

ABSTRACT

PRUITT, AMY FORISTER. Heat Induced Reporter and Therapeutic Gene Expression in Cancer Gene Therapy. (Under the direction of Marlene Hauck.)

Gene therapy for cancer is an important research focus, with most approved gene therapy clinical trials involving cancer patients. This reflects, in part, the inadequacies of conventional treatment for advanced, refractory solid tumors. However, significant problems remain to be solved before gene therapy will become a first line modality in the clinical setting. Two challenges to overcome are the need for targeted transgene expression and noninvasive evaluation of transgene expression. In this study, we hypothesized that the use of the heat inducible heat shock protein 70 promoter (HSP) in combination with the human somatostatin receptor type 2 (hSSTR2) reporter gene will allow for the *in vivo*, non-invasive monitoring and quantification of targeted therapeutic gene (murine Interleukin 12) expression. Replication-incompetent adenoviral vectors containing either the therapeutic gene (AdHSPmIL-12) or the reporter gene (AdHSPsstr2) under control of the HSP were constructed and their function first assessed *in vitro*. Cells were infected with both vectors and then heated at temperatures that ranged from 38-42° C. Uninfected cells and infected cells at 37° C served as controls. Twenty-four hours after heating, radiolabeled somatostatin analogue was added to the wells, and radioligand receptor binding was measured with a gamma counter. A murine interleukin-12 (mIL-12) ELISA kit was used to measure mIL-12 levels in the supernatants. The adenoviral vectors function was also assessed *in vivo*. Balb/c and C57BL/6 mice with subcutaneous mammary adenocarcinoma and murine melanoma tumors respectively received intratumoral co-injection of 1×10^8 pfu each of AdHSPsstr2 and AdHSPmIL-12 followed by either no hyperthermia, hyperthermia for 40 minutes at 41.5°C or hyperthermia for 40 minutes at 42.5°C. Additional animals not receiving intratumoral vector injection served as controls. Radioligand was injected via the tail vein 48 h

after heating, and animals were sacrificed one hour after tail vein injection. Tissues were collected and counted in a gamma counter. Tumors were also disaggregated in lysis buffer using a Mini-Beadbeater, and murine IL-12 concentrations in tumor extracts were determined using a mIL-12 ELISA kit. In the *in vitro* experiments, a statistically significant heat-dose response of radioligand binding for the reporter gene and mIL-12 production for the therapeutic gene was detected, with minimal expression observed in the controls. Results demonstrated a strong correlation between radioligand uptake and mIL-12 production in response to heat treatment (Pearson coefficient 0.95, $p = < 0.0001$). A statistically significant heat-dose response of tumor radioligand uptake for the reporter gene and tumor mIL-12 production for the therapeutic gene was also detected *in vivo*, with minimal expression observed in the controls. Normal tissue biodistribution of radioligand was similar between all treatment groups with no significant difference between the infected groups and the non-infected control. Results demonstrated a strong correlation between tumor radioligand uptake and tumor mIL-12 production in response to heat treatment (Pearson coefficient 0.85, $p = < 0.0001$). The results of this study indicate that gene expression from both vectors could be induced *in vitro* and *in vivo* by hyperthermia in a temperature dependent manner and that induced expression from the reporter and therapeutic gene vectors were highly related. Normal tissue biodistribution of radioligand was similar between all treatment groups with no significant difference between the infected groups and the non-infected controls demonstrating the ability of the HSP promoter to limit transgene expression to the tumor. Results have the potential to pave the way for evaluation of heat induced gene therapy with hSSTr2 reporter monitoring in animals and humans.

Heat Induced Reporter and Therapeutic Gene Expression in Cancer Gene Therapy

by
Amy Forister Pruitt

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Comparative Biomedical Sciences

Raleigh, North Carolina

2008

APPROVED BY:

Mark Dewhirst

Jill Barnes

Jorge Piedrahita
Co-Chair of Advisory Committee

Marlene Hauck
Chair of Advisory Committee

BIOGRAPHY
CURRICULUM VITAE

Amy Forister Pruitt

Education

Doctor of Philosophy, May 2008.

North Carolina State University, College of Veterinary Medicine,
Raleigh, North Carolina.

Residency in Radiation Oncology, 1999-2001

North Carolina State University, College of Veterinary Medicine,
Raleigh, North Carolina.

Internship in Small Animal Medicine and Surgery, 1998-1999.

University of Pennsylvania, College of Veterinary Medicine, Philadelphia,
Pennsylvania.

Doctor of Veterinary Medicine, May, 1998.

North Carolina State University, College of Veterinary Medicine, Raleigh, North
Carolina.

Bachelor of Arts in Biology, *Magna Cum Laude*, December 1993.

University of North Carolina at Greensboro, Greensboro, North
Carolina.

Licenses and Specialty Board Certification

Diplomate, American College of Veterinary Radiology (Radiation Oncology), 2002

Licensed for practice of veterinary medicine and surgery, North Carolina, 2002

Employment

Clinical Assistant Professor of Radiation Oncology

North Carolina State University, College of Veterinary Medicine,
Raleigh, North Carolina. July 2005 - present

Research Associate - Hyperthermia and perfusion effects in cancer therapy.

North Carolina State University, College of Veterinary Medicine,
Raleigh, North Carolina. July 2001 – June 2005

Professional Organization Membership

American College of Veterinary Radiology

Veterinary Cancer Society

American Veterinary Medical Association

TABLE OF CONTENTS

LIST OF TABLES_____	iv
LIST OF FIGURES_____	v
INTRODUCTION_____	1
HISTORY OF GENE THERAPY_____	1
PROBLEMS TO OVERCOME IN GENE THERAPY_____	4
VECTORS USED IN GENE THERAPY_____	5
ADENOVIRUSES_____	6
RETROVIRUSES_____	8
ADENO-ASSOCIATED VIRUS_____	9
HERPES SIMPLEX VIRUS_____	10
CANCER GENE THERAPY_____	10
IMUNOPOTENTIATION_____	11
SUICIDE GENE THERAPY_____	14
TUMOR SUPPRESSOR GENES AND ONCOGENES_____	15
ANTIANGIOGENESIS_____	17
CONDITIONALLY REPLICATIVE ONCOLYTIC VIRUSES_____	18
TARGETTING TRANSGENE EXPRESSION_____	19
PHYSICAL TARGETTING_____	20
PASSIVE TARGETTING_____	21
TRANSDUCTIONAL TARGETTING_____	22
TRANSCRIPTIONAL TARGETTING_____	26
INDUCIBLE PROMOTERS_____	31
HEAT SHOCK PROTIEEN PROMOTER_____	33
NON-INVASIVE MONITORING OF TRANSGENE EXPRESSION_____	37
REPORTER GENES_____	38
SOMATOSTATIN RECEPTOR REPORTER GENE_____	40
INTRODUCTION SUMMARY_____	42
<i>IN VITRO</i> CORRELATION OF HEAT INDUCED REPORTER AND THERAPEUTIC GENE EXPRESSION_____	43
INTRODUCTION_____	46
MATERIAL AND METHODS_____	49
RESULTS_____	52
DISCUSSION_____	55
<i>IN VIVO</i> CORRELATION OF HEAT INDUCED REPORTER AND THERAPEUTIC GENE EXPRESSION_____	72
INTRODUCTION_____	75
MATERIALS AND METHODS_____	78
RESULTS_____	82
DISCUSSION_____	85
SUMMARY/FUTURE CONCEPTS_____	98
LITERATURE CITATIONS_____	102

LIST OF TABLES

IN VITRO CORRELATION OF HEAT INDUCED REPORTER AND THERAPEUTIC GENE EXPRESSION

TABLE 1. *In Vitro* Spearman rank-order correlation coefficients and Pearson correlation coefficients for mIL-12 with radioligand binding_____70

TABLE 2. *In Vitro* Regression analysis results_____70

IN VIVO CORRELATION OF HEAT INDUCED REPORTER AND THERAPEUTIC GENE EXPRESSION

TABLE 3. Biodistribution of [¹²⁵I] Gluc-TOCA in mice bearing 4T1 murine mammary tumors evaluating the HSP promoter at different temperatures_____93

TABLE 4. Biodistribution of [¹²⁵I] Gluc-TOCA in mice bearing 4T1 murine mammary tumors comparing the HSP promoter with the CMV promoter_____94

TABLE 5. *In Vivo* Spearman rank-order correlation coefficients and Pearson correlation coefficients for mIL-12 with radioligand binding in tumors_____95

TABLE 6. *In Vivo* Regression analysis in tumors_____95

LIST OF FIGURES

IN VITRO CORRELATION OF HEAT INDUCED REPORTER AND THERAPEUTIC GENE EXPRESSION

FIGURE 1. Radioligand uptake (surface-bound and internalized) reported in counts per minute (CPM) as a function of incubation time with (125 I)Gluc-TOCA_____ 62

FIGURE 2. Comparison of radioligand uptake in counts per minute from B16 cells infected with 10 pfu/cell of either AdCMVhsstr2 or AdHSPhsstr2_____ 63

FIGURE 3. Radioligand uptake and mIL-12 production as a function of viral vector dose_____ 64

FIGURE 4. Radioligand uptake and mIL-12 production from infected and non-infected B16 cells at 42° C or remaining at 37° C_____ 66

FIGURE 5. Radioligand uptake in counts per minute (CPM) and mIL-12 production in pg/ml as a function of heat dose_____ 68

FIGURE 6. Correlation of radioligand uptake and mIL-12 production from B16 cells and 4T1 cells coinfecting with 15 pfu/cell of each AdHSPhsstr2 and Ad HSPmIL-12 and exposed to variable heat doses_____ 71

IN VIVO CORRELATION OF HEAT INDUCED REPORTER AND THERAPEUTIC GENE EXPRESSION

FIGURE 7. Data from Balb/c mice bearing 4T1 mammary tumors that were co-injected with AdHSPhsstr2 and AdHSPmIL-12 at 1×10^8 pfu each and non-infected controls_____ 96

FIGURE 8. Correlation of tumor radioligand uptake in % injected dose/gram tumor and tumor mIL-12 concentration in pg mIL-12/gram tumor from Balb/c mice bearing 4T1 mammary tumors and from C57BL/6 mice bearing B16.F10 melanomas_____ 97

INTRODUCTION

Rapid advances in molecular sciences and the elucidation of the human genome over the past decade have fostered remarkable insights into the molecular basis for many diseases and into the development of novel therapies at the genetic level. Continued improvements in recombinant DNA technologies and in vector systems, identification of genes associated with specific diseases and the development of novel mouse models of human diseases have lead to significant progress in the field of gene therapy (1-3). A form of molecular medicine, gene therapy has the potential to provide new treatments for a considerable number of inherited and acquired diseases. The goal of gene therapy is to successfully introduce genetic material into target cells with the intent to either cure or slow the progression of disease (3, 4).

HISTORY OF GENE THERAPY

The results of the first gene transfer experiment involving the delivery of gene-marked tumor-infiltrating lymphocytes to patients receiving treatment for malignant melanoma were published in 1990 (5). Since that time the number of gene therapy clinical trials has increased dramatically, with a recent report stating that over 1,340 clinical gene therapy trials had been completed, were ongoing or approved worldwide (6). Gene therapy was initially developed for treating inherited monogenic disorders by inserting a normal copy of a mutant or deleted gene. Examples of gene therapy for monogenic disorders that have been evaluated include severe combined immunodeficiency (SCID), cystic fibrosis, hemophilia, familial hypercholesterolemia, Duchenne muscular dystrophy and Gaucher's disease (1, 7). While originally envisioned for the treatment of monogenic disorders, the interest in and application of gene therapy soon broadened. Much attention has been directed

at the potential gene therapy has to offer for treatment of more complex, multifactoral diseases such as cardiovascular disorders, neurologic disorders and cancer (1, 2, 8)

While gene therapy has great potential to improve human health, progress in this new medical field suffered from a couple of serious adverse events. In 1999, the death of a patient during a gene therapy clinical trial at the University of Pennsylvania caused several trials to be temporarily halted by the US Food and Drug Administration (9-11). The patient experienced an exaggerated and unexplained innate immune response to a second-generation E1 and E4-deleted adenovirus vector during a clinical safety trial for the treatment of ornithine transcarbamylase deficiency, a genetic disorder that affects the body's ability to get rid of ammonia. Eighteen patients were treated in the trial with the adenovirus vector infused into the right hepatic artery. The patient who died received the highest dose of the vector used in the trial, as did an additional patient who experienced no adverse side effects. The question that remains unclear is why the one patient suffered such severe side effects, whereas the other patient received a similar dose of the vector and tolerated it well. There is some indication that previous exposure to wild-type virus infection might have sensitized his immune system to the vector. The case highlighted the substantial patient-to-patient variation in immune responses to systemically administered vectors and the need for further study of the host immune response to these vectors (12).

Enthusiasm for gene therapy returned when the first gene therapy success was reported in 2000. It involved the successful treatment of two patients suffering from X-linked severe combined immunodeficiency (SCID-X1), an inherited disease characterized by an early block in T and natural killer lymphocyte differentiation (13). Between 1999 and 2002, 10 patients were enrolled in this gene therapy clinical trial, and treatment was

successful in 9 out of the 10 patients (14). However, the initial enthusiasm turned to alarm in 2003 when reports were published that 2 of the 10 patients had developed a leukemia-like, clonal T-cell lymphocyte proliferation that became clinically overt after 3 years (15). It was subsequently shown that this serious complication resulted from retroviral vector genome integration near the LMO2 proto-oncogene promoter, leading to aberrant transcription and expression of LMO2 (16). Insertional mutagenesis resulting in clonal T-cell proliferation ultimately occurred in 3 patients, leading to the death of 1 patient while chemotherapy resulted in complete remissions for the other 2 patients (4). However, this was a deadly disease with imperfect alternatives. These complications emphasize the need for continued evaluation of the risk/benefit ratio in clinical trials and for continued research seeking to make existing vectors safer and prevent such incidents in the future (17).

For a period after these two serious adverse events, gene therapy maintained a low profile stemming from decreased public confidence in the field. However, progress was being made in many areas of gene therapy. To date, 17 out of 20 SCID-X1 patients enrolled in gene therapy clinical trials have been successfully treated (4). Likewise, adenosine deaminase deficiency (ADA-SCID), another form of SCID, has been successfully treated using a similar approach in two recent clinical trials (18, 19). While gene therapy for SCID is the most significant success story thus far, there has been promising developments in gene therapy for a range of diseases (6, 20, 21). Clinical trials have been conducted evaluating gene therapy for skin diseases, eye diseases, neurological disorders, HIV/AIDS, ornithine transcarbamylase deficiency, α 1-antitrypsin deficiency, haemophilia, cystic fibrosis, muscular dystrophies, cardiovascular disease and cancer (20, 21). Of particular note, evidence of clinical efficacy has been demonstrated for: junctional epidermolysis bullosa, a

severe recessive skin blistering condition (22), chronic granulomatous disease, an inherited primary immunodeficiency that affects phagocytes (23), Parkinson's disease, a devastating degenerative disorder of the central nervous system marked by progressive loss of dopamine producing cells in the midbrain (24, 25), and cancer, with successful immunotherapy treatment of two patients with metastatic melanoma (26) .

PROBLEMS TO OVERCOME IN GENE THERAPY

While these positive results are encouraging, significant problems remain to be solved before gene therapy will become a first line therapy. These include the lack of efficient gene transfer, lack of sustained or regulated gene expression, host immune responses, vector safety issues (insertional mutagenesis, severe immune/inflammatory response), inadequate targeting of transgene expression to appropriate cells and the need for noninvasive evaluation of transgene expression (7, 27, 28). The degree of importance of each problem may depend on the specific therapeutic application.

One of the major impediments to the successful, widespread application of gene therapy strategies is the development of safe and effective vectors for gene delivery and expression in cells (3). Development of vectors that can selectively and efficiently deliver a gene to target cells with minimal toxicity will largely determine the success of gene therapy (29). An ideal vector should be easy to produce at high titer and amenable to commercial production. It should be able to produce sustained or regulated transgene expression. Stable, sustained gene expression would be desirable for treatment of a monogenic disease like hemophilia but may result in increased toxicity in a cancer cytotoxic gene therapy approach where gene expression may need to be more tightly regulated. The vector should be capable of transducing both dividing and non-dividing cells. Vector components should not illicit an

immune response after transduction. A local inflammatory response can result in lysis of transduced cells and decreased duration of transgene expression, while development of virus-neutralizing antibodies limits the potential for repeated administrations of the vector.

Marked activation of an innate immune response can be life-threatening. When attempting to achieve sustained transgene expression using integrating vector systems, it is important to consider the potential hazards of insertional mutagenesis. In this instance, vectors capable of site-specific integration will be appealing (3, 30).

VECTORS USED IN GENE THERAPY

Vectors fall into two broad categories: nonviral and viral vectors (31). Some examples of nonviral vectors include direct injection of naked DNA, liposomes (cationic lipids mixed with nucleic acids) and molecular conjugates (cationic polymer/nucleic acid complexes). While nonviral vectors are easy to produce in relatively large quantity and are less likely to present toxic or immunologic problems, they so far have suffered from poor transfection efficiencies compared to viral vectors, and expression of the transgene tends to be transient (29, 31, 32). Viruses are efficient in transducing cells and so far have been the most suitable vehicles for efficient gene transfer.

The most commonly used vectors are retroviral and adenoviral vectors which combined have been used in almost 50% of human clinical trials. Retroviral vectors were the first to be used as gene therapy vectors, and until recently, had been the most commonly used vector. The decrease in the proportion of trials using retroviral vectors may be the consequence of the serious adverse events seen in the SCID clinical trial. Adenoviruses are now the most commonly used vector in human clinical trials (6). Adeno-associated viruses and Herpes

simplex virus-1 (HSV-1) have also been studied to a great extent and have been evaluated in many preclinical and some clinical gene therapy applications.

ADENOVIRUSES

Adenoviruses are DNA viruses, with 42 serotypes known to infect humans. Most gene therapy vectors are based on serotype 5, with deletions in one or two viral early genes (E1 and E3) to prevent replication. More recent adenoviral vectors have also had additional deletions in other early genes (E2 and/or E4) to reduce host immune response to the vector (33). Adenovirus vectors are relatively limited by the size of the transgene that can be inserted, with current vectors capable of inserting 8-9 kb of foreign DNA (30 kb for helper dependent vectors deleted for all viral genes). Most human cells are susceptible to adenovirus infection, and adenoviral vectors can efficiently transduce both dividing and non-dividing cells. The adenovirus vectors have been described as, arguably, the most efficient category of vector for transgene delivery (33). Cell entry of the adenoviral vector starts with binding of the virus fiber knob to the primary receptor, the coxsackie-adenovirus receptor (CAR). Internalization of the virus is mediated by the interaction of a penton-base Arg-Gly-Asp (RGD) motif and cellular $\alpha_v\beta_3$ & 5 integrins, which leads to endocytosis of the virion via clathrin-coated pits (34). Because most human cells are susceptible to adenovirus infection, a potential disadvantage of this vector system is the lack of target cell selectivity. However, various strategies have been evaluated to modify adenovirus tropism in order to target cell transduction. These include the use of adaptor molecules that can retarget the adenovirus binding to alternative receptors and genetic modification of the receptor-binding knob domain to achieve CAR-independent binding to target cell associated receptors (34, 35). The adenoviral life cycle does not require integration into the host cell genome. Instead

it has an extrachromosomal genome that replicates as an episomal element in the host cell nucleus. Typically with adenoviral vector systems, there is transient transgene expression. This is due in part to the lack of integration into the host DNA, but the transient expression also results from the host's immune response to the vector (33). T-cell mediated response against adenoviral proteins causes a local inflammatory response that can result in lysis of transduced cells and consequent decreased duration of transgene expression *in vivo*. The development of virus-neutralizing antibodies limits the potential for repeated administrations of the vector. The strong immunogenicity and resultant transient transgene expression of adenoviral vectors are a disadvantage for many gene therapy applications. However, for some gene therapy applications, transient expression may be an advantage, such as in the treatment of vascular and coronary artery disease and cancer (33). In the case of cancer treatment, immunogenicity and cellular toxicity may enhance the antitumor effect (36). The major safety issue with adenoviral vectors is the possibility of provoking a severe immune and inflammatory response as was the case in the ornithine transcarbamylase deficiency clinical trial (6). Strategies that are currently being examined to avoid this drawback of adenoviral vectors include immunosuppression, immunomodulation, serotype switching, use of targeted adenoviral vectors with restricted tropism, microencapsulation of adenoviral vectors, use of helper-dependent adenoviral vectors, and development of nonhuman adenoviral vectors (37). In addition, because adenoviruses are relatively stable and resistant to physical manipulations, an advantage is that high titer viral stocks can be generated and sample aliquots can be frozen for use at later times.

RETROVIRUSES

Retroviruses are single stranded RNA viruses. Retroviral vectors also have a relatively limited size capacity to carry transgenes (approximately 8 kilobases). They target actively dividing cells with a high degree of efficiency, an example being the C-type retrovirus, Moloney murine leukemia retrovirus (Mo-MuLV). Their inability to infect non-dividing cells has been the main limitation of retroviral vectors, restricting in vivo applications to actively dividing tissues like stem cells and cancer cells (3). An exception to this are the lentiviruses (members of the retrovirus family) which can replicate in dividing and non-dividing cells, with the most extensively studied for clinical applications being the human immunodeficiency virus type-1 (HIV-1) based vectors (30). While lentiviral vectors fulfill many of the criteria for an ideal vector, their move into gene therapy clinical trials has been slow due to fear of the possibility of recombination and generation of infectious HIV. However, newer vectors that are based on HIV retain <5% of the parental genome, and <25% of the genome is incorporated into packaging constructs. This minimizes the risk of generating revertant replication-competent HIV. Also, the development of self-inactivating vectors has further increased their biosafety (33). Self-inactivating vectors contain deletions of the regulatory elements in the downstream long terminal- repeat sequence (LTR), which eliminates the transcription of the packaging signal that is required for vector mobilization (38). The Mo-MuLV is one of the earliest gene therapy vectors. Because it is an ecotropic virus it normally only infects murine cells due to a specific interaction of a viral glycoprotein and a murine cell surface transporter. The Mo-MuLV envelope glycoprotein is pseudotyped with the G-protein of vesicular stomatis virus (VSV-G), with its receptors found on most human cells (33). Selective target cell transduction can be achieved by generating hybrid

envelope proteins that fuse a portion of the *env* gene with a sequence encoding a ligand for a specific cell surface receptor of the target cells (3). Retroviral vectors integrate into target cell DNA and have low immunogenicity. Both of these qualities could lead to long-term transgene expression. However, retroviral vector-mediated insertional mutagenesis could induce oncogenesis (33). The SCID-X1 clinical trial showed that this previously hypothetical risk of insertional mutagenesis due to the random integration of the retroviral genome into the host genome was indeed a very real risk. Development of site-specific integration vectors and self-inactivating vectors hopefully will decrease this risk in the future.

ADENO-ASSOCIATED VIRUS

Adeno-associated virus (AAV) vectors have very limited carrying capacity, being able to accommodate only about 4 kb of DNA insert. AAV can infect both dividing and non-dividing cells of many different cell types (different serotypes show different efficiencies). AAV vectors have been reported to display long-term and efficient transgene expression in various cell types. Adeno-associated virus DNA can integrate into the host cell genome. However, most studies report AAV vector genomes as episomal about 90% and integrated about 10% of the time due to gene deletions within the vectors (33). AAV-2, the predominant serotype from which most AAV vectors have been derived, has a broad tissue tropism as a result of AAV-2 attachment to the ubiquitously expressed cell surface heparan sulfate proteoglycan. AAV vectors pose little toxicity because the wild-type virus has never been shown to cause any pathologic effects in humans and AAV integrates specifically into chromosome 19 of the human genome which decreases the risk of insertional mutagenesis. However, virus vectors deleted of the open reading frame (ORF), Rep (to allow maximal insert size), will have lost the ability of site-specific integration and thus integrate at random

if at all. AAV vectors have low immunogenicity usually because of the deletion of the Rep and Cap ORFs, and therefore, the lack of viral protein expression (30, 33).

HERPES SIMPLEX VIRUS

One of the most important features of the replication-deficient herpes simplex viral vector (HSV-1) is its ability to carry up to 40-50 kb of foreign DNA. HSV-1 is naturally neurotrophic and also infects epithelial cells. A characteristic of HSV-1 infection is the establishment of a latent state in neurons that can be reactivated with the proper stimulus resulting in entry into the lytic cycle (3). Most HSV-1 vectors have gene deletions making them replication-incompetent. HSV-1 vector has an episomal genome and does not integrate into the host cell genome. There is transient transgene expression in cells other than neurons due to induction of an antiviral immune response. It is an attractive vector system for use in neuropathological conditions because it can establish long-term expression in these cells. While it has a strong tropism for neurons, it can deliver genes to a wide range of cell types. There is the possibility of recombination and reversion to a wild-type phenotype with defective viruses grown in complementary cell lines. Given that wild-type HSV-1 infection is associated with neurologic sequelae, recombination is a serious concern (30, 33).

CANCER GENE THERAPY

Although gene therapy strategies are being evaluated for the treatment of many different diseases, thus far two-thirds of the gene therapy clinical trials have been aimed at the treatment of cancer (6). This partly reflects the lack of effective conventional treatments for advanced refractory solid tumors. Solid tumors are often difficult to control due to various tumor factors, including tumor size, heterogeneity, location, vascularization and inherent chemo- and/or radio-therapy resistance. These factors can limit the effectiveness of

conventional treatment modalities such as surgery, radiation and chemotherapy.

Experimental therapies often provide the only alternative for patients with advanced disease who are refractory to conventional treatments. Also, strategies for cancer gene therapy are not as limited by some of the technical difficulties that challenge gene-replacement therapies of monogenetic diseases, such as the need for sustained, long-term gene expression and evading host immune response (2, 39).

The American Cancer Society projected a total of 1,444,920 new cancer cases and 559,650 deaths from cancers in the United States in 2007 (40). Due to the limitations of current cancer therapies, continued research evaluating novel cancer treatment strategies is essential, and many encouraging gene-directed therapies for cancer are being explored.

Cancer immunotherapy is the major cancer gene therapy strategy that has been investigated and accounts for almost forty percent of the genes transferred in human clinical trials (6).

Immunotherapy approaches have involved cytokine or antigen gene transfer to tumor cells or host immune cells. Other cancer gene therapy strategies involve cytotoxic therapy with ‘suicide gene’ transfer to tumor cells allowing the conversion of nontoxic prodrugs into toxic drugs, replacement of defective tumor suppressor genes such as p53, inhibition of expression of oncogenes by antisense mechanisms, anti-angiogenesis strategies and the use of conditionally replicative oncolytic viruses (21, 41-43).

CANCER GENE THERAPY - IMMUNOPOTENTIATION

The enhancement of the immune systems ability to destroy cancer cells through gene transfer is commonly referred to as immunopotential, and targets identified for gene transfer have included dendritic cells, effector T cells and tumor cells (44, 45). Dendritic cells are antigen presenting cells that can be modified either to present specific tumor

antigens to immune effector cells or to more efficiently activate an antitumor immune response (21, 41). Transduction of tumor protein into dendritic cells as a means to express tumor antigens for extended periods and to allow normal antigen processing and presentation of a full range of peptides from that particular tumor has been evaluated. The goal is to achieve stronger and sustained antigen presentation leading to a more potent antitumor immune response than can be achieved with transient presentation of a single peptide (41). Recently, the FDA recommended approval for the first genetically modified dendritic cell vaccine for the treatment of prostate cancer (21). Attempting to optimize the immunostimulatory efficacy of dendritic cell vaccines, current early phase clinical studies augment dendritic cells by adding expression of costimulatory molecules such as CD40 ligand, enhancing their secretion of immunostimulatory cytokines or by increasing their lifespan (21, 46).

Genetic modification of effector T cells is another promising approach to immunopotentialization of the host antitumor immune response. Tumor specificity can be induced in T lymphocytes isolated from patients by gene transfer and expression of tumor specific receptors (T cell receptors), which have an antigen specificity toward the targeted tumor (41). These cells are expanded *ex vivo* and reinfused into the patient. A recent study demonstrated the ability to specifically confer tumor recognition by autologous lymphocytes from peripheral blood by using a retrovirus that encodes a T cell receptor. High sustained levels of circulating, engineered cells were observed for one year after treatment in two patients, both of whom had complete tumor regression for over one year (26).

Gene therapy immunopotentialization can increase antigenic stimulation from tumor cells and their microenvironment to enhance tumor 'visibility' to the immune system (21) Cancer

cells are immunogenic naturally, however, the regular immune response is not sufficient to eradicate tumors. There are several reasons why tumors are able to evade the immune system including tumor secretion of immunosuppressive factors, tumor down regulation of antigen and major histocompatibility complex expression, and lack of co-stimulation since antigen is presented by the tumor cell rather than antigen presenting cells (42). The antitumor effects of systemically administered cytokines can be substantial. However, severe systemic side effects often result due to the high doses required. In an attempt to achieve high local cytokine levels necessary for antitumor immune response with minimal systemic effects, gene therapy approaches have been explored (41, 47). Modification of malignant cells using genes encoding immunostimulatory cytokines (e.g. IL-2, IL-4, IL-12, IFN- γ , TNF- α , GM-CSF), or co-stimulatory molecules (e.g. B7-1, B7-2, CD40 ligand) augments *in vivo* tumor antigen presentation helping to recruit tumor-specific T cells and B cells (21). Strategies for introduction of immunostimulatory genes include *in vivo* injection of tumors with a vector containing an immunostimulatory gene or *ex vivo* transduction of tumor cells, followed by irradiation and re-administration of the cells to achieve the desired cytokine production.

Tumor cell transfection with the cytokine Interleukin-12 gene has been the focus of intensive research. Interleukin-12 (IL-12) plays an essential role in the interactions between the innate and adaptive arms of the immune system (48). IL-12 acts on T lymphocytes (T cells) and natural killer cells (NK cells) by enhancing the generation and activity of cytotoxic lymphocytes and inducing the production of many cytokines, especially interferon- γ (IFN- γ) (49). Differentiation of T helper 1 cells, which are potent producers of IFN- γ , is mediated by IL-12. In turn, IFN- γ acts as a potent positive feedback mechanism by enhancing the ability

of phagocytes and dendritic cells to produce IL-12 (50). IFN- γ is also thought to play a major role in the anti-tumor actions of IL-12 (51, 52). IL-12 strongly inhibits angiogenesis, and its induction of IFN- γ appears to play a critical role as a mediator of the antiangiogenic effects of IL-12 (49). There is also evidence for a role of IL-12 in humoral immunity (53). IL-12 has been found to eradicate tumors, metastases, elicit sustained anti-tumor immunity (54-56) and inhibit angiogenesis (57, 58). After successful interleukin-12 gene transfection into tumor cells, complete tumor regression in rat animal models was observed in hepatocellular carcinoma and adenocarcinoma (55, 59). Several studies have been published on the use of heat-inducible IL-12 genes (60-62), and in one study, injection of an adenovirus containing a heat inducible mIL12 gene into a mouse melanoma model followed by local hyperthermia significantly retarded tumor growth (60).

