang et al., 2009b).

Herein I showed that the *Grp78* promoter driving *HSVtk* gene expression in the context of double-targeted RGD4C AAVP led to dramatic 9L and U87 cell death *in vitro*. More interestingly this *in vitro* therapeutic effect of the RGD4C AAVP/*Grp78*-*HSVtk* proved more efficient than that of the targeted AAVP/*CMV*-*HSVtk* as measured at day 9 post- vector transduction. This finding is consistent with our luciferase quantification experiments that showed better persistence of *Grp78* promoter activity overtime in these cells. Our results are also in agreement with previous reports that demonstrated the superiority of retroviral vectors driving *HSVtk* expression under *Grp78* promoter control over retroviral vectors carrying the *HSVtk* gene under the control of the murine leukemia virus (MuLV) long-terminal repeat (LTR) promoter (Huang et al., 2000, Azatian et al., 2009).

In conclusion, I have shown that the *Grp78* promoter is functional and retains its inducible characteristic in the context of the AAVP phage viral vector. Thus, I have generated a novel double-targeted vector that proved efficient to target the α_v integrin receptors on the cell surface as well as target transgene expression at the transcriptional level *in vitro*. I found that

the double-targeted AAVP/*Grp78* vector provides better long-term transgene expression in tumour cells *in vitro* than the original targeted AAVP/*CMV* vector. Finally, the therapeutic efficacy of the double-targeted RGD4C AAVP/*Grp78*-*HSVtk* proved more efficient on tumour cells *in vitro*.

Chapter 4

Long-term Grp78 and CMV promoter activity in the context of the AAVP vector in vitro

4.1 Introduction

The *CMV* promoter has been largely utilized in many gene therapy vectors, because of its ability to drive strong and constitutive expression of transgenes both *in vitro* and *in vivo*. However, one problematic feature of this promoter is its nonspecific nature; the promoter itself does not significantly discriminate between different cell types or conditions as its activity is high in a range of mammalian tissues (Cheng et al., 1993). In addition to the above issue, the *CMV* promoter has been reported to undergo silencing by mammalian host cells (Brooks et al., 2004, Grassi et al., 2003, Prosch et al., 1996). Although the exact mechanisms of silencing of viral DNAs have remained mainly unknown, several studies have demonstrated the association of DNA methylation and histone deacetylation with inactivation of the *CMV* promoter (Swindle and Klug, 2002, Murphy et al., 2002). Generally DNA methylation/demethylation and histone acetylation/deacetylation play major roles in epigenetic gene regulation through providing accessibility of transcription factors to gene promoters.

DNA methylation is mediated by the DNA methyltransferase enzymes DNMT1, DNMT3A and DNMT3B. Using S-adenosyl-methionine as the methyl donor, DNMTs can covalently methylate a cytosine residue adjacent to guanosine in the 5' direction of DNA (CpG islands) (Singal and Ginder, 1999). Following DNA methylation, particular proteins containing a

methylcytosine-binding domain (MBD) will be recruited to the methylated DNA and consequently repress the gene transcription by obstructing the access of transcription factors to the gene promoter (Jin et al., 2011). 55% of all human gene promoters contain CpG islands while the presence of CpG islands in human genome is quite infrequent. This proportion demonstrates the significant role of DNA methylation in the regulation of gene expression.

Drugs such as 5-Azacytidine (5-Aza) which can incorporate into the DNA of dividing cells instead of deoxycytosine and covalently binds and inhibits the DNMT activity, was shown to promote the expression of previously silenced genes. By inhibition of DNMT activity, the MBD proteins cannot bind to DNA and as a result there is access for transcription factors to bind to gene promoters. 5-Aza has successfully been used to reactivate silenced gene expression from several gene therapy vectors such as retroviral and adeno-associated viral vectors (Jaenisch et al., 1985, Gram et al., 1998).

Previous studies demonstrated complete repression of *CMV* promoter activity by *Spiroplasma* methyltransferase SssI, a prokaryotic DNA methyltransferase, which mediates DNA methylation through 5' CpG islands (Prosch et al., 1996). In the same study, they partially reversed *CMV* repression by simultaneously transfecting cells with highly methylated DNA which suggests a repressive control via a trans-acting factor that binds to 5'CpG methylated DNA (Prosch et al., 1996). Moreover, other reports also confirmed the inactivation of the *CMV* promoter in transfected cells *in vitro* by methylation. In addition, *CMV*-driven human transgenes delivered to rat muscle via first-generation adenovirus underwent a 53- fold decrease in expression over 24 hours (Brooks et al., 2004). In the same study, bisulfite sequencing revealed extensive methylation of both 5'CpG and non-CpG sites in the promoter region, with 5'CpA methylation predominating among the non-CpG sites. Notably, hypermethylation of tumour suppressor genes is an attribute of many cancers, with 95% of acute leukemias displaying hypermethylated calcitonin, and more than 40 genes are being hypermethylated in breast cancer (Das and Singal, 2004). This raises the question of whether cancer cells, with their tendency toward extensive methylation, are predisposed to *CMV* methylation in particular (Robertson and Jones, 2000, Herman and Baylin, 2003).

Another mechanism which has been implicated in modifying gene expression epigenetically is histone acetylation/ deacetylation. The histone acetylation status and consequently regulation of gene transcription are maintained by a balance between acetylation by histone

acetyltransferase (HATs) and deacetylation by histone deacetylase enzymes (HDACs) (de Ruijter et al., 2003). HATs mediate histone hyperacetylation which assist the relaxed form of chromatin and activate gene transcription, while HDACs function in opposition to HATs. HDACs can catalyse removal of acetyl groups from specific lysine residues located in N-terminal parts of the histone tail, resulting in chromatin condensation, and therefore prevention of recruitment of transcription factors on gene promoters leading to silencing of gene expression.

The link between transcriptional repression and HDAC activity was first demonstrated by the identification of the first HDAC (HDAC1) which shares significant homology to budding yeast Rpd3p that has previously been implicated in transcriptional repression (Taunton et al., 1996).

In mammalian cells, eighteen HDACs have been identified so far and based on their homology to budding yeast's HDACs, they are classified into four groups (de Ruijter et al., 2003, Blander and Guarente, 2004, Kim and Bae, 2011). Members of class I (HDAC1, 2, 3 and 8) have homology to yeast reduced potassium dependency 3 (Rpd3) which are located within the nucleus. Class II members (HDAC4, 5, 6, 7, 9 and 10) share homology to yeast histone deacetylase 1 (Hda1) and are located in both nucleus and cytoplasm.

Based on the sequence homology and domain organization, Class II is further subdivided into class IIa (HDACs 4, 5, 7, and 9) and class IIb (HDACs 6 and 10). Class IIa is composed of a highly conserved C-terminal deacetylase catalytic domain, while Class IIb is characterized by having two deacetylase domains (Verdin et al., 2003).

HDAC11 is the sole member of class IV which shares sequence similarity with catalytic core regions of class I and class II members, however since it does not show enough similarity it has been placed in class IV. Classes I, II, and IV require a zinc molecule as a cofactor in their active site for their deacetylase activity; hence, their activities are inhibited by Zn^{2+} binding HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA). The classification of the eleven Zn^{2+} dependent HDACs is shown in Table 4.1.

Class III HDACs (SIRT1, 2, 3, 4, 5, 6 and 7) were first found with the discovery of yeast silent information regulator 2 (Sir2) which exhibits NAD-dependent histone deacetylase activity (Imai et al., 2000). Since this class of HDACs requires NAD^+ as a cofactor, their activity cannot be inhibited by Zn^{2+} binding HDAC inhibitors.

The involvement of histone deacetylation in gene silencing might occur in conjugation with DNA methylation. It has been reported that DNMT and MBD proteins can recruit HDACs following DNA methylation. Several studies demonstrated the key function of gene hypermethylation and histone deacetylation in human malignancies as well as silencing and protection against viral DNA sequences such as the *CMV* promoter (Baylin, 2005, Cedar and Bergman, 2009).

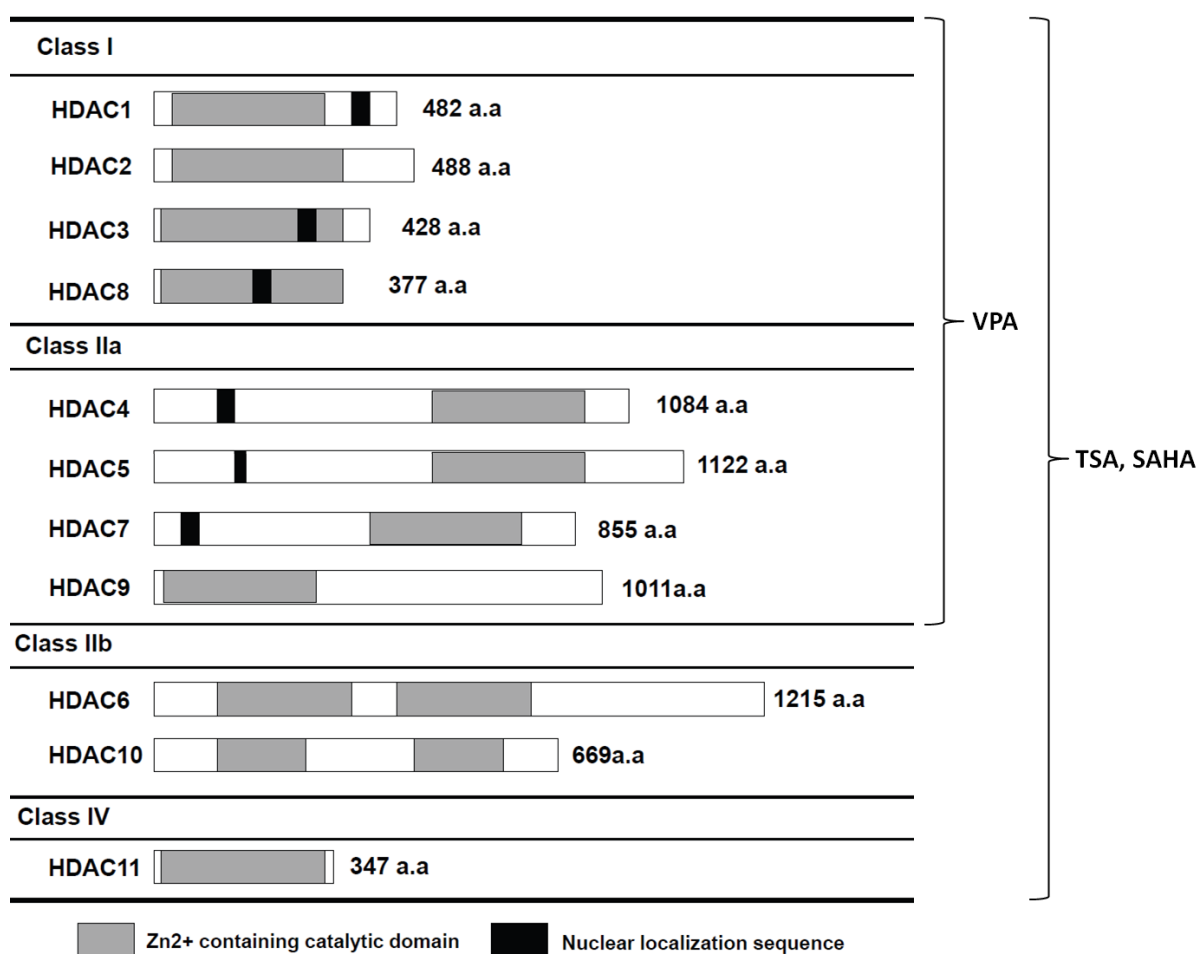
The first characterized organic HDAC inhibitor drug, TSA (Yoshida et al., 1990) has been widely utilized to study the reactivation of silenced viral constructs. Also SAHA, structurally similar to TSA, has been categorized as a histone deacetylase inhibitor. Both chemicals can mediate inhibition of HDACs of class I and II by binding and blocking the catalytic site of the HDACs (Bi and Jiang, 2006).

Previous studies demonstrated that removal of trichostatin-A (TSA), a histone deacetylase inhibitor, from the medium of HeLa cells transduced with a recombinant AAV/*CMV-LacZ* construct resulted in chromatin remodelling and transcriptional repression of the *LacZ* gene. This change in chromatin structure was rapid, with 90% of silencing occurring within 4hrs of TSA removal (Chen and Townes, 2000). Although the *CMV* promoter silencing has involved considerable controversy, these data and others suggest that, regardless of the mechanism, silencing of the *CMV* promoter region over time will lead to reduced transgene expression. Consequently, consideration of alternate promoters will be necessary for a number of gene therapy pursuits requiring long-term transgene expression.

In chapter 3, our data demonstrated a marked decrease in *CMV* promoter activity in 9L, U87 and MCF-7 cells over 7-10 days post RGD4C AAVP/*CMV-Luc* vector transduction. In contrast *Grp78*- regulated *Luc* expression increased over 7-10 days post RGD4C AAVP/*Grp78-Luc* vector transduction. Unfortunately due to a dilution effect of proliferating transduced cells within a larger population of proliferating nontransduced cells (Hajitou et al., 2008) I could not further monitor the promoter's activity beyond that time point. Therefore, much of the effort in this chapter was devoted to characterizing both *CMV* and *Grp78* promoter activity over a long time course. For this purpose I first cloned a puromycin resistance cassette into the AAVP vector to enable generation of stably transduced cells. The results of this chapter address the *CMV* promoter silencing in the context of AAVP whose activity I consequently tried to further restore by treatment with the DNA methylation inhibitor 5-Aza, as well as histone deacetylation inhibitors such as TSA and SAHA which are

known to inhibit class I and II HDACs, and valporic acid (VPA) which inhibits class I and nicotinamide which inhibits Class III HDAC enzyme activity (Johnstone, 2002) (Table 4.1). It is noteworthy to mention that the work presented in sections 4.2.4 and 4.2.5 was undertaken with the assistance of Miss Nastasia Nianiaris, MSc student in Molecular Medicine at Imperial College.

Table 4.1. Schematic representation of the Zn^{2+} dependent histone deacetylase family. (VPA: valporic acid, TSA: trichostatin A and SAHA: suberoylanilide hydroxamic acid. Adapted from Kim, H.J. & Bae, S.C., 2011.



4.2 Results

4.2.1 Generation of an AAVP vector containing a puromycin resistance cassette

4.2.1.1 RGD4C AAVP/Grp78-GFP-puro^R and RGD4C AAVP/CMV-GFP-puro^R

To generate the double-targeted RGD4C AAVP/ *Grp78-GFP-puro^R* and targeted RGD4C AAVP/CMV-GFP-puro^R vector, a puromycin resistance cassette was cloned into the *SacI* site of AAVP located next to the AAV transgene cassette (Fig. 4.1). First, the 1168bp fragment containing the puromycin resistance gene under the control of SV40 early enhancer/ promoter was released from the pGL4.20 plasmid (promega) by *BamHI* and *SalI* double restriction digestion. The purified puromycin resistance cassette fragment from the agarose gel was blunted with DNA polymerase and further ligated to *SacI* linkers. After restriction digestion with *SacI*, the puromycin resistance cassette was ligated into the *SacI* site of the RGD4C AAVP/CMV-GFP and RGD4C AAVP/Grp78-GFP plasmids (Fig. 4.1a and 4.1b). The unique *SacI* site in the RGD4C AAVP/GFP is located next to the AAV transgene cassette. The positive recombinants were identified by *SacI* digestion and agarose gel electrophoresis.

4.2.1.2 RGD4C AAVP/Grp78-Luc-puro^R and RGD4C AAVP/CMV-Luc-puro^R

To insert the puromycin resistance cassette into AAVP carrying *Luc* as a reporter gene, a different strategy was performed as the *SacI* restriction site is located within the *Luc* region. Briefly, AAVP/Grp78-GFP-puro^R plasmid was digested with *NotI* and *SphI* in order to remove a fragment containing *GFP* and replaced by a *NotI*- *SphI* *Luc*-containing fragment from the AAVP/Grp78-*Luc* (Fig. 4.2). The same strategy was applied for the RGD4C AAVP/CMV-*Luc*-puro^R construct.

The positive recombinants were identified by *NotI*- *SphI* digestion and agarose gel electrophoresis.

4.2.1.3 RGD4C AAVP/Grp78-HSVtk-puro^R and RGD4C AAVP/CMV-HSVtk-puro^R

To generate RGD4C AAVP/Grp78-HSVtk-puro^R, a different strategy from the previous constructs was adapted since both *SacI* and *SphI* restriction sites are located within the *HSVtk* gene. Briefly, the AAVP/Grp78-GFP-puro^R plasmid was digested with *SacII* in order to

remove the *Grp78-GFP* fragment. This fragment was subsequently replaced by a *SacII* *HSVtk*-containing fragment from the AAVP/*Grp78-HSVtk* (Fig. 4.3). The correct orientation of the *Grp78-HSVtk* fragment within AAVP was evaluated by *BamHI* digestion. The same procedure was performed to generate the RGD4C AAVP/*CMV-HSVtk-puro*^R vector.

All the generated vectors were amplified, isolated and purified from the culture supernatant of host bacteria (*E.coli* K91) as described in materials and methods.

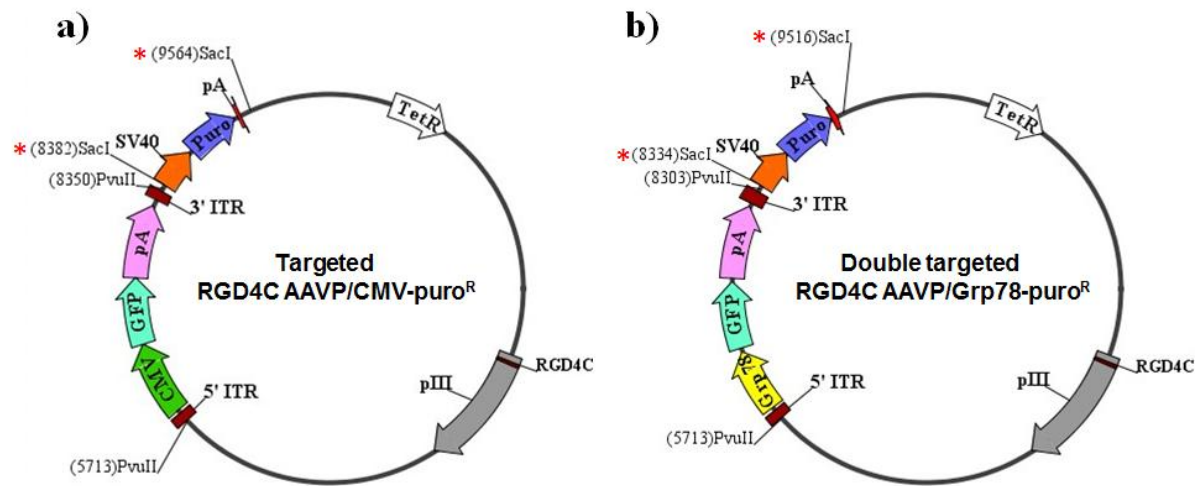


Figure 4.1. Schematic chart of the a) RGD4C AAVP/CMV-GFP-puro^R and b) RGD4C AAVP/Grp78-GFP-puro^R vectors. The puromycin resistance gene under the control of the SV40 promoter was released from the pGL4.20 by *BamHI* and *SalI* digestion and then ligated to *SacI* linkers. This fragment was subsequently inserted into the unique *SacI* site of the RGD4C AAVP/CMV-GFP and RGD4C AAVP/Grp78-GFP plasmids. (Puro: puromycin-resistance gene, TetR: tetracycline resistance gene, ITR: inverted terminal repeat, pA: polyadenylation site).

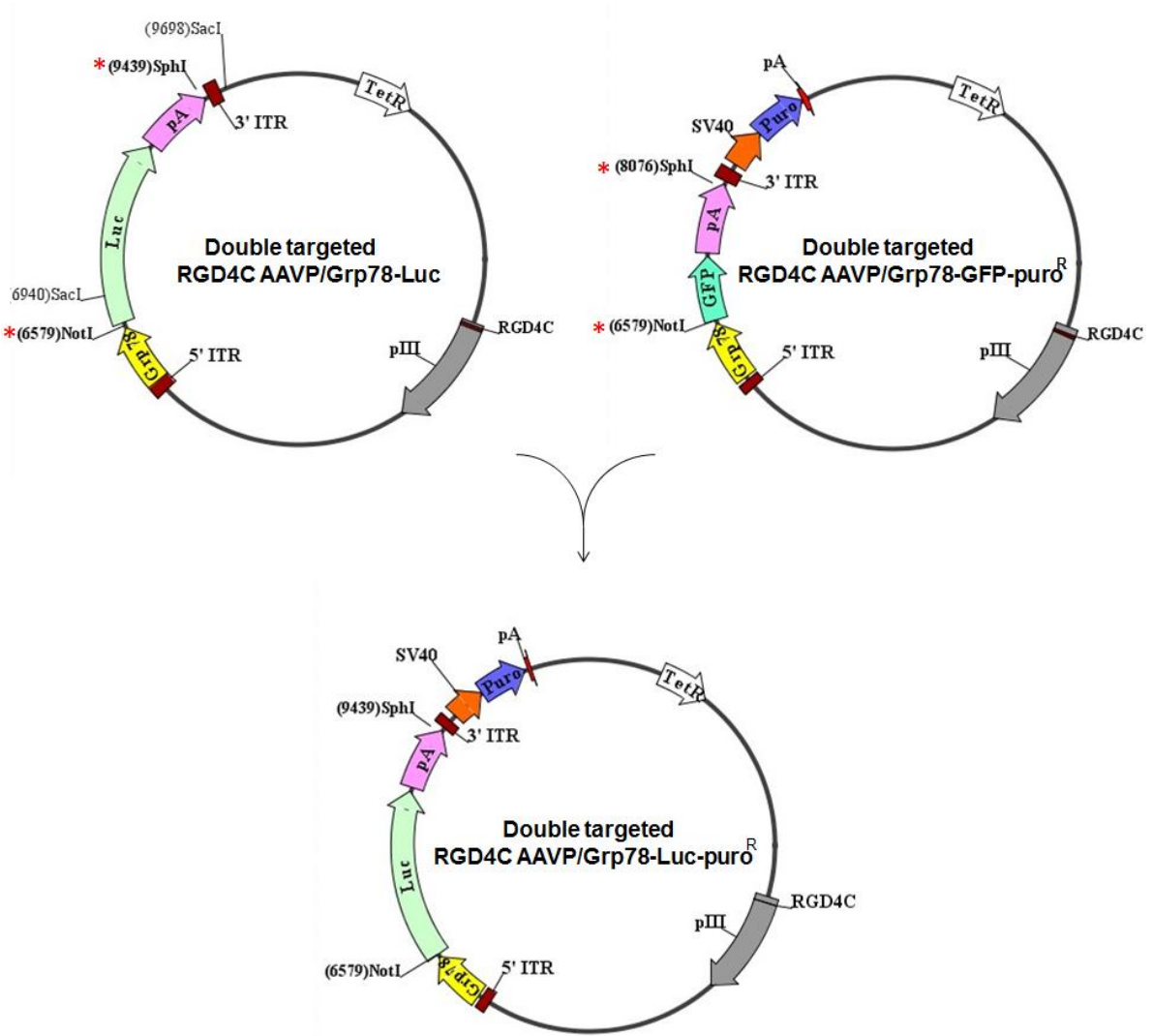


Figure 4.2. Schematic chart describing construction of the RGD4C AAVP/Grp78-Luc-puro^R. The *Luc* gene flanked by *Sph*I and *Not*I was released from the AAVP/Grp78-Luc plasmid and ligated to the RGD4C AAVP/Grp78-GFP-puro^R plasmid digested with *Sph*I and *Not*I to replace *GFP*. (Puro: puromycin-resistance gene, TetR: tetracycline resistance gene, ITR: inverted terminal repeat, pA: polyadenylation site).

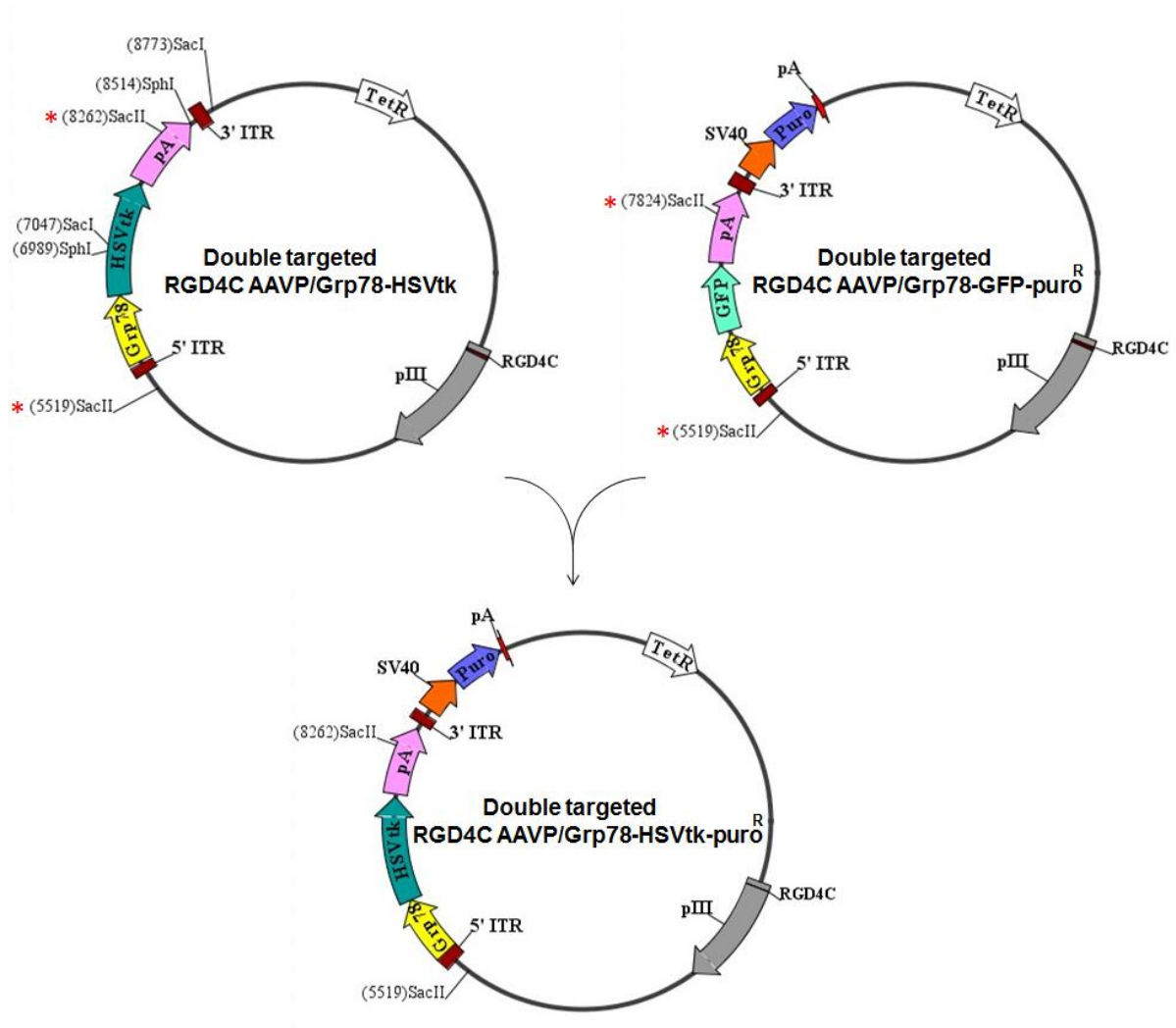


Figure 4.3. Schematic chart describing construction of the RGD4C AAVP/Grp78-HSVtk-puro^R. The *Grp78-HSVtk* fragment flanked by *SacII* was released from the RGD4C AAVP/Grp78-HSVtk plasmid and ligated to the RGD4C AAVP/Grp78-GFP-puro^R plasmid digested with *SacII* to replace the *Grp78-GFP* fragment. (Puro: puromycin-resistance gene, TetR: tetracycline resistance gene, ITR: inverted terminal repeat, pA: polyadenylation site).

4.2.2 TG treatment of glioblastoma cells induces Grp78 promoter activity at both the endogenous and AAVP vector level

In the previous chapter, I showed an induction of endogenous Grp78 following TG treatment in 9L and U87 cells. Herein, I aimed at assessing the *Grp78* promoter activity in the context of double-targeted AAVP vector after TG treatment, an inducer of ER stress, *in vitro*. Thus, Western blot analysis of stably transduced 9L as well as U87 cells with RGD4C AAVP/*Grp78*-HSVtk-puro^R or RGD4C AAVP/*CMV*-HSVtk-puro^R following treatment with 300nM TG for 16 hours was performed to evaluate both endogenous *Grp78* and transgene *HSVtk* expression. Non-transduced parental cells were also used as controls. As shown in figure 4.4, following TG treatment, endogenous Grp78 expression increased in parental as well as stably transduced 9L and U87 cells.

At the AAVP vector level, as expected, no effect of TG was observed on HSVtk expression in both 9L and U87 cells stably transduced with RGD4C AAVP/*CMV*-HSVtk-puro^R. In contrast, TG treatment for 16 hours, resulted in increased expression of HSVtk from the *Grp78* promoter in both cells transduced with the RGD4C AAVP/*Grp78*-HSVtk-puro^R vector. No HSVtk expression was detected in parental 9L and U87 cells containing no vector (Fig. 4.4).

These results show the induction of the rat *Grp78* promoter in the context of the AAVP vector following TG treatment in both rat 9L and human U87 glioblastoma cells.

Moreover, Western blot analysis of HSVtk expression driven by *Grp78* in 9L cells stably transduced with the RGD4C AAVP/*Grp78*-HSVtk-puro^R vector showed higher activity of the *Grp78* promoter over *CMV*. In contrast, *CMV* promoter activity was much higher than that of the *Grp78* promoter in stably transduced U87 cells (Fig. 4.4). These data are in agreement with our previous results demonstrating higher activity of the *Grp78* promoter in rapidly growing 9L cells compared to moderately fast growing U87 cells.

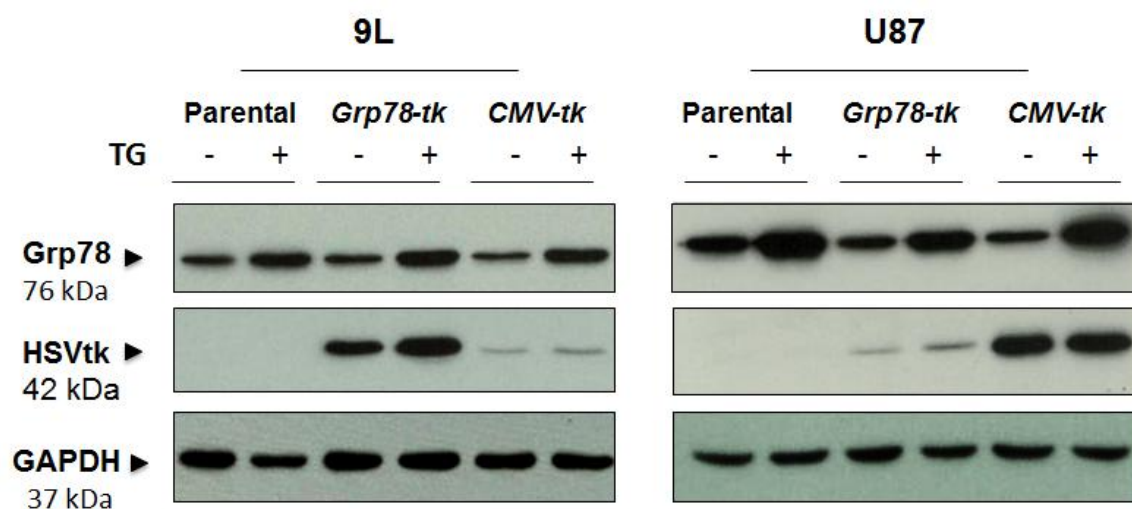


Figure 4.4. Thapsigargin induces *Grp78* promoter activity in the context of the AAVP vector in stably transduced 9L and U87 glioblastoma cells. Stably transduced 9L and U87 cells with RGD4C AAVP/*Grp78*-HSVtk-puro^R or RGD4C AAVP/*CMV*-HSVtk-puro^R as well as parental cells, were grown in the presence or absence of 300 TG for 16 hours. Then, equal amounts of treated and untreated cell lysates were analysed by Western blot with antibodies to Grp78, HSVtk and GAPDH (n=3).

4.2.3 RGD4C AAVP/*Grp78* provides long-term GFP transgene expression in glioblastoma cells

Data obtained in chapter 3 showed that *Grp78*- regulated *Luc* expression increased in 9L, U87 and MCF-7 cells over 7-10 days post RGD4C AAVP/*Grp78*-*Luc* vector transduction; in contrast, *Luc* expression driven by the *CMV* promoter decreased over the same period post RGD4C AAVP/*CMV*-*Luc* vector transduction. Unfortunately, due to the dilution effect of proliferating transduced cells within a larger population of proliferating non-transduced cells (Hajitou et al., 2008) I could not further monitor the promoter activity beyond that time point. To overcome the above limitation and enable further characterization of both *CMV* and *Grp78* promoter activities over a long time course, 9L and U87 glioblastoma cell lines were stably transduced with targeted RGD4C AAVP/*CMV*-GFP-puro^R or double-targeted RGD4C AAVP/*Grp78*-GFP-puro^R AAVP vectors, and selected in the presence of puromycin. As negative controls, parental cells were grown in the presence of puromycin alongside the

transduced cells. Next, puromycin-resistant clones were monitored over time using a fluorescent microscope.

As an initial analysis of transgene expression efficiency, we counted and monitored GFP positive as well as GFP negative puromycin-resistance cell clones over time. Counting of 9L stably transduced clones started at 2 weeks post-vector transduction when they were visible under microscope, while U87 clones were visible at 17 days post-vector transduction. Two representative images of GFP positive and GFP negative cell clones under the microscope are shown in figure 4.5.

At two weeks post-vector transduction, 92% of the 9L clones transduced by the RGD4C AAVP/*Grp78-GFP-puro^R* construct (n= 846 of 959 clones) were GFP positive, compared to 83% of clones transduced by the RGD4C AAVP/*CMV-GFP-puro^R* (n= 845 of 1258 clones). At four weeks post-vector transduction, we detected the presence of GFP expression in 83% of the RGD4C AAVP/*Grp78-GFP-puro^R* clones, while only 59% of the clones transduced by the RGD4C AAVP/*CMV-GFP-puro^R* vector were GFP positive (Fig. 4.6a). Figure 4.7 represents an example of complete silencing of the *CMV* promoter over two weeks in one of the 9L cell clones stably transduced with RGD4C AAVP/*CMV-GFP-puro^R*. Also, more representative images depicting the initiation stage of the *CMV* promoter silencing in 9L clones stably transduced with the RGD4C AAVP/*CMV-GFP-puro^R* vector are presented in figure 4.8.

As for U87 cells, at 17 days post-vector transduction, out of a total of 276 clones transduced with RGD4C AAVP/*Grp78-GFP-puro^R* 67.4 % were GFP positive compared to only 47.3% out of a total of 300 clones transduced with RGD4C AAVP/*CMV-GFP-puro^R* (Fig. 4.6b). No significant change in GFP expression was observed in U87 cell clones at four weeks post-vector transduction with either RGD4C AAVP/*Grp78-GFP-puro^R* or RGD4C AAVP/*CMV-GFP-puro^R* (Fig. 4.6b).

To further evaluate promoter activity in the course of time, all cell clones were pooled after 4 weeks post-vector transduction and GFP expression was analysed by flow cytometry. The examples of histograms shown in figure 4.9a, represent the silencing of stably transduced cells over a time-course study. At day 39 post-vector transduction, 57% of 9L cells transduced by the RGD4C AAVP/*Grp78-GFP-puro^R* vector were positive for GFP expression and this number increased drastically to 85% towards day 97 (Fig. 4.9b). In contrast, only 37% of cells transduced by RGD4C AAVP/*CMV-GFP* expressed GFP at day

39 post-vector transduction, followed by a marked drop to 11% at day 97 (Fig. 4.9b). Moreover, this decrease in GFP positive cells was associated with ~10- fold drop of the mean fluorescent intensity (MFI) of RGD4C AAVP/*CMV-GFP-puro*^R cells from day 39 towards day 97 post-vector transduction, while MFI remained stable in cells transduced with RGD4C AAVP/*Grp78-GFP* (Fig. 4.9c). Also, representative fluorescence pictures depict a drastic decrease in GFP positive cells stably transduced by the RGD4C AAVP/*CMV-GFP-puro*^R from day 39 towards day 97 post-vector transduction (Fig. 4.9d).

Similarly, FACS analyses showed that GFP expression in U87 cells transduced with RGD4C AAVP/*Grp78-GFP-puro*^R increased from 40% at day 39 to 66.3% at day 75 post-vector transduction (Fig. 4.10a and 4.10b). In contrast, 59% of cells transduced with RGD4C AAVP/*CMV-GFP-puro*^R were positive for GFP expression at day 39, and this number decreased remarkably to 5.6% towards day 75 post-vector transduction (Fig. 4.10a and 4.10b). Although, U87 cells transduced with RGD4C AAVP/*CMV-GFP-puro*^R presented significantly higher MFI than that of RGD4C AAVP/*Grp78-GFP-puro*^R cells at day 39, their MFI decreased by 17.6- fold towards day 75 post-vector transduction. Interestingly MFI remained constant in RGD4C AAVP/*Grp78-GFP-puro*^R cells over the indicated times (Fig 4.10c). In figure 4.10d, fluorescence pictures show a marked decrease in GFP positive cells stably transduced by the RGD4C AAVP/*CMV-GFP-puro*^R from day 39 to day 75 post-vector transduction, whereas, GFP positive cells stably transduced by the RGD4C AAVP/*Grp78-GFP-puro*^R remained constant over the indicated times.

