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Annual Report
PCRP Idea Development Award
W81XWH-04-1-0867
A cMyc-Driven in vivo Model of Human Prostate Cancer

Introduction
The long-term goal of the work proposed here is to generate, characterize and interrogate human epithelial cell-based in vivo models of prostatic carcinogenesis. These models will allow an examination of processes involved in carcinogenesis, tumor growth and metastasis. Since the tumors are themselves of human origin they represent an in vivo testbed to examine both tumor biology and the application of therapeutic agents.

The first two aims will generate data on cancer development, androgen dependence, gene expression and patterns of metastatic spread prostate samples overexpressing cMyc. The third specific aim will use this information to target specific genes and normalize their expression in the Myc tumor. If a positive phenotype results the gene product may be a potential therapeutic target.

We are using a model in which human prostatic epithelial cells (huPrE) are grown in a tissue recombinant with rat urogenital sinus mesenchyme (rUGM) and grafted back into the in vivo environment of an intact male athymic rat host. Manipulations of the huPrE allow us to examine the effects of retroviral transfection with cMyc within the huPrE. Our original C7-Myc model forms aggressive tumors, hence, as originally proposed we are making lower expressing clones that will allow us to follow the progressive events in cancer initiation and progression. It is not possible to recover such samples from human patients, as it would require continued repeat biopsies with no medical/pharmaceutical intervention. It is highly desirable to study prostate cancer formation/progression in human prostatic epithelial cells, in an in-vivo setting, in order to minimize cell culture artifacts and more fully understand prostate cancer in vivo.

Body
The initial work proposed was a characterization of the C7-myc model of prostate cancer. The highly aggressive nature of the tumors resulting from the introduction of C7-myc into primary cultures of human prostatic epithelium, has been described in our first paper on this subject (Williams et al., 2005) (appended). These data strongly indicated that the development of models which moderated this effect was desirable if we were to generate data with relevance to human disease, rather than to an esoteric model system. Such an approach was proposed in specific aim 2. The development of such models early in the life of the award has been pursued aggressively as our initial findings suggest that this provides a better basis to understand the questions raised in aim 1, in a disease-relevant context, than the use of C7-myc cells as originally proposed. To this end we have generated and applied a tetracycline-regulatable lentiviral-based system as shown in figure 1. As shown this allows tetracycline-regulated expression of cMyc in a human prostate cell line. We have successfully infected primary cultures of huPrE with pLenti/TO/Myc and generated tissue recombinant grafts that are at present in animals. These experiments are ongoing to produce enough grafts for the experimental time points outlined in the grant proposal. Once these recombinants have aged expression of cMyc will be initiated by manipulating tetracycline levels in drinking water and the disease progression will be followed.

In addition to the regulatable cMyc vector a series of color marked vectors using the same basic construct have been generated as shown in figure 2.
Figure 1. Tetracycline regulated lentiviral system for controlled expression of cMyc. Left panel. Plasmid map of pLenti/TO/Myc. This plasmid is constitutively active in absence of TR vector. Right panel. Western showing Myc expression from lentiviral infected human prostate epithelial cells. TR is the tet repressor which suppresses Myc expression in the absence of tetracycline.

Three modified pLenti vectors have been created pLenti/TO/V5 DEST EGFP, pLenti/TO/V5 DEST ECFP, pLenti/TO/V5 DEST DsRed2

Figure 2. PLenti4/TO/v5-Dest clones carrying different color markers. This allows us to use multiple vectors in one graft. The different color markers allow us to track cells and EGFP and DsRed have specific antibodies raised against them (the closely related green and blue colors cannot be separated immunohistochemically). Color markers allow us to visualize infection efficiencies and are unaffected by the presence of TR vector or tetracycline.

In order to more efficiently track metastatic lesions, as proposed in specific aim 1 we have utilized a trifusion protein generated at Stanford University (Ray et al., 2004). This protein has been designed for cell and metastasis tracking and provides a number of technical advantages over the GFP-based method proposed in the original application. Thus this development does not change the aims but should increase the ability to achieve them. The trifusion protein has been introduced into a lentiviral vector (figure 3) and has been successfully tested in vitro. In vivo testing is about to commence.
Figure 3. The Lenti Tri fusion was developed from the pcDNA trifusion vector (kind gift from Dr. Ray, Stanford) This vector was created to allow us to follow metastatic spread in the C7-Myc or other newly-developed cell lines. The Vector gives strong red fluorescence and is positive on luciferase assays. We have not yet tested our construct in mice but the original construct has been used by Dr. Ray’s group to image lesions.

As an alternative promoter to drive cMyc expression we are testing the ARR2PB promoter (Zhang et al., 2000) (figure 4). This promoter, developed by Dr. Robert Matusik at Vanderbilt, should allow for androgen-dependent expression of cMyc in tissue recombinants. This provides another alternative to the proposed cmv and tet-regulated systems. Should this prove unsuccessful the PSA promoter also exists as an alternative.

Figure 4. The ARR2PB-Myc system is being developed. The ARR2PB promoter is an androgen dependent prostate specific promoter that will drive high expression of Myc only in the luminal cells of the prostate grafts. This will be developed into either a lentivirus or retroviral construct.

In specific aim 3 we proposed to suppress the expression of PTEN. We have designed and validated retroviral vectors to drive shRNA for the suppression of PTEN. This approach results in malignant transformation in SV40T immortalized cells and is now undergoing initial testing in primary cultures of human prostatic epithelial cells.

A collaboration has been established with Dr. Chalfant, Virginia Commonwealth University, to use the C7-Myc virus in a grant proposal on Sphingolipid Regulation of Caspase 9 Alternative Splicing.
Technical Modifications:

The use of lentiviral as compared to retroviral approaches has been pursued. This reflects technological advances in viral methodologies and the use of a system in which infectivity is more efficient. The general approaches to be used are unchanged by this modification.

The use of a trifusion peptide rather than GFP as originally proposed allows increased sensitivity and more efficient tracking of metastatic lesions. Again this does not affect the overall aims of the proposal but rather the general trend of improvement in the tools available.

