AD_____

Award Number: W81XWH-04-1-0753

TITLE: Retrotransposon Targeting of Tumor Cells

PRINCIPAL INVESTIGATOR: Dongdong Wu, M.D., Ph.D.
George DeVaux

CONTRACTING ORGANIZATION: Biostar, LLC
McLean, VA 22101-5653

REPORT DATE: October 2005

TYPE OF REPORT: Final

20060223 035

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-10-2005	Final	15 Sep 2004 – 14 Sep 2005

4. TITLE AND SUBTITLE
Retrotransposon Targeting of Tumor Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0753

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dongdong Wu, M.D., Ph.D.
George DeVaux

E-mail: dongdongwu@hotmail.com

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Biostar, LLC
McLean, VA 22101-5653

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Cancer gene therapy might provide highly selective, treatment without systemic toxicity. Cancer gene therapy techniques include oncogene inactivation, tumor suppressor gene replacement, inhibition of angiogenesis, immunopotentiation, molecular chemotherapy, and transfer of drug resistance genes. These treatments have enjoyed only limited success.
We propose to use suicide gene therapy transfection of cancer cells with genes that encode enzymes able to activate nontoxic pro-drugs in situ to form cytotoxic products. After insertion of the suicide gene constructs into the cancer cells, we will apply high doses of a relatively non-toxic pro-drug. Intracellular conversion of the pro-drug into the cytotoxic form will result in high concentrations within the cell, and kill the cancer cell. We designed and codon optimized the desired vectors. We constructed a vector to deliver special suicide gene (TK) into tumor cell lines. The vector with selectable marker gene, promoter and codon optimized suicide gene(s) were transfected into breast cancer cell lines. We examined the expression of the transfected gene in the cell culture by RT-PCR and special colony assay. Adequate levels of expression were not attained in these experiments.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction

Gene therapy is a new method of cancer treatment that involves the manipulation of intracellular DNA in order to control or destroy cancer cells. Cancer gene therapy has the potential to provide highly selective, curative cancer treatment without systemic toxicity. However, application of this type of cancer treatment requires an understanding of tumor biology; methods for gene delivery to specific cell types; and strategies to regulate the level and duration of gene expression. The biological uniqueness of each tumor cell has led to the development of a variety of techniques for cancer gene therapy including oncogene inactivation, tumor suppressor gene replacement, inhibition of angiogenesis, immunopotentiation, molecular chemotherapy, and transfer of drug resistance genes.

Body

Suicide gene therapy:

One approach to chemotherapy is the transfection of cancer cell with genes that encode for the production by the cancer cell of an enzyme able to activate non-toxic pro-drugs *in situ* to form cytotoxic products (suicide gene therapy). After insertion of the suicide gene constructs into the cancer cells, treatment with high doses of a relatively non-toxic, pro-drug ensures that high concentrations of the cytotoxic drug are being produced by the transfected cancer cells. As the intracellular concentration of the cytotoxic product increases, survival of the transfected cell is maintained by diffusion of the chemical through the gap junction into adjacent non-transfected cancer cells. Once the toxin reaches high enough concentrations in the contiguous cancer cell mass, it kills both the transfected cells and their neighbors (bystander effect). Thus, suicide gene therapy can cause tumor regression even when only a fraction of the tumor cells are transfected.

One technique for suicide gene therapy is the use of herpes simplex virus (HSV)-thymidine kinase (TK), followed by treatment with ganciclovir (GCV). GCV is a substrate for viral TK but is not recognized by the analogous mammalian enzyme. Viral TK phosphorylate GCV. Monophosphate GCV is converted to the triphosphate form by cytoplasmic enzymes. GCV triphosphate is incorporated into replicating DNA and stops chain elongation resulting in cell death. Another strategy is to transfect with the E. coli gene coding for cytosine deaminase (CD) followed by systemic use of 5-fluorocytosin (5-FC). CD transforms the non-toxic pro-drug 5-FC into the cytotoxic drug 5-fluorouracil (5-FU). A recent phase I study of adenovirus (Ad)-mediated double suicide gene therapy (HSV-TK and CD) for the treatment of locally recurrent prostate carcinoma demonstrated that this technique can be safely and successfully used in humans. A number of other suicide gene therapies have been developed and tested in cell culture, laboratory animals, and some early clinical trials. The LINE-1 (L1) retrotransposons are suppressed in primary somatic cells but elevated in many tumor cell types, including breast cancer cells. The ability of a highly active L1 vector to deliver selectively into mammary tumor cells 'suicide' genes which will be expressed only upon retrotransposition and integration into the genome was tested.