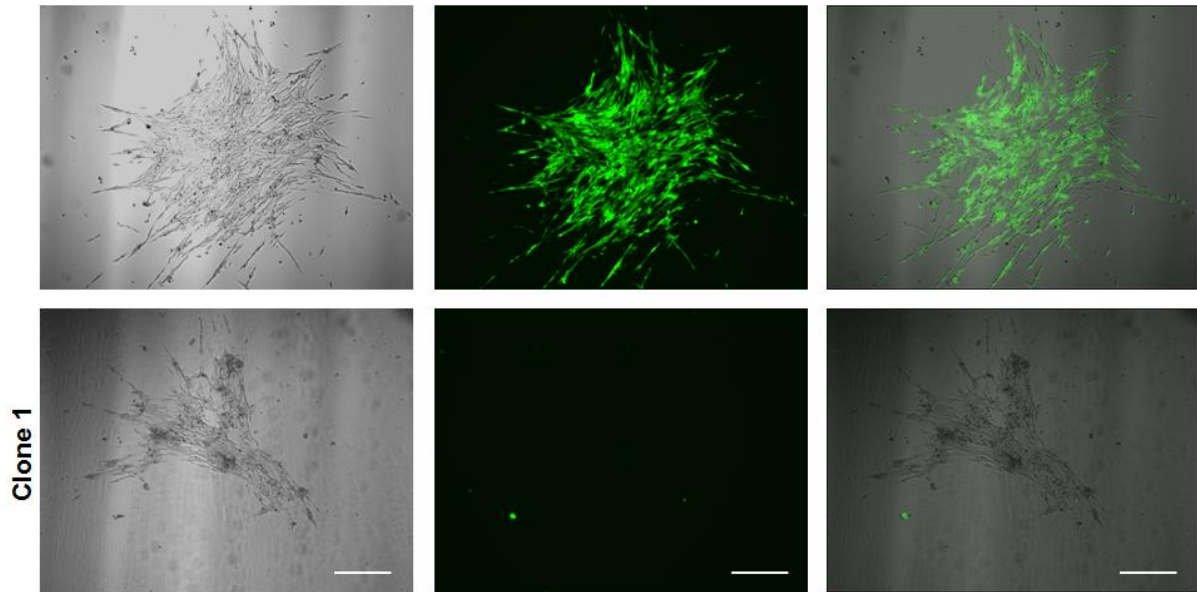


Figure 4.5. Representative images of GFP positive and GFP negative clones. 9L cells (70-80% confluent in a 6-well plate) were transduced with targeted RGD4C AAVP/*CMV-GFP-puro^R*. At day 3 post-vector transduction cells were diluted 1:10 and grown in puromycin- containing medium. Representative phase contrast and fluorescence microscopy images captured at day 14 post-vector transduction depict GFP positive and GFP negative clones (n=5). Magnification= x4. Scale bar = 200 μ m.

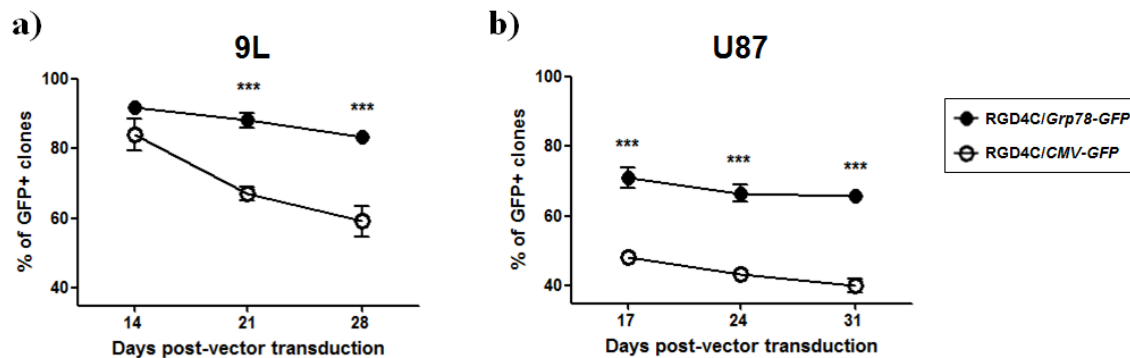


Figure 4.6. Evaluation of *Grp78* and *CMV* promoter activity in the context of AAVP in glioblastoma cells. a) 9L and b) U87 cells (70-80% confluent in a 6-well plate) were transduced with either targeted RGD4C AAVP/*CMV-GFP-puro^R* or double-targeted RGD4C AAVP/*Grp78-GFP-puro^R*. At day 3 post-vector transduction, cells were diluted 1:10 and grown in puromycin- containing medium. The total GFP positive and negative clones were counted under the microscope over the indicated times. The statistic analysis was performed using a 2-way ANOVA between two variants at each time point. ***p < 0.001.

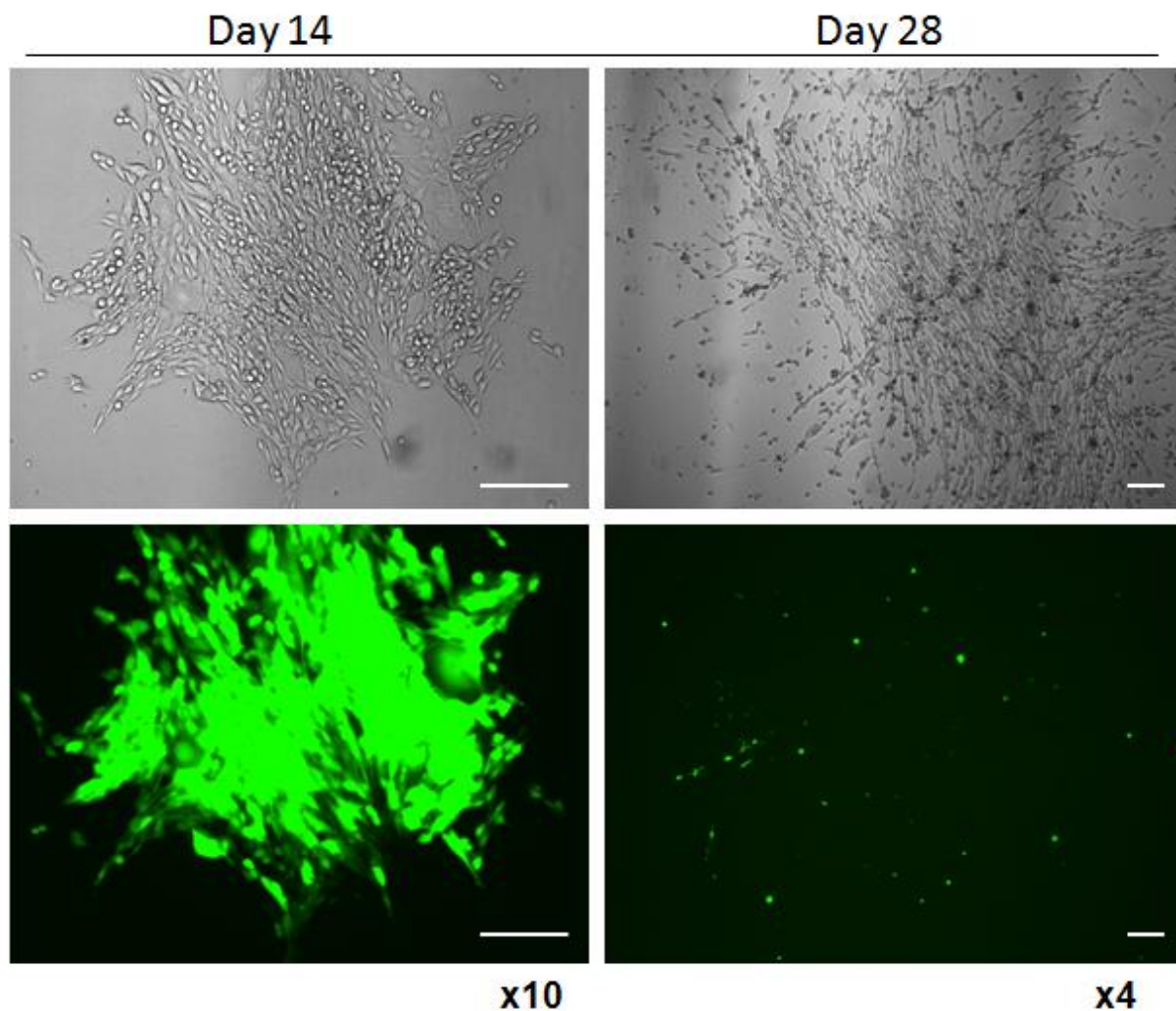


Figure 4.7. CMV promoter activity silences over time during the puromycin selection process in 9L cell clones transduced with RGD4C AAVP/CMV-GFP-puro^R. 9L cells (70-80% confluent in a 6-well plate) were transduced with the targeted RGD4C AAVP/CMV-GFP-puro^R vector. At day 3 post-vector transduction cells were diluted 1:10 and grown in puromycin- containing medium. The GFP positive clones were monitored under the microscope over time. Representative images were captured at days 14 and 28 post-vector transduction from the same clone (n=5). Scale bar = 200μm.

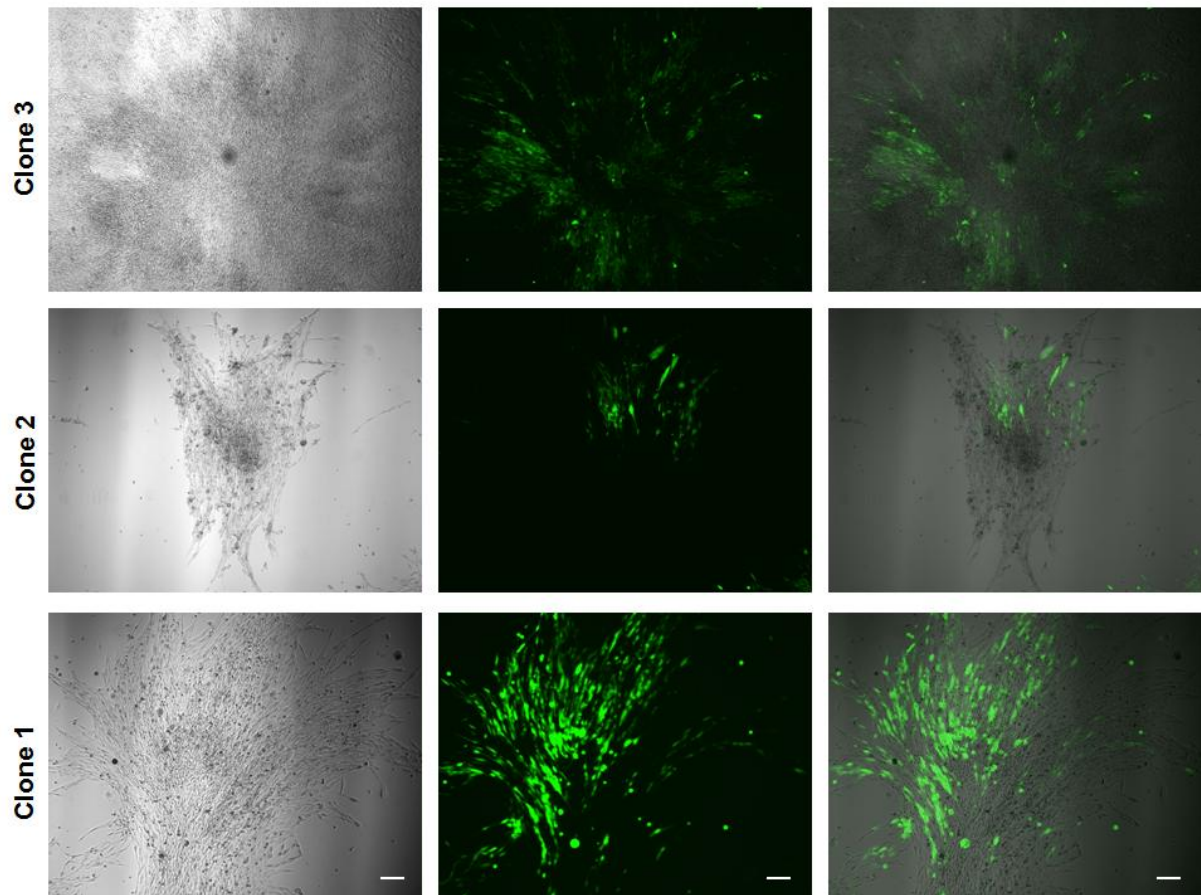
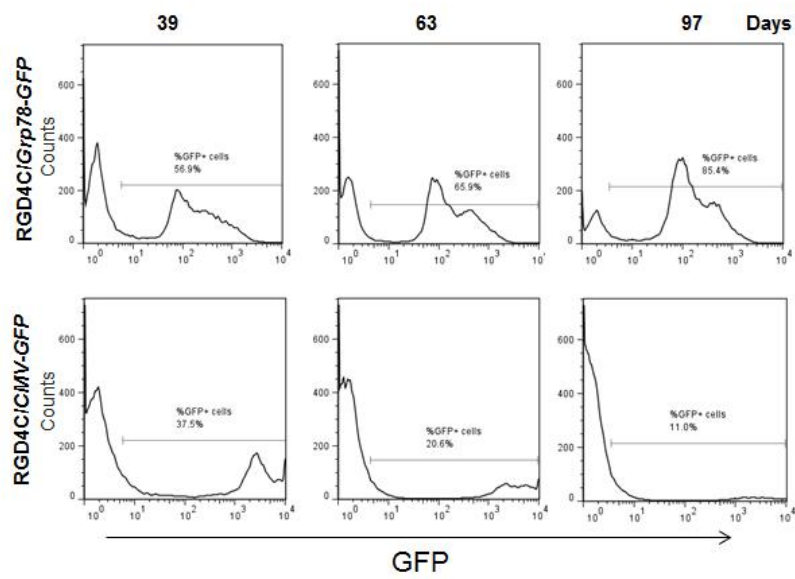
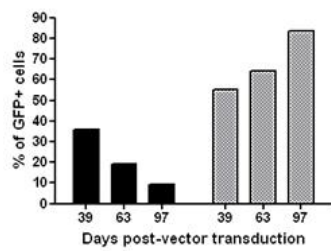


Figure 4.8. Initiation of *CMV* promoter silencing within puromycin resistant 9L clones. 9L cells (70-80% confluent in a 6-well plate) were transduced with targeted RGD4C AAVP/*CMV*-GFP-puro^R. The GFP positive clones were monitored over time under the microscope. Representative images captured at day 21 post-vector transduction depict initiation of *CMV* promoter silencing within stably transduced clones. Magnification= x4. Scale bar = 200μm.

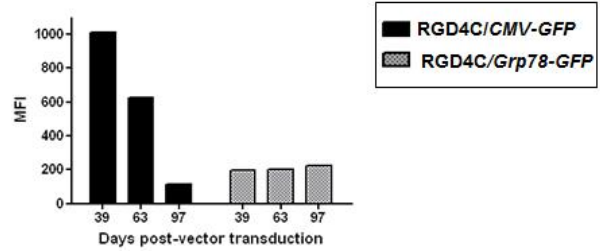
a) 9L



b)



c)



d)

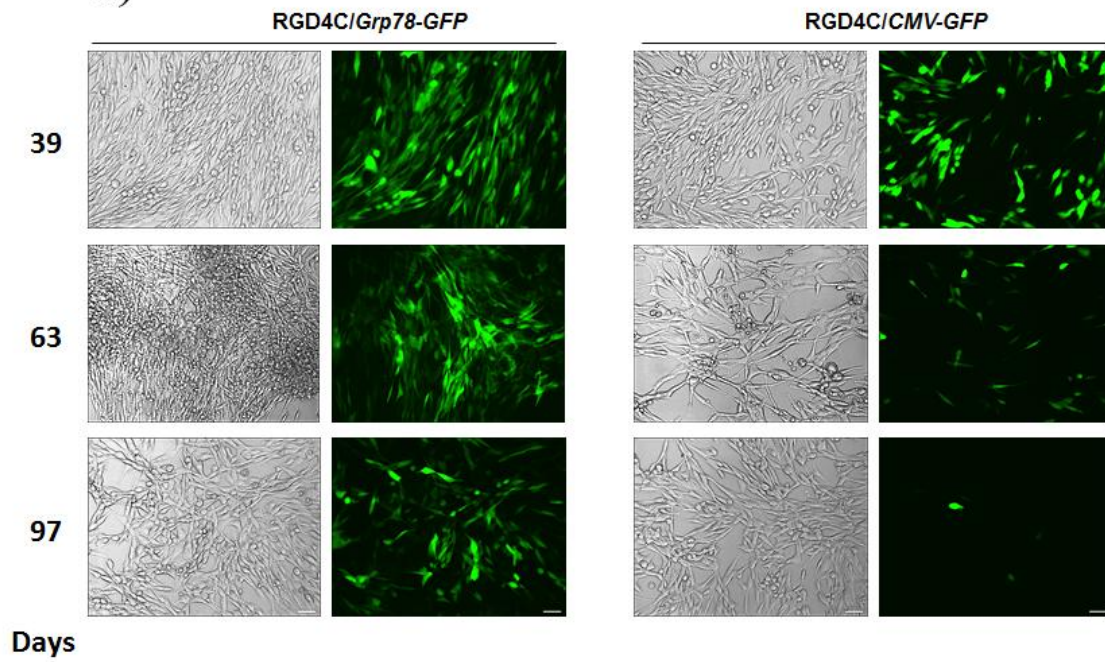


Figure 4.9. Persistence of *Grp78* promoter activity in 9L rat glioblastoma cells upon transduction with RGD4C AAVP/*Grp78*-GFP- *puro*^R. **a)** Time course FACS analysis was performed to quantify GFP expression in the 9L cell population of pooled cell clones obtained with either RGD4C AAVP/*Grp78*-GFP or RGD4C AAVP/*CMV*-GFP. **b)** Percentage of GFP positive cells and **c)** Mean fluorescence intensity (MFI) derived by either *Grp78* or *CMV* promoter over the indicated times. **d)** The pictures are representative of GFP positive cell change overtime. These data are representative of two independent experiments. Scale bar = 45µm.

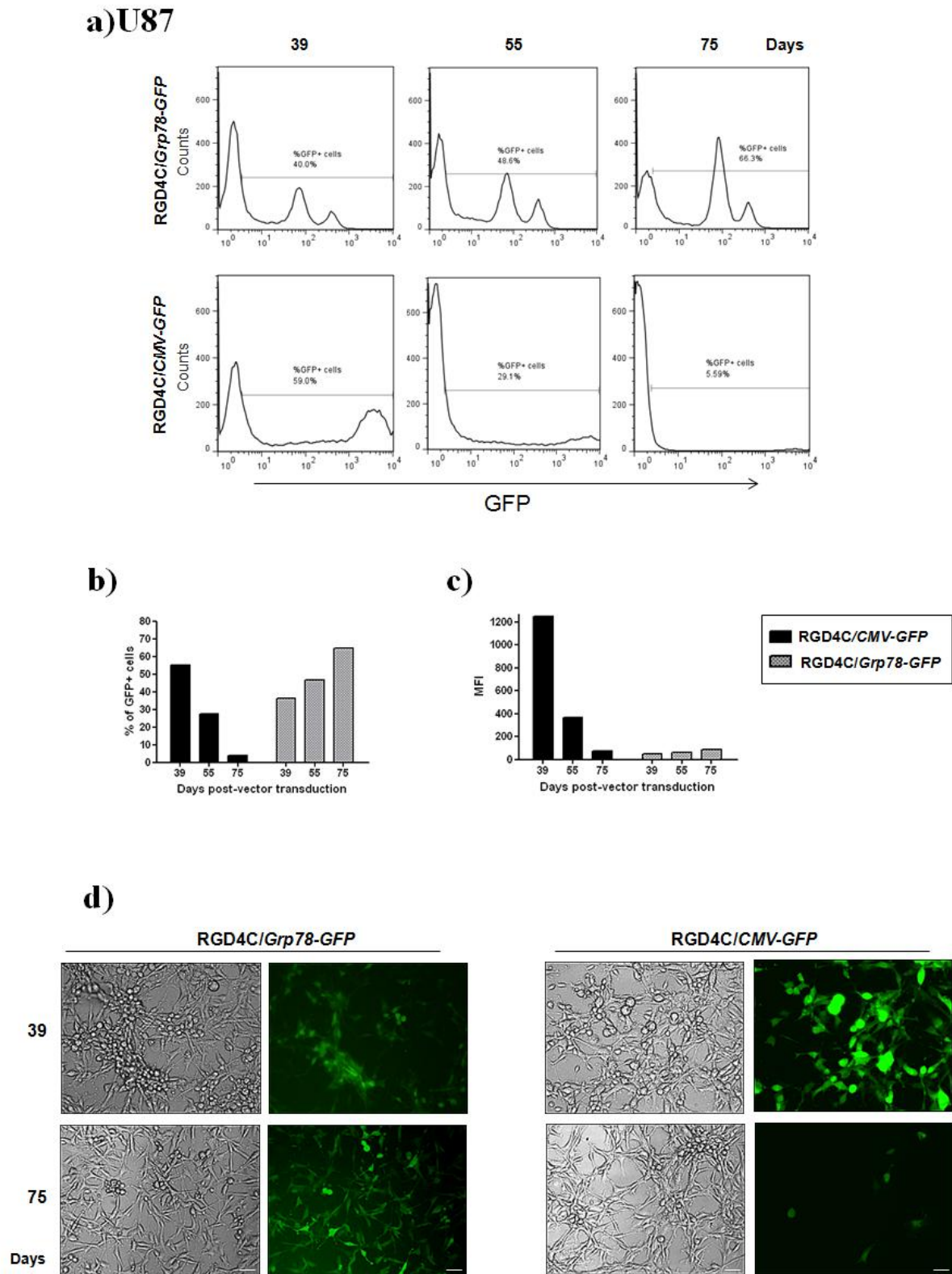


Figure 4.10. Persistence of *Grp78* promoter activity in U87 human glioblastoma cells upon transduction with RGD4C AAVP/*Grp78*-GFP-*puro*^R. **a)** Time course FACS analysis was performed to quantify GFP expression in the U87 cell population of pooled cell clones

obtained with either RGD4C AAVP/*Grp78-GFP* or RGD4C AAVP/*CMV-GFP*. **b)** Percentage of GFP positive cells and **c)** Mean fluorescence intensity (MFI) derived by either *Grp78* or *CMV* promoter activity over the indicated times. **d)** The pictures are representative of GFP positive cell change overtime. These data are representative of two independent experiments. Scale bar = 45µm.

4.2.4 RGD4C AAVP/*Grp78* mediates long-term HSVtk suicide gene expression in glioblastoma cells

In order to confirm the long-term activity of the *Grp78* promoter, we examined suicide gene *HSVtk* expression over time, controlled by either *CMV* or *Grp78* promoters. Tumour cells were transduced with RGD4C AAVP/*Grp78-HSVtk-puro^R* or RGD4C AAVP/*CMV-HSVtk-puro^R* vectors and stably transduced cells were generated under puromycin selection. 9L and U87 stably transduced cells were lysed and harvested at one month intervals beginning at days 47 and 66 post-vector transduction, respectively. Then, *HSVtk* expression was evaluated by Western blot analysis.

Consistent with FACS analysis experiments, western blot analysis showed higher level of *HSVtk* expression in 9L cells transduced by RGD4C AAVP/*Grp78-HSVtk-puro^R* compared to cells transduced with RGD4C AAVP/*CMV-HSVtk-puro^R* and increased gradually over time (Fig. 4.11a). In contrast, a dramatic decrease of *HSVtk* levels was observed in cells transduced with RGD4C AAVP/*CMV-HSVtk-puro^R* from day 47 towards day 78 post-vector transduction, at day 109 post-vector transduction the *HSVtk* level was barely detectable (Fig. 4.11a). Quantification of the *HSVtk* band intensities and normalization to loading control GAPDH showed ~ 6.5- fold decrease in *HSVtk* expression driven by the *CMV* promoter (Fig. 4.10a).

In U87 cells, consistent with FACS data, specifically MFI analysis, although *HSVtk* expression was initially stronger in cells stably transduced with RGD4C AAVP/*CMV-HSVtk-puro^R*, the levels of *HSVtk* decreased gradually from day 66 towards day 137 post-vector transduction. In contrast, *HSVtk* expression stayed constant over time in cells stably transduced with RGD4C AAVP/*Grp78-HSVtk-puro^R* (Fig. 4.11b). After normalizing *HSVtk* level to GAPDH ~ 3.3- fold decrease in *HSVtk* expression driven by the *CMV* promoter was detected.

As a control, parental cell lines containing no vector were also subjected to similar analysis and no *HSVtk* expression was detected (Fig. 4.11c).

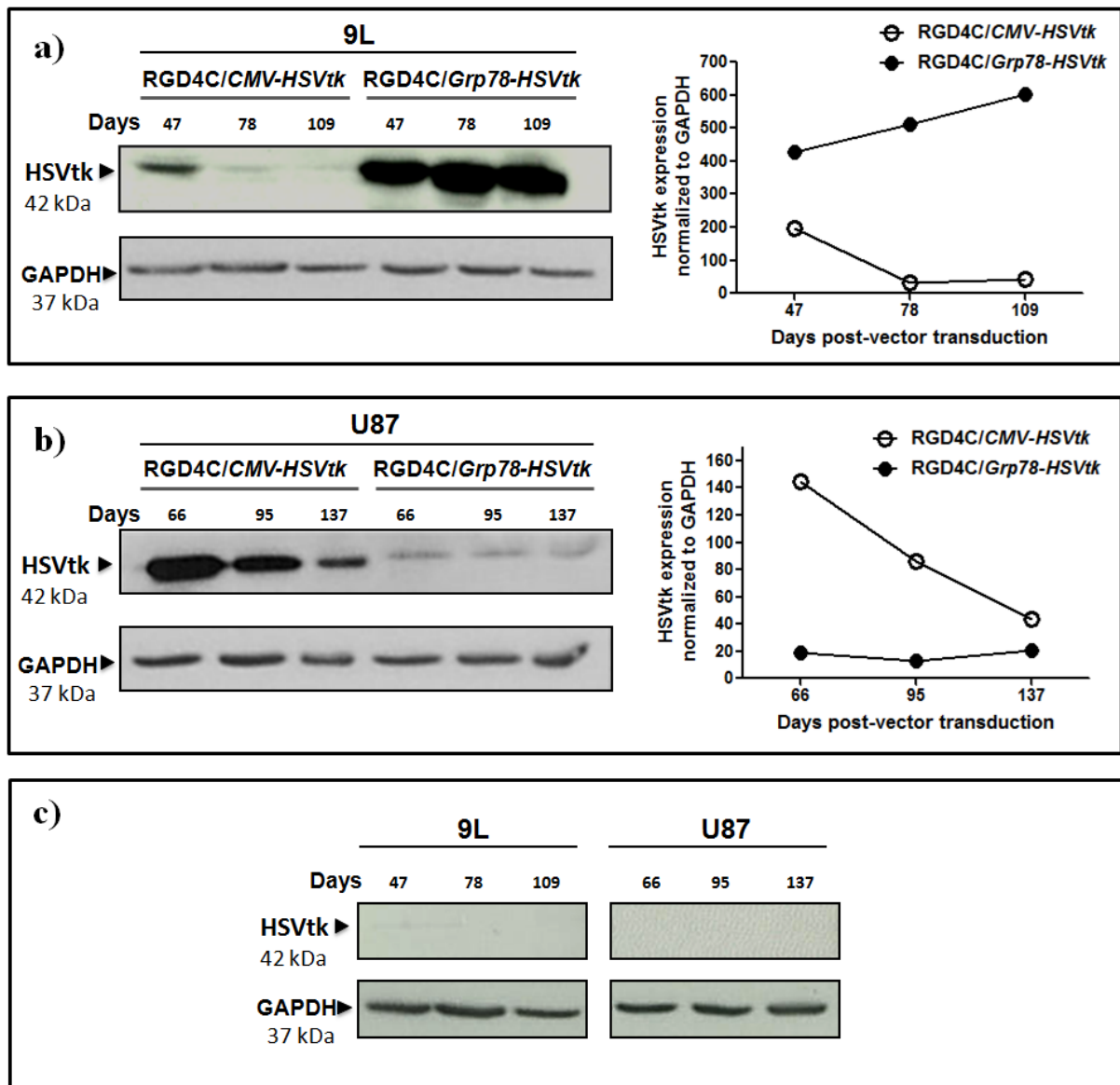


Figure 4.11. Long-term HSVtk expression driven by either *Grp78* or *CMV* promoter in the context of AAVP. Whole cell lysates of **a)** 9L and **b)** U87 cells stably transduced with either targeted RGD4C AAVP/*CMV-HSVtk-puro*^R or double-targeted RGD4C AAVP/*Grp78-HSVtk-puro*^R were collected at one month intervals for three months beginning at 44 and 66 days post-vector transduction for 9L and U87 cells, respectively. **c)** Parental 9L and U87 cells were collected alongside the stably transduced cells as a control. HSVtk protein levels were determined by western blot analysis. The intensity of Grp78 protein bands were measured and normalised to GAPDH intensity. These data are representative of two independent experiments.

4.2.5 RGD4C AAVP/Grp78-HSVtk plus GCV treatment mediates strong and constant tumour cell killing

4.2.5.1 Stably transduced 9L cells with RGD4C AAVP/Grp78-HSVtk-puro^R vector showed strong cell death in combination with GCV

Next, stably transduced 9L cells with RGD4C AAVP/Grp78-HSVtk-puro^R or RGD4C AAVP/CMV-HSVtk-puro^R were used to assess tumour cell killing efficacy by vectors overtime once at day 47 (t1) and then at day 109 (t2) post-vector transduction. HSVtk suicide gene therapy was induced at each time point by treatment with GCV for 24hr, 48hr, 72hr or 96 hr. Live cells were counted using the trypan blue method. The results are shown as the number of surviving cells post GCV treatment normalized to the number of cells grown in the absence of GCV at each time point. Addition of GCV at t1 resulted in significantly higher death of 9L cells transduced with RGD4C AAVP/Grp78-HSVtk-puro^R relative to those transduced with RGD4C AAVP/CMV-HSVtk-puro^R vector at each of the four time points (Fig. 4.12). More interestingly, consistent with Western blot experiments of HSVtk expression over time (Fig. 4.11a), at a later time point (t2), we observed a significant decrease in the efficacy of tumour cell killing by RGD4C AAVP/CMV-HSVtk-puro^R compared to t1 (Fig. 4.12), in which between t1 and t2 the CMV promoter activity decreased by 3.3, 4.2 and 12.7 times at 24, 48 and 96 hours post GCV treatment, respectively. In contrast to CMV promoter efficacy, Grp78 promoter activity remained stable at both t1 and t2 (Fig 4.12).

Representative phase contrast images were also taken at each 24 hour interval up to 96 hours following GCV treatment for each of the time points (Fig. 4.13). The images also depict substantial cell death difference between t1 and t2 time points from 9L cells stably transduced with RGD4C AAVP/CMV-HSVtk-puro^R (Fig. 4.13a and 4.13b). In contrast, dramatic cell death at both t1 and t2 time points were observed in the 9L cells stably transduced with RGD4C AAVP/Grp78-HSVtk-puro^R (Fig. 4.13a and 4.13b).

4.2.5.2 Stably U87 transduced cells by the RGD4C AAVP/Grp78-HSVtk-puro^R vector mediated strong cell death overtime in combination with GCV

To assess long-term promoter activity in the context of AAVP in U87 cells, stably transduced U87 cells with RGD4C AAVP/Grp78-HSVtk-puro^R or RGD4C AAVP/CMV-HSVtk-puro^R

were subjected to GCV *in vitro*. Cells were counted comparably to 9L cells described in section 4.3.2.1. The U87 stably transduced cells were first assessed at 66 days post-vector transduction (t1) then passaged and reassessed at day 137 (t2).

Results demonstrated less survival in U87 cells transduced with RGD4C AAVP/*Grp78-HSVtk-puro*^R relative to RGD4C AAVP/*CMV-HSVtk-puro*^R transduced cells at each of the four 24 hour intervals of GCV treatment (Fig. 4.14). This experiment was done twice and each time results indicated more death in cells stably transduced with RGD4C AAVP/*Grp78-HSVtk-puro*^R than in RGD4C AAVP/*CMV-HSVtk-puro*^R transduced cells; however, in both experiments the differences between the two populations were not statistically significant at all time points.

Interestingly, consistent with the Western blot experiment of HSVtk expression over time (Fig. 4.11b), at the later time point, t2, tumour cell death was significantly decreased in U87 cells stably transduced with RGD4C AAVP/*CMV-HSVtk-puro*^R compared to t1 (Fig. 4.14a). While in contrast there was no significant difference in tumour cell killing efficiency in U87 cells stably transduced with RGD4C AAVP/*Grp78-HSVtk-puro*^R at both t1 and t2 time point (Fig. 4.14b).

Also, representative phase contrast images depict more cell death with RGD4C AAVP/*Grp78-HSVtk* at both time points relative to RGD4C AAVP/*CMV-HSVtk* in stably transduced U87 cells (Fig. 4.14c).

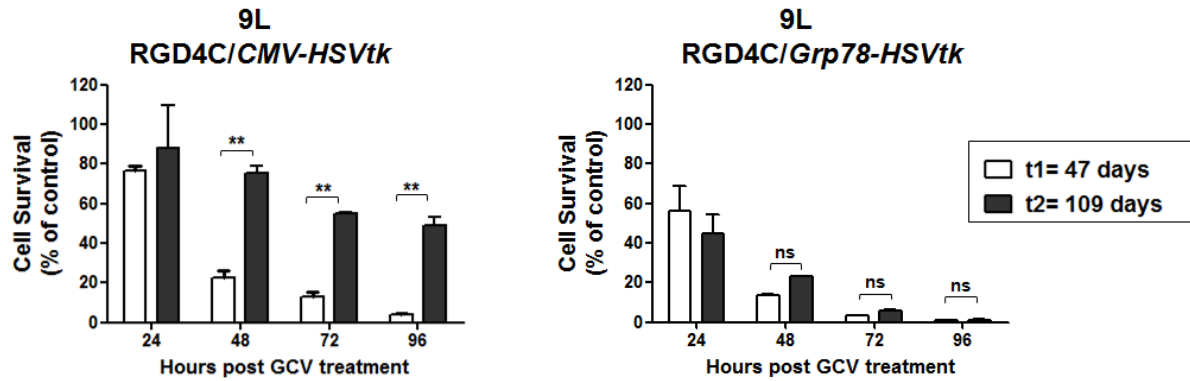


Figure 4.12. Long-term cell killing efficiency of RGD4C AAVP/CMV-HSVtk and RGD4C AAVP/Grp78-HSVtk in transduced 9L cells. 9L cells stably transduced with RGD4C AAVP/CMV-HSVtk and RGD4C AAVP/Grp78-HSVtk at t1= 47 days and t2=109 days post-vector transduction, were plated at 70,000 cells/well in 24-well plates. Treatment with GCV commenced 48 hours after plating. Cells were counted in triplicates for each condition in 24 hour intervals over 96 hours post GCV treatment. The results are shown as the number of surviving cells post GCV treatment normalized to the number of cells grown in the absence of GCV at each time point. Data represent the standard error of the mean obtained from triplicate wells and are representative of two independent experiments. **p < 0.01 and ns: non-significant.

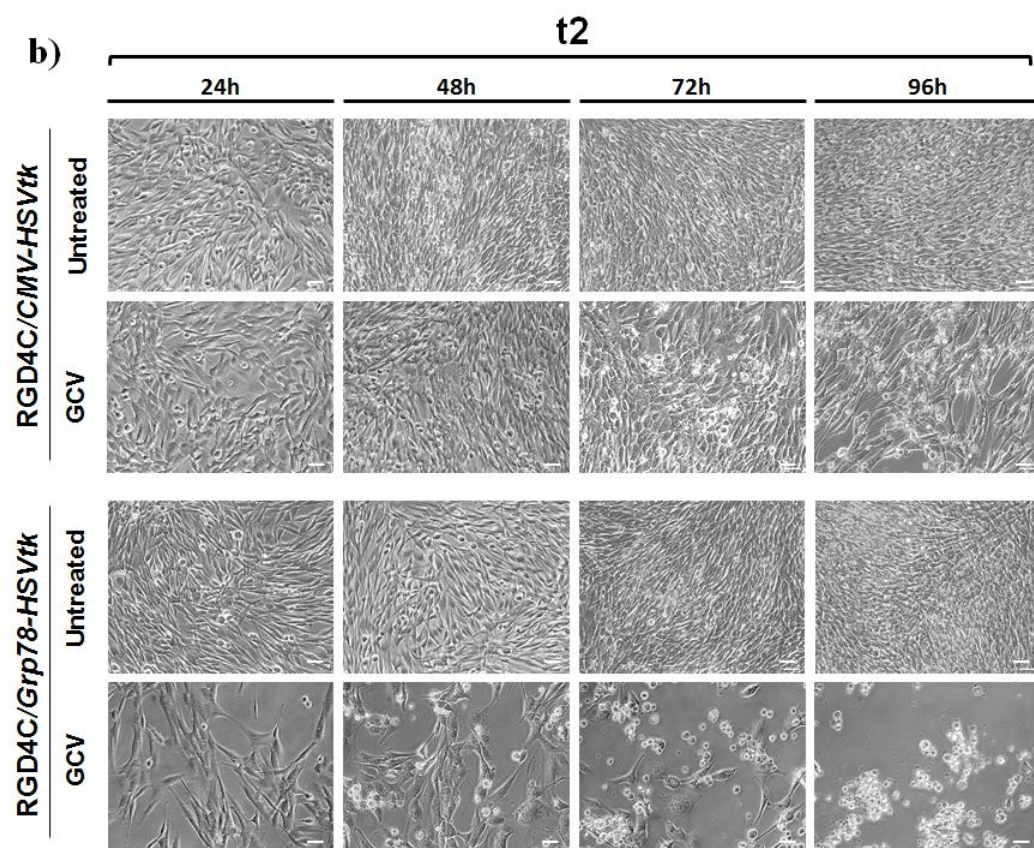
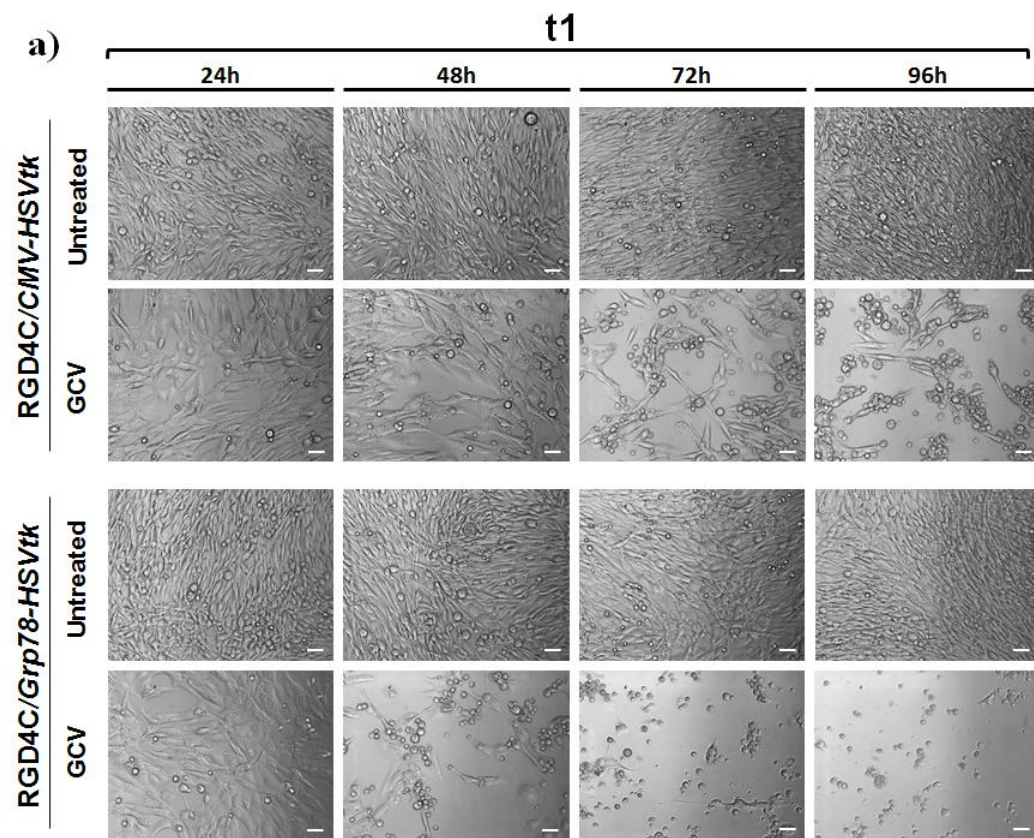


Figure 4.13. The double-targeted RGD4C AAVP/*Grp78*-*HSVtk* mediated long-term cell death in 9L tumour cells *in vitro*. Stably transduced 9L tumour cells (70-80% confluent in a 24-well plate) were plated at **a)** 47 days (t1) and **b)** at 109 days (t2) post-vector transduction. GCV treatment commenced 48 hours after plating. Cells were photographed using a 20x objective at 24 hour intervals over 96 hours post GCV treatment. Scale bar = 45µm.

