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29 May 2012

Abstract

Title of Dissertation:

"Pathogenetic Influences of Human Herpesvirus 8 (HHV-8) in Prostate Cancer Progression"

Justin Garner Mygatt, Doctor of Philosophy, 2012

Thesis Directed by:

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The mechanisms that give rise to androgen-independent prostate cancer (AIPC) are incompletely understood, resulting in a lack of treatment options for this fatal form of the disease. It is believed that most cases of AIPC are the result of constitutive activation of the androgen receptor (AR) pathway, leading us to hypothesize that chronic, prostatic infection could promote cancer progression as a result of pathogen virulence factors activating cell signaling pathways known to induce AR signaling. We found that human herpesvirus-8 (HHV-8) infection of androgen-sensitive LNCaP cells promotes AI cell proliferation. Contrary to our initial hypothesis, we found that, despite enhanced AI cell proliferation, HHV-8-infected LNCaP (LNCaPv219) cells have decreased expression of both AR and AR-regulated genes such as prostate-specific antigen (PSA), implying that the phenotype induced by HHV-8 occurs concomitantly with decreased AR signaling. It is known that HHV-8 maintains latency by epigenetically suppressing lytic gene expression though histone methyltransferase activity by the Polycomb group protein

EZH2, which coincidentally has been shown to be up-regulated in advanced, metastatic prostate cancer tissues. We found that increased EZH2 expression in LNCaPv219 cells results in epigenetic silencing of two tumor-suppressing proteins, microseminoprotein-β (MSMB) and DAB2-interacting protein (DAB2IP). Additionally, we found that LNCaPv219 cells have adopted a pro-mesenchymal phenotype, evidenced by increased expression of mesenchymal markers (N-cadherin and vimentin) and decreased expression of the epithelial marker E-cadherin. Collectively, these results suggest that HHV-8 infection promotes an AI phenotype that mimics the EZH2-mediated transcriptional profile of poorly differentiated prostate tumors.

Pathogenetic Influences of Human Herpesvirus 8 (HHV-8) in Prostate Cancer Progression

By

Justin Garner Mygatt

Dissertation submitted to the Faculty of the Department of Microbiology and Immunology and the Emerging Infectious Disease Graduate Program of the Uniformed Services University of the Health Sciences F. Edward Hèbert School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2012

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Dedication

This thesis is dedicated to my wife, Elisabeth, whose patience, understanding, support and love have been limitless during my medical education.

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Chapter 1

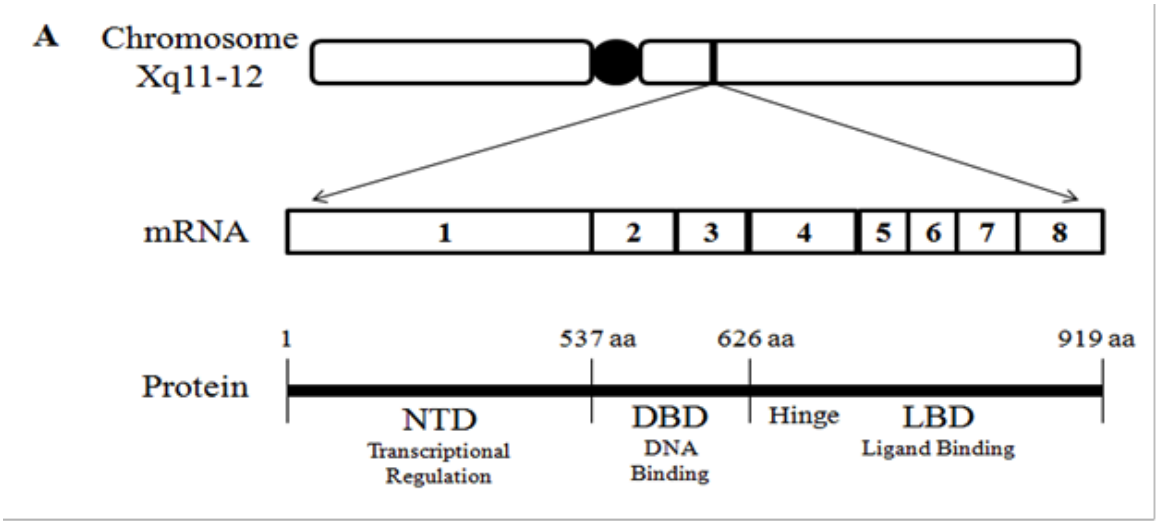
Introduction

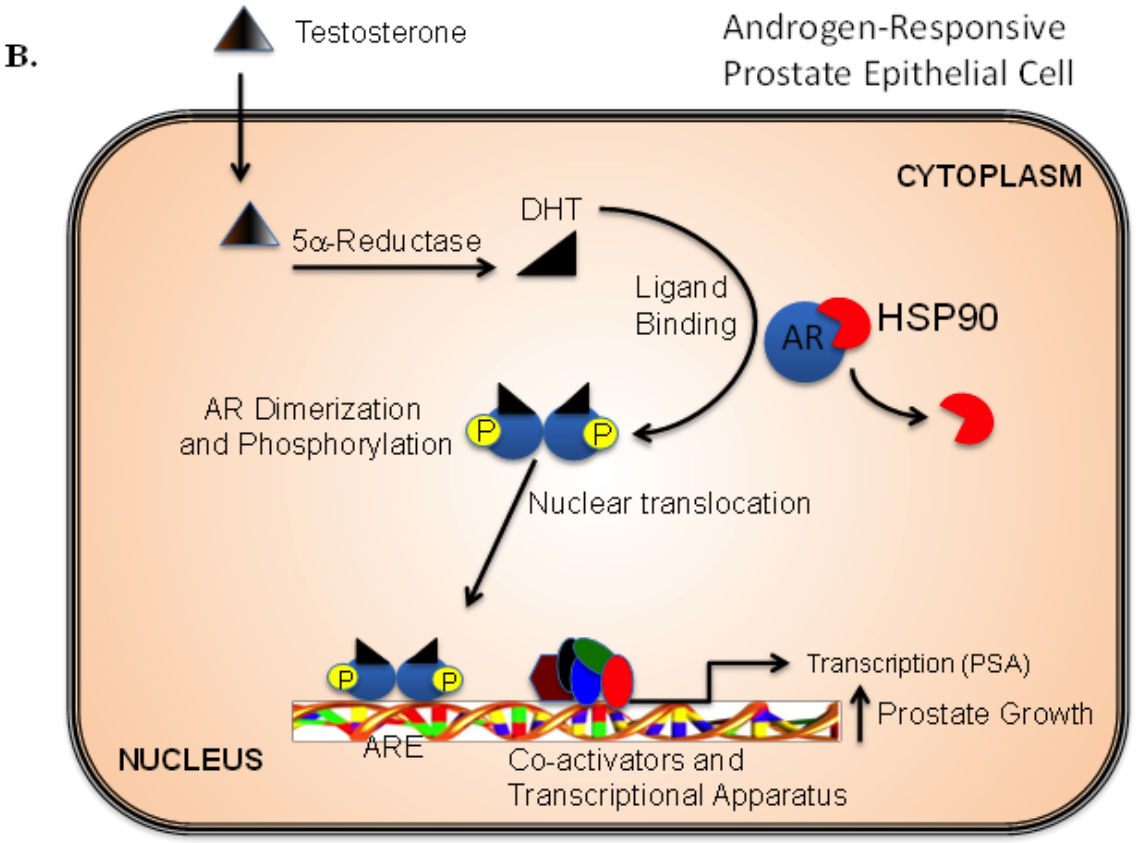
The Prostate Gland and the Androgen Receptor

The male accessory sex organs include the seminal vesicles, the bulbourethral gland and the prostate gland. Completely encircling the urethra, the prostate is located at the base of the bladder. As an exocrine gland, the prostate's primary function is the production of prostatic secretions, which make up a majority of the total volume of the seminal fluid (1). The prostate is composed of a fibromuscular stroma that surrounds glandular lumens formed by an epithelium, which itself is made up of a bilayer of luminal secretory cells and an underlying basal cell layer. The luminal cells express prostate-specific antigen (PSA), prostatic acid phosphatase (ACPP) and microseminoprotein-β (MSMB), the three most common proteins in the prostatic fluid (2), which also contains potassium, citric acid, zinc, amino acids and prostaglandins, all of which contribute to the motility and viability of the sperm (1). While basal cells generate the basal membrane, it is also thought that there are stem cells located within the basal cell layer that are capable of replenishing the secretory cell population (3,4). Lastly, neuroendocrine (NE) cells, the least common cellular constituent of the prostate, are a population of terminally differentiated, non-proliferating cells that possess dendritic outgrowths and express neuroendocrine peptides, such as chromogranin A and neuron-specific enolase (5-7). The exact role for NE cells in the prostate is not completely understood, but they most likely regulate growth in a paracrine manner through the release of NE peptides (6).

The male sex hormones, which as a group are referred to as androgens, are essential for both prostatic development and function and regulate prostate biology by binding to the androgen receptor (AR) (8,9). The testes are the primary source of testosterone in the male. Testosterone within the prostate is converted by the enzyme 5α-reductase to the more potent 5α-dihydrotestosterone (DHT). In conditions such as 5α-reductase deficiency, in which DHT is not biosynthesized, or androgen-insensitivity syndrome, a condition where AR mutations prevent ligand binding, the prostate fails to develop (10, 11). Located on chromosome Xq11-12 (12), *AR* encodes a ligand-induced transcription factor that belongs to the steroid hormone gene family. *In utero,* DHT and testosterone are responsible for driving male development of the fetus. During puberty, DHT and testosterone cause secondary sexual characteristics and thereafter are necessary for fertility and overall male reproductive function (13). Androgens elicit these effects through the AR and AR-regulated gene expression. The AR protein is transcribed from eight exons that encode four distinct functional domains (14; **Figure 1 Panel A**). Exon 1 encodes the N-terminal domain (NTD), which regulates transcriptional activity. The DNA-binding domain (DBD) is encoded by exons 2 and 3 and contains two zinc fingers that recognize specific DNA sequences referred to as androgen response elements (ARE). A hinge domain encoding a nuclear localization signal connects the DBD with the ligand-binding domain (LBD), which is encoded by exons 4-8 (rev in 13, 14). In the absence of ligand, the AR **(Figure 1 Panel B)** is bound in a complex to heat-shock proteins and kept in an inactive state in the cytoplasm. Following DHT binding to AR protein, conformational changes in the receptor result in release of the heat-shock proteins.

Figure 1. Genomic organization of the gene encoding the androgen receptor (AR) and the AR signaling in prostate epithelial cells. Panel A: The transcribed and processed AR mRNA contains 8 exons, which, following translation, make up specific domains of the AR protein. This figure is adapted from (13, 14). **Panel B:** Inactive AR is bound by heat-shock proteins (HSP) that dissociate following androgen binding. Activated receptors dimerize and translocate to the nucleus. The AR binds specific androgen response elements (ARE) and regulates the transcription of AR-dependent genes. The figure is adapted from (9, 16).


A
Chromosome
Xq11-12
mRNA
1
2
3
4
5
6
7
8
Protein
1
537 aa
626 aa
919 aa
NTD
Transcriptional
Regulation
DBD
DNA
Binding
Hinge
LBD
Ligand Binding


B.
Testosterone
Androgen-Responsive
Prostate Epithelial Cell
CYTOPLASM
5α-Reductase
DHT
Ligand
Binding
AR
HSP90
AR Dimerization
and Phosphorylation
P
P
Nuclear translocation
Transcription (PSA)
Prostate Growth
ARE
NUCLEUS
Co-activators and
Transcriptional Apparatus

Hormone-bound, activated receptors form homodimers that then translocate to the nucleus, bind AREs and initiate transcription of AR-regulated genes (15, rev in 16).

Prostate Cancer: Epidemiology and Risk Factors

In the United States, approximately one man out of every six will be diagnosed with prostate cancer in his lifetime (17). Excluding malignancies of the skin, prostate cancer remains the most commonly diagnosed neoplasm in men in the United States. For example, in addition to the 217,000 cases diagnosed in 2010, it was estimated that over 32,000 men in the U.S. died from prostate cancer. Only cancers of the lungs and bronchus caused more cancer-related deaths (18). On a global scale, prostate cancer is the second most frequently diagnosed malignancy, with the highest incidence rates occurring in North America, Western Europe, Australia and New Zealand, where cancer screening is commonly performed (19). The exact cause(s) behind prostate cancer development and progression remain incompletely understood; however, increasing age, family history and race, namely African descent, are well-established risk factors for developing the disease (20).

Prostate cancer is the prototypical malignancy of aging. Fewer than 10% of cases are identified in men younger than 55 years old, whereas 30.7% and 35.3%, or two-thirds of prostate cancer diagnoses, are made in men in the 55-64 and 65-74 age groups, respectively (17). Another risk factor for prostate cancer is family history. Studies have shown that a positive family history for prostate cancer in a first-degree relative increases an individual's risk twofold, and this risk grows larger as the number of affected relatives increases (21). The last clearly defined risk factor for prostate cancer is race. In the United States, African-American men have the highest incidence of prostate cancer (18),

and African-Americans, in comparison to white men in the U.S., are more commonly diagnosed with more aggressive cancer and have higher age-adjusted mortality rates (22). The reason for this disease disparity is unknown, but genetics, metabolism, diet, lifestyle and health care accessibility have all been hypothesized to play a role (23).

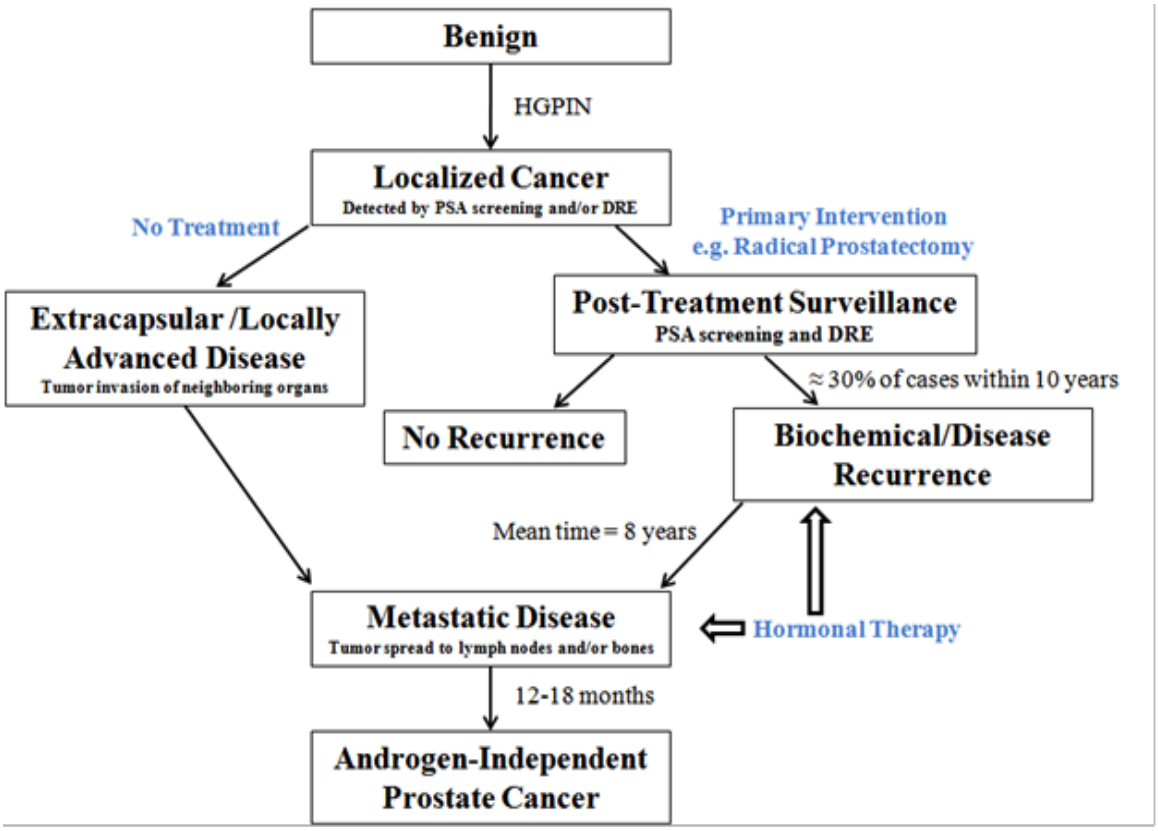
Numerous studies have attempted to identify specific genes and genetic alterations that have direct, causal roles in prostate cancer development. Although polymorphisms in several genes, including *RNASEL* (24), *HPC2/ELAC2* (25) and *MSR1* (26) to name a few, have been reported to confer a higher risk for prostate cancer development, the gene fusion between the *TMPRSS2* promoter and the coding region for the *ERG* gene (*TMPRSS2-ERG)* is the most prevalent and best supported genetic alteration that occurs in prostate cancer (27, 28). The pathological significance of the *TMPRSS2-ERG* gene fusion will be discussed in detail in Chapter 5. Another potential causal factor that has been examined in regards to prostate cancer risk is diet, and numerous epidemiological studies have examined the effect of diet on prostate cancer development and disease progression. It has been suggested that tomatoes, cruciferous vegetables, vitamin E, selenium and carotenoids may provide protective benefits, whereas diets heavy in dairy products, red meat, saturated fats and grilled meats may increase one's risk of prostate cancer and/or aggressive cancer (rev in 29). Related to personal diet, it has been speculated that obesity is another modifiable risk factor that contributes to prostate cancer risk, but studies examining the role obesity plays have been inconclusive (30).

Disease Course and Clinical Symptoms

The evolution of benign prostate tissue to prostatic adenocarcinoma and eventually to metastatic disease is summarized in **Figure 2**. A precursor lesion to prostatic adenocarcinoma is high-grade prostatic intraepithelial neoplasia (HGPIN), a term used to designate proliferating, atypical epithelial cells found in benign prostatic tissue (31, 32). HGPIN is cytologically similar to prostate cancer, and the two conditions have numerous molecular and genetic markers in common (32). Invasive prostate cancer begins when the basal cell layer is breached, and malignant cells enter the stroma. While the natural history of prostate cancer is incompletely understood, it is generally considered to be an indolent disease that can remain localized within the prostate and may be clinically insignificant for years, even decades. Nonetheless, prostate cancer is also a heterogeneous malignancy, and some tumors can be aggressive, a fact that complicates clinical management of prostate cancer (33, 34).

The silent nature of prostate cancer is best understood by the startling fact that in the first half of the twentieth century over 90% of men with prostate cancer presented with metastatic disease (35). Fortunately, today in the United States, most cases of prostate cancer are diagnosed from biopsy specimens obtained following an abnormal digital rectal exam and PSA test while the tumor is still localized (36). Localized prostate cancer, referring to tumors that remain confined within the prostate gland, is most often asymptomatic. When symptoms do occur, they present late in the disease course and represent cancers that have spread beyond the prostatic capsule and invade neighboring organs (37). If the tumor grows superiorly into the urethra or neck of the bladder, a patient may present with a collection of symptoms referred to as “prostatism,” which includes polyuria (increase in urinary frequency), dysuria (difficulty urinating) and slow

Figure 2. Disease course for adenocarcinoma of the prostate. This schematic shows the disease progression of prostate cancer from benign tissue through metastatic and androgen-independent disease. The approximate time between stages of disease progression are also depicted.


Benign
HGPIN
Localized Cancer
Detected by PSA screening and/or DRE
No Treatment
Primary Intervention
e.g. Radical Prostatectomy
Extracapsular /Locally Advanced Disease
Tumor invasion of neighboring organs
Post-Treatment Surveillance
PSA screening and DRE
≈ 30% of cases within 10 years
No Recurrence
Biochemical/Disease Recurrence
Mean time = 8 years
Metastatic Disease
Tumor spread to lymph nodes and/or bones
Hormonal Therapy
12-18 months
Androgen-Independent Prostate Cancer

urinary stream. It should be noted that these symptoms are not specific to prostate cancer and are also seen in benign prostatic hyperplasia (37). Other more infrequent symptoms include hematuria (blood in the urine), hematospermia (blood in the semen), caused by tumor spread into the ejaculatory ducts, and impotence, due to invasion of neurovascular structures (37).

Metastasis occurs when prostate cancer cells penetrate the lymphatic and/or the vascular systems, an event associated more commonly with higher grade and larger volume tumors (38, 39). Berges *et al.* calculated that the doubling time for localized prostate cancer cells is approximately 475 days but only 33 days for metastatic cells in the lymph nodes (40), a finding that illustrates the aggressive nature of metastatic prostate carcinoma cells and the need to find biomarkers to identify aggressive tumors early in the disease course. The pelvic lymph nodes, the pelvis and the bones of the axial skeleton are the most common sites for prostate cancer metastasis (36). Sometimes metastatic spread to bone results in the first symptoms of prostate cancer (37) and can result in significant pain, fractures and compression of the spinal cord (41). Metastatic prostate cancer confers a poor prognosis, with mean survival times of three to five years (42, 43).

Clinical Management of Localized Prostate Cancer

Treatment options for localized prostate cancer include active surveillance, radical prostatectomy or radiation therapy. To help guide clinical management, pathological staging and histological grading of prostate tumor tissue obtained by biopsy is conducted. Prostate cancer staging utilizes the TNM system, which assesses the primary tumor (T), whether lymph nodes (N) are involved and whether metastases (M) are present (44). Developed by Dr. Donald Gleason in the 1960's, the Gleason score, which grades tumors

based on the degree of glandular differentiation and architecture, is the most widely used grading system for prostate cancer and is a very powerful determinant of prognosis for prostate cancer (45), as demonstrated by studies that show how Gleason tumor grading correlates with aggressiveness and survival (46, 47). For an individual with prostate cancer, the treatment strategy takes into account the TNM classification (tumor stage), the Gleason score (tumor grade) and the age and health of the patient, with the goal being to concurrently minimize both the risk of treatment side effects and the possibility of future advanced disease (34, 44).

Studies assessing the watchful waiting approach for men with localized, low-grade prostate cancer have shown a 10-year survival rate around 90%. In the United States, watchful waiting is employed for men with low-grade, low-stage cancer and a life expectancy less than a decade (34, 44, 46). In contrast, for younger men with a longer life expectancy and with localized, low-grade prostatic carcinoma, curative intent is recommended, and radical prostatectomy has become the primary intervention of choice (34, 48). Potential complications following surgery include incontinence and impotence, the risk of which can be lessened with nerve-sparing approaches (49), but cancer eradication and long-term survival rates are excellent (48, 50-52). Radiation therapy is generally used for localized tumors in cases when the patient is older and less healthy and involves external beam radiotherapy or brachytherapy, a procedure in which radioactive seeds are implanted in cancerous tissue (48). Following primary intervention, patients are monitored by PSA testing for disease recurrence. Studies have shown that on average approximately one in three men will have PSA/biochemical recurrence within ten years

of primary intervention (43, 50-52). Following detection of recurrence, Pound *et al.* have reported that the median time to the development of metastatic disease is eight years (43).

Androgen Deprivation Therapy for Advanced and Metastatic Disease

While primary interventions such as radical prostatectomy are the mainstay for treating localized prostate cancers, cases involving advanced and metastatic disease present different challenges. The objective for treating prostate cancer that has extended beyond the prostatic capsule involves controlling further tumor progression in order to increase a patient's survival and maintain quality of life. How best to achieve these goals for patients with locally advanced prostate cancer is controversial, and numerous strategies involving surgery or radiation with or without hormonal therapy have been utilized (53). For the patient with biochemical recurrence or with metastatic disease, hormonal therapy, also known as androgen deprivation therapy (ADT), is the foundation for any treatment regimen (48).

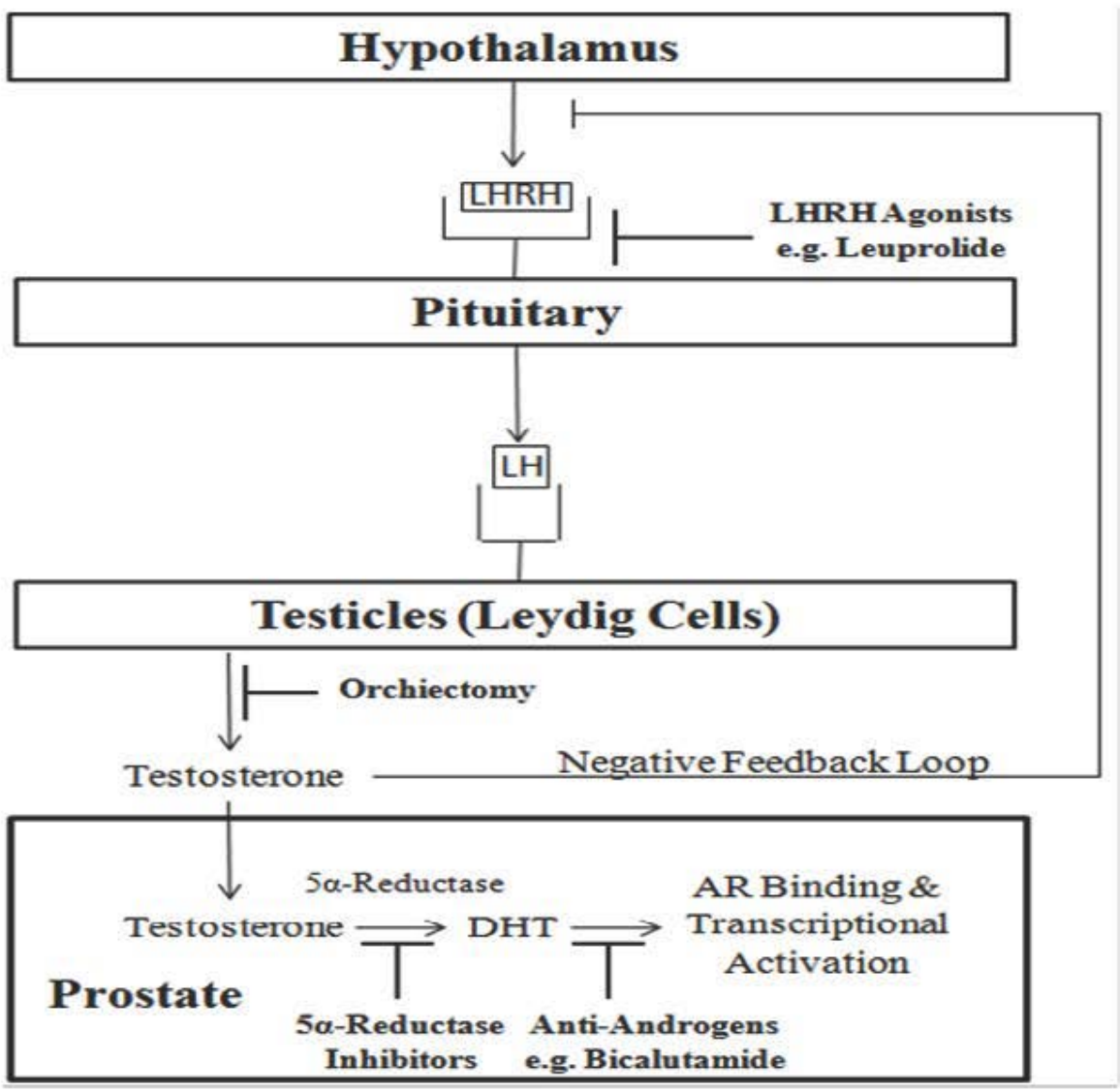
In 1941, Huggins and Hodges demonstrated that patients with metastatic prostate cancer following bilateral orchiectomy (surgical removal of the testes) or estrogen treatment experienced alleviation of cancer-related symptoms (54). This work, which exposed prostate cancer dependence on androgens, won the researchers a Nobel Prize and engendered the concept of ADT for advanced and metastatic prostate cancer (55). Subsequently, randomized trials conducted in order to compare orchiectomy with oral estrogen treatments for treating metastatic prostate cancer revealed that oral estrogen therapy, while as effective as orchiectomy, resulted in significant, increased risk for cardiovascular and thromboembolic events (55). Fortunately, research on the

hypothalamic-pituitary-gonadal (HPG) axis and the discovery and characterization of the AR enabled the development of other pharmacological interventions for ADT.

