CREATING A BUILT-IN SUICIDE SYSTEM IN ADENO ASSOCIATED VIRAL CONSTRUCTS AS A SAFETY CHECK MECHANISM FOR TREATMENT OF POSSIBLE TUMOR DEVELOPMENT IN LONG-TERM GENE THERAPY STUDIES

by

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Abstract

Adeno associated virus (AAV) is proving to be a powerful tool for gene therapy. However, a drawback of this tool reported in experimental studies is that a small percentage of the administered virus can integrate into host deoxyribonucleic acid (DNA) sequences, sometimes resulting in oncogenesis. Suicide gene therapy (SGT), a strategy for gene-directed killing of cells, is one of the approaches that could be well utilized to augment existing gene therapies by providing a safety element in the event of tumor development. This technique has not been used in AAV based gene therapy experiments. One of the successful SGT system utilizes Herpes Simplex virus thymidine kinase gene (HSVtk) which confers ganciclovir (GCV) sensitivity to the transduced cells. We hypothesized that placing HSVtk/GCV under the control of the HSP70 (Heat Shock Protein 70) promoter will results in apoptosis of HSVtk expressing cells, only when heat is applied.

We developed a dual promoter-transgene AAV viral construct (AAV-70-tk-CAG-GFP) carrying an inducible HSP70 promoter for the expression of HSVtk and chicken β-actin (CAG) (a ubiquitous promoter), driving green fluorescent protein (GFP). Human embryonic kidney cells (HEK293 cells) were stably transfected with the study plasmid and analyzed for protein expression. The experimental and control cells were exposed to normal (37°C) and high temperature (41°C) to measure cell survival at different concentrations of GCV.

The survival of stably transfected cells was markedly reduced with GCV treatment at 41°C, compared to untransfected HEK 293 cells. However, an unexpected decrease in cell survival was also noted at 37°C, indicating the promoter was also active at normal temperature. In summary, our experiment was the first step to test cell killing with SGT under control of inducible promoter in an AAV construct. The results showed cell killing both at high and normal temperatures. This provides a preface for future research exploring HSP70 induction physiology and use of other inducible promoters in the HSVtk/GCV suicide gene system.

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List of Abbreviations

5-FC: 5–Fluorocytosine

5-FU: 5-Fluorouracil

ACV: Aciclovir

AAV: Adeno Associated Virus

BLAST: Basic Local Alignment Search Tool

BRCA1: Breast cancer gene 1

CD: Cytosine deaminase

CAG: Chicken beta-actin

CFTR: Cystic Fibrosis Transmembrane Conductance Regulator

CPA: Carboxypeptidase A

CPG2: Carboxypeptidase G2

CYP: Cytochrome P450

DAAO: D-amino-acid oxidase

dCK: Deoxycytidine kinase

dGTP: 2 Deoxyguanosine triphosphate

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribonucleic acid

DRNK: Deoxyribonucleotide

ECL: Enhanced chemiluminescence

EGFP: Enhanced green fluorescent protein

ErbB: Erythroblastic leukemia

FACS: Fluorescence-activated cell sorting

FAS: Fatty-acid synthase

FBS: Fetal bovine serum

FDA: Food and Drug Administration

GCV: Ganciclovir

GDEPT: Gene-Directed Enzyme Prodrug Therapy

GFP: Green fluorescent protein

G418: Geneticin

GPAT: Gene prodrug activation therapy

GvHD: Graft versus host disease

HCC: Hepatocellular carcinoma

HEK293: Human embryonic kidney 293 cells

HeLa: Henrietta Lacks – Cervical cancer cell line

hPSC: Human pluripotent stem cell

HRP: Horseradish peroxidase

HSP: Heat Shock Protein

HSVtk: Herpes simplex virus thymidine kinase

iCasp9: Inducible Caspase 9

Kan/Neo: Kanamycin/Neomycin

K-ras: Kristen Rat sarcoma virus

LTR: Long terminal repeat

MDAE: Multiple-drug activation enzyme

MDR1: Multi-drug resistance 1

MTS: 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NCBI: National Center for Biotechnology Information

NR: Nitro reductase

PBS: Phosphate-buffered saline

PGA: Penicillin-G amidase

PCR: Polymerase Chain Reaction

PMSF: Phenylmethylsulfonyl fluoride

PNP: Purine nucleoside phosphorylase

PI: Propidium iodide

rAAV: Recombinant Adeno Associated Virus

Rep: Replication inhibitor protein

RB: Retinoblastoma

Rian: RNA imprinted and accumulated in nucleus

RPE65: Retinal pigment epithelium-specific 65 kDa protein

scAAV: Self-complementary Adeno Associated Virus

SCID: Severe combined immunodeficiency

SGT: Suicide gene therapy

shRNA: Small hairpin RNA

TBS: Tris-buffered saline

TK: Thymidine kinase

TP: Thymidine phosphorylase

UPRT: Uracil phosphoribosyl transferase

VDEPT: Virus-directed enzyme prodrug therapy

VEGF: Vascular endothelial growth factor

XGPRT: Xanthine–guanine phosphoribosyl transferase

β-Gal: β-Galactosidase

β-Glu: β-Glucuronidase

β-L: β-Lactamase

Chapter 1

Introduction

The journey of human gene therapy started in 1989 when the first human gene transfer experiment was conducted and since then, has continued to develop and progress (Hasan & Saini, 2014). New applications for gene therapy continue to be found for a broad mixture of acquired and inherited diseases, such as malignant neoplastic diseases, severe combined immunodeficiency, and other life threatening diseases (Hasan & Saini, 2014). The advent of gene therapy brings with it promise, but has also generated many concerns and possible side effects. These challenges ranged from immunogenic reactions, to the development of tumors. One of the unresolved concerns about safety of gene therapy is the possibility of insertional mutagenesis; leading to the activation of proto-oncogenes or inactivation of a tumor-suppressing gene, resulting in tumorigenesis (Kay, 2007). Malignancies secondary to chemotherapy and radiation therapy are not uncommon in cancer therapeutics. Although gene therapy is considered as miracle for mankind and results in comparatively fewer secondary malignancies then other available therapies, gene therapy related tumorigenesis appeared early after treatment and had fatal results (Brower, 2013; Kamran et al., 2016). This resulted in a loss of confidence and a delay in successful clinical implementation. Retrovirus, lentivirus and transposon vectors, the predecessors of AAV, were shown to have relatively high rates of insertional mutagenesis and oncogenesis (Valdmanis et al., 2012). AAV, however, entered late in gene therapy experimentation but was considered safer. However, the discovery of the oncogenic potential in AAV mediated transduction has led further exploration in this field to enhance the safety of this vector.

1.1 Insertional Mutagenesis

The therapeutic potential of gene therapy depends upon the introduction of genetic factors into the cellular machinery leading to its modification. In the case of some diseases, like cancer, only transient expression of the genetic material is required as the purpose is to destroy neoplastic cells. However, there are diseases, especially genetic disorders, where long term stable transgene expression is essential to correct a phenotype, and does so by providing functional copies of a defective gene. Gene delivery systems that can integrate the therapeutic gene into the nucleus of a target cell lead to more stable and long term gene expression (Romano et al., 2000; Romano, 2003). However, this integration could possibly alter natural chromosomal arrangements and possibly lead to the development of malignancies.

After the two successful clinical trials of gene therapy in 1990 in the USA, hundreds of gene therapy trials were put forward all around world (Rosenberg et al., 1990; Blaese et al., 1995; Muul et al., 2003). However, this success was not sustained and very soon transformed into a safety risk when patients enrolled in France/United Kingdom for treatment of SCID developed leukemia after the successful gene therapy of their primary disease (Hacein-Bey-Abina et al., 2003; Noguchi, 2003; Chinen & Puck, 2004; Cavazzana-Calvo & Fischer, 2007; Baum, 2007b; Hacein-Bey-Abina et al., 2008; Howe et al., 2008; Baum 2011). These trials used retroviral vectors, which are now thought to be the most oncogenic and well understood for mechanisms of insertional mutagenesis. The underlying mechanism was found to be activation of proto-oncogenes as the vector had been inserted adjacently to activate oncogenes (Biasco et al., 2012). This was specifically studied in retrovirus based hemopoietic gene therapy (Baum, 2007a). The mechanism studied suggested that genotoxic effects are not limited to inactivation at the integration site, but that the enhancers and promoters of the vector also cause dysregulation of neighboring genes, again affecting the gene expression (Baum et al., 2011; Romano, 2012).

1.1.1 Factors Contributing to Insertional Mutagenesis

Oncogenesis, caused by insertional mutagenesis, is a recognized safety concern, as briefly mentioned above. This has resulted in the development of acute lymphoid leukemia in five patients of SCID, four patients of Wiskott-Aldrich syndrome, and myelodysplasias in chronic granulomatous disease (Stein et al., 2010; Gaspar et al., 2011; Seymour & Thrasher, 2012). Several factors have been studied and labelled as risk factors for vector integration. Vector associated factors are the most common cause of mutagenesis. Moreover, among the various causes, vector design is by far the most prevalent cause of this toxicity (Romano, 2012). The choice of vector backbone is, therefore, important. To date, retroviral and lentiviral vectors are commonly associated with the highest risk as compared to adenovirus or recombinant adeno associated virus (rAAV). A strong enhancer/promoter like long terminal repeat (LTR) favours activation of oncogenesis by initiating transcription (Uren et al., 2005). This has led to the use of self-inactivating LTR or a moderately active promoter in vector design to decrease risk of oncogenesis.

The site of vector integration also plays an important role. Vectors with a tendency to be inserted near a transcriptional region are associated with a high probability of oncogenesis. This risk can be modulated with the use of sequence-specific endonucleases (Biasco et al., 2011). Chromatin insulators can act as a block to prevent enhancer/promoter interaction, yet their value is limited to specific vector designs (Aiuti et al., 2013).

Beside the vector itself, maturity of target cell is considered as inversely related to risk of oncogenicity. For example, fetal tissue has a higher risk of mutagenicity as compared to adult tissue. Similarly, induction of oncogenesis in human pluripotent cells has been observed (Biasco et al., 2011; Themis et al., 2005).

1.2 Adeno Associated Virus (AAV)

AAV is an emerging vector for many types of gene therapies with its current applications in more than 180 clinical trials ("Gene Therapy Clinical Trials Worldwide," 2017). AAV is a non-enveloped single-stranded virus belonging to *parvoviridae* family with a genome size of approximately 4.8kb, efficient gene transfer capability and an ability to infect both dividing and non-dividing cells. Additionally, AAV is not associated with human disease even though most humans are sero-positive for one or more serotypes, as well as having a low immunogenic response towards it. In addition, failure to propagate when the helper virus is absent is a natural safety feature that prevents its spread (Samulski & Muzyczka, 2014; Naso et al., 2017) .

Recombinant adeno associated virus (rAAV) is the genetically engineered form of the wild-type virus. It is a protein-based nano-particle which lacks viral DNA and can traverse the cell membrane to deliver its DNA to the cellular nucleus (Choi et al., 2006). rAAV is proving to be a potent tool in the field of gene therapy for many genetic and non-genetic conditions. The first rAAV human experiment was performed almost 25 year ago (Flotte et al., 1996). Some of the effective clinical examples include hemophilia, alpha 1 antitrypsin and spinal muscular atrophy. Long term safety has been established with a single injection of rAAV carrying factor IX in hemophilia B (Nathwani et al., 2011; Nathwani et al., 2014). Additionally, many preclinical, proof-of-concept studies are ongoing with rAAV in monogenic metabolic and skeletal disorders (ElMallah et al., 2014; Borel et al., 2015; Stoica et al., 2016).

1.3 Biology and Oncogenic Potential of Recombinant Adeno Associated Virus (rAAV)

Post-infection, AAV uncoats itself in the target cell nucleus and its single-stranded genome is converted to double-stranded deoxyribonucleic acid (DNA) either by host-cell DNA synthesis, in the case of conventional rAAV vectors, or by intrastrand base pairing, if it is self-

complementary AAV (scAAV) (Nakai et al., 2000). The host cell DNA repair process converts these double-stranded vector genomes into circular forms. The beauty of rAAV is the lack of active genes which are responsible for active viral gene expression and which amplify immunogenicity (Basner-Tschakarjan & Mingozzi, 2014).

Previously, rAAV was thought to be both non-integrating and non-oncogenic, especially in pre-clinical studies (Valdmanis et al., 2012). The carcinogenesis related to wild type AAV is thought to be associated with the Replication inhibitor protein (*Rep)* gene which is responsible for integration. Although rAAV lacks the *Rep* gene, there is clearly a small percentage (<0.5%) that randomly integrates into host chromosomal sequences via non- homologous recombination (Miller et al., 2003; Miller et al., 2004; McCarty et al., 2004; Miller et al., 2005; Nakai et al., 2005; Inagaki et al., 2007; McCarty, 2008; Gaj et al., 2016).

The carcinogenic potential of rAAV was first noted in 2001 in a mouse model of mucopolysaccharidosis type VII (Donsante et al., 2001). Subsequent analysis by the same group identified a potential locus as an insertion site of rAAV for hepatocellular carcinoma (HCC) (Donsante et al., 2007). Later, many other groups reported genotoxicity of rAAV. In a retrospective analysis of rAAV treatment in a mouse model of ornithine transcarbamylase deficiency, an association of liver tumors with vector carrying the lacZ gene was found, but not with the therapeutic ornithine transcarbamylase gene (Bell et al., 2005). Nevertheless, the authors doubted the true relation of this association because of the genetic background of the ornithine transcarbamylase mice, B6C3F1, being a 'liver tumor-prone' strain. In a third study, in which excess tumors were associated with rAAV treatment in a mouse model of phenylketonuria, the oncogenic effects were thought to be related to the expression of a woodchuck hepadnavirus X-protein coding region in the vector constructs (Embury et al., 2008). At the same time, other studies specifically looking at AAV-associated tumorigenesis showed no increased risk. For example, a large cohort study showed a similar incidence of malignancy in the control and study

mice (n=695) which received different types of AAV vectors at two-week intervals (Bell et al., 2005). Further support of non-carcinogenicity was shown in p53-deficient mice with inherent risk of malignancy; not exhibiting increased tumor formation with AAV (Schuettrumpf et al., 2007). The use of multiplex linear amplification-mediated polymerase chain reaction (PCR) assay by Gil-Farina et al. to detect integration events in rAAV genome yielded no integration clusters in genes which were suspected to link to hepatocellular carcinoma in previous studies (Gil-Farina et al., 2016).

The analysis of these studies provided contradictory results as some vector constructs are more prone to genotoxicity than others, and all the features that might contribute to this effect were not clear. Rosas et al. studied the insertion pattern of scAAV and identified three common insertion sites associated with proto-oncogenes in liver tumors (Rosas et al., 2012). As the efforts continued to establish a cause-and-effect relationship, more reports confirming affinity of rAAV gene therapy with hepatocellular carcinoma have been published. A recent study of systemic treatment of GM2 gangliosidosis using rAAV9 reported tumors in 8 out of 10 Sandhoff disease and control neonatal mice; seven animals had liver tumors and one had multiple lung tumors (Walia et al., 2015). The vector integration analysis confirmed integration mainly at RNA imprinted and accumulated in nucleus (Rian) locus, among other loci.

Interestingly, Chandler et al. reported similar insertional sites in their mouse model treated with AAV. They used a sensitive high-throughput integration site capture technique and global expression analysis for a large cohort of treated and control mice with methylmalonic-acidemia, which were followed for 22 months (Chandler et al., 2015). This study also concludes that the AAV vector dose, enhancer/promoter selection, codon optimization and the timing of gene delivery are all important variables for HCC incidence after AAV gene delivery. The study concluded that the vector design (including promoter and codon optimization of the transgene) influences hepatic genotoxicity after AAV gene therapy.

Another new addition to this series of studies came out recently in a report showing a linkage between human HCC and AAV infection, which was not studied previously. Nault et al reported 11 out of 193 cases of human HCC were caused by AAV2 integration (Nault et al., 2015), though it was argued against the causal relationship, in a follow-up study (Berns et al., 2015).

1.4 Development of Safer Gene Delivery System

To overcome the concern of potential oncogenicity of gene therapy and to achieve success in genetic therapeutic potential, various methods of safe gene delivery have been studied. These methods are based on three overarching models: improved genetic integration; better episomal gene delivery models, which can result in long stable transgene expression (Vargas et al., 2004B; Terskikh et al., 2005; Philippe et al., 2006; Hu et al., 2009; Negri et al., 2010); and development of alternatives like genetically modified nuclease/protein to correct the defect (Dhanasekaran et al., 2006; Papapetrou et al., 2006; Dent et al., 2007).

In relation to safer gene integration models, most of these strategies deal with alterations in choice of vector backbone in which the vector does not affect the physiological arrangement in transduced cell. These include promoter-enhancer sequences; use of chromatin insulator to prevent promoter-enhancer interaction; expression specific to cell type; and targeted transduction (Aiuti et al., 2013). A suicidal gene can be co-transduced or co-expressed under the control of an inducible promoter, an approach called suicide gene therapy (SGT).These safer methodologies have been well-studied in models of retrovirus and lentivirus-based vectors, and now, with the discovery of the oncogenic potential of rAAV, there is a need to develop experimental methodology to make this safer as well (Yi et al., 2011).

1.4.1 Role of Suicide Gene Therapy (SGT)

SGT is among one of many initial strategies used in gene therapy for cancer treatment. It is based on converting a non-toxic compound into a toxic drug by viral or bacterial gene

introduction into tumor cells. One of the most powerful SGT systems utilizes Herpes Simplex virus thymidine kinase gene (HSVtk) which induces ganciclovir (GCV) sensitivity to the transduced or transfected cells. This results in expression of the HSVtk gene in the target cell. The resulting viral thymidine kinase metabolizes GCV to its monophosphate form, which acts as a cytotoxic agent by causing cell cycle arrest. The effectiveness of SGT for treatment of cancer has been validated by *in vitro* and *in vivo* experiments and in a series of preclinical studies. A small number of clinical trials for prostate cancer, glioblastoma, mesothelioma and gastrointestinal cancer have already been carried out using the HSVtk/GCV system with some success (Rajab et al., 2013). This strategy could be very well-utilized to supplement existing gene therapy by providing a safety factor in the event of detrimental outcomes (Duarte et al., 2012). Co-expression of the HSVtk suicide gene along with a therapeutic gene can serve as a built-in safety switch, so that if there is an abnormal growth of transduced cells, treatment with ganciclovir can eradicate all the transduced cells. This strategy has been applied in experiments for an allogenic bone marrow transplantation trial in animals to reduce graft versus host disease (GvHD). In allo-transplants, the donor T cells are desirable, initially, to resolve anti-leukemic effect. However, they become undesirable later when they also induce GvHD. In one study, a retroviral transduction of HSVtk into donor T-cells was performed before transplantation and as soon as GvHD appeared, T-cells were shown to be destroyed following GCV treatment (Bonini et al., 1997).

