THE EFFECT OF CYCLIN G ASSOCIATED KINASE ON ANDROGEN RECEPTOR
FUNCTION AND PROSTATE CANCER PROGRESSION

by

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Abstract

The mechanism by which prostate cancer progresses from androgen dependence (AD) to androgen independence/castration resistance (AI/CR) is currently a major focus of prostate cancer-related research. Prostate cancers that progress to a state of AI/CR are typically resistant to most standard types of treatments. Due to its primary role in driving normal prostate cell growth and proliferation, the androgen receptor (AR) is believed to play a key role in progression. Coregulators, or any proteins which may either enhance or abrogate AR activity, are considered to be one of the potential mechanisms by which AR function may become impaired. Cyclin G-associated kinase (GAK) was initially identified as a potential coregulator of AR in a Tup 1 repressed transactivation system. A LNCaP cDNA library was screened for proteins which interacted with the NH2-terminus of AR. GAK was isolated from three independent library clones using two different AR baits (AR 1-549 and AR 1-646). This interaction was confirmed via GST pulldown and coimmunoprecipitation experiments, and preliminary luciferase assays suggested that GAK activates AR in a hormone dependent manner.

In this study, my objectives were to validate GAK's role as a coregulator of AR and to determine if overexpressing GAK affects progression to AI. In vitro luciferase assays whereby GAK was either overexpressed or knocked down in both LNCaP and PC3 cells did not significantly affect AR activity. Xenograft experiments utilizing a doxycycline (DOX) inducible lentiviral LNCaP-GAK overexpressing stable cell line demonstrated that while GAK may not play a significant role in modulating AR activity, it may adopt a more subtle role enhancing tumour take and tumour volume growth rate in vivo. While these results could not confirm GAK to be a direct coregulator of AR, it is entirely possible that GAK may influence prostate cancer progression, albeit indirectly. Recent publications report a growing amount of evidence suggesting GAK's involvement in the critical cellular process of clathrin coated vesicle endocytosis, the dysregulation of which could potentially indirectly affect AR regulated genes.

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3β-HSD	β-hydroxysteroid dehydrogenase
ACTH	adrenocorticotropic hormone
AD	androgen dependence
AF-1	activation function 1
AF-2	activation function 2
AI	androgen independent
AMP	ampicillin
AR	androgen receptor
ARA	androgen receptor-associated protein
ARE	androgen response element
ARK-1	actin regulating kinase
ATA GAK	pcDNA3.1-GAK V5 ATA mutated plasmid
BCRA1	breast and ovarian cancer susceptibility gene-1
BMI	body mass index
BPH	benign prostatic hyperplasia
CAPB	cancer of the prostate and brain
CBP	cAMP-response element binding protein/p300
CCV	clathrin coated vesicle
cdc25B	Cdk-activating phosphatase 25B
CO_2	carbon dioxide
CR	castration resistance
CRH	corticotrophin-releasing hormone
CSS	charcoal serum stripped
DBD	DNA binding domain
DHEA	dehydroepiandrosterone
DHT	5α-dihydrotestosterone
DMEM	Dulbecco's Modified Eagles's Media
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECL	electrochemiluminescence
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EV	pcDNA3.1 control plasmid
FAK	focal adhesion kinase
FBS	fetal bovine serum
FSH	follicle stimulating hormone
GAK V5	pcDNA3.1-GAK V5 plasmid
GAK	cyclin G associated kinase
hER	estrogen receptor
hGR	glucocorticoid receptor
hMR	mineralocorticoid receptor
HPC1	hereditary prostate cancer gene 1

HPV	human papilloma virus
hPR	progesterone receptor
HRP	horseradish peroxidase
IGF	insulin-like growth factor
IGF-1R	insulin-like growth factor type I receptor
IL-12	novel interleukin 12 interacting protein
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilodalton
LB	Luria-Bertani
LBD	ligand binding domain
LH	lutenizing hormone
LHRH	lutenizing hormone-releasing hormone
LNCaP-DEST	LNCaP-DEST control cell line
LNCaP-GAK	LNCaP-GAK overexpressing cell line
MSR1	macrophage scavenger receptor
N_2	nitrogen
NHT	neoadjuvant hormone therapy
nmt55	nuclear matrix protein
NTD	N-terminal domain
P/S	penicillin-streptomycin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC3	prostate carcinoma cell line
PCAP	predisposing to prostate cancer
PI3K	phosphatidylinosital kinase
PSA	prostate-specific-antigen
PSF	PTB-associated splicing factor
PTEN	phosphatase and tensin homolog
PVDF	polyvinylidene difluoride membrane
R1881	synthetic androgen
Rb	retinoblastoma protein
RLU	relative luciferase units
RNASEL	ribonuclease L
RPMI	RPMI 1640 Media
RREB-1	Ras responsive element binding protein-1
SDS	sodium dodecyl sulfate
SHBG	sex hormone-binding globulin
SRC1	steroid receptor coactivator
T	testosterone
TGF	transforming growth factor
X	times

Acknowledgements

Finally, I would like to extend endless amounts of love and gratitude to my family. I have an enormous amount of respect for their sacrifices and I am immensely proud of their accomplishments. My parents instilled in me the value of hard work, integrity, and commitment. To my mom, Louise Emsley, who taught me to never, ever give up, no matter what. To my brother, Jack, who has always been there for me and who has become one of my best friends. And finally to my dad, Doug Leik, who passed away suddenly January 11, 2008, whose quiet strength, compassion for people, and gentle manner I will miss immensely. He always told me to keep it simple, to do what you love. I have learned that lesson well over the past few years. I would like to dedicate this thesis to him, in his loving memory, whose quiet strength I admired so much and without whom I would not be here, completing this difficult chapter, and very much looking forward to the next. From the bottom of my heart, thank you for everything you did for me and for giving me the strength to do this now. I love you and you have my word, the next chapter will be full of passion and purpose.

To my dad, Doug Leik
March 06, 1950 – January 11, 2008

That's Truckin'

Chapter 1   Introduction

1.1   Overview of Prostate Cancer

Prostate cancer is the most commonly diagnosed cancer in Canadian men, and the the second leading cause of cancer-related death in North America [1]. It is estimated that in 2008, 24,700 Canadian men will be diagnosed with prostate cancer, and 4,300 will die from it (*www.cancer.ca*). Furthermore, one in eight men will develop prostate cancer sometime during his lifetime [2]. Overall, incidence of prostate cancer has been dramatically increasing, peaking in 1993, the year PSA testing was introduced (*www.cancer.ca*). In contrast to incidence, mortality rates have been gradually decreasing at a rate of 2.9% annually between 1995 and 2004 (*www.cancer.ca*).

The prostate is a small, walnut sized exocrine gland consisting of two semicircular lobes which surrounds the urethra, the tube which carries urine from the bladder to the tip of the penis. It functions to produce and store seminal fluid. The development of the prostate is regulated by androgens, or male sex hormones, such as testosterone (T) and dihydroxytestosterone (DHT). These activate the androgen receptor (AR) which regulates genes that are responsible for promoting prostate cell growth. When this process becomes dysfunctional, the result is uncontrollable cell growth, metastasis to bone tissue, and eventual fatality.

AI/CR prostate cancer is known to be resistant to standard types of treatment such as surgery, radiotherapy, and androgen withdrawal therapy, all of which block the growth promoting effects of androgens and activate apoptosis. If detected early, prostate cancer can be cured by surgery or radiotherapy [3] - [4]. However, many patients present with locally advanced or metastatic prostate cancer for which there are currently no curative treatment options [5]. More advanced disease is treated with androgen withdrawal therapy, also referred to as neoadjuvant hormone therapy (NHT) [6]. Patients are treated with drugs which either prevent the synthesis of androgens or irreversibly bind to the androgen receptor respectively, therefore reducing the ability of the androgen receptor to

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continue driving the expression of growth promoting genes within the prostate. Lutenizing hormone releasing hormone (LHRH) analogues, such as goserelin (Zolodex ®), leuprorelin (Prostap®), and triptorelin (Decapeptyl®), inhibit the release of follicle stimulating hormone (FSH) and lutenizing hormone (LH) from the pituitary gland [7]. Under normal physiological cricumstances, FSH and LH act synergistically on the testes to promote androgen production, ultimately playing a substantial regulatory role in male development, growth, pubertal maturation, and reproductive processes. Alternatively, anti-androgens such as flutamide (Chimax®, Drogenil®), bicalutamide (Casodex®), and cyproterone acetate (Cyprostat®) function by attaching themselves to AR, effectively inhibiting androgen binding and stimulation of AR related activity [8].

Despite these treatment options, a hallmark of prostate cancer is its ability to progress to a lethal state which is variably referred to as androgen independent (AI), hormone refractory, or more recently castration resistant (CR) prostate cancer. Castration resistance refers to the observation that residual androgens are quite often detectable within prostate tumours of castrate individuals [9]. These androgens are believed to originate from either the conversion of androgens generated by the adrenal glands or de novo biosynthesis of androgens from cholesterol precursors within the prostate [10]. Either way, androgens having an intracrine origin are thought to play a critical role in facilitating the androgen receptor-mediated signaling pathways leading to disease progression. It has been demonstrated that several androgen-metabolizing genes within castrate-resistant metastases are upregulated [11], suggesting that endogenous steroidogenic pathways are also upregulated and may be contributing to the negative outgrowth of castration-adapted tumors. Patients that acquire androgen independent/castration resistant (AI/CR) prostate cancer have a median survival of ~19 months [5], [12], [13]. Survival can be briefly extended through the administration of docetaxel-based chemotherapy [14]; however, more effective treatment strategies must be developed in order to prevent the lethality affiliated with AI/CR prostate cancer.

Progression to AI/CR may be due to several possible molecular mechanisms. Loss of AR does not appear to be a contributing factor, as AR expression is retained

throughout progression [15]. In fact, 80% of AI/CR prostate cancers possess high levels of AR [16]. It has been suggested that inactivation of AR may play a role [15] - [17]. In fact, *in vivo* knockdown of AR has been shown to delay progression to AI/CR [18]. While there are several mechanisms by which prostate cancer may progress to a state of AI/CR, including amplification of AR [19] - [20] and functional mutations [21], [22] - [23] prevailing evidence suggests that the most commonly occurring mechanisms for progression to AI most likely involve ligand-independent activation of AR either through deregulation of cell signaling pathways and/or altered activity and expression of AR coregulators [15], [24] - [25]. This thesis focuses on the inappropriate activation of AR by coregulators, or proteins which interact with AR to enhance or reduce transactivation of target genes [26]. Coregulators achieve this via numerous mechanisms, namely facilitating chromatin remodeling, recruiting basal transcription factors, modulating the appropriate folding of AR, ligand binding to AR, or modulating the N-terminal/C-terminal interaction of AR, stabilizing the AR and/or its interaction with DNA, or aiding in translocation [27]. Aberrant expression/activity of these coregulators is thought to be closely associated with the development and maintenance of the AI/CR phenotype in human prostate cancer.

1.2   Prostate Cancer Epidemiology

Prostate cancer is the sixth most common cancer world wide and the second leading cause of cancer-related death in North America [1]. In the developed parts of the world, 15.3 % of cancers are prostate related [28]. The incidence of prostate cancer is highest in US, Canada, Scandanavia and lowest in Asian countries such as China [29]. Asians have the lowest incidence (107.2 cases per 100,000), while African Americans have the highest incidence (275.3 per 100,000), which is 60 % higher than Caucasians (172.9 per 100,000) [30].

The causes of prostate cancer are not well understood but contributing factors such as age, geographical region, genetic predisposition, and diet are all thought to play a

role [31] - [32]. Prostate cancer is infrequent in men below 40 years of age, lending merit to the observation that prostate cancer is an age related disease [33]. In the US, 13.7 % of the prostate cancer cases are diagnosed in men between the ages of 60 and 79 years, whereas only 2.2% of the cases are diagnosed in men between the ages of 40 and 59 years [34]. Evidence from autopsies illustrates that 50% of men over 70 years old have some sign of prostate cancer, while the same is true for 20% of men over 50 [34]. Different geographical regions have also been shown to yield variable rates of incidence. For example, Japanese men who have migrated to the West at a younger age have a higher risk of acquiring prostate cancer than do men who migrated later on in life, or not at all [35].

Genetic predisposition to prostate cancer is believed to be one of the more prominent factors involved in determining cause. Men with a familial history have a 2-4 times higher incidence of prostate cancer [36]. Furthermore, men with a familial history of breast cancer possess a higher risk, and interestingly, men who acquire prostate cancer also have a higher risk of developing central nervous system tumours [37]. In terms of specific genetic abnormalities linked to prostate cancer, chromosomal rearrangement of 21q has been observed in over 50% of prostate cancers [38]. Furthermore, linkage studies have been conducted to determine which genes are linked to an increased susceptibility of prostate cancer. Some of the possible candidates are hereditary prostate cancer gene 1 (HPC1), predisposing to prostate cancer (PCAP), cancer of the prostate and brain (CAPB), ribonuclease L (RNASEL), and macrophage scavenger receptor (MSR1) [39]. Genetic polymorphism of AR, whereby the transcript contains a decreased number of CAG and GGC repeats within exon 1 of the transactivation domain, has also been linked to an increased incidence of prostate cancer [40].

Diet is another possible contributing factor. Increased risk of prostate cancer is affiliated with consumption of dietary fat found in meat and dairy products [31], [41]. High-fat diets are rich in n-6 fatty acids [32], which are often found in meat and dairy products. Grilling or frying meat generates heterocyclin amines and polycyclic aromatic hydrocarbons, and these chemical compounds have both been linked with increased risk

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of postate cancer [32]. Postive correlation of dairy products and red meat with prostate cancer could also involve alpha-methylacyl-CoA racemase activity. Alpha methyl coenzyme-m reductase is an enzyme which is abundant in dairy products and red meat, and which functions in the peroxidation of branched fatty acids. The byproduct of this enzyme, hydrogen peroxide, may damage DNA [42]. Diets rich in calcium are also linked to an increased risk of prostate cancer [43], and this enzyme is required for beta-oxidation of phytanic acid present in dairy products [32]. It has also been reported that diets rich in fat allow for an increased consumption of insulin like growth factor (IGF), which is linked to increased cell proliferation, decreased rates of apoptosis, and up to 4.3 times increased risk of developing prostate cancer [44].

Decreased risk of prostate cancer is linked to low-fat diets high in n-3 fatty acids [32]. Furthermore, there are several compounds which are affiliated with a decreased risk of incidence, these being lycopenes [45], selenium [46], vitamin E and betacarotene [47] with a 16%, 66%, 40%, and 40% decrease in incidence respectively. Incidentally, soya beans, which contain the isoflavones genistin and daidzin, are thought to reduce the incidence of prostate cancer. These two enzymes inhibit tyrosine kinases which play large roles in cell proliferation and angiogenesis [48]. There is currently inadequate evidence to suggest that vegetables, fruit, carotenoids, and vitamins A and C reduce risk [31].

Gene fusions are another potential cause of prostate cancer. Recently it was demonstrated that 41% of prostate cancer patients present with a TMPRSS2-ERG gene fusion, and 82% of fusion postive prostate cancers were Gleason Grade 6 or 7. The recurrent gene fusion involves the 5' untranslated region of transmembrane serine protease 2 (TMPRSS2), an androgen regulated gene, and a member of the ETS (E26 transformation-specific) family of genes which includes ERG, ETV1, or ETV4 [49]. This distinct class of fusion gene rearrangements demonstrates that dormant oncogenes have the potential to be activated in prostate cancer by juxtaposition to tissue-specific or ubiquitously active genomic loci [49]. TMPRSS2/ERG gene fusion products possess

variable biological activities all of which promote tumor development and progression [50].

Viral infections may also play a role in prostate carcinogenesis. Recently, it has been hypothesized that human polyomavirus BK (BKV) may be a possible candidate because of its transforming properties. Out of 26 patients analyzed, BKV-DNA was discovered in 54%, 31%, and 85% of urine, plasma, and fresh prostate cancer specimens, respectively [51]. Another virus that has been linked to prostate cancer is xenotropic murine leukemia virus-related virus (XMRV), which has been identified in prostate cancer tissue from patients homozygous for a reduced-activity variant of the antiviral enzyme RNase L [52]. Forty percent of prostate cancer patients homozygous for a mutation in RNase L (R462Q) are positive for XMRV, while the virus is rarely detected in patients heterozygous for R462Q or those carrrying the wild type allele [53].

Aside from age, genetic predisposition, diet, gene fusions, and viral etiology, there are only a few more commonly accepted risk factors. A body mass index (BMI) between the range of 35.5 and 39.9 yields an increased risk of up to 34% [54]. Some studies have demonstrated that an increased number of sexual partners correlates positively with the risk of prostate cancer [55], much like it was discovered that infection with human papilloma virus (HPV) is directly correlated with the risk of cervical, vaginal, and rectal cancer. Interestingly, one consistent finding was that men from rural areas who farm or work in agriculture present with higher stage and grade prostate cancer than do men from urban areas [56]. Furthermore, men who partake in heavy physical labor are at higher risk, as are those involved in heavy industry, rubber manufacturing, and newspaper printing. This is presumably due to the exposure these men have to certain chemical compounds in their occupations [57]. Social class, smoking [58], alcohol consumption [59], physical [60] and sexual activity [61], and whether or not an individual has had a vasectomy [62] appear to have no correlation with the risk of acquiring prostate cancer.

1.3   Biology and Function of the Prostate Gland

The prostate gland is a small, walnut sized exocrine gland located at the base of the bladder anterior to the rectum (Figure 1). It consists of three glandular zones: a peripheral zone, a central zone, and a transitional zone, all of which differ in their composition and susceptibilities to cancer (Figure 2). The peripheral and transition zones are the most prone to cancer, presumably due to the higher rates of cell turnover in those regions [63].   Cancers originating from the peripheral zone are more frequently associated with histological features of progression, such as extracapsular extension and seminal vesicle invasion, than transition zone cancers [64].   The prostate gland is comprised of fibromuscular stroma and glandular epithelia [65].   These two compartments are separated by the basement membrane. The stromal tissue consists of fibroblasts, smooth muscle, and endothelial cells, while the glandular epithelia consist of basal, secretory luminal, and neuroendocrine cells. Disturbances to the homeostatic mechanisms which control the normal sequence of proliferation, differentiation, and apoptosis often result in neoplastic transformation [65].

The primary function of the prostate gland is to produce and store a slightly alkaline fluid that consists of 10-30% of the total volume of semen, which consists of spermatozoa and seminal fluid. The other 70-90% of the volume of semen is produced in the seminal vesicles, which are located right above the prostate gland. The composition of semen itself is species specific, with variations occurring in the type of simple sugars and basicity of the fluid. Human seminal fluid contains proteolytic enzymes, acid phosphatases, PSA, zinc, citric acid, among many other substances [66]. Furthermore, proteomic analysis of semen identified multiple post-translational variants of the majority of the proteins. The urethra, which runs through the prostate gland to the tip of the penis, is responsible for transporting both urine and semen from the bladder and seminal vesicles/prostate gland, respectively. Smooth muscles and nerves, which help with ejaculation and control erectile function respectively, run alongside the prostate gland [67].
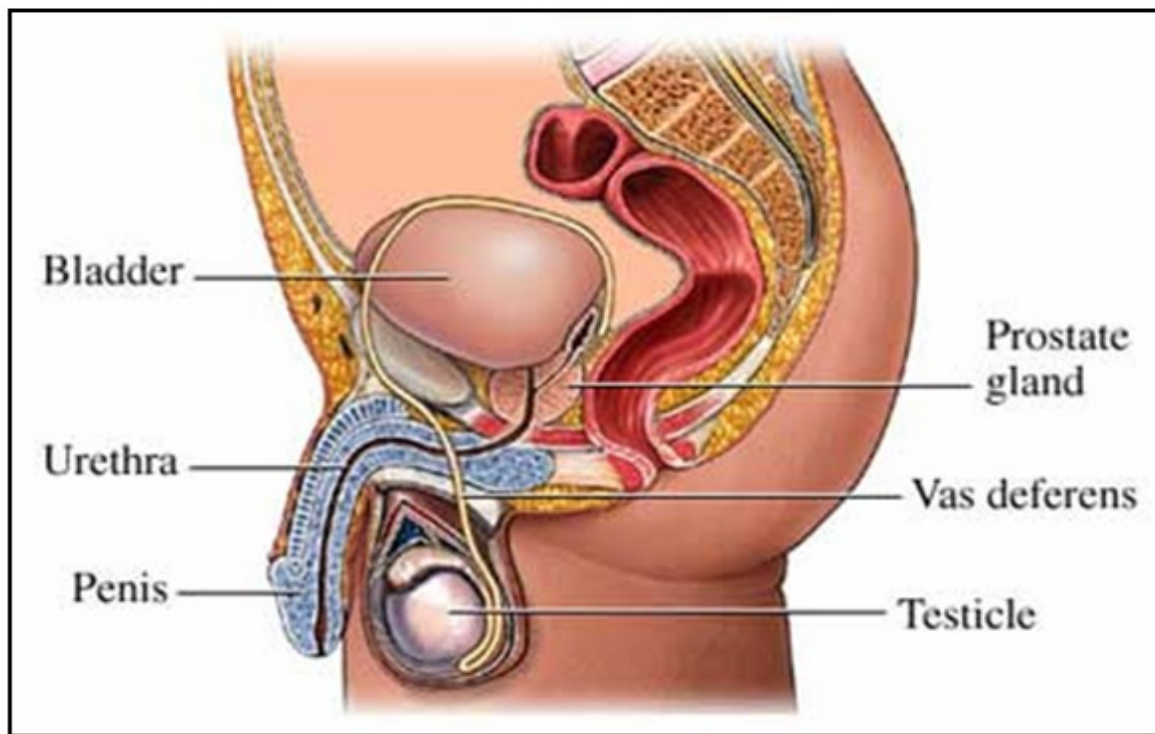
Figure 1 - The male reproductive system.

The prostate gland is located at the base of the bladder anterior to the rectum. The urethra runs through the prostate gland to the tip of the penis and is responsible for transporting both urine and semen from the bladder and seminal vesicles/prostate gland *(taken from http://www.bchealthdept.org/prostate.html).*
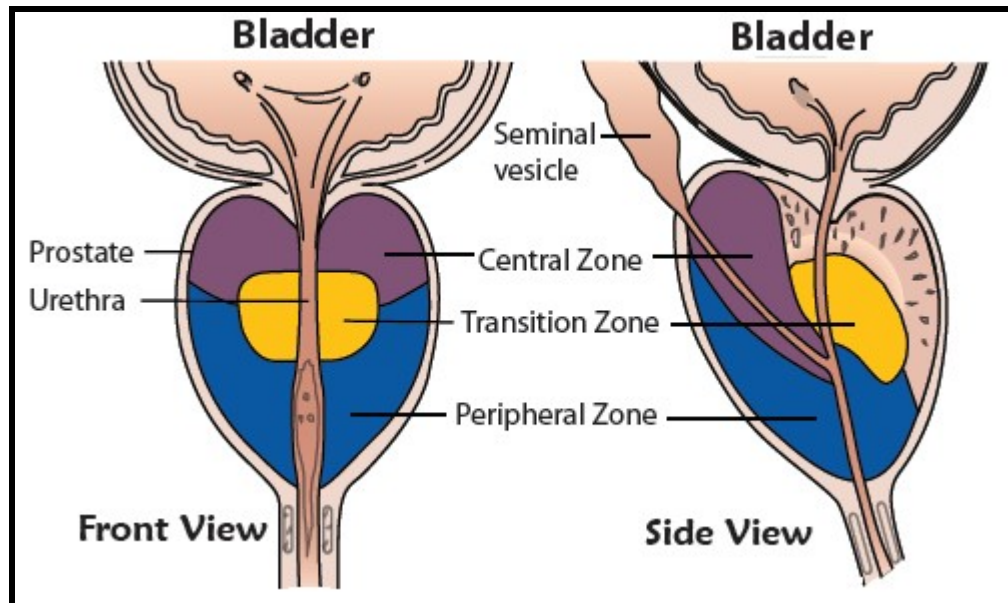
Figure 2 - The zones of the prostate gland.

The prostate consists of three zones, the central zone, the transitional zone, and the peripheral zone, each of which varies in its cellular composition and susceptibility to prostate cancer. The peripheral and transition zones exhibit higher proliferation rates than the central zone, and appear to be more prone to prostate cancer *(taken from http://www.meducator.org/archive/20070320/04fig1.jpg)*.

1.4 Disorders of the Prostate

There are a few main disorders that affect the prostate gland, the symptoms of which are often overlapping, making diagnosis sometimes difficult. Prostatitis is the first condition whereby the prostate gland becomes inflamed. Approximately 2-10% of men are afflicted with this condition [68]. There are several different forms of prostatitis: acute bacterial, chronic bacterial, chronic non-bacterial, and asymptomatic inflammatory prostatiti [69]. Acute and chronic prostatitis are clearly defined by the detection of microbial agents and they are often treated with antibiotics [70]. Chronic non-bacterial prostatitis, also referred called chronic pelvic pain syndrome, is the third type of prostatitis which is responsible for over 95% of all cases [71]. Chronic non-bacterial prostatitis is further subdivided into inflammatory and non-inflammatory categories, and is often treated with alpha blockers, physical therapy, antihistamines, and nerve modulators [72]. The etiology of chronic non-bacterial prostatitis is characterized by pain and urinary/ejaculatory symptoms. The fourth type, asymptomatic inflammatory prostatitis, does not appear to possess any clinical significance and usually does not require treatment [73].

Benign prostatic hyperplasia (BPH) is another disorder of the prostate that affects many older men [74]. The prostate enlarges to a point where urination becomes difficult, resulting in urinary hesitancy, weak stream, nocturia, incontinence, and recurrent urinary tract infections [74]. The exact cause of BPH is not known, however, BPH itself is not a malignant condition. It is believed to originate predominantly in the periurethral zones, an overall area which comprises less than 2% of the entire mass of the normal prostate [75]. BPH progresses in three distinct stages. In the earliest stage, microscopic nodules form as a result of glandular budding and branching, resulting in a subtle enlargement of the prostate without any clinical manifestations. In the second stage, the microscopic nodules develop into macroscopic nodules, causing a distortion of the original anatomy of the prostate; however, symptoms may not yet be apparent. In the final stage, it is hypothesized that an acute event, such as a prostatic infarction, result in clinical

symptoms, as enlargement alone is not sufficient to obstruct the urinary tract [75]. Spiro *et. al.* demonstrated by serially sectioning prostates taken from over 100 patients with and without acute urinary retention that over 85% of the patients suffering from acute urinary retention possessed significant prostatic infarcts [76]. For men with mild symptoms, the appropriate course of action is watchful waiting paired with annual reassessment [74]. When treatment is necessary, BPH has been traditionally treated surgically via transurethral resection, whereby an instrument is placed up the urethra to remove tissue which is blocking and restricting the flow of urine [77]. However, over the past decade, other remedies have been employed such as the use of alpha blockers and newer laser-based surgical techniques, which have been reported to be as effective as transurethral resection [78] - [79].

The third disorder of the prostate is prostate cancer itself. It is one of the most common concerns for aging men in developing countries. The relationship between prostate cancer and BPH is not well established. BPH is not considered a precursor to prostate cancer, as most prostate cancers originate in the external, peripheral zone as opposed to the transurethral zone. Furthermore, the largest study conducted to date utilizing over 85,000 BPH patient samples demonstrated only marginally elevated risk of prostate cancer among these individuals [80]. Nevertheless, because prostate cancer and BPH share common risk factors, it is difficult to differentiate how one influences the onset of the other. Prostate cancer itself is most often asymptomatic. Most men are completely unaware that they are afflicted with the disease. Symptoms of prostate cancer generally occur in advanced stages, making early detection that much more vital [81]. Symptoms of prostate cancer are weak urine flow, frequent urination, urgency, inablity to urinate, pain or burning of the urethra, blood in the semen, pain in the back or hips, and painful ejaculation [81].

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1.5   The Production of Androgens

The production of androgens, or male steroid hormones, is regulated by the hypothalamic-pituitary-gonadal hormone axis (Figure 3).   The organs which are specifically involved in this axis are the hypothalamus, pituitary glands, adrenal glands, and the testes.   Prostate cancer treatments often exploit the endocrine regulatory mechanisms that control androgen biosynthesis.   Activation of the androgen signaling cascade begins in the hypothalamus whereby two important peptide hormones are synthesized: lutenizing hormone-releasing hormone (LHRH) and corticotrophin-releasing hormone (CRH) [82] - [83].   LHRH and CRH are released into circulation where they eventually stimulate the anterior pituitary to release several hormones, including lutenizing (LH), follicle stimulating hormone (FSH), adrenocorticotrophic hormone (ACTH), and prolactin (PRL) [84] - [85].   From the pituitary, LH and FSH are released into the circulation and eventually they stimulate *de novo* testosterone biosynthesis within the Leydig cells of the testes [86].   Negative feedback loops control the overall production of testosterone, regulating the release of LHRH and CRH from the hypothalamus [87].

Prostate growth, development, and maintenance are regulated by androgens [88]. Androgens, specifically testosterone (T) and dihydrotestosterone (DHT), are predominantly produced from a cholesterol precursor [89].   Testosterone is a steroid hormone which was initially discovered in 1935 [90].   While the majority of circulating testosterone is produced in the testicles by the Leydig cells [91], small amounts are also generated by the adrenal, brain, muscle, skin, and prostate tissues themselves [92].

Testosterone biosynthesis is iniated in the mitochondria.   Cholesterol is converted to pregnenolone by the P-450 side-chain-cleavage enzyme (P-450$_{SCC}$) in a rate limiting reaction (Figure 4).   In a secondary reaction, pregnonolone (which is a precursor of multiple steroids such as T, estrogen, progesterone, cortisol, and aldosterone) is converted
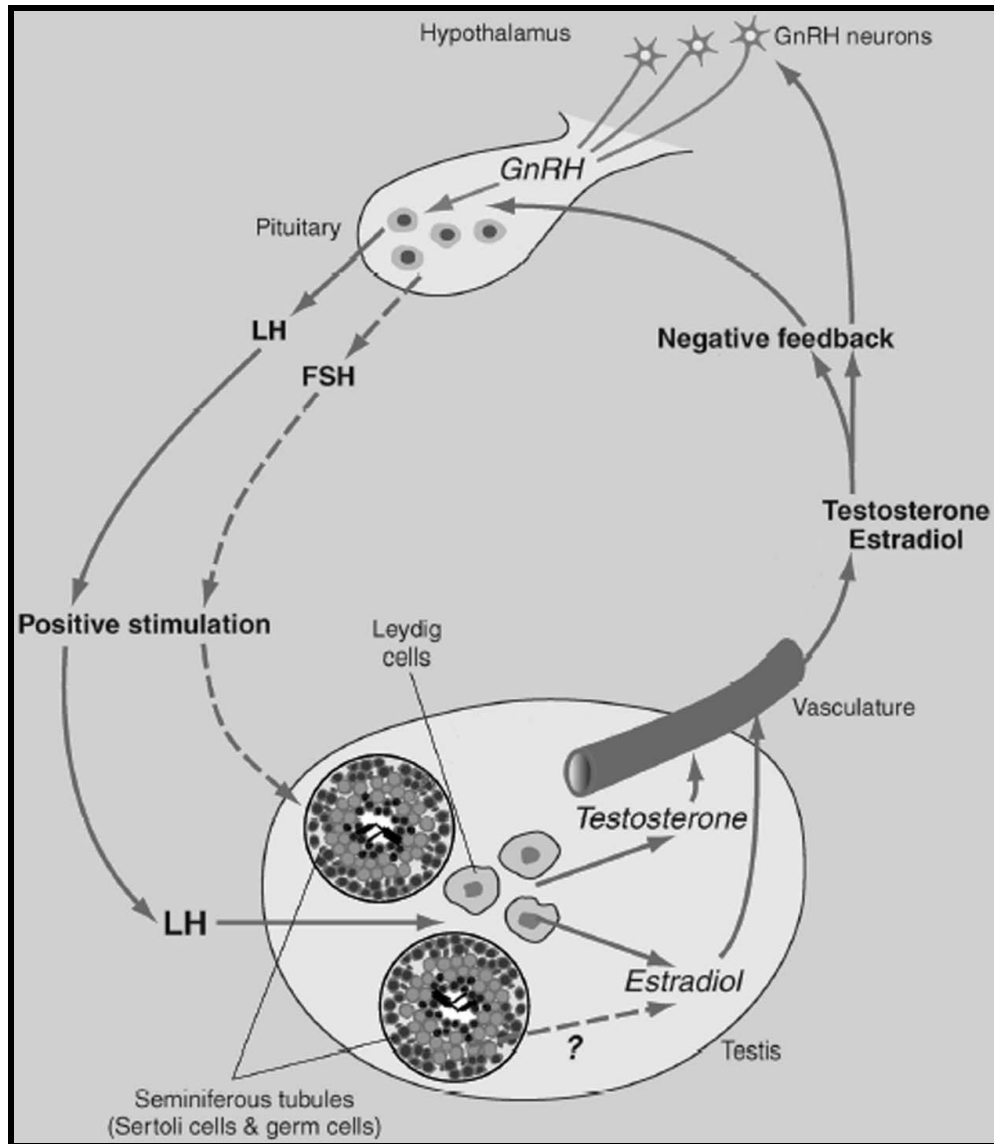
Figure 3 - The hypothalamus pituitary gonadal axis.

