Integrated, Molecular Engineering Approaches to Develop Prostate Cancer Gene Therapy

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

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

INTRODUCTION

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

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

BIOLOGY OF HUMAN PROSTATE CANCER

Prostate cancer is the second leading cause of cancer deaths in American men. In 2002, diagnosis of about 189,000 new cases of prostate cancer have been estimated 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 are present with metastatic disease [Christiano, 2000]. The ominous nature of recurrent metastatic disease is signified by the fact that the majority (nearly 60%) of these patients die within five years of recurrence [Smaletz, 2002].

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

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

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 hydroxylflutamide, which are ligands that are inactive in wild-type AR. Another clear example of expanded AR ligand specificity was demonstrated in the MDA PCs 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.](image-url)
between growth factor and AR pathways rather than bypassing 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 transgenic mouse models of prostate cancer and xenograft models; however, no model is perfect at this time, yet each system has its advantages. Two issues that should caution researchers against fully relying on one mouse model to study human disease are: 1) the developmental processes of the rodent and human prostate are different; and 2) few rodent models recapitulate the complete spectrum of human disease progression. At this time, there is a severe shortage of prostate cancer animal models that will spontaneously disseminate to the bone and fully mimic the osteoblastic lesions of skeletal metastasis observed in clinical situation [Zhau, 2000].

In the transgenic adenocarcinoma of mouse prostate (TRAMP) model, prostate-restricted expression of potent oncogenic SV40 large T-antigen and small T-antigen results in an aggressive reproducible murine prostatic carcinoma [Greenberg, 1995]. The TRAMP mice develop high-grade dysplasia and carcinoma within 12 weeks of age, and ultimately develop metastases by 28 weeks, primarily to the lungs and lymph nodes, and less often, to bone [Greenberg, 1995; Gingrich, 1996]. Androgen ablation by castration results in decreased tumor burden compared to non-castrated TRAMP animals early in the course of disease. However, aggressive androgen-independent tumors emerge in castrated mice at a much higher rate than in non-castrated controls in later stages of disease [Gingrich, 1997]. In a similar "LADY" transgenic model, a larger region of the prostate-specific rat probasin promoter was utilized to drive the expression of the SV40 large T-antigen [Kasper, 1998]. Interestingly, cancer development and progression in the LADY model is less aggressive than in TRAMP mice [Kasper, 1998; Masumori, 2001]. These time-dependent progression models, highly reminiscent of human disease, provide a fertile ground to investigate growth regulatory pathways involved in murine prostatic carcinoma and potential chemoprevention and therapeutic interventions [Kaplan, 1999; Huss, 2001; Mentor-Marcel, 2001].

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

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

Several other institutions have established useful human prostate cancer xenograft models with a spectrum of androgen responsiveness, AR mutation status, and PSA expression levels. The University of Rotterdam, Netherlands has acquired the most extensive experience, with more than ten long-term established models [van Weerden, 2000; van Weerden, 1996]. A distinguishing feature of the Rotterdam models is that no reconstituted extracellular matrix
(Matrigel) was used to establish the subcutaneous tumors, unlike procedures at other institutions. The majority of the Rotterdam models expresses wild-type AR and is androgen-responsive [van Weerden, 2000]. Interestingly, PC-295 and PC-310 are two androgen-dependent models that can be induced to exhibit neuroendocrine differentiation upon androgen deprivation treatment [Jongsma, 1999; Jongsma, 2002]. LuCaP models developed at the University of Washington exhibit a range of growth patterns in response to castration, including minimal responsiveness with continued tumor growth, intermediate responsiveness exhibiting a small decline in growth and eventual progression, and sustained growth arrest [Ellis, 1996]. Interestingly, intratibial-implanted LuCaP 35 induces an osteoblastic bone growth response, which is reminiscent of clinical metastatic bone lesions [Corey, 2002]. In 1991, Pretlow et al. [Pretlow, 1991] generated the CWR22 model derived from a primary tumor, which is a well-studied model with a hormone refractory subline, CWR22R [Nagabhushan, 1996]. Interestingly, the AR in CWR22 harbors a missense mutation in the ligand-binding domain (H874Y), which results in expanded tropism similar to that in the LNCaP cell line [Tan, 1997]. Availability of multiple xenografts, covering a wide spectrum of clinical manifestations, will greatly benefit the investigation of prostate cancer biology and potential therapeutic interventions.