Personnel Changes

None

Key Research Accomplishments

One of the important proposed methods was to develop a tetracycline-inducible system to examine prostate cancer progression. This has been achieved. The tissue recombinant grafts of tetracycline inducible Myc are in animals, these will constitute the longest time point of possibly close to 1 year.

In a development from the proposed technology, reflecting technological improvements in the field, lentiviral (rather than retroviral) constructs have been constructed that allow transfer of Myc in a tetracycline responsive plasmid (figure 1). The original clone created had a 2bp insert immediately adjacent to the ATG start site that was not initially discovered on DNA sequencing. A new clone has now been extensively DNA sequenced and expression of Myc verified by RT-PCR and Western blot. (figure 1)

We have successfully Infected the prostatic BPH1 cell line with pLenti Myc and shown repression of Myc expression with introduction of TR vector. We have constructed several color expression clones for use in this and other projects. These expand our potential use of the commercially available vectors that at present contain only Zeo resistance.

We have created a lentiviral clone specifically for imaging of metastatic lesions. The luciferase can be imaged using light detectors. Ttk allows PET scanning and mRFP emits a wavelength of light that can penetrate deeper into the mouse tissues to give a signal from internal sites, especially the bone grafts our host mice carry.

We have characterized the use of orthotopic grafting as an improvement on the use of sub-renal capsule grafting to examine the metastatic process. These studies indicate that the orthotopic site has considerable advantages in such applications, and will therefore be used in some specific applications as required by experimental protocols.
Reportable Outcomes.

Papers

Presentations

Karin Williams, Kenichiro Ishii and Simon W. Hayward. (2004) c-Myc Overexpression In Human Prostate Initiates Cancer In Vivo. 10th Prouts Neck Prostate Cancer Meeting. Portland Maine Nov. 4-7


Cell lines
Human prostatic epithelial cells overexpressing cMyc under a cmv promoter (C7-myc) have been generated and are described in the published paper (Williams et al, 2005 - appended). These cells are available for distribution to non-profit institutions upon request. In the event of requests from for-profit organizations appropriate licensing agreements will be established.

Conclusions.
This work is proceeding slightly behind the predicted timeline. A number of changes to the specific details of the original statement of work are noted. These reflect technical methodological improvements that enhance the overall quality of the proposal. None of these changes alters the overall aims and long-term goal of the proposed work but have required that we delay some proposed experiments while developing new tools. The tool development stage is now effectively completed, subject to technological changes which may occur, and the in vivo biological studies have started.

References


Unopposed c-MYC Expression in Benign Prostatic Epithelium Causes a Cancer Phenotype

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BACKGROUND. We have sought to develop a new in vivo model of prostate carcinogenesis using human prostatic epithelial cell cultures. Human prostate cancers frequently display DNA amplification in the 8q24 amplicon, which leads to an increase in the copy number of the c-MYC gene, a finding that suggests a role for c-MYC in human prostate carcinogenesis. In addition overexpression of c-MYC in transgenic mouse models results in prostatic carcinogenesis.

METHODS. We took advantage of the ability of retroviruses to integrate foreign DNA into human prostatic epithelium (huPrE) to generate cell lines that overexpress the c-MYC protooncogene. These cells were recombined with inductive rat urogenital sinus mesenchyme and grafted beneath the renal capsule of immunocompromised rodent hosts.

RESULTS. The resultant tissue displayed a phenotype consistent with a poorly differentiated human prostatic adenocarcinoma. The tumors were rapidly growing with a high proliferative index. The neoplastic cells in the tumor expressed both androgen receptors (AR) and prostate-specific antigen (PSA), both characteristic markers of human prostate cancers. Microarray analysis of human prostatic epithelial cells overexpression c-MYC identified a large number of differentially expressed genes some of which have been suggested to characterize a subset of human cancers that have myc overexpression. Specific examples were confirmed by Western blot analysis and include upregulation of c-Myb and decreased expression of PTEN. Control grafts using either uninfected huPrE or using huPrE cells infected using an empty vector expressing a green fluorescent protein tag gave rise to well differentiated benign prostatic glandular ducts.

CONCLUSIONS. By using a retroviral infection strategy followed by tissue recombination we have created a model of human prostate cancer that demonstrates that the c-MYC gene is sufficient to induce carcinogenesis. Prostate 63: 369–384, 2005. © 2004 Wiley-Liss, Inc.

KEY WORDS: myc; tissue recombination; retroviral gene transfer; prostate cancer

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*Correspondence to: Karin Williams, Department of Urologic Surgery, A1302 MCN, Vanderbilt University Medical Center, Nashville, TN 37212-2765. E-mail: karin.williams@vanderbilt.edu Received 25 May 2004; Accepted 7 September 2004 DOI 10.1002/pros.20200 Published online 21 December 2004 in Wiley InterScience (www.interscience.wiley.com).

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INTRODUCTION

Prostate cancer is the single most diagnosed cancer in men and a major cause of mortality/morbidity within North America and Europe [1–3]. The introduction of routine PSA testing has resulted in earlier detection of prostate cancer and appears to be resulting in a decrease in disease-specific death rates [4]. While certain proteins such as TSPY have been found to display altered expression in very early cancer [5], there has not been sufficient characterization of PCa to identify many potential progression pathways that characterize prostate cancer [6]. Therefore, unlike the well-characterized pathway of acquired mutations displayed by colon cancer, there is not a clearly defined pathway for the progression and development of malignant disease in the prostate.

C-MYC is a transcription factor that belongs to the myc/mad/max family of Basic-helix-loop-helix-zipper (bHLHZ) proteins. Three closely related members make up the MYC family (c-MYC, L-MYC, N-MYC) and although they have very distinct patterns of expression, evidence exists that the proteins are able to compensate, to some extent, for the loss of one family member [7]. However, both c-MYC and N-myc knockout mice exhibit embryonic lethality [8,9].

Myc forms a heterodimeric transcription factor complex with its partner Max [10]. In this state Myc/Max is capable of binding to its DNA recognition site, the so-called E-box [core sequence (CACGTG)]. Max/Mad heterodimers also bind the E-box and act as transcriptional repressors presumably repressing genes induced by Myc/Max [11]. Like myc, both Max and Mad have related family members, capable of modulating this pattern of induction/repression by binding to each other and modulating the availability of the E-box. Myc also appears to be capable of binding and sequestering several other regulatory factors such as Sp-1 and Miz-1, causing transcriptional modulatory effects not associated with E-box binding [12–14].