CANCER GENE THERAPY – SUICIDE GENE THERAPY

Suicide gene therapy involves the transfer of genes that encode an enzyme product that converts relatively nontoxic prodrugs administered systemically into highly toxic metabolites selectively in the cancer cells. The suicide gene is delivered to the target cells, where it is not normally present, usually by injecting a viral vector containing the suicide gene directly into the tumor mass. Most of the strategies have employed transduction of genes encoding non-mammalian enzymes to tumor cells to selectively increase conversion of prodrugs to their toxic metabolites in tumors (63). Survival of the transduced cell, as intracellular concentrations of the cytotoxic product increase, is maintained by diffusion of the cytotoxin through gap junctions into adjacent non-transduced cancer cells (64). Once the cytotoxin concentration is sufficiently high in the adjoining cancer cell mass, it kills both the transduced cells and their neighboring cells. Thus, there is a 'bystander effect' of transduced

cells on nontransduced cells, and tumor regression can occur even when only a fraction of the tumor cells are transduced (65). Translation into human clinical trials has included herpes simplex virus - thymidine kinase (HSV-TK) gene transfer to induce susceptibility to acyclovir, ganciclovir or valacyclovir; bacterial cytosine deaminase (CD) gene transfer to induce susceptibility to 5-fluorocytosine; and mammalian cytochrome P450 2B (CYP2B) gene transfer to enhance chemosensitivity to cyclophosphamide and ifosfamide, among others (21).

CANCER GENE THERAPY – TUMOR SUPPRESSOR GENES AND ONCOGENES

Tumor suppressor genes lost or mutated in human cancer include p53, retinoblastoma gene Rb, p16INK/CDKN2 and PTEN. They encode a variety of proteins that regulate the cell cycle and mediate DNA repair pathways. Normally, when a cell is damaged, cell cycle arrest occurs with initiation of mechanisms for DNA repair, or, if the damage cannot be repaired, induction of programmed cell death (apoptosis) (44). By inhibiting the expression of tumor suppressor genes, cancer cells with DNA mutations can continue to proliferate, acquire new gene mutations, become less differentiated, and avoid apoptosis (44). The most commonly inactivated tumor suppressor gene in cancer cells is p53 which is seen in many different types of tumors (66). Inactivation of p53 can lead to loss of tumor suppressor function, loss of mutational repair, increased drug resistance, increased tumor angiogenesis, cell proliferation, and inhibition of apoptosis (41, 45). *In vitro* and preclinical *in vivo* tumor models have shown reversal of these effects and induction of apoptosis after p53 gene transfer to tumor cell (45). Phase I-II clinical trials evaluating p53 transfer have shown excellent safety profiles with some reports of p53 mediated tumor regression/growth arrest and increased effectiveness of radiation therapy and chemotherapy (45, 67, 68).

During fetal development, the expression of proto-oncogenes is required for various organs development. Afterwards, proto-oncogenes are silenced to prevent abnormal tissue growth. By activating and amplifying proto-oncogenes (called oncogenes) cancer cells can undergo unregulated proliferation (44). Among the most important oncogenes altered in human tumors are the genes *ras*, *c-myc*, *c-erbB-2*, *abl*, and *bcl-2* (45). The targeted disruption of tumor oncogenes is a form of cancer gene therapy being researched, and can be accomplished either by reduction of mRNA translation into protein or by inhibition of oncogene transcription into mRNA (42, 44). Translation can be blocked by using antisense oligonucleotides; short single-stranded nucleic acid (DNA, RNA or a chemical analogue) designed to hybridize to complementary mRNA sequences encoding target protein, effectively turning that gene "off", as mRNA must be single stranded for translation. The double-stranded RNA complexes are subsequently destroyed by intracellular enzymes (44, 69). Transcription can be blocked by using DNA oligonucleotides designed to bind to specific oncogene promoter regions, forming triple helix structures that prevent oncogene transcription (44, 70, 71). Several phase I studies targeting *ras*, *c-myc*, *c-erbB-2*, *abl*, and *bcl-2* genes have been carried out or are in progress, and a trial using an antisense oligonucleotide against *bcl-2* reported negligible toxicity and several clinical responses in nine patients who had relapsed non-Hodgkin lymphoma (45, 72). Another technique targeting oncogene mRNA uses specialized RNA molecules called ribozymes that bind and cleave targeted mRNA sequences, preventing subsequent gene translation. A variety of mRNAs over-expressed in leukemias, gliomas, breast, prostate, gastric, and ovarian cancers have been targeted with specially designed ribozymes (44, 73-75).

CANCER GENE THERAPY - ANTIANGIOGENESIS

Proliferation of cancer cells is associated with vasculature growth from existing blood vessels (i.e. angiogenesis) because tumor growth and survival depends on angiogenesis to provide oxygen and nutrients to cancer cells (76). Interfering with this process could arrest tumor growth and induce cell death. Gene therapy strategies have been developed to impede tumor angiogenesis with the main approaches involving inhibition of angiogenesis inducers such as vascular endothelial growth factor (VEGF) and angiopoietin or introduction of angiogenic inhibitors such as angiostatin, endostatin, interleukin-12 and p53 (42, 77). For example, no tumor growth was observed when 90– 100% of hepatocellular carcinoma cells were transduced in vivo with the angiostatin gene (78), and significant tumor regression was reported when only 5% of human breast cancer cells were transduced in vivo with the p53 gene (79). In the latter study, tumor regression was associated with 60% reduction in blood vessels. Hypoxia inducible factor-1 (HIF-1) is a transcription factor induced by low oxygen concentrations, as well as additional tumor microenvironment effects, that can stimulate angiogenesis via VEGF induction (80-83). HIF-1 (alpha subunit) over-expression has been reported in a broad range of human cancers (colon, brain, breast, prostate, cervical, esophageal, gastric, oropharyngeal, and ovarian) and is correlated with treatment failure and mortality (44, 84, 85). Gene therapy using antisense oligonucleotides to HIF-1 α targeted antisense oligonucleotide gene therapy was found to down regulate VEGF, decrease tumor microvessel density, and cause rejection of lymphomas in a mouse model (86). The easy accessibility to blood vessel endothelial cells and its applicability to different tumor types are advantages of the antiangiogenesis cancer gene therapy approach (42).

CANCER GENE THERAPY – CONDITIONALLY REPLICATIVE ONCOLYTIC VIRUSES

Conditionally replicative oncolytic viruses exploit the cytolytic capacity of virus. Viruses induce cell death by cytolysis as part of their normal life cycle (87). In cancer gene therapy, replication is selectively induced in tumor cells by controlling expression of early viral genes needed for replication with tumor-specific/tissue-specific or inducible promoters (88, 89). Thus, oncolytic viruses can multiply selectively within cancers, causing cell death and release of its progeny viral particles that can then infect neighbouring cells. During natural infection, viruses subvert the same tumor suppressor and cell defense mechanisms that are lost in carcinogenesis (21). The first formal clinical trials tested the E1B-55K deleted adenovirus Onyx-015. The adenovirus early protein E1B binds and inactivates the host cell's p53 protein to promote its own replication (90). In p53 positive cells, adenoviruses without E1B are incapable of replication. It was hypothesised that Onyx would replicate selectively in p53 negative tumor cells. Khuri et al. reported better overall tumor response rate, complete response rate and time to tumor progression rate after combined chemotherapy and Onyx treatment compared to chemotherapy alone (91). However, after completion of approximately 15 clinical trials using Onyx, single agent activity has been shown to be poor, with minimal systemic efficacy, and p53 status was unrelated to Onyx activity (21). Three clinical trials have been completed in hormone-refractory prostate cancer using transcriptionally regulated adenoviruses, with the prostate-specific antigen (PSA) promoter/enhancer elements driving E1 expression. CV706 and CG7870 were administered intratumorally and intravenously respectively with no significant toxicities and some transient PSA responses (92). These initial phase one trials confirmed replication safety and

suggested efficacy. In one study, 20 men with locally recurrent prostate cancer who had failed radiation therapy received single injections into the prostate of CV706 (93). Thirteen patients experienced more than a 30% decrease in PSA levels, and five patients receiving the highest doses had more than 50% decrease in PSA, suggesting efficacy. Several oncolytic viruses have been tested in clinical trials, with impressive safety records. All can induce anti-tumor responses after direct injection, but systemic activity remains a challenge (21). Strategies for the future will include arming viruses with transgenes that increase virus production or cellular spread and combining oncolytic virus with chemotherapy and/or radiotherapy.

An extensive variety of gene therapy strategies for cancer treatment have shown impressive preclinical success. However, with a few exceptions, the difficulty has been in translating this preclinical success into effective clinical therapies. As with gene therapy in general, there remain many challenges to overcome before the goal of cancer gene therapy is fully realized. In particular, two major challenges for gene therapy strategies directed at cancer treatment are the need for 1) targeted transgene expression, (94-96), and 2) noninvasive evaluation of transgene expression (28, 97-99).

TARGETING TRANSGENE EXPRESSION

One of the main limitations in cancer gene therapy is the inability to target transgene expression to the tumor cells, while limiting or excluding expression in normal cells. Achieving tumor cell targeting of transgene expression is extremely important for successful cancer gene therapy strategies where the goal is often to introduce a therapeutic cytotoxic gene specifically to cancer cells. Effective targeting of cancer cells increases the therapeutic

window and chance of successful treatment, and it is essential for safe and effective *in vivo* human gene therapy (94-96).

Many strategies have been used in an attempt to target transgene expression to specific cell types *in vivo*. This can involve the use of viral vectors or non-viral vectors (including naked DNA such as plasmids or antisense oligonucleotides, cationic liposomes, cationic polymers). Targeting approaches involve targeting at either the level of gene delivery by selective vector infection of target cells or at the level of gene expression with selective transcription induction within target cells (96)

Targeting at the level of gene delivery can involve physical, passive or transductional delivery of nucleic acids. Physical targeting utilizes physical forces such as mechanical force, hydrodynamic pressure, electric or magnetic fields and light or thermal effects (100). Passive targeting exploits the enhanced permeability and retention effects characteristic of solid tumor microenvironments (101), while transductional targeting employs specific ligand-receptor interactions that distinguish target cells from non-target cells (94, 102).

PHYSICAL TARGETTING

Physical targeting relies on the spatial focus of physical methods to enhance nucleic acid delivery at a specific site. These methods include direct injection of nucleic acid, the gene gun, electroporation, magnetofection, ultrasound and hyperthermia (100, 103). Initially, targeting transgene expression locally to tumor cells was attempted with intratumoral injections of the gene therapy vector. However, systemic vector leakage has been reported following intratumoral injection which can lead to potentially toxic systemic levels of transgene product (61, 62). The gene gun uses DNA coated microscopic gold beads that are shot into the target tissue with a helium gas propelled gun and is often used in DNA

vaccination and cytokine gene therapy applications (100, 104). *In vivo* electroporation is a technique involving the application of electrical pulses to target tissues after local nucleic acid administration to enhance their cellular uptake (103). It is thought that cell membranes are destabilized following electric stimulus allowing direct passage of the nucleic acid into the cytoplasm and avoiding the endocytosis pathway (105). Magnetofection is a technique in which nucleic acid is reversibly attached to superparamagnetic nanoparticles and a high energy magnetic field is applied to focus the particles to the target tissue (106). Sonoporation is a term referring to ultrasound mediated increase in cell permeability and is used for gene delivery after intratumoral gene injection (107). It is assumed that the formation of short-lived pores in the plasma membrane (up to 100 nm in effective diameter) associated with acoustic cavitation is the dominant mechanism (108). Ultrasound waves cause microbubbles to cavitate as a result of the interaction between ultrasonic waves and a gaseous inclusion in an aqueous medium (109). Ultrasound enhanced gene transfer (UEGT) has also been successfully achieved *in vivo*, with reports of spatially restricted and therapeutically relevant levels of transgene expression (108, 110-112). Finally, it has been demonstrated that the application of heat at target sites increase the permeability of tumor vessels and the extravasation of liposomes out of the tumor microvasculature (113-115). The combination of nonviral gene therapy vectors with hyperthermia was recently initiated with encouraging results *in vitro* (116).

PASSIVE TARGETTING

Passive targeting of nucleic acids to tumors takes advantage of unique biological and physiological characteristics of solid tumors. Unrestricted tumor cell proliferation results in the need for a tumor to develop its own blood supply. It does this through angiogenesis in

which new capillaries are developed from a previously established network (83). However, newly formed blood vessels in tumors are abnormal with blood vessels displaying severe structural and functional abnormalities. They are characterized as having a chaotic vascular geometry with hyperpermeable vessels due to incomplete vessel linings and basement membranes (117-119). The imperfect and leaky tumor vasculature combined with an inadequate lymphatic drainage results in an effect termed “enhanced permeability and retention” (EPR effect) (101). This EPR effect can allow preferential accumulation of sufficiently long circulating, shielded (lipid-based or polymer-based systems) particles in tumor tissue (100). Passive targeting involves exploiting the enhanced permeability and retention effect in tumor vasculature for tumor-selective macromolecular drug targeting.

TRANSDUCTIONAL TARGETING

Transductional targeting involves imparting to a gene delivery vector the capacity to distinguish target cells from non-target cells using ligands directed to receptors specific for the target tissue (34, 94, 120). There has been substantial progression in the development of targeting technologies in recent years for several types of vectors, particularly viral vectors (94), which have been used in 67% of gene therapy clinical trials as recently reported (6). Although there has been some promising preclinical results using non-viral vectors, they present greater challenges with regard to gene-transfer efficiency. However, non-viral vectors can be retargeted by incorporating ligands into their lipid or protein shells, and the targeting approaches discussed below are also likely to be applicable to non-viral vectors once their efficiencies approach those of viral vectors (94). A major technical challenge in facilitating transductional targeting is that the native tropism of the virus often does not meet the therapeutic need. A viral vector may be inefficient in transducing the types of cells

needing treatment or may be too efficient in transducing a broad range of cells, which may result in deleterious effects if the therapeutic gene is delivered to unintended cells. Thus, the safety and efficacy of gene transfer can potentially be improved with ligand-directed targeting (94, 120). Most progress in targeted vector development has been achieved using adenovirus, adeno-associated virus (AAV) and retroviruses (particularly lentiviruses), and the technical difficulties encountered in the development of targeting methods are similar for most viral vector systems. The main approaches that have been applied to transductional targeting of vectors include vector targeting by pseudotyping, vector targeting using adaptors, and vector targeting by genetic incorporation of targeting ligands (94).

Pseudotyping was the first method used to alter viral vector tropism and involves transferring viral attachment proteins either between strains within a family of viruses or between virus families (121). Pseudotyping can be achieved by co-transfection of plasmids (one plasmid encoding the attachment protein to be pseudotyped and separate one encoding all other vector components), expression of the viral attachment protein in trans from the production cell line, or genetic incorporation of the viral attachment protein into the viral genome (94). Pseudotyping is often used with retroviral vectors to improve their host cell range (121, 122). The most widely used retroviral vector pseudotypes are those that incorporate the attachment glycoprotein of the vesicular stomatitis virus (VSV-G) (94). This modification results in the production of high-titer vector stocks and confers a broad host range. With pseudotyping, it is technically easier to manipulate binding specificity without negatively impacting the efficiency of cell transfection. However, the number of pseudotypes for which receptors are expressed exclusively and abundantly on target cells is limited. To overcome this limitation by genetic modifications requires extensive viral

structural knowledge since modifications to vector structure could be detrimental to vector production or gene transfer (94).

Vector targeting can also be achieved using adaptor systems. Adaptor systems are two-component systems that involve a bifunctional adaptor or bridging molecule that binds to the viral attachment protein and the receptor on the target cell (123-125). Different types of adaptor systems include: receptor–ligand approach in which a native viral receptor is fused to the targeting ligand (94, 126, 127); bispecific antibody approach in which two antibodies are coupled, with the resulting molecule having specificity for the vector and the target (94, 128, 129); chemical linkage approach where the targeting moiety is bound to the vector by chemical means (94, 130, 131); avidin–biotin approach exploits the strong bond between avidin and biotin where biotin is coupled to the vector and then bound to the avidin– ligand complex (94, 132-134); antibody approach where antibody binds to a genetically incorporated immunoglobulin binding domain of the vector (94, 135, 136). As an example of an adaptor system, an adenoviral antibody may be fused to a targeting ligand, (such as epidermal growth factor - EGF), of a specific cell receptor on the target cells (EGF receptor which is over expressed in many cancers). The antibody-ligand fusion protein binds to the adenoviral vector fiber and blocks interaction with cellular coxsackie-adenovirus receptors (CAR). The ligand fused to the antibody then redirects the viral vector to cells expressing that ligand's receptor (EGF receptor) (34). Advantages of the adaptor approaches include its great flexibility allowing different adaptors to be easily coupled to the same vector, and the fact that it does not require changes in vector structure that could result in unfavorable effects on vector production or gene transfer (94). Adaptor systems allow easy screening of several target receptors, which makes them particularly useful for preclinical feasibility studies

(137). One disadvantage of adaptor systems that might limit their clinical use for gene therapy concerns the potential for suboptimal stability of the vector–adaptor complex *in vivo*, resulting in the dissociation of adaptor from vector. The strong binding avidin–biotin complex and chemical conjugation are thought the least likely to be affected by this (94). Also, since two molecules must be produced separately, the adaptor approach is considered more cumbersome for clinical applications, and the adaptor and the virus are considered to be separate drugs by regulatory agencies making it more difficult to get approval for use in clinical trials (94).

Genetic systems used for vector targeting incorporate a polypeptide into the vector by genetic means to facilitate transduction. The genetic incorporation of targeting ligands into viral vectors avoids the potential complexities of adaptor systems. Genetic systems are single-component systems that produce a single virus molecule that recognizes the target cell. While more technically challenging than the use of adaptors, single-component systems overcome the regulatory issues of two-component systems and facilitates high-titer production by eliminating the need to create a separate adaptor molecule (94). Different approaches to vector targeting by genetic incorporation of targeting ligands include: serotype switching by using a different serotype from within the same virus family (94, 138, 139); small targeting motifs in which small peptides are inserted into the capsid or viral attachment protein (94, 140-142); single-chain antibody that is incorporated into the viral attachment protein (94, 143, 144); mosaic viral attachment proteins involve combining two viral attachment proteins with different properties, allowing targeting, production or imaging in parallel (94, 145, 146); and ablation of native tropism by mutation of the amino acids responsible for native tropism (94, 147, 148). As an example, genetic modification of the

adenovirus receptor-binding knob domain can achieve CAR-independent binding to tumor associated receptors. Studies have utilized a truncated CAR as a knob binding component in a single recombinant fusion molecule of truncated-CAR fused to EGF to direct vector to target cancer cells over expressing the EGF receptor. Also, the complete knob can be deleted and replaced with a retargeting ligand, or other adenovirus capsid proteins can be genetically modified, such as the pentose base or hexon, in attempt to redirect vectors to target cells (34).

TRANSCRIPTIONAL TARGETTING

A second strategy used to target transgene expression to specific cell types *in vivo* is directed at the level of gene expression with selective transcription induction within target cells (27, 95, 149, 150). While transductional targeting approaches attempt to increase vector delivery to target cells and reduce gene transfer to non-target cells, transcriptional targeting does not change the tropism of viruses but instead restricts transgene transcription induction to target cells. As has been demonstrated with many promising delivery systems, there will be some unavoidable gene delivery to local and distant normal tissues (149). Therefore, additional targeting methods are necessary to ensure that gene delivery to non-target tissues does not result in significant gene expression and potential toxicity. Transcriptional targeting is achieved by placing the viral gene or transgene under control of a tissue-specific, tumor-specific or inducible promoter, allowing very tight control of gene expression at the transcriptional level. The viruses are able to infect various cell types, but the viral genes or transgene are expressed only in the cell types that actively express the transcription factors needed for the promoter activity.

Transcription of a therapeutic gene in a tissue-specific manner can be achieved by utilizing tissue-specific transcriptional control elements present in the various cell types (for

example, hepatic, melanocyte, neuronal). Likewise, therapeutic gene transcription in a tumor-specific manner can be achieved by employing tumor-specific promoters/enhancers that are activated either in the malignant process or as a result of being exposed to an unfavorable tumor-associated microenvironment (for example, hypoxia) (150). The use of tissue and tumor specific cellular promoters usually results in the constitutive expression of the transgene in the target tissue. However, it may be preferable to more tightly regulate the duration and level of transgene expression for some therapeutic strategies. This may be achieved by the use of exogenously controlled cellular promoters that are induced to drive transgene expression in target cells under certain conditions. Examples include promoters induced by ionizing radiation, heat, and systemically administered small molecules (150).

While most of the cells in the body have the same genotype, there are often considerable differences in cellular phenotype as the result of differences in patterns of gene expression in the cells belonging to the various tissues. Certain tissues express proteins, which are, more or less, specific to that tissue and the promoters that switch on these genes have been called tissue specific promoters (TSP) (102, 149). Some well characterized tissue specific promoters and their target tissues include: tyrosinase promoter (melanocyte/melanoma) (151-156); prostate-specific antigen, probasin, human glandular kallikerin (prostate/prostatic carcinoma) (157-162); myelin basic protein, glial fibrillary acidic protein, myelin proteolipid protein (glial/glioma) (163-166); albumin (liver/hepatocellular carcinoma) (167-169); thyroglobulin (thyroid/thyroid carcinoma) (170-172). In the context of cancer treatment, one of the main limitations of tissue-specific promoters is that transgene expression may lead to cytotoxic effects in normal as well as tumor tissue derived from that same cell type (150). Therefore, if employing tissue-specific

promoters for cancer gene therapy, the target tissue (for example, prostate, melanocytes, breast or thyroid) must be expendable due to potentially significant normal tissue toxicity (173). Whereas if the tissue/organ is critical (liver or neuronal tissues), a similar approach could cause significant damage with potentially devastating consequences (149). In those cases, additional levels of targeting are necessary such as the direct delivery of the transgene to the tumor or the use of retroviral vectors that do not efficiently infect non-dividing cells, such as the liver and neural tissue (150). Also, use of a tissue-specific promoter does not guarantee transgene expression will be exclusively limited to the target tissue, and depending on the transgene being used, low level background gene expression in other tissues may be associated with toxic side effects (149).

Tumor specific promoters include certain promoters that are either silent or have very low background activity in normal tissues but are highly active in tumors. Tumor-specific promoters have been described in the following subgroups (149, 150): promoters considered cancer-specific, that show no tissue specificity but are specific for the malignant process; promoters considered tumor-type specific that encode oncofetal antigens and are related with tissue specificity; promoters responsive to conditions associated with the tumor microenvironment (hypoxia and glucose regulated); promoters that are more active in the tumor vasculature compared to normal vasculature.

Cancer-specific promoters are associated with certain genes that are up-regulated as part of the malignant process in a broad range of tissue nonspecific tumors. As such, cancer-specific promoters may provide the opportunity to target therapeutic genes to a wide variety of tumor types (173). The development of generic cancer specific vectors would offer potential for targeting a wide range of different tumor types without the need to design

unique promoters on an individual, patient by patient, basis (149). The telomerase gene is probably the only gene currently that can be classified as cancer specific. Not expressed in normal tissues (except germ cells and stem cells), telomerase is abnormally reactivated in all major cancer types (174-176). Telomerase enables tumor cells to maintain telomere length and circumvent the process of senescence, whereas normal cells senesce after a set number of population doublings. The human telomerase reverse transcriptase subunit (hTERT) promoter is being used to drive the expression of transgenes selectively in a wide variety of tumor cells (176-178). The apoptotic pathway was successfully targeted in vivo using Bax as a transgene, driven by the hTERT promoter (179, 180). The expression of transgenes necessary for viral replication has been controlled by hTERT promoter for selective replication of oncolytic viruses in tumor cells (181). The hTERT promoter was recently used to drive expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in human pancreatic tumors in the livers of nude mice, which was associated with suppressed tumor growth and no liver toxicity (182).

Tumor-type specific promoters encode oncofetal antigens which are expressed during fetal life as a part of normal development but which are silenced in normal adult tissues. However, in certain malignant conditions, these proteins can be re-expressed (183). The best characterized promoters of these tumor-specific genes are carcinoembryonic antigen (CEA), which is expressed by a number of adenocarcinomas including colorectal, breast and lung cancers, and alpha fetoprotein (AFP), which is expressed by hepatocellular carcinomas and malignant testicular teratomas (149, 150). There are many other genes that are overexpressed in certain tumor types (150), and their promoters are also being evaluated for transcriptional regulation of therapeutic gene expression. Other tumor-specific promoters

that have been identified and extensively evaluated include: MUC1 (breast, cholangiocarcinomas, ovarian) (184-186), ErbB2 (breast, pancreatic, ovarian) (186-188), and osteocalcin (prostate, ovary, lung, brain, osteoblasts) (150, 189, 190).

Tumor specific promoters can also include promoters that respond to the tumor microenvironment and physiology. Many aspects of the tumor microenvironment are unique and present opportunities to specifically target tumor tissue (191). Tumor hypoxia is a common characteristic of many tumor microenvironments due to the defective blood vessel network that exists in tumors as a result of accelerated growth (192, 193). For tumors to grow, they must stimulate angiogenesis which requires the proliferation of endothelial cells. A number of genes have been reported to be upregulated in proliferating endothelium of tumor blood vessels. The endothelial-specific kinase insert domain receptor (KDR/flk-1) (194-197) and E-selectin (194, 196, 198) have been shown to be upregulated in tumor endothelium, and regions of these promoters have been used for targeting transgene expression to the tumor vasculature. Targeting the tumor vasculature, could potentially lead to the death of a large number of dependent tumor cells in a type of anatomical bystander effect (149). The promoters of human preproendothelin-1 (199-201) and human von Willebrand factor (202) have also been used to drive transgene expression selectively in proliferating endothelial cells (150). Genes involved in angiogenesis, anaerobic metabolism, vascular permeability, and inflammation are activated in response to hypoxia (192). Transcription is usually initiated by the binding of the transcription factor hypoxia-inducible factor (HIF) to hypoxia responsive elements (HREs). Hypoxia-targeting of gene therapy to induce selective transgene activation within hypoxic tumor regions has been evaluated by utilizing promoters containing HREs (193). Hypoxia response elements found within the

hypoxia-responsive genes of vascular endothelial growth factor (VEGF) (203-206), erythropoietin (Epo) (207) and phosphoglycerate kinase-1 (PGK-1) (208) have been tested for their use in targeted cancer gene therapy. Targeting the hypoxic population of tumor cells is attractive since they are inherently resistant to radio- or chemotherapy (209), and increased expression of HIF-1 and its target genes appear to play a critical role in the malignant progression of tumors (84, 85). Finally, in addition to oxygen starvation due to the aberrant tumor vasculature, tumors can also be deprived of glucose resulting in increased expression of genes involved in glucose metabolism. The GRP78 promoter (210-213) and hexokinase II promoter (214) associated with glucose metabolism are also being used to drive transgene expression specifically within a tumor.

INDUCIBLE PROMOTERS

Some general problems with the use of tissue and tumor specific promoters are the challenge of identifying unique promoters for different tissue or tumor types, the relative weakness of existing promoters, the lack of clear restriction of gene expression to the target tissue (referred to as promoter “leakiness”), and the lack of temporal control of transgene expression (60, 96, 149, 173). Therefore, inducible promoters in which the transcription of transgenes introduced into cells can be controlled by administration of external physical or chemical agents have gained attention as mediators of transient transgene activation. These systems, often referred to as “gene switches” (149), are particularly valuable for the temporal control of gene expression. A number of agents including drugs, radiation and heat, can be used to control these switches (173).

One strategy for an inducible transgene expression system requires chemical signals where small molecules are used to selectively activate genes that have been delivered by

virus injection into target tissues. The tetracycline inducible or suppressible gene expression systems (Tet-ON or Tet-OFF) is the most widely evaluated chemically inducible system and is based on the bacterial tetracycline operon (95). Tetracycline is nontoxic at doses required for gene activation and it is rapidly metabolized and cleared from the body, properties that make it an ideal drug for an inducible system (95). Two different forms of the Tet repressor exist due to allosteric changes that alter their DNA binding properties, and as a result, the two different repressors respond differently to the binding of tetracycline. When tetracycline is present in cells the Tet-OFF system is silent, but when tetracycline is withdrawn from the cells, 2-5-fold induction of transgene expression can be attained (215, 216). The Tet-ON system is only active when tetracycline is present in the cells with up to 1,000-fold induction of transgene expression reported after addition of tetracycline to cells *in vitro* (217). Other chemically-based inducible systems that have been evaluated include muristerone (218), mifepristone (219), and rapamycin (220).

Inducible gene expression systems that are based on external physical agents have a unique advantage over other regulatable gene expression systems in the ability to focus the inducing agent in a spatially defined manner. When evaluating chemically inducible promoters, gene expression is usually targeted through intratumoral vector injection. However, as previously stated, vector dissemination into the systemic circulation has been reported with this approach (62, 221). Systemic injection of the promoter-activating agents of chemically induced promoters does not permit correction for leakage of vector outside of the target tissue and can lead to ectopic expression of transgene in distant organs, such as the liver, spleen and lung (60, 221). To acquire tumor specific transgene expression using

chemically induced promoters it is likely necessary to target the virus to specific cell types by incorporating a targeting delivery technique (222).

The physical inducing agents that have been most evaluated for gene therapy are radiation and hyperthermia. Exposure of cells to ionizing radiation is associated with the transcriptional activation of a variety of genes driven by a radiation-inducible promoter, including, c-jun, NFB, EGR1, and p21^{WAF1/CIP1} (150). Radiation can be delivered in a way that allows precise spatial and temporal control of transgene expression (223). Most work has been performed with the early growth response (Egr-1) promoter which binds to DNA through specific CC[A/T]₆GG (CArG) domains (224, 225). Radiation-mediated gene therapy has included strategies involving placing the radio-sensitizing cytokine TNF- α gene (226-228) and the HSTK therapeutic suicide gene (229, 230) under the control of the Egr-1 promoter. Combined treatment with Egr-1 promoter-driven TNF- α expression and fractionated radiotherapy resulted in increased tumor control without increased toxicity in a prostate cancer xenograft model (231). In vivo data have also been presented for the combination of Egr-1 promoter-driven HSVtk expression and radiation in a hepatocellular carcinoma model (232).