The hypothalamus synthesizes and releases luteinizing hormone (LH)-releasing hormone (LHRH), which signals the pituitary to produce LH. In Leydig cells in the testes, LH signaling induces testosterone synthesis. Current pharmacological androgen ablation interventions are designed to disrupt the HPG axis **(Figure 3)**. Andrew Schally elucidated the structure of LHRH and using synthesized LHRH agonists discovered that persistent LHRH signaling paradoxically inhibited LH release. By giving LHRH agonists to men with advanced prostate cancer, Schally's group demonstrated that LHRH agonists strongly reduced serum testosterone and significantly relieved bone pain caused by metastatic disease. LHRH agonists, including leuprolide, goserelin and nafarelin, have since been developed and are used for ADT (56-58). Although LHRH inhibitors are for patients psychologically more palatable than bilateral orchiectomy, both treatment strategies have similar, unpleasant side effect profiles, including menopausal symptoms, such as hot flashes, sweating, weight gain, loss of libido and osteoporosis (59).

Another approach to ADT resulted from determination of the structure and function of the AR, which enabled screening for compounds that can directly bind to and block AR signaling. Cyproterone, a steroid compound, was the first anti-androgen, but it was soon discovered that the drug binds AR both in the prostate and in the hypothalamus and pituitary, thereby inhibiting the negative feedback mechanism and ultimately leading to LH release and increased testosterone levels (55). To circumvent this problem and to reduce discomforting, sexual side effects caused by surgical or medical castration, drug companies developed non-steroidal anti-androgens, including flutamide and

Figure 3. The hypothalamic-pituitary-gonadal endocrine axis and points of clinical intervention in androgen deprivation therapy. This schematic shows the hypothalamic-pituitary-gonadal axis specific for the biosynthesis of testosterone and includes the prostate, an androgen-regulated organ. Also depicted are the negative feedback loop that regulates male sex hormone synthesis and the intra-prostatic conversion of testosterone to the more potent androgen, DHT, which induces AR transcriptional activity. This figure is adapted from (55).


Hypothalamus
LHRH
LHRH Agonists
e.g. Leuprolide
Pituitary
LH
Testicles (Leydig Cells)
Orchiectomy
Testosterone
Negative Feedback Loop
5α-Reductase
Testosterone
DHT
AR Binding &
Transcriptional
Activation
Prostate
5α-Reductase
Inhibitors
Anti-Androgens
e.g. Bicalutamide

bicalutamide, but randomized trials have demonstrated that these drugs are not as effective as castration or LHRH inhibitors (60). Clinical trials have also been performed to assess the effectiveness of combining treatments, for example using a LHRH agonist with a non-steroidal anti-androgen, but meta-analyses have shown that such an approach confers only modest to no benefit versus therapy with a single drug (61, 62). Overall, approximately 80% of men with advanced prostate cancer will respond positively to hormonal treatment. Nevertheless, ADT, while palliative, is not curative and approximately 12-22 months after initiating therapy, patients with metastatic prostate cancer will experience disease progression despite androgen ablation. At this point the cancer is considered hormone-refractory or androgen-independent, and the prognosis is dismal, with survival measured in months (9, 42, 48).

Pathogenesis of Androgen-Independent Prostate Cancer (AIPC)

As previously mentioned above, despite improvements in detecting and treating prostate cancer, approximately 30% of men will experience biochemical recurrence within a decade of primary treatment. Time to biochemical recurrence following primary intervention, the rate at which the PSA value rises (measured as PSA-doubling time) and tumor Gleason score have all been shown to correlate with time to metastatic disease, which fortunately can take years to develop (43). Unfortunately, however, ADT cannot cure metastatic disease, and AIPC, the lethal form of prostate cancer, invariably develops. It is accepted that in a majority of cases AIPC is the result of constitutive activation of the AR-signaling pathway despite androgen ablation (rev in 16, 63). There is a dearth of effective treatments for AIPC, but research efforts have identified several pathogenic

mechanisms that enable AR-signaling even when androgens are not available to tumor cells, findings that could facilitate the development of new treatment options.

Maintenance of intratumoral androgens. Despite the effectiveness of ADT in radically reducing serum testosterone levels, it has been shown that androgens are still detectable within prostate tumors, and it has been demonstrated that even though DHT levels within tumor tissue can be significantly reduced, the remaining hormone is still adequate to activate AR signaling (64). One possible source for intratumoral androgens in AIPC tumors is up-regulated expression of enzymes involved in steroidogenesis. Indeed, Montgomery *et al.* showed that androgen-independent and metastatic tumors compared to localized prostate tumors had increased expression of genes such as *CYP17A1* and *AKR1C3*, which are capable of biosynthesizing testosterone from cholesterol precursors, thus allowing AR signaling to continue unabated despite pharmacological blockade of normal testosterone and DHT production (65). To combat this route of androgen biosynthesis, abiraterone acetate, an irreversible CYP17 inhibitor, has been developed and has shown promising antitumor activity in approximately three-quarters of patients with advanced, androgen-independent disease (66).

Increased AR expression. A second mechanism believed to maintain AR-signaling in androgen deprived conditions is increased AR expression, which occurs as an adaptation to low levels of androgens. In animal models, increased AR expression is sufficient for the development of AIPC, as the increased levels of receptor allow the tumor cells to adapt to decreased levels of ligand (67). Only a minority, approximately 25%, of androgen-independent tumors have *AR* gene amplification (68, 69), but it has been demonstrated that increased AR protein expression occurs commonly in androgen-

independent tumors (70). Furthermore, in addition to enabling androgen-independent growth, increased AR expression levels in androgen-independent tumor samples have been shown to be predictive of increased risk for biochemical recurrence and future disease progression (71, 72).

AR Mutations. Numerous *AR* mutations have been identified (Androgen Receptor Gene Mutations Database www.mcgill.ca/androgendb/). Although quite rare in primary tumors, the prevalence of *AR* mutations increases with disease stage, which suggests that *AR* mutations enable or predispose for disease progression (73). Mutations in the LBD have been shown to increase sensitivity for androgens, allow binding and activation by other hormones, and even allow anti-androgens to serve as receptor agonists (rev in 9, 14).

Ligand-Independent AR Activation. Research has shown that growth factors, such as insulin-like growth-factor-1 (IGF-1), keratinocyte growth factor (KGF), and epidermal growth factor (EGF), activate the AR and induce AR transcriptional activity (74). Subsequent studies demonstrated that cellular signal transduction pathways and transcription factors are responsible for ligand-independent AR activation. Protein kinases, such as the mitogen-activated protein kinase (MAPK) and AKT, drive signaling pathways that are involved in cellular functions such as cell-cycle progression, anti-apoptotic/survival signals and cellular proliferation. Deregulated, constitutive activity of the MAPK and AKT pathways has been documented in numerous malignancies (75, 76), and in prostate cancer, both the MAPK and the AKT pathways both independently and in tandem have been shown to promote tumor cell proliferation (77-79). Activation of MAPK in particular has been shown to directly promote AR signaling (80, 81), and the

degree of MAPK activation within tumor specimens has been shown to correlate directly with the stage of the tumor, Gleason score and risk of disease progression (82, 83).

Downstream of both MAPK and AKT, the transcription factor nuclear factor-κB (NF-κB), which regulates the transcription of genes involved in inflammation, cell proliferation, apoptosis and cell migration, is constitutively active in numerous cancer types (rev in 84). In addition to being implicated in ligand-independent AR activation and inducing expression of AR-regulated genes (85, 86), NF-κB activation, in studies utilizing immunohistochemistry, is found more commonly in prostate cancer lymph node metastases than in localized tumors. In fact, NF-κB activation status in conjunction with Gleason scoring accurately identifies patients at high risk for disease progression (87, 88). Interestingly, NF-κB regulates the expression of interleukin-6 (IL-6), a pleiotropic, pro-inflammatory cytokine that can also function as a growth factor for prostate cancer and prostate cancer cell lines (89, 90). Clinically, serum IL-6 levels correlate with tumor burden, metastasis (91) and AIPC (92). IL-6 activates signal transducers and activators of transcription 3 (STAT3), another transcription factor involved in ligand-independent AR activation (81). Like NF-κB, the level of STAT3 activation in tumor samples has been shown to correlate with higher grade tumors, and STAT3 is active in three-quarters of lymph node metastases and two-thirds of bone metastases, respectively (93, 94).

Prostate Infections: Potential Drivers of Prostate Cancer Progression?

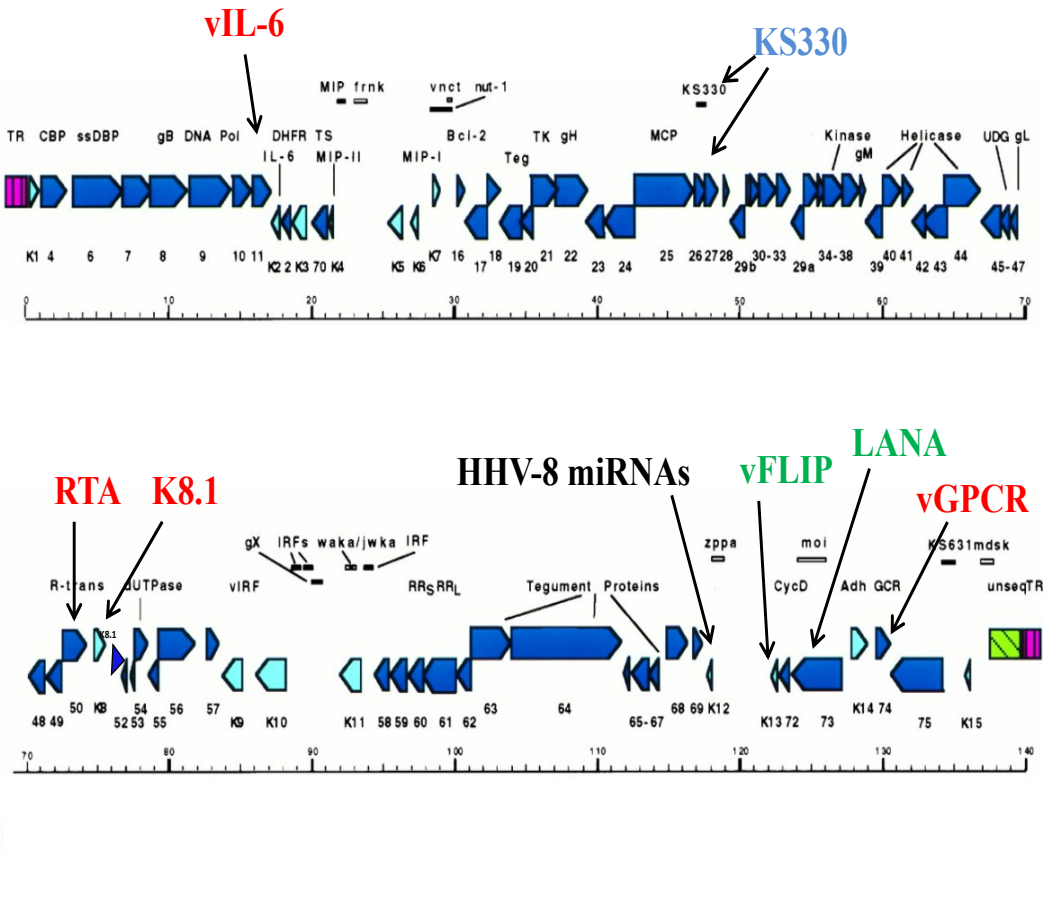
It is widely accepted that constitutive activation of cell signal transduction pathways can result in ligand-independent AR activation, prostate cancer progression and the development of AIPC. Host cell signaling pathways are also commonly targeted by microbial and viral pathogenic mechanisms. Two well-studied oncogenic pathogens,

Helicobacter pylori and Hepatitis C virus, which are linked to the development of gastric adenocarcinoma and hepatocellular carcinoma, respectively, both express an array of virulence factors and peptides that interact with and subvert multiple host signaling cascades and transcription factors that when deregulated lead to malignant transformation (rev in 95, 96). Gram-negative bacteria, such as uropathogenic *Escherichia coli,* the most common infection of the prostate (97), express on their outer membrane lipopolysaccharide (LPS), a virulence factor recognized by Toll-like receptor 4 (TLR4) on the host cellular surface. Binding of LPS by TLR4 results in activation of several signal transduction pathways, including MAPK, and activates transcription factors, such as NF-κB (rev in 98). These examples illustrate the capacity for infections to negatively affect normal cellular function and in extreme cases drive cellular transformation and cancer progression.

Human herpesvirus 8 (HHV-8)

Besides bacterial infections, some human viruses have been found in the prostate, including human herpesvirus-8 (HHV-8) (99, 100). In regard to this work, it is important to note that an association between HHV-8 and prostate cancer has not been established. HHV-8 belongs to the same gammaherpesvirus subfamily as Epstein-Barr virus, and is the etiological agent for the most common AIDS-related malignancy, Kaposi's sarcoma (KS), a tumor of endothelial and lymphatic origin (101), and several other lymphoproliferative disorders, such as body-cavity based lymphoma (102). The HHV-8 episome (**Figure 4)** is between 165-170 kilobases in size (103) and encodes 87 open reading frames and over a dozen microRNAs (104). Similar to other herpesviruses, HHV-8 establishes persistent infection by constructing a circular, double-stranded DNA

Figure 4. The HHV-8 episome. HHV-8 encodes 87 open reading frames and over a dozen miRNAs. KS330 is a viral genetic sequence from a gene encoding the minor capsid protein and is used clinically to identify the presence of the virus. Latent genes (green) include LANA, which tethers the viral episome to the host genome (107, 108), and oncogenic genes such as vFLIP. Lytic genes (red), such as vGPCR and vIL-6, also possess transformative properties and are known to activate multiple host cell signaling molecules, including MAPK, AKT and STAT3 (116-118, 120, 121). The replication and transcriptional activator (RTA) initiates the viral lytic cycle (159) and induces the expression of lytic genes such as K8.1, which encodes a viral envelope glycoprotein (160). Each vertical mark along the scale denotes one kilo-basepair. This figure is adapted from (103).


vIL-6
KS330
RTA
K8.1
HHV-8 miRNAs
vFLIP
LANA
vGPCR
TR CBP ssDBP gB DNA Pol DHFR TS
IL-6 MIP-II MIP-I Bcl-2 TK gH
Teg MCP Kinase gM Helicase UDG gL
MIP frnk vnct nut-1 KS330
R-trans dUTPase vIRF gX IRFs waka/jwka IRF
RRS RRL Tegument Proteins zppa
moi CycD Adh GCR KS631mdsk unseqTR

episome that binds to the host genome (105) and has distinct transcriptional programs that dictate latent and lytic viral cycles. The identification of both latent and lytic HHV-8 transcripts within the prostate by *in situ* hybridization (106) indicates that the virus is able to establish persistent, latent infections and can also enter the lytic phase and replicate within this target organ, an important finding, because both latent and lytic viral proteins target host cell signaling proteins and contribute to HHV-8-related pathology. For example, the latently expressed latency-associated nuclear antigen (LANA) is best known for tethering the viral episome to the host genome (107, 108), but LANA also has been shown to affect cell proliferation and apoptosis pathways by interfering with two of the most critical tumor suppressor genes, p53 and Rb (109, 110). Another latent viral transcript is the viral FADD-like interferon converting enzyme inhibitory protein (vFLIP). vFLIP is known to activate the NF-κB pathway (111, 112) and induces the expression of numerous pro-inflammatory cytokines, such as IL-6 (113, 114), that both contribute to KS pathogenesis, and, in the context of prostate cancer, have been shown to serve as cancer cell growth factors (89, 90). In support of this notion, Montgomery and co-workers found that HHV-8 infection in the prostate does indeed induce localized inflammation (115). Despite not being ubiquitously expressed by HHV-8 infected cells, some HHV-8 lytic proteins have been described to possess pro-oncogenic characteristics. The viral G protein-couple receptor (vGPCR) is a lytic protein that promotes cellular proliferation by simultaneously activating several signal transduction pathways, including MAPK, AKT and the transcription factor NF-κB (116-118). A second lytic protein, viral IL-6 (vIL-6) is a structural and functional homolog to cellular IL-6 (119) and through activation of the STAT3 pathway also contributes to cellular growth (120, 121).

Statement of Problem

Prostate cancer is exceedingly common, yet compared to other malignancies mortality is modest, due to the indolent clinical course observed in most cases and improved diagnostics and treatment. Death from prostate cancer is exclusively the result of metastatic, castration resistant disease. The most significant clinical problem for the patient with localized prostate cancer is the inability to distinguish indolent, low-risk tumors from aggressive tumors with potential for future disease progression. At the present time, the Gleason grading system, tumor stage and PSA collectively provide the best assessment of prognosis. As such, identification of biomarkers and risk factors that increase an individual's risk of developing advanced, metastatic, androgen-independent disease would vastly improve the clinical management of prostate cancer.

Since host cell signaling pathways are commonly targeted by microbial and viral pathogenic mechanisms, we hypothesized that infectious agents could serve as model systems to explore the cellular processes and pathobiology of prostate cancer progression. We asked whether a pathogen within the prostate tumor microenvironment could induce cellular conditions that are conducive to the development of an androgen-independent growth state analogous to the phenotype commonly seen in advanced disease. Using *in vitro* and *in vivo* prostate infection models, other groups have shown that *Propionibacterium acnes* and *Escherichia coli* infections of benign prostate cells induce significant changes in cell proliferation rates (122-124). However, to our knowledge, no studies have assessed how a chronic infection of malignant but otherwise androgen-sensitive prostate cells would affect disease course. Therefore, the goal of this study was to establish androgen-sensitive LNCaP cells that are persistently infected with HHV-

8, a herpesvirus that has been detected in the prostate (106, 115, 125). Using HHV-8 to study how an infection affects prostate cancer progression is a novel concept with potential implications for biomarker discovery due to the fact that this virus encodes for several proteins that activate pathways believed to be intrinsic both to prostate oncogenesis and the development of castration resistance.

Hypothesis

We hypothesize that prostatic infection with HHV-8 is a risk factor for prostate cancer progression and for the development of an androgen-independent phenotype. Furthermore, we predict that an HHV-8 infection within the prostate tumor microenvironment could modulate cell signaling pathways and alter gene expression patterns that promote cancer progression through activation of the AR signaling pathway, thereby promoting an androgen-independent phenotype. Two specific aims were developed to test this hypothesis.

Specific Aim #1: Demonstrate that prostate cancer cell lines are permissive to infection with HHV-8 and examine the impact of viral infection on markers for prostate cancer progression.

Specific Aim #2: Examine the impact of HHV-8 infection on prostate cancer gene expression, focusing on the expression of genes that have been shown to correlate with cancer progression.

Chapter 2

Materials and Methods

Cell Lines. Table 1 provides an overview and characteristics of the cell lines used in this project. The transformed prostate epithelial cell line, RWPE2-W99 (RWPE2; 126), and the prostate cancer cell lines LNCaP, DU145 and PC3 were obtained from the American Type Culture Collection (Manassas, VA). RWPE2 cells were maintained in K-SFM (Invitrogen, Carlsbad, CA), supplemented with bovine pituitary extract (0.05 mg/ml; Invitrogen) and human recombinant epidermal growth factor (5 ng/ml; Invitrogen). LNCaP and PC3 cells were cultured in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). DU145 cells were cultured in E-MEM (Quality Biological, Gaithersburg, MD) with 10% FBS. Infected cell lines were maintained in the appropriate medium with puromycin (1.0 μg/ml; Calbiochem, EMD Chemicals, Inc., Gibbstown, NJ). All cells were incubated at 37°C in 5% CO_2. Androgen deprivation experiments used RPMI-1640 supplemented with 10% charcoal-stripped FBS (CS-FBS; Invitrogen). BCBL-1, a body cavity-based lymphoma cell line that is persistently infected with KSHV, was provided by Michael McGrath and Don Ganem, from the National Institutes of Health AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MD) and was cultured in RPMI-1640 supplemented with 10% FBS.

Virus and Infections. All infections were established with rKSHV.219, a recombinant HHV-8 virus encoding both the green fluorescent protein (GFP) under the

Table 1. Cell lines used in this project.

Cell Line	Source	AR/Androgen Sensitivity
RWPE2-W99	Prostate, normal epithelium transformed with Ki-ras	AR+/Grow in serum-free media
LNCaP	Prostatic adenocarcinoma, supraclavicular lymph node metastasis	AR+/Androgen-sensitive
DU145	Prostatic adenocarcinoma, brain metastasis	AR-/Androgen-insensitive
PC3	Prostatic adenocarcinoma, androgen-independent bone metastasis	AR-/Androgen-insensitive

control of the constitutively active human elongation factor 1-α promoter and red fluorescent protein (RFP) driven strictly by the HHV-8 lytic polyadenylated nuclear RNA promoter. rKSHV.219 also carries a gene for puromycin resistance, which allows for selection and the maintainence of the viral episome in infected cells (127). Viral inoculums were made by reactivating rKSHV.219-infected Vero cells with 1.5 mM of valproic acid (Sigma-Aldrich, St. Louis, MO) and monitoring for the expression of RFP. Four to five days later, cells and supernatants were harvested and collected by centrifugation at 2500 rpm for 10 minutes at 4°C. Supernatant with free KSHV particles was kept on ice. The cell pellet was resuspended in 3 mL of Opti-mem (Invitrogen) and frozen and thawed three times to release cell-associated virus. The lysate was then centrifuged at 2500 rpm for 10 minutes at 4°C to remove cellular debris and combined with the original culture supernatant, sterile-filtered through a 0.45 μm membrane filter and stored at -80°C. Cells to be infected were seeded in 6 well plates in the appropriate growth medium. When the cultures were around 60-70% confluent, media was removed and 1 mL of the rKSHV.219 inoculum was added to each well. Three hours later, an additional 1 mL of growth media was added, and the cells and inoculums were incubated for 24 hours at 37°C. Cell cultures were monitored for GFP expression. When GFP was observed, 0.5 μg/ml puromycin was added to the cell cultures to begin selection. For all experiments involving LNCaP and LNCaPv219 cells, only cells of a low (< 20) passage number were used.

Microscopy. Images were captured using an AxioCam MRm digital camera (Carl Zeiss North America), attached to a Zeiss Axio Observer A.1 inverted fluorescent

microscope (Carl Zeiss). Image analysis and pseudo-coloring was performed with the AxioVision Release 4.6 software.

DNA Isolation and Polymerase Chain Reaction (PCR). DNA was isolated from infected and uninfected cell lines using the DNeasy Blood and Tissue DNA Extraction Kit (Qiagen, Germantown, MD). PCR was performed with the Platinum PCR SuperMix High Fidelity system (Invitrogen). PCR products were run on a 1.5% agarose gel with ethidium bromide and visualized with UV transillumination. Primer sequences used were: **KS330-F:** 5' AGC CGA AAG GAT TCC AAA TTG TGC 3' **KS330-R:** 5' CCG TGT TGT CTA CGT CCA GAC GAT A 3' **GAPDH-F**: 5' GAA GGT GAA GGT CGG AGT CA 3' **GAPDH-R:** 5' TTC ACA CCC ATG ACG AAC AT 3'.

Immunofluoresence Assays. 2.5 x 10^5 cells were seeded per well in eight-well chamber slides (Lab-Tek Chamber Slide System, Nunc, Rochester, NY) and were incubated at 37ºC for 48 hours. Cells were fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS for 30 minutes and then permeabilized in 2% paraformaldehyde and 0.1% Triton X-100 (Calbiochem, La Jolla, CA) in PBS for an additional 30 minutes. Following fixation and permeabilization, the cells were washed once with PBS and incubated in blocking buffer (2% FBS in PBS) for one hour at 37ºC. Blocking buffer was then removed, rat anti-LANA primary antibody LN35 (Abcam, Cambridge, MA) in fresh blocking buffer was applied and the cells were incubated overnight at 37ºC. The next morning cells were washed five times with PBS and then incubated with fluorescently-labeled anti-rat Alexa Fluor-647® secondary

antibody (Invitrogen) for one hour at 37ºC. Secondary antibody was removed and the cells were washed again five times with PBS. Finally, coverslips were mounted onto the slides using Vectashield Mounting Medium for Fluorescence with DAPI (Vector Laboratories, Burlingame, CA). Cells were visualized using fluorescence microscopy.

RNA Isolation and Semi-Quantitative Reverse Transcription PCR (sqRT-PCR). RNA was isolated using the *GenCatch* Total RNA Miniprep Kit (Epoch Biolabs, Sugar Land, TX). Genomic DNA contamination was eliminated by treating the RNA samples with 2U DNase for 30 minutes at 37°C (TURBO DNA-free, Ambion, Austin, TX). sqRT-PCR was performed using the SuperScript One-Step kit (Invitrogen), which combines reverse transcription and thermal cycling into one reaction. Following the 45 minute reverse transcription run at 55°C and 2 minute initial denaturation at 94°C, the samples were ran at 94°C for 30 seconds, 55°C for 30 seconds and 68°C for 60 seconds for 35 cycles, followed by a final extension at 68°C for seven minutes. sqRT-PCR products were run on a 1.5% agarose gel with ethidium bromide and visualized with UV transillumination. Primer sequences were: **RTA-F:** 5' GGG AGT TGC CTG TAA TGT CAG C 3' **RTA-R:** 5' CCT CTC TTT GCT TCT CTG CTT TCG 3' **K8.1-F:** 5' CGT GGA TCC CTC CTA ATC CTA TGC TTT GTC TGG T 3' **K8.1-R:** 5' TGC GGA ATT CAT GGG TCC GTA TTT CTG CAT TGT AGT GCG 3' **GAPDH-F**: 5' GAA GGT GAA GGT CGG AGT CA 3' **GAPDH-R:** 5' TTC ACA CCC ATG ACG AAC AT 3'

Immunoblotting. Whole cell extracts were made using a high salt extraction buffer (courtesy of Dr Mary Lou Cutler, USUHS, Bethesda, MD) containing 400 mM

NaCl, 10 mM Hepes pH 7.5, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 5% glycerol, 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and 10 μL/ml HALT Protease Inhibitor (Thermo Scientific, Waltham, MA). Samples were separated on 4%-12% NuPage Bis-Tris gels (Invitrogen), transferred to nitrocellulose membranes and blocked in SuperBlock Blocking Buffer in TBS (Thermo Scientific) for 2 hours at room temperature. Blots were then incubated with primary antibody and 0.1% Tween-20 overnight at 4°C. Primary antibodies used were: **AR** (1:1000 Cell Signaling Technology, Danvers, MA), **PSA** (1:500; Dako, Glostrup, Denmark), **p44/42 MAPK** (1:1000; Cell Signaling), **phospho-p44/42 MAPK** (1:1000; Cell Signaling), **NF-κB p65** (1:1000; Cell Signaling), **phospho-NF-κB p65** (1:1000; Cell Signaling), **EZH2** (1:1000; Cell Signaling), **SUZ12** (1:1000; Cell Signaling), **Tri-methyl H3K27** (1:1000; Cell Signaling) and **GAPDH** (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). Blots were incubated in secondary antibody at room temperature for 45 minutes, followed by five 10 minute washes in PBS-T and two 10 minute washes in PBS. Bands were detected with SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific).

Cell Proliferation Assays. Cells to be used in the assay were grown in the appropriate media, harvested, washed, and re-suspended in growth media with 10% FBS or media with 10% CS-FBS for LNCaP androgen-independence studies. Cells were seeded in a 96 well plate at a density of 10^4 cells/well in 100 μL of media. Plates were then incubated at 37°C, and media was changed 48 hours after seeding. Cell proliferation was determined using the CellTiter AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. The assay utilizes a MTS tetrazolium compound that is reduced by viable cells to a formazan product, the

quantity of which is directly proportional to the number of living cells in culture. Absorbance at 490 nm was measured using a Versamax microplate reader (Molecular Devices, Sunnyvale, CA) two hours after the addition of the tetrazolium compound. Background absorbance of culture media was accounted for by measuring absorbance in control wells containing culture media alone.

