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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The purpose of this proposal is to evaluate the ability of a replication-restrictive adenovirus(Ad-OC-E1a)to specifically target and lyse cells of an androgen independent prostate cancer osseous metastasis, which account for a majority of the morbidity and mortality experienced by men with prostate cancer. The scope of this project is to perform the studies outlined in the proposal to prove the hypothesis that a conditional replication under the guidance of the osteocalcin promoter can exert a prostate cancer-specific cell kill in well defined pre-clinical models of human androgen independent prostate cancer metastases. Specific Aim I was designed to evaluate the in vitro ability of Ad-OC-E1a to specifically lyse human androgen independent prostate cancer cells and demonstrated up to a 100 fold killing differential favoring OC+ cell lines (LNCaP, C4-2, PC-3) and over OC- cell lines (LOVO, PrSC). Specific Aim II evaluates the growth inhibition of human prostate cancer xenografts after intralesional or systemic administration of Ad-OC-E1a, as well as, the tissue distribution and toxicity profile after administration. Ad-OC-E1a has demonstrated the ability to destroy human xenografts of both androgen-dependent and androgen-independent with limited toxicity.

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Table of Contents

Introduction to Final Report

The **subject** of this proposal is to develop and test the ability to a genetically modified common cold virus to destroy androgen-independent prostate cancer cells. Androgen-independent prostate cancer cells account for 100% of the mortality associated with prostate cancer. The **purpose** of this proposal is to evaluate the ability of replication-restrictive adenovirus which specifically targets and lyses cells of an androgen independent prostate cancer osseous metastasis. The **scope** of this project to perform the studies outline in the two specific aims to prove the hypothesis that conditional replication under the guidance of the osteocalcin promoter can exert a prostate cancer-specific cell kill in well defined pre-clinical models of human androgen independent prostate cancer metastases. More specifically, **Specific Aim I** is designed to evaluate the specificity of the tumor-restrictive replication of Ad-OC-E1a using in vitro assays on prostate and non-prostate cancer cells. Based on the reviewers comments from the first annual review suggested that prostate specific antigen (PSA) expression by prostate cancer cell lines may be down regulated by adenoviral infection a series of experiments were completed to address this question in vitro. **Specific Aim 2** evaluates the growth inhibition of human prostate cancer xenografts attributable to Ad-OC-E1a administration, as well as, the tissue distribution and toxicity profile of such injections. The request for a revised second Annual Report did not request a revised State of Work but the have provided results of additional experiments supported under this grant. Since the first Annual Report Review raised several format and editorial issues, the final report expands on the first and second annual report including the entire report with corrected grammatical text per the reviewer's comments. ***At the completion of this funding period several reportable outcomes have been uncovered which have supported several presentations and publications (Appendix I-III). Additionally, the findings of this study will support further pre-clinical development of Ad-OC-E1a and an application to the NCI for a Phase I clinical trial of this vector for men with metastatic prostate cancer.***

Body

Metastatic prostate cancer remains a daily challenge for the urologist, oncologist and radiation oncologist. The relatively unique pathophysiology underlying the formation of osteoblastic lesions predominately isolated to the bone of men with metastatic prostate cancer has allowed us to transcriptionally target these cancer cells with an osteoblastic promoter, osteocalcin. We have previously utilized a replication-defective adenovirus containing the osteocalcin promoter driving toxic gene expression to target osseous metastases in pre-clinical models[1] and a phase I clinical trial (Ad-OC-TK)[2]. The transcription regulation of transgene expression using tumor- and tissue-specific promoters within adenoviral vectors has been shown to impart tumor or tissue specificity. The osteocalcin promoter has been demonstrated to effectively and safely target prostate cancer[1] and osteosarcoma[3-5] based on the shared osteoblastic phenotype, using a suicide gene therapy approach in preclinical and phase I testing[2].

The lytic replicative cycle of the adenovirus was initially used shortly after the discovery of the adenovirus for the treatment of cancer[6]. The greater understanding of the adenoviral genetic make-up and function has led to the ability to construct conditionally replicating adenoviruses. Restrictive adenoviral replication has been used previously to target p53 mutated[7] cells and more recently PSA producing cells[8]. In this report we demonstrate that previously defined transcriptional specificity of the osteocalcin promoter can be used to destroy prostrate cancer cells by harnessing this adenoviral lytic replication cycle both in vitro and in vivo using relevant models of human hormone-refractory prostate cancer. This is achieved by placing the

E1a gene under the transcriptional regulation of the osteocalcin promoter. By constructing an adenoviral vector that has the E1a gene under the control of the murine osteocalcin promoter the osseous metastases, which account for most of the morbidity and eventual mortality attributable to prostate cancer, can be effectively targeted.

Illustration 1 demonstrates the rational underlying this proposal. The osteocalcin promoter has the ability to transcriptionally regulate the production of the E1a protein in osteocalcin positive cell types. The production of this essential protein then directs adenoviral replication and eventual cell lysis. The lytic life cycle of the adenovirus is then allowed to proprogate throughout a tumor mass. The propagation wave will potentially continue until normal osteocalcin negative cells are encountered at the periphery of the tumor.

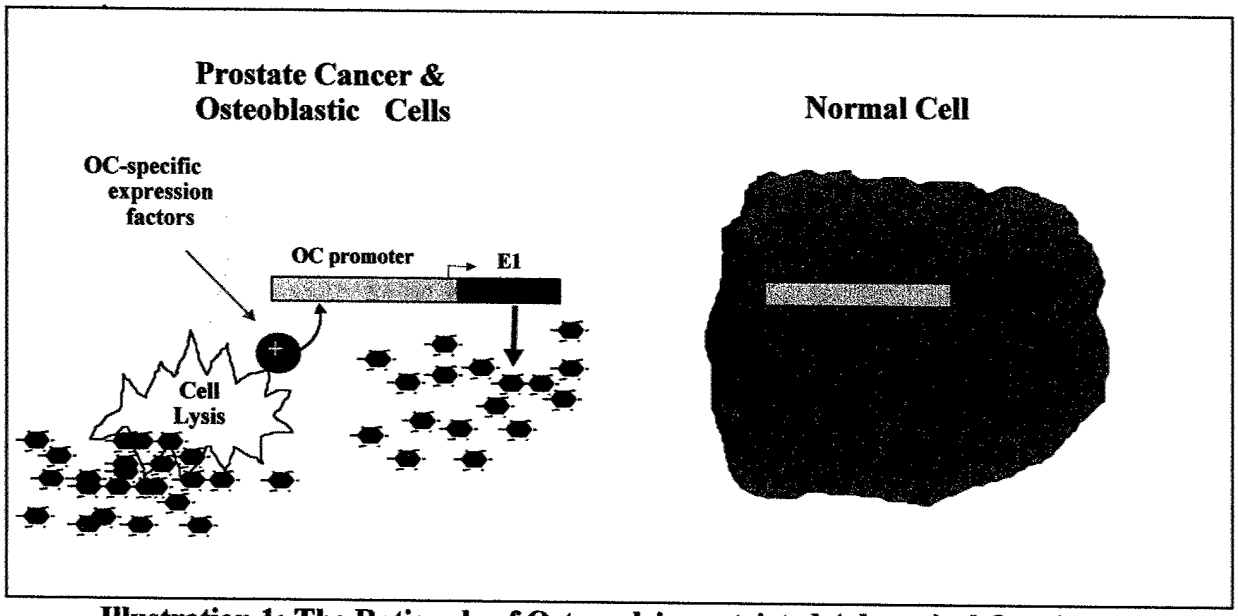


Illustration 1: The Rationale of Osteocalcin-restricted Adenoviral Oncolysis

The completion of the tasks outlined by the Statement of Work of the initial proposal is being performed with slight modifications of the timing as described below. In general, some of the In vivo studies projected for the second year of the proposal were initiated at the 6-month mark and results will be presented. This was a result of equipment difficulties that have been resolved that delayed the initiation of 3-dimensional studies and the addition of other studies postponed completion of the 3-dimensional studies. The research findings will be broken down by Task # as per the "Format Requirements for Preparing Reports".

Task 1 was to amplify, purify, quantify titer and confirm the activity of sufficient viral stocks of Ad-OC-E1a and Ad-CMV-Bgal. This task is complete and the PI and other members of the research team continue to improve on the technique in several ways. The PI and others have developed a novel production technique that allows for adenoviral production using a serum-free hollow fiber system[9]. Currently, sufficient Ad-OC-E1a and Ad-CMV-Bgal has been produced to perform the next 6 months of experiments. The adenoviral production continues on schedule. One anticipated problem, which can occur during the amplification of any recombinant adenovirus, was the regeneration of wild-type Ad5, which occurred during the amplification process of Ad-CMV-B-Gal. This contaminant required re-isolation of the virus and subsequent re-amplification of the Ad-CMV-B-Gal virus.

Virus construction and production

The shuttle plasmid pOCE1a was constructed by starting with the shuttle vector pΔE1sp1B, provided by Dr. Frank Graham (McMasters University, Hamilton, Ontario, Canada). pΔE1sp1B contains the right end of the adenovirus type 5 genome, nucleotides 28 to 347, encoding several minor E1a promoters. To stop the transcription initiated from these minor E1a promoters, an SV40 polyadenylation signal (170 bp, Cla I – Hind III fragment, from pXCMVPA, obtained from Dr. Wei-Wei Chang) was cloned into pΔE1sp1B to generate pΔBE1sp1BPA. The Ad5 E1 region, from pX548c (also provided by Frank Graham), was cloned into pΔBE1sp1BPA. These subcloning procedures created the shutter vector pΔE1, which contains the 5' -end of the adenovirus type 5 genome, from nucleotides 28 to 347 and 549 to 5852, with multiple cloning sites between sequences 347 to 549. pΔE1 contains the majority of the E1 region except part of the E1a promoter, nucleotides 348 to 548. A mouse osteocalcin promoter (1370 bp, Not I-EcoR I fragment) from pII1.5, including TATA box, was cloned between the SV40 polyadenlyation site and E1a sequence of pΔE1 to generate **pΔOCE1a**, which has E1a under the transcriptional regulation of the osteocalcin promoter. [pII1.5 was provided by Dr. Gerard Karsenty of the University of Texas M. D. Anderson Cancer Center, Houston Texas.] The sequence of pΔOCE1a was generated by known sequence information and analyzed by restriction enzyme digestion. The virus was generated using traditional method [10] and amplified using both the traditional amplification on monolayers of 293 cells and a hollow fiber production method developed by the investigator[9].

Task 2 was to perform DNA quantification and time course experiments with Ad-OC-E1a using dot blot experiments. The emphasis of this proposal has been shifted to the in vivo studies to prepare for a clinical trial of this virus. This task remains ongoing and results will be presented in the final report.

Task 3 and 4 was to perform in vitro killing assays in a variety of human cell lines. This task has been accomplished and the material, methods, results and discussion follow:

Methods and Materials

Cell lines and cell cultures:

The LNCaP cell line was kindly supplied by Dr. Gary Miller (University of Colorado, Denver, CO). C4-2 was established from LNCaP tumors propagated in castrated hosts[11; 12]. PC-3 [13]was obtained from the American Type Culture Collection (Rockville, MD). ROS 17/2.8 (ROS), a rat osteoblastic osteosarcoma cell line was generously supplied by Dr. Cindy Farrach-Carson (The University of Texas Dental Branch, Houston, TX). Prostate stromal cells (PrSC) were obtained from Clonetics (Walkersville, MD). LNCaP, C4-2, and PC-3 cell lines were maintained in T-medium [80% Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY), 20% F12K (Irving Scientific, Santa Ana, CA), 3 gm/L $NaHCO_3$, 100 units/mL penicillin G, 100μg/ml streptomycin, 5 μg/ml insulin, 13.6 pg/ml triiodothyronine, 5 μg/ml transferrin, 0.25 μg/ml biotin, and 25 μg/ml adenine] with 10% fetal bovine serum (FBS; Sigma Chemical Company, St. Louis, MO). ROS cells were maintained in DMEM (GIBCO, Grand Island, NY) supplemented with penicillin (100 units/mL), streptomycin (100mg/ml), and 10% fetal bovine serum (FBS; Sigma Chemical Company, St. Louis, MO). PrSC cells were maintained in Stromal cell basal media, supplemented with the Stromal cell growth media BulletKit (Clonetics, Walkersville, MD). All cell cultures were maintained at 37° C in a humidified atmosphere of 95% air and 5% carbon dioxide. The cells were fed three times per week with fresh growth media.

MTT proliferation assay:

Cells were plated in 24 well plates at the following initial seeding densities in cells/well based on growth rates and prior experience in other in vitro assay on these cells: ROS (10,000), PC-3 (10,000) LNCaP (15,000), PrSC (15,000), C4-2 (40,000). Twenty-four hours after seeding, fresh

media was placed on the cells and the cells were exposed to variable concentrations of the Ad-OC-E1a vector (0.01, 0.1, 1, 10, and 100 viral particles/cell; 4 wells each dilution) dissolved in PBS. An additional 4 wells that were treated with vector-free PBS served as controls. The media on all wells was changed every two days. Relative cell numbers were determined at intervals were by incubating the cells with MTT (thiazolyl blue). Briefly, cells were then solubilized in a solution of 10% sodium dodecyl sulfate and 0.1 N hydrochloride for 16 hours. Absorbance was measured at wavelength 550 nm as per the manufactures protocol. The in vitro cell-killing activity of Ad-OC-E1a ranged from 0.01 to100 viral particles per cell was evaluated on an androgen-independent and metastatic human prostate cancer C4-2 cell line from 0 to 7 days.

Task 3 and 4 Results and Discussion:

The viral lytic effect of Ad-OC-E1a is demonstrated in LNCaP (OC-positive) cells at five day after infection with 1 vp/per cell. The left panel of Figure 1 demonstrates LNCaP cells five days after 1 vp/cell of Ad-CMV-B-Gal exposure. The right panel of Figure 1 demonstrates the significant lytic ability of Ad-OC-E1 on LNCaP five days after 1 vp/cell exposure. The typical cytopathic effect (CPE) is seen in the right panel while absent in the left panel.

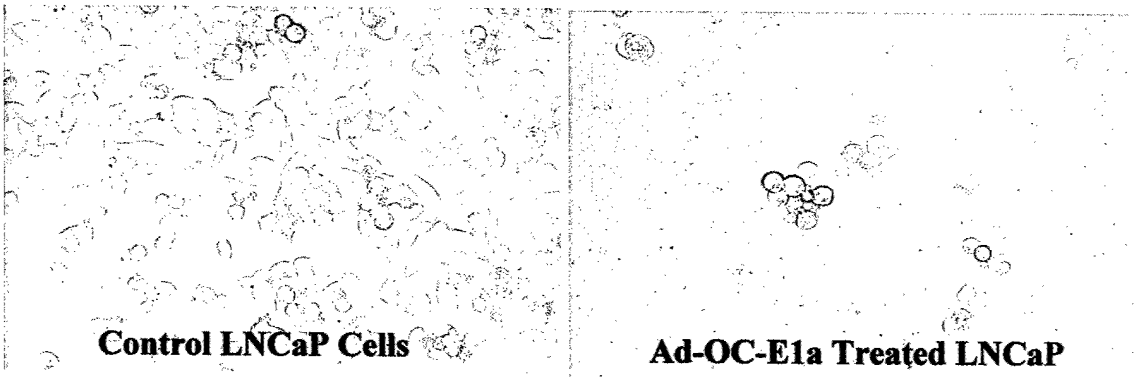


Figure 1. CPE 5 days after Exposure of LNCaP to Ad-OC-E1a (1 vp/cell)

The osteocalcin positive cell lines LNCaP, C4-2 and PC-3 all demonstrate a dose-dependent cell lysis as evaluated by MTT assay. The PrSC serve as an osteocalcin-negative relevant human cell line and demonstrates no cell lysis at day 7. The ROS cell line, expresses higher levels of osteocalcin, but serves a negative control for viral replication since the human adenovirus cannot replicate in rodent cells. (**Figure**

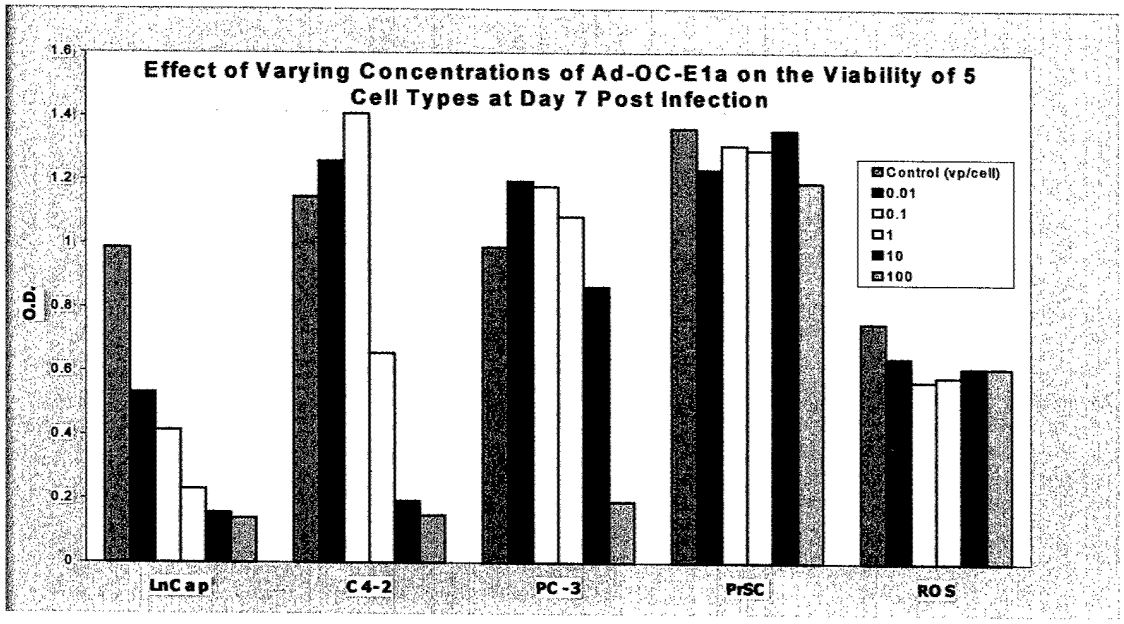


Figure 2: Osteocalin-Resistricted and Viral Particle-Dependent Cell Lysis by MTT Assay

Figures 3 and 4 demonstrate a time course of the Ad-OC-E1a dependent lysis in osteocalcin positive C4-2 cells and osteocalcin negative LOVO cells, respectively. A 100 fold differential is seen between the OC+ C4-2 cell and the OC- LOVO cells. Quantitative PCR will be performed on the DNA extracts from Days 0, 1, 3, 5, 7 to generate the viral production levels and time course.

Figure 3: Time Course of Ad-OC-E1a Osteocalcin Dependent Cell Lysis in C4-2 Cells as Measured by MTT Assay

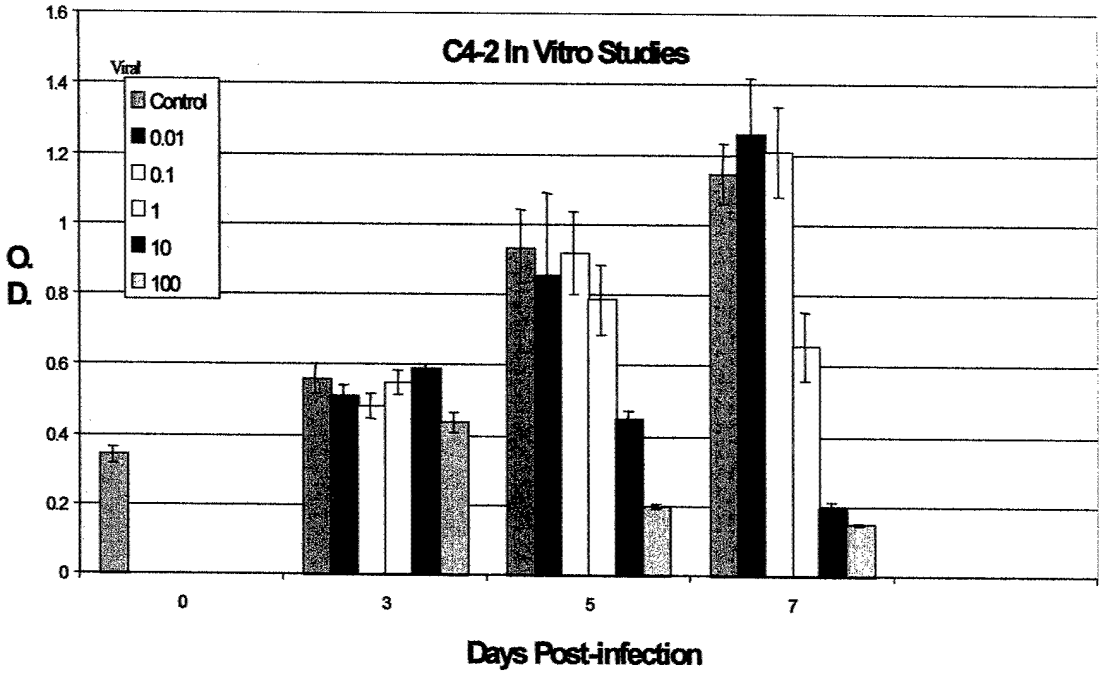


Figure 4: Time Course of Ad-OC-E1a Osteocalcin Dependent Cell Lysis in LOVO Cells as Measured by MTT Assay

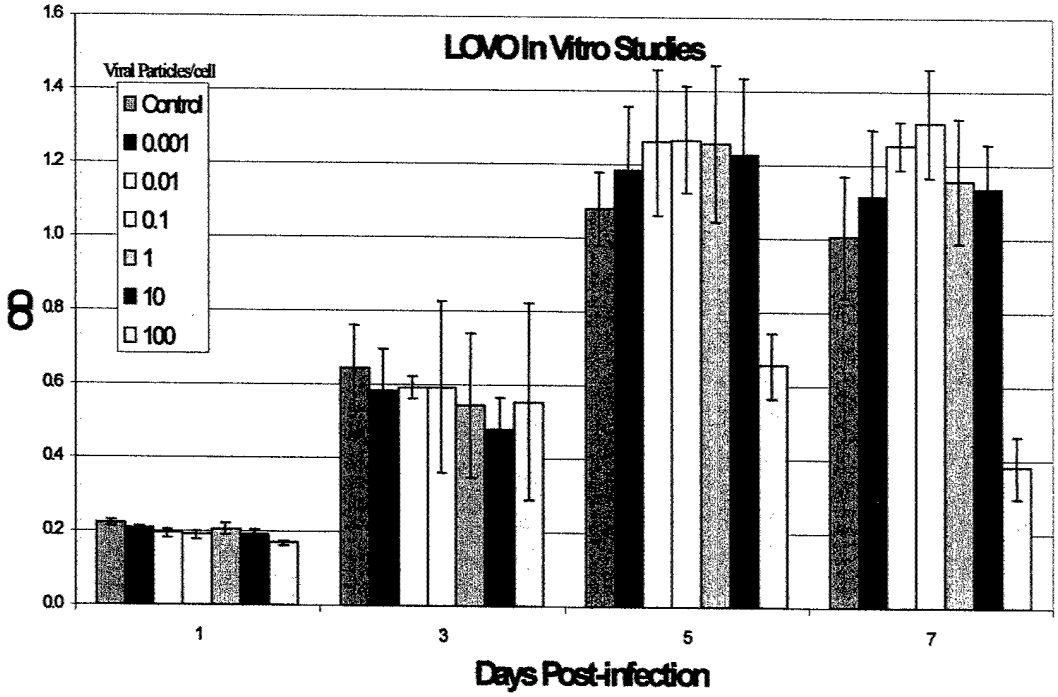


Figure 5 and 6 would suggest that PSA production by both PSA producing human cell lines LNCaP and C4-2 is not down regulated by adenoviral infection and again confirms the lytic potential of Ad-OC-E1a. Figure 5 illustrates the mean PSA readings in ng/dl and cell counts per 1000 of 4 replicates of LNCaP and C4-2 human prostate cancer cell lines at day 0, 2, 4, 6, 8. LNCaP cells were culture for six days post infection while C4-2 cells were assayed for 8 days post exposure. Four experimental groups were studied. The Control Group was exposed to only normal media, the Control Ly were similar but were exposed to lysis buffer on designated Day, Ad-CMV-GFP Group were exposed to 10 vp/cell on Day O and AD-OC-E1a Group were exposed to 10 vp per cell with media and cells being collected on Day 0, 2, 4, 6, 8 as designated on the x axis. Examination of the cell counts on day 6 for LNCaP and Day 8 for C4-2 again confirms the specific lytic ability of the Ad-OC-E1a virus has on prostate cancer cells.

