

AD \_\_\_\_\_

Award Number: DAMD17-03-1-0094

TITLE: Integrating Molecular Imaging Approaches to Monitor  
Prostate Targeted Suicide and Anti-Angiogenic Gene  
Therapy

PRINCIPAL INVESTIGATOR: Makoto Sato, Ph.D.

CONTRACTING ORGANIZATION: University of California, Los Angeles  
Los Angeles, California 90024-1406

REPORT DATE: February 2004

TYPE OF REPORT: Annual Summary

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.

20040706 037

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

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 Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY</b> (Leave blank)		<b>2. REPORT DATE</b> February 2004	<b>3. REPORT TYPE AND DATES COVERED</b> Annual Summary (1 Feb 2003 - 31 Jan 2004)	
<b>4. TITLE AND SUBTITLE</b> Integrating Molecular Imaging Approaches to Monitor Prostate Targeted Suicide and Anti-Angiogenic Gene Therapy			<b>5. FUNDING NUMBERS</b> DAMD17-03-1-0094	
<b>6. AUTHOR(S)</b> Makoto Sato, Ph.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> University of California, Los Angeles Los Angeles, California 90024-1406  E-Mail: makotos@ucla.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b> Original contains color plates: All DTIC reproductions will be in black and white.				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited			<b>12b. DISTRIBUTION CODE</b>	
<b>13. ABSTRACT (Maximum 200 Words)</b> Our goal of this awarded study is to develop combined approach of prostate-targeted suicide and anti-angiogenic gene therapy into a new effective treatment for prostate cancer (CaP). To accomplish these two distinct approaches, three recombinant adenoviruses, AdTSTA-tk, AdCMV-TSP-1 and AdCMV-METH-1 have been propagated. The binary TSTA system in AdTSTA-tk is composed of the GAL4-VP16 potent synthetic activator regulated by a modified prostate-specific antigen (PSA) promoter/enhancer, and a GAL4 responsive promoter driven tk gene. As expected from results by the combination of AdTSTA-FL (firefly luciferase) and bioluminescence imaging. AdTSTA-tk showed a promising result as a PET reporter as well as a therapeutic gene. In vitro infection study to CaP cell lines exhibited that AdTSTA-tk expresses as robust as AdCMV-tk, but it is androgen regulated. Intratumoral injection of AdTSTA-tk or AdCMV-tk to human prostate cancer xenografted mice gave us a positive signal from tumor only or tumor and liver, respectively. In a pilot suicide gene therapy study, AdTSTA-tk exhibited extensive tumor cell killing and superior liver toxicity over AdCMV-tk. On the other hand, dose-and time-dependent expression of TSP-1 and METH-1 were confirmed by western analysis of cultured supernatant and cell lysate of cells infected with AdCMV-TSP-1 or AdCMV-METH-1.				
<b>14. SUBJECT TERMS</b> No Subject Terms Provided.			<b>15. NUMBER OF PAGES</b> 37	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

**Table of Contents**

**Cover..... 1**

**SF 298..... 2**

**Introduction..... 4**

**Body.....4-6**

**Key Research Accomplishments..... 7**

**Reportable Outcomes..... 7**

**Conclusions..... 7**

**References..... 8**

## Introduction

The scope of this project is to develop combined approach of selective suicide and anti-angiogenic gene therapy into a new effective targeted treatment for prostate cancer (CaP). We propose to incorporate a highly potent and specific two-step transcriptional amplification (TSTA) system to mediate prostate-targeted thymidine kinase (tk) gene expression in our vectors. In diagnostic applications, this targeted vector will be utilized as a PET reporter gene. The hypotheses are; 1) this PET imaging reporter virus could be useful to illuminate prostate metastatic cells in living animals, 2) this selective tk virus could also be efficient therapeutic vector in suicide gene therapy approach, and 3) combinatorial approach of this suicide gene therapy and anti-angiogenesis could be more efficient to eradicate CaP.

## Body

### Task 1a. Amplify and propagate AdTSTA-tk up to large scale.

The recombinant adenovirus (Ad), AdTSTA-tk was constructed by AdEasy method (1), and propagated successfully up to large scale using suspension culture system of human embryonic kidney cell line 293 N3S. Average titer of two large scale preparations was about  $1 \times 10^{10}$  pfu per ml. We may need more optimization of virus growing condition so that we will be able to inject higher dose in a smaller volume in *in vivo* experiments.

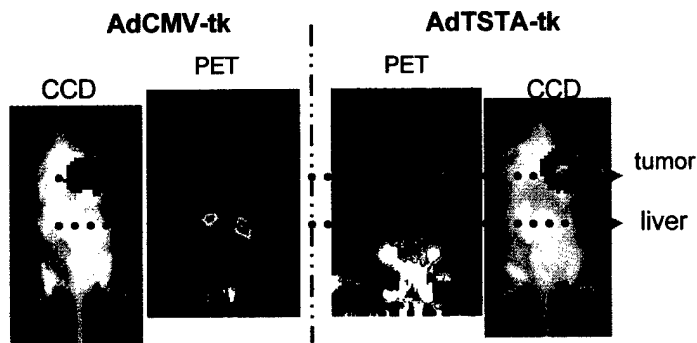
### Task 1b. Perform both imaging for naïve animals.

### Task 1c. Perform both imaging for tumor bearing mice.

Findings relevant to these tasks were reported in following manuscript (2).

1. Sato M, Johnson M, Zhang L, Zhang B, Le K, Gambhir SS, Carey M and Wu L. Optimization of Adenoviral Vectors to Direct Highly Amplified Prostate-Specific Gene Expression for Imaging and Gene Therapy. 2003, Molecular Therapy, 8: 726-737. This article reported that the potency of the prostate-specific TSTA adenoviral vector exceeds the strong constitutive CMV driven vector. However, significant androgen responsive and prostate-specific activity of this vector is retained. This study further supports the application of this transcriptional-targeted gene expression imaging approach to diagnostic imaging.

In our pilot study of microPET imaging to LAPC-4 (3), human prostate tumor-bearing mice with intratumoral injection of AdTSTA-tk or AdCMV-tk, signals were similar to those from optical

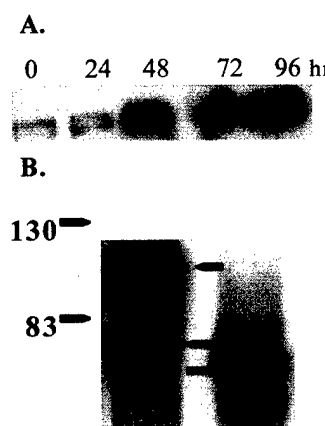


**Figure 1. PET imaging mediated by tk gene.**  $10^8$  infectious units of Ad expressing variant HSV tk (sr39tk) gene controlled by CMV promoter or prostate specific TSTA system was injected into LAPC-4 tumors. The tumor cells have been transduced with a lentivirus expressing optical reporter renilla luciferase gene. Mice were imaged 12 days post viral injection.

imaging with AdTSTA-FL and AdCMV-FL (Figure 1). AdCMV-tk administration resulted in tk expression in the liver as well as the tumor, while AdTSTA-tk mediated expression was more restricted to the tumor. We have frequently observed the dissemination of AdCMV-FL into systemic circulation after intratumoral injection, resulting in prominent liver expression (4). The hepatobiliary and renal excretion route of the tracer  $^{18}\text{F}$ -FHBG leads to high intensity PET signal in the lower abdomen.

**Task 2a. Evaluate anti-angiogenic effect of Ad with anti-angiogenic factors.**

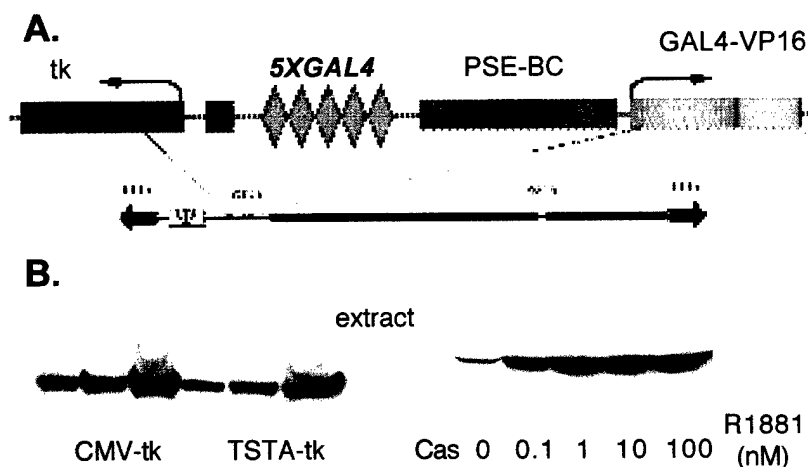
Two different potent anti-angiogenic factors TSP-1 (5) and METH-1 (6) were incorporated into Ad as same way as Task 1a, both driven by constitutive active CMV promoter. Each virus was propagated up to large scale. In preliminary data, we confirmed that both TSP-1 and METH-1 proteins are robustly expressed from the cell lines infected with the virus and secreted in the culture media (Figure 2A, B). Functional analyses of these anti-angiogenic factors are currently underway.



**Figure 2. Vector mediated expression of anti-angiogenic factors.** A. Culture media from infected LNCaP prostate cancer cells (5 infectious AdCMV-TSP1/cell) were harvested at specified time, concentrated and blot with anti-TSP1 antibody. B. A549 lung cells infected with AdCMV-METH1 were harvested at 48 hr. Western blot of cell pellet (P) or supernatant (S) were shown. The unprocessed 110kd and the proteolytic cleaved 87kd and 65kd are the major METH1 products detected.

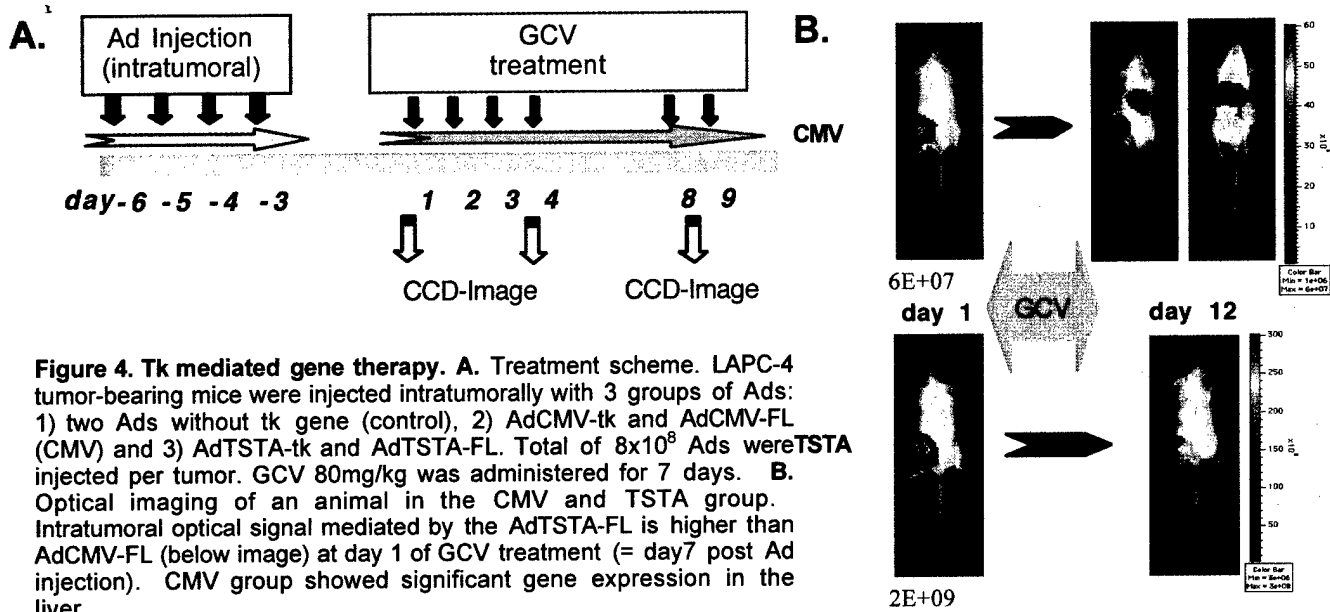
**Task 2b. Evaluate cytotoxic effect of AdTSTA-tk-mediated suicide gene therapy.**

The prostate-specific TSTA expression system was applied in herpes simplex virus thymidine kinase (tk) gene-mediated suicide gene therapy. The tk enzyme mediates the first phosphorylation step of prodrug ganciclovir (GCV), which is further modified to the toxic triphosphate form that causes DNA chain termination and apoptosis. The tk gene utilized in our study is an active site variant, SR39tk, which was engineered to bind prodrug acyclovir (ACV) and GCV with increased affinity to improve therapeutic efficacy. The magnitude of AdTSTA-tk (Figure 3A) mediated tk expression is similar to AdCMV-tk, but it is androgen-regulated (Figure 3B). A pilot gene therapy study comparing the therapeutic effects of AdCMV-tk and AdTSTA-tk, using the LAPC-4 human prostate cancer xenografts was conducted (Figure 4A). The CMV- or TSTA-FL-expressing vector was co-injected with the corresponding promoter driven tk vector to allow facile monitoring of transgene expression *in vivo*. Optical imaging of FL expression during treatment indicated significant dissemination of the Ad into systemic circulation, resulting prominent hepatic transgene

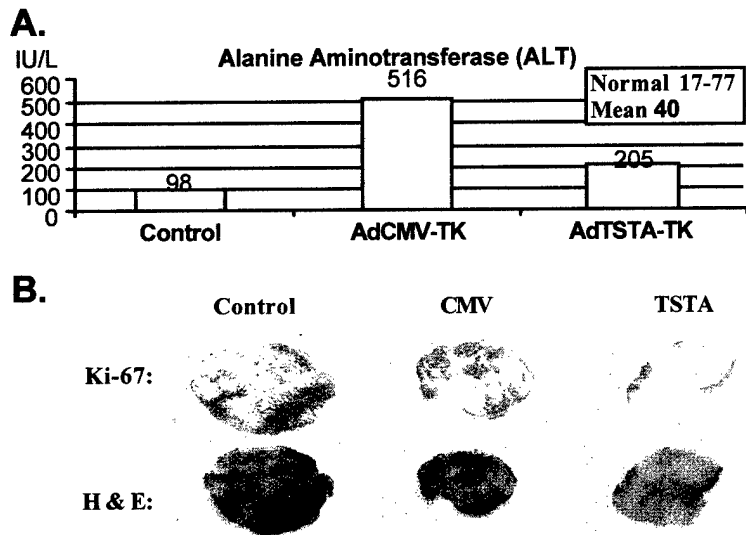


**Figure 3. Tk expression mediated by AdTSTA-tk.** A. The genomic structure of AdTSTA-tk. The transgene is inserted into the E1 region of the first generation Ad. B. The protein expression was evaluated by western blot of LNCaP cells infected with AdCMV-tk or AdTSTA-tk at 1 infectious unit per cell. Synthetic androgen R1881 at varying concentration or anti-androgen Casodex (10µM) was added.

expression in the AdCMV group (Figure 4B). In contrast, the prostate-specificity of the TSTA Ads restricted expression in non-targeted organs such as liver. From the imaging results, one could predict that the animals in the CMV group might sustain hepatic cytotoxic injury after GCV administration. In fact, hepatocellular damage indicated by the elevated serum liver transaminase (ALT) (Figure 5A), histology and TUNEL staining (data not shown) was noted in the CMV group.



Tumor histology 12 days after GCV treatment revealed substantial tumor cell killing in both treatment groups. A small rim of Ki67 positive proliferating cells remained in the tumor periphery in the TSTA group (Figure 5B). The residual living tumor cells could be due to limited gene transduction by the intratumoral vector injection method. However, these results still suggest that the amplified prostate-specific TSTA gene expression system exhibits improved safety and equivalent or better efficacy compared to constitutive CMV promoter driven Ad.



## **Key Research Accomplishments**

- Adenoviral vectors carrying TSTA-tk, CMV-TSP-1 and CMV-METH-1 are constructed and prepared as high titer stocks.
- AdTSTA-tk as a microPET imaging vector exhibits similar signal with AdTSTA-FL in optical bioluminescence imaging, no liver signal from the AdTSTA-tk injected animal.
- Adenoviral vectors created for anti-angiogenesis therapy successfully express their transgenes TSP-1 and METH-1 in culture supernatant from the infected cells.
- AdTSTA-tk exhibits similar level expression of AdCMV-tk while maintaining androgen regulation.
- Pilot suicide gene therapy employing AdTSTA-tk showed superior liver toxicity and comparable or higher tumor cell killing over AdCMV-tk.

## **Reportable Outcomes**

Manuscripts: (Enclosed in appendix)

- 1) Sato M, Johnson M, Zhang L, Zhang B, Le K, Gambhir SS, Carey M and Wu L. Optimization of Adenoviral Vectors to Direct Highly Amplified Prostate-Specific Gene Expression for Imaging and Gene Therapy. 2003, *Molecular Therapy*, 8: 726-737.
- 2) Wu L, and Sato M. Integrated, Molecular Engineering Approaches to Develop Prostate Cancer Gene Therapy. 2003, *Current Gene Therapy*, 3: 452-467.

Abstracts:

- 1) Sato M, Johnson M, Zhang L, Zhang B, Carey M, Gambhir SS and Wu L. In Vivo Regulation And Specificity Of Adenoviral Vectors With Two-Step Transcriptional Amplification System For Prostate Cancer Gene Therapy. AACR annual meeting 7/2003.
- 2) Sato M\*, Johnson M\*, Zhang B, Carey M, Gambhir SS and Wu L. Non-Invasive Imaging Of A Highly Amplified Prostate-Targeted (TSTA) Gene Expression System In Suicide Cancer Gene Therapy. AACR annual meeting 7/2003. (\*=equally contributed)
- 3) Sato M, Johnson M, Zhang L, Zhang B, Carey M and Wu L. Prostate-specific and titratable transgene expression from adenovirus with two-step transcriptional amplification system. ASGT 6/2003.
- 4) Sato M, Johnson M, Gambhir SS, Carey M and Wu L. TSTA mediated multi-modality gene-expression imaging in hormone refractory prostate cancer. ASGT 6/2004.

## **Conclusions**

We have had all viral constructs propagated and analyzed their transgene expression. We confirmed the TSTA system as a useful tool for microPET imaging, as well as an efficient suicide gene therapy vector in human prostate tumor xenografted mice model. Anti-angiogenesis Ads in addition to AdTSTA-tk will be excellent tool to facilitate efficient and safe combinatorial therapy of suicide gene and anti-angiogenesis gene therapy.

## References

1. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. *A simplified system for generating recombinant adenoviruses*. Proc Natl Acad Sci U S A. 1998, 95(5): 2509-2514.
2. Sato M, Johnson M, Zhang L, Zhang B, Le K, Gambhir SS, Carey M, Wu L. *Optimization of adenoviral vectors to direct highly amplified prostate-specific expression for imaging and gene therapy*. Mol Ther. 2003, 8(5): 726-737.
3. Klein KA, Reiter RE, Redula J, Moradi H, Zhu XL, Brothman AR, Lamb DJ, Marcelli M, Belldegrun A, Witte ON, Sawyers CL. *Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice*. Nat Med. 1997, 3(4): 402-408.
4. Adams JY, Sato M, Johnson M, Berger F, Gambhir SS, Carey M, Iruela-Arispe ML, Wu L. *Visualization of Advanced Human Prostate Cancer Lesions in Living Mice by a Targeted Gene Transfer Vector and Optical Imaging*. Nat Med, 2002, 8: 891-897.
5. Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, Bouck NP. *A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin*. Proc Natl Acad Sci U S A. 1990, 7(17): 6624-6628.
6. Vazquez F, Hastings G, Ortega MA, Lane TF, Oikemus S, Lombardo M, Iruela-Arispe ML. *METH-1, a human ortholog of ADAMTS-1, and METH-2 are members of a new family of proteins with angiogenic-inhibitory activity*. J Biol Chem. 1999, 274(33): 23349-23357.

# Optimization of Adenoviral Vectors to Direct Highly Amplified Prostate-Specific Expression for Imaging and Gene Therapy

Makoto Sato,<sup>1</sup> Mai Johnson,<sup>1</sup> Liqun Zhang,<sup>2</sup> Baohui Zhang,<sup>1</sup> Kim Le,<sup>2</sup> Sanjiv S. Gambhir,<sup>3\*</sup> Michael Carey,<sup>2,3</sup> and Lily Wu<sup>1,3,†</sup>

<sup>1</sup>Department of Urology, <sup>3</sup>Crump Institute of Molecular Imaging and Department of Molecular and Medical Pharmacology, and <sup>2</sup>Department of Biological Chemistry, University of California at Los Angeles School of Medicine, Los Angeles, California 90095

\*Present address: Department of Radiology and Bio-X Program, Stanford University, Stanford, CA.

†To whom correspondence and reprint requests should be addressed. Fax: (310) 206-5343. E-mail: LWu@mednet.ucla.edu.

Gene expression-based imaging coupled to gene therapy will permit the prediction of therapeutic outcome. A significant challenge for successful gene therapy is to achieve a high-level of specific gene expression; however, tissue-specific promoters are weak. We postulate that if the weak activity of tissue-specific promoters can be amplified to the levels of strong viral promoters, which have been successful in preclinical scenarios, while retaining specificity, the therapeutic index of gene therapy can be greatly augmented. With this in mind, we developed a two-step transcriptional activation (TSTA) system. In this two-tiered system, a modified prostate-specific antigen promoter was employed to drive a potent synthetic transcriptional activator, GAL4-VP2. This, in turn, activated the expression of a GAL4-dependent reporter or therapeutic gene. Here we demonstrate that recombinant adenoviral vectors (Ads) in which we have incorporated prostate-targeted TSTA expression cassettes retain cell specificity and androgen responsiveness in cell culture and in animal models, as measured by noninvasive optical bioluminescence imaging. We investigated the mechanism of TSTA in different adenoviral configurations. In one configuration, both the activator and the reporter components are inserted into a single Ad (AdTSTA-FL). The activity of AdTSTA-FL exceeds that of a cytomegalovirus promoter-driven vector (AdCMV-FL), while maintaining tissue specificity. When the activator and reporter components are placed in two separate Ads, androgen induction is more robust than for the single AdTSTA-FL. Based on these findings, we hope to refine the TSTA Ads further to improve the efficacy and safety of prostate cancer gene therapy.

**Key Words:** prostate-specific expression, two-tiered amplification, androgen regulation, adenoviral vector, optical imaging

## INTRODUCTION

Metastatic and recurrent hormonal refractory prostate cancer (HRPC) account for an estimated loss of one life every 17 minutes in the United States [1]. Androgen ablation is the main treatment for advanced disease and can induce an initial remission and achieve symptomatic improvement in 80–90% of patients [2–4]. However, progression to HRPC is inevitable even in the absence of circulating androgen. Currently, there is no effective treatment for HRPC, and median survival is approximately 12 months. Gene-based therapy is a promising possibility for HRPC [5,6]. However, an important prereq-

uisite for developing a safe and effective therapy is to achieve high levels of prostate-specific gene expression *in vivo* [6].

The use of tissue-specific promoters to express transgenes is an attractive approach that is particularly suitable for prostatic tissue, because it is one of the organs other than the pancreas and breast that expresses an unusually high number of unique genes. A survey of the Cancer Genome Anatomy Project database published by the National Cancer Institute (<http://www.ncbi.nlm.nih.gov/ncicgap/>) lists more than 2000 prostate-specific genes, although the majority of them are not fully characterized at this time. Many prostate-specific genes, including pros-

tate-specific antigen (PSA) and prostate-specific membrane antigen, are well characterized [7–12]. The PSA gene is regulated by testosterone (T) and dihydrotestosterone (DHT), which bind androgen receptor (AR). The ligand-bound AR binds directly to sites within the PSA promoter and enhancer, thus activating PSA gene expression [7,10,11]. Clinical findings indicate that AR and PSA are expressed in all stages of prostate cancer and in distant metastases, even after androgen-deprivation therapy [13–16]. Currently, serum PSA measurements remain the most reliable means to detect recurrent HRPc [17]. Numerous studies support the likelihood that the AR pathway is still functioning in HRPc at castrated levels of DHT and T. Several mechanisms have been proposed to facilitate AR function under androgen-deprived conditions [6,18], such as AR overexpression [19]; increased expression of the nuclear receptor transcriptional coactivators, SRC-1 and SRC-2 [20]; AR mutations that confer expanded ligand specificity [21]; or cross talk between other signaling cascades and AR pathways [22].

