Regulation of the Physiological Consequences of Neuroendocrine Differentiation in Prostate Cancer by Kinase and Phosphatase Cross Talk

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies
(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2011

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Abstract

Prostate cancer is a leading health concern among Canadian males, with one in seven Canadian men developing the disease in their lifetime and one in 27 dying from it. Localized prostatic disease can be treated with surgery, but once metastasis occurs, clinicians rely on the hormone dependent nature of the tumor for treatment. Androgen withdrawal therapy is very effective at limiting tumor growth; however, progression of the tumor to an androgen independent state is inevitable, and at the present time there is no effective therapy for this form of the disease.

Neuroendocrine (NE) cells are post-mitotic, secretory cells found distributed throughout both normal and malignant prostate tissue. Increased NE cell content is associated with hormone refractory disease, and it is suspected that these NE cells play a role in the adaptation of surrounding cells to androgen withdrawal conditions through the secretion of paracrine factors. The research in this thesis was aimed towards understanding the biochemical mechanisms by which trans-differentiation of an adenocarcinoma cell to a NE cell occurs. By understanding the nature of this process, rationally designed therapeutic reagents can be developed that either block transdifferentiation of adenocarcinoma cells and inhibit the increase in NE cell content following androgen withdrawal or block the actions of NE cells that promote tumor progression.

I used the Kinetworks™ Phospho-Site Screen KPSS 1.1 as well as the Human Operon Version 3.0 microarray to broadly profile changes in protein kinase regulation and mRNA expression levels occurring during NE differentiation of the human prostate cancer cell line model, LNCaP. I found that agents that induce NE differentiation in
LNCaP cells cause a perturbation in the phospho-state of two downstream targets of the mammalian target of rapamycin (mTORC1), the ribosomal S6 kinase S6K1 and Rb, as well as increasing vascular endothelial growth factor (VEGF) mRNA expression. Both of these phenomena appear to involve the cAMP-dependent protein kinase PKA and a protein phosphatase PP2A family member. Since mTORC1 is considered to be a critical component in the control of tumourigenicity, and since increased VEGF is associated with advanced tumor progression, these findings appear to address some of the processes by which transdifferentiation of adenocarcinoma cells to NE cells may regulate prostate cancer progression.
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<th>Description</th>
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<tbody>
<tr>
<td>3D-CRT</td>
<td>3 dimensional conformal external beam radiotherapy</td>
</tr>
<tr>
<td>4EBP1</td>
<td>4E binding protein-1</td>
</tr>
<tr>
<td>5'TOP</td>
<td>5' transcriptional start site oligo-pyrimidine tract</td>
</tr>
<tr>
<td>AI</td>
<td>androgen independence</td>
</tr>
<tr>
<td>AIPC</td>
<td>androgen-independent prostate cancer</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ARE</td>
<td>androgen response element</td>
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<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
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<tr>
<td>BARK</td>
<td>beta adrenergic receptor kinase</td>
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<tr>
<td>bHLH</td>
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<td>chromogranin A</td>
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<td>castrate resistant</td>
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<td>castrate resistant prostate cancer</td>
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<td>Cqr</td>
<td>constitutively active mutant of the PKA catalytic subunit</td>
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<td>CREB</td>
<td>cAMP response element-binding protein</td>
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<td>eNOS</td>
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<td>FK506 binding protein</td>
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<td>FKBP12-rapamycin binding domain</td>
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<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
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<td>Description</td>
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<tr>
<td>GδL</td>
<td>G protein β-subunit-like protein</td>
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<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<td>growth hormone receptor</td>
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<td>gonadotropin-releasing hormone</td>
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<td>GSK</td>
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<td>HIF</td>
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<tr>
<td>HMEC</td>
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<td>HRE</td>
<td>hypoxic response element</td>
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<td>horseradish peroxidase</td>
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<td>kilodalton</td>
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<td>Kyoto Encyclopedia of Genes and Genomes</td>
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<td>LHRH</td>
<td>luteinizing hormone-releasing hormone</td>
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<tr>
<td>LY</td>
<td>LY-294002</td>
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<td>MAPK</td>
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<td>mammalian target of rapamycin complex</td>
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<tr>
<td>NE</td>
<td>neuroendocrine</td>
</tr>
<tr>
<td>NED</td>
<td>neuroendocrine differentiation</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NSE</td>
<td>neuron specific enolase</td>
</tr>
<tr>
<td>NT</td>
<td>no treatment</td>
</tr>
<tr>
<td>OA</td>
<td>okadaic acid</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PCa</td>
<td>prostate cancer</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDK</td>
<td>3-phosphoinositide-dependent protein kinase</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide-3 kinase</td>
</tr>
<tr>
<td>PIN</td>
<td>prostatic intra-epithelial neoplasia</td>
</tr>
<tr>
<td>PIP3</td>
<td>phosphatidylinositol (3,4,5)-trisphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PKR</td>
<td>protein kinase R</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2 A</td>
</tr>
<tr>
<td>PPP2R2A</td>
<td>PP2A regulatory subunit B, α isoform</td>
</tr>
<tr>
<td>PRAS40</td>
<td>proline-rich Akt substrate 40 kDa</td>
</tr>
<tr>
<td>PRR5L</td>
<td>proline rich protein 5-like protein</td>
</tr>
<tr>
<td>PSA</td>
<td>prostate specific antigen</td>
</tr>
<tr>
<td>PtdIns</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PTHrP</td>
<td>parathyroid hormone related peptide</td>
</tr>
<tr>
<td>pVHL</td>
<td>von-Hippel-Lindau tumor suppressor protein</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>quantitative real time PCR</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator for nuclear factor κ B ligand</td>
</tr>
<tr>
<td>Rap</td>
<td>rapamycin</td>
</tr>
<tr>
<td>Rb</td>
<td>retinoblastoma</td>
</tr>
<tr>
<td>Rheb</td>
<td>Ras homolog enriched in brain</td>
</tr>
<tr>
<td>RT</td>
<td>real time</td>
</tr>
<tr>
<td>S6 or rpS6</td>
<td>40S ribosomal protein S6</td>
</tr>
<tr>
<td>S6K</td>
<td>40S ribosomal subunit protein kinase</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SH2</td>
<td>src homology 2 domain</td>
</tr>
<tr>
<td>SHBG</td>
<td>sex hormone-binding globulin</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2-containing inositol phosphatase</td>
</tr>
<tr>
<td>SPARC</td>
<td>secreted protein, acidic and rich in cysteine</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activator of transcription</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline tween-20</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TNFRSF</td>
<td>tumor necrosis factor receptor superfamily</td>
</tr>
<tr>
<td>TNM</td>
<td>tumor, nodes, metastasis</td>
</tr>
<tr>
<td>TOS</td>
<td>TOR signalling</td>
</tr>
<tr>
<td>TP/A</td>
<td>transiently proliferating and amplifying</td>
</tr>
<tr>
<td>TSC</td>
<td>tuberous sclerosis complex</td>
</tr>
<tr>
<td>UHR RNA</td>
<td>universal human reference RNA</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>WCL</td>
<td>whole cell lysate</td>
</tr>
</tbody>
</table>
Acknowledgements

First and foremost I would like to acknowledge Dr. Michael Cox, a mentor and a friend, who has taught me more than I realize. Everything he does is done with integrity and kindness. Thank you.

I would like to thank all of those at the Prostate Centre who have assisted and befriended me over the years. I would especially like to thank Dr. Jodie Palmer, Clare Gardiner, Elaine Vickers and Jim Peacock for their assistance, advice and, most of all, their company.

I would like to thank Manuel Altamirano-Dimas for all of this work on the microarrays, Dr. Steven Pelech for providing the Kinetworks™ Phospho-Site Screen, Elaine Vickers for assistance with the VEGF ELISAs. I would like to thank my graduate committee, Dr. Vincent Duronio and Dr. Steven Pelech, for providing feedback. I would like to acknowledge project funding from the Canadian Prostate Cancer Research Initiative, The Terry Fox Foundation, and the National Cancer Institute of Canada.

Finally, I would like to thank Steve for his patience and encouragement during this long process.
1. Introduction

1.1. The Prostate

1.1.1. Location and Function of the Prostate

The prostate is a walnut sized fibromuscular exocrine gland surrounding the urethra between the bladder and the genitourinary membrane. It functions as an accessory sex gland that plays a critical role in the mammalian male reproductive system by contributing about 25-30% of the volume of seminal fluid. Semen is composed of spermatozoa and the seminal plasma that is made up of over a hundred protein and peptide components and provides nutrients and a protective environment for the sperm [1]. Secretions from the seminal vesicle comprise the majority of seminal plasma and provide amino acids and sugars necessary for the survival of the sperm as well as phosphorylcholine and prostaglandins that protect the sperm from the vaginal immune response. The bulbourethral gland contributes galactose and mucus that are responsible for the texture of semen, providing a favorable swimming environment for the sperm.

The prostate secretes an alkaline fluid that adds proteolytic enzymes, acid phosphatases, prostate specific antigen (PSA), citric acid, fibrinolysin and zinc to the seminal plasma. These additions help stabilize DNA within the spermatozoa and protect from denaturation in the acidic environment of the vaginal canal as well as liquefy semen to provide a favorable swimming environment. Ejaculatory ducts pass through the prostate and enter the prostatic urethra at the verumontanum [2]. The proteolytic solution from the prostate is secreted into the urethra during ejaculation.

The structure of the prostate is described either in terms of lobes or zones. The lobular classification, first described by Lowsley in 1912 [3], divides the prostate into five
lobes: anterior, median, posterior and two lateral lobes. A more contemporary zonal
description, first established in the 1960’s and made clinically popular in the 1980’s, takes a
histological approach dividing the prostate into the anterior fibromuscular stroma, the
periurethral glandular tissue, and the transition zone and central zones which together make
up the central gland, and the peripheral zone [2].

The prostate gland can be divided into glandular and non-glandular components. The
non-glandular components consist of the prostatic urethra and the anterior fibromuscular
stroma. The glandular component is comprised of the inner prostate (periurethral glands and
transition zone) and the outer prostate (central zone and peripheral zone) [2]. The prostate
primarily consists of secretory acini lined by epithelium. The peripheral and transition zones
are composed of small round acini, while the central zone which surrounds the ejaculatory
ducts is made up of large acini [4].

1.1.2. Development of the Prostate

Up until the 7th week of fetal development, the human male and female urogenital
systems are identical. At 10 weeks prostatic buds begin to develop from the urogenital sinus
and signal the beginning of prostate development. Testicular androgens act on androgen
receptors found in the urogenital sinus mesenchyme stimulating epithelial budding as well as
proliferation and differentiation of the prostatic bud to form ductal structures [5]. In response
to a variety of andromedins produced by the developing prostatic mesenchyme, the epithelial
cells differentiate into the various cell types of the prostate discussed below, while
proliferation and differentiation of the urogenital sinus mesenchyme forms prostatic smooth
muscle and interfascicular fibroblasts [5, 6].
1.1.3. **Cellular Makeup of the Prostate**

Prostate epithelium is composed of three types of cells: basal, secretory luminal and neuroendocrine (NE). The basal cells are undifferentiated cells with a high mitotic index that constitute the proliferative component of the prostate epithelium. The secretory luminal cells are differentiated cells that produce components of the seminal fluid: PSA and prostatic acid phosphatases [7]. The NE cells comprise a minor fraction of the prostate epithelium and are non-proliferative, terminally differentiated cells whose function and lineage is still in question. However, it is believed that they may regulate the growth and development of the prostate via secretory products.

The various cells of the prostate epithelium appear to have been derived from a common basal stem cell population [7]. This was determined by analyzing the subgroups of keratins present in each cell type at various stages of differentiation. The basal stem cells, through asymmetric cell division, replenish themselves while at the same time giving rise to a population of more committed intermediate cells termed TP/A (transiently proliferating and amplifying) [7]. It appears that these intermediate TP/A cells give rise to the luminal cells and the NE cells; however, the true origin of NE cells is still in debate and will be discussed later [7].

1.1.4. **Androgen and the Prostate**

Androgens are steroid hormones that bind to the androgen receptor (AR) resulting in the development and maintenance of primary and secondary sex characteristics in males. Testosterone is the principle androgen in males and is secreted by the testis under the control
of the hypothalamo-pituitary-gonadal axis. The majority of this secreted testosterone is bound by the plasma proteins Sex Hormone Binding Globulin and albumin, leaving only about 2% in the active unbound form. It is only this unbound form that can be taken up by target cells. Once in the target cell, testosterone is converted into its active intracellular form, dihydrotestosterone (DHT) by one of two isoforms of the enzyme 5α-reductase, only one of which is found in prostate [8].

Androgen-mediated gene transcription is initiated by the binding of DHT to the ligand binding domain of the AR. The AR is a member of the steroid receptor subgroup of the nuclear receptor superfamily. A single copy of the AR gene is located on the X chromosome at position q11-12 in humans [9]. It is a ligand dependent transcription factor that regulates a profile of genes involved in the survival, growth and differentiation of prostatic cells [10].

The binding of appropriate ligand, namely DHT, to AR induces a conformational change in the receptor resulting in disassociation from AR-interacting heat shock proteins. This disassociation allows receptor dimerization and translocation of the receptor from the cytoplasm to the nucleus. Once in the nucleus, the AR homodimer can bind two neighboring, asymmetric six base pair nucleotide sequences termed ARE1 and ARE2 (androgen response element) and, in conjunction with co-activator and co-repressor proteins, alter transcription of AR target genes such as PSA [11]. The AR DNA binding domain, containing two zinc finger motifs, is essential for both receptor dimerization and subsequent translocation to the nucleus as well as for the binding of AR to ARE within the promoters of AR regulated genes [12-14].
Many of the genes regulated by the AR are critical for the development and maintenance of the prostate. However, of the components of the prostatic epithelium, the secretory luminal cells are the only androgen responsive component [15]. The basal and NE cells are androgen independent, and, unlike the luminal cells, they do not express the AR [16].

1.1.5. Prostatic Diseases

The prostate is considered one of the most disease-prone organs of the body. The three primary prostatic diseases are prostatitis, benign prostatic hyperplasia (BPH) and prostate cancer (PCa). These are distinct diseases. Prostatitis is inflammation of the prostate and is a common occurrence in aging men which can become a chronic condition [17]. The most common symptoms are pelvic pain and urinary problems [18]. In a proportion of cases, prostatitis is caused by a bacterial infection; however, in the majority of cases the cause is unknown [19]. A history of prostatitis and sexually transmitted disease has been correlated with an increased risk of PCa, which implicates prostatic inflammation as a contributor to carcinogenesis [20, 21].

In older men, there is often an increase in the size of the prostate due to either prostatic stromal expansion or mixed expansion of stromal and epithelial components within the organ. This is termed BPH. This hyperplasia is androgen dependent and results in the formation of expanding nodules in the periurethral portion of the prostate. These nodules, over time, can compress the urethra causing urinary problems. The resulting partial or complete block of urinary flow can lead to increased urinary tract infections, bladder stones and bladder and kidney damage. While BPH is the most common human prostatic tissue
abnormality, this condition is not believed to be associated with the development of malignant PCa.

A third prostatic pathological condition, prostatic intra-epithelial neoplasia (PIN), is considered the precursor lesion that can progress to development of malignant PCa. PIN results from increased proliferation and anaplasia of surrounding prostatic ducts and acini in the peripheral zones of the prostate [4]. PIN is classified into low- and high-grade, with high-grade PIN being considered the earliest stage of carcinogenesis and possessing many of the morphologic and phenotypical traits of cancer [22]. During the progression from high-grade PIN to invasive carcinoma, there is an increased disruption of the basal cell layer of the prostatic ducts and acini as well as stromal invasion that results in invasive carcinoma development [23].

1.2. Prostate Cancer

1.2.1. Epidemiology

PCa is a leading health concern among Canadian males, with one in 7.4 Canadian men developing the disease in their lifetime and one in 27 dying from it [24]. It is the most common cancer detected in Canadian males with the third highest mortality rate following lung and colorectal cancer. It is projected that there will be 24,600 newly diagnosed cases and 4,300 deaths from PCa in Canadian men in 2010 [24]. Incidence rates for the disease vary geographically. Canada, along with parts of Western Europe, is considered to have an intermediate rate of PCa incidence [25]. The highest rates are found in the United States and the lowest in Asia and Northern Africa [26]. These geographical differences could be
attributed to genetic variations found more frequently in one part of the world than another compounded by epigenetics of environment such as lifestyle, diet and environmental exposures. However, it is important to also consider that these observed geographical trends could be due to differential use of screening and detection methods as well as reporting and documentation [27]. Of note, incidence rates of PCa have been rising more rapidly in Asia than in North America [28]. This could be due to an increase in screening and early detection in Asia or to increased pollution and the globalization of an American style diet. The prostate cancer incidence rate of immigrants tends to shift to that of their host country with the length of time in the host country influencing the extent of that shift. Once again, a change in diet, as well as possible changes in environmental exposure to risk factors, may play a role [27].

1.2.2. Risk Factors

There are many identified risk factors for PCa. One of the most widely known and undisputed risk factors is age. Prostate cancer is generally a disease associated with older men. It is most frequently diagnosed in the 60-70 age group and rarely shows up before 40 [24, 27]. In Canada, most deaths from PCa occur in the 80 plus age group [24]. This strong age correlation indicates that accumulated genetic damage, such as exposure to oxidative stresses and other environmental risk factors over the course of one's life, plays a significant role in the development of the disease [27].

A family history of PCa has also been considered a considerable risk factor [29, 30]. The risk increases with a greater number of relatives with PCa, especially first-degree relatives. Although it could be speculated that such a correlation could be due to more
aggressive screening of individuals with a family history of PCa, this correlation was seen before the wide spread use of modern screening methods such as PSA (described later) [27].

Another well established risk factor is race. Despite no difference seen in the incidence of latent PCa discovered in autopsies, a racial difference is seen in clinically detected PCa [27]. In the United States, African-America men are at the highest risk for dying from PCa [31]. This has been in part attributed to genetic predispositions such as polymorphic variations or differences in allelic frequencies of microsatellites in the AR. Another contributing factor may be dietary differences. However, other factors which might influence this are socio-economic differences that exist between African-American and white American men [32]. A key factor may include access to medical care including early detection of PCa as well as follow-up. To put these trends in perspective, African-American men and women have been shown to have a greater rate of incidence and death than white Americans in all cancers combined: 15% higher incidence and 48% higher death in men; and 9% lower incidence but 18% higher death in women [33, 34].

Several studies indicate that diet and weight may be important risk factors in PCa carcinogenesis, and that obesity can modulate PCa progression by affecting levels of insulin and associated IGF pathway as well as sex steroids [35, 36]. Ongoing work in this field is discovering the nuance of this complicated association. It appears that obesity increases risk of high grade PCa but decreases risk of low grade [37]. There appears to be an association between high serum IGF-1 levels and high-grade PIN and risk of PCa [38]. While the use of circulating IGF-I levels has yet to be developed as a reliable screen for PCa, emerging evidence indicates that combined levels of IGF-1 and certain IGF-binding proteins may prove useful for predicting the risk of developing advanced stage disease [39].
Many studies have looked at correlations between fat consumption and PCa incidence. There has been considerable data supporting a positive correlation; however, the details remain unclear. Some studies claim that this correlation is due to total fat consumption. Others claim that it is the consumption of a specific type of fat: polyunsaturated fats, animal fats, fats from dairy, fats specifically from hamburgers and meatballs. In contrast, others have found that certain fats have a negative correlation with PCa: saturated fats and fats from fish. A few studies have shown that the correlation seen between fats and PCa was negated once corrected for total energy intake [27].

Another proposed risk factor is the concentration of circulating levels of hormone and well as environmental exposure to hormones. It has been suggested that elevated levels of androgens and DHT over a lifetime may lead to an increased risk of PCa; however, data relating to this has been inconsistent and inconclusive. Similarly inconclusive data exists for other hormones [27]. This risk factor could tie in with the racial risk factor as it has been reported that young African-American males have higher levels of circulating testosterone as well as SHBP than young white Americans [40, 41]. In contrast, young Japanese men have a lower activity of 5α-reductase than either white or African-Americans [42].

There are many other potential risk factors that have been studied in context of PCa that, to date, have yielded inconclusive data. These include physical activity, human papilloma virus and levels of sexual activity during puberty. Another debated risk factor is environmental exposure to various chemicals and hormones. This includes endocrine disrupting chemicals found in some pesticides, such as vincozolin, and the production of plastics and rubber [27]. These risk factors, as well as others, can be used to determine how
closely a man should be screened for PCa and, if cancer is detected, how treatment should be approached.

1.2.3. Detection of Prostate Cancer

PCa usually begins as a single small focus, or several foci, of cancerous cells in the periphery of the prostate [43]. This stage of the disease is asymptomatic and remains dormant in most men. In some cases this latent disease becomes active and the tumor grows. Occasionally this can obstruct the urethra and interfere with urination; however, the tumor usually goes undiagnosed for some time. If undiagnosed, metastasis might occur: the tumor spreads to the lymph nodes and bones leading to a greatly diminished prognosis for the patient. Incidental discovery during prostate biopsies and autopsies have shown that the majority of aging men will develop microscopic foci of prostatic disease [43]. However, in only a small portion of these men will the slow-growing tumor take on the properties of an invasive PCa [25].

Initial detection of PCa is usually by digital rectal examination (DRE) and PSA screening. PSA screening measures the serum concentration of the glycoprotein produced by the prostate gland. This is a first round screening and is used to detect patients who may have undetected prostatic disease. Serum PSA concentration is usually low, under 4 ng/ml. Increased PSA is often indicative of prostatic disease or inflammation. PSA serum levels can vary quite a bit between healthy men and those with prostatic disease, so it is a combination of absolute serum concentration and the change in concentration over time that is diagnostically important. Patients with PSA measurements of over 4 ng/ml and a PSA increase of 0.75 ng/ml/yr are generally considered to be candidates for a prostate biopsy.
There is debate as to whether these numbers should be lower, to potentially detect prostatic disease at an earlier stage; or if they should be higher, to prevent over-diagnosis of clinically insignificant prostatic disease [44, 45].

Circulating PSA is present in two forms: free, unbound PSA or bound to serum protease inhibitors. Assessing the ratio of free to total PSA helps distinguish between BPH and PCa and can reduce the rate of unnecessary biopsies by about half [46] [47]. PSA density is another way in which PSA serum levels can be analyzed. PSA density refers to PSA serum levels in relation to the size of the prostate. PSA density has been shown to be predictive for determining the aggressiveness of clinically localized PCa [48].

Once detected the clinical stage of the disease is classified from needle biopsies by Gleason grade, TNM staging, as well as the patient serum level of PSA. Gleason grading describes the carcinoma in terms of gland differentiation and stromal invasion by scoring the two most prevalent histological components of the tumor section [49]. Scoring for each of these sections is from 1 to 5. These two scores are added together to get a Gleason grade from 2 to 10. Lower scores in the 2 to 4 range signify a well-differentiated tumor. Scores in the 5 to 7 range signify moderately differentiated tumors, while higher scores from 8 to 10 represent poorly differentiated tumors. Clinical prognosis worsens with increase in Gleason grade. TNM is a standardized staging protocol used for describing a broad array of cancer types. TNM staging is based on the size of the primary tumor, the degree to which it has spread to regional lymph nodes, and the presence or absence of metastasis [50].
1.2.4. Initial Treatment of Prostate Cancer

Low risk PCa is classified as a tumor that is confined within the prostate gland and usually involves only one lobe of the gland. It has a Gleason grade of 2 to 6 and a patient PSA serum level of 10 ng/ml or less. Treatment decisions for low risk PCa are primarily based on the patient’s life expectancy. Patients usually undergo “active surveillance.” This involves regular monitoring of the tumor by DRE and biopsies as well as monitoring the patients PSA levels. Since low-grade tumors progress slowly and patients generally have a 10 yr disease survival rate, treatment is often deferred until such time as the tumor shows signs of becoming more advanced. This is especially the case where a patient has a natural life expectancy of less than five years. Patients with a longer life expectancy may undergo radical prostatectomy or radiotherapy, by way of brachytherapy or 3-D conformal external beam radiotherapy (3D-CRT). The clinical aim with a low-grade tumor is to balance the maintenance of patient quality of life with the prevention of progression to a metastatic disease.

Intermediate risk PCa is still confined within the prostate gland, but may involve both lobes of the gland. It has a Gleason grade of 7 and a PSA of 10-20 ng/ml. Encapsulated, bilobed disease is usually treated with prostatectomy. High risk PCa extends through the prostate capsule and may invade the seminal vesicles. Despite being encapsulated the disease is still regionally confined. It has a Gleason grade of 8-10 and a PSA level of greater than 20 ng/ml. Patients with high risk PCa are generally treated with radiation or a combination of surgery and radiation. Non-localized disease is usually treated with androgen ablation therapy with or without 3D-CRT. Surgical and anti-androgen treatments can prove successful in those with localized cancer, and in approximately 75% of men it will reduce
disease symptoms [51]. However, almost all will experience a re-occurrence with the possibility of subsequent metastasis. Unlike the initial androgen sensitive disease, the relapsed disease is tolerant to castrate levels of androgens. This makes subsequent treatment difficult, since further hormone therapy is not effective. The median survival of men with androgen-independent metastatic disease is about 9 to 12 months [51], and at the present time there is no effective therapy for this form of the disease [52].

1.2.5. Androgen Ablation Therapy

In the 1940’s Huggins and Hodges demonstrated that prostate tumor cell growth is responsive to androgens [53, 54]. Since then, androgen ablation therapy has been the main therapeutic treatment for high risk and non-localized PCa. Depriving the tumor of androgens is achieved by one of several mechanisms. Testicular androgens can be eliminated by bilateral orchiectomy. However, the more common method is by “chemical castration.” This can be accomplished by inhibiting testicular androgen production by endocrine therapy targeting the pituitary-hypothalamic gonadotrophic signalling. LHRH (luteinizing hormone-releasing hormone) agonists, such as Lupron and Zoladex, down-regulate pituitary LHRH receptors and disrupt the pulsatile nature of LHRH stimulation on the pituitary making the pituitary refractory to hypothalamic regulation. Gonadotropin, and subsequent testosterone production, has an initial surge and then tapers off. Treatment with non-steroidal anti-androgens such as flutamide, bicalutamide, or cyproterone acetate along side LHRH agonists, augments androgen ablation by inhibiting the intracellular response to circulating androgens. Augmentation of androgen ablation is also possible through inhibiting the synthesis of androgen using the steroidogenesis inhibitor ketoconazole or inhibiting the conversion of
testosterone to its more bioactive form DHT with the use of 5α-reductase inhibitors, such as finasteride [55]

1.2.6. Castrate Resistant Progression of PCa

Treatment for metastatic PCa usually involves the withdrawal of androgen in some form. As the tumor is responsive to levels of androgen for survival and growth, androgen ablation results in tumor regression. However, over time, the selective pressures of androgen ablation inevitably lead to physiologic adaptations of the tumor and the acquisition of androgen independence.

Several mechanisms have been proposed for transformation from an androgen responsive to androgen independent (AI) tumor or, more accurately, castration-resistant PCa (CRPC). One such mechanism has been termed “the hypersensitivity pathway” whereby the tumor gains an increased sensitivity to very low levels of androgen. This can be brought about by an over-expression of the AR, as seen in approximately 30% of tumors; an increased sensitivity of AR; or by increased local androgen levels [56]. Local androgen levels can be boosted by an increase in the activity of 5α-reductase as well as by activation of steroid biosynthesis machinery in prostate cancer cells [8, 57]. While “the hypersensitivity pathway” is not technically complete AI as low levels of hormone are involved, this mechanism can enable tumor cells to evade death induced by androgen ablation therapy.

Another mechanism for progression to AI has been termed “the promiscuous pathway.” This involves mutations in the AR gene that reduce the ligand binding specificity of the receptor allowing it to respond to non-androgen steroids such as progestin, oestrogens, and others [56]. An example of this is the threonine to alanine mutation seen at position 877
of the AR gene in the PCa cell line LNCaP [58]. Along the same lines, over-expression of AR co-activators or a reduced expression of AR co-repressors can have a similar outcome [56].

A third mechanism is “the outlaw pathway” which describes the activation of AR, as well as other steroid hormone receptors, by ligand-independent mechanisms. Signalling through growth factor or receptor tyrosine kinase activated pathways, as well as the Akt pathway, can activate AR independent of androgen binding [56]. An increase in intracellular cAMP or signalling through the interleukin (IL) -6 pathway can also trigger AR signalling [58].

“The bypass pathway,” which describes alternate signalling pathways that can bypass AR signal to promote the growth and survival of the cell, is yet another mechanism by which a tumor can become AI [56]. Lastly, there is “the lurker cell pathway.” This theoretical mechanism is based on the idea that cells within the prostate tumor inherently vary in their level of dependence upon androgens. Upon androgen ablation therapy, androgen responsive cells are eliminated but AI cells, such as the epithelial stem cells, remain viable. Malignant transformation and clonal expansion of these AI epithelial cells provide a mechanism by which the tumour can overcome androgen withdrawal [56].

1.2.7. Current Therapeutic Approaches Addressing CRPC

To delay the onset of CRPC, intermittent androgen withdrawal therapy can be used. Successive rounds of androgen ablation are interrupted by periods of no treatment. The optimal length of time on or off of therapy is determined by careful monitoring of the patient’s PSA levels and plasma testosterone. The value of this approach is that the time off
of treatment allows the cells to retain some sensitivity to androgen and thus maintain a
degree of apoptotic response to subsequent periods of androgen withdrawal. An additional
advantage to intermittent treatment is that it can improve the patient’s quality of life by
allowing time off of treatment. Despite delaying the onset of AI, it does not do so
indefinitely. AI is still an inevitable outcome.

CRPC has proved therapeutically problematic, and at this time, it is not clear how to
best treat this form of the disease. However, some biological reagents, in combination with
cytotoxic drugs, have proved promising. These treatments generally fall into the following
broad categories: angiogenesis inhibitors, agents that stimulate apoptosis, matrix
metalloproteinase inhibitors, and signal transduction modulators that disrupt pathways
promoting growth [59]. In addition, complementary and alternative therapies can be
employed. In some cases treatments are used purely for their palliative benefits without the
hope of increasing survival. These palliative treatments include the radioisotopes strontium
and samarium as well as the chemotherapeutic agents mitoxantrone and prednisone [60].