Cloning, Expression Vectors, and Transfection. To clone MSMB, the complete open reading frame of MSMB was reverse transcribed and amplified from LNCaP mRNA using the following primers: **MSMB-F:** 5' CGG GGA TCC ATG AAT GTT CTC CTG GGC AGC GTT 3' **MSMB-R:** 5' CTG CTC GAG TTA GAT TAT CCA TTC ACT GAC AGA 3'. MSMB cDNA was then cloned into the eukaryotic expression vector pcDNA3.1/Hygro (Invitrogen). Transfection of prostate cancer cell lines was done using jetPRIME transfection system (Polyplus-Transfection Inc., New York, NY), according to the manufacturer's instructions. Derivation of MSMB-expressing cell lines was achieved by hygromycin (Invitrogen) antibiotic selection and stably-expressed recombinant MSMB was confirmed by real-time quantitative RT-PCR.

Real-time Quantitative Reverse Transcription PCR (qRT-PCR). 5×10^5 LNCaP and rKSHV.219-infected LNCaP were seeded in either FBS- or CSFBS-supplemented RPMI media. After 4-5 days of growth, cells were harvested and RNA was extracted using the *GenCatch* Total RNA Miniprep Kit (Epoch Biolabs). As for sqRT-PCR, genomic DNA contamination in the RNA template used for qRT-PCR was eliminated by treating the samples with 2U DNase for 30 minutes at 37°C (TURBO DNA-free, Ambion). cDNA was synthesized from 1.5 μg of total RNA according to the

manufacturer's instructions with SuperScript III Reverse Transcriptase (Invitrogen). Each qRT-PCR reaction was carried out in triplicate in a 25 μL reaction with the SYBR GreenER qPCR SuperMix kit (Invitrogen). Using the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA), reactions were run for 15 minutes at 95°C for denaturation, followed by 40 cycles of 95°C for 30 seconds and 60°C for 30 seconds. For each experiment, a PCR reaction was run without template for a negative control. C_t values for the gene of interest (GOI) were normalized to β_2-microglobulin (β_2M) C_t (ΔC_t = C_t GOI – C_t β_2M) to generate $\Delta\Delta C_t$ values. RQ values to determine relative expression of each GOI were calculated by raising 2 to the negative value $\Delta\Delta C_t$ for each sample. Primer sequences used were: **AR-F:** 5' ATG GTG AGC AGA GTG CCC TAT C 3' **AR-R:** 5' ATG GTC CCT GGC AGT CTC CAA A 3' **PSA-F:** CGC AAG TTC ACC CTC AGA AGG T 3' **PSA-R:** 5' GAC GTG ATA CCT TGA AGC ACA CC 3' **MSMB-F:** 5' AAC TCG GAG TGG CAG ACT GAC A 3' **MSMB-R:** 5' CCT CCT TCT TGA AGA TTC TTT GGC 3' **PTHrP-F:** 5' GAA CTC GCT CTG CCT GGT TAG A 3' **PTHrP-R:** 5' GTC CTT GGA AGG TCT CTG CTG A 3' **E-cadherin-F:** 5' GCC TCC TGA AAA GAG AGT GGA AG 3' **E-cadherin-R:** 5' TGG CAG TGT CTC TCC AAA TCC G 3' **N-cadherin-F:** 5' CCT CCA GAG TTT ACT GCC ATG AC 3' **N-cadherin-R:** 5' GTA GGA TCT CCG CCA CTG ATT C 3' **Vimentin-F:** 5' AGG CAA AGC AGG AGT CCA CTG A 3' **Vimentin-R:** 5' ATC TGG CGT TCC AGG GAC TCA T 3' **Zeb1-F:** 5' GGC ATA CAC CTA CTC AAC TAC GG 3' **Zeb1-R:** 5' TGG GCG GTG TAG AAT CAG AGT C 3' **Snail-F:** 5' TGC CCT CAA GAT GCA CAT CCG A 3' **Snail-R:** 5' GGG ACA GGA GAA GGG CTT CTC 3'

Statistical analyses. Error bars in graphical data represent mean ± standard deviation. Unless otherwise noted in the figure legend, the Student's t-test was used to calculate the statistical relevance between groups, with $P < 0.05$ considered to be significant.

Chapter 3

Human herpesvirus-8 (HHV-8) promotes an androgen-independent phenotype in the androgen-sensitive LNCaP cell line

Introduction

It is estimated that in 2010 there were over 200,000 new cases of prostate cancer in the United States, making it the most commonly diagnosed, non-cutaneous malignancy for men in the U.S. (18). Following primary interventions to treat prostate cancer, approximately one in three men will develop biochemical recurrence (43, 50-52). Ever since Huggins and Hodges demonstrated tumor dependence on androgens, androgen deprivation therapy has been the primary treatment modality for cancer recurrence (54). Despite initial efficacy, many cases over time become resistant to treatment, at which point the cancer is considered androgen-independent prostate cancer (AIPC). How prostate cancer becomes androgen-independent is incompletely understood, resulting in few therapeutic options for AIPC, which is the metastatic and fatal form of the disease. Recently, inflammation has been proposed as a contributing factor to both prostate cancer initiation and progression (99, 100), and it has been demonstrated that *P. acnes* and uropathogenic *E. coli* infections have been shown to strongly elicit inflammatory responses and enhance prostate cell proliferation (122-124). Furthermore, pathogens often activate host cell signaling pathways, a phenomenon supported by Fehri *et al.* who showed that *P. acnes* infection of a prostate epithelial cell line resulted in NF-κB and STAT3 activation (122), both of which have been shown to cause ligand-independent activation of the AR signaling pathway and drive prostate cancer progression (81, 85,

86). Thus, in the setting of prostate cancer, an underlying infection could alter cellular growth properties and activate signal transduction pathways that promote and enable disease progression. We hypothesized that prostatic infection with HHV-8, a herpesvirus that infects the prostate (106, 115, 125), could have a profound influence on prostate cancer progression and the development of an androgen-independent phenotype. Although there is no evidence to support an association between HHV-8 infection and prostate cancer, presence of the virus in the prostate is notable. HHV-8 encodes several proteins, including vFLIP, vIL-6 and a viral G protein-coupled receptor that activate the same host cell signaling pathways that are critical in prostate cancer progression (111-113, 116, 117, 120, 121). To test the contribution of HHV-8 to prostate cancer progression, we established chronic HHV-8 infection in the androgen-sensitive LNCaP cell line and assessed the effect of infection on markers for prostate cancer progression.

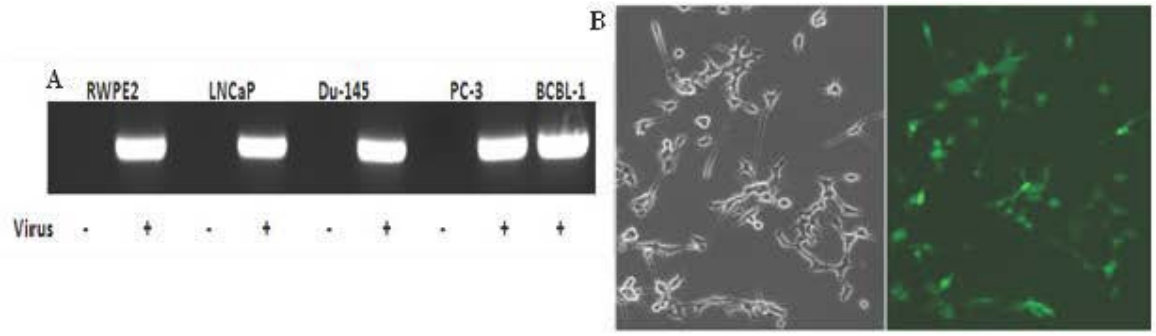
Results

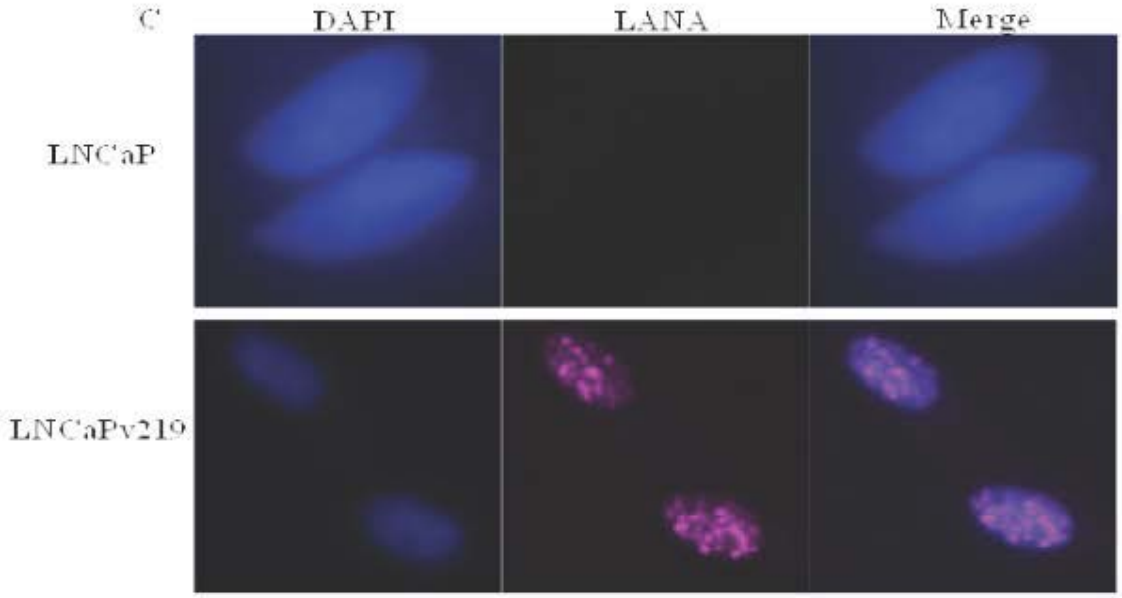
Prostate cancer cell lines are permissive to rKSHV.219 infection. A number of infectious agents have been detected in prostate epithelium (rev in 99), leading to the notion that infections may contribute to conditions that support prostate cancer initiation and/or progression. Murine and *in vitro* cell culture models have been established for studying bacterial infection of the prostate and examining whether pathogen-induced inflammation contributes to prostate cancer development (99, 100, 122-124), but none of them have provided insight into infections and cancer progression. Despite evidence showing HHV-8 infection of human prostate specimens (106, 115, 125), to our knowledge, no efforts have been made to determine whether HHV-8 can establish chronic infection in prostate epithelial or prostate cancer cell lines *in vitro*, which could serve as a model for examining the role of this virus in cancer progression. To determine

whether prostate epithelial and prostate cancer cell lines are susceptible to HHV-8 infection, a panel of prostate epithelial and prostate cancer-derived cell lines was infected with rKSHV.219, a recombinant HHV-8 virus. rKSHV.219 expresses GFP during viral latency and carries a gene for puromycin resistance, enabling selection and establishment of rKSHV.219-infected cell lines (127). We monitored *de novo* infection and subsequent establishment of latency based on long-term GFP expression.

Our results show that rKSHV.219 can infect and establish latency in all prostate cell lines tested, including the transformed prostate epithelial cell line RWPE2, the androgen-sensitive prostate cancer cell line LNCaP, and the androgen-independent prostate cancer cell lines, DU145 and PC3. To confirm infection, DNA was isolated from both uninfected and infected cell lines, and PCR was performed using the KS330 primer set that targets the HHV-8 minor capsid protein (128). We were able to detect viral DNA in infected cells but not in uninfected cells **(Figure 5 Panel A)**. **Figure 5 Panel B** is representative image of rKSHV.219-infected LNCaP (LNCaPv219) showing phase and corresponding GFP fluorescence in the same field of view. Latent infection of these cells was further confirmed by immunoflurosence assays designed to detect expression of HHV-8 open reading frame 73, which encodes the virus-specific latency-associated nuclear antigen (LANA). This antigen localizes to the nucleus of infected cells and is essential for maintaining latency by tethering the viral episome to the host chromosome (107, 108). In latently–infected cells, immunofluoresence detects LANA as distinct foci in a characteristic punctate distribution in the nucleus (129), a pattern that we observed in rKSHV.219-infected LNCaP cells **(Figure 5 Panel C)**.

Figure 5. The recombinant HHV-8 virus, rKSHV.219, infects and establishes chronic, latent infections in prostate epithelial and prostate cancer cell lines. Panel A: DNA was extracted from both uninfected and rKSVH.219-infected RWPE2, LNCaP, DU145 and PC3 cell lines and was used for PCR amplification with primers specific for KS330, a viral capsid protein. DNA from BCBL-1, a chronically infected cell line derived from a body-cavity based lymphoma was used as a HHV-8 positive control for viral genome template. **Panel B:** Representative photomicrograph of virus-infected LNCaPv219 cells (left) and GFP expression (right) indicating establishment of viral latency. **Panel C:** Immunofluoresence assay showing the characteristic punctate nuclear staining for LANA in rKSHV.219-infected LNCaP cells but not in uninfected cells.

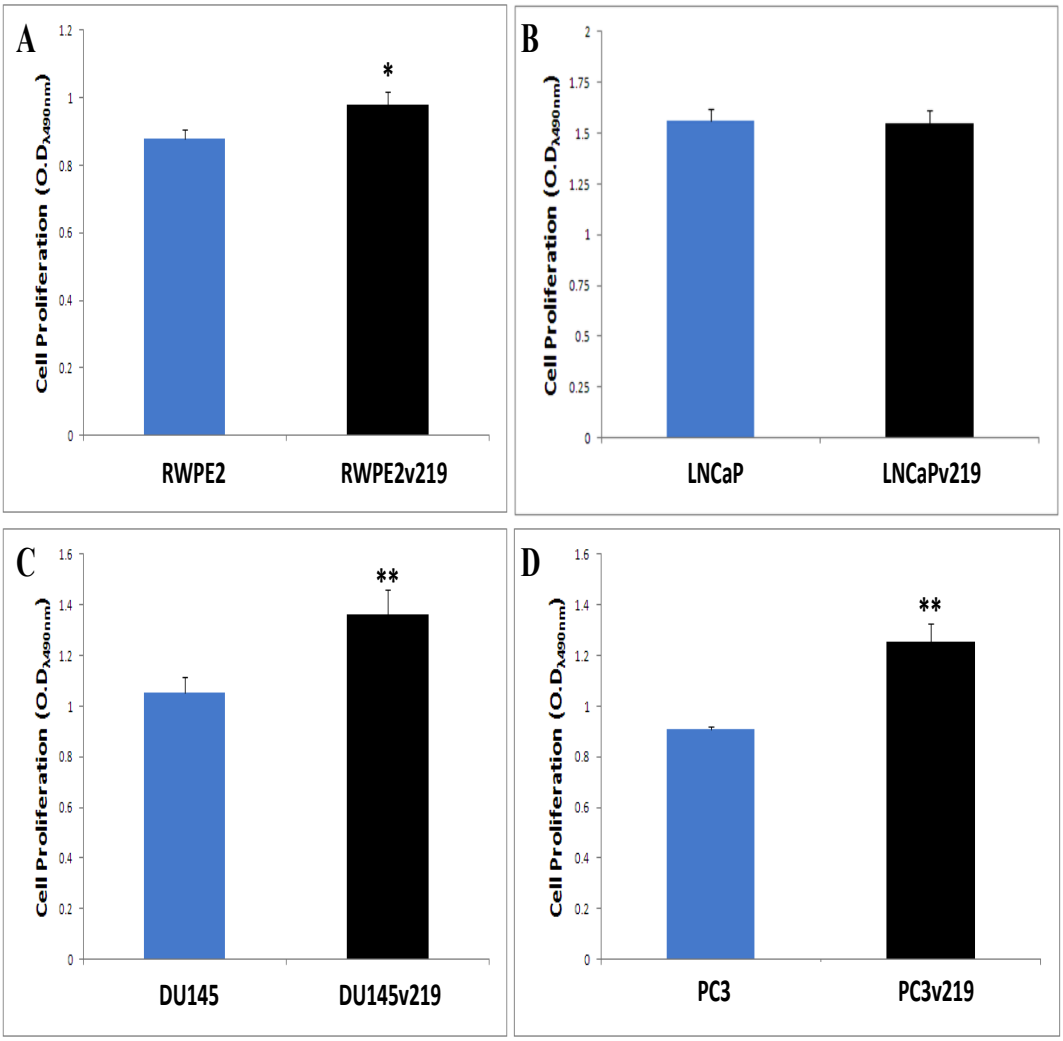

A
RWPE2
LNCaP
Du-145
PC-3
BCBL-1
Virus - + - + - + - + +
B


C
DAPI
LANA
Merge
LNCaP
LNCaPv219

rKSHV.219 infection alters cell proliferation. As mentioned above, a few studies have examined how microbial infections alter the growth properties of prostate cells. In one study, *P. acnes* infection increases the growth of the prostate epithelial cell line, RWPE1 (122). Furthermore, in mouse models, *E. coli* infection has been shown to induce prostate epithelial hyperplasia and increase cell proliferation (123, 124). Based on these observations in other systems, we performed cell proliferation assays with uninfected and rKSHV.219-infected cell lines in order to determine whether chronic HHV-8 infection affected the growth of prostate epithelial and prostate cancer cell lines. We found that rKSHV.219 infection of RWPE2 (RWPE2v219) results in significantly increased cell proliferation compared to non-infected controls **(Figure 6 Panel A)**. The increase in proliferation was even more profound in rKSHV.219-infected androgen-insensitive prostate cancer cell lines, DU145 (DU145v219) and PC3 (PC3v219), implying the existence of interactions between HHV-8 and host pathways that control cell proliferation **(Figure 6 Panels C and D)**. Interestingly, the only cell line for which we did not observe increased cell proliferation following rKSHV.219 infection was the androgen-sensitive LNCaP line **(Figure 6 Panel B),** suggesting that the virus could impact specific host pathways that would enable proliferation in androgen-deprived conditions.

HHV-8-infected LNCaP cells have enhanced *in vitro* androgen-independent growth. The observation that rKSHV.219 infection greatly enhanced cell proliferation in the androgen-insensitive DU145 and PC3 cell lines but not in the androgen-sensitive LNCaP cell line prompted us to test whether rKSHV.219 infection would impact LNCaP cell proliferation if the cells were cultured in an androgen-independent environment. We

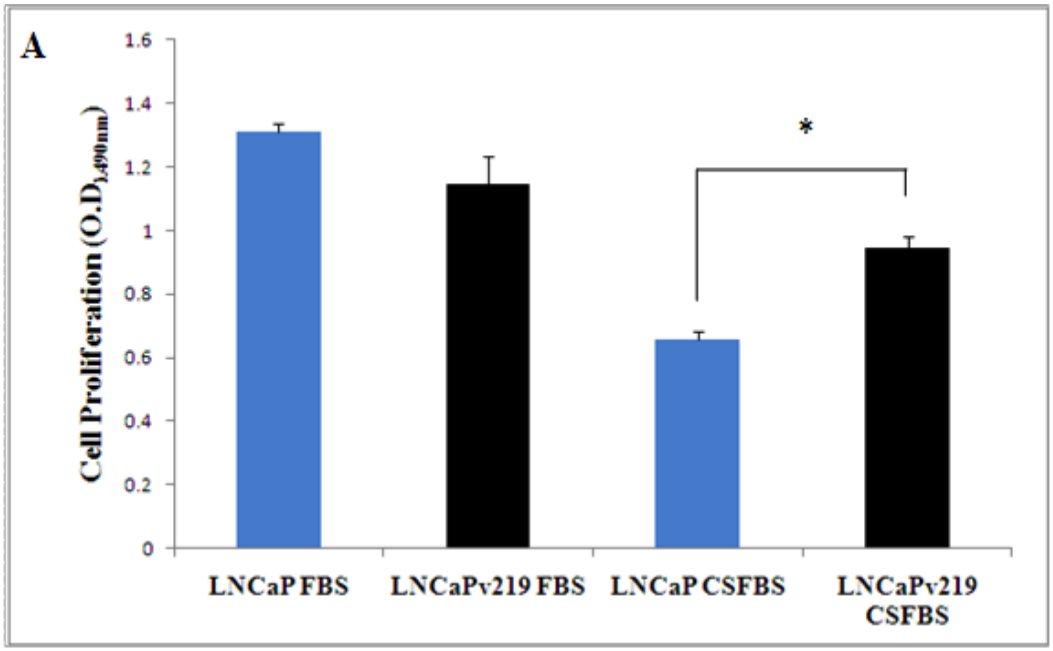
Figure 6. Prostate and prostate cancer-derived cell lines infected with rKSHV.219 exhibit increased cellular proliferation. The individual panels show proliferation rates for the prostate epithelial cell line, RWPE2 **(Panel A),** the androgen-sensitive prostate cancer cell line LNCaP **(Panel B)**, the androgen-insensitive prostate cancer cell lines, DU145 and PC3 **(Panels C and D)** and their rKSHV.219-infected counterparts. All cells were seeded in a 96-well plate; however, due to different doubling times between cell lines, the following cell culture scheme was adopted prior to measurement of cell proliferation in each case: 0.5×10^4 RWPE2 and rKSHV.219-infected RWPE2 (RWPE2v219) cells were seeded and allowed to grow for four days (**Panel A),** whereas 1.0×10^4 LNCaP and LNCaPv219 cells were allowed to grow for five days (**Panel B).** Similarly, 0.5×10^4 DU145 and DU145v219 cells (**Panel C)** and 0.5×10^4 PC-3 and PC-3v219 cells **(Panel D)** were allowed to grow for three days. At the end of each culture time, cell proliferation was analyzed using the Promega Cell-Titer kit according to the manufacturer's protocol. Values are the mean of independent readings from six wells of each sample set ± SD. *, $P = 0.005$; **, $P < 0.001$.

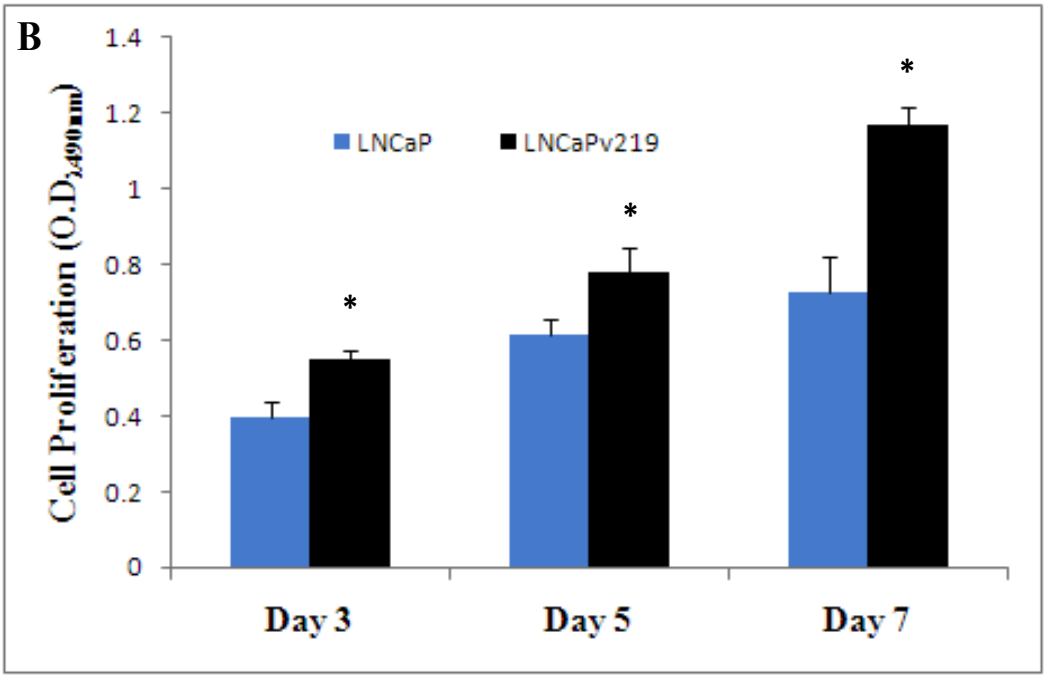

A
Cell Proliferation (O.D λ490nm)
1.2
1
0.8
0.6
0.4
0.2
0
*
RWPE2
RWPE2v219
B
Cell Proliferation (O.D λ490nm)
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
LNCaP
LNCaPv219
C
Cell Proliferation (O.D λ490nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
DU145
DU145v219
D
Cell Proliferation (O.D λ490nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
PC3
PC3v219

compared the growth kinetics of LNCaP and LNCaPv219 cells seeded in media supplemented with charcoal-stripped fetal bovine serum (CSFBS). Charcoal stripping of FBS removes lipophilic compounds such as steroid hormones and is used as a standard procedure to simulate androgen deprivation in *in vitro* assays. As expected, culturing androgen-sensitive LNCaP cells in media supplemented with CSFBS reduced growth by 50% **(Figure 7 Panel A)**. Whereas no difference in cell proliferation was observed between LNCaP and LNCaPv219 when cultured in normal growth media **(Figure 7 Panel A Left)**, when both cell lines are cultured in CSFBS, LNCaPv219 cells had a significantly higher cellular proliferative capacity than uninfected LNCaP **(Figure 7 Panel A Right)**. Although androgen-deprivation impaired LNCaPv219 cell proliferation, the reduction in growth of these cells is not nearly as drastic as observed for uninfected LNCaP cultured in the absence of androgens.

In order to quantify the temporal nature of this virological influence under androgen-deprived conditions, we assayed androgen-independent cell proliferation over a seven-day period of culture in CSFBS media. As shown in **Figure 7 Panel B**, LNCaPv219 cells had significantly better androgen-independent cell growth at each of the successive days of the assay, as revealed by the progressive increase in the magnitude of cell proliferation between uninfected and LNCaPv219 cells over the course of the experiment. These results suggest that rKSHV.219 infection of LNCaP cells either renders them less susceptible to the growth inhibitory effects of androgen-deprivation, or the presence of virus promotes an androgen-independent phenotype by significantly reducing the requirement of androgens for growth and survival.

Figure 7. HHV-8 infection enhances LNCaP cell proliferation in androgen-deprived conditions. Panel A: 1.0 x 10^4 uninfected LNCaP and rKSHV.219-infected LNCaP (LNCaPv219) cells were seeded in a 96 well plate in growth media supplemented with either 10% FBS (left) or 10% CSFBS (right), as indicated. Plates were incubated at 37°C for four days, at which point a cell proliferation assay was performed. Values are a mean of six independent samples ± SD. *, $P < 0.0001$. **Panel B:** 1.0 x 10^4 LNCaP and LNCaPv219 cells were seeded in a 96 well plate in growth media supplemented with 10% CSFBS. Plates were then incubated at 37°C for three, five and seven days before cell proliferation assays were performed. Values are a mean of five samples ± SD for each time point shown. Statistical analysis was done by two-way ANOVA with Bonferroni posttest *, $P < 0.0001$.

