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Circumventing Recombination Events Encountered with Production of a Clinical-Grade Adenoviral Vector with a Double-Expression Cassette

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ABBREVIATIONS: HSVtk, herpes simplex virus thymidine kinase; hSSTR2, human somatostatin receptor subtype-2; CMV, cytomegalovirus; SV40, Simian Virus 40; BGH, bovine growth hormone; CAR, coxsackie-adenovirus receptor; RGD-4C, arginine-glycine-aspartic motif; GCV, gancyclovir; HEK 293, human embryonic kidney 293 cell line; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PCR, polymerase chain reaction; TCID₅₀, 50% tissue culture infective dose assay; MOI, multiplicity of infection; pfu, plaque forming units; HBSS, Hank's balanced salt solution.

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ABSTRACT

Delivery of multiple exogenous genes into target cells is important for a broad range of gene therapy applications, including combined therapeutic gene expression and noninvasive imaging. Previous studies (1) described the adenoviral vector RGDTKSSTR with a double-expression cassette that encodes herpes simplex virus thymidine kinase (HSVtk) for molecular chemotherapy and human somatostatin receptor subtype-2 (hSSTR2) for indirect imaging. In this vector, both genes are inserted in place of the E1 region of the adenoviral genome and expressed independently from two cytomegalovirus (CMV) promoters. During production of clinical-grade RGDTKSSTR, we found that the CMV promoters and SV40 polyA regions located in both expression cassettes provoked homologous recombination and deletion of one of the cassettes. To resolve this problem, we designed a strategy for substituting the duplicate promoters and polyA regions. We placed the hSSTR2 gene in the new Ad5.SSTR/TK.RGD vector under the control of a CMV promoter with a bovine growth hormone (BGH) polyA region, whereas the SV40 promoter, enhancer, and polyA signal controlled HSVtk expression. This use of different regulatory sequences allowed independent expression of both transgenes from a single adenoviral vector and circumvented the recombination problem. Reconstruction of the vector with a double-expression cassette enables its use in human clinical trials.

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INTRODUCTION

Adenovirus-mediated gene therapy is promising for advanced cancers refractory to other treatment modalities because it enables the delivery of therapeutic genes directly into the target cells. However, to date, clinical trials of adenovirus-mediated gene therapy have had limited success. One of the primary obstacles to the realization of the full potential of this promising modality is the inability of currently used adenoviral vectors to efficiently transduce target cancer cells. Inefficient transduction is principally caused by inconsistent expression of coxsackie-adenovirus receptor (CAR), which is often low or absent in primary cancer cells (1,2). Investigators have performed extensive research in an effort to enhance the transduction capacity of adenoviral vectors (3,4). An important corollary to this line of research is development of the means to monitor gene transfer in vivo.

Several studies have suggested the use of a noninvasive imaging approach to observe the efficiency of gene transfer and expression over time. This approach is based on the coupled expression of therapeutic and reporter genes: the level of expression of the therapeutic gene is predicted by the level of expression of the reporter gene. In general, researchers have used four approaches to link the expression of two genes in the context of an adenoviral vector. One approach is based on the use of two adenoviral vectors (5,6). However, this approach cannot guarantee uniform infection of all cells with both vectors because of the relative inefficiency of gene transfer procedures. Another approach involves the use of bicistronic vectors co-expressing the genes linked by an internal ribosomal entry site (7,8). Use of this method results in valid correlations in expression, but the absolute level of expression of the second gene is generally reduced. The third approach is expression of multiple gene products from a single cistron through a fusion protein that can be postsynthetically cleaved and processed into individual

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biologically active proteins (9) or that can itself have two or more functions (10). The fourth approach involves the insertion of multiple expression cassettes into a single vector, with each cassette having its own independent promoter and polyA signal. Different groups have employed this approach, including ours (11,12). Specifically, we developed the infectivity-enhanced adenoviral vector RGDTKSSTR, which incorporates a genetically modified fiber with an arginine-glycine-aspartic (RGD-4C) motif in the HI loop of the knob (11). The RGD-4C modification allows the virus to use cellular integrins, which are frequently overexpressed in ovarian tumors (13) and other types of tumors (14-16), for binding and internalization. Thus, the vector can circumvent dependence on CAR. For a therapeutic effect, HSVtk/gancyclovir (GCV) suicide system (17) was applied. A reporter gene, hSSTR2 was used to provide high-sensitivity imaging with positron emission tomography and single-photon emission computed tomography. Both hSSTR2 and HSVtk expression cassettes in RGDTKSSTR were driven by CMV promoters and included SV40 polyA regions. This gene therapy approach had cytotoxic effects on both ovarian cell lines and primary cancer cells. Moreover, in a subsequent study, we were able to detect the virus for more than 2 weeks in an ovarian cancer animal model, and we showed that the RGD-4C modification can enhance the in vivo effects of the HSVtk/GCV approach (18). Based on the promising in vitro and in vivo data, we suggested that the RGDTKSSTR vector could be a useful agent in the treatment of ovarian cancer and set the stage for clinical testing.

In the study described herein, while upscaling RGDTKSSTR for a clinical trial in patients with ovarian cancer, we found that the presence of homologous promoters and polyA regions in expression cassettes induced recombination and led to the deletion of one of two transgenes. This defect rendered the use of this vector in its original configuration impossible in human clinical

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trials. We hypothesized that to resolve this problem, we had to reconstruct the vector by replacing the duplicate promoters and polyA signals with nonhomologous ones.