A common treatment for men with advanced disease is a combination of an AR blocker
and the microtubule disruptor and mitotic inhibitor taxane. The median increase in life
expectance with this combination therapy is 6 weeks. This number reflects a dramatic
positive response in some patients while other patients having no marked increase in life
expectancy [61]. Like with most PCa treatments, we do not currently have a reliable way to
predict who will or will not respond to this treatment.
1.2.8. Over Treatment in Prostate Cancer

A significant problem with the current clinical approach to PCa is that of over treatment. Earlier diagnosis of PCa, enabled by the widespread use of PSA screening, improves the likelihood that the disease will be caught at the pre-metastatic stage and thus improves the potential for successful treatment. On the other hand, these advancements in early detection have led to another problem: over treatment. PCa is generally a slow growing cancer with a doubling time of approximately 3-4 years [27]. In most men these slow growing microscopic foci of prostatic disease will not progress to an invasive cancer in their lifetime.

Many factors are considered when determining the therapeutic approach for a patient. These include the risk factors discussed above: age, current health and life expectancy of the patient; as well as the stage and tumor volume of the disease detected. However, these are often not sufficient to determine the most appropriate treatment strategy. We currently do not have sufficient diagnostic tools to stratify patients into those who will develop invasive cancer in their expected lifetime and those who will not. Although much effort has been directed towards the discovery of biomarkers to differentiate between patients with an increased or a decreased likelihood of developing a fatal disease, there is currently no conclusive evidence that these are reliable.

The overall goal during treatment is to appropriately balance providing the most effective treatment to prevent progress of the disease with the maintenance of the patient’s quality of life during the process. Without appropriate biomarkers to assist with these decisions, clinicians as well as patients tend to err on the side of over treatment. Klotz and colleagues [62] argue that active surveillance, with selective delayed intervention when
needed, is the most appropriate therapeutic approach for patients with low risk PCa. They have shown that virtually all men with low risk PCa under active surveillance die of unrelated causes [62]. Despite this, many patients who would likely be better off with active surveillance, when given the choice, usually opt for a more aggressive treatment. It is estimated that only one out of every twenty men that undergoes treatment for PCa, actually benefits from it. The others unnecessarily sacrifice quality of life. With appropriate and reliable biomarkers, and a way to classify PCa into different subclasses, smarter treatment decisions could be made. Lapointe et al. [63] point out that PCa develops from a limited number of alternative preferred genetic pathways. Further understanding the genetic mechanisms behind these abhorrent pathways may lead to reliable categorization for PCa. Recent developments along these lines look promising.

1.2.9. Genetic Mechanisms and Genetic Markers for Prostate Cancer

The “multi hit hypothesis” states that development of PCa, as with other cancers, involves an accumulation of genetic changes [64]. These genetic changes allow a cell to acquire the six hallmarks of cancer described by Weinburg and Hanahan [65]: self-sufficiency in growth signals; insensitivity to growth inhibitory signal; evasion of apoptosis; limitless replicative potential; sustained angiogenesis; and tissue invasion and metastasis. Since the number of basal gene mutations found in human cells is too low to account for the frequency of multi-hit mutations seen in many tumors, it is suspected that many of the initial mutations that predispose to cancer actually increase the genetic instability of a cell [64]. Appreciation of these recurrent genetic adaptations has led to evaluation of the genetic “fingerprints” of specific disease types.
Genetic aberrations and chromosomal rearrangements are used for the diagnosis and sub-classification of many types of tumors [66-68]. Recently there has been considerable excitement as tracking genetic translocations involving the androgen-responsive gene TMPRSS2 with members of the Ets family of transcription factors are emerging as a very promising approach to classifying prostate tumors [69]. Epidemiologic studies have shown that these translocations are widespread with approximately half of PCa samples showing some variation of a TMPRSS2:Ets translocation.

The ETS family of highly conserved oncogenic transcription factors includes 27 human family members that regulate the expression of target genes involved in an array of biological processes such as proliferation, apoptosis, angiogenesis, development, differentiation and transformation, [70]. Translocations of Ets family genes have been previously implicated in Ewing’s sarcomas as well as acute and chronic myeloid leukemia [71, 72]. The ETS family members ETV1 and ERG are receiving considerable attention in the PCa field with fusions of TMPRSS2 and ERG or ETV1 described in both hormone-dependent and independent PCa xenograft lines [73]. These TMPRSS2:Ets translocations correlate with poor PCa prognosis, and an increased risk is observed when the translocated locus is amplified [74, 75]. Presumably this is because tumors containing these translocations represent a subclass of PCa in which genomic rearrangements promote aberrant AR-driven expression of potential oncogenes. In addition, it has been proposed that these gene fusions may be involved in the progression of PCa from a hormone dependent to independent disease [69, 76]. There is emerging evidence that these TMPRSS2:Ets translocations may be useful for classification of patients who will likely develop clinically
aggressive PCa, but the molecular details of how these gene fusion events impact disease etiology remain to be resolved. [63, 73].

1.2.10. PTEN, a Canonical Mutation in Prostate Cancer

One long-appreciated recurrent molecular signature of advanced PCa is lost expression of the tumor suppressor, PTEN (phosphatase-tensin homologue). PTEN is found mutated in a broad range of human cancers. PTEN acts as a negative regulator of cell migration, cell survival and progression through the G1 phase of the cell cycle by opposing the PI3K/Akt signalling pathway which is described below [77]. In addition to effects on cell survival, proliferation and migration, PTEN loss is associated with an increased resistance to chemotherapy, and increased angiogenesis [78, 79]. In general, loss of PTEN in PCa correlates with poor prognosis [77].

Approximately half of patients who present with PCa are PTEN null. Amongst those patients who are not PTEN null, many exhibit loss of one PTEN allele [80]. There is a well-characterized correlation of increased loss of the PTEN genetic locus as the cancer progresses [81]. It has been demonstrated that PTEN is a haploinsufficient tumor suppressor and that PCa progression, latency and biology can be dictated in a dose dependent manner by the extent of PTEN inactivation [80]. This dose dependence appears to function at the level of both dose determined by allelic variations and dose determined by the concentration of PTEN compromised cells in a tumor [80].
1.3. Signalling Through the PI3K/Akt/mTOR Pathway

1.3.1. PI3K Generates the Second Messenger PIP3

PI3K (phosphoinositide 3-kinase) is a heterodimeric enzyme composed of a p110 catalytic domain and a p85 regulatory domain. The regulatory subunit contains two tyrosine binding SH2 domains. Binding of the regulatory subunit to phosphorylated tyrosine residues on the intracellular component of activated receptors results in a conformational change disabling autoinhibitory constrains on the catalytic domain [82]. Once inhibition is released, PI3K can phosphorylate the phosphatidylinositols PtdIns(4)P and PtdIns(4,5)P2 at the 3’ position of the inositol ring to generate the second messengers PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (PIP3). PIP3 recruits many critical cell signalling components, such as Akt and phosphoinositide dependent kinase (PDK) 1, to the cell membrane via Pleckstrin Homology (PH) domain binding [82].

1.3.2. Loss of PTEN Leads to Constitutive Signalling Through the PI3K/Akt Pathway

The PTEN tumor suppressor gene, mapped to 10q23, encodes a dual specificity phosphatase [83, 84]. PTEN is one of two lipid phosphatases, the other being SHIP2, that opposes the action of PI3K by dephosphorylating PI3K-generated PIP3 (Fig 1.1). PTEN dephosphorylates the D3 position of the inositol ring of PIP3 while SHIP2 dephosphorylates the D5 position. The result is reduced availability of the hyper-phosphorylated second messengers and reduced potential for membrane translocation of proteins containing PH binding domains. In the absence of PTEN, there is reduced negative regulation on PIP3
production and, therefore, an amplification of basal levels of upstream receptor activation. In agreement with PTEN’s role as a negative regulator of PI3K, loss of PTEN results in elevated levels of phosphorylated Akt and downstream S6K1 activity [85, 86].

Figure 1.1: PI3K and Opposing Phosphatases Regulate Levels of PIP3
Schematic representation of the opposing regulation of PtdIns phosphorylation by the kinase PI3K and the phosphatases PTEN and SHIP2.

1.3.3. Akt Signalling

Akt, also known as protein kinase B (PKB), is a family of protein-serine/threonine kinases composed of Akt1, Akt2 and Akt3. Akt is an oncogene associated with cell survival, proliferation and protein synthesis and is one of the most deregulated proteins in a wide array of cancers. The activity of Akt, like many other kinases, is dependent on both localization and activation. Akt contains a PH domain that binds the phosphoinositide PIP3 and is thereby recruited to the cell membrane. Localization at the cell membrane positions Akt to be phosphorylated by its activating kinases –PDK1 and PDK2. Akt is phosphorylated on
T308 in the activation loop by PDK1 facilitating a subsequent phosphorylation at S473 within the carboxy terminus hydrophobic motif by PDK2. The identity of PDK2 has stimulated much debate. PDK2 activity has been attributed to PDK1, MAPKAP kinase-2, ILK and autophosphorylation, amongst others [87-89]. More recent work by Sarbassov et al. [90] has identified the mTOR signalling complex mTORC2 (described below), which itself lies downstream of Akt, as the primary, and perhaps solitary, PDK2 [90].

1.3.4. mTOR Signalling

mTOR is a 290 kDa protein-serine/threonine kinase member of the PI-kinase-related family that is critical for cell growth and proliferation [91, 92]. The mTOR protein is the singular mammalian equivalent of the two S. cerevisiae proteins TOR1 and TOR2. mTOR acts as a central controller of cell growth and division by detecting and integrating information regarding cellular nutrient sufficiency, energy levels and mitogenic signalling and relaying these proliferative signals to critical players involved in protein translation initiation and entry into the cell cycle [93-96]. Two of these downstream players, the 40S ribosomal subunit protein kinase (S6K1) and the eukaryotic initiation factor 4E binding protein-1 (4EBP1) are critical for ribosomal biosynthesis and the production of proteins required for G1/S transition [92, 97].
1.3.5. Multi-protein Complexes Determine mTOR Signalling - mTORC1 and mTORC2

The activation of mTOR substrates is believed to be dependent on scaffolding proteins that facilitate mTOR-catalyzed phosphorylation [98]. In vivo, mTOR appears to exist in two complexes (Fig 1.2): the rapamycin-sensitive mTOR complex 1 (mTORC1) and the rapamycin-insensitive mTOR complex 2 (mTORC2) with the proportion of mTOR contained within mTORC1 and mTORC2 varying amongst cell types [99]. Various proteins, such as the mTOR complex stabilization protein mLST8 (mammalian lethal with sce-13; also known as GβL, G protein β-subunit-like protein), have been described in association with both mTORC1 and mTORC2; however, at this time the full complement of interacting proteins and their functional significance is still unknown. Currently, the main distinguishing compositional aspect of these complexes is that mTORC1 contains a subunit known as raptor (regulatory associated protein of mTOR) while mTORC2 contains a subunit known as rictor (rapamycin-insensitive companion of mTOR) [99]. mTOR, mLST8, raptor and rictor all contain repeating HEAT (huntington, elongation factor 3, A subunit of protein phosphatase 2A and TOR1) and WD-40 sequences which presumably mediate protein-protein interactions [100]. Recent studies have demonstrated distinct roles for raptor and rictor, indicating that the formation of these multi-protein mTORC1 and mTORC2 complexes provides tight regulation on the actions of mTOR.

mTORC1 is the primary controller of cell growth. The 150 kDa binding protein raptor forms a complex with mTOR as well as binding S6K1 and 4EBP1 through short highly conserved TOS (TOR signalling) motifs. These motifs, located at the N terminus of S6K1 and the C terminus of 4EBP1, act as mTOR/raptor docking sites and are critical for
mTOR dependent regulation of downstream proteins *in vivo* [98, 101]. Down-regulation of raptor inhibits mTOR activation of S6K1 and 4EBP1 resulting in decreased growth factor stimulated protein synthesis [74]. In contrast to raptor’s ability to facilitate the phosphorylation of mTOR’s downstream targets S6K1 and 4EBP1, binding of raptor has been shown to have the ability to suppress mTOR’s kinase activity and mTOR-dependent phosphorylation of Akt [102]. Two additional components of mTORC1 are mLST8 and PRAS40 (proline-rich Akt substrate 40kDa). mLST8 is bound to the kinase domain of mTOR and appears to stabilize its interaction with raptor as well as increase its kinase activity [103] while the exact role of PRAS40 is still unclear (and will be discussed later in this chapter).

mTORC2 is involved in activation of Akt, cytoskeletal organization via activation of PKCα as well as other rapamycin-insensitive effects of mTOR [90, 104]. mTORC2 is characterized by the association of mTOR and mLST8 with the p200 protein rictor, as well as mSin1 (also known as mitogen-activated protein kinase-associated protein 1) and the recently identified PRR5L[105]. Rictor and mSin1 appear to be unique to mTORC2 and are only seen in association with mTOR in the absence of raptor [106]. Sarbassov et al. [90, 99, 106] have demonstrated the ability, and possibly the necessity, for an mTOR-riotor-mSin1 complex in direct phosphorylation of Akt at S473 [90, 99, 106]. Down-regulation of rictor inhibits Akt phosphorylation at S473, while having no effect on the phosphorylation of the Akt substrate tuberin. In addition, down-regulation of rictor stimulated phosphorylation of S6K1 and 4EBP1 and a subsequent increase in protein synthesis in a growth factor independent manner [99, 107]. This indicates that rictor is required for mTOR-mediated phosphorylation of Akt and is inhibitory for mTOR-mediated activation of S6K1 and 4EBP1.
In summary, mTOR’s binding partners determine mTOR-mediated control of biological processes by focusing mTOR’s course of action either toward Akt or S6K1/4EBP1 [108].
Figure 1.2: Multi-protein mTOR Complexes mTORC1 and mTORC2
Schematic representation of the subunits comprising the mTORC1 and mTORC2 complexes as well as some of the signaling proteins that regulate or are regulated by these complexes.
1.3.6. **Akt Regulation of mTOR**

mTOR intriguingly lays both upstream and downstream of Akt. In recent years, considerable advances have been made in understanding the mechanism by which Akt signals to mTOR. Historically it was believed that Akt directly phosphorylated mTOR at a COOH-terminal site S2448 [109, 110]. Subsequently, an auto-phosphorylation event led to the phosphorylation of mTOR at S2481 in a His-Ser-Phe motif near the conserved COOH-terminal tail [91].

More recently, it has become evident that there is intermediate signalling between Akt and mTOR that regulates mTOR activity and substrate specificity (Fig 1.3). TSC1 and TSC2 are genes that when lost result in the genetic disorder Tuberous Sclerosis Complex. The genes encode the Hamartin and Tuberin proteins which form the tumor suppressing TSC heterodimer that inhibits mTOR signalling and phosphorylation of S6K1 and 4EBP1 [111, 112]. The TSC1/TSC2 heterodimer acts as a GTPase activating protein (GAP), inhibiting the small G protein Ras family member, Rheb (Ras homolog enriched in brain) by promoting Rheb guanosine triphosphate (GTP) to guanosine diphosphate (GDP) conversion and suppressing effector binding [111]. Rheb-GTP directly binds the endogenous mTOR inhibitor FKBP38 (FK506 binding protein 38) and prevents its association with mTOR [113]. FKBP38 acts in a similar manner to the FKBP12-rapamycin complex described later. TSC1/TSC2 Rheb-GAP activity therefore suppresses mTOR activity by releasing the inhibitory FKBP38 protein. Phosphorylation of TSC2 by Akt inactivates the TSC1/TSC2 complex by destabilizing the interaction between the two proteins [114, 115]. This in turn allows for Rheb to return to the GTP state and promote S6K1 and 4EBP1 activation in an mTOR dependent fashion.
In addition to regulation by inhibitory Akt phosphorylation, TSC2 is also phosphorylated by AMPK (AMP-activated protein kinase) and MAPK (mitogen activated protein kinase). MAPK phosphorylation destabilizes complex formation and activates mTOR, while AMPK phosphorylation promotes TSC1/TSC2 complex formation and, therefore, inhibits mTOR [116, 117]. AMPK phosphorylates TSC in low nutrient conditions then ATP levels are low. This promotion of TSC formation by AMPK and inhibition of formation by Akt and MAPK demonstrates the pivotal role of TSC2 and mTOR in integrating information about cellular nutrient levels and mitogenic stimulation.

Recent studies by Vander Haar [118] describe TSC-independent regulation of mTOR by Akt. They show that the mTORC1 protein PRAS40 may provide a point of direct Akt regulation of mTORC1 [118]. PRAS40 binds mTORC1 via raptor and is believed to inhibit mTOR auto-phosphorylation as well as phosphorylation of mTORC1 substrates [105]. Phosphorylation of this mTORC1 inhibitory protein by Akt generates a 14-3-3 binding site resulting in 14-3-3-mediated disassociation of PRAS40 from mTORC1 and relief from inhibition [118].

Akt deregulation in cancer is common and occurs via multiple mechanisms: PTEN mutations or deletions; amplification and over-expression of PI3K; activating mutations of PI3K subunits; activating mutations of Ras; and Akt over-expression (reviewed by Bhaskar et al. [119]). Studies by Skeen et al. [120] have demonstrated that key tumorigenic aspects of Akt over-activity, cell proliferation and oncogenic transformation, are dependent on Akt’s regulation of mTORC1. They show that, in the context of Akt deficiency, hyperactivity of mTORC1 is sufficient to induce the proliferation and oncogenic transformation associated with Akt signalling, indicating the significance of mTOR in cancer biology.
Figure 1.3: Akt Regulation of mTOR
Schematic representation of Akt regulation of mTOR.
**1.3.7. Inhibitors of mTOR**

Downstream targets of the PI3K/Akt pathway, such as mTOR and its substrate S6K1, have been implicated in controlling cellular proliferation. For this reason, recent studies have explored the use of mTOR inhibitors as a way to prevent the increased proliferation seen in PTEN null systems. Pharmacological mTOR inactivation is successful in suppressing S6K1 activity as well as being sufficient to reduce tumor size and inhibit cell growth and proliferation [85, 86].

Pharmacological inactivation of mTOR is achieved using the naturally occurring immunosuppressive, antifungal, and anti-proliferative macrolide rapamycin or one of its derivatives. Rapamycin is highly specific and known only to inhibit mTOR [96]. It works by binding to the small protein immunophilin FK506 binding protein 12 (FKBP12) [92, 121]. It is the resulting FKBP12-rapamycin complex that inhibits mTOR activity by binding to the FKBP12-rapamycin binding domain and, consequently, leads to cell cycle arrest at the G1/S transition phase by blocking activation of S6K1 and 4EBP1 [92, 121]. Other mechanisms by which rapamycin induces cell cycle arrest include preventing cyclin-dependent kinase activation, accelerating the turnover of cyclin D1 causing a reduction of active cdk4/cyclin D1 complexes, and inhibiting phosphorylation of retinoblastoma (Rb) protein [92].

Rapamycin has been predominantly described as an inhibitor of mTORC1 and its downstream targets. However, despite the inability of the FKBP12-rapamycin complex to interact with mTORC2, there is some evidence for long term rapamycin treatment blocking mTORC2 assembly and the phosphorylation of Akt [122-124].

Rapamycin is used as an immunosuppressive agent to prevent organ rejection following transplants and was first found to have anti-proliferative activity in numerous
systems by the U.S. National Cancer Institute in the late 1970’s [96]. It has since demonstrated growth inhibition in cell lines representing the following cancers: small-cell lung cancer, rhabdomyosarcoma, B-cell lymphoma, and human pancreatic cancer [125]. More recently, the small molecule rapamycin analogues CCI-779, RAD001 and AP23573, have been developed for pharmaceutical purposes to address the issue of poor solubility and stability of rapamycin [92, 126, 127]. Several rapamycin analogues have been shown to have significant growth inhibitory effects in a vast array of cancers in preclinical and early clinical evaluations [92]. Another function of rapamycin and CCI-779 is their ability to reverse the resistance of PTEN-null tumors to the chemotherapy treatment doxorubicin [78]. This indicates the possibility that rapamycin derivatives may prove useful in increasing the response of patients with PTEN-null cancers to chemotherapy.

Until recently, mTOR appeared to be an attractive molecular target for therapeutics in PTEN deficient systems, since PTEN-null tumors show greater sensitivity to the rapamycin ester CCI-779 than PTEN-positive control tumors [86, 128]. This differential sensitivity, possibly due to up-regulated mTOR activity in PTEN-deficient cells, could prove valuable in developing cancer treatments with reduced side effects. However, despite promising outcomes in model systems, clinical use of rapamycin analogues as anti-tumor drugs has shown only modest success. This has been attributed to increased Akt phosphorylation in response to treatment. O’Reilly et al. [129] have shown that inhibition of mTOR abrogates feedback inhibition pathways that down-regulate receptor tyrosine kinase signalling in systems with constitutive mTOR signalling. Treatment with rapamycin analogues induces insulin receptor substrate-1 expression resulting in activation of Akt via insulin growth factor 1 receptor signalling. Investigating downstream targets of mTOR for therapeutic potential
may provide a way in which to inhibit the transforming abilities of constitutively active Akt/mTOR signalling without the interfering with feedback inhibition on Akt.

1.3.8. mTORC1 Regulation of S6K1 and 4EBP1

mTORC1 achieves its role as central controller of cell growth and proliferation by relaying proliferative signals from the cells environment to critical players in the control of the cell cycle regulatory machinery, S6K1 and 4EBP1 (Fig 1.4). Studies involving amino acid deprivation and re-introduction have shown perturbation of S6K1 activity within 15 minutes [97, 130, 131]. In addition, deprivation of amino acids or treatment with an mTORC1 inhibitor results in a rapid dephosphorylation and inactivation of S6K1 and 4EBP1 [93]. Despite solid evidence for mTORC1’s role as a regulator of S6K1 and 4EBP1, there is some debate as to whether mTORC1’s regulation of S6K1 and 4EBP1 is by direct phosphorylation or whether it is facilitated by convergence with other downstream elements such as protein phosphatases [97].
Figure 1.4: mTORC1 Regulation of S6K1 and 4EBP1
Schematic representation of mTORC1 regulation of S6K1 and 4EBP1.
1.3.9. PP2A as the Intermediate between mTOR and its Downstream Targets

The phosphorylation state of a protein is determined not only by protein kinases, but also by opposing protein phosphatases. It is unknown whether mTOR directly phosphorylates S6K and 4EBP1 or whether their phosphorylation stoichiometry is elevated by an mTOR-dependent suppression of an intermediate phosphatase in conjunction with other impinging kinases. While many studies make conclusions with respect to the former simplified model, there is considerable experimental evidence that supports the latter mechanism [130].

PP2A, a ubiquitous protein-serine/threonine phosphatase, is a likely candidate as the intermediate player between mTOR and its substrates for several reasons. In vitro experiments have shown that mTOR can phosphorylate PP2A inhibiting its phosphatase activity [93]. Also, PP2A can dephosphorylate S6K1 and 4EBP1 [93, 132]. Both rapamycin treatment and amino-acid deprivation abolish the ability of mTOR to silence PP2A [93]. In Jurkat cells there is increased activity of PP2A towards 4EBP1 upon rapamycin treatment [93]. In addition, inactivation of S6K1 by rapamycin treatment can be partially relieved by calyculin A (CalA) [93]. Finally, PP2A has been shown to associate with wild type S6K1, but not with an S6K1 mutant that is resistant to rapamycin-induced dephosphorylation [93, 133].

1.3.10. PP2A

PP2A is a holoenzyme made up of three subunits: 36 kDa catalytic C subunit, 65 kDa regulatory A subunit, and a 48-72 kDa structural B subunit [133, 134]. The core enzyme consists of just the A and C subunits [135]. Each of these subunits exists as two closely
related isoforms: alpha and beta [135]. There are, therefore, four variations of the core enzyme: AαCα, AαCβ, AβCα, AβCβ. Of interest is the observation that mutations in both isoforms of the A subunit have been found in a variety of human cancers including lung and colorectal [134, 136].

The holoenzyme is formed by the core enzyme plus one of the B subunits. There are four seemingly unrelated families of B subunits designated B, B’ (also known as B56), B” and B’’’ each of which is made up of multiple family members [135]. The B subunits designate subcellular localization and substrate specificity [137]. This large variation of subunits theoretically could form over 70 different holoenzymes; however, it is unknown how many, and in what cells, these isoforms actually exist in vivo [134].

Dephosphorylation by PP2A has been implicated in a vast array of cellular functions including transcription and translation, metabolism, cell growth, differentiation and development [133]. The various roles of PP2A indicate that a tight level of regulation would be necessary. It is likely that a significant portion of this PP2A regulation comes from the substrate specificity and subcellular localization conferred by each B regulatory subunit [133]. In addition to subunit binding, PP2A activity is also regulated by post-translational modifications, including phosphorylation and carboxymethylation of the subunits [133].

It has been proposed that another mechanism by which PP2A is regulated is by association with other proteins and the formation of protein kinase-PP2A signalling modules [133]. Isolation and analysis of PP2A-containing multi-protein complexes has show them to contain such proteins as S6K1, p21-activated kinases PAK1 and PAK3, Ca2+/calmodulin-dependent protein kinase 4, and casein kinase 2α [93, 133]. It appears that these multi-protein complexes determine phosphatase specificity by restricting the localization of the
phosphatase to specific microenvironments, optimally positioning the enzymes so as to respond to and control specific intracellular signalling cascades [133].

1.3.11. S6K1

S6K1 is a protein-serine/threonine kinase known to control cell size, growth and proliferation [138]. Two coordinately regulated isoforms of S6K, collectively termed S6K1, are derived from alternate start sites on the same transcript. The p70 isoform is predominantly cytoplasmic, whereas p85 is exclusively nuclear due to a 23-amino-acid nuclear localization sequence found at its amino terminus [138, 139].

S6K1 controls cell size and proliferation via the regulation of the 40S ribosomal protein S6 at multiple phosphorylation sites [139]. This in turn regulates the translation of a family of mRNA transcripts, termed 5’TOP mRNAs, containing an oligo-pyrimidine tract at their 5’ transcriptional start site. These 5’TOP mRNAs encode ribosomal proteins, translational elongation factors and other components that make up the translational apparatus and hence are critical for cell growth and cell cycle progression [138, 139]. Despite the fact that these 5’TOP mRNAs represent only 100 to 200 genes, they can account for 20-30% of the total cellular mRNA [138]. There is considerable published evidence supporting the role of S6K1 in controlling cell size, growth and proliferation. Inhibition of the mitogen-induced activation of S6K1 in vivo slows cell growth and reduces the ability of cells to progress through the G₁ phase of the cell cycle [138, 139]. Embryonic stem cells lacking S6K grow at a reduced rate [140]. In Drosophila, mutated dS6K results in a fly with cells that grow and multiply slower than in wild-type [141].

The activation of S6K1 takes place in a hierarchical manner and is dependent on multiple upstream kinases. The carboxy-terminus of the protein contains a putative auto-
inhibitory domain. This domain has significant homology with the region of phosphorylation of 40S ribosomal protein S6 and kinase activation can be inhibited by low concentrations of synthetic peptides modeled after this region [138]. Contained within this region are four phosphorylation sites: S411, S418, T421, and S424. All four sites are phosphorylated to some extent in quiescent cells and their phosphorylation increases upon mitogen stimulation. The S418 site is believed to be necessary for substrate recognition [138]. These are believed to be the first sites phosphorylated in the activation of S6K1, and their phosphorylation facilitates the next step in the hierarchy.

Next to the auto-inhibitory domain lays the linker domain that connects the carboxyl tail to the amino-terminal serine/threonine kinase catalytic domain. The linker domain contains the sites T389 and S404, which are the next to be phosphorylated. T389 is the mTOR dependent site and is critical for activation of S6K1. Phosphorylation of T389 is thought to disrupt protein folding in a way that allows the next phosphorylation site to be exposed. This phosphorylation site, T229 is necessary for S6K1 activation, and is found in the activation T-loop of the catalytic domain. It is believed to be phosphorylated by PDK-1. T229 appears unregulated in vivo; it is phosphorylated as long as T389 is phosphorylated. For this reason, the phosphorylation state of T389 is indicative of the activation state of S6K1. The final region of S6K1 is the highly acidic amino terminus. This region appears to be responsible for S6K1’s sensitivity to rapamycin [139].

1.3.12. 4EBP1

In addition to its role in regulating S6K1, mTOR also regulates the phosphorylation status of the elongation initiation factor 4E (eIF4E) binding protein-1, 4EBP1. As with
S6K1, it is unclear as to whether this is by direct phosphorylation or by an indirect mechanism. Biologically, 4EBP1 is a central regulator of translation. In its unphosphorylated form 4EBP1 binds the eukaryotic translation initiation factor eIF4E and prevents its incorporation into the RNA-binding complex eIF4F [142]. Upon phosphorylation, 4EBP1 releases eIF4E.

The eIF4F complex is made up of three eukaryotic translation initiation factors, eIF4E, eIF4A and eIF4G (25, 46 and 220 kDa, respectively). This complex acts to bring mRNA into close association with ribosomes as well as translation initiation factors [142]. eIF4E binds the 5’-terminal 7-methyl-GTP cap of the mRNA; eIF4A, an RNA helicase subunit, unwinds mRNA secondary structure; and eIF4G is the scaffolding protein that binds the subunits together. In addition to the binding of the aforementioned subunits, eIF4G has a binding site for eIF3 that links the eIF4F complex and associated RNA to the 40S ribosomal subunit [142].

eyF4E is the least abundant of the eIF4F proteins and is thus believed to be the rate-limiting component of the eIF4F complex [143]. This limited pool of eIF4E is further reduced via sequestering by 4EBP1, which binds at a region that overlaps the eIF4G-binding site [144]. Phosphorylation of eIF4E at S209 provides additional regulation of translation initiation and is correlated with high levels of protein synthesis [142, 145]. Unlike the phosphorylation of 4EBP1, which is regulated via the PI3K/Akt/mTOR pathway, phosphorylation of eIF4E is via Mnk1 in the Ras/MAPK pathway [142, 146]. It has been shown that Mnk1 may form part of the eIF4F complex and thus when the unphosphorylated 4EBP1 pulls eIF4E from the complex, eIF4E is distanced from Mnk1 resulting in reduced phosphorylation at the S209 site [145]. Since eIF4E and its inhibitory binding protein
4EBP1 are regulated by different pathways, the eIF4F complex proves to be another site of coordinate control over translation and over subsequent G1 cell cycle progression [147].

