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PRINCIPAL INVESTIGATOR: Lily Wu, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of California, Los Angeles
Los Angeles, California 90095-1406

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Molecular Engineering of Vector-Based Oncolytic and Imaging Approaches for Advanced Prostate Cancer

Lily Wu, Ph.D.

University of California, Los Angeles
Los Angeles, California 90095-1406

E-Mail: lwu@mednet.ucla.edu

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

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Hormone refractory and metastatic prostate cancer are not well understood. Better animal models, diagnostic and treatment modalities are sorely needed for these advanced stages of disease. We have coupled non-invasive optical imaging to develop metastatic prostate cancer animal models, and vector-based diagnostic and therapeutic approaches. A highly potent and prostate-specific transcriptional regulatory system (TSTA) has been utilized to restrict the expression of our adenoviral vector specifically to prostate or prostate cancer cells. In the diagnostic approach, this TSTA system will be applied to express optical imaging reporter gene. Alternatively, the TSTA system will be applied to regulate the expression of viral replication proteins in the therapeutic approach. In doing so, the viral amplification and cell lysis will be directed in a prostate-specific manner. The progress of this project has been interesting and according to plan in the first year. Several human prostate xenograft models have been marked with the optical luciferase reporter gene. This approach allows facile monitoring of cancer dissemination. We observed distinctly different metastatic potentials in different models. These metastatic animal models will be the bases for us to investigate the efficacy of our diagnostic and oncolytic therapeutic viral vectors.

Metastasis, oncolytic adenovirus, gene therapy, imaging
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Introduction:

The scope of this project is to develop adenoviral vectors that are capable of mediating gene expression specifically in prostate and prostate cancer cells. We propose to incorporate a highly potent and specific two-step transcriptional amplification (TSTA) system to mediate prostate-targeted gene expression in our vectors. In diagnostic applications, this targeted vector will be utilized to express optical reporter luciferase gene. The hypothesis is that administration of this imaging reporter virus could illuminate prostate metastatic cells in living animals. In a therapeutic approach, the TSTA system will be employed to regulate viral replication, which leads to specific lysis of prostate tumor cells. Creation of consistent and easy to follow metastatic prostate cancer models will be very useful towards the evaluation of the proposed vector-based diagnostic and therapeutic approaches.

Body:

Task 1A: Determine sensitivity of vector-based imaging.

Findings relevant to this task were reported in two manuscripts.

1) Wu L, Johnson M, and Sato M. Transcriptionally-targeted Gene Therapy to Detect and Treat Cancer. 2003, Trends in Molecular Medicine, 9:421-429. In this review article, we address the issue of the sensitivity of optical CCD imaging in mice. The conclusion is that robust optical signals can be detected from approximately 1000 to 10000 tumor cells expressing luciferase gene.

2) 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.

![Figure 1. Real time monitoring of metastasis in prostate xenograft models.](image)

Task 1B: To generate optically marked metastatic prostate tumor models to allow detection by prostate-targeted imaging vector.

We have also made significant progress in these tasks (unpublished data). There are several pre-existing models of human prostate cancer xenografts designated as LAPC series, established by Dr. Charles Sawyers' group (1). A metastatic subline of LAPC-4 was also generated (2). The metastatic events of these tumor models have been difficult to characterize or compare in living animals. Therefore, we elected to mark the different LAPC tumor cells by transducing with a renilla luciferase (RL)-expressing lentiviral vector...
in the pRRL backbone (3). The growth and dissemination of the stably marked tumors can be monitored in real-time by optical CCD imaging (4, and Figure 1). Different prostate xenografts exhibit differential metastatic potential. The metastatic subline of LAPC-4 was capable of dissemination to multiple organs such as spine, liver and lung as detected by optical imaging (Figure 1). Micrometastatic lesions in the lungs have been verified by immunohistochemistry using human-specific cytokeratin antibody (4, Figure 2). Detailed histological analyses of metastasis in other organs are underway. Establishment of these optically marked models will be extremely useful towards future evaluation of therapeutic effects of our targeted gene therapy and tumor biology.

**Task 2A: Generation of therapeutic oncolytic adenovirus.**
We have initiated the construction of the oncolytic virus. We modified the location of renilla luciferase (RL) transgene (see Figure 3), placing it downstream to E1A as a second cistron (5). This will allow the optical RL signals to be reflective of E1A level. To confirm that the bi-direction expression of two genes can be regulated by the centrally located Gal4-VP16 activators, we have created a virus that expresses firefly luciferase (FL) and herpes simplex thymidine kinase (TK) gene in divergent orientation (Figure 4). Co-infection of this bi-directional reporter virus with a Gal4-VP16 activator-expressing virus (AdBC-VP2, 6) resulted in expression of both FL and TK proteins (Figure 4). Robust FL activity was also detected (data not shown). This result supports that the design of our oncolytic virus should lead to regulated
expression of both E1A and E1B gene.

Key Research Accomplishments:

- An adenoviral vector containing the prostate-targeted TSTA system is able to mediate robust and specific gene expression in vivo.
- Non-invasive optical imaging is very useful to monitor vector-mediated gene expression in living animals.
- Human prostate xenograft models exhibit differential metastatic potentials.
- Optically marked tumor cells can facilitate monitoring of the metastatic process in living animals.
- The Gal4-VP16 mediated divergent transcription of two transgenes (bi-directional construct) in a viral vector is functional.