We were interested in generating systems for delivery of therapeutic and imaging genes to prostate cancer. We designed our systems around the PSA promoter because of its ability to function in early androgen-dependent prostate cancer and in advanced-stage HRPc [23] and metastasis. Although the native PSA regulatory elements confer tissue selectivity, their activity is too weak to mediate efficient vector-based gene expression *in vivo* [6]. Therefore, we have undertaken two strategies to augment the activity of the PSA promoter/enhancer, while maintaining its specificity. First, the upstream enhancer core of PSA was duplicated in a construct designated PSE-BC, which achieved 20-fold enhancement of activity compared to the native PSA enhancer and promoter construct [24,25]. An adenoviral vector (Ad) bearing this PSE-BC promoter-driven firefly luciferase (FL) gene was able to achieve targeted expression in distant metastatic prostate cancer cells in living mice [26]. In a second approach, we employed a two-step transcriptional amplification (TSTA) system both to elevate and to modulate the activity of the PSA enhancer/promoter over a 1000-fold range [27,28]. In this two-tiered system illustrated in Fig. 1a, the PSA regulatory region was employed to express the potent synthetic transcription activator, GAL4-VP2, which in turn activates a GAL4-responsive reporter. In tissue culture transfection studies, optimal TSTA constructs displayed levels of activity significantly higher than the cytomegalovirus immediate early promoter (CMV), while maintaining prostate cell specificity and ligand responsiveness [27,28].

Imaging of vector-mediated transgene expression provides a critical assessment of the *in vivo* capabilities of targeted gene transfer. Rapid advances in imaging technology have allowed repetitive monitoring of the loca-

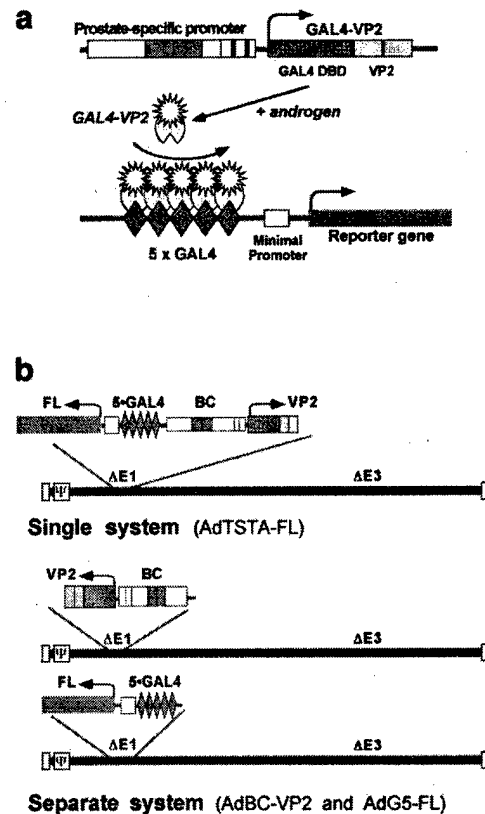


FIG. 1. Schematic representation of TSTA system. (a) Illustration of the two-step transcriptional activation process. In the first step, GAL4-VP2 activator proteins (fusion of GAL4 DNA binding domain and two copies of the VP16 transactivation domain) are expressed under the control of a prostate-specific promoter (an augmented PSA promoter, PSE-BC), which is activated by androgen. In the second step, GAL4-VP2 binds to a GAL4-responsive promoter and activates the expression of the FL reporter gene. (b) The two different TSTA configurations in Ad. In the single TSTA Ad (AdTSTA-FL), both activator and reporter are inserted into the E1 region of the same Ad in a head-to-head orientation. In the separate TSTA Ads (AdBC-VP2 and AdG5-FL), activator and reporter components are incorporated into the E1 region of two separate Ads with the transcription oriented toward the left end of the viral genome. BC is the abbreviation of the PSE-BC prostate-specific promoter [25].  $\psi$  denotes the packaging signal of adenovirus and open rectangles at both termini denote inverted terminal repeats of the viral genome.

tion, magnitude, and kinetics of reporter gene expression in small living animals [29–31]. Optical bioluminescence imaging (BLI) is particularly suitable for small animal studies, with the distinct advantage of low background signal, rapid scanning time, and low cost in comparison to radionuclide imaging. The *in vivo* expression of the popular FL reporter gene can be monitored by a highly sensitive cooled charge-coupled device (CCD) camera after the administration of the relatively nontoxic d-luciferin substrate in living animals [32,33]. Because imaging can provide real-time information on *in vivo* biological processes, the BLI technology was used to monitor estro-

gen receptor function under physiological conditions and during pharmacological intervention [34].

To assess the potential of the TSTA system in gene therapy applications, we incorporated the system into an Ad, which is an efficient *in vivo* gene delivery vehicle. The purpose of this study was to investigate the *in vivo* specificity of and the parameters necessary to achieve optimal regulation of the TSTA system in different Ad configurations. In the AdTSTA-FL construct, the activator and reporter component were inserted into the Ad in a divergently linked head-to-head configuration. Alternatively, two Ads that separately express the GAL4-responsive FL and the PSE-BC-regulated GAL4-VP2 activator were also generated. We analyzed the prostate-specific expression and androgen regulation of the separate TSTA Ads in comparison to the single AdTSTA-FL *in vitro* and *in vivo*. We found that separate Ads elicited a more robust response to androgen versus the single Ad.

## RESULTS

### Generation of Adenovirus Vectors Containing the TSTA System

The TSTA system is schematically represented in Fig. 1a. We previously determined the combination of activator and reporter plasmid TSTA constructs that achieves the highest levels of activity in prostate cancer cells using transfection studies [28]. Based on these results, we generated TSTA Ads, utilizing the bacterial recombination AdEasy methodology [35]. The activator is composed of an augmented prostate-specific PSE-BC promoter/enhancer [25] controlling the expression of the chimeric activator protein, GAL4-VP2 (the GAL4 DNA-binding domain fused to two tandem repeats of the herpes simplex virus VP16 activation domain) [28]. The reporter component consists of five repeats of the 17-bp GAL4 binding sites positioned upstream of a minimal promoter containing the adenovirus E4 gene TATA box driving FL. We inserted the activator (BC-VP2) and reporter (G5-FL) components linked in a divergent head-to-head orientation into the E1 region of the Ad, resulting in the AdTSTA-FL vector (Fig. 1b). We also constructed two Ads, designated AdBC-VP2 and AdG5-FL, which harbor the BC-VP2 activator and the G5-FL reporter, respectively (Fig. 1b).

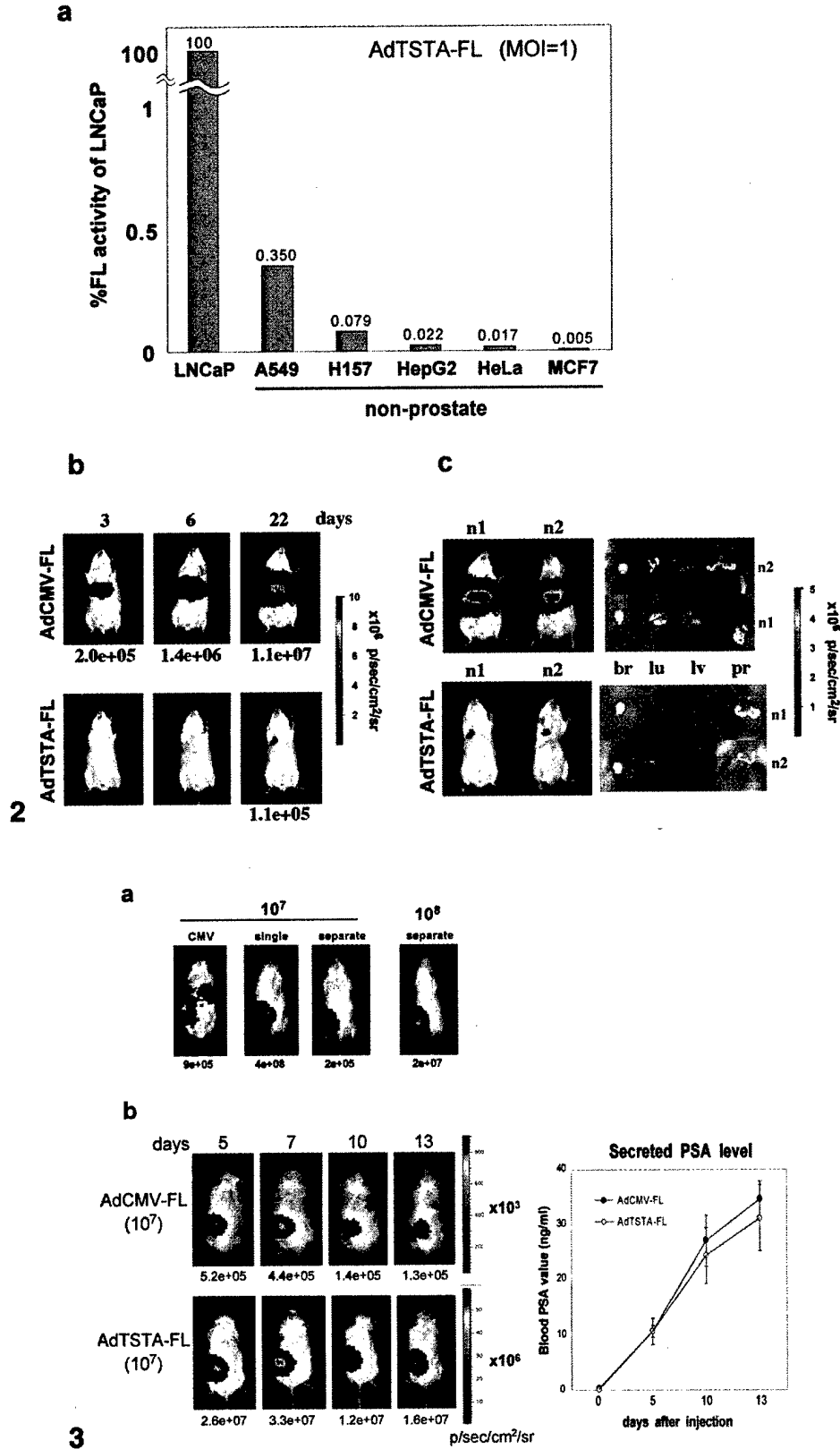
### Specificity of the TSTA Vectors

We evaluated the capability of AdTSTA-FL to direct prostate-specific expression by infecting a variety of cell lines derived from different tissues. Human serotype 5 Ad exhibits wide cell-type viral tropism. However, the susceptibility of a cell line to Ad infection is modulated by the cell surface expression of coxsackievirus and adenovirus receptor (CAR) [36,37] and  $\alpha$ v integrins [38]. Thus, a measurement of infectivity in different cell lines is needed to assess the activity of AdTSTA-FL. Initially, we determined relative infectivity of different cells by normalization to

the activity of AdCMV-FL, in which the FL expression is driven by the constitutively active CMV promoter. Our view was that similar pfu (plaque-forming unit) amounts of AdTSTA-FL and AdCMV-FL should display similar infectivity. Thus, differences in the activity of AdCMV-FL in different lines would represent a standard for normalizing infectivity of AdTSTA-FL. However, two findings alerted us to the potential inaccuracy of this measurement: (1) a 3- to 4-fold androgen induction was noted in the AdCMV-FL-infected prostate cell lines (data not shown) and (2) a greater than 30-fold difference in activity was observed between the most and the least active cell lines infected with equivalent doses of AdCMV-FL (data not shown). Discrepancy between luciferase activity and physical viral DNA measurement mediated by AdCMV-luc in different cell types has been reported [39]. An ideal assay to measure infectivity is not available.

In this study we elected to use the viral DNA uptake in the cells as a measurement of infectivity. We harvested the internalized viral DNA from infected cells and quantified the FL DNA by real-time PCR. We determined the infectivities of LNCaP and LAPC-4 (prostate carcinoma), H157 and A549 (lung cancer), MCF-7 (breast carcinoma), HepG2 (liver cancer), and HeLa (cervical carcinoma) cell lines by this viral DNA uptake approach. Among the panel of cell lines that we tested, HeLa cells were the least susceptible to infection and their infectivity was designated as 1. The infectivities of LNCaP, H157, A549, MCF7, LAPC-4, and HepG2 cells were 1.7-, 1.6-, 1.5-, 1.3-, 1.1-, and 1.1-fold higher than that of HeLa cells, respectively. Differential CAR expression in different stages of prostate carcinoma [40] might contribute to the enhanced infection in LNCaP cells.

We evaluated the activity of AdTSTA-FL in several prostate cancer cell lines, including two androgen-responsive cell lines (LNCaP and LAPC-4 [41]) and two AR-negative lines (DU145 and PC-3). Infection was carried out at a calculated AdTSTA-FL dosage of 1 infectious unit (pfu) per cell (m.o.i. 1). The normalized FL activity in LAPC-4 was 4.4-fold lower than LNCaP (see Fig. 5b and data not shown). Conversely, the FL activity in AR-negative prostate cancer lines was negligible (nearly 500-fold lower than in LNCaP cells, data not shown). For simplicity, we compared the normalized FL activities to that of the LNCaP cell line, set at 100% (Fig. 2a). The activity in A549, H157, HepG2, HeLa, and MCF7 cells was 290-, 1200-, 4500-, 6000-, and 20,000-fold lower than in LNCaP cells, respectively (Fig. 2a). The FL activity in nonprostate cell lines and AR-negative prostate cancer lines was not induced by androgen (data not shown). We also observed consistent diminished cell-specific expression in infections. At higher m.o.i. the cell specificity became less apparent due to higher androgen-independent or basal activity, an effect that we do not completely understand (data not shown). For example, AdTSTA-FL-mediated activity in HeLa cells at m.o.i. 10 was 660-fold lower than in



LNCaP cells, compared to 6000-fold at m.o.i. 1 (data not shown; see Discussion).

We next investigated the specificity of the single AdTSTA-FL *in vivo*. We compared its activity to that of AdCMV-FL, because vector DNA quantitation studies in animals are less well controlled. We employed CCD imaging to monitor *in vivo* expression over a 22-day period. Fig. 2b illustrates the optical imaging profiles of animals that received systemic administration of AdTSTA-FL or AdCMV-FL. A robust signal emanating from the midsection of mice injected with AdCMV-FL via the tail vein was seen as early as 3 days postinjection, which we determined was due to efficient liver transduction as assessed by imaging of isolated organs (Figs. 2b and 2c). In contrast, the AdTSTA-FL-injected animals did not have detectable signals until a late time point (day 22), which signals then appeared in the lung (Figs. 2b and 2c); however, this signal is more than 3 orders of magnitude lower than tumor-directed expression (see Fig. 3). The absence of optical signal in the prostate after tail vein injection of AdTSTA-FL is unclear at this time. However, limitations of *in vivo* Ad distribution that result in low gene transfer to organs other than mouse liver have been well documented [42]. We expand on these issues under Discussion.

We next evaluated intratumoral activity mediated by both single and separate TSTA Ads in LAPC-4 xenografts, which were derived from a lymph node metastatic lesion from a patient [41]. LAPC-4 expresses PSA and AR and exhibits androgen-responsive gene expression and growth. Fig. 3a shows that intratumoral injection of  $10^7$  pfu of the single AdTSTA-FL resulted in a robust signal at 4 days postinjection, compared to AdCMV-FL. In a cohort of four animals, the average activity of AdTSTA-FL was 110-fold higher than that seen with AdCMV-FL ( $P = 0.06$ ).

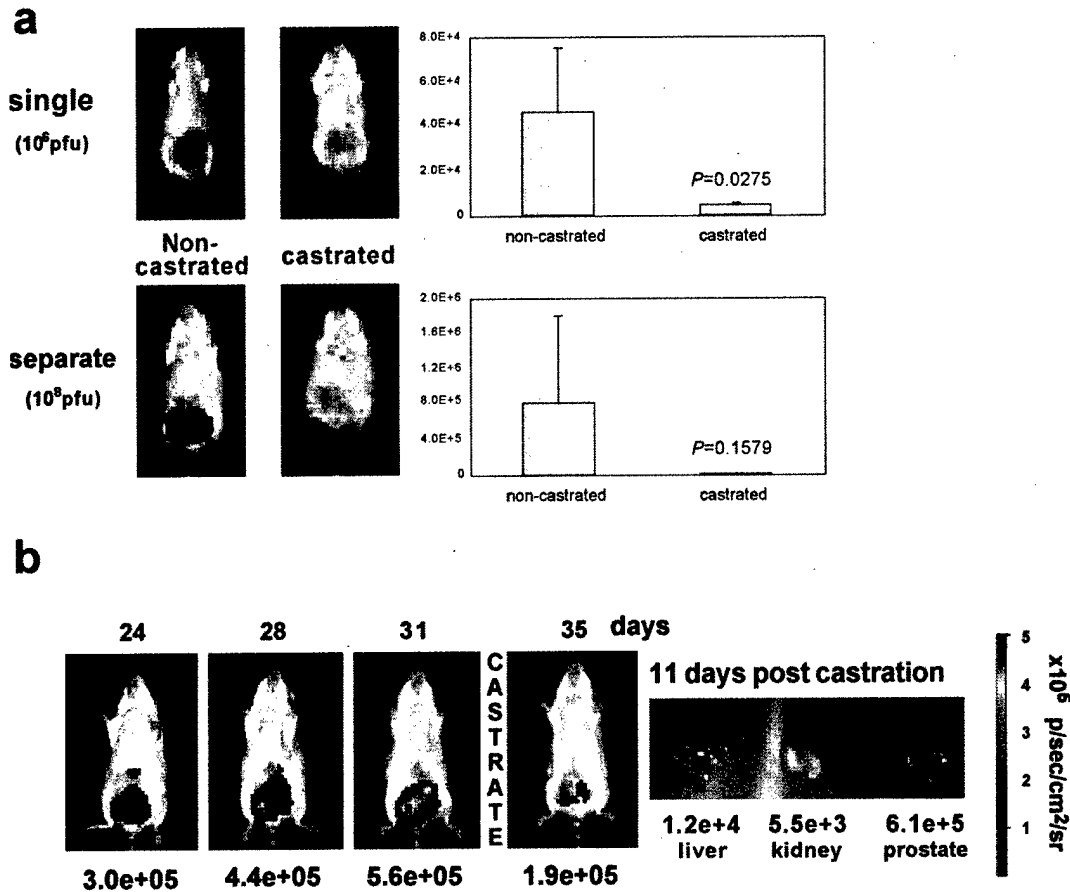
Cancer-specific gene therapy based on activation of a toxic gene by the Cre/lox recombination system delivered by separate two Ads has been reported to work in animals

[43,44]. However, *in vivo* transduction of the two paired TSTA Ads, AdBC-VP2 and AdG5-FL, into the same cell, is anticipated to be less effective than delivery of a single vector containing both elements. Thus, it is not surprising that injection of  $10^7$  pfu of each of the two Ads resulted in lower optical signal ( $2 \times 10^5$  photons/s/cm<sup>2</sup>/sr) versus single Ad ( $4 \times 10^8$  photons/s/cm<sup>2</sup>/sr). When the dose of the two paired TSTA Ads was increased to  $10^8$  pfu each, expression level ( $2 \times 10^7$  photons/s/cm<sup>2</sup>/sr) higher than that of  $10^7$  pfu of AdCMV-FL ( $9 \times 10^5$  photons/s/cm<sup>2</sup>/sr) was achieved (Fig. 3a). However, this magnitude of activity is still lower than can be achieved by  $10^7$  pfu of AdTSTA-FL ( $4 \times 10^8$  photons/s/cm<sup>2</sup>/sr).

We examined the kinetics of expression after intratumoral delivery of  $10^7$  pfu of AdCMV-FL and AdTSTA-FL into LAPC-9 tumors. The LAPC-9 xenograft expresses AR and PSA and was derived from a bone metastasis [41]. Sequential optical images between 5 and 13 days post-viral injection were recorded (Fig. 3b). The TSTA vector displayed 50- to 100-fold higher levels of FL activity than the AdCMV-FL during this period (Fig. 3b). The increasing serum PSA levels in both groups likely reflect the increase in tumor mass over the duration of the time course (Fig. 3b, right). However, despite the consistent increase in serum PSA levels the intratumoral FL signals gradually decayed after day 7 in both the AdCMV and the AdTSTA cohorts (Fig. 3b) due to the transient nature of Ad-mediated gene expression. The LAPC-9 tumors, like the LAPC-4 tumors, show a propensity for vector leakage into systemic circulation. However, we consistently observed a greater magnitude of leakage in LAPC-4 tumors, which was manifested as prominent signals in the liver after intratumoral injection of AdCMV-FL [26]. Because both vectors (TSTA and CMV) are serotype 5 adenovirus with the same deletion of the E1 and E3 genes, their biodistribution in mice should not differ. Intratumoral injection of AdTSTA-FL should result in the same extent of vector leakage as AdCMV-FL. However, no detectable liver signal was observed after intratumoral injection of AdTSTA-FL.

**FIG. 2.** Cell specificity of the TSTA Ad. (a) *In vitro* cell specificity of AdTSTA-FL. The prostate cell line LNCaP and nonprostate cell lines were infected with AdTSTA-FL at m.o.i. 1. Cells were harvested and subjected to an FL assay 48 h after infection. FL activity was normalized to cell numbers and infectivity of each cell line as assayed by real-time PCR (see Materials and Methods). FL activity was plotted for each cell line using LNCaP (an androgen-responsive prostate carcinoma line) as a normalization standard, set at 100%. The activities in nonprostate cell lines are more than 290-fold lower than in LNCaP. (b) *In vivo* tissue specificity of AdTSTA-FL.  $10^7$  pfu (plaque-forming units = infectious units) of Ad was injected into naive mice via the tail vein and FL expression was sequentially monitored by optical imaging at days 3, 6, and 22. Robust liver signals were noted in the AdCMV-FL-injected animals starting at day 3 and increasing from that point onward. The AdTSTA-FL-injected animals remained transcriptionally silent until day 22, when a weak signal was noted in the lung. Numbers below the images are the maximal activities in the region of interest as photons (p) acquired per second per square centimeter per steradian (sr). (c) Optical activities in the isolated organs. Two additional animals (n1 and n2) from the AdCMV-FL- or AdTSTA-FL-injected group were sacrificed at day 22, and the isolated organs were reimaged. The liver is the predominant site of expression in AdCMV-FL-injected animals. Low level of expression in the lung was observed in the AdTSTA-FL-injected animals (br, brain; lu, lung; lv, liver; and pr, prostate).

**FIG. 3.** *In vivo* FL expression mediated by single and separate TSTA Ads in LAPC-4 xenografts and prostates. (a) Optical signals after injections of the respective Ads in LAPC-4 tumors.  $10^7$  or  $10^8$  pfu of Ads (as specified) was injected. The injection of separate Ads denotes the coadministration of both AdBC-VP2 and AdG5-FL at the specified dosage. CCD images of representative animals analyzed at 4 days postinjection were shown. (b) Kinetics of FL expression in LAPC-9 tumors.  $10^7$  pfu of AdCMV-FL or AdTSTA-FL was injected intratumorally. Optical signals were monitored on the specified days after viral injection. The number below each image represents the maximal signal over the tumor. The graph on the right represents the averaged serum PSA level measured in the animals at the specified days post-viral injection.



**FIG. 4.** Intraprostatic signals in intact and castrated animals. (a) AdTSTA-FL ( $10^6$  pfu) or AdBC-VP2 and AdG5-FL ( $10^8$  pfu each) were injected into the prostate of intact and castrated male SCID (7-days postcastration). The images were taken 4 days after viral injection. In each group,  $n$  is  $\geq 3$  animals. The averages and standard errors are plotted on the right. The FL signals in the castrated animals were  $4.7 \times 10^3$  and  $9.6 \times 10^3$  with single and separate TSTA Ad, respectively. The significance between intact and castrated animals is denoted in each graph as the  $P$  value. (b) The FL signal mediated by AdTSTA-FL in the same animal before and after castration.  $10^7$  pfu of AdTSTA-FL was injected into prostate in intact SCID mice. Days post-intraprostatic viral injection are indicated above the mouse images. The animal was castrated at day 31 and reimaged at day 35 (4 days postcastration). The animal was sacrificed at 11 days postcastration (day 42). The prostate gland was the predominant site of expression. The optical activity is specified below each image.

This finding supports our view that the prostate specificity of TSTA is able to prevent expression of FL in the liver.

#### Androgen Regulation of the TSTA Ads

To determine if TSTA Ads respond to androgen regulation *in vivo* we assessed FL expression in the prostates of intact and castrated male SCID mice (Fig. 4). We injected  $10^6$  pfu of AdTSTA-FL or  $10^8$  pfu each of AdBC-VP2 and AdG5-FL into the prostate glands of cohorts of either intact male mice or mice castrated 7 days prior to injection (androgen-deprived group). The intact males infected with the single- or two-virus TSTA vectors displayed significant optical signals compared to the castrated mice. We conclude that both vector systems are responding to androgen depletion *in vivo* (Fig. 4a). The FL expression level of a 100-fold higher dose of the separate TSTA Ads was 20-fold greater than that of the single AdTSTA-FL (Fig. 4a,

graphs). We also observed androgen regulation of AdTSTA-FL in the prostate gland when castration was performed 30 days postinjection, after FL expression had stabilized. In this case a 3-fold drop in expression was observed 3 days after castration (Fig. 4b).

