ANALYSIS OF NOVEL TARGETS IN THE PATHOBIOLOGY OF PROSTATE CANCER

by

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The process of developing a greater understanding of the fundamental molecular mechanisms involved in prostate carcinogenesis will provide insights into the questions that still plague the field of prostate cancer research. The goal of this study was to identify altered genes that may have utility either as biomarkers, for improved diagnostic or prognostic application, or as novel targets important in the pathobiology of prostate cancer. We hypothesize that an improved understanding of the genomic and proteomic alterations associated with prostate cancer will facilitate the identification of novel biomarkers and molecular pathways critical to prostate carcinogenesis. In order to enhance our knowledge of the molecular alterations associated with prostate cancer, our laboratory performed microarray analysis comparing gene expression in healthy normal prostate to that in prostate cancer tissue. Of the greater than 400 genes with significantly altered expression identified in our study, MT2A, Tacc2, Nell2, FosB, PCP4, and Cyr61 were selected for further evaluation to confirm expression changes and evaluate their potential impact in prostate cancer. Analysis of MT2A, Tacc2, and Nell2 expression patterns failed to demonstrate significant changes between prostate cancer and donor prostate tissue and, therefore, these results do not support their further development as prostate cancer biomarkers. We demonstrated that PCP4 was expressed predominently in the stromal compartment of the prostate and was expressed at similar levels in the stroma of normal and prostate cancer tissue. Interestingly, protein expression of PCP4 in a panel of colon cancer tissues was dramatically

higher in adenoma and adenocarcinoma tissues compared to donor and benign colon tissue and, consequently, we feel that PCP4 has more potential as a biomarker in colon cancer than in prostate cancer. We also demonstrated that FosB and Cyr61 were upregulated in prostate cancer tissues over donor prostate tissues. Based on expression analysis of FosB and expression and functional analysis of Cyr61, we believe that these two targets have the greatest potental to be functionally significant in the etiology of prostate cancer.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

I would like to thank Dr. Robert Getzenberg for his support throughout my training and belief in my potential as a scientist and for reminding me along the way that earning a PhD is more about learning to think, question, and problem solve than about how the data comes together. I would also like to thank my committee for their time and input in the development of this project, for their insights and encouragement. There are many individuals at the University of Pittsburgh and at Johns Hopkins whose guidance has been indespensible and whose support and encouragement helped me to persevere through the difficult points along the way in particular, my lab mates and the members of both Departments of Urology.

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1.0 INTRODUCTION

1.1 PROSTATE GLAND

The prostate (Figure 1) is a walnut shaped and sized organ in men that is located at the base of the bladder through which the urethra passes. It is a tubuloalveolar gland composed of stroma and secretory acini, and produces fluids that contribute to semen (1). Hormonal signaling carefully regulates normal prostate development and function (2).

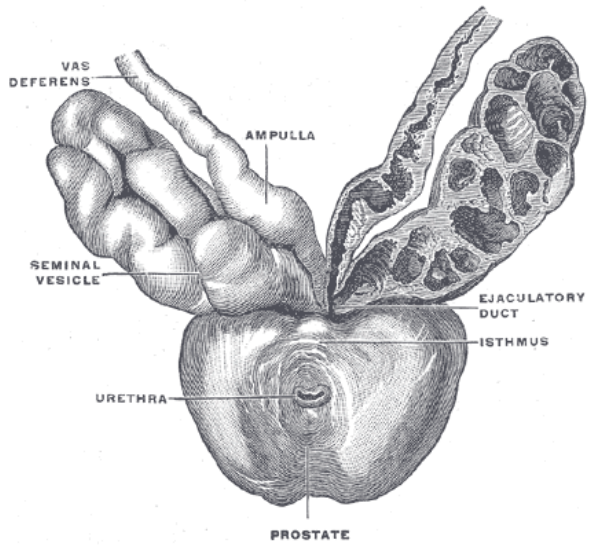


Figure 1. The prostate

http://www.enlargedprostateremedy.com (3)

The prostate is comprised of five zones (1). The anterior zone consists of predominantly smooth muscle. The preprostatic zone plays a role in ejaculation, preventing the flow of semen back up the urethra. The central zone, adjacent to the seminal vesicle, is the least likely to develop prostate cancer. The transitional zone consists of the area surrounding the proximal urethra. BPH and about one quarter of prostate cancer originates in the transitional zone (4). The peripheral zone, the area that most commonly develops prostate cancer, is the portion of the prostate posterior to the urethra and contains the highest concentration of prostate glands (4).

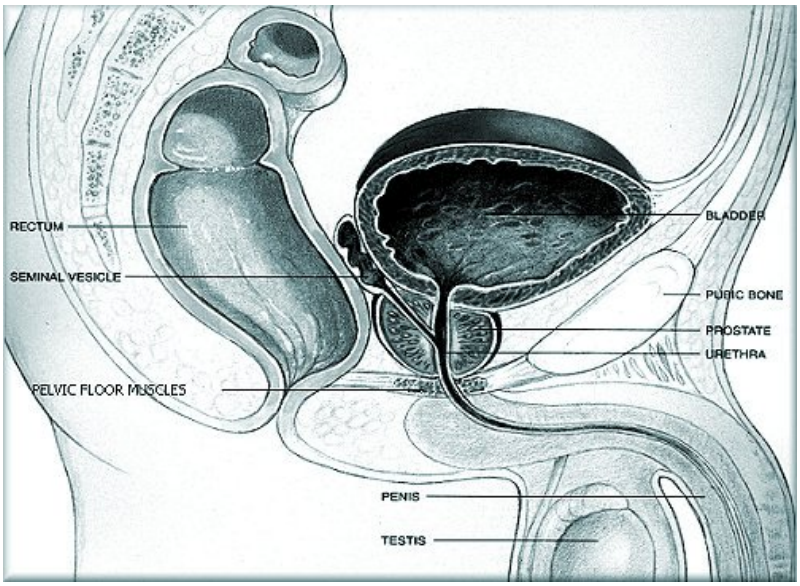


Figure 2. The prostate and associated anatomy

http://www.keyholeurology.co.uk/prostatecancer.html (5)

The prostate is predominantly composed of glands, which produce the simple sugars, enzymes and other components that contribute to semen, and the supporting stroma, which is made up of smooth muscle, nerves, blood vessels and fibroblasts (Figure 3). The architecture of the prostatic gland is characterized by the presence of a basement membrane separating the

epithelial from the stromal compartment. Prostate gland epithelium is comprised of a basal and luminal secretory layer of cells. The basal cells, which have a cuboidal morphology, lie adjacent to the basement membrane. These cells express p63, cytokeratins 5 and 14, and very little, if any, androgen receptor (AR) (6). The basal cell layer also contains a small number of prostate stem cells that maintain the basal layer as well as differentiate into the rare, terminally differentiated neuroendocrine cells. When stimulated by stromal-secreted paracine growth factors, basal cells undergo proliferation and further differentiation into transient-amplifying (TA) cells (6). Eventually TA cells lose expression of p63 and cytokeratins 5 and 14, and increase expression of cytokeratins 8 and 18 as well as AR (6,7), resulting in fully differentiated, luminal, secretory epithelial cells. Luminal epithelial cells, unlike basal cells, exhibit a stratified morphology, lack the capacity to self-renew or proliferate, and express AR-regulated genes such as prostate specific antigen (PSA) and human kalikrein 2 (hK2) (8). As a result of their dependence on androgen-stimulated stromal-secretion of survival factors, these luminal epithelial cells, unlike basal cells, undergo apoptosis as a result of androgen withdrawal. The stromal compartment, composed of fibroblasts, smooth muscle, nerves and supporting vasculature, plays a critical role in maintaining normal homeostasis within the prostate (9-11). Smooth muscle contraction of the glands is necessary for the expulsion of prostatic fluids during ejaculation but, later in life, the smooth muscle plays a role in the very common prostate disease benign prostatic hyperplasia (BPH) (4,12). Fibroblasts provide support by producing the basement membrane and matrix that supports the epithelial gland structures.

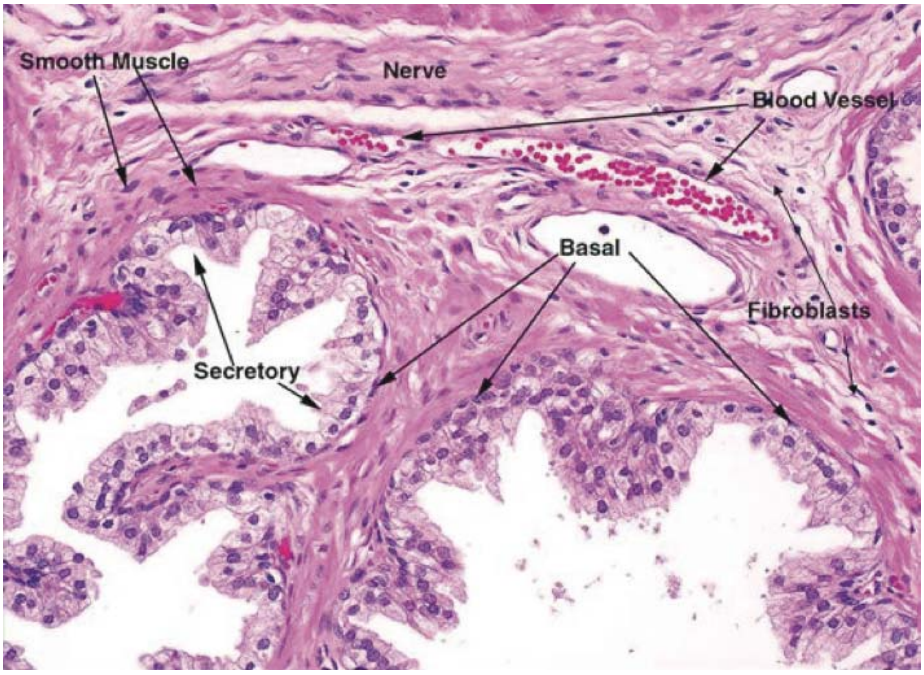


Figure 3. Cellular heterogeneity within the normal prostate

Histology of the normal prostate; the epithelial glands supported by stroma. (6)

The regulation of the prostate begins with the release of luteinizing hormone-releasing hormone (LHRH) from the hypothalamus, which signals the pituitary to secrete luteinizing hormone (LH) (13). LH circulates through the blood resulting in the production of testosterone by the testes. Testosterone, though mainly produced in the testes, is also made by the adrenal glands and is necessary for the growth and maintenance of the prostate gland (4). Taken up from the blood, cells of the prostate convert testosterone into the more active androgen dihydrotestosterone (DHT) through a reaction catalyzed by 5-α reductase. DHT and, with lower affinity, testosterone bind to the AR in the cytoplasm, the resulting ligand/AR complexes dimerize, shuttle into the nucleus, and interact with DNA at androgen response elements (ARE) in gene promotor/enhancer regions to effect transciption of downstream genes (4,6).

AR signaling in smooth muscle cells and fibroblasts induces the secretion of peptide growth factors including IGF, KGF, and FGF-10. These growth factors diffuse through the basement membrane and bind basal and luminal epithelial cell surface receptors to support proliferation and survival, respectively (14-17).

1.2 MALE REPRODUCTIVE SYSTEM

The organs of the male reproductive system include the prostate, testes, epididymis, penis, bulbourethral gland, ductus deferens, and seminal vesicles. Sperm production, which occurs in the seminiferous tubules of the testes, occurs as a continuous process in the post-pubescent male (1). Sperm mature as they pass through the epididymis and are either expelled through ejaculation or phagocytized by the epithelial cells of the epididymis. During ejaculation the ductus deferens and ejaculatory duct transport sperm from the epididymis to the prostate gland where the sperm enter the urethra and mix with the accessory gland secretions to produce semen (1). The accessory glands, including the seminal vesicles, bulbourethral gland and prostate gland, supplement sperm with fluid containing the nutrients and enzymes necessary to survive in the female reproductive tract (1). The seminal vesicles contribute about 60% of the total volume of semen; seminal fluid primarily contains fructose, ascorbic acid, and prostaglandins and is alkaline in nature. The prostate gland secretions, which constitute about 33% of semen volume, contain a high concentration of citrate, zinc, and various enzymes including fibrinolysin, hyaluronidase, acid phosphatase, and PSA (18). The secretions of the bulbourethral gland precede ejaculation in order to coat the urethra to neutralize residual acidic urine. Finally, this

mix of sperm and fluid travel through the urethra, which extends the length of the penis, and is expelled via the spinal reflex-controlled contraction of the bulbospongiosus muscle fibers (1).

1.3 PROSTATE DEVELOPMENT

Much of our knowledge of normal prostate development stems from the groundbreaking tissue recombination work of Gerald Cunha in the 1980s and 1990s. The studies illustrate the role of various hormonal, cellular and molecular mechanisms that regulate prostatic development. The prostate develops from the ambisexual endodermal urogenital sinus (UGS) and urogenital sinus mesenchyme (UGM), which also differentiates into the bladder and seminal vesicles (14). In the presence of fetal testicular androgens, the androgen receptor-positive (AR+) UGM induces the AR-negative (AR-) UGS to develop into prostatic epithelial buds that proliferate and grow out into the UGM (11). Through this process of branching morphogenesis, the UGS and UGM grow together differentiating to form the progenitor prostate through bud extension and branching that requires each tissue to respond to gradient signals dictating the type and direction of growth. Tissue recombination studies of chimeric prostates developed from UGM and UGE of wild-type and Testicular Feminized Mice (AR-null) established that mesenchymal-epithelial interactions tightly regulate early prostatic development (19). Androgens induce the UGM to initiate prostate epithelia determination of UGS and epithelial bud formation and branching, and promote cell differentiation into secretory epithelium (2,20). Later recombination studies demonstrated the necessity of UGE AR expression, triggered by UGM paracrine signaling, for the production of AR-dependent secretory proteins (21,22), which in turn induce the maturation of the UGM into the supporting stromal compartment (23). In the prostate there exists a fine balance between

epithelial cell proliferation and apoptosis, which is controlled by mesenchymal-epithelial paracrine signaling (10); a loss in the coordination of these hormonal, cellular and molecular interactions tips the scale in favor of proliferation, resulting in the development of prostate adenocarcinoma.

1.4 PROSTATIC DISEASE

Most men are thought to give little consideration to their prostate until symptoms arise later in life that generally manifest as difficulty with urination: urgency, frequency, flow rate, pain etc. There are three diseases most often discussed when considering prostate health: prostatitis, benign prostatic hyperplasia (BPH), and prostate cancer. Infections and inflammation of the prostate are classified as prostatitis. BPH refers to benign enlargement of the prostate gland. Lastly, prostate cancer is the abnormal growth of prostate cells leading to tumors.

1.4.1 Prostatitis

Prostatitis, characterized by inflammation of the prostate, is a common problem and the only one of the three prostate diseases that can affect men at any age (24). Symptoms of prostatitis include the frequent and urgent need to urinate, pain when urinating, and sometimes blood in the urine (25). These can be accompanied by fever, painful ejaculation and pelvic, groin or lower back pain. The National Institutes of Health (NIH) have subdivided prostatitis into four categories (4) :

- Category 1 – acute bacterial prostatitis

        - This is generally treated with antibiotics. Unchecked, an infection that originates in the prostate can spread to the bladder. Bacteria that are part of the colon's natural flora are often the cause.
    - Category 2 – chronic bacterial prostatitis
        - Also treated with antibiotics. Chronic infections can arise following an episode of acute prostatitis or as a result of repetitive stress to the urinary system: catheter tubes, biking or horseback riding.
    - Category 3 – chronic or nonbacterial prostatitis/chronic pelvic pain syndrome
        - Treatment aimed to relieve symptoms, not intended to target the cause. Although symptoms are similar to those for bacterial prostatitis, antibiotics are not effective. Though the cause is not always known, various factors contribute including viral infection, heavy lifting, interstitial cystitis, physical activity, and structural abnormalities of the urinary tract.
    - Category 4 – asymptomatic inflammatory prostatitis
        - This generally goes undiagnosed unless the doctor is looking at the prostate for another reason and is left untreated.

Prostatitis most often develops in men younger than age 40 but can develop later in life as well. It is not always clear why prostatitis develops and can be difficult to diagnose due to the fact that symptoms of the disease resemble those of a bladder infection, bladder or prostate cancer, even BPH. HIV infected men also are at increased risk of bacterial prostatitis (4,25).

1.4.2 Benign Prostatic Hyperplasia

BPH is a complex of symptoms more appropriately termed Lower Urinary Tract Symptoms (LUTS). One of the most notable aspects is a benign enlargement of the transitional zone of the prostate that develops as men age (Figure 4). Symptoms of BPH rarely develop before age 40 but become more common with each passing decade and include frequent urination, urgency, difficulty and pain while urinating, weak urine flow, and even acute urinary retention (4,26). BPH develops due to growth in either cell size or cell number of the transitional zone of the prostate.

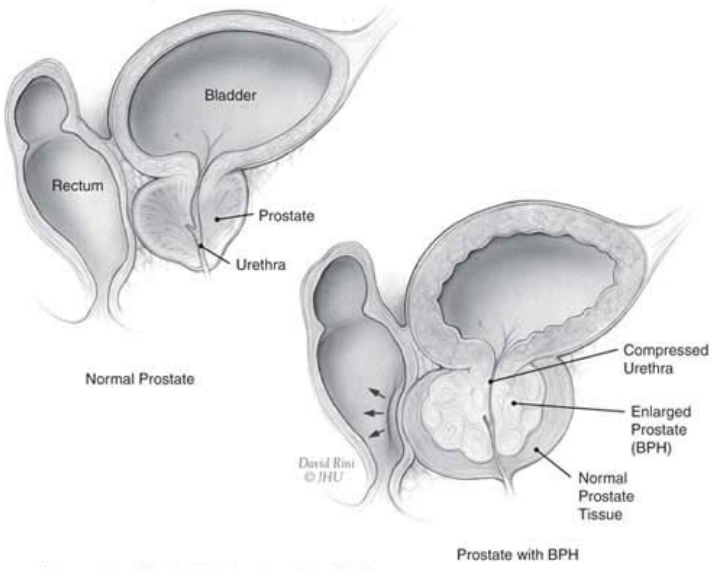


Figure 4. Benign prostatic hyperplasia

http://www.prostatecancerfoundation.org/site/c.itIWK2OSG/b.4091215/k.A28D/Prostatitis.htm (24)

BPH is a disease that can involve dysregulation of both the stroma and epithelium (27). Two mechanisms proposed to play a role in BPH are the hyper-sensitization of prostate cells to testosterone and the destabilization of epithelial-stromal interactions (28). Symptoms present

differently in each man depending on the individual's anatomy, the extent to which the urethra is pinched as the prostate expands, and the level of smooth muscle involvement (4). Treatment strategies take into account the level of smooth muscle involvement and size of the prostate. Alpha-blockers (doxazosin, alfuzocin, etc.) target the smooth muscle causing muscle relaxation aimed to decrease the pressure on the urethra and are particularly effective in those who have symptoms of BPH without evidence of an overly enlarged prostate gland (4). Another method used to treat BPH, particularly in those with substantial enlargement of the prostate, targets testosterone to block androgen signaling. 5-alpha reductase inhibitors (dutasteride and finasteride) prevent the conversion of testosterone into DHT leading to prostate involution (4). When symptoms become severe, BPH can require surgery. The most common type of surgery performed is transurethral resection of the prostate (TURP) where the prostate is removed in small pieces through the urethra (4). Another option, transurethral incision of the prostate (TUIP), differs from TURP in that incisions in the prostate are made to relieve pressure but no tissue is removed. Minimally invasive therapy options include transurethral microwave therapy (TUMP), in which targeted microwaves shrink the prostate, and interstitial laser therapy, during which overgrown tissue is targeted by laser (4,24).

The relationship between BPH and prostate cancer has long been a question and, although BPH and prostate cancer are two distinct diseases, evidence suggests that they may share some molecular similarities (29). Given that BPH, like prostate cancer, is a disease involving abnormal prostate growth, study of the molecular pathobiology of BPH may lead to insights that also enhance our understanding of prostate cancer.

1.5 PATHOBIOLOGY OF CANCER

In 1971 President Richard Nixon declared "War on Cancer" and, after almost four decades, clinicians and researchers still struggle to gain the upper hand against this disease (30). The diversity in the genetic background and environmental exposures of the human population and the ability of cancer cells to adapt when confronted with novel treatment methods complicate the study of this disease. Cancer develops when cells no longer grow, differentiate, or die in response to the conventional molecular cues resulting in an abnormal accumulation of cells. While great diversity exists from cancer to cancer, certain pathways and molecules are particularly vulnerable in the transition to and the maintenance of neoplastic growth. DNA repair, cell cycle and apoptotic regulation, growth factors with their receptors and a wide variety of other pathways become dysregulated in cancer (31,32).

1.5.1 Expression Changes, A Hallmark of Cancer

Dysregulation of the body's natural mechanisms essential in maintaining growth, differentiation, development and survival is a paramount step in the successful establishment of neoplastic growth. Changes in the genetic, epigenetic, and proteomic makeup of a cell all contribute to the process of cellular transformation (32-34). Although not all three of these mechanistic changes are required for cancer initiation, progression to more advanced disease is characterized by the accumulation of additional molecular alterations. The balance between tumor suppressor gene and oncogene expression and the function of the resulting proteins is critical in maintaining cellular and molecular homeostasis (32). During cancer initiation and progression, this balance is altered, thus favoring enhanced oncogene function in the face of lost tumor suppression. The

molecular pathways most often affected involve genes that encode regulators of the cell cycle and apoptosis, DNA damage repair proteins, transcription factors, growth factors and their receptors, and proteins that enhance metastatic potential (32). Tumor suppressor genes encode proteins, including p53, Rb (retinoblastoma protein), PTEN (phosphatase and tensin homolog), APC (adenomatosis polyposis coli), p14, p15, p16, VHL (Von Hippel-Lindau), GSTπ (glutathione S-transferase pi), Bad, Bax, BRCA1 (breast cancer 1), ERα (estrogen receptor alpha), etc., that prevent or slow the development and/or progression of tumor growth (31,32,35-37). Expression of these proteins is either decreased or blocked completely in cancer cells by a variety of means including deletion, inactivating mutation, and epigenetic silencing (promotor hypermethylation or histone modification). Oncogenes encode proteins whose up-regulated expression in cancer enhances tumor development or progression, such as Myc (myelocytomatosis viral oncogene), Akt (protein kinase B), Ras, NF-κB (Nuclear Factor kappaB), Bcl-xl (basal cell lymphoma-extra large), Bcl-2 (B-cell CLL/lymphoma 2), PI3-kinase (phosphatidylinositol 3-kinase) mTOR (mammalian target of rapamycin), ERK (extracellular signal-regulated kinase), BCR-ABL ("breakpoint cluster region" fused to the Abelson gene), etc (31,32,35-37). Elevated expression occurs by many different mechanisms including gene duplication, genomic amplification, chromosomal translocations, activating mutations or insertions, which may impact the promoter region or the coding sequence, and epigenetic modifications leading to expression.

Genetic alterations are believed to develop over the course of a lifetime as a result of lifestyle, diet, and environmental exposures as well as the normal effects of aging (33,38). The inheritance of an affected allele may also result in a predisposition to develop cancer (32). For

example inherited mutations in *BRCA1* and *BRCA2* are associated with an elevated risk of breast and ovarian cancer in women and prostate cancer in men (39-41).

More recent discoveries regarding epigenetic mechanisms of gene expression have been thrust to the forefront of cancer research. Defined as heritable changes in gene expression, which are not linked or accompanied by changes in the actual DNA sequence, epigenetic regulation of gene expression is essential for proper development as well as cancer initation and progression. The four primary components of epigenetics in cancer are DNA methylation, histone modifications, nucleosomal rearrangements and non-histone chromosomal proteins (33,42). Cells that deviate from their normal programming exhibit signs of tumor suppressor loss due to promoter hypermethylation, activation of oncogenic retrotransposon elements, and alterations in histone organization that alter RNA polymerase access to the DNA. Examples of epigenetically silenced tumor suppressor genes include Rb, VHL, APC, p16, BRCA1, and ERα (33).

Many proteomic changes can be attributed to the downstream impact of genetic and epigenetic changes, for example gene mutation leads to altered protein sequence and, potentially, function. In addition, post-translational modification (phosphorylation, glycosylation, acetylation, etc.) and protein localization (cytoplasmic vs. nuclear, intracellular vs. extracellular) greatly impacts proper protein function (43,44).

1.5.2 Metastasis

The evolution of metastatic potential in cancer is pivotal and often a most deadly transition (34). In order to develop metastases, cancers cells must develop a variety of abilities that healthy cells, even most cancer cells, generally do not possess (Figure 5). Cancer cells must break through the

basement membrane, migrate through the extracellular matrix (ECM), enter, survive, and exit the blood stream, as well as, survive and proliferate in a foreign tissue microenvironment.

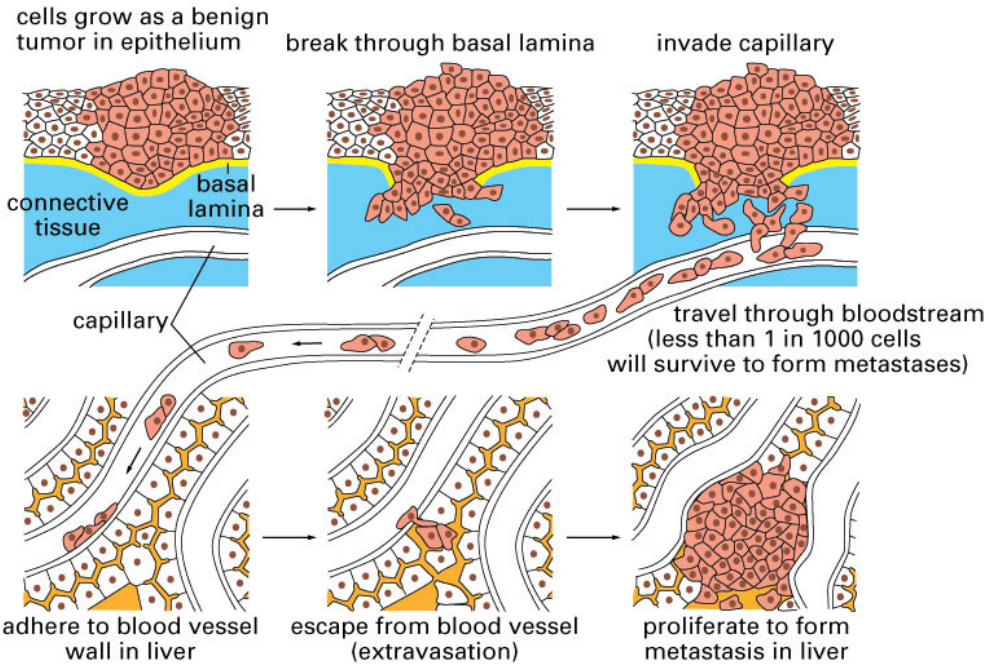


Figure 5. Steps involved in cancer metastasis

http://64.202.120.86/upload/image/articles/2008/smart-bombing-cancer/cell-grow.jpg (45)

Most healthy cells require survival signals, the maintainance of an intact basement membrane, and a tight association with neighboring cells but, in metastasis, the ability to survive without these external survival signals and to respond to the unique growth and survival factor milieu present at secondary sites is essential (31,38,46). In order to metastasize, tumor cells must digest the basement membrane by producing the necessary proteolytic enzymes, such as the matrix metalloproteinase, MMPs, and then alter expression of adhesion and surface recognition molecules to facilitate migration away from neighboring cells, into the connective tissue,

between the capillary endothelial cells (intravasation and extravasation), and into secondary sites (31,38,46).

Once the cancer cells become metastatic, cells disseminate throughout the body via the blood vasculature and lymphatics. While less than 0.01% of circulating tumor cells establish a lesion of cancer, millions of tumor cells are shed daily into the circulation and almost 30% of patients have clinically detectable metastases at the time of initial diagnosis (46). The signaling pathways involved in metastasis are prime therapeutic targets and targeted therapies under development include growth factor signaling blockade (EGFR or VEGF), MMP inhibitors, and taxanes, which interrupt microtubule cycling, to name a few (34).

1.6 PROSTATE CANCER

About 1 in 6 men will be diagnosed with the prostate cancer during their lifetime (4). Although not to diminish the number of lives lost due to prostate cancer, the truth is that many more men will die with prostate cancer than from the disease. Prostate cancer is typically a slow progressing cancer and, on average, ten years will pass between the time of diagnosis and when the cancer becomes life threatening.

Prostate cancer is a type of adenocarcinoma (cancer of glandular tissue) that develops most often in men over the age of fifty and generally produces slow growing tumors (4). With an estimated 186,000 new cases of prostate cancer diagnosed in 2008, prostate cancer is the most widely diagnosed cancer and the second leading cause of cancer deaths in men in the United States (47). In the early-mid 1990s, the acceptance of PSA as a diagnostic tool revolutionized the prostate cancer field. Since the advent of PSA testing, most prostate cancer does not present

with symptoms. If symptoms are present they generally resemble those of BPH and include frequent urination, blood in the urine, and pain while urinating (4,24,26). However, most men are diagnosed following an abnormal digital rectal exam (DRE) or an elevated PSA test that leads to biopsy of the prostate. Final diagnosis of prostate cancer requires histologic analysis of prostate biopsy tissue. In the post-PSA era the proportion of cancers identified after metastases develop dropped by 56% (from 14.9 in 1985 to 6.6 per 100,000 in 1995) (48). The most common treatment options include watchful waiting, radical prostatectomy, radiation therapy, androgen ablation therapy, and chemotherapy. Surgical removal of the prostate via nerve sparing radical prostatectomy, which is the most common treatment for organ-confined disease, not only offers a curative outcome but also limits adverse side effects, including impotence and incontinence which previously accompanied prostatectomy. In localized disease, external beam radiation therapy can yield similar outcomes as surgery (4). Additional therapeutic approaches for the treatment of prostate cancer include brachytherapy, chemotherapy for non-localized disease, and watchful waiting. Despite recent improvements in the diagnosis and treatment of prostate cancer, we are still unable to differentiate between indolent and lethal forms of the disease. As a result, many thousands of men worldwide, and particularly in the United States, are unnecessarily diagnosed and treated for a disease that they will never die from. This issue of overdiagnosis continues to frustrate both clinicians as well as research scientists. Therefore, identification and validation of more specific and sensitive biomarkers is critically needed in order to provide more accurate, minimally invasive diagnosis of the lethal forms of prostate cancer.

During prostate tumorigenesis the balance of stromal-regulated epithelial cell turnover changes, resulting in neoplastic growth (6). Originating from the seminal clinical studies by

Huggins and Hodges in the 1940s (49), the role of hormone action, more specifically the interplay of androgens and the AR, in prostate carcinogenesis has been the center of intense investigation. In the normal prostate, diffusion of androgens from the blood triggers AR signaling in the stromal compartment, leading to the secretion of growth and survival factors (andromedins), which act on both the stromal cells (autocrine) as well as epithelial cells (paracrine) (Figure 6) (50) (51-53). AR-stimulated paracrine secretion of andromedins functions in promoting basal epithelial cell proliferation and maturation into secretory luminal epithelial cells (6). Once differentiated, AR activation of luminal epithelial cells suppresses proliferation as demonstrated by p27 expression (54), induces terminal differentiation (6), and at the same time, represses transforming growth factor-β receptor (TGF-βR) expression (55). In contrast, AR signaling in prostate cancer cells exhibits "gain of function" molecular changes from a suppressor to that of a stimulator of growth (56). As part of this malignant conversion, AR activation within the nucleus of prostate cancer cells autonomously stimulates cell proliferation, independently of stromally-derived andromedins (Figure 6). One such novel "gain of function" change involves DNA rearrangements such that the promoter of the TMPRSS2 gene, which contains androgen response elements, is translocated to confer androgen responsiveness upon select members of the ETS transcription factor gene family (57,58).