Figure 5 PSA Values and Cell Counts

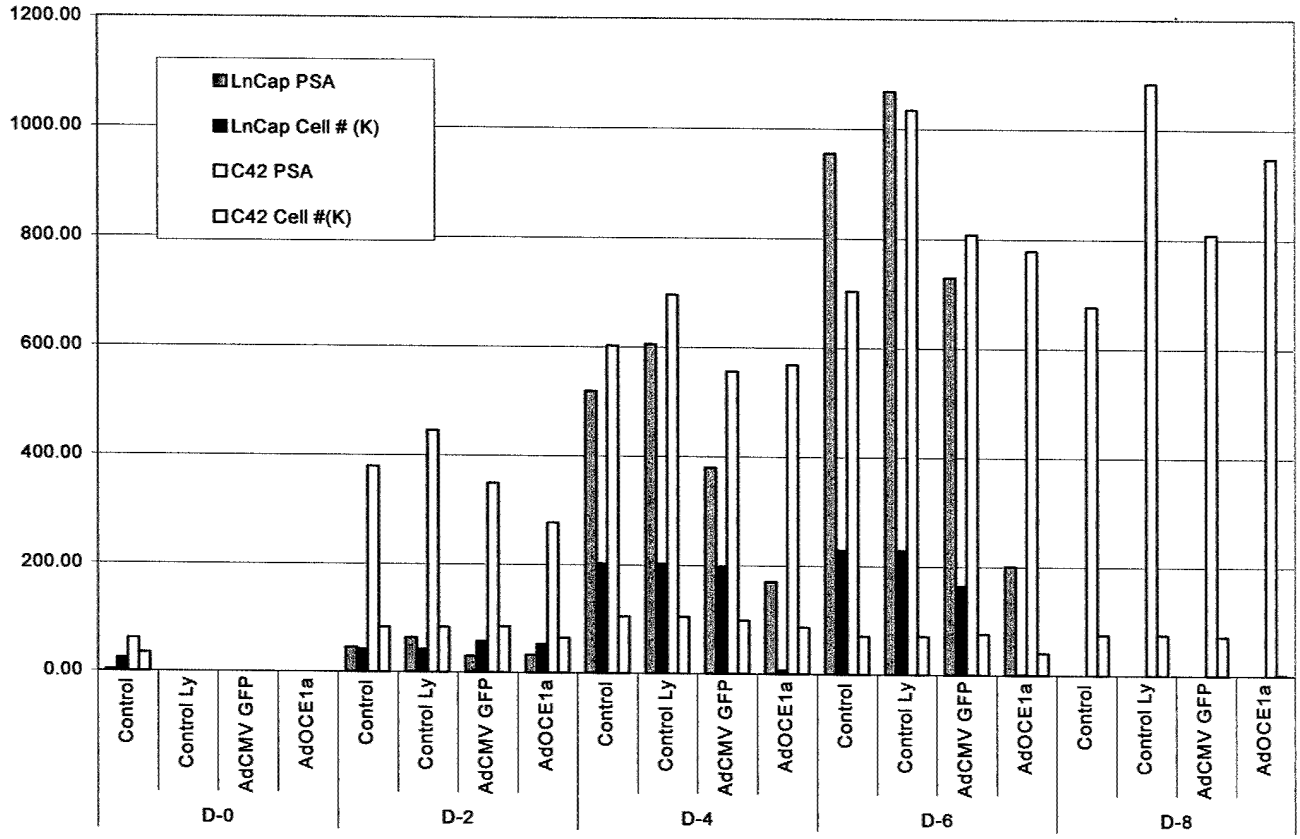
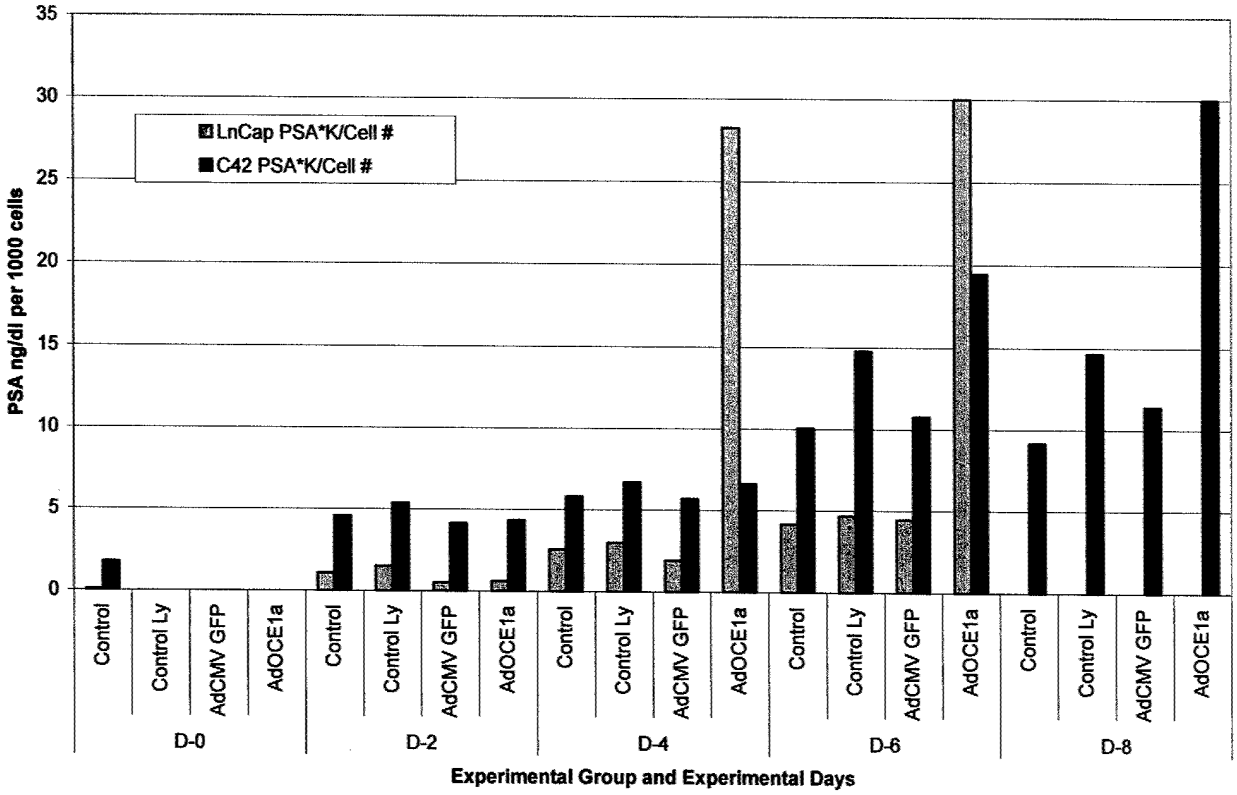


Figure 6 illustrates the PSA production per 1000 human prostate cancer cells for clarity. In particular the PSA production in the media per cell does not change with the control GFP expressing reporter virus but does increase in within the Ad-OC-E1a group when viral mediated lysis is occurring. An in vivo time course study is proposed to address the PSA expression in the serum of animal with established tumors.

Figure 6. PSA Expression and Adenoviral Infection

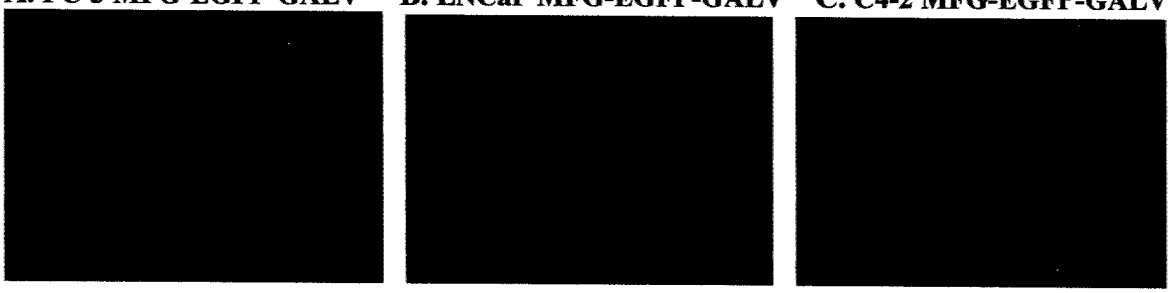


Task 5 is to analyze the results of the first 12 months to allow completion of this annual report. The biostatistician of the cancer center is reviewing the complete statistical analysis of this data presented in this annual report and complete statistical analysis will be included in the final report.

Task 6 is to conduct the microgravity experiments to assess the lytic ability of Ad-OC-E1a on various human cancer organiods. This task remains ongoing; therefore, the final results will be included in the final report. One unexpected occurrence was a malfunction of the microgravity chamber that required repair by the company and ultimately a new apparatus. I have been using these apparatus for the last 12 months without further malfunction. The initial analysis based on cellular morphology of the organoids was difficult *despite repeated experiments*. To facilitate the completion of the proposed experiments a series of GFP labeled and human prostate cancer cells have been established (figure 7). The intracellular markings will allow for more accurate assessment of the treatment effect.

Figure 7 demonstrates stable GFP expression of human prostate cancer cell line PC-3 (A), LNCaP (B) and C4-2 (C).

A. PC-3 MFG-EGFP-GALV **B. LNCaP MFG-EGFP-GALV** **C. C4-2 MFG-EGFP-GALV**



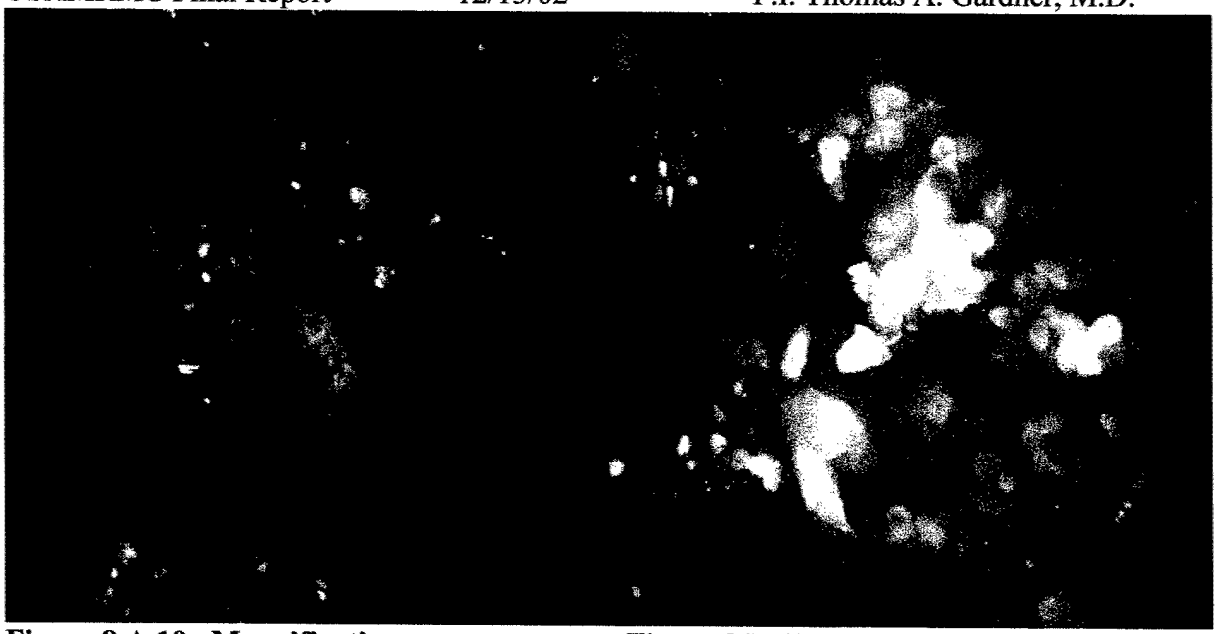


Figure 8 A 10x Magnification **Figure 8 B 60x Magnification**

Figure 8 illustrates the ability of the GFP expressing cell lines to form xenographs similar to none GFP expressing cell lines. Figures 8 A (10x) and B (60 x) are intravital images by 3-D dual photon confocal microscopy with green cells corresponding to C4-2 GFP expressing cells and the red illustrating the vascular markings after infusion with Rhodamine Dextran (500,000 MW).

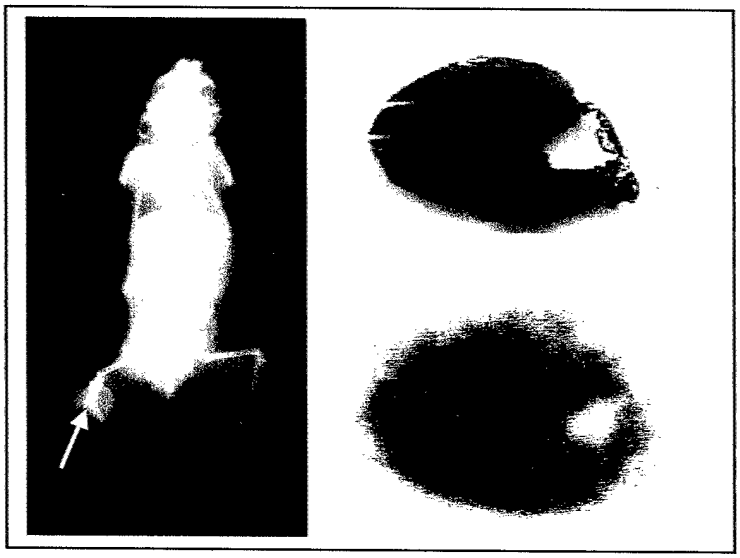
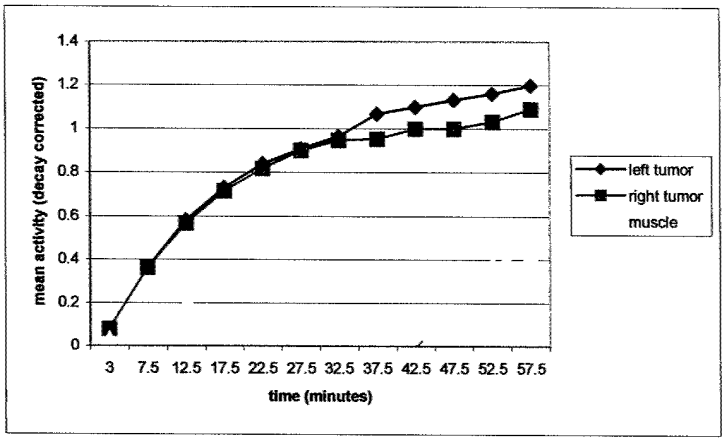


Figure 9. illustrates microPET imaging of prostate tumor xenograft in nude mouse model. Using a nude mouse C4-2 interosseous model of human androgen-independent prostate cancer the potential of microPET imaging is demonstrated by figure 9. Figure 9(left panel) is a plain radiograph of a male nude mouse 8 weeks after intra-osseous injection of 1 million C4-2(GFP) cells into the tibia. The arrow points to the osteoblastic tibial lesion accounting for a serum PSA of 250 ng/dl, which can be contrasted to the contralateral tibia. Figure 9 (Right top panel). is a photomicrograph of the frozen section whole mount cross-section of the tibia at necropsy. Figure 9 (Right bottom panel) represents an autoradiograph of the same frozen cross-sectional slice after ^{18}F-FDG administration. The strong radioactive signal from this tissue attests to the biological activity of this androgen independent osteoblastic intraosseous human prostate cancer. Figures 8 and 9 demonstrate the maintained ability of the GFP expressing cells to form subcutaneous and intraosseous xenografts. Additionally the figures demonstrate novel techniques to evaluate actual tumor viability with the model systems being utilized. Although not described in the initial Statement of Work these establishment and validation of these non-invasive techniques will provide more meaningful biological information of the model system and the treatment effect demonstrated by the Ad-OC-E1a Virus.

Task 7 is to conduct subcutaneous xenograft experiments confirming the in vivo lytic activity post Ad-OC-E1a administration. This task is complete and the results are summarized in appendix 1. This demonstrates the significant potential growth differential of PC-3 and LOVO tumors[14]. MicroPET will be used to confirm tumor volume with viable tissue after exposure to the control and therapeutic virus. Additional studies outside this current proposal are evaluating the subcutaneous model with a non-invasive Choline-based PET imaging at several time points. Attempts to initially use FDG based PET were not easy to evaluate.

Figure 10-A
Mean Activity vs Time after Injection



Muscle Activity vs Time after Injection

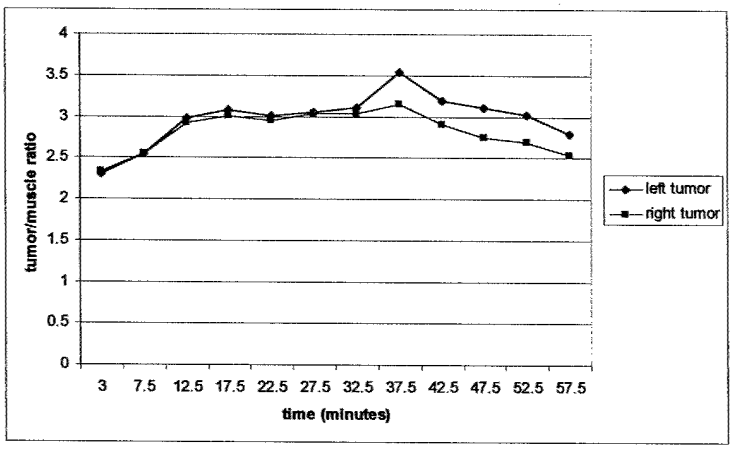


Figure 10-C MicroPET/MicroCT
Figure 10-B Tumor Activity to

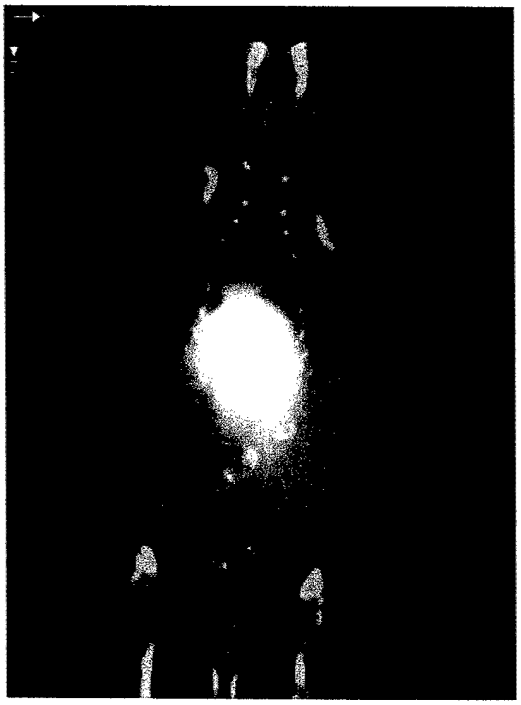


Figure 10 A-C illustrates the potential of PET imaging and prostate cancer using a ^{11}C-Choline based radiotracer. Several experiments were performed to test the ability of FDG based MicroPET/MicroCT imaging to detect prostate cancer xenografts. Similar findings to fiqure 9 could be detected but quantified in vivo imaging of xenografts was not detected thus still requiring sacrifice of the animals and one time point analysis. The initial evaluation of a ^{11}C-Choline based radiotracer would suggest a substantial improvement as demonstrated by the Figures 10 A-C above. Figure 10-A demonstrates the typical activity uptake of a radiotracer of region of interests outlined by blue circles in Fiqure 10-C. The ability to detect a differential uptake of ^{11}C-Choline based radiotracer would allow in vivo quantification of xenograft volume prior to and at several time points after administration of an adenoviral vector or any other therapeutic. These findings have laid the foundation to the submission of a biodistribution study in men with localized and metastatic prostate cancer to validate this approach in men. The support from this grant will be acknowledged in the pending manuscript submission.

Task 8 is to evaluate the viral distribution time course and growth inhibition of intraosseous xenograft model. A portion of this task is completed, the methods, materials, results and discussion follow:

Methods and Materials: Intraosseous Xenografts with C4-2

Using an intraosseous model of androgen-independent prostate cancer, C4-2 cells were directly inoculated in the tibia or femur of nude mice and serum PSA was followed weekly until greater 5 ng/dl.[12]. When the Serum PSA was greater than 5 ng/dl these mice bearing intraossoeous xenografts were then treated with intralesional administration of Ad-OC-E1a of a control reporter virus Ad-CMV-B-Gal 1 x 10^9 pfu per lesion on one occasion. These animals were followed with weekly serum PSA's, radiographic findings and necropsy at 10 weeks post injection.

Results and Discussion Task 8

Figure 11 demonstrates the establishment of stable intraosseous xenografts using the C4-2 model. The y-axis is the serum PSA in ng/dl with each bar representing one mice on Day 0 of the intralesional study. The seven bars on the left received Ad-OC-E1a injections and the seven bars on the right received Ad-CMV-B-Gal injections. The starting PSA values were comparable in each group.

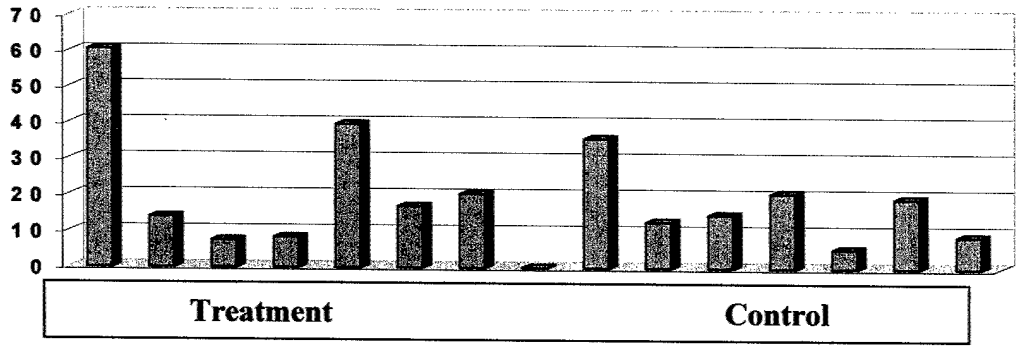


Figure 11 Day 0 Serum PSA (ng/dl) in Intraosseous C4-2 Model

Figure 12 and 13 show the weekly PSA readings for the control and treatment group, respectively. As predicted the control animals have increasing PSA values until sacrifice despite receiving Ad-CMV-B-Gal injections on day 0. Note that the scale of the y-axis on a 0-1000 scale while the treatment group is on a 0-100 scale to avoid dwarfing the treatment group's non-existent PSA values.

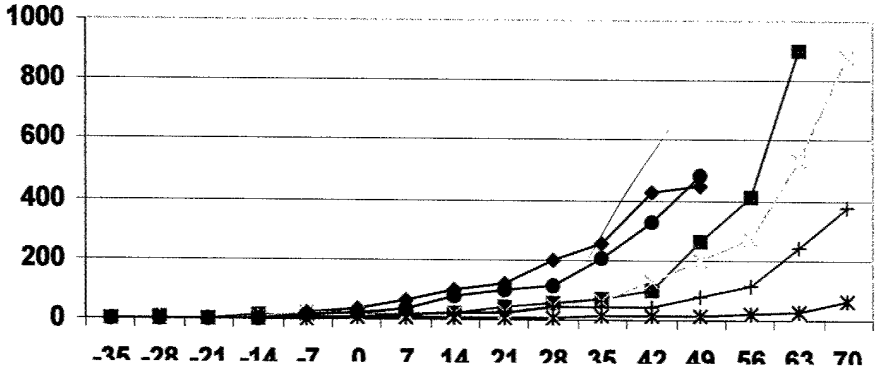


Figure 12: Serum PSA (ng/dl) of Immunocompromised Mice with Intraosseous C4-2 after One Intralesional Ad-CMV-B-Gal (1 x 10^9 PFU) on Day 0.

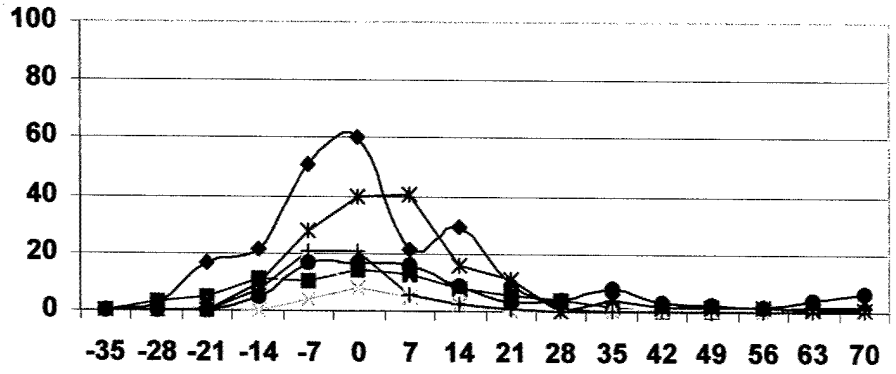


Figure 13: Serum PSA (ng/dl) of Immunocompromised Mice with Intraosseous C4-2 after One Intralesional Ad-OC-E1a (1 x 10^9 PFU) on Day 0

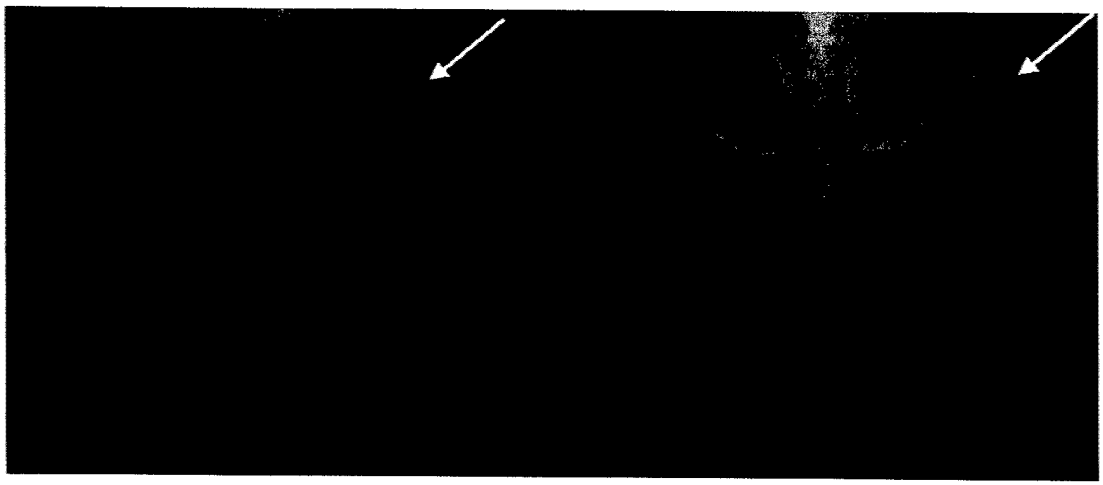


Figure 13 represents radiographs at sacrifice of one control (left) and one treated (right) mice, respectively. These radiographs illustrate the significant growth inhibition of intraosseous C4-2 tumors as evidenced by both PSA decline and normalization of radiograph after intralesional Ad-OC-E1a. Note the large tumor on the tibia of the control animal and the normal radiographic picture of the mouse after receiving the injection of Ad-OC-E1a.

Task 9 is to examine the histopathological correlations with viral distribution study of task #8. The histopathological correlations became less important once the bimodal pharmacokinetics of this virus in rodents previously thought to be unable to replicate the virus took precedence. This examination remains ongoing and results will be reported in a subsequent manuscript which acknowledges the support of this funding.

Task 10 is to analysis the results prior to submission of the 2nd annual report is complete for all experiments completed at that time.