To investigate androgen regulation of the TSTA Ads in more detail, we employed cell culture infection studies, in which the concentration of androgen and its antagonists could be carefully manipulated. We infected two androgen-dependent prostate cancer cell lines, LNCaP and LAPC-4, with TSTA Ads at different m.o.i. and androgen concentrations. Androgen levels in the medium were manipulated by adding R1881 (methyltrienolone), a synthetic androgen that is more stable than DHT under culture conditions. The antagonist Casodex was used to minimize residual androgen activity in the charcoal-

stripped serum because even low androgen levels activate the highly sensitive TSTA system. Both the activator and the reporter TSTA components are required to generate detectable FL in the two-virus system (Fig. 5a). Additionally, androgen stimulated the FL activity for both the separate and the single TSTA Ads, with the highest activity observed between 1 and 10 nM R1881. In Fig. 5b, AdTSTA-FL demonstrates a clear m.o.i.- and R1881-dependent increase in FL activity. In the presence of 1 nM R1881, activity increased 27-fold from m.o.i. 0.1 to m.o.i. 1 in LNCaP cells and 96-fold from m.o.i. 1 to m.o.i. 10 in LAPC-4 cells.

We also quantitated the androgen response of TSTA Ads by calculating the fold induction, based on the ratio of the highest activity at 10 nM R1881 over the basal activity in the presence of Casodex. Both separate and single TSTA Ads exhibited high levels of androgen induction in LNCaP and LAPC-4 cells. The androgen induction observed in infections at m.o.i. 1 and 5 of separate Ads were 672- and 915-fold, respectively, in LNCaP cells, and 52- and 67-fold, respectively, in LAPC-4 cells. The androgen induction mediated by the single AdTSTA-FL was 117- and 101-fold at m.o.i. 1 and 5 in LNCaP cells, respectively, and 35- and 24-fold in LAPC-4 cells at m.o.i. 1 and 5, respectively. The single AdTSTA-FL displayed diminished androgen inducibility compared to separate TSTA Ads. This point is illustrated in Fig. 5c by a plot of the relative induction ratio of separate Ads to single AdTSTA-FL in LNCaP and LAPC-4 cells at the two different m.o.i. The lower inducibility of AdTSTA-FL is not due to a lower maximal activity, but to a higher basal activity (in the presence of Casodex). Because this higher basal activity could potentially contribute to reduced specificity, we investigated the activation mechanism of TSTA Ads in more detail.

#### Investigating the Activation Mechanism in Single and Separate TSTA Ads

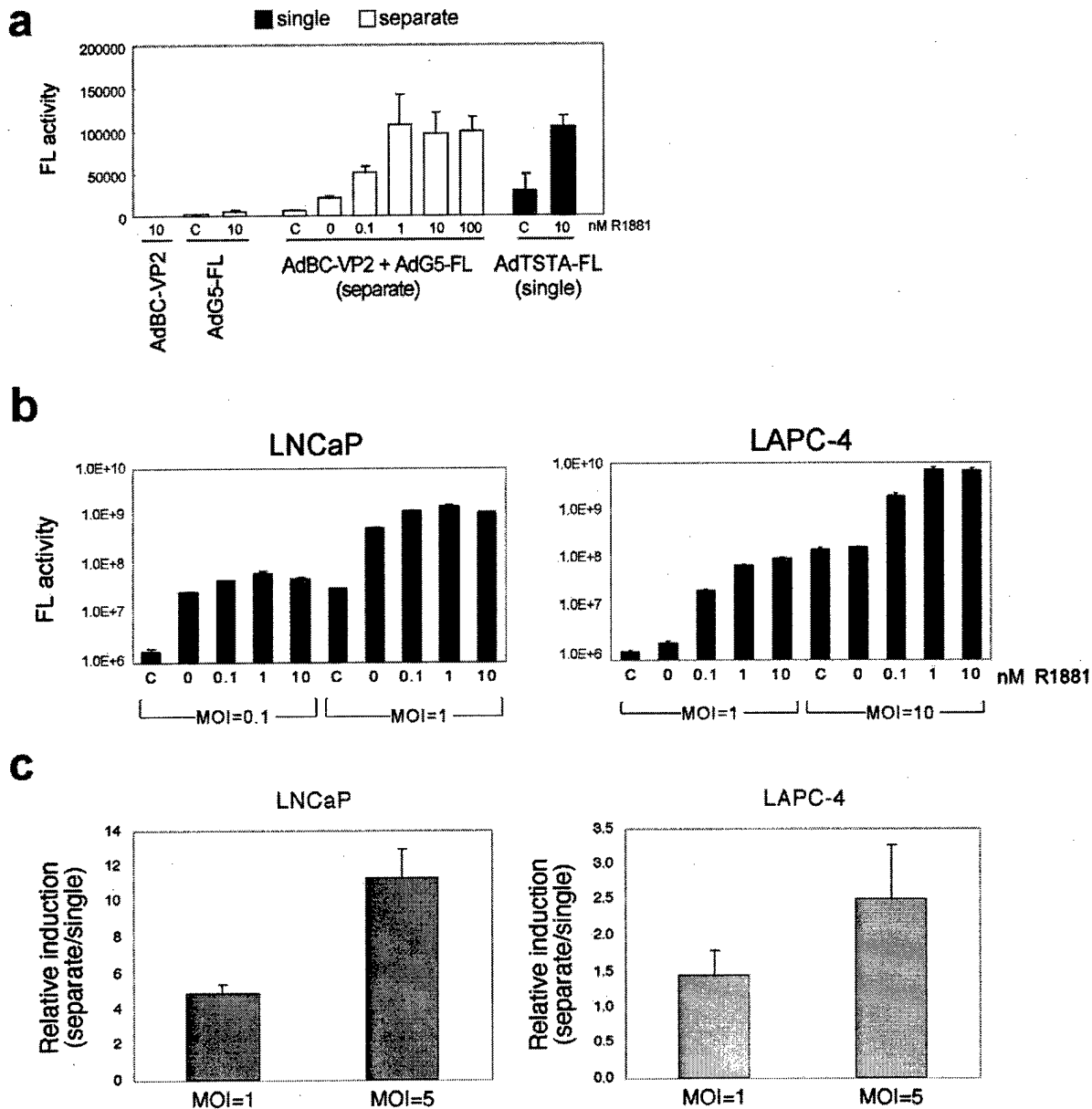
To investigate the mechanism responsible for differences in androgen induction, we analyzed FL activity and activator protein expression profiles over a wide range of infection ratios. LNCaP cells were infected with AdTSTA-FL at m.o.i. of 50, 16.7, 5.6, 1.9, 0.62, and 0.21 (threefold serial dilutions). For the separate system, we added AdBC-VP2 and AdG5-FL at the indicated m.o.i. to generate levels of activator and reporter gene delivery equivalent to those in the single AdTSTA-FL infections. We demonstrated by Southern blotting that equal m.o.i. of AdTSTA-FL and AdBC-VP2 led to equivalent amounts of vector delivery (data not shown). We then examined FL activity and activator expression 48 h postinfection, as shown in Fig. 6. It is difficult to compare directly the levels of FL activity of single and separate Ad infections, especially at low m.o.i., due to the limited codelivery. However, both systems exhibited m.o.i.-dependent increases in activity as indicated in earlier figures. We ob-

served a saturation of activity in both single and separate TSTA vectors. Overall, the magnitude of FL activity corresponded very well with the level of GAL4-VP2 expression measured by Western blotting. In the single AdTSTA-FL infections, the GAL4-VP2 expression reached a maximum at m.o.i. 16.7. We did not observe a plateau of activator expression in the separate TSTA infections at the range of m.o.i. tested. A surprising finding was that at each m.o.i., the activator expression in the single AdTSTA-FL was considerably higher than that mediated by the AdBC-VP2 in the separate system, despite comparable levels of activator (BC-VP2) gene delivery (data not shown). Given the fact that the same PSE-BC promoter-driven GAL4-VP2 expression cassette was inserted into both the single and the separate TSTA Ads, the different protein levels observed imply that a property of the vector genome context or design is influencing GAL4-VP2 expression. We propose in the Discussion that a self-perpetuating feedforward loop may be activated by the head-to-head orientation of AdTSTA-FL. A positive feedback loop could explain the higher basal activity observed for the single virus AdTSTA-FL even in the presence of Casodex (Fig. 5).

#### DISCUSSION

The key objectives of this study were to investigate the regulation of the TSTA system in different Ad configurations and to define the dynamic range of this system in different *in vivo* settings. Published and ongoing studies by our groups have demonstrated that TSTA technology is an effective approach to augment the activity of a weak tissue-specific promoter [27, 28]. Our goal is to develop these targeted gene expression systems for diagnostic and therapeutic applications in clinical settings. Thus, we have incorporated our PSA promoter-based TSTA system into an adenoviral gene delivery vector. Inserting the two components of TSTA, the activator and the reporter component, into a single vector does improve the functional efficiency of this system *in vivo*. In fact, the activity of the first single vector we generated, AdTSTA-FL, is quite impressive, as its activity consistently exceeded AdCMV-FL in all AR-expressing prostate cancer cell lines and tumors tested ([23] and this study), and it also displayed significant prostate specificity in cell culture studies, achieving 290-fold or higher levels of tissue discrimination (Fig. 2a). AR-mediated expression is a critical component of the PSA-based promoter in the TSTA system. We utilized the optical signal produced by AdTSTA-FL in tumors to monitor the dynamics of AR function during prostate cancer progression [23].

To understand the mechanism of activation of TSTA in adenoviral vectors better, we examined and compared the activity of single and separate TSTA configured Ads. The activity of this TSTA system is fully dependent on the GAL4-VP2 activator (Fig. 6). A second interesting finding is that despite equivalent promoter (PSE-BC) and gene



**FIG. 5.** *In vitro* expression and regulation of the single and separate TSTA Ads. (a) Expression of TSTA Ads in LNCaP. LNCaP cells were infected with Ad at m.o.i. 10. 48 h postinfection the cells were harvested and assayed. Infections with AdBC-VP2 or AdG5-FL exhibited minimal activity. The FL activities of co-infection of separate TSTA Ads increased with increasing amount of synthetic androgen (R1881, nM). C denotes the addition of 10  $\mu$ M Casodex (anti-androgen). (b) Androgen regulation of AdTSTA-FL. LNCaP and LAPC-4, two androgen-dependent prostate cell lines, were infected with AdTSTA-FL at the indicated m.o.i. in the presence of Casodex or R1881. The FL activities assayed at 48 h postinfection are shown. (c) Relative androgen induction ratio of the separate to the single TSTA Ads. The cells were infected at m.o.i. 1 or 5. Fold induction of activity was calculated based on the ratio of the peak activity (in 10 nM R1881) to the basal activity (in 10  $\mu$ M Casodex). The relative induction ratio was calculated by dividing the androgen induction in the separate TSTA Ads infection by the induction in the single-AdTSTA-FL-infected cells. The ratio shows that separate TSTA Ads exhibit higher androgen induction than the single Ad.

delivery of the activator in the single and separate TSTA Ads, the single AdTSTA-FL consistently expressed an elevated level of GAL4-VP2 activator. This finding indicates that the head-to-head configuration in the single Ad promotes an increase in GAL4-VP2 and, hence, FL expres-

sion. This result could also contribute to the slightly elevated basal activity mediated by AdTSTA-FL in androgen-depleted cell culture medium. We hypothesize that a feedforward loop might be at play in the single AdTSTA-FL. A schematic illustration of this idea is shown in

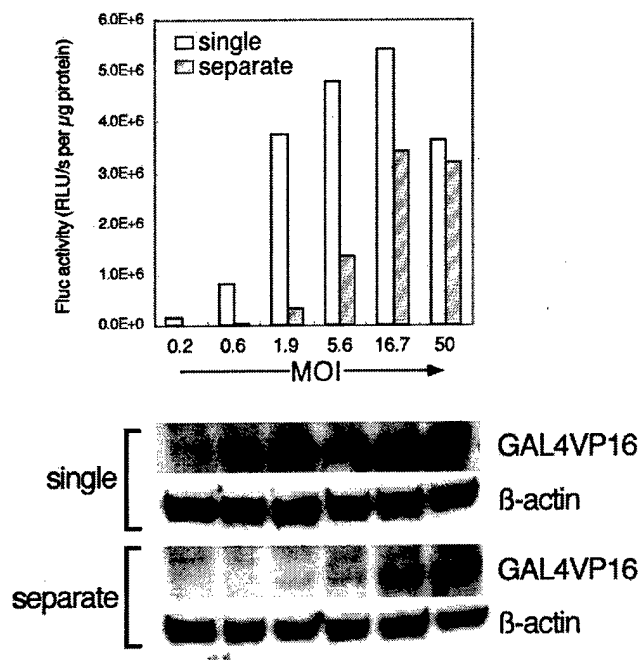


FIG. 6. The activation mechanism in TSTA Ads. LNCaP cells were infected with AdTSTA-FL at specified m.o.i. For the separate system, both AdBC-VP2 and AdG5-FL were infected at the denoted m.o.i. The FL activities and GAL4-VP2 activator expression were examined at 48 h postinfection. Western blot analysis is shown at the bottom. The GAL4-VP2 activator was probed with anti-GAL4 polyclonal antibody (see Materials and Methods).  $\beta$ -Actin is shown as a control.

Fig. 7. The initial expression of GAL4-VP2 (step 1) is regulated by the PSE-BC promoter. In step 2, GAL4-VP2 binds to the GAL4 sites and activates FL gene expression. However, in the head-to-head configuration, the multiple GAL4-VP2 activators could also stimulate transcription in the direction of the PSE-BC promoter in an enhancer-like manner (step 3), further enhancing the synthesis of GAL4-VP2 (step 4). The feedforward loop leads to a perpetuating cycle of activator production that exceeds the natural capability of the PSE-BC promoter. This phenomenon could also contribute to "leaky" expression in non-prostate cells when the TSTA vector is administered at high m.o.i. From these results, we would postulate that functional separation of the activator and reporter components in a single vector might achieve tighter regulation of the TSTA system.

A transcriptionally targeted gene expression approach [reviewed in 45] could reduce the potential side effects of Ad-mediated cytotoxic cancer gene therapy such as that mediated by the herpes simplex virus thymidine kinase (HSV-TK) gene [46]. After intratumoral injection of Ad constitutively expressing luciferase or other reporter gene, leakage of the vector into systemic circulation resulted in transgene expression in the liver [26,47]. From this finding liver toxicity can be anticipated after intratumoral

injection of CMV-driven HSV-TK Ad following administration of the prodrug ganciclovir. When a tissue- or cancer-specific promoter is employed to drive HSV-TK, the same extent of vector delivery to the liver is expected to occur. However, HSV-TK expression in the liver will be restricted by the tissue-specific promoter and therefore transgene-mediated liver toxicity should be reduced.

The gene-expression targeting approach employed in this study will not alter the *in vivo* liver distribution observed of Ad5 [26,48]. This preferential Ad transduction has contributed to liver toxicity [48–50] due to the innate immune response to viral capsid proteins [51] and cell-mediated immunity against viral gene products [52,53]. Utilization of a specific promoter to drive transgene expression was shown to reduce both the immune response against the Ad and the associated liver toxicity [54]. In addition, the potent gene expression mediated by the TSTA system could potentially reduce the amount of vector needed to transduce cancer cells *in vivo* compared to nonamplified tissue-specific vectors. Reducing the input dosage of Ad has been documented to reduce liver toxicity [49–52]. A second consequence of Ad sequestration in the liver [48,49] is that viral distribution to other organs such as kidney and intestine was nearly 1000-fold lower [55]. To improve *in vivo* gene transduction to other organs or tumors, many studies are under way to divert the natural adenovirus tropism away from the liver by ablation of CAR- and integrin-mediated interactions [48,55,56].

The inability to detect prostatic expression after tail vein administration of  $10^7$  pfu of AdTSTA-FL (Figs. 2b and 2c) could be due to liver sequestration. We have detected FL expression in the prostate after intravenous administration of  $1.8 \times 10^9$  pfu of AdPSE-BC-FL [25]. The AdTSTA-FL is estimated to be about 50-fold more active than AdPSE-BC-FL [23,28]. However, the 180-fold lower dosage of AdTSTA-FL used in this study compared to AdPSE-BC-FL [25] might be below the threshold of detection for

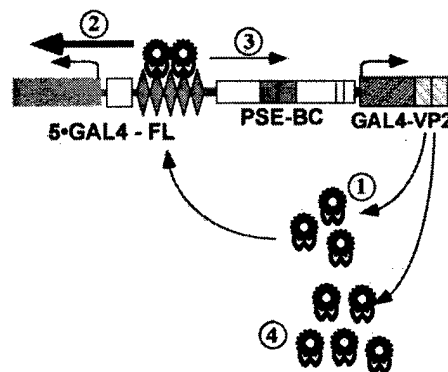


FIG. 7. Schematic representation of the activation mechanism. The feedforward loop mechanism postulates that GAL4-VP2 expression is upregulated by the activator's binding to the designed GAL4 sites in a self-activated manner.

optical imaging. Moreover, by an intraperitoneal route of vector delivery we have been able to detect specific optical signals in the prostate (M. Johnson and L. Wu, unpublished data).

The mechanism for the low level of expression observed in lung tissue is unclear (Figs. 2b and 2c). We speculate that there could be transcription factors common to both lung and prostate that partially contribute to the regulation of the PSA promoter. For example, GATA zinc finger transcription factors have been shown to bind to the PSA promoter [57], and they are involved in lung development [58] and transcription of lung-specific promoters [59]. A large family of Ets transcription factors could also participate in epithelial-specific expression in the lung and prostate [60–62]. Investigating these intriguing possibilities might lead to a better understanding of tissue-specific gene regulation.

It is quite evident that the TSTA approach can amplify the activity of many other weak tissue-specific promoters. Dr. Gambhir's group has demonstrated that the activity of the hypoxia-inducible VEGF promoter can be amplified [63]. A similar approach has been employed to amplify the carcinoembryonic antigen promoter in a binary adenovirus system [64]. This approach exhibited increased therapeutic index compared to constitutive viral RSV-driven HSV-TK suicide gene therapy [64]. To adapt the TSTA system to other promoters, it will be necessary to adjust the various components of TSTA (i.e., the potency of the specific promoter, the strength of the activator, and the number of GAL4 sites) to achieve optimal regulation and expression dictated by the specific applications.

The modular and titratable nature of the TSTA system also makes it particularly attractive for a variety of gene therapy applications [28]. Transgene levels needed to achieve therapeutic efficacy in different gene therapy strategies might vary greatly; for example, p53 tumor suppressor expression in genetic corrective strategies might need to be higher than cytokine expression in immune-mediated tumor rejection. The various adjustable constituents of TSTA can be fine-tuned to achieve the most effective and least toxic therapeutic result. We have shown that transcriptionally targeted Ad (AdPSE-BC-luc) can achieve cell-specific expression to localize metastatic prostate cancer lesions in living mice, using optical CCD imaging [26]. To translate this finding to clinical diagnostic settings, a higher energy imaging modality will be needed to circumvent the loss of optical signal observed with increased tissue depth. Positron emission tomography (PET) is a radionuclide imaging modality widely used in clinical settings. Our institution has acquired substantial experience in adapting this modality to gene-based imaging in small animals, using the HSV-TK or the dopamine type 2 receptor reporter genes [29,30,65]. Compared to optical imaging, PET has the distinct advantage of providing tomographic quantitative image signals and adaptability for human imaging. However, optical imag-

ing is several orders of magnitude higher in sensitivity than PET in small animal applications [66]. Thus, the highly amplified and prostate-specific expression mediated by TSTA will likely permit the development and successful implementation of gene-based PET imaging to detect metastasis *in vivo*.

Many of our studies have demonstrated that the TSTA system is a promising tool to create future targeted gene-based diagnostic and therapeutic applications. With an in-depth understanding of its functional properties and fine-tuning of various components of TSTA, a truly safe, effective, and specific treatment can be developed for metastatic or hormone-refractory prostate cancer.

## MATERIALS AND METHODS

**Adenovirus constructs.** AdCMV-FL was constructed as previously described [25,33]. The single AdTSTA-FL and separate TSTA Ads, AdBC-VP2 and AdG5-FL, were constructed with the AdEasy system [35]. The head-to-head fragment of activator and reporter in the single virus was derived from PBCVP2G5-L [28]. The construction of AdTSTA-FL has been previously described [23]. A *NotI* fragment containing the PSE-BC-driven GAL4-VP2 expression cassette was cloned into the *NotI* site of pShuttle to generate AdBC-VP2. For the construction of AdG5-FL, an *Asp718-SalI* fragment with five GAL4 binding sites upstream of the minimal adenovirus E4 and FL genes was blunted and ligated into the *EcoRV* site of pShuttle. All the pShuttle expression plasmids were used for recombination with pAdEasy-1 in the BJ5183 rec<sup>+</sup> bacteria strain to generate the full-length recombinant virus-containing plasmid. The viruses were propagated in 293 cells, purified on a CsCl gradient, and titered by plaque assays on 293 monolayers. Viruses are stored in 10 mM Tris-HCl, 1 mM MgCl<sub>2</sub>, and 10% glycerol at –80°C until use.

**Cell culture and infection studies.** The human prostate cancer cell lines, LNCaP and LAPC-4, were grown in RPMI 1640 and Iscove's modified DMEM, respectively, and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution. PC-3, Du145, HeLa, MCF7, HepG2, A549, and H157 cells were cultured in RPMI 1640 (Mediatech, Herndon, VA) with 10% FBS and 1% penicillin/streptomycin. For FL assays, the cultured cells were plated onto 24-well plates at  $5 \times 10^4$  cells per well, and cells were counted at the day of infection to calculate m.o.i. For prostate cell lines, medium was replaced with 10% charcoal-stripped serum for 2 days prior to infection. The cells were infected with AdTSTA-FL or co-infected with AdBC-VP2 and AdG5-FL at certain m.o.i. Following infection, the synthetic androgen methylenetriolone (R1881; NEN Life Science Products, Boston, MA) or anti-androgen bicalutamide (Casodex) was added to samples as indicated. At 48 h postinfection, the cells were harvested and lysed, using passive lysis buffer (Promega, Madison, WI). Levels of FL activity were measured according to the manufacturer's instructions (Promega), using a luminometer (Berthold Detection Systems, Pforzheim, Germany) with a 10-s integration time. Each value was calculated as the average of triplicate samples.

Real-time PCR was performed to quantify the amount of intracellular viral DNA. HeLa, MCF7, HepG2, H157, A549, and LNCaP cells were infected with AdG5-FL at m.o.i. 0.1 or 1 36 h after plating. After 12 h, cells were harvested and lysed. The total DNA was prepared with the DNeasy Tissue Kit (Qiagen, Valencia, CA). Opticon2 (MJ Research, Boston, MA) real-time PCR was performed, using these DNAs as template and the DyNamo SYBR Green qPCR Kit (Finnzymes, Espoo, Finland). The viral FL sequences were detected by the following primer set: FL-a (5'-GAGAT-ACGCCCTGGTTCTCTG-3') and FL-b (5'-GCATACGACGATTCTGT-GATTG-3'). Infectivity was calculated based on the copy number of internalized viral DNA divided by cell number. The relative infectivities of all cells are in reference to HeLa cells, which were set as 1, as they are the least susceptible to infection among the cell lines we tested.

**Animal experiments with CCD imaging.** Animal care and procedures were performed in accordance with the University of California Animal Research Committee guidelines. Eight- to ten-week-old male SCID mice (ICRSC-M, ~25 g, Taconic Farms, Germantown, NY) were used in these studies. Human prostate tumor xenografts were generated in SCID mice as previously described [41]. The LAPC-4 xenograft was originally provided by Dr. Charles Sawyers at UCLA. We passaged the tumor by implanting small tumor fragments mixed 1:1 with Matrigel (Collaborative Research, Bedford, MA) subcutaneously into the flanks of male SCID mice.