The L1 possesses several unique features making it attractive as a gene delivery vector. L1-encoded proteins show a strong cis-preference for their own encoding RNA (Wei et al., 2001). It is therefore very unlikely that proteins expressed by an L1-delivery vector would comobilize endogenous L1s. Also, L1 proteins are not foreign, and when expressed from a vector, are unlikely to provoke an immune response. If immunogenicity is not an issue, repeated administration of the L1 vector should improve therapeutic effect. Finally, L1-mediated genomic insertion permits long-term expression of a therapeutic gene, important if the gene product is not toxic but rather one requiring prolonged secretion, such as an angiogenesis inhibitor. Moreover, some cancer cells, such as those of the breast, prostate, and

colon, are frequently slow growing, necessitating prolonged suicide gene expression and long-term administration of the selected pro-drug to achieve adequate killing

OBJECTIVES

1) Construct a vector containing LINE-1 gene and HSV-tk gene capable of delivering a suicide gene into tumor cells in culture.
2) If the above the aim is successful, test the effect of the suicide vectors on the growth of tumor xenografts in mice.

Method & Results:

HSV-tk is the most widely studied enzyme used for suicide gene strategies (Yazawa et al. 2002). Tumor cells transduced with HSV-tk are sensitive to treatment with the nucleoside analog ganciclovir (GCV) due to the ability of HSV-tk to catalyze the phosphorylation of GCV, thereby converting it to a cytotoxic DNA analog which interferes with replication. A bystander killing effect has been reported for HSV-tk both in vivo and in vitro, mediated by the transfer of phosphorylated GCV between cells through gap junctions, (Vrionis et al .1997). In animal models of HSV-tk and GCV therapy, it has been shown that expression of the HSV-tk protein by as few as 10% of the tumor cells is sufficient to attain complete killing of the tumor (Freeman et al. 1993).

Construction of plamid vector which contained L1 and HSV-tk genes

The vector used was pIRES (BD Biosciences Clontech, Palo Alto, CA). The L1 gene was synthesized with codon optimization (GeneArt, Germany) and designed to containing unique cloning sties NheI/EcoRI. The pIRES vector multiple cloning site A was chosen for insertion of L1 gene. The vector was digested with NheI/EcoRI and gel-purified. The same enzymes digested, gel-purified L1 gene was ligated into the pIRES vector and transformed into E. coli DH5a to making pIRES-L1. The plasmid pIRES-L1 was purified with column (Qiagen, Valencia, CA). The size and orientation of the fragment in the selected clones were verified through restriction mapping, and the insert was sequenced from both directions.

The HSV-tk gene was also synthesized with codon optimization (GeneArt, Germany) which contained open reading frame and cloned into pUC18 plasmid. The product digested with XmaI and Sma I. The gel-purified fragment was ligated into the pRIES-L1 multiple cloning site B which was digested with XmaI and Sma I and gel-purified. The size and orientation of the fragment in the selected clones were verified through restriction mapping, and the insert was sequenced from both directions.

Figure 1. Construction of plamid vector which contained L1 and HSV-tk genes

Transfection of vector into tumor cell lines.

Optimize the transfection components:

In order to obtain optimum transfection efficiencies, the reporter vector pSV-ß-Galactosidase (Progema, Madison, WI) and Lipofectemine^{TM} 2000 (Invitrogen, Carlsbad, CA) were used. The plasmid was purified on miniprep columns (Qiagen). The medium used in transfection process was

Opti-MEM I reduced serum medium (Invitrogen). We assessed how different ratio of DNA and liposome, and cell number affect transfection rate and toxicity. ß-Galactosidase enzyme assay system kit (Progema, Madison, WI) were used for examining the transfer rate. The more detailed method was followed the manufacture's protocol (see Fig 2-5).

