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Nejat Düzgüneş *Editor*

Suicide Gene Therapy

Methods and Protocols

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Suicide Gene Therapy

Methods and Protocols

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Preface

The concept of suicide gene therapy most likely originated in 1986 with the work of Frederick Moolten at the Hubert H. Humphrey Cancer Research Center at Boston University. He exposed neoplastic BALB/c murine cell lines carrying the herpes simplex virus thymidine kinase gene to the herpes thymidine kinase-specific substrate 9-{[2-hydroxy-1-(hydroxymethyl)ethoxy] methyl}guanine, which inhibited the clonogenic potential of the cells in vitro. Tumors produced by these cell lines in BALB/c mice underwent complete regression following exposure to this drug.

Subsequently, in 1988, Ronald Evans and collaborators at the Salk Institute in La Jolla used a procedure to kill transfected cells in culture or to selectively delete immune cells in transgenic mice upon administration of acyclovir or 1-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)-5-iodouracil (FIAU). In 1991, Knudsen and Karlström at the University of Copenhagen used the *Escherichia coli relF* gene driven by *lac*-derived promoters to devise a biological containment system for genetically engineered bacteria. In the same year, the Pardoll laboratory at Johns Hopkins University in Baltimore utilized the HSV-*tk* + ganciclovir system in tumor cell vaccines as a means to eliminate the cells in case the immune system was unable to clear them. Klatzman and colleagues at the Hôpital de la Pitié-Salpêtrière, Paris, in 1992, employed the HSV-*tk* + ganciclovir system to kill liver tumor cells in vivo. They also engineered CD4+ T-lymphocytes to express HSV-*tk* under the control of the HIV promoter (5′-LTR) and showed the elimination of these cells upon HIV infection. The Blaese laboratory at the National Cancer Institute showed in 1994 that tumor cells expressing the cytosine deaminase gene are eliminated in vivo by the administration of 5-fluorocytosine, which is converted to the toxic compound, 5-fluorouracil.

This volume includes the methods used for most of the recent approaches to suicide gene therapy of cancer. It also contains chapters describing methods to improve the safety of cell therapy and those utilizing bone marrow mesenchymal cells. The goal of cancer treatment is to eliminate, or at least to greatly reduce, the number of cancer cells without harming normal cells. In addition to utilizing cell surface markers overexpressed on cancer cells, targeted suicide gene therapy exploits promoters that are specific to cancer cells, thereby ensuring (or greatly increasing the likelihood) that the therapeutic gene is expressed only in cancer cells.

In the first chapter, we outline the origins of the different systems used for suicide gene therapy. In Chapter 2, Fehse, Miletic, and collaborators provide guidelines for the preparation of high-titer 3rd-generation lentiviral vectors that encode a genetically improved HSV-*tk* version (TK.007) and its application in vitro and in vivo. The success of suicide gene therapy can be greatly enhanced by the use of tissue-specific or tumor-specific gene expression and efficient gene delivery. Thus, Chung and Hsieh and co-authors provide the details of osteonectin promoter-mediated suicide gene therapy of prostate cancer in Chapter 3. Pedroso de Lima and collaborators describe in Chapter 4 the methods for the use of the HSV-*tk*/ganciclovir system to achieve antitumor activity both in cultured oral cancer cells and in orthotopic and subcutaneous murine models of oral squamous cell carcinoma, using ligand-associated lipoplexes for enhancing therapeutic delivery.

Although chimeric antigen receptor (CAR)-redirected T-cells can be used for the treatment of cancers, they can have adverse effects such as the cytokine release syndrome and off-target effects that can cause organ damage. In Chapter 5, Di Stasi and co-authors describe an inducible caspase 9 suicide gene system that can eliminate a large percentage of CAR T-cells when necessary. Altaner and Altanerova outline their methods to prepare exosomes derived from human mesenchymal stem cells engineered to express cytosine deaminase and uracil phosphoribosyl transferase mRNA, and their use in inhibiting the growth of cancer cells following the administration of 5-fluorocytosine as a prodrug (Chapter 6). In Chapter 7, Saydam and colleagues describe a similar system, which also expresses the green fluorescent protein in HEK293T cells, to produce extracellular vesicles that they have used to inhibit the growth of glioblastoma cell lines and spheroids, and glioblastoma tumors in vivo.

Viral vectors with suicide genes are very difficult to produce, because trace amounts of toxins (such as diphtheria toxin or the enzyme barnase) can kill the cells used for viral vector production. In Chapter 8, Chen describes a method to overcome this problem. In this method, insect cells are used, since mammalian introns are not recognized by these cells, and the open reading frames of the toxic genes are not broken up. Thus, the insect cells do not produce the toxins but generate normal levels of baculovirus and adeno-associated viral vectors carrying the toxin genes. Bhatia and Shi (Chapter 9) describe a vector containing resveratrol-responsive CC(A+T rich)6GG (referred to as a "CArG box") elements from the promoter of the early growth response protein 1 (Egr-1) and the GADD45α open reading frame. After delivery into lung cancer cells and treatment with resveratrol, the vector expresses GADD45α, leading to cell cycle arrest.

Suicide gene expression may be used as a safety mechanism in regenerative medicine. Unwanted multipotent stem cells could thus be removed before they cause side effects, such as teratoma formation. The location of such cells can be monitored by imaging approaches. Himmelreich and co-authors describe in Chapter 10 how therapeutic cells can be engineered to express a suicide gene as well as genes that can be used for visualization in vivo. Demidyuk and colleagues provide the methods to create and evaluate the cytotoxic action of a bicistronic plasmid expressing the yeast cytosine deaminase/uracil phosphoribosyltransferase fusion protein and the hepatitis A virus 3C protease (Chapter 11).

Suicide gene therapy can result in the elimination of cancer cells within a tumor beyond those cells that actually express the transgene, in a process termed the "bystander effect." This is achieved by the spread (via gap junctions) of cytotoxic anti-metabolites produced by the transgene to neighboring cells that may not have been transfected or transduced. Neschadim and Medin have developed a suicide gene therapy system that mediates a highly effective bystander effect, based on a variant of human deoxycytidine kinase that can phosphorylate the unusual nucleoside analogs bromovinyl deoxyuridine and L-deoxythymidine, which they describe in Chapter 12. Hirschberg and colleagues describe a modified form of photodynamic therapy that enhances the delivery of the cytosine deaminase gene into tumor cells and the cytotoxic effect of the locally produced 5-FU (Chapter 13). Finally, in Chapter 14, we provide the methods for TransfeX-mediated transfection of the plasmid pNGVL1-*tk* encoding HSV-tk under the control of the CMV promoter into HeLa cervical carcinoma, HSC-3 and H357 oral squamous cell carcinoma, and FaDu pharyngeal carcinoma cell lines, and the resulting cytotoxicity upon administration of ganciclovir.

I would like to thank Professor John Walker, the series editor, for his patient and unwavering support and advice in moving the editing along. I am also grateful to my wife Diana and my children Avery and Maxine who always supported me in my editing and writing endeavors.

I would like to dedicate this volume to the memory of my Ph.D. advisor, Professor Shinpei Ohki at the State University of New York at Buffalo (now University at Buffalo).

San Francisco, CA, USA ***Nejat Düzgüneş***

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Chapter 1

Origins of Suicide Gene Therapy

Nejat Düzgüneş

Abstract

"Tumor chemosensitivity" can be achieved by the expression of the herpes simplex virus thymidine kinase gene in cells, followed by the conversion of the "prodrug" ganciclovir into the therapeutic drug inside the cells. This system presaged other combinations of suicide genes and prodrugs, including cytosine deaminase/5-fluorocytosine, purine nucleoside phosphorylase/6-methylpurine deoxyriboside, and horseradish peroxidase/indole-3-acetic acid.

Key words Suicide gene therapy, Prodrug, Herpes simplex virus thymidine kinase, Cytosine deaminase, Purine nucleoside phosphorylase, Horseradish peroxidase

1 Introduction

The concept of suicide gene therapy was introduced initially as "tumor chemosensitivity" via the stable introduction of the herpes simplex virus thymidine kinase gene into BALB/c murine cell lines, followed by treatment with 9-([2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)guanine, later called "ganciclovir" [1, 2]. In addition, tumors were generated in BALB/c mice using these cell lines, and the mice were treated with ganciclovir. This treatment caused the regression of the tumors. These studies opened the field of suicide gene therapy. The many in vitro and in vivo studies on suicide gene therapy have been reviewed by Lal et al. [3], Duarte et al. [4], Zarogoulidis et al. [5], and Karjoo et al. [6]. In this chapter we describe some of the major systems used for suicide gene therapy.

2 Systems Used for Suicide Gene Therapy

2.1 HSV Thymidine Kinase and Ganciclovir

A variety of pyrimidine deoxyribo- and arabino-nucleosides inhibit the replication of a strain of herpes simplex virus ($HSV\text{-}1_{KOS}$) that induces deoxythymidine-deoxycytidine kinase activity in cultured rabbit and human cells. However, these agents do not inhibit the

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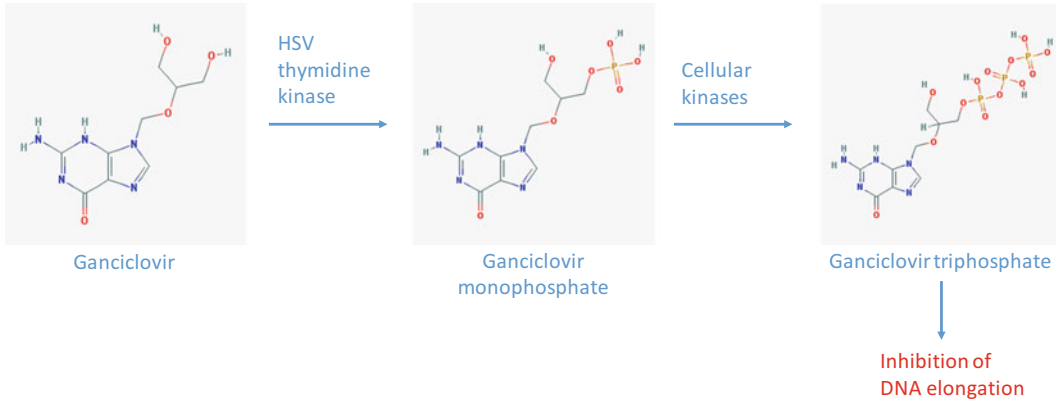

Fig. 1 Conversion of ganciclovir to toxic ganciclovir triphosphate. Delivery of the HSV-*tk* gene to target cells results in the expression of viral thymidine kinase, which selectively phosphorylates ganciclovir (GCV). Monophosphorylated GCV is further phosphorylated by cellular enzymes into an active triphosphate compound. The molecular structures were adapted from PubChem

replication of HSV-2_{333}, which does not induce kinase activity [7]. These findings indicate that thymidine kinase is necessary for the replication of HSV. After an initial lytic phase, the virus establishes latency in sensory ganglia. Reactivation of the latent virus requires thymidine kinase activity. Specific inhibitors of HSV thymidine kinase, N2-phenyl-2′-deoxyguanosine and N2-(m-trifluoromethylphenyl) guanine, inhibit the reactivation of the virus from explant cultures of latently infected murine trigeminal ganglia [8].

These observations indicated that nucleoside analogs, such as ganciclovir, could be used as substrates for HSV-thymidine kinase after the introduction of its gene into cancer cells destined for destruction. HSV-thymidine kinase normally converts deoxythymidine into deoxythymidine monophosphate; cellular kinases then diphosphorylate this product into deoxythymidine triphosphate. In HSV-infected cells treated with ganciclovir, the viral thymidine kinase converts the prodrug into ganciclovir monophosphate, and cellular kinases diphosphorylate this product into ganciclovir triphosphate (Fig. 1). Ganciclovir triphosphate is incorporated into DNA by DNA polymerase, and causes chain termination because of the absence of the deoxyribose, and hence of 3′-OH. Chain termination then triggers the apoptotic signals.

One of the issues in the use of the HSV-thymidine kinase/ganciclovir system is how to achieve tumor-specific expression of the gene. Regardless of the vector for the delivery of the gene (e.g., viral or nonviral vectors), it would be highly desirable to have the gene under the control of tumor-specific promoters. For example, the promoter for the FBJ murine osteosarcoma viral oncogene homolog (FOS) in an adenoviral vector facilitated much higher transcriptional activity in glioma cell lines, compared to that in normal astrocytes [9]. A high percentage of serous and

endometrioid epithelial ovarian cancer cells express the human epididymis protein 4 (HE4), which is an important factor in the adhesion and motility of ovarian cancer cells. An adenoviral construct expressing HSV-thymidine kinase driven by the HE4 promoter achieved significant killing of cisplatin-sensitive and cisplatin-resistant ovarian cell lines [10]; however, the HE4 promoter-driven expression of HSV-thymidine kinase in non-cancer cells was not evaluated. Normal human somatic cells have very low levels of telomerase activity and have a limited life span resulting from the transcriptional silencing of telomerase during differentiation. By contrast, telomerase activity is observed in a high percentage of tumors and also in stem cells and adult germline tissues [11]. The promoter for the human telomerase reverse transcriptase (hTERT) can be used to control HSV-thymidine kinase expression in an adenovirus vector. Following ganciclovir treatment, this vector decreased the viability of a renal cell carcinoma cell line, but not normal fibroblasts [12]. It also inhibited the growth of xenograft tumors in mice more effectively than a vector expressing the thymidine kinase with a cytomegalovirus promoter.

2.2 Cytosine Deaminase and 5-Fluorocytosine

The enzyme cytosine deaminase cloned from *Escherichia coli* can deaminate cytosine and 5-fluorocytosine [13]. Thus, the nontoxic prodrug 5-fluorocytosine can be converted to the toxic molecule 5-fluorouracil (Fig. 2). The introduction of the cytosine deaminase gene into a human colorectal carcinoma cell line mediated the inhibition of cell growth by 5-fluorocytosine at an IC_{50} that was 566-fold lower than that required for the parental cell line without the cytosine deaminase [13]. Studies with a colorectal carcinoma cell line, WiDr, engineered to express either cytosine deaminase or HSV-thymidine kinase, and used to produce xenograft tumors, indicated that both systems can result in completely tumor-free animals after treatment with ganciclovir or 5-fluorocytosine, respectively [14]. When the tumors were composed of 90% wild-type cancer cells and only 10% HSV-thymidine kinase-expressing cells, ganciclovir treatment was ineffective, suggesting that no bystander effect occurred in this case. However, with tumors containing 4% cytosine deaminase-expressing cells and 96% wild-type cells, 60% of the animals became tumor-free, suggesting that the 5-fluorouracil produced could enter neighboring cells not expressing the enzyme and cause cytotoxicity [14].

Retroviruses encoding the cytosine deaminase gene directed by promoters derived from the carcinoembryonic antigen promoter regions were used to transduce human colorectal carcinoma cells, which became susceptible to treatment with 5-fluorocytosine [15]. Cells producing these retroviruses were used to treat intraperitoneally disseminated colorectal carcinoma cells, together with 5-fluorocytosine. This treatment prolonged the survival of the animals compared to treatment with cells producing cytosine

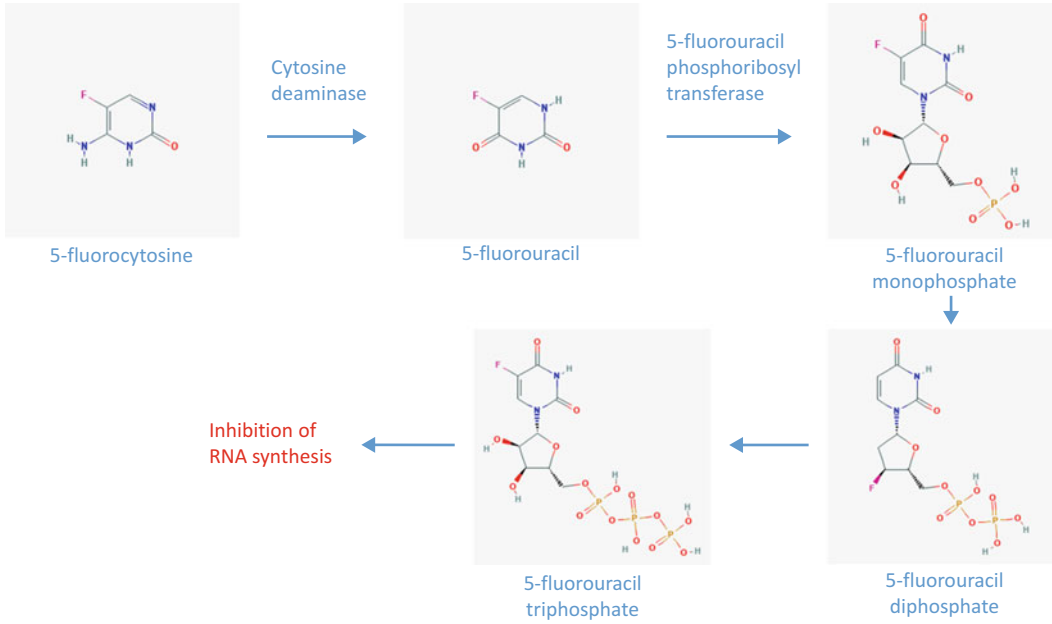

Fig. 2 Conversion of 5-fluorocytosine to 5-fluorouracil triphosphate. Cytosine deaminase cloned from *Escherichia coli* deaminates 5-fluorocytosine to 5-fluorouracil, which is phosphorylated by 5-fluorouracil phosphoribosyl transferase. Monophosphorylated 5-fluorouracil is further phosphorylated by cellular enzymes into an active triphosphate compound. The molecular structures were adapted from PubChem

deaminase without the specific promoters, and also avoided bone marrow suppression observed with the non-targeted retrovirus.

In a very different approach to gene therapy, mesenchymal stem cells expressing the cytosine deaminase gene were used to create a bystander, cytotoxic effect on neighboring C6 glioma cells, in the presence of 5-fluorocytosine, and this effect was proportional to the enzyme produced by the stem cells [16]. The cytosine deaminase-expressing mesenchymal stem cells were employed in the treatment of early-stage C6 glioma tumors and reduced the tumor mass as a function of the 5-fluorocytosine dose. Suppression of the growth of established tumors required multiple injections of the mesenchymal stem cells.

One of the limitations of cytosine deaminase is its low affinity for 5-fluorocytosine relative to cytosine. Mutant enzymes have been identified that have a much lower activity toward cytosine, and a slightly higher activity against 5-fluorocytosine; for example, the mutant D314A has about 20-fold higher activity toward 5-fluorocytosine than cytosine [17]. When delivered by a lentiviral vector, this mutant inhibited the growth of colon carcinoma cell xenografts by a factor 6 compared to the wild-type cytosine deaminase, in the presence of 5-fluorocytosine [18]. The F186W mutant caused cytotoxicity in A549 human lung cancer cells at a significantly lower IC_{50} of 5-fluorocytosine compared to that with mild type cytosine deaminase [19].

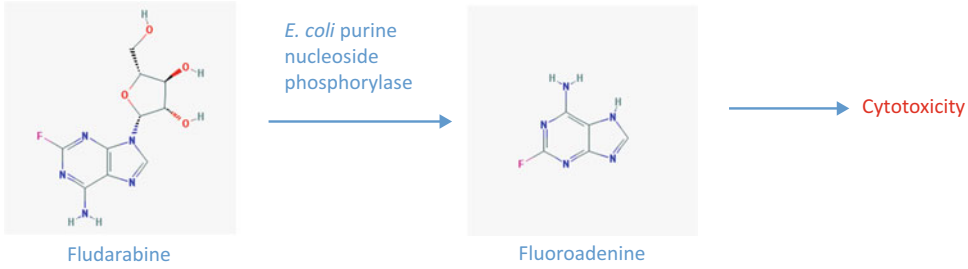

Fig. 3 Conversion of fludarabine to toxic fluoroadenine by purine nucleoside phosphorylase of *Escherichia coli*. The molecular structures were adapted from PubChem

2.3 Purine Nucleoside Phosphorylase and 6-Methylpurine Deoxyriboside or Fludarabine

The enzyme purine nucleoside phosphorylase of *Escherichia coli* catalyzes the conversion of deoxyadenosine analogs that are non-toxic to toxic adenine analogs (Fig. 3). When this enzyme was expressed in even less than 1% of human colonic carcinoma cells and the culture is treated with the deoxyadenosine analog 6-methyl-purine-2′-deoxyribonucleoside, essentially all the bystander cells were killed [20]. By contrast, control cells that were not transfected or mock-transfected were not affected appreciably, particularly since this analog is not a substrate for human purine nucleoside phosphorylase.

This system was applied to the treatment of human malignant glioma tumors that expressed the *E. coli* purine nucleoside phosphorylase gene in nude mice [21]. The mice were treated with the substrates of the enzyme (6-methylpurine-2′-deoxyriboside or arabinofuranosyl-2-fluoroadenine monophosphate). The products of enzyme action were toxic to the tumors, without producing apparent systemic toxicity to the animals. The administration of 3 doses of 6-methylpurine-2′-deoxyriboside essentially eradicated the tumors.

In a phase I clinical trial, the purine nucleoside phosphorylase gene was introduced into tumors by direct injection of an adenoviral vector and the prodrug fludarabine was given intravenously [22]. Significant tumor regression was observed in most patients receiving the highest doses of fludarabine. Fluoroadenine was produced locally, and was not found in serum.

2.4 Horseradish Peroxidase and Indole-3-Acetic Acid

The plant hormone indole-3-acetic acid (auxin) can be converted by horseradish peroxidase into the toxic product, 3-methylene-2-oxindole [23] (Fig. 4). When the cDNA for horseradish peroxidase was inserted in a mammalian expression vector and transfected into human T24 bladder carcinoma cells, treatment with indole-3-acetic acid resulted in cytotoxicity [24, 25]. Treatment with auxin alone or horseradish peroxidase alone at equivalent concentrations had no effect. The toxic product is likely to affect the nitrogenous DNA bases and thiol groups on proteins, and can also penetrate cell

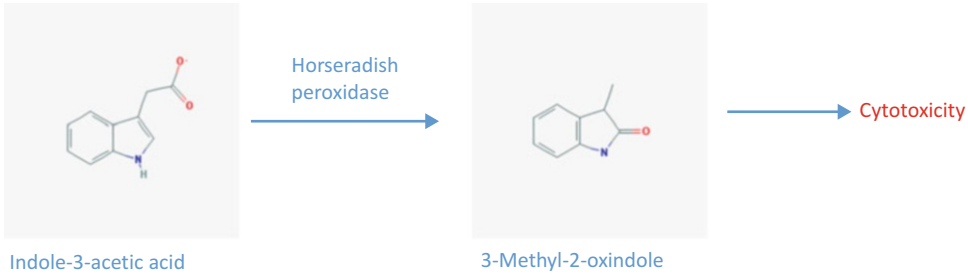

Fig. 4 Conversion of indole-3-acetic acid to toxic 3-methyl-2-oxindole by horseradish peroxidase. The molecular structures were adapted from PubChem

membranes. Thus cell–cell contact is not necessary for the bystander effect.

This suicide gene therapy modality was tested in SCID mice injected with FaDu human nasopharyngeal squamous cell carcinoma cells expressing horseradish peroxidase [26]. Millimolar concentrations of indole-3-acetic acid or 5-bromo-indole-3-acetic acid could be achieved in tumor tissue when administered intraperitoneally at nontoxic doses. However, only a modest growth delay of the tumors could be achieved with this system. In cell culture experiments, the stably transfected FaDu cells showed higher activity than transiently transfected cells, but the horseradish peroxidase/indole-3-acetic acid system was not as effective in the stably transfected cells [26].

Dai et al. [27] produced an adenovirus expressing horseradish peroxidase isoenzyme C under the control of the alpha-fetoprotein promoter (pAdv-AFP-HRPC), transduced hepatocellular carcinoma cells (H22, Hep-G2, Huh 7, Hep-B3) with this construct, and treated the cells with indole-3-acetic acid. Cytotoxicity increased as a function of the dose of indole-3-acetic acid and the time of incubation. Control Vero African green monkey kidney epithelial cells were not affected. The same system was used to treat H22 hepatoma xenografts, and the size and weight of the tumors were inhibited compared to a control group. Horseradish peroxidase was expressed in the tumor, but not in other organs [27].

2.5 Other Systems

Jounaidi et al. [28] described a suicide gene therapy system based on the activation of oxazaphosphorine drugs, such as cyclophosphamide, by cytochrome P450. The genes for the cytochrome P450 2B6 enzyme and for P450 reductase expressed in 9L gliosarcoma cells enhanced the cytotoxic effect of cyclophosphamide in vitro and in vivo.

Bridgewater et al. [29] transduced NIH3T3 cells with the *E. coli* nitroreductase gene using a recombinant retrovirus, and treated the cells with the prodrug CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide). The nitroreductase converts the prodrug into a

bifunctional alkylating agent, which kills the cells. This system was effective in human melanoma, mesothelioma, ovarian carcinoma, colorectal and pancreatic cancer cells [29, 30].

The human carboxylesterase gene was introduced into A549 human lung adenocarcinoma cells by means of an adenovirus vector, to convert irinotecan (CPT-11) into the active metabolite 7-ethyl-10-hydroxy-camptothecin (SN-38) that inhibits topoisomerase I [31]. This treatment arrested the growth of cells in vitro and reduced the size of subcutaneous xenografts in nude mice.

3 Suicide Gene Therapy in Perspective

One of the main problems with suicide gene therapy of solid tumors is considered to be the low transduction ability of the vectors delivering the therapeutic genes [6]. The inaccessibility of the vector to the cells at the core of the tumor, the potentially low or short-term expression of the suicide gene, and the rate of conversion of the prodrug to the active drug may all be contributing factors.

One of the early studies on gene expression in tumors following injection of naked DNA or its complex with cationic liposomes showed that gene expression was localized around the injection site [32]. This could be one of the limiting factors in achieving therapeutic efficacy. Nevertheless, binding of transferrin to cationic liposomes before the addition of DNA to form ternary complexes generates vectors that are net negatively charged that are likely to penetrate deeper into tumor tissue [33]. Such a vector was used in the treatment of oral squamous cell carcinoma tumors, whose growth was arrested by the intratumoral delivery of HSV-*tk* followed by ganciclovir administration [34].

Additional issues that may need improvement for successful clinical applications of suicide gene therapy are localization of the therapeutic gene in malignant cells or metastatic tumors, and the achievement of a substantial bystander effect without significant toxicity to non-cancer cells in the vicinity of the tumor.

An intriguing hypothesis regarding suicide gene therapy of cancer was put forward by Duarte et al. [4]. They proposed that the localized production of cytotoxic drugs by cancer stem cells transfected or transduced by a suicide gene will be more effective in treating cancer than the systemic administration of the drug. Cancer stem cells are resistant to apoptosis, have an active DNA repair process, express high levels of drug transporters, and are relatively quiescent, properties that may render them resistant to conventional chemotherapy [35]. These cells can therefore act as local drug-producing cells, thereby eliminating the neighboring "differentiated" tumor cells. The hypothesis further proposes that when the cancer stem cells finally divide, the drug they produce (e.g., phosphorylated ganciclovir) will finally kill them.

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Chapter 2

Cancer Suicide Gene Therapy with TK.007

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Abstract

Cancer is a devastating disease characterized by uncontrolled and aggressive cell growth. Suicide gene therapy (SGT) facilitating induction of malignancy-specific cell death represents a novel therapeutic approach to treat cancer, which has been investigated in several cancer types with very promising results. In addition, SGT has been suggested as a safeguard in adoptive immunotherapy and regenerative-medicine settings. Generally, SGT consists of two steps—vector-mediated delivery of suicide genes into tumors and subsequent activation of the suicide mechanism, e.g., by administration of a specific prodrug. This chapter provides a framework of protocols for basic and translational research using the Herpes-simplex-virus thymidine kinase (HSV-TK)/ganciclovir (GCV) system, the most widely used suicide gene approach. The protocols provide standard guidelines for the preparation of high-titer third-generation lentiviral vectors encoding a genetically improved HSV-TK version known as TK.007 and its application in in vitro and in vivo treatment setups.

Key words Cancer gene therapy, Suicide gene therapy, HSV-TK, Lentiviral vectors, Glioblastoma

1 Introduction

Thymidine kinase (TK) is an important enzyme in the thymidine salvage pathway. Human cells contain two distinct TKs: cell-cycle-dependent cytosolic TK1 and cell-cycle-independent mitochondrial TK2 [1, 2]. Herpes simplex viruses (HSVs) and some other types of DNA viruses also contain a thymidine kinase gene in their genome [2]. The TK from HSV-1 (HSV-TK), which has probably evolved distinctly from TK1 [3], demonstrates wider recognition of substrates. Apart from catalyzing thymidine, HSV-TK can also recognize various other pyrimidines, pyrimidine analogs and purine analogs. These purine and pyrimidine analogs can be phosphorylated by HSV-TK to mono-phosphate forms. Within cells additional phosphorylation is carried out by the cellular nucleotide mono-phosphate kinase (or by HSV-TK, depending on the analog) [2]. Subsequently, tri-phosphorylated forms of the nucleoside analogs can be generated by the action of various cellular nucleotide di-phosphate kinases. Tri-phosphorylated nucleoside analogs are

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cytotoxic due to interference with DNA elongation [4]. Furthermore, triphosphates such as GCV-TP (ganciclovir triphosphate) can directly inhibit the DNA polymerase, which in consequence also results in the inhibition of chain synthesis and cell death [5, 6]. These properties of the analogs and their recognition by the viral TKs underpinned the development of various classical antiherpetic drugs. At the same time, the substrate promiscuity of HSV-TK and substrate specificity of TK1 can also be harnessed to eliminate tumor cells in a selective fashion. By introducing HSV-TK into tumor cells Moolten and colleagues showed that tumor cells could be targeted selectively upon administration of nucleoside analogs HHEMG [7] and ganciclovir [8]. This concept of selectively conferring chemosensitivity has led to the development of suicide gene therapy (SGT) approaches, whereby an introduced gene would lead to selective cell killing upon prodrug (e.g., a nucleoside analog) exposure.

A variety of useful suicide genes with corresponding prodrugs have been introduced over the years [9], with HSV-TK being most extensively used and studied. Among several suitable prodrugs for the HSV-TK system, ganciclovir (GCV), acyclovir (ACV), and brivudin (BVDU) have been most widely tested. Based on comprehensive parameters such as potency of cytotoxicity, performance as substrate for HSV-TK and the capacity of inter-cellular diffusion (the basis for the so-called bystander effect), GCV was found to be the most effective prodrug for HSV-TK SGT and thus is most commonly used [4, 10–12]. Importantly, GCV has been applied for many years in the treatment of CMV infections and has a well-established safety profile. Moreover, data from numerous preclinical and clinical studies indicates that the use of the HSV-TK/GCV system for SGT is safe and feasible [13–17].

HSV-TK.007 (TK.007) is a novel recombinant version of the wild-type HSV-TK that has been developed to mend several shortcomings of HSV-TK: (1) higher affinity and catalytic efficiency toward endogenous thymidine compared to GCV [2, 18], (2) presence of cryptic promoter and splice sites resulting in truncated products due to anomalous transcription [19] or splicing [20], and (3) relatively slow kinetics for cytotoxicity [21]. TK.007 harbors a point mutation at its 168th amino acid position (A168H) that imparts the enzyme with greater specificity toward GCV compared to thymidine [18]. In enzyme assays, Balzarini et al. showed that this mutant has higher efficiency (V_{max}/K_m) for GCV than wild-type HSV-TK [18]. Furthermore, TK.007 has been codon-optimized to eradicate cryptic sites and improve expression. Indeed, increased expression and lower non-specific toxicity of TK.007 compared to a splice-corrected HSV-TK version [22] were shown in hematopoietic cells [21]. Later we also confirmed the superior performance of the new suicide gene in cancer gene

therapy settings using our lentiviral LeGO vector [23] platform for delivery [24].

In the subsequent sections, we will provide a comprehensive protocol of cancer gene therapy with TK.007 using lentiviral gene transfer. As a disease model, we apply the malignant brain tumor glioblastoma multiforme (GBM) that is studied both in vitro and in vivo.

1.1 Basic Issues with Lentiviral Vectors and Animal Works

Lentiviral vectors are generated in so-called producer cell lines based on the complementation principle [25, 26]. To this aim, several plasmids encoding different components of the viral particles are co-expressed together with a virus-derived vector genome that lacks all viral genes. This means that the individual plasmids encoding viral proteins are noninfectious, but lentiviral vector particles generated upon co-transfection of all necessary plasmids in producer cells are infectious but non-replicative, genome-integrating agents [for more information: www.LentiGO-Vectors.de]. Since these lentiviral particles are dedicated to infect/transduce human cells, they must be handled under Biosafety-Level (BSL) 2 conditions with dedicated sets of instruments. Everything that comes in contact with vector-containing solutions or aerosols should be decontaminated (by autoclave/bleach/virkon/70% ethanol, depending on the production material) or contained in biohazard bags before exiting the biosafety cabinet. After intracranial injections, infectious lentiviral vector particles might still be present in rodents for a few days, although the likelihood is very low. Check with your local biosafety officer, from which time point treated animals can be housed under biosafety level 1. Note that any intervention in research animals is subject to national laws and regulations.

2 Materials

1. Plasmids—Lentiviral vector expressing TK.007: pRRL.PPT.CMV.TK.007-GFP.pre* (*see* **Note 1**); Packaging plasmids—(1) pMDLgpRRE (Addgene # 12251) [26] (2) pRSV-Rev (Addgene # 12253) [26] (3) phCMV-VSV-G [27] (*see* **Note 2**).
2. Cell lines: HEK 293T (ATCC # CRL-11268), U87-MG (ATCC # HTB-14), P3 [28].
3. Culture Media—DMEM supplemented with 10% FBS, 2% (100 U/mL) Penicillin/(100 μg/mL) Streptomycin [Pen/Strep], 2 mM Glutamax, 0.5 μg/mL Plasmocin; NB (Gibco) supplemented with 1× B27 (Invitrogen), 20 ng/mL bFGF, 2 mM Glutamax and 1% Pen/Strep (50 U/mL Penicillin and 50 μg/mL Streptomycin).

4. 0.05% Trypsin-EDTA solution.
5. Accutase (Innovative Cell Technologies).
6. T-175 tissue culture flask, 15 cm tissue culture dish.
7. X-tremeGENE DNA transfection reagent (Roche).
8. Optimem I (Gibco).
9. 50-mL Falcon tube.
10. 20-μm and 70-μm cell strainer.
11. 0.45-μm syringe filters.
12. Sterile glass bottle.
13. Digital balance (readability 10 mg or better).
14. Ultracentrifuge (Beckman & Coulter, Optima LE80K).
15. SW 28 rotor and bucket set (Beckman & Coulter # 342204, 342217), thick-wall polycarbonate tube (Beckman & Coulter # 355631).
16. Benchtop centrifuge.
17. Phosphate buffered saline (PBS) and Hank's balanced salt solution (HBSS).
18. Microcentrifuge tubes.
19. 6-well and 96-well cell culture plates.
20. Ganciclovir (Sigma).
21. Plate reader (Thermo Scientific Multiskan FC).
22. Isoflurane or Sevoflurane.
23. Marcaine (Bupivacaine).
24. Temgesic (Buprenorphine).
25. MRI (7T Pharmascan, Bruker Biospin, Ettlingen, Germany) equipped with surface or volume coil set up.
26. Stereotactic frame with anesthesia mask.
27. Two sets of surgical instruments:
    (a) Needle holders.
    (b) Forceps.
    (c) Scissors.
    (d) Scalpel holders.
    (e) Clamps.
    (f) Vessel clips (AgnThos # 13-320-035).
28. Suture 4-0.
29. Automated injector (World Precision Instruments, UltraMicroPump).
30. Hamilton gastight syringe (ga 22 s/51 mm/pst2, 25 μL).

31. Hydrophobic barrier pen (Vector).
32. Fetal calf serum.
33. Horse serum.
34. Goat serum.
35. Anti-HSV-1 TK antibody (Santa Cruz # 28038).
36. Biotinylated horse anti-goat antibody (Vector Labs # BA9500).
37. Anti-GFP antibody (Merck Millipore # AB3080).
38. Biotinylated Goat anti-rabbit antibody (Vector Labs # BA1000).
39. ABC reagent (Vector Labs).
40. Bovine serum albumin.
41. Chromogen solution DAB$^+$ (DAKO).
42. Hematoxylin.
43. Light microscope (Nikon Eclipse E600, Nikon, Japan), fluorescence microscope.
44. Cell sorter, e.g., FACSAria III (BD Bioscience), flow cytometer, e.g., Accuri C6 (BD Bioscience), equipped with appropriate filter sets to detect eGFP.

3 Methods

3.1 Production and Collection of Lentiviral Particles

1. Seed 5×10^6 293T cells (per dish) in two to four 15-cm tissue culture dishes with 20 mL complete DMEM/dish. Swirl the dish to allow uniform distribution of the cells. Incubate in a humidified cell culture incubator at 37 °C with 5% CO_2. After 16 h of seeding the cells, proceed with transfection of the cells with vector-producing plasmids by using X-tremeGENE reagent (*see* **Notes 3** and **4**).
2. Add plasmids in 1 mL prewarmed Optimem I (for one dish) according to the table below:

Plasmid	Amount (μg)
pRRL.PPT.CMV.TK.007-GFP.pre	5.6
pMDLgpRRE	4.8
pRSV-Rev	2.4
phCMV-VSV-G	1.2

3. Gently tap the tube and briefly spin to allow uniform distribution of the plasmids. Incubate for 5 min at room temperature and then gently pipette 42 μL of X-tremeGENE reagent into

the tube. Leave the tube at room temperature for 30 min. Meanwhile, replace the old medium of 293T cells by fresh and prewarmed 20 mL DMEM.

4. After 30 min of incubation with X-tremeGENE, slowly add 500 μL of the mixture to the 293T cells in one half of the dish drop by drop. Repeat the procedure with the rest of the mixture in the other half of the dish in a similar manner. Gently shake or swirl twice to promote uniform distribution of the mixture within the dish. Put the dish back to the cell culture incubator.
5. Collect first round of supernatant after 24 h of transfection in a sterile glass bottle by straining it through a 10- or 20-μm strainer to minimize filter-clogging (by removing cell debris) in later filtration (*see* **Notes 5** and **6**). Place the bottle on ice. Promptly add 20 mL prewarmed DMEM into the dish and put it back into the incubator. Filter the viral supernatant through a 0.45-μm filter into a new sterile glass bottle. Keep the supernatant in +4 °C (*see* **Note 7**).
6. Collect supernatant every 12 h afterward for two more times, according to the same procedure and pool together the supernatants (*see* **Note 8**). After final collection, proceed to concentration of viral particles by ultracentrifugation.

3.2 Concentration of Lentiviral Particles

1. Fill thick-walled polycarbonate ultracentrifuge tubes that are compatible with SW 28 swinging bucket rotors with 26 mL of viral supernatant (*see* **Note 9**). Carefully balance the tubes, so that the weight difference between two opposing tubes is not greater than 0.05 g. Gently slide the tubes into corresponding buckets.
2. After installing the rotor onto the driver inside the centrifuge chamber, close the door, switch on *vacuum*, and wait until the temperature reaches 4 °C (*see* **Note 10**). Set the run speed to 103,679 × *g* (24,000 rpm) for 90 min. Start the run (*see* **Note 11**).
3. After the centrifuge run, decant the supernatant carefully and place the tube upside-down on a sterile piece of tissue paper for 3–5 min.
4. Pour 100 μL ice-cold 1× HBSS over pellet, which should remain at the bottom of the tube (*see* **Notes 12** and **13**). Leave it on ice for 5 min and then slowly pipette up and down for 10–20 times without making any bubbles. Use the same dissolving medium in the same way to dissolve and pool all pellets from the other tubes. Finally, aliquot the suspension in small tubes and freeze down at −80 °C until further need (*see* **Note 13**).

3.3 Titration of Functional Lentiviral Particles by FACS

1. Seed 5×10^4 U87 cells/well (or another cell line used as standard for virus titration) in total 12 wells of a 24-well plate in a final volume of 500 μL complete DMEM. Gently swirl the plates to make the cells distribute uniformly.
2. After 10–16 h add 1 μL, 10^{-1} μL, 10^{-2} μL, 10^{-3} μL, and 10^{-4} μL of vector suspensions serially diluted in 10 μL DMEM into the wells. Take each amount of suspension in duplicates. Leave two wells without adding viral suspension to be used as control. Wrap plastic paraffin film (parafilm) around the plate and spin at $720 \times g$ for 1 h at 31 °C (spinoculation). Take the plate back into the incubator (*see* **Note 14**).
3. After 5 days, remove medium and wash the cells with 1× PBS. Add 100 μL trypsin and incubate for 3 min at 37 °C. Harvest the cells in 300 μL cold DMEM in a microcentrifuge tube or FACS tube (*see* **Note 15**). Determine the percentage of GFP^+ cells in each dilution by FACS analysis (*see* **Note 16**).
4. Calculate the titer by the following equation (*see* **Note 17**):

$$\text{Titer (transducing units/mL)} = \left\{5 \times 10^4 \times (\text{percentage of GFP}^+\text{cells}/100)\right\} \div \text{Volume of supernatant (in mL)}$$

The detailed procedure outlined above routinely results in titers of more than 1×10^8 transducing units per mL.

3.4 In Vitro GCV Sensitivity Assay

1. Seed target cells (e.g., U87) in each well of a 6-well plate at a density of 3×10^5 cells in 2 mL complete DMEM per well.
2. After 12–16 h, transduce the cells with thawed viral supernatant at an MOI of 0.25–1 (*see* **Note 18**). Leave two wells untreated to serve as controls. After adding the viral supernatant, follow the spinning protocol as described in Subheading 3.3, **step 2** (*see* **Note 19**).
3. After 3 days, harvest the cells in a 15-mL falcon tube by trypsinization, spin at $170 \times g$ for 3 min, and remove the supernatant (*see* **Note 20**). Dissolve the pelleted cells in fresh and prewarmed DMEM in a T-175 flask and put them back into the cell culture incubator.
4. After 3 additional days, sort the cells by FACS on the basis of eGFP expression. Culture the sorted cells (U87.TK.007-eGFP) further for 1 or 2 weeks (*see* **Note 21**).
5. Seed U87.TK.007-eGFP and U87 cells separately in 48 individual wells of a 96-well plate at a density of 2×10^4 cells in 50 μL DMEM per well. Additionally, mix these two cell types in two ratios: (1) 50% U87.TK.007-eGFP cells + 50% U87 cells, (2) 10% U87.TK.007-eGFP cells + 90% U87 cells and seed the cell-mixtures separately in 48 individual wells into the same or a second 96-well plate in similar density.

6. Prepare DMEM without GCV and with three different concentrations of GCV, namely 2 μM GCV, 20 μM GCV, and 200 μM GCV. Add 50 μL of this freshly prepared DMEM in such a way that each cell type or mixture type receives 6 wells of normal DMEM, 6 wells of DMEM + 2 μM GCV, 6 wells of DMEM + 20 μM GCV, and 6 wells of DMEM + 200 μM GCV (*see* **Note 22**). Place the cells back into the cell culture incubator. Prepare another 96-well plate by adding 100 μL of cell-free DMEM (with 0 μM GCV, 1 μM GCV, 10 μM GCV, and 100 μM GCV; 6 replicates each) to serve as blank for absorbance reading. Keep it in the same cell culture incubator.
7. After 72 h, add 10 μL Wst-1 reagent to each well. After a gentle 1-min shake incubate at 37 °C for 1 h.
8. Measure absorbance at 440 nm by using a microplate reader. Calculate mean absorbance of each sample type in each treatment condition from the six replicates, subtract the corresponding mean blank values, and compare with the corresponding values derived of U87 cells (*see* **Note 23**).

3.5 Treatment with TK.007 in Experimental Brain Tumor Model

1. Prepare P3 cells (or cells from another glioblastoma/brain tumor cell line) by detaching from culture flask using accutase (trypsin can be used if the medium contains serum). Count the cells and collect 100,000 cells in 5 μL PBS (*see* **Note 24**).
2. Anesthetize nude rats by using 5% isoflurane at an oxygen flow rate of 1 L/min. Alternatively, 8% sevoflurane can be used (*see* **Note 25**). When the animal is sleeping and respiration frequency is approximately 30/min, transfer the animal to stereotactic frame followed by confirmatory reflex checking (*see* **Note 26**). Secure the anesthesia mask properly to the mouthpiece on the frame and set the vaporizer to 1% (isoflurane) or 3% (sevoflurane) (*see* **Note 27**).
3. Inject 0.5 mL of Marcain subcutaneously (s.c.) into the scalp as local anesthesia and in addition 0.1 mL Temgesic s.c. as analgesic over the flank (*see* **Note 28**). Eye-balm in both eyes should also be provided. Shave the operation site and disinfect using 70% ethanol-chlorhexidine (2%). After 2 min, make a longitudinal incision by sterile scalpel. Gently expose the skull sutures by using sterile cotton-tipped applicators and scalpel. To get an uninterrupted view of the skull, fix two vessel clamps to the incised skin on two sides and let the clamps fall so that the two parts of the incised skins on two sides are restrained away from each other.
4. Inject 5 μL P3 cell suspension at the following coordination: 3 mm right of the sagittal suture, 1 mm posterior of the bregma and 2.5 mm beneath the dura by using a glass syringe from Hamilton (ga 22 s/51 mm/pst2) (*see* **Note 29**).

5. After injection, wait for 5 min and then slowly retract the injection needle. Close the wound by sterile suture (size 4-0). Administer the animal with 0.9% NaCl (1 mL per 100 g) s.c. and keep in a heating chamber until it wakes up. Animals should be fed with temgesic-containing gels or injected with temgesic postoperatively for 2–3 days (*see* **Note 30**).
6. Follow the tumor growth by T2-weighted anatomical MRI sequences (fast spin echo, TE/TR: 36 ms/3500 ms, in-plane resolution: 0.137 Å ~0.137 mm per pixel, slice thickness: 1 mm). Generally, after 3–4 weeks (for P3 cells) the tumor volume reaches a reasonable and detectable size (app. 10–15 mm^3) when intratumoral vector injection should be carried out. Analyze the most recent image and determine the distance of the center of the tumor mass from dural surface (*see* **Note 31**).
7. Anesthetize the animals and prepare the site according to Subheading 3.5, **steps 2** and **3**. Clear the connective tissues over the injection hole of the last operation. Inject 10 μL TK.007-eGFP lentiviral suspension (titer should be in the range of 1×10^8–1×10^{10}) by using an automated injector (microprocessor controlled stereotactic infusion) according to the following velocity to allow widened distribution through the tumor tissue.

Volume (nL)	Speed (nL/min)
2000	200
4000	400
4000	800

8. Follow Subheading 3.5, **step 5** to close the wound.
9. After 5–7 days, start treating the animals with GCV. Each animal should get daily intraperitoneal injection (i.p.) of GCV at a dose of 50 mg/kg/day for 3 weeks (*see* **Note 32**). Follow tumor reduction weekly by using MRI scanning. A treatment duration of 3–4 weeks should result in remission on MRI.

3.6 Analysis of In Vitro TK.007 Expression by Western Blotting

Extract cell lysates from U87.TK.007-eGFP (Subheading 3.4, **step 4**). Follow standard immunoblot procedures with the lysates. Identify the TK.007-eGFP fusion protein by using antibodies directed against HSV 1-TK or GFP.

3.7 Analysis of In Vivo TK.007 Expression by Immunostaining with Anti-HSV-1 TK Antibody

1. Euthanize TK.007-injected rats with CO_2 and collect the whole brain after intracardiac perfusion with 0.9% NaCl. Preserve the brain tissue by snap freezing (*see* **Note 33**). Fix the mounted frozen brain sections (12 μm) in acetone for 10 min followed by chloroform for 7 min. Make a circle around the sections by using a hydrophobic barrier pen and block with 5%

horse serum and 5% fetal calf serum in 5% BSA/PBS for 20 min. Incubate with primary goat anti-HSV 1-TK antibody diluted (1:200) in PBS for 1 h at room temperature. After washing with PBS incubate with secondary biotinylated horse anti-goat antibody (1:300) for 1 h at room temperature.

2. Wash again with PBS and add a couple of drops of ABC complex. Incubate for 30 min and then wash with PBS again. Apply DAB color development solution for detection of TK.007 and stop the reaction until sufficient coloring is observed under a light microscope. Counterstain with hematoxylin and follow standard dehydration steps according to the following sequence: ddH_2O for 2 min/96% ethanol 2 × 3 min/100% ethanol 2 × 3 min/xylene 2 × 5 min. After the dehydration steps, place a cover glass on the sections by using a xylene-based mounting medium and let it dry under a hood. The dried sections should be stable and can be used for detailed analysis for several years.