For the naïve mouse experiments,  $10^7$  pfu of Ad was injected via the tail vein ( $n = 3$ ). *In vivo* expression was monitored sequentially over time. For the LAPC-4 xenografts, tumors were allowed to grow for 3 weeks prior to injection and reached a diameter of approximately 1 cm. AdBC-VP2 and AdG5-FL ( $10^8$  pfu each) or AdTSTA-FL ( $10^7$  pfu) was injected at three sites on each tumor at  $10 \mu\text{l}$  per site ( $n = 3$ ). Optical CCD imaging was performed at the indicated days postinjection. Intraprostatic injections were performed 7 days after castration. Both castrated and noncastrated animals received injection of  $10^8$  infectious units each of the paired TSTA Ads or  $10^6$  infectious units of the single TSTA Ad in both posterior lobes of the prostate ( $n = 4$  per group). For each imaging session, the mice were anesthetized with ketamine/xylazine (4:1), and the d-luciferin substrate (150 mg/kg in PBS, Xenogen) was given intraperitoneally at a volume of 200  $\mu\text{l}$ , with a 20-min incubation period prior to imaging. CCD images were obtained using a cooled IVIS CCD camera (Xenogen, Alameda, CA), and images were analyzed with IGOR-PRO Living Image Software, as described [26,28], in units of photons acquired per second per square centimeter per steradian.

**Western blot analysis of GAL4-VP2 expression.** LNCaP cells were grown in 60-mm dishes and infected with AdTSTA-FL or co-infected with AdBC-VP2 and AdG5-FL at m.o.i. 0.21, 0.62, 1.9, 5.6, 16.7, or 50 (threefold serial dilution). For co-infection, each Ad was administered at the m.o.i. listed above. Forty-eight hours later, the cells were harvested and lysed with RIPA buffer (1% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.5), protease inhibitor cocktail (Sigma, St. Louis, MO)). The samples were fractionated on 8–16% gradient acrylamide gels (Gradipore, Frenchs Forest, Australia) and subjected to immunoblot analysis with rabbit polyclonal antibodies generated against intact GAL4-VP2 or  $\beta$ -actin A5316 (Sigma). Detection was done by visualization of bands with HRP-labeled secondary antibody and ECL (Amersham Pharmacia Biotech, Piscataway, NJ).

#### ACKNOWLEDGMENTS

We appreciate the technical support of Erika Billick, the assistance of Wendy Aft on manuscript preparation, and helpful discussions with Drs. Harvey Herschman and Helen Brown. This work is supported by Department of Defense (DOD) CDMRP PC020536 (to L.W.), California Cancer Research Program 3N10226 (to L.W.), NIH R01 CA101904 (to L.W.), CapCURE (to M.C., S.S.G.), DOD PC 991019 (to Charles Sawyers and M.C.), DOD PC020177 (to M.C.), an interdisciplinary seed grant from the JCCC (to M.C., L.W., and S.S.G.), R01 CA82214 (to S.S.G.), SAIRP R24 CA92865 (to S.S.G.), and Department of Energy Contract DE-FC03-87ER60615 (to S.S.G.). M.S. is supported by a DOD CDMRP postdoctoral fellowship (PC020531).

RECEIVED FOR PUBLICATION JUNE 16, 2003; ACCEPTED AUGUST 20, 2003.

#### REFERENCES

- American Cancer Society (2002). Cancer Facts & Figures 2002. Am. Cancer Soc., Atlanta, pp. 3–15.
- Denis, L., and Murphy, G. P. (1993). Overview of phase III trials on combined androgen treatment in patients with metastatic prostate cancer. *Cancer* 72: 3888–3895.
- Hellerstedt, B. A., and Pienta, K. J. (2002). The current state of hormonal therapy for prostate cancer. *CA Cancer J. Clin.* 52: 154–179.
- Labrie, F. (2002). Androgen blockade in prostate cancer in 2002: major benefits on survival in localized disease. *Mol. Cell. Endocrinol.* 198: 77–87.
- Mabjeesh, N. J., Zhong, H., and Simons, J. W. (2002). Gene therapy of prostate cancer: current and future directions. *Endocr. Relat. Cancer* 9: 115–139.
- Wu, L. and Sato, M. (2003). Integrated, molecular engineering approaches to develop prostate cancer gene therapy. *Curr. Gene Ther.* 3: 452–467.
- Cleutjens, K. B., van der Korput, H. A., van Eekelen, C. C., van Rooij, H. C., Faber, P. W., and Trapman, J. (1997). An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. *Mol. Endocrinol.* 11: 148–161.
- Noss, K. R., Wolfe, S. A., and Grimes, S. R. (2002). Upregulation of prostate specific membrane antigen/folate hydrolase transcription by an enhancer. *Gene* 285: 247–256.
- O'Keefe, D. S., et al. (1998). Mapping, genomic organization and promoter analysis of the human prostate-specific membrane antigen gene. *Biochim. Biophys. Acta* 1443: 113–127.
- Pang, S., et al. (1997). Identification of a positive regulatory element responsible for tissue-specific expression of prostate-specific antigen. *Cancer Res.* 57: 495–499.
- Schuur, E. R., Henderson, G. A., Kmetec, L. A., Miller, J. D., Lamparski, H. G., and Henderson, D. R. (1996). Prostate-specific antigen expression is regulated by an upstream enhancer. *J. Biol. Chem.* 271: 7043–7051.
- Watt, F., et al. (2001). A tissue-specific enhancer of the prostate-specific membrane antigen gene, FOLH1. *Genomics* 73: 243–254.
- Hobisch, A., Culig, Z., Radmayr, C., Bartsch, G., Klocker, H., and Hittmair, A. (1995). Distant metastases from prostatic carcinoma express androgen receptor protein. *Cancer Res.* 55: 3068–3072.
- Koivisto, P. A., and Helin, H. J. (1999). Androgen receptor gene amplification increases tissue PSA protein expression in hormone-refractory prostate carcinoma. *J. Pathol.* 189: 219–223.
- Sweat, S. D., Pacelli, A., Bergstralh, E. J., Slezak, J. M., Cheng, L., and Bostwick, D. G. (1999). Androgen receptor expression in prostate cancer lymph node metastases is predictive of outcome after surgery. *J. Urol.* 161: 1233–1237.
- van der Kwast, T. H., and Tetu, B. (1996). Androgen receptors in untreated and treated prostatic intraepithelial neoplasia. *Eur. Urol.* 30: 265–268.
- Bok, R. A., and Small, E. J. (2002). Bloodborne biomolecular markers in prostate cancer development and progression. *Nat. Rev. Cancer* 2: 918–926.
- Feldman, B. J., and Feldman, D. (2001). The development of androgen-independent prostate cancer. *Nat. Rev. Cancer* 1: 34–45.
- Visakorpi, T., et al. (1995). In vivo amplification of the androgen receptor gene and progression of human prostate cancer. *Nat. Genet.* 9: 401–406.
- Gregory, C. W., et al. (2001). A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. *Cancer Res.* 61: 4315–4319.
- Zhou, Z. X., Lane, M. V., Kempainen, J. A., French, F. S., and Wilson, E. M. (1995). Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. *Mol. Endocrinol.* 9: 208–218.
- Abreu-Martin, M. T., Chari, A., Palladino, A. A., Craft, N. A., and Sawyers, C. L. (1999). Mitogen-activated protein kinase kinase 1 activates androgen receptor-dependent transcription and apoptosis in prostate cancer. *Mol. Cell. Biol.* 19: 5143–5154.
- Zhang, L., et al. (2003). Interrogating androgen receptor function in recurrent prostate cancer. *Cancer Res.* 63: 4552–4560.
- Latham, J. P., Searle, P. F., Mautner, V., and James, N. D. (2000). Prostate-specific antigen promoter/enhancer driven gene therapy for prostate cancer: construction and testing of a tissue-specific adenovirus vector. *Cancer Res.* 60: 334–341.
- Wu, L., et al. (2001). Chimeric PSA enhancers exhibit augmented activity in prostate cancer gene therapy vectors. *Gene Ther.* 8: 1416–1426.
- Adams, J. Y., et al. (2002). Visualization of advanced human prostate cancer lesions in living mice by a targeted gene transfer vector and optical imaging. *Nat. Med.* 8: 891–897.
- Iyer, M., Wu, L., Carey, M., Wang, Y., Smallwood, A., and Gambhir, S. S. (2001). Two-step transcriptional amplification as a method for imaging reporter gene expression using weak promoters. *Proc. Natl. Acad. Sci. USA* 98: 14595–14600.
- Zhang, L., et al. (2002). Molecular engineering of a two-step transcription amplification (TSTA) system for transgene delivery in prostate cancer. *Mol. Ther.* 5: 223–232.
- Blasberg, R. G., and Tjuvajev, J. G. (1999). Herpes simplex virus thymidine kinase as a marker/reporter gene for PET imaging of gene therapy. *Q. J. Nucl. Med.* 43: 163–169.
- Gambhir, S. S. (2002). Molecular imaging of cancer with positron emission tomography. *Nat. Rev. Cancer* 2: 683–693.
- Massoud, T., and Gambhir, S. S. (2003). Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev.* 17: 545–580.
- Rehmentulla, A., et al. (2000). Rapid and quantitative assessment of cancer treatment response using in vivo bioluminescence imaging. *Neoplasia* 2: 491–495.
- Wu, J. C., Sundaresan, G., Iyer, M., and Gambhir, S. S. (2001). Noninvasive optical imaging of firefly luciferase reporter gene expression in skeletal muscles of living mice. *Mol. Ther.* 4: 297–306.
- Ciana, P., et al. (2003). In vivo imaging of transcriptionally active estrogen receptors. *Nat. Med.* 9: 82–86.
- He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998). A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* 95: 2509–2514.
- Bergelson, J. M., et al. (1997). Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* 275: 1320–1323.
- Roelvink, P. W., Mi Lee, G., Einfield, D. A., Kovessi, I., and Wickham, T. J. (1999).

- Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae. *Science* **286**: 1568–1571.
38. Wickham, T. J., Mathias, P., Cheres, D. A., and Nemerow, G. R. (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell* **73**: 309–319.
  39. Fechner, H., et al. (2000). Trans-complementation of vector replication versus coxsackie-adenovirus-receptor overexpression to improve transgene expression in poorly permissive cancer cells. *Gene Ther.* **7**: 1954–1968.
  40. Rauen, K. A., et al. (2002). Expression of the coxsackie adenovirus receptor in normal prostate and in primary and metastatic prostate carcinoma: potential relevance to gene therapy. *Cancer Res.* **62**: 3812–3818.
  41. Klein, K. A., et al. (1997). Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. *Nat. Med.* **3**: 402–408.
  42. Fechner, H., et al. (1999). Expression of coxsackie adenovirus receptor and alphav-integrin does not correlate with adenovector targeting in vivo indicating anatomical vector barriers. *Gene Ther.* **6**: 1520–1535.
  43. Kijima, T., et al. (1999). Application of the Cre recombinase/loxP system further enhances antitumor effects in cell type-specific gene therapy against carcinoembryonic antigen-producing cancer. *Cancer Res.* **59**: 4906–4911.
  44. Ueda, K., et al. (2001). Carcinoembryonic antigen-specific suicide gene therapy of cytosine deaminase/5-fluorocytosine enhanced by the Cre/loxP system in the orthotopic gastric carcinoma model. *Cancer Res.* **61**: 6158–6162.
  45. Wu, L., Johnson, M. and Sato, M. (2003). Transcriptionally-targeted gene therapy to detect and treat cancer. *Trends Mol. Med.* **9**: 421–429.
  46. Shalev, M., et al. (2000). Suicide gene therapy toxicity after multiple and repeat injections in patients with localized prostate cancer. *J. Urol.* **163**: 1747–1750.
  47. Lohr, F., Huang, Q., Hu, K., Dewhirst, M. W., and Li, C. Y. (2001). Systemic vector leakage and transgene expression by intratumorally injected recombinant adenovirus vectors. *Clin. Cancer Res.* **7**: 3625–3628.
  48. Alemany, R., and Curiel, D. T. (2001). CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. *Gene Ther.* **8**: 1347–1353.
  49. Tao, N., et al. (2001). Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Mol. Ther.* **3**: 28–35.
  50. Morral, N., et al. (2002). Lethal toxicity, severe endothelial injury, and a threshold effect with high doses of an adenoviral vector in baboons. *Hum. Gene Ther.* **13**: 143–154.
  51. Zhang, Y., et al. (2001). Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. *Mol. Ther.* **3**: 697–707.
  52. Yang, Y., Su, Q., and Wilson, J. M. (1996). Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. *J. Virol.* **70**: 7209–7212.
  53. Jooss, K., Yang, Y., Fisher, K. J., and Wilson, J. M. (1998). Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *J. Virol.* **72**: 4212–4223.
  54. Pastore, L., et al. (1999). Use of a liver-specific promoter reduces immune response to the transgene in *Ads. Hum. Gene Ther.* **10**: 1773–1781.
  55. Nakamura, T., Sato, K., and Hamada, H. (2003). Reduction of natural adenovirus tropism to the liver by both ablation of fiber-coxsackievirus and adenovirus receptor interaction and use of replaceable short fiber. *J. Virol.* **77**: 2512–2521.
  56. Einfeld, D. A., et al. (2001). Reducing the native tropism of adenovirus vectors requires removal of both CAR and integrin interactions. *J. Virol.* **75**: 11284–11291.
  57. Perez-Stable, C. M., Pozas, A., and Roos, B. A. (2000). A role for GATA transcription factors in the androgen regulation of the prostate-specific antigen gene enhancer. *Mol. Cell. Endocrinol.* **167**: 43–53.
  58. Laverriere, A. C., MacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B., and Evans, T. (1994). GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. *J. Biol. Chem.* **269**: 23177–23184.
  59. Bruno, M. D., Korfhagen, T. R., Liu, C., Morrissey, E. E., and Whitsett, J. A. (2000). GATA-6 activates transcription of surfactant protein A. *J. Biol. Chem.* **275**: 1043–1049.
  60. Neve, R., et al. (1998). The epithelium-specific ets transcription factor ESX is associated with mammary gland development and involution. *FASEB J.* **12**: 1541–1550.
  61. Oettgen, P., et al. (2000). PDEF, a novel prostate epithelium-specific ets transcription factor, interacts with the androgen receptor and activates prostate-specific antigen gene expression. *J. Biol. Chem.* **275**: 1216–1225.
  62. Tymms, M. J., et al. (1997). A novel epithelial-expressed ETS gene, ELF3: human and murine cDNA sequences, murine genomic organization, human mapping to 1q32.2 and expression in tissues and cancer. *Oncogene* **15**: 2449–2462.
  63. Wang, Y., Iyer, M., Wu, L., Carey, M., and Gambhir, S. S. (2002). A two-step transcriptional approach for imaging of vascular endothelial growth factor (VEGF) gene expression using a bioluminescent reporter gene. *Mol. Imaging Biol.* **4**: 542.
  64. Qiao, J., et al. (2002). Tumor-specific transcriptional targeting of suicide gene therapy. *Gene Ther.* **9**: 168–175.
  65. Gambhir, S. S., et al. (2000). Imaging transgene expression with radionuclide imaging technologies. *Neoplasia* **2**: 118–138.
  66. Ray, P., Wu, A. M., and Gambhir, S. S. (2003). Optical bioluminescence and positron emission tomography imaging of a novel fusion reporter gene in tumor xenografts of living mice. *Cancer Res.* **63**: 1160–1165.

# Integrated, Molecular Engineering Approaches to Develop Prostate Cancer Gene Therapy

Lily Wu\* and Makoto Sato

Department of Urology, Jonsson Comprehensive Cancer Center and Crump Institute for Molecular Imaging, David Geffen School of Medicine at UCLA, Los Angeles CA 90095, USA

**Abstract:** Gene therapy is a translational science, with the ultimate goal of cancer gene therapy research being to develop effective and safe treatments for patients. In the new millennium, it is imperative to tailor a therapeutic strategy for a particular disease, based on clinical management issues. The desirable regulatory features and therapeutic strategies need to be fully considered before proceeding with molecular engineering of the gene delivery vector. Issues, such as cell-targeted expression, *in vivo* monitoring of gene delivery and expression, therapeutic strategies, and vector selection that targets the particular disease stage should be addressed. During the validation phase of the study, an objective evaluation in relevant animal models should determine whether the vector meets the desired specifications. Meeting the predetermined criteria should propel the product towards the clinical phase of evaluation. This review will present the conceptual framework that has been applied to developing an integrated and targeted gene therapy for prostate cancer.

**Keywords:** Prostate-specific expression, gene therapy, molecular imaging, hormone-refractory prostate cancer, metastasis, transgenic prostate cancer models, human prostate cancer xenograft, adenoviral vector.

## INTRODUCTION

Gene therapy offers the unique prospect of selectively introducing genes into cancer cells. Multiple biological processes can be targeted and exploited for gene-based therapeutic approaches [McCormick, 2001], including induction of cytotoxic and apoptotic responses, correction of aberrant growth regulation, and enhancement of anti-tumor immune responses in the tumor. However, the reality is that achieving efficient *in vivo* gene delivery to the targeted disease tissue is very complex. Heralded as safe in its inception, unanticipated side effects of gene therapy have occurred recently, and prompted the public and scientific community to question the value of clinical gene therapy [Marshall, 2000; Podsakoff, 2001; NIH report, 2002]. However, recent advances in defining the genetic alterations in cancer, in gene regulation, and in gene delivery vectorology could all be applied towards improving the efficacy of gene therapy.

The concept of cancer gene therapy is fundamentally sound, and it offers extraordinary potential to radically alter the outcome of cancer. At this juncture of justifiable concern and enthusiasm about the development of gene therapeutic approaches to cancer, it is an opportune time to critically review what we have learned, and to discuss what directions should be taken to realize the full potential of cancer gene therapy. In this review, we will focus on prostate cancer, and assimilate the information that portrays the clinical disease, governs prostate-specific expression, enables *in vivo* imaging of gene expression, and addresses issues in therapeutic genes and gene delivery vectors. The hope is that effective gene-

based therapeutic approaches could be tailor-made for this disease in the near future.

## BIOLOGY OF HUMAN PROSTATE CANCER

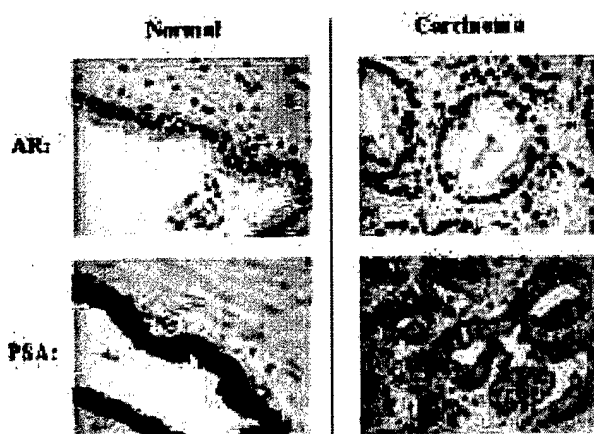
Prostate cancer is the second leading cause of cancer deaths in American men. In 2002, diagnosis of about 189,000 new cases of prostate cancer have been estimated, and that more than 30,000 American men have been died from this disease (American Red Cross, <http://prostate-help.org/castats.htm>). Since the mid-1980s, the incidence of prostate cancer has increased, likely due to the better detection method of the sensitive serum prostate-specific antigen (PSA) screening test [Papsidero, 1980]. More recently, since 1992, both the incidence and mortality rates have shown a downward trend [Newcomer, 1997; Han, 2001]. Due to the general protracted natural history of prostate cancer development and potential side effects of different treatments, therapeutic decisions for patients and physicians are not straightforward [Hegarty, 1999]. Radical prostatectomy and radiation therapy are the two most common therapeutic modalities [Harlan, 2001]. Up to one-third of patients with the localized disease, who have undergone treatments with curative intent [Han, 2003; Coen, 2002] will experience disease recurrence and metastasis, as determined by elevated levels of serum PSA. Moreover, nearly 20% of newly diagnosed patients are present with metastatic disease [Christiano, 2000]. The ominous nature of recurrent metastatic disease is signified by the fact that the majority (nearly 60%) of these patients die within five years of recurrence [Smaletz, 2002].

A better understanding of the molecular pathogenesis of prostate cancer, and in particular, hormone-refractory disease progression will be vital for the development of an effective treatment. Much research activity has been focused on the androgen receptor (AR) signaling pathway, as AR is the

\*Address correspondence to this author at the Departments of Urology, Jonsson Comprehensive Cancer Center and Crump Institute for Molecular Imaging, David Geffen School of Medicine at UCLA, Los Angeles CA 90095 Box 951738, USA; Tel: 310/794-4390; Fax: 310/206-5343; E-mail: [lwu@mednet.ucla.edu](mailto:lwu@mednet.ucla.edu)

critical mediator of the biological effects of androgen [Gelman, 2002]. In androgen-dependent prostate cancer cells, the ligand-bound AR translocates from the cytoplasm into the nucleus and binds to androgen-responsive elements (AREs) to activate the target genes. The PSA gene is one such prostate-specific AR-regulated target gene that has served as an invaluable serological biomarker to detect prostate cancer and monitor treatment response [Bok, 2002]. As shown in (Fig. 1), both AR and PSA are expressed in normal and carcinomatous prostatic epithelia; in fact, expression of AR and PSA is detected in all stages of prostate cancer, including hormone-refractory prostate cancer (HRPC) and distant metastases [van der Kwast, 1996; Sweat, 1999; Hobisch, 1995; Koivisto, 1999].

Over 60 years ago, Huggins and Hodges [Huggins, 1941] first reported that removal of testicular androgen by castration induces prostate cancer regression. The key concept is that removal of androgen would impede AR function and the expression of prostatic growth regulatory genes. Thus, androgen ablation remains the primary mode of treatment for high-grade, recurrent, or metastatic prostate cancer [Hellerstedt, 2002; Labrie, 2002]. In 80-90% of patients, this hormonal therapy induces an initial remission, with symptomatic relief and reduction in PSA levels. However, the response is usually transient, sustaining a median progression-free interval of up to 33 months [Denis, 1993], followed by the inevitable progression to HRPC. At this time, the patient's median survival time is approximately 12 months. Due to the fact that PSA expression is AR-regulated, it serves as an indicator of AR function. Therefore, elevated serum PSA levels during androgen ablation are equated to the emergence of HRPC, and it is the most reliable means to detect recurrent disease [Bok, 2002].



**Fig. (1).** Androgen receptor and PSA expression in normal and cancerous prostate epithelia. Paraffin-embedded sections obtained from archives of clinical samples were stained with anti-AR- $\beta$  antibody (Upstate, Lake Placid, NY) and anti-PSA antibody (Novocastra, Newcastle upon Tyne, UK), as described previously [Adams 2002]. The brown positive staining is localized mainly to the nucleus for AR and to the cytoplasm for PSA. Several samples of high-grade and metastatic lesions also stained positive for both markers (data not shown).

The precise mechanism by which AR functions in the androgen-deprived environment of HRPC is not fully understood. Several recent reviews [Feldman, 2001; Navarro, 2002; Balk, 2002; Debes, 2002] have covered this important topic. Feldman *et al.* [Feldman, 2001] designated the three best-supported mechanisms as “hypersensitive AR”, “promiscuous AR”, and “outlaw pathway”. The “hypersensitive AR” mechanism proposes that overexpression of AR or the co-activators of nuclear receptors could restore AR signaling in the presence of very low intracellular levels of androgen. In support of this concept, AR gene amplification and overexpression was detected in up to 30% of hormone-refractory recurrent tumors, whereas none of the primary tumors from the same patients exhibited the AR amplification prior to androgen ablation [Visakorpi, 1995; Koivisto, 1997]. There are two additional strategies that HRPC could utilize to achieve hypersensitive AR function in castrated levels of androgen. Gregory *et al.* have shown that AR protein is stabilized in recurrent prostate cancer xenografts [Gregory, 2001b], and many recurrent prostate cancer clinical samples display elevated expression levels of AR, as well as two nuclear receptor coactivators, SRC1 and SRC2 [Gregory, 2001a].

The “promiscuous AR” concept proposes that acquisition of mutations that broadened the AR ligand specificity is a means by which HRPC activates AR pathways in androgen-deprived conditions. Recent studies using microdissected metastatic tumor samples [Marcelli, 2000] confirmed an increased incidence of AR mutations in advanced cancer as compared to primary tumors. Results from clinical investigations [Marcelli, 2000; Taplin, 1995; Tilley, 1996] and transgenic mouse models [Han G, 2001; Buchanan, 2001] indicate that AR mutations may play a role in cancer progression. The T877A AR point mutation identified in LNCaP cells [Veldscholte, 1992; Gaddipati, 1994] allows this mutated AR to activate gene expression in response to progesterone, estrogen, and even anti-androgen hydroxyflutamide, which are ligands that are inactive in wild-type AR. Another clear example of expanded AR ligand specificity was demonstrated in the MDA PCa 2a and 2b cell lines established from bone metastasis by Navone *et al.* at the M.D. Anderson Cancer Center [Navone, 1997]. This cell line harbors T877A and L701H double-mutations in the ligand-binding domain of AR, which exhibits reduced androgen-binding affinity. Interestingly, this double-mutated AR can activate gene expression and cell growth in response to glucocorticoids [Zhao, 1999; Zhao, 2000]. These results support the hypothesis that under selective pressure of androgen deprivation, alternative ligands could be activating the altered AR to promote growth in advanced stages of the disease.