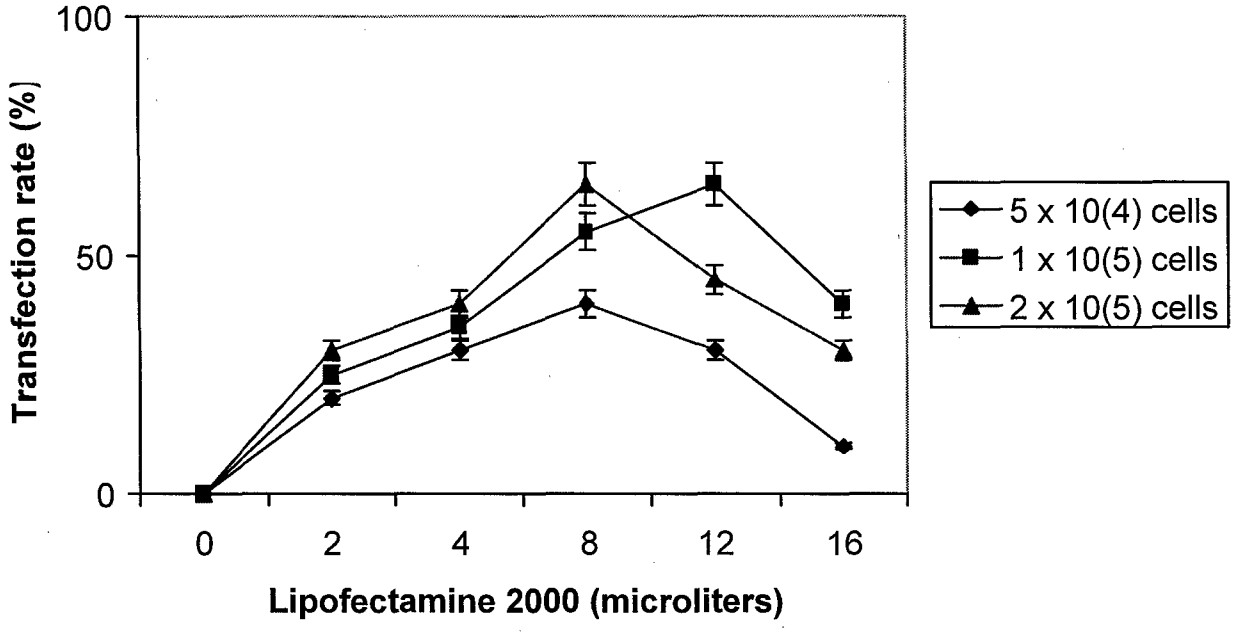

Figure 2. Effect of MCF7 cell number and lipid concentration on transfection rate. MCF7 cells were transfected with 1 μg of pSV-β-Galatosidase plasmid DNA. Error bars indicate standard deviation determined in triplicate experiments.

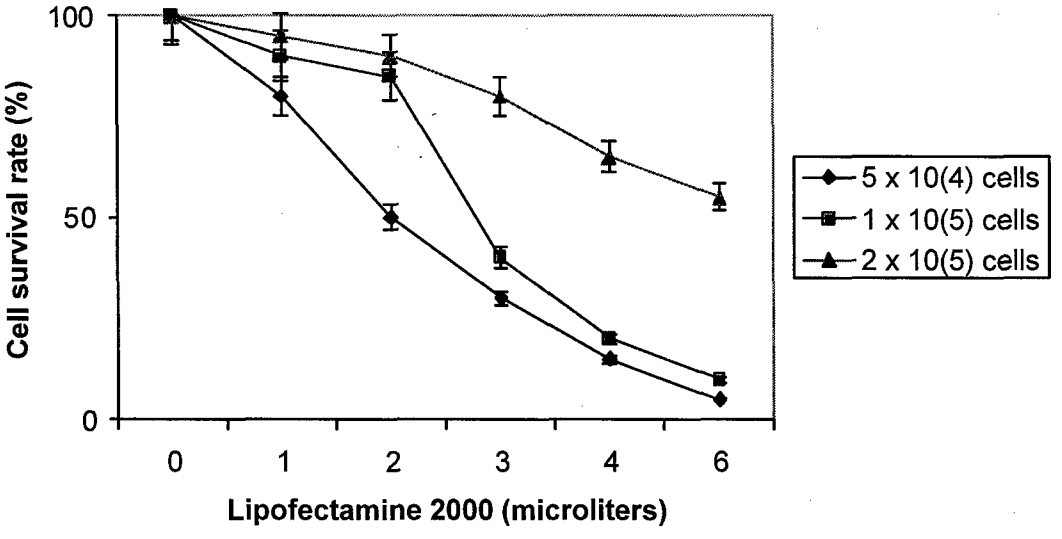

Figure 3. Toxicity effect of MCF7 cell number and lipid concentrations on transfection cells. Cells were transfected with 1 μg of pSV-β-Galatosidase plasmid DNA. Error bars indicate standard deviation determined in triplicate experiments.

