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Advances in Preclinical Investigation of Prostate Cancer Gene Therapy

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Abstract

Treating recurrent prostate cancer poses a great challenge to clinicians. Research efforts in the last decade have shown that adenoviral vector-based gene therapy is a promising approach that could expand the arsenal against prostate cancer. This maturing field is at the stage of being able to translate many preclinical discoveries into clinical practices. At this juncture, it is important to highlight the promising strategies including prostate-targeted gene expression, the use of oncolytic vectors, therapy coupled to reporter gene imaging, and combined treatment modalities. In fact, the early stages of clinical investigation employing combined, multimodal gene therapy focused on loco-regional tumor eradication and showed promising results. Clinicians and scientists should seize the momentum of progress to push forward to improve the therapeutic outcome for the patients.

INTRODUCTION

Like many cancers, prostate cancer is a complex disease, and different kinds of therapeutic strategies will be required in order to demonstrate a benefit in a particular patient cohort. Most of the ~29,000 men who succumb to prostate cancer each year in the United States die of metastatic disease and this highlights the need for better systemic therapies.[1] Currently, the best available systemic therapies for prostate cancer, including hormone therapy and the recently approved Taxotere (docetaxel), are not curative, and result only in an extension (sometimes modest) of survival. It is probably for these reasons that vaccine-based gene therapy approaches were the first to be attempted in the clinic and have progressed the furthest. Not only are these approaches seductive because of their potential to impact metastatic disease, they also have scientific merit and have produced provocative results in Phase II trials. What is needed, in the long-term, are therapies that will work towards the eradication of both local and systemic disease, ultimately resulting in a reduction in the annual number of prostate cancer deaths.

As a therapeutic tactic, gene therapy offers the opportunity to target diverse molecular pathways in cancer cells. According to the Genetic Modification Clinical Research Information System, [2] 72 gene therapy protocols targeting prostate cancer have been submitted to National Institutes of Health's Recombinant DNA and Advisory Committee for review. However, only a fraction of these have progressed to the stage of clinical trials and even fewer have published results. Besides the logistical issues (*i.e.*, lack of resources, regulatory barriers, etc.), what are the

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reasons for this meager progress? From the investigations that have taken place during the past decade it is clear that we need to overcome several limitations in order to foster the future success of prostate cancer gene therapy. How can we improve upon the inefficient *in vivo* gene transduction? Can gene therapy be directed specifically at the cancerous prostate gland without damaging normal tissues? Can the process of *in vivo* gene transfer and therapy be visualized or monitored non-invasively? How can we augment the efficacy of current therapeutic approaches? We will discuss specifically: how we can harness the potency of viral replicative power to promote the killing of cancerous prostate cells; how we can build upon current prostate-specific transcriptional amplification systems for improving gene expression efficiency; how we can integrate multiple therapeutic modalities and/or molecular imaging to achieve higher efficacy of gene therapy; and how we can acquire the ability to monitor the efficacy of gene therapy in preclinical applications with a view to ultimately translating developed approaches to the clinic. In this report we will give an overview of several therapeutic approaches that are currently being developed and appear to be promising in pre-clinical settings. We will also address the technical advancements that could guide continual progress in this field. The intent of this review is to focus on the potential of transcriptionally-targeted gene therapy and to place it within the framework of newly-developing strategies that are available to cancer therapists, so that it may one day earn a place among the effective treatment modalities for prostate cancer.

TECHNICAL DEVELOPMENTS FOR ADVANCING PROSTATE-TARGETED THERAPY

Prostate-targeted gene expression and amplification strategies

A major limitation of human cancer gene therapy is a lack of significant efficacy. Systemic delivery, specific introduction, and specific expression of the target gene are the major issues to be managed in order to establish a clinically relevant treatment strategy. Although non-specific constitutive viral promoters (cytomegalovirus (CMV), respiratory syncytial virus) have been utilized with success in clinical applications *in situ*, the use of prostate-specific transcriptional activity could improve the efficacy of both *in situ* and systemic gene therapies, and ensure that the latter is safe to administer. Transcriptional targeting refers to the use of a particular cell-specific regulatory element (promoters or promoter/enhancers) to restrict gene expression to a particular tissue or cell type. Many prostate-specific gene regulatory regions are well characterized and several of them have been assessed in pre-clinical and clinical therapeutic studies (Table 1).

Promoters to direct prostate-specific gene expression

The prostate-specific promoter most extensively studied to date is derived from the prostate-specific antigen (PSA or human glandular kallikrein 3, *hK3*) gene, which encodes a serine protease.[3] Since PSA is expressed in normal prostate as well as at all stages of prostate carcinogenesis, it remains an excellent candidate for directing therapeutic gene expression specifically toward prostate tissue. Putative recognition sites such as for the androgen receptor (AR), activating protein-1, and c-fos transcription factors span the larger enhancer regions. However, a minimal 440 base pair core enhancer region was found to be most crucial in conferring strong activity and cell specificity. This enhancer core contains one high affinity androgen response element[4] and at least four non-consensus androgen response elements with variable AR affinity,[5] and these elements contribute to the synergistic activation of *PSA* gene expression. Extensive studies have been performed of methods to boost the inherently weak, though specific, activity of the native PSA promoter and enhancer.[6,7] The most effective amplification methods to boost the activity of PSA promoter will be discussed in the next section.

Both PSA (*hK3*) and the *hK2* belong to the same large kallikrein family and share similar gene regulatory elements that confer prostate-restricted gene expression.[8–10] *hK2* gene expression is increased in prostate carcinoma,[11] suggesting that this element also can be used for achieving target gene expression in prostate cancer. Approaches taken to enhance the PSA and the rat probasin promoter,[12] such as increasing androgen responsive elements,[13] have also been effective in increasing the activity and androgen inducibility of the hK2 promoter.[14] In the past, concerns had been raised that these androgen- and AR-regulated promoters (*i.e.*, PSA, hK2, and probasin) might not function in patients with androgen-independent (AI) prostate cancer. However, current findings indicate that the AR remains active in AI or metastatic stage of disease, and therefore mechanisms such as mutation or amplification of AR, enhanced co-activator activity, or modulation by other signaling pathways, such as interleukin-6 (IL-6) and insulin-like growth factors, can all compensate for the absence or low level of the activating ligand dihydrotestosterone.[15] Thus, the concept that AR-dependent promoters can have application in gene therapy even in recurrent AI prostate cancer is supported by the current understanding of prostate cancer progression.[16]

As we have mentioned earlier, several of the most extensively studied prostate-specific promoters are AR-dependent. However, the two notable exceptions are the osteocalcin (OC) promoter[17] and the prostate-specific membrane antigen (PSMA).[18] Epithelial and stromal interactions contribute to the establishment of prostate cancer bone metastasis.[19] It is interesting to note that prostate cancer cells begin to express bone specific proteins such as OC, osteopontin, and bone sialoprotein after metastasizing to bone.[20] *OC* gene expression is controlled by multiple elements, including an osteo-specific element 2, the vitamin D-responsive element, and cyclic adenosine monophosphate response element (CRE).[21–23] These osteomimetic properties provide a golden opportunity to target bony metastases of prostate cancer.[24–30] In the case of PSMA, it encodes an integral membrane protein that is expressed in primary prostate cancer and lymph node metastases.[31] Interestingly, *PSMA* gene expression is induced by androgen deprivation. Hence, PSMA promoter-driven cytotoxic gene therapy has been developed specifically to eradicate AI prostate cancer in the face of hormone ablation treatment.[32] However, recent studies have suggested that the induced gene expression is not completely restricted to the prostate,[33] and this leaky property might limit its potential use in clinical applications.