Reportable Outcomes:

Manuscripts: (Enclosed in appendix)


Abstracts:

1) Makoto Sato, Mai Johnson, JoAnn Zhang, Christina Zhang, Michael Carey, Sanjiv Gambhir and Lily Wu. In Vivo Regulation And Specificity Of Adenoviral Vectors With Two-Step Transcriptional Amplification System For Prostate Cancer Gene Therapy. AACR annual meeting 7/03.
3) Makoto Sato, Michael Carey, Lily Wu. Prostate-specific and titratable transgene expression from adenovirus with two-step transcriptional amplification system. ASGT 6/03.

Presentations:

1) “Prostate Cancer Gene Therapy Coupled to Imaging” presented by Lily Wu in NCI/NIH MMHCC Symposium UCLA, 1-04.

Funding applied for:

1) DOD PC '04 Idea Development Award. “PROSTATE GENE THERAPY COUPLED WITH MULTIMODAL MOLECULAR IMAGING IN DOGS.”, 2-04.

Conclusions:

We have demonstrated that the TSTA approach can greatly augment prostate-specific gene expression. Imaging and therapeutic TSTA adenoviral vectors mediate targeted gene expression in living animals. We have also generated metastatic prostate
cancer models marked with sensitive optical reporter gene. These models will facilitate the investigation of tumor biology and serve as excellent models to investigate our vector-based imaging and oncolytic therapeutic strategy for metastatic disease.

References:


Transcriptionally targeted gene therapy to detect and treat cancer

Lily Wu, Mai Johnson and Makoto Sato

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

The greatest challenge in cancer treatment is to achieve the highest levels of specificity and efficacy. Cancer gene therapy could be designed specifically to express therapeutic genes to induce cancer cell destruction. Cancer-specific promoters are useful tools to accomplish targeted expression; however, high levels of gene expression are needed to achieve therapeutic efficacy. Incorporating an imaging reporter gene in tandem with the therapeutic gene will allow tangible proof of principle that gene expression occurs at the correct location and at a sufficient level. Gene-based imaging can advance cancer detection and diagnosis. By combining the cancer-targeted imaging and therapeutic strategies, the exciting prospect of a ‘one-two punch’ to find hidden, disseminated cancer cells and destroy them simultaneously can potentially be realized.

Multiple genetic alterations that confer growth advantages to tumor cells are accumulated during the transformation from normal to neoplastic growth [1]. The loss of growth suppressive genes or gain of oncogenes constitutes a common mechanism of oncogenesis. Based on this information, a rational therapeutic approach is to reintroduce the defective growth control genes into tumor cells. In addition, approaches of inducing apoptotic responses and enhancing anti-tumor immune responses have also been employed [2]. Due to the availability of multiple flexile therapeutic strategies, gene therapy is being actively investigated in clinical settings.

Among the ~40 ongoing gene therapy clinical trials for cancer (searched via www.clinicaltrials.gov/), 18 of the trial protocols involve an immune activating scheme, ten studies employ a genetic correction strategy and five use a cytotoxic gene. With regard to genetic correction strategies, p53 is a major target. The recombinant adenovirus is the dominant viral gene delivery vector, and it is employed in 18 protocols. However, none of 40 protocols use a cancer-specific gene expression strategy. An oncolytic adenovirus containing prostate-specific antigen (PSA) promoter driven viral replication [3] is being evaluated in phase II clinical trial for prostate cancer [4].

Because the goal of cancer gene therapy is to eradicate cancer cells, many therapeutic genes could be detrimental if unintentionally expressed in normal cells. Selectively targeting the cancer cells is useful to achieve safety and efficacy, especially when the gene therapy vector is directly delivered into patients. Based on features that distinguish cancerous from normal cells, three targeting strategies could be employed. Transcriptional targeting takes advantage of the fact that some cancer cells express a subset of exclusive genes, and uses these cancer-specific promoters to express the desired transgenes [5]. Transductional targeting refers to surface modification on the gene delivery vehicle to enhance interactions with the cancer cell membrane antigen, thereby improving gene transfer into the cancerous cell. A third promising approach is to exploit cancer-associated cellular pathways to activate therapy. For example, the attenuated adenovirus dl1520 (ONXY-015), lacking viral E1B 55k protein, was reported to selectively replicate and kill p53 deficient tumor cells and not normal cells (2 and references within). In another example, the cytotoxic activity of a fusogenic glycoprotein (GalV) was engineered to be activated by matrix metalloproteinase (MMP) cleavage of a blocking domain [6]. This modulated GalV exhibited selective cytotoxicity to MMP-expressing glioma cells, while sparing normal human astrocytes.

In this review, we will focus on transcriptional targeting for cancer, and discuss strategies to amplify the magnitude of specific expression and the use of imaging modalities to monitor transgene expression in living animals.

Transcriptional targeting

The transcriptional regulatory regions of a gene control the kinetics and levels of mRNA production. Typically, the gene regulatory regions can be subdivided into proximal promoter and distal enhancer elements, gauged by the distance from the start site of transcription [7]. A complex array of transcription factors bind to these regulatory regions. Complex coordinated actions of the activators recruit the RNA polymerase II general machinery to the promoter and initiate transcription of the gene. Some activators are ubiquitously expressed, whereas others are restricted to certain cell types [7]. Cell-specific expression can be thought of as being mediated by a unique subset of ubiquitous and specific activators present in the cell. Transcriptional targeting is feasible because the tissue- or cancer-specific promoter can be activated in the targeted cancer cell in the presence of the proper subset of activators but would remain silent in the non-targeted cell (Figure 1).