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MATERIALS AND METHODS

Cells

The human embryonic kidney 293 (HEK 293) cell line used for propagation of recombinant adenoviruses was obtained from Magenta Corporation (Rockville, MD, USA). The lung cancer cell line A549 was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM):Ham's F-12 (50:50 mix) medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. FBS was purchased from GIBCO-BRL (Gaithersburg, MD, USA), and media and supplements were purchased from Mediatech (Herndon, VA, USA). Cells were propagated at 37°C in a 5% CO₂ atmosphere.

Production of Clinical-Grade RGDTKSSTR

Production of the RGDTKSSTR vector, in which expression of the hSSTR2 and HSVtk genes is driven by CMV promoters, was described previously (11). Small-scale virus propagation was performed in 175-cm² flasks. Further cell expansion and upscaled adenovirus production in 850-cm² roller bottles (Corning, Acton, MA, USA) were performed as described previously. In all, three rounds of virus amplification were performed. Adenoviral vectors were isolated from infected cells and purified by equilibrium centrifugation in CsCl gradients.

Recombination Assay

Viral particles (10⁷) were used for polymerase chain reaction (PCR) analysis with pairs of primers located in the region, including hSSTR2 and HSVtk expression cassettes: P1-Ad5.F277 (CGCGGGAAACTGAATAAGA) and P2-pShuttle.R (GCGCGTTGTCAAATATGAG), P3-

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SSTR.F1014 (GGAGCGGAGTGACAGTAAGC) and P4-TK.R11129 (AGCCTCCCCCATCTCCC), P1 and P6-TK.R150 (CAGTAGCGTGGGCATTTTCT), and P2 and P5-SSTR.F79 (TCAACCAACACCTCAAACCA). The primers were designed to amplify both full-size DNA fragments corresponding to double-expression cassettes and potentially truncated fragments corresponding to recombinant genomes with only one expression cassette. Localization of the primers is shown in Figure 1A.

Genetic Reengineering of Ad5.SSTR/TK.RGD

The HSVtk gene was amplified from pORF-HSVtk (InvivoGen, San Diego, CA, USA) by using sticky-end PCR (44). This was accomplished by using two pairs of primers: HindIII.F.L (AGCTTGCCACCATGGCTTCGTACCCC) and XbaI.R.S (ATCATTCACCTCAGTTAGCCTCCCCCAT) and HindIII.F.S (TGCCACCATGGCTTCGTACCCC) and XbaI.R.L (CTAGATCATTCACCTCAGTTAGCCTCCCCCAT). The PCR products were mixed in equimolar amounts, denatured, annealed, and ligated with a *HindIII-XbaI*-digested pGL3-control vector (Promega, Madison, WI, USA). The resultant plasmid was designated pGL-TK.

Sequences corresponding to the CMV promoter and hSSTR2 gene were amplified from pDC.SSTR2 plasmid (1), and the BGHpA region was amplified from pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). In all cases, the sticky-end PCR technique was employed. Next, pairs of primers were used for amplification: for the CMV promoter, NotI.F.L (GGCCGCGATCTAATTCCCTGGCAT) and AgeI.R.S (TCGGTCCCGGTGTCTTCTAT) and NotI.F.S (GCGATCTAATTCCCTGGCAT) and AgeI.R.L (CCGGTCGGTCCCGGTGTCTTCTAT); for the hSSTR2 gene, AgeI.F.L

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(CCGGTGCAACCATGGACATGGCGGA) and BstBI.R.S
(TCATTCACCTCAGATACTGGTTTGGAGGTC) and AgeI.F.S
(TGCAACCATGGACATGGCGGAT) and BstBI.R.L
(CGAATCATTCACTCAGATACTGGTTTGGAGGTC); and for the BGHpA signal, BstBI.F.L
(TTCGAAGACTGTGCCTTCTAGTTGC) and NheI.R.S (CGGTGGGCTCTATGGG) and
BstBI.F.S (AAGACTGTGCCTTCTAGTTGC) and NheI.R.L
(CTAGCCCATAGAGCCCACCGC). The PCR products for each structure were mixed in
equimolar amounts, denatured, and annealed. Then, they were combined and ligated with an
NheI-SalI fragment from pGL-TK containing the HSVtk expression cassette and an *NotI-SalI*--
digested pShuttle vector (Quantum Biotechnologies, Montreal, Quebec, Canada). The final
plasmid was designated pShuttle.SSTR/TK.

To generate recombinant adenoviral genomes containing hSSTR2/HSVtk-expressing
cassettes in place of the E1 region and a fiber gene incorporating an RGD-4C--encoding peptide,
homologous DNA recombination with *PmeI*-digested pShuttle.SSTR/TK and an E1, E3-deleted
pVK554 vector was performed (11). The resultant plasmid was designated pNB557.

Ad5.SSTR/TK.RGD Vector

The Ad5.SSTR/TK.RGD vector containing hSSTR2 and HSVtk expression cassettes
(driven by CMV and SV40 promoters, respectively) and an RGD-4C--incorporating fiber gene
was rescued after transfection of HEK 293 cells with *PacI*-digested pNB557. The virus was
amplified three times consecutively in the HEK 293 cells and purified by equilibrium
centrifugation in CsCl gradients by using a standard protocol. Determination of the virus particle
titer was accomplished spectrophotometrically by using a conversion factor of 1.1×10^{12} viral

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particles per absorbance unit at 260 nm. The infectious titer of the vector in HEK 293 cells was determined by using a standard 50% tissue culture infective dose (TCID₅₀) assay (Quantum).