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

PROSTATE-SPECIFIC GENE EXPRESSION

The use of a prostate-targeted vector with greatly enhanced transcriptional activity, as well as highly accurate cell-specific discrimination should improve the efficacy and safety of prostate cancer gene therapy. An effective approach is transcriptional targeting (i.e., restricting expression to prostate-specific tissue by using gene regulatory elements), which is particularly suitable to target tissue of prostate origin because the prostate is one of the organs (in addition to pancreas and breast) that express an unusually high number of unique genes. Many of these prostate-specific gene regulatory regions, such as PSA and prostate-specific membrane antigen (PSMA), are well characterized (Table 1). A survey of the Cancer Genome Anatomy Project (CGAP) database published by the National Cancer Institute (http://www.ncbi.nlm.nih.gov/ncicgap/) lists more than 2000 prostate-specific genes, although the majority of them are not fully characterized at this time.

The current strategies of prostate cancer gene therapy are covered in several recent reviews [Mabjeesh, 2002; Lu, 2001; Hsieh, 2001; Koeman, 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; Oettgen, 2000]. GATA transcription factors have been shown to induce the androgen-responsive activity of the PSA enhancer and promoter [Perez-Stable, 2000]. Other factors, such as AP-1, c-Fos, and CREB, might also contribute to prostate-specific regulation, as putative binding sites have been identified in the PSA upstream gene regulatory region [Schuur, 1996]. PDEF is a prostate epithelium-specific Ets factor that has been shown to interact with AR and activate PSA gene expression [Oettgen, 2000]. Cell culture transfections [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>
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<tr>
<th>promoter</th>
<th>experiment</th>
<th>transgene; result summary</th>
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<td><strong>human origin</strong></td>
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<td>PARP (apoptotic); androgen-inducible DNA-damaging</td>
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<td>HSV-TK (therapeutic); cell-killing in vitro and inhibition of growth in tumor model</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>DTA (therapeutic); cell killing in vitro, specific EGFP expression in tumor model</td>
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<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>Oettgen (2000)</td>
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<tr>
<td>human origin</td>
<td>transfection</td>
<td>CAT (reporter); expression in prostate cancer cell lines and androgen regulation</td>
<td>Murtha (1993), Mitchell (2000)</td>
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<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>transfection</td>
<td>luciferase (reporter); expression in LNCaP</td>
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<td><strong>rodent origin</strong></td>
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<td>transfection</td>
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<td>bcl-2, c-myc (oncogene); limited expression and hyperplasia</td>
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<td>rat C3(1)</td>
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<td>E1A (oncolytic); inhibition of the growth of prostate cancer cell lines and tumor</td>
<td>Matsubara (2002)</td>
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Abbreviations: PARP, poly(ADP-ribose) polymerase; HSV-TK, herpes simplex virus thymidine kinase; E1, adenovirus immediate early gene 1; DTA, diphtheria toxin A; lacZ, beta-galactosidase; CAT, chloramphenicol acetyl transferase; EGFP, enhanced
demonstrated the prostate specificity and androgen-responsive activity of PSA regulatory regions.

In our experience, the magnitude of the native PSA promoter and enhancer (PSE) [Wu, 2001] might not be sufficient to mediate robust expression in vector-based gene therapy applications. As illustrated in (Fig. 2), due to the weak activity of PSE, differential RNA expression levels in prostate versus non-prostate cells was difficult to confirm. Two useful strategies have been applied to augment the activity of the PSA promoter, while maintaining its specificity. In the first strategy, synergistic activation of the PSA upstream enhancer can be achieved by insertion of four tandem copies of synthetic AREs [Wu, 2001], or by duplication of the enhancer core [Wu, 2001; Latham, 2000]. We demonstrated that the PSE-BC construct with a duplicated enhancer was nearly 20-fold more active than the native PSE construct [Wu, 2001]. In a second approach, a two-step transcriptional amplification (TSTA) system was employed to boost the activity of the PSA enhancer/promoter over a range of 1000-fold [Zhang, 2002; Iyer, 2001]. In this system, illustrated in (Fig. 3), the PSA regulatory region was used to express the potent artificial transcription activator, GAL4-VP16, which acts upon a GAL4-responsive reporter. Optimal TSTA constructs displayed activity levels 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; Xie, 2001a; Yu, 1999b]. In addition, the increased hK2 expression in carcinoma, as compared to benign tissues, supports its utility as a prostate cancer biomarker [Yousef, 2002]. Future studies are needed to compare the utility of PSA and hK2, both as biomarkers and in targeted gene therapy applications.