Many tissue-specific promoters have been applied to targeted gene therapy (Table 1). Testing in animal models showed that specific promoters exhibit a clear advantage of
Figure 1. Schematic representation of transcriptionally targeted gene expression. Tissue- or cancer-specific promoter-driven reporter or therapeutic gene is incorporated into a gene delivery vector (depicted as recombinant adenovirus here). Gene transfer can occur in both targeted cancer cells and non-targeted normal cells. However, transgene expression can only occur in cancer cells due to the presence of transcription factors competent to mediate expression of the specific promoter. Red squares denote the endogenous gene product expressed from the specific promoter. The swirls denote the reporter/therapeutic gene product.

Reduced cytotoxicity, compared with a strong constitutive promoter such as the human cytomegalovirus (CMV) promoter currently used in clinical trials. For example, when Fas ligand expression was driven by neuronal tissue-specific promoters such as glial fibrillary acidic protein (GFAP) or neuronal-specific enolase (NSE), no hepatocyte apoptosis manifested as acute liver hemorrhage was observed [8]. Despite the tumor-directed injection of the CMV-driven interleukin (IL)-12 adenoviral vector, serious side effects resulted from systemic immune activation [9].

Table 1. Tissue- and cancer-specific promoters used in targeted gene therapy*

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Target tumor type</th>
<th>Summary</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>Glioma</td>
<td>Ad with GFAP or NSE promoter driving FasL exhibited diminished liver toxicity</td>
<td>(7)</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>Melanoma</td>
<td>Tissue-specific expression combined with E1A mutation exhibited highly selective oncology of melanoma</td>
<td>(9)</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate cancer</td>
<td>Ad with E1A driven by PSA promoter destroyed LNCaP tumors</td>
<td>(10)</td>
</tr>
<tr>
<td>ALA</td>
<td>Breast cancer</td>
<td>Ad with ALA or β-lactoglobulin promoter driving HSV-TK exhibited regression of breast cancer in animal model</td>
<td>(12)</td>
</tr>
<tr>
<td>CEA</td>
<td>Digestive tract cancer</td>
<td>Cytotoxic gene therapy approach with viral vector using CEA promoter</td>
<td>(23,24)</td>
</tr>
<tr>
<td>AFP</td>
<td>Hepatocellular carcinoma</td>
<td>Cytotoxic or immunotherapy for hepatocellular carcinoma with viral vector regulated by AFP promoter</td>
<td>(20,21)</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Tumor (fibrosarcoma)</td>
<td>Specific expression with hypoxia-responsive element in HT1080 transfectant in vitro and in vivo</td>
<td>(30)</td>
</tr>
<tr>
<td>HTERT</td>
<td>Tumor (glioma)</td>
<td>Constitutive active caspase-6 driven by HTERT promoter suppressed glioma in nude mice</td>
<td>(35)</td>
</tr>
<tr>
<td>E2F</td>
<td>Tumor</td>
<td>Tumor-selective oncolytic adenovirus</td>
<td>(38)</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>Prostate cancer</td>
<td>Oncolytic effect of conditional-replicating Ad co-targeting tumor and associated osteoblasts kills cancer cells in vitro and skeletal metastasis in animal model</td>
<td>(39)</td>
</tr>
<tr>
<td>Muc-1</td>
<td>Breast cancer</td>
<td>Ad with E1A driven by DF3/MUC1 promoter and CMV promoter driven TNF induced tumor regression</td>
<td>(40)</td>
</tr>
<tr>
<td>(DF3)</td>
<td>Ovarian cancer</td>
<td>Ad with the BAX driven by DF3 promoter showed cytotoxicity in vitro and in vivo</td>
<td>(41)</td>
</tr>
<tr>
<td>L-plastin</td>
<td>Breast and ovarian cancer</td>
<td>Conditional-replicating Ad with E1A driven by truncated L-plastin promoter showed cytotoxic effect in animal model</td>
<td>(10)</td>
</tr>
</tbody>
</table>

*Abbreviations: ALA, α-lactalbumin; BAX, pro-apoptotic member of the Bcl-2 family; DF3/Muc-1, mucin core protein-1; E2F, E2A-binding factor; GFAP, glial fibrillary acidic protein; NSE, neuronal-specific enolase; PSA, prostate-specific antigen; CEA, carcinoembryonic antigen; AFP, α-fetoprotein; HIF-1α, α-subunit of hypoxia-inducible transcription factor-1; HTERT, human telomerase reverse transcriptase; CMV, cytomegalovirus; TNF, tumor necrosis factor.

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The use of an inducible promoter based on the heat shock proteins (hsp70B) greatly limited IL-12 transgene-mediated toxicity [9].

Tissue-targeted gene therapy has been investigated for melanoma [10,11], prostate cancer [12] and breast cancer [13]. Tyrosinase, a key enzyme in melanin synthesis, is highly expressed in melanoma cells; therefore, its promoter has been used in melanoma targeted gene therapy [14]. The fidelity of the tyrosinase promoter was demonstrated in a melanoma-targeted oncolytic scheme, in which restricted adenoviral E1A gene expression led to greater than 200-fold selective viral replication and cytolyis [10].