1.3.13. Retinoblastoma Protein

S6K1 and 4EBP1 are the canonical downstream targets for mTOR; however, retinoblastoma protein (Rb) is also regulated by mTOR [148]. Rb is dysregulated in a vast array of cancers. In PCa, a causal relationship has been described between the loss of heterozygosity of the Rb locus and development of early stage disease [149]. Rb was the first protein described to be a tumor suppressor and is a critical controller of cell cycle progression, regulating progression through the G1 phase of the cell cycle. In its unphosphorylated state, Rb acts at a tumor suppressor and binds and inhibits E2F family proteins of transcription factors preventing progression through G1. Phosphorylation of Rb results in the release of E2F and, thus, inhibition of the tumor suppressor qualities of Rb. Like S6K1 and 4EBP1, PP2A is also known to dephosphorylate Rb [150], so it is unclear if mTOR regulation of Rb is direct or via PP2A.

1.4. Neuroendocrine Differentiation in Prostate Cancer

1.4.1. Neuroendocrine Cells in Normal, Disease Free Prostate

Epithelial compartment neuroendocrine (NE) cells, first described by Pretl in 1944 [151], are fully differentiated, post-mitotic secretory cells found in several organ systems including the prostate, lung, pancreas and gastrointestinal tract [151]. The exact function of NE cells in the normal prostate is unclear, but in analogy with their analogues in other
epithelia, they are presumed to be involved with growth and homeostasis of the organ [152]. The cellular origin of prostatic NE cells is not fully understood. They may arise from the neural crest during embryogenesis, from the same stem cells as other prostatic epithelial cells, or through trans-differentiation of other cells into NE cells [151]. During human male development, NE cells are first detected in the primordial urogenital sinus mesenchyme between embryonic week 13 to 21, and NE cell density remains fairly constant until puberty [153]. The number of NE cells in the normal prostate varies during different stages of male development. At birth, NE cells that were present throughout the fetal prostate rapidly disappear from the periphery [154]. However, after puberty the number of NE cells increase to the basal level seen in adult males where they remain until the mid-fifties when they trail off [155].

In the fully developed prostate, NE cells show heterogeneity in size and shape but can be classified into two main morphologically distinct classes of NE cells: the “open” and the “closed” cell type. The “open” cells are flask shaped with long, slender extensions reaching the lumen; whereas, the “closed” cells lack these extensions [156]. A key feature found in all NE cells is the presence of cytoplasmic dense core granules involved in storage, proteolytic processing, and secretion of endogenously active compounds such as peptide hormones and biogenic amines. The granules within a single cell differ with respect to the products secreted and whether that secretion is in response to a stimuli or constitutive. Similarly NE cells differ from each other in terms of which specific secretory products they release [151].
1.4.2. **Neuroendocrine Cells in Prostatic Disease**

NE cells are found distributed throughout both normal and malignant prostate tissue with greatest density in the major prostatic ducts [151]. Clinical studies have demonstrated that expression of focal NE differentiation, determined by immunoreactivity of NE markers such as chromogranin A (CgA) and neuron specific enolase (NSE), is common in prostatic adenocarcinomas and is associated with hormone refractory disease [156-160]. Although they comprise only a minor fraction of the normal epithelial cell layer, increased NE cell content in prostatic malignancies correlates with increased tumor grade and decreased mean survival [156, 161] and, it is thus argued, can be used as a diagnostic and prognostic indicator [162].

Despite uncertainty as to the exact role of NE cells in PCa and progression of the disease, there seems to be an association with NE cells and poor disease prognosis. For this reason NE cell content, measured either by biopsy, immunoreactivity, or by serum levels of NE secreted factors such as CgA, has been considered for use as a predictor of disease and provide clues as to which men are best suited for which treatment [163]. A significant problem in the current approach to treating PCa is over-treatment. We need tools to identify which men would benefit from treatment and which would not. Thus far it is uncertain as to whether NE cell content can assist in this problem. The data shows mixed outcomes. For example, on the one hand an increase in serum CgA levels appears to precede PSA elevation during androgen withdrawal treatment providing an early marker for treatment failure [164]. NSE has also been shown to be a predictive marker [165]. On the other hand, increase of NE elements are not predictive for outcomes with the cytotoxic therapeutic agents.
estramustine/etoposide and carboplatin [166]. Clearly this is an aspect of NE biology that needs further work.

1.4.3. Neuroendocrine Cells and Androgen Independence

A strong link has been made with NE cells and androgen independent progression. It is suspected that NE cells may play a role in the development of AI disease [151, 160]. NE cells are more abundant after androgen ablation therapy, and it is thought that long-term androgen deprivation may induce NE differentiation [167, 168]. Another theory is that androgen blockade, which induces the regression of androgen-sensitive carcinoma cells but not androgen-insensitive NE cells, may result in a tumor with a high NE density by subtraction of non-NE cells rather than by expansion of the NE population.

A feature of NE cells that may contribute to AIPC is their secretion of various peptide hormones and biogenic amines into the surrounding environment [169]. Some of the secretory products of NE cells include gastrin-releasing peptide, serotonin, PTHrP (parathyroid hormone related peptide), CgA, calcitonin, somatostatin and VEGF (vascular endothelial growth factor) [167]. Because of this, it has been proposed that NE cells may enhance the proliferative index of surrounding neoplastic cells in a paracrine fashion, thus diminishing the requirement for androgen.

There are two proposed mechanisms by which NE cell content increases during progression to androgen independence. The first proposes that NE cells act as lurker cells. As described earlier in this chapter, lurker cells are androgen-independent cells found within the prostate that are able to endure the pressures of androgen withdrawal. These cells become selected for during androgen withdrawal and therefore end up making up a larger
proportion of the tumor than they did before. The other proposed mechanism is that androgen withdrawal induces a trans-differentiation of adenocarcinoma cells into NE adenocarcinoma cells. Evidence exists for both of these mechanisms. Understanding these mechanisms is important when discussing potential therapies to address androgen independent disease. On the one hand, if increased NE differentiation upon androgen withdrawal is the result of NE lurker cells, specifically targeting NE cells prior to or during androgen withdrawal treatment may prevent the high NE content seen after androgen ablation treatment and, therefore, increase the time to acquisition of androgen independence. On the other hand, if increased NE cell content is the result of trans-differentiation, then understanding the mechanisms by which trans-differentiation is occurring could also provide invaluable knowledge for the design of therapeutic agents to block this event.

1.4.4. Model Systems for Studying Neuroendocrine Differentiation

There are several established androgen-sensitive and androgen-insensitive human cell lines used in PCa research each, of course, with their own advantages and limitations. A few of the most well known of these are PC-3, DU145 and LNCaP. The androgen-responsive LNCaP cell line is useful for studying NE differentiation since it acquires a NE phenotype in response to a number of conditions: long-term androgen deprivation, exposure to IL-6 and IL-1β [161], and stimulation with agents that increase intracellular cAMP levels [152, 157, 170]. This induced acquisition of NE characteristics is reversible and is identified by an increase in NE biomarkers. These include CgA [161], NSE [156] and serotonin. In addition to biomarkers, NE differentiation in LNCaP cells can be detected by morphological characteristics: the rounding up of the cell body; the development of long, branched
processes; and an increase in secretory vesicles [170]. The ability to induce trans-differentiation of prostatic cells in the lab provides credence to models describing prostatic NE trans-differentiation \textit{in vivo}.

The LNCaP cell line is a commonly used model for studying androgen-responsive PCa. The line was originally isolated in 1977 from the left supraclavicular lymph node metastasis of a 50 year old man with prostatic adenocarcinoma. It has proved to be a valuable \textit{in vitro} model due to the following features. The cell line grows readily \textit{in vitro} and leads to tumor growth at the injection site in athymic nude mice [171]. The development of these tumors requires the co-inoculation of the cells with either Matrigel or matrix derived from bone or urogenital sinus mesenchyme [172, 173]. LNCaP cells exhibit a human male karyotype that is significantly different from HeLa cells [171]. The cell line has maintained the malignant properties of the cancer, expresses AR in both the cytosol and nuclear fractions, and is hormonally responsive. In addition, they are responsive to non-specific steroid binding due to a T877A mutation in the AR. The frequency of \textit{in vivo} tumor growth correlates with serum androgen levels and, in accordance with this, tumors develop earlier and more frequently in male mice [171]. The LNCaP cell is rendered PTEN-null due to a loss of one PTEN allele and a frameshift mutation in the other allele caused by a two base pair deletion at codon 6 [174]. The majority of the work described in this thesis uses the LNCaP model. The primary reasons for this were that they are the only prostatic androgen responsive system available capable of NE trans-differentiation and that, as with approximately half of clinical prostate tumors, they are PTEN null [174, 175].

Transfection of LNCaP cells with a constitutively active mutant of the PKA catalytic subunit enabled Cox \textit{et al.} [169] to show that PKA was sufficient to induce the
morphological changes, reduced mitotic arrest and expression of NSE characteristic of NE differentiation. The constitutively active PKA catalytic subunit, termed Cqr, contains point mutations H87Q and W196R rendering Cqr unable to bind the PKA regulatory subunit even in the absence of cAMP. Transfection of LNCaP cells with a dominant negative PKA regulatory subunit demonstrated the necessity for PKA in NE differentiation, measured by morphological change, induced by agents that increase intracellular cAMP [176]. To facilitate further studies of PKA-stimulated aspects of NE differentiation in the lab, they generated tetracycline-inducible clonal cell lines (LNCaP Cqr) that express FLAG-tagged Cqr when stimulated with doxycyclin [170]. The LNCaP Cqr cells allow induction of NE differentiation with doxycyclin, an agent that has no effect on cells not containing a tetracycline-inducible plasmid. This enables the use of conditioned media, co-culture and in vivo experiments without contamination with NE-inducing agents. Using the LNCaP Cqr cells, Deeble et al. [169] demonstrated that conditioned media from NE differentiated cells can increase the mitotic index of surrounding cells, co-culture of NE differentiated cells can increase the soft agar colony formation of LNCaP cells, and the presence of NE differentiated cells can enhance tumor growth in castrated mice in reduced androgen conditions.

The PC-3 cell line can also be induced to undergo NE differentiation upon increased intracellular cAMP. PC-3 cells, established in 1979, were derived from a bone metastasis of a 62 year old Caucasian male with grade IV prostatic adenocarcinoma [177]. PC-3 cells are PTEN and p53 null, show anchorage-independent growth, and form subcutaneous tumors in nude mice without a requirement for Matrigel [177]. The cell line is AR-negative and displays characteristics of a poorly differentiated adenocarcinoma [177].
The DU145 cell line shows high levels of NE differentiation and displays NE features such as the secretion of the NE markers. Unpublished data shows that DU145 cannot be induced to undergo further NE differentiation with cAMP stimulation (M.E. Cox, unpublished observations). The DU145 cell line, established in 1976 by K.R. Stone, was derived from a brain lesion of a 69 year old Caucasian male with metastatic prostate carcinoma and a three year history of lymphocytic leukemia [178]. This rapidly growing cell line is PTEN wild type but p53 null. It does not express PSA and is AR-negative.

1.5. cAMP/PKA Signalling

1.5.1. Epinephrine Increases Intracellular cAMP

Epinephrine is a hormone released by the adrenal medulla. Like norepinephrine and dopamine, it is a biogenic amine synthesized from the amino acid tyrosine. Epinephrine’s effects are mediated by its interaction with adrenergic receptors and subsequent downstream signalling (Fig 1.5). Adrenergic receptors are G protein-coupled receptors that bind epinephrine and norepinephrine. When agonist binds, a conformational change occurs in the receptor, enabling an exchange of GDP for GTP on the Gα subunit of the tripartite G protein. This guanine nucleotide exchange causes a disassociation of Gα from Gβγ. There are three subtypes of adrenergic receptors: α1 which signals through Gq; α2 which signals through Gi (inhibitory); and β which signals through Gs (stimulatory) [179, 180]. Gq activates phospholipase C and signals through inositol-phospholipid and calcium pathways. Gs predominately activates adenylate cyclase which catalyses the conversion of ATP to the second mesenger cAMP causing an increase in intracellular cAMP; however, there have also
been reports of Gs mediated activation of calcium signalling [181]. Gi inhibits both inositol-phospholipid and calcium pathways as well as adenylate cyclase.

This thesis focuses on the effects of epinephrine-induced increases in intracellular cAMP via the β2 adrenergic receptor and adenylate cyclase. In the cell, cAMP is readily converted into AMP by degradation of the phosphodiester bond by cyclic nucleotide phosphodiesterases. This plays a key role in modulating the amplitude and duration of signalling via these second messengers. Inhibitors of phosphodiesterases can be used both in the lab and clinically as a way to enhance and prolong the effects of cyclic nucleotides. Isobutylmethylxanthine (IBMX) is a non-specific phosphodiesterase inhibitor that is used in the lab for this purpose [182, 183].
1.5.2. cAMP Signals Through PKA and EPAC

Increased intracellular cAMP has two main consequences: activation of protein kinase A (PKA) and activation of Epac. PKA, also known as cAMP-dependent protein kinase, is a key effector of cAMP signalling. It is a holoenzyme consisting of four subunits: two regulatory and two catalytic. The regulatory subunits each have two cAMP binding
Upon cAMP binding, the regulatory subunits undergo a conformational change resulting in release of the catalytic subunits. When bound, the catalytic components of the catalytic subunits are blocked; however, once released, it can catalyse the phosphorylation of downstream substrates on threonine and, more commonly, serine residues recognised by a Arginine-Arginine-X-(Serine/Threonine) motif.

Up until the discovery of Epacs (exchange proteins activated by cAMP) in the 1990’s, PKA was considered the main cAMP target [184]. However, there is a growing body of literature to support a critical Epac mediated component of cAMP signalling. Aspects of cAMP signalling that were previously attributed to PKA are now being shown to be Epac mediated. cAMP analogues specific for either PKA or Epac have been developed, despite significant sequence identity in the PKA and Epac binding sites, and have aided in these discoveries [185].

Epacs are a class of guanine nucleotide exchange factors (GEFs) that include the closely related Epac1 and Epac2. These exchange proteins activate Rap1, a small Ras family GTPase, by catalyzing the release of GDP thereby allowing GTP to bind [184]. Epac signalling appears to be involved with a vast array of biological processes including the following: cell proliferation, cell differentiation, cell survival, cell polarization, cell-cell adhesion, calcium handling, ion transport, gene transcription, and neuronal signalling. These biological outcomes are the result of Epac signalling via a growing list of effectors: Ras, Rho GTPases, MAPK, Akt, phospholipase D, phospholipase C, and ion channels [186].

Despite distinct roles for PKA and Epac in cAMP signalling, it appears that coordinate signalling through both is necessary for certain biological processes [187, 188]. Cox et al. [176] have demonstrated that PKA is sufficient to induce morphological
transformations in LNCaP cell characteristics of NE differentiation. However, Epac may also contribute to aspects of cAMP-induced NE differentiation.

1.5.3. Spatiotemporal Regulation of cAMP/PKA Signalling by AKAPs

Two key elements of cell signalling are localization of the signalling element and regulation of the duration of the signal transmitted. A-kinase-anchoring proteins (AKAPs) are a family of over 50 functionally related scaffolding proteins that are integral to the spatiotemporal regulation of cAMP/PKA signalling [189]. While the role of AKAPs has traditionally been studied in the context of cAMP/PKA signalling, there is evidence they may play a similar role in cAMP/Epac signalling [190].

AKAPs contain a conserved anchoring domain that binds the regulatory subunit of PKA as well as unique subcellular targeting domains that localize PKA to specific microdomains within the cell. In addition, AKAPs contain binding sites for other signalling proteins, resulting in the formation of PKA-containing signalling complexes [189]. The formation of these signalling complexes ensures cAMP/PKA signalling specificity and duration by bringing the enzyme into proximity with target proteins as well as regulatory elements such as phosphatases, other kinases and cAMP phosphodiesterases (PDEs). PDEs within the complex are key to regulation of signal duration as they function to terminate cAMP signalling by degrading cAMP to AMP [191].
1.6. **VEGF in Prostate Cancer and Neuroendocrine Differentiation**

1.6.1. **VEGF**

VEGF, also known as VEGF-A, is a secreted protein involved in both vasculogenesis and angiogenesis that has been shown to be up-regulated upon increased levels of intracellular cAMP. There is evidence for not only increased VEGF expression and secretion, but also VEGF-induced angiogenesis in response to cAMP signalling [192, 193]. VEGF, along with other genes that promote angiogenesis, are secreted from cells into the tumor microenvironment. Up-regulation of these angiogenic genes is believed to be an important early event in the development of metastatic disease [194].

The HIF1 (hypoxia inducible factor 1) transcription factor is one of the prime controllers of VEGF and is primarily known for its role in cellular adaptation to oxygen availability. This adaptation involves the regulation of not only VEGF, but also a long list of hypoxia-responsive genes including erythropoietin, glucose transporters, glycolytic enzymes, Nitric Oxide Synthase 2, and the VEGF receptor Flt-1 [195, 196]. The HIF1 transcription factor is composed of two bHLH subunits: HIF1α and HIF1β (Fig 1.7). HIF1β (also known as the aryl hydrocarbon receptor nuclear translocator, ARNT) is constitutively present within the cell and dimerizes with various bHLH-binding partners. In contrast, HIF1α is continually synthesized but its availability is controlled by oxygen regulated ubiquitination [195]. Under normal conditions, where oxygen is available, HIF1α is targeted for destruction via polyubiquitination by a von-Hippel-Lindau tumor suppressor protein (pVHL) containing E3 ubiquitin ligase. Binding of pVHL - a protein implicated in cell cycle control, differentiation, extracellular matrix dynamics and angiogenesis - occurs when a conserved
proline residue at the core of the HIF1α peptide is hydrolyzed [197]. Hydrolysis of the proline residue requires molecular oxygen and, hence, may be a key step in cellular oxygen sensing [198]. Under hypoxic conditions there is a decreased interaction of pVHL and HIF1α, allowing rapid accumulation of HIF1α protein [199]. In addition to protein degradation, HIF1α is also regulated via hypoxia induced rapid nuclear localization signal (NLS) dependent nuclear localization as well as de-repression of the transactivation domain leading to recruitment of CBP/p300 [196]. Dimerization of HIF1α with HIF1β to form HIF1 leads to activation of the hypoxic response elements (HREs) of target genes and their transcription. In the case of VEGF, expression levels are regulated by the binding of HIF-1 to a 47bp cis-acting hypoxia-response element found 1 kb 5′ to the transcriptional start site of the VEGF gene [200]. Up-regulated expression of these hypoxia-responsive genes is associated with increased angiogenesis, glycolytic metabolism, and resistance to apoptosis – key processes involved in tumor growth and metastasis [201].

VEGF mRNA levels increase in hypoxic cells as a result of both increased transcriptional activation and mRNA stabilization [202]. Hypoxia results in an overall inhibition of protein synthesis; however, VEGF mRNA is efficiently translated under these conditions [202]. Due to a long 1,014 bp 5′UTR, VEGF is not efficiently translated by ribosomal scanning. However, the presence of a functional internal ribosome entry site (IRES) in the 5′UTR allows efficient cap-independent translation that is maintained under hypoxic conditions [203].
Figure 1.6: Regulation of VEGF by Hypoxia and HIF1
Schematic representation of VEGF regulation by hypoxia and HIF1.

1.6.2. VEGF and HIF1 in Prostate Cancer

Over-expression of HIF-1 and VEGF has been reported in various tumors, including human PCa, with dramatically increased levels of VEGF found in primary and metastatic tumors compared to normal prostate [195, 204]. HIF1α mutants have been found in PCa and provide a possible mechanism for enhanced HIF1 activity that leads to increased tumor susceptibility and increased tumor aggressiveness [205]. However, a more prevalent occurrence is VEGF over-expression in PCa elevated by the PI3K/Akt/mTOR pathway that is chronically activated in PTEN null PCa [202]. PI3K and mTOR inhibition, using LY-
294002 or rapamycin, respectively, blocks HIF1 expression and expression and cell secretion of VEGF [202, 206]. Dominant-negative Akt or PI3K, or wild-type PTEN, blocks HIF1 accumulation and HIF1-dependent gene transcription and HIF1 accumulation [206]. In contrast, constitutively active Akt or PCa dominant negative PTEN induces HIF1-dependent transcription [206, 207]. Further indication of the relevance of the Akt pathway in HIF1/VEGF regulation is that in, PTEN loss of function correlates with an increase in angiogenesis [206].

1.6.3. VEGF and HIF1 in Neuroendocrine Disease

VEGF expression in prostatic tissue samples, as measured by immunohistochemistry, has been shown to correlate with Gleason score and disease-specific survival [208, 209]. PCa specimens stain positively for VEGF with the highest levels being found in NE cells. In contrast, VEGF staining in normal tissue is usually negative or low [208, 210]. Levels of VEGF can be reduced by androgen ablation therapy; however, VEGF levels in the NE cell are resistant to this reduction [208]. VEGF staining in prostate tissue sections, including both primary tumors and lymph node metastasis, has revealed confined VEGF staining in CgA and/or serotonin positive NE cells [211]. Neuroendocrine cells appear to be a significant source of VEGF in prostate tumors, and that may be significant in the role of NE cells in disease progression [212].

Angiogenic potential is an important prognostic factor for patients with PCa [208]. The induction of genes involved in angiogenesis, such as VEGF, is an important early event in the development of androgen-insensitive metastatic disease and VEGF over-expression has been shown to correlate with metastasis in PCa [194, 212]. Angiogenic factors are
released by the tumor cells into the surrounding microenvironment and have been shown to be able to act directly on PCa cells in vitro [211]. The increased expression and subsequent secretion of VEGF by NE cells may contribute to the ability of NE cells to promote growth and survival of surrounding cells and progression to AI.

1.7. **Hypothesis, Project Rational and Approach**

1.7.1. **Hypothesis**

Induction of neuroendocrine differentiation by induction of cAMP accumulation in PCa cells affects the activation states of intracellular signalling modules and alters gene expression patterns that contribute to disease progression.

1.7.2. **Rational and Approach**

There is currently no effective treatment for CRPC. NE cell content is associated with poor prognosis and progression towards AI. Androgen withdrawal increases NE cell content within the tumor, and in turn the NE cells assist surrounding cells to escape androgen withdrawal by promoting survival and proliferation. Of the two mechanisms that have been proposed by which androgen withdrawal increases NE cell content, lurker cell or trans-differentiation, the findings in our lab are more consistent with the latter mechanism describing transdifferentiation of adenocarcinoma cells into NE cells. To address the question of targeted therapeutics for AIPC, I have focused my studies on understanding the biochemical mechanisms by which trans-differentiation of an adenocarcinoma cell to a NE cell occurs. By understanding the biochemical nature of this process, rationally designed therapeutic reagents can be developed that either block transdifferentiation of
adenocarcinoma cells and inhibit the increase in NE cell content following androgen withdrawal or block the actions of NE cells that promote tumor progression.

To profile biochemical changes occurring during NE differentiation induced in the lab at the protein activation and the mRNA levels I used the Kinetworks™ phospho protein array KPSS 1.1 as well as the Human Operon Version 3.0 microarray. I found altered expression in a wide array of genes as well as significant perturbations in protein phospho-status. There were two observations that I chose for subsequent follow-up: agents that induce NE differentiation in LNCaP cells cause a perturbation in the phospho-state of two downstream targets of mTORC1; and Epi/IBMX causes an increase in VEGF mRNA expression. Since mTORC1 is considered to be a critical component in the control of tumorigenicity, and since increased VEGF is associated with advanced tumor progression, these findings appear to address some of the processes by which transdifferentiation of adenocarcinoma cells to NE cells may regulate PCa progression. The remainder of the thesis focuses on dissecting the mechanism by which treatment with the NE inducing agents Epi/IBMX alters control of downstream targets of mTOR and the angiogenic factor VEGF.
2. Materials and Methods

2.1. Cell Culture

2.1.1. Cell Culture

Human prostate cancer LNCaP (from Leland W. K. Chung, Emory University), LNCaP Cqr and PC3 (from ATTC) cells were maintained in RPMI (Invitrogen) supplemented with 5% fetal bovine serum (FBS; GibcoBRL). HMEC cells (from Aly Karsan, British Columbia Cancer Agency Research Centre) were maintained in MCDB 131 (Invitrogen) supplemented with 10% FBS (GibcoBRL). Cells were cultured at 37°C in a humidified 5% CO₂ environment. Cells were cultured on pre-coated cell culture dishes to minimize cell detachment. Cells were generally passaged at 60-80% confluence, using trypsin/0.25% EDTA (GibcoBRL) to detach cells from tissue culture dishes.

2.1.2. Treatments and Reagents

The reagents and concentrations used to treat cells are outlined in Table 2.1. Unless otherwise stated, treatments occurred in RPMI with 5% FBS. Cells were treated in the presence of normal growth medium containing serum by directly adding the differentiation agents to the culture medium. Serum containing medium was used for the experiments in this thesis in order to study NE differentiation under optimal androgenic and growth conditions for the cell. Another reason for the use of normal growth medium is because hormone deprivation, a consequence of serum deprivation, can induce NE differentiation [213]. Potential steroid-like effects of phenol red within the RPMI media are insignificant in the context of experiments conducted in full serum.
Optimization for reagent concentrations, stimulation times, as well as inhibitor pretreatment times was based on previously described experimental protocols, product specification sheets and established use within our lab [170, 214-216].

Table 2.1: Reagents
The following reagents were used at the concentration listed according to manufacturer’s instructions:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine (Epi)</td>
<td>10 µM</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Isobutylmethylxanthine (IBMX)</td>
<td>100 µM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Interleukin-6 (IL6)</td>
<td>20 nM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>LY-294002 (LY)</td>
<td>40 µM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Rapamycin (Rap)</td>
<td>10 nM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Okadaic Acid (OA)</td>
<td>10 nM</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Calyculin A (CalA)</td>
<td>10 nM</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>H-89</td>
<td>10 nM</td>
<td>Seikagaku Corporation</td>
</tr>
<tr>
<td>Doxycycline (Dox)</td>
<td>2 µg/ml</td>
<td>Clonetech</td>
</tr>
<tr>
<td>U0126</td>
<td>10 µM</td>
<td>Promega and Biomol</td>
</tr>
<tr>
<td>PD98059 (PD)</td>
<td>10 µM</td>
<td>Biomol</td>
</tr>
</tbody>
</table>

2.2. Protocols for Examining Mitotic Index and Migration

2.2.1. Mitotic Index: BrdU Labeling

LNCaP cells were cultured on cover slips in 5% serum containing medium for 24 hr to allow cells to adhere. Cells were treated with NE differentiating agents for 3 days. 100 µM bromodeoxyuridine (BrdU) (Sigma) was added to the culture medium during the last 18 hr of treatment. BrdU incorporation into LNCaP DNA was assessed by immunofluorescence microscopy of methanol-fixed cells using anti-BrdU-fluorescein isothiocyanate-conjugated antibody (Boeringer-Mannheim). Mitotic activity was calculated as a percent of BrdU
positive cells relative to total cell number. Between 100-200 cells were counted for each of three biological replicates for each treatment condition. BrdU pulse duration was optimized to 18 hr to allow sufficient incorporation of BrdU into slower growing treated samples without saturation in untreated samples [170]. Previous studies in the lab have shown no cytotoxicity in response to these labeling conditions.

2.2.2. **Migration: Scratch Assay**

HMEC cells were seeded to form a confluent monolayer on pre-coated tissue culture plates and left to adhere. A “scratch” was created by scraping a p200 pipette tip along the plate. Specific points along the “scratch” were monitored and photographed at the indicated time points. The size of the “scratch” was measured and recorded at each time point for each field monitored and charted as percentage of “scratch” closed over time.

2.3. **Protocols for Examining Proteins**

2.3.1. **Western Blotting and Immunoprecipitation**

2.3.1.1. **Preparation of Cell Lysates**

Cells were grown to 70% confluency in RPMI with 5% FBS. Following treatment, cells were washed with phosphate-buffered saline (50 mM sodium phosphate, 150 mM NaCl, pH7.4) and lysed on ice in one of the following buffers: HO buffer (50 mM HEPES, 100 mM NaCl, 1% NP-40, 2 mM EDTA, 1 µg/ml leupeptin, 2 µg/ml aprotinin, 0.5 mM sodium vanadate, 2MM microcystin, pH7.2); RIPA (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA 50 mM Tris, pH 7.5); or Passive Lysis Buffer (Promega). Lysates were subjected to centrifugation at 10,000 x g for 10 min at 4°C. The
supernatant was collected and protein concentration was determined using BCA Protein Assay Kit (Pierce) according to manufacturers instructions.

2.3.1.2. Immunoprecipitation

Immunoprecipitations were performed from 500 µg-1 mg of total protein lysate using 1 µg of antibody collected on 40 µl of Protein A/G PLUS-Agarose immunoprecipitation reagent (Santa Cruz Biotechnology) using a rotating platform at 4°C for 1 hr. Immune complexes were separated from lysate by centrifugation and washed three times with lysis buffer.

2.3.1.3. Gel Electrophoresis and Western Blotting

Between 10-50 µg of protein, or washed immune complexes from immunoprecipitation, were processed for SDS-polyacrylamide gel electrophoresis by boiling for 5 min in 2x reducing protein sample buffer (100 mM Tris, 2% SDS, 5% β-mercaptoethanol, 15% glycerol, bromophenol blue, pH 6.8). Samples were elecrophoresed in 8% polyacrylamide (29:1 Bis Ratio) gels (12% when looking for 4EBP1 or rpS6) with 5% stacking gels at 100V using either Hoeffer Electrophoresis Unit SE600 Series (Hoeffer) or Bio-Rad Mini-PROTEAN® 3 Cell (Bio-Rad) with 1x SDS-PAGE running buffer (6 g/l Tris base, 28.8 g/l glycine, 0.1 % SDS). Proteins were transferred to nitrocellulose membrane (Biorad, Hercules, CA.) using an Owl Panther™ semi dry Electroblotting (Thermo Scientific) at 0.8 mA/cm² of membrane for 1.5-2 hr in SDS-PAGE buffer spiked to 10% with MeOH.
Immunoblotting was performed using antibodies listed in Table 2.2 according to manufacturer’s recommendations following a 1 hr blocking incubation in 4% skim milk diluted in TBST (20 mM Tris-HCl pH7.6, 140 mM NaCl, 0.1% Tween). Excess antibody was removed by washing 3 x 8 min in TBST. Immunoblots were visualized either by ECL chemiluminescence kit (Amersham) using horseradish peroxidase (HRP) conjugated secondary antibodies or by Li-Cor Odyssey Infrared Imaging System (Li-Cor Biosciences) using fluorescent conjugated secondary antibodies listed in Table 2.3.