Activation of androgen signaling begins in the hypothalamus where two critical peptides, LHRH and CRH, are produced. LHRH and CRH act on the anterior pituitary to stimulate the release of LH, FSH, and ACTH. LH, FSH, and ACTH are subsequently released into circulation, eventually inducing the testes and the adrenal glands to produce testosterone *(taken from http://dels.nas.edu/ilar_n/ilarjournal/45_4/graphics/45_4_471f1.jpg).*

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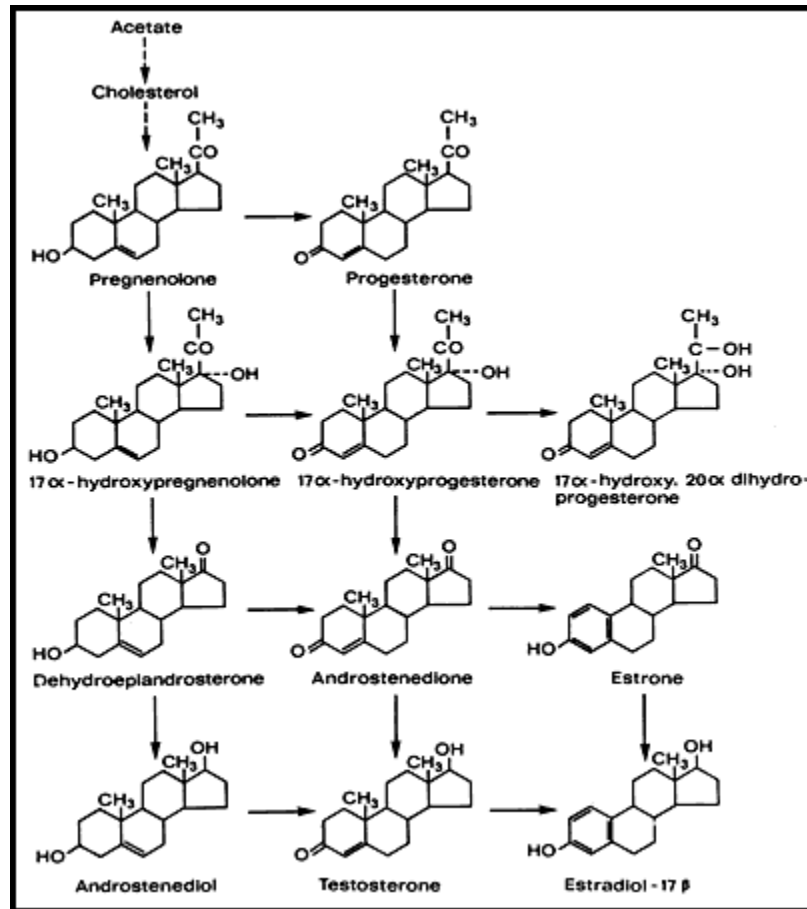
Figure 4 - The production of testosterone.

Testosterone is produced from a cholesterol precursor in Leydig cells. One of the key rate limiting reactions in the production of testosterone is the conversion of cholesterol to pregnenolone by the P-450 side chain cleavage enzyme, P-450SCC. This reaction occurs in the mitochondria. Pregnenolone is then converted to dehydroepiandrosterone (DHEA) in the endoplasmic reticulum, which then diffuses into the bloodstream *(taken from http://www.endotext.org/male/male1/figures/figure13.png)*.

to dehydroepiandrosterone (DHEA) within the endoplasmic reticulum of the Leydig cells. DHEA is then converted to testosterone (T) by an enzyme called 3 β-hydroxysteroid dehydrogenase (3β-HSD) [89]. After synthesis, T is released into circuation whereby it rapidly interacts with specific carrier proteins such as albumin and sex hormone-binding globulin (SHBG), both of which either retard androgen uptake by cells or facilitate the diffusion of steroids out of the cell, respectively [93]. Only about 1-2% of circulating T exists in a free, unbound state, and it is this free T that enters prostate cells [94]. The amount of serum T that remains unbound equates to approximately 1 nM [95].

Circulating free T diffuses across the plasma membrane of prostate cells and is irreversibly converted into dihydrotestosterone (DHT) by the membrane-bound 5α-reductase type 2 enzyme (Figure 5). Interestingly, there are two isoforms of 5-alpha reducatase: type I and II [96]. Immunostaining experiments demonstrated that the type I isoform, 5alphaR1, is increased is malignant prostate cancer; conversely, expression of the type II isoform, 5alphaR2, is decreased [97]. There also appears to be a differential localization of these two enzymes as the prostate stroma appears to express both isoforms, whereas the prostate epithelium only expresses 5alphaR1 [98]. Finasteride, one of the major agents used to combat prostate cancer in androgen withdrawal therapy, acts against the type II isoform [99]. Dutasteride is another 5alpha-reductase inhibitor which inhibits both isoforms, translating into a greater degree and consistency of DHT suppression compared with finasteride [100]. The activity of these two enzymes is a critical step in the production of DHT, a compound for which AR has 5X as much affinity for compared to T [101].

In the absence of androgens, AR remains in the cytoplasm as part of an inactive complex with heat shock proteins [102] - [103]. However, in the presence of androgens, AR binds DHT, initiating a conformational change; heat shock proteins are shed and AR homodimerizes, and translocates to the nucleus where it binds to specific palindromic androgen response elements (AREs) in the genome [104]. This receptor-DNA complex interacts with coregulators and basal transcriptional machinery, thereby initiating
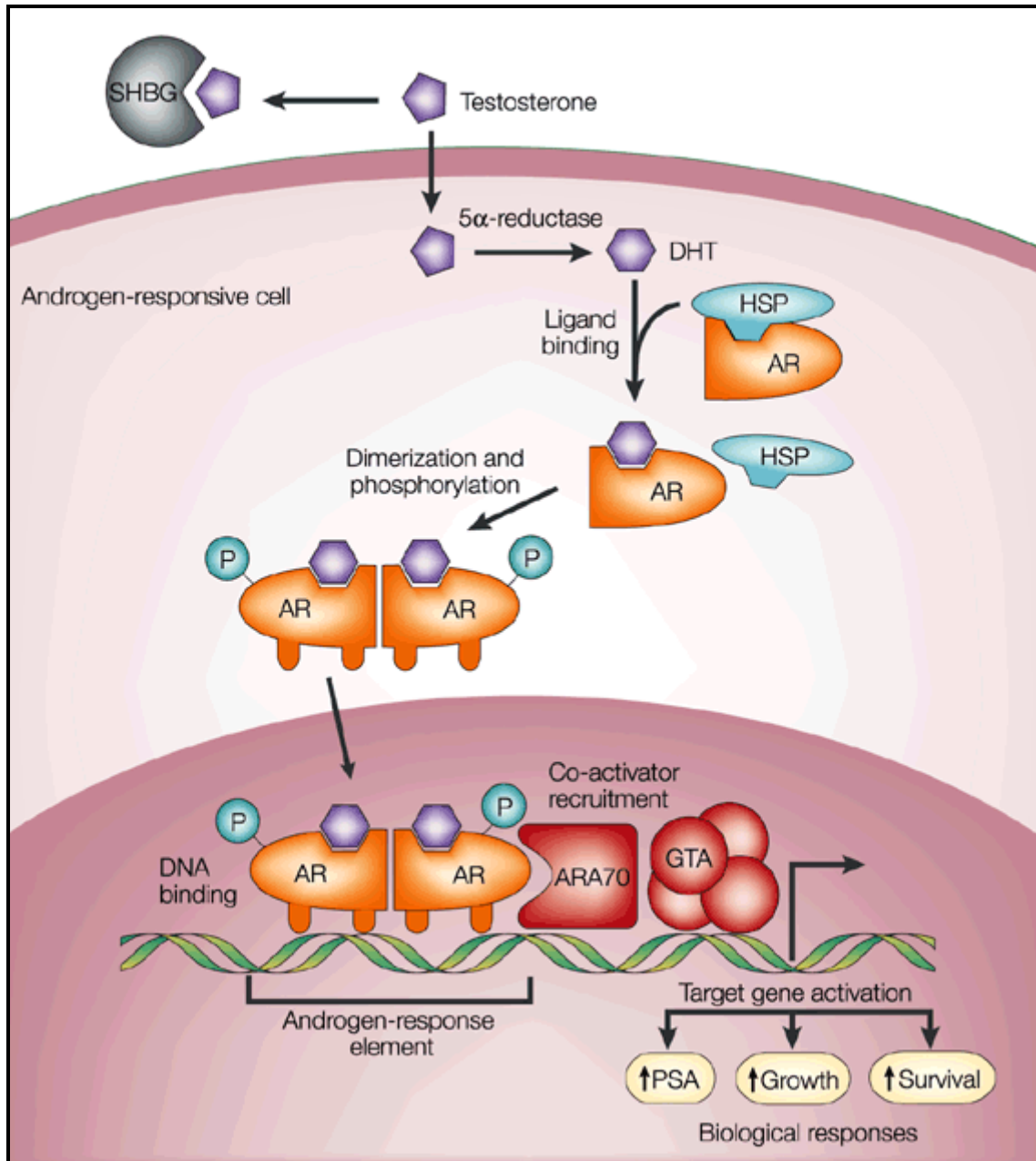
Figure 5 - Androgen receptor signaling.

Testosterone produced in the Leydig cells passes through the plasma membrane, whereby the enzyme 5α-reductase converts testosterone to DHT. DHT binds AR, causing it to dimerize and subsequently pass through the nuclear membrane, where it binds to AREs within the genome, resulting in growth and proliferation.

(take from http://www.nature.com/nrc/journal/v1/n1/images/nrc1001-034a-f1.gif)

downstream effects such as translation and transcription of protein products which ultimately result in proliferation, differentiation, and anti-apoptotic responses in prostate cells. Deregulation of this pathway by any mechanism results in prostate cancer. Chemical compounds which block this pathway have become prevalent methods of treating this disease. AR's ability to promote growth makes it the central focus of the vast majority of prostate cancer related research. Determining alternate mechanisms by which AR is activated or by which prostate cell proliferation and apoptosis is controlled is key to discovering potential cures.

1.6 Detection and Treatment of Prostate Cancer

A hallmark of prostate cancer is its ability to transition from an AD to an AI phenotype (Figure 6), placing addition emphasis on our ability to detect and treat prostate cancer in its initial stages. This also poses definite treatment challenges if the cancer is detected at a later stage. There are 4 main methods of detecting prostate cancer: digital rectal exams, prostate specific antigen (PSA) tests, transrectal ultrasounds, and biopsies[81], [105], [106]. Despite the fact that early detection and subsequent treatment is critical, at present Canadian medical plans do not cover these pre-screening methods like they do for breast and cervical cancers (*www.bccancer.bc.ca*). In conjunction with digital rectal exams, PSA testing is capable of detecting prostate cancers that are clinically significant at an early stage, and therefore potentially curable. However, multiple factors affect PSA levels, and certainly it has become a well known fact that it is possible for PSA levels to be normal even in the presence of prostate cancer [107]. Although PSA-based screening has led to a dramatic increase in prostate cancer detection, and is correlated with a significant downward stage migration, there is still no concrete evidence to suggest that it reduces mortality from prostate cancer [108].

Factors affecting prognosis and subsequent treatment are stage, grade, the patient's age and health, as well as whether or not the cancer has just been detected or recurring. Unfortunately approximately one third of patients present with advanced disease leaving only a limited number of treatment options [109]. In its early stages,

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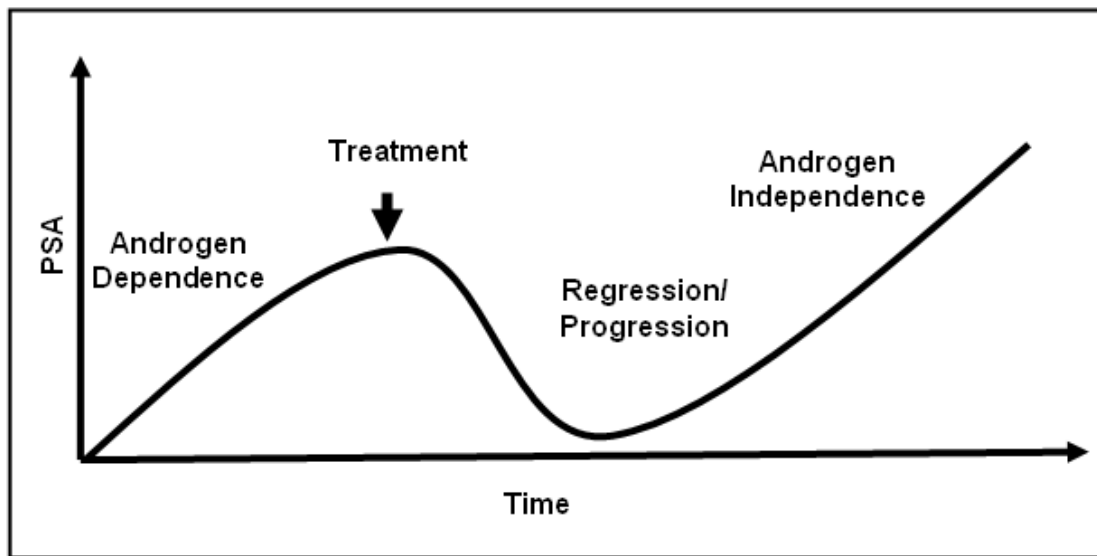
Figure 6 - Progression to androgen independence.

Treatment of prostate cancer results in an initial regression of the disease; however, progression towards androgen independent is inevitable, and ultimately fatal in most cases.

prostate cancer is treated by radical prostatectomy or radiation ablation of the prostate gland [81]. However, once the disease escapes the prostatic capsule and metastatic cells disseminate throughout the body, treatment becomes much more difficult. For those patients who are older and whose health may be in question in terms of ability to withstand more aggressive treatment, active surveillance is the recommended strategy. There appears to be some degree of controversy as to whether or not this particular strategy is effective. Holmberg demonstrated that at a median time of 6.2 years follow up post-diagnosis of 656 patients, there did not appear to be any significant difference in the overall survival between those individuals that received treatment versus those that did not [110]. Furthermore, another study conducted by Wong reported an overall improvement in the survival of treated patients over 30% compared to those that prescribed to watchful waiting [111]. Alternatively, for those individuals who are in good health, surgery or radiation may be an option. Radiation can be administered either externally or internally. Prostate brachytherapy is the procedure whereby radioactive beads are surgically implanted into the prostate. It was recently reported that modern brachytherapy using transperineal interstitial permanent radioactive seeds offered men a convenient outpatient treatment of up to 10-15 years with biochemical relapse free survival rates ranging from 67-87% [112].

If detected at the more advanced stage of the disease, androgen withdrawal therapy is an effective means of abrogating the growth promoting effects of androgens. Huggins invented androgen ablation therapy in 1941, for which he won the Nobel Peace Prize [88]. Androgen withdrawal therapy removes hormones, prevents androgen growth related effects, and encourages apoptosis resulting in regression. However, even during regression prostate cells still require androgens for survival and growth, and these very same cells inevitably bypass the requirement for androgenenic growth stimuli and acquire the ability to grow in the absence of androgens, essentially becoming androgen independent [113]. Androgen withdrawal therapy maybe achieved via orchioectomy, however there are numerous pharmacological agents available. LHRH agonists such as leuprolide acetate prevent the testicles from manufacturing testosterone. Leuprolide acetate down regulates LHRH receptors in the anterior pitiutary, resulting in repression of

LH and FSH release and therefore removing the stimulatory signals which ultimately encourage T production in the Leydig cells [114]. Using LHRH agonists alone, however, does not completely ablate the the level of androgens in the system, as androgens are still produced in the adrenal glands. Therefore to achieve total androgen ablation, LHRH agonists should be combined with AR antogonists to inhibit androgen binding [115].

Another treatment option is the use of AR antagonists, or chemical compounds which effectively prevent androgen-induced conformational change and activation of AR [116]. AR antagonists are either steroidal (cyproterone acetate) or nonsteroidal (hydroxyflutamide, bicalutamide and nilutamide) compounds [117]. Anti-androgens such as flutamide or bicalutamide effectively bind up circulating testosterone. Some compounds such as ketoconazole and aminoglutethimide prevent androgen production by the adrenal glands. Studies report that androgen ablation ablation therapy reduces plasma T levels to approximately 10% [118] - [119]. Intermittent androgen withdrawal therapy is where patients are subjected to multiple rounds of hormone therapy followed by phases of no treatment. This prolongs progression to AI [120]. For patients undergoing intermittant androgen suppression treatment, average time to AI is extended to 48 months [121].

However, androgen withdrawal therapy is not without its own disadvantages, the main one being that prostate cancer cells become AI over time, and androgen ablation therapy sometimes does not produce a clinically detectable response [122]. Furthermore, it has been demonstrated that compounds such as bicalutamide can switch from having an antagonistic to agonist effect during long-term androgen withdrawal, as shown in prostate cancer LNCaP cells [116].

Surgery and radiation are the two of the most common forms of treatment. However, treatment of prostate cancer often disrupts normal urinary, bowel, and sexual functioning in men [123]. Under normal conditions, the urinary sphincter at the base of the bladder prevents urine leakage; during urination this sphincter relaxes and urine flows from the bladder, through the urethra, and out. When the prostate is surgically removed

via prostatectomy, the bladder is pulled downwards and connected to the urethra where the prostate originally resided [124]. If the sphincter is damaged during this procedure, urinary incontinence may result. If erectile nerves are damaged during this procedure, the ability to achieve an erection is difficult, whilst sexual desire is not affected. Seminal vesicle invasion is observed in 12% of men diagnosed with prostate cancer; this is essentially a condition whereby the cancer has extended through the prostatic capsule into the seminal vesicles [124]. In these instances, the seminal vesicles are removed along with the prostate, leading to infertility.

Radiation may lead to side effects such as rectal damage, resulting in rectal bleeding, urinary incontinence, urgency, impotence, diarrhea, and hair loss [125]. Although these treatments may prevent prostate cancer from spreading if caught early, there are often strong, psychological effects which remain well beyond the course of treatment. Incontinence and erectile dysfunction often lead to feelings of loss of masculinity, and ultimately depression [126]. For this reason, prostate cancer challenges not only a man's physiological health, but also his mental and social well-being and life satisfaction.

Finally, although not a popular option among men, an orchioectomy, or the surgical removal of the testicles, may be performed [127]. Finally, estrogens may be administered to prevent testosterone production. Estrogens prevent prostate cancer growth indirectly by inhibiting LHRH relrease from the hypothalamus, leading to lower serum testosterone and castrate-like conditions [128]. However this method is also not commonly utilized due to its serious side effects, ranging from impaired sexual function, loss of sexual desire, and weakened bones and cardiovascular function.

1.7   The Androgen Receptor

AR plays a pivotal role in the growth and survival of normal prostate epithelium and prostate carcinoma. AR is a member of the steroid and nuclear receptor family

which has over 150 members [129]. The estrogen (hER), progesterone (hPR), glucocorticoid (hGR), and mineralocorticoid (hMR) receptors are all members of this family [130]. AR is mainly expressed in prostate, adrenal, skeletal muscle, liver, and central nervous system tissue [131]. Under normal physiological conditions, AR gene expression is responsible for male sexual differentiation *in utero* and male prepubertal changes. In adult males, AR is responsible for libido, spermatogenesis, muscle mass, strength, bone mineral density, and erthropoisis [132].

It was not until 1981 that the AR gene was localized to the X chromosome [133]. Only one AR gene was discovered in the human genome. It was subsequently cloned in 1988 by Chang and Kokontis [134]. Located on chromosome Xq11-12, the AR gene spans 90 kilobases. The gene is comprised of eight exons [135]. Exon 1 (1586 bp) encodes the N-terminal domain (NTD), while exons 2 and 3 encode the DNA binding domain (DBD) (152 and 117 bp, respectively). The ligand binding domain (LBD) and the hinge region are encoded by the remaining five exons ranging in size from 131 to 288 bp [136].

Due to differential splicing in the 3' untranslated region of the transcript, three androgen receptor mRNA species of approximately 11 kb, 8.5 kb, and 4.7 kb, respectively, have been identified in LNCaP cells [137], all of which encode protein products 110 kDa is size. These multiple transcripts contain variable lengths of a polymorphic CAG region in exon 1. Studies done on this polymorphic region in human populations have determined that the length of this region is inversely proportional to the risk of prostate cancer. Expansion of this tract has also been linked to spinal bulbar dystrophy [138].

The protein structure of AR has been fully characterized. The 919 amino acid protein contains 11 α-helices and two short β-turns which form an α-helical sandwich [139]. AR consists of four major domains: N-terminal domain (NTD), the ligand binding domain (LBD), DNA binding domain (DBD), and the hinge region [140] (Figure 7). The NTD is comprised of 532 amino acids, the sequence of which is not conserved amongst

nuclear receptor family members [141]. The NTD encodes the constitutively active AF1 transactivation function of AR which is essential for its activity [142]. The NTD interacts with the LBD in a hormone dependent manner, serving to stabilize the AR dimer complex and/or stabilize the ligand/receptor complex. The LBD, which contains the ligand independent AF2 function, is approximately 250 amino acids in length and is the second most conserved domain within the AR [143]. It is connected to the DBD by the hinge region. Upon hormone binding, a conformation change in the LBD generates a docking platform onto which AR coregulators may assemble [144]. The AF2 transactivation function in the LBD is strongly dependent on the presence of these coregulators. Deletions in the LBD abolish hormone binding completely. Mutations detected in the LBD impair the ability of AR to bind hormone, generating a molecular basis for androgen insensitivity that may be involved in prostate cancer progression. This regulatory function of the AR domain in the absence of hormone, is not unique for the androgen receptor, and has been reported also for the glucocorticoid receptor.

The DBD is the highest conserved domain within the nuclear receptor family [145]. It is characterized by a high content of basic amino acids and by nine signature cysteine residues. The DBD has a compact, globular structure containing two substructures, each of which centrally encloses one zinc atom [146]. These zinc atoms each interact with four cysteine residues. The α-helix of the most N-terminal zinc cluster interacts directly with nucleotides of the androgen response element in the major groove of the DNA. Three amino acid residues (Gly, Ser, Val) at the N-terminus of this α-helix are responsible for the specific recognition of the DNA-sequence of the responsive element [147]. These three amino acid residues, referred to as to the P-box, are identical in the androgen, progesterone, glucocorticoid, and mineralocorticoid receptors. It is not surprising, therefore, that these receptors share the ability to recognize the same response elements.

Finally, the hinge region of AR is between the DBD and the LBD. It encodes a bipartite nuclear localization sequence. This nuclear localization sequence partially spans
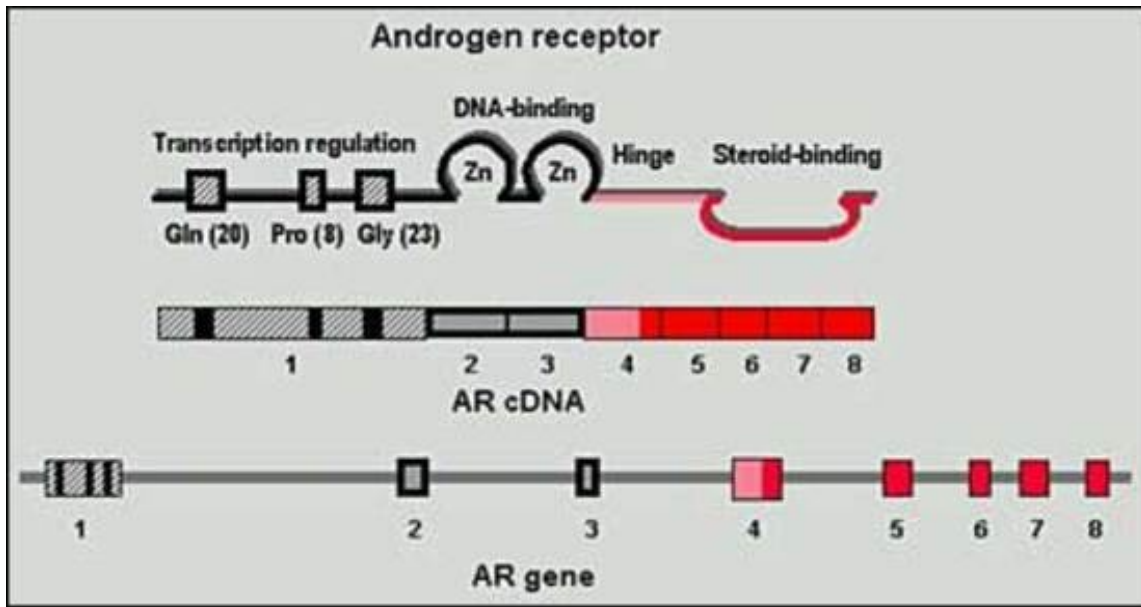
Figure 7 - The androgen receptor gene.

The AR gene, located on chromosome Xq11-12, is comprised of eight exons which encode a protein product that consists of four major domains: the transactivation domain, the DNA binding domain, the hinge region, and the ligand binding domain
(taken from http://www.endotext.org/Pediatrics/pediatrics7/figures/figure26.jpg).

the DBD. The signal responsible for nuclear import is encoded by amino acid residues 608 to 625 [148].

In addition to the intrinsic regulatory structural features of AR, there are several post-translational modifications that regulate its activity as well. Post-translational modifications affect receptor stability and activity, and provide potential mechanisms for cell and/or gene-specific regulation. Newly synthesized unbound AR is associated with heat shock proteins, namely Hsp 90, 70, 56, and 54. These heat shock proteins interact with the LBD [149]. Upon androgen binding, AR goes through a series of conformational changes which facilitate dimerization of the receptor, translocation into the nucleus, and binding to androgen response elements in the nucleus characterized by a six nucleotide half site consensus sequence 5'TGTTCT-3' spaced by three random nucleotides. This consensus sequence is located in the promoter and enhancer region of AR target genes [27]. Furthermore, covalent additions such as acetylation, ubiquitylation, and sumoylation are all examples of AR modifications which have been reported to modulate this complicated process.

1.8 Molecular Mechanisms Involved In Progression of Prostate Cancer

The vast majority of prostate cancer research has focused on determining the molecular mechanisms by which the disease transitions from AD to AI/CR. These molecular mechanisms are currently not well understood, but they are largely responsible for the lethality of prostate cancer. As previously discussed, AR regulates the transcription of target genes related to growth and development of the prostate [27], and therefore any inappropriate activation of AR may ultimately drive prostate cancer progression. Enhanced AR activity triggered by androgens is most likely not a major factor involved in progression. *In vitro* studies have demonstrated that androgens have the capacity to inhibit growth in addition to promoting cellular differentiation [150]. Loss of AR expression is also most likely not a contributing factor to progression, as 80% of prostate cancers retain high levels of AR [151]. Bone metastases often exhibit higher levels of AR than primary tumors [152]. I*n vivo* knockdown of AR using short hairpin

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siRNAs has been demonstrated to inhibit tumor growth and delay progression to AI [18]. While loss of androgens and loss of AR may not be responsible for progression to AI, the molecular mechanisms potentially involved will be discussed in detail in this next section.

1.8.1   AR Mutations

Mutations in AR are one of several potential molecular mechanisms which may contribute to AI/CR. Recently there has been a surge of experimental evidence to support this theory. Over 30% of prostate tumours possess AR mutations and several different AR variants have been identified which confer loss of receptor specificity in the absence of conventional ligands [153]. Mutant receptors activated by alternative ligands other than androgens drive growth via different cellular pathways than those traditionally activated by wild type AR. Consequently, these tumours may behave differently based on the predominant mutation present. A highly conserved sequence at the C-terminal end of AR is prone to point mutations in prostate cancer [154]. These mutations are believed to contribute to the failure of hormonal therapy, although the mechanism by which this occurs is largely unknown [155]. Three of the most commonly identified AR variants with amino acid substitutions in tumours are H874Y, T877A, and T877S [153]. The H874Y mutation has been shown to enhance AR binding and transactivation activity with all three members of the p160 family of coactivator proteins [154]. The structure of the H874Y mutant is not drastically different from that of wildtype AR, however it does appear to possess an increased sensitivity to protease digestion in the absence of hormone. Therefore in low androgen environments, this particular mutant may confer a selective advantage to tumour cells during androgen withdrawal therapy. The T877A mutant has been linked to cell growth and survival promoting effects. In contrast to wild type AR, the T877A mutant continues to grow in the absence of androgens and exhibits androgen independent AR activity [156]. Furthermore, the mutant AR T877A exhibits decreased binding affinities for ligands such as bicalutamide, mifepristone, DHT, and R1881, possibly explaining the altered responses of cells to treatment in androgen-independent prostate cancers [157]. A recent publication demonstrated that these two AR

mutants, H874Y and T877A, are also activated by the pesticide 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (DDE), a compound that is readily found in the environment [158]. DDE induces mutant AR recruitment to the PSA promoter in the absence of androgens, ultimately resulting in enhanced cell proliferation via the MAPK kinase pathway. Interestingly, DDE is not able to induce AR activation in cells which express wild type AR.

Two other examples of point mutations discovered in patients with prostate cancer are S296R and P340L. The point mutation (S296R), located in the N-terminal domain of AR, decreases its ligand specificity and alters its interaction with the corepressor N-coR [159], a protein involved in transcriptional regulation of AR. N-coR regulates the magnitude of the hormone response by competing with other coactivators of AR [160]. Another mutation which alters the function of AR is P340L, which results in reduced transcriptional responses to ART-27. ART-27 is an AR N-terminal coactivator that is involved in mediating growth inhibition [161]. While the vast majority of AR mutations seem to be increase AR activation in response to ART-27, the AR P340L mutation suppresses AR activity, and may contribute to progression via alterations in AR activity [162].