1.4.2 Role of Inducible Promoter in SGT

Systemic treatment of AAV vectors with ubiquitous promoters brings widespread expression of transgenes, in a random fashion (Naso et al., 2017). SGT gene induction in such vector systems can induce widespread damage of tissues with GCV treatment, which is highly undesirable. To induce targeted, localized, and controlled therapeutic gene expression, one approach could be to use stress-inducible promoters. Heat shock protein (HSP), one of the most

notably studied stress proteins, is highly expressed in tumor tissues in response to environmental and pathophysiological stimuli, such as glucose deprivation, anoxia, and acidic pH 6.0-8.0. HSP promoters, particularly HSP70, have been used quite often in gene therapy strategies because they are both heat-inducible and efficient (Kaur et al., 2000; Rome et al., 2005). Incorporation of the HSP70 promoter for HSVtk expression, thus for SGT, can potentially provide inducible and location-specific expression of HSVtk and will also address the safety concern by localized and controlled killing of tumors (Isomoto et al., 2006).

1.5 Study Rationale

The success of rAAV in gene therapy pre-clinical trial holds potential for future gene therapy, but on the other side, requires development of a system of safe delivery. Most of the work in this field has been to improve vector backbones on both the retrovirus and lentivirus, and has shown significantly improved results. Consequently, we planned to devise a similar proof-of-concept experiment for the rAAV gene delivery system. SGT-based treatment has already been used successfully to overcome the risk of carcinogenesis; therefore, the aim of this study was to test this method in an rAAV vector. The objective was to develop a targeted HSVtk system which can be used as a safety mechanism with therapeutic gene delivery and inducible only when needed. When the therapeutic gene expression is needed for long-term, then the cells harbouring the gene will keep producing the therapeutic protein as desired. However, if the transduced cells become oncogenic, only tumor-forming cells will be killed by inducing HSVtk with local and targeted heating of the tumor (planned to be achieved by high frequency ultrasound waves or laser treatment) along with systemic GCV treatment.

This study was the initial step in the development of a “safety switch” towards making rAAV viral vector gene therapy safer. This construct, if proven to work, would be a beneficial tool for rAAV based gene therapy carrying therapeutic genes with a built-in safety switch to address the concerns of insertional mutagenesis and oncogenesis.

1.6 Hypothesis

My hypothesis is that the use of the HSP70 promoter in a dual promoter-transgene construct will result in high temperature-inducible transcription of HSVtk and apoptosis of only HSVtk expressing cells in a confined and targeted way. This targeted suicidal gene therapy can be applied as a safety switch in cases of tumorigenesis induced by rAAV gene therapy.

1.7 Aims and Objective

My study objectives are: 1) to construct a bicistronic dual-promoter AAV vector which can mediate expression of two transgenes – a. green fluorescent protein (GFP) under a ubiquitous promoter - chicken β-actin (CAG), and b. HSVtk under the HSP70 promoter 2) to test the function of the newly-constructed vector *in vitro* using a stably-transfected cell line to demonstrate selective, inducible cell killing.

Chapter 2

Literature Review

2.1 Gene Therapy

Gene therapy is defined as administration of genetic material in human cells which alters expression of a gene or biological product of the gene for therapeutic purpose (FDA, 2018). The concept of gene therapy was first seen during the 1970's when it was discovered that mutations in genes result in diseases. However, the first clinical breakthrough experiment in humans with gene therapy was done in 1990 (Rosenberg et al., 1990). Since then, it has progressed rapidly and is the most promising future therapy for various diseases (Boye et al., 2013). Early on, gene therapy was effective only in diseases caused by a single gene mutation. However, further research has led to its application in multifactorial and somatic genetic diseases, such as cancer (Barzon et al., 2004; Aiuti et al., 2009; Boye et al., 2013; Testa et al., 2013; Kumar et al., 2016).

2.1.1 Process of Gene Therapy

The process of gene therapy begins with localizing the defective/non-functional gene which is responsible for disease causation. The next step is to create a gene which can simulate function of non-functioning gene. This experimental gene is then introduced into targeted cells to improve function and overcome disease process. The function can be improved by replacing the disease producing gene or inhibit the disease-causing gene. The details of the techniques and types which are commonly employed in gene therapy are also described below in a summarized manner. In simple words, process of gene therapy involves adding a new gene to compensate for defective or missing gene in the body. The process is illustrated as a simplified diagram in Figure 1 below.

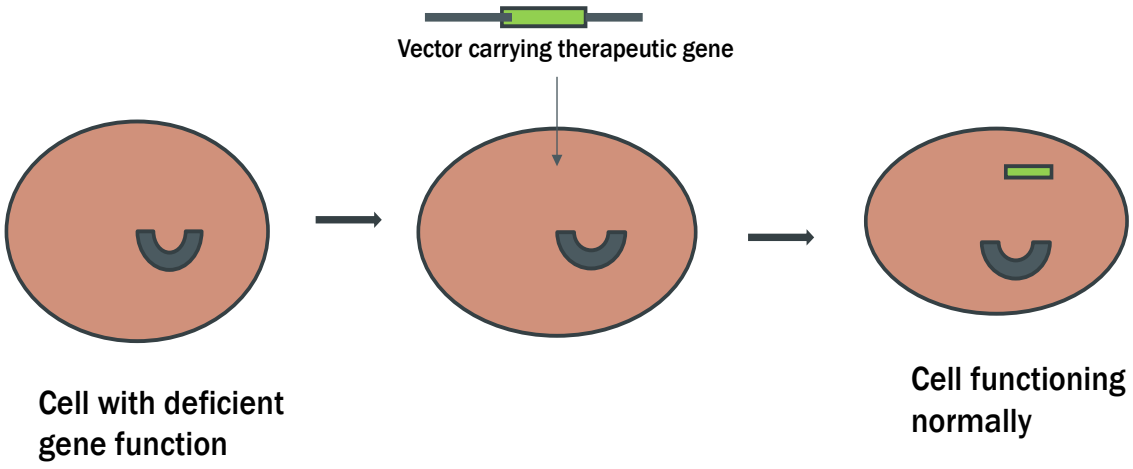


Figure 1. Gene Therapy

A simplified diagram showing the process of gene therapy. The cell on the left side of the diagram contains half circle representing a defective gene. In the gene therapy, new DNA represented by a rectangle in a vector is introduced into the cell with a deficient gene. The new vector integrates into the cell to help modify disease outcome and help the cell to return to its normal state of function.

2.1.2 Techniques of Gene Therapy

There is a rapid increase in the number of ongoing experiments in the field of gene therapy, the new trials not only include new diseases but more and more different designs and strategies of gene therapy are being investigated. However, there are three basic techniques in conventional gene therapy which depend upon the type of mutation and its effect in causing a disease. The three types of conventional gene therapy are: gene replacement therapy, gene inhibition therapy, and gene therapy as a cell killer.

Further to this, there has been rapid progress made in the field of genome editing. Genome editing is a very precise technique which allows for targeted cleavage and repair, only at the defective site (Gupta & Musunuru, 2014).

2.1.2.1 Gene Replacement Therapy

This is the most basic and initial form of gene therapy. In cases where a known mutation is responsible and disease results as mutation results in production failure of functioning protein; a replacement of mutated gene with functional gene would treat the disease (Figure 2). As an example, cystic fibrosis is caused by defect in cystic fibrosis transmembrane conductance regulator (CFTR) gene; this gene is responsible for production of salt regulating protein. In gene therapy trial, adenovirus carrying CFTR will insert this gene in targeted cell resulting in correction of defect and normal production of required protein (Quon & Rowe, 2016).

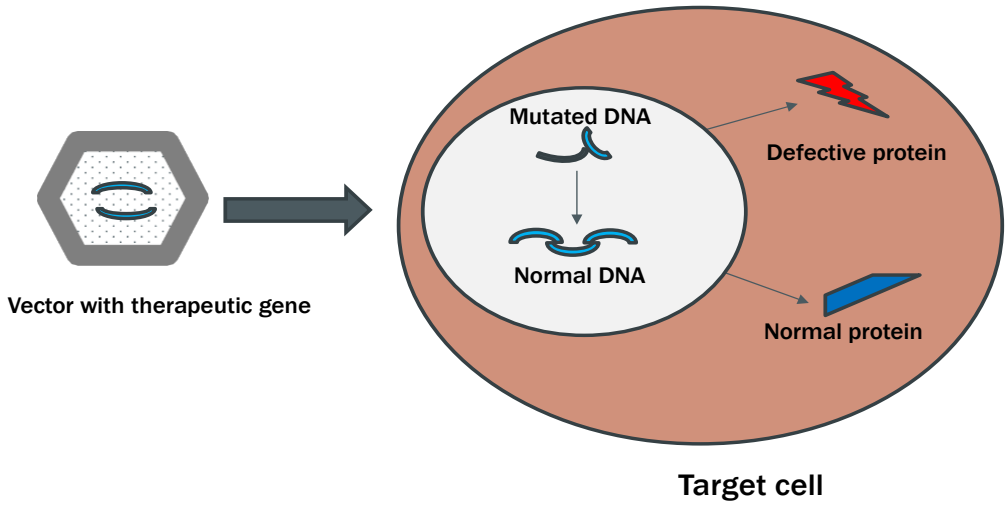


Figure 2. Process of Gene Replacement

The therapeutic gene introduced in gene therapy will replace a disease-causing mutated gene. Once integrated into the cellular DNA, the new normally functioning gene leads to the production of the normal protein, resulting in elimination of the disease.

2.1.2.2 Gene Inhibition Therapy

Certain diseases result from the failure of normal inhibitory process of gene expression or the product of a gene. This type of therapy aims at inhibition of ongoing defective activity. The typical example is carcinogenesis whereas stimulation of oncogene shifts cellular production

from controlled manner to abnormal growth. A new functional gene can interfere this process either by inhibiting the expression of the stimulatory gene or upregulation of the inhibitory gene which can counter act the activity of faulty gene (Figure 3).

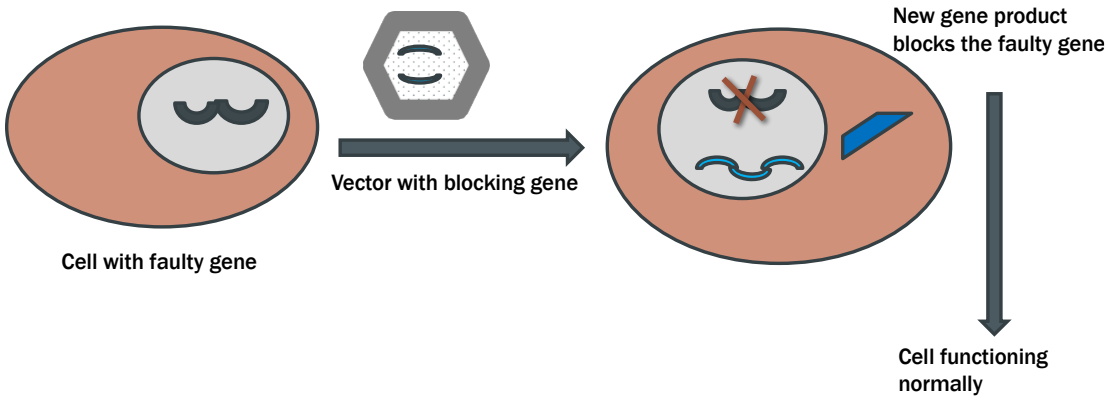


Figure 3. Process of Gene Inhibition

The therapeutic gene introduced in gene therapy will deactivate the disease-causing mutated gene. This process is valuable where a disease-producing gene is causing defects due to excessive production of either normal or abnormal proteins. Once integrated into the cellular DNA, the inhibition leads to the production of the normal protein, resulting in elimination of the disease.

2.1.2.3 Gene Therapy as Cell Killer

The third technique is one in which gene therapy is directed to kill cells. The aim is that newly inserted DNA will lead to cellular destruction. Again, this technique is valuable in disease resulting from excessive growth like cancer or infection. This can be implemented in various ways. One of them is using pre-existing immune response to target the defective cell. The newly inserted DNA could express proteins that would make cells more vulnerable to the immune system. Now the immune system could easily identify them as a foreign body and activate an immune reaction that leads to cellular death. One example of this is gene immunotherapy in cancer. Usually cancer cells are deficient in immune recognition, which allows these cells to escape from the immune system. When the appropriate gene is introduced into the cancer cell, the

resultant protein expression on the cell surface can be identified by the immune system (Rangel-Sosa et al., 2017). Correct identification allows the immune system to destroy cancer cells using antigen-antibody reaction and/or other pathways. A second form of directed killing is cell 'suicide'. The inserted DNA could either stimulate apoptotic pathways for cell death or convert a non-toxic product to toxic to kill the diseased cell (Figure 4).

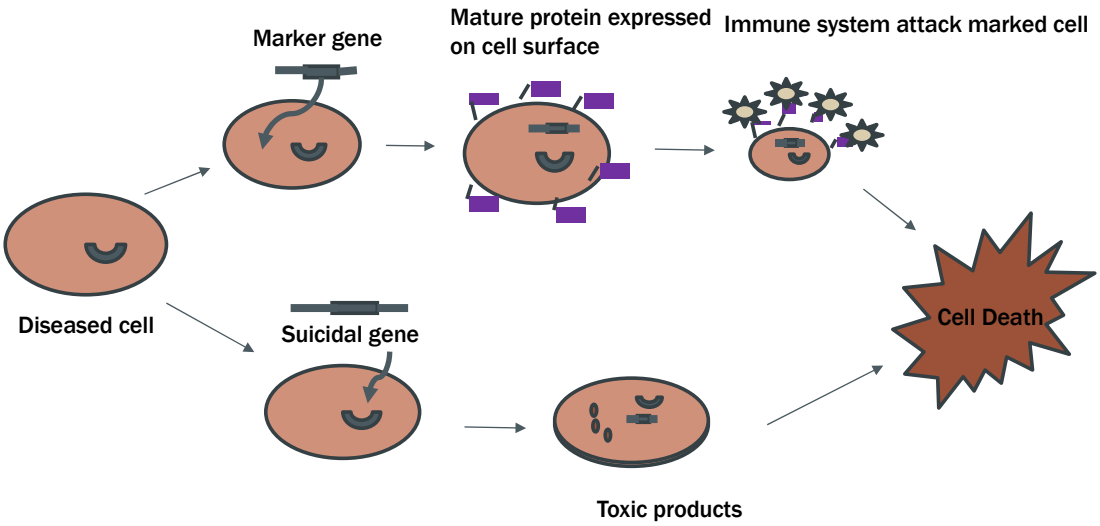


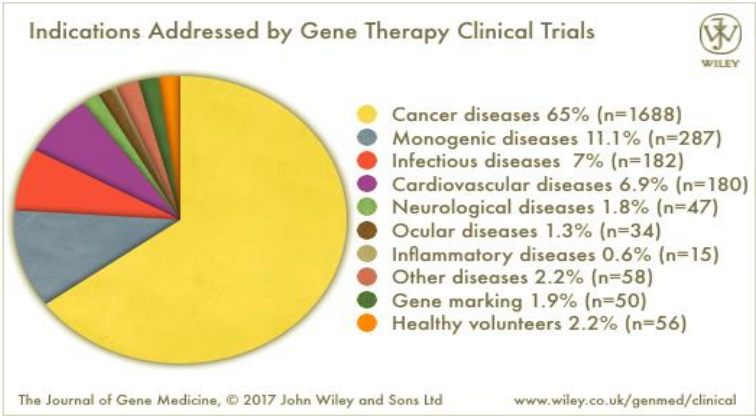
Figure 4. Process of Cell Killing with Gene Therapy

The therapeutic gene will lead to death of the receiver cell by different mechanism; this is important in case of excessive cellular growth. The upper part of diagram indicates use of marker gene as an example of cancer immunotherapy. The recipient cell expresses marker on cell surface which makes it attractive to immune system leading to destruction. In the bottom part of diagram, a suicidal gene is introduced which could stimulate cell apoptosis leading to cell death.

2.1.3 Applications of Gene Therapy

The role of gene therapy is consistently growing. Nearly 2000 trials have been enrolled in gene therapy to date since 1989, in almost every field of medicine. Of these, 65% have been in cancer; followed by monogenic diseases, which account for 11%, and infectious diseases accounting for 7% (Hanna et al,. 2017). The more recent applications are in cardiovascular and

neurological diseases. The use of gene therapy in inflammatory diseases is quite interesting as these were not typically considered with genetic background. The Journal of Gene Medicine provides extensive data regarding current and past ongoing genetic trials ("Gene Therapy Clinical Trials Worldwide Database"). Figure 5 shows cancer being the most commonly studied group.



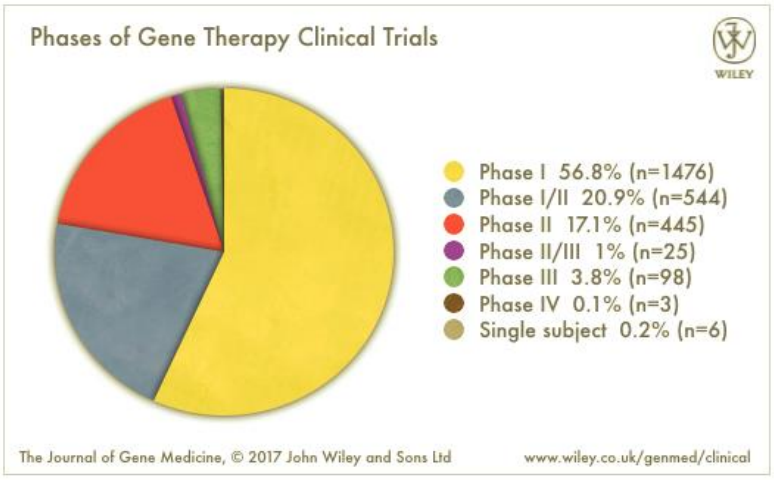
Indications	Gene Therapy Clinical Trials	
	Number	%
Cancer diseases	1688	65
Cardiovascular diseases	180	6.9
Gene marking	50	1.9
Healthy volunteers	56	2.2
Infectious diseases	182	7
Inflammatory diseases	15	0.6
Monogenic diseases	287	11.1
Neurological diseases	47	1.8
Ocular diseases	34	1.3
Others	58	2.2
Total	2597	

Figure 5. Current Gene Therapy Indications

Data accessed from **http://www.abedia.com/wiley/indications.php** on 22 Feb. 2018 with permission from Wiley on line library. To date two thousand five hundred ninety-seven trials have been registered for gene therapy, out of which, sixty five percent are for cancer related diseases followed by monogenic disorders, with ongoing work also occurring in cardiovascular, neurological, ocular and infectious diseases.