Task 11 is to conduct the viral distribution after a variety of delivery techniques. A baseline viremia study of Ad-OC-E1a has been carried out and serum samples from a series of animals are being tested using quantitative PCR to discern the viral copy number in the blood and tissues. These preliminary studies will set-up the standards for the remainder of the viral distribution studies. This viremia study was performed in immune intact rats to allow for serial blood samples to validate the PCR assay. The initial results revealed a second wave of viremia at day 14, which was unexpected due to the rodents' inability to replicate the human adenovirus in rodents. The PCR analysis is being repeated by a collaborator using independent funding to confirm this finding of replication or delayed release in the rodent model. The completion of this task is beyond this proposal's funding.

Task 12 is the final data compilation, statistical analysis, manuscript preparation and final report preparation. To facilitate continued development of a clinical trial using Ad-OC-E1a some of the results were published Cancer Research as an "Advances in Brief". This delayed the more detailed manuscript to Molecular Therapy with the title being **"Osteocalcin promoter-based adenoviral oncolytic obliteration of human prostate cancer metastatic models."** This collaborative manuscript awaits results of several other experiments prior to submission. The funding of this proposal will be acknowledged in this manuscript.

Key Research Accomplishments of Proposal

- **Confirmation of In Vitro specificity of OC promoter in OC+ cell lines.**
- **Confirmation of reproducibility of C4-2 intraosseous model.**
- **Development of GFP marked PC-3, LNCaP and C4-2 human prostate cancer cell lines.**
- **Confirmation of Intraosseous and Subcutaneous Xenografts formation of GFP-marked cell lines by MicroPET and Dual Photon Confocal Microscopy.**
- **Confirmation of continued PSA production by human prostate cancer cells in culture after adenoviral infection.**
- **Confirmation of intraosseous tumor growth inhibition after intralesional injection of Ad-OC-E1a.**
- **Confirmation of intraosseous tumor growth inhibition after intravenous administration of Ad-OC-E1a.**
- **Initial demonstration of bimodal viremia of Ad-OC-E1a in immune intact rodents.**
- **Early justification of a Choline based MicroPET/CT validation of tumor volume.**

Reportable Outcomes

- **Gardner TA, Wada Y, Shirakawa T, Ko S-C, Kao C, Kim SJ, Yang L, Chung LWK. Osteocalcin promoter restricted adenoviral replication as a potential treatment of prostate cancer metastasis. Presented at the 8th International Conference on Gene Therapy of Cancer, San Diego, CA, December 1999. 1999 Vical Best Abstract Award**

- **Gardner TA, (PI) OBA Gene Transfer Protocol #0010-426 "Phase I study of intratumoral injections of OCaP1(Ad-OC-E1a) for metastatic or locally recurrent prostate cancer, Part 1: Dose finding, Part 2: Index lesion escalation"**

- **Matsubara, S., Wada, Y., Gardner, T. A., Egawa, M., Park, M. S., Hsieh, C. L., Zhau, H. E., Kao, C., Kamidono, S., Gillenwater, J. Y. & Chung, L. W. (2001). A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. *Cancer Res* 61, 6012-9. (Appendix I) This was submitted as an Advance in Brief and final page proofs were submitted by Dr. Matsubara without additional grant support added.**

- **Gardner TA, Sloan J, Raikwar SP, Kao C. Prostate cancer gene therapy: Past experience and future promise. *Cancer Metastasis Reviews* 21:137-145, 2002. (Appendix II).**

- **Raikwar SP, Kao C, Gardner TA. Chapter 9:Targeted adenoviral vectors II: Transcriptional targeting , pp247- 286. *Adenoviral Vectors for Gene Therapy* Eds. Curiel and Douglas, Elsevier Scientific Press, 2002. (Appendix III)**

- **Gardner TA, Wada Y, Sukay M, Yang L, Brown L, Ko S-C, Cheng L, Chung LWK, Kao C. Osteocalcin promoter-based adenoviral oncolytic obliteration of human prostate cancer metastatic models. Molecular Therapy (in preparation submission held for additional PET Data).**

- **Gardner TA, Salvatto D, Shalhav M, Kao C. PSA expression by human prostate cancer cell lines after adenoviral mediated gene therapy: In vitro confirmation of clinical finding. Cancer Gene Therapy (in preparation).**

Conclusions

This proposal is designed to test the hypothesis the adenoviral lytic replication cycle can be placed under the transcriptional regulation of the osteocalcin promoter. Since the initiation of the work the osteocalcin promoter continues to perform well in osteoblastic diseases such as osteosarcoma and prostate cancer in both pre-clinical and clinical settings[2; 14]. The work described above further illustrates the specificity of this promoter. The results to date can be simply divided into in vitro and in vivo results. In the in vitro assays demonstrate at a 100 fold better cell kill in the OC+ (LNCaP, C4-2, PC-3) and OC- (LOVO and PrSC). The completion of these in vitro experiments supported the early investigation in the in vivo setting. The in vivo finding of near-complete ablation of serum PSA in mice with established intraosseous C4-2 tumors compared to controls also provides strong evidence to support the current hypothesis under investigation.

The findings to date can be further supported by a recent clinical study from MD Anderson Cancer Center which demonstrated a true benefit to a therapy which targets the osteoblastic component of a prostate cancer mass[15]. This clinical trial performed by investigators from M. D. Anderson demonstrated improvements in median survival from 17 to 28 months in men with hormone-refractory prostate cancer receiving bone targeted therapy combined with a similar chemotherapy. This study combined with the findings to date would suggest that a Phase I trial of Ad-OC-E1a may confirm the pre-clinical findings above and allow for the verification of the safety of Ad-OC-E1a as a novel therapeutic to target prostate cancer metastases.

Finally, the SOW initially outlined has been completed with two exceptions. First, the microgravity experiments were initially delay and eventually replaced by the dual photon confocal microscopy and microPET experiments due to the greater ability for pre-clinical validation of animal models and the potential clinical ability of a noninvasive imaging technique would have for men with prostate cancer in adding with diagnosis, staging, and prognosis after therapy. Secondly, the observation of a second wave of adenoviral production in rodents after multiple routes of administration Ad-OC-E1a questioned the need for a murine viral distribution study. Several additional and unexpected outcomes have also been uncovered which will require additional investigation beyond the time and funding scope of this grant. First, initial testing of a choline based microPET/CT assessment of animal models could decrease the animal requirements for testing novel therapeutics as well as become a valuable tool in the diagnosis and assessment of prostate cancer in men suffering from all stages of this disease. At the completion of this proposal it is likely that the information generated will support grant application to the NCI and the National Gene Vector Laboratory to initiate a Phase I trial confirming the above preclinical findings. The completion of the investigations outline in the initial grant and it's modifications have lead to proposal to study this lytic adenoviral vector in men with metastatic and locally recurrent prostate cancer.

Most importantly for men with prostate cancer the results of the investigations will support initiation of a phase I clinical trial and forms the foundation for further investigations into osteocalcin based transcriptional regulation of viral vectors alone and in combination with other conventional and molecular strategies. These findings are heading to the clinic in the phase I trial of Ad-OC-E1a for men with metastatic prostate[16]. Additionally, the confirmation of choline-based PET imaging has allowed for submission of a biodistribution of ^{11}C-Choline based radiotracer study to confirm detection and quantification of prostate cancer in patients. The trials referred to in this annual report will be funded through independent funding mechanisms and are beyond the scope of current funding.

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[CANCER RESEARCH 61, 6012–6019, August 15, 2001]

Advances in Brief

A Conditional Replication-competent Adenoviral Vector, Ad-OC-E1a, to Cotarget Prostate Cancer and Bone Stroma in an Experimental Model of Androgen-independent Prostate Cancer Bone Metastasis[1]

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Abstract

Prostate cancer has a high propensity to metastasize to bone, which often resists hormone, radiation, and chemotherapies. Because of the reciprocal nature of the prostate cancer and bone stroma interaction, we designed a cotargeting strategy using a conditional replication-competent adenovirus to target the growth of tumor cells and their associated osteoblasts. The recombinant Ad-OC-E1a was constructed using a noncollagenous bone matrix protein osteocalcin (OC) promoter to drive the viral early *E1a* gene with restricted replication in cells that express OC transcriptional activity. Unlike Ad-PSE-E1a, Ad-OC-E1a was highly efficient in inhibiting the growth of PSA-producing (LNCaP, C4–2, and ARCaP) and nonproducing (PC-3 and DU145) human prostate cancer cell lines. This virus was also found to effectively inhibit the growth of human osteoblasts and human prostate stromal cells *in vitro*. Athymic mice bearing s.c. androgen receptor-negative and PSA-negative PC-3 xenografts responded to a single intratumoral administration of 2×10^9 plaque-forming unit(s) of Ad-OC-E1a. In SCID/bg mice, intraosseous growth of androgen receptor-positive and PSA-producing C4–2 xenografts responded markedly to i.v. administrations of a single dose of Ad-OC-E1a. One hundred percent of the treated mice responded to this systemic Ad-OC-E1a therapy with a decline of serum PSA to an undetectable level, and 80% of the mice with PSA rebound responded to the second dose of systemic Ad-OC-E1a. Forty percent of the mice were found to be cured by systemic Ad-OC-E1a without subsequent PSA rebound or tumor cells found in the skeleton. This cotargeting strategy shows a broader spectrum and appears to be more effective than systemic Ad-PSE-E1a in preclinical models of human prostate cancer skeletal metastasis.

Introduction

Genetic therapy for prostate cancer has been applied in preclinical animal models and in patients with localized and metastatic diseases (1–16). The prevailing approach is to target a single cell compartment such as the tumor epithelial or associated endothelial compartment (1, 2, 5–7). Examples of transgenes delivered to tumor cells include suicide genes (2–5), tumor suppressor (6, 8), antiangiogenesis (7), and immune modulators (9–13). Recently, Henderson *et al.* (1, 14, 15) demonstrated the efficacy of conditional replication-competent adenovirus with viral replication driven by a tissue-specific promoter, PSE,[3] for the treatment of prostate cancer. In this article, we present a novel strategy for cotargeting both tumor epithelial and bone stromal cells using a conditionally replicating adenovirus driven by a tissue-specific but tumor-restrictive promoter, OC (4, 16). This strategy was based on the well-established reciprocal cellular interaction that occurs between prostate cancer and prostate or bone stromal cells (17, 18). Evidence suggests that permanent phenotypic and genotypic alterations are induced in prostate cancer and bone stromal cells subsequent to tumor-stromal interaction (17, 19). The cotargeting strategy accomplishes maximal cell kill by eliminating not only the growth of tumor epithelium but also by interrupting the intercellular communication and reciprocal induction between prostate tumor and bone or prostate stromal cells (17–19).

OC, a noncollagenous Gla protein, was found to be produced exclusively by differentiated osteoblasts and is deposited onto bone matrices at the time of bone mineralization (4, 20, 21). The OC promoter contains several species-specific and overlapping regulatory elements (22–29). The "osteocalcin box" contains sites to bind factors such as homeobox MSX proteins, AP-1, AP-2, NF-1, viral core enhancer, c-AMP, vitamin-D, and glucocorticoid receptors (22–29). The osteoblast-specific *cis*-acting element OSE2 binds to the transcription activator of osteoblast differentiation, Osf2/Cbfa1 (28). Mouse OC promoter contains an additional OSE1 *cis*-acting DNA element (29) but has a nonfunctional vitamin D responsive element (22). The current study used mouse OC promoter to drive viral replication through the regulation of E1a, an adenoviral early gene required for viral replication (30). We described this cotargeting strategy by demonstrating: (1) Ad-OC-E1a is a highly efficient inhibitor of the growth of prostate cancer and bone and prostate stromal cells *in vitro*; (2) Ad-OC-E1a has a broad spectrum of cell kill activity that caused lysis in PSA-producing and -nonproducing prostate tumor, bone, and prostate stromal cells *in vitro*; (3) systemic administration of Ad-OC-E1a inhibited the growth of human prostate tumor established previously in the skeleton; and (4) the cotargeting strategy is superior to targeting a single cell compartment in which only the growth of prostate cancer cells is affected. Results of this study demonstrate for the first time that systemically administered Ad-OC-E1a induced regression of preexisting human prostate cancer growth in the skeleton irrespective of their prior PSA and AR status.

Materials and Methods

Cells and Cell Culture. LNCaP, an androgen-responsive, AR-positive, PSA-secreting human prostate cancer cell line, was derived from a cervical lymph node metastasis by Horoszewicz *et al.* (31). From this parental cell line,

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[3] The abbreviations used are: PSE, prostate-specific antigen enhancer; OC, osteocalcin; PSA, prostate-specific antigen; AR, androgen receptor; pfu, plaque-forming unit(s); FBS, fetal bovine serum; MOI, multiplicity of infection.

we derived a series of androgen-independent (defined as cells that are capable of forming PSA-secreting solid tumors when inoculated in castrated athymic male mice without the supporting stromal cells or extracellular matrices) and lineage-related LNCaP sublines (32, 33). One of the sublines, C4–2, remains AR- and PSA-positive and acquires osseous metastatic potential when inoculated either s.c. or orthotopically (32, 33). ARCaP is an androgen-repressed, low AR- and PSA-expressing human prostate cancer cell line established by our laboratory (34). This cell line is highly tumorigenic and metastatic and is a model to study advanced human prostate cancer (34). PC-3 is an androgen-independent, AR- and PSA-negative human prostate cancer cell line established by Kaighn *et al.* (35) from the bone marrow aspirates of a patient with confirmed metastatic disease. DU-145 is an androgen-independent, AR- and PSA-negative human prostate cancer cell line established by Stone *et al.* (36) from a patient with prostate cancer brain metastasis. Lovo, a colon cancer cell line, was established by Drewinko *et al.* (37) from a localized colon tumor tissue specimen and was kindly provided by Dr. L. Y. Yang, University of Texas M. D. Anderson Cancer Center, Houston, TX. WH, a cell line derived from a human bladder transitional cell carcinoma specimen, was established by Zhau *et al.* (38). 293 is a transformed human embryonic kidney cell line established by Graham *et al.* (39) with the cells expressing a complementing adenoviral E1 region that supports adenoviral replication. A human prostate fibroblast cell line, 9096F, was established by our laboratory from a surgical prostate biopsy specimen (40). A human bone stromal cell line, MG-63, was established from an osteosarcoma specimen and was obtained from the American Type Culture Collection (Rockville, MD). The PC-3, DU-145, and 293 cell lines were also obtained from American Type Culture Collection. In this study, C4–2 and 9096F cells were maintained in T medium (Life Technologies, Inc.) containing 10% FBS as described previously (32, 33). LNCaP, PC-3, DU-145, ARCaP, WH, and MG-63 cells were all maintained in T medium (Life Technologies, Inc.) containing 5% FBS. Lovo cells were maintained in F-12 Nutrient Mixture (Life Technologies, Inc.) containing 10% FBS. 293 cells were maintained in MEM (Life Technologies, Inc.) containing 10% FBS. The cells were fed three times per week with fresh growth medium and maintained at 37°C in 5% CO_2.

Construction and Production of the Replication-competent Ad-OC-E1a. All of the plasmids were constructed according to standard published protocols (41). Briefly, a *Bam*HI-XcaI fragment containing the backbone of an Ad5 vector from 549 bp to 5792 bp was digested from pXC 548C, a derivative of plasmid pXC1 (42), and inserted into pΔE1sp1B (obtained as a gift from Dr. Frank Graham, MacMaster University, Hamilton, Ontario, Canada) between the *Bam*HI and XcaI site to create a pΔBPAEII shuttle vector. A pOCE1a was constructed by inserting a 1370-bp fragment of murine OC promoter, which was cut from pII1.5 TK using *Xho*I and *Sal*I enzymes, into the *Xho*I site of pΔBPAEII to drive the Ad5 *E1a* gene. The shuttle pOCE1a vector was cotransfected with a replication-defective recombinant Ad vector, pJM17, into 293 cells by the *N*-[1-(2,3-dioleoyloxyl)propyl]-*N,N,N*-trimethylammonium-methyl sulfate (Boehringer Mannheim Biochemicals)-mediated transfection method (43) to generate a partially E3-deleted replication-competent adenovirus, Ad-OC-E1a. The resulting Ad-OC-E1a was demonstrated to replicate in a restricted manner only in cells that expressed OC promoter activity. The culture medium of the 293 cells showing complete cytopathic effect was collected and centrifuged at 1000 × *g* for 10 min. The pooled supernatants were aliquoted and stored at −80°C as primary viral stock. Viral stocks were propagated in 293 cells, and selected clones of Ad-OC-E1a virus were obtained by plaque purification according to the method of Graham and Prevec (44). One of the viral clones was selected, propagated in 293 cells, harvested 36–40 h after infection, pelleted, resuspended in PBS, and lysed. Cell debris was removed by subjecting the cells to centrifugation, and the virus in the cell lysate was purified by CsCl gradient centrifugation. Concentrated virus was dialyzed, aliquoted, and stored at −80°C. The viral titer was determined by plaque assay as described previously (2, 8, 45). Other control viruses used in this study, Ad-CMV-pA and Ad-CMV-β-gal, were constructed, plaque purified, and propagated in 293 cells using a similar procedure. The specificity of Ad-OC-E1a replication in cells was assessed by determining the titer of the virus after infecting 293 (7.7 ± 3.2 pfu/cell), C4–2 (15 ± 8 pfu/cell), and WH (0.047 ± 0.021 pfu/cell) cells (n = 3).

Immunohistochemical Staining of Primary and Metastatic Human Prostate Tumor Specimens. Deparaffinized primary human prostate cancer specimens and lymph node and bone metastatic specimens were obtained from the Department of Urology and Pathology, University of Virginia School of Medicine, Charlottesville, VA and McGill University, Montreal, Quebec, Canada. Tissues were treated with 3% H_2O_2, blocked with SuperBlock (Scytek Laboratories, Logan, UT), and reacted with a monoclonal IgG OC antibody (5–16 μg/ml; OC 4–30 antibody purchased from Takara Shuzo, Otsu, Japan). The antibody staining signals were amplified by a biotinylated peroxidase-conjugated streptavidin system (Bio-Genex Laboratories, San Ramen, CA). Background and negative control staining were routinely obtained by the use of a purified control mouse IgG and a clinical human colon cancer specimen, respectively. OC stain was visualized after reacting the conjugated peroxidase with either an AEC Chromogen, 3-amino-9-ethylcarbazole, or a diaminobenzidine as described previously (2, 8, 34, 38, 45). Positive OC is defined by ≥15% of the cell populations reacted positively with the OC antibody.

***In Vitro* Cell Growth Assay.** LNCaP, C4–2, PC-3, DU-145, ARCaP, 293, WH, Lovo, MG-63 or 9096F cells (5×10^3) were plated in 24-well plates. After 24 h, the cells were infected with Ad-OC-E1a with a range of concentrations from 0.01 to 5 MOI (or pfu/cell, which was estimated to be 0.2–100 virus particles/cell) for 2 h. Cells infected with Ad-CMV-pA or Ad-CMV-β-gal served as negative controls. Cell numbers were measured 3 days later by the crystal violet assay using an automated E max spectrophotometric plate reader (Molecular Devices Corp., Sunnyvale, CA) as described previously (2, 8, 45).

Assessment of Adenoviral Infectivity in Mouse and Human Bones. To determine whether normal mouse or healthy human bones are susceptible to Ad infection, we performed two studies. An Ad-CMV-β-gal (1×10^9 pfu) was injected into the femur of an adult mouse, and the bone was harvested 3 days later for histochemical analysis of β-gal activity using a method established previously (2, 8, 44). Additionally, a normal bone specimen harvested from a 69-year-old man with bone fracture was cultured in T medium containing 0.6% soft agar. A human prostate cancer PC-3 xenograft cultured similarly in 0.6% soft agar was also infected and secured as a control. The tissue specimens were exposed to Ad-CMV-β-gal (1×10^9 pfu) and were processed 3 days after infection. After harvesting bone and prostate tumor specimens, tissues were first washed in PBS and fixed in 0.05% glutaraldehyde at 4°C for 24 h. Bone specimens were put in PBS for 24 h after fixing and decalcified with 0.25 M EDTA in PBS (pH 7.4) at 4°C for 5 days. After decalcification, the specimens were stained overnight in a solution of 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS. Prostate tumor specimens were processed as described previously and were stained similarly as described above for β-galactosidase activity (2, 8, 44).

***In Vivo* Animal Experiment.** To demonstrate oncolytic activity and tumor specificity of Ad-OC-E1a, athymic mice (20–25 g) were inoculated s.c. with 1×10^6 PC-3 or Lovo cells suspended in 100 μl T medium containing 5% FBS. When the tumor became palpable (4–5 mm in diameter), the animals were randomly assigned to two experimental groups: group 1, Ad-OC-E1a; and group 2, Ad-CMV-β-gal. A single dose of virus (2×10^9 pfu) was injected intratumorally in mice. After administration of the test viruses, tumor sizes were measured and recorded.

To evaluate the effect of systemic Ad-OC-E1a in the intraosseous prostate tumor model, 1×10^6 C4–2 cells were injected into the bone marrow space of the right tibial bone in castrated male SCID/bg mice according to procedures published previously (46). Blood specimens (~100 μl) were obtained from the tail vein for PSA assay once per week. Serum PSA was determined by microparticle ELISA using an Abbott IMx machine (Abbott Laboratories, Abbott Park, IL). After the detection of serum PSA elevation, a single dose of 25 μl Ad-OC-E1a, 2×10^9 pfu (or 4×10^{10} virus particles)/animal, was administered i.v. to mice. When serum PSA rebound had occurred, animals were treated with the second or third i.v. injection of the same dose of the test virus at the specified time points as indicated. Serum PSA was monitored weekly, and histopathology and X-ray of the tumors were routinely assessed when the animals were sacrificed.

Results

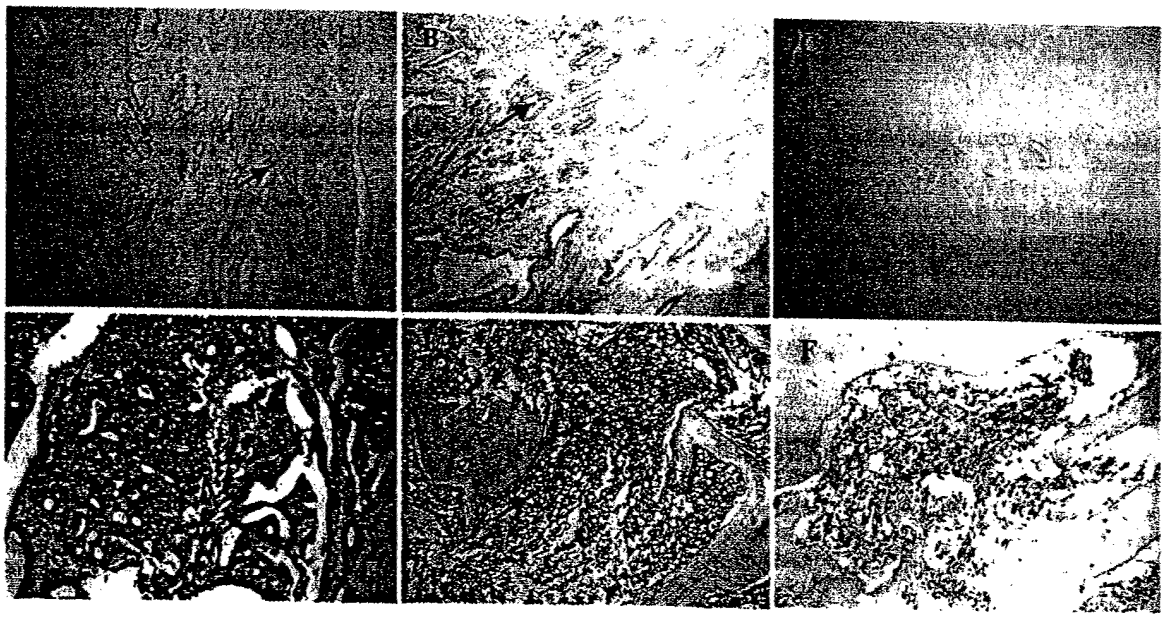
Immunohistochemical Staining of Primary and Metastatic Human Prostate Tumor Specimens. Whereas OC has been shown to be a specific marker expressed exclusively in osteoblast-lineage cells, OC was also found to be expressed in calcified normal smooth muscle tissues, vascular endothelial pericytes, and benign tumors

(47–50). We demonstrated OC expression (see *arrows*) by immunohistochemistry in a primary prostate tumor stroma (Fig. 1*A*), primary prostate tumor epithelium and stroma (Fig. 1*B*), a prostate tumor lymph node (Fig. 1*D*), and a bone (Fig. 1*E*) metastatic specimen. Positive OC stain was found in 85% (23/27) of the primary prostate tumor specimens and in 100% of the prostate tumor lymph node (12/12) and bone (10/10) metastatic specimens. OC stains are generally less intense in the primary prostate cancer but are very intense in metastatic prostate cancer (lymph node and bone) specimens. Background immunohistochemical staining of OC was demonstrated by the use of a control mouse IgG in a primary human prostate tumor (Fig. 1*C*), a human colon cancer (data not shown), and human prostate tumor bone metastatic specimens (Fig. 1*F*). Positive immunostaining of OC was also demonstrated in normal human bone specimens (data not shown) and tumor-associated osteoblasts (Fig. 1*E*).

Cytotoxicity of Ad-OC-E1a to Prostate Cancer Cell Lines *in Vitro*: Independent of Endogenous PSA and AR Status. To assess the cytotoxicity of Ad-OC-E1a, we exposed a number of human prostate cancer cell lines, LNCaP, C4–2, PC-3, DU-145, and ARCaP, *in vitro* to a wide range (0.01–5 MOI) of Ad-OC-E1a vector. We used 293, or WH and Lovo cells as positive or negative controls, respectively. We observed that whereas exposure of LNCaP and C4–2 cells to 5 MOI of Ad-OC-E1a inhibited the growth of these cells by 70% (Fig. 2*a*), this same dose of Ad-OC-E1a was ineffective in blocking the growth of WH and Lovo cells, which exhibit barely detectable or nondetectable OC promoter activity (data not shown). Cells infected similarly by the control viruses, either without an insert (Ad-CMV-pA) or with β-gal insert (Ad-CMV-β-gal), were also unaffected even when exposed to 5 MOI of the virus (Fig. 2*a*). Next, we evaluated the efficacy of Ad-OC-E1a in several other human prostate cancer cell lines that either expressed a very low level (*e.g.*, ARCaP) or nondetectable (*e.g.*, PC-3 and DU-145) level of PSA and AR. Fig. 2*b* shows that all of the tested human prostate cancer cell lines were sensitive to Ad-OC-E1a-induced cell lysis *in vitro* irrespective of their intrinsic levels of PSA and AR expression. In addition, we also evaluated the effects of Ad-OC-E1a on the growth of a human prostate fibroblast and a human osteosarcoma cell line *in vitro*. As demonstrated in Fig. 2*c*, Ad-OC-E1a infection induced significant cell lysis in both cultured human prostate fibroblast (*e.g.*, 9096F) and osteoblast (MG-63) cell lines.