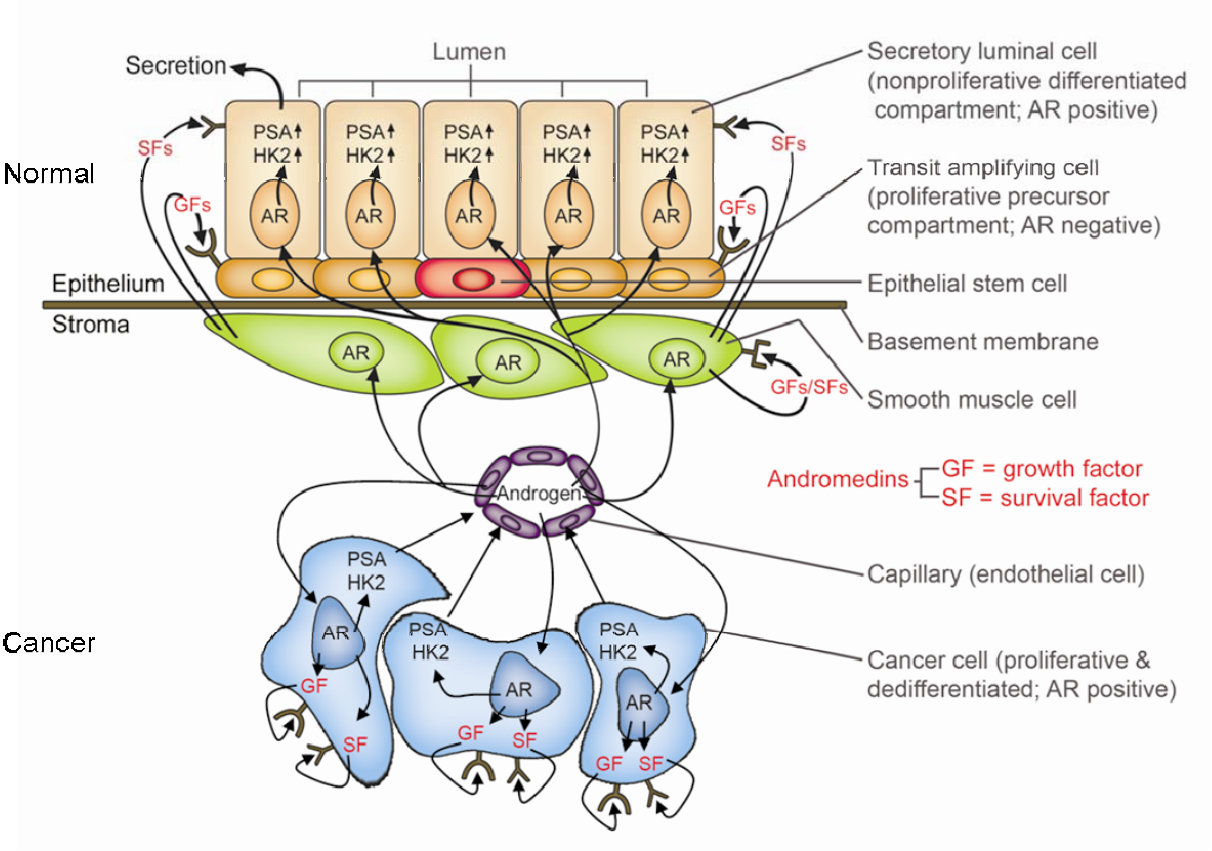


Figure 6. Dysregulation in prostate cancer

The disruption of normal architecture and hormone regulation in prostate cancer (50)

Based on the groundbreaking work of Huggins and Hodges in 1941, hormone ablation therapy has been the mainstay treatment for advanced, metastatic prostate cancer (49). Surgical castration, now typically achieved via chemical disruption of testosterone production, induces regression of the prostate luminal epithelial compartment, and therefore can provide significant therapeutic benefit. However, the majority of these patients will, in time, develop hormone-refractory disease (13). Many hypotheses have been proposed to explain this phenomenon and ongoing research continues to identify potential mechanisms that contribute to the adaptations that facilitate prostate cancer growth in a low androgen environment (59,60).

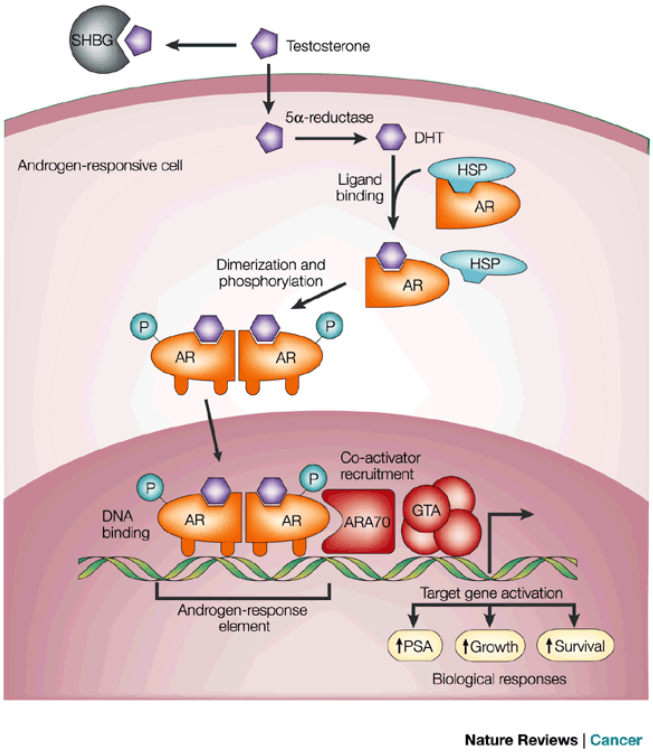


Figure 7. Normal androgen signaling and transcriptional activation

http://www.nature.com/nrc/journal/v1/n1/images/nrc1001-034a-f2.gif (61)

In cancer, strict regulation of growth of the prostate epithelial compartment is lost. With the loss of circulating testosterone, as in the case of surgical or medical castration, stromal andromedin secretion is replaced by transforming growth factor-β (TGF-β) secretion leading to TGF-βR-induced apoptosis of luminal epithelial cells (15,62,63) and subsequent regression of prostate epithelia and prostate cancer; molecular mechanisms that offer explanation for previous observations put forth by Huggins and Hodges in 1941 and Coffey *et al*. in 1968 (49,62).

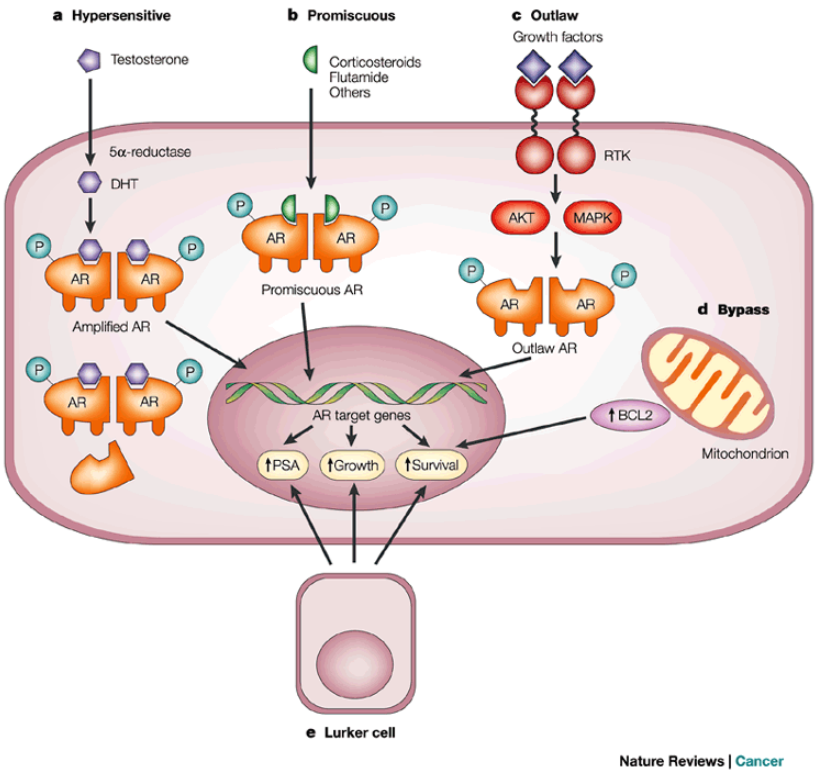


Figure 8. Possible pathways leading to androgen independence

http://www.nature.com/nrc/journal/v1/n1/images/nrc1001-034a-f2.gif (61)

Changes in AR levels, mutations that enhance AR function, amplification of AR copy number, and alterations in expression and function of AR co-regulators play a role in prostate cancer progression; however, additional mechanisms likely exist that contribute to cancer cell survival and the development of castration resistant prostate cancer (Figure 8) (31,59).

1.6.1 Risk Factors

Risk factors associated with prostate cancer include age, race, ethnicity, family history, environment, and diet (4). Of these age, race, and family history, i.e., genetic susceptibility, represent the primary risk factors influencing the development and progression of the disease.

Numerous epidemiological studies focusing on the potential benefits of dietary factors such as lycopene, selenium, vitamin E, vitamin D, and the potential harmful effects of diets high in red meat and exposure to charred meat by-products, such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PHIP) (64), yielded mixed results. Recently, studies looking at serum calcium levels as a predictive factor established a positive association between high serum calcium (Ca^{2+}) levels and prostate cancer incidence (65). However, studies such as these described above are confounded by variations in geography, accurate reporting of diet, smoking and medication use, as well as the inherent difficulties in extrapolating conclusions from small study populations, e.g., the Icelandic and Swedish studies.

A current trend in medicine is to focus on disease prevention but, in prostate cancer, the interplay of environmental and genetic factors have made this difficult. Studies show that a family history of prostate cancer is a significant risk factor and this, of course, cannot be altered, but factors like diet, lifestyle, and geographic location also play a potentially important role (66). Although data linking specific foods and dietary supplements with prostate cancer incidence remains unclear, the impact of diet on prostate cancer development should not be ignored (66). Additionally, prostate cancer incidence rates in those who immigrate from Japan, where rates are low, to the United States, where incidence rates are very high, rise within the first generation and increase further in second generation immigrants to levels equivalent seen in men in the United States (4). Although researchers attribute the increasing rate of prostate cancer to dietary changes, few specific differences have been discovered that contribute to prostate cancer susceptibility. When evaluated independently, physical activity, vitamin levels, outdoor exposure and the few dietary factors currently known, only elevate prostate cancer risk modestly and a better understanding of how life choices and environmental exposures impact the risk of

developing prostate cancer has the potential to decrease prostate cancer incidence without requiring pills, invasive tests, or surgery (65-75).

1.6.2 Diagnostics

Initial methods of screening for prostate cancer include digital rectal exams (DRE) and the PSA test but, since these tests are not definitive, prostate cancer must be confirmed by histological analysis of prostate biopsies. Prior to the advent of PSA testing DRE was the primary method of prostate cancer screening and is still employed by clinicians to evaluate prostate health and assess the presence and extent of cancer within the prostate. For men of African descent, and anyone with a family history of prostate cancer, PSA testing is recommended beginning at age 40. For all other men, annual PSA testing is recoomended after the age of 50, with a few exceptions (4). One of the nuances of prostate cancer is its slow progression. As such, life expectancy should be taken into account when screening patients. Specifically, men of advanced age, who are likely to die from other causes and therefore will not benefit from treatment for prostate cancer, may no longer need active screening.

1.6.3 Stage and Grade

Clinically, prostate cancer is described in two ways: stage and grade (Gleason score). Disease stage describes the location of the tumor, whether it is localized to a small area of the prostate, or has spread throughout the organ, to regional lymph nodes, or to other organs. Cancer staging is based on the recently updated TNM staging system: T represents local disease contained within

the prostate, and is further subdivided as described in Table 1; N indicates metastasis to regional lymph nodes; and M indicates the presence of distant metastases (4).

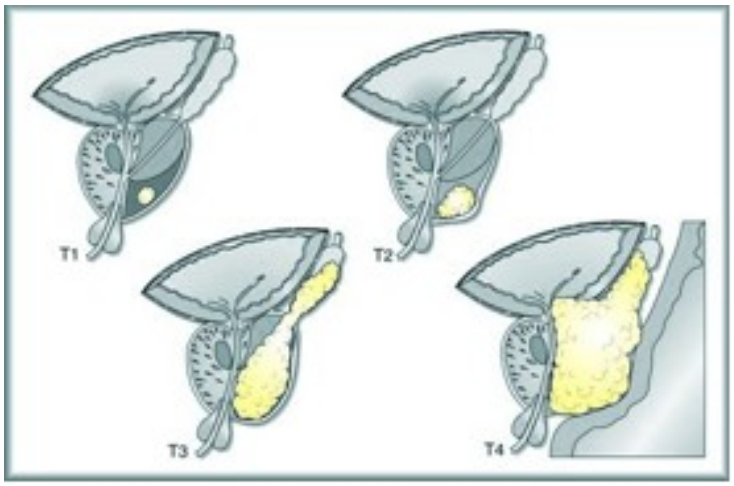


Figure 9. Stages of prostate cancer

http://www.prostate-cancer.org/education/staging/Dowd_GleasonScore.html (5)

Table 1. TNM Staging System

Stage	Description
T1a	Not palpable; found incidentally, 5 percent or less of the removed tissue is cancerous.
T1b	Not palpable; found incidentally, greater than 5 percent of the tissue removed by the TUR is cancerous.
T1c	Not palpable; identified by needle biopsy because of elevated PSA.
T2a	Palpable; involves less than half of only one of the lobes of the prostate.
T2b	Palpable; involves more than half of one lobe but not both lobes of the prostate.
T2c	Palpable; involves both lobes of the prostate.
T3, T4	Palpable; invades through the wall of the prostate and/or involves the seminal vesicles.
N+	Has spread to the lymph nodes.
M+	Has spread to bone.

Prostate cancer grade describes the histologic appearance of the cancer, which is based on analysis of biopsy tissue and referred to as clinical stage. However, this designation may be re-evaluated or corrected following radical prostatectomy, thereafter referred to as pathological stage. In general histologic appearance reflects the level of cellular dedifferentiation (76). In normal prostate, secretory glands are comprised of a single layer of well-differentiated, luminal epithelial cells. In regions of cancer, structures of the prostate strikingly change in appearance reflecting progressive cellular de-differentiation. Alterations in prostate architecture include loss of the basement membrane and basal cell compartment, changes in luminal cell nuclear morphology, as well as development of luminal epithelial cell hyperplasia. The Gleason grading system, developed by Donald F. Gleason in 1966, employs a scale of 1-5 to represent the most common histologic patterns seen in prostate cancer: 5 being the most aggressive and 1 the least (Figure 10) (77,78). For each individual, the Gleason score represents the sum of the Gleason grades for the two most common histologic patterns. Most men identified by needle biopsy today present with Gleason 5, 6, or 7 cancer while about 8% harbor high grade cancer (8, 9, or 10) (4). Lower Gleason scores are typically associated with organ-confined, localized disease, whereas higher grade cancers often present once cancer cells have metastasized to regional lymph nodes, seminal vesicles or other tissues. In recent years, Gleason 7 disease is often further stratified into Gleason 3+4 or 4+3 disease. 4+3 disease is considered more aggressive because treatment outcomes are typically less favorable compared to those for 3+4 disease.

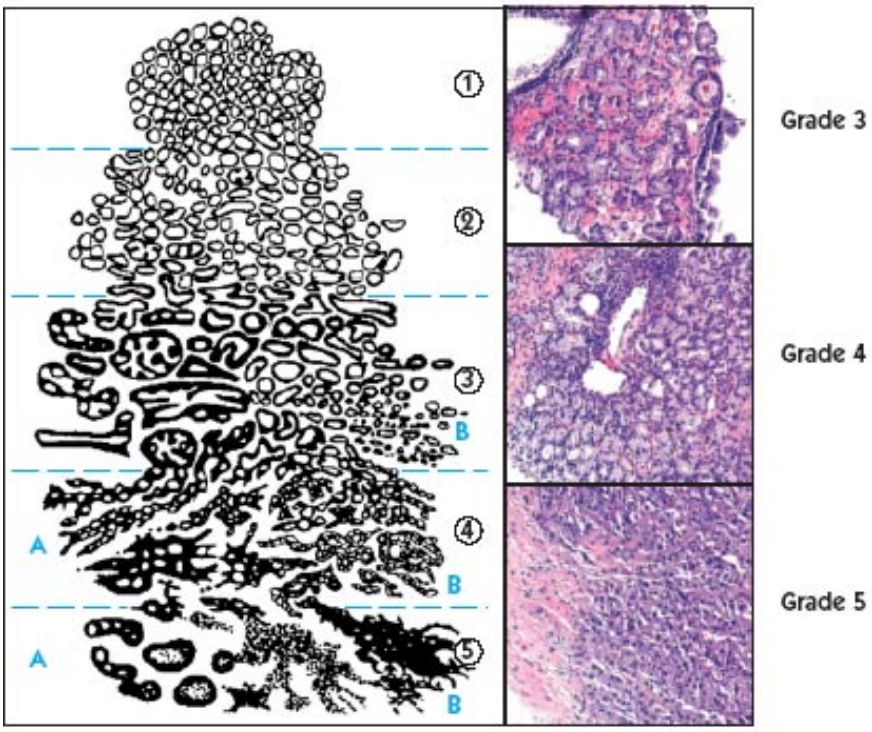


Figure 10. Gleason grading system diagram

http://www.prostate-cancer.org/education/staging/Dowd_GleasonScore.html (78)

The Partin Tables, developed in 1993 and updated in 2007, represent a combination of PSA testing, clinical staging and Gleason scoring, and facilitate more accurate pre-surgery prediction of pathologic stage, i.e., the likelihood of organ-confined disease (79-81). Similarly, the Kattan nomograms, the first of which was generated in 1999, are algorithms to predict 1) pre-treatment, a patient's probability of survival prior to primary treatment 2) post-radical prostatectomy, the probability of survival following prostatectomy 3) following recurrence, the probability of treatment success with salvage radiation therapy and 4) following recurrence, the probability of treatment success with hormone-refractory disease (82-91).

1.6.4 Genetics

Although the most recent genome wide analysis studies (GWAS) have not yielded a unique genetic signature for prostate cancer, the list of genes altered in prostate cancer, via both genetic and epigenetic mechanisms, continues to grow (92-94). Nonetheless, GWAS have revealed an association between particular single nucleotide polymorphisms (SNPs) and prostate cancer risk (2p15, 3p12, 6q25, 7p15, 7p21, 8q24, 9q33, 10q11, 10q26, 11q13, 17q12, 17q24.3, 19q13, and Xp11) (92,93,95-101). In the early stages of cellular transformation, gene expression changes in *MYC* (v-myc myelocytomatosis viral oncogenes), *MET* (met proto-oncogene), *TERT* (telomerase reverse transcriptase), *AMACR* (alpha-methylacyl-CoA racemase) and cell cycle regulators such as *CDKN1B* (cyclin-dependent kinase inhibitor 1B, $p27^{Kip1}$) are among the most common (102). The most prominent epigenetic modification observed in disease initiation is hypermethylation of the *GSTP1* promoter, rendering this gene silent (37,103). In patients with clinically diagnosed prostate cancer, *TP53* (tumor protein p53) is the most commonly mutated gene, along with a host of other less prevalent genetic alterations including *BCL2* (B-cell CLL/lymphoma 2), *PTEN*, *RB1*, *CDKN2A* (cyclin-dependent kinase inhibitor 2A, p16), and *TGFB1* (102). In stages of more advanced and hormone-refractory disease, mutated and amplified *AR* is the most consistent change observed both at the genetic as well as at the protein level (104). Fusion of the TMPRSS2 gene with members of the ETS family (ETS, ERG, ETV1, and ETV4) is also commonly seen in prostate cancer (105-108).

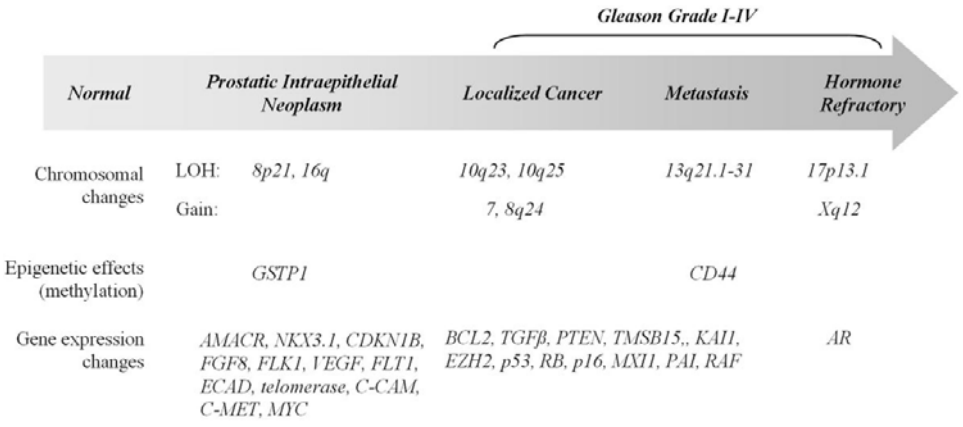


Figure 11. Alterations associated with prostate cancer progression

http://www.molecular-cancer.com/content/figures/1476-4598-3-9-8-l.jpg (102)

1.6.5 Treatment

When diagnosed early while disease is still locally confined, prostate cancer is most often treated by surgery (radical prostatectomy), radiation therapy, or expectant management (4). Factors that influence treatment include age at diagnosis, life expectancy, and quality of life considerations (issues of incontinence and impotence). Two methods of radiation therapy are used to treat prostate cancer: brachytherapy and external beam radiation therapy. Expectant management, also referred to as active surveillance or watchful waiting, involves a program designed to closely monitor the disease, without invasive treatment, until there are signs of cancer progression (4). Another option, far less common, is cryo- or thermal ablation therapy (24).

If prostate cancer is not diagnosed while it is still confined within the prostate or if there is evidence that prostate cancer has returned (rising PSA) following surgery, treatment options include radiation therapy, hormone therapy, and chemotherapy (4). Radiation therapy targets a particular area and therefore is most useful when metastases are local, to the seminal vesicles or

pelvic lymph nodes. Hormone therapy, also known as androgen-deprivation therapy (ADT), results in significant androgen-dependent apoptosis, which substantially reduces prostate size and tumor burden, for most men (6). ADT targets three main points in the signaling cascade leading to androgen action in the prostate: by disrupting the pulsitile manner of the hypothalamic-pituitary connection (LHRH agonists), by inhibiting testosterone synthesis (5α reductase inhibitors like finasteride), or by blocking androgen/AR signaling using AR antagonists (Figure 12) (13). However, progression to castrate-resistant disease generally occurs within 12-33 months (13).

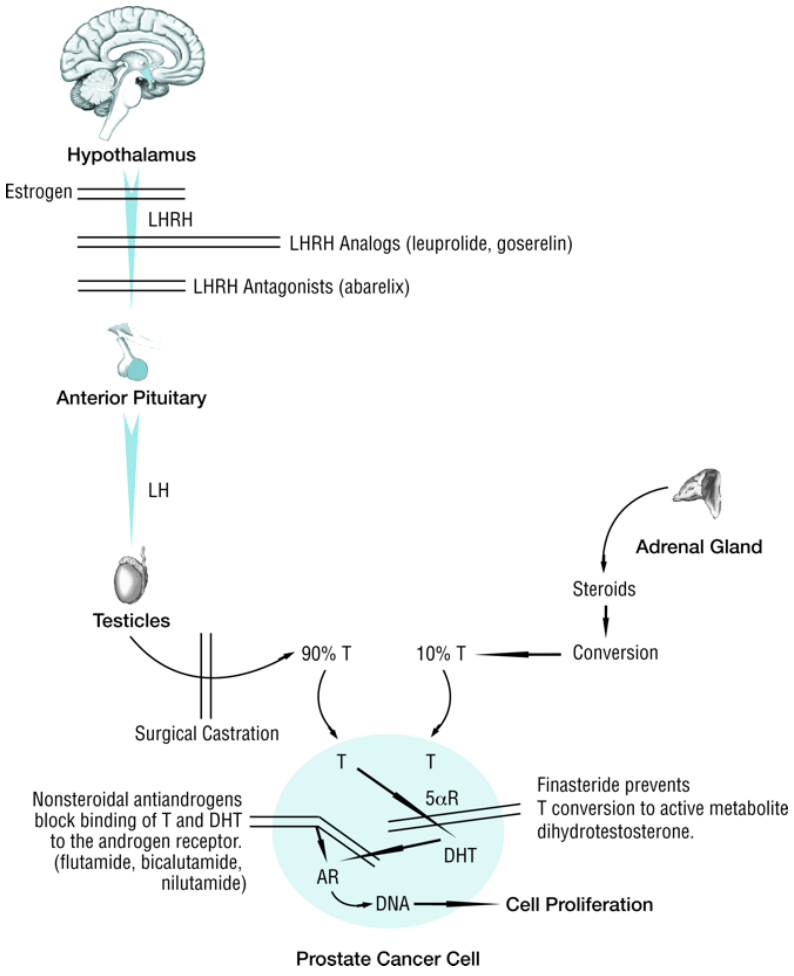


Figure 12. Strategies for androgen deprivation

1.7 PROSTATE CANCER BIOMARKERS

The utility of prostate cancer biomarkers is underscored by the transition that has occurred over the past few decades during which time the likelihood that prostate cancer will be diagnosed after metastasis has declined (4,48). In 1988, before PSA testing was a common clinical practice, of the men diagnosed with prostate cancer 20% already had metastatic disease (4). Currently only about 5% of prostate cancer is metastatic at the time of diagnosis (4). The increasing proportion of men presenting with low-grade, low-stage cancer is refered to as "stage migration" (109). Despite the clear benefits of PSA testing, this biomarker is far from perfect. PSA level in the serum can be elevated by a variety of other prostate conditions including prostatitis and BPH and, in fact, has been shown to increase slowly in the normal course of aging. Great effort has been made in the pursuit of novel prostate cancer biomarkers. Although some (PCA3, AMACR, and EPCA2) have been characterized, none are currently utilized clinically to the same extent as PSA.

1.7.1 The Issue of Over-Treatment

Of significant concern is the issue of over-treatment of prostate cancer that will never progress or disease that will not progress during a man's lifetime. As previously mentioned, a great number of men currently diagnosed with prostate cancer undergo radical prostatectomy or other treatment for prostate cancer when it is unclear that their disease has the capacity to progress and eventually kill. Many feel that a large subset of prostate cancer that develops is indolent and lacks the potential to metastasize. No good markers currently exist in clinical use with the ability

to differentiate ‘good’ cancer from ‘bad’ cancer, although some have been recently discovered (110).

In response to the concern regarding over-treatment, many institutions are implementing expectant management programs that allow patients with low grade cancers to enroll in a program designed to closely monitor disease progress. Definitive treatment is only undertaken after it becomes clear the tumor is growing. The hope, in those men with no evidence of disease progression, is to avoid unpleasant treatment and associated side effects from a disease that may not become a danger.

1.7.2 Prostate Specific Antigen

PSA, also known as kallikrein 3, is the most widely known cancer biomarker. It is an androgen-regulated serine protease that is secreted by the normal prostate predominantly into the prostatic lumen (1,111). Produced by the ductal and acinar epithelial cells, PSA functions in semen to cleave semenogelin I and II (111). When the prostate is diseased or experiences trauma PSA can leak into the bloodstream (4). Prostatitis and BPH, in addition to prostate cancer, allow PSA to escape the prostate gland. Although most PSA is produced by the prostate, low levels of PSA have been reported in the paraurethral and perianal glands, apocrine sweat glands, breast, thyroid, and placenta but extra-prostatic sources contribute little to circulating PSA (111). Taken together with the fact that not all prostate cancers exhibit elevated serum PSA, reliance on clinical testing of serum PSA levels is confounded by limited sensitivity and specificity to prostate cancer.

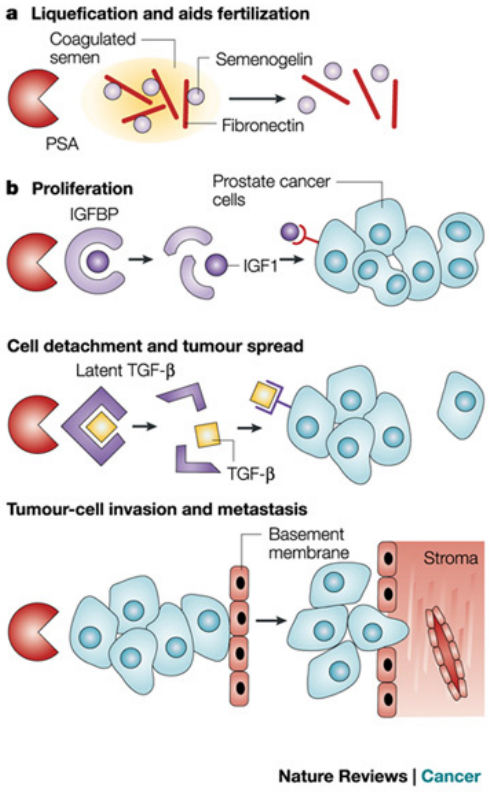


Figure 13. Function of PSA in a) the normal prostate and b) prostate cancer carcinogenesis

http://www.nature.com/nrc/journal/v2/n12/images/nrc951-f2.jpg (112)

Most studies regarding PSA focus on its utility as a biomarker, whereas only a few investigate mechanisms by which PSA might contribute to carcinogenesis (Figure 13) (112). In prostate cancer, PSA cleaves insulin-like growth-factor-binding protein 3 (IGFBP3) releasing insulin-like growth factor 1 (IGF-1), which acts as a mitogenic signal for prostate cancer cells (113,114). Also, PSA may activate TGF-β-induced cell detachment (115) or may cleave proteins in the ECM and basement membrane, thus facilitating metastasis (116).

Early PSA testing assayed only the amount of circulating total PSA but more recent screening techniques differentiate between free vs. total and free vs. bound PSA, as well as address PSA velocity and age-adjusted PSA cut-off points. Elevated free PSA is characteristic

of BPH while a comparatively high level of bound PSA is suggestive of prostate cancer (4). By studying the ratio of free to bound PSA, clinicians are better able to differentiate between cases likely to be BPH from prostate cancer (109). Studies previously demonstrated that PSA velocity, the rate of change in PSA from year to year, correlates with disease aggressiveness (117). Another limitation in the utility of PSA as a diagnostic tool is that, based on the results of the large Prostate Cancer Prevention Trial (PCPT) trial, only a quarter of men with abnormally high PSA levels ($\geq$ 4.0 ng/mL) have prostate cancer on biopsy (118). However, about 15% of men whose PSA level is considered normal (<1.0 ng/mL) do have prostate cancer found by biopsy (100,118,119). Currently, some leading urologists favor a sliding scale for circulating PSA and PSA velocity that lowers cut-offs for younger men, which, evidence shows, allows increased sensitivity without a substancial decrease in specificity, and raises cut-offs for older men, a population whose baseline PSA tends to be higher on average (120,121). The use of PSA as a regular screening tool is quite controversial and few large-scale studies have been undertaken with enough long-term follow-up to accurately weigh the survival benefits against the morbitity associated with treatment (122). Sceptics question whether the side-effects following prostate cancer treatment outweigh the benefits based on two main points: 1) in order to prevent a single death due to prostate cancer, many more men need to be treated for the disease and 2) definitive proof is lacking that the PSA sceening has lead to improved rates of mortality from prostate cancer. This impacts both the patients' quality of life and increases healthcare burden. The controversy rages on as two large, randomized trials published their conflicting results. The US-based Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial does not showed clear benefit in reducing mortality as a result of PSA testing and found that a similar portion of men in each group developed advanced disease (123). Additionally, the trial allowed men

previously screened by PSA test to enroll, did not ensure that men identified with elevated PSA during the course of the study received subsequent treatment by a urologist, and only had a median follow-up of 7 years. As a consequence this study may undervalue PSA screening. The European Randomized Study of Screening for Prostate Cancer (ERSPC) trial found a 20% relative reduction in mortality from prostate cancer with a mean follow-up of 9 years (124). Interpretation of this study is complicated by the fact that participating countries followed similar but not exactly identical protocols and factors like, randomization strategy, PSA cut-off, and follow-up varied between countries. Both studies reported findings prior to the 15-20 years of follow-up that many feel is necessary to truly evaluate the impact of PSA screening on mortality. Despite the controversy surrounding PSA screening in the general population, few doubt that PSA screening is beneficial particularly in the screening of men at high risk of developing prostate cancer. Other areas where the PSA test is particularly useful and effective are as a marker of disease recurrence and in monitoring the effectiveness of ADT (111).

1.7.3 Prostate Cancer Antigen 3

A urine test for prostate cancer gene 3 (PCA3), which is a non-coding mRNA sequence that more reliably identifies prostate cancer than serum PSA testing, is currently under development (125). PCA3 (*DD3*), originally identified in 1999, is encoded at 9q21 and includes 4 exons that yield, by alternative splicing, more than one mRNA transcript (126). The urine PCA3 test performed better than serum PSA at predicting prostate cancer in men undergoing repeat biopsy (125). When evaluated in conjunction with current diagnostic parameters in the PCPT trial, PCA3 was found to improve the accuracy of the PCPT risk calculator, an algorithm that predicts prostate cancer risk based on PSA, DRE, family and biopsy history, race and age (127). In two

studies of the standardized PCA3 urine test looking at large cohorts of men, the receiver operator curve (ROC) was about 0.69 (128-130). Although promising, the PCA3 urine test might be best utilized to supplement currents screening methods in order to improve the accuracy of prostate cancer diagnostics (125,131-134).

1.7.4 Alpha-methylacyl-CoA Racemase

In an effort to more confidently diagnose difficult-to-discriminate cases of prostate cancer, expression of AMACR, in addition to expression of basal cell markers, e.g., p63, is evaluated by immunohistochemical analysis in biopsy samples (135-139). In prostate cancer, AMACR expression is elevated while basal cell marker expression is lost.