Cell proliferation, differentiation, and apoptosis are all responses regulated by myc expression. The Myc protein acts as a cell activator that relies on other accessory proteins to specify the nature of the response. The proliferation pathway is mediated by Myc’s ability to activate several cyclins, including cyclin E [15] and cyclin D2 [16,17]. The activation of cyclin D2 causes sequestering of p27kip from cyclin E and driving the cell into S phase. Myc also indirectly reduces expression of p21WAF1 and p16ink4a [18,19], both of which are involved in cell cycle arrest.

Myc overexpression/deregulation has been implicated in numerous neoplastic transformations both in human disease and transgenic mouse models [20–28]. Furthermore, inactivation of the myc gene has been shown to elicit regression of Myc-induced tumors in the absence of novel mutations [23,29,30].

C-MYC was the first oncogene to be recognized as being overexpressed in human prostate cancer [28]. However, the precise role played by c-MYC in human prostate cancer is unclear in part due to the amplification of the 8q24 amplicon. This amplicon is particularly rich in genes, several of which [e.g., c-MYC [31], NOV [nephroblastoma overexpressed gene], EIF3S3 [eukaryotic translation initiation factor 3 subunit 3], HAS2 [hyaluronan synthase2] [32], KIAA0196 [33], and PSCA [34]] are expressed in prostate and have either oncogenic or tumor suppressor potential. FISH analysis has identified amplification of the 8q24 amplicon [32,35–37] in a large percentage of human adenocarcinomas [38,39] and some prostate intraepithelial neoplasias (PIN) [37].

Mouse models of prostatic neoplasia have been generated in which the c-MYC gene was expressed from either the probasin promoter [20] or C(3)1 promoter [22]. Two probasin promoter variants were used in one study; these promoters share the same prostate specificity but differ in promoter activity. Both the Hi-Myc (ARR3PB-myc) and Low-Myc (sPB-myc) animals develop mouse PIN (mPIN) and invasive carcinoma but the time to development and progression differs by approximately 6 months [20]. While these probasin-Myc transgenic mice apparently progress from mPIN to prostatic adenocarcinoma the relationship of PIN to adenocarcinoma in humans remains unsubstantiated. The C(3)1 promoter is a weaker promoter than the probasin constructs. Mice carrying the C(3)1-Myc transgene fail to develop adenocarcinoma within their lifetime although they do develop mPIN-like lesions [22]. These data suggest that a low level of myc expression correlates with mPIN development while the progression to adenocarcinoma requires elevated c-MYC levels.

Given that c-MYC is reported to be overexpressed and the gene amplified in human prostate cancer, and because transgenic mouse models overexpressing c-MYC in the prostate have a dose related progression towards malignancy, we decided to test the ability of cMYC to transform human prostatic epithelium. To do so in vivo a tissue recombination model was used to follow prostatic carcinogenesis in response to overexpression of c-MYC.

MATERIALS AND METHODS

Human Cell Culture

Human prostate tissue samples were obtained from the Vanderbilt Tissue Acquisition Core via the Department of Pathology in accordance with Vanderbilt IRB protocols. Cores 6 mm in diameter were removed from
radical prostatectomy samples and were sampled by histologic analysis on frozen sections to determine the nature (benign vs. malignant vs. severe inflammation) of the tissue contained within the core. Benign tissue was cut into 2 mm cubes using sterile scalpels. After washing in RPMI (Gibco, Carlsband, CA) 5% FCS (Atlanta Bioscience Atlanta, GA) the tissue was plated on Primaria™ tissue culture flasks with sufficient medium to wet the plate and create a strong surface tension (1.2 ml/25 cm²). After cell attachment had taken place (~12 hr) the volume of medium was increased. Tissue obtained from tissue recombination grafts was reintroduced into culture in an identical manner.

**Tissue Culture Medium**

Tissue culture medium for human prostatic epithelial cells (huEpi mix) consisted of: [RPMI 1640, 1% ITS (Insulin Transferrin Selenium), 1% Antibiotic/Antimycotic (all from Gibco)], 2.5% charcoal stripped serum (Atlanta Bioscience), BPE (Bovine Pituitary Extract) 1:250 (Hammond Cell Tech, Winsor CA), Cholera toxin (1 μg/ml), and Epidermal Growth Factor (0.01 μg/ml) (Sigma, St. Louis, MO).

**LZRS Retroviral Plasmid Construct**

The plasmid LZRS c-MYC/EGFP (Fig. 1) was constructed utilizing the LZRS-EGFP backbone (Nolan Laboratory, Stanford, CA). The CMV promoter was excised from pIRE-EGFP (Clontech, Palo Alto, CA) as a BgIII/BamHI fragment. The fragment was ligated into the BamHI site of the LZRS-EGFP backbone to give C7A.

The human c-MYC cDNA clone (BC000917) was obtained from ATCC (Rockville, MD) and amplified by PCR using a 5' primer specific to translational start site and a 3' primer containing an XhoI restriction site and the consensus sequence for the translational stop site with subsequent deletion of any polyadenylation sites. After PER amplification, the product was gel purified, and cloned into poem T-Easy (Promega, Madison, WI). Following DNA sequence verification of the cloned product the c-MYC coding region was excised using EcoRI/XhoI and sub cloned into the EcoRI/XhoI sites of pLZRS-EGFP to give C7-Myc.