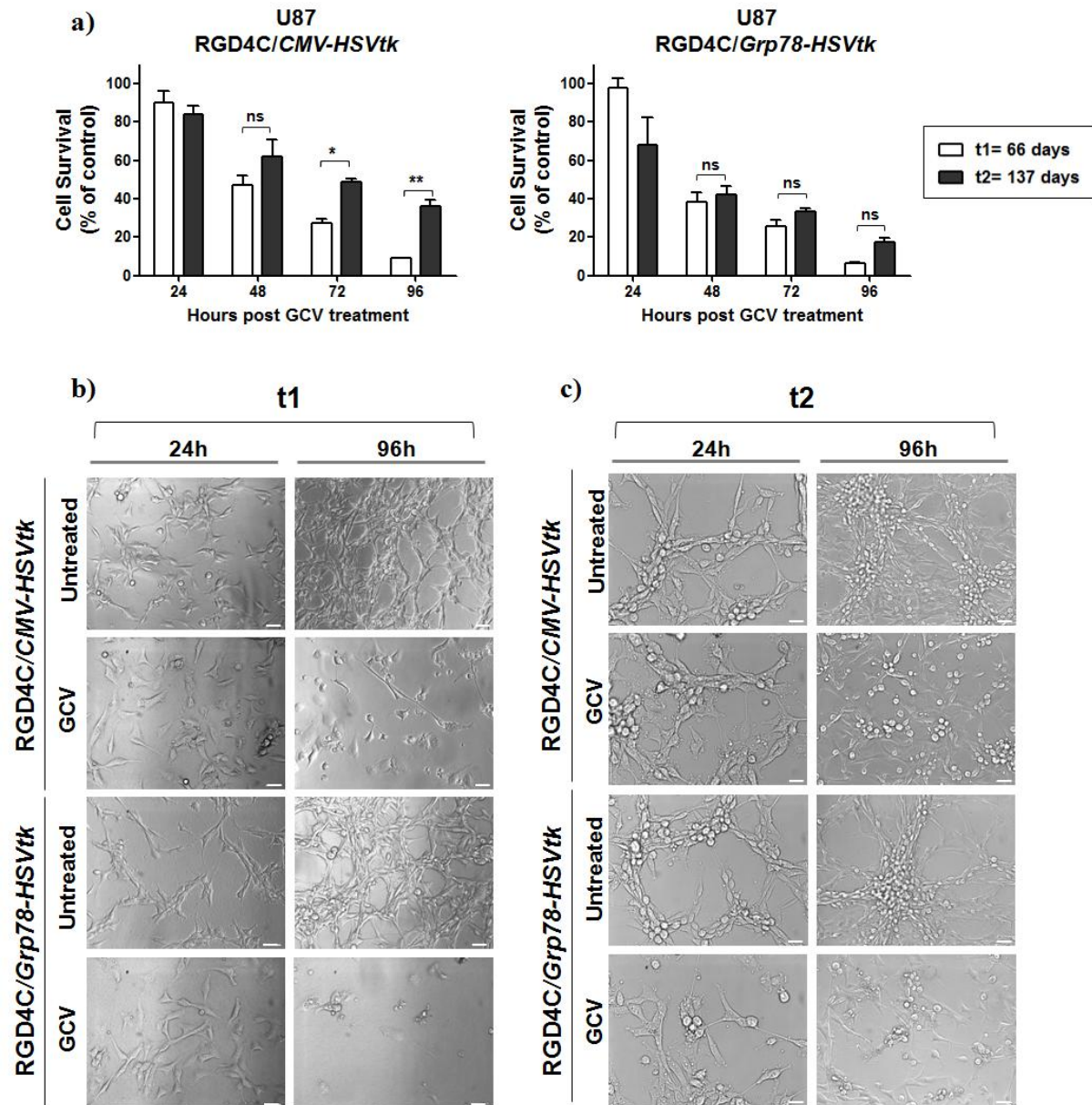


Figure 4.14. Long-term cell killing efficiency of RGD4C AAVP/CMV-HSVtk and RGD4C AAVP/Grp78-HSVtk in transduced U87 cells. 9L cells stably transduced with RGD4C AAVP/CMV-HSVtk and RGD4C AAVP/Grp78-HSVtk at t1= 66 days and t2=137 days post-vector transduction, were plated at 70,000 cells/well in 24-well plates. Treatment with GCV commenced 48 hours after plating. **a)** Cells were counted in triplicate for each condition in 24 hour intervals over 96 hours post GCV treatment. Data were calculated by dividing the percent conversion under GCV by that under non-GCV conditions. Data represent the standard error of the mean obtained from triplicate wells and are representative of two independent experiments. *p < 0.05, **p < 0.01 and ns: non-significant. Representative phase contrast images of stably transduced U87 cells at **b)** t1 and **c)** t2. Cells were photographed using a 20x objective at 24 and 96 hours post GCV treatment. Scale bar = 45µm.

4.2.6 The effect of *in vitro* cell growth and confluence on Grp78 promoter activity

We showed that the *Grp78* promoter activity increases over time in 9L and U87 cells *in vitro*. Then, we raised the question whether cell growth and confluence *in vitro* could mediate *Grp78* induction. To verify this effect, 9L and U87 cells were stably transduced with RGD4C AAVP/*Grp78-GFP-puro*^R or RGD4C AAVP/*CMV-GFP-puro*^R vectors. Populations of pooled clones as well as two randomly isolated clones were grown in the presence of puromycin and plated in 6 well plates until 70% confluent. GFP expression was analysed by flow cytometry every other day, initiated at day 1 post cells plating. At days 7 and 10 post cell plating, 9L and U87 cells, respectively, reached confluent state and the experiment was terminated.

As expected, the percentage of GFP positive cells in 9L cells stably transduced with RGD4C AAVP/*Grp78-GFP-puro*^R remained constant over time. In addition to GFP expression, the MFI level either remained stable or slightly decreased (Fig. 4.15). Similar results were also observed in 9L cells stably transduced with RGD4C AAVP/*CMV-GFP-puro*^R (Fig. 4.15).

In U87 cells stably transduced with RGD4C AAVP/*Grp78-GFP-puro*^R, the percentage of GFP positive cells showed almost the same pattern as 9L cells stably transduced with RGD4C AAVP/*Grp78-GFP-puro*^R; however, the MFI level was fluctuating slightly over time (Fig. 4.16). Again the same effect was detected in U87 cells stably transduced with RGD4C AAVP/*CMV-GFP-puro*^R (Fig. 4.16).

These data prove that cell confluence status *in vitro* does not affect the *Grp78* activity.

9L

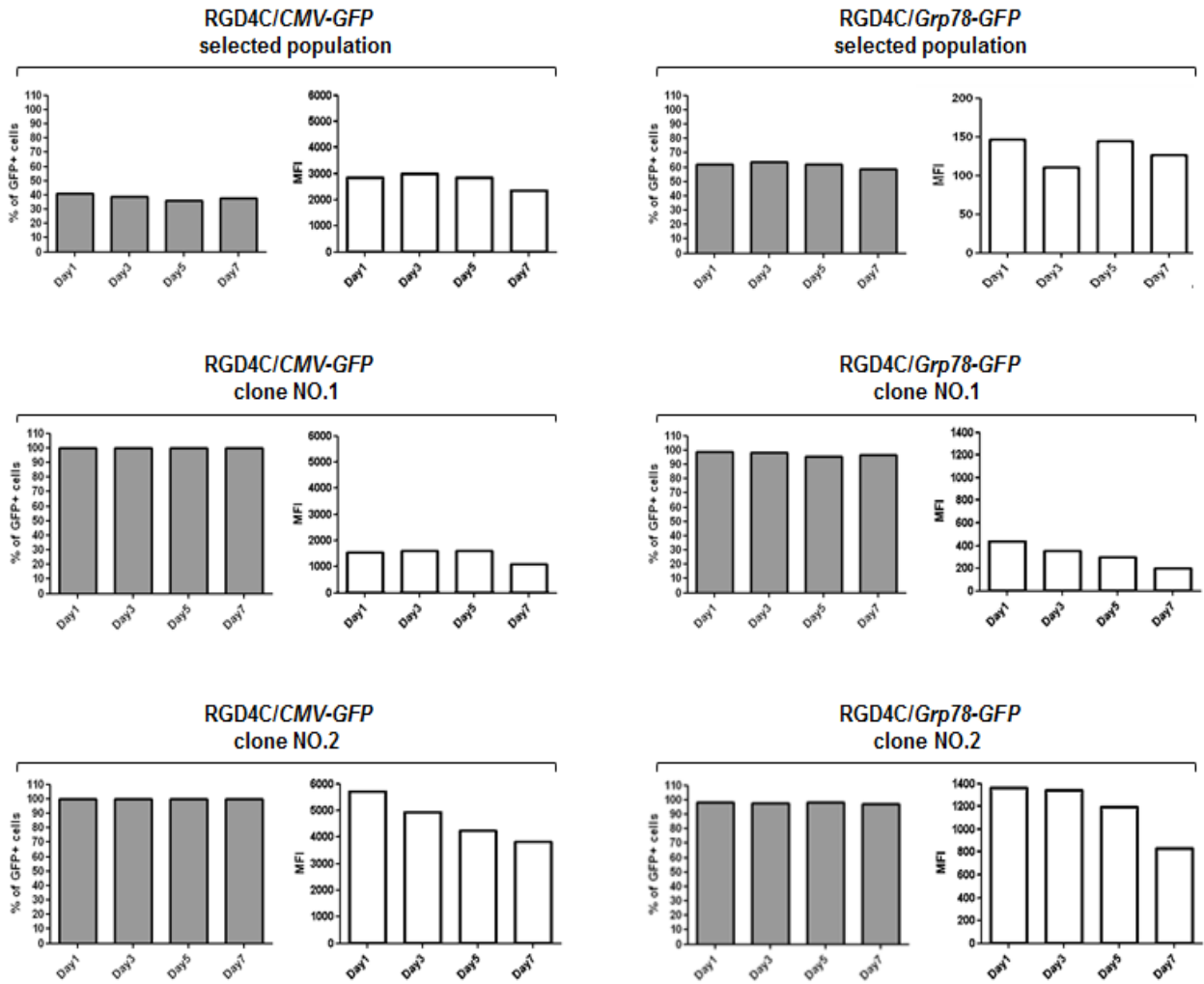


Figure 4.15. Assessment of the effect of cell confluence on *Grp78* activity in stably transduced 9L cells. 9L isolated clones as well as a population of pooled clones stably transduced with RGD4C AAVP/CMV-GFP-*puro*^R or RGD4C AAVP/*Grp78*-GFP-*puro*^R, were plated at 300,000 cells/well in 6-well plates and FACS analysis was performed to quantify GFP expression over the indicated times. Shown are percentage of GFP positive cells and MFI derived from either the *Grp78* or CMV promoter over the indicated days post cell plating.

U87

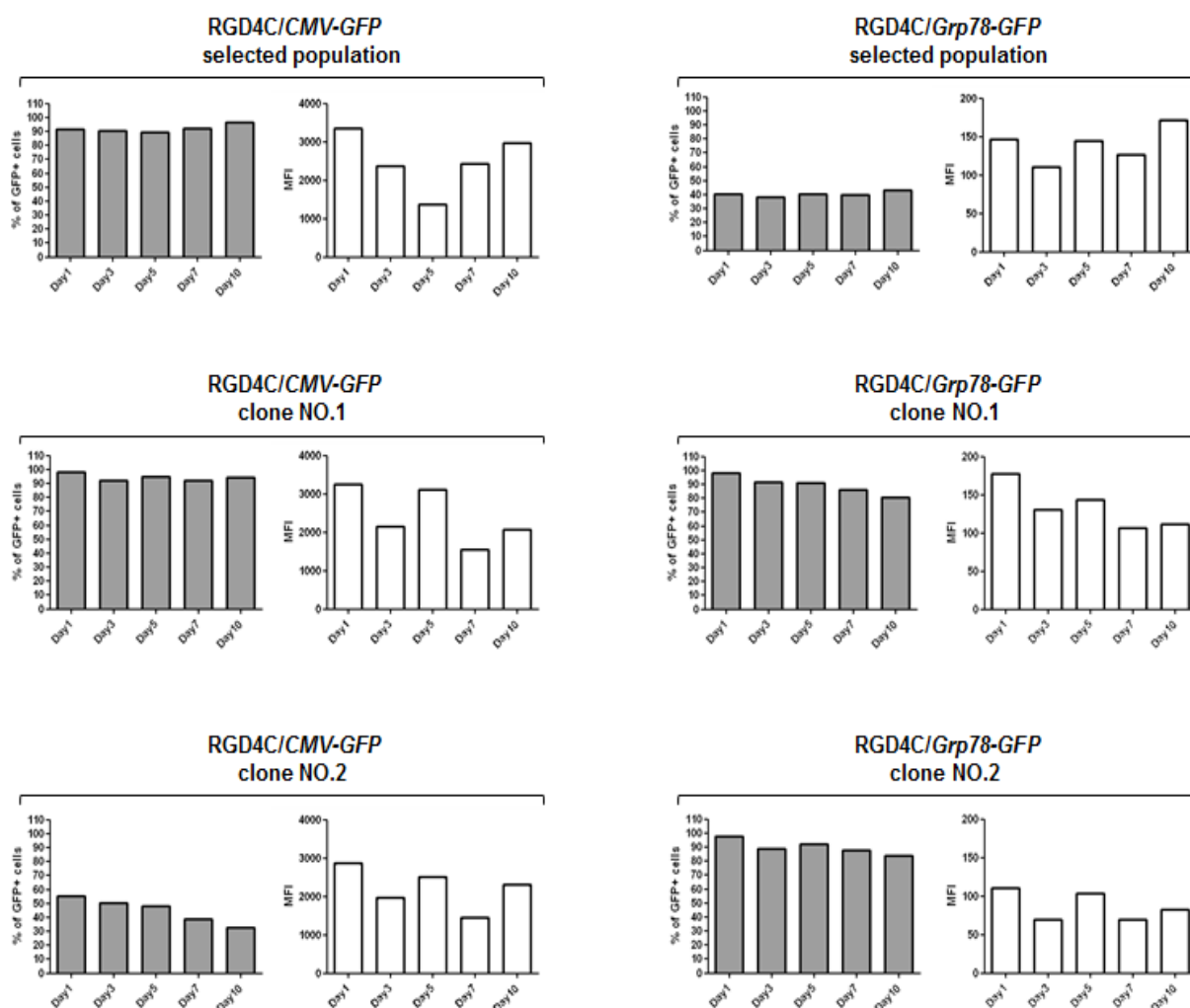


Figure 4.16. Effect of cell confluency on *Grp78* activity in stably transduced U87 cells. U87 isolated clones or a population of pooled clones stably transduced with RGD4C AAVP/CMV-GFP- *puro*^R and RGD4C AAVP/*Grp78*-GFP- *puro*^R, were plated at 300,000 cells/well in 6-well plates and FACS analysis was performed to quantify GFP expression. Shown are percentage of GFP positive cells and MFI derived from either the *Grp78* or CMV promoter over the indicated days post cell plating.

4.2.7 Investigation of the molecular mechanism of the CMV promoter silencing

Transcriptional silencing of many virally transduced genes has been reported by several studies; however, the exact mechanism of silencing still remains unclear. Earlier in this chapter, we showed that in the rat 9L and human U87 cell lines stably transduced with RGD4C AAVP, expression of the *GFP* or *HSVtk* transgene under the control of the *CMV* promoter was suppressed after a few weeks. As DNA methylation and histone deacetylation are known to play an important role in epigenetic gene silencing; herein, we aimed at inhibiting DNA methylation and histone deacetylation in order to evaluate their effect on reactivation of the *CMV* promoter.

4.2.7.1 DNA methylation effect on CMV and Grp78 promoter activity

To investigate the mechanism regulating the *CMV* promoter silencing and determine if the silencing of the *CMV* promoter can be rescued in the context of AAVP, the promoter methylation status was first characterised. Thus, 9L and U87 cells stably transduced with RGD4C AAVP/*CMV-GFP-puro*^R were passaged and grown until the *CMV* promoter silencing was detected by fluorescent microscope analysis. Next, cells were incubated with different concentrations of the DNA methylation inhibitor 5-Azacytidine (5-Aza) for three days. As 5-Aza has been shown to have a short half-life, growth medium containing 5-Aza was replaced on a daily basis. To quantify the effect of 5-Aza on the *CMV* promoter activity, flow cytometry was performed on treated and untreated cells. As previously explained in material and methods (2-10) both the percentage of GFP positive cells and the MFI were calculated by normalizing the results to parental non-transduced cells.

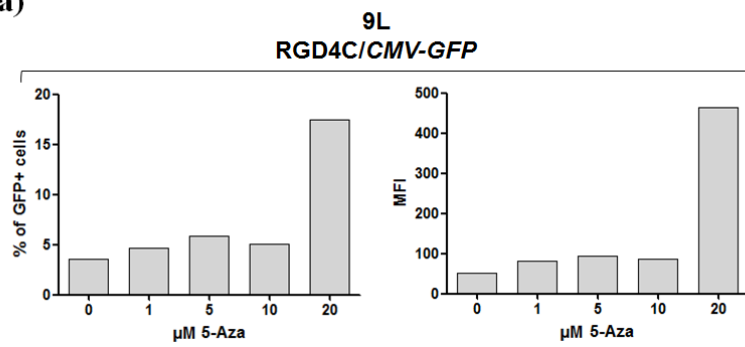
5-Aza treatment of 9L cells stably transduced with RGD4C AAVP/*CMV-GFP-puro*^R resulted in a dose dependent increase in both percentage of GFP positive cells and MFI (Fig. 4.17a).

As shown in figure 4.17a, only 3.5% of control untreated cells were positive for GFP expression; however, this number increased to 17.5% after 3 days of treatment with 20μM of 5-Aza. More interestingly, this increase in GFP positive cells following treatment with 5-Aza, was associated with an 8.9- fold increase of the MFI compared to untreated cells.

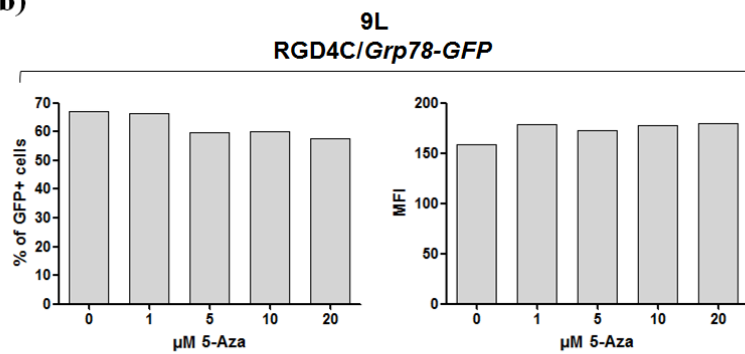
In contrast to the *CMV* promoter, 5-Aza treatment of 9L cells stably transduced with RGD4C AAVP/*Grp78-GFP-puro*^R, resulted in no change in GFP expression. However, a slight increase in MFI was spotted; starting at 1μM of 5-Aza and this effect was not enhanced further by increasing 5-Aza concentrations (Fig. 4.17b).

While the above results clearly depict the efficacy of 5-Aza to rescue the *CMV* promoter in 9L cells, no specific effect was observed in U87 cells stably transduced by RGD4C AAVP/*CMV-GFP-puro*^R following 5-Aza treatment. As shown in figure 4.17c, while the percentage of GFP positive U87 cells stably transduced by RGD4C AAVP/*CMV-GFP-puro*^R remained the same following 5-Aza treatment, only 1.4- fold increase in MFI was detected upon treatment with 1 μ M 5-Aza. Similar to 9L cells, 5-Aza treatment of RGD4C AAVP/*Grp78-GFP-puro*^R stably transduced U87 cells, resulted in no effect on *Grp78* promoter activity (Fig. 4.17d).

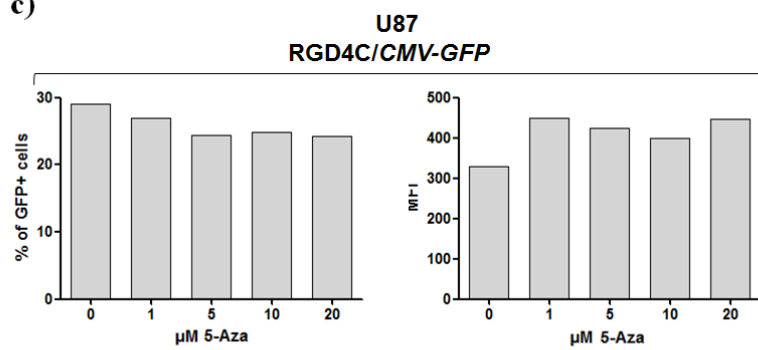
a)



b)



c)



d)

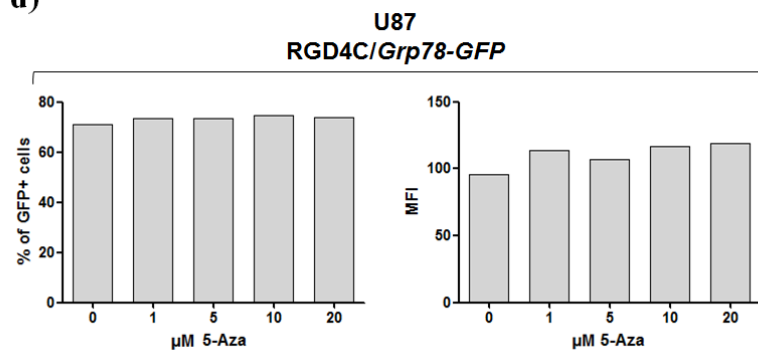


Figure 4.17. Effect of 5-Aza on CMV and Grp78 promoter activity. 9L cells stably transduced with **a)** RGD4C AAVP/CMV-GFP -puro^R or **b)** RGD4C AAVP/Grp78-GFP -puro^R as well as U87 cells stably transduced with **c)** RGD4C AAVP/CMV-GFP -puro^R or **d)** RGD4C AAVP/Grp78-GFP -puro^R were plated in 6 well plates and 24 hours later, 5-Aza was added to cells at 4 different concentrations as indicated. At day 3 post 5-Aza treatment, cells were collected and GFP positive cells as well as the MFI were analysed by flow cytometry. These data are representative of three independent experiments.

4.2.7.2 Effect of the HDAC inhibitors on the CMV and Grp78 promoter activity

4.2.7.2.1 Assessment of the effect of HDAC inhibitors on the CMV and Grp78 promoter activity in stably transduced 9L cells

After demonstrating the effect of DNA methylation on CMV promoter silencing, I next aimed to study the effect of histone deacetylation on long-term CMV promoter activity. Thus, 9L cells stably transduced with RGD4C AAVP/CMV-GFP-puro^R were passaged and grown until the CMV promoter silencing was detected by fluorescent microscope analysis. Next, cells were treated with different concentrations of the HDAC inhibitor TSA for 3 days and GFP expression was analysed by flow cytometry. The data showed no change in CMV promoter activity in 9L cells upon TSA treatment when compared to control untreated cells (Fig. 4.18a). In contrast to the CMV promoter, treatment of 9L cells stably transduced with RGD4C AAVP/Grp78-GFP-puro^R with 0.2 μ M TSA resulted in a 1.5- fold increase of the MFI while the percentage of GFP positive cells remained stable (Fig. 4.18b).

As no specific effect from TSA treatment was observed on CMV reactivation, I further aimed to evaluate the effect of other HDAC inhibitor drugs; SAHA, VPA and Nicotinamide. Both SAHA and TSA mediate inhibition of class I and II HDAC family members while VPA can only inhibit HDAC class I. The fourth HDAC inhibitor candidate nicotinamide, can inhibit class III HDAC family members. Interestingly, treatment of 9L cells stably transduced with RGD4C AAVP/CMV-GFP-puro^R with SAHA, VPA and nicotinamide for three days resulted in no change in CMV promoter activity (Fig. 4.19a).

In contrast, 3 days of treatment of 9L cells stably transduced by RGD4C AAVP/Grp78-GFP-puro^R with SAHA, resulted in a 3.1- fold increase of the MFI compared to untreated cells (Fig. 4.19b). While the increase in MFI was demonstrated, the percentage of GFP positive

cells remained stable upon treatment. VPA and nicotinamide treatment resulted in no specific change in *Grp78* promoter activity (Fig. 4.19b).

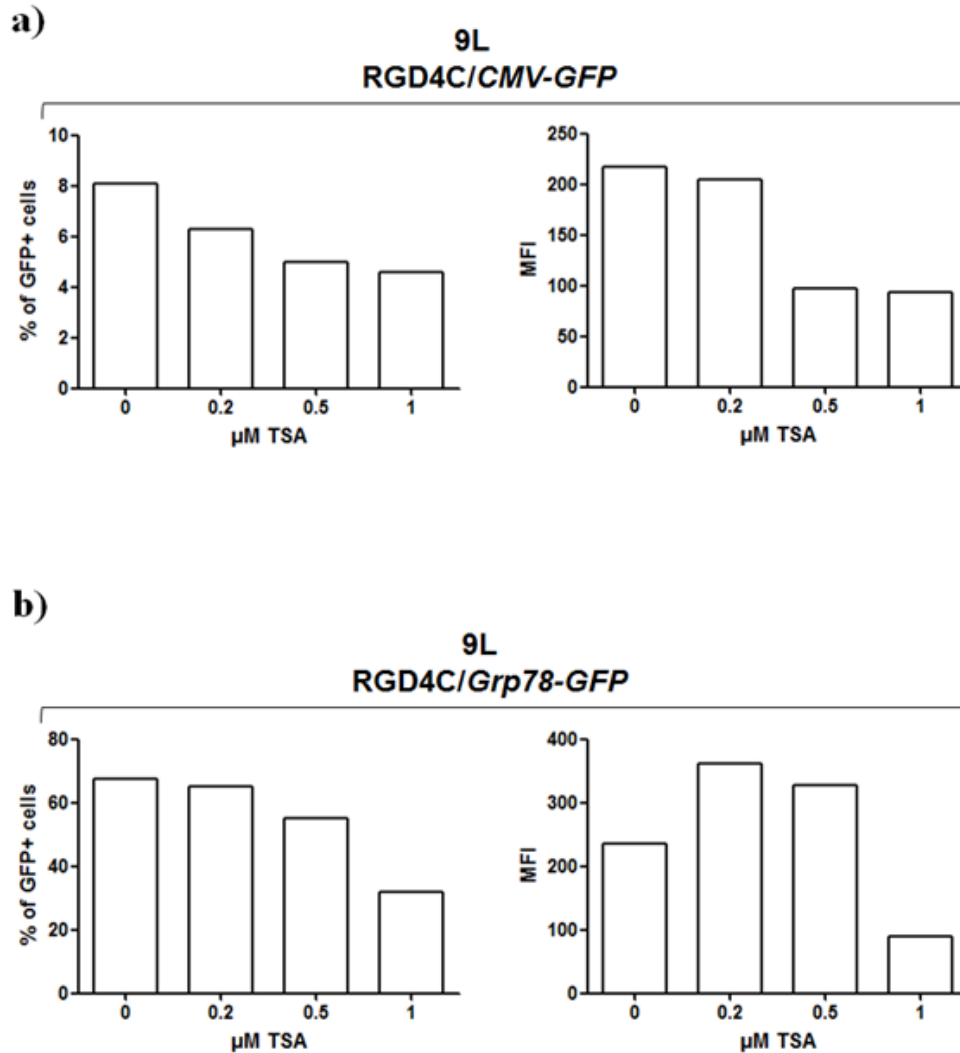


Figure 4.18. The effect of the HDAC inhibitor TSA on CMV and *Grp78* promoter activity in stably transduced 9L cells. 9L cells stably transduced with **a)** RGD4C AAVP/CMV-GFP-puro^R and **b)** RGD4C AAVP/*Grp78*-GFP-puro^R were plated in 6 well plates and 24 hours later, TSA was added to cells at 3 different concentrations as indicated. At day 3 post TSA treatment, cells were collected and GFP positive cells as well as the relative MFI were analysed by flow cytometry. These data are representative of two independent experiments.

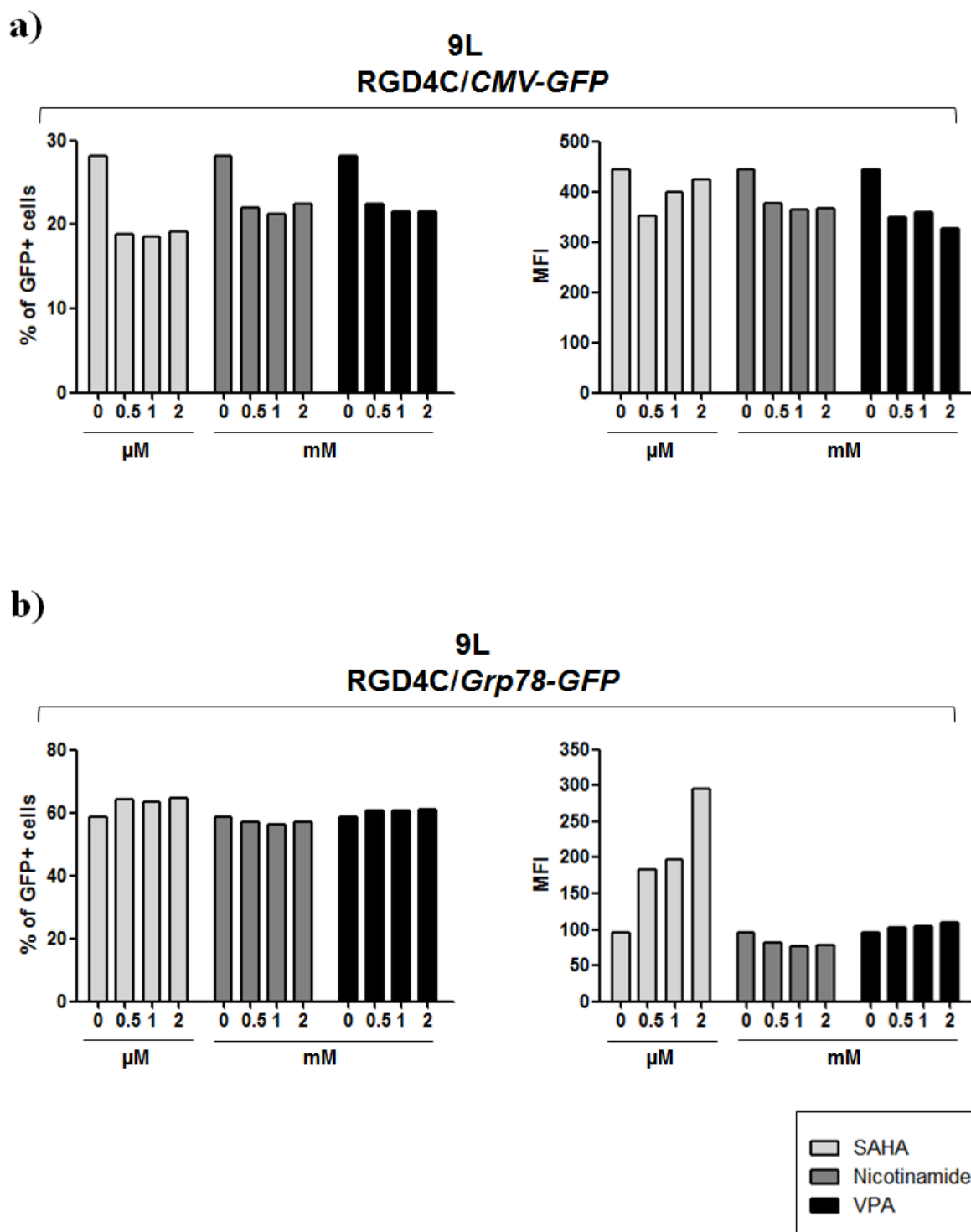


Figure 4.19. Effect of HDAC inhibitor drugs on CMV and Grp78 promoter activity in stably transduced 9L cells. Stably transduced 9L cells with **a)** RGD4C AAVP/CMV-GFP-puro^R and **b)** RGD4C AAVP/Grp78-GFP-puro^R were plated in 6 well plates and 24 hours later, cells were treated with either the HDAC inhibitor drug SAHA, Nicotinamide or VPA. Three different concentration of each compound were applied as indicated. At day 3 post drug treatment, cells were collected and GFP positive cells as well as relative MFI were analysed by flow cytometry. These data are representative of two independent experiments.

4.2.7.2.2 Effect of HDACs inhibitors on CMV and Grp78 promoter activity in stably transduced U87 cells

After evaluation of the effect of HDAC inhibitors on *CMV* and *Grp78* promoter activity in stably transduced 9L cells, I further aimed to find out whether this effect is also detectable in other U87 cells. Thus, U87 cells stably transduced with RGD4C AAVP/*CMV-GFP-puro*^R or RGD4C AAVP/*Grp78-GFP-puro*^R were treated with different concentrations of HDAC inhibitor drugs for 3 days. As shown in figure 4.20a, in contrast to 9L cells, TSA and SAHA treatment of U87 cells stably transduced with RGD4C AAVP/*CMV-GFP-puro*^R resulted in a dose dependent reactivation of the silenced *CMV* promoter. Treatment with 1 μ M TSA resulted in 1.8- and 3- fold increases in the percentage of GFP positive cells and MFI respectively. Moreover, following SAHA treatment, the percentage of GFP positive cells and MFI were elevated 2.2- and 2.8- fold, respectively (Fig. 4.20a). VPA treatment did not show any change in *CMV* promoter activity. Also, treatment with nicotinamide resulted in a minor increase in the percentage of GFP positive cells but not MFI (Fig. 4.20a).

In previous experiments, an induction in *Grp78* promoter activity upon TSA and SAHA treatment in 9L cells stably transduced by RGD4C AAVP/*Grp78-GFP-puro*^R was observed. Interestingly in agreement with the previous study, *Grp78* activity was shown to increase in U87 cells stably transduced by RGD4C AAVP/*Grp78-GFP-puro*^R upon TSA and SAHA treatment (Fig. 4.20b). Consistent with the 9L study, only the MFI was increased upon treatment and the percentage of GFP positive cells remained stable over treatment. No effect from either VPA or Nicotinamide on *Grp78* promoter activity was observed (Fig. 4.20b).

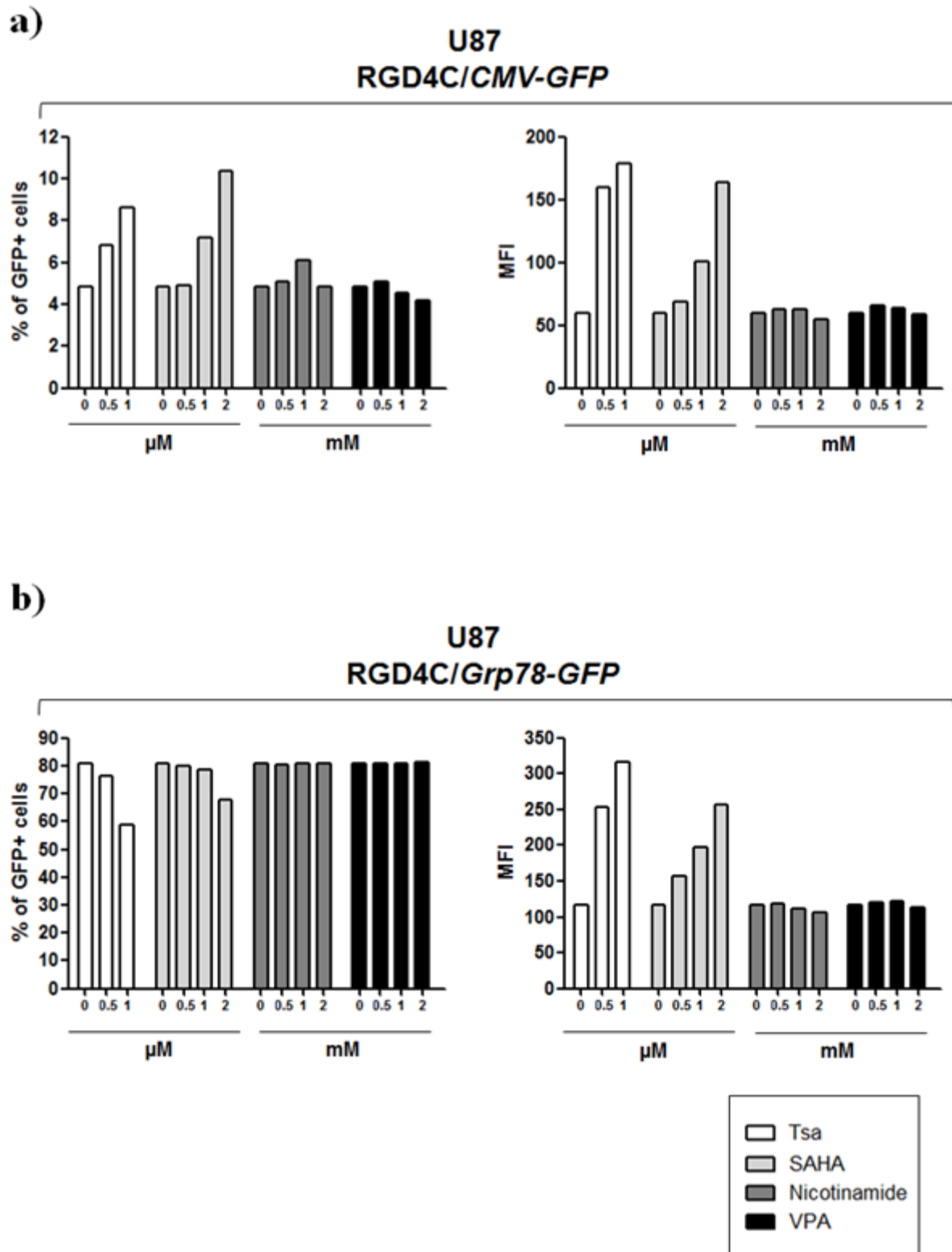


Figure 4.20. Effect of HDAC inhibitor drugs on CMV and Grp78 promoter activity in stably transduced U87 cells. U87 cells stably transduced with **a)** RGD4C AAVP/CMV-GFP-puro^R or **b)** RGD4C AAVP/Grp78-GFP-puro^R were plated in 6 well plates and 24 hours later, different concentrations of either HDAC inhibitors TSA, SAHA, Nicotinamide or VPA were added to cells as indicated. At day 3 post drug treatment, cells were collected and

GFP positive cells as well as relative MFI were analysed by flow cytometry. These data are representative of two independent experiments.