Cytotoxicity Assay

A549 cells were plated in 96-well plates (3×10^3 cells per well). The next day, cells were infected with an adenovirus diluted in 5% FBS-DMEM/F-12 medium at a multiplicity of infection (MOI) of 5 pfu/cell. Medium without a virus was added to the control wells. After 4 h, GCV was added to the cells to a final concentration of 0.25, 0.50, and 0.75 mM. Plates were incubated in a CO₂ incubator for 4 more days, and the medium was replaced with 100 μ L of phosphate-buffered saline buffer. Cytotoxicity was analyzed by using a colorimetric method with the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) as described by the manufacturer. Absorbance was measured at a wavelength of 490 nm by using an Emax spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA). Each data point was set in triplicate and calculated as the mean of three determinations.

hSSTR2 Expression

Experiments were performed with adherent cells in 96-well plates with each treatment evaluated in five separate wells. A total of 5×10^3 A549 cells per well were infected with an adenovirus diluted in 5% FBS-DMEM/F-12 medium at an MOI of 5 pfu/cell. After 96 h, the medium was removed, and cells were incubated with an internalization medium alone. Detection and measurement of in vitro gene transfer was performed by using gamma camera imaging. Two groups were set with an internalization medium or with internalization medium containing 2 μ g of unlabeled (or cold) P2045 peptide (Diatide Research Laboratories, Londonderry, NH,

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USA) per well. Unlabeled P2045 was used as a competitor for blocking labeled (or hot) P2045 to bind to the expressed hSSTR2. After blocking for 5 min, technetium (Tc) 99m-P2045 (specific activity, approximately 1000 $\mu\text{Ci}/\mu\text{g}$) was added to all of the wells on the plate (15 $\mu\text{Ci}/\text{well}$; final concentration, 11.5 nM) and incubated for 2 h. After incubation, the cells were washed twice with ice-cold Hank's balanced salt solution (HBSS) (pH 7.2) and lysed with 1 M NaOH. The radioactivity in the lysed cells was measured by using a gamma-ray counter (MINAXI γ Auto-gamma[®] 5000 series gamma counter; Packard Instrument Company, Grove, Downers Grove, IL, USA). All of the data were normalized for protein concentration measured in the samples by Lowry assay (Bio-Rad, Hercules, CA, USA).

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RESULTS

The Homologous Regions in RGDTKSSTR Provoke Recombination and Internal Deletions

We upscaled RGDTKSSTR (11) for use in a phase I clinical trial in patients with ovarian cancer, and we examined the stability of the adenoviral genome, particularly the E1-modified region, by using PCR analysis with primers located in that region (Figure 1A). We clearly detected truncated PCR products corresponding to the genome but only one expression cassette (hSSTR2 or HSVtk; Figure 1B). Thus, because of the presence of the homologous regions corresponding to the CMV promoter (414 bp) and SV40 polyA (165 bp) sequences in both the hSSTR2 and HSVtk cassette, homologous recombination occurred and induced internal deletion. Consequently, the viral population was heterogeneous and included viral particles with genomes encoding both transgenes as well as particles with only one expression cassette. This defect hindered our ability to use the original configuration of RGDTKSSTR in human clinical trials.

Reconstruction of Ad5.SSTR/TK.RGD

To resolve the problem of recombination, we designed a strategy to replace duplicate promoters and polyA regions in the RGDTKSSTR vector with nonhomologous ones. To that end, we placed the hSSTR2 gene in the first expression cassette under the control of the CMV promoter and BGHpA, whereas we placed the HSVtk gene in the second expression cassette under the control of the SV40 promoter with the SV40 enhancer and SV40 polyA signal (Figure 2). We combined the two cassettes in a pShuttle vector and then transferred them by homologous recombination into an E1, E3-deleted pVK555 backbone. We rescued the new vector, named Ad5.SSTR/TK.RGD, after transfection of HEK 293 cells with the resultant adenoviral genome. We consecutively amplified Ad5.SSTR/TK.RGD on HEK 293 cells three times and used CsCl-

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purified virions for PCR analysis. The analysis of Ad5.SSTR/TK.RGD did not reveal any truncated products (Figure 2). Thus, use of the different regulatory sequences circumvented the problem of recombination experienced with the vector containing two expression cassettes under the control of identical regulatory regions.

Validation of Ad5.SSTR/TK.RGD Transgene Expression

We confirmed the cytotoxic effect of HSVtk by using a cytotoxicity assay (Figure 3A). This assay showed that Ad5.SSTR/TK.RGD effectively killed A549 cells. The percentage of surviving cells depended on the GCV concentration and varied from 39% to 55%. Although the numbers of viable cells were higher with Ad5.SSTR/TK.RGD compared with the positive control vector, Ad5.CMV-TK, in which expression of the HSVtk gene was driven by the CMV promoter, much lower than two negative control groups, Ad5Luc1 and no virus.