Abolishing s.c. PC-3 Tumor Growth *in Vivo* with Intratumoral Ad-OC-E1a. To establish the specificity of Ad-OC-E1a in inhibiting prostate tumor growth *in vivo*, we compared the activity of this virus on the growth of s.c. human prostate PC-3 tumors established previously with that of human colon Lovo tumors (serve as a negative control) *in vivo*. Fig. 3 shows that Ad-OC-E1a effectively inhibited the growth of PC-3 but not Lovo tumors when injected intratumorally. PC-3 tumors injected similarly with Ad-CMV-β-gal (data not shown) and Lovo tumors with Ad-OC-E1a exhibited a slight inhibitory effect on tumor volumes. These data are consistent with the observation that OC promoter activity is present in PC-3 but not in Lovo cells and that Ad-OC-E1a induced marked cytotoxicity in PC-3 but not Lovo cells *in vitro* (Fig. 2, *a* and *b*).

Systemic Ad-OC-E1a Eliminated C4–2 Human Prostate Tumors Established Previously in the Skeleton. A PSA-secreting and androgen-independent human LNCaP prostate cancer subline, C4–2, was chosen to evaluate the efficacy of systemic Ad-OC-E1a. Prostate tumors established previously in the skeleton with increased



Prostate Cancer Specimens	Positive/Total (%)
Primary Prostate Cancer	23/27 (85%)
Lymph Node Metastasis	12/12 (100%)
Bone Metastasis	10/10 (100%)

Fig. 1. Immunohistochemical demonstration of the presence of OC in primary and metastatic human prostate cancer specimens. Positive OC stain (*arrows*) was detected in primary cancer-associated stroma (*A*) and both prostate stroma and tumor epithelium (*B*). Positive immunostaining of OC was also found in lymph node (*D*) and bone (*E*) metastasis. Background immunostaining was found in control primary (*C*) and bone metastatic (*F*) prostate cancer when these specimens were stained by mouse IgG. Accumulated incidences of OC-positive specimens (positive/total) are expressed on *bottom panel*.

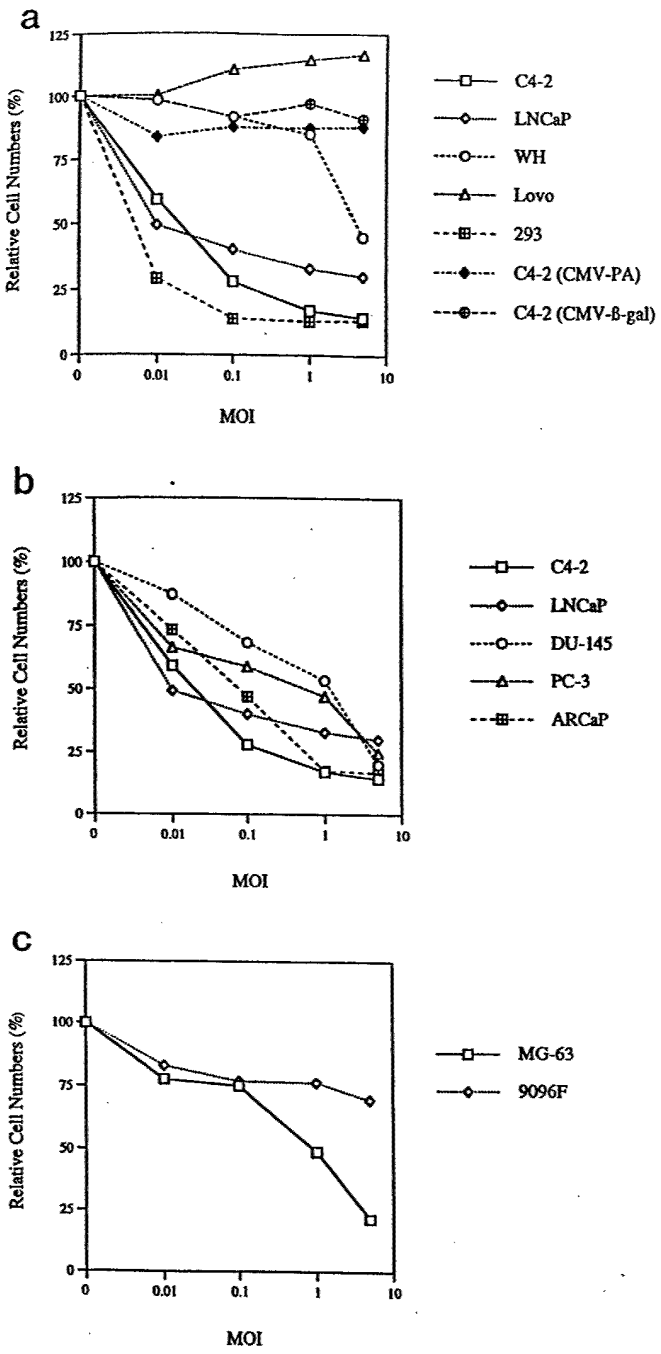


Fig. 2. Inhibition of human prostate cancer and bone and prostate stromal cell growth *in vitro* by the replication-competent Ad-OC-E1a. Cell growth was assessed *in vitro* in the presence of Ad-OC-E1a, Ad-CMV-β-gal, or Ad-CMV-pA. The percentage of cell viability was measured on day 3 after infection of the test virus (range, 0.01–5 MOI or pfu/cell). Results showed that although Ad-CMV-pA and Ad-CMV-β-gal did not affect the growth of C4–2, Ad-OC-E1a inhibited cell growth of C4–2 and 293 in a viral concentration-dependent manner. Ad-OC-E1a was not effective in inhibiting cell growth of WH and Lovo cells, because these cells lack OC promoter activity and OC expression. Results also showed the comparative aspect of efficacy of Ad-OC-E1a-induced cell lysis in C4–2, LNCaP, PC-3, ARCaP, and DU-145 human prostate cancer cell lines. Note PSA- and AR-positive (LNCaP, C4–2), PSA-negative (PC-3, DU-145), and marginally PSA- and AR-positive (ARCaP) human prostate cancer cell lines are all susceptible to Ad-OC-E1a-induced cell lysis. Additionally, results showed both human prostate stromal cells and osteoblasts are also sensitive to Ad-OC-E1a-induced cell lysis. Data represent the average of triplicate experiments determined with SD within 15% of the mean.

serum PSA were subjected to systemic Ad-OC-E1a administration. Serum PSA was followed weekly, and upon PSA rebound, Ad-OC-E1a treatment was repeated on animals. Six animals were evaluated in this study. Fig. 4*a* shows that in one control untreated mouse, serum PSA underwent marked elevation from a basal 10 ng/ml at 6 weeks to 630 ng/ml at 15 weeks (*Panel A*). This profile of rapid PSA rise is consistent with our previous reports (2, 33, 46). Serum PSA profiles of Ad-OC-E1a-treated mice are shown in Fig. 4*a* from *Panel B* to *F*. Several variants of the PSA responses were noted. Mice 2 and 3 responded to systemic Ad-OC-E1a treatment with a complete regression of the skeletal tumors (Fig. 4, *b* and *c*), and a PSA nadir (*i.e.*, nondetectable PSA) was achieved for >15 weeks (see *Panels B* and *C* of Fig. 4*a*). These two mice are considered as cured by systemic Ad-OC-E1a treatment, because there was no subsequence PSA rebound, and no tumor was detected in the skeleton. Mice 4 and 5 responded to systemic Ad-OC-E1a with a marked and rapid PSA decline. PSA nadir in these mice was maintained for a variable period ranging from 1 to 6 weeks (see *Panels D* and *E* of Fig. 4*a*). These mice appear to have variable rebound of PSA during subsequent observation. Mouse 6 responded favorably to systemic Ad-OC-E1a initially with a PSA nadir lasting for 5 weeks. However, this mouse gradually escaped from systemic Ad-OC-E1a growth inhibition and appeared less responsive to the second and third dose of Ad-OC-E1a treatment (Fig. 4*a, Panel F*).

Gross Morphology, Histopathology, and Immunohistochemistry of Prostate Tumor Xenografts Harvested from the Skeleton in Mice Treated with Systemic Ad-OC-E1a. Marked gross anatomical differences were found between the control mice and mice responsive to Ad-OC-E1a (Fig. 4*b*). As shown by X-ray and gross anatomy, systemic Ad-OC-E1a induced marked regression of prostate tumors in

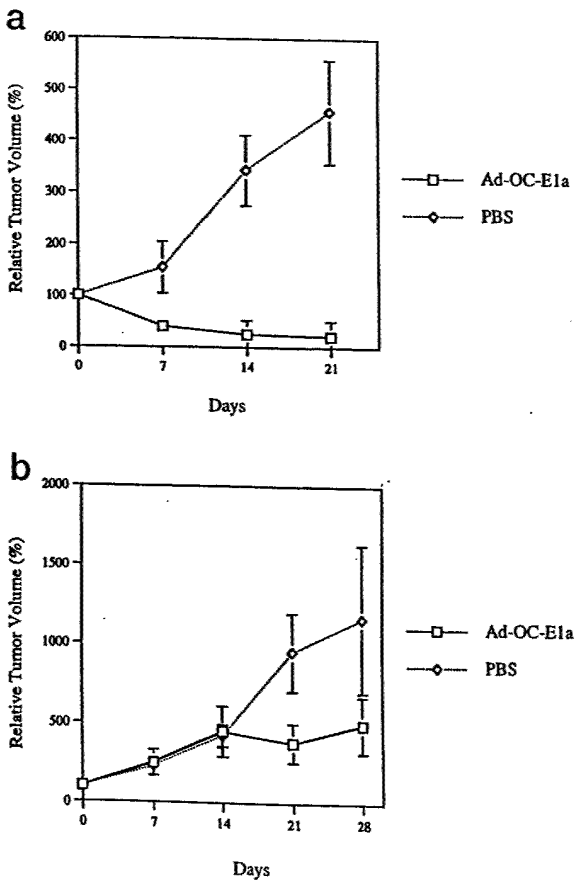
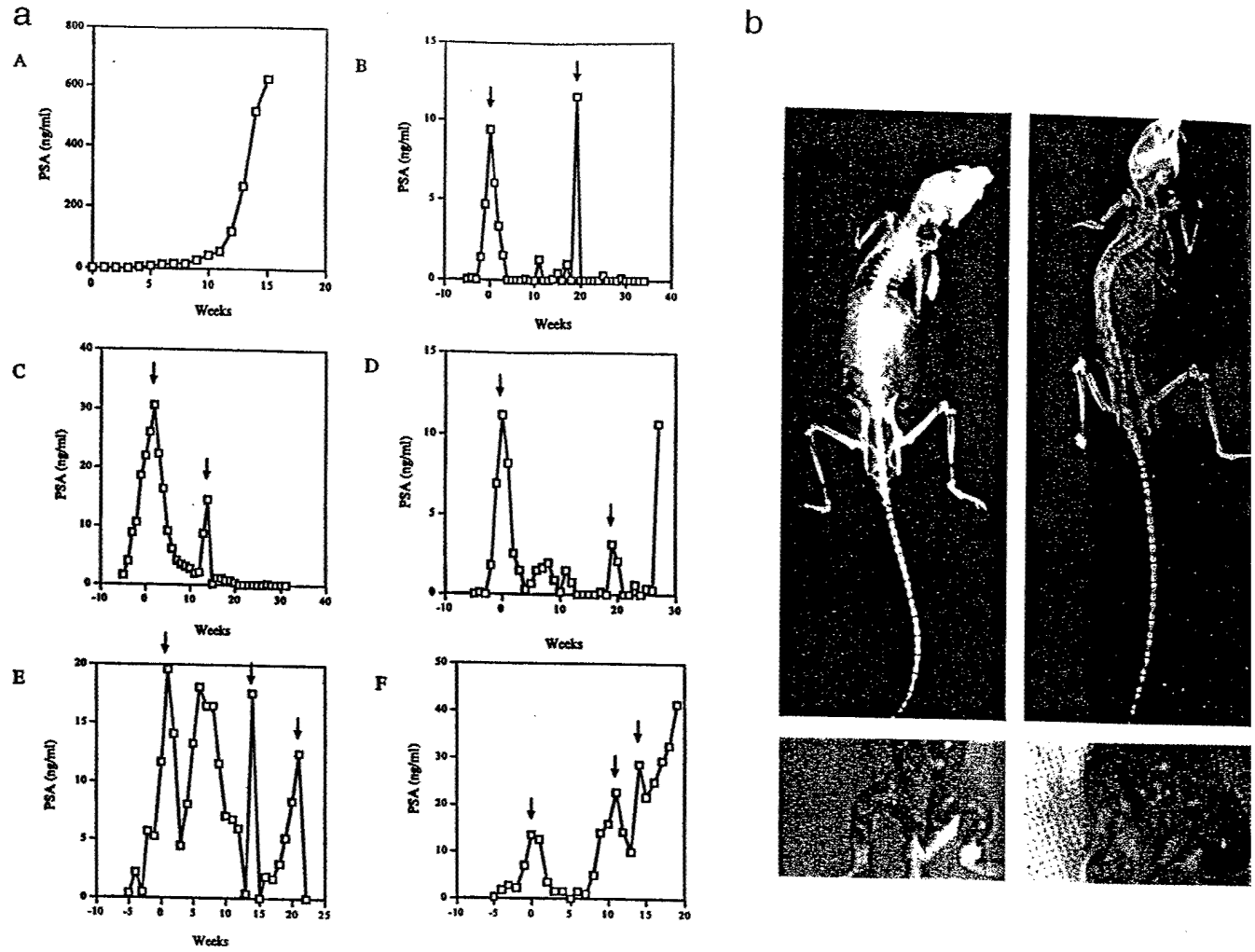
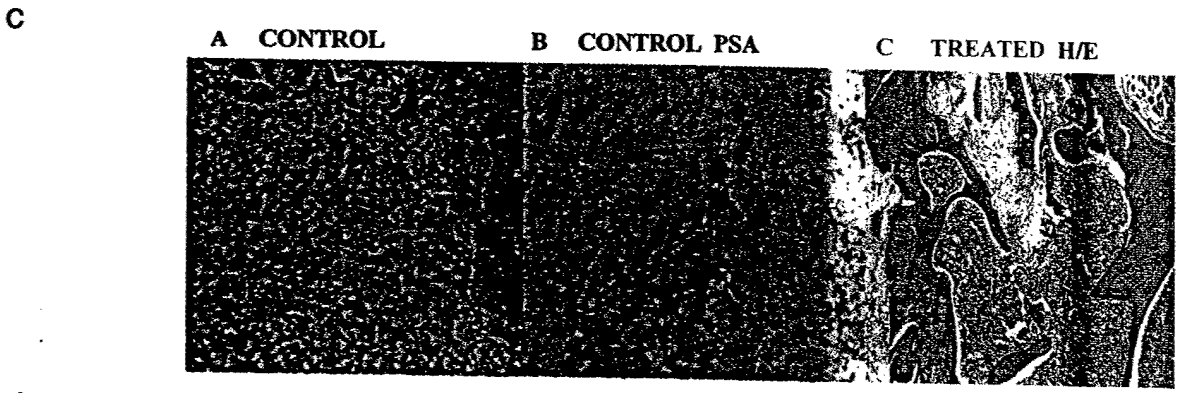


Fig. 3. Treatment of tumor xenografts with recombinant adenoviruses. Tumor xenografts were grown s.c. in athymic nude mice. Tumors were treated with recombinant viruses and PBS by intratumoral injection on day 0 and measured weekly. The data represent mean; *bars*, ± SD. *a*, PC-3 xenograft tumors were treated with Ad-OC-E1a and PBS (n = 4). *b*, Lovo xenograft tumors were also treated with Ad-OC-E1a and PBS (n = 4).





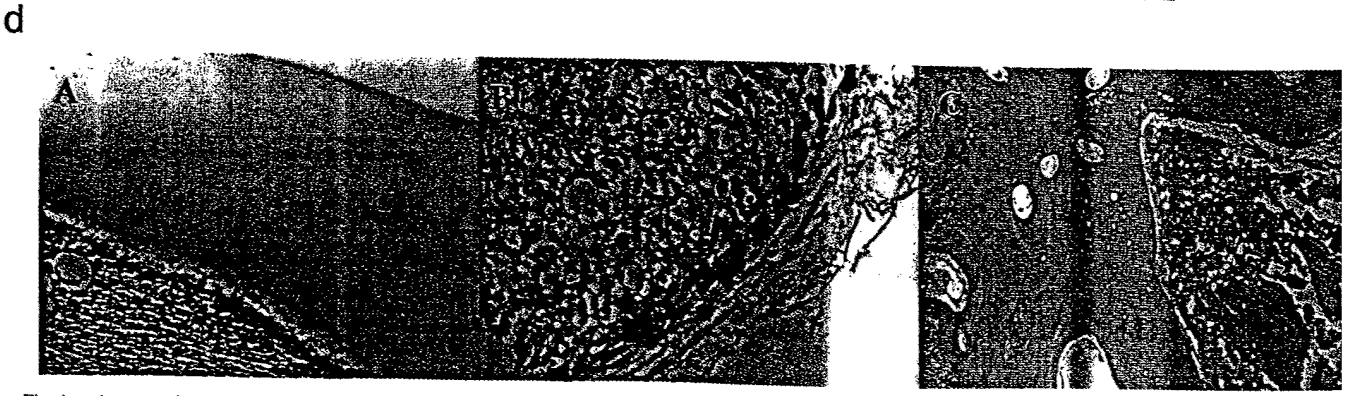


Fig. 4. *a*, demonstration of i.v. Ad-OC-E1a on serum PSA levels in SCID/bg mice injected intraosseously with C4-2 cells. *Panel A*, serum PSA level of an untreated control mouse after intraosseous injection of 1×10^6 C4-2 cells. Note exponential rise of serum PSA in the untreated mouse (mouse 1). *Panels B–F*, mouse 2–6 serum PSA levels of animals injected

the tibia. This marked improvement was confirmed by examining the histopathological section of the tumors obtained from the control and Ad-OC-E1a treated animals. Fig. 4*c* shows that in comparison to the systemic Ad-OC-E1a, the untreated mice have large tumors and strained positively by PSA antibody in the skeletal specimens (Fig. 4*c, Panels A* and *B*), whereas the prostate tumors cured by systemic Ad-OC-E1a failed to yield positive histopathology in the skeleton (Fig. 4*c, Panel C*) or positive immunohistochemical staining of PSA in the representative specimens (data not shown). We also compared the adenoviral infectivity in mouse bone *in situ*, human prostate PC-3 xenografts, and human bone maintained *in vitro* in soft agar. Results of this study showed that a single intraosseous administration of Ad-CMV-β-gal infected effectively the mouse bone cells without affecting the cortical bone (Fig. 4*d, Panel A*). *In vitro* Ad-CMV-β-gal efficiently infected upper layers of PC-3 tumor cells (Fig. 4*d, Panel B*) but not human bone cells (Fig. 4*d, Panel C*) maintained as explants in soft agar.

Discussion

Cancer therapies using adenoviral vectors can be divided into two broad categories, replication-defective and replication-competent (51). Because of the difficulties in infecting all of the cancer cells with adenoviral vectors, numerous laboratories have designed various versions of viral constructs with the primary goal of increasing the efficiency of viral infectivity (or transgene expression) or viral replication in competent tumor cells without damaging the normal tissues. One such approach relies on the ability of "bystander" genes such as hsv-TK or cytosine deaminase incorporated into replication-defective adenoviral vectors to convert prodrugs into biologically active growth-inhibitory products that elicit efficient cell kill even in cells that were not transduced with virus-bearing genes (2–5, 52, 53). The construction of replication-competent ONYX-015 lacking E1b, a MR 55,000 protein, can conceptually replicate in tumor cells that lack functional p53 protein (54). Conditional activation of viral gene expression and replication have also been achieved using tissue-specific promoters such as PSA or PSE for prostate cancer (1, 14, 15), α-fetal protein for liver cancer (55), and tyrosinase for melanoma (56, 57). Modification of adenoviral gene structure by introducing adenoviral death protein has achieved higher efficiency of viral replication (58). In the present study, we explored the possibility of using a novel tissue-specific (*i.e.*, osteoblast-specific) and tumor-restrictive (*i.e.*, restricted to calcified benign and malignant tumors) OC promoter to drive adenovirus replication in cells that contain OC promoter activity. This version of adenoviral vector allows the virus to replicate in both tumor epithelium (Fig. 2, *a* and *b*) and its supporting stromal cells including a human prostate stromal cell line (Fig. 2*c*) and a human osteoblast cell line (Fig. 2*c*). Thus, Ad-OC-E1a could potentially inflict maximal cell kill through, primarily, viral replication in tumor epithelium, and secondarily, by destruction of intercellular communication between tumor and stroma, inducing cell lysis in prostate fibromuscular stromal cells, osteoblasts, and potentially vascular endothelial pericytes (47–49). In experimental coculture studies both *in vitro* and *in vivo*, induction of osteoblast cell death by hsv-TK/acyclovir or ganciclovir also markedly inhibited the growth of prostate tumor cells.[4]

Because OC expression is highly restricted to maturing osteoblasts (20–29, 47), Ad-OC-E1a may potentially damage bone and alter the balance between rate of bone resorption and formation. This concern has been addressed, and our study is summarized below. First, the cortical bone of both mouse and human restricts adenoviral infection. We observed that whereas mouse bone marrow is highly susceptible to adenoviral infection, human bone marrow including maturing osteoblasts appeared to be more resistant to adenoviral infection (Fig. 4*d*). Therefore, it is possible that in humans, Ad-OC-E1a replication may be limited to proliferating and maturing osteoblasts, which express OC promoter activity. Second, intraosseous administration of Ad-OC-hsvTK plus i.p. ganciclovir in intact adult mice did not induce any abnormal histopathology of the skeleton (4, 59). In fact, OC has been shown as an inhibitor of bone mineralization by preventing the growth of mineral crystals in an *in vitro* assay (60). This role of OC is consistent with the transgenic OC knockout mouse model where the destruction of OC-expressing cells by hsv-TK resulted in increased bone mass and bone formation (61, 62).

The tissue-specific and tumor-restrictive OC promoter potentially has several advantages over other prostate-specific promoters such as PSA or PSE enhancer (1, 15), human kallikrein 2 (hK2; 14), or prostate-specific membrane antigen (63). One advantage is that OC is expressed prevalently by human primary and metastatic prostate cancers with expression found in both tumor epithelium and/or surrounding stromal compartment (see Fig. 1). Another advantage is that OC expression is not limited to prostate tumors and was also found expressed by other calcified benign and malignant tissues such as smooth muscle plaques associated with heart valve and blood vessels (48, 49); benign tumors (50); and malignant osteosarcoma, brain, thyroid, breast, lung, and ovarian tumors (unpublished results) irrespective of their basal PSA and AR status. This is significant because it was estimated that ~20% of prostate cancer patients do not have elevated PSA despite the detection and progression of the disease (64). In addition, although AR gene amplification and overexpression were detected in almost 30% of the clinical prostate cancer specimens (65), AR-mutant or AR-null prostate cancer cells and tissues were nevertheless commonly observed (64–67). On the basis of the above observations, it is possible that PSA and/or AR-negative tumors may be responsive to Ad-OC-E1a but not to Ad-PSA-E1a-induced cell lysis.

Several previous publications demonstrated that an Ad vector-mediated toxic gene, hsv-TK, expression driven by OC promoter, inhibited the growth of osteosarcoma (16, 45, 68) and its metastasis (69) and inhibited prostate tumor growth both *in vitro* and *in vivo* (4, 16). Although intratumoral administration of Ad-OC-TK was used in most of these earlier studies, we observed significant remission of osteosarcoma lung metastasis and improvement of survival by systemic administration of Ad-OC-TK (69). The ability of systemic Ad-OC-TK to exert antitumor effects on osteosarcoma pulmonary

[4] C-L. Hsieh, *et al.*, unpublished observation.

with Ad-OC-E1a (*arrows*, 2×10^9 pfu of Ad vector was administered via tail vein after detecting a rising serum PSA in animals that received intraosseous injection of 1×10^6 C4-2 cells). Systemic Ad-OC-E1a administration induced rapid decline of serum PSA, and 4/5 (80%) animals responded markedly to repeated Ad-OC-E1a treatment. *b*, gross morphology and X-ray evidence indicating the capability of Ad-OC-E1a in eradicating the growth of intraosseous human prostate tumor xenografts. Administration of Ad-OC-E1a via tail vein induced marked tumor regression in the treated mouse (*left panels*). *c*, regression of prostate tumors by Ad-OC-E1a is supported by histopathological evidence of the absence of prostate tumor cells in the skeleton (*Panel C*). In the untreated animals, both tumor cells (*Panel A*) and PSA (*Panel B*) were observed in the skeletal specimen. *d*, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining of normal mouse and human bone infected with Ad-CMV-β-gal (1×10^9 pfu). Note whereas the mouse bone marrow cells are susceptible to Ad-CMV-β-gal infection, the cortical bone of the mouse is resistant to Ad infection (*Panel A*). *Panel B*, human PC-3 prostate tumor is susceptible to Ad-CMV-β-gal infection when maintained as explants on soft agar. Normal human bone exposed to this virus appears to exhibit background β-gal activity suggesting that normal human bone cells may be resistant to Ad vector infection (*Panel C*).

metastasis without causing liver toxicity (59, 69) indicated the importance of considering the selection of tumor- or tissue-specific promoters to drive the expression of therapeutic genes or viral replications for cancer therapy. In this context, it is clear that conditional replication-competent adenovirus may have the advantage of amplifying the input of oncolytic virus and help the spread of agents to adjacent cells in a highly promoter- and cell-dependent manner. (1, 5, 14, 15, 51, 54, 58). In this study, we demonstrated substantial efficacy of systemic Ad-OC-E1a for the treatment of androgen-independent prostate cancer skeletal xenografts. However, we demonstrated that to eliminate the preexisting human prostate tumor xenografts in the bone, Ad-OC-E1a administration must be repeated. We obtained evidence that all of the mice responded initially to Ad-OC-E1a therapy (as judged by serum PSA response) and only one mouse (20%) escaped Ad-OC-E1a effects gradually and became an Ad-OC-E1a nonresponder. Forty percent (2/5) of the Ad-OC-E1a-treated mice have undergone complete tumor regression and are considered "cured" in the present protocol. Reasons why mice may lose their response to Ad-OC-E1a are presently unclear, but it is reasonable to suggest that Ad-OC-E1a infectivity may be reduced in the resistant tumors through a decreased coxsackie adenoviral receptor on tumor cell surface or a rapid clearance of Ad vectors at tumor sites from systemic circulation. Whereas the current protocol may be applicable to the treatment of clinical prostate cancer skeletal metastasis, some precautions need to be observed: (*a*) Ad-OC-E1a replication in normal human tissues requires more extensive testing, and human bone and human prostate cancer chimeric xenografts grown s.c. may be ideal for this evaluation (70); and (*b*) serum PSA response may be an indication but not the proof of tumor regression (71). Even if there is a potential pitfall in using altered serum PSA as the indicator for an antitumor effect, it is firmly established that serum PSA response does correlate with improved survival, pain relief, increased hemoglobin level, normalization of bone-derived alkaline phosphatase, weight gain, or improved performance status of prostate cancer patients (72). Smith *et al.* (73) found that a decrease in the serum PSA level of $\geq$50% at 8 weeks was correlated with significantly increased survival. Such data validate the use of changes in the serum PSA level as a response parameter in trials of therapy in prostate cancer. In this study, we have shown that the PSA response correlated well with the histopathologies of prostate tumors in the skeleton and demonstrated the efficacy of systemic OC promoter-driven conditional replication-competent adenovirus in abolishing the growth of preexisting prostate tumors in bone.