Another study demonstrated that nonsense mutations that lead to C-terminal truncated ARs are found at high frequency in metastatic prostate cancer [163]. Expressing a mutant AR (Q640X) in LNCaP cells was accompanied by increase in PSA production and strong, ligand independent transcriptional activity. Furthermore, expression of this Q640X mutant AR leads to enhanced nuclear localization of the endogenous AR protein in neighboring cells in the absence of androgens; this paracrine mechanism of activating neighboring cells appears to be ligand independent. The exclusive cytoplasmic action of an AR mutant, AR23, which is a splice variant of normal AR, has also been reported [164]. The last 69 nucleotides of the second intron are retained in this mutant, leading to the insertion of 23 additional amino acids in between the two zinc fingers in the DBD. The AR23 mutant cannot be imported into the nucleus, resulting in the formation of the cytoplasmic and perinuclear aggregates that partially

colocalize with ER; these AR proteins were devoid of genomic action. Although AR mutations are a possible mechanism by which prostate cancer may progress towards AI, it is but only one of several mechanisms proposed in theory, and not necessarily the most contributory, as most evidence suggests that AI/CR prostate cancers possess normal AR.

1.8.2   AR Amplification

AR amplification is another potential mechanism which may encourage androgen independent. It has been demonstrated via *in situ* hybridization and ki67 labelling (an antibody that labels cells which effectively express Mib1) that progression to AI/CR is associated with AR gene amplification and increased cell proliferation in one third of tumours analyze. The most common DNA copy number aberrations are losses in chromosomes 5q, 6q, 8p, 10q,13q, 16q, 17p, 18q and gains in 7p, 7q, 8q, 9p, Xq.

Observations linking the overexpression of the AR gene with the acquisition of hormone resistance have been observed *in vitro* ever since 1995. Visakorpi conducted comparative genomic hybridization experiments which demonstrated that the Xq11-q13 region of AR is amplified in tumours which reoccur during androgen withdrawal therapy [165]. In fact, over 30% of recurrent tumours exhibited this trait, whereas tumours taken from patients who had not undergone treatment did not exhibit any AR amplification. This was the first evidence to suggest that AR amplification emerges during androgen withdrawal therapy. Koivisto *et. al.* studied whether or not the AR gene is structurally intact, and whether tumours with AR amplification have distinct biological and clinical characteristics compared to those without AR amplifications. This group produced results which reflected previous findings, that 28% of the recurrent androgen resistant tumours (but none of the untreated tumours) contained AR amplification as determined by *in situ* hydribization [165]. The AR gene was demonstrated to be predominantly wild type, however AR amplification was associated with substantially increased mRNA levels. Furthermore, median survival time was two times greater for patients with AR amplification in comparison to no amplification after recurrence. Koivisto suggested that the failure of androgen withdrawal therapy may actually be caused by clonal

expansion of tumour cells possessing the capability to grow in an androgen dependent manner even at low levels of androgens, or essentially that AR amplification creates hypersensitivity to low levels of androgens. Using real time PCR to compare AR expression levels between AR-dependent and AR-independent prostate cancer, all hormone refractory tumours analyzed expressed AR and showed 6-fold higher expression than androgen-dependent tumours [166]. Furthermore, the enhancement of AR was demonstrated not only at the translational level, but at the transcriptional level as well, as these tumours had 2-fold higher AR protein levels [166].

Finally, in a more recent study, it was demonstrated that the simultaneous overexpression of AR and activation of the Wnt pathway promote prostate cancer cell growth and transformation at castrate levels of androgens. AR overexpression appears to potentiate the transcriptional activities of the Wnt-beta catenin pathway [167]. Upon Wnt signaling, AR and beta catenin, a coactivator of AR, are recruited to the promoter and enhancer regions of the PSA gene. Interestingly, physiological levels of androgens inhibit these effects. In this way, AR overexpression has been shown to promote prostate cell malignancy in a ligand-independent manner.

1.8.3   AR Independent Mechanisms of Growth

Progression may also be caused by the cellular mechanisms which directly bypass the classic growth promoting affects of AR. In normal prostate tissue, the rate of apoptosis is 1-2% per day which is balanced by 1-2% of proliferation per day [168]. Any inbalance of this ratio may occur via the dysregulation of different signaling cascades which may directly or indirectly alter AR activity, or enhance growth independently of AR. There are several AR independent mechanisms in which progression towards AI may be promoted. Neuroendocrine differentiation, phosphorylation of AR or its coregulators by growth factor and cytokines, and epigenetic changes are just a few possibilities by which growth may be promoted [169] - [170].

Of these, one of the major AR independent mechanisms that is currently the focal point of a substantial amount of research is the acquisition of neuroendocrine phenotype by prostate cancer cells. This neuroendocrine phenotype has recently become a promising diagnostic factor of AI [171]. Neuroendocrine cells derived from non-neuroendocrine prostate cancer cells secrete factors that act in a paracrine manner to stimulate a variety of cellular processes such as growth, survival, motility, and metastasis [169]. Factors such as IL6, epinephrine, and forskolin promote neuroendocrine differentiation, resulting in increases in cellular cAMP and protein kinase A, and reductions in intracellular calcium levels [172] - [173]. Prostate cells that have acquired neuroendocrine features often possess increased levels of activated transcription factors such as STAT3, CREB, EGR1, c-fos, and NF-kappa B [174] - [175]. Furthermore, neuroendocrine prostate cancer cells also secrete neuropeptides such as bombesin, neurotensin, PTHrP, serotonin, and calcitonin, all of which trigger growth and survival in androgen-independent prostate cancer cells [176] - [177]. These neuropeptides activate signal transduction cascades involving Src, focal adhesion kinase (FAK), ERK, PI3K/Akt, Elk-1, and c-myc, the result of which is the regulation and expression of a multitude of genes which are directly involved in proliferation, anti-apoptosis, migration, metastasis, and angiogenesis [178].

Progression towards AI is often affiliated with alteration of growth factor or growth factor receptor expression within the tumour. Growth factors and cytokines initiate signaling cascades that ultimately result in the phosphorylation of AR or coregulators of AR [179]. Growth factors and cytokines may either enhance AR activity, therefore promoting proliferation, or they may suppress AR activity, thus selecting for clonal populations that can survive in an AR independent manner [180].

Transforming growth factor (TGF)-β, a polypeptide growth factor, is a specific example of a protein which is directly involved in enhancing prostate cell growth in an AR independent manner. In normal prostate, TGF-beta has multiple different functions. It functions as a growth inhibitor of prostate epithelial cells, a differentiation factor of prostate stromal cells, as well as a mediator of castration-induced epithelial apoptosis

[181]. Prostate cancer cells secrete large quantities of TGF-beta, and high levels of TGF-beta paired with the loss of the TGF-beta receptor are affiliated with poor prognosis. Elevated TGF-beta levels are also affiliated with elevated PSA serum levels, suggesting an interaction between the two pathways in prostate cancer [182]. In androgen dependent prostate cancer, TGF-beta stimulates cell differentiation while simultaneously inducing cell death [183]. TGF-beta has also been reported to enhance growth and metastasis by promoting angiogenesis and inhibiting immune responses against tumour cells. TGF-beta is mediated by the Smad proteins, which regulate its activity via phosphorylation. AR has been reported to interact with Smad 3. Cotransfection of AR and TGF-beta results in increased AR transactivation, and the addition of Smad 3 further enhances this transactivation [184]. It has been reported that Smad 4 counters Smad 3 activity, and that potentially the loss of Smad 4 may allow Smad 3 to enhance AR transcription continually, resulting in deregulated growth and proliferation of prostate cancer cells [185].

Insulin-like growth factor type I receptor (IGF-1R) is another example of a specific protein which is believed to play an important role in prostate cancer progression [186]. Recent research indicates that IGF-1R may alter AR compartmentalization, therefore altering AR activity in prostate cells. Inhibition of IGR-IR results in cytoplasmic retention of AR and a significant change in androgen-regulated gene expression [186]. Furthermore, translocation of AR to the nucleus may be associated with IGF-induced phosphorylation.

IL6 is a cytokine which regulates differentiation, proliferation, and growth inhibition in prostate cells simultaneously. Serum IL-6 levels are increased in men with hormone refractory prostate cancer [187]. IL6 induces the MAPK pathway via two different methods. Firstly, IL6 activates JAK, which is an upstream activator of Ras, which then in turn activates the MAPK pathway [187]. IL6 also induces Her2 and gp130 association, which results in MAPK pathway activation [188]. In addition to the MAPK pathway, IL6 also stimulates the PI3 kinase pathway [187]. Inhibition of this pathway causes apoptosis in LNCaP cells. Interestingly, although it is established that IL6

activates a variety of different cellular pathways, all of which result in cell growth and proliferation, transactivation assays attempting to determine whether IL6 regulates AR are conflicting, and this may very well be due to the fact that IL6 activates numerous different pathways.

Epigenetic changes in DNA are another AR independent mechanism by which growth is enhanced in prostate cancer cells. Epigenetic mechanisms are essential for development and progression, and they also permit the stable inheritance of cellular properties without changes in DNA sequence [189]. One such mechanism is DNA methylation, which affects up to 30 different genes involved in prostate cancer. Hypomethylated genes are affiliated with progression towards AI, while hypermethylation occurs during early carcinogenesis [190]. The end result of DNA methylation is histone modification, increased expression of histone methyltransferase, and changes in the interaction between corepressors and coactivators and DNA, all of which have the capability of facilitating growth in prostate cancer cells.

1.8.4 Coregulators

Aberrant interactions between AR and coregulator proteins may contribute to progression of prostate cancer and androgen insensitivity. Coregulators are any such factors that serve to either enhance or repress AR activity [26]. These are referred to as coactivators or corepressors, respectively. Coregulators are classified as type I or type II according to the mechanism by which they function [27]. Type I coregulators modulate transcription through chromatin remodeling and recruitment of basal transcription machinery. Type II coregulators utilize other mechanisms to modulate AR activity such as facilitating appropriate AR folding, ligand binding, and N and C termini interactions. Type II coregulators may also disrupt intramolecular interactions of AR and modulate nuclear AR translocation [27].

There are a number of AR coregulators that have been identified, many of which have the capability of behaving either as coactivators or corepressors depending on the

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physiological circumstances. SRC1 (steroid receptor coactivator), TIF2 (SCR2), SRC3, PTEN (phosphatase and tensin homolog), retinoblastoma protein (Rb), CBP (cAMP-response element binding protein)/p300, ARA (androgen receptor-associated protein)-24, 54, 55, 70, 160, 267, Hsp 70, 90, 54, 56, cyclin D1, caveolin, β-catenin, and DDC have all been shown to coregulate AR in through one mechanism or another [191] - [192].

SRC1, TIF-2 (also referred to as SRC-2), and SRC-3, are the most prominent coregulators of AR [193] - [194], and each of these are overexpressed in prostate cancer. SCR1, the first identified and most well characterized coregulator of AR, is overexpressed in up to 50% of all androgen-dependent prostate cancers [195] and expression levels appear to correlate positively with tumour aggressiveness [196]. Androgen-independent cancers overexpress SRC1 and TIF2 in 63% of all tissue biopsies analyzed [197]. Increases in SRC3 levels have been positively correlated with an increase in prostate cancer grade and stage, and a decrease in disease-free survival [198]. In terms of function, members of the SRC family have been shown to interact with the N-terminal of AR and the AF2 of the LBD to enhance ligand dependent transactivation of AR [199] - [200]. SRCs also modulate AR activity via intrinsic HAT activity [201] by directly enhancing AR binding to chromatin [202]. SRCs may also act as platforms for recruitment of secondary transcription factors such as CBP/p300 and PCAF which function to remodel chromatin [203]. SRCs are also involved in RNA polymerase II recruitment to distant enhancer elements of target genes such as PSA [204].

Phosphatase and tensin homolog (PTEN) is another well known coregulator of AR. PTEN is a phosphatase that negatively regulates the activity of PI3K and Akt. Loss of PTEN correlates positively with an increase in tumour stage and grade, as over 20% of tumours possessing Gleason grade of 7-9 do not produce PTEN [205]. In addition, in 20-27% of all prostate cancers, loss of PTEN is associated with a decreased survival rate [205]. Loss of PTEN is also correlated with an enhancement of AR transactivation and resistance to cell death in tumour cells [206]. Furthermore, it has been demonstrated that prostate specific deletion of PTEN induces metastatic prostate cancer [207]. In the absence of PTEN, PI3K and Akt activity is increased, resulting in an increase in cellular

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proliferation and a corresponding decrease in apoptosis [208]. PTEN inhibits AR function by promoting AR degradation [209], therefore, in the absence of PTEN, normal AR turnover may be impaired, indirectly contributing to an increase in AR transcriptional activity.

Retinoblastoma protein (Rb) is another prominent coactivator of AR. Phosphorylated Rb protects cells from apoptosis, however, the mechanism by which this is accomplished is not well understood. It has been shown that hyperphosphorylated Rb interacts with and sequesters pp32, a pro-apoptotic nuclear factor that is commonly increased in cancer [210]. Inhibition of the interaction between AR and retinoblastoma protein decreases AR activity [211].

In addition to these very well known AR coregulators, there is an ever growing pool of coregulators that are being documented. ART-27 is an AR N-terminal coregulator that is associated with AR-mediated growth inhibition [212]. Mutations in AR have been reported to interfer with normal ART-27 activity. The vast majority of AR mutations result in increased AR activity in response to ART-27; however, two AR mutations, AR P340L and AR E2K, have been proven to diminish AR activity in the presence of ARA-27. Ras activation may play a causal role in progression of prostate cancer toward a more malignant, hormone insensitive phenotype. Mukhopadhyay *et. al.* identified RREB-1 (Ras responsive element binding protein-1) as a binding partner and coregulator of AR [213]. Transient expression of RREB-1 down regulates AR mediated promoter activity and suppresses expression of PSA. Inhibition of RREB-1 expression by RNA interference enhanced the effect of Ras on PSA promoter activity. Collectively these studies showed that RREB-1 acts as a repressor of AR and further implicates the Ras/MAPK kinase pathway in progression.

Yeh and Chang demonstrated that ARA-70 increases AR-dependent transcriptional activity [214]. Cdk-activating phosphatase (cdc25B) has been identified as an AR coactivator and is overexpressed in prostate cancer [215]. Tip60 is another coregulator which increases in expression during androgen withdrawal. This particular

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coregulator also exhibits enhanced AR nuclear localization during progression [198]. Nuclear matrix protein (nmt55) is upregulated in prostate cancer, resulting in an increase in PSA expression with overexpression [216]. Beliakoff and Sun identified two novel coregulators, ZIMP7 and ZIMP10, which are protein inhibitors of activated STATs [217]. AR activity is reportedly enhanced through interaction with BCRA1 (breast and ovarian cancer susceptibility gene) [218]. BCRA2 has been shown to increase AR transcriptional activities through TIF2, a member of the p160 family of nuclear receptor coactivators [219].

L-Dopa decarboxylase (DDC) is yet another coregulator of the AR, however unlike all the proteins previously mentioned, DDC, in addition to GAK, was identified via the Tup 1 repressed transactivation system [220]. Because the NTD of AR possesses intrinsic transactivation activity, conventional yeast two-hybrid systems cannot be used with any peptides that possess intrinsic transactivation activity. The Tup 1 repressed transactivation system is specifically designed for transactivator bait proteins such as AR and it provides a means of identifying only those proteins which interact with the NTD, two of which are DDC and GAK. Using this system, DDC was detected multiple times. Furthermore, transfection of LNCaP cells with DDC enhanced ligand dependent AR activity which could subsequently be antagonized by anti-androgen bicalutamide. Using a tetracycline-inducible LNCaP-DDC prostate cancer stable cell line, several downstream target genes of DDC were identified using oligonucleotide microarray analysis [220]. Overexpressing DDC illustrated a number of changes in the expression levels of androgen regulated genes, twenty of them being upregulated and ten of them being down regulated. One in particular, TMEPAI, is a well known coregulator of the AR, providing further evidence that DDC does in fact function as a classical coregulator or AR. Finally, DDC was also shown to be a neuroendocrine marker of human prostate adenocarcinoma, and much like GAK, DDC expression level is increased after hormone ablation therapy and AI.

1.9 Cyclin G Associated Kinase

Cyclin G associated kinase (GAK), the primary topic of this thesis, has been recently reported to be a potential coregulator of AR [142]. GAK is a 144 kDa protein transcribed from a 4331 base pair gene located on chromosome 4p16. GAK is localized to the cytoplasm and perinucleus, and is expressed ubiquitously, with the highest expression level occurring in the testes [221]. GAK is classified as an Ark family kinase as a result of its homology with actin regulating kinase, ARK-1. It possesses a serine/threonine kinase domain, an N-terminal tensin/auxilin domain, a clathrin binding domain, a C-terminal J domain, as well as a highly conserved tyrosine phosphorylation target site. GAK was initially discovered through its interaction with cyclin G and CDK5 *in vivo* [222]. Cyclin G is a SRC kinase family member that is a target of the tumour suppressor p53, while CDK5 is activated by the binding of p35, and plays an important role in the control of neurogenesis.

GAK has largely been implicated in the disassembly of clathrin coated vesicles (CCVs) during endocytosis. Clathrin-mediated endocytosis is the most well characterized mechanism of cellular transport, and it is responsible for the internalization of receptors, growth factors, antigens, pathogens, and nutrients. GAK possesses a high sequence homology to auxilin, a cofactor found specifically in neuronal tissue that is required for Hsc70-mediated CCV uncoating. Hsc70 is a constitutively expressed version of Hsp70 and a known binding partner of AR. One potential role suggested for GAK is that it is required for the uncoating of CCVs by Hsc70 in non-neuronal cells. Liver CCVs, which do not express auxilin but which do express GAK, are uncoated by Hsc70, supporting the theory that GAK may act as an auxilin homolog in non-neuronal cells. This theory was supported by Eisenberg and Greene who recently demonstrated that ATP dependent dissociation of clathrin from CCVs by Hsc70 requires J domain cofactors proteins, and that both auxilin and GAK induce CCVs to bind to Hsc 70 [122].

Immunolocalization studies conducted in rat liver, bovine testes, and bovine brain tissue demonstrate that GAK is primarily a cytosolic protein concentrated in the

perinuclear region and trans-Golgi network, two cellular regions where CCV budding occurs [223]. GAK was also shown to phosphorylate μ2, a protein component of the CCV machinery which links cargo proteins to be internalized with clathrin [224]. Interestingly, GAK does possess a clathrin binding domain which has been reported to induce clathrin polymerization. To support the growing amount of evidence linking GAK's role to cellular transport, the early stages of CCV endocytosis are inhibited when GAK is knocked down [225]. Furthermore, depletion of GAK was shown to inhibit the internalization of epidermal growth factor (EGF), as well as reducing the quantities of perinuclear clathrin associated with TGN [226].

Zhang *et. al.* demonstrated that down regulation of GAK using small hairpin siRNAs had two pronounced effects [227]. Firstly, the levels of EGFR expression and tyrosine kinase activity were enhanced by 50-fold. Secondly, downstream signalling was significantly altered, most notably a large increase in the amount of extracellular signal-regulated kinase 5 and Akt. In CV1P cells, down-regulation of GAK resulted in outgrowth of cells in soft agar, suggesting that lack of GAK promotes tumourigenesis.

A more recent study on GAK identified GAK as a novel interleukin 12 (IL-12) interacting protein via yeast two hybrid and coimmunoprecipitation studies. IL-12 mediates critical molecular mechanisms in activated T-cells. Furthermore, overexpression of GAK in activated T-cells suppresses IL-12 IFN-gamma production but has no recognizable effects on cellular proliferation. However, knocking down GAK increases IFN-gamma production in T cells, suggesting GAK may play a regulatory role in IL-12 signalling pathways [228].

Kametaka demonstrated that GAK interacts with the gamma subunit of AP1, the adaptor protein complex that is a heterotetramer that participates in cargo sorting into CCVs at the TGN [229]. Mutations of the GAK ear domain which binds AP1 or siRNA interference directed against AP1 decreases the association of GAK with the TGN *in vivo*. Furthermore, siRNA to GAK inhibits sorting of acid hydrolase and cathepsin D to lysosomes.

In a follow up study, total internal reflectance microscopy was used to determine the timing of GAK binding relative to dynamin and clathrin binding during invagination of clathrin coated pits. It was demonstrated that large amounts of GAK are transiently recruited to developing CCVs following dynamin binding and that this recruitment of GAK is dependent on its auxilin/tensin domain which binds to phospholipids [230].

Recently GAK has been implicated in prostate cancer progression [142]. In this study, a Tup 1 repressed transactivation system which exploits the intrinsic transactivation properties of the AR-NTD was utilized to identify novel AR interacting proteins. Three GAK clones were found to interact with AR 1-549 and AR 1-646. This interaction was confirmed using GST pull down assays and coimmunoprecipitation studies. Pull down assays demonstrated that the strongest interaction occurred between GAK and the AR-LBD. This interaction does not appear to be dependent on the kinase or J domains of GAK; however the auxilin/tensin domain does appear to be critical. It was also reported that the interaction between GAK and AR as demonstrated by coimmunoprecipitation is enhanced 2.3X in the presence of hormone. Additionally, GAK appeared to enhance AR transcriptional levels at low levels of androgens. Transactivation assays were conducted in PC3 cells transfected with AR and increasing quantities of GAK expression plasmid. In the presence of hormone, GAK enhanced AF1 activity of AR up to 5X in a dose dependent manner. Finally, neoadjuvant tissue microarray analysis illustrated that GAK expression was enhanced during progression to prostate cancers.

1.10 Hypothesis

As discussed previously, initial findings by Ray *et. al.* [142] suggest that GAK may play a valuable role in prostate cancer progression towards AI/CR. It is therefore possible that GAK interacts directly with AR to ultimately cause progression to AI/CR by enhancing AR activity and facilitating transcription of growth related genes regulated by AR.

1.11 Objectives

This thesis aims to provide supplementary evidence to support the initial finding that GAK modulates AR activity and to determine if overexpressing GAK affects progression to AI. Transactivation assays overexpressing and knocking down GAK, combined with a series of xenograft experiments, were the primary methods utilized to accomplish this.

Chapter 2   Materials and Methods

2.1   Cell Lines and Culture Conditions

PC3 and LNCaP cells were used to determine the effect of GAK on prostate cancer progression. The PC3 cells were acquired from AATC and the LNCaP cells were generously obtained from Dr. Martin Gleave.   PC3 cells were maintained in Dulbecco's Modified Eagles's Media (DMEM) (#12500-096; Invitrogen) supplemented with 10 % fetal bovine serum (FBS) (#12483-020; Invitrogen) and 100 units/ml of penicillin-streptomycin (P/S) (#15070-063; Invitrogen).   LNCaP cells were grown in RPMI 1640 Media (RPMI) (#12633-020; Invitrogen) supplemented with 10% FBS and 100 units/ml of P/S. All cell lines were grown in 5% carbon dioxide (CO_2) environment at 37ºC in 96 well, 12 well, 24 well, 10 cm (Corning) or 15 cm plates (NUNC).

2.2   Plasmids and siRNA

The plasmid for GAK expression was inherited from Ray *et. al.* [142], the initial template of which was provided by Dr. H. Hojima (Osaka University, Japan). The ATA mutated cyclin G associated kinase (pcDNA3.1-ATA GAK V5), a control construct in which the ATG start codon is mutated to an ATA leucine residue which effectively hinders protein translation, was produced in the lab via site directed mutagenesis by Dr. Jason Read.

The pSuper and pSuper-GAK 545 siRNA plasmids used in this study were a generous gift from Zhang *et. al.* [227]. The retroviral pSuper vector used in the study was the same as that described by Brummelkamp *et. al.* [231]. This retroviral pSuper vector contains a hairpin construct capable of generating a 19-nt duplex RNAi oligonucleotide corresponding to the human GAK sequence starting at the 525th or 145th base (denoted 545 or 145 respectively) in the coding sequence. The sequences for 545 and 145 are as follows: (aggct gagttcctct tgct) and (cgggag cccgagcccg acc). Briefly,

selected sequences were submitted to BLAST searches against the human genome sequence to ensure only GAK mRNA was targeted.

2.3   Transactivation Assays

To examine the effect of GAK overexpression on AR activity, PC3 and LNCaP cells were grown in 6-well plates and cotransfected with pcDNA3.1-hAR, pARR3-tk-Luc reporter plasmid, renilla pRL-TK vector, and one of pcDNA3.1, pcDNA3.1-GAK V5, or pcDNA3.1-ATA GAK V5 to a total DNA mass of 9 µg total/plate.   The pARR3tk-Luc reporter plasmid has 3 tandem ARRs upstream of the thymadine kinase (tk) promoter, and thus expression of the luciferase gene is under the control of AR.   The renilla reporter plasmid functions as a genetic reporter immediately following translation.   It generates a monomeric 36 kDa protein which catalyzes coelenterate-luciferin oxidation to produce light. To assess the effect of GAK knockdown on AR activity, GAK siRNA 545 or its corresponding control plasmid pSuper, were cotransfected with the above aforementioned plasmids. Transfected cells were stimulated with 1 nm R1881, a potent, synthetic androgen, or with an ethanol control in media containing 10% charcoal serum stripped (CSS)-FBS for 24 hours prior to analysis.   Transfection efficiency was normalized to renilla luciferase expressed from the pRL-TK vector and/or to protein concentration depending on the cell line.   Renilla and firefly activities were assayed with the Dual Luciferase Assay Kit (#TM040; Promega).   20 µl of cell lysate per well was analyzed for luciferase activity using the MicroLumiatPlus luminometre (EG&G Berthold). Lysates were analyzed for GAK, AR, and actin expression by western blot.

2.4   Western Blotting

Western blotting was carried out as previously described [220]. Briefly, protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 8.5% or 10% gels, and transferred to a polyvinylidene difluoride (PVDF) membrane (#IPVH00010; Millipore).   Markers used were See Blue Plus 2 Prestained

Standard (1X) (#LC5925; Invitrogen) and PageRuler^{TM} Prestained protein Ladder (#SM0671; Fermentus). Membranes were blocked in TBS-T (20 mM Tris-CL, pH 7.6, 137 mM NaCl, and 0.05% Tween 20) with 5% skim milk for 1 hour. Membranes were then incubated overnight in primary antibodies (final concentrations vary depending on antibody), washed in TBS-T, and then incubated with horseradish peroxidae (HRP)-conjugated secondary antibody for 1 hour. Membranes were washed again in TBS-T, and then developed using a chemiluminescence (ECL) kit (#RPN2108; Amersham). Primary antibodies used in the western blot analysis were mouse monoclonal anti-V5 [68-0705: Invitrogen], mouse monoclonal AR (441) (sc-7305; Santa Cruz Technology), rabbit polyclonal AR (C-19) (sc-815; Santa Cruz Biotechnology), and rabbit polyclonal anti-actin ((#2066; Sigma). Secondary antibodies (1/1000 dilution) used during western blotting were goat anti-mouse IgG-conjugated HRP (sc-2005; Santa Cruz Biotechnology), goat anti-rabbit IgG-conjugated HRP (sc-2054; Santa Cruz Biotechnology), and anti-mouse True Blot (#18-8877-31; eBioscience).

2.5   LNCaP Cells Expressing Tetracycline-Inducible GAK

LNCaP cells stably expressing tetracycline-inducible GAK (LNCaP-GAK) and the corresponding control cell line (LNCAP-DEST) were produced by Dr. Latif Wafa, as was done with DDC [11]. Briefly, the ViraPower T-REX Lentiviral Expression System and Gateway Technology vectors were utilized to generate these two cells lines according to the manufacturer's protocol (#K4967-00; Invitrogen). Using Lipofectamine 2000 reagent (#11668-019; Invitrogen), 3 μg of each lentiviral vector (pLenti4/TO/V5-DEST carrying the *GAK* gene, the corresponding pLenti4/TO/V5-DEST control, and the pLenti6/TR containing the TetR gene) together with 9 μg of the ViraPower packaging mix, were transfected into 293T cells. The DNA- Lipofectamine 2000 complexes diluted in Opti-MEM I Medium (#31985; Gibco-BRL) were allowed to form for 20 min at room temperature before addition to 293T cells. The cells were then incubated for 24 hours at 37°C and 5% CO_2 before removing the DNA-Lipofectamine 2000 complex containing media and replacing it with supplemented DMEM media (10% FBS, 2 mM L-glutamine,

0.1 mM MEM Non-Essential Amino Acids, 1% penicillin/streptomycin, and 1 mM MEM Sodium Pyruvate). The resulting retroviral particles were harvested by removing the media 72 hours after transfection and using them to generate stably co-transduced LNCaP cell lines. Two cell lines were created: the LNCaP-GAK line, which expresses tetracycline-inducible GAK, and the LNCaP-DEST line, which contains the empty vector control and the tetR gene. The tetracycline analogue, doxycycline hyclate (DOX) was used in subsequent concentration experiments to determine the optimal concentration required to induce GAK expression. 1 µg/ml of DOX (#D9891-25G; Sigma Aldrich) was added to the cell culture media for *in vitro* studies while 200 ug/ml was used in the *in vivo* experiments.

2.6   MTS Assays

Stably transfected DOX inducible LNCaP-GAK cells, and the control counterpart, LNCaP-DEST cells, were seeded in replicates of 6 into five 96 well plate at a density of 3000 cells/plate in RPMI + 5% CSS-FBS. Three days later once the cells have had an opportunity to attach to the plate, 100 µl of CSS-FBS containing media containing the following treatments was added to the cells: -DOX/-R1881, -DOX/+R1881, +DOX/-R1881, and +DOX/+R1881. DOX was added at the optimal concentration of 5 µg/ml and R1881 was added at its optimal concentration of 1 nM. The first plate was removed from the set on day #3 and cell proliferation was quantified using the CellTitre 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (#TB169; Promega) following the manufacturers procedures. Cell proliferation in the four remaining plates was quantified on days 5, 7, 9, and 11.

2.7   Soft Agar Assays

The LNCaP-GAK and LNCaP-DEST cell lines were grown *in vitro* as previously described. The base agar was made by microwaving 1% agarose in distilled water, mixing it with an equal volume of 2X RPMI + 20% FBS, and then plating 1.5 ml of it out

on gridded 35 x 10 mm plates (#83.1800.001; Starstedt) to make a final base agar consisting of 0.5% agar in 1X RPMI containing 10% FBS. The top agar was produced in a very similar fashion, except the final top agar contained 0.35% agar in 1X RPMI containing 10% FBS and 10, 000 cell/plate. Once plated, the dishes were incubated in humidified 15 cm plates containing 5 ml of distilled water. After one week, cells were counted using a light microscope (Wilovert A Hund, Fisher Scientific).