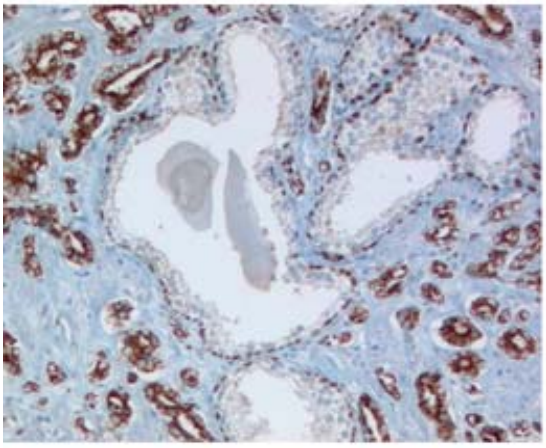


Figure 14. Prostate cancer stained for AMACR

Expression of AMACR is specific to regions of cancer (140)

The AMACR enzyme is involved in fatty acid metabolism, specifically peroxisomal β-oxidation of branched-chain fatty acids and bile acid intermediates (141). In 2002, it was

demonstrated that expression of *AMACR* is upregulated in 88% of prostate cancer tissues (141) and subsequent studies observed the same pattern at the protein level (142). Since then, staining for AMACR and p63, to help diagnose cases with abnormal histology, is becoming increasingly more commonplace (109,135-138,143-146).

1.7.5 Early Prostate Cancer Antigen 2

Changes in the expression and organizatin of nuclear matrix proteins is characteristic of cancer (147). Early Prostate Cancer Antigen (EPCA) 2 is a nuclear matrix protein identified previously by our group that differentiates between prostate cancer and non-cancerous serum samples by ELISA with higher sensitivity and specificity, 94% and 92% respectively, than PSA (110). Additionally, EPCA-2 expression was also better able to differentiate between men with organ-confined and non-organ-confined prostate cancer (109,110). Further validation studies are underway as well as the development of a test that could be utilized clinically.

2.0 PROJECT BACKGROUND AND METHODS

2.1 HYPOTHESIS

The process of developing a greater understanding of the fundamental molecular mechanisms involved in prostate cancer will provide insights into the questions that still plague the field of prostate cancer research. In order to improve our understanding of the molecular alterations associated with prostate cancer, our laboratory performed microarray analysis comparing gene expression in healthy normal prostate to that in prostate cancer tissue. The goal of this study was to identify altered genes that may have utility either as biomarkers, for improved diagnosis or with prognostic importance, or as novel targets important to the pathobiology of prostate cancer. Of the greater than 400 genes with significantly altered expression, a small number were selected as interesting targets and further evaluated to confirm expression changes and evaluate potential impact in prostate cancer. **We hypothesize that an improved understanding of the genomic and proteomic alterations associated with prostate cancer will facilitate the identification of novel biomarkers and molecular pathways critical to prostate carcinogenesis.**

2.2 TARGET GENES MODIFIED IN PROSTATE CANCER

Of the genes that appear most intriguing from our genomic analysis, a number are described in this chapter and thoughout the remainder of this thesis. These represent the genes considered to be most relevant to our understanding of prostate cancer.

2.2.1 Metallothionein 2A

The metallothioneins (MT) are a family of proteins that safeguard cells from environmental insults through the intracellular transport of metals, primarily zinc but also cadmium, copper, cobalt, mercury, silver, gold, platinum, lead, arsenic, technetium and bismuth (148,149). The intracellular metal concentration requires tight regulation because the availability of metals, zinc in particular, impacts enzymatic activity; some enzymes are inactive or have low activity in the absence of zinc while others are inhibited by the presence of zinc (148). These low molecular weight (~7kD) proteins are uniquely structured to accommodate metal binding. The α and β domains each form cysteine rich clusters, unique to the metallothionein family of proteins, that bind molecules of zinc (or other metals) (148). MTs also effectively quench free radicals and are up-regulated in the presence of oxidative stress (150). Zinc deficiency impacts growth and has been linked to certain malignant tumors (151). Normal prostate contains very high levels of zinc but, during carcinogenesis, a significant decrease has been reported (152) and prostate cancer cells undergo apoptosis in response to zinc exposure, unlike normal prostate (153). In humans, 10 functional and 7 nonfunctional isoforms of MT are encoded by a family of genes located at 16q13 (149). MT-1A, -1B, 1E, 1F, 1G, 1H, 1X and MT-2A are expressed in most adult tissues. Expression of MT-3, once thought to be uniquely expressed by the central nervous system, is

also found in kidney, breast, pancreas, intestine, bladder, and prostate cancer, whereas expression of MT-4 is limited to stratified squamous tissues and during cell differentiation (148,153). BPH, prostate cancer cells, and prostate cancer tissues express low levels of MT1/2 but exposure of prostate cancer cells to high concentrations of zinc induces expression of MT1/2 (153). The restoration of proper intracellular zinc levels through up-regulation of MT is possibly one mechanism by which the proper balance between growth and apoptosis may be re-established in prostate diseases (153,154). In a recent study, Yamasaki *et al*. demonstrate that MT-1X and -2A are up-regulated in prostate cancer cells exposed to hypoxia and that MT expression elevates the resistance of cells to radiation and chemotherapeutic agents like cisplatin (154).

2.2.2 Transformin, acidic coiled-coiled containing protein 2

Between 1999 and 2003 the three human members of the transforming, acidic coiled-coil containing (Tacc) family were cloned (155-157). Through genomic sequence analysis and investigation of proposed protein interactions, a few early studies have shed light on the proposed function of Tacc proteins in the regulation of microtubule dynamics. The Tacc family is evolutionarily conserved; homologs exist in organisms ranging from yeast to mammals but only in mammals and insects does alternative splicing play a role (158,159). All Tacc proteins are highly acidic, contain an N-terminal proline-rich and a serine-rich region, along with a C-terminal coiled-coil domain (160,161), and a potential tyrosine phosphorylation site (157). Gergely *et al.* found that the *D. melanogaster* homolog, D-tacc, interacts with microtubules and is critical in normal spindle function (162-164). Human Tacc proteins also interact with the spindle apparatus and other microtubule structures. Tacc2 associates with the centrosome-spindle apparatus during cell cycling and can directly bind to the nuclear hormone receptor

RXRβ (161). *In vitro*, Tacc2 has been shown to bind histone acetyltransferases (HAT), and this interaction has been confirmed *in situ* with HAT protein hGCN5L2 (165). According to Still *et al.* "the function of the TACC proteins may have evolved from performing assembly or coordination functions in the centrosome to include a more intimate role in the functional evolution of chromatin remodeling, transcriptional and posttranscriptional complexes in the cell (161)."

Recently, the importance of Tacc2, particularly its role as an oncogene, has been called into question by the development of phenotypically normal Tacc2 knockout mice (166). The role of Tacc proteins in cancer remains unclear. Tacc1 and 3 are commonly modified in cancer but expression may be up- or down-regulated depending on the type of cancer (159,161); however, Tacc2 expression is commonly lost in breast cancer and forced expression decreases tumorigenicity of breast cancer cell lines (160). Tacc2 is encoded on chromosome 10q26.13 (167), an area commonly lost in prostate cancer. MXI1 and PTEN are tumor suppressors encoded at nearby loci within the region of 10q23-26 allele loss (168). The Tacc2 knockout mouse was not found to have an increased incidence of tumors in the first fifteen months of life (166), calling into question the role of Tacc2 in cancer.

2.2.3 Nell2

In large part, past research of neural epidermal growth factor like-2, Nell2, has focused on expression and function in the nervous system (169-173). Originally identified as a homolog of the chicken protein nel1, this protein is expressed throughout the chick embryo prior to hatching, after which, expression becomes limited to neurons (173). Initial studies report that, outside of development, Nell2 expression is also confined in humans to the nervous system (173) but, in

fact, subsequent studies demonstrate high expression in the prostate and pancreas. This raises the possibility that Nell2 plays a similar role in these tissues in proliferation, growth inhibition, and differentiation (167). This 816 amino acid protein is encoded by a gene located at 12q13.1 (167). Nell2 contains 6 epidermal growth factor (EGF)-like domains, 5 von Willebrand factor C domains, and an N-terminal TSP-1 domain (169,171,174). Both a heavily glycosylated and an unglycosylated form exist in neural cells but the impact of glycosylation on the function of Nell2 remains unclear (172). Expression of Nell2 is predominantly cytoplasmic but in some neural cells the secretion of a homotrimeric form has been reported (169,172,174). Nell2 phosphorylation is induced by estrogen and PKC signaling (169,172,174,175). Aihara *et al.* found that Nell2 activates JNK leading to survival in neural cells (169). The Nell2 knockout mouse grows normally except for decreased plasticity of the hippocampus; no abnormal growth or disease was noted in the knockout mouse prostate (171).

Two studies evaluating the expression of Nell2 in BPH have found that Nell2 expression is up-regulated in BPH and also expressed by basal epithelial cells (176,177). Nell2 may be involved in regulating epithelial-stomal homeostasis and, through its impact on growth rate, Nell2 may contribute to the pathobiology of prostate cancer.

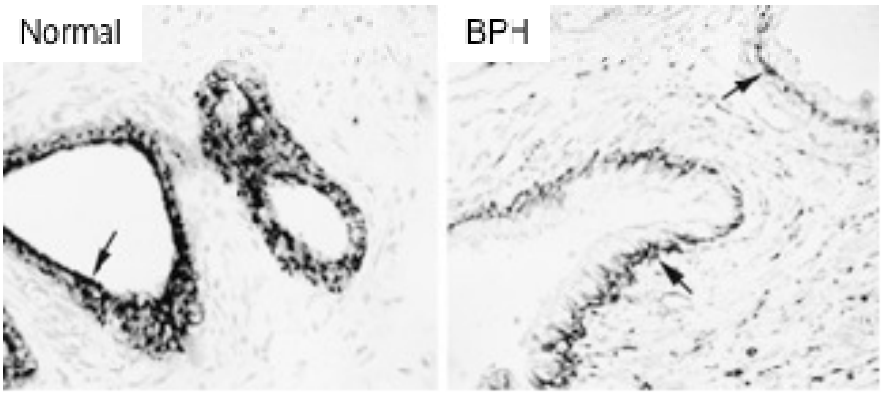


Figure 15. Nell2 expression decreases in BPH

In a recent study, the copy number of Nell2 was determined by Affymetrix 11.5K single-nucleotide polymorphism (SNP) arrays (94). The DNA of seven BPH tissue samples was extracted and copy number analysis was performed as previously reported (178). Briefly, DNA was digested with *XbaI* and ligated to a single primer. Linkers were ligated to the XbaI fragments using T4 DNA ligase. The linkers provide a primer site for the subsequent PCR reaction. PCR amplification under conditions favoring the generation of 200 base pair amplicons was then performed. These amplicons were fragmented, fluorophore-labeled, and hybridized to an Affymetrix SNP array containing over 400,000 probes interrogating over 11,560 SNP loci (179). Using the informatics platform dChip (180), signal intensities at each probe locus were analyzed, compared to a normal prostate tissue, representing (178) DNA from 12 individuals without any prostatic disease. The copy number at each SNP locus was determined.

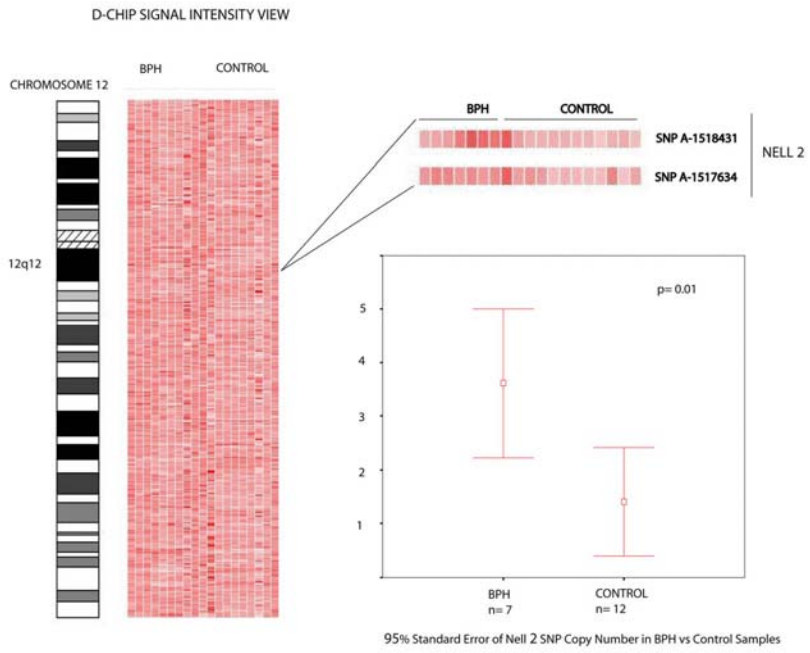


Figure 16. Loss of Heterozygosity of the Nell2 gene in prostate cancer (94)

Two SNPs specifically target the Nell2 locus. The copy number of Nell2 was evaluated by measuring the intensity of expression of both SNP loci within the region spanned by Nell2, (Figure 16). On average, a copy number increase was observed at Nell2 loci in BPH samples compared to normal prostate (T-test, p=0.01) (94). This suggests that Nell2 is involved in prostatic disease. Considering these gene copy increases in conjunction with the microarray data showing expression level changes, we surmise that Nell2 is involved in prostate cancer and BPH, each involving changes in cellular proliferation.

2.2.4 FosB

The FBJ murine osteosarcoma viral oncogene homolog B, FosB, is a member of the Fos family of transcription factors that form AP-1 dimers (181,182). These are leucine zipper proteins capable of interacting with DNA at the TRE, TPA response element (182). The members of this

family fall into one of two groups. Fos proteins, c-Fos, FosB, Fra-1, and Fra-2, heterodimerize with Jun proteins to bind the AP-1 binding elements of DNA. The Jun proteins, c-Jun, JunB, and JunD, can either heterodimerize with Fos proteins or homodimerize and interact with DNA (182). Fos/Jun complexes are more stable and have a higher affinity for AP-1 binding elements than Jun/Jun complexes. The most stable complex is FosB/JunD (181). AP-1 family members are induced and activated by growth factors, cytokines, and numerous other physiological and pathological stimuli (182). The ratio of proteins as well as the expression levels of the different Fos and Jun proteins impacts gene expression. There are two forms of FosB. The full length protein is 65 kDa and is able to interact with Jun proteins, bind DNA, and activate transcription (183). There is also a splice variant of 55 kDa able to complex with Jun proteins and bind DNA but is incapable of activating transcription (184-188). Jochum *et al.* demonstrated that the splice variant, ΔFosB, acts as a negative regulator of gene transcription and that abnormal expression of ΔFosB in osteoblasts promotes differentiation (189).

There has been a significant amount of research into the AP-1 proteins since the discovery that a number have oncogenic potential. c- and v-Fos and c- and v-Jun have transforming capability in some cell lines (182). Knockout mice and overexpressing mice have been developed but no overt phenotype exists beyond a nurturing defect and somewhat diminutive size (189). In breast cancer, it has been noted that FosB is more highly expressed in well differentiated tumors than in poorly differentiated breast cancers (183). Bamberger *et al.* report a correlation between expression of FosB and well-differentiated estrogen receptor positive breast cancer (181). If the same correlation exists in prostate cancer, increased expression of FosB may have potential as a biomarker of more advanced or aggressive prostate cancer. Identification of patients with higher FosB expression may indicate a more aggressive

disease enabling clinicians to formulate a different treatment plan than that required of early localized disease.

2.2.5 Purkinje Cell Protein 4

At present, only a limited body of research exists regarding Purkinje cell protein 4 (PCP4). In 1986, this 61 amino acid peptide, also known as neuropeptide-19 or peptide-19, was identified and thought to be a neural specific protein (190). Similar to Nell2, PCP4 expression has subsequently been recognized in a number of tissues; most abundantly in brain, kidney, and prostate, less prominently in uterus, heart, and pancreas (190,191). PCP4 is a calmodulin binding protein involved in regulating intracellular Ca^{2+} levels by binding and sequestering Ca^{2+}-free calmodulin (191). Kanamori *et al.* postulate that PCP4 may interfere with apoptosis (191). Although it is not yet clear which molecules are activated downstream, Slemmon *et al.* demonstrate that PCP4 signals independently of protein kinase C (192). Like other proteins selected, PCP4 is involved in dysregulation of growth but there is no previously published link with cancer. The over-expression of PCP4 has been identified in human uterine leiomyoma by mRNA and immunohistochemistry (191). The potential role in apoptosis makes PCP4 an interesting protein to address in terms of prostate cancer.

2.2.6 Cysteine-rich, angiogenic inducer, 61

2.2.6.1 CCN protein family

CCN1, the first member discovered in the CCN (Cyr61/connective tissue growth factor/nephroblastoma over-expressed) family is more commonly known as cysteine-rich,

angiogenic inducer 61, Cyr61 (193). Other members of the CCN family include CTGF (CCN2), Nov (CCN3), WISP1 (CCN4), WISP2 (CCN5), and WISP3 (CCN6). CCN proteins are induced in response to various serum growth factors, cytokines, and environmental stresses (194,195). CCN proteins secreted into the extracellular matrix exert their influence on cells through interactions with integrins in processes like development, wound healing, tissue regeneration, angiogenesis, and fibrosis (194). Each protein in this family is composed of four structural domains: insulin-like growth factor binding protein domain, von Willeband type C domain, thrombospondin-1 domain (TSP1), and a C-terminal domain with a cysteine knot motif required for activity (196). Many members of the family, including Cyr61, possess an N-terminal signaling peptide. Cyr61 is named for its high concentration of cysteine residues, 10% by mass or 38 residues, higher than other members of this family (196).

2.2.6.2 The normal role of Cyr61

Expression of Cyr61, as its name indicates, has been affiliated with angiogenesis. Specifically, it promotes endothelial cell growth, migration, adhesion, and survival *in vitro*; it is a growth factor-inducible immediate early gene that is a positive regulator of growth (197). It is important in the development of the kidneys, nervous system, muscle, cartilage, bone marrow, and bone (197). Brigstock *et al.* suggest that Cyr61 modulates hormonal pathways or hormone-regulated processes making it an interesting target for prostate research (196). Interestingly, Cyr61 appears to respond to Vitamin D in human fetal osteoblasts to induce differentiation (198) but this has not yet been evaluated in prostate. Cyr61 binds cell surface integrins, specifically $\alpha_V\beta_3$ and $\alpha_{IIb}\beta3$, and heparin sulfate proteoglycans (HSPCs) located in the ECM to induce intracellular signaling events that include kinase activation and gene transcription (196). The Cyr61 knockout mouse is embryonic lethal (196,199).

2.2.6.3 Cyr61 in disease

Alterations in Cyr61 expression have been noted in a number of diseases. Cyr61 expression decreases in endometriosis (200) and up during acute renal failure (193) and in atherosclerotic and arteriosclerotic vessels (196). Cyr61 may be involved in disrupting the stromal-epithelial balance. This was demonstrated in experiments in which Cyr61 specific antibodies blocked Cyr61's ability to stimulate cell proliferation (201).

2.2.6.4 Cyr61 in cancer

In genomic studies of various cancers, Cyr61 commonly exhibits altered expression (202-214). These expression changes have been linked with, depending on the type of cancer, either enhanced or inhibited tumor growth. Modulation of Cyr61 expression has been observed in melanoma, breast, ovarian, hepatocellular, lung, colorectal cancer, and esophageal squamous cell carcinoma. In early studies Pilarskey *et al.* found that Cyr61 mRNA is down-regulated in prostate cancer compared to normal adjacent-to-cancer tissue (215). More recently, in our study comparing BPH to healthy donor prostates, symptomatic BPH samples exhibited moderately elevated levels of Cyr61 mRNA while a subset of prostate cancer samples also evaluated exhibited expression greater than either BPH or donor prostates (29). In additional analysis of expression in the prostate, Sakamoto *et al.* showed that mRNA expression of Cyr61, by *in situ* hybridization, localizes to the basal cells in normal prostate tissues (216). Additional evidence substantiating the importance of Cyr61 in prostate cancer was recently published by Sun *et al.*; the expression of Cyr61 in prostate cancer cells was found to enhance migration, invasion, and proliferation and showed that Cyr61 regulates Rac1 signaling (217), a mechanism by which Cyr61 potentially may exert its influence on cell growth and motility.

2.3 MATERIALS AND METHODS

2.3.1 Cell Culture

LNCaP, PC3, and LAPC4 prostate cancer cells were obtained from ATCC and maintained in RPMI with 10% FBS and 5% penicillin/streptomycin, P/S. LAPC4 prostate cancer cells were maintained in IMDM media with 10% FBS and 5% penicillin/streptomyin supplemented with testosterone. CWR22R.v1 were generously provided by the lab of Dr. John Isaacs and maintained in RPMI with 10% FBS and 5% P/S. All cells were maintained at 37°C and 5% CO_2. Cells were fed every 3-4 days and passaged when cells reached about 90-95% confluence.

2.3.2 Tissues and Tissue Microarrays

Healthy normal donor, prostate cancer, and normal adjacent to prostate cancer tissue samples, for RNA and protein isolation, were obtained from the University of Pittsburgh Medical Center Tissue Bank. Paraffin embedded donor prostate tissue was obtained from organ donors. The tissue bank provided >500 mg of tissue which was snap frozen in liquid nitrogen within 30 min of excision and stored at -80°C. All samples were submitted for pathological evaluation.

Tissue microarray analysis (TMA) sections were obtained from the TMA facility in the Department of Pathology at the Johns Hopkins School of Medicine. Tissue specimens used for making the 5 high-density TMAs were obtained from 200 consecutive radical retropubic prostatectomies performed at the Johns Hopkins Hospital between 2000-2001. All specimens consisted of tissue dissected immediately after surgical removal and immersed in 10% neutral

buffered formalin prior to paraffin embedding and processing for IHC. High-density TMAs were utilized to determine 1) cellular distribution of staining for Cyr61 in NAT (histologically normal tissue from a tumor bearing prostate), high-grade prostate intraepithelial neoplasia (PIN), and prostate cancer tissue and 2) in evaluation of Cyr61 expression in a unique nested case-control TMA set established to evaulate prostate cancer progression. Additionally, 13 BPH tissues obtained by transurethral resection of the prostate (TURP) due to BPH were analyzed. Histopathological evaluation was first performed on each H&E stained TMA spot and BPH slide by a urologic pathologist to confirm final target diagnosis. Tissue used for real time PCR analysis was acquired from the University of Pittsburgh Medical Center. Samples (>500 mg) were excised and snap frozen in liquid nitrogen within 30 min of excision and stored at −80°C until extraction of RNA. All samples were submitted for pathological evaluation.

2.3.3 Immunohistochemistry

Cyr61 (H-78) rabbit polyclonal IgG antibody epitope 163-240 (sc-13100) (Santa Cruz Biotechnologies, Santa Cruz, CA) or α-PCP4 rabbit polyconal antibody developed against the peptide sequences IQSQFRKFQKKK, amino acids 46-57 (α-PCP4 pep2), or GQKKVQEEFDIDMD, amino acids 22-35 (α-PCP4 pep3) (Sigma-Genosys, now Sigma-Aldrich, St. Louis, MO) were used for staining tissue sections and tissue microarray slides. Immunostaining, including deparaffinization and antigen retrieval, was performed on a Ventana Benchmark XT autostainer (Ventana Medical Systems, Inc. Tucson, AZ) using the I-view DAB detection Kit (Ventana Medical Systems, Inc., Tuscon, AZ). The slides were incubated for 90 minutes with EDTA buffer for antigen retrieval and then incubated with primary antibodies (1:200) for 32 minutes at room temperature. The reaction was visualized by peroxide/

diaminobenzidine tetrahydrochloride (DAB). The slides were then counterstained with hematoxylin.

2.3.4 Scoring and TMA Analysis

Due to the diffuse and uniform intensity of expression of CYR61 by all glandular epithelial cells in a given (tumor, PIN or NAT) TMA spot, assessment of area of staining (percentage of cells stained) in each TMA spot was not required. A visual intensity score was assigned for each spot by a urologic pathologist using a three tier intensity system. Strong intensity staining was scored as 3, moderate as 2, weak as 1, and no staining as 0. BPH slides were analyzed by the same method and pathologist.

In order to validate the accuracy of the above visual scoring method, the semiquantitative visual intensity score results, for one of the five TMA sections, were compared to a quantitative analysis of the Cyr61 staining intensity obtained by image analysis. The latter was performed by capturing TMA core images using the SCANScope XT scanner (Aperio Technologies, Vista, CA). Images were then imported into the TMAJ Image application (http://tmaj.pathology.jhmi.edu) as described previously. Subsequently, the average intensity and area of staining in each TMA core image was calculated using FrIDA (Framework for Image Dataset Analysis), a custom open source image analysis software package (available at http://sourceforge.net/projects/friddajhu/) as described in Gurel *et al.* (218). To eliminate any potential bias due to variation in epithelial to stromal components from core to core, the total area of epithelial cells in each spot was first calculated and the ratio of the (sum of intensity of

Cyr61 expression/total area of epithelial cells) was used as the final FrIDA analysis intensity score in each TMA spot.

2.3.5 Nested Case-Control Study: Rational, Parameters and Tissues

Rationale: The identification of a common set of cases and matched controls would facilitate collaborative basic, clinical, and applied research on prognostic and risk factors for progression after prostatectomy.

Study Design: Case-control study nested in a cohort of radical prostatectomy patients with incidence density sampling of matched controls

Parent Cohort: 1) Database maintained by Dr. Alan Partin. 2) 4,860 men who underwent radical prostatectomy for a diagnosis of clinical organ-confined prostate cancer in 1993 or later

Exclusions (total of 365 men excluded; Table 8): 1) Excluded men (n=7) who had follow-up data (variable name=exposure) that was longer than possible based on the time that had elapsed between RRP date and 2004 (i.e., exposure > 11 years or exposure > 2004 – RRP date).

2) Excluded men (n=171) who had treatment (hormonal, radiation, saw palmetto) prior to surgery (variable name = pre_RRP_treatment = 1), leaving men with missing values for pre_RRP_treatment as well as those who are a definitive 'no' for radiation treatment.

3) Excluded men (n=62) with missing follow-up time (variable name =exposure = .).

4) Excluded men (n=11) with missing or unknown pathologic Gleason sum (variable name = postgl in . , 0, 98, 99).

5) Excluded men (n=10) with missing or unknown pathologic stage, which occurred when all of the stage and margins variables (ln, sv, ecp, fcp, oc, sm) was equal to 0 when ln, sv, ecp, fcp, and oc all had a value of 0, but sm had a value of . or 1.

6) Excluded men (n=6) who had radiation therapy after prostatectomy (variable name = status), which may have obscured observation of progression or time to progression

41=rad tx with later local recurrence
42=rad tx with later distant mets
43=rad tx with later both local & distant mets

7) Excluded men (n=98) with oc = 1 and sm = 1 or . because it is unclear whether the tumor was truly organ confined.

Case and Control Definitions:

1) Cases are men who progressed as indicated by the following:
30=increase in PSA
31=local recurrence
32=distant mets
33=both local and distant mets
40=decrease in PSA through rad tx
60=died from prostate cancer
70=died from another cause but with prostate cancer recurrence

2) Possible controls are men with the following status variable values:
20=no recurrence
50=died from any non-cancer related cause
or who progressed (status=30, 31, 32, 33, 40, 60, 70) in a subsequent time interval

New Variables: 1) Grouped stage variable (variable name = sstage):
sstage = 3 if ln = 1 or sv = 1 (N1+T3b)
sstage = 2 if ecp = 1 or fcp = 1 (T3a)
sstage = 1 if oc = 1 and sm = 0 (T2)

2) Grouped grade variable (variable name = pgl):
pgl = 5 if postgl in (2, 3, 4, 5)
pgl = 6 if postgl = 6
pgl = 7 if postgl = 7
pgl = 8 if postgl in (8, 9, 10)

3) Grouped race variable (variable name = race): recoded unknown, other, and missing race (0, 5, .) to race = 5

Control Sampling: 1) Cases (as defined above) occurred over the course of nine years (1993 onward). Exact progression times were not recorded in part because they could not be determined for biochemical progression. All men's exposure times were left aligned at the date of prostatectomy with case failure times given in 1 year intervals of time since prostatectomy.

Nine 1-year wide risk sets were created defined by the exposure variable. Sampling of controls was done within those nine year intervals. A man selected as a control in an earlier risk set was eligible to be sampled again in a later risk set as long as he was still alive and under follow-up, and had not yet progressed. A man who progressed was eligible to be selected as a control in an earlier risk set. See the figure below for graphical representation of the sampling approach. These sampling rules are parallel to the statistical analysis performed when using Cox proportional hazards regression.

2) For each year two temporary datasets were created for use in (3)

a) a set of all cases who were diagnosed in that year since prostatectomy; those cases who were selected as controls in previous years were included as cases during the year since prostatectomy in which they progressed

b) a set of all possible controls who had not progressed by that year since prostatectomy, who were still alive, and still under follow up; men selected as a control in a prior year since prostatectomy were eligible to be selected again in subsequent years as a controls or to become a case

3) The matching variables were: pathologic stage (stage), pathologic Gleason sum (pgl), race, and age. To optimize the closeness of the matching of the controls to the cases, a variable for the difference in the value between the case and the possible control for each matching variables was generated. A SAS macro (Tassoni *et al.* "One-to-one matching of case/controls using SAS software") was used to optimize the closeness of the matching. The control with the smallest difference from a given case on all four matching variables was selected. Once that control was selected from the risk set, he could not be selected as a control for another case during the same one year-interval since prostatectomy, although he could be selected as a control

in an earlier or later year interval. Because stage and grade are such strong predictors of progression and because the goal is to study factors that influence progression independent of stage and grade, we matched the cases and controls very closely on stage and grade. When an exact match could not be obtained, matching was loosened in the order as follows: age only (up to ± 10 years); race only; age and race; Gleason sum only (± 1); Gleason sum (± 1) and age (up to ± 10 years); Gleason sum (± 1) and race; Gleason sum (± 1), age (up to ± 10 years), and race; stage only (± 1), stage (± 1) and age (up to ± 10 years); stage (± 1) and race; stage (± 1), age (up to ± 10 years), and race; any other loosening that will produce a match.

2.3.6 Protein Isolation

Total cell protein lysates were obtained by three methods. First, total cell protein lysates were obtained by washing cells once in PBS, and scraping cells in ice cold PBS + sodium vanadate. Cells and cellular debris were centrifuged at 3,000 RPM for 5 minutes, the supernatant removed and the pellet re-suspended in RIPA Buffer (1%Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 55 mM Tris-HCl, ph7.5) with 1x protease inhibitor cocktail (Promega, Madison, WI). Following 6 freeze-thaw cycles (15 minutes at -80°C and thawing on ice), lysates were centrifuged at 13,000 RPM for 10 minutes at 4°C. Supernatants were collected, and protein concentrations measured. Second, from cell pellets of various prostate cell lines, 6S, BPH1, BRF-55T, LAPC4, VCaP, DuCaP, PC3, Du145, E006AA, E006AA-T and E006AA-T AR transfected cells (provided by the lab of Dr. John Isaacs), total protein lysates were obtained by repeated shearing the of cells using a 26-guage needle lysis in Triton-X100 buffer (1% triton-X100, 10mM Tris pH7.4, 150mM NaCl, 1mM EDTA) containing Protease Inhibitor Cocktail

tablets (Roche, Indianapolis, IN) and Aprotonin and PMSF. Lysates were centrifuged at 13,000 RPM for 10 minutes at 4°C. Supernatants were collected, and protein concentrations measured. Last, in experiments validating Cyr61 expression in all transfections, cells were trypsonized and counted. Cell pellets were resuspended in lysis buffer (20mM Tris, 140mM NaCl, 1mM EDTA, and, added fresh before each use, 1% NP-40, 1mM DTT, 1% sodium deoxycholate, 1x Phospho-Stop (Roche, Indianapolis, IN) with protease inhibitors, complete mini. EDTA-free protease inhibitor (Roche, Indianapolis, IN), for $1x10^6$ cells, 50µl lysis buffer. Pellets were resuspended and incubated on ice for 15 min before they were spun at 14,000 rpm at 4°C. The supernatants were transferred and stored at -20°C.

2.3.7 Immunoblot Analysis

Lysates (30µg) were subjected to SDS-polyacrylamide gel electrophoresis (4-15% gel) and electrotransferred onto PVDF (Millipore, Bedford, MA). Membranes were blocked in 5% milk in PBST and probed with anti-Cyr61 primary polyclonal antibody (1:200 dilution) (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-HGF monocolonal antibody (1:200 dilution) (Santa Cruz Biotechnologies, Santa Cruz, CA), or (1:20,000 dilution) β-actin monoclonal antibody (Sigma, St. Louis, MO) for 1 hour. After washing with PBST, blots were incubated for 1 hr with peroxidase-conjugated anti-rabbit IgG (1:5000 dilution) or anti-mouse IgG (1:40,000 dilution) (Santa Cruz Biotechnologies, Santa Cruz, CA) for Cyr61 or β-actin, respectively, washed again and detected by enhanced chemiluminescence, ECL (Thermo Fisher, Rockford, IL).