In summary, we have established a novel replication-competent adenoviral therapy using a tissue-specific and tumor-restrictive OC promoter to drive the replication of adenovirus for the treatment of prostate cancer metastasis in an experimental human prostate cancer skeletal xenograft model. Ad-OC-E1a was shown to be effective in eliminating preexisting androgen-independent prostate tumors in the bone without adverse effects on mouse bone. This study establishes for the first time that cotargeting prostate cancer and bone stroma may be an effective strategy for destroying human prostate tumor skeletal metastasis.

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Prostate cancer gene therapy: Past experiences and future promise

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Abstract

Gene therapy has been used to target prostate cancer with excellent pre-clinical efficacy but limited clinical efficacy. The concept of delivering genetic material to prostate cancer cells to alter their phenotype and ultimately their behavior has been demonstrated in the laboratory over the last decade. Translating those pre-clinical findings into novel therapies for prostate cancer has been difficult. The stigma of gene therapy and the aggressive regulation of clinical trials involving transfer of genetic material to patients are two major impediments to clinical successes in gene therapy. This review hopes to provide a snapshot of prior gene transfer protocol findings and forecast the exciting future directions investigators are heading.

1. Introduction – why gene therapy for prostate cancer

In 2002 it is estimated the prostate cancer will account for the most new cancer diagnoses at 189,000 men and will be second only to lung cancer for number of cancer related deaths at 30,200 men. The steady decline of the annual age-adjusted death rates in males according to the SEER data base over the past five years would suggest improvements in detection and treatment of clinically organ confined prostate cancer. The 1998 annual age-adjusted death rate now approximates that of the 1940 death rate of 20 per 100,000 person years [1]. The elucidation of prostate cancer's innate hormone dependency 60 years ago was the last big breakthrough in the treatment of the lethal form of prostate cancer that metastasizes to osseous sites. The greater understanding of the molecular events underlying the development of metastatic disease allows gene therapy approaches to be developed that specifically target these molecular events. For this reason, our group has focused on developing tumor-specific promoters, such as the osteocalcin (OC) promoter, based on gene therapy that specifically targets osseous metastases, the most lethal form of the disease [2–4].

2. Basics of gene therapy for prostate cancer

Gene therapy can be defined as the transfer of genetic material with therapeutic intent. This raises several major questions: (1) Who to target with gene therapy? (2) How to transfer genetic material? (3) Which genetic material to transfer? and (4) What route to administer?

Starting with the first question of 'who' to target with prostate cancer gene therapy. Several stages of prostate cancer are both amenable and appropriate for current gene therapy approaches. Most of the stages in the progression from localized disease to metastatic disease have been targeted, as well as several others that could be targeted as improvements in the molecular understanding of prostate cancer and developments of vectors occur. Men with locally advanced, locally recurrent or metastatic prostate cancer are natural groups to target due to the lack of conventional therapeutics. Additionally, those men at high risk for local or distant failure have been targeted for neoadjuvant and adjuvant approaches. As with most therapies the ability to treat men with low volume disease should enhance the success of the therapy. This is probably a critical point for both the immune-based and cytoreductive

approaches discussed below. For example, a patient with a detectable PSA recurrence after definitive conventional therapy may be the perfect patient for either an immune-based strategy or systemically administered and targeted cytoreductive approach.

In the future, a corrective approach could be used for men with high grade PIN if a genetic determinant of prostate cancer progression could be identified. A gene therapist with the knowledge of continually improved vectors, with enhanced targeting and greater killing ability, can easily envision the realization of a 'molecular prostatectomy' for the treatment of localized disease or even better a 'prophylactic molecular prostatectomy' to prevent morbidity and mortality associated with both benign prostatic hypertrophy and prostate cancer.

The currently available methods of 'how' to transfer genetic material are listed in Table 1. Each vector has been used in prostate cancer clinical gene transfer protocols. Each of the vector has advantages and disadvantages as couriers of genetic information. For example, the 'common cold virus' or adenovirus can transfer a large amount of genetic information with high efficiency, regardless of cell cycle considerations and without toxicity to the cellular genome (genotoxicity). Unfortunately, this virus results in only transient expression of the genetic material and most individuals will have innate immunity due to prior exposure to the 'common cold' which limits multiple dosing or systemic administration. The 'gutless' adenovirus has less immunogenicity and can transfer three times the amount of DNA as the conventional one.

The answer to the 'what DNA' question depends on the objective of the therapy. Gene therapy for cancer has been either corrective, allowing for the replacement of a defective tumor suppressor gene (i.e. p53, p16, etc.) or cytoreductive, allowing for direct cell killing (i.e. suicide, oncolytic) or indirect cell killing by immunomodulation (i.e. IL-2, GM-CSF, IL-12). Table 2 lists some common paradigms used in prostate cancer gene transfer protocols.

For example, the genetic material from the herpes simplex virus encoding for the thymidine kinase (TK) enzyme has been utilized in a number of gene transfer protocols for prostate cancer listed in Table 3 [5]. The HSV-TK enzyme encoded by this viral DNA segment remains the main pharmaceutical target for treating herpes viral infections. The viral form of the TK enzyme can convert a number of well-tolerated clinically approved pro-drugs (i.e. ganciclovir (GCV), acyclovir, valacyclovir, etc.) to a potent intracellular toxin, which interferes with DNA replication. Due to the activated pro-drugs effect on dividing DNA, this form of suicide gene therapy will effectively kill a cell when it attempts to proliferate or divide, but is limited to the cells infected by the virus and those immediately surrounding the infected cell ('bystander-effect').

The final question raised was by 'which' route to administer the gene therapy. This question can only be answered after answering the first three questions of whom, how and what. Currently, the vector being used generally dictates the route of administration. For instance, it would be illogical to treat locally recurrent prostate cancer after external beam radiotherapy using an intravenous delivery of adenoviral suicide approach. First, a majority of the dose of a currently available replication defective adenovirus will be filtered by the liver only leaving a small fraction of the dose to be delivered to the radiated prostate bed containing the recurrent disease. Second, the ability to visualize

Table 1. Comparison of vector systems in gene therapy. This table lists the important attributes of the currently used gene therapy vectors for prostate cancer. The bold items are felt to be the disadvantage of the particular vector

Attribute	Retrovirus/ lentivirus ('HIV')	Adenovirus/ 'gutless'	Adeno-associated virus	Vaccinia/fowl pox virus	Non-viral (liposome)
In vivo gene transfer rate	**Low**/high	High	High	High	Low
Gene transfer cell cycle independent	**Yes**/No	No	No	No	No
Size limit of DNA transfer	8 kb	10/35 kb	**<5 kb**	>30 kb	? Limit
Genome integration/genotoxicity	**Yes**	No	**Yes**	No	No
Period of DNA expression	Stable	Transient/ stable	Stable	Transient	Transient
Immunoreactive	No	**Yes**/No	No	Yes	No
Large scale production	Yes	Yes*/**No**	Yes*	Yes	Yes
Clinical safety profile	Yes/**No**	Yes/**No**	No	Yes	Yes

* Significant difficulties still persist in clinical grade large scale production.

Table 2. Current approaches for prostate cancer gene therapy

Strategy	Vector(s) used	DNA transferred
Immunotherapy	Retrovirus	GM-CSF
	Vaccina/ fowlpox	IL-2
		IL-12
	Liposome	PSA
	RNA	Tumor RNA
	AAV	
	Adenovirus	
Corrective/tumor suppressor	Retrovirus	P53
	Adenovirus	P16
		C-MYC
Suicide/toxic pro-drug	Adenovirus	TK
		CD
		TK/CD
Oncolytic	Adenovirus	OC promoter
		PB/PSA promoter
		PB/PSE promoter

the prostate and directly inject the prostate using ultrasound would be the obvious route for such a vector with limited abilities.

3. Past approaches

In the past seven years a variety of gene therapy paradigms have been used to target prostate cancer and can broadly be classified into immunotherapy, tumor suppressor therapy, suicide gene therapy and oncolytic therapy (Table 2). Each of these approaches has a strong foundation of pre-clinical data allowing for such clinical studies to be approved. Currently, 45 gene transfer protocols targeting prostate cancer can be found on the Office of Biologic Activities Protocol List (web page) out of 314 protocols targeted at cancer. Therefore, prostate cancer gene therapy trials account for 14% of all cancer gene therapy trials to date (Table 3) [5]. The details of each of the protocols listed in Table 3 can be found at the reference web address. Each investigator and all co-investigators involved in these protocols should be commended for their exhaustive efforts in getting a gene therapy protocol to the clinic. Prostate cancer remains a focus of the gene therapist because the prostate is accessible, and limited availability of treatments for locally advanced or metastatic disease states, represents a large enough population/market to obtain NIH/corporate dollars. Prostate cancer gene therapy protocols listed in Table 3 exemplify the translation nature of trial development in the 21st century. Within the table there are several examples of trials that have come to the clinic based on pre-clinical findings which then lead to further refinement and improved therapeutic trials, demonstrating bi-directional flow from the clinic and the basic research labs [2–4].

3.1. Immune-mediated gene therapy

Multiple mechanisms allow tumors to go unrecognized by the host immune system. Immunomodulatory therapy can enhance the hosts' anti-tumor immune response. Fifty-six percent or 25 of the 45 clinical prostate cancer gene therapy protocols utilize an immune-based approach. Initially, after demonstrating that immunostimulatory molecules could transform cancer cells previously able to hide from the immune system to activate a tumor-directed immune response, the first gene therapy trial in prostate cancer demonstrated that autologous prostate cancer cells from patients could be amplified, retrovirally transduced to express GM-CSF, irradiated and given back to patients to elicit an immune response toward metastatic prostate cancer cells. Unfortunately, this required a number of *ex vivo* steps, which limited the successful vaccination rate to 73% [6].

This trial led to the development of a simple vaccine composed of irradiated prostate cancer cell line allografts expressing GM-CSF. This approach will allow for multiple dosing. Several centers have put forth protocols to evaluate this approach further as listed in Table 3.

More recently, *in vivo* immunomodulatory approaches are being used. These include the use of both vaccina or fowlpox viruses to illicit an immune response against prostate cancer-specific molecules PSA, PSMA, MUC-1 alone or in conjunction with immunumodulating molecules (i.e. B7) and the direct intralesional injections of adenovirus, adeno-associated virus expressing a multitude of cytokines, including IL-2, GM-CSF and IL-12. The goal of each approach is to achieve a systemic anti-tumor response. These more recent *in vivo* methods avoid the complexities associated with *ex vivo* gene transfer approaches. The *in vivo* approach has the potential of making the tumor increasingly immunogenic because the vector carrying a gene to produce a cytokine is directly placed into the patient's tumor cells allowing the patient's own tumor cell to trigger the immune response to itself and other prostate cancer cells. Several trials have been

Table 3. Prostate cancer gene therapy trials (OBA Protocol List 11/19/01) [5]

Principal investigator	Institution	Vector	Genetic material	Year reviewed	Ref.
1. Simons JW	Johns Hopkins, MD	Retro	GM-CSF	94	[6]
2. Steiner MS	Vanderbilt Univ., TN	Retro	c-myc	95	
3. Chen AP	Nat. Naval Med, MD	Vaccinia	PSA	95	
4. Paulson DF	Duke Univ., NC	Liposome	IL-2	95	
5. Scardino PT	MSKCC, NY	Adeno	HSV-TK	96	[17,18]
6. Eder JP	Dana-Farber, MD	Vaccinia	PSA	96	
7. Sanda MG	Univ. of Michigan, MI	Vaccinia	PSA	97	
8. Belldegrun AS	UCLA, CA	Liposome	IL-2	97	
9. Hall SJ	Mt. Sinai, NY	Adeno	HSV-TK	97	
10. Belldegrun AS	UCLA, CA	Adeno	P53	97	[19]
11. Simons JW	Johns Hopkins, MD	Retro	GM-CSF	97	
12. Logothetis CJ	MD Anderson, TX	Adeno	p53	97	
13. Kadmon D	Baylor, TX	Adeno	HSV-TK	98	
14. Simons JW	Johns Hopkins, MD	Adeno	PSA	98	[13]
15. Figlin RA	UCLA, CA	Vaccinia	MUC-1/IL-2	98	
16. Gardner TA	Univ. of Virginia, VA	Adeno	OC promoter-HSV-TK	98	[3]
17. Eder JP	Dana-Farber Cancer Inst., MD	Vaccinia/fowlpox	PSA	99	[20]
18. Small EJ	UCSF, CA	Retro	GM-CSF	99	
19. Kaufman HL	Albert Einstein, NY	Vaccinia/fowlpox	PSA	99	
20. Vieweg J	Duke Univ., NC	RNA	PSA	99	
21. Belldegrun AS	UCLA, CA	Liposome	IL-2	99	
22. Small EJ	UCSF, CA	Retro	GM-CSF	99	
23. Kim JH	Henry Ford Hosp, MI	Adeno	CD/TK	99	
24. Aguilar-Cordova E	Harvard Univ., MA	Adeno	TK	99	
25. Gingrich JR	Univ. of Tenn., TN	Adeno	p16	99	
26. Terris MK	Stanford Univ., CA	Adeno	PSA	99	
27. Wilding G	Univ. of Wisc., WI	Adeno	PSA	99	
28. Belldegrun AS	UCLA, CA	Liposome	IL-2	99	
29. Dahut WL	NIH/NCI	Vaccinia/fowlpox	PSA	99	
30. Arlen PM	NIH/NCI	Vaccinia/fowlpox	PSA	00	
31. Vieweg J	Duke Univ., NC	RNA	Total tumor RNA	00	
32. Pollack A	Univ. of Texas, TX	Adeno	p53	00	
33. Gardner TA	Indiana Univ., IN	Adeno	OC promoter	00	
34. Freytag SO	Henry Ford Hosp., MI	Adeno	CD/TK	00	
35. Lubaroff DM	Univ. of Iowa	Adeno	PSA	01	
36. Miles BJ	Baylor College, TX	Adeno	IL-12	01	
37. DeWeese TL	Johns Hopkins, MD	Adeno	PB/PSE promoter	01	
38. Small EJ	UCSF, CA	Adeno	PB/PSE promoter	01	
39. Dula E	West Coast Clinical Res.	AAV	GM-CSF	01	
40. Freytag SO	Henry Ford, MI	Adeno	CD/TK	01	
41. Scher H	MSKCC, NY	Liposome	PSMA	01	
42. Corman J	VA Puget Sound, WA	AAV	GM-CSF	01	
43. Pantuck AJ	UCLA, LA	Vaccinia	MUC-1/IL-2	01	
44. Vieweg J	Duke, NC	RNA	hTeRT	01	
45. Corman J	VA Puget Sound, WA	Adeno	PSE promoter	01	

initiated and limited published results are available for further comment.

3.2. *Corrective gene therapy*

Replacement of normal tumor suppressor genes (i.e. p53, p16) can reestablish the normal cell cycle progression and enhance apoptosis of abnormal cancer cells. Unfortunately, multiple genetic sites, as opposed to a single locus, have been implicated in the development and progression of prostate cancer. However, mutations in the p53 gene have been identified in multiple prostate cancer cell lines and clinical specimens. Replacement of wild type p53, or other tumor

suppressor genes, *in vitro* and in animal models have resulted in inhibited tumor growth [7]. Clinical studies using replacement of p53 alone and also in combination with radiation therapy are being investigated (Table 3).

The over expression of particular oncogenes may be combated by the introduction of anti-sense mRNA to inhibit the translation of the oncogenic protein(s). The introduction of anti-sense c-myc mRNA into prostate cancer cell lines, such as LNCaP, result in decreased translation of the oncogenic protein. Mouse studies indicate that this approach results in tumor shrinkage. Clinical trials are also in progress.

3.3. *Suicide gene therapy*

The efficacy of suicide gene therapy relies on the conversion of a non-toxic pro-drug to a lethal metabolite by the enzymatic product of a previously delivered gene. Fortunately, the toxic effect is not limited to cells transfected by a suicide gene (i.e. TK, CD, etc.), but extends to nearby cells via the 'bystander effect'. The bystander effect is mediated primarily by intercellular communication (i.e. gap junctions) and phagocytosis of debris from dying cells by the neighboring cells.

The most well characterized suicide gene therapy utilizes the TK from the herpes simplex virus and one of several clinically available anti-herpetic medications (i.e. GCV, acyclovir, valacyclovir, etc.) to activate the suicide mechanism. Unlike its human counterpart, the viral DNA encodes for the TK enzyme, which can phosphorylate the un-phosphorylated nucleoside guanine or the guanine analogues (i.e. GCV, acyclovir, valacyclovir, etc.). Therefore, when a pro-drug such as GCV is administered it will be phosphorylated and incorporated into the DNA at the time of replication. This incorporation occurs at a guanine site and causes abnormal DNA replication, which in turn generates an apoptotic signal and kills the cell when it tries to divide.

The TK/pro-drug system is the most studied because it is a safe approach for cancer gene therapy for a number of reasons. First, suicide of the infected cell occurs only when they divide thus targeting cancer cells, which divide more rapidly then non-cancerous cells. Second, the toxic effect only occurs when the pro-drug is administered allowing the cessation of the pro-drug in the event of serious toxicities. Third, several anti-herpetic drugs used as pro-drugs are clinically available, therefore simplifying the approval process for the clinical trail. Finally, the bystander effect is limited to surrounding cells that can take up the toxin produced by within the dying cell. All of these attributes allowed the pioneers of this form of gene therapy to apply and receive approval to conduct clinical trials in patients with advanced cancer.

Initially, TK was placed under the regulation of a universal promoter (i.e. retroviral LTR, RSV or CMV). The universal promoter is very active but it is activated in all cell types. This allows for the TK to be active in normal and cancer cells that are transduced by the vector. These vectors required direct injection or intralesional delivery due to the effect they would exert on normal cells trying to divide if administered systemically. The initial prostate cancer TK trial used a replication-defective adenovirus to transport RSV-TK followed by GCV in men with locally recurrent prostate cancer after definitive external beam radiotherapy. This trial realized the potential of this therapy by demonstrating anti-cancer activity as evidenced by sustained decreases in serum PSA and improved biopsies from these men with recurrent tumors. As a result of some of the adenovirus leaving the injection site and reaching the liver, several of the patients experienced a self-limiting toxicity and one patient experienced a grade III hepatic dysfunction and grade IV thrombocytopenia.

Our group tried to harness the abilities of TK and to allow for systemic therapy for men with metastatic disease by exchanging the universal promoter for a tumor-specific OC promoter and PSA promoter. After a series of pre-clinical studies demonstrating the specificity of the OC promoter, we performed a phase I trial by directly injecting post-surgical recurrences ($n = 2$) or metastatic prostate cancer lesions ($n = 9$) with a replication-defective adenovirus to transport the OC promoter driving TK expression followed by valacyclovir administration.

This trial demonstrated several novel principles upon which we are now building, first, an adenoviral vector that could be safely and repeatedly injected into either a prostate cancer osseous metastatic (Figure 1) or a post-surgical recurrence. Second, anti-tumor effect could be seen by transient declines in PSA or improved post-treatment biopsies in several patients. Third, despite transient detection of live viral particles in the serum of these men, valacyclovir administration did not lead to several hepatic toxicities, suggesting that the hepatocytes could not activate the OC promoter and did not produce TK even though exposed to the Ad–OC–TK virus and were unaffected by valacyclovir. Finally, TK gene expression could be detected in the biopsy specimens after treatment, demonstrating target tissue gene transfer and protein expression.

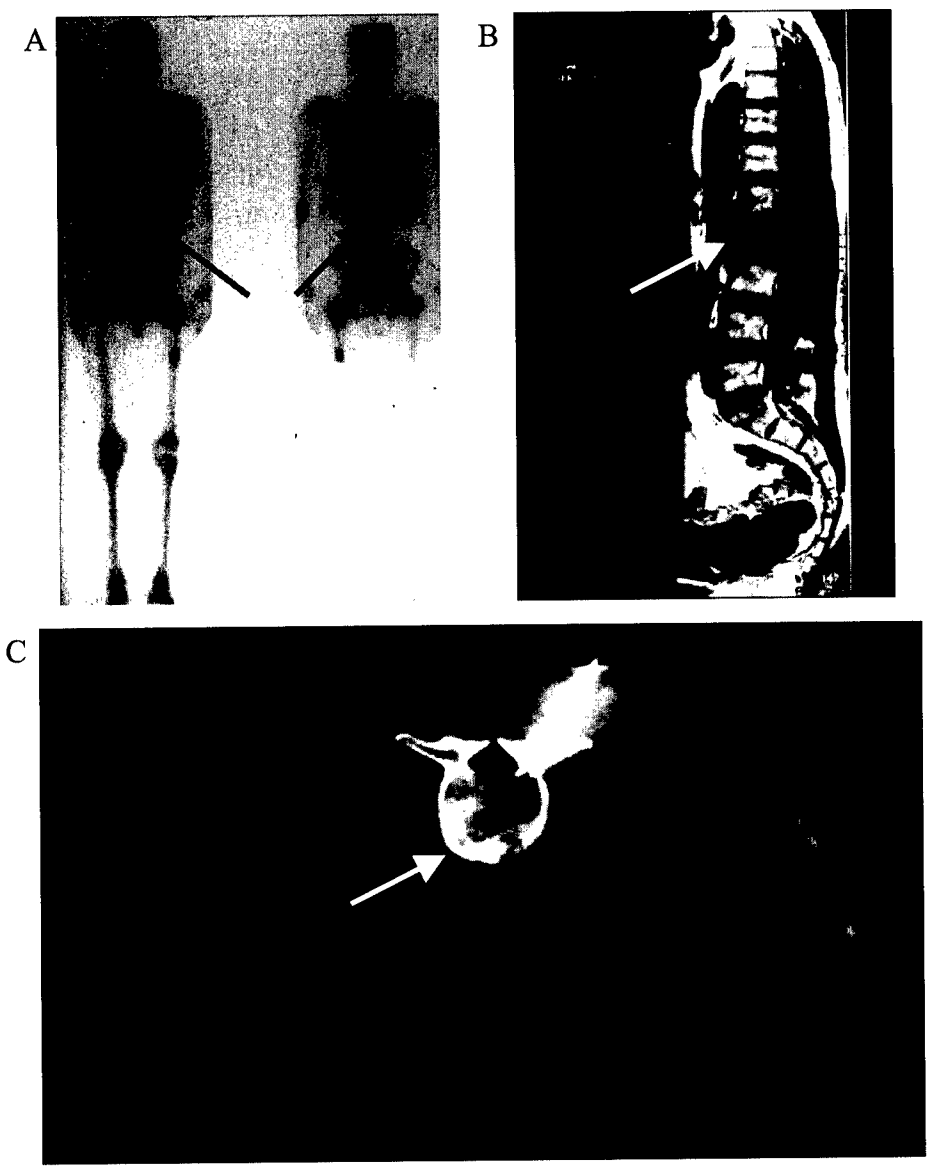

Figure 1. Intralesion administration of Ad–OC–TK to 2nd lumbar vertebral body osseous metastases delineated with an arrow by (A) Bone Scan, (B) MRI and (C) CT Scan during the actual injection.

It is important to note that as with many of these trials it took three years after demonstrating efficacy in pre-clinical models to produce and test cGMP grade Ad–OC–TK at a cost of $200,000 and perform GLP grade toxicology testing in mice at a cost of $100,000. The completion of this 11 patient trial including clinical monitoring, and personnel cost (PI and CRN) was $700,000 or about $91,000 per patient enrolled.

Fortunately, we received a gift from the Kluge Foundation to cover these costs or this trial may never have been performed. It is also important to note that this trial was conducted prior to the unfortunate events at the University of Pennsylvania that have led to additional viral testing and toxicology requirements now being requested by the regulatory agencies.

This trail demonstrated that the prostate tumor-specific promoter OC could be used to target prostate cancer cells and protect normal surrounding cells. Phase II testing of this vector was proposed, but due to viral production delays and the development of an

improved OC based oncolytic vector (see below) with more potential clinical efficacy based on pre-clinical studies, the phase II trial was appropriately shelved by the sponsor.