2.8   *In Vivo* GAK Xenograft Studies

The DOX inducible GAK overexpressing lentiviral stable cell line, LNCaP-GAK, and the control cell line, LNCaP-DEST, were grown in eighteen 175 cm^2 cell culture flasks (Corning) containing RPMI and 10% FBS. Cells were trypsinized with 3 ml of trypsin/flask; trypsin was deactivated by adding 7 ml of RPMI + 10% FBS. 10 ml of cell suspension per flask were pooled into 50 ml conical tubes (Starstedt) and spun at 1000 rpm for 4 minutes using a Fisher Scientific accuSpinR centrifuge.   The media was aspirated immediately after the spin and the cell pellets were resuspended in 50 ml of RPMI + 10 % FBS. Cells were counted using a hemocytometer, both cell lines were normalized to ensure the same number of cells would be injected into each mouse, and cells were spun down one more time, leaving enough media to comprise half the final volume required for injections, approximately 2.5 ml. Cell suspensions containing equal numbers of cells were resuspended in 2.5 ml of Matrigel (cell preparation and injections were by Mary Bowden). Approximately 100 ul of cell suspension/Matrigel was injected into each flank, comprising 2.0 x 10^6 cells. Mice were monitored weekly from the day of injection for body mass, tumour volume, and PSA serum levels via tail bleeds. Enzyme-linked immunoabsorbent assay (ELISA) was used to quantify the level of PSA produced; all samples were taken in a minimum of duplicates.

Three types of xenograft experiments were conducted, each varying in terms of overall objective and consequently experimental design. In the first xenograft, all mice were castrated at the point in time at which serum PSA reached a threshold of 75 ng/μl.

At the time of castration, 200 µg/ml DOX was administered in the drinking water to half of the mice in each treatment group. The DOX water was changed every 4 days. In the second xenograft experiment, at the point in time when serum PSA levels reached 75 ng/µl, only half of the mice were castrated; however, unlike the previous experiment, all of the mice were treated with DOX to alleviate the issue of uninduced GAK expression, a phenomenon referred to as "leakiness".

In the third xenograft experiment, mice were injected with either the LNCaP-GAK or the LNCaP-DEST cell line and half of each group of mice were immediately treated with DOX, yielding four different groups (DEST-DOX, DEST+DOX, GAK-DOX, and GAK+DOX) all of which have been treated right from the time of injection as opposed to the time of castration at 75 ng/µl of serum PSA, as conducted in the previous two experiments.

For all three types of experiments, when the tumour burden for each mouse reached 10% of its body mass, the mouse was sacrificed and tumours were excised from both sites, pooled, and frozen at -80°C. Tumour samples were later used for western analysis and to build a tissue microarray for immunohistochemical analysis.

2.9 ELISA Assays

To quantify the amount of PSA in the serum of each mouse, ELISA assays were conducted, following the manufacturers protocol (#TM-107; ClinPro International). Briefly, 50 µl of each standard was loaded in duplicate onto the rabbit anti-PSA coated microtitre plate provided, along with a well containing a distilled water blank, and three control wells containing samples of known PSA concentrations (#1085-12; Abbott Laboratories). 10 µl of each serum samples was diluted into 40 µl of distilled water, and then loaded onto the plate in duplicate. 50 µl of zero buffer was added to each well, mixed gently, and the plate was allowed to incubate at room temperature for 60 minutes. The plate was washed 5X with distilled water, and excess water was removed by striking

the plate against absorbent paper towel. 100 µl of enzyme conjugate reagent was then added to each well, mixed briefly, and then allowed to incubate for 60 minutes. Once again, the plate was washed 5X with distilled water, and the excess water was removed by striking the plate against absorbent paper towel. 100 µl of TMB reagent was added to each well, and after 20 minutes of incubation, the reaction was stopped by adding 100 µl of stop solution. The plate was read at 450 nm using a PowerWaveX microtitre plate reader (Bio-Tek Laboratories) and KC4 Kineticalc software for Windows (Bio-Tek Laboratories).

2.10 Protein Extraction From Tumours

A protocol designed in house was utilized to extract protein from the tumours. 100 mg of tumour tissue was weighed out, placed into an ice cold eppendorf, and 200 ul of RIPA buffer (0.5% sodium deoxycholate, 150 mM sodium chloride, 50 mM Tris (pH 8.0) 1% NP40, 0.1% SDS) containing protease inhibitor cocktail (#1836145; Complete), 0.1 mM PMSF, and phosphatase inhibitors (60 mM ß-glycerol phosphate, 4 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 30 mM sodium fluoride) was added to it immediately. The tissue was then ground up using a 2 ml dounce (Kontes Glass Company) and once the tissue was completely dounced, samples were spun at maximum speed for 60 minutes at 4°C. The supernatant was collected and stored at -80°C. Protein concentrations were quantified using the BSA kit (#23227; Pierce). 50 µg of protein was run out on 8.5% or 10% SDS-PAGE gels and western blotted for various proteins.

2.11 Immunohistochemistry and Tissue Microarray Analysis

Mice were sacrificed by Dr. Rob Snoek when the tumour burden reached 10% of the mouse's body mass, tumours were extracted, and embedded in paraffin blocks. Twelve cores per tumour were used to construct a tissue microarray (protocols as per those provided and conducted generously by Dr. Ladan Fazli). The cores, each 0.6 mm in diameter, were arranged in rectilinear pattern and organized via treatment group. A

conventional deparaffinization and dehydration sequence was followed to prepare the sections for staining. Sections were incubated 2X in xylene for 5 minutes, 2X in 100% absolute ethanol for 3 minutes, 2X in 95% ethanol for 3 minutes, 1X in 70% ethanol for 3 minutes, and rinsed 3X with PBS for 5 minutes. Once the sections had been re-hydrated, they were not allowed to dry. One drop of 0.2% Triton for every 10 ml of PBS was applied for 10 minutes. Sections were washed with PBS 3X for 5 minutes. The slides were then placed in pre-warmed steamer (60-90°C) with citrate buffer in a Caplan jar for 30 minutes, and then removed and allowed to cool to room temperature for 20 minutes. Sections were then washed with PBS 3X for 5 minutes. Sections were then incubated for 30 minutes with blocking solution (3% BSA) to eliminate the background and then washed 3X again with PBS for 5 minutes. 3% H_2O_2 was applied for 10 minutes and sections were washed 3X in PBS for 5 minutes.

Sections immunostained for AR (# PA1-111A; Affinity Bioreagents) used a dilution series of 1/50, 1/100, 1/200, 1/400, 1/800 diluted with 1% BSA and were incubated overnight at room temperature. Sections were washed 3X with PBS for 5 minutes. Sections were incubated in anti-rabbit secondary antibody HRP (# K4011; DAKO Envision System) for 15 minutes, and then washed 3X with PBS for 5 minutes. Nova-red (#SK-4800; Vector) was applied for 5 minutes and then washed with distilled water 3X for 5 minutes. Sections were counterstained with hematoxylin (# H-3404, Vector) for 3 minutes and then washed with distilled water 3X for 5 minutes. Sections were dehydrated by rapidly dipping them into 70%, 95%, and 100% ethanol and then cleared in xylene for 3 minutes and covered with a cover glass.

Sections immunostained for GAK (#M057-3: Medical and Biological Laboratories) used a dilution series of 1/10, and 1/20 diluted with 1% BSA and were incubated overnight at room temperature. Sections were washed 3X with PBS for 5 minutes. Sections were incubated in biotinylated secondary antibody (# K0690; DAKO LSAB) for 15 minutes, and then washed 3X with PBS for 5 minutes. Sections were then soaked in strepavidin-peroxidase conjugate (#K0690; DAKO LSAB + HRP) for 15 minutes at room temperature, and then washed with PBS 3X for 5 minutes. Nova-red

(#SK-4800; Vector) was applied for 5 minutes and then washed with distilled water 3X for 5 minutes. Sections were counterstained with hematoxylin (# H-3404, Vector) for 3 minutes and then washed with distilled water 3X for 5 minutes. Sections were dehydrated by rapidly dipping them into 70%, 95%, and 100% ethanol and then cleared in xylene for 3 minutes and covered with a cover glass.

2.12 Statistical Analysis

Statistical analyses were conducted on all results generated. Students t-tests were utilized to determine statistically significant differences between treatment groups in the luciferase, proliferation, tumourigenicity experiments. Any values less than the p value of 0.05 were determined to be significant. Linear regression analyses of slopes was conducted on all data generated from the xeongraft experiments. Differences between treatment groups were determined to be significant if the p value was calculated to be less than 0.05.

Chapter 3   Results

3.1   The Effect of GAK On AR Transactivation

3.1.1   Overexpressing GAK In PC3 Cells

Transactivation assays conducted by Ray reported that GAK enhances the AF-1 activity of AR [142]. To verify this observation, initial experiments were conducted in PC3 prostate carcinoma cells. PC3 cells were chosen because they consistently yield high transfection efficiencies and generate reproducible results. PC3 cells were cotransfected with expression vectors for full length human AR (pcDNA3.1-hAR), pARR3-tk-Luc reporter, renilla pRL-TK reporter, and one of the following in both the presence and absence of hormone: pcDNA3.1 (EV), pcDNA3.1-GAK V5 (GAK V5), or pcDNA3.1-ATA GAK V5 (ATA GAK V5). pcDNA3.1 served as the empty vector control, pcDNA3.1-GAK V5 overexpressed a V5-tagged version of GAK, and pcDNA3.1-ATA GAK V5 functioned as a secondary control vector whereby the ATG start codon had been mutated to ATA. The ATA codon encodes for a leucine residue and this mutation hinders translation and ultimately the production of a protein product. An important point to note is that there is substantial difficulty affiliated with detecting endogenous GAK levels via western blot analysis. Consequently, transfecting cells with pcDNA.3.1-GAK V5 takes advantage of the ease in which V5-tagged GAK can be visualized. The results presented demonstrate the effect of exogenous sources of GAK on AR levels and activity. Transfected cells were grown in culture and stimulated with 1 nM of synthetic androgen, R1881, 24 hours prior to via western blotting and luciferase assay analysis. All experiments were done at a concentration of 1 nM R1881 and were done in triplicate. Statistical analysis was conducted by means of a t-test to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

Western blotting analysis confirmed V5-tagged GAK overexpression in cells transfected with pcDNA3.1 GAK V5 (Figure 8). As expected, neither of the control vectors, pcDNA3.1 or pcDNA3.1-ATA GAK V5, generated any visible protein product.
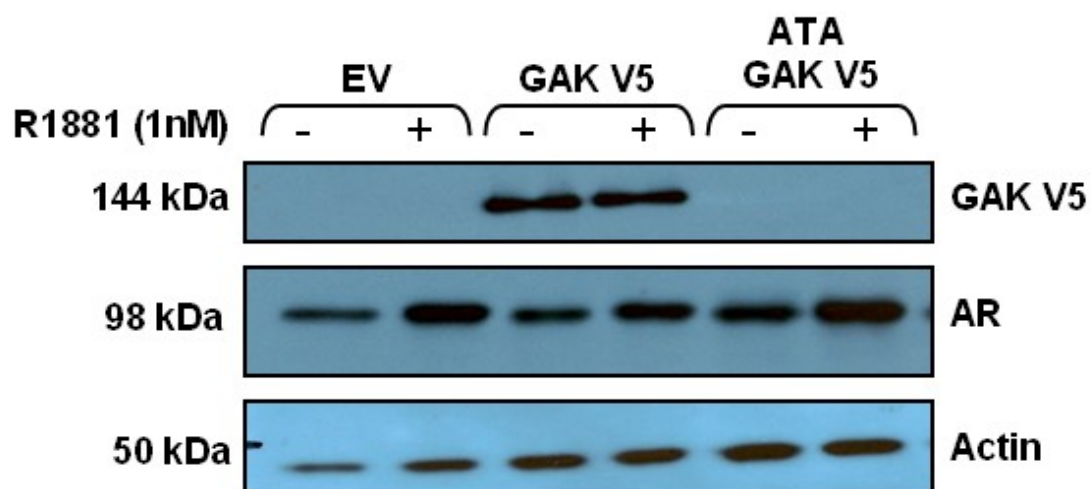
Figure 8 - Western blot analysis of PC3 cells overexpressing GAK.

PC3 cells were transfected with pcDNA3.1 (EV), pcDNA3.1-GAK V5 (GAK), and pcDNA3.1-ATA GAK V5 (ATA GAK V5) in both the presence (+) and absence (-) of hormone. Western blot analysis was conducted and blots were probed for V5-tagged GAK, AR, and actin.

Several attempts were made to determine endogenous GAK levels, but generating reproducible results utilizing the original antibody cited by Ray proved to be difficult [142]. AR levels were visibly enhanced in the presence of hormone, but no noticeable differences were observed between cells transfected with pcDNA3.1, pcDNA3.1-GAK V5, or pcDNA3.1-ATA GAK V5, suggesting that overexpressing GAK does not modulate AR expression.

The effect of GAK on AR activity was quantified using the dual luciferase reporter assay. Initially it appeared as if overexpressing GAK positively enhanced AR activity compared to the pcDNA3.1 control vector in the presence of hormone (Figure 9). However, the pcDNA3.1-ATA GAK V5 control vector, which produces a transcript but fails to produce protein, induced AR activity to a similar degree as the pcDNA3.1-GAK V5 plasmid in the presence of hormone. Several trials demonstrated that the pcDNA3.1-GAK V5 and pcDNA3.1-ATA GAK V5 vectors consistently generated enhanced AR activity levels compared to the pcDNA3.1 control vector to a degree that was highly significant ($p < 0.01$). Interestingly, the initial transactivation experiments conducted by Ray did not integrate the use of this type of control [142].

Given that the pcDNA3.1-ATA GAK V5 plasmid enhances AR activity despite the fact that it does not produce a GAK V5 protein product, either GAK does indeed regulate AR activity as previously reported, or the pcDNA3.1 vector suppresses AR activity, therefore creating the artificial impression that GAK transactivates AR. These results in PC3 cells are highly reproducible, but from these experiments alone, it is difficult to conclude definitively whether or not GAK modulates AR activity.

As mentioned previously, Ray reported that overexpressing GAK results in an increase in AR activity [142]. According to this paper, increasing amounts of GAK enhances AR transactivation up to 5.3-fold in the presence of hormone. PC3 cells in the above studies were transfected following the same protocol adopted by Ray. However, it should be noted that in these previous studies, the pcDNA3.1-GAK V5 expression vector was added in quantities anywhere from 0-500 ng/well, using the pcDNA3.1 vector as a

Vector	EV	GAK V5	ATA GAK V5
pARR3tk-Luc	1.0	1.0	1.0
renilla pRL-TK	0.5	0.5	0.5
pcDNA3.1-hAR	1.5	1.5	1.5
pcDNA3.1	6.0	-	-
pcDNA3.1-GAK V5	-	6.0	-
pcDNA-ATA GAK V5	-	-	6.0

Figure 9 - The effect of overexpressing GAK on AR activity in PC3 cells.
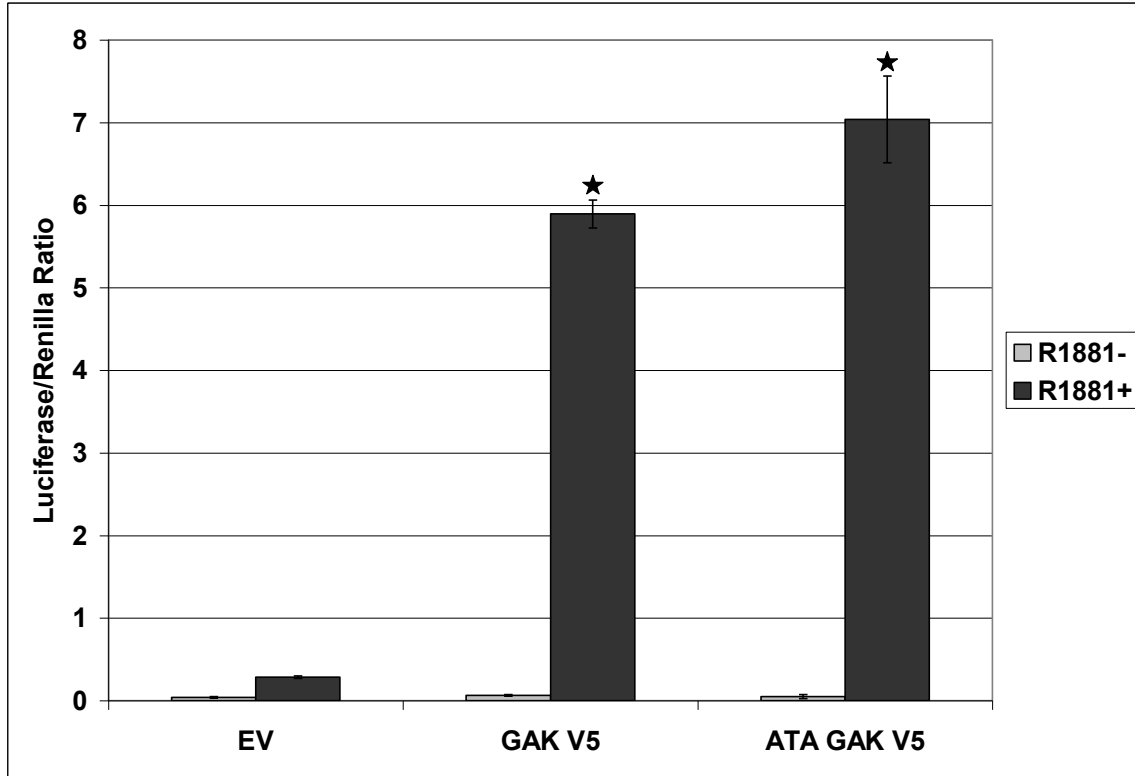
PC3 cells were transfected with pcDNA3.1 (EV), pcDNA3.1-GAK V5 (GAK V5), and pcDNA3.1-ATA GAK V5 (ATA GAK V5) in both the presence and absence of R1881. AR activity was quantified using the luciferase reporter assay, and results were normalized to renilla. Experiments were conducted in triplicate and statistical analysis was conducted by means of a t-test to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

filler to maintain the amount of DNA transfected equivalent for each sample at 1ug/well. Knowing that the pcDNA3.1-ATA GAK V5 vector can enhance AR activity despite the fact that it does not produce protein, questions arise as to whether or not GAK really does in fact does transactivate AR in a dose-dependent manner.

3.1.2   Testing The Empty Vector Effect In PC3 Cells

To test the hypothesis that the pcDNA3.1 vector may have an inhibitory effect on AR activity, transactivation assays were conducted in PC3 cells whereby increasing amounts of GAK were transfected into cells, offset by decreasing amounts of control pcDNA3.1 vector. PC3 cells were transfected with equivalent quantities of pcDNA3.1-hAR, pARR3tk-Luc, renilla pRL-TK, and increasing amounts of pcDNA3.1-GAK V5 ranging from 0-1.0 µg/well. Total DNA of 1 µg/well was achieved by supplementing with decreasing amounts of pcDNA3.1 (1 to 0 µg respectively). As expected, increasing quantities of pcDNA3.1-GAK V5 generated a significant enhancement of AR activity in PC3 cells in the presence of R1881 ($p < 0.05$) in a dose dependent manner (Figure 10). Conversely, increasing quantities of pcDNA3.1 produced lower levels of AR activation, supporting the possibility that the empty vector has an inhibitory effect on AR activity ($p < 0.05$).

It is worth noting that AR activity in PC3 cells transfected with the two reporters is comparable to AR activity transfected in cells with 0.75 to 1.0 µg of pcDNA3.1-GAK V5, providing additional supporting evidence that the pcDNA3.1 may in fact be having an inhibitory effect. There does not appear to be a statistical difference in AR activity generated in cells transfected with the reporters compared to those transfected with 0.75 µg and 1.0 µg of pcDNA3.1-GAK V5 ($p = 0.4923$ and $p = 0.4594$ respectively). These results are compelling; however, further experimentation knocking down GAK is required to provide conclusive evidence that GAK does not modulate AR activity.
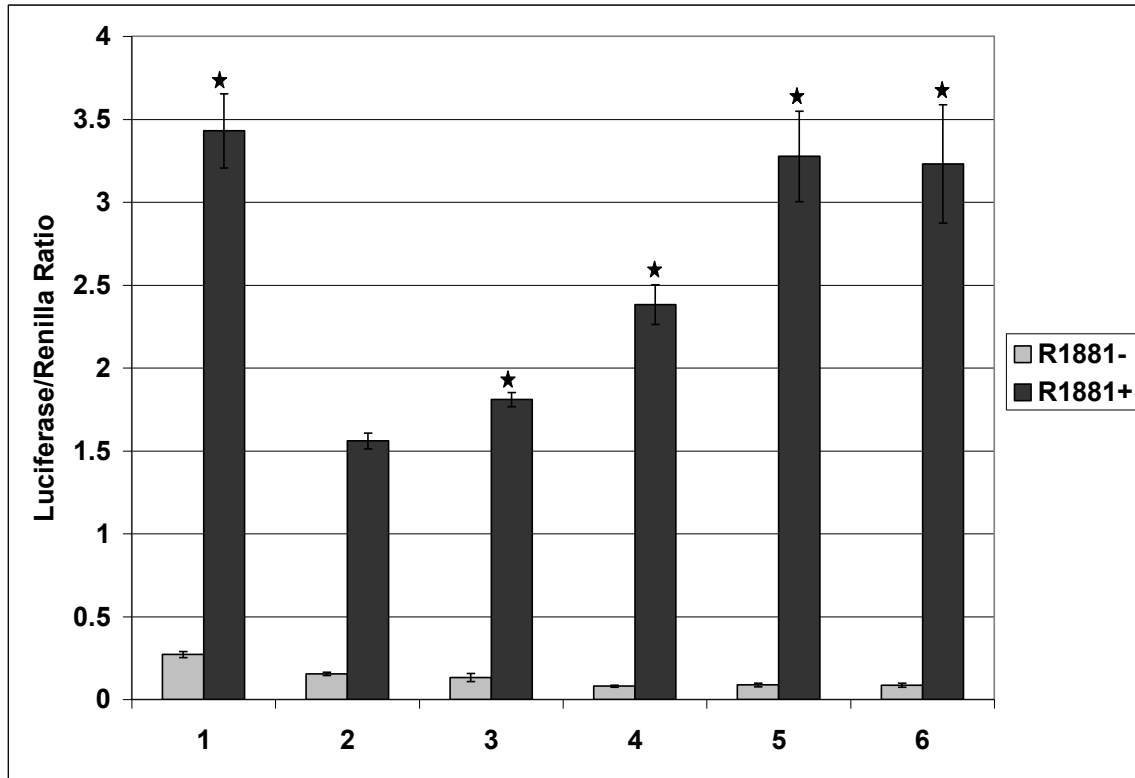
Vector	1	2	3	4	5	6
pARR3tk-Luc	1.0	1.0	1.0	1.0	1.0	1.0
renilla pRL-TK	0.5	0.5	0.5	0.5	0.5	0.5
pcDNA3.1-hAR	1.5	1.5	1.5	1.5	1.5	1.5
pcDNA3.1	0	1	0.75	0.50	0.25	0
pcDNA3.1-GAK V5	0	0	0.25	0.50	0.75	1

Figure 10 - The effect of pcDNA3.1 on AR activity in PC3 cells.

PC3 cells were cotransfected with increasing quantities of pcDNA3.1-GAK V5 (GAK V5) and decreasing quantities of pcDNA3.1 (EV) in both the presence and absence of hormone. AR activity was quantified using the luciferase reporter assay and results were normalized to renilla. Experiments were conducted in triplicate and statistical analysis was conducted by means of a t-test to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

3.1.3   Overexpressing GAK In LNCaP Cells

Slightly modified experiments were carried out in LNCaP cells. LNCaP cells, unlike PC3 cells, express endogenous AR. Consequently, the results generated using LNCaP cells are a reflection of endogenous AR activity levels, as opposed to those derived from an artificial source of AR. LNCaP cells were transfected with the two reporters, pARR3-tk-Luc and renilla pRL-TK, and one of pcDNA3.1, pcDNA3.1-GAK V5, or pcDNA3.1-ATA GAK V5 in both the presence and absence of hormone.   Cells were cultured, harvested, and assayed as previously described. Unlike in PC3 cells where the standard practice is to normalize the data to the luminescence produced from the internal renilla control, protein quantities are utilized in LNCaP cells instead. In LNCaP cells, renilla values fluctuate in the presence of hormone, indicating that there may be some difference in the manner in which renilla is processed in PC3 cells compared to LNCaP cells. However, when the data are normalized to protein in LNCaP cells, there does not appear to be any statistical difference in AR activity levels between cells transfected with either of the control vectors, pcDNA3.1 or pcDNA3.1-ATA GAK-V5, and cells which have been transfected with pcDNA3.1-GAK V5 ($p > 0.05$) (Figure 11). These results support the rationale that GAK may not modulate AR activity as initially reported.

3.1.3   Inhibition of GAK Using Small Interfering RNA In PC3 Cells

To definitively determine whether or not GAK modulates AR activity, and more specifically, whether or not the enhancement of AR activity observed during GAK overexpression is valid or is in fact an artificial enhancement caused by the suppression of AR activity in cells transfected with the pcDNA3.1 control vector, transactivation assays were conducted in PC3 cells knocking down GAK. PC3 cells were transfected with pcDNA3.1-hAR, pARR3tk-Luc, renilla pRL-TK, and either pcDNA3.1, pcDNA3.1-GAK V5, or pcDNA3.1-ATA GAK V5 in conjunction with one of either the pSuper control vector or GAK siRNA 545. Cells were transfected, harvested, and luciferase
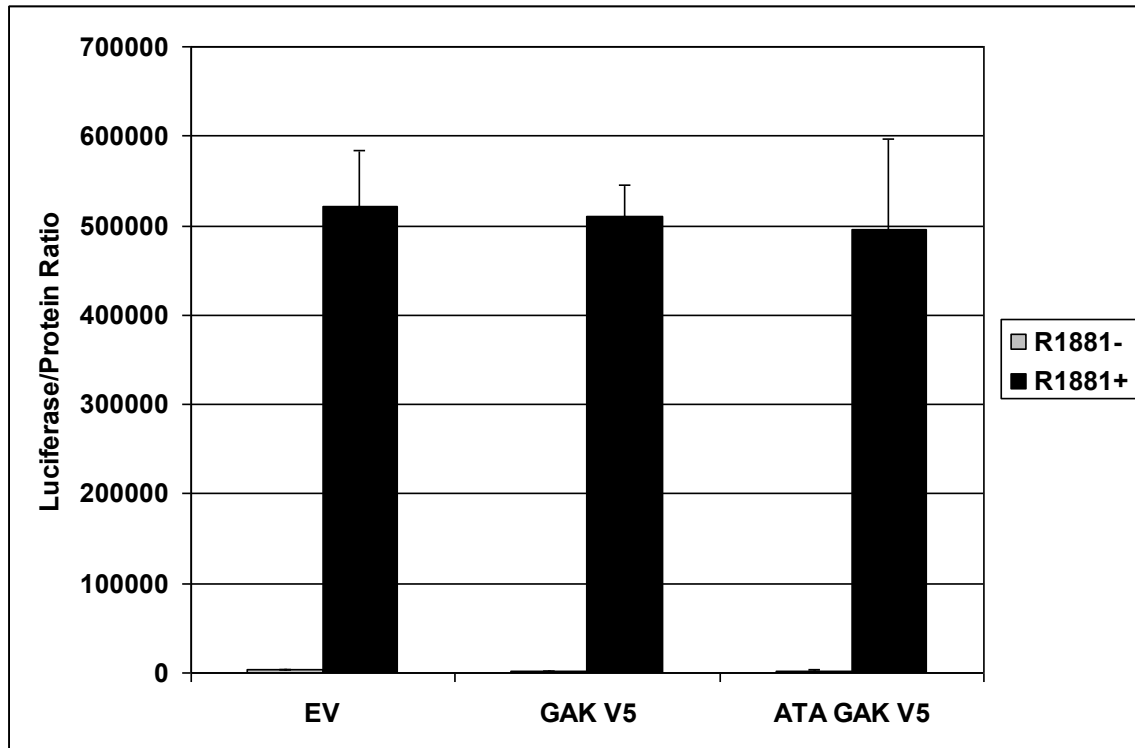
Vector	EV	GAK V5	ATA GAK V5
pARR3tk-Luc	1.0	1.0	1.0
renilla pRL-TK	0.5	0.5	0.5
pcDNA3.1	6.0	-	-
pcDNA3.1-GAK V5	-	6.0	-
pcDNA-ATA GAK V5	-	-	6.0

Figure 11 - The effect of overexpressing GAK on AR activity in LNCaP cells.

LNCaP cells were transfected with pcDNA3.1 (EV), pcDNA3.1-GAK V5 (GAK), and pcDNA3.1-ATA GAK V5 (ATA GAK V5) in both the presence and absence of hormone. AR activity was quantified using the luciferase reporter assay, and results were normalized to protein. Experiments were conducted in triplicate and statistical analysis was conducted by means of a t-test to determine if there were any statistical differences amongst treatment groups.

results were normalized to renilla. Statistical analyses were conducted as previously described.

Western blot analysis confirmed GAK-V5 overexpression, as well as successful V5-tagged GAK knockdown in both the presence and absence of hormone (Figure 12). As expected and previously demonstrated, PC3 cells transfected with either of the two control vectors, pcDNA3.1 and pcDNA3.1-ATA GAK V5, did not produce any V5-tagged signal, while the pcDNA3.1-GAK V5 plasmid expressed GAK in both the presence and absence of hormone. The addition of the pSuper siRNA control did not appear to have any effects in terms of the overall expression level of V5-tagged GAK; however, GAK siRNA 545 generated a potent knockdown of V5-tagged GAK in the presence and absence of hormone.   Knocking down GAK appeared to have little effect on AR expression levels, confirming previous conclusions that GAK most likely does not modulate AR quantities.

In terms of AR activity, as shown previously, transfecting PC3 cells with the pcDNA3.1-GAK V5 and pcDNA3.1-ATA GAK V5 plasmids appeared to significantly enhance AR activity in comparison to the pcDNA3.1 control vector ($p < 0.01$) (Figure 13). Cotransfecting the pSuper vector appeared to have no effect on the pattern of AR activity generated by these three plasmids. Interestingly, when PC3 cells transfected with pcDNA3.1, pcDNA3.1-GAK V5, or pcDNA3.1-ATA GAK V5 were also transfected with GAK 545 siRNA, which knocks down GAK derived from the pcDNA3.1-GAK V5 plasmid, and in theory, should also knock down endogenous GAK, the overall pattern of AR transactivation observed was very similar to that produced by cells transfected with either of these three vectors in conjunction with the pSuper control. Cells transfected with pcDNA3.1-ATA GAK V5 and pcDNA3.1-GAK V5 in addition to GAK 545 siRNA generated AR activity levels that were significantly enhanced compared to cells transfected with pcDNA3.1 and GAK 545 siRNA ($p < 0.01$).   Perhaps the most intriguing result was that cells transfected with the two control vectors, pcDNA3.1 and pSuper, generated AR activity levels that were no
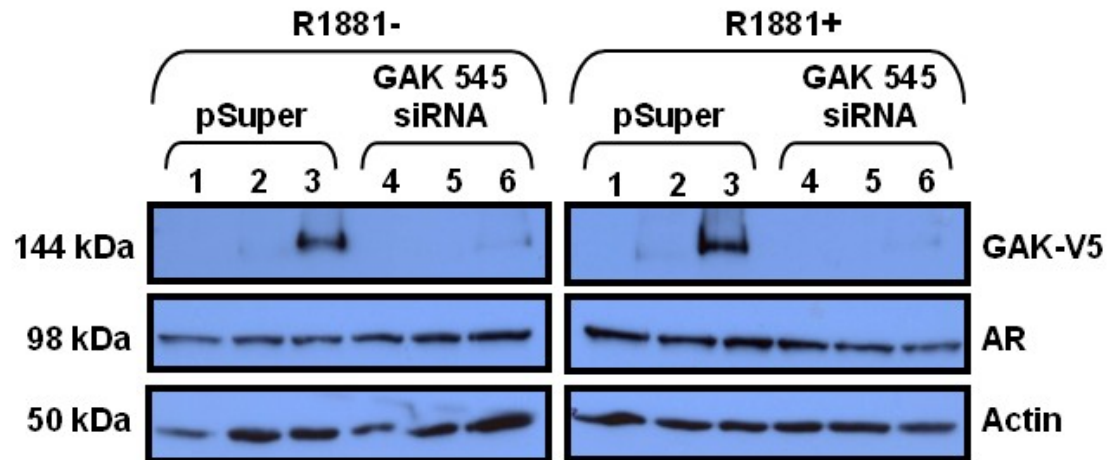
Figure 12 - Western blot analysis of PC3 cells knocking down GAK.