2.3.8 RNA Isolation

Frozen BPH, donor, normal adjacent and tumor tissues, obtained from the University of Pittsburgh Department of Pathology as described previously, were minced and homogenized on ice using a rheostat homogenizer in RLT buffer and centrifuged at 10,000 rpm at 4°C. Total RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Briefly, an equal volume of 70% ethanol was added to the RLT/supernatant and the mixture was loaded on the RNeasy column. Following centrifugation, the column was washed with Buffer RW1 and twice with RPE, each wash separated by centrifugation. RNA was released from the column by RNase free water. After centrifugation, the flow-through was stored at -20°C. RNA was quantified by spectrophotometer at OD 260/280 nm. For RNA samples to be analyzed by qPCR at the University of Pittsburgh Clinical Genomics Facility, RNA samples were treated with the DNase I kit (Ambion, Austin, TX) to remove genomic DNA contamination. For RNA isolated from CWR22 transient and stable transfected cells for quantitative PCR analysis, Qiagen's on-column DNase digestion was performed. Briefly, The Buffer RW1 wash of the column was divided, half the volume before DNase treatment and half after. 80 µl of DNase I incubation mix (10 µl of DNase I stock solution with 70 µl Buffer RDD mixed in advance) was added to each column and incubated for 15 min at room temperature. The second ½ volume of Buffer RW1 was added to the column before resuming the protocol described above.

2.3.9 Semi-quantitative PCR

RT first strand synthesis. cDNA was synthesized using the Active Motif reverse transcription kit (Carlsbad, CA).

Semi-quantitative PCR. PCR reactions were performed on a Eppendorf Mastercycler Gradient machine in a total reaction volume of 50 µl containing 25 µl RedMix Plus 1.5 mM $MgCl_2$ master mix (GeneChoice, Frederick, MD), 1µl each forward and reverse primer, and 1µl cDNA.

Gene	Forward Primer , 5'–3'	Reverse Primer , 5'–3'	Amplicon
FosB Full	GAGGAAGAGGAGAAGCGAAGG	CAGGTGAGGACAAACGAAG	262
FosB Both	CCACTGCCATCGGACAGGAGGA	AGGACTCCAGCCCACCCCACAG	468
Nell2	CCTGTATTGCCGCTAATGTGTG	GCGGGCAGCACTCATTCTCT	660
Cyr61	GGCTGCGGCTGCTGTAAGGTC	GTTCGGGGGATTTCTTGGTCT	739
PCP4	CGGGACTGAGCTGTTGAGTTAGA	TTGCAGGAGGAATGAAAATGG	454
Tacc2	ACTGGAGAGGCAGGTGTCAG	CTGGGATGGTCTCTGCTCTC	451

Amplification was performed under the following conditions: Nell2, Cyr61, and PCP4 - 94°C for 3 minutes, and 35 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 3 minutes; Tacc2 - 94°C for 3 minutes, and 35 cycles of 94°C for 30 seconds, 57°C for 45 seconds, and 72°C for 3 minutes; FosB Full, FosB Both - 94°C for 3 minutes, and 30 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 3 minutes. Amplification products were separated by electrophoresis through a 1% agarose gel with ethidium bromide and visualized by UV illumination. GapDH was used as a positive control: forward – CGTGGAGTCTACTGGTGTCTTCACC and reverse – GATGGCATGGACTGTGGTCATGAGC.

2.3.10 Quantitative PCR

RT first strand synthesis. Hex-RT was performed on 1µg of total RNA, 10 U/µl of MMLV RT enzyme (Epicentre, Madison, WI), 40 U/µl of RNase Inhibitor (Promega, Madison, WI), 1.25 mM hexamer primers, 25 mM of dNTPs, 10µl of 10x PCR buffer and 75mM $MgCl_2$.

Quantitative PCR. Quantitative gene expression analysis experiments analyzing tissue samples were performed at the Clinical Genomics Facility of the University of Pittsburgh Cancer Institute. Primer Express (Applied Biosystems, Foster City, CA) was used to design all primers. RT-PCR was used to confirm q-PCR primer specificity prior to use in real time qPCR.

Gene	Forward Primer (5'–3')	Reverse Primer (5'–3')	Probe (5'–3')
FosB Full	AACCTGACGGCTTCTCTCTTTACA	GGGCAGGTGAGGACAAACG	CTTCCCCGTTGTTAACCCTTCGTACACTTC
FosB Both	CAGGCGGAGACAGATCAGTTG	GCACAAACTCCAGACGTTCCTT	CGGAGATCGCCGAGCTCCAAAA
Cyr61	GCAGCCTGAAAAAGGGCAA	AACATCCAGCGTAAGTAAACCTGAC	GCAAGACCAAGAAATCCCCCGAACC
Nell2	CCATGGAGTCTCGGGTCTTACT	AGGGAAGGGTCCACACCAA	TCTTCGGTCTCGGAGCAGTTTGGG
PCP4	GAGCGACAAGGTGCTGGG	CTTGAACTTTCTTCTGTCCATCATTTT	AATGATGGACAGAAGAAAGTTCAAGA
Tacc2	CCTTGTACCTTATGTTTGACACTTCTCA	TCAAAACTTGACCCTGAACACG	TGTCAAGTCATCTCCCGTCCGCATG
ß-GUS	CTCATTTGGAATTTTGCCGATT	CCGAGTGAAGATCCCCTTTTTA	TGAACAGTCACCGACGAGAGTGCTGC

Amplicons: FosB Full = 104bp; FosB Both = 94bp; Cyr61 = 78bp; Nell2 = 85bp; PCP4 = 80bp; Tacc2 = 100bp. Expression of genes of interest were normalized against the housekeeping gene Glucuronidase β (GUS). 5' FAM fluorescent dye and 3' TAMRA quencher dye. Each 50µl amplification reaction contained 1x Taqman universal PCR mastermix (containing AmpliTaq Gold DNA polymerase, 25 mM dNTPs, passive reference (ROX), and optimized buffer components including 3.5 mM $MgCl_2$) with 5µl cDNA, 200nM primers and 100nM probe. Amplification was performed by 95°C for 12 minutes, and 40 cycles of 15sec at 95°C, and 1min at 60°C run on the ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). Reactions were carried out at two concentrations (1x and 1/4x) for each sample, along with a no-template control containing water and a positive control containing cDNA. Expression of genes of interest was normalized for unknown samples by comparison of RNA loading as determined expression of the housekeeping gene GUS. For quantification, analysis was done with the Δ cycle threshold (Ct) value (Ct gene of interest – Ct housekeeping gene) to generate relative expression. For quantification, results were analyzed by the ΔΔCt method (Δ cycle threshold (Ct) sample - ΔCt value calibrator). Conversion between ΔΔ Ct and relative gene expression is fold induction = 2-ΔΔ Ct. A master cDNA mix (derived from a mix of normal, BPH, and prostate cancer tissue) was used in all primer/probe optimization experiments. Gene expression analysis of CWR22 transient and stable transfections were performed on the Bio-Rad iCycler (Bio-Rad, Hercules, CA) at the Brady Urological Institute of the Johns Hopkins School of Medicine using the same Cyr61 and Gus primers described above. Experiments were performed using a similar protocol to the one described above, including the Hex-RT and the same primers, but with the modification that with the exception that iTaq Supermix with ROX (Bio-Rad,

Hercules, CA) used instead of the Taqman universal PCR mastermix and reaction volume was cut from 50µl to 25µl.

2.3.11 Cloning

Cyr61, gene accession BC16952, was obtained in the pCMV-Sport6 expression plasmid (OpenBiosystems, Huntsville, AL). Cyr61 sequence was confirmed by sequencing (Seqwright, Houston, TX) and restriction digest with BstX1 and Eag1. To obtain a plasmid able to express Cyr61 in mammalian cells with G418 antibiotic resistance, Cyr61 was PCR cloned into a pdsRed vector (Invitrogen, Carlsbad, CA). Primers were designed with the guidance of Dr. Sushant Kachhap such that an XhoI and EcoRI site were added up and downstream of the Cyr61 open reading frame: forward primer: 5'-ATTATCTCGAGATGAGCTCCCGCATCGCCAGGGC-3' and reverse primer: 5'-CATCGGAATTCCTAGTCCCTAAATTTGTGAATGTCAT-3'. The 25 µl PCR reaction with platinum taq, a high fidelity DNA polymerase, and was performed at 95°C for 10 minutes followed by 35 cycles of 30 sec at 95°C, 30 sec. at 60°C and 1 min at 70°C. The product of this reaction was run on 1.5% Agarose DNA gel (100V for 1hour). A single band was visualized by Sybersafe (Invitrogen, Carlsbad, CA) and cut from the gel. The DNA was isolated using the Gel Extraction Kit (Qiagen, Valencia, CA). Restriction digest for 2 hours at 37°C with Xho1 and EcoR1 of the purified PCR product and pcDNA3.1 plasmid resulted in the desired fragments (Cyr61 flanked by EcoRV and Nco1 sticky ends and pcDNA3.1zeo with the MCS cut at the EcoRV and Nco1). These were visualized on a 1% agarose gel and purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA) by the manufacturer's instructions. Briefly, buffer QG was added in 3:1 ratio to the volume of the gel (the excised band) and the mixture incubated at 50 for 10min. 1 gel volume of isopropanol was added, the total volume applied to

the QIAquick column, and then spun for 1 minute at 13,000 RPM. The column was washed once with 0.5mls of buffer QG, washed with 0.75ml of buffer PE, and, following an extra spin to ensure removal of all ethanol, the column was transferred to a clean microcentrifuge tube and eluted with TE. The Cyr61 fragment was ligated into pdsRed using NEB's Quick ligation T4 DNA ligase kit (NEB, Ipswich, MA) to form pdsRed-Cyr61. Sequence and orientation were confirmed by restriction digest with Sca1. pdsRed was generously provided by Dr. Sushant Kachhap of the Levitsky lab. All plasmids were transformed into One Shot Top10'compotent bacteria (Invitrogen, Carlsbad, CA) by incubation of 1µg of plasmid DNA in 50µl of bacteria at 30°C for 30 minutes, 42°C for 45 seconds, and placed back on ice before adding 450µl of warm SOCS media. The whole reaction was incubated with shaking at 225 rpm in a 37°C incubator for 1hr. The transformed bacteria were plated on pre-warmed LB plates with the appropriate antibiotic and grown overnight in a 37°C incubator. Individual colonies were selected and cultured overnight in either 5 or 125 mls of LB+Ampicillin depending on the desired yield and plasmid preps performed using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) or the PureLink HiPure Plasmid Maxiprep Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2.3.12 Transient and Stable Transfection

CWR22 cells were plated in 6 well plates and allowed to grow up a minimum of 48 hours to about 75% confluence. Lipofectamine 2000 reagent was used according to the protocol suggested by Invitrogen: per well - 10µl Lipofectamine 2000 in 50µl of Optimem Media mixed with 2.5µg of pdsRed-Cyr61, or p-dsRed (visual control of transfection efficiency) in 500µl of Optimem Media. The 100µl mixture was incubated for 25 minutes and brought up to 500µl with

Optimem Media and added to cells, allowed to incubate for 4 hours after which 1.5mL of RPMI with 10% FBS. For validation of the effect of transient transfection, protein and RNA were harvested at 48, 72, or 96 hours post-transfection. For stable transfections, CWR22 cells were grown in RPMI with 10% FBS and 1% P/S plus 400μg/ml G418 (Invitrogen, Velencia, CA). Media + G418 was refreshed every 2-3 days during selection process and every 2 days thereafter.

2.3.13 Microarray Analysis

Genes of interest were selected based on results of microarray analysis performed at the University of Pittsburgh School of Medicine. Samples were hybridized to the 42K Affymetrix HuGeneFL array and the raw data was analyzed with Affymetrix software, GENE CHIP V.3.0 with ANOVA, principal component analysis (PCA), and hierarchical clustering analysis performed using EXPERIMENTAL DATA MINING TOOL V.1.0 S-Plus. For the PCA, the correlation matrix on nontransformed expression values was used.

For the evaluation of gene expression by CWR22 Cyr61 clones, aliquotes of DNase treated RNA from the CWR22RV1 parental line (in duplicate) and 5 transfected clones (2 that express low and 3 that express high levels of the Cyr61 protein) were provided to the Johns Hopkins Microarray Core facility for hybridization to the Human Exon 1.0 ST Array (Affymatrix, Santa Clara, CA).

2.3.14 Growth and Survival Assays

CWR22 cells (parental line, Cyr61, and dsRed control clones), were cultured in growth media (RPMI plus 10% FBS, 1% P/S) and plated at 10,000 cells/well in triplicate in 96-well tissue culture plates (Becton Dickinson, San Jose, CA) and measured each day for 6 days by WST1 assay following the manufacturer's protocol (Roche, Indianapolis, IN). Briefly, cells were plated in 100µl on day 0. 10µl of WST1 reagent was added to each well and plates were returned to the 37°C incubator. After 3 hours, plates were shaken for 5 minutes and the reduction of the WST-1 reagent results in a color shift read on a microplate reader at 450nm. Survival advantage was assessed by exposing cells to 25µM Cisplatin (48 and 72 hours, DMF control) or 10nM Docetaxel (24 and 48 hours, DMSO control) or to media made with low (2%), no, or charcoal stripped FBS (72 hours).

2.3.15 Cell Doubling Time

CWR22RV1 cells (parental, Cyr61, and control clones) were cultured in growth media (RPMI plus 10% FBS, 1% Pen/Strep) and plated at 50,000 per well in 6-well plates. Cells were counted, duplicate wells counted in duplicate, via a Nexelcom Cellometer (Lawrence, MA) every day for 6 days. Cell growth was analyzed graphically and doubling time was determined by calculating cell doubling time (hr) = inv (((log (final count) – log (initial count)) x 3.32) / time).

CWR22RV1 (50,000 cells/well), LNCaP (50,000 cells/well), PC3 (20,000 cells/well), and E006-T cells (10,000 cells/well) were plated in duplicate wells in growth media (phenol red free RPMI plus 10% charcoal stripped FBS, 1% Pen/Strep) supplemented with 2µg/ml, 200ng/ml, 20ng/ml

or 0ng/ml recombinant Cyr61 (PeproTech Inc., Rocky Hill, NJ). After four days of growth, cells were counted via cellometer. Doubling time was determined by calculating cell doubling time (hr) = inv (((log (final count) – log (initial count)) x 3.32) / time).

2.3.16 Migration and Invasion

The BD BioCoat Matrigel Invasion Chambers (BD Biosciences, Bedford, MA) were utilized to perform invasion assays. The matrigel invasion chambers were allowed to warm to room temperature and then re-hydrated in serum free media for 2 hours and after which 0.75 ml of media with 10% FBS was added to the bottom well. Control wells were also prepared with 0.75 ml of media in the bottom well. During re-hydration, cells were trypsinized, counted on the Nexelcom Cellometer and re-suspended at 500,000 cells/mL. 100µl of the cell suspension (50,000 cells) was diluted up to 500µl of media containing 2% FBS and added to the control and matrigel inserts in duplicate. The chambers were incubated for 22 hours before the membranes were stained. The non-invaded cells were removed by scrubbing the inside of the inserts with sterile cotton-tipped applicators. The inserts were stained in 0.5ml of 0.5% crystal violet/25% methanol for 5+ minutes, washed with water and visualized on a Nikon Eclipse TE200-E microscope. The invaded cells in 4 separate frames were counted for each membrane at 20x magnification (2 wells per cell type with 4 counts per well).

CWR22RV1, LNCaP, PC3, and E006-T cells were plated at 25,000 cells/well in duplicate wells in growth media (phenol red free RPMI plus 10% charcoal stripped FBS, 1% Pen/Strep) in the insert. 0.75 ml of growth media (phenol red free RPMI plus 10% charcoal stripped FBS, 1% Pen/Strep) supplemented with 2µg/ml, 200ng/ml, 20ng/ml or 0ng/ml Cyr61 or 100ng/ml EGF

(as a positive control of migration) was plated in the bottom well. Cells were allowed to migrate for 22 hours before staining and invaded cells were counted, as described above.

2.3.17 Statistical Analysis

Statistical analysis for the TMA studies was performed by Dr. Elizabeth Platz and Alison Mondul, of the Johns Hopkins School of Public Health. To test the correlation between FrIDA analysis intensity score and pathologist assigned visual intensity score, a correlation coefficient was calculated by pairwise correlation analysis using Stata 9.0 statistical package (Stata Corp., College Station, TX).

The median staining intensity by spot type was determined by first calculating the median staining intensity of all spots of a given type for a single individual and then calculating the median across all individuals. Paired analyses of median staining intensity between spot types were conducted using the Wilcoxon signed rank test. Paired analyses of whether at least one spot for an individual met a threshold value comparing between spot types were conducted using McNemar's test. Paired analyses were also conducted using a repeated measures modeling approach that used a robust variance, which yielded very similar results (data not shown). Analyses comparing median staining intensity in cancer spots between men with Gleason sum $\geq$ 8 and those with Gleason sum <8 were conducted using the Wilcoxon rank sum test.

3.0 GENE EXPRESSION CHANGES IN PROSTATE CANCER

3.1 INTRODUCTION

In the pursuit of novel biomarkers of prostatic disease (BPH and prostate cancer), an evaluation of genomic expression in healthy donor, symptomatic BPH, asymptomatic BPH, and histologically diagnosed BPH adjacent to prostate cancer tissues (NAT) was undertaken by Affymetrix gene expression analysis (29). To narrow in on novel targets important in the etiology of prostate cancer, gene expression in 10 healthy donor prostate tissues was compared to expression in tissue from 6 men with prostate cancer by microarray analysis and, of the over 400 differentially expressed genes, a panel of genes whose expression was significantly and consistently altered in prostate cancer were selected for further analysis. Also after consulting the literature at the time, the list of targets was narrowed further to those with potential impact in prostate cancer.

Table 2. Genes of interest

Protein	Gene Expression	Chromosomal Location	Protein Size	Protein Function
MT2A	Down-regulated	16q13	6.5 kD	Heavy metal chaperone
Tacc2	Down-regulated	10q26.13	30.1 kD	Heterochromatin Organization
PCP4	Down-regulated	21q22.2	7 kD	Calcium Homeostasis
Nell2	Up-regulated	12q13.11	9 kD	Intracellular Signaling
FosB	Up-regulated	19q13.32	36 kD	Transcription Factor
Cyr61	Up-regulated	1p22.3	37-41 kD	Extracellular Matrix Signaling

3.2 RESULTS

3.2.1 Expression of MT2A in prostate cancer

To confirm results from the gene expression microarray analysis (Figure 17) (29) for MT2A in the prostate, semi-quantitative PCR (Figure 18) was employed to compare gene expression in healthy donor prostate tissue with paired tumor/normal adjacent prostate tissue.

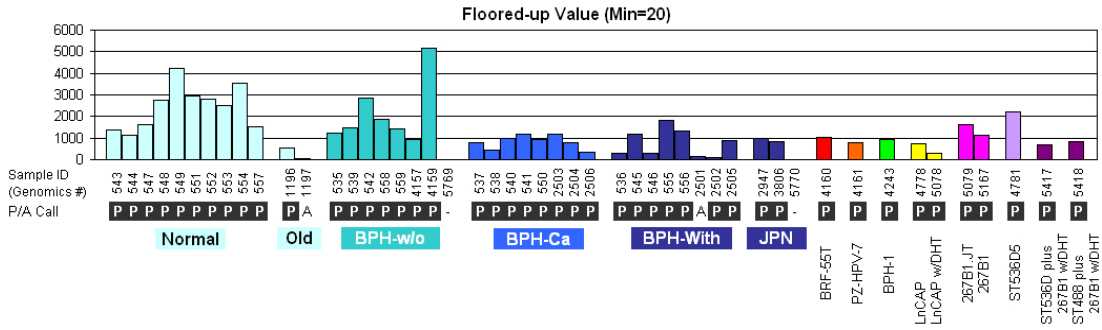


Figure 17. Genomic analysis of MT2A expression in prostate tissues and cell lines

From the semi-quantitative analysis, expression of MT2a mRNA was virtually identical in prostate donor, adjacent-to-tumor, and tumor tissues; no differences were evident. This expression was considered semi-quantitative given that expression of the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GapDH) was comparable between the six tissues (data not shown).

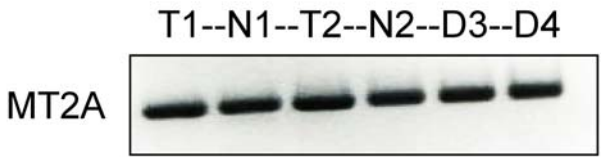


Figure 18. Expression analysis of MT2A in prostate tissues

Semi-quantitative PCR analysis of 2 paired tumor/normal and 2 healthy donor prostate tissues

Additionally, the many sets of gene expression data submitted to Oncomine do not show significantly altered expression of MT2A in prostate cancer (219). One study demonstrated decreased expression in metastatic compared to primary prostate cancer (219) and this suggests that MT2A may have a metastasis suppressor properties that warrant further investigation. However, given that the differental expression of MT2A seen in the microarray analysis was not reproducible in primary prostate cancer tissues, further analysis of MT2A as a potential diagnostic biomarker in prostate cancer was not pursued.

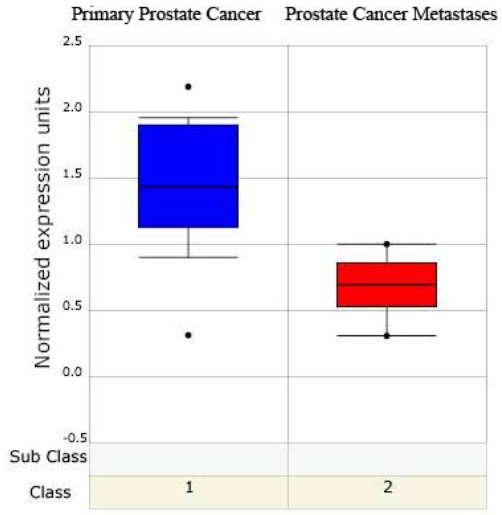


Figure 19. Expression of MT2A is decreased in metastasis compared to primary prostate cancer

3.2.2 Expression of Tacc2 in prostate cancer

To confirm gene microarray analysis (Figure 20) (29) and more fully characterize Tacc2 expression in the prostate, semi-quantitative PCR (Figure 21) and quantitative PCR analyses (Figure 22) were employed to compare gene expression in healthy donor prostate tissue with tissues obtained from prostates that contained cancer.

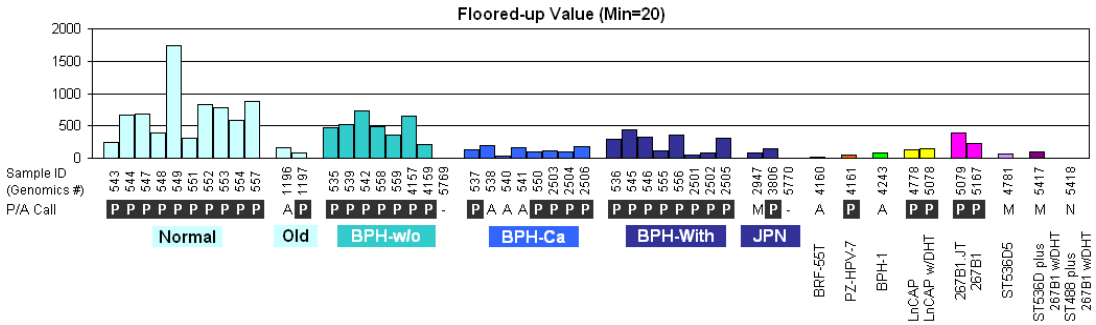


Figure 20. Genomic analysis of Tacc2 expression in prostate tissues and cell lines

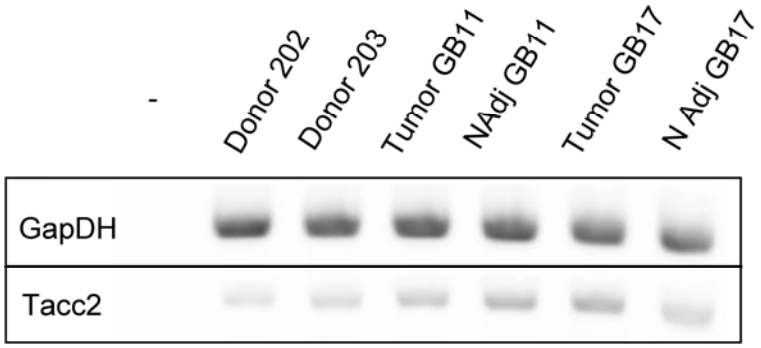


Figure 21. Expression of Tacc2 in prostate tissue

Semi-quantitative PCR analysis of 2 paired tumor/normal and 2 healthy donor prostate tissues

Semi-quantitative expression analysis of Tacc2 illustrated a moderate increase in adjacent-to-tumor and tumor tissues. This pattern is the opposite of that reported in the preliminary microarray analysis (Figure 19). Expression analysis was considered semi-quantitative given that expression of the housekeeping gene GapDH was comparable between the six tissues. To determine Tacc2 expression quantitatively, taqman qPCR was performed and, contrary to the pattern observed in semi-quantitative expression analysis but in agreement with the array data, Tacc2 mRNA expression decreases mildly but not significantly in prostate cancer compared to donor prostate tissue (N=10; Figure 22).

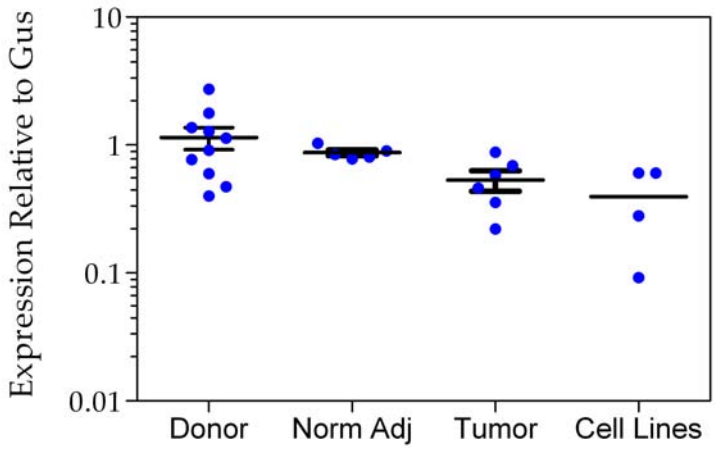


Figure 22. Quantitative gene expression analysis of Tacc2 in prostate tissues

Each circle represents the relative mRNA abundance (Tacc2 message normalized to Gus expression) for prostate tumor, adjacent-to-tumor or healthy donor prostate tissues or cell line (LNCaP, PC3, BPH1, and 267B1.

To look for data supporting the notion of altered Tacc2 expression in prostate cancer, a query of the Oncomine database was performed but none of the studies report altered expression

of Tacc2 in prostate cancer although comparison of Tacc2 expression in nomal tissues revealed that prostate is among the tissues with highest expression of Tacc2 (219).

3.2.3 Expression of Nell2 in prostate cancer

Nell2 was selected as a target for further analysis based on gene expression analysis (Figure 23) (29) and, to more fully characterize the expression of Nell2 in prostate, semi-quantitative (Figure 24 *top*) and quantitative gene expression analyses (Figure 24 *bottom*) were employed to compare gene expression in healthy donor prostate tissue and prostate cancer tissues.

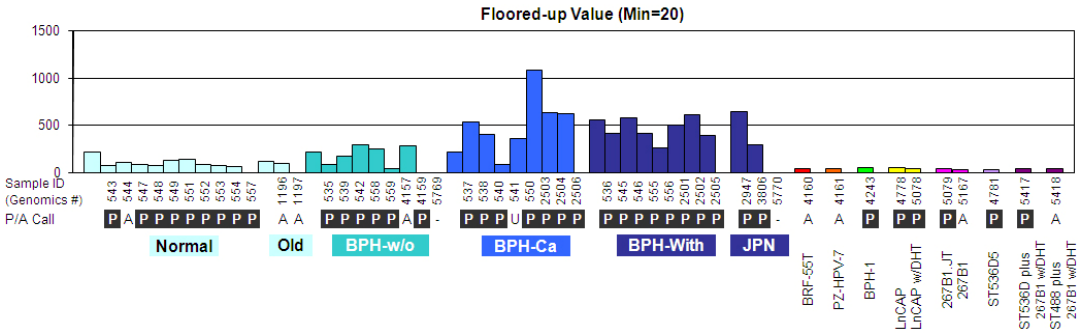


Figure 23. Genomic analysis of Nell2 expression in prostate tissues and cells

Semi-quantitative PCR analysis of Nell2 expression in paired tumor/normal adjacent-to-tumor prostate tissue compared to donor prostate found higher expression in histologically normal tissue from tumor bearing prostates than in the matched tumor tissue or from donor prostate tissue. To quantitatively evaluate this, taqman qPCR was performed and expression of Nell2 was found to be elevated in 4/5 (80%) normal adjacent to prostate cancer tissues in comparison to the expression in donor tissues (N=9; Figure 24 *bottom*) (0.25 ± 0.32 for donor tissues vs. 2.08 ± 2.57 for normal adjacent-to-tumor; $p<0.05$).

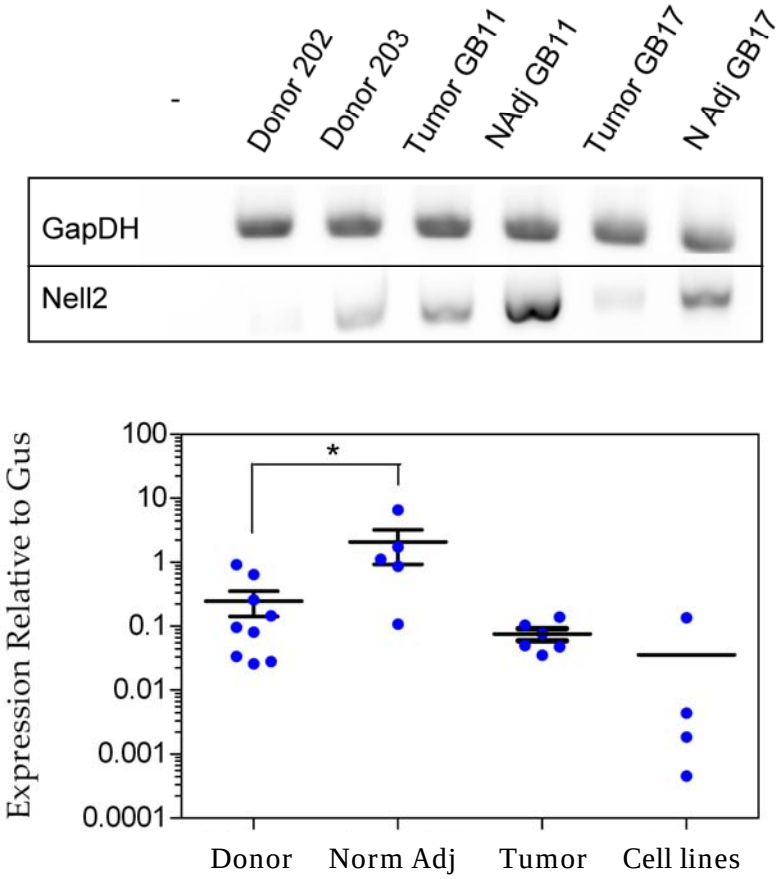


Figure 24. Gene expression analysis of Nell2 in prostate

(Top) Semi-quantitative PCR analysis of 2 paired tumor/normal and 2 healthy donor prostate tissues *(Bottom)* Quantitative PCR analysis - each circle represents the relative mRNA abundance (Nell2 message normalized to Gus expression) for prostate tumor, adjacent-to-tumor or healthy donor prostate tissues or cell line (LNCaP, PC3, BPH1, and 267B1.

To further explore the expression of Nell2 in prostate cancer, a query of the Oncomine database was performed and no studies report altered expression of Nell2 in prostate cancer but three independent studies report that expression of Nell2 in metastatic prostate cancer is lower than in primary prostate cancer (219).

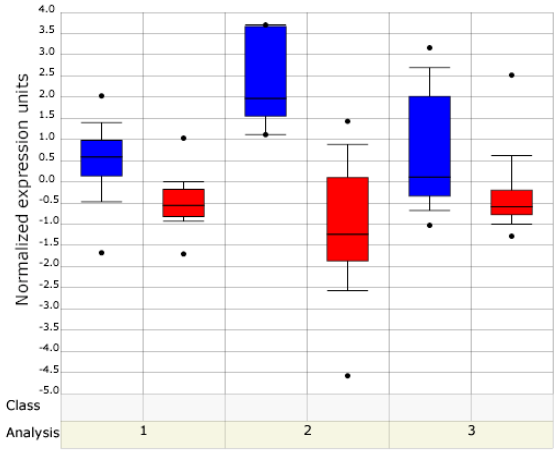


Figure 25. Nell2 expression is lower in prostate cancer metastases (red) than in primary prostate cancer (blue) in three independent studies submitted to Oncomine

3.2.4 Expression of FosB in prostate cancer

From the genes differentially expressed in the microarray analysis of various normal, BPH, and prostate cancer tissues, expression of FosB appears significantly and consistently elevated in prostate tissues from men with prostate cancer (Figure 26) (29).