HEAT SHOCK PROTIEN PROMOTER

The heat shock response provides another opportunity for an inducible promoter. Heat shock response is an evolutionarily conserved response to cellular stresses that involves the rapid and transient induction of cytoprotective proteins (heat shock proteins - HSP) (233). While heat application induces the most robust heat shock response, cellular stresses other than heat can also activate the heat shock response. These stressors include oxidative stress, hypoxia, ischemia, acidosis, cytokines, fever or inflammation (60, 221). Most tumor

microenvironments have many features that would lead to the induction of HSPs, including having areas with hypoxic and acidic conditions, tissue damage, inflammation and cell death. All these factors can induce the expression of HSPs in a tumor, and in fact, heat shock proteins have been found to be expressed or overexpressed in many types of tumors (234, 235).

Heat shock proteins are also transcriptionally regulated by a variety of physiologic processes not typically associated with cell stress such as the cell cycle, cell proliferation and differentiation, growth factor activity, tissue development and hormonal stimulation (234, 236). In addition, HSP expression is modulated by many of the conditions leading to apoptosis. Thus, the increased cellular proliferation, with cell cycle and apoptotic pathway deregulation, present in tumors may induce HSP expression.

For tumor growth and survival, there are advantages to having HSP overexpression. Apoptosis resistance and tumor cell survival have been associated with high expression levels of HSPs (237). HSPs associate with key apoptotic proteins at multiple levels in both the intrinsic and extrinsic apoptotic pathways. HSPs can inhibit the activity of pro-apoptotic Bcl-2 proteins and prevent caspase activation and induction of apoptosis. Thus, HSPs coordinated modulation of multiple events within the apoptotic pathways help sustain cell survival (238). Studies have shown that a decrease in HSP 70 levels achieved with antisense HSP70 inhibits proliferation and induces apoptosis in tumor cells (235). In addition, HSPs are involved in the phenomenon of thermotolerance in which cells exposed to nonlethal elevated temperatures acquire a transient resistance to a subsequent heat shock. In particular HSP 70 has been shown to have a specific role in thermotolerance and to confer a cytoprotective effect against cell death in certain tumor cells (239).

For the cancer cell, the main disadvantage of HSP overexpression is the immunologic properties HSP possess that can induce specific protection against cancer (240-242). Observations in animal tumor models have demonstrated the importance of molecular chaperones in tumor recognition and subsequent tumor regression (235). Some immunologic characteristics of the HSPs include the following (242): HSP 70 participates in anti-tumor immune responses as a carrier of antigenic tumor peptides and interacts with professional antigen presenting cells (APC); HSP-peptide complexes can be cross-presented to CD8+ T cells through the endogenous MHC-I pathway of professional antigen-presenting cells; HSPs can induce dendritic cell maturation and pro-inflammatory cytokine release by APCs . Finally, the ability to stimulate the innate immune system is independent of the peptides they carry, which defines the HSPs as natural endogenous adjuvants (242).

The heat inducible hsp70 promoter has been found to be one of the most efficient inducible regulatory systems (222, 243, 244). Strategies combining hyperthermia with gene therapy by using the promoter of the HSP 70 gene have been developed to selectively activate therapeutic transgenes expression following hyperthermia treatment, allowing control of the location, duration and level of transgene expression. Advantages of the heat inducible promoter system over radiation inducible promoters systems are the ability to safely administer the hyperthermia treatment, minimal baseline transgene expression and strong induction of transgene expression at 300-1000 fold over background and in a heat dose dependent manner (60, 61, 245). Whereas, the ERG-1 promoter that is induced by ionizing radiation (a carcinogen) has high baseline transgene expression, is only inducible 10-fold *in vitro* without additional modification, and requires a high, single radiation dose (223, 224). No long-term side effects have been observed in more than 20 years of

hyperthermia use in experimental cancer treatment at temperatures in the range shown to be most effective for heat-induced transgene expression (60). Therefore, the heat inducible promoter system is potentially suitable for treatment of diseases other than cancer.

The HSP70B promoter has been used to drive expression of suicide gene (HSV- tk) therapy where significant tumor regression was observed in HSP-tk-transduced tumors following hyperthermic therapy and injection of ganciclovir (246). An HSV-tk/cytosine deaminase (cd) fusion gene and replication-competent E1B-attenuated adenoviral vector effectively suppressed tumor cell proliferation by the cytopathic effect of the virus and cytotoxicity in the presence of the prodrugs (247). Also, combined treatment using both cd and tk suicide genes controlled by hsp70 promoters was shown to be highly effective against heat- and radiation-resistant breast tumor cells (248). An adenovirus vector expressing heat induced antisense KU70 RNA effectively attenuated KU70 protein expression in FSA-II tumors as well as significantly radiosensitized the tumors (249). Several studies have also been published on the use of heat-inducible IL-12 genes (60-62, 250). In one such study, injection of an adenoviral vector containing a heat-inducible IL12 gene into a mouse melanoma model in combination with hyperthermia resulted in significant tumor growth delay (60). Adenovirus mediated intratumoral expression of IL-12 under the control of a HSP70B promoter in combination with hyperthermia resulted in an improved response to radiotherapy without apparent systemic toxicity in another study (61). Use of an inducible system in which expression can be modulated *in vivo* is critical for the advancement of cancer gene therapy in human patients. This heat inducible expression system has great potential to enhance gene therapy of many diseases where localized or transient therapeutic gene expression is desired.

NON-INVASIVE MONITORING OF TRANSGENE EXPRESSION

In addition to the need for targeted transgene expression, the need for *in vivo*, non-invasive monitoring of transgene expression is another major problem hindering the clinical progression of cancer gene therapy. Currently, in the clinical setting, techniques to determine transgene expression are difficult to perform because they involve unacceptable repetition of invasive procedures and provide only very limited information since they do not allow for evaluation of transgene expression throughout the body (251). Improved *in vivo* imaging modalities that accurately detect the location, magnitude and temporal change of transgene expression will greatly facilitate the transition of preclinical animal studies to human clinical trials (97, 98, 252-255).

Since most therapeutic genes have no ligands or substrates for direct imaging analysis, techniques for indirect monitoring of therapeutic gene expression with the use of reporter genes coupled to therapeutic genes have been developed. Several imaging modalities have been utilized to detect various reporter gene products with most work involving the following three non-invasive imaging technologies (98, 99, 253, 256): 1) nuclear imaging (positron emission tomography (PET), single photon emission computed tomography (SPECT), gamma camera and autoradiography) (28, 257, 258), 2) magnetic resonance imaging (259-261), and 3) optical imaging (262, 263). Each imaging modality has its own advantages and disadvantages. For example, *in vivo* optical imaging using green fluorescence protein or luciferase as reporter genes is quite sensitive and relatively inexpensive, but is largely limited to imaging small animals (mice/rats), due to tissue absorption of visible photons, and it lacks tomographic resolution (253, 256). Magnetic resonance has the advantage of very high spatial resolution of approximately 50 μ m

compared to optical imaging (3-5 mm) and SPECT/PET (1-2mm), but is expensive and currently more limited with respect to the number and variety of molecular and reporter gene assessments (256, 264). Nuclear imaging has the benefit of high sensitivity and tomographic capability with relatively easy quantification of signal information that can be directly transferable to large animals and humans (253, 264). Compared with gamma camera and SPECT imaging, PET is considered a superior detection technique with respect to sensitivity, resolution and quantification (265). However, PET nuclear imaging technology can also be very expensive, requiring a cyclotron and radiochemistry resources (256).

REPORTER GENES

Nuclear imaging methods have been used for noninvasive imaging of gene transfer in numerous animal models. Intravenous injection of a radiotracer results in its accumulation at sites of reporter gene expression that can then be detected using gamma camera, SPECT or PET imaging techniques. Among the genes that can serve as radiologically detectable reporters, the human type 2 somatostatin receptor (hSSTr2), the herpes simplex virus type 1 thymidine kinase (HSTK), the type 2 dopamine receptor (D₂R) and the sodium iodide symporter (NIS) have been most intensively investigated (98, 253, 266).

Reporter genes can be placed in three broad categories: enzymes (HSTK), receptors (hSSTr2, D₂R) and transporters (NIS). HSTK is one of the most widely used reporter genes for gene therapy imaging (253, 267). The first report on imaging expression of successful gene transduction *in vivo* involved the use of a recombinant retrovirus containing the HSTK reporter gene (257). HSTK is a model for enzyme-based reporter systems where the reporter gene encodes an enzyme that chemically modifies the signal substrate (probe), fixing its biodistribution. A single molecule of reporter enzyme can interact with many probe

molecules, thereby multiplying the signal (268). HSTK has been investigated using various fusion proteins and bicistronic vector constructions, and specific accumulation of its substrates (acycloguanosine and uracil nucleoside derivatives) has been demonstrated in HSTK expressing cells (267, 269-274). Two studies have recently demonstrated direct correlation between HSTK and D₂R reporter gene expression, and HSTK and luciferase reporter gene expression (275, 276).

The NIS is probably the best model for transporter-substrate reporter systems where the transporter concentrates the signal substrate within a cell but does not chemically alter the substrate (268). Endogenous expression of the NIS is mainly in the thyroid, although lower levels of expression are present in the salivary glands, stomach, thymus, and a few other tissues (267). Several early studies demonstrated NIS gene expression using ¹²³I or ^{99m}TcO₄⁻ as imaging agents with γ scintigraphy in xenograft tumors transduced with the NIS gene (277-279). Recently, the NIS was used as a reporter gene to noninvasively image therapeutic gene (p53 or MnSOD) transfer using a dual-expressing adenoviral vector *in vitro*. Results showed a direct positive correlation between therapeutic gene expression and NIS function (253).

The D₂R and hSSTr2 genes are examples of reporter genes that encode for a cell membrane receptor. In the receptor–ligand reporter model, one signal molecule (the ligand) binds with high affinity to the reporter receptor, which has a stationary biodistribution over the period of imaging. Unlike enzyme systems, there is a fixed stoichiometry determined by the number of receptors available, and thus, high specificity and affinity of the receptor–ligand interaction along with receptor density are key determinants for success with this type of reporter imaging (268). D₂R has limited expression in the body, mainly in the nigrostriatal

system of the brain. *In vivo* studies have demonstrated that tumors expressing the transfected D2R reporter gene retain significantly more radiolabeled probe than control tumors (280, 281)

SOMATOSTATIN RECEPTOR REPORTER GENE

The hSSTr2 belongs to a family of G protein coupled receptors that bind the peptide somatostatin (somatotropin release inhibitory factor). To date, five human somatostatin receptor subtypes have been cloned and partially characterized, with subtype 2 most extensively studied and used as a reporter gene (282-284). Somatostatin has a wide variety of biologic functions including acting as a neuromodulator, a modulator of endocrine and exocrine functions and a regulator of cell differentiation and proliferation. Biodistribution of the endogenous receptor includes the brain, pituitary, pancreas, stomach, kidneys and other neuroendocrine tissues (283, 284). Somatostatin receptors have also been found with a very high incidence, and often a high density, in neuroendocrine tumors, particularly pituitary adenomas (285, 286), gastroenteropancreatic tumors (287, 288), pheochromocytomas (289, 290), neuroblastomas (291, 292), and to a lesser extent in medullary thyroid cancers (293) and small cell lung cancers (294). Some nonneuroendocrine tumors to have been shown to express somatostatin receptors include brain tumors such as meningiomas, medulloblastomas and gliomas (295-298), renal cell cancers (299), breast (300), prostatic (301), and ovarian carcinomas (302). In several studies, it has been shown that an inhibitory signal is produced when somatostatin and related analogues bind to somatostatin receptors. In cancer cells, this inhibitory signal is antiproliferative, and it has been reported to inhibit tumor growth in many animal tumor models and cultured cell lines (303-305). In a primary and metastatic pancreatic cancer model, transfection of tumors with hSSTr2 resulted in a significant

antitumor effect characterized by an increase of apoptosis and an inhibition of cell proliferation (306). Somatostatin has also been shown to be an inhibitor of neovascularization in several experimental models where somatostatin receptors, particularly, hSSTr2, have been found to be expressed on endothelial cells and up-regulated during the angiogenic switch (305). The use of radiolabeled hSSTr2 specific agonists was shown to display potent antineoplastic activity by inhibiting the growth of hSSTr2-positive vascular endothelial cells in hSSTr2-negative tumors (307, 308). The clinical relevance of this growth inhibition remains to be defined but suggests the use of hSSTr2 as a reporter gene may also provide some anticancer benefit (255).

Because many tumors have been found to express hSSTr2, radiolabeled somatostatin analogs (e.g., ^{111}In -pentetreotide [OctreoScan; Mallinckrodt Inc.] and $^{99\text{m}}\text{Tc}$ -depreotide [Neotect; Diatide Inc.]) have been used for clinical imaging of patients with hSSTr2-positive tumors (309, 310). In addition, the peptides have been labeled with various radionuclides for therapeutic applications performed on preclinical animal models (310, 311) and on cancer patients with hSSTr2-positive tumors (312, 313). In addition, the hSSTr2 gene has been evaluated as a gene therapy reporter gene in several studies and has been used to successfully image gene transfer by use of gamma-camera imaging and SPECT (255, 314-318). Specific, localized expression of hSSTr2 reporter gene in human tumor xenografts has been demonstrated in mice after gene transfer using intratumorally injected adenovirus vector. Reporter gene expression was detected after intravenous injection of radiolabeled somatostatin analogue using γ camera imaging and immunohistochemistry (266, 319-321), and there was good agreement between imaging analyses and gamma counter measurements (319). Also, excellent sensitivity and linearity were reported by the same group of

investigators in a mouse xenograft model where hSSTr2 gene expression resulting from vector doses as low as 1×10^6 plaque forming units was consistently detected and tumor uptake of radiolabeled analogue correlated well with adenoviral vector dose. In contrast, when the HSTK reporter gene system was evaluated in the same study, there was unreliable detection of HSTK expression and no correlation between radiolabeled ligand accumulation and vector dose (266). Thus, hSSTr2 reporter gene imaging may be useful for quantification of transgene expression in addition to spatial localization. Recently, somatostatin analogs radiolabeled with PET radionuclides have been evaluated, with hsstr2 gene transfer detected in mice using microPET imaging (322). This is a more sensitive imaging modality compared to gamma camera/SPECT imaging and holds much promise for future reporter gene studies with the hsstr2 gene.

INTRODUCTION SUMMARY

In summary, there has been much progress in the field of gene therapy despite several serious setbacks. In particular, many promising gene therapy strategies are emerging that are directed at the treatment of cancer. While many problems hindering the clinical application of these gene therapy strategies still remain, two important challenges pertaining to cancer gene therapy are the need to target transgene expression to the tumor cells exclusively and to non-invasively monitor the level, location and duration of transgene expression. The *in vitro* and *in vivo* experiments described in the next sections evaluate the use of the HSP 70 promoter combined with the hSSTr2 reporter gene and mIL-12 therapeutic gene to achieve both 1) targeting of reporter and therapeutic transgene expression and 2) correlation of therapeutic transgene expression with reporter gene detection.

In Vitro Correlation of Heat Induced Reporter and Therapeutic Gene Expression.

Amy F. Pruitt¹, Chuan-Yuan Li², Xiuwu Zhang², Michael R. Zalutsky³, Jeff Johnson⁴, Mark W. Dewhirst², Marlene L. Hauck¹

¹Department of Clinical Sciences, North Carolina State University, College of Veterinary Medicine, Raleigh, North Carolina and Departments of ²Radiation Oncology, ³Radiology and Statistics, Duke University Medical Center, Durham, North Carolina.

Corresponding author:

Amy F. Pruitt

Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606.

Phone: 919-513-6322; Fax: 919-513-6669. E-mail: amy_pruitt@ncsu.edu

First Author: Amy F. Pruitt

Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606.

Phone: 919-513-6322; Fax: 919-513-6669. E-mail: amy_pruitt@ncsu.edu

In training: graduate student

Word count: 6,485

Supported in part by: Department of Health and Human Services, NIH grant P01CA42745.

Foot line: Heat Induced Reporter Gene Expression

Abstract

Gene therapy for cancer is an important research focus, with most approved gene therapy clinical trials involving cancer patients. This reflects, in part, the inadequacies of conventional treatment for advanced, refractory solid tumors. However, significant problems remain to be solved before gene therapy will become a first line modality in the clinical setting. Two challenges to overcome are the need for targeted transgene expression and noninvasive evaluation of transgene expression. In this study, we hypothesized that the use of the heat inducible heat shock protein 70 promoter (HSP) in combination with the human somatostatin receptor type 2 (hSSTr2) reporter gene will allow for the non-invasive monitoring and quantification of targeted therapeutic gene (murine Interleukin 12) expression. **Methods:** Replication-incompetent adenoviral vectors containing either the therapeutic gene or the reporter gene under control of the HSP were constructed and their function assessed *in vitro*. Cells were infected with both vectors and then heated at temperatures that ranged from 38-42° C. Uninfected cells and infected cells at 37° C served as controls. Twenty-four hours after heating, radiolabeled somatostatin analogue was added to the wells, and radioligand receptor binding was measured with a gamma counter. A murine interleukin-12 (mIL-12) ELISA kit was used to measure mIL-12 levels in the supernatants. The results of the radioligand and ELISA assays were compared to determine if gene expression from the reporter gene vector and the therapeutic gene vector could be correlated. **Results:** A statistically significant heat-dose response of radioligand binding for the reporter gene and mIL-12 production for the therapeutic gene was detected, with minimal expression observed in the controls. Results demonstrated a strong correlation between radioligand uptake and mIL-12 production in response to heat treatment (Pearson

Coefficient 0.95, p-value = <0.0001). **Conclusion:** The results of this study indicate that gene expression from both vectors could be induced by hyperthermia and that induced expression from the reporter and therapeutic gene vectors were highly related. Additional *in vivo* testing in a mouse model is warranted. Results have the potential to pave the way for evaluation of heat induced gene therapy with hSSTr2 reporter monitoring in animals and humans.

Key words: Heat inducible gene therapy, Imaging, Somatostatin receptor

Introduction

Rapid advances in molecular sciences over the past decade have fostered remarkable insights into the molecular basis for many diseases and into the development of novel therapies at the genetic level. Continued improvements in recombinant DNA technologies and in vector systems have lead to significant progress in the field of gene therapy, a promising therapeutic modality with the potential to improve human health (1, 2, 94, 95, 267). Based on a recent report, over 1340 gene therapy clinical trials have been completed, are ongoing or have been approved worldwide (6). Gene therapy in the treatment of cancer has become an important research focus due to limitations of current conventional cancer therapies for advanced refractory solid tumors (2, 39). In fact, about two thirds of the clinical trials in gene therapy have been aimed at the treatment of cancer (6).

Despite significant preclinical success in gene therapy, its clinical applications remain limited, although clinical trials have highlighted some of the main challenges that remain to be solved before gene therapy will become a first line therapy. These include the need for 1) targeted transgene expression, and 2) noninvasive evaluation of transgene expression (28, 94-98).

A major goal of gene therapy is to target transgene expression to the correct cells, at the correct level and time, while limiting or excluding expression in non-target cells. Achieving selective targeting becomes even more important when considering cancer gene therapy, where the goal is often to introduce a therapeutic cytotoxic gene specifically to cancer cells. Safe, specific and controlled expression of therapeutic genes is essential for safe and effective *in vivo* human gene therapy (94-96).

Intratumoral vector injection for local gene therapy is one approach to targeting transgene expression that has been tested in clinical trials. Systemic leakage of the vector has

been reported following intratumoral injection, leading to potentially toxic systemic levels of transgene product (61, 62). Other approaches involve transductional targeting by selective vector infection of target cells (94) or transcriptional targeting by selective transcription induction within target cells (95, 96). However, with transcriptional and transductional targeting strategies, there is the challenge of identifying either unique promoters for different tissue/tumor types or ligand-receptor interactions that distinguishes tumor and normal cells and are likely unique for each tumor type. In addition, the duration and level of transgene expression are not controllable with constitutive tissue-specific or tumor-specific promoters (60, 96).

A more adaptable method for controlled, targeted transgene expression involves inducible promoters that are activated by physical or chemical agents. Inducible promoters permit tight spatial and temporal control of transgene expression (60). A promising targeted gene therapy approach exploits the heat shock response, an evolutionarily conserved response to cellular stress involving the rapid and transient induction of cytoprotective proteins (heat shock proteins). The heat inducible hsp70 promoter (HSP) has been found to be one of the most efficient inducible regulatory sequences (222, 243). Previous studies have shown the ability of the hsp70 promoter to target transgene expression both spatially and temporally after intratumor injection and local hyperthermia in animal models (61, 62, 250).

A major limitation in preclinical and clinical cancer gene therapy trials is difficulty monitoring gene expression and distribution noninvasively. Improved *in vivo* imaging modalities that accurately detect the location, magnitude and temporal change of transgene expression will greatly facilitate the translation of preclinical animal studies to human clinical trials (97, 253-255). Several imaging modalities have been utilized to detect various reporter

gene products, including nuclear imaging, optical imaging and magnetic resonance imaging, each having various advantages and disadvantages (253). Several reporter genes, mainly the human type 2 somatostatin receptor (hSSTr2), the herpes simplex virus type 1 thymidine kinase, the type 2 dopamine receptor and the sodium iodide symporter (NIS), are currently being successfully used with gamma camera, single photon emission computed tomography, and positron emission tomography imaging (253, 266, 322).

The hSSTr2 belongs to a family of G protein coupled receptors that bind the peptide somatostatin (somatotropin release inhibitory factor) (283, 323). Detection of hSSTr2 reporter gene expression in vitro and after intratumoral adenovirus vector injection in mice with human tumor xenografts has been reported in several studies, with good agreement between imaging analyses and gamma counter measurements (316, 319-321). Also, excellent sensitivity and linearity was reported in a mouse xenograft model where tumor uptake of radiolabeled analogue correlated well with adenoviral vector dose (266). Reporter gene imaging using hSSTr2 may be useful for quantification of therapeutic transgene expression in addition to spatial localization.

In this study, we hypothesized that the use of the heat inducible hsp70 promoter in combination with the hSSTr2 reporter gene will allow for the non-invasive monitoring and quantification of targeted therapeutic gene (murine Interleukin 12) expression. Characterization of the relationship between therapeutic gene expression and reporter gene imaging is necessary for the noninvasive monitoring of transgene expression, while use of a heat inducible promoter would allow temporal and spatial targeting of transgene expression.

Materials and Methods

Cell Culture

4T1 mouse mammary cancer cells and B16.F10 melanoma cells were obtained from American Type Culture Collection (Manassas, VA). The 4T1 cells were maintained in RPMI and the B16.F10 cells were maintained in DMEM, both supplemented with 10% FBS and 1% Penicillin/Streptomycin (Mediatech, Inc. Cellgro®, Herndon, Va). HEK 293 human transformed primary embryonal kidney cells, also obtained from American Type Culture Collection, were used for adenovirus propagation and were maintained in DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin (Mediatech, Inc. Cellgro®, Herndon, Va). All cells were cultured at 37°C in a humidified atmosphere with 5% CO₂.

Adenoviral vectors

The replication-incompetent adenoviral vectors used in this study were based on an Ad5 recombinant system. The human somatostatin receptor type 2 (hsstr2) gene (cloned by RT-PCR from the human fibroblast cell strain 1522) was placed under control of either the human hsp70B promoter (HSP) for heat-inducible gene expression or under control of a constitutively active CMV promoter as a control to derive the AdHSP_{hSSTR2} and AdCMV_{hSSTR2} vectors, respectively. In construction of the heat-inducible murine interleukin 12 (mIL-12) gene, an internal ribosomal entry site was placed in between the cDNAs for both mIL-12 subunits (p40 and p35) so that both units can be translated from the same mRNA molecule. The gene was then placed under control of the HSP promoter to construct the AdHSP_{mIL12} vector. All vectors were then packaged by use of the AdEasy system (Stratagene, La Jolla, CA) (324). The viruses were propagated in 293 cells, and purification by CsCl-banding was performed according to a standard protocol (325).

Radiolabeled Peptide

The somatostatin receptor avid peptide used in this study was glucose-octreotate (Gluc-TOCA), an octreotide analogue with COOH-terminal oxidation and NH₂-terminal carboxylation. Radioiodination of Gluc-TOCA was performed using Chloramine-T as previously reported (298). Briefly, 20 µl of 0.05 M phosphate buffer (pH 7.5) and a solution of peptide in 0.05 M acetic acid (14 µg in 20 µl) was added to a solution of sodium [¹²⁵I]iodide (approximately 1 mCi in 3 µl of 0.1 N NaOH; PerkinElmer Life and Analytical Sciences, Billerica, MA) in a Reacti vial. This mixture was vortexed for 30 sec and then a solution of Chloramine-T in the above phosphate buffer (1.6 µg in 20 µl) was added. The reaction occurred at room temperature over 1 minute of vortexing. The labeled peptide was isolated from the reaction mixture by reversed-phase HPLC using a linear gradient (10% to 40% B over 30 min) consisting of solvents 0.1% trifluoroacetic acid in water (A) and 0.1% trifluoroacetic acid in acetonitrile (B) at a flow rate of 1 ml/min. The HPLC fraction containing the labeled peptide was passed through an activated solid-phase cartridge (C18ENV; Waters). The cartridge was washed with 5 ml of water and 1 ml of 0.5 M HOAc, and then eluted with 5 × 0.25 ml ethanol. The ethanol fractions containing the radioiodine activity were pooled, evaporated with a stream of argon at room temperature, and reconstituted in a solution BSA and PBS (pH 7.14) for use in the cell culture experiments.

In Vitro protocol

Both B16.F10 melanoma cells and 4T1 mouse mammary cancer cells were seeded in 96-well cell culture plates (Costar®, Corning Costar, Corning, NY) with the concentration of added cells adjusted to ensure 85–90% confluency after 48 h (3×10^3 cells/well). Cells were infected with either one or both of the heat inducible vectors 12 h later. Infection occurred

over 24 h at 37° C at the specified plaque forming units (pfu)/cell. Cells were then heated at temperatures ranging from 38-42° C for 1 h in a humidified atmosphere with 5% CO₂. Additional infected cells remained at 37° C, serving as unheated controls to determine baseline HSP activity. Uninfected cells for each treatment group also served as controls to determine constitutive or non-viral, heating related gene expression. Twenty-four hours after heating, [¹²⁵I]Gluc-TOCA (0.05 µCi/well) was added to the wells infected with AdHSPhsstr2 and the uninfected controls. Incubation was for specified time periods (1–10 h) at 37°C in a humidified atmosphere with 5% CO₂. At the appropriate time, the supernatant was aspirated and discarded and the cells were washed twice with ice cold PBS. Individual cell wells were separated after drying and counted in a LKB 1282 dual-channel gamma counter (Wallac, Turku, Finland). For cells infected with AdHSPmIL12, mIL-12 concentration in the supernatant was determined using a commercially available murine interleukin-12 (mIL-12) ELISA kit (Quantikine mouse IL-12p70, R&D Systems, Minneapolis, MN) 24 h after heating. For cells infected with AdCMVhsstr2, [¹²⁵I]Gluc-TOCA was added to the wells 24 h after infection and processed as described for AdHSPhsstr2. All experiments were done in triplicate and performed in two cell lines (B16 and 4T1).

Statistical Analysis

The Student's *t* test (two-tailed, unpaired) was performed when only two experimental treatment groups were compared and in comparing various vector doses, with *P* < 0.05 being considered significantly different. One-way analysis of variance (ANOVA) was performed using SAS v.9.1 to test for differences among treatment groups. Results presented in graphs report untransformed data; however, all ANOVA analysis was performed on log transformed data. For the mIL-12 data, the Kruskal-Wallis test was performed as a check on

the ANOVA results due to data distribution. Spearman rank-order correlations and Pearson correlation coefficients were computed for mIL-12 production and radioligand binding over infected treatment groups at 39-42 °C. Regression analysis, using radioligand uptake as a predictor of mIL-12 production was also performed on these data.

Results

To determine the optimal incubation time with radioligand [125 I]Gluc-TOCA], cells infected with 10 pfu/cell of AdHSP ϕ sstr2 and heated at 42° C were incubated with [125 I]Gluc-TOCA for 1, 2, 4, 6, 8 and 10 h (Figure 1). Additional infected cells that remained at 37° C and uninfected cells (37° C, 42° C) were used as controls. Specific radioligand uptake was calculated by correcting for [125 I]Gluc-TOCA accumulation in uninfected (37° C, 42° C) control cells (constitutive or non-viral, heating related gene expression) and infected 37° C control cells (baseline HSP activity). There was a statistically significant difference in radioligand uptake between incubations of 2-4 and 8-10 h. No statistically significant difference in radioligand uptake at 4, 6 and 8 h was observed. Based on these results, a 6 h incubation with [125 I]Gluc-TOCA was used in all subsequent experiments.

Comparisons of [125 I]Gluc-TOCA uptake were made in parallel experiments between the heat-inducible AdHSP ϕ sstr2 vector and the constitutively expressing AdCMV ϕ sstr2 vector (Figure 2). A statistically significant difference in radioligand uptake was seen between the constitutive CMV promoter vector and the inducible HSP promoter vector, consistent with previous reports (61, 62).

Inducible gene expression as a function of viral vector dose was evaluated next to determine the optimal viral dose for *in vitro* experiments. Results of these experiments are shown in Figures 3A (AdHSP ϕ sstr2) and 3B (AdHSPmIL-12). Differences in both

radioligand uptake and mIL-12 production between the different viral doses were statistically significant, with 20 pfu/cell identified as the optimal viral dose for use in single vector experiments. For experiments involving co-infection of cells with both heat inducible vectors, a viral dose of 15 pfu/cell of each vector was used based on cytotoxicity seen at higher viral doses. All experiments consisted of at least four treatment groups as described in Figures 4A and 4B and included cells that were 1) infected, heated, 2) infected, not heated, 3) not infected, heated, 4) not infected, not heated. The difference in radioligand uptake (Fig. 4A) between infected cells at 42° C and 37° C was significant based on an unpaired, two-tailed Student *t* test. There was not a significant difference between non-infected control cells at 37° C and 42° C. There was a statistically significant difference in radioligand uptake between infected cells at 37° C and the non-infected controls. However, the magnitude of the increase in radioligand uptake (2.3 fold) in the 37° C infected cells was small when compared to the 134 fold increase observed in infected cells heated at 42° C. Similar results were seen with mIL-12 production (Fig. 4B) where mIL-12 supernatant concentration (mean 232 pg/ml) from 42° C treated, infected cells was significantly different from the mIL-12 supernatant concentration (mean 1.3 pg/ml) from infected cells that remained at 37° C. A statistically significant difference between infected cells at 37° C and the non-infected controls was not detected with ELISA. Similar to the non-infected control cells, the non-heated infected control cells showed cytokine levels that were at or below the detection limits of the ELISA assay.