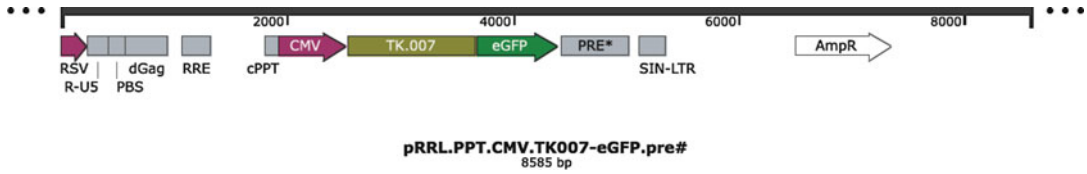
3.8 Analysis of In Vivo TK.007 Expression by Immunostaining with Anti-GFP Antibody

1. Collect the brain of a TK.007-injected rat according to Subheading 3.7, **step 1** and embed sections in paraffin. After following standard deparaffinization procedure (*see* **Note 34**) for paraffin-embedded sections, carry out antigen retrieval by placing the slides in 10 mM citrate buffer (pH 6.0) at 95 °C for 25 min. After cooling down for 15 min at room temperature, get rid of the citrate buffer by running tap water for 15 more minutes. By using a hydrophobic barrier pen make circles around the tissue and block the sections with 5% goat serum/TBST and 5% BSA for 30 min. Wash with TBST and incubate with primary anti-GFP antibody (1:200) at 4 °C overnight. The next day, after washing off the primary antibody solution, add 3% hydrogen peroxide in PBS and incubate for 10 min. Wash with TBST and incubate with biotinylated anti-rabbit antibody (1:100) at room temperature for 1 h.
2. Follow Subheading 3.7, **step 2**.

4 Notes

1. The plasmid is designed as an HIV-1-based third-generation SIN vector expressing TK.007 fused with eGFP. The TK.007 segment with Kozak sequence was cloned from LeGO-iG2-NeoOpt-TK.007 [24] and inserted into pRRL.PPT.EFS.GFPpre* (kind gift from Prof. Axel Schambach, Hannover, Germany) under control of a CMV promoter obtained from M488 (kind gift from Prof. Dorothee von Laer, Innsbruck, Austria). Finally, an eGFP PCR fragment was fused to TK.007

in the same reading frame to yield 8585-bp long pRRL.PPT.CMV.TK.007-GFP.pre*. The schematic map is shown below, and full sequence can be shared upon request.



2. 2nd-generation packaging plasmids can also be used to produce viral particles with pRRL.PPT.CMV.TK.007-GFP.pre*. Both types of packaging plasmids are available in the nonprofit repository Addgene (*addgene.org*), but variants can be procured from various commercial sources. A different VSV-G encoding plasmid, known as pMD2-G, is available from Addgene (Addgene # 12259) which works as good as phCMV-VSV-G.
3. Transfection can also be done by traditional $CaPO_4$ transfection method. It should not matter which transfection method is being used as long as the reagent is effective with minimal or no toxicity and the corresponding protocol is followed properly. Ready-made commercial reagent (i.e., X-tremeGENE) is more expensive but less cumbersome. Using different transfection methods, it might be necessary to titrate the total amount of plasmids used. For instance, more than double amounts of plasmids are needed (relative to the amount mentioned in this protocol) for the traditional $CaPO_4$ transfection method.
4. Ensure that the cells are ~70% confluent during X-tremeGENE transfection. The reagent should be at room temperature before adding to Optimem I. After adding to the tube, do not vortex or pipette up and down. It can be tapped, but very gently.
5. 14–16 h post transfection, vector production starts and viral particles bud out of the transfected cells' plasma membrane and become deposited in the medium. Maximal production continues from approximately 20–48 h.
6. Cell debris can alternatively be removed by centrifuging the collected supernatant at 2000 × *g* for 2–3 min at 4 °C.
7. Using 0.2-μm filter can reduce yield.
8. Collection strategy can be simplified by harvesting supernatants only two times: 24 h and 48 h post transfection. However, this might lower the titer, as lentiviral particles are sensitive to higher temperature (i.e., inside the cell culture incubator).

9. Other swinging bucket rotors and compatible tubes can be used as long as they support the speed of 24,000 rpm (about 80,000 × *g*) or more.
10. Before installing the rotor onto the driver hub inside the centrifuge chamber make sure that neither the rotor nor the buckets are scratched or malformed and the O-rings are in place. Buckets must be hooked into the correspondingly designated places in the rotor. Proper installment in the driver hub should make a confirmatory faint click. To further confirm, gently turn the rotor clockwise by hand (e.g., up to 100–150 rpm). Read the operation instructions of the centrifuge carefully, since ultracentrifuges are a serious source of danger if used improperly.
11. Concentration can also be carried out by overnight normal centrifugation at 8000 × *g* at 4 °C.
12. Pellet mostly consists of cell debris and medium components. It may be faintly visible or invisible. In any case, the pellet should remain at the nethermost point of the tube.
13. Amount of dissolving medium can vary upon necessity and depends on the total amount of pooled supernatant. For instance, 50–150 μL/tube for 30 mL original volume is optimal. Instead of HBSS, PBS (with Ca^{2+}, Mg^{2+}) or normal PBS will also work. Dissolving period can also be increased from 5 min up to several hours to minimize retention of particles in the tube. Small aliquots (e.g., 20 μL) are recommended, because lentiviral particles are highly sensitive to freeze-thaw cycles (one cycle can potentially result in up to 50% titer loss). Although the suspension is quite stable at 4 °C up to 3–4 days, it makes sense to titrate from a frozen batch unless the whole lot is being used nonfrozen immediately after production.
14. Spinoculation is an effective method for transduction with lentiviral particles. Transduction efficiency can further be increased by using positively charged polycations such as polybrene, DEAE-dextran, and protamine sulfate that promote favorable juxtaposition between the cells and the vectors. Of note, the use of any additives improving transduction will influence the measured infectious titer of the tested supernatant. About 24–48 h after spinoculation, eGFP expression becomes strong enough to be detectable in the newly transduced cells, especially with higher load of viral particles. The maximum brightness is reached after about 5 days.
15. Transduced cells can be analyzed directly 3 days after spinoculation, if the flow cytometer is situated in a BSL2 facility. Alternatively, the cell pellets can be incubated with 1–2% formaldehyde in PBS for 5 min to inactivate remnant viral particles before transferring them to a FACS facility that belongs to a BSL1

premise. This treatment will also fix the cells. It might reduce the brightness of eGFP fluorescence significantly, but in most cases transduced cells remain bright enough to be detected.

16. In case of unavailability of a flow cytometer/FACS, percentage of cells can be counted with lower precision by analyzing several random fields of view using a fluorescence microscope.
17. Use a dilution that yields 2–25% of GFP^+ positive cells. In this range, most transduced cells contain one single vector integration only. Calculation of the titer based on higher transduction rates will lead to apparently lower titers because of multiple vector integrations within single cells.
18. Viral suspension needed for a particular MOI can be calculated by the following formula:

    Volume of viral suspension required (μL) = {Number of cells seeded × MOI × 1000} ÷ titer (from Subheading 3.3, **step 4**). An MOI of 0.25–1 will increase the likelihood of single (and thus equal) copy numbers per cell within the majority of transduced cells (reviewed by Fehse et al. [29]). For purposes where copy numbers are not an important issue, a higher MOI can be used.
19. The same spinoculation protocol (with or without polycations) can be applied, that also has been used for titration. However, lentiviral transduction is a very complex process that will significantly vary from cell line to cell line and from in vitro to in vivo.
20. There may be some viral particles still active at this point, so the passaging should be done in a hood dedicated for BSL2 viral work.
21. In case of unavailability of a cell sorter/FACS, use higher MOI (e.g., MOI 4–5) that would likely result in near 99% transduction rate with varying numbers of integration events per cell. Before starting the cytotoxicity experiment, approximately confirm expected eGFP expression according to **Note 16**.
22. Final GCV concentration becomes: 0 μM GCV, 1 μM GCV, 10 μM GCV, and 100 μM GCV.
23. To calculate the absolute number of cells survived, a standard graph can be made separately by seeding different numbers of U87 cells per well and plotting the absorbance against the corresponding cell number.
24. The reason for using P3 as a model for in vivo SGT for brain tumors is that P3 is a stem-like glioma cell line that is cultured in a defined neurobasal medium. This system is reported to be a more reliable preclinical model for GBM research than many classical serum-based glioma cell lines (reviewed in [30]).

25. Use of N_2O flow is not mandatory and should rather be avoided due to potential health hazards. Injectable anesthetics can also be used as an alternative.
26. "Pedal withdrawal reflex" can be examined by pinching the foot-pad on both hind feet. Lack of response would indicate that adequate level of anesthesia has been obtained. "Falling sensation test" is also a powerful test in rats where a forced falling-sensation makes the rat straighten its tail in case of suboptimal anesthesia.
27. Change the direction of gas-flow. Continuously monitor (by, e.g., respiration, temperature, and pedal withdrawal reflex) the animal and adjust the settings depending on the level of consciousness and respiration frequency.
28. Rub after marcain injection.
29. First lower the needle up to 3 mm from the dural surface and then retract 0.5 mm to make a small "pouch" for injection. For a reproducible implantation procedure it is recommended to use an automated injector at a speed of 500 nL/min.
30. All surgical instruments should be autoclaved before surgical intervention. For sequential surgical intervention of multiple animals (especially nude rats), it is recommended to sterilize the used surgical tools by putting them inside a hot bead sterilizer for 1 min before engaging with the next animal. After operation, animals should be kept separately for 2–3 days; otherwise they may disturb each other's wound area.
31. Volume of the tumor can be ascertained by quantifying total "area" of the tumor mass that is visible in the MRI sequences by using ImageJ software. Multiplication of the obtained "area" with the thickness of MRI sequences (i.e., 1 mm in this protocol) will equal the volume of the tumor mass.
32. Do not perform i.p. injections in the same side everyday. For example, injection in the lower left-quadrant can be followed by lower right-quadrant on the next day and further likewise.
33. The HSV 1-TK antibody mentioned here does not work on paraffin sections in our hands. Therefore, the use of cryosection is preferred.
34. Chronological reversal of the dehydration procedure, i.e., xylene 2 × 5 min/100% ethanol 2 × 3 min/96% ethanol 2 × 3 min/ddH_2O for 2 min.

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Chapter 3

Osteonectin Promoter-Mediated Suicide Gene Therapy of Prostate Cancer

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Abstract

Suicide gene therapy using the herpes simplex virus thymidine kinase (HSV-*tk*) gene, combined with the prodrug ganciclovir (GCV) medication, is a promising approach for the treatment of malignant tumors, including prostate cancer. The success of this therapeutic strategy requires tissue- or tumor-specific gene expression and efficient gene delivery. In this chapter, we describe the experimental protocols of key methodologies, including promoter construction, reporter assay, adenoviral vector construction and preparation, HSV-*tk* enzymatic assay and cytotoxicity assay to evaluate the specificity and efficacy of osteonectin promoter-mediated HSV-*tk*/GCV suicide gene therapy of prostate cancer.

Key words Osteonectin promoter construction, Suicide gene therapy, Prostate cancer, Adenoviral vector, HSV-*tk* enzymatic assay

1 Introduction

The end-stage of prostate cancer is commonly associated with osseous metastasis, which causes significant mortality and morbidity in affected patients [1, 2]. Unfortunately, there are few treatment options available for men who develop hormonal refractory bone metastasis. Novel therapeutic strategies need to be developed to improve the survival of prostate cancer patients with skeletal metastasis. The promoter of osteonectin (ON), known as BM-40 and SPARC, a major noncollagenous protein in bone, was employed here to direct the expression of herpes simplex virus thymidine kinase (HSV-*tk*) in both prostate tumor epithelium and supporting bone stromal cells. ON promoter-mediated suicide gene therapy is effective in controlling the growth of both localized and metastatic prostate tumors [3].

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2 Materials

2.1 Osteonectin Promoter Assay Using Firefly Luciferase Reporter

2.1.1 Genomic DNA Isolation from Cultured Cells

1. DU145 human prostate cancer cells are available from the American Type Culture Collection (ATCC).
2. Cell growth medium: RPMI 1640 medium (Invitrogen Cat. no. 11875085 or other equivalent) supplemented with 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Store media in 4 °C and warm to 37 °C prior to use.
3. Sterile phosphate buffered saline (PBS, 1×) without Ca^{2+} and Mg^{2+}.
4. Trypsin-EDTA (2.5 g Trypsin and 0.2 g EDTA in 1 L PBS). Stir until solution clear. Filter solutions with 0.22 μm pore size filter membranes.
5. Digestion buffer: 10 mM Tris (pH 7.5), 25 mM EDTA (pH 8.0), 100 mM NaCl, 0.5% SDS, and 0.1 mg/mL Proteinase K.
6. 25:24:1 (v/v) phenol/chloroform/isoamyl alcohol. Store at 4 °C.
7. 24:1 (v/v) chloroform/isoamyl alcohol.
8. 3 M Sodium acetate.
9. 100% and 70% ethanol.
10. DNA resuspension buffer: TE (10 mM Tris, pH 7.4, 0.5 mM EDTA, pH 8.0).
11. Centrifuge (Eppendorf model 5810 R).
12. Microcentrifuge (Eppendorf model 5430 R).
13. Incubator (Labnet model 311DS).
14. Rotor (Biosan Bio model RS-24).
15. −20 °C freezer.

2.1.2 Osteonectin Promoter Construction Using TOPO TA Cloning System

1. Genomic DNA template and primers for PCR product (*see* **Note 1**).
2. Taq polymerase or a mixture of polymerase containing Taq and a proofreading DNA polymerase supplemented with 10× reaction buffer.
3. 10 mM dNTPs (10 mM of each dATP, dCTP, dTTP, and dGTP).
4. TOPO TA cloning kit containing pCR2.1-TOPO vector (Invitrogen, Cat. no. 450641).
5. DH-5α or other desired chemically E. coli competent cells.
6. LB plates containing 50 μg/mL ampicillin or 50 μg/mL kanamycin.

7. S.O.C. medium.
8. Thermocycler (Applied Biosystems model Veriti 96-Well Thermal Cycler).
9. 37 °C shaking incubator.
10. 42 °C water bath.

2.1.3 Osteonectin Promoter-Driven Luciferase Reporter (ON-Luc) Assay

1. Human prostate cancer cell lines, such as LNCaP, PC3, and DU145, are available from the American Type Culture Collection (ATCC).
2. Cell growth medium: RPMI 1640 medium (Invitrogen, Cat. no. 11875085) or other equivalent supplemented with 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Store media in 4 °C and warm to 37 °C prior to use.
3. pGL3-basic vector (Promega, Cat. no. E1751) or other *firefly* luciferase reporter plasmids.
4. pRL-TK vector (Promega, Cat. no. E2241) or other *renilla* luciferase control reporter plasmid.
5. Lipofectamine 2000 (Invitrogen) or other desired transfection reagent.
6. Opti-MEM/ Reduced-Serum Medium (Invitrogen Cat. no. 31985062) or other equivalent.
7. Dual luciferase reporter assay system (Promega, Cat. no. E1960).
8. 6-well cell culture plate.
9. Falcon polystyrene round bottom tubes with snap cap (BD Bioscience Cat. no. 352054).
10. Cell scraper.
11. 1.5 mL microcentrifuge tube.
12. Microcentrifuge (Eppendorf 5424R).
13. Plate shaker (Lab-Line model 4625 - 11).
14. Luminometer (Promega model GloMax 20/20).

2.2 ON-HSV-tk Adenoviral Vector (Ad-ON-HSV-tk) Construction and Preparation

1. pAd/PL-DEST Gateway Vector Kit (Invitrogen Cat. no. V49420).
2. pENTR 1A (Invitrogen Cat. no. 11813-011) containing osteonectin promoter-driven HSV-tk expression cassette (*see* **Note 2**).

2.2.1 Ad-ON-HSV-tk Construction Using Gateway System

3. Gateway® LR Clonase II Enzyme Mix (Invitrogen Cat. no. 11791-020).
4. TE Buffer, pH 8.0.
5. 37 °C water bath.

6. Endotoxin-Free Plasmid DNA Isolation Kit (Geneaid Cat. no. PIE25 or from other manufactures).
7. Restriction enzyme Pac I (New England Biolabs Cat. no. R0547S).
8. Lipofectamine 2000 or other transfection reagent.
9. 293A cells (Invitrogen, Carlsbad, CA, USA).
10. 293A culture medium: DMEM supplemented with 10% heat-inactivated fetal calf serum, 0.1 mM Non-Essential Amino Acids (NEAA), 2 mM L-glutamine, and 1% Pen-Strep.
11. Dry ice.
12. Methanol.

2.2.2 *Ad-ON-HSV*-tk Amplification and Purification

1. 1× PBS.
2. $CsCl_2$ solution (*see* **Note 3**):
   (a) 1.5 g/mL: 30 g $CsCl_2$, ultrapure (Invitrogen Cat. no. 15507023, or other equivalent) into a final volume of 42.5 mL PBS.
   (b) 1.33 g/mL: 15 mL of (a) to a final volume of 21 mL with PBS.
   (c) 1.25 g/mL: 11 mL of (a) to a final volume of 20 mL with PBS.
   (d) 1.33 g/mL: 15 mL of (a) to a final volume of 22.7 mL with PBS.

   Filter sterilize with 0.22 μm filter and store at room temperature.
3. Dialysis buffer: 10% glycerol, 10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, and 150 mM NaCl. Three litters are needed for one purification. Sterilize by autoclaving and store at 4 °C.
4. 37 °C water bath.
5. Refrigerated centrifuge (Eppendorf 5810R).
6. Ultracentrifuge (Beckman Optima LE-80K) with SW40Ti swing bucket rotor (Beckman Coulter, Fullerton, CA, USA).
7. Beckman Coulter ULTRA-CLEAR thinwall tube (BK344059).
8. Spectra/Pro 7 dialysis tubing (28 mm; 50,000 MWCO; Spectrum no. 132129).
9. Dialysis tubing closures (35 mm width; Spectrum no. 132726).
10. Magnetic stir bar. Sterilize by autoclaving.
11. Sterilized 1 L glass beaker.

2.2.3 Determine the Virus Titer by Plaque Assay

1. 293A cells.
2. 293A culture medium: DMEM supplemented with 10% heat-inactivated fetal calf serum, 0.1 mM Non-Essential Amino Acids (NEAA), 2 mM L-glutamine, and 1% Pen-Strep.
3. Plaque assay medium: 2× 293A culture medium.
4. 24-well plate.
5. 1.2% sterile SeaPlague Agarose (Lonza Cat. No. 50101 or equivalent) in distilled water (autocleaved).
6. 1× PBS.
7. 3.7% formaldehyde solution by diluting the 37% formaldehyde stock (Sigma-Aldrich, Cat. no. F-1268) with 1× PBS.
8. 0.5% Crystal violet: dissolve 0.5 g crystal violet powder (Sigma-Aldrich Cat. no. C-6158) in 100 mL 25% methanol.
9. 65 °C water bath.
10. Microwave oven.
11. Vortex.

2.3 Prostate Cancer Suicide Gene Therapy by Ad-ON-HSV-tk

1. Ad-ON-HSV-tk.
2. 3H-GCV (8 μCi/mL, 100 μM; Moravek Biochemicals, CA, USA).
3. $MgCl_2$.
4. 10 mM ATP (New England Biolabs, Beverly, MA, USA).
5. Bovine serum albumin (Sigma-Aldrich Cat. no. A7906).
6. 1 M phosphate buffer (pH 6.5).
7. Whatman DE-81 filter disk.
8. 50% ethanol.
9. 0.1 N HCl.
10. 37 °C water bath.
11. Liquid scintillation cocktails.
12. Scintillation vials.
13. Scintillation counter (Beckman Coulter model LS6500).

2.3.1 HSV Thymidine Kinase Enzymatic Assay

2.3.2 Cytotoxicity Assay

1. Ad-ON-HSV-tk.
2. Dulbecco's Phosphate Buffered Saline containing calcium and magnesium (D-PBS).
3. 37 °C Incubator.
4. 10 μg/mL GCV.
5. 3.7% formaldehyde: dilute the 37% formaldehyde stock (Sigma-Aldrich, Cat. no. F-1268) with 1× PBS.

6. 0.5% Crystal violet: dissolve 0.5 g crystal violet powder (Sigma-Aldrich, Cat. no. C-6158) in 100 mL 25% methanol.
7. Sorenson's buffer: 0.03 M trisodium citrate and 50% ethanol. Adjust pH level to 4.2 using 10 N HCl.
8. Titer plate shaker (Lab-Line, Melrose Park, IL).
9. Microplate reader (SPECTROstar Omega; BMG Labtech, Ortenberg, Germany).

3 Methods

3.1 Osteonectin Promoter Assay Using Firefly Luciferase Reporter

3.1.1 Genomic DNA Isolation from Cultured Cells

1. Harvest DU145 prostate cancer cells from 100 mm culture dish (~5 × 10^6 cells) by trypsinization, and inactivate the trypsin with growth medium.
2. Pellet cells by centrifuging 5 min at 300 × *g* at 4 °C.
3. Wash cells with ice-cold PBS and re-pellet.
4. Add 500 μL digesting buffer to resuspend cell by gentle pipetting. Transfer the sample to 1.5 mL microcentrifuge tube.
5. Incubate samples with rotating at 50 °C overnight.
6. Add equal volume (500 μL) of Phenol/Chloroform/Isoamylalcohol (25:24:1) and mix gently by inverting for 5 min.
7. Centrifuge at 1700 × *g* for 10 min at 25 °C.
8. Transfer aqueous phase to a new tube.
9. Repeat **steps 6–8**.
10. Add equal volume (500 μL) of Chloroform/Isoamylalcohol (24:1) and mix gently by inverting for 5 min.
11. Repeat **steps 6** and 7.
12. Add 1/10 volume (50 μL) of 3 M sodium acetate, and mix well by vortexing.
13. Add 2 volume (1 mL) of ice-cold 100% ethanol to sample, and mix by inversion.
14. Place the tube at −20 °C overnight to precipitate DNA.
15. Centrifuge the tube at 1700 × *g* for 30 min at 4 °C to pellet the DNA. Remove supernatant carefully.
16. Add 150 μL 70% ethanol for wash and centrifuge the tube at 1700 × *g* for 10 min. Remove as much supernatant and the remaining ethanol as possible. Air-dry the pellet and dissolve DNA pellet in appropriate volume of deionized water. DNA is stored at −20 °C.

3.1.2 Osteonectin Promoter Construction Using TOPO TA Cloning System

1. Design PCR primers properly to ensure that you obtain the product you need for your studies (*see* **Note 4**).
2. Set up the following 50 μL PCR reaction.

Genomic DNA from **3.1.1**	100 ng
10× PCR buffer	5 μL
10 mM dNTPs	1.25 μL
Forward primers (10 μM)	0.5 μL
Reverse primers (10 μM)	0.5 μL
Water add to a final volume of	49 μL
Taq polymerase (1 unit/μL)	1 μL
Total volume	50 μL

3. Set an optical PCR program including a 7 min extension at 72 °C after the last cycle to ensure that all PCR products are full length and 3′ adenylated.
4. Check the PCR product by agarose gel electrophoresis, and a single band should be seen.
5. For TA cloning, fresh PCR product should be used. Set up the following 6 μL reaction according to manufacturer's instruction.

Fresh PCR product	3 μL
Salt solution	1 μL
Water	1 μL
TOPO® vector	1 μL
Total volume	6 μL

6. Mix the reaction gently and incubate for 5 min at room temperature, then place the reaction on ice for further transformation.
7. For transform DNA to DH-5α competent cells, add 5 μL of TA cloning product from previous procedure to 100 μL DH-5α competent cells. Mix the reaction gently by stirring with the tip. Incubate on ice for 15 min.
8. Heat-shock the cells for 45 s at 42 °C without shaking. Immediately transfer the tubes to ice for 2 min.
9. Add 900 μL of room temperature S.O.C. medium and shake the tube horizontally (200 rpm) at 37 °C for 1 h.
10. Spin down the cell pellet and remove 900 μL supernatant, then plate transformation to the prewarmed LB plate containing 100 μg/mL ampicillin and incubate at 37 °C for 16 h.

11. Set up the following 10 μL colony PCR reaction. Mix together the following on ice. For multiple samples, make a large master mix and aliquot 10 μL in each PCR tube.

10× PCR buffer	1 μL
10 mM dNTPs	0.25 μL
Forward primers (10 μM)	0.1 μL
Reverse primers (10 μM)	0.1 μL
Water	8.5 μL
Taq polymerase (1 unit/μL)	0.05 μL
Total volume	10 μL

12. Touch a signal colony by pipette tip and streak cells to a new selective LB plate, then mix the remand cells into PCR tube by pipetting up and down. Incubate the selective LB plate at 37 °C for 6 h. Set the PCR program as **step 10**.
13. Check the PCR product by agarose gel electrophoresis, and select optical clone for DNA extraction and further sequencing.

3.1.3 Osteonectin Promoter-Driven Luciferase Reporter Assay

1. Subclone osteonectin promoter DNA fragment from TOPO vector (Subheading 3.1.2) to a desired *firefly* luciferase reporter vectors (e.g., pGL-3-basic) using appropriate cloning methods designed by individual laboratory. The resulting plasmid is designated as pON-Luc.
2. Extract ON-Luc using endotoxin-free DNA isolation kit, such as the Geneaid™ Midi Plasmid Kit Endotoxin Free according to manufacturer's instruction.
3. One day before transfection, plate prostate cancer cells into 6-well plates at about 70–80% confluent (~2 × 10^5 cells in 2 mL growth medium) at the time of use.
4. For each transfection sample at triplicate wells, prepare DNA-Lipo complexes as follows (*see* **Note 5**):
   (a) Mix 6 μg of DNA consisting of pON-Luc and a *renilla* luciferase control reporter plasmid (e.g., pRL-TK) at 20:1 molar ratio with 750 μL Opti-MEM in a sterilized micro-centrifuge tube by pipetting.
   (b) Mix 15 μL of lipofectamine 2000 with 750 μL Opti-MEM in a Falcon 5 mL polystyrene tube by gently inverting several times, and then stand for 5 min at room temperature.

(c) Transfer DNA solution (a) into diluted lipofectamine 2000 (b) and gently inverting the tube five times, following by incubation for 20 min at room temperature to allow DNA-Lipo complexes formation.

5. Slowly add 0.5 mL DNA-Lipo complexes (**step 4c**) to each cell culture well drop by drop with gently hand rocking the plate back and forth.
6. Incubate cells for 6 h at 37 °C in CO_2 incubators, then replace with 2 mL growth medium for additional 48 h culture.
7. Remove the growth medium from cultured cells. Rinse cells with 2 mL PBS twice and remove all solutions.
8. Add 300 μL of 1× PLB (dilute 5× Passive Lysis Buffer supplied in the Dual Luciferase Reporter Assay kit with 4 volume distilled water) to each well and incubate for 15 min at room temperature with rocking on plate shaker.
9. Harvest cell lysate using cell scraper and transfer to 1.5 mL microcentrifuge tube. Vortex the tube for 15 s, then centrifuge at 13,000 rpm for 2 min at 4 °C.
10. Add 50 μL of LAR II luciferase substrate solution (supplied in the Dual Luciferase Reporter Assay kit) in a new 1.5 mL microcentrifuge tube for each assay sample plus one for background control.
11. Transfer 10 μL of cell lysate supernatant (**step 9**) into the tube containing LAR II (**step 10**) and quickly mix by pipetting up and down three times. For background control tube, add 10 μL of 1× PLB.
12. Immediately after adding samples, insert tubes into Luminometer and measure luminescence (refereed as firefly luminescence).
13. Add additional 50 μL of Stop & Glo reagent (supplied in the Dual Luciferase Reporter Assay kit) into the tube and quickly mix by pipetting up and down three times. Immediately measure luminescence (refereed as renilla luminescence). Perform **steps 11–13** for only one sample at a time.
14. Calculate osteonectin promoter-driven luciferase reporter activity as relative light units (RLU) using the following formula:

(Sample firefly luminescence − Background firefly luminescence) ÷ (Sample renilla luminescence − Background renilla luminescence)

3.2 ON-HSV-tk Adenoviral Vector (Ad-ON-HSV-tk) Construction and Preparation

3.2.1 Ad-ON-HSV-tk Construction Using Gateway System (*See* **Note 6**)

1. Subclone the HSV-tk gene from laboratory available HSV-tk cloning or expression vector into pON-Luc (Subheading 3.1.3) by replacing the *firefly* luciferase gene with HSV-tk using appropriate cloning strategies designed by individual laboratory. The resulting plasmid is designated as pON-HSV-tk.
2. Subclone ON-HSV-tk expressing cassette from pON-HSV-tk into pENTR-1A vector by replacing the *ccdB* gene located within the multiple cloning sites with ON-HSV-tk using appropriate cloning strategies designed by individual laboratory. The resulting plasmid is designated as pON-HSV-tk/ENTR.
3. Set up an LR recombination as follows and mix well by vortexing.

pON-HSV-tk/ENTR	150 ng
pAd/PL-DEST	300 ng
TE Buffer, pH 8.0	to 8 μL
LR Clonase II enzyme mix	2 μL
Total volume	10 μL

4. Incubate reactions at room temperature for 1 h.
5. Add 2 μL of proteinase K solution (supplied with LR Clonase® II Enzyme Mix) to the reaction. Incubate at 37 °C for 10 min.
6. Transform 5 μL of the LR recombination reaction into to 100 μL of DH-5α competent cells and select the expression clones by growing transformed cells on LB agar plates containing 100 μg/mL ampicillin as describe in procedure Subheadings 3.1.2, **steps 7–10**.
7. Confirm the expressing clone containing the correct sequence of ON-HSV-tk (designated as pAd-ON-HSV-tk) by sequencing using the following primer set.

   pAd forward primer: 5′-GACTTTGACCGTTTACGTGGAGAC-3′

   pAd reverse primer: 5′-CCTTAAGCCACGCCCACACATTTC-3′

8. Digest 5–10 μg purified pAd-ON-HSV-tk plasmid with Pac I in 200 μL of reaction at 37 °C for 1 h (*see* **Note 7**).
9. Clean up the digested DNA using chloroform/isoamylalcohol extraction without gel purification followed by ethanol precipitation (Subheading 3.1.1, **steps 6–16**). Resuspend DNA in 20 μL TE buffer (pH 8.0).
10. The day before transfection, seed 1×10^6 293A cells with 5 mL growth medium in a 6 cm culture dish.

11. Prepare DNA-lipo complexes by mixing 5 μg of the digested pAd-ON-HSV-tk in 0.5 mL Opti-MEM with 12.5 μL lipofectamine 2000 in 0.5 mL Opti-MEM following the protocol in Subheading 3.1.3, **step 4c**.
12. Slowly add 1 mL DNA-Lipo complexes to cell culture drop by drop with gently hand rocking the plate back and forth. After 5–6 h, remove the medium and replace with 4 mL of culture media.
13. After 3 days of incubation, subculture the transfected cells from the 6 cm dish and split into two 100 mm culture dishes with 10 mL fresh culture medium per each dish. Monitor cytopathic effect (CPE), which are areas with enlarged and rounded cells that start to lose contact from the surface, under a light microscope daily.
14. When above 70% of cells show CPE (appropriately within 5 days), scrape cells off without removing the culture medium, and collect all (totally about 20 mL from two dishes) into a 50 mL conical centrifuge tube.
15. Immediately freeze the cell suspension in dry ice/methanol bath, and thaw in a 37 °C water bath for three cycles.
16. Centrifuge at 5000 × *g* for 10 min at 4 °C, transfer the supernatant (P0 virus stock) into new tubes and store at −80 °C for further amplification and purification.

3.2.2 Ad-ON-HSV-tk Viral Amplification and Purification

1. The day before viral infection, seed 2×10^7 293A cells in a 150 mm culture dish for 16 plates and allow to grow overnight to achieve 80% confluence.
2. On the day of infection, remove the P0 viral stock (Subheading 3.2.1) from the freezer and completely thaw them on ice. Dilute desired amount of P0 virus stock with culture medium at a ratio of 1:4.
3. After removing original medium from cells, add 5 mL of the diluted viral supernatant to each plate. Incubate the infected cells at 37 °C for 1 h with gently rock the plates every 15 min to allow an even distribution of virus.
4. Add 15 mL fresh culture medium to each plate and continue to incubate the cells at 37 °C in 5% CO_2 till CPE are completely formed. It usually can be observed on day 2 or day 3 post infection.
5. Harvest the cells by gently pipetting medium over the cells and transfer them into 50 mL conical centrifuge tubes. Centrifuge at 1000 × *g* for 5 min at room temperature.
6. Discard the supernatant and resuspend all cell pellets in a total of 10 mL of PBS, and lyse cells by three cycles of freeze and thaw procedure (Subheading 3.2.1, **step 15**).

7. Centrifuge at 5000 × *g* for 10 min at 4 °C, Aliquot supernatant (P1 virus stock) and store at −80 °C or go to $CsCl_2$ purification as follows.
8. Prepare Beckman ULTRA-CLEAR thinwall tubes. The lysate from 16 plates should be placed in 2 centrifuge tubes.
9. Add 0.5 mL of 1.5 g/mL $CsCl_2$ solution to the bottom of each tube.
10. To prepare gradient, slowing layer 3 mL of the 1.35 g/mL $CsCl_2$ solution on top of 1.5 g/mL $CsCl_2$, followed by layering 3 mL of 1.25 g/mL $CsCl_2$ solution, and then 5 mL of lysate (P1 virus stock).
11. Fill to within 3 mm of the top and balance with PBS.
12. Load tubes symmetrically in a SW40Ti rotor. Centrifuge at 14,000 × *g* for 1 h in a Beckman ultracentrifuge at 4 °C. Adenoviruses should appear as a light blue band at the interface between 1.25 g/mL and 1.35 g/mL $CsCl_2$.
13. Prepare new Beckman tubes. Add 5 mL of 1.33 g/mL $CsCl_2$ solution to the bottom of each tube.
14. Discard the upper portion of viral band by suction, and then carefully remove the band with p1000 pipette to the new tube (**step 13**). Fill to within 3 mm of the top and balance with PBS.
15. Centrifuge at 14,000 × *g* for 16–18 h at 4 °C with deceleration profile no brake.
16. After overnight spin, discard the solution above viral band, and carefully collect the viruses and transfer them into the dialysis bag (with one end of bag already clipped). Squeeze out all air from bag and seal with a second clip.
17. Place the bag in a 1 L beaker containing 1 L of pre-chilled dialysis buffer. Cover the beaker with aluminum foil to protect the light. Place on a magnetic stirrer and gently stir at 4 °C for overnight with two times of dialysis buffer exchange once later on the first day and once in the morning of the second day.
18. In the afternoon of the second day, carefully take out the solution from the bag with p200 pipette and aliquot into sterile 1.5 mL microcentrifuge tubes in quantities enough for one experiment to avoid freeze-thawing. Store at −80 °C.

3.2.3 Determine the Virus Titer by Plaque Assay

1. Seed 293A cells at 2×10^5 per well in 24-well cell culture plate and allow to grow overnight. Cells should be 70–80% confluent the day of infection.
2. Perform tenfold serial dilution of purified adenovirus ranging from 10^{-4} to 10^{-8} in D-PBS.
3. Remove growth medium from 293A cells, and add 100 μL of diluted adenoviral solution in triplicate to each well. Incubate

the infected cells at 37 °C for 1 h and shake the plates gently every 15 min during this adsorption period.

4. Melt 1.2% sterile agarose in microwave oven for liquefying, then cool to 65 °C in a water bath. Prewarm plaque assay medium to 37 °C.
5. Mix 1:1 warm plaque assay medium and 1.2% agarose.
6. After 1 h of viral absorption, remove the virus-containing media from the cells and gently add 1 mL of the agarose/media mixture to each well without dislodging any cells. Leave cells at room temperature for at least 20 min. Once the agarose overlay turns solid, add 1 mL of the culture medium to each well and return plates to 37 °C incubator.
7. Monitor the plaque formation daily. Plaques should be visible under a microscope within 7–10 days.
8. Once the plaques have fully developed, remove culture medium and add 1 mL of 3.7% formaldehyde to each well for fixation. Stand at room temperature for at least 2 h.
9. Lift off the agarose overlay with a quick inversion, rinse the monolayers with distilled water.
10. Add 1 mL of 0.5% crystal violet solution to each well. Stand at room temperature for 15 min. Wash out the crystal violet with tap water.
11. Count plaques and calculate the concentration of the initial viral suspension in PFU/mL.
    Example: If you placed 100 μL of a 10^{-8} dilution on a well and you see 10 plaques, the titer from that well is 1.0×10^{10} PFU/mL.

3.3 Prostate Cancer Suicide Gene Therapy by Ad-ON-HSV-tk

3.3.1 HSV Thymidine Kinase Assay by Ganciclovir Phosphorylation Activity

1. Lyse Ad-HSV-tk injected cells with 300 μL of Reporter Lysis Buffer (Promega, WI, US) and then centrifuge for 15 min at 12,000 × *g* at 4 °C.
2. Collect supernatants and measure the protein concentration ($Conc_{sample}$). Samples can be assayed immediately or store in aliquots at −80 °C.
3. Prepare substrate mixture (at least 125 μL for one sample):
   10 μM ^{3}H-GCV, 3 mM $MgCl_2$, 3 mM ATP, 1 mg/mL bovine serum albumin, and 50 mM phosphate buffer (pH 6.5).
4. Warm up substrate mixture and sample separately in 37 °C water bath for 5 min.
5. At time zero, mix 125 μL sample and 125 μL substrate mixture, and continue to incubate in 37 °C water bath.
6. At various time point (Tn), put 25 μL of reaction mixture onto DE-81 disk.
7. Wash DE-81 disks twice with 50% ethanol on a shaker for 15 min.

8. Put an extra 25 μL of reaction mixture on a DE-81 disk for unwashed total.
9. Put washed and unwashed DE-81 disks in scintillation vials.
10. Incubate in 1 mL of 0.1 N HCl for 1 h.
11. Add 10 mL scintillation fluid and count in scintillation counter (CPM).
12. Calculate HSV-tk enzyme kinetics (pmol/min/mg protein) using the following formula:

$$[(\mathrm{CPM}_{\text{washed disc}} - \mathrm{CPM}_{\text{washed blank}}) \div (\mathrm{CPM}_{\text{unwashed total}} - \mathrm{CPM}_{\text{unwashed blank}})] \times 10\,\mu M \div \mathrm{Tn\ (min)} \div \mathrm{C}_{\text{sample}}(\mathrm{mg}/\mu L)$$

3.3.2 Cytotoxicity Assay

1. Seed prostate cancer cells at 2×10^4 per well in a 24-well plate for triplicate experiment and incubate at 37 °C in 5% CO_2 the day before Ad vector infection.
2. For the range of 10–100 Multiplicity of Infection (MOI) adenovirus infection, serial dilute adenovirus ranging from 2×10^5 to 2×10^6 pfu in 100 μL DPBS for a total of 350 μL for each concentration.
3. Remove the culture medium from cells and add 100 μL diluted virus to each well. Treat one group of wells with 100 μL DPBS only (no virus) for normalization.
4. Incubate cells at 37 °C in 5% CO_2 for 2 h adsorption. Shake the plates gently every 15 min during this adsorption period.
5. After 2 h of viral absorption, remove the media and replace with fresh culture medium.
6. After 24 h of incubation, add 10 μg/mL GCV to cells and incubate for 5 days.
7. Remove the medium from cells and fix cells with 1 mL 3.7% formaldehyde in each well. Stand at room temperature for 15 min.
8. Remove 3.7% formaldehyde and add 1 mL of 0.5% crystal violet solution (include blank wells for background). Stand at room temperature for 15 min.
9. Wash the plate in tap water for about 30 s. Drain off excess water and lightly blot dry on a paper towel.
10. Add 500 μL Sorenson's buffer in each wells for dye extraction and incubate for 30 min at room temperature with gentle shaking on a titer plate shaker.
11. Transfer 100 μL solution from 24-well culture plate to 96-well microplate and measure absorbance at 570 nm using a microplate reader.

12. Subtract the absorbance readings of background wells from that of experimental wells.
13. Divide the experimental wells by the control wells (no Ad virus) to normalize the data.

4 Notes

1. Cloning method for insertion of ON-HSV-tk expression cassette into pENTR 1A vector is varied depending on the experimental design of individual laboratory.
2. Cloning method for insertion of expression cassette into pENTR 1A vector is varied depending on the experimental design of individual laboratory.
3. To ensure accuracy, weigh a sample of each solution after filtration, adjusting as necessary.
4. The primer sets that we used for cloning a 522 bp of osteonectin promoter with deleted GGA-boxes 1 and 2 by recombinant PCR are the follows:

   Set 1:

   522-N: ACTAGTAGCAGCTTGTCTTGTC
   spdel-C: CTTCTCCCCTGTCTCTGTCTT3′

   Set 2:

   spdel-N: AAGACAGAGACAGGGGAGAAG
   Exon-C: CAGGCAGGCAGGCGGCAG

5. The transfection protocol is based on the Lipofectamine 2000 (Invitrogen) manufacturer's instruction with some modification.
6. The adenoviral vector construction protocol is majorly based on the manual of ViraPower™ Adenoviral. Expression System (Invitrogen) with some modification.
7. Ensure complete digestion by running a small sample of the digested product on 0.8% agarose gel to see two distinct bands approximately 2.1 kb and 35 kb.

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Chapter 4

Suicide Gene Therapy for Oral Squamous Cell Carcinoma

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Abstract

Suicide gene therapy has been tested for the treatment of a variety of cancers, including oral cancer. Among the various suicide gene therapy approaches that have been reported, the Herpes Simplex Virus thymidine kinase (HSV-tk)/ganciclovir (GCV) system is one of the most extensively studied systems, holding great promise in cancer therapy. In this chapter, we describe methods to use the HSV-tk/GCV system to achieve antitumor activity, both in cultured oral cancer cells and in orthotopic and subcutaneous murine models of oral squamous cell carcinoma, using ligand-associated lipoplexes for enhancing therapeutic delivery.

Key words Cationic liposomes, Lipoplexes, HSV-tk/GCV suicide gene therapy, Oral carcinoma, Modulation of gap junctions, Antitumor activity

1 Introduction

Head and neck squamous cell carcinoma is a disease with a high rate of morbidity and mortality. Despite recent advances in early detection and diagnosis, current treatment options for oral cancer are unsatisfactory, and the 5-year survival rate has not significantly improved over the last two decades [1–3].

In this regard, suicide gene therapy has attracted a special attention as a promising therapeutic approach for cancer treatment, including oral squamous cell carcinoma [4–8]. Among the different suicide gene therapy strategies, the HSV-*tk*/GCV approach is the most commonly used [4, 5]. The delivery of the HSV-*tk* expressing plasmid to cancer cells results in the expression of viral thymidine kinase, which selectively phosphorylates GCV. Monophosphorylated GCV is further phosphorylated by endogenous cellular kinases into the highly toxic triphosphate purine analog which incorporates into cellular DNA, causing chain termination and cell death [9, 10]. Importantly, this enzyme/prodrug combination has the capacity to promote cell death, not only in the recipient cells but also in the neighboring cells, by the so-called "bystander effect" [11–13]. In this regard, our results from the application of

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HSV-tk/GCV therapy in cultured oral squamous cancer cells have shown that tumor cell death occurs mainly by an apoptotic process and the observed high cytotoxicity is due to the bystander effect, which is promoted by the diffusion of the toxic agent into neighboring cells via gap junctions [13]. Moreover, our studies in syngeneic orthotopic and subcutaneous murine models for squamous cell carcinoma of the head and neck revealed that intratumoral administration of the HSV-tk gene mediated by transferrin- or folate-associated lipoplexes, followed by intraperitoneal injection of ganciclovir, results in a potent antitumor effect [13–15].

In this chapter we will present several protocols and assays that are fundamental to evaluate, both in vitro and in vivo, the therapeutic potential of the HSV-tk/GCV suicide gene therapy strategy in oral squamous cell carcinoma.

2 Materials

2.1 Cationic Liposomes

1. 1,2-dioleoyl-3-trimethylammonium-propane (**DOTAP**), 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (EPOPC), and cholesterol (Chol) (Avanti Polar Lipids, Alabaster, AL) are dissolved at 100 mg/mL in chloroform and stored at −20 °C.
2. Lipid films are made in a Heidolph VV 2000 rotatory evaporator (Heidolph Instruments GmbH & Co, Schwabach, Germany).
3. Lipid film hydration is carried out with deionized water (*see* **Note 1**).
4. Lipid film hydration is accomplished in a vortex mixer.
5. Liposome sonication is performed using a Sonorex RK100H sonicator (Bandelin Electronic, Berlin, Germany).
6. Liposome extrusion is performed in a mini-extruder (Avanti Polar Lipids, Alabaster, AL).
7. Polycarbonate membranes of 50 nm pore-diameter (Whatman, Maidstone, UK).
8. Filters of 0.22 μm pore-diameter (Schleicher & Schuell, Dassel, Germany).

2.2 Lipid Concentration

1. The Infinity™ cholesterol liquid stable commercial reagent (Thermo Electron Corporation, Waltham, MA, USA) is stored at 4 °C and protected from light.
2. Cholesterol (Cholesterol–methyl-β-cyclodextrin) (Sigma) standard solutions (0.0625; 0.125; 0.250; 0.5; 1 mg/mL) are prepared with deionized water and stored at 4 °C.
3. Absorbance is measured in a SPECTRAmax PLUS384 spectrophotometer (Molecular Devices, Union City, CA).

2.3 Cationic Liposome/DNA Complexes

1. HEPES-buffered saline (HBS): 100 mM NaCl, 20 mM HEPES, pH 7.4. Store at 4 °C.
2. Solution of plasmid pCMV.tk (National Gene Vector Laboratory at the University of Michigan, Ann Arbor, MI), encoding the Herpes Simplex Virus thymidine kinase (HSV-tk), is prepared at 10 mg/mL in HBS and kept at 4 °C.
3. Transferrin (Tf) and folate (FA) (both from Sigma, St. Louis, MO) solutions are prepared in HBS at 640 μg/mL and kept at 4 °C.

2.4 Cell Culture

1. Culture medium: Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). Store at 4 °C and warm to 37 °C before addition to cells.
2. Solutions of 0.05% (w/v) trypsin and 1 mM ethylenediamine tetraacetic acid (EDTA). Store at 4 °C and warm to 37 °C before addition to cells.
3. Cells are cultured in 75 cm^2 flasks (Corning Costar Corporation, Cambridge, MA, USA).
4. Phosphate-buffered saline (PBS): Prepare 10× stock solutions containing 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4 (adjust to pH 7.4 with HCl) and autoclave before storage at room temperature. Prepare the working solution by dilution of one part with nine parts of deionized water.

2.5 In Vitro Antitumor Activity (Alamar Blue)

1. SCC-VII and HSC-3 cells are seeded in 48-well culture plates (Corning Costar Corporation).
2. Transfection medium: Dulbecco's modified Eagle's medium high glucose (DMEM-HG) without serum and antibiotics. Store at 4 °C and warm to 37 °C before use.
3. Tf-DOTAP:Chol/DNA lipoplexes are prepared with 1 μg of pCMV.tk plasmid DNA.
4. Solutions of ganciclovir (GCV) (Roche, Grenzach-Wyhlen, Germany) are freshly prepared at different concentrations (10, 25, 50, and 100 μM) in DMEM-HG medium and warmed to 37 °C before addition to cells.
5. Resazurin solution: 10% (v/v) resazurin in DMEM-HG (prepared from a 0.1 mg/mL stock solution of resazurin, stored at −20 °C). The solution is prepared immediately before use and warmed to a working temperature of 37 °C.
6. Absorbance is measured in a SPECTRAmax PLUS384 spectrophotometer (Molecular Devices, Union City, CA).

2.6 Effect of Gap Junctions Modulation on the HSV-tk/GCV Therapeutic Strategy

1. SCC-VII and HSC-3 cells are seeded in 48-well culture plates (Corning Costar Corporation).
2. Solutions of α-glycyrrhetinic acid (AGA) (25 and 50 μM) (Sigma, St Louis, MO, USA), dibutyryl adenosine 3′,5′-cyclic monophosphate (db-cAMP) (250 and 500 μM) (Sigma, St Louis, MO, USA), and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (10 μM) (Biomol International L.P, PA, USA) are freshly prepared immediately before use.
3. Transfection medium: Dulbecco's modified Eagle's medium high glucose (DMEM-HG) without serum and antibiotics. Store at 4 °C and warm to 37 °C prior use.
4. Tf-DOTAP:Chol/DNA lipoplexes are prepared with 1 μg of pCMV.tk plasmid DNA.
5. Solutions of GCV (Roche, Grenzach-Wyhlen, Germany) are freshly prepared in DMEM-HG medium (50 μM GCV for experiments with AGA and 25 μM GCV for experiments with db-cAMP) and warmed to 37 °C before addition to cells.
6. Resazurin (Sigma) solution: 10% (v/v) resazurin in DMEM-HG (prepared from a 0.1 mg/mL stock solution of resazurin, stored at −20 °C). The solution is prepared immediately before use and warmed to a working temperature of 37 °C.
7. Absorbance is measured in a SPECTRAmax PLUS384 spectrophotometer (Molecular Devices, Union City, CA).