In the “outlaw pathway”, other growth factor pathways not related to steroid hormone receptors serve to activate AR signaling and induce the expression of AR target genes in the absence of androgen. Many growth regulatory pathways, such as insulin-like growth factor-1 (IGF-1), keratinocyte growth factor (KGF), epidermal growth factor (EGF), HER-2/neu, and protein kinase A (PKA), can induce androgen-responsive genes such as PSA [Culig, 1994; Craft, 1999a; Sadar, 1999]. Interestingly, AR protein is still required in these HRPC studies, indicating a phenomenon of cross talk

between growth factor and AR pathways rather than by-passing of the AR function.

The three mechanisms discussed above are not mutually exclusive. Thus, HRPC cells might likely utilize a combination of these strategies to overcome androgen-deprived growth conditions.

What can we extrapolate from the extensive knowledge of prostate cancer biology and apply to future gene-based therapeutic approaches? Firstly, future therapy should attempt to target advanced disease, such as recurrent HRPC or metastatic disease, since no effective therapy is available at this time. Secondly, AR-mediated pathways are likely to be active in HRPC, and are thus amenable as therapeutic targets.

### ANIMAL MODELS OF PROSTATE CANCER

Whether the aim is to study cancer biology or to develop effective treatments, valid clinically relevant animal models of human prostate cancer are needed. In addition to mice bearing human prostate cancer xenografts [van Weerden, 2000; Reiter, 2001], many rodent prostate carcinoma models have been developed by methods, such as hormone treatment [Noble, 1977], spontaneous development [Dunning, 1963], transgenic prostate-specific oncogene expression [Greenberg, 1995], and knockout of tumor suppressor genes [Podsypanina, 1999]. Interested readers should direct their attention to several recent reviews that cover the topic of animal models of prostate cancer in detail [van Weerden, 2000; Reiter, 2001; Abate-Shen, 2002; Castrillon, 2001; Gendler, 2001; Zhau, 2000, Thompson, 2000; Navone, 1998]. This review will cover the salient features of transgenic mouse models of prostate cancer and xenograft models; however, no model is perfect at this time, yet each system has its advantages. Two issues that should caution researchers against fully relying on one mouse model to study human disease are: 1) the developmental processes of the rodent and human prostate are different; and 2) few rodent models recapitulate the complete spectrum of human disease progression. At this time, there is a severe shortage of prostate cancer animal models that will spontaneously disseminate to the bone and fully mimic the osteoblastic lesions of skeletal metastasis observed in clinical situation [Zhau, 2000].

In the transgenic adenocarcinoma of mouse prostate (TRAMP) model, prostate-restricted expression of potent oncogenic SV40 large T-antigen and small T-antigen results in an aggressive reproducible murine prostatic carcinoma [Greenberg, 1995]. The TRAMP mice develop high-grade dysplasia and carcinoma within 12 weeks of age, and ultimately develop metastases by 28 weeks, primarily to the lungs and lymph nodes, and less often, to bone [Greenberg, 1995; Gingrich, 1996]. Androgen ablation by castration results in decreased tumor burden compared to non-castrated TRAMP animals early in the course of disease. However, aggressive androgen-independent tumors emerge in castrated mice at a much higher rate than in non-castrated controls in later stages of disease [Gingrich, 1997]. In a similar "LADY" transgenic model, a larger region of the prostate-specific rat probasin promoter was utilized to drive the

expression of the SV40 large T-antigen [Kasper, 1998]. Interestingly, cancer development and progression in the LADY model is less aggressive than in TRAMP mice [Kasper, 1998; Masumori, 2001]. These time-dependent progression models, highly reminiscent of human disease, provide a fertile ground to investigate growth regulatory pathways involved in murine prostatic carcinoma and potential chemoprevention and therapeutic interventions [Kaplan, 1999; Huss, 2001; Mentor-Marcel, 2001].

As information about genetic and molecular alterations in human prostate cancer is being accumulated at a rapid pace, transgenic manipulation is becoming the preferred approach to verify the involvement of a particular molecular pathway in oncogenesis. For example, elevated serum IGF-1 levels are correlated with increased risk for prostate cancer [Chan, 1998]. In the corresponding transgenic model, targeted IGF-1 expression in basal epithelial cells by the keratin 5 promoter results in dysplastic and neoplastic changes in the prostate, starting at around six months of age in 50% of the transgenic mice [DiGiovanni, 2000]. To clarify whether AR plays a direct oncogenic role, a transgenic model was developed in which prostate-targeted AR expression was controlled by the minimal rat probasin promoter. The phenotypic alteration of this model is quite mild, with hyperplastic and dysplastic changes noted at one year of age [Stanbrough, 2001]. It would be interesting to investigate whether overt overexpression of AR could result in a more aggressive oncogenic process.

Until recently, few human prostate cancer cell lines, with the exception of LNCaP, were androgen-responsive and expressed PSA and AR. Several investigators have established xenografts whereby clinical prostate cancer samples, most commonly derived from metastatic lesions, were implanted into and propagated directly in immunodeficient mice [van Weerden, 2000; Reiter, 2001]. The xenografts recapitulate *in vivo* many of the clinical features of human prostate cancer [Klein, 1997; Craft, 1999b]. Two well-characterized xenografts established at UCLA, LAPC-4 and LAPC-9, both have intact AR transcription pathways and produce easily detectable levels of PSA in the serum. They grow as androgen-dependent (AD) tumors in male mice, respond to androgen ablation treatment, then eventually progress to a hormone-refractory stage. Subcutaneous LAPC-4 tumors were documented to metastasize to regional lymph nodes, the blood stream, and the lung, as determined by a PCR technique [Klein, 1997]. Injected LAPC-4 cells homed to human bone implants *in vivo* and established osteolytic lesions [Tsingotjidou, 1999]. Moreover, a cell line that can be maintained in tissue culture has been adapted from the LAPC-4 xenograft [Klein, 1997], and it retains the androgen-responsive features of the xenograft.

Several other institutions have established useful human prostate cancer xenograft models with a spectrum of androgen responsiveness, AR mutation status, and PSA expression levels. The University of Rotterdam, Netherlands has acquired the most extensive experience, with more than ten long-term established models [van Weerden, 2000; van Weerden, 1996]. A distinguishing feature of the Rotterdam models is that no reconstituted extracellular matrix

(Matrigel) was used to establish the subcutaneous tumors, unlike procedures at other institutions. The majority of the Rotterdam models expresses wild-type AR and is androgen-responsive [van Weerden, 2000]. Interestingly, PC-295 and PC-310 are two androgen-dependent models that can be induced to exhibit neuroendocrine differentiation upon androgen deprivation treatment [Jongsma, 1999; Jongsma, 2002]. LuCaP models developed at the University of Washington exhibit a range of growth patterns in response to castration, including minimal responsiveness with continued tumor growth, intermediate responsiveness exhibiting a small decline in growth and eventual progression, and sustained growth arrest [Ellis, 1996]. Interestingly, intratibial-implanted LuCaP 35 induces an osteoblastic bone growth response, which is reminiscent of clinical metastatic bone lesions [Corey, 2002]. In 1991, Pretlow *et al.* [Pretlow, 1991] generated the CWR22 model derived from a primary tumor, which is a well-studied model with a hormone refractory subline, CWR22R [Nagabhushan, 1996]. Interestingly, the AR in CWR22 harbors a missense mutation in the ligand-binding domain (H874Y), which results in expanded tropism similar to that in the LNCaP cell line [Tan, 1997]. Availability of multiple xenografts, covering a wide spectrum of clinical manifestations, will greatly benefit the investigation of prostate cancer biology and potential therapeutic interventions.

In the development of prostate cancer-targeted gene therapy approaches, what would be a rational approach to utilize some of these available models productively? The approach that could be taken to evaluate a prostate-targeted vector is to first assess proper regulation in human prostate cancer cell lines, such as LNCaP and LAPC-4. By comparing expression levels of a vector in prostate and non-prostate cell lines, the cell type-discriminating ability of the vector could be established. Next, evaluation of vector expression profiles in prostate cancer xenografts and/or transgenic mouse models would be very beneficial to defining the capability of the targeted gene delivery vehicle *in vivo*. The transgenic models also provide a competent immune system in which to evaluate the vector. We have followed this systematic approach to evaluate our PSA promoter-based prostate-targeted vectors [Wu, 2001; Zhang, 2002; Iyer, 2001; Adams, 2002].

#### PROSTATE-SPECIFIC GENE EXPRESSION

The use of a prostate-targeted vector with greatly enhanced transcriptional activity, as well as highly accurate cell-specific discrimination should improve the efficacy and safety of prostate cancer gene therapy. An effective approach is transcriptional targeting (i.e., restricting expression to prostate-specific tissue by using gene regulatory elements), which is particularly suitable to target tissue of prostate origin because the prostate is one of the organs (in addition to pancreas and breast) that express an unusually high number of unique genes. Many of these prostate-specific gene regulatory regions, such as PSA and prostate-specific membrane antigen (PSMA), are well characterized (Table 1). A survey of the Cancer Genome Anatomy Project (CGAP) database published by the National Cancer Institute (<http://www.ncbi.nlm.nih.gov/ncicgap/>) lists more than 2000

prostate-specific genes, although the majority of them are not fully characterized at this time.

The current strategies of prostate cancer gene therapy are covered in several recent reviews [Mabjeesh, 2002; Lu, 2001; Hsieh, 2001; Koenen, 2001; Pantuck, 2001; Steiner, 2000]. Here we emphasize prostate transcriptionally targeted approaches and introduce strategies to amplify gene expression. It is important to keep in mind that *in vivo* application of a transcriptionally targeted vector does not diminish gene transduction to non-targeted tissue; rather, only the gene expression step is restricted. The targeted vector inadvertently delivered to normal tissues will remain transcriptionally silent, and thus reduce potential side effects of any cytotoxic gene therapy. Due to the fact that the frequently utilized promoters are prostate tissue-specific but not prostate cancer-specific, the normal prostate gland would be affected by this therapy; however, two clinical facts reduce the concerns about damaging normal prostate tissues. In the older patient population that is diagnosed with prostate cancer, the prostate gland is expendable, as it does not serve any essential function [Steiner, 2000]. Moreover, if the recurrent or metastatic disease is the intended treatment target, the primary prostate gland would likely have already undergone surgical resection or radiation therapy.

Table 1 compiles the findings on several frequently utilized prostate-specific promoters of human and rat origin. The best studied is the PSA gene (hk3), which encodes a serine protease [Aumüller, 1990]. Since PSA is expressed in all stages of cancerous prostatic epithelial cells, its gene regulatory regions serve as prime candidates to direct prostate-specific gene expression [Schoor, 1996; Cleutjens, 1997b; Pang, 1997]. The precise mechanism by which the PSA regulatory elements orchestrate accurate prostate-specific transcription is not completely understood. The PSA 600 base-pair proximal promoter contains a TATA box and two functionally important AREs (binding sites for AR) [Riegman, 1991; Zhang, 1997]. The PSA enhancer core (nt -4366 to -3874) is a functionally important region that contains a cluster of at least four AREs [Huang, 1999], which can be further subdivided into two types. The class I and II AREs each have distinctive nucleotide variations and confer differential AR binding affinity and patterns [Reid, 2001]. The overall effect of having two classes of AREs in the PSA enhancer core is to facilitate the cooperative AR-AR interaction and synergistic transcriptional activation [Huang, 1999; Reid, 2001]. A combination of general and prostate-specific transcription factors is likely working in concert to mediate PSA cell-specific gene expression [Schoor, 1996; Perez-Stable, 2000; Oettgen, 2000]. GATA transcription factors have been shown to induce the androgen-responsive activity of the PSA enhancer and promoter [Perez-Stable, 2000]. Other factors, such as AP-1, c-Fos, and CREB, might also contribute to prostate-specific regulation, as putative binding sites have been identified in the PSA upstream gene regulatory region [Schoor, 1996]. PDEF is a prostate epithelium-specific Ets factor that has been shown to interact with AR and activate PSA gene expression [Oettgen, 2000]. Cell culture transfections [Schoor, 1996; Cleutjens, 1997b; Pang, 1997; Riegman, 1991; Zhang, 1997], viral vector transduction in animal models [Wu, 2001; Gotoh, 1998], and transgenic mouse studies [Cleutjens, 1997a; Wei, 1997] have

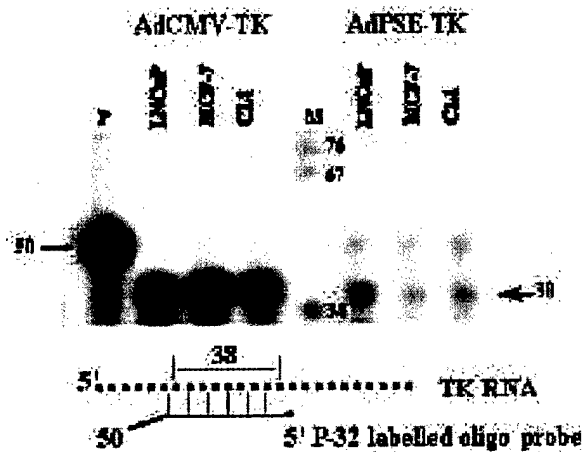
Table 1. Prostate-Specific Gene Expression

promoter	experiment	transgene; result summary	Reference
<i>human origin</i>			
PSA	transfection	reporters; prostate specific expression and androgen regulation	Riegman (1991), Schuur (1996), Cleutjens (1997), Pang (1997), Zhang (1997)
		PARP (apoptotic); androgen-inducible DNA-damaging	Trofimova (2002)
	adenovirus	HSV-TK (therapeutic); cell-killing in vitro and inhibition of growth in tumor model	Huang (1999)
		E1A (oncolytic); selective replication in LNCaP, regression in tumor model	Reid (2001)
	lentivirus	DTA (therapeutic); cell killing in vitro, specific EGFP expression in tumor model	Yu (2001)
	transgenic mice	lacZ (reporter); prostate specific activity and a decline with castration	Perez-Stable (2000)
human PSA; prostate specific expression		Oettgen (2000)	
kallikrein 2 (hK2/hKLK2)	transfection	CAT (reporter); expression in prostate cancer cell lines and androgen regulation	Murtha (1993), Mitchell (2000)
	adenovirus	EGFP (reporter); specific expression in tumor model	Xie (2001)
		E1 protein (oncolytic); expression in PSA(+), selective replication in prostate tumor cells	Yu (1999)
PSMA	transfection	luciferase (reporter); expression in LNCaP	O'Keefe (1998)
		luciferase (reporter); expression in prostate cells, negative regulation with androgen	Watt (2001), Noss (2002)
		CD (therapeutic); expression in PMSA(+), cell killing in vitro (108) and in vivo (193)	O'Keefe (2000), Uchida (2001)
PSCA	transfection	luciferase, GFP (reporter); cell specific and androgen responsive expression	Jain (2002)
	transgenic mice	EGFP (reporter); expression in the prostate and prostate cancer	Watabe (2002)
<i>rodent origin</i>			
rat C3(1)	transgenic mice	SV40 T-Ag (oncogene); development of hyperplasia and adenocarcinoma	Maroulakou (1994)
		bcl-2, c-myc (oncogene); limited expression and hyperplasia	Zhang (1997), Zhang (2000)
rat probasin	transfection	CAT (reporter); androgen regulation in PC-3	Rennie (1993)
	adenovirus	iCaspase-9 (apoptotic); expression and apoptosis in LNCaP and tumor model	Xie (2001)
		E1A (oncolytic); selective replication in PSA(+) cell lines	Yu (1999)
		Bax (apoptotic); cell-specific expression and cell death in LNCaP	Lowe (2001)
	transgenic mice	SV40 T-Ag (oncogene); development of prostatic disease (PIN to metastatic cancer) <TRAMP model>	Dunning (1963)
SV40 T-Ag (oncogene); development of prostatic disease (PIN to cancer) <LADY model>		Gingrich (1997)	
PSP94	transgenic mice	SV40 T-Ag (oncogene); tumor development in the prostate	Gabril (2002)
<i>bone/cancer target</i>			
Osteocalcin	adenovirus	HSV-TK (therapeutic); Phase I clinical trial	Koeman (2000)
		E1A+E1B (oncolytic); growth inhibition of AD and AI cells and in tumor model	Hsieh (2002)
		E1A (oncolytic); inhibition of the growth of prostate cancer cell lines, tumor suppression with tumor model	Matsubara (2002)

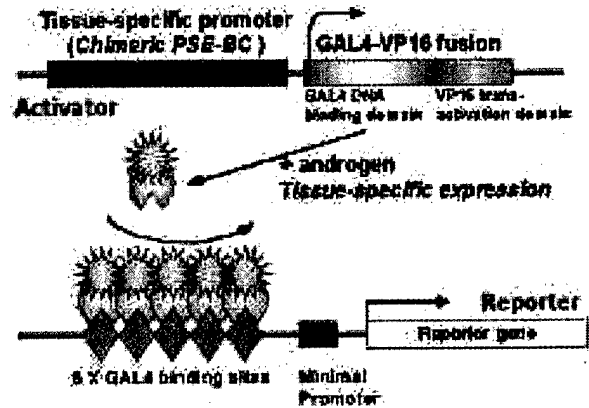
Abbreviations: PARP, poly(ADP-ribose) polymerase; HSV-TK, herpes simplex virus thymidine kinase; E1, adenovirus immediate early gene 1; DTA, diphtheria toxin A; lacZ, beta-galactosidase; CAT, chloramphenicol acetyl transferase; EGFP, enhanced

demonstrated the prostate specificity and androgen-responsive activity of PSA regulatory regions.

In our experience, the magnitude of the native PSA promoter and enhancer (PSE) [Wu, 2001] might not be sufficient to mediate robust expression in vector-based gene therapy applications. As illustrated in (Fig. 2), due to the weak activity of PSE, differential RNA expression levels in prostate versus non-prostate cells was difficult to confirm. Two useful strategies have been applied to augment the activity of the PSA promoter, while maintaining its specificity. In the first strategy, synergistic activation of the PSA upstream enhancer can be achieved by insertion of four tandem copies of synthetic AREs [Wu, 2001], or by duplication of the enhancer core [Wu, 2001; Latham, 2000]. We demonstrated that the PSE-BC construct with a duplicated enhancer was nearly 20-fold more active than the native PSE construct [Wu, 2001]. In a second approach, a two-step transcriptional amplification (TSTA) system was employed to boost the activity of the PSA enhancer/promoter over a range of 1000-fold [Zhang, 2002; Iyer, 2001]. In this system, illustrated in (Fig. 3), the PSA regulatory region was used to express the potent artificial transcription activator, GAL4-VP16, which acts upon a GAL4-responsive reporter. Optimal TSTA constructs displayed activity levels



**Fig. (2).** HSV-TK RNA expression mediated by prostate-targeted or constitutive adenoviral vector. Cells were infected with AdCMV-TK or AdPSE-TK- at an MOI of 5. Post-infection (48 hrs), cytoplasmic RNA was harvested and subjected to S1 nuclease protection analysis. RNA (10 and 50  $\mu$ g) from AdCMV-TK- and AdPSE-TK-infected cells, respectively, was analyzed. The unprotected TK 5' end-labeled oligonucleotide probe is 50 bases in length. TK mRNA transcribed from the CMV or PSE promoter would protect a 38-base probe fragment. TK RNA expression mediated by the CMV promoter was similar in all cells, and was 100X higher than that mediated by the PSE promoter in LNCaP and CL1 cells (after correcting for input RNA concentrations). TK RNA from PSE in LNCaP is 3-5X higher than in MCF-7. P=probe alone; M=marker; LNCaP is an androgen-dependent prostate cancer line; MCF-7 is a breast carcinoma cell line; and CL1 is an androgen-independent cell line derived from LNCaP [Tso 2000].



**Fig. (3).** A schematic diagram of the two-step transcriptional amplification (TSTA) system. In this prostate-specific expression scheme, the chimeric PSE-BC promoter derived from PSA drives the expression of the artificial fusion protein, GAL4-VP16. This synthetic fusion protein is composed of two components, the DNA-binding domain of the yeast transcriptional activator, GAL4, and the transcriptional activator of herpes simplex virus, VP16. In turn, this GAL4-VP16 protein binds specifically to multiple repeats of GAL4-binding sites upstream of a reporter gene. The extensive dynamic range of transcriptional amplification can be modulated by: 1) the variety of tissue-specific promoters utilized; 2) the multiplicity of the VP16 activator domain; and 3) the number of GAL4-binding sites.

significantly higher than the cytomegalovirus (CMV) promoter-driven firefly luciferase, while maintaining prostate cell specificity and ligand responsiveness [Zhang, 2002; Iyer, 2001].

PSA (hk3) belongs to a large 15-member human kallikrein gene family, located on chromosome 19q13.4 [Yousef, 2002]. The large number of kallikrein genes, likely resulting from gene duplication, is all differentially expressed in endocrine-related malignancies, such as prostate, breast, and ovarian and testicular cancers. The human glandular kallikrein 2 (hk2) gene located 12 kb away from the PSA gene in the genome also exhibits restricted expression in prostate epithelium. The hk2 gene is an androgen-activated gene, and its upstream regulatory regions contain AREs highly homologous to the known AREs of PSA [Mitchell, 2000]. DNA transfection and adenoviral vector transduction studies have confirmed the androgen-responsive and cell-specific nature of the hk2 promoter [Mitchell, 2000; Xie, 2001a; Yu, 1999b]. In addition, the increased hk2 expression in carcinoma, as compared to benign tissues, supports its utility as a prostate cancer biomarker [Yousef, 2002]. Future studies are needed to compare the utility of PSA and hk2, both as biomarkers and in targeted gene therapy applications.

PSMA [Fair, 1997] and prostate stem cell antigen (PSCA) [Reiter, 1998] are two integral membrane proteins that exhibit preferential prostate expression. PSMA was first identified by a monoclonal antibody to be expressed in primary prostate cancer and lymph node metastases [Horoszewicz, 1987]. Detailed immunohistochemical

analyses revealed that PSMA expression is not restricted to prostatic tissue alone [Silver, 1997]. Interestingly, PSMA promoter-driven cytotoxic gene expression is induced by androgen deprivation and is more effective in eradicating androgen-independent prostate cancer cells [O'Keefe, 2000]. The PSCA expression profile also appears to be less restricted than is originally anticipated [Amara, 2001]. The PSCA gene regulatory regions contain both androgen-dependent and -independent components [Jain, 2002]. Combined with the finding that PSCA expression is increased in advanced stage and metastatic disease [Gu, 2000], it might be advantageous to utilize the PSCA enhancer in gene therapeutic settings for advanced disease. The normal biological roles of PSMA and PSCA are unclear, but their potential contributions in prostate development and oncogenesis are of substantial interest.

Several rodent prostate promoters have been utilized frequently in transgenic mouse studies, resulting in the generation of many successful prostate-targeted knockout and carcinogenesis models, including the rat C3 and probasin promoters [Greenberg, 1995; Podsypanina, 1999; Kasper, 1998; Maroulakou, 1994], and recently, the mouse PSP94 gene regulatory regions [Gabril, 2002]. These rodent-based promoters are less well characterized in gene expression and therapy studies in human models. The probasin promoter is highly androgen-responsive and contains multiple AREs [Kasper, 1994]. Several recent studies have shown that probasin-based adenoviral vectors can mediate prostate-selective transgene expression and therapeutic responses in human prostate cells [Xie, 2001; Yu, 1999a; Lowe, 2001]. Since PSP94 is a prostate secretory protein that is conserved across mammalian species, its gene regulatory regions might be useful to delineate the transcriptional machinery that governs prostate-specific expression both in rodents and humans.

Selective targeting with the prostate tumor-specific promoter is not feasible at this time, because no gene has yet been identified that is unique to prostate cancer. However, one promising approach employed by Dr. Leland Chung's group is to target both the bone stromal component and malignant prostatic epithelial cells, using the osteocalcin promoter [Yeung, 2002]. Osteocalcin is a bone matrix protein produced exclusively by osteoblasts, and its expression is regulated by multiple elements, including the osteo-specific OSE2 element and the vitamin D-responsive element [Lian, 1999; Hsieh, 2002]. Interestingly, primary and metastatic prostate cancer cells were shown to express osteocalcin [Matsubara, 2001]. Osteocalcin promoter-activated adenoviral replication in an oncolytic strategy was capable of suppressing growth of prostate tumors in animals [Hsieh, 2002; Matsubara, 2001]. Whether employing a tissue- or tumor-targeted approach, both high-magnitude of expression and specificity are very important for achieving effective *in vivo* therapeutic responses. In the oncolytic scheme, key viral regulator E1A and E1B proteins are not needed at high levels to induce adenovirus replication, which results in a 3- to 4-order amplification of infectious virus [Yu, 1999a; Matsubara, 2001]. Thus, oncolytic therapy with improved tissue or tumor selectivity is being actively pursued [Hawkins, 2002].