Given the heterogeneity of prostate tumors, a legitimate concern is that the use of a cell-specific promoter could limit transgene expression to a subset of the tumor cells, thereby reducing therapeutic efficacy. To address this issue, investigators have developed chimeric promoters by fusing crucial transcriptional elements from distinct prostate-specific promoters. Combining hK2 and PSA enhancer elements enhanced the promoter activity in AR-positive prostate cancer cells.[34] Another interesting approach is to create chimeric fusions from promoters with disparate functional activities. A novel prostate-specific enhances PSES chimeric promoter, composed of regulatory elements of *PSA* and *PSMA* genes, remained silent in non-prostate cell lines, but mediated high levels of expression in PSA- and PSMA-expressing prostate cancer cell lines in the presence and absence of androgen.[35] Thus, the PSES element exhibits promising activity in targeting AI prostate cancer. The most recently described chimeric promoter construct (PPT) is the most complex, combining the T-cell-receptor gamma-chain alternate reading frame protein promoter, the PSMA enhancer, and the PSA enhancer.[36] It was reported to be highly active in testosterone-deprived prostate cancer cells (PC-346C tumor model). An advantage of the PSES and the PPT promoters is their ability to remain active both in the presence and in the absence of androgen.[36]

Despite the high specificity of many prostate-specific regulatory regions developed to date, the magnitude of expression directed by these promoters may not be sufficient to mediate robust expression in vector-based *in vivo* gene therapy applications. To compensate for the low gene

transfer efficiency, it is critically important to incorporate the highest gene expression potency into each vector construct. Several successful approaches have been developed to amplify transcription from cell-specific promoters,[37] and the ones examined in prostate cancer applications will be discussed in the next section.

Transcriptional amplification strategies for increasing prostate-specific gene expression

The ubiquitously active strong viral promoters (*e.g.*, cytomegalovirus CMV enhancer/promoter or SV40 promoter) have been the benchmark promoters used in gene therapy. Hence, a goal in augmenting the activity of tissue-specific promoters is to achieve levels comparable to the CMV promoter, while retaining cell discriminatory activity. A useful two-step transcriptional amplification system has been developed to boost the activity of the weak but tissue-specific chimeric PSA promoter/enhancer (PSE-BC).[7] This two-tiered amplification methodology has been extensively tested and validated in amplifying expression from the PSA promoter or its variants,[7,16,38–42] and also for non prostate-specific promoters such as vascular endothelial growth factor, carcinoembryonic antigen and Mucin-1.[38,43,44] In the two-step transcriptional amplification system, a cell-specific promoter controls the expression of a potent transcriptional activator, which acts on a responsive construct to activate expression of a reporter/therapeutic gene (Figure 1a). The potent activator is the GAL4-VP16 fusion protein, comprising the DNA binding domain from the yeast transcription activator GAL4 and the transactivation domain from the herpes simplex virus 1 (HSV1) viral protein 16 (VP16). This GAL4-VP16 fusion protein binds specifically to multiple repeats of GAL4-binding sites upstream of the gene of interest. This transcriptional amplification system presents an extensive dynamic range that can be modulated by increasing the number of VP16 activator domains expressed in the fusion protein, and/or the number of GAL4 binding sites in the reporter/therapeutic construct. For instance, increasing the activator binding sites from one to five amplified the activity of the system more than 200-fold.[7] Several effective adenoviral vectors were generated, containing all the two-step transcriptional amplification components in a contiguous DNA insert and, in prostate tumors, they displayed activity levels higher than the CMV promoter.[7,16,38–42] Additionally, the heightened activity of two-step transcriptional amplification vectors enables sufficient expression of gene products to mediate robust positron emission tomography (PET) signals in the prostate tumor (Figure 2).

A novel regulation system involves a positive feedback loop with prostate specificity (Figure 1b). This systems incorporates a tet-responsive element upstream of the prostate-specific ARR2PB promoter driving a tet-transactivator to enhance its activity with tet regulation *in vitro*.[45] The expressions of both green fluorescent protein (GFP) and tet-transactivator were placed under the control of these TRE-ARR2PB promoters, and a positive feedback loop was demonstrated specifically in prostate cells. The innovation of this design lies in the combination of drug-inducible and tissue-selective transcriptional control to achieve even greater regulation of expression levels in prostate cancer cells.

A different conditional approach to enhancing tissue-specific promoter activity involves the bacteriophage P1-derived Cre-lox system (Figure 1c), a powerful and versatile tool for *in vivo* DNA recombination. When Cre recombinase expression is targeted using a tissue-specific promoter, conditional “knockout” of a target gene can be achieved in a particular tissue or cell type. This system can also be applied for enhancing tissue- or tumor-specific promoter activities in cancer gene therapy. Target cells are transduced with a vector harboring the therapeutic transgene separated from a strong constitutive promoter by a translational stop cassette flanked by two loxP sites. Co-transduction with a vector expressing Cre from a tissue-specific promoter results in excision of the stop cassette and expression of the transgene in a highly tissue-specific manner.[46] The Cre-lox system has been applied to enhance the activity of prostate-specific promoters such as PSA and PSMA.[47–49] In this approach, the Ad vector expressing Cre driven

by the PSA promoter-enhancer serves to activate the expression of the secondary CMV-loxP-stop-luciferase vector. As expected, this PSA promoter-based system was regulated by androgen, and its use in a cytosine deaminase (CD) suicide gene therapy inhibited LNCaP tumor growth in nude mice.[47] The efficiency of this binary system might be limited by the need for two vectors to transduce the same cell in order to achieve activation. As a result, the combined PSA promoter/enhancer and Cre-loxP system exhibited modest increases in activity (3× stronger) when compared with a direct PSA promoter/enhancer-driven vector. When a liposome-mediated gene transfer was used, the combination of the Cre/lox system with the PSMA promoter/enhancer (PEPM) greatly enhanced the efficacy of HSV-tk suicide gene therapy in $PSMA^+$ cells, as compared with the performance of the PEPM promoter alone.[48] Because the PSMA promoter is induced by androgen depletion, the PEPM-Cre/CMV-lox system also exhibited a stronger inhibitory effect on tumor growth in castrated mice as compared with intact non-castrated mice. These findings indicate that the PEPM- and Cre-lox mediated cytotoxic therapy may be applicable to patients under androgen deprivation therapy.[49] Another interesting strategy involved an altered form of Cre recombinase (CRE-M) that displays an androgen-induced activity. CRE-M was generated by fusion of Cre to the AR ligand binding domain, and three different forms of ligand binding domain (wild-type, non-ligand binding, and Thr → Ala mutant) were examined.[50] The activity of CRE-M-LBDwt incorporated in adenovirus was stimulated tenfold by the addition of androgen. Interestingly, CRE-M-LBD mutant activity was inducible by both androgen and the anti-androgen flutamide, suggesting that the CRE-M-AR-LBD system can provide an additional level of regulation to Cre/loxP-mediated prostate gene therapy applications.[50]

The utility and potential benefits of transcriptional-targeted expression and amplification strategies have been demonstrated largely in preclinical animal models. The incorporation of transductional-targeted strategies will likely enhance the efficacy of gene therapy modalities for the treatment of prostate cancer. Transductional-targeting approaches have shown great potential for systemic delivery in treating metastastic disease. These approaches have included redirecting Ad tropism to prostate cancer cells via alpha6beta1 integrins,[51] epidermal growth factor receptor,[52] and PSMA,[53] either incorporated into the Ad vector or coupled to a polymer coating. Clearly, the efficacy of these newer transcriptionally and transductionally targeted approaches in clinical settings will need to be assessed in comparison with traditional vectors in future clinical trials.