**Table 2.2: Primary Antibodies**

The following primary antibodies were used for Western blotting and/or immunoprecipitation according to manufacturer’s instructions:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
</tr>
</thead>
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<td>NSE</td>
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<tr>
<td>βIII tubulin (TuJ1)</td>
<td>A. Frankfurter, University of Virginia</td>
</tr>
<tr>
<td>Phospho-S6K1 (T421/S424)</td>
<td>Cell Signalling Technologies</td>
</tr>
<tr>
<td>Phospho-S6K1 (T389)</td>
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<td>S6K1</td>
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<td>Rb</td>
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<td>4EBP1</td>
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<tr>
<td>Phospho-CREB (S133)</td>
<td>Millipore</td>
</tr>
<tr>
<td>CREB</td>
<td>Millipore</td>
</tr>
<tr>
<td>Phospho-MAPK</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Erk2 MAPK (1B3B9)</td>
<td>Upstate Biotechnology</td>
</tr>
</tbody>
</table>
Table 2.3: Secondary Antibodies
The following secondary antibodies were used for Western blotting according to manufacturer’s instructions:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat Anti-Mouse HRP</td>
<td>DAKO</td>
</tr>
<tr>
<td>Goat Anti-Rabbit HRP</td>
<td>DAKO</td>
</tr>
<tr>
<td>AlexaFluor®680 Goat Anti-Mouse</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>AlexaFluor®680 Goat Anti-Rabbit</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>IR Dye 800 Conjugated Goat Anti-Mouse</td>
<td>Rockland</td>
</tr>
<tr>
<td>IR Dye 800 Conjugated Goat Anti-Rabbit</td>
<td>Rockland</td>
</tr>
</tbody>
</table>

2.3.2. **Kinetworks™ Multi-Immunoblotting Analysis**

Total LNCaP cell lysates were prepared with sonication in a MOPS based lysis buffer (20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 60 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 3 mM benzamidine, 5 µM pepstatin, 10 µM leupeptin, 1 mM dithiothreitol, pH 7.2) as outlined in the Kinexus Service Customer Information Package (see www.kinexus.ca). Lysates were sent to Kinexus for total and phospho-protein profiling using the Kinetworks™ KPSS1.1 screen.

2.3.3. **Measuring Secreted VEGF**

LNCaP cells were seeded with approximately 60% confluency onto either 6 well plates, 10 cm dishes or 100 cm dishes in RPMI with 5% FBS. On the following day the media was removed and replaced with fresh media containing the treatments described in the experiments. This fresh media was serum free unless otherwise stated. Media was collected after one day in all cases except for time course experiments, centrifuged, and stored at -20°C until ELISA analysis. Conditioned media was analyzed for accumulation of secreted VEGF.
using the Human VEGF ELISA kit (Biosource, California, USA) according to manufacturer’s instructions. Data is presented as the median ng/ml with accompanying standard deviation (SD) obtained from quadruplicate samples. Statistical differences were analyzed by the Student’s t-test with a $P$ value equal or greater than 0.05 considered not significant. Each experiment was performed 2 or 3 times with graphical representation displaying either the accumulation of these repeats or a representative experiment from these repeats.

2.4. Protocols for Examining RNA

2.4.1. Quantitative Real Time PCR

RNA was extracted from cells using either Trizol (Invitrogen Life Technologies, Inc.) followed by DNase treatment with TURBO DNA-free (Ambion, Inc.), or E.Z.N.A. Total RNA Kit (Omega Bio-Tek) with on-membrane DNase step. Single stranded cDNA was synthesized using random heximer primer SuperScript II First Strand cDNA Synthesis System (Invitrogen, Inc.) according to manufacturer’s instructions. Quantitative Real Time PCR (Q-PCR) using the comparative cycle time (Ct) method described by Bookout et al. [217] was performed with SYBR® Green (Applied Biosystems) according to manufacturer’s instructions using the following primers designed with Primer Express™ Software (Applied Biosystems):

VEGF forward primer 5’- CTACCTCCACCATGCCAGT-3’
VEGF reverse primer 5’-TGATTCTGCCCTCCTCCTT-3’
β-actin forward primer 5’-GCTCTTTTCCAGCCTTCCTT-3’
β-actin reverse primer 5’-CGGATGTCAACGTCACACTT-3’
Unique primer specificity was confirmed by a BLAST of each primer sequence.

β-actin was used as a reference gene for normalization of inconsistent PCR reaction loading. A template titration assay, as described by Bookout et al. [217], with a 5-fold dilution series of cDNA was used to determine the validity of using β-actin as the endogenous normalizer for VEGF in this system. A slope difference of <0.1 between the gene of interest, VEGF, and the reference gene, β-actin, on the standard curve of the template titration assay showed similar amplification efficiencies for the two genes validating the use of the β-actin as the reference gene. A single peak seen following dissociation curve analysis outlined by Bookout et al. [1] indicated no non-specific binding of primers.

Total reaction volume was 25 μl with the following cycling conditions: 50°C for 2 min, 95°C for 10 min, 95° 15 sec, 60° 1 min, 40 repeats. Comparative threshold cycle was measured using the GeneAmp 7900HT Sequence detection System and accompanying SDS version 2.2 software (Applied Biosystems, Foster City, CA). “No reverse transcriptase” controls, “no RNA” controls and “no cDNA” controls were used for each experiment. The resulting values were normalized to β-actin, and data is presented as the mean ratio with accompanying standard deviation (SD) of experimental sample to NT (no treatment sample) obtained from three replicates. Statistical differences were analyzed by the student’s t-test with a P value equal or greater than 0.05 considered not significant. Each experiment was performed 2 or 3 times with graphical representation displaying either the accumulation of these repeats or a representative experiment from these repeats.
2.4.2. Gene Microarray

2.4.2.1. Microarray Probe Preparation and Hybridization

Cells were treated with Epi/IBMX for 0, 2 hr or 24 hr in full serum conditions. RNA was extracted from LNCAP cells by Trizol (Invitrogen Life Technologies, Inc.) according to the manufacturer’s instructions. Seven biological RNA replicates were used for each of the three treatment conditions, for a total of 21 samples. cDNA was generated from 10µg of RNA using Superscript II (Stratagene). Experimental RNA was labeled with a commercial RT dT primer from the Array 350 Expression Array Detection Kit for microarrays (Genisphere). cDNA was hybridized to the microarray Human Operon Version 3.0 containing 34 912 sequence-specific gene target oligonucleotides spotted on the glass slides (Gene Array Facilities, Prostate Center, Vancouver Hospital). Labeled cDNA from a commercial RNA preparation made from a combination of 10 cell lines and designated as Universal Human Reference (UHR) RNA (Stratagene) was used as a normalization control. Each slide was hybridized with cDNA from the UHR control as well as cDNA from one of the 21 experimental samples.

2.4.2.2. Microarray Scanning

After hybridization, microarrays were scanned using GenePix 4000B (Axon). Independent grayscale 16-bit TIFF images were generated for each array pair to be compared (each experimental samples versus UHR RNA). These images were analyzed to identify the arrayed sequence-specific gene target oligonucleotides and to measure the relative fluorescence intensities for each element. Signal median intensities and background correction were quantitated using the computer program Imagene 7.0.0.
Slide and experimental quality validation was performed by plotting the signal intensities of each one of the oligonucleotide spots in the microarray to select only those slides in which the ratio of signal to noise was higher than 3.0 in at least 40 % of the total number of oligonucleotide spots. 9 slides passed this quality control and were used for analysis. These 9 slides represented triplicate for each of the experimental conditions.

The Human Operon Version 3.0 microarray is known to contain probes that do not represent verified genes. We, therefore, considered only the 22,814 oligonucleotides present on the slide that belong to genes present in the curated Source database (Stanford University) for further analysis.

2.4.2.3. Microarray Normalization

Gene expression was evaluated with the aid of the Gene Spring 7.3.1 program. The intensity of each oligonucleotide spot from the experimental sample was compared against the intensity of the UHR RNA at that same position in order to obtain a normalized signal ratio working value. Normalization adjusts the individual hybridization intensities to compensate for unequal quantities of starting RNA, differences in labelling or detection efficiencies between the fluorescent dyes used as well as systematic biases in the measured expression levels. The final expression level and associated p-value for each gene under each experimental condition represents a normalized value calculated from all 3 biological replicates.
2.4.2.4. Microarray Analysis

Volcano analysis was used to identify genes with a 2-fold change in mRNA expression in the experimental conditions compared to the untreated sample. Genes with greater than 2-fold increase or decrease in expression relative to control with a $P$ value of less than 0.05 determined by $t$-test, were considered to have altered regulation upon experimental treatment. Pathway analysis was performed using Onto-Tools Pathway Express online software publicly available at http://vortex.cs.wayne.edu/home.htm.
3. Characterizing Kinase Activation Changes in LNCaP Cells Undergoing NE Differentiation: Increased cAMP Induces a Transient Reduction in Phosphorylation of mTOR Target Genes

3.1. Introduction

The progression of PCa to androgen independence is one of the largest problems facing prostate cancer therapeutics. Since the discovery of the androgen responsiveness of PCa, androgen withdrawal treatment has been the standard therapeutic approach [53, 54]. Once the tumor inevitably progresses to an androgen independent state, we have no effective treatment for the disease. It has been shown that there is an increase in foci of neuroendocrine cells within the prostatic tumor after androgen withdrawal treatment [167, 168]. This concentration of NE cells appears to aid in the progression to androgen independent prostate cancer, presumably through the secretion of factors that promote growth and survival of surrounding cells [167, 169].

Both the mechanisms underlying transdifferentiation of adenocarcinoma cells to a neuroendocrine-like state as well as their involvement in the progression to AIPC are poorly understood. I anticipate that upon induction of prostatic tumor cells to undergo NE differentiation I would see an altered activation state of a number of protein kinases. To understand what other changes in kinase signalling cascades occur during NE differentiation of prostatic cells, I used the Kinetworks™ Phospho-Site Screen KPSS 1.1 to identify changes in expression and phosphorylation of a large panel of kinases and kinase targets in
the LNCaP model system during the transition to a NE phenotype. As outlined in Chapter 1, LNCaP cells are an appropriate model for studying NE differentiation in prostate cancer as they exhibit a strong prostatic adenocarcinoma phenotype under standard culture conditions and can be induced to acquire a spectrum of NE characteristics by treatment with agents that increase intracellular cAMP and with the cytokine IL-6 [152, 157, 161, 170]. Identifying genes involved, and understanding their expression pattern and regulation, could provide a broader understanding of the nature of prostatic NE differentiation and potentially identify molecular targets for the rational design of therapeutic agents capable of blocking cellular signalling events that underlie prostate cancer progression.

3.2. Results

3.2.1. Confirmation that Treatment with Epi/IBMX and/or IL-6 Induces NE Differentiation of LNCaP Cells

Epi/IBMX and/or IL-6 have been shown to induce NE differentiation in LNCaP cells [170]. Fig 3.1 demonstrates this differentiation by way of temporal morphologic changes as well as altered expression of NE markers. LNCaP cells cultured in 5% serum were treated with Epi/IBMX, IL-6 or Epi/IBMX/IL-6. After 24 hr treatment morphological signs of NE differentiation, rounding up of the cell body and the development of branched neuritic extensions, were seen in response to all treatments. The combined Epi/IBMX/IL-6 treatment resulted in the most dramatic morphological change.

After 3-day stimulation, increased protein expression of NE markers βIII tubulin and NSE could be seen using Western blot analysis. This section of the figure is taken from an
experiment that I performed in Deeble et al. [170]. βIII tubulin, a neuronal isoform of
tubulin, was undetectable in untreated samples, slightly elevated in Epi/IBMX treated
samples, and dramatically increased in IL-6 and Epi/IBMX/IL-6 treated samples. NSE was
detectable at low levels in untreated samples, slightly elevated in IL-6 treated samples and
dramatically increased in Epi/IBMX and Epi/IBMX/IL-6 treated samples (Erk2 MAPK was
used as a loading control). These results demonstrate that Epi/IBMX and IL-6 alone and in
combination can induce NE differentiation in LNCaP cells. However, the preferential
increase of βIII tubulin by IL-6 and of NSE by Epi/IBMX shows that differential
biochemical responses were activated by each treatment.

Induction of NE differentiation has been reported to be accompanied by a reduction
in mitotic index [170]. Fig 3.2 shows confirmation of a reduction in mitotic index in
response to Epi/IBMX and IL-6. LNCaP cells were treated with Epi/IBMX, IL-6 or
Epi/IBMX/IL-6 in 5% serum containing media for 3 days. Cells were incubated with BrdU
for the last 18 hr, fixed and stained with a fluorescein isothiocyanate-conjugated anti-BrdU
antibody. Mitotic activity is represented as the percentage of BrdU-positive cells. The
average percentage of BrdU labeled cells for each treatment was determined by counting
between 100 to 200 cells in each of 4 biological replicates. This is a representative of three
experiments. 72% of untreated cells were BrdU positive while 51% of Epi/IBMX treated
cells, 41% of IL-6 treated cells and 37% of Epi/IBMX/IL-6 treated cells were BrdU positive.
Using p < 0.05 as a determinant of statistical significance, t-test analysis shows that all of the
treated samples are statistically different from the untreated control, while none of the treated
samples are statistically significant from each other. Therefore, our data confirm that
treatment with Epi/IBMX and IL-6 alone or in combination results in a reduction in mitotic index in LNCaP cells.

Figure 3.1: Epi/IBMX and IL-6 Induce NE Differentiation in LNCaP Cells

LNCaP cells were stimulated with NT control, Epi (10 μM)/IBMX (100 μM), IL-6 (20 nM) or combined Epi/IBMX/IL-6 in full serum for A) 24 hr and B) 3 d. A) Representative phase contrast photomicrographs. B) Immunoblots using anti-βIII tubulin, total tubulin, anti-NSE and Erk2 MAPK antibodies were performed as described in Materials and Methods. These are representative blots of three replicates.
Figure 3.2: Induction of NED is Accompanied by a Reduction in Mitotic Index

LNCaP cells were stimulated with NT control, Epi (10 μM)/IBMX (100 μM), IL-6 (20 nM) or combined Epi/IBMX/IL-6 in full serum for 3 d. Cells were pulsed with 100μM BrdU for 18 hr, fixed, and stained with fluorescein isothiocyanate-conjugated anti-BrdU antibody. Mitotic index is determined by the percentage of BrdU positive cells by counting 100-200 cells per random field in triplicate samples. This experiment is representative of trends seen in three replicate experiments. P-values were obtained by Student’s t-test. * P < 0.05 relative to NT.

3.2.2. Kinetworks™ Screen Showed Changes in LNCaP Phospho-profile in Response to Short Term Treatment with the NE Differentiation Factors Epi/IBMX and IL-6

Deeble et al. [170] have demonstrated increased phosphorylation of STAT3 and MAPK upon induction of LNCaP NE differentiation with Epi/IBMX and IL-6, with the increased MAPK phosphorylation showing co-ordinate regulation by both of these agents.
However, these studies did not interrogate the impact of Epi/IBMX and IL-6 stimulation on activation of other parallel or convergent kinase cascades. The following studies were designed to look at combinatorial regulation of known downstream components of the Epi/IBMX or IL-6 pathways. To investigate, in an unbiased manner, what other alterations in kinase activation state occur in response to treatment with agents that induce NE differentiation, I used the medium throughput Kinetworks™ Phospho-Site Screen KPSS 1.1 (Kinexus Bioinformatics Corporation, Vancouver, B.C., Canada). Total protein lysates from LNCaP cells treated +/- Epi/IBMX and IL-6 for 20 min were screened for the phosphorylation state profiles of 33 phosphoproteins (Appendix A). Lysates consisted of pooled samples from three biological replicate experiments assayed on a single array run.

The KPSS phospho-screen showed increased phosphorylation of the Erk1 and Erk2 MAPK as well as CREB, and decreased phosphorylation of adducin α, GSK3 α and β, Akt, four PKC isoforms, Rb and S6K1 (Table 3.1). These changes represent alterations of the phosphoproteome from basal state during acute phase stimulation at one time point. The 20 min time point for stimulation was based on studies by Deeble et al. [170] describing early event signalling changes in response to NE inducing agents. This acute-phase stimulation represents responses that are occurring in direct response to primary stimulation and not as a downstream consequence of regulatory loops and adaptive changes. The purpose of the phospho-site screen was to find potentially interesting changes in kinase regulation for further follow up. Despite the screen showing only a snapshot at one time, proteins that I determined to be interesting for follow-up were assessed over multiple time points as described later in this chapter.
### Table 3.1: Altered Protein Phospho-Status Following Short Term Treatment with Epi/IBMX/IL-6 seen by Kinetworks™ Phospho-Site Screen KPSS 1.1

List of the 26 phosphorylation sites on 20 phospho-proteins detected on the KPSS phospho-screen comparing LNCaP cells treated with or without Epi (10 μM)/IBMX (100 μM), IL-6 (20 nM) for 20 min as described in Materials and Methods. Trace quantities of phosphorylated protein detected in each band for the untreated and the Epi/IBMX/IL6 treated samples are shown as well as the ratio of these two treatments. Blue indicates greater than two-fold increase in phosphorylation; red indicates greater than two-fold decrease in phosphorylation. Lysates consisted of pooled samples from three biological replicate experiments assayed on a single array run.

<table>
<thead>
<tr>
<th>FULL NAME OF PROTEIN</th>
<th>EPITOPE</th>
<th>TRACE QUANTITY</th>
<th>TRACE RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NT</td>
<td>Epi/IBMX/IL6</td>
</tr>
<tr>
<td>Erk1 MAPK</td>
<td>T202/Y204</td>
<td>822</td>
<td>increase</td>
</tr>
<tr>
<td>Erk2 MAPK</td>
<td>T185/Y187</td>
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<td>increase</td>
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<td>CREB</td>
<td>S133</td>
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<td>736</td>
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<td>Adducin gamma</td>
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<td>1417</td>
<td>1375</td>
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<tr>
<td>Raf 1</td>
<td>S259</td>
<td>1034</td>
<td>852</td>
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<tr>
<td>Cyclin-dependent kinase 1 (cdc2)</td>
<td>Y15</td>
<td>1557</td>
<td>1244</td>
</tr>
<tr>
<td>Src</td>
<td>Y529</td>
<td>3570</td>
<td>2455</td>
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<tr>
<td>Jun</td>
<td>S73</td>
<td>610</td>
<td>405</td>
</tr>
<tr>
<td>JNK</td>
<td>T183/Y185</td>
<td>2522</td>
<td>1507</td>
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<tr>
<td>Raf 1</td>
<td>S259</td>
<td>2381</td>
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<tr>
<td>PKC alpha</td>
<td>S657</td>
<td>1764</td>
<td>987</td>
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<tr>
<td>Akt</td>
<td>T308</td>
<td>8067</td>
<td>4021</td>
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<tr>
<td>PKC</td>
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<td>650</td>
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<td>S9</td>
<td>1839</td>
<td>737</td>
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<tr>
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<td>T638/641</td>
<td>1201</td>
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<td>S21</td>
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<td>1310</td>
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<tr>
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<td>534</td>
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<td>6098</td>
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<tr>
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<td>S780</td>
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<tr>
<td>dsRNA dependent protein kinase</td>
<td>T451</td>
<td>2677</td>
<td>decrease</td>
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</tbody>
</table>
3.2.3. **Epi/IBMX/IL-6 Treatment Results in a Greater Proportion of Proteins with Reduced Phosphorylation than Increased Phosphorylation**

To gain a global perspective of the extent of altered phospho-status upon treatment with NE differentiation agents, I first looked at general trends in the Kinetworks™ KPSS 1.1 screen. Ninety-five immunoblot bands were detectable by the KPSS screen for the untreated sample compared to 104 for the treated sample. Some of these bands were unclassified in terms of protein and phospho-site identity. For the purposes of this study, I filtered these out of the analysis; however, they may represent phospho-proteins of interest that have epitope matches for the phospho-site antibodies used in the screen. The remaining identified KPSS bands represent 26 phosphorylation sites on 20 proteins (Table 3.1).

The KPSS screen analysis shows “trace quantity” of phosphorylated protein detected in each band for the untreated and the Epi/IBMX treated samples. The “trace quantity” of the treated sample is divided by that of the untreated sample to give a ratio representing the fold change of phosphorylation upon treatment. The data were sorted into proteins that show increased phosphorylation in response to the treatment, those that show decreased phosphorylation, and those that show no change. Proteins were further categorized into those showing a greater than two-fold increase or decrease. The KPSS analysis revealed the following: three proteins with increased phosphorylation, including two proteins with greater than two-fold increase; and 16 proteins with decreased phosphorylation, including 10 proteins with greater than two-fold decrease (Table 3.1). Change was defined as anything greater or equal to a 0.1 fold increase or decrease. Phospho-sites with undetectable trace
quantities in either NT or Epi/IBMX/IL-6 were classified in the greater than two-fold change categories.

It was quite surprising to observe that the majority of changes to the phosphoproteome were the results of decreased phosphorylation. There were 5 times as many proteins with reduced phosphorylation in response to Epi/IBMX/IL-6 than there were with increased phosphorylation and 4.5 times as many proteins with greater than two-fold decrease in phosphorylation compared to two-fold increase (Table 3.1). These data, indicating a net global decrease in protein phospho-status in LNCaP cells in response to agents that induce NE differentiation, were surprising since IL-6 and Epi/IBMX are primarily characterized to initiate activation of kinase cascades. These results implicate a role for two underappreciated aspects of Epi/BMX/IL-6 mediated intracellular signalling in NED of LNCaP cells: decreased enzymatic activity of a multitude of upstream kinases, and/or increased activity of one or more key phosphatases. This concept is discussed further in Section 3.2.12.

### 3.2.4. Validation of KPSS Screen by Western Blot: CREB, MAPK and Akt

In previous studies, combined Epi and IL-6 treatments have been shown to activate CREB and MAPK [170]. To validate the capacity of the Kinetworks™ KPSS screen to identify Epi/IBMX/IL-6 induced changes in intracellular kinase signalling, I compared the KPSS results to the literature and to traditional Western blots. CREB and Erk1/Erk2 MAPK were the only proteins detected on the screen that showed a greater than two-fold increase in phosphorylation upon Epi/IBMX/IL-6 treatment (Table 3.1). This mirrors what has
previously been described in the literature and what I saw using traditional Western blots (Fig. 3.3).

Another protein that I checked for phosphorylation changes using traditional Western blotting was Akt. In contrast to the decrease in phosphorylation seen in the Kinetworks™ KPSS screen (Table 3.1), I did not detect a change in Akt phosphorylation at either the T308 or S473 sites by Western blot analysis in subsequent experiments (Fig. 3.3). It is possible that cross-reactive proteins in the KPSS screen may have confounded the results or there may have been sample variation. Validation of these three proteins indicates that the Kinetworks™ KPSS screen is capable of detecting Epi/IBMX/IL-6 induced changes in LNCaP cells, but it is essential to verify the results using other methods.
LNCaP cells were stimulated with DMSO control, Epi (10 µM)/IBMX (100 µM), IL-6 (20 nM) or combined Epi/IBMX/IL-6 for 20 minutes. Immunoblots using A) anti-phospho-Erk1/Erk2 MAPK antibody or anti-Erk2 MAPK antibody B) anti-phospho-CREB antibody or anti-CREB antibody and C) anti-phospho-Akt antibodies or anti-Akt antibody were performed as described in Materials and Methods. Phospho-ATF-1, a CREB related protein, is also detected by the anti-phospho-CREB antibody. These are representative blots of three replicates.

Figure 3.3: Western Blots Confirm Changes in Erk1/Erk2 MAPK and CREB Phospho-State in Response to Epi/IBMX/IL-6 Treatment, but Not Akt Phospho-State
3.2.5. Selection of Proteins from KPSS Screen for Validation and Further Studies: S6K1 and Rb

The purpose of the immunoblotting screens was to identify potentially novel intracellular signalling events occurring during NE differentiation as modeled by LNCaP cells. I saw greater than two-fold decreases in phosphorylation of GSK3α and β, four PKC isoforms, adducin α, dsRNA-dependent protein kinase, Akt, Rb and S6K1 (Table 3.1). The two proteins from this list that I chose for more detailed follow-up were Rb and S6K1. These two proteins were selected, because they both have a common upstream regulatory element, mTOR, known to be a key protein in PTEN null PCa tumor progression.

Since loss of PTEN is such a critical and characteristic mutation in a large proportion of PCa cases, and since a key consequence of loss of PTEN is chronic up-regulation of signalling through the PI3K/Akt/mTOR pathway, it was very intriguing to see a decreased phosphorylation of S6K1 and 4EBP1 in response to our treatments. As a critical controller of transcription and cell cycle, mTOR has received considerable attention as a target for therapeutics especially in the context of PTEN null prostate cancer. Much of this work has focused on blocking mTOR signalling in the context of constitutive signalling through Akt. Despite promising outcomes in model systems, disruption of feedback loops to Akt by inhibition of mTOR results in only modest success in the clinical use of rapamycin analogues [129].

The reduction in phosphorylation of two downstream targets of mTORC1, S6K1 and Rb, in response to early stage NE differentiation of LNCaP cells may indicate that progression to NE might regulate this key node involved in PTEN null PCa tumor progression.
progression. Understanding the mechanism by which targets of mTOR are regulated could assist in designing alternate therapeutic approaches or appropriate combination therapies for androgen-independent prostate cancer and perhaps elucidate ways in which this can be done without disrupting the feedback loops that have proved the shortfall for the use of rapamycin analogues in therapeutics.

3.2.6. Increased Levels of Intracellular cAMP Leads to a Decrease in Phosphorylation of S6K1

The Kinetworks™ Phospho-Site Screen shows a decrease in phosphorylation of S6K1 in LNCaP cells treated with the neuroendocrine differentiating agents Epi/IBMX and IL-6 to 45% of that seen in untreated cells (Table 3.1). To validate this observation from the screen, and to determine if it was the Epi/IBMX or IL-6 components separately or their combined treatments that caused this phenomenon, I carried out Western blot analysis using antibodies against both the T389 and the T421/S424 phosphorylation sites of S6K1. LNCaP cells were treated with Epi/IBMX or IL-6 alone, or the combination of these treatments in full serum for 20 min (Fig 3.4A). The untreated LNCaP sample showed robust phosphorylation at both the p70 band of S6K1 detected by the T389 antibody and doublet band detected by the T421/S424 antibody. In contrast, the 20 min Epi/IBMX/IL-6 sample showed remarkably reduced phosphorylation as detected by both of the phospho-antibodies, confirming what was seen in the Kinetworks™ multi-immunoblot array. The 20 min Epi/IBMX sample had the same immunoreactivity profile as that of the Epi/IBMX/IL-6 sample; however, the 20 min IL-6 sample had a profile similar to that of the untreated sample. None of the treatments affected S6K1 total protein levels. This indicates that
intracellular signalling events downstream of Epi/IBMX stimulation, and not IL-6 stimulation, are responsible for reduced S6K phosphorylation.

During the course of my experiments, I found the T421/S424 and the T389 antibodies to report parallel results. Therefore, for the remainder of the experiments I focused on T421/S424 to monitor phosphorylation changes on S6K1. T421 and S424 are located in the pseudo-substrate domain of S6K1 and are the first phosphorylation events in the multi-step activation of S6K1 (Fig 3.5).

This Epi/IBMX induced decrease in S6K1 phosphorylation could be due to an increase in intracellular cAMP and subsequent downstream signalling events, or could arise from some other aspects of epinephrine signalling, such as signalling pathways modulated by the βγ-subunit of the β-adrenergic receptor-coupled heterotrimeric G-protein complex released upon Gs-α activation. Therefore, I performed a parallel immunoblot analysis on lysates from LNCaP cells stimulated with the cell permeable cAMP analogue, dbcAMP, for 20 min to investigate the role of cAMP (Fig 3.4B). Much like the Epi/IBMX treated sample, the 20 min dbcAMP treated sample shows reduced immunoreactivity at the p70 doublet band visualized using the T421/S424 S6K1 antibody. This was seen in the context of unaltered signal from the S6K1 total protein antibody. This indicates that increased cAMP was sufficient to cause the changes seen upon Epi/IBMX treatment.
Figure 3.4: Increased cAMP Causes a Decrease in S6K1 Phosphorylation

LNCaP cells were stimulated with A) DMSO control, Epi (10 µM)/IBMX (100 µM), IL-6 (20 nM) or combined Epi/IBMX/IL-6 or B) with or without dbcAMP (100 µM), for 20 min. Immunoblots using anti-phospho-S6K1 antibodies or anti-S6K1 antibody were performed as described in Materials and Methods. These are representative blots of three replicates.

Figure 3.5: S6K1 Phosphorylation Sites

Schematic representation of phosphorylation sites and domain contained in S6K1 protein based on review by G. Thomas [139].
3.2.7. cAMP-Induced Reduction of S6K1 Phosphorylation is Transient

The above results apply to a short term, 20 min treatment with Epi/IBMX. This was to mirror our initial observations stemming from the Kinetwoks™ multi-immunoblotting screen with 20 min treatments of NE inducing agents. To determine if the observed changes in S6K1 phospho-protein stoichiometry were persistent or transient, I examined the kinetics of S6K1 phosphorylation in LNCaP cells over a 3-day time course of Epi/IBMX treatment (Fig 3.6A). Western blot analysis using the T389 S6K1 phospho-antibody showed reduced phosphorylation following 20 min treatment with Epi/IBMX compared to NT. This phosphorylation recovered and surpassed basal levels by 18 hr. This elevated phosphorylation stoichiometry was sustained for up to 3 days. The data demonstrate that Epi/IBMX caused a transient decrease in S6K1 phosphorylation in LNCaP cells followed by a subsequent rebound to levels above steady state levels observed in cells cultured in full serum.

I was very intrigued by this previously undescribed bimodal kinetics of cAMP-mediated S6K1 phosphorylation. cAMP-induced activation of S6K1 has been previously reported [218-220], but the bimodal regulation that I have described is novel. While many cell signalling studies describe increases in phosphorylation, decreases in phosphorylation are likely just as important for the overall biology of a cell. Therefore, this is the aspect of cAMP mediated S6K1 signalling that I pursued for the remainder of this project.
Figure 3.6: cAMP Mediated Reduction of S6K1 Phosphorylation is Transient and Seen in Both LNCaP and PC-3

A) LNCaP cells and B) PC-3 cells were stimulated with Epi (10 μM) and IBMX (100 μM) for varying lengths of time. Immunoblots using anti-phospho-S6K1 or anti-S6K1 were performed as described in Materials and Methods. These are representative blots of three replicates.