2.7 In Vivo Antitumor Activity of the HSV-tk/GCV Therapeutic Strategy

2.7.1 Orthotopic Tumor Implantation and Treatment

1. Six- to eight-week-old female C3H/HeJ mice (Charles River Laboratories, Barcelona, Spain).
2. Mixtures of chlorpromazine (2 mg/kg) and ketamine (100 mg/kg) are freshly prepared.
3. SCC-VII cells are used for tumor implantation.
4. Tf-DOTAP:Chol/DNA lipoplexes are prepared with 40 μg of pCMV.tk plasmid DNA.
5. Solution of 40 μg of naked pCMV.tk plasmid DNA is prepared in PBS.
6. Solutions of GCV (5.0 mg/mL) (Roche, Grenzach-Wyhlen, Germany) are prepared in PBS immediately before use.
7. Tumor size measurement is performed with calipers.

2.7.2 Subcutaneous Tumor Implantation and Treatment

1. Six- to eight-week-old female C3H/HeJ mice (Charles River Laboratories, Barcelona, Spain).
2. SCC-VII cells are used for tumor implantation.
3. EPOPC:Chol/DNA (+/−) (2/1) (plain lipoplexes) or FA-EPOPC:Chol/DNA (+/−) (2/1) (FA-lipoplexes) lipoplexes are prepared with 40 μg of pCMV.tk plasmid DNA.

4. Solutions of valganciclovir (VGCV) (7.5 mg/mL) (Roche, Grenzach-Wyhlen, Germany), an l-valyl ester of GCV, are prepared in PBS immediately before use.
5. Tumor size measurement is performed with calipers.

3 Methods

3.1 Cationic Liposomes

1. Small unilamellar cationic liposomes (SUV) are prepared by extrusion of multilamellar liposomes (MLV) composed of 1:1 (mol ratio) mixtures of either 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (EPOPC) or 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and cholesterol (Chol).
2. The required volumes of the stock lipids, dissolved in chloroform at the referred concentration, are measured using Hamilton syringes and mixed at the desired molar ratio in glass tubes, previously washed with chloroform. The glass tubes containing the lipid mixtures are placed in the rotatory evaporator (220 rpm) until a thin film is formed, which is then allowed to dry under vacuum (−0.8 bar) for 1 h at 37 °C to remove any residual chloroform.
3. The dried lipid films are hydrated with 1 mL of deionized water under constant vortexing in order to promote the formation of MLV.
4. The resulting MLV are sonicated in a bath-type sonicator for 3 min and extruded 21 times through two stacked polycarbonate filters of 50 nm pore-diameter, using the referred mini-extruder (Subheading 2.1), to obtain a suspension of SUV with uniform size distribution. The SUV are then diluted with deionized water to the desired concentration and filter-sterilized using 0.22 μm pore-diameter filters. The liposome suspension is stored at 4 °C until use after flushing the top part of the tube with dried nitrogen.

3.2 Lipid Concentration

1. The lipid concentration of SUV containing DOTAP/Chol or EPOPC/Chol is determined by quantification of cholesterol using the Infinity™ cholesterol liquid stable commercial reagent [16] (*see* **Note 2**).
2. The standard solutions with different cholesterol concentrations (0.0625; 0.125; 0.250; 0.5; 1 mg/mL) are prepared by diluting the cholesterol standard solution (3 mg/mL) with deionized water and stored at 4 °C.
3. One milliliter of the Infinity™ cholesterol reagent is placed in spectrophotometer cuvettes, followed by addition to each of the cuvettes of: 10 μL of the liposome suspension (sample); or 10 μL of deionized water (blank); or 10 μL of each of the standard solutions (standard).

4. After gentle mixing, followed by incubation for 15 min at room temperature, the absorbance of the solutions in each cuvette is measured at 500 nm in a SPECTRAmax PLUS384 spectrophotometer (*see* **Note 3**).
5. The cholesterol content of the sample is assessed from a standard curve obtained from the absorbances determined for the standard cholesterol solutions.

3.3 Cationic Liposome/DNA Lipoplexes

1. Cationic liposome/DNA complexes are prepared in HBS from liposomal suspensions of known concentrations and solutions of DNA (10 μg/mL for in vitro experiments and 2 mg/mL for in vivo studies), and Tf (640 μg/mL for in vitro experiments and 128 mg/mL for in vivo studies) or FA (800 μg/mL for in vitro experiments and 160 mg/mL for in vivo studies) (*see* **Note 4**). All the solutions are previously filtered under aseptic conditions.
2. For the preparation of the complexes, sterile polypropylene tubes are used in order to avoid interactions between DNA and the tube walls. The concentration of DNA is maintained constant (5 μg/mL for in vitro experiments and 1 mg/mL for in vivo studies) independently of the total volume of the prepared complexes. For instance, for in vitro experiments, complexes without Tf or FA (plain lipoplexes), corresponding to 1 μg of DNA, are prepared by sequentially adding 100 μL of HBS, 100 μL of the DNA solution (10 μg/mL), and the necessary volume of liposomes, which is dependent on the liposome concentration. For complexes containing Tf or FA, part of the HBS volume is replaced by the volume of Tf or FA solutions, in order to obtain 32 μg of Tf/μg of DNA or 40 μg of FA/μg of DNA [13–15].
3. Plain lipoplexes are prepared by sequentially mixing the established HBS volume with the necessary volume of liposomes and DNA solution, the latter being added gently to the liposome suspension. The mixture is shaken and incubated for 15 min at room temperature to allow complex formation through the establishment of electrostatic interactions between the cationic liposomes and DNA. For complexes containing Tf or FA, liposomes are preincubated with the ligand (Tf or FA) for 15 min to allow interaction between the two components, followed by slow addition of the DNA solution and a further 15 min incubation of the resulting mixture at room temperature. Complexes are used immediately after preparation [13–15].

3.4 Cell Culture

1. SCC-VII cells (murine oral squamous cell carcinoma) and HSC-3 (human oral squamous cell carcinoma) are cultured in DMEM-HG medium supplemented with 10% (v/v) heat-

inactivated FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) in 75 cm^2 flasks and maintained at 37 °C, under 5% CO_2 (*see* **Note 5**).

2. When cells reach 80–90% of confluence, the culture medium is removed and cells are washed with 5 mL of PBS (*see* **Note 6**). After this washing step, cells are detached from the plastic by adding 3 mL of fresh trypsin solution followed by incubation at 37 °C, for 2–3 min (*see* **Note 7**). Cells are then resuspended in 10 mL of DMEM-HG medium and counted in a hemocytometer to determine the cell suspension density.
3. The cell suspension is diluted with DMEM-HG medium to obtain a final density of 100×10^3 cells/mL, for in vitro experiments, and 2×10^6 cells/mL, for in vivo studies. For studies on in vitro antitumoral activity and gap junction modulation, cells are plated 24 h before the experiments in 48-multiwell plates, at 50×10^3 cells/well and 100×10^3 cells/well, respectively. The multiwell plates are then placed in the incubator at 37 °C, under 5% CO_2. For in vivo studies, the cell suspension is prepared immediately before administration into the animals. All the steps involved in cell preparation are performed under sterile conditions in a bioguard hood.

3.5 In Vitro Antitumor Activity (Alamar Blue)

1. Twenty-four hours before transfection, 50×10^3 SCC-VII or HSC-3 cells in DMEM-HG culture medium are added to each well of a 48-multiwell plate.
2. At the time of transfection, the culture medium is removed, and the cells are washed once with DMEM-HG medium without serum and antibiotics, and then covered with 0.3 mL of the same medium.
3. The cationic liposome/DNA complexes are prepared by using the needed volumes of the solutions of HBS, Tf, and DNA, and of the liposomal suspension. After a period of 15 min for complex maturation, the volume of the DOTAP:Chol/DNA/Tf (3/2 (+/−) charge ratio) complexes (Tf-lipoplexes), corresponding to 1 μg of pCMV.tk plasmid DNA, is immediately added to each well.
4. Lipoplexes are incubated with the cells for 4 h at 37 °C and then the medium is replaced with DMEM-HG medium containing different concentrations of ganciclovir (10, 25, 50, 100 μM), which is replaced every day during 5 or 7 days for SCC-7 and HSC-3 cells, respectively.
5. Cell viability and proliferation are evaluated by a modified Alamar Blue assay. For this purpose, 300 μL of DMEM-HG medium containing 10% (v/v) Alamar Blue are added to each

well and cells are incubated at 37 °C for 1 h in a 5% CO_2 humidified atmosphere [13, 15].

6. One-hundred fifty microliters of supernatant are collected from each well, and carefully transferred to 96-well plates in order to avoid air bubble formation.
7. Absorbance is measured at 570 (A_{570}) and 600 nm (A_{600}) and cell viability (as a percentage of untreated control cells) is calculated according to the following equation:

$$(A_{570} - A_{600}) \text{ of treated cells} \times 100/(A_{570} - A_{600}) \text{ of control cells.} \quad (1)$$

An example of the obtained results is shown in Fig. 1.

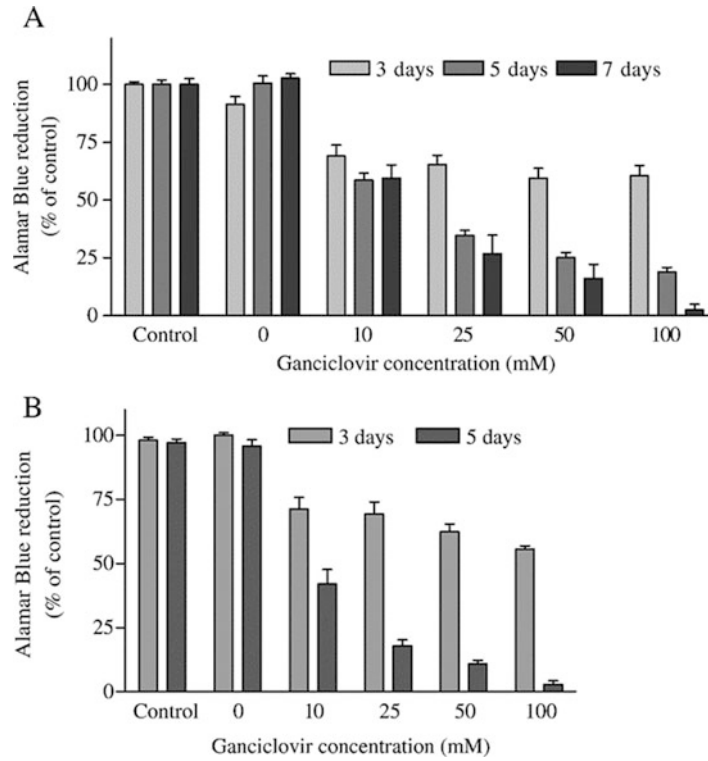

Fig. 1 Effect of GCV concentration and incubation time on the viability of HSC-3 cells (**a**) and SCC-7 cells (**b**) transfected with lipoplexes containing the HSV-tk gene. Cells were transfected with Tf-lipoplexes composed of DOTAP:Chol/pCMV-tk /transferrin, (3/2 (+/−) charge ratio) followed by GCV treatment at the indicated doses and incubation times. Cell viability was evaluated by the Alamar Blue assay. Control represents cells (non-transfected) treated with 100 μM GCV. Data represent the mean ± SD of at least three independent experiments performed in triplicate (Reproduced from ref. 13)

3.6 Effect of Gap Junction Modulation on the HSV-tk/GCV Therapeutic Strategy

1. Twenty-four hours before transfection, 100×10^3 SCC-VII or HSC-3 cells in DMEM-HG culture medium are added to each well of a 48-multiwell plate and treated with the gap junctions modulators: α-glycyrrhetinic acid (AGA) (25 and 50 μM), an inhibitor of gap junctions; or with dibutyryl adenosine 3′,5-′-cyclic monophosphate (db-cAMP) (250 and 500 μM), an activator of gap junctions, in the presence of the cAMP phosphodiesterase 4 selective inhibitor, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (10 μM) [13].
2. The cationic liposome/DNA complexes are prepared as described above (Subheading 3.2) and immediately used after a period of 15 min for complex maturation. Cells are transfected with Tf-lipoplexes, containing 1 μg of pCMV.tk plasmid DNA, as described in Subheading 3.4.
3. Lipoplexes are incubated with the cells for 4 h at 37 °C and the medium is then replaced with DMEM-HG medium containing ganciclovir (25 μM, when cells are treated with db-cAMP, or 50 μM, when cells are treated with AGA) and the different concentrations of gap junction modulators. This medium is replaced every day during 5 days.
4. Cell viability resulting from the application of the HSV-tk/GCV antitumor strategy is evaluated by a modified Alamar Blue assay. Absorbance is measured at 570 and 600 nm and cell viability (as a percentage of untreated control cells) is calculated according to Eq. 1. An example of the obtained results is shown in Fig. 2.

3.7 In Vivo Antitumor Activity of HSV-tk/GCV Therapeutic Strategy

3.7.1 Orthotopic Tumor Implantation and Treatment

1. In vivo experiments are performed using a syngeneic orthotopic murine model for squamous cell carcinoma of the head and neck developed by O'Malley et al. [17, 18]. All the surgical procedures and the care given to the animals are in accordance with the European guidelines.
2. After being detached with trypsin and washed two times with PBS, SCC-VII cells were resuspended in PBS saline buffer, to obtain a final density of 2×10^6 cells/mL. The tumor model is established by injecting 100×10^3 SCC-7 cells, in a volume of 50 μL, directly into the floor of 6- to 8-week-old female C3H/HeOuJ mice which are previously anesthetized upon intramuscular injection of a mixture of chlorpromazine (2 mg/kg) and ketamine (100 mg/kg).
3. Five days after cell implantation, mice are anesthetized, as before, and the developed tumors are injected with 40 μL of Tf-lipoplexes, containing 40 μg of pCMV.tk plasmid DNA or 40 μg of pCMV.SPORT-LacZ plasmid DNA, or 40 μg of naked pCMV.tk plasmid DNA (in 40 μL of PBS) (*see* **Note 8**). PBS alone is also injected into control animals.

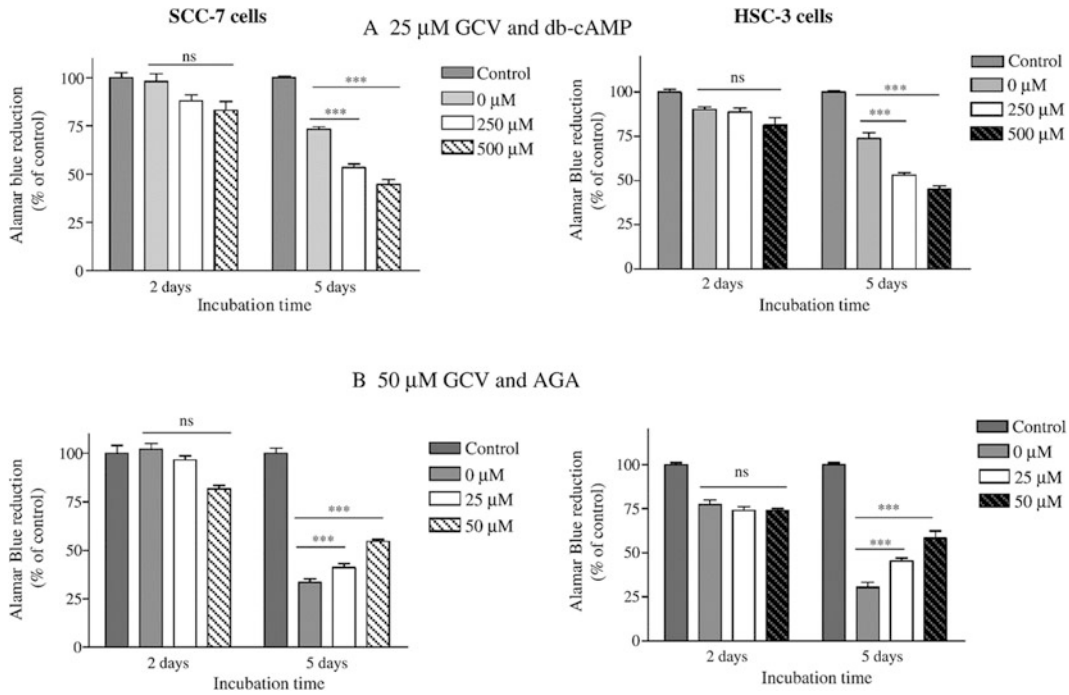

Fig. 2 Effect of modulating oral cancer cells gap junctions on the bystander effect. Cells were transfected with Tf-lipoplexes following treatment with (**a**) various concentrations of dbAMPc and then incubated with 25 μM GCV or (**b**) various concentrations of AGA and then incubated with 50 μM GCV. Cell viability was evaluated by the Alamar Blue assay. Data represent the mean ± SD of at least three independent experiments performed in triplicate. Statistical significance between experimental groups was determined by one-way ANOVA analysis (***$P < 0.001$; ns $P > 0.05$) (Reproduced from ref. 13)

4. Four hours after, mice are intraperitoneally injected with ganciclovir (50 mg/kg daily) during 8 days and sacrificed 13 days following tumor implantation. The tumors are measured in three dimensions everyday with calipers [13, 14].

3.7.2 Subcutaneous Tumor Implantation and Treatment

1. In vivo experiments are performed using a subcutaneous murine model for squamous cell carcinoma of the head and neck, as we have previously described [15]. All the procedures and the care given to the animals are in accordance with the European guidelines.
2. After being detached with trypsin and washed two times with PBS, SCC-VII cells are resuspended in PBS saline buffer, to obtain a final density of 2×10^6 cells/mL. Fifty microliters of this cell suspension (100×10^3 cells) are immediately injected subcutaneously in the left flank of 6- to 8-week-old female C3H/HeOuJ mice (*see* **Note 9**).
3. When the tumor volume reaches approximately 0.025 cm^3, usually 8 days after cell injection, the animals are submitted to different treatments. These treatments consist of four

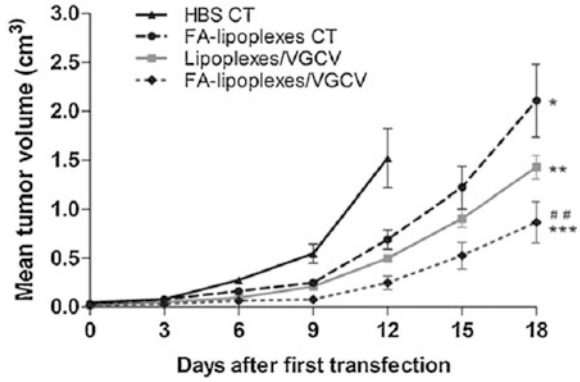

Fig. 3 Antitumor effect of HSV-tk/VGCV gene therapy strategy in a subcutaneous mouse model of oral carcinoma. Eight days after SCC-VII cell implantation, tumors were submitted to four intratumor administrations of plain lipoplexes or FA-lipoplexes containing 40 μg of plasmid DNA coding for HSV-tk, at days 0, 2, 4, and 6. Two groups of animals, one treated with the FA-lipoplexes and the other treated with plain lipoplexes, were submitted to ten intraperitoneal administrations of VGCV (75 mg/kg). Control (CT) mice were injected with HBS or with FA-lipoplexes in the absence of VGCV. Results represent the tumor size after treatment (mean ± SEM) of different experimental groups ($n = 4$). Statistical significance between experimental groups was determined by one-way ANOVA analysis. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ indicate values that differ significantly from those measured in the HBS control group; $^{\#\#}P < 0.01$ indicate values that differ significantly from those measured in the FA-lipoplexes control group (Reproduced from ref. 15)

intratumor administrations of 40 μL of plain lipoplexes or FA-lipoplexes, containing 40 μg of pCMV.tk plasmid DNA, at days 0, 2, 4, and 6 (*see* **Note 8**).

4. In two groups of animals, one treated with the FA-lipoplexes and the other treated with plain lipoplexes, mice are submitted to intraperitonial administrations of VGCV (75 mg/kg) performed from the first day after the first treatment and during 10 consecutive days (*see* **Note 10**). Tumor growth is monitored every 3 days by measuring two perpendicular tumor diameters with a caliper. Mice are sacrificed when the tumor volume reaches approximately 1.5–2 cm^3. An example of the obtained results is shown in Fig. 3.

4 Notes

1. All solutions should be prepared in water with a resistivity of 18.2 MΩ-cm and total organic content of less than five parts per billion.

2. The method for assessment of cholesterol used here is based on the enzymatic method for determination of cholesterol described by Allain et al. [16]. This method relies on the cholesterol oxidation by cholesterol oxidase, resulting in the production of hydrogen peroxide, which, after reacting with 4-aminoantipyrine and phenol in the presence of peroxidase, yields the chromogen quinoneimine that displays maximum absorption at 500 nm.
3. The time of incubation can be reduced to 10 or 5 min by increasing the temperature to 30 °C or 37 °C, respectively.
4. These solutions should be prepared the day before the beginning of the experiment and stored at 4 °C.
5. SCC-7 and HSC-3 cells grow as monolayers and should be detached by treatment with a trypsin solution (0.05%) before reaching 100% confluence in order to be maintained in the exponential growth phase.
6. The washing procedure facilitates the complete removal of the culture medium (which inhibits trypsin action due to the presence of serum), including Ca^{2+} and Mg^{2+}, which are necessary to the cell attachment.
7. During cell detachment, vigorous shaking of the cells should be avoided in order to prevent the formation of cell clusters, which can hamper the determination of cell density.
8. Since most primary and recurrent lesions in oral cancer are accessible to injection, this type of cancer is a particularly appropriate target for treatment by intratumor injection.
9. Subcutaneous xenograft tumors do not face the animal morbidity problems of orthotopic oral tumors, which interferes with animal feeding and may limit their survival for the expected duration of therapy response evaluation.
10. Since the production of ganciclovir was discontinued, its prodrug valganciclovir (VGCV), an L-valyl ester of GCV, is used instead.

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Chapter 5

Generation of Suicide Gene-Modified Chimeric Antigen Receptor-Redirected T-Cells for Cancer Immunotherapy

Kentaro Minagawa, Mustafa Al-Obaidi, and Antonio Di Stasi

Abstract

Chimeric antigen receptor (CAR)-redirected T-cells are a powerful tool for the treatment of several type of cancers; however, they can cause several adverse effects including cytokine release syndrome, off-target effects resulting in potentially fatal organ damage or even death. Particularly, for CAR T-cells redirected toward acute myeloid leukemia (AML) antigens myelosuppression can be a challenge. The previously validated inducible Caspase9 (iC9) suicide gene system is one of the approaches to control the infused cells in vivo through its activation with a nontherapeutic chemical inducer of dimerizer (CID). We performed a preclinical validation using a model of $CD33^+$ AML, and generated iC9 CAR T-cells co-expressing a CAR targeting the AML-associated antigen CD33 and a selectable marker (ΔCD19). ΔCD19 selected (sel.) iC9-CAR.CD33 T-cells were effective in controlling leukemia growth in vitro, and could be partially eliminated (76%) using a chemical inducer of dimerization that activates iC9. Moreover, to completely eliminate residual cells, a second targeted agent was added. Future plans with these methods are to investigate the utility of iC9-CAR.CD33 T-cells as part of the conditioning therapy for an allogeneic hematopoietic stem cell transplant. Additional strategies that we are currently validating include (1) the modulation of the suicide gene activation, using different concentrations of the inducing agent(s), to be able to eliminate CAR T-cells modified by a regulatable gene, ideally aiming at preserving a proportion of the infused cells (and their antitumor activity) for mild to moderate toxicities, or (2) the co-expression of an inhibitory CAR aiming at sparing normal cells co-expressing an antigen not shared with the tumor.

Key words AML, Chimeric antigen receptor, CD33, Suicide gene, Safety switch, Dimerizing agent, AP1903, Inducible caspase 9

1 Introduction

1.1 Chimeric Antigen Receptor (CAR) T-Cells for Acute Myeloid Leukemia (AML)

Chimeric antigen receptor (CAR)-redirected T-cells are a powerful tool for the treatment of several type of cancers [1]. Particularly, anti-CD19 genetically redirected CAR T-cells have shown an impressive rate of complete response in patients with relapsed/refractory acute lymphoid leukemia (ALL). CAR T-cells have also been tested against CD123 [2–11], CD44v6 [12], CD33 [5, 8, 13–16], Lewis Y (LeY) difucosylated carbohydrate antigen [17],

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https://doi.org/10.1007/978-1-4939-8922-5_5,

PR1 peptide [18], and folate receptor β [19, 20] acute myeloid leukemia (AML) associated antigens.

For clinical applications with CAR T-cells to refractory AML, only results from two small clinical trials have been reported. In one trial targeting the Le-Y antigen, 4 AML patients were enrolled, and one patient achieved cytogenetic remission [17]. The other study was a pilot trial with CAR T-cells redirected for CD33. With this trial a patient with refractory AML experienced a marked but transient decrease in the percentage of bone marrow blasts [15]. Currently several clinical trials are going on, including those with CD123 (www.clinicaltrials.gov NCT02159495, NCT02623582, NCT02937103, NCT03114670) and CD33 (NCT01864902, NCT02958397, NCT03126864) targeting CAR T-cells.

One of the factors limiting the translation to CAR T-cell therapies for AML antigens is the resulting myelosuppression, since the targeted antigen is often shared between normal stem cells and/or myeloid progenitor cells [21].

Although incorporation of suicide genes is a promising strategy that could result in the elimination on demand of the infused cells [1, 11], it is not clear that it would be effective when the toxicity is already manifest. Furthermore, it could result in the loss of the therapeutic effect. Novel strategies are investigated to circumvent this "on-target off-tumor activation" such as repeated doses of transiently expressed "biodegradable" mRNA CAR [11, 21]. Additionally, co-expression of an inhibitory CAR signaling with PD-1 or CTLA-4 intracellular domain could limit the cytotoxic effect toward some antigens expressed on some normal tissues [22].

Here we describe our experience and protocols for the generation and characterization of anti-CD33-redirected CAR T-cells co-expressing the iC9 suicide gene and a selectable marker (ΔCD19), and express our considerations for potential additional strategies to enhance the safety of CAR T-cell therapies.

1.2 Inducible Caspase 9 Safety Switch

A suicide gene co-expressed in CAR T-cells would allow elimination on demand of the introduced cells in case of toxicity. Our group has previously validated the iC9 suicide gene system, and tested the efficacy in clinical trials [23–25]. The iC9 suicide gene consists of FKBP12-F36V domain linked, via a flexible Ser-Gly-Gly-Gly-Ser linker to ΔCaspase 9, which is caspase without the physiological dimerization domain (CARD). FKBP12-F36V consists of a FKBP domain with a substitution at residue 36 of phenylalanine for valine, binding synthetic dimeric ligands, AP1903 (in vivo), and closely related AP20187 (in vitro), with high selectivity and affinity. This construct also linked a truncated CD19 (ΔCD19) molecule as a selectable marker by 2A self-cleaving peptide. Previous studies showed a single 10 nM dose of the chemical inducer of dimerization (CID) induces apoptosis in up to 99% of

high transgene expressing T-cells. Also, this system containing CAR CD19 can eliminate these gene-modified T-cells in a dose-dependent manner in an in vivo mouse model [26]. One limitation of this approach would be the loss of the therapeutic effects. Therefore, additional strategies under investigation in our lab involve (1) a regulatable system to control the adverse event without completely eliminating the cells, or (2) the co-expression of an inhibitory CAR aiming at sparing normal cells co-expressing an antigen nonshared with the tumor.

1.3 In Vitro *Preclinical Validation of iC9 CAR.CD33 T Cells*

We genetically modified human activated T-cells from healthy donors or patients with acute myeloid leukemia with retroviral supernatant encoding the iC9 suicide gene, a ΔCD19 selectable marker, and a humanized third generation chimeric antigen receptor recognizing human CD33 (Fig. 1). We reported on the ability of these cells to effectively control leukemia growth in vitro, and the ability to conditionally and partially eliminate (76%) the gene-modified cells through activation of the suicide gene alone with the nontherapeutic dimerizing agent, or to completely eliminate them through activation of the suicide gene in combination with a second targeted agent, especially aiming at eliminating eventual resistant cells (BCL-2 inhibitor (ABT-199), pan-BCL inhibitor (ABT-737), or Mafosfamide). All these agents are suitable for clinical application.

Albeit never performed in the clinical setting, our future plan is to investigate the utility of iC9-CAR.CD33 T-cells as part of the conditioning therapy for an allogeneic hematopoietic stem cell transplant for acute myeloid leukemia, together with other myelosuppressive agents, while the activation of the inducible Caspase9 suicide gene would grant elimination of the infused gene-modified T-cells prior to stem cell infusion to reduce the risk of engraftment failure as the CD33 is also expressed on a proportion of the donor stem cell graft.

Here, we describe the protocol for generating gene-modified T cells targeting human CD33, co-expressing the iCaspase 9 suicide gene system and the ΔCD19 selectable marker, and describe the in vitro functional assay by us employed to characterize those cells.

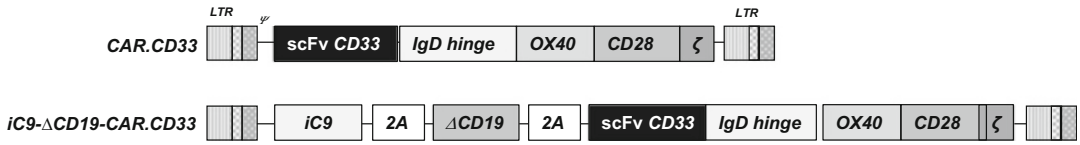

Fig. 1 The structure of the iC9.2A.ΔCD19.2A.CARCD33 transgene. The transgene consists of the inducible caspase 9 (iC9) suicide gene, the truncated CD19 (Δ CD19) selectable marker, and a chimeric antigen receptor redirected toward human CD33 all separated by a 2A-like sequence. iC9 consists of a drug-binding domain (FKBP12-F36V) connected via a short linker (SGGGS) to human caspase 9. The sequence cassette is then incorporated into the SFG retroviral vector. *LTR* long terminal repeat, *Ψ* packaging signal, *scFv* single chain variable fragment, *ζ* Zeta chain of the T-cell receptor

2 Materials

2.1 Common Materials and Reagents

1. T cell medium: 45% Advanced RPMI 1640 (Thermo Fischer Scientific), 45% Click's medium (Irvine Scientific), 10% FBS (GE Healthcare Life Science), 2 mM L-glutamine (Thermo Fischer Scientific).
2. Human interleukin-2 (working solution 200 IU/μL) (Miltenyi Biotec).
3. Trypsin (Thermo Fischer Scientific).
4. D-PBS (Thermo Fischer Scientific).
5. DMSO (Fischer Scientific).
6. 10 mL syringe (BD Biosciences).
7. 15 and 50 mL conical tubes (Falcon).
8. Sterile tubes (Eppendorf).
9. T25 and T75 tissue culture flasks with vented caps (Corning).
10. 24-Well plates (Corning).
11. 96-Well flat-bottom plates (Corning).
12. Cryovials (Nunc).
13. 18G and 20G needles (BD Biosciences).
14. Appropriate antibody for flow cytometry staining and acquisition (see text).

2.2 Retrovirus Supernatant

1. 293T producer cells (ATCC).
2. Cationic liposome formulation (Life technologies).
3. 90% IMDM medium (Life technologies), 10% FBS (GE Healthcare Life.

   Science), 2 mM L-glutamine (Thermo Fischer Scientific).
4. Opti-MEM (Life technologies).
5. *Peg-pam-3e* plasmid containing the sequence for *MoMLV gag-pol*.
6. *RDF* plasmid containing the sequence for the *RD114*.
7. SFG-iC9.2A.ΔCD19.2A.CARCD33 plasmid, SFG-CARCD33 plasmid,

   SFG-GFP-firefly luciferase (ffLuc) plasmid.
8. 10 cm cell culture dish (Corning).
9. 0.25 μm syringe filter (Fisher Scientific).

2.3 Peripheral Mononuclear Cells (PBMCs)

1. Donor peripheral blood.
2. Lymphoprep (stem cell).

2.4 Transduction

1. No azide/low endotoxin anti-CD3 and CD28 antibodies (BD Biosciences).
2. Retronectin (Takara).
3. Non-tissue culture treated 24 well plates (Costar).

2.5 MACS Enrichment

1. MACS column (Miltenyi Biotec).
2. MACS column holder and magnet (Miltenyi Biotec).
3. MACS antihuman CD19 beads (Miltenyi Biotec).
4. MACS buffer (Milteny Biotec).

2.6 Luciferase and Flow Cytometry-Based Coculture Cytotoxicity Assays

1. X-VIVO medium (Lonza).
2. Flat-bottom 96 well assay plate with black plate, clear bottom (Costar).
3. Bright-Glo™ Luciferase Assay System (Promega).
4. PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Phanos Technologies).

2.7 Killing Assay

1. Chemical inducer of dimerization (CID), AP20187 (Clontech).
2. Mafosfamide (Sant Cruz Biotech).
3. ABT-199 (ApexBio Technology).
4. ABT-737 (Santa Cruz Biotech).
5. Annexin-PE assay kit (BD Biosciences).

3 Methods

3.1 Preparation of Retrovirus Supernatant

1. On Day 0, plate 1.5×10^6 293T cells in 10 cm culture dish in total of 12 mL

   IMDM medium containing 10% FBS and 2 mM L-glutamine.
2. Next day (Day 1), for each plate pipette 470 μL of serum-free media (e.g., opti-MEM) to a sterile eppendorf tube. Then, add 30 μL of a cationic liposome formulation and gently mix by pipetting up and down. Incubate for 5 min at RT.
3. After 5 min incubation, add total 12.5 μg DNA per plate to cationic liposome formulation/opti-MEM mixture of the following plasmids.
   (a) RDF 3.125 μg/plate
   (b) Peg-Pam-3e 4.6875 μg/plate
   (c) SFG-iC9.2A.ΔCD19.2A.CARCD33 4.6875 μg/plate

   Gently mix by pipetting up and down and incubate for 15 min at RT.

4. After 15 min incubation, "drop-wise" add this mixture onto the 293T cells and gently swirl to distribute evenly. Return 293T cells to the incubator.
5. At 48 h (Day 2), 293T cells should be 80–90% confluent. Harvest your supernatant and filter the supernatant through a 0.45 μm syringe filter. This may result in loss of titer.
6. Aliquot 5 mL in 15 mL conical tubes. Label the tubes with printer labels.

   Snap freeze retrovirus supernatant in dry ice/ethanol bath and preserve supernatant at −80 °C.
7. Replace the media with fresh complete medium.
8. At 72 h (Day 3), 293T cells should be 100% confluent. Harvest your supernatant and snap freeze again.

3.2 Preparation of PBMC Cells

1. Dilute heparinized peripheral blood 1:1 in D-PBS.
2. Carefully overlay 10 mL Lymphoprep with 20 mL of diluted blood in a 50 mL centrifuge tube.
3. Centrifuge at 400 × *g* for 40 min at room temperature without break.
4. Harvest mononuclear cells with aspirating pipet and resuspend in equal volume of D-PBS (*see* **Note 1**).
5. Centrifuge at 450 × *g* for 10 min at room temperature.
6. Aspirate supernatant.
7. Loosen pellet by "finger-flicking" and resuspend in 20 mL of D-PBS. Remove 10 μL of cells, add 10 μL of appropriate counting solution containing 50% red cell lysis buffer, and count using a hemocytometer (*see* **Note 2**).

3.3 Cryopreservation (Optional)

1. Calculate the number of vials to be frozen, ideally at 5–10 × 10^6 cells/mL and at least 5 × 10^6 cells/vial. Prepare appropriate volume of freezing medium consisting of 40% RPMI, 50% FBS, and 10% DMSO, and place on ice.
2. Centrifuge cells for 5 min at 400 × *g*.
3. Resuspend cells at 5–10 × 10^6 cells/mL (20 × 10^6 cells/mL) in ice-cold freezing medium.
4. Place the pellet on ice for at least 10 min.
5. Immediately transfer to labeled cryovials.
6. Labels should record donor #, cell type, amount, concentration, and date.
7. Immediately transfer the vials to a Nalgene freezing container (*see* **Note 3**).

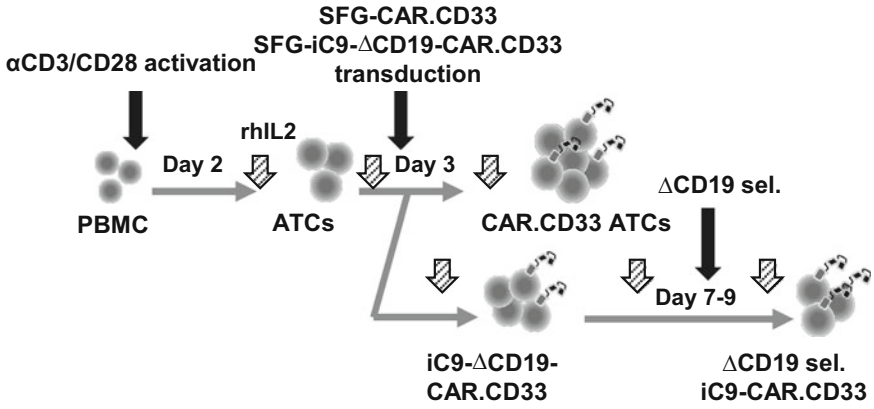

Fig. 2 Overview of the manufacturing process. PBMCs were activated by CD3 and CD28 stimulation, and then transduced with the retroviral vector encoding the transgene of interest on Day 3. Immunomagnetic selection was performed on Day 7–9; the positive fraction could be expanded and cryopreserved. The meshed arrows indicate the times of adding recombinant human interleukin-2 to the culture. *rhIL2* recombinant human interleukin-2

8. Transfer immediately the Nalgene container to a −80 °C freezer.
9. Next day, transfer the vials to liquid nitrogen tank (*see* **Note 4**).
10. Record location and cell information in the freezer inventory form.

3.4 Generation and Transduction of Human-Activated T-cells

1. The start date for CD3/CD28 activation for peripheral blood mononuclear cells (PBMC) is designated "Day 0." The main protocol will run from Day 0 to Day 28 (Fig. 2).
2. The average time for generation of ΔCD19 selected (sel.) iC9-CAR.CD33 T-cells takes 2–3 weeks. Expanded T cells can be frozen for later experiments.
3. Calculation of the expansion of donor PBMC is required to estimate how much number of cells from donor PBMC is needed.

3.4.1 Activation of PBMC Cells with CD3/CD28 Antibodies

1. Plate 0.5 mL of H_2O containing 1 μg of antihuman CD3 antibody and 1 μg of antihuman CD28 antibody per well (*see* **Note 5**).
2. Incubate for at least 4 h in 37 °C incubator (*see* **Note 6**).
3. Aspirate CD3/CD28/H_2O from each well of the 24 well plates. Add 1 mL of T-cell medium to each well or and incubate for 15–30 min at 37 °C.
4. Thaw frozen PBMCs when CD3/CD28-coated plate is ready.
5. Warm 10 mL of complete medium per 1 mL frozen cells in a 15 mL conical tube.

6. Remove the cryopreserved PBMC from liquid nitrogen (*see* **Note 7**).
7. Thaw cryopreserved PBMCs in a 37 °C water bath.
8. Transfer the cell suspension from all vials (*see* **Note 8**).
9. Centrifuge at 400 × *g* for 5 min.
10. Aspirate supernatant, loosen the pellet by "finger-flicking," resuspend the pellet in D-PBS, and count cell number with hemocytometer (*see* **Note 9**).
11. Centrifuge at 400 × *g* for 5 min.
12. Aspirate supernatant, loosen the pellet by "finger-flicking," and resuspend in T-cell medium at 1×10^6 cells/mL.
13. Aspirate media from plate, and aliquot cells into a 24 well plate at 2 mL/well and place cells in incubator.

3.4.2 IL-2 Feeding

1. On Day 2, put 1 μL of medium containing 200 units/μL of IL-2 into each well.
2. Return to incubator.

3.4.3 Retroviral Transduction

1. Prepare 1 mL of H_2O containing 7 μL/mL of retronectin stock for each well to be coated (*see* **Note 10**).
2. Aliquot 1 mL retronectin solution per well.
3. Label plate, mark coated wells, and seal with parafilm. Store in 4 °C.
4. Prepare the required volume of retroviral supernatant (each 2.0 mL/well), and transport the retroviral supernatant on ice.
5. Transfer retronectin plates from the refrigerator. Aspirate retronectin from each well. Wash each well with 0.5 mL complete media (*see* **Note 11**).
6. Place 0.5 mL of thawed retroviral supernatant in each retronectin-coated well and incubate for approximately 30 min in the incubator at 37 °C (*see* **Note 12**).
7. Harvest the T-cells to be transduced from 24 wells plates into a sterile centrifuge tube. After cell collection, there are still cells adherent to the wells. Add 0.5 mL of cell dissociation media to each well and incubate for 5 min at room temperature. Then, add 1 mL of cold D-PBS. Harvest the rest of T-cells and transfer this suspension to the previously pooled cells.
8. Wash the cells by centrifugation at 400 × *g* for 5 min.
9. Aspirate the supernatant and resuspend the cell pellet in 10 mL of complete medium. Count cells and record results in worksheet.
10. Wash the cells by centrifugation at 400 × *g* for 5 min.

11. Adjust the cell suspension volume to 1×10^6 cells/mL by adding additional complete medium.
12. Aspirate half of the supernatant from retronectin plates/wells.
13. Plate 0.5 mL (0.5×10^6 cells) of cell suspension per well. Set aside an appropriate number of non-transduced (NT) cells as control (0.5 mL cell suspension per well, and bring up to 1.5 mL with complete medium and add IL-2 to achieve a final concentration of 100 IU/mL).
14. Add 1.5 mL of retroviral supernatant per well.
15. Add IL-2 to achieve a final concentration of 100 IU/mL.
16. Label as appropriate, and return the plates to the incubator.
17. Harvest cells by gently pipetting and pool cells suspension in to 10 mL conical centrifuge tubes 1–3 days after transduction (*see* **Note 13**).
18. Centrifuge at $400 \times g$ for 5 min. Aspirate supernatant and resuspend in a volume of complete medium estimated to give 1×10^6 cells/mL.
19. Count cells.
20. Centrifuge at $400 \times g$ for 5 min. Adjust cell concentration to 1×10^6 mL.
21. Add IL-2 to 100 IU/mL and plate cells in 24 well tissue culture plate, at $1–2 \times 10^6$ cells/well.
22. Return to 37 °C incubator.
23. At 3–4 days of Intervals you can expand cells subsequently. Gently resuspend cells by pipetting and transfer 1 mL of cells to the new well of 24 well plate.
24. Add 1 mL of the new complete media containing IL-2 to each well.
25. After expansion, perform flow cytometry characterization at least for lymphocyte, and memory markers, and transgene expression (Fig. 3) (*see* **Note 14**).

3.5 Δ CD19 Selection

1. Harvest iC9.2A.ΔCD19.2A.CARCD33 transduced T cells in 50 mL tube and spin at $400 \times g$ for 5 min in D-PBS (*see* **Note 15**).
2. Wash again with D-PBS and centrifuge at $400 \times g$ for 5 min.
3. Add 1 μL of anti CD19 beads with 9 μL of MACS buffer/10^6 cells in 50 mL tube, mix well, and incubate 30 min at 4 °C (*see* **Note 16)**.
4. Add MACS buffer about 10 mL/10^7 cells and spin $300 \times g$ for 10 min (*see* **Note 17**).

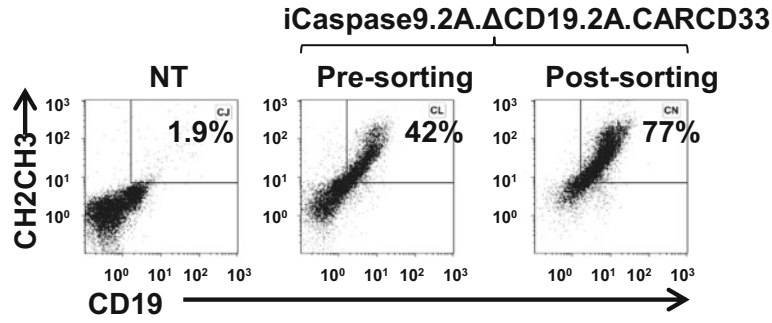

Fig. 3 CAR expression on suicide gene-modified ATCs. T cells were transduced with iC9.2A.ΔCD19.2A.CARCD33 retroviral supernatant and analyzed by flow cytometry (center panel). Cells were stained with Alexa Fluor® 647 AffiniPure F $(ab')_2$ Fragment Goat Antihuman IgG (H + L) polyclonal antibody (Jackson ImmunoResearch) which recognize the IgG1 Fc portion of the CAR, (*y*-axis) followed by staining with PE conjugated CD19 mice monoclonal antibody (*x*-axis); (all antibodies for flow cytometry from BD Biosciences unless otherwise specified). NT control T cells are shown in the left panel. After CD19 sorting, $CH_2CH_3^+CD19^+$ cells were enriched (right panel)

5. Set MS column to the magnet system and apply 1 mL of MACS buffer and discard (*see* **Note 18**).
6. Label one 15 mL tube for negative fraction and one for positive fraction.
7. After centrifugation, resuspend cells in 500 μL of cold MACS buffer and apply cells to the column.
8. Wait until the fluid go through and collect the flow through in the negative fraction tube.
9. Apply 500 μL of cold MACS buffer three times and collect in the same negative fraction tube.
10. Remove columns, apply 1 mL cold MACS buffer and plunge cells in the positive fraction tube.
11. Count cells.
12. Add additional MACS buffer, spin, and resuspend with complete medium (*see* **Note 19**).

3.6 Luciferase- and Flow Cytometry-Based Coculture Cytotoxicity Assays

3.6.1 Luciferase-Based Coculture Cytotoxicity Assay [27]

1. Harvest target cells (MV4-11-eGFP-ffLuc), wash once, and resuspend 0.25×10^6/mL in X-VIVO medium.
2. While incubating targets, harvest and resuspend each effector NT, CARCD33, or ΔCD19 sel. iC9-CAR.CD33 activated T-cells at 2.5×10^6 cells/mL in T-cell complete medium. Add 200 μL to wells designated as Effector/Target ratio (E:T = 10:1) (2.5×10^5/100 μL/well), in clear-flat-bottom 96 well assay plate.

3. Perform serial dilution from the wells of "E:T = 10:1" by mixing, collecting 100 μL and plate in next well and repeat, until the last dilution (E:T = 0.6:1) where you will discard 100 μL.
4. Add 100 μL X-VIVO media in the well designated as "maximum," and add 200 μL X-VIVO media in the well designated as "minimum."
5. After having resuspended target cells at 0.25×10^6/mL, add 100 μL to each well (2.5×10^4/100 μL/well), and add to the well designated as "maximum."
6. Mix each well with multichannel pipet without changing tips from the wells of "E:T = 0.6:1" to the wells of "E:T = 10:1."
7. Incubate overnight.
8. Next day, carefully take the supernatant up to 150 μL from each well.
9. For measuring cell-associated luciferase activity, 50 μL of the prepared Bright-Glo™ Luciferase Assay System was added to each well.
10. Incubated for 5 min for the cells to be completely lysed.
11. The lysed mixture was directly measured for luminescence with a luminometer.
12. The percentage of specific lysis was calculated after subtraction of the minimum value of medium only with the following formula: [(maximum value of labeled targets only (max)−experimental)/max × 100] (Fig. 4).