Note. See permission attached as appendix.

Another important finding to note is that, although number of trials has increased over the years, more than half are still in phase one and less than one percent are currently reaching the phase five stage ("Gene Therapy Clinical Trials Worldwide Database"), as shown in Figure 6.



Phase	Gene Therapy Clinical Trials	
	Number	%
Phase I	1476	56.8
Phase I/II	544	20.9
Phase II	445	17.1
Phase II/III	25	1
Phase III	98	3.8
Phase IV	3	0.1
Single subject	6	0.2
Total	2597	

Figure 6. Gene Therapy Clinical Trials

Data accessed from http://www.abedia.com/wiley/phases.php

on 22 Feb. 2018 with permission from Wiley on line library. Although total registered trials are two thousand five hundred ninety-seven, more than half were as phase I, with less than five percent reaching stages beyond phase III.

Note. See permission attached as appendix.

This is also reflective in practical therapeutic strategies. Although the first trial was done in 1989, beyond trials, the first gene therapy was not approved for clinical application until recently: 2012 in Europe and 2013 in USA. There are only a few genetic therapies which are being approved for clinical use. The FDA approved the first genetic therapy strategy in 2013; Kymriah which is a chimeric antigen receptor T cell therapy approved for the treatment with B-cell precursor acute lymphoblastic leukemia (ALL) which are either refractory or in relapse. Last year in 2017, there are two other gene therapies that got approval from FDA to be used as a part of clinical settings. This included Imlygic, which is a local treatment of unresectable melanoma. And Luxturna, for patients with a specific form of mutation-associated retinal dystrophy (FDA, 2018).This situation is similar in Europe, where only three therapeutic strategies have been approved for clinical use (Touchot & Flume, 2017).

2.2 Cancer Gene Therapy

Cancer is an acquired genetic disease where mutations in genes allow cells to gain an ability to proliferate in an uncontrolled manner and invade surrounding cells. Over the last few decades, a better understanding of mechanisms on the molecular level has resulted in newer strategies to treat cancer. Conventional therapies to treat cancer diseases include chemotherapy, radiotherapy, and surgical resection. However, many cancers remain resistant to these treatments, or the prognosis remains poor.

Gene therapy has been used in multiple ways to treat cancer cells or to synergistically enhance the current treatment. This approach is based on modifying the cellular genome to introduce benefits related to cancer therapy. The various ways in which gene therapy has been used include: mutational correction (for known mutations), immune response enhancement against the tumor cell, tumor cell lysis by selective replicate viruses causing targeted lysis of tumor cells and suicidal gene therapies, and tumor vasculature inhibition. It has also been used in

chemo protective therapy by protecting bone marrow cells from the effects of high doses of chemotherapy (Cross & Burmester, 2006).

2.2.1 Molecular Basis of Carcinogenesis

Cancer cells develop once normal cells suffer DNA damage due to several external stimuli. Normal cells have a mechanism of DNA repair, however unsuccessful repair leads to mutations in genomes, which lead to either activation of growth promoting oncogenes or inactivation of cancer suppression genes. Under normal conditions, suppression of aberrant growth is maintained by a balance between inhibition of a tumor-suppressing gene and activation of the proto-oncogene, while an imbalance of these, results in malignant proliferation of cells. The characteristics features of cancer cells include independence in growth signals, poor response to anti-growth signals, unlimited replication, continued angiogenesis, inability to repair DNA, apoptotic dysfunction and metastasis (Fouad & Aanei, 2017). Another pathway of acquiring mutant genes is by inheritance, which can also result in malignant transformation.

2.2.2 Cancer as a Genetic Disease

Cancer is considered a genetic disorder for multiple overlapping reasons, including; the discovery of somatic mutations and genetic selection in single cells, inherited germline mutations in rare familial cancer syndromes, increase in mutation rate or genomic instability increases cancer frequency, and that aneuploidy is a hallmark of cancer cells. For these reasons, gene therapy has emerged as an appropriate tool approach for mechanistic identification and the treatment of cancer.

2.2.3 Different Methods of Cancer Gene Therapy

Gene therapy has been used in multiple ways to treat cancer cells or to enhance the current treatment by synergism. It is based on modifying the cellular genome for benefits related to cancer therapy. The various ways gene therapy has been used is listed below in Table1.

Table 1. Gene Therapy in Cancer

Techniques of gene therapy used in experiment to treat cancers.

Gene therapy technique	Mechanism	Gene as an example	Related cancer
Tumor Suppressor Gene (Kuball, 2002)	Stopping the tumor growth by restoring normal copy of tumor suppressor gene	p53	Glioma, head and neck cancer
		Breast cancer gene (BRCA1)	Breast cancer
		Retinoblastoma (RB)	Retinoblastoma
Oncogene (Alvarez et al., 2000)	Correcting genetic related cancer	Erythroblastic leukemia (ErbB)	Erythroblastic leukemia
		Kristen Rat sarcoma virus (K-ras)	Lung cancer
Anti-angiogenesis (Im et al., 2001)	Inhibition of tumor vasculature leading to ischemic death	Vascular endothelial growth factor (VEGF)	Non-small lung cancer
Immunotherapy (Rosenberg et al., 2014)	Destruction of tumor cell by immune mediated pathway	Interleukin-2	Renal cancer
Chemo-protective therapy (Adair et al., 2012)	Bone marrow protection from toxic effect of chemotherapy	P140K	Glioblastoma
Oncolytic Biotherapy (Wollmann et al., 2012)	Rapid viral replication leading to target cell death	Herpes simplex virus	Glioblastoma
		Herpes virus	Brain tumor
Suicide gene therapy (Rajab et al., 2013)	Expression of prodrug gene –active drug causing cell death	HSVtk	Hepatocellular carcinoma

2.3 Suicide Gene Therapy

'*A suicide gene is a genetically encoded molecule that allows selective destruction of transduced cells or gene modified cell'* (Jones et al., 2014).

2.3.1 Types of Suicide Gene Therapy

Suicide gene therapy is divided into three groups according to mechanisms of action. Jones et al. grouped them into gene-directed enzyme prodrug therapy (GDEPT), dimerization

induction, and therapeutic monoclonal antibody mediated, as shown in Figure 7 (also adapted from Jones et al., 2014).

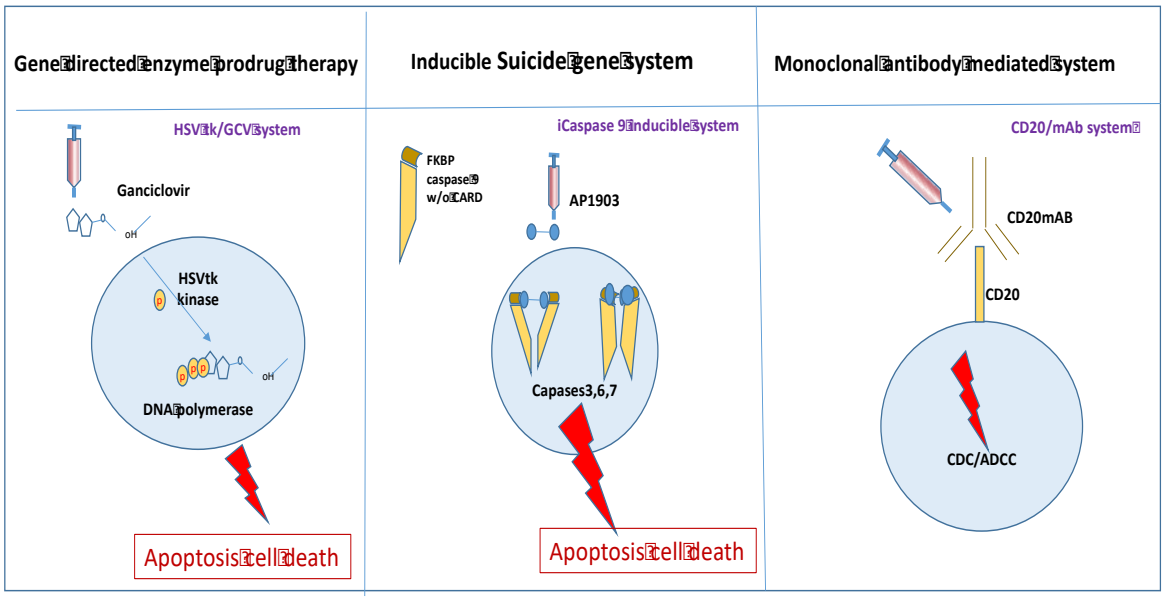


Figure 7. Different Types of Suicide Gene Therapy

Three types of suicide gene therapy are displayed. The GDEPT is based on enzyme which can activate a prodrug into toxic form which leads to cell death. Inducible suicide gene therapy relies on cellular pre-existing machinery and transduced enzymes will activate killer pathway while antibody mediated system depends on immunogenic cascade to kill cell.

Note. Adapted from Jones et al 2014.

2.3.1.1 Gene-directed Enzyme Prodrug Therapy (GDEPT)

GDEPT is also known as virus-directed enzyme prodrug therapy (VDEPT) (Huber et al., 1995) and gene prodrug activation therapy (GPAT) (Eaton et al., 2001). GDEPT is the most well-known and broadly researched type of suicide gene technology and term GDEPT is synonymously used as suicide gene therapy (Stedt et al., 2015). This technique aims to lyse

genetically modified cells by converting a nontoxic compound into the lethal drug; it was first introduced in 1986 in K3T3 sarcoma tumor cell lines, mouse model (Moolten, 1986).

GDEPT is comprised of two basic steps. The first is to introduce a gene into the tumor cell using a vector (delivery system) that encodes for the enzyme. Then a less toxic prodrug is systematically administered. This causes lysis of the transduced tumor and neighbouring unmodified cells, due to the bystander effect (described in detail in chapter 2.3.3.5).

2.3.1.2 Inducible Suicide Gene System

This technique relies on endogenous apoptosis machinery to induce cell death (Jones et al., 2014). The main advantage of an inducible gene is that it does not depend on the cell cycle or needs a dividing cell to act, which is very vital for slow growing tumors. It also bypasses the step of prodrug activation to active drug for cell lysis. Rather, it uses chimeric proteins, which can link with components of the apoptotic pathways resulting in dimerization and induction of the process. An example includes the inducible Fatty-acid synthase (FAS) or the inducible Caspase 8 system (Zhou et al., 2014).

2.3.1.3 Monoclonal Antibody Mediated System

This system is based on protein expression in the plasma membrane. A genetically modified cell expresses a protein on the surface and is then removed by a specific monoclonal antibody. One example is the cytosine deaminase CD20/anti CD20 system. This is where a retrovirus is used to deliver CD20 into T cells, which is then followed by anti CD20 monoclonal antibody post T cell infusion (Griffioen et al., 2009).

2.3.2 Pros and Cons of Various Suicide Gene Systems

Marine et al. designed an experiment to compare the *in vitro* effectiveness of all three types. The system from each type used included: HSVtk, iCasp9, and CD 20 Codon. Interestingly, results proved that they were equally effective in killing transduced T cells;

however, with respect to time, immediate killing was observed using iCasp9 and CD20 while HSVtk/GCV demonstrated an effect after three days (Marin et al., 2012).

The design of suicide gene therapy should be selected according to application and consider other factors like target cell, source of the suicide gene (GDEPT are viral based while an inducible gene is always human), and onset of action. Currently, only GDEPT and iCasp system have been utilized as a part of clinical trials (Jones et al., 2014).

2.3.3 Gene-Directed Enzyme Prodrug Therapy (GDEPT)

Suicidal gene therapy is often used synonymously with GDEPT as it is the most common technique used in cancer gene therapy.

2.3.3.1 Steps Involved in GDEPT

There are three basic steps involved in GDEPT. The first step is construction of a recombinant vector which carries the cloned gene and delivery of a recombinant product to target cells using a carrier. The use of promoters, which are unique to tumor cells to restrict gene expression to the target cells, is important at this step. Once the vector is ready, and transcription and translation of the gene into an enzyme occurs inside the target cell, the last step is systemic administration of a prodrug. The prodrug is taken up by cells and is converted to a toxic compound only in cells carrying specific enzymes. As such, even if healthy cells were to take up the prodrug, they would be at minimal risk as the conversion to a toxic drug is preferentially tumor-cell specific (Zhang et al., 2014).

2.3.3.2 Prerequisite of Efficient and Effective Suicide Gene Therapy

Among the prerequisites for GDEPT, the first and foremost is the choice of suicidal gene/enzyme system to be used. There are some specific requirements of the enzymes, which are being used in a suicide gene therapy system: (i) enzyme activity should result in full activation of the prodrug without any intermediate products, (ii) the enzyme should have high catalytic

activity, and (iii) the enzyme should not have any similarity with intrinsic enzymes and should not express in healthy tissues. The second prerequisite is the selection of prodrug and the corresponding toxic drug, a very important choice. The combination of prodrug and the toxic drug should carry some specific characteristics (Niculescu-Duvaz et al, 2004). The prodrug should have good pharmacokinetic and pharmacological properties, it should be minimally toxic, chemically stable in all physiological conditions, and highly diffusible in tumor tissue. The prodrug should be activated effectively by the enzyme system in use yet be of low toxicity; while the active product should have maximal toxicity and potency for tumor-targeted cells. The active compound should be converted into active drug only by transduced enzyme and not by any endogenous enzyme, and should have a long half-life and high diffusibility to be efficient for the bystander effect. Ideally, the active drug should be effective in all phases of the cell cycle i.e. dividing and quiescent. The third important consideration is the choice of vector/carrier to deliver the chosen suicide gene. The vector should be non-immunogenic and non-toxic in ideal conditions, and should be efficient in transduction of tumor cells for high levels of expression (Niculescu-Duvaz etal., 1998).

2.3.3.3 Enzyme Prodrug System

With respect to the choice of an enzyme prodrug system, both mammalian and non-mammalian enzymes are available. However, non-mammalian systems are more frequently used, although their main drawback is being immunogenic. The system is usually selected by the characteristics of the targeted tumor cell. Examples for non-mammalian enzymes include viral thymidine kinase (TK), bacterial cytosine deaminase (CD), bacterial carboxypeptidase G2 (CPG2), purine nucleotide phosphorylase (PNP), thymidine phosphorylase (TP), nitro reductase (NR), D-amino-acid oxidase (DAAO), xanthine–guanine phosphoribosyl transferase (XGPRT), penicillin-G amidase (PGA), β-lactamase (β-L), multiple-drug activation enzyme (MDAE), β-galactosidase (β-Gal), horseradish peroxidase (HRP), and deoxy ribonucleotide kinase (DRNK).

Groups of mammalian enzymes used in GDEP include deoxycytidine kinase (dCK), carboxypeptidase A (CPA), β-glucuronidase (β-Glu), and cytochrome P450 (CYP) (Niculescu-Duvaz & Springer, 2005). Although they have the benefit of being non-immunogenic, transfected enzymes need to be modified to prevent activation in non-tumor cells.

There are two common ways to compare the therapeutic efficacy between different GDEPT systems, including kinetic parameters like K_M, $V_{max.}$ and biological parameters (Springer & Niculescu-Duvaz, 2000). The values of K_M and V_{max} indicate the amount of substrate required to reach half of the reaction's maximum velocity, and maximum velocity of the activation reaction respectively. In general, a low K_M and the higher V_{max} represents a trustable system (Springer & Niculescu-Duvaz, 2000). See Table 2 for details of common GDEPT systems with Kinetics.

Table 2. Commonly Used GDEPT Systems

ENZYME/ORIGIN	PRODRUG	DRUG	K_M[18]	V_{MAX}[18]	MECHANISM
Herpes simplex virus thymidine kinase (HSVtk) (Viral)	Ganciclovir (GCV)	Ganciclovir triphosphate (GCV-3P)	11–15.8	1.3–22 x 10–3	Inhibit DNA polymerase, leading to replication failure and cell death. -apoptosis
Cytosine deaminase (CD) (E. coli / Yeast)	5-Fluorocytosine (5-FC)	5-Fuorouracil (5-FU)	17,900 / 800	11.7 / 68	Inhibition of thymidylate synthase, leading to inhibition of protein synthesis and DNA breakdown. - Apoptosis
Hepatic cytochrome p 450 (human)	Cyclophosphamide Ifosamide	Phosphoramide mustard	300	39.1	Alkylating agent - Apoptosis and necrosis
Carboxyl esterase (CE) (Human/Rabbit)	Irinotecan	SN-38	23-52.9	1.43 X 10^{-3}	Inhibition of topoisomerase 1. Inhibition of DNA transcription
Varicella –Zoster virus thymidine kinase (viral)	ara-M	ara -ATP	56	680b	Pyramidine nucleoside pathway
Nitro reductase (NR) (E. coli)	CB1954	N-acetoxy derivatives			Induce rapid cell death by forming interstrand DNA cross-links
Carboxypeptidase G2 (CPG2) (bacterial)	CMDA	CMBA,	3.4		DNA cross linking

2.3.3.3.1 Herpes Simplex Virus Thymidine Kinase-Ganciclovir (HSVtk/GCV)

The HSVtk/GCV system is the most widely used combination both *in vivo* and *in vitro* experiments. The recombinant plasmid containing Herpes Simplex Virus Thymidine kinase is transfected into tumor cells. Following the expression of the gene, the viral thymidine kinase is transcribed and translated. The efficacy of viral thymidine kinase is 1000 times more than its mammalian counterparts as it phosphorylates guanine-ganciclovir (GCV) into GCV monophosphate, which is then converted into its ultimate toxic products. This final conversion of GCV monophosphate to triphosphate is conducted by cellular kinases. GCV is an acyclovir derived prodrug 9-([hydroxyl-1-hydroxymethyl] methyl) guanine (Altaner et al., 2008).