3.2.8. cAMP-Induced Transient Reduction of S6K1 Phosphorylation Also Occurs in the Prostate Cancer Cell Line PC-3

To determine how broadly applicable this phenomenon of Epi/IBMX induced reduction in S6K1 phosphorylation may be, I looked at another commonly studied prostate cancer cell line: PC-3. I chose a PTEN null prostate cell line for this study so that basal
S6K1 levels would likely be highly phosphorylated due to constitutive Akt/mTOR signalling. Highly phosphorylated S6K1 would allow us to more easily observe reductions in phosphorylation in response to treatment. PC-3 cells were treated with Epi/IBMX for varying time points from 20 min to 10 hr and Western blotted for S6K1 phosphorylation in the manner described above (Fig 3.6B). The robust S6K1 phosphorylation that I saw in the untreated sample, represented by an immunoreactive band at 70 kDa using the S6K1 T421/S424 phospho-antibody, was barely visible in the 20 min Epi/IBMX treated sample. Unlike LNCaP cells that took over 2 hr to recover from this reduction, recovery began as early as 45 minutes in PC3 cells reaching near basal levels by 10 hr. This demonstrates that the cAMP induced phenomenon of a transient reduction in S6K1 phosphorylation is not unique to the LNCaP cell line. The different kinetics seen in the different cell types is not surprising since PC3 cells have a much faster doubling time than LNCaP cells [171, 177].

3.2.9.  **Rb Shows a Similar Phosphorylation Profile to S6K1 Upon Epi/IBMX Treatment**

Another protein that showed decreased phosphorylation in LNCaP cells upon acute Epi/IBMX/IL-6 treatment in the Kinexus screen was the cell cycle regulatory protein Rb. Rb phosphorylation is known to be a critical regulator of G1/S phase cell cycle progression. Intriguingly, there is evidence that Rb phospho-state can also be regulated by mTOR [221]. Because of the observed correlation between Epi/IBMX treatment, cell cycle arrest and modulation of targets of mTOR, I looked to confirm the decreased phosphorylation seen in the Kinetworks™ multi-immunoblotting screen and determine if Epi/IBMX would exert a similar temporal effect on Rb phosphorylation kinetics as was seen with S6K1. To do this I
treated LNCaP cells with Epi/IBMX for varying time points from 20 min to 3 days and
Western blotted using an antibody against total Rb as well as phospho-antibodies recognizing
the S807/811 and S780 sites of Rb (Fig 3.7A). Consistent with phosphorylation changes
seen for S6K1, the 110 kDa immunoreactive bands, seen using both the Rb S807/811 and
S780 antibodies, show decreased phosphorylation upon 20 min Epi/IBMX treatment
compared to the untreated sample. P-Rb S807/811 immunoreactivity was almost
undetectable between 20 min and 2 hr of Epi/IBMX treatment, whereas P-Rb S780
immunoreactivity decreased by 2-fold during this time frame. Mirroring the bimodal kinetics
observed for S6K1 phosphorylation, by 14 hr post Epi/IBMX treatment immunoreactivity of
both Rb phospho-site antibodies had recovered and surpassed basal intensity and this
increased phosphorylation stoichiometry was sustained throughout the 3 day time course.

Since bimodal phospho-kinetics of S6K1 and Rb paralleled each other, and since both
have been reported to be regulated by mTORC1, I tested to see if Rb phosphorylation is, in
fact, regulated by mTORC1 in this context by treating LNCaP cells with agents that block the
PI3K/Akt/mTOR pathway. I treated with Epi/IBMX for 20 min, with the PI3K inhibitor LY-
294002 (LY) for 2.5 hr, and with the mTORC1 inhibitor rapamycin (Rap) for 30 min.
Western blots using Rb phospho-antibodies showed, once again, a reduction in
phosphorylation after 20 min Epi/IBMX treatment that was most readily visualized with the
S807/811 Rb antibody but still visible with the S780 Rb antibody (Fig 3.7B). Blocking
mTORC1 with rapamycin caused a significant decrease in phosphorylation as seen with both
the S807/811 and S780 antibodies. A less dramatic decrease was seen following LY
treatment. These data confirmed that Rb is situated downstream of mTORC1.
Figure 3.7: cAMP Mediated Reduction of Rb Phosphorylation is Transient

LNCaP cells were stimulated with A) Epi (10 μM) and IBMX (100 μM) for varying lengths of time and B) Epi (10 μM)/IBMX (100 μM) for 20 min, Rap (10 nM) for 30 min or LY (40 μM) for 2.5 hr. Immunoblots using anti-phospho-Rb antibodies or anti-Rb antibody were performed as described in Materials and Methods. These are representative blots of three replicates.

3.2.10. Increased Levels of Intracellular cAMP Also Lead to a Decrease in Phosphorylation of 4EBP1 - Another mTORC1 Target

S6K1 and Rb lie downstream of mTORC1 in the PI3K/Akt pathway, and I have shown the phospho-states of both of these proteins to be altered upon Epi/IBMX stimulation. Another protein that is targeted by mTORC1 is 4EBP1 [97]. To determine whether elevated cAMP levels selectively suppress S6K1 and Rb phosphorylation or generally suppress
phosphorylation of mTORC1 substrates, I examined the effects of Epi/IBMX on the phosphorylation of another target of mTORC1, 4EBP1. 4EBP1 is phosphorylated on at least 5 residues (i.e. T36, T45, S64, T69 and S82 in human 4EBP1) by mTORC1. The most highly phosphorylated form of 4EBP1 migrates slowly through SDS-PAGE compared to the least phosphorylated form due to a higher charge to mass ratio. This enables the detection of 4EBP1 phospho-status by band-shifts using an antibody against 4EBP1 total protein [222, 223]. Again, LNCaP cells were treated with or without Epi/IBMX for 20 min and analyzed by Western blotting using an anti-4EBP1 total antibody (Fig 3.8). In LNCaP cells cultured under standard conditions, the 4EBP1 antibody detected two protein bands at around 15 and 17 kDa indicative of the presence of hyper- and hypo-phosphorylated protein. In contrast, following 20 min Epi/IBMX stimulation, only the lower 15 kDa band was detected, which indicated the presence of only the hypo-phosphorylated protein. These results indicate that increased cAMP leads to a decreased phosphorylation of the mTORC1 target 4EBP1 as well as S6K1 and Rb. Treatment with Epi/IBMX appears to generally regulate downstream targets of mTORC1.

![Figure 3.8: Increased cAMP Causes a Decrease in 4EBP1 Phosphorylation](image)

LNCaP cells were stimulated with Epi (10 µM)/IBMX (100 µM) for 20 min. Immunoblots using an anti-4EBP1 antibody were performed as described in Materials and Methods. A band shift shows reduced hyperphosphorylation upon Epi/IBMX treatment. This is a representative blot of three replicates.
3.2.11. cAMP Mediated Reduction of S6K1, 4EBP1 and Rb Phosphorylation is Via a Mechanism Other Than Through the PI3K/Akt/mTOR Pathway

I have shown that increased cAMP leads to a reduced phosphorylation of S6K1, 4EBP1 and Rb, three downstream targets of the PI3K/Akt/mTOR pathway. I reasoned that suppression of S6K1, 4EBP1 and Rb phosphorylation by cAMP was likely to be due to a decreased activity of a common upstream kinase in the PI3K/Akt/mTOR pathway. To test this hypothesis, I compared the phosphorylation state of Epi/IBMX treated LNCaP cells to that of LNCaP cells treated with agents that block the PI3K/Akt/mTOR pathway to determine if Epi/IBMX treatment mimicked a block at some point along that pathway. I treated cells with Epi/IBMX for 20 min, with LY294002 (LY) for 2.5 hr to inhibit PI3K activity, and with rapamycin for 30 min to inhibit mTORC1 (Fig 3.9A). The phosphorylation state of S6K1 in cells treated with LY294002 or rapamycin differed from that seen after treatment with Epi/IBMX. As expected, both the Akt and mTORC1 inhibitors disrupt the phosphorylation of the T389 site that has been reported to be an mTOR-dependent site [224]. However, these inhibitors also cause complete disruption of phosphorylation at the T421/S424 sites. Despite T389 being regarded as the primary mTOR dependent phospho-site, there is evidence for insulin induced phosphorylation of the T421/S424 sites that is disrupted by rapamycin [225, 226]. This indicates a role for mTOR in phosphorylation of these sites, and could account for the effect of LY and Rap on S6K1 phosphorylation. In contrast, treatment with Epi/IBMX caused a very dramatic reduction in phosphorylation at the T389 site but a less dramatic reduction at the T421/S424 sites. Total S6K1 protein levels remained constant throughout. These results show that Epi/IBMX treatment did not mimic a block of the upstream PI3K/Akt/mTOR pathway and indicates that
the mechanism by which Epi/IBMX causes a reduction in S6K1 phosphorylation (and potentially of Rb and 4EBP1) is distinct from direct inhibition of PI3K/Akt/mTOR pathway components.

Supporting this notion that Epi/IBMX is not disrupting upstream PI3K signalling, I carried out Western blotting using antibodies against Akt, mTOR and their phospho-sites. The treatments were the same as described above. The results showed no change in Akt or mTOR phosphorylation upon Epi/IBMX treatment (Fig 3.9B and C). Reduction in phosphorylation of Akt and, to a lesser extent, mTOR by LY but not by Rap proved that these phospho-antibodies are appropriate reagents to view changes in Akt and mTOR phospho-status and that LY in these conditions effectively blocks the PI3K pathway. It is not surprising that Rap does not have clear inhibitory effects on Akt and mTOR phospho-levels at these short stimulation times as Rap inhibition of the Akt/mTOR pathway is via mTOR binding and not by direct dephosphorylation (see section 1.3.7 of this thesis).

The total Akt antibody blot shows a very strong band in the LY-treated sample (Fig 3.9B). This band has a mobility consistent with the faster migrating band in the NT, E/I and Rap treated samples. This is partially due to a change in Akt phospho-state. Under basal conditions, the stoichiometry of phospho-Akt in LNCaP cells is very high compared to unphosphorylated Akt as can be seen by the darker upper band in the total Akt blot of the untreated LNCaP sample. When treated with LY, the block in PI3K results in essentially all of the phosphorylated Akt returning to the dephosphorylated state. The increased mobility of this unphosphorylated Akt compared to the phosphorylated form results in a concentration of Akt in lower band in the LY-treated sample. It can be argued that the intensity of this lower band is greater that that would be expected when combining the Akt in the upper and lower
bands of the untreated sample; however, this increase in intensity of the band is an artifact of the antibody as described previously by Cox/Deeble whereby the Akt total antibody appears to be better at detecting the dephosphorylated form of the protein. The observations that Epi/IBMX did not perturb the phosphorylation status of Akt nor mTOR, indicated that cAMP can induce dephosphorylation of S6K1 and 4EBP1 without interfering with the basally elevated PI3K/Akt/mTOR signalling levels found in LNCaP cells.
Figure 3.9: Epi/IBMX Induced Reduction in S6K1 Phosphorylation is Via a Mechanism Other Than Through PI3K/Akt/mTOR Pathway

LNCaP cells were stimulated with Epi (10 µM)/IBMX (100 µM) for 20 min, Rap (10 nM) for 30 min or LY (40 µM) for 2.5 hr. Immunoblots using A) anti-phospho-S6K1 antibodies or anti-S6K1 antibody B) anti-phospho-Akt antibodies or anti-Akt antibody C) anti-phospho-mTOR antibodies or anti-mTOR antibody were performed as described in Materials and Methods. These are representative blots of three replicates.
3.2.12. NE-Inducing Agents Cause a Net Reduction in Protein Phosphorylation -

PP2A as a Likely Intermediate Candidate

So far I have shown that upon short term Epi/IBMX stimulation of LNCaP cells there was reduced phosphorylation of multiple proteins at multiple sites. Two possible explanations are that treatment of Epi/IBMX decreases the enzymatic activity of a multitude of protein kinases, or it may increase the activity of one or more key phosphatases. However, despite a decrease in phosphorylation of the downstream targets S6K1, Rb and 4EBP1, I saw phosphorylation of components of the common upstream PI3K/Akt/mTOR pathway remain intact. In addition, the net reduction in protein phosphorylation seen using the Kinetworks™ KPSS screen discussed in Section 3.2.3 (Table. 3.1) also hinted at an increase in phosphatase activity. I therefore hypothesized that there may be an increase in activity of a common phosphatase that caused this dephosphorylation rather than a decrease in activation of various different upstream kinases.

The phosphatase PP2A is a likely candidate for causing the dephosphorylation that I have shown in these studies. cAMP regulation of PP2A has been previously described [227, 228], and the literature describes known associations and interactions between S6K1 and PP2A [229] as well as Rb and PP2A [230]. There is also considerable evidence in the literature for PP2A to act as an intermediate between mTOR and its downstream targets S6K1 and 4EBP1 [93, 133]. This caused me to wonder whether there are known associations and interactions with PP2A and the other proteins that showed decreased phosphorylation in the Kinetworks™ KPSS screen. I reviewed the scientific literature to ascertain what proportion of the de-phosphorylated proteins in the Kinetworks™ KPSS screen have been described as regulated by PP2A. Intriguingly, there is evidence for a PP2A association or
regulation with all of the proteins shown to have a greater than two-fold reduction in phosphorylation in the Kinetronks™ screen: Adducin-α [231], PKC-α [232], PKC-β [233], PKC-δ [234], PKC-ε [235], Akt [236, 237], GSK3β (indirectly through PP2A regulation of PI3K/Akt) [236, 238], Rb [230], PKR [239] and S6K1 [229]. Such consistent observations strongly implicate activation or localization of PP2A as a transiently stimulated process in response to agents that induce NED.

3.2.13. Epi/IBMX Does Not Affect PP2A Phosphorylation

One way in which PP2A is regulated is by inhibitory Src-mediated phosphorylation at Y307 [240, 241]. To determine if treatment with Epi/IBMX alters the PP2A phospho-state, I treated LNCaP cells with Epi/IBMX for varying times ranging from 0 to 21 hr. Using a PP2A antibody specific for the protein phosphorylated at Y307, I observed a doublet band at 35 kDa. If altered phosphorylation of this inhibitory site was responsible for the reduced phosphorylation of S6K1, I would expect to see a reduction of Y307 phosphorylation around the 20 min E/I treatment time. I did not see this (Fig 3.10A). These data indicated that Epi/IBMX did not alter the Src-mediated Y307 phosphorylation of PP2A and, therefore, did not cause a reduction in S6K1 phosphorylation by relieving the inhibition of Y307 phosphorylation on PP2A.
Figure 3.10: Epi/IBMX Does Not Alter PP2A Phosphorylation, but Does Induce the Formation of a Kinase-Phosphatase Complex Containing S6K1, PKA and PP2A

LNCaP cells were stimulated with A) Epi (10 μM) and IBMX (100 μM) for times ranging from 0 to 24 hr B) and C) Epi (10 μM) and IBMX (100 μM) for 20 min in the presence of a 5 hr pretreatment with OA (10 nM). A) Immunoblots using anti-phospho-PP2A antibody or anti-PP2A antibody were performed as described in Materials and Methods. B) Immunoblots using anti-PKA catalytic subunit antibody was performed on proteins immunoprecipitated with an anti-S6K1, anti-PKA catalytic subunit, anti-PP2A and anti-4EBP1 antibodies as well as on WCL and C) immunoblots using anti-S6K1 antibody was performed on proteins immunoprecipitated with and anti-S6K1 and anti-PKA catalytic subunit antibodies as well as on WCL as described in Materials and Methods. Aliquots used for each immunoprecipitation were from the same sample lysate. WCL acts as a control to verify that protein levels are not altered with treatment. These are representative blots of three replicates.
3.2.14. **S6K1, PKA and PP2A Form a Complex with Increased Association Upon Epi/IBMX Treatment**

While PP2A activity can be modulated by post-translational modifications such as phosphorylation and methylation, the primary regulation of PP2A activity is by localization [242]. Specifically, it is thought that localization, targeting, and the assembly of protein kinase-PP2A signalling modules is a general mechanism for regulation of PP2A [93, 133]. PP2A’s catalytic activity is dependent on which regulatory subunit it is associated with [133]. A key role of the regulatory subunit is to localize the phosphatase. For this reason, I looked to see if PP2A associated with signalling molecules involved in S6K1 dephosphorylation using immunoprecipitation. I treated the LNCaP cells with or without Epi/IBMX for 20 min, in the presence or absence of a 5 hr pretreatment with the PP2A inhibitor, Okadaic Acid (OA), and immunoprecipitated for S6K1, PKA catalytic subunit, PP2A and 4EBP1 (Fig 3.10B). These immunoprecipitates, as well as the corresponding whole cell lysates, were blotted using an antibody against the PKA catalytic subunit. Each immunoprecipitations and WCL are all from aliquots from the same sample and we use the WCL as a loading control. As a positive control, I was able to readily detect a 40 kDa band corresponding to PKA catalytic subunit in cognate immunoprecipitations and in immunoblots of whole cell lysates. In the S6K1 IP lanes, as well at the 4EBP1 and PP2A IP lanes, I barely visualized bands at 40 kDa in the samples that were not treated with Epi/IBMX; however, I saw clearly visible bands in the samples treated with Epi/IBMX. This was true in the presence or absence of a pretreatment with the PP2A inhibitor OA. Similarly, I treated the LNCaP cells with or without Epi/IBMX for 20 min, in the presence or absence of a 5 hr pretreatment with OA and
immunoprecipitated for S6K1 and PKA catalytic subunit (Fig 2.10C). These immunoprecipitates, as well as the corresponding whole cell lysates, were immunoblotted using an S6K1 total antibody. Each immunoprecipitations and WCL are all from aliquots from the same sample and we use the WCL as a loading control. In the lanes with lysates immunoprecipitated for S6K1, dark bands were seen at 70 kDa confirming that the immunoprecipitation did pull down the appropriate protein. In addition, 70 kDa S6K1 bands were seen in all of the whole cell lysate lanes. In the PKA catalytic subunit immunoprecipitates, 70 kDa S6K1 bands were barely visible in the samples that were not treated with Epi/IBMX. However, the samples that were treated with Epi/IBMX showed clear bands at 70 kDa. This was true in the presence or absence of a pretreatment with the PP2A inhibitor OA. This indicated that cAMP induced increased association of PKA with S6K1, 4EBP1, and PP2A and that this association was independent of PP2A activity. These data are consistent with the idea that signalling from cAMP through PKA and PP2A to S6K1 is facilitated by co-association of these proteins and that co-association can be altered upon treatment of agents that increase intracellular cAMP levels.

3.2.15. PP2A-Family Phosphatase Inhibitor Increases S6K1 Phosphorylation and Prevents Epi/IBMX from Decreasing It

If PP2A dephosphorylates S6K1 in LNCaP cells, blocking PP2A should cause an increase in S6K1 phosphorylation. There are two main techniques currently used to elucidate the effects of abrogated PP2A activity: phosphatase inhibition and siRNA knock-down of PP2A catalytic subunit. I made the decision to use OA to block PP2A activity instead of siRNA for two key reasons. Unlike siRNA, OA has the ability to acutely repress PP2A.
More significantly, the use of OA is more likely to mimic future therapeutic intervention on PP2A as a drugable target.

The classic use of OA as a way in which to study the effects of removing PP2A activity in a system is based on its phosphatase inhibition specificity. Unlike the phosphatase inhibitor CalA which has similar specificity for PP1 and PP2A, OA has a greater specificity for PP2A and can be used at low concentrations as a PP2A specific inhibitor [243]. OA inhibits the phosphatase activity of PP2A with IC50 of 1nM, while it inhibits PP1 with IC50 of 0.1-0.5µM and PP2B with IC50 of 4-5µM. Concentrations as high as 10µM are insufficient to inhibit PP2C as well phosphotyrosyl phosphatases, alkanine phosphatases, acid phosphatases and inositol triphosphate phosphatases [244]. It is these variations in IC50 that allow selective concentrations of OA to be used to distinguish between the actions of various phosphatases, especially the highly sensitive PP2A [243]. One potential problem with using OA is that despite its common use as a PP2A specific inhibitor, it may also have effects on the PP2A-family phosphatases PP4 and PP6. Despite the results from co-immunoprecipitation experiments in my studies that suggest a role for PP2A in my studies, in my experiments looking at the loss of PP2A activity using OA I will use the term “PP2A-family phosphatase” to encompass the possibility that PP4 or PP6 could also be affected.

I treated LNCaP cells with Epi/IBMX in the presence or absence of a 5 hr pretreatment with the PP2A phosphatase inhibitor OA. By Western blotting with S6K1 T421/S424 antibody, I saw that in the absence of OA there was a 70 kDa phosphoprotein band in the untreated sample at the expected size of S6K1, and in the Epi/IBMX treated sample its phosphorylation was substantially decreased (Fig. 3.11). In the samples pretreated with 1 nM OA, the pattern was the same as those that were not pre-treated: the untreated
sample contained an immunoreactive band at 70 kDa that decreased in intensity by 80% in the Epi/IBMX treated sample. However, in the samples treated with 10 nM OA, the 70 kDa band was very dark and this band intensity did not decrease in the Epi/IBMX treated sample. Due to the dramatic differences in phosphorylation levels for samples treated with 0 or 1 nM OA compared to the highly phosphorylated 10 nM OA pretreated samples, a relatively low exposure of this blot is presented to show the 10 nM OA treated samples below levels of signal saturation. This has the effect of making the NT S6K1 phosphorylation look lower than in other figures present in this thesis with longer exposures.

It is to be noted that the two lanes containing 10 nM pretreated sample display lower signal for total S6K1. This is likely an artefact of blotting for total protein following phospho-protein on the same membrane. In my lab we have seen many examples demonstrating that when we induce super-physiological phosphorylation levels and blot for total protein following phospho-protein, the ability to detect the total protein is compromised. This is likely due to antibody competition. The alternative would be to blot two different membranes containing the same samples; however, the downfall of that approach is that there is the possibility that the two membranes will not be identical replicates with exactly the same sample volumes present.

The dramatic increase in S6K1 phosphorylation seen upon OA pretreatment indicates that a PP2A-family phosphatase actively dephosphorylates S6K1 in LNCaP cells growing in serum. This indicates that dephosphorylation by a PP2A-family phosphatase plays a role in keeping basal phospho-S6K1 levels in check in these cells. In addition, the observation that OA prevents Epi/IBMX mediated dephosphorylation of S6K1 indicates that there may be a
requirement for a PP2A-family phosphatase in the Epi/IBMX induced dephosphorylation of S6K1.

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**Figure 3.11: PP2A Inhibitor Increases S6K1 Phosphorylation and Prevents Epi/IBMX's Ability to Decrease it**

LNCaP cells were stimulated with Epi (10 µM) and IBMX (100 µM) for 20 min following 5 hr pre-treatment with either 0, 1 nM or 10 nM of OA. Immunoblots using anti-phospho-S6K1 or anti-S6K1 were performed as described in Materials and Methods. These are representative blots of three replicates.

## 3.3. Conclusion

I have shown that in the LNCaP prostate cancer model treatment with agents that increase intracellular cAMP levels can regulate the phosphorylation of three signalling proteins downstream of mTOR: S6K1, 4EBP1 and Rb. I see an initial decrease in phosphorylation of S6K1 and Rb, followed by an increase in phosphorylation that surpasses basal levels.

Cross-talk between cAMP signalling and S6K1 regulation has been previously described [220, 245]; however, a mechanism for such regulation remains unclear. My data indicates that the reduced phosphorylation of these proteins occurs without alterations of the phospho-status of the upstream proteins Akt and mTOR. I show the formation of a dynamic signalling complex containing PKA, S6K1, PP2A and demonstrate that a PP2A-family
phosphatase is a necessary signalling intermediate involved in the reduced phosphorylation of S6K1. A more detailed discussion of the possible mechanisms for and consequences of this bimodal regulation is presented in Chapter 6.
4. Characterizing Gene Expression Profile Changes in LNCaP Cells Undergoing NE Differentiation

4.1. Introduction

Altered gene expression is involved in many aspects of cancer progression and regulation: the control of cell cycle regulation; secretion of autocrine and paracrine regulatory factors; up- or down-regulation of key signalling pathways [246, 247]. To observe the temporal program of transcription that underlies the progression of a prostatic cell towards a neuroendocrine-like state, I employed the commercially available Human Operon Version 3.0 gene microarray.

The microarray was used for discovery-based investigation of mRNA expression changes during the progression of LNCaP cells to a NE-like state. The purpose was to create a dataset from which to select genes that show perturbations during NE differentiation to use as the basis for further work in our lab. These future studies will first entail validation of specific gene expression changes using qPCR.

4.2. Results

4.2.1. Treatments

Both Epi and IL-6 are able to independently induce NE differentiation with each treatment inducing differentiation in a different manner with a distinct but overlapping set of NE markers [170]. The distinct nature of Epi/IBMX, compared to IL-6, induction of NE differentiation is described by Deeble et al. [170] in the form of MAPK signalling as well as in Chapter 3 of this thesis in the form of regulation of S6K1. The studies described in...
Chapter 3 showing an Epi/IBMX dependent, IL-6 independent aspect LNCaP cell signalling directed the remainder of this project to focus on aspects of Epi/IBMX induced NE differentiation. Therefore, the aim of this microarray study was to characterize transcriptional changes during Epi/IBMX induced NE differentiation.

The total cellular RNA from three treatments of LNCaP cells was analyzed in this microarray experiment: untreated, 2 hr Epi/IBMX treatment, and 24 hr Epi/IBMX treatment. These two time points were chosen in order to see the transcription of immediate early genes, which are transcribed rapidly and transiently in response to stimuli, as well as late response genes, which are only transcribed following the synthesis of early response gene products. Deeble et al. [170], as well as previous lab optimization, has shown that a 1 to 2 hr Epi/IBMX time range is appropriate for analysis of immediate early genes, such as c-Fos, and the 24 hr time point parallels the time at which morphological changes become clearly visible (Fig 3.1).

4.2.2. Array Preparation and Normalization

cDNA from the three treatment conditions was hybridized to the Human Operon Version 3.0 microarray containing 34,912 sequence-specific gene target oligonucleotides spotted on glass slides (as described in Materials and Methods). Seven biological RNA replicates were used for each of the three treatment conditions, for a total of 21 samples. In addition to one of the 21 experimental samples, Human Reference (UHR) RNA was hybridized to each slide as a normalization control.

Array normalization adjusts individual hybridization intensities to balance them appropriately so that meaningful biological comparisons can be made. There are a number of
reasons why array data must be normalized, including unequal quantities of starting RNA, differences in labeling or detection efficiencies between the fluorescent dyes used, and systematic biases in the measured expression levels. A key aspect in microarray studies is the choice of a normalization control. UHR RNA, a commercially available RNA preparation made from a combination of 10 human cell lines [248], was used as an alternative to conventional dye flip normalization. Dye flip normalization involves each treatment being compared to another treatment on the same microarray slide using two different fluorophores. The slides are then re-probed on another slide with the dye used for each treatment being flipped to negate the effects of different fluorescent intensities with different fluorophores. Using reference RNA normalization, each sample is compared to the UHR RNA on a single slide. The intensity of each spot on the array is then represented as a signal ratio of the experimental sample compared to the UHR RNA. Hence, the UHR RNA becomes the common reference point with which treatments can be compared to each other [248]. When comparing multiple treatments, using the dye-flip normalization method can be costly compared to using a reference RNA due to the greater number of microarray slides needed. In addition, the use of the UHR RNA leaves open the potential for comparing data amongst experiments [248].

After hybridization, microarrays were scanned using GenePix 4000B (Axon) to generate a greyscale image for each experimental sample and each UHR RNA sample from which to measure the relative fluorescence intensities for each element. The Imagene 7.0.0 program was used to quantitate median signal intensities and background corrections.
4.2.3. **Quality Control**

A total of 21 Human Operon Version 3.0 gene microarray slides were probed, representing seven replicates for each of the three Epi/IBMX time points. Slide quality control analysis was performed by plotting the signal intensities of each of the oligonucleotide spots on the microarray to select only those slides in which the ratio of signal to noise was higher than 3.0 in at least 40% of the total number of oligonucleotide spots. This quality control criterion is based on guidelines from the Microarray Facility at the Prostate Centre, Vancouver General Hospital. Nine of the 21 slides passed this quality control and were used for analysis. These 9 slides represented triplicates for each of the experimental conditions.

Each glass array slide was spotted with 34,912 sequence-specific gene target oligonucleotides printed in duplicate. However, I only analyzed the 22,814 oligonucleotide spots belonging to genes present in Stanford University’s Source database. The reason for this is that the Human Operon array is known to contain artifacts that do not represent actual genes; whereas, Stanford’s Source database is a curated database that only contains previously documented genes. These 22,814 oligonucleotide spots represent 16,648 single genes with no repeats.

4.2.4. **Volcano Analysis: Epi/IBMX Induces Altered Genes Expression**

**Measured by Microarray Analysis**

Volcano plots are Cartesian plots that simultaneously show biological significance in the form of fold change and statistical significance in the form of p-values. The -log10(p-
value) is plotted on the ordinate and log2(fold change) on the abscissa. For the purposes of this study, I chose to consider genes showing a greater than 2-fold change in experimental versus control conditions with a p-value of less than 0.05, calculated by way of t-test. The normalized expression levels and associated p-values for each gene in each of the 3 experimental conditions were calculated from 3 biological replicates using Gene Spring software as described in the Chapter 2.

Two vertical lines on the volcano plot, one at 0.5-fold change and one at 2-fold change, separate the genes into three categories: greater than 2-fold decrease, no change, and greater than 2-fold increase in mRNA expression in experimental versus control samples. A horizontal line at the threshold p-value of 0.05 divides the plot into genes whose fold change is statistically significant and those whose is not. Therefore, the upper corners of the plot show genes with a statistically significant fold change of greater than 2-fold. I conducted two sets of volcano analysis: 2 hr Epi/IBMX vs. NT, and 24 hr Epi/IBMX vs. NT. Genes reaching threshold levels for both fold change and statistical significance in either the 2 hr or the 24 hr samples compared to NT were considered for further analysis.

Changes in excess of the threshold of 2-fold with p < 0.05 were observed for 242 genes (Appendix B). To gain perspective on overall trends in gene expression change in response to Epi/IBMX, I looked at the proportion of genes up- and down-regulated at the two time points. All 242 of the genes filtered by Volcano analysis showed greater than 2-fold change at 2 hr with 118 (49%) being up-regulated and 124 (51%) being down-regulated. At 24 hr, a greater than 2-fold change compared to NT was seen in 110 genes with 48 (44%) up-regulated and 62 (56%) down-regulated. Of the genes upregulated at 2 hr, 73% remained at those levels at 24 hr, 26% begin to return to basal levels by 24 hr, and only 2% of genes
increase further. Of the genes downregulated at 2 hr, 50% remain at those levels at 24 hr, 42% begin to return to basal levels by 24 hr, and 8% decrease further.