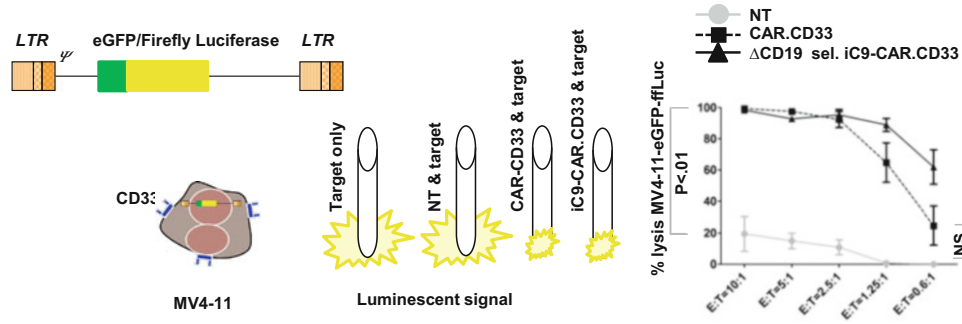

Fig. 4 Luciferase-based coculture cytotoxicity assays. (*Right panel*) T cells transduced with CARCD33, or iC9.2A.ΔCD19.2A.CARCD33 were cocultured with the target cells bearing a GFP-firefly luciferase construct, and the intensity of luminescence analyzed with luminometer. Cells were lysed with Bright-Glo™ Luciferase Assay System. Target cells only (maximum luminescence, i.e. no-killing), and the appropriate experimental control. The respective luminescent values were calculated after subtraction of the background value. (*Left panel*) A representative data of coculture assay with the acute leukemia cell lines (MV4-11) were shown with several Effector/Target ratio (E:T). Percentage of lysis was calculated based on the luminescence of each well

3.6.2 Flow Cytometry-Based Coculture Cytotoxicity Assay [16, 28]

1. Harvest target cells (ex. patient samples), wash once, and resuspend 2×10^6/100 μL in Diluent C of the PKH26 Red Fluorescent Cell Linker Kit.
2. Dilute 1 μL of PKH26 Red Fluorescent dye to 250 μL of Diluent C in the different tube.
3. Take 100 μL of diluted PKH26 Red Fluorescent dye, and add it to the target cells.
4. Incubate at room temperature for 5 min.
5. Add 10 mL of RPMI containing 10% FBS to stop the reaction.
6. Spin and count the cells. Resuspend the cells to 0.25×10^6/mL.
7. While staining the targets, harvest and resuspend each effector NT, CARCD33, or ΔCD19 sel. iC9-CAR.CD33 activated T-cells) at 1×10^6 cells/mL in T-cell complete medium. Add 100 μL to each well (E:T = 4:1) (1×10^5/100 μL/well), in flat-bottom 96 well assay plate.
8. After having resuspended target cells at 0.25×10^6/mL, add 100 μL to each well (2.5×10^4/100 μL/well), and make the well of "only target cells" to add 100 μL of the medium without effector cells.
9. Mix each well with multichannel pipet.
10. Incubate overnight.
11. Next day, carefully take the supernatant up to 150 μL from each well for the cytokine assay, if necessary.
12. Directly stain with several antibodies according to the experiments.
13. Acquire by flow cytometry (Fig. 5).

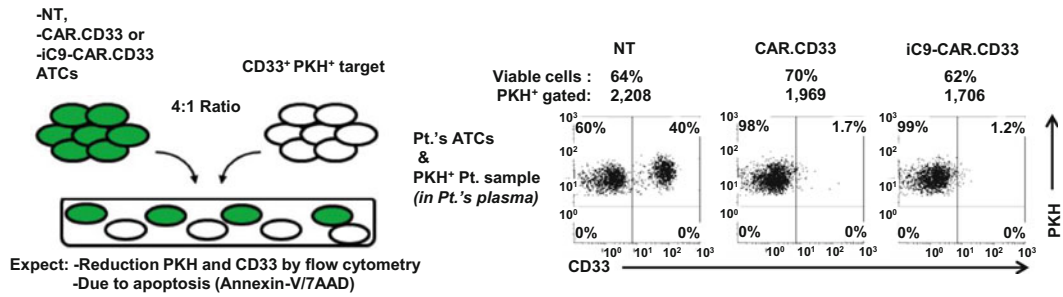

Fig. 5 Flow cytometry-based coculture cytotoxicity assays. (*Right panel*) NT-activated T cells (ATCs), ATCs transduced with CAR-CD33, or iC9.2A.ΔCD19.2A.CARCD33 (iC9-CAR.CD33) were cocultured with PHK26 (PKH) stained $CD33^+$ target cells to E:T = 4:1 ratio and analyzed by flow cytometry after overnight incubation. (*Left panel*) Each effector cell was cocultured with PHK26 (PKH) stained $CD33^+$ target acute myeloid leukemia patient's samples and analyzed after overnight incubation with patient's plasma containing media. APC conjugated anti CD33 mouse monoclonal antibodies were used for the detection of CD33 (*x*-axis). PKH staining was shown in PE channel (*y*-axis). CD33 positive tumor cells were dramatically decreased

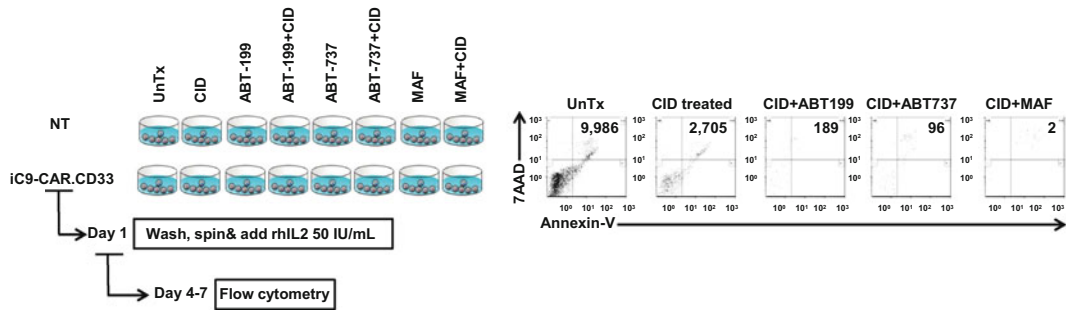

Fig. 6 Method overview, and flow cytometry dot plot of the killing assay. (*Left panel*) NT and CD19 sorted iC9.2A.ΔCD19.2A.CARCD33 transduced T cells (iC9-CAR.CD33) were treated overnight with ABT-199 or ABT-737, or mafosfamide with or without the CID. Next day (Day 1), cells were harvested, stained with Annexin V/7-AAD and analyzed by flow cytometry. Thereafter cells were washed by centrifugation and cultured for additional 4–7 days in the presence of culture medium supplemented with IL-2 (recombinant human IL-2, rhIL2). (*Right panel*) Annexin-V/7AAD dot plots from one representative for iC9.2A.ΔCD19.2A.CARCD33 ATC killing assay. For each experimental condition, it was acquired the same number of events (20,000) including viable and dead cells. Viable cell numbers (Annexin-V^{neg}/$7AAD^{neg}$ cells) are reported in each figure. UnTx: untreated; CID treated (AP20187 10 nM), CID+ABTs (ABT199 or ABT737 10 μM), CID + MAF (2 μg/mL)

3.7 Conditional Elimination of iC9.2A.Δ CD19.2A.CARCD33 Transduced T Cells [16, 23]

1. Seed 0.5×10^6 cells/200 μL of non, or ΔCD19 sel. iC9-CAR.CD33 transduced T cells in 96 well plate.
2. Add CID [10 nM] and/or the BCL-2 inhibitor (ABT-199 [2 or 10 μM]), and/or with the pan-BCL inhibitor (ABT-737 [2 or 10 μM]), and/or mafosfamide [0.5 or 2 μg/mL] (*see* **Note 20**).
3. After overnight culture, take half of T cells and wash cells by centrifugation.
4. Percentage of cell killing is estimated with annexin V/7-AAD staining with the following formula: 100% − (%Viability treated/% Viability non-treated cells) (Fig. 6).
5. Remaining cells were washed by centrifugation, and cultured for 4~7 additional days in the presence of IL-2.
6. After 4~7 days, the cells were harvested, and assessed for percentage of cell killing again.

3.8 Future Directions

Adoptive immunotherapy strategies are attracting notable interest for the treatment of cancer, due to the potential of potent anti-tumor activity. However, novel toxicology challenges are emerging. In fact, the Foundation for the Accreditation of Cellular Therapy (FACT) has published a draft of the 1st edition FACT Standards for Immune Effector Cell Administration. These Standards are intended to promote quality in administration of immune effector cells, including CAR T-cells and therapeutic vaccines, and will be incorporated into a voluntary FACT accreditation in this field.

As the manufacture of those complex therapeutics requires considerable funding and infrastructure, until the commercialization of dedicated products, one possibility would be the formation and/or the use of centralized manufacturing laboratories. For example, one interesting initiative is the Production Assistance for Cellular Therapies to facilitate the transition of laboratory research developments into clinical research products for use in regenerative medicine as well as the novel treatments for cardiac, pulmonary, and blood diseases, the diseases under the auspices of the NHLBI.

The implementation of some automated systems, eventual gene modification systems alternative to viral-derived ones can also contribute to a more stream lined, cost-effective, and safe therapeutic products [29].

4 Notes

1. During centrifugation, higher density cells pass through the barrier and cells with buoyant density of less than 1.077 g/mL (including lymphocytes, monocytes, and platelets) form an interface directly above the barrier.
2. Cells concentration is calculated as follows: the average count per square × the dilution factor × 10^4/mL. For total number of cells, cell concentrations are multiplied by the total volume.
3. Nalgene freezing container can be used up to five times without changing the isopropanol.
4. Cells should not be kept for longer than 5 days at −80 °C, ideally overnight.
5. Coat the cells with non-tissue culture treated 24 well plates. If stock concentration of antihuman CD3 and CD28 antibody is 1 mg/mL, put each 2 μL of stock antibody into 1 mL of H_2O. The number of coated wells is to be determined based on the PBMC number prepared. We typically plate PBMC at 1×10^6 cells/well.
6. Anti-CD3/CD28 coated plate can alternatively be incubated overnight at 4 °C.
7. Keep the vial on dry ice until you are ready to thaw. ATCs can be generated from either frozen or fresh PBMCs.
8. Once the vial is thawed, clean it with alcohol wipes and place in the bio safety cabinet.
9. When thawing cells, loss of 10–20% of total cells is expected.
10. Prepare retronectin-coated wells for transduction on the previous day (Day 1). Alternatively, retronectin-coated plate can be incubated for 4 h at 37 °C.

11. Put back retronectin in the tube and keep at 4 °C. We reuse it once within 30 days.
12. Alternatively, you can centrifuge plates at 1000 × *g* for 5 min at room temperature.
13. Transduction occurs mostly within 16~24 h. When you harvest the cells, use nonenzymatic cell dissociation media to completely collect the cells.
14. After expansion, you can freeze those cells for later use. Every week perform cell count to assess expansion rate. We usually check the expression of the transgene by flow cytometry on Day 9~11 after transduction.
15. We typically use 10~20 × 10^6 transduced cells for each selection.
16. To aliquot the beads, with a syringe and sterile needle disinfect rubber part of the vial of CD19 beads and aspirate ~150 μL (need 1 μL/10^6 cells) in the hood. Put it in a cryovial (glass preferred) and label it appropriately. You can keep the vial at 4 °C.
17. Make sure centrifuge is set to "no break" to treat pellet gently.
18. Be careful to remove columns without touching the plunger. Put the plunger in a place in hood out-of-touch.
19. We usually plate cells with 1~2 × 10^6/mL.
20. To make final concentration of CID. First add 1 μL of CID, AP20187 stock solution (0.5 mM), in complete medium 9 μL (50 μM). Then add 3 μL of last dilution to 147 μL of complete medium (1 μM). Finally, add 2 μL of last dilution to 200 mcL of cells. For ABT-199 (1 μg/μL), add 1.74 μL in 200 μL of complete medium (10 μM). For ABT737 (2.5 μg/μL), add 0.65 μL in 200 μL of complete medium (10 μM). For mafosfamide (1 μg/μL), add 0.4 μL of stock solution into 200 μL of complete media (2 μg/mL) (Alternatively, dilute 1 μL of stock solution in 9 μL of complete medium, then use 4 μL of that dilution solution.)

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Chapter 6

Mesenchymal Stem Cell Exosome-Mediated Prodrug Gene Therapy for Cancer

Cestmir Altaner and Ursula Altanerova

Abstract

Exosomes derived from human mesenchymal stem cells (MSCs) engineered to express the suicide gene yeast cytosine deaminase::uracil phosphoribosyl transferase (yCD::UPRT) represent a new therapeutic approach for tumor-targeted innovative therapy. The yCD::UPRT-MSC-exosomes carry mRNA of the suicide gene in their cargo. Upon internalization by tumor cells, the exosomes inhibit the growth of broad types of cancer cells in vitro, in the presence of a prodrug. Here we describe the method leading to the production and testing of these therapeutic exosomes. The described steps include the preparation of replication-deficient retrovirus possessing the yCD::UPRT suicide gene, and the preparation and selection of MSCs transduced with yCD::UPRT suicide gene. We present procedures to obtain exosomes possessing the ability to induce the death of tumor cells. In addition, we highlight methods for the evaluation of the suicide gene activity of yCD::UPRT-MSC-exosomes.

Key words Mesenchymal stem cells, Retrovirus vectors, Suicide gene, Therapeutic stem cells, Conditional medium, Exosomes, Cancer cells, Tumor cell growth inhibition

1 Introduction

Mesenchymal stem cells (MSCs) possess the ability to migrate to the site of injury and secrete a variety of factors capable of a number of functions inducing and supporting regenerative processes in the damaged tissue, inducing angiogenesis, protecting cells from apoptotic cell death and modulating the immune system (reviewed in [1]). The tumor, being a "wound that does not heal" [2], attracts MSCs. The MSCs home in the tumor and, together with other cells, form the tumor stroma. MSCs tumor tropism inspired us to develop a prodrug suicide gene therapy mediated by various types of MSCs. Prodrug cancer suicide gene therapy mediated by mesenchymal stem/stromal cells (MSCs) represents an attractive tool to activate the prodrug directly within the tumor, thus avoiding systemic toxicity [3–5]. We have developed two prodrug suicide gene therapy systems: MSCs engineered to express fused yeast cytosine

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deaminase::uracil phosphoribosyl transferase (yCD::UPRT) with 5-fluorocytosine (5-FC) as a prodrug—yCD::UPRT-MSC/5-FC system [3] and MSCs expressing thymidine kinase of Herpes simplex virus with ganciclovir as a prodrug—tk*HSV*-MSC/ganciclovir system [6].

Suicide genes were efficiently expressed due to strong retrovirus promoters in both vectors. Both prodrug gene therapeutic systems express enzymes for conversion of a nontoxic prodrug to an efficient cytotoxic compound from a provirus integrated into cellular DNA. MSCs transduced with suicide genes sustained their tumor-tropic behavior. MSCs expressing fused yCD::UPRT gene convert nontoxic 5-FC to 5-fluorouracil (5-FU) at the site of the tumor. Such cells have been designated "therapeutic mesenchymal stem/stromal cells" (Th-MSCs) [5]. The design of our retrovirus vector being a bicistronic construct with suicide gene separated by internal ribosome entry site sequence from neo gene allows selecting homogenous population of transduced cells by cell selection with G418 antibiotic (Fig. 1). Therapeutic potential of the MSCs-driven yCD::UPRT/5-FC suicide gene therapy system was found

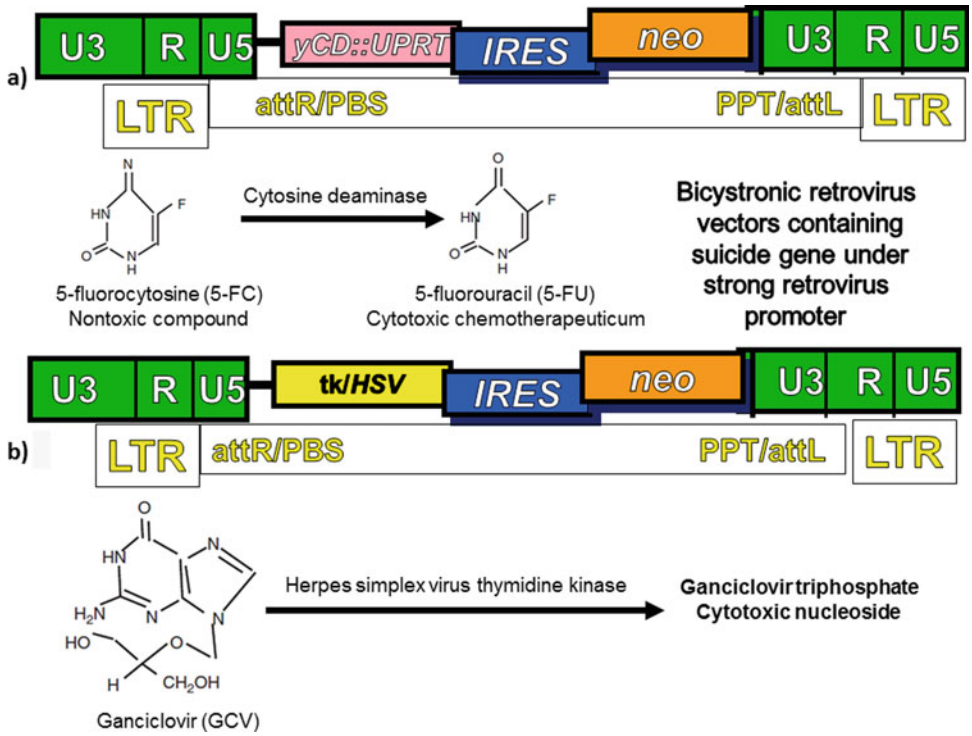

Fig. 1 Bicystronic retrovirus vectors containing suicide genes used in our laboratory. Presence of an internal ribosome entry site facilitates simultaneous expression of genes under retrovirus promoter. Product of *neo* gene allows selection of positively transduced cells in medium containing antibiotic G418. (**a**) Yeast cytosine deaminase::uracil phosphoribosyltransferase (yCDUPRT) synthetic fused gene vector. (**b**) *Herpes simplex virus* thymidine kinase vector

to be quite effective. By showing a strong bystander cytotoxic effect toward xenografts of human colorectal carcinoma cells and melanoma both in vitro and on nude mice in vivo [3, 7] we demonstrated the universality of the therapeutic ability of expanded yCD::UPRT-AT-MSCs (ThMSCs).

In a pilot preclinical study with nude mice, we demonstrated that the human yCD::UPRT-AT-MSCs were effective in significantly inhibiting subcutaneous xenografts of human bone metastatic prostate cells. The ThMSCs were administered intravenously with systemic delivery of 5-FC. Tumor regression by ThMSCs was dose dependent and repeated application improved the therapeutic outcome [8]. A positive therapeutic effect of the autologous and/or human yCD::UPRT-AT-MSCs cells was proven in the autochthonous prostate adenocarcinoma in TRAMP mice, which spontaneously develop aggressive prostate cancer [9]. Intracranial administration of ThMSCs gene therapy system has been shown in a preclinical study effective in the treatment of intracerebral rat C6 glioblastoma [10–12]. The treatment leads to complete tumor regression in a significant number of animals. Systemic Th-MSCs administration resulted in therapeutic cell homing into subcutaneous tumors and mediated tumor growth inhibition (reviewed in [4]). It has been increasingly observed that the transplanted MSCs did not necessarily engraft and differentiate at the site of injury but might exert their therapeutic effects through paracrine/endocrine manner inducing endogenous reparatory processes [13]. Rich MSCs' secretome together with extracellularly released vesicles—exosomes participate on the paracrine cross-talk between cells by delivering molecular information that leads to biological consequences [14]. Main progress in the field of MSC-driven suicide gene therapy for cancer brought our finding of exosomes released from the therapeutic stem cells engineered to express the suicide gene [15–17]. We found release of exosomes possessing the mRNA of suicide gene in their cargo, thus expanding the interpretation to combined action of bystander effect and internalized exosomes. Size-exclusion chromatography of yCD::UPRT-MSCs secretome revealed that the biological activity was clearly localized the exosome fractions (Fig. 2).

Evaluation of tumor cell death induction of exosomes with suicide gene mRNA in their cargo in a dose-dependent manner is presented in Fig. 3. High therapeutic potential of human yCD::UPRT-AT-MSCs can be attributed to several factors. Cumulating evidence supports the notion that soluble factors and/or exosomes secreted by MSCs act in a paracrine/endocrine manner, executing functions similar to those of MSCs. Exosomes from suicide gene modified mesenchymal stem cells have the potential to be a new class of highly selective cancer-targeted therapeutics. Composition of exosomes released from MSCs might be purposely modified to create therapeutically interesting exosomes for cancer treatment

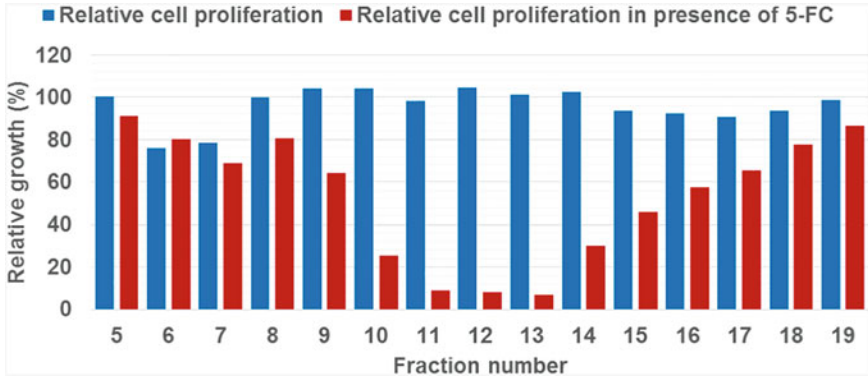

Fig. 2 Fractionation of secretome by size-exclusion chromatography. One milliliter of CM harvested from yCD::UPRT-AT-MSCs cells was separated on a Sephacryl 500 HR column at 4 °C. Each fraction was tested for stimulation of tumor cell proliferation in the absence of 5-FC and for growth inhibiting activity in the presence of 5-FC using the Incucyte system

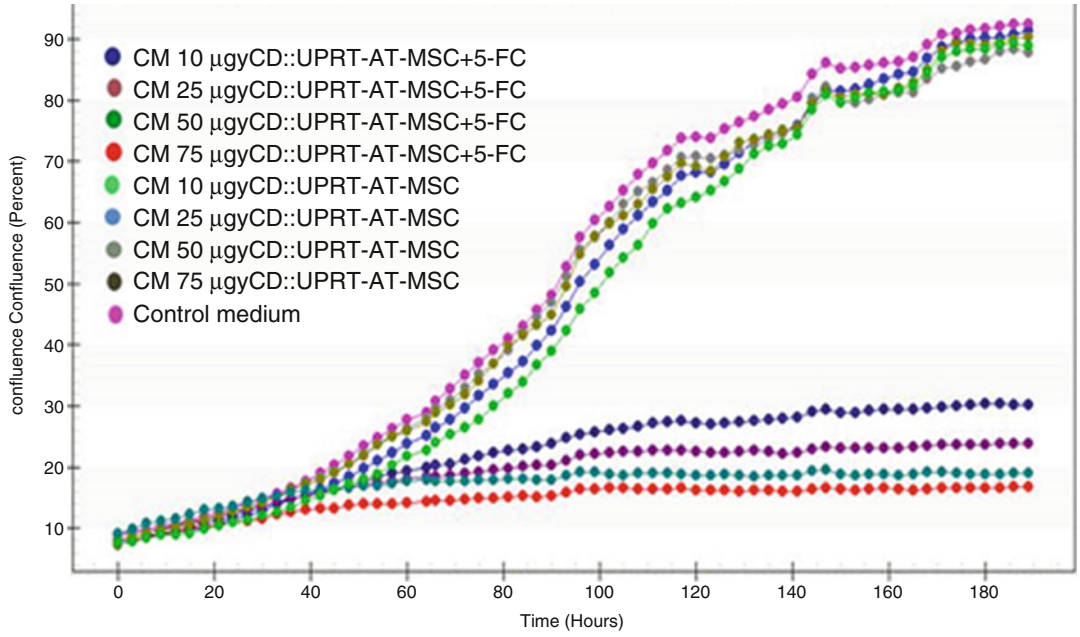

Fig. 3 Exosomes in conditional medium kill human breast cancer cells in a dose-dependent manner. Growth curves of HeLa cells treated with CM from yCD::UPRT-AT-MSCs in presence and/or absence of 5-FC. HeLa cells (3×10^3) were plated in wells of the 96 well plate. Next day, indicated µg of CM were added to growth medium in each well either with prodrug 5-FC or without 5-FC. The course of growth/inhibition was monitored by the Incucyte system

[18]. Recently we reported a dual action of MSCs-yCD-UPRT-MSCs exosomes possessing beside mRNA of suicide gene also iron oxide nanoparticles. These nanoparticles were able to act both as a prodrug-dependent therapeutic and as hyperthermia-inducing factor when tumor cell was exposed to alternating magnetic field [19].

In conclusion, we believe to be innovative cancer therapy curative, it must differ from standard therapies, which just alleviate symptoms of the disease. The innovative cancer therapy has to be targeted not only to tumor, but also specifically to tumor cells and the therapy has to act intracellularly. This could avoid side effects of standard therapies. The therapeutic exosomes possessing mRNA message of suicide genes in their cargo might be the novel approach to curative cancer therapy. The advantages of nano-sized exosomes compared to the administration of exogenous MSCs are multiple. Exosomes are easier to preserve and be transferred, have none immunogenicity and therefore are safer for therapeutic administration (reviewed in [20]).

In this chapter we intend to describe in detail how to:

- Prepare replication-deficient retrovirus possessing yCD::UPRT suicide gene
- Prepare MSCs transduced with yCD::UPRT suicide gene
- Harvest conditional medium from yCD::UPRT-MSCs
- Isolate of biologically active exosomes possessing mRNA of suicide gene yCD::UPRT by size-exclusion chromatography
- Evaluate suicide gene activity of yCD::UPRT exosomes on tumor cells

2 Preparation of Replication-Deficient Retrovirus Possessing yCD::UPRT Suicide Gene

2.1 Materials

1. Dulbecco's Modified Eagle's Medium, high glucose with 2 μM GlutaMAX and 5% fetal calf serum.
2. GP+E-86 helper cells.
3. GP+envAM12 helper cells.
4. Plasmid designated pST2 (DNA of bicistronic retrovirus construct with yCD::UPRT gene separated by IRES sequence from neo gene).
5. G418 solution.
6. Plastic dishes (Corning Life Sciences).
7. 0.05% trypsin/EDTA (Thermo Fisher Scientific).
8. Sterile transfer pipettes.
9. Sterile centrifuge tubes.
10. Aspirating pipette.
11. Cryovials.
12. Dimethyl sulfoxide.

13. Freezing medium: DMEM containing 10% dimethyl sulfoxide and 10% human serum albumin.
14. NIH3T3 cells.

2.2 Methods

1. Transfect GP+E-86 helper cells with plasmid 1 μg using Effectene (Qiagen).
2. Select cells with G418.
3. Harvest virus-containing medium from G418-resistant GP+E-86/pST2 cells.
4. Filter harvested medium through syringe 0.45 μm filter.
5. Infect GP+envAM12 cells with harvested medium supplemented with 100 μg/mL protamine sulfate.
6. Harvest virus-containing medium from G418-resistant GP +envAM12/pST2 cells.
7. Filter harvested medium through 0.45 μm syringe filter.
8. Ping-pong the infection and G418 selection procedures three times.
9. Harvest final virus-containing medium from G418-resistant GP+E-86 cells.
10. Distribute the harvested and filtered medium in cryovials for storage at −80 °C freezer.
11. Freeze the virus-producing cells in freezing medium and store them in liquid nitrogen container.
12. Determine the virus titer on NIH2T3 cells by counting of G418-resistant cell colonies.

3 Preparation of MSCs Transduced with yCD::UPRT Suicide Gene

3.1 Materials

1. Dulbecco's Modified Eagle's Medium, low glucose with 2 μM GlutaMAX, 5% human platelet extract.
2. MSCs isolated from various human tissues (bone marrow, adipose tissue, dental pulp, umbilical cord).
3. Final virus-containing medium from G418-resistant GP + E-86 cells.
4. Protamine sulfate.
5. G418 solution.
6. Plastic dishes (Corning Life Sciences).
7. 0.05% trypsin/EDTA (Thermo Fisher Scientific).
8. Sterile transfer pipette.
9. Sterile centrifuge tubes.
10. Aspirating pipette.

11. Syringe filter 0.22 μm.
12. Cryovials.
13. Dimethyl sulfoxide.
14. Freezing medium: DMEM containing 10% dimethyl sulfoxide, and 10% human serum albumin.

3.2 Methods

1. Preparation of platelet extract (PE). Expired bag of human platelets of healthy tested donors obtained from the blood bank is quickly frozen to −80 °C on a metal plate. This takes about 15 min. Place the frozen bag in a 37 °C water bath to thaw. Repeat this procedure three times. Distribute the disintegrated platelets in tubes and centrifuge the platelets extract at 900 × *g* for 30 min at 4 °C. Filter through 0.22 μm filter unit and freeze in aliquots on −20 °C. Ensure that platelet donors have run infectious disease testing and risk assessment.
2. Infect MSCs with final virus-containing medium from G418-resistant GP+E-86 cells. Supplement the medium with 100 μg/mL protamine sulfate.
3. Repeat infection three to five times.
4. Select cells with G418 in pretested concentration of G418 to eliminate all non-transduced cells. (G418 concentration is in range of 0.5–1.2 mg/mL).
5. Make at least two transfer of cells under G418.
6. Test transduced cells for sensitivity to 5-FC. The cells should die.
7. Designate cells yCD::UPRT-MSCs—Therapeutic stem cells (ThSC).
8. Expand cells by cultivation.
9. Count the cells and determine their viability using an automated cell counter (Countess).
10. Distribute cells into cryovials at concentration of five million of cells per ml in freezing medium.
11. Freeze the cells in liquid nitrogen container.

4 Preparation of Conditional Medium from yCD::UPRT-MSCs

4.1 Materials

1. yCD::UPRT-MSCs cells.
2. Growth medium (Dulbecco's Modified Eagle's Medium, low glucose with 2 mM GlutaMAX, 4% PE).
3. PBS.
4. Plastic dishes (Corning Life Sciences).
5. Syringe filter 0.22 μm.

6. Sterile transfer pipette.
7. Sterile centrifuge tubes.
8. Aspirating pipette.
9. Eppendorf tubes.
10. Syringe filter 0.45 μm.

4.2 Methods

1. Plate yCD::UPRT-MSCs cells to plastic dishes.
2. Grow them to semi-confluence in the growth medium.
3. Remove the growth medium.
4. Wash cells with PBS.
5. Add DMEM without growth supplements.
6. Cultivate cells for another 24 h.
7. Harvest 24 h conditional medium (CM).
8. Centrifuge the CM at 900 × *g* for 5 min at room temperature.
9. Filter CM through 0.22 μm syringe filter.
10. Distribute CM to Eppendorf tubes for future use.
11. Store CM at −80 °C.

5 Isolation of Biologically Active Exosomes Possessing mRNA of Suicide Gene yCD::UPRT by Size-Exclusion Chromatography

5.1 Materials

1. 24 h or 48 h conditional medium (CM).
2. Sephacryl S-500 gel (GE Healthcare).
3. PD-10 column (GE Healthcare).
4. Peristaltic pump P-1 (Pharmacia LKB, Uppsala, Sweden).
5. PBS.
6. Sterile Eppendorf tubes.
7. Syringe filter 0.22 μm.
8. Sterile pipette tips.
9. Nanodrop instrument.
10. Nanosight instrument.

5.2 Methods

1. Fill PD-10 column with Sephacryl S-500 gel.
2. Wash gel by PBS to remove gel conserving fluid.
3. Apply carefully 1 mL of CM on the top of gel.
4. Start to pump PBS by means of peristaltic pump on the column.
5. Collect 0.5 mL fractions.
6. Determine protein content in each fraction by Nanodrop.

7. Quantify number of suicide gene exosomes by Nanosight.
8. Estimate tumor cell growth inhibition of fractions (in the presence of 5-FC) or tumor cell progression (in the absence of 5-FC in medium) by Incucyte IncuCyte® S3 Live-Cell Analysis System (*see* Fig. 2).
9. Freeze left fractions for further evaluation.

6 Evaluation of Suicide Gene Activity of yCD::UPRT Exosomes on Tumor Cells

6.1 Materials

1. 24 h or 48 h conditional medium (CM).
2. Collected fractions of Sephacryl S-500 gel separation run.
3. Human tumor cells.
4. 96-well microplate (Corning 3595).
5. DMEM high glucose with 2 mM GlutaMAX, 5% FBS.
6. DMEM high glucose with 2 mM GlutaMAX, 5% FBS plus 5-FC.
7. 5–fluorocytosine (5-FC), 100 mg/mL.
8. Sterile pipette tips.
9. 8-canal micropipette.
10. IncuCyte® S3 Live-Cell Analysis System.
11. CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega).

6.2 Methods

1. Plate tumor cells into 96-well microplate dish 1 day before the testing (for instance, 3×10^3/well of PC3 cells in 200 μL of growth medium).
2. Next day add fractionated CM and fractions to medium in the well, use increasing amounts if you would like to titrate the tumor cell death inducing capacity.
3. Incubate tumor cells with added fractions with or without 5-FC for 5 days.
4. Use Incucyte IncuCyte® S3 Live-Cell Analysis System for monitoring of tumor cell growth inhibition (in the presence of 5-FC) or tumor cell progression (in the absence of 5-FC in medium) (*see* Fig. 3).
5. Measure final tumor cell viability by standard MTS proliferation assay in each fraction and controls.

7 Notes

Valid for all procedures.

1. Check all materials for cell cultivation (growth supplements, serum, platelets extracts) and cells for presence of mycoplasma contamination.
2. MSCs as well as yCD::UPRT-MSCs should be split, harvested, and cryopreserved before they reach full confluence.
3. All procedures intended to be used therapeutically should be manufactured under good manufacturing practices (GMPs).
4. Therapeutic stem cells yCD::UPRT-MSCs can be frozen at any passage without affecting their cell viability or subsequent cultivation to expand them.
5. Exosomes in conditional medium are biologically active at 4 °C for several days.

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Chapter 7

Extracellular Vesicles as Carriers of Suicide mRNA and/or Protein in Cancer Therapy

Erdogan Pekcan Erkan, Nurten Saydam, Clark C. Chen, and Okay Saydam

Abstract

Gene therapy involves the introduction of genes (termed transgenes) into cells to compensate for a deficiency or to make a beneficial protein. Gene therapy can used as a form of cancer treatment. A particularly attractive paradigm in this regard involves the selective introduction of transgenes into cancer cells that converts inactive prodrugs into active chemotherapeutic agents, thereby triggering the death of cancer cells. Since prodrugs are inactive, they tend not to cause significant side-effects and are well-tolerated by patients relative to conventional chemotherapy. Several viral and nonviral vectors have been used as delivery tools for suicide gene therapy. Extracellular vesicles (EVs) are now recognized as a promising class of nonviral delivery vectors. Here, we describe a method in which a suicide fusion gene construct is loaded into EVs derived from a non-tumorigenic cell line. Delivery of these modified EVs to glioblastoma cell lines and spheroids decreases glioblastoma cell viability, induces apoptotic cell death, and inhibits tumor growth in vivo.

Key words Extracellular vesicles, Cancer therapy, Suicide mRNA, Suicide protein, Glioblastoma

1 Introduction

Suicide gene therapy aims to kill cancer cells by delivering foreign genes (usually of viral or bacterial origin), which would then promote self-destruction of recipient cells through induction of apoptotic cell death. Among several suicide gene therapy systems, Herpes simplex virus thymidine kinase/Ganciclovir (HSK-TK/GCV) and cytosine deaminase/5-fluorocytosine (CD/5-FC) [1–4] have been characterized extensively. A further improvement to the latter strategy has been brought by the introduction of yeast uracil phosphoribosyl transferase (UPRT), and the resulting CD-UPRT/5-FC system has enhanced efficiency [5–7], and is able to overcome 5-FU-resistance in cancer cells [8].

Extracellular vesicles represent an evolutionarily conserved mechanism of intercellular communication and transport [9]. The research on EVs has skyrocketed in the past decade, and several

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groups have focused on the utilization of EVs as natural delivery vectors for therapeutic purposes [10–12]. Their superior biological properties make EVs a promising class of nonviral vectors [13]. In the context of cancer therapy, most efforts have focused on modification of EVs with small RNAs. It is possible to modify EVs before or after isolation; successful implementation of both strategies has been reported in several proof-of-principle studies, majority of which has relied on introduction of small interfering RNAs (siRNAs) [14, 15] or microRNAs (miRNAs) [16, 17]. Having said that, both strategies have certain technical limitations (*see* ref. 18 for a detailed discussion).

Here, we describe a protocol that involves incorporation of CD-UPRT mRNA and/or protein into EVs. Briefly, the protocol involves transient transfection of the non-tumorigenic HEK293T cells with a plasmid encoding CD-UPRT-EGFP fusion gene construct. We have previously demonstrated that transfection with CD-UPRT-EGFP plasmid results in enrichment of CD-UPRT-EGFP mRNA and protein in host cell EVs [12]. Following transfection, ultracentrifugation is used to isolate EVs from 293T cells. Isolated EVs have an average size of 100 nm (Fig. 1a), and also express the surface marker CD63 (Fig. 1b). Treatment of glioblastoma cell lines or glioblastoma tumor spheroids with CD-UPRT EVs and 5-FC reduces cell viability by triggering apoptosis (Fig. 2a) and spheroid volume (Fig. 2b), and also inhibits glioblastoma tumor growth in mice (Fig. 2c).

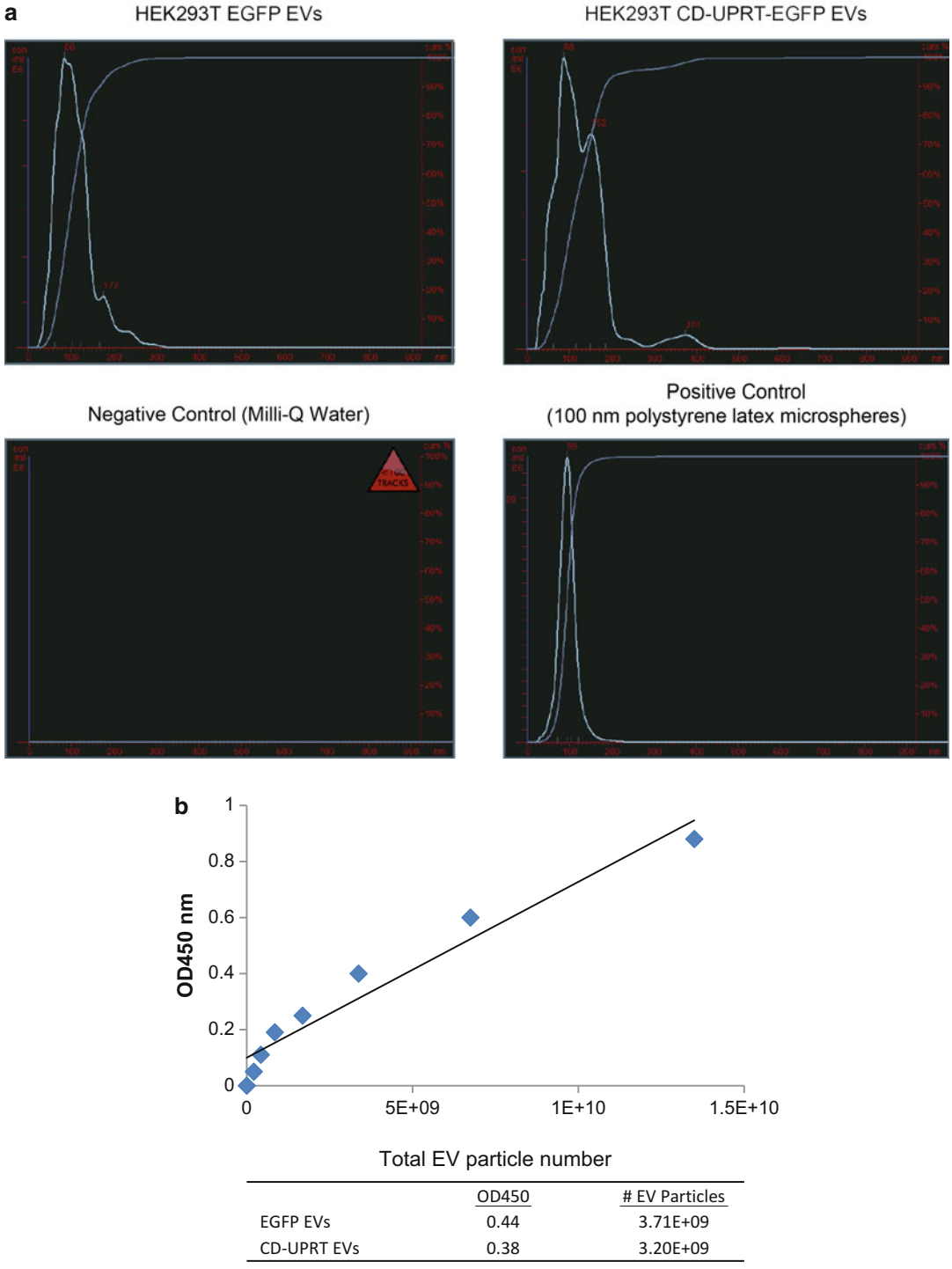
2 Materials

2.1 Transfection

1. Cationic transfection reagent.
2. Reduced serum medium.
3. pCD-UPRT-EGFP,
4. pEGFP-N1.

2.2 Cell Culture

1. HEK-293T cells: Purchase from American Tissue Culture Collection.
2. U87-MG cells: Purchase from American Tissue Culture Collection.
3. Fetal bovine serum (FBS).
4. EV-depleted FBS (*see* **Note 1**).
5. Dulbecco's modified Eagle's medium (DMEM).
6. 0.25% Trypsin solution.
7. Penicillin/streptomycin solution.
8. Flasks or dishes suitable for cell culture.
9. Trypan blue solution, 0.4%.



	OD450	# EV Particles
EGFP EVs	0.44	3.71E+09
CD-UPRT EVs	0.38	3.20E+09

Fig. 1 Characterization of EVs isolated from HEK293T cells transfected with pEGFP-N1 or pCD-UPRT-EGFP. (**a**) Isolated EVs were diluted in PBS, and particle number and size distribution were analyzed on a NanoSight NS500 instrument. Polystyrene latex microspheres (100 nm) and Milli-Q water were used as positive and negative controls, respectively. (**b**) Characterization of EVs by CD63 expression. CD63 ExoELISA (System Biosciences, CA, USA) was used to characterize CD63 expression on EVs, and total number of CD63-positive particles was calculated from the standard curve. Images taken from SI Figs. 1 and 2 in the original paper in Cancer Gene Therapy [11]

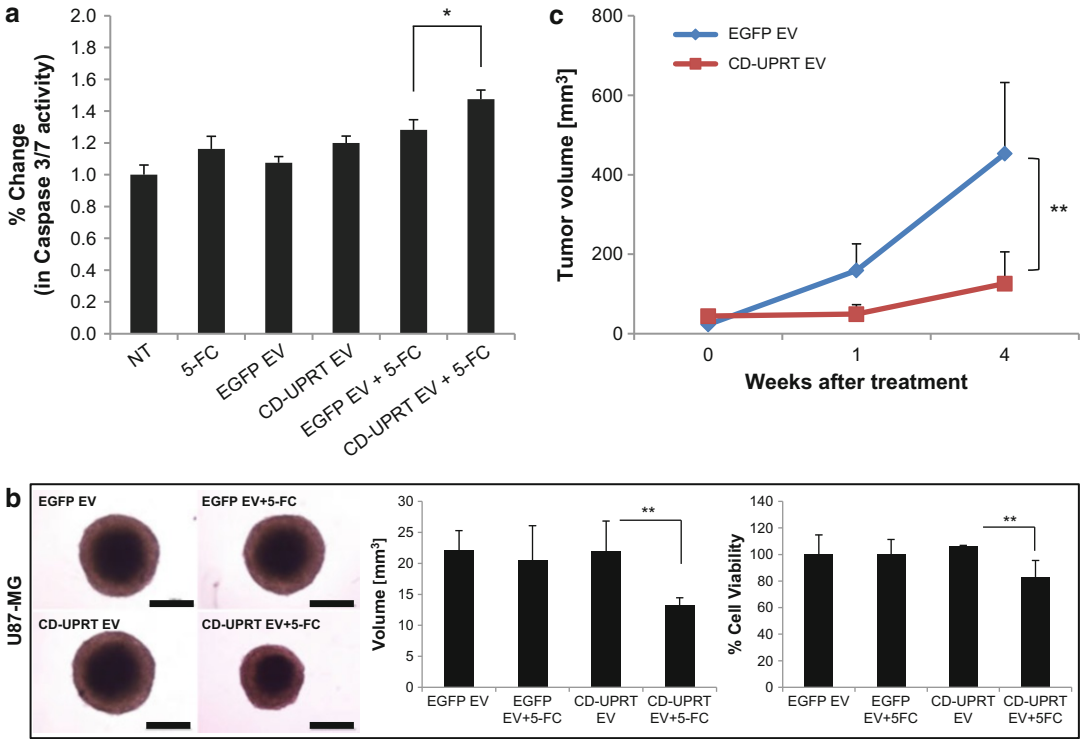


Fig. 2 Effects of CD-UPRT-EVs and 5-FC treatment on glioblastoma tumor growth. (**a**) Combination treatment with CD-UPRT EVs and 5-FC induces apoptotic cell death in glioblastoma cells in vitro. A commercial caspase-3/7 assay was used to determine the level of apoptotic cell death. (**b**) Under similar experimental conditions, glioblastoma spheroids were treated with CD-UPRT EVs and 5-FC, which resulted in a significant reduction in spheroid volume. (**c**) Combination treatment with CD-UPRT EVs and 5-FC inhibits tumor growth in a subcutaneous xenograft model of glioblastoma in mice. Images taken from Figs. 3c, 4a, and 5b in the original paper in Cancer Gene Therapy [11]

2.3 EV Isolation

1. Ultracentrifuge capable of reaching 110,000 × *g*.
2. Ultracentrifuge tubes.
3. Sterile syringe filters, 0.8 μM.
4. Phosphate-buffered saline (PBS), suitable for cell culture.

2.4 EV Quantification

1. NanoSight NS500 (Malvern Instruments, Malvern, UK) or similar instrument capable of performing dynamic light scattering (DLS) analysis.
2. PBS, suitable for cell culture.
3. CD63 ExoELISA: Purchase from System Biosciences, CA, USA.

2.5 Cell Viability Assay

1. Trypan blue solution, 0.4%.
2. Hemocytometer.
3. 5-FC: Purchase from Invivogen, CA, USA.

2.6 Caspase-3/7 Activity Assay

1. 5-FC.
2. Caspase-3/7 ELISA: Purchase from Promega, WI, USA.

2.7 In Vivo Glioblastoma Model

1. Nude, SCID mice, female.
2. Matrigel Matrix, high concentration: Purchase from Corning, MA, USA.

3 Methods

Experiments involving cultured cells should be performed in a Class II biosafety laminar flow hood. Disposal of waste materials should be done in accordance with institutional regulations. All centrifugation steps in EV isolation protocol should be performed at 4 °C.

3.1 Transfection with pCD-UPRT-EGFP and pEGFP-N1

1. Seed early-passage HEK-293T cells at a density of 5×10^6 cells per 10 cm dish (*see* **Note 2**). Allow the cells to attach to the surface by incubating them overnight at 37 °C in a humidified atmosphere containing 5% CO_2.
2. Prepare transfection mixtures by mixing pEGFP-N1 or pCD-UPRT-EGFP (7 μg) with a cationic transfection reagent in 1:3 ratio in 1 mL of reduced serum medium (*see* **Note 3**). Mix thoroughly, and let it sit at RT for 15 min.
3. Remove the cell media, and wash the cells once with PBS.
4. Add transfection mixture dropwise. Add 4 mL of reduced serum medium onto the cells to achieve a total volume of 5 mL.
5. Six hours after transfection, replace transfection medium with DMEM supplemented with 5% EV-depleted FBS.

3.2 Isolation and Characterization of EVs

1. Following Subheading 3.1, collect culture media 72 h after transfection. Centrifuge at 300 × *g* at 4 °C for 10 min.
2. Filter the supernatant through a 0.8 μM filter.
3. Then, centrifuge the filtered supernatant at 2000 × *g* at 4 °C for 20 min.
4. Transfer the final supernatant to an ultracentrifuge tube, and centrifuge at 110,000 × *g* at 4 °C for 90 min.
5. Resuspend the pellet in 50 μL ice-cold phosphate-buffered saline. Store at 4 °C or −20 °C until further analysis (*see* **Note 4**). EVs isolated from cells that were transfected with pEGFP-N1 will be referred to as "EGFP EVs," and EVs isolated from cells that were transfected with pCD-UPRT-EGFP will be referred to as "CD-UPRT EVs."
6. Dilute the EVs in an appropriate volume of PBS (0.5–1 mL). Determine particle size and number on a NanoSight NS500 (Malvern Instruments, Malvern, UK) or similar instrument (Fig. 1).