3.4. *Oncolytic gene therapy*

To overcome some of the limitations of replication-deficient viral vectors used in suicide gene approach, such as the incomplete delivery of virus to every tumor cell, viruses have been engineered that harness the natural lytic life cycle of the adenovirus under the regulation of tumor-specific promoter. Several oncolytic vectors have demonstrated strong and specific tumor killing in animal models of aggressive human prostate cancers and are currently being tested in the clinic.

Initially, our group [8] and others [9] demonstrated the prostate cancer-specific gene regulation of the PSA promoter in animal models of prostate cancer as has been demonstrated in other tumor models such as CEA promoter in colon cancer [10,11]. This finding combined with the advanced understanding of the adenoviral genes and their regulatory functions allowed for the creation of a series of conditionally replicative adenoviruses.

Rodriquez et al. demonstrated that a portion of the PSA promoter could provide prostate cancer-specific viral replication both *in vitro* and in animal models. Additionally, the element from the PSA promoter could be enhanced if androgens were applied [12]. This virus has been used in a clinical trial of men with recurrent prostate cancer after failed definitive external beam radiotherapy [13]. This trial demonstrated several important findings about tumor-specific oncolysis. Limited adverse events throughout the dose escalation in all 20 men, combined with 5 responders demonstrating a greater than 50% decline in serum PSA would suggest that the portion of the PSA promoter allowed conditional replication of the adenovirus only within prostate cancer cells (Table 3, #14). Additional modifications [14] to the above virus has demonstrated enhanced specificity and strength in pre-clinical models and is currently being tested in clinical trial at several centers (Table 3, #26, 27, 37, 38, 45).

Due to our groups continued interest in the OC promoter to provide gene expression in advanced prostate cancer cells and the supportive bone cells of osseous metastasis, we created an adenovirus that would replicate intracellularly and lyse them if the cell was able to activate the OC promoter (Figure 2). The Ad–OC–E1a virus is a conditionally replicative adenovirus which has performed well in pre-clinical animal models of human prostate cancer [4]. A trial paralleling the design Ad–OC–TK trial with this virus has been proposed and is awaiting final approvals from regulatory agencies (Table 3, #33).

4. Future directions in prostate cancer gene therapy

The immediate future of gene therapy for prostate cancer appears to be found in four major paradigms as follows: (1) immune-based therapy (2) novel vectors (3) synthetic oncolytic viruses and (4) a combination of gene therapy with conventional therapies.

Immunotherapeutic approaches to prostate cancer still account for greater than 50% of the current gene transfer trials. The safety of these approaches has been demonstrated although the efficacy remains to be seen. The ability to target extremely low volume disease and the continual discovery of new prostate-specific targets permits continued optimism for this approach.

Several new vectors are being tested in pre-clinical models including oncolytic herpes viral vectors [15] and lenti viral vectors [16] containing prostate-specific regulatory elements (e.g. PSA promoter). Two adeno-associated virus trials are proposed and should provide great insight into stable gene transfer to prostate cancer cells (Table 3, #39, 42).

The third major focus will be to create synthetic or designer vectors that are genetically engineered to exert their DNA transfer to the target cell only. This will allow the gene therapist to keep the desired attributes of a vector while altering the undesired attributes. For example, the knob protein of the adenovirus binds to the Coxsackie-adenovirus receptor (CAR) on the surface of a cell and allows an infection of the cell to occur. The toxicity associated with systemic administration of adenoviral vectors is thought to be secondary to the presence of CAR on normal cells (e.g. hepatocytes). We are currently testing an oncolytic virus which has been modified at the knob protein to attach to a RGD sequence found on the surface of most cancer cells and less common on normal cells (e.g. hepatocytes). The combination of our ability to manipulate the viral genomes and the information about cancer cells is allowing us to design vectors that will specifically target and destroy prostate cancer cells with precision.

Finally, as demonstrated by several of the recent submission to the Office of Biologic Activities is the

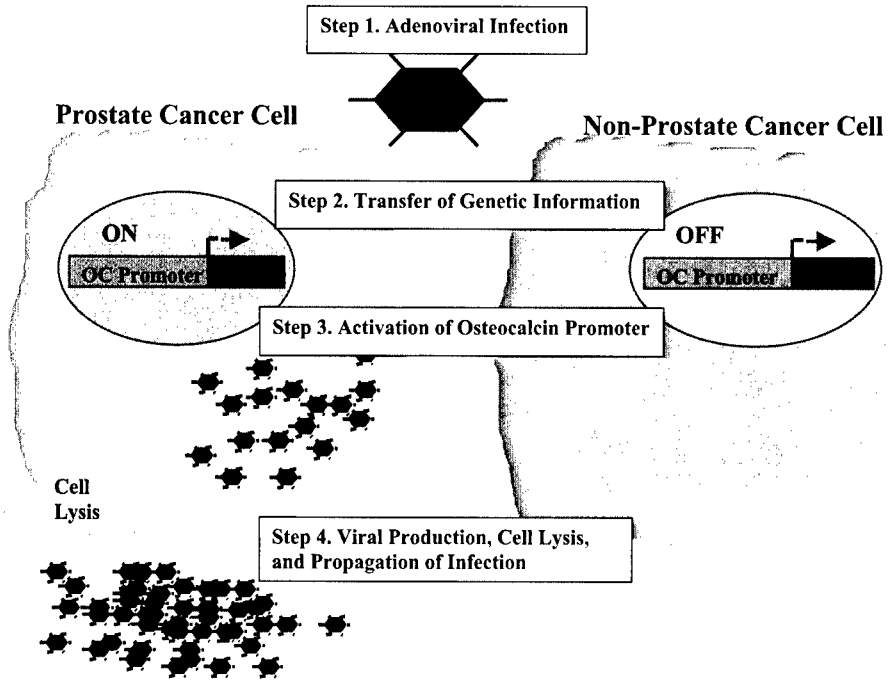

Figure 2. Steps of Ad–OC–E1a directed prostate cancer-specific oncolysis. The molecular steps required for all oncolytic viruses using conditionally replicating adenovirus employing the OC promoter to obtain prostate cancer specificity are illustrated. Step 1: the viral infection occurs in both the prostate cancer and non-prostate cancer cell. Step 2: the transfer of genetic material of cellular internalization of the DNA packaged in the virus occur in both cell type. Step 3: the activation of the OC promoter and subsequent initiation of new viral replication is specific to the prostate cancer due to the inability of the non-prostate cancer cell to activate the OC promoter. Step 4: the lytic cell death and the propagation of the adenoviral infection to neighboring cells can only occur in the prostate cancer cells therefore minimizing toxicities to the normal cells.

combination of a gene therapy strategy with more conventional therapy such as radiation or chemotherapy. There have been several elegant pre-clinical studies that demonstrate the ability of combining chemo-gene therapy and radio-gene therapy that have led to the proposed clinical trials (Table 3, #38) and (Table 3, #37, 40), respectively.

5. Conclusion

Gene therapy has been shown to be safe and effective in patients with prostate cancer. Principle investigators are currently using all available vectors and employing all available approaches as illustrated by Table 3. The paucity of gene therapy-related toxicities in the trials published to date would suggest that they could be added to conventional therapies with potentially additive or even synergistic improvements in efficacy without overlapping toxicity profiles. The main limitations to advances in this field are the time and expense producing and testing these biologics to meet the continually rising regulatory hurdles. The ability to target the delivery of vectors to prostate cancer cells as well as to limit the desired gene transfer to prostate cancer cells, currently exists in numerous labs throughout the world, but several years will pass before these non-traditional vectors will make it to patients in need of therapy.

Acknowledgements

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CHAPTER 9

Targeted Adenoviral Vectors II: Transcriptional Targeting

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I. Introduction: Rationale of Transcriptional Targeting

Gene therapy is an innovative approach aimed at introducing genetic material into an organism for therapeutic intent. Still in its infancy, this novel concept has witnessed fundamental preclinical success with numerous ongoing clinical trials to confirm these findings. Critical to the success of gene therapy trials are issues relating to specific delivery of physiologically active biomolecules at therapeutically significant concentrations. Initially this was achieved by using direct intralesional injections of vectors to localize the delivery to the target tissue and universal promoters to maximize expression at that site. Over the past several years, we and several other investigators have investigated the potential of tumor-specific promoters to transcriptionally regulate gene expression in the laboratory and in clinical trials. The safety demonstrated by these trials using tumor/tissue-specific promoters has led to the recent approval of a trial administering a conditionally replicative adenovirus systemically for the treatment of metastatic prostate cancer.

In order for gene therapy to be widely applicable, there is an urgent need to develop a new generation of viral vectors capable of achieving these goals of targeted delivery and controlled gene expression at the target site. The aim of this chapter is to discuss various potential strategies that have been utilized to achieve tissue/tumor-specific expression using adenoviral vectors. A better understanding of tissue specific gene expression necessitates a basic review of the eukaryotic transcription process at the molecular level. Consequently, we

ADENOVIRAL VECTORS FOR GENE THERAPY

begin by examining the molecular architecture of DNA and its relationship with the transcriptional mechanism.

II. Regulation of Transcription in Eukaryotes

To fully understand the complexity underlining transcriptional targeting a brief review of the mammalian transcriptional process follows:

A. Molecular Organization of DNA

During interphase the genetic material in association with proteins is dispersed throughout the nucleus in the form of chromatin. At the onset of mitosis, chromatin condensation takes place and during prophase it undergoes further compression into recognizable chromosomes. The associated proteins are basic, positively charged (lysine- and arginine-containing) histones and less positively charged nonhistones including high-mobility group (HMG) proteins. Histones play a key role in chromatin structural organization and are subject to various posttranslational modifications like acetylation, phosphorylation, and ubiquitination. Histones constitute nearly half of all the chromatin protein by weight and can be divided into six types: H1, H2A, H2B, H3, H4, and H5. DNA is incorporated into a 100 Å nucleosomal fiber comprising of two molecules each of H2A, H2B, H3, and H4 which form the core histone octamer along with one linker histone H1 or H5. The nucleosome core particle consists of 146 base pairs of DNA while the core histone octamer interacts with about 200 base pairs of DNA. While the histones function by interacting with DNA to form nucleosome, the nonhistone proteins are responsible for performing diverse functions including tissue-specific transcription. The 100-Å nucleosomal fiber is arranged into a higher order structure termed a 300-Å supercoiled filament or solenoid. Evidence indicates that certain nonhistone proteins including topoisomerase II bind to chromatin every 60–100 kilobases and tether the supercoiled, 300-Å filament into structural loops. Further interaction with other nonhistone proteins leads to gathering of loops into rosettes, which in association with additional nonhistones undergo condensation forming a scaffold. This is known as the radical loop–scaffold model of compaction. Special, irregularly spaced repetitive base sequences associate with nonhistone proteins to define chromatin loops. These stretches of DNA are known as scaffold-associated regions (SARs). In order to be competent for transcription, the 300-Å chromatin filament must undergo decondensation.

B. The Central Dogma

According to the central dogma, the genetic information flows from (1) DNA to DNA during genomic replication and (2) DNA to protein during

gene expression. Gene expression can be simply defined as a phenomenon by which the genetic information stored in DNA is transferred to a protein. It involves two distinct processes. The process by which cells convert genetic information from DNA to RNA is called *transcription* and the decoding of RNA information to generate a specific sequence of amino acids is called *translation*. In addition, the flow of genetic information from RNA to DNA has been demonstrated in the case of retroviruses. Thus, the flow of genetic information from DNA to RNA is sometimes reversible. However, this flow is unidirectional from RNA to protein and irreversible since, normally, the genetic information within the messenger RNA (mRNA) intermediate is not altered. However, a few exceptions in the form of RNA editing seem to challenge the present concept. RNA editing has been shown to alter the information content of the gene transcripts by changing the structures of individual bases and by inserting or deleting uridine monophosphate residues.

Gene expression in eukaryotes is a spatially and temporally regulated process. Gene expression is regulated at multiple levels including transcription, posttranscriptional processing, nucleocytoplasmic transport, mRNA stability, translation, posttranslational modification, and intracellular trafficking of the protein.

C. Transcription (Fig. 1)

In eukaryotes, transcription occurs in the nucleus with the help of RNA polymerase to generate a single-stranded RNA molecule that is complementary in base sequence to the DNA template strand. There are three different types of RNA polymerases for the transcription of different types of genes. *RNA polymerase I* functions to transcribe ribosomal RNA (rRNA) genes to generate a large rRNA primary transcript which undergoes processing within the nucleolus to generate a 28S rRNA, a 5.8S rRNA, and an 18S rRNA. *RNA polymerase II* transcribes all of the protein coding genes into primary transcripts called pre-mRNAs that upon posttranscriptional processing generate mRNAs. While *RNA polymerase III* is known to transcribe transfer RNAs (tRNAs), 5S rRNA, and small nuclear RNAs (snRNAs).

There are two main types of *cis*-acting elements in all polII-transcribed genes: promoters and enhancers. The promoter, which is in close proximity to the protein coding region, consists of nucleotide sequences spanning approximately −40 and +50 nucleotides relative to a transcription initiation site. A typical core promoter consists of four distinct elements: (1) A unique sequence called Goldberg–Hogness or the TATA box which has a consensus sequence TATAAAA and is located about −25 to −30 nucleotides upstream of the transcription initiation site. The TATA box alone is sufficient for independently directing a low-level polII-mediated transcription. (2) An initiator element that is functionally analogous to the TATA box and directly

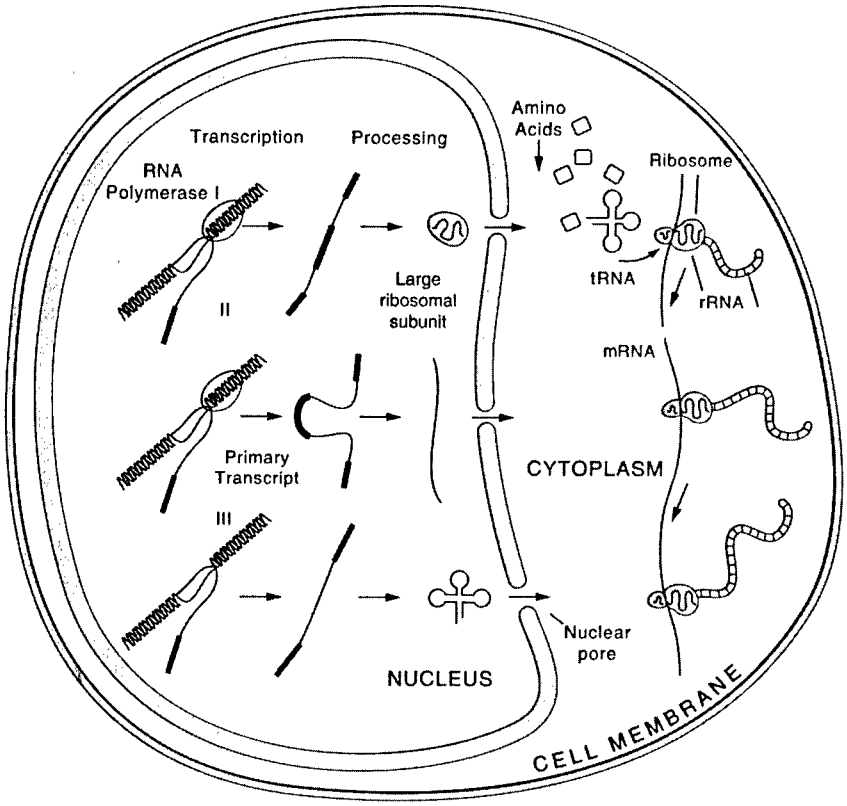

Figure 1 The transcriptional process of the RNA polymerase I, II, and III transcribing unwound DNA to rRNA, mRNA, and tRNA, respectively.

overlaps the transcription start site and has the loose consensus sequence PyPyA+1NT/APyPy. (3) The downstream core promoter element which is located approximately at position +30 downstream of the initiation site and acts in conjunction with the initiator element to direct transcription initiation. (4) The TFIIB recognition element, which has the consensus sequence $^{G}/_{C}{}^{G}/_{C}{}^{G}/_{A}$CGCC and is located from −32 to −38 upstream of the TATA box. Another cis-acting element called the CAAT box has a consensus sequence GGCCAATCT and is located near position −70 to −80 relative to transcription initiation site. Mutagenesis studies suggest a critical role of the CAAT box in modulating the promoter's ability to facilitate transcription.

In addition, polII promoters often contain two conserved sequences, the SP1 or GC box (GGGCGG) at about position −110 and the octamer box (ATTTGCAT); however, their positions are variable and they may occur either singly or in multiple copies. These consensus sequences are known to influence

the efficiency of the promoter in initiating transcription. In addition, the regulatory regions termed enhancers are located farther upstream, downstream, or within the gene. The activity of the enhancers is independent of the location, orientation, and gene type. Although they may not be involved directly in template binding, they are capable of modulating highly efficient transcription initiation.

The promoter regions are normally sequestered within the nucleosome and thus are rarely able to bind to basal transcription factors and RNA polymerase, thereby leading to transcriptional repression or silencing. In order for transcription initiation to occur, the sequestered promoter must be exposed so that it can readily bind basal factors and this is achieved by chromatin remodeling. The DNA in highly compacted chromatin is relatively resistant to nuclease DNaseI digestion. Thus, sensitivity of the DNA to DNaseI reveals the degree of chromatin condensation and is directly proportional to the transcriptional activity of a particular gene. Chromatin remodeling by acetylation and deacetylation of the histone proteins represents a major regulatory mechanism during gene activation and repression, respectively. The acetylation of histones by histone acetylase causes neutralization of the lysine basic charge, which in turn causes relaxation of contacts between the histones and the DNA. Thus, acetylated histones are preferentially found in active or potentially active genes where the chromatin is less tightly packed. Further, treatment of cultured cells with compounds like sodium butyrate, which enhances histone acetylation, leads to activation of previously silenced cellular genes.

The normal chromatin in the nucleosomal conformation can be converted into highly condensed heterochromatin which is transcriptionally inactive by the addition of methyl groups to a series of cytosine residues in the CpG dinucleotides found in tissue-specific genes. Thus, methylation and demethylation may play a crucial role in tissue-specific gene regulation. Locus control regions (LCRs) are specialized regulatory sequences located several kilobases upstream of the gene and capable of modulating transcription of gene clusters by influencing the chromatin structure. An assembled LCR–transcription factor complex is called an enhanceosome and if any of the components of this complex are missing, transcriptional activation of the gene cluster cannot occur. Insulators or boundary elements are regulatory sequences located in the vicinity of junctions between condensed and decondensed chromatin, which represent transcriptionally active and inactive loci, respectively. Insulators do not enhance transcription and are responsible for position-independent effects, but can prevent transcription when placed between an enhancer and a promoter.

D. Mechanism of Transcription

Eukaryotic transcription by RNA polII involves five stages: (a) formation of the preinitiation complex, (b) initiation, (c) promoter clearance, (d) elongation, and (e) termination. RNA polII cannot interact directly with the promoter to initiate transcription but requires recruitment to the promoter by interacting

with transcription factors. Transcription initiation is precisely controlled by the binding of a variety of *trans*-acting proteins termed transcription factors to the promoter and the enhancer. Transcription factors that assist the binding of RNA polymerase II to the promoter and initiate low levels of transcription are called basal factors, while other transcription factors are termed activators and repressors by binding to the enhancers. The transcription factors that bind to the TATA box are known as the TATA-binding protein (TBP) and are essential to the initiation of transcription from all pol II genes. A number of other basal factors that associate with TBP are called TBP-associated factors (TAF_{II}s) and help in the assembly and binding of the complex to the promoter, which in turn leads to transcription initiation.

The first event in the formation of preinitiation complex involves recognition of the TATA box by a multisubunit TFIID complex. A complex consisting of TBP and TAF_{II}s called TFIID specifically binds to the TATA box to induce conformational changes that favor the binding of other transcription factors like TFIIA and TFIIB, both of which can interact directly with TFIID. TFIIB serves two critical roles in transcription initiation: (a) It acts as a bridge and recruits TFIIF/RNA polII to the promoter; and (b) it aids in the selection of the transcriptional start site. TFIIB interacts asymmetrically with TFIID–DNA and contacts the phosphodiester backbone of DNA both upstream and downstream of TATA box. The position of the amino terminus of TFIIB in the DNA–TFIID–TFIIB complex is located near the transcription start site, which might explain the role of TFIIB in stabilizing the melting of the promoter prior to RNA synthesis.

Following the assembly of the DNA–TFIID–TFIIA–TFIIB complex, RNApolII is recruited to the promoter by TFIIF. TFIIF has two subunits: (1) the larger subunit, RAP74, which has an ATP-dependent DNA helicase activity which may catalyze the local unwinding of the DNA to initiate transcription, and (2) the smaller subunit, RAP38, by which it binds tightly to the RNA polII. This is followed by binding of TFIIE to the DNA downstream from the transcriptional start point. Two other factors, TFIIH and TFIIJ are recruited to the initiation complex but their locations in the complex are unknown. The interaction of the preinitiation complex with the core promoter alone is not sufficient to initiate transcription. A sequence of events beginning with the phosphorylation of the carboxy-terminal domain of RNApolII by TFIIF followed by ATP hydrolysis set the stage for DNA melting, initiation of synthesis and promoter clearance. Most of the TFII factors dissociate before RNApolII leaves the promoter. The carboxy-terminal domain coordinates processing of RNA with transcription.

The general process of transcription initiation is similar to that catalyzed by bacterial RNA polymerase. Binding of the RNApolII generates a closed complex, which is converted at a later stage to an open complex in which the DNA strands have been separated. TFIIE and TFIIH are involved in an

extension of the unwound region of the DNA to allow the polymerase to begin transcription elongation. Several elongation factors including TFIIF, SII, SIII, ELL, and P-TEFb function to suppress or prevent premature pausing of RNApolII as it traverses the DNA template. Early in the elongation process when the growing RNA chains are about 30 nucleotides long, the 5′ ends of the pre-mRNAs are modified by the addition of 7-methyl guanosine caps. The 7-methyl guanosine cap contains an unusual 5′–5′ triphosphate linkage and two methyl groups are added posttranscriptionally. The 7-methyl guanosine caps are recognized by protein factors involved in the initiation of translation and also help by protecting the growing RNA chains from degradation by nucleases.

The 3′ ends of the RNA transcripts are produced by endonucleolytic cleavage of the primary transcripts rather than by the termination of transcription. The transcription termination occurs at multiple sites located 1000 to 2000 nucleotides downstream from the site that will eventually become the 3′ end of the mature transcript. The endonucleolytic cleavage occurs 11 to 30 nucleotides downstream from the conserved consensus sequence AAUAAA, which is located near the end of the transcription unit. Following endonucleolytic cleavage, the enzyme poly(A) polymerase adds about a 200-nucleotide-long poly(A) tail to the 3′ ends of the transcript in a process termed polyadenylation.

E. Structural Motifs (Fig. 2)

The transcription factors are modular in nature and contain characteristic structural motifs. The DNA binding domain as the name implies, binds to the DNA sequences present in the promoters and enhancers while the *trans*-activation domain is responsible for the activation of transcription via protein–protein interactions. The DNA binding domains have characteristic three-dimensional motifs, which result from associations between amino acids present within the polypeptide chains. Thus far, at least five types of DNA binding motifs have been extensively characterized. These include (1) helix–turn–helix (HTH) motif, (2) leucine zipper motif, (3) helix–loop–helix (HLH) motif, (4) zinc-finger motif, and (5) steroid hormone-binding motif.

1. Helix–turn–helix motif (HTH) was first discovered as the DNA-binding domain of phage repressor proteins. It is characterized by a geometric conformation that consists of two α-helical regions separated by a turn of several amino acids, which enable it to bind to DNA. Unlike other DNA binding motifs, HTH cannot function alone, but as part of a larger DNA-binding domain it fits well into the major groove of the DNA. The HTH motif has been identified in a 180-bp sequence called the homeobox, which specifies a 60-amino-acid homeodomain sequence in a large number of eukaryotic transcription factors involved in developmentally regulated genes.

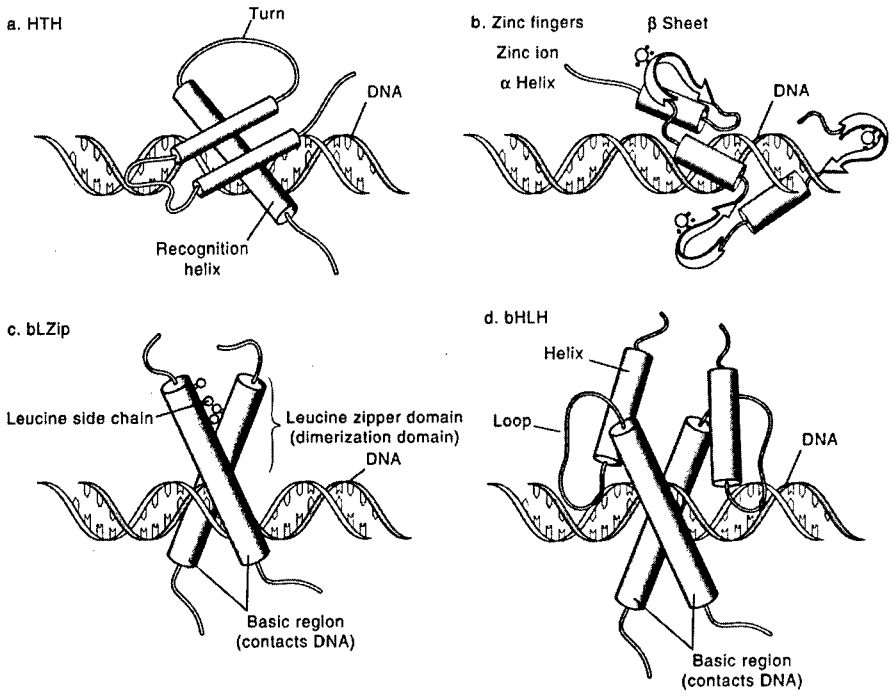

Figure 2 The structural motifs exhibited by transcriptional factors.