From this volcano analysis, I saw that treatment with Epi/IBMX for either 2 hr or 24 hr resulted in greater than 2-fold expression level changes of a substantial number of genes. These changes in gene expression represent both up- and down-regulated genes, and the proportion of up- to down-regulated genes was fairly even with just under half being up-regulated and just over half being down-regulated. Of the genes with altered expression at 2 hr, I observed a subset of genes that began to return to basal levels at 24 hr. A greater proportion of downregulated genes returned to baseline levels by 24 hr compared to upregulated genes. This may represent an initial response to the Epi/IBMX treatment followed by desensitization.

4.2.5. Pathway Analysis

Filtering using Volcano analysis generated a list of 242 genes with altered gene expression in response to Epi/IBMX. From an initial ad hoc analysis, it was clear that the genes identified that changed in response to Epi/IBMX treatment are implicated in many aspects cellular physiology. To translate this diverse list of altered gene expression into an orderly choreography of expression changes, I further analyzed the Volcano-selected genes set using the Onto-Tools Pathway Express online software publicly available at http://vortex.cs.wayne.edu/home.htm [249]. The Onto-Tools series of online data analysis software was developed by the Intelligent Systems and Bioinformatics Laboratory at Wayne State University. The Pathway Express software allows a user to enter a microarray data set and receive a comprehensive list of cell signalling pathways likely altered by the
experimental conditions studied using an unbiased clustering set of algorithms. The output
describes pathway significance based on several parameters: number of genes perturbed per
pathway, proportion of genes perturbed per pathway, and “impact factor” (discussed below).
Onto-Tools Pathway Express uses the KEGG (Kyoto Encyclopedia of Genes and Genomes)
database of pathway diagrams (KEGG PATHWAY) [250]. KEGG is a publicly available
database of biological systems created and curated by the Kanehisa Laboratories in the
Bioinformatics Center of the Kyoto University and the Human Genome Center of the
University of Tokyo. The database can be accessed from http://www.genome.jp/kegg.
KEGG PATHWAY is continuously updated according to current literature and as of March
2009 contained 91,970 pathways generated from 335 reference pathways

A key feature of the Onto-Tools Pathway Express is the “impact analysis” of
pathways developed by Draghici et al. [249]. Impact analysis incorporates multiple analysis
parameters into a complex calculation, providing a more biologically meaningful analysis at
the pathway and systems level than most other currently available analytical approaches.
The impact analysis for each pathway incorporates these key factors: the number of
perturbed genes per pathway normalized to size of the pathway; the magnitude of fold
change of perturbed genes within the pathway; the position of perturbed genes within the
pathway; the interactions of genes within the pathway. In this way it reflects the pathway-
specific biology and relative importance of each perturbed gene. In their 2007 paper,
Draghici et al. [249] provide a detailed description and rationale of the statistics used in the
impact analysis and demonstrate the benefit of using this analytical approach.
Pathway Express analysis of 2 hr Epi/IBMX treated samples shows alterations in genes contained in 59 pathways. However, impact analysis reveals that only 7 of these pathways were significant using a 5% confidence interval (Table 4.1). These pathways are antigen processing and presentation, circadian rhythm, adherens junction, T-cell receptor signalling, regulation of actin cytoskeleton, VEGF signalling and focal adhesion. Table 4.2 shows the differentially regulated genes in each of these pathways.

Three pathways related to the cytoskeleton are amongst the 7 pathways: adherens junction, regulation of actin cytoskeleton and focal adhesion. Alterations in these pathways could contribute to the morphological changes seen in LNCaP cells upon Epi/IBMX treatment and to the increased metastatic potential of NE cells [169, 170, 194]. Adherens junctions are involved in epithelial intercellular adhesions, intracellular cytoskeletal interactions and regulation, as well as being associated with cellular morphogenesis and differentiation [251, 252].

Table 4.1: Pathways Significantly Altered Following 2 hr Treatment with Epi/IBMX Determined Using Pathway Express Analysis

The total cellular RNA from LNCaP cells treated with or without 2 hr Epi (10 μM)/IBMX (100 μM) was analyzed by microarray and pathway analysis as described in Materials and Methods. Table lists the 7 pathways determined to be significantly altered upon Epi/IBMX treatment based on impact analysis using a 5% confidence interval.

<table>
<thead>
<tr>
<th>Pathway Name</th>
<th>Impact Factor</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen processing and presentation</td>
<td>99.63</td>
<td>5.42E-42</td>
</tr>
<tr>
<td>Circadian rhythm</td>
<td>25.80</td>
<td>1.68E-10</td>
</tr>
<tr>
<td>Adherens junction</td>
<td>14.07</td>
<td>1.17E-05</td>
</tr>
<tr>
<td>T cell receptor signalling pathway</td>
<td>13.31</td>
<td>2.37E-05</td>
</tr>
<tr>
<td>Regulation of actin cytoskeleton</td>
<td>8.82</td>
<td>1.45E-03</td>
</tr>
<tr>
<td>VEGF signalling pathway</td>
<td>5.29</td>
<td>0.03</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>5.25</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Table 4.2: Differentially Regulated Genes Contained within Pathways Significantly Altered Following 2 hr Treatment with Epi/IBMX Determined Using Pathway Express Analysis

The total cellular RNA from LNCaP cells treated with or without 2 hr Epi (10 μM)/IBMX (100 μM) was analyzed by microarray and pathway analysis as described in Materials and Methods. Table lists the significantly altered genes, as well as their fold change, within the 7 pathways determined to be significantly altered upon Epi/IBMX treatment described in Table 4.1.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen Processing and Presentation Pathway</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-DQB1</td>
<td>major histocompatibility complex, class II, DQ beta 1</td>
<td>3.92</td>
</tr>
<tr>
<td>KIR3DL3</td>
<td>killer cell immunoglobulin-like receptor, three domains, long</td>
<td>0.10</td>
</tr>
<tr>
<td>Circadian Rhythm Pathway</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PER1</td>
<td>period homolog 1</td>
<td>2.96</td>
</tr>
<tr>
<td>Adherens Junction Pathway</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMO7</td>
<td>LIM domain 7</td>
<td>4.69</td>
</tr>
<tr>
<td>T Cell Receptor Signalling Pathway</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTPRC</td>
<td>protein tyrosine phosphatase, receptor type, C</td>
<td>9.59</td>
</tr>
<tr>
<td>Regulation of Actin Cytoskeleton Pathway</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITGA7</td>
<td>integrin, alpha 7</td>
<td>3.53</td>
</tr>
<tr>
<td>VEGF Signalling Pathway</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRKCG</td>
<td>protein kinase C, gamma</td>
<td>0.24</td>
</tr>
<tr>
<td>VEGFA</td>
<td>vascular endothelial growth factor A</td>
<td>6.55</td>
</tr>
<tr>
<td>Focal Adhesion Pathway</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITGA7</td>
<td>integrin, alpha 7</td>
<td>3.53</td>
</tr>
<tr>
<td>MAPK8</td>
<td>mitogen-activated protein kinase 8</td>
<td>2.89</td>
</tr>
<tr>
<td>PRKCG</td>
<td>protein kinase C, gamma</td>
<td>0.24</td>
</tr>
<tr>
<td>VEGFA</td>
<td>vascular endothelial growth factor A</td>
<td>6.55</td>
</tr>
</tbody>
</table>
4.2.6. Epi/IBMX Induces an Up-regulation of VEGF Expression Levels and Perturbation at Several Points Along the VEGF Pathway

Identifying follow-up decisions from microarray analysis based solely on fold change is a very simplistic approach. Current microarray analysis leans towards the use of more sophisticated contextual and systematic approaches to determining promising array leads for follow up. Systematic analysis, integrating array fold-change with physiological and pathway information, is a more relevant way to look at data than simply the highest fold change.

There are three main reasons that I was interested in changes in VEGF expression during the NE progression of LNCaP cells. Firstly, gene expression fold-change ratios as well as pathway analysis highlighted VEGF as an interesting target for follow-up. The microarray analysis showed a significant alteration in VEGF expression, with 2 hr fold change ratios being in the top 10% of the Volcano filtered genes. The array showed a 6.55-fold increase in VEGF (also known as VEGF-A) after 2 hr Epi/IBMX treatment that declined to a 1.1-fold increase after 24 hr (Table 4.1). In addition, the VEGF signalling pathway was one of only 7 pathways shown to be significantly affected using the Pathway Express impact factor analysis with a 5% confidence interval, (Table 4.2). The VEGF gene was also shown to be significant in 2 of these 7 pathways: the VEGF signalling pathway and the focal adhesion pathway (Table 4.2).

Secondly, there is strong evidence in the literature supporting of an important role for VEGF in the progression of PCa. The induction of genes that promote angiogenesis is believed to be linked with the progression of prostate cancer to CRPC [253, 254]. More specifically, VEGF over-expression correlates with prostatic cancer metastasis [211] and androgen independence [254]. VEGF has been shown to be associated with NE cell content,
and NE cells have been cited as a major source of VEGF in prostate tumors [194, 209, 211]. Despite VEGF mainly being associated with angiogenesis, there is evidence that it may play other roles in prostate cancer. For example, VEGF has been shown to act directly on the prostatic cell line PC-3, triggering tyrosine phosphorylation of FAK and stimulating cell motility [211, 212].

Finally, a key deciding factor in focusing on VEGF instead of other significant expression changes seen in this array was that its analysis complements general themes studied in my lab. It has been suggested that secretion from NE cells influences surrounding prostatic cells in a paracrine fashion [169, 255]; however, this has not been shown. Perhaps the secretion of VEGF is a mechanism by which NE cells influence both surrounding prostatic cells and angiogenesis. Induction of an Epi/IBMX induced transient increase in VEGF was confirmed and is discussed further in Chapter 5.

4.3. Discussion

Complete analysis and validation of all significant changes in gene expression detected by the microarray does not fall within the scope of this thesis. The purpose of performing this microarray analysis was to profile changes in gene expression during NE differentiation. Validation and follow-up of changes seen with regard to specific genes, proteins and pathways, will provide the basis for future projects stemming from my lab.

The microarray showed up- and down-regulated genes in response to treatment with Epi/IBMX as well as an in-silica signalling pathway categorization of these transcriptional profile changes using Onto-Tools Pathway Express. Once I reduced the dataset to only those genes determined by Volcano analysis to have at least a 2-fold change from NT upon
Epi/IBMX stimulation, I detected a general pattern. There was an initial perturbation in genes at 2 hr with a trend towards returning to basal levels at 24 hr.

Array observations of VEGF gene transcript increases as well as regulation of the VEGF pathway at multiple points, in combination with related information in the literature and current lab interests, led me to further investigate the effect of NE differentiating agents on VEGF message levels in LNCaP cells, as described in Chapter 5:
5. cAMP, via PKA, Increases the Expression and Secretion of VEGF in a PP2A-Family Phosphatase and PI3K Dependent Manner

5.1. **Introduction**

Increased NE cell content is associated with poor prognosis and progression to CRPC. NE cells are thought to influence their surrounding tumor micro-environment through the secretion of paracrine factors; however, this has not been rigorously shown. They may be assisting other androgen-sensitive cells to survive in an androgen independent environment by secreting proliferative and survival signals, thereby bypassing their need for androgens. Amongst the survival factors known to be produced by NE cells is the angiogenic protein VEGF [211, 212].

Data from Chapter 4 of this thesis revealed an increase in VEGF mRNA upon Epi/IBMX treatment suggesting that cAMP signalling in LNCaP cells regulates expression of the angiogenic and tumorigenic growth factor VEGF. This was of interest because VEGF over-expression correlates with prostatic cancer metastasis [212] and has been shown to be associated with increased NE cell content [194, 209, 211]. In addition, there is a correlation with NE differentiation and neovascularization within the tumor [256]. My aim was to confirm this increase in VEGF mRNA upon Epi/IBMX treatment, to determine if the increased mRNA translates to increased secreted VEGF protein, and to elucidate the mechanism by which this phenomenon was occurring.
5.2. Results

5.2.1. cAMP Induces an Increase in VEGF Expression

The array data described in Chapter 4 showed a 6.6-fold increase in VEGF expression after 2 hr Epi/IBMX treatment declining to a 1.1-fold increase after 24 hr (Appendix B). To validate these observations, I conducted a time course of Epi/IBMX treatment and analyzed VEGF mRNA expression using quantitative PCR (Q-PCR). To examine an acute stimulation response as well as a prolonged stimulation response, I treated LNCaP cells with Epi/IBMX for 0, 2, 6, 24 and 48 hr, extracted RNA and monitored VEGF expression levels. The Q-PCR analysis was conducted using SYBR® Green double stranded (ds)DNA Binding Dye with primers specific for VEGF. β-actin primers were utilized as a reference control. To test the response to treatments I used relative quantification, comparing the quantity of nucleic acid target sequence in treated samples to the no treatment (NT) control, to show the change in VEGF cDNA levels upon Epi/IBMX treatment (Fig 5.1). I observed a 3.7-fold increase in VEGF expression following 2 hr treatment with Epi/IBMX compared to the untreated control. VEGF expression decreased to 2.2-fold of the NT expression levels after 6 hr treatment and leveled off to 1.3-fold of the NT expression levels after 24 and 48 hr treatment. Preliminary experiments incorporating time points of 1 hr and of 72 hr were consistent with this trend with a peak at 2 hr and stabilization near basal levels after about 24 hr. This demonstrated that Epi/IBMX induced an increase in VEGF expression within 1 hour, reached a peak at 2 hr, and trailed off to near basal levels by 24 hr. These results mirror the VEGF results seen in the microarray in Chapter 4 in directional change, but differ in the magnitude of change. In both the microarray and using QPCR I do see a significant increase in VEGF
expression levels following 2 hr treatment with Epi/IBMX and a return to levels close to those of basal conditions by 24 hr.

Since epinephrine can trigger signalling through both adenylate cyclase and the βγ-subunits of the β-adrenergic receptor-coupled heterotrimeric G protein complex, I wanted to confirm that the Epi/IBMX induced increase in VEGF was actually due to an increase in cAMP. To do this I treated LNCaP cells with the cAMP analogue dbcAMP and looked at VEGF expression using Q-PCR (Fig 5.2). I observed a 2.2-fold increase in VEGF expression following 2 hr dbcAMP treatment. This rose to a 3.2-fold increase at 6 hr, and dropped back down to near basal levels of 1.1-fold change by 24 hr. The data showed that dbcAMP increases VEGF expression; however, it did so with slower kinetics than Epi/IBMX peaking at about 6 hr.
Figure 5.1: Transient Increase in VEGF Expression Upon Treatment with Epi/IBMX

LNCaP cells were treated with Epi (10 µM)/IBMX (100 µM) from 0 to 48 hr and analyzed for expression of VEGF using Q-PCR. VEGF expression was normalized to beta-actin and is presented as a ratio to NT in A) histogram and B) line chart. Error bars represent standard deviation of three biological replicates. P-values were obtained by Student’s t-test. * P < 0.05 relative to NT; ** P > 0.05 relative to 24 hr.
Figure 5.2: Transient Increase in VEGF Expression Upon Treatment with dbcAMP

LNCaP cells were treated with dbcAMP (100 µM) from 0 to 24 hr and analyzed for expression of VEGF using Q-PCR. VEGF expression was normalized to beta-actin and is presented as a ratio to NT in A) histogram and B) line chart. Error bars represent standard deviation of three biological replicates. P-values were obtained by Student’s t-test. * P < 0.05 relative to NT.
5.2.2. Epi/IBMX Induces an Increase in VEGF Expression in PC-3 and DU145 Cells

To determine whether cAMP-induced VEGF expression is specific to the PTEN null, androgen-responsive LNCaP cell line or a more widespread phenomena, I looked at two well characterized androgen-independent prostate cancer cell lines: the PTEN null PC-3 cell line; and the PTEN wild type DU145 cell line. PC-3 and DU145 cells were treated with Epi/IBMX, RNA was extracted and Q-PCR analysis was conducted as described above. VEGF expression levels increased by 5.7-fold over NT upon 2 hr Epi/IBMX treatment in PC-3 cells (Fig 5.3A) and by 4.1-fold in DU145 (Fig 5.3B). These results did not necessarily reflect the peak of Epi/IBMX-induced VEGF expression, since the 2 hr time point established in LNCaP may not be optimal in these other cell lines. However, they did show that increased VEGF expression was seen in both PTEN null PC-3 cells and PTEN wild type DU145 cells. This indicates that cAMP-induction of VEGF expression was not unique to LNCaP cells and that it was independent of PTEN or androgen dependence status.
Figure 5.3: Treatment with Epi/IBMX Results in Increased VEGF Expression in PC-3 and DU145 cells

A) PC-3 and B) DU145 cells were treated with Epi (10 µM)/IBMX (100 µM) for 2 hr and analyzed for expression of VEGF using Q-PCR. VEGF expression was normalized to beta-actin and is presented as a ratio to NT. Error bars represent standard deviation of three biological replicates. P-values were obtained by Student’s t-test. * P < 0.05 relative to NT.
5.2.3. **PKA is Necessary for Epi/IBMX Induced VEGF Expression**

Increased cAMP caused by Epi/IBMX stimulation signals through both PKA and Epac. To determine if PKA was necessary for the cAMP-induced increase in VEGF expression, I pretreated LNCaP cells with the PKA inhibitor H89 for 30 min prior to Epi/IBMX treatment (Fig 5.4). I normalized the results and assessed the ratio of Epi/IBMX-induced levels of VEGF expression relative to NT for treatments without inhibitor and for Epi/IBMX induced levels of VEGF expression relative to NT for treatments with H89 (Fig 5.4 B). E/I treatment increased VEGF expression levels 6.4-fold over basal levels, while pretreatment with H89 enabled Epi/IBMX to increase VEGF levels by only 1.7-fold over NT. While the analysis shown in Fig 5.4 B is not dramatically different from that shown in Fig 5.4 A, this comparison becomes useful in later figures and is included here as a benchmark figure for subsequent comparisons. H89 alone had no effect on un-stimulated VEGF levels (Fig 5.4 A). These data indicated that blocking PKA had no effect on basal VEGF expression levels; however, it almost completely reduced the ability of Epi/IBMX to induce VEGF expression. This indicated that cAMP acted via PKA to stimulate VEGF expression in LNCaP cells.
**Figure 5.4: PKA is Necessary for Epi/IBMX Induced Increase in VEGF Expression Levels**

LNCaP cells were pretreated with H89 (10 nM) for 30 min before 2 hr treatment with Epi (10 \( \mu \text{M} \))/IBMX (100 \( \mu \text{M} \)) and analyzed for expression of VEGF using Q-PCR. VEGF expression was normalized to beta-actin and is presented as A) a ratio to NT and B) the fold change of E/I to NT for treatments with or without H89. Error bars represent standard deviation of three biological replicates. P-values were obtained by Student’s t-test. * P < 0.05 relative to NT or None.
5.2.4. **Epi/IBMX Induced Increases in VEGF Expression are Dependent on an Intact PI3K/Akt Pathway**

The PI3K/Akt/mTOR pathway is known to regulate HIF1 and VEGF [257, 258]. I used inhibitors of PI3K and mTOR to determine whether the cAMP dependent increase in VEGF was dependent or independent of the Akt pathway. I pretreated LNCaP cells with the PI3K inhibitor LY for 2.5 hr or with the mTORC1 inhibitor rapamycin for 30 min before stimulating with Epi/IBMX for 2 hr. Q-PCR analysis revealed that Epi/IBMX induced an 11.5-fold increase in VEGF expression relative to NT, whereas pretreatment with rapamycin reduced this to 8.1-fold and pretreatment with LY reduced this to 3.5-fold (Fig 5.5A). However, rapamycin and LY both also reduced basal VEGF levels: rapamycin to 0.7 and LY to 0.5 of NT (Fig 5.5A and B). Since these results indicated that there was a basal level of signalling through the PI3K/Akt/mTOR pathway, we also normalized the results and assessed the ratio of Epi/IBMX-induced levels of VEGF expression relative to NT for treatments without inhibitor, for Epi/IBMX induced levels of VEGF expression relative to NT for treatments with rapamycin, and for Epi/IBMX induced levels of VEGF expression relative to NT for treatments with LY. The ratio of Epi/IBMX to NT with no inhibitor was 11.5, with rapamycin it was 11.8, and with LY it was 6.4 (Fig 5.5C). When analyzed in this manner, it becomes clear that only the PI3K inhibitor LY, and not the mTORC1 inhibitor rapamycin, was able to inhibit the ability of Epi/IBMX to increase VEGF expression. This indicated that an intact PI3K/Akt pathway was necessary for maximal Epi/IBMX effect in LNCaP cells, but mTORC1 was not required. I also saw that blocking either PI3K or mTORC1 inhibited basal VEGF expression levels; however, it did not completely abolish
VEGF expression indicating PI3K/Akt/mTOR independent-mechanisms by which basal VEGF is regulated.
Figure 5.5: LY, Not Rap, Inhibits Epi/IBMX Induced Increase in VEGF Expression Levels

LNCaP cells were pretreated with Rap (10 nM) for 30 min or LY 40 µM) for 2.5 hr before 2 hr treatment with Epi (10 µM)/IBMX (100 µM) for 2 hr and analyzed for expression of VEGF using Q-PCR. VEGF expression was normalized to beta-actin and is presented as A) a ratio to NT B) a ratio of NT highlighting changes in samples without E/I and C) the fold change of E/I to NT for each inhibitor. Error bars represent standard deviation of three biological replicates. P-values were obtained by Student’s t-test. * P < 0.05 relative to NT or None. ** P < 0.05 relative to Rap.
5.2.5. Epi/IBMX Induced Increases in VEGF Expression are Dependent on PP2A-Family Phosphatase Activity

In Chapter 3 I demonstrated that induction of NE differentiation led to widespread reduction in target protein phosphorylation and that Epi/IBMX could increase the co-association of PKA with the phosphatase PP2A. Here I examined whether PP2A-family phosphatase activity was necessary for the Epi/IBMX induced induction of VEGF expression. I pre-treated LNCaP cells with the protein phosphatase inhibitors CalA (2 hr) or OA (overnight) before a 2 hr stimulation with Epi/IBMX and undertook Q-PCR analysis of VEGF expression. Epi/IBMX caused a 7.4-fold increase in VEGF expression compared to NT. This was reduced to 1.1-fold with OA pretreatment and to 1.3-fold with CalA (Fig 5.6A). These inhibitors did affect basal VEGF expression: 1.6-fold NT for OA and 2.4-fold NT for CalA. When plotted as ratios of Epi/IBMX-induced VEGF expression compared to untreated VEGF expression, samples without inhibitor pretreatment were 7.4-fold compared to NT, samples pretreated with OA were 0.7-fold NT and samples pretreated with CalA were 1.3-fold NT (Fig 5.6B). These data show that both inhibitors prevented Epi/IBMX induced VEGF expression. While these inhibitors are relatively broad-spectrum inhibitors of PP1/PP2 phosphatases, at these concentrations OA is known to not inhibit PP1 activity [243]. Therefore, the data indicate a necessity for PP2A-family phosphatase activity in Epi/IBMX attenuation of VEGF expression.
**Figure 5.6: Phosphatase Inhibitors Prevent Epi/IBMX Induced Increase in VEGF Expression Levels**

LNCaP cells were pretreated with OA (10 nM) overnight or CalA (10 nM) for 2 hr before 2 hr treatment with Epi (10 µM)/IBMX (100 µM) and analyzed for expression of VEGF using Q-PCR. VEGF expression was normalized to beta-actin and is presented as A) a ratio to NT and B) the fold change of E/I to NT for each inhibitor. Error bars represent standard deviation of three biological replicates. P-values were obtained by Student’s t-test. * P < 0.05 relative to NT or None; ** P < 0.05 relative to E/I.
5.2.6. **Inhibition of MEK1/MEK2 Did Not Affect the Ability of Epi/IBMX to Induce VEGF Expression**

Treatment with Epi/IBMX, leading to the progression to a NE like state, is known to involve increased activation of MAPK [170]. To determine if Erk1/Erk2 MAPK is involved in Epi/IBMX-stimulated VEGF expression, I pretreated LNCaP cells with the MEK1/MEK2 inhibitor U0126 for 30 min before stimulating with Epi/IBMX for 2 hr. Pre-treatment with U0126 for 30 min prior to treatment with NE-inducing agents is adequate to block induction of Erk1/Erk2 MAPK activity in LNCaP cells (Fig 5.7A). Treatment with Epi/IBMX induced a 3.4-fold increase in VEGF expression compared to NT, U0126 alone induced a 10% reduction in expression, while Epi/IBMX with pretreatment of U0126 caused a 3.0-fold increase (Fig 5.7B). Once again, I normalized for effects on basal VEGF levels by taking ratios of Epi/IBMX compared to NT for the samples without inhibitor and for those with U0126 pretreatment. These normalized data showed that without inhibitor Epi/IBMX caused a 3.4-fold increase in VEGF expression and that U0126 pretreatment did not significantly affect Epi/IBMX-induced VEGF mRNA expression (3.3-fold increase; Fig 5.8C). This demonstrated that inhibition of MEK1/MEK2 did not affect the ability of Epi/IBMX to induce increased VEGF expression, indicating MEK1/MEK2 and the downstream Erk1/Erk2 MAPK were not involved in signaling from cAMP to VEGF in this context.
Figure 5.7: U0126 Does Not Block Epi/IBMX Induced Increase in VEGF Expression Levels

A) LNCaP cells were pretreated with or without U0126 (10 μM) for 30 min before stimulation with NE-inducing agents Epi (10 μM)/IBMX (100 μM)/IL-6 (2nM) for 5, 10 or 20 min. Immunoblots using anti-phospho-Erk1/Erk2 MAPK antibody or anti-Erk2 MAPK antibody were performed as described in Materials and Methods. B and C) LNCaP cells were pretreated with or without U0126 (10 μM) for 30 min before 2 hr stimulation with Epi (10 μM)/IBMX (100 μM) and analyzed for expression of VEGF using Q-PCR. VEGF expression was normalized to beta-actin and is presented as B) a ratio to NT and C) the ratio of E/I to NT for samples treated with or without U0126. Error bars represent standard deviation of four biological replicates. P-values were obtained by Student’s t-test. * P < 0.05 relative to NT.
A

E/I + IL6

UO126 (µM)

<table>
<thead>
<tr>
<th></th>
<th>NT</th>
<th>10 min</th>
<th>20 min</th>
<th>20 min</th>
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</thead>
<tbody>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
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40 kDa

B

![Graph showing VEGF expression relative to NT.](image)

C

![Graph showing fold change in VEGF expression for E/I vs NT.](image)
5.2.7. **Epi/IBMX Increases Secretion of VEGF by LNCaP cells**

I have shown that Epi/IBMX induces an increase in VEGF expression in a PKA-dependent but MEK1/2-independent manner; however, an increase in mRNA expression does not necessarily translate into an increase in VEGF protein levels. Also, VEGF is a secreted protein; therefore, increased transcript and protein production may not necessarily correlate with increased bio-available VEGF in the absence of appropriate regulation of post-translational processing and secretion.

To determine if the increase in VEGF transcript level seen upon Epi/IBMX stimulation resulted in an increase of VEGF protein secreted by the cells, I assayed VEGF levels in conditioned media using a VEGF specific ELISA. Conditioned media was obtained from LNCaP cells incubated with either NT or Epi/IBMX for either 1 or 3 days in 5% serum-containing media and assayed for VEGF using an ELISA. The 1 day conditioned media from untreated cells contained 0.9 ng/ml of VEGF and from Epi/IBMX treated cells contained 2.3 ng/ml VEGF, showing a 2.6 fold increase upon Epi/IBMX treatment (Fig 5.8). The 3 day conditioned media from untreated cells contained 2.4 ng/ml VEGF and from Epi/IBMX treated cells contained 4.1 ng/ml of VEGF, showing a 1.7 fold increase upon Epi/IBMX treatment. These data show that Epi/IBMX treatment increased VEGF secretion in LNCaP cells, and that this increase was sustained for at least 3 days.
Figure 5.8: Epi/IBMX Treatment Induces an Increase in VEGF Secretion

Conditioned media from LNCaP cells incubated with or without Epi/IBMX for 1 or 3 days was analyzed for VEGF secretion using a VEGF ELISA. The data is presented as ng/ml VEGF in conditioned media. Error bars represent standard deviation of four biological replicates. P-values were obtained by Student’s t-test.
* P < 0.05 relative to 1 d NT; ** P < 0.05 relative to 3 d NT.
5.2.8. PKA is Sufficient to Increase VEGF Secretion by LNCaP Cells

Using inhibitors as loss-of-function modalities, I have shown that PKA activity was necessary for Epi/IBMX induced VEGF secretion. To determine if PKA was sufficient to induce an increase in VEGF secretion, I used the PKA gain-of-function model LNCaP Cqr cell line [176]. LNCaP Cqr cells are stably transfected with a tetracycline-regulatable promoter and a VP17-modified tet response factor system to inducibly express constitutively active PKA upon treatment with doxycyclin (Dox). Dox-induced expression of PKA results in the acquisition of an NED phenotype in LNCaP Cqr cells, including the extension of neuritic processes, reduced mitotic activity, and expression of the NED marker NSE [176].

Conditioned media was collected from LNCaP Cqr cells treated with Dox for 1 or 2 days and compared to conditioned media from untreated Cqr cells and Cqr cells treated with Epi/IBMX for 1 day. For this experiment, all cells were cultured in the media collected for analysis for 48 hr for all of the samples in order to normalize for basal PKA activity and resulting expression and secretion of VEGF by Cqr cells. For the 48 hr Dox sample, treatment commenced when the media was added. For the 24 hr Dox and 24 hr Epi/IBMX samples, treatment was added for the last 24 hr. After 48 hr, there was 1.3 ng/ml of VEGF accumulation in the untreated sample (Fig 5.9). There was 2.0 ng/ml VEGF accumulation in the sample spiked with Dox for 24 hr and 3.1 ng/ml in the sample spiked with Dox for 48 hr. The sample spiked with E/I for 24 hr contained 3.3 ng/ml of accumulated VEGF. Relative to NT, 24 hr Dox caused a 1.5-fold increase in VEGF accumulation, 48 hr Dox caused a 2.5-fold increase and 24 hr E/I caused a 2.6-fold increase. VEGF accumulation levels from 48 hr Dox treatment compared to 24 hr E/I treatment were indistinguishable, which indicated that these two treatments had an equivalent effect on VEGF secretion levels. Dox treatment took
48 hr to cause a similar level of secreted VEGF as E/I did in 24 hr, showing that Dox induced VEGF temporally lagged behind E/I induced VEGF. However, induction of PKA expression by Dox treatment in Cqr cells is delayed, which may account for this lagging VEGF effect compared to the faster acting Epi/IBMX. These data demonstrated that PKA was sufficient to induce an increase in VEGF secretion.