The PSA gene encodes a serine protease that is expressed in normal and cancerous prostatic epithelial cells, and is an important serum marker for prostate cancer [12]. Due to its highly specific nature, the PSA promoter has been frequently used in prostate cancer-targeted gene therapy approaches [3,15,16]. A modified PSA promoter-driven luciferase reporter adenoviral vector construct showed preferential expression in prostate gland and tumors, by three orders of magnitude, compared with liver tissue [17,18]. Human α-lactalbumin (hαLA) is an enzyme involved in lactose production, and it is expressed in the lactating mammary gland and in a high proportion of breast cancer cases [19]. Selected expression in breast cancer cells was demonstrated with an hαLA promoter-driven reporter and therapeutic gene [13]. Tissue-specific promoter-based cytotoxic gene therapy can damage normal tissue at the site where the promoter is active. This therapeutic approach is feasible in situations in which the normal targeted tissue is dispensable.

The carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) are embryonic proteins that become reactivated in carcinomas. Because these two genes are dormant in normal adult tissues, their promoters are highly tumour selective. The AFP promoter has been employed to target hepatic cancers in many therapeutic strategies, including expression of cytosine deaminase [20], the immunostimulatory IL-2 gene [21] and in oncolytic adenovirus [22]. The CEA promoter can be applied to diverse cancers in which this gene is overexpressed, such as gastric carcinoma [23] and colorectal cancer [24]. Adenoviral vectors carrying CEA promoter-driven therapeutic genes were able to mediate targeted expression, tumor regression and prolonged survival in CEA + tumor-bearing mice [25], with minimal liver toxicity [26]. Because AFP and CEA are used as serum diagnostic markers in several carcinomas [27,28], employing these promoters as targets would be supported in expression-positive carcinomas.

Promoters that are upregulated in cancer-specific conditions can be exploited for targeting. The abnormal tumor microenvironment can induce altered gene expression; for example, inadequate vascular supply relative to the rapid growth of cancer cells leads to hypoxia [29], which initiates a cascade of gene expression mediated by the hypoxia-inducible transcription factor (HIF). The α-subunit of HIF-1 is the inducible component of the heterodimeric HIF, which binds to the hypoxia response element (HRE) and activates target gene expression in response to hypoxia [30,31]. Incorporation of HRE into the adenovirus E1A gene regulatory region resulted in a virus that replicates and lyses tumor cells in a hypoxia-dependent manner [32].

Telomerase plays an important role in cell immortalization and tumorigenesis, and its activity is highly dependent on the catalytic subunit, human telomerase reverse transcriptase (hTERT) [33]. High levels of hTERT expression regulated at the transcription step are observed in malignant tumors but not in normal cells [34]. Therefore, use of the hTERT promoter has achieved targeted therapeutic results in experimental bladder cancer and glioma models [35].

Dysregulated cell cycle control and unrestricted growth of cancer is frequently caused by disruption of the retinoblastoma (Rb)/E2F/p16 pathway [36], which in turn activates E2F. E2F is a transcription factor that activates its own promoter and other genes involved in cell-cycle transition [37]. This elevated E2F-1 activity in cancer cells was exploited to control adenoviral E1A gene expression and viral replication. E2F appears to be a feasible target to mediate tumor lysis of multiple Rb-defective cancers [38,39]. A similar cancer-targeted oncolytic virus has been developed with the osteocalcin promoter for prostate cancer [40], the Muc-1 promoter for breast cancer [41] and the L-plasmin promoter for breast and ovarian cancer [11]. It is desirable to use a specific promoter to direct viral lytic replication because an intrinsic amplification of therapeutic response is incorporated into this approach. Upon viral replication, a burst of progeny viruses can infect additional tumor cells.

Augmenting cancer-specific expression

Although the use of specific promoters will be likely to improve the safety of gene-based therapy, the activities of specific promoters are weaker than the current benchmark CMV promoter. Given that in vivo gene delivery to the tumor cells might be limited, a concern for employing weak specific promoters is that therapeutic efficacy might decline. However, any attempt to enhance the potency of promoters will need to retain the specificity of the promoter in order to maintain potential therapeutic benefits.

Simple manipulations of known regulatory elements, such as removal of a negative and inert regulatory sequence or multimerization of positive elements, can promote synergistic and cooperative interactions of activators to enhance transcription. For example, the activity of native PSA promoter and enhancer (PSE) can be augmented by modifying the androgen receptor (AR) elements, which serve key activating functions for the PSA gene [15,16]. By insertion of four tandem copies of the synthetic androgen-responsive element, or by duplication of a 400-base pair enhancer core element, a nearly 20-fold enhancement of activity over the parental PSE was achieved [17]. Similar approaches have been successful in improving the activity of the tyrosinase promoter [14] and the CEA promoter [42]. An interesting and more extreme approach would be to generate a complete synthetic promoter by multimerization and shuffling of known regulatory elements, then selecting the most active and properly regulated construct. This approach has been applied to the chicken skeletal α-actin promoter to achieve
muscle-specific expression that exceeds the level of the CMV promoter [43].