4.3 Discussion

In this chapter I characterised the long-term activity of both promoters in the context of AAVP *in vitro*. Thus, initially stably U87 and 9L transduced cells with RGD4C AAVP/*Grp78-puro*^R and RGD4C AAVP/*CMV-puro*^R vectors were generated and the levels of expression of either the *GFP* or the *HSVtk* transgenes driven by *CMV* or *Grp78* promoters were quantified over time. To monitor GFP expression, stably transduced 9L and U87 cells with RGD4C AAVP/*Grp78-GFP-puro*^R and RGD4C AAVP/*CMV-GFP-puro*^R were grown in the presence of puromycin until the individual cell clones were noticeable. Initially GFP expression was monitored by counting the GFP positive and negative individual selected clones under a fluorescent microscope over two weeks time. Subsequently, due to growth of clones, this process was further followed by flow cytometry analysis of the pooled clones. For *HSVtk* expression, initially cells were stably transduced with RGD4C AAVP/*Grp78-HSVtk-puro*^R and RGD4C AAVP/*HSVtk-GFP-puro*^R and Western blot analysis was performed on cell lysates at one month intervals. Interestingly, by utilizing a different experimental approach, I confirmed over time silencing of the *CMV* promoter in the context of AAVP in both 9L and U87 cells, while a sustained or even increased activity of the *Grp78* promoter was observed. Our findings regarding silencing of the *CMV* promoter in both 9L and U87 cells in culture are in agreement with previous studies demonstrating gradual silencing of the *CMV* promoter over a few months *in vitro* (Choi et al., 2005, Knust et al., 1989, Palmer et al., 1991, Verma and Somia, 1997).

In stably transduced 9L cells, FACS analysis of GFP expression as well as Western blot studies of *HSVtk* protein level driven by the *Grp78* promoter demonstrated higher activity of this promoter than that of the *CMV*, but an opposite pattern was observed in the U87 cell line. While, *CMV*- controlled *GFP* or *HSVtk* transgene expression in stably transduced U87 cells was higher than that under the control of *Grp78*, transgene expression eventually reached levels beneath those in RGD4C AAVP/*Grp78* in the course of time. Collectively, these data justify research into alternate promoters, such as *Grp78*, when long-term transgene expression is of primary importance.

In addition to investigating *CMV* promoter silencing overtime, we further aimed to confirm the superior effectiveness of RGD4C AAVP/*Grp78-HSVtk* over RGD4C AAVP/*CMV-HSVtk* in suicide gene therapy of cancer *in vitro*, after GCV treatment. Interestingly, the obtained data are consistent with previous results described in chapter 3, section 3.3.3 showing superior therapeutic effect of AAVP/*Grp78-HSVtk* over AAVP/*CMV-HSVtk* in non-selected transduced cells.

As shown in chapter 3 and in agreement with previous reports, *Grp78* is up-regulated in highly proliferating 9L cells compared to the less aggressive U87 cells. Thus, within 9L cells, the *Grp78* promoter may ultimately upregulate *HSVtk* expression in the context of AAVP. Consequently, a higher level of *HSVtk* expression in RGD4C AAVP/*Grp78-HSVtk* transduced 9L cells relative to RGD4C AAVP/*CMV-HSVtk* allows more rapid phosphorylation, and thus activation, of the GCV prodrug to the toxic GCV triphosphate.

In U87 cells, *GFP* and *HSVtk* expression regulated by *CMV* surpasses that regulated by *Grp78* suggesting that overall activity of the *Grp78* promoter is lower in this cell line. Interestingly, although a lower activity of *Grp78* relative to *CMV* in this cell line was observed, more cell death was caused by AAVP/*Grp78-HSVtk* than AAVP/*CMV-HSVtk*. This finding raised the question of whether the toxic form of GCV is capable of inducing ER stress and consequently activates the UPR pathway. This objective will be further addressed in the next chapter.

Previous studies have shown that silencing and down regulation of the *CMV* promoter leads to reduced transgene expression (Brooks et al., 2004, Chen and Townes, 2000). Consistently, our data suggest that *CMV* silencing in both 9L and U87 cells has led to a progressive decrease in *HSVtk* expression and therefore cell killing becomes a far less effective endeavour.

This is not the first study to suggest that the *CMV* promoter is silenced. While the promoter is popular due to its constitutive activity in most mammalian tissues (Cheng et al., 1993), a number of silencing mechanisms including DNA methylation and histone deacetylation have been implicated in its down regulation (Grassi et al., 2003, Prosch et al., 1996, Chen and Townes, 2000). In this chapter, I further aimed to characterise the *CMV* promoter silencing and evaluate its activity in the presence of 5-Aza inhibitor of DNA methylation, and HDAC inhibitors TSA, SAHA, VPA and nicotinamide.

Interestingly following *in vitro* treatment with 5-Aza, a DNA methyltransferase inhibitor, the *CMV* promoter was induced and its activity restored in stably transduced 9L cells as both percentage of GFP positive cells and MFI were increased overtime. However, in contrast to

the effect of 5-Aza on *CMV* promoter reactivation, treatment of RGD4C AAVP/*CMV-GFP* stably transduced 9L cells with either of HDACs inhibitor drugs resulted in no relevant differences in the *CMV* reactivation trend. Interestingly different sets of results were observed in U87 cells.

It has been demonstrated that the mechanism of *CMV* promoter inactivation can be mediated by methylation dependent or independent pathways and are cell-type specific (Radhakrishnan et al., 2008). Thus, this report might explain the fact that different mechanisms of *CMV* silencing are involved in U87 and 9L cells.

In a previous study (Grassi et al., 2003), a correlation between *CMV* activity and 5-Aza concentration was reported at days 2 and 5 post treatment in U87 cells. Within the same study, two days of treatment of U87 cells stably transfected with GFP under the control of the *CMV* promoter with 1 μ M 5-Aza resulted in approximately 1.5- fold increase in MFI. Interestingly, in our study after treating cells with the same concentrations of 5-Aza for 3 days, 1.4- fold increases in MFI are detected. Although this part of our study was consistent with previous work, I could not detect any effect on the percentage of GFP positive cells. Unfortunately, it is important to note, the previous study had not shown the percentage of GFP positive cells following 5-Aza treatment.

Regarding the effect of TSA in *CMV* promoter activity in U87 cells, our data are in agreement with previous reports which have demonstrated an effect of TSA on the *CMV* promoter activity (Grassi et al., 2003).

As mentioned before, other research has shown extensive methylation of both 5'CpG and non-CpG sites in the *CMV* promoter region and moreover complete silencing of *CMV* promoter due to methylation was observed (Das and Singal, 2004, Prosch et al., 1996). Interestingly, another study showed that over a period of 60 days, the expression of AAV transduced *CMV-lacZ* genes was silenced in 80% of HeLa cell clones (Chen et al., 1997). Surprisingly, the same as our observation in U87 cells stably transduced with RGD4C AAVP/*CMV-GFP*, treatment with TSA but not 5-Aza resulted in dramatic reactivation of transgene expression (Chen et al., 1997).

As discussed, only TSA and SAHA which both are Zn^{2+} binding HDAC Inhibitors mediate inhibition of HDACs class of I and II (Table 4.1), could increase both the percentage of GFP positive cells and MFI driven by the *CMV* promoter in U87 cells. Neither VPA, an inhibitor of class I HDACs nor nicotinamide, a class III HDAC inhibitor, could affect the *CMV* promoter activity. Based on these observations we can postulate the possible involvement of HDAC family class II in *CMV* promoter silencing in U87 cells.

In contrast to U87 cells, reactivation of the *CMV* promoter in 9L cells was achieved by 5-Aza treatment while treatment with HDAC inhibitors resulted in no change of the *CMV* activity demonstrating that in this cell line, hypoacetylation of histones is not a mechanism involved in *CMV* silencing.

Another interesting observation was a dose-dependent induction of the *Grp78* promoter by TSA and SAHA in both 9L and U87 cells. However, the mechanism of this function remains unclear and necessitates further investigation. Nevertheless, overexpression of the *Grp78* promoter in response to the HDAC inhibitor TSA in a variety of cancer cells from colon, breast to brain tumours have been previously reported (Baumeister et al., 2009). Also in addition to *in vitro* study, induction of *Grp78* by HDAC inhibitors has been detected in xenograft models. Moreover this induction was also detectable in biopsies from breast cancer patients receiving TSA treatment (Baumeister et al., 2009). Since *Grp78* has been characterised as a major effector of drug resistance, the induction of *Grp78* upon HDAC inhibitor treatment might address resistance of solid tumours against HDAC inhibitors in clinical trials. Indeed, as *Grp78* promotes tumour growth and at the same time inhibits apoptosis induced by stress (Dong et al., 2008). *Grp78* gene knock down using siRNA results in a significant increase in cell death upon TSA treatment (Baumeister et al., 2009).

HDAC deregulation has been associated with several cancers. Also usage of HDAC inhibitors proved to mediate cell growth inhibition and induce terminal differentiation. Moreover administration of HDAC inhibitors has been shown to mediate inhibition of tumour formation in mice models, thus HDAC inhibitors are considered as potential candidates for cancer treatment. SAHA has recently been approved by the FDA as a new class of anti-cancer drugs with great therapeutic potential against cutaneous T cell lymphoma (CTCL) (Grant et al., 2007). SAHA has also been used in phase I and II clinical trials for hematological malignancies and solid tumours (Modesitt et al., 2008, Garcia-Manero et al., 2008, Galanis et al., 2009). Although HDAC inhibitors are clinically effective against hematologic cancers, they are solely considered poor anti-cancer agents against solid tumours (Modesitt et al., 2008, Luu et al., 2008).

Based on our data, the *Grp78* promoter can increase the therapeutic benefits of HDACs inhibitors as these drugs are able to induce overexpression of therapeutic transgenes under the control of the *Grp78* promoter within solid tumours. In addition to sustained long-term *Grp78* activity, these data validate another beneficial usage of this promoter in cancer gene therapy.

Chapter 5

HSVtk/GCV suicide gene therapy induces Grp78 promoter activity

5.1 Introduction

In eukaryotic cells, ER is the main organelle in charge of protein synthesis and modification. Grp78, which belongs to the HSP70 family, is a highly abundant ER chaperone. The primary function of this protein is to bind to hydrophobic regions of nascent ER polypeptides and assist their proper folding. Along with its role in protein folding, Grp78 is also known as an important component to promote degradation of proteins if their misfolding is irreversible.

Regulation of *Grp78* expression is controlled by a conserved signalling cascade termed UPR (unfolded protein response) pathway. In non-stressed cells, Grp78 is bound to three endoplasmic reticulum (ER) transmembrane proteins from the ER lumen side and maintain them in inactive forms. These three proteins which represent three arms of the UPR are: protein kinase- like ER kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) (Hetz et al., 2011, Zhang and Zhang, 2010).

However, in a condition referred to as ER stress caused by accumulation of unfolded or misfolded proteins load within the ER, Grp78 dissociates from the PERK, IRE1 and ATF6, and further binds to unfolded proteins to facilitate their proper folding and thus re-establish ER homeostasis. Dissociation of Grp78 from the ATF6, IRE1 and PERK proteins will also mediate initiation of the UPR signalling cascade. UPR mainly assists cell desensitization to ER stress by reducing overall translation (Zhang and Zhang, 2010). Moreover this pathway will subsequently induce the up-regulation of ER chaperone protein expression such as Grp78 which has been shown to prevent apoptosis in stress-induced cell death.

However under conditions in which UPR cannot re-establish the ER homeostasis, the UPR is capable of inducing programmed cell death (Pfaffenbach and Lee, 2011). Upon dissociation of Grp78, the 90 kDa activating transcription factor 6 (ATF6-90 kDa) is released and transported to the Golgi where it is cleaved by site-1 and site-2 proteases (S1P and S2P) to generate and release the 50 kDa ATF6 (ATF6-p50) into the cytosol (Fig. 5.1) (Haze et al., 1999, Zhang and Zhang, 2010). ATF6-p50 is the active nuclear form of ATF6. Previous studies showed that upon ER stress, S2P-mediate proteolytic cleavage of the transcription factor ATF6, which targets the ERSE region of the *Grp78* promoter and thus strictly activates transcription of *Grp78* (Ye et al., 2000, Lee et al., 2002).

As explained in chapter 1, transcriptional activation of the *Grp78* promoter is mediated and controlled by highly conserved elements located in the *Grp78* promoter which is referred to as ER stress response element (ERSE). In the mammalian *Grp78* promoter, ERSE contribute to ER stress-induced transcriptional activation (Roy and Lee, 1999). Apart from ATF6-p50, other transcription factors including TFII-I, YY1 and NF-Y bind to ERSE and regulate the *Grp78* promoter activity.

Further, when it is no longer bound to Grp78, inositol-requiring enzyme 1 (IRE1) dimerizes and this will activate its endoribonuclease activity, thus resulting in splicing of the mRNA encoding X-box binding protein 1 (XBP1) (Calton et al., 2002, Samali et al., 2010). Spliced XBP1 protein is a highly active transcription factor, and like ATF6-p50, it can bind to ERSE sequences and induce the transcription of ER chaperone genes (Fig. 5.2) (Yoshida et al., 2001). XBP1 is present in cells in both, spliced and unspliced form, with the unspliced form acting as a dominant negative (Wang et al., 2010). Spliced XBP1 has greater stability than its unspliced counterpart (Samali et al., 2010). The IRE1-XBP1 arm of the UPR regulates genes responsible for differentiation, inflammation, lipogenesis, and apoptotic pathways (Wang et al., 2010). Increased expression of spliced XBP1 is correlated with tumour survival, whereas XBP1 knockout and XBP1 unspliced-expressing cells demonstrated an increased occurrence of apoptosis (Wang et al., 2010). These data suggest an anti-apoptotic role for Grp78 via the downstream effector spliced XBP1 in tumour cells.

Upon dissociation of Grp78 from pancreatic ER kinase (PERK), the third arm of UPR, this transmembrane protein will homooligomerize and subsequently phosphorylate Ser51 of α subunit of the eukaryotic initiation factor 2 α (eIF2 α). Phosphorylated-eIF2 α can selectively trigger translation of the transcription factor ATF4 which mediates activation of the *Grp78* promoter independent of the ERSE (Fig. 5.3). In mammalian cells, CRE/ATF sequence localized upstream of the ERSE within the *Grp78* promoter has been described as an ATF4

binding site (Luo et al., 2003). Phosphorylated-eIF2 α can also attenuate mRNA translation and thus prevent a new load of polypeptides from entering the ER lumen (Fig. 5.3) (Back et al., 2009).

Each of these three arms plays a cytoprotective role in the UPR pathway, ultimately upregulating pro-survival genes such as Grp78 via transcriptional activation in the nucleus.

In chapter 3 and chapter 4, I showed that in both 9L and U87 cells, the *Grp78* promoter driving the *HSVtk* transgene in the context of AAVP leads to dramatic cell death compared to that of the targeted RGD4C AAVP/*CMV-HSVtk* after GCV treatment. In contrast, Western blot analysis of *HSVtk* expression, (before GCV treatment) under the control of either promoter, as well as GFP analysis by flow cytometry in U87 cells showed higher activity of the *CMV* relative to the *Grp78* promoter.

In summary although, lower activity of the *Grp78* promoter relative to the *CMV* promoter was observed in the U87 cell line, more cell death was achieved by the RGD4C AAVP/*Grp78-HSVtk* vector after GCV treatment. Thus, an important question arises whether the *Grp78* promoter exhibits higher activity over the *CMV* promoter upon *HSVtk*/GCV treatment. I previously explained that *HSVtk* can mediate phosphorylation of the GCV prodrug and subsequently leading to the formation of toxic GCV triphosphate which is able to incorporate into DNA and result in cell death. Thus toxic GCV triphosphate might play a role as an inducer of *Grp78*.

To date no study has addressed this phenomenon; thus, for the first time I aimed to investigate the effect of the *HSVtk* plus GCV cytotoxic gene therapy on the activity of *Grp78* promoter at both the endogenous and AAVP/*Grp78* vector level. Moreover, I further aimed to study the possible mechanism involved in *Grp78* overexpression upon *HSVtk*/GCV therapy. Since expression of *Grp78* is tightly controlled by the UPR pathway, my goal was to gain insight into UPR activation upon *HSVtk*/GCV treatment.

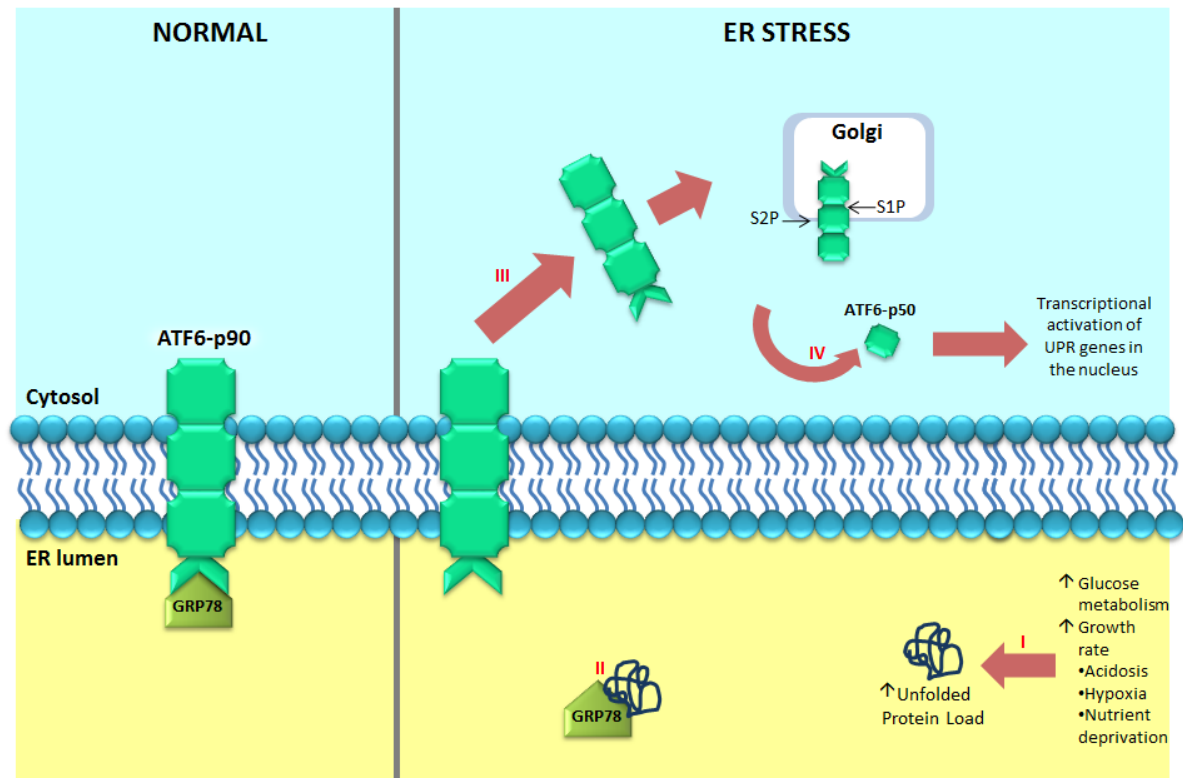


Figure 5.1. The ATF6 arm of the UPR. In normal conditions, ATF6 is maintained in an inactive state through an association with Grp78. However in the presence of ER stress I) the unfolded protein load will increase and II) Grp78 will dissociate from ATF6, then binds the unfolded proteins, thus III) ATF6 will be activated and translocate into the Golgi apparatus. IV) In the Golgi, ATF6 is cleaved and a 50kDa ATF6 fragment (ATF6-p50) is released into the cytosol. ATF6-p50 migrates to the nucleus and binds ERSEs to activate transcription of ER chaperon genes.

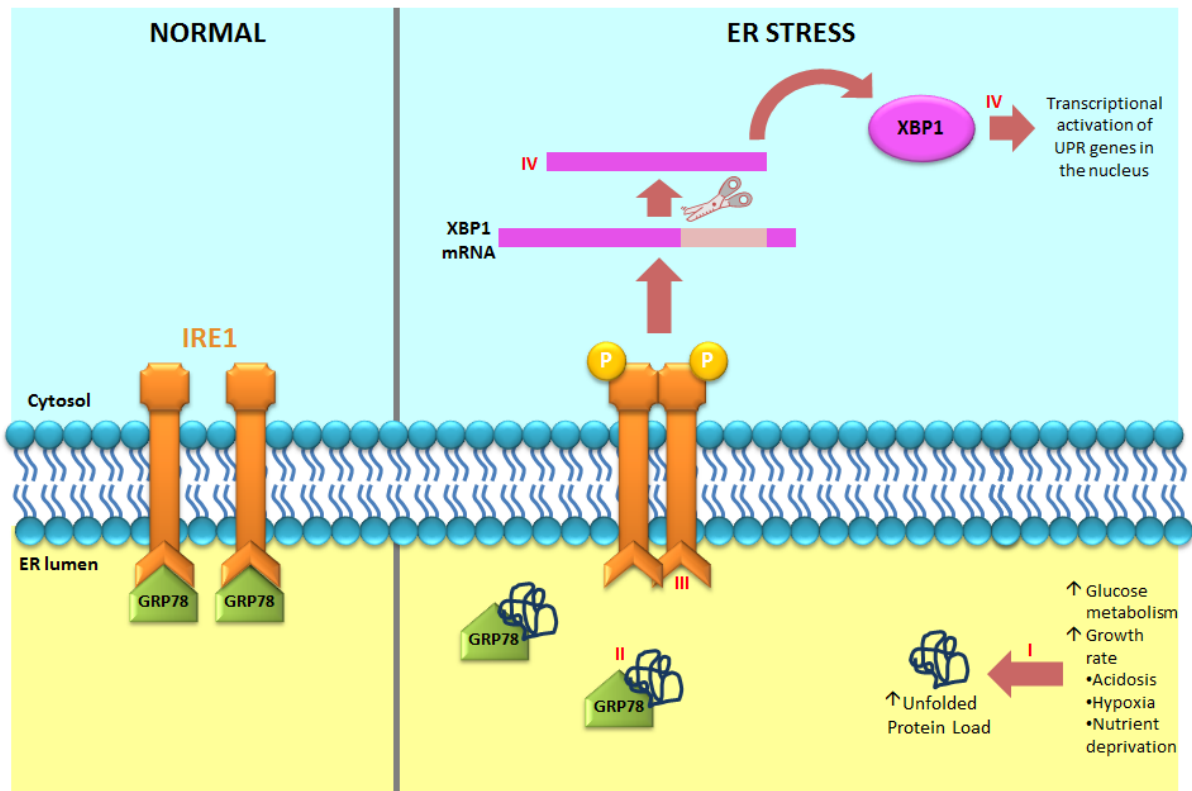


Figure 5.2. The IRE1 arm of the UPR. In non-stressed cells, IRE1 is inactive through an association with Grp78. I) upon an increase in unfolded protein load due to ER stress II) Grp78 dissociates from PERK to bind unfolded proteins, III) leading to spontaneous dimerization and phosphorylation of IRE and also activation of its RNase. IV) The endogenous endoribonuclease activity of IRE1 splices XBP1 mRNA. The spliced mRNA is translated into the functional XBP1 protein, which migrates to the nucleus and binds the ERSE regions of chaperone proteins such as Grp78.

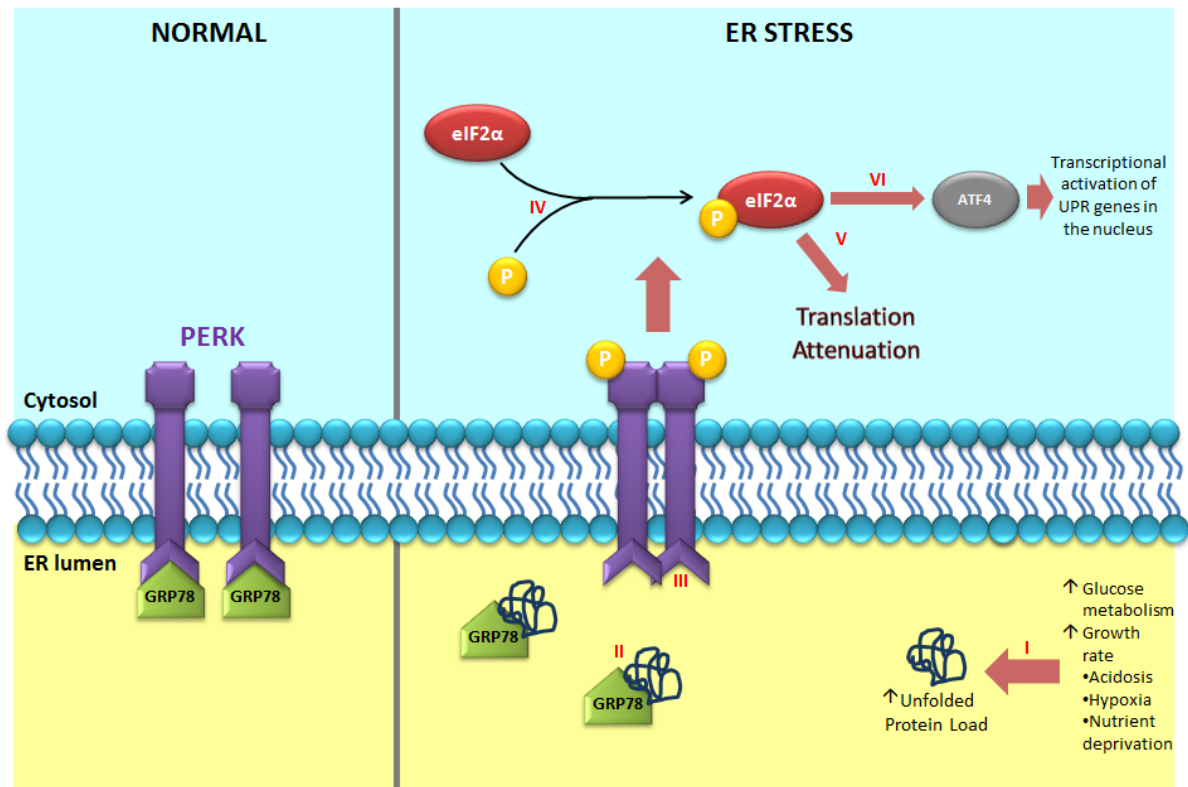


Figure 5.3. The PERK arm of the UPR. In normal conditions, Grp78 is bound to PERK and maintains it in an inactive state. Upon ER stress and thus I) an increase in unfolded protein load, II) Grp78 dissociates from PERK and binds the unfolded proteins, III) permitting homooligomerization and phosphorylation of PERK. IV) PERK facilitates the phosphorylation of eIF2 α , which then V) attenuates translation and VI) stimulates the translation of ATF4. The ATF4 migrates to the nucleus where it transcriptionally activates the ER chaperone genes.

5.2 Results

5.2.1 Induction of *Grp78* promoter activity upon *HSVtk/GCV* treatment in 9L cells

To gain further insight into the understanding of the beneficial effects by the double-targeted RGD4C/*Grp78*-*HSVtk* vector, I investigated the effect of *HSVtk* plus GCV cytotoxic gene therapy on the activity of the *Grp78* promoter, both at the endogenous *Grp78* and RGD4C AAVP/*Grp78* vector level. 9L cells stably transduced with RGD4C AAVP/*Grp78*-*HSVtk-puro*^R or RGD4C AAVP/*CMV*-*HSVtk-puro*^R were incubated with 20μmol/L of GCV from 1hr to 12hr. Untreated cells were used as a negative control. Total cell lysates were prepared from 9L stably transduced cells under normal conditions or GCV treatment, and equal amounts of protein were separated by SDS-PAGE and then subjected to Western blot analysis. As shown in figure 5.4, *HSVtk* in combination with GCV treatment can mediate endogenous *Grp78* induction in 9L cells transduced with RGD4C/*Grp78*-*HSVtk-puro*^R or RGD4C/*CMV*-*HSVtk-puro*^R; a gradual increase of endogenous *Grp78* level with GCV treatment was observed over-time. *Grp78* level normalized to the loading control GAPDH has demonstrated ~3- fold increase in endogenous *Grp78* levels after 12 hours of incubation with GCV.

More interestingly, in cells in which the *HSVtk* transgene was driven by the *Grp78* promoter, *HSVtk* level was increased upon GCV treatment, with ~2.5- fold increase detected at 12 hours post GCV treatment (Fig. 5.4a). In contrast no effect of GCV on *HSVtk* expression driven by the *CMV* promoter was observed in cells stably transduced with RGD4C/*CMV*-*HSVtk-puro*^R (Fig. 5.4b). These data show that *HSVtk/GCV* suicide therapy can induce both promoters of endogenous *Grp78* and of the double-targeted RGD4C AAVP/*Grp78* vector. Thus, induction of the representative UPR marker *Grp78* indicates that GCV in combination with *HSVtk* might be a novel inducer of ER stress.

downstream protein target eIF2 α . Activated PERK, upon dissociation of Grp78, will subsequently phosphorylate eIF2 α which blocks general protein synthesis by suppressing mRNA translation. However, Phosphorylated eIF2 α will selectively promote translation of the transcription factor ATF4 which will subsequently translocate into the nucleus and induce Grp78 transcription. To detect the phosphorylated eIF2 α after GCV treatment, 9L cells stably transduced with RGD4C/Grp78-HSVtk-puro^R were incubated with GCV from 1 to 12 hours. Cell lysates were analyzed by Western blot with an antibody against phospho- eIF2 α , which can detect phosphorylated eIF2 α only when phosphorylation occurs at the Ser51 position, mediated by activated PERK. Interestingly, the phosphorylated eIF2 α , a downstream target of PERK, was first detected after 9 hours and maintained at least through 12 hours of GCV treatment (Fig. 5.5). Detection of phosphorylated eIF2 α after GCV treatment demonstrates the induction of the PERK pathway upon GCV/HSVtk treatment of 9L cells.

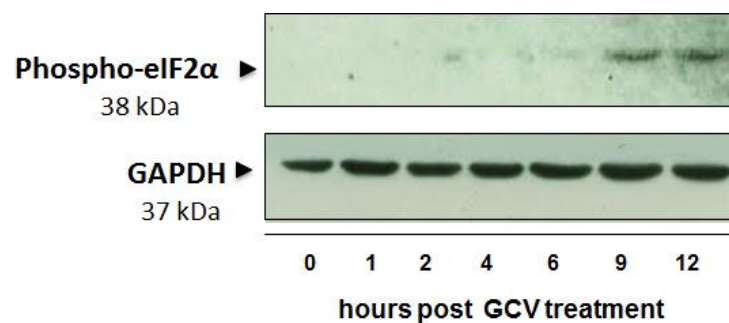


Figure 5.5. HSVtk/GCV treatment induces phosphorylation of eIF2 α . 9L cells stably transduced with RGD4C/Grp78-HSVtk-puro^R were grown in the presence of GCV for the indicated times. Next, equal amount of cell lysates were analysed by Western blot using antibodies against phosphor-eIF2 α and GAPDH as control. The above Western blots are representative of three independent experiments.

5.2.2.2 Activation of the ATF6 arm of UPR

Upon Grp78 dissociation, ATF6 will be converted from a 90 kDa protein (ATF6- p90) to a 50 kDa protein (ATF6-p50) which can translocate to the nucleus and subsequently activate transcription of target genes such as *Grp78*. To evaluate ATF6 activation after GCV

treatment, 9L cells stably transduced with RGD4C/*Grp78-HSVtk-puro*^R were incubated with GCV from 1 to 12 hours. Due to technical difficulties, we were unable to detect the 50-kDa ATF6 by Western blot analysis; however, results indicated an increase in ATF6-p90 level after treatment with GCV in the 9L cells stably transduced with RGD4C/*Grp78-HSVtk-puro*^R (Fig. 5.5). The induction became evident after 6 hours of GCV treatment and persisted as well as intensified, at least, through the 12 hours of treatment (Fig. 5.5). Although we were unable to detect the 50-kDa ATF6, a previous study reported that an increase in 90-kDa ATF6 level after GCV treatment demonstrates ER stress and UPR activation (Teske et al., 2011).

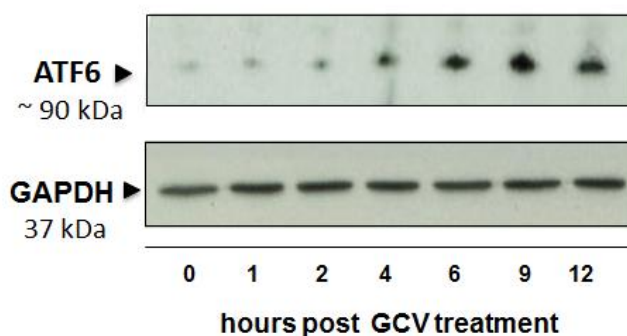


Figure 5.6. *HSVtk*/GCV treatment induces ATF6-p90 expression. 9L cells stably transduced with RGD4C/*Grp78-HSVtk-puro*^R were grown in the presence of GCV for the indicated times. Then equal amount of cell lysates were analysed by Western blot using antibodies against ATF6 and GAPDH as control. The above Western blot is representative of three independent experiments.

5.2.2.3 Activation of the IRE1 arm of UPR

The induction of the ATF6 and PERK arms of the UPR pathway has been confirmed in 9L cells after *HSVtk*/GCV treatment. Next, I aimed to characterise the effect of GCV triphosphate on IRE1, the third arm of UPR. As detection of IRE1 is not possible due to lack of efficient commercial antibody, I tried to analyse its downstream protein target XBP1. Activated IRE1 upon ER stress can splice a 26-nucleotide intron from XBP1 mRNA which

leads to a shift in the open reading frame. Spliced mRNA is an effective transcription factor which can mediate activation of *Grp78* promoter activity. To detect the spliced XBP1 mRNA, 9L cells stably transduced with RGD4C/*Grp78-HSVtk-puro*^R were incubated with GCV from 1 to 12 hours and semiquantitative RT-PCR was performed by using a set of primers which were able to detect both the spliced and unspliced form of XBP1 mRNA (Samali et al., 2010).

Semi-quantitative RT-PCR results showed that the level of mRNA of *XBP1* was constitutively spliced in 9L cells, similar to both positive control TG treated and negative control untreated cells, and GCV treatment did not induce any further increase in spliced XBP1 mRNA level (Fig. 5.7a). Similar to 9L cells, constitutive expression of spliced XBP1 mRNA has been reported in C-26 colon adenocarcinoma cells and some human melanoma cell lines (Nowis et al., 2007, Jiang et al., 2009) . Moreover, the important role of the IRE1/XBP1 arm of UPR in tumour growth and also tumour survival has been described (Romero-Ramirez et al., 2004, Lin et al., 2007, Feldman et al., 2005).

In contrast to 9L cells, in U87 cells stably transduced with RGD4C/*Grp78-HSVtk-puro*^R, the spliced XBP1 mRNA was only detectable in TG treated cells but not in GCV treated or untreated cells. Taken together, I can state that *HSVtk*/GCV treatment does not have any effect on the IRE1 arm of the UPR pathway; however, I could reveal the constant expression of spliced XBP1 in 9L cells but not in U87, which can address the more stress resistant character and high proliferation rate of 9L cells over U87 cells.

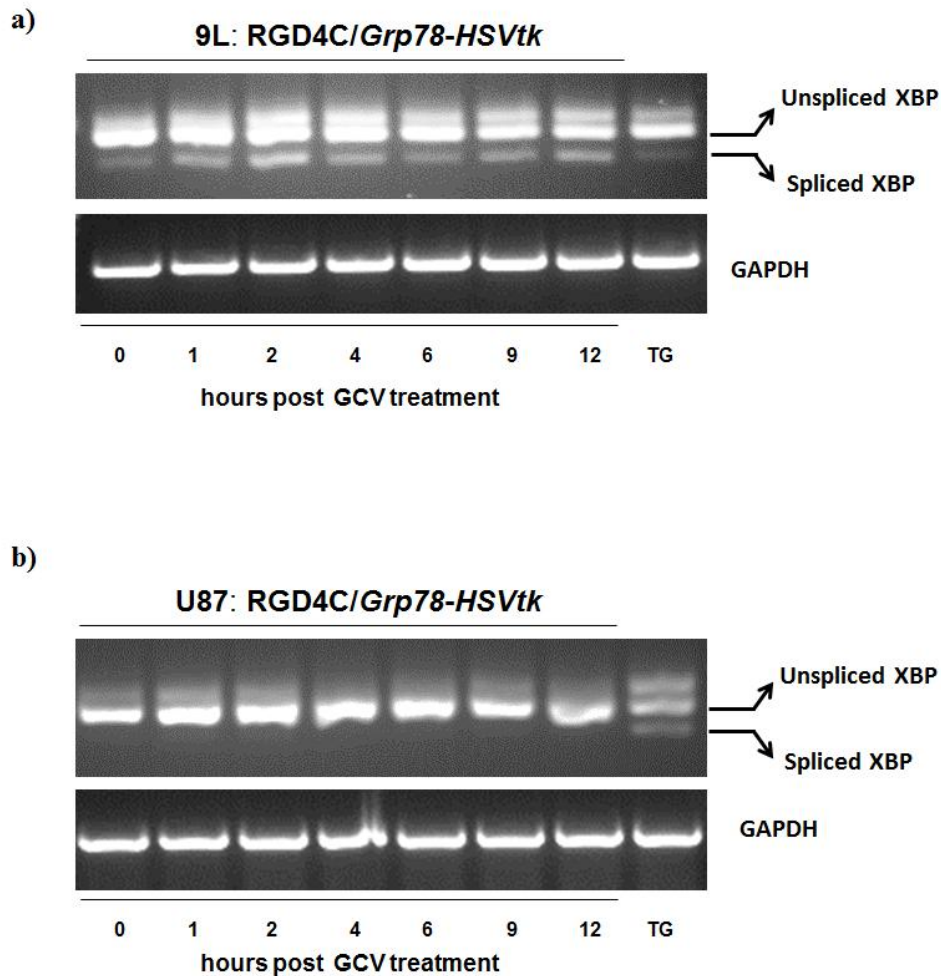


Figure 5.7. Constitutive expression and splicing of XBP1 in 9L cells. Total RNA from **a)** 9L cells stably transduced with RGD4C AAVP/*Grp78-HSVtk-puro*^R and **b)** U87 cells stably transduced with RGD4C AAVP/*Grp78-HSVtk-puro*^R grown in the presence of GCV for the indicated times or TG was isolated and analysed by semiquantitative RT-PCR. PCR products were separated by 1.5% agarose gel for GAPDH or 3% agarose gel for XBP1. Size of PCR products: unspliced XBP1= 289 bp and spliced XBP1= 263 bp. Shown are results of two independent experiments.