PSMA [Fair, 1997] and prostate stem cell antigen (PSCA) [Reiter, 1998] are two integral membrane proteins that exhibit preferential prostate expression. PSMA was first identified by a monoclonal antibody to be expressed in primary prostate cancer and lymph node metastases [Horoszewicz, 1987]. Detailed immunohistochemical
analyses revealed that PSMA expression is not restricted to prostatic tissue alone [Silver, 1997]. Interestingly, PSMA promoter-driven cytotoxic gene expression is induced by androgen deprivation and is more effective in eradicating androgen-independent prostate cancer cells [O'Keefe, 2000]. The PSCA expression profile also appears to be less restricted than is originally anticipated [Amara, 2001]. The PSCA gene regulatory regions contain both androgen-dependent and –independent components [Jain, 2002]. Combined with the finding that PSCA expression is increased in advanced stage and metastatic disease [Gu, 2000], it might be advantageous to utilize the PSCA enhancer in gene therapeutic settings for advanced disease. The normal biological roles of PSMA and PSCA are unclear, but their potential contributions in prostate development and oncogenesis are of substantial interest.

Several rodent prostate promoters have been utilized frequently in transgenic mouse studies, resulting in the generation of many successful prostate-targeted knockout and carcinogenesis models, including the rat C3 and probasin promoters [Greenberg, 1995; Podsyppanina, 1999; Kasper, 1998; Maroulakou, 1994], and recently, the mouse PSP94 gene regulatory regions [Gabril, 2002]. These rodent-based promoters are less well characterized in gene expression and therapy studies in human models. The probasin promoter is highly androgen-responsive and contains multiple AREs [Kasper, 1994]. Several recent studies have shown that probasin-based adenoviral vectors can mediate prostate-selective transgene expression and therapeutic responses in human prostate cells [Xie, 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 prostatic epithelial cells, using the osteocalcin promoter [Yeung, 2002]. Osteocalcin is a bone matrix protein produced exclusively by osteoblasts, and its expression is regulated by multiple elements, including the osteo-specific OSE2 element and the vitamin D-responsive element [Lian, 1999; Hsieh, 2002]. Interestingly, primary and metastatic prostate cancer cells were shown to express osteocalcin [Matsubara, 2001]. Osteocalcin promoter-activated adenoviral replication in an oncolytic strategy was capable of suppressing growth of prostate tumors in animals [Hsieh, 2002; Matsubara, 2001]. Whether employing a tissue- or tumor-targeted approach, both high-magnitude of expression and specificity are very important for achieving effective in vivo therapeutic responses. In the oncolytic scheme, key viral regulator E1A and E1B proteins are not needed at high levels to induce adenovirus replication, which results in a 3- to 4-order amplification of infectious virus [Yu, 1999a; Matsubara, 2001]. Thus, oncolytic therapy with improved tissue or tumor selectivity is being actively pursued [Hawkins, 2002].

Vector delivery by targeting cell surface molecules specifically expressed on prostate cancer cells (i.e., transductional targeting) is a powerful tool and an active field of investigation. Cell surface targets can be based on pre-determined membrane antigens (e.g., PSMA or PSCA) 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 threedimensional 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 1.8x10^9 infectious units of AdPSE-BC-luc, as previously described [Adams 2002]. Serial optical images were acquired on designated day post-viral injection (noted above each image). Signal intensities are represented by a color scale (red, highest, to blue, weakest). Note that each image was acquired with a slightly different scale, as specified by the maximal signal listed below each image. Extra-tumoral signals were detected at 21 days post-injection. The animals were sacrificed on the last day of imaging, and the isolated organs were re-imaged. Sections (5-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 sr39 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, sr39tk showed improved therapeutic efficacy in response to acyclovir (ACV) and GCV administration in prostate cancer cell lines and tumor xenografts [Pantuck, 2002]. In a preclinical therapy model, HSV-TK-mediated PET imaging prior to GCV instillation should indicate the magnitude of HSV-TK gene expression in the tumor. If the treatment is effective after GCV administration, the HSV-TK-transduced tumor cells should be eradicated; consequently, PET signals should drop precipitously.

Molecular imaging should play an important role in gene therapy studies. Firstly, it can help to verify the activity of a prostate-targeted gene expression vector in vivo. Measuring the expression levels of therapeutic genes by indirect methods (reporter genes) or direct methods (HSV-TK with PET) will permit modulation of gene delivery to achieve optimal expression to the tumor target, which should enhance the gene-based therapeutic response. Imaging approaches could be developed to assess treatment response in real-time (e.g., apoptosis [Laxman, 2002]) or to specifically detect disseminated cancer cells [Adams, 2002].