A heat dose response was initially evaluated for each heat inducible vector in parallel experiments using variable heat doses (38° C – 42° C), and a strong correlation between radioligand uptake and mIL-12 production was observed (data not shown). Based on the

results of these experiments performed in parallel, additional experiments were performed with co-infection of B16 cells with both AdHSP α sstr2 and AdHSPmIL-12 vectors. Figure 5 shows the results of an experiment designed to evaluate the heat dose response after co-infection of B16 cells with 15 pfu/cell of each Adhsphsstr2 and AdhspmIL12 vectors and exposure to variable heat doses (38° C – 42° C). Figure 5A shows radioligand uptake in counts per minute (CPM) as a function of heat dose. A significant increase in radioligand uptake was detected with increasing heat dose starting at 39° C. Differences in radioligand uptake among heat doses in the 38-42° C range were significant. There was not a significant difference in radioligand uptake between infected cells at 37° C and 38 ° C, nor was there a significant difference between non-infected control cells at 37° C and 42° C. ANOVA was used to test for differences among treatment groups and the p-value for an overall difference among treatments among infected cells was $p < 0.0001$. A test for a linear increase in means as temperature increased among the infected cells was performed with the p-value for the test for trend being $p < 0.0001$. A statistically significant difference in radioligand uptake between infected cells at 37° C and the non-infected controls was observed. Again, the magnitude of the increase in radioligand uptake (2.7 fold) in the 37° C infected cells was small when compared to the 23-133 fold increase observed in infected cells heated at 39-42° C respectively. Figure 5B shows mIL-12 production in pg/ml as a function of heat dose. As with radioligand uptake results, an increase in mIL-12 production was detected with increasing heat dose starting at 39 °C, and differences between each heat dose (38-42° C) were significant. The p-value for an overall difference among treatments among infected cells was $p < 0.0001$. The p-value for the test for trend was $p < 0.0001$. The p-value from the Kruskal-Wallis test for an overall difference among treatments among infected cells also

was $p < 0.0001$, which supports the results from the ANOVA. Also, similar to results from the single vector experiments, a significant difference between the non-heated infected cells and the non-infected controls was not detected with ELISA. These experiments were also performed with the 4T1 cell line, and the results from the statistical tests for the 4T1 cell line were identical to those for the B16 cell line (data not shown). Spearman rank-order correlations and Pearson correlation coefficients were computed for mIL-12 and radioligand binding over infected treatment groups at 39-42 degrees. The correlation coefficients and p-values are reported in Table 1. Regression analysis, using radioligand uptake as a predictor of IL-12 production was also performed on these data. Table 2 shows the results from these analyses. A strong correlation between radioligand uptake and mIL-12 production in response to heat treatment was observed (Figure 6).

Discussion

Two important goals in gene therapy are to target transgene expression to the appropriate cells only and to non-invasively monitor the location, magnitude and duration of transgene expression. Numerous studies evaluating various approaches to address each of these challenges have been reported. A system that would allow both targeting and non-invasive monitoring of therapeutic transgene expression has great potential to enhance gene therapy of diseases where localized and transient therapeutic gene expression is desired. However, limited studies have evaluated this combined approach of correlating reporter gene expression with therapeutic gene expression in an inducible system. In this study, a gene therapy approach was evaluated that has the potential to achieve both of these goals. The AdHSP_{hsstr2} and AdHSP_{mIL-12} vectors were used to demonstrate *in vitro* that 1) level of therapeutic gene (mIL-12) expression was measurable by monitoring *hsstr2* activity with

radioligand uptake, and 2) the level of expression of both vectors was controlled by the heat dose administered.

After heat treatment, the AdHSP α str2 and AdHSP α mIL-12 vectors resulted in the uptake of radioligand and the production of mIL-12 respectively in a temperature and viral dose dependent manner. There was excellent linearity between viral dose and radioligand uptake as previously reported with the α str2 reporter gene (266). With the AdHSP α str2 vector, there was negligible radioligand uptake in the non-infected control cells, whether left at 37° C or exposed to a heat dose of 42° C. There was a statistically significant difference in radioligand uptake between infected cells left at 37° C when compared to the the non-infected control cells. However, the magnitude of the increase in radioligand uptake (2.3 - 2.7 fold) in the 37° C infected cells was small and considered clinically insignificant when compared to the 23-134 fold increase observed in infected cells heated from 39-42° C, respectively. The increase in radioligand uptake in the unheated, infected cells was likely due to the development of other cellular stresses (nutritional, crowding) during in vitro growth as discussed in a previous report (326). Whether this would occur within the tumor micro-environment is not known but systemic expression in normal tissues would seem unlikely (326). With the AdHSP α mIL-12 vector, there was not a significant difference between non-infected control cells at 37° C and 42° C or between infected cells at 37° C and the non-infected controls. After coinfection of cells with both AdHSP α str2 and AdHSP α mIL-12 vectors, followed by exposure to variable heat doses, results showed that therapeutic mIL-12 gene expression measured by ELISA was strongly, positively correlated with α str2 activity as determined by radioligand uptake. These results support the

hypothesis that this heat inducible reporter system may be capable of monitoring and quantifying inducible therapeutic transgene expression non-invasively *in vivo*.

The therapeutic gene used in this study was the gene encoding the potent immunomodulatory cytokine interleukin-12 (IL-12), which has significant antitumor activity in preclinical tumor models reported in previous studies (60). However, the specific therapeutic gene used was not directly related to the objectives of this study, and this approach could be utilized with many different therapeutic genes and for clinical applications outside of cancer treatment. Inducible gene expression systems that are based on external physical agents have some unique advantages, foremost of which is the ability to focus the inducing agent in a spatially defined manner. In the case of chemically inducible promoters, gene expression is usually targeted through vector injection *in situ* intratumorally, but vector dissemination into the systemic circulation has been reported (62, 221). Systemic injection of the promoter-activating agents of chemically induced promoters does not permit correction for leakage of vector outside of the target tissue and can lead to ectopic expression of transgene in distant organs, such as the liver, spleen and lung (60, 221). Advantages of the heat inducible promoter system over other physically inducible promoter systems (i.e. radiation inducible) are the ability to safely administer the hyperthermia treatment, minimal baseline transgene expression, and strong induction at 300-1000 fold over background in a heat dose dependent manner (60, 61, 245). Whereas the ERG-1 promoter that is induced by ionizing radiation (a carcinogen) has high baseline transgene expression, is only inducible 10-fold *in vitro* without additional modification, and requires a high, single radiation dose. Hyperthermia has been used in experimental cancer treatment for more than 20 years with no

long-term side effects observed for temperatures in the range shown to be most effective for heat-induced transgene induction (60).

There are also potential disadvantages of a hyperthermia inducible gene therapy approach. Some sites in the body may not be amenable to hyperthermia treatment due to the difficulty in heating specific areas to the desired temperatures (60, 61). However, continued technological advances in the development of the equipment used for planning, implementing and monitoring hyperthermia treatments have significantly improved the ability to heat more target areas in the body. Patients with breast, cervix, head and neck cancers and glioblastoma multiforme have been treated in several positive randomized clinical trials combining hyperthermia with radiation therapy (60, 327-330). Another potential problem is that the heat shock response may be activated by other cellular stresses. These stressors could include oxidative stress, hypoxia, ischemia, acidosis, cytokines, fever or inflammation (60, 221). However, in most reported studies, only minimal basal expression of HSP promoter controlled genes have been observed, with strong induction of gene expression after hyperthermia treatment (62, 221). Still, it may be necessary to control body temperature below 39°C in certain clinical situations (60).

In this study, the reporter gene and the therapeutic gene were delivered by two separate adenoviral vectors, which were identical in every way except that one carried the reporter gene and the other the therapeutic gene. The ideal way to monitor therapeutic gene expression would be to use a specific imaging probe that can directly detect its expression such as with the herpes simplex virus thymidine kinase gene (267, 331). However, a direct imaging probe is not available for most therapeutic genes, and the development and in vivo validation of a new probe for each therapeutic gene would be time-consuming, expensive and

not possible for many genes. As a result, the development of a few well-characterized reporter genes for indirect imaging of therapeutic gene expression has become a highly researched alternative. Linking the expression of a therapeutic gene to a reporter gene can be done through a variety of means including fusion protein constructs, bidirectional promoter vectors, dual promoter constructs, bicistronic vectors or simply co-vector administration (276, 331).

The co-vector administration used in the current study is the most straightforward. The rationale behind this approach is that if both vectors are identical and the expression of both the reporter gene and therapeutic gene is regulated by the same promoter, when they are co-administered in equal amounts, correlation of expression of both genes at the target tissue should be attainable (267). The host responses to a gene therapy vector should be the same whether it is carrying the therapeutic or reporter gene, and thus, either vector should have an equal probability of uptake by the target cells (276). Although any single cell may not be infected with equal amounts of the two vectors, this should not pose a significant problem given the spatial resolution limits of most clinical imaging modalities (267). The greatest advantage of co-vector administration is that a single reporter gene vector can be used to evaluate the expression of many different therapeutic genes, eliminating the need to produce a new construct for every new therapeutic gene (267, 276).

A potential limitation of the double vector approach is the concomitant increase of viral burden and a greater chance of immunological reactions. However, with the continued development of improved, less immunogenic adenoviral vectors, the increased viral burden may not be a problem (37, 276). Another problem that may affect correlation between the reporter and therapeutic genes involves having both genes regulated by identical promoters

on separate vectors(276). *Cis*-acting mechanisms of transcription regulation could affect correlation between the expression of both genes due to positive and negative feedback from either the therapeutic or reporter gene. The bicistronic vector, bidirectional promoter or fusion protein approaches may eliminate this problem as any affect on the promoter will affect the transcription of both the therapeutic and the reporter gene(276). Future optimization of the heat inducible reporter system used in this study may involve development of a single vector construct containing both the therapeutic and reporter gene. However, all the approaches, except the fusion protein approach, may encounter loss of correlation between therapeutic and reporter gene protein products at the translational and posttranslational level. On the other hand, with the fusion protein approach, the final folding of the fusion protein might affect the function of the therapeutic or imaging component (267, 276).

Conclusion

In summary, the results of this study demonstrate the feasibility of this heat inducible reporter system approach with *in vitro* results showing a strong, positive correlation between therapeutic gene expression and *hsstr2* expression that was regulatable by heat dose. Validation of this approach *in vivo* is necessary and those studies are currently being conducted. The use of inducible systems of gene expression and the development of sensitive and quantitative methods for *in vivo* monitoring of gene therapy trials will be critical to the future advancement of this new field of medicine.

Acknowledgments

Supported in part by: Department of Health and Human Services, NIH grant P01CA42745.

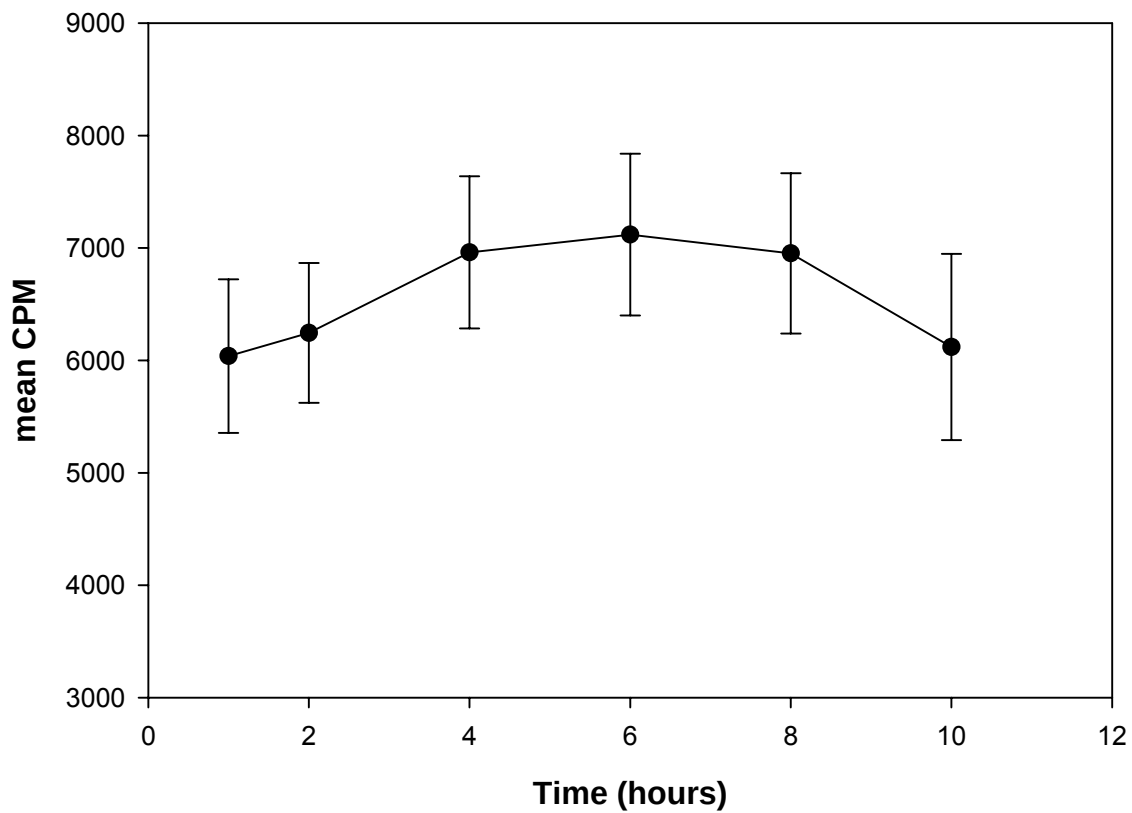


Figure 1: Radioligand uptake (surface-bound and internalized) reported in counts per minute (CPM) as a function of incubation time with (^{125}I)Gluc-TOCA. B16 cells infected with 10 pfu/cell of AdHSPsstr2 were exposed to a heat dose of one hour at 42 ° C twenty-four hours after infection. (^{125}I)Gluc-TOCA was added to the cells twenty-four hours after heating and incubation was for variable time periods (1, 2, 4, 6, 8, 10 hours). Bars, $\pm$ SD

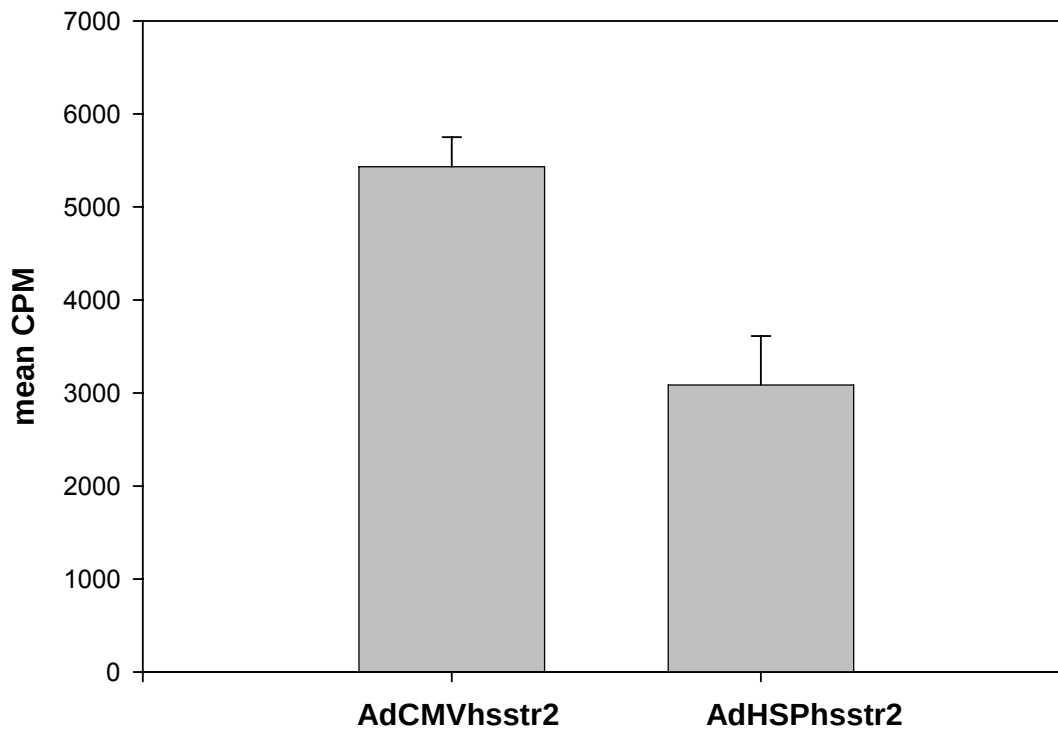
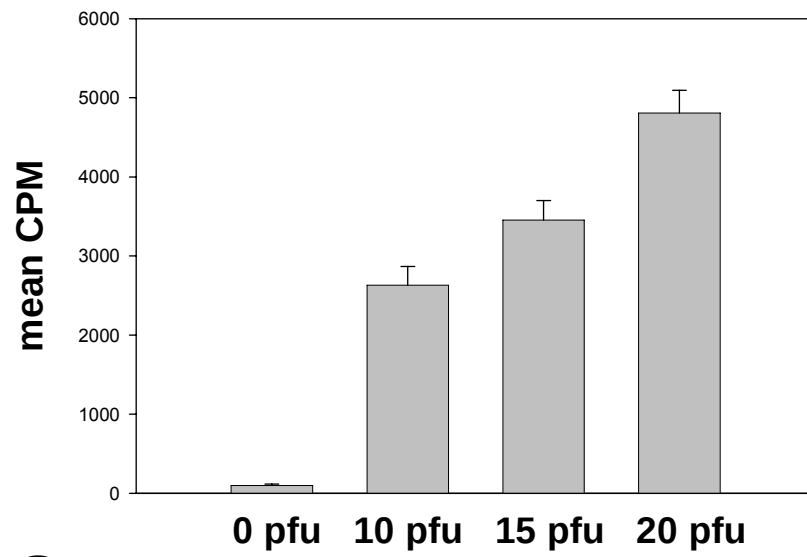


Figure 2: Comparison of radioligand uptake in counts per minute from B16 cells infected with 10 pfu/cell of either AdCMVhsstr2 or AdHSPhsstr2. A six hour incubation with (125 I)Gluc-TOCA occurred 24 hours after infection with AdCMVhsstr2. Cells infected with AdHSPhsstr2 were heated 24 hours after infection for one hour at 42° C, with addition of radioligand 24 hours after heating. A statistically significant difference in radioligand uptake ($P = 0.000002$) was seen between the constitutive CMV promoter vector and the inducible HSP promoter vector. bars, $\pm$ SD

Figure 3: A) Radioligand uptake in counts per minute (CPM) as a function of viral vector dose. B16 cells were infected with AdHSP_{hsstr2} at variable doses (0, 10, 15 and 20 pfu/cell). Differences in radioligand uptake between each of the four viral doses were significant. P-values comparing each vector dose (starting with 0 pfu) with the immediate next increase in dose were $P=0.000001$, $P=0.00015$, $P=0.000006$. B) mIL-12 production in pg/ml as a function of viral vector dose. B16 cells were infected with AdHSP_{mIL-12} at variable doses (0, 10 and 20 pfu/cell). Twenty-four hours after infection, the cells were heated for one hour at 42° C. Supernatant was removed 24 hours after heating and mIL-12 production was determined using a murine IL-12 ELISA kit. Differences in mIL-12 production between the three viral doses were significant. P-values comparing each vector dose (starting with 0 pfu) with the immediate next increase in dose were $P=0.005$, $P=0.019$. bars, $\pm$ SD

A



B

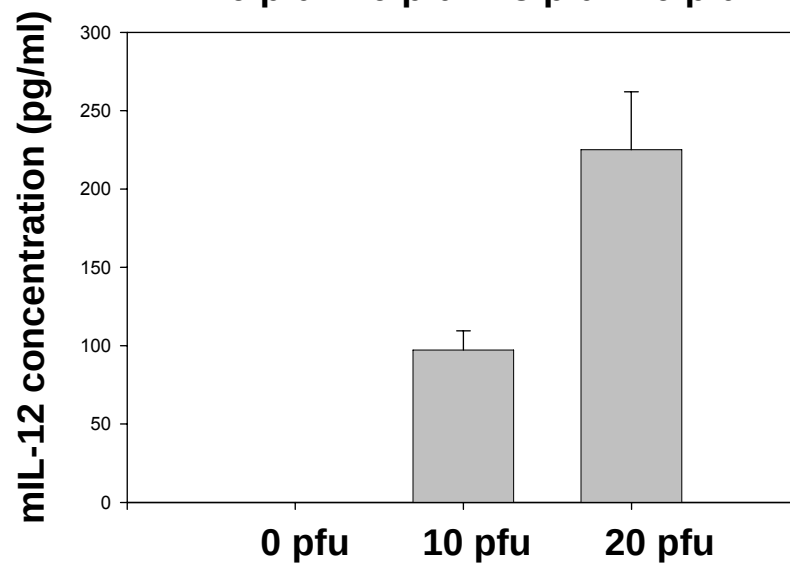
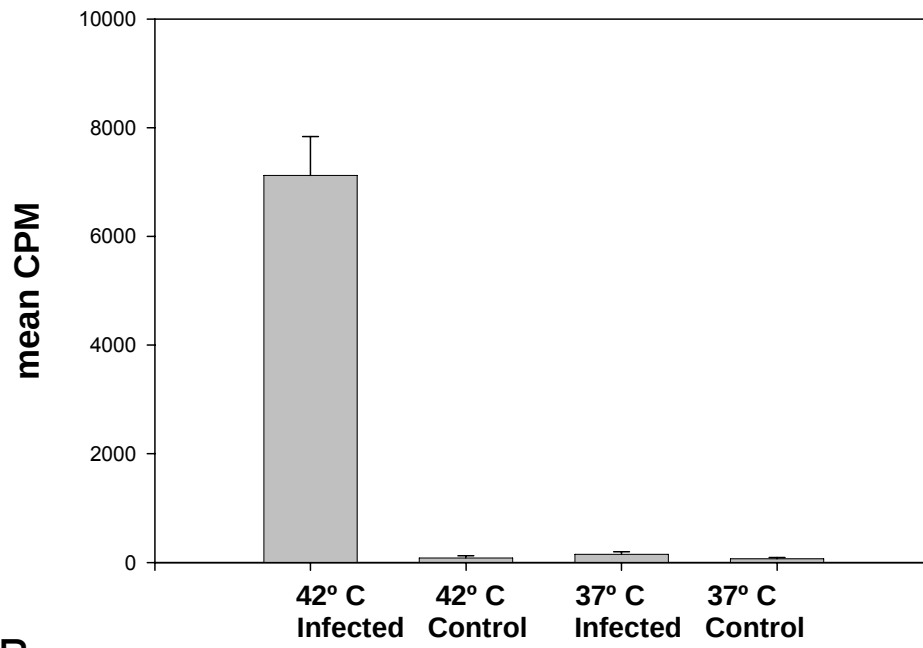


Figure 4: A) Radioligand uptake in counts per minute (CPM) from infected and non-infected B16 cells at 42° C or remaining at 37° C. Cells were infected with 20 pfu/cell of AdHSP_{hsstr2} with non-infected cells as controls. Difference in radioligand uptake between infected cells at 42° C and 37° C was significant ($P= 0.0000002$). There was not a significant difference between non-infected control cells at 37° C and 42° C. There was a statistically significant difference in radioligand uptake between infected cells at 37° C and the non-infected controls ($P= 0.001$). B) mIL-12 production in pg/ml from infected and non-infected B16 cells treated at 42° C or remaining at 37° C. Cells were infected with 20 pfu/cell of AdHSP_{mIL12} with non-infected cells as controls. Supernatant was removed 24 hours after heating and mIL-12 production was determined. Difference in mIL-12 production between infected cells at 42 ° C and 37 ° C was significant ($P= 0.003$). There was not a significant difference between non-infected control cells at 37° C and 42° C or between infected cells at 37° C and the non-infected controls. bars, $\pm$ SD

A



B

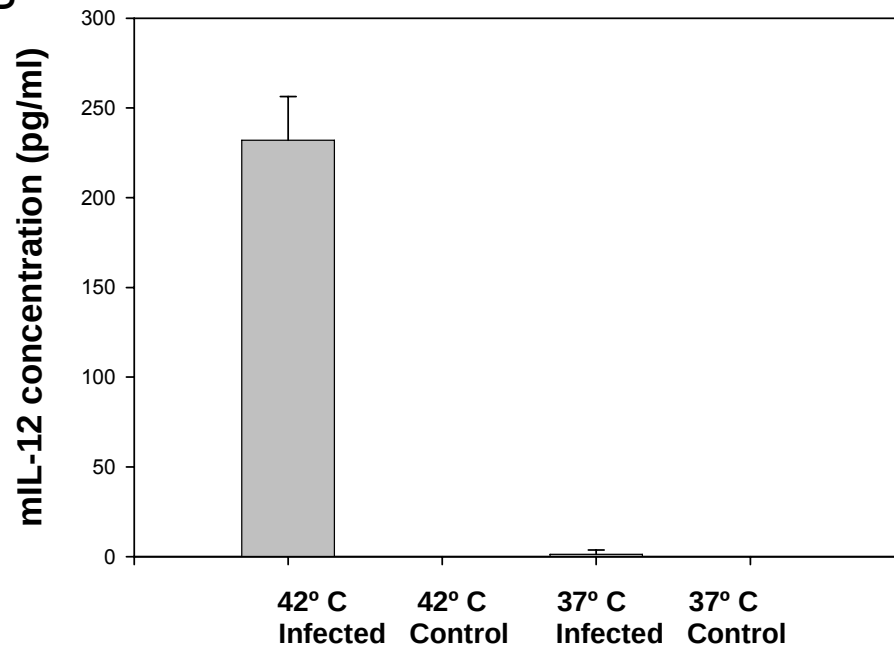


Figure 5: A) Radioligand uptake in counts per minute (CPM) as a function of heat dose.

B16 cells were co-infected with 15 pfu/cell of each Adhsphsstr2 and AdhspIL12 and either exposed to variable heat doses (38° C – 42° C) or remained at 37° C. Non-infected cells (ni) exposed to 42° C and 37° C were used as controls. The p-value for an overall difference among treatments among infected cells was $p < 0.0001$. A test for a linear increase in means as temperature increased among the infected cells was performed. The p-value for the test for trend is $p < 0.0001$. Data is from two separate experiments with 8 replicates per group from each experiment. B) mIL-12 production in pg/ml as a function of heat dose. The p-value for an overall difference among treatments among infected cells was $p < 0.0001$. The p-value for the test for trend was $p < 0.0001$. The p-value from the Kruskal-Wallis test for an overall difference among treatments among infected cells also was $p < 0.0001$, which supports the results from the ANOVA. Data is from two separate experiments with 8 replicates from each experiment in each treatment group.