5.3 Discussion

Current available drugs for cancer treatment such as chemotherapeutic agents are not tumour specific as they cannot discriminate in their mode of action. Thus, suicide genes such as *HSVtk* have gained an increasing interest in cancer treatment as this approach proved to have no significant systemic toxicity (Shen and Nemunaitis, 2006). Suicide gene therapy also known as prodrug therapy requires the presence of prodrugs for their action. In particular, *HSVtk* suicide gene therapy is induced by administration of the GCV prodrug. The protein product of the *HSVtk* suicide gene which has almost 1000 times more affinity for the prodrug GCV than the host cell tk (Elion et al., 1977) can phosphorylate GCV and thus convert it into toxic metabolites resulting in significant cell death. In addition, the bystander effect of toxic GCV makes this system more efficient for cancer treatment. Indeed in the original AAVP/*CMV* vector, the *HSVtk* suicide gene was used for cancer treatment and proved efficient to mediate cell death in solid tumours *in vivo* after GCV administration (Hajitou et al., 2006b).

In this project, to assess systemic gene therapy efficacy of the double-targeted RGD4C AAVP/*Grp78* vector, I inserted the *HSVtk* gene under the control of *Grp78* promoter. To our surprise, despite the fact that the *Grp78* was a weaker promoter than *CMV* in U87 cells, the efficacy of the therapeutic *HSVtk* gene driven by *Grp78* promoter in the context of AAVP vector proved more efficient than the original vector and resulted in higher rate cell death in both U87 and 9L *in vitro*. Our data are also consistent with previous work reporting the stronger tumour cell killing efficacy of *HSVtk* under the *Grp78* promoter *in vivo* in comparison to viral promoter *LTR*, although a higher activity of *LTR* over *Grp78* promoter *in vitro* has been reported (Azatian et al., 2009). The authors stated that *in vivo* the tumour microenvironment can strongly induce *Grp78* promoter activity.

However, since in this project our first goal was to characterise the therapeutic efficacy of our novel double-targeted vector *in vitro* prior *in vivo* study, I observed that higher expression of *HSVtk* under the control of *Grp78* in glioblastoma cells is not limited by the tumour microenvironment *in vivo* and indeed it can be achieved *in vitro* by using cancer cells. This fact raised the question of whether toxic GCV triphosphate can mediate ER stress and thus induce *Grp78* promoter activity. To further characterize and address the effect of *HSVtk*/GCV in *Grp78* induction, 9L cells stably transduced by RGD4C AAVP/*Grp78*-*HSVtk-puro*^R, were incubated with GCV and our results revealed the induction of the *Grp78* promoter activity for both endogenous *Grp78* level and *HSVtk* level driven by the *Grp78* promoter.

Numerous recent studies have shown that treatment with several drugs such as chemotherapeutic drugs and HDACs inhibitor can stimulate *Grp78* expression (Lin et al., 2011, Jiang et al., 2009, Baumeister et al., 2009, Pyrko et al., 2007, Mandic et al., 2003). Yet, this is the first report to show that *HSVtk/GCV* suicide gene therapy induces *Grp78* at both the endogenous and vector level, leading to increased *HSVtk* expression and subsequently to enhanced tumour cell killing. Our discovery that GCV in combination with HSVtk is an inducer of Grp78 suggests that it might mediate ER stress.

As *Grp78* expression is tightly controlled by the UPR pathway, our next goal was to verify the effect of GCV/HSVtk treatment on this conserved pathway. PERK, ATF6 and IRE1, the three arms of UPR, get activated upon ER stress and each initiates the downstream signalling pathway itself which results in activation of a set of transcription factors that will subsequently induce the *Grp78* expression. Technically, the detection of phosphorylated PERK and IRE1 are quite difficult and is not advisable due to the low quality of commercially available antibodies (Samali et al., 2010); hence, I tried to assess their activation upon *HSVtk/GCV* treatment by analysing their downstream protein targets such as phosphorylated eIF2 α and spliced XBP1 mRNA, respectively.

Indeed, incubation of stably transduced 9L cells by RGD4C AAVP/*Grp78-HSVtk-puro*^R with the GCV prodrug resulted in phosphorylation of eIF2 α which was detectable after 9 hours of GCV treatment and maintained through 12 hours. This result confirmed the activation of the PERK pathway upon GCV treatment. In addition, an increase in ATF6-p90 level was observed through GCV treatment. As some other studies suggested, detection of ATF6-p50, an active transcription factor, is quite difficult as this cleaved fraction is maintained at very low level within cells and, moreover, there is currently a lack of good commercial antibodies for accurate detection.

Indeed Teske et al. have shown that activated PERK/phosphorylated-eIF2 α can mediate the activation of *ATF6* transcription and also control its translation in response to ER stress (Teske et al., 2011). Through this process there will be sufficient amounts of newly synthesized ATF6 available for continued processing into the ATF6-p50 or ATF6 active form in the presence of ER stress. In this scenario an increase in ATF6-p90 level detected upon GCV treatment is an indication of ER stress induction.

In contrast to PERK and ATF6 activation upon *HSVtk/GCV* treatment, no specific induction was observed for the IRE1 arm of UPR. IRE1 induction was characterised by detection of its downstream target, spliced mRNA XBP1. To our surprise, semiquantitative RT-PCR analysis revealed the constant presence of the spliced XBP1 mRNA in 9L tumour cells, which acts as

a transcription factor for the *Grp78* promoter. In contrast I could not detect spliced XBP1 mRNA in U87 tumour cells neither in untreated cells nor in the presence of GCV. Indeed, the presence of spliced XBP1 in 9L cells can address its higher Grp78 level relative to U87 cells, as discussed in chapter 3.

The main roles of XBP1 in tumour growth and tumour survival have been established (Romero-Ramirez et al., 2004, Lin et al., 2007, Feldman et al., 2005). Moreover, a recent study has argued the fact that through induction of *Grp78*, XBP1 may prevent apoptosis in cells surviving drug treatment. They also demonstrated prolonged survival of the quiescent fraction upon Grp78 induction (Schewe and Aguirre-Ghiso, 2009).

As shown in chapter 3, 9L cells grown in medium containing 10% FBS, depict higher proliferation rate relative to U87 cell over time. Here, to further investigate the effect of spliced XBP1 on quiescent cells, 9L and U87 cells were incubated with 0.5% FBS-containing medium. As shown in figure 5.8, consistently with previous studies, 9L cells could survive and proliferate once they have entered a prolonged quiescent state (G0-G1 arrest) while quiescent U87 cells could not survive over-time. Based on previous studies and our data obtained in this chapter I can address the correlation between the constant presence of spliced XBP1 and proliferation of quiescent 9L cells.

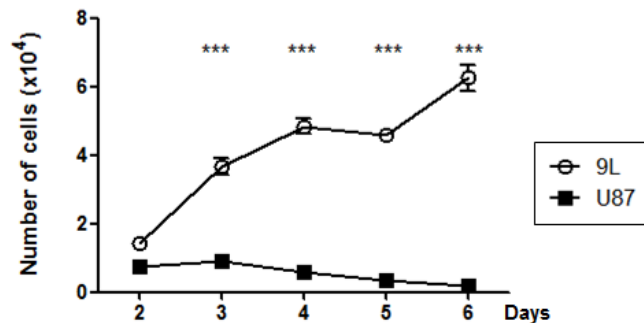


Figure 5.8. Proliferation of quiescent 9L cells over time. Proliferation rate of 9L and U87 glioblastoma quantified by counting the cells over time. For growth curves, 1×10^4 cells of 9L and U87 cell lines were plated in 24-wells plates and grown in DMEM medium supplemented with 0.5% FBS. Triplicates were collected on days 2, 3, 4, 5 and 6 using trypsin- EDTA, and counted using the trypan blue. This experiment was performed twice. Error bars represent standard error of the mean (s.e.m). *** $p < 0.001$.

In summary for the first time I show induction of the *Grp78* expression upon *HSVtk*/GCV treatment. Moreover, I confirmed this induction by showing ER stress mediated by toxic GCV as I could successfully detect UPR activation through induction of the PERK and ATF6 pathways known to control and induce Grp78 expression.

In this manner, cells transduced by RGD4C AAVP/*Grp78*-*HSVtk* unintentionally facilitate their own death upon GCV administration. Consequently, our studies also provide proof of concept supporting the usage of our novel double-targeted vector for cancer treatment. I also strongly believe that this system might mediate treatment against therapy-resistant tumours; a quest that has long been a challenge in oncology.

Chapter 6

In vivo efficacy of double-targeted vector RGD4C AAVP/ Grp78 in tumour-bearing mice

6.1 Introduction

To date, the usage of the *Grp78* promoter in cancer gene therapy has been tested in the context of retroviral vectors. Therapeutic efficacy of a retroviral vector carrying the *HSVtk* suicide gene under the control of the *Grp78* promoter followed by administration of GCV proved efficient to eradicate murine tumours and human xenografts in mice with no recurrence of tumour growth (Dong et al., 2004, Chen et al., 2000). Moreover, by using the same vector another group demonstrated gastroesophageal junction and gastric adenocarcinoma cell death *in vitro* and tumour regression *in vivo* (Azatian et al., 2009). However, in all the previous studies, tumour cells were first transduced with a vector carrying a *Grp78* transgene expression cassette, and then implanted in mice. Thus no vector administration was applied directly in mice.

This is the first study designed to utilise the *Grp78* promoter in a ligand targeted AAVP vector for systemic cancer gene therapy. After demonstrating that transgene expression in tumour cells *in vitro* is clearly improved in the context of the double-targeted RGD4C AAVP/*Grp78* vector and most importantly after full characterisation of the therapeutic efficacy of this novel vector carrying *HSVtk* gene, our next goal was to evaluate the specificity and efficacy of gene delivery into tumours *in vivo* after systemic administration of

the RGD4C AAVP/*Grp78* vector and compare it alongside the original RGD4C AAVP/*CMV* vector. In addition, to the specificity of the double-targeted vector *in vivo*, I aimed to evaluate the long-term activity of the *Grp78* promoter, in living tumour-bearing mice following single intravenous administration (IV) of vector.

As an initial preclinical model, we used immunodeficient nude mice bearing subcutaneous tumours derived from either 9L rat glioblastoma or U87 human glioblastoma cells. In this chapter for non-invasive monitoring of *Luc* expression in living tumour-bearing mice following intravenous administration (IV) of *Luc*-expressing vectors, we used a standard experimental setup for *in vivo* imaging of the firefly *Luc* reporter transgene. By using repetitive bioluminescence imaging (BLI), we have visualized and quantitated *Luc* expression after a single systemic administration of RGD4C AAVP/*Grp78-Luc* or RGD4C AAVP/*CMV-Luc*, or control insertless vectors in 9L or U87-derived tumours and other tissues in nude mice.

In addition to the above studies, we will evaluate the therapeutic efficacy of the double-targeted vector *in vivo* using both 9L glioblastoma cells as an aggressive tumour model and U87 cells as a fast-moderate tumour model.

6.2 Results

6.2.1 Bioluminescence imaging reveals activation of the *Grp78* promoter in growing tumours overtime

In chapter 4, I demonstrated long-term activity of the *Grp78* promoter activity in stably transduced 9L and U87 cells. Interestingly, in contrast to the *Grp78* promoter, silencing of *CMV* promoter in the 9L and U87 cells transduced with RGD4C AAVP/*CMV-puro*^R was observed over time. These results were obtained by monitoring either the *GFP* reporter gene or *HSVtk* suicide gene expression under the control of either promoter in the context of the AAVP vector. After demonstrating both promoter activities overtime in tumour cells *in vitro*, we further aimed to examine the possibility of improved transgene expression in RGD4C AAVP/*Grp78-puro*^R stably transduced cells overtime in an *in vivo* context.

Thus, 9L cells were first stably transduced with RGD4C AAVP/*Grp78-puro*^R or RGD4C AAVP/*CMV-puro*^R vectors expressing the *Luc* reporter transgene. At day 25 post-vector transduction, stably selected 9L cells were subcutaneously implanted into immunodeficient

nude mice. At day 14 post-cell implantation, the BLI of whole living tumour-bearing mice was initiated and continued at the indicated days to measure the luciferase signal driven by the *Grp78* promoter within stably transduced 9L tumour-bearing mice and was compared to the *CMV* promoter (Fig. 6.1a). Note that in figure 6.1, the same scale bars have been applied for all the images and thus comparisons are valid. Within the initial tumour growth phase, the luciferase signal was comparable between the two groups of tumours. However, long-term, the luciferase signal increased significantly in tumours derived from 9L cells stably transduced by RGD4C AAVP/*Grp78-Luc* in which at day 36 post imaging, luciferase activity in the *Grp78* tumour was on average 6 times greater than in RGD4C AAVP/*CMV-Luc* derived 9L tumours (Fig. 6.1b). The direct effect of the tumour microenvironment on *Grp78* induction has been reported by several studies and indeed our results demonstrate the effect of *in vivo* growing 9L tumours on the *Grp78* promoter induction in the context of AAVP. In contrast, luciferase expression driven by the *CMV* promoter in stably transduced 9L tumours was only detectable in one edge of the tumour. The *CMV* activity was completely silenced in other parts of the tumour as no specific luciferase signal could be observed (Fig. 6.1a). As the implanted tumour cells were previously stably transduced and selected with RGD4C AAVP/*CMV-Luc-puro^R*, the increase in luciferase expression upon tumour growth was expected, while in contrast luciferase expression driven by the *CMV* promoter remained at the same level overtime (Fig. 6.1b).

Interestingly the above long-term *in vivo* imaging results directly correlate with our *in vitro* study demonstrating the *CMV* promoter silencing over time.

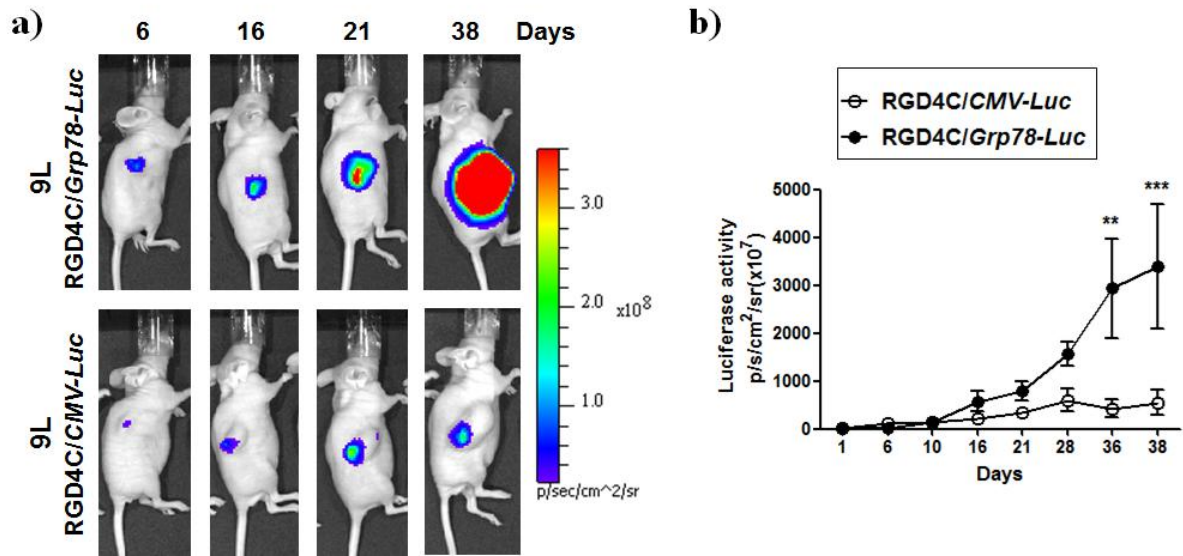


Figure 6.1. Evaluation of *Grp78* and *CMV* promoter activity *in vivo* over time. **a)** *In vivo* bioluminescent imaging (BLI) of immunodeficient nude mice (n=5) bearing 9L glioblastoma cells stably transduced with either RGD4C AAVP/*Grp78-Luc-puro*^R or RGD4C AAVP/*CMV-Luc-puro*^R vector over indicated days of bioluminescence imaging. **b)** Luciferase expression driven by either *Grp78* or *CMV* promoter was measured at the indicated times. **p < 0.01 and ***p < 0.001.

6.2.2 Systemic tumour targeting of gene delivery *in vivo* by the double-targeted vector RGD4C AAVP/*Grp78*

6.2.2.1 RGD4C AAVP/*Grp78* targeting 9L glioblastoma xenografts

To evaluate AAVP vector-mediated transgene expression after systemic delivery in tumour-bearing mice, we first established 9L glioblastoma tumours by injecting 10^6 9L cells into the right flank of immunodeficient nude mice. Then, a single dose (5×10^5 TU) of targeted RGD4C AAVP/*CMV* or double-targeted RGD4C AAVP/*Grp78* as well as control insertless vectors carrying the *Luc* reporter gene was administered intravenously (IV) to nude mice bearing subcutaneous 9L glioblastomas ($\sim 100 \text{ mm}^3$).

The BLI technique was used to monitor the *Luc* reporter transgene expression in living tumour-bearing mice after RGD4C AAVP administration. The BLI of *Luc* expression is a very sensitive method for reporter gene imaging in mice and has almost no nonspecific background activity in the images (Gross and Piwnica-Worms, 2005, Gelovani Tjuvajev and Blasberg, 2003, Hajitou et al., 2006b). Serial *in vivo* BLI measurement with D-luciferin substrate (on days 0, 3, 5, 7, 14 and 18) was performed. We have visualized and quantitated *Luc* expression within the 9L-derived tumour and other organs and tissues of nude mice, at the indicated days post systemic administration of RGD4C AAVP/*Grp78-Luc* or RGD4C AAVP/*CMV-Luc*, or control insertless vectors (Fig. 6.2).

Consistent with previous reports (Hajitou et al., 2007), luciferase expression within 9L tumours was detectable at very low levels at day 3 after RGD4C AAVP/*CMV-Luc* administration (Fig. 6.2a and 6.2b). While low luciferase expression at day 3 was followed by a linear increase that reached a maximum of transgene expression at day 5, this short time increase in *CMV* activity within 9L tumours was followed by a marked decrease at day 7 post-vector administration. In sharp contrast to mice administered with the RGD4C AAVP/*CMV-Luc* vector, a significantly higher luciferase expression level within tumour cells was achieved at day 3 post IV administration of the RGD4C AAVP/*Grp78-Luc* vector, followed by a gradual and much longer stabilization of luciferase expression levels towards day 8 (Fig. 6.2a and 6.2b).

Although both promoters proved to have similar activity within the 9L tumour, the *Grp78* promoter operated earlier and lasted longer compared to the *CMV* promoter (Fig. 6.2b). Finally, no tumour-associated bioluminescent signals were observed in mice receiving control vectors (Fig. 6.2a).

Moreover, consistent with a previous study no bioluminescence was observed in normal organs of mice such as lung, liver, spleen, or kidneys from all experimental groups.

However, luciferase expression was detected in animal's paws and also at the site of IV injection of the RGD4C AAVP/*CMV-Luc* vector, while mice administered with double-targeted RGD4C AAVP/*Grp78-Luc* vector did not show such an effect (Fig. 6.2a).

Although $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are barely detectable in quiescent vessels, their up-regulation during wound -induced angiogenesis has been previously reported (Max et al., 1997, Christofidou-Solomidou et al., 1997, Sheppard, 1996, Hodivala-Dilke et al., 2003). Thus, the non-specific expression of the RGD4C AAVP/*CMV-Luc* vector in mice tails and paws might be mediated by targeting the α_v integrins overexpressed on injured endothelial cell surfaces and further constitutive expression of *Luc* under the control of *CMV* promoter.

Interestingly, *Luc* expression driven by the *Grp78* promoter was only detectable within the tumour as evidenced by the absence of specific BLI signals in mice paws and tail (Fig. 6.2a). In agreement with previous reports, these data indicate quiescent activity of the *Grp78* promoter in non-malignant cells *in vivo*, proving its significant role in controlling transgene expression within the solid tumour and tumour endothelial cells only.

6.2.2.2 RGD4C AAVP/*Grp78* targeting of U87 glioblastoma xenografts

Having shown that systemic delivery of the RGD4C AAVP/*Grp78* vector could successfully mediate transgene expression solely in tumours in mice bearing 9L glioma, we further aimed to evaluate the AAVP vector-mediated transgene expression in another tumour model. Thus, as a second preclinical model we used immunodeficient nude mice bearing subcutaneous tumours derived from U87 glioblastoma cells.

To visualise *Luc* transgene expression in mice after vector administration, serial *in vivo* BLI was performed on days 0, 3, 5, 7 and 10 post AAVP injection. To our surprise and in contrast to 9L xenografts, no U87 tumour-associated bioluminescent signal was observed after systemic administration of the targeted RGD4C AAVP/*CMV* or double-targeted RGD4C AAVP/*Grp78* vector over time (Fig. 6.3a). Also, no luciferase signal was detected in mice receiving control insertless vectors.

Interestingly, although no luciferase signal was detected within the tumour areas (Fig. 6.3a and 6.3b), RGD4C AAVP/*CMV-Luc* vector mediated luciferase activity was detectable in paws and tail of injected mice (Fig. 6.3a). Moreover, non-specific transduction of RGD4C AAVP/*CMV-Luc* vector was observed in different parts of mice bearing U87 tumours, mainly localized close to the site of injection (Fig. 6.3a). Quantified luciferase expression within the whole animal body demonstrated non-specific transgene expression after systemic administration of RGD4C AAVP/*CMV-Luc* vector while in sharp contrast, IV administration of the RGD4C AAVP/*Grp78-Luc* vector resulted in no vector-mediated expression in mice bearing U87 glioblastoma (Fig. 6.3c).

The lack of detection of luciferase in U87 tumours by BLI is consistent with our *in vitro* data showing better and stronger transgene expression of the RGD4C AAVP/*Grp78* vector in 9L cells compared to U87 cells.

However, despite the fact that no tumour-associated bioluminescent signals were observed and detected in mice bearing U87 tumours, RGD4C AAVP/*Grp78* proved safer and more reliable than the original RGD4C AAVP/*CMV* vector.

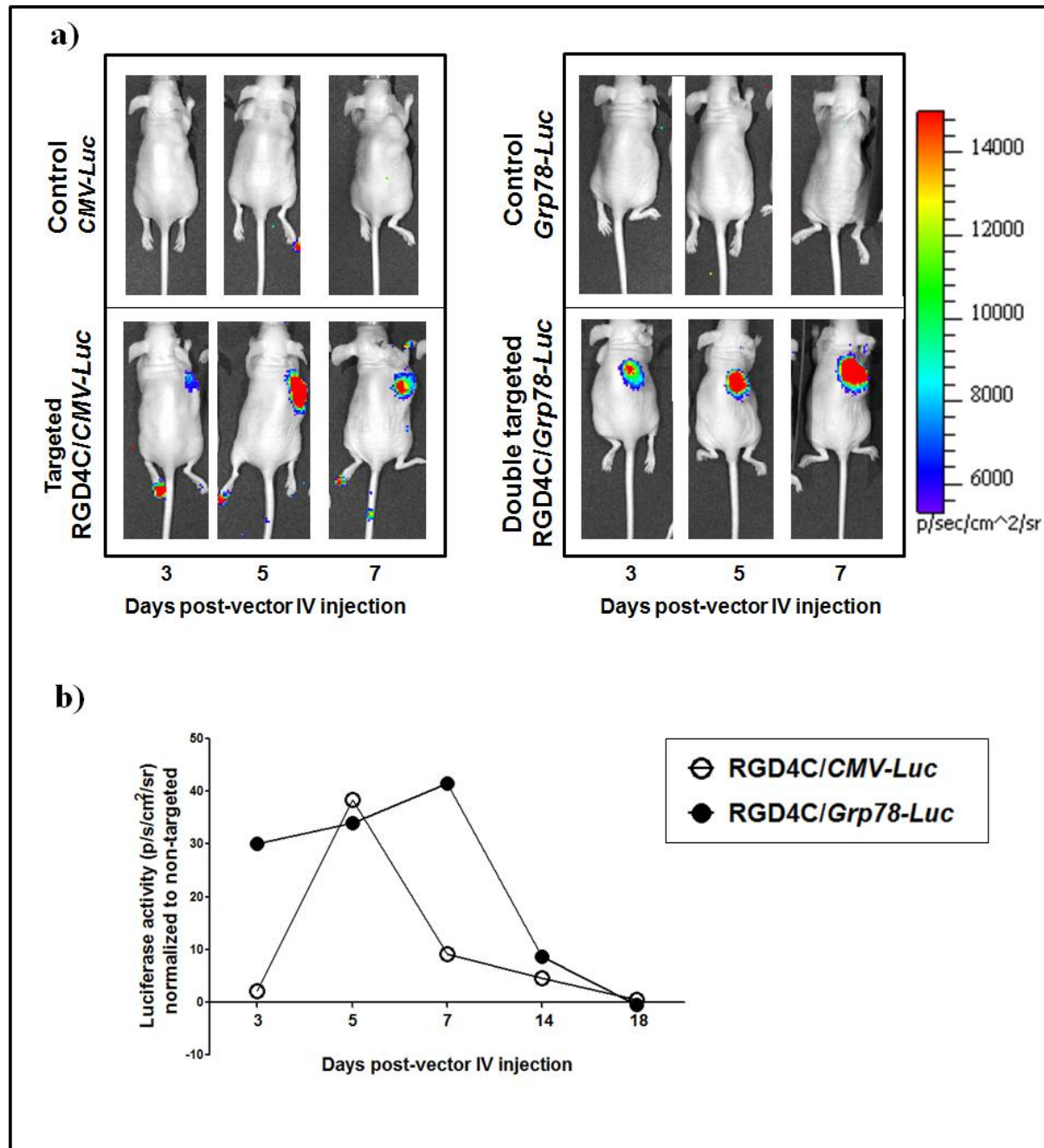


Figure 6.2. Systemic administration of the RGD4C AAVP/*Grp78* mediates early and durable expression of the *Luc* reporter transgene in 9L tumours. a) *In vivo* bioluminescent imaging (BLI) of *Luc* expression. Immunodeficient nude mice bearing size-matched 9L subcutaneous xenografts ($\sim 100 \text{ mm}^3$) received a single intravenous (IV) dose of the double-targeted RGD4C AAVP/*Grp78*-*Luc* or targeted RGD4C AAVP /*CMV*-*Luc* as well as control insertless vectors (5×10^{10} TU/mouse). *Luc* transgene expression was monitored at the indicated days post-vector administration. A standard calibration scale is provided. **b)** Serial real-time quantification of *Luc* expression in tumour-bearing mice. *Luc* expression

from RGD4C AAVP/*Grp78-Luc* and RGD4C AAVP/*CMV-Luc* was normalised to that of control insertless AAVP/*Grp78-Luc* and AAVP/*CMV-Luc*, respectively.

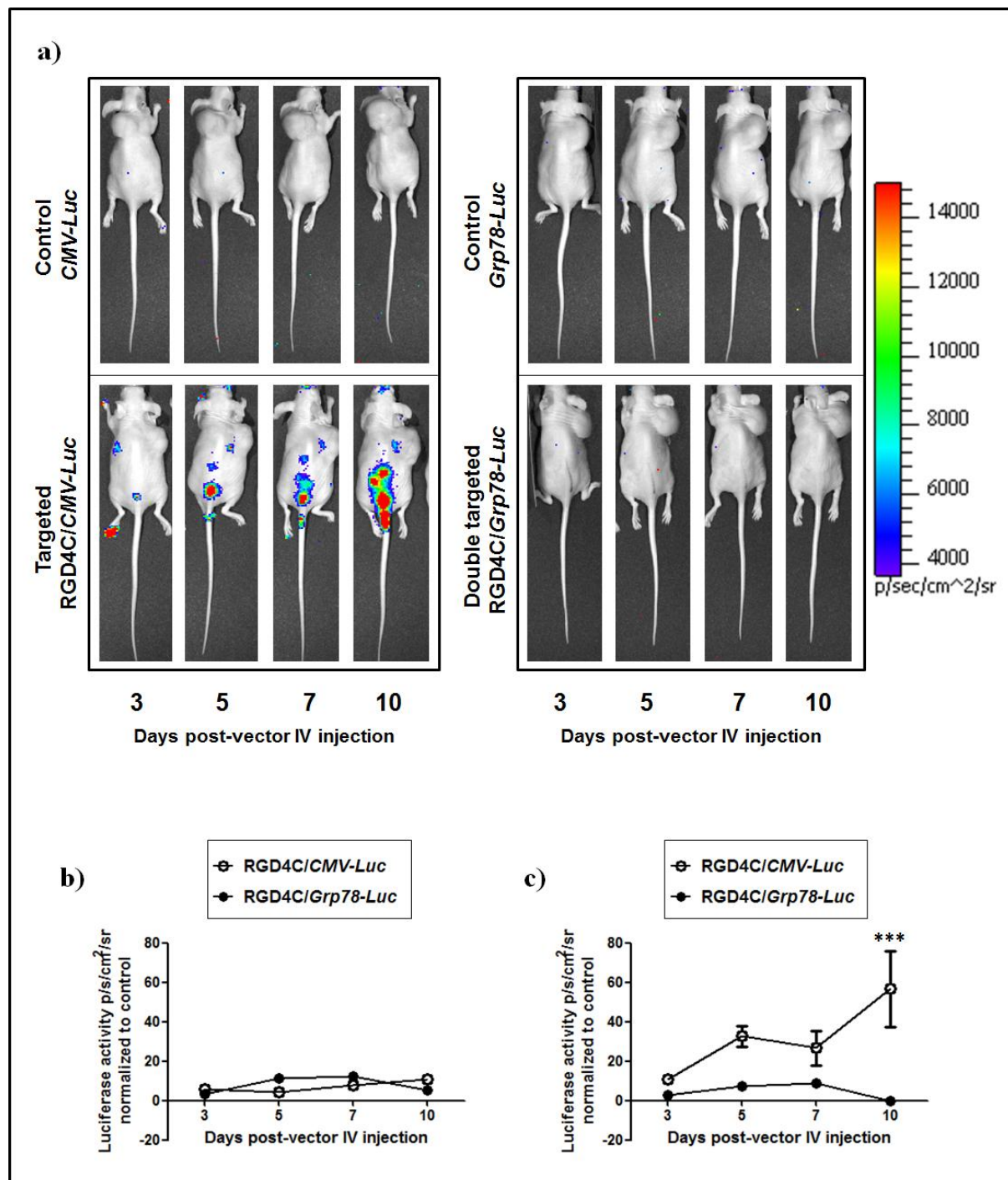


Figure 6.3. Undetectable luciferase activity in U87 tumours upon systemic administration of the RGD4C AAVP/*Grp78-Luc*. **a)** *In vivo* bioluminescent imaging (BLI) of *Luc* expression. Immunodeficient nude mice bearing size-matched U87 subcutaneous xenografts, received a single intravenous (IV) dose of the double-targeted RGD4C AAVP/*Grp78-Luc* or targeted RGD4C AAVP /*CMV-Luc* as well as control insertless vectors

(5×10^{10} TU/mouse). *Luc* transgene expression was monitored at the indicated days post-vector administration. A standard calibration scale is provided. Serial real-time quantification of *Luc* expression in **b)** tumour area **c)** whole mouse body. *Luc* expression from RGD4C AAVP /*Grp78-Luc* and RGD4C AAVP /*CMV-Luc* was normalised to that of control insertless AAVP/*Grp78-Luc* and AAVP/*CMV-Luc*, respectively. *** $p < 0.001$.

6.3 Generation of SV40- pGL4.20 plasmid

To assess therapeutic efficacy of the RGD4C AAVP/*Grp78-HSVtk* vector *in vivo*, in addition to tumour size we sought to measure the tumour viability before and after *HSVtk/GCV* treatment. Thus, stably luciferase-labelled 9L and U87 cells were implanted into the flank of nude mice. To generate cells stably expressing the *Luc* reporter gene (2.8.6), the pGL4.20 (2.2) was used. The pGL4.20 vector encodes the Luciferase reporter gene *Luc2* (*Photinus pyralis*); however, as this plasmid does not contain any promoter to mediate *Luc* expression prior to cell transfection using this plasmid, we had to insert a constitutive promoter upstream the *Luc* gene. For this purpose, the *SV40* promoter was removed from pPUR plasmid (2.2) by *PvuII* and *HindIII* double digestion and subsequently ligated to pGL4.20 digested with *EcoRV* and *HindIII*, located in multiple cloning sites, to control *Luc* transgene expression (*PvuII* can ligate to *EcoRV*) (Fig. 6.4).

In order to find the positive clones and release the *SV40* fragment, *HindIII* and *NheI* double digestion was performed (Fig. 6.4).

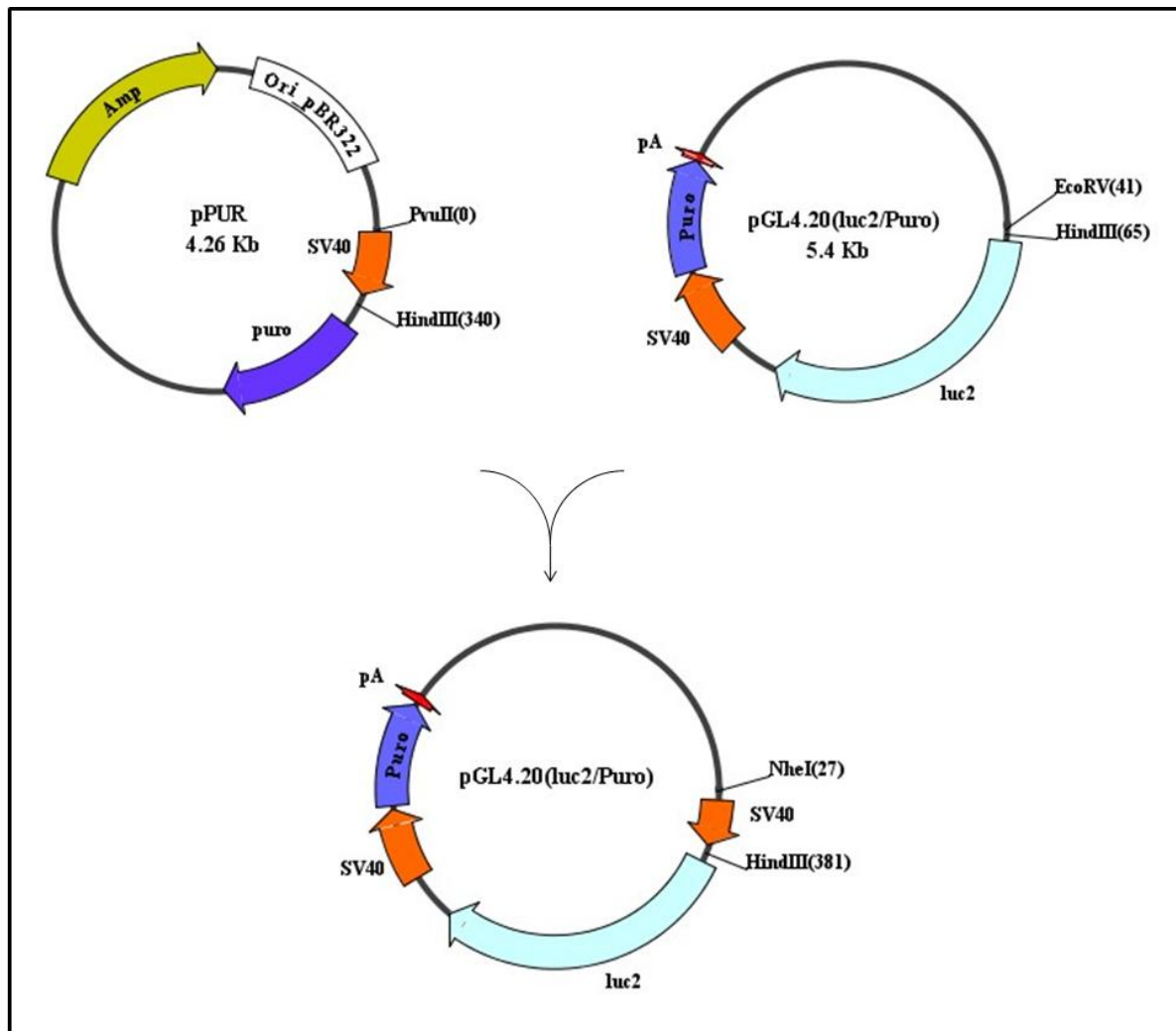


Figure 6.4. Schematic chart describing construction of the pGL4.20- SV40 plasmid. the SV40 promoter flanked by *PvuII* and *HindIII* was released from pPUR plasmid and ligated to the pGL4.20 plasmid digested with *EcoRV* and *HindIII* to control Luciferase gene expression. (Amp: ampicillin resistance gene, Puro: puromycin-resistance gene, Luc2: Luciferase reporter gene).

6.4 Therapeutic efficacy of the double-targeted vector carrying HSVtk suicide gene in vivo in small size glioblastoma

6.4.1 Systemic administration of the double-targeted RGD4C AAVP/Grp78- HSVtk mediated 9L tumour growth suppression in mice after GCV treatment

We next aimed to use the RGD4C AAVP/Grp78 vector encoding the HSVtk as a therapeutic gene to determine therapeutic efficacy of this vector *in vivo* in combination with GCV treatment. To this end, *Luc*-expressing 9L rat glioblastoma cells which were previously stably transfected with pGL4.20- SV40 plasmid were subcutaneously implanted into the flank of nude mice. Cohorts of mice bearing size-matched *Luc*-expressing 9L tumours (~100 mm³) received a single IV dose (5×10^5 TU) of either RGD4C AAVP/Grp78-HSVtk or RGD4C AAVP/CMV-HSVtk. Also as a control, insertless AAVP/Grp78-HSVtk and AAVP/CMV-HSVtk vectors were systemically administered. At day 3 post-vector delivery, daily GCV treatment started for all groups of mice.