7. Perform CD63 ExoELISA according to manufacturer's instructions (Fig. 2).

3.3 Cell Viability Assay

1. Seed U87-MG cells at a density of 1×10^4 per well in a 24-well plate. Allow the cells to attach to the plate by incubating them overnight at 37 °C in a humidified atmosphere containing 5% CO_2.
2. Preincubate the cells with either CD-UPRT EVs or with control EGFP EVs (MOI: 10) for 24 h (*see* **Note 5**).
3. Add 5-FC to a final concentration of 250 μg/mL into the culture media (*see* **Note 6**), and incubate the cells for another 24 h.
4. Replace EVs and 5-FC, and incubate cells for an additional 24 h.
5. Harvest the cells with 0.25% trypsin solution. Mix thoroughly to make a homogenous cell suspension.
6. Take 10 μL of cell suspension, and stain with an equal volume of 0.4% trypan blue solution. Count the cells on a hemocytometer.
7. Use the following formula to calculate relative cell viability:

$$\text{Cell viability } (\%) = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100$$

3.4 Caspase-3/7 Activity Assay

1. Seed U87-MG cells at a density of 5×10^3 cells per well in a 96-well plate.
2. Preincubate the cells with CD-UPRT EVs or the control EGFP EVs (MOI: 20) for 24 h.
3. Add 5-FC to a final concentration of 250 μg/mL.
4. Replace EVs and 5-FC every 24 h.
5. Seventy-two hours after incubation with EVs, measure caspase-3/7 activity with a commercial ELISA-based kit. Calculate relative caspase-3/7 activity to determine the level of apoptotic death.

3.5 Cell Cycle Analysis

1. Seed U87-MG cells at a density of 4×10^5 cells per well in 6-well plates, and incubate at 37 °C overnight in a humidified atmosphere containing 5% CO_2.
2. Preincubate the cells with CD-UPRT EVs or the control EGFP EVs (MOI 20) for 24 h.
3. Add 5-FC to a final concentration of 250 μg/mL.

4. Replace EVs and 5-FC every 24 h.
5. Harvest cells after 72 h of incubation with EVs.
6. Add 1 mL of 85% ice-cold ethanol in a drop wise manner while shaking the tube (*see* **Note 7**).
7. Wash cells once with PBS.
8. Add PI solution containing RNAse A (50 μg/mL), and keep the sample in dark until analysis.
9. Perform cell cycle analysis on a flow cytometer. Make sure to have sufficient number of biological replicates and appropriate experimental controls (*see* **Note 8**).

3.6 Tumor Spheroid Assay

1. Generate tumor spheroids as described previously [11].
2. Preincubate spheroids with CD-UPRT EVs or EGFP EVs (MOI: 100) for 24 h.
3. Add 5-FC to a final concentration of 250 μg/mL.
4. After 48 h, replace EVs and 5-FC, and incubate spheroids for an additional 24 h.
5. Image and measure circumference of at least ten spheroids. Use the following formula to calculate spheroid volume:

$$V = \frac{4}{3}\pi r^3, \quad \text{where } r = \frac{C}{2\pi}$$

3.7 In Vivo Glioblastoma Model

The researchers should get approval from the institutional ethics committee before proceeding with experiments involving animals. We recommend performing a pilot study with a small number of animals to determine possible adverse effects related to tumor progression, and to identify humane endpoints.

1. Implant 2×10^6 U87-MG cells in 50 μL of Matrigel into the flanks of nude SCID mice (female, 10 weeks old) (*see* **Notes 9** and **10**).
2. Ten days after the implantation, assign the animals randomly into two groups.
3. Inject EGFP EVs to the animals in the control group, and CD-UPRT EVs to the animals in the treatment group. Injections are performed intratumorally at an MOI of 100.
4. Forty-eight hours after EV injection, inject 5-FC (250 μg/mL) intraperitoneally.
5. Continue this treatment cycle twice a week for a period of 4 weeks.

6. Measure the tumor volume regularly with caliper measurements.
7. Use the following formula to calculate the tumor volume:

$$V = 0.5 \times \left(L \times W^2\right),$$

where L is the length of the tumor, and W is the width of the tumor.

4 Notes

1. High level of endogenous EVs in FBS may have a negative effect on downstream assays and interpretation of experimental results [19]. Therefore, it is crucial to use EV-depleted FBS to supplement the cell culture medium. Commercial EV-depleted FBS are available from different vendors. An alternative to commercial EV-depleted FBS is to use ultracentrifugation. However, it should be noted that the number of remaining EV particles after ultracentrifugation is considerably high, compared to commercial EV-depleted FBS.
2. It is important to use early-passage HEK-293T cells (maintained within ten passages) for transfection and subsequent EV isolation experiments, as prolonged culturing may lead to differences in quantity and quality of EVs isolated from the cells.
3. The structural integrity of EVs and the optimal storage conditions remain as two major open questions. Storing EVs at 4 °C or room temperature have detrimental effects on the number of EVs, which occur rapidly (within 2–3 h). Certain reports have argued that EV-derived DNA and miRNA molecules are highly stable under different storage conditions [20, 21].
4. For optimal transfections, the ratio of transfection reagent to plasmid should be determined for each cell type and transfection reagent. HEK293T cells are easy to transfect, and a transfection reagent-to-plasmid ratio of 3:1 yields good transfection efficiency (>95%) for this cell type.
5. The indicated MOIs were determined empirically for the cell lines used in this protocol. For functional assays with other cell lines, it is important to determine the optimum MOI to eliminate undesired cytotoxicity due to EVs.
6. The 5-FC concentration described in this protocol was determined previously for the cell lines tested. We recommend performing a dose-response curve experiment to determine the optimal 5-FC concentration (and to exclude potential cytotoxicity) before implementing the protocol on other cell types.

7. Fixation step is prone to errors, and the most common problem is formation of cell clumps, which may result in loss of cells. After freezing, it is possible to store the cell suspension at −20 °C for several weeks.
8. Cell cycle analysis experiments should be performed with at least three biological replicates.
9. Prior to use, Matrigel matrix should be thawed overnight in a refrigerator or cold room (2–6 °C). Once thawed, it should be kept on ice. It is recommended to minimize the number of freeze-thaw cycles, and to use pre-cooled pipette tips when working with Matrigel.
10. Performing power analysis and sample size calculation, in light of the 3R principle in animal research, is the recommended approach before starting any experiment involving animals.

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Chapter 8

Production of Viral Vectors with Suicide Genes by Utilizing the Intron-Splicing Mechanism of Insect Cells

Haifeng Chen

Abstract

Viral vectors carrying suicide genes such as diphtheria toxin, Pseudomonas exotoxin, or barnase are very useful tools in cancer gene therapy and cell ablation research. However, such viral vectors are extremely difficult to produce due to the fact that trace amounts of the toxin will kill any cells used for viral vector production. To overcome this obstacle, we inserted mammalian introns that are not recognized by insect cells to break up the open reading frames (ORFs) of the toxic genes and successfully produced at normal levels of baculoviral and adeno-associated viral (AAV) vectors carrying these toxic genes. Once these viral vectors were used to infect mammalian cells, the introns were spliced out and toxic proteins expressed to kill the target cells.

Key words Adeno-associated virus, Baculovirus, Insect cells, Toxin, Suicide genes, Cancer therapy, Cell ablation, Purification, Intron-splicing

1 Introduction

Suicide genes have been used in cancer gene therapy research [1, 2] or in cell ablation applications [3] by utilizing their ability to kill cells. Though plasmid DNA containing toxic gene under control of eukaryotic promoter can be easily obtained due to the inactivity of eukaryotic promoter in bacteria, viral vectors containing the toxic gene are extremely difficult to produce because of the toxic effect exerted on the producer cells. Several attempts tried to limit the toxicity during viral vector production have yielded limited success. One is to use specific cell line that can tolerate certain level of toxin due to mutation on the gene of eukaryotic elongation factor 2 (eEF-2) and adenoviral, and lentiviral vectors were produced at limited yield [2, 4, 5]. The second is to utilize inducible promoter so that toxin expression during viral vector production can be shutoff. However, due to the fact that inducible promoter is not 100% tight and there is still certain level of toxin synthesized, only very limited AAV vectors were produced [6]. The third is to use

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specific promoter that is not active in insect cells so that recombinant baculovirus (rBV) was produced [7]. We developed a novel method by exploiting the intron-splicing mechanism of insect cells and succeeded in production of baculoviral and AAV vectors carrying any toxic genes at normal level [1]. The protocol provided here describes the detailed steps on how to produce these viral vectors in insect cells and apply these vectors in mammalian cells to study the cell killing effect of these toxic genes.

2 Materials

2.1 Designing and Cloning of Toxic Gene with Intron Insertion

1. DNA processing software such as SnapGene, Vector NTI, or other DNA manipulating software.
2. Synthetic DNA sequence corresponding to the whole toxic gene with intron insertion.
3. BamHI and AgeI restriction enzymes.
4. T4 DNA ligase.
5. LB media.
6. Agar plates.
7. Ampicillin.
8. pFastBac shuttle plasmid harboring AAV ITRs with multiple cloning sites (Virovek, Inc., Hayward, California, USA).

2.2 Preparation of Recombinant Bacmid DNA

1. DH10Bac competent cells (Invitrogen, Carlsbad, California, USA).
2. Plasmids pFB-inCap2-inRep, pFB-inCap6-inRep, pFB-CMV-inDTA(hGH), pFB-hTERT-inDTA(hGH), pFB-CMV-inBar (SV40)-GFP, etc. (Virovek, Inc.)
3. Antibiotics-containing LB media: 50 μg/mL kanamycin, 7 μg/mL gentamycin, and 10 μg/mL tetracycline.
4. Antibiotics-containing LB plates: 50 μg/mL kanamycin, 7 μg/mL gentamycin, 10 μg/mL tetracycline, 100 μg/mL X-gal, and 40 μg/mL IPTG (isopropyl-γ-D-thiogalactopyranoside).
5. DNA miniprep buffers.
6. 100% Isopropanol and 70% ethanol.

2.3 Generation of rBVs and Production of rAAV Vectors Carrying Toxic Gene

1. *Spodoptera frugiperda* Sf9 cell line adopted in ESF921 media (Expression Systems, Davis, California, USA).
2. ESF921 media (Expression Systems).
3. GenJet In Vitro DNA Transfection Reagent (SignaGen Laboratories, Rockville, Maryland, USA).
4. Penicillin Streptomycin Solution 100×.

5. Corning Easy-Grip Storage Bottles with volume sizes of 150 mL, 500 mL, and 1000 mL.
6. Corning PP Centrifuge Tubes with volume sizes of 250 mL and 500 mL.

2.4 Purification of rAAV Vectors Carrying Toxic Gene with CsCl-Gradient Ultracentrifugation

1. Sf9 lysis buffer: 50 mM Tris–HCl, pH 7.8, 50 mM NaCl, 2 mM $MgCl_2$, 1% sarkosyl, and 1% triton X-100.
2. Benzonase Endonuclease.
3. Dulbecco's Phosphate Buffered Saline (DPBS).
4. Cesium chloride (CsCl) solutions of 1.32 g/cc, 1.1.38 g/mL, and 1.55 g/mL prepared in DPBS buffer.
5. 5 M NaCl.
6. SimplyBlue SafeStain (Thermo Fisher Scientific, Fremont, California, USA).
7. Falcon 50 mL Conical Centrifuge tubes.

2.5 Salt Removal and Buffer Exchange Using PD-10 Desalting Columns

1. Equilibration buffer: DPBS, 0.001% pluronic F-68, sterile filtered.
2. Disposable PD-10 desalting columns and LabMate PD-10 buffer reservoirs (GE Healthcare Bio-Sciences Corp., Piscataway, New Jersey, USA).
3. Vac-Man Vacuum Manifold (Promega,Madison, Wisconsin, USA).
4. 0.2 μm Low-protein binding syringe filters (Pall Corporation, Ann Harbor, Michigan, USA).

2.6 Verification of Cell Killing Effect with rBV and rAAV Vectors Carrying Toxic Gene

1. WI38 and HEK293 cell lines (ATCC, Manassas, Virginia, USA).
2. EMEM and DMEM media.
3. Fetal Bovine Serum (FBS).
4. Cell lysis buffer: 5 mM Tris–HCl, pH 8.0, 1% triton X-100, and 20 mM EDTA.
5. CellTiter-Glo Reagent (Promega).
6. Tecan Ultra 384 Multi-Detection Microplate Reader (Tecan, Switzerland).

3 Methods

3.1 Designing and Cloning of Toxic Gene with Intron Insertion

1. In a DNA processing software (Snapgene, Vector NTI, or other DNA manipulation software) select the toxic gene and choose "AGG" sequence located after the start codon and before the half size of the toxic ORF. The intron insertion site is between the two "G" nucleotides (*see* **Note 1**).

AGGCCTgccacc**atg**ggcgctgatgatgttgttgattcttctaaatcttttgtgatggaaaacttttcttcgtaccac
gggactaaacctggttatgtagattccattcaaaaagGTAAGCGCCCCTAAAATCCCTTTGGCACAAT
GTGTCCTGAGGGGAGAGGCAGCGACCTGTAGATGGGACGGGGGCACTAACCCTCAGG
GTTTGGGGTTCTGAATGTGAGTATCGCCATGTAAGCCCAGTATTTGGCCAATCTCAGAAA
GCTCCTGGCTCCCTGGAGGATGGAGAGAGAAAAACAAACAGCTCCTGGAGCAGGGAG
AGTGCTGGCCTCTTGCTCTCCGGCTCCCTCTGTTGCCCTCTGGTTTCTCCCCAGgtatacaa
aagccaaaatctggt---//---ggaaatcgtgtcaggcga**tga**GGATCC

Fig. 1 Diagram of human growth hormone (hGH) intron inserted into the diphtheria toxin fragment A (DT-A) sequence. Underlined upper-cases indicate the restriction sites AgeI and BamHI; lower-cases indicate the DT-A coding sequence; bold lower-cases indicate the start and stop codons of DT-A; upper-cases indicate the hGH intron

2. Choose the intron sequence that is not spliced in insect cells but can be spliced in mammalian cells. The human growth hormone (hGH) and SV40 large T antigen (SV40-T) introns have been tested in Virovek and the insertion of hGH intron in the ORF of diphtheria toxin A fragment (DT-A) is shown as an example (Fig. 1 and *see* **Note 2**).
3. Synthesize the whole toxic gene containing the inserted intron with appropriate restriction enzyme sites (BamHI and AgeI were used here) on both ends and clone it into pFastBac shuttle plasmid. The identity of the plasmid can be verified through restriction digestions and DNA sequencing analysis. The plasmid containing the DT-A with hGH intron is shown as an example (Fig. 2).

3.2 Preparation of Recombinant Bacmid DNA

1. Label two 1.5-mL microfuge tubes, one for plasmid carrying the toxic gene (for example pFB-SURV-inDTA) and the other for plasmid carrying the rep and cap genes for AAV packaging (for example, pFB-inCap6-inRep) and put in an ice bucket. Thaw one vial of MAX Efficiency DH10Bac competent cells on ice. Mix gently and dispense 20 μL into each tube. Aliquot the rest of the competent cells into 20 μL/vial and store at −80 °C for later use (*see* **Note 3**).
2. Dilute pFB-SURV-inDTA and pFB-inCap6-inRep plasmids, respectively, into 1 ng/μL in TE buffer. Transfer 2 μL of each diluted plasmid into the corresponding tube in the ice bucket. Gently mix the DNA with the competent cells by flicking the microfuge tube several times and incubate on ice for 30 min.
3. Heat shock the competent cells at 42 °C for 30 s and chill on ice for 2 min. Add 500 μL of SOC medium and incubate with agitation at 37 °C for 4 h to allow recombination happen.
4. Label four 1.5-mL microfuge tubes, two for each construct. Transfer 2.5 μL and 25 μL of the transformation mix, respectively, into each tube. Add 97.5 μL and 75 μL of SOC medium,

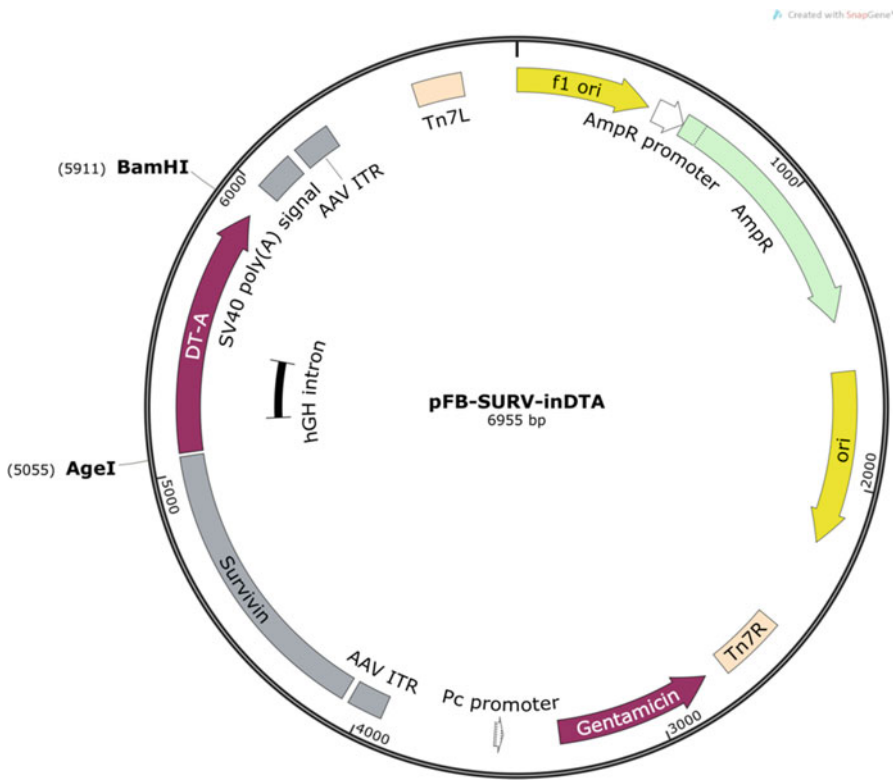

Fig. 2 Diagram of the shuttle plasmid pFB-SURV-inDTA. The DT-A coding sequence containing the hGH intron was synthesized and inserted into the AgeI and BamHI sites of a pFB-AAV shuttle plasmid. This shuttle plasmid was used to generate recombinant baculovirus with the Bac-to-Bac Baculovirus Expression System and subsequently to produce AAV vectors carrying the toxic gene

respectively, to the tubes to obtain 100 μL. Spread each 100 μL of diluted transformation mix into each LB plate containing kanamycin, gentamicin, tetracycline, X-gal, and IPTG. Incubate the LB plates in a plastic bag for 2 days (40–48 h) to allow the development of color (*see* **Note 4**).

5. Pick 2 white colonies from each transformation with sterile pipet tips and culture them at 37 °C overnight in 14-mL culture tubes with 3 mL of LB medium containing kanamycin, gentamicin, and tetracycline.
6. The next morning, label 4 microfuge tubes and pour 1.5 mL culture, respectively, into each tube and centrifuge at >13,000 × *g* for 30 s. Discard the supernatant and pour the rests culture into the same microfuge tube. Centrifuge again at the same speed for 30 s and discard the supernatant. Resuspend pelleted bacterial cells in 200 μL of Buffer P1 through vortexing. Add 200 μL of Buffer P2 and gently invert the tube ten times to mix. Incubate at room temperature for 5 min. Then add 200 μL of Buffer 3 and gently invert the tube ten times to mix (*see* **Note 5**).

7. Centrifuge at >13,000 × *g* for 5 min at room temperature to pellet the cell debris. Label 4 microfuge tubes, and add 450 μL of 100% isopropanol to each tube. Pour the supernatant into each tube and invert the tube ten times to mix. Incubate at room temperature for 5 min.
8. Centrifuge at >13,000 × *g* for 15 min at room temperature to pellet the bacmid DNA. Now transfer all the tubes into a BioSafety Cabinet (BSC). Carefully pour out the supernatant immediately after centrifugation and slowly add 1 mL of 70% ethanol. Gently invert the tube once and centrifuge at >13,000 × *g* for 1 min. In the BSC, Gently pour out the supernatant and close the tube lid. Centrifuge at the same speed again for 1 min. In the BSC, use pipet tip to remove the residue ethanol and let stand for 5 min to evaporate (*see* **Note 6**).
9. Add 50 μL sterile TE buffer to each tube and use pipet tip to rinse the bacmid DNA on tube wall. Let the tubes stay inside the BSC for 30 min or longer so that the bacmid DNA is fully dissolved before use.
10. Verify the presence of gene inserts by PCR assays using primers 5'-CTTTGAAAGCAGTCGAGGGG-3′ (forward) and 5'-ATAGGGCGAATTGGGTACCG-3′ (reverse) for bacmid carrying DT-A, and 5'-ATAGGACCCTGCAGGTATAC-3′ (forward) and 5'-AGCTCTTCAACATCCAAGTC-3′ (reverse) for bacmid carrying capsid 6. Pick the correct bacmid DNA and proceed to next step for generation of recombinant baculovirus (rBV) or store at −20 °C if not use immediately.

3.3 Generation of rBVs and Production of AAV Carrying Toxic Gene

1. Thaw one vial of Sf9 cells and rinse the cells with ESF921 media containing antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin). Resuspend the cells in 25 mL ESF921 media containing antibiotics and transfer to a 250-mL Corning Storage bottle. Culture the cells in a shaker incubator with sticky matt on the platform (*see* **Note 7**) at 28 °C with agitation of 150 rpm until the cell density reaches between 6×10^6 and 1×10^7 cells/mL. Maintain the cell culture by splitting the cells at 1:4 ratios.
2. Seed low passage number of Sf9 cells at 1.5×10^6 cells/well in 6-well plate with 2 mL ESF921 culture media without antibiotics and allow the cells to attach for at least 30 min in an incubator at 28 °C before transfection. Label four sterile microfuge tubes and Dilute 10 μL of each bacmid DNA into 100 μL of ESF921 media without antibiotics in each tube. Dilute 20 μL of GenJet into 400 μL of ESF921 media without antibiotics in a sterile microfuge tube and mix by pipetting up and down three times. Transfer 100 μL of the diluted GenJet to

each tube containing the diluted bacmid DNA and pipet up and down for three times to mix. Let incubate at room temperature for 30–45 min.

3. Add 0.8 mL of ESF921 media without antibiotics to each tube containing the bacmid-GenJet mixture. Aspirate media from the cells and transfer the bacmid-GenJet mixture to each corresponding well. Incubate overnight at 28 °C in the incubator. The next morning add 1 mL ESF921 media with two-fold concentration of antibiotics (200 U/mL of penicillin and 200 μg/mL of streptomycin) to each well and continue incubate at 28 °C for a total of 4 days.
4. Harvest rBVs (P_0 stocks) by tilting the 6-well plate to collect the media into 3-mL sterile tube. Take a 50-μL aliquot and perform quantitative PCR (QPCR) with primers 5'-ATTT GACTTGGTCAGGGCCG-3′ (forward) and 5'-TGTTACG CAGCAGGGCAGTC-3′ (reverse) to determine the rBV titer (multiplicity of infection or moi) and verify the rBVs. Store the rBVs at 4 °C under dark for short-term (less than 1 month) or add sodium citrate to 100 mM and store at −80 °C for long-term storage (*see* **Note 8**).
5. Label two 500-mL size Corning bottles, one for rBV-inCap6-inRep and the other for rBV-SURV-DTA. Dilute Sf9 cell culture (usually grown at 8×10^6 cells/mL or higher) to 2×10^6 cells/mL with fresh ESF921 media containing antibiotics and transfer 200 mL to each flask. Add 0.05–0.1 moi of each P_0 stock to the corresponding bottle and incubate in a shaker incubator at 28 °C with agitation of 150 rpm for 3 days.
6. Pour the cultures into 250-mL sterile centrifuge tubes, respectively, and centrifuge at 900 × *g* for 10 min in a Beckman GS-6 Centrifuge. Collect the supernatants. These are the amplified passage 1 (P_1) rBVs. Perform QPCR assay to determine the rBV titer and store the P_1 rBVs at 4 °C under dark.
7. Label two 1000-mL Corning bottles. Dilute Sf9 cell culture (usually grown at 8×10^6 cells/mL or higher) with equal volume of fresh ESF921 media containing antibiotics and transfer 300 mL each to the labeled bottles. Calculate the required amounts of rBV-inCap6-inRep (10 moi) and rBV-SURV-DTA (5 moi). Mix both rBVs in a sterile tube and then pour the mixed rBVs into the 1000-mL Corning bottles. Infect for 3 days with shaking at 150 rpm, 28 °C in a shaker incubator.
8. Pour the cell cultures into two 500-mL centrifuge tubes and centrifuge at 2750 × *g* for 10 min in a Beckman GS-6 Centrifuge. Discard the supernatant and store the cell pellets at −20 °C if not processed immediately.

3.4 Purification of AAV Vectors Carrying Toxic Gene with CsCl-Gradient Ultracentrifugation

1. Add 16 mL of Sf9 lysis buffer to each cell pellet and benzonase to final 125 units/mL of lysate. Resuspend thoroughly by vortexing. Set the Output Control of Branson Sonifier 150 to 7 and sonicate at room temperature for 20 s. If there are still visible clumps, sonicate for another 20 s.
2. Incubate the lysates at 37 °C for 1 h to digest the nucleic acids. At the end of the incubation, add 1.8 mL of 5 N NaCl to each lysate to adjust the NaCl concentration to about 500 mM. Mix and centrifuge at 7750 × *g* for 20 min in the Avanti J-25 centrifuge with a conical rotor.
3. Transfer each cleared lysate to a clear ultracentrifuge tube for SW28 rotor. The volume of each lysate should be about 22 mL. Use a syringe with long needle that can reach the bottom of the centrifuge tube and add 10 mL of 1.32 g/cc CsCl solution, followed by 5 mL of 1.55 g/cc CsCl solution. Mark the interface between heavy and light densities of the CsCl solution. Centrifuge at 141,000 × *g* overnight (17–20 h) at 15 °C in a Beckman Optima LE-80 K Ultracentrifuge.
4. At the end of centrifugation, carefully take out the centrifuge tubes and assemble the tube one at a time in a stand. Shine a beam light at the bottom of the tube to assist visualization of AAV band. Turn all the room lights off to get better view.
5. Insert a syringe needle (18G × 1 gauge) slightly below the interface mark and slowly draw the AAV band. Collect about 6 mL samples from each tube and transfer all 12 mL to a clear ultracentrifuge tube for 70 ti rotor. Add additional CsCl solution of 1.38 g/mL to fill to the neck of the tube. Balance with another ultracentrifuge tube filled with CsCl solution of 1.38 g/mL. Seal both tubes.
6. Centrifuge at 387,331 × *g* overnight (17–20 h) at 15 °C. Carefully take out the tubes and assemble the tube one at a time in a stand. Shine a beam light at the bottom of the tube to aim the visualization of the AAV band. Turn off all lights to get a better view.
7. Carefully puncture a small hole at the top of the centrifuge tube to release pressure. Insert a syringe needle (18G × 1 gauge) at about 3 mm below the AAV band and slowly draw the AAV band completely into the syringe (1.5–2.5 mL). Transfer the AAV sample to a 15-mL Falcon tube and store at 4 °C if not used immediately.

3.5 Salt Removal and Buffer Exchange Using PD-10 Desalting Columns

1. Cut the tip of a PD-10 Desalting column and pour out the storage solution. Assemble the column with reservoir on a Vac-Man Vacuum Manifold and add 25 mL of sterile final AAV buffer to equilibrate the column. Turn on the vacuum

Table 1
Production yield of AAV vectors carrying DTA or GFP

Clone no.	AAV Full name	Titer (vg/mL)	Total volume (mL)	Total culture (mL)	Total yield per liter culture
V151	AAV2-CMVen-hTERT-inDTA (hGH)	2.63E + 13	12	200	1.58E + 15
v152	AAV2-CMVen-hTERT-GFP	2.78E + 13	10	200	1.39E + 15
V153	AAV2-CMV-inDTA(AAV)	2.12E + 13	15	200	1.59E + 15
V168	AAV2-hcXR4-DTA	2.26E + 13	15	200	1.70E + 15
V173	AAV2-SURV-DTA	2.33E + 13	12	200	1.40E + 15
V174-1	AAV2-hSURV-inDTA389 (hGH)-GPC3	2.25E + 13	14	200	1.58E + 15
V174-3	AAV2-hSURV-inDTA389 (hGH)-GPC3	2.23E + 13	13	200	1.45E + 15
V174-5	AAV2-hSURV-inDTA389 (hGH)-GPC3	2.32E + 13	12	200	1.39E + 15
V170	AAV2-hLP-inDTA(hGH)	2.14E + 13	7	100	1.50E + 15
v175	AAV2-PSA-inDTA(hGH)	2.08E + 13	6.7	100	1.39E + 15
V176	AAV2-SURV-inDTA389(hGH)-GPC3	2.24E + 13	6.5	100	1.46E + 15
V173	AAV8.2-SURV-DTA	2.44E + 13	20	300	1.63E + 15
V179	AAV2-APoE-hAAT-inDTA	2.26E + 13	13	200	1.47E + 15
V180	AAV2-ApoE-hAAT-inDTA-miR3	2.55E + 13	15	200	1.91E + 15

and control the flow rate at 10–15 mL/min until the buffer just run out.

2. Assemble the column with an adaptor and put into a 50-mL Falcon tube and centrifuge at 900 × *g* for 2 min in Beckman GS-6 Centrifuge to remove excessive buffer from the column.
3. Transfer the column to a fresh sterile 50-mL Falcon tube and add the AAV sample (not more than 2.5 mL) to the column and centrifuge at 900 × *g* for 2 min in Beckman GS-6 Centrifuge to collect the AAV sample. More than 99% of the salt and detergent residues are removed at this step.
4. Assemble the used PD-10 column with reservoir on the Vac-Man Vacuum Manifold. Wash the used column with 25 mL final AAV buffer and desalt the AAV as described in Steps from 1 to 3 for the same AAV sample to remove additional trace amount of the salt and detergent residues. Now the AAV sample is fully exchanged in the final buffer.

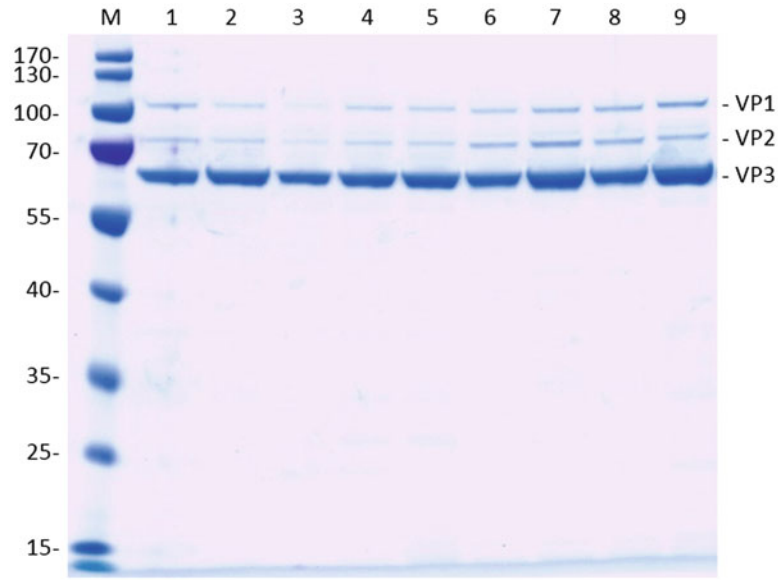

Fig. 3 Representative gel image of SDS-PAGE and SafeStain showing the purity of AAV vectors after 2 rounds of CsCl ultracentrifugation. M, protein ladders; lane 1, AAV9 control vector loaded with 1e + 11 vg/lane; lanes 2 through 9, various AAV vectors loaded at different amounts per lane

5. In a BioSafety Cabinet, equilibrate a sterile syringe filter of 0.2 μm (low-protein binding properties) with 0.5 mL of the final AAV buffer and push out excessive buffer. Transfer the desalted AAV sample to the syringe and filter sterilize the AAV sample. Collect the AAV sample in a sterile 15-mL Falcon tube and store it at 4 °C when performing characterization assays. Once the assays are completed, aliquot and store purified AAV at −80 °C for long-term storage.
6. Determine the AAV genome copy numbers by quantitative PCR (qPCR) assay, dilute the AAV vectors to a desired concentration (usually 2e + 13 vg/mL). Examples of producing AAV vectors carrying toxin gene at normal level are shown in Table 1.
7. Examine the purity of AAV sample with SDS-PAGE followed by SimplyBlue SafeStain. An example of gel stain image is shown in Fig. 3.

3.6 Verification of Cell Killing Effect with AAV Vectors Carrying Toxic Gene

1. Grow Su86.86 cells (ATCC CRL-1837) at 37 °C in RPMI media containing 10% FBS and antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin) until confluent and split 1:4 weekly for maintenance.
2. Seed 96-well plates at cell density of 3.2e + 4 cells/well in 100 μL RPMI media containing 10% FBS and antibiotics and grow at 37 °C overnight.

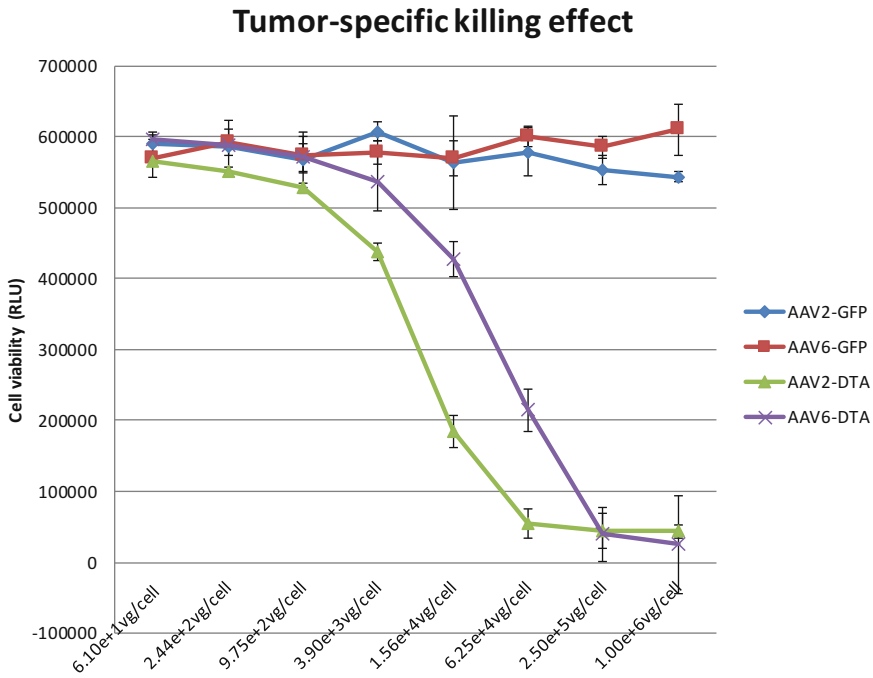

Fig. 4 Dose-response curve showing the killing effect of DT-A on pancreatic cancer cell line Su86.86. AAV2 or AAV6, adeno-associated virus serotype 2 or 6; GFP, green fluorescent protein; DTA, diphtheria toxin fragment A

3. The next morning prepare fourfold serial dilutions from 3.2e + 11 vg/mL to 1.95e + 7 vg/mL of the rAAV vectors in serum-free RPMI media. Remove the old media from the wells and carefully add 100 μL/well of the diluted rAAV vectors.
4. After incubation at 37 °C overnight, add 100 μL/well of RPMI media containing 20% FBS and 2× antibiotics. Continue incubation at 37 °C for additional 5 days.
5. Remove the old media and add 100 μL/well of cell lysis buffer. Shake gently for 10 min to lyse the cells. Add 100 μL/well of CellTiter-Glo Reagent and gently mix for 2 min.
6. Measure cell viability with Tecan Ultra 384 Multi-Detection Microplate Reader. An example of cell killing effect is shown in Fig. 4.

4 Notes

1. The reason to choose "AGG" as the intron insertion site is based on the intron-splicing consensus sequence in which the 5′ splice site (donor site) is frequently linked to "AG" and the 3′ splice site (acceptor site) to "G" nucleotides (5′-----**AG**:GT---intron---AG:**G**----3′). Selection of other sites may also work

but may not be as efficient for intron splicing. The rationale to insert the intron after the start codon and before the half size of the toxic gene is to avoid the possibility of generating functional partial toxin. If the intron is inserted close to the N-terminal, one needs to make sure that there is no in-frame start codon ATG after the insertion site.

2. Empirical studies need to be performed if you want to select different introns than the introns we tested. Some mammalian introns are functional in insect cells for splicing even though the splicing efficiency may be low. For example, AAV intron has low level of splicing in Sf9 cells and cannot be used for this purpose because trace amount of the toxin will kill the Sf9 cells and no baculovirus carrying the toxin gene can be produced.
3. pFB-inCap6-inRep was used together with pFB-SURV-inDTA as an example to produce AAV6 vectors carrying the DT-A gene. We have tested other AAV serotypes such as AAV1, 2, 5, 6, 8, and 9 and all can be produced at normal level. MAX Efficiency DH10Bac competent cells come as 100 μL/via. We aliquot it into 5 vials and it works very well.
4. The transformation efficiency of different constructs is different. Plating different amounts of transformation mix on the LB plates ensures to have good separation of white from blue colonies. Incubate the LB plates in a plastic bag to maintain the humidity and therefore the appropriate growth of bacteria.
5. Use all 3 mL of bacterial culture for bacmid preparation yields higher concentration of bacmid DNA, which increases the transfection efficiency to generate recombinant baculovirus. The baculoviral genome is 143 kb long and strong physical forces should be avoided to minimize damaging to the DNA once the DNA is released from the cells.
6. When you perform centrifugation in this step, orient the lid hinge of the microfuge tubes facing outward so that you know the location of the bacmid DNA pellet. Do not disturb the DNA pellet when adding 70% ethanol. Put the microfuge tubes in the same direction with lid hinge facing outward for centrifugation. It is necessary to keep the bacmid DNA sterile by working inside a BioSafety cabinet. The microfuge tubes contacted with isopropanol and 70% ethanolis sterile. Change pipet tips when removing residue ethanol from the tubes to avoid possible cross-contamination. The miniprep bacmid DNA concentration prepared with this protocol is about 0.1 μg/μL.
7. Corning Easy-Grip storage bottle has larger air space than shake flask to promote cell growth and it can sit securely on the sticky matt. Be sure to loosen the cap for air ventilation. Using sticky matt instead of the metal spring flask holders can save space and accommodate more culture bottles.

8. We use QPCR primers corresponding to the gentamicin gene that presents only in the rBVs. The QPCR assay serves two purposes: one is to verify the rBVs and second to determine rBV titers. We empirically determined that approximately 20 viral genomes (vg) equal to 1 moi. For rBV storage at −80 °C, we tested several reagents for preserving baculovirus infectivity after repeated freeze-thawing and found out that 100 mM sodium citrate works the best not only in preserving rBV infectivity but also in producing AAV vectors.

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Chapter 9

Resveratrol-Responsive CArG Elements from the Egr-1 Promoter for the Induction of GADD45α to Arrest the G2/M Transition

Qiwen Shi and Deepak Bhatia

Abstract

Suicide gene therapy is based on the introduction of a foreign gene into tumor cells to sensitize cells to treatment, to convert a nontoxic compound into a lethal drug, or to produce a cytotoxic effect. We have constructed a suicide gene therapy vector that contains resveratrol-responsive CArG elements from the Egr-1 promoter and the GADD45α open reading frame. CArG elements are utilized as a "molecular switch" to drive the expression of GADD45α. When transfected into lung cancer cells, the vector is able to express GADD45α upon resveratrol treatment, and subsequently leads to cell cycle arrest at the G2/M transition. In this chapter, we describe a detailed protocol for vector construction, transfection, cell viability assay, and cell cycle analysis.

Key words Cancer gene therapy, CArG elements, Resveratrol, GADD45α

1 Introduction

Reinstitution of a tumor suppressor gene by gene transfer technology has been shown to result in cancer cell death, tumor regression, and angiogenesis inhibition [1]. Growth arrest and DNA damage-inducible 45 alpha (GADD45α) is a ubiquitously expressed protein involved in the regulation of DNA repair, cell cycle progression, senescence, and stress-induced signaling transduction, and has been identified as a tumor suppressor [2]. Deficiency in GADD45α is associated with the initiation and development of malignancy and is regarded as a "second genetic hit" in tumorigenesis [3, 4]. Importantly, upregulation of GADD45α is necessary for many anticancer agents to exhibit their proapoptotic and antigrowth effects in cancer cells [5, 6]. Therefore, we chose GADD45α as the suicide gene and hypothesized that overexpression of GADD45α leads to tumor cell growth arrest and apoptosis.

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CArG elements are the serum response elements (SREs) or 10-nucleotide motifs of the consensus sequence $CC(A/T)_6GG$ in the Egr-1 promoter that are responsive to ROS generated by radiation and chemotherapy [7]. Both deletion construct containing the CArG elements in Egr-1 5′ distal enhancer region and synthetic promotes consisting of isolated CArG motifs are able to work as a "molecular switch" in the presence of radiation or chemotherapeutic agents to initiate the expression of cDNA engineered in the therapy vector [8, 9]. Resveratrol has been implicated to induce Egr-1 promoter activity and to exert antitumor effects via Egr-1 activation [10, 11]. Altogether, we assumed that resveratrol is sufficient to trigger CArG-based promoter to drive the transcription of cloned cDNA.

The suicide gene therapy vector we designed relies on the use of CArG elements as an inducible promoter to transcriptionally activate the expression of tumor suppressor gene GADD45α. When combined with resveratrol, the vector increases cellular GADD45α protein level and subsequently results in cell growth inhibition and cell cycle arrest at G2/M transition.

2 Materials

2.1 Materials for Cell Culture and Transfection

1. Culture medium for cell growth and maintenance: Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin.
2. Sterile phosphate-buffered saline (PBS; 1×): dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 mL of deionized H_2O. Adjust the pH to 7.4 with HCl, and then add deionized H_2O to 1 L. Sterilize the solution by autoclave. Store at room temperature.
3. Trypsin: 0.25%.
4. Culture medium for plasmid transfection: Opti-MEM containing 10% FBS and 1% NEAA without antibiotics.

2.2 Materials for Plasmid Construction

1. LB/Ampicillin plates: Dissolve 40 g of LB agar (Miller) in 1 L of deionized H2O in a 2 L flask. Cover with aluminum foil and autoclaved. Add ampicillin sodium sulfate (50 mg/mL; Sigma-Aldrich) to a final concentration of 100 μg/mL when the temperature is about 50 °C. Pour approximately 10 mL into each Petri dish. Swirl to coat the plates. Allow the agar to solidify at room temperature. Seal the plates with tape. Store at 4 °C.
2. IPTG stock solution: Add deionized water to 1.2 g IPTG to 50 mL final volume. Filter-sterilize and store at 4 °C.

3. X-Gal stock solution: dissolve 100 mg of X-Gal in 2 mL of *N*, *N*′-dimethyl formamide (DMF). Cover with aluminum foil and store at −20 °C.
4. LB/Ampicillin/IPTG/X-Gal plates: Spread 100 μL of 100 mM IPTG and 20 μL of 50 mg/mL X-Gal over the surface of LB/Ampicillin plate and dry the plate for 30 min at room temperature.
5. Tris-acetate-EDTA (TAE) buffer (50×): Dissolve 242 g of Tris base in 800 mL of deionized water. Add 57.1 mL of glacial acetic acid and 100 mL of 500 mM EDTA (pH 8.0) solution, and deionzied water to a final volume of 1 L.
6. Agarose gel (1%): Dissolve 1 g of agarose in 100 mL of 1× TAE buffer.
7. 0.5% GTG™ agarose gel: Dissolve 0.5 g of GTG™ agarose in 100 mL of 1× TAE buffer.
8. LB/Ampicillin broth: Dissolve 10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, and 5 g of NaCl in 1 L of deionized water. Adjust pH to 7.0 with NaOH. Add ampicillin to a final concentration of 100 μg/mL.
9. 10× oligo annealing buffer: Dissolve 100 μL of 1 M Tris–HCl (pH 8.0), 200 μL of 5 M NaCl, and 20 μL of 500 mM EDTA in 1 L of deionized water.
10. SOC Medium: Purchased from Promega (Wisconsin, USA).

3 Methods

3.1 Growth and Maintenance of Cells

A549, a human lung adenocarcinoma epithelial cell line, was passaged when confluency was reached, usually every 2–4 days.

3.2 Subcloning of Luciferase Reporter Gene into Gene Therapy Plasmid Vector pTarget

3.2.1 Cloning of Luciferase Reporter Gene from pPK-CMV-F3 Fusion Vector

1. Double restriction enzyme digestion of pPK-CMV-F3 fusion vector.
   (a) Combine the following reaction component at room temperature (*see* **Note 1**):

Water, nuclease-free	11 μL
10× FastDigest green buffer	2 μL
pPK-CMV-F3 fusion vector (~150 ng/μL)	5 μL
FastDigest XhoI	1 μL
FastDigest NotI	1 μL
Total volume:	20 μL

   (b) Mix gently and spin down.

(c) Incubate the reaction mix at 37 °C in a thermal cycler for 10 min. Then inactivate the enzyme by heating at 80 °C for 5 min.

2. Run the product on 0.5% GTG™ agarose gel to separate DNA fragments (*see* **Note 2**).
3. Excise the luciferase reporter gene band (*see* **Note 3**), and purify the DNA fragment with a gel purification kit (*see* **Note 4**).

3.2.2 pTarget Vector Ligation and Transformation

1. Briefly centrifuge the pTarget vector and control insert DNA tubes to collect the contents at the bottom of the tube.
2. Vortex the thawed T4 DNA ligase 10× buffer before use (*see* **Note 5**).
3. Prepare the following ligation reaction mix (*see* **Note 6**):

T4 DNA ligase 10× buffer	1 μL
pTarget (60 ng/μL)	1 μL
Control insert DNA (4 ng/μL)	2 μL
T4 DNA ligase (3 Weiss units/μL)	1 μL
Deionized water	5 μL
Total volume:	10 μL

4. Incubate above mix overnight at 4 °C (*see* **Note 7**).
5. Transformation of the ligation mix.
   (a) Prepare LB/Ampicillin/IPTG/X-Gal plates.
   (b) JM109 competent cells were thawed on wet ice. Mix the cells by gently flicking the tube.
   (c) Transfer 50 μL of cells into a sterile 1.5 mL microcentrifuge tube, and add 2 μL of above ligation mix to the same tube. Gently flick the tube to mix cells and ligation mix, and incubate the tube on ice for 20 min.
   (d) Heat shock at exactly 42 °C for 45 s (*see* **Note 8**).
   (e) Immediately place the tube on ice for 2 min.
   (f) Add 950 μL of SOC medium and incubate in the 37 °C shaker at 225 rpm for 1.5 h.
   (g) Spread 100 μL of above cell-containing culture medium on agar plate prepared in step a, and let the medium dry for 10 min.
   (h) Incubate the plate overnight at a 37 °C incubator (*see* **Note 9**).

6. Next day, pick up single white colonies, and incubate each colony in 3 mL of LB broth containing Ampicillin in the 37 °C shaker at 225 rpm overnight.
7. Next day, isolate recombinant plasmid using minipreps DNA purification system.
8. Make glycerol stocks.
   (a) Remove 425 μL of overnight culture and add 75 μL of sterile glycerol.
   (b) Snap freeze in dry ice/ethanol bath.
   (c) Store stocks in −80 °C.

3.2.3 Cloning of Luciferase Reporter Gene into pTarget Vector

1. Obtain linear pTarget vector by double restriction enzyme digestion of ligated pTarget vector using XhoI and NotI as described in Subheading 3.2.1.
2. Ligate linear pTarget with luciferase reporter gene (final concentration is ~ 50 ng) using T4 ligase as described in Subheading 3.2.2.
3. Transform ligation mix into JM109 cells as described in Subheading 3.2.2 to create pT.luc.

3.3 Cloning of Resveratrol Responsive-Promoter from PCR Product (For Natural Promoter) or Oligonucleotides (For Synthetic Promoter) into pT.luc/pTarget

3.3.1 Cloning of Natural Egr-1 Promoter into pT.luc/pTarget

1. Egr-1 primers were designed to amplify 460 bp of Egr-1 promoter upstream of start site.

   E460 infusion forward primer:
   5′- ATGGCTCGACAGATCTGCTTGGAACCAGGGAGAG-3′
   E460 infusion reverse primer:
   5’-TCAACGGGGCGGGCGATCGCGGCCTCATTTGAAGGGTCTGG-3’
2. Perform the following PCR reaction:

Temperature and time	Number of cycles
95 °C, 5 min	1
94 °C, 15 s; 53 °C, 60 s; 72 °C, 60 s	5
94 °C, 15 s; 60 °C, 60 s; 72 °C, 60 s	25
72 °C, 10 min	1
4 °C, ∞	–

3. Run the PCR product on GTG™ 0.5% agarose gel.
4. Excise the DNA band at ~460 bp and purify the fragment using Qiagen gel extraction kit.
5. Set up the following reaction to create linear pT.luc/pTarget:

pT.luc/pTarget (400 ng/μL)	10 μL
10× FastDigest green buffer	4 μL
FastDigest Bgl II	4 μL
FastDigest AsiSI	4 μL
Deionized water	18 μL
Total volume:	40 μL

6. Incubate the reaction mix in a thermal cycler at 37 °C for 15–20 min.
7. Cool and spin down the mix. Gel purify the linear vector as described in Subheading 3.2.1.
8. Prepare the following reaction to ligate promoter E460 with linear pT.luc/pTarget:

E460 DNA (25 ng/μL)	2 μL
Linearized pT.luc/pTarget (~50 ng/μL)	4.85 μL
Deionized water	3.15 μL
Total volume:	10 μL

9. Add 10 μL of ligation mix to one In-Fusion HD EcoDry pellet, and mix by pipetting up and down.
10. Incubate the reaction mix for 15 min at 37 °C, followed by 15 min at 50 °C, to construct pE460.luc/pE460, and then place on ice for transformation and plasmid purification as described in Subheading 3.2.2.