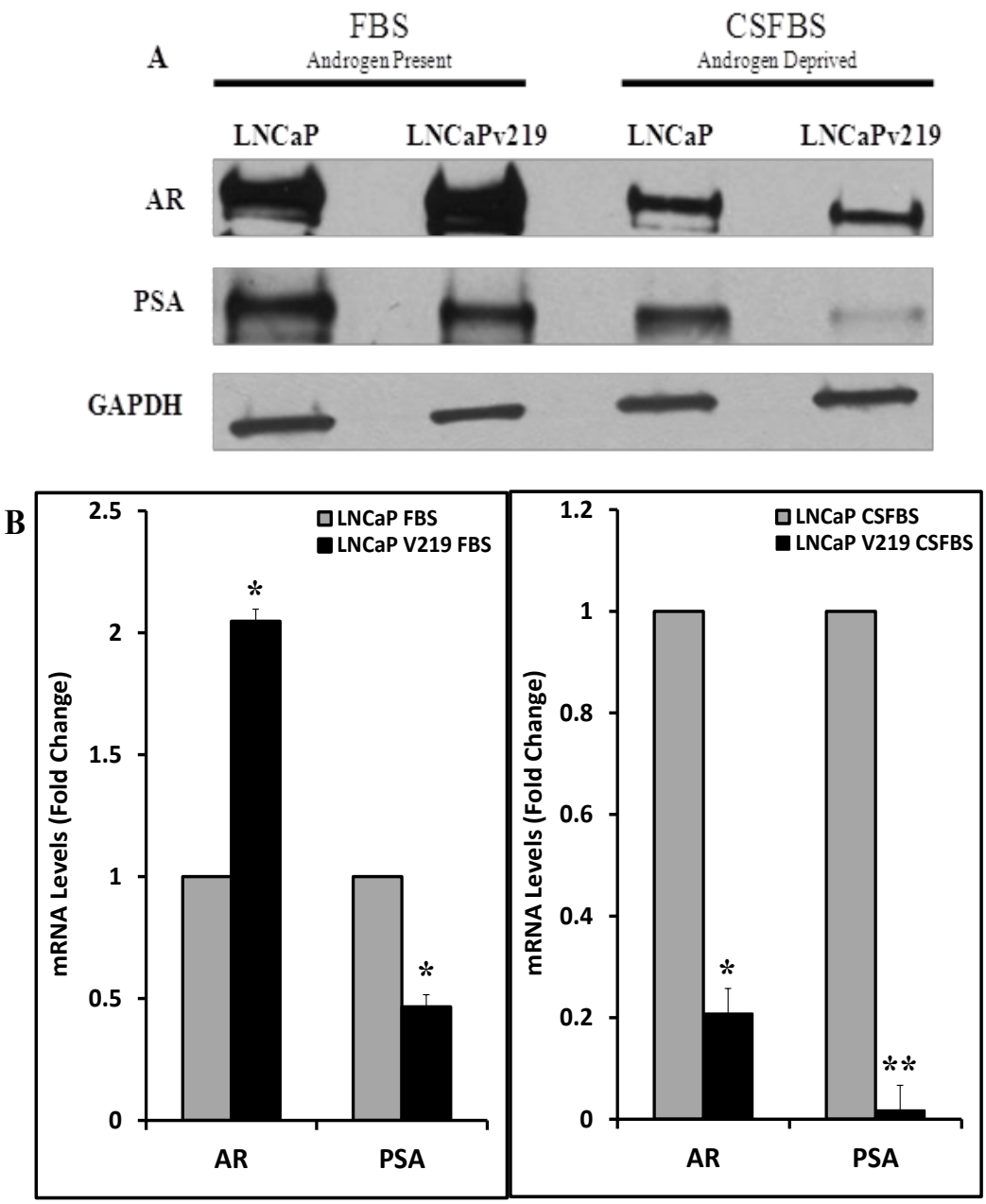

A
Cell Proliferation (O.D490nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
LNCaP FBS
LNCaPv219 FBS
LNCaP CSFBS
LNCaPv219 CSFBS


B
Cell Proliferation (O.D490nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
LNCaP
LNCaPv219
*
*
*
Day 3
Day 5
Day 7

AR and PSA expression levels are down-regulated in LNCaPv219 cells despite enhanced androgen-independent cell proliferation. It is understood that constitutive activation of the AR signaling pathway commonly enables prostate cancer progression (rev in 16, 63), and it has been shown that increased AR expression alone is sufficient to promote an androgen-independent phenotype (67). Therefore, to identify the mechanism(s) by which HHV-8 promotes an androgen-independent phenotype, we used Western blot analysis to compare the level of AR expression in LNCaP and LNCaPv219 cells cultured in either normal growth media with FBS or in androgen-deprived CSFBS media. Since AR regulates PSA expression, measuring both mRNA and protein levels of PSA allows for the assessment of AR transcriptional activity. When cultured in normal growth media, LNCaPv219 exhibited increased AR expression but lower PSA expression **(Figure 8 Panel A)**, implying that while there may be increased AR protein, HHV-8 infection does not appear to induce AR transcriptional activity. As expected, expression of both AR and PSA is greatly reduced when LNCaP are cultured in androgen-deprived CSFBS media. Interestingly, compared to uninfected LNCaP cells, AR and PSA expression levels are even further reduced in LNCaPv219-CSFBS. The AR and PSA protein data are corroborated by qRT-PCR analysis of mRNA expression levels for AR and PSA in LNCaP and LNCaPv219 cells in both culture conditions **(Figure 8 Panel B)**. Together these results suggest that HHV-8 promotes an androgen-independent phenotype that does not require activation of and is potentially independent of the AR signaling pathway.

HHV-8 infection increases MAPK ERK1/2 activity in LNCaP cells and inhibiting MAPK signaling impairs HHV-8 induced androgen-independent cell

Figure 8. AR and PSA expression levels are down-regulated in LNCaPv219 cells despite enhanced androgen-independent cell proliferation. 5 x 10^5 LNCaP and LNCaPv219 cells were grown either in media with FBS (left) or in media supplemented with CSFBS (right) to simulate androgen-deprivation. Cells were then harvested and analyzed protein and mRNA expression profiles with whole cell lysates or total RNA, respectively. **Panel A:** Western blot analysis for AR and PSA expression in LNCaP and LNCaPv219 cells cultured in either FBS or CSFBS. GAPDH was used as a loading control. **Panel B:** Graphic representation of the fold change in AR and PSA mRNA levels measured by qRT-PCR in LNCaP (gray bars) and LNCaPv219 (black bars) cells cultured in normal growth media (left) or in androgen-deprived CSFBS-supplemented media (right). *, $P < 0.01$; **, $P < 0.0001$.


A
FBS
Androgen Present
CSFBS
Androgen Deprived
LNCaP
LNCaPv219
LNCaP
LNCaPv219
AR
PSA
GAPDH
B
LNCaP FBS
LNCaP V219 FBS
mRNA Levels (Fold Change)
2.5
2
1.5
1
0.5
0
*
*
AR
PSA
LNCaP CSFBS
LNCaP V219 CSFBS
mRNA Levels (Fold Change)
1.2
1
0.8
0.6
0.4
0.2
0
*
**
AR
PSA

proliferation. Deregulated signal transduction pathways and constitutive activation of transcription factors have been shown to be involved in the development of an androgen-independent phenotype (rev in 16, 63). As the pathogenic mechanisms of infectious agents commonly involve subversion of host cell signaling pathways and transcription factors, we hypothesized that the androgen-independent phenotype we observed in LNCaPv219 cells could be the result of HHV-8 induction of cell signaling pathways involved in prostate cancer progression. Using activation-specific antibodies, we compared the activation status of some of the most commonly activated pathways in cancer, MAPK ERK1/2, NF-κB p65, AKT and STAT3 in both uninfected and rKSHV.219-infected LNCaP cells using lysates from cells cultured in both normal growth media (FBS) and in androgen-deprived (CSFBS) conditions.

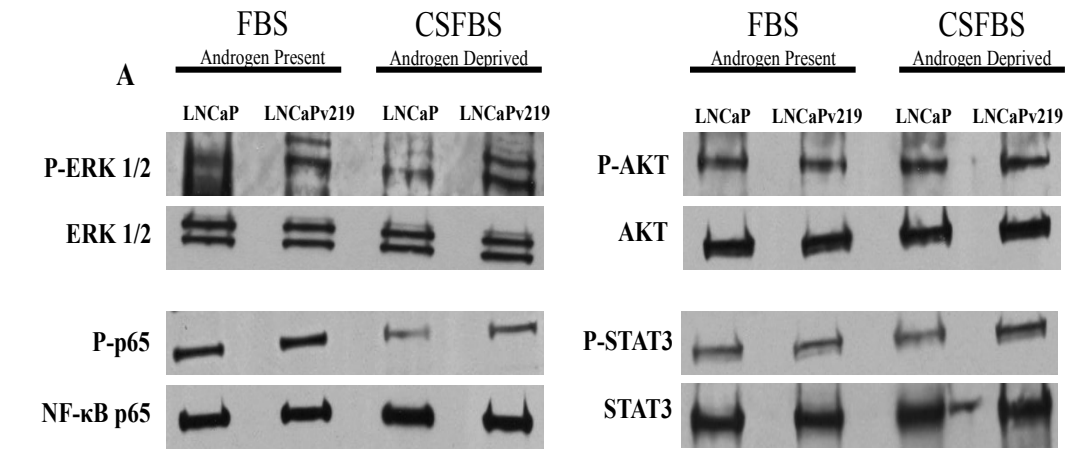
We detected varying degrees of background activation of NF-κB p65, STAT3 and AKT in LNCaP and LNCaPv219 cells. When maintained in androgen-deprived conditions (CSFBS), we observed no difference in AKT activation between LNCaP and LNCaPv219, but we did find moderately increased NF-κB p65 and STAT3 activity in LNCaPv219. In addition, we detected pronounced activation of the MAPK ERK1/2 pathway in rKSHV.219-infected cells cultured in androgen-deprived conditions **(Figure 9 Panel A)**, which appears to be specific to HHV-8 infection as there is no comparable MAP ERK1/2 activity in LNCaP-CSFBS. To test whether the androgen-independent phenotype of LNCaPv219 is dependent on the activation of MAPK ERK1/2, we repeated cell proliferation assays using cells treated with the selective MAP kinase inhibitor PD98059. Our results show that treatment of LNCaPv219 with PD98059 reduced androgen-independent cell proliferation in a dose dependent manner **(Figure 9 Panel B).**

Additionally, as **Figure 9 Panel C** illustrates, LNCaPv219 cells have increased androgen-independent proliferation compared to uninfected LNCaP, a result consistent with our previous observation **(Figure 7 Panel A)**. Treatment of LNCaP and LNCaPv219 with 50 μM of PD98059 resulted in a significant reduction in cell growth only in LNCaPv219 ($P < 0.0001$). It is important to note that PD98059 does not affect growth of LNCaP, as the reduction in growth rates of LNCaP cells treated with the drug is just outside of statistical significance ($P = 0.0508$). This experiment shows that LNCaPv219 cells are much more sensitive to MAPK ERK1/2 inhibition and suggests that MAPK ERK1/2 activation may be one of the mechanisms by which HHV-8 contributes to the androgen-independent phenotype of LNCaPv219.

Discussion

Death from prostate cancer occurs almost exclusively when the cancer metastasizes and develops growth mechanisms that no longer depend on the availability of androgens. The pathogenic mechanisms of AIPC are incompletely understood, and it remains difficult to distinguish localized cancers that will remain indolent from those that will become advanced, aggressive and potentially lethal. It is recognized that on both the clinical and molecular levels, prostate cancer is a heterogeneous disease. The numerous genetic and molecular alterations that occur in the malignant state coupled with the tumor microenvironment and external factors, such as treatment regimens and diet, dictate the disease course. Other groups have demonstrated that *P. acnes* and uropathogenic *E. coli* infections of prostate epithelium incite strong inflammatory responses, induce hyperplasia and increase cell proliferation, supporting a possible link between pathogen-induced inflammation and prostate cancer initiation (122-124). In contrast to other studies that explore the idea of infections as etiologic agents of prostate carcinogenesis, in

Figure 9. HHV-8 infection increases MAPK ERK1/2 activity in LNCaP cells and inhibiting MAPK signaling impairs HHV-8 induced androgen-independent cell proliferation. Panel A: Whole cell lysates from LNCaP and LNCaPv219 cells cultured in FBS or CSFBS-supplemented media were used in Western blot analysis to assess activation of MAPK ERK1/2, NF-κB p65, AKT and STAT3 using phospho-specific antibodies. **Panel B:** PD98059, a potent inhibitor of the MAPK signaling pathway, reduced LNCaPv219 androgen-independent cell proliferation in a dose-dependent manner. Values represent the mean of six independent samples ± SD. Statistical analysis was done using the Kruskal-Wallis test. *, $P = 0.0016$. **Panel C:** LNCaP and LNCaPv219 cells were cultured in CSFBS- supplemented media for three days and then were treated with either no drug or with 50 μM of PD98059 for 24 hours before assaying for cell proliferation. Values are a mean of six independent samples ± SD. *, $P = 0.051$; **, $P < 0.0001$.


A
FBS
Androgen Present
CSFBS
Androgen Deprived
FBS
Androgen Present
CSFBS
Androgen Deprived
LNCaP
LNCaPv219
LNCaP
LNCaPv219
LNCaP
LNCaPv219
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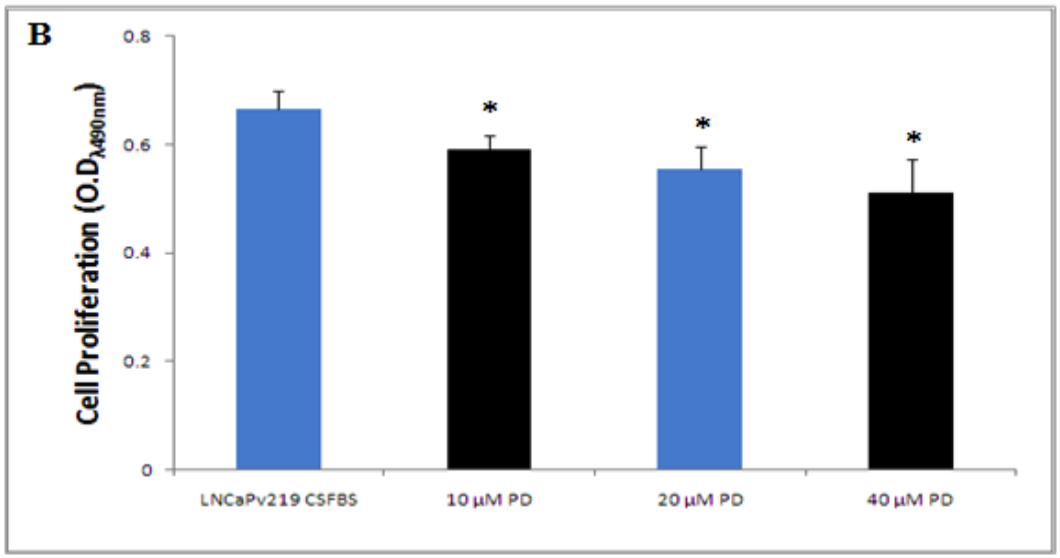
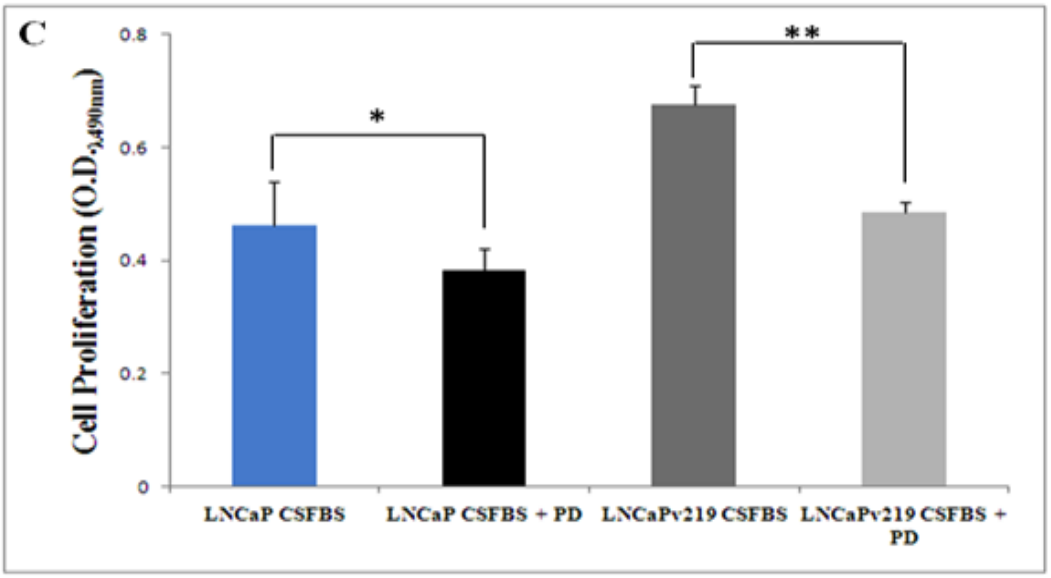
NF-κB p65
P-STAT3
STAT3


B
Cell Proliferation (O.D490nm)
0.8
0.6
0.4
0.2
0
*
*
*
LNCaPv219 CSFBS
10 μM PD
20 μM PD
40 μM PD


C
Cell Proliferation (O.D490nm)
0.8
0.6
0.4
0.2
0
*
**
LNCaP CSFBS
LNCaP CSFBS + PD
LNCaPv219 CSFBS
LNCaPv219 CSFBS + PD

this study we wanted to see how an infection affects prostate cancer progression. To study the effect of infection on prostate cancer phenotype, we used the androgen-sensitive LNCaP cell line and established cells that are persistently infected with HHV-8, a herpesvirus that has been shown to infect the prostate, as a tool for dissecting the role of prostatic infections in activating pathologic networks involved in prostate cancer progression. The rationale for our approach is supported by the fact that HHV-8 encodes several proteins that target and activate host cell signaling pathways, including those relevant to prostate cancer.

We demonstrate that prostate cancer cell lines are permissive for HHV-8 infection **(Figure 5)** and can support the establishment of latency, during which the most notable oncogenic viral genes are expressed (rev in 130). In addition to the LNCaP cell line, we have successfully established persistent rKSHV.219 infection in RWPE2, an immortalized prostate epithelial cell line, and in the androgen-independent DU145 and PC-3 cell lines. As several reports have shown how infections can alter the rate of growth in benign prostate epithelium (122-124) we first examined how HHV-8 infection would alter cellular proliferation rates in chronically infected cell lines. Our results show that HHV-8 infection significantly increased the proliferative rates of RWPE2, DU145, and PC3 **(Figure 6)**. Surprisingly, LNCaPv219 was the only infected cell line that did not have increased growth rates compared to uninfected cells; however, when cell proliferation was assayed in androgen-deprived conditions, LNCaPv219, compared to uninfected LNCaP, had significantly increased rates of proliferation **(Figure 7)**. LNCaPv219 cells appear to be more resistant to the growth-impairing effects of androgen-deprivation. In the presence of androgens, LNCaP and LNCaPv219 most

likely adopt a default cell growth pattern regulated by androgens available in the media. When androgens are not available, we hypothesize that HHV-8 infection provides alternative mechanisms for cell growth and proliferation.

It is understood that most cases of AIPC are the result of increased AR expression and/or constitutive signaling of the AR pathway despite androgen deprivation (16, 63, 67). Therefore, to test whether HHV-8 promotes an androgen-independent phenotype by up-regulating AR expression, we used qRT-PCR and Western blotting to measure AR expression in LNCaP and LNCaPv219 at both the mRNA and protein levels, respectively. In the presence of androgens (FBS), LNCaPv219 cells have increased AR mRNA and protein expression, but PSA expression, which is transcriptionally regulated by AR, is decreased **(Figure 8)**. AR and PSA expression were also measured from LNCaP and LNCaPv219 cell lines that were maintained in androgen-deprived (CSFBS) culture conditions. LNCaPv219, compared to uninfected LNCaP, actually have decreased expression of both AR and PSA, despite having increased androgen-independent proliferation. We had hypothesized that HHV-8-encoded proteins could potentially induce AR expression and transcriptional function in a ligand-independent manner by activating host cell signaling pathways. For example, HHV-8 open reading frame (ORF) K13 encodes a viral homolog of FADD-like interferon-converting enzyme inhibitory protein (vFLIP) that prevents death receptor-mediated apoptosis. vFLIP has also been shown to activate the NF-κB (111) and JNK/AP-1 pathways, both of which induce cellular IL-6 expression (113, 114). In the context of prostate cancer, increased NF-κB activity and cellular IL-6 signaling have both been shown to be ligand-independent activators of AR expression and transcriptional activity (81, 85). Contrary to

what we predicted, however, our results suggest that the androgen-independent phenotype induced by HHV-8 in LNCaP cells is independent of the AR signaling pathway. Based on our data, it appears that HHV-8 infection of LNCaP cells results in a cell line in which AR transcription is attenuated, in a manner similar to the androgen-independent DU145 and PC3 cell lines, both of which lack expression of the AR.

A large body of research has demonstrated that prostate cancer progression is associated with the aberrant activation of numerous signal transduction pathways and transcription factors. Deregulated activation of MAPK ERK1/2, AKT, NF-κB p65 and STAT3 have all been correlated with prostate cancer progression (77-80, 82, 83, 86-88) and are commonly targeted by HHV-8 proteins during infection (130). Using lysates from LNCaP and LNCaPv219 cells cultured in either normal growth media or CSFBS-supplemented media, we employed Western blot analysis to measure the activation status of MAPK ERK1/2, NF-κB p65, AKT, and STAT3 using phospho-specific antibodies. We were most interested in the CSFBS results, as our goal was to identify specific pathway(s) contributing to the androgen-independent phenotype of HHV-8-infected LNCaP. LNCaPv219-CSFBS showed only a modest increase in the activation of several molecules, including NF-κB p65 and STAT3, which was also a bit surprising since HHV-8 encodes two proteins, vFLIP and vIL-6, that when expressed not only prevent apoptosis but also specifically maintain NF-κB (111, 112) and STAT3 (120, 121) signaling, respectively, as a survival strategy for infected cells. However, our data show that HHV-8 infection has a marked impact on MAPK ERK1/2 activation under CSFBS conditions **(Figure 9 Panel A)**. The MAPK ERK1/2 pathway is a pivotal regulator of cell growth and is commonly found to be associated with cancer pathogenesis and progression (rev in

75). Accordingly, our studies also suggest that activation of the MAPK ERK1/2 pathway is crucial to the androgen-independent phenotype induced by HHV-8 infection. Inhibiting MAPK ERK1/2 with PD98059 severely impaired LNCaPv219 proliferation in androgen-deprived conditions **(Figure 9 Panels B and C)**, whereas uninfected cells were far less sensitive to PD98059 treatments. Although this finding implicates MAPK ERK1/2 activation, we do not believe that this signaling pathway is the only one that has a crucial role in HHV-8-induced androgen-independent cell survival. It is entirely possible that other pathways such as NF-κB and STAT3 are contributing, but perhaps only modestly, to the phenotype in the context of HHV-8 infection. On the other hand, it is recognized that HHV-8 infection induces a profound alteration of the host cell transcriptional program (131, 132). As such, further studies are needed to identify mechanisms and the host genes that are modulated by HHV-8 that in turn drive the development of an androgen-independent phenotype in this model system.

To our knowledge, this is the first report of an infectious agent promoting an androgen-independent phenotype in an androgen-sensitive prostate cancer cell line. While HHV-8 may not be a common infection of the prostate, given the low sero-prevalence of this virus in North American and Western European populations, our work highlights the potential for certain infections as possible risk factors for the development of aggressive prostate cancer. Prostatic infections are assumed to be fairly common yet are never diagnosed in the majority of cases (97, 133, 134). Studies should be undertaken to determine how frequently malignant prostates harbor infections and how other infectious agents, such as *P. acnes* and *E. coli*, affect the tumor microenvironment and disease course.

Chapter 4

HHV-8 Down-Regulates Microseminoprotein-β and Induces a Mesenchymal Phenotype in the LNCaP Cell Line: Mechanisms for Decreased Androgen-Sensitivity

Introduction

In the 1940's, Huggins and Hodges showed that prostate cancer growth is dependent on androgens (54). This finding engendered androgen deprivation therapy, which is still utilized for treating men diagnosed with recurrent and advanced prostate cancer. This treatment strategy, however, is not curative, and over time tumors become refractory to hormonal deprivation. For the majority of cases, it has been shown that androgen independence is the result of constitutive signaling of the AR pathway (rev in 16, 63), but emerging evidence suggests that there may be other mechanisms that could contribute to this phenotype. For instance, in previous work, we have shown that chronic infection of the androgen-sensitive LNCaP prostate cancer cell line with the recombinant HHV-8 virus, rKSHV.219, induces enhanced androgen-independent cell proliferation. Analogous to the androgen-independent DU145 and PC3 cell lines, we found that LNCaPv219 have attenuated AR transcriptional activity, suggesting that the androgen-insensitive phenotype induced by the virus in LNCaPv219 occurs independently of the AR signaling pathway.

Recently, Yu and colleagues showed that the *TMPRSS2-ERG* gene fusion, the most commonly occurring genetic mutation in prostate cancer (27, 28), and subsequent over-expression of ERG resulted in reduced AR expression, interruption of AR

transcriptional activity and yet promoted androgen-independent cell proliferation in LNCaP cells by causing cellular dedifferentiation (135). The authors showed that ERG mediated these effects by directly blocking AR signaling and by activating enhancer of zeste homlog 2 (EZH2), a member of the Polycomb group of proteins that specifically tri-methylate lysine 27 of histone 3 (H3K27), an event that has been shown to be responsible for epigenetically repressing genes involved in stem cell differentiation (136-138). This finding is significant as it has been shown that the level of EZH2 expression in tumor specimens directly correlates with worsening prognosis and is most strongly expressed in metastatic and androgen-independent cases (139, 140). Additionally, it has been shown that EZH2 epigenetically inhibits the expression of two critical tumor-suppressor proteins, microseminoprotein-beta (MSMB) and DOC-2/DAB2 interactive protein (DAB2IP) (141-143). MSMB has recently received attention for its potential as a prostate cancer biomarker (rev in 144), while DAB2IP is a Ras GTPase-activating protein that regulates intracellular signal transduction and inhibits prostate cancer cell growth (143, 145). As a consequence of increased EZH2 activity and transcriptional repression, the loss of both MSMB and DAB2IP expression is strongly associated with and is a common feature of high grade, metastatic and androgen-independent prostate tumors (143, 146).

Interestingly, HHV-8 researchers have found that the virus employs EZH2 to epigenetically repress expression of lytic viral proteins in order to maintain latent infection (147, 148). Additionally, it is understood that latent HHV-8 infection results in a pronounced modification of the host cellular transcriptome (131, 132), as recently demonstrated by the fact that HHV-8-infected endothelial cells lose expression of

endothelial markers and begin expressing mesenchymal ones (149, 150), a phenomenon known as virus-induced endothelial-to-mesenchymal transition. For a variety of tumor types, including prostate cancer, the loss of an epithelial phenotype and the acquisition of mesenchymal characteristics occurs during cancer progression, enables metastasis and imparts tumor cells with stem cell-like phenotypes (151-156). Thus, in this study we asked whether the androgen-independent phenotype of LNCaPv219 cells is the result of the virus promoting cellular dedifferentiation as a consequence of HHV-8 using EZH2 to maintain latency. We show that LNCaPv219 cells have increased EZH2 expression and elevated levels of the repressive tri-methylated H3K27 (H3K27me3) mark, which can explain the down-regulation of MSMB and DAB2IP, two critical tumor-suppressor proteins that are epigenetically repressed by EZH2 methyltransferase activity in aggressive prostate cancers. We also show that HHV-8 infection up-regulates expression of markers for EMT, suggesting that HHV-8 infection is profoundly altering the phenotype of the androgen-sensitive LNCaP cell line. We contend that the LNCaPv219 cell line could be a useful model to study the pathophysiology and cellular mechanisms involved in prostate cancer progression.