The Effects of HSVtk/GCV therapy were evaluated on tumour size by using calliper measurement (Fig. 6.5a) as well as on tumour viability by monitoring *Luc* expression of tumours (Fig. 6.5b).

As shown in figure 6.5, similar tumour growth suppressive effects were initially observed after GCV treatment in both tumour-bearing mice receiving RGD4CAAVP/Grp78-HSVtk or RGD4CAAVP/CMV-HSVtk as compared to control mice treated with control insertless vectors. As both vectors demonstrated the same therapeutic efficacy in the small size 9L tumours, we then aimed to stop GCV treatment in recurrent tumours from day 10 until day 13 post-vector injection; hence, we could assess the therapeutic efficacy of vectors in larger tumours. Interestingly, repeated daily treatment with GCV after day 13 post-vector injection resulted in complete growth suppression of the large growing back tumours (~400 mm³) in mice that received double-targeted RGD4C AAVP/Grp78-HSVtk compared to mice administered with control insertless vector (Fig. 6.5a). In contrast, as shown in figure 6.5a, daily administration of GCV showed no effect on the large recurrent tumours which received the original RGD4C AAVP/CMV-HSVtk vector.

Moreover, in addition to tumour size, BLI of the *Luc* reporter gene expression in tumours showed better suppressive effects with the RGD4C AAVP/Grp78-HSVtk vector against the large growing back tumours than the original vector RGD4C AAVP/CMV-HSVtk (Fig. 6.5b).

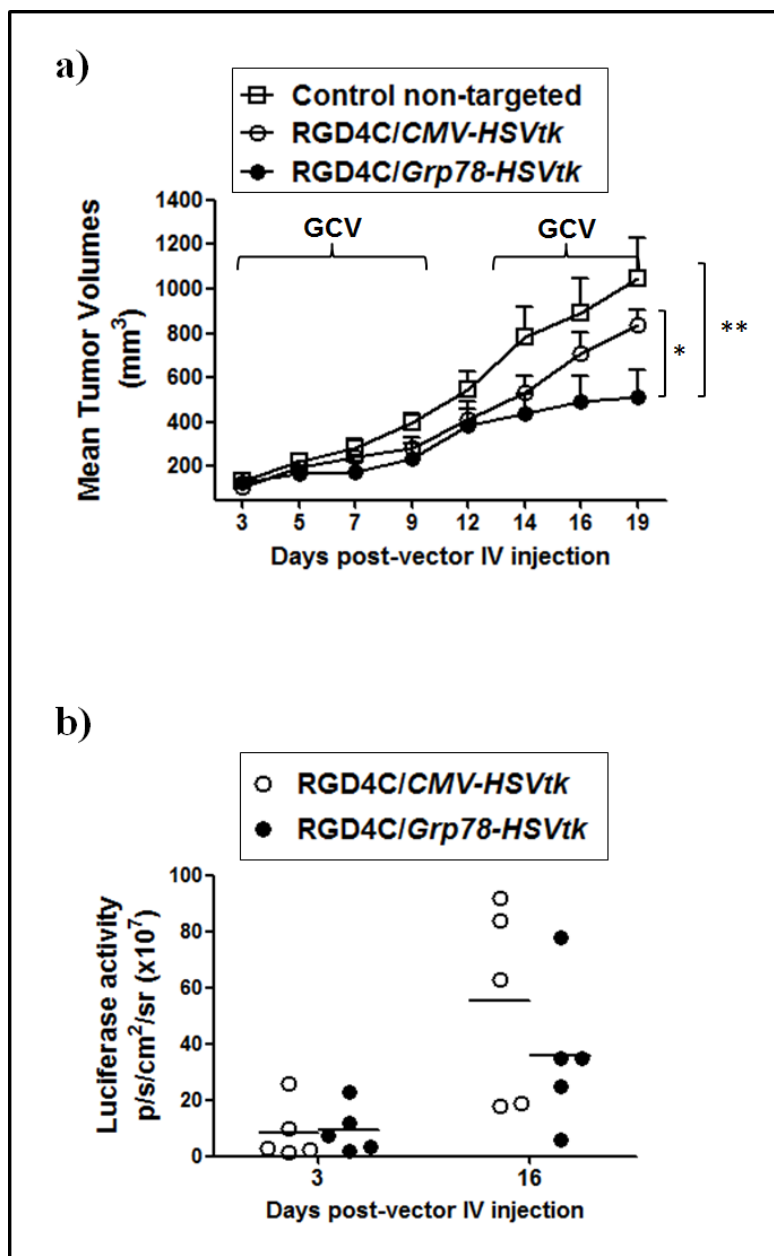


Figure 6.5. Therapeutic efficacy of the RGD4C AAVP/Grp78-HSVtk vector in small size 9L xenografts after GCV treatment. **a)** Cohorts of nude mice with established 9L tumours (~100 mm³) received a single intravenous administration of either double-targeted RGD4C AAVP/Grp78-HSVtk or RGD4C AAVP/CMV-HSVtk, or control insertless vectors (5×10^{10} TU). GCV (75 mg/kg/day) was injected from day 3 posttreatment until day 10, and then re-administered from day 13 until the end of the experiments. Shown are the mean tumour volumes $\pm$ standard errors of the mean (s.e.m). * $p < 0.05$ and ** $p < 0.01$. **b)** Changes in tumour

viability after GCV therapy were assessed with BLI of luciferase expression in 9L tumour cells stably expressing the *Luc* transgene. Error bars represent standard error of the mean (s.e.m). (n=5).

6.4.2 U87 human glioblastoma growth suppression in mice after systemic administration of RGD4C AAVP/Grp78- HSVtk

To rule out the possibility that the observed antitumour effects obtained in 9L tumour xenografts were tumour specific, we further aimed to analyse efficacy of the RGD4C AAVP/Grp78-HSVtk vector on the human U87-derived xenografts. To determine therapeutic

effects, we started intraperitoneal injections of GCV at day 3 post IV injection of the vector and this treatment continued for 23 days. Tumour size was measured by calliper. *In vivo* BLI of *Luc*- expressing U87 cells was also applied to validate tumour cell viability during therapy assessment.

As shown in figure 6.6, results demonstrated rapid growth of tumours in mice receiving either insertless AAVP/*CMV-HSVtk* or insertless AAVP/*Grp78-HSVtk* with no therapeutic effect observed in these groups (Fig. 6.6a).

Interestingly, similar to 9L tumours, comparable antitumour effects were initially detected following GCV treatment of mice administered with RGD4C AAVP/*Grp78-HSVtk* or RGD4C AAVP/*CMV-HSVtk* as compared to control mice injected with insertless vectors (Fig. 6.6). Next, we sought to confirm whether the *Grp78* promoter retained its activity in recurrent tumours. Thus, GCV treatment was stopped between days 17 to 22 post-vector injection. Yet again, when tumours grew back (around 300 mm³ in size), repeated GCV treatment resulted in clear regression of the large volumes of the growing back tumours from mice that received the double-targeted RGD4C AAVP/*Grp78-HSVtk* vector while tumour growth continued in mice administered with targeted RGD4C AAVP/*CMV-HSVtk* vector (Fig. 6.6).

As shown in figure 6.6a and 6.6b, tumour viability was reduced at day 26 post-vector injection in mice that received RGD4C AAVP/*Grp78-HSVtk* vector as evidenced by loss of *Luc* activity of the *Luc*-expressing U87 tumours. Moreover, consistent with tumour viability, tumour size decreased in mice administered with RGD4C AAVP/*Grp78-HSVtk* (Fig. 6.6c). In contrast, administration of RGD4C AAVP/*CMV-HSVtk* vector initially resulted in reduced tumour growth after 5 days of GCV treatment. However, such an effect was not observed in the long-term (day 26 post-vector injections) as clear tumour growth as well as increase in tumour luciferase signals were observed (Fig. 6.6). These results in addition to previous data obtained from 9L xenografts demonstrate that no significant effect with the original vector RGD4C AAVP/*CMV-HSVtk* on recurrent tumours were obtained while in contrast the double-targeted vector RGD4C AAVP/*Grp78-HSVtk* in combination with GCV treatment resulted in significant tumour regression.

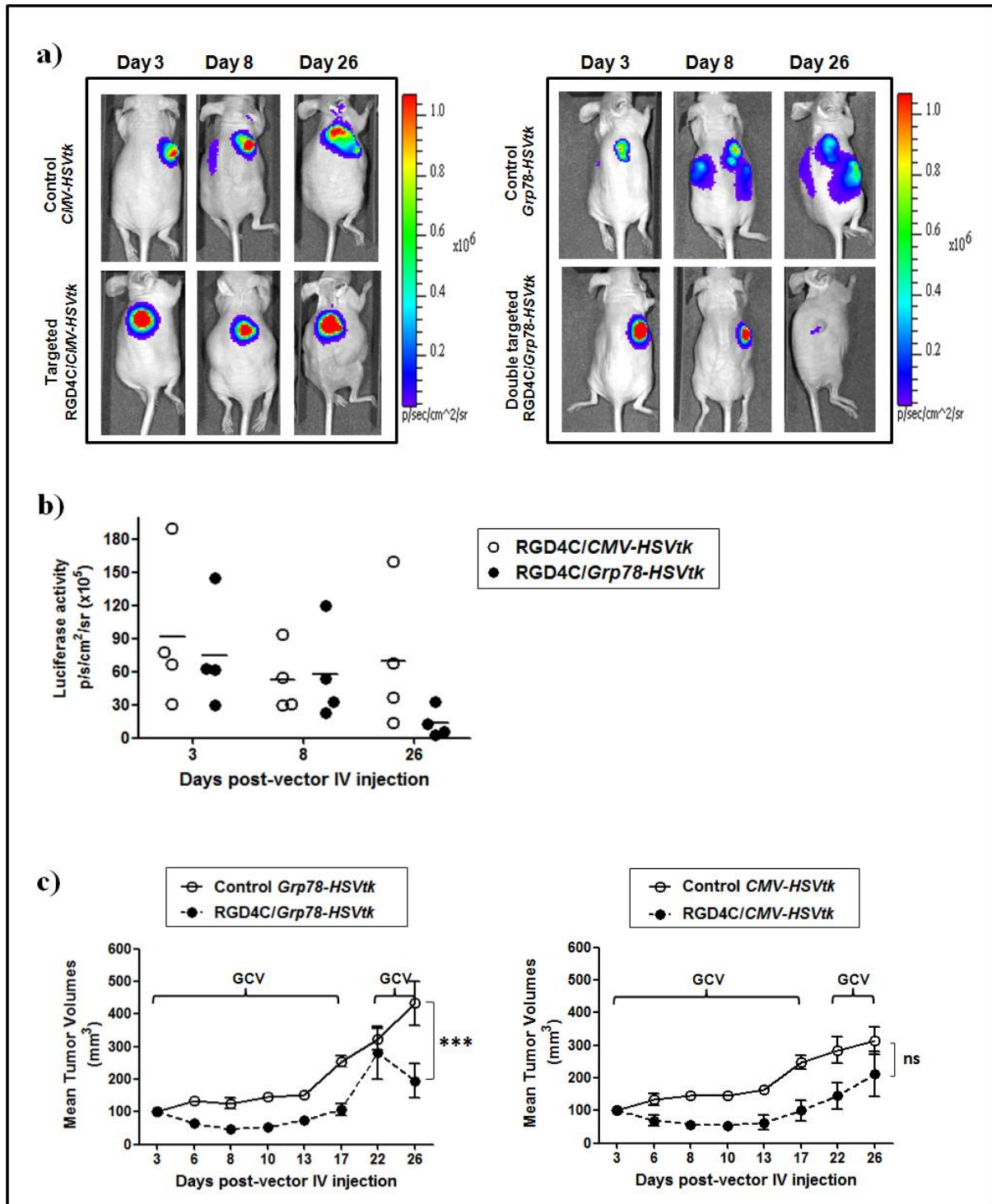


Figure 6.6. Therapeutic efficacy of RGD4C AAVP/*Grp78*-HSVtk vector in the human U87. Nude mice bearing subcutaneous U87 xenografts derived from *Luc*-expressing U87 cells were intravenously administered with a single dose (5×10^{10} TU/mouse) of RGD4C AAVP/*Grp78*-HSVtk or RGD4C AAVP/*CMV*-HSVtk as well as insertless control *Grp78*-HSVtk or *CMV*-HSVtk vectors. GCV treatment (75 mg/kg/day) started when tumours reached $\sim 100 \text{ mm}^3$ and was given daily until day 24 post-vector transduction, then re-administered

again at day 29 and continued until the end of experiment. **a)** Representative images of *in vivo* BLI showing tumour size and tumour luciferase activity in tumour-bearing mice at the indicated time points post-vector administration. **b)** Changes in tumour viability before and after GCV treatment as measured by BLI of tumour luciferase activity. **c)** Tumour growth curves after GCV treatment. Error bars represent standard errors of the mean (s.e.m). *** $p < 0.001$ and ns: non-significant.

6.5 Systemic administration of double-targeted RGD4C AAVP/Grp78-HSVtk and GCV treatment induces regression of aggressive and large tumours

Our results using the double-targeted RGD4C AAVP/*Grp78-HSVtk* vector proved more efficient than original vector to suppress large tumours after vector administration. This remarkable result was achieved in both human U87 and rat 9L glioblastoma models *in vivo*.

However in previous therapy assessments using both U87 and 9L models in mice, prodrug GCV treatment was started randomly at day 3 post-vector administration. To avoid this uncontrolled GCV administration, we aimed to set up a new experiment to be able to detect *Grp78* activity within tumours prior to GCV treatment.

To monitor *Grp78* activity during the growth of 9L tumours in mice prior to GCV treatment, 9L cells were first stably transduced with the RGD4C AAVP/ *Grp78-Luc-puro*^R vector and selected in the presence of puromycin to express the *Luc* transgene under the control of the *Grp78* promoter. Next nude mice were subcutaneously injected with 1×10^6 *Grp78-Luc* expressing 9L cells. When tumour volume reached around 250-300 mm³, a single dose of either double-targeted or targeted as well as control insertless vectors driving the *HSVtk* therapeutic gene were intravenously administered to tumour-bearing mice. At day 3 post-vector injection, tumours grew rapidly to reach large size (~400-450 mm³) and luciferase activity of 9L tumour cells driven by the *Grp78* promoter was evaluated using the IVIS imaging system. BLI of *Luc* expression revealed high activity of the *Grp78* promoter within the 9L tumours (Fig. 6.7a). Therefore, GCV treatment was immediately initiated and continued for 4 days at which time the experiment was terminated.

As shown in figures 6.7a and 6.7b, following 4 days of GCV treatment, the double-targeted RGD4C AAVP/*Grp78-HSVtk* vector resulted in marked regression of the large volume tumours, while no effect on tumour suppression was observed in tumour-bearing mice

administered with RGD4C AAVP/*CMV-HSVtk* vector. Moreover, no effect on tumour growth was observed in mice treated with control insertless vectors (Fig. 6.7a and 6.7b)

Consistently, the RGD4C AAVP/*Grp78-HSVtk* vector administration resulted in less viable tumours after 4 days of GCV treatment (Fig. 6.7c). In contrast, mice administered with RGD4C AAVP/*CMV-HSVtk* vector showed less tumour cell viability after GCV treatment, although there was no effect on tumour size (Fig. 6.7c). No therapeutic effect was observed in mice bearing tumour administered with control insertless vector as tumour cell viability increased at the same rate as tumour growth.

To check for post-treatment effects, tumour tissues were snap frozen after 4 days of GCV treatment, sectioned and stained with Hematoxylin and Eosin (H&E). The stained tumour sections revealed extensive tumour destruction by the single systemic administration of the double-targeted RGD4C AAVP/*Grp78-HSVtk* vector plus GCV treatment. Most of the tumour destruction was observed in the central area of tumours. In contrast to the double-targeted RGD4C AAVP/*Grp78-HSVtk* vector, slight tumour disruption resulted from the original targeted RGD4C AAVP/*CMV-HSVtk* vector (Fig. 6.7d). No effect resulted from control insertless vectors.

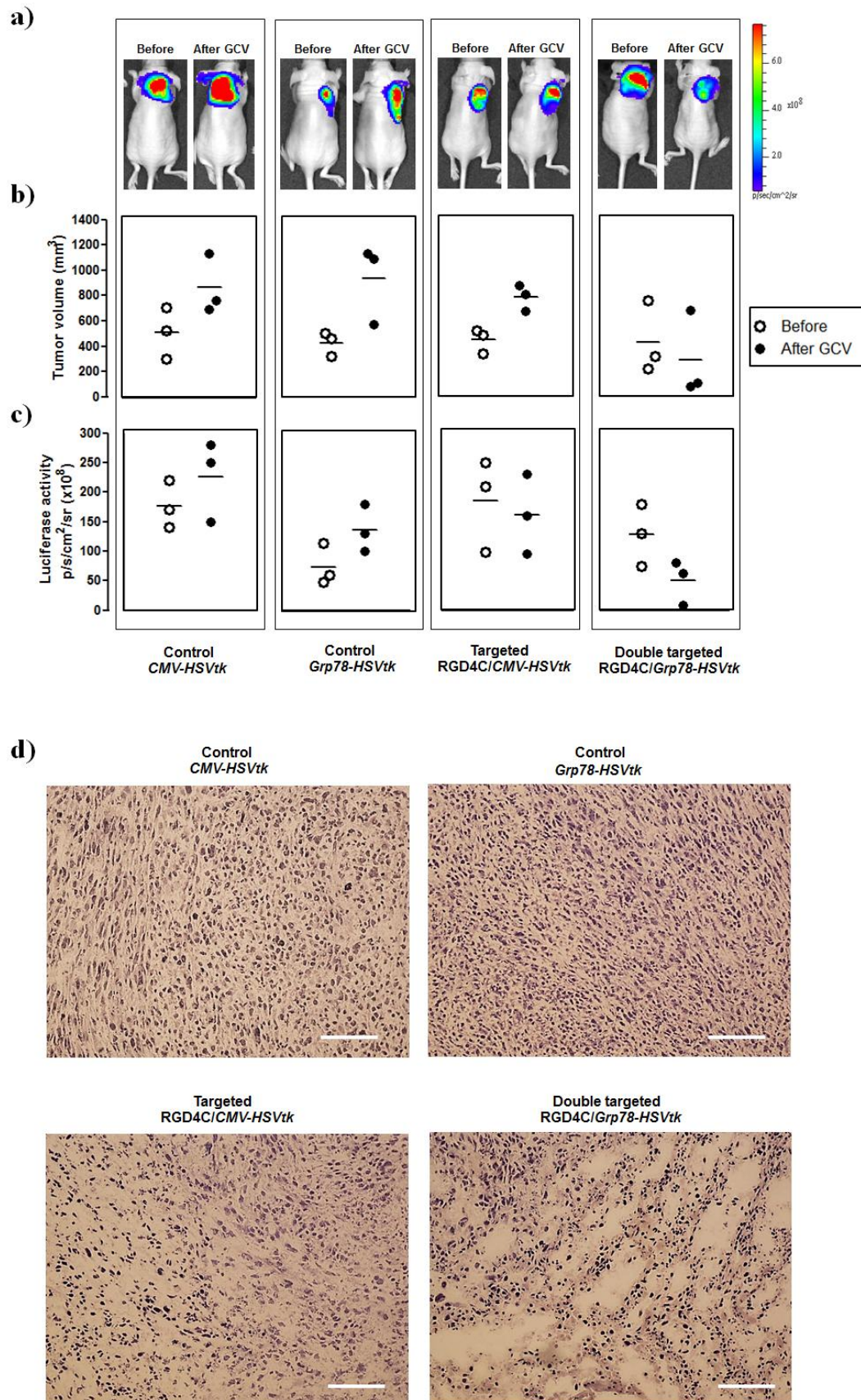


Figure 6.7. Regression of large-size 9L tumours in mice by a single intravenous dose of RGD4C AAVP/*Grp78*-HSVtk plus GCV. **a)** Representative tumour-bearing mice from all the experimental groups before and after GCV therapy. Immunodeficient nude mice were subcutaneously implanted with 9L cells stably expressing the *Luc* transgene reporter under the *Grp78* promoter to establish tumours. Next, mice bearing size-matched (~400-450 mm³) 9L xenografts received an intravenous dose of either RGD4C AAVP/*Grp78*-HSVtk or RGD4C AAVP/*CMV*-HSVtk, or control insertless vectors (5×10^{10} TU). GCV was administered to mice at day 3 post-vector delivery and continued for 4 days. *Luc* transgene expression was monitored before and after GCV treatment. **b)** Individual tumour volumes are shown. **c)** Tumour viability before and after GCV treatment as measured by BLI of tumour luciferase activity before (open circles) and after (filled circles) GCV treatment. **d)** Hematoxylin and eosin (H&E) staining of representative tumour sections from all the experimental groups (scale bar = 100µm).

6.6 Discussion

As discussed before, the original vector RGD4C AAVP/*CMV* has been previously administered for cancer gene therapy and proved efficient to express therapeutic genes within the tumour models such as Kaposi's sarcoma, bladder carcinomas, prostate carcinomas and soft tissue sarcoma and more interestingly resulted in marked suppression of tumour growth in mice and dogs (Hajitou et al., 2006b, Hajitou et al., 2008), (Tandle et al., 2009, Paoloni et al., 2009).

Indeed while the use of a viral *CMV* promoter is the preferred choice to drive transgene expression, its usage in some tissues proved to be problematic due to strong silencing of transgenes. In mouse tissues, the *CMV* promoter exhibits different expression pattern, while it is active in testis, neurons and some other known tissues (Fritschy et al., 1996, Schmidt et al., 1990, van den Pol and Ghosh, 1998), the *in vivo* silencing of this promoter has been reported by several studies (Choi et al., 2005, Meier, 2001, Grassi et al., 2003, Mehta et al., 2009, Brooks et al., 2004).

In fact, we showed in chapter 4, that 9L and U87 cells stably transduced by RGD4C AAVP/*CMV* demonstrated a dramatic decrease in transgene expression over time while in contrast both cells stably transduced by RGD4C AAVP/*Grp78* demonstrated either stable or increasing transgene expression in the long-term. This superiority of *Grp78* over the viral *CMV* promoter was achieved by monitoring the GFP reporter gene, and Western blot analysis of *HSVtk*.

In this chapter as a proof of principle our first aim was to elucidate both promoter activity in the context of AAVP *in vivo* over time. Interestingly, consistent with our previous *in vitro* studies, serial *in vivo* BLI of *Luc* in mice implanted with 9L cells stably transduced with targeted RGD4C AAVP/*CMV-Luc-puro*^R vector demonstrated loss of the *CMV* viral promoter activity over-time. In contrast to the *CMV* promoter, *Grp78* promoter activity within the stably transduced 9L tumours with RGD4C AAVP/*Grp78-Luc-puro*^R increase over time as the tumour size increased.

After validating the silencing of the *CMV* promoter in the context of AAVP in glioblastoma *in vivo* and proving the strong activity of *Grp78* promoter within the large tumours over time, our next aim was to determine the efficacy and safety of the RGD4C AAVP/*Grp78* vector after systemic IV administration.

Interestingly in mice bearing 9L tumours, transgene expression by RGD4C AAVP/*Grp78 - Luc* not only started at about 2 days earlier than the original vector, but also lasted longer than that. The drop of *Luc* expression was observed in both RGD4C AAVP/*CMV-Luc* and, at a later time point, with RGD4C AAVP/*Grp78-Luc*, which might be due to dilution effects of the proliferating transduced cells within a larger population of proliferating non-transduced cells (Hajitou et al., 2008).

In contrast to 9L tumour xenografts, no luciferase signal was detected within the U87 tumours after systemic administration of either vector. Although *Grp78* is reported to be highly expressed in glioma (Lee et al., 2008), its expression shows a positive association with tumour growth rate, with rapidly growing tumours expressing more *Grp78* than those that proliferate more slowly (Pfaffenbach and Lee, 2011).

Indeed, the higher endogenous *Grp78* levels in the 9L cells, which are a more aggressive cell line and has a greater proliferation rate than U87 has been previously demonstrated. Moreover, spliced XBP1 predominates in 9L, suggesting chronic ER stress and activation of the UPR. In the U87 cell line, however, spliced XBP1 levels were undetectable. These data imply that U87 cells undergo less ER stress relative to 9L cells and this fact might explain the lack of detection of luciferase within the U87-derived tumours.

In addition to stability and to higher transgene expression by the double-targeted vector within 9L xenografts or even no detectable transgene expression within U87 xenografts, this vector proved safer than the original vector as luciferase expression was solely detectable within the tumour tissue while in mice administered with targeted RGD4C AAVP/*CMV-Luc*

vector apart from the tumour tissue, luciferase expression was observed in mice paws and at the site of vector injection. This effect was observed in both 9L and U87 tumour-bearing mice following IV administration of the RGD4C AAVP/*CMV-Luc* vector.

Indeed, while $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are barely detectable in quiescent vessels, their upregulation during wound -induced angiogenesis has been previously reported (Max et al., 1997, Sheppard, 1996, Hodivala-Dilke et al., 2003, Christofidou-Solomidou et al., 1997). In addition to injury, inflammation can also play an important role in altering the local concentration of integrins (Sheppard, 1996). Thus RGD4C AAVP vectors can mediate transduction of injured endothelial cells and subsequently the constitutive *CMV* promoter can mediate transgene expression within these cells, while the inducible *Grp78* promoter can tightly control the expression of the transgene within the tumour tissues as this promoter is quiescent in non-tumour cells.

After confirming the efficacy and safety of the double-targeted vector to deliver transgene expression in tumours after systemic vector administration, we subsequently aimed to evaluate therapeutic effects of the double-targeted vector carrying *HSVtk* in preclinical models of glioblastoma. Interestingly, a single IV administration of the RGD4C AAVP/*Grp78-HSVtk* or RGD4C AAVP/*CMV-HSVtk* vector in mice bearing small size 9L or U87 tumours ($\sim 100 \text{ mm}^3$) and daily prodrug GCV treatment resulted in similar tumour suppression at early time points. Although *Grp78* promoter activity was shown to start earlier than that of the *CMV* promoter in 9L tumours (Fig. 6.2), it was expected to obtain a faster and better antitumour activity by the double-targeted vector; however, the comparable antitumour effects of both vectors at early time points after GCV treatment might be due to the bystander effect of the *HSVtk/GCV* as the *CMV* promoter could increase its activity to reach *Grp78* level.

Next at a later time point, when the tumour was growing back, once again the RGD4C AAVP/*Grp78-HSVtk* vector resulted in marked tumour suppression; while, the RGD4C AAVP/*CMV-HSVtk* had no such effect.

This data is consistent with our previous results of silencing of the *CMV* promoter which may explain why the original vector had no effect on tumour suppression at a late time point.

Moreover, previous studies have shown that, unlike most of the popular constitutive promoters utilised in gene therapy vectors such as *LTR* or *CMV* promoters, which are active in small size tumour and lose their potency when the tumours grow large, inducible *Grp78* promoter activity elevates as tumour size increases (Dong et al., 2004, Chen et al., 2000). The

above data establish the potential of the *Grp78* promoter in the context of AAVP for systemic treatment of aggressive tumours.

Next, we aimed to monitor the *Grp78* inducible promoter activity using a non-invasive imaging procedure prior to GCV treatment with the prospect to control GCV treatment and to predict response to therapy; thus, stably transduced 9L cells with RGD4C AAVP/*Grp78-Luc-puro*^R were implanted in nude mice. Single IV administration of RGD4C AAVP/*Grp78-HSVtk* in mice bearing large size tumours (~400-450 mm³) and 4 days of GCV treatment, initiated after *Grp78* promoter activity within the tumours, resulted in significant tumour regression. Also luciferase quantification of tumours administered with the RGD4C AAVP/*CMV-HSVtk* vector showed some reduction in tumour viability after GCV treatment; however, this amount of tumour cell killing was not sufficient to suppress tumour growth.

In summary, here for the first time we reported a unique and superior antitumour effect of the double-targeted vector over the original vector after systemic vector administration into mice bearing therapy resistant as well as large glioblastoma tumours. These effects from the double-targeted vector might be explained by: (i) the tumour environment in large aggressive tumours, such as insufficient blood supply, an acidic pH, glucose deprivation and anoxia which will induce the *Grp78* promoter activity, and (ii) our previous findings that *HSVtk*/GCV treatment stimulates the UPR stress pathway leading to increased *Grp78* activity.

In addition to the advantage of the *Grp78* promoter to drive and control the expression of the therapeutic gene *HSVtk*, we found that the dual targeting design mediates selective and long-term transgene expression within the tumour tissues while leaving normal tissues virtually safe.

In conclusion, the above *in vivo* studies demonstrate the effectiveness of the double-targeted RGD4C AAVP/*Grp78* vector for systemic targeted gene therapy directed against large malignant and therapy resistant tumours. More importantly, our studies show the potential for this novel double-targeted vector in human cancer gene therapy.

Chapter 7

Chemotherapeutic drugs induce Grp78 promoter activity in the context of the double-targeted AAVP vector

7.1 Introduction

Conventional cytotoxic therapies, such as radiation and chemotherapy, are currently the methods of choice for cancer treatment. However, both therapies have had little success, significantly against solid and metastatic cancers and are often highly toxic with a wide variety of severe side effects. For glioblastoma tumour treatment, chemotherapy has been extensively used for recurrent glioma after surgery and radiotherapy. Unfortunately glioblastoma is greatly resistant to the effects of chemotherapy and thus, its treatment remains a major challenge. This effect is not limited to glioblastoma which is the most frequent and aggressive brain tumour, but many other cancers have shown chemoresistance. Although chemotherapeutic drugs must effectively work on fast growing tumours as these agents mediate cell death by impairing cell division, fast growing malignant gliomas confer resistance to apoptosis after chemotherapy treatment.

Several studies using human cancer cells have demonstrated a correlation between Grp78 overexpression and chemoresistance (Wey et al., 2012, Lee et al., 2011, Al-Rawashdeh et al., 2010, Lee, 2007). In fact anti-cancer drugs that target and eradicate the tumour vasculature are intended to starve the tumour of oxygen and nutrients. However, these drugs consequently induce the UPR, and thus *Grp78* expression, which aids the tumour cells to become resistant to these drug (Lee, 2007). Upon consideration of this knowledge, it is clear

that *Grp78* induction is one of the mechanisms by which cells counteract the cytotoxicity of chemotherapeutic drugs.

Previous studies have shown that malignant glioma tumour vasculature develop chemoresistance through upregulation of *Grp78* (Virrey et al., 2008). More interestingly, the authors have shown that upregulation of *Grp78* in normal endothelial cells is also capable of rendering them chemotherapy-resistant (Virrey et al., 2008). Knockdown of *Grp78* or blockage of the ATPase domain of *Grp78* with (-)-epigallocatechin gallate was able to increase chemosensitivity of these endothelial cells (Virrey et al., 2008). The *Grp78* level has also been used as a marker to predict breast cancer patient response to chemotherapy, with *Grp78*-positive cancers having a decreased sensitivity to the drugs and an increased risk of recurrence (Lee et al., 2006).

Amongst many clinically used chemotherapeutic drugs, the effects of cisplatin and temozolomide on UPR activation and thus *Grp78* induction have been demonstrated by numerous studies (Jiang et al., 2009, Lin et al., 2011, Virrey et al., 2008, Pyrko et al., 2007).

7.1.1 Cisplatin

Cisplatin is a widely used chemotherapeutic drug to treat various types of cancers such as testicular, ovarian, bladder, cervical, head and neck, and small lung cancer cells (Cepeda et al., 2007, Boulikas and Vougiouka, 2004).

This agent is known to induce apoptosis by causing DNA damage. As an electrophile in aqueous solution, it reacts with nucleophilic DNA, RNA, and protein sites to form adducts. By binding to genomic DNA, cisplatin can inhibit DNA replication and/or transcription which subsequently lead to cancer cell death (Gonzalez et al., 2001, Cepeda et al., 2007).

Despite the success of cisplatin in treatment of some cancer cells, others such as melanoma cells are resistant to cisplatin. Interestingly, similar to glioblastoma cells, constitutive expression of spliced XBP1 and high level of *Grp78* have been reported in melanoma cells, indicating constant UPR activation (Jiang et al., 2009).

In addition to the fact that the level of endogenous *Grp78* is constantly elevated within melanoma cells, incubation of these cells with cisplatin has been shown to induce the expression of this chaperon protein. Indeed incubation of melanoma cells with cisplatin resulted in 2.6- fold increase in *Grp78* protein expression (Mandic et al., 2003). In addition to the above reports, a dramatic increase in the *Grp78* expression following cisplatin treatment has been shown in other tumour cells such as hepatocellular carcinoma (Chen et al., 2011).

Cisplatin has been shown to mediate ER stress in many cell types and induce the activation of the *Grp78* promoter through the generation of reactive oxygen species (Miyajima et al., 1999, Benhar et al., 2001, Adler et al., 1999).

The activation of UPR and thus expression of *Grp78* have been reported to be involved in resistance of tumour cells to cisplatin. In glioblastoma cells, *Grp78* overexpression has been reported to protect tumour cells from cisplatin-induced apoptosis; however, silencing of *Grp78* could significantly increase cell sensitivity to cisplatin treatment (Lee et al., 2008). Also, it has been reported that *Grp78* can promote tumour cell resistance to cisplatin by inhibiting the caspases 4 and 7 (Jiang et al., 2009). Moreover, *Grp78* was shown to be required for downstream phosphorylation of the serine/threonine protein kinase Akt, which is required for cisplatin resistance in lung cancer cells (Lin et al., 2011).

7.1.2 Temozolomide

Temozolomide is a DNA-methylating chemotherapeutic drug, which by deposition of methyl groups on DNA guanine bases can induce formation of nicks in the DNA and activate the mismatch repair mechanisms. Recognition of recurring mismatches by cells, leads to programmed cell death (Nagasubramanian and Dolan, 2003, Wesolowski et al., 2010).

Due to its small size (194 Da), temozolomide can easily penetrate and pass the blood brain barrier. Thus temozolomide monotherapy or in combination with other chemotherapeutic drugs such as cisplatin have been widely used for glioblastoma multiforms treatment (Villano et al., 2009, Friedman et al., 2000, Zustovich et al., 2009).

Previous research has shown that treatment of glioblastoma tumour cells with temozolomide induces *Grp78* expression (Pyrko et al., 2007). Moreover they have shown that knockdown of *Grp78* can sensitize cells to temozolomide. These data suggest that, much like cisplatin, temozolomide induces ER stress and thus its cytotoxic activity is antagonized in tumour cells by *Grp78*-regulated chemoresistance mechanisms.

As both Cisplatin and temozolomide chemotherapy agents have been previously shown to induce *Grp78* promoter activity and also used for glioblastoma tumour treatment, we aimed to administer cisplatin and temozolomide as two chemotherapy drug candidates, to glioblastoma cells *in vitro* and assess their effects on *Grp78* activity both at endogenous and RGD4C AAVP/*Grp78* vector level.

It is noteworthy to mention that the work presented in this Chapter was undertaken with the assistance of Miss Nastasia Nianiaris, MSc student in Molecular Medicine at Imperial College.

7.2 Results

7.2.1 Chemotherapeutic drug Cisplatin treatment of glioblastoma cells induces Grp78 and boosts transgene expression mediated by RGD4C AAVP/Grp78

Numerous recent studies demonstrate an increase in Grp78 level in tumour cells following cisplatin treatment, implicating cisplatin as an ER stress inducer (Lin et al., 2011, Jiang et al., 2009, Mandic et al., 2003). To further assess the effect of cisplatin on the *Grp78* promoter in the context of the AAVP vector, 9L cells stably transduced with RGD4C AAVP/*Grp78*-*HSVtk* as well as control 9L cells stably transduced by RGD4C AAVP/*CMV*-*HSVtk* were treated with increasing cisplatin concentrations (10-75 μ M) for 12 hours. Subsequently to analyse the expression level of the *HSVtk* gene controlled by either the *CMV* or *Grp78* promoters or of the endogenous Grp78, equal amounts of protein sample were subjected to Western blot analysis (Fig. 7.1a and 7.1b). Figures 7.1a, 7.1b and 7.1c show that a dose dependent increase in endogenous Grp78 expression resulted from both 9L populations. This result is in agreement with previous studies demonstrating *Grp78* induction upon cisplatin treatment (Lee et al., 2008).

Moreover quantification of the *HSVtk* band intensity and normalization to control GAPDH, revealed a dose dependent increase in *HSVtk* expression driven by the *Grp78* promoter (Fig. 7.1d), while in cells stably transduced with RGD4C AAVP/*CMV*-*HSVtk-puro*^R, cisplatin treatment resulted in a slight increase in *HSVtk* expression. However, this increase generally remained below the 2- fold mark from the control (0 μ M cisplatin). *HSVtk* expression in RGD4C AAVP/*Grp78*-*HSVtk-puro*^R stably transduced cells appeared to exponentially increase with drug concentration, even surpassing a 4- fold increase in one instance. The average increase of *HSVtk* expression driven by the *Grp78* promoter at 75 μ M was approximately 3- fold from the control.

The above data indicate that cisplatin can induce both promoters of endogenous *Grp78* and the RGD4C AAVP/*Grp78* vector in 9L cells.