Two strategies have frequently been employed to amplify weak promoter activity in a two-tiered manner (Figure 2). In an approach known as 'two-step transcriptional activation' (TSTA, Figure 2a), the specific promoter directed the potent transcription activator, GAL4-VP16, which in turn acted upon a second GAL4-responsive reporter or therapeutic gene. This TSTA approach, based on the original 'enhancer trap' methodology to study gene expression in Drosophila melanogaster development, can boost the activity of the PSA promoter over a range of up to 1000-fold [44,45]. Optimal TSTA constructs displayed activity levels significantly higher than those of the CMV promoter, while maintaining prostate cell specificity and androgen responsiveness [44,45]. The fidelity of this prostate-targeted TSTA-firefly luciferase (FL) expression cassette was maintained when inserted into an adenoviral vector, AdTSTA-FL [46]. Many applications of this two-tiered amplification strategy have been documented, including amplification of PSA promoter-mediated polyglutamine expression to treat prostate cancer [47], enhancement of the CEA promoter [48] and Muc-1-mediated expression for colon cancer [49].

In another approach, a cancer-specific promoter controls the expression of Cre site-specific recombinase [50], which activates the reporter or therapeutic gene expression in a second step (Figure 2b). The desired transgene is linked to a strong constitutive promoter that is interrupted by expression termination sequences flanked by two loxP

**Figure 2.** Binary approaches to amplify activity of weak promoter. (a) Two-step transcriptional amplification. The specific promoter drives expression of a synthetic transcriptional activator, GAL4-VP16, which in turn binds to and activates the GAL4 responsive promoter of the reporter or therapeutic gene. Multiple GAL4-VP16 activators work in concert to synergistically activate transcription of the downstream gene (denoted by upward sweeping arrow). (b) Cre-mediated activation of gene expression. Cre recombinase expression is regulated by a tissue- or cancer-specific promoter. Activation of transgene expression is induced by removal of the translational inhibition sequence via a Cre-specific recombination between the two loxP sites.

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sites, the cognate site of Cre. Commonly, the two components of this scheme are incorporated into two separate adenoviral vectors [51–54]. By co-infection into target cells, the cell-specific, Cre-dependent activation of transgene expression was demonstrated in CEA-targeted systems [51], in thyroid carcinoma-targeted therapy [52], in a growth hormone promoter-mediated strategy targeting pituitary tumor [53] and in AFP promoter-based therapy for liver tumor [54]. The specificity of this system is maintained when the Cre and the loxP component are inserted in two separate vectors. However, the activation of this system requires co-delivery of two vectors into the same cell, which could be inefficient in vivo [54].

**Imaging-specific expression in animals**

Non-invasive gene-based imaging is a powerful tool to assess the performance of targeted gene therapy in vivo. Due to the multitude of therapeutic genes used, direct imaging of each therapeutic gene is not feasible. Thus, an imaging reporter gene delivered and expressed in conjunction with the therapeutic gene becomes a generalizable approach 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 animals [55]. Two such modalities that will be discussed, luciferase-based bioluminescence imaging (BLI) and positron emission tomography (PET) have frequently been applied in preclinical small animal models.

BLI [56] has the distinct advantage of low background signal, ease of use, and low cost, in comparison with radionuclide imaging; however, it is limited by light scatter and absorption, presenting difficulties in detecting and localizing signals in deep tissues. Luciferase is a generic term for a family of photo-proteins that can be isolated from insects, marine organisms and prokaryotes [57]. Biochemically, all luciferases are oxygenases that use 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 emission spectra ranging between 400 and 620 nm [57]. Imaging of FL expression in living mice has been accomplished using a highly sensitive charged coupled device (CCD) camera [56,58]. Light is produced through the interaction of FL with its substrate, D-luciferin, injected peritoneally in the presence of magnesium and ATP [56]. An estimate of the sensitivity of this FL-based CCD imaging is illustrated in Figure 3. Optical signals from $10^4$ or more cells expressing FL driven by the CMV promoter can be easily detected.

The specificity of transcriptionally targeted gene delivery vectors can be investigated by BLI. One advantage of non-invasive imaging is that sequential detection of signals in the same animal in a time-dependent manner often alleviates some of the uncertainty due to inter-animal technical variations. To overcome the limitation of precise three-dimensional signal localization in the animal, post-mortem imaging of isolated organs was applied [18]. The specificity of a prostate-targeted adenoviral vector (AdPSE-BC-FL) has permitted detection of metastatic lesions in living mice [18]. Because these vector-based gene imaging approaches are relatively new, extra caution is needed to assure the reliability of the optical signals. As shown in Figures 4 and 5, the positive imaging signals were confirmed with detailed histological and pathological analyses of the tissues [18] (Figure 4) and with the sensitive polymerase chain reaction technology (Figure 5). Moreover, the highly amplified prostate-specific AdTSTA-FL can be applied to interrogate AR function during prostate cancer progression [46]. The real-time optical signals mediated by AdTSTA-FL correlated with cellular transcription complex formation by chromatin immuno-precipitation and AR cellular localization [46] (Figure 6). Depletion of testicular androgen in castrated animals resulted in rapid decay of the AdTSTA-FL mediated signal, which correlated with diffusion of AR into the cytoplasm from the nucleus (Figure 6).

Multiple distinct luciferases could potentially be developed for BLI. This exciting development means that it will be possible simultaneously to monitor multiple pathways or cell populations in the animal. In fact, Gambhir’s group has demonstrated the feasibility of this principle by simultaneously monitoring Renilla luciferase (RL) and FL [59]. 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. Thus, by injecting coelenterazine or D-luciferin, respective levels of RL and FL expression can be imaged simultaneously in the same mouse, to track two different cell populations or gene therapy vectors [59]. Although BLI is widely applicable to investigating many biological processes in small
animals [46], it cannot be applied to human studies because of the loss of signal penetration with increased tissue depth. To translate optical imaging results in animals to clinical settings, a high-energy imaging modality will be needed.