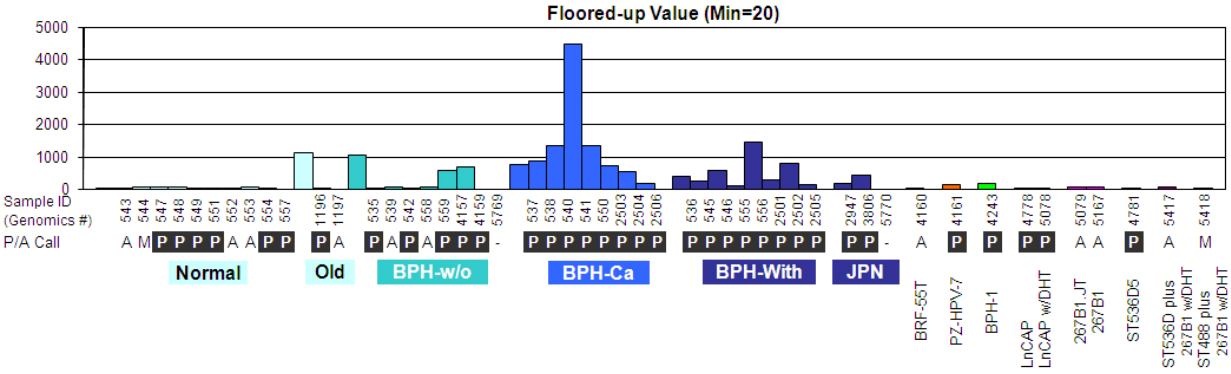


Figure 26. Genomic analysis of FosB expression in prostate tissue and cell lines

In order to confirm these array results, FosB expression in healthy donor prostate and prostate cancer tissue was evaluated using semi-quantitative (Figure 27) and quantitative PCR techniques (Figure 28) were employed to compare gene expression. Both show a significant increase in FosB expession in prostate cancer tissue compared to healthy donor tissue.

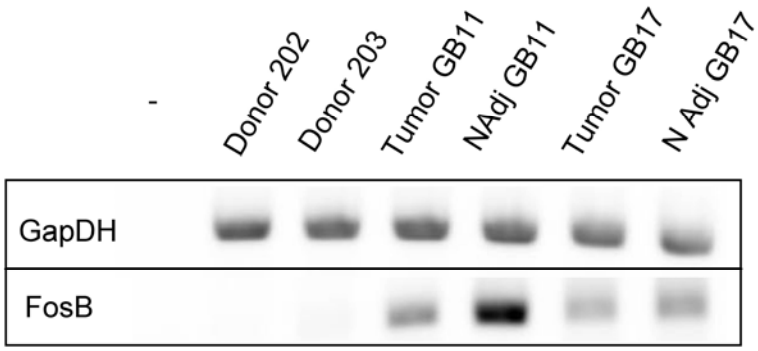


Figure 27. Expression analysis of FosB in prostate tissues

Semi-quantitative PCR analysis of 2 paired tumor/normal and 2 healthy donor prostate tissues

From semi-quatitative analysis it was noted that, depending on the primers used, PCR amplification resulted in either one or two bands, the smaller being about 100 base pairs shorter than the other (the larger being the band anticipated based on primer design) (data not shown). This smaller product represents ΔFosB, a splice variant of FosB lacking the C-terminal 101 amino acids. Given that the prostate expresses both FosB and ΔFosB, taqman primers and probe were designed to differentiate between the two variants (FosB-both transcripts or ΔFosB alone). Taqman qPCR demonstrated elevated expression of FosB-both transcripts in 10/11 (91%) prostate cancer tissues in comparison to donor tissues (N=10; Figure 28 *top*) (0.07 ± 0.09 for donor tissues vs. 1.43 ± 1.03 for tumor; $p<0.05$). Expression of ΔFosB alone was also elevated in 10/11 (91%) prostate cancer tissues in comparison to the expression in donor tissues (N=10;

Figure 28 *bottom*) (0.12 ± 0.09 for donor tissues vs. 1.80 ± 1.98 for tumor; $p<0.05$). Primer efficiency was optimized prior to evaluating expression in tissues with the goal that expression of ΔFosB could be subtracted from FosB-both transcripts yielding the relative expression of FosB full-length transcript. The efficiency must not have been similar enough because higher expression was detected for ΔFosB alone than for ΔFosB and FosB together and therefore the expression of full length FosB could not be evaluated independently of ΔFosB.

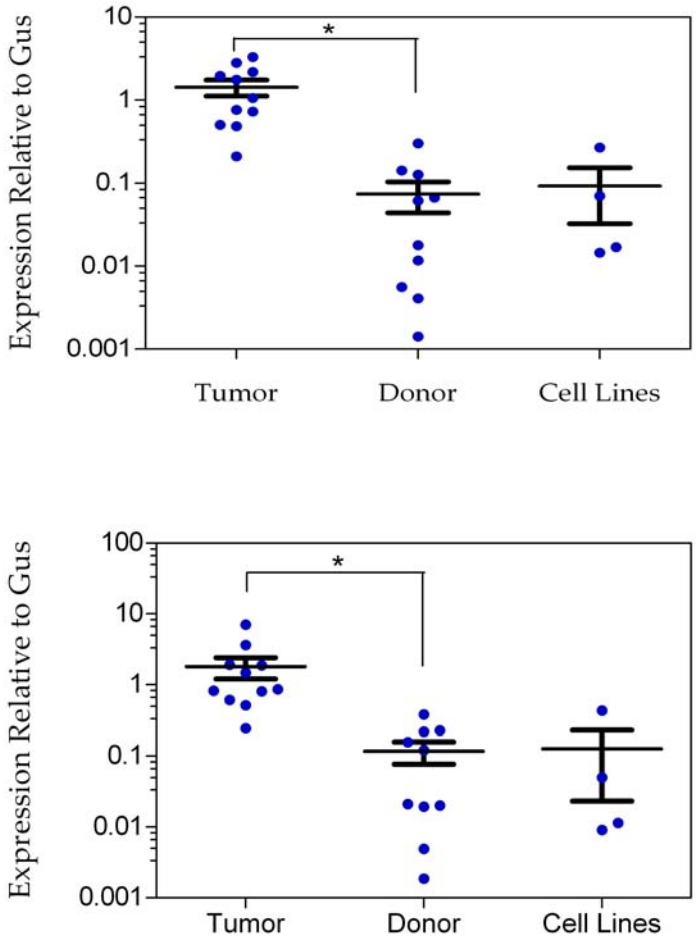


Figure 28. Quantitative analysis of FosB and ΔFosB expression

(top) primers dectect full length FosB & ΔFosB transcripts *(bottom)* – primers detect only full length FosB transcript. Each circle represents the relative mRNA abundance (PCP4 message normalized to Gus expression) for prostate tumor, adjacent-to-tumor or healthy donor prostate tissues or cell line (LNCaP, PC3, BPH1, and 267B1).

To further substantiate this pattern of elevated FosB expression in prostate cancer, a query of the Oncomine database was performed and two studies report elevated expression of FosB in prostate cancer (219). In addition, seven independent studies report that FosB expression in metastatic prostate cancer is lower than in primary prostate cancer (219). These microarray studies do not differentiate between full length FosB and ΔFosB transcripts.

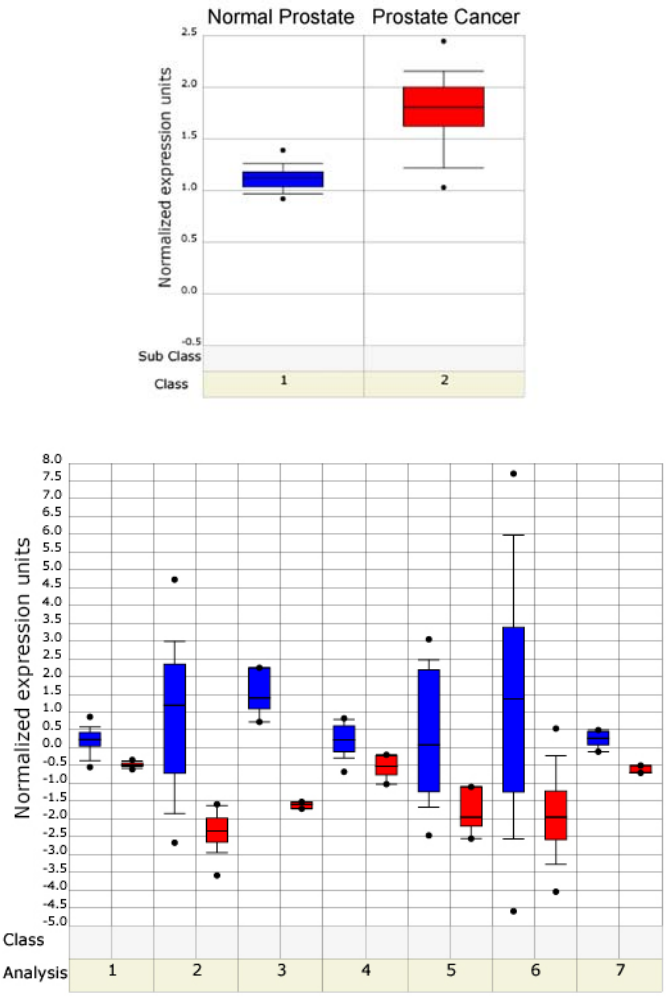


Figure 29. FosB expression in microarray analyses submitted to Oncomine

(top) FosB expression is up-regulated in prostate cancer compared to normal prostate and *(bottom)* lower in metastatic prostate cancer than in primary lesions. Seven independent studies show decreased expression of FosB in metastatic (red) compared to primary prostate cancer (blue).

3.3 CONCLUSIONS

In this series of experiments the expression changes in MT2a, Tacc2, Nell2, and FosB were evaluated in normal and prostate cancer tissues as well as prostate derived cell lines. Results show disparity between the expression patterns of MT2a and Tacc2 by microarray analysis and expression patterns obtained for PCR analysis. These differences in expression reported by microarray analysis may be attributable to 'noise' in this type of study (220,221). Microarray analysis was undertaken with a goal of hypothesis generation and it was anticipated that some genes that appear promising by microarray would not translate to be viable targets upon subsequent analysis. Decreased expression of MT2a seen in microarray analysis was not replicated by semi-quantitative PCR or validated by data reported in the Oncomine database. We conclude that MT2A expression is not altered in primary prostate cancer.

Decreased expression of Tacc2 observed in the microarray analysis was not statistically significant by quantitative PCR, although there was a trend toward lower expression in cancer and this decreased expression was also true in prostate cancer cell lines. Conversely, semi-quantitative PCR shows slightly elevated Tacc2 expression in prostate cancer tumor and adjacent-to-tumor tissues. While evaluation of the Oncomine database shows that expression of Tacc2 is relatively high in prostate compared to other normal tissue types, no altered expression was reported in prostate cancer and thus we conclude that, while Tacc2 probably is an important protein in prostate biology, altered expression does not appear to be characteristic in prostate cancer.

The increased expression of Nell2 reported by microarray analysis was reproduced in semi-quantitative and quantitative PCR analyses. An interesting aspect of the expression pattern seen for Nell2 is that expression appears higher in histologically normal tissue adjacent-to-tumor

tissue than in donor or tumor tissue by both semi-quantitative and quantitative analyses. This reflects a characteristic of the population from which targets were selected. In the original microarray analysis, the BPH-cancer group, tissues from which targets for this project were selected, included tissue enriched, not for regions of tumor but instead, for the adjacent hyperplastic tissue (essentially adjacent-to-tumor tissue). Consequently, genes identified in this group are likely to include those that reflect field effect changes in a prostate with dysregulated neoplastic growth or possibly those that are involved in BPH. In the case of Nell2, a protein previously demonstrated to be upregulated in BPH, the latter explanation seems the most plausible. The low expression of Nell2 in prostate cancer derived cell lines also supports this hypothesis. The pattern of higher expression in adjacent-to-tumor tissue compared to tumor tissue was also seen for FosB and Cyr61 (data not shown) but prostate donor tissue expression of these two genes was lower still than tumor tissue.

Increased expression of FosB was consistently found by microarray, semi-quantitative and quantitative expression analysis and supported by data reported in the Oncomine database. A number of studies in Oncomine report that FosB expression is lower metastatic lesions than in primary prostate cancer and the low expression of FosB in prostate cancer cell lines may be a product of this fact given that most these cell lines are derived from lesions of metastasis. The study of FosB is complicated by two factors: 1) existence of redundancy among the AP-1 transcription factors and 2) the dominant negative splice variant ΔFosB which, when expressed, is able to dimerize with Jun proteins but not able to interact with the DNA to initiate transcription. Comparison of FosB and ΔFosB transcript levels in tumor and donor tissue was not possible here because the qPCR primer efficiencies varied enough to confound the results. Based on these findings, elevated expression of FosB is characteristic in prostate cancer and I

feel that, despite the complications, the role of FosB in prostate cancer warrants further analysis and is likely to be important in the pathobiology of prostate cancer.

We decided to pursue further expression and functional analysis of Cyr61 and PCP4 rather than MT2A, Tacc2, Nell2, and FosB. Cyr61 and PCP4 were evaluated similarly by microarray, semiquantitative PCR, and qPCR (results presented in chapters 4 and 5). Based on the consistent pattern observed in the expression analyses and reported Oncomine data, Cyr61 and PCP4 expression warrant further analysis.

4.0 PCP4 EXPRESSION IN COLON AND PROSTATE CANCER

4.1 INTRODUCTION

Currently there is a lack of knowledge regarding the biological function of PCP4 in the prostate. Our preliminary microarray analysis demonstrated significantly diminished PCP4 mRNA expression in prostate cancer compared to healthy donor prostate tissue (29). In this portion of the project we set out to evaluate the expression and utility of PCP4 as a potential biomarker for prostate cancer diagnosis. As described below, our data demonstrate that PCP4 in prostate cancer is largely expressed by the stroma and therefore might not be a useful marker in prostate cancer, a disease of the epithelial compartment. However, our data suggests that PCP4 might serve as a more apt biomarker in colon cancer.

4.2 RESULTS

4.2.1 PCP4 gene expression in prostate

From the genes differentially expressed in microarray analysis of various normal, BPH, and prostate cancer tissues, expression of PCP4 appears significantly and consistently downregulated

in prostate tissues from men with prostate cancer (Figure 30) (29). For this reason PCP4 was selected for further analysis.

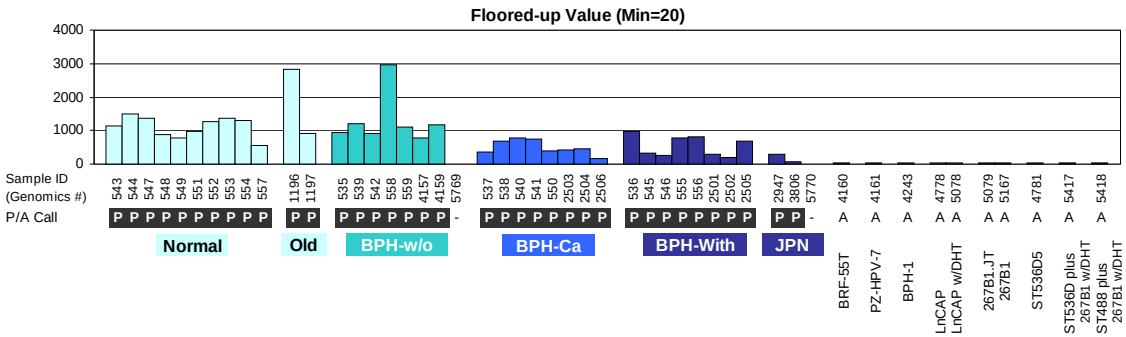


Figure 30. Genomic analysis is PCP4 expression in prostate tissues and cell lines

In order to confirm this, semi-quantitative (Figure 31) and quantitative gene expression analyses (Figure 32) were employed to compare gene expression in healthy donor prostate and prostate cancer tissue. By semi-quantitative analyses, both tumor tissues show a decrease in PCP4 expession, one modest and one striking, in prostate cancer tissue compared to adjacent to cancer and healthy donor tissue. In quantitative analysis, the trend toward decreased PCP4 expression in tumor compared to adjacent-to-tumor and healthy donor tissue was again demonstrated but the results are not statistically significant because the expression of PCP4 in healthy donor tissue varied over a relatively broad spectrum.

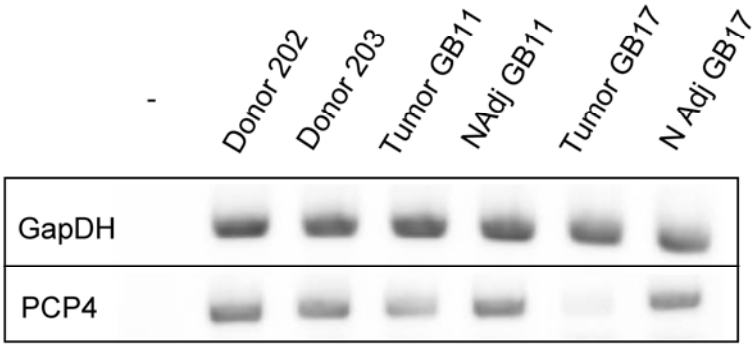


Figure 31. Expression of PCP4 in prostate tissues

Semi-quantitative PCR analysis of 2 paired tumor/normal and 2 healthy donor prostate tissues

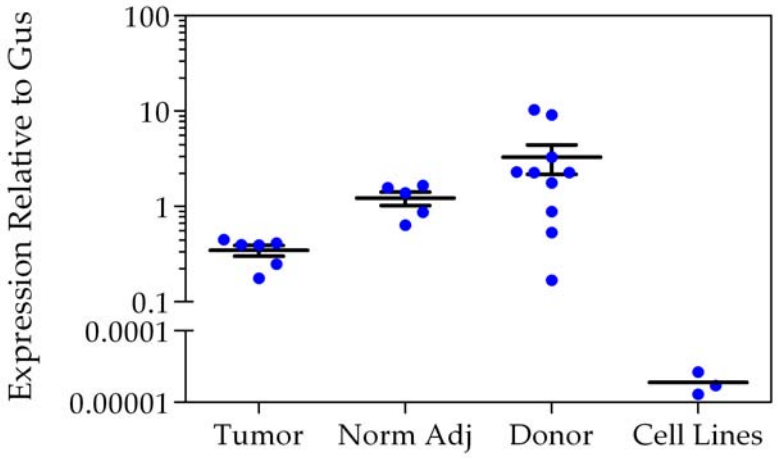


Figure 32. PCP4 gene expression in prostate tissues

Each circle represents the relative mRNA abundance (PCP4 message normalized to Gus expression) for prostate tumor, adjacent-to-tumor or healthy donor prostate tissues or cell line (LNCaP, PC3, BPH1, and 267B1). PCP4 expression is absent in LNCaP.

To further substantiate this pattern of decreased PCP4 expression in prostate cancer, a query of the Oncomine database was performed and six studies report decreased expression of PCP4 in prostate cancer (222).

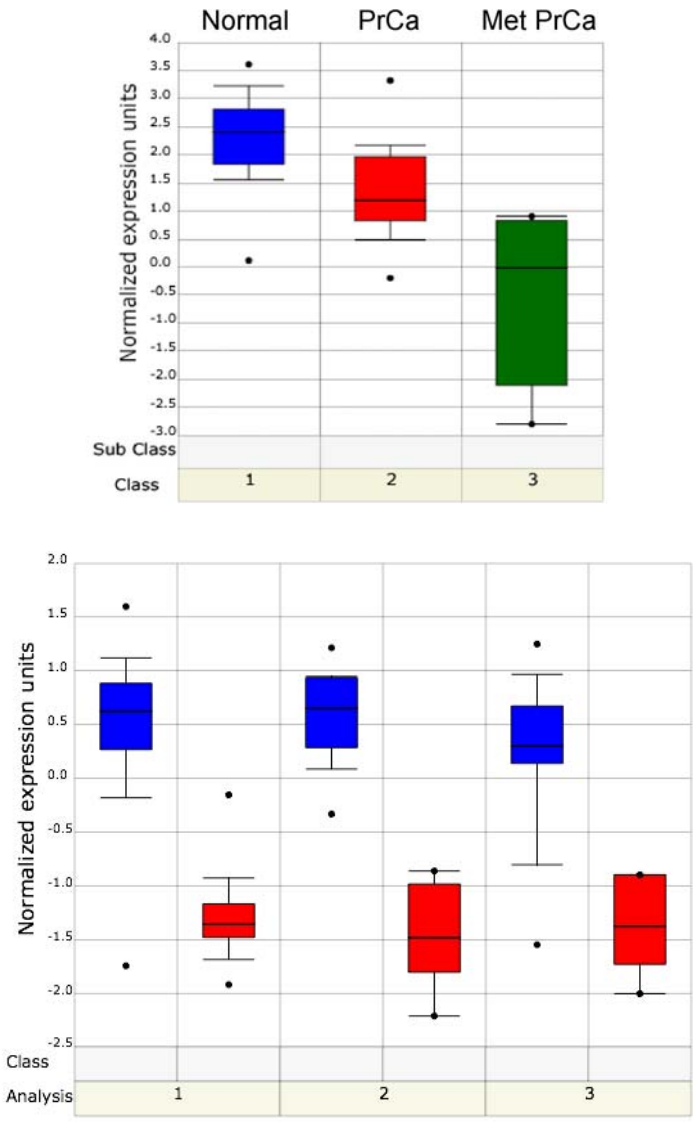


Figure 33. Compared to normal prostate tissue, expression of PCP4 is decreased in prostate cancer (blue) and lower still in metastatic prostate cancer (red)

PCP4 mRNA expression is lower in prostate cancer tissues than in normal prostate and lower still in metastatic prostate cancer tissues (Figure 33). The same trend was demonstrated in seven independent genomic analyses submitted to Oncomine contributed (219).

4.2.2 Validation of PCP4 Antibody

In order to have an ample supply of PCP4 antibody, we raised rabbit polyclonal antibodies against the peptide sequences IQSQFRKFQKKK - AA 46-57 (α-PCP4 pep2) and GQKKVQEEFDIDMD – AA 22-35 (α-PCP4 pep3). The specificity of these antibodies for peptide was established by ELISA. Peptide curves suggest that α-PCP4 pep2 has more potential as a specific antibody against PCP4 (Figure 34).

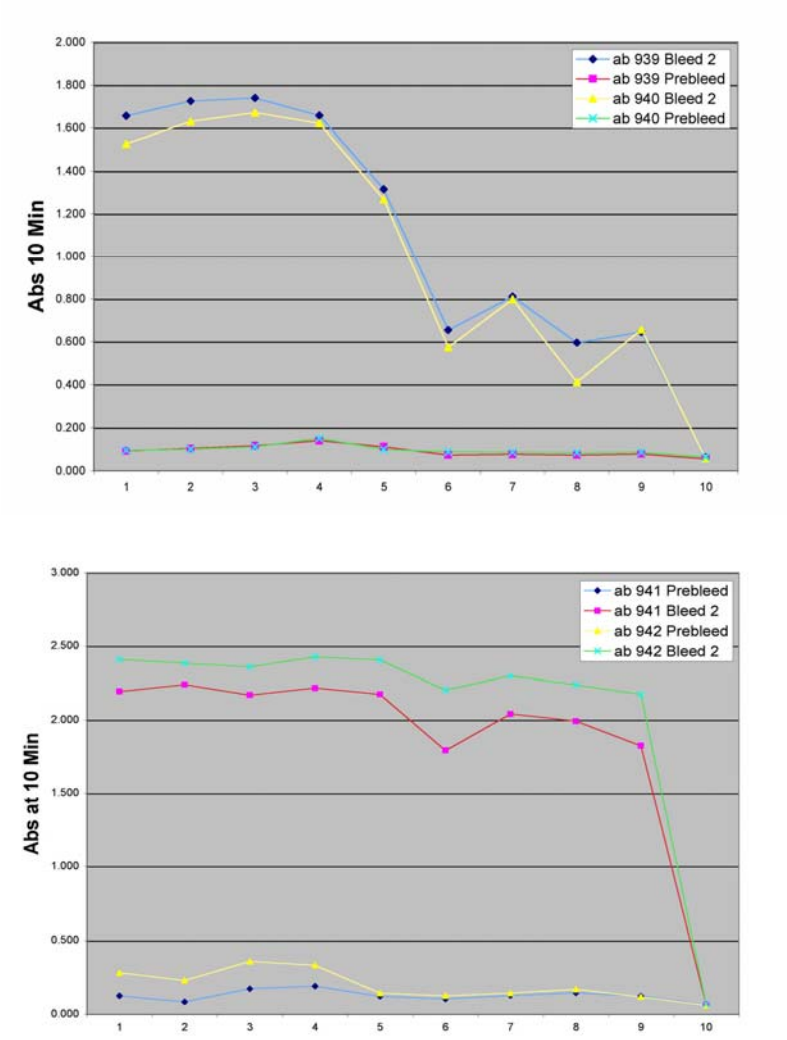


Figure 34. α-PCP4 pep2 and α-PCP4 pep3 reactivity

Peptide curve dilution: 1mg/ml - 1pg/ml A) α-PCP4 pep2 (*upper graph*) B) α-PCP4 pep3 (*lower graph*)

These antibodies were compared by IHC with α-Pep19, a polyclonal antibody developed in the lab of James Morgan (St. Jude's Children's Hospital, Memphis, TN). Brain tissue was stained as a positive control given that the most well studied site of PCP4 expression is the brain. Lung, an organ reported to express little to no PCP4 in the healthy adult, was evaluated as a negative control.

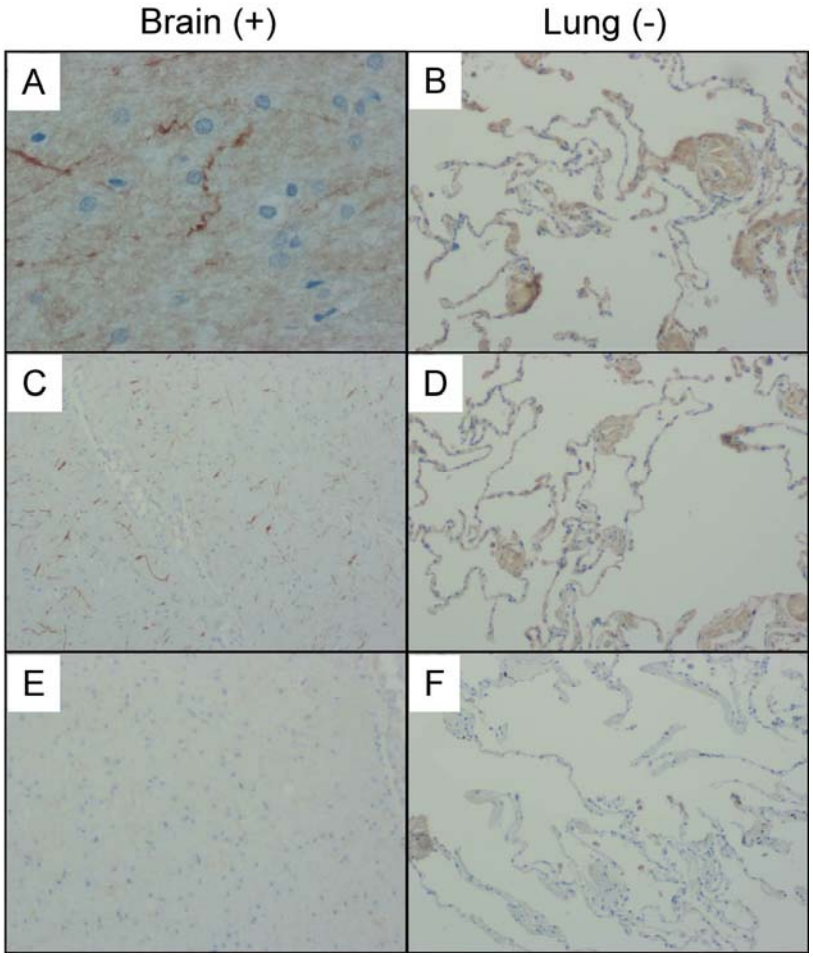


Figure 35. Generation of PCP4 antibodies

The specificity of α-PCP4 pep2 (C & D) and α-PCP4 pep3 (E & F) were compared against α-Pep19 (A & B) in brain (positive control) and lung (negative control).

Although α-Pep19 nicely stained the axons in brain tissue, non-specific background staining was also evident in the brain and lung. α-PCP4 pep2 resulted in similar strong staining of axons with low background. α-PCP4 pep3 was unable to detect PCP4 expression. Based on these results α-PCP4 pep2 was used for all further IHC analysis.

4.2.3 PCP4 is expressed by prostate stroma

In the evaluation of the role of PCP4 in prostate cancer, it is important to also be familiar with the expression of PCP4 in normal prostate tissue. Four independent genomic analyses submitted to Oncomine that compare expression in normal prostate epithelia and stroma demonstrate that PCP4 expression is predominantly stromal (Figure 36 top) (219).

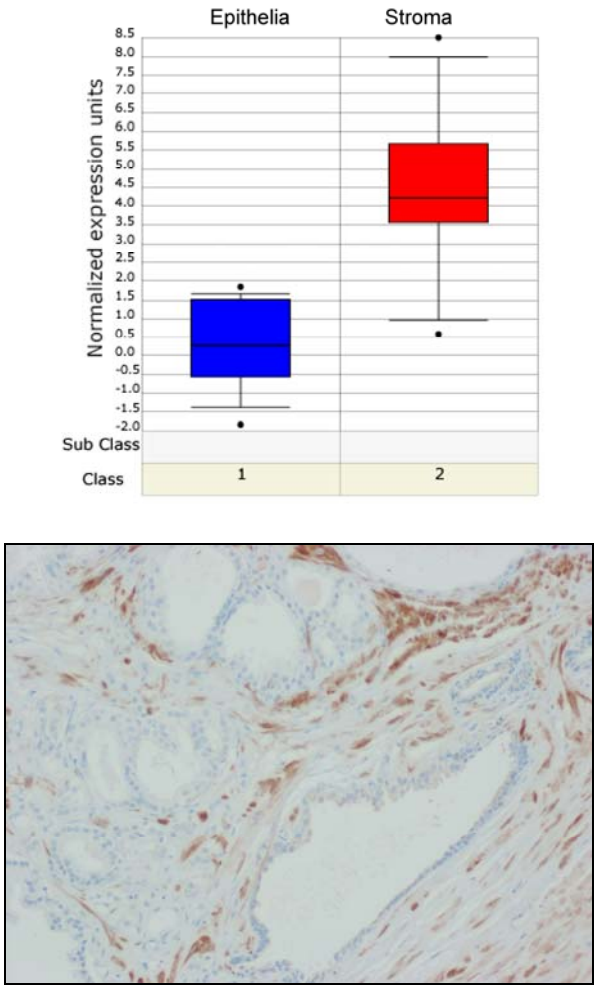


Figure 36. PCP4 is predominently expressed by the prostate stroma *(top)* mRNA; representative 1 of 4 microarray analysis studies from Oncomine *(bottom)* protein

This finding was confirmed by IHC staining of prostate tissue (Figure 36 bottom). Little to no expression of PCP4 was evident in the glands of healthy prostate (0%; N=10) or prostate cancer (4.4%; N=90), while the stroma of all normal prostate tissue (100%; N=10) and the majority of tumor associated stroma (93.3%; N=90) expressed PCP4.

Table 3. Expression of PCP4 in Normal Prostate and Prostate Cancer Tissue

	Benign Prostate	Prostate Cancer
Stroma	10/10	84/90
Epithelia	0/10	4/90

From these tissue-staining results no clear pattern existed to support the initial hypothesis that decreased expression of PCP4 is a functionally relevant alteration in prostate cancer.

4.2.4 Expression of PCP4 in various cancers

Given that PCP4 expression in the prostate is predominantly stromal in nature, we hypothesized that this protein may play an interesting role in sarcomas. To assess this, we evaluated PCP4 expression in various types of cancer by staining a 'various cancers' tissue array. Expression of PCP4, appears common in cancers of the gastrointestinal (GI) tract (Figure 37) as well as in small cell lung carcinoma (data not shown). Colon, gastric, and pancreatic cancer lesons all exhibit positive staining for PCP4 in regions of cancer. To further substantiate this pattern of elevated PCP4 expression in GI cancers, a query of the Oncomine database was performed but no studies report altered expression of PCP4 in gastric or pancreatic cancer (223). Contradictory to our findings, a single study reports decreased expression of PCP4 in colon cancer (219).