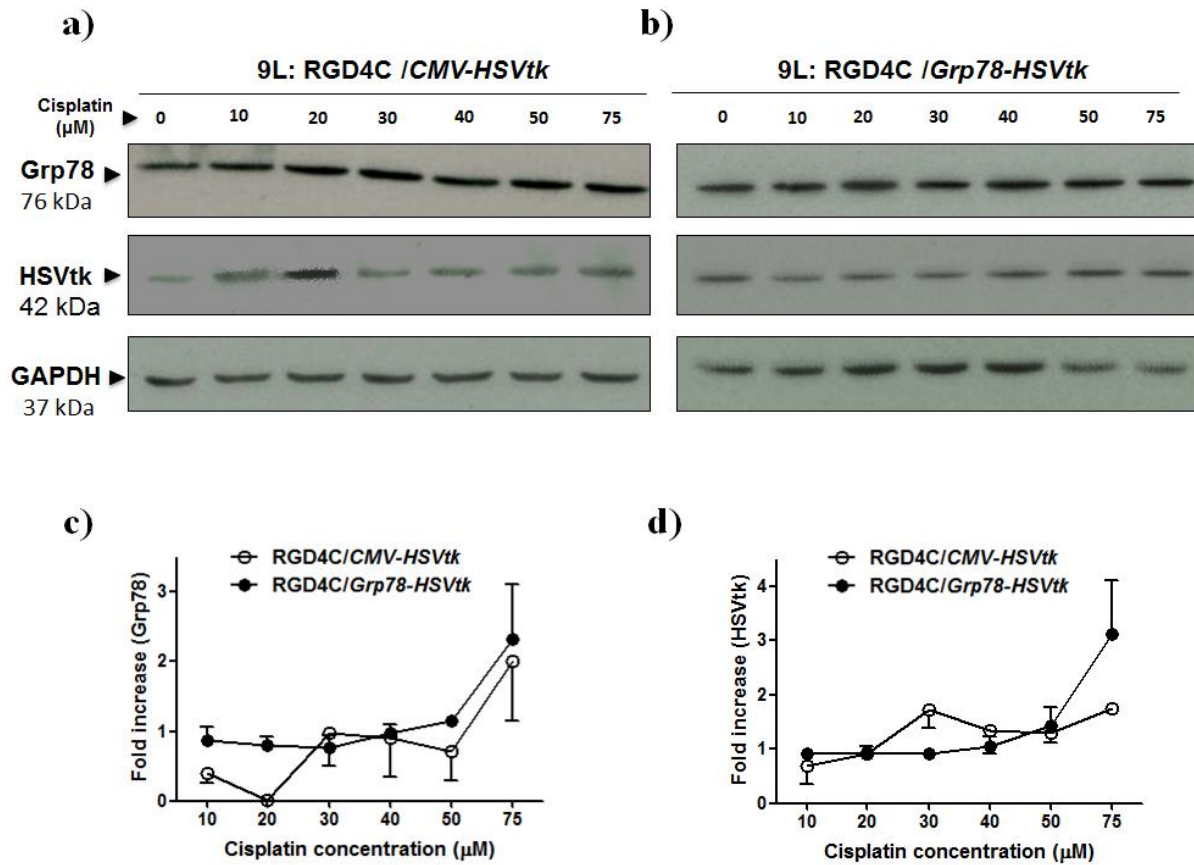


Figure 7.1. Chemotherapeutic drug cisplatin induces *Grp78* promoter activity in 9L glioblastoma cells. Stably transduced 9L cells with **a)** RGD4C AAVP/*CMV-HSVtk-puro*^R or **b)** RGD4C AAVP/*Grp78-HSVtk-puro*^R were grown in the presence of increasing doses of cisplatin for 12 hours. Then equal amount of cell lysate were subjected to Western blot and analysed with antibodies against Grp78, HSVtk and GAPDH as control. **c)** Endogenous Grp78 and **d)** HSVtk levels were measured and normalized to GAPDH. Fold increase of both Grp78 and HSVtk over control untreated cells (0 μ M cisplatin) was calculated and graphed (n=3).

7.2.2 Temozolomide treatment of glioblastoma cells induces *Grp78* and boosts transgene expression mediated by RGD4C AAVP/*Grp78*

Temozolomide is an effective and widely used drug for glioblastoma treatment and its effect to induce the *Grp78* promoter has been previously reported (Pyrko et al., 2007, Friedman et al., 2000, Stupp et al., 2005). Herein, we further aimed to assess the *Grp78* promoter activity in the context of the double-targeted AAVP vector after temozolomide treatment *in vitro*. Thus Western blot analysis of 9L cells stably transduced with RGD4C AAVP/*Grp78*-HSVtk or RGD4C AAVP/*CMV*-HSVtk following treatment with various doses of temozolomide was performed to evaluate both endogenous *Grp78* and transgene *HSVtk* expression.

Figure 7.2 shows that, endogenous *Grp78* expression increases in 9L cells stably transduced by RGD4C AAVP/*Grp78*-HSVtk following temozolomide treatment. However, the results demonstrated a slight increase in endogenous *Grp78* in RGD4C AAVP/*CMV*-HSVtk stably transduced 9L cells following temozolomide treatment, which then returned to basal control levels between 200 μ M and 250 μ M temozolomide (Fig 7.2c).

As expected, no effect of temozolomide was observed on HSVtk expression in cells stably transduced with RGD4C AAVP/*CMV*-HSVtk. In contrast, following temozolomide drug treatment, HSVtk expression driven by the *Grp78* promoter increased exponentially in a dose dependent manner. The induction of *HSVtk* expression under the control of the *Grp78* promoter became evident at 150 μ M of temozolomide and further intensified and reached maximum level at 250 μ M.

Our results are in agreement with previous studies indicating the role of temozolomide in inducing ER stress (Pyrko et al., 2007), yet this is the first report showing that the chemotherapeutic drug temozolomide can mediate *Grp78* promoter induction in the context of the RGD4C AAVP/*Grp78* vector in 9L glioblastoma cells.

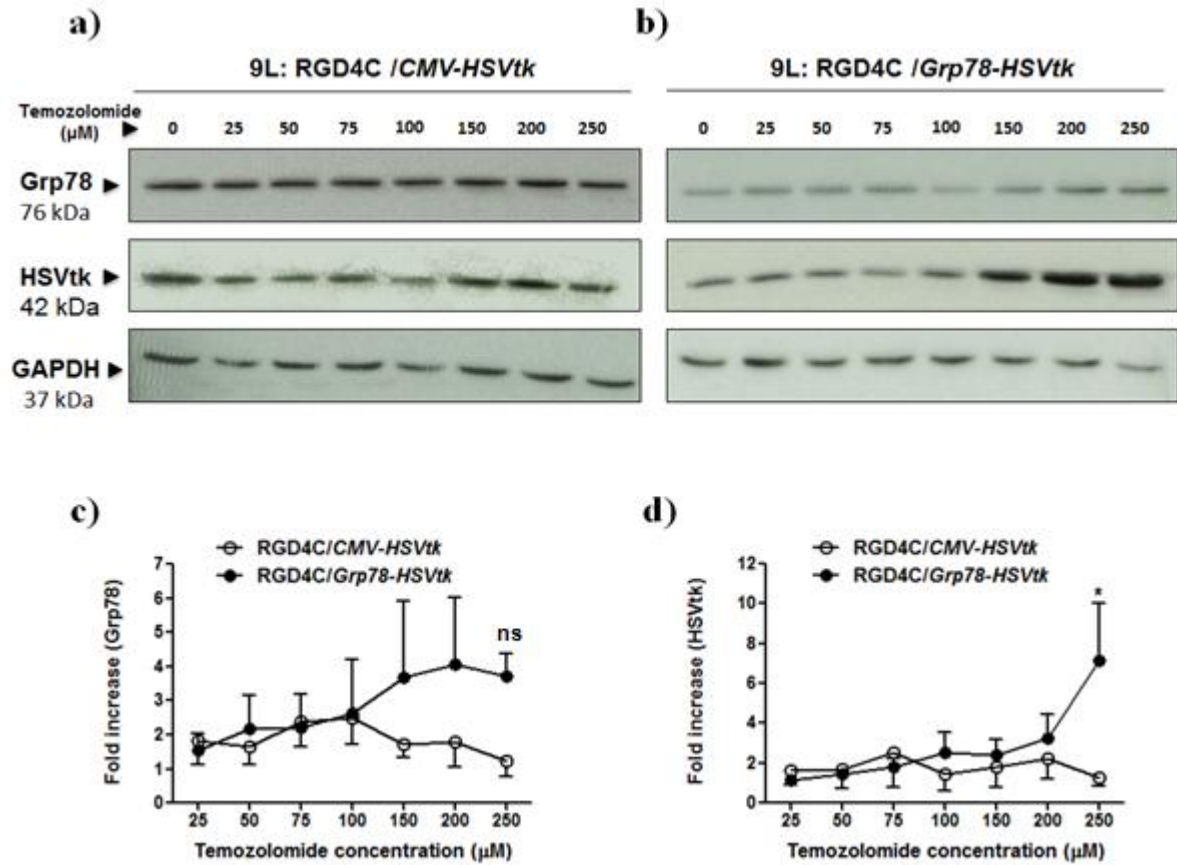


Figure 7.2. The chemotherapeutic drug temozolomide induces *Grp78* promoter activity in 9L glioblastoma cells. Stably transduced 9L cells with **a)** RGD4C AAVP/*CMV-HSVtk-puro*^R or **b)** RGD4C AAVP/*Grp78-HSVtk-puro*^R were grown in the presence of increasing doses of temozolomide for 12 hours. Then equal amount of cell lysate were analysed by Western blot with antibodies to Grp78, HSVtk and GAPDH. **c)** Endogenous Grp78 and **d)** HSVtk levels were measured and normalized to GAPDH. Fold increase of both Grp78 and HSVtk over control untreated cells (0μM cisplatin) was calculated and graphed (n=3). *p < 0.05 and ns: non-significant.

7.3 Discussion

Resistance of some cancers, in particular glioblastoma multiform, to chemotherapeutic drugs is a major obstacle to effective treatment (Giles, 1995). It has been previously shown that overexpression of Grp78 within tumour cells after treatment with chemotherapeutic agents can increase tumour resistance against chemotherapy. Induction of UPR activation and Grp78 expression in a variety of human tumour cells and tumour vasculature upon treatment with chemotherapeutic drugs such as cisplatin and temozolomide, have been reported (Jiang et al., 2009, Lin et al., 2011, Pyrko et al., 2007, Mandic et al., 2003). Herein, using glioblastoma cells, we attempted to further assess the effect of the above chemotherapy agents on *Grp78* promoter activity to drive the *HSVtk* therapeutic gene in the context of the double-targeted vector.

Consistent with previous reports, endogenous Grp78 expression increased *in vitro* in 9L cells upon treatment with either cisplatin or temozolomide indicating that both drugs are UPR inducers. Next, we assessed the *HSVtk* expression by the *Grp78* promoter after treating 9L cells with either drug. The results revealed dose-dependent induction of the *Grp78* promoter following treatment with temozolomide and cisplatin; with temozolomide showing the highest effect on the *Grp78* promoter activity. Note that statistical analysis of Western blots showed some variability in the fold increase of *HSVtk* expression driven by the *Grp78* promoter, which might be due to natural heterogeneity of the *Grp78* promoter activity.

In summary both cisplatin and temozolomide induce activation of *Grp78* at both endogenous and vector level in 9L cells *in vitro*.

In contrast to the *Grp78* promoter, *CMV* activity was not induced upon temozolomide treatment as the transgene expression under the control of *CMV* stayed at the same level or even decreased through treatment. However following treatment with cisplatin a slight increase in *HSVtk* expression driven by the *CMV* promoter was detected. This result is in agreement with a previous study showing the induction of the *CMV* promoter activity both *in vitro* and *in vivo* after cisplatin treatment (Kamensek et al., 2011).

Although Cisplatin and temozolomide have been shown to induce *Grp78* in 9L cells, more studies are needed to find out whether this observation is applicable to other glioma cells as well.

Moreover, further *in vivo* studies will be necessary to confirm the induction of the *Grp78* promoter in the context of the AAVP vector *in vivo* using glioblastoma xenografts following chemotherapy treatment.

In previous studies, systemic administration of the original vector RGD4C AAVP/*CMV-HSVtk* in mice bearing tumours inhibited the tumour growth in mice after GCV treatment; however, the viable outer tumour rim was intact following completion of therapy (Hajitou, 2010, Hajitou et al., 2008). Moreover, it is of primary interest to accelerate suicide gene therapy to reduce the time duration of human exposure to potentially harmful drugs, such as GCV. As chemotherapeutic drugs cisplatin and temozolomide can mediate the induction of *Grp78* activity within the AAVP vector and thus a combination therapy regiment, using AAVP vector carrying the *HSVtk* and GCV treatment in addition to chemotherapy, has the potential to address both issues mentioned above.

Based on our obtained data in this part of the study we can hypothesize that administration of low-doses of chemotherapy drugs in combination with the AAVP/*Grp78-HSVtk* vector might prove efficient to enhance tumour cell death following GCV treatment. The administration of low dose of chemotherapeutics will be both cost effective and will lower the side effects associated with the treatment (Giaccone, 2000).

Chapter 8

Discussion and Conclusions

For successful cancer gene therapy, the designed vector must be able to deliver and mediate sustained expression of the desired therapeutic gene within the tumour after systemic administration. In this thesis I showed that the double-targeted RGD4C AAVP/*Grp78* vector is a promising gene therapy tool that implements ligand-directed and transcriptional targeting into a single particle for effective cancer treatment.

The results of this project for the first time demonstrate the efficacy of systemic gene therapy with the *Grp78* promoter and show that the double-targeted RGD4C AAVP/*Grp78* vector is superior to the original targeted RGD4C AAVP/*CMV* vector in providing striking persistence of gene expression in tumour cells. Furthermore, a clear advantage of the *HSVtk* and GCV suicide gene therapy of cancer was observed by the double-targeted RGD4C AAVP/*Grp78* vector in which this effect was shown to persist long-term, while in contrast silencing of the *CMV* promoter in the context of AAVP was detected.

The *in vitro* long-term transgene expression mediated by the double-targeted RGD4C AAVP/*Grp78* vector was demonstrated using both FACS analysis of the GFP reporter transgene expression and Western blot analysis of *HSVtk* showing persistence of gene expression by *Grp78* in glioblastoma cells. In contrast, silencing of the *CMV* promoter was observed. In addition to the *in vitro* studies, silencing of the *CMV* promoter was spotted *in vivo* by serial imaging of the *Luc* expression under the control of the *CMV* promoter. Yet this is not the first report demonstrating the silencing of the *CMV* promoter *in vitro* and *in vivo* by mammalian host cells (Grassi et al., 2003, Brooks et al., 2004, Prosch et al., 1996, Chen and Townes, 2000).

In the first part of the thesis I showed that the overall activity of the *Grp78* promoter is lower in moderate- slowly growing tumour cells such as MCF-7 and U87 compared to aggressive-

fast growing 9L glioblastoma cells. These data are in agreement with a previous study showing a positive correlation between cell proliferation rate and *Grp78* activity (Pfaffenbach and Lee, 2011). Moreover, we showed that U87 cells are under less ER stress compared to 9L cells which are under constitutive ER stress, and that the proliferation rate of U87 is lower than that of 9L. Although in U87 cells, the primary activity of the *Grp78* promoter in the context of AAVP was lower than the constitutive *CMV* promoter, more cell death induced by RGD4C AAVP/*Grp78*-*HSVtk* than RGD4C AAVP/*CMV*-*HSVtk* was observed following GCV treatment. This phenomenon can be explained by the fact that those U87 cells expressing the *HSVtk* suicide transgene might experience ER stress following GCV treatment. Indeed this is the first report to show that *HSVtk*/GCV suicide gene therapy activates *Grp78* at both the endogenous and vector level, leading to increased *HSVtk* expression and subsequently to enhanced cell killing. Moreover, these data were confirmed by the induction of the UPR stress pathway upon overexpression of the *HSVtk* and GCV treatment which consequently led to *Grp78* induction. In this manner, cells transduced by RGD4C AAVP/*Grp78*-*HSVtk* unintentionally facilitate their own death upon GCV treatment by activating one of their most effective stress-managing mechanisms that lead to the UPR stress pathway, then *Grp78* activation. These data are also consistent with previous work reporting the stronger tumour cell killing efficacy of *HSVtk* under the control of the *Grp78* promoter in comparison to viral promoters (Azatian et al., 2009).

In addition to *in vitro* studies presented in the first part of the thesis, the superiority of *Grp78* to the popular and commonly used *CMV* viral promoter in cancer gene therapy was observed *in vivo*, when both promoters were systemically targeted to tumours.

In vivo systemic administration of the double-targeted RGD4C AAVP/*Grp78*-*Luc* mediated higher transgene expression with a more stable pattern than the original RGD4C AAVP/*CMV* vector. Moreover the *Grp78* promoter appeared to be selective and active within the tumour tissues in animal models as no transgene expression or related toxicity was detected following systemic administration of the double-targeted RGD4C AAVP/*Grp78*-*Luc* vector, while in addition to tumours, the original vector containing the constitutive *CMV* promoter resulted in transgene expression in animal paws and tail vein at the site of vector injection.

Interestingly comparable antitumour effects were obtained at early time points on small size tumours, which could be explained by the increased expression levels of transgene expression reached by *CMV* within few days and the bystander effect of the *HSVtk*/GCV. On the contrary, the effect of the double-targeted RGD4C AAVP/*Grp78*-*HSVtk* was clearly demonstrated on large 9L tumours. In contrast to small size tumours, the double-targeted

RGD4C AAVP/*Grp78*-*HSVtk* vector proved more efficient than the original targeted RGD4C AAVP/*CMV*-*HSVtk* vector in reducing large size 9L tumours following GCV treatment.

Given that specific activation of the endogenous *Grp78* promoter has been reported in aggressive and poorly perfused malignant tissues, including tumour-associated vasculature and tumour cells themselves (Virrey et al., 2008), the results obtained from the large size tumours are consistent with the literature.

Taken together, there are three explanations for the unique antitumour effect of the double-targeted RGD4C AAVP/*Grp78* on therapy-resistant and recurring tumours. First, being a stress-inducible gene, *Grp78* encodes a potent anti-apoptotic protein that plays a critical role in tumour survival and resistance to therapy. Second, *Grp78* is specifically over-active in aggressive cancers. Third, I found that *HSVtk*/GCV treatment stimulated the UPR stress pathway leading to increased *Grp78* activity.

In the last part of the thesis I have clearly shown the effect of the temozolomide and cisplatin chemotherapeutic drugs on *Grp78* induction in the context of the AAVP vector. Thus a combination therapy using both a double-targeted AAVP vector carrying suicide gene such as *HSVtk* and chemotherapy might accelerate treatment of cancer in particular the aggressive, chemotherapy-resistant tumours such as glioblastomas.

In conclusion I showed that the RGD4C AAVP/*Grp78* can potentially eradicate large malignant and therapy-resistant tumours, after systemic administration, a route applicable for both localised and metastatic tumours. Moreover, dual targeting ensures increased tumour targeting while leaving normal tissues virtually safe. Toxicity to healthy tissues is an ever-present problem with systemic cancer therapies, like chemotherapy.

Our studies prove in principle the efficacy of combining homing ligands and a mammalian tumour specific promoter in the context of bacteriophage in order to generate a novel chimeric vector for targeted systemic gene delivery to cancer. Based on this study, it seems that the translation of the double-targeted RGD4C AAVP/*Grp78* particle may lead to realistic gene therapy with the *Grp78* promoter in cancer patients. Moreover, and given that any of our ligands could also target angiogenic vasculature in addition to tumour cells, these advances are likely to extend the potential of disease-specific gene therapy targeting to other applications and human angiogenesis-related diseases. Indeed, tissue-double-targeted particles can also be developed for targeted systemic gene delivery to normal tissues and other human diseases. The *in vivo* phage display technology is thus a powerful tool to be used to isolate and identify phage clones able to target, diffuse and achieve internalization in the

target cells after systemic administration. The selected phage clone displaying a specific ligand can then be directly combined with a tissue specific promoter to generate a double-targeted phage particle for systemic gene delivery.

Other Achievements

Publications:

Paper: **Dual systemic tumor targeting with ligand-directed phage and Grp78 promoter induces tumor regression.** Molecular Cancer Therapy 2012. (A copy of the paper can be found at the end of the thesis).

Poster: **‘Targeted Systemic Gene Therapy Against Glioblastoma with a Dual Tumour Targeted Bacteriophage’** Presented at the British Neuro-Oncology Society (BNOS) International Meeting 2012, Manchester, UK.

Awards: Winer of the award for Best Science at British Neuro-Oncology Society (BNOS) International Meeting 2012, Manchester, UK.

References

- ADLER, V., YIN, Z., TEW, K. D. & RONAI, Z. 1999. Role of redox potential and reactive oxygen species in stress signaling. *Oncogene*, 18, 6104-11.
- AL-RAWASHDEH, F. Y., SCRIVEN, P., CAMERON, I. C., VERGANI, P. V. & WYLD, L. 2010. Unfolded protein response activation contributes to chemoresistance in hepatocellular carcinoma. *Eur J Gastroenterol Hepatol*, 22, 1099-105.
- ARAP, M. A., LAHDENRANTA, J., MINTZ, P. J., HAJITOU, A., SARKIS, A. S., ARAP, W. & PASQUALINI, R. 2004. Cell surface expression of the stress response chaperone GRP78 enables tumor targeting by circulating ligands. *Cancer Cell*, 6, 275-84.
- AZATIAN, A., YU, H., DAI, W., SCHNEIDERS, F. I., BOTELHO, N. K. & LORD, R. V. 2009. Effectiveness of HSV-tk suicide gene therapy driven by the Grp78 stress-inducible promoter in esophagogastric junction and gastric adenocarcinomas. *J Gastrointest Surg*, 13, 1044-51.
- BACK, S. H., SCHEUNER, D., HAN, J., SONG, B., RIBICK, M., WANG, J., GILDERSLEEVE, R. D., PENNATHUR, S. & KAUFMAN, R. J. 2009. Translation attenuation through eIF2alpha phosphorylation prevents oxidative stress and maintains the differentiated state in beta cells. *Cell Metab*, 10, 13-26.
- BAO, R., SELVAKUMARAN, M. & HAMILTON, T. C. 2002. Targeted gene therapy of ovarian cancer using an ovarian-specific promoter. *Gynecol Oncol*, 84, 228-34.
- BARROW, P. A. & SOOTHILL, J. S. 1997. Bacteriophage therapy and prophylaxis: rediscovery and renewed assessment of potential. *Trends Microbiol*, 5, 268-71.
- BAUERSCHMITZ, G. J., NETTELBECK, D. M., KANERVA, A., BAKER, A. H., HEMMINKI, A., REYNOLDS, P. N. & CURIEL, D. T. 2002. The flt-1 promoter for transcriptional targeting of teratocarcinoma. *Cancer Res*, 62, 1271-4.
- BAUMEISTER, P., DONG, D., FU, Y. & LEE, A. S. 2009. Transcriptional induction of GRP78/BiP by histone deacetylase inhibitors and resistance to histone deacetylase inhibitor-induced apoptosis. *Mol Cancer Ther*, 8, 1086-94.
- BAUMEISTER, P., LUO, S., SKARNES, W. C., SUI, G., SETO, E., SHI, Y. & LEE, A. S. 2005. Endoplasmic reticulum stress induction of the Grp78/BiP promoter: activating mechanisms mediated by YY1 and its interactive chromatin modifiers. *Mol Cell Biol*, 25, 4529-40.
- BAYLIN, S. B. 2005. DNA methylation and gene silencing in cancer. *Nat Clin Pract Oncol*, 2 Suppl 1, S4-11.

- BENHAR, M., DALYOT, I., ENGELBERG, D. & LEVITZKI, A. 2001. Enhanced ROS production in oncogenically transformed cells potentiates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase activation and sensitization to genotoxic stress. *Mol Cell Biol*, 21, 6913-26.
- BI, G. & JIANG, G. 2006. The molecular mechanism of HDAC inhibitors in anticancer effects. *Cell Mol Immunol*, 3, 285-90.
- BLANDER, G. & GUARENTE, L. 2004. The Sir2 family of protein deacetylases. *Annu Rev Biochem*, 73, 417-35.
- BOSSLET, K., STRAUB, R., BLUMRICH, M., CZECH, J., GERKEN, M., SPERKER, B., KROEMER, H. K., GESSON, J. P., KOCH, M. & MONNERET, C. 1998. Elucidation of the mechanism enabling tumor selective prodrug monotherapy. *Cancer Res*, 58, 1195-201.
- BOUARD, D., ALAZARD-DANY, D. & COSSET, F. L. 2009. Viral vectors: from virology to transgene expression. *Br J Pharmacol*, 157, 153-65.
- BOULIKAS, T. & VOUGIOUKA, M. 2004. Recent clinical trials using cisplatin, carboplatin and their combination chemotherapy drugs (review). *Oncol Rep*, 11, 559-95.
- BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72, 248-54.
- BRAND, K., LOSER, P., ARNOLD, W., BARTELS, T. & STRAUSS, M. 1998. Tumor cell-specific transgene expression prevents liver toxicity of the adeno-HSVtk/GCV approach. *Gene Ther*, 5, 1363-71.
- BROCCOLI, D., YOUNG, J. W. & DE LANGE, T. 1995. Telomerase activity in normal and malignant hematopoietic cells. *Proc Natl Acad Sci U S A*, 92, 9082-6.
- BROOKS, A. R., HARKINS, R. N., WANG, P., QIAN, H. S., LIU, P. & RUBANYI, G. M. 2004. Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. *J Gene Med*, 6, 395-404.
- BUNING, H., PERABO, L., COUTELLE, O., QUADT-HUMME, S. & HALLEK, M. 2008. Recent developments in adeno-associated virus vector technology. *J Gene Med*, 10, 717-33.
- BURTON, E. R. & LIBUTTI, S. K. 2009. Targeting TNF-alpha for cancer therapy. *J Biol*, 8, 85.
- CALFON, M., ZENG, H., URANO, F., TILL, J. H., HUBBARD, S. R., HARDING, H. P., CLARK, S. G. & RON, D. 2002. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature*, 415, 92-6.

- CAO, G., KURIYAMA, S., GAO, J., KIKUKAWA, M., CUI, L., NAKATANI, T., ZHANG, X., TSUJINOUE, H., PAN, X., FUKUI, H. & QI, Z. 1999. Effective and safe gene therapy for colorectal carcinoma using the cytosine deaminase gene directed by the carcinoembryonic antigen promoter. *Gene Ther*, 6, 83-90.
- CEDAR, H. & BERGMAN, Y. 2009. Linking DNA methylation and histone modification: patterns and paradigms. *Nat Rev Genet*, 10, 295-304.
- CEPEDA, V., FUERTES, M. A., CASTILLA, J., ALONSO, C., QUEVEDO, C. & PEREZ, J. M. 2007. Biochemical mechanisms of cisplatin cytotoxicity. *Anticancer Agents Med Chem*, 7, 3-18.
- CHEN, R., DAI, R. Y., DUAN, C. Y., LIU, Y. P., CHEN, S. K., YAN, D. M., CHEN, C. N., WEI, M. & LI, H. 2011. Unfolded protein response suppresses cisplatin-induced apoptosis via autophagy regulation in human hepatocellular carcinoma cells. *Folia Biol (Praha)*, 57, 87-95.
- CHEN, W. Y., BAILEY, E. C., MCCUNE, S. L., DONG, J. Y. & TOWNES, T. M. 1997. Reactivation of silenced, virally transduced genes by inhibitors of histone deacetylase. *Proc Natl Acad Sci U S A*, 94, 5798-803.
- CHEN, W. Y. & TOWNES, T. M. 2000. Molecular mechanism for silencing virally transduced genes involves histone deacetylation and chromatin condensation. *Proc Natl Acad Sci U S A*, 97, 377-82.
- CHEN, X., ZHANG, D., DENNERT, G., HUNG, G. & LEE, A. S. 2000. Eradication of murine mammary adenocarcinoma through HSVtk expression directed by the glucose-starvation inducible grp78 promoter. *Breast Cancer Res Treat*, 59, 81-90.
- CHENG, L., ZIEGELHOFFER, P. R. & YANG, N. S. 1993. In vivo promoter activity and transgene expression in mammalian somatic tissues evaluated by using particle bombardment. *Proceedings of the National Academy of Sciences*, 90, 4455-4459.
- CHOI, K. H., BASMA, H., SINGH, J. & CHENG, P. W. 2005. Activation of CMV promoter-controlled glycosyltransferase and beta -galactosidase glycoconjugates by butyrate, trichostatin A, and 5-aza-2'-deoxycytidine. *Glycoconj J*, 22, 63-9.
- CHRISTOFIDOU-SOLOMIDOU, M., BRIDGES, M., MURPHY, G. F., ALBELDA, S. M. & DELISSER, H. M. 1997. Expression and function of endothelial cell alpha v integrin receptors in wound-induced human angiogenesis in human skin/SCID mice chimeras. *Am J Pathol*, 151, 975-83.
- CORREA, T. C., BROHEM, C. A., WINNISCHOFER, S. M., DA SILVA CARDEAL, L. B., SASAHARA, R. M., TABOGA, S. R., SOGAYAR, M. C. & MARIA-ENGLER, S. S. 2006. Downregulation of the RECK-tumor and metastasis suppressor gene in glioma invasiveness. *J Cell Biochem*, 99, 156-67.
- CROSS, D. & BURMESTER, J. K. 2006. Gene therapy for cancer treatment: past, present and future. *Clin Med Res*, 4, 218-27.

- DAS, P. M. & SINGAL, R. 2004. DNA Methylation and Cancer. *Journal of Clinical Oncology*, 22, 4632-4642.
- DE RUIJTER, A. J., VAN GENNIP, A. H., CARON, H. N., KEMP, S. & VAN KUILENBURG, A. B. 2003. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J*, 370, 737-49.
- DENMEADE, S. R. & ISAACS, J. T. 2005. The SERCA pump as a therapeutic target: making a "smart bomb" for prostate cancer. *Cancer Biol Ther*, 4, 14-22.
- DI GIOVINE, M., SALONE, B., MARTINA, Y., AMATI, V., ZAMBRUNO, G., CUNDARI, E., FAILLA, C. M. & SAGGIO, I. 2001. Binding properties, cell delivery, and gene transfer of adenoviral penton base displaying bacteriophage. *Virology*, 282, 102-12.
- DONG, D., DUBEAU, L., BADING, J., NGUYEN, K., LUNA, M., YU, H., GAZIT-BORNSTEIN, G., GORDON, E. M., GOMER, C., HALL, F. L., GAMBHIR, S. S. & LEE, A. S. 2004. Spontaneous and controllable activation of suicide gene expression driven by the stress-inducible grp78 promoter resulting in eradication of sizable human tumors. *Hum Gene Ther*, 15, 553-61.
- DONG, D., NI, M., LI, J., XIONG, S., YE, W., VIRREY, J. J., MAO, C., YE, R., WANG, M., PEN, L., DUBEAU, L., GROSHEN, S., HOFMAN, F. M. & LEE, A. S. 2008. Critical role of the stress chaperone GRP78/BiP in tumor proliferation, survival, and tumor angiogenesis in transgene-induced mammary tumor development. *Cancer Res*, 68, 498-505.
- DONG, D., STAPLETON, C., LUO, B., XIONG, S., YE, W., ZHANG, Y., JHAVERI, N., ZHU, G., YE, R., LIU, Z., BRUHN, K. W., CRAFT, N., GROSHEN, S., HOFMAN, F. M. & LEE, A. S. 2011. A critical role for GRP78/BiP in the tumor microenvironment for neovascularization during tumor growth and metastasis. *Cancer Res*, 71, 2848-57.
- DONSANTE, A., MILLER, D. G., LI, Y., VOGLER, C., BRUNT, E. M., RUSSELL, D. W. & SANDS, M. S. 2007. AAV vector integration sites in mouse hepatocellular carcinoma. *Science*, 317, 477.
- DORNER, A. J., WASLEY, L. C. & KAUFMAN, R. J. 1990. Protein dissociation from GRP78 and secretion are blocked by depletion of cellular ATP levels. *Proc Natl Acad Sci U S A*, 87, 7429-32.
- DUAN, D., SHARMA, P., YANG, J., YUE, Y., DUDUS, L., ZHANG, Y., FISHER, K. J. & ENGELHARDT, J. F. 1998. Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J Virol*, 72, 8568-77.
- ELION, G. B., FURMAN, P. A., FYFE, J. A., DE MIRANDA, P., BEAUCHAMP, L. & SCHAEFFER, H. J. 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl) guanine. *Proc Natl Acad Sci U S A*, 74, 5716-20.

- FECKER, L. F., RUCKERT, S., KURBANOV, B. M., SCHMUDE, M., STOCKFLETH, E., FECHNER, H. & EBERLE, J. 2011. Efficient melanoma cell killing and reduced melanoma growth in mice by a selective replicating adenovirus armed with tumor necrosis factor-related apoptosis-inducing ligand. *Hum Gene Ther*, 22, 405-17.
- FELDMAN, D. E., CHAUHAN, V. & KOONG, A. C. 2005. The unfolded protein response: a novel component of the hypoxic stress response in tumors. *Mol Cancer Res*, 3, 597-605.
- FERNANDEZ, P. M., TABBARA, S. O., JACOBS, L. K., MANNING, F. C., TSANGARIS, T. N., SCHWARTZ, A. M., KENNEDY, K. A. & PATIERNO, S. R. 2000. Overexpression of the glucose-regulated stress gene GRP78 in malignant but not benign human breast lesions. *Breast Cancer Res Treat*, 59, 15-26.
- FOLKMAN, J. 1997. Addressing tumor blood vessels. *Nat Biotechnol*, 15, 510.
- FREEMAN, S. M., ABBOUD, C. N., WHARTENBY, K. A., PACKMAN, C. H., KOEPLIN, D. S., MOOLTEN, F. L. & ABRAHAM, G. N. 1993. The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res*, 53, 5274-83.
- FRIEDMAN, H. S., KERBY, T. & CALVERT, H. 2000. Temozolomide and treatment of malignant glioma. *Clin Cancer Res*, 6, 2585-97.
- FRIEDMANN, T. 1992. A brief history of gene therapy. *Nat Genet*, 2, 93-8.
- FRITSCHY, J. M., BRANDNER, S., AGUZZI, A., KOEDOOD, M., LUSCHER, B. & MITCHELL, P. J. 1996. Brain cell type specificity and gliosis-induced activation of the human cytomegalovirus immediate-early promoter in transgenic mice. *J Neurosci*, 16, 2275-82.
- FU, Y. & LEE, A. S. 2006. Glucose regulated proteins in cancer progression, drug resistance and immunotherapy. *Cancer Biol Ther*, 5, 741-4.
- GADDAMEEDHI, S. & CHATTERJEE, S. 2009. Association between the unfolded protein response, induced by 2-deoxyglucose, and hypersensitivity to cisplatin: a mechanistic study employing molecular genomics. *J Cancer Res Ther*, 5 Suppl 1, S61-6.
- GALANIS, E., JAECKLE, K. A., MAURER, M. J., REID, J. M., AMES, M. M., HARDWICK, J. S., REILLY, J. F., LOBODA, A., NEBOZHYN, M., FANTIN, V. R., RICHON, V. M., SCHEITHAUER, B., GIANNINI, C., FLYNN, P. J., MOORE, D. F., JR., ZWIEBEL, J. & BUCKNER, J. C. 2009. Phase II trial of vorinostat in recurrent glioblastoma multiforme: a north central cancer treatment group study. *J Clin Oncol*, 27, 2052-8.

- GARCIA-MANERO, G., YANG, H., BUESO-RAMOS, C., FERRAJOLI, A., CORTES, J., WIERDA, W. G., FADERL, S., KOLLER, C., MORRIS, G., ROSNER, G., LOBODA, A., FANTIN, V. R., RANDOLPH, S. S., HARDWICK, J. S., REILLY, J. F., CHEN, C., RICKER, J. L., SECRIST, J. P., RICHON, V. M., FRANKEL, S. R. & KANTARJIAN, H. M. 2008. Phase 1 study of the histone deacetylase inhibitor vorinostat (suberoylanilide hydroxamic acid [SAHA]) in patients with advanced leukemias and myelodysplastic syndromes. *Blood*, 111, 1060-6.
- GEIER, M. R., TRIGG, M. E. & MERRIL, C. R. 1973. Fate of bacteriophage lambda in non-immune germ-free mice. *Nature*, 246, 221-3.
- GEOVANI TJUVAJEV, J. & BLASBERG, R. G. 2003. In vivo imaging of molecular-genetic targets for cancer therapy. *Cancer Cell*, 3, 327-32.
- GHAEMI, A., SOLEIMANJAH, H., GILL, P., HASSAN, Z., JAHROMI, S. R. & ROOHVAND, F. 2010. Recombinant lambda-phage nanobioparticles for tumor therapy in mice models. *Genet Vaccines Ther*, 8, 3.
- GIACCONE, G. 2000. Clinical perspectives on platinum resistance. *Drugs*, 59 Suppl 4, 9-17; discussion 37-8.
- GILES, G. G. 1995. The epidemiology of central nervous system tumours; trends and risk factors. *J Clin Neurosci*, 2, 191-205.
- GONZALEZ, V. M., FUERTES, M. A., ALONSO, C. & PEREZ, J. M. 2001. Is cisplatin-induced cell death always produced by apoptosis? *Mol Pharmacol*, 59, 657-63.
- GORDON, E. M., CORNELIO, G. H., LORENZO, C. C., 3RD, LEVY, J. P., REED, R. A., LIU, L. & HALL, F. L. 2004. First clinical experience using a 'pathotropic' injectable retroviral vector (Rexin-G) as intervention for stage IV pancreatic cancer. *Int J Oncol*, 24, 177-85.
- GOTOH, A., KO, S. C., SHIRAKAWA, T., CHEON, J., KAO, C., MIYAMOTO, T., GARDNER, T. A., HO, L. J., CLEUTJENS, C. B., TRAPMAN, J., GRAHAM, F. L. & CHUNG, L. W. 1998. Development of prostate-specific antigen promoter-based gene therapy for androgen-independent human prostate cancer. *J Urol*, 160, 220-9.
- GRAM, G. J., NIELSEN, S. D. & HANSEN, J. E. 1998. Spontaneous silencing of humanized green fluorescent protein (hGFP) gene expression from a retroviral vector by DNA methylation. *J Hematother*, 7, 333-41.
- GRANT, S., EASLEY, C. & KIRKPATRICK, P. 2007. Vorinostat. *Nat Rev Drug Discov*, 6, 21-2.
- GRASSI, G., MACCARONI, P., MEYER, R., KAISER, H., D'AMBROSIO, E., PASCALE, E., GRASSI, M., KUHN, A., DI NARDO, P., KANDOLF, R. & KUPPER, J. H. 2003. Inhibitors of DNA methylation and histone deacetylation activate cytomegalovirus promoter-controlled reporter gene expression in human glioblastoma cell line U87. *Carcinogenesis*, 24, 1625-35.