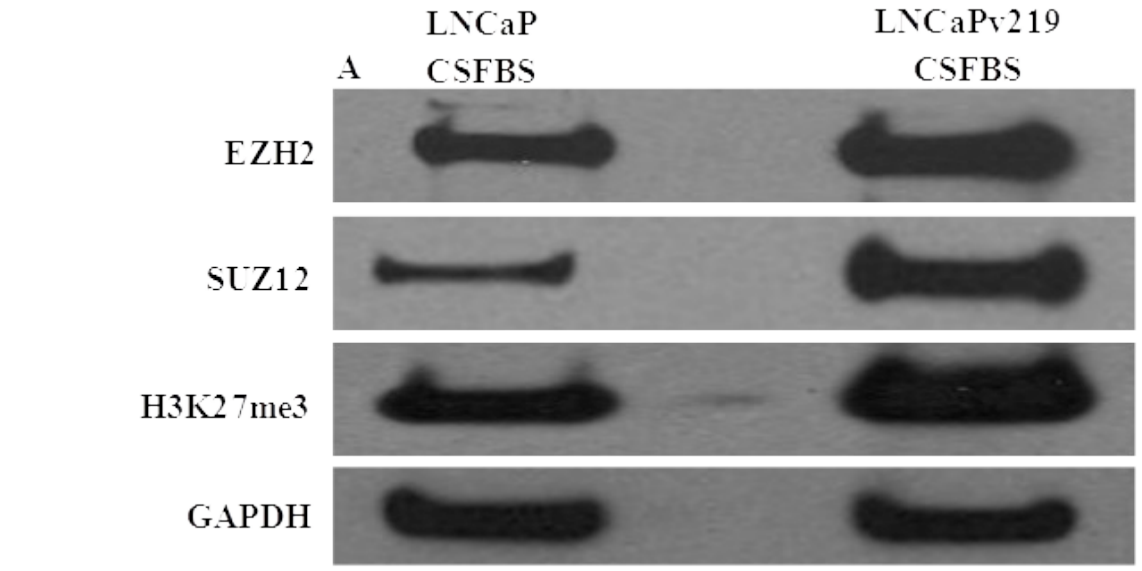
Results

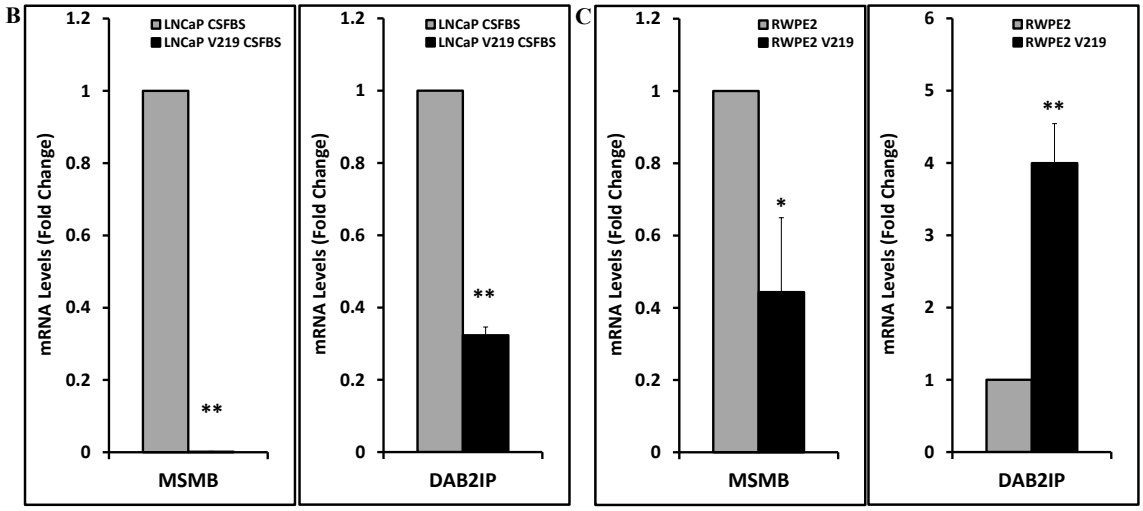
HHV-8 infection of LNCaP cells induces expression and activity of Polycomb group proteins, resulting in increased methylation of H3K27 and decreased expression of MSMB and DAB2IP. Over-expression of EZH2 occurs frequently in metastatic prostate cancer and confers a poor prognosis (139, 140). Since HHV-8 latency is maintained by EZH2-mediated suppression of viral lytic genes, we hypothesized that viral-induced EZH2 expression and subsequent methyltransferase activity in LNCaPv219 cells could result in epigenetic modifications that alter the normal transcriptional program

and promote androgen-independent proliferation. Using lysates from uninfected LNCaP and LNCaPv219 cells cultured in CSFBS-supplemented media, we measured by Western blot EZH2 expression and the expression of SUZ12, another Polycomb group protein that is essential for EZH2-mediated H3K27 methylation (157). EZH2 methyltransferase activity was assessed using an antibody specific for H3K27me3. Our results show that both EZH2 and SUZ12 protein expression is increased in rKSHV.219-infected LNCaP cells **(Figure 10 Panel A)**. We also found an increased level of H3K27me3 in LNCaPv219, which is indicative of increased EZH2 methyltransferase activity and the potential for epigenetic modifications that have an impact on the phenotype of these cells.

To determine whether the increase we observed in EZH2 and H3K27me3 expression was modifying the transcriptome of LNCaPv219 cells, we used qRT-PCR to measure the mRNA expression levels of MSMB and DAB2IP in both LNCaP and LNCaPv219 cells that were cultured in androgen-deprived conditions. MSMB is one of the most highly expressed proteins secreted by the prostate into the seminal fluid (2, 158). Several groups have shown there is reduced MSMB expression in malignant prostatic glands compared with benign prostate tissue (rev in 144), and a recent study found that decreased MSMB expression in tumor specimens is strongly predictive of prostate cancer progression and recurrence (146). As discussed above, DAB2IP is a Ras GTPase-activating protein that has been shown to possess tumor-suppressing characteristics, such as repressing prostate cancer cell growth and metastasis (143, 145). Consistent with these observations, we found that expression of both MSMB and DAB2IP is significantly down-regulated in LNCaPv219 compared to uninfected LNCaP cells **(Figure 10 Panel B)**. To confirm that this phenomenon is not specific to one particular cell line, we also

Figure 10. HHV-8 infection of LNCaP cells induces expression and activity of Polycomb group proteins, resulting in increased methylation of H3K27 and decreased expression of MSMB and DAB2IP. Panel A: Whole cell lysates were collected from LNCaP and LNCaPv219 cells cultured in androgen-deprived CSFBS-supplemented media and were used in Western blot analysis to assess the expression of the Polycomb group proteins EZH2 and SUZ12. The methylation status of H3K27 was also assessed using an antibody specific for the tri-methylated form of the protein. GAPDH was used as a loading control. **Panel B:** Graphic representation of the fold-change in mRNA expression levels of MSMB and DAB2IP measured by qRT-PCR in LNCaP (gray bars) and LNCaPv219 (black bars) cells under androgen-deprived (CSFBS) conditions. **Panel C:** Graphic representation of the fold-change in mRNA expression levels of MSMB and DAB2IP in RWPE2 (gray bars) and RWPE2v219 (black bars) cells. *, $P < 0.01$; ** $P < 0.0001$.


A
LNCaP CSFBS
LNCaPv219 CSFBS
EZH2
SUZ12
H3K27me3
GAPDH


B
LNCaP CSFBS
LNCaP V219 CSFBS
mRNA Levels (Fold Change)
MSMB
DAB2IP
C
RWPE2
RWPE2 V219
mRNA Levels (Fold Change)
MSMB
DAB2IP

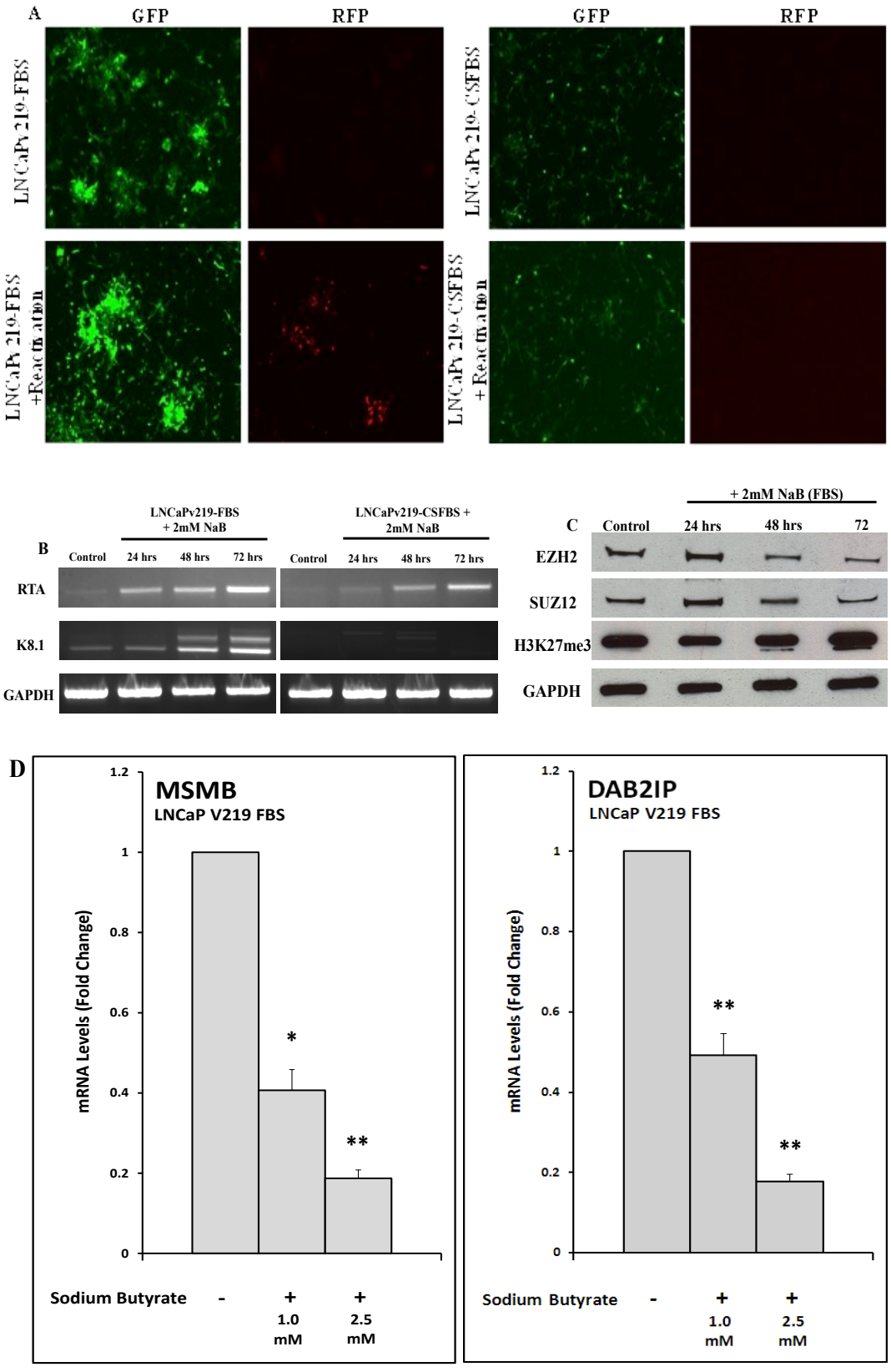
measured MSMB and DAB2IP expression in RWPE2 and RWPE2v219 cells. We found that MSMB mRNA expression levels are also down-regulated in RWPE2v219 cells **(Figure 10 Panel C)**, implying that down-regulation of MSMB could be a generalized consequence of HHV-8 infection of prostate cells. Unexpectedly, DAB2IP expression was actually increased in RWPE2v219 compared to uninfected cells. Why DAB2IP is up-regulated is not clear, although it should be noted that the RWPE2 cell line was established by transformation with the Ki-ras oncogene (126), which could affect expression of DAB2IP, a protein intimately involved in regulating the Ras pathway (143, 145).

Induction of the HHV-8 lytic cycle does not relieve transcriptional repression of MSMB and DAB2IP expression. Decreased EZH2 methyltransferase activity and the subsequent loss of the H3K27me3 mark is associated with the transcription of viral genes that initiate and drive the lytic cycle and HHV-8 replication (147, 148). If the decreased expression of MSMB and DAB2IP in LNCaPv219 cells is directly the result of EZH2-mediated maintenance of viral latency, we reasoned that inducing the viral lytic cycle would impair methyltransferase activity, result in decreased levels of the repressive H3K27me3 mark and therefore possibly relieve epigenetic transcriptional repression of MSMB and DAB2IP expression. To test this hypothesis, we cultured LNCaPv219 cells in both normal and CSFBS-supplemented media and treated them with 2mM sodium butyrate (NaB), a histone deacetylase inhibitor that induces viral reactivation and has been shown to reduce the levels of the H3K27me3 mark along the HHV-8 genome (148). We monitored the cells over a period of several days for red fluorescent protein (RFP) expression, which is used as a marker for lytic reactivation of rKSHV.219 (127).

Whereas increased RFP expression was evident as early as 48 hours of NaB treatment of LNCaPv219 cells cultured in FBS-supplemented media, we did not observe evidence of viral reactivation in LNCaPv219 cells growing in CSFBS-supplemented media within the same time frame (**Figure 11 Panel A)**. Next, we used semi-quantitative RT-PCR to measure the expression levels of HHV-8 lytic gene transcripts **(Figure 11 Panel B)**. RTA, the replication and transcriptional activator, is sufficient to drive the HHV-8 lytic cycle and the expression of other lytic genes (159). K8.1 is a late-lytic gene that encodes a viral envelope-associated structural glycoprotein that is incorporated into the mature virion (160). Next, we cultured LNCaPv219 cells in either FBS- or CSFBS-supplemented media and treated the cells with 2 mM NaB for 24, 48 or 72 hours. Consistent with the RFP fluorescence data, **Figure 11 Panel B** shows that upon treatment with NaB, LNCaPv219 cells grown in FBS exhibited robust expression viral lytic genes, and additionally these results clearly demonstrate that whereas we achieve viral reactivation with NaB treatment in LNCaPv219-FBS cells, the ability to induce viral reactivation is impaired in androgen-deprived culture conditions. This finding was indeed surprising, and it is unclear as to why NaB-induced viral reactivation occurs much more efficiently in FBS-supplemented media than in CSFBS-supplemented media.

Since suppression of RTA expression is due to tri-methylation of H3K27 at the RTA promoter (147, 148), the presence of transcripts for RTA and other lytic genes represents evidence of histone demethylation on the viral genome, which allows RTA transcription. To confirm this, we used lysates from NaB-treated LNCaPv219-FBS cells and measured the expression of EZH2, SUZ12 and H3K27me3 by Western blot. As **Figure 11 Panel C** shows, viral reactivation resulted in decreased expression of EZH2

Figure 11. Induction of the HHV-8 lytic cycle does not relieve EZH2-mediated transcriptional repression of MSMB and DAB2IP. Panel A: Representative photomicrograph showing lytic reactivation in NaB-treated LNCaPv219 cells, as evidenced by RFP expression, cultured in either FBS- (left) or CSFBS-supplemented media (right). **Panel B:** RNA was extracted from LNCaPv219 cells cultured in FBS- (right) or CSFBS-supplemented (left) media that were treated with 2 mM NaB for 24, 48 or 72 hours. The expression of HHV-8 lytic genes was assessed by sqRT-PCR. RNA from untreated cells was used as a control. **Panel C:** Whole cell lysates were made from LNCaPv219-FBS cells treated with no drug or 2 mM NaB for 24, 48 and 72 hours and were used in Western blot analysis to assess the expression of EZH2, SUZ12 and tri-methylated H3K27. GAPDH was used as a loading control. **Panel D:** Graphic representation of fold-change in expression levels of MSMB (left) and DAB2IP (right) measured by qRT-PCR in LNCaPv219 cells treated with either no drug, 1.0 mM or 2.5 mM NaB. *, $P = 0.015$; **, $P < 0.01$.


A
GFP
RFP
GFP
RFP
LNCaPv 219-FBS
LNCaPv 219-CSFBS
+Reactivation
+Reactivation
B
LNCaPv219-FBS
+ 2mM NaB
LNCaPv219-CSFBS +
2mM NaB
Control
24 hrs
48 hrs
72 hrs
Control
24 hrs
48 hrs
72 hrs
RTA
K8.1
GAPDH
C
+ 2mM NaB (FBS)
Control
24 hrs
48 hrs
72
EZH2
SUZ12
H3K27me3
GAPDH
D
MSMB
LNCaP V219 FBS
mRNA Levels (Fold Change)
1.2
1
0.8
0.6
0.4
0.2
0
*
**
Sodium Butyrate
-
+
1.0
mM
+
2.5
mM
DAB2IP
LNCaP V219 FBS
mRNA Levels (Fold Change)
1.2
1
0.8
0.6
0.4
0.2
0
**
**
Sodium Butyrate
-
+
1.0
mM
+
2.5
mM

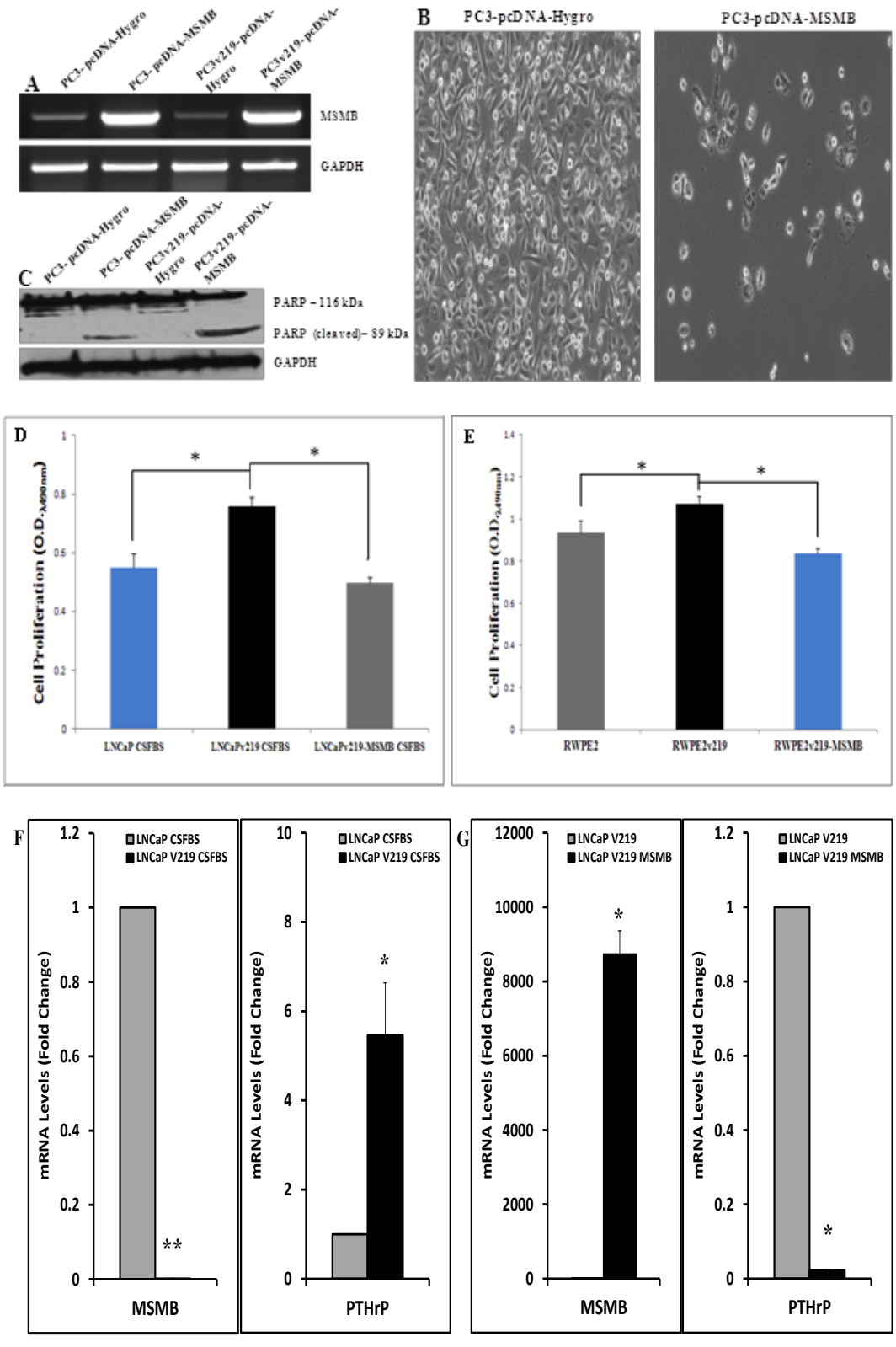
and SUZ12, but paradoxically the levels of H3K27me3 increased. Using RNA from LNCaPv219 cells treated with increasing concentrations of NaB we measured by qRT-PCR the expression levels of MSMB and DAB2IP in reactivated cell lines **(Figure 11 Panel D)**, and found decreased expression for both, consistent with increased tri-methylation of H3K27 and further epigenetic suppression of transcription, verifying the H3K27me3 protein data **(Figure 11 Panel C)**. These results suggest that during NaB-induced viral reactivation while histone demethylation has occurred on the viral genome, enabling RTA expression, the repressive H3K27me3 mark is maintained on the host genome, resulting in continued silencing of MSMB and DAB2IP expression.

Ectopic expression of MSMB induces apoptosis in PC3 cells and inhibits LNCaPv219 androgen-independent growth. It is believed that MSMB limits prostate cellular proliferation by regulating apoptosis (161), a hypothesis consistent with the observation that MSMB expression is highest in benign tissue, reduced in malignant tissues and then progressively declines as prostate cancers evolve to higher grade and more aggressive tumors (146, 162). Given its role in regulating prostate cellular proliferation, we therefore sought to determine the contribution that down-regulation of MSMB has on LNCaPv219 androgen-independent proliferation. We cloned MSMB into the pcDNA3.1/Hygro expression vector in order to ectopically express the protein in uninfected and rKSHV.219-infected prostate cancer cell lines. It has been shown that MSMB inhibits growth of the androgen-insensitive PC3 cell line by inducing apoptosis (161). To test the biological functionality of our MSMB construct, we transfected PC3 and PC3v219 cells with either empty vector (pcDNA-Hygro) or with MSMB (pcDNA-MSMB). As **Figure 12 Panel A** shows, there is low basal expression of MSMB in both

the PC3 and PC3v219 cell lines, but we were able to achieve strong MSMB expression upon transfection of both cell lines with pcDNA-MSMB, but not with the empty pcDNA-Hygro vector. In agreement with previous findings, we noticed that MSMB-expressing PC3 and PC3v219 cells quickly died and could not be maintained in culture **(Figure 12 Panel B)**. Moreover, Western blot analysis showed cleavage of nuclear poly (ADP-ribose) polymerase (PARP), a marker of apoptosis, only in cells over-expressing MSMB **(Figure 12 Panel C)**. To test the hypothesis that HHV-8-mediated down-regulation of MSMB promoted androgen-independent cell proliferation, we next transfected the MSMB construct into LNCaP, LNCaPv219, RWPE2 and RWPE2v219 cell lines. Remarkably, stable MSMB-expressing LNCaP and RWPE2 cell lines could not be established as the cells did not survive in culture. However, we were able to generate and serially passage LNCaPv219 and RWPE2v219 cells that stably express MSMB (LNCaPv219-MSMB and RWPE2v219-MSMB). Based on this result, we tested using cell proliferation assays whether the forced expression of the protein in LNCaPv219 cells would attenuate the androgen-independent proliferative capacity of the cell line. As we have shown before, LNCaPv219, compared to uninfected LNCaP, have enhanced androgen-independent proliferation, but this experiment demonstrates that restoration of MSMB expression significantly impairs androgen-independent growth of LNCaPv219 cells **(Figure 12 Panel D)**. The growth inhibition caused by ectopic MSMB expression appears to be a generalizable theme as we also observed reduced rates of cell growth in RWPE2v219-MSMB cells **(Figure 12 Panel E)**.

Additionally, we assessed the level of parathyroid hormone-related protein (PTHrP), which is a known growth factor for prostate cancer cells and is directly

Figure 12. Ectopic expression of MSMB induces apoptosis in PC3 cells and inhibits LNCaPv219 androgen-independent growth. Panel A: PC3 and PC3v219 cells were transfected with either empty vector (pcDNA-Hygro) or with MSMB-plasmids (pcDNA MSMB) for 48 hours. The level of MSMB RNA expression was measured by sqRT-PCR. GAPDH was used as a loading control. **Panel B:** Photomicrograph taken 48 hours post-transfection of PC3-pcDNA-Hygro cells (left) and depletion of the PC3-pcDNA-MSMB cell population (right). **Panel C:** Western blot analysis for PARP expression in MSMB-transfected PC3 and PC3v219 cells shows increased apoptosis in MSMB-expressing cell lines. **Panel D:** 1.0 x 10^4 LNCaP, LNCaPv219 and LNCaPv219-MSMB cells were seeded in a 96 well plate in growth media supplemented with 10% CSFBS. Cells were allowed to grow for seven days, at which point a cell proliferation assay was performed. Values are a mean of six samples ± SD. Kruskal-Wallis test. *****, $P < 0.001$. **Panel E:** 0.5 x 10^4 RWPE2, RWPE2v219 and RWPE2v219-MSMB cells were seeded in a 96 well plate. Cells were allowed to grow for three days, at which point a cell proliferation assay was performed. Values are a mean of six samples ± SD. Kruskal-Wallis test. *****, $P < 0.001$. **Panel F:** Graphic representation of the fold-change in mRNA levels of MSMB and PTHrP measured by qRT-PCR in LNCaP-CSFBS (gray bars) and LNCaPv219-CSFBS (black bars) cells. *, $P = 0.003$; **, $P < 0.0001$ **Panel G:** Graphic representation of the fold-change in mRNA levels of MSMB and PTHrP in LNCaPv219 (gray bars) and LNCaPv219-MSMB (black bars). *, $P < 0.0001$.