PC3 cells were transfected with pcDNA3.1 (1), pcDNA3.1-ATA GAK V5 (2), and pcDNA3.1-GAK V5 [19] in both the presence and absence of hormone and one of either pSuper or GAK 545 siRNA. Western blot analysis was conducted and cell lysates were probed for V5-tagged GAK, AR, and actin.
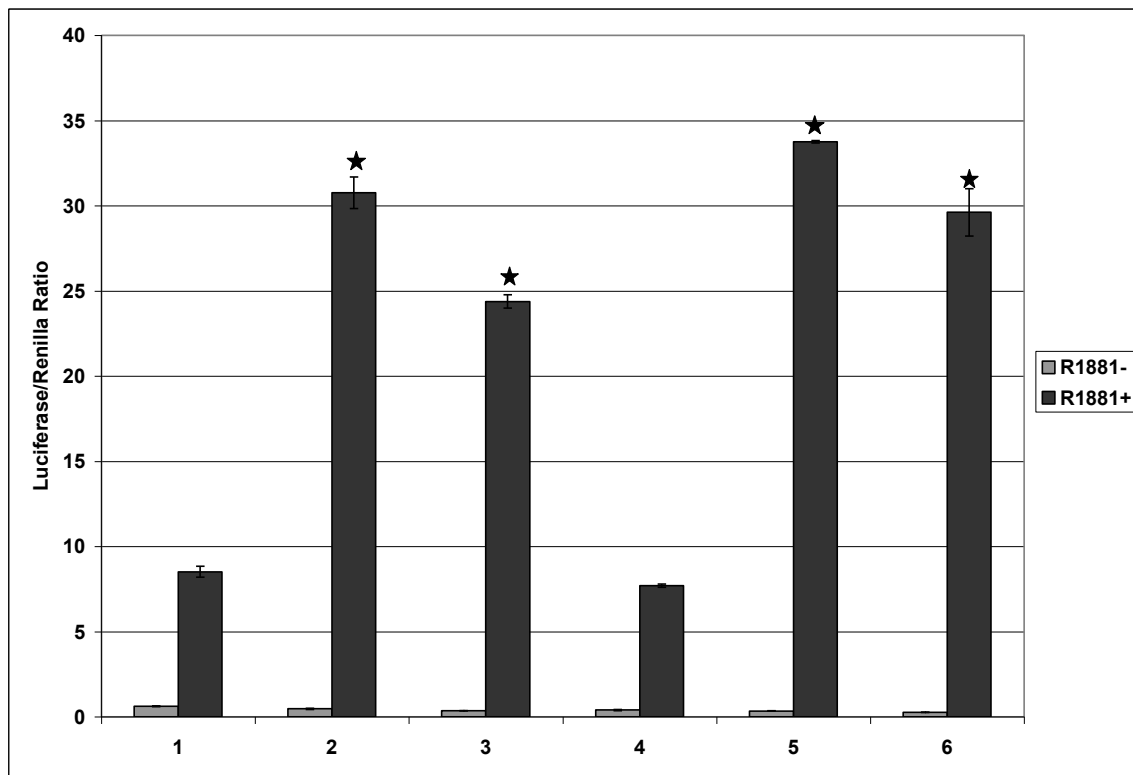
Vector	1	2	3	4	5	6
pARR3tk-Luc	1.0	1.0	1.0	1.0	1.0	1.0
renilla pRL-TK	0.5	0.5	0.5	0.5	0.5	0.5
pcDNA3.1-hAR	1.5	1.5	1.5	1.5	1.5	1.5
pcDNA3.1	6.0	-	-	6.0	-	-
pcDNA3.1-GAK V5	-	6.0	-	-	6.0	-
pcDNA3.1-ATA GAK V5	-	-	6.0	-	-	6.0
pSUPER	6.0	6.0	6.0	-	-	-
GAK 545 siRNA	-	-	-	6.0	6.0	6.0

Figure 13 - Comparing the effect of overexpressing and knocking down GAK on AR activity in PC3 cells.

PC3 cells were transfected with pcDNA3.1 (EV), pcDNA3.1-ATA GAK V5 (ATA GAK V5), and pcDNA3.1-GAK V5 (GAK) and one of either pSuper or GAK 545 siRNA in both the presence and absence of hormone. AR activity was quantified using the luciferase reporter assay and results were normalized to renilla. Experiments were conducted in triplicate and statistical analysis was conducted by means of a t-test to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

different than cells transfected with pcDNA3.1 and GAK545 siRNA, in which endogenous GAK is theoretically knocked down (p = 0.5317). Overall, despite the fact that there is a very apparent knockdown of V5-tagged GAK generated by GAK 545 siRNA, there does not appear to be any significant difference in the level of AR transactivation, providing strong supporting evidence that GAK is most likely not involved in modulating AR activity, and that the pcDNA3.1 vector does indeed suppress AR activity.

3.1.4   Inhibition of GAK Using Small Interfering RNA In LNCaP Cells

In an effort to ensure that the results generated were not cell-type specific, GAK knockdown experiments were also conducted in LNCaP cells. Initial experiments were conducted whereby LNCaP cells were transfected with equivalent quantities of pcDNA3.1-GAK V5 in addition to equivalent quantities of one of the following: pSuper control vector, GAK siRNA 145, or GAK siRNA 545.   It is important to note that GAK 545 siRNA induces a more potent knockdown of GAK than the GAK 145 siRNA and thus was utilized for subsequent transactivation assays. Cells were transfected, harvested, and results were normalized to protein for reasons previously described.   Interestingly, there were no statistical differences in AR transactivation levels between LNCaP cells transfected with the pSuper control vector, GAK 145 siRNA, or GAK 545 siRNA (p > 0.05) (Figure 14), supporting the findings previously generated in PC3 cells. Western blot analysis confirmed V5-tagged GAK overexpression, as well as a very effective knockdown generated by GAK 145 and GAK 545 siRNA in both the presence and absence of hormone (Figure 15). Knocking down GAK did not appear to effect AR levels in LNCaP cells, mirroring previous observations made in PC3 cells.

3.1.5   Summary

The transactivation assay results generated from the overexpression and knockdown of GAK in both PC3 and LNCaP cells suggest that GAK does not modulate

Vector	pSUPER	GAK 145	GAK 545
pARR3tk-Luc	1.0	1.0	1.0
Renilla pRL-TK	0.5	0.5	0.5
pcDNA3.1-GAK V5	6.0	6.0	6.0
pSUPER	6.0	-	-
GAK 145	-	6.0	-
GAK 545	-	-	6.0

Figure 14 - The effect of knocking down GAK on AR activity in LNCaP cells.

LNCaP cells were transfected with equivalent quantities of pSuper, GAK 145 siRNA, or GAK 545 siRNA in both the presence and absence of hormone. AR activity was quantified using the luciferase reporter assay and results were normalized to protein. Experiments were conducted in triplicate and statistical analysis was conducted by means of a t-test to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).
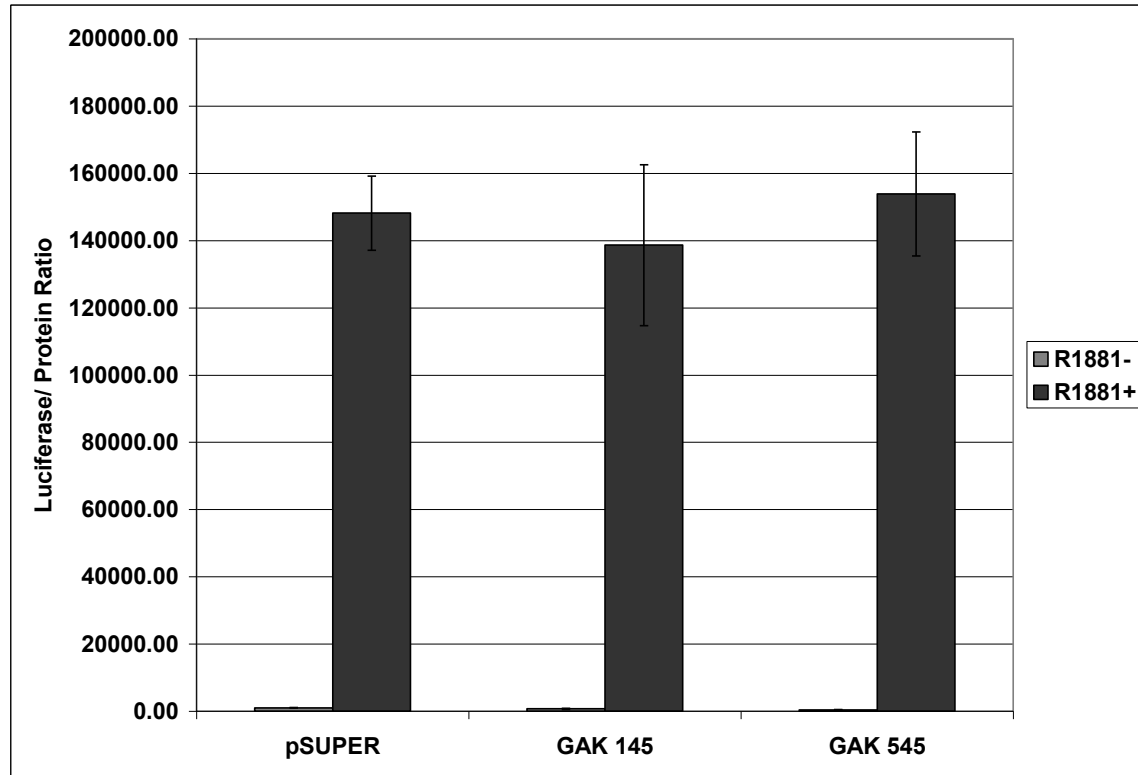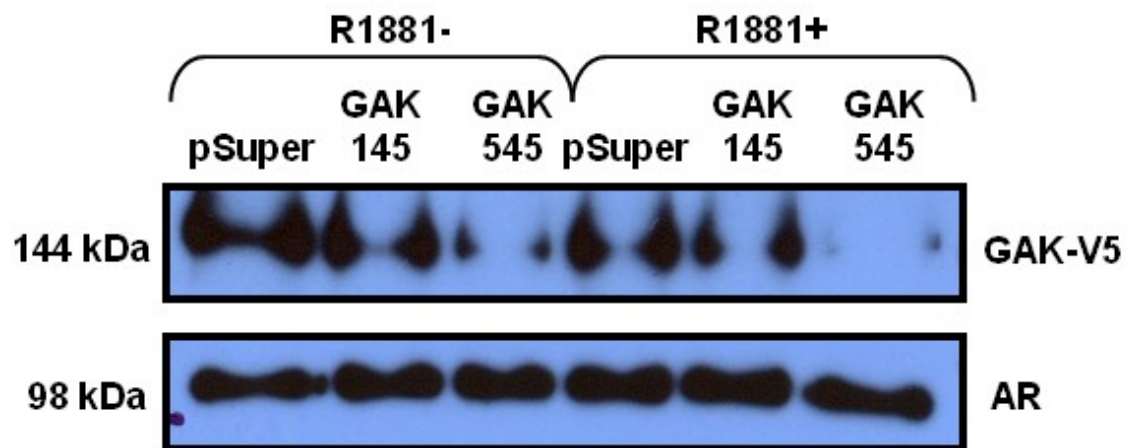
Figure 15 - Western blot analysis of LNCaP cells knocking down GAK.

LNCaP cells were transfected with pSUPER, GAK 145 siRNA, or GAK 545 siRNA in the presence and absence of hormone. Western blot analysis was conducted and cell lysates were probed for V5-tagged GAK and AR.

AR activity, and that the activation observed when cells overexpress GAK is most likely due to a phenomenon whereby the pcDNA3.1 plasmid inhibits AR activity in comparison to the pcDNA-ATA GAK V5 and pcDNA3.1-GAK plasmids. Compared to the pcDNA3.1 control plasmid, overexpressing GAK in PC3 cells demonstrates an enhancement of AR activity which is similar to that produced by the pcDNA3.1-ATA GAK V5 control vector, a plasmid which was verified via western blot analysis to not produce any viable V5-tagged protein product. Given that AR activity is observed in the absence of protein, this suggests that the pcDNA3.1 control vector is having an inhibitory effect on AR activity, as opposed to GAK having any real stimulatory effect on AR. This possibility was verified in an experiment that demonstrated that increasing quantities of pcDNA3.1-GAK V5 plasmid offset by decreasing quantities of pcDNA3.1 plasmid generates an artificial enhancement of AR activity. This phenomenon has since been referred to as the "empty vector effect" and was previously reported, as well as confirmed via personal communications with Latif Wafa [220], and provides some convincing evidence that GAK does not in fact modulate AR activity as previously suggested.

3.2   Confirmation of GAK Overexpression In The LNCaP-GAK Cell Line

Although initial results failed to positively confirm GAK's role as a coregulator, it is still possible that GAK may play a role in progression to AI, albeit an indirect one that does not involve AR. To test this hypothesis, LNCaP cells stably expressing tetracycline-inducible GAK (LNCaP-GAK) and the corresponding control cell line (LNCAP-DEST) were produced and generously provided by Latif Wafa (2007) for all subsequent experiments. Before proceeding, it was important to verify that the LNCaP-GAK cell line overexpressed V5-tagged GAK upon DOX induction. The LNCaP-GAK and the control LNCAP-DEST cells were cultured in standard conditions, induced with DOX, and analyzed via western blot for V5. Two versions of the DOX inducible GAK cells were initially constructed, denoted N1 and N2. Both of these cell lines were tested to determine which line yielded a higher level of GAK expression upon DOX induction. As expected, the LNCAP-DEST control cell line produced no trace of V5-tagged protein.
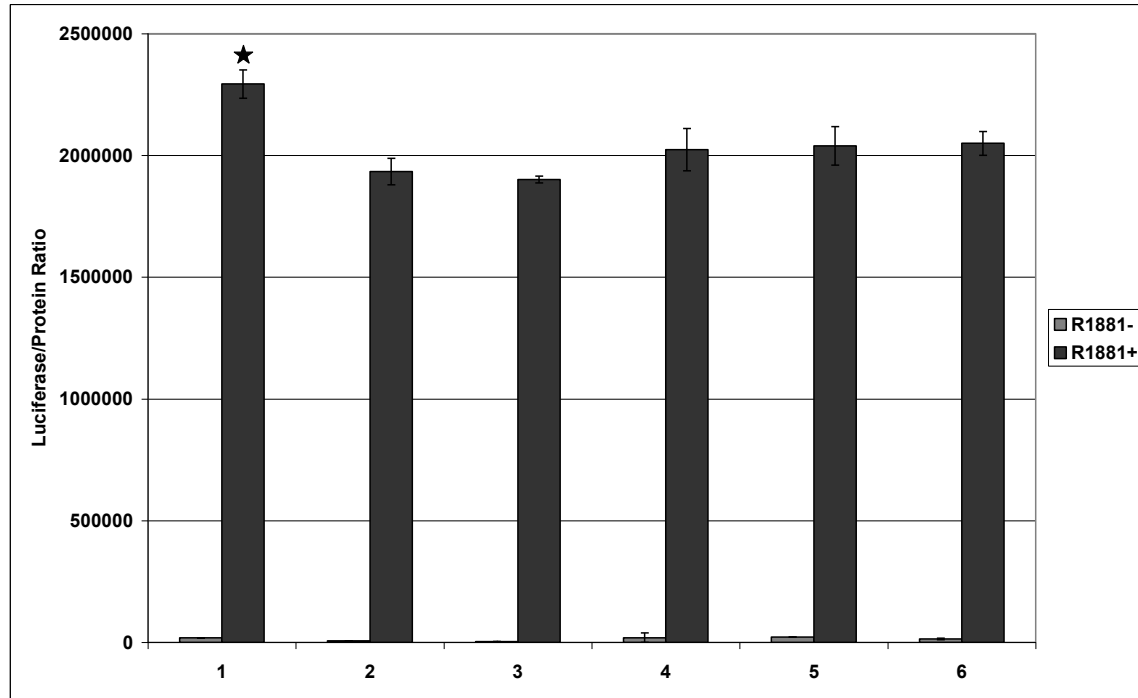
Vector	1	2	3	4	5	6
pARR3tk-Luc	1.0	1.0	1.0	1.0	1.0	1.0
renilla pRL-TK	0.5	0.5	0.5	0.5	0.5	0.5
pcDNA3.1	6.0	-	-	6.0	-	-
pcDNA3.1-GAK V5	-	6.0	-	-	6.0	-
pcDNA3.1-ATA GAK V5	-	-	6.0	-	-	6.0
pSUPER	6.0	6.0	6.0	-	-	-
GAK 545 siRNA	-	-	-	6.0	6.0	6.0

Figure 16 - Comparing the effect of overexpressing and knocking down GAK on AR activity in LNCaP cells.

LNCaP cells were transfected with pcDNA3.1 (EV), pcDNA3.1-ATA GAK V5 (ATA GAK V5), and pcDNA3.1-GAK V5 (GAK), and one of either pSuper or GAK 545 siRNA in both the presence and absence of hormone. AR activity was quantified using the luciferase reporter assay and results were normalized to protein. Experiments were conducted in triplicate and statistical analysis was conducted by means of a t-test to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

Both the N1 and N2 LNCaP-GAK cell lines overexpressed GAK in the presence of DOX. It is important to note that they also exhibited quantities of V5-tagged GAK in the absence of DOX (Figure 17). This is subsequently referred to as "leakiness". The N1 version of the LNCaP-GAK cell line exhibited a smaller degree of leakiness compared to the N2 version of the LNCaP-GAK cell line, and as a result, the N1 version was utilized in the subsequent studies.

3.2   The Effect of GAK On Cell Proliferation

To determine if overexpressing GAK effects cell proliferation rates *in vitro*, MTS assays were conducted comparing LNCaP-GAK cells to the control LNCAP-DEST cells in the presence and absence of hormone. Cells were grown *in vitro* and growth was measured at incremental time points. Proliferation was quantified by adding MTS reagent to the cells. MTS is dehydrogenated by enzymes within viable cells into a formazan product, which is subsequently quantified using a spectrophotometer. Observations were made over an 11 day period. Experiments were conducted in triplicate and statistical analysis was conducted by means of a linear regression followed by a one way ANOVA to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

In the uninduced state, in both the presence and absence of hormone, there appears to be no discernable statistical difference between the proliferation profiles of the LNCaP-GAK or the LNCaP DEST cell lines (Figure 18). Hormone itself appeared to be the only factor which enhanced the proliferation rates of both cell lines to any degree of significance. In the presence of DOX, although it appears that GAK may enhance growth in both the presence and absence of hormone between the time of seeding and the 4 day time point, the only statistically significant effect was that of hormone itself. This seems to indicate that while it is possible GAK may promote cell proliferation, it does not significantly enhance growth, at least not *in vitro* over the course of 11 days (Figure 19).
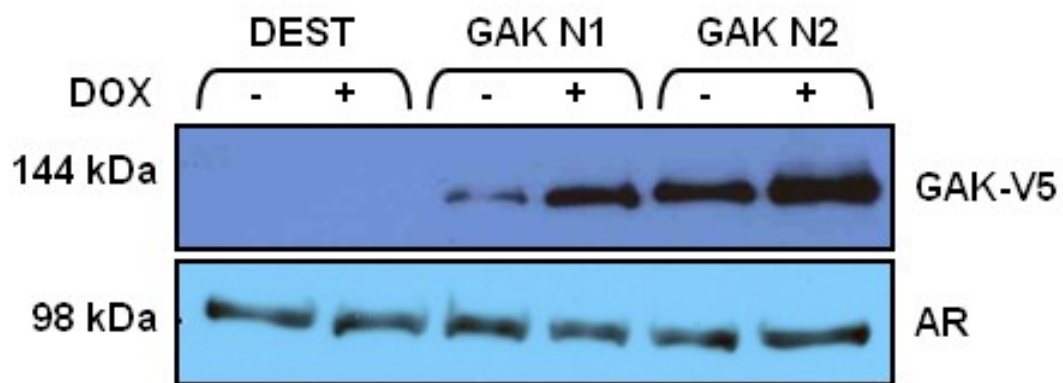
Figure 17 - Confirmation of GAK-V5 overexpression in LNCaP-GAK cells.

LNCaP-DEST and GAK N1 and N2 stable cells were cultured, uninduced (-) or induced with 5 µg/µl DOX (+), and cell lysates were western blotted for V5 and AR.
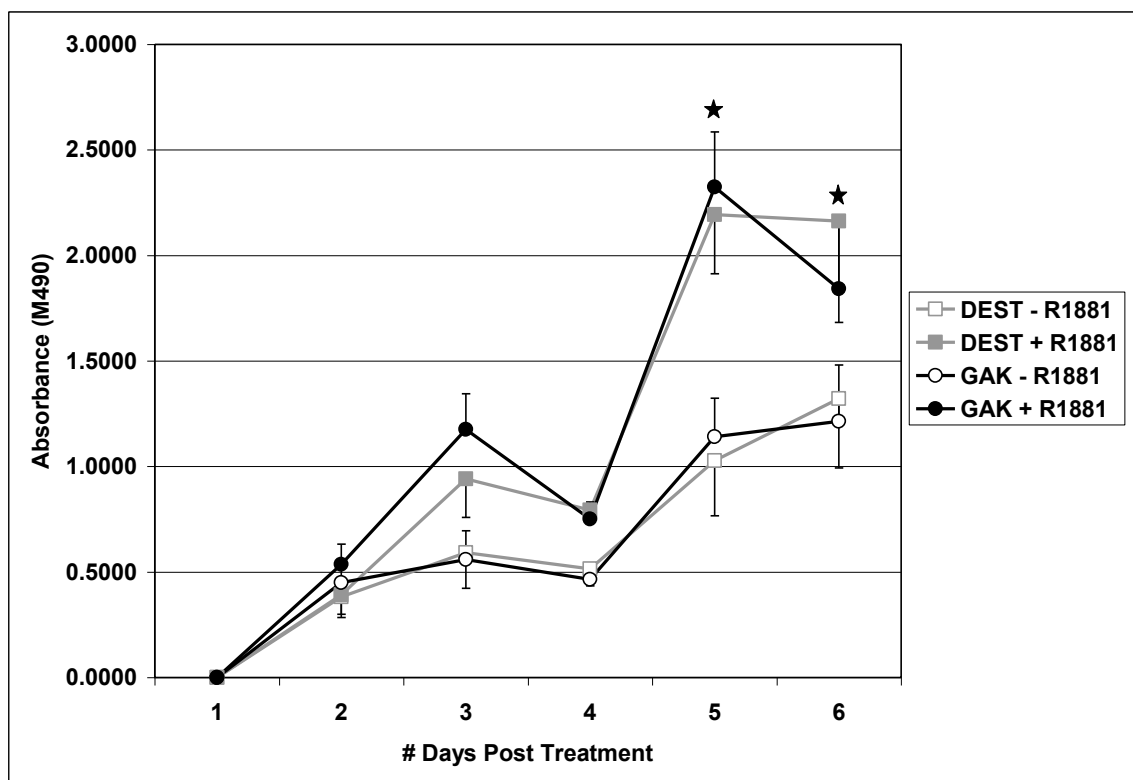
Figure 18 - Comparing *in vitro* growth of uninduced LNCaP-GAK and LNCaP-DEST cells +/- hormone.

Stably transfected DOX inducible LNCaP-GAK cells and the control LNCaP-DEST cells were seeded in replicates of 6 into five 96 well plate at a density of 3000 cells/well. Cells were treated with 1 nM of R1881 at specified time points and cell proliferation was quantified using the CellTitre 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (#TB169; Promega) following the manufacturers procedures. Experiments were conducted in triplicate and statistical analysis was conducted by means of a linear regression analysis followed by a one way ANOVA to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).
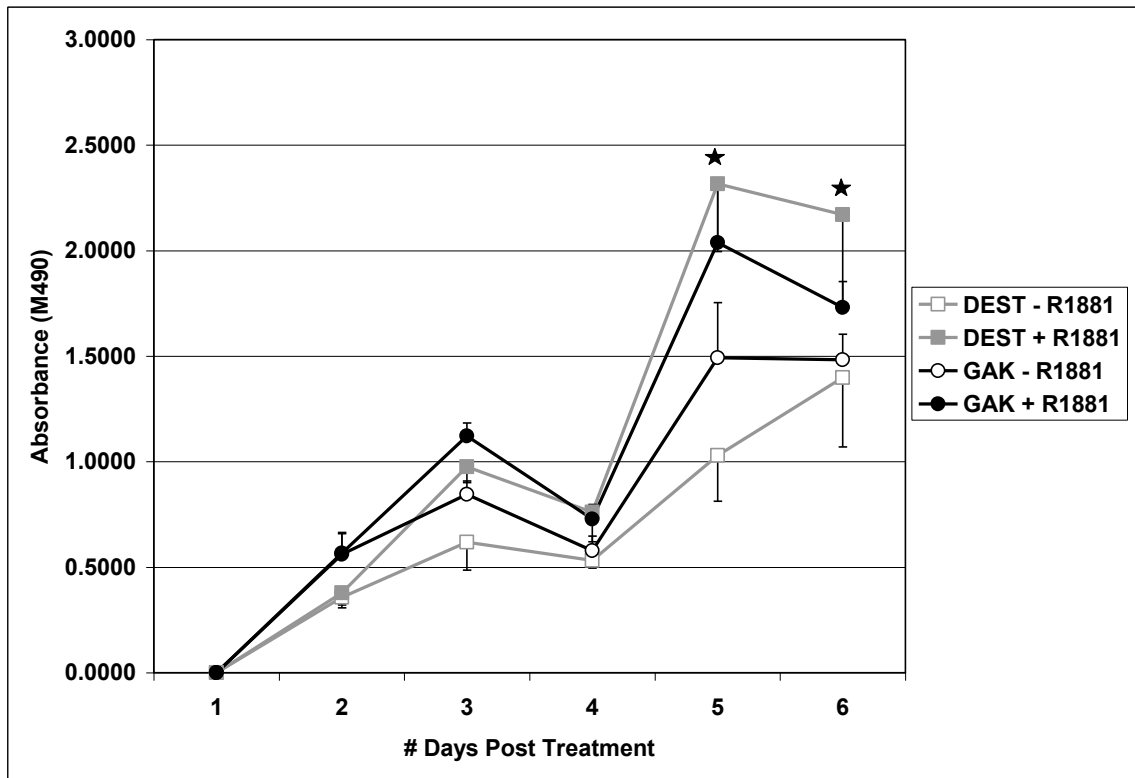
Figure 19 - Comparing *in vitro* growth of DOX induced LNCaP-GAK and LNCaP-DEST cells +/- hormone.

Stably transfected DOX inducible LNCaP-GAK cells and the control LNCaP-DEST cells were seeded and treated with 1 nM of R1881 as previously mentioned. 5 ng/ml DOX was utilized to induce GAK expression and at specified time points, cell proliferation was quantified as previously described. Experiments were conducted in triplicate and statistical analysis was conducted by means of a linear regression followed by a one way ANOVA to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

The drop in cell proliferation observed at day 4 is due to the change in media and subsequent loss of cells disturbed during this process. The reproducibility of these results suggests that GAK does not possess any robust growth promoting effects *in vitro*. This may be either a function of time or growth conditions, or a combination of both.

3.3   The Effect of GAK On Tumourigenicity

LNCaP-GAK and LNCaP-DEST cells were also grown in soft agar in an assay used primarily to gauge tumourigenicity of different cell types. Once again, both cell lines were grown in the presence and absence of DOX. Cells were plated at a density of 100,000 cells/dish, and allowed to grow for 10 days in a humidified 15 cm plate in the incubator. Colonies were counted and the data was graphed accordingly.

The GAK+DOX treatment group generated a slightly higher number of colonies compared to the GAK-DOX group, indicating that GAK may play a role in enhancing tumourigenicity ($p < 0.05$) (Figure 20). Interestingly, the DEST-DOX group generated more colonies that the DEST+DOX group, suggesting that DOX itself may play a mild inhibitory role ($p < 0.05$).   If this is the case, any subtle effects generated by GAK may be masked by the addition of DOX. Despite this possibility, GAK+DOX produced significantly more colonies than DEST+DOX, lending additional evidence to the argument that GAK may enhance tumourigenicity. There was no significant difference between the GAK-DOX and DEST-DOX treatment groups.

3.4   The Effect of GAK On Progression To AI

3.4.1   Experimental Overview

Despite our inability to successfully confirm GAK's role as a coregulator of AR *in vitro*, three xenograft mouse experiments were conducted in an effort to determine
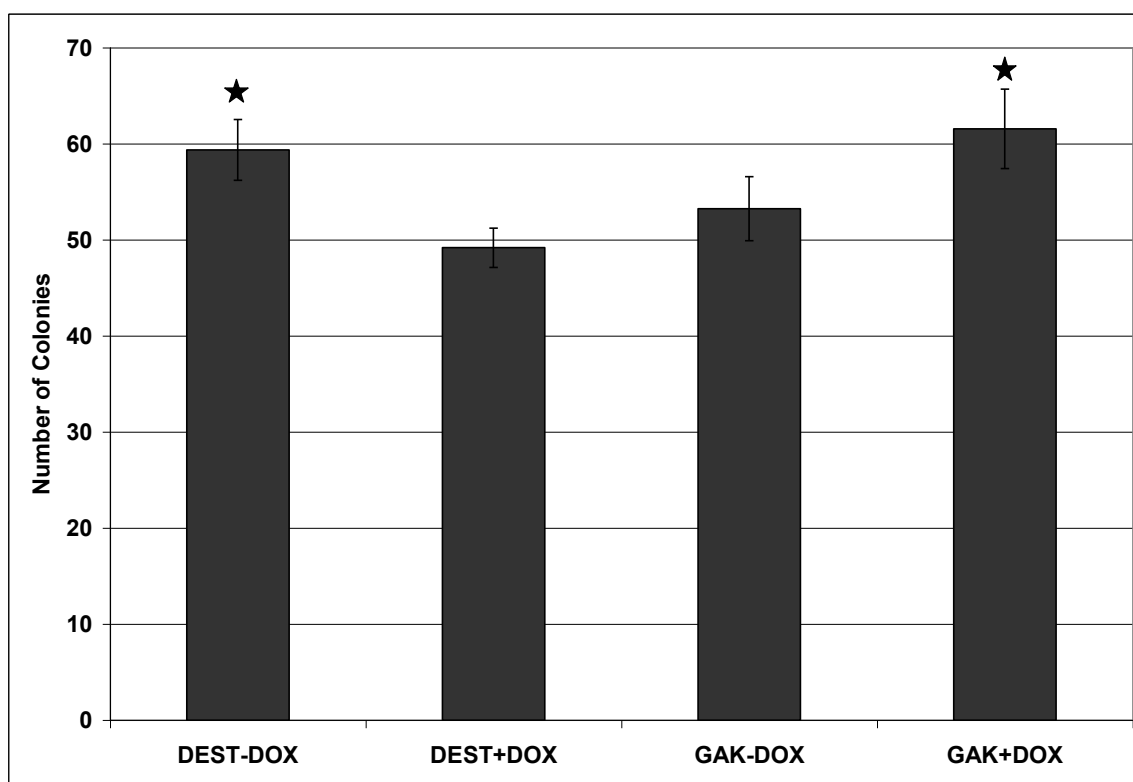
Figure 20 - Comparing *in vitro* colony formation of LNCaP-GAK and LNCaP-DEST cells.