Coupling molecular imaging with gene therapy

The basis of “molecular” imaging is to exploit specific molecular probes to achieve image contrast in order to differentiate target tissue from normal or background tissue in the subject.[54] Molecular imaging is particularly beneficial in cancer gene therapy,[55] because it can answer many hitherto unanswered questions about the efficacy of gene delivery and expression *in vivo.* Has the gene reached its target? Is the gene product being expressed in the target, and if yes, what is the magnitude? More importantly, how long does the gene expression persist *in vivo*? Figure 2 is a synopsis of using *in vivo* imaging to assess the location, duration, and magnitude of gene transfer, as well as therapeutic activity.

The most common method to query *in vivo* transgene expression is to use imaging reporter genes. Optical imaging technologies based on bioluminescent (*e.g., luciferase* genes) and fluorescent proteins produced in nature have been applied widely as imaging reporters, allowing efficient, non-invasive and rapid assessment of transgene expression in pre-clinical small animal models (Figure 2a). However, the attenuation and scattering of visible light as it traverses through tissue severely hinders the use of optical imaging for applications in humans. The high-energy radionuclide imaging modality such as PET has the distinct advantage of producing 3D localized signals, which can be translated from small animal to human subjects

(Figure 2b). Two categories of PET reporter systems have been developed that either utilize intracellular enzymes (*e.g.*, HSV1 thymidine kinase (HSV1-tk)), or cell membrane receptors (*e.g.*, dopamine, somatostatin, transferin, and sodium iodide symporter (NIS)). We will focus our discussion on the *HSV1-tk* and *NIS* genes because they have the dual capacity to provide imaging, and to serve a potentially therapeutic function.

The *HSV1-tk* gene is currently one of the most frequently used PET reporter genes. The enzymatic activity of HSV1-TK is also the cornerstone of anti-viral treatment[56] and suicide gene therapy to treat cancer.[57] In PET applications, the phosphorylation of radioactive guanine derivatives leads to the trapping and accumulation of the radiolabeled tracer in cells expressing HSV1-TK. Because the tracer level of the radiolabeled probe used in PET is 3–4 orders of magnitude lower than the pharmacological dosage in cytotoxic therapy it causes no harmful effects. Extensive research in chemical synthesis has been devoted to developing the ideal radionuclide PET probe or substrate for HSV1-TK.[58–63] Among the fluorinated analogs developed to date, [^{18}F]-9-(4- fluoro-3-hydroxymethylbutyl)guanine exhibits the most favorable characteristics for *in vivo* applications.[64]

Improvements were made to the *HSV1-tk* gene for imaging applications by carrying out genetic engineering on the thymidine kinase (TK) protein active site to increase the affinity towards acycloguanosine analogs while decreasing the affinity for thymidine,[65,66] a natural substrate and competitor. A particular mutant, HSV1-sr39tk, exhibited a greater-than-twofold enhancement in imaging sensitivity for [^{18}F]-9-(4-fluoro-3-hydroxymethylbutyl)guanine when compared with the wild type *HSV1-tk* gene.[67] Among the different reporter gene-and-probe combinations studied, HSV1-sr39tk/[^{18}F]-9-(4-fluoro-3-hydroxymethylbutyl)guanine offered the maximum sensitivity for *in vivo* imaging,[68] enabling the real-time quantification of Ad mediated gene transfer to murine liver[61] and prostate tumors.[41] In suicide gene therapy, the ability to monitor gene transfer to the targeted tumor in living animals provided credence to the therapeutic effects observed (Figure 2b). Importantly, this PET-coupled *HSV1-tk*-mediated suicide gene therapy has proven to be feasible in the clinical setting for treating patients with hepatic carcinoma.[69]

The NIS is a good example of a transporter protein that has been exploited for gene therapy imaging by virtue of its ability to specifically incorporate a labeled tracer into the cell (reviewed in ref. [70]). Endogenous NIS is a transmembrane protein that is expressed mainly in the thyroid, the stomach, and the salivary glands.[71] In the thyroid, the NIS protein transports iodide into the cytoplasm and concentrates it 20- to 40-fold, driven by the sodium gradient across the basal membrane.[72] NIS-mediated radioisotope uptake has been used clinically for decades in the diagnosis and therapy of thyroid disease.[73] The cloning of its complementary DNA initiated the use of *NIS* as an imaging reporter gene[74–77] and a cancer therapeutic gene,[78] including in prostate cancer.[79] One distinct advantage of using *NIS* in the dual role of reporter gene and therapeutic gene is that the biodistribution, metabolism, and toxicity of many radiotracers are well known from decades of clinical experience. Although isotopes of iodide ($^{123/124/131}$I) are its most common substrates, NIS transports a wide range of other isotopes including ^{99} mTc Pertechnetate, $^{188/186}$Re Perrhenate, and ^{211}At, which may be employed for many diagnostic and therapeutic purposes. Most recently, several studies demonstrated the utility of the *NIS* gene in modeling gene transfer into the prostate glands of larger animal subjects such as dogs. For instance, Ad vector-mediated NIS expression was successfully imaged by single photon emission computed tomography.[80,81] The magnitude of spreading of an oncolytic Ad vector within the dog prostate gland was assessed by NIS-mediated accumulation of radioactive tracer.[80] Collectively, the promising activities of *HSV-tk* and *NIS* genes suggest that imaging of prostate cancer gene therapy in action might be on the horizon.

THERAPEUTIC STRATEGIES

Immune-based gene therapy approaches

The major focus of this treatment is to modulate the immune system to reject prostate tumor cells. It is a well-tolerated modality, and has progressed the furthest along among the various therapeutic options under clinical investigation.[82,83] The three common approaches to generating prostate cancer-targeted immune response are (i) tumor cell vaccines, (ii) vector-based vaccines, and (iii) dendritic cell (DC)-based vaccines.