The gamma camera imaging assay with the hSTTr2-avid Tc 99m-P2045 peptide confirmed that the hSSTR2 gene was also biologically active (Figure 3B). Only A549 cells infected with Ad5.SSTR/TK.RGD bound the Tc 99m-P2045 peptide; unlabeled P2045 specifically blocked this binding. This meant that the functional hSSTR2 receptor was expressed on the surface of infected cells. Noninfected cells and cells infected with the control Ad5Luc1 virus, which expressed the luciferase gene, did not demonstrate significant binding. Also, planar gamma camera imaging detected binding of Tc 99m-P2045 to the Ad5.SSTR/TK.RGD-infected cells (data not shown).

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DISCUSSION

In this study, we upscaled the adenoviral vector RGDTKSSTR for a clinical trial involving patients with ovarian cancer and tested the stability of the adenoviral genome. PCR analysis showed that genome was unstable, and recombination events occurred in the homologous regions of the expression cassettes. We reconstructed the vector by replacing the duplicate promoters and polyA regions with nonhomologous ones. We did not detect any recombinant events, and both hSSTR2 and HSVtk were functional in the new Ad5.SSTR/TK.RGD construct. Thus, we achieved independent expression of two transgenes and genetic stability in the dual-expression cassette region of a single adenoviral vector with different regulatory sequences, allowing the use of this new vector in human clinical trials.

Investigators have developed a number of approaches to link the expression of two genes in a single adenoviral vector. One approach that has been employed by several groups is based on the independent expression of two transgenes from two CMV promoters (11). In the present study, we found that the adenoviral genome carrying the double-expression cassette was unstable and that one of the cassettes was deleted by homologous recombination during vector upscaling. This finding led us to conclude that even relatively small repeated sequences (about 150 bp) in the viral genome can participate in recombination and induce deletion of the sequence located between the repetitive elements.

Researchers observed a similar phenomenon when two CMV promoters were located in two different vectors (helper-dependent and helper vectors) (19). The recombination occurred when the CMV promoters were positioned close to one end of the vector and the promoters from both vectors were oriented in the same direction, resulting in the appearance of and dominating new vector species. The emergence of replication-competent adenoviruses during large-scale

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production of replication-deficient adenoviral vectors is another example of homologous recombination, which in this case occurs between vector DNA and a homologous sequence in the genome of E1-complementing HEK 293 cells (20). Consequently, to create a stable genome, one should avoid homologous sequences within one adenoviral vector or between the vector and the cell genome as well as co-replicating adenoviral vectors. If these sequences are located too close to each other within one genome, adenoviral genomes with a deletion should be properly packed into adenoviral particles so that the final viral population will be heterogeneous.

The combined delivery of two transgenes is important for a number of biomedical applications. Use of cooperatively interacting immunomodulatory genes or the combination of an immunomodulatory gene and a tumor antigen is a promising approach to immunotherapy for cancer in vivo (21). Also, linkage of angiogenic, preapoptotic, and chemotherapeutic genes with reporter gene expression may enable monitoring of the magnitude, location, and duration of therapeutic gene expression with the use of indirect imaging in vivo. Our finding that an adenoviral vector with a dual-expression cassette has a stable genome and functional transgenes when they are placed under the control of different regulatory sequences can be used for generation of adenoviral vectors for any of these applications.

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REFERENCES

- Ahmed N, Riley C, Rice G and Quinn M (2005) Role of Integrin Receptors for Fibronectin, Collagen and Laminin in the Regulation of Ovarian Carcinoma Functions in Response to a Matrix Microenvironment. *Clin Exp Metastasis* **22**:391-402.
- Anton M, Wittermann C, Haubner R, Simoes M, Reder S, Essien B, Wagner B, Henke J, Erhardt W, Noll S, Hackett NR, Crystal RG, Schwaiger M, Gansbacher B and Bengel FM (2004) Coexpression of herpesviral thymidine kinase reporter gene and VEGF gene for noninvasive monitoring of therapeutic gene transfer: an in vitro evaluation. *J Nucl Med* **45**:1743-1746.
- Buchsbaum DJ (2004) Imaging and therapy of tumors induced to express somatostatin receptor by gene transfer using radiolabeled peptides and single chain antibody constructs. *Semin Nucl Med* **34**:32-46.
- Caruso M, Pham-Nguyen K, Kwong YL, Xu B, Kosai KI, Finegold M, Woo SL and Chen SH (1996) Adenovirus-mediated interleukin-12 gene therapy for metastatic colon carcinoma. *Proc Natl Acad Sci U S A* **93**:11302-11306.
- Cooper CR, Chay CH and Pienta KJ (2002) The role of alpha(v)beta(3) in prostate cancer progression. *Neoplasia (New York, N.Y.)* **4**(3):191-194.
- Danen EH (2005) Integrins: regulators of tissue function and cancer progression. *Current pharmaceutical design*. **11**(7).
- Fillat C, Carrio M, Cascante A and Sangro B (2003) Suicide gene therapy mediated by the Herpes Simplex virus thymidine kinase gene/Ganciclovir system: fifteen years of application. *Curr Gene Ther* **3**:13-26.

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Gaken J, Jiang J, Daniel K, van Berkel E, Hughes C, Kuiper M, Darling D, Tavassoli M, Galea-Lauri J, Ford K, Kemeny M, Russell S and Farzaneh F (2000) Fusogene vectors: a novel strategy for the expression of multiple genes from a single cistron. *Gene Ther* **7**:1979-1985.

Goldberg I, Davidson B, Reich R, Gotlieb WH, Ben-Baruch G, Bryne M, Berner A, Nesland JM, Kopolovic J and Department of Pathology SMCT-HI (2001) Alphav integrin expression is a novel marker of poor prognosis in advanced-stage ovarian carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research*. **7(12)**:4073-4079.