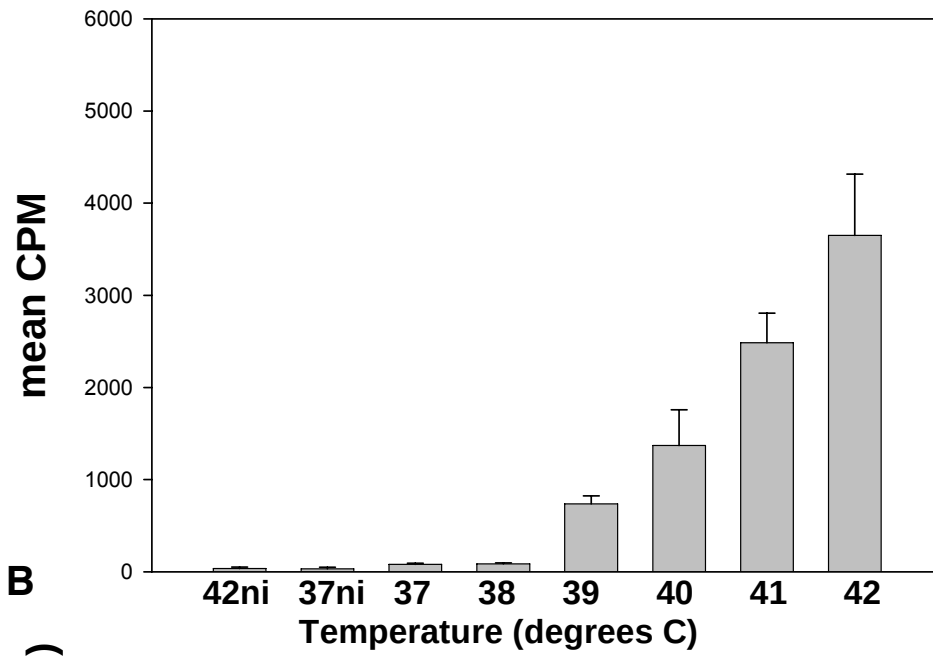
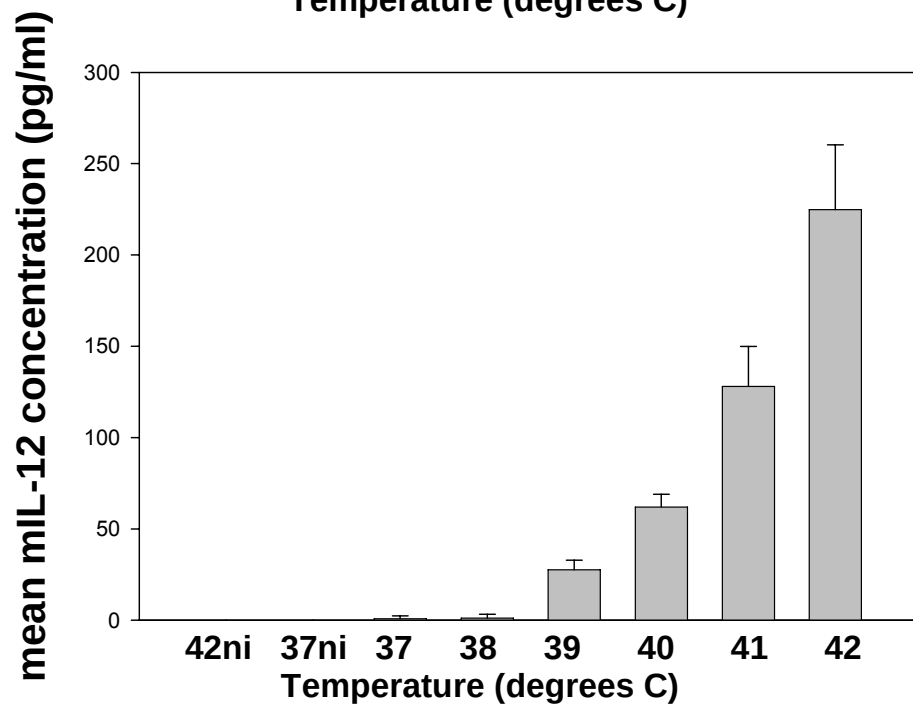
A**B**

Table 1 – *In Vitro* Spearman rank-order correlation coefficients and Pearson correlation coefficients for mIL-12 with radioligand binding

Cell Line	Spearman Coefficient	p-value	Pearson Coefficient	p-value
B16	0.93	<0.0001	0.95	<0.0001
4T1	0.90	<0.0001	0.87	<0.0001

Table 2 – *In Vitro* Regression analysis results

Cell Line	Intercept	Coefficient	R-Square	Model p-value
B16	-3.70	1.09	0.90	<0.0001
4T1	-1.50	0.86	0.75	<0.0001

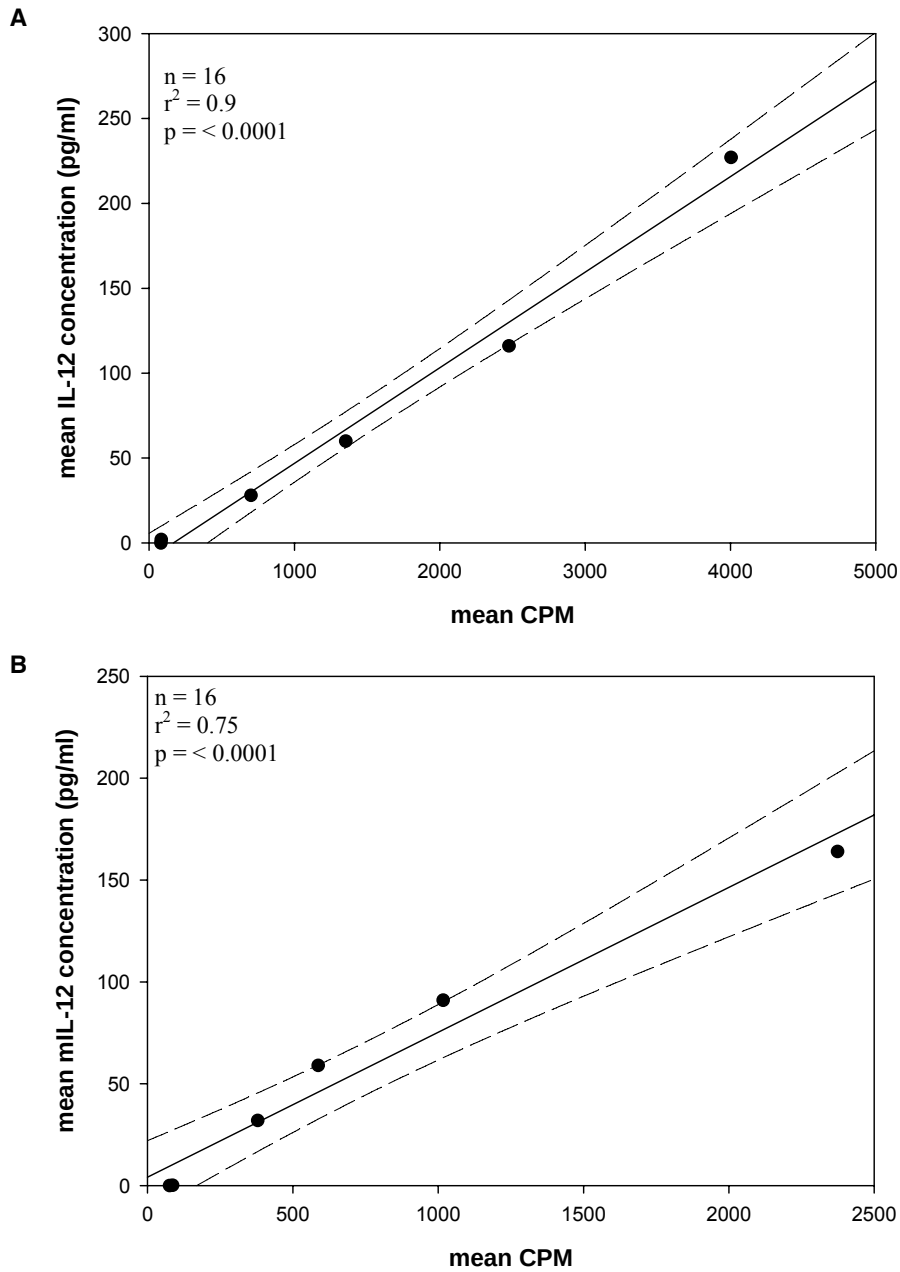


Figure 6: A) Correlation of radioligand uptake in counts per minute (CPM) and mIL-12 supernatant concentration in pg/ml from B16 cells coinfectd with 15 pfu/cell of each AdHSPsstr2 and Ad HSPmIL-12 and exposed to variable heat doses (37° C - 42° C in one degree increments) as described in Figure 5. B) The same experiment was repeated with 4T1 cells. Results also demonstrate strong correlation between radioligand uptake and mIL-12 production in response to heat treatment.

In Vivo Correlation of Heat Induced Reporter and Therapeutic Transgene Expression

Amy F. Pruitt¹, Chuan-Yuan Li², Xiuwu Zhang², Michael R. Zalutsky³, Jeff Johnson⁴, Mark W. Dewhirst², Marlene L. Hauck¹

¹Department of Clinical Sciences, North Carolina State University, College of Veterinary Medicine, Raleigh, North Carolina and Departments of ²Radiation Oncology, ³Radiology and Statistics, Duke University Medical Center, Durham, North Carolina.

Corresponding author:

Amy F. Pruitt

Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606.

Phone: 919-513-6322; Fax: 919-513-6669. E-mail: amy_pruitt@ncsu.edu

First Author: Amy F. Pruitt

Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606.

Phone: 919-513-6322; Fax: 919-513-6669. E-mail: amy_pruitt@ncsu.edu

In training: graduate student

Supported in part by: Department of Health and Human Services, NIH grant P01CA42745.

Foot line: Heat Induced Reporter Gene Expression

Abstract

A wide variety of gene therapy strategies for cancer treatment have shown impressive preclinical success. However, the difficulty has been in translating this preclinical success into effective clinical therapies. There are many challenges to overcome before the goal of cancer gene therapy is fully realized. These include the need for 1) targeted transgene expression, and 2) noninvasive evaluation of transgene expression. In this study, we hypothesized that the use of the heat inducible heat shock protein 70 promoter (HSP) in combination with the human somatostatin receptor type 2 (hSSTR2) reporter gene will allow for the *in vivo* monitoring and quantification of targeted therapeutic gene (murine Interleukin 12) expression. **Methods:** Replication-incompetent adenoviral vectors containing either the therapeutic gene (AdHSPmIL-12) or the reporter gene (AdHSPhsstr2) under control of the HSP were constructed and their function assessed *in vivo*. Balb/c and C57BL/6 mice with subcutaneous mammary adenocarcinoma and murine melanoma tumors respectively received intratumoral co-injection of 1×10^8 pfu each of AdHSPhsstr2 and AdHSPmIL-12 followed by either no hyperthermia, hyperthermia for 40 minutes at 41.5°C or hyperthermia for 40 minutes at 42.5°C. Additional animals not receiving intratumoral vector injection served as controls. Radioligand was injected via the tail vein 48 h after heating, and animals were sacrificed one hour after tail vein injection. Tissues were collected and counted in a gamma counter. Tumors were also disaggregated in lysis buffer using a Mini-Beadbeater, and murine IL-12 concentrations in tumor extracts were determined using a commercially available murine interleukin-12 (mIL-12) ELISA kit. The results of the radioligand and ELISA assays were compared to determine if gene expression from the reporter gene vector and the therapeutic gene vector could be correlated. **Results:** A statistically significant heat-

dose response of tumor radioligand uptake for the reporter gene and tumor mIL-12 production for the therapeutic gene was detected *in vivo*, with minimal expression observed in the controls. Normal tissue biodistribution of radioligand was similar between all treatment groups with no significant difference between the infected groups and the non-infected control. Results demonstrated a strong correlation between tumor radioligand uptake and tumor mIL-12 production in response to heat treatment (Pearson Coefficient 0.85, p-value = <0.0001). **Conclusion:** The results of this study indicate that gene expression from both vectors could be induced *in vivo* by hyperthermia in a temperature dependent manner and that induced expression from the reporter and therapeutic gene vectors were highly related. Normal tissue biodistribution of radioligand was similar between all treatment groups with no significant difference between the infected groups and the non-infected controls demonstrating the ability of the HSP promoter to limit transgene expression to the tumor. Results have the potential to pave the way for evaluation of heat induced gene therapy with hSSTr2 reporter monitoring in animals and humans.

Key words: Heat inducible gene therapy, Imaging, Somatostatin receptor

Introduction

Over the past decade, rapid progress in molecular sciences, along with the unraveling of the human genome, have lead to the emergence and development of molecular medicine with significant advancement in the field of gene therapy (1, 2, 94, 95, 264, 267). Edelstien *et al.* recently reported that over 1,340 gene therapy clinical trials have been completed, are ongoing or have been approved worldwide (6). In particular, gene therapy in the treatment of cancer has become an important research focus, with about two thirds of the gene therapy clinical protocols aimed at the treatment of cancer (6). This is partly due to the lack of effective conventional treatments for advanced refractory solid tumors and that strategies for cancer gene therapy are not as limited by some technical difficulties (such as achieving sustained gene expression in a sufficient number of rare, long-lived cells) that challenge gene-replacement therapies of inherited monogenetic diseases (2, 39).

A wide variety of gene therapy strategies for cancer treatment have shown impressive preclinical success. However, the difficulty has been in translating this preclinical success into effective clinical therapies (332). There are many challenges to overcome before the goal of cancer gene therapy is fully realized. These include the need for 1) targeted transgene expression, (94-96), and 2) noninvasive evaluation of transgene expression (28, 97-99).

One of the major limitations in cancer gene therapy is the inability to target transgene expression to the tumor cells, while limiting or excluding expression in normal cells. Achieving selective tumor cell targeting is extremely important for successful cancer gene therapy strategies where the goal is often to introduce a therapeutic cytotoxic gene specifically to cancer cells. Effective targeting of cancer cells increases the therapeutic

window, chance of successful treatment, and is essential for safe and effective *in vivo* human gene therapy (94-96).

Many different approaches at targeting transgene expression have been evaluated. Initially, targeting transgene expression locally to tumor cells was attempted with intratumoral injections of the gene therapy vector. However, systemic vector leakage has been reported following intratumoral injection which can lead to potentially toxic systemic levels of transgene product (61, 62). Other targeting approaches include transductional targeting by selective vector infection of target cells (94) or transcriptional targeting by selective transcription induction within target cells (95, 96). However, identifying either unique tissue/tumor type promoters for transcriptional targeting or unique ligand-receptor interactions for transcriptional targeting is challenging. Additionally, with constitutive tissue-specific or tumor-specific promoters, the duration and level of transgene expression are not controllable (60, 96).

Inducible promoters that are activated by physical or chemical agents provide an opportunity for a more adaptable method of controlled, targeted transgene expression. Inducible promoters have a distinct advantage in their ability to permit tight spatial and temporal control of transgene expression (60). The heat shock promoter, one of several inducible promoters, has been found to be one of the most efficient inducible regulatory systems (222, 243, 244) and has shown promise as a targeting tool in many preclinical cancer gene therapy models (60-62, 249). This approach exploits the heat shock response, an evolutionarily conserved response to cellular stress involving the rapid and transient induction of cytoprotective proteins (heat shock proteins) (233). With minimal baseline expression, the heat inducible hsp70 promoter can induces transgene expression at 300-1000

fold over background *in vitro* and *in vivo* after moderate hyperthermia for relatively short duration (60, 61, 249). Previous studies in animal models have shown the ability of the hsp70 promoter to target transgene expression both spatially and temporally after intratumoral adenoviral vector injection and local hyperthermia (61, 62, 250).

Another major limitation impeding the clinical translation of cancer gene therapy strategies is the difficulty in monitoring transgene expression and distribution noninvasively. Improved *in vivo* imaging modalities that can globally monitor and quantify transgene expression will greatly facilitate the testing of emergent gene therapy systems (97, 253-255). Several radiotracer imaging techniques have been used for noninvasive, *in vivo* imaging of gene transfer in animal models. Intravenous injection of a radiotracer results in its accumulation at sites of reporter gene expression that can then be detected using gamma camera, single photon emission computed tomography (SPECT) and positron emission tomography (PET) imaging techniques. The four reporter gene products that have been most researched and detected *in vivo* are the human type 2 somatostatin receptor (hsstr2), the herpes simplex virus type 1 thymidine kinase (HSTK), the type 2 dopamine receptor and the sodium iodide symporter (NIS) (98, 253, 266).

Specific, localized expression of hsstr2 reporter gene in human tumor xenografts has been demonstrated in mice after gene transfer using intratumorally injected adenovirus vector. Reporter gene expression was detected after intravenous injection of radiolabeled somatostatin analogue using gamma camera imaging and immunohistochemistry, with good agreement between imaging analyses and gamma counter measurements (316, 319-321). In a study by the same group of investigators also using a mouse xenograft model, tumor uptake of radioligand correlated well with adenoviral vector dose (266). Thus, hSSTr2 reporter gene

imaging may be useful for quantification of transgene expression in addition to spatial localization.

In this study, we hypothesized that the use of the heat inducible hsp70 promoter in combination with the hSSTr2 reporter gene will allow for the *in vivo* monitoring and quantification of targeted therapeutic gene (murine Interleukin 12) expression. In a previous study (paper in review), the feasibility of this heat inducible reporter system approach was demonstrated *in vitro* with results showing 1) a strong, positive correlation between therapeutic gene expression and hsstr2 reporter gene expression and 2) a strong heat dose response relationship. Characterization of the relationship between therapeutic gene expression and reporter gene imaging would be of considerable value in noninvasive monitoring of transgene expression, while use of a heat inducible promoter would allow temporal and spatial targeting of transgene expression.

Materials and Methods

Tumor models/Cell Culture

B16.F10 (American Type Culture Collection (ATCC), Manassas, VA) is a transplantable, spontaneously arising, murine melanoma cell line and is syngeneic in C57BL/6 mice. 4T1 (ATCC) is a transplantable, spontaneously arising, murine mammary adenocarcinoma cell line and is syngeneic in Balb/c mice. The 4T1 cells were maintained in RPMI and the B16.F10 cells were maintained in DMEM, both supplemented with 10% FBS and 1% Penicillin/Streptomycin (Mediatech, Inc. Cellgro®, Herndon, Va). HEK 293 human transformed primary embryonal kidney cells, also obtained from ATCC, were used for adenovirus propagation and were maintained in DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin (Mediatech, Inc. Cellgro®, Herndon, Va). All cells were

cultured at 37°C in a humidified atmosphere with 5% CO₂. Balb/c and C57BL/6 mice were obtained from the National Cancer Institute.

Adenoviral vectors

The replication-incompetent adenoviral vectors used in this study were based on an Ad5 recombinant system and consisted of AdHSP_{hsstr2}, AdCMV_{hsstr2} and AdHSP_{mIL-12}. The human somatostatin receptor type 2 (*hsstr2*) gene was cloned by RT-PCR from the human fibroblast cell strain 1522. It was placed under control of either the inducible human *hsp70B* (HSP) promoter or under control of a constitutively active CMV promoter to derive the AdHSP_{SSTr2} and AdCMV_{hSSTr2} vectors respectively. The murine IL-12 gene was obtained as described (60). In construction of the heat-inducible murine interleukin 12 (mIL-12) gene, the cDNAs for both mIL-12 subunits (p40 and p35) were linked together through the internal ribosome entry site sequence so that both subunits were expressed from a single promoter. The gene was then placed under control of the HSP promoter to construct the AdHSP_{mIL12} vector. All vectors were then packaged by use of the AdEasy system (Stratagene, La Jolla, CA) (324). The individual gene expression cassettes were transferred into the pShuttle plasmid of the AdEasy system. The viruses were propagated in 293 cells, and purification by CsCl-banding was performed according to a standard protocol (325).

Radiolabeled Peptide

The somatostatin receptor avid peptide used in this study was glucose-octreotate (Gluc-TOCA), an octreotide analogue with COOH-terminal oxidation and NH₂-terminal carbohydration. Radioiodination of Gluc-TOCA was performed using Chloramine-T as previously reported (298). Briefly, 20 µl of 0.05 M phosphate buffer (pH 7.5) and a solution of peptide in 0.05 M acetic acid (14 µg in 20 µl) was added to a solution of sodium

[¹²⁵I]iodide (approximately 1 mCi in 3 µl of 0.1 N NaOH; PerkinElmer Life and Analytical Sciences, Billerica, MA) in a Reacti vial. This mixture was vortexed for 30 sec and then a solution of Chloramine-T in the above phosphate buffer (1.6 µg in 20 µl) was added. The reaction occurred at room temperature over 1 minute of vortexing. The labeled peptide was isolated from the reaction mixture by reversed-phase HPLC using a linear gradient (10% to 40% B over 30 min) consisting of solvents 0.1% trifluoroacetic acid in water (A) and 0.1% trifluoroacetic acid in acetonitrile (B) at a flow rate of 1 ml/min. The HPLC fraction containing the labeled peptide was passed through an activated solid-phase cartridge (C18ENV; Waters). The cartridge was washed with 5 ml of water and 1 ml of 0.5 M HOAc, and then eluted with 5 × 0.25 ml ethanol. The ethanol fractions containing the radioiodine activity were pooled, evaporated with a stream of argon at room temperature, and reconstituted in a solution BSA and PBS (pH 7.14) for use in animal experiments.

Animal Biodistribution Studies

All animal studies were performed in accordance with institutional guidelines. Approximately 10⁶ mammary adenocarcinoma or melanoma cells in 50 µL PBS were transplanted subcutaneously in the right hindlimbs of Balb/c or C57BL/6 mice respectively. Treatment was initiated when tumors were 4-6 mm in diameter (about 6 days), and each treatment group consisted of 5 animals. Adenoviral vectors were injected intratumorally using a 30-gauge needle at a dosage of 1 × 10⁸ pfu (in 50 µL PBS) either alone or, in the case of the HSP promoter vectors, combined. Animals that received intratumoral injections of AdHSP_{hsstr2} +/- AdHSP_{mIL-12} were treated with local hyperthermia 24 h after vector injection. Tumors were heated to 41.5° C or 42.5° C for 40 min by immersion of the right hindlimb in a circulating water bath while mice were under pentobarbital anesthesia (1.5

mg/20-g mouse i.p.). Additional animals received intratumoral injection of AdHSP β str2 and/or AdHSP β IL-12 but remained at 37° C to serve as unheated controls to determine baseline HSP promoter activity. Mice not receiving intratumoral adenoviral vector injection for each treatment group also served as controls to determine constitutive gene expression. Based on a previous study (250) that detected the highest level of heat induced gene expression 48 h after tumor heating, 5 μ Ci of [125 I] Gluc-TOCA was injected via the tail vein 48 h after heating in all experiments. All animal treatment groups were sacrificed one hour after tail vein injection of [125 I] Gluc-TOCA based on a previous study (298) that reported the highest tumor to normal tissue ratios for Gluc-TOCA were generally seen one hour post-injection. Animals that received intratumoral injections of AdCMV β str2 were injected with 5 μ Ci of [125 I] Gluc-TOCA via the tail vein 48 h after vector injection. All mice were killed with an overdose of halothane, dissected, and tissues of interest were isolated. Weights of blot-dried tissues were determined, and the tissues were counted along with dose standards in a LKB 1282 dual-channel gamma counter (Wallac, Turku, Finland). The results were expressed as % injected dose/gram tissue. For animals that received intratumoral injections of AdHSP β IL-12, tumors of sacrificed animals were disaggregated in a 2 ml microcentrifuge tube (BioSpec Products, Inc., Bartlesville, OK) containing 1.0 ml of 1.0 mm zirconia beads (BioSpec Products) and 500 μ L lysis buffer consisting of mammalian protein extraction reagent (M-PER®, Pierce Biotechnology, Rockford, IL) and Halt protease inhibitor cocktail (Pierce Biotechnology) at a 10 μ L Halt/mL concentration. The tube was placed in a Mini-Beadbeater (BioSpec Products) and shaken 2-3 cycles at 4,800 rpm for 1 min with cooling on ice between cycles. Centrifugation for 10 minutes at 10,000 rpm followed and a micropipette was used to remove supernatant from the vial. Murine IL-12 concentrations in tumor

extracts were determined using a commercially available murine interleukin-12 (mIL-12) ELISA kit (Quantikine mouse IL-12p70, R&D Systems, Minneapolis, MN). The results were expressed as pg mIL-12/gram tumor. Blood and tissue sampling for mIL-12 levels was not believed necessary due to the reports in previous papers of minuscule systemic levels of IL-12 after intratumor injection of the vector containing the IL-12 gene under control of the HSP promoter (61, 62).

Statistical Analysis

One-way analysis of variance (ANOVA) was used to test for differences among treatment groups. A log transformation was used prior to analyzing the tumor radioligand uptake and tumor mIL-12 production data. The Kruskal-Wallis test was used to compare mIL-12 levels in non-infected vs. infected tumor cells at 37 degrees. ANOVA tests were performed on untransformed data for radioligand uptake by treatment by normal tissue type for each tissue separately. Multiple comparisons tests using the Bonferroni adjustment were performed to test for pair-wise differences among treatment groups. Spearman rank-order correlations and Pearson correlation coefficients were computed for mIL-12 production and radioligand binding over treatment groups in tumor cells. Regression analysis using radioligand uptake as a predictor of mIL-12 production was also performed on these data.

All analyses were performed using SAS v.9.1.

Results

A heat dose response was initially evaluated for each heat inducible vector individually in experiments using Balb/c mice with subcutaneous mammary adenocarcinoma. Treatment groups received intratumoral injection of 1×10^8 pfu of AdHSP β str2 or AdHSPmIL-12 and either no hyperthermia, hyperthermia for 40 minutes at 41.5°C or

hyperthermia for 40 minutes at 42.5°C. A significant difference in tumor radioligand uptake and mIL-12 production was observed between the different heat doses (data not shown). Based on the results of these experiments evaluating the inducible vectors individually, additional experiments were performed to evaluate intratumoral co-injection of both AdHSPhsstr2 and AdHSPmIL-12 vectors.

Table 3 shows the biodistribution of [¹²⁵I]Gluc-TOCA in Balb/c mice bearing 4T1 mammary tumors. AdHSPhsstr2 and AdHSPmIL-12 were co-injected intratumorally at 1 X 10⁸ pfu each. Experiments consisted of four treatment groups as described in Table 3 and included animals with tumors that were 1) not infected, not heated, 2) infected, not heated, 3) infected, heated at 41.5°C, 4) infected, heated at 42.5°C. Data is from two separate experiments with 5 animals per group from each experiment. A heat dose response was observed with tumor uptake of [¹²⁵I] Gluc-TOCA occurring in a temperature dependent manner. A significant increase in tumor radioligand uptake was detected between 37°C and 41.5°C and between 41.5°C and 42.5°C for tumors infected with AdHSPhsstr2. There was not a significant difference in tumor radioligand uptake between the infected and non-infected groups at 37°C. The p-value for an overall difference among means among the infected cells at the 3 different temperatures was $p < 0.0001$. The test for an increasing trend in mean levels with temperature was also significant at $p < 0.0001$. Normal tissue biodistribution of [¹²⁵I] Gluc-TOCA was similar between all treatment groups with no significant difference between the infected groups and the non-infected control.

A separate experiment was conducted with mice receiving intratumoral injection of AdCMVhSSTr2 containing the constitutive CMV promoter for comparison with mice receiving intratumoral injection of AdHSPhSSTr2 containing the inducible HSP promoter.

The experiment consisted of four treatment groups as described in Table 4 and included animals with tumors that were 1) not infected, heated at 42.5°C (to determine affect of heating alone on radioligand uptake), 2) HSP infected, not heated, 3) HSP infected, heated at 42.5°C, 4) CMV infected. Data is from a single experiment with 5 animals per treatment group. There was not a significant difference in tumor radioligand uptake in the non-infected 42.5°C group when compared with the infected 37°C group, or when compared with the non-infected 37°C groups from other experiments. A significant increase in tumor radioligand uptake was detected in the infected 42.5°C group compared to the non-infected and infected control groups. Normal tissue biodistribution of [¹²⁵I] Gluc-TOCA was similar between all AdHSPhSSTr2 treatment groups with no significant difference between the infected groups and the non-infected, heated control. There was a significant increase in [¹²⁵I] Gluc-TOCA uptake in the liver, spleen and lungs of mice treated with the AdCMVhSSTr2 vector compared to all other treatment groups. Tumor [¹²⁵I] Gluc-TOCA uptake in the CMV group was significantly greater than in the infected 42.5°C group.

Figure 7 contains data from the combined experiments described above for Table 3. This figure shows tumor mIL-12 production as a function of heat dose compared with tumor radioligand uptake as a function of heat dose. Similar to results described in Table 3 for tumor radioligand uptake, a significant increase in tumor mIL-12 production was detected between 37°C and 41.5°C and between 41.5°C and 42.5°C for tumors infected with AdHSPmIL-12. There was also not a significant difference in tumor mIL-12 production between the infected and non-infected groups at 37°C. The p-value for mIL-12 levels in non-infected vs. infected tumor cells at 37°C p=0.086. The p-value for an overall difference among means among the infected cells at the 3 different temperatures was p < 0.0001. The

test for an increasing trend in mean levels with temperature was also significant at $p < 0.0001$.

Spearman rank-order correlations and Pearson correlation coefficients were computed for tumor mIL-12 and radioligand binding over treatment groups. The correlation coefficients and p-values are reported in Table 5. Regression analysis, using radioligand uptake as a predictor of IL-12 production was also performed on these data. Table 6 shows the results from these analyses. Results demonstrated a strong correlation between tumor radioligand uptake and mIL-12 production in response to heat treatment after intratumor co-injection of AdHSP_{hsstr2} and AdHSPmIL-12 as shown in Figure 8A. This experiment (two separate experiments with 5 animals per group from each experiment) was repeated in C57BL/6 mice bearing B16.F10 melanomas (Figure 8B), and the results obtained were similar to those described for the 4T1 mammary adenocarcinoma.

Discussion

For the clinical advancement of many promising gene therapy strategies, two important goals must be addressed. They are the need to target transgene expression to the intended cells only and to noninvasively monitor and quantify transgene expression. Different approaches to address each of these challenges have been evaluated and reported in numerous studies. The development of an approach that would allow both targeted transgene expression and noninvasive monitoring has great promise to accelerate the development and testing of new gene therapies for many diseases. However, studies evaluating a combined approach that targets transgene expression and correlates reporter gene expression with therapeutic gene expression have been limited. In this study, we evaluated a gene therapy approach that has the potential to achieve both of these goals. We used AdHSP_{hsstr2} and AdHSPmIL-12 vectors to demonstrate *in vivo* that 1) level of therapeutic gene (mIL-12)

expression was measurable by monitoring hsstr2 activity with radioligand uptake, and 2) the level of expression of both vectors was controllable by the heat dose administered.

After intratumoral vector co-injection and heat treatment, AdHSPhsstr2 and AdHSPmIL-12 resulted in tumor radioligand uptake and mIL-12 production respectively in a temperature dependent manner. A significant increase in tumor radioligand uptake and mIL-12 production was detected between each treatment temperature. There was not a significant difference in tumor radioligand uptake between the infected and non-infected tumors that remained at 37°C demonstrating little to no baseline HSP promoter activity. Normal tissue biodistribution of radioligand was similar between all treatment groups with no significant difference between the infected groups and the non-infected controls confirming the ability of the HSP promoter to limit transgene expression to the tumor. In contrast, biodistribution studies after intratumoral injection of AdCMVhSSTr2, containing the constitutive promoter, showed a significant increase in radioligand uptake in the liver, spleen and lungs of treated mice. These results are consistent with previous studies reporting systemic vector leakage after intratumoral vector injection (61, 62), and it highlights the potential for systemic toxicity when using constitutive promoters. Tumors in one group of mice were heated at 42.5°C but were not injected with vector to determine the effect of heat alone on radioligand uptake and mIL-12 production, with results showing no effect of heat alone. Therapeutic mIL-12 gene expression measured by ELISA was strongly, positively correlated with hsstr2 activity as determined by radioligand uptake. These results support the hypothesis that use of the heat inducible hsp70 promoter in combination with the hsstr2 reporter gene can be used to 1) regulate therapeutic and reported transgene expression in a temperature dependent manner, and 2) monitor and quantify, non-invasively, induced therapeutic transgene

expression based on reporter activity. These results are significant as the development of both regulatable transgene expression systems and noninvasive molecular imaging systems that can measure therapeutic transgene expression both at the target site and on a whole body level are essential for the rational progress of gene therapy (221, 333). Recently, somatostatin analogs radiolabeled with PET radionuclides have been evaluated, with *hsstr2* gene transfer detected in mice using microPET imaging. This is a more sensitive imaging modality compared to gamma camera/SPECT imaging and holds much promise for future studies with *hsstr2* (322).

In this study the gene encoding the immunomodulatory cytokine interleukin-12 (IL-12) was used as the therapeutic gene. Previous studies evaluating IL-12 in preclinical tumor models have demonstrated that it has significant antitumor activity (60). However, any therapeutic gene could be utilized with the gene therapy approach evaluated in this study. A unique advantage of inducible gene therapy approaches that are based on external physical agents over other inducible gene therapy approaches is the ability to focus the inducing agent in a spatially defined manner. When evaluating chemically inducible promoters, intratumoral vector injection is often employed to target gene expression. However, as previously stated, this approach has been associated with systemic vector dissemination (62, 221). Leakage of vector outside of the target tissue can lead to ectopic expression of transgene in distant organs because systemic injection of promoter-activating agents used with chemically induced promoters does not permit correction for this leakage (60, 221). The heat inducible promoter system offers several advantages over other physically inducible promoter systems such as the radiation inducible promoter system. The *hsp70* promoter has been shown to have minimal baseline transgene expression and strong induction at 300-1000

fold over background in a heat dose dependent manner (60, 61, 245). In contrast, the ERG-1 promoter that is induced by ionizing radiation (a carcinogen) has high baseline transgene expression and is only inducible 10-fold *in vitro* without additional modification (223, 224). Also, the hyperthermia treatment can be safely administered. No long-term side effects have been observed in over 20 years of hyperthermia use in experimental cancer treatment at temperatures in the range shown to be most effective for heat-induced transgene expression (60). Therefore, the heat inducible promoter system is potentially suitable for treatment of diseases other than cancer.