In an actively dividing cell, the mechanism of death is attributed to apoptosis. GCV triphosphate inhibits DNA polymerase and competes with its analogue, 2-deoxyguanosine triphosphate (dGTP), which leads to termination of strand formation in S-phase. This can result in early apoptotic death or slow death due to necrotic sub G1 DNA fragmentation. Several experimental models have shown the efficiency of this system including glioma, leukemia, bladder cancer, liver cancer, oral, and colon adenocarcinoma. The success of preclinical experiments leads towards some clinical trials, however positive results were yet to be obtained as there is still a need for improvement of this system (Rajab & Nelson, 2013). The limitations are related to gene targeting and targeted drug delivery. This is because its toxic product is only useful in dividing cells, with limited efficacy in slow growing or solid tumors, where only a limited number of cells are in the dividing phase. There are also issues with drug concentration and toxicity. Bone marrow depression is one of the side effects if GCV is used in high concentrations. When the prodrug is used in a low concentration to prevent this side effect, it results in poor concentration within the target cells due to high diffusibility. This is opposite to its active toxic form, which is insoluble with poor diffusibility limiting its bystander effect. The only way for GCV triphosphate to have a bystander effect is through gap junctions. *In vitro*

experimentation demonstrated an interesting effect; as active GCV was released from dying cells, it was taken up by non-transduced cells through phagocytosis, resulting in an effect similar to the bystander effect. The HSVtk/GCV system and its by stander effect is shown in Figure 8.

There are several efforts underway to improve this system, which include experiments to enhance gene expression using radiation to upregulate promoters, increase solubility of the system to get more penetration by combining it with collagenase, which can degrade the extracellular matrix. Another attempt to increase anti-tumor activity has been made to combine with multi-drug resistance-1 (MDR-1) targeted small hairpin RNA (shRNA) to inhibit p-glycoprotein, which can increase intracellular drug accumulation and sensitivity to the drug (Park et al., 2008). The experiments were also performed using different prodrug with acyclovir and valacyclovir to achieve high efficacy. The results were equivocal. Attempts were also made to improve the bystander effect using fused protein i.e. proteins to promote intercellular export/import combined with HSVtk, as well as by increasing intracellular drug accumulation and sensitivity to a drug (Zhang et al., 2014).

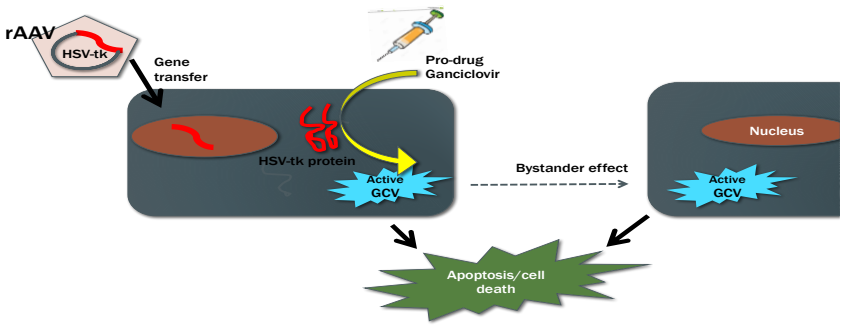


Figure 8. HSVtk Suicide Gene Therapy and Bystander Effect.

HSVtk is carried to cell using carrier /vector. Once inside the cell, after transduction the HSVtk enzyme is transcribed. Now if GCV prodrug is given, only the cells with HSVtk enzymes will convert GCV into its active toxic form and will kill the cell.

2.3.3.3.2 Cytosine Deaminase/5-Fluorocytosine Prodrug; CD/5-FC

Another commonly used, non-mammalian enzyme is cytosine deaminase (bacterial or fungal in origin) system. Cytosine deaminase converts cytosine into uracil, which is an important component of the nucleotide salvage pathway. It uses 5–fluorocytosine (5-FC) as a prodrug, which is converted into a toxic chemotherapeutic agent, 5 –fluorouracil (5-FU). The main benefit is the ability to mitigate the toxicity of 5-FU by targeting it specifically into cancer cells. Once 5-FU is formed, it needs cellular enzymes to be converted into three active antimetabolites of the pyrimidine pathway (5-fluorodeoxyuridine mono and triphosphate, 5-fluorodine triphosphate). This can lead to cell death in three distinct pathways, but primarily through apoptosis, which is enhanced further by inhibiting thymidylate synthase irreversibly or by incorporating in DNA and RNA complexes. 5-FU is a small molecule and is very easily diffusible, thus making it efficient in the bystander effect (Altaner, 2008; Rajab & Nelson, 2013; Zhang et al., 2014). *In vivo* and *in vitro* success has been seen for this system in breast cancer, hepatic metastasis of colon carcinoma and prostate cancers. The observed efficacy of CD/5-FC and HSVtk/GCV was the same in hepatocellular carcinoma, but superior in renal and colorectal carcinoma.

2.3.3.4 Vector Delivery Systems Used for Suicidal Gene Therapy

Once a suitable enzyme/prodrug system is selected, the next step is to find the delivery vector. Suicidal gene therapy has been tried with both viral and non-viral vectors. Usually, viral vectors are preferred due to prolonged efficiency. Tumor selectivity can be increased by vector design.

2.3.3.4.1 Viral Vectors

As viruses exist in nature, they have already derived the mechanisms by which to get access to a cell's genome. For specific gene delivery, this mechanism is exploited and they are designed to replace a non-essential genomic part with a foreign therapeutic gene. The commonly used viral vectors include adenoviruses, retroviruses, vaccinia virus, poxviruses, adeno associated

viruses, herpes simplex and lentiviruses. The biggest side effects are mutagenic integration or insertional mutagenesis (retroviral and lentivirus) and inflammatory toxicity (adenovirus). Different strategies like promoter-enhancer sequence, use of chromatin insulator to prevent enhancer-promoter interaction, cell type specific expression and targeted transduction and alterations in choice of backbone have been suggested to prevent insertional mutagenesis (Kawat et al., 1991; Huber et al.,1994; Hacein-Bey-Abina, 2003).

2.3.3.4.2 Non-Viral Vectors

Due to the aforementioned safety concerns of viral vectors, researchers are becoming interested in the development of non-viral vectors. Non-viral vectors can be used in the form of naked DNA, physical techniques and chemical methods. Currently, several trials registered with naked DNA have similar results like adeno or lentivirus but the main disadvantage is low uptake and that they can be cleared very rapidly. Physical techniques like hydrodynamic, gene gun, and electroporation are in use as well (Kawakami et al., 2008; Al-Dosari & Gao, 2009). Chemical methods using cationic components of vectors like in cationic liposomes & cationic polymers are the most commonly used non-viral gene delivery systems. These positively charged particles use their electrostatic interactions with negatively charged DNA to form either lipoplex or polyplex compounds. Advantages of lipoplexes include physiochemical versatility, which can cause numerous modifications, easy and inexpensive large-scale production and low immunogenic response. Although extensive work has been done using lipoplexes and clinical trials have been registered, poor *in vivo* transfections limit their use. Their second major limitation is toxicity due to the potential of making aggregates after systemic administration (Simões et al., 2005).

2.3.3.4.3 Cellular Vehicles

Recently, some mammalian cells like mesenchymal stem cells, bone marrow stromal cells, and neural stem cells have been considered as delivery vehicles due to their tumor–tropism.

Another attractive group of cells is endothelial progenitor and blood outgrowth endothelial cells as they can target tumor vasculature (Dudek, 2010; Kim, 2010).

2.3.3.4.4 Miscellaneous

Other effective vehicles that have been used include other non-viral biological agents like bacteria, bacteriophages, virus-like particles, erythrocyte ghosts and exomes and nanoparticle-based delivery systems. Bacterial oncolytic therapy uses the ability of obligate anaerobic bacteria to colonize in the hypoxic area of the tumor. These bacterial vectors can be altered to carry bacterially-expressed DNA, which later can encode for the gene of interest in the target cell (Seow & Wood, 2009). Nano particles have a unique ability of enhanced permeability and retention effect, which results in accumulation of these particles in solid tumors after systemic administration (Jabr-Milane et al., 2008).

2.3.3.5 Bystander Effect

The bystander effect is an important mechanism of cell death. This is where cell death occurs in the cells surrounding the transfected cells (Edwards et al., 2004). This mechanism is an important characteristic of suicidal gene therapy and the efficacy of the system depends on the effective bystander effect of an active drug. Both local and distant bystander effects have been observed during in *vitro* and *in vivo* experiments (Duarte et al., 2012).

Several mechanisms have been established to explain how this effect works, including: passive diffusion, use of gap junctions, endocytosis of apoptotic vesicles, release of soluble factors and activation of the immune response (Duarte et al., 2012).

2.3.3.5.1 Local Bystander Effect

The local bystander effect could be in various forms like non-facilitated diffusion, gap junction mediated, phagocytosis etc. as shown in Figure 9. In non-facilitated diffusion, the movement in and out of cells does not require cell-to-cell contact. This has been observed

strongly in CD/5-FC system in a colorectal mouse model. Although only 4% of cells expressed the enzyme encoding gene, tumor regression was effective. Soluble factors can also induce the bystander effect, independent of cell-to-cell contact. On the other hand, in gap-junction mediated diffusion, it is essential to have cell-to-cell contact. In the case of the HSVtk/GCV system, the bystander effect is mediated through gap junctions, as active GCV cannot diffuse due to poor solubility. Another interesting pathway is when non-transduced cells phagocytose the apoptotic bodies, as these get generated from dying cells and follow the same fate after engulfing them. It is also observed in the HSV tk/GCV system (Freeman et al., 1993). The local bystander effect can also be mediated using a cellular vehicle expressing a suicidal gene. This was found in mouse embryonic endothelial progenitor cells, human adipose tissue- derived mesenchymal stem cells and neural stem cells.

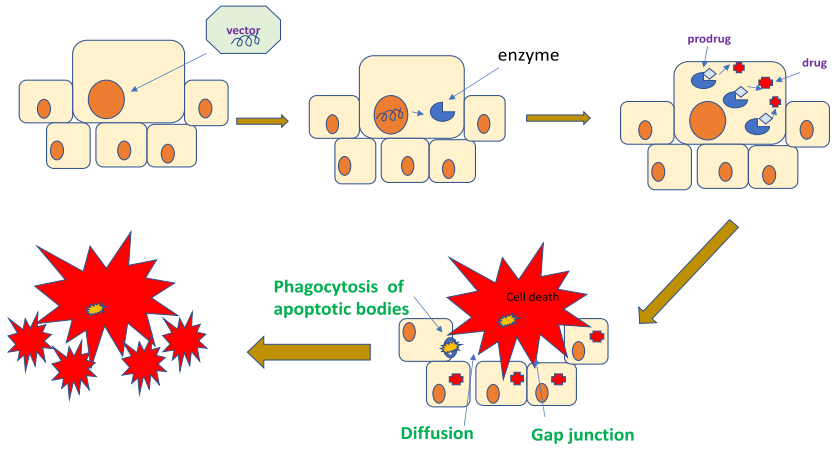


Figure 9: Bystander Effects in Gene-Directed Enzyme Prodrug Therapy (GDEPT)

GDEPT exerts local and distant by-stander effect by which the neighboring cells which are not transduced but receive toxic drug are killed. This is achieved through leaky gap junctions, diffusion and phagocytosis of apoptotic bodies containing toxic material.

Note. Adapted from Zhang J et al., 2015.

2.3.3.5.2 Distant Bystander Effect

The killing effect seen in cells distant from therapeutic gene expressing cells is called distant bystander effect. It is a commonly observed phenomenon in the HSVtk /GCV system. The mechanism is still not clear, although involvement of the immune system is the most common hypothesis. Researchers found T-cell infiltrates within the tumor when experiments were performed thus proving that the immune system might play a role in destruction of far cells (Wilson et al., 1996).

2.3.4 Strategies to Improve Suicide Gene Therapy

Although GDEPT has been known to scientists for more than two decades, its clinical success and application is still low, there is a focus towards improvement of these systems in the form of either increasing the efficacy of the enzyme, optimizing an efficient activation process, or creating an effective prodrug.

The different strategies adopted to increase enzyme efficiency include extracellular expression of enzymes and site-directed mutagenesis. The latter technique is based on alteration in the active site of the enzyme, which serves to increase the catalytic effect for the substrate. This has been used for HSVtk to raise sensitivity to GCV and ACV. It has also been described for carboxypeptidase A and the tyrosine prodrug system. Another way to make GDEPT an efficient system is to combine genes and do co-transfections in cases where several enzymes are involved in a sequential pattern. For example, the HSVtk /GCV system needs three kinases to produce an active product from GCV to GCV triphosphate. A similar approach was also successful in the CD/5-FC system. A few other approaches using the same concept include transduction of two copies of the same gene in the target cell and transfecting the target cell with two different suicide genes so two different prodrugs can be activated and synergetic effects can be obtained. Furthermore, designing or experimenting with a new drug analogue to find prodrugs with low toxicity in the old system is another approach for finding a safe system. For example, the most

extensively used HSVtk/GCV system was used with less genotoxic alternative, pencicolvir (Niculescu-Duvaz & Springer, 2004).

2.3.5 Suicidal Gene Therapy Combination with other Therapies (Potentiation and Synergism)

Another technique to enhance cell death could be to use two prodrug systems in combination. One example is sequential prodrug treatment using HSVtk/GCV followed by CD/5-FC (Boucher et al., 2006). Radiotherapy in combination with suicidal gene therapy has been used in several clinical trials. They confirm the potential benefits and safety of using radiotherapy along with suicidal gene therapy (Rogulski et al., 2000). Suicide genes used in conjunction with oncolytic viral vectors (Leveille et al., 2011) and conventional chemotherapy has resulted in increased anti-tumor activity demonstrated both *in vivo* and *in vitro* models (Zeng et al., 2005).

2.3.6 Suicidal Gene Therapy as a Safety Switch

Although changes in vector backbone are well studied to mitigate the risk of oncogenesis, addition of a safety-control switch within a vector system is another promising technique. Conditional expression of a suicidal gene incorporated into a therapeutic vector could eliminate the oncogenic cell upon administration of a prodrug (Painter et al., 2005). The most common use of suicidal gene therapy outside cancer is for adoptive T-cell therapy (Kochenderfer et al., 2011; Brentjens et al., 2013). GVHD is a complication seen after allogenic stem cell transplants in patients with leukemia. The donor lymphocyte activates the autoimmune mechanism and results in destruction of transplanted cells. Genetically modified T-cells expressing the HSVtk gene can be killed by administering the prodrug ganciclovir (GCV) to induce apoptosis at the first sign of GvHD (Zhan et al., 2013).

The other example of using suicidal gene therapy as safety switch is in therapies using human pluripotent stem cell (hPSC). These cells have great potential in regenerative medicine but comes with risk of forming germ layer tumors. The introduction of a safety switch has been well

studied to enhance their use in hPSC (Sułkowski et al., 2018; Yagyu et al.,2015).The Figure 10 below explains that if hPSCs changes into tumor cells, selective killing could be achieved.

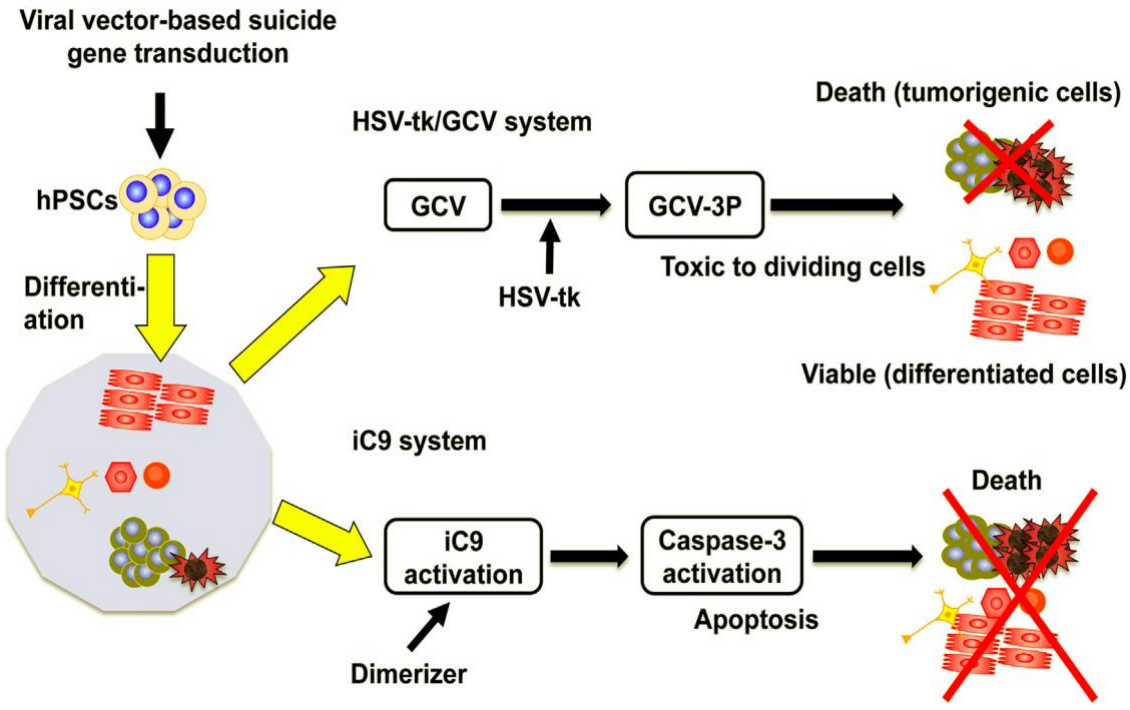


Figure 10. **Selective Killing of Tumorigenic hPSCs by Two Types of Suicide Genes.**
Herpes simplex virus thymidine kinase (HSVtk) or inducible caspase 9 (iC9) gene is virally transduced into hPSCs. In the former, a nontoxic prodrug ganciclovir (GCV) is phosphorylated by HSVtk expression, and ultimately GCV-triphosphate induces death in dividing tumorigenic hPSCs, but not in differentiated cells. In the latter, dimerizer treatment activates iC9, followed by caspase-3 activation. This ultimately results in apoptosis of the transduced cells.
Note. Reprinted from Mitsui et al., 2017. Copyright under the terms of the Creative Commons Attribution License

Chapter 3

Material and Methods

This section is set out to show sequence of events, reflecting each of the steps by which this study was conducted. Figure 11 illustrates an overall sketch of steps planned and followed to conduct the study experiment.