Early in the development of whole tumor cell vaccines, irradiated autologous tumor cells engineered to express granulocyte-macrophage colony-stimulating factor were used to treat patients who had metastatic prostate cancer at the time of surgery.[84] This autologous vaccine strategy was greatly hampered by the laborious process required for cell preparation and the low volume of tumor cells that could be harvested. Therefore the focus of development was shifted to allogeneic vaccines using established prostate cancer cell lines, such as GVAX, a vaccine composed of a mixture of PC3 and LNCaP cells transduced with granulocyte-macrophage colony-stimulating factor.[84,85] Another allogeneic whole cell vaccine is Onyvax-P (Onyvax, London, UK), composed of OnyCap23, LNCaP and P4E6 cells, that also exhibited the ability to delay time to progression in a small cohort of patients with AI prostate cancer.[86] The advantages of the allogeneic vaccine are its “off the shelf” feature and the option of adding co-stimulatory molecules and cytokines to augment immune responses. On the other hand, the main drawbacks of this approach are the potential interference from the multitude of antigens in the vaccine, and the fact that it is impossible to assess specific immunological responses during the treatment.

The vector-based vaccine strategy involves the use of viral vectors expressing tumor antigen and immune stimulatory molecules. The development of this strategy has been focused on selecting the proper viral vector, co-stimulatory molecules, cytokine cocktails, and immunization regimes for achieving optimal antigen presentation and T-cell activation *in vivo*. The vaccination strategy against PSA is achieved by priming with live recombinant vaccinia and boosting with recombinant fowl pox and is among the most well-studied approaches.[87–89] Although PSA-specific T-cell responses were demonstrated in these studies, significant clinical benefits were not established.[87–89] Taken together, the immune-based therapies hold promise in eliciting a targeted response against disseminated disease. But they are unlikely to be effective in eradicating bulky diseases.[83] Hence, the future investigations would be to determine the optimal combination of immune-activating approaches and cytotoxic therapies to improve patient outcomes.

Because of their proficiency in antigen presentation, DCs have been widely exploited in cancer vaccine applications, including in prostate cancer.[90] To elicit anti-tumor immunity, the autologous DCs isolated from patients were pulsed *ex vivo* with messenger RNA, DNA or peptides of tumor antigens, and infused back into the patients. Studies with small cohorts of patients have been completed, employing autologous DCs pulsed with PSMA,[91,92] PSA,[93,94] or recombinant prostatic acid phosphatase fused to granulocyte-macrophage colony-stimulating factor.[95,96] Immunological responses against the targeted antigen were demonstrated in these studies, but clinical benefits to patients were difficult to substantiate. The challenges of processing patient-derived DCs and the inherent non-uniformity of the therapeutic agent are the shortcomings of the DC-based vaccination strategy.

Cytotoxic gene therapy approaches

Cytotoxic or cytolytic gene therapy can involve the transfer of drug-susceptible (“suicide”) genes, or proapoptotic genes. The “suicide” gene strategy uses a gene encoding an enzyme that

converts a nontoxic prodrug into a cytotoxic form when transfected into tumor cells. Two common suicide gene therapy systems are the classic *HSV1-tk*, and the *Escherichia coli CD* genes. HSV1-TK converts nontoxic nucleoside analogs such as ganciclovir into phosphorylated compounds that act as chain terminators of DNA synthesis, while the prodrug for CD is 5-fluorocytosine. Tumor cell killing is achieved by necrosis and apoptosis.

The *E. coli* nitroreductase (NTR) is another example of a suicide gene which efficiently kills tumor cells upon reduction of a systemically administered prodrug CB1954 [5-(aziridin1-yl)-2,4-dinitrobenzamide] to a DNA-crosslinking derivative at the tumor site. The effect is independent of cell cycle, and a crucial bystander killing effect is observed on untransduced tumor cells. Testing *in vivo* by Ad-NTR administration in a murine prostate tumor model induced necrosis and upregulation of heat shock hsp25 and hsp70 proteins.[97] In the same study, combining NTR with hsp70 expression elicited enhanced protection over NTR alone for both the treated tumor and a subsequent re-challenge via $CD4^+$ and $CD8^+$ T-cell responses. Ad-NTR/hsp70 therefore comprises a promising cytotoxicplus-immunomodulatory gene combination for treating prostate tumors. It should be interesting to see whether Ad-NTR/hsp70 therapy can be further enhanced by cytokines.

In the proapoptotic gene strategy, Ad vectors expressing tumor necrosis factor family genes such as Fas ligand (*FasL*) and *TRAIL* (tumor necrosis factor–related apoptosis-inducing ligand) are employed commonly. Two particular issues that need to be addressed in using this therapeutic mechanism are (i) to overcome the resistance that some tumor cells develop, and (ii) to restrict the cytotoxic effects to tumor cells only. Low dose chemotherapeutic agents can induce sensitivity of prostate tumor cells to TRAIL by down regulation of an intrinsic anti-apoptotic pathway[98] by downregulation of the intrinsic anti-apoptotic X-linked mammalian inhibitor of apoptosis protein pathway.[99] Recently, a histone deacetylase inhibitor (depsipeptide) was also shown to augment Ad-TRAIL cytotoxic activity in human prostate tumor cells by increasing levels of TRAIL-R1 and R2 in membrane lipid rafts.[100] Interestingly, depsipeptide treatment also augmented the expression of the coxsackie Ad receptor, which provided an additional mechanism to boost the efficacy of Ad-mediated TRAIL gene therapy. A prostate-directed inducible strategy was developed to restrict the cytotoxic effects of FasL to prostate tumor. In this approach, the mouse *Tnfsf6 (FasL)-GFP* fusion gene was controlled by a tetracycline-responsive promoter.[101] This intricate expression system will be described in more detail in the next section. Systemic delivery of these vectors showed that the inducible FasL-GFP system was better tolerated at doses lethal to Balb/c mice for a CMV-driven vector, suggesting that inducibility plus prostate-restricted transgene expression may improve the safety and efficacy of cytotoxic gene therapy.

A new class of cancer-specific apoptosis-inducing genes is represented by the *mda-7/IL-24* (melanoma differentiation associated gene-7) gene. *In vitro*, Ad-mda7 induces G2/M arrest through the inhibition of cdc25c pathway and apoptosis in a cancer-specific manner, without affecting normal cells.[102,103] These effects are mediated through mitochondrial pathways involving bcl-2, and independent of Bax and p53.[102] Furthermore, expression of Ad-mda7/IL-24 in normal cells can induce direct anti-tumor and radiation-enhancing effects that are dependent on the presence of receptors for the IL-24 cytokine on tumor cells.[104] The combination of secreted mda7/IL-24 and radiation results in a bystander antitumor effect not only in mda7/IL-24 and radiation-sensitive cancer cells, but also in tumor cells overexpressing anti-apoptotic proteins bcl2 or bcl-xL, and in those resistant to either treatment. Another promising gene that exhibited anti-tumor activity in a pre-clinical model of prostate cancer is the mouse *RTVP-1* gene (related to testes specific, vespid, and pathogenesis proteins), which is a direct target of p53.[105] Administration of the Ad-RTVP1 in an orthotopic metastatic murine prostate model resulted in extension of animal survival by diverse effects such as reduced metastasis to lung, suppression of tumoral angiogenesis, and increased infiltration of

macrophages, DCs, and $CD8^+$ T cells into the tumor. The ability to induce a plethora of anti-tumoral effects such as proapoptotic, antiangiogenic, and immunemodulatory functions will be the hallmark of a promising candidate gene, because these are important not only for the eradication of localized tumors but also in the suppression of systemic metastases of prostate cancer, especially in treating high-risk localized disease.