Hall SJ, Canfield SE, Yan Y, Hassen W, Selleck WA and Chen SH (2002) A novel bystander effect involving tumor cell-derived Fas and FasL interactions following Ad.HSV-tk and Ad.mIL-12 gene therapies in experimental prostate cancer. *Gene Ther* **9**:511-517.

Hamstra DA, Lee KC, Tychewicz JM, Schepkin VD, Moffat BA, Chen M, Dornfeld KJ, Lawrence TS, Chenevert TL, Ross BD, Gelovani JT and Rehemtulla A (2004) The use of 19F spectroscopy and diffusion-weighted MRI to evaluate differences in gene-dependent enzyme prodrug therapies. *Mol Ther* **10**:916-928.

Havenga MJ, Vogels R, Bout A and Mehtali M (2002) Pseudotyping of adenoviral vectors. *Vector Targeting for Therapeutic Gene Delivery* **edited by Curiel D.T. and Douglas J.T.**:89-121.

Hemmi S, Geertsens R, Mezzacasa A, Peter I and Dummer R (1998) The presence of human coxsackievirus and adenovirus receptor is associated with efficient adenovirus-mediated transgene expression in human melanoma cell cultures. *Hum Gene Ther* **9**:2363-2373.

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- Hemminki A, Belousova N, Zinn KR, Liu B, Wang M, Chaudhuri TR, Rogers BE, Buchsbaum DJ, Siegal GP, Barnes MN, Gomez-Navarro J, Curiel DT and Alvarez RD (2001) An adenovirus with enhanced infectivity mediates molecular chemotherapy of ovarian cancer cells and allows imaging of gene expression. *Mol Ther* **4**:223-231.
- Hemminki A, Zinn KR, Liu B, Chaudhuri TR, Desmond RA, Rogers BE, Barnes MN, Alvarez RD and Curiel DT (2002) In vivo molecular chemotherapy and noninvasive imaging with an infectivity-enhanced adenovirus. *J Natl Cancer Inst* **94**:741-749.
- Isayeva T, Kotova O, Krasnykh V and Kotov A (2003) Advanced Methods of Adenovirus Vector Production for Human Gene Therapy: Roller Bottles, Microcarriers, and Hollow Fibers. *BioProcessing Journal* **2**:75-81.
- Kawakami Y (2000) New cancer therapy by immunomanipulation: development of immunotherapy for human melanoma as a model system. *Cornea*. **19(3) Suppl**:S2-6.
- Krasnykh VN, Douglas JT and van Beusechem VW (2000) Genetic targeting of adenoviral vectors. *Mol Ther* **1**:391-405.
- Kwong YL, Chen SH, Kosai K, Finegold M and Woo SL (1997) Combination therapy with suicide and cytokine genes for hepatic metastases of lung cancer. *Chest* **112**:1332-1337.
- Li Y, Pong RC, Bergelson JM, Hall MC, Sagalowsky AI, Tseng CP, Wang Z and Hsieh JT (1999) Loss of adenoviral receptor expression in human bladder cancer cells: a potential impact on the efficacy of gene therapy. *Cancer Res* **59**:325-330.
- Liang Q, Gotts J, Satyamurthy N, Barrio J, Phelps ME, Gambhir SS and Herschman HR (2002) Noninvasive, repetitive, quantitative measurement of gene expression from a bicistronic message by positron emission tomography, following gene transfer with adenovirus. *Mol Ther* **6**:73-82.

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- Okegawa T, Li Y, Pong RC, Bergelson JM, Zhou J and Hsieh JT (2000) The dual impact of coxsackie and adenovirus receptor expression on human prostate cancer gene therapy. *Cancer Res* **60**:5031-5036.
- Okegawa T, Pong RC, Li Y, Bergelson JM, Sagalowsky AI and Hsieh JT (2001) The mechanism of the growth-inhibitory effect of coxsackie and adenovirus receptor (CAR) on human bladder cancer: a functional analysis of car protein structure. *Cancer Res* **61**:6592-6000.
- Putzer BM, Hitt M, Muller WJ, Emtage P, Gauldie J and Graham FL (1997) Interleukin 12 and B7-1 costimulatory molecule expressed by an adenovirus vector act synergistically to facilitate tumor regression. *Proc Natl Acad Sci U S A* **94**:10889-10894.
- Ray P, De A, Min JJ, Tsien RY and Gambhir SS (2004) Imaging tri-fusion multimodality reporter gene expression in living subjects. *Cancer Res* **64**:1323-1330.
- Rots MG, Curiel DT, Gerritsen WR and Haisma HJ (2003) Targeted cancer gene therapy: the flexibility of adenoviral gene therapy vectors. *J Control Release* **87(1-3)**:159-165.
- Sandig V, Youil R, Bett AJ, Franlin LL, Oshima M, Maione D, Wang F, Metzker ML, Savino R and Caskey CT (2000) Optimization of the helper-dependent adenovirus system for production and potency in vivo. *Proc Natl Acad Sci U S A* **97**:1002-1007.
- Sinkovics JG, Horvath JC and Cancer Institute SJsHTFLUSA (2000) Vaccination against human cancers (review). *International journal of oncology*. **16(1)**:81-96.
- Tjuvajev JG, Joshi A, Callegari J, Lindsley L, Joshi R, Balatoni J, Finn R, Larson SM, Sadelain M and Blasberg RG (1999) A general approach to the non-invasive imaging of transgenes using cis-linked herpes simplex virus thymidine kinase. *Neoplasia* **1**:315-320.