Vector delivery by targeting cell surface molecules specifically expressed on prostate cancer cells (i.e., transductional targeting) is a powerful concept and an active field of investigation. Cell surface targets can be based on pre-determined membrane antigens (e.g. PSMA or PSCA) or identified by selecting in phage-displayed peptide libraries for phages capable of homing in onto the prostate [Arap, 2002]. Since the adenoviral vector is a popular cancer gene therapy vehicle, several approaches have been developed to re-direct its tropism via specific cell surface molecules. Modifications of viral fiber protein through either genetic alterations [Krasnykh, 2001; Stevenson, 1997] or through conjugation with bi-specific antibodies [Haisma, 2000; Wickham, 1996] have shown promising results. Interestingly, cells from advanced prostate cancer might be more favorable for adenoviral-mediated transduction, because Coxsackie adenoviral receptor (CAR), the endogenous cellular adenoviral receptor, is overexpressed in metastatic lesions [Rauen, 2002].

Prostate-targeted gene delivery and expression approaches are continually being refined. Although dependent upon the particular therapeutic strategy, the precise magnitude of gene product needed could vary greatly; for example, in strategies with amplification effects, such as immune stimulatory approaches or viral oncolysis, a high level of expression is probably not necessary. However, for growth suppressive or cytotoxic gene therapy schemes, achieving cancer-restricted expression equivalent or above CMV levels might be essential [Gerdes, 2000]. Using a combinatorial strategy might be fruitful for attaining further restricted expression. For example, an artificial chimeric enhancer derived from PSA and PSMA elements has been generated that exhibits high tissue specificity [Lee, 2002]. Moreover, combining transcriptional and transduction targeted approaches might likely result in an added level of specificity [Reynolds, 2001]. It would be very important to evaluate the capabilities of future prostate-targeted vectors in relevant animal models with accurate quantitative assays.

#### NON-INVASIVE IMAGING ASSAYS FOR SMALL ANIMALS

Imaging transgene expression *in vivo* is a powerful tool to assess and optimize targeted gene therapy approaches. Due to the multitude of therapeutic genes utilized, direct imaging of each therapeutic gene is not feasible. Thus, an imaging reporter gene delivered and expressed in conjunction with the therapeutic gene becomes an important general approach taken to monitor expression *in vivo*. Rapid advances in imaging technologies have accomplished repetitive monitoring of detailed location, magnitude, and kinetics of reporter gene expression in living small animals [Ray, 2000; Gambhir, 2002; Blasberg, 1999]. Complete coverage of these imaging advances is beyond the scope of this review; instead, we will focus on the few imaging modalities that have frequently been applied in pre-clinical gene therapy models.

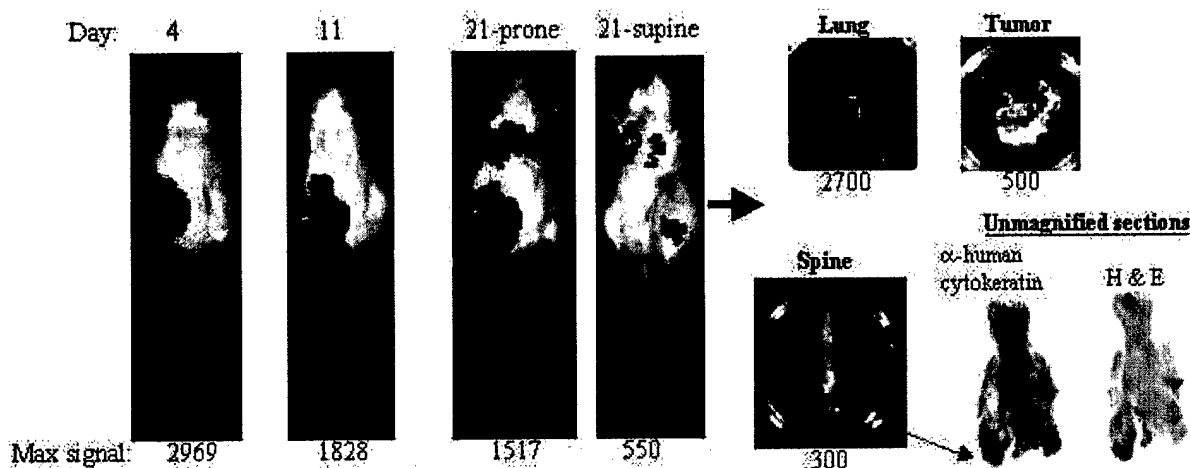
Bioluminescence imaging (BLI) that takes the advantage of the light-producing properties of disparate proteins produced in nature has become a favorable modality in many mouse cancer models. All optical approaches have the

distinct advantage of low background signal, ease of use, and low cost, in comparison to radionuclide imaging; however, they are limited by light scatter and absorption, presenting difficulties in detecting and localizing signals in deep tissues. Two recently developed optical imaging systems based either on the firefly luciferase or green fluorescent protein (GFP) gene enable visualization of gene expression non-invasively in living animals [Contag, 1998; Yang, 2000]. GFP fluorescent approaches have been used to track metastasis and angiogenesis in marked tumor models [Hoffman, 2002], but an additional external source of light is needed to excite the GFP in fluorescence imaging [Hoffman, 2002].

Luciferase is a generic term for a family of photoproteins that can be isolated from insects, marine organisms, and prokaryotes [Hastings, 1996]. Biochemically, all luciferases are oxygenases that utilize molecular oxygen to oxidize a substrate, with the formation of product in an electronically excited state. The bioluminescent systems are not evolutionarily conserved; thus, each luciferase isolated from a particular organism catalyzes a unique substrate, with the emission spectra ranges between 400 and 620 nm [Hastings, 1996]. Imaging of firefly luciferase (FL) gene expression in living animals has been carried out using a highly sensitivity charged coupled device (CCD) camera [Contag, 1998; Wu J, 2001]. Light is produced through the interaction of FL with its substrate, D-luciferin, injected peritoneally in the presence of magnesium and ATP [Contag, 1998]. This FL optical imaging approach has been used to monitor tumor cell growth and metastasis [Adams, 2002; Edinger, 1999; Honigman, 2001] and vector-mediated gene delivery and expression [Adams, 2002; Wu J, 2001; Honigman, 2001; Lipshutz, 2001].

We have successfully employed a prostate-targeted adenoviral vector and FL optical imaging to detect metastatic lesions in living mice [Adams, 2002]. An example of this approach is shown in (Fig. 4). One key advantage of a non-invasive imaging is that sequential detection of signals in the same animal in a time-dependent manner often alleviates some uncertainty due to inter-animal technical variations. However, to overcome the limitation of precise three-dimensional signal localization in the animal, we rely on post-mortem imaging of isolated organs (Figure 4) and further histological and pathological analyses of the tissues [Adams, 2002].

Multiple distinct luciferases could potentially be developed for BLI. This exciting development means that it will be possible to simultaneously monitor multiple pathways or cell populations in the animal. In fact, Dr. Sanjiv Gambhir's group has demonstrated the feasibility of this principle by simultaneously monitoring Renilla luciferase (RL) and FL [Bhaumik, 2002]. RL is purified from sea pansy, a bioluminescent soft coral. This enzyme has an origin, enzyme structure and substrate requirements distinct from FL, and it catalyzes coelenterazine oxidation [Inouye 1997]. Thus, by injecting coelenterazine or D-luciferin, respective levels of RL and FL expressions can be imaged simultaneously in the same mouse to track two different cell populations or gene therapy vectors [Bhaumik, 2002]. Although BLI is widely applicable to investigate many biological processes in small animals [Ciana, 2003], it cannot yet be generalized to human studies. To evaluate the feasibility of clinical imaging modalities in small animals, our institution has adapted the reporter gene concept to radionuclide imaging of positron emission tomography (PET) [Gambhir, 2002].



**Fig. (4). Detection of metastatic prostate cancer lesions by optical imaging.** Mice bearing LAPC-4 xenografts in the left flank received intratumoral injection of  $1.8 \times 10^9$  infectious units of AdPSE-BC-luc, as previously described [Adams 2002]. Serial optical images were acquired on designated day post-viral injection (noted above each image). Signal intensities are represented by a color scale (red, highest, to blue, weakest). Note that each image was acquired with a slightly different scale, as specified by the maximal signal listed below each image. Extra-tumoral signals were detected at 21 days post-injection. The animals were sacrificed on the last day of imaging, and the isolated organs were re-imaged. Sections (5- $\mu$ m) of the animals' spine were analyzed by hematoxylin, and eosin staining (H&E) and immunohistochemistry, using a human specific anti-cytokeratin antibody (BioGenex Laboratories, San Ramon, CA). The details of the methodology have been described previously [Adams 2002].

One widely used reporter gene system is based on the herpes simplex virus type I thymidine kinase (HSV-TK) gene. In contrast to human thymidine kinase, which phosphorylates thymidine selectively, HSV-TK has a relaxed substrate specificity for other nucleoside analogs, and can phosphorylate a variety of acycloguanosine and uracil derivatives. Radionuclide reporter probes derived from uracil (FIAU labeled with radioactive iodine,  $^{131}\text{I}$ ) and from guanosine ( $^{18}\text{F}$ -labeled penciclovir PCV) have been applied to single photon emission computed tomography (SPECT) and PET. The success of these imaging approaches in many mouse models is based on HSV-TK's ability to selectively phosphorylate and sequester the probes in cells expressing this gene [Gambhir, 2002; Blasberg, 1999; Gambhir, 2000a]. Active site HSV-TK variants were generated by random mutagenesis of the binding site amino acids, and were selected for increased affinity for the acycloguanosine analogs as compared to thymidine [Black 2001, Black 1996]. One particular HSV-TK variant, designated sr39 tk, displayed enhanced  $^{18}\text{F}$ -labeled PCV substrate uptake compared to wild-type HSV-TK, which resulted in improved sensitivity of PET imaging [Gambhir, 2000b].

The same HSV-TK enzyme activity has been widely exploited for suicide cancer gene therapy strategy for more than 10 years, with several ongoing clinical trials [Hall, 1997; Hassan, 2000; Herman, 1999]. Therefore, the HSV-TK gene has the unique property that it can function both as an imaging reporter gene and as a cytotoxic therapeutic gene. In practice, different dosages of HSV-TK substrate are used to achieve the two different modes of action. The tracer level of  $^{18}\text{F}$ -labeled PCV administered for PET imaging is at a 3- to 4-order of magnitude below the toxic pharmacological dose of the acycloguanosine analog, ganciclovir (GCV), used for suicide gene therapy. The toxic effect of GCV is a result of HSV-TK-mediated conversion to GCV monophosphate. The monophosphorylated GCV undergoes further phosphorylation by endogenous cellular kinases into the corresponding nucleoside triphosphate, which is incorporated into cellular DNA and prevents DNA synthesis; this ultimately results in cell death by several proposed mechanisms [Mesnil, 2000; Rubsam, 1999; Wallace, 1996]. We have demonstrated that among a panel of adenoviral vectors expressing either the wild-type HSV-TK or active site variants, sr39tk showed improved therapeutic efficacy in response to acyclovir (ACV) and GCV administration in prostate cancer cell lines and tumor xenografts [Pantuck, 2002]. In a preclinical therapy model, HSV-TK-mediated PET imaging prior to GCV instillation should indicate the magnitude of HSV-TK gene expression in the tumor. If the treatment is effective after GCV administration, the HSV-TK-transduced tumor cells should be eradicated; consequently, PET signals should drop precipitously.

Molecular imaging should play an important role in gene therapy studies. Firstly, it can help to verify the activity of a prostate-targeted gene expression vector *in vivo*. Measuring the expression levels of therapeutic genes by indirect methods (reporter genes) or direct methods (HSV-TK with PET) will permit modulation of gene delivery to achieve optimal expression to the tumor target, which should enhance the gene-based therapeutic response. Imaging approaches could be developed to assess treatment response

in real-time (e.g., apoptosis [Laxman, 2002]) or to specifically detect disseminated cancer cells [Adams, 2002].

## THERAPEUTIC APPROACHES AND GENE DELIVERY VEHICLES

We believe that the therapeutic gene and gene delivery vehicle utilized are the two critical determinants of eventual therapeutic success. We will cover these two topics broadly to convey the conceptual framework, rather than the specific details, for two reasons. Firstly, the number of available gene-based therapeutic options is great, and an ever-increasing number are being refined. Secondly, there are many recent reviews that covered these topics in detail [Mabjeesh, 2002; Lu, 2001; Amalfitano, 2002; Hemminki, 2002].

Therapeutic strategies can be subdivided into four categories: 1) correction of genetic alterations; 2) introduction of cytotoxic pathways; 3) viral-mediated oncolysis; and 4) induction of the immune response. In particular, for the first two approaches (corrective and cytotoxic), efficient transduction to as many tumor cells as possible would be a prerequisite for success. An intrinsic amplification of therapeutic response is incorporated into the third oncolytic approach and the fourth immune-mediated strategy, thus alleviating the requirement for a high percentage of tumor cell transduction. Since the second and third strategies invoke cytotoxic effects, crucial targeting control switches need to be incorporated. Tumor-targeted expression of tumor suppressors or cell cycle regulatory genes is also desirable in the first strategy.

More genetic alterations are being defined as the understanding of prostate oncogenesis increases. This trend also leads to an ever-expanding array of gene-based therapeutic options. Functional loss of tumor suppressor and cell cycle regulatory genes, such as p53, PTEN, retinoblastoma (Rb), p16, and p27, has been well documented in prostate cancer [MacGrogan, 1997; Whang, 1998; Cordon-Cardo, 1998]. Gene-based therapies designed to replenish these genetic deficits have shown promising results in animal tumor models [Davies, 2002; Steiner, 2000; Katner, 2002]. In spite of the extension of therapeutic effects to non-transduced cells, the so-called "bystander effect", has been documented as a genetic correction approach, the magnitude of this effect is attenuated [Rizk, 1999], and improving transduction efficiency might be a critical determinant of success. Alternatively, this approach could be feasible in a combined therapeutic strategy, given that toxic side effects mediated by tumor suppressor genes on normal cells are not anticipated. However, vector versus gene-mediated toxic effects need to be carefully evaluated [Zhang, 1995; Morrissey, 2002].

On the opposite end of the oncogenic spectrum, overexpression of several oncogenes, such as c-Myc, bcl-2, and Her2/neu, has also been implicated in prostate cancer oncogenesis or progression [Craft, 1999; Furuya, 1996; Buttyan, 1987]. Several gene-based approaches to suppress these activated oncogenic pathways have disrupted tumor growth [Steiner, 1998; Gleave, 1999; Dorai, 1999]. The concern with the suppression of a particular gene function is

the level of diminution in expression that is adequate to achieve the desired result. Use of anti-sense RNA or a dominant-negative protein has been used to interfere with a particular gene function. RNA interference (RNAi) is an evolutionarily conserved mechanism whereby a 21-23-nt double-stranded RNA can mediate homology-dependent degradation of mRNA [Hannon, 2002]. Ever since this mechanism was shown to efficiently silence gene expression in mammalian cells, it has become a tool to investigate the gene-function relationship. Recently, the polycomb EZH2 gene was shown to be up-regulated in metastatic HRPC [Varambally, 2002]. Reducing EZH2 expression in prostate cancer cells by the RNAi approach resulted in the inhibition of cell proliferation *in vitro* [Varambally, 2002]. The prospect of discovering novel genes involved in the oncogenic process is overwhelming, but means that new therapeutic targets are likely to be forthcoming. Gene-based approaches should be translated to develop novel therapy, but importantly, can also support the biology of neoplastic transformation.

As referred to in the last section, suicide gene therapy is a generic term that refers to the application of exogenous enzymes to convert a specific prodrug to toxic metabolites that kill cancer cells. Two of the most widely utilized enzyme/prodrug systems are HSV-TK/GCV and cytosine deaminase (CD)/5-fluorocytosine (5-FC). Both systems are currently being evaluated in clinical trials for prostate [Mabjeesh, 2002] and several other cancers. Many other enzyme/prodrug combinations are also being refined (see list in [Kirn, 2002]). Molecular engineering to improve HSV-TK enzyme kinetics that favor prodrug activation has improved therapeutic efficacy [Black, 1996; Black, 2001; Pantuck, 2002]. From our experience, achieving high levels of HSV-TK enzyme expression in targeted cancer cells is a critical determinant of effective cell killing [Pantuck, 2002]. Mindful of the fact that expression of the HSV-TK gene in normal cells will invoke cytotoxic side effects, we have utilized the highly amplified prostate-specific TSTA system to express this suicide gene. Our preliminary results indicate that TSTA-driven HSV-TK in an adenoviral vector expresses higher levels of transgene and induces more extensive tumor cell killing and less systemic toxicity than the comparable CMV-driven system in LAPC-4 tumor xenograft models ([Zhang, 2002] and unpublished data). Employing a strict and potent prostate-specific expression system should also boost the therapeutic index of other promising cytotoxic approaches, such as FasL, TRAIL [Norris, 2001], and viral oncolytic therapy [Yu, 1999a; Kirn, 2002; Hsieh, 2002; Matsubara, 2001].

The recent review by Mabjeesh *et al.* [Mabjeesh, 2002] thoroughly covers many topics of prostate cancer gene therapy, particularly immune therapy. Immunotherapy is a safe therapeutic modality that is under constant refinement to define the most appropriate prostate cancer antigen and vaccination protocol to improve efficacy. Its application might be most suitable for minimal residual disease settings rather than large bulky tumors. As the limitations and advantages of different gene-based therapies are being defined, designing rational combined therapeutic strategies could achieve a synergistic response. The concept of a multimodality approach by combining radiotherapy and double-

suicide gene therapy [Freytag, 1998] employing a CD-TK fusion gene is currently being evaluated. The initial clinical trial results have indicated that this approach is safe in intra-prostatic administration, and it also shows signs of biological activity [Freytag, 2002].

The biggest challenge in the next decade for gene therapy is to develop an ideal gene delivery vehicle capable of achieving efficient gene transduction *in vivo*. In addition to the previously discussed transcriptional regulatory approaches that could be incorporated to improve specificity, an ideal vehicle for cancer therapy should have the following characteristics: 1) non-toxic to normal cells; 2) easily produced in large quantities; 3) capable of being modified on the surface to achieve transductional targeting; 4) easily modified to incorporate multiple genetic elements; 5) capable of efficient gene transfer into targeted cells; 6) widely distributed throughout the subject upon systemic administration; and 7) non-immunogenic. Modified viruses, exploited for their natural ability to efficiently introduce genetic material into host cells, are the most commonly utilized gene delivery vehicles. Inactivated RNA retrovirus-based vectors include murine leukemia virus (MuLV) and lentiviruses. The DNA virus-based vectors include human adenovirus (Ad), adeno-associated virus (AAV), and herpes simplex virus (HSV).

Twenty of 38 currently ongoing clinical trials of prostate cancer gene therapy utilize Ad vectors. Ad-based vectors have taken a leading role for cancer therapy because they possess the first five desired characteristics listed above. The inability to mediate sustained expression is not a concern in cancer gene therapy. It is not necessary for *in situ* Ad gene transfer (i.e., intra-prostatic injection) to overcome distribution issues. Systemic administration of Ads frequently encounters vector sequestration in the liver, leading to the reduced delivery to organs in the arterial circulation [Tao, 2001]. Efforts to modify the viral surface to achieve targeted transduction [Krasnykh, 2001; Stevenson, 1997; Haisma, 2000; Wickham, 1996] could potentially de-target the liver. Anti-Ad immune responses are greatly reduced in the helper-dependent or "gutless" Ad with all viral coding sequences removed [Amalfitano, 2002, O'Neal, 2000]. Another advantage of the gutless Ad is that its capacity for incorporating exogenous genes is greatly expanded, exceeding 30 kb. Simultaneous expression of multiple therapeutic genes and control elements in gutless Ads is quite feasible.

A non-viral gene carrier can be generated by modifying DNA with cationic lipids (liposome) or polymers to form condensed DNA complexes [Niidome, 2002]. These DNA-based synthetic gene delivery vehicles are both non-immunogenic and non-toxic; however, one consistent drawback is the poor *in vivo* gene transduction efficiency. Interestingly, a recent report described the use of integrin-targeted synthetic nanoparticles to deliver cytotoxic gene selectively to tumor blood vessels [Hood 2002]. Nanoparticles are synthetic submicronic (<1  $\mu$ m) colloidal particles for drug delivery. The key advantage of a nanoparticle in cancer treatment is that its surface can be decorated with molecules to target cancer cells [Brigger, 2002]. With the advent of improved targets for prostate

tumor homing [Arap, 2002], prostate-specific gene transduction and expression might soon be realized.

## FUTURE CONSIDERATIONS

Targeted therapy is a prerequisite for medicine in the new millennium. The road for improving prostate-targeted gene therapy is wide open. At this time, PSA-based expression systems appear feasible to target even advanced HRPc. However, it is quite clear that successful *in vivo* gene therapy will require robust and specific gene expression. Applying easy, well-tested, non-invasive imaging modalities to monitor or verify targeted gene expression *in vivo* should be very rewarding. Once a prostate-targeted gene expression system is developed and refined, the magnitude of differential expression in targeted tumor cells versus normal tissues needs to be carefully evaluated in cell culture studies, as well as in animal models of prostate cancer. This information should provide guidance on the efficacy and safety of the particular targeted gene therapy strategy. Since the options of favorable therapeutic genes are quite numerous and ever expanding, many approaches should be tested. Potentially, combining different treatment strategies to suppress growth or mediate cell destruction could lead to more effective therapeutic responses. Meticulous design and stringent testing of prostate-targeted gene therapy in preclinical settings should facilitate a clear path for future clinical applications.

## ACKNOWLEDGMENTS

We deeply appreciate the excellent technical support of Jason Adams and Mai Johnson. This work is supported by the California Cancer Research Program (3NI0226) and the Department of Defense CDMRP (PC020536). M.S. is supported by a Department of Defense CDMRP postdoctoral fellowship (PC020531).