Potential disadvantages of a hyperthermia inducible gene therapy approach include difficulty heating some tumor locations and non-specific activation of HSP promoter. It is sometimes difficult to heat specific sites in the body to the desired temperatures (60, 61). However, recently there has been significant improvement in the ability to heat more target areas in the body. This has resulted from continued technological advances in equipment development for planning, implementing and monitoring hyperthermia treatments. Hyperthermia treatments have been successfully administered to patients with a variety of tumor types including breast, cervix, head and neck cancers and glioblastoma multiforme (60, 327-330). Another potential problem is activation of the heat shock response by cellular stresses other than heat application. These stressors could include oxidative stress, hypoxia, ischemia, acidosis, cytokines, fever or inflammation (60, 221). However, only minimal basal expression of HSP promoter controlled genes have been observed in most reported studies (62, 221), and there was no difference in radioligand uptake between the infected and non-infected control mice in this study. Nevertheless, monitoring and controlling body temperature below 39°C may be necessary in certain clinical situations (60).

While the use of a specific imaging probe that can directly detect therapeutic gene expression would be the ideal way to monitor therapeutic gene expression, a direct imaging probe is not available for most therapeutic genes (267, 331). In addition, new probe development and in vivo validation for each therapeutic gene would be time-consuming and expensive. Instead, research has concentrated on the development of a few well-characterized reporter genes for indirect imaging of therapeutic gene expression. A variety of methods exist for linking the expression of a therapeutic gene to a reporter gene including fusion protein constructs, bidirectional promoter vectors, dual promoter constructs, bicistronic vectors or simply co-vector administration (276, 331).

The most straightforward of these approaches is co-vector administration. The reporter gene and therapeutic gene used in this study were delivered by two separate but identical adenoviral vectors. If both vectors are identical and the same promoter regulates both the reporter and therapeutic gene, then co-administration of equal amounts of both vectors should allow correlation of expression of both genes at the target tissue (267) which was achieved in the current study. There should be equal probability of uptake by the target cells for each vector because the host responses to a gene therapy vector should be the same whether it is carrying the therapeutic or reporter gene (276). The two vectors will not infect any single cell in equal amounts but this should not pose a significant problem given the spatial resolution limits of most clinical imaging modalities (267). The greatest advantage of co-vector administration is that many different therapeutic genes can be evaluated with a single reporter gene vector (267, 276).

A potential limitation of the double vector approach is the potential for greater immunological reactions due to the concomitant increase of viral burden (267, 276).

However, the risk of this potential problem may be reduced with the continued development of improved, less immunogenic adenoviral vectors (37). Having both the therapeutic gene and reporter gene regulated by identical promoters on separate vectors could pose a problem that may affect correlation between the reporter and therapeutic gene (276). Additional mechanisms of transcription regulation could reduce the correlation between the expression of the therapeutic and reporter gene due to positive and negative feedback from either gene (267, 276). However, this simple approach of vector co-administration has been validated in a previous study using an adenovirus vector and CMV promoter (276). In this study using an adenoviral vector and HSP promoter, we also found a strong correlation between reporter and therapeutic gene expression after intratumoral co-injection of AdHSP β gal and AdHSPmIL-12. The bicistronic vector, bidirectional promoter or fusion protein approaches may eliminate this potential problem of loss of correlation associated with the use of two vectors because the transcription of both the therapeutic and the reporter gene will be affected since both are regulated by a single promoter (276). Future optimization of the heat inducible reporter system used in this study may involve development of a single vector construct containing both the therapeutic and reporter gene. However, the results of this study demonstrate the feasibility of using AdHSP β gal and AdHSPmIL-12 co-injection for monitoring transgene expression in vivo. In addition, loss of correlation between therapeutic and reporter gene protein product at the translational and posttranslational level can occur with any of these approaches except for the fusion protein approach where therapeutic and reporter product function may be lost with the final folding of the fusion protein (267, 276).

Conclusion

In summary, the results of this study demonstrate the feasibility of this heat inducible reporter system approach with *in vivo* results showing a strong, positive correlation between therapeutic gene expression and hsstr2 reporter gene expression that was regulated by heat dose. Comparing tumor radioligand uptake in % injected dose/gram tumor and tumor mIL-12 concentration in pg mIL-12/gram tumor is the gold standard for evaluating correlation between reporter and therapeutic gene expression. Imaging was not performed in this study, but having now demonstrated a positive correlation between reporter and therapeutic gene expression with direct tissue measurements, imaging may be evaluated in future studies. However, one would expect that the strong correlation identified would be persistent with imaging studies. Others have reported good agreement between imaging analyses and gamma counter measurements in detection of hSSTr2 reporter gene expression after intratumoral adenovirus vector injection in mice with human tumor xenografts (316, 319-321). The use of inducible gene therapy systems that allow tight control of both spatial and temporal transgene expression and the development of sensitive, quantitative methods for *in vivo* monitoring of transgene expression will be critical for the future advancement of gene therapy.

Acknowledgments

Supported in part by: Department of Health and Human Services, NIH grant P01CA42745.

Table 3: Biodistribution of [125 I] Gluc-TOCA in mice bearing 4T1 murine mammary tumors evaluating the HSP promoter at different temperatures.

% Injected dose/gram Tissue^a

Tissue	37° C Non-infected	37° C Infected	41.5° C Infected	42.5° C Infected
Liver	2.33 +/- 0.35	2.37 +/- 0.31	2.41 +/- 0.60	2.40 +/- 0.46
Spleen	2.04 +/- 0.47	2.09 +/- 0.37	2.10 +/- 0.35	2.21 +/- 0.48
Lungs	10.26 +/- 1.58	10.18 +/- 1.28	10.33 +/- 1.62	9.96 +/- 1.96
Heart	1.11 +/- 0.16	1.13 +/- 0.16	1.14 +/- 0.12	1.12 +/- 0.12
Kidneys	5.65 +/- 0.49	5.80 +/- 0.38	5.95 +/- 0.67	5.75 +/- 0.57
Stomach	12.59 +/- 1.68	11.29 +/- 1.99	11.67 +/- 1.98	12.40 +/- 2.86
Thyroid ^b	0.24 +/- 0.05	0.25 +/- 0.07	0.29 +/- 0.06	0.30 +/- 0.08
Muscle	0.30 +/- 0.08	0.32 +/- 0.03	0.33 +/- 0.06	0.32 +/- 0.09
Blood	1.08 +/- 0.22	0.98 +/- 0.3	1.08 +/- 0.39	1.03 +/- 0.30
Brain	0.10 +/- 0.03	0.11 +/- 0.03	0.13 +/- 0.03	0.11 +/- 0.02
Tumor ^c	1.11 +/- 0.12	1.16 +/- 0.15	3.62 +/- 0.36	5.43 +/- 0.55

^a %ID/g tissue; mean $\pm$ SD for n = 10

^b % injected dose per organ

^c The p-value for the test for differences between infected and non-infected tumors at 37 degrees was p=0.46. The p-value for an overall difference among means among the infected cells at the 3 different temperatures was p < 0.0001. The test for an increasing trend in mean levels with temperature was also significant at p < 0.0001.

Table 4: Biodistribution of [¹²⁵I] Gluc-TOCA in mice bearing 4T1 murine mammary tumors comparing the HSP promoter with the CMV promoter.

% Injected dose/gram Tissue^a

Tissue	42° C Non-infected	37° C Infected	42.5° C Infected	CMV Infected
Liver ^b	2.40 ± 0.41	2.31 ± 0.44	2.50 ± 0.32	5.69 ± 0.47
Spleen ^c	2.21 ± 0.62	2.15 ± 0.90	2.15 ± 0.41	4.93 ± 0.23
Lungs ^d	10.40 ± 1.86	10.57 ± 1.14	10.83 ± 1.27	16.50 ± 1.05
Heart	1.06 ± 0.24	1.08 ± 0.32	1.04 ± 0.24	1.07 ± 0.45
Kidneys	5.14 ± 0.36	4.85 ± 0.79	5.72 ± 0.54	5.83 ± 0.61
Stomach	9.51 ± 1.20	10.78 ± 1.14	11.48 ± 1.61	11.91 ± 1.05
Thyroid ^e	0.14 ± 0.06	0.16 ± 0.05	0.17 ± 0.04	0.16 ± 0.08
Muscle	0.27 ± 0.11	0.30 ± 0.03	0.31 ± 0.04	0.31 ± 0.07
Blood	0.86 ± 0.12	0.79 ± 0.06	0.91 ± 0.14	1.01 ± 0.13
Brain	0.11 ± 0.02	0.13 ± 0.02	0.12 ± 0.02	0.11 ± 0.01
Tumor ^f	0.96 ± 0.57	1.19 ± 0.45	5.64 ± 0.83	6.54 ± 0.62

^a %ID/g tissue; mean ± SD for n = 5

^{b, c, d} Values for the CMV group were significantly higher than values for the 42.5° C group (P = <0.0001, P = <0.0001, P = 0.0006 respectively).

^e % injected dose per organ

^f Value for CMV group was significantly higher than value for 42.5° C group (P = 0.033).

Table 5 – *In Vivo* Spearman rank-order correlation coefficients and Pearson correlation coefficients for mIL-12 with radioligand binding in tumors

Cell Line	Spearman Coefficient	p-value	Pearson Coefficient	p-value
4T1	0.87	<0.0001	0.89	<0.0001
B16	0.83	<0.0001	0.85	<0.0001

Table 6 – *In Vivo* Regression analysis in tumors

Cell Line	Intercept	Coefficient	R-Square	Model p-value
4T1	-65.07	75.60	0.79	<0.0001
B16	-32.06	70.66	0.72	<0.0001

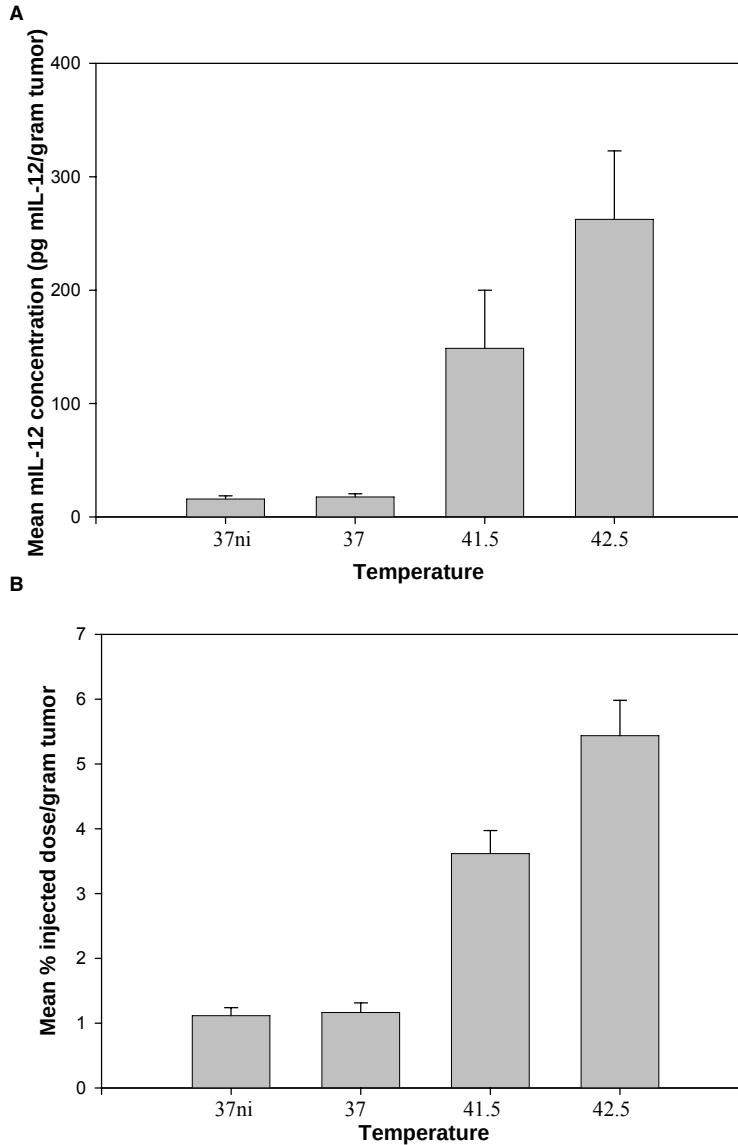


Figure 7: Data from Balb/c mice bearing 4T1 mammary tumors that were co-injected with AdHSP_{hsstr2} and AdHSP_{mIL-12} at 1×10^8 pfu each and non-infected controls (ni). A) mL-12 production in pg mL-12/gram tumor as a function of heat dose. The p-value for an overall difference among means among the infected cells at the 3 different temperatures was $p < 0.0001$. The test for an increasing trend in mean levels with temperature was also significant at $p < 0.0001$. B) Radioligand uptake in % injected dose/gram tumor as a function of heat dose. Data from Table 3; bars, $\pm$ SD

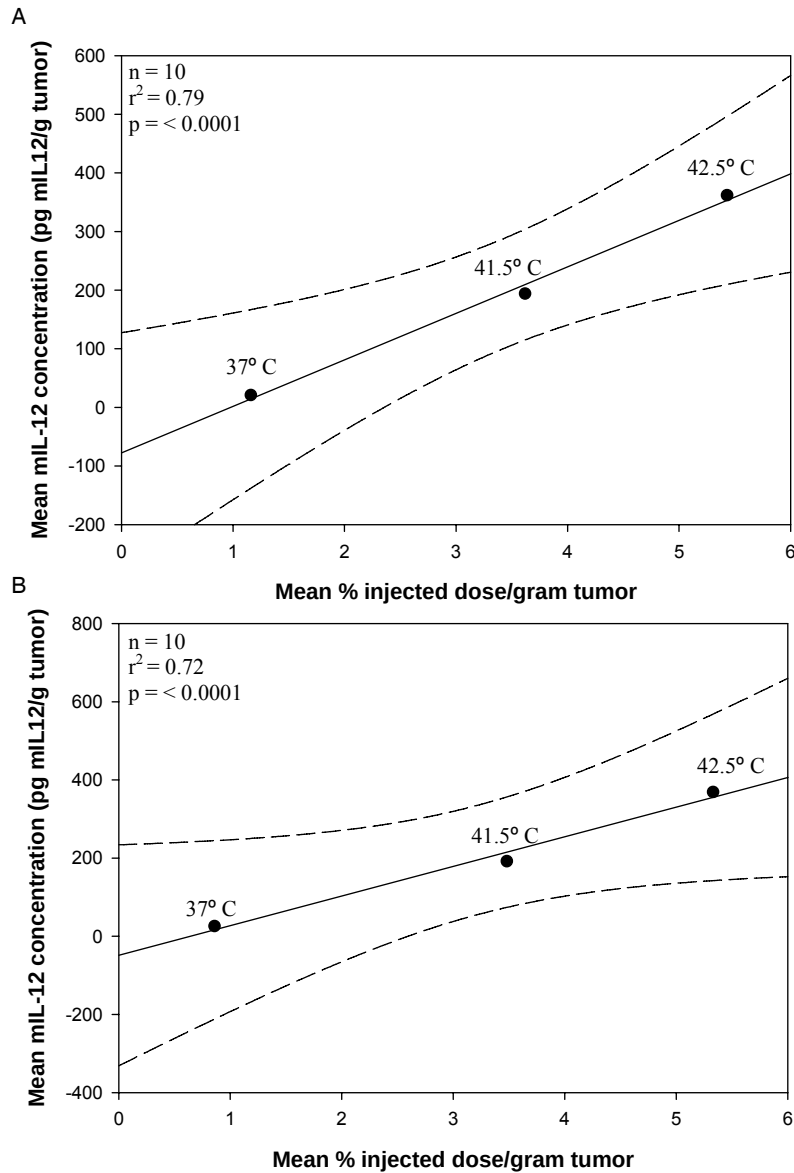


Figure 8: A) Correlation of tumor radioligand uptake in % injected dose/gram tumor and tumor mIL-12 concentration in pg mIL-12/gram tumor from Balb/c mice bearing 4T1 mammary tumors as described in Figure 7. Results demonstrate strong correlation between tumor radioligand uptake and mIL-12 production in response to heat treatment. B) The same experiment was repeated in C57BL/6 mice bearing B16.F10 melanomas. Results also show strong correlation between radioligand uptake and mIL-12 production in response to heat treatment.

SUMMARY/FUTURE CONCEPTS

The goal of this research was to evaluate a heat inducible gene therapy strategy to target both reporter and therapeutic transgene expression to tumor cells exclusively and to correlate the expression of therapeutic transgene expression with reporter gene detection. The *in vitro* results showed a strong, positive correlation between therapeutic gene expression and hsstr2 reporter gene expression that was regulatable by heat dose. These results were validated with *in vivo* experiments where therapeutic mIL-12 gene expression measured by ELISA was strongly, positively correlated with hsstr2 activity as determined by radioligand uptake. These *in vivo* results support the hypothesis that use of the heat inducible hsp70 promoter in combination with the hsstr2 reporter gene can be used to 1) regulate therapeutic and reported transgene expression in a temperature dependent manner, and 2) monitor and quantify induced therapeutic transgene expression based on reporter activity. While the results obtained in this mouse model were positive and encouraging, validation of this system in a large animal spontaneous tumor model would better predict the successful translation of this approach in people. Pets with cancer are excellent models of human cancer, and positive studies in large animal spontaneous tumor models may expedite future human clinical investigations. Imaging was not performed in this study because comparing tumor radioligand uptake in % injected dose/gram tumor and tumor mIL-12 concentration in pg mIL-12/gram tumor is the gold standard for evaluating correlation between reporter and therapeutic gene expression. Having now demonstrated a positive correlation between reporter and therapeutic gene expression with direct tissue measurements, imaging may be evaluated in future studies. However, one would expect that the strong correlation identified would be persistent with imaging studies. The recent reports of somatostatin analogs

radiolabeled with PET radionuclides for use in evaluating *hsstr2* gene transfer in mice using microPET imaging will likely prove to be a significant development for future studies. This is a more sensitive imaging modality compared to gamma camera/SPECT imaging and holds much promise for future studies with *hsstr2*.

While the inducible gene therapy reporter system evaluated in this research could be used in many clinical settings for many different diseases, it may be ideal in cancer immunotherapy. In this research, I used a replication-incompetent adenovirus vector based on an Ad5 recombinant system, with deletion of the E1 and E3 viral genes. My vector included a therapeutic gene for *mIL-12* and a reporter gene for the human type 2 somatostatin receptor, both under the control of heat shock protein promoters. The recombinant adenovirus vector was injected intratumorally, and transgene transcription was induced by the administration of local hyperthermia. This vector system efficiently transduces both dividing and non-dividing cells which is ideal in the setting of tumors that can contain a heterogeneous cell population of both dividing and quiescent cells. The strong immune response against adenoviral vectors can be a disadvantage in certain applications. However, when used in cancer gene therapy, the cellular toxicity and immunogenicity might enhance antitumor effects. This is particularly true with the therapeutic gene used in this research project which encoded the potent immunostimulatory cytokine *IL-12*. Also, the local hyperthermia used to induce transcription of the therapeutic and reporter gene induces the expression of heat shock proteins that may also play a role in tumor-specific immune response. In addition, several studies have shown that an inhibitory signal is produced when somatostatin and related analogues bind to somatostatin receptors, producing an inhibitory

signal that is antiproliferative and reported to inhibit tumor growth in many animal tumor models.

Direct tumor injection of heat inducible gene therapy vectors followed by local hyperthermia has been effective in various preclinical studies. However, there may be a limited number of accessible sites for direct injection and local hyperthermia administration. Also, the serious problem of metastatic disease cannot be addressed with this local treatment approach. Future studies could look at double targeting where transductional and transcriptional targeting can be combined to create a double-targeted vector. This approach could potentially be synergistic with regard to safety (transcriptional) and efficacy (transductional). Transductional targeting could allow systemic vector administration to address tumor sites not accessible for direct injection and to address metastatic disease. Transcriptional targeting can regulate the magnitude and duration of gene expression within the transduced cancer cells. With the inducible reporter system used in this study, addition of transductional targeting followed by whole body hyperthermia would allow for treatment of distant metastatic sites. This is the approach I would take to improve this system, especially in regards to cancer gene therapy. If cancer gene therapy vectors can be both targeted to transduce cancer cells and designed to undergo tumor-specific transgene expression, then the level of transgene product within tumor can be greatly increased while sparing normal tissues.

I could see a role for gene expression profiling by microarray in future studies. Studies have shown that many types of tumors express or overexpress HSP70. Additionally, somatostatin receptors have also been shown to be present and overexpressed in a number of tumors. Thus, gene expression profiling could be used to determine those tumors types that

have overexpression of HSP70 and consequently may show increased transcription of both transgenes even in non heated controls due to tumor microenvironment activation of the HSP70 promoter. Likewise, if the somatostatin receptors are already present or overexpressed in certain tumors, results may show no correlation between reporter gene and therapeutic gene expression when there may in fact be a correlation. Gene expression profiling could determine which tumor types would be better suited for this heat inducible reporter gene therapy application. Gene expression profiling by microarray technology could also be used to determine tumor specific ligand-receptor interactions for transductional targeting.

The results from the research reported here are significant because the development of both regulatable transgene expression systems and noninvasive molecular imaging systems that can measure therapeutic transgene expression both at the target site and on a whole body level are essential for the rational progression of gene therapy into human clinical trials.

LITERATURE CITATIONS

1. Leiden JM. Human gene therapy: the good, the bad, and the ugly. *Circulation research*. May 12 2000;86(9):923-925.
2. Dranoff G. Cancer gene therapy: connecting basic research with clinical inquiry. *J Clin Oncol*. Jul 1998;16(7):2548-2556.
3. Verma IM, Weitzman MD. Gene therapy: twenty-first century medicine. *Annual review of biochemistry*. 2005;74:711-738.
4. Cavazzana-Calvo M, Fischer A. Gene therapy for severe combined immunodeficiency: are we there yet? *The Journal of clinical investigation*. Jun 2007;117(6):1456-1465.
5. Rosenberg SA, Aebbersold P, Cornetta K, et al. Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *The New England journal of medicine*. Aug 30 1990;323(9):570-578.
6. Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2007--an update. *The journal of gene medicine*. Oct 2007;9(10):833-842.
7. Verma IM, Somia N. Gene therapy -- promises, problems and prospects. *Nature*. Sep 18 1997;389(6648):239-242.
8. Russell SJ. Science, medicine, and the future. Gene therapy. *BMJ (Clinical research ed)*. Nov 15 1997;315(7118):1289-1292.
9. Couzin J, Kaiser J. Gene therapy. As Gelsinger case ends, gene therapy suffers another blow. *Science (New York, NY)*. Feb 18 2005;307(5712):1028.
10. Vogel G. Gene therapy. FDA moves against Penn scientist. *Science (New York, NY)*. Dec 15 2000;290(5499):2049-2051.
11. Marshall E. Gene therapy death prompts review of adenovirus vector. *Science (New York, NY)*. Dec 17 1999;286(5448):2244-2245.
12. Raper SE, Chirmule N, Lee FS, et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Molecular genetics and metabolism*. Sep-Oct 2003;80(1-2):148-158.
13. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science (New York, NY)*. Apr 28 2000;288(5466):669-672.

14. Cavazzana-Calvo M, Lagresle C, Hacein-Bey-Abina S, Fischer A. Gene therapy for severe combined immunodeficiency. *Annual review of medicine*. 2005;56:585-602.
15. Hacein-Bey-Abina S, von Kalle C, Schmidt M, et al. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *The New England journal of medicine*. Jan 16 2003;348(3):255-256.
16. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science (New York, NY)*. Oct 17 2003;302(5644):415-419.
17. Williams DA, Baum C. Medicine. Gene therapy--new challenges ahead. *Science (New York, NY)*. Oct 17 2003;302(5644):400-401.
18. Gaspar HB, Bjorkegren E, Parsley K, et al. Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning. *Mol Ther*. Oct 2006;14(4):505-513.
19. Aiuti A, Slavin S, Aker M, et al. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science (New York, NY)*. Jun 28 2002;296(5577):2410-2413.
20. Alton E. Progress and Prospects: Gene Therapy Clinical Trials (Part 1). *Gene therapy*. 14(20):1439-1447.
21. Griesenbach U. Progress and Prospects: Gene Therapy Clinical Trials (Part 2). *Gene therapy*. 14(22):1555-1563.
22. Mavilio F, Pellegrini G, Ferrari S, et al. Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells. *Nature medicine*. Dec 2006;12(12):1397-1402.
23. Ott MG, Schmidt M, Schwarzwaelder K, et al. Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nature medicine*. Apr 2006;12(4):401-409.
24. Kaplitt MG, Feigin A, Tang C, et al. Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. *Lancet*. Jun 23 2007;369(9579):2097-2105.
25. Kordower JH, Herzog CD, Dass B, et al. Delivery of neurturin by AAV2 (CERE-120)-mediated gene transfer provides structural and functional neuroprotection and neurorestoration in MPTP-treated monkeys. *Annals of neurology*. Dec 2006;60(6):706-715.

26. Morgan RA, Dudley ME, Wunderlich JR, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science (New York, NY)*. Oct 6 2006;314(5796):126-129.
27. Walther W, Stein U. Cell type specific and inducible promoters for vectors in gene therapy as an approach for cell targeting. *Journal of molecular medicine (Berlin, Germany)*. Jul 1996;74(7):379-392.
28. Gambhir SS, Barrio JR, Herschman HR, Phelps ME. Assays for noninvasive imaging of reporter gene expression. *Nuclear medicine and biology*. Jul 1999;26(5):481-490.
29. Li S, Huang L. Nonviral gene therapy: promises and challenges. *Gene therapy*. Jan 2000;7(1):31-34.
30. Somia N, Verma IM. Gene therapy: trials and tribulations. *Nature reviews*. Nov 2000;1(2):91-99.
31. Boulaiz H, Marchal JA, Prados J, Melguizo C, Aranega A. Non-viral and viral vectors for gene therapy. *Cellular and molecular biology (Noisy-le-Grand, France)*. 2005;51(1):3-22.
32. Brannon-Peppas L, Ghosn B, Roy K, Cornetta K. Encapsulation of nucleic acids and opportunities for cancer treatment. *Pharmaceutical research*. Apr 2007;24(4):618-627.
33. Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. *Nature reviews*. May 2003;4(5):346-358.
34. Kanerva A, Hemminki A. Modified adenoviruses for cancer gene therapy. *International journal of cancer*. Jul 1 2004;110(4):475-480.
35. Douglas JT. Adenoviral vectors for gene therapy. *Molecular biotechnology*. May 2007;36(1):71-80.
36. Yang ZR, Wang HF, Zhao J, et al. Recent developments in the use of adenoviruses and immunotoxins in cancer gene therapy. *Cancer gene therapy*. Jul 2007;14(7):599-615.
37. Bangari DS, Mittal SK. Current strategies and future directions for eluding adenoviral vector immunity. *Current gene therapy*. Apr 2006;6(2):215-226.
38. Zufferey R, Dull T, Mandel RJ, et al. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *Journal of virology*. Dec 1998;72(12):9873-9880.
39. Roth JA, Cristiano RJ. Gene therapy for cancer: what have we done and where are we going? *Journal of the National Cancer Institute*. Jan 1 1997;89(1):21-39.

40. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA: a cancer journal for clinicians*. Jan-Feb 2007;57(1):43-66.
41. Wadhwa PD, Zielske SP, Roth JC, Ballas CB, Bowman JE, Gerson SL. Cancer gene therapy: scientific basis. *Annual review of medicine*. 2002;53:437-452.
42. El-Aneed A. Current strategies in cancer gene therapy. *European journal of pharmacology*. Sep 13 2004;498(1-3):1-8.
43. Dong JY, Woraratanadharm J. Gene therapy vector design strategies for the treatment of cancer. *Future oncology (London, England)*. Jun 2005;1(3):361-373.
44. Hughes RM. Strategies for cancer gene therapy. *Journal of surgical oncology*. Jan 2004;85(1):28-35.
45. Rochlitz CF. Gene therapy of cancer. *Swiss Med Wkly*. Jan 12 2001;131(1-2):4-9.
46. Hanks BA, Jiang J, Singh RA, et al. Re-engineered CD40 receptor enables potent pharmacological activation of dendritic-cell cancer vaccines in vivo. *Nature medicine*. Feb 2005;11(2):130-137.
47. Li CY, Huang Q, Kung HF. Cytokine and immuno-gene therapy for solid tumors. *Cellular & molecular immunology*. Apr 2005;2(2):81-91.
48. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annual review of immunology*. 1995;13:251-276.
49. Colombo MP, Trinchieri G. Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine & growth factor reviews*. Apr 2002;13(2):155-168.
50. Ma X, Chow JM, Gri G, et al. The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. *The Journal of experimental medicine*. Jan 1 1996;183(1):147-157.
51. Brunda MJ, Luistro L, Hendrzak JA, Fountoulakis M, Garotta G, Gately MK. Role of interferon-gamma in mediating the antitumor efficacy of interleukin-12. *J Immunother Emphasis Tumor Immunol*. Feb 1995;17(2):71-77.
52. Segal JG, Lee NC, Tsung YL, Norton JA, Tsung K. The role of IFN-gamma in rejection of established tumors by IL-12 : source of production and target. *Cancer research*. Aug 15 2002;62(16):4696-4703.
53. Metzger DW, McNutt RM, Collins JT, Buchanan JM, Van Cleave VH, Dunnick WA. Interleukin-12 acts as an adjuvant for humoral immunity through interferon-gamma-

dependent and -independent mechanisms. *European journal of immunology*. Aug 1997;27(8):1958-1965.

54. Liu Y, Ehtesham M, Samoto K, et al. In situ adenoviral interleukin 12 gene transfer confers potent and long-lasting cytotoxic immunity in glioma. *Cancer gene therapy*. Jan 2002;9(1):9-15.

55. Shi F, Rakhmievich AL, Heise CP, et al. Intratumoral injection of interleukin-12 plasmid DNA, either naked or in complex with cationic lipid, results in similar tumor regression in a murine model. *Molecular cancer therapeutics*. Sep 2002;1(11):949-957.

56. Golab J, Zagozdzon R. Antitumor effects of interleukin-12 in pre-clinical and early clinical studies (Review). *International journal of molecular medicine*. May 1999;3(5):537-544.