- GREEN, N. K. & SEYMOUR, L. W. 2002. Adenoviral vectors: systemic delivery and tumor targeting. *Cancer Gene Ther*, 9, 1036-42.
- GROSS, S. & PIWNICA-WORMS, D. 2005. Monitoring proteasome activity in cellulo and in living animals by bioluminescent imaging: technical considerations for design and use of genetically encoded reporters. *Methods Enzymol*, 399, 512-30.
- HAJITOU, A. 2010. Targeted Systemic Gene Therapy and Molecular Imaging of Cancer: Contribution of the Vascular-Targeted AAVP Vector. In: RENATA, P. (ed.) *Advances in Genetics*. Academic Press.
- HAJITOU, A., LEV, D. C., HANNAY, J. A., KORCHIN, B., STAQUICINI, F. I., SOGHOMONYAN, S., ALAUDDIN, M. M., BENJAMIN, R. S., POLLOCK, R. E., GELOVANI, J. G., PASQUALINI, R. & ARAP, W. 2008. A preclinical model for predicting drug response in soft-tissue sarcoma with targeted AAVP molecular imaging. *Proc Natl Acad Sci U S A*, 105, 4471-6.
- HAJITOU, A., PASQUALINI, R. & ARAP, W. 2006a. Vascular Targeting: Recent Advances and Therapeutic Perspectives. *Trends in Cardiovascular Medicine*, 16, 80-88.
- HAJITOU, A., RANGEL, R., TREPEL, M., SOGHOMONYAN, S., GELOVANI, J. G., ALAUDDIN, M. M., PASQUALINI, R. & ARAP, W. 2007. Design and construction of targeted AAVP vectors for mammalian cell transduction. *Nat Protoc*, 2, 523-31.
- HAJITOU, A., TREPEL, M., LILLEY, C. E., SOGHOMONYAN, S., ALAUDDIN, M. M., MARINI, F. C., 3RD, RESTEL, B. H., OZAWA, M. G., MOYA, C. A., RANGEL, R., SUN, Y., ZAOUI, K., SCHMIDT, M., VON KALLE, C., WEITZMAN, M. D., GELOVANI, J. G., PASQUALINI, R. & ARAP, W. 2006b. A hybrid vector for ligand-directed tumor targeting and molecular imaging. *Cell*, 125, 385-98.
- HAJITOU, A., TREPEL, M., LILLEY, C. E., SOGHOMONYAN, S., ALAUDDIN, M. M., MARINI, F. C., RESTEL, B. H., OZAWA, M. G., MOYA, C. A., RANGEL, R., SUN, Y., ZAOUI, K., SCHMIDT, M., VON KALLE, C., WEITZMAN, M. D., GELOVANI, J. G., PASQUALINI, R. & ARAP, W. 2006c. A Hybrid Vector for Ligand-Directed Tumor Targeting and Molecular Imaging. *Cell*, 125, 385-398.
- HARDING, H. P., ZHANG, Y., BERTOLOTTI, A., ZENG, H. & RON, D. 2000. Perk is essential for translational regulation and cell survival during the unfolded protein response. *Mol Cell*, 5, 897-904.
- HARRINGTON, K. J., LINARDAKIS, E. & VILE, R. G. 2000. Transcriptional control: an essential component of cancer gene therapy strategies? *Adv Drug Deliv Rev*, 44, 167-84.
- HATEFI, A. & CANINE, B. F. 2009. Perspectives in vector development for systemic cancer gene therapy. *Gene Ther Mol Biol*, 13, 15-19.

- HAZE, K., YOSHIDA, H., YANAGI, H., YURA, T. & MORI, K. 1999. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol Biol Cell*, 10, 3787-99.
- HENDERSHOT, L. M. 2004. The ER function BiP is a master regulator of ER function. *Mt Sinai J Med*, 71, 289-97.
- HERMAN, J. G. & BAYLIN, S. B. 2003. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med*, 349, 2042-54.
- HERZ, K., HEINEMANN, J. C., HESSE, M., OTTERSBAACH, A., GEISEN, C., FUEGEMANN, C. J., ROLL, W., FLEISCHMANN, B. K. & WENZEL, D. 2012. Live monitoring of small vessels during development and disease using the flt-1 promoter element. *Basic Res Cardiol*, 107, 1-14.
- HETZ, C., MARTINON, F., RODRIGUEZ, D. & GLIMCHER, L. H. 2011. The Unfolded Protein Response: Integrating Stress Signals Through the Stress Sensor IRE1{alpha}. *Physiol Rev*, 91, 1219-43.
- HODIVALA-DILKE, K. M., REYNOLDS, A. R. & REYNOLDS, L. E. 2003. Integrins in angiogenesis: multitasking molecules in a balancing act. *Cell Tissue Res*, 314, 131-44.
- HONG, M., LIN, M. Y., HUANG, J. M., BAUMEISTER, P., HAKRE, S., ROY, A. L. & LEE, A. S. 2005. Transcriptional regulation of the Grp78 promoter by endoplasmic reticulum stress: role of TFII-I and its tyrosine phosphorylation. *J Biol Chem*, 280, 16821-8.
- HUANG, G. B., CHEN, Y. Q. & BABRI, H. A. 2000. Classification ability of single hidden layer feedforward neural networks. *IEEE Trans Neural Netw*, 11, 799-801.
- IMAI, S., ARMSTRONG, C. M., KAEBERLEIN, M. & GUARENTE, L. 2000. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature*, 403, 795-800.
- ISHIKAWA, H., NAKATA, K., MAWATARI, F., UEKI, T., TSURUTA, S., IDO, A., NAKAO, K., KATO, Y., ISHII, N. & EGUCHI, K. 1999. Utilization of variant-type of human alpha-fetoprotein promoter in gene therapy targeting for hepatocellular carcinoma. *Gene Ther*, 6, 465-70.
- JAENISCH, R., SCHNIEKE, A. & HARBERS, K. 1985. Treatment of mice with 5-azacytidine efficiently activates silent retroviral genomes in different tissues. *Proc Natl Acad Sci U S A*, 82, 1451-5.
- JAGGAR, R. T., CHAN, H. Y., HARRIS, A. L. & BICKNELL, R. 1997. Endothelial cell-specific expression of tumor necrosis factor-alpha from the KDR or E-selectin promoters following retroviral delivery. *Hum Gene Ther*, 8, 2239-47.
- JEMAL, A., BRAY, F., CENTER, M. M., FERLAY, J., WARD, E. & FORMAN, D. 2011. Global cancer statistics. *CA Cancer J Clin*, 61, 69-90.

- JIANG, C. C., MAO, Z. G., AVERY-KIEJDA, K. A., WADE, M., HERSEY, P. & ZHANG, X. D. 2009. Glucose-regulated protein 78 antagonizes cisplatin and adriamycin in human melanoma cells. *Carcinogenesis*, 30, 197-204.
- JIN, B., LI, Y. & ROBERTSON, K. D. 2011. DNA methylation: superior or subordinate in the epigenetic hierarchy? *Genes Cancer*, 2, 607-17.
- JOHNSTONE, R. W. 2002. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. *Nat Rev Drug Discov*, 1, 287-99.
- KAMENSEK, U., SERSA, G., VIDIC, S., TEVZ, G., KRANJC, S. & CEMAZAR, M. 2011. Irradiation, Cisplatin, and 5-Azacytidine Upregulate Cytomegalovirus Promoter in Tumors and Muscles: Implementation of Non-invasive Fluorescence Imaging. *Molecular Imaging and Biology*, 13, 43-52.
- KAY, M. A. 2011. State-of-the-art gene-based therapies: the road ahead. *Nat Rev Genet*, 12, 316-28.
- KIM, H. J. & BAE, S. C. 2011. Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. *Am J Transl Res*, 3, 166-79.
- KIM, N. W., PIATYSZEK, M. A., PROWSE, K. R., HARLEY, C. B., WEST, M. D., HO, P. L., COVIELLO, G. M., WRIGHT, W. E., WEINRICH, S. L. & SHAY, J. W. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science*, 266, 2011-5.
- KNUST, B., BRUGGEMANN, U. & DOERFLER, W. 1989. Reactivation of a methylation-silenced gene in adenovirus-transformed cells by 5-azacytidine or by E1A trans activation. *J Virol*, 63, 3519-24.
- LANG, L. H. 2006. FDA approves use of bacteriophages to be added to meat and poultry products. *Gastroenterology*, 131, 1370.
- LATHAM, J. P., SEARLE, P. F., MAUTNER, V. & JAMES, N. D. 2000. Prostate-specific antigen promoter/enhancer driven gene therapy for prostate cancer: construction and testing of a tissue-specific adenovirus vector. *Cancer Res*, 60, 334-41.
- LEE, A. S. 1992. Mammalian stress response: induction of the glucose-regulated protein family. *Curr Opin Cell Biol*, 4, 267-73.
- LEE, A. S. 2001. The glucose-regulated proteins: stress induction and clinical applications. *Trends Biochem Sci*, 26, 504-10.
- LEE, A. S. 2007. GRP78 induction in cancer: therapeutic and prognostic implications. *Cancer Res*, 67, 3496-9.
- LEE, E., NICHOLS, P., GROSHEN, S., SPICER, D. & LEE, A. S. 2011. GRP78 as potential predictor for breast cancer response to adjuvant taxane therapy. *Int J Cancer*, 128, 726-31.

- LEE, E., NICHOLS, P., SPICER, D., GROSHEN, S., YU, M. C. & LEE, A. S. 2006. GRP78 as a Novel Predictor of Responsiveness to Chemotherapy in Breast Cancer. *Cancer Research*, 66, 7849-7853.
- LEE, H. K., XIANG, C., CAZACU, S., FINNISS, S., KAZIMIRSKY, G., LEMKE, N., LEHMAN, N. L., REMPEL, S. A., MIKKELSEN, T. & BRODIE, C. 2008. GRP78 is overexpressed in glioblastomas and regulates glioma cell growth and apoptosis. *Neuro Oncol*, 10, 236-43.
- LEE, K., TIRASOPHON, W., SHEN, X., MICHALAK, M., PRYWES, R., OKADA, T., YOSHIDA, H., MORI, K. & KAUFMAN, R. J. 2002. IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev*, 16, 452-66.
- LI, J. & LEE, A. S. 2006. Stress induction of GRP78/BiP and its role in cancer. *Curr Mol Med*, 6, 45-54.
- LI, J., LEE, B. & LEE, A. S. 2006. Endoplasmic reticulum stress-induced apoptosis: multiple pathways and activation of p53-up-regulated modulator of apoptosis (PUMA) and NOXA by p53. *J Biol Chem*, 281, 7260-70.
- LI, S. D. & HUANG, L. 2006. Gene therapy progress and prospects: non-viral gene therapy by systemic delivery. *Gene Ther*, 13, 1313-9.
- LI, W. W., ALEXANDRE, S., CAO, X. & LEE, A. S. 1993. Transactivation of the grp78 promoter by Ca²⁺ depletion. A comparative analysis with A23187 and the endoplasmic reticulum Ca(2+)-ATPase inhibitor thapsigargin. *J Biol Chem*, 268, 12003-9.
- LILGE, L. D., ET AL., 2002. PDT-induced apoptosis: investigations using two malignant brain tumor models. *Proc. SPIE*, 4612, 136-142.
- LIM, L. Y., VIDNOVIC, N., ELLISEN, L. W. & LEONG, C. O. 2009. Mutant p53 mediates survival of breast cancer cells. *Br J Cancer*, 101, 1606-12.
- LIN, J. H., LI, H., YASUMURA, D., COHEN, H. R., ZHANG, C., PANNING, B., SHOKAT, K. M., LAVAIL, M. M. & WALTER, P. 2007. IRE1 signaling affects cell fate during the unfolded protein response. *Science*, 318, 944-9.
- LIN, Y., WANG, Z., LIU, L. & CHEN, L. 2011. Akt is the downstream target of GRP78 in mediating cisplatin resistance in ER stress-tolerant human lung cancer cells. *Lung Cancer*, 71, 291-297.
- LITTLE, E., RAMAKRISHNAN, M., ROY, B., GAZIT, G. & LEE, A. S. 1994. The glucose-regulated proteins (GRP78 and GRP94): functions, gene regulation, and applications. *Crit Rev Eukaryot Gene Expr*, 4, 1-18.
- LIU, F. S. 2009. Mechanisms of chemotherapeutic drug resistance in cancer therapy--a quick review. *Taiwan J Obstet Gynecol*, 48, 239-44.

- LU, W., ZHOU, X., HONG, B., LIU, J. & YUE, Z. 2004. Suppression of invasion in human U87 glioma cells by adenovirus-mediated co-transfer of TIMP-2 and PTEN gene. *Cancer Lett*, 214, 205-13.
- LU, Y. & MADU, C. O. 2010. Viral-based gene delivery and regulated gene expression for targeted cancer therapy. *Expert Opin Drug Deliv*, 7, 19-35.
- LUNA, M. C., CHEN, X., WONG, S., TSUI, J., RUCKER, N., LEE, A. S. & GOMER, C. J. 2002. Enhanced photodynamic therapy efficacy with inducible suicide gene therapy controlled by the grp promoter. *Cancer Res*, 62, 1458-61.
- LUO, S., BAUMEISTER, P., YANG, S., ABCOUWER, S. F. & LEE, A. S. 2003. Induction of Grp78/BiP by translational block: activation of the Grp78 promoter by ATF4 through and upstream ATF/CRE site independent of the endoplasmic reticulum stress elements. *J Biol Chem*, 278, 37375-85.
- LUU, T. H., MORGAN, R. J., LEONG, L., LIM, D., MCNAMARA, M., PORTNOW, J., FRANKEL, P., SMITH, D. D., DOROSHOW, J. H., WONG, C., APARICIO, A., GANDARA, D. R. & SOMLO, G. 2008. A phase II trial of vorinostat (suberoylanilide hydroxamic acid) in metastatic breast cancer: a California Cancer Consortium study. *Clin Cancer Res*, 14, 7138-42.
- MANDIC, A., HANSSON, J., LINDER, S. & SHOSHAN, M. C. 2003. Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signaling. *J Biol Chem*, 278, 9100-6.
- MAX, R., GERRITSEN, R. R., NOOIJEN, P. T., GOODMAN, S. L., SUTTER, A., KEILHOLZ, U., RUITER, D. J. & DE WAAL, R. M. 1997. Immunohistochemical analysis of integrin alpha vbeta3 expression on tumor-associated vessels of human carcinomas. *Int J Cancer*, 71, 320-4.
- MCCORMICK, F. 2000. ONYX-015 selectivity and the p14ARF pathway. *Oncogene*, 19, 6670-2.
- MEHTA, A. K., MAJUMDAR, S. S., ALAM, P., GULATI, N. & BRAHMACHARI, V. 2009. Epigenetic regulation of cytomegalovirus major immediate-early promoter activity in transgenic mice. *Gene*, 428, 20-4.
- MEIER, J. L. 2001. Reactivation of the human cytomegalovirus major immediate-early regulatory region and viral replication in embryonal NTera2 cells: role of trichostatin A, retinoic acid, and deletion of the 21-base-pair repeats and modulator. *J Virol*, 75, 1581-93.
- MERRITT, J. A., ROTH, J. A. & LOGOTHETIS, C. J. 2001. Clinical evaluation of adenoviral-mediated p53 gene transfer: review of INGN 201 studies. *Semin Oncol*, 28, 105-14.
- MESNIL, M. & YAMASAKI, H. 2000. Bystander Effect in Herpes Simplex Virus-Thymidine Kinase/Ganciclovir Cancer Gene Therapy: Role of Gap-junctional Intercellular Communication1. *Cancer Research*, 60, 3989-3999.

- MILLER, A. D. 1992. Human gene therapy comes of age. *Nature*, 357, 455-60.
- MISRA, U. K., DEEDWANIA, R. & PIZZO, S. V. 2006. Activation and cross-talk between Akt, NF-kappaB, and unfolded protein response signaling in 1-LN prostate cancer cells consequent to ligation of cell surface-associated GRP78. *J Biol Chem*, 281, 13694-707.
- MIYAJIMA, A., NAKASHIMA, J., TACHIBANA, M., NAKAMURA, K., HAYAKAWA, M. & MURAI, M. 1999. N-acetylcysteine modifies cis-dichlorodiammineplatinum-induced effects in bladder cancer cells. *Jpn J Cancer Res*, 90, 565-70.
- MIYATAKE, S., IYER, A., MARTUZA, R. L. & RABKIN, S. D. 1997. Transcriptional targeting of herpes simplex virus for cell-specific replication. *J Virol*, 71, 5124-32.
- MODESITT, S. C., SILL, M., HOFFMAN, J. S. & BENDER, D. P. 2008. A phase II study of vorinostat in the treatment of persistent or recurrent epithelial ovarian or primary peritoneal carcinoma: a Gynecologic Oncology Group study. *Gynecol Oncol*, 109, 182-6.
- MOOLTEN, F. L. 1986. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res*, 46, 5276-81.
- MUNRO, S. & PELHAM, H. R. 1986. An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell*, 46, 291-300.
- MURPHY, J. C., FISCHLE, W., VERDIN, E. & SINCLAIR, J. H. 2002. Control of cytomegalovirus lytic gene expression by histone acetylation. *EMBO J*, 21, 1112-20.
- NAGASUBRAMANIAN, R. & DOLAN, M. E. 2003. Temozolomide: realizing the promise and potential. *Curr Opin Oncol*, 15, 412-8.
- NI, M., ZHOU, H., WEY, S., BAUMEISTER, P. & LEE, A. S. 2009. Regulation of PERK signaling and leukemic cell survival by a novel cytosolic isoform of the UPR regulator GRP78/BiP. *PLoS One*, 4, e6868.
- NOWIS, D., MCCONNELL, E. J., DIERLAM, L., PALAMARCHUK, A., LASS, A. & WOJCIK, C. 2007. TNF potentiates anticancer activity of bortezomib (Velcade) through reduced expression of proteasome subunits and dysregulation of unfolded protein response. *Int J Cancer*, 121, 431-41.
- OHLFEST, J. R., DEMOREST, Z. L., MOTOOKA, Y., VENGCO, I., OH, S., CHEN, E., SCAPPATICCI, F. A., SAPLIS, R. J., EKKER, S. C., LOW, W. C., FREESE, A. B. & LARGAESPADA, D. A. 2005. Combinatorial antiangiogenic gene therapy by nonviral gene transfer using the sleeping beauty transposon causes tumor regression and improves survival in mice bearing intracranial human glioblastoma. *Mol Ther*, 12, 778-88.

- OOSTERHOFF, D., PINEDO, H. M., WITLOX, M. A., CARETTE, J. E., GERRITSEN, W. R. & VAN BEUSECHEM, V. W. 2005. Gene-directed enzyme prodrug therapy with carboxylesterase enhances the anticancer efficacy of the conditionally replicating adenovirus AdDelta24. *Gene Ther*, 12, 1011-8.
- PALMER, T. D., ROSMAN, G. J., OSBORNE, W. R. & MILLER, A. D. 1991. Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. *Proc Natl Acad Sci U S A*, 88, 1330-4.
- PAOLONI, M. C., TANDLE, A., MAZCKO, C., HANNA, E., KACHALA, S., LEBLANC, A., NEWMAN, S., VAIL, D., HENRY, C., THAMM, D., SORENMO, K., HAJITOU, A., PASQUALINI, R., ARAP, W., KHANNA, C. & LIBUTTI, S. K. 2009. Launching a novel preclinical infrastructure: comparative oncology trials consortium directed therapeutic targeting of TNFalpha to cancer vasculature. *PLoS One*, 4, e4972.
- PASQUALINI, R., KOIVUNEN, E. & RUOSLAHTI, E. 1997. Alpha v integrins as receptors for tumor targeting by circulating ligands. *Nat Biotechnol*, 15, 542-6.
- PASQUALINI, R. & RUOSLAHTI, E. 1996. Organ targeting in vivo using phage display peptide libraries. *Nature*, 380, 364-6.
- PATIERNO, S. R., TUSCANO, J. M., KIM, K. S., LANDOLPH, J. R. & LEE, A. S. 1987. Increased expression of the glucose-regulated gene encoding the Mr 78,000 glucose-regulated protein in chemically and radiation-transformed C3H 10T1/2 mouse embryo cells. *Cancer Res*, 47, 6220-4.
- PFAFFENBACH, K. T. & LEE, A. S. 2011. The critical role of GRP78 in physiologic and pathologic stress. *Current Opinion in Cell Biology*, 23, 150-156.
- PIERSANTI, S., CHERUBINI, G., MARTINA, Y., SALONE, B., AVITABILE, D., GROSSO, F., CUNDARI, E., DI ZENZO, G. & SAGGIO, I. 2004. Mammalian cell transduction and internalization properties of lambda phages displaying the full-length adenoviral penton base or its central domain. *J Mol Med (Berl)*, 82, 467-76.
- PLUMB, J. A., BILSLAND, A., KAKANI, R., ZHAO, J., GLASSPOOL, R. M., KNOX, R. J., EVANS, T. R. & KEITH, W. N. 2001. Telomerase-specific suicide gene therapy vectors expressing bacterial nitroreductase sensitize human cancer cells to the pro-drug CB1954. *Oncogene*, 20, 7797-803.
- PROSCH, S., STEIN, J., STAAK, K., LIEBENTHAL, C., VOLK, H. D. & KRUGER, D. H. 1996. Inactivation of the very strong HCMV immediate early promoter by DNA CpG methylation in vitro. *Biol Chem Hoppe Seyler*, 377, 195-201.
- PYRKO, P., SCHONTHAL, A. H., HOFMAN, F. M., CHEN, T. C. & LEE, A. S. 2007. The unfolded protein response regulator GRP78/BiP as a novel target for increasing chemosensitivity in malignant gliomas. *Cancer Res*, 67, 9809-16.

- RACEK, T., BUHLMANN, S., RUST, F., KNOLL, S., ALLA, V. & PUTZER, B. M. 2008. Transcriptional repression of the prosurvival endoplasmic reticulum chaperone GRP78/BIP by E2F1. *J Biol Chem*, 283, 34305-14.
- RADHAKRISHNAN, P., BASMA, H., KLINKEBIEL, D., CHRISTMAN, J. & CHENG, P. W. 2008. Cell type-specific activation of the cytomegalovirus promoter by dimethylsulfoxide and 5-aza-2'-deoxycytidine. *Int J Biochem Cell Biol*, 40, 1944-55.
- RASMUSSEN, H., RASMUSSEN, C., LEMPICKI, M., DURHAM, R., BROUGH, D., KING, C. R. & WEICHSELBAUM, R. 2002. TNFerade Biologic: preclinical toxicology of a novel adenovector with a radiation-inducible promoter, carrying the human tumor necrosis factor alpha gene. *Cancer Gene Ther*, 9, 951-7.
- REDDY, R. K., MAO, C., BAUMEISTER, P., AUSTIN, R. C., KAUFMAN, R. J. & LEE, A. S. 2003. Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. *J Biol Chem*, 278, 20915-24.
- RESENDEZ, E., JR., WOODEN, S. K. & LEE, A. S. 1988. Identification of highly conserved regulatory domains and protein-binding sites in the promoters of the rat and human genes encoding the stress-inducible 78-kilodalton glucose-regulated protein. *Mol Cell Biol*, 8, 4579-84.
- RICHARDS, C. A., AUSTIN, E. A. & HUBER, B. E. 1995. Transcriptional regulatory sequences of carcinoembryonic antigen: identification and use with cytosine deaminase for tumor-specific gene therapy. *Hum Gene Ther*, 6, 881-93.
- ROBERTSON, K. D. & JONES, P. A. 2000. DNA methylation: past, present and future directions. *Carcinogenesis*, 21, 461-7.
- ROBSON, T. & HIRST, D. G. 2003. Transcriptional Targeting in Cancer Gene Therapy. *J Biomed Biotechnol*, 2003, 110-137.
- RODRIGUEZ, R., SCHUUR, E. R., LIM, H. Y., HENDERSON, G. A., SIMONS, J. W. & HENDERSON, D. R. 1997. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res*, 57, 2559-63.
- ROMERO-RAMIREZ, L., CAO, H., NELSON, D., HAMMOND, E., LEE, A. H., YOSHIDA, H., MORI, K., GLIMCHER, L. H., DENKO, N. C., GIACCIA, A. J., LE, Q. T. & KOONG, A. C. 2004. XBP1 is essential for survival under hypoxic conditions and is required for tumor growth. *Cancer Res*, 64, 5943-7.
- ROY, B. & LEE, A. S. 1999. The mammalian endoplasmic reticulum stress response element consists of an evolutionarily conserved tripartite structure and interacts with a novel stress-inducible complex. *Nucleic Acids Res*, 27, 1437-43.
- RUOSLAHTI, E. 1996. RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol*, 12, 697-715.

- SADEGHI, H. & HITT, M. M. 2005. Transcriptionally targeted adenovirus vectors. *Curr Gene Ther*, 5, 411-27.
- SAMALI, A., FITZGERALD, U., DEEGAN, S. & GUPTA, S. 2010. Methods for monitoring endoplasmic reticulum stress and the unfolded protein response. *Int J Cell Biol*, 2010, 830307.
- SAMBROOK (ed.) 1989. *Molecular cloning (A laboratory manual)*: US. Cold spring Harbor laboratory.
- SAMULSKI, R. J., CHANG, L. S. & SHENK, T. 1987. A recombinant plasmid from which an infectious adeno-associated virus genome can be excised in vitro and its use to study viral replication. *J Virol*, 61, 3096-101.
- SCHEWE, D. M. & AGUIRRE-GHISO, J. A. 2009. Inhibition of eIF2 α dephosphorylation maximizes bortezomib efficiency and eliminates quiescent multiple myeloma cells surviving proteasome inhibitor therapy. *Cancer Res*, 69, 1545-52.
- SCHMIDT, E. V., CHRISTOPH, G., ZELLER, R. & LEDER, P. 1990. The cytomegalovirus enhancer: a pan-active control element in transgenic mice. *Mol Cell Biol*, 10, 4406-11.
- SELVAKUMARAN, M., BAO, R., CRIJNS, A. P., CONNOLLY, D. C., WEINSTEIN, J. K. & HAMILTON, T. C. 2001. Ovarian epithelial cell lineage-specific gene expression using the promoter of a retrovirus-like element. *Cancer Res*, 61, 1291-5.
- SENATUS, P. B., LI, Y., MANDIGO, C., NICHOLS, G., MOISE, G., MAO, Y., BROWN, M. D., ANDERSON, R. C., PARSA, A. T., BRANDT-RAUF, P. W., BRUCE, J. N. & FINE, R. L. 2006. Restoration of p53 function for selective Fas-mediated apoptosis in human and rat glioma cells in vitro and in vivo by a p53 COOH-terminal peptide. *Mol Cancer Ther*, 5, 20-8.
- SENZER, N., MANI, S., ROSEMURGY, A., NEMUNAITIS, J., CUNNINGHAM, C., GUHA, C., BAYOL, N., GILLEN, M., CHU, K., RASMUSSEN, C., RASMUSSEN, H., KUFEL, D., WEICHSELBAUM, R. & HANNA, N. 2004. TNFerade biologic, an adenovector with a radiation-inducible promoter, carrying the human tumor necrosis factor alpha gene: a phase I study in patients with solid tumors. *J Clin Oncol*, 22, 592-601.
- SHAY, J. W. & WRIGHT, W. E. 2001. Ageing and cancer: the telomere and telomerase connection. *Novartis Found Symp*, 235, 116-25; discussion 125-9, 146-9.
- SHEN, Y. & NEMUNAITIS, J. 2006. Herpes simplex virus 1 (HSV-1) for cancer treatment. *Cancer Gene Ther*, 13, 975-92.
- SHEPPARD, D. 1996. Epithelial integrins. *Bioessays*, 18, 655-60.
- SHU, C. W., SUN, F. C., CHO, J. H., LIN, C. C., LIU, P. F., CHEN, P. Y., CHANG, M. D., FU, H. W. & LAI, Y. K. 2008. GRP78 and Raf-1 cooperatively confer resistance to endoplasmic reticulum stress-induced apoptosis. *J Cell Physiol*, 215, 627-35.

- SINGAL, R. & GINDER, G. D. 1999. DNA methylation. *Blood*, 93, 4059-70.
- SMITH, G. P. & SCOTT, J. K. 1993. Libraries of peptides and proteins displayed on filamentous phage. *Methods Enzymol*, 217, 228-57.
- SONG, J. S. & KIM, H. P. 2004. Adenovirus-mediated HSV-TK gene therapy using the human telomerase promoter induced apoptosis of small cell lung cancer cell line. *Oncol Rep*, 12, 443-7.
- STUPP, R., MASON, W. P., VAN DEN BENT, M. J., WELLER, M., FISHER, B., TAPHOORN, M. J., BELANGER, K., BRANDES, A. A., MAROSI, C., BOGDAHN, U., CURSCHMANN, J., JANZER, R. C., LUDWIN, S. K., GORLIA, T., ALLGEIER, A., LACOMBE, D., CAIRNCROSS, J. G., EISENHAUER, E. & MIRIMANOFF, R. O. 2005. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*, 352, 987-96.
- SU, H., LU, R., CHANG, J. C. & KAN, Y. W. 1997. Tissue-specific expression of herpes simplex virus thymidine kinase gene delivered by adeno-associated virus inhibits the growth of human hepatocellular carcinoma in athymic mice. *Proc Natl Acad Sci U S A*, 94, 13891-6.
- SWINDLE, C. S. & KLUG, C. A. 2002. Mechanisms that regulate silencing of gene expression from retroviral vectors. *J Hematother Stem Cell Res*, 11, 449-56.
- SWISHER, S. G., ROTH, J. A., KOMAKI, R., GU, J., LEE, J. J., HICKS, M., RO, J. Y., HONG, W. K., MERRITT, J. A., AHRAR, K., ATKINSON, N. E., CORREA, A. M., DOLORMENTE, M., DREILING, L., EL-NAGGAR, A. K., FOSSELLA, F., FRANCISCO, R., GLISSON, B., GRAMMER, S., HERBST, R., HUARINGA, A., KEMP, B., KHURI, F. R., KURIE, J. M., LIAO, Z., MCDONNELL, T. J., MORICE, R., MORELLO, F., MUNDEN, R., PAPADIMITRAKOPOULOU, V., PISTERS, K. M., PUTNAM, J. B., JR., SARABIA, A. J., SHELTON, T., STEVENS, C., SHIN, D. M., SMYTHE, W. R., VAPORCIYAN, A. A., WALSH, G. L. & YIN, M. 2003. Induction of p53-regulated genes and tumor regression in lung cancer patients after intratumoral delivery of adenoviral p53 (INGN 201) and radiation therapy. *Clin Cancer Res*, 9, 93-101.
- TANDLE, A., HANNA, E., LORANG, D., HAJITOU, A., MOYA, C. A., PASQUALINI, R., ARAP, W., ADEM, A., STARKER, E., HEWITT, S. & LIBUTTI, S. K. 2009. Tumor vasculature-targeted delivery of tumor necrosis factor-alpha. *Cancer*, 115, 128-39.
- TAUNTON, J., HASSIG, C. A. & SCHREIBER, S. L. 1996. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. *Science*, 272, 408-11.
- TEN HAGEN, T. L. & EGGERMONT, A. M. 2003. Solid tumor therapy: manipulation of the vasculature with TNF. *Technol Cancer Res Treat*, 2, 195-203.

- TESKE, B. F., WEK, S. A., BUNPO, P., CUNDIFF, J. K., MCCLINTICK, J. N., ANTHONY, T. G. & WEK, R. C. 2011. The eIF2 kinase PERK and the integrated stress response facilitate activation of ATF6 during endoplasmic reticulum stress. *Mol Biol Cell*.
- TING, J. & LEE, A. S. 1988. Human gene encoding the 78,000-dalton glucose-regulated protein and its pseudogene: structure, conservation, and regulation. *DNA*, 7, 275-86.
- TREPEL, M., STONEHAM, C. A., ELEFTHEROHORINO, H., MAZARAKIS, N. D., PASQUALINI, R., ARAP, W. & HAJITOU, A. 2009. A heterotypic bystander effect for tumor cell killing after adeno-associated virus/phage-mediated, vascular-targeted suicide gene transfer. *Mol Cancer Ther*, 8, 2383-91.
- VAN DEN POL, A. N. & GHOSH, P. K. 1998. Selective neuronal expression of green fluorescent protein with cytomegalovirus promoter reveals entire neuronal arbor in transgenic mice. *J Neurosci*, 18, 10640-51.
- VARDA-BLOOM, N., SHAISH, A., GONEN, A., LEVANON, K., GREENBERGER, S., FERBER, S., LEVKOVITZ, H., CASTEL, D., GOLDBERG, I., AFEK, A., KOPOLOVITC, Y. & HARATS, D. 2001. Tissue-specific gene therapy directed to tumor angiogenesis. *Gene Ther*, 8, 819-27.
- VERDIN, E., DEQUIEDT, F. & KASLER, H. G. 2003. Class II histone deacetylases: versatile regulators. *Trends Genet*, 19, 286-93.
- VERMA, I. M. & SOMIA, N. 1997. Gene therapy -- promises, problems and prospects. *Nature*, 389, 239-42.
- VILE, R. G. & HART, I. R. 1993. In vitro and in vivo targeting of gene expression to melanoma cells. *Cancer Res*, 53, 962-7.
- VILLANO, J. L., SEERY, T. E. & BRESSLER, L. R. 2009. Temozolomide in malignant gliomas: current use and future targets. *Cancer Chemother Pharmacol*, 64, 647-55.
- VIRREY, J. J., DONG, D., STILES, C., PATTERSON, J. B., PEN, L., NI, M., SCHÖNTHAL, A. H., CHEN, T. C., HOFMAN, F. M. & LEE, A. S. 2008. Stress Chaperone GRP78/BiP Confers Chemoresistance to Tumor-Associated Endothelial Cells. *Molecular Cancer Research*, 6, 1268-1275.
- WAEHLER, R., RUSSELL, S. J. & CURIEL, D. T. 2007. Engineering targeted viral vectors for gene therapy. *Nat Rev Genet*, 8, 573-87.
- WANG, G., YANG, Z. Q. & ZHANG, K. 2010. Endoplasmic reticulum stress response in cancer: molecular mechanism and therapeutic potential. *American journal of translational research*, 2, 65-74.
- WANG, M., WEY, S., ZHANG, Y., YE, R. & LEE, A. S. 2009. Role of the unfolded protein response regulator GRP78/BiP in development, cancer, and neurological disorders. *Antioxid Redox Signal*, 11, 2307-16.

- WANG, Q., HE, Z., ZHANG, J., WANG, Y., WANG, T., TONG, S., WANG, L., WANG, S. & CHEN, Y. 2005. Overexpression of endoplasmic reticulum molecular chaperone GRP94 and GRP78 in human lung cancer tissues and its significance. *Cancer Detect Prev*, 29, 544-51.
- WEI, S. J., CHAO, Y., HUNG, Y. M., LIN, W. C., YANG, D. M., SHIH, Y. L., CH'ANG, L. Y., WHANG-PENG, J. & YANG, W. K. 1998. S- and G2-phase cell cycle arrests and apoptosis induced by ganciclovir in murine melanoma cells transduced with herpes simplex virus thymidine kinase. *Exp Cell Res*, 241, 66-75.
- WESOŁOWSKI, J. R., RAJDEV, P. & MUKHERJI, S. K. 2010. Temozolomide (Temodar). *AJNR Am J Neuroradiol*, 31, 1383-4.
- WEY, S., LUO, B., TSENG, C. C., NI, M., ZHOU, H., FU, Y., BHOJWANI, D., CARROLL, W. L. & LEE, A. S. 2012. Inducible knockout of GRP78/BiP in the hematopoietic system suppresses Pten-null leukemogenesis and AKT oncogenic signaling. *Blood*, 119, 817-25.
- WU, L., MATHERLY, J., SMALLWOOD, A., ADAMS, J. Y., BILLICK, E., BELLDEGRUN, A. & CAREY, M. 2001. Chimeric PSA enhancers exhibit augmented activity in prostate cancer gene therapy vectors. *Gene Ther*, 8, 1416-26.
- XIONG, J., SUN, W. J., WANG, W. F., LIAO, Z. K., ZHOU, F. X., KONG, H. Y., XU, Y., XIE, C. H. & ZHOU, Y. F. 2012. Novel, chimeric, cancer-specific, and radiation-inducible gene promoters for suicide gene therapy of cancer. *Cancer*, 118, 536-48.
- YE, J., RAWSON, R. B., KOMURO, R., CHEN, X., DAVE, U. P., PRYWES, R., BROWN, M. S. & GOLDSTEIN, J. L. 2000. ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol Cell*, 6, 1355-64.
- YOSHIDA, H., MATSUI, T., YAMAMOTO, A., OKADA, T. & MORI, K. 2001. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell*, 107, 881-91.
- YOSHIDA, M., KIJIMA, M., AKITA, M. & BEPPU, T. 1990. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. *J Biol Chem*, 265, 17174-9.
- YU, Z., LUO, H., FU, W. & MATTSON, M. P. 1999. The endoplasmic reticulum stress-responsive protein GRP78 protects neurons against excitotoxicity and apoptosis: suppression of oxidative stress and stabilization of calcium homeostasis. *Exp Neurol*, 155, 302-14.
- ZHANG, G., LIU, T., CHEN, Y. H., CHEN, Y., XU, M., PENG, J., YU, S., YUAN, J. & ZHANG, X. 2009a. Tissue specific cytotoxicity of colon cancer cells mediated by nanoparticle-delivered suicide gene in vitro and in vivo. *Clin Cancer Res*, 15, 201-7.

- ZHANG, J., JIANG, Y., JIA, Z., LI, Q., GONG, W., WANG, L., WEI, D., YAO, J., FANG, S. & XIE, K. 2006. Association of elevated GRP78 expression with increased lymph node metastasis and poor prognosis in patients with gastric cancer. *Clin Exp Metastasis*, 23, 401-10.
- ZHANG, J. X., ZHAO, P., LI, R., SHI, L., CHENG, Z. H., YOU, Y. P. & FU, Z. 2009b. [Antiglioma activity of endothelial progenitor cells transduced with HSV-TK via inhibiting angiogenesis in vitro and in vivo]. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi*, 26, 170-4.
- ZHANG, L., WANG, S., WANGTAO, WANG, Y., WANG, J., JIANG, L., LI, S., HU, X. & WANG, Q. 2009c. Upregulation of GRP78 and GRP94 and its function in chemotherapy resistance to VP-16 in human lung cancer cell line SK-MES-1. *Cancer Invest*, 27, 453-8.
- ZHANG, L. H. & ZHANG, X. 2010. Roles of GRP78 in physiology and cancer. *J Cell Biochem*, 110, 1299-305.
- ZHANG, R., STRAUS, F. H. & DEGROOT, L. J. 2001. Adenoviral-mediated gene therapy for thyroid carcinoma using thymidine kinase controlled by thyroglobulin promoter demonstrates high specificity and low toxicity. *Thyroid*, 11, 115-23.
- ZUSTOVICH, F., LOMBARDI, G., DELLA PUPPA, A., ROTILIO, A., SCIENZA, R. & PASTORELLI, D. 2009. A phase II study of cisplatin and temozolomide in heavily pre-treated patients with temozolomide-refractory high-grade malignant glioma. *Anticancer Res*, 29, 4275-9.