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 herpes simplex virus thymidine kinase (HSV-TK) or the dopamine type 2 receptor gene [60]. Compared with optical imaging, PET has the distinct advantage of providing tomographic, quantitative image signals and adaptability for human imaging [60]. However, optical imaging is several orders of magnitude higher in sensitivity than PET [61]. Thus, gene expression amplification such as TSTA might be required to successfully implement PET imaging to monitor vector-mediated transgene expression in vivo.

One widely used PET reporter gene system is based on the 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 [2'-fluoro-2'-deoxy-1-β-D-arabinofuranosyl-5-iodouracil (FIAU) labeled with radioactive iodine

Figure 4. Verification of positive optical signals. Animals bearing prostate tumor received 3.6 or 7.2 x 10^7 infectious units of prostate-targeted adenovirus, AdPSE-BC-FL, administered via the tail vein. (a) Histological analysis to assess positive signal. Twelve days after administration, an optical signal was observed in the lung (left panel) of an animal that received 3.6 x 10^7 infectious units of virus. The positive optical signal in this animal was correlated with the presence of metastatic human cancer cells in the lung (right panel). Cancer cells in the lung sections were visualized by confocal microscopy using CV-3 conjugated (red) human-specific pan-epithelial antibody (BioGenex Laboratories). Lung blood vessels were visualized by FITC-lectin (green).

Figure 5. Reverse transcriptase polymerase chain reaction (RT-PCR) to detect the presence of tumor cells in the lung. Semi-quantitative RT-PCR was used to detect the presence of prostate-specific antigen (PSA)-expressing human cancer cells. The animals in the left and right panel received 3.6 x 10^7 and 7.2 x 10^7 infectious units of AdPSE-BC-FL, respectively. At 21 days after viral injection, the animal in the left and right panel displayed positive and negative lung signals, respectively, after organ isolation. Thirty-five cycles of PCR amplifications performed on the lung extract revealed a low-intensity positive band detected by PSA-specific primers (the animal in the left panel). No PSA transcripts were detected in the lung of the optically silent animal (right panel). Androgen receptor RNA was detected in the lung of non-tumor bearing (naive) animals (negative control). Tumor tissue extract served as positive control.

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(²³¹⁴) and from guanosine (¹⁸F-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 the ability of HSV-TK to selectively phosphorylate and sequester the probes in cells expressing this gene [60]. Active site HSV-TK variants have been generated by random mutagenesis of the binding site amino acids, and selected for increased affinity for the acycloguanosine analogs, compared with thymidine [62]. One HSV-TK variant, sr39tk, displayed enhanced ¹⁸F-labeled PCV substrate uptake and improved sensitivity of PET imaging compared with wild-type HSV-TK [63].

The HSV-TK gene has been used in suicide cancer therapy for more than ten years, with ongoing clinical trials [64]. Therefore, the HSV-TK gene has the unique
property of being able to 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 modes of action. The $^{32}$P-labeled PCV level administered for PET imaging is three to four orders of magnitude lower than the toxic pharmacological dose of ganciclovir (GCV). The toxicity of GCV is a result of HSV-TK-mediated conversion to GCV monophosphate, which undergoes further phosphorylation to the triphosphate form; this in turn disrupts DNA synthesis and induces apoptosis [65,66]. 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 GCV in prostate cancer cell lines and tumors [67].

Molecular imaging should play an important role in gene-based therapeutic and diagnostic studies. Imaging the expression levels of therapeutic genes by indirect methods [reporter genes] or direct methods (HSV-TK with PET) can assess the performance and verify the specificity of the cancer-targeted vector in vivo. For example, the magnitude of HSV-TK-mediated PET imaging signals correlate directly with the gene expression level [60]. Thus, PET imaging before GCV instillation should permit localization and assessment of transduction efficiency of HSV-TK gene therapy in vivo. Based on this information, the magnitude of transgene expression can be modulated to achieve optimal expression in the tumor target, to enhance therapeutic efficacy. Moreover, imaging approaches could be developed to assess treatment response in real time. In the case of suicide gene therapy, the HSV-TK-transduced tumor cells should be eradicated after GCV administration; consequently, PET signals should drop precipitously. The kinetics of PET signal diminution can be monitored in real time. In addition, if the cancer-targeted gene expression vector is truly specific in vivo, imaging of the reporter gene can be adapted to detect disseminated cancer cells [18]. By combining targeted gene-based imaging and therapeutic approaches, the potential to detect and treat metastatic cancer could be developed in the future.

Concluding remarks
To achieve highly precise cell-specific targeting remains a great challenge in the future of cancer therapy. Transcriptional targeting is a feasible means of improving the specificity and efficacy of gene therapy. However, significant obstacles remain in seeking out and destroying the hidden metastatic cancer cells in the whole organism. The well documented abnormal tumor angiogenesis [68] might prevent sufficient drug and gene therapy vector delivery to the tumor [69]. To further enhance tumor selectivity, approaches which target cell surface or vascular antigens [70] or biochemical pathways unique to tumors can be incorporated. We foresee that combining different targeting strategies into the cancer-specific vector could achieve synergistic selectivity. Non-invasive imaging will be a useful tool to assess the performance of the targeted vector in vivo. Meticulous design and stringent testing of cancer-targeted gene therapy in preclinical settings should facilitate a clear path for future applications in clinics.