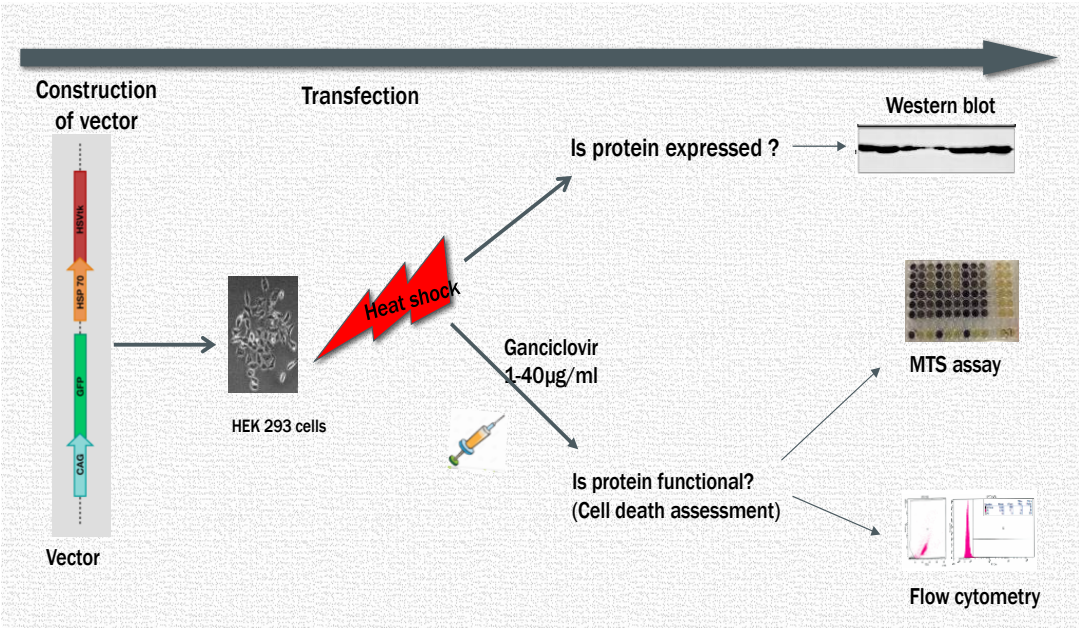


Figure 11. Overview of Experimental Design

The experiment started with construction of the study plasmid, and its subsequent co-transfection with (pEGFP-C2) which contains Kan^r/Neo^r resistance gene into HEK 293 cells to achieve stable cell lines. Effective transfections were confirmed using western blot analysis to detect the protein of interest. Subsequently, functional assessment was performed using the MTS essay and Flow cytometry.

3.1 Reagents and Chemicals

As my study involved various techniques, each using specific protocol and reagents; the

detailed list presented in Table 3 enlists the chemicals and protocols used in different time lines during experiments.

Table 3. Chemicals and Reagents Used in Research Experiments

List of chemicals used at different stages in experiments.

Chemical/reagent/strains	Protocol used	Experiment
Restriction enzymes and cut smart buffer	Addgene	Restriction digest
E. coli DH-5α	Invitrogen Cat. No. 18265017	Transformation
QIAprep spin Miniprep Kit	Qiagen, Hilden, Germany	Colony selection during transformation
HEK 293	293 [HEK293] (ATCC® CRL-1573™)	Transfection
HeLa Cells	HeLa ATCC (CCL-2)	Western Blot
lipofectamine 2000	(Invitrogen cat No.11668027	Transfection
pre-mixed 2X Taq FroggaMix	FroggaBio scientific solutions, Canada	PCR
RIPA Lysis Buffer (with Inhibitor PMSF)	Thermo Fisher (catalog no 89900)	Western blot
Bradford assay reagent	Thermo Fisher (catalog no 23238)	Protein Assays
primary antibody, goat polyclonal HSV-1 Thymidine Kinase antibody	(Santa Cruz Biotechnology, Inc, USA sc-28038)	Western blot
donkey anti-goat IgG-HRP	(Santa Cruz Biotechnology, Inc, USA sc-2020	Western blot
Anti-HSP70	(Abcam cat # ab2787)	Western blot
donkey Anti-Mouse IgG-HRP	EMD Millipore cat # AP192P	Western blot
MTS Cell Proliferation Assay kit	Abcam ab 197010	MTS
Propidium Iodide	Thermo Fisher (catalog no. P1304 MP)	Flow cytometry
Ganciclovir(GCV)	Sigma (G2536)	Cytotoxic drug in SGT
Geneticin (G418)	Fisher BioReagents (FLBP6731)	Selection of stable clones

3.2 Plasmids

Three plasmids were used in these experiments: (1) the plasmid of interest (pAAV-70-tk-CAG-GFP); (2) the positive control plasmid (pUNO1-HSVtk), which lacked the HSP70 promoter; and, (3) the plasmid, with GFP transgene and Kan^r/Neo^r resistance gene which was utilized to establish stable transfection in experimental cells (pEGFP-C2). In addition, two more plasmids were used to develop the study plasmid and one was created to further optimize results (Table 3).

Table 4. Plasmids Used in Current Study

List of plasmids used in study experiment with their resources.

Plasmid	size	origin	Purpose
pUC57-HSP70-HSVtk	1593bp	Biobasics	Source of HSVtk with HSP70 promoter
pAAV-CAG-GFP-WPRE-SV40	5439 bp	Vector Core, University of Pennsylvania.	Act as vector backbone containing GFP
pAAV-70-tk-CAG-GFP	7015 bp	Constructed during experiment	Study plasmid
pUNO1-HSV1tk	4317 bp	InvivoGen	Positive control plasmid without HSP70
pEGFP-C2	4700 bp	BD Biosciences Clontech (GenBank Accession # U57606)	Used to achieve stable transfection, contains Kan^r/Neo^r gene
pUNO1-HSP70-EGFP	3660 bp	GenScript	Used to confirm activity of HSP70 promoter

3.3 Construction of Plasmids

The study plasmid (pAAV-70-tk-CAG-GFP) was created using segments from two already available plasmids in our lab. The snap gene viewer was used to plan, visualize and document these plasmids (SnapGene® software (from GSL Biotech; available at snapgene.com). The HSVtk gene under the HSP70 minimal promoter was cloned into pUC57 (Biobasics); the

sequence was prepared by Evan Woodley (a graduate from Walia lab) and is illustrated in Figure 12. The second vector used, pAAV-CAG-GFP-WPRE-SV40 is shown in Figure 13. This plasmid was ordered from Vector Core, University of Pennsylvania. This plasmid was constructed with AAV as backbone and served as the backbone of our experimental plasmid.

Figure 12. pUC57-HSP70-HSVtk plasmid

Structure and elements of pUC 57 plasmid. HSVtk gene under the control of HSP70 minimal promoter was cloned into pUC57 expression plasmid by Biobasics.

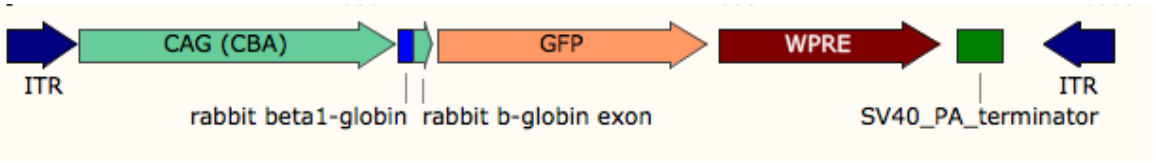


Figure 13. pAAV-CAG-GFP-WPRE-SV40 Plasmid

This vector, ordered from Vector Core, University of Pennsylvania was used as the back bone of main study vector –the original GFP with ubiquitous promoter CAG was kept in the study vector. HSP70.HSVtk segment was extracted from pUC 57 plasmid was cloned into this vector to get the study plasmid.

3.3.1 Plasmid Digestion and Ligation

The study plasmid was constructed using standard subcloning techniques. The pUC57 was digested to isolate HSP70-HSVtk using restriction enzymes *Mfe*I and *Hpa*I. The same sites were digested on pAAV-CAG-GFP-WPRE-SV40. Independent restriction reactions were performed with each enzyme, both with 1 µg of DNA, 1 µL of each enzyme, 5 µL of 10X outsmart buffer and 19.5 µL of water to make a final volume of 25 µL per reaction. The reaction

mixtures were incubated for 1 hour at 37°C and the digested mixture was analyzed by gel electrophoresis, to verify the restriction patterns. Subsequently, the linear fragment of the HSP70-HSVtk cassette from the pUC57 plasmid was recovered and ligated into the target GFP plasmid using 10x T4 DNA Ligase buffer followed by incubation at 16°C overnight and heat inactivation at 65°C for 10 minutes before transforming to get the final experimental vector product, pAAV-70-tk-CAG-GFP (Figure 14). The final construct is displayed in Figure 15.

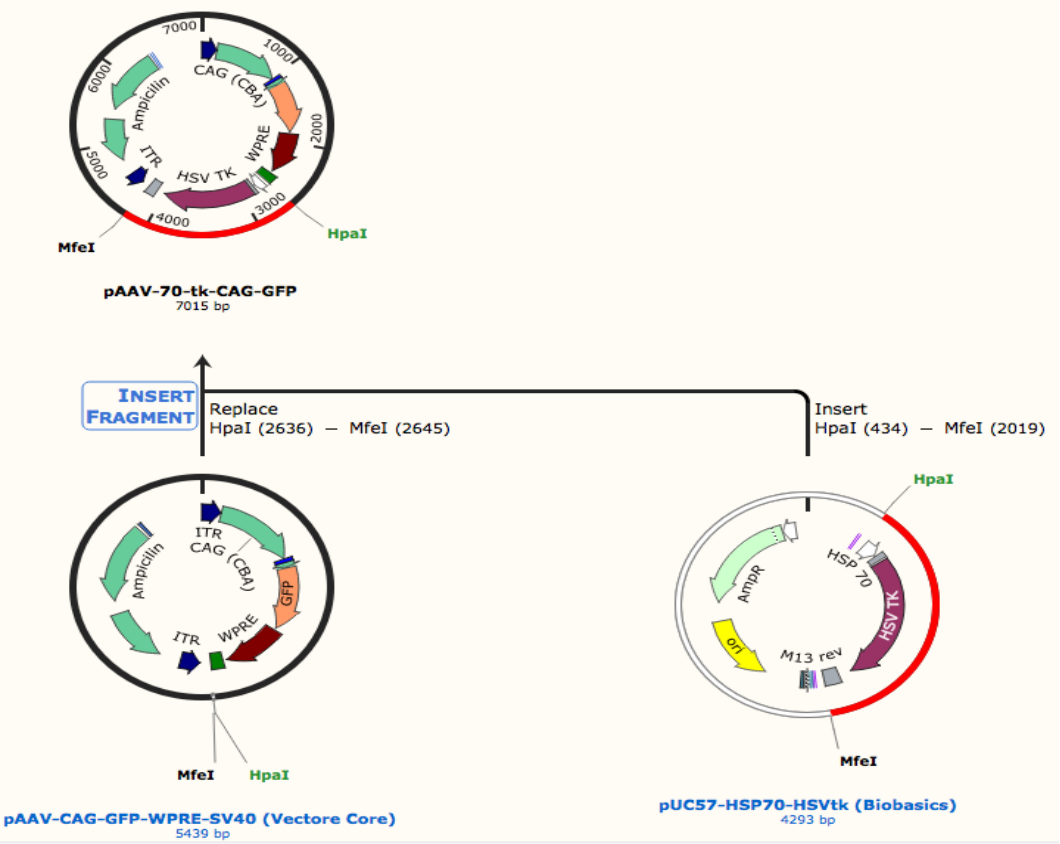


Figure 14. Procedure for Construction of pAAV-70-tk-CAG-GFP Plasmid

The study plasmid was prepared by using standard cloning procedures from two other plasmids. HSVtk with HSP70 was digested using restriction enzymes at *Mfe*I and *Hpa*I sites from pUC57 plasmid and then ligated at same sites in target vector pAAV-CAG-GFP-WPRE-SV40.

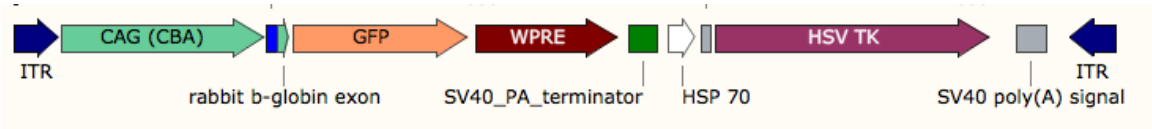


Figure 15. pAAV-70-tk-CAG-GFP Construct

The study plasmidwith dual promoter-GFP under CAG and HSVtk under HSP70 promoter. The ubiquitous CAG promoter causes widespread expression of GFP in stably transfected cells and HSP70 serves as an inducible promoter which controls HSVtk expression. The entire sequence was 4300 base pairs (ITR TO ITR) in length.

A simplified map of the study plasmid is displayed in Figure 16.

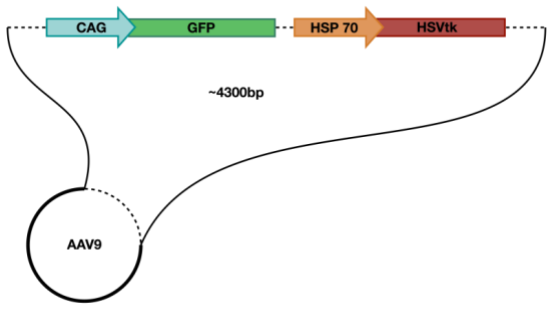


Figure 16. Plasmid with Dual Promoter

A self-drew picture of plasmid used in experiment whereas HSVtk was under HSP70 promoter and GFP was under CAG

3.3.2 Transformation and Colony Selection

The cloned experimental plasmid was transformed into competent *E. coli* DH-5α (Invitrogen Cat. No. 18265017) using the standard protocol as described by Forger et al. (Froger & Hall, 2007). The selected colonies were picked and plasmid was extracted and purified using a QIAprep spin Miniprep Kit (Qiagen, Hilden, Germany) as per protocol given by Zhang et al.

(Zhang & Cahalan, 2007). The orientation of the resultant inserts was confirmed through restriction enzyme digestion and subsequent sequencing.

3.3.3 pEGFP-C2

The pEGFP-C2 plasmid (BD Biosciences Clontech), shown in Figure 17, was used to co-transfect with the experimental plasmid to obtain stable transfectants. This plasmid carries the Neo/Kan resistance marker which was needed for stable selection of clones after transfection.

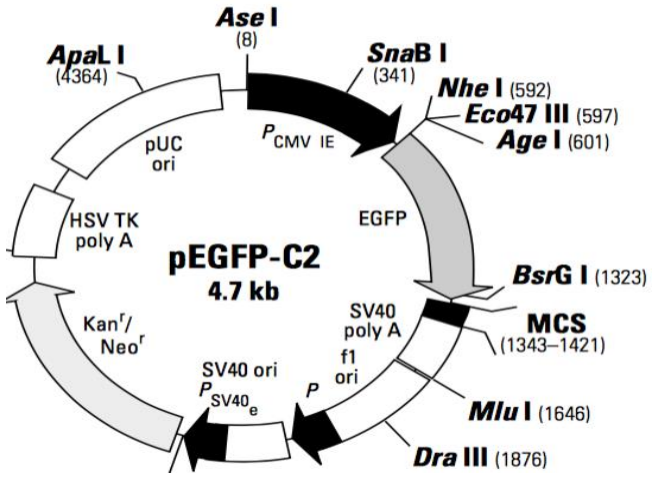


Figure 17. pEGFP-C2 Plasmid

Vector map of the pEGFP-C2 plasmid showing position of the Kan^r/Neo^r resistance gene required for selection of stably transfected cells, (BD Biosciences Clontech GenBank Accession # U57606).

3.3.4 pUNO1-HSV1tk

The pUNO1-HSV1tk plasmid (marketed and prepared by InvivoGen as an expression plasmid) was purchased from Invivogen. Figure 18 illustrates the map of pUNO1-HSV1tk and this plasmid served as a positive control for my study plasmid for HSVtk expression. The gene of interest, HSVtk here is under control of EF1α/HTLV (elongated factor 1 α and human T-cell leukemia virus) promoter. The gene of interest in the experimental plasmid pAAV-70-tk-CAG-GFP (shown in Figure 15) is under control of HSP70 promoter.

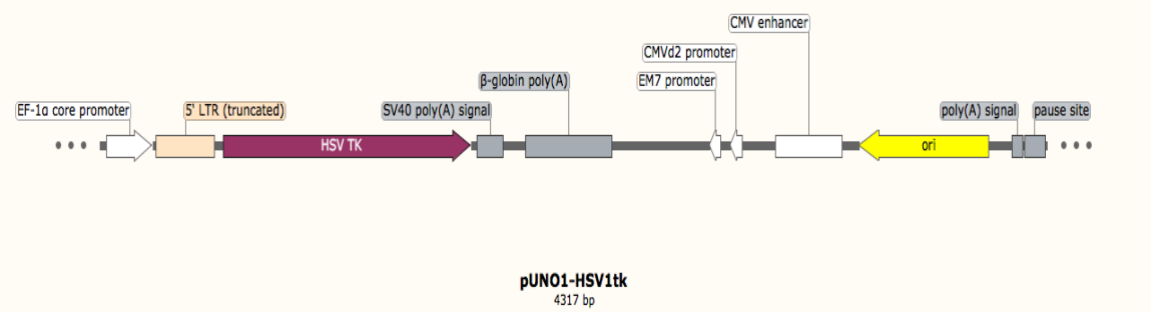


Figure 18. pUNO1-HSV1tk Plasmid

This pUNO1-HSVtk plasmid purchased from Invivogen contains HSVtk gene without HSP70 promoter to serve as a positive experimental control for HSVtk expression.