**Viral Production**

Amphotrophic φNXA packaging cells were obtained from ATCC [under an MTA from the Nolan laboratory Stanford (www.stanford.edu/group/nolan)]. These cells were maintained in 5% FCS/RPMI 1,640 with antibiotic surveillance. The φNX packaging cell lines were reselected with both hygromycin B and dipheria toxin (Sigma) every 3–4 months. The LZRS retroviral constructs were transfected into φNX cells that were 70%–80% confluent. Routine transfection took place using 25 cm² flasks. Lipofectamine 2000 (Invitrogen, Grand Island, NY) was optimally used at a

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**Fig. 1.** Retroviral constructs, control vector C7Δ and LZRS C7-MYC/EGFP. pLZRS is a retroviral vector derived from the Moloney murine Leukemia virus (MoMuLV). The gene of interest (c-MYC) and the Enhanced Green Fluorescent Protein (EGFP) are expressed from a bicistronic message under the control of the cmv promoter. The 5' viral LTR controls expression of the transcript that contains V (the extended viral packaging signal). The retroviral particle contains, and integrates into the genome, only the genetic information between and including the 5' and 3' LTRs. pLZRS does not contain the structural genes necessary for viral formation and replication, these are provided by the packaging cell line φNX. Other components of pLZRS such as the puromycin resistance gene and EBNA (EBV episomal functions) contribute to stability and selection of the plasmid in the packaging cell line. pLZRS also includes the pUC origin of replication and E. coli Amp gene for propagation and antibiotic selection in bacteria.
final concentration of 3 \mu{l}/ml according to manufacturer's protocols. Eight hours post transfection the \( \psi \)NX cells were given fresh RPMI/5% FCS and incubated overnight at 37°C. Viral supernatant was removed in the morning and the cells split if necessary and moved to a 32°C incubator to ensure greater viral stability. The viral supernatant was spun at 3,000 rpm and passed through a 45 \( \mu \)m filter to ensure the absence of contaminating \( \psi \)NX cells. The viral supernatant was then supplemented with 4 \mu{g}/ml Polybrene (hexadimethrine bromide, Sigma) and either stored at -70°C or used immediately.

**Viral Infection**

The viral supernatant containing the c-MYC/EGFP or C7-delta control retrovirus was diluted 1:1 with huEpi mix that was double strength with respect to the added constituents and the polybrene concentration was corrected to 4 \mu{g}/ml. The viral medium was then placed on the huPrE cells (three patients from which both TZ and PZ cores were put into independent culture dishes in duplicate) in culture and replaced 8 hr later. Primary cells were incubated at 37°C as their rate of cell division was severely compromised at 32°C, the increased stability of the viral particles at 32°C therefore could not be utilized when working with huPrE but proved useful when certain immortal cell lines were infected, data not shown. Successive rounds of infection over 5 days were employed to generate infected cells. Owing to the growth patterns of the huPrE we found that daily infection would ensure the presence of virus during the initial outgrowth of cells and therefore generate the maximum number of infected cells. Infection rates were monitored using fluorescence microscopy of the bicistronic EGFP tag. After infection the cells were maintained in culture until use. The cells were examined by a clinical pathologist to assess any phenotypic differences in the infected cells.

**Culture of Infected Cells**

Following infection, a proportion of the cells were used as a source of epithelium in tissue recombinants. The remaining cells were maintained in culture for a further 16 passages with batches frozen in liquid nitrogen at different points. All remaining cells were frozen after passage 16. Cells were examined for the continued expression of the EGFP tag using fluorescence microscopy; expression of AR, PSA, and c-MYC was monitored using Western blot analysis.

**Tissue Recombination**

Tissue recombinants were prepared, as previously described [40,41]. Briefly, pregnant rats were obtained from Harlan (Harlan, Indianapolis). Rat urogenital sinus mesenchyme (UGM) was prepared from 18-days embryonic fetuses (plug date denoted as day 0). Urogenital sinuses were dissected from fetuses and separated into epithelial and mesenchymal components by trypsin digestion, as previously described. UGM was then further reduced to single cells by a 90 min digestion at 37°C with 187 U/ml collagenase (Gibco). Following digestion, the cells were washed extensively with RPMI-1640 tissue culture medium. Viable cells were then counted using a hemacytometer, with viability determined by trypan blue exclusion. Epithelial cells were released from tissue culture plates using trypsin. Trypsin was neutralized and the cells washed and counted using a hemacytometer. Cell recombinants were prepared by mixing 100,000 epithelial cells with 300,000 stromal cells. Cells were pelleted and resuspended in 50 \mu{l} of neutralized type I rat tail collagen prepared as previously described [42]. The recombinants were allowed to gel at 37°C for 15 min and were then covered with growth medium and cultured overnight. They were then grafted beneath the renal capsule of adult male severe combined immunodeficient (SCID) mice [C.B.17/IcrHsd-scid mice (Harlan)].

**Subcutaneous Grafting**

Epithelial cells (100,000) were pelleted and resuspended in 50 \mu{l} of neutralized type I rat tail collagen prepared, as described previously [42] and placed under the skin of adult male SCID mice [C.B.17/IcrHsd-scid mice (Harlan)]. Some of these subcutaneous grafts were surrounded in matrigel (BD Biosciences, Bedford, MA) at the time of grafting.

**Tissue Recovery, Fixation, and Processing**

Mice were sacrificed by Isoflurane inhalation followed by cervical dislocation according to Vanderbilt animal care protocols. The kidney and attached graft together with internal organs were removed and examined at the gross level. The c-MYC grafts owing to their substantial size were divided into several pieces. Those fragments containing the kidney were fixed in 10% neutral buffered formalin, as were the internal organs of the host. The remaining tissue was then divided to give representative portions for (1) RNA extraction, (2) Protein extraction, and (3) for further tissue culture. Tissue destined for RNA extraction was cut into small pieces and immersed in RNA LATER (Ambion, Austin, TX) according to manufacturer's instructions. Tumor tissue for protein extraction was snap frozen on dry ice and stored at -70°C until required. The third portion of the tumor was removed...
for tissue culture or frozen [43] to allow extraction of viable cells.

**Culture of Tumor-Derived Epithelial Cells**

Tumor tissue was minced and placed in culture in a minimal volume of tissue culture medium (as per primary culture). Cells were passaged using trypsin and then frozen. Continued transduced gene expression in these cells was confirmed by fluorescence microscopy to detect the expression of EGFP and by Western blotting to confirm continued expression of c-MYC.