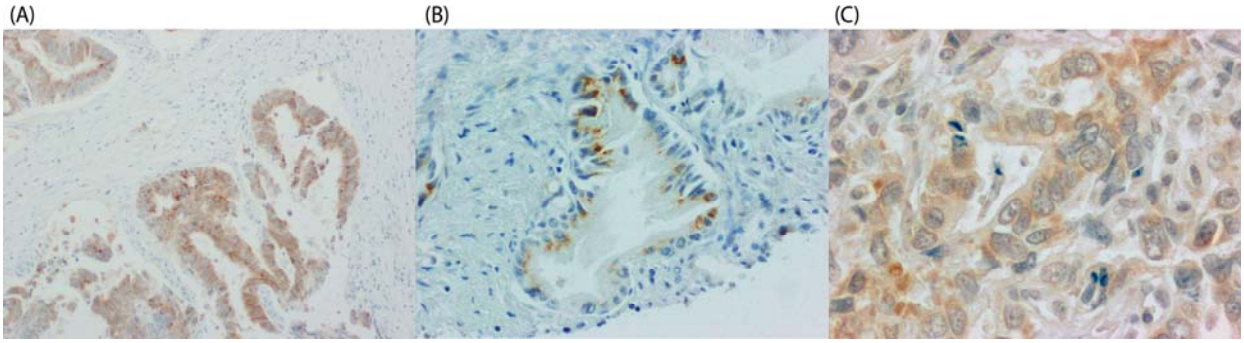


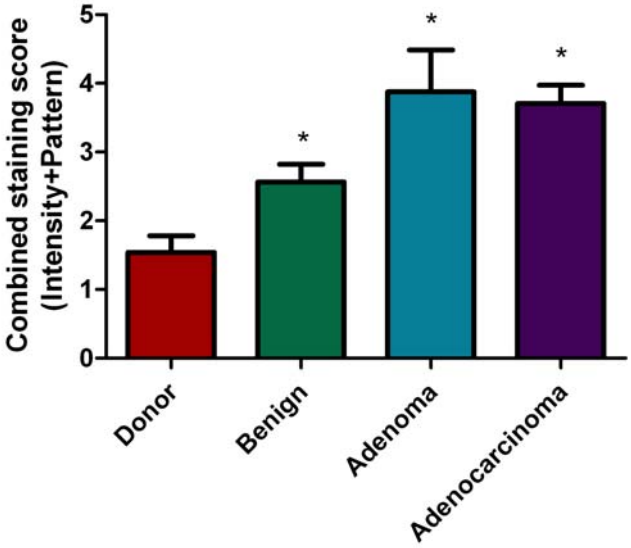
Figure 37. Expression of PCP4 in colon, gastric, and pancreatic cancer

4.2.5 PCP4 expression is elevated in colon cancer

To more fully evaluate the expression of PCP4 in cancers of the GI tract, we made use of a colon cancer TMA to evaluate expression of PCP4 in a panel of normal colon, colon polyp (benign growths), adenoma, and colon cancer tissues. Tissues were assigned an intensity (0=no staining, 1=light, 2=moderate, 3=intense staining) and a pattern score (0= no staining, 1= focal, 2=diffuse). Intensity and pattern were each evaluated independently and in combination (intensity+pattern) and the same pattern existed by all three methods. Tissue from benign polyps, adenomas, and adenocarcinomas all express significantly more PCP4 than donor colon tissue; in addition adenomas and adenocarcinomas also express significantly more PCP4 than benign polyps (Table 4 and Figure 38). There is no discernable difference between PCP4 expression in adenomas compared to adenocarcinomas.

Table 4. PCP4 expression in colon cancer - average combined staining score

	Donor	Benign	Adenoma	Adenocarcinoma
Average Staining Score	1.5	2.6	3.9	3.7
N	13	24	8	17



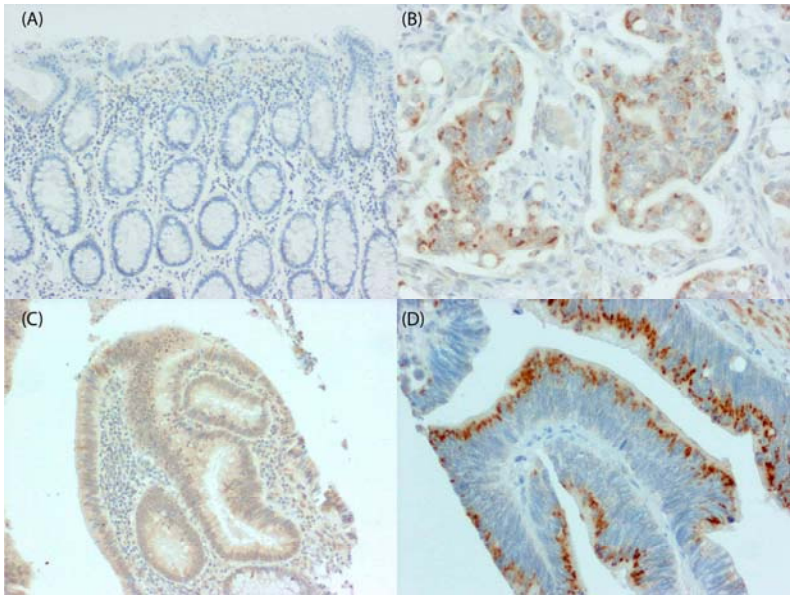


Figure 38. Comparison of PCP4 protein expression in colon tissue by IHC

(top) Intensity on a scale of 0-3; Pattern on a scale of 0-2 (0= none; 1=focal; 2=diffuse); Maximum combined staining score of 5. Graphed independently Intensity and Pattern each yield a similar pattern: increased staining intensity and a more diffuse pattern in adenoma and adenocarcinoma tissues. *(bottom)* IHC of colon – A) benign colon; B) adenocarcinoma; C and D) adenomas and examples of the two staining patterns seen for adenoma and adenocarcinoma (diffuse cytoplasmic and diffuse punctate staining).

4.3 CONCLUSIONS

In this series of experiments the expression of PCP4 was evaluated in prostate cancer as well as in a variety of other types of cancer, of particular interest colon cancer. Decreased expression of PCP4 observed in microarray analysis and supported by PCR analysis, although not statistically significant, indicated a trend toward lower expression in cancer by quantitative and semi-quantitative PCR and prostate cancer cell lines express similarly low levels of PCP4. Additionally, in evaluation of the Oncomine database, multiple studies confirm that expression of PCP4 is lower in prostate cancer compared to normal prostate tissue and lower still in metastatic prostate cancer than normal prostate.

The α-PCP4 pep2 antibody was successfully developed and showed strong specificity for PCP4 with low background staining. IHC analysis of normal prostate and prostate cancer tissues revealed that PCP4 expression is predominantly stromal and this is confirmed by microarray results reported in the Oncomine database. PCP4 may have future utility as a marker of prostate stromal cells. Additionally, the decreased expression reported in expression analysis in these studies and those reported by Oncomine may in part or whole reflect the amount of stroma in the analyzed tissue rather than altered expression by epithelial cells transitioning to cancer. In the microarray analysis, unless highly pure samples (isolated by laser capture) are collected for epithelia or cancer than the percentage of the stromal contribution will have a major impact on the amount of PCP4 detected in each sample.

In staining the 'various cancers' tissue array, high expression of PCP4 was noted in small cell lung carcinoma (SCLC) and in GI cancers (colon, gastric, and pancreatic cancer). The high expression of PCP4 in SCLC is quite interesting given the fact that normal lung tissue expresses little to no PCP4 and was used as a negative control in the selection of α-PCP4 antibodies.

Evaluation of PCP4 expression in a larger cohort of SCLC tissue is needed to substantiate the preliminary finding of elevated PCP4 expression in SCLC. High expression of PCP4 in colon cancer was confirmed by evaluation of the colon cancer TMA. PCP4 is expressed the highest intensity and the most diffusely in colon adenomas and adenocarcinomas compared to benign polyps or donor colon tissue.

In conclusion, PCP4 is expressed highly in both normal and prostate cancer stroma and thus is not likely to play an important role in prostate cancer. The fact that various GI cancers express PCP4 in regions of cancer suggests the possibility of a shared mechanism; potentially the reactivation of a quiescent developmental signaling pathway but this has yet to be investigated.

5.0 CYR61 EXPRESSION AND ROLE IN PROSTATE CANCER

(Portions adapted from manuscript: Katherine B. D'Antonio, Antoun Toubaji, Roula Albadine, Alison M. Mondul, Elizabeth A. Platz, George J. Netto, and Robert H. Getzenberg, Brady Urological Institute, the Sidney Kimmel Cancer Center, and the Departments of Pathology, Johns Hopkins University School of Medicine, and the Department of Epidemiology, Johns Hopkins Scholl of Public Health *submitted for publication*)

5.1 INTRODUCTION

Cysteine-rich angiogenic inducer 61 (Cyr61), also known as CCN1, is an extracellular matrix protein involved in the transduction of growth factor and hormone signaling and mediation of mechanical stress responses, in some systems. Generally Cyr61 is implicated as a molecule that keeps cells connected to changes in their cellular environment. The CCN family of proteins (named after the earliest identified family members: Cyr61, CTGF, and Nov), is characterized by four common conserved domains: a von Willeband factor type C repeat, a thrombospondin type 1 repeat, an insulin-like growth factor binding protein (IGFBP) motif, and a cysteine-knot motif. The members of this family have highly regulated expression, are important in the regulation of growth and development, and have been linked to a variety of pathologic disorders, including cancer. In particular, Cyr61 has been found to participate in regulating many pathways including cell adhesion, migration, proliferation, differentiation, and survival (196,212,224-233). Despite over a decade of research, the specific function of Cyr61 remains largely unknown; its biological

activity is believed to be contextual and cell type-dependent (234). Cyr61 signals through interactions with integrins and, depending on the combination of integrins bound and the tissue type, the downstream effects vary greatly (211,214,227,235-241).

In genomic studies of various cancers, Cyr61 commonly exhibits altered expression (202-214). These expression changes have been linked with, depending on the type of cancer, either enhanced or inhibited tumor growth. Modulation of Cyr61 expression has been observed in breast, ovarian, hepatocellular, lung, and colorectal cancer. In early studies Pilarskey *et al.* found that Cyr61 mRNA is down-regulated in prostate cancer compared to normal adjacent to cancer tissue (215). More recently, in our study comparing benign prostatic hyperplasia (BPH) to healthy donor prostates, symptomatic BPH samples exhibited moderately elevated levels of Cyr61 mRNA while a subset of prostate cancer samples also evaluated exhibited elevated expression higher than either BPH or donor prostates (29). In additional analysis of expression in the prostate, Sakamoto *et al.* showed that mRNA expression of Cyr61, by in situ hybridization, localizes to the basal cells in normal prostate tissues (216). Additional evidence substantiating the importance of Cyr61 in prostate cancer was recently published by Sun *et al.*; the expression of Cyr61 in prostate cancer cells was found to enhance migration, invasion, and proliferation and showed that Cyr61 regulates Rac1 signaling, a mechanism by which Cyr61 potentially may exert its influence on cell growth and motility (217).

5.2 RESULTS

5.2.1 Elevated Cyr61 gene expression in prostate cancer

To confirm gene expression microarray analysis (Figure 39) (29) and more fully characterize the expression of Cyr61 in the prostate, semi-quantitative (Figure 40) and then quantitative gene expression analyses (Figure 41) were employed to compare gene expression in healthy donor prostate tissue and tissues obtained from prostates that contained foci of cancer.

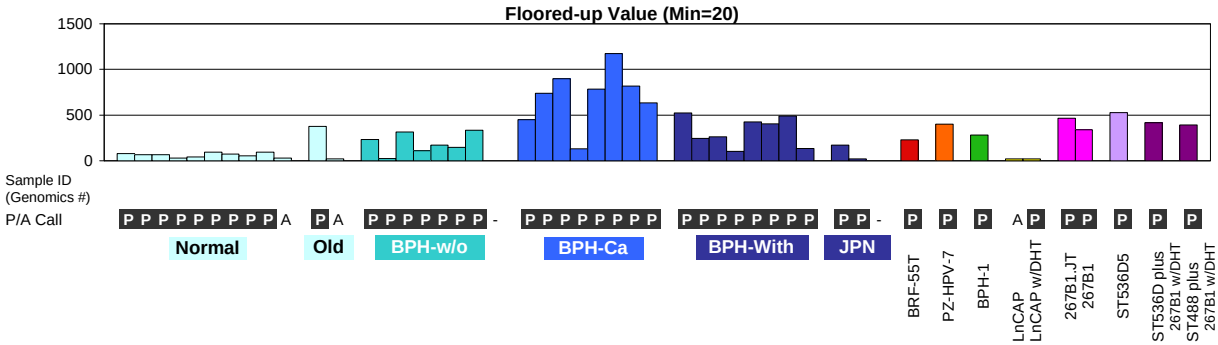


Figure 39. Genomic analysis of Cyr61 expression in prostate tissues and cell lines

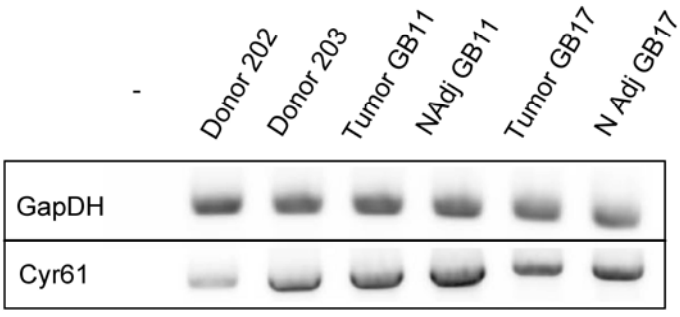


Figure 40. Expression of Cyr61 in prostate tissues

Semi-quantitative PCR analysis of 2 paired tumor/normal adjacent and 2 healthy donor prostate tissues

In semi-quantitative analysis of paired tumor/normal adjacent prostate tissue, expression of Cyr61 was higher in normal adjacent compared to matched tumor tissue and donor prostate tissue expressed Cyr61 at or below levels in tumor tissue. This expression was considered semi-quantitative given that expression of the housekeeping gene GapDH is comperable between the six tissues. To quantitatively evaluate this, taqman qPCR was performed and expression of Cyr61 is elevated in 10/11 (91%) prostate cancer tissues in comparison to the expression in donor tissues (N=10; Figure 41) (1.84 ± 1.34 for donor tissues vs. 9.05 + 5.44 for tumors; $p<0.05$).

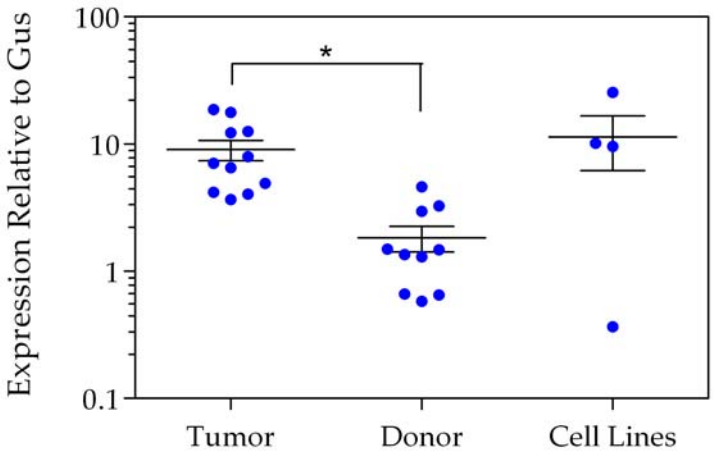


Figure 41. Quantitative gene expression of Cyr61

Each circle represents the relative mRNA abundance (Cyr61 message normalized to Gus expression) for prostate tumor or healthy donor prostate tissues or cell line (LNCaP, PC3, BPH1, and 267B1). LNCaP expression of Cyr61 is notably lower than other cell lines.

To support the notion of altered Cyr61 expression in prostate cancer, a query of the Oncomine database was performed. Cyr61 is up-regulated in prostate cancer tissue in two

studies in comparison with either healthy prostate tissue or prostatic intraepithelial neoplasia (PIN); additionally, in nine studies where expression in primary prostate cancer was compared to metastases, the expression of Cyr61 is lower in metastases than in primary cancer lesions (Figure 42 bottom – one representative study) (223).

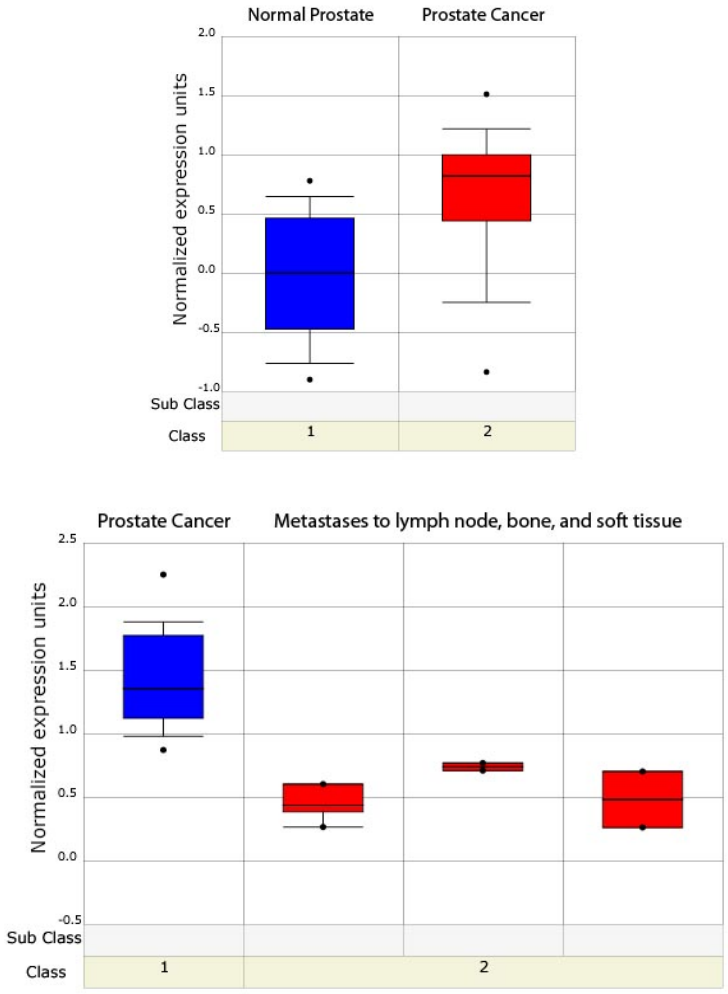


Figure 42. Oncomine studies support the finding that Cyr61 is *(top)* up-regulated in prostate cancer over normal prostate and *(bottom)* lower in metastases than in primary prostate cancer

5.2.2 Cyr61 protein expression in prostate

5.2.2.1 Up-regulation of Cyr61 in prostate cancer tissues

Initial Cyr61 immunohistochemical analysis of routine prostate tissue sections obtained from 18 radical prostatectomy specimens was performed to characterize the Cyr61 localization in the prostate. Cyr61 protein appeared significantly up-regulated in prostate cancer lesions compared with adjacent histologically normal glands (data not shown). Expression was of consistent intensity within each lesion and was of diffuse finely granular nature and cytoplasmic in location. No evidence of Cyr61 expression in the stroma was observed.

Based on these preliminary findings, tissue microarray analysis of 1,366 spots representing tissue from the prostates of 197 consecutive RRPs patients were analyzed for Cyr61 protein expression and localization (Figure 43 and Table 5).

Table 5. Demographics of Individuals whose Tissue was Included in the TMA

# of Patients	200
Race	
Caucasian	86%
African American	9.5%
Other	4.5%
Patient Age at RRP	
Mean±SD	57.89±6.88
Median	58
Range	37-74
Stage	
pT2	131 (65.5%)
pT3a	52 (26%)
pT3b	12 (6%)
N1	4(2%)
Gleason	
5-6	110 (55%)
7	67 (33.5%)
8-10	23 (11.5%)

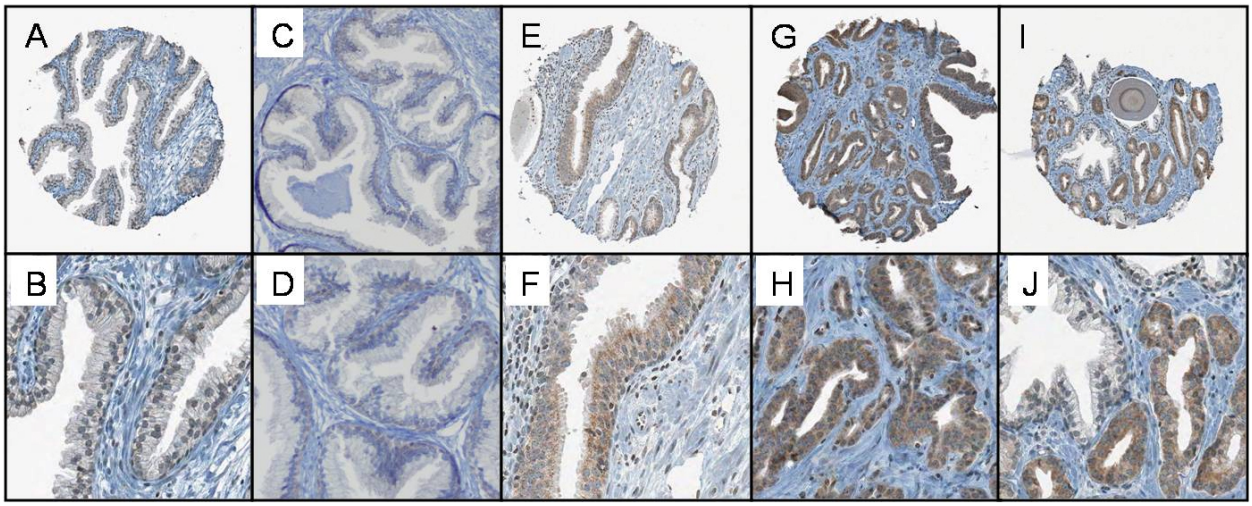


Figure 43. Immunohistochemistry analysis of Cyr61 protein expression in prostate tissue

Images representative of staining observed in prostate cancer, adjacent to prostate cancer, or BPH tissues. A & B. Low and high power images of NAT. C & D. Low and high power images of BPH. E & F. Low and high power images of high grade PIN. G & H. Low and high power images of tumor. I & J. Low and high power images of tumor with adjacent NAT.

Of these, there were 174 matched tumor/normal adjacent-to-tumor tissues, 25 matched PIN/normal adjacent-to-tumor tissues, and 23 matched PIN/Tumor tissues. Similar cytoplasmic staining pattern was observed in the TMAs as that of the above described in routine tissue sections; only weak Cyr61 staining was detected in histologically normal prostate tissue while areas of cancer generally exhibited a relatively more intense Cyr61 staining.

Table 6. Paired comparisons of Normal staining with atrophy, PIN and Cancer

	Normal	**Atrophy**	**PIN**	**Cancer**
# Spots per Man				
Median	4	1	1	3
# Pairs				
N	-	40	25	174
Median Staining Intensity				
median [§]	0	1	2	2
p value [*]	*ref*	*0.0009*	*<0.0001*	*<0.0001*
% Spots with Staining Intensity:				
≥ 1	60.3%[‡]	70.0%	96.0%	98.3%
p value[†]	*ref*	*0.21*	*0.003*	*<0.0001*
≥ 2	21.3%[‡]	15.0%	64.0%	85.1%
p value[†]	*ref*	*0.32*	*0.0009*	*<0.0001*
≥ 3	1.7%[‡]	5.0%	20.0%	60.9%
p value[†]	*ref*	*0.56*	*0.18*	*<0.0001*

Table 7. Paired comparisons of PIN and Cancer

	PIN	Cancer
# Spots per Man		
median	1	3
# Pairs		
N	-	23
Median Staining Intensity		
median ‡	2	2.5
*p value**	*ref*	-
% Spots with Staining Intensity:		
≥ 1	100%	95.7%
p value†	*ref*	-
≥ 2	65.2%	78.3%
p value†	*ref*	*0.32*
≥ 3	17.4%	60.9%
p value†	*ref*	*0.01*

The median staining intensity, on a scale of 0-3, across individuals was 0 for normal tissue, 1 for atrophy, 2 for PIN, and 2 for cancer (*p trend<0.0001*) (Figure 44). The area under the ROC curve was 0.928 comparing the median staining intensity for cancer and normal spots (Figure 45). For cancer spots, 98.3% had a staining intensity of ≥ 1, 85.1% had ≥ 2, and 60.9% had ≥ 3, and for normal spots 60.3% had a staining intensity of ≥ 1, 21.3% had ≥ 2, and 1.7% had ≥ 3 (*p<0.0001)*. In those men diagnosed with prostate cancer with a Gleason sum of 8 and higher, the median Cyr61 staining intensity was higher in their cancer spots compared to those with Gleason sum below 8 (*p=0.01*) (Figure 44), although no threshold value could statistically differentiate between higher and lower Gleason sum cancers (data not shown).

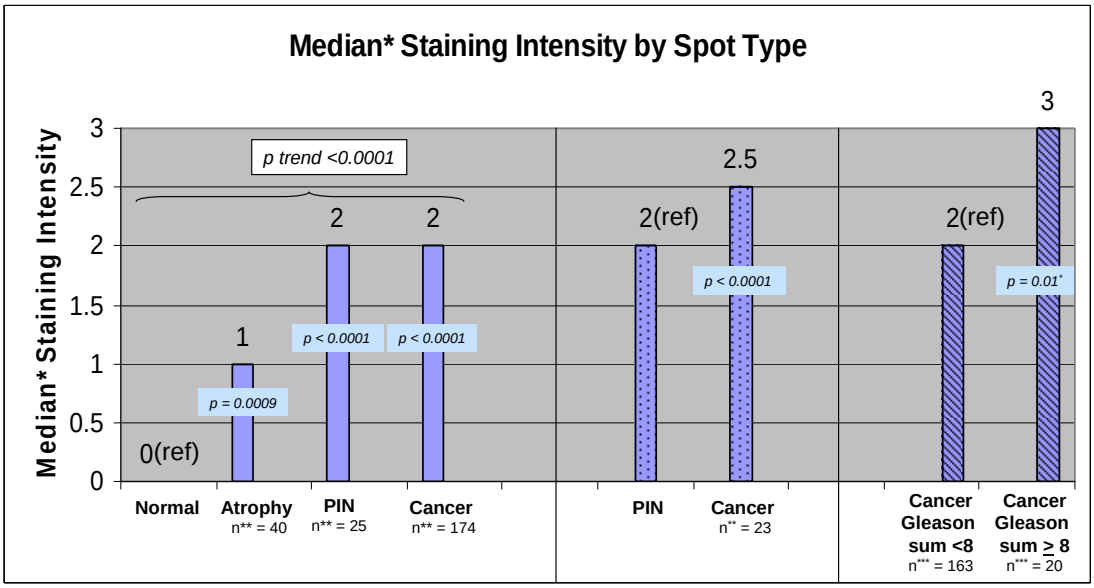


Figure 44. Protein expression of Cyr61 by IHC scoring of TMA spots

Comparison of the median staining intensity for matched pairs by spot type.

Comparison of the FriDA intensity and visual intensity measurements in one of the five TMAs revealed a strong correlation by pair wise correlation analysis (correlation coefficient = 0.7166; *p<0.0001)*.

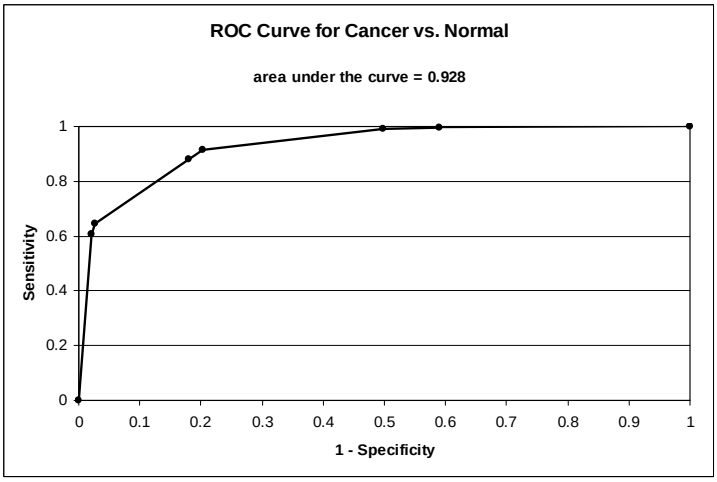


Figure 45. ROC Curve for Cyr61 separation of prostate cancer from adjacent-to-tumor tissue

Cyr61 expression in BPH tissue sections obtained from 13 TURP specimens was analyzed by IHC to characterize Cyr61 localization within benign prostatic lesions. No evidence of elevated Cyr61 expression was observed in any of the BPH tissues in either the glands or the stroma.

5.2.2.2 Cyr61 expression in Progression - TMA analysis

Given that Cyr61 expression is elevated in Gleason 8 and higher cancers compared to Gleason 7 and lower cancers, we decided to evaluate whether there is a correlation between Cyr61 staining and incedence of prostate cancer progression. To answer this question, a set of 10 prostate cancer TMAs were stained for Cyr61 expression. These TMAs were designed as a case-control study nested in a cohort of radical prostatectomy patients with incedence density sampling of matched controls in order to facilitate research on prognostic and risk factors for progression following prostatectomy. The database from which cases and controls were selected includes 4,860 men who underwent radical prostatectomy for a diagnosis of clinical organ-confined prostate cancer between the years 1993 and 2004. Data from this study is currently undergoing statistical analysis with the goal that it will be complete for inclusion in thesis revisions before final submission.

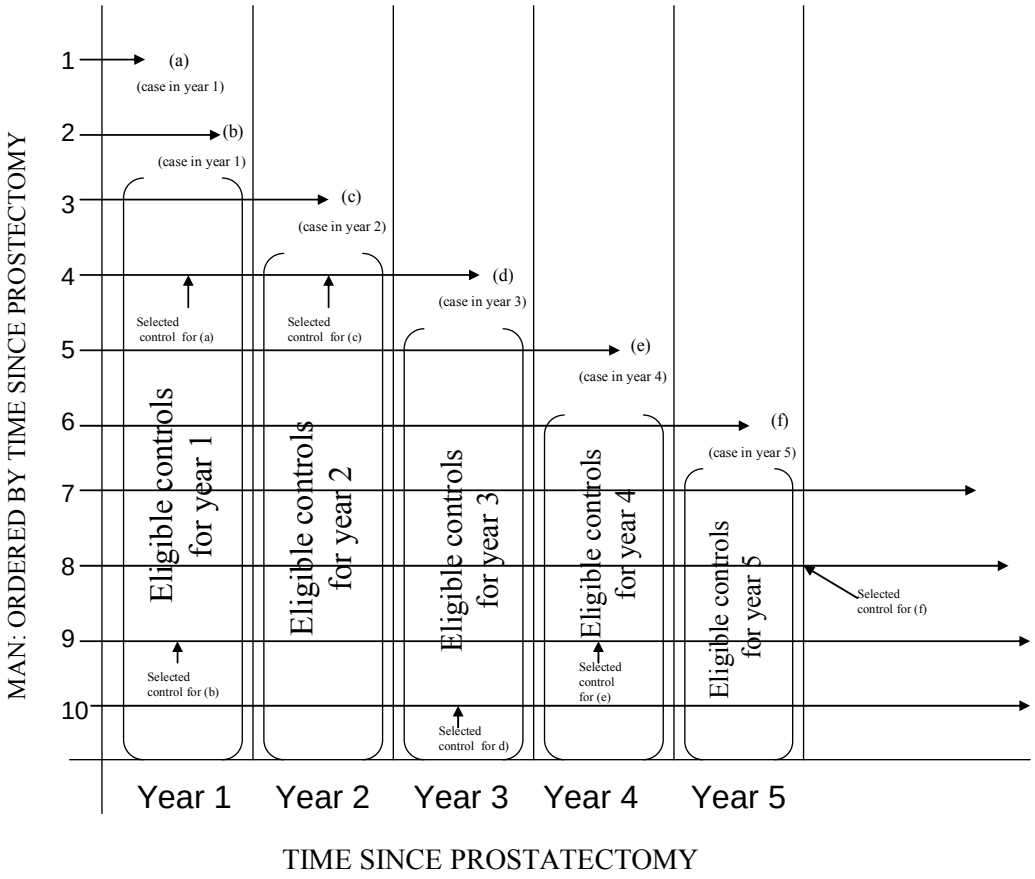


Figure 46. Schematic illustration of case/control selection

Table 8. Characteristics of eligible and ineligible men who underwent radical prostatectomy (N=4860) at Johns Hopkins Hospital, 1993 – 2004

	Eligible N=4495	Ineligible N=365						
		Implausibly long FU time N=7	Had tx prior to RP N=171	Missing on follow-up time N=62	Missing on pathologic Gleason sum N=11	Missing on pathologic stage N=10	Subsequent tx didn't allow obs of progression N=6	Unclear if organ confined N=98
Mean age (years)	57.8	62.9	58.7	57.9	60.3	56.8	58.7	57.1
Race (%)								
White	89.0	100	83.6	93.6	100	80.0	33.3	80.6
Black	5.9	0	5.3	3.2	0	0	16.7	9.2
Hispanic	0.7	0	0.6	0	0	0	0	0
Asian	0.4	0	1.2	0	0	10.0	0	0
Other	4.0	0	9.4	3.2	0	10.0	50.0	10.2
Mean PSA before RP (ng/mL)	7.6	11.7	6.8	6.9	5.7	6.3	6.2	6.8
Mean pathologic Gleason sum	6.4	6.4	6.5	6.3	Missing	6.2	7.8	6.3
Pathologic stage (%)								
T2	64.4	100	63.7	74.2	72.7	0	16.7	0
T3a	28.8	0	26.9	24.2	27.3	0	66.7	0
T3b/N1	6.8	0	9.4	1.6	0	0	16.7	0
Missing	0	0	0	0	0	100	0	100
Mean time to progre-ssion or last follow-up (yrs)	3.1	12.7	2.2	Missing	3.8	2.9	1.2	3.4

5.2.2.3 Protein expression varies in prostate cell lines

The protein expression of Cyr61 in prostate cell lines, including primary, immortalized epithelial, and prostate cancer derived lines, was assessed and found to be present in most prostate lines (15/18) (Figure 47). All lines tested express Cyr61 with the exception of VCaP, CWR22-R1, and 267B1 cells.