## REFERENCES

- Abate-Shen, C., Shen, M.M. Mouse models of prostate carcinogenesis. (2002) *Trends Genet.*, **18**: S1-5.
- Adams, J.Y., Johnson, M., Sato, M., Berger, F., Gambhir, S. S., Carey, M., Iruela-Arispe, M.L., Wu, L. (2002) Visualization of Advanced Human Prostate Cancer Lesions in Living Mice by a Targeted Gene Transfer Vector and Optical Imaging. *Nat. Med.*, **8**: 891 - 897.
- Amalfitano, A., Parks, R.J. (2002) Separating fact from fiction: assessing the potential of modified adenovirus vectors for use in human gene therapy. *Curr. Gene Ther.*, **2**: 111-33.
- Amara, N., Palapattu, G.S., Schrage, M., Gu, Z., Thomas, G.V., Dorey, F., Said, J., Reiter, R.E. (2001) Prostate stem cell antigen is overexpressed in human transitional cell carcinoma. *Cancer Res.*, **61**: 4660-5.
- Arap, W., Haedicke, W., Bernasconi, M., Kain, R., Rajotte, D., Krajewski, S., Ellerby, H.M., Bredesen, D.E., Pasqualini, R., Ruoslahti, E. (2002) Targeting the prostate for destruction through a vascular address. *Proc. Natl. Acad. Sci. USA*, **99**: 1527-31.
- Aumüller, G., Seitz, J., Lilja, H., Abrahamsson, P.A., von der Kammer, H., Scheit, K.H. (1990) Species- and organ-specificity of secretory proteins derived from human prostate and seminal vesicles. *Prostate*, **17**: 31-40.
- Balk, S.P. (2002) Androgen receptor as a target in androgen-independent prostate cancer. *Urology*, **60**: 132-8.
- Bhaumik, S., Gambhir, S.S. (2002) Optical imaging of Renilla luciferase reporter gene expression in living mice. *Proc. Natl. Acad. Sci. USA*, **99**: 377-82.
- Black, M.E., Kokoris, M.S., Sabo, P. (2001) Herpes simplex virus-1 thymidine kinase mutants created by semi-random sequence mutagenesis improve prodrug-mediated tumor cell killing. *Cancer Res.*, **61**: 3022-6.
- Black, M.E., Newcomb, T.G., Wilson, H.M., Loeb, L.A. (1996) Creation of drug-specific herpes simplex virus type 1 thymidine kinase mutants for gene therapy. *Proc. Natl. Acad. Sci. USA*, **93**: 3525-9.
- Blasberg, R.G., Tjuvajev, J.G. (1999) Herpes simplex virus thymidine kinase as a marker/reporter gene for PET imaging of gene therapy. *Q. J. Nucl. Med.*, **43**: 163-9.
- Bok, R.A., Small, E.J. (2002) Bloodborne biomolecular markers in prostate cancer development and progression. *Nat. Rev. Cancer*, **2**: 918-26.
- Brigger, I., Dubernet, C., Couvreur, P. (2002) Nanoparticles in cancer therapy and diagnosis. *Adv. Drug Deliv. Rev.*, **54**: 631-51.
- Buchanan, G., Yang, M., Harris, J.M., Nahm, H.S., Han, G., Moore, N., Bentel, J.M., Matusik, R.J., Horsfall, D.J., Marshall, V.R., Greenberg, N.M., Tilley, W.D. (2001) Mutations at the boundary of the hinge and ligand binding domain of the androgen receptor confer increased transactivation function. *Mol. Endocrinol.*, **15**: 46-56.
- Buttayan, R., Sawczuk, I.S., Benson, M.C., Siegal, J.D., Olsson, C.A. (1987) Enhanced expression of the c-myc protooncogene in high-grade human prostate cancers. *Prostate*, **11**: 327-37.
- Castrillon, D.H., DePinho, R.A. (2001) Modeling prostate cancer in the mouse. *Adv. Cancer Res.*, **82**: 187-204.
- Chan, J.M., Stampfer, M.J., Giovannucci, E., Gann, P.H., Ma, J., Wilkinson, P., Hennekens, C.H., Pollak, M. (1998) Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science*, **279**: 563-6.
- Christiano, A.P., Yoshida, B.A., Solloff, M., Rinker-Schaeffer, C.W. (2000) Development of markers of prostate cancer metastasis. Review and perspective. *Urol. Oncol.*, **5**: 217-223.
- Ciana, P., Raviscioni, M., Mussi, P., Vegeto, E., Que, I., Parker, M.G., Lowik, C., Maggi, A. (2003) In vivo imaging of transcriptionally active estrogen receptors. *Nat. Med.*, **9**: 82-6.
- Cleutjens, K.B., van der Korput, H.A., Ehren-van Eekelen, C.C., Sikes, R.A., Fasciana, C., Chung, L.W., Trapman, J. (1997a) A 6-kb promoter fragment mimics in transgenic mice the prostate-specific and androgen-regulated expression of the endogenous prostate-specific antigen gene in humans. *Mol. Endocrinol.*, **11**: 1256-1265.
- Cleutjens, K.B., van der Korput, H.A., van Eekelen, C.C., van Rooij, H.C., Faber, P.W., Trapman, J. (1997b) An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. *Mol. Endocrinol.*, **11**: 148-161.
- Coen, J.J., Zietman, A.L., Thakral, H., Shipley, W.U. (2002) Radical radiation for localized prostate cancer: local persistence of disease results in a late wave of metastases. *J. Clin. Oncol.*, **20**: 3199-205.
- Contag, P.R., Olomu, I.N., Stevenson, D.K., Contag, C.H. (1998) Bioluminescent indicators in living mammals. *Nat. Med.*, **4**: 245-7.

- Cordon-Cardo, C., Koff, A., Drobnyak, M., Capodiceci, P., Osman, I., Millard, S.S., Gaudin, P.B., Fazzari, M., Zhang, Z.F., Massague J., Scher, H.I. (1998) Distinct altered patterns of p27KIP1 gene expression in benign prostatic hyperplasia and prostatic carcinoma. *J. Natl. Cancer Inst.*, **90**: 1284-91.
- Corey, E., Quinn, J.E., Bladou, F., Brown, L.G., Roudier, M.P., Brown, J.M., Buhler, K.R., Vessella, R.L. (2002) Establishment and characterization of osseous prostate cancer models: intra-tibial injection of human prostate cancer cells. *Prostate*, **52**: 20-33.
- Craft, N., Shostak, Y., Carey, M., Sawyers, C.L. (1999a) A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. *Nat. Med.*, **5**: 280-5.
- Craft, N., Chhor, C., Tran, C., Belldgrun, A., DeKernion, J., Witte, O.N., Said, J., Reiter, R. E. and Sawyers, C.L. (1999b) Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process. *Cancer Res.*, **59**: 5030-6.
- Culig, Z., Hobisch, A., Cronauer, M.V., Radmayr, C., Trapman, J., Hittmair, A., Bartsch, G., Klocker, H. (1994) Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res.*, **54**: 5474-8.
- Davies, M.A., Kim, S.J., Parikh, N.U., Dong, Z., Bucana, C.D., Gallick, G.E. (2002) Adenoviral-mediated expression of MMAC/PTEN inhibits proliferation and metastasis of human prostate cancer cells. *Clin. Cancer Res.*, **8**: 1904-14.
- Debes, J.D., Tindall, D.J. (2002) The role of androgens and the androgen receptor in prostate cancer. *Cancer Lett.*, **187**: 1-7.
- Denis, L., Murphy, G.P. (1993) Overview of phase III trials on combined androgen treatment in patients with metastatic prostate cancer. *Cancer*, **72**: 3888-95.
- DiGiovanni, J., Kiguchi, K., Frijhoff, A., Wilker, E., Bol, D.K., Beltran, L., Moats, S., Ramirez, A., Jorcano, J., Conti, C. (2000) Deregulated expression of insulin-like growth factor 1 in prostate epithelium leads to neoplasia in transgenic mice. *Proc. Natl. Acad. Sci. USA*, **97**: 3455-60.
- Dorai, T., Perlman, H., Walsh, K., Shabsigh, A., Goluboff, E.T., Olsson, C.A., Buttyan, R. (1999) A recombinant defective adenoviral agent expressing anti-bcl-2 ribozyme promotes apoptosis of bcl-2-expressing human prostate cancer cells. *Int. J. Cancer*, **82**: 846-52.
- Dunning, W.F. (1963) Prostate cancer in the rat. *Monogr. Natl. Cancer Inst.*, **12**: 351-69.
- Edinger, M., Sweeney, T.J., Tucker, A.A., Olomu, A.B., Negrin, R.S., Contag, C.H. Noninvasive assessment of tumor cell proliferation in animal models. *Neoplasia*, **1**: 303-10, 1999.
- Ellis, W.J., Vessella, R.L., Buhler, K.R., Bladou, F., True, L.D., Bigler, S.A., Curtis, D., Lange, P.H. (1996) Characterization of a novel androgen-sensitive, prostate-specific antigen-producing prostatic carcinoma xenograft: LuCaP 23. *Clin. Cancer Res.*, **2**: 1039-48.
- Fair, W.R., Israeli, R.S., Heston, W.D. (1997) Prostate-specific membrane antigen. *Prostate*, **32**: 140-8.
- Feldman, B.J., Feldman, D. (2001) The development of androgen-independent prostate cancer. *Nat. Rev. Cancer*, **1**: 34-45.
- Freytag, S.O., Khil, M., Stricker, H., Peabody, J., Menon, M., DePeralta-Venturina, M., Nafziger, D., Pegg, J., Paielli, D., Brown, S., Barton, K., Lu, M., Aguilar-Cordova, E., Kim, J.H. (2002) Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res.*, **62**: 4968-76.
- Freytag, S.O., Rogulski, K.R., Paielli, D.L., Gilbert, J.D., Kim, J.H. (1998) A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, and radiotherapy. *Hum. Gene Ther.*, **9**: 1323-33.
- Furuya, Y., Krajewski, S., Epstein, J.I., Reed, J.C., Isaacs, J.T. (1996) Expression of bcl-2 and the progression of human and rodent prostatic cancers. *Clin. Cancer Res.*, **2**: 389-98.
- Gabril, M.Y., Onita, T., Ji, P.G., Sakai, H., Chan, F.L., Koropatnick, J., Chin, J.L., Moussa, M., Xuan, J.W. (2002) Prostate targeting: PSP94 gene promoter/enhancer region directed prostate tissue-specific expression in a transgenic mouse prostate cancer model. *Gene Ther.*, **9**: 1589-99.
- Gaddipati, J.P., McLeod, D.G., Heidenberg, H.B., Sesterhenn, I.A., Finger, M.J., Moul, J.W., Srivastava, S. (1994) Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. *Cancer Res.*, **54**: 2861-4.
- Gambhir, S.S., Bauer, E., Black, M.E., Liang, Q., Kokoris, M.S., Barrio, J.R., Iyer, M., Namavari, M., Phelps, M.E., Herschman, H.R. (2000a) A mutant herpes simplex virus type 1 thymidine kinase reporter gene shows improved sensitivity for imaging reporter gene expression with positron emission tomography. *Proc. Natl. Acad. Sci. USA*, **97**: 2785-90.
- Gambhir, S.S., Herschman, H.R., Cherry, S.R., Barrio, J.R., Satyamurthy, N., Toyokuni, T., Phelps, M.E., Larson, S.M., Balatoni, J., Finn, R., Sadelain, M., Tjuvajev, J., Blasberg, R. (2000b) Imaging transgene expression with radionuclide imaging technologies. *Neoplasia*, **2**: 118-38.
- Gambhir, S.S. (2002) Molecular imaging of cancer with positron emission tomography. *Nat. Rev. Cancer*, **2**: 683-93.
- Gelmann, E.P. (2002) Molecular biology of the androgen receptor. *Clin. Oncol.* **20**: 3001-15.
- Gendler, S.J., Mukherjee, P. (2001) Spontaneous adenocarcinoma mouse models for immunotherapy. *Trends Mol. Med.*, **7**: 471-5.
- Gerdes, C.A., Castro, M.G., Lowenstein, P.R. (2000) Strong promoters are the key to highly efficient, noninflammatory and noncytotoxic adenoviral-mediated transgene delivery into the brain *in vivo*. *Mol. Ther.*, **2**: 330-8.
- Gingrich, J.R., Barrios, R.J., Kattan, M.W., Nahm, H.S., Finegold, M.J., Greenberg, N.M. (1997) Androgen-independent prostate cancer progression in the TRAMP model. *Cancer Res.*, **57**: 4687-91.
- Gingrich, J.R., Barrios, R.J., Morton, R.A., Boyce, B.F., DeMayo, F.J., Finegold, M.J., Angelopoulou, R., Rosen, J.M., Greenberg, N.M. (1996) Metastatic prostate cancer in a transgenic mouse. *Cancer Res.*, **56**: 4096-102.
- Gleave, M., Tolcher, A., Miyake, H., Nelson, C., Brown, B., Beraldi, E., Goldie, J. (1999) Progression to androgen independence is delayed by adjuvant treatment with antisense Bcl-2 oligodeoxynucleotides after castration in the LNCaP prostate tumor model. *Clin. Cancer Res.*, **5**: 2891-8.
- Gotoh, A., Ko, S.C., Shirakawa, T., Cheon, J., Kao, C., Miyamoto, T., Gardner, T.A., Ho, L.J., Cleutjens, C.B., Trapman, J., Graham, F.L., Chung, L.W. (1998) Development of prostate-specific antigen promoter-based gene therapy for androgen-independent human prostate cancer. *J. Urology*, **160**: 220-229.
- Greenberg, N.M., DeMayo, F., Finegold, M.J., Medina, D., Tilley, W.D., Aspinall, J.O., Cunha, G.R., Donjacour, A.A., Matusik, R.J., Rosen, J.M. (1995) Prostate cancer in a transgenic mouse. *Proc. Natl. Acad. Sci. USA*, **92**: 3439-43.
- Gregory, C.W., He, B., Johnson, R.T., Ford, O.H., Mohler, J.L., French, F.S., Wilson, E.M. (2001a) A mechanism for androgen

- receptor-mediated prostate cancer recurrence after androgen deprivation therapy. *Cancer Res.*, **61**: 4315-9.
- Gregory, C.W., Johnson, R.T. Jr., Mohler, J.L., French, F.S., Wilson, E.M. (2001b) Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. *Cancer Res.*, **61**: 2892-8.
- Gu, Z., Thomas, G., Yamashiro, J., Shintaku, I.P., Dorey, F., Raitano, A., Witte, O.N., Said, J.W., Loda, M., Reiter, R.E. (2000) Prostate stem cell antigen (PSCA) expression increases with high gleason score, advanced stage and bone metastasis in prostate cancer. *Oncogene*, **19**: 1288-96.
- Haisma, H.J., Grill, J., Curiel, D.T., Hoogeland, S., van Beusechem, V.W., Pinedo, H.M., Gerritsen, W.R. (2000) Targeting of adenoviral vectors through a bispecific single-chain antibody. *Cancer Gene Ther.*, **7**: 901-4.
- Hall, S.J., Mutchnik, S.E., Chen, S.H., Woo, S.L., Thompson, T.C. (1997) Adenovirus-mediated herpes simplex virus thymidine kinase gene and ganciclovir therapy leads to systemic activity against spontaneous and induced metastasis in an orthotopic mouse model of prostate cancer. *Int. J. Cancer*, **70**: 183-7.
- Han, G., Foster, B.A., Mistry, S., Buchanan, G., Harris, J.M., Tilley, W.D., Greenberg, N.M. (2001) Hormone status selects for spontaneous somatic androgen receptor variants that demonstrate specific ligand and cofactor dependent activities in autochthonous prostate cancer. *J. Biol. Chem.*, **276**: 11204-13.
- Han, M., Partin, A.W., Piantadosi, S., Epstein, J.I., Walsh, P.C. (2001) Era specific biochemical recurrence-free survival following radical prostatectomy for clinically localized prostate cancer. *J. Urol.*, **166**: 416-9.
- Han, M., Partin, A.W., Zahurak, M., Piantadosi, S., Epstein, J.I., Walsh, P.C. (2003) Biochemical (prostate specific antigen) recurrence probability following radical prostatectomy for clinically localized prostate cancer. *J. Urol.*, **169**: 517-23.
- Hannon, G.J. (2002) RNA interference. *Nature*, **418**: 244-251.
- Harlan, L.C., Potosky, A., Gilliland, F.D., Hoffman, R., Albertsen, P.C., Hamilton, A.S., Eley, J.W., Stanford, J.L., Stephenson, R.A. (2001) Factors associated with initial therapy for clinically localized prostate cancer: prostate cancer outcomes study. *J. Natl. Cancer Inst.*, **93**: 1864-71.
- Hassan, W., Sanford, M.A., Woo, S.L., Chen, S.H., Hall, S.J. (2000) Prospects for herpes-simplex-virus thymidine-kinase and cytokine gene transduction as immunomodulatory gene therapy for prostate cancer. *World J. Urol.*, **18**: 130-5.
- Hastings, J.W. (1996) Chemistries and colors of bioluminescent reactions: a review. *Gene*, **173**: 5-11.
- Hawkins, L.K., Lemoine, N.R., Kim, D. (2002) Oncolytic biotherapy: a novel therapeutic platform. *Lancet Oncol.*, **3**: 17-26.
- Hegarty, N.J., Fitzpatrick, J.M., Richie, J.P., Scardino, P.T., deVere White, R.W., Schroder, F.H., Coffey, D.S. (1999) Future prospects in prostate cancer. *Prostate*, **40**: 261-8.
- Hellerstedt, B.A., Pienta, K.J. (2002) The current state of hormonal therapy for prostate cancer. *CA Cancer J. Clin.*, **52**: 154-79.
- Hemminki, A., Alvarez, R.D. (2002) Adenoviruses in oncology: a viable option? *Bio. Drugs*, **16**: 77-87.
- Herman, J.R., Adler, H.L., Aguilar-Cordova, E., Rojas-Martinez, A., Woo, S., Timme, T.L., Wheeler, T.M., Thompson, T.C., Scardino, P.T. (1999) In situ gene therapy for adenocarcinoma of the prostate: a phase I clinical trial. *Hum. Gene Ther.*, **10**: 1239-49.
- Hobisch, A., Culig, Z., Radmayr, C., Bartsch, G., Klocker, H., Hittmair, A. (1995) Distant metastases from prostatic carcinoma express androgen receptor protein. *Cancer Res.*, **55**: 3068-72.
- Hoffman, R.M. Green fluorescent protein imaging of tumour growth, metastasis and angiogenesis in mouse models. *Lancet Oncol.*, **3**: 546-556, 2002.
- Honigman, A., Zeira, E., Ohana, P., Abramovitz, R., Tavor, E., Bar, I., Zilberman, Y., Rabinovsky, R., Gazit, D., Joseph, A., Panet, A., Shai, E., Palmon, A., Laster, M. and Galun, E. (2001) Imaging transgene expression in live animals. *Mol. Ther.*, **4**: 239-249.
- Hood, J.D., Bednarski, M., Frausto, R., Guccione, S., Reisfeld, R.A., Xiang, R., Cheresch, D.A. (2002) Tumor regression by targeted gene delivery to the neovasculature. *Science*, **296**: 2404-7.
- Horoszewicz, J.S., Kawinski, E., Murphy, G.P. (1987) Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer patients. *Anticancer Res.*, **7**: 927-35.
- Hsieh, C.L., Chung, L.W. (2001) New perspectives of prostate cancer gene therapy: molecular targets and animal models. *Crit. Rev. Eukaryot. Gene Expr.*, **11**: 77-120.
- Hsieh, C.L., Yang, L., Miao, L., Yeung, F., Kao, C., Yang, H., Zhou, H.E., Chung, L.W. (2002) A novel targeting modality to enhance adenoviral replication by vitamin D(3) in androgen-independent human prostate cancer cells and tumors. *Cancer Res.*, **62**: 3084-92.
- Huang, W., Shostak, Y., Tarr, P., Sawyers, C., Carey, M. (1999) Cooperative assembly of androgen receptor into a nucleoprotein complex that regulates the prostate-specific antigen enhancer. *J. Biol. Chem.*, **274**: 25756-25768.
- Huggins, C., Hodges, C.V. (1941) Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *J. Urol.*, **2002** **168**: 9-12.
- Huss, W.J., Hanrahan, C.F., Barrios, R.J., Simons, J.W., Greenberg, N.M. (2001) Angiogenesis and prostate cancer: identification of a molecular progression switch. *Cancer Res.*, **61**: 2736-43.
- Inouye, S., Shimomura, O. (1997) The use of Renilla luciferase, Oplophorus luciferase, and apoaquorin as bioluminescent reporter protein in the presence of coelenterazine analogues as substrate. *Biochem. Biophys. Res. Commun.*, **233**: 349-53.
- Iyer, M., Wu, L., Carey, M., Smallwood, A. and Gambhir, S.S. (2001) Two-Step Transcriptional Amplification as a Method for Imaging Reporter Gene Expression Utilizing Weak Promoters. *Proc. Natl. Acad. Sci. USA*, **98**: 14595-14600.
- Jain, A., Lam, A., Vivanco, I., Carey, M.F., Reiter, R.E. (2002) Identification of an androgen-dependent enhancer within the prostate stem cell antigen gene. *Mol. Endocrinol.*, **16**: 2323-37.
- Jongsma, J., Oomen, M.H., Noordzij, M.A., Van Weerden, W.M., Martens, G.J., van der Kwast, T.H., Schroder, F.H., van Steenbrugge, G.J. (1999) Kinetics of neuroendocrine differentiation in an androgen-dependent human prostate xenograft model. *Am. J. Pathol.*, **154**: 543-51.
- Jongsma, J., Oomen, M.H., Noordzij, M.A., Van Weerden, W.M., Martens, G.J., van der Kwast, T.H., Schroder, F.H., van Steenbrugge, G.J. (2002) Different profiles of neuroendocrine cell differentiation evolve in the PC-310 human prostate cancer model during long-term androgen deprivation. *Prostate*, **50**: 203-15.
- Kaplan, P.J., Mohan, S., Cohen, P., Foster, B.A., Greenberg, N.M. (1999) The insulin-like growth factor axis and prostate cancer: lessons from the transgenic adenocarcinoma of mouse prostate (TRAMP) model. *Cancer Res.*, **59**: 2203-9.
- Kasper, S., Rennie, P.S., Bruchovsky, N., Sheppard, P.C., Cheng, H., Lin, L., Shiu, R.P., Snoek, R., Matusik, R.J. (1994) Cooperative binding of androgen receptors to two DNA sequences is required for androgen induction of the probasin gene. *J. Biol. Chem.*, **269**: 31763-9.

- Kasper, S., Sheppard, P.C., Yan, Y., Pettigrew, N., Borowsky, A.D., Prins, G.S., Dodd, J.G., Duckworth, M.L., Matusik, R.J. (1998) Development, progression, and androgen-dependence of prostate tumors in probasin-large T antigen transgenic mice: a model for prostate cancer. *Lab. Invest.*, **78**: 319-33.
- Katner, A.L., Hoang, Q.B., Gootam, P., Jaruga, E., Ma, Q., Gnarra, J., Rayford, W. (2002) Induction of cell cycle arrest and apoptosis in human prostate carcinoma cells by a recombinant adenovirus expressing p27(Kip1). *Prostate*, **53**: 77-87.
- Kim, D., Niculescu-Duvaz, I., Hallden, G., Springer, C.J. (2002) The emerging fields of suicide gene therapy and virotherapy. *Trends Mol. Med.*, **8**: S68-73.
- Klein, K.A., Reiter, R.E., Redula, J., Moradi, H., Zhu, X.L., Brothman, A.R., Lamb, D.J., Marcelli, M., Belldegrun, A., Witte, O.N. and Sawyers, C.L. (1997) Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. *Nat. Med.*, **3**: 402-8.
- Koeman, K.S., Hsieh, J.T. (2001) The prospect of gene therapy for prostate cancer: update on theory and status. *Curr. Opin. Urol.*, **11**: 489-94.
- Koeman, K.S., Kao, C., Ko, S.C., Yang, L., Wada, Y., Kallmes, D.F., Gillenwater, J.Y., Zhou, H.E., Chung, L.W., Gardner, T.A. (2000) Osteocalcin-directed gene therapy for prostate-cancer bone metastasis. *World J. Urol.*, **18**: 102-10.
- Koivisto, P., Kononen, J., Palmberg, C., Tammela, T., Hyytinen, E., Isola, J., Trapman, J., Cleutjens, K., Noordzij, A., Visakorpi, T., Kallioniemi, O.P. (1997) Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. *Cancer Res.*, **57**: 314-9.
- Koivisto, P.A., Helin, H.J. (1999) Androgen receptor gene amplification increases tissue PSA protein expression in hormone-refractory prostate carcinoma. *J. Pathol.*, **189**: 219-23.
- Krasnykh, V., Belousova, N., Korokhov, N., Mikheeva, G., Curiel, D.T. (2001) Genetic targeting of an adenovirus vector via replacement of the fiber protein with the phage T4 fibritin. *J. Virol.*, **75**: 4176-83.
- Labrie, F. (2002) Androgen blockade in prostate cancer in 2002: major benefits on survival in localized disease. *Mol. Cell Endocrinol.*, **198**: 77-87.
- Latham, J.P., Searle, P.F., Mautner, V., James, N.D. (2000) Prostate-specific antigen promoter/enhancer driven gene therapy for prostate cancer: construction and testing of a tissue-specific adenovirus vector. *Cancer Res.*, **60**: 334-41.
- Laxman, B., Hall, D.E., Bhojani, M.S., Hamstra, D.A., Chenevert, T.L., Ross, B.D., Rehemtulla, A. (2002) Noninvasive real-time imaging of apoptosis. *Proc. Natl. Acad. Sci. USA*, **99**: 16551-5.
- Lee, S.J., Kim, H.S., Yu, R., Lee, K., Gardner, T.A., Jung, C., Jeng, M.H., Yeung, F., Cheng, L., Kao, C. (2002) Novel prostate-specific promoter derived from PSA and PSMA enhancers. *Mol. Ther.*, **6**: 415-21.
- Lian, J.B., Stein, G.S., Stein, J.L., van Wijnen, A.J. (1999) Regulated expression of the bone-specific osteocalcin gene by vitamins and hormones. *Vitam. Horm.*, **55**: 443-509.
- Lipshutz, G.S., Gruber, C.A., Cao, Y., Hardy, J., Contag, C.H., Gaensler, K.M. In utero delivery of adeno-associated viral vectors: intraperitoneal gene transfer produces long-term expression. *Mol. Ther.*, **3**: 284-92, 2001.
- Lowe, S.L., Rubinchik, S., Honda, T., McDonnell, T.J., Dong, J.Y., Norris, J.S. (2001) Prostate-specific expression of Bax delivered by an adenoviral vector induces apoptosis in LNCaP prostate cancer cells. *Gene Ther.*, **8**: 1363-71.
- Lu, Y. (2001) Viral based gene therapy for prostate cancer. *Curr. Gene Ther.*, **1**: 183-200.
- Mabjeesh, N.J., Zhong, H., Simons, J.W. (2002) Gene therapy of prostate cancer: current and future directions. *Endocr. Relat. Cancer*, **9**: 115-39.
- MacGrogan, D., Bookstein, R. (1997) Tumour suppressor genes in prostate cancer. *Semin. Cancer Biol.*, **8**: 11-9.
- Marcelli, M., Ittmann, M., Mariani, S., Sutherland, R., Nigam, R., Murthy, L., Zhao, Y., DiConcini, D., Puxeddu, E., Esen, A., Eastham, J., Weigel, N.L., Lamb, D.J. (2000) Androgen receptor mutations in prostate cancer. *Cancer Res.*, **60**: 944-9.
- Maroulakou, I.G., Anver, M., Garrett, L., Green, J.E. (1994) Prostate and mammary adenocarcinoma in transgenic mice carrying a rat C3(1) simian virus 40 large tumor antigen fusion gene. *Proc. Natl. Acad. Sci. USA*, **91**: 11236-40.
- Marshall, E. (2000) Gene Therapy on Trial. *Science*, **288**: 951-7.
- Masumori, N., Thomas, T.Z., Chaurand, P., Case, T., Paul, M., Kasper, S., Caprioli, R.M., Tsukamoto, T., Shappell, S.B., Matusik, R.J. (2001) A probasin-large T antigen transgenic mouse line develops prostate adenocarcinoma and neuroendocrine carcinoma with metastatic potential. *Cancer Res.*, **61**: 2239-49.
- Matsubara, S., Wada, Y., Gardner, T.A., Egawa, M., Park, M.S., Hsieh, C.L., Zhou, H.E., Kao, C., Kamidono, S., Gillenwater, J.Y., Chung, L.W. (2001) 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 Res.*, **61**: 6012-9.
- McCormick, F. (2001) Cancer gene therapy: fringe or cutting edge. *Nat. Rev. Cancer*, **1**: 130-141.
- Mentor-Marcel, R., Lamartiniere, C.A., Eltoun, I.E., Greenberg, N.M., Elgavish, A. (2001) Genistein in the diet reduces the incidence of poorly differentiated prostatic adenocarcinoma in transgenic mice (TRAMP). *Cancer Res.*, **61**: 6777-82.
- Mesnil, M. and H. Yamasaki. (2000) Bystander effect in herpes simplex virus-thymidine kinase/ganciclovir cancer gene therapy: role of gap-junctional intercellular communication. *Cancer Res.*, **60**: 3989-99.
- Mitchell, S.H., Murtha, P.E., Zhang, S., Zhu, W., Young, C.Y. (2000) An androgen response element mediates LNCaP cell dependent androgen induction of the hK2 gene. *Mol. Cell Endocrinol.*, **168**: 89-99.
- Morrissey, R.E., Horvath, C., Snyder, E.A., Patrick, J., Collins, N., Evans, E., MacDonald, J.S. (2002) Porcine toxicology studies of SCH 58500, an adenoviral vector for the p53 gene. *Toxicol. Sci.*, **65**: 256-65.
- Murtha, P., Tindall, D.J., Young, C.Y. (1993) Androgen induction of a human prostate-specific kallikrein, hK2: characterization of an androgen response element in the 5' promoter region of the gene. *Biochemistry*, **32**: 6459-64.
- Nagabhushan M., Miller CM., Pretlow TP., Giaconia JM., Edgehouse NL., Schwartz S., Kung HJ., de Vere White RW., Gumerlock PH., Resnick MI., Amini SB., Pretlow TG. (1996) CWR22: the first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both *in vivo* and *in soft agar*. *Cancer Res.*, **56**: 3042-6.
- Navarro, D., Luzardo, OP., Fernandez, L., Chesa, N., Diaz-Chico, B.N. (2002) Transition to androgen-independence in prostate cancer. *J. Steroid. Biochem. Mol. Biol.*, **81**: 191-201.
- Navone, N.M., Logothetis, C.J., von Eschenbach, A.C., Troncoso, P. (1998-99) Model systems of prostate cancer: uses and limitations. *Cancer Metastasis Rev.*, **17**: 361-71.