**Antibodies**

For immunolocalization studies the following antisera were used. Androgen receptors (AR) were detected using a rabbit polyclonal antibody (sc-816) raised against a peptide within the N-terminal domain of hAR, EGFP was detected using a mouse monoclonal to the full length GFP that detects all GFP variants (sc-9996). c-Myb (sc-8412), PTEN (sc-7974sc-9996), p63 (sc-8343) (Santa Cruz Biotechnology, Santa Cruz, CA). c-MYC was detected using a mouse monoclonal (NCL-c-MYC) raised against full-length recombinant human protein obtained from Novocastra (Burlingam, CA). Ki67 (M-7240), PSA (A-0562), broad-spectrum Keratin antibodies (Z-0622) were obtained from DAKO (Carpinteria, CA), and keratin 8, 14, and 18 (clones LE41, LL001, and LE61 gifts from Prof. E.B.Lane, Dundee University, UK [44,45]).

**Protein Extraction**

Tissue extracts of human prostate epithelial cells maintained in tissue culture for one passage, and c-MYC infected huPrE cells were prepared by homogenization in 400 µl cold buffer A (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 0.1 mM DTT; 1 x protease complete (Roche, Indianapolis, IN)). The cells were allowed to swell on ice for 15 min, after which 25 µl of a 10% solution of Nonidet NP-40 (Sigma) was added and the tube vortexed vigorously for 10 sec. The homogenate was then centrifuged for 30 sec in a Microfuge. The supernatant was snap frozen and stored at −70°C.

**Western Blotting Analysis**

Tissue extracts from huPrE epithelial cells grown in culture and their c-MYC infected counterparts as well as extracts from the BPH1 prostatic epithelial cell line were run on denaturing mini gels containing an acrylamide gradient from 4%−20% (w/v) polyacrylamide (Invitrogen). Gels were run in MOPS/SDS running buffer (50 mM 3-[N-morpholino] propane sulfonic acid [MOPS], 50 mM Tris base, 0.1% SDS, 1.025 mM EDTA [pH 7.7] for 35 min at 200 mA. Samples were blotted onto PVDF membrane (Invitrogen) using transfer buffer (25 mM bicine, 25 mM Bis-Tris, 1.025 mM EDTA, 50 mM choralbutanol [pH 7.2] (Invitrogen) in the mini gel tank according to the manufacturer's instructions. Thereafter, membranes were blocked for 2−3 hr at room temperature in BLOTTO (5% nonfat dried milk powder [Difco] dissolved in Phosphate buffered saline (Sigma) containing 0.1% Tween-20 (PBST). Membranes were incubated overnight in Blotto with the any one of the antibodies (anti c-MYC 1:800, AR 1:1,000, c-Myb 1:600, GFP 1:2,000, E-Cad 1:1,000, PTEN 1:800). Bound antibodies were detected using appropriate secondary antibodies (1:4,000 peroxidase conjugated Donkey anti rabbit/Sheep anti mouse [Amersham Pharmacia Biotech, Piscataway, NJ] and 1:2,000 rabbit anti goat [Santa Cruz]) and the enhanced chemiluminescence visualization system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

**Immunohistochemistry**

Deparaffinized, slide mounted sections were rehydrated and then subjected to heat induced antigen retrieval using the commercial retrieval buffer (H-3300) from Vector Laboratories, Brlingham, CA. The sections were microwaved for 15 min at a power that ensured continuous but not excessive boiling. Slides were permitted to cool to room temperature prior to incubation with 3% hydrogen peroxide in methanol for 15 min to block endogenous peroxidase. After washing in PBS the slides were blocked in Clean Vision™ from ImmunoVision Technologies for 15 min. This blocking optimized the use of monoclonal antibodies on tissue recombinants in which an immunocompromised mouse host was the graft host. The antibodies towards GFP (Santa Cruz), Ki67, PSA, and broad spectrum Keratin (DAKO) were all used at 1:200 (p63 used at 1:1,000) [diluted in 1:4 normal swine serum (broad spectrum Keratin, PSA, p63) or normal rabbit serum (GFP, Ki67, keratins 8, 14, 18) in PBS/5% BSA). All antibodies were incubated on sections overnight at 4°C. Sections were then incubated with the appropriate biotinylated secondary antibodies for 1 hr. (Broad spectrum Keratin, p63 and PSA, swine anti rabbit (DAKO), and for GFP, keratin 8, 14, 18 and Ki67 rabbit anti mouse, (DAKO), both of which were diluted 1:500 in the appropriate normal serum (see above)]. After appropriate washing steps the sections were incubated in ABC-HRP complex (Vector) for 30 min and washed extensively. Bound antibodies were then visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride (liquid DAB, DAKO). Sections were counter-
stained with hematoxylin. Images were captured onto a computer using a Zeiss microscope equipped with an AxioCam camera (Zeiss) and software.

**Identification in Histological Sections of Species Origin of Cells in a Tissue Graft**

Staining with the Hoechst 33258 dye (Sigma) was performed, as previously described [46]. The Hoechst dye Sections were examined by fluorescence microscopy. Host mouse cells contain several small discrete intranuclear fluorescent bodies, which are absent in cells from either rat or human allowing us to confirm that the tumor is not derived from the host mouse.

**RNA Isolation**

Tissue was rapidly excised from the outer portions of the graft and placed in 10x v/v RNA LATER (Ambion, Austin, TX). The tissue was then refrigerated prior to use 1-5 days after removal from the host. The tissue was dissected in a Petri dish containing RNA LATER to remove any kidney tissue (none was actually visible in any of the tissues) or obviously necrotic tissue (this tissue was very soft and white, resembling cotton candy).

RNA was isolated using the Qiagen mini RNA Easy kit according to the manufacturers instructions (Qiagen, Valencia, CA). The RNA was DNAse treated again using Qiagen reagents as detailed in the RNA easy protocol. The RNA concentration was then determined spectrophotometrically and the RNA aliquoted and snap frozen at -70°C.

**RNA Labeling and Hybridization**

All RNA and cDNA manipulations and cDNA array hybridizations were undertaken by technical support staff within the Vanderbilt Microarray Shared Resource (http://array.mc.vanderbilt.edu) using the protocols outlined on their website http://array.mc.vanderbilt.edu/support/protocols.htm. The use of tissue culture cells permitted the use of the standard cDNA labeling without amplification of the RNA. The microarray used for our experiments was the human 11K comprised of the Research Genetics human clone set.