Cells were plated in 0.35% agar in 1X RPMI containing 10% FBS at a density of 10,000 cell/plate. Plates were incubated in humidified 15 cm plates containing 5 ml of distilled water and colonies were counted one week later using a light microscope (Wilovert A Hund, Fisher Scientific). Experiments were conducted in triplicate and statistical analysis was conducted by means of a t-test to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

GAK's role in progression to AI. The purpose of the first xenograft experiment was to determine the effect of overexpressing GAK on progression towards AI in castrate mice. Thirty four nude BALB/c male mice were injected with one of the two stably transfected doxycycline inducible cell lines. Mice injected with either the LNCaP-GAK cell line or the LNCAP-DEST control cell line were treated at the time of castration, predetermined to be the point in time at which serum PSA reached 75 ug/µl, +/-DOX to induce GAK overexpression (Figure 21). Body mass, tumour volume, and serum PSA levels were measured on a weekly basis. Results were graphed on the basis of treatment group from the time of castration. At the point in time when tumour volume reached 10% of body mass, the experiment was concluded and both tumours were extracted and pooled. Tumours were frozen at -80°C or embedded in paraffin for subsequent western blot or immunohistochemical analysis, respectively. Linear regression paired with a one way ANOVA or t-test was utilized to determine if there were any statistical differences amongst the data sets.

3.4.2   Western Blot Analysis of LNCaP Xenograft Tumour Tissue

Protein was extracted from the tumours and western blotted for V5-tagged GAK, AR, and actin. As expected, 15/15 mice which were injected with the LNCAP-DEST control cell line in the absence and presence DOX showed no trace of V5 tagged GAK (Figure 22). Furthermore, 7/7 of the tumours excised from mice which were injected with the LNCaP-GAK cell line and subsequently induced with DOX expressed V5-tagged GAK. Not surprisingly, the "leakiness" initially observed *in vitro* during the initial DOX induction confirmation experiments was also observed *in vivo*. 4/7 mice which were injected with the LNCaP-GAK cell line but not treated with DOX expressed V5-tagged GAK. This observation indicates that the "leakiness" initially observed *in vitro* is in fact worth noting, as over 50% of the mice in the GAK-DOX group express V5-tagged GAK.

Western blot analysis of AR levels did not show any noticeable differences
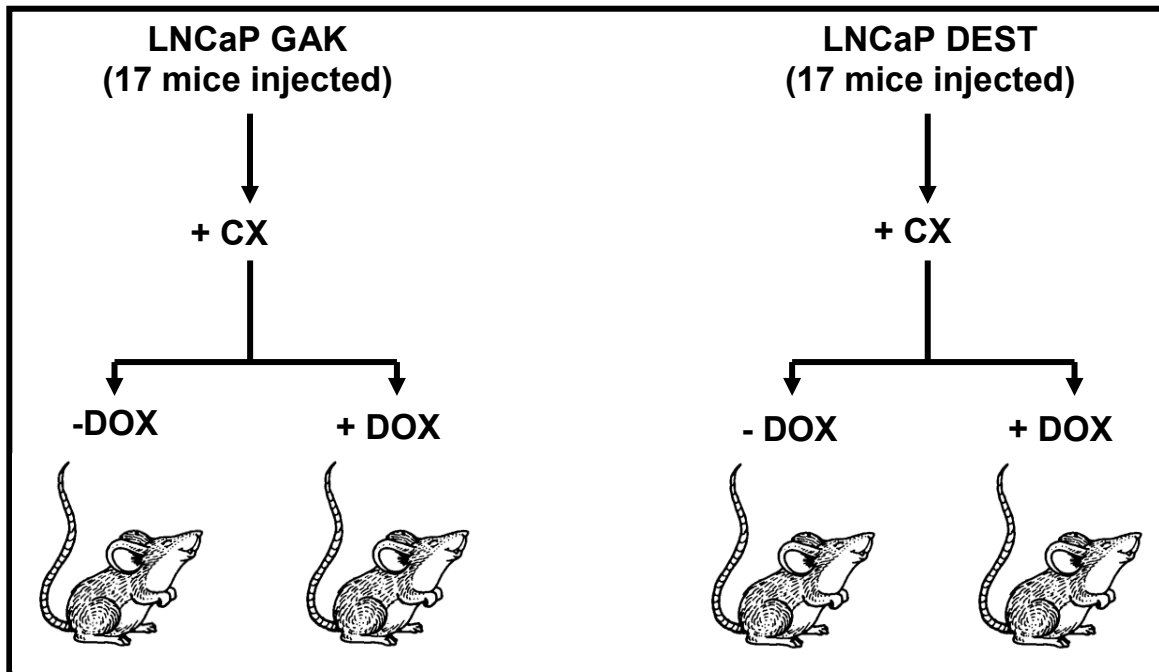
Figure 21 - Experimental design of xenograft experiment #1.

Nude BALB/c mice (17) were injected with either the LNCaP-GAK or the LNCaP-DEST cell line. Serum PSA was measured on a weekly basis until 75 ng/µl was reached, at which point all mice were castrated and treated plus (+) or minus (-) DOX. Mice were sacrificed when tumor volume reached 10% of body mass.
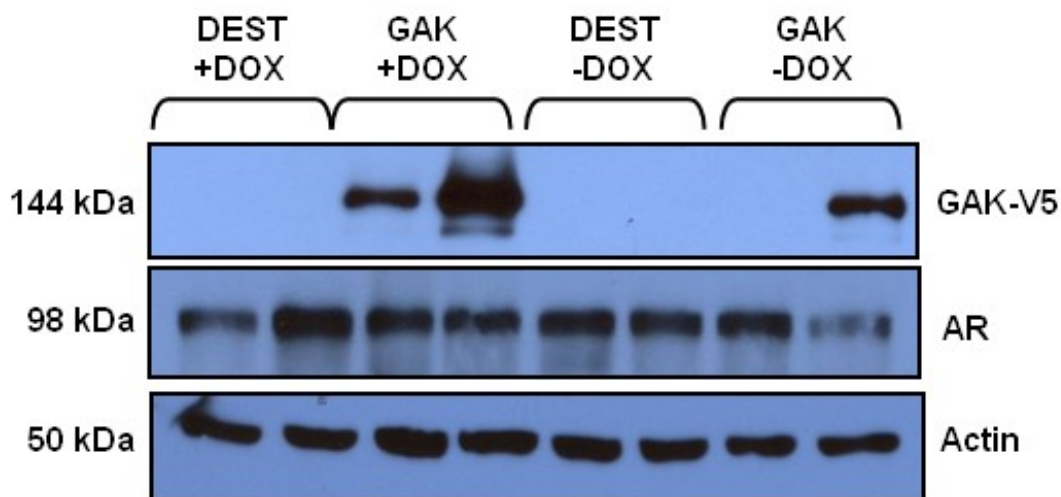
Figure 22 - Western blot analysis of LNCaP xenograft tumour tissue overexpressing GAK.

Two mice per treatment group were examined. Tumours were extracted at the point in time when total tumour volume reached 10% of the total body mass, protein was extracted, and western blot analysis was conducted. Lysates were probed for V5-tagged GAK, AR, and actin.

between any of the four treatment groups, indicating that overexpressing GAK does not regulate AR transcription or translation *in vivo*. The same observation was made previously during the *in vitro* transactivation assays. Granted, the GAK-DOX group exhibits "leakiness", but this does not preclude the fact that the AR expression levels observed in the GAK+DOX group are similar to those observed in the DEST+DOX group. For reasons not yet determined, the AR bands appeared fainter and barely detectable despite the fact that the same antibody was utilized throughout the *in vivo* and *in vitro* experimentation. Furthermore, the antibody did not appear to generate its usual robust signal when compared to similar western blots analyzing cells grown *in vitro*. This may be due to the fact that this particular mouse monoclonal AR antibody may have an affinity for circulating mouse IgGs which infiltrate the tumour tissue, which are not normally present *in vitro*, thus reducing the actual AR signal. Overall, western blot analysis confirmed GAK overexpression, leakiness in 4/7 mice in the GAK-DOX group, and stable AR levels in mice injected with either cell line.

3.4.3 Immunohistochemical Analysis of LNCaP Xenograft Tumour Tissue

Immunohistochemical analysis was conducted on the tumours excised from mice injected with either the LNCaP-GAK or LNCaP-DEST cell lines. A total of 34 mice were initially injected, once on each flank, resulting in two tumours per mouse. Both of these tumours were pooled at the time of sacrifice, and samples were embedded in paraffin for immunohistochemical and tissue microarray analysis. Sections stained for V5 confirmed GAK expression in the GAK+DOX, as well as leakiness in the GAK-DOX group compared to the two control groups, DEST+DOX and DEST-DOX (Figure 23). In comparison, staining for AR yielded no visual differences between any of the four treatment groups, reconfirming the *in vitro* observations that GAK overexpression does not appear to affect AR levels.

Tissue microarray analysis reaffirmed these findings. Twelve cores per mouse were utilized to construct a tissue microarray which grouped the mice on the basis of

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Figure 23 - Immunohistochemical staining of LNCaP xenograft tumour tissue probed for V5 and AR.

Tumours were excised when total tumour volume reached 10% of body mass, tumour tissue from each tumour was pooled, and samples were embedded in paraffin. Sections were stained with V5 and AR and visualized at 20X magnification.

treatment group. The tissue microarray was then probed for V5 and AR and results were scored by a pathologist (Dr. Ladan Fazli). Quantifying the staining intensities of V5-tagged GAK in each of the four treatment groups confirmed GAK overexpression in the GAK+DOX group and leakiness in the GAK-DOX group to a degree which was highly significant ($p < 0.001$) compared to DEST counterparts. Even though significant elevation in AR in the presence of the GAK vector irrespective of DOX was observed, this is most likely due to the scoring system being utilized ($p < 0.05$) (Figure 24 and 25). Overall, the results generated from the immunohistochemical and tissue microarray analysis support those produced via western blot analysis.

3.4.4 The Effect of Overexpressing GAK On Tumour Volume

To determine if overexpressing GAK has an effect on the rate of tumour growth, tumour volume was measured on a weekly basis throughout the duration of the experiment. Mice were injected on each flank, and each tumour was measured from the point at which it first became visible to the point at which the total volume for both tumours exceeded 10% of body weight, at which point the animal was sacrificed. The data were plotted from the time of injection and from the time from of treatment when the mice were castrated and DOX treatment was initiated

From the time of injection, the two groups of mice which were injected with LNCaP-GAK cells (GAK-DOX and GAK+DOX) possessed very similar rates of tumour growth, both of which were higher than either of the rates of growth of the LNCAP-DEST control groups (Figure 26). This suggests that GAK promotes growth, and certainly the fact that the GAK cell line is leaky in the absence of DOX would support this. In the absence of DOX, there is a significant difference between the GAK-DOX and the DEST-DOX treatment group ($p < 0.05$). Interestingly, in the presence of DOX, there appears to be a highly significant difference between the GAK+DOX and DEST+DOX treatment groups ($p < 0.001$). This seems to suggest that GAK is in fact impacting tumour growth. The leakiness observed in the absence of DOX supports this observation.
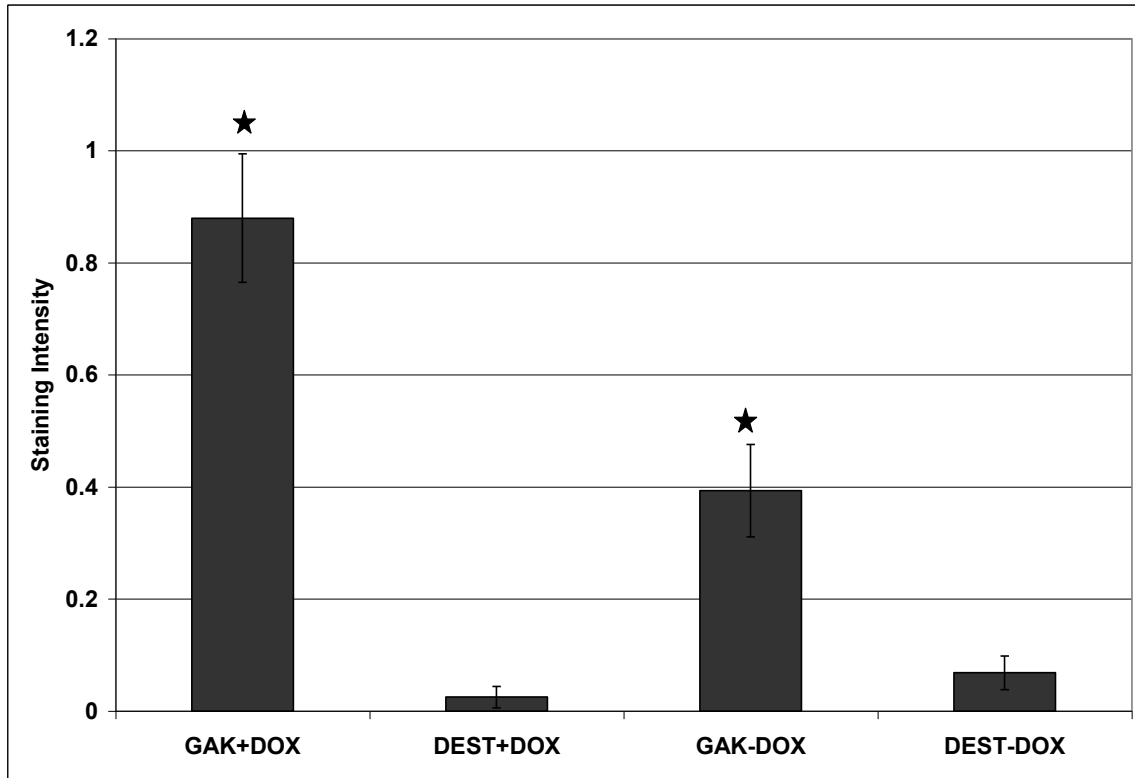
Figure 24 - Immunohistochemical staining intensity of V5-tagged GAK in LNCaP xenograft tumour tissue.

Tumours were excised when total tumour volume reached 10% of body mass, tumour tissue from each tumour was pooled, and samples were embedded in paraffin. Twelve cores per mouse were utilized to construct a tissue microarray. Cores were stained with V5, scored by a pathologist, and results were analyzed on the basis of treatment group. Statistical analysis was conducted by means of a t-test to determine if there were any statistical differences amongst treatment groups ($p < 0.001$).
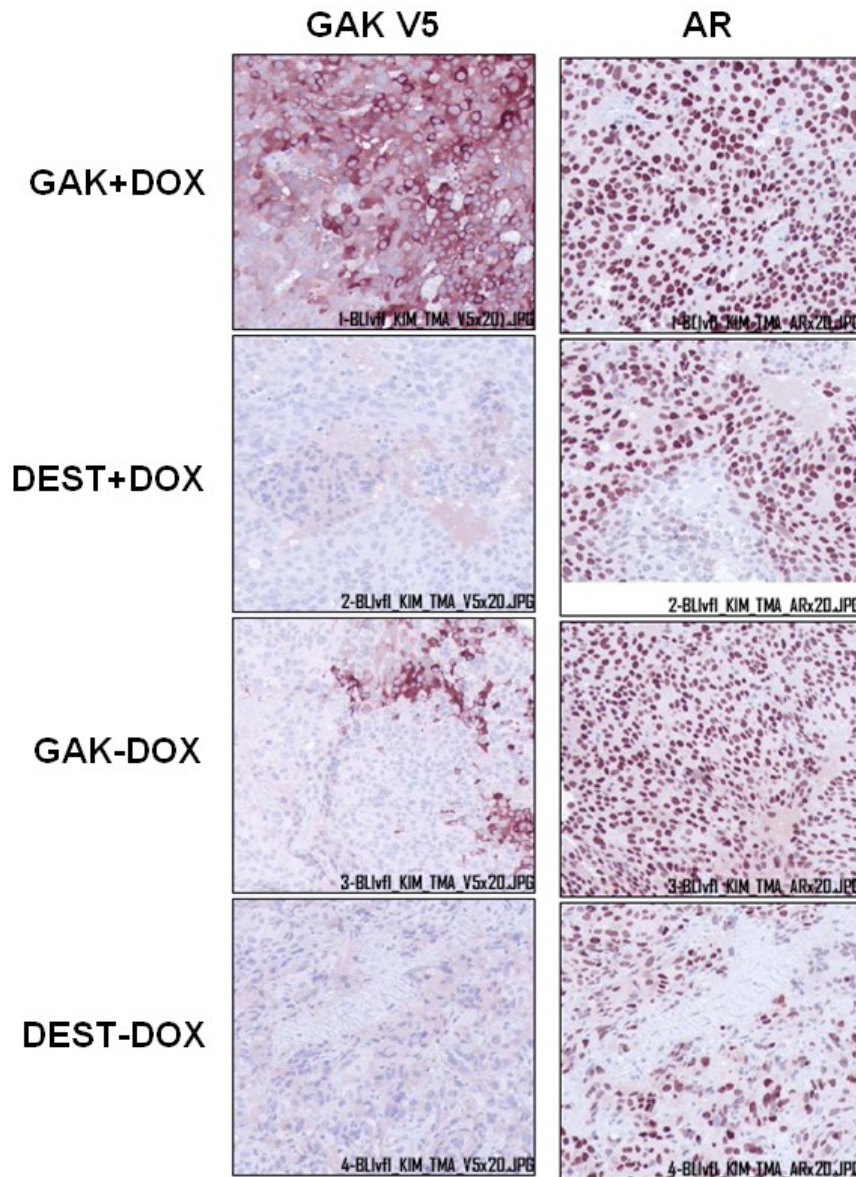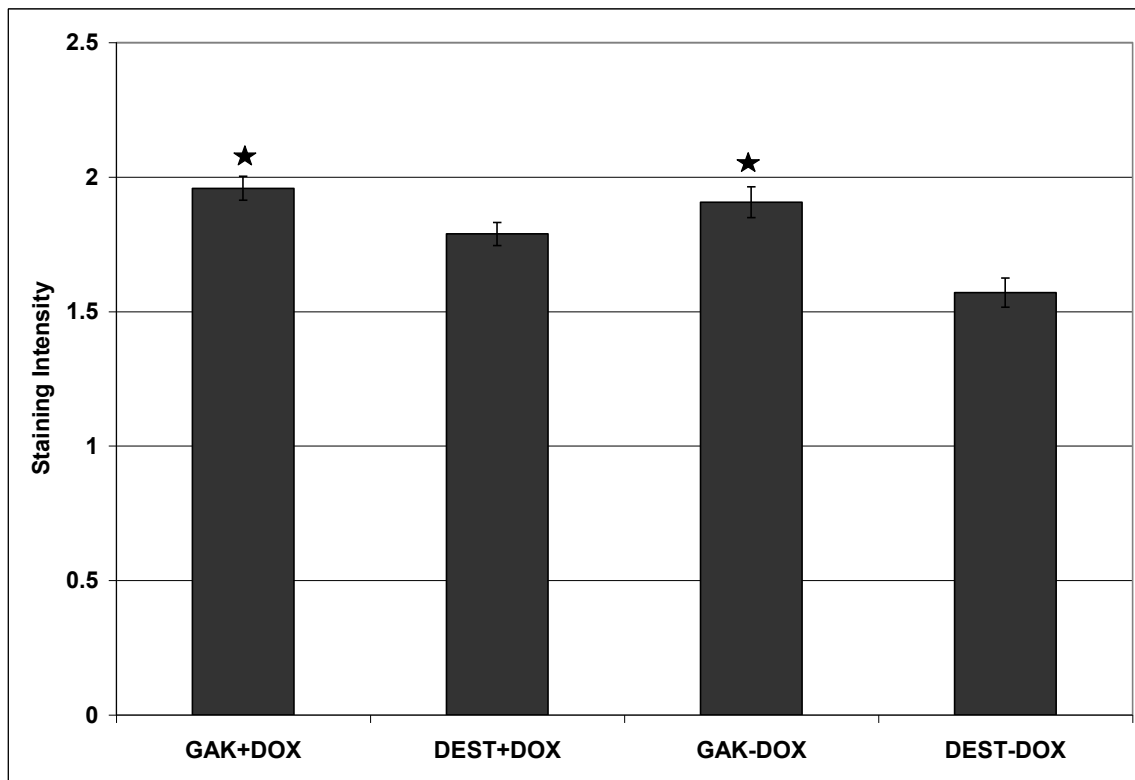
Figure 25 - Immunohistochemical staining intensity of AR in LNCaP xenograft tumour tissue.

Tumours were excised when total tumour volume reached 10% of body mass, tumour tissue from each tumour was pooled, and samples were embedded in paraffin. Twelve cores per mouse were utilized to construct a tissue microarray. Cores were stained with AR, scored by a pathologist, and results were analyzed on the basis of treatment group. Statistical analysis was conducted by means of a t-test to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).
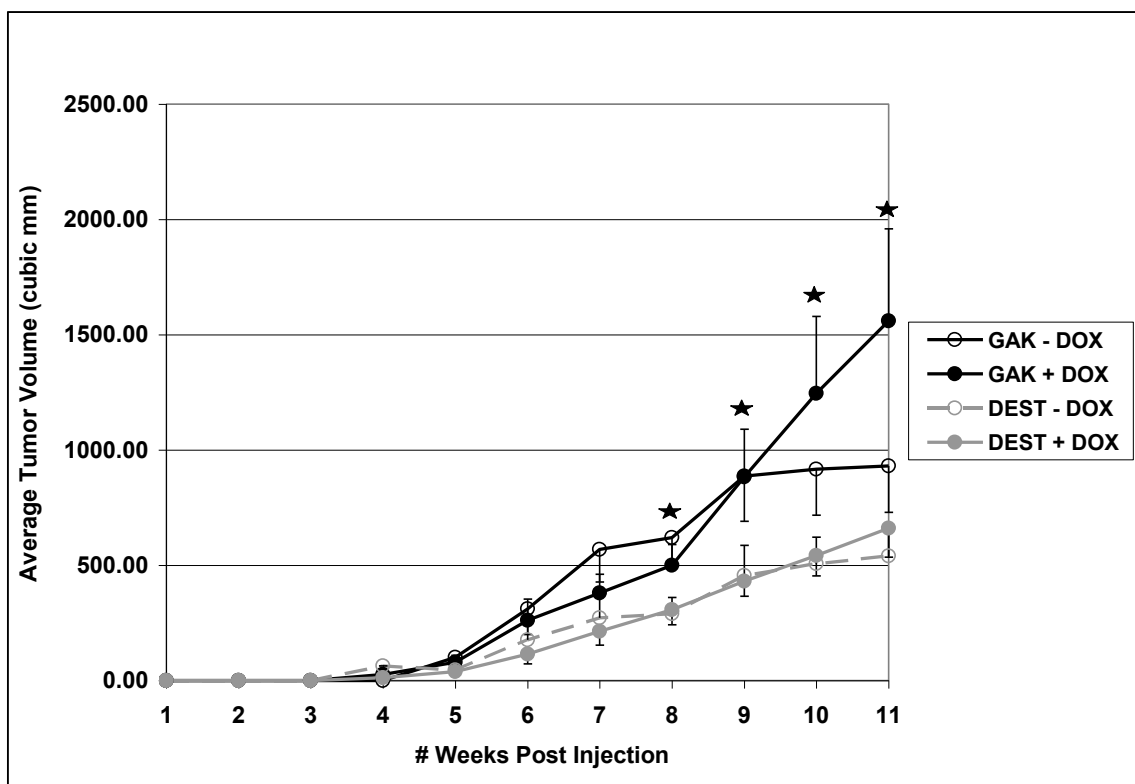
Figure 26 - The effect of GAK overexpression on tumour volume from the time of injection.

Mice were injected with either the LNCaP-GAK or the LNCaP-DEST cell line on both flanks. Tumour volume at both sites was measured on a weekly basis with calipers. Total tumour volume per mouse was averaged on the basis of treatment group and plotted as a function of time. Statistical analysis was conducted by means of a linear regression followed by a one way ANOVA to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

However, these results do not exclude the possibility that this observation may in fact be a cell line difference as opposed to a GAK induced enhancement of growth. Furthermore, plotting the data from the time of injection does not take into account the fact that GAK may be expressed prior to castration or that mice were treated at varying time points across the x-axis, as not all mice reached 75 ug/µl serum PSA simultaneously.

The data were then graphed from the time of castration. Once again there appears to be a highly significant difference between the GAK+DOX and DEST+DOX treatment groups ($p < 0.01$), supporting the previous observation made plotting the data from the time of injection (Figure 27). Interestingly, from the time of castration, there does not appear to be a significant difference between the GAK-DOX and DEST-DOX group ($p > 0.05$), contradicting the previous observation made from the time of injection which suggested that leakiness may play a significant role in enhancing tumour volume.

Finally, the data were plotted from the time of castration, whereby all the GAK expressors were compared to the GAK non-expressors within the GAK-DOX group. The objective of plotting the data in this manner was to determine if leakiness was in fact having a significant impact on tumour volume (Figure 28). Interestingly, the four mice in the GAK-DOX group which did express GAK possessed a higher percentage change in tumour volume than those three mice which did not express GAK ($p = 0.02053$). The fact that GAK expressing mice within the GAK-DOX group exhibited faster tumour growth than those that do not certainly suggests that any growth promoting effects observed are in fact most likely GAK specific as to opposed to a cell line specific effects.

3.4.5   The Effect of Overexpressing GAK On Serum PSA

The gene for prostate specific androgen (PSA) is regulated by AR, and is thus a prime candidate to monitor in terms of determining whether or not GAK regulates AR *in vivo*. Serum PSA was monitored by ELISA on a weekly basis from tail bleed samples.

Figure 27 - The effect of GAK overexpression on the percentage change in tumour volume from the time of castration.

Mice were injected with either the LNCaP-GAK or the LNCaP-DEST cell line on both flanks. Tumour volume was measured on a weekly basis with calipers. Total tumour volume per mouse was averaged on the basis of treatment group and plotted as a function of time. Statistical analysis was conducted by means of a linear regression followed by a one way ANOVA to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).
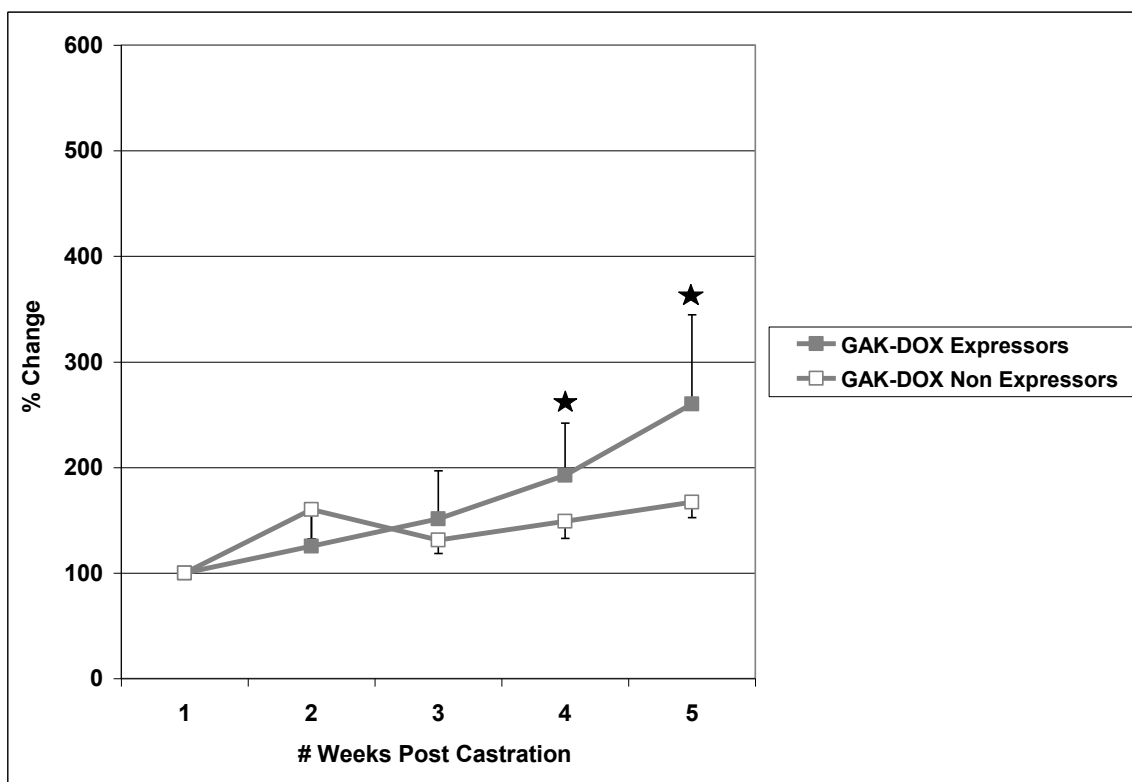
Figure 28 - Comparing tumour volume between the GAK expressors and non-expressors within the GAK-DOX treatment group from the time of treatment.

The data depicted are derived only from those mice which were injected with the LNCaP-GAK cell line. Tumour volume at both sites was measured on a weekly basis with calipers. Total tumour volume per mouse was averaged on the basis of GAK expression, as indicated via western blot analysis, and plotted as a function of time. Statistical analysis was conducted by means of a linear regression followed by a one way ANOVA to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

The data were analyzed based on treatment group from the time of castration. Linear regression analysis demonstrates that there is no statistically significant differences present between any of the four treatment groups ($p > 0.05$) (Figure 29). This is not surprising, as the *in vitro* luciferase assays overexpressing and knocking down GAK failed to demonstrate that GAK enhance AR activity, which would ultimately result in the expression of AR regulated genes such as PSA. However, despite no differences in PSA expression, the point in time at which each of these treatment groups nadirs, or reaches AI as denoted by when pre-castrate levels of PSA are achieved, does differ. All treatments except for the GAK+DOX group reach nadir at four weeks post-castration. The GAK+DOX group nadirs one week before any of the other three treatment groups, at week three, suggesting that GAK may play a role in facilitating the rate at which AI/CR is achieved. This may suggest that while GAK may not directly upregulate AR activity to facilitate progression to AI through AREs, it may do so by some other mechanism independent of AR.