57. Voest EE, Kenyon BM, O'Reilly MS, Truitt G, D'Amato RJ, Folkman J. Inhibition of angiogenesis in vivo by interleukin 12. *Journal of the National Cancer Institute*. Apr 19 1995;87(8):581-586.

58. Duda DG, Sunamura M, Lozonschi L, et al. Direct in vitro evidence and in vivo analysis of the antiangiogenesis effects of interleukin 12. *Cancer research*. Feb 15 2000;60(4):1111-1116.

59. Barajas M, Mazzolini G, Genove G, et al. Gene therapy of orthotopic hepatocellular carcinoma in rats using adenovirus coding for interleukin 12. *Hepatology (Baltimore, Md)*. Jan 2001;33(1):52-61.

60. Huang Q, Hu JK, Lohr F, et al. Heat-induced gene expression as a novel targeted cancer gene therapy strategy. *Cancer research*. Jul 1 2000;60(13):3435-3439.

61. Lohr F, Hu K, Huang Q, et al. Enhancement of radiotherapy by hyperthermia-regulated gene therapy. *International journal of radiation oncology, biology, physics*. Dec 1 2000;48(5):1513-1518.

62. Lohr F, Huang Q, Hu K, Dewhirst MW, Li CY. Systemic vector leakage and transgene expression by intratumorally injected recombinant adenovirus vectors. *Clin Cancer Res*. Nov 2001;7(11):3625-3628.

63. Yazawa K, Fisher WE, Brunicardi FC. Current progress in suicide gene therapy for cancer. *World journal of surgery*. Jul 2002;26(7):783-789.

64. Wygoda MR, Wilson MR, Davis MA, Trosko JE, Rehemtulla A, Lawrence TS. Protection of herpes simplex virus thymidine kinase-transduced cells from ganciclovir-mediated cytotoxicity by bystander cells: the Good Samaritan effect. *Cancer research*. May 1 1997;57(9):1699-1703.

65. Freeman SM, Abboud CN, Whartenby KA, et al. The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. *Cancer research*. Nov 1 1993;53(21):5274-5283.
66. Gottesman MM. Cancer gene therapy: an awkward adolescence. *Cancer gene therapy*. Jul 2003;10(7):501-508.
67. Roth JA. Adenovirus p53 gene therapy. *Expert opinion on biological therapy*. Jan 2006;6(1):55-61.
68. Roth JA, Nguyen D, Lawrence DD, et al. Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer. *Nature medicine*. Sep 1996;2(9):985-991.
69. Collis SJ, DeWeese TL. Enhanced radiation response through directed molecular targeting approaches. *Cancer metastasis reviews*. Aug-Dec 2004;23(3-4):277-292.
70. Kim HG, Reddoch JF, Mayfield C, et al. Inhibition of transcription of the human c-myc protooncogene by intermolecular triplex. *Biochemistry*. Feb 24 1998;37(8):2299-2304.
71. Kim HG, Miller DM. A novel triplex-forming oligonucleotide targeted to human cyclin D1 (bcl-1, proto-oncogene) promoter inhibits transcription in HeLa cells. *Biochemistry*. Feb 24 1998;37(8):2666-2672.
72. Webb A, Cunningham D, Cotter F, et al. BCL-2 antisense therapy in patients with non-Hodgkin lymphoma. *Lancet*. Apr 19 1997;349(9059):1137-1141.
73. Snyder DS, Wu Y, McMahon R, Yu L, Rossi JJ, Forman SJ. Ribozyme-mediated inhibition of a Philadelphia chromosome-positive acute lymphoblastic leukemia cell line expressing the p190 bcr-abl oncogene. *Biol Blood Marrow Transplant*. Oct 1997;3(4):179-186.
74. Bi F, Zhang X, Hui H. [Reversion of the malignant phenotype of gastric cancer by c-erbB-2 specific ribozyme]. *Zhonghua yi xue za zhi*. Apr 1999;79(4):295-297.
75. Jiang WG, Grimshaw D, Lane J, et al. A hammerhead ribozyme suppresses expression of hepatocyte growth factor/scatter factor receptor c-MET and reduces migration and invasiveness of breast cancer cells. *Clin Cancer Res*. Aug 2001;7(8):2555-2562.
76. Folkman J. What is the evidence that tumors are angiogenesis dependent? *Journal of the National Cancer Institute*. Jan 3 1990;82(1):4-6.
77. Chen QR, Zhang L, Gasper W, Mixson AJ. Targeting tumor angiogenesis with gene therapy. *Molecular genetics and metabolism*. Sep-Oct 2001;74(1-2):120-127.

- 78.** Ishikawa H, Nakao K, Matsumoto K, et al. Antiangiogenic gene therapy for hepatocellular carcinoma using angiostatin gene. *Hepatology (Baltimore, Md.* Mar 2003;37(3):696-704.
- 79.** Xu M, Kumar D, Srinivas S, et al. Parenteral gene therapy with p53 inhibits human breast tumors in vivo through a bystander mechanism without evidence of toxicity. *Human gene therapy.* Jan 20 1997;8(2):177-185.
- 80.** Semenza G. Signal transduction to hypoxia-inducible factor 1. *Biochemical pharmacology.* Sep 2002;64(5-6):993-998.
- 81.** Dewhirst MW, Cao Y, Li CY, Moeller B. Exploring the role of HIF-1 in early angiogenesis and response to radiotherapy. *Radiother Oncol.* Jun 2007;83(3):249-255.
- 82.** Semenza GL. HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends in molecular medicine.* 2002;8(4 Suppl):S62-67.
- 83.** Brahimi-Horn C, Berra E, Pouyssegur J. Hypoxia: the tumor's gateway to progression along the angiogenic pathway. *Trends in cell biology.* Nov 2001;11(11):S32-36.
- 84.** Maxwell PH, Pugh CW, Ratcliffe PJ. Activation of the HIF pathway in cancer. *Current opinion in genetics & development.* Jun 2001;11(3):293-299.
- 85.** Zhong H, De Marzo AM, Laughner E, et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer research.* Nov 15 1999;59(22):5830-5835.
- 86.** Sun X, Kanwar JR, Leung E, Lehnert K, Wang D, Krissansen GW. Gene transfer of antisense hypoxia inducible factor-1 alpha enhances the therapeutic efficacy of cancer immunotherapy. *Gene therapy.* Apr 2001;8(8):638-645.
- 87.** Russell WC. Update on adenovirus and its vectors. *The Journal of general virology.* Nov 2000;81(Pt 11):2573-2604.
- 88.** Lin E, Nemunaitis J. Oncolytic viral therapies. *Cancer gene therapy.* Oct 2004;11(10):643-664.
- 89.** Curiel DT. The development of conditionally replicative adenoviruses for cancer therapy. *Clin Cancer Res.* Sep 2000;6(9):3395-3399.
- 90.** Alemany R. Cancer selective adenoviruses. *Molecular aspects of medicine.* Feb 2007;28(1):42-58.
- 91.** Khuri FR, Nemunaitis J, Ganly I, et al. a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nature medicine.* Aug 2000;6(8):879-885.

- 92.** Small EJ, Carducci MA, Burke JM, et al. A phase I trial of intravenous CG7870, a replication-selective, prostate-specific antigen-targeted oncolytic adenovirus, for the treatment of hormone-refractory, metastatic prostate cancer. *Mol Ther.* Jul 2006;14(1):107-117.
- 93.** DeWeese TL, van der Poel H, Li S, et al. A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer research.* Oct 15 2001;61(20):7464-7472.
- 94.** Waehler R, Russell SJ, Curiel DT. Engineering targeted viral vectors for gene therapy. *Nature reviews.* Aug 2007;8(8):573-587.
- 95.** Goverdhana S, Puntel M, Xiong W, et al. Regulatable gene expression systems for gene therapy applications: progress and future challenges. *Mol Ther.* Aug 2005;12(2):189-211.
- 96.** Peng KW. Strategies for targeting therapeutic gene delivery. *Molecular medicine today.* Oct 1999;5(10):448-453.
- 97.** Weissleder R, Mahmood U. Molecular imaging. *Radiology.* May 2001;219(2):316-333.
- 98.** Min JJ, Gambhir SS. Gene therapy progress and prospects: noninvasive imaging of gene therapy in living subjects. *Gene therapy.* Jan 2004;11(2):115-125.
- 99.** Shah K. Current advances in molecular imaging of gene and cell therapy for cancer. *Cancer biology & therapy.* May 2005;4(5):518-523.
- 100.** Wagner E, Kircheis R, Walker GF. Targeted nucleic acid delivery into tumors: new avenues for cancer therapy. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie.* Apr 2004;58(3):152-161.
- 101.** Maeda H. The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. *Advances in enzyme regulation.* 2001;41:189-207.
- 102.** McCormick F. Cancer gene therapy: fringe or cutting edge? *Nat Rev Cancer.* Nov 2001;1(2):130-141.
- 103.** Russ V, Wagner E. Cell and tissue targeting of nucleic acids for cancer gene therapy. *Pharmaceutical research.* Jun 2007;24(6):1047-1057.
- 104.** Wang J, Murakami T, Hakamata Y, et al. Gene gun-mediated oral mucosal transfer of interleukin 12 cDNA coupled with an irradiated melanoma vaccine in a hamster model:

successful treatment of oral melanoma and distant skin lesion. *Cancer gene therapy*. Oct 2001;8(10):705-712.

105. Wells DJ. Gene therapy progress and prospects: electroporation and other physical methods. *Gene therapy*. Sep 2004;11(18):1363-1369.

106. Scherer F, Anton M, Schillinger U, et al. Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo. *Gene therapy*. Jan 2002;9(2):102-109.

107. Wagner E. Advances in cancer gene therapy: tumor-targeted delivery of therapeutic pDNA, siRNA, and dsRNA nucleic acids. *J Buon*. Sep 2007;12 Suppl 1:S77-82.

108. Newman CM, Bettinger T. Gene therapy progress and prospects: ultrasound for gene transfer. *Gene therapy*. Mar 2007;14(6):465-475.

109. Miller DL, Pislaru SV, Greenleaf JE. Sonoporation: mechanical DNA delivery by ultrasonic cavitation. *Somatic cell and molecular genetics*. Nov 2002;27(1-6):115-134.

110. Dittmar KM, Xie J, Hunter F, et al. Pulsed high-intensity focused ultrasound enhances systemic administration of naked DNA in squamous cell carcinoma model: initial experience. *Radiology*. May 2005;235(2):541-546.

111. Sakakima Y, Hayashi S, Yagi Y, Hayakawa A, Tachibana K, Nakao A. Gene therapy for hepatocellular carcinoma using sonoporation enhanced by contrast agents. *Cancer gene therapy*. Nov 2005;12(11):884-889.

112. Hauff P, Seemann S, Reszka R, et al. Evaluation of gas-filled microparticles and sonoporation as gene delivery system: feasibility study in rodent tumor models. *Radiology*. Aug 2005;236(2):572-578.

113. Gaber MH, Wu NZ, Hong K, Huang SK, Dewhirst MW, Papahadjopoulos D. Thermosensitive liposomes: extravasation and release of contents in tumor microvascular networks. *International journal of radiation oncology, biology, physics*. Dec 1 1996;36(5):1177-1187.

114. Kong G, Dewhirst MW. Hyperthermia and liposomes. *Int J Hyperthermia*. Sep-Oct 1999;15(5):345-370.

115. Matteucci ML, Anyarambhatla G, Rosner G, et al. Hyperthermia increases accumulation of technetium-99m-labeled liposomes in feline sarcomas. *Clin Cancer Res*. Sep 2000;6(9):3748-3755.

116. Zintchenko A, Ogris M, Wagner E. Temperature dependent gene expression induced by PNIPAM-based copolymers: potential of hyperthermia in gene transfer. *Bioconjugate chemistry*. May-Jun 2006;17(3):766-772.

- 117.** Dewhirst MW. Concepts of oxygen transport at the microcirculatory level. *Seminars in radiation oncology*. Jul 1998;8(3):143-150.
- 118.** Rofstad EK. Microenvironment-induced cancer metastasis. *International journal of radiation biology*. May 2000;76(5):589-605.
- 119.** Xie K, Huang S. Regulation of cancer metastasis by stress pathways. *Clinical & experimental metastasis*. 2003;20(1):31-43.
- 120.** Wickham TJ. Ligand-directed targeting of genes to the site of disease. *Nature medicine*. Jan 2003;9(1):135-139.
- 121.** Cronin J, Zhang XY, Reiser J. Altering the tropism of lentiviral vectors through pseudotyping. *Current gene therapy*. Aug 2005;5(4):387-398.
- 122.** Schnierle BS, Stitz J, Bosch V, et al. Pseudotyping of murine leukemia virus with the envelope glycoproteins of HIV generates a retroviral vector with specificity of infection for CD4-expressing cells. *Proceedings of the National Academy of Sciences of the United States of America*. Aug 5 1997;94(16):8640-8645.
- 123.** Dmitriev I, Kashentseva E, Rogers BE, Krasnykh V, Curiel DT. Ectodomain of coxsackievirus and adenovirus receptor genetically fused to epidermal growth factor mediates adenovirus targeting to epidermal growth factor receptor-positive cells. *Journal of virology*. Aug 2000;74(15):6875-6884.
- 124.** Kim J, Smith T, Idamakanti N, et al. Targeting adenoviral vectors by using the extracellular domain of the coxsackie-adenovirus receptor: improved potency via trimerization. *Journal of virology*. Feb 2002;76(4):1892-1903.
- 125.** Douglas JT, Rogers BE, Rosenfeld ME, Michael SI, Feng M, Curiel DT. Targeted gene delivery by tropism-modified adenoviral vectors. *Nature biotechnology*. Nov 1996;14(11):1574-1578.
- 126.** Pereboev AV, Nagle JM, Shakhmatov MA, et al. Enhanced gene transfer to mouse dendritic cells using adenoviral vectors coated with a novel adapter molecule. *Mol Ther*. May 2004;9(5):712-720.
- 127.** Snitkovsky S, Young JA. Targeting retroviral vector infection to cells that express heregulin receptors using a TVA-heregulin bridge protein. *Virology*. Jan 5 2002;292(1):150-155.
- 128.** Bartlett JS, Kleinschmidt J, Boucher RC, Samulski RJ. Targeted adeno-associated virus vector transduction of nonpermissive cells mediated by a bispecific F(ab'gamma)₂ antibody. *Nature biotechnology*. Feb 1999;17(2):181-186.

- 129.** Wurdinger T, Verheije MH, Raaben M, et al. Targeting non-human coronaviruses to human cancer cells using a bispecific single-chain antibody. *Gene therapy*. Sep 2005;12(18):1394-1404.
- 130.** Eto Y, Gao JQ, Sekiguchi F, et al. PEGylated adenovirus vectors containing RGD peptides on the tip of PEG show high transduction efficiency and antibody evasion ability. *The journal of gene medicine*. May 2005;7(5):604-612.
- 131.** Kreppel F, Gackowski J, Schmidt E, Kochanek S. Combined genetic and chemical capsid modifications enable flexible and efficient de- and retargeting of adenovirus vectors. *Mol Ther*. Jul 2005;12(1):107-117.
- 132.** Roux P, Jeanteur P, Piechaczyk M. A versatile and potentially general approach to the targeting of specific cell types by retroviruses: application to the infection of human cells by means of major histocompatibility complex class I and class II antigens by mouse ecotropic murine leukemia virus-derived viruses. *Proceedings of the National Academy of Sciences of the United States of America*. Dec 1989;86(23):9079-9083.
- 133.** Maguire CA, Sapinoro R, Girgis N, et al. Recombinant adenovirus type 5 vectors that target DC-SIGN, ChemR23 and alpha(v)beta3 integrin efficiently transduce human dendritic cells and enhance presentation of vectored antigens. *Vaccine*. Jan 30 2006;24(5):671-682.
- 134.** Arnold GS, Sasser AK, Stachler MD, Bartlett JS. Metabolic biotinylation provides a unique platform for the purification and targeting of multiple AAV vector serotypes. *Mol Ther*. Jul 2006;14(1):97-106.
- 135.** Gigout L, Rebollo P, Clement N, et al. Altering AAV tropism with mosaic viral capsids. *Mol Ther*. Jun 2005;11(6):856-865.
- 136.** Tai CK, Logg CR, Park JM, Anderson WF, Press MF, Kasahara N. Antibody-mediated targeting of replication-competent retroviral vectors. *Human gene therapy*. May 20 2003;14(8):789-802.
- 137.** Parrott MB, Adams KE, Mercier GT, Mok H, Campos SK, Barry MA. Metabolically biotinylated adenovirus for cell targeting, ligand screening, and vector purification. *Mol Ther*. Oct 2003;8(4):688-700.
- 138.** Stone D, Ni S, Li Z-Y, et al. Development and Assessment of Human Adenovirus Type 11 as a Gene Transfer Vector. Vol 79; 2005:5090-5104.
- 139.** Wu Z, Asokan A, Samulski RJ. Adeno-associated virus serotypes: vector toolkit for human gene therapy. *Mol Ther*. Sep 2006;14(3):316-327.
- 140.** Gollan TJ, Green MR. Redirecting retroviral tropism by insertion of short, nondisruptive peptide ligands into envelope. *Journal of virology*. Apr 2002;76(7):3558-3563.

- 141.** Hajitou A, Trepel M, Lilley CE, et al. A hybrid vector for ligand-directed tumor targeting and molecular imaging. *Cell*. Apr 21 2006;125(2):385-398.
- 142.** Mahasreshti PJ, Kataram M, Wu H, et al. Ovarian cancer targeted adenoviral-mediated mda-7/IL-24 gene therapy. *Gynecologic oncology*. Mar 2006;100(3):521-532.
- 143.** Chowdhury S, Chester KA, Bridgewater J, Collins MK, Martin F. Efficient retroviral vector targeting of carcinoembryonic antigen-positive tumors. *Mol Ther*. Jan 2004;9(1):85-92.
- 144.** Hedley SJ, Auf der Maur A, Hohn S, et al. An adenovirus vector with a chimeric fiber incorporating stabilized single chain antibody achieves targeted gene delivery. *Gene therapy*. Jan 2006;13(1):88-94.
- 145.** Stachler MD, Bartlett JS. Mosaic vectors comprised of modified AAV1 capsid proteins for efficient vector purification and targeting to vascular endothelial cells. *Gene therapy*. Jun 2006;13(11):926-931.
- 146.** Pereboeva L, Komarova S, Mahasreshti PJ, Curiel DT. Fiber-mosaic adenovirus as a novel approach to design genetically modified adenoviral vectors. *Virus research*. Sep 15 2004;105(1):35-46.
- 147.** Morizono K, Xie Y, Ringpis GE, et al. Lentiviral vector retargeting to P-glycoprotein on metastatic melanoma through intravenous injection. *Nature medicine*. Mar 2005;11(3):346-352.
- 148.** Nicklin SA, Wu E, Nemerow GR, Baker AH. The influence of adenovirus fiber structure and function on vector development for gene therapy. *Mol Ther*. Sep 2005;12(3):384-393.
- 149.** Harrington KJ, Linardakis E, Vile RG. Transcriptional control: an essential component of cancer gene therapy strategies? *Advanced drug delivery reviews*. Nov 15 2000;44(2-3):167-184.
- 150.** Robson T, Hirst DG. Transcriptional Targeting in Cancer Gene Therapy. *J Biomed Biotechnol*. 2003;2003(2):110-137.
- 151.** Vile RG, Hart IR. In vitro and in vivo targeting of gene expression to melanoma cells. *Cancer research*. Mar 1 1993;53(5):962-967.
- 152.** Vile RG, Hart IR. Use of tissue-specific expression of the herpes simplex virus thymidine kinase gene to inhibit growth of established murine melanomas following direct intratumoral injection of DNA. *Cancer research*. Sep 1 1993;53(17):3860-3864.

- 153.** Cao G, Zhang X, He X, Chen Q, Qi Z. A safe, effective in vivo gene therapy for melanoma using tyrosinase promoter-driven cytosine deaminase gene. *In vivo (Athens, Greece)*. Mar-Apr 1999;13(2):181-187.
- 154.** Emiliusen L, Gough M, Bateman A, et al. A transcriptional feedback loop for tissue-specific expression of highly cytotoxic genes which incorporates an immunostimulatory component. *Gene therapy*. Jul 2001;8(13):987-998.
- 155.** Nahde T, Muller K, Fahr A, Muller R, Brusselbach S. Combined transductional and transcriptional targeting of melanoma cells by artificial virus-like particles. *The journal of gene medicine*. Jul-Aug 2001;3(4):353-361.
- 156.** Bazzoni F, Regalia E. Triggering of antitumor activity through melanoma-specific transduction of a constitutively active tumor necrosis factor (TNF) R1 chimeric receptor in the absence of TNF-alpha. *Cancer research*. Feb 1 2001;61(3):1050-1057.
- 157.** Yu DC, Sakamoto GT, Henderson DR. Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. *Cancer research*. Apr 1 1999;59(7):1498-1504.
- 158.** Pang S, Taneja S, Dardashti K, et al. Prostate tissue specificity of the prostate-specific antigen promoter isolated from a patient with prostate cancer. *Human gene therapy*. Nov 1995;6(11):1417-1426.
- 159.** Rodriguez R, Schuur ER, Lim HY, Henderson GA, Simons JW, Henderson DR. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer research*. Jul 1 1997;57(13):2559-2563.
- 160.** Wu L, Matherly J, Smallwood A, et al. Chimeric PSA enhancers exhibit augmented activity in prostate cancer gene therapy vectors. *Gene therapy*. Sep 2001;8(18):1416-1426.
- 161.** Latham JP, Searle PF, Mautner V, James ND. Prostate-specific antigen promoter/enhancer driven gene therapy for prostate cancer: construction and testing of a tissue-specific adenovirus vector. *Cancer research*. Jan 15 2000;60(2):334-341.
- 162.** Suzuki S, Tadakuma T, Asano T, Hayakawa M. Coexpression of the partial androgen receptor enhances the efficacy of prostate-specific antigen promoter-driven suicide gene therapy for prostate cancer cells at low testosterone concentrations. *Cancer research*. Feb 15 2001;61(4):1276-1279.
- 163.** Miyao Y, Shimizu K, Moriuchi S, et al. Selective expression of foreign genes in glioma cells: use of the mouse myelin basic protein gene promoter to direct toxic gene expression. *Journal of neuroscience research*. Nov 1 1993;36(4):472-479.

- 164.** Miyao Y, Shimizu K, Tamura M, et al. Usefulness of a mouse myelin basic protein promoter for gene therapy of malignant glioma: myelin basic protein promoter is strongly active in human malignant glioma cells. *Jpn J Cancer Res.* Jul 1997;88(7):678-686.
- 165.** Vandier D, Rixe O, Brenner M, Gouyette A, Besnard F. Selective killing of glioma cell lines using an astrocyte-specific expression of the herpes simplex virus-thymidine kinase gene. *Cancer research.* Oct 15 1998;58(20):4577-4580.
- 166.** Shinoura N, Ohashi M, Yoshida Y, et al. Adenovirus-mediated overexpression of Fas induces apoptosis of gliomas. *Cancer gene therapy.* Feb 2000;7(2):224-232.
- 167.** Kuriyama S, Yoshikawa M, Ishizaka S, et al. A potential approach for gene therapy targeting hepatoma using a liver-specific promoter on a retroviral vector. *Cell structure and function.* Dec 1991;16(6):503-510.
- 168.** Kuriyama S, Sakamoto T, Masui K, et al. Tissue-specific expression of HSV-tk gene can induce efficient antitumor effect and protective immunity to wild-type hepatocellular carcinoma. *International journal of cancer.* May 2 1997;71(3):470-475.
- 169.** Cao G, Kuriyama S, Du P, et al. Complete regression of established murine hepatocellular carcinoma by in vivo tumor necrosis factor alpha gene transfer. *Gastroenterology.* Feb 1997;112(2):501-510.
- 170.** Zeiger MA, Takiyama Y, Bishop JO, Ellison AR, Saji M, Levine MA. Adenoviral infection of thyroid cells: a rationale for gene therapy for metastatic thyroid carcinoma. *Surgery.* Dec 1996;120(6):921-925.
- 171.** Shimura H, Suzuki H, Miyazaki A, et al. Transcriptional activation of the thyroglobulin promoter directing suicide gene expression by thyroid transcription factor-1 in thyroid cancer cells. *Cancer research.* May 1 2001;61(9):3640-3646.
- 172.** Zhang R, Straus FH, DeGroot LJ. Adenoviral-mediated gene therapy for thyroid carcinoma using thymidine kinase controlled by thyroglobulin promoter demonstrates high specificity and low toxicity. *Thyroid.* Feb 2001;11(2):115-123.
- 173.** Haviv YS, Curiel DT. Conditional gene targeting for cancer gene therapy. *Advanced drug delivery reviews.* Dec 17 2001;53(2):135-154.
- 174.** Shay JW. Telomerase in cancer: diagnostic, prognostic, and therapeutic implications. *The cancer journal from Scientific American.* May 1998;4 Suppl 1:S26-34.
- 175.** Buys CH. Telomeres, telomerase, and cancer. *The New England journal of medicine.* Apr 27 2000;342(17):1282-1283.
- 176.** Kyo S, Takakura M, Inoue M. Telomerase activity in cancer as a diagnostic and therapeutic target. *Histology and histopathology.* Jul 2000;15(3):813-824.

- 177.** Horikawa I, Cable PL, Afshari C, Barrett JC. Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. *Cancer research*. Feb 15 1999;59(4):826-830.
- 178.** Takakura M, Kyo S, Kanaya T, et al. Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. *Cancer research*. Feb 1 1999;59(3):551-557.
- 179.** Gu J, Kagawa S, Takakura M, et al. Tumor-specific transgene expression from the human telomerase reverse transcriptase promoter enables targeting of the therapeutic effects of the Bax gene to cancers. *Cancer research*. Oct 1 2000;60(19):5359-5364.
- 180.** Gu J, Andreeff M, Roth JA, Fang B. hTERT promoter induces tumor-specific Bax gene expression and cell killing in syngenic mouse tumor model and prevents systemic toxicity. *Gene therapy*. Jan 2002;9(1):30-37.
- 181.** Huang Q, Zhang X, Wang H, et al. A novel conditionally replicative adenovirus vector targeting telomerase-positive tumor cells. *Clin Cancer Res*. Feb 15 2004;10(4):1439-1445.
- 182.** Jacob D, Davis JJ, Zhang L, Zhu H, Teraishi F, Fang B. Suppression of pancreatic tumor growth in the liver by systemic administration of the TRAIL gene driven by the hTERT promoter. *Cancer gene therapy*. Feb 2005;12(2):109-115.
- 183.** Wepsic HT. Overview of oncofetal antigens in cancer. *Annals of clinical and laboratory science*. Jul-Aug 1983;13(4):261-266.
- 184.** Manome Y, Abe M, Hagen MF, Fine HA, Kufe DW. Enhancer sequences of the DF3 gene regulate expression of the herpes simplex virus thymidine kinase gene and confer sensitivity of human breast cancer cells to ganciclovir. *Cancer research*. Oct 15 1994;54(20):5408-5413.
- 185.** Tai YT, Strobel T, Kufe D, Cannistra SA. In vivo cytotoxicity of ovarian cancer cells through tumor-selective expression of the BAX gene. *Cancer research*. May 1 1999;59(9):2121-2126.
- 186.** Stackhouse MA, Buchsbaum DJ, Kancharla SR, et al. Specific membrane receptor gene expression targeted with radiolabeled peptide employing the erbB-2 and DF3 promoter elements in adenoviral vectors. *Cancer gene therapy*. May-Jun 1999;6(3):209-219.
- 187.** Maeda T, J OW, Matsubara H, et al. A minimum c-erbB-2 promoter-mediated expression of herpes simplex virus thymidine kinase gene confers selective cytotoxicity of human breast cancer cells to ganciclovir. *Cancer gene therapy*. Nov 2001;8(11):890-896.

- 188.** Harris JD, Gutierrez AA, Hurst HC, Sikora K, Lemoine NR. Gene therapy for cancer using tumour-specific prodrug activation. *Gene therapy*. May 1994;1(3):170-175.
- 189.** Matsubara S, Wada Y, Gardner TA, et al. A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. *Cancer research*. Aug 15 2001;61(16):6012-6019.
- 190.** Benjamin R, Helman L, Meyers P, Reaman G. A phase I/II dose escalation and activity study of intravenous injections of OCaP1 for subjects with refractory osteosarcoma metastatic to lung. *Human gene therapy*. Aug 10 2001;12(12):1591-1593.
- 191.** McCarthy HO, Worthington J, Barrett E, et al. p21((WAF1))-mediated transcriptional targeting of inducible nitric oxide synthase gene therapy sensitizes tumours to fractionated radiotherapy. *Gene therapy*. Feb 2007;14(3):246-255.
- 192.** Greco O, Marples B, Joiner MC, Scott SD. How to overcome (and exploit) tumor hypoxia for targeted gene therapy. *Journal of cellular physiology*. Dec 2003;197(3):312-325.
- 193.** Greco O, Scott S. Tumor hypoxia and targeted gene therapy. *International review of cytology*. 2007;257:181-212.
- 194.** Jaggar RT, Chan HY, Harris AL, Bicknell R. Endothelial cell-specific expression of tumor necrosis factor- α from the KDR or E-selectin promoters following retroviral delivery. *Human gene therapy*. Dec 10 1997;8(18):2239-2247.
- 195.** Szary J, Kalita K, Przybyszewska M, et al. KDR promoter can transcriptionally target cytosine deaminase suicide gene to cancer cells of nonendothelial origin. *Anticancer research*. Sep-Oct 2001;21(5):3471-3475.
- 196.** Modlich U, Pugh CW, Bicknell R. Increasing endothelial cell specific expression by the use of heterologous hypoxic and cytokine-inducible enhancers. *Gene therapy*. May 2000;7(10):896-902.
- 197.** Heidenreich R, Kappel A, Breier G. Tumor endothelium-specific transgene expression directed by vascular endothelial growth factor receptor-2 (Flk-1) promoter/enhancer sequences. *Cancer research*. Nov 1 2000;60(21):6142-6147.
- 198.** Walton T, Wang JL, Ribas A, Barsky SH, Economou J, Nguyen M. Endothelium-specific expression of an E-selectin promoter recombinant adenoviral vector. *Anticancer research*. May-Jun 1998;18(3A):1357-1360.
- 199.** Jager U, Zhao Y, Porter CD. Endothelial cell-specific transcriptional targeting from a hybrid long terminal repeat retrovirus vector containing human prepro-endothelin-1 promoter sequences. *Journal of virology*. Dec 1999;73(12):9702-9709.