**THERAPEUTIC APPROACHES AND GENE DELIVERY VEHICLES**

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

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

More genetic alterations are being defined as the understanding of prostate oncogenesis increases. This trend also leads to an ever-expanding array of gene-based therapeutic options. Functional loss of tumor suppressor and cell cycle regulatory genes, such as p53, PTEN, retinoblastoma (Rb), p16, and p27, has been well documented in prostate cancer [MacGrogan, 1997; Whang, 1998; Cordon-Cardo, 1998]. Gene-based therapies designed to replenish these genetic deficits have shown promising results in animal tumor models [Davies, 2002; Steiner, 2000; Katner, 2002]. In spite 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 oncogenes, such as c-Myc, bcl-2, and Her2/neu, has also been implicated in prostate cancer oncogenesis or progression [Craft, 1999; Furuya, 1996; Buttyan, 1987]. Several gene-based approaches to suppress these activated oncogenic pathways have disrupted tumor growth [Steiner, 1998; Gleave, 1999; Dorai, 1999]. The concern with the suppression of a particular gene function is
the level of diminution in expression that is adequate to achieve the desired result. Use of anti-sense RNA or a dominant-negative protein has been used to interfere with a particular gene function. RNA interference (RNAi) is an evolutionarily conserved mechanism whereby a 21-23-nt double-stranded RNA can mediate homology-dependent degradation of mRNA [Hannon, 2002]. Ever since this mechanism was shown to efficiently silence gene expression in mammalian cells, it has become 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 redefined (see list in [Kirn, 2002]). Molecular engineering to improve HSV-TK enzyme kinetics that favor prodrug activation has improved therapeutic efficacy [Black, 1996; Black, 2001; Pantuck, 2002]. From our experience, achieving high levels of HSV-TK enzyme expression in targeted cancer cells is a critical determinant of effective cell killing [Pantuck, 2002]. Mindful of the fact that expression of the HSV-TK gene in normal cells will invoke cytotoxic side effects, we have utilized the highly amplified prostate-specific TSTA system to express this suicide gene. Our preliminary results indicate that TSTA-driven HSV-TK in an adenoviral vector expresses higher levels of transgene and induces more extensive tumor cell killing and less systemic toxicity than the comparable CMV-driven system in LAPC-4 tumor xenograft models ([Zhang, 2002; 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 FasL, TRAIL [Norris, 2001], and viral oncolytic therapy [Yu, 1999a; Kirn, 2002; Hsieh, 2002; Matsubara, 2001].

The recent review by Mabjeesh et al. [Mabjeesh, 2002] thoroughly covers many topics of prostate cancer gene therapy, particularly immune therapy. Immunotherapy is a safe therapeutic modality that is under constant refinement to define the most appropriate prostate cancer antigen and vaccination protocol to improve efficacy. Its application might be most suitable for minimal residual disease settings rather than large bulky tumors. As the limitations and advantages of different gene-based therapies are being defined, designing rational combined therapeutic strategies could achieve a synergistic response. The concept of a 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 transcriptional regulatory approaches that could be incorporated to improve specificity, an ideal vehicle for cancer therapy should have the following characteristics: 1) non-toxic to normal cells; 2) easily produced in large quantities; 3) capable of being modified on the surface to achieve transductionsal targeting; 4) easily modified to incorporate multiple genetic elements; 5) capable of efficient gene transfer into targeted cells; 6) widely distributed throughout the subject upon systemic administration; and 7) non-immunogenic. Modified viruses, exploited for their natural ability to efficiently introduce genetic material into host cells, are the most commonly utilized gene delivery vehicles. Inactivated RNA retrovirus-based vectors include murine leukemia virus (MuLV) and lentiviruses. The DNA virus-based vectors include human adenovirus (Ad), adeno-associated virus (AAV), and herpes simplex virus (HSV).

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

A non-viral gene carrier can be generated by modifying DNA with cationic lipids (liposome) or polymers to form condensed DNA complexes [Niidome, 2002]. These DNA-based synthetic gene delivery vehicles are both non-immunogenic and non-toxic; however, one consistent drawback is the poor in vivo gene transduction efficiency. Interestingly, a recent report described the use of integrin-targeted synthetic nanoparticles to deliver cytotoxic gene selectively to tumor blood vessels [Hood 2002]. Nanoparticles are synthetic submicron (<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 [Brigger, 2002]. With the advent of improved targets for prostate
tumor homing [Arap, 2002], prostate-specific gene transduction and expression might soon be realized.

FUTURE CONSIDERATIONS

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

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