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- Wang Y, Iyer M, Annala AJ, Chappell S, Mauro V and Gambhir SS (2005) Noninvasive monitoring of target gene expression by imaging reporter gene expression in living animals using improved bicistronic vectors. *J Nucl Med* **46**:667-674.
- Wu JC, Chen IY, Wang Y, Tseng JR, Chhabra A, Salek M, Min JJ, Fishbein MC, Crystal R and Gambhir SS (2004) Molecular imaging of the kinetics of vascular endothelial growth factor gene expression in ischemic myocardium. *Circulation* **110**:685-691.
- Yaghoubi SS, Wu L, Liang Q, Toyokuni T, Barrio JR, Namavari M, Satyamurthy N, Phelps ME, Herschman HR and Gambhir SS (2001) Direct correlation between positron emission tomographic images of two reporter genes delivered by two distinct adenoviral vectors. *Gene Ther* **8**:1072-10780.
- Yu Y, Annala AJ, Barrio JR, Toyokuni T, Satyamurthy N, Namavari M, Cherry SR, Phelps ME, Herschman HR and Gambhir SS (2000) Quantification of target gene expression by imaging reporter gene expression in living animals. *Nat Med* **6**:933-937.
- Zeng G (1998) Sticky-end PCR: new method for subcloning. *Biotechniques* **25**:206-208.
- Zhu J, Grace M, Casale J, Chang AT, Musco ML, Bordens R, Greenberg R, Schaefer E and Indelicato SR (1999) Characterization of replication-competent adenovirus isolates from large-scale production of a recombinant adenoviral vector. *Hum Gene Ther* **10**:113-121.
- Zinn KR, Chaudhuri TR, Buchsbaum DJ, Mountz JM and Rogers BE (2001) Simultaneous evaluation of dual gene transfer to adherent cells by gamma-ray imaging. *Nucl Med Biol* **28**:135-144.

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FIGURE LEGENDS

Figure 1. Analysis of the RGDTKSSTR genome structure. (A) Schema of the RGDTKSSTR genome organization. The E1 and E3 regions were deleted and replaced with two expression cassettes: an “imaging” cassette encoding the hSSTR2 receptor and a “suicide” cassette encoding HSVtk. Both cassettes included CMV promoters and SV40 polyA regions as regulatory sequences. A fiber gene was modified to incorporate an RGD-4C--encoding motif to increase the viral infectivity to CAR-negative, integrin-positive cancer cells. Localization of primers P1-P5 is indicated by the arrows. (B) PCR analysis of the CsCl-purified virions after the third round of upscaling. Pairs of primers were used for PCR: lane 1, P1 and P2; lane 2, P3 and P4; lane 3, P1 and P6; lane 4, P2 and P5. The arrows point to truncated PCR products. Wild-type Ad5 was used as a control.

Figure 2. Reconstruction and analysis of the double-expression cassette in the Ad5.SSTR/TK.RGD vector. (A) Schema of the double-expression cassette in Ad5.SSTR/TK.RGD. Expression of the hSSTR2 gene in the first expression cassette was placed under the control of a CMV promoter and BGHpA sequence. Expression of the HSVtk gene in the second expression cassette was regulated by an SV40 promoter, SV40 enhancer, and SV40 late polyA signal. The arrows indicate unique restriction endonucleases in pShuttle.SSTR/TK. (B) Diagnostic PCR. To imitate the conditions of RGDTKSSTR analysis, the Ad5.SSTR/TK.RGD vector was subjected to three rounds of amplification and used for PCR with the primers employed for RGDTKSSTR: lane 1, P1 and P2; lane 2, P3 and P4; lane 3, P1 and P6; lane 4, P2 and P5. Ad5Luc1 was used as a control.

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Figure 3. Analysis of transgene activity in A549 cells infected with Ad5.SSTR/TK.RGD. (A)

Validation of HSVtk activity by cytotoxicity assay. A549 cells grown in 96-well plates were infected with viruses at an MOI of 5 pfu/cell. After 4 h, GCV was added to the cells to a final concentration of 0.25, 0.50, and 0.75 mM. After 4 days, the medium was replaced with a phosphate-buffered saline buffer, and an MTS/PMS solution was added to each well. The absorbance was measured 1 h later at a wavelength of 490 nm. One hundred percent of viable cells points to absorbance in the absence of GCV. Each data point is the mean of three determinations. (B) Imaging of hSSTR2. A549 cells grown in 96-well plates were infected with viruses at an MOI of 5 pfu/cell. After 96 h, cold P2045 peptide as a competitor for blocking hot P2045 peptide to bind to expressed hSSTR or an internalization medium was added to the plates. Five minutes later, labeled Tc 99m-P2045 (11.5 nM) was applied. Five minutes after that, the control wells were washed and subjected to the first round of imaging. After 2 h, all cells were washed, lysed with 1 M NaOH, and imaged. The data were normalized on protein concentrations measured in the samples by Lowry assay.