To further assess whether the increased expression of VEGF was dependent on PKA activation, I used the PKA inhibitor H-89. Cells were treated with or without Epi/IBMX in the presence or absence of H-89 for 24 hr. Conditioned media from untreated cells contained 3.2 ng/ml VEGF (Fig 5.10). This was increased by 1.7-fold to 5.5 ng/ml with Epi/IBMX treatment. Conditioned media from cells treated with H89 contained 2.9 ng/ml VEGF. Epi/IBMX treatment in the presence of H89 contained 3.5 ng/ml VEGF. This was a 1.2-fold increase from cells treated with H89 alone (not deemed to be a statistically significant increase by Student’s t-test; p < 0.05) compared to the 1.7-fold change induced by E/I in the absence of H89. These data demonstrated that inhibition of PKA reduced the ability of Epi/IBMX to increase VEGF secretion and provided further evidence that PKA was required for optimal VEGF protein secretion as well as for the increase in VEGF transcription described earlier.
Figure 5.9: Treatment of LNCaP Cqr Cells with Dox Results in Increased VEGF Secretion

Conditioned media for 48 hr from LNCaP Cqr cells stimulated with Dox for 24 or 48 hr or Epi/IBMX for 24 hr was analyzed for VEGF secretion using a VEGF ELISA. Data is presented as ng/ml VEGF in conditioned media in A) a histogram or B) a line chart. Error bars represent standard deviation of four biological replicates. P-values were obtained by Student’s t-test.
* $P < 0.05$ relative to NT;  ** $P > 0.05$ relative to E/I 24 hr.
Figure 5.10: H89 Blocks Epi/IBMX Induced Increase in VEGF Secretion

Conditioned media from LNCaP cells pretreated with H89 (10 nM) for 30 min and treated with Epi (10 µM)/IBMX (100 µM) for 24 hr was analyzed for VEGF secretion using a VEGF ELISA. Data is represented as A) ng/ml VEGF in conditioned media and B) fold change of VEGF in conditioned media from cells treated with compared to without E/I. Error bars represent standard deviation of four biological replicates. P-values were obtained by Student’s t-test.

* P < 0.05 relative to NT or None; ** P < 0.05 relative to E/I.
5.2.9. PI3K, but Not mTOR, Activity is Necessary for PKA Induced VEGF Secretion

I have already described a role for PI3K/Akt in Epi/IBMX-induced increase in VEGF mRNA expression and have shown that mTORC1 activity was not required. To determine if a similar trend was observed for VEGF secretion, I treated LNCaP cells with or without Epi/IBMX for 24 hr in the presence or absence of the PI3K inhibitor LY or the mTORC1 inhibitor rapamycin (Fig 5.11). Conditioned media from untreated LNCaP cells contained 3.3 ng/ml VEGF. Epi/IBMX treatment increase in VEGF expression to 6.5 ng/ml. In the presence of rapamycin, conditioned media contained 2.5 ng/ml which was increased to 5.2 ng/ml with Epi/IBMX co-treatment. In the presence of LY, LNCaP conditioned media contained 2.6 ng/ml VEGF, which increased to 3.5 ng/ml with Epi/IBMX co-treatment. The ratio of Epi/IBMX to NT with no inhibitor was 2, with rapamycin it was 2.1, and with LY it was 1.4 (Fig 5.11 B). This analysis shows that only the PI3K inhibitor LY, and not the mTORC1 inhibitor rapamycin, was able to stunt the ability of Epi/IBMX to increase VEGF secretion. Despite E/I inducing a modest increase in VEGF secretion in LY treated cells, the presence of LY did reduce the ability or E/I to induce VEGF secretion compared to samples without inhibitor with rapamycin. These data show that an active PI3K/Akt pathway is involved in Epi/IBMX induced increase in VEGF secretion, but mTORC1 activity is not necessary. This mirrored what I have shown with respect to VEGF mRNA expression changes.
Figure 5.11: LY-294002, but Not Rapamycin, Reduces Epi/IBMX Induced Increase in VEGF Secretion

Conditioned media from LNCaP cells treated with Rap (10 nM) or LY (40 µM) and Epi (10 µM)/IBMX (100 µM) for 24 hr was analyzed for VEGF secretion using a VEGF ELISA. Data is presented as A) ng/ml in conditioned media and B) VEGF concentration fold change upon Epi/IBM co-treatment. Error bars represent standard deviation of four biological replicates. P-values were obtained by Student’s t-test.
* P < 0.05 relative to None; ** P < 0.05 relative to Rap.
A

![Graph showing VEGF Concentration (pg/ml) for different treatments: NT, E/I, Rap, Rap + E/I, LY, LY + E/I. Error bars are present for each bar.]

**T-Test Table:**

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B

![Graph showing Fold Change VEGF for different inhibitors: None, Rap, LY. Error bars are present for each bar. Symbols ** and * are used to indicate statistical significance.]

**Inhibitor**

- None
- Rap
- LY

**Legend:**

- **:** Indicates statistical significance at a 0.01 level.
- *: Indicates statistical significance at a 0.05 level.
5.2.10. A PP2A-Family Phosphatase is Necessary for Epi/IBMX Induced VEGF Secretion

I have also demonstrated that a PP2A-family phosphatase was necessary for an Epi/IBMX induced increase in VEGF mRNA expression. To determine if phosphatase activity is also necessary for Epi/IBMX induced increase in VEGF secretion, I treated cells with or without Epi/IBMX in the presence or absence of the PP1/PP2A inhibitor CalA for 1 day. Conditioned media from untreated LNCaP cells contained 3.5 ng/ml VEGF (Fig 5.1). This was increased by 2.0-fold to 6.7 ng/ml with Epi/IBMX co-treatment. Conditioned media from LNCaP cells incubated with CalA contained 3.3 ng/ml VEGF, which decreased to 2.7 ng/ml with Epi/IBMX co-treatment. The parallel experiment using OA instead of CalA, at PP2A-family phosphatase specific concentrations, showed the following. LNCaP conditioned media contained 1.8 ng/ml VEGF (Fig 5.1). This increased 3.7-fold to 6.5 ng/ml with Epi/IBMX co-treatment. Conditioned media from LNCaP cells treated with OA contained 2.2 ng/ml VEGF which increased by 1.4-fold to 3.0 ng/ml VEGF with Epi/IBMX co-treatment. These data show that blocking PP2A-family phosphatase activity blocked Epi/IBMX induced secretion of VEGF, which revealed the necessity of a PP2A-family phosphatase in this process. Once again, this data mirrored that seen above for mRNA expression.
Figure 5.12: Calyculin A Blocks Epi/IBMX Induced Increase in VEGF Secretion

Conditioned media from LNCaP cells treated with CalA (10 nM) and Epi (10 µM)/IBMX (100 µM) for 24 hr was analyzed for VEGF secretion using a VEGF ELISA. Data is presented as A) ng/ml VEGF in conditioned media and B) fold change in VEGF concentration with compared to without E/I treatment. Error bars represent standard deviation of four biological replicates. P-values were obtained by Student’s t-test.

* P < 0.05 relative to NT or None; ** P < 0.05 relative to E/I; *** P > 0.05 relative to CalA.
Conditioned media from LNCaP cells treated with OA (10 nM) and Epi (10 µM)/IBMX (100 µM) for 1 day was analyzed for VEGF secretion using a VEGF ELISA. Data is presented as A) ng/ml VEGF in conditioned media and B) fold change in VEGF concentration compared to without E/I treatment. Error bars represent standard deviation of four biological replicates. P-values were obtained by Student’s t-test.

* P < 0.05 relative to NT or None; ** P < 0.05 relative to E/I; *** P > 0.05 relative to OA.
5.2.11. MEK1/Erk1 MAPK Activity is Needed for Epi/IBMX Induced VEGF Secretion

I showed using Q-PCR that the MEK1/MEK2 inhibitor U0126 did not block the Epi/IBMX induced increase in VEGF mRNA expression. To determine if this is also true for VEGF protein secretion, I treated LNCaP cells with or without Epi/IBMX for 1 day in the presence or absence of the MEK1/MEK2 inhibitor U0126. Conditioned media from untreated LNCaP cells contained 3.5 ng/ml VEGF (Fig 5.14). This increased 2.0-fold to 6.7 ng/ml with Epi/IBMX co-treatment. Conditioned media from LNCaP cells exposed to U0126 contained 3.4 ng/ml VEGF. This increased 1.1-fold to 3.8 ng/ml with Epi/IBMX co-treatment. Pre-treatment with the MEK1 inhibitor PD98059 (PD) for 5 min prior to treatment with NE-inducing agents is also adequate to block induction of Erk1/Erk2 MAPK activity in LNCaP cells (Fig 5.15A). The parallel experiment to that shown in Fig 5.14, substituting the MEK1 inhibitor PD for U0126, showed the following. Conditioned media from untreated LNCaP cells contained 1.8 ng/ml VEGF with a 3.4-fold increase to 6.0 ng/ml with Epi/IBMX co-treatment (Fig 5.15B). Incubation of LNCaP cells with PD resulted in 2.4 ng/ml VEGF that remained unchanged upon co-treatment with Epi/IBMX. These data show that blocking MEK1 and the downstream Erk1 MAPK inhibited the Epi/IBMX induced increase in VEGF secretion. This indicates that the MEK1/Erk1 MAPK pathway, while not regulating VEGF transcription, is involved in Epi/IBMX’s effect on VEGF secretion. This is contrary to what is seen in relation to Epi/IBMX induced mRNA expression that I discussed above. Inhibition of MEK1 did not affect Epi/IBMX induced mRNA expression, but it did affect secretion of VEGF.
Figure 5.14: U0126 Blocks Epi/IBMX Induced Increase in VEGF Secretion

Conditioned media from LNCaP cells treated with U0126 (10 µM) and Epi (10 µM)/IBMX (100 µM) for 1 day was analyzed for VEGF secretion using a VEGF ELISA. Data is presented as A) ng/ml VEGF in conditioned media and B) fold change in VEGF concentration with compared to without E/I. Error bars represent standard deviation of four biological replicates. P-values were obtained by Student’s t-test. * P < 0.05 relative to NT or None; ** P < 0.05 relative to E/I; *** P > 0.05 relative to U0126.
Figure 5.15: PD98059 Blocks Epi/IBMX Induced Increase in VEGF Secretion

A) LNCaP cells were pretreated with or without PD98059 (10 μM) for 5 min before stimulation with NE-inducing agents Epi (10 μM)/IBMX (100 μM) or IL-6 (2nM) or combined Epi (10 μM)/IBMX (100 μM), IL-6 (2nM) treatment for 20 min. Immunoblots using anti-phospho-Erk1/Erk2 MAPK antibody or anti-Erk2 MAPK antibody were performed as described in Materials and Methods. B and C) Conditioned media from LNCaP cells treated with PD (10 μM) and Epi (10 μM)/IBMX (100 μM) for 1 day was analyzed for VEGF secretion using a VEGF ELISA. Data is presented as A) ng/ml VEGF in conditioned media and B) fold change in VEGF concentration with compared to without Epi/IBMX. Error bars represent standard deviation of four biological replicates. P-values were obtained by Student’s t-test. * P < 0.05 relative to NT or None
5.2.12. **Conditioned Media from NED LNCaP Cells Increases Migration of HMEC Cells**

There is discussion of NE cells *in vivo* promoting tumorigenesis by stimulating surrounding cells in a paracrine-like manner; however, no one has rigorously proved these theories. Therefore, a key question to address is whether or not NED LNCaP cells secrete factors that influence other cells. I have shown that treatment with the NED-inducing agents Epi/IBMX increases secretion of VEGF. To test the biological impact of secreted factors from NED LNCaP cells, I used conditioned media from 4 d Dox treated LNCaP Cqr cells as well as conditioned media from untreated LNCaP Cqr cells. I assessed the influence of conditioned media from NED LNCaP cells on Human Mammary Epithelial Cell (HMEC) migration using a scratch assay (Fig 5.16). HMEC cells were seeded on pre-coated 6-well tissue culture plates and left to adhere. Conditioned media from Dox-induced or untreated LNCaP Cqr cells was added to the cells so that the conditioned media comprised 50% of the total media. A “scratch” was created by scraping away a strip of cells using a p200 pipet tip. The scratch was monitored and photographed at 0, 6, 25 and 30 hr. I used conditioned media from Dox treated LNCaP Cqr cells instead of from Epi/IMBX treated LNCaP cells so that the NED-inducing agents contained in the media would not have an effect on the HMEC cells.

HMEC cells migrated to fill the scar more quickly in the presence of cultured media from Dox-treated LNCaP Cqr cells (CM-Dox) compared to conditioned media from untreated LNCaP Cqr cells (CM-NT) (Fig 5.16). T-test analysis with p < 0.05 showed significant difference in the percentage of the “scratch” filled by HMEC cells in CM-Dox.
compared to CM-NT at all three time points. After as little as 6 hr, CM-Dox cultured HMEC cells had filled 57% of the scar, while CM-NT cultured cells had only filled 36% of the scar. CM-Dox cultured cells had essentially closed the scar by 25 hr (95%), while CM-NT cultured cells had yet to achieve scar closure by 30 hr (87%). This shows that conditioned media from Dox-triggered induction of PKA in LNCaP Cqr cells causes an increase in HMEC migration. This demonstrates that that NED LNCaP cells can influence surrounding cells in a paracrine manner.
Figure 5.16: Conditioned Media from NED LNCaP Cells Increases Migration of HMEC Cells

HMEC cells were seeded to form a confluent monolayer on pre-coated tissue culture plates and left to adhere. A “scratch” was created by scraping a p200 pipet tip along the plate and cells were incubated in CM-NT (NT) or CM-Dox (Dox) for 30 hr. Specific points along the “scratch” were monitored and photographed at the indicated time points. The size of the “scratch” was measured and recorded at each time point by drawing lines to correspond with the edge of the filled scratch and comparing to the original scratch and charted as percentage of “scratch” closed over time. A) Representative phase contrast photomicrographs and B) percentage of “scratch” filled. Error bars represent standard deviation of three replicate experiments. P-values were obtained by Student’s t-test. * P < 0.05 relative to NT.
5.3. Conclusion

Here I have shown that Epi/IBMX induces an increase in VEGF expression and secretion in LNCaP cells. I demonstrate the involvement of PKA, PI3K, a PP2A-family phosphatase in this phenomenon and show that mTOR is not required. In addition, I show that Epi/IBMX regulates VEGF at both the level of transcription and post transcription. PKA, PI3K and a PP2A-family phosphatase are involved in the regulation of VEGF transcription, while MEK1/ MAPK is required for Epi/IBMX induced post-transcriptional control. This regulation of post-translational control could be at the level of VEGF transcript stabilization or at the level of regulation of exocytosis. Cox et al. [259] have previously described MAPK regulation of cytokine exocytosis in neuroendocrine chromaffin cells of the adrenal medulla and Hellmich et al. [260] have reported this in human NE-like BON cells and suggested that this regulation may be via maintenance of calcium levels. This may tie in to observations that calcium levels can control VEGF secretion [261, 262].

A role for cAMP in regulating VEGF has previously been described [263, 264], as has the regulation of VEGF by the PI3K/Akt pathway [265]. However, the requirement for intact PI3K/Akt signalling in cAMP-dependent regulation of VEGF is a novel observation. Likewise, the requirement for a PP2A-family phosphatase in cAMP-dependent regulation of VEGF has not previously been described. Finally, cAMP regulation of VEGF has not previously been described to occurs at two levels, transcription and post-transcription.

I also show that conditioned media from NED LNCaP Cqr cells causes increased HMEC migration. Cell migration is critical cellular process often deregulated during tumor progression. This shows that prostatic NED cells can influence surrounding cells in a paracrine-like, pro-tumorigenic manner.
6. General Discussion and Future Directions

6.1. Overview

Castration-resistant progression of PCa following anti-androgen treatments is the major issue confronting management of disseminated disease. Upon androgen withdrawal therapy, prostatic tumors become enriched with NE cells, and these NE cells are presumed to aid in progression towards CR through the secretion of factors that promote growth and survival of surrounding cells [255]. The important role of NE cells in CR progression points to the value of NE cells as a potential target for therapeutic intervention.

Despite this compelling epidemiologic evidence, the mechanisms underlying both the transdifferentiation of adenocarcinoma cells to a neuroendocrine-like state, as well as their involvement in the progression to CRPC, are poorly comprehended. If we could understand the process of NE differentiation and the actions of NE cells, then perhaps we would be able to address the issue of androgen independence in two ways. Firstly, by blocking adenocarcinoma transdifferentiation to an NE-like state during androgen withdrawal. Secondly, by inhibiting the ability of NE cells to secrete growth and survival factors that protect surrounding cells from the effects of androgen withdrawal. For these reasons, the overarching objective of this study was to contribute to the understanding of the process of NE differentiation by profiling changes in gene expression and protein activation during transdifferentiation and then to follow up on potentially novel concepts related to NE progression and regulation.

The process of neuroendocrine differentiation, like any other biological process, requires elaborate coordination at both the level of protein production and activation. I used the Kinetworks™ Phospho-Site Screen KPSS 1.1 and Human Operon Version 3.0 gene
microarray to profile changes in the LNCaP model system during the transition to a NE phenotype. Identifying genes involved in differentiation, and understanding their expression pattern and regulation, could provide a broader understanding of the nature of prostatic NE differentiation and potentially identify molecular targets for the rational design of therapeutic agents capable of blocking cellular signalling events that underlie prostate cancer progression.

By following up on key observations in the Kinetworks Screen and the microarray, I describe two outcomes of stimulation with the NE inducing agents Epi/IBMX in LNCaP cells, and I dissect out signalling mechanisms involved in these outcomes. The two outcomes that I describe are i) a transient dephosphorylation of targets of mTORC1 - S6K1, 4EBP1 and Rb and ii) an increase in VEGF production and secretion. My first observation, the decreased phosphorylation of mTORC1 targets following stimulation with NE inducing agents, addresses the first aim of this project which is to elucidate aspects of the biochemical mechanism by which transdifferentiation of an adenocarcinoma cell to a NE cell occurs. My second observation, the increased production and secretion of VEGF following stimulation with NE inducing agents, addresses the second aim of this project which is to elucidate ways in which transdifferentiation of adenocarcinoma cells to NE cells may regulate PCa progression.
6.2. **Array Analysis of LNCaP Cells Induced to Undergo NED**

6.2.1. **Kinetworks™ Phospho-Site Screen and Microarray Analysis Show**

Reduced Protein Phosphorylation, Alterations in Gene Expression Levels and Perturbations of Signalling

The first array screen used in this project was the Kinetworks™ Phospho-Site Screen. Amongst the proteins screened in the Kinetworks™ Phospho-Site Screen, I found that stimulation with NE inducing factors caused a net reduction in protein phosphorylation. This observation was interesting to us because many studies in the field of cell signalling focus on kinases and activation of proteins by phosphorylation. Despite the importance of these activating phosphorylation events, turning off these signalling events by de-phosphorylation is equally significant.

The second array screening used in this study was in the form of an oligonucleotide microarray. The data from the microarray show alterations in gene expression. We saw a greater than two fold change in expression levels of 242 genes. These data can be useful for finding trends and for discovering phenomena of interest for validation and follow-up. However, without further investigation we do not know what these changes in gene expression actually mean to the cell. Changes in mRNA expression may not translate to similar changes in protein levels. In addition, changes in protein levels may not affect the biological impact of that protein depending on regulation (phosphorylation, ubiquitination, localization etc.). The transcriptional changes we saw in the array require direct validation using Q-PCR or Northern blot analysis as well as further follow up to determine if the changes in mRNA expression translate to changes in protein levels and biological activity of the protein. Validation and follow-up of all of the expression changes seen did not fit within
the scope of this thesis. I have focused on changes in VEGF transcript levels, as discussed in Chapter 5, based on previously described roles for VEGF in PCa metastasis and neovascularization [211]. Validation and determination of the biological significance of the remaining genes of interest provide a starting point for further projects.

While at the most fundamental level microarray studies show changes in expression levels for individual genes, I believe that the real strength of this technology lies in taking the data provided for the vast numbers of individual genes and plotting these to see alterations in signalling pathways. Using pathway analysis, I found significant alterations in the following pathways: antigen processing and presentation; circadian rhythm; adherens junction; T-cell receptor signalling; regulation of actin cytoskeleton; VEGF signalling; and focal adhesion. Perturbation of the VEGF pathway, as well as increased expression of VEGF mRNA, led me to investigate the role of NE-inducing factors in VEGF regulation for the studies in this thesis. However, another interesting observation to come out of this pathway analysis is that almost half of the pathways shown to have significant alterations (adherens junction, regulation of actin cytoskeleton, focal adhesion) are associated with aspects of cytoskeleton and cell adhesion. Cytoskeletal and cell adhesion pathways are associated with morphology changes and the formation of cell extensions, as well as with secretion. These are key characteristics of NED.

Microarray analysis of NED LNCaP cells has also been conducted by Mori et al. [266]. This study used Affymetrix GeneChips to array LNCaP cells induced to undergo NED by the following treatments: IL-6 for 7 d; Epi for 48 hr; genistein for 48 hr; charcoal stripped media for 7 d. While the Mori et al. study also used KEGG based pathway analysis of Epi-induced NED LNCaP cells, my analysis reveals a novel set of pathways and genes
altered during NED. This is likely due to the length of exposure of the LNCaP cells to Epi. Mori et al. studied 48 hr Epi exposure. The arrays discussed in this thesis studied LNCaP cells with 2 hr and 24 hr Epi/IBMX exposure, with the primary focus for analysis on the changes in immediate early genes represented in the 2 hr treatment.

6.2.2. Future Direction: Identifying Common Transcription Factors that Regulate the Changes in Gene Expression

In this project I have used two powerful profiling tools to assess changes in LNCaP cells undergoing NE differentiation: an early time point phospho-kinase screen and a later time point gene expression array. Perturbations in phosphorylation status of many intracellular signalling molecules at the short time point may contribute to alterations in mRNA expression later on. Looking at the Kinetworks™ screen, I saw that the greatest increase in phosphorylation was seen for the transcription factor CREB and mitogenic agent Erk, which can activate the transcription factor Elk [267]. Being able to identify common transcription factors that regulate the changes in gene expression seen in the microarray, would give insight into which transcription factors play a role in the transformation of LNCaP cells to a NE-like state. Of course, only a fraction of transcription factors and regulating proteins were represented on the Kinetworks™ screen. For this reason a future direction would be to analyze the promoter regions of these genes for commonalities and for known transcription factor regulation motifs. This analysis could be facilitated by the use of Onto-Tools Promoter-Express web-accessible transcription factor binding site mining tool [268]. In addition, other Kinetworks™ multi-immunoblotting screens and Kinexus™
antibody microarrays could be tested to reveal other candidate proteins that are affected by Epi/IBMX treatment of prostate cancer cells.

6.3. **Increased cAMP Regulates VEGF Production**

6.3.1. **Agents that Increase Intracellular cAMP Levels Regulate Phosphorylation of mTOR Targets in LNCaP Cells**

Loss of PTEN, and the subsequent up-regulation of growth and survival signals through the PI3K/Akt pathway, is a common feature in prostate cancer. Up-regulation of the PI3K pathway can prove to be a therapeutic challenge, providing resistance to chemotherapeutics as well as endocrine therapies. Several studies have shown that co-treatment with mTORC1 inhibitors can overcome this resistance. For example, Grunwald *et al.* [78] have shown that PCa resistance to doxorubicin conferred by PTEN status can be reversed with mTORC1 inhibitors. Also, Beeram *et al.* [269] have shown that resistance to antiestrogen treatment in breast tumors with high levels of Akt can be overcome by co-treatment with the mTORC1 inhibitor RAD-001.

I have shown that in a PTEN null prostate cancer system, with constitutively elevated growth and survival signals through the PI3K/Akt pathway, treatment with agents that increase intracellular cAMP levels regulates the phosphorylation of three signalling proteins downstream of mTORC1: S6K1, 4EBP1 and Rb. These three proteins are associated with regulation of protein translation initiation and entry into the cell cycle. Perhaps these signalling events that I describe in this study, outlining the possibility of altering signalling downstream of PI3K/Akt without disrupting critical upstream feedback loops, will contribute
alternate ways to overcome chemotherapeutic resistance and providing another tool for optimizing combination therapies.

6.3.2. **Hypo-Phosphorylation as a Reset Mechanism**

In this study I show that the overall long-term effect of stimulation with NE-inducing factors was an increased phosphorylation of the mTORC regulated proteins S6K1 and RB; however, the initial reduction in their phospho-state is intriguing. Despite it being transient in nature, this reduced phosphorylation may play an important biological role.

The significance of the hypo-phosphorylation of mTORC1 targets is unclear; however, I speculate that it may function as a reset mechanism to re-sensitize the system to new signalling stimulation. In a PTEN null cell with constitutively robust signalling through the PI3K/Akt/mTOR pathway, it is possible that downstream targets of mTORC1 are desensitized to the strength of this signal and cannot respond to subsequent signalling without some sort of reset mechanism. For example, it has been demonstrated that in cardiac cells signalling from Akt to S6K1 is impaired following prolonged Akt activation [270]. A dephosphorylation event may provide the re-sensitization needed by reducing phosphorylation to allow subsequent increases in phosphorylation to be recognized (Fig 6.1).

Self regulation by signalling pathways to shut off signalling when appropriate is well known and seen in MAPK cascades, BARK, and GnRH [271-274]. Hupfeld *et al.* [275] demonstrated an example closely related to what I see. They describe cAMP regulating the dephosphorylation of β-Arrestin1, a G protein-coupled receptor regulating protein, via PP2A in 3T3-L1 adipocytes. They describe this cAMP-PP2A mediated dephosphorylation as a regulatory balance to prevent insulin-induced desensitization of β-Arrestin1-dependent G
protein-mediated MAPK signalling. Intriguingly, they show that cAMP led to an increased association between β-Arrestin1 and PP2A and that treatment with okadaic acid caused an increase in phosphorylation of β-Arrestin1 [275]. This is reminiscent of both the cAMP induced association between S6K1 and PP2A and the okadaic acid sensitive cAMP induced de-phosphorylation of S6K1 that I show in this study.

Negative feedback loops keep signals precise and regulate their duration. However, the cAMP-induced dephosphorylation events that I saw appear to be a different situation. It seems that signalling via cAMP has a reset mechanism built in. Instead of relying on previous signalling events to be turned off by negative regulation of some sort, it first ensures that the system is in a state to recognize its message by having an initial dephosphorylation event. Data presented here have led us to propose the following two-step signalling process culminating in increased phosphorylation of S6K1 and other targets of mTOR by cAMP (Fig 6.1). The first step is a de-phosphorylation event involving the formation of a kinase-phosphatase complex including the PKA catalytic subunit and PP2A that appears not to effect the upstream mTORC. The second step is phosphorylation of these mTORC target proteins.
Figure 6.1: Reset Model for S6K1 Activation

Schematic representation of a model proposing a two-step signalling process culminating in increased phosphorylation of S6K1 (and other targets of mTOR) by cAMP. The first step is a dephosphorylation event involving the formation of a kinase-phosphatase complex including PKA catalytic subunit and PP2A that appears not to affect the upstream mTORC. The second step is phosphorylation of these mTORC target proteins.

6.3.3. Future Direction: A Balance Between PKA and Epac

The effects of cAMP have been shown to differ depending on cell type, acting either as a mitogen or inhibiting proliferation [245]. As previously mentioned, cAMP can signal through PKA or Epac. Mei et al. [276] have suggested that these cell type specific divergent roles of cAMP can be attributed to a balance between PKA and Epac signalling. For example, Hochbaum et al. [277] have demonstrated synergistic PKA and Epac signalling in cAMP-mediated mitogenesis, while Kiermayer et al. [188] have demonstrated that in PC12
cells the Epac signalling can affect the duration of PKA signalling and change the biological outcome from proliferative to differentiating. They show that activation of PKA alone is sufficient to induce MAPK activation; however, concurrent activation of Epac plays a role in sustaining this MAPK activation. They demonstrated that cAMP could only induce cell proliferation in the absence of an Epac signal.

In this study I have described several NE-related consequences of increased cAMP signalling in LNCaP cells. The next step would be to determine whether the cAMP-induced transient decrease and subsequent increase in S6K1 phosphorylation is regulated via PKA or Epac. I theorize that it is the balance between PKA and Epac signalling that regulates this signalling flux. Perhaps the initial reduction in phosphorylation is mediated by one of the cAMP signalling targets and the subsequent increase is controlled by the other (Fig 6.1). The combination of what I have shown, a co-association of PP2A with PKA, as well as previously described PKA-PP2A connections, leads me to speculate that PKA may be the cAMP agent responsible for the initial dephosphorylation of S6K1. Reports of Epac-dependent and PKA-independent cAMP-induced activation of Akt and S6K1 are consistent with Epac causing the subsequent S6K1 phosphorylation [219, 220, 278].

From these observations, I suggest two key questions that should be addressed in future directions. Firstly, through which signalling intermediate, PKA or Epac, does cAMP cause a decrease and subsequent increase in S6K1 phosphorylation? Secondly, is the initial reduction in phosphorylation of the mTORC1 target proteins that I described necessary for the subsequent increase in phosphorylation? To address these questions relating to the roles of PKA and Epac in cAMP mediated regulation of S6K1 and the necessity of the first “priming step,” one could use commercially available PKA and Epac specific analogues of
cAMP to determine the sufficiency of each of these signalling agents to induce changes in S6K1 activation state. Using these analogues, one could first look to see which one is sufficient to cause the initial decrease in S6K1 phosphorylation. One could then look to see if the same analogue is sufficient to also induce the subsequent increase in phosphorylation, or if phosphorylation levels remain repressed or return to baseline levels.

6.3.4. Future Direction: Subsequent Increase in Phosphorylation May be Via Akt Pathway

A recent paper by Wu et al. [279] has outlined a requirement for Akt in NE differentiation both by androgen withdrawal and by Epi treatment. This paper shows a similar increase in S6K1 phosphorylation seen after long term epinephrine treatment to that described in this thesis. It also states that long term epinephrine treatment causes an increase in Akt phosphorylation. In addition, Webster et al. [218] have described a 3-fold cAMP-mediated increase in S6K1 phosphorylation in hepatocyte cells. They went on to show that both basal and cAMP regulated S6K1 phosphorylation could be inhibited by rapamycin, indicating the necessity for mTORC1 in this process.