3.4.6 Summary

The first xenograft experiment demonstrated that GAK may play a significant role in facilitating progression to AI. Not only did GAK expression appear to enhance tumour volume from the time of castration, as well as from the time of injection, but this data is also supported by the overall observation that mice injected with the LNCaP-GAK cell line appeared to progress through the experiment at a faster rate than mice injected with the LNCaP-DEST cell line. Furthermore, most mice within the GAK+DOX reached the 75 ug/µl PSA treatment threshold prior to DEST mice, and tumours originating from the GAK mice reached 10% of the mouse's mass sooner than those of the DEST variety. Interestingly, mice in the GAK+DOX group reached pre-castrate levels of PSA (nadir) before any of the other three treatment groups despite the fact serum PSA levels amongst treatment groups were not significantly different from one another. Further experimentation was pursued in an attempt to confirm these preliminary findings, and subsequent experiments were redesigned to take into account the confounding leakiness issue.
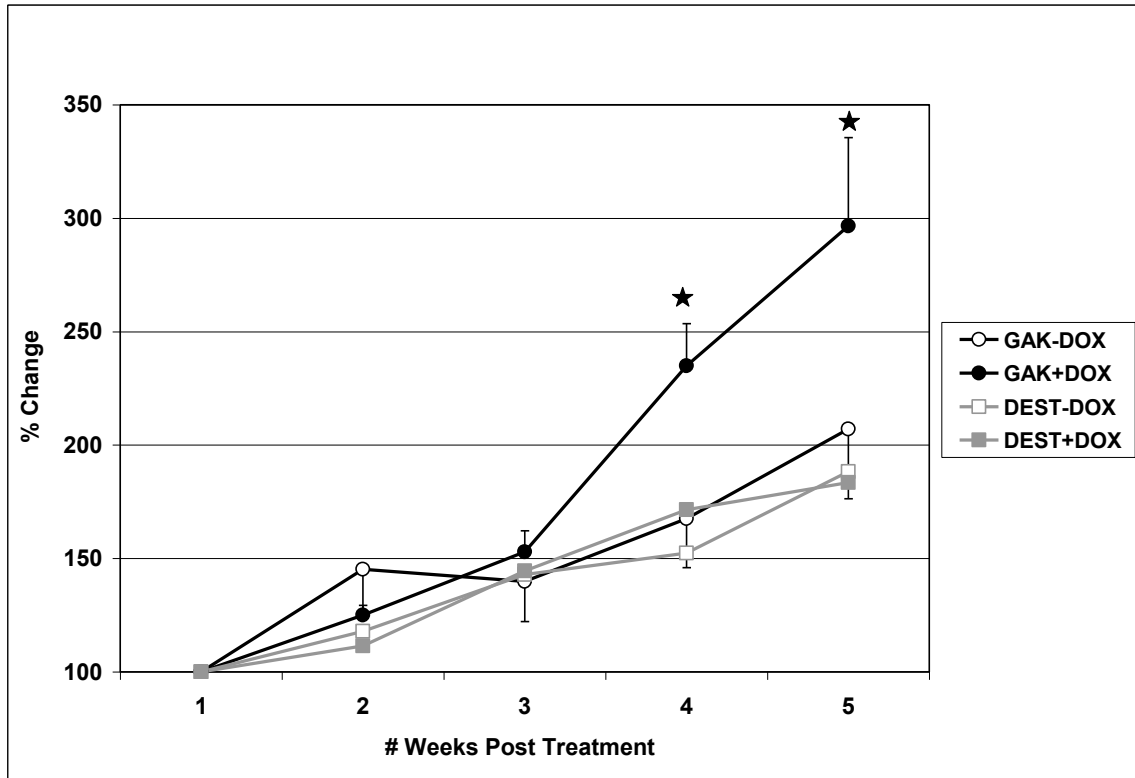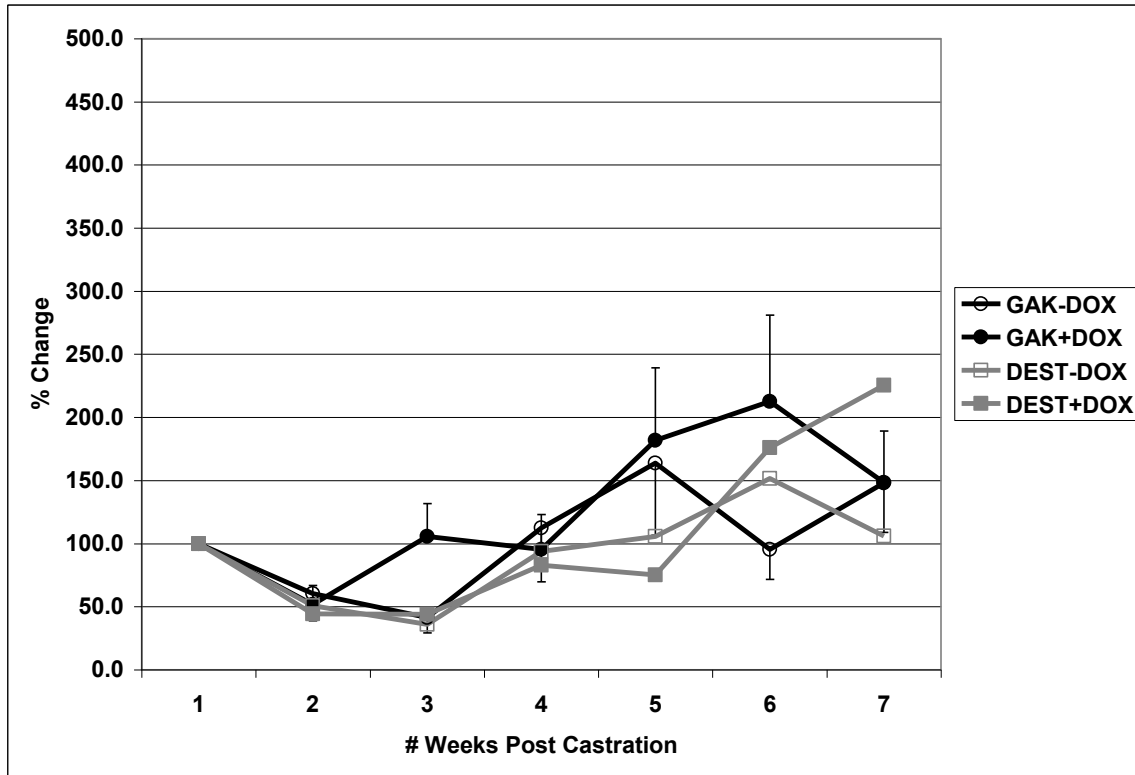
Figure 29 - The effect of GAK overexpression on average serum PSA from the time of castration.

Mice were injected with either the LNCaP-GAK or the LNCaP-DEST cell line on both flanks. Serum PSA was measured on a weekly basis using ELISA from blood samples taken from tail bleeds. PSA was measured in triplicate, averaged per mouse, and then averaged on the basis of treatment group and plotted against the number of weeks post castration. Statistical analysis was conducted by means of a linear regression followed by a one way ANOVA to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

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3.5   Hormone Dependency of GAK On Progression To AI

3.5.1   Experimental Overview

A second xenograft experiment was conducted in order to determine if the growth promoting effects of GAK observed in the first xenograft experiment were androgen dependent. A total of 37 nude male BALB/c mice were injected with either the control LNCAP-GAK line (19 mice) or the LNCaP-DEST cell line (18 mice) (Figure 30). Body mass, tumour volume, and serum PSA were monitored on a weekly basis as in the previous experiment. When serum PSA reached 75 ng/µl, half of the mice injected with either cell line were randomly castrated and all of the mice were treated with DOX to eliminate leakiness. Despite the fact that the data generated from this particular experiment could be interpreted without the confounding effects of leakiness occurring post-treatment, it does not, however, account for any effects GAK expression prior to treatment may be having on tumour development.

3.5.2   Western Blot Analysis of LNCaP Xenograft Tumour Tissue

Western blot analysis was conducted on tumour tissue samples excised and prepared as previously mentioned. In both the presence and absence of castration, mice injected with the LNCaP-GAK cell line generated V5-tagged GAK. Indeed, mice which had been injected with the LNCaP-GAK cell line and which had not been castrated showed abundant quantities of V5-tagged GAK. Mice which had been castrated possessed lower quantities of V5-tagged GAK compared to those which had not been treated, presumably because either the stable construct is androgen responsive or more plausibly that the regions from which tumor samples were taken randomly produced less V5-tagged GAK than other regions within the tumor (Figure 31). Both control groups, DEST-CX and DEST+CX showed no trace of V5-tagged GAK. AR levels among treatment groups appeared to remain unchanged, however, as mentioned in the previous xenograft, the AR antibody did not appear to be generating as strong a signal from *in vivo* samples as it otherwise would from *in vitro* samples, presumably because tumours are
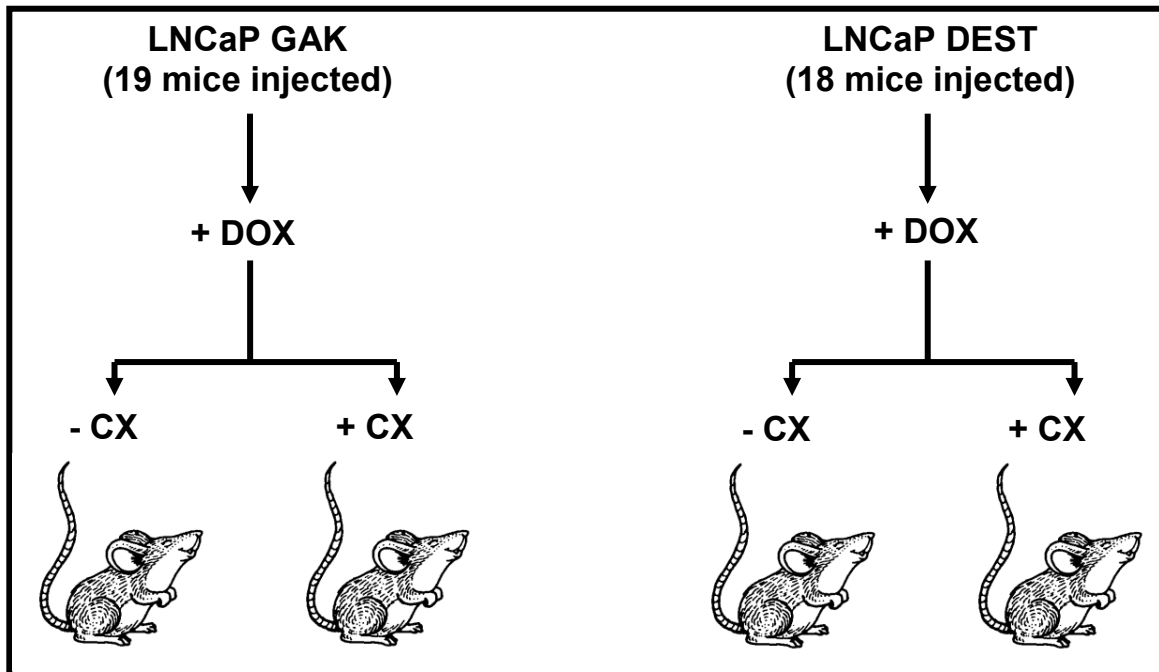
Figure 30 - Experimental design of xenograft experiment #2.

Nude BALB/c mice were injected with either the LNCaP-GAK or the LNCaP-DEST cell line. Serum PSA was measured on a weekly basis until 75 ng/µl was reached, at which point all mice were administered DOX and treated plus (+CX) or (-CX) castration. Mice were sacrificed when tumor volume reached 10% of body mass.
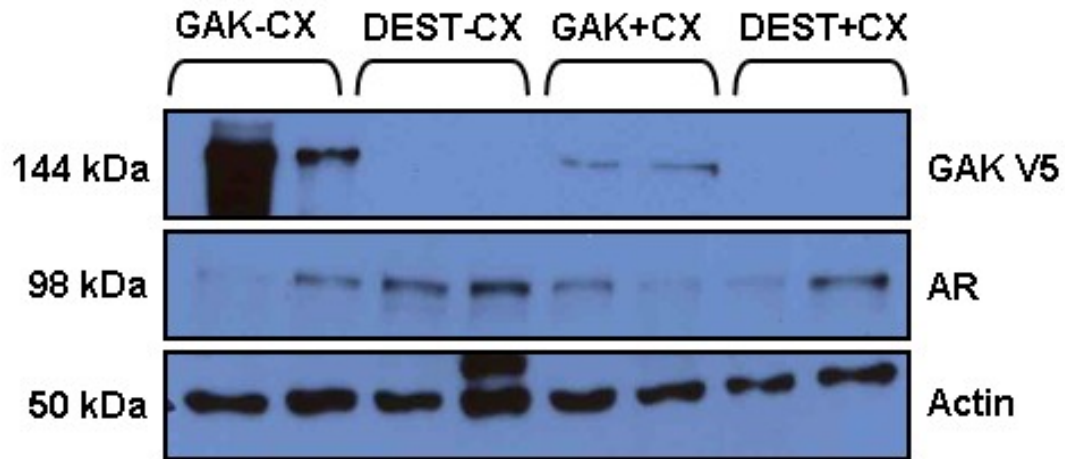
Figure 31 - Western blot analysis of LNCaP xenograft tumour tissue overexpressing GAK +/- castration.

Two mice representative of each treatment group were examined. Tumours were excised at the point in time when total tumour volume reached 10% of the total body mass, protein was extracted, and western blot analysis was conducted. Lysates were probed for V5-tagged GAK, AR, and actin.

infiltrated with mouse IgGs which potentially cross react with the antibody. Despite this technical issue, there does not appear to be any trend identified in terms of the intensity of AR signal between any of the treatment groups.

3.5.3 Immunohistochemical Analysis of LNCaP Xenograft Tumour Tissue

Immunohistochemical analysis was conducted on tumour tissue samples excised and prepared as previously mentioned. Six cores per tumour extracted from each mouse were probed with antibodies to V5 and AR. Predictably mice injected with the LNCaP-GAK cell line expressed significantly higher levels of V5-tagged GAK in both castrate and non-castrate conditions compared to the DEST control groups ($p < 0.001$) (Figure 32). Western blot analysis utilizing tumor samples from two mice per treatment depicted a difference between castrate and non-castrate mice injected with the LNCAP-GAK cell line; however, compiling data from 6 cores per tumor from each mouse within the treatment group eliminated the possibility that the GAK construct is androgen responsive, and demonstrated that the difference previously shown via western blot analysis is most likely due to regional variations of GAK expression within the tumour. Furthermore, even though there were measurable significant differences both within cell lines and between treatment groups in terms of AR expression ($p < 0.05$), these differences are most likely due to the scoring method being utilized (Figure 33).

3.5.4 Hormone Dependent Effects of GAK On Tumour Volume

To assess whether GAK's ability to enhance tumour growth is androgen regulated, tumour volume was analyzed on the basis of treatment group from both the time of injection and from the time of treatment. As anticipated, from the time of injection the effect of castration is observed in the GAK-CX and DEST-CX groups compared to the GAK+CX and DEST-CX groups, as indicated by the fact that the castrated mice possessed lower tumour volume growth rates than the those in non-castrated groups (Figure 34). There appears to be no measurable statistical difference in

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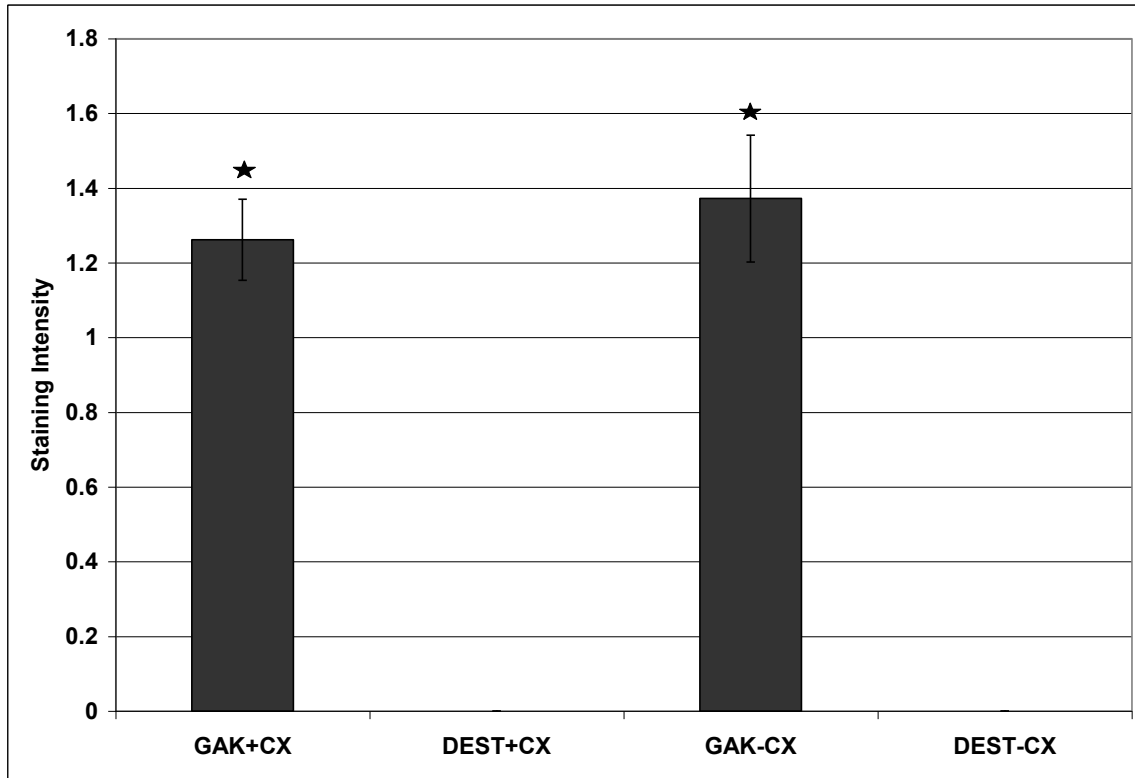
Figure 32 - Immunohistochemical staining intensity of V5-tagged GAK in LNCaP xenograft tumour tissue.

Tumours were excised when total tumour volume reached 10% of the mouse's body mass, tumour tissue from each tumour was pooled, and samples were embedded in paraffin. Twelve cores per mouse were utilized to construct a tissue microarray. Cores were stained with V5, scored by a pathologist, and results were analyzed on the basis of treatment group. Statistical analysis was conducted by means of a t-test to determine if there were any statistical differences amongst treatment groups ($p < 0.001$).
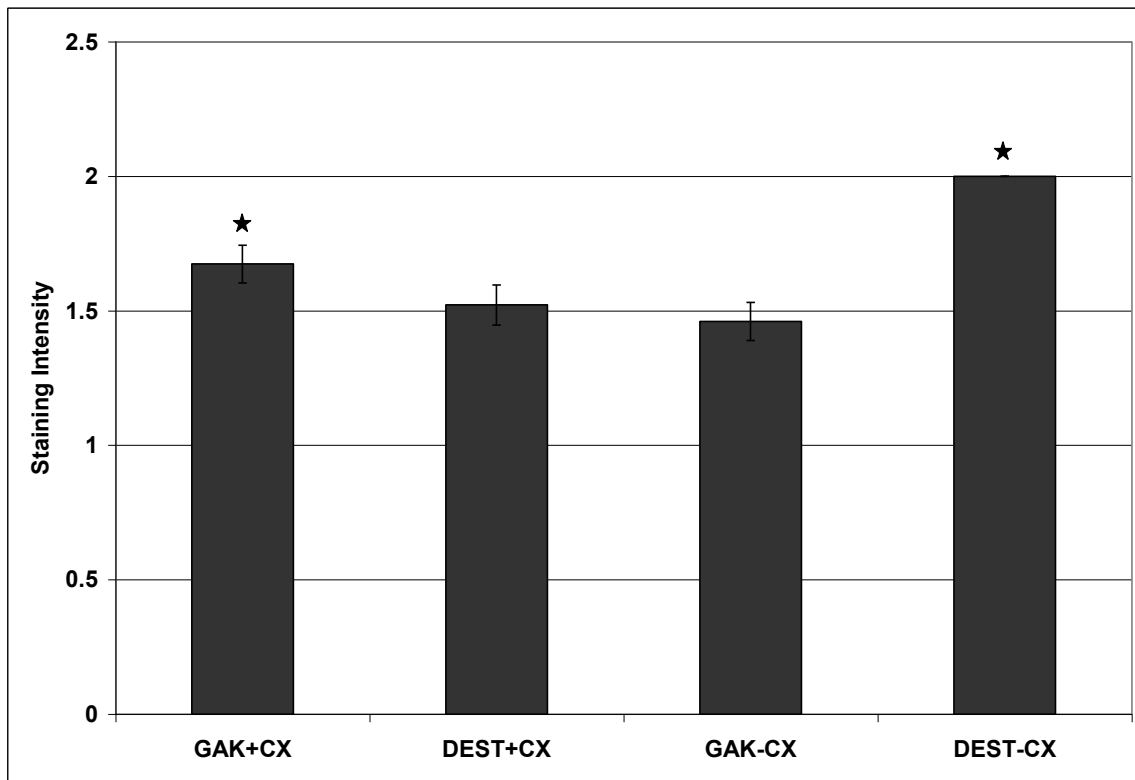
Figure 33 - Immunohistochemical staining intensity of AR in LNCaP xenograft tumour tissue.

Tumours were excised when total tumour volume reached 10% of the mouse's body mass, tumour tissue from each tumour was pooled, and samples were embedded in paraffin. Twelve cores per mouse were utilized to construct a tissue microarray. Cores were stained with AR, scored by a pathologist, and results were analyzed on the basis of treatment group. Statistical analysis was conducted by means of a t-test to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

Figure 34 - The effect of GAK overexpression on tumour volume +/- castration from the time of injection.

Mice were injected with either the LNCaP-GAK or the LNCaP-DEST cell line on both flanks. Tumour volume at both sites was measured on a weekly basis with calipers. Total tumour volume per mouse was averaged on the basis of treatment group and plotted as a function of time. Statistical analysis was conducted by means of a linear regression followed by a one way ANOVA to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

the average tumour growth rate between GAK+CX and DEST+CX from the time of injection, suggesting that androgens are most likely required to observe any GAK specific effects that are present ($p > 0.05$). As expected, there is a measurable statistical difference in average tumour volume between the GAK+CX and the GAK-CX groups due to the effects of castration ($p < 0.01$). There is no statistical difference between the GAK-CX to DEST-CX treatments, however, there does seem to be a very obvious separation between the two trendlines, suggesting that while not significant, there does appear to be some GAK specific effect occuring under castrate conditions. However, it should be noted that presenting the data from the time of injection is not ideal due to the fact that at any given point along the x-axis, any number of mice may or may not have been treated in each treatment group and that leakiness is a possibility in any number of the mice injected with the GAK cell line prior to the time of treatment, or 75 ng/µl of serum PSA.

Graphing tumour volume from the time of treatment is a much more accurate depiction of the data set due to the fact that the experimental design is much more suited to this type of analysis. Here it is reconfirmed that castration significantly reduces the tumour growth rate in both the GAK+CX and DEST+CX groups ($p < 0.05$) compared to the non castrate conditions, GAK-CX and DEST-CX. It also appears that there is no difference between the two cell lines in their response to castration ($p > 0.05$) (Figure 35). Overall this data suggests that GAK may only enhance tumour volume growth rate in an androgen dependent manner, at least from the time of castration. And that while GAK may accelerate the rate of tumor volume growth from the time of injection, this acceleration may be either an artifact of experimental design and subsequent analysis, or a viable observation that that GAK does enhance tumour volume growth, that this is most likely dependent on the presence of androgens, and that further experimentation is required to definitively determine whether or not GAK enhances tumour volume. As mentioned before, this particular experiment was not designed to quantify the difference in tumor volume growth from the time of injection, as the time of treatment varies in each mouse as per when the 75 ng/µl serum PSA was achieved. Data were graphed from the time of injection nonetheless as noticeable differences were observed between mice
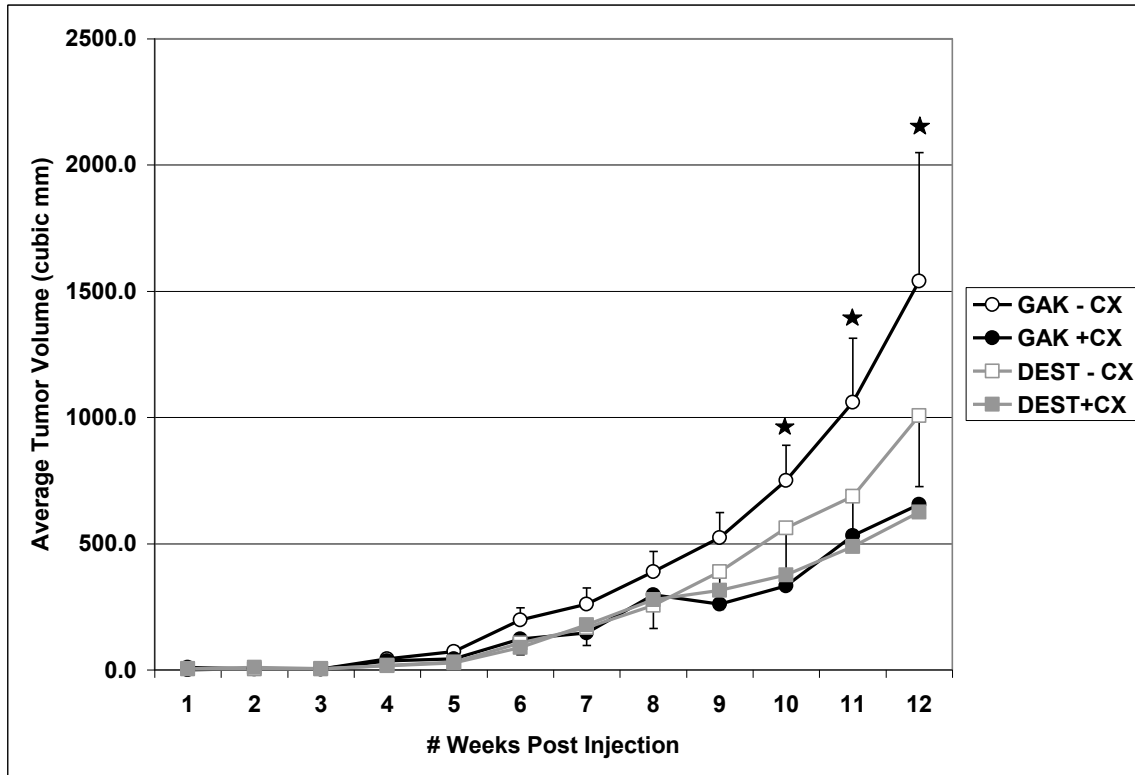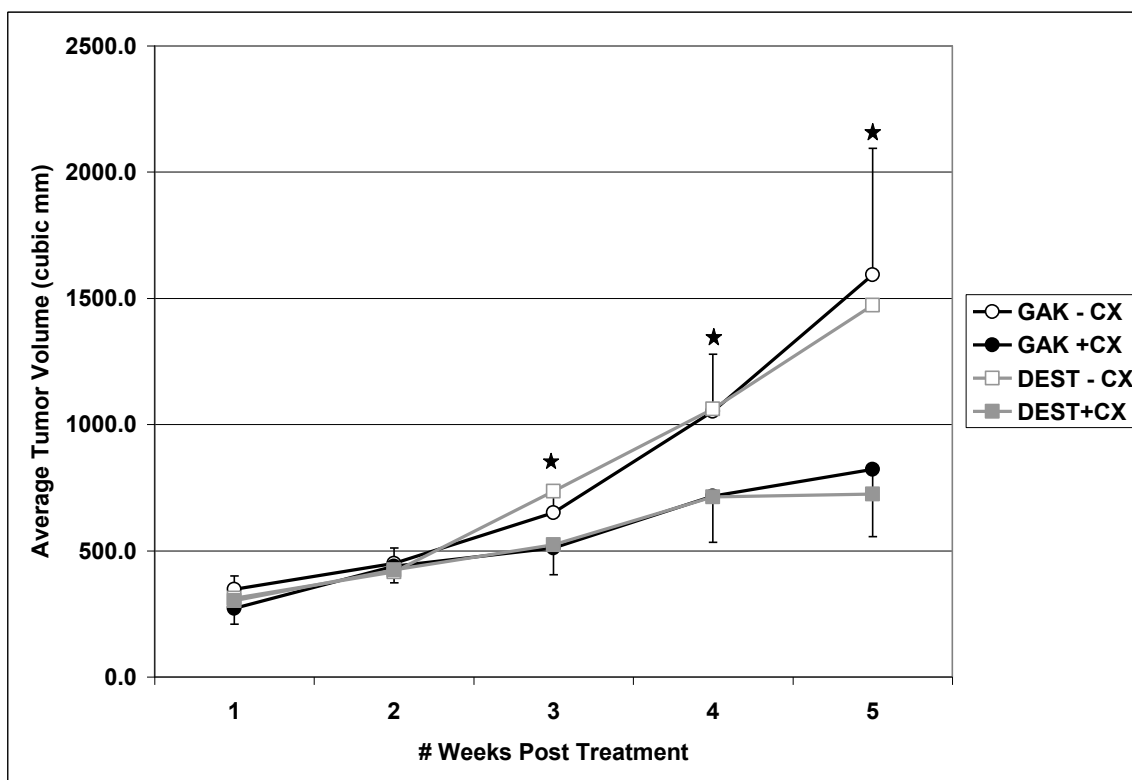
Figure 35 - The effect of GAK overexpression on tumour volume +/- castration from the time of treatment.

Mice were injected with either the LNCaP-GAK or the LNCaP-DEST cell line on both flanks and all mice were treated with DOX in the presence or absence of castration. Tumour volume at both sites was measured on a weekly basis with calipers. Total tumour volume per mouse was averaged on the basis of treatment group and plotted as a function of time. Statistical analysis was conducted by means of a linear regression followed by a one way ANOVA to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

injected with the LNCaP-GAK cell line and the LNCaP-DEST cell line in terms of the rate at which they progressed through the experiment. Mice injected with the LNCaP-GAK cell line reached the 75 ng/µl serum PSA threshold sooner and tumors reached 10% of body mass sooner, indicating that GAK may play a role in enhancing progression to AI.

3.5.5 Hormone Dependent Effects of GAK On Serum PSA

Average serum PSA production on the basis of treatment group from the time of treatment was analyzed. As expected, castration significantly reduced average PSA production post-treatment in both the GAK+CX and DEST+CX groups ($p < 0.001$) (Figure 36). Unfortunately there does not appear to be any difference between the GAK-CX and DEST-CX groups under castrate or non-castrate conditions ($p > 0.05$), suggesting GAK has no significant effect on PSA levels *in vivo*. This conclusion is not particularly surprising as *in vitro* luciferase assay results overexpressing and knocking down GAK demonstrated little effect on AR activity. However, it should be noted that the tumors within the GAK-CX and DEST-CX did not respond to castration as well as they did in the initial experiment. Regardless, significant differences amongst the treatment groups due to anything other than castration were absent.