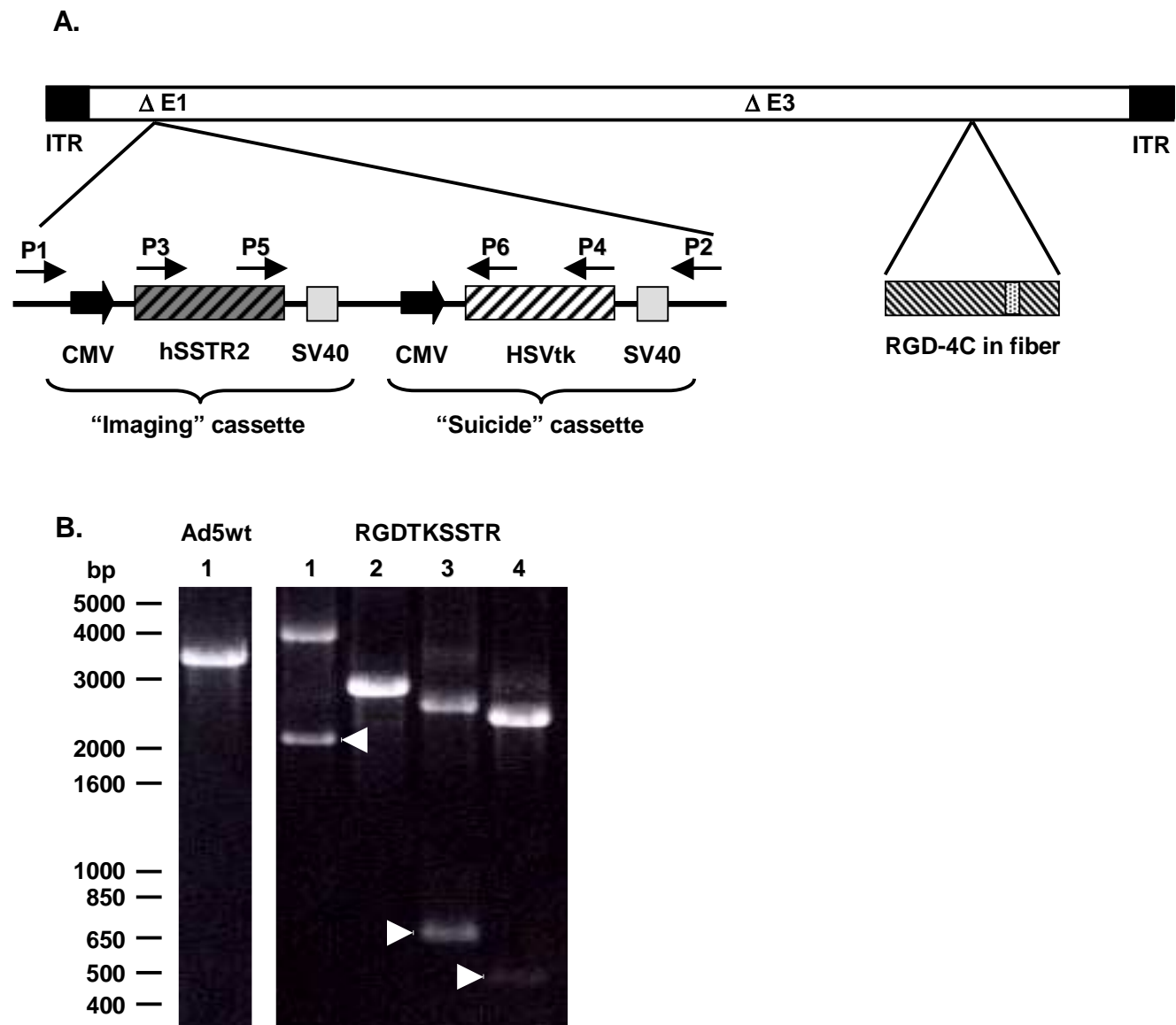


Figure 1

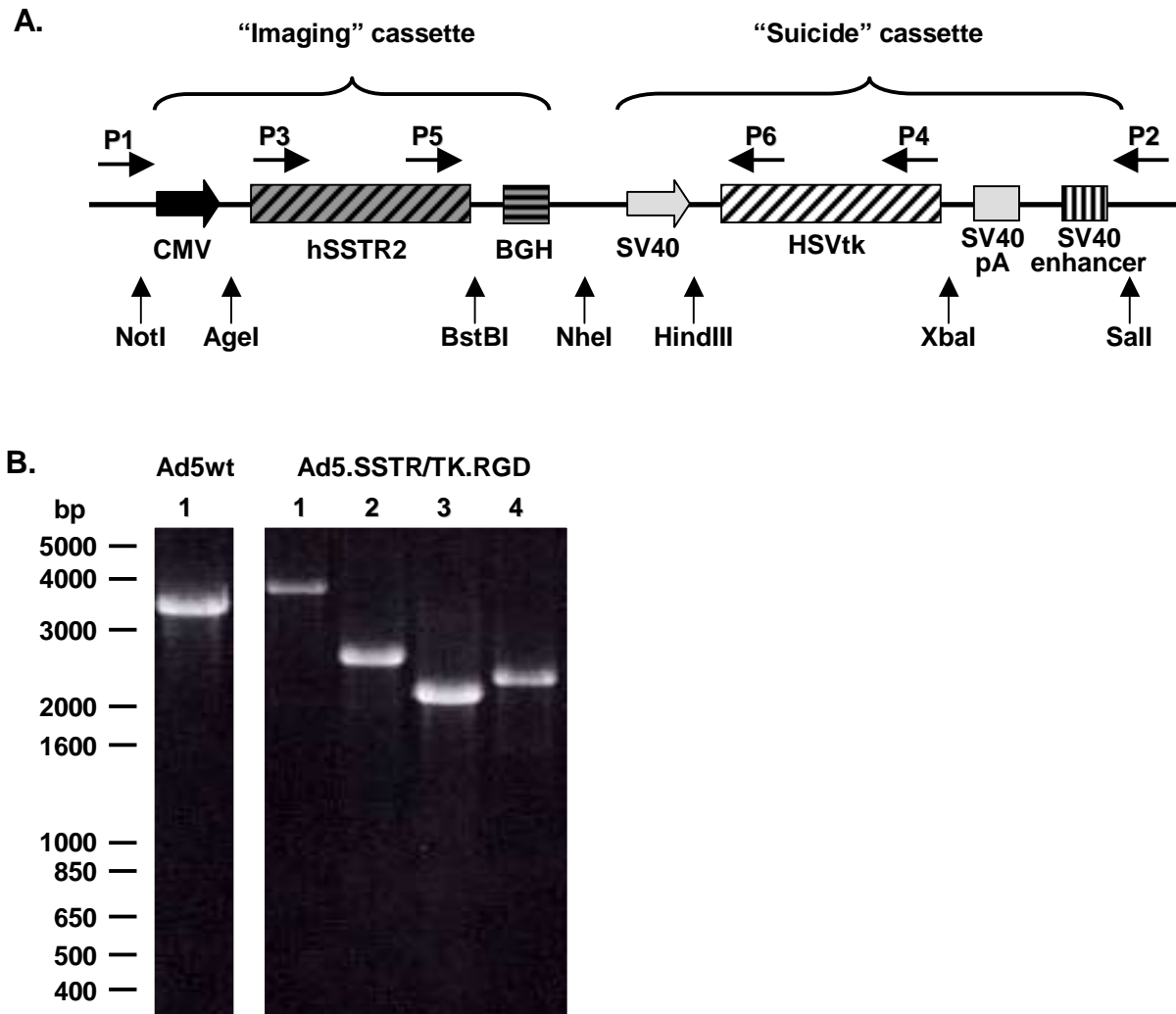