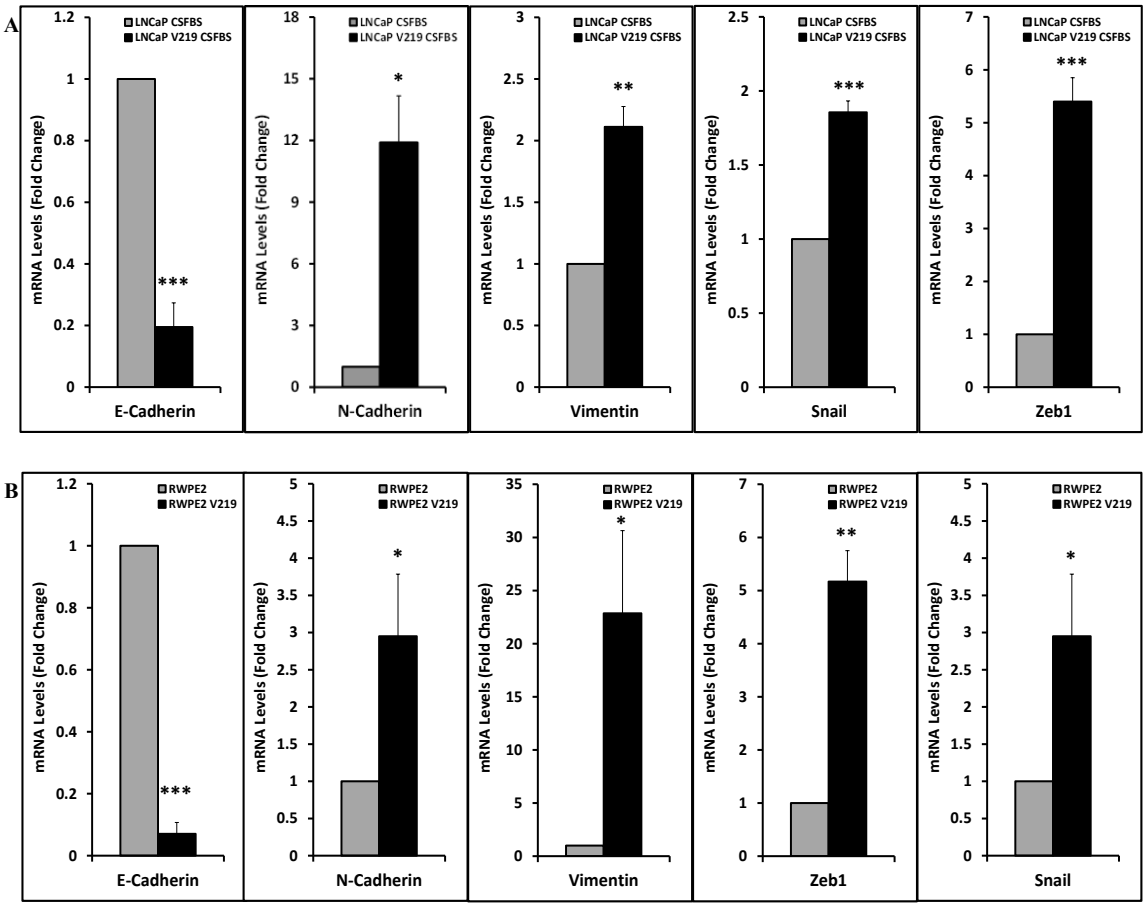

A
PC3-pcDNA-Hygro
PC3-pcDNA-MSMB
PC3v219-pcDNA-Hygro
PC3v219-pcDNA-MSMB
MSMB
GAPDH
B
PC3-pcDNA-Hygro
PC3-pcDNA-MSMB
C
PC3-pcDNA-Hygro
PC3-pcDNA-MSMB
PC3v219-pcDNA-Hygro
PC3v219-pcDNA-MSMB
PARP – 116 kDa
PARP (cleaved)– 89 kDa
GAPDH
D
Cell Proliferation (O.D.A490nm)
LNCaP CSFBS
LNCaPv219 CSFBS
LNCaPv219-MSMB CSFBS
E
Cell Proliferation (O.D.A490nm)
RWPE2
RWPE2v219
RWPE2v219-MSMB
F
LNCaP CSFBS
LNCaP V219 CSFBS
mRNA Levels (Fold Change)
MSMB
PTHrP
G
LNCaP V219
LNCaP V219 MSMB
mRNA Levels (Fold Change)
MSMB
PTHrP

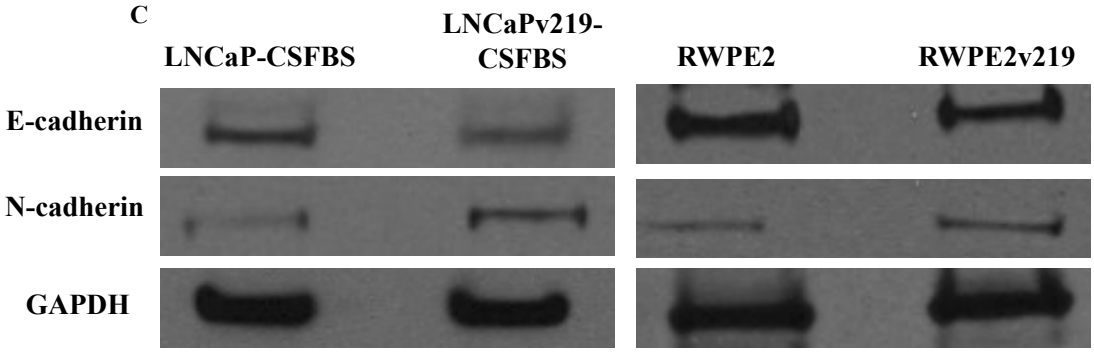
involved in the development of skeletal metastases (163-167). It has been shown that MSMB limits tumor growth and inhibits metastasis in part by directly inhibiting PTHrP expression in prostate cancer cells (168, 169). Therefore, in addition to the cell proliferation data, we measured by qRT-PCR the mRNA expression of PTHrP. In LNCaPv219 cells, which have low levels of MSMB expression, we found that PTHrP expression is up-regulated five-fold compared to uninfected LNCaP **(Figure 12 Panel F)**. However, ectopic expression of MSMB in LNCaPv219 cells dramatically inhibits PTHrP expression, a finding that supports previous reports that an inverse relationship between MSMB and PTHrP expression exists **(Figure 12 Panel G)**. Since re-expressing MSMB in LNCaPv219 severely curtailed cellular proliferation in androgen-deprived conditions, these results suggest that the loss of MSMB expression in LNCaPv219 cells, possibly via a virus-induced up-regulation of EZH2 histone methyltransferase activity, contributes to their enhanced androgen-independent proliferative capacity.

Markers for EMT are expressed in LNCaPv219-CSFBS cells. The loss of MSMB and DAB2IP expression, coupled with our previous finding that AR and PSA protein expression is decreased in LNCaPv219-CSFBS cells led us to investigate whether HHV-8 infection of LNCaP cells was causing a dedifferentiation event. Specifically, we were interested in determining whether HHV-8 infection of LNCaP cells was inducing EMT, a phenomenon whereby epithelial cells dedifferentiate and acquire a mesenchymal phenotype. While EMT is a normal and important process in embryology, in the setting of cancer, EMT is associated with tumor progression and metastasis for several malignancies, including prostate cancer (151, 152, 154-156). HHV-8 has been shown to induce a mesenchymal phenotype in endothelial cells (149, 150), leading us to ask

whether a similar shift in phenotype underlies the androgen-independent proliferation observed in LNCaPv219. We isolated RNA from LNCaP and LNCaPv219 cells cultured in androgen-deprived, CSFBS-supplemented media, and used qRT-PCR to compare the mRNA expression levels of several known EMT markers, including the epithelial marker, E-cadherin, mesenchymal markers, N-cadherin and vimentin, as well as transcription factors responsible for driving the mesenchymal phenotype, Snail and Zeb1 **(Figure 13 Panel A)**. We also measured in LNCaP-CSFBS and LNCaPv219-CSFBS cells E-cadherin and N-cadherin protein expression by Western blot **(Figure 13 Panel C)**. Our data show that in androgen-deprived conditions, LNCaPv219 cells, compared to uninfected LNCaP, have decreased expression of E-cadherin and increased N-cadherin expression at both the level of mRNA and protein expression **(Figure 13 Panels A and C)**, a phenomenon commonly referred to as "cadherin switching" that characterizes EMT (151). The loss of E-cadherin expression in tumors has been correlated with increased invasiveness and metastatic potential (170). Additionally, LNCaPv219 cells have increased mRNA levels of vimentin, another marker of EMT, and increased expression of the transcription factors Snail and Zeb1. We also found that rKSHV.219 infection induces a pro-mesenchymal phenotype in the RWPE2 cell line as well, indicating that HHV-8-induced EMT is not limited to a single prostatic cell line **(Figure 13 Panels B and C)**. These results show that HHV-8 infection of prostate cancer cells results in adoption of a pro-mesenchymal phenotype, a well-established pathological feature of aggressive and castration-resistant prostate cancers (154-156).

Figure 13. rKSHV.219-infected LNCaP cells have increased expression of EMT markers. Graphic representation of the fold change in mRNA expression levels measured by qRT-PCR of the epithelial marker, E-cadherin, EMT markers N-cadherin and vimentin and transcriptional promoters of EMT, Zeb1 and Snail in LNCaP-CSFBS (gray bars) and LNCaPv219-CSFBS (black bars) cells (**Panel A**) and RWPE2 (gray bars) and RWPE2v219 (black bars) cells (**Panel B**). *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. **Panel C:** Whole cell lysates were collected from LNCaP and LNCaPv219 cells (left) cultured in CSFBS-supplemented media and from RWPE2 and RWPE2v219 cells (right) and were used in Western blot analysis to assess expression of E-cadherin and N-cadherin. GAPDH was used as a loading control.


A
LNCaP CSFBS
LNCaP V219 CSFBS
mRNA Levels (Fold Change)
E-Cadherin
N-Cadherin
Vimentin
Snail
Zeb1
B
RWPE2
RWPE2 V219
E-Cadherin
N-Cadherin
Vimentin
Zeb1
Snail


C
LNCaP-CSFBS
LNCaPv219-CSFBS
RWPE2
RWPE2v219
E-cadherin
N-cadherin
GAPDH

Discussion

In previous work, we demonstrated that LNCaP cells chronically infected with HHV-8 have enhanced growth in androgen-deprived conditions. We hypothesized that the expression of viral proteins would result in activation of cell signaling pathways and transcription factors that would subsequently induce AR expression and maintain constitutive activation of the AR-signaling pathway, thereby enabling androgen-independent cell proliferation. However, we found that whereas HHV-8 infection caused activation of pathways known to drive prostate cancer progression, notably the MAPK ERK1/2 signaling cascade, the androgen-independent phenotype observed in LNCaPv219 cells was occurring independently of the AR-signaling pathway, as analysis of mRNA and protein levels in LNCaPv219 cells revealed decreased expression of both AR and PSA, an AR-regulated gene **(Chapter 2 Figure 8)**. While it is understood that AR signaling drives prostatic cellular differentiation, growth and proper organ function (rev in 8, 9), it is also known that AR signaling contributes to prostate carcinogenesis, and it has generally been believed that in the setting of androgen deprivation sustained AR signaling and transcriptional activity are responsible for the development of androgen-independent prostate cancer. However, some tumors that develop androgen-independence dispense with the AR pathway for other signaling pathways that can drive prostate cancer progression. In support of this notion, clinical trials using the latest AR-inhibiting drugs show that a number of patients have little to no response to treatment (171-173), which suggests that these patients have tumors that acquired androgen-independence by mechanisms other than constitutive AR signaling. These findings are corroborated by autopsy studies that have shown that advanced, androgen-independent

tumors are composed of diverse cell populations, where some cells over-express AR while others have no detectable AR expression (174, 175). Additionally, the loss of expression of two other prostatic proteins, PSA and MSMB, occurs frequently in high-grade, advanced cancers (146, 162, 176-178), which suggests that prostate cancer cells do undergo dedifferentiation in some cases.

In androgen-sensitive prostate cancer cell lines, it was recently shown that the *TMPRSS2-ERG* gene fusion promotes prostate cancer progression by disrupting AR signaling and promoting cellular dedifferentiation through activation of the H3K27 methyltransferase, EZH2 (135). In embryonic stem cells the H3K27me3 mark silences the expression of genes required for cellular differentiation (137, 138), and results from gene expression profiling of prostate cancer specimens have demonstrated that EZH2 expression is significantly up-regulated in metastatic prostate cancer compared to localized tumors and benign tissue (139), which results in a stem cell-like transcriptional program that is predictive of poor outcome (140). Coincidentally, during viral latency EZH2 and H3K27me3 co-exist along the HHV-8 genome and maintain latency by epigenetically repressing viral genes involved in lytic reactivation. This led us to speculate that latent HHV-8 infection and EZH2-mediated epigenetic modifications could affect the host genome as well and cause a profound transcriptional reprogramming of the host cell, mirroring what occurs at the transcriptional level during prostate cancer progression and the development of metastatic, androgen-independent disease. In support of this hypothesis, we found that LNCaPv219 cells, compared to uninfected LNCaP cells, have increased expression not only of EZH2 but also SUZ12, a second Polycomb group protein that is necessary for EZH2 methyltransferase activity (157).

Furthermore, we detected increased levels of H3K27me3 in LNCaPv219 cells, which is indicative of EZH2-mediated histone methylation and transcriptional repression of target genes **(Figure 10 Panel A).** In addition to promoting a stem-cell like expression profile (140), EZH2 methylation of histone proteins has been shown to epigenetically silence the transcription of two prostate-growth regulating proteins, MSMB and DAB2IP (141-143). By qRT-PCR we show that in LNCaPv219 cells both MSMB and DAB2IP expression is sharply down-regulated **(Figure 10 Panel B)**, a finding that shows that a virus-induced increase in EZH2 activity and increased deposition of the H3K27me3 mark may be occurring on both the viral and host genomes and consequently altering the transcriptome of LNCaPv219 cells. Our attempts to relieve transcriptional repression of MSMB and DAB2IP by inducing viral reactivation revealed that chemical induction of viral reactivation resulted in decreased expression of both EZH2 and SUZ12, two critical components of the histone methylation complex. Paradoxically, our results showed increased levels of H3K27me3, which we found by qRT-PCR resulted in further transcriptional repression of MSMB and DAB2IP. Although we expected, as others have shown, that NaB treatment and viral reactivation would decrease the levels of H3K27me3, more detailed experimental approaches are clearly needed to analyze the histone modifications associated with both the viral and cellular genome during viral latency and reactivation. It is interesting to speculate, however, that during viral reactivation distinct chromatin modification programs exist between the viral and host genomes that concurrently allow the expression of the necessary viral lytic genes and that maintain repression of certain cellular genes, for instance MSMB and DAB2IP.

Our finding that MSMB is down-regulated in LNCaPv219 cells is a significant finding in the context of prostate cancer progression. Along with PSA, MSMB is one of the most common seminal proteins produced by the prostate gland (2, 158), and several tumor-suppressor functions have been identified for MSMB. For example, MSMB limits prostate cancer cell growth by modulation of apoptotic pathways (161), inhibits neoangiogenesis by blocking vascular endothelial growth factor signaling (179) and impairs the development of prostate cancer bone metastases by decreasing tumor expression of PTHrP (168, 169). The biological functions thus far identified for MSMB are consistent with reports demonstrating that intraprostatic MSMB expression and MSMB serum levels are reduced in men with prostate cancer compared to controls, and protein expression and serum values continues to decline progressively as prostate cancer evolves from localized, androgen-sensitive tumors to aggressive, invasive and castration-resistant disease (162, 180). Dahlman *et al.* recently showed that patients with tumors that maintain MSMB expression have an overall better prognosis and their tumors are associated with numerous positive clinicopathological parameters, including lower Gleason scores and lower stage disease (146).

Since MSMB plays a critical role in regulating prostate cell proliferation, we hypothesized that one mechanism whereby LNCaPv219 cells are able to proliferate in androgen-deprived conditions is by down-regulation of MSMB. We tested this idea by over-expressing MSMB in LNCaPv219 cells guided by the hypothesis that re-expressing MSMB would impair androgen-independent proliferation. Using LNCaPv219-MSMB-expressing cells, we performed cell proliferation assays to test whether MSMB expression plays a role in the androgen-independent phenotype observed in LNCaPv219.

Our data show that ectopic MSMB expression significantly limited the proliferative ability of LNCaPv219 **(Figure 12 Panel D)** to a level comparable with uninfected LNCaP. Our results therefore provide good evidence that viral-induced inhibition of MSMB expression contributes to androgen-independent proliferation. Additionally, we demonstrate an inverse relationship in regards to MSMB expression and PTHrP expression. LNCaPv219 cells, which have dramatically decreased MSMB expression, and have increased PTHrP expression **(Figure 12 Panels F and G)**; however, in the LNCaPv219-MSMB-expressing cell line, we show that re-establishing MSMB expression strongly inhibited PTHrP expression, a finding in agreement with a previous report (168). PTHrP is a known growth factor for prostate cancer cell lines and is a critical molecule for the development of skeletal metastases (163-167). The increased expression of PTHrP, due to down-regulation of MSMB, raises the possibility that PTHrP is an important contributor to the enhanced androgen-independent growth of LNCaPv219 cells. Future studies using neutralizing antibodies to PTHrP will help define the role PTHrP has in LNCaPv219 androgen-independent proliferation. An interesting question that should be explored is whether PTHrP has any role in HHV-8 pathology in general, and whether this peptide contributes to the disease processes of other known HHV-8-related malignancies.

DAB2IP, like MSMB, has several tumor-suppressor functions. DAB2IP has been shown to suppress the Ras-signaling pathway and to inhibit NF-κB transcriptional activity, two regulatory functions that have been shown to limit prostate cancer growth (143, 145). Using a mouse prostate cancer model, Min and colleagues showed that the loss of DAB2IP enabled tumor proliferation and metastatic spread, and, by examining

DAB2IP expression in human prostate cancer specimens, they found an inverse relationship between DAB2IP expression and tumor grade and that low DAB2IP expression was predictive of disease progression and poor prognosis (143). Additionally, the loss of DAB2IP in prostate cancer has been shown to result in EMT (143, 181). In recent years, the correlation between EMT and the development of aggressive malignancies has begun to be appreciated. During EMT, the loss of normal cellular polarity and the deconstruction of cell-cell adhesions collectively impart to tumor cells invasive and metastatic phenotypes (151, 152). Two recent publications have demonstrated that HHV-8 induces the adoption of mesenchymal properties in endothelial cells (149, 150), and, consistent with these reports, we show that HHV-8 induces EMT in prostate cells, which we propose to play a significant role in imparting the androgen-independent phenotype we have observed in rKSHV.219-infected LNCaP cells.

Our results provide several lines of evidence to support the hypothesis that HHV-8-induced EMT is contributing to the androgen-independent phenotype in LNCaPv219 cells. We found that LNCaPv219-CSFBS cells have decreased expression of E-cadherin and increased N-cadherin expression as well as increased expression of EMT-promoting transcription factors, such as Zeb1 and Snail **(Figure 13 Panel A).** Cadherin switching is a classic characteristic of EMT and is of considerable importance in terms of cancer phenotype. E-cadherin is an important tumor-suppressing protein, and the loss of E-cadherin expression contributes to invasiveness and metastatic potential (151, 170). Clinical studies evaluating E-cadherin/N-cadherin expression in prostate cancer specimens have found that high N-cadherin expression is found in high grade, poorly differentiated tumors (155, 156) and, additionally, patients with tumors with low E-

cadherin expression and high N-cadherin expression had increased rates of disease recurrence, skeletal metastases and death from prostate cancer (154). Tanaka and colleagues in a recent publication found that compared to androgen-dependent xenografts, androgen-independent xenografts have increased expression of N-cadherin, and ectopic expression of N-cadherin in LNCaP cells resulted in increased invasiveness (182). Furthermore, the authors observed that N-cadherin-expressing LNCaP cells had enhanced *in vitro* androgen-independent cell proliferation and decreased AR expression, suggesting that N-cadherin-induced androgen independence is mechanistically independent of the AR signaling pathway (182). These findings are in line with what we have observed in LNCaPv219 cells and suggest that the enhanced androgen-independent phenotype induced by HHV-8 occurs concurrently with the virus promoting a pro-mesenchymal phenotype.

The central role that EZH2-mediated epigenetic modifications have in prostate cancer progression cannot be understated, and we contend that the androgen-independent and pro-mesenchymal phenotype promoted by HHV-8 infection is directly the result of the virus using EZH2 to maintain latency, which has profound consequences on the phenotype of the infected cells. HHV-8 infection results in the epigenetic repression of both MSMB and DAB2IP, two critical growth-regulating proteins, and the development of a pro-mesenchymal phenotype, evidenced by increased expression of N-cadherin and down-regulated E-cadherin expression. Like other malignancies, prostate cancer progression occurs concomitantly with the gradual loss of expression of genes that regulate numerous functions, including cellular proliferation, cell-cell adhesion, intracellular signaling and cellular differentiation. We believe that HHV-8-infected

LNCaP cells provide a platform to study these changes and can provide new insights into the mechanisms that underlie prostate cancer progression and the development of an androgen-independent phenotype.

Chapter 5

Discussion

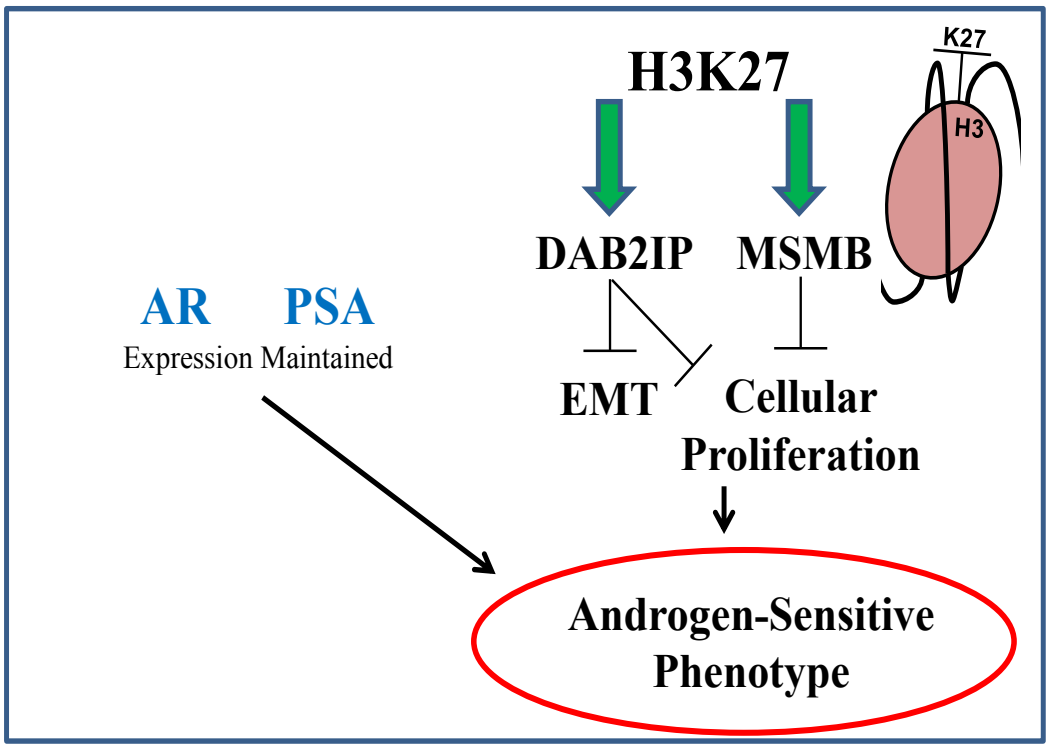
Excluding skin cancers, prostate cancer is the most common malignancy diagnosed in American and Western European men. A heterogeneous disease, prostate cancer is associated with a wide spectrum of clinical outcomes ranging from indolent, slow growing tumors, which are successfully treated with primary interventions, to tumors that are aggressive, metastasize and can result in the death of the patient. Despite significant improvements in detecting and treating localized prostate cancer, challenges still remain, including the lack of means to delineate low-risk, indolent tumors from potentially aggressive malignancies and the need for effective therapies for metastatic disease and AIPC, all of which requires a better understanding of the cellular and genetic mechanisms that enable disease progression and the development of androgen-independence.

In this study, we established androgen-sensitive LNCaP cells that are persistently infected with HHV-8 in order to examine the effect of chronic infection on prostate cancer phenotype. We predicted that HHV-8, which encodes several oncogenic proteins that cause persistent activation of cell signaling pathways, could induce constitutive activation of the AR signaling pathway and thereby promote an androgen-independent phenotype. While we found that HHV-8-infected LNCaP cells compared to uninfected cells have enhanced cell proliferation in androgen-deprived conditions, this phenotype occurred independently of the AR signaling pathway, as AR and PSA expression was down-regulated in LNCaPv219 cells cultured in androgen-deprived conditions. This

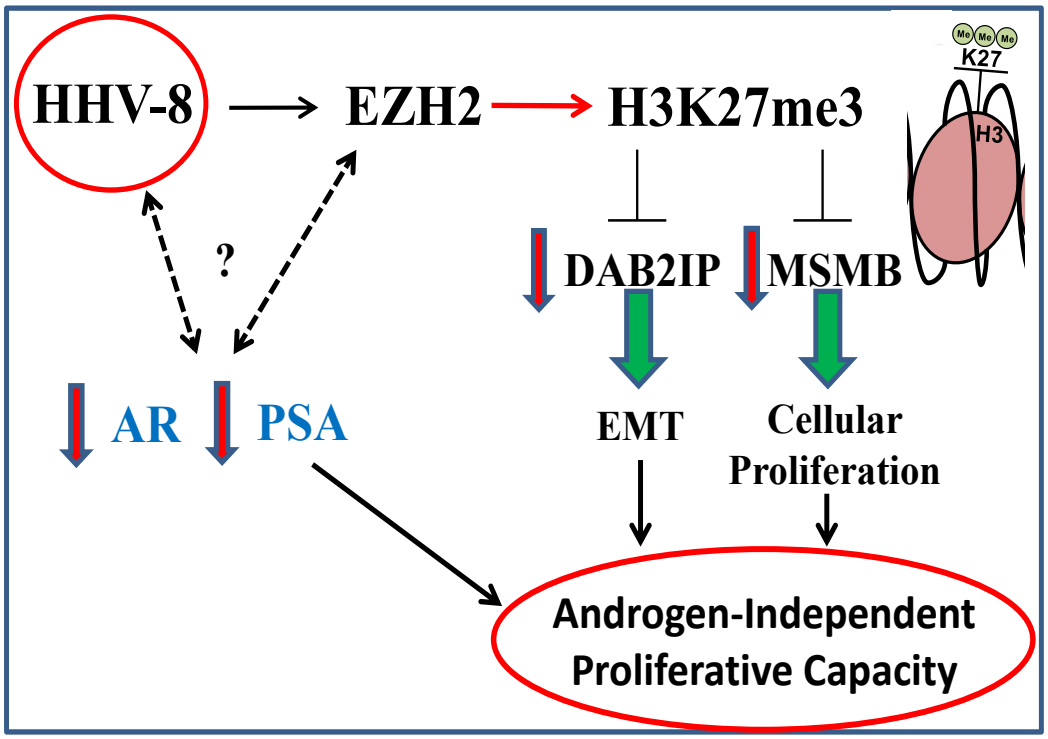
finding prompted us to uncover how HHV-8 infection supports cellular proliferation of the LNCaP cell line in androgen-deprived conditions and whether the mechanisms are analogous to those associated with the development of androgen-independence in prostate cancer patients. **Figure 14** is a proposed model for how we believe HHV-8 promotes an androgen-androgen independent phenotype in the LNCaP cell line. Instead of inducing constitutive AR activation to promote androgen-independent proliferation, HHV-8 infection of LNCaP cells results in attenuation of AR transcriptional activity and induces a profound alteration of the cellular transcriptional program, which we contend occurs as a consequence of the virus using the histone methyltransferase EZH2 to epigenetically repress lytic gene expression in order to maintain a latent infection. Viral activation of this repressive complex has significant implications in the context of prostate cancer cell biology. Compared to localized prostate cancer, EZH2 expression is significantly up-regulated in metastatic specimens (139), and the stem cell-like gene expression signature induced by EZH2 epigenetic modifications is predictive of clinical outcome (140). EZH2 has been shown to epigenetically silence expression of two prostate tumor-suppressing proteins, MSMB (141) and DAB2IP (142), a phenomenon replicated in LNCaPv219 cells and that we show contributes, at least in the case of MSMB, to the androgen-independent phenotype of the cell line. Another line of evidence that supports the notion that HHV-8 is causing LNCaP dedifferentiation is the increased expression of mesenchymal markers in LNCaPv219 cells. We show that HHV-8 infection results in decreased expression of the epithelial marker, E-cadherin, and increased expression of N-cadherin and vimentin, a finding that implies that LNCaPv219 cells undergo EMT. EMT has garnered much attention as a contributor to cancer

Figure 14. Proposed model for how HHV-8 induces an androgen-independent phenotype in the LNCaP cell line. Panel A: In uninfected LNCaP cells, cellular proliferation remains dependent on the availability of androgens. The expression of prostate-differentiating genes, such as AR and AR-regulated genes (e.g. PSA), is maintained, as is the expression of genes such as DAB2IP and MSMB, which play important roles in regulating cell proliferation. **Panel B:** Upon HHV-8 infection, the virus induces activation of the histone methyltransferase, EZH2, in order to establish a latent infection. This results in increased levels of the repressive H3K27me3 mark, which causes transcriptional silencing and decreased expression of DAB2IP and MSMB. The loss of DAB2IP and MSMB expression results in EMT and enhanced cell proliferation. Additionally, we found that HHV-8 infection attenuates (by an as of now undetermined mechanism) AR transcriptional function, which we propose enables dedifferentiation of the cell line. Collectively, these virus-mediated events facilitate androgen-independent cellular proliferation.

Panel A: Uninfected LNCaP



Panel B: HHV-8-infected LNCaP



progression and the development of metastatic disease. Numerous reports have shown that prostate cancer cells that undergo EMT become more invasive and adopt an androgen-independent phenotype (143, 154-156, 181, 182). Additionally, and in agreement with our findings, the development of a pro-mesenchymal phenotype results in decreased AR expression, reflecting the loss of expression of prostatic differentiation markers.

Limitations and Future Directions. Based on our data, we conclude that HHV-8 infection promotes an androgen-independent phenotype by inducing host epigenetic modifications and alterations to the cellular transcriptional program that together drive prostate cellular dedifferentiation, evidenced by decreased expression of AR, PSA and MSMB and increased expression of markers of EMT. The most obvious critique of this work pertains to the virus itself. While we believe that HHV-8 infection of LNCaP cells could be a useful tool to study prostate cancer progression, whether or not the virus is a common enough infection of the prostate to be an actual risk factor for prostate cancer progression remains to be determined. Nonetheless, a number of pathogens are capable of infecting the prostate, and prostatic infection is a fairly common event (rev in 99). Arguably one of the most important aspects of this work is that if chronic HHV-8 infection can deleteriously affect the tumor phenotype, then other infectious agents could very likely do the same. Our findings warrant new research initiatives that address the idea of chronic infection contributing to prostate cancer progression.