It is intuitively logical to use prostate- or prostatic cancer-specific promoters in directing potent cytolytic and oncolytic gene therapy. Restricting the expression of cytotoxic genes to the targeted tumor cells could potentially improve the dose-limiting toxicity of a therapeutic vector by reducing systemic side effects.

Conditionally replicating oncolytic adenoviruses

From investigations carried out during the past decade it is clear that we need to overcome the inefficient *in vivo* gene transduction in order to achieve successful cancer gene therapy. Adenovirus is a lytic virus, capable of producing up to 10,000 progeny viruses per host cell. Thus, the rationale for using replication-competent, rather than replication-defective adenoviruses as gene therapy vectors is because: (i) replication-competent adenoviruses are cytolytic and have demonstrated anti-tumor activity in both preclinical tumor models and humans, (ii) replication-competent adenoviruses have the potential to replicate to high copy, thereby resulting in significantly greater therapeutic gene expression *in vivo*, and (iii) replication-competent adenoviruses have a greater potential to spread locally and therefore could infect a greater number of tumor cells. All of these potential advantages have now been demonstrated in large animal models or humans.[106–111] In attempting to harness this potent viral replicative power towards prostate tumor destruction, a key consideration is how to "turn on" this process specifically in prostate tumors. One approach is to restrict viral replication only in prostate tumor cells. This prostate-restricted approach can safeguard against viral replication in normal non-prostate cells in case of viral spillage into systemic circulation during local therapy.[9] If proven effective and specific, the prostate-targeted strategy has the potential to be a therapeutic option for metastatic prostate cancer.

The first step in the strategy to develop an oncolytic adenovirus is to take advantage of viral replication pathways that could discriminate between cancer cells and normal ones. It is known that Ad 55 kd E1b protein can inactivate tumor suppressor p53, and p53 is frequently inactivated in tumors. This gave rise to the interesting line of reasoning that a 55 kd-E1b deleted adenovirus called "dl1520" or "ONYX-015" would replicate only in p53-defective tumor cells but not in normal cells.[80] This strategy has been intensively investigated, including 16 phase I and II clinical trials for head and neck carcinoma, pancreatic cancer, ovarian cancer, colorectal cancer, hepatobiliary cancer, gastric cancer and gliomas.[112–118] In clinical investigations of ONYX-015, modest responses were observed when it was used as a single agent. Recent findings support the view that the replication of ONYX-015 is probably dependent not on p53 inactivation or p19ARF status,[119–122] but rather on late viral messenger RNA export.[123] Further investigation to determine the underlying targeting mechanism or pathway used by ONYX-015 will enhance its usefulness in cancer therapeutics.

A second strategy involving the use of oncolytic virus that has worked particularly well in prostate cancer is to employ a tissue-specific promoter to control the expression of adenovirus E1A and E1B proteins, which are key regulators of viral life cycle. The E1A proteins are the first viral gene products generated, and they initiate and coordinately regulate the intricate cascade of viral DNA replication and gene expression. The large E1A protein transactivates all other early and late viral genes. The small *E1A* and *E1B* gene products modulate the activity of tumor suppressors (*e.g.*, pRB and p53) and apoptotic pathway in the host cell in order to benefit viral propagation. Hence, deletion of E1 genes is the mainstay of creating a replication-defective adenovirus. Conversely, placing E1 gene expression under prostate-specific

promoter should restrict virus replication in a tissue-specific manner. As discussed in the previous section, many prostate-specific gene regulatory systems have been developed (Table 1). Among them PSA, PSMA, and OC are biomarkers of prostate cancer, and their promoters are excellent candidates to control prostate-specific Ad replication.

The first prostate-restricted replicative adenovirus was developed by Rodriguez *et al.*[106] Calydon virus (CV706) was engineered by placing the Ad *E1A* gene under the control of the minimal PSA promoter and enhancer sequences. The E1A expression of CV706 was limited to PSA-positive LNCaP human prostate cancer cells, and anti-tumor activity was demonstrated against LNCaP tumor xenografts.[106] When combined with radiation therapy, a significant synergistic effect was demonstrated both in cell culture and xenograft models.[107] In a clinical trial, it was found that it is safe to administer CV706 at less than the maximum tolerable dose. A drop in serum PSA greater than 50% was achieved in patients treated with the highest doses of CV706.[108] In order to refine the viral oncolytic efficacy in a second generation virus CV787 (also known as CG7870), the viral E3 region was retained to enhance viral spread, and both *E1A* and *E1B* genes were driven by prostate-specific promoters; E1A was controlled by the rat probasin promoter and E1B by the human PSA promoter/enhancer. By restricting the expression of both viral early genes (*E1A* and *E1B*) to prostate-specific cells, further improvement was achieved in the tissue-selectivity of viral replication as compared with CV706, in which only the E1A expression is restricted.[109] CV787 replicated as efficiently as wild-type Ad5 and were successful in eliminating LNCaP xenograft tumors when administered by tail vein injection.[109] Further *in vivo* analysis revealed that the effect of CV787 is synergistically enhanced both with radiation therapy[110] and with chemotherapeutic agents such as paclitaxel and docetaxel.[111]

Improved transcriptional-based strategies have been developed for targeting AI or metastatic prostate cancer cells. In particular, the OC promoter and the novel chimeric PSES promoter have been utilized to drive oncolytic viral replication.[124] Matsubara *et al.* utilized the murine OC promoter to restrict the expression of E1A to prostate epithelia and its supporting bone stroma in osseous metastases of prostate cancer. This virus, named Ad-OC-E1A, appears to be more effective than PSA-controlled virus at killing a broader spectrum of prostate cancer cells, including LNCaP, C4-2, and ARCaP (PSA-positive) as well as PC-3 and DU-145 (PSA-negative). Intratumoral injection of Ad-OC-E1A effectively obliterated subcutaneous AI PC-3 athymic mouse xenograft models. In addition, intraosseous C4-2 prostate cancer xenografts responded very well to the systemic administration of Ad-OC-E1A. One hundred percent of the treated mice responded with a drop in serum PSA below detectable levels. At the conclusion of the study, 40% of the treated mice were cured, with no evidence of prostate cancer cells in the skeleton.[125] To improve upon this technology, Hsieh *et al.* developed Ad-hOC-E1, employing a centrally located human OC promoter to transcript both the E1A and E1B genes.[22] Under the control of this vitamin D-responsive promoter, Ad-hOC-E1 induced tenfold higher replication and cytotoxicity than wild-type adenovirus upon administration of vitamin D.[22] Vitamin D treatment alone can exert mild anti-proliferative effect on prostate cancer cells.[126,127] However, the systemic administration of a combination of vitamin D and Ad-hOC-E1 showed a synergistic effect, with repression of tumor growth being better achieved than with either treatment given alone.[22]

In applying PSES promoter to oncolytic technology, Li *et al.* created AdE4PSESE1A with both *E1A* and *E4* genes regulated by the prostate-specific promoter.[128] The *E4* gene is an essential viral early gene that modulates viral transcription and messenger RNA transport. AdE4PSESE1A replicated and lysed PSA/PSMA-positive tumor cells with the same efficiency as wild-type adenovirus did. As expected, replication of AdE4PSESE1A was severely impaired in PSA/PSMA-negative cells. AdE4PSESE1A effectively suppressed the growth of AI CWR22rv mouse xenograft tumors.[128] In order to obtain a better understanding of the kinetics

of the oncolytic process, the GFP driven by the CMV promoter was inserted into AdE4PSESE1A, enabling the use of intraviral imaging for monitoring viral replication and spread within the tumor. Three days after AdE4PSESE1A injection, a big boost in GFP expression was observed, indicating rapid replication and viral spreading, decreasing 1 week after injection. Although subsequent rebound in GFP expression indicative of renewed viral amplification was observed, tumor growth and viral oncolysis reached equilibrium at 2 weeks time. After this point, tumor growth exceeded the killing rate.[128] Despite the high oncolytic activity of the virus, several studies have reported limited viral spread and incomplete tumor eradication.[129,130] What are the mechanisms that hinder the oncolytic process *in vivo* and how can we improve its efficacy?