2. Leucine zipper motif consists of a stretch of amino acids with a leucine residue in every seventh position. The leucine-rich regions form an α-helix with a leucine residue protruding at every other turn and when two such molecules dimerize, the leucine residues zip together. The dimer contains two alpha-helical regions adjacent to the zipper, which bind to phosphate residues and specific bases in DNA, giving it a scissors-like appearance. The transcription factor AP1 has two major components: Jun and Fos polypeptides encoded by c-*jun* and c-*fos* genes, respectively. Both Jun and Fos contain leucine zippers in their dimerization domains. A Jun leucine zipper can interact with another Jun leucine zipper to form a homodimer or with a Fos leucine zipper to form a heterodimer; however, a Fos leucine zipper is unable to interact with another Fos leucine zipper to form a homodimer. Neither Jun nor Fos alone can bind to DNA and thus in their monomeric forms, they are unable to act as transcription factors. However, Jun–Jun homodimers or Jun–Fos heterodimers are both transcription factors and bind to DNA with the same target specificity but with different affinities. The ability to form homo- or heterodimers greatly increases the repertoire of potential transcription factors a cell can assemble from a limited number of gene products.

3. Helix–loop–helix motif consists of a stretch 40–50 amino acids containing two amphipathic α-helices separated by a 12- to 28-amino-acid long nonhelical loop. The proteins bearing HLH form both homodimers and heterodimers by means of interactions between the hydrophobic residues on the corresponding faces of the two helices. The HLH proteins that contain a stretch of highly basic amino acids adjacent to the HLH motif are termed bHLH proteins. These bHLH proteins are of two types: Class A consists of proteins that are ubiquitously expressed (e.g., mammalian E12/E47), while, Class B consists of proteins that are expressed in a tissue-specific manner (e.g., mammalian MyoD, myogenin, and Myf-5).

4. Zinc-finger motif was first recognized in the *Xenopus* RNA polIII transcription factor TFIIIA. There are several types of zinc-finger proteins, however, the classic zinc-finger consists of about 23 amino acids with a loop of 12 to 14 amino acids between the Cys and His residues and a 7–8-amino-acid linker between the loops. The consensus sequence of a typical zinc finger is Cys-X_{2-4}-Cys-X_3-Phe-X_3-Leu-X_2-His-X_3-His. The interspersed cysteine and histidine residues covalently bind a single zinc ion to form a tetrahedral structure thereby folding the amino acids into loops. The crystal structure analysis of DNA bound by zinc fingers suggests that the C-terminal part of each finger forms α-helices that bind DNA while the N-terminal part forms a β-sheet. Three α-helices fit into one turn of the major groove and each α-helix makes two sequence-specific contacts with DNA. A zinc finger transcription factor may contain anywhere from 2 to 13 zinc fingers. Thus zinc fingers bind to DNA and also control the specificity of dimerization. Therefore, a zinc finger motif offers a novel strategy to design an artificial sequence-specific DNA-binding protein aimed at regulating specific gene expression.

Recent studies indicate that it is possible to engineer zinc finger protein-based gene switches for precise and specific regulation of gene expression. Beerli *et al* [1] have utilized zinc-finger domains to design a polydactyl protein specifically recognizing 9- or 18-bp sequences in the 5′ untranslated region of the erbB-2/HER-2 promoter. They have evaluated the efficacy of gene regulation by converting the polydactyl finger into a transcriptional repressor by fusion with Kruppel-associated box (KRAB), ERF repressor domain (ERD), or mSIN3 interaction domain (SID) repressor domains. Transcriptional activators were generated by fusion with the HSV VP16 activation domain or with a tetrameric repeat of VP16's minimal activation domain, termed VP64. Their results indicate that both gene repression and activation can be achieved by targeting designed proteins to a single site within the transcribed region of a gene. Kang and Kim [2] examined the ability of designer zinc-finger transcription factors to regulate transcription *in vitro* using an ecdysone-inducible system. They constructed a 268/NRE chimeric peptide by linking the three-finger peptide from Zif268, which recognizes the site 5′-GCGTGGGCG-3′, and the three-finger NRE peptide (a variant of the Zif268 peptide), which binds specifically to part

of a nuclear hormone response element 5′-AAGGGTTCA-3′. By incorporating a 19-bp binding site for the 268/NRE near the transcriptional start site in the luciferase reporter vectors >99% repression of activated transcription was observed *in vivo*. Earlier studies have shown that 268/NRE peptide binds to the 19-bp recognition sequence about 6000-fold more tightly than the Zif268 peptide [3]. Imanishi *et al.* [4] utilized zinc fingers to create six-zinc-finger proteins Sp1ZF6$(Gly)_n$ by connecting two DNA-binding domains of transcription factor Sp1 with flexible polyglycine peptide linkers. These peptides were capable of inducing specific DNA bending by binding to two GC boxes and may provide an optimized approach to control gene expression by changing the DNA bending direction.

Corbi *et al.* [5] engineered a novel gene, "Jazz," that encodes for a three-zinc-finger peptide capable of binding the 9-bp DNA sequence 5′-GCTGCTGCG-3′ present in the promoter region of the human and murine utrophin genes. Chimeric transcription factors Gal4-Jazz and Sp1-Jazz were able to drive the expression of luciferase from the human utrophin promoter. Moore *et al.* [6] addressed the issue of zinc-finger DNA-binding specificity by altering the way in which zinc-finger arrays are constructed. Their results suggest that by linking three two-finger domains rather than two three-finger units, far greater target specificity and binding with picomolar affinity can be achieved through increased discrimination against mutated or closely related sequences. Taken together, the overall results suggest the potential utility of zinc-finger-based designer transcription factors in achieving regulation of gene-specific expression in diverse applications including gene therapy, functional genomics, and transgenic organisms.

F. Regulation of Adenoviral DNA Transcription Process

The adenovirus is a double-stranded DNA virus that has evolved to infect a host cell, transport its DNA into the nucleus of the host, replicate its DNA, use the host transcriptional apparatus to produce necessary structural proteins for replication, assemble itself, and destroy the host to release the newly formed infectious particles to perpetuate the process further. This process has been described in detail in Chapter 3.

III. Approaches of Transcriptional Regulation

A. Prior Rationale: Universal Promoters

Several universal promoters have been utilized to attempt to maximize gene expression. The LTR, CMV, and RSV promoters were isolated from Maloney retrovirus, cytomegalovirus, and Rous sarcoma virus, respectively.

These promoter elements were used because of the universal transcriptional activation over a broad host range. This universal transcription allowed for excellent but nondiscriminatory gene transcription and subsequent transgene expression. Because of the high levels of gene expression within several DNA constructs (i.e., viruses, cosmids, plasmids, etc.), these promoters are still used daily throughout the scientific community to test hypotheses which require uniform and high-level gene transcription. These were the promoters utilized in the first wave of gene therapy clinical trials, which focused on maximal gene expression and used local injection techniques to control the region of gene expression achieved. The LTR promoter was used to control herpes simplex virus thymidine kinase (HSV-TK) expression in a retroviral vector by placing retroviral producer cells into residual brain tumors to confer TK expression to the brain tumor, which could lead to conversion of a prodrug and subsequent tumor cell death. The CMV promoter was used in a replication-deficient adenovirus to deliver p53 gene expression after intralesional delivery to patients with both lung and head and neck tumors and is still under clinical investigation. The RSV promoter was employed to express HSV-TK after intralesional delivery in patients with several different tumor types.

B. Current Rationale of Tissue-Specific Promoters

A major challenge facing gene therapy is to generate vectors capable of achieving tissue- or tumor-specific expression. Initial gene therapy strategies utilized universal promoters that demonstrated gene transfer, but were associated with toxicity associated with nonspecific gene transduction (section III.A, above). Tissue-specific promoters offer a novel approach to developing transcriptionally targeted viral vectors with enhanced potential for human gene therapy applications as described below. Several important characteristics are required to develop a tissue/tumor-specific strategy for a particular disease. Fortunately, the recent explosion in our understanding of molecular events that are present in a variety of disease processes has simplified the identification of suitable promoters. Additionally the completion of the genome project and the utilization of microarray technology have enhanced the development of tissue- or tumor-specific promoters by allowing for the identification of novel but specific molecules associated with a particular disease (e.g., cancer). The advancements in molecular cloning techniques (e.g., PCR) has allowed the investigator to extract regulatory sequences from genomic DNA and evaluate each component through site directed mutagenesis analysis in plasmid expression vectors. Additionally, the development of luciferase and green fluorescent protein as well as other quantifiable transgenes has enabled the investigator to test the tissue- or tumor-specific nature of a particular promoter.

To illustrate the concept and utility of a tissue/tumor-specific promoter five such promoters have been selected from Table I. The basic rationale for

Table I
Gene Therapy Applications Of Tissue-Specific Promoters for Transcriptional Targeting

Promoter	Tissue-Specificity	Transgene	Vector	References
AFP	HCC	HSV-TK	Adenoviral	[27, 126, 134]
		CD, IL-2	Adenoviral	[28, 30]
		E1A	Adenoviral	[133]
Albumin	Liver	factor VIII	Adenoviral	[135, 136]
α-Actin	Muscle	GHRH	Nonviral	[137]
α-Lactalbumin	Breast cancer	CD	Adenoviral	[138]
β-Lactoglobulin	Breast cancer	HSV-TK	Adenoviral	[139]
β-Globin	Erythroid cells	β-globin	Retroviral	[140]
c-erbB2	Breast and pancreatic cancer	HSV-TK	Adenoviral	[141, 142]
CEA	Breast, pancreatic, lung, and colorectal carcinoma	HSV-TK, Cre H-ras mutant	Adenoviral	[15, 19, 21]
Egr-1	Radiation induced	TNF-α, LacZ	Adenoviral	[143, 144]
E-Selectin	Tumor endothelium	TNF-α	Retroviral	[145]
Flt-1	Vascular endothelial growth factor receptor type-1	Luciferase	Adenoviral	[146]
GFAP	Glial cells	FasL	Adenoviral	[147, 148]
		TH	Retroviral	
Grp78 (BIP)	Anoxic/acidic tumor tissue	HSV-TK	Adenoviral	[149, 150]
		HSV-TK	Retroviral	
hAAT	Hepatocytes	FactorIX	Nonviral	[151]
HGH and HGPH-α	Pituitary	HSV-TK,	Adenoviral	[152, 153]
HIF-1α/HRE	Hypoxia inducible	Erythropoietin	Nonviral	[154]
hK2	Prostate	EGFP, E1A, E1B	Adenoviral	[132, 155, 156]
HSP	Heat induced	p53, TNF-α	Nonviral	[157]
Hybrid ERE-HRE	Breast Cancer	Harakiri	Adenoviral	[158]
L-Plastin	Epithelial tumors	LacZ	Adenoviral	[159]
MBP	Oligodendrocytes	Caspase 8	Adenoviral	[160]
		GFP	AAV	[161]
MCK	Undifferentiated muscle	LacZ	Adenoviral	[162]
MMTV-LTR	Prostated cancer	antisense c-myc	Retroviral	[163]
MN/CA9	Renal cell carcinoma	E1A	Adenoviral	[164, 165]
MUC1 (DF3)	Breast cancer	E1A	Adenoviral	[166]
		HSV-TK	Retroviral	[167]
Nestin	Glioma, glioblastoma	Cre, LacZ	Adenoviral	[168]

Table I
(continued)

Promoter	Tissue-Specificity	Transgene	Vector	References
NSE	Neurons	FasL,	Adenoviral	[169]
		BDNF	AAV	[170]
Osteocalcin	Osteosarcoma	HSV-TK	Adenoviral	[77, 79, 171–173]
	Prostate	HSV-TK	Adenoviral	[174–176]
		E1A	Adenoviral	[177]
PEPCK	Hepatocytes	Insulin	Adenoviral	[178]
PSA	Prostate	Nitroreductase	Adenoviral	[46, 48, 49]
		HSV-TK, PNP	Adenoviral	
Preproenkephalin	CNS	LacZ	HSV	[179]
Probasin	Prostate	E1A	Adenoviral	[156, 180]
		Caspase 9		
Prolactin	Pituitary lactotrophic cells	HSV-TK	Adenoviral	[181]
SLPI	Ovarian, cervical carcinoma	HSV-TK	Nonviral	[182]
SM22α	Smooth muscle cells	LacZ	Adenoviral	[183]
Surfactant protein C	Respiratory epithelium	HSV-TK	Adenoviral	[184]
Tyrosinase	Melanocytes	Luc, PNP	Nonviral	[185, 186]
		GALV-FMG	Retroviral	
Tyrosine hydroxylase	Sympathetic nervous system	LacZ	HSV	[122]

selection, *in vitro* and *in vivo* laboratory investigation and the clinical testing associated with each, will be briefly reviewed below.

1. Carcinoembryonic Antigen (CEA) Promoter

a. Rationale Carcinoembryonic antigen is a 180-kDa cell surface glycoprotein overexpressed in 90% of gastrointestinal malignancies, including colon, gastric, rectal, and pancreatic tumors, 70% of lung cancers, and about 50% of breast cancers [7]. Thompson *et al.* [8] initially reported on the molecular cloning of the CEA gene from a human genomic library. Subsequently, Schrewe *et al.* [9] also isolated and characterized a cosmid clone containing the entire coding region of the CEA gene including its promoter. The CEA promoter region encompasses 400 bp upstream of the translational start site and is known to confer tissue-specific CEA expression. Hauck and Stanners [10] demonstrated that the CEA promoter region located between −403 and −124 bp upstream of the translational initiation site is capable of directing high levels of gene expression in CEA-expressing human colon cancer CRC cells. Chen *et al.* [11] showed the CEA promoter region to lie between −123 and −28 bp upstream from the transcriptional start site and have demonstrated

the presence of SP1 and upstream stimulatory factor binding sites. According to Richards *et al.* [12] the CEA promoter is located between −90 and +69 bp upstream from the transcriptional start site and the essential sequences of the CEA promoter reside between −90 and −17 bp upstream of the transcriptional start site of the CEA gene. Cao *et al.* [13] compared the CEA core promoter regions between −135 and +69 bp isolated from human colorectal carcinoma and normal adjacent mucosa and found that both the sequences were identical and without any mutations. Taking advantage of this fact, various studies have suggested the potential utility of the CEA promoter for restricted expression of heterologous genes (14, 10, 12).

b. In Vitro and in Vivo Experiments with CEA Promoter Takeuichi *et al.* [15] demonstrated that an adenoviral vector encoding a CEA promoter-driven N116Y dominant-negative H-Ras mutant was capable of suppressing liver metastasis by the human pancreatic cancer cell line PCI-43 in a nude mice model. Lan *et al.* [16, 17] demonstrated successful adenoviral-mediated transduction of *E. coli* cytosine deaminase (CD) *in vitro* as well as in an immunodeficient *in vivo* model of MKN45 gastric carcinoma. As compared to an adenoviral vector in which CD expression is driven by the constitutive CAG promoter, the expression of CD under the control of CEA promoter was confined to tumor xenografts. However, the reduction in tumor burden by AdCEA-CD/5-fluoro-cytosine (5FC), although significant, was not as great as that induced by AdCAG-CD/5FC. In fact, the CEA promoter was shown to be 200 times less active than the CAG promoter.

Similar results have been described in mice bearing xenografts that were transfected with CEA-CD constructs and subsequently treated with 5-FC (18, and 12). Tanaka and colleagues [19] have used the CEA promoter sequence located between −424 and −2 bp upstream of translational start site to generate an adenoviral vector expressing HSV-TK and examined its efficacy in killing CEA-producing cancer cells *in vitro* and *in vivo*. By employing intratumoral Ad-CEA-TK injection and gancyclovir (GCV) administration, the growth of the tumors was inhibited by 20% as compared to untreated tumors. Brand *et al.* [20] have used the CEA promoter (−296 to +102 bp with respect to transcriptional start site) to drive the expression of HSV-TK in an adenoviral vector. Their results indicate that the CEA promoter was active in several human and rat tumor-derived cell lines but not in rat primary hepatocytes and in mouse liver, while the CMV promoter was highly active in all cell types. Although the CEA promoter-driven TK expression was less, it was sufficient to kill 100% of cancer cells, indicating a significant bystander effect. Treatment of subcutaneous tumors in SCID mice with Ad-CEA-TK was able to significantly reduce tumor growth and the tail vein injection of a high dose of this virus caused no side-effects in the liver.

Kijima *et al.* [21] have utilized a novel Cre-lox-based strategy to achieve enhanced antitumor effect against CEA-producing human lung and colon

cancer cell lines. Their strategy involved generation of two recombinant adenoviral vectors: one expressing the Cre recombinase gene under the control of the CEA promoter while the second adenoviral vector is designed to express HSV-TK gene from the CAG promoter only after Cre excises the neomycin resistance gene (inserted between the CAG promoter and HSV-TK) in a loxP site-specific manner. (Cre recombinase derived from bacteriophage P1 mediates site-specific excisional deletion of a DNA sequence that is flanked by a pair of loxP sites composed of 34 nucleotides.) This novel approach requires simultaneous infection of a cell by the two adenoviral vectors. Using this approach, a CEA-producing human cancer cell line was rendered 8.4-fold more sensitive to GCV than infection by Ad-CEA-TK alone. Intratumoral injection of Ad-CEA-Cre along with Ad-lox-TK followed by GCV treatment almost completely eradicated CEA-producing tumors in an athymic subcutaneous tumor model, whereas intratumoral injection of Ad-CEA-TK with GCV treatment showed reduced tumor growth.

2. α-Fetoprotein (AFP) Promoter

a. Rationale The human AFP gene is developmentally regulated and is expressed at high levels in the fetal liver but its transcription declines rapidly after birth and is barely detectable in adult life [22, 23]. However, overexpression of the AFP gene is a characteristic feature of human hepatocellular carcinoma. The human AFP gene is about 20 kb long and contains 15 exons and 14 introns [24]. The cap site is located 44 nucleotides upstream of the translation initiation site and the TATA box is located 27 nucleotides upstream from the cap site and is flanked by sequences with dyad symmetry. Other sequences in the 5′ untranslated region include a CCAAC pentamer, a 14-bp enhancer-like sequence, a 9-bp sequence homologous to the glucocorticoid responsive element, a 90-bp direct repeat, and several alternating purine/pyrimidine sequences.

The AFP promoter is 200 bp upstream of the transcriptional start site. It is regulated by hepatocyte nuclear factor 1 (HNF1), nuclear factor 1 (NF1), and CCAAT/enhancer binding protein (C/EBP). The human AFP enhancer is located between −4.9 and −3.0 kb upstream of the transcriptional start site and consists of at least two functional domains designated A and B which have binding sites for at least four transcription factors, including HNF1, HNF3, HNF4, and C/EBP. The domain B is located at −3.7 to −3.3 kb upstream of the transcriptional start site and is solely responsible for typical enhancer effects, but maximum enhancer activity is observed together with domain A located at −5.1 to −3.7 kb. A hepatoma-specific nuclear factor termed AFP1 is known to bind to an AT-rich sequence, TGATTAATAATTACA, in the B domain of the human AFP enhancer. The AFP enhancer plays a critical role in enhancing AFP gene expression in the fetal liver as well as in hepatocellular carcinoma. The AFP silencer, which is a negative *cis*-acting element with a consensus sequence, 5′-CTTCATAACCTAATACTT- 3′, has been identified [25]. Two

transcriptional silencer elements have been identified: the proximal silencer which contains a single copy of the consensus sequence at −0.31 kb and the distal silencer at −1.75 kb which carries four copies of the consensus sequence. Of the two silencers, the distal silencer, exhibits a higher suppressive activity than the proximal silencer. The silencer activity is manifested only when the silencer is located downstream of the enhancer and upstream of the promoter. An inverse correlation exists between the silencer activity and the AFP expression levels in hepatocellular carcinoma cell lines, thereby suggesting the role of the silencer in downregulating the level of AFP expression.

b. In Vitro and in Vivo Experiments with the AFP Promoter Because of its tissue-specific nature, the AFP promoter has been used in adenoviral vectors for transcriptional targeting of suicide genes in AFP-producing hepatocellular carcinoma (HCC) cells *in vitro* as well as *in vivo*. Kaneko *et al.* [26] developed adenoviral vectors using either the 4.9-kb AFP promoter (Av1AFPTK1) or RSV promoter (Av1TK1) to express HSV-TK gene. *In vitro* and *in vivo* cell-specific killing was observed in AFP-producing HuH7 hepatocellular carcinoma cells transduced with Av1AFPTK1 and treated with GCV. In contrast to HuH7 tumors, AFP-nonproducing hepatocellular carcinoma SK-Hep-1 cells did not show complete regression when treated with Av1AFPTK1. Av1TK1 was able to cause complete regression in SK-Hep-1 tumors. Using a similar approach, Kanai *et al.* [27] developed adenoviral vectors by incorporating AFP enhancer domains A and B (−4.0 to −3.3 kb) and a 0.17-kb AFP promoter to drive the expression of HSV-TK. These vectors conferred cell-specific killing in AFP-producing HuH-7 and HepG2 cell lines but not in non-AFP-producing HLE and HLF cell lines. Kanai *et al.* [28] have also reported on the development of adenoviral vectors in which the expression of *E. coli* CD is driven by the AFP promoter. These vectors were capable of causing regression of HCC xenografts following treatment with 5FC. Arbuthnot *et al.* [29] analyzed *in vitro* and *in vivo* cell-specific expression of the nuclear β-galactosidase using adenoviral vectors containing transcriptional elements derived from either rat AFP or the human insulin-like growth factor II genes. Their results indicate hepatoma cell-specific expression using AFP promoter; however, primary hepatoma cells were poorly infected by these adenoviral vectors. Bui *et al.* [30] compared adenoviral vector-mediated expression of IL-2 under the transcriptional control of murine AFP promoter and CMV promoters for the treatment of established human hepatocellular xenografts in CB-17/SCID mice. Intratumoral injection of these adenoviral vectors resulted in growth retardation and regression in a majority of animals but with a wider therapeutic index and less systemic toxicity using the AFP vector. Using the AFP promoter and cre-lox based approach Sato *et al.* [31] were able to achieve strictly tissue-specific expression of LacZ in AFP-producing cells *in vitro* as well as *in vivo* in nude mice bearing AFP-producing tumor xenografts.

3. Prostate-Specific Antigen (PSA) Promoter

a. Rationale The gene for prostate-specific antigen, a member of the glandular kallikrein family, was independently characterized by Riegman *et al.* [32, 33] and Lundwall [34] from a human genomic library. The gene contains five exons and is located on the long arm of chromosome 19, in the region *q*13.3–*q*ter [33]. The gene is 7130 bp long and includes 633 bp of 5′ and 639 bp of 3′ flanking sequence. The promoter region contains a variant TATA box (TTTATA) at position −28 to −23, a GC box at −53 to −48, a CACCC box at −129 to −125. An imperfect palindromic sequence (AGAACAGCAAGTGCT) closely related to the reverse complement of the consensus sequence for steroid hormone receptor binding (TGTACANNNTGTC/TCT), is found at position −170 to −156. In addition, GGGAGGG and CAGCCTC repeats are located in the region −123 to −72. Expression of PSA is primarily detected in human prostate [35–37]. Further, PSA expression has been shown to be androgen-responsive [38]. This is achieved by several transcription factors that are involved in regulating prostate-specific antigen gene.

Two functionally active androgen receptor-binding sites or androgen response elements (AREs) have been identified at positions −170 (ARE-I) and −394 (ARE-II) [38–41]. Cleutjens *et al.* have identified a complex, androgen-regulated 440-bp enhancer (−4366 to −3874) which contains a high-affinity AR-binding site, ARE-III (5′-GGAGGAACATATTGTATCGAT-3′), at position −4200. In subsequent studies, a 6-kb PSA promoter fragment has been shown to confer prostate-specific and androgen-regulated expression of β-galactosidase in transgenic mice [42]. Pang *et al.* [43] identified an 822-bp PSA gene regulatory sequence, PSAR which when combined with the PSA promoter (PCPSA-P) exhibited an enhanced luciferase activity in LNCaP cells. Upon stimulation with 10 to 100 nM dihydrotestosterone, a more than 1000-fold increase in expression was observed as compared to androgen-negative controls. Their studies further suggest that this 822-bp sequence alone could serve as a promoter, thereby indicating that the complete PSA promoter contains two functional domains: a proximal promoter and a distal promoter, which can also function as an enhancer.

Yeung *et al.* [44] have identified two *cis*-acting elements within the 5.8-kb PSA promoter that are essential for the androgen-independent activity of the PSA promoter in prostate cancer cells. Their studies provide evidence that androgen-independent activation of the PSA promoter in the androgen-independent prostate cancer cell line C4-2 involves two distinct regions, a 440-bp AREc and a 150-bp pN/H, which are responsible for upregulation of the PSA promoter activity by employing two different pathways. AREc confers high basal PSA promoter activity in C4-2 cells, while pN/H is a strong AR-independent positive-regulatory element of the PSA promoter in both LNCaP and C4-2 cells. Further, a 17-bp RI fragment within the pN/H region was identified as the key *cis* element, which interacts with a 45-kDa

prostate cancer cell-specific transcription factor to mediate androgen- and AR-independent transcriptional activation of the PSA promoter. By juxtaposing AREc and pN/H, a chimeric PSA promoter has been created that exhibits 2- to 3-fold higher activity than wild-type PSA promoter in both LNCaP and C4-2 cells. Oettgen *et al.* [45] have identified a novel prostate epithelial-specific Ets transcription factor, PDEF, that is involved in PSA gene regulation and acts as a coregulator of AR. PDEF acts as an androgen-independent transcriptional activator of the PSA promoter. It also directly interacts with the DNA-binding domain of AR and enhances androgen-mediated activation of the PSA promoter. Thus, strong tissue-specificity of the PSA promoter makes it an ideal candidate for prostate cancer gene therapy. Latham [46] compared tissue-specific expression of luciferase reporter vectors by employing PSA, human glandular kallikrein (hKLK2), and CMV promoters in PSA-positive LNCaP and PSA-negative CoLo320, DG75, A2780, and Jurkat cells. Their studies revealed that minimal 628-bp PSA and hKLK2 promoters showed only low-level androgen-independent expression in both PSA-positive and PSA-negative cell lines. Tandem duplication of the PSA promoter slightly increased expression in LNCaP cells. Addition of the CMV enhancer upstream of the PSA or hKLK2 promoter led to substantially enhanced and nonspecific luciferase expression in all the cell lines. By placing a 1455-bp PSA enhancer sequence upstream of either the PSA or the hKLK2 promoter, a 20-fold increase in tissue-specific luciferase expression was observed. Tandem duplication of the PSA enhancer increased the expression 50-fold higher than either promoter while retaining tissue specificity. The expression from all the enhancer constructs was 100-fold above the basal levels upon induction with androgen dihydrotestosterone.

b. In Vitro and in Vivo Experiments with the PSA Promoter These enhancer sequences were incorporated in adenoviral vectors to express enhanced green fluorescent protein (EGFP) and nitroreductase. The results indicate low-level expression of EGFP by PSA enhancer promoter in LNCaP cells and no expression in non-PSA-producing EJ cells when compared with CMV promoter-driven EGFP. However, the PSA enhancer promoter was able to direct expression of comparable levels of nitroreductase in a tissue-specific manner in LNCaP cells alone. These transduced LNCaP cells upon treatment with CB1954 exhibited cytotoxicity. A replication-competent adenoviral vector CN706 in which the E1A gene is under the transcriptional control of the PSA enhancer/promoter has been shown to exhibit selective toxicity toward PSA-expressing prostate cancer cells [47]. Martinello-Wilks *et al.* [48] examined the efficacy of adenoviral vectors with a 630-bp PSA promoter-driven HSV-TK and *E. coli* purine nucleoside phosphorylase (PNP) genes for their ability to kill androgen-insensitive prostate cancer cell line PC-3 tumor xenografts in a nude mouse model. Both HSV-TK and *E. coli* PNP-expressing adenoviral vectors were able to achieve significant tumor regression *in vivo* following

GCV or 6MPDR treatment. Gotoh *et al.* [49] developed transcriptionally targeted recombinant adenoviral vectors by incorporating either 5837-bp long or 642-bp short PSA promoter elements to drive the expression of HSV-TK. The long PSA promoter was shown to have superior activity over the short promoter and was more active in C4-2 cells than in LNCaP cells. *In vitro* expression of TK conferred marked killing of C4-2 cells upon acyclovir treatment. Administration of this virus in an *in vivo* subcutaneous C4-2 tumor model, followed by acyclovir treatment, revealed significant inhibition of tumor burden. Lee *et al.* [50] demonstrated tissue-specific growth suppression of PSA-positive and -negative cell lines by transfecting PSA promoter enhancer-driven p53 tumor-suppressor genes. Recently, human prostate cancer- and tissue-specific genes P503, P540S, and P510S have been identified using a combination of cDNA library subtraction and high-throughput microarray screening by Xu *et al.* [51]. It would be interesting to characterize the promoter region of these genes and use it in developing transcriptionally targeted adenoviral vectors.