As for this project specifically, several questions remain that must be addressed in future experiments. First, although we demonstrate that LNCaPv219 cells have enhanced androgen-independent proliferative ability, our findings are limited to a degree as all

experiments were *in vitro*, and stronger evidence that HHV-8 promotes an androgen-independent phenotype would come from the incorporation of animal studies. Several mouse models for prostate cancer exist (rev in 183), but all are limited by the inability to reliably model metastasis to bone. This consequently limits the ability to study the factors required for skeletal metastases, which is a significant source of morbidity for advanced prostate cancer. We have found that LNCaPv219 cells possess several features that have been shown to contribute to metastatic disease, such as increased PTHrP expression, EZH2-mediated epigenetic regulation and a pro-mesenchymal phenotype, and it would be of great interest to see whether castrated mice with LNCaPv219 xenografts would develop metastatic disease.

Our data show that the androgen-independent phenotype induced by HHV-8 infection occurs as a result of the virus attenuating AR signaling and by activating EZH2-mediated epigenetic modifications that drive dedifferentiation of the cell line, which mechanistically mirrors what has been shown for *TMPRSS2-ERG*. Admittedly it is intriguing to speculate that HHV-8 mimics *TMPRSS2-ERG* and induces androgen-independent cell growth by causing EZH2-mediated cellular dedifferentiation. However, whereas Yu and colleagues demonstrated with chromatin immunoprecipitation and sequencing assays that ERG physically binds to the AR and abrogates transcription of AR-dependent genes (135), the relationship between HHV-8 infection and AR function needs to be determined. In both normal and androgen-deprived conditions we found that LNCaPv219 cells, compared to uninfected LNCaP cells, had decreased expression of PSA, which suggests that HHV-8 infection impairs AR transcriptional function. How and to what degree the virus interferes with AR signaling should be addressed in future

studies as well. Since the establishment and maintenance of viral latency is EZH2-mediated, the full impact of viral-induced epigenetic modifications on the host transcriptional profile should also be determined. In addition to MSMB, DAB2IP and E-cadherin, the expression of other genes is certainly going to be affected by the virus promoting EZH2 histone methylation events, as demonstrated by this work. In metastatic prostate cancer samples, ChIP-on-chip analysis has been used to identify the location of H3K27me3 marks across the entire genome, which enabled the identification of a panel of genes silenced by EZH2 epigenetic repression that both characterized metastatic tumors and resembled a stem cell-like expression pattern (140). A similar approach should be taken using LNCaPv219 cells to see how HHV-8 infection impacts the host transcriptional profile in regards to prostate-specific differentiation expression patterns.

Lastly, it will be important to determine how individual HHV-8-endoded proteins affect prostate cancer cell biology and the contributions each make in promoting an androgen-independent phenotype. The pathogenic mechanisms of the latently expressed viral proteins, LANA, vCyclin, vFLIP, miRNAs and Kaposins, have been elucidated in regards to HHV-8-related malignancies, but how these proteins influence and/or modulate AR signaling, for example, needs to be determined. Studies currently underway in our laboratory are addressing the role HHV-8 viral miRNAs have in latency and regulation of histone demethylases, and hopefully this work will reveal in further detail how HHV-8 modulates cellular histone modifications and the cellular transcriptional profile.

Implications for the HHV-8 Field. Establishing prostate cancer cell lines that are persistently infected with HHV-8 was intended to examine how chronic infection affects tumor cell phenotype, but in conducting our experiments this system also revealed new and interesting insights into HHV-8 biology that demand further study. To show that the establishment of viral latency, which requires EZH2-mediated epigenetic silencing of lytic genes, is the mechanism by which the virus also represses both MSMB and DAB2IP expression, we pharmacologically induced the viral lytic cycle by treating LNCaPv219 cells with NaB. We reasoned that NaB treatment, which has been shown to relieve the repressive H3K27me3, would concurrently enable the expression of viral lytic genes and re-establish MSMB and DAB2IP expression. This seemingly straightforward experiment revealed two very interesting phenomena. First, the ability of NaB to reactivate HHV-8 and induce the lytic cycle appears to be dependent on an unknown factor(s) that is present in FBS-supplemented media but has been removed during the process of charcoal-stripping FBS. One intriguing possibility for this discrepancy is that viral reactivation requires the availability of steroid hormones. As opposed to histone methylation, which represses gene expression, histone acetylation is associated with active transcription of genes. Chen and co-workers showed that steroid hormone signaling and expression of hormonally-regulated genes requires ligand-bound hormone receptors to complex with histone acetylases, which acetylate histones located at gene promoter regions and enable hormone receptors to access and transcribe target genes (184). It has been observed that chromatin remodeling, specifically histone acetylation of the RTA promoter is required for the induction of viral reactivation (147, 148, 185, 186). Interestingly, within the transcriptional activation domain of RTA is an LXXLL motif

that is also utilized by and exposed on hormone receptors following ligand binding, which enables receptor-coactivator interactions and transcription of steroid hormone target genes (184, 185). Thus, it seems reasonable to hypothesize that ligand-bound hormone receptors facilitate the recruitment of and/or chaperone histone acetylases to the RTA promoter and enable RTA transcription and viral reactivation. This scenario could be an explanation as to why we observe diminished viral reactivation in HHV-8 infected cell lines cultured in CSFBS-supplemented media. With the removal of steroid hormones in CSFSB media, ligand is not available for the receptor and consequently hormone receptor-coactivator complexes cannot form and thus cannot efficiently activate RTA transcription. In this context, a requirement for specific steroid hormones for viral reactivation could help explain the striking 10:1 disparity in KS incidence classically observed between men and women.

The second interesting finding from the virus reactivation experiments also involves chromatin remodeling. Toth and colleagues showed that histone demethylases decreased levels of the repressive H3K27me3 mark, which enables RTA expression and viral reactivation (147). Günther *et al.* found that NaB treatment decreases H3K27me3 levels at the RTA promoter (148), so we reasoned that treating LNCaPv219 cells with NaB, in addition to inducing viral reactivation, would potentially result in increased expression of MSMB and DAB2IP, which are silenced by EZH2-mediated histone methylation. We achieved viral reactivation and observed diminished expression of the histone methyltransferases EZH2 and SUZ12 following NaB treatment. Paradoxically, we found that global H3K27me3 protein levels increase, and by qRT-PCR we found that this resulted in further decreases in MSMB and DAB2IP expression. Beyond these

observations, the employment of more sophisticated assays will enable a comprehensive analysis and more precise identification of the histone modifications induced by viral reactivation along both the viral and cellular genomes. To clarify this issue, more detailed studies should attempt to map activating and repressive histone modifications for both the HHV-8 and host genomes during latency and during viral reactivation. During viral reactivation, it appears that the virus is able to direct chromatin remodeling to ensure that lytic genes are transcribed and that repression of certain cellular genes continues. The identification of such genes whose expression is further repressed during viral reactivation could reveal insights into HHV-8 pathogenesis and reveal potential avenues for pharmacological intervention.

Finally, from the perspective of viral evolution, this work has brought into focus the common biological themes shared by the pathogenic mechanisms of HHV-8 and the pathological consequences in prostate cancer oncogenesis and progression, especially those associated with the *TMPRSS2-ERG* gene fusion and *ERG* over-expression. **Table 2** summarizes some of these similarities. As noted in the introduction, over-expression of *ERG* is observed in tumor cells of a majority of prostate cancer patients, and this has been shown to be caused by the fusion of the *TMPRSS2* promoter to the coding sequence of *ERG* (27, 28). A member of the *ETS* gene family, *ERG* encodes a transcription factor that is expressed in endothelial and hematopoietic cells and is required for driving and sustaining endothelial and lymphatic cellular differentiation (187-190). As Sreenath *et al.* note in their review of the biological significance of ERG expression in prostate cancer pathology, the ERG protein is not normally expressed in any epithelial tissue, but its expression in such tissues results in activation of the MAPK pathways and cellular

Table 2. Summary of similarities between ERG and HHV-8

ERG	HHV-8
Transcription factor – expressed exclusively in endothelial, lymphatic, hematopoietic tissues.[187-190]	Herpesvirus – etiological agent for Kaposi's sarcoma (KS), tumor of endothelial and lymphatic origin[101]
ERG expression critical for lineage specific differentiation of endothelial cells, angiogenesis and expression of endothelial markers[187-190]	KS lesions composed of "spindle cells," express endothelial and lymphatic markers; virus induces expression of numerous pro-angiogenic proteins[130]
TMPRSS2-ERG gene fusion most prevalent genetic mutation in prostate cancer[27,28] - transforms epithelial cells in part by activating MAP kinases[191,192]	Virus-encoded proteins activate numerous host cell signaling pathways critical to KS development[130]
Oncogenic fusion induces NF-κB activation and inflammation through TLR4 signaling[199]	HHV-8 strong inducer of inflammation; activation of NF-κB critical in KS pathology[111-113]
TMPRSS2-ERG mediates epigenetic regulation through EZH2[140]	Viral latency maintained by EZH2[147,148]
TMPRSS2-ERG induces epithelial-mesenchymal transition[196-198]	Induces epithelial-mesenchymal transition[149,150]

transformation (187, 191-193). In prostate cancer, ERG expression causes prostate epithelial dedifferentiation and the loss of expression of prostate-defining genes, such as *PSA* (194, 195). This ERG-induced epithelial dedifferentiation occurs as the result of ERG activating EZH2-mediated epigenetic modifications (140). Lastly, ERG has been shown to promote EMT and to up-regulate transcription factors that drive EMT, including Zeb1/2 and Snail1/2 (196-198). The mechanisms by which ERG promotes the development and progression of prostate cancer concern many of the same themes that this project has identified as mechanisms by which HHV-8 promotes an androgen-independent phenotype. Classic Kaposi's sarcoma and prostate cancer, although seemingly unrelated maladies that affect aging males, are perhaps more similar than one may initially suspect. An interesting feature of this thesis project, in our opinion, is how cross-disciplinary research and the merger of two different scientific disciplines have the potential to reveal new insights and contribute to our understanding of both fields.

Chapter 6

Bibliography

1. **Partin AW, Coffey DS.** The molecular biology, endocrinology, and physiology of the prostate and seminal vesicles. In: Walsh PC, Retik AB, Vaughn ED Jr, *et al*, eds. Campbell's Urology 8th ed. Philadelphia: Saunders; 2002. pp. 1237-1296.
2. **Lilja H, Abrahamsson PA.** Three predominant proteins secreted by the human prostate gland. Prostate 1988;12:29-38.
3. **Collins AT, Habib FK, Maitland NJ, Neal DE.** Identification and isolation of human prostate epithelial stem cells based on alpha(2)beta(1)-integrin expression. J Cell Sci 2001;114:3865-3872.
4. **Robinson EJ, Neal DE, Collins AT.** Basal cells are progenitors of luminal cells in primary cultures of differentiating human prostatic epithelium. Prostate 1998;37:149-160.
5. **di Sant'Agnese PA, de Mesy Jensen KL.** Somatostatin and/or somatostatinlike immunoreactive endocrine-paracrine cells in the human prostate gland. Arch Pathol Lab Med 1984;108:693-696.
6. **di Sant'Agnese PA.** Neuroendocrine differentiation in prostatic carcinoma. Recent findings and new concepts. Cancer 1995;75:1850-1859.
7. **Bonkhoff H, Stein U, Remberger K.** Endocrine-paracrine cell types in the prostate and prostatic adenocarcinoma are postmitotic cells. Hum Pathol 1995;26:167-170.
8. **Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby RM, Higgins SJ, Sugimura Y.** The endocrinology and development biology of the prostate. Endocr Rev 1987;8:338-362.
9. **Heinlein CA, Chang C.** Androgen receptor in prostate cancer. Endocr Rev 2004;25:276-308.

10. **Imperato-McGinley J, Guerrero L, Gautier T, Peterson RE.** Steroid 5α-reductase deficiency in man: an inherited form of male pseudohermaphroditism. Science 1974;186:1213-1215.
11. **Wilson JD, Griffin JE, Leshin M, George FW.** Role of gonadal hormones in development of the sexual phenotypes. Hum Genet 1981;58:78-84.
12. **Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM**. Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science 1988;240:327-330.
13. **McEwan IJ.** Molecular mechanisms of androgen receptor-mediated gene regulation: structure-function analysis of the AF-1 domain. Endocr Relat Cancer 2004;11:281-293.
14. **Gelmann EP**. Molecular biology of the androgen receptor. J Clin Oncol 2002;20:3001-3015.
15. **Brinkmann AO, Blok LJ, de Ruiter PE, Doesburg P, Steketee K, Berrevoets CA, Trapman J.** Mechanisms of androgen activation and function. J Steroid Biochem Mol Biol 1999;69:307-313.
16. **Feldman BJ, Feldman D.** The development of androgen-independent prostate cancer. Nat Rev Cancer 2001;1:34-45.
17. **National Cancer Institute Surveillance Epidemiology and End Results.** http://seer.cancer.gov/statfacts/html/prost.html.
18. **Jemal A, Siegel R, Xu J, Ward E.** Cancer statistics, 2010. CA Cancer J Clin 2010;60:277-300.
19. **Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D.** Global cancer statistics. CA Cancer J Clin 2011;61:69-90.
20. **Platz EA, Giovannucci E.** Prostate cancer. In: Schottenfeld D, Fraumeni JF Jr, eds. Cancer Epidemiology and Prevention. 3rd ed. New York: Oxford University Press; 2006. pp 1151-1165.

21. **Walsh PC, Partin AW.** Family history facilitates the early diagnosis of prostate carcinoma. Cancer 1997;80:1871-1874.

22. **Moul JW, Douglas TH, McCarthy WF, McLeod DG.** Black race is an adverse prognostic factor for prostate cancer recurrence following radical prostatectomy in an equal access health care setting. J Urol 1996;155:1667-1673.

23. **Aronson WJ, Freedland SJ.** Can we lower the mortality rate of black men with prostate cancer? J Urol 2000;163:150-151.

24. **Rokman A, Ikonen T, Seppala EH, Nupponen N, Autio V, Mononen N, Bailey-Wilson J, Trent J, Carpten J, Matikainen MP, Koivisto PA, Tammela TLJ, Kallioniemi OP, Schleutker J.** Germline alterations of the *RNASEL* gene, a candidate *HPC1* gene at 1q25, in patients and families with prostate cancer. Am J Hum Genet 2002;70:1299-1304.

25. **Stanford JL, Sabacan LP, Noonan EA, Iwasaki L, Shu J, Feng Z, Ostrander EA.** Association of *HPC2/ELAC2* polymorphisms with risk of prostate cancer in a population-based study. Cancer Epidemiol Biomarkers Prev 2003;12:876-881.

26. **Xu J, Zheng SL, Komiya A, Mychaleckyj JC, Isaacs SD, Chang B, Turner AR, Ewing CM, Wiley KE, Hawkins GA, Bleecker ER, Walsh PC, Meyers DA, Isaacs WB.** Common sequence variants of the macrophage scavenger receptor 1 gene are associated with prostate cancer risk. Am J Hum Genet 2003;72:208-212.

27. **Petrovics G, Liu A, Shaheduzzaman S, Furasato B, Sun C, Chen Y, Nau M, Ravindranath L, Chen Y, Dobi A, Srikantan V, Sesterhenn IA, McCleod DG, Vahey M, Moul JW, Srivastava S.** Frequent overexpression of *ETS*-related gene-1 *(ERG1)* in prostate cancer transcriptome. Oncogene 2005;24:3847-3852.

28. **Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM.** Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 2005;310:644-648.

29. **Chan JM, Gann PH, Giovannucci EL.** Role of diet in prostate cancer development and progression. J Clin Oncol 2005;23:8152-8160.

30. **Hsing AW, Sakoda LC, Chua Jr SC.** Obesity, metabolic syndrome, and prostate cancer. Am J Clin Nutr 2007;86(suppl):843S-857S.

31. **Humphrey PA.** Prostatic intraepithelial neoplasia. In: Humphrey PA. Prostate pathology. Chicago: American Soceity for Clinical Pathology Press; 2003. pp. 182-217.

32. **Brawer MK**. Prostatic intraepithelial neoplasia: an overview. Rev Urol 2005;7(suppl 3):S11-S18.

33. **Rosenberg MT, Froehner M, Albala D, Miner MM.** Biology and natural history of prostate cancer and the role of chemoprevention. Int J Clin Pract 2010;64:1746-1753.

34. **Johansson J, Andren O, Andersson S, Dickman PW, Holmberg L, Magnuson A, Adami H.** Natural history of early, localized prostate cancer. JAMA 2004;291:2713-2719.

35. **Gleaves RC, Militzer RE.** Carcinoma of the prostate with metastases. J Urol 1935;33:235-251.

36. **Humphrey PA.** Clinical aspects of prostatic carcinoma, with histopathological correlations. In: Humphrey PA. Prostate pathology. Chicago: American Soceity for Clinical Pathology Press; 2003. pp. 226-257.

37. **Kim ED, Grayhack JT.** Clinical symptoms and signs of prostate cancer. In: Vogelzang NJ, Scardino PT, Shipley WU, et al, eds. Comprehensive Textbook of Genitourinary Oncology. Baltimore: Williams & Wilkins; 557-564, 1996.

38. **McNeal JE, Yemoto CEM.** Significance of demonstrable vascular space invasion for progression of prostatic adenocarcinoma. Am J Surg Pathol 1996;20:1351-1360.

39. **Salomoa DR, Graham SD, Bostwick DG.** Microvascular invasion in prostate cancer correlates with pathologic stage. Arch Pathol Lab Med 1995;119:1050-1054.

40. **Berges RR, Vukanovic J, Epstein JI, CarMichel M, Cisek L, Johnson DE, Veltri RW, Walsh PC, Isaacs JT.** Implication of cell kinetic changes during the progression of human prostatic cancer. Clin Cancer Res 1995;1:473-480.

41. **Smith JA Jr, Soloway MS, Young MJ.** Complications of advanced prostate cancer. Urology 1999;54(suppl 6A):8-14.

42. **Sternberg CN.** Systemic chemotherapy and new experimental approaches in the treatment of metastatic prostate cancer. Ann Oncol 2008;19(suppl 7):vii91-vii85.

43. **Pound CR, Partin AW, Eisenberger MA, Chan DW, Pearson JD, Walsh PC.** Natural history of progression after PSA elevation following radical prostatectomy. JAMA 1999;281:1591-1597.

44. **Catalona WJ, Whitmore WF Jr.** New staging systems for prostate cancer. J Urol 1989;142:1302-1304.

45. **Epstein JI.** An update of the Gleason grading system. J Urol 2010;183:433-440.

46. **Chodak GW, Thisted RA, Gerber GS, Johansson J, Adolfsson J, Jones GW, Chisholm GD, Moskovitz B, Livne PM, Warner J.** Results of conservative management of clinically localized prostate cancer. N Engl J Med 1994;330:242-248.

47. **Lu-Yao GL, Albertsen PC, Moore DF, Shih W, Lin Y, DiPaola RS, Barry MJ, Zeitman A, O'Leary M, Walker-Corkery E, Yao S.** Outcomes of localized prostate cancer following conservative management. JAMA 2009;302:1202-1209.

48. **Catalona WJ.** Management of cancer of the prostate. N Engl J Med 1994;331:996-1004.

49. **Walsh PC.** Anatomic radical retropubic prostatectomy. In: Walsh PC, Retik AB, Vaughn ED Jr, et al eds. Campbell's Urology. 8th ed. Philadelphia: Saunders; 2002. pp. 3107-3129.

50. **Zincke H, Oesterling JE, Blute ML, Bergstralh EJ, Myers RP, Barrett DM.** Long-term (15 years) results after radical prostatectomy for clinically localized (stage T2c or lower) prostate cancer. J Urol 1994;152:1850-1857.

51. **Roehl KA, Han M, Ramos CG, Antenor JV, Catalona WJ.** Cancer progression and survival rates following anatomical radical retropubic prostatectomy in 3,478 consecutive patients: long-term results. J Urol 2004;172:910-914.

52. **Han M, Partin AW, Zahurak M, Piantadosi S, Epstein JI, Walsh PC.** Biochemical (prostate specific antigen) recurrence probability following radical prostatectomy for clinically localized prostate cancer. J Urol 2003;169:517-523.

53. **Lerner SE, Blute ML, Zincke H.** Primary surgery for clinical stage T3 adenocarcinoma of the prostate. . In: Vogelzang NJ, Scardino PT, Shipley WU, et al, eds. Comprehensive Textbook of Genitourinary Oncology. Baltimore: Williams & Wilkins; 1996. pp. 557-564.

54. **Huggins C, Hodges CV.** Studies on prostatic cancer: the effects of castration, of estrogen, and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. Cancer Res 1941;1:293-297.

55. **Denmeade SR, Isaacs JT.** A history of prostate cancer treatment. Nat Rev Cancer 2002;2:389-396.

56. **Schally AV, Comaru-Schally AN, Plonowski A, Nagy A, Halmos G, Rekasi Z.** Peptide analogs in the therapy of prostate cancer. Prostate 2000;45:159-166

57. **Vilchez-Martinez JA, Pedroza E, Arimura A, Schally AV.** Paradoxical effects of D-Trp6-leutinizing-hormone-releasing hormone on the hypothalamic-pituitary-gonadal axis in immature female rats. Fertil Steril 1979;31:677-682.

58. **Tolis G, Ackman D, Stellos A, Mehta A, Labrie F, Fazekas AT, Comaru-Schally AN, Schally AV.** Tumor growth inhibition in patients with prostatic carcinoma treated with leutinizing hormone-releasing hormone agonists. Proc Natl Acad Sci USA 1982;79:1658-1662.

59. **McCleod DG, O'Brien ME.** Hormonal management of metastatic prostate cancer and quality of life issues. In: Vogelzang NJ, Scardino PT, Shipley WU, et al, eds. Comprehensive Textbook of Genitourinary Oncology. Baltimore: Williams & Wilkins; 854-874, 1996.

60. **Seidenfeld J, Samson DJ, Hasselblad V, Aronson N, Albertson PC, Bennett CL, Wilt TJ.** Single-therapy androgen suppression in men with advanced prostate cancer: a systematic review and meta-analysis. Ann Intern Med 2000;132:566-577.

61. **Anonymous.** Maximum androgen blockade in advanced prostate cancer: an overview of the randomised trials. Prostate Trialists' Collaborative Groups. Lancet 2000;355:1491-1498.

62. **Schmitt B, Wilt TJ, Schellhammer PF, DeMasi V, Sartor O, Crawford ED, Bennett CL.** Combined androgen blockade with nonsteroidal antiandrogens for advanced prostate cancer: a systematic review. Urology 2001;57:727-732.

63. **Scher HI, Sawyers CL.** Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. J Clin Oncol 2005;23:8253-8261.

64. **Mohler JL, Gregory CW, Ford OH III, Kim D, Weaver CM, Petrusz P, Wilson EM, French FS.** The androgen axis in recurrent prostate cancer. Clin Cancer Res 2004;10:440-448.

65. **Montgomery BR, Mostaghel EA, Vessella R, Hess DL, Kalhorn TF, Higano CS, True LD, Nelson PS.** Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth. Cancer Res 2008;68:4447-4454.

66. **Attard G, Reid GH, Olmos D, de Bono JS.** Antitumor activity with CYP17 blockade indicates that castration-resistant prostate cancer frequently remains hormone driven. Cancer Res 2009;69:4937-4940.

67. **Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, Rosenfeld MG, Sawyers CL.** Molecular determinants of resistance to antiandrogen therapy. Nat Med 2004;10:33-39.

68. **Bubendorf L, Kononen J, Koivisto P, Schraml P, Moch H, Gasser TC, Willi N, Mihatsch MJ, Sauter G, Kallioniemi OP.** Survey of gene amplifications during prostate cancer progression by high-throughput fluorescence *in situ* hybridization on tissue microarrays. Cancer Res 1999;59:803-806.

69. **Linja MJ, Savinainen KJ, Saramaki OR, Tammela TLJ, Vessella RL, Visakorpi T.** Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Res 2001;61:3550-3555.

70. **Holzbeierlein J, Lal P, LaTulippe E, Smith A, Satagopan J, Zhang L, Ryan C, Smith S, Scher H, Scardino P, Reuter V, Gerald WL.** Gene expression analysis of human prostate carcinoma during hormonal therapy identifies androgen-responsive genes and mechanisms of therapy resistance. Am J Pathol 2004;164:217-227.

71. **Rosner IL, Ravindranath L, Furusato B, Chen Y, Gao C, Cullen J, Sesterhenn IA, McLeod DG, Srivastava S, Petrovics G.** Higher tumor to benign ratio of the androgen receptor mRNA expression associates with prostate cancer progression after radical prostatectomy. Urology 2007;70:1225-1229.

72. **Li R, Wheeler T, Dai H, Frolov A, Thompson T, Ayala G.** High level of androgen receptor is associated with aggressive clinicopathologic features and decreased biochemical recurrence-free survival in prostate: cancer patients treated with radical prostatectomy. Am J Surg Pathol 2004;28:928-934.

73. **Marcelli M, Ittmann M, Mariani S, Sutherland R, Nigam R, Murthy L, Zhao Y, DiConcini D, Puxeddu E, Esen A, Eastham J, Weigel NL, Lamb DJ.** Androgen receptor mutations in prostate cancer. Cancer Res 2000;60:944-949.

74. **Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, Bartsch G, Klocker H.** Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-1, keratinocyte growth factor, and epidermal growth factor. Cancer Res 1994;54:5474-5478.

75. **Downward J.** Targeting Ras signaling pathways in cancer therapy. Nat Rev Cancer 2003;3:11-22.

76. **Vivanco I, Sawyers CL.** The phosphatidylinositol 3-kinase-AKT pathway in human cancer. Nat Rev Cancer 2002;2:489-501.

77. **Uzgare AR, Isaacs JT.** Enhanced redundancy in Akt and mitogen-activated protein kinase-induced survival of malignant versus normal prostate epithelial cells. Cancer Res 2004;64:6190-6199.

78. **Malik SN, Brattain M, Ghosh PM, Troyer DA, Prihoda T, Bedolla R, Kreisberg JI.** Immunohistochemical demonstration of phosphor-Akt in high Gleason grade prostate cancer. Clin Cancer Res 2002;8:1168-1171.

79. **Kinkade CW, Castillo-Martin M, Puzio-Kuter A, Yan J, Foster TH, Gao H, Sun Y, Ouyang X, Gerald WL, Cordon-Cardo C, Abate-Shen C.** Targeting AKT/mTOR and ERK MAPK signaling inhibits hormone-refractory prostate cancer in a preclinical mouse model. J Clin Invest 2008;118:3051-3064.

80. **Yeh S, Lin HK, Kang HY, Thin TH, Lin MF, Chang C.** From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. Proc Natl Acad Sci USA 1999;96:5458-5463.

81. **Ueda T, Bruchovsky N, Sadar MD.** Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. J Biol Chem 2002;277:7076-7085.

82. **Gioeli D, Mandell JW, Petroni GR, Frierson Jr HF, Weber MJ.** Activation of mitogen-activated protein kinase associated with prostate cancer progression. Cancer Res 1999;59:279-284.

83. **Mukherjee R, McGuinness DH, McCall P, Underwood MA, Seywright M, Orange C, Edwards J.** Upregulation of MAPK pathway is associated with survival in castrate-resistant prostate cancer. Br J Cancer 2011;

84. **Karin M, Cao Y, Greten FR, Li ZW. NF-κB in cancer: from innocent bystander to major culprit.** Nat Rev Cancer 2002;2:301-310.

85. **Zhang L, Altuwaijri S, Deng F, Chen L, Lal P, Bhanot UK, Chang C, Scher HI, Gerald WL.** NF-κB regulates androgen receptor expression and prostate cancer growth. Am J Pathol 2009;175:489-499.