3.3.2 Cloning of Synthetic CArG Elements into pT.luc/pTarget

Synthetic CArG enhancers E5, E6, and E9NS were prepared using the following oligonucleotides (ODNs) [9]:

E5ODN1: 5′-[Phos]GATCT(CCTTATTTGG)$_5$GCGAT-3’
E5ODN2: 5′-[Phos]CGC(CCAATAAAGG)$_5$A-3’
E6ODN1: 5′-[Phos]GATCT(CCTTATTTGG)$_6$GCGAT-3’
E6ODN2: 5′-[Phos]CGC(CCAATAAAGG)$_6$A-3’
E9NSODN1: 5′-[Phos]GATCT(CCATATAAGG)$_9$GCGAT-3’
E9NSODN2: 5′-[Phos]CGC(CCTTATATGG)$_9$A-3’

1. ODNs were dissolved in deionized H_2O to obtain 200 μM stock concentration of each ODN.
2. Set up the following reaction for each pair of ODNs:

ODN1 (200 μM)	5 μL
ODN2 (200 μM)	5 μL
10× oligo annealing buffer	2 μL
Deionized water	8 μL
Total volume:	20 μL

3. Incubate the reaction mix at 95 °C for 4 min. Remove the reaction mix from heating and cool it for 5–10 min at room temperature. Spin to collect the droplets. The reaction mix is now 50 μM stock of double ODN (dODN).
4. Remove 1 μL of dODN stock (50 μM) and dilute to 100 μL with deionized H_2O. The mix is now 500 nM stock.
5. Dilute 500 nM stock to 10 nM working solution (1 μL of 500 nM dODN, 5 μL of 10× oligo annealing buffer and 44 μL of dH_2O).
6. Linearize pT.luc/pTarget with FastDigest Bgl II and AsiSI enzymes as described in Subheading 3.3.1 (*see* **Note 10**).
7. Prepare the following reaction mix:

dODN (10 nM)	10 μL
Linearized pT.luc/pTarget (50 ng/μL)	1 μL
T4 DNA ligase (3 Weiss units/μL)	2 μL
T4 DNA ligase 10× buffer	2 μL
Deionized water	5 μL
Total volume:	20 μL

8. Incubate the above mix at 22 °C for 1 h to ligate dODN with linear pT.luc/pTarget to create pE5.luc/pE5, pE6.luc/pE6 and pE9NS.luc/pE9NS, respectively. Then heat the mix at 70 °C for 5 min, followed by transformation and plasmid purification as described in Subheading 3.2.2.

3.4 Evaluating Promoters for Resveratrol Induction by DLR Assay

1. Seed A549 cells in Opti-MEM® containing 10% fetal bovine serum and 1% NEAA without antibiotics at a density of 3×10^4 in 100 μL per well in 96-well plates (*see* **Note 11**).
2. Next day, transfect cells using Lipofectamine™ 2000 and CombiMag reagent prepared as follows (*see* **Note 12**):
   (a) DNA solution: 0.2 μg plasmid is diluted to 25 μL Opti-MEM.
   (b) Lipofectamine™ 2000 solution: Gently mix the reagent before use. Dilute 0.3 μL of Lipofectamine™ 2000 in 25 μL of Opti-MEM in a new eppendorf. Incubate for 5 min at room temperature.
   (c) CombiMag reagent: vortex the reagent before each use. Transfer 0.2 μL of CombiMag into a new eppendorf (do not dilute with any culture medium).
   (d) Complexes formation: add 175 μL of diluted Lipofectamine™ 2000 to 175 μL of dilute plasmid containing tube. Mix gently and add the 350 μL of DNA/Lipofectamine™

2000 mixture immediately to the 1.4 μL of CombiMag containing eppendorf tube.

(e) Mix gently and incubate for 20–25 min at room temperature.

3. Add 50 μL of complexes dropwise onto the cells growing in serum-containing culture medium and homogenize by rocking the plate back and forth (total volume per well should be ~150 μL).
4. Incubate the cells 20 min on the magnetic plate at 37 °C in a CO_2 incubator, and then remove the magnetic plate (*see* **Note 13**).
5. Incubate cells at 37 °C in a 5% CO_2 incubator for 36–48 h.
6. Treat cells with 100 μM resveratrol. Incubate overnight.
7. Aspirate the media and add 30 μL of M-Per lysis solution per well and incubate on rocker for 15 min.
8. Read luminescence using DLR buffers.

3.5 Cloning of GADD45α Gene into pTarget Containing Resveratrol-Responsive Promoter

GADD45α ORF clone was amplified by high-fidelity hot-start PCR using Qiagen HotStar HiFidelity Polymerase Kit.

3.5.1 Amplification of GADD45α ORF Clone

1. Activate HotStar HiFidelity DNA Polymerase at 95 °C for 5 min.
2. Thaw 5× HotStar HiFidelity PCR Buffer, primer solution and 5× Q-solution. Mix the solutions completely before use.
3. Prepare the following reaction mix (*see* **Note 14**).

Components of reaction mix	Volume	Final concentration
5× HotStar HiFidelity PCR buffer	10 μL	1×
5× Q-solution	10 μL	1×
Forward primer (10 μM)	5 μL	1 μM
Reverse primer (10 μM)	5 μL	1 μM
HotStar DNA polymerase	1 μL	2.5 units
Water, nuclease-free	17 μL	–
GADD45α ORF clone (25 ng/μL)	2 μL	1 ng/μL
Total volume:	50 μL	

GADD45α Fusion Forward Primer (*see* **Note 15**):

5'-GGGCGAATTCGGATCCGCCACCATGACTTTGGAGGAATTCTCG-3′

GADD45α Fusion Reverse Primer:

5'-TTGGAATTCGCGGCCGCTCACCGTTCAGGGAGATTAAT-3'

4. Perform PCR reaction as described in Subheading 3.3.1.

3.5.2 Cloning of GADD45α ORF into pTarget, pE460, pE5, pE6, and pE9NS

1. Prepare the following reaction to linearize pTarget, pE460, pE5, pE6, and pE9NS, respectively:

10× FastDigest Green Buffer	2 μL
FastDigest BamH1	1 μL
FastDigest Not1	1 μL
Deionized water	11 μL
pTarget/pE460/pE5/pE6/pE9NS (~150 ng/μL)	5 μL
Total volume:	20 μL

2. Get linear vectors as described in Subheading 3.2.1.
3. Prepare the following cloning reaction solution:

Purified GADD45α ORF (25 ng/μL)	2 μL
Linear pTarget/pE460/pE5/pE6/pE9NS (10 ng/μL)	8 μL
Total volume:	10 μL

4. Mix 10 μL of above cloning reaction solution with one In-Fusion HD EcoDry pellet by pipetting up and down.
5. Incubate the reaction solution for 15 min at 37 °C, followed by 15 min at 50 °C, to create pT.G45α, pE460.G45α, pE5.G45α, pE6.G45α, and pE9NS.G45α.
6. Place the vectors on ice for following transformation and plasmid purification as described in Subheading 3.3.2.
7. Follow the PCR protocol described in Subheading 3.1.3 to verify GADD45α insert (*see* **Note 16**).

3.6 Assaying Growth Inhibition of Tumor Cells Treated with Suicide Gene Therapy

3.6.1 Cell Viability After Vector Transfection and Resveratrol Induction

1. Seed A549 cells at a density of 1×10^4 per well in 96-well plates.
2. Next day, transfect cells with pE460.G45α, pE5.G45α, pE6.G45α, or pE9NS.G45α as described in Subheading 3.4.
3. Day 3, treat cells with or without 100 μM of resveratrol for 24 h.
4. Add 20 μL of 5 mg/mL MTT to each well, and incubate for 4 h. Discard media and add 100 μL of Dimethyl Sulfoxide (DMSO).
5. Incubate on a shaker at room temperature for 15 min and read the absorbance at 570 nm.

3.6.2 Cell Cycle Analysis by Flow Cytometry

1. Seed A549 cells at a density of 5×10^5 in 2 mL per well in 6-well plates.
2. Next day, transfect cells with pE460.G45α, pE5.G45α, pE6.G45α, or pE9NS.G45α as described in Subheading 3.4.

3. Day 3, treat cells with or without 100 μM of resveratrol for 24 h.
4. Day 4, harvest cells with 500 μL of trypsin for about 3 min, and stop trypsinization by adding 500 μL of culture medium. Transfer cells to 1.5 mL epi tubes.
5. Centrifuge the cells at 1000 rpm (100 × *g*), 4 °C for 5 min and discard supernatant.
6. Resuspend cell pellet in 1 mL of cold PBS and pipette cells up and down gently to wash the cells. Repeat **step 5**.
7. Wash the cells as described in **step 6** again (*see* **Note 17**).
8. Add 500 μL of PBS containing 50 μg/mL PI, 50 μg/mL RNase, and 0.1% Triton X-100 and mix with cells by pipetting up and down gently. Incubate at room temperature for 15 min in the dark.
9. Perform flow cytometry using BD Accur™ C6 and analyze data by ModFit.

4 Notes

1. FastDigest enzymes bought from Thermo Scientific are able to digest DNA in 5–15 min. Any combination of restriction enzymes can work simultaneously in one reaction tube. FastDigest Green Buffer includes two tracking dyes (in a 1% agarose gel, blue dye migrates with 3–5 kb DNA fragments and yellow dye migrates faster than 10 bp DNA fragments). Therefore, the reaction product can be directly loaded on a gel for electrophoresis when FastDigest Green Buffer is used.
2. The whole targeted DNA fragment has to be recycles. In order to reduce the volume recycled gel, do not make the gel too thick, or use a wide and thin comb to make the wells. Make fresh electrophoresis buffer and gel every time to avoid DNA contamination. Use a DNA marker to find targeted DNA fragment since the size of the DNA fragment has already been known.
3. Use UV light to excise DNA band. Clean plastic wrap and blade without DNA contamination should be used in order to prevent exogenous DNA contamination.
4. If the gel is unable to dissolve, the heating time can be increased and the tube can be turned upside down several times to facilitate dissolution. Additionally, the gel can be cut into small pieces if it is too big.
5. T4 DNA Ligase 10× buffer should be aliquoted to avoid ATP degradation caused by multiple freeze-thaw cycles.

6. The molar ratio of PCR product to pTarget Vector may be optimized. Ratios from 3:1 to 1:3 are initial parameters. pTarget is 5.67 kb in size and is supplied at a concentration of 60 ng/μL. The appropriate amount of inserted PCR product can be calculated according to the equation provided in manufacturer's protocol.
7. The minimum time for incubation is 3 h. Shorter incubation time may result in fewer colonies.
8. Do not shake during heat shock.
9. Generally, white colonies contain inserts. Longer incubations or storing plates at 4 °C after 37 °C overnight incubation may facilitate blue/white screening.
10. DNA sequencing can be conducted with PCIN2 forward primer and CMV reverse primer to confirm the insert of E5, E6, and E9NS.

    PCIN2 forward primer: 5'-CCACCTCTGACTTGAGCG TCG-3'
    CMV reverse primer: 5'-GGTTCACTAAACGAGCTCTGC-3'
11. The cells should be 90–95% confluent before transfection. Do not use medium containing antibiotics for transfection because antibiotics may cause cell death.
12. Transfection can be conducted with or without CombiMag. CombiMag can improve transfection rates. The ratio of DNA, CombiMag and Lipofectamine™ may be optimized according to manufactures' instruction.
13. The medium can be changed at this step in order to improve transfection efficiency and minimize potential cytotoxicity. Leave the cells on the magnetic plate, remove the culture medium, and replace it with fresh culture complete medium.
14. HotStar HiFidelity DNA polymerase is inactive at room temperature. Therefore, it is not necessary to keep reaction tube on ice.
15. The Tm of GADD45α Fusion Forward Primer is 58.3 °C and is calculated from ATG onward. The Tm of GADD45α Fusion Reverse Primer is 58.5 °C and is calculated from TCA onward. The size of PCR fragment is 531 bp.
16. Colony PCR can be performed before plasmid purification to determine whether the colony has desired plasmid. Use a sterile pipette tip to pick up a colony, stir the tip around the bottom of a PCR tube, and put the tip into a culture tube containing 3 mL of LB broth and 100 μg/mL ampicillin. Bacteria in the PCR tube are used for colony PCR and bacteria remain on the tip are used for incubation to obtain more bacteria. Add the following reaction mix into the PCR tube

containing colony: 6 μL of nuclease-free water, 10 μL of 2× Taq, 2 μL of 10 μM forward primer, 2 μL of 10 μM of reverse primer. GADD45α Fusion primers are used to amplify GADD45α ORF. PCIN2 forward primer and CMV reverse primer are used to amplify natural Egr-1 promoter or synthetic CArG elements. Amplification parameters are: denaturation at 94 °C for 10 min, followed by 25 cycles of 94 °C, 30 s; 52 °C, 90 s; 72 °C, 90 s, and full extension at 72 °C for 5 min. GADD45α is ~511 bp in size. Natural Egr-1 promoter and synthetic CArG elements are ~277 bp in size.

17. Fixation can be added before proceeding cells to staining and analysis. For fixation, add 300 μL of cold PBS and pipette up and down to resuspend cells and make single cells. Then add 700 μL of cold ethanol dropwise while vortexing. Fix cells in this 70% ethanol solution for at least 30 min. The cells can remain in 70% ethanol for up to 1 week. Before staining, spin the fixed cells at 2000–2200 rpm (380–460 × *g*) for 10 min and wash twice with PBS. A higher speed of centrifuge is needed to prevent significant cell loss.

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Chapter 10

Noninvasive Monitoring of Suicide Gene Therapy by Using Multimodal Molecular Imaging

Bryan Holvoet, Cindy Leten, Christophe M. Deroose, and Uwe Himmelreich

Abstract

Cells expressing suicide genes can be used as therapeutic vehicles for difficult-to-treat tumors, for example, if stem cells are used that are able to track infiltrating tumor cells. An alternative application of suicide gene expression is their use as a safety switch in regenerative medicine where the presence of a few pluripotent stem cells could potentially cause unwanted side effects like the formation of teratoma. One potential bottleneck of these applications is that information on the initiation of cell suicide is needed early on, for example, when therapeutic cells have reached infiltrating tumor cells or when teratomas are formed. Therefore, in vivo imaging methods are needed that provide information on target location, (stem) cell location, (stem) cell viability, pathology, and suicide gene expression. This requires multimodal imaging approaches that can provide this information longitudinally and in a noninvasive way. Here, we describe examples of how therapeutic cells can be modified so that they express a suicide gene and genes that can be used for in vivo visualization.

Key words Suicide gene, Magnetic resonance imaging, Positron emission tomography, Bioluminescence imaging, Stem cells

1 Introduction

Stem cells have gained increased attention over the last decade not only in regenerative medicine but also in the field of tumor therapy [1–3]. Hereby, their potential to track infiltrating tumor cells and to form gap junctions is essential. The expression of a suicide gene by the therapeutic stem cells not only results in suicide of the therapeutic cells but also of the tumor cells, which is also called bystander killing effect [4–7]. The selection of the most suitable stem cell type depends on the application (regenerative medicine, oncology, etc.) but also intrinsic stem cell characteristics.

For regenerative applications of stem cells, their potential to differentiate into a broad variety of cell types is not only essential for tissue regeneration but also poses potential safety risks as the transplanted cells can proliferate longitudinally and can also differentiate

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into cell types that might cause unwanted side effects. Unfortunately, differentiation protocols of embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) do not yield 100% purity and still have the risk of containing undifferentiated cells in the mixture and hence forming neoplastic lesions. These safety risks include the formation of teratomas by embryonic stem cells (ESC) [8] and induced pluripotent stem cells (iPSC) [9] or the formation of yolk sac-like tumors by bone marrow-derived stem cells [10]. Suicide gene expression of therapeutic stem cells has been suggested as a safety switch in case of formation of tumorigenic masses [10].

Among various suicide genes that have been suggested in experimental models, the Herpes Simplex virus-thymidine kinase (HSV-TK) and the cytosine deaminase are the most extensively studied systems [11]. Hereby, either the pro-drug ganciclovir (GCV) is converted by HSV-TK into GVC-monophosphate, which is transformed into the cytotoxic GCV-triphosphate, or 5-fluorocytosine is converted by the cytosine deaminase gene into the cytotoxin cytotoxic 5-fluorouracil, respectively. Among the more recently suggested approaches is the overexpression of the somatostatin receptor type 2 (hSSTr2) in combination with ^{177}Lu-DOTATATE therapy [12], which has already demonstrated promising results for the treatment of neuroendocrine tumors [13].

For most applications of suicide gene expressing cells, the administration of a co-drug or other therapeutic compounds is required to trigger cell killing. The time point after cell engraftment for initiating this cell suicide is highly variable and depends on

1. The cells reaching their target (tumor therapy), or
2. The occurrence (or absence) of unwanted side effects (teratoma formation for regenerative applications).

Therefore, it is necessary to monitor both pathology and the location of therapeutic cells longitudinally and in a noninvasive manner to be able to decide on co-drug administration. Many previous studies have focused on the assessment of the treatment rather than on guiding therapy by using in vivo imaging methods [14]. Hereby, magnetic resonance imaging (MRI) and positron emission tomography (PET) are the most frequently used methods in a clinical setting. In preclinical research, bioluminescence imaging (BLI) is frequently used in addition to PET and MRI [15, 16]. For the guidance of therapy, cell imaging methods can be used to optimize the therapeutic response and to identify therapeutic windows. In order to visualize cells against the background of host tissue, either contrast agents or tracers are used for prelabeling cells or cells are genetically modified to express an imaging reporter gene [5, 15–19]. In order to overcome disadvantages of individual imaging methods, combining different techniques in

multimodal approaches has become common practice in preclinical research [1, 5, 7]. For example, MRI has an excellent resolution and provides detailed information on anatomical background. Using superparamagnetic iron oxide particles for cell labeling offers a method to follow those prelabeled cells longitudinal with high resolution [1, 7, 17, 20]. However, MRI provides only limited information on cell viability or suicide gene expression. BLI of luciferase expressing cells can provide information on the viability of cells and possibly the expression of a suicide gene [7, 10, 12, 15, 16]. PET is a highly sensitive method that provides information on suicide gene expression and indirectly on cell viability [5, 12, 16, 18, 19, 21, 22]. Combining those imaging methods provides a comprehensive picture of treatment models that involve suicide gene expressing stem cells. For example, the cell location, cell viability, and suicide gene expression can be monitored by MRI, BLI, and PET ([^{18}F] hydroxymethyl-butyl-guanine, FHBG) by using iron oxide labeled transgenic cells that express firefly luciferase and the HSV-tk gene, respectively [7]. If these cells are used in a preclinical model of tumor treatment, the growth/shrinkage of the tumor can be monitored by MRI and the viability of *Renilla* luciferase expressing tumor cells by BLI, respectively [7].

In this chapter, we focus on how to generate transgenic cells to express a suicide gene and an imaging reporter gene and how to image those cells in vivo in preclinical disease models using a recently introduced approach [12].

2 Materials

2.1 Cell Culture

1. 24- and 6-well plates (Corning, Corning, NY, USA).
2. Cell culture laboratory, including normoxic cell culture incubator (37 °C; 5% CO_2), cell culture hood, laminar flow, sterile pipettes, and labware.
3. Colony picking needle.
4. Benchtop centrifuge.
5. Warm water bath.
6. Vortex.
7. Human ESC line H9 (WA09) (WiCell Research Institute, Madison, WI, USA).
8. mTesR1 cell culture medium (StemCell Technologies, Vancouver, Canada).
9. Enzyme-free dissociation reagent (We have used dReLeSR™ (StemCell Technologies)).
10. hESC-qualified Matrigel (Corning, NY, USA).

11. Dulbecco's modified Eagle medium (DMEM)/nutrient F-12 (DMEM/F12, Sigma-Aldrich, St. Louis, MO, USA).
12. RevitaCell™ Supplement (Thermo Fisher Scientific, MA, USA).

2.2 Genome Editing of Human ESC

1. As described in a previous publication [23].
2. Donor DNA plasmid containing puromycin resistance gene and a reporter gene that can function both as imaging and suicide gene. (We have used a plasmid containing hSSTr2 suicide gene (^{177}Lu-DOTATATE and ^{68}Ga-DOTATATEPET) and firefly luciferase (Fluc, for BLI, *see* Fig. 1)).
3. Puromycin (Sigma-Aldrich).

2.3 Imaging Equipment

1. Bioluminescence Imaging system (We have used an IVIS Spectrum (PerkinElmer, Waltham, MA, USA)).
2. Gamma counter (Wizard, Perkin Elmer).
3. Small-animal PET system (We have used a Focus 220 micro-PET system (Siemens Medical Solutions, Knoxville, TN, USA)

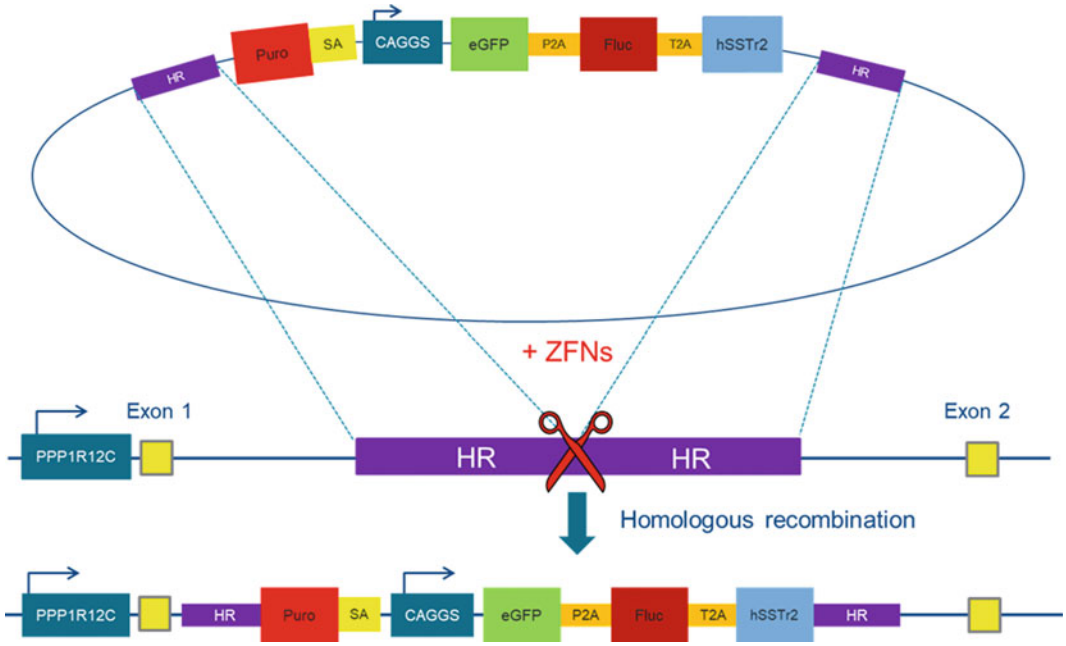

Fig. 1 Schematic representation of gene editing approach and donor DNA plasmid for multimodal imaging. The donor DNA plasmid contains homology regions (HR) to an intron region of the protein phosphatase 1 regulatory subunit 12C (PPP1R12C) gene. After a double-strand DNA break, induced by zinc finger nucleases (ZFNs), site-specific integration of the triple imaging reporter gene construct occurred via homologous recombination. The triple imaging reporter gene construct contained enhanced green fluorescent protein (eGFP), Fluc and hSSTr2. The puromycin resistance gene (Puro) was placed under control of the endogenous PPP1R12C promoter to enhance selection of correctly targeted cells. SA: splice acceptor

or a Bruker Albeira Si PET insert for a 7 Tesla Bruker Biospec 70/30 MRI scanner (Bruker Biospin, Ettlingen, Germany)).

4. Magnetic Resonance Imaging system (We have used a Bruker BioSpec 70/30 MRI scanner (Bruker Biospec)).
5. Isoflurane sedation system.
6. Insulin syringes.

2.4 Substrate and PET Tracers

1. D-Luciferin (126 mg/kg in PBS for in vivo, 15 mg/L for in vitro (Promega, Madison, WI, USA)).
2. ^{68}Ga-DOTATATE.
3. ^{177}Lu-DOTATATE.

3 Methods

3.1 Validation of Functional Reporter Gene Incorporation After Genome Editing

All cell culture experiments need to be performed with sterile material and under a sterile laminar flow unless specified otherwise.

1. After genome editing, as described [23], the functionality of the reporter gene was assessed (*see* **Note 1**). Hereby, 24 well plates were coated with 250 μL of qualified matrigel (diluted 1:100 in DMEM:F12) and placed for at least 30 min in an incubator at 37 °C (*see* **Note 2**). During this period, mTeSR1 media was placed at room temperature (RT) under the flow in an aliquot (*see* **Note 3**).
2. After the incubation period has finished, the excess of qualified matrigel was removed from the wells and the mTeSR1 medium at RT was put in the wells.
3. The genome edited ESC were dissociated from the plate by washing the cells twice with PBS. RelesR™ was added to the cells (sufficient amount to cover the surface of the well). ReLesR™ was aspirated within 1 min (*see* **Note 4**). The plate was incubated at 37 °C for 5–7 min. Afterward, mTeSR1 medium was added. The colonies can be detached by placing the plate on a vortexer at 1200 rpm (80 × *g*) for 2 min at RT. Transfer the detached cells to a sterile 15 mL tube and dissociate colonies by gentle pipetting up and down (~3 times) until colonies have a size of 50–200 μm. Plate the cell mixture onto the previously coated wells containing mTeSR1. Place the plate in the incubator and move the plate quickly back-and-forth and side-to-side to equally distribute the cells. Do not move the plate in the first 24 h. Change mTeSR1 medium at RT daily. *See* **Notes 5–8**.
4. Let the cells grow until they reach 100% confluence before starting the in vitro validation of the reporter gene. For firefly luciferase (Fluc), aspirate the media, rinse, and incubate the

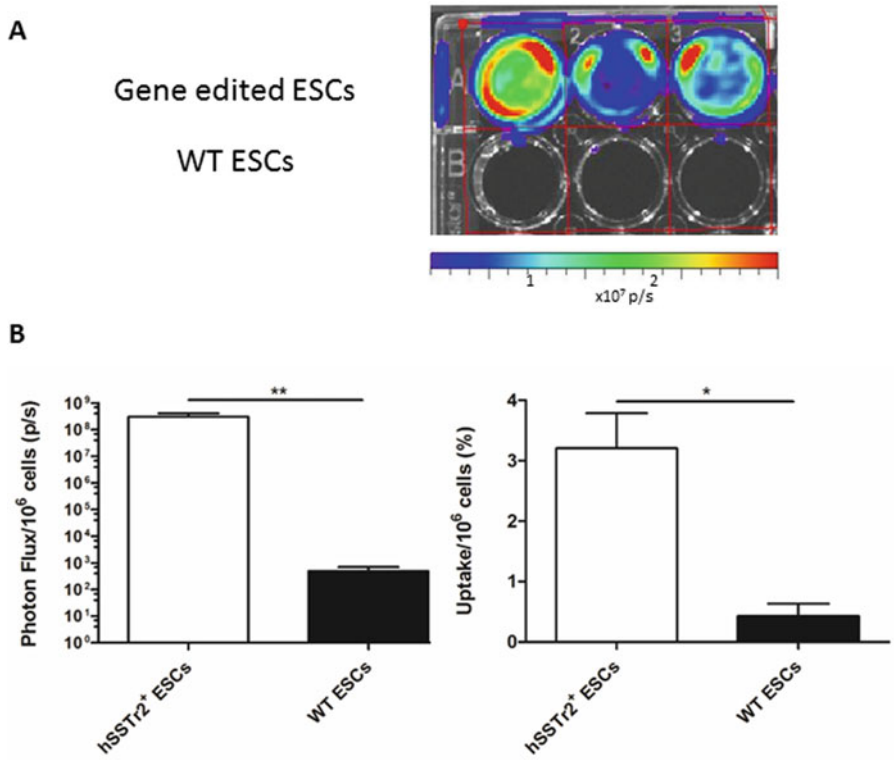

Fig. 2 Functional imaging reporter gene expression after gene editing. Robust BLI signal was observed in gene edited ESCs but was absent in wild-type (WT) ESCs (**a**). Significantly higher ^{68}Ga-DOTATATE uptake in hSSTr2^{+} ESCs was seen compared to WT ESCs (*$p < 0.05$) (**b**)

cells with D-luciferin (15 mg/L). Place the 24 well plate into the appropriate plate holder of the bioluminescence system (IVIS Spectrum). The settings should be on luminescence and auto-detection of the binning and exposure time to allow optimal detection of the light signal without saturation. Afterward, a 24-well grid was properly placed over the photographic picture and superimposed BLI image to determine the photon per seconds that are released from each well (*see* Fig. 2). Measurements should be re-performed until maximal BLI signal was obtained. Afterward, cells were collected and counted to correct the BLI signal to the total amount of viable cells. For uptake experiments, aspirate the media, rinse, and incubate the cells with ^{68}Ga-DOTATATE(0.74 MBq/mL) for 1 h (or shorter depending on the half-life of the tracer). The next step does not need to be performed under a laminar flow: Supernatant was collected and cells were rinsed three times with ice-cold PBS. These rinsing fluids were collected in the same Eppendorf as the supernatant. Afterward, cells were collected in a separate Eppendorf and all samples were counted on the gamma counter. After gamma counting, the amount of cells in each

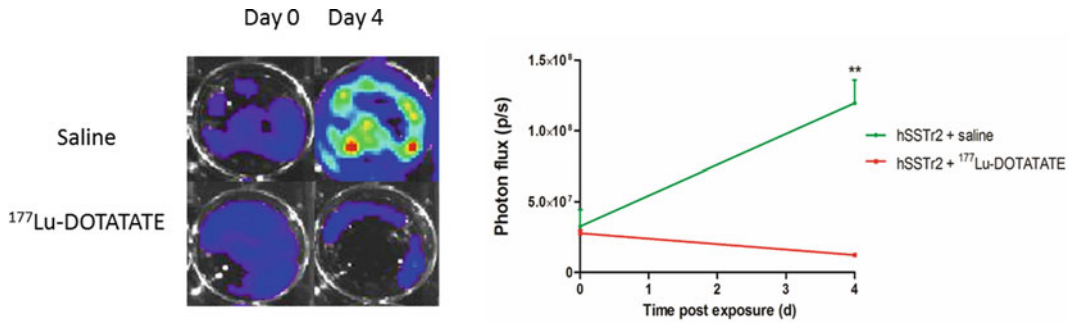

Fig. 3 Selective killing of hSSTr2$^+$ ESCs after in vitro exposure to ^{177}Lu-DOTATATE. Baseline BLI was performed before exposure to either saline or ^{177}Lu-DOTATATE and 4 days later a BLI scan was re-performed. hSSTr2$^+$ ESCs exposed to saline had an increase in BLI signal over time (cell proliferation), while hSSTr2$^+$ ESCs exposed to ^{177}Lu-DOTATATE had a reduction in BLI signal (cell killing)

Eppendorf was determined to normalize the uptake percentage to the total count of cells. *See* **Note 9**.

3.2 In Vitro Testing of Suicide Gene

1. Cells were plated in 24 wells as previously described; the only difference is that cells do not need to be confluent, but should be around 25% confluence at the start of the experiment. A baseline in vitro BLI scan (as previously described) was performed before adding ^{177}Lu-DOTATATE or PBS. Afterward, cells were incubated with mTeSR1 containing ^{177}Lu-DOTATATE (activity determined by previous pilot experiment or immediate by an activity escalation study) or mTeSR1 containing same volume of PBS. On consecutive days, the same plates were tested for their in vitro BLI signal and noninvasive quantitative information on cell death is obtained (Fig. 3). Additional terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and ki67 staining can be performed to validate previously obtained results. *See* **Note 1**.

3.3 In Vivo Testing of Suicide Gene Therapy

1. Cells were expanded in vitro until sufficient cell numbers were obtained for in vivo transplantation. Cells were detached and made a single cell suspension. Cells were counted and one to two million genome edited cells were added into a 2 mL Eppendorf tube. Cells were centrifuged (5 min 1200 rpm, 80 × *g*) and supernatant was discarded. Cells were resuspended in 1:1 PBS and qualified matrigel with supplement RevitaCell (1/100). The total volume in our experiment was 100 μL. *See* **Note 1**.
2. As the cells are from human origin, immune deficient mice were used. Animals were sedated with Isoflurane (1–3%) in 100% oxygen (airflow 1–2 L/min). The cell-matrigel mixture was resuspended before injection with a P100 pipet to generate a homogenous cell mixture. This mixture was injected subcutaneously via an insulin syringe.

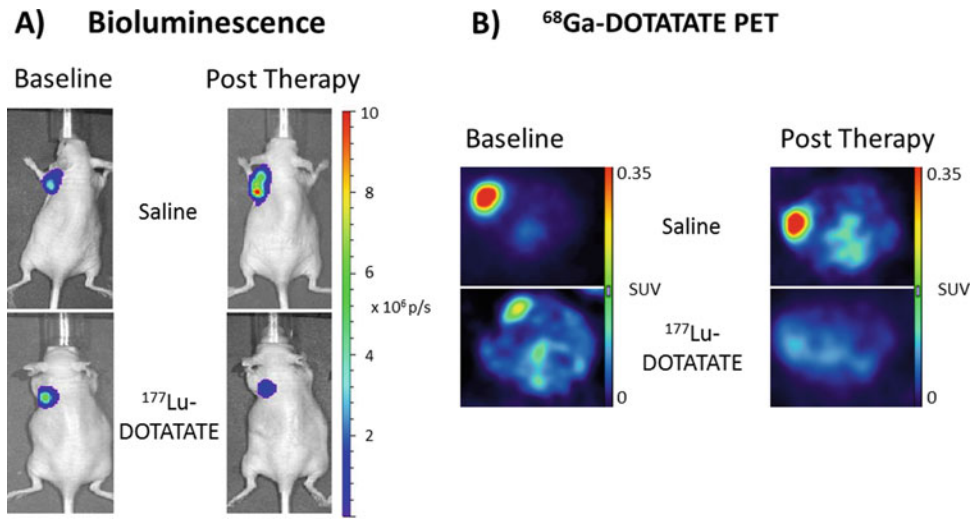

Fig. 4 Potential of hSSTr2 as an imaging and suicide reporter gene in vivo. Nude mice were subcutaneously engrafted with hSSTr2^{+} ESCs. Before administration of either saline or ^{177}Lu-DOTATATE a baseline BLI was taken, 20 days later a new BLI scan was performed. Saline treated animals had an increase in BLI signal and volume, while ^{177}Lu-DOTATATE treated animals had a reduction in BLI signal (**A**). The same animals were also monitored before and after therapy with ^{68}Ga-DOTATATE PET and transverse images clearly indicated focal accumulation of ^{68}Ga-DOTATATE at both time points in saline treated animals, while in ^{177}Lu-DOTATATE treated animals the focal ^{68}Ga-DOTATATE accumulation was lost at the post therapy time point (**B**)

3. Cell injected animals were monitored via BLI a couple of days after injection to visualize successful cell engraftment. This was performed by sedating the animals with Isoflurane (1–3%) in 100% oxygen and placing them inside the IVIS Spectrum. Afterward, D-luciferin was administered (intra peritoneal, subcutaneous or intravenous; 126 mg/kg, *see* **Note 2**) and BLI signals were acquired until maximal BLI signal was obtained. Region of interests were manually placed around the cell injection sites. Proper teratoma formation and hence increase in BLI signal can be followed with BLI over time (Fig. 4).
4. Additionally, animals were scanned with MRI to visualize teratoma development.
5. Teratoma growth was furthermore macroscopically monitored via caliper measurements.
6. After a period of time, a clear macroscopic visible teratoma was present and a baseline BLI scan and small-animal PET scan was performed (*see* **Note 3**). For the small-animal PET scan, animals were again sedated with (1–3%) Isoflurane and IV injected with 5.5–7.4 MBq ^{68}Ga-DOTATATE. 1 h after injection a static 20 min PET scan was performed. *See* **Note 4**.
7. The next day animals either received daily PBS or ^{177}Lu-DOTATATE (55 MBq) and were monitored via BLI, MRI,

small-animal PET, and macroscopic measurements via caliper for the effect on teratoma size (Fig. 4).

8. After a determined end-point, teratomas were isolated and additional histological examination was performed (H&E, TUNEL, ki67 staining).

4 Notes

To Subheading 3.1

1. To select for the correctly targeted hESCs, it is better to determine the concentration of puromycin on your cells (in our case 1 μg/mL) that result in 100% cell death after 3–5 days in non-puromycin resistance gene expressing cells. If all cells die before 2 days it might be that the concentration is even too toxic for cells with the puromycin resistance gene. If the cells do not tolerate antibiotic selection it is also feasible to include a fluorescent protein in the reporter gene construct to select via fluorescence-associated cell sorting (FACS). Take into account that an ROCK inhibitor (in our case RevitaCell; 1/100) needs to be added to the medium to minimize the impact of stress upon single cell passaging of pluripotent stem cells.
2. Do not let the qualified matrigel stand too long (>3 h) in the incubator as it can dry out.
3. Do NOT warm up mTesR1 in a warm water bath.
4. Incubation time with ReLeSR™ needs to be optimized for each cell line.
5. If you want to keep cells in culture also foresee extra plate as the cells used for the reporter gene validation will not be sterile anymore.
6. Do not touch the plate within the first 20 h after plating, as it will disturb the cell attachment. Change the media every 24 h, leave the light out in the flow as mTeSR1 is not stable.
7. Before starting with the in vitro validation of Fluc, already start by initializing the IVIS Spectrum as it needs time until the charge-coupled device of the camera is cooled until appropriate temperature to reduce thermal noise (−90 °C). If you start first with cell culture work the majority of D-luciferin will already be converted to oxyluciferin and you will miss the peak in BLI signal. Cover the tube with D-luciferin with, for example, aluminum foil to prevent light exposure as D-luciferin is unstable upon light exposure.

8. Either black well plates should be used or black ink can be added to the outsides of the well to prevent light scattering from one well toward the other.
9. Always wear a dosimeter if you work with radioactivity, make a lead wall when performing the uptake experiment and put absorbent paper on the bench to prevent radioactive contamination.

To Subheading 3.2

1. First test in vitro different concentrations of ganciclovir on cells expressing hsv-tk1 and non-genome edited hESCs to determine the optimal concentration that hsv-tk1 expressing cells die over time while it should not be toxic to non-genome edited hESCs.

To Subheading 3.3

1. Qualified matrigel should be thawed on ice and kept always on ice to prevent solidification of the matrigel. After adding the matrigel, cells with matrigel should be transported on ice. Also upon injection of the cells place the syringes on ice before to prevent the matrigel to solidify inside the syringe.
2. Intravenous administration achieves the highest bloodstream concentrations of D-luciferin, and might be advised for cell visualization in the brain. However, it is associated with some disadvantages as the peak BLI signal is immediate after substrate infusion and in the IVIS Spectrum it is difficult to inject as it is a dark chamber so the time to transport the animal from IV injection site toward the IVIS spectrum should be as short as possible. Furthermore, it should be performed by an expert in IV injection as partial injection with result in high BLI signal variabilities. In addition, the injection has to be performed quickly as the substrate is unstable in a light environment. Therefore, intraperitoneal or subcutaneous administration of D-luciferin can be used. The disadvantage is that it takes longer until the maximal BLI signal is obtained, roughly 20 min. The main importance is to consistently use the same D-luciferin administration procedure.
3. It is important to perform the BLI scan before the small-animal PET scan or perform the BLI scan after the radionuclide had decayed as the remaining radionuclide can produce Cherenkov luminescence [12, 18].
4. Similar approaches can also be taken using different suicide and imaging genes. For example, the expression of HSV-TK can be used to induce cell suicide after administration of ganciclovir. HSV-tk phosphorylates GVC. GVC-triphosphate is—in contrast to GVC—a cytotoxin. HSV-TK also results in intracellular accumulation of the PET tracer [^{18}F] FHBG, which can be used for in vivo PET imaging. The example in Fig. 5 shows

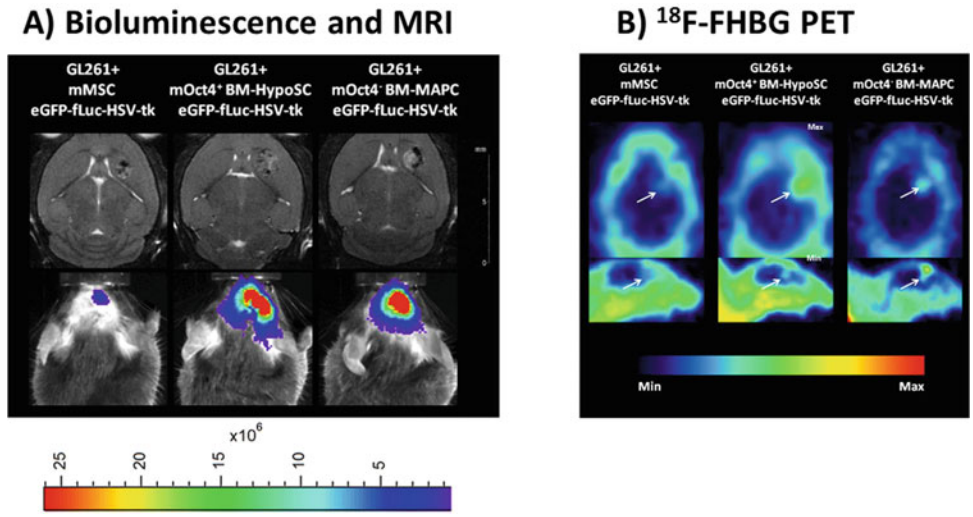

Fig. 5 Suicide and imaging reporter gene expression for monitoring tumor therapy in vivo. HSV-TK acts as a suicide gene by phosphorylating the otherwise nontoxic GCV into the cytotoxin GCV-triphosphate. It also results in the intracellular accumulation of the PET tracer [^{18}F] FHBG. Due to the capacity of stem cells to track infiltrating brain tumor cells and to form gap-junctions, they can be used as vehicles for bystander killing of tumor cells (*see* also [1, 5, 7]). Images below show a GL261 brain tumor model in mice that received HSV-TK and Fluc expressing stem cells. MRI and BLI measurements of GL261 brain tumor bearing mice injected with 5×10^5 mouse mesenchymal stem cells (mMSC's), mouse bone marrow-derived stem cells with high Oct 4 expression (mOct4$^+$ BM-HypoSC's) or mouse bone marrow-derived multipotent adult progenitor cells with low Oct4 expression (mOct4$^-$ BM-MAPC's) showed that stem cells could be detected by MRI after labeling with superparamagnetic iron oxide nanoparticles (SMG^2-mPEGSi, hypointense (dark) contrast around tumor lesion) and BLI (fLuc). Tumor growth/therapy can be monitored by MRI (hyperintense lesion) (**a**). Based on stem cell location surrounding the tumor lesion, decisions can be made on co-drug administration (GCV) and initiation of suicide therapy. BLI provides information on stem cell viability (success of suicide). [^{18}F] FHBG PET scan on the same animals mentioned in (**a**) showed that HSV-TK expressing stem cells could also be detected using PET (**b**). Hereby, PET provides information on the expression of the suicide gene relative to the tumor location (detected by MRI)

brain tumor bearing mice treated with different types of stem cells expressing fLuc for BLI and the suicide gene HSV-TK, also used for [^{18}F] FHBG PET. MRI was acquired for monitoring tumor growth/treatment and the location of iron oxide labeled stem cells (seen as hypointense contrast in Fig. 5).

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Chapter 11

In Vitro Assay for the Evaluation of Cytotoxic Effects Provided by a Combination of Suicide and Killer Genes in a Bicistronic Vector

Alexey A. Komissarov, Sergey V. Kostrov, and Ilya V. Demidyuk

Abstract

When using bicistronic expression constructs the issue arises concerning proper evaluation of the cytotoxic efficiency of a combination of therapeutic genes. For this purpose, an approach can be applied based on the transient transfection of cultured human cells with a specifically designed set of mono- and bicistronic expression constructs and on the comparison of their cytotoxic effects. Here the application of this approach is described using an example of the evaluation of the combined cytotoxic action of bifunctional yeast cytosine deaminase/uracil phosphoribosyltransferase fusion protein (FCU1) and hepatitis A virus 3C protease in a bicistronic plasmid construct.

Key words Suicide gene therapy, Bicistronic expression vector, Transient transfection, Colorimetric cell viability assay, Bifunctional yeast cytosine deaminase/uracil phosphoribosyltransferase fusion protein FCU1, Human hepatitis A virus 3C protease

1 Introduction

Gene therapy represents a promising approach to the treatment of a wide range of human pathological conditions [1, 2] including oncological diseases [3]. Cancer gene therapy involves different strategies, suicide gene therapy being one of the most promising among them. This strategy consists of introducing into tumor cells of a gene providing for the conversion of a nontoxic prodrug into lethal compounds [4, 5]. Despite the success of in vivo studies using several animal models, the application of suicide gene therapy systems to patients has not yet reached desirable clinical significance. Accordingly, attempts to improve these systems represent an actively developing trend.

One of the approaches to improve suicide gene therapy systems consists in combining them with other genes. Such combination

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may result in synergistic enhancement of the therapeutic response compared to the individual application of the genes [6–18]. To provide simultaneous expression of two transgenes in a cell, bicistronic genetic constructs seem to be preferable to monocistronic systems, since they are notably more compact, which often improves genetic material delivery into cells and nuclei [19–21]. This is especially important when using nonviral gene delivery systems which possess better safety profiles and easier large scale production, but lower efficiency of delivery compared to viruses [22–24].

When using bicistronic expression constructs the issue arises concerning proper evaluation of the cytotoxic efficiency of a combination of therapeutic genes. In this case it is required to characterize the cytotoxic action mediated by each of the genes used and to estimate the mutual influence of their protein products. For this purpose an approach can be applied based on the transient transfection of cultured human cells with a specifically designed set of mono- and bicistronic expression constructs and on the comparison of the cytotoxic effects provided by them using colorimetric cell viability assays [25]. Here the application of the given approach is described on the example of the evaluation of the combined cytotoxic action of bifunctional yeast cytosine deaminase/uracil phosphoribosyltransferase fusion protein (FCU1) [16] and hepatitis A virus 3C protease (3C) [26] in a bicistronic plasmid construct.

2 Materials

2.1 Expression Constructs

Set of the plasmid constructs (*see* Fig. 1 and **Note 1**) that were amplified in *Escherichia coli* TG1 cells, purified using Plasmid Miniprep kit (Evrogen, Russia), and stored at −20 °C (*see* **Note 2**).

2.2 Cultivation and Transfection of Human Cells

1. Tissue culture treated 96-well plates.
2. CO_2 incubator.
3. Automatic cell counter or hemocytometer.
4. Laboratory microscope.
5. Laboratory thermostat set at 37 °C.
6. Growth medium appropriate for the cell lines used. For HEK293 and HeLa cells it is DMEM/F-12 (1:1) medium supplemented with 10% fetal bovine serum (FBS) and 0.3 mg/mL glutamine. Store the complete growth medium and components at 4 °C.

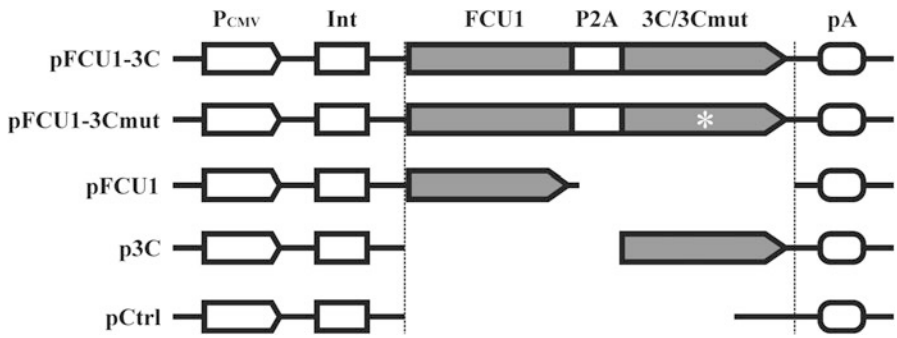

Fig. 1 Schematic representation of the expression cassettes in the plasmids used. The regions lying below each other represent identical nucleotide sequences. Vertical dotted lines delimit the region where the used constructs vary. Gaps correspond to deletions in pFCU1, p3C, and pCtrl. P_{CMV}, cytomegalovirus immediate-early enhancer/promoter; Int, chimeric human b-globin/IgG intron; FCU1, bifunctional yeast cytosine deaminase/uracil phosphoribosyltransferase fusion gene; P2A, porcine teschovirus-1 2A-peptide coding sequence; 3C/3Cmut, 3C protease (intact or mutant, respectively) of human hepatitis A virus, the asterisk (*) indicates the Cys172-Ala substitution; pA, SV40 late polyadenylation signal. Reproduced from ref. [25] with permission from Springer

7. TurboFect transfection reagent (Thermo Fisher Scientific, USA) and Gibco OptiMEM medium with GlutaMAX supplement (Thermo Fisher Scientific, USA) or another serum-free medium proper for transfection mixture preparation (*see* **Note 3**).
8. Dry 5-fluorocytosine (5-FC) powder.