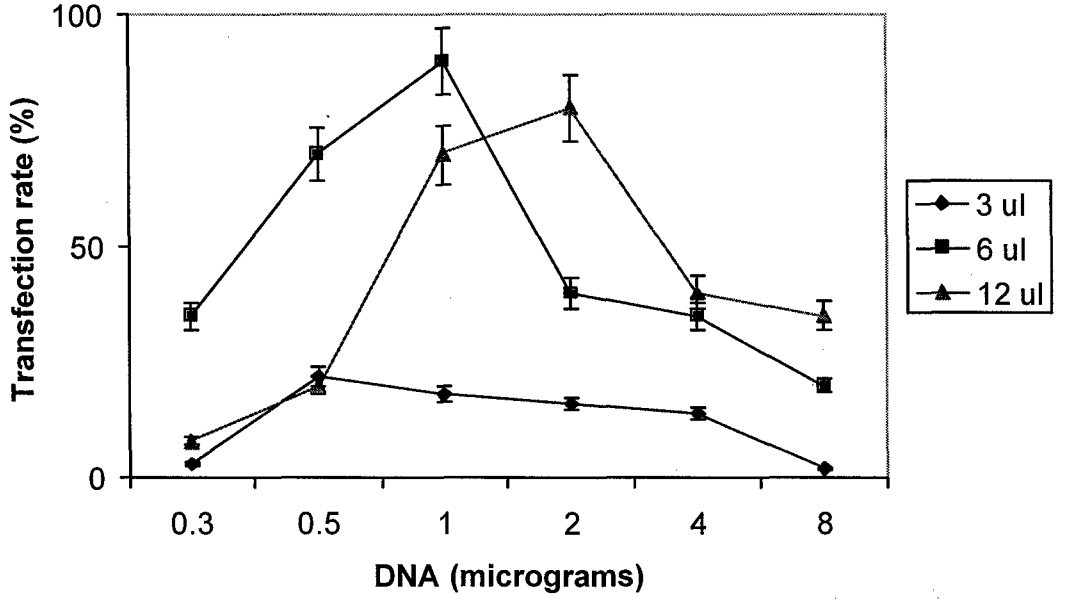

Figure 4. Effect of DNA and Lipid concentrations on transfection rate. 1 x 10^5 MCF7 cells/well/6 well plate were transfected with pSV-β-Galatosidase plasmid DNA. Error bars inicatestandard deviation determined in triplicate experiments.

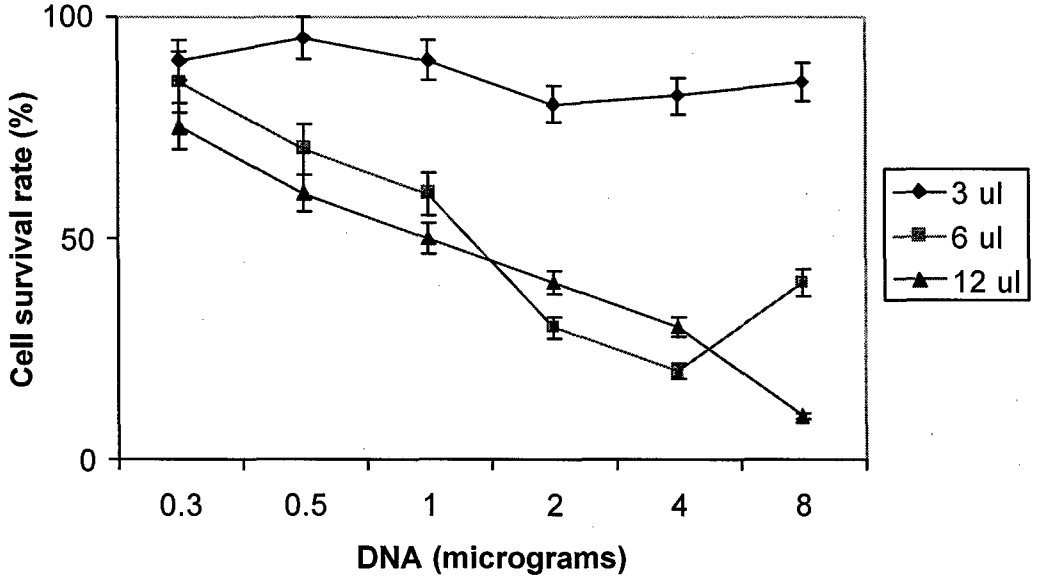

Figure 5. Toxicity effect of DNA and lipid concentrations on transfection cells.
1 x 10^5 MCF7 cells/well/6 well plate were transfected with pSV-β-Galatosidase plasmid DNA. Error bars indicate standard deviation determined in triplicate experiments.

Breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (ATCC, HTB-22). MCF-7 was maintained in DMEM medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 1 mM sodium pyruvate, at 37° C, 5% CO_2 in air.

Transfection of vector into tumor cell lines

A total of 1 x 10^5 exponentially growing MCF-7 cells cultured in 6-well dishes were grown to 80% confluency in DMEM complete medium. Plasmid DNA was purified on maxiprep columns (Qiagen) and diluted in sterile millipore water to 1 μg/ul. Cells were transfected with 2 μg of recombinant plasmid vector DNA with 6 μg of Lipofectemine 2000 in Opti-MEM I Medium (Invitrogen) following the above optimized protocol. After 24 hours of culturing, the transfected cells were selected with G418 (0.5 mg/ml, active) (Invitrogen), for 2 weeks with medium exchange every week.

Three individual neo^R colonies were isolated by limiting dilution and further established as stable cell lines and propagated in DMEM medium with 10% fetal bovine serum (Hyclone).

Detection of genomic DNA with HSV-tk cDNA incorporation and RNA expression

To examine the integrated HSV-tk gene in the transfected cell line, total genomic DNA was isolated for PCR amplification of HSV-tk cDNA. To determine the expression of HSV-tk, cellular RNA isolated with RNAzol reagent (Tel-Test, Friendswood, TX) was extracted 4 times with phenol chloroform, ethanol-precipitated and resuspended in diethylpyrocarbonte-treated water to reach a final concentration of 0.5 mg/ml. Contaminated DNA was removed by incubating the RNA mixture with DNase 950U/0.5mg RNA) at 37° C for 1 hr. The reaction was terminated by adding 100 ul of 0.1M EDTA (pH 8.0) and extracting the RNA twice with phenol chloroform. RNA was ethanol-precipitated, washed once with 80% ethanol and resuspended in 2 ml of diethylpyrocarbonate-treated (DEPC) water. Primer for PCR was used at an excess concentration of 500 ng in a 50 ul final reaction volume. PCR was performed using AmpliTaq DNA polymerase, as described in the Clontech (Palo Alto, CA) Advantage RT-for-PCR kit protocol (25 cycles of 94°C for 45 sec. 60°C for 45 sec. 72°C for 2 min; 7 min final extension at 72°C), and the amplified product separated in a 0.7% agarose gel.

Key Research Accomplishments:

- Codon optimized genes for construction.
- Constructed plasmid vectors containing L1 and HSV-tk genes.
- Included neo-resistant gene to allow selection of the transfected cells.
- Studied human tumor cell transfer rate.
- Achieved high transfer rates – 70% to 75% - by varying amount of DNA, cell count, and amount of liposome.
- Established several neo-resistant clones.
- PCR analysis indicated that the vector was inserted into the genome of the transfected cells.
- Did not detect expression of the HSV-tk gene in the transfected cells by RT-PCR.
- Did not detect expression of the HSV-tk gene in cell culture *in vitro*.

Reportable Outcomes

No manuscripts or presentations have been developed based on this study.

Conclusions:

Because we did not achieve expression of the HSV-tk gene in cell culture *in vitro*, we did not proceed to animal testing. Because of the high promise of suicide gene therapy, we believe that additional efforts in vector construction and expression in vitro are desirable. Once these efforts are successful, animal tests can be reevaluated.

References:

Boissinot S, Chevret P, and Furano AV. 2000 (LINE-10 retrotransposon evolution and amplification in recent human history. Mol. Biol. Evol. 17: 915-28.

Bratthaucer GL, Cardiff RD, and Fanning TG. 1994. Expression of LINE-1 retrotransposons in human breast cancer. Cancer 73: 2333-2336.

Brouha B, et al. 2002 Evidence consistent with human L1 retrotransposition in maternal meiosis I. Am. J. Hum. Genet. 71:327-36.