One plausible explanation is that conditions within the established tumor become unfavorable for viral replication. As tumor cell lysis occurs, the tumor environment becomes highly necrotic and hypoxic. Several recent studies observed a reduced level of viral replication in hypoxic conditions, attributed to diminished levels of E1A protein expression without concomitant decrease of messenger RNA.[131,132] On the basis of these findings, future investigations aimed at understanding the mechanism that modulates E1A protein stability (and how to stabilize E1A in hypoxia) may improve the efficacy of viral oncolytic therapy. Extensive investigation by Wold and colleagues has demonstrated that the 11.6 kd adenovirus death protein encoded in the E3 region enhances viral spreading and cytolytic activity of replication-competent adenovirus in cell culture studies.[133,134] The E3 region is involved in modulating the host immune response to Ad infection and is non-essential for *in vitro* viral replication. Thus, quite often the E3 region is deleted in oncolytic Ad vectors due to space constraints in the E1-containing viral genome. A recent study demonstrated that the incorporation of adenovirus death protein into a multimodality replication-competent adenovirus enhanced the viral spread and the distribution of expressed transgene within the prostate glands of dogs.[80] Clearly, replication-restricted viral oncolysis is a promising and emerging technology in treating prostate cancer, but additional modifications and improvements are required for maximum effectiveness. There has been extensive investigation regarding the integration of expression cassettes for therapeutic genes, such as *TRAIL*, *CD/TK*, *FasL*, antiangiogenic factor, and immune modulators into the conditional replication-competent virus in order to enhance therapeutic efficiency.

CONSIDERATIONS IN COMBINED TREATMENT MODALITIES

In cancer therapeutics, and especially in treating advanced stage prostate cancer, rarely is a cure achievable by using only one form of treatment. Due to intratumoral cell heterogeneity, combined modality therapies may achieve additive or synergistic therapeutic activity. As gene therapy matures as a treatment option, an important consideration is about the kinds of rational and beneficial combined treatments that investigators should explore. Combined therapies can take on the form of (i) simultaneous delivery of multiple therapeutic genes, (ii) combining gene therapy and radiation therapy, and (iii) treating with both gene therapy and chemotherapy. In considering simultaneous delivery of multiple gene therapies, let us follow up on the earlier discussion about oncolytic therapy. In the scenario where a hypoxic environment is created by viral therapy within the tumor, the expression of vascular endothelial growth factor A and tumor angiogenesis would be induced, and the resulting neovasculature would promote tumor growth. If this mechanism is in play, then the use of an antiangiogenic factor as an adjuvant to oncolytic therapy should promote tumor eradication. Recently, Jin *et al.* demonstrated the effectiveness of a combination of Ad-hOC-E1 with Ad-Flk1-Fc, a vector delivering the secreted form of the antiangiogenic factor Flk-1, also known as vascular endothelium growth factor receptor 2. When used as single agents, Ad-hOC-E1 and Ad-Flk1-Fc reduced C4-2 mouse xenograft models by 60 and 40%, respectively. However, when co-infection with both viruses was carried out, the average reduction in tumor size was 90% in 8 weeks, with 30% of

the tumors completely regressing by week three.[135] Liu *et al.* combined antiangiogenesis with an E1B-55K-deleted oncolytic virus expressing the apoptosis inducer, TRAIL. In this study, Ad-k5, a replication-deficient Ad delivering the fifth kringle domain of plasminogen, which inhibits angiogenesis by regulating the intrinsic angiogenic factor pathway, was co-injected with ZD55-hTRAIL into colorectal carcinoma mouse xenografts. This combined therapy resulted in the complete eradication of all treated tumors.[136] The future designs of replicative adenoviruses could include an antiangiogenic gene engineered into the same vector.

Other multi-gene therapies that have demonstrated enhanced efficacy in comparison with single modality therapies include dual suicide genes for enzyme/prodrug strategies (HSV1-tk and CD),[137,138] suicide-plus-apoptotic therapies (HSV1-tk and p53),[139] and combined cytotoxic (HSV-tk) and immune-activation (Flt-3).[140] Combined administration of paclitaxel and p53 gene revealed therapeutic synergy in prostate DU145 xenografts.[141] The combined therapeutic approach using prostate-specific oncolytic Ad CV787 (or CG7870) and Taxotere displayed significant synergistic antitumor activity in mouse tumor models of prostate cancer.[110] Because early clinical studies on suicide gene therapies showed low gene transduction rate and limited beneficial results, the multi-gene combined treatment has begun to transition into clinical investigations. Freytag *et al.* integrated a double suicide gene, a CD/TK fusion gene, into ONYX-015 conditional replication-competent adenovirus (Ad5-CD/TK*rep*), and further combined gene therapy with radiotherapy.[141–143] Following a single intraprostatic adenovirus injection, subjects received 5-fluorocytosine and valganciclovir prodrug therapy and a standard course of conformal radiotherapy.[144] The multi-modal treatment was well tolerated, with no dose-limiting toxicities. However this strategy was limited by two factors. First, it was based on ONYX-015 whose replication was neither p53-dependent nor tumor-restricted as discussed earlier. This makes it difficult to predict which of the patients will respond well to the therapy. Second, this strategy could be applied only to treat local disease, because it used a strong universal CMV to control the expression of the CD/TK fusion gene. The use of a prostate-specific promoter, such as OC, PSES and PPT, could broaden its use in treating metastatic lesions. The OC promoter has been applied to control TK gene expression and thereby to inhibit the growth of advanced stage AI prostate cancer cells.[125] A Phase I clinical study with Ad-OC-TK demonstrated the safety of the therapy and showed some therapeutic effects in treating bone metastasis.[145] In the light of these promising pre-clinical and early clinical findings, clinical trials employing multi-gene therapeutic strategies in combination with standard radiation or chemotherapy will be the logical way forward to improve the management of advanced-stage prostate cancer.