**cDNA Array Analysis**

Data was normalized by Lowess sub grid and Gene Pix Pro Software was used to analyze differentially expressed genes between the two analysis groups. The groups consisted of huPrE grown in culture and infected with the LZRS-myc virus.

**RESULTS**

**Infection of Hupre With a Retrovirus Containing C-Myc/Egfp Elicits Phenotypic Changes In Vitro**

The huPrE cells grown on tissue culture plastic have a characteristic cobblestone appearance (Fig. 2). The presence of high levels of cholera toxin severely suppressed fibroblastic growth resulting in an almost pure epithelial cell population, as determined by visual examination. The rate of emergence of epithelial cell sheets from individual tissue fragments was highly variable both with respect to ‘flask to flask’ variation using tissue from a single patient and with respect to

![Fig. 2](image-url)

*Fig. 2. Phase contrast photomicrograph of human prostate epithelium in cell culture 4 days after infection (Left panel). Note the typical cobblestone morphology in lower left hand side compared to the denser cells present in the upper right hand side. (Middle panel) Fluorescent image of human prostate epithelium culture 4 days post infection. Note that approximately one half of the cultured cells strongly express EGFP due to infection by the C7-Myc retrovirus. (Right panel) overlay, confirming that the small densely packed cells express EGFP while those with the cobblestone morphology do not.*
microscope the nests displayed uniform EGFP fluorescence. Recombinants composed of C7A PrE and rUGM

...tissues carried myc-expressing grafts were sacrificed after 28 days due to the large size of the graft. The nests had well defined borders and were surrounded by cells displaying the expected normal phenotype. There were discreet nests of tightly packed cells, composed of 100,000 epithelial cells and 300,000 rUGM monolayer of epithelial cells with a cobblestone appearance. Instead of a uniform monolayer of epithelial cells with a cobblestone appearance, there were discreet nests of tightly packed cells. The nests had well defined borders and were surrounded by cells displaying the expected normal phenotype (Fig. 2). When visualized on an inverted fluorescence microscope the nests displayed uniform EGFP fluorescence while the surrounding cells did not express EGFP.

All of the c-MYC/EGFP infected cell cultures observed (three patients from which both TZ and PZ cores were put into independent culture dishes in duplicate, a total of 12 independent cultures) exhibited similar morphological changes. The culture consisted of EGFP expressing epithelial cells that varied widely in their fluorescence (and by subsequent analysis c-MYC expression). Over time the EGFP expression became more uniform but a range of EGFP expression has always been observed throughout the cultures. This suggests that the cultures represent a variety of clones resulting from multiple initial infections.

HuPrE infected with the LZRS C7A (the 'empty vector') exhibited normal cobblestone morphology and typically infect with relatively low efficiency ≤1% at low passage number, the control infections are less efficient than infections with the C7-Myc retrovirus. The Green EGFP infected cells were indistinguishable from their uninfected neighbors under phase contrast and could only be identified by EGFP expression. The C7A-infected cells could be passaged a maximum of five times before they became senescent.

The C7 Myc infected cells were maintained through 16 passages and showed no signs of senescence during this period. They continued to express both EGFP and c-MYC as well as prostatic epithelial markers including AR and PSA.

Hupre/C-Myc-EgfP Plus RugmTissue Recombinants Form Rapidly Growing Adenocarcinomas Expressing Human Prostatic Markers

Tissue recombinants of huPrE and rUGM were prepared. Control grafts contained either uninfected or C7-A-infected epithelium. Recombinants using C7-myc contained different percentages of c-MYC-infected cells (from 10% to 50% dependent on the time post infection that the cells were grafted). All grafts were composed of 100,000 epithelial cells and 300,000 rUGM cells. Host mice carrying myc-expressing grafts were sacrificed after 28 days due to the large size of the graft, which exceeded the size of the normal kidney (Fig. 3). Recombinants composed of C7A PrE and rUGM grafted to the contralateral kidneys of experimental hosts were very small and poorly developed at 28 days post grafting. In separate experiments, using a 3-month time point fully differentiated prostatic structures expressing PSA and AR were seen. Control grafts maintained a benign histology throughout (Fig. 3). The phenotype and timing of developmental events in these control grafts is consistent with previously published results using human prostatic epithelial organoids [47].

Initial inspection of the large c-MYC expressing grafts, indicated several very large blood vessels located on the surface of the graft and areas of white necrotic tissue in areas devoid of obvious blood vessels (Fig. 3). The kidney tissue was readily apparent upon subsequent dissection and was essentially normal.

C7-Myc cells grafted subcutaneously into the flank of SCID mice developed large tumors after 6 weeks (data not shown) that were indistinguishable from C7-Myc recombinated with rUGM. The slightly longer time frame probably reflects the slower recruitment of blood vessels to a subcutaneous graft site compared to the subrenal graft site.

Thin smears of surface cells from the tumors expressed EGFP when exposed to fluorescent light (data not shown). These cells were placed into tissue culture to confirm EGFP expression and their human origin.

Expression from the CMV promoter is maintained throughout the growth of the graft. We have previously observed promoter downregulation (unpublished data) in cells carrying genes under the CMV promoter introduced via retroviral integration possibly via methylation events consistent with those described in transgenic mice [48,49]. The EGFP levels within the cells placed into reculture maintained a range of EGFP expression consistent with cultures prior to grafting suggesting that such repression had not occurred in these tumors.

In all the huPrE/c-MYC tumors the grafts consisted of cells strongly staining with hematoxylin. The cells characteristically exhibited large irregular nuclei, pro-
Fig. 3. Gross anatomy of mouse kidney with a \( c\)-MYC overexpressing prostate epithelial cell recombinant xenograft (top left). Note the encasement of the kidney by the tumor (black arrow). The control kidney carrying the \( C7\Delta\) Pr\(E\)/rUGM graft is displayed on the right, the small xenograft is barely visible (red arrow). Photomicrograph showing the poorly differentiated \( c\)-MYC expressing human prostatic tumors (middle/bottom panel) strongly express enhanced green fluorescent protein (middle left) PSA [(center)* indicates necrotic tissue]. The high mitotic rate observed in the tumor was confirmed by a very high index of staining with Ki67 (middle right). Bottom panel (left to right) AR, and p63.