Chiu CC, et al., 2002. Ectopic expression of herpes simplex virus-thymidine kinase gene in human non-small cell lung cancer cells conferred caspase-activated apoptosis sensitized by ganciclovir. Int. J. Cancer 102: 328-33.

Dass CR. et al. 1997. Cationic liposomes and gene therapy for solid tumors. Drug Delivery 4: 151-165.

Deonarain MP, et al. 1995. Genetic delivery of enymes for cancer therapy. Cancer Gene Ther 2: 235-244.

Ding I, et al. 2001. Intratumoral administration of endostatin plasmid inhibits vascular growth and perfusion in Mca-4 murine mammary carcinomas. Cancer Res. 61: 526-531.

Ellion GB, et al. 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. Proc. Natl. Acad. Sci. USA. 74: 5716-5720.

Esnault C, Maestre J, and Heidmann T. 2000. Human LINE retrotransposonsgenerate processed pseudogenes. Nat. Genet. 24: 363-67.

Freeman SM, et al. 1993. The "bystander effect": Tumor regression when a fraction of the tumor mass is genetically modified. Cancer Res 53: 5274-5283.

Freytag SO, et al. 2002. Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of loally recurrent prostate cencer. Cancer Res. 62: 4968-4976.

Huber BE, et al. 1994. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. Proc. Natl. Acad. Sci. USA. 91: 8302-8306.

Hughes RM. 2004. Strategies for cancer gene therapy. J. Surg. Onco. 85: 28-35.
Kimberland ML, et al. 1999. Full-length human L1 inertions retain he capacity for high frequency retrotransposition in cultured cells. Hum. Mol. Genet. 8:1557-60.
Li Z, et al. 1997. enzyme/prodrug gene therapy approach for breast cancer using a recombinant adenovirus expressing Escherichia coli cytosine deaminase. Cancer Gene Ther. 4: 113-117.

Moolten F. 1986. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. Cancer Rec. 46: 5276-5281.
Moran JV, Et al. 1996. High frequency retrotransposition in cultured mammalian cells. Cell 87:917-927.

Park ET, et al., 2003. Tracking an embryonic L1 retrotransposition event. Proc. Natl. Acad. Sci. USA. 100: 1832-1837.

Rubsam LZ, et al. 1999. Cytotoxicity and accumulation of ganciclovir triphosphate in bystander cells cocultured with herpes simplex virus type 1 thymidine kinase-expressing human glioblastoma cells. Cancer Res. 59: 669-675.

Shi W, et al. 2002. Adeno-asociated virus-mediated gene transfer of endostatin inhibits angiogenesis and tumor growth in vivo. Cancer Gene Ther. 9:513-521.

Skowronski J and Singer MF. 1985. Expression of a cytoplasmic LINE-1 transcript is regulated in a human teratocarcinoma cell line. Proc. Natl. Acad. Sci. USA. 82: 6050-6054.

Soifer H, et al. 2001. Stable integration of transgenes delivered by a retrotransposon-adenovirus hybrid vector. J. Human Gene Therapy 12: 1417-1428.

Symer DE, et al. 2002. Human L1 retrotransposition is associated with genetic instability in vivo. Cell 110: 327-338.

Trelogan SA and Martin SL. 1995. Tightly egulated, developmentally specific expression of the first open reading frame from LINE-1 during mouse embryogenesis. Proc. Natl. Acad. Sci. USA. 92: 1520-1524.

Vrionis FD, et al. 1997. The bystander effect exerted by tumor cells expressing the herpes simplex virus thymidine kinase (HSV-tk) gene is dependent on connexin and cell communication via gap junctions. Gene Therapy 4: 577-585.

Wei W, et al. 2000. A transient assay reveals that cultured human cells can accommodate multiple LINE-1 retrotransposition events. Anal. Biochem. 284: 435-438.

Wygoda MR, et al. 1997. Protection of herpes simplex virus thymidine kinase-transduced cells from ganciclovir-mediated cytotoxicity by bystander cell: the good Samaritan effect. Cancer Res. 57: 1699-1703.

Yazawa K, Fisher WE, and Brunicardi FC. 2002. Curreent progress in suicide gene therapy for cancer. World J. Surg. 26: 783-789.

Yerushalmi N, et al 2000. Attenuating the growth of tumors by intratumoral administration of DNA encoding pseudomonas exotoxin via cationic liposomes. Cancer Gene Ther. 7: 91-96.