2.3 Cell Viability Assay

1. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4 (*see* **Note 4**).
2. CellTiter 96 AQueous One Solution assay kit (Promega, USA).
3. Microplate reader able to measure the light absorption at 490 nm.

3 Methods

Carry out all procedures at room temperature unless otherwise specified. All procedures concerning cell cultures should be done according to the standard aseptic technique.

3.1 Determination of the Cell Seeding Conditions for Transfection

1. Plates 5000, 10,000, 20,000, 30,000, 40,000 and 50,000 HEK293 or HeLa cells (*see* **Note 5**) in 100 μL of preincubated at 37 °C growth medium per well of 96-well plate. Incubate for 20–24 h at 37 °C in humidified atmosphere with 5% CO_2.

2. Prepare transfection mixtures for all the constructs just before the transfection following the manufacturer's protocol (*see* **Note 6**). Add the mixtures dropwise into appropriate wells and incubate the plate at 37 °C in humidified atmosphere with 5% CO_2 for 24 h.
3. Assay the cell viability in the plate (*see* Subheading 3.2).
4. Analyze the results of the viability test and choose the optimal number of cells to be plated in a well for each cell line (*see* **Note 7**).

3.2 Colorimetric Cell Viability Assay

1. Remove the medium from the plate and gently add 100 μL of PBS (*see* **Note 8**) per well along the well wall.
2. Add 20 μL of CellTiter 96 AQueous One Solution Reagent (Promega, USA) (*see* **Note 9**) per well using multichannel pipette or automatic injector (if provided with the microplate reader). Avoid vigorous adding since it may result in monolayer damaging and producing of bubbles in the wells.
3. Incubate the plate at 37 °C in humidified atmosphere with 5% CO_2 for 1–2 h recording the absorbance at 490 nm (A_{490}) in wells every 10–20 min starting immediately after CellTiter 96 AQueous One Solution Reagent addition, i.e., from zero time point (*see* **Note 10**).
4. Analyze the results obtained. Choose the time point where the A_{490} values are in a linear range for all wells; subtract the background (A_{490} value at zero time point) for each well.

3.3 Evaluation of Sensitivity of the Transfected Cells to the Prodrug

1. Plate 20,000 of HEK293 or HeLa cells (or another experimentally defined quantity, *see* Subheading 3.1) in 100 μL of pre-incubated at 37 °C fresh growth medium per well of 96-well plate. Incubate for 20–24 h at 37 °C in humidified atmosphere with 5% CO_2.
2. Prepare transfection mixtures for all the constructs just before the transfection, following the manufacturer's or experimentally optimized protocol. Add the mixtures dropwise into corresponding wells and incubate the plate at 37 °C in humidified atmosphere with 5% CO_2 for 24 h.
3. Change the medium into the plate (*see* **Note 11**) with the fresh one without the prodrug or with different concentrations of 5-FC ranging from 10^{-3} to 10^{4} μM in increments of tenfold. Examine the wells using a microscope on the absence of microbial contamination and monolayer damage. Repeat these procedures every 48 h until the experiment reaches 168 h post transfection (p.t.) or another required time point (*see* **Note 12**).

4. Assay the cell viability as described in Subheading 3.2. Normalize the obtained A_{490} values for the cultures exposed to various 5-FC concentrations against the values for the corresponding cultures without the prodrug administration (*see* **Note 13**).
5. Analyze the results (Fig. 2). Compare the obtained cell viability curves for all the constructs (*see* **Note 14**).
6. If it is required perform the experiment again using a shorter concentration range of the prodrug and/or smaller increments. Otherwise just repeat the experiment to gather statistics.

3.4 Time Course Analysis of Cytotoxic Effects

1. Plate 20,000 HEK293 or HeLa cells (or another experimentally defined quantity, *see* Subheading 3.1) in 100 μL of pre-incubated at 37 °C fresh growth medium per well of 96-well plates (*see* **Note 15**). Incubate for 20–24 h at 37 °C in humidified atmosphere with 5% CO_2.
2. Prepare transfection mixtures for all the constructs just before the transfection following the manufacturer's or experimentally optimized protocol. Add the mixtures dropwise into corresponding wells and incubate the plate at 37 °C in humidified atmosphere with 5% CO_2 for 24 h.
3. Assay the cell viability in one plate as described in Subheading 3.2. Normalize the obtained A_{490} values for the transfected cultures against that for the untransfected ones.
4. Change the medium in the remaining plates with the fresh one without the prodrug or with 100 μM 5-FC (*see* **Note 11**). Examine the wells using a microscope on the absence of microbial contamination and monolayer damage.
5. Repeat **steps 3** and **4** every 48 h until the experiment reaches 168 h p.t. or another determined time point (*see* **Note 16**).
6. Analyze the results obtained (Fig. 3). Compare the cell viability profiles for all constructs (*see* **Note 17**).
7. If it is required perform the experiment again using a different prodrug concentration. Otherwise just repeat the experiment to gather statistics.

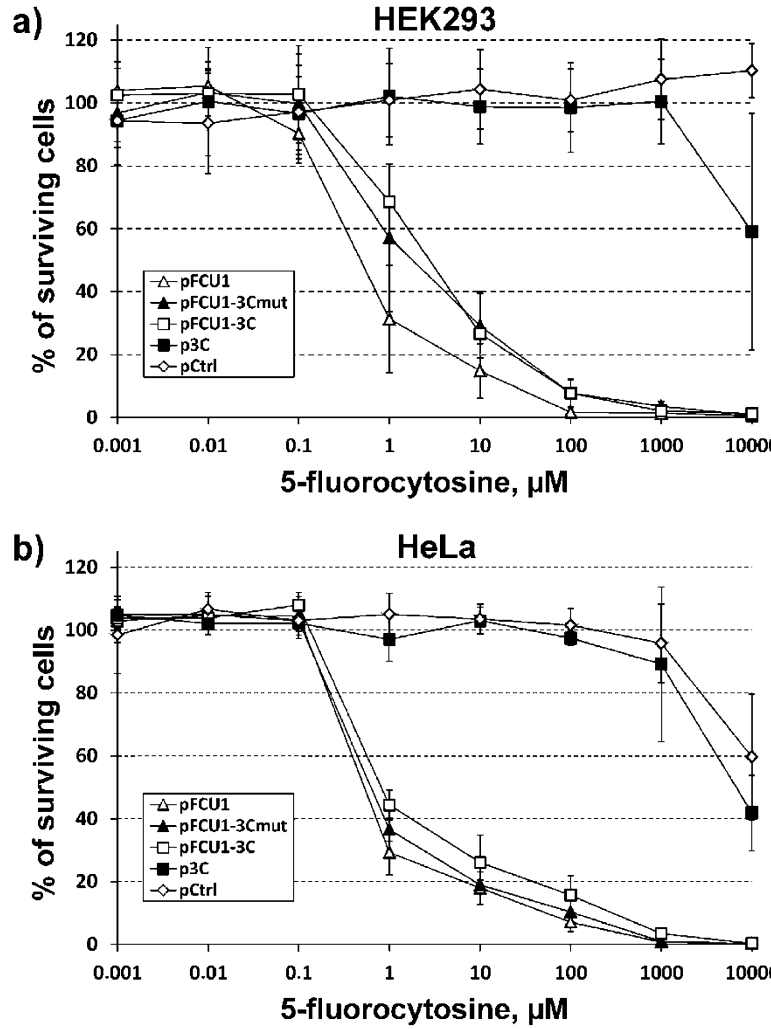

Fig. 2 Sensitivities to 5-FC of human cells transfected with mono- and bicistronic constructs. HEK293 (**a**) and HeLa (**b**) cells were transfected with the indicated plasmids and exposed to various concentrations of 5-FC for 7 days. Results are expressed as the percentage of viable cells relative to cells transfected with the corresponding construct but without exposure to 5-FC. Values are represented as mean $\pm$ SD of two individual experiments with triplicates ($n = 6$). Reproduced from ref. [25] with permission from Springer

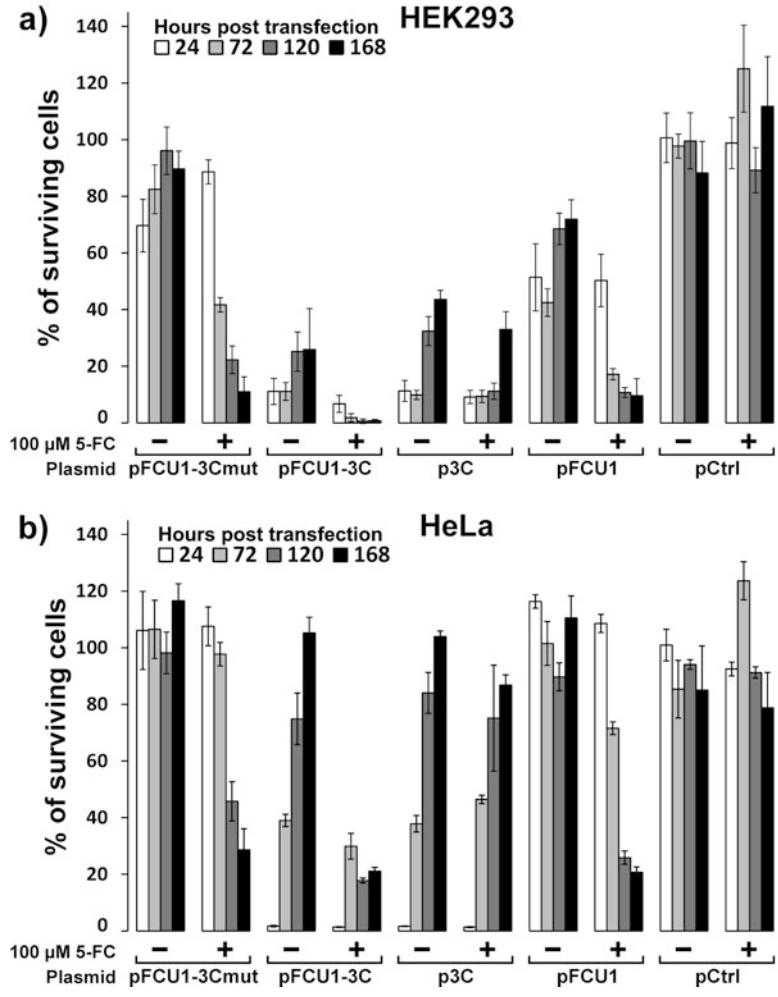

Fig. 3 Time course analysis of cytotoxic effects mediated by mono- and bicistronic constructs. HEK293 (**a**) and HeLa (**b**) cells were transfected with the indicated plasmids and cultured in the presence or absence of 100 µM 5-FC for 7 days. Results are expressed as the percentage of viable cells relative to non-transfected cultures without or with exposure to 5-FC, respectively. Values are represented as mean ± SD of two individual experiments with triplicates ($n = 6$). Reproduced from ref. [25] with permission from Springer

4 Notes

1. For a comprehensive analysis of the cytotoxic activity provided by a bicistronic vector containing a combination of suicide and killer genes a set of additional expression constructs should be created (Fig. 1). (1) An «empty» construct without any transgene. It is used in order to estimate cytotoxic side-effects (e.g., the effect of a chosen transfection method) of the experimental system. For this purpose the intact expression vector on the basis of which the bicistronic construct have been created can be used, or this construct should be generated individually. In the given example it is the **pCtrl** plasmid. (2) For the estimation of individual cytotoxic effects provided by the genes used two monocistronic constructs for the individual expression of the genes should be created. In the given example they are **pFCU1** and **p3C** plasmids. (3) Since manifestation of the cytotoxic activity of a suicide gene (*FCU1* in the given example) requires a prodrug administration, the individual action of a killer gene (*3C* in the given example) in a chosen bicistronic expression system can be estimated using the main plasmid construct (i.e., the **pFCU1-3C** plasmid). But the killer gene cannot be switched off so easily. Thus, when the individual action of the suicide gene in the bicistronic system should be characterized the additional bicistronic construct which provides for the expression of the suicide and a mutant killer gene encoding inactivated protein should be created. In the given example it is the **pFCU1-3Cmut** plasmid encoding the killer enzyme without catalytic activity (*3Cmut*).

   The construction procedures for the plasmids used in the given example are presented in Komissarov et al. [25]. The bicistronic expression system applied was created using 2A-peptide of porcine teschovirus-1. Principals and general methods of bicistronic vector construction using viral 2A-peptides are described in Szymczak-Workman et al. [27].
2. For plasmid DNA purification another proper method can be applied. The method should mainly provide for the obtaining of DNA samples of good quality. Check the integrity of plasmids using agarose gel electrophoresis and the A_{260}/A_{280} value (should be higher than 1.8). Do not use DNA solutions with concentrations less than 0.15 μg/μL. Otherwise it will result in addition of too much volume of a DNA storage buffer into transfection mixtures which may influence the transfection efficiency.
3. Another transfection method or reagent can be used. The method should provide for the sufficient transfection efficiency with minor or no side cytotoxicity. Choosing a transfection

reagent pay attention on its compatibility with the DNA type used, since several reagents are sensitive to DNA size and/or topology [28].

4. We use the phosphate buffered saline tablets from PanEco Company (Russia).
5. The number of cells to be plated per well of a 96-well plate should be defined experimentally for each cell line. This number depends on the cell line features and transfection method/reagent used. Since transfection is a cytotoxic process the culture to be transfected should reach the optimal confluence that provides for minimal unwanted cytotoxicity.
6. In most cases transfection reagent manufacturers supply their products with protocols which suit for a wide range of cell lines. But sometimes the protocol should be optimized to yield better results concerning the particular cell lines used. Recommendations for the transfection optimization can be found elsewhere (for example, *see* the reference [29]). If the expression constructs used significantly differ in their size the use of «stuffer» DNA may be required to equalize the amount of target DNA added to the cells [30].
7. The proportion of viable cells in cultures transfected with **pCtrl** construct should be similar to that of untransfected cultures. At the same time the significant cytotoxic effects in cultures transfected with constructs bearing active killer gene (i.e., **p3C** and **pFCU1-3C**) should be observed. In general the side cytotoxic effects should also be undetectable in cultures transfected with **pFCU1** and **pFCU1-3Cmut** since the prodrug has not been added. Otherwise it may indicate that the expression of the given proteins is harmful for the cells. This observation is valuable itself and can be helpful for correct interpretation of the further experiments.
8. According to the manufacturer's protocol the cell viability assay using CellTiter 96 AQueous One Solution Reagent can be performed in a growth medium supplemented with FBS and phenol red. But we recommend the assay to be done in PBS, since we have found that components of FBS are able to convert the reagent, and phenol red absorbs the light emitted at 490 nm with different efficiency depending on pH. This results in spreading of A_{490} values and in lowering the reproducibility of the results.
9. The loss of mitochondrial activity always accompanies cell death and is regarded as "point of no return" [31, 32]. Thus, to evaluate the cell viability we estimated the mitochondria metabolic activity in transfected cultures. For this purpose we used CellTiter 96 AQueous One Solution assay based on the application of the water-soluble tetrazolium compound MTS

(5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt), which is converted outside the cell in the medium presumably by NADPH and NADH produced by cells with metabolic active mitochondria [33]. Because the product of MTS conversion is water-soluble the given assay requires fewer steps than procedures that use MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide), other common tetrazolium compound.

10. We highly recommend recording the absorbance in time course and not in end point mode since A_{490} dynamic provides for the information that the assay works properly in the current well and that the values are in a linear range. For this purpose, we use Infinite M200 PRO microplate reader (Tecan, Switzerland) with CO_2 gas module. But, as an alternative, the plate can be kept in the cell CO_2 incubator between A_{490} measurements.
11. One of the main problems concerning long-term cultivation of adherent cell cultures is that high density cell monolayers tend to lose adhesion to the substrate and induce cell cycle arrest or death via contact inhibition. Thus, it is very important to be as gentle as possible when handling the plates, especially at late time points. In order to enhance the reproducibility of the results we recommend using an aspirator to remove the medium from wells, and to add the fresh medium along the well wall, not in a dropwise manner.
12. The duration of the experiment can be predefined on the basis of the published data or can be chosen during the testing. For the latter examine the wells using a microscope every day and pay attention to the color of the growth medium. Wells with different amount of viable cells will have different pH (and the indicator color) changing kinetics. Thus when you see that the color of growth medium in wells after the replacement is not changing it means that the cells are metabolically inactive, i.e., dead. The experiment can be finished when the situation mentioned above is observed for the wells with cultures exposed to the highest prodrug concentrations.
13. This is not in the case of 3C protease but, theoretically, the expression of a killer gene may enhance the cell sensitivity to the prodrug. This effect can be estimated by comparison of the cell viability curves for **pCtrl** and **p3C**. Thus, we recommend using all the obtained expressing constructs in this experiment and not only bearing the suicide gene.
14. Compare the cell viability curves for **pFCU1** and **pFCU1-3Cmut**; it will reflect the difference in the cytotoxic effects between mono- and bicistronic systems expressing the suicide gene. The comparison of the curves for **pFCU1** and **pFCU1-**

3C, as well as for **pFCU1-3Cmut** and **pFCU1-3C** will characterize the influence of the killer gene mediated cytotoxicity on the effects of the suicide gene. For the quantitative estimation of these effects and differences calculate the IC50 values for the constructs, e.g., according to the recommendations in ref. 34, and compare them.

15. For long-term experiments we highly recommend to use individual plates for each time point. This will yield more reproducible results since the cell cultures will not be disturbed until their time to be assayed has come.
16. The prodrug concentration should be chosen on the basis of the results obtained in the experiment described in Subheading 3.3. It can be determined as a concentration which provides for the significant cytotoxic effects in transfected cultures by the end of the experiment.
17. Compare the cell viability profiles for the constructs with **(+5-FC)** and without **(−5-FC)** prodrug exposure. (1) The profiles for **pFCU1-3Cmut/(−5-FC)** and **pFCU1/(−5-FC)** should be similar with that for **pCtrl/(−5-FC)** and **pCtrl/(+5-FC)** since none of the genes manifests its activity. (2) The profiles for **pFCU1-3Cmut/(+5-FC)** and **pFCU1/(+5-FC)** should be similar since only FCU1 manifests its cytotoxic activity. (3) The profiles for **pFCU1-3C/(−5-FC)**, **p3C/(−5-FC)** and **p3C/(+5-FC)** should be similar since only 3C protease manifests its cytotoxic activity. (4) Finally, the profile for **pFCU1-3C/(+5-FC)** where the products of both genes manifest their cytotoxic activity should be compared to other profiles to conclude whether the combined action works properly and possess synergy or not.

Acknowledgments

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Chapter 12

Engineered Thymidine-Active Deoxycytidine Kinase for Bystander Killing of Malignant Cells

Anton Neschadim and Jeffrey A. Medin

Abstract

Suicide transgenes encode proteins that are either capable of activating specific prodrugs into cytotoxic antimetabolites that can trigger cancer cell apoptosis or are capable of directly inducing apoptosis. Suicide gene therapy of cancer (SGTC) involves the targeted or localized delivery of suicide transgene sequences into tumor cells by means of various gene delivery vehicles. SGTC that operates via the potentiation of small-molecule pharmacologic agents can elicit the elimination of cancer cells within a tumor beyond only those cells successfully transduced. Such "bystander effects", typically mediated by the spread of activated cytotoxic antimetabolites from the transduced cells expressing the suicide transgene to adjacent cells in the tumor, can lead to a significant reduction of the tumor mass without the requirement of transduction of a high percentage of cells within the tumor. The spread of activated cytotoxic molecules to adjacent cells is mediated primarily by diffusion and normally involves gap junctional intercellular communications (GJIC). We have developed a novel SGTC system based on viral vector-mediated delivery of an engineered variant of human deoxycytidine kinase (dCK), which is capable of phosphorylating uridine- and thymidine-based nucleoside analogues that are not substrates for wild-type dCK, such as bromovinyl deoxyuridine (BVdU) and L-deoxythymidine (LdT). Since our dCK-based SGTC system is capable of mediating strong bystander cell killing, it holds promise for clinical translation. In this chapter, we detail the key procedures for the preparation of recombinant lentivectors for the delivery of engineered dCK, transduction of tumor cells, and evaluation of bystander cell killing effects in vitro and in vivo.

Key words Suicide gene therapy (SGT), Cancer, Tumor xenografts, Deoxycytidine kinase (dCK), Bromovinyl deoxyuridine (BVdU), L-Deoxythymidine (LdT)

1 Introduction

Injection of viral vectors to deliver the expression of a suicide transgene directly into tumors is a viable and safe strategy to reduce significantly the tumor burden of inoperable tumors with pharmacologic therapy, and is generally termed Suicide Gene Therapy of Cancer (SGTC). In its most common embodiment, a viral gene delivery vector is injected directly into the tumor mass that engineers the expression of the suicide transgene. A prodrug is coadministered systemically. The suicide transgene encodes an enzyme that

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promotes the conversion of a normally nontoxic prodrug into a cytotoxic antimetabolite that ultimately induces apoptosis in the cells expressing the suicide transgene. Achieving functional transduction of the entire tumor has proven to be a major challenge; the typical result is a marbled pattern of expression of the suicide transgene throughout the targeted mass [1]. Yet this approach is of particular interest in the armamentarium of therapies for solid tumors because it can achieve damage to tumor cells that far exceeds the extent of original transduction because of "bystander cell killing" [2]. Bystander cell killing is mediated by the diffusion of activated cytotoxic antimetabolites from the transduced cells to neighboring tumor tissue. Such transfer typically takes place via gap junctional intercellular communications (GJIC), which the small-molecule antimetabolites can traverse [3, 4]. As a result, substantial areas of tumor cell killing are established within the malignant mass, emanating from the successfully transduced cells (*see* Fig. 1).

SGTC approaches have been well studied, particularly with the canonical system: the Herpes Simplex Virus-derived thymidine kinase (HSV-*tk*) suicide transgene and aciclovir (ACV), valaciclovir (VCV), or ganciclovir (GCV) as the prodrug [5, 6]. The HSV-tk system has shown utility in the clinic with some promising outcomes, including an ongoing Phase 3 trial in localized prostate cancer (NCT01436968). Clinical evidence suggests that viral vector-mediated SGTC also acts as an immunotherapy by virtue of inducing significant apoptosis and necrosis within the tumor mass, and expression of foreign proteins, which can drive systemic antitumor immunity (known as Gene-Mediated Cytotoxic Immunotherapy or GMCI) [7–9].

The HSV-*tk* system, despite being the most extensively studied, is not very robust and can be hampered by poor prodrug activation. There are three main reasons for this. First, HSV-*tk* catalyzes the monophosphorylation of GCV, but it is the phosphorylation of GCV monophosphate to GCV diphosphate by the human guanylate kinase (GMPK) that is the rate-limiting step in the activation pathway of GCV to its antimetabolite form [10–12]. Second, GCV-mediated cell killing relies solely on inhibition of DNA replication and is less effective in slowly proliferating tumor cells [13]. Third, GCV has poor lipophilicity and thus a poor ability to cross the blood-brain barrier, limiting applicability in SGTC aimed at brain tumors [14]. The latter issue is somewhat overcome with the use of VCV and other more lipophilic prodrugs, however.

To overcome the limitations of the HSV-*tk* suicide gene therapy axis, we have developed alternative systems based on engineered human kinases, such as human thymidylate monophosphate kinase (tmpk) [15] and deoxycytidine kinase (dCK) [16]. In this chapter, we will describe SGTC based on an

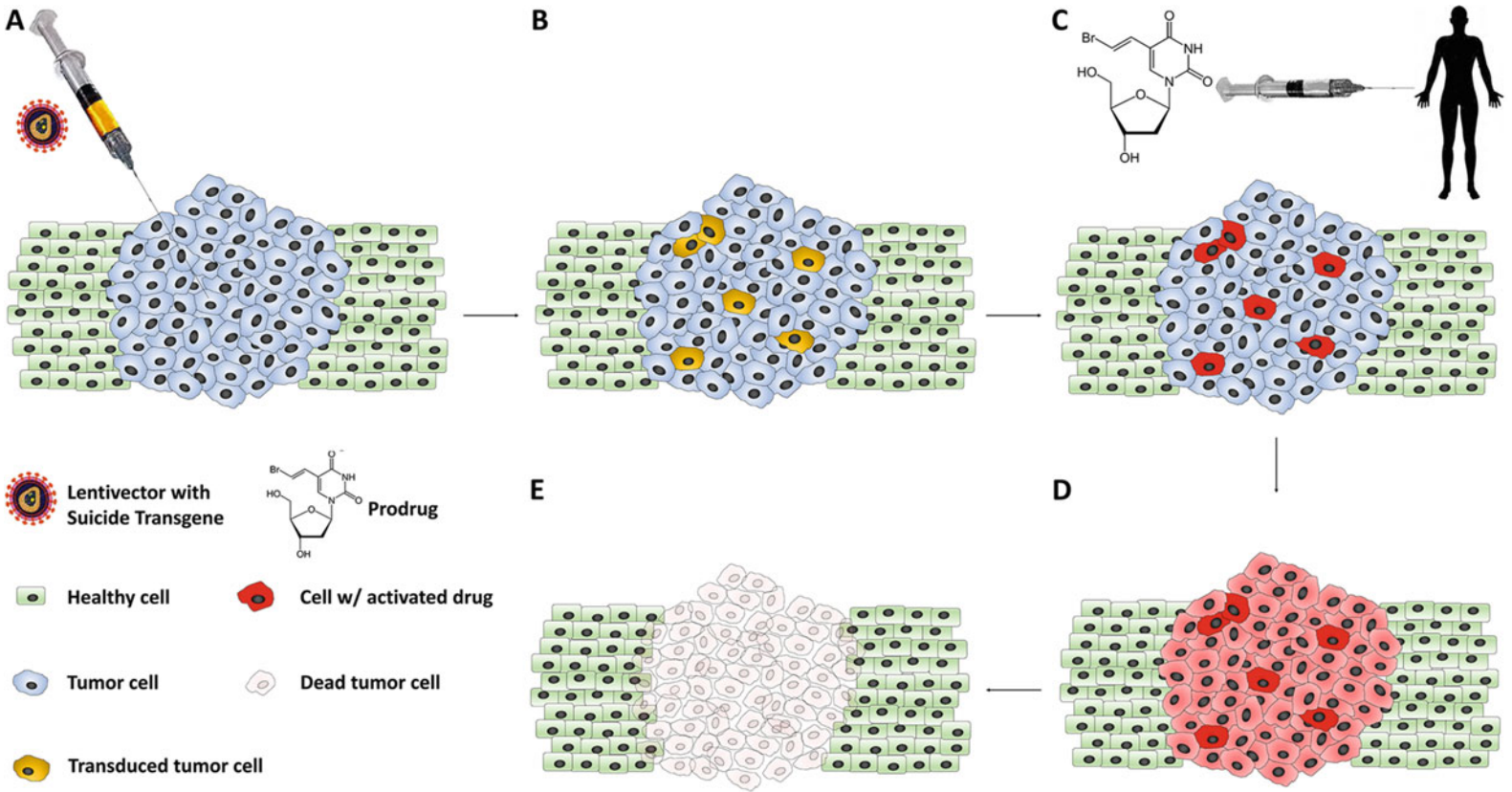

Fig. 1 SGTC and the mechanism of bystander cell killing. (**a**) A recombinant lentivector carrying the suicide transgene is injected directly into multiple areas of the tumor (blue-colored cells). (**b**) Some, but not all cells in the tumor are successfully transduced and express the suicide transgene product (yellow-colored cells). (**c**) The prodrug is administered systemically, but is only activated in the cells expressing the suicide gene prodrug (red-colored cells). Activated drug induces apoptosis in transduced cells. (**d**) Activated drug diffuses from the transduced cells into neighboring, non-transduced bystander cells via Gap Junctional Intercellular Communications (GJIC) and triggers apoptosis in those cells (pink-colored cells). (**e**) Activated drug diffuses throughout the tumor cells interconnected via GJIC, ultimately inducing cell death in the tumor (transparent cell ghosts)

engineered human dCK (triple mutant R104M, D133A and S74E), which is capable of phosphorylating uridine- and thymidine-based nucleoside analogues that are not substrates for wild-type dCK. The R104M.D133A mutation confers additional substrate specificity to dCK and enables it to activate such prodrugs as bromovinyl deoxyuridine (BVdU) and L-deoxythymidine (LdT), while the S74E mutation mimics an activating phosphorylation of dCK that enhances its activity [17]. Viral vector-mediated delivery of dCK.R104M.D133A.S74E, or dCK.DM.S74E, into a variety of cancer cell types renders them sensitive to BVdU and LdT [17]. Inclusion of truncated, non-signaling human CD19 molecules (CD19Δ), expressed downstream off of an internal ribosomal entry site (IRES) element included in the vector construct, enables rapid enrichment of transgene-positive cells by flow cytometric and magnetic enrichment techniques [15]. Unlike HSV-*tk*, dCK is the rate-limiting enzyme in the phosphorylation pathway of nucleosides in the cell and overcomes poor prodrug activation kinetics [17]. Cells expressing this engineered dCK can be visualized directly in situ by micro-PET imaging, which would enable one to monitor the progress and outcomes of SGTC in patients [18]. Furthermore, unlike HSV-*tk*-activated prodrugs, dCK-activated BVdU can elicit cell killing of nondividing cells by a unique additional mechanism to inhibition of DNA synthesis—via the inhibition of thymidylate synthase [17]. With respect to permeability through the blood-brain barrier, BVdU is also substantially more lipophilic than GCV [14]. SGTC based on dCK elicits significant bystander cell killing effects in vitro and in solid tumors in vivo [16].

Here we detail the key procedures for the preparation of recombinant lentivectors (LVs) for the delivery of engineered dCK, injection such of LVs to achieve transduction of tumor cells, and evaluation of bystander cell killing effects in cell culture in vitro and animal xenograft models in vivo.

2 Materials

2.1 Cell Culture

1. Culture media: Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L of glucose and supplemented with 10% Fetal Calf Serum (FCS), penicillin and streptomycin (P/S) (1× or 100 U/mL and 100 μg/mL, respectively), and 2 mM L-glutamine. Media is stored at 4°C.
2. Trypsin-EDTA 0.05%.
3. Dulbecco's PBS without calcium and magnesium chloride (Ca^{-}/Mg^{-} DPBS).

4. Cell lines:
   (a) Human U87 mg glioblastoma-astrocytoma cells (HTB-14, ATCC).
   (b) Human embryonic kidney-derived epithelial 293T cells (CRL-3216, ATCC), low-passage.
5. Second-generation lentiviral packaging plasmids (16, 17):
   (a) Gene delivery plasmid pHR'-EF-dCK.DM.S74E-IRES-huCD19Δ-WPRE-SIN for LV-dCK.
   (b) Gene delivery plasmid pHR'-EF-eGFP-WPRE-SIN (eGFP, enhanced green fluorescent protein) for LV-eGFP.
   (c) Envelope glycoprotein-encoding plasmid pMD.G.
   (d) Lentiviral packaging plasmid pCMV-ΔR8.91.

2.2 Transfection and Transduction

1. 10 mM polyethyleneimine (PEI), high molecular weight, water-free (preferred molecular weight of 25,000) (Sigma). *See* **Note 1** for preparation.
2. 150 mM NaCl, filter-sterilized.
3. 0.1% BSA-containing PBS, filter-sterilized.
4. Transduction reagent: 4 mg/mL protamine sulfate (Sigma) in water, filter-sterilized.

2.3 SDS-PAGE and Western Blotting

1. Polyacrylamide: 30% Acrylamide/Bis Solution, 37.5:1.
2. Polyvinylidene difluoride (PVDF) membrane (EMD Millipore).
3. 10× Tris-Glycine and 10× Tris-Glycine-SDS buffer concentrate.
4. Nonfat dry milk (NFDM) powder.
5. Antibodies:
   (a) Anti-dCK monoclonal antibody (Clone # 2243C2; Abcam).
   (b) Sheep anti-mouse immunoglobulin G antibody conjugated to horseradish peroxidase (Amersham).
6. Immobilon Western Chemiluminescent HRP Substrate or equivalent (EMD Millipore).
7. Kodak X-Omat LS Film or equivalent, or a luminescence image analyzer such as the LAS-1000 system (Fujifilm).
8. Wash buffer: 20 mM Tris–HCl (pH 7.4) with 0.05% Tween-20 (TBS-T).
9. Blocking buffer: 5% (w/v) NFDM in 20 mM Tris–HCl (pH 7.4) with 0.05% Tween-20 (TBS-T).
10. RIPA or Laemmli lysis buffers.

2.4 Other Buffers and Reagents

1. FACS buffer: DPBS containing 2.5% (v/v) FCS, and, optionally, 1 mM EDTA.
2. Prodrug: BVdU ((E)-5-(2-bromovinyl)-2′-deoxyuridine, Alfa Aesar), 5 mg/mL stock solution in DPBS, filter-sterilized. Stored aliquoted at −20°C.
3. Anti-CD19-PE or anti-CD19-APC antibodies (BD Biosciences).
4. Annexin V conjugated with allophycocyanin (Annexin V-APC) (BD Biosciences).
5. Annexin V binding buffer: 10 mM HEPES-NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4.
6. Dimethyl sulfoxide (DMSO).
7. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent kit (Promega).
8. 100 mM carbenoxolone (Sigma) in DMSO.
9. 100 mM 18-β-glycyrrhetinic acid (Sigma) in ethanol.

2.5 Experimental Animals

1. Nonobese/diabetic severe combined immunodeficient (NOD/SCID) mice (male, 6–10 weeks old) (Jackson Laboratories).

2.6 Consumables and Equipment

1. Hamilton syringe (Sigma).
2. PES filter units, 0.45 μm and 0.22 μm (EMD Millipore).
3. Lab-Tek 16 Chamber Glass Slide for cell culture (Thermo Fischer Scientific).
4. T25 tissue culture-treated flasks.
5. 6-well tissue culture-treated plates.
6. 10-cm and 15-cm diameter tissue culture-treated plates.
7. Transwell culture plates (0.4 mm pore size; BD Falcon).
8. Polypropylene tubes (15 mL and 50 mL).
9. Polyallomer conical SW28 centrifuge tubes (Beckman Coulter).
10. Tissue-culture cell strainers, 40 μm, sterile.
11. Absorbance microplate reader (Fisher Scientific).
12. Biosafety Level 2 tissue culture hood.
13. Humidified tissue culture CO_2 incubator.
14. Ultracentrifuge and SW28 ultracentrifuge rotor (Beckman Coulter).
15. Flow cytometry instrument (FACSCalibur, BD).

3 Methods

3.1 Cell Culture

1. Maintain the adherent 293T and U87 mg cells in tissue culture plates or flasks in DMEM medium supplemented with 10% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2 mM L-glutamine at 37 °C in 5% CO_2 atmosphere at constant humidity.
2. To passage (for cells maintained in a 10-cm plate), gently rinse cells with 5 mL of DPBS.
3. Aspirate DPBS and incubate with 1 mL of 0.05% trypsin-EDTA at 37°C, in CO_2 incubator for 3–5 min, until cells have completely dislodged.
4. Quench trypsinization with culture media and collect cells by centrifugation in a 15 mL polypropylene tube at 300 × *g* for 5–10 min at 4°C.
5. Following the centrifugation, aspirate and resuspend the cell pellet in fresh media. Seed cells at the desired concentration onto new tissue culture plates. For cells maintained in other culture vessels, scale the above volumes appropriately.

3.2 Production of Recombinant Lentivector

1. Seed 4.5 × 10^6 293T cells in a 10-cm plate in 10 mL of supplemented DMEM and incubate at 37°C in a humidified 5% CO_2 incubator for 24 h.
2. Check the morphology of the cells using a phase-contrast microscope and ensure that the confluence is less than 50%.
3. Refresh media 2 h prior to transfection.
4. Prepare DNA plasmids for transfection by combining the following preparations in a 15 mL polypropylene tube in a total volume of 500 μL of 150 mM NaCl (*see* **Note 2**):

   10 μg of the gene delivery vector (pHR' backbone for LV-dCK or LV-eGFP).

   10 μg of pCMVΔR8.91 (packaging plasmid).

   5 μg of pMD.G (VSV-g envelope plasmid).
5. Dilute 91 μL of 10 mM PEI with 409 μL of 150 mM NaCl in a separate 15 mL polypropylene tube. Mix well by vortexing. Add the diluted PEI solution dropwise into the DNA mixture while vortexing continuously (*see* **Note 3**).
6. Incubate the resulting transfection mixture for 20 min at room temperature.
7. Add the transfection mixture dropwise to the cells. Mix by gently rocking the plate and incubate for 12–16 h at 37°C in a humidified CO_2 incubator.
8. Aspirate, replacing media with 10 mL of fresh DMEM media.

9. Collect the viral supernatant at 24–48 h after the media change, pass through a 0.45 μm PES filter unit and concentrate if desired. Media can also be collected at both the 24- and 48-h time points, if desired.
10. Scale accordingly for LV production from multiple plates.
11. Concentrate by ultracentrifugation in polyallomer conical SW28 centrifuge tubes and using a SW28 rotor at 72,128 × *g* (20,000 rpm) for 2 h at 4°C. An alternative ultracentrifuge setup can also be used. Although it is preferable to use the recommended speed or higher, the centrifugation time can be increased to concentrate at lower speeds.
12. Resuspend in 1/1,000th volume of the original supernatant or so in 0.1% BSA-containing DPBS and keep on ice for 2 h to slowly resuspend the LV (assist resuspension by repeated pipetting). The resuspended LV can be aliquoted and stored at −80°C until use. Avoid repeated freeze-thaw cycles to maximize LV viability.

3.3 Determination of LV Titer

1. Seed 2×10^5 293T cells per well in 6-well tissue culture-treated plates and incubate overnight at 37°C in a humidified CO_2 incubator.
2. Remove DMEM culture media and add 1 mL of fresh culture media (1 well) or 1 mL of serially diluted LV supernatant in culture media (minimum of 4 wells). Collect and count cells in the spare sixth well.
3. Add protamine sulfate to each well to a final concentration of 8 μg/mL.
4. Incubate the cells for 24 h at 37°C in a humidified CO_2 incubator.
5. Determine percent transduction by analyzing cells on a flow cytometer:
   (a) LV-eGFP-transduced cells can be washed in FACS buffer and analyzed unstained for green fluorescence using the appropriate fluorescence channel on the flow cytometer.
   (b) LV-dCK-transduced cells can be washed in FACS buffer, stained with the anti-CD19-PE or anti-CD19-APC antibody (1:20 dilution), washed in FACS buffer, and analyzed for PE or APC fluorescence using the appropriate fluorescence channel on the flow cytometer.
6. Calculate the titer at each dilution: LV titer (infectious units or IU/mL) = (# of cells on day of transduction) × (% of positive cells) × (dilution factor) and use linear fit to determine the titer of the concentrated supernatant in the linear region of the resulting curve.

3.4 Transduction of U87 mg Cells In Vitro and Analysis of Transduction Efficiency

1. Seed 3×10^5 U87 mg cells per well in 6-well tissue culture-treated plates and incubate overnight at 37°C in a humidified CO_2 incubator.
2. Infect cells by incubation with the concentrated LV stocks (LV-eGFP or LV-dCK), diluted to achieve a multiplicity of infection (MOI) of 10, in the presence of protamine sulfate at a final concentration of 8 μg/mL (*see* **Note 4**). Incubate cultures overnight at 37°C in a humidified CO_2 incubator.
3. Clone transduced cells by limiting dilution or another preferred method such as single-cell sorting to generate single-cell clones.
4. Expand cells at 37°C in a humidified CO_2 incubator to obtain sufficient number for subsequent experiments as needed.
5. Transgene expression in the transduced U87 mg cells can be confirmed indirectly by flow cytometric analysis of eGFP or CD19Δ-positive expression:
   (a) LV-eGFP-transduced cells can be washed in FACS buffer and analyzed unstained for green fluorescence using the appropriate fluorescence channel on the flow cytometer.
   (b) LV-dCK-transduced cells can be washed in FACS buffer, stained with the anti-CD19-PE or anti-CD19-APC antibody (1:20 dilution), washed in FACS buffer, and analyzed for PE or APC fluorescence using the appropriate fluorescence channel on the flow cytometer..
6. Alternatively, transgene expression can be confirmed by Western blot analysis:
   (a) Prepare total cell lysates from transduced cells using RIPA or Laemmli lysis buffer.
   (b) Resolve lysate using 8–12% SDS-PAGE and transfer onto a PVDF membrane.
   (c) Block membrane with PBS-T with 5% NFDM for 2 h at room temperature or overnight at 4 °C.
   (d) Probe membrane with an anti-dCK monoclonal antibody diluted to 0.5 μg/mL in PBS-T with 5% NFDM for 4 h at room temperature.
   (e) Wash membrane with PBS-T at least 3 times for a total time of 30 min.
   (f) Probe with the secondary sheep anti-mouse immunoglobulin G antibody conjugated to horseradish peroxidase diluted 1:10,000 in PBS-T with 5% NFDM for 1 h at room temperature.
   (g) Wash membrane with PBS-T at least 3 times for a total time of 30 min.

(h) Develop with the Immobilon Western Chemiluminescent HRP Substrate following manufacturer's instructions or an alternative chemiluminescence kit. Expose onto film in a dark room or image using a luminescence image analyzer.

(i) Confirm equal protein loading by probing or stripping and re-probing with a murine anti-human GAPDH antibody or antibody for another housekeeping protein, if needed.

3.5 Evaluation of Prodrug-Induced Cell Killing In Vitro by the Colorimetric MTS Cell Proliferation Assay

1. Seed parental U87 mg cells or LV-eGFP-transduced U87 mg cells and LV-dCK-transduced U87 mg cells in 6-well plates at a concentration of 5×10^4 cells/well in 0.5 mL of DMEM medium.
2. Add 0.5 mL of media containing increasing concentrations of BVdU or LdT to each well (for example, 0 μM, 0.2 μM, 2 μM, 20 μM, 200 μM, and 2 mM). Final treatment concentrations will then be 0 μM, 0.1 μM, 1 μM, 10 μM, 100 μM, and 1 mM, respectively. Set up each incubation and drug treatment in triplicate (at a minimum).
3. Incubate for 5 days. Refresh the treatment medium daily or once every 2 days.
4. As an additional positive control, untreated cells can be killed by incubation with 10% ethanol 1–2 h prior to the proliferation assay. After 5 days of culture, add 200 μL of stock solution of the MTS reagent to each well and incubate for 1 to 3 h at 37 °C, observing color conversion every 30 min.
5. Once sufficient color has developed, mix wells by gentle shaking and transfer 200 μL from each 6-well plate well into a 96-well plate.
6. Measure the absorbance at 490 nm in each well of the 96-well plate using an absorbance plate reader. The absorbance values are proportional to the number of remaining viable cells in each well. The values can be normalized to those obtained from untreated wells (0% cell killing) and 10% ethanol-treated wells (100% cell killing).

3.6 Evaluation of Prodrug-Induced Apoptosis In Vitro

1. Seed parental U87 mg cells or LV-eGFP-transduced U87 mg cells and LV-dCK-transduced U87 mg cells in 6-well plates at a concentration of 5×10^4 cells/well in 1–2 mL of DMEM medium.
2. Add 0.5 mL of media containing increasing concentrations of BVdU or LdT to each well (for example, 0 μM, 0.2 μM, 2 μM, 20 μM, 200 μM, and 2 mM). Final treatment concentrations will then be 0 μM, 0.1 μM, 1 μM, 10 μM, 100 μM, and 1 mM, respectively. Set up each treatment in triplicate (at a minimum).

3. Incubate for 2–4 days. Refresh the treatment medium daily or once every two days.
4. As an additional positive control, untreated cells can be killed by incubation with 10% ethanol 1–2 h prior to the proliferation assay.
5. After incubation, collect cells by trypsinization and stain with Annexin V (APC version) and propidium iodide (PI) following the manufacturer's protocol. Wash cells at least twice with Annexin V binding buffer (*see* **Note 5**).
6. Analyze cells using flow cytometry for Annexin V-APC and PI fluorescence. Determine the apoptotic index as the ratio of the percentage of apoptotic cells to the percentage of non-apoptotic cells in treated and untreated cultures.

3.7 Evaluation of AZT-Induced Apoptosis in Bystander Cells In Vitro in Mixed Cocultures

1. Seed parental U87 mg cells or LV-eGFP-transduced U87 mg cells and LV-dCK-transduced U87 mg cells in 6-well plates at a concentration of 2×10^5 cells/well in 1–2 mL of DMEM medium. Additionally, set up several cocultures of LV-dCK-transduced U87 mg cells to non-transduced or LV-eGFP-transduced U87 mg cells. Sample ratios can be 1:10, 4:1, 1:1, 1:4, 1:10 (ranging from approximately 10–50% of LV-dCK-transduced cells).
2. Culture cells untreated or treated with the desired concentration of prodrug (for example, 100 μM of BVdU or 1 mM of LdT) for 3 days. Significant bystander effects can be observed at or above the prodrug concentration that achieves the cell killing IC50 in vitro in LV-dCK-transduced cells.
3. To confirm the involvement of GJICs in the bystander cell killing induced, 100 μM carbenoxolone and/or 35 μM 18-β-glycyrrhetinic acid inhibitors can be added simultaneously with the prodrug to additional cultures.
4. Set up each treatment in triplicate (at a minimum).
5. Incubate for 3 days. Refresh the treatment medium daily or at least once.
6. As an additional positive control, untreated cells can be killed by incubation with 10% ethanol 1–2 h prior to the proliferation assay.
7. After incubation, collect cells by trypsinization and stain with Annexin V (APC version) and propidium iodide (PI) following the manufacturer's protocol. Wash cells at least twice with Annexin V binding buffer (*see* **Note 5**).
8. Analyze cells using flow cytometry for Annexin V-APC and PI fluorescence. Determine the apoptotic index as the ratio of the percentage of apoptotic cells to the percentage of non-apoptotic cells in treated and untreated cultures for each coculture ratio.

3.8 Evaluation of Prodrug-Induced Bystander Cell Killing In Vivo in a Mixed Tumor Xenograft Model

1. Inoculate nonobese/diabetic severe combined immunodeficiency (NOD/SCID) mice (6–10 weeks old) subcutaneously (into the right dorsal flank) with U87 mg-derived mixed tumor xenografts comprising a 1:1 mixture of LV-dCK-transduced cells (1×10^6 cells resuspended in D-PBS) and LV-eGFP-transduced cells (1×10^6 cells resuspended in D-PBS).
2. In vivo tumor cell growth is monitored weekly by measuring tumor volume with a caliper (calculated as either $\pi/6 \times \text{length} \times \text{width}^2$ or $\pi/6 \times \text{length} \times \text{width} \times \text{height}$ to yield mm^3).
3. Treat one group of animals daily with BVdU by intraperitoneal injection (a dose of 60 mg/kg of BVdU in DPBS can be used). Another group of animals is treated with vehicle control (DPBS only).
4. Once vehicle-treated control tumors reach maximum size allowed by the Animal Use Protocol, euthanize the animals and harvest the tumors.
5. Disaggregate tumors into single-cell suspensions (can be accomplished by incubation with trypsin or passage through a cell strainer).
6. Wash cells in FACS buffer, stain for CD19Δ expression with anti-CD19-PE or anti-CD19-APC antibody (1:20 dilution), wash in FACS buffer, and analyze by flow cytometry for eGFP (green fluorescence) and CD19Δ expression (PE or APC fluorescence). Determine the relative percentages of the bystander LV-eGFP-expressing tumor cell populations to the LV-dCK-transduced (eGFP-negative) cell populations in each of the recovered tumors to assess the magnitude of the bystander effect as a result of prodrug treatment.