Grossly, the tumors were well-vascularized, with prominent nucleoli, and dense cytoplasm. A large number of dividing cells were present in a single field and many of the mitotic figures were clearly abnormal (Fig. 3). No fibromuscular stroma was present in the grafts nor was any apparent at the graft extremities. Irregular areas of necrotic tissues extended throughout the grafts and predominated in the interior, but did not interfere with the kidney. Areas of living tissue surrounded the few blood vessels that populated the graft interior. Immunohistochemical analysis of the \( C7\)-myc induced tumors revealed that these retained expression of two key markers of prostate tissue, AR and PSA. In addition, the tumors expressed EGFP confirming their origin. Tumors expressed keratins 8 and 18 and lacked p63 and keratin 14 staining suggesting a luminal rather than basal cell origin. This is consistent with the profile seen
in human prostate cancer. The proliferation rate of the cells was extremely high as indicated by the almost universal presence of Ki67 in the nuclei (Fig. 3). This was consistent with the observation of extremely rapid tumor growth.

No infiltration was observed into the kidney, a well delineated border was observed and the kidney morphology was normal with no obvious compression or damage (Fig. 3). Hoechst 33258 staining confirmed that none of the epithelial cells observed were of mouse origin and that no 'non mouse' cells were present in the kidney (data not shown). Some areas displaying p63 positive cells were observed within the graft, these were determined (by a trained pathologist) to be mouse kidney structures caught in cross section, providing a good internal positive control for the tumor cells, which were universally negative for basal cell markers (Fig. 3).

**Molecular Characteristics of Tumor-Derived Cells**

A microarray analysis comparison of the cells derived from the C7-myc tumors and of primary epithelial cultures from the same patient revealed a number of changes (Table 1). As might be expected many genes were seen to be regulated in response to c-MYC overexpression. Attention in this analysis was focused upon genes known to be regulated in tumors. Of particular note was the observation that PTEN and E-cadherin expression was seen to be suppressed and c-Myb expression was observed to be upregulated in the tumorigenic cells. This observation was confirmed by Western blotting to examine expression of these proteins (Fig. 5). Western blotting analysis also confirmed the continued overexpression of c-MYC, and the expression of both AR and EGFP.

**DISCUSSION**

The present study demonstrates that c-MYC overexpression is sufficient to drive normal human prostatic epithelium to a metastatic tumor within a tissue recombination model. Retroviral infection of normal prostate epithelium with the EGFP expressing empty vector resulted in benign prostatic architecture indicating that MYC overexpression not infection/integration

![Fig. 4. Histopathologic appearance of metastatic lesions. Multiple metastatic lesions were found in mice as focal growths predominantly following the line of the intestinal tract. H&E high magnification of tumor immediately adjacent to the intestine (left panel top & bottom). Low magnification composite (right panel) showing three metastatic nodules that were attached to the host mouse intestine.](image-url)
TABLE I. Microarray Data Set of mRNA Showing 2-fold Differences in Levels Between C7-Myc PrE and C7A PrE

<table>
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<tr>
<th>Category</th>
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<th>Description</th>
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<td>Transcription factor 7</td>
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<td>FOSL2</td>
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<td>Dimerizes with JUN forming the transcription of factor complex AP-1</td>
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<td>Adaptor protein involved in transducing signals from receptor tyrosine kinases to downstream recipients such as RAS</td>
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<td>Caspase 3 apoptosis-related protease</td>
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<td>Extracellular matrix</td>
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<td>A disintegrin and metalloproteinasn domain 15 (metargidin)</td>
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<td>TIMP1</td>
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<td>Tissue inhibitor of MMP1</td>
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<td>MMP14</td>
<td>NM_00499</td>
<td>Matrix metalloproteinasn activates MMP2</td>
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<td>Cell cycle</td>
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<td>Couples activated growth factor receptors to a signaling pathway</td>
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<td>Integral membrane protein, which belongs to the Claudine family</td>
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TABLE 1. (Continued)

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<td>p8 protein (candidate of metastasis)</td>
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of the EGFP expressing retrovirus induces the cancer phenotype. The tumors formed in this model retain some key characteristics of human prostate cancer, notably the expression of AR and PSA, and the ability to metastasize.

The c-MYC overexpression combined with a large percentage of infected cells produces a cancer phenotype within our xenograft model that is aggressive and progressive. The cells were obtained from tissue derived from aging males and may already have accumulated genetic hits that, of themselves, are insufficient to alter histology. The levels of c-MYC expressed by a CMV-driven retroviral delivery system would certainly constitute a very large additional molecular hit. The resulting tumors resemble a poorly differentiated advanced carcinoma, however, the growth rate is accelerated in comparison with the human disease. Myc expression can be a pivot point in the cellular decision-making process that determines if a cell will undergo proliferation or apoptosis [50-55]. Myc overexpression within the primary huPrE appears to be exhibiting a proliferative effect demonstrated by the extremely large number of Ki67 positive cells present within the renal capsule graft and the metastatic lesions. The lack of expression of the basal cell markers keratin 14 and p63 indicate that the c-Myc tumor, like human prostate adenocarcinoma, loses basal cells during tumor formation.

Similar c-MYC-based retroviral rodent models created by Thompson and co-workers [25-27,56-59] in prostate and by Edwards [60] in the breast lacked the aggressiveness of our model unless activated ras was also present. Less than 0.1% of epithelial cells carried the retrovirally introduced genes in the mouse TR's while our grafts contained 10%-50% infected cells thus altering the environment considerably as normal cells

![Fig. 5. Western blot analysis of C7-Myc and C7Δ infected cells.](image-url) Western blot of human prostatic epithelial cells infected with C7-Myc or C7Δ (empty vector). All lanes contain 100 μg of protein except PTEN (250 μg). EGFP (Top left) is detected only in C7-Myc infected cells, C7Δ infected cells made up approximately 1% of the culture and while fluorescent cells were visible no signal was detected by western analysis. E-cadherin 120 kDa (top middle), Androgen receptor 130 kDa (Top right), cMyb 75 kDa (bottom left) c-MYC 67 kDa (bottom middle) PTEN 60 kDa (bottom right).
have the potential to inhibit the growth of transformed cells [61]. Microarray data do not indicate increased levels of ras mRNA within our C7-Myc tumors. Mouse strain backgrounds modify the effects of the oncogenes myc and ras [27] within the retrovirally infected TR's. By virtue of using human tissue, the present study examines an outbred epithelial cell population.