3.3.5 pUNO1-HSP70-EGFP

The pUNO1-HSP70-EGFP plasmid as shown in Figure 19 was not a part of the original protocol for this study. This plasmid contains GFP transgene under HSP70 promoter. This was developed using two pre-existing plasmids such that the HSVtk cassette in pUNO1-HSV1tk was replaced by EGFP with the HSP70 promoter.

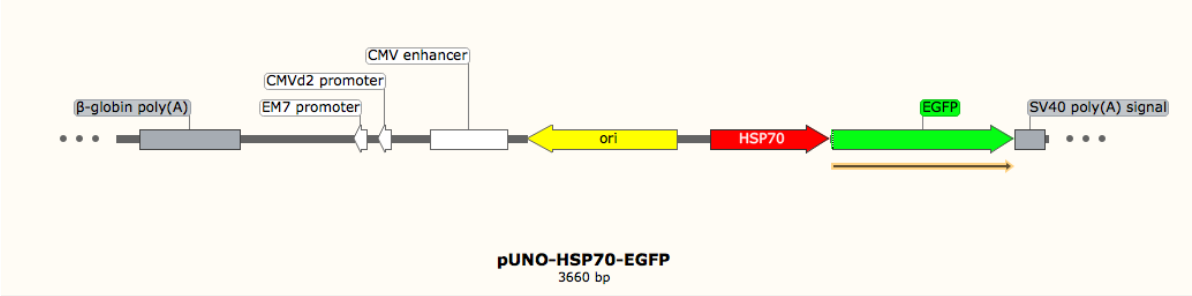


Figure 19. pUNO1-HSP70-EGFP Plasmid

This plasmid was developed using two pre-existing plasmids such that the HSVtk cassette in pUNO1-HSV1tk was replaced by EGFP with the HSP70 promoter.

3.4 Cell Culture

The HEK 293 cell line (ATCC) was used for these experiments. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in a 5 % CO_2 humidified incubator. The cultured cells were maintained up to 80% confluency and then routinely passaged by trypsinization.

3.5 *In vitro* Transfections

The general procedure for transfections involved seeding of HEK293 cells 24 hours prior to transfection in a 6-well plate at a density that resulted in approximately 80 to 90% confluence on the day of transfection. The cell medium was exchanged for fresh growth medium (with serum) directly prior to transfection. The HEK293 cells were transfected with lipofectamine 2000 (Invitrogen cat No.11668027). Cells were incubated at 37°C overnight. After incubation, medium was removed and replaced with fresh complete medium. The cells were allowed to grow for another 36-48 hours and transfected cells were checked by viewing under a fluorescent microscope. Testing was also performed to evaluate the transfection efficiency using different timelines post transfection and to optimize the amount of pDNA and lipofectamine using the protocol as described by Avci-Adali et al. (Avci-Adali et al., 2014).

3.5.1 Co-transfection

HEK293 cells were seeded in duplicate in 6-well plates and co transfected with

a. pAAV-70-tk-CAG-GFP (5 parts)
b. pEGFP-C2 (Kan^r/Neo^r) (1 part)

Post transfection (36-48 hours) images were obtained under fluorescence microscope. These cells were then trypsinized and split, letting them re-plate overnight, before proceeding with the expansion and selection of stable clones.

3.5.2 Stable Transfection

Stable cell line generation is essential for sustained gene expression. Thus, stable clones were selected through limiting dilutions to get a genetically homogenous and clonal population. Generation of stable cell lines required use of positive selection markers such as any antibiotic resistance and gene of interest which were both on separate plasmids (in trans approach). The trans method of co-transfection is a good alternative in instances where the target construct does not have the antibiotic-resistance gene in the vector backbone. In such cases, a plasmid mixture containing 5 to 10 parts gene expression plasmids and 1 part antibiotic selection marker plasmid can be introduced into cells. This plasmid ratio helps to ensure that the selected cells will express both the gene of interest and the selection marker.

Figure 20 displays an overall layout and timelines to create stably transfected clones.

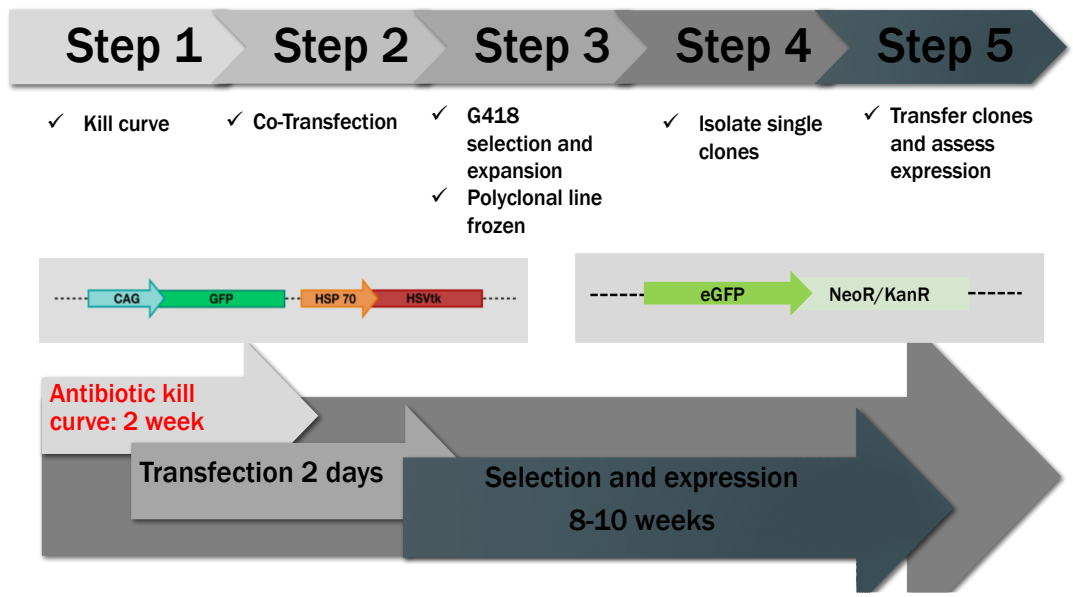


Figure 20. Process of Creating Stable Clones

The process of creating stable clones firstly requires the minimal toxic dose to be established, using kill curve analysis of the transfected cell line. This is followed by expansion of polyclonal cells and then isolating single stable clones. The process of selection and expression of stable clones takes 8-10 weeks.

3.5.2.1 Kill Curve

Step 1 in creating stable clones was to generate a kill curve to determine the optimal selection of antibiotic concentration Geneticin (G418). HEK293 cells were seeded in triplicate and cultured overnight, followed by addition of G418 in varying concentrations from 0-1200 µg/ml. The medium containing selection antibiotic was replaced every 48 hours for up to two weeks. The lowest concentration of G418 (600 µg/ml) that killed all the plated cells by 14 days was selected for selection of stable clones. The result obtained are plotted on kill curve displayed in Figure 21.

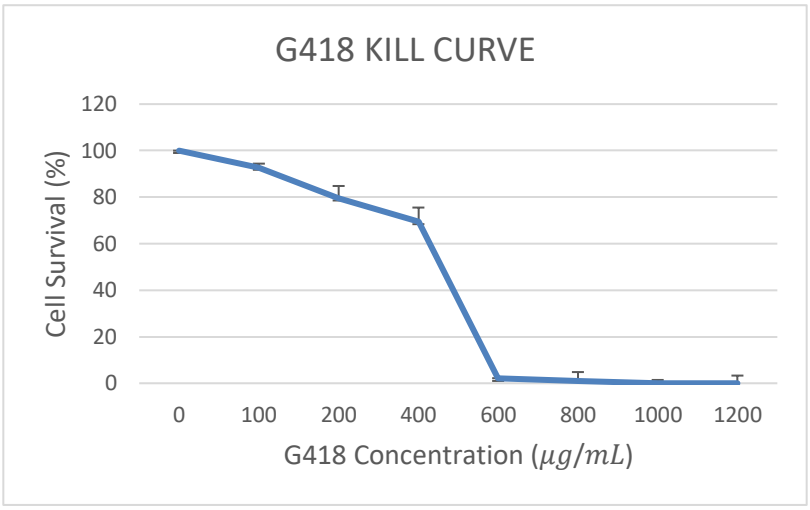


Figure 21. Kill Curve to Determine Antibiotic Concentration

HEK 293 cells were exposed to increasing concentrations of Geneticin. This is to determine the lowest possible concentration which can kill the cells; this dose was later used to select cell for stable transfection. The y axis represents cell survival in percentage and X axis shows increasing concentration of Geneticin. G418 (600 µg/ml) was found as the minimal concentration required to kill the cells.

3.5.2.2 Selection and Expansion

Steps 2 and 3 in this process involved selection of transiently co-transfected cells with G418 and expansion for polyclonal colonies. The medium containing the appropriate G418

concentration (600 µg/ml) was changed twice a week. Surviving cells were allowed to proliferate until they reached high confluence. Upon reaching confluence, they were frozen down as a polyclonal line while an aliquot of the cells was plated for selection of single cell clones.

3.5.2.3 Isolation of Single Clones

Step 4 in the process of creating stable cell lines was to identify single clones by limited dilution and expansion. The polyclonal cells from the selection step were plated at a density of 10 cells/ml in a 96-well tissue culture plate and into 10/15 cm plates. The number of cells per well was assessed every 24 hours; wells containing only one cell were noted and followed. Colony numbers were verified each week until the well, which had contained one cell, had reached a high level of confluence.

3.5.2.4 Clone Assessment of Expression

In Step 5, clones were transferred and assessed for expression of gene of interest. The wells which had been expanded from a selected single-colony in the 96-well tissue culture plate were grown to a high level of confluence, and then transferred to a 12-well tissue culture plate. Once the 12-well tissue culture plate clones had expanded to high confluence, they were passaged to a 6-well tissue culture plate. The highly expressing clones were assessed further and frozen down for future stock and experiments.

3.6 Structural and Functional Analysis of Stable Clones

Stably transfected clones were assessed for presence of HSVtk by polymerase chain reaction (PCR) and western blot analysis was used to detect protein expression.

3.6.1 PCR Analysis of Stable Single Cell Clones for HSVtk Expression

Stable single cell clones carrying HSVtk were analyzed by PCR. Primers specific to the HSVtk insert were designed using NCBI/Primer-Blast tool and were then used to amplify the HSVtk gene in different single cell clones obtained after stable transfection. The forward primer

5′-GCAGAAAATGCCCACGCTAC-3′ and reverse primer 5′- GGTCGATGTGTCTGTCCTCC-3′ were purchased from Eurofins Genomics. The pre-mixed 2X Taq FroggaMix (FroggaBio scientific solutions, Canada) was used as per manufacturer's instructions in the protocol for amplification, with the thermal conditions being the initial activation at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute and final extension at 72°C for 10 min. The amplification product was then analyzed by electrophoresis on an agarose gel.

3.6.2 Western Blot Analysis

The expression of HSVtk and HSP70 was evaluated by western blot analysis. The cell lysate preparation involved the culturing of adherent cells to approximately 80% confluence on 10cm plates. The cell medium was aspirated and plates kept on ice for all steps. The cells were gently washed twice with 10 ml ice cold PBS and 500 µl of complete RIPA Lysis Buffer (with Inhibitor PMSF) was added to each plate and swirled gently to distribute buffer. Using a cold cell scraper, the cells were scraped and the lysate transferred into a pre-cooled 1.5 ml tube and incubated on ice for 15 minutes. Thereafter, the lysate was sonicated three times at an amplitude of 20 % for 10 secs each, subsequently incubated on ice for an additional 15 minutes, and then centrifuged at 12,000 rpm for 10 minutes at 4°C. The clarified supernatant was collected into new cold micro tubes and protein concentration was determined by Bradford assay. Samples were aliquoted and stored at -20°C to avoid multiple freeze/thaw cycles.

For western blot analysis, lysate samples were boiled for 5 minutes and cell proteins were separated by protein electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel, loading the equal amounts of protein from each sample. The proteins were then transferred to a nitrocellulose membrane (Bio-Rad laboratories cat. # 162-0115) using a standard wet transfer. The membrane was blocked with 5% skim milk in TBS-Tween (TBST) at 4°C overnight. The membrane was washed 3 times with TBST and incubated with primary antibody: goat polyclonal HSV-1

Thymidine Kinase antibody (Santa Cruz Biotechnology, Inc, USA sc-28038), at a dilution of 1:200 dissolved in 3% skim milk for 1 hour at room temperature. After washing three times with TBST, the membrane was then blocked with the secondary antibody, donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Inc, USA sc-2020), at a dilution of 1:2500 for 1 hour. The membrane was then washed with TBST 3 times, developed by using ECL reagents and proteins bands were visualized on Azure C600 imager (azure biosystems), in the faculty of department of biomedical and molecular sciences, at exposure times ranging between 20-60 seconds. For detection of HSP70, western blot analysis was performed using the anti-HSP70 antibody as the primary antibody at a dilution of 1:1000 (Abcam cat # ab2787) followed by a donkey anti-Mouse IgG-HRP, as the secondary antibody, at a dilution of 1: 10,000 (EMD Millipore cat # AP192P). As an internal control, the B-Actin antibody was used at a dilution of 1:1000 to block the membrane after washing with TBST and the same procedure was used to visualize the bands.

3.7 Heat Shock Treatment

Heat shock parameters including time, temperature and the method of heat shock were optimised by using different experiments. The cell culture dishes were sealed with paraffin wax and were heat shocked in a calibrated water bath at 41°C for 1 hour and then returned to incubator at 37˚C for the remainder of the experiment.

3.8 MTS Assay

The MTS (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium is a tetrazolium based calorimetric assay used in different viability and cytotoxicity studies. This assay is based on reduction of tetrazolium salt into formazan crystals by viable cells. The quantity of formazan product measured by absorbance at 485 nm is directly proportional to the number of living cells in culture.

Assays were carried out in 96-well microtiter plates in triplicate as per manufacturer's instructions in the protocol. The cells (20,000) were cultured with a final volume of 200 µl per

well by incubating the plates for 24 hours before addition of the GCV and heat shock. Treated for 72 hours with various concentration of GCV (0–40 μg/ml) and 20 μl MTS reagent was added into each well with incubation time of 3 hours, followed by measurement of absorbance of treated and untreated cells at 485 nm on the plate reader. The percentage of cell survival against different concentrations of GCV was calculated using the formula provided by Palizban et al. (Palizban et al., 2010).

$$\% \text{ cell survival} = \frac{(At-Ab)}{(A_c-A_b)} \times 100$$

At: *Mean absorbance of the test drug (GCV)*

Ab: *Mean absorbance of the blank (Medium)*

Ac: *Mean absorbance of the negative control*

3.9 Flow Cytometry

The transfected cells were also analyzed for viability by flow cytometry using the FC500 system at Queens University Biomedical Imaging Centre. This technique was used only later during the experiments with the newly constructed plasmid (details are given in Chapter 5). The transfected cells were prepared for flow cytometry using a standard technique of staining with Propidium Iodide (PI). HEK293 cells were transfected with pUNO.HSP70. The EGFP plasmid was harvested by washing it with PBS and then adding trypsin to detach the transfected cells. These cells were then resuspended in growth medium after 10-minute incubation at 37°C. Cells were then centrifuged at 300-400 *x g* for 5 minutes at room temperature. The supernatant was discarded and the cell pellet was resuspended in PBS, centrifuged again at 300-400 *x g* for 5 minutes at room temperature. The supernatant was then discarded, and the pellet resuspended in PBS. The cells were then counted under a microscope using a hemocytometer ($1x10^6$ cells/ml).

The cells were transferred into FACS tubes with 50 μl of propidium iodide added into each tube before being analyzed on the flow cytometer.

3.10 Statistical Analysis

For each clone, cells were plated in triplicate with varying concentrations of GCV ranging from 0 to 40 μg/ml. The absorbance was measured at 485 nm for the triplicates at each level of GCV, and the cell survival rate was found for each well by using the formula mentioned above. The survival rate was displayed as mean of triplicate with standard error of mean. The significance in survival difference between heated and unheated cells at selected GCV concentrations within one cell was calculated using student t-test. The significance level was set at $P < 0.05$ obtained from two tailed t- test.

Chapter 4

Results

The results are presented here in a format that reflects the overall study objectives. The first part of the research experiment was to construct a bicistronic dual-promoter AAV vector which can mediate expression of two transgenes a. Green fluorescent protein (GFP) under a ubiquitous promoter - Chicken β-actin (CAG), and b. HSVtk under HSP70 promoter. The development of the vector itself has been described in chapter 3.3. The expression of the transgene was verified using fluorescence imaging (Chapter 4.1), PCR (Chapter 4.2) and western blot analysis (Chapter 4.3). The second part of the experiment was to test the function of newly constructed vector construct *in vitro* using a stably transfected cell line to prove selective inducible cell killing (Chapter 4.5).

4.1 Verification of *in vitro* Transfection

HEK 293 cells were co-transfected with the dual promoter plasmid and another plasmid bearing neomycin/kanamycin resistant marker that was required for selection of stable cell clones. Both plasmids contained GFP as expression marker allowing the visualization of the green color of GFP under fluorescence microscope to reflect efficient transfection. Following identification of transfected cells, stable colonies were selected as described in Chapters 3.5.2.2 and 3.5.2.3. Four clones C1, C7, C9, C10 were found positive for HSVtk and remaining clones C2, C3, C5, C6 and C8 were negative for HSVtk. The images of transiently transfected HEK293 cells and untransfected HEK293 cells, taken under fluorescence microscope, are shown below in Figure 22.