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

Makoto Sato,¹ Mai Johnson,¹ Liqun Zhang,² Baohui Zhang,¹ Kim Le,² Sanjiv S. Gambhir,³* Michael Carey,²,³ and Lily Wu¹,³†

¹Department of Urology, ²Crump Institute of Molecular Imaging and Department of Molecular and Medical Pharmacology, and ³Department 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 prerequisite 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 therapeautic 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 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 system 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, FSE-BC), which is activated by androgens. In the second step, GAL4-VP2 binds to a GAL4-responsive promoter and activates the expression of 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]. E designates the packaging signal of adenosine 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 αv 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 cell lines 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 measure 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 10⁷ 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 AdGS-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 10⁷ pfu of each of the two Ads resulted in lower optical signal (2 × 10⁵ photons/s/cm²/sr) versus single Ad (4 × 10⁶ photons/s/cm²/sr). When the dose of the two paired TSTA Ads was increased to 10⁸ pfu each, expression level (2 × 10⁷ photons/s/cm²/sr) higher than that of 10⁷ pfu of AdCMV-FL (9 × 10⁶ photons/s/cm²/sr) was achieved (Fig. 3a). However, this magnitude of activity is still lower than can be achieved by 10⁷ pfu of AdTSTA-FL (4 × 10⁸ photons/s/cm²/sr).

We examined the kinetics of expression after intratumoral delivery of 10⁷ 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. 10⁷ pfu (plaque-forming units = infectious units) of Ad was injected into naïve 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; lv, lung; lv, liver; and pr, prostate).

FIG. 3. In vivo FL expression mediated by single and separate TSTA Ads in LAPC-4 xenografts and prostate. (a) Optical signals after injections of the respective Ads in LAPC-4 tumors. 10⁷ or 10⁸ pfu of Ads (as specified) was injected. The injection of separate Ads denotes the coadministration of both AdBC-VP2 and AdGS-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. 10⁷ 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.
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 10⁶ pfu of AdTSTA-FL or 10⁶ pfu each of AdBC-VP2 and AdGS-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 (three-fold 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 observed 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 infection 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 adenooviral 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 promotor 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 adenooviral 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 LAPC-4. LAPC-4 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 μM 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 μM 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 a 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 expression. 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 was shown at the bottom. The GAL4-VP2 activator was probed with anti-GAL4 polyclonal antibody (see Materials and Methods). β-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 nonprostate 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 Ads [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 10⁷ 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 × 10⁹ 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 may 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 cardinoembryonic 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 components 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 imaging is several orders of magnitude higher in sensitivity than PET in small animal applications [66]. Thus, the highly amplified 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 [25,35]. The single AdTSTA-FL and separate TSTA Ads, AdBC-VP2 and AdG5-FL, were constructed with the AdEasy system [35]. The head-to-tail fragment of activator and reporter in the single virus was derived from pBCV1PG-FL [28]. The construction of AdTSTA-FL has been previously described [23]. A Nol fragment containing the PSE-BC-driven GAL4-VP2 expression cassette was cloned into the Nol 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 pAdEasy1 in the BSI413 rec+ bacteria strain to generate the full-length recombinant virus-containing plasmid. The viruses were propagated in 293 cells, purified on a CsCl gradient, and tethered by plaque assays on 293 monolayers. Viruses are stored in 10 mM Tris–HCl, 1 mM MgCl2, 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, Herdon, VA) with 10% FBS and 1% penicillin/streptomycin. For FL assays, the cultured cells were plated onto 24-well plates at 5 × 104 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 methylidenieniolone (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 1.6 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 DyNaNo SYBR Green qPCR Kit (Finnzymes, Espoo, Finland). The viral FL sequences were detected by the following primer set: FL-a (5'-GAGATACGCGCTCGTCTGCG-3') and FL-b (5'-GATACGAGATCCGATTG-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 Sawyer 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 naive mouse experiments, 10^5 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 AdGS-FL (10^5 pfu each) or AdTSTA-FL (10^5 pfu each) was injected at three sites on each tumor at 10 μl 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 10^5 infectious units each of the paired TSTA Ads or 10^5 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 μl 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 IGROR-PRO Living Image Software, as described [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 AdGS-FL at m.o.i. 0.2, 0.6, 1.2, 3.6, 16.7, or 50 (threefold serial dilution). For each 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 (Gradient, Frenchs Forest, Australia) and subjected to immunoblot analysis with rabbit polyclonal antibodies generated against intact GAL4-VP2 or β-actin AS5316 (Sigma). Detection was done by visualization of bands with HRP-labeled secondary antibody and ECL (Amersham Pharmacia Biotech, Piscataway, NJ).

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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; Podskaloff, 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 with a projected mortality rate of more than 30,000 American men per year (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 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
critical mediator of the biological effects of androgen [Gelmann, 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].

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.

![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-β 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).](image-url)
between growth factor and AR pathways rather than bypassing 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; Zhu, 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 [Zhu, 2000].