More recent transgenic mouse studies have demonstrated that the degree of response to c-MYC overexpression in mouse prostatic epithelium is dose related, with increased expression giving rise to more severe phenotypes [20,22]. All three described prostate-specific c-MYC-overexpressing mice develop mPIN. Mice expressing c-MYC using the Probasin promoter progress to invasive cancer in a time frame that is determined by the relative strength of the promoter construct used (ARR1PB or sPB) [20]. Low levels of c-MYC expression are thought to cause prostatic dysplasia in the prostates of rats treated in the neonatal period with DES [62]. Progression beyond PIN is not observed within the lifetime of the C(3)Myc mouse. As serial recombination experiments using the C(3)-Myc mouse or the DES treated prostates have not been performed it is not known if these PIN-like lesions would progress over time as is thought to happen in humans. Our model possibly shows such an accelerated progression because the epithelium is already primed by both age related genetic changes and environmental/physiological events within the diseased prostate combined with expression of c-MYC from an extremely strong constitutive promoter.

Human prostatic epithelium expresses prostate specific antigen (PSA), in vivo this weak protease belongs to the large family of Kallikreins, and is not, despite the name, totally specific for the prostate. However, PSA is the main basis of the blood tests used to detect and monitor prostate cancer. PSA is androgen-regulated in normal and low-grade prostate cancers, however, as cancers progress to an androgen-independent state, PSA levels climb marking disease progression. Primary cultures of benign prostate cells initially express PSA as do the established cell line LNCaP [63]. However, it is commonly observed that cells in culture rapidly lose expression of steroid receptors, including AR, and consequently lose expression of steroid regulated gene products such as PSA. Tissue recombinants composed of normal prostatic epithelium and rUGM express both AR and PSA [47], as would be expected, since these genes are both regulated by the context in which the cell is growing. More remarkably Myc overexpressing cells express PSA and maintain their androgen receptor expression in culture. Tumors derived from these cells continue to express PSA and AR in vivo adding an important element to the relevance of this model.

Sawyers and co-workers [20] have identified a molecular signature that identifies both the c-MYC overexpressing prostate tumors present in their transgenic mouse model and a subset of human prostate tumors that overexpress Myc. Our cDNA analysis identified several targets that were subsequently analyzed by Western blotting, as our cDNA array differed from that used by the Sawyers group we were unable to compare their molecular signature with the one we obtained. We were however able to use the microarray data to identify proteins previously shown to be involved in tumorigenesis which were regulated in this model. Western blot analysis was used to confirm microarray data on the loss of PTEN and E-cadherin in the c-MYC expressing cells. Both of these proteins are implicated as playing a tumor suppressive role in cancer development and show a marked decrease in expression compared to control cells. Decreased or absent E-cadherin expression is a frequent occurrence in human prostate cancer [64] and this is recapitulated within our myc overexpressing tumor. The undetectable levels (by Western blotting) of E-cadherin present in our myc tumor cells in culture may represent a general loss of adhesion, which would in part explain the low adhesion of the cells both to the tissue culture plastic and other cells resulting in loss of the cobblestone morphology and lack of strong junctions between cells. Immunohistochemistry on the tumor tissue failed to pick up E-cadherin staining on the cell membranes within the tumor (data not shown).

PTEN is a tumor suppressor gene situated at 10q25, a deletion hot spot in prostate cancer [65]. PTEN Knock-out mice are embryonic lethal [66] but tissue specific homozygotes (0% gene dose in prostate 100% in most other tissues) and the newer hypomorphic mice (25%–35% gene dose) [67–70] display mPIN that progresses to invasive carcinoma over time. Heterozygote PTEN deletion results in mPIN but requires the presence of a second genetic lesion to progress to an invasive cancer phenotype [71,72]. PTEN encodes a lipid phosphatase that inhibits the PI3 kinase/AKT pathway. The AKT pathway is a pivotal point in the control of cellular homeostasis. Many pathways including TGFβ, IGF, EGF and Ras interact with AKT. The activation of AKT appears to indirectly promote androgen-independent survival of prostate cancer cells, but the mechanism is as yet undetermined [73,74]. PTEN and AKT are upstream of c-MYC; PTEN loss causes upregulation of AKT that in turn upregulates c-MYC. The role of AKT on AR and subsequent repercussions on c-MYC are unknown but the importance of AKT and by extension PTEN in androgen independent growth of prostate cancer correlates with the role of c-MYC.

Microarray data verified by Western blot analysis demonstrated upregulation of the cMyb oncogene in
the C7-Myc model. Myb is usually regarded as a proto-oncogene within the hematopoietic line where it functions as a cell cycle promoter. c-Myb inhibits expression of p15INK4b while transactivating c-MYC, Bcl-2, COX2, IGF-1, and IGF-IR [75–77]. While c-Myb has primarily been studied in the hematopoietic lineage it is also selfless support.

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This work demonstrates the application of retroviral infection strategy followed by tissue recombination to examine the contribution of individual gene products to carcinogenesis. The approach can be used on any of the regulated products within the 8q24 amplicon, or those identified in the cDNA microarray analysis, both alone and in combination to determine their contribution to the final graft phenotype. This in vivo model of prostate cancer based upon human prostatic epithelium has many interesting and potentially useful features. However, the rapid progression and extremely high proliferative rate limits the ability to examine early tumorigenic events. The model retains many characteristics of human prostate cancer including continued expression of AR and PSA. The model also demonstrates downregulation of two tumor suppressors (PTEN and E-cadherin), which are suppressed in human prostate cancer and shows upregulation of Myb, which is known to be upregulated in the human disease. Metastasis is also an important component of the in vivo model. Cell cultures derived from both the initial viral infections and from the tumors retain many of these important characteristics and represent a potentially useful resource to examine prostate cancer biology. We are working to modify the model using a series of weaker constitutive, conditional, and regulatable promoters that will better allow us to follow stages of progression.

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