4. Osteocalcin (OC) Promoter (Fig. 3)

a. Rationale Osteocalcin (bone γ-carboxyglutamic acid (Gla)-containing protein (BGP)) is a 50-amino-acid, 5.8-kDa, major noncollagenous protein found in adult bone and has been shown to be transcriptionally regulated by 1,25-dihydroxyvitamin D_3 [52, 53]. The human, rat, and murine osteocalcin genes have been cloned and each consists of four exons and three introns [54–57]. Montecino [58] reported that the key promoter elements are located in two DNase I-hypersensitive sites. The proximal hypersensitive site (−170 to −70) includes sequence motifs that specifically interact with basal transcription factors such as Msx [59–61], HLH protein Id-1 [62], AP-1 [63], a bone-specific nuclear-matrix-associated protein, NMP-2 [64],

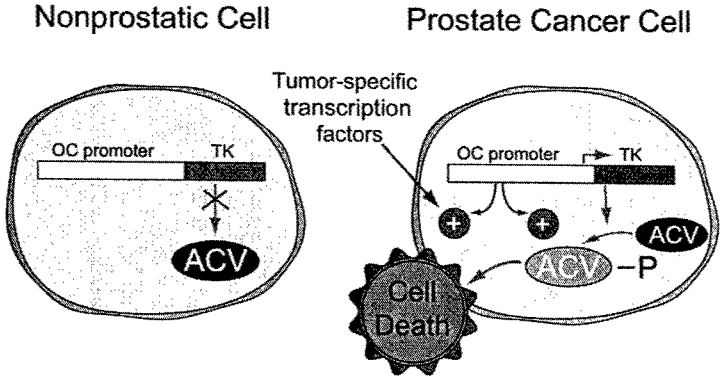

Figure 3 The specific ability of a tissue specific promoter such as the osteocalcin promoter to produce HSV-TK in a prostate cancer allowing cell death on prodrug (ACV) administration while sparing nonprostate cell by not allowing osteocalcin promoter activation.

and a member of the AML family of transcription factors [65, 66, 71]. The distal hypersensitive domain (−600 to −400) contains the vitamin D-responsive element (VDRE, −465 to −437), which interacts with the VDR-RXRα complex in a ligand-dependent manner [67–69]. Montecino *et al.* [70] have demonstrated that the promoter segment −343 to −108 is critical for inducing both proximal nuclease hypersensitivity and basal transcriptional activity and the DNase I hypersensitivity at −600 is not essential for vitamin D-dependent transcriptional upregulation. Two additional NMP-2 sites (site A, −604 to −599; site B, −440 to −435) have been identified in the sequences flanking the distal DNase I-hypersensitive domain that might support specific interactions between the nuclear matrix and the OC gene promoter [64, 71]. Analysis of the 5′ flanking sequence of rat osteocalcin gene reveals a modular organization of the promoter consisting of the TATAAAA sequence between −31 and −25 and the CCAAT sequence between −92 and −88 [72]. Lian *et al.* [55] identified a 24-nucleotide regulatory sequence, 5′-ATGACCCCCAATTAGTCCTGGCAG-3′, in the proximal promoter region with a CAAT motif as a central element, and have designated this sequence as an osteocalcin (OC) box since only two nucleotide substitutions are found in the rat and human osteocalcin genes in this region. Hoffman *et al.* [59] reported that the OC box is located at nucleotide positions between −99 and −76 and TATA box containing a consensus glucocorticoid-responsive element (GRE) between −44 and −31. The stimulation of osteocalcin gene expression by 1,25-dihydroxyvitamin D_3 is associated with sequence-specific binding of nuclear factors to a 26-bp sequence, 5′-CTGGGTGAATGAGGACATTACTGACC-3′, located between −462 and −437. This sequence contains a region of hyphenated dyad symmetry and shares homology with consensus steroid-responsive elements.The promoter region has been shown to contain two sites of an E-box motif (a consensus binding site for HLH proteins) termed OCE1 (CACATG at −102) and OCE2 (CAGCTG at −149) [62]. Mutagenesis studies have indicated that osteoblastic-specific gene transcription is regulated via the interaction between certain E-box binding transcription factors in osteoblasts and the OCE1 sequence in the promoter region of the osteocalcin gene. Banerjee *et al.* [63] demonstrated that an AML-1 binding sequence within the proximal promoter (nt −138 to −130) contributes to 75% of the level of osteocalcin gene expression. The promoter region is not GC-rich and does not contain a consensus sequence for the SP1 binding site [73]. Theofan *et al.* [74] performed a detailed analysis of the BGP promoter region. Three regulatory elements that share partial homology with the consensus sequence for the GRE have been identified at nucleotide positions −356, −178, and −68, respectively. In addition, two sequences related to the consensus sequence for the metal ion-responsive element (MRE) have been identified at positions −190 and −143. An octanucleotide sequence, TGCAGTCA, is located directly adjacent 3′ to the second MRE. Two other sequences that share homology

with the cAMP-responsive element are found at −437 (TGAGGACA) and −392 (TCACGGCA). The BGP promoter region also contains several pairs of inverted repeat sequences that form regions of dyad symmetry. Three particularly long regions of imperfect dyad symmetry are located between −523 and −504, −234 and −214, and −51 and −28. An octanucleotide palindromic sequence from −134 to −127 partially overlaps both a putative MRE and a cAMP-responsive element. A short sequence, GCAG, or its complement, CTGC, is repeated 17 times. A region of alternating purines and pyrimidines at location −90 to −81 from the CAT box has the potential to form a Z-DNA structure which may be important in gene regulation. A 7-bp osteocalcin silencer element, 5′-TGGCCCT-3′, has been located between +29 and +35 position in the first exon of the human osteocalcin gene, while two silencer elements, 5′-CCTCCT-3′ (nt +106 to +111 and +135 to +140) and 5′-TTTCTTT-3′ (nt +118 to +124), have been located in the first intron of the rat osteocalcin gene [75, 76].

b. In Vitro and in Vivo Experiments with the OC Promoter Ko *et al.* [77] developed an osteocalcin-promoter-driven TK-expressing recombinant adenoviral vector to achieve tissue-specific killing of osteosarcoma cells in experimental animal model. Administration of this vector followed by acyclovir treatment led to a significant growth inhibition of osteosarcoma in an experimental animal model. Cheon *et al.* [78] used a chemogene therapy approach by combining OC-promoter-driven TK expression and acyclovir with a methotrexate treatment regimen in nude mice bearing either subcutaneous human osteosarcoma (MG-63) or rat osteosarcoma (ROS) xenografts. Their results indicate that osteosarcoma tumor growth was more efficiently inhibited due to synergistic effects of combined methotrexate and acyclovir treatment. Shirakawa *et al.* [79] further demonstrated the potential utility of an adenoviral osteocalcin promoter-mediated suicide gene therapy for osteosarcoma pulmonary metastasis in nude mice. Hou *et al.* [80] demonstrated osteoblast-specific gene expression in adherent bone marrow cells using a 1.7-kb rat OC-CAT gene. Recipient mice were shown to be positive for osteoblast-specific expression following bone marrow transplantation.

Using a replication-defective adenovirus, Ad-OC-TK, we have completed a phase I clinical trial that demonstrated the expected safety profile and gene transfer that we expected. Eleven men with recurrent or metastatic prostate cancer were enrolled in a phase I intralesional dose-escalating trial, combining two Ad-OC-TK injections with 3 weeks of valacyclovir administration. In summary, this was well tolerated at all doses reaching a maximum of 5×10^{10} pfu (or 1×10^{12} vp) in patients in the high-dose group. Viral distribution studies revealed that after intralesional administration the patients demonstrated a measurable viremia for 2–3 days. Despite the presence of viral particles at these time points, no patient demonstrated hepatotoxity with valacyclovir administration. This is in direct contrast to intralesional delivery of Ad-RSV-TK

to the prostatic recurrence, in which patients experience hepatotoxicity upon prodrug administration. Finally, comparison of biopsy specimens prior to the first (day 0) and second (day 7) injection and at the end of the study (day 30) revealed successful gene transfer at day 7 by immunohistochemical staining for HSV-TK and some evidence of tumor destruction by day 30. These expected and encouraging results have led us to propose a phase I trial to test the transcriptional ability of the osteocalcin promoter to regulate adenoviral replication in a similar format.

5. MN/CA9 Promoter

a. Rationale The human MN/CA9 gene has been isolated, sequenced, and characterized by Opavsky [81]. This gene is a member of the carbonic anhydrase (CA) family, which codes for a diverse group of catalysts of the reversible conversion of carbon dioxide to carbonic acid. MN/CA9 expression has been detected in several types of carcinomas including renal, ovarian, and cervical, as well as in normal gastric mucosa [82–85]. The complete genomic sequence of the MN/CA9 gene including the 5′-flanking region encompasses 10.9 kb with a coding sequence comprising of 11 exons. The MN/CA9 protein contains 459 amino acids with a molecular weight ranging from 54 to 58 kDa. MN displays CA activity and binds zinc [86]. The nucleotide sequence close to the 5′ end shows 91.4% sequence homology to the U3 region of the long terminal repeats (LTRs) of the human HERV-K endogenous retroviruses [87]. This LTR-like sequence is 222 bp long with an A-rich tail at its 3′ end. Analysis of the MN/CA9 promoter region between −507 and +1 upstream of the transcription initiation site indicates that despite the presence of 60% GC residues, the additional features of TATA-less promoters are absent, but the presence of consensus sequences for AP1, AP2, and p53 transcription factor binding sites has been demonstrated [88–90]. Functional characterization of the 3.5-kb MN 5′ upstream region by deletion analysis led to the identification of −173 to +31 fragment as the MN promoter. The promoter region lacks the CpG-rich islands that are typical for TATA-less promoters but contains two nonoverlapping consensus initiator sequences required for promoter activity.

b. In Vitro and in Vivo Experiences with MN Initial *in vitro* studies with this promoter driving luciferase expression demonstrated tumor specificity for both renal cell carcinoma and cervical carcinoma. Based on the expression assays, we have constructed an oncolytic adenovirus with the MN promoter which has demonstrated 40- to 100-fold increased killing in human renal cell carcinomas compared to control cell lines not expressing this promoter activity. We are currently evaluating this oncolytic vector in animal models of human renal cell carcinoma.

C. Inducible Transcription

The ability to precisely regulate spatial and temporal expression of a particular gene is likely to have a significant impact in the field of human gene therapy. In order to be effective, such an approach must necessarily fulfill several criteria, including: (1) biological safety, (2) ease of administration, (3) low basal expression, (4) high and gene-specific inducibility, (5) reversibility, and (6) (preferably) of human origin to minimize immunogenicity. A wide variety of inducible systems for regulating gene expression have been developed. These include the use of metal response promoter [91], heat-shock promoter [92], the glucocorticoid-inducible promoter [93], IPTG-inducible lac repressor/operator system [94, 95], tetracycline-inducible system [96], RU486-inducible system [97], ecdysone-inducible system [100], FK506/rapamycin-inducible system [101], hypoxia-inducible factor 1 system [102], radiation-inducible system [103], and the tamoxifen-inducible system [104]. It is beyond the scope of this chapter to provide in-depth information on all of the above-mentioned inducible systems. Consequently, we will focus on those inducible systems that might have the greatest potential for human gene therapy applications.

1. Tetracycline-Inducible System

The tet-inducible system originally developed by Bujard and coworkers [94, 105] is widely used to regulate gene expression. The tet-inducible system is based on the tetracycline resistance operon of *E. coli*. The system utilizes the specificity of the tet repressor (tetR) for the tet operator sequence (tetO), the sensitivity of tetR to tetracycline, and the potent transactivator function of herpes simplex virus protein VP16. The system is based upon the concept of negatively regulating the transcription of the bacterial resistance gene by tetR protein binding to tetO DNA sequences. Addition of tetracycline or doxycycline causes derepression by binding to the tetR protein, thereby allowing transcription to proceed. This has been achieved by employing a tet transactivator (tTA) which is a chimeric tetracycline-repressed transactivator generated by fusing the carboxy terminal of tetR protein to the carboxy terminal 127 amino acids of VP16. The tTA, when bound to tetracycline, is prevented from binding to seven copies of tetO sequences, which are juxtaposed upstream of a minimal human cytomegalovirus promoter, thereby selectively turning off the transcription of the gene in question. Removal of tetracycline results in binding of tTA to the tetO sequences in the tet-inducible promoter, following which the VP16 moiety of tTA transactivates the target gene by promoting assembly of a transcriptional initiation complex, thereby selectively turning on the gene expression. A recent modification of this system allows for selective induction of gene expression in the presence of tetracycline. In this strategy, a mutated tetR, called reverse tTA (rtTA), has been generated by incorporating 4 amino acid changes into tTA, thereby facilitating rtTA

binding to the tetO sequence in the presence of tetracycline. Another variation involves fusion of tTA with the KRAB repressor domain of the human zinc-finger protein Kox1. Upon binding to tetO sequences, this protein is capable of blocking transcription as far as 3 kb downstream [106]. A further variation has revealed that by placing two minimal promoters in opposite orientations on either side of the tetO sequences, it is possible to simultaneously regulate the expression of two genes from a single plasmid [107]. Massie *et al.* [108] used the tet-inducible system to generate a recombinant adenoviral vector encoding a deletion in the R1 subunit of the herpes simplex virus type 2 ribonucleotide reductase. Topical and tetracycline-inducible gene expression in transgenic mice carrying a gene under the tet-inducible promoter has been achieved by adenovirus mediated gene transfer and expression of tTA [109]. Rubinchik *et al.* [110] developed a tet-inducible, double recombinant adenoviral vector expressing a fusion of murine FasL and green fluorescent protein. In this virus, the tet-responsive element and the transactivator element are built into opposite ends of the same vector to avoid enhancer interference. The *in vitro* expression of FasL-GFP in various cell lines could be conveniently regulated by tetracycline or doxycycline in a dose-dependent manner.

2. FK506/Rapamycin-Inducible System

The latest in the armamentarium of inducible gene expression systems are the chemical dimerizers that rely upon drug-dependent recruitment of a transactivation domain to a basal promoter to drive the expression of the therapeutic gene. The strategy is based upon generating a genetic fusion composed of a heterologous DNA-binding domain and an activation domain with the drug binding domain, thereby enabling a bivalent drug to crosslink the two proteins and reconstitute an active transcription factor. This is achieved by using small cell-permeable immunosuppressive molecules, FK506, rapamycin, and cyclosporine, to bind members of the immunophilin family. The FK506 molecule binds tightly to the cellular protein, FKBP12, while FK1012, a synthetic dimer of FK506, causes dimerization of several chimeric proteins containing FKBP12 [111]. Another synthetic compound, FKCsA, created by fusion between FK506 and cyclosporine A, binds with high affinity to FKBP12 and cyclophilin and has been used for inducible transcription of exogenous genes [112]. However, the most promising results have been obtained using the heterodimerizer rapamycin, which binds simultaneously to the human proteins FKBP and FRAP [113, 114]. In this system, transcriptional activation is achieved through rapamycin induced reconstitution of a transcription factor complex formed by coupling of (a) a unique DNA-binding domain, ZFHD, genetically fused to FKBP and (b) the activation domain of the p65 subunit of nuclear factor kappa B (NFκB), fused with the rapamycin-binding domain of FRAP. This novel approach has been successfully utilized for stable *in vivo* delivery of secreted alkaline phosphate, murine erythropoietin

and human growth hormone using eukaryotic expression vectors, adenoviral, retroviral, and adeno-associated viral vectors [115–117]. One of the limitations of this approach is the growth inhibitory and immunosuppressive activity of rapamycin which is due to the inhibition of endogenous FRAP activity [114]. This limitation can be overcome by nonimmunosuppressive analogs (rapalogs) of rapamycin by incorporating mutations in the FRAP domain that accommodate modified drugs [118, 119]. Considerable progress has also been made in designing novel synthetic dimerizers of the ligand for human FKBP12 and mutated FKBP [119–121]. These studies are suggestive of the potential utility of this novel approach for human gene therapy applications.

3. RU 486

Wang *et al.* [97] developed a novel regulated transcriptional activator consisting of a truncated ligand-binding domain of the human progesterone receptor, the DNA-binding domain of yeast transcriptional activator GAL4, and a C-terminal fragment of the herpes simplex virus VP16 transcriptional regulator protein. This novel transcriptional activator binds with high affinity to the synthetic progesterone antagonist RU 486 but binds very poorly to progesterone. In conjunction with the target gene containing four copies of the consensus GAL4 binding site, the gene expression was activated only in the presence of RU 486 [97, 98]. Wang *et al.* [99] also developed an inducible repressor system by substituting the KRAB transcriptional repressor domain for the VP16 transactivation domain. In addition to RU 486, this system can be activated by other synthetic progesterone antagonists at low concentration. The efficacy of this system has been demonstrated using an *ex vivo* transplantation approach in which cells containing stably integrated chimeric regulator GLVP and a target gene (tyrosine hydroxylase) were grafted in rats. One of the caveats of this system is the low but distinctive basal activity of the GAL4-responsive promoter in the absence of RU 486. Consequently, this system has been refined by designing a synthetic transcription factor which contains a 35-amino-acid truncation of the progesterone receptor rather than the 42-amino-acid truncation [123]. This system exhibits two- to threefold lower basal activities as compared to the earlier version.

IV. Enhanced Control of Transgene Expression

A. Safety Improvements

Prior to initiating our clinical trial with Ad-OC-TK, we performed a distribution study that measured TK activity in a variety of organs harvested 3 days after intravenous (iv) injection of Ad-CMV-TK (2×10^9 pfu) or

Ad-OC-TK (2×10^9 pfu) with three mice per group. TK enzymatic activity was detected only in the AdCMV-TK group (liver and spleen only), but not the Ad-OC-TK group. Next we performed a comparative study in which 10 C57/BL mice received one iv injection of 2×10^9 pfu of Ad-OC-TK or Ad-CMV-TK and intraperitoneal (ip) GCV. Significant mortality with severe hepatic histopathology was observed in the Ad-CMV-TK/GCV group (90% mortality), while the Ad-OC-TK/GCV administration did not affect survival of the treated animals (100% survival). These data and the above tissue distribution studies support the hypothesis that, in syngeneic hosts, the OC promoter is tissue-specific for tumors, since Ad-OC-TK inhibits tumor growth as effectively as do RSV-TK and CMV-TK, but without the generalized toxicity observed with these universal promoters. These findings paralleled the formal GLP toxicology study in mice and our toxicology profile in our clinical trial. Others have demonstrated the lethal effects of both universal promoter HSV-TK viruses in mice and rats and hepatotoxicity in humans after intraprostatic injections.

B. Potency Concerns

The initial concern with a tumor-specific promoter is that the magnitude of the transgene expression would decrease because of the specificity of the promoter. To address this issue, we compared the *in vivo* growth inhibition associated with intralesional administration of Ad-OC-TK with that of Ad-CMV-TK using a rat osteosarcoma (ROS 17/2.8) subcutaneous model. Ten athymic nude mice were injected with 1×10^6 ROS cells per site in four subcutaneous locations. After establishment of tumor growth at greater than 5 mm diameter, Ad-CMV-TK or Ad-OC-TK were injected intralesionally into five animals (or 20 tumors) each. After viral injection, the animals received ip GCV (three mice, 12 tumors) or phosphate-buffered saline (PBS; two mice, 8 tumors) for a 2-week period. The animals received one additional adenoviral injection 7 days after the first. The tumors were measured weekly and the animals were sacrificed after the second week of GCV or PBS administration. Both Ad-OC-TK and Ad-CMV-TK forms of therapy demonstrated a greater growth-inhibitory effect than was observed with PBS administration. The growth inhibition was superior with the Ad-OC-TK adenovirus. Therefore, the OC promoter has high intrinsic activity rivaling that of the strong universal CMV promoter, at least in ROS cells.

V. Future Directions

A. Enhancement of Weak But Specific Promoters

A wide variety of highly tissue-specific promoters have been evaluated for achieving transcriptional targeting, however, their applicability has been

hampered due to weak transcriptional activity. Enhancement of weak tissue specific promoters can be achieved by employing several different strategies. One of the simplest approach involves (a) deletion of those sequences from the promoter that do not contribute to tissue specificity or transcriptional activity and (b) incorporation of multiple copies of the enhancer and positive regulatory elements. This approach has been successfully used in the case of PSA promoter [43], tyrosinase promoter [124, 125], and CEA promoter [12].

Another approach involves generation of activating point mutations within the promoter region as has been in the case of AFP promoter [126] and the MDR 1 promoter [127]. Yet another strategy involves selective combination of multiple positive regulatory and tissue-specific elements to achieve enhancement of weak promoters. This strategy has shown promising results in augmenting melanoma-specific gene expression when the tyrosinase promoter, either alone or in combination with single or dual, tandem melanocyte-specific enhancer, was used to drive the expression of luciferase and the *E. coli* purine nucleoside phosphorylase gene.

Transient expression studies indicated 5- to 500-fold increase in luciferase activity following incorporation of either single or tandem enhancer elements. In another example, when 5–20 muscle-specific transcriptional elements were randomly assembled and linked to the minimal chicken α-actin promoter, sixfold higher activity was observed as compared to the CMV promoter [128]. In case of adenoviral vectors it might be possible to selectively increase specific expression from exogenous promoters by coexpression of modified VAI genes. Using this approach, Eloit *et al.* [129] were able to achieve 12.5- to 502-fold increased reporter gene expression. The fact that activity of certain E2F-responsive promoters in tumor cells exceeds that achieved in mitotically active normal cells has been exploited for tumor-selective transgene expression using an adenoviral vector in a malignant glioma model [130]. A novel approach involves development of dual-specificity promoters that are both cell-type-specific and cell-cycle-regulated. In this approach the transgene is under the transcriptional control of an artificial heterodimeric transcription factor whose DNA binding domain is expressed from a tissue-specific promoter, whereas the transactivating subunit is transcribed from a cell-cycle-regulated promoter. The feasibility of this approach has been successfully tested in a transient transfection system [131].

Transcriptional targeting of viral replication for selective killing of tumor cells can be achieved by deletion of adenoviral E1B/55-kDa protein which is essential for viral replication but is dispensable in p53-deficient tumor cells. An alternate approach involves generation of a replication-competent adenoviral vector in which E1A or E1A and E1B genes are under the transcriptional control of tumor-specific promoters like PSA, kallikrein-2, or AFP [47, 132, 133].

B. Improving Specificity with Multiple Promoter Segments

Several investigators have placed combinations of promoter sequences in tandem to derive more specific transgene expression. The authors of the following chapter in this book have both laboratory and clinical experience with this approach and this topic is well covered in their chapter.

C. Tumor-Specific Oncolysis

Several different approaches have been designed to achieved cancer-cell-specific adenoviral replication and subsequent tumor lysis. Based on our previous work in the laboratory and the clinic, we have designed an adenoviral vector that would only replicate in cells, which could activate the osteocalcin promoter. We have recently received approval for OBA #426 using the osteocalcin promoter to transcriptionally regulate adenoviral replication for the treatment of men with metastatic and recurrent prostate cancer. This approach is thoroughly reviewed elsewhere in this volume (see Chapter 10).

D. Combined Targeting Approaches

The preceding chapter describes elegant methods to achieve transductional targeting. These approaches will allow for the concentration of adenovirus at metastatic tumor deposits after a systemic administration. In collaboration with these investigators we have begun to combine both transductional and transcriptional targeting to allow for both tumor-specific concentration and tumor-specific oncolysis. This approach combines many of the individual strides achieved in adenoviral gene therapy in the past decade and holds great promise for the future of adenoviral cancer gene therapy.

VI. Summary

In summary, we believe that the success of gene therapy and its general applicability to medicine will be partially linked to the development of effective transcriptional targeting strategies. The main purpose of this chapter was to illustrate to the reader the benefits of transcriptional targeting and how this approach can be used to generate tumor- or tissue-specific gene expression. The main example of the osteocalcin promoter was used because of our laboratory's significant investigation of this promoter.

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