86. **Chen C, Sawyers CL.** NF-κB activates prostate-specific antigen expression and is upregualted in androgen-independent prostate cancer. Mol Cell Biol 2002;22:2862-2870.

87. **Lessard L, Mes-Masson AN, Lamarre L, Wall L, Lattouf BJ, Saad F.** NF-κB nuclear localization and its prognostic significance in prostate cancer. BJU Int 2003;91:417-420.

88. **Ismail HA, Lessard L, Mes-Masson AM, Saad F.** Expression of NF-κB in prostate cancer lymph node metastases. Prostate 2004;58:308-313.

89. **Giri D, Ozen M, Ittmann M.** Interleukin-6 is an autocrine growth factor in human prostate cancer. Am J Pathol 2001;159:2159-2165.

90. **Okamoto M, Lee C, Oyasu R.** Interleukin-6 as a paracrine and autocrine growth factor in human prostatic carcinoma cells *in vitro*. Cancer Res 1997;57:141-146.

91. **Adler HL, McCurdy MA, Kattan MW, Timme TL, Scardino PT, Thompson TC.** Elevated levels of circulating interleukin-6 and transforming growth factor-β1 in patients with metastatic prostatic carcinoma. J Urol 1999;161:182-187.

92. **Drachenberg DE, Elgamal AAA, Rowbotham R, Peterson M, Murphy GP.** Circulating levels of interleukin-6 in patients with hormone refractory prostate cancer. Prostate 1999;41:127-133.

93. **Mora LB, Buettner R, Seigne J, Diaz J, Ahmad N, Garcia R, Bowman T, Falcone R, Fairclough R, Cantor A, Muro-Cacho C, Livingston S, Karras J, Pow-Sang J, Jove R.** Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. Cancer Res 2002;62:6659-6666.

94. **Abdulghani J, Gu L, Dagvadorj A, Lutz J, Leiby B, Bonuccelli G, Lisanti MP, Zellweger T, Alanen K, Mirtti T, Visakorpi T, Bubendorf L, Nevalainen MT.** Stat3 promotes metastatic progression of prostate cancer. Am J Pathol 2008;172:1717-1728.

95. **Jones KR, Whitmire JM, Merrell DS.** A tale of two toxins: Helicobacter pylori CagA and VacA modulate host pathways that impact disease. Front Microbio 2010;1:1-17.

96. **Banerjee A, Ray RB, Ray R.** Oncogenic potential of hepatitis C virus proteins. Viruses 2010;2:2108-2133.

97. **Lipsky BA, Byren I, Hoey CT.** Treatment of bacterial prostatitis. Clin Infect Dis 2010;50:1641-1652.

98. **Lu YC, Yeh WC, Ohashi PS.** LPS/TLR4 signal transduction pathway. Cytokine 2008;42:145-151.

99. **Sutcliffe S, Platz EA.** Inflammation and prostate cancer: a focus on infections. Curr Urol Rep 2008;9:243-249.

100. **De Marzo AM, Platz EA, Sutcliffe S, Xu J, Grönberg H, Drake CG, Nakai Y, Isaacs WB, Nelson WG.** Inflammation in prostate carcinogenesis. Nat Rev Cancer 2007;7:256-269.

101. **Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, Moore PS.** Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science 1994;266:1865-1869.

102. **Cesarman E, Chang Y, Moore PS, Said JW, Knowles DM.** Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N Engl J Med 1995;332:1186-1191.

103. **Russo JJ, Bohenzky RA, Chien MC, Chen J, Yan M, Maddalena D, Parry JP, Peruzzi D, Edelman IS, Chang Y, Moore PS.** Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proc Natl Acad Sci USA 1996;93:14862-14867.

104. **Cai X, Lu S, Zhang Z, Gonzalez CM, Damania B, Cullen B.** Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. Proc Natl Acad Sci USA 2005;102:5570-5575.

105. **Verma SC, Lan K, Robertson E.** Structure and function of latency-associated nuclear antigen. Curr Top Microbiol Immunol 2007;312:101-136.

106. **Diamond C, Brodie SJ, Krieger JN, Huang ML, Koelle DM, Diem K, Muthui D, Corey L.** Human herpesvirus 8 in the prostate glands of men with Kaposi's sarcoma. J Virol 1998;72:6223-6227.

107. **Rainbow L, Platt GM, Simpson GR, Sarid R, Gao SJ, Stoiber H, Herrington CS, Moore PS, Schulz TF.** The 222- to 234-kilodalton latent nuclear protein (LNA) of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) is encoded by orf73 and is a component of the latency-associated nuclear antigen. J Virol 1997;71:5915-5921.

108. **Ballestas ME, Chastis PA, Kaye KM.** Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. Science 1999;284:641-644.

109. **Friborg J, Kong WP, Hottiger MO, Nabel GJ.** p53 inhibition by the LANA protein of KSHV protects against cell death. Nature 1999;402:889-894.

110. **Radkov SA, Kellam P, Boshoff C.** The latent nuclear antigen of Kaposi's sarcoma-associated herpesvirus targets the retinoblastoma-E2F pathway and with the oncogene *Hras* transforms primary rat cells. Nature Med 2000;6:1121-1127.

111. **Liu L, Eby MT, Rathore N, Sinha SK, Kumar A, Chaudhary PM.** The human herpesvirus 8-encoded viral FLICE inhibitory protein physically associates with and persistently activates the IκB kinase complex. J Biol Chem 2002;277:13745-13751.

112. **Keller SA, Hernandez-Hopkins D, Vider J, Ponomarev V, Hyjek E, Schattner EJ, Cesarman E.** NF-κB is essential for the progression of KSHV- and EBV-infected lymphomas in vivo. Blood 2006;107:3295-3302.

113. **Matta H, Surabhi RM, Zhao J, Punj V, Sun Q, Schamus S, Marzzacurati L, Chaudhary PM.** Induction of spindle cell morphology in human vascular endothelial cells by human herpesvirus 8-encoded viral FLICE inhibitory protein K13. Oncogene 2007;26:1656-1660.

114. **An J, Sun Y, Sun R, Rettig MB.** Kaposi's sarcoma-associated herpesvirus encoded vFLIP induces cellular IL-6 expression: the role of NF-κB and JNK/AP1 pathways. Oncogene 2003;22:3371-3385.

115. **Montgomery JD, Jacobson LP, Dhir R, Jenkins FJ.** Detection of human herpesvirus 8 (HHV-8) in normal prostates. Prostate 2006;66:1302-1310.

116. **Arvanitakis L, Geras-Raaka E, Varma A, Gershengorn MC, Cesarman E.** Human herpesvirus KSHV encodes a constitutively active G-protein-couple receptor linked to cell proliferation. Nature 1997;385:347-350.

117. **Bais C, Santomasso B, Coso O, Arvanitakis L, Raaka EG, Gutkind JS, Asch AS, Cesarman E, Gershengorn MC, Mesri EA.** G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. Nature 1998;391:86-89.

118. **Martin D, Gutkind JS.** Kaposi's sarcoma virally encoded, G-protein coupled receptor: a paradigm for paracrine transformation. Methods Enzymol 2009;460:125-150.

119. **Moore PS, Boshoff C, Weiss RA, Chang Y.** Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. Science 1996;274:1739-1744.

120. **Molden J, Chang Y, You Y, Moore PS, Goldsmith MA.** A Kaposi's sarcoma-associated herpesvirus-encoded cytokine homolog (vIL-6) activates signaling through the shared gp130 receptor subunit. J Biol Chem 1997;272:19625-19631.

121. **Chen D, Sandford G, Nicholas J.** Intracellular signaling mechanisms and activities of human herpesvirus 8 interleukin-6. J Virol 2009;83:722-733.

122. **Fehri LF, Mak TN, Laube B, Brinkmann V, Ogilve LA, Mollenkopf H, Lein M, Schmidt T, Meyer TF, Brüggemann H.** Prevalence of *Propionibacterium acnes* in diseased prostates and its inflammatory and transforming activity on prostate epithelial cells. Int J Med Microbiol 2010;301:69-78.

123. **Boehm BJ, Colopy SA, Jerde TJ, Loftus CJ, Bushman W.** Acute bacterial inflammation of the mouse prostate. Prostate 2011;72:307-317.

124. **Elkahwaji JE, Hauke RJ, Brawner CM.** Chronic bacterial inflammation induces prostatic intraepithelial neoplasia in mouse prostate. Br J Cancer 2009;101:1740-1748.

125. **Staskus KA, Zhong W, Gebhard K, Herndier B, Wang H, Renne R, Beneke J, Pudney J, Anderson DJ, Ganem D, Haase AT.** Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. J Virol 1997;71:715-719.

126. **Bello D, Webber MM, Kleinman HK, Wartinger DD, Rhim JS.** Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis 1997;18:1215-1223.

127. **Vieira J, O'Hearn PM.** Use of the red fluorescent protein as a marker of Kaposi's sarcoma-associated herpesvirus lytic gene expression. Virology 2004;325:225-240.

128. **Moore PS, Chang Y.** Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and without HIV. N Engl J Med 1995;332:1181-1185.

129. **Simpson GR, Schulz TF, Whitby D, Cook PM, Boshoff C, Rainbow L, Howard MR, Gao SJ, Bohenzky RA, Simmonds P, Lee C, de Ruiter A, Hatzakis A, Tedder RS, Weller IVD, Weiss RA, Moore PS.** Prevalence of Kaposi's sarcoma associated herpesvirus infection measured by antibodies to recombinant capsid protein and latent immunofluorescence protein. Lancet 1996;349:1133-1138.

130. **Mesri EA, Cesarman E, Boshoff C.** Kaposi's sarcoma and its associated herpesvirus. Nat Rev Cancer 2010;10:707-719.

131. **Moses AV, Jarvis MA, Raggo C, Bell YC, Ruhl R, Luukkonen BGM, Griffith DJ, Wait CL, Druker BJ, Heinrich MC, Nelson JA, Fruh K.** A functional genomics approach to Kaposi's sarcoma. Ann NY Acad Sci 2002;975:180-191.

132. **Poole LJ, Yu Y, Kim PS, Zheng QZ, Pevsner J, Hayward GS.** Altered patterns in cellular gene expression in dermal microvasvular endothelial cells infected with Kaposi's sarcoma-associated herpesvirus. J Virol 2002;76:3395-3420.

133. **Hochreiter WW, Duncan JL, Schaeffer AJ.** Evaluation of the bacterial flora of the prostate using a 16S rRNA gene based polymerase chain reaction. J Urol 2000;163:127-130.

134. **Krieger JN, Riley DE.** Bacteria in the chronic prostatitis-chronic pelvic pain syndrome: Molecular approaches to critical research questions. J Urol 2002;167:2574-2583.

135. **Yu J, Yu, J, Mani RM, Cao Q, Brenner CJ, Cao X, Wang X, Wu L, Li J, Hu M, Gong Y, Cheng H, Laxman B, Vellaichamy A, Shankar S, Li Y, Dhanasekaran SM, Morey R, Barrette T, Longiro RJ, Tomlins SA, Varambally S, Qin ZS, Chinnaiyan AM.** An integrated network of androgen receptor, polycomb, and TMPRSS2-ERG gene fusions in prostate cancer progression. Cancer Cell 2010;17:443-454.

136. **Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D.** Histone methyltransferase activity associated with a human multiprotein complex containing the enhancer of zeste protein. Genes Dev 2002;16:2893-2905.

137. **Kirmizis A, Bartley SM, Kuzmichev A, Margueron R, Reinberg D, Green R, Farhnam PJ.** Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes Dev 2004;18:1592-1605.

138. **Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, Kumar RM, Chevalier B, Johnstone SE, Cole MF, Isono K, Koseki H, Fuchikami T, Abe K, Murray HL, Zucker JP, Yuan B, Bell GW, Herbolsheimer E, Hannett NM, Sun K, Odom DT, Otte AP, Volkert TL, Bartel DP, Melton DA, Gifford DK, Jaenisch R, Young RA.** Control of developmental regulators by polycomb in human embryonic stem cells. Cell 2006;125:301-313.

139. **Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RGAB, Otte AP, Rubin MA, Chinnaiyan AM.** The

polycomb group protein EZH2 is involved in prostate cancer progression. Nature 2002;419:624-628.

140. **Yu J, Yu J, Rhodes DR, Tomlins SA, Cao X, Chen G, Mehra R, Wang X, Ghosh D, Shah RB, Varambally S, Pienta KJ, Chinnaiyan AM.** A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. Cancer Res 2007;67:10657-10663.

141. **Beke L, Nuytten M, Van Eynde A, Beullens M, Bollen M.** The encoding the prostatic tumor suppressor PSP94 is a target for repression by the Polycomb group protein EZH2. Oncogene 2007;26:4590-4595.

142. **Chen H, Tu S, Hseih J.** Down-regulation of human DAB2IP gene expression mediated by polycomb Ezh2 complex and histone deacetylase in prostate cancer. J Biol Chem 2005;280:22437-22444.

143. **Min J, Zaslavsky A, Fedele G, McLaughlin SK, Reczek EE, De Raedt T, Guney I, Strochlic DE, MacConaill LE, Beroukhim R, Bronson RT, Ryeom S, Hahn WC, Loda M, Cichowski K.** An oncogene-tumor suppressor cascade drives metastatic prostate cancer by coordinately activating Ras and nuclear factor-κB. Nat Med 2010;16:286-295.

144. **Whitaker HC, Warren AY, Eeles R, Kote-Jarai Z, Neal DE.** The potential value of microseminoprotein-β as a prostate cancer biomarker and therapeutic target. Prostate 2010;70:333-340.

145. **Wang Z, Tseng CP, Pong RC, Chen H, McConnell JD, Navone N, Hsieh JT.** The mechanism of growth-inhibitory effect of DOC-2/DAB2 in prostate cancer. J Biol Chem 2002;277:12622-12631.

146. **Dahlman A, Rexhepaj E, Brennan DJ, Gallagher WM, Gaber A, Lindgren A, Jirstrom K, Bjartell A.** Evaluation of the prognostic significance of MSMB and CRISP3 in prostate cancer using automated image analysis. Mod Pathol 2011;24:708-719.

147. **Toth Z, Maglinte DT, Lee SH, Lee HR, Wong LY, Brulois KF, Lee S, Buckley JD, Laird PW, Marquez VE, Jung JU.** Epigenetic analysis of KSHV latent and lytic genomes. PLoS Pathog 2010;6:e1001013.

148. **Günther T, Grundhoff A.** The epigenetic landscape of latent Kaposi sarcoma-associated herpesvirus genomes. PLoS Pathog 2010;6:e1000935.

149. **Gasperini P, Espigol-Frigole G, McCormick PJ, Salvucci O, Maric D, Uldrick TS, Polizzotto MN, Yarchoan P, Tosato G.** Kaposi sarcoma herpesvirus promotes endothelial-to-mesenchymal transition through Notch-depending signaling. Cancer Res 2012;72:1157-1169.

150. **Cheng F, Pekkonen P, Laurinavicius S, Sugiyama N, Henderson S, Guenther T, Rantanen V, Kavianto E, Aavikko M, Sarek G, Hautaniemi S, Biberfeld P, Aaltonen L, Grundhoff A, Boshoff C, Alitalo K, Lehti K, Ojala PM.** KSHV-initiated Notch activation leads to membrane-type-1 matrix metalloproteinase-dependent lymphatic endothelial-to-mesenchymal transition. Cell Host Microbe 2011;10:577-590.

151. **Thiery JP.** Epithelial-mesenchymal transitions in tumor progression. Nat Rev Cancer 2002;2:442-454.

152. **Arias AM.** Epithelial mesenchymal interactions in cancer and development. Cell 2001;105:425-431.

153. **Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA.** The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008;133:704-415.

154. **Gravdal K, Halvorsen OJ, Haukaas SA, Akslen LA.** A switch from E-cadherin to N-cadherin expression indicates epithelial to mesenchymal transition and is of strong and independent importance for the progress of prostate cancer. Clin Cancer Res 2007;13:7003-7011.

155. **Tomita K, van Bokhoven A, van Leenders GJLH, Ruijter ETG, Jansen CFJ, Bussemakers MJG, Schalken JA.** Cadherin switching in human prostate cancer progression. Cancer Res 2000;60:3650-3654.

156. **Jaggi M, Nazemi T, Abrahams NA, Baker JB, Galich A, Smith LM, Balaji KC.** N-cadherin switching occurs in high Gleason grade prostate cancer. Prostate 2006;66:193-199.

157. **Cao R, Zhang Y.** SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. Mol Cell 2004;15:57-67.

158. **Dube JY, Frenette G, Paquin R, Chapdelaine P, Tremblay J, Tremblay RR, Lazure C, Seidah N, Chretien M.** Isolation from human seminal plasma of an abundant 16-kDa protein originating from the prostate, its identification with a 94-residue peptide originally described as β-inhibin. J Androl 1987;8:182-189.

159. **Sun R, Lin SF, Gradoville L, Yuan Y, Zhu F, Miller G.** A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. Proc Natl Acad Sci USA 1998;95:10866-10871.

160. **Chandran B, Bloomer C, Chan SR, Zhu L, Goldstein E, Horvat R.** Human herpesvirus-8 ORF K8.1 gene encodes immunogenic glycoproteins generated by splice transcripts. Virology 1998;249:140-149.

161. **Garde SV, Basrur VS, Li L, Finkelman MA, Krishan A, Wellham L, Ben-Josef E, Haddad M, Taylor JD, Porter AT, Tang DG.** Prostate secretory protein (PSP94) suppresses the growth of androgen-independent prostate cancer cell line (PC3) and xenografts by inducing apoptosis. Prostate 1999;38:118-125.

162. **Hyakutake H, Sakai H, Yogi Y, Tsuda R, Minami Y, Yushita Y, Kanetake H, Nakazono I, Saito Y.** Beta-microseminoprotein immunoreactivity as a new prognostic indicator of prostatic carcinoma. Prostate 1993;22:347-355.

163. **Iwamura M, Abrahamsson PA, Foss KA, Wu G, Cockett ATK, Deftos LJ.** Parathyroid hormone-related protein: a potential autocrine growth regulator in human prostate cancer cell lines. Urology 1994;43:675-679.

164. **DaSilva J, Gioeli D, Weber MJ, Parsons SJ.** The neuroendocrine-derived peptide parathyroid hormone-related protein promotes prostate cancer cell growth by stabilizing the androgen receptor. Cancer Res 2009;69:7402-7411.

165. **Sepulveda VAT, Falzon M.** Parathyroid hormone-related protein enhances PC-3 prostate cancer cell growth via both autocrine/paracrine and intracrine pathways. Regul Pept 2002;105:109-120.

166. **Rabbani SA, Gladu J, Harakidas P, Jamison B, Goltzman D.** Over-production of parathyroid hormone-related peptide results in increased osteolytic skeletal metastasis by prostate cancer cells in vivo. Int J Cancer 1999;80:257-264.

167. **Deftos LJ, Barken I, Burton DW, Hoffman RM, Geller J.** Direct evidence that PTHrP expression promotes prostate cancer progression in bone. Biochem Biophys Res Commun 2005:327:468-472.

168. **Shukeir N, Arakelian A, Kadhim M, Garde S, Rabbani SA.** Prostate secretory protein PSP-94 decreases tumor growth and hypercalcemia of malignancy in a syngenic *in vivo* model of prostate cancer. Cancer Res 2003;63:2072-2078.

169. **Shukeir N, Arakelian A, Chen G, Garde S, Ruiz M, Panchal C, Rabbani SA.** A synthetic 15-mer peptide (PCK3145) derived from prostate secretory protein can reduce tumor growth, experimental skeletal metastases, and malignancy-associated hypercalcemia. Cancer Res 2004;64:5370-5377.

170. **Perl AK, Wilgenbus P, Dahl U, Semb H, Christofori G.** A causal role for E-cadherin in the transition from adenoma to carcinoma. Nature 1998;392:190-193.

171. **Lassi K, Dawson NA.** Emerging therapries in castrate-resistant prostate cancer. Curr Opin Oncol 2009;21:260-265.

172. **Attard G, Reid AHM, Olmos D, de Bono JS.** Antitumor activity with CYP17 blockade indicates that castration-resistant prostate cancer frequently remains hormone driven. Cancer Res 2009;69:4937-4940.

173. **Scher HI, Beer TM, Higano CS, Anand A, Taplin ME, Efstathiou E, Rathkopf D, Shelkey J, Yu EY, Alumkal J, Hung D, Hirmand M, Seely L, Morris MJ, Danila DC, Humm J, Larson S, Fleisher M, Sawyers CL, Prostate Cancer Foundation/Department of Defense Prostate Cancer Clinical Trails Consortium.** Antitumor activity of MDV3100 in castration-resistant prostate cancer: a phase 1-2 study. Lancet 2010;375:1437-1446.

174. **Roudier MP, True LD, Higano CS, Vesselle H, Ellis W, Lange P, Vessella RL.** Phenotypic herterogeneity of end-stage prostate carcinoma metastatic to bone. Hum Pathol 2003;34:646-653.

175. **Shah RB, Mehra R, Chinnaiyan AM, Shen R, Ghosh D, Zhou M, Macvicar GR, Varambally S, Harwood J, Bismar TA, Kim R, Rubin MA, Pienta KJ.** Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. Cancer Res 2004;64:9209-9216.

176. **Aihara M, Lebovitz RM, Wheeler TM, Kinner BM, Ohori M, Scardino PT.** Prostate specific antigen and Gleason grade: an immunohistochemical study of prostate cancer. J Urol 1994;151:1558-1564.

177. **Weir EG, Partin AW, Epstein JI.** Correlation of serum prostate specific antigen and quantitative immunohistochemistry. J Urol 2000;163:1739-1742.

178. **Erbersdobler A, Isbarn H, Steiner I, Schlomm T, Chun F, Mirlacher M, Sauter G.** Predictive value of prostate-specific antigen expression in prostate cancer: a tissue microarray study. J Urol 2009;74:1169-1173.

179. **Lamy S, Ruiz MT, Wisniewski J, Garde S, Rabbani SA, Panchal C, Wu JJ, Annabi B.** A prostate secretory protein94-derived synthetic peptide PCK3145 inhibts VEGF signaling in endothelial cells: implications in tumor angiogenesis. Int J Cancer 2006;118:2350-2358.

180. **Nam RK, Reeves JR, Toi A, Dulude H, Trachtenberg J, Emami M, Daigneault L, Panchal C, Sugar L, Jewett MAS, Narod SA.** A novel serum marker, total prostate secretory protein of 94 amino acids, improves prostate cancer detection and helps identify high grade cancers at diagnosis. J Urol 2006:175:1291-1297.

181. **Xie D, Gore C, Liu J, Pong RC, Mason R, Hao G, Long M, Kabbani W, Yu L, Zhang H, Chen H, Sun X, Boothman DA, Min W, Hsieh J.** Role of DAB2IP in modulating epithelial-to-mesenchymal transition and prostate cancer metastasis. Proc Natl Acad Sci USA 2010;107:2485-2490.

182. **Tanaka H, Kono E, Tran CP, Miyazaki H, Yamashiro J, Shimomura T, Fazli L, Wada R, Huang J, Vessella RL, An J, Horvath S, Gleave M, Rettig MB, Wainberg ZA, Reiter RE.** Monoclonal antibody targeting N-cadherin inhibits prostate cancer growth, metastasis and castration resistance. Nat Med 2010 16:1414-1421.

183. **Abate-Shen C, Shen MM.** Mouse models of prostate carcinogenesis. Trends Genet 2002;18:S1-S5.

184. **Chen H, Lin RJ, Xie W, Wilpitz D, Evans RM.** Regulation of hormone-induced histone hyperacteylation and gene activation via acetylation of an acetylase. Cell 1999;98:675-686.

185. **Gwack Y, Byun H, Hwang S, Lim C, Choe J.** CREB-binding protein and histone deacetylase regulate the transcriptional activity of Kaposi's sarcoma-associated herpesvirus open reading frame 50. J Virol 2001;75:1909-1917.

186. **Lu F, Zhou J, Wiedmer A, Madden K, Yuan Y, Lieberman PM.** Chromatin remodeling of the Kaposi's sarcoma-associated herpesvirus ORF50 promoter correlates with reactivation from latency. J Virol 2003;77:11425-11435.

187. **Reddy ES, Rao VN, Papas TS.** The *ERG* gene: A 2 human gene related to the ets oncogene. Proc Natl Acad Sci USA 1987;84:6131–6135.

188. **Mohamed AA, Tan SH, Mikhalkevich N, Ponniah S, Vasioukhin V, Bieberich CJ, Sesterhenn IA, Dobi A, Srivastava S, Sreenath TL.** Ets family protein, *ERG* expression in developing and adult mouse tissues by a highly specific monoclonal antibody. J Cancer 2010;1:197–208.

189. **McLaughlin F, Ludbrook VJ, Cox J, von Carlowitz I, Brown S, Randi AM.** Combined genomic and antisense analysis reveals that the transcription factor *ERG* is implicated in endothelial cell differentiation. Blood 2001;98:3332–3339.

190. **Birdsey GM, Dryden NH, Amsellem V, Gebhardt F, Sahnan K, Haskard DO**, **Dejana E, Mason JC, Randi AM.** Transcription factor *ERG* regulates angiogenesis and endothelial apoptosis through VE-cadherin. Blood 2008;111:3498–3506.

191. **Sreenath TL, Dobi A, Petrovics G, Srivastava S.** Oncogenic activation of *ERG*: a predominant mechanism in prostate cancer. J Carcinog 2011;10:37.

192. **Hart AH, Corrick CM, Tymms MJ, Hertzog PJ, Kola I.** Human *ERG* is a proto-oncogene with mitogenic and transforming activity. Oncogene 1995;10:1423–1430.

193. **Sementchenko VI, Schweinfest CW, Papas TS, Watson DK.** ETS2 function is required to maintain the transformed state of human prostate cancer cells. Oncogene 1998;17:2883–2888.

194. **Sun C, Dobi A, Mohamed A, Li H, Thangapazham RL, Furusato B, Shaheduzzaman S, Tan SH, Vaidyanathan G, Whitman E, Hawksworth DJ, Chen Y, Nau M, Patel V, Vahey M, Gutkind JS, Sreenath T, Petrovics G, Sesterhenn IA, McLeod DG, Srivastava S.** TMPRSS2-*ERG* fusion, a common genomic alteration in prostate cancer activates C-MYC and abrogates prostate epithelial differentiation. Oncogene 2008;27:5348–5353.

195. **Tomlins SA, Laxman B, Varambally S, Cao X, Yu J, Helgeson BE, Cao Q, Prensner JR, Rubin MA, Shah RB, Mehra R, Chinnaiyan AM.** Role of the TMPRSS2-ERG gene fusion in prostate cancer. Neoplasia 2008;10:177–188.

196. **Gupta S, Iljin K, Sara H, Mpindi JP, Mirtti T, Vainio P,Rantala J, Alanen K, Nees M, Kallioniemi O.** FZD4 as a mediator of *ERG* oncogene-induced WNT signaling and epithelial-to-mesenchymal transition in human prostate cancer cells. Cancer Res 2010;70:6735–6745.

197. **Leshem O, Madar S, Kogan-Sakin I, Kamer I, Goldstein I, Brosh R, Cohen Y, Jacob-Hirsch J, Ehrlich M, Ben-Sasson S, Goldfinger N, Loewenthal R, Gazit E, Rotter V, Berger R**. TMPRSS2/*ERG* promotes epithelial to mesenchymal transition through the ZEB1/ZEB2 axis in a prostate cancer model. PLoS ONE 2011;6:e21650.

198. **Drake JM, Strohbehn G, Bair TB, Moreland JG, Henry MD.** ZEB1 enhances transendothelial migration and represses the epithelial phenotype of prostate cancer cells. Mol Biol Cell 2009;20:2207–2217.

199. **Wang J, Cai Y, Shao LJ, Siddiqui J, Palanisamy N, Li R, Ren C, Ayala G, Ittmann M.** Activation of NF-{kappa}B by TMPRSS2-ERG fusion isoforms through Toll-like receptor 4. Cancer Res 2011;71:1325-1333.