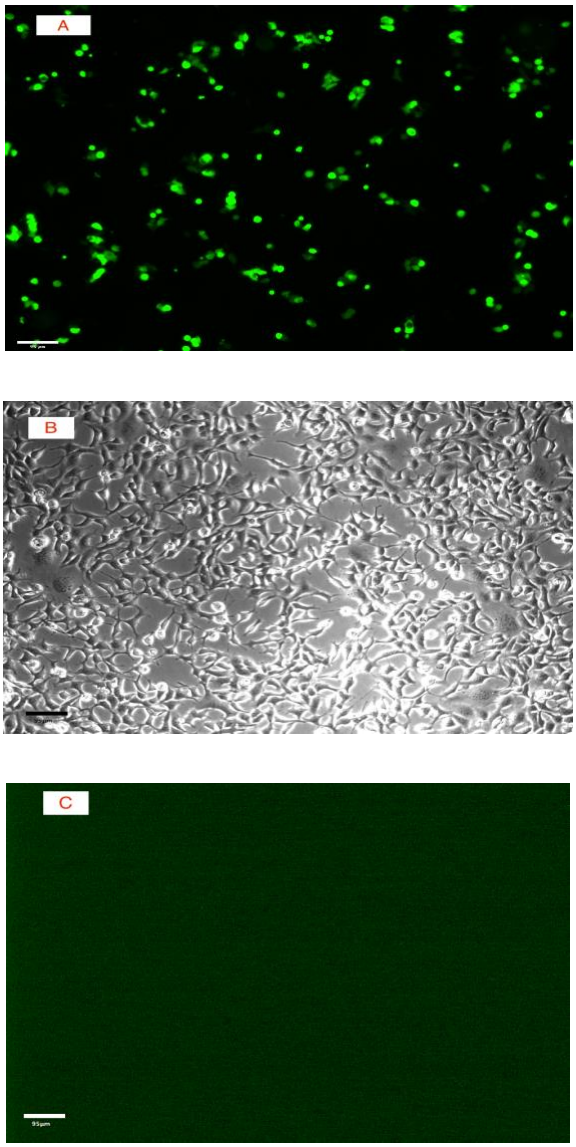


Figure 22. HEK293 under Fluorescence Microscope after Co-transfection

Fluorescence microscopy was used to verify transfection of HEK293 cells by observing the expression of GFP protein. (A) GFP expression in proliferating HEK 293 cells transfected with two plasmids pAAV-70-tk-CAG-GFP and pEGFP-C2 and images taken after 36 hours of transfection. (B) Bright field image of untransfected HEK293 cells (-ve control). (C) Untransfected HEK293 cells (-ve control) as seen using GFP filter showing no GFP expression. (Scale bars representing 95 μm)

4.2 Confirmation of Single Stable Cell Clones Using PCR Analysis

The stable single clones carrying HSVtk were analyzed by PCR for confirmation of gene presence. Primers were designed specific to the HSVtk insert used to amplify the HSVtk gene in different single cell clones obtained after stable transfection. The amplified PCR product using the HSVtk primer was calculated to be 518 base pairs. Analysis of the products by gel electrophoresis (Figure 23) positively demonstrated four stable clones (C1, C7, C9, C10) carrying HSVtk, while the other five (C2, C3, C5, C6, C8) were negative for HSVtk insert. Further experiments were carried out using the C1, C10 as the experimental clone cells while C8 was used as a negative control.

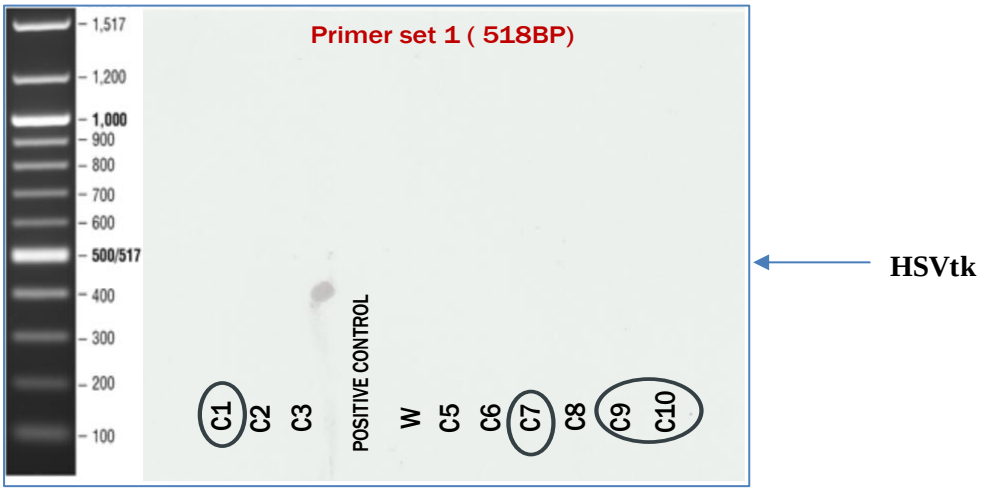


Figure 23. PCR Electrophoresis with Stable Clones

For the process of PCR, HSVtk specific primer was used with 518bp. The stable transfected clones were amplified using PCR and then analyzed on gel electrophoresis and the image was taken. Clones C1, C7, C9 and C10 showed in the picture were considered as positive clones for HSVtk. The clones C2, C3, C5, C6 and C8 were negative for HSVtk.

4.3 Western Blot Analysis

The expression of HSVtk was evaluated by western blot analysis. The proteins bands were visualized with exposure times ranging between 20-60 seconds. The HSVtk protein expression was detected in the positive control plasmid (pUNO1-HSVtk) only. The stably transfected clones C1 and C10 did not show any expression (Figure 24).

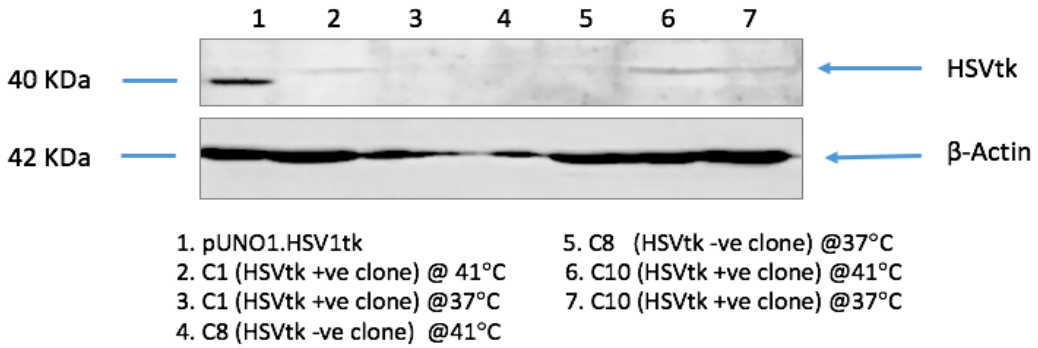


Figure 24. Western Blot Analysis for HSVtk Expression

The lysate samples were boiled for 5 minutes and cell proteins were separated by protein electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel. Ten µg of protein from each sample was loaded, transferred and then incubated with primary antibody (goat polyclonal HSV-1 Thymidine Kinase antibody) followed by blocking with secondary antibody (donkey anti-goat IgG-HRP). The membrane was then washed and developed. HSVtk was only expressed as band of 40 kDa is in positive control plasmid (1st lane) while there was no expression observed against our positive experimental clones (C1, C10). The lower blot shows Beta-Actin loaded as an internal control and expressed as 42 kDa band across all lanes.

4.4 Control Experiments

Experiments were performed with a HEK 293 cell line and a negative control to estimate cell killing when heat shock is given with GCV. It was done to confer the survival difference in cells with or without transfected plasmid. These include effect of heat on HEK293 proliferation (heat alone does not kill HEK293 cells), effect of GCV on positive control cells (cells with

HSVtk only plasmid), effect of GCV and heat shock on stably transfected clone C8 (negative control cells with no HSVtk).

In the experiments below unheated refers to conditions at 37°C and heated refers to a temperature of 41°C (heat shock treatment was described in Chapter 3.7).

4.4.1 HEK293 Proliferation Assay (MTS Assay)

Proliferation of HEK293 cells was observed with and without heat shock. This was to rule out any effect of heat on the experimental cell line. Proliferation was followed by measuring the absorbance at a wavelength of 485 nm, and showed a steady increase with increase in number of cells, that was similar in both, with or without heat shock (Figure 25). Thus, heat shock was concluded to have no effect on the proliferation of wild-type HEK293 cells.

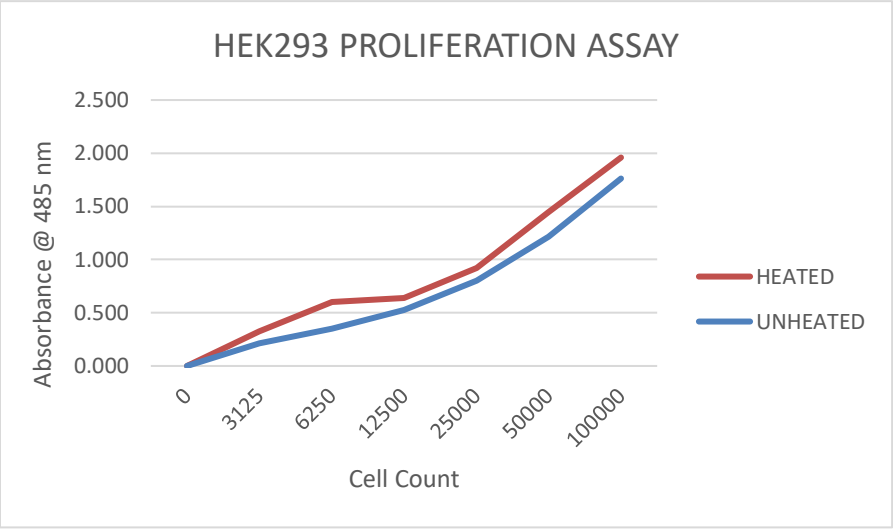


Figure 25. Effect of Heat Shock on Proliferation of HEK293

The effects of heat on proliferation of HEK 293 cells. This experiment was performed as a control. The increase in absorbance on the Y axis demonstrates cell survival. The response to normal temperature and at high temperature is consistent, reflecting that heat shock has no effect on proliferation in wild-type HEK293 cells

4.4.2 GCV Treatment of Positive Control Plasmid

In another control experiment, HEK 293 cells were transfected with the positive control plasmid (pUNO1-HSV1tk) which contains HSVtk without heat shock promoter. The transfected cells were then treated with GCV at different doses (0 µg/ml to 80 µg/ml) to observe the killing effect on transfected cells without heat shock (these cells can produce HSVtk without heat shock, which can convert GCV into a fatally toxic drug). A direct proportional decrease in cell survival was noticed with increasing doses of GCV (Figure 26).

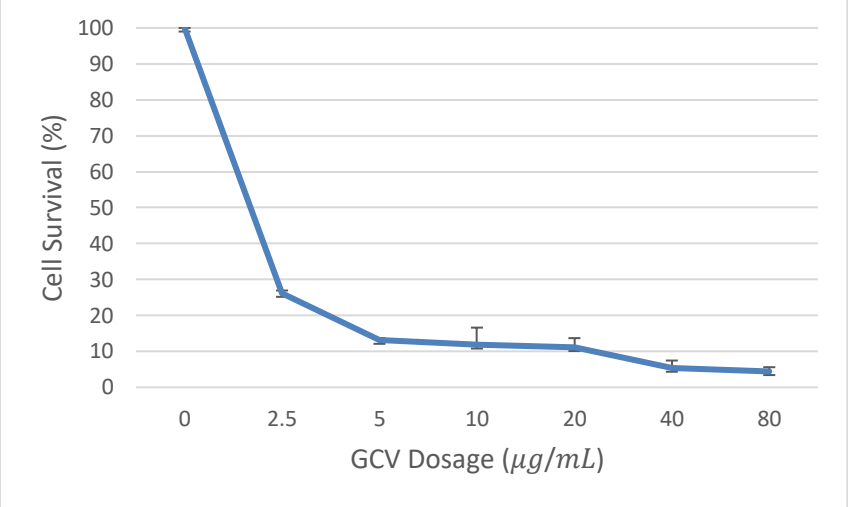


Figure 26. Effect of GCV on Positive Control Plasmid without HSP70 Promoter

The effect of GCV was observed on HEK293 cells transfected with plasmid, without HSP70 promoter, with increasing dose concentrations. The GCV dose was increased from 0 µg/ml to 80 µg/ml. A direct proportional relationship with increase cell survival on lower doses and poor survival at higher doses was observed.

4.4.3 Heat Shock and GCV Treatment of C8

C8 is a stable single cell clone but did not contain HSVtk as shown by PCR analysis.

As such, the C8 cell line was predicted to behave like the wild-type HEK293 cells with no effect of heat treatment and GCV. Figure 27 illustrates the survival curve of C8 clones against increasing concentration of GCV. The cell survival was not significantly different between heated and unheated cells at GCV concentration of 5μg/ml (P = 0.2), 20 μg/ml (P= 0.1) and 40 μg/ml (P= 0.13).

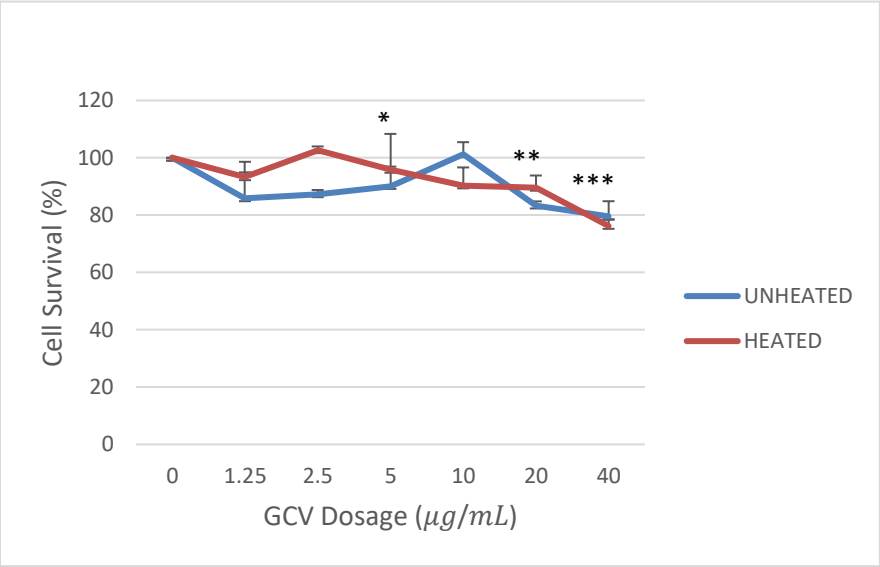


Figure 27. Effect of Heat Shock and GCV Treatment on Stably Transfected Clone C8 without HSVtk (negative control)

The study experiment was conducted with stably transfected C8 clones (HSVtk –ve). The cells were heat shocked in a calibrated water bath at 41°C for 1 hour and then returned to the incubator at 37˚C for the remainder of the experiment. Then increasing dosage of GCV was introduced in culture media for 48 hours. Cell survival assay was used to calculate percentage survival. As this clone was without HSVtk, there was no effect of GCV on cell survival with or without heat shock conditions.

* (P = 0.2) **(P= 0.1) ***(P= 0.13)

4.5 *In vitro* Test of the Function to Prove Selective Inducible Cell Killing

4.5.1 GCV and Heat Shock Treatment of C1

C1 is one of the stable experimental single cell clones that carries HSVtk under heat shock promoter (HSP70). These cells were treated with either heat shock and GCV, or GCV alone at various dose concentrations, and cell survival was measured. A dose response curve was constructed (Figure 28) showing cell survival as a function of GCV dosage. The GCV dose varies from (0 µg/ml to 40 µg/ml) without any significant difference in survival between heated vs unheated cells. The *P* values obtained at 5µg/ml, 20µg/ml, 40 µg/ml were *P* = 0.3, *P*= 0.9 and *P*= 0.19 respectively.

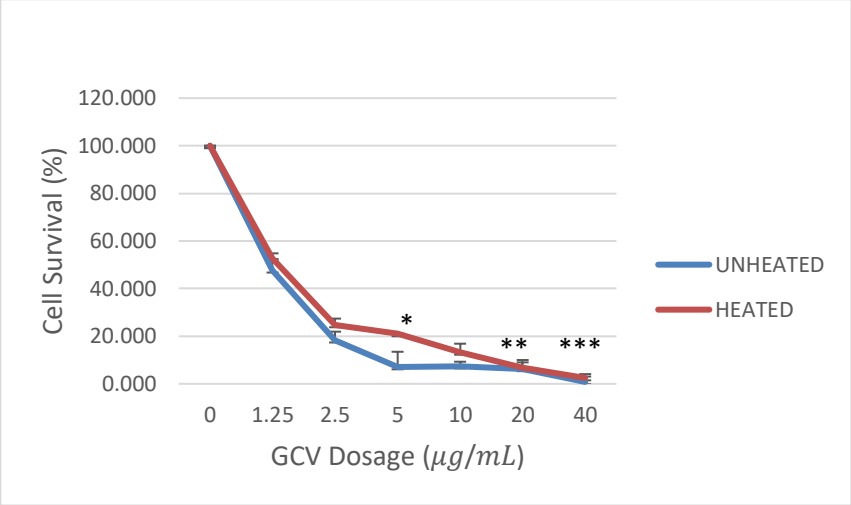


Figure 28. Effect of Heat Shock and GCV Treatment on Stably Transfected Clone C1

The study experiment was conducted with stably transfected C1 clones (HSV tk +ve). The cells were heat shocked in a calibrated water bath at 41°C for 1 hour and then returned to incubator at 37˚C for the remainder of the experiment. Then increasing dosage of GCV was introduced in culture media for 48 hours. Cell survival assay was used to calculate percentage survival.

* (*P* = 0.3) ** (*P*= 0.9) ***(*P*= 0.19)

4.5.2 GCV and Heat Shock Treatment of C10

A similar experiment was revised with another positive stable clone C10. Figure 29 illustrates similar results as with the C1 clone with heat shock and GCV treatment. The cell survival was not significantly different between heated and unheated cells at GCV concentration of 5µg/ml (*P* = 0.4), 20µg/ml (*P*= 0.6) and 40 µg/ml (*P*= 0.1).