3.9 Evaluation of Prodrug-Induced Bystander Cell Killing Effect In Vivo in a Xenograft Mouse Model by Intratumoral Injection of dCK Lentivirus

1. Inoculate nonobese/diabetic severe combined immunodeficiency (NOD/SCID) mice (6–10 weeks old) subcutaneously (into the right dorsal flank) with naive U87 mg cells or LV-eGFP-transduced U87 mg cells (a total of 2×10^6 cells resuspended in D-PBS) to derive tumor xenografts.
2. In vivo tumor cell growth is monitored weekly by measuring tumor volume with a caliper (calculated as either $\pi/6 \times \text{length} \times \text{width}^2$ or $\pi/6 \times \text{length} \times \text{width} \times \text{height}$ to yield mm^3).
3. Once tumors become palpable and reach a volume of 100 mm^3 or more (approximately 2 weeks past inoculation), anesthetize animals and inject each tumor with up to 10 μL of concentrated lentiviral supernatant of LV-dCK ($>1 \times 10^8$ IU/mL or 1×10^6 IU/injection). Use the Hamilton syringe for the accurate injection of such a small volume and hold the tumors with tweezers to guide the needle into the approximate center of the tumor mass.

4. Treat one group of animals with BVdU daily, starting the next day following the transduction, by intraperitoneal injection (a dose of 60 mg/kg of BVdU in DPBS can be used). Another group of animals is treated with a vehicle control (DPBS only).
5. Transduction efficiency in vivo can be evaluated using a small, separate group of xenograft-bearing mice, harvesting the tumor xenografts 24 h following the LV infection, dissociating the tumor cells (by trypsinization or passage through a cell strainer), and analyzing expression of CD19Δ and/or eGFP by flow cytometry. Wash the cells in FACS buffer, stain for CD19Δ expression with anti-CD19-PE or anti-CD19-APC antibody (1:20 dilution), wash in FACS buffer, and analyze by flow cytometry for eGFP (green fluorescence) and CD19Δ expression (PE or APC fluorescence).
6. Once vehicle-treated control tumors reach maximum size allowed by the Animal Use Protocol, euthanize the animals and harvest tumors, if desired, for analysis of eGFP and dCK (by CD19Δ) expression.

4 Notes

1. Weigh 43 mg of PEI, add water (~80 mL), and warm up to 37 °C in a water bath to dissolve. Adjust pH to 7.00 with HCl and adjust the final volume to 100 mL. Filter the solution through a 0.22 μm PES filter to sterilize and store at 4 °C.
2. Concentrated plasmid preparations of high quality are required for a successful transfection. For LV that will be used in vivo use endotoxin-free plasmid preparations.
3. The nitrogen-to-phosphorus ratio (N/P) is a measure of the ionic balance of the DNA-PEI complexes. The positive charge of PEI originates from the nitrogen of the repeat unit of PEI, $NHCH_2CH_2$. The negative charge in the plasmid DNA backbone arises from the phosphate group of the deoxyribose nucleotides. The ratio of N/P is critical for optimal transfection and is a measure of the ionic balance of the DNA-PEI complexes. Use an N/P value of 12 for the transfection or optimize as needed.
4. A functional MOI of 10–20 is recommended but a lower MOI can also be used (MOI = ratio of IU to the number of cells being transduced). Ensure that the concentrated viral preparation does not comprise more than 10% of the diluted virus media for transduction, as cell viability can decline when incubating highly concentrated LV preparations with cells due to VSV-g toxicity.

5. When staining cells with Annexin V, make sure that the Annexin V staining buffer is used throughout the procedure. Divalent cations are required to facilitate and retain Annexin V binding to phosphatidylserine moieties on cells. Work quickly and analyze the cells within 1 h of staining.

Acknowledgments

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Chapter 13

Photochemical Internalization Enhanced Nonviral Suicide Gene Therapy

Chung-Ho Sun, Kristian Berg, and Henry Hirschberg

Abstract

Nonviral gene transfection overcomes some of the disadvantages of viral vectors, such as undesired immune responses, safety concerns, issues relating to bulk production, payload capacity, and quality control, but generally have low transfection efficiency. Here we describe the effects of a modified form of photodynamic therapy (PDT), i.e., photochemical internalization (PCI) to: (1) greatly increase nonviral cytosine deaminase gene (CD) transfection into tumor cells, significantly increasing the conversion of 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU), and (2) enhance the toxic efficacy of the locally produced 5-FU to induce cell death on both transfected and non-transfected bystander cells.

Key words Nonviral gene transfection, Cytosine deaminase gene, Suicide gene therapy, Photodynamic therapy, Photochemical internalization, Endosomal escape

1 Introduction

One important limitation for suicide gene therapy, in particular when nonviral gene carriers are employed, is the inability of the gene to transfect a sufficient number of tumor cells. This in turn sets a limit to the amount of on-site conversion of prodrug to active drug. The well-established suicide gene therapy modality, utilizing the transfection of the cytosine deaminase (CD) gene, which codes for an enzyme that converts the antifungal agent 5-fluorocytosine (5-FC) into its antimetabolite 5-fluorouracil (5-FU) was used in the development of this protocol [1–3]. Additionally the bystander effect, where active drug is exported from the transfected cancer cells into the tumor microenvironment, plays an important role by inhibiting growth of adjacent non-transfected tumor cells. Methods to enhance the initial gene transfection efficiency and the efficacy of the locally produced and consequently exported drug would therefore offer a distinct advantage. A modified form of

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photodynamic therapy (PDT), photochemical internalization (PCI), has been shown to greatly enhance gene insertion efficiency for both viral and nonviral gene transfection protocols as well as significantly increasing the efficacy of a variety of anticancer agents [4–8]. DNA polyplexes or drugs, which are internalized into cells via endocytosis, end up inside and trapped in the intracellular endosomes and lysosomes. The concept of PCI is based on using photosensitizers which localize to endosomes and lysosomes. When light is applied, the photosensitizer will react with oxygen causing endo-lysosome membrane rupture releasing the trapped macromolecules into the cell cytosol, avoiding lysosomal degradation. The released gene or drug can therefore exert its full biological activity, in contrast to being degraded by lysosomal hydrolases.

Here we describe an in vitro three-dimensional multicell glioma spheroid tumor model [9] that has proved useful in exploring the ability of PCI to: (1) greatly increase the subpopulation of CD gene transfected cells within the spheroid, resulting in increased amounts of 5-FU converted from 5-FC, and (2) significantly increase the toxic efficacy of the locally produced and exported 5-FU both in the CD transfected and the non-transfected bystander cells.

Due to the rapid attenuation of light in tissue, this enhanced gene transfection efficiency and increased drug efficacy would be limited to the illuminated targeted tumor site, thus reducing damage to off target normal tissue. Thus, the light-based therapy PCI can both increase gene transfection efficiency and the efficacy of the compounds converted by the suicide gene-induced enzyme, in a targeted site- and time-specific manner.

2 Materials

2.1 CD Gene Plasmid

1. Plasmid pPH36, Addgene (Cambridge MA) cat #35101. The plasmid is received as bacteria *E. coli* (high copy number DH5alpha strain).
2. LB agar ampicillin-100, SigmaAldrich Cat# L5667, Prepoured LB agar plates with 100 μg/mL ampicillin. Store at 2–8 °C.
3. LB Broth, Mediatech Cellgro from Fisher Scientific Cat#MT-46-050-CM.
4. Ampicillin, EMD Millipore Calbiochem from Fisher Scientific Cat# 17-125-520ML, 20 mL at 10 mg/mL H_2O, sodium salt, sterile and tissue culture grade.
5. Inoculation loop 1 μL, SARSTEDT Cat# 86.1567.050, white, 48 sterile pieces packed in the bag. SARSTEDT (Germany) with sale office at Sparks NV USA.

6. Plain Polycarbonate Fisherbrand Shaker Flasks with vented polypropylene closure, the vent is with 0.22 μm PTFE pore. Both flasks and closures are autoclavable.
7. Thick wall Polypropylene 50 mL tube for Beckman JA-20 rotor, Beckman Coulter Cat# 357005.

2.2 CD Gene DNA Isolation

1. QIAGEN Plasmid Midi Kit Cat# 12143. This kit can be stored at room temperature (15–25 °C) for up to 2 years.
2. Buffer P1 (suspension buffer): 50 mM Tris–HCl, pH:8.0, 10 mM EDTA (inactivate DNase activity by chelating out Ca^{++} and Mg^{++} which are essential cofactors), 100 μg/mL RNase A, resuspend before each use when LyseBlue is added.
3. Buffer P2 (lysis buffer): 200 mM sodium hydroxide (alkaline mixture to rupture the cells, break down the cell wall and denature dsDNA to ssDNA), 1% SDS w/v (breaks apart the lipid membranes and solubilizes cellular proteins, keep the bottle closed when not in use to avoid acidification of the buffer by CO_2 in the air).
4. Buffer P3 (neutralization buffer): Neutralize to allow DNA strands to renature which is easy for plasmid DNA: (acetic acid), 3.0 M potassium acetate pH:5 (precipitate SDS out along with cellular debris and genomic DNA, *E. coli* chromosomal DNA while plasmid DNA remains in solution).
5. Buffer QBT (equilibrium buffer): isopropanol 15% (v/v), 0.15% Triton X 100 (v/v), 750 mM NaCl, 10 mM MOPS, pH: 7.0.
6. Buffer QC (wash buffer): isopropanol 15% (v/v), 1.0 M NaCl, 50 mM MOPS, pH:7.0.
7. Buffer QF (elution buffer): isopropanol 15% (v/v), 1.0 M NaCl and 50 mM Tris–HCl, pH:8.5.
8. RNase A.
9. LyseBlue.
10. QIAGEN-tip 100: anion exchange resin, plasmid DNA binds to the resin under appropriate low salt and pH condition, RNA, proteins and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer followed by isopropanol precipitation to concentrate and desalt the plasmid DNA.

2.3 CD Gene DNA/Gene Carrier Polyplex

1. DI water (Nuclease-free water (Fisher Scientific, Pittsburgh, PA).
2. Protamine sulfate (PS, 5.1 kDa), Fisher Scientific (Pittsburgh, PA).
3. Malvern Zetasizer Nano ZS (Malvern Instruments, Westborough, MA).

2.4 Tumor Spheroids

1. *Cell lines*: Rat F98 glioma cell line obtained from the American Type Culture Collection (Manassas, VA, USA).
2. Ultra-low attachment surface 96-well round-bottomed plates (Corning In., NY).
3. Advanced DMEM medium (Invitrogen Corp., Carlsbad CA., Cat. # 35101).
4. TrypLE Express.
5. Tissue Culture Flask T25.
6. Serological Pipettes 5 mL.
7. P10.
8. Disposable plastic hemocytometer C-chip.

2.5 Laser Light Treatment

1. Photosensitizer $AlPcS_{2a}$ (Frontier Scientific, Inc., Logan, UT).
2. Laser fiber Frontal light distributor Model FD1 (Med*light* Ecublens Switzerland).
3. 670 nm Diode laser; Intense-HPD 7404 (Intense HPD, New Jersey, USA).

3 Methods

3.1 CD Gene Plasmid Generation

1. Take out one LB agar plate with 100 μg/mL ampicillin from refrigerator and label the bottom of the plate with pPH36, ampicillin 100, date and your initial. Bring the labeled agar plate, pPH36 stab, and a pack of sterile 1 μL inoculation loop to the clean bench. Insert and touch the punctured area of the stab culture with the sterile loop and hold the loop at an angle to make several broad zigzag strokes to spread the bacteria over about a one-third section of the surface of the plate to generate streak #1. Take a new sterile loop and drag through streak #1 at an slight angle and spread the bacteria over a second section of the plate in the broad zigzag stroke to generate streak #2. Take a third new sterile loop and drag through streak #2 at an angle and spread the bacteria over the third and last section of the plate in the broad zigzag stroke to generate streak #3.
2. Place the inoculated plate in a 37 °C incubator overnight (12–18 h). Next morning, single colonies looking like white dots should be visible on the solid agar plate. Each single colony is composed of millions of genetically identical bacteria grown from a single bacterium. Store the plate in the refrigerator until ready for expansion in liquid bacteria culture.
3. Add 20 mL LB broth into a sterile 125 mL shaker flask with vented closure. Label the flask the same as the plate with new date. Add total 200 μL of ampicillin (EMD Millipore Calbiochem Fisher Scientific Cat# 17-125-520ML). Use a sterile loop

to pick a single clone from the inoculated agar plate. Dip and swirl the tip of the loop into the 125 mL shaker flask containing the 20 mL LB broth and ampicillin. Cap the flask with the vented closure and hook up the flask onto a shaking incubator. Incubate the liquid bacteria culture overnight at 37 °C.

4. After incubation, the LB broth should look cloudy. For incubation to obtain bacteria culture at log phase of growth, check the density of the culture at OD_{600}.
5. Further expanding the culture by preparing 20 of 125 mL shaker flasks with 40 mL LB broth supplemented with 400ul ampicillin each. Transfer 1 mL of the initial liquid culture to each of the new sterile shaker flasks and incubate overnight at 37 °C in a shaking incubator. Transfer the bacteria culture in log phase from each shaker flask to a thick wall polypropylene 50 mL tube (Beckman Coulter cat# 357005). Centrifuge at 6000 × *g* (7000 rpm for Beckman J2–21 centrifuge with JA-20 rotor) for 15 min at 4 °C, discard the supernatant and store the bacteria pellets at −20 °C until ready to isolate the plasmid DNA.

3.2 Isolation of CD Gene Plasmid DNA (See Note 1)

1. Thaw out the bacteria pellets from −20 °C freezer at room temperature for 10 min. Recentrifuge at 6000 × *g* (7000 rpm) for 10 min. Remove residue LB broth by inverting the tubes on a paper towel covered with alcohol wipe. This step is not necessary if thawed pellet is relatively dry. Resuspend the bacteria pellet in each 50 mL tube in 4 mL Buffer P1. Add 4 mL Buffer P2, mix thoroughly by vigorously inverting 4–6 times and incubate at room temperature (15–25 °C) for 5 min. If using LyseBlue reagent, the solution will turn blue. Maximum number of tubes to use each time is 8 when using Beckman J2–21 with JA-20 rotor. Add 4 mL prechilled Buffer P3, mix thoroughly by vigorously inverting 4–6 times. Incubate on ice for 15 min. If using LyseBlue reagent, mix the solution until it is colorless.
2. Equilibrate a QIAGEN-tip 100 by applying 4 mL Buffer QBT, and allow the column to empty by gravity flow into a sterile 50 mL conical tube. Use the blue column holder. Set up the same number of column with the number of bacteria pellet containing tubes. Centrifuge all the 50 mL tubes in Beckman J2–21 centrifuge with JA-20 rotor at 20,000 × *g* (13,000 rpm) for 30 min at 4 °C. Recentrifuge at the same speed for 15 min if the supernatant is not clear. Pellets contain genomic DNA, proteins, cell debris and potassium salt of SDS is discarded. Apply by dumping directly the supernatant from each tube into each QIAGEN-tip 100 column. Allow the supernatant to enter the resin by gravity flow.

3. Wash the QIAGEN-tip 100 with 2 × 10 mL Buffer QC. Allow Buffer QC to move through the QIAGEN-tip by gravity flow. Elute plasmid DNA with 5 mL Buffer QF just removed from 65 °C water bath from the QIAGEN-tip 100 column into a sterile Beckman 50 mL tube. For constructs larger than 45 kb like ours, pre-warming the elution buffer to 65 °C may help increase the yield.
4. Precipitate plasmid DNA by adding 3.5 mL room-temperature isopropanol (room temperature to minimize salt precipitation) and mix. Solution should look a little bit hazy. Centrifuge again in Beckman J2–21 centrifuge with JA-20 rotor at minimum 15,000 × *g* for 30 min at 4 °C. Carefully decant the supernatant. Keep the precipitates.
5. Wash the plasmid DNA pellet with 2 mL room-temperature 70% ethanol (prepared with 7 mL 100% ethanol and 3 mL autoclaved double deionized water adjusted to pH:8.0). This step is to remove precipitated salt and replace isopropanol with the more volatile ethanol) and centrifuge at minimum 15,000 × *g* for 10 min. Carefully decant supernatants. Air-dry plasmid DNA pellet for 5–10 min and redissolve DNA in a suitable volume of autoclave ddH_2O at pH: 8.0. We use 150 μL for each tube. DNA dissolves better in alkaline condition. Overdrying will make DNA difficult to redissolve. Be careful not shearing DNA by over pipetting to promote resuspension.
6. Save 2 μL of plasmid DNA sample from each tube and use Nanodrop 1000 to check the plasmid DNA concentration and quality based on the absorbance at 280 nm, 260 nm, 230 nm and the ratio of 260 nm/280 nm and the ratio of 260 nm/230 nm. The unit uses 340 nm for baseline normalization and 50 ng-cm/μL as a constant for dsDNA. The read out unit of the plasmid DNA concentration is ng/μL. The result is acceptable when the ratio of 260/280 is in the range of 1.8–2.0 and the ratio of 260/230 is in the range of 2.0–2.2 (*see* **Note 2**).

3.3 Polyplex Preparation

Protamine sulfate (PrS)/DNA polyplexes formed as follows (*see* **Note 3**).

1. Disperse 20 μg of pDNA in 500 μL of DI water.
2. Add dropwise pDNA in water to protamine sulfate solution while vortexing.
3. Leave the resulting polyplex undisturbed for 30 min to allow polyplex formation. Refrigerate prior to use for efficient complexation. N/P ratio of the PrS/DNA polyplexes, 10:1 (molecular ratio of amines of polyamine to phosphates of DNA).

4. Characterize the size and surface charge of the prepared polyplex by dynamic light scattering and zeta-potential analysis (Malvern Zetasizer Nano ZS).

3.4 Cell Preparation

1. Grow the tumor cells (F98) as adherent monolayers in a T25 culture flask in Advanced MEM medium (Gibco) supplemented with GlutaMax (Gibco), 2% heat-inactivated fetal Bovine serum (FBS) and GS/AB (Gibco) in a humidified CO_2 incubator at 37 °C and 7.5% CO_2 (*see* **Note 4**). When the cells have reached sub-confluence at late log growth phase, remove the entire growth medium from the T25 flask along with any floating F98 cells.
2. Add 2 mL TrypLE Express with flask standing with cap on top and tilted at a slight angle to avoid direct contact of TrypLE Express with the cell monolayer. Place the flask under the inverted microscope with the cell side down to allow direct contact of TrypLE Express with the cell monolayer under the microscope. Observe the cells and turn the flask with cap on top as soon as some of the cells start to detach from each other. Keep the TrypLE Express from direct contact with the cell monolayer and remove all the TrypLE Express with a serological pipette.
3. Place the flask in the 37C and 7.5% CO_2 incubator for 10 min. Check under the inverted microscope to make sure all the cells have rounded up. Tap the side of the flasks gently to dislodge all the cells before adding 2 mL fresh medium to wash down the cells. Transfer the cell suspension to a 15 mL. Pipette the cells up and down gently several times to generate single cell suspension.
4. Count the cell number either manually (C-chip or Neubauer improved hemocytometer) or with a Coulter counter. Resuspend the cells in culture medium at 25×10^4 cells/mL.

3.5 PCI Phase 1: Initial PCI-Mediated CD Gene Transfection (Fig. 1)

1. Aliquot 2.5×10^3 F98 cells per well in all wells. Spin the plate 1000 rpm for 30 min. Incubate the plate for 48 h (37 °C, 7.5% CO_2) (*see* **Note 5**).
2. Measure the diameters of each spheroid using an inverted microscope with a calibrated eyepiece micrometer, and record the measurements (*see* **Note 6**).
3. Aliquot 0.5 μg/mL of the photosensitizer $AlPcS_{2a}$ and the PrS/CD DNA polyplexes into the wells that will be receiving light treatment. A typical plate layout is shown in Fig. 2. Loosely wrap the plate in aluminum foil (to shield it from ambient light). Incubated for 18 h (*see* **Note 7**).

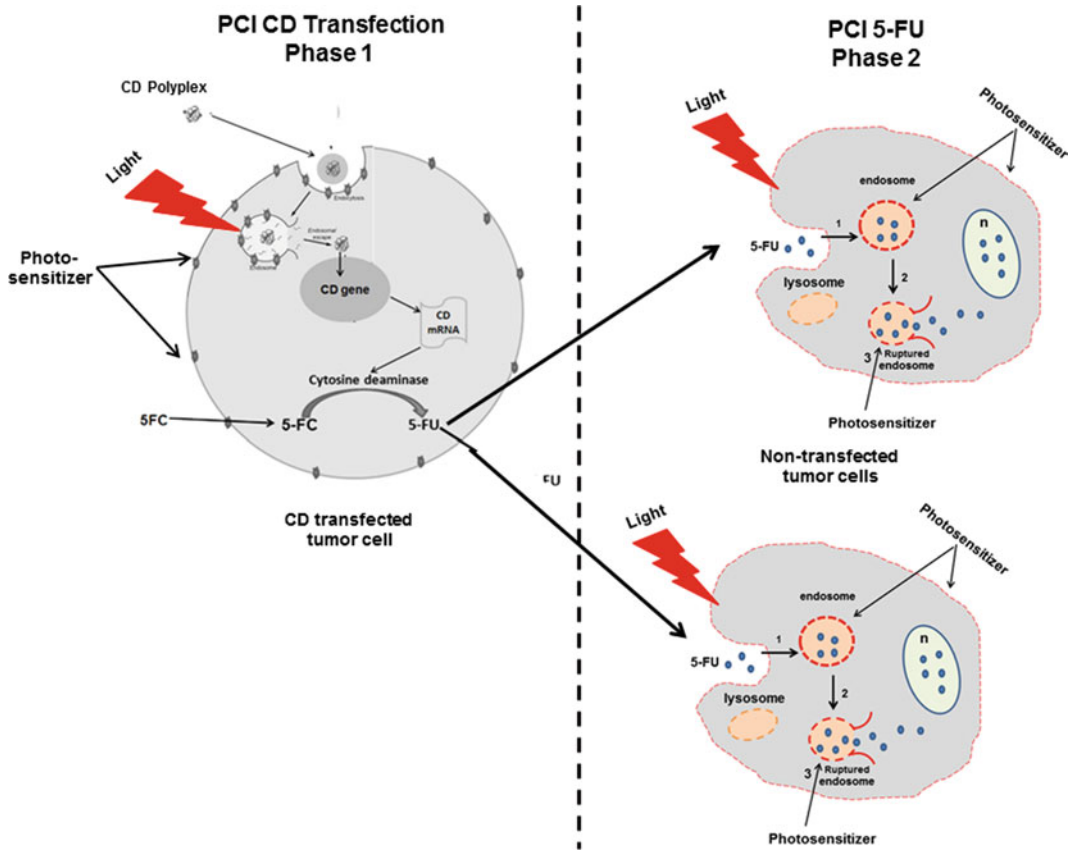

Fig. 1 Basic concept of PCI enhanced gene transfection followed by PCI enhanced drug efficacy. Phase 1: (1) Cell exposed to photosensitizer. (2) CD gene and carrier polyplex taken into cells via endocytosis. (3) Transported to endosome; released from entrapment by light treatment, 40 Induction of CD enzyme, converts 5-FC 5-FU 5) 5-FU exported to bystander non-transfected tumor cell; Phase 2: Second light treatment synergistically enhances the effect of the toxic metabolites of 5-FU, leading to enhanced transfected and non-transfected tumor cell death

4. Replace half of the medium in each well (100 µl) with fresh medium. Repeat 3 times with 10 min between cycles (*see* **Notes 7** and **8**). Incubate the plates for 4 h 37 °C, 7.5% CO_2 following the washing procedure. The incubator must have port for laser fiber insertion (*See* Fig. 3 for setup details).
5. Initiate light treatment, covering the entire plate (12 cm beam) for 8 min exposures at an irradiance of 2 mW/cm^2, administered from $\lambda = 670$ nm light from a diode laser (*see* **Notes 9** and **10**).

3.6 PCI Phase 2: Efficacy Enhancement of Converted 5-FU from 5-FC (Fig. 1)

1. Following light treatment, incubate the plate for 18 h. Each well should contain 200 µL. Replace 100 µL in each well with 100 µL of medium containing 50 µM 5-FC (*see* **Note 7**). Incubate the plate for 4 h.

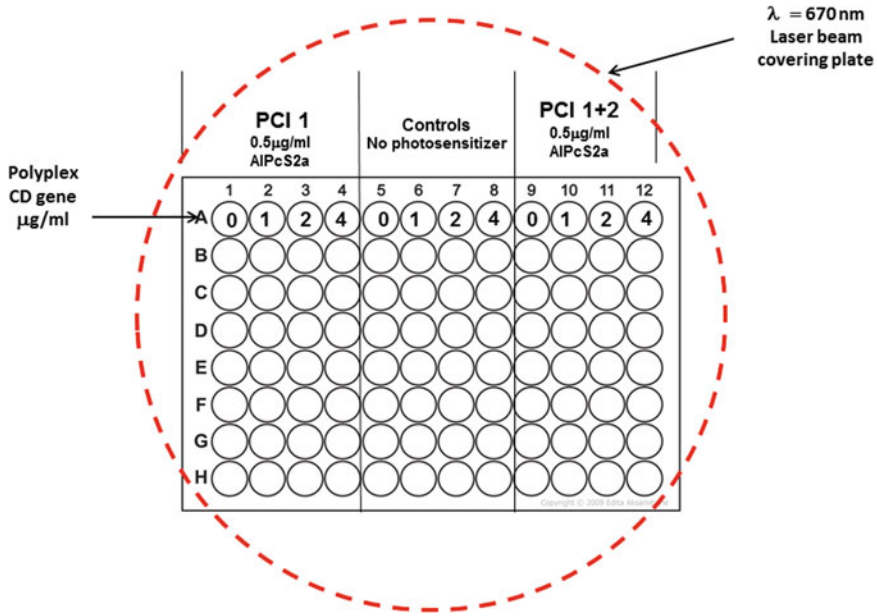

Fig. 2 Micro plate layout tumor for gene DNA titration. Spheroids formed in the wells. CD gene polyplexes at increasing concentrations and photosensitizer added as shown. First light treatment covers the entire plate (PCI phase 1). 5-FC added to all wells. Second light treatment administered only to columns 9–12

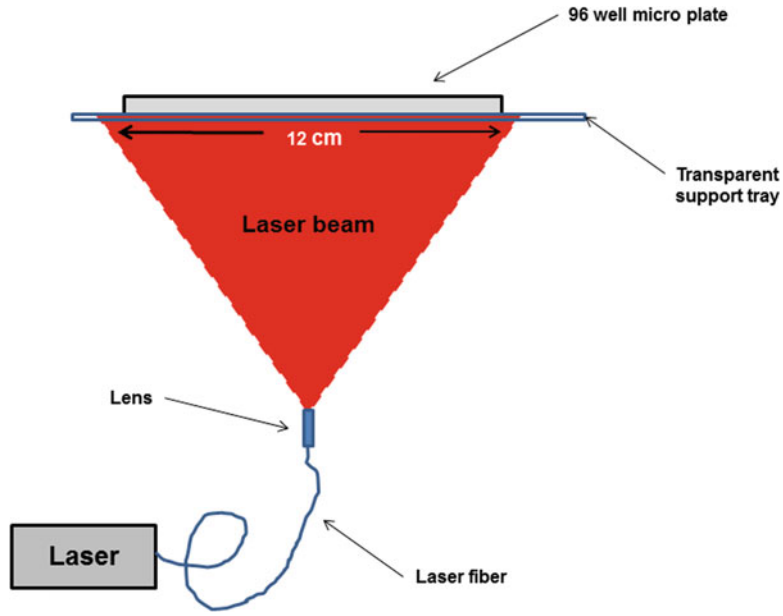

Fig. 3 Light treatment. Irradiance exposure time intervals generate various fluence levels at a constant irradiance of 2 mW/cm^2 administered with $\lambda = 670$ nm light from a diode laser (Intense HPD model 7404, New Jersey, USA). Laser light is coupled into a 200 μm dia. optical fiber containing a micro-lens at the output end (Frontal light distributor Med lightSA, Switzerland). The lens will form conical light beam. The distance from the micro-lens to the microplate is adjusted to form a 12 cm diameter beam pattern at the bottom of the plate. Irradiation is performed through the plate bottom with the micro plate supported on a transparent plastic plate

2. Place a rectangle of aluminum foil under the micro plate shielding columns 1–8 from the beam (only columns 9–12 should receive irradiance). Initiate light treatment with the laser beam only illuminating columns 9–12, for 8 min exposures at an irradiance of 2 mW/cm^2, $\lambda = 670$ nm.
3. Incubate the plate for 3 days (wrapped in foil). Do not expose to light during this interval (*see* **Note 11**).
4. Measure the diameters of each spheroid using an inverted microscope with a calibrated eyepiece micrometer, and record the measurements. Repeat the measurement every 3–5 days. Replace half of the culture medium in the wells every third day with fresh medium taking care not to accidently remove the spheroid. Terminate the plates 14 days after initiation.
5. Calculate the volume of the spheroids assuming a perfect sphere ($4/3\pi r^3$).
6. Figure 4 illustrates typical results following both phase 1 and 2 of PCI treatment with calculated volumes.

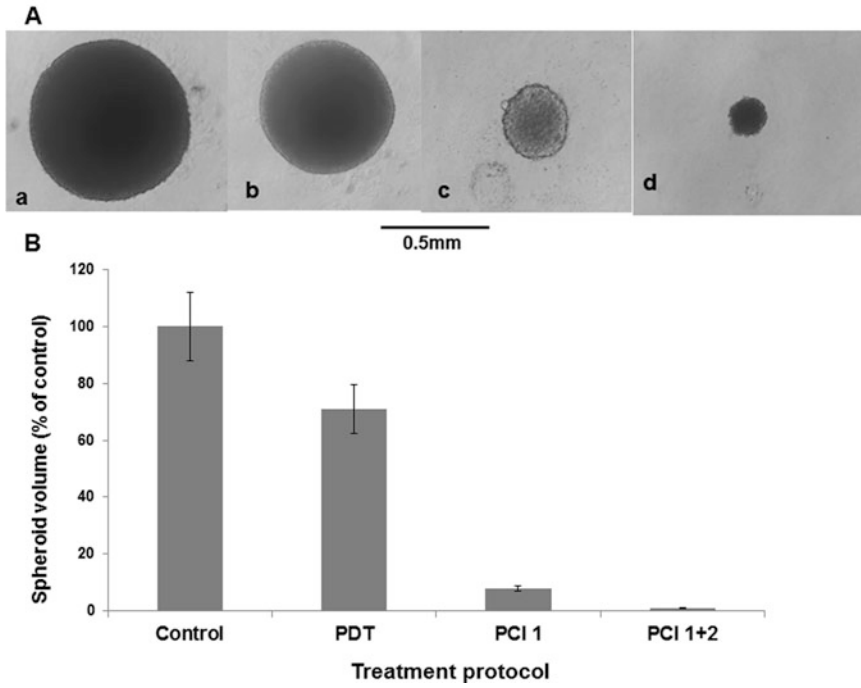

Fig. 4 Effects of PCI-mediated suicide CD gene therapy on spheroid growth. (**A**); Light micrograph of F98 tumor spheroids 14 days post treatment. (*a*) Non-treated control, (*b*) PDT control (column 9, Fig. 2), (*c*) PCI phase 1 CD transfection, 2 μg/mL DNA + 5-FC (column 3 Fig. 2), (*d*) PCI phase 1 + 2 CD transfection, 2 μg/mL DNA + second light treatment PCI drug enhancement (column 11 Fig. 2) (**B**); Calculated spheroid volumes from the measured diameters (shown in A) expressed as a % of non-treated controls 14 days post treatment

4 Notes

1. Preparation for Isolation of CD gene plasmid DNA: Place Buffer QF from QIAGEN Plasmid Midi Kit (QIAGEN Cat# 12143) in a 65 °C waterbath and make sure the bottle is secured to stay upright.

   Add RNase A solution to Buffer P1, mix, and store at 2–8 °C. Label as RNaseA added with the date. The combined buffer is good for 6 months. Add LyseBlue reagent to Buffer P1 with RNase A at a ratio of 1:1000. Check buffer P2 for SDS precipitation and prechill Buffer P3 at 4 °C.
2. Aromatic amino acids side chains within the proteins are responsible for the absorbance at 280 nm. The aromatic base moieties within the structure of nucleic acids are responsible for the peak absorbance of purines and pyrimidines at 260 nm. Many organic compounds have strong absorbance at around 225 nm along with peptide bonds in proteins absorb light between 200 and 230 nm. The 260/280 ratio is used to assess the purity of DNA and RNA with a ratio of ~1.8 for DNA and a ration of ~2.0 for RNA. A lower ratio indicates the sample is contaminated with proteins. The ratio of 260/230 is used as a secondary measure of nucleic acid purity with expected 260/230 ratio in the range of 2.0–2.2.
3. The gene carrier PrS Protamine Sulfate was used here due to its low toxicity [10]. Alternate carriers, such as branched polyethylenimine (bPEI), MW 25,000, (Sigma, St. Louis, MO), can also be used.
4. The F98 cells have a short doubling cycle and require splitting often. One day after confluence, they are no longer useable.
5. Other cell lines that easily form spheroids and can be used in place of the rat F98 cell line are, human; glioma U87, squamous cell carcinoma FaDu, and breast tumor MDA-MB-231 (all obtained from the American Type Culture Collection Manassas, VA, USA). If other cell lines are to be used a light dose titration should be done (PDT control). PCI is most efficient when PDT (i.e., no drug) cell or spheroid viability is 70–80%.
6. Spheroids usually form 48 h following centrifugation but occasionally an extra day of incubation is required.
7. All handling of the plates after the addition of the photosensitizer must be done in subdued external room and bench lighting. A dim blue light source can be used to facilitate work on the plate.
8. Careful washing of the spheroids is required to remove excess photosensitizer while avoiding the accidental removal during the washing process.

9. All light treatment should be done at 37 °C in an incubator that will allow accesses for the laser fiber.
10. The laser power required given in mW is: fluence rate x the area of the laser beam to cover the plate. In the protocol described here with a 12 cm diameter beam gives: 2 mW × 113 = 226 mW.
11. Do not expose the plates to external light either ambient or by observation through the inverted microscope until 3 days following light treatment.

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Chapter 14

Suicide Gene Therapy of Oral Squamous Cell Carcinoma and Cervical Carcinoma In Vitro

Nejat Düzgüneş, Jennifer Cheung, and Krystyna Konopka

Abstract

Suicide gene therapy induced by the Herpes Simplex Virus thymidine kinase/ganciclovir (HSV-tk/GCV) system has been utilized to successfully treat various cancers. We describe TransfeX-mediated transfection of pCMV.Luc into HeLa cervical carcinoma and HSC-3, FaDu, and H357 oral squamous cell carcinoma (OSCC) cell lines in the presence of 10% serum. This method has proved to be highly efficient, with low nonspecific cytotoxicity. The plasmid pNGVL1-tk encoding HSV-tk under the control of the CMV promoter was delivered to the cells in vitro via TransfeX, a cationic liposomal reagent, followed by treatment with ganciclovir. The Alamar Blue cell viability assay was used to determine levels of the suicide effect.

Key words Herpes simplex virus thymidine kinase, Ganciclovir, Oral squamous cell carcinoma, Cervical carcinoma, Transfection

1 Introduction

Oropharyngeal squamous cell carcinomas (OSCCs) that originate in the tissues that line the mouth and lips are some of the most common cancers worldwide. OSCC is the eighth most common cancer in the world, representing 2–4% of annually diagnosed cancers [1–4]. The 5-year survival rate is about 52%, a rate that has not improved over many years [5]. OSCCs develop generally from premalignant lesions of the oral mucosa [6]. Despite the fact that oral lesions occur at easily accessible sites, diagnosis is often made at late stages. Even with current treatments—chemotherapy, surgery, and radiotherapy—the recurrence rate for oral cancer has not improved, ranging from 30% to 47% for SCCs [1]. Cervical cancer is the third most common cause of cancer in women, and the second most common cancer in women in the developing world [7].

Cancer gene therapy aims to introduce genetic material into cells for therapeutic purposes. If a high percentage of cancer cells can be transfected, and the introduced gene can be expressed over a

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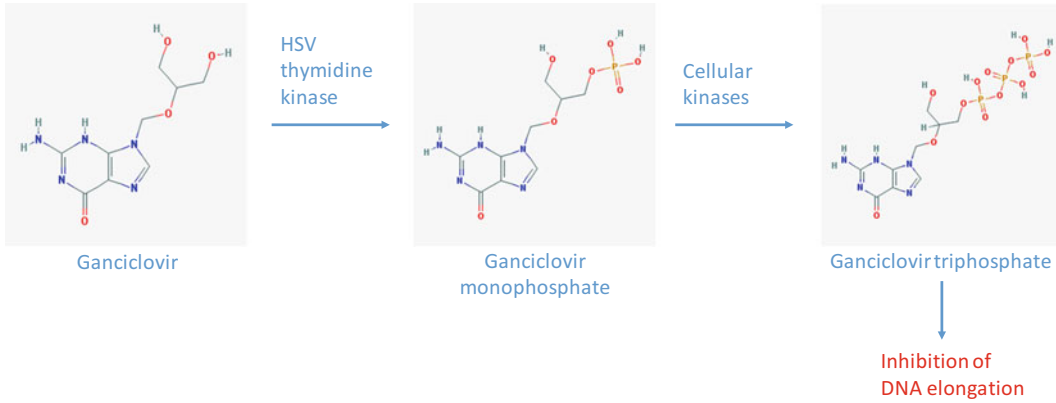

Fig. 1 Mechanism of action of ganciclovir. Delivery of the HSV-*tk* gene to target cells results in the expression of viral thymidine kinase, which selectively phosphorylates ganciclovir (GCV). Monophosphorylated GCV is further phosphorylated by cellular enzymes into an active triphosphate compound. The molecular structures were adapted from PubChem

relatively long period of time, it may be possible to induce apoptosis in the cancer cells by delivering a suicide gene, such as the herpes simplex virus thymidine kinase gene, HSV-*tk* [8]. Oral cancer is a particularly appropriate target for gene therapy, since direct injection of most primary and recurrent lesions is possible with this approach. Delivery of the HSV-*tk* gene to target cells results in the expression of viral thymidine kinase, which selectively phosphorylates ganciclovir (GCV). Monophosphorylated GCV is further phosphorylated by cellular enzymes into an active triphosphate compound (Fig. 1; *see* **Note 1**). The end product is incorporated into cellular DNA during replication, causing chain termination and inhibition of replication. In addition, the HSV-*tk*/GCV approach to gene therapy benefits from the bystander effect, in which neighboring, non-HSV-*tk* expressing cells receive monophosphorylated GCV via gap junctional intercellular communication [9–12].

A number of viral vectors have been used in transducing oral cancer cells in vitro or in vivo [13, 14]. Although generally efficient in transducing cells, viral vectors suffer from problems of immunogenicity, toxicity, limits in the size of exogenous DNA, and the risk of inducing tumorigenic mutations and generating active viral particles through recombination [14–16]. Synthetic cationic liposome-DNA (lipoplexes) or cationic polymer-DNA complexes (polyplexes) constitute an alternative to the use of viral vectors and provide a simple means of transferring DNA into target cells. They are advantageous, because they can accommodate large expression cassettes, they do not self-replicate, and the plasmids they carry can be made recombination-defective. In addition, liposomal complexes are noninfectious, cost-effective, and easy to administer [15–17].

Here we describe TransfeX-mediated transfection [18] of the pNGVL1-*tk* plasmid, expressing thymidine kinase under the control of the CMV promoter, and the pEEV plasmid, expressing HSV-*tk* under the CAG promoter (cytomegalovirus enhancer + beta-actin promoter + beta-globin splice acceptor), using HeLa cervical carcinoma cells and HSC-3 oral squamous carcinoma cells. Mock-transfected and transfected cells are incubated in the absence and the presence of ganciclovir (GCV). Cell viability (ganciclovir-mediated cytotoxicity) is quantified by a modified Alamar Blue assay [19, 20].

2 Materials

2.1 Cell Culture

1. Cell lines: HeLa cervical carcinoma cells (ATCC, Manassas, Virginia) and HSC-3 OSCC cells provided by Dr. T. Chino (University of the Pacific).
2. Incubator set to 37 °C under 5% CO_2.
3. Sterile phosphate buffered saline (PBS) (UCSF, San Francisco, CA; or Thermo Fisher Scientific, Waltham, MA).
4. Accutase Cell Detachment Solution (Innovative Cell Technologies, San Diego, CA).
5. High glucose Dulbecco's modified Eagle's medium containing L-glutamine (Thermo Fisher Scientific), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), and 1% Antibiotic-Antimycotic (Thermo Fisher Scientific) (designated DME/10).
6. Tissue culture flasks T-25 and T-75 (Thermo Fisher Scientific).
7. 48-well culture plates (Grenier Bio-One CELLSTAR, Monroe, NC).
8. Countess Cell Counter (Thermo Fisher Scientific).
9. Trypan Blue solution (Sigma).

2.2 Lipoplex Complexation

1. TransfeX™ (ATCC®, Manassas, VA) transfection reagent.
2. Plasmid expressing thymidine kinase under the control of the CMV promoter (pNGVL1-*tk*) obtained from the National Gene Vector Laboratory at the University of Michigan (Ann Arbor, MI).
3. EEV plasmid expressing thymidine kinase under the CAG promoter (cytomegalovirus enhancer + beta-actin promoter + beta-globin splice acceptor) (pEEV-HSV-*tk*) custom-made by System Biosciences (SBI) (Palo Alto, CA).
4. 12 × 75 mm polystyrene tubes (BD Biosciences, San Jose, CA).

5. High glucose Dulbecco's modified Eagle's medium containing L-glutamine (DMEM) (without FBS).
6. Sterile PBS.

2.3 Cell Viability Assay

1. Alamar Blue dye (Life Technologies, Carlsbad, CA).
2. DMEM/10.
3. 96-well plates (Grenier Bio-One, Kremsmünster, Austria).
4. VersaMax Tunable ELISA Microplate Reader (Molecular Devices, Sunnyvale, CA).

2.4 Ganciclovir-Mediated Cytotoxicity

1. Ganciclovir (GCV) Synonyms: 9-(1,3-Dihydroxy-2-propoxymethyl)guanine; 2′-Nor-2′-deoxyguanosine; 2′-NDG; BIOLF-62; DHPG; BW-759 U) (Sigma-Aldrich, St. Louis, MO).
2. Sterile PBS.
3. DME/10.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Cell Culture

1. Incubate HeLa and HSC-3 cells in tissue culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2, and passage them 1:6 twice a week in DME/10.
2. Remove the medium (DME/10) from the culture flask, wash with 5 mL of PBS, remove the PBS, and add 1 mL Accutase Cell Detachment Solution. Tilt the flask until the cells are detached, add 9 mL of DME/10, and transfer 2 mL into a flask containing 10 mL of fresh medium.
3. Evaluate cell morphology by inverted phase contrast microscopy at 25× magnification.
4. One day prior to transfection, and before the cells are diluted for passaging, mix 20 μL of the cells with 20 μL Trypan blue solution, place in a Countess chamber slide, and read on the Countess Cell Counter.
5. According to the cell count, prepare a cell suspension of 1.5×10^5 cells/mL DME/10, and place 1 mL of the suspension into each well of a 48-well cell culture plate.
6. The next day, check that the cells are at approximately 85% confluence, and proceed with the transfection.

3.2 Transfection Procedure

1. Commercially available TransfeX is a cationic lipid formulation suspended in an aqueous solution.
2. The pNGVL1-*tk* and pEEV-HSV-*tk* plasmids are stored at −20 °C. Thaw both plasmids at room temperature before the experimental procedure.
3. Mix 2 μL TransfeX and 1 μg pNGVL1-*tk* or pEEV-HSV-*tk* and allow to complex at room temperature for 15 min in 12 × 75 mm polystyrene tubes in a total of 100 μL DMEM (supplemented with L-glutamine, but without FBS or antibiotics).
4. Prepare the required quantity of complexes of TransfeX and plasmids, depending on the number of wells being used in the experiment. For example, for 13 wells (12 wells + extra for 1 well to accommodate losses), use 1272.43 μL DME (transfection media) + 1.57 μL pNGVL1tk stock plasmid (stock concentration: 8.285 μg/μL) + 26 μL TransfeX (added last); allow 15 min to complex.
5. While waiting for complexation, replace media in control wells (without lipoplexes) with fresh DME/10. For lipoplex conditions, replace the media with 900 μL of DME/11 (i.e., 11% serum).
6. Add 100 μL of the lipoplexes to each well containing 900 μL of DME/11.
7. Incubate cells for 24 h at 37 °C with lipoplexes before the addition of GCV. Cells incubated with medium alone serve as controls.
8. Determine cell viability at 48 and 96 h after the addition of GCV, using the Alamar blue procedure.

3.3 Ganciclovir-Mediated Cytotoxicity

1. Prepare a stock solution of GCV in PBS (1 mg/mL) and store at 2–8 °C.
2. Add 20 μL of the GCV solution to one half of the control and of the transfected cells (final concentration: 20 μg GCV/mL) (+GCV conditions).
3. Add 20 μL of sterile PBS to the remaining wells with control and transfected cells (–GCV conditions).
4. Incubate cells in the absence and the presence of GCV for 48 and 96 h, and measure cell viability after each of these time points (*see* **Note 2**).

3.4 Cell Viability Assay

1. Prepare 10% (v/v) Alamar Blue dye in DMEM/10 medium.
2. Remove completely the medium from wells incubated with medium ± lipoplexes and ± ganciclovir.
3. Add 1 mL of 10% (v/v) Alamar Blue dye in DMEM/10 medium to each well and incubate at 37 °C under 5% CO_2 for approximately 90 min.

Table 1
Suicide gene therapy in HeLa and HSC-3 cells. Cell viability as expressed by OD_{570}-OD_{600} in the Alamar Blue assay

	HeLa cells	HSC-3 cells
Control cells − GCV	0.329 ± 0.005	0.332 ± 0.014
Control cells + GCV	0.308 ± 0.008	0.335 ± 0.023
pNGVL1-tk − GCV	0.323 ± 0.008	0.299 ± 0.009
pNGVL1-tk + GCV	0.112 ± 0.006	0.022 ± 0.005
pEEV − GCV	0.325 ± 0.005	0.286 ± 0.035
pEEV + GCV	0.184 ± 0.006	0.194 ± 0.017

Sensitivity of HeLa cervical carcinoma cells and HSC-3 oral squamous carcinoma cells transfected with the plasmid pNGVL1-*tk*, expressing thymidine kinase under the CMV promoter, and the pEEV plasmid, expressing HSV-*tk* under the CAG promoter (cytomegalovirus enhancer + beta-actin promoter + beta-globin splice acceptor) using a cationic liposomal transfection reagent, TransfeX. Mock-transfected and transfected cells were incubated in the absence and the presence of ganciclovir (GCV). Cell viability was quantified by the modified Alamar Blue assay 96 h post-transfection. Results are expressed as OD_{570}-OD_{600} values. Data represent the mean ± standard deviation obtained from triplicate wells.

4. Following incubation, collect 200 μL of the supernatant from each well and transfer to the wells of a 96-well plate.
5. Measure the absorbance at 570 nm and 600 nm, using the VersaMax Tunable ELISA Microplate Reader.
6. Under these conditions, the control OD_{570}-OD_{600} values ± GCV are usually in the range from 0.300 to 0.350 with both HeLa and HSC-3 cells (Table 1).
7. Calculate cell viability (as a percentage of mock-treated control cells) according to the formula [(A570–A600) of test cells] × 100/[(A_{570}–A_{600}) of control cells].
8. The relative percentage changes define the cytotoxic efficacy of the experimental conditions used throughout transfection procedures.
9. For less metabolically active cells, extend the time of incubation with the Alamar Blue dye to 2–3 h.
10. After the Alamar Blue assay, remove the dye, add fresh medium with the same concentration of GCV, and continue the incubation until the next time-point.

4 Notes

1. The genetic approach to the treatment of cancer is based on the hypothesis that the transfer and expression of the therapeutic genes in target cells will cause a cytotoxic effect or mediate apoptosis and/or necrosis. The idea that has received recently considerable attraction for cancer therapy is targeted suicide

gene therapy (gene directed enzyme prodrug therapy; GDEPT). GDEPT is a two-step process where the cancer cells are first transduced by a gene coding for a nontoxic enzyme (suicide gene), followed by administration of a nontoxic prodrug [21, 22]. The HSV-*tk*/GCV system is the most abundantly used enzyme/prodrug combination in preclinical suicide gene therapy studies.

2. Achieving cytotoxicity with the HSV-*tk*/GCV system is not always straightforward. Conditions including the GCV concentration, the amount of DNA, the TransfeX to DNA ratio, and transfection time, and the duration of incubation with GCV can all affect the ability of the system to effect cytotoxicity. These conditions should be optimized for each cell system utilized. Low transfection efficiency (the percentage of cells that are measurably transfected), measured by the expression of the green fluorescent protein gene or ß-galactosidase staining, may not necessarily result in low cytotoxicity in the HSV-*tk*/GCV system, because of the bystander effect where the initial phosphorylated product of GCV can be transferred to neighboring cells [11, 12]. The cytotoxicity of just the transfection procedure, using the same plasmid, but without GCV, should be checked before the suicide gene therapy experiments are carried out.

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