CONCLUDING REMARKS

Contrary to public perception, gene therapy has demonstrated an outstanding safety profile in humans. Although it has yet to demonstrate a significant improvement in outcome in a randomized, controlled trial of prostate cancer, the strategy of combining multi-modal gene therapy with conventional radiation or chemotherapy has generated promising results in early stage trials in select patient groups. To achieve the most effective control of the disease, gene therapy should be considered as one arm of a multi-pronged approach to improve the outcome of first-line therapies such as surgery or radiotherapy. Although novel systemic therapies (*e.g.,* GVAX, improved androgen blockage therapy) have showed some promise, the management of metastatic disease still poses great challenges for clinicians. As a collective group of investigators with the common goal of improving the health of prostate cancer patients, we cannot sit idle and rely on yet unproven promises. We need to maintain diligence and continue to improve our technologies. Transcriptional targeting is a means to further improve specificity and efficacy of gene therapy. Amplifying the magnitude of transgene expression, boosting copies of a vector genome in a replication-competent system, and combining multiple therapeutic modalities including gene therapy, are all proven strategies to

achieve synergistic augmentation of therapeutic efficacy. Coupling non-invasive imaging to gene therapy will not only provide better transparency of the therapeutic process *in vivo* but will also provide an early confirmation of gene transfer, which should be predictive of therapeutic efficacy. Clearly, the numerous improvements achieved in the last 5 years in gene therapy for prostate cancer will promote the translation of many promising therapies to the clinics.

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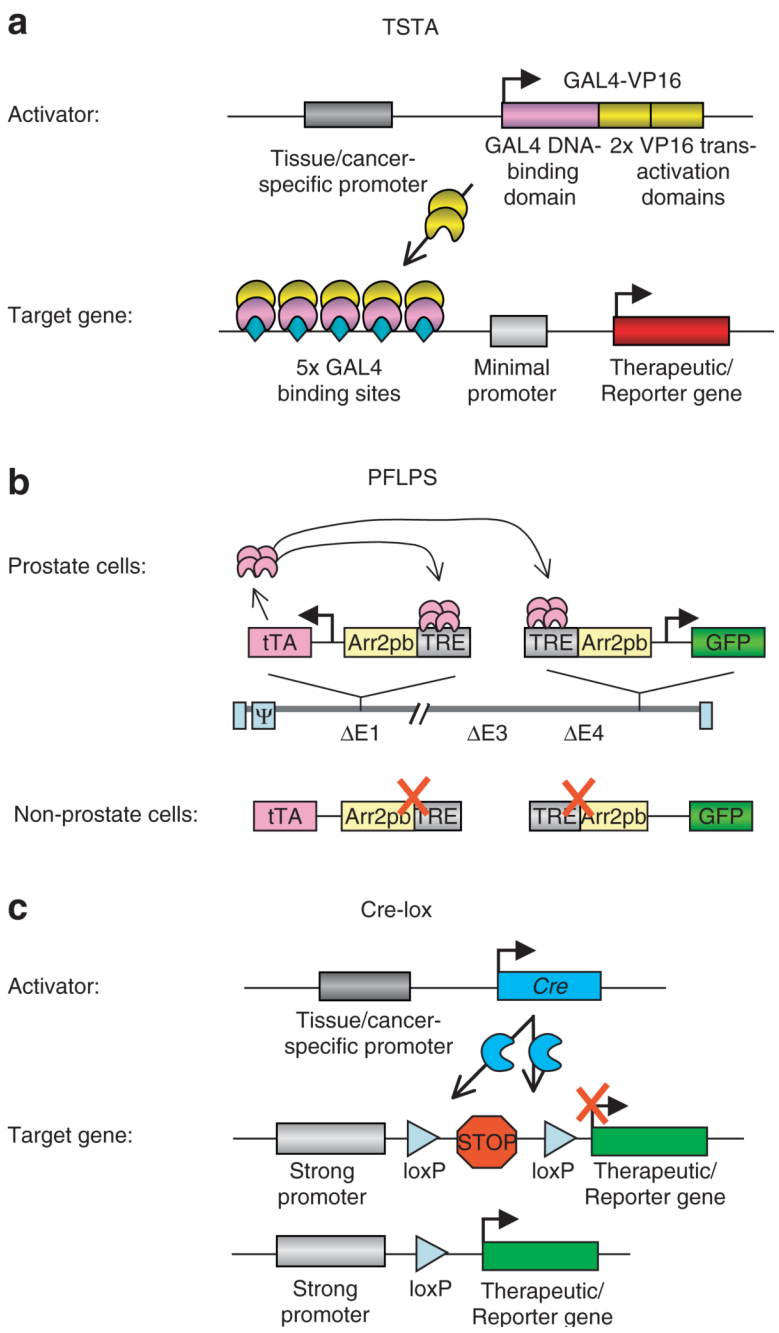

Figure 1. Transcription amplification targeting systems

(**a**) The two-step transcriptional amplification (TSTA) system consists of a two-step transcriptional activation process. In the first step, the artificial GAL4-VP16 activator is expressed in a tissue-specific manner by virtue of regulation by a tissue- or cancer-specific promoter (*e.g.*, prostate-specific antigen promoter). In the second step, the GAL4-VP16 protein binds to five GAL4 DNA binding sites upstream of a minimal promoter, and activates expression of a reporter gene or a therapeutic gene. (**b**) A new amplification system, a novel positive feedback loop with prostate specificity (PFLPS).[45] Both tet-transactivator (*tTA*) and green fluorescent protein (*GFP*) genes are expressed in a tissue-specific manner by ARR2PB promoters. Binding of tTA protein to the tet-responsive element (TRE) sequences upstream of

the ARR2PB promoters can induce GFP and tTA expression further, initiating a positive feedback loop. In non-prostate cells, where the ARR2PB promoter is inactive, gene expression would not be induced. (**c**) Cre-lox system-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 by a Cre-specific recombination between the two loxP sites.