I have shown that the initial reduction in phosphorylation of S6K1, Rb and 4EBP1 appears to be mTORC1-independent; however, perhaps an mTORC1-independent event is necessary for subsequent mTORC1-dependent signalling through S6K1 as seen in these other systems. From these observations, I suggest the following question should be addressed in the future: Is this later increase in phosphorylation via the Akt pathway? To address this question, asking whether the increase in S6K1 phosphorylation that I saw was via Akt/mTOR, one could use appropriate inhibitors such as LY-294002 and rapamycin.
6.3.5. **Future Direction: How Wide Spread is the Phenomenon of a cAMP-Mediated Transient Reduction in the Phosphorylation of mTOR Targets**

The observations in this study focus on the prostate cell line LNCaP. I have also shown similar cAMP induced phosho-state kinetics in PC-3 cells to that seen in LNCaP cells. PC-3 cells are also PTEN null, so perhaps this phenomenon is specific to the PTEN null condition. Alternately, perhaps it is just more readily detectable in PTEN null cells due to their elevated basal PI3K/Akt signalling. Signalling by cAMP may recruit a phosphatase to a kinase only in situations with high levels of phosphorylation, or perhaps it regulates phosphatase actions regardless of activation state.

Another question for future directions that stems from the observations in Chapter 3 asks how widespread this phenomenon of a cAMP-mediated transient reduction in the phosphorylation of mTOR targets is. Stimulating PI3K/Akt signalling in PTEN wild type cells and then elevating cAMP levels may be a way to determine if the bi-modal phospho-kinetics I have described is a PTEN null related phenomenon or a mechanism employed regardless of PTEN status. If it is the case that this cAMP-induced suppression of phosphorylation is widespread, then this phenomenon is more significant than a signalling nuance found in NE differentiation of PCa. It may describe a built in reset mechanism that is a general aspect of cAMP signalling.
6.4. Increased cAMP Regulates VEGF Production

6.4.1. Epi/IBMX Induces an Increase in VEGF Expression and Secretion in LNCaP Cells at Both the Transcriptional and Post-Transcriptional Level

In Chapter 5, I have shown that Epi/IBMX induces an increase in VEGF expression and secretion in LNCaP cells and that it does so at both the transcriptional and post-transcriptional level. It has been suggested that secretions from NE cells influence surrounding prostatic cells in a paracrine fashion. Co-culture experiments by Wang et al. show that secretions from LNCaP cells induced to undergo NED by long term treatment with IL-6, suppress proliferation of the prostatic cell lines LNCaP, PC-3 and DU145 [280]. Xerograph experiments by Deeble et al. show that secretions from LNCaP Cqr cells induced to undergo NED by constitutive activation of PKA, enhances growth of PCa tumor cells and xerographs [169]. Perhaps the secretion of VEGF is a way in which NE cells influence both surrounding prostatic cells and angiogenesis. This relates back to our main objective, as understanding how NE differentiation can regulate the production and secretion of VEGF may elucidate ways in which to prevent NE cells from being able to protect surrounding cells from the apoptotic cues of androgen withdrawal.

6.4.2. Transcriptional Regulation of VEGF by PI3K/Akt, but Not mTORC1

In this study I have shown that stimulation with Epi/IBMX leads to an increase in VEGF expression and that this increase is dependent on signalling through PI3K/Akt and PP2A (Fig. 6.2). VEGF expression can be regulated by cAMP and by Akt; however, the
observation that an intact PI3K/Akt pathway is required for cAMP induced increases in VEGF expression has not previously been described [193, 206, 263].

One intriguing aspect of what I found is that, despite the necessity for signalling through PI3K/Akt, signalling through mTORC1 is not required for the cAMP-mediated increase in VEGF expression (Fig 6.2). Studies looking at the role of the tumor suppressor protein TSC2 in VEGF regulation have shown both mTOR-dependent and independent mechanisms [257, 281]. Under normal conditions, TSC2 regulates HIF1α through suppression of mTORC1; whereas, in TSC2/-/- conditions there is an accumulation of HIF1α as well as an up-regulation of HIF1 responsive genes [257]. In contrast TSC2/-/- up-regulation of VEGF through mTORC1-independent means may involve chromatin remodeling and appears more specific to VEGF as opposed to HIF1 regulated genes in general [257]. Since I see a PI3K dependent, mTORC1 independent up-regulation of VEGF by Epi/IBMX, this may occur via TSC2 and chromatin remodeling as described above.
Figure 6.2: Model for Regulation of VEGF Expression and Secretion

Schematic representation of a model for regulation of VEGF expression and secretion by Akt, PKA and Epac in LNCaP cells.
6.4.3. Transcriptional Regulation of VEGF by PKA

I have shown that PKA is necessary for this VEGF induced increase in expression and secretion and is sufficient to induce increased secretion. I used PKA-inducible Cqr cells as well as the PKA inhibitor H-89 to demonstrate this. H-89 has been reported to have non-specific activity towards S6K1 due to a homologous region of the activation loop of PKA and S6K1 [282]. Since I have demonstrated in the previous chapter that Epi/IBMX can regulate S6K1 signalling, I felt that it was important to verify that PKA, and not S6K1, was responsible for blocking Epi/IBMX induced increases in VEGF. Use of the Cqr cells showed that PKA was sufficient to cause an increase VEGF secretion and; therefore, the inhibitor H-89 was likely inhibiting the increase in VEGF by blocking PKA.

6.4.4. Transcriptional and Post Transcriptional Regulation of VEGF by PP2A and MEK1/Erk1 MAPK

My studies show the necessity for the activity of a PP2A-family phosphatase in cAMP induction of VEGF expression (Fig 6.2). This is further indication that phosphatase activity may play an important role in NE-differentiation. I have also shown the necessity for MEK1 activity in the Epi/IBMX induced secretion of VEGF, but not the increased mRNA expression (Fig 6.2). This is interesting because all of the other signalling components that I looked at in this study showed effects on VEGF secretion that mirrored mRNA expression, which indicates that control is at the level of altered transcription. VEGF gene expression can be controlled via both transcriptional and post-transcriptional means [283]. MEK1/Erk1 MAPK appears necessary for secretion but not for mRNA expression, which indicated that
MEK1/Erk1 MAPK control of Epi/IBMX induced changes in VEGF is at a post-transcriptional level.

Key aspects of post-transcriptional control of VEGF are transcript stability and efficiency of translation. Both aspects are regulated by mRNA binding proteins acting on the cis elements of VEGF untranslated regions (UTR) [284]. Some of these binding proteins are stabilizing and some destabilizing. Essafi-Benkhadir et al. [285] have described a role for MAPK in VEGF transcript stabilization. They established MAPK’s control over a VEGF destabilizing protein tristetraprolin. Tristetraprolin binds 3’ UTR elements in VEGF, recruiting decay machinery and resulting in transcript degradation and reduced VEGF gene expression. MAPK’s control of tristetraprolin results in promotion of VEGF mRNA stability [285]. The MEK1/Erk1 MAPK dependent post-translational control of VEGF that I see upon Epi/IBMX stimulation in LNCaP cells could be at the level of transcript stabilization, as described above.

VEGF is regulated at the transcriptional, translational and, post-translational level. MEK1/Erk1 MAPK may be necessary for Epi/IBMX induced post-translational regulation of VEGF. MAPK has been previously described as a regulator of cytokine exocytosis in chromaffin cells [259]. The MEK1/Erk1 MAPK- dependent increased accumulation of VEGF in the conditioned media of LNCaP cells stimulated with Epi/IBMX may be due to regulation of exocytosis and, hence, VEGF secretion.

6.4.5. Regulation of VEGF in Monocytic Cells

My work on regulation of VEGF in this thesis has focused on cAMP induced expression of VEGF in LNCaP cells undergoing NED. However, I also studied regulation of
VEGF in the monocytic cell line THP-1 by relaxin. This data can be found in a paper published by Kevin Figueiredo, Michael Cox and myself [286].

Relaxin is a peptide hormone that is implicated in PCa and the development of CRPC [287]. Relaxin upregulation, which has been demonstrated to occur during NED of PCa cells [288], is associated with increased angiogenesis, tumor volume and tumor cell survival [289]. Relaxin is known to stimulate the adhesion and migration of monocyte cells [290]. Since leukocytic infiltration is a contributor to tumor growth and progression [291], stimulation of monocytic cells by relaxin upregulation may be a factor in the role of NED in PCa progression. By analyzing VEGF expression levels in monocytic THP-1 cells using Q-PCR, I showed that relaxin increases VEGF expression in THP-1 cells as early as 2 hr after stimulation and that this increase is sustained for at least 24 hr. Since relaxin, like Epi, is known to elevate intracellular cAMP levels [292] levels, whether some of the same signalling requirements that I describe for Epi/IBMX induced VEGF expression in LNCaP cells are mirrored in relaxin induced VEGF expression in monocytic cells may be worthy of exploration.

6.4.6. **Future Direction: Effects of Secreted Factors from NE-Induced LNCaP Cells on Surrounding Cells**

In Chapter 5, I analyzed the conditioned media from cells induced to undergo NE differentiation and showed an increase in VEGF protein content. I also showed that conditioned media from NED LNCaP cells can increase epithelial cell migration. It would be valuable to go on to assess other effects of this NED LNCaP conditioned media on cells in
culture. The following are two key questions that I see coming out of these studies. Is VEGF critical for the increased migration of HMEC cells exposed to CM-Dox? Anti-VEGF antibodies that block VEGF receptor could be used in combination with scratch assays to answer this question. Do NED LNCaP cells secrete factors that also influence proliferation and survival of surrounding cells? This can be addressed by incubating cells in CM-Dox and CM-NT and comparing survival and proliferation of the cells using flow cytometry and BrdU or tridiated thymidine assay.

Anticipation of these further studies influenced our decision to use Dox induced Cqr cells to study PKA-mediated increases in VEGF. I predicted that when assessing the effects of conditioned media on endothelial cells, there may be changes in the endothelial cells caused by the lingering presence of Epi/IBMX in the conditioned media. Some of my preliminary experiments showed a clear Epi/IBMX effect on HMEC cells. For this reason, in our studies looking at increases in VEGF secretion, I wanted to establish that promotion of NE differentiation by Dox induced PKA activation in Cqr cells was sufficient for VEGF regulation. However, using Dox induced LNCaP Cqr cells is not completely parallel to using Epi/IBMX-induced LNCaP cells. Since induction of PKA activity in LNCaP Cqr cells is direct, instead of via a G protein, this method does not show the potential effects of signalling downstream of either G protein beta-gamma subunit nor Epac. Despite the majority of research relating to cAMP signalling focusing on PKA, the effects of Epac may be just as significant in aspects of NE differentiation of LNCaP cells.
6.4.7. Future Direction: VEGF in Progression to Androgen Independence

Using the LNCaP Cqr model, Deeble et al. [169] studied the consequence of the presence of Cqr NE-like cells on tumor growth rate in athymic mice. They found that the presence of Cqr NE-like cells resulted in only modest, not statistically significant, increases in tumor growth rate in gonad-intact mice. However, the presence of Cqr NE-like cells significantly increased tumor growth rates, motility and invasion in castrated mice, supporting a role for PKA-induced NE cells in the progression to androgen independence [169].

To test the necessity of VEGF in the PKA-NE-induction of androgen independence, I anticipate the use of the potent small molecule VEGF receptor inhibitor AG-013736. The first step would involve in vitro apoptosis and proliferation assays on LNCaP cells co-cultured with doxycyclin-induced Cqr cells in serum free media treated with AG-013736 to determine if VEGF is necessary for the NE cells to promote survival and proliferation in androgen withdrawal conditions. Subsequent in vivo studies would involve use of the experimental protocol described by Deeble et al. [169] with the addition of AG-013736.

6.4.8. Future Direction: VEGF and Bone Metastasis

NE has been implicated in metastatic progression. Increased NE content in PCa has been shown to correlate with increased metastasis [293]. However, the molecular requirements for this remain unclear. Bone is the primary site for PCa metastasis. Kitagawa et al. [294] have shown that VEGF found in PCa cell conditioned media is sufficient to induce osteoblast differentiation and that an inhibitor of VEGF was sufficient to block PCa-induced osteoblastic activity in vivo and reduce metastatic tumor burden. This VEGF-
induced increase in osteoblastic activity appears to be regulated by bone morphogenetic proteins [204].

It is believed that components of the bone matrix are responsible for attraction and integration of metastatic PCa cells into the bone environment. SPARC (secreted protein, acidic and rich in cysteine) is one such component of the bone matrix that is believed to attract and anchor metastatic tumor cells within the bone. De et al. [295] have shown that secreted VEGF can regulate functional activity of αV containing integrins (αVβ3 and αVβ5) on the cell surface of LNCaP cells. Activated αV integrins mediate migration, recognition and integration of PCa cells to bone through associations with SPARC. Integrin/SPARC interactions then stimulate further VEGF production that induces neovascularization and assists in development of the metastatic tumor [295]. It appears that regulation of the αVβ3 integrin receptor in PCa is required for tumor growth within bone and tumor-induced bone transformation [296]. In addition, increased VEGF expression has been shown to correlate with increased bone degradation, which supports a role for VEGF in bone remolding [297].

The significance of VEGF in the establishment of metastatic prostatic tumors has been described in other studies [298, 299]. Perhaps the increased expression and secretion of VEGF during NE differentiation is relevant, possibly through a VEGF/αV integrin/SPARC axis, to the increase in metastatic potential of tumors with high NE cell content. Intriguingly, changes in αVβ3 expression have been shown to correlate with progression to androgen independence in PCa [300]. This leads me to speculate about the possibility that increased VEGF secretion by NE cells, and subsequent activation of αV integrins on surrounding adenocarcinoma cells, may be a mechanism by which NE cells contribute to both increased bone metastasis as well as androgen independence in prostate cancer.
To test the necessity of VEGF in NE-induced bone metastasis, I anticipate the use of ST-2 murine bone marrow stromal cells for in vitro analysis and direct intra-tibial injections for mouse studies. ST-2 cells are a stromal cell line derived from murine fetal liver that can differentiate into osteoblasts, adipocytes and hematopoietic supporting cells [301]. ST-2 cells can be used to assess osteoblastic activity because they do not exhibit osteoblastic characteristics in standard culture conditions, but they differentiate into osteoblast-like cells and deposit calcium phosphate mineral upon exposure to osteoinductive stumuli [302]. Osteoblastic differentiation can be measured by assessing both alkaline phosphatase activity in conditioned media using commercially available kits and mineralization by staining for calcium phosphate using silver nitrate as described previously [302]. Alkaline phosphatase is indicative of early stage osteoblastic differentiation while mineralization is characteristic of late stage differentiation.

Firstly, to test if PKA-induced NE differentiation can mediate osteoblastic activity, I suggest either the co-treatment of LNCaP-Cqr and ST-2 cells or the treatment of ST-2 cells with doxycyclin-induced LNCaP-Cqr conditioned media. The necessity of VEGF in this process would be assessed using the experiments described with the addition of the VEGF receptor inhibitor AG-013736. The ability of PKA induced NE cells to regulate bone turnover, and the necessity of VEGF, would be assessed using intra-tibial injections of LNCaP and LNCaP-Cqr cells in immunodeficient mice with or without AG-013736. Following tumor growth, radiological and histological evidence for osteolytic and osteoblastic lesions would be assessed as previously described [302, 303].
6.5. Concluding Remarks

I have shown that induction of NE differentiation in the lab, by treatment with cAMP elevating agents, leads to altered activation state of several protein kinases implicated in tumor biology as well as changes in mRNA expression of a wide array of genes. Mapping these changes in gene expression to known cell signalling pathways shows perturbation of pathways involved in PCa that may potentially contribute to the ability of PCa cells to adapt to androgen independent conditions and enhance metastatic potential.

Two key novel observations described in this thesis are the ability of increased cAMP to cause bi-modal regulation of S6K1, 4EBP1 and Rb – three proteins that lie downstream of a critical tumor protein complex mTORC1; as well as increased mRNA expression and protein secretion of the angiogenic growth factor VEGF. Regulation of the targets of mTORC1 that I have described appeared to be via interaction with a PP2A-family phosphatase instead of by regulation of mTORC1. Studies by Skeen et al. [120] showing the necessity and sufficiency of mTORC1 in oncogenic transforming abilities of Akt highlight the importance of mTORC1 in cancer biology especially in the context of a PTEN null system. Since small molecule inhibitors of mTORC1 have shown limited clinical success [129], understanding alternate ways of regulating targets of mTORC1 could aid in the design of therapeutics directed at PTEN null prostatic cancer.

The up-regulation and increased protein secretion of VEGF by LNCaP cells that I have described in response to increased cAMP occur through altered transcriptional and post-transcriptional regulation. NE cells have been described as major producers of VEGF in PCa tumors [211], and this may be a way in which NE cells contribute to the survival of surrounding cells and enhance adaptation to androgen independence. I have also shown the
ability of secretions from NED LNCaP cells to increase migration of epithelial cells. This trait may be one of the ways in which NE cells can increase the metastatic potential of a tumor. Understanding signalling events involved in the stages of VEGF production and secretion by NE cells may elucidate ways to block some of the paracrine-like traits of NE cells and prevent the progression of clinically manageable PCa to lethal androgen-independent metastatic disease.
References


Appendices

A. Phosphoproteins Tracked by Kinetworks™ Phospho-Site Screen KPSS 1.1

Phosphorylation sites tracked for each protein are indicated in parenthesis.

Adducin (S662)
CDK1 (Y15)
CREB (S133)
ERK1/2 (T202/Y204)
Gsk3α/β (S21/S9)
Gsk3α/β (Y279/Y216)
cJun (S73)
MEK1 (S217/S221)
MEK3/6 (S189/S207)
MSK1 (S376)
NR1 (S896)
P38α (T180/Y182)
p70S6K (T389)
PKBα (T308)
PKBα (S473)
PKCα (S657)
PKCα (T638)
PKCδ (T505)
PKCε (S719)
PKR (T451)
Raf1 (S259)
RB1 (S780)
RB1 (S809/S811)
Rsk1 (T360/S364)
SAPK/JNK (T183/Y185)
Smad1 (S463/S465)
Src (Y418)
Src (Y529)
STAT1 (Y701)
STAT3 (S727)
STAT5 (S694)
B. Altered Gene Expression Following 2 hr and 24 hr Treatment with Epi/IBMX Seen by Human Operon Version 3.0 Microarray
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<th>Gene Symbol</th>
<th>Name</th>
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<th>24 hr</th>
<th>Fold Change from NT to 2 hr</th>
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<td>10.89</td>
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<td>1.08</td>
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| CYP4F3      | Gamma-amino- 
  butyric acid (GABA) B receptor, 2                  | 0.31    | 1.06  | 2.38                       | 3.42                        | 7.69                          | 2.25                          |
<p>| GABBR2      | Family with sequence similarity 148, member C                     | 0.39    | 1.32  | 1.32                       | 3.37                        | 3.36                          | 1.00                          |
| ZNF703      | Zinc finger protein 703                                           | 0.51    | 1.72  | 0.98                       | 3.34                        | 1.90                          | 0.57                          |
| RHOBTB1     | Rho-related BTB domain containing 1                               | 0.68    | 2.24  | 1.18                       | 3.31                        | 1.75                          | 0.53                          |
| LOC285300   | Hypothetical protein LOC285300                                     | 0.79    | 2.38  | 1.69                       | 3.28                        | 1.38                          | 0.42                          |
| TAFAP2A     | Transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha) | 0.35    | 1.16  | 1.07                       | 3.26                        | 3.03                          | 0.93                          |
| ZNF606      | Zinc finger protein 660                                           | 0.44    | 1.43  | 1.11                       | 3.25                        | 2.52                          | 0.78                          |
| CENPN       | Centromere protein N                                               | 0.49    | 1.60  | 0.99                       | 3.25                        | 2.01                          | 0.62                          |
| RNFL91      | Ring finger protein 219                                            | 0.36    | 1.18  | 2.69                       | 3.24                        | 7.42                          | 2.29                          |
| MAGEC3      | Melanoma antigen family C, 3                                      | 0.81    | 2.57  | 0.73                       | 3.16                        | 0.90                          | 0.29                          |
| TMEM66      | Transmembrane protein 66                                           | 0.53    | 1.66  | 1.02                       | 3.12                        | 1.91                          | 0.61                          |
| PKP4        | Plakophilin 4                                                     | 0.69    | 2.11  | 0.60                       | 3.04                        | 0.87                          | 0.29                          |
| R09MTD2     | RNA (guanine-9-) methyltransferase domain containing 2            | 0.73    | 2.19  | 0.69                       | 2.99                        | 0.94                          | 0.32                          |
| MDN1        | MDN1, midasin homolog (yeast)                                     | 0.37    | 1.10  | 1.05                       | 2.98                        | 2.83                          | 0.95                          |
| PER1        | Period homolog 1 (Drosophila)                                     | 0.79    | 2.32  | 0.45                       | 2.96                        | 0.57                          | 0.19                          |
| SFRED1      | Sprouty-related, EVH1 domain containing 1                         | 0.31    | 0.92  | 1.48                       | 2.95                        | 4.75                          | 1.61                          |
| LOC285484   | Hypothetical protein LOC285484                                   | 1.00    | 2.94  | 0.59                       | 2.94                        | 0.59                          | 0.20                          |
| FHD3        | Formin homology 2 domain containing 3                              | 0.62    | 1.81  | 0.57                       | 2.94                        | 0.92                          | 0.31                          |
| CD40        | CD40 molecule, TNF receptor superfamily member 5                  | 0.59    | 1.73  | 0.61                       | 2.94                        | 1.04                          | 0.35                          |
| ZNF267      | Zinc finger protein 267                                           | 0.62    | 1.83  | 0.53                       | 2.93                        | 0.85                          | 0.29                          |
| GDPD3       | Glycerophosphodiester phosphodiesterase domain containing 3       | 0.41    | 1.19  | 1.98                       | 2.93                        | 4.86                          | 1.66                          |
| ZBTB10      | Zinc finger and BTB domain containing 10                          | 0.82    | 2.39  | 0.66                       | 2.91                        | 0.81                          | 0.28                          |
| MED5        | Methy1-CpG binding domain protein 5                                | 0.63    | 1.82  | 0.48                       | 2.90                        | 0.77                          | 0.26                          |
| MAPK8       | Mitogen-activated protein kinase 8                                  | 0.39    | 1.12  | 1.93                       | 2.89                        | 5.00                          | 1.73                          |
| TBX5        | T-box 5                                                            | 0.77    | 2.23  | 2.11                       | 2.87                        | 2.73                          | 0.95                          |
| PROK2       | Prokacinelic 2                                                    | 0.76    | 2.18  | 1.80                       | 2.85                        | 2.36                          | 0.83                          |
| CPEBI       | Cytoplasmic polyadenylation element binding protein 1              | 0.44    | 1.24  | 0.82                       | 2.85                        | 1.89                          | 0.66                          |
| ABC2C       | ATP-binding cassette, sub-family C (CFTR/MPR), member 2            | 0.57    | 1.63  | 3.44                       | 2.85                        | 6.01                          | 2.11                          |
| TRA@        | YME1-like 1 (S. cerevisiae)                                        | 0.85    | 2.39  | 0.46                       | 2.80                        | 0.53                          | 0.19                          |
| UTP3        | UTP3, small subunit (SSU) processome component, homolog (S. cerevisiae) | 0.55    | 1.52  | 3.01                       | 2.76                        | 5.47                          | 1.98                          |
| DBX2        | Developing brain homeobox 2                                        | 0.63    | 1.73  | 0.79                       | 2.74                        | 1.25                          | 0.46                          |
| SFRS2IP     | Splicing factor, arginine/serine-rich 2, interacting protein       | 0.90    | 2.46  | 0.82                       | 2.74                        | 0.91                          | 0.33                          |
| MRP3S25     | Mitochondrial ribosomal protein S25                                | 0.87    | 2.39  | 0.52                       | 2.73                        | 0.60                          | 0.22                          |
| RBM27       | RNA binding motif protein 27                                        | 0.71    | 1.89  | 1.39                       | 2.68                        | 1.96                          | 0.73                          |</p>
<table>
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<th>Gene Symbol</th>
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<td>BRSK1</td>
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<td>2.04</td>
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<td>DLGAP3</td>
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<td>2.03</td>
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<td>FRP3 pre-mRNA processing factor 3 homolog (S. cerevisiae)</td>
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<td>0.95</td>
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<td>0.47</td>
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<td>Protein kinase, AMP-activated, beta 2 non-catalytic subunit</td>
<td>1.78</td>
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<td>0.87</td>
<td>0.45</td>
<td>0.49</td>
<td>1.09</td>
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<td>Shugoshin-like 1 (S. pombe)</td>
<td>1.56</td>
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<td>0.44</td>
<td>0.98</td>
<td>2.21</td>
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<td>ERECC5</td>
<td>(Cockayne syndrome))</td>
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<td>0.56</td>
<td>1.02</td>
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<td>0.44</td>
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<td>Zinc finger, FYVE domain containing 1</td>
<td>1.23</td>
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<td>0.44</td>
<td>0.87</td>
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<td>0.43</td>
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<td>Unc-119 homolog B (C. elegans)</td>
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<td>0.73</td>
<td>0.43</td>
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<td>0.43</td>
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<td>Transcription factor AP-4 (activating enhancer binding protein 4)</td>
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<td>DAR32</td>
<td>Aspartyl-tRNA synthetase 2, mitochondrial</td>
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<td>Solute carrier family 25 (carnitine/acyl carnitine translocase), member 29</td>
<td>1.53</td>
<td>0.65</td>
<td>1.23</td>
<td>0.42</td>
<td>0.81</td>
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<td>Amine oxidase, copper containing 2 (retina-specific)</td>
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<td>0.68</td>
<td>0.84</td>
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<td>2.58</td>
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<td>0.41</td>
<td>0.13</td>
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<td>0.46</td>
<td>3.68</td>
<td>0.41</td>
<td>3.28</td>
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<td>1.34</td>
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<td>0.76</td>
<td>1.90</td>
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<td>0.40</td>
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<td>0.39</td>
<td>0.61</td>
<td>1.57</td>
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<td>0.92</td>
<td>0.39</td>
<td>0.75</td>
<td>1.96</td>
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<td>0.71</td>
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<td>1.55</td>
<td>0.37</td>
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<td>3.63</td>
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<td>PPF2R2B</td>
<td>Protein phosphatase 2 (formerly 2A), regulatory subunit B, beta isoform</td>
<td>2.38</td>
<td>0.95</td>
<td>0.86</td>
<td>0.37</td>
<td>0.33</td>
<td>0.91</td>
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<td>DMD</td>
<td>Dystrophin (muscular dystrophy, Duchenne and Becker types)</td>
<td>1.95</td>
<td>0.71</td>
<td>0.54</td>
<td>0.36</td>
<td>0.28</td>
<td>0.77</td>
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<td>N-acetyltransferase 2 (arylamine N-acetyltransferase)</td>
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<td>0.65</td>
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<td>0.36</td>
<td>0.65</td>
<td>1.79</td>
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<td>JFT74</td>
<td>(Chlamydomonas)</td>
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<td>1.95</td>
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<td>24 hr</td>
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<tr>
<td>C9orf24</td>
<td>Chromosome 9 open reading frame 24 Glutamate receptor, ionotropic, N-methyl-D-aspartate-like 1A</td>
<td>1.39</td>
<td>0.49</td>
<td>1.47</td>
<td>0.35</td>
<td>1.06</td>
<td>3.01</td>
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<tr>
<td>ORIL1A</td>
<td>G protein-coupled receptor 1 (GPR1A)</td>
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<td>0.70</td>
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<td>0.35</td>
<td>0.58</td>
<td>1.66</td>
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<td>TXNIP</td>
<td>Thioredoxin interacting protein</td>
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<td>0.89</td>
<td>0.57</td>
<td>0.35</td>
<td>0.22</td>
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<td>ORC4L</td>
<td>Origin recognition complex, subunit 4-like (yeast) Myeloid/lymphoid or mixed-lineage leukemia</td>
<td>1.80</td>
<td>0.62</td>
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<td>(trithorax homolog, Drosophila)</td>
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<td>0.35</td>
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<td>2.92</td>
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<td>Cell division cycle 7 homolog (S. cerevisiae)</td>
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<td>1.71</td>
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<td>Hypothetical protein LOC100131941</td>
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<td>0.32</td>
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<td>0.91</td>
<td>0.59</td>
<td>0.32</td>
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<td>CTD (carboxy-terminal domain, RNA polymerase)</td>
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<td>0.40</td>
<td>1.49</td>
<td>0.31</td>
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<td>1.00</td>
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<td>0.76</td>
<td>1.00</td>
<td>0.30</td>
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<td>0.78</td>
<td>0.28</td>
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<tr>
<td>LIPF</td>
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<td>0.53</td>
<td>0.85</td>
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<td>0.26</td>
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<td>0.26</td>
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<td>PTBP2</td>
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<td>0.26</td>
<td>0.31</td>
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<td>Glutamate-ammonia ligase (glutamate synthase) domain containing 1</td>
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<td>0.56</td>
<td>0.37</td>
<td>0.24</td>
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<td>PKCG</td>
<td>Protein kinase C, gamma</td>
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<td>0.43</td>
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<td>0.25</td>
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<td>Solute carrier family 16, member 8 (monocarboxylic acid transporter 3)</td>
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<td>0.49</td>
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<td>0.64</td>
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<td>Name</td>
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<td>24 hr</td>
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<td>Fold Change from NT to 24 hr</td>
<td>Fold Change from 2 hr to 24 hr</td>
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<tr>
<td>SFTPB</td>
<td>Surfactant, pulmonary-associated protein B</td>
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<td>0.22</td>
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<td>TGM3</td>
<td>Transglutaminase 3 (E polypeptide, protein-glutaminyl-γ-glutamyltransferase)</td>
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<td>0.22</td>
<td>1.29</td>
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<td>TRU1</td>
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<td>0.21</td>
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<td>TARKF</td>
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<td>MAOA</td>
<td>Monoamine oxidase A</td>
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<td>Myosin 13A</td>
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<td>1.04</td>
<td>0.17</td>
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<td>IL12A</td>
<td>Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)</td>
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<td>3.06</td>
<td>0.16</td>
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<td>0.25</td>
<td>1.15</td>
<td>0.16</td>
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<td>GNAZ</td>
<td>Guanine nucleotide binding protein (G protein), alpha z polypeptide</td>
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<td>0.31</td>
<td>0.89</td>
<td>0.14</td>
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<td>0.55</td>
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<td>0.81</td>
<td>0.06</td>
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