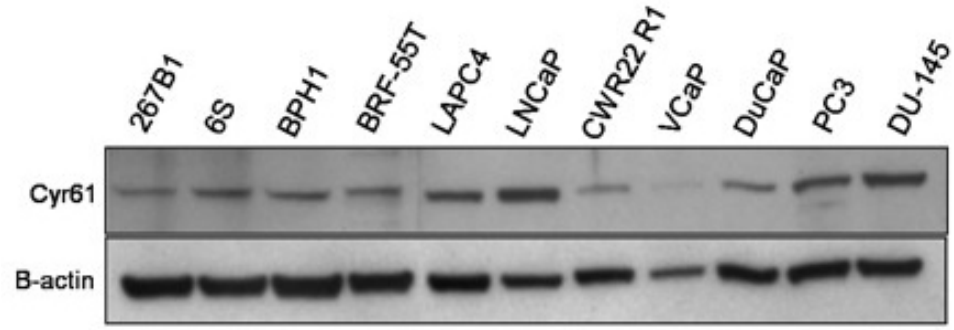


Figure 47. Immunoblot analysis of Cyr61 protein in prostate cell lines

Total protein from various prostate cell lines was analyzed.

5.2.3 Modulation of Cyr61 expression in prostate cancer cells

In order to develop an understanding of the role of Cyr61 in prostate and how it might contribute to the pathobiology of prostate cancer, we transfected VCaP and CWR22R.v1 prostate cells with pdsRed-Cyr61 vector to introduce higher levels of Cyr61 and transfected PC3 and CWR22R.v1 cells with shRNA or siRNA to Cyr61 to knockdown protein expression. VCaP and CWR22R.v1 were selected for their low basal expression of Cyr61 (Figure 43); PC3 cells express higher levels of Cyr61 protein making that cell line ideal for knock-down experiments. Transient tranfection of VCaP cells did not result in elevated Cyr61 protein expression (Figure 48) and therefore, subsequent experiments focused on CWR22R.v1 cells only.

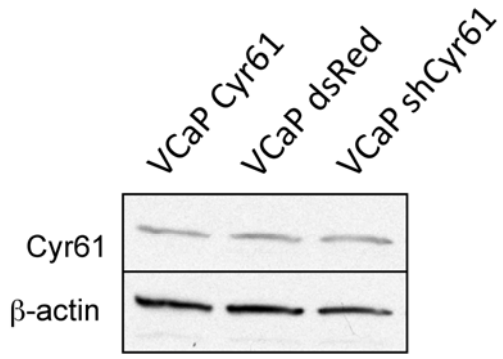


Figure 48. Transient transfection of VCaP cells does not alter Cyr61 expression

In transiently transfected CWR22R.v1 cells mRNA expression of Cyr61 was similar in mock transfected, dsRed transfected, and shNonSilencing cells and significantly upregulated in Cyr61 and shCyr61 transfected cells (Figure 49). The same was found when transfecting Hek293T cells (data not shown).

Figure 49. mRNA expression of Cyr61 in transiently transfected CWR22R.v1 cells

In transiently transfected PC3 cells, mRNA expression of Cyr61 was similar in mock transfected and siNonSilencing-transfected cells and significantly down-regulated in siCyr61-transfected cells in a dose dependent manner (Figure 50 top). The decreased expression of Cyr61 was confirmed at the protein level by immunoblot but the dose dependent decrease in mRNA expression did not translate to a dose dependent decrease in protein given that Cyr61protein expression level appears similar in cells exposed to a low or high concentration of siRNA (Figure 50 bottom).

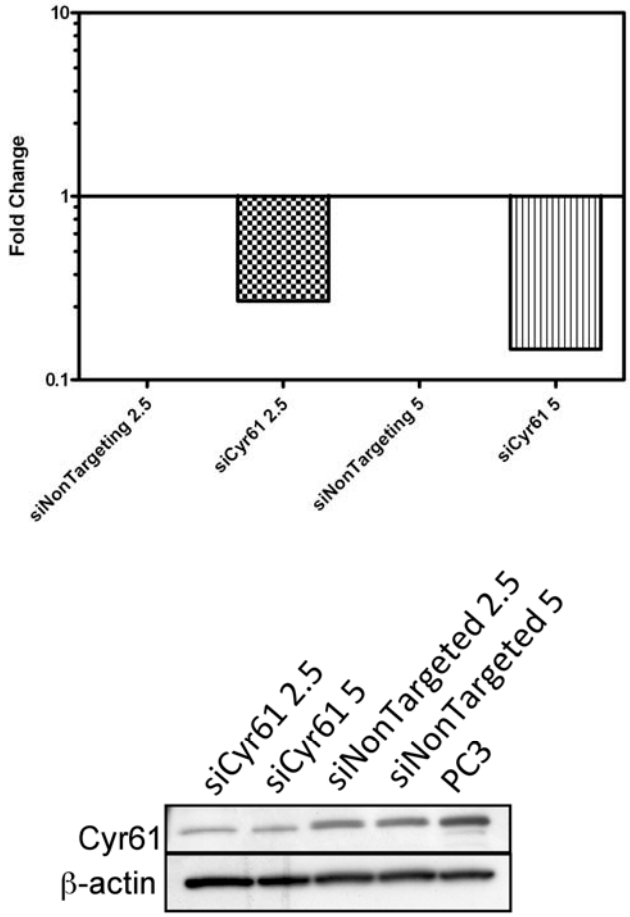


Figure 50. Transient knockdown of Cyr61in PC3

(top) Dose dependent knockdown of Cyr61 mRNA by qPCR; siNonTargeting fold change=1 *(bottom)* Decreased Cyr61 protein expression by siRNA knockdown

Given that transient transfection of Cyr61 into CWR22R.v1 cells resulted in a significant increase in Cyr61 expression, CWR22R.v1 cells were again transfected with Cyr61 and stable clones were selected by G418 resistance. mRNA expression Cyr61 was elevated in pooled Cyr61 clones compared to pooled dsRed clones (Figure 51) and parental CWR22R.v1 cells.

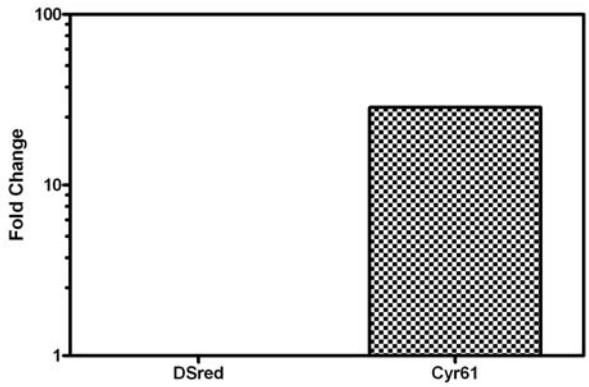


Figure 51. mRNA expression of Cyr61 in pooled stable clones of CWR22R.v1 cells

Individual clones were propagated and expression of Cyr61 mRNA (data not shown) and protein (Figure 52) was evaluated. A few clones with elevated Cyr61 mRNA and protein expression were selected for subsequent functional studies, in particular CWR22 Cyr61 3, 5, 14, 16, and 23. Protein loading of CWR22 Cyr61 23 is low compared to other clones based on b-actin and, thus, the expression of Cyr61 is likely even higher than the moderate increase shown here.

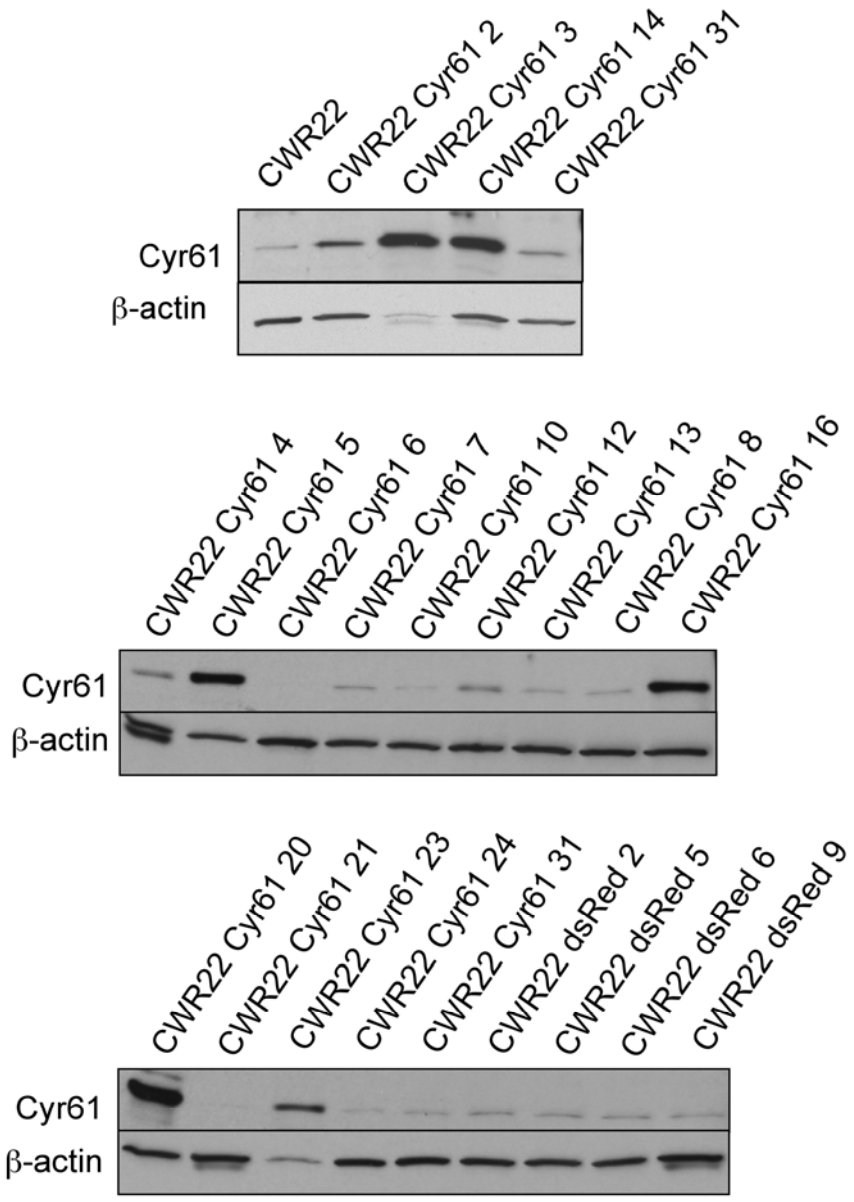


Figure 52. Expression of Cyr61 by CWR22R.v1 Cyr61 stable clones

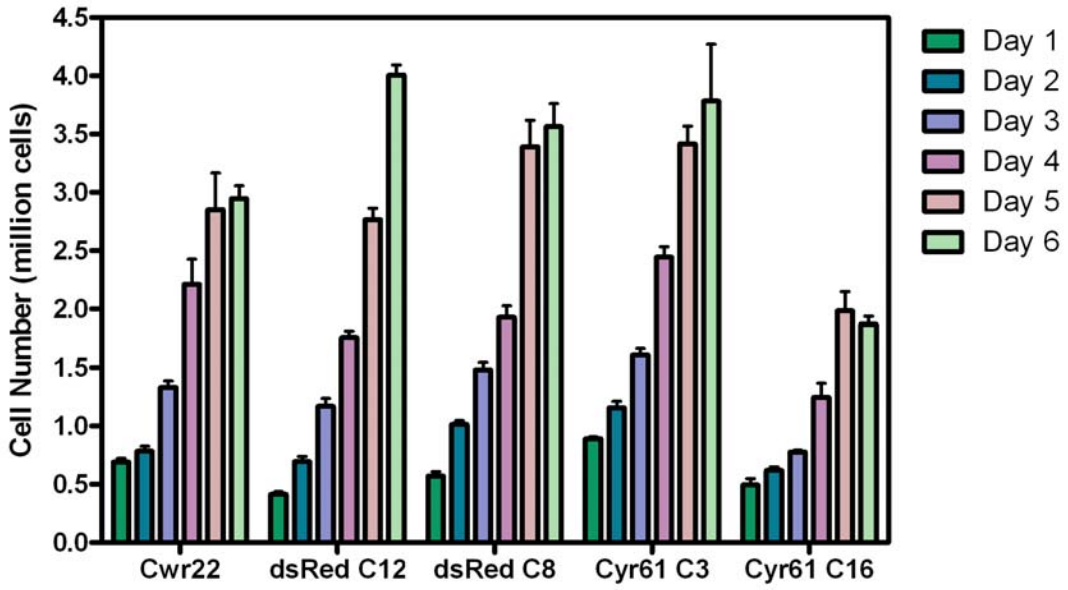


Figure 53. Growth curve of CWR22 - Cyr61 clones

In other organ systems Cyr61 induces cells to undergo proliferation at an increased rate (194,216,217,229,242-244). To evaluate whether Cyr61 induces a similar response in prostate, for 6 days cell counts were recorded for CWR22R.v1 parental cells and Cyr61 expressing clones. This experiment was repeaded more than five times and extreme variability confounded the recorded results. CWR22 Cyr61 3 and CWR22 Cyr61 16 differ in that proliferation rate of CWR22 Cyr61 3 cells does not significantly differ from parental and control transfected cells while the proliferation CWR22 Cyr61 16 is lower than parental or control transfected clones. Reporting the results for other CWR22 Cyr61 clones was a problem due to variability in counts between experiments but the trend for those clones was that Cyr61 expression decreased proliferation rate (similar to Cyr61 C16). CWR22R.v1 - Cyr61 do not appear to have a growth advantage over control transfected cells or CWR22R.v1 parental cells.

5.2.3.1 Microarray analysis of CWR22 – Cyr61 clones

In an effort to understand the impact of Cyr61 expression and signaling on prostate cancer cells, microarray analysis of CWR22 Cyr61 clones was performed. CWR22 Cyr61 3, 14, and 23 were evaluated on the Affymetrix Human Exon 1 array and compared against control transfected CWR22R.v1 cells. 48 genes were significantly up-regulated in CWR22 Cyr61 cells (Table 9) including HGF, FosB, Jun, INSR, and MMP16. 288 genes were significantly down-regulated in CWR22 Cyr61 cells (Table 10) including IGF1R, Arrestin, Map3K5, Furin, Nkx3.1, Tp53, GEMIN6, Sox9, and AnnexinA4.

Table 9. Genes up-regulated in CWR22-Cyr61

Unique Locus	UniqueGene Name	RefSeq	p-val	Lin(FC)	Log2(FC)
SLFN13	schlafen family member 13	NM_144682	0.002	43.538	5.444
HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	NM_000601	0.008	32.404	5.018
SEMA3D	sema domain, Ig domain, short basic domain, secreted, 3D	NM_152754	0.000	18.194	4.185
ATP1B2	ATPase, Na+/K+ transporting, beta 2 polypeptide	NM_001678	0.001	15.014	3.908
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	NM_004105	0.001	14.332	3.841
SLC7A5	solute carrier family 7 member 5	NM_003486	0.031	13.845	3.791
ASB9	ankyrin repeat and SOCS box-containing 9	NM_024087	0.002	13.250	3.728
PRLR	prolactin receptor	NM_000949	0.002	12.406	3.633
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	NM_006732	0.070	11.706	3.549
PTENP1	phosphatase and tensin homolog pseudogene 1	NR_023917	0.005	11.496	3.523
SYT11	synaptotagmin XI	NM_152280	0.015	9.440	3.239
CBWD3	COBW domain containing 3	NM_201453	0.016	8.573	3.100
FLJ42957	FLJ42957 protein	BC122872	0.024	8.402	3.071
PNMA2	paraneoplastic antigen MA2	NM_007257	0.039	8.228	3.041
INSR	insulin receptor	NM_000208	0.001	7.894	2.981
ASB11	ankyrin repeat and SOCS box-containing 11	NM_080873	0.001	7.693	2.944
C8orf57	chromosome 8 open reading frame 57	AL136588	0.010	7.162	2.840
NID1	nidogen 1	NM_002508	0.027	7.050	2.818
SLIT2	slit homolog 2 (Drosophila)	NM_004787	0.005	6.917	2.790
AQP3	aquaporin 3 (Gill blood group)	NM_004925	0.045	6.736	2.752
SLFN11	schlafen family member 11	NM_001104587	0.023	6.693	2.743
LIN7A	lin-7 homolog A (C. elegans)	NM_004664	0.002	6.571	2.716
FREM2	FRAS1 related extracellular matrix protein 2	NM_207361	0.043	6.508	2.702
ALDH1A1	aldehyde dehydrogenase 1 family, member A1	NM_000689	0.048	6.179	2.627
SMPDL3A	sphingomyelin phosphodiesterase, acid-like 3A	NM_006714	0.006	6.144	2.619
PLA1A	phospholipase A1 member A	NM_015900	0.018	6.108	2.611
EID3	EP300 interacting inhibitor of differentiation 3	NM_001008394	0.002	5.709	2.513
ANKRD30A	ankyrin repeat domain 30A	NM_052997	0.033	5.692	2.509
SLC3A2	solute carrier family 3 member 2	NM_001012661	0.023	5.472	2.452
IFI30	interferon, gamma-inducible protein 30	NM_006332	0.010	5.410	2.436
KIF16B	kinesin family member 16B	NM_024704	0.008	5.295	2.405
CDON	Cdon homolog (mouse)	NM_016952	0.027	5.273	2.399
TTLL6	tubulin tyrosine ligase-like family, member 6	NM_173623	0.013	5.193	2.376
MMP16	matrix metallopeptidase 16 (membrane-inserted)	NM_005941	0.002	5.130	2.359
RCN3	reticulocalbin 3, EF-hand calcium binding domain	NM_020650	0.000	5.083	2.346
CA12	carbonic anhydrase XII	NM_001218	0.008	4.987	2.318
LAMB1	laminin, beta 1	NM_002291	0.035	4.960	2.310
HLA-DMB	major histocompatibility complex, class II, DM beta	NM_002118	0.015	4.946	2.306
GPRC5B	G protein-coupled receptor, family C, group 5, member B	NM_016235	0.004	4.648	2.216
FAM130A2	family with sequence similarity 130, member A2	NM_024969	0.047	4.618	2.207
ULBP1	UL16 binding protein 1	NM_025218	0.024	4.544	2.184
WDR79	WD repeat domain 79	NM_018081	0.002	4.505	2.171
LPHN3	latrophilin 3	NM_015236	0.011	4.468	2.159
IFITM1	interferon induced transmembrane protein 1 (9-27)	NM_003641	0.002	4.375	2.129
AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	NM_032717	0.002	4.311	2.108
FN1	fibronectin 1	NM_212482	0.007	4.303	2.105
JUN	jun oncogene	NM_002228	0.052	4.163	2.058
MME	membrane metallo-endopeptidase	NM_007288	0.031	4.154	2.055
DUSP6	dual specificity phosphatase 6	NM_001946	0.048	4.142	2.050
SQLE	squalene epoxidase	NM_003129	0.002	4.140	2.050
APLP1	amyloid beta (A4) precursor-like protein 1	NM_001024807	0.005	4.073	2.026

Table 10. Genes down-regulated in CWR22-Cyr61

Unique Locus	UniqueGene Name	RefSeq	p-val	Lin(FC)	Log2(FC)
F3	coagulation factor III (thromboplastin, tissue factor)	NM_001993	0.001	-33.996	-5.087
AQP4	aquaporin 4	NM_001650	0.003	-26.573	-4.732
ARHGAP29	Rho GTPase activating protein 29	NM_004815	0.001	-23.484	-4.554
RAB25	RAB25, member RAS oncogene family	NM_020387	0.029	-15.362	-3.941
INHBA	inhibin, beta A	NM_002192	0.011	-10.716	-3.422
CPNE4	copine IV	NM_130808	0.023	-10.396	-3.378
ZNF592	zinc finger protein 592	NM_014630	0.000	-7.606	-2.927
PSD3	pleckstrin and Sec7 domain containing 3	NM_015310	0.000	-7.150	-2.838
ME1	malic enzyme 1, NADP(+)-dependent, cytosolic	NM_002395	0.000	-7.127	-2.833
TNFSF15	tumor necrosis factor (ligand) superfamily, member 15	NM_005118	0.041	-7.051	-2.818
RASEF	RAS and EF-hand domain containing	NM_152573	0.006	-6.520	-2.705
ACPP	acid phosphatase, prostate	NM_001099	0.008	-6.393	-2.676
PMEPA1	prostate transmembrane protein, androgen induced 1	NM_020182	0.000	-6.211	-2.635
IGF1R	insulin-like growth factor 1 receptor	NM_000875	0.001	-5.591	-2.483
IMP3	IMP3, U3 small nucleolar ribonucleoprotein, homolog	NM_018285	0.022	-5.485	-2.456
TOM1L2	target of myb1-like 2 (chicken)	NM_0010335	0.026	-4.842	-2.275
PPP1R3B	protein phosphatase 1, regulatory (inhibitor) subunit 3B	NM_024607	0.003	-4.772	-2.255
TRERF1	transcriptional regulating factor 1	ENST000003	0.006	-4.731	-2.242
AK5	adenylate kinase 5	NM_174858	0.028	-4.567	-2.191
ARRB1	arrestin, beta 1	NM_004041	0.002	-4.557	-2.188
HIST1H4L	histone cluster 1, H4l	NM_003546	0.002	-4.514	-2.174
HIST1H4H	histone cluster 1, H4h	NM_003543	0.004	-4.453	-2.155
B3GALNT1	beta-1,3-N-acetylgalactosaminyltransferase 1	NM_0010386	0.008	-4.443	-2.152
HIST1H2BK	histone cluster 1, H2bk	NM_080593	0.024	-4.347	-2.120
SKIL	SKI-like oncogene	NM_005414	0.003	-4.339	-2.117
PRSS21	protease, serine, 21 (testisin)	NM_006799	0.008	-4.334	-2.116
ZFAND6	zinc finger, AN1-type domain 6	NM_019006	0.001	-4.310	-2.108
EGLN3	egl nine homolog 3 (C. elegans)	NM_022073	0.002	-4.302	-2.105
PTPRM	protein tyrosine phosphatase, receptor type, M	NM_0011052	0.014	-4.300	-2.104
LCE1D	late cornified envelope 1D	NM_178352	0.044	-4.249	-2.087
MAP3K5	mitogen-activated protein kinase kinase kinase 5	NM_005923	0.020	-4.039	-2.014
RORC	RAR-related orphan receptor C	NM_005060	0.008	-3.919	-1.970
SPATA13	spermatogenesis associated 13	NM_153023	0.001	-3.793	-1.923
ST20	suppressor of tumorigenicity 20	NM_0011008	0.012	-3.785	-1.920
PARP8	poly (ADP-ribose) polymerase family, member 8	NM_024615	0.008	-3.778	-1.918
LINGO4	leucine rich repeat and Ig domain containing 4	NM_0010044	0.004	-3.702	-1.888
PSD3	pleckstrin and Sec7 domain containing 3	NM_015310	0.000	-3.658	-1.871
P2RX7	purinergic receptor P2X, ligand-gated ion channel, 7	NM_002562	0.009	-3.596	-1.846
PTPN9	protein tyrosine phosphatase, non-receptor type 9	NM_002833	0.000	-3.584	-1.842
FURIN	furin (paired basic amino acid cleaving enzyme)	NM_002569	0.006	-3.556	-1.830
GADD45G	growth arrest and DNA-damage-inducible, gamma	NM_006705	0.009	-3.514	-1.813
HOMER2	homer homolog 2 (Drosophila)	NM_199330	0.001	-3.512	-1.812
SPATA18	spermatogenesis associated 18 homolog (rat)	NM_145263	0.005	-3.451	-1.787
SMPD3	sphingomyelin phosphodiesterase 3, neutral membrane	NM_018667	0.000	-3.396	-1.764
HOPX	HOP homeobox	NM_032495	0.000	-3.373	-1.754
C1orf59	chromosome 1 open reading frame 59	NM_144584	0.004	-3.337	-1.739
SOHLH2	spermatogenesis and oogenesis specific basic helix-loop-helix 2	NM_017826	0.040	-3.253	-1.702
SERPINB6	serpin peptidase inhibitor, clade B (ovalbumin), member 6	NM_004568	0.006	-3.231	-1.692
PHLDA3	pleckstrin homology-like domain, family A, member 3	NM_012396	0.001	-3.197	-1.677
TP53	tumor protein p53	NM_000546	0.021	-3.180	-1.669
NR3C1	nuclear receptor subfamily 3, group C, member 1	NM_000176	0.014	-3.069	-1.618
NKX3-1	NK3 homeobox 1	NM_006167	0.011	-3.021	-1.595
SEC11A	SEC11 homolog A (S. cerevisiae)	NM_014300	0.000	-2.978	-1.574
AP3S2	adaptor-related protein complex 3, sigma 2 subunit	NR_023361	0.002	-2.965	-1.568
LOC124220	similar to common salivary protein 1	NM_145252	0.022	-2.948	-1.560
SLC44A3	solute carrier family 44, member 3	NM_0011141	0.003	-2.941	-1.556
RPL4	ribosomal protein L4	NM_000968	0.022	-2.900	-1.536
C18orf1	chromosome 18 open reading frame 1	NM_181481	0.022	-2.877	-1.525
MID1IP1	MID1 interacting protein 1 (gastrulation specific G12 homolog)	NM_021242	0.000	-2.841	-1.506
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_078467	0.017	-2.815	-1.493
TTLL13	tubulin tyrosine ligase-like family, member 13	NM_0010299	0.001	-2.809	-1.490
PXN	paxillin	NM_0010808	0.002	-2.796	-1.484
DNAJC12	DnaJ (Hsp40) homolog, subfamily C, member 12	NM_021800	0.016	-2.793	-1.482
VPS24	vacuolar protein sorting 24 homolog	NM_016079	0.004	-2.775	-1.473
ITGB5	integrin, beta 5	NM_002213	0.040	-2.750	-1.459
MRPL46	mitochondrial ribosomal protein L46	NM_022163	0.006	-2.703	-1.435
ERRFI1	ERBB receptor feedback inhibitor 1	NM_018948	0.027	-2.684	-1.424

Unique Locus	UniqueGene Name	RefSeq	p-val	Lin(FC)	Log2(FC)
BLOC1S2	biogenesis of lysosomal organelles complex-1, subunit 2	NM_0010013	0.007	-2.671	-1.417
CAP2	CAP, adenylate cyclase-associated protein, 2 (yeast)	NM_006366	0.034	-2.663	-1.413
C8orf4	chromosome 8 open reading frame 4	NM_020130	0.022	-2.653	-1.408
FAH	fumarylacetoacetate hydrolase (fumarylacetoacetase)	NM_000137	0.001	-2.649	-1.405
DSTN	destrin (actin depolymerizing factor)	NM_0010115	0.003	-2.647	-1.404
ARF6	ADP-ribosylation factor 6	NM_001663	0.036	-2.643	-1.402
TTRAP	TRAF and TNF receptor associated protein	NM_016614	0.011	-2.623	-1.391
SYK	spleen tyrosine kinase	NM_003177	0.011	-2.622	-1.391
UNC45A	unc-45 homolog A (C. elegans)	NM_0010396	0.000	-2.590	-1.373
SLC5A8	solute carrier family 5 (iodide transporter), member 8	NM_145913	0.004	-2.584	-1.369
CIB1	calcium and integrin binding 1 (calmyrin)	NM_006384	0.002	-2.581	-1.368
GEMIN6	gem (nuclear organelle) associated protein 6	NM_024775	0.010	-2.579	-1.367
MTHFS	5,10-methenyltetrahydrofolate synthetase	NM_006441	0.001	-2.576	-1.365
HSBP1	heat shock factor binding protein 1	NM_001537	0.006	-2.570	-1.362
GDF15	growth differentiation factor 15	NM_004864	0.027	-2.554	-1.353
WWC1	WW and C2 domain containing 1	NM_015238	0.002	-2.549	-1.350
C3orf34	chromosome 3 open reading frame 34	NM_032898	0.001	-2.530	-1.339
SOX9	SRY (sex determining region Y)-box 9	NM_000346	0.013	-2.529	-1.339
CAPN2	calpain 2, (m/II) large subunit	NM_001748	0.004	-2.524	-1.336
ANAPC13	anaphase promoting complex subunit 13	NM_015391	0.010	-2.516	-1.331
EEF2	eukaryotic translation elongation factor 2	NM_001961	0.005	-2.507	-1.326
ACTA2	actin, alpha 2, smooth muscle, aorta	NM_001613	0.011	-2.500	-1.322
THG1L	tRNA-histidine guanylyltransferase 1-like	NM_017872	0.010	-2.498	-1.321
C8orf45	chromosome 8 open reading frame 45	AK302672	0.003	-2.488	-1.315
C22orf27	chromosome 22 open reading frame 27	BC042980	0.049	-2.480	-1.310
ANXA4	annexin A4	NM_001153	0.033	-2.476	-1.308
UFC1	ubiquitin-fold modifier conjugating enzyme 1	NM_016406	0.033	-2.463	-1.301
PSD4	pleckstrin and Sec7 domain containing 4	NM_012455	0.003	-2.457	-1.297
VPS33B	vacuolar protein sorting 33 homolog B (yeast)	NM_018668	0.002	-2.455	-1.296
FABP6	fatty acid binding protein 6, ileal	NM_0010404	0.046	-2.454	-1.295
RAB39B	RAB39B, member RAS oncogene family	NM_171998	0.000	-2.446	-1.290
C12orf31	chromosome 12 open reading frame 31	BC107780	0.034	-2.418	-1.274
GPR110	G protein-coupled receptor 110	NM_153840	0.011	-2.407	-1.267
ZSCAN20	zinc finger and SCAN domain containing 20	NM_145238	0.047	-2.406	-1.267
LAD1	ladinin 1	NM_005558	0.002	-2.395	-1.260
NAP1L2	nucleosome assembly protein 1-like 2	NM_021963	0.001	-2.395	-1.260
TNKS1BP1	tankyrase 1 binding protein 1, 182kDa	NM_033396	0.003	-2.377	-1.249
DOK4	docking protein 4	NM_018110	0.013	-2.377	-1.249
FEZ1	fasciculation and elongation protein zeta 1	NM_005103	0.038	-2.375	-1.248
TDRD9	tudor domain containing 9	NM_153046	0.027	-2.362	-1.240
TMEM186	transmembrane protein 186	NM_015421	0.020	-2.361	-1.239
WDR61	WD repeat domain 61	NM_025234	0.003	-2.357	-1.237
ACSL1	acyl-CoA synthetase long-chain family member 1	NM_001995	0.027	-2.354	-1.235
FBXO22	F-box protein 22	NM_147188	0.000	-2.350	-1.232
CCDC121	coiled-coil domain containing 121	NM_024584	0.026	-2.347	-1.231
FAM50B	family with sequence similarity 50, member B	NM_012135	0.017	-2.340	-1.226
HCCS	holocytochrome c synthase (cytochrome c heme-lyase)	NM_005333	0.024	-2.339	-1.226
AP1M2	adaptor-related protein complex 1, mu 2 subunit	NM_005498	0.003	-2.338	-1.225
LSM10	LSM10, U7 small nuclear RNA associated	NM_032881	0.039	-2.336	-1.224
HDGFRP3	hepatoma-derived growth factor, related protein 3	NM_016073	0.004	-2.336	-1.224
CDK2AP1	CDK2-associated protein 1	NM_004642	0.015	-2.335	-1.223
FREQ	frequenin homolog (Drosophila)	NM_014286	0.010	-2.326	-1.218
PFDN1	prefoldin subunit 1	NM_002622	0.017	-2.323	-1.216
RAB3B	RAB3B, member RAS oncogene family	NM_002867	0.020	-2.321	-1.215
MAP2K2	mitogen-activated protein kinase kinase 2	NM_030662	0.004	-2.316	-1.212
BTBD1	BTB (POZ) domain containing 1	NM_025238	0.002	-2.311	-1.209
CABP4	calcium binding protein 4	NM_145200	0.000	-2.310	-1.208
DAPK2	death-associated protein kinase 2	NM_014326	0.003	-2.310	-1.208
DND1	dead end homolog 1 (zebrafish)	NM_194249	0.029	-2.309	-1.207
HYDIN	hydrocephalus inducing homolog (mouse)	NM_032821	0.004	-2.300	-1.202
Homo sapiens cDNA clone MGC:9921 IMAGE:3872056, complete cds.		AK023856	0.001	-2.290	-1.195
SERTAD4	SERTA domain containing 4	NM_019605	0.002	-2.287	-1.194
TGIF2LY	TGFB-induced factor homeobox 2-like, Y-linked	NM_139214	0.001	-2.281	-1.190
DENND2D	DENN/MADD domain containing 2D	NM_024901	0.001	-2.279	-1.188
C2orf37	chromosome 2 open reading frame 37	NM_025000	0.016	-2.278	-1.188
SNUPN	snurportin 1	NM_005701	0.003	-2.275	-1.186
SEPW1	selenoprotein W, 1	NM_003009	0.001	-2.273	-1.185

5.2.3.2 Cyr61 expression in Progression - TMA analysis

Given that microarray analysis of CWR22 Cyr61 clones shows a significant increase in hepatacyte growth factor (HGF) mRNA expression, we sought to confirm this at the protein level. By immunoblot analysis, no increased expression of HGF was evident in CWR22 Cyr61 clones (Figure 54).