In the transgenic adenocarcinoma of mouse prostate (TRAMP) model, prostate-restricted expression of potent oncosgenic SV40 large T-antigen and small T-antigen results in an aggressive reproducible murine prostate 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 [Tsintogiudis, 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 [Nagabhusan, 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 prostrate-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/ncigap/) 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; Koeneman, 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 [Schuur, 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 [Schuur, 1996; Perez-Stable, 2000; Oetten, 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 [Schuur, 1996]. PDEF is a prostate epithelium-specific Ets factor that has been shown to interact with AR and activate PSA gene expression [Oetten, 2000]. Cell culture transfections [Schuur, 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

<table>
<thead>
<tr>
<th>promoter</th>
<th>experiment</th>
<th>transgene; result summary</th>
<th>Reference</th>
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<tr>
<td><strong>human origin</strong></td>
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<td>PARP (apoptotic); androgen-inducible DNA-damaging</td>
<td>Trofimova (2002)</td>
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<td>adenovirus</td>
<td>HSV-TK (therapeutic); cell-killing in vitro and inhibition of growth in tumor model</td>
<td>Huang (1999)</td>
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<td>E1A (oncolytic); selective replication in LnCaP, regression in tumor model</td>
<td>Reid (2001)</td>
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<td></td>
<td>lentivirus</td>
<td>DTA (therapeutic); cell killing in vitro, specific EGFP expression in tumor model</td>
<td>Yu (2001)</td>
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<td></td>
<td>transgenic mice</td>
<td>lacZ (reporter); prostate specific activity and a decline with castration</td>
<td>Perez-Stable (2000)</td>
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<td>human PSA; prostate specific expression</td>
<td>Oetjen (2000)</td>
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<td>kallikrein 2 (hK2/hKL2)</td>
<td>transfection</td>
<td>CAT (reporter); expression in prostate cancer cell lines and androgen regulation</td>
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<td>adenovirus</td>
<td>EGFP (reporter); specific expression in tumor model</td>
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<td>E1 protein (oncolytic); expression in PSA(+), selective replication in prostate tumor cells</td>
<td>Yu (1999)</td>
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<td>PSMA</td>
<td>transfection</td>
<td>luciferase (reporter); expression in LnCaP</td>
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<td>luciferase (reporter); expression in prostate cells, negative regulation with androgen</td>
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<td>PSCA</td>
<td>transfection</td>
<td>luciferase, GFP (reporter); cell specific and androgen responsive expression</td>
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<td>transgenic mice</td>
<td>EGFP (reporter); expression in the prostate and prostate cancer</td>
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<td><strong>rodent origin</strong></td>
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<td>rat C3(1)</td>
<td>transgenic mice</td>
<td>SV40 T-Ag (oncogene); development of hyperplasia and adenocarcinoma</td>
<td>Maroulakou (1994)</td>
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<td>bcl-2, c-myc (oncogene); limited expression and hyperplasia</td>
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<td>transfection</td>
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<td>Rennie (1993)</td>
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<td>Caspase-9 (apoptotic); expression and apoptosis in LnCaP and tumor model</td>
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<td>adenovirus</td>
<td>E1A (oncolytic); selective replication in PSA(+) cell lines</td>
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<td>Bax (apoptotic); cell-specific expression and cell death in LnCaP</td>
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<td>rat probasin</td>
<td>transgenic mice</td>
<td>SV40 T-Ag (oncogene); development of prostatic disease (PIN to metastatic cancer) &lt;TRAMP model&gt;</td>
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<td>SV40 T-Ag (oncogene); development of prostatic disease (PIN to cancer) &lt;LADY model&gt;</td>
<td>Kasper (1998)</td>
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<td>transgenic mice</td>
<td>SV40 T-Ag (oncogene); tumor development in the prostate</td>
<td>Gabri (2002)</td>
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<td><strong>bone/cancer target</strong></td>
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<td>Osteocalcin</td>
<td>adenovirus</td>
<td>HSV-TK (therapeutic); Phase I clinical trial</td>
<td>Koeneman (2000)</td>
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<td>E1A+E1B (oncolytic); growth inhibition of AD and AI cells and in tumor model</td>
<td>Hsieh (2002)</td>
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<td></td>
<td></td>
<td>E1A (oncolytic); inhibition of the growth of prostate cancer cell lines and tumors</td>
<td>Matsubara (2002)</td>
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</table>

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 green fluorescent protein; CD, cytosine deaminase; SV40 T-Ag, simian virus 40 T antigen
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 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; 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]. 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 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, 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'Keeffe, 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 prosin 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 prosin promoter is highly androgen-responsive and contains multiple AREs [Kasper, 1994]. Several recent studies have shown that prosin-based adenoviral vectors can mediate prostate-selective transgene expression and therapeutic responses in human prostate cells [Xie, 2001b; 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 prostate 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 such as estrogen receptor function [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 1.8x10^6 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-μm) of the animals’ spine were analyzed by hematoxylin and eosin staining (H&E) and immunohistochemistry, using a human specific anti-cytokeration 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, $^{131}$I) and from guanosine ($^{18}$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 s39 tk, displayed enhanced $^{18}$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 $^{18}$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, s39tk 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 the extension of therapeutic effects to non-transduced cells, the so-called "bystander effect", has been documented in the 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 oncoproteins, such as c-Myc, bel-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; Doria, 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 an useful 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 [Krn, 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; Zhang, 2003] 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 Fastl., TRAIL [Norris, 2001], and viral oncolytic therapy [Yu, 1999a; Knir, 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 multi-modality 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 transcriptionsal 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), aden-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 [Amalifiato, 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 [Nidiome, 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 integrat-targeted synthetic nanoparticles to deliver cytotoxic gene selectively to tumor blood vessels [Hood 2002]. Nanoparticles are synthetic submicronic (<1 μm) 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 [Brieger, 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.

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REFERENCES


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