- 200.** Mavria G, Jager U, Porter CD. Generation of a high titre retroviral vector for endothelial cell-specific gene expression in vivo. *Gene therapy*. Mar 2000;7(5):368-376.
- 201.** Varda-Bloom N, Shaish A, Gonen A, et al. Tissue-specific gene therapy directed to tumor angiogenesis. *Gene therapy*. Jun 2001;8(11):819-827.
- 202.** Ozaki K, Yoshida T, Ide H, et al. Use of von Willebrand factor promoter to transduce suicidal gene to human endothelial cells, HUVEC. *Human gene therapy*. Aug 20 1996;7(13):1483-1490.
- 203.** Shibata T, Akiyama N, Noda M, Sasai K, Hiraoka M. Enhancement of gene expression under hypoxic conditions using fragments of the human vascular endothelial growth factor and the erythropoietin genes. *International journal of radiation oncology, biology, physics*. Nov 1 1998;42(4):913-916.
- 204.** Shibata T, Giaccia AJ, Brown JM. Development of a hypoxia-responsive vector for tumor-specific gene therapy. *Gene therapy*. Mar 2000;7(6):493-498.
- 205.** Shibata T, Giaccia AJ, Brown JM. Hypoxia-inducible regulation of a prodrug-activating enzyme for tumor-specific gene therapy. *Neoplasia (New York, NY)*. Jan-Feb 2002;4(1):40-48.
- 206.** Koshikawa N, Takenaga K, Tagawa M, Sakiyama S. Therapeutic efficacy of the suicide gene driven by the promoter of vascular endothelial growth factor gene against hypoxic tumor cells. *Cancer research*. Jun 1 2000;60(11):2936-2941.
- 207.** Ruan H, Su H, Hu L, Lamborn KR, Kan YW, Deen DF. A hypoxia-regulated adeno-associated virus vector for cancer-specific gene therapy. *Neoplasia (New York, NY)*. May-Jun 2001;3(3):255-263.
- 208.** Dachs GU, Patterson AV, Firth JD, et al. Targeting gene expression to hypoxic tumor cells. *Nature medicine*. May 1997;3(5):515-520.
- 209.** Bussink J, Kaanders JH, van der Kogel AJ. Tumor hypoxia at the micro-regional level: clinical relevance and predictive value of exogenous and endogenous hypoxic cell markers. *Radiother Oncol*. Apr 2003;67(1):3-15.
- 210.** Gazit G, Kane SE, Nichols P, Lee AS. Use of the stress-inducible grp78/BiP promoter in targeting high level gene expression in fibrosarcoma in vivo. *Cancer research*. Apr 15 1995;55(8):1660-1663.
- 211.** Gazit G, Hung G, Chen X, Anderson WF, Lee AS. Use of the glucose starvation-inducible glucose-regulated protein 78 promoter in suicide gene therapy of murine fibrosarcoma. *Cancer research*. Jul 1 1999;59(13):3100-3106.

- 212.** Lee AS. The glucose-regulated proteins: stress induction and clinical applications. *Trends in biochemical sciences*. Aug 2001;26(8):504-510.
- 213.** Luna MC, Chen X, Wong S, et al. Enhanced photodynamic therapy efficacy with inducible suicide gene therapy controlled by the grp promoter. *Cancer research*. Mar 1 2002;62(5):1458-1461.
- 214.** Katabi MM, Chan HL, Karp SE, Batist G. Hexokinase type II: a novel tumor-specific promoter for gene-targeted therapy differentially expressed and regulated in human cancer cells. *Human gene therapy*. Jan 20 1999;10(2):155-164.
- 215.** Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences of the United States of America*. Jun 15 1992;89(12):5547-5551.
- 216.** Gossen M, Bujard H. Anhydrotetracycline, a novel effector for tetracycline controlled gene expression systems in eukaryotic cells. *Nucleic acids research*. Sep 11 1993;21(18):4411-4412.
- 217.** Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. *Science (New York, NY)*. Jun 23 1995;268(5218):1766-1769.
- 218.** No D, Yao TP, Evans RM. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America*. Apr 16 1996;93(8):3346-3351.
- 219.** Tsai SY, O'Malley BW, DeMayo FJ, Wang Y, Chua SS. A novel RU486 inducible system for the activation and repression of genes. *Advanced drug delivery reviews*. Mar 2 1998;30(1-3):23-31.
- 220.** Pollock R, Issner R, Zoller K, Natesan S, Rivera VM, Clackson T. Delivery of a stringent dimerizer-regulated gene expression system in a single retroviral vector. *Proceedings of the National Academy of Sciences of the United States of America*. Nov 21 2000;97(24):13221-13226.
- 221.** Rome C, Couillaud F, Moonen CT. Spatial and temporal control of expression of therapeutic genes using heat shock protein promoters. *Methods (San Diego, Calif)*. Feb 2005;35(2):188-198.
- 222.** Li CY, Dewhirst MW. Hyperthermia-regulated immunogene therapy. *Int J Hyperthermia*. Nov-Dec 2002;18(6):586-596.
- 223.** Hallahan DE, Mauceri HJ, Seung LP, et al. Spatial and temporal control of gene therapy using ionizing radiation. *Nature medicine*. Aug 1995;1(8):786-791.

- 224.** Datta R, Rubin E, Sukhatme V, et al. Ionizing radiation activates transcription of the EGR1 gene via CArG elements. *Proceedings of the National Academy of Sciences of the United States of America*. Nov 1 1992;89(21):10149-10153.
- 225.** Scott SD, Greco O. Radiation and hypoxia inducible gene therapy systems. *Cancer metastasis reviews*. Aug-Dec 2004;23(3-4):269-276.
- 226.** Weichselbaum RR, Hallahan DE, Beckett MA, et al. Gene therapy targeted by radiation preferentially radiosensitizes tumor cells. *Cancer research*. Aug 15 1994;54(16):4266-4269.
- 227.** Seung LP, Mauceri HJ, Beckett MA, Hallahan DE, Hellman S, Weichselbaum RR. Genetic radiotherapy overcomes tumor resistance to cytotoxic agents. *Cancer research*. Dec 1 1995;55(23):5561-5565.
- 228.** Mauceri HJ, Hanna NN, Wayne JD, Hallahan DE, Hellman S, Weichselbaum RR. Tumor necrosis factor alpha (TNF-alpha) gene therapy targeted by ionizing radiation selectively damages tumor vasculature. *Cancer research*. Oct 1 1996;56(19):4311-4314.
- 229.** Joki T, Nakamura M, Ohno T. Activation of the radiosensitive EGR-1 promoter induces expression of the herpes simplex virus thymidine kinase gene and sensitivity of human glioma cells to ganciclovir. *Human gene therapy*. Dec 1995;6(12):1507-1513.
- 230.** Takahashi T, Namiki Y, Ohno T. Induction of the suicide HSV-TK gene by activation of the Egr-1 promoter with radioisotopes. *Human gene therapy*. May 1 1997;8(7):827-833.
- 231.** Chung TD, Mauceri HJ, Hallahan DE, et al. Tumor necrosis factor-alpha-based gene therapy enhances radiation cytotoxicity in human prostate cancer. *Cancer gene therapy*. Nov-Dec 1998;5(6):344-349.
- 232.** Kawashita Y, Ohtsuru A, Kaneda Y, et al. Regression of hepatocellular carcinoma in vitro and in vivo by radiosensitizing suicide gene therapy under the inducible and spatial control of radiation. *Human gene therapy*. Jun 10 1999;10(9):1509-1519.
- 233.** Leppa S, Sistonen L. Heat shock response--pathophysiological implications. *Annals of medicine*. Feb 1997;29(1):73-78.
- 234.** Jolly C, Morimoto RI. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *Journal of the National Cancer Institute*. Oct 4 2000;92(19):1564-1572.
- 235.** Sarto C, Binz PA, Mocarelli P. Heat shock proteins in human cancer. *Electrophoresis*. Apr 2000;21(6):1218-1226.
- 236.** Morimoto RI, Kroeger PE, Cotto JJ. The transcriptional regulation of heat shock genes: a plethora of heat shock factors and regulatory conditions. *Exs*. 1996;77:139-163.

237. Beere HM. Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *The Journal of clinical investigation*. Oct 2005;115(10):2633-2639.
238. Beere HM. "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. *Journal of cell science*. Jun 1 2004;117(Pt 13):2641-2651.
239. Barnes JA, Dix DJ, Collins BW, Luft C, Allen JW. Expression of inducible Hsp70 enhances the proliferation of MCF-7 breast cancer cells and protects against the cytotoxic effects of hyperthermia. *Cell stress & chaperones*. Oct 2001;6(4):316-325.
240. Todryk SM, Gough MJ, Pockley AG. Facets of heat shock protein 70 show immunotherapeutic potential. *Immunology*. Sep 2003;110(1):1-9.
241. Manjili MH, Wang XY, Park J, et al. Cancer immunotherapy: stress proteins and hyperthermia. *Int J Hyperthermia*. Nov-Dec 2002;18(6):506-520.
242. Menoret A, Chaillot D, Callahan M, Jacquin C. Hsp70, an immunological actor playing with the intracellular self under oxidative stress. *Int J Hyperthermia*. Nov-Dec 2002;18(6):490-505.
243. Borrelli MJ, Schoenherr DM, Wong A, Bernock LJ, Corry PM. Heat-activated transgene expression from adenovirus vectors infected into human prostate cancer cells. *Cancer research*. Feb 1 2001;61(3):1113-1121.
244. Blackburn RV, Galoforo SS, Corry PM, Lee YJ. Adenoviral-mediated transfer of a heat-inducible double suicide gene into prostate carcinoma cells. *Cancer research*. Apr 1 1998;58(7):1358-1362.
245. Dreano M, Brochot J, Myers A, et al. High-level, heat-regulated synthesis of proteins in eukaryotic cells. *Gene*. 1986;49(1):1-8.
246. Braiden V, Ohtsuru A, Kawashita Y, et al. Eradication of breast cancer xenografts by hyperthermic suicide gene therapy under the control of the heat shock protein promoter. *Human gene therapy*. Dec 10 2000;11(18):2453-2463.
247. Lee YJ, Galoforo SS, Battle P, Lee H, Corry PM, Jessup JM. Replicating adenoviral vector-mediated transfer of a heat-inducible double suicide gene for gene therapy. *Cancer gene therapy*. Jun 2001;8(6):397-404.
248. Brade AM, Szmitko P, Ngo D, Liu FF, Klamut HJ. Heat-directed suicide gene therapy for breast cancer. *Cancer gene therapy*. Apr 2003;10(4):294-301.

- 249.** Li GC, He F, Shao X, et al. Adenovirus-mediated heat-activated antisense Ku70 expression radiosensitizes tumor cells in vitro and in vivo. *Cancer research*. Jun 15 2003;63(12):3268-3274.
- 250.** Siddiqui F, Li CY, Larue SM, et al. A phase I trial of hyperthermia-induced interleukin-12 gene therapy in spontaneously arising feline soft tissue sarcomas. *Molecular cancer therapeutics*. Jan 2007;6(1):380-389.
- 251.** Penuelas I, Boan J, Marti-Climent JM, et al. Positron emission tomography and gene therapy: basic concepts and experimental approaches for in vivo gene expression imaging. *Mol Imaging Biol*. Jul-Aug 2004;6(4):225-238.
- 252.** Blasberg RG. Molecular imaging and cancer. *Molecular cancer therapeutics*. Mar 2003;2(3):335-343.
- 253.** Niu G, Anderson RD, Madsen MT, Graham MM, Oberley LW, Domann FE. Dual-expressing adenoviral vectors encoding the sodium iodide symporter for use in noninvasive radiological imaging of therapeutic gene transfer. *Nuclear medicine and biology*. Apr 2006;33(3):391-398.
- 254.** Massoud TF, Gambhir SS. Integrating noninvasive molecular imaging into molecular medicine: an evolving paradigm. *Trends in molecular medicine*. May 2007;13(5):183-191.
- 255.** Zinn KR, Chaudhuri TR. The type 2 human somatostatin receptor as a platform for reporter gene imaging. *European journal of nuclear medicine and molecular imaging*. Mar 2002;29(3):388-399.
- 256.** Blasberg R. PET imaging of gene expression. *Eur J Cancer*. Nov 2002;38(16):2137-2146.
- 257.** Tjuvajev JG, Stockhammer G, Desai R, et al. Imaging the expression of transfected genes in vivo. *Cancer research*. Dec 15 1995;55(24):6126-6132.
- 258.** Tjuvajev JG, Finn R, Watanabe K, et al. Noninvasive imaging of herpes virus thymidine kinase gene transfer and expression: a potential method for monitoring clinical gene therapy. *Cancer research*. Sep 15 1996;56(18):4087-4095.
- 259.** Weissleder R, Lee AS, Fischman AJ, et al. Polyclonal human immunoglobulin G labeled with polymeric iron oxide: antibody MR imaging. *Radiology*. Oct 1991;181(1):245-249.
- 260.** Weissleder R, Simonova M, Bogdanova A, Bredow S, Enochs WS, Bogdanov A, Jr. MR imaging and scintigraphy of gene expression through melanin induction. *Radiology*. Aug 1997;204(2):425-429.

- 261.** Louie AY, Huber MM, Ahrens ET, et al. In vivo visualization of gene expression using magnetic resonance imaging. *Nature biotechnology*. Mar 2000;18(3):321-325.
- 262.** Rehemtulla A, Stegman LD, Cardozo SJ, et al. Rapid and quantitative assessment of cancer treatment response using in vivo bioluminescence imaging. *Neoplasia (New York, NY)*. Nov-Dec 2000;2(6):491-495.
- 263.** Bennett J, Duan D, Engelhardt JF, Maguire AM. Real-time, noninvasive in vivo assessment of adeno-associated virus-mediated retinal transduction. *Investigative ophthalmology & visual science*. Dec 1997;38(13):2857-2863.
- 264.** Kristian Raty J, Liimatainen T, Unelma Kaikkonen M, Grohn O, Jumaní Airene K, Ylä-Herttuala S. Non-invasive Imaging in Gene Therapy. *Mol Ther*. 2007;15(9):1579-1586.
- 265.** Schottelius M, Poethko T, Herz M, et al. First (18)F-labeled tracer suitable for routine clinical imaging of sst receptor-expressing tumors using positron emission tomography. *Clin Cancer Res*. Jun 1 2004;10(11):3593-3606.
- 266.** Zinn KR, Chaudhuri TR, Krasnykh VN, et al. Gamma camera dual imaging with a somatostatin receptor and thymidine kinase after gene transfer with a bicistronic adenovirus in mice. *Radiology*. May 2002;223(2):417-425.
- 267.** Penuelas I, Haberkorn U, Yaghoubi S, Gambhir SS. Gene therapy imaging in patients for oncological applications. *European journal of nuclear medicine and molecular imaging*. Dec 2005;32 Suppl 2:S384-403.
- 268.** Britz-Cunningham SH, Adelstein SJ. Molecular targeting with radionuclides: state of the science. *J Nucl Med*. Dec 2003;44(12):1945-1961.
- 269.** Deng WP, Yang WK, Lai WF, et al. Non-invasive in vivo imaging with radiolabelled FIAU for monitoring cancer gene therapy using herpes simplex virus type 1 thymidine kinase and ganciclovir. *European journal of nuclear medicine and molecular imaging*. Jan 2004;31(1):99-109.
- 270.** Kim YJ, Dubey P, Ray P, Gambhir SS, Witte ON. Multimodality imaging of lymphocytic migration using lentiviral-based transduction of a tri-fusion reporter gene. *Mol Imaging Biol*. Sep-Oct 2004;6(5):331-340.
- 271.** Soling A, Theiss C, Jungmichel S, Rainov NG. A dual function fusion protein of Herpes simplex virus type 1 thymidine kinase and firefly luciferase for noninvasive in vivo imaging of gene therapy in malignant glioma. *Genet Vaccines Ther*. Aug 4 2004;2(1):7.
- 272.** Verwijnen SM, Sillevs Smith PA, Hoeben RC, et al. Molecular imaging and treatment of malignant gliomas following adenoviral transfer of the herpes simplex virus-thymidine kinase gene and the somatostatin receptor subtype 2 gene. *Cancer biotherapy & radiopharmaceuticals*. Feb 2004;19(1):111-120.

273. Alauddin MM, Shahinian A, Park R, Tohme M, Fissekis JD, Conti PS. Synthesis of 2'-deoxy-2'-[18F]fluoro-5-bromo-1-beta-D-arabinofuranosyluracil ([18F]-FBAU) and 2'-deoxy-2'-[18F]fluoro-5-chloro-1-beta-D-arabinofuranosyl-uracil ([18F]-FCAU), and their biological evaluation as markers for gene expression. *Nuclear medicine and biology*. May 2004;31(4):399-405.
274. Alauddin MM, Shahinian A, Gordon EM, Conti PS. Direct comparison of radiolabeled probes FMAU, FHBG, and FHPG as PET imaging agents for HSV1-tk expression in a human breast cancer model. *Mol Imaging*. Apr 2004;3(2):76-84.
275. Wang Y, Iyer M, Annala AJ, Chappell S, Mauro V, Gambhir SS. Noninvasive monitoring of target gene expression by imaging reporter gene expression in living animals using improved bicistronic vectors. *J Nucl Med*. Apr 2005;46(4):667-674.
276. Yaghoubi SS, Wu L, Liang Q, et al. Direct correlation between positron emission tomographic images of two reporter genes delivered by two distinct adenoviral vectors. *Gene therapy*. Jul 2001;8(14):1072-1080.
277. Mandell RB, Mandell LZ, Link CJ, Jr. Radioisotope concentrator gene therapy using the sodium/iodide symporter gene. *Cancer research*. Feb 1 1999;59(3):661-668.
278. Spitzweg C, O'Connor MK, Bergert ER, Tindall DJ, Young CY, Morris JC. Treatment of prostate cancer by radioiodine therapy after tissue-specific expression of the sodium iodide symporter. *Cancer research*. Nov 15 2000;60(22):6526-6530.
279. Boland A, Ricard M, Opolon P, et al. Adenovirus-mediated transfer of the thyroid sodium/iodide symporter gene into tumors for a targeted radiotherapy. *Cancer research*. Jul 1 2000;60(13):3484-3492.
280. MacLaren DC, Gambhir SS, Satyamurthy N, et al. Repetitive, non-invasive imaging of the dopamine D2 receptor as a reporter gene in living animals. *Gene therapy*. May 1999;6(5):785-791.
281. Liang Q, Satyamurthy N, Barrio JR, et al. Noninvasive, quantitative imaging in living animals of a mutant dopamine D2 receptor reporter gene in which ligand binding is uncoupled from signal transduction. *Gene therapy*. Oct 2001;8(19):1490-1498.
282. Reubi JC. Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocrine reviews*. Aug 2003;24(4):389-427.
283. Reisine T, Bell GI. Molecular biology of somatostatin receptors. *Endocrine reviews*. Aug 1995;16(4):427-442.
284. Patel YC. Somatostatin and its receptor family. *Frontiers in neuroendocrinology*. Jul 1999;20(3):157-198.

- 285.** Reubi JC, Landolt AM. High density of somatostatin receptors in pituitary tumors from acromegalic patients. *The Journal of clinical endocrinology and metabolism*. Dec 1984;59(6):1148-1151.
- 286.** Reubi JC, Heitz PU, Landolt AM. Visualization of somatostatin receptors and correlation with immunoreactive growth hormone and prolactin in human pituitary adenomas: evidence for different tumor subclasses. *The Journal of clinical endocrinology and metabolism*. Jul 1987;65(1):65-73.
- 287.** Reubi JC, Hacki WH, Lamberts SW. Hormone-producing gastrointestinal tumors contain a high density of somatostatin receptors. *The Journal of clinical endocrinology and metabolism*. Dec 1987;65(6):1127-1134.
- 288.** Reubi JC, Kvols LK, Waser B, et al. Detection of somatostatin receptors in surgical and percutaneous needle biopsy samples of carcinoids and islet cell carcinomas. *Cancer research*. Sep 15 1990;50(18):5969-5977.
- 289.** Epelbaum J, Bertherat J, Prevost G, et al. Molecular and pharmacological characterization of somatostatin receptor subtypes in adrenal, extraadrenal, and malignant pheochromocytomas. *The Journal of clinical endocrinology and metabolism*. Jun 1995;80(6):1837-1844.
- 290.** Reubi JC, Waser B, Khosla S, et al. In vitro and in vivo detection of somatostatin receptors in pheochromocytomas and paragangliomas. *The Journal of clinical endocrinology and metabolism*. May 1992;74(5):1082-1089.
- 291.** Moertel CL, Reubi JC, Scheithauer BS, Schaid DJ, Kvols LK. Expression of somatostatin receptors in childhood neuroblastoma. *American journal of clinical pathology*. Dec 1994;102(6):752-756.
- 292.** Prevost G, Veber N, Viollet C, et al. Somatostatin-14 mainly binds the somatostatin receptor subtype 2 in human neuroblastoma tumors. *Neuroendocrinology*. Feb 1996;63(2):188-197.
- 293.** Reubi JC, Chayvialle JA, Franc B, Cohen R, Calmettes C, Modigliani E. Somatostatin receptors and somatostatin content in medullary thyroid carcinomas. *Laboratory investigation; a journal of technical methods and pathology*. Apr 1991;64(4):567-573.
- 294.** Sagman U, Mullen JB, Kovacs K, Kerbel R, Ginsberg R, Reubi JC. Identification of somatostatin receptors in human small cell lung carcinoma. *Cancer*. Nov 15 1990;66(10):2129-2133.

- 295.** Reubi JC, Maurer R, Klijn JG, et al. High incidence of somatostatin receptors in human meningiomas: biochemical characterization. *The Journal of clinical endocrinology and metabolism*. Aug 1986;63(2):433-438.
- 296.** Reubi JC, Lang W, Maurer R, Koper JW, Lamberts SW. Distribution and biochemical characterization of somatostatin receptors in tumors of the human central nervous system. *Cancer research*. Nov 1 1987;47(21):5758-5764.
- 297.** Fruhwald MC, O'Dorisio MS, Pietsch T, Reubi JC. High expression of somatostatin receptor subtype 2 (sst2) in medulloblastoma: implications for diagnosis and therapy. *Pediatric research*. May 1999;45(5 Pt 1):697-708.
- 298.** Vaidyanathan G, Friedman HS, Affleck DJ, Schottelius M, Wester HJ, Zalutsky MR. Specific and high-level targeting of radiolabeled octreotide analogues to human medulloblastoma xenografts. *Clin Cancer Res*. May 2003;9(5):1868-1876.
- 299.** Reubi JC, Kvols L. Somatostatin receptors in human renal cell carcinomas. *Cancer research*. Nov 1 1992;52(21):6074-6078.
- 300.** Reubi JC, Waser B, Foekens JA, Klijn JG, Lamberts SW, Laissue J. Somatostatin receptor incidence and distribution in breast cancer using receptor autoradiography: relationship to EGF receptors. *International journal of cancer*. Sep 15 1990;46(3):416-420.
- 301.** Reubi JC, Waser B, Schaer JC, Markwalder R. Somatostatin receptors in human prostate and prostate cancer. *The Journal of clinical endocrinology and metabolism*. Sep 1995;80(9):2806-2814.
- 302.** Halmos G, Sun B, Schally AV, Hebert F, Nagy A. Human ovarian cancers express somatostatin receptors. *The Journal of clinical endocrinology and metabolism*. Oct 2000;85(10):3509-3512.
- 303.** Moody TW, Chan D, Fahrenkrug J, Jensen RT. Neuropeptides as autocrine growth factors in cancer cells. *Current pharmaceutical design*. 2003;9(6):495-509.
- 304.** Schally AV. Oncological applications of somatostatin analogues. *Cancer research*. Dec 15 1988;48(24 Pt 1):6977-6985.
- 305.** Dasgupta P. Somatostatin analogues: multiple roles in cellular proliferation, neoplasia, and angiogenesis. *Pharmacology & therapeutics*. Apr 2004;102(1):61-85.
- 306.** Vernejoul F, Faure P, Benali N, et al. Antitumor effect of in vivo somatostatin receptor subtype 2 gene transfer in primary and metastatic pancreatic cancer models. *Cancer research*. Nov 1 2002;62(21):6124-6131.

- 307.** Gulec SA, Gaffga CM, Anthony CT, Su LJ, O'Leary JP, Woltering EA. Antiangiogenic therapy with somatostatin receptor-mediated in situ radiation. *The American surgeon*. Nov 2001;67(11):1068-1071.
- 308.** Gulec SA, Drouant GJ, Fuselier J, et al. Antitumor and antiangiogenic effects of somatostatin receptor-targeted in situ radiation with (111)In-DTPA-JIC 2DL. *The Journal of surgical research*. May 15 2001;97(2):131-137.
- 309.** Blum JE, Handmaker H, Rinne NA. The utility of a somatostatin-type receptor binding peptide radiopharmaceutical (P829) in the evaluation of solitary pulmonary nodules. *Chest*. Jan 1999;115(1):224-232.
- 310.** Buchsbaum DJ, Chaudhuri TR, Zinn KR. Radiotargeted gene therapy. *J Nucl Med*. Jan 2005;46 Suppl 1:179S-186S.
- 311.** Lewis JS, Lewis MR, Cutler PD, et al. Radiotherapy and dosimetry of ⁶⁴Cu-TETA-Tyr3-octreotate in a somatostatin receptor-positive, tumor-bearing rat model. *Clin Cancer Res*. Nov 1999;5(11):3608-3616.
- 312.** de Jong M, Krenning E. New advances in peptide receptor radionuclide therapy. *J Nucl Med*. May 2002;43(5):617-620.
- 313.** De Jong M, Valkema R, Jamar F, et al. Somatostatin receptor-targeted radionuclide therapy of tumors: preclinical and clinical findings. *Seminars in nuclear medicine*. Apr 2002;32(2):133-140.
- 314.** Buchsbaum DJ. Imaging and therapy of tumors induced to express somatostatin receptor by gene transfer using radiolabeled peptides and single chain antibody constructs. *Seminars in nuclear medicine*. Jan 2004;34(1):32-46.
- 315.** McCart JA, Mehta N, Scollard D, et al. Oncolytic vaccinia virus expressing the human somatostatin receptor SSTR2: molecular imaging after systemic delivery using ¹¹¹In-pentetreotide. *Mol Ther*. Sep 2004;10(3):553-561.
- 316.** Zinn KR, Chaudhuri TR, Buchsbaum DJ, Mountz JM, Rogers BE. Simultaneous evaluation of dual gene transfer to adherent cells by gamma-ray imaging. *Nuclear medicine and biology*. Feb 2001;28(2):135-144.
- 317.** Zinn KR, Chaudhuri TR, Buchsbaum DJ, Mountz JM, Rogers BE. Detection and measurement of in vitro gene transfer by gamma camera imaging. *Gene therapy*. Feb 2001;8(4):291-299.
- 318.** Yang D, Han L, Kundra V. Exogenous gene expression in tumors: noninvasive quantification with functional and anatomic imaging in a mouse model. *Radiology*. Jun 2005;235(3):950-958.

319. Zinn KR, Buchsbaum DJ, Chaudhuri TR, Mountz JM, Grizzle WE, Rogers BE. Noninvasive monitoring of gene transfer using a reporter receptor imaged with a high-affinity peptide radiolabeled with ^{99m}Tc or ¹⁸⁸Re. *J Nucl Med.* May 2000;41(5):887-895.
320. Chaudhuri TR, Rogers BE, Buchsbaum DJ, Mountz JM, Zinn KR. A noninvasive reporter system to image adenoviral-mediated gene transfer to ovarian cancer xenografts. *Gynecologic oncology.* Nov 2001;83(2):432-438.
321. Rogers BE, McLean SF, Kirkman RL, et al. In vivo localization of [(111)In]-DTPA-D-Phe1-octreotide to human ovarian tumor xenografts induced to express the somatostatin receptor subtype 2 using an adenoviral vector. *Clin Cancer Res.* Feb 1999;5(2):383-393.
322. Rogers BE, Parry JJ, Andrews R, Cordopatis P, Nock BA, Maina T. MicroPET imaging of gene transfer with a somatostatin receptor-based reporter gene and (94m)Tc-Demotate 1. *J Nucl Med.* Nov 2005;46(11):1889-1897.
323. Csaba Z, Dournaud P. Cellular biology of somatostatin receptors. *Neuropeptides.* Feb 2001;35(1):1-23.
324. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. *Proceedings of the National Academy of Sciences of the United States of America.* Mar 3 1998;95(5):2509-2514.
325. Graham FL, Prevec L. Methods for construction of adenovirus vectors. *Molecular biotechnology.* Jun 1995;3(3):207-220.
326. Siddiqui F, Li CY, Zhang X, et al. Characterization of a recombinant adenovirus vector encoding heat-inducible feline interleukin-12 for use in hyperthermia-induced gene-therapy. *Int J Hyperthermia.* Mar 2006;22(2):117-134.
327. Vernon CC, Hand JW, Field SB, et al. Radiotherapy with or without hyperthermia in the treatment of superficial localized breast cancer: results from five randomized controlled trials. International Collaborative Hyperthermia Group. *International journal of radiation oncology, biology, physics.* Jul 1 1996;35(4):731-744.
328. Harima Y, Nagata K, Harima K, Ostapenko VV, Tanaka Y, Sawada S. A randomized clinical trial of radiation therapy versus thermoradiotherapy in stage IIIB cervical carcinoma. *Int J Hyperthermia.* Mar-Apr 2001;17(2):97-105.
329. Valdagni R, Amichetti M. Report of long-term follow-up in a randomized trial comparing radiation therapy and radiation therapy plus hyperthermia to metastatic lymph nodes in stage IV head and neck patients. *International journal of radiation oncology, biology, physics.* Jan 1 1994;28(1):163-169.
330. Sneed PK, Stauffer PR, McDermott MW, et al. Survival benefit of hyperthermia in a prospective randomized trial of brachytherapy boost +/- hyperthermia for glioblastoma

multiforme. *International journal of radiation oncology, biology, physics*. Jan 15 1998;40(2):287-295.

331. Li GC, He F, Ling CC. Hyperthermia and gene therapy: potential use of microPET imaging. *Int J Hyperthermia*. May 2006;22(3):215-221.

332. Palmer DH, Young LS, Mautner V. Cancer gene-therapy: clinical trials. *Trends in biotechnology*. Feb 2006;24(2):76-82.

333. Penuelas I, Mazzolini G, Boan JF, et al. Positron emission tomography imaging of adenoviral-mediated transgene expression in liver cancer patients. *Gastroenterology*. Jun 2005;128(7):1787-1795.