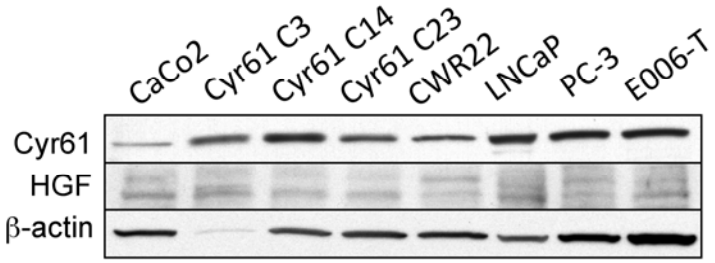


Figure 54. HGF expression in CWR22 - Cyr61 clones

5.2.3.3 Androgen receptor expression induced expression of Cyr61

E006AA is a hypertriploid cell line established from primary PCa cells from an African-American patient, which demonstrates androgen-sensitive growth in culture (245-247). The expression of Cyr61was evaluated in E006AA and E006AA-T cells along with the impact of elevated or decreased AR expression on Cyr61 protein expression. E006AA-T line is a tumorigenic varient of E006AA cells passaged through a NOG/SCID mouse. Cyr61 expression is elevated in E006AA-T cells compared to E006AA and expression was elevated slightly more in AR transfected E006AA-T cells but not significantly (Figure 55).

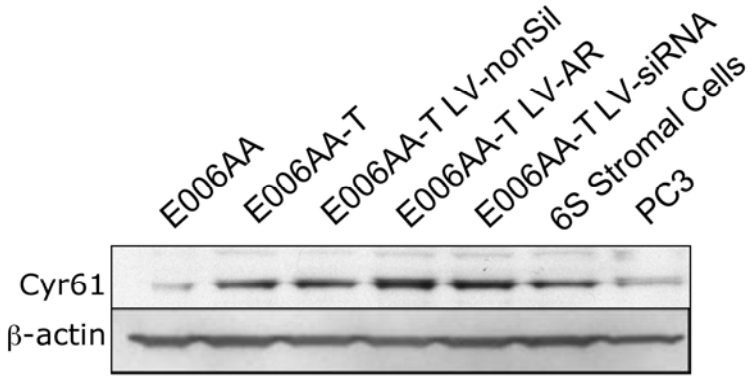


Figure 55. Cyr61 expression in E006AA-T prostate cancer cells

Cyr61 is up-regulated in the more tumorigenic version of this cell type and mildly elevated further in cells transfected to express active AR.

5.2.4 The Effect of Cyr61 on Proliferation

As discussed previously Cyr61 induces proliferation in other organ systems (194,216,217,229,242-244). To evaluate whether Cyr61 induces a similar response in prostate CWR22R.v1, LNCaP-C42B, PC3, and E006AA-T cells were grown in media containing 20ng/ml, 200ng/ml, or 2µg/ml recombinant Cyr61. For each cell line an increase in proliferation was seen for cells grown in Cyr61 containing media (Figure 56). More specifically, CWR22R.v1, LNCaP-C42B, and E006AA-T cell proliferation increased the most in 20 and 200 ng/ml Cyr61 but this effect on proliferation does not appear to be maintained at higher concentrations of Cyr61 as cell counts were somewhat lower for all four cell lines when grown in media containing 2µg/ml Cyr61. The effect of Cyr61 on proliferation of PC3 cells is variable and, while there is a modest increase in proliferation of cells grown in media containing 20 ng/ml Cyr61, this is sustained for cells grown in 200 ng/ml or 2µg/ml.

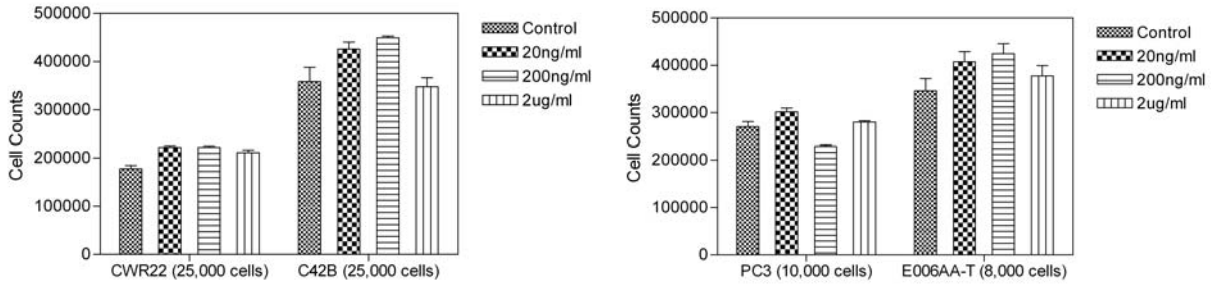


Figure 56. Prostate cancer cell proliferation in Cyr61 containing media

Cell counts after *(left)* 4 days for CWR22 and LNCaP C42B and *(right)* 3 days for PC3 and E006AA-T

5.2.5 The Effect of Cyr61 on Invasion

Migration, in many systems, is also impacted by Cyr61 expression (194,217,227-229,248-250). In matrigel invasion assays, the concentration of Cyr61 in the media does not appear to significantly or consistently impact cell migration (Figure 57). In order to address the fact that Cyr61 is generally a matrix associated protein, this assay was performed two ways, the first was to rehydrate matrigel in regular media and the second to rehydrate matrigel in Cyr61 containing media. This did not significantly impact migration (data not shown).

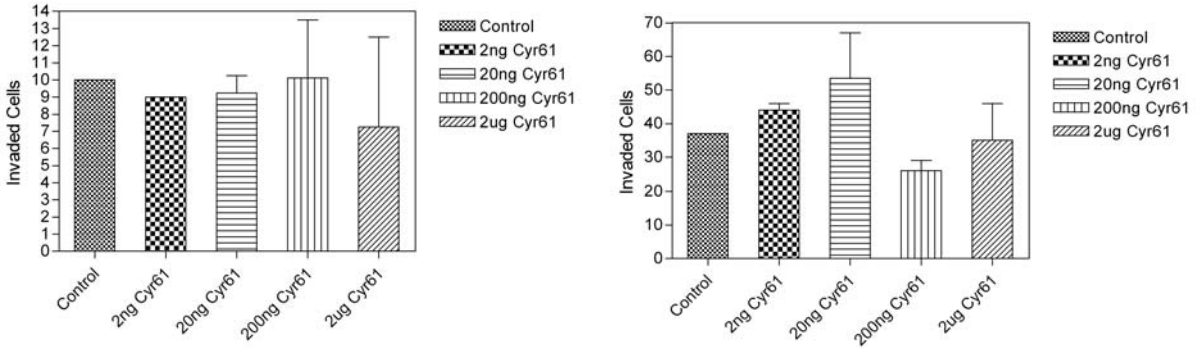


Figure 57. Cyr61 does not significantly impact migration of *(left)* CWR22R.v1 or *(right)* LNCaP-C42B cells

5.3 CONCLUSIONS

In this series of experiments it was shown that Cyr61 expression is up-regulated in prostate cancer compared to expression in normal prostate. Increased expression of Cyr61 transcript is consistently up-regulated in tumor by microarray and PCR analysis and these studies suggest that expression in normal adjacent tissues is higher than in areas of tumor. By IHC this is not the case for protein expression; Cyr61 protein is predominantly in regions of cancer while low expression is seen in some normal prostate or adjacent to cancer prostate epithelium. Additionally, in evaluation of the Oncomine database, multiple studies confirm that expression of Cyr61 is elevated in prostate cancer compared to normal prostate tissue but lower in metastatic prostate cancer compared to primary prostate cancer. This suggests that, in prostate cancer, Cyr61 is likely important to the development and/or maintenance of the disease, rather than metastasis or invasion.

The TMA results presented here suggest that Cyr61 may have clinical utility as a prostate cancer biomarker. Cyr61 expression is elevated in prostate cancer compared to adjacent-to-tumor and BPH tissue. Although this study was not specifically designed to address this point, using Gleason sum as a prognostic indicator, Cyr61 appears to differentiate the most aggressive prostate cancer cases from the less aggressive. To evaluate Cyr61 expression in prostate cancer progression, another set of TMAs were evaluated for Cyr61 expression. The TMAs were designed as a case-control study nested in a cohort of radical prostatectomy patients with incedence density sampling of matched controls in order to facilitate research of prognostic markers nad risk factors for progression following prostatectomy. The results of this study are currently under evaluation.

All prostate cell lines screened express Cyr61 protein. Expression was highest in LNCaP, PC3, and Du145 and lowest in VCaP, CWR22R.v1, and 267B1. Transient transfection of CWR22R.v1 cells resulted in a robust up-regulation of Cyr61 transcript and but knockdown with an shRNA vector against Cyr61 also resulted in robust up-regulation of Cyr61 transcript, thus, in subsequent experiments, siRNA was utilized and resulted in significant decreases in Cyr61 transcript and protein. Stable expression of an elevated amount of Cyr61 was achieved in CWR22Rv.1 cells and microarray analysis demonstrated the upregulation of 48 genes and down-regulation of 288 genes.

CWR22R.v1, LNCaP-C42B, PC3, E006AA-T cells proliferation increases moderately when cells are incubated in media containing 20 or 200ng/ml recombinant Cyr61. In a matrigel invason assay, no clear pattern was observed for these cells at 20ng/ml, 200ng/ml, or 2μg/ml. The increased rate of proliferation is supported by the findings of Lv et al. and Sun et al., which demonstrate that Cyr61 causes increased proliferation of prostate cells (217,243). However, these published studies also demonstrate increase in migration in responses to Cyr61 signaling which contradicts the data presented here. One possible reason for this difference could be the stability of the recombinant protein. Sun et al. produced recombinant Cyr61 in their own lab (217)and therefore had a ready stock of fresh recombinant protein. Cyr61 used for these studies was obtained from Peprotech Inc. as a lyophilized powder and this difference might have impacted the bioactivity of Cyr61 used in these studies.

In summary, up-regulation of Cyr61 is evident both the mRNA and protein level. Our study demonstrating elevated Cyr61 expression in prostate tissue and increased Cyr61-induced proliferation in prostate cells, in addition to recent publications regarding the role of Cyr61 in prostate cancer demonstrate that Cyr61 is an exciting new target which, with further analysis,

may result in clinically useful advances in prostate cancer. The TMA expression study in particular suggests a potential role for Cyr61 in the characterization of hard to diagnose prostate cancer cases, though extensive analysis of this marker is needed, and currently underway, to evaluate whether a correlation exists between Cyr61 expression and prognosis or disease progression.

6.0 DISCUSSION AND FUTURE DIRECTIONS

Two recent clinical trials published in the New England Journal of Medicine call into question the impact of PSA testing on prostate cancer related mortality and bring to light the absolute necessity in the field of prostate cancer research for improved biomarkers (123,124). In particular, prostate cancer treatment would be revolutionized by the discovery of a biomarker able to distinguish between lethal and indolent prostate cancer or a panel of biomarkers that signify a lethal genotype. There are two ways of thinking about the origin of lethal prostate cancer; 1) from the outset, prostate cancer falls into one of two categories: indolent disease or aggressive cancer that, given enough time, will ultimately be lethal and 2) all prostate cancer has the potential to be lethal given enough time. Some cancers transition to aggressive disease sooner than others and, if we could identify that point, treatment (i.e., surgery or radiation) could be postponed until that time delaying the need for procedures, which can have significant quality of life implications. These two hypotheses call for rather different biomarkers and screening. For the first hypothesis, early disease diagnosis remains essential and necessitates the identification of a novel biomarker that, upon diagnosis of prostate cancer, delineates between indolent and lethal disease. Men with lethal disease would receive immediate intervention while those with indolent cancer would enroll in a minimal program of watchful waiting. The second is the approach currently taken in active surveillance programs; the initial diagnosis of prostate cancer does not warrant intervention until the presence of disease progression is detected.

Currently clinicians depend primarily on PSA and DRE to determine when intervention is necessary; however, significant variability exists in how these are applied to determine when intervention should be initiated. A novel biomarker of progression would greatly enhance both sensitivity and specificity while at the same time providing a greater degree of uniform intervention. The search for biomarkers specific to the lethal phenotype remains complicated because, in proportion to the number of men diagnosed, few men have disease that progresses and the identification of disease progression occurs years after diagnosis necessitating extensive follow-up.

This work was undertaken to identify novel targets with clinical utility in disease diagnosis or in the evaluation of prognosis and to determine their role in prostate carcinogenesis. In the search for novel prostate cancer biomarkers, genomic analysis of donor prostate and prostate cancers was performed and the genes that appear most intriguing from our genomic analysis have been characterized more fully.

6.1 METALLOTIONEINS IN PROSTATE CANCER

Although the studies presented here do not demonstrate MT2A expression differences between prostate cancer and normal prostate tissue, a further exploration of other members of the MT protein family in prostate cancer would be a worthwhile undertaking and some evidence exists that MTs are differentially expressed in prostate cancer (29,151). The role of MTs in mediating enzymatic activity through zinc availability and the management of oxidative stress make MTs a point of susceptibility in cancer.

One hypothesis by which MTs might impact the development of prostate cancer involves selenium, an element thought to be protective against prostate cancer. Though still controversial, epidemiologic evidence suggests that men exposed to higher amounts of selenium develop prostate cancer less frequently and that their cancer may be less aggressive (70,75,251,252). Selenium containing peptides modulate the oxidoreduction reactions involved in the MT redox cycle enabling the reactions to take place in the reducing environment of the cytosol (148). The catalytic role of selenium derivatives in controlling the release and binding of zinc may be essential.

6.2 TACC2 IN THE PROSTATE

While Tacc2 does not appear to play a role in prostate cancer carcinogenesis, it is likely important to prostate biology given that Tacc2 expression is highly expressed the prostate. Thus, the prostate may be a good model system for further studying the role of Tacc2 in cells. Given that very little is know regarding the function of Tacc2, a logical step toward unraveling Tacc2 cell biology would be to over-express Tacc2 and use knockdown or siRNA to evaluate which pathways are impacted by altered Tacc2 expression. There is evidence in the literature that Tacc2 interacts with HAT (165). Additional interactions could be elucidated by doing co-immuno-precipitation (co-IP) with antibodies against Tacc2 followed by mass-spectrometry analysis to identify associated proteins or by chromatin immunoprecipitation (ChIP) to evaluate Tacc2/DNA binding sites.

6.3 NELL2 IN PROSTATE CANCER

Expression analysis of Nell2 in the small number of samples studied here was variable and analysis of a larger subset of prostate cancers is necessary to confidently characterize whether Nell2 expression changes are common in prostate cancer or are more indicative of BPH. In support of our hypothesis that Nell2 expression changes are common and important in prostate cancer, Luo et al. found that Nell2 is over-expressed in prostate cancer in a study that evaluated the therapeutic influence of finasteride on prostate cancer by microarray analysis of normal, BPH, and prostate cancer tissues (177). This supports the microarray and semi-quantitative results presented here which also show that Nell2 expression is higher in tumor than in healthy donor tissue. Interestingly, in analysis of urinary bladder cancer, Nell2 was identified in a panel of biomarkers able to identify bladder cancer patients from healthy controls by blood gene expression (253). This suggests that upregulation of Nell2 is not a prostate cancer specific phenomenon.

Another study published since the completion of the Nell2 expression analysis reports the existence of a cytosolic splice variant of Nell2 (cNell2) (254). Nell2 is localized to the golgi and endoplasmic reticulum intracellular before being secreted as a heavily glycosylated protein while cNell2 lacks the secretory signal sequence and is evenly distributed throughout the cytoplasm and does not get secreted. Hwang et al. focused on rat Nell2, not human (254), but, if humans express a similar variant, this may have complicated gene expression studies as the primers against Nell2 were not designed to specifically detect one variant versus the other.

6.4 FOSB IN PROSTATE CANCER

Results presented in this thesis demonstrate robust, consistent upregulation of FosB in prostate cancer and I believe this expression change to be one of the most findings of this line of experiments. Although complicated by the existence of ΔFosB and their sequence similarity, the differential expression of FosB in prostate cancer should be more thoroughly expolored in order to evaluate its biological significance. One mechanism by which increased FosB expression might contribute to prostate cancer carcinogenesis is through the altered composition of AP-1 transcription factor complexes thus altering AP-1 transcriptional activation in prostate cells. In 1998 Feng *et al*. demonstrate that c-Fos deficient mice are resistant to castration dependant regression of prostate luminal epithelial cells (255). After castration, cells remain intact and secretory activity is continues. When the composition of AP-1 transcription factor complexes were analysed, the AP-1 complexes of control mice consisted of c-Fos, FosB, Fra-2, and Jun-D while c-Fos deficient mice consisted of FosB and Fra-2 and JunD. If this study is valid than over-expression of FosB may competitively decrease the contribution of c-Fos to AP-1 complexes in prostate cancer cells and thus could be a novel mechanism of prostate cancer cell resistance to apoptosis. This could be assessed through apoptosis assays in untranfected and FosB transfected prostate cancer cell lines. Additionally, evaluation of FosB expression in normal prostate and prostate cancer tissue should be performed by IHC staining of a panel of prostate cancers.

Contradictory to Oncomine data illustrating that FosB expression in metastatic prostate cancer is lower than in primary prostate cancer, a biomedical literature search tool, Lecture Lab, looks for associative information in published data sets and found a strong association between

increased FosB expression and metastatic prostate cancer, a finding subsequently confirmed by immunoblot analysis (256).

PCP4 IN CANCER

6.4.1 ERG fusion products and PCP4

Recent studies demonstrate that gene fusions involving the ETS family of transcription factors affects the vast majority of prostate cancer (108). Prostate cancers with TMPRSS2-ERG fusion have a more aggressive phenotype and a distinct expression pattern (257-263). Interestingly, the PCP4 gene is encoded on chromosome 21 in the region lost in the formation of the ERG-TMPRSS2 fusion product. Given the frequency of this fusion occurs, about in 50 to 70% of prostate cancers (106,264), the decreased expression of PCP4 is understandable. This deletion may be inadvertent, a simple consequence of the formation of the ERG-TMPRSS2 product, or may impact the pathobiology of prostate cancer, e.g., a deletion that confers an advantage to the developing tumor. A better understanding of the role of the PCP4 protein in normal prostate and cancer is needed and could help illuminate whether PCP4 loss contributes to the distinct phenotype of TMPRSS2-ERG rearranged prostate cancers.

6.4.2 PCP4 in colon cancer

To my knowledge, the data presented here is the first assessment of PCP4 in colon cancer. We demonstrate that expression of PCP4, a protein involved in sequestering Ca^{2+}-free calmodulin

and regulating the rate with which calmodulin binds and releases Ca^{2+}, is elevated in colon adenoma and adenocarcinoma. This finding plays nicely into the emerging story regarding Ca^{2+}/calmodulin signaling in colon cancer. A number of studies in recent years highlight that some calmodulin-regulated proteins are overexpressed in colon cancer (265,266). Also, Shim *et al.* were able to inhibit colon cancer cell proliferation with a novel calcium (Ca^{2+})/calmodulin antagonist, (4-{3,5-bis-[2-(4-hydroxy-3-methoxy-phenyl)-vinyl]-4,5-dihydro-pyrazol-1-yl}-phenyl)-(4-methyl-piperazin-1-yl)-methanone (HBC), a derivative of curcumin (267,268). Taken together these studies suggest that alterations to the Ca^{2+}/calmodulin signaling pathway may be more common in colon cancer than previously appreciated. The altered expression of PCP4, noted in these studies, represents another aspect of Ca^{2+}/calmodulin signaling dysregulation in colon cancer.

To further evaluate the role of PCP4 in colon cancer, transient or stable knockdown of PCP4 expression in colon cancer cells (e.g., Caco2) followed by functional analysis particularly evaluating proliferation rate would be a logical next step. In order to more fully assess whether dysregulation of the Ca^{2+}/calmodulin signaling pathway is a common phenomenon in colon cancer, the gene expression of proteins involved in calmodulin signaling could be evaluated by microarray analysis of colon cancer tissues or by query of the Oncomine database. Also, functional studies of colon cancer cells grown in media with varying Ca^{2+} concentration might also be revealing. Although the expression results in this thesis are not extensive enough to determine whether up-regulated PCP4 expression in colon adenomas and adenocarcinomas might have diagnostic utility, a study by Johansson *et al*. that assessed neuropeptide expression, but not PCP4 specifically, in ulcerative colitis (UC), colon cancer, and healthy controls found

higher plasma levels of other neuropeptides in the plasma of individuals with colon cancer (269). It would be interesting to look into plasma levels of PCP4.

6.5 CYR61 IN PROSTATE CANCER

One mechanism proposed to explain the initiation of prostate cancer is that the balance established during organogenesis between the stroma and epithelia, reciprocally regulating growth and development of the prostate, becomes altered by or during cancer pathogenesis. Disruption of epithelial-stromal interactions most likely involves aberrant signaling between cell types and is an important component of prostate cancer. Current understanding of epithelial-stromal crosstalk remains limited and further study is needed to identify key proteins in this dynamic relationship. With this hypothesis in mind, the extracellular matrix associated protein, Cyr61, holds unique potential in that this protein gauges the extracellular environment, transmitting signals which alter many pathways involved in tumorigenesis including proliferation and migration.

6.5.1 Expression of Cyr61 as a biomarker in prostate cancer

While the majority of prostate cancers can be diagnosed based on histologic analysis of H&E stained needle biopsy tissue, a subset of diagnostically challenging cases require further characterization for accurate diagnosis. For these, IHC analysis of AMACR and basal cell markers, such as high molecular weight cytokeratins or p63, are used to help differentiate prostate cancer from benign disease. AMACR, also known as racemase, is characteristically up-

regulated in 80-100% of prostate cancer (137). This marker also stains as much as 21% of benign prostatic glands and stains up to 79% of partial atrophy lesions, which can potentially lead to misinterpretation of partial atrophy as adenocarcinoma (137). Though supplementing H&E staining with these IHC markers has clearly improved accuracy of prostate cancer diagnosis, additional prostate cancer specific marker able to compliment current markers differentially staining benign prostate glands that currently might be misdiagnosed or identifying prostate cancer that is not detected by currently methods. Unlike current prostate cancer IHC markers, an ideal marker would have prognostic value as well.

The study presented here demonstrates similarities between Cyr61 and AMACR including the elevated expression of the respective proteins in lesions of prostate cancer compared to histologically normal adjacent tissue or normal donor prostate tissue. Both Cyr61 and AMACR are primarily expressed in lesions of cancer and in some PIN cases. Although this question was not specifically addressed in this manuscript, based upon the resemblance in expression and staining patterns between Cyr61 and AMACR, we propose that immunohistochemical evaluation of Cyr61 expression has similar potential to aid in the diagnosis of problematic atypical cases. The uniform pattern of cytoplasmic Cyr61 staining in a given cancer focus, has a potential advantage over the variable intensity of AMACR staining in individual cancer glands within a given focus.

In a variety of tissues, Cyr61 has been shown to influence many signaling pathways involved in the development and regulating normal physiological functions. The biological mechanisms through which Cyr61 exerts its influence on the cellular environment remain elusive. This may in part be a consequence of context dependent signaling by Cyr61 resulting in the activation of different downstream pathways in response to varying stimuli or in differing

cells or tissues. Clearly further study is necessary to define the pathways both up- and downstream of Cyr61. Developing a better understanding of the role of this protein is even more vital given the number of studies documenting the altered expression of Cyr61 in diseases ranging from cardiovascular disease, to arthritis, to cancer (196,203,205,208,212,241,270-275). For example the up-regulation of Cyr61 in prostate cancer highlighted in this study brings into focus the need for further analysis of the role of Cyr61 in both prostate cancer and normal prostate tissue. The analysis of Cyr61 in prostate cell lines by Sun *et al*. found that Cyr61 can enhance the proliferation of prostate cells and suggests that Rac1 signaling may be a mechanism by which Cyr61 exerts its influence (217). Further analysis of the impact of Cyr61 over-expression and an evaluation of the pathways effected is necessary to define how Cyr61 contributes to prostate cancer development and progression.

Interestingly, the Cyr61 expression pattern in BPH tissues presented here does not agree with the previous literature that found elevated expression in BPH, particularly the stroma where we are unable to detect staining in any of our tissue samples, donor, BPH, or cancer. The majority of previous studies concerning BPH and Cyr61 in tissue involved analysis of mRNA (201,216,276) or in cell lines through stimulation by FBS (216,276). Thus the differences in the previously published data may be indicative of altered mRNA expression that does not translated to elevated protein expression. In dogs Oliveira *et al*. observed elevated Cyr61 protein expression in BPH but not prostatitis (277). Though species differences may account for this difference, the lack of consistency regarding Cyr61 expression in BPH warrants further analysis.

The findings presented here raise a number of questions that warrant further study. In spite of the benefits of studying Cyr61 expression in a series of consecutive RRP cases (providing a clean snapshot of the expression of Cyr61 in the average man who presents with

prostate cancer), this study provided limited prognostic information. Additional analysis of Cyr61 expression in a population with longer follow up and greater proportion of individuals whose disease progressed is necessary to assess the prognostic potential of Cyr61 and these studies are ongoing.

Cyr61 may also have utility as a blood-based biomarker in the evaluation of prostate cancer. Altered expression of Cyr61 is reported in a variety of types of cancer and, therefore, has limited utility as a diagnostic marker for prostate cancer or for any other type of cancer. However, in an individual previously diagnosed with prostate cancer, the amount of serum Cyr61 may be used to evaluate disease progression or to differentiate between aggressive and non-aggressive cancers. Additional studies are needed to demonstrate that Cyr61 can be detected in the serum as well as whether and how serum Cyr61 is altered as prostate cancer develops.

6.5.2 Effects of Cyr61 on CWR22 cells

In an effort to understand the impact of Cyr61 expression and signaling on prostate cancer cells, microarray analysis of CWR22 Cyr61 clones was performed. 48 genes were significantly up-regulated in CWR22 Cyr61 cells (Table 9) including HGF, FosB, MMP16, Sema3D, and SLC7A5. Many of these expression changes support or enhance the prostate cancer neoplastic phenotype. HGF is a protein normally expressed by prostate cells and secreted as a component of prostatic secretions (communication with W.B. Isaacs) that is over-expressed in prostate cancer and contribute to invasive potential (278,279). In addition, HGF has been proposed as a biomarker prostate cancer (280-282). It was quite interesting to see that FosB was up-regulated in the CWR22 – Cyr61 clones. As discussed previously, our expression analysis, and Oncomine data, demonstrate that FosB is up-regulated in prostate cancer but this, to my knowledge, is the

first evidence that the expression of FosB may be driven by Cyr61 signaling. Though there is no published literature regarding MMP16 in the normal prostate or prostate cancer, many studies illustrate the contribution of matrix metalloproteinases in prostate cancer (283-287). Semaphorin3D (Sema3D) belongs to a family of proteins bind neuropilin receptors that mediate adhesion and migration in malignant cells (288,289). Solute Carrier Family 7 (SLC7A5), also called LAT1, was recently identified as a potential biomarker of high-grade malignancy in prostate cancer (290).

288 genes were significantly down-regulated in CWR22 Cyr61 cells (Table 10) and some of the interesting genes affected include ARHGAP29, RAB25, TNFSF15, IGF1R, SKIL, and Tp53. Rho GTPase activating protein 29 (ARHGAP29), also known as PARG1, is involved in Rac1 activation with a proposed tumor suppressor role (291,292). RAB25, a member of the RAS oncogene family, is involved in integrin-induced migration (293). Tumor necrosis factor superfamily, member 15 (TNFSF15), commonly refered to as VEGI in the literature, is a regulator of angiogenesis and endothelial growth and promotes activation of caspases and apoptosis (294). In addition, reduced expression is associated with more aggressive cancers (295-297). Currently, targeting the insulin-like growth factor-1 receptor (IGF1R) signaling pathway is gaining momentum cancer theraputics due to the involvement of IGF1R in carcinogenesis (298). SKI-like oncogene (SKIL) is involved in cell division and differentiation in response to extracellular signals through negative regulation of TGF-β (299-302). TGF-β regulates prostate growth and acts as a tumor promoter in prostate cancer (303). The decreased expression of SKIL in CWR22 Cyr61 clones could have a tumor promoting effect given that SKIL tightly regulates TGF-β and this may a be a mechanims supporting the tumor promoting effects of TGF-β in prostate cancer. p53 is a tumor suppressor whose gene, Tp53, is the most

commonly mutated gene in prostate cancer which results in diminished tumor suppressor function (35). Also, expression of some prostate related genes were decreased including Nkx3.1, prostatic acid phosphatase, prostate transmembrane protein, and androgen induced 1. This suggests a possible de-differentiation from a prostate phenotype. PSA and KLK2 expression appeared downregulated as well but narrowly missed achieving statistical significance. The majority of the expression changes described above would suggest that the phenotype of these cells might be more tumorigenic. Suprisingly these alterations did not translate in functional analyses to yield result in more proliferative cells with higher invasive potential.

An interesting future study would be to evaluate the tumorigenic behavior of these Cyr61 over-expressing cells *in vivo*, compared to parental CWR22R.v1 parental and mock-transfected control cells. Cells would be re-suspended in various delivery vehicles, including Matrigel and collagen, to address the potental impact of Cyr61 on prostate cancer cell tumorigenicity. The study wuld likely involve sub-cutaneous injections of cells into male nude mice. Various endpoints would include time to tumor development, longitudinal analysis of tumor progrogression, and presence of metastases in distant organs. Additional *in vivo* studies would include the incorporation of chemotherapeutic agents such as docetaxel, cisplatin, and ADT via castration.

6.5.3 The role of Cyr61 in cellular response to mechanical stress

In a recent Science article, del Rio *et al.* published on the molecular mechanisms involved in relaying mechanical stimulus to the cell, the conversion of a mechanical stimulus into a chemical response (304). Specifically these studies show that the application of force on a single talin rod resulted in a shift in the protein structure of vinculin, a focal adhesion protein. This relates to the

Cyr61 story quite nicely given the recent publication by Lv *et al*., which showed that prostate cells with down-regulated expression of Cyr61 developed an abnormal accumulation of mature focal adhesions, and by Tamura *et al.* who found that Cyr61 is involved in transmitting mechanical stress signals from the extracellular environment to the cell (243,305). Vinculin has been studied for its role in cancer for almost three decades and recent publications link alterations in expression of vinculin with prostate cancer, particularly metastatic disease (306-315). Both Cyr61, an ECM protein, and vinculin, an intracellular membrane/cytoskeletal associated protein, interact with integrins, play an important role in focal adhesions, and influence migration and adhesion. To date, there is no literature that directly links these two proteins but it would be very interesting to evaluate whether the role of Cyr61 in cellular response to extracellular mechanical forces is mediated, via stimulation, by integrins, of vinculin and talin signaling.

6.6 CONCLUSIONS

The process of developing a greater understanding of the fundamental molecular mechanisms involved in prostate cancer will provide insights into the questions that still plague the field of prostate cancer research. In order to improve our understanding of the molecular alterations associated with prostate cancer, our laboratory characterized expression changes identified through microarray analysis comparing healthy normal prostate to prostate cancer tissue. The goal of this study was to identify altered genes that may have utility either as biomarkers, for improved diagnosis or with prognostic importance, or as novel targets important to the pathobiology of prostate cancer. Of the greater than 400 genes with significantly altered

expression, we selected MT2A, Tacc2, Nell2, FosB, PCP4, and Cyr61. Expression of PCP4 proved to have more potential as a biomarker in colon cancer than in prostate cancer given that expression in prostate is prodominently stromal and is expressed similarly in the stomal of normal prostate and prostate cancer tissue. We found Cyr61 and FosB expression changes to be the most likely to be functionally significant in the etiology of prostate cancer.

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