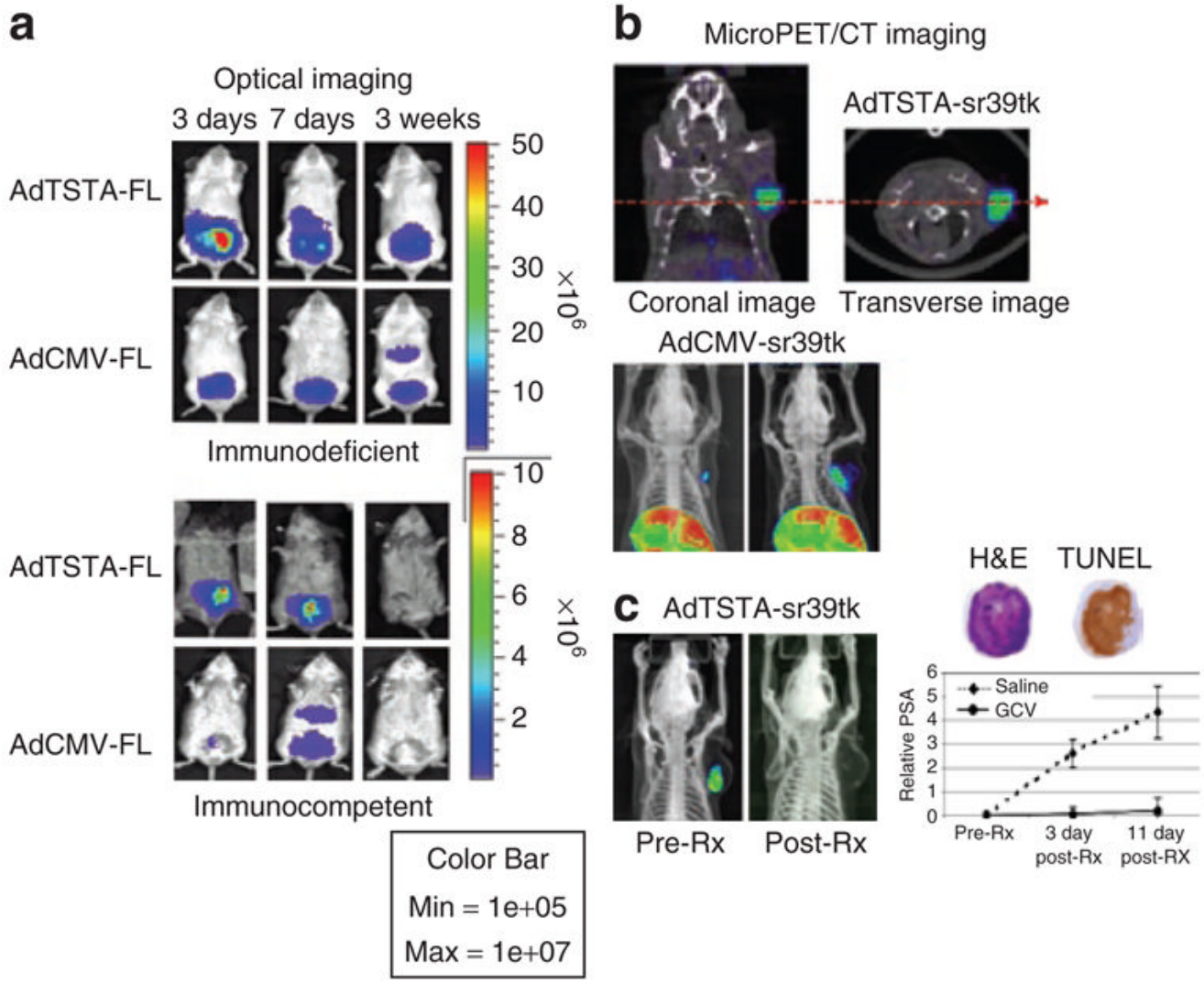

Figure 2. Utility of coupling *in vivo* imaging to prostate-targeted gene therapy

(**a**) Location and duration of gene expression as followed by optical imaging for firefly luciferase (FL) activity. After intraprostatic administration of 1×10^7 infectious plaque forming units (pfu) of AdTSTA-FL or AdCMV-FL into immune competent (Balb/c × 129) and severe combined immune deficient mice, FL expression directed by the prostate-specific AdTSTA-FL was localized to the prostate gland, whereas AdCMV-FL produced a detectable level of expression in the liver. Trans-gene expression mediated by each of the vectors was higher and more persistent in immune-deficient than in the immune-competent mice. Liver and prostate signals were confirmed by *ex vivo* organ imaging (data not shown). (**b**) Magnitude and location of gene expression by vector mediated [^{18}F]-9-(4-fluoro-3-hydroxymethylbutyl)guanine positron emission tomography (FHBG-PET). Robust HSV-sr39tk-dependent PET signal was detected in the LAPC-4 prostate tumor 8 days after intratumoral injection of 4×10^9 pfu of AdTSTA-sr39tk. The coronal and transverse images of a PET/computed tomography (CT) study are represented. The lower panel refers to two animals, both of which received similar 4×10^9 pfu of AdCMV-sr39tk injections directly into the tumor. However, their levels of transgene expression on day 8, reflected by FHBG-PET signals, were different. Unexpectedly, strong PET signals were observed in the livers of both animals. This finding is attributed to

spillage of AdCMV-sr39tk into systemic circulation despite the tumor-directed viral administration.[41] (**c**) Monitor suicide gene therapy by PET. The paired images are the PET/CT of one representative animal injected with AdTSTA-sr39tk, pre- and post-treatment with ganciclovir (GCV). The tumor-localized PET signal declined post GCV treatment. The graph on the right shows that the serum prostate-specific antigen (PSA) of the control group (saline treatment) continued to rise, while the serum PSA of GCV-treated cohort showed no increase, thereby indicating that tumor growth was halted in this group. Immunohistochemical analysis of the treated tumor showed substantial apoptosis, indicated by TUNEL-positive staining.[41] Ad, adenoviral; CMV, cytomegalovirus; H&E, hematoxylin and eosin; TSTA, two-step transcriptional amplification; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick end labelling.

Table 1

Prostate-specific regulatory promoter/enhancers applied in Ad vector-mediated interventions *in vivo*

Regulatory element configuration	Gene therapy; summary of results	References
(a) Native promoters		
PSA (prostate-specific antigen)	• *HSV-tk* (therapeutic); cell killing *in vitro* and growth inhibition in tumor model	• Huang[5]
hK2 (Kallikrein-2, PSA-related)	• E1 protein (oncolytic); expression in PSA^+, prostate tumor-selective replication	• Yu[9]
OC (osteocalcin; prostatic metastasis and bone cell-specific)	• *HSV-tk* (therapeutic); Phase I clinical trial	• Koeneman[30]
	• *E1a* and *E1b* (oncolytic); conditional replication competence in OC-expressing cells, growth inhibition in tumor model	• Hsieh[22]
(b) Improved promoters		
PSE (PSA promoter/enhancer)	• *Luciferase* (detection of metastasis); expression (PSE-BC) higher in AI than in AD tumors and excluded from liver. Non-invasive bioluminescence imaging showed that the adenovirus could locate and illuminate lung and spine metastases. Systemic injection of low Ad doses also illuminated lung metastasis	• Adams[146]
	• *Nitroreductase* (therapeutic); expression (PSE) in PSA^+ cells inducible by androgen and comparable to that of CMV vector	• Latham[14]
Rat Probasin (ARRPB2)	• *E1a* (oncolytic); selective replication in PSA^+ cells	• Yu[109]
	• *Bad* (apoptotic); cell-specific expression and cell death *in vitro*; tumor size reduction in tumor models	• Zhang[147]
	• *HSV-tk* (therapeutic); retinoid inducible; growth suppression of AI tumor model	• Furuhata[148]
(c) Chimeric elements		
PSMA/PSES (prostate-specific membrane antigen/artificial chimeric enhancer)	• *E1a* (oncolytic); replication-competent virus, exclusively in prostatic AI cells, efficient killing of $PSMA^+$ cells *in vitro* and in tumor model	• Lee[35]
	• *E1a* and *E4* (oncolytic); controlled viral vector replication and specific cell killing in prostate cancer cell lines and tumor model	• Li[128]
PPT (PSMA enhancer/PSA enhancer/ T-cell receptor gamma-chain alternate reading frame protein)	• *Luciferase* (reporter gene); high prostate-specificity and expression levels in the presence and absence of testosterone *in vitro* and *in vivo*; higher activity and selectivity than the CMV promoter following intravenous virus administration	• Cheng[36]

Abbreviations: AD tumors, androgen-dependent tumors; AI tumors, androgen-independent tumors; CMV, cytomegalovirus; HSV-tk, herpes simplex virus thymidine kinase.