Figure 2

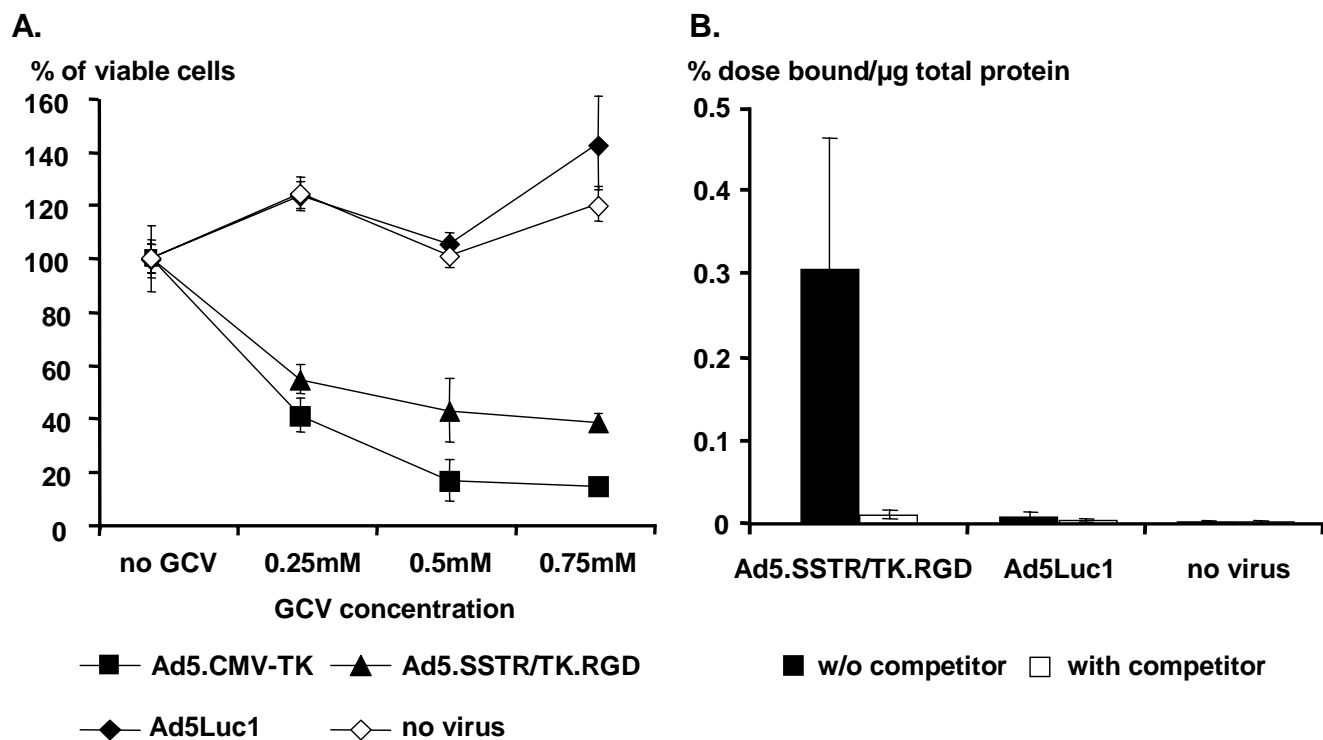


Figure 3