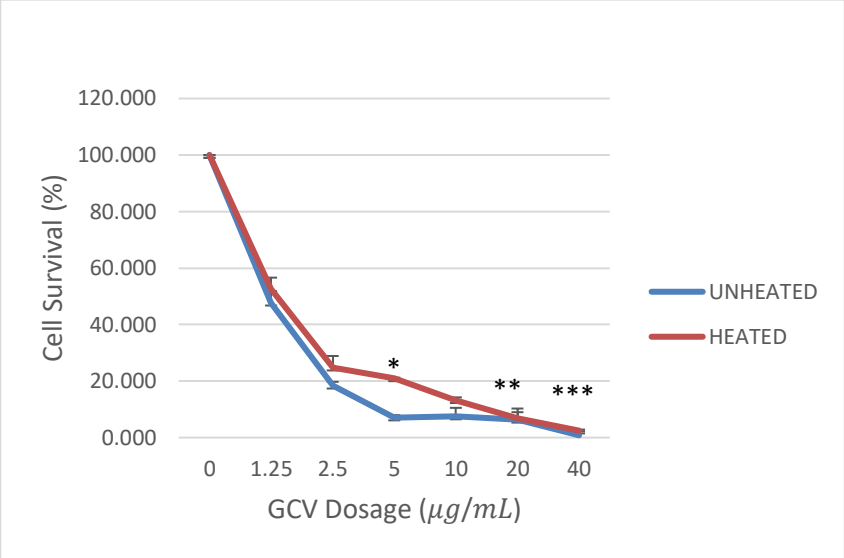


Figure 29. Effect of Heat Shock and GCV Treatment on Stably Transfected Clone C10

The study experiment was conducted with C10 clones (HSV tk +ve). The cells were heat shocked in a calibrated water bath at 41°C for 1 hour and then returned to incubator at 37˚C for the remainder of the experiment. Then increasing dosage of GCV was introduced in culture media for 48 hours. A cell survival assay was performed to calculate percentage survival.

*(*P* = 0.4) **(*P*= 0.6) ***(*P*= 0.1)

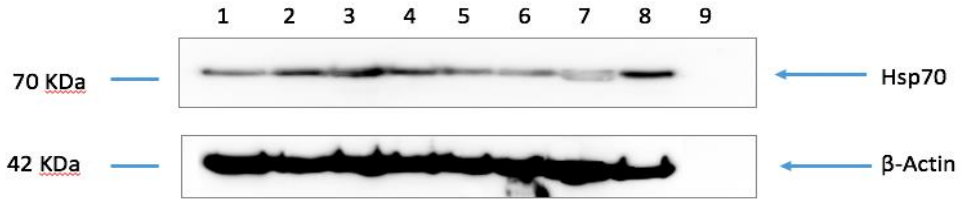
4.6 Trouble Shooting of Principal Findings.

The above-mentioned results demonstrated that cell killing was achieved in transfected cells, however few unexpected results were observed as well; cell survival was reduced at both normal and heated temperature. This led to further continuation of work to determine if this was because of some inherent characteristics of material used in the experimental design.

4.6.1 Heat Shock Promoter Activity in HEK293 and HeLa Cells

Expression of HSP70 was observed in two different cell lines over various time ranges to determine if the cell line being used in our research experiment already contains endogenous HSP70 which could be activated at lower temperatures. The HSP70 expression was verified by western blot analysis using an antibody against HSP70 (Figure 30). Two cell lines were examined: HEK293 and HeLa cell lines. HEK293 were used as they were used in my primary experiment; these were compared to HeLa cell lines, as it is a cancer cell line which might have more endogenous HSP70 activity.

The HEK293 and HELA cell lines were maintained at 37°C and 41°C degrees for different time periods ranging from 24 hours to 72 hours. Subsequently, HSP70 expression was determined by western blot analysis using the HSP70 antibody. We found very interesting results as HSP70 was detected in both cell lines and at all temperatures. This could be one possible explanation of our initial findings that cell killing was high even at normal temperature; both cell lines have active HSP70 at normal body temperature, which may have been expressed due to other stress factors during preparation of these cell line.



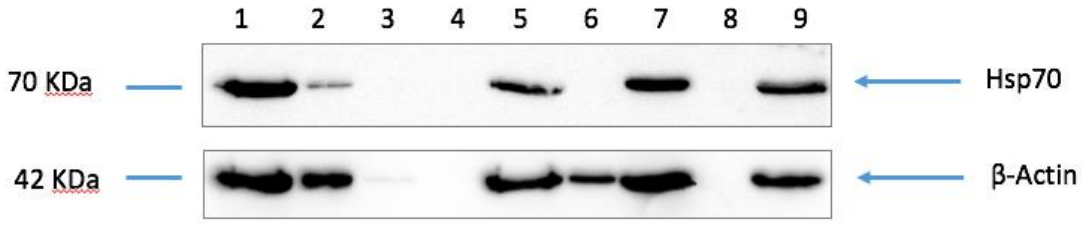
1- HEK293 cells 37°C
2- HEK293 cells 41°C @ 24 hours
3- HEK293 cells 41°C @ 48hours
4- HEK293 cells 41°C @ 72 hours
5- HeLa cells 37°C
6- HeLa cells 41°C @ 24 hours
7- HeLa cells 41°C @ 48 hours
8- HeLa cells 41°C @ 72 hours
9-Water

Figure 30. Western Blot of HSP70 Expression in HEK 293 and HeLa Cells

The lysate samples for HEK293 and HeLa cells were prepared at 37°C and 41°C for varying time periods. An equal amount of each sample was loaded onto SDS-10% polyacrylamide gel, transferred and incubated with primary antibody (mouse monoclonal anti-HSP70 antibody) followed by blocking with secondary antibody (donkey anti-Mouse IgG-HRP). HSP70 bands were visualized in all lanes other than –ve control.

4.6.2 Preparation of a Plasmid with EGFP Under the Control of the HSP70 Promoter and Western Blot Analysis

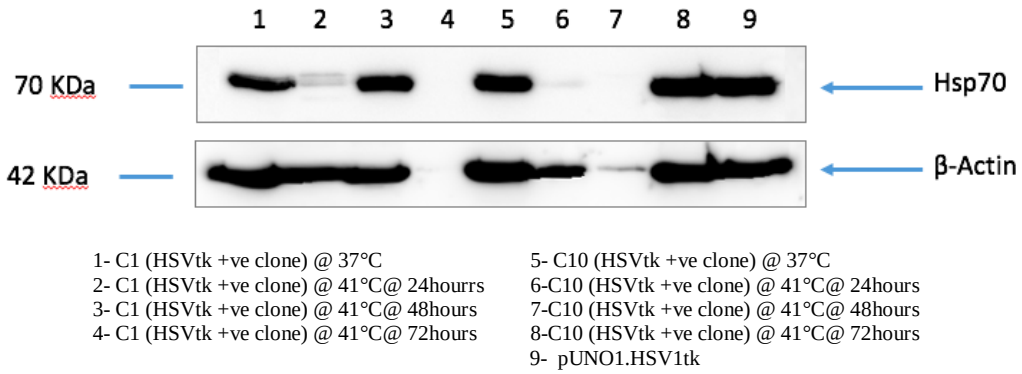
Another experiment was carried out to see if HSVtk is being driven by some other strong promoter like chicken β-actin (CAG) being a very strong promoter present in the backbone, rather than HSP70, which was leading to cell death at all temperatures. An indirect way to do this was to construct a new plasmid with a different backbone into which EGFP was cloned under the control of the HSP70 promoter. This plasmid was created using a plasmid which already existed in the lab. The HSVtk cassette from the positive control plasmid (pUNO1-HSV1tk) was replaced by HSP70.EGFP. (GenScript) (Figure 19). This was then transfected into HEK293 cells as per the procedures mentioned in Chapter 3.5 to obtain transiently transfected clones alongside those transfected with the study plasmid. Western blot analysis was performed in parallel to detect HSP70 in these respective clones (Figure 31 and 32).



1- HSP-GFP-HEK293 cells 37°C
2- HSP-GFP-HEK293 cells 41°C @ 24hours
3- HSP-GFP-HEK293 cells 41°C @ 48hours
4- HSP-GFP-HEK293 cells 41°C @ 72hours
5- HSP-HSVtk-HEK293 cells 37°C
6- HSP-HSVtk-HEK293 cells 41°C @ 24hours
7- HSP-HSVtk-HEK293 cells 41°C @ 48hours
8- HSP-HSVtk-HEK293 cells 41°C @ 72 hours
9- pUNO1.HSV1tk

Figure 31. Western Blot of HSP70 Expression in HEk293 Cells Transfected with HSP70-EGFP and HSP70-HSVtk Plasmids

HEK293 cells were transfected with each plasmid HSP70-EGFP and HSP70-HSVtk and lysates were prepared at 37°C and 41°C for varying time periods. An equal amount of each sample was loaded onto SDS-10% polyacrylamide gel, transferred and incubated with primary antibody (mouse monoclonal anti-HSP70 antibody) followed by blocking with secondary antibody (donkey anti-Mouse IgG-HRP). HSP70 bands were visualized in lane 1,2,5,7 and 9.



1- C1 (HSVtk +ve clone) @ 37°C
2- C1 (HSVtk +ve clone) @ 41°C@ 24hourrs
3- C1 (HSVtk +ve clone) @ 41°C@ 48hours
4- C1 (HSVtk +ve clone) @ 41°C@ 72hours
5- C10 (HSVtk +ve clone) @ 37°C
6-C10 (HSVtk +ve clone) @ 41°C@ 24hours
7-C10 (HSVtk +ve clone) @ 41°C@ 48hours
8-C10 (HSVtk +ve clone) @ 41°C@ 72hours
9- pUNO1.HSV1tk

Figure 32. Western Blot of HSP70 Expression in Stable Clones C1 and C10

The lysate samples for stably transfected clones C1 and C10 were prepared at 37°C and 41°C for varying time periods. An equal amount of each sample was loaded onto SDS-10% polyacrylamide gel, transferred and incubated with primary antibody (mouse monoclonal anti-HSP70 antibody) followed by blocking with secondary antibody (donkey anti-Mouse IgG-HRP) HSP70 bands were visualized in lane 1,2,3,5,8 and 9.

The results were similar to what we had seen so far as HSP70 was detected in both above mentioned clones at temperature 37°C and 41°C.

4.6.3 Flow Cytometry for GFP expression with and without heat shock

This time further confirmation of gene expression was obtained using flow cytometry. The HEK293 cells were transiently transfected with pUNO1-HSP70-EGFP plasmid and cells were prepared for flow cytometry using the method described in Chapter 3.9. We checked EGFP expression in transfected cells with and without heat shock conditions. GFP was expressed to similar level under both conditions, thus confirming HSP70 being activated at 37°C as shown in Figure 33.

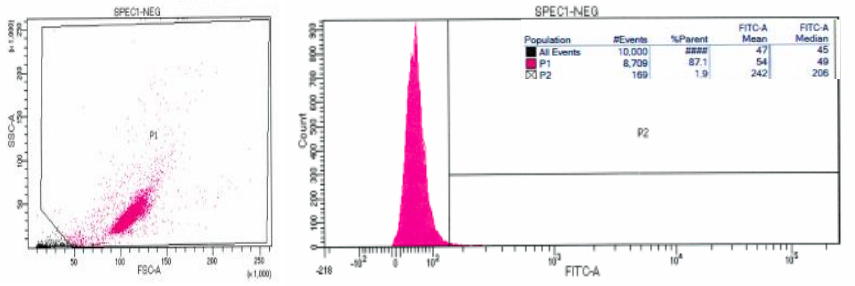


Figure 33a: Control HEK293 Cells – No GFP

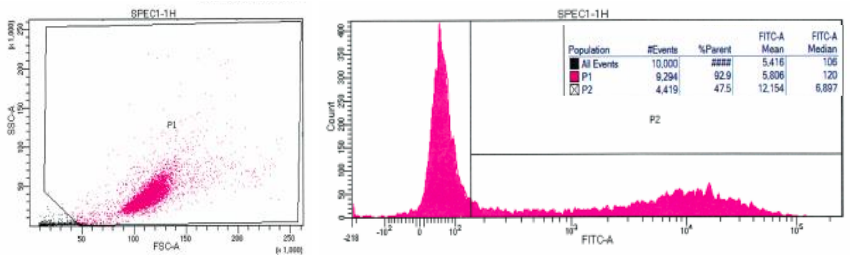


Figure 33b: GFP Expression at 41°C

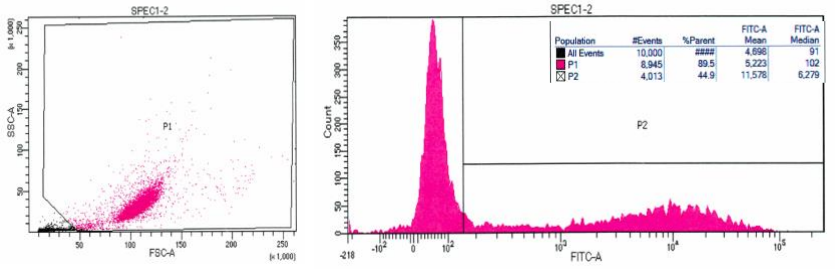


Figure 33c: GFP Expression at 37°C

Figure 33. Flow Cytometry for HSP70-EGFP

FACS analysis of GFP expression in HEK293 cells transfected with HSP70-EGFP plasmid using propidium iodide with and without heat shock. Untransfected HEK293 cells (-ve control) and transfected cells were first gated on a dot plot with forward scatter along X-axis and side scatter along Y-axis. The histogram plot shows the fluorescence recorded along X-axis against the number of cells along Y-axis. Figure 33(a) shows no fluorescence as these were untransfected HEK293 cells and served as –ve control. Figure 33(b) and 33(c) show GFP expression at both temperatures of 41°C as well as 37°C.

Chapter 5

Discussion

This study was the first step towards the development of a safety switch for AAV-based therapies. Although this technique is being utilized with some other vectors, this was the first to demonstrate a proof-of-concept in the use of the rAAV back bone. The use of an inducible promoter provides a unique feature of targeted gene expression rather than generalized expression as seen with a ubiquitous promoter. HSP70 is a temperature sensitive promoter. Thus, upon exposure to high temperature, heat shock factor 1 is released, which binds to heat shock elements in the promoter leading to transcriptional activation (Shoji et al., 2008). As the current experiments with rAAV are showing concerns of carcinogenesis, we aimed to develop this safety switch which can only be activated when needed to kill tumor cells. With this potential idea in mind, use of HSP70 made sense for our experiment as it can be activated using a local heat source. Local application of heat like high-intensity focused ultrasound has been successfully used (Plathow et al., 2005) in raising temperature and subsequently inducing gene expression. HSVtk expression under HSP70 models have been studied using an adenovector backbone (Lee et al., 2001). Another study also used HSVtk/GCV under the HSP70 promoter in a mouse mammary cancer cell line and gastric cancer cell line (Braiden et al., 2000; Isomoto et al., 2006).

5.1 Principal Findings

Our results confirmed that stable transfection with HSVtk under the control of the HSP70 promoter is feasible, as shown by PCR. Also, that stably transfected clones showed significant killing/decreased survival at 41°C, as expected, when treated with GCV, relative to the untransfected cells. However, we encountered some unexpected results. Following heat shock treatment, the comparative curves were similar to what was observed in the absence of heat shock (Figure 28, 29).

The unheated cells were treated at 37°C, this temperature is considered "normal body temperature" and, thus, should not activate HSP70 to initiate gene expression. In the control experiments, we already showed that survival of our positive and negative controls was not affected by heat shock (Chapter 4.4). This finding helped us to concentrate only on the stably transfected cell line containing HSVtk and HSP70 to find out the cause of cell death at 37°C.

5.2 Discussion of Principal Findings

The aforementioned results can be explained if we assume that HSP70 was activated at 37°C, that HSP70 is activated by factors other than temperature, or that HSVtk expression was initiated by the other promoter in this dual promoter vector. Heat shock proteins are molecular chaperones and expressed in cells under many stressful conditions. The other well-studied factors that could lead to activation of HSP70 include hypoxia, stress, and chemicals like heavy metals ions, and arsenite (Benjamin et al., 1990; Giaccia et al., 1992; Ryan and Hightower, 1996; Kiang and Tsokos, 1998).

Beside the potential role of stress-related factors in activation, the response of HSP70 to heat shock also depends upon a few other factors. Cell growth prior to heat application was found to modulate the effects of heat shock. A study performed in HeLa cells gave a differential response to the same temperature at different stages of growth (Abravaya et al., 1991) HSP70 activation is also found to be attenuated depending upon the type of stimulus. This phenomenon is well-studied in pulmonary cells, where HSP70 was activated by heat shock and cadmium but not by ozone, paraquat, and parathion (Wirth et al., 2002).

Furthermore, in previous studies, the activation temperature for heat shock proteins varies in different species. For instance, in fruit flies, growth temperature is 25°C so 35–37°C is considered as heat shock. In contrast, normal body temperature for human or mouse cells is 37 °C, and the HSP is induced when the temperature is raised to several degrees above this i.e. 41–42°C.

These findings point out that further work is required to understand the biology of HSP70 in a co-transfection model and optimization of induction related to temperature. To answer the principal findings in my project, further work was done to narrow down the results. With our trouble shooting experiments we checked endogenous expression of heat shock protein in HEK293 and Hela cells both are commonly used cell line in gene therapy experiments. HSP70 was expressed in both cell lines at normal temperature and at high temperature with different time. This was one step in confirmation that HSP70 is activated with other stimuli in these experimental cells. In the second series of experiment a new plasmid was constructed where EGFP expression was checked under HSP70. This was to see if in our original experiments HSVtk was at fault and getting expressed under the influence of other factors. However, EGFP expression was observed again at normal and heated temperature in similar manner as in original study.

These finding demonstrate that HSP70 is activated at normal body temperature and, thus we conclude that it did not act as an inducible promoter for our experiments. However, it is worth mentioning that each experiment is designed in the environment with differences in vector backbones and factors which cannot be simulated in the other experiments.

5.3 Future Directions

In the future, our study hopes to extend itself to determine causes for and optimize the inducible potential of HSP70 like the factors that can result in stimulation of HSP70. The potential of other inducible/specific promoters in HSVtk /GCV suicide gene system will also be explored.

5.4 Conclusion

Gene therapy is evolving but safety challenges still exist and developing a safety mechanism to deal with insertional mutagenesis is needed. This study sought to investigate the

potential for a built-in safety switch in the event of gene therapy induced tumorigenesis, and to investigate the *in vitro* effect of a HSVtk/ GCV system using a heat inducible promoter- driven AAV vector system. Unfortunately, the desired results were not being achieved. However, our study has provided a platform for future work to understand HSP70 biology and find out factors which are responsible for its activation at different temperature ranges in commonly used experimental cells.

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