- Navone, N.M., Olive, M., Ozen, M., Davis, R., Troncoso, P., Tu, S.M., Johnston, D., Pollack, A., Pathak, S., von Eschenbach, A.C., Logothetis, C.J. (1997) Establishment of two human prostate cancer cell lines derived from a single bone metastasis. *Clin. Cancer Res.*, **3**:2493-500.
- Newcomer, L.M., Stanford, J.L., Blumenstein, B.A., Brawer, M.K. (1997) Temporal trends in rates of prostate cancer: declining incidence of advanced stage disease, 1974 to 1994. *J. Urol.*, **158**: 1427-30.
- NIH report. (2002) Assessment of adenoviral vector safety and toxicity: report of the National Institutes of Health Recombinant DNA Advisory Committee. *Hum. Gene Ther.*, **13**: 3-13.
- Niidome, T., Huang, L. (2002) Gene therapy progress and prospects: nonviral vectors. *Gene Ther.*, **9**: 1647-52.
- Noble, R.L. (1977) The development of prostatic adenocarcinoma in Nb rats following prolonged sex hormone administration. *Cancer Res.*, **37**: 1929-33.
- Norris, J.S., Hyer, M.L., Voelkel-Johnson, C., Lowe, S.L., Rubinchik, S., Dong, J.Y. (2001) The use of Fas Ligand, TRAIL and Bax in gene therapy of prostate cancer. *Curr. Gene Ther.*, **1**: 123-36.
- Noss, K.R., Wolfe, S.A., Grimes, S.R. (2002) Upregulation of prostate specific membrane antigen/folate hydrolase transcription by an enhancer. *Gene*, **285**: 247-56.
- O'Keefe, D.S., Su, S.L., Bacich, D.J., Horiguchi, Y., Luo, Y., Powell, C.T., Zandvliet, D., Russell, P.J., Molloy, P.L., Nowak, N.J., Shows, T.B., Mullins, C., Vonder, Haar, R.A., Fair, W.R., Heston, W.D. (1998) Mapping, genomic organization and promoter analysis of the human prostate-specific membrane antigen gene. *Biochim. Biophys. Acta*, **1443**: 113-27.
- O'Keefe, D.S., Uchida, A., Bacich, D.J., Watt, F.B., Martorana, A., Molloy, P.L., Heston, W.D. (2000) Prostate-specific suicide gene therapy using the prostate-specific membrane antigen promoter and enhancer. *Prostate*, **45**: 149-57.
- O'Neal, W.K., Zhou, H., Morral, N., Langston, C., Parks, R.J., Graham, F.L., Kochanek, S., Beaudet, A.L. (2000) Toxicity associated with repeated administration of first-generation adenovirus vectors does not occur with a helper-dependent vector. *Mol. Med.*, **6**: 179-95.
- Oettgen, P., Finger, E., Sun, Z., Akbarali, Y., Thamrongsak, U., Boltax, J., Grall, F., Dube, A., Weiss, A., Brown, L., Quinn, G., Kas, K., Endress, G., Kunsch, C., Libermann, T.A. (2000) PDEF, a novel prostate epithelium-specific ets transcription factor, interacts with the androgen receptor and activates prostate-specific antigen gene expression. *J. Biol. Chem.*, **275**:1216-1225.
- Pang, S., Dannull, J., Kaboo, R., Xie, Y., Tso, C.L., Michel, K., deKernion, J.B., Belldegrun, A.S. (1997) Identification of a positive regulatory element responsible for tissue-specific expression of prostate-specific antigen. *Cancer Res.*, **57**: 495-499.
- Pantuck, A., Matherly, J., Zisman, A., Nguyen, D., Berger, F., Gambhir, S.S., Black, M.E., Belldegrun, A.S., Wu, L. (2002) Optimizing Prostate Cancer Suicide Gene Therapy Using Herpes Simplex Virus Thymidine Kinase Active Site Variants. *Hum. Gene Ther.*, **13**: 777-789.
- Pantuck, A.J., Zisman, A., Henderson, D., Wilson, D., Schreiber, A., Belldegrun, A. (2001) New biologicals for prostate cancer prevention: Genes, vaccines, and immune-based interventions. *Urology*, **57**: 95-9.
- Papsidero, L.D., Wang, M.C., Valenzuela, L.A., Murphy, G.P., Chu, T.M. (1980) A prostate antigen in sera of prostatic cancer patients. *Cancer Res.*, **40**: 2428-32.
- Perez-Stable, C.M., Pozas, A., Roos, B.A. (2000) A role for GATA transcription factors in the androgen regulation of the prostate-specific antigen gene enhancer. *Mol. Cell Endocrinol.*, **167**:43-53.
- Podsakoff, G.M. (2001) Lentiviral vectors approach the clinic but fall back: National Institutes of Health Recombinant DNA Advisory Committee review of a first clinical protocol for use of a lentiviral vector. *Mol. Ther.*, **4**: 282-3.
- Podsypanina, K., Ellenson, L.H., Nemes, A., Gu, J., Tamura, M., Yamada, K.M., Cordon-Cardo, C., Catoretti, G., Fisher, P.E., Parsons, R. (1999) Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. *Proc. Natl. Acad. Sci. USA*, **96**:1563-8.
- Pretlow, T.G., Delmoro, C.M., Dilley, G.G., Spadafora, C.G., Pretlow, T.P. (1991) Transplantation of human prostatic carcinoma into nude mice in Matrigel. *Cancer Res.*, **51**: 3814-7.
- Rauen, K.A., Sudilovsky, D., Le, J.L., Chew, K.L., Hann, B., Weinberg, V., Schmitt, L.D., McCormick, F. (2002) Expression of the coxsackie adenovirus receptor in normal prostate and in primary and metastatic prostate carcinoma: potential relevance to gene therapy. *Cancer Res.*, **62**: 3812-8.
- Ray, P., Bauer, E., Iyer, M., Barrio, J.R., Satyamurthy, N., Phelps, M.E., Herschman, H.R., Gambhir, S.S. (2001) Monitoring gene therapy with reporter gene imaging. *Semin. Nucl. Med.*, **31**: 312-20.
- Reid, K.J., Hendy, S.C., Saito, J., Sorensen, P., Nelson, C.C. (2001) Two classes of androgen receptor elements mediate cooperativity through allosteric interactions. *J. Biol. Chem.*, **276**: 2943-52.
- Reiter, R., Sawyers, C. (2001) Xenograft models and the molecular biology of human prostate cancer. In *Prostate Cancer Biology* Eds Chung LWK *et al. Humana Press*, pp. 163-174.
- Reiter, R.E., Gu, Z., Watabe, T., Thomas, G., Szigeti, K., Davis, E., Wahl, M., Nisitani, S., Yamashiro, J., Le Beau, M.M., Loda, M., Witte, O.N. (1998) Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. *Proc. Natl. Acad. Sci. USA*, **95**: 1735-40.
- Rennie, P.S., Bruchovsky, N., Leco, K.J., Sheppard, P.C., McQueen, S.A., Cheng, H., Snoek, R., Hamel, A., Bock, M.E., MacDonald, B.S. (1993) Characterization of two cis-acting DNA elements involved in the androgen regulation of the probasin gene. *Mol. Endocrinol.*, **7**: 23-36.
- Reynolds, P.N., Nicklin, S.A., Kaliberova, L., Boatman, B.G., Grizzle, W.E., Balyasnikova, I.V., Baker, A.H., Danilov, S.M., Curiel, D.T. (2001) Combined transductional and transcriptional targeting improves the specificity of transgene expression *in vivo*. *Nat. Biotechnol.*, **19**: 838-42.
- Riegman, P.H., Vlietstra, R.J., van der Korput, J.A., Brinkmann, A.O., Trapman, J. (1991) The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. *Mol. Endocrinology*, **12**: 1921-1930.
- Rizk, N.P., Chang, M.Y., El Kouri, C., Seth, P., Kaiser, L.R., Albelda, S.M., Amin, K.M. (1999) The evaluation of adenoviral p53-mediated bystander effect in gene therapy of cancer. *Cancer Gene Ther.*, **6**: 291-301.
- Rubsam, L.Z., Boucher, P.D., Murphy, P.J., KuKuruga, M., Shewach, D.S. (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**: 669-75.
- Sadar, M.D. (1999) Androgen-independent induction of prostate-specific antigen gene expression via cross-talk between the androgen receptor and protein kinase A signal transduction pathways. *J. Biol. Chem.*, **274**: 7777-83.

- Schuur, E.R., Henderson, G.A., Kmetec, L.A., Miller, J.D., Lamparski, H.G., Henderson, D.R. (1996) Prostate-specific antigen expression is regulated by an upstream enhancer. *J. Biol. Chem.*, **271**: 7043-7051.
- Silver, D.A., Pellicer, I., Fair, W.R., Heston, W.D., Cordon-Cardo, C. (1997) Prostate-specific membrane antigen expression in normal and malignant human tissues. *Clin. Cancer Res.*, **3**: 81-5.
- Smaletz, O., Scher, H.I. (2002) Outcome predictions for patients with metastatic prostate cancer. *Semin. Urol. Oncol.*, **20**: 155-63.
- Stanbrough, M., Leav, I., Kwan, P.W., Buble, G.J., Balk, S.P. (2001) Prostatic intraepithelial neoplasia in mice expressing an androgen receptor transgene in prostate epithelium. *Proc. Natl. Acad. Sci. USA*, **98**: 10823-10828.
- Steiner, M.S., Anthony, C.T., Lu, Y., Holt, J.T. (1998) Antisense c-myc retroviral vector suppresses established human prostate cancer. *Hum. Gene Ther.*, **9**: 747-55.
- Steiner, M.S., Gingrich, J.R. (2000) Gene therapy for prostate cancer: where are we now? *J. Urol.*, **164**: 1121-36.
- Steiner, M.S., Wang, Y., Zhang, Y., Zhang, X., Lu, Y. (2000) p16/MTS1/INK4A suppresses prostate cancer by both pRb dependent and independent pathways. *Oncogene*, **19**: 1297-306.
- Stevenson, S.C., Rollence, M., Marshall-Neff, J., McClelland, A. (1997) Selective targeting of human cells by a chimeric adenovirus vector containing a modified fiber protein. *J. Virol.*, **71**: 4782-90.
- Sweat, S.D., Pacelli, A., Bergstralh, E.J., Slezak, J.M., Cheng, L., Bostwick, D.G. (1999) Androgen receptor expression in prostate cancer lymph node metastases is predictive of outcome after surgery. *J. Urol.*, **161**: 1233-7.
- Tan, J., Sharief, Y., Hamil, K.G., Gregory, C.W., Zang, D.Y., Sar, M., Gumerlock, P.H., deVere White, R.W., Pretlow, T.G., Harris, S.E., Wilson, E.M., Mohler, J.L., French, F.S. (1997) Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells. *Mol. Endocrinol.*, **11**: 450-9.
- Tao, N., Gao, G.P., Parr, M., Johnston, J., Baradet, T., Wilson, J.M., Barsoum, J., Fawell, S.E. (2001) Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Mol. Ther.*, **3**: 28-35.
- Taplin, M.E., Buble, G.J., Shuster, T.D., Frantz, M.E., Spooner, A.E., Ogata, G.K., Keer, H.N., Balk, S.P. (1995) Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. *N. Engl. J. Med.*, **332**: 1393-8.
- Thompson, T.C., Timme, T.L., Park, S.H., Yang, G., Ren, C. (2000) Mouse prostate reconstitution model system: A series of *in vivo* and *in vitro* models for benign and malignant prostatic disease. *Prostate*, **43**: 248-54.
- Tilley, W.D., Buchanan, G., Hickey, T.E., Bentel, J.M. (1996) Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. *Clin. Cancer Res.*, **2**: 277-85.
- Trofimova, I., Dimtchev, A., Jung, M., Rosenthal, D., Smulson, M., Dritschilo, A., Soldatenkov, V. (2002) Gene therapy for prostate cancer by targeting poly(ADP-ribose) polymerase. *Cancer Res.*, **62**: 6879-83.
- Tsingotjidou, A.S., Zotalis, G., Jackson, K.R., Sawyers, C., Puzas, J.E., Hicks, D.G., Reiter, R. and Lieberman, J.R. (2001) Development of an animal model for prostate cancer cell metastasis to adult human bone. *Anticancer Res.*, **21**: 971-8.
- Tso, C.L., McBride, W.H., Sun, J., Patel, B., Tsui, K.H., Paik, S.H., Gilitz, B., Caliliw, R., van Ophoven, A., Wu, L., deKernion, J., Belldegrun, A. (2000) Androgen deprivation induces selective outgrowth of aggressive hormone-refractory prostate cancer clones expressing distinct cellular and molecular properties not present in parental androgen-dependent cancer cells. *Cancer J.*, **6**: 220-33.
- Uchida, A., O'Keefe, D.S., Bacich, D.J., Molloy, P.L., Heston, W.D. (2001) *In vivo* suicide gene therapy model using a newly discovered prostate-specific membrane antigen promoter/enhancer: a potential alternative approach to androgen deprivation therapy. *Urology*, **58**: 132-9.
- van der Kwast, T.H., Tetu, B. (1996) Androgen receptors in untreated and treated prostatic intraepithelial neoplasia. *Eur. Urol.*, **30**: 265-8.
- van Weerden, W.M., de Ridder, C.M., Verdaasdonk, C.L., Romijn, J.C., van der Kwast, T.H., Schroder, F.H., van Steenbrugge, G.J. (1996) Development of seven new human prostate tumor xenograft models and their histopathological characterization. *Am. J. Pathol.*, **149**: 1055-62.
- van Weerden, W.M., Romijn, J.C. (2000) Use of nude mouse xenograft models in prostate cancer research. *Prostate*, **43**: 263-71.
- Varambally, S., Dhanasekaran, S.M., Zhou, M., Barrette, T.R., Kumar-Sinha, C., Sanda, M.G., Ghosh D., Pienta, K.J., Sewalt, R.G., Otte, A.P., Rubin, M.A., Chinnaiyan, A.M. (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature*, **419**: 624-9.
- Veldscholte, J., Berrevoets, C.A., Ris-Stalpers, C., Kuiper, G.G., Jenster, G., Trapman, J., Brinkmann, A.O., Mulder, E. (1992) The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. *J. Steroid Biochem. Mol. Biol.*, **41**: 665-9.
- Visakorpi, T., Hyytinen, E., Koivisto, P., Tanner, M., Keinänen, R., Palmberg, C., Palotie, A., Tammela, T., Isola, J., Kallioniemi, O.P. (1995) *In vivo* amplification of the androgen receptor gene and progression of human prostate cancer. *Nat. Genet.*, **9**: 401-6.
- Wallace, H., Clarke, A.R., Harrison, D.J., Hooper, M.L., Bishop, J.O. (1996) Ganciclovir-induced ablation non-proliferating thyrocytes expressing herpesvirus thymidine kinase occurs by p53-independent apoptosis. *Oncogene*, **13**: 55-61.
- Watabe, T., Lin, M., Ide, H., Donjacour, A.A., Cunha, G.R., Witte, O.N., Reiter, R.E. (2002) Growth, regeneration, and tumorigenesis of the prostate activates the PSCA promoter. *Proc. Natl. Acad. Sci. USA*, **99**: 401-6.
- Watt, F., Martorana, A., Brookes, D.E., Ho, T., Kingsley, E., O'Keefe, D.S., Russell, P.J., Heston, W.D., Molloy, P.L. (2001) A tissue-specific enhancer of the prostate-specific membrane antigen gene, FOLH1. *Genomics*, **73**: 243-54.
- Wei, C., Willis, R.A., Tilton, B.R., Looney, R.J., Lord, E.M., Barth, R.K., Frelinger, J.G. (1997) Tissue-specific expression of the human prostate-specific antigen gene in transgenic mice: implications for tolerance and immunotherapy. *Proc. Natl. Acad. Sci. USA*, **94**: 6369-6374.
- Whang, Y.E., Wu, X., Suzuki, H., Reiter, R.E., Tran, C., Vessella, R.L., Said, J.W., Isaacs, W.B., Sawyers, C.L. (1998) Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. *Proc. Natl. Acad. Sci. USA*, **95**: 5246-50.
- Wickham, T.J., Segal, D.M., Roelvink, P.W., Carrion, M.E., Lizonova, A., Lee, G.M., Kovesdi, I. (1996) Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. *J. Virol.*, **70**: 6831-8.
- Wu, L., Matherly, J., Smallwood, A., Adams, J.Y., Billick, E., Belldegrun, A., Carey, M. (2001) Chimeric PSA enhancers exhibit augmented activity in prostate cancer gene therapy vectors. *Gene Ther.*, **8**: 1416-1426.

- Wu, J.C., Sundaresan, G., Iyer, M. and Gambhir, S. S. (2001) Noninvasive optical imaging of firefly luciferase reporter gene expression in skeletal muscles of living mice. *Mol. Ther.*, **4**: 297-306.
- Xie, X., Zhao, X., Liu, Y., Young, C.Y., Tindall, D.J., Slawin, K.M., Spencer, D.M. (2001a) Robust prostate-specific expression for targeted gene therapy based on the human kallikrein 2 promoter. *Hum. Gene Ther.*, **12**: 549-61.
- Xie, X., Zhao, X., Liu, Y., Zhang, J., Matusik, R.J., Slawin, K.M., Spencer, D.M. (2001b) Adenovirus-mediated tissue-targeted expression of a caspase-9-based artificial death switch for the treatment of prostate cancer. *Cancer Res.*, **61**: 6795-804.
- Yang, M., Baranov, E., Moossa, A.R., Penman, S., Hoffman, R.M. Visualizing, gene expression by whole-body fluorescence imaging. *Proc. Natl. Acad. Sci. USA*, **97**: 12278-82, 2000.
- Yeung, F., Chung, L.W. (2002) Molecular basis of co-targeting prostate tumor and stroma. *J. Cell Biochem. Suppl.*, **38**: 65-72.
- Yousef, G.M., Diamandis, E.P. (2002) Expanded human tissue kallikrein family-a novel panel of cancer biomarkers. *Tumour Biol.*, **23**: 185-92.
- Yu, D., Chen, D., Chiu, C., Razmazma, B., Chow, Y.H., Pang, S. (2001) Prostate-specific targeting using PSA promoter-based lentiviral vectors. *Cancer Gene Ther.*, **8**: 628-35.
- Yu, D.C., Chen, Y., Seng, M., Dilley, J., Henderson, D.R. (1999a) The addition of adenovirus type 5 region E3 enables calydon virus 787 to eliminate distant prostate tumor xenografts. *Cancer Res.*, **59**: 4200-3.
- Yu, D.C., Sakamoto, G.T., Henderson, D.R. (1999b) 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 Res.*, **59**: 1498-504.
- Zhang, J., Zhang, S., Murtha, P.E., Zhu, W., Hou, S.S., Young, C.Y. (1997) Identification of two novel cis-elements in the promoter of the prostate-specific antigen gene that are required to enhance androgen receptor-mediated transactivation. *Nucleic Acids Res.*, **25**: 3143-3150.
- Zhang, L., Adams, J.Y., Billick, E., Ilagan, R., Iyer, M., Le, K., Smallwood, A., Gambhir, S.S., Carey, M. and Wu, L. (2002) Molecular Engineering of a Two-step Transcription Amplification (TSTA) System for Transgene Delivery in Prostate Cancer. *Mol. Ther.*, **5**: 223-232.
- Zhang, W.W., Alemany, R., Wang, J., Koch, P.E., Ordonez, N.G., Roth, J.A. (1995) Safety evaluation of Ad5CMV-p53 *in vitro* and *in vivo*. *Hum. Gene Ther.*, **6**: 155-64.
- Zhang, X., Chen, M.W., Ng, A., Ng, P.Y., Lee, C., Rubin, M., Olsson, C.A., Buttyan, R. (1997) Abnormal prostate development in C3(1)-bcl-2 transgenic mice. *Prostate*, **32**: 16-26.
- Zhang, X., Lee, C., Ng, P.Y., Rubin, M., Shabsigh, A., Buttyan, R. (2000) Prostatic neoplasia in transgenic mice with prostate-directed overexpression of the c-myc oncoprotein. *Prostate*, **43**: 278-85.
- Zhao, X.Y., Boyle, B., Krishnan, A.V., Navone, N.M., Peehl, D.M., Feldman, D. (1999) Two mutations identified in the androgen receptor of the new human prostate cancer cell line MDA PCa 2a. *J. Urol.*, **162**: 2192-9.
- Zhao, X.Y., Malloy, P.J., Krishnan, A.V., Swami, S., Navone, N.M., Peehl, D.M., Feldman, D. (2000) Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor. *Nat. Med.*, **6**: 703-6.
- Zhou, H.E., Li, C.L., Chung, L.W.K. (2000) Establishment of human prostate carcinoma skeletal metastasis models. *Cancer*, **88**: 2995-3001.