3.5.6 Summary

The results from this second xenograft experiment suggest that GAK does not enhance tumour volume in a hormone dependent fashion. It also reconfirms previous observations that suggest that GAK does not regulate AR, and this stands true in both the presence and absence of androgens. One consistant observation made in the first and second xenografts was that the mice in the GAK groups appeared to progress through the experiment at a faster rate, both in terms of the rate at which they reached the treatment threshold of 75 ug/µl and the rate at which the tumours reached 10% of the mouse's body mass. To determine if GAK truly does promote tumour volume growth, a third xenograft
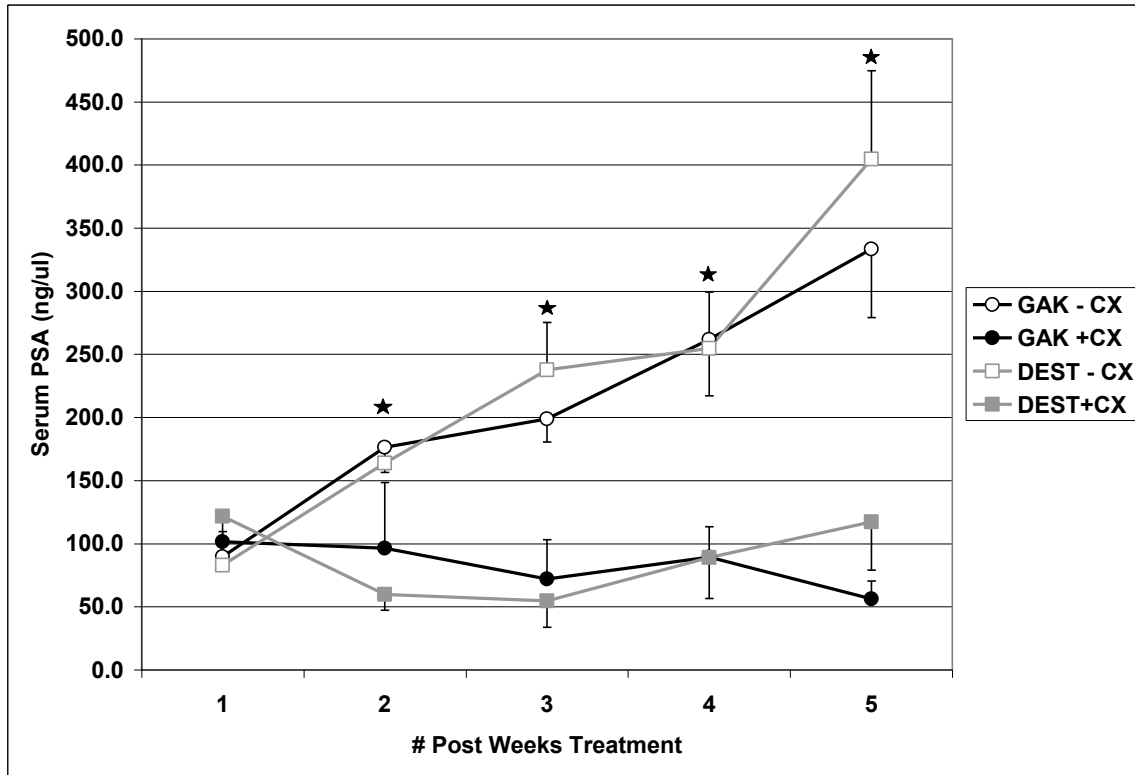
Figure 36 - The effect of GAK overexpression on serum PSA +/- castration from the time of treatment.

Mice were injected with either the LNCaP-GAK or the LNCaP-DEST cell line on both flanks. Serum PSA was measured on a weekly basis using ELISA from blood samples taken from tail bleeds. PSA was measured in triplicate, averaged per mouse, and then averaged on the basis of treatment group and plotted against the number of weeks post treatment. Statistical analysis was conducted by means of a linear regression followed by a one way ANOVA to determine if there were any statistical differences amongst treatment groups ($p < 0.05$).

experiment removing the confounding effects of treatment as well as pre-treatment leakiness was conducted.

3.6   The Effect of GAK On Tumour Take and Tumour Volume

3.6.1   Experimental Overview

A third xenograft experiment was conducted with the sole objective of determining whether or not GAK overexpression enhanced tumour take and tumour volume growth rate. Fifteen mice were injected with the LNCaP-GAK cell line, and another fifteen mice were injected with the LNCaP-DEST cell line (Figure 37). Four treatment groups were established: GAK+DOX, GAK-DOX, DEST+DOX, and GAK-DOX. DOX treatment was initiated from the time of injection, thus alleviating the confounding effects caused by the leakiness in all of the treatment groups with the exception of the GAK-DOX group. Mice were not castrated at any point time during this experiment. Body mass and tumour volume were monitored on a weekly basis as in the previous two experiments.

3.6.2   The Effect of GAK Overexpression On Tumour Take

A major objective of this experiment was to determine if GAK expression enhanced the rate of tumour take. Despite the fact that the previous two experiments were not designed to assess tumour take, general observations did suggest that GAK may enhance tumour take, as mice injected with the GAK cell line seemed to progress through the experiment at a faster rate. However, because DOX addition occurred at the time of treatment in both of the previous experiments, it was difficult to accurately assess the effect of GAK on tumour take when confounding factors such as leakiness prior to treatment most likely existed. In this experiment, DOX addition occurred at the time of injection.

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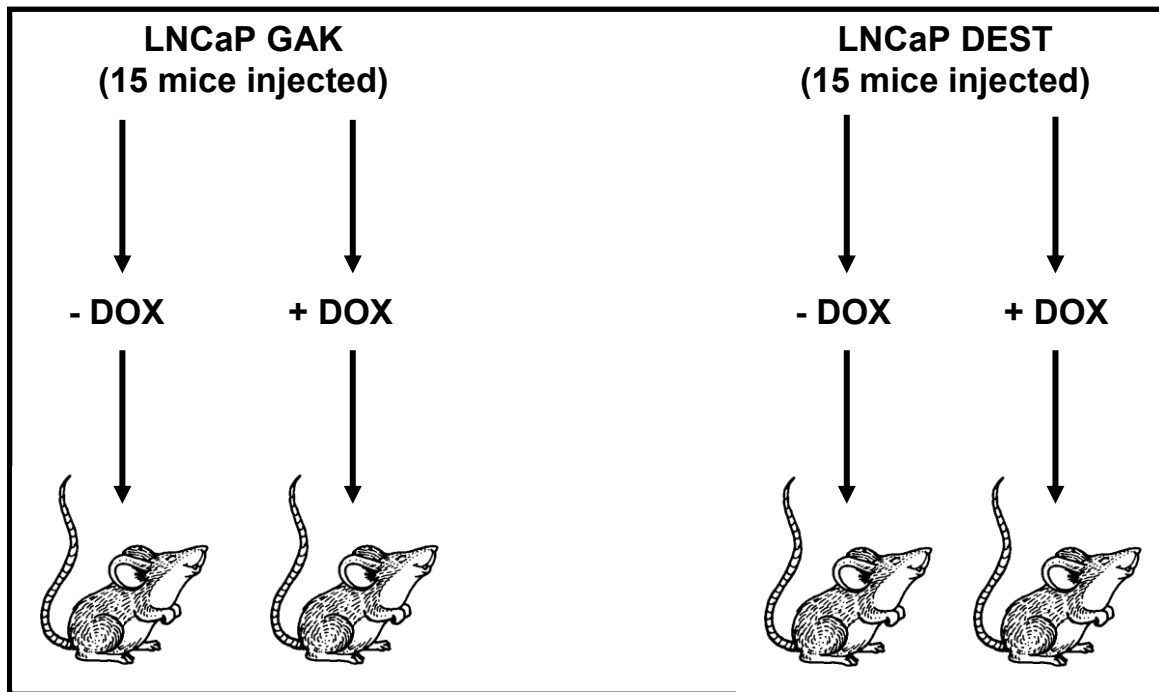
Figure 37 - Experimental design of xenograft experiment #3.

Nude BALB/c mice (30) were injected with either the LNCaP-GAK or the LNCaP-DEST cell line and immediately treated at the time of injection plus (+) or (-) DOX. Tumor volume was measured on a weekly basis until tumor volume reached 10% of body mass, at which point mice were sacrificed.

Interestingly, the DEST-DOX group possesses an enhanced rate of tumour take compared to the DEST+DOX group to a degree that is highly significant (p < 0.001) (Figure 38). This indicates that DOX inhibits tumour take. It has been previously published that DOX has an inhibitory effect on cell growth *in vitro,* and if that is the case, it is entirely possible that any growth promoting effects of GAK may be countered by the addition of DOX [232]. Interestingly, the GAK+DOX group exhibits a substantially higher tumour take compared to the DEST+DOX control group (p <0.0001), suggesting that irrespective of DOX, GAK is responsible for an enhanced rate of tumour take (Figure 39). These results confirm previous observations which suggested mice in the GAK group appeared to progress through the experiment at a faster rate than mice in the DEST group. The confounding effects of treatment and leakiness are not factors which affect the results.

3.6.3   The Effect of GAK Overexpression On Tumour Volume

The other variable that was analyzed in the third xenograft experiment was tumour volume and the rate at which it accelerated within each treatment group. Much like the tumour take results, the DEST-DOX group possessed an enhanced rate of tumour growth compared to the DEST+DOX group (p < 0.001) (Figure 40), indicating a possible inhibitory effect of DOX. Furthermore, the GAK+DOX group exhibited a faster rate of tumour growth than the DEST+DOX group to a degree that was highly significant (p < 0.0001) (Figure 41). These results confirm that even in the presence of DOX, which appears to play an inhibitory role, GAK enhances tumour volume.

3.6.4   Summary

As previously mentioned, prior xenograft experiments were not designed to measure tumour take or tumour volume from the time of injection. However, this particular experiment was designed to specifically measure tumour take and tumour
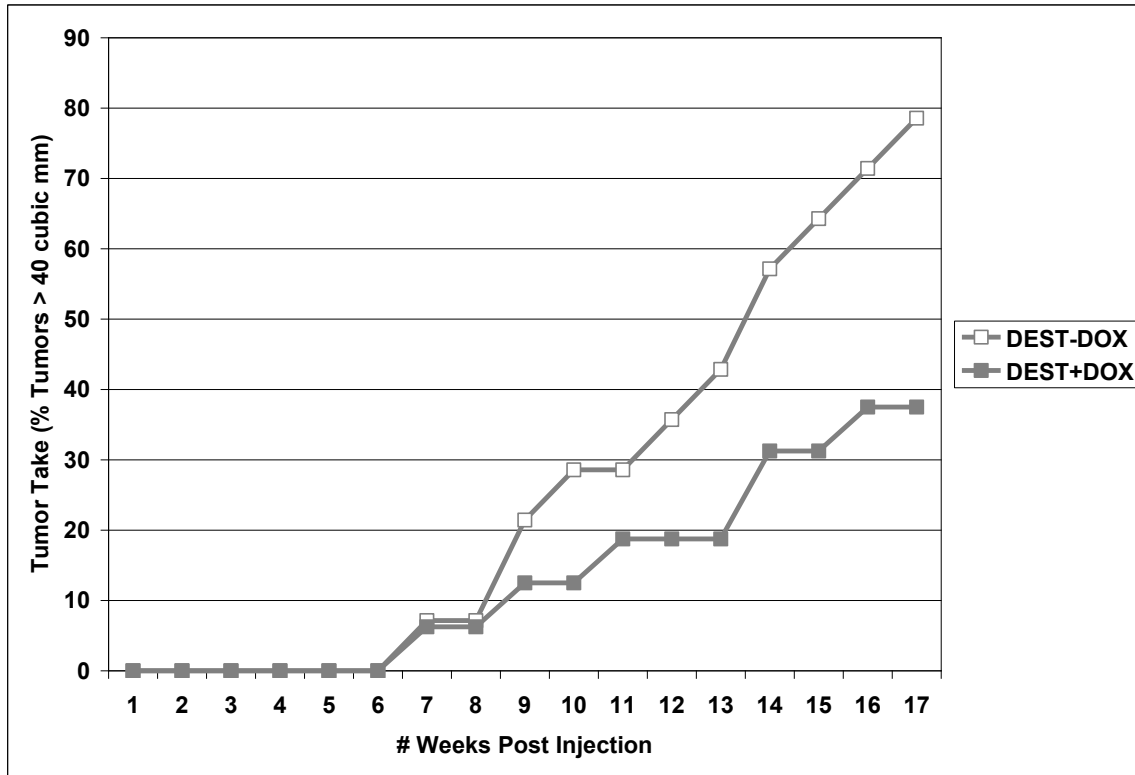
Figure 38 - The effect of DOX on tumour take.

Mice were injected with either the LNCaP-DEST cell line on both flanks and treated +/-
DOX. Tumour volume was measured on a weekly basis with calipers. Tumour take was
calculated as the total number of sites per treatment group as a percentage of the total
number of mice injected with either LNCaP-GAK or LNCaP-DEST cells that produced
sizable tumours over 40 cubic mm3. Statistical analysis was conducted by means of a
linear regression followed by a one way ANOVA to determine if there were any
statistical differences amongst treatment groups ($p < 0.001$).
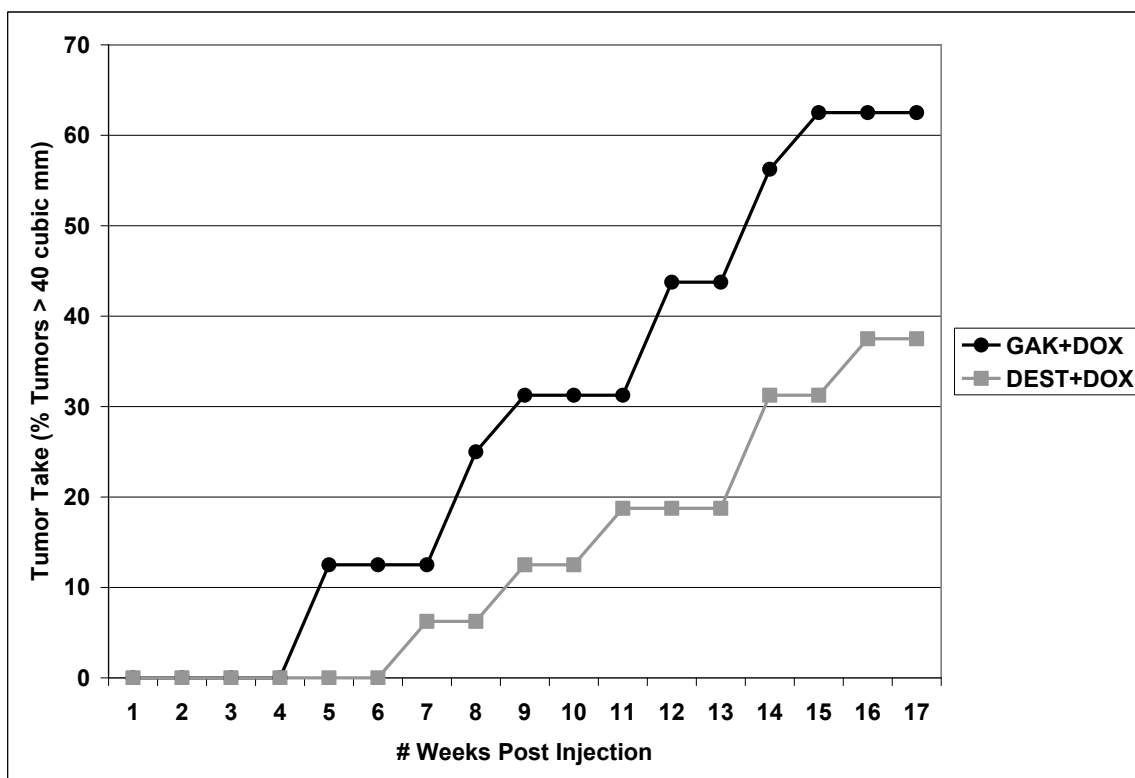
Figure 39 - Comparing tumour take of GAK+DOX and DEST+DOX LNCaP xenograft tumours.

Mice were injected with either the LNCaP-GAK or the LNCaP-DEST cell line on both flanks and tumour take was measured and results were recorded as previously described. Statistical analysis was conducted by means of a linear regression followed by a one way ANOVA to determine if there were any statistical differences amongst treatment groups ($p < 0.0001$).
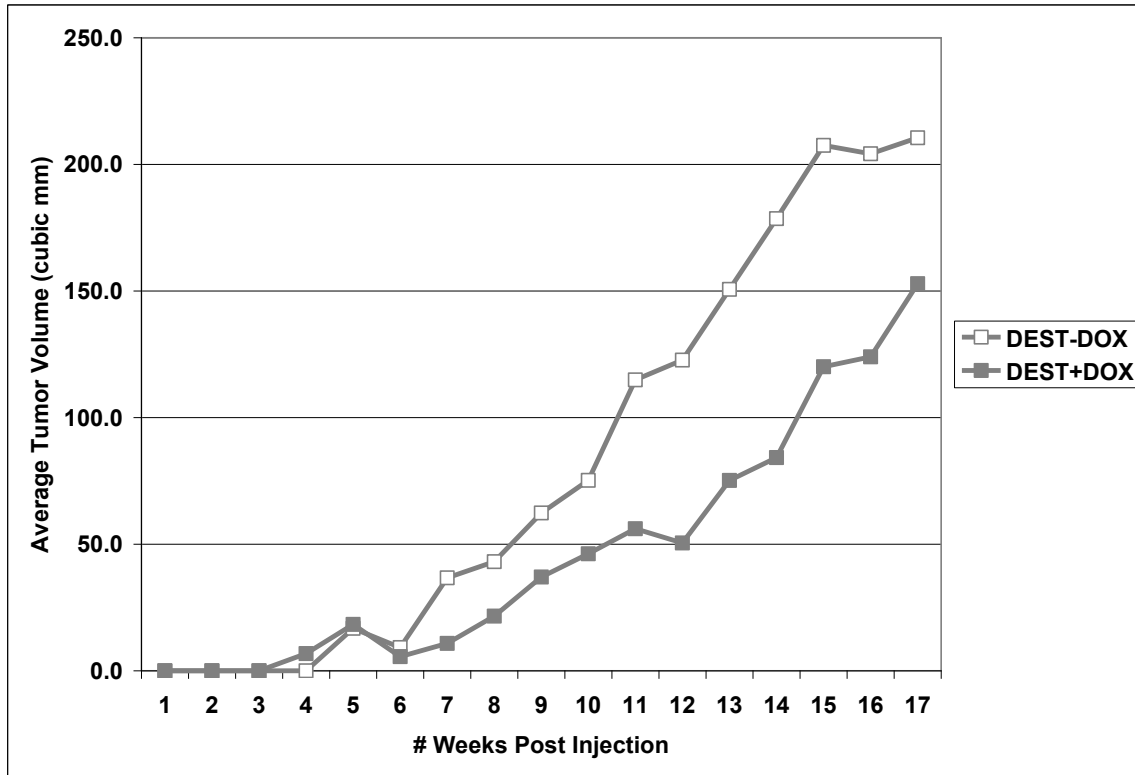
Figure 40 - Comparing tumour volume of DEST-DOX and DEST+DOX LNCaP xenograft tumours.

Mice were injected with either the LNCaP-GAK or the LNCaP-DEST cell line on both flanks and tumour volume was measured and results were recorded as previously described. Statistical analysis was conducted by means of a linear regression followed by a one way ANOVA to determine if there were any statistical differences amongst treatment groups ($p < 0.001$).
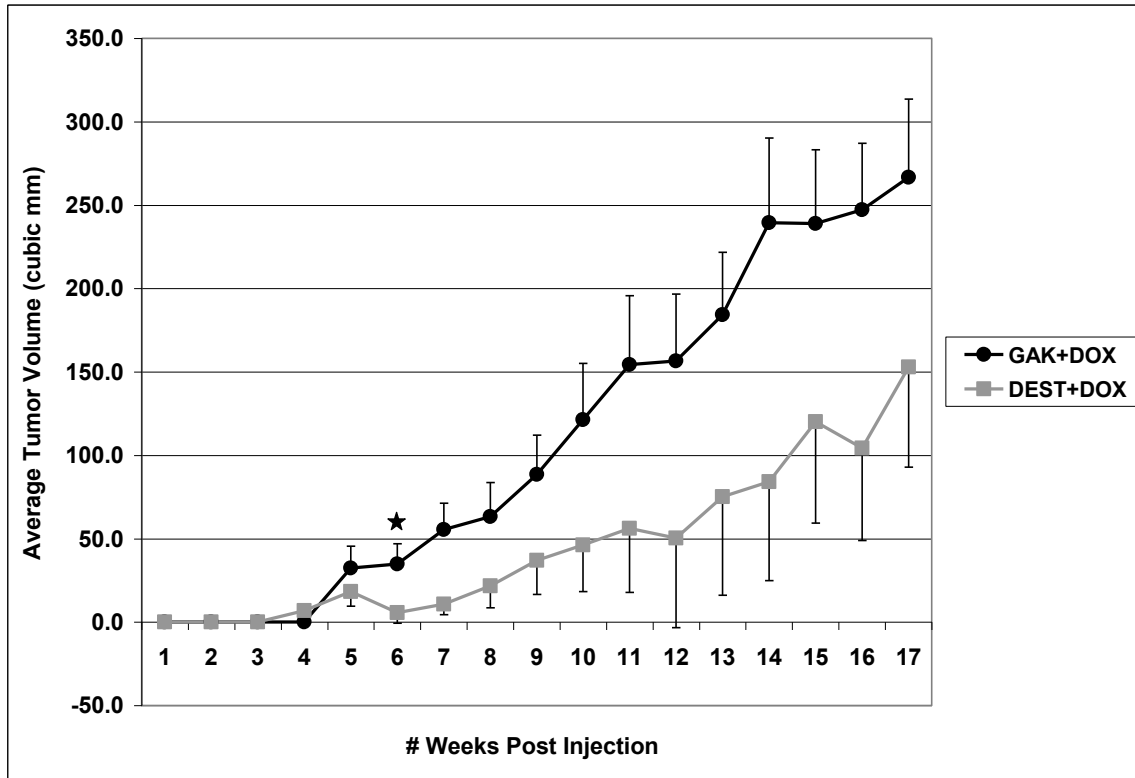
Figure 41 - Comparing tumour volume of GAK+DOX and DEST+DOX LNCaP xenograft tumours.

Mice were injected with either the LNCaP-GAK or the LNCaP-DEST cell line on both flanks and tumour volume was measured and results were recorded as previously described. Statistical analysis was conducted by means of a linear regression followed by a one way ANOVA to determine if there were any statistical differences amongst treatment groups ($p < 0.001$).

volume from the time of injection without the confounding effects of leakiness and treatment, and as expected, GAK expression significantly enhanced the rate of tumour take compared to the DEST+DOX group. GAK expression also significantly enhanced the rate of tumour growth compared to the DEST+DOX group. Overall these results suggest that while GAK may not regulate AR, nor is its activity androgen regulated, overexpression of GAK does enhance the rate at which tumours take and subsequently develop *in vivo*.

Chapter 4   Conclusions and Future Directions

Prostate cancer is the most commonly diagnosed cancer in both North American and European men and the second leading cause of cancer related death (Backman, 2004). At this point time, there is no curative treatment for advanced and metastatic prostate cancer, and as such, research directed towards determining the mechanism by which prostate cancer progresses from a state of AD to AI is of high priority. In the present study, our objectives were to validate GAK's role as a coregulator of AR and to determine if overexpressing GAK affects progression to AI. While we were unable to confirm GAK's ability to regulate AR activity, GAK does appear to play a role in promoting tumour take and tumour growth *in vivo*.

Preliminary transactivation assays in PC3 and LNCaP cells suggested that overexpressing GAK enhances of AR activity *in vitro*. Follow up studies utilizing the same methodology were able to confirm AR transactivation in the presence of overexpressed GAK; however, the use of a secondary pcDNA3.1-ATA GAK V5 control plasmid, which in theory produces an RNA transcript but which fails to produce a protein product, also generated AR activity to a level that was significantly higher than that generated by the original control pcDNA3.1 plasmid, and comparable to the level of AR activity generated by the plasmid which overexpressed GAK (Figure 9). Indeed, experiments involving the transfection of PC3 cells with increasing quantities of control plasmid pcDNA3.1 supplemented with decreasing quantities of pcDNA3.1 GAK V5 seemed to support the theory that the pcDNA3.1 vector itself has an inhibitory effect on

AR activity (Figure 10). These results conflicted with the preliminary findings which suggested that GAK enhances AR activity *in vitro*.

In an effort to determine if the enhancement of AR activity in the presence of overexpressed GAK is indeed an artificial result caused by the false suppression of AR activity induced by the pcDNA3.1 vector itself, subsequent experiments were conducted *in vitro* which utilized siRNA technology. Knocking down GAK failed to generate significant differences in the level of AR transactivation compared to cells which overexpressed GAK (Figure 13), despite the fact that western blot analysis confirmed a very apparent knockdown of V5-tagged GAK generated by the GAK 545 siRNA construct (Figure 12). These results were not only reproducible, but the same conclusions were drawn in both PC3 and LNCaP cells, overall providing strong supporting evidence that GAK is most likely not involved in modulating AR activity.

Despite being unable to confirm GAK's role as a coregulator of AR, steps were taken to determine whether or not GAK plays a role in progression to AI using two DOX inducible cell lines, LNCaP-GAK and LNCaP-DEST, generously provided by Dr. Latif Wafa. Unfortunately, Western blot analysis confirmed that the LNCaP-GAK cell line produced some V5-tagged GAK in the absence of DOX (Figure 17). Nonetheless, these cells were utilized in subsequent experimentation. MTS assays were conducted in order to determine whether or not overexpressing GAK affected cell proliferation *in vitro*. Unfortunately, there were no measurable statistical differences between induced and uninduced GAK cell lines in both the presence and absence of hormone (Figure 18 and 19). Hormone was the only factor that appeared to significantly enhance cell proliferation *in vitro*. The results from soft agar colony formation assays were somewhat more encouraging. Not only did LNCaP-GAK cells, which overexpressed GAK, possess significantly enhanced colony formation ($p < 0.05$), but it was determined between the DEST-DOX and DEST+DOX treatment groups that DOX itself most likely plays an inhibitory role to a degree that is statistically significant (Figure 20). Certainly it follows that any colony forming effects caused by GAK may be offset by the inhibitory effects of DOX.

Of notable concern is the fact that these *in vitro* experiments were conducted at 1 nM R1881, a concentraion that is known to produce maximal AR activity. Consequently it could be argued that it would be very difficult to determine any GAK related effects on AR activity if the hormonal treatment was already inducing AR to the highest degree. In hindsight, and in future, experiments should be designed and conducted at varying R1881 concentrations to determine if GAK has any subtle effects on AR activity that most likely were not observed here. To assume that GAK would be able to induce AR activity over and above that which is optimally produced by 1 nM R1881 should be seriously considered in future. However, while this is a concern, it should be noted that the previous experiments conducted by Ray *et. al.* *** as well as most other experimentation in our laboratory use a concentration of R1881 nM where applicable.

To determine if GAK effects progression to AI, a series of xenograft experiments were conducted, each specifically designed to answer a particular question about the physiological role GAK may or may not play, given its subtle ability to enhance colony formation *in vitro*. The first xenograft experiment was aimed at determining whether or not overexpressing GAK affected tumour volume growth and serum PSA production *in vivo*. Western blot analysis supplemented by immunohistochemical analysis confirmed GAK overexpression in tumours extracted from the mice at the point of sacrifice, confirming the viability of the system (Figures 21, 22, and 23). It was also able to confirm that the leakiness initially observed *in vitro* was in fact exhibited *in vivo* as well. Minor differences in AR were observed via immunohistochemical analysis, but this was attributed to the manner in which the samples were in fact scored, as opposed to any real differences amongst treatment groups (Figure 24). The effect of GAK overexpression on tumour volume from both the time of injection and the time of castration was assessed. Interestingly, from the time of treatment, mice in the GAK+DOX and GAK-DOX groups possessed rates of average tumour volume growth which were significantly higher than that of the DEST+DOX and DEST-DOX groups respectively (Figure 25). From the time of castration, there is a highly significant difference between the GAK+DOX and DEST+DOX treatment groups supporting the previous observation made in the data plotted from the time of injection (Figure 26). However, from the time of castration, there does not appear to be any significant difference between the GAK-DOX and DEST-

DOX group, which contradicts previous data plotted from the time of injection which suggest the leakiness may play a significant role in enhancing tumour volume. That said, it is a well established fact that a portion of the mice comprising the GAK-DOX group expressed V5-tagged GAK. Plotting the data from the time of castration, whereby all the GAK expressors were compared to the GAK non-expressors within the GAK-DOX group itself indicated that the 4 out of 7 GAK expressing mice in the GAK-DOX group possessed a significantly higher percentage change in tumour volume than those 3 mice which did not express GAK (Figure 27). The fact that GAK expressing mice within the GAK-DOX group exhibit faster tumour growth than those that do not certainly suggests that this may in fact be a GAK specific effect as opposed to a cell line specific effect. Overall this seemed to support the general observation that mice injected with the LNCaP-GAK cell line appeared to progress through the experiment at a faster rate. These mice reached the 75 ug/µl PSA threshold at a faster rate than mice injected with the DEST cell line (Figure 28), and the tumour volumes of these mice also seemed to reach 10% of their body mass faster than those in the DEST group.

A second xenograft experiment was conducted with the specific aim of determining whether or not the growth promoting effects of GAK were in fact androgen regulated. Western blot and immunohistochemical analysis confirmed the findings previously generated in the first xenograft (Figure 29 and 30). GAK overexpression was achieved in the presence of DOX, and this overexpression did not appear to affect AR levels to any considerable degree.

Tumour volume was analyzed from both the time of injection and from the time of castration as in the previous experiment. However, this experiment was specifically designed to test the effects of castration on GAK's ability to enhance tumour volume growth. Not surprisingly, from the time of injection mice in the GAK-CX and DEST-CX control groups possess higher tumour volume growth rates than the those in castrated groups, GAK+CX and DEST+CX (Figure 32). There was no measurable statistical difference in average tumour volume between the GAK+CX and DEST+CX groups from the time of injection, any effects GAK may have on progression to AI are in fact androgen independent. Furthermore, a measurable difference in average tumour volume

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between the GAK+CX and the GAK-CX groups suggested that GAK itself appears to promote growth. Graphing tumour volume from the time of treatment supports these findings and demonstrates that there appears to be no difference between the two cell lines in their response to castration (Figure 33). Overall this data suggests that the initial enhancement of tumour growth generated by GAK is most likely not hormone dependent.

The effect of castration on serum PSA generated the same results as the previous experiment. Average serum PSA production on the basis of treatment group from the time of treatment was analyzed and as expected, castration significantly reduced average PSA production post-treatment in both the GAK+CX and DEST+CX groups (Figure 34). Unfortunately no differences were observed between the GAK-CX and DEST-CX groups under castrate or non-castrate conditions, supporting the recurring theme that GAK most likely does not affect PSA levels via AR modulation *in vivo*. Monitoring PSA levels in both the first and second xenograft experiments seems to substantiate the conclusions made *in vitro*, whereby luciferase assay results overexpressing and knocking down GAK demonstrated little effect on AR activity. Overall these findings seem to support the idea that GAK does not appear to modulate AR activity as previously shown.

A third xenograft experiment was conducted to determine the effect of overexpressing GAK on tumour take and tumour volume, minus the confounding effects of pre-treatment leakiness and treatment itself. As in the first xenograft experiment, the mice within the GAK group reached the 75 ug/µl PSA threshold at a faster rate than those mice within the DEST group, and the tumour volumes of these mice also seemed to reach 10% of their body mass faster than those in the DEST group. However both the first and second xenograft experiments were not designed to specifically analyze these parameters. In both the previous experiments, DOX and castration was administered at the point in time in which serum PSA reached 75 ug/µl. The third xenograft attempted to alleviate these confounding factors in order to definitely determine whether or not the general observation made regarding the speed at which the GAK mice progressed through the experiment were in fact real. DOX was administered right from the time of injection and castration was omitted for the purposes of this experiment.

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With respect to tumour take, two critical observations were made. Firstly, tumour take was significantly enhanced in the GAK+DOX group compared to the DEST+DOX group, suggesting that GAK plays a role in tumour development *in vivo* (Figure 36). Secondly, tumour take was drastically reduced in mice within the DEST+DOX group compared to the mice in the DEST-DOX group, indicating that DOX is in fact inhibiting tumour development (Figure 37). This suggests that any growth promoting effects induced by GAK may in fact be masked by the presence of DOX.

In terms of tumour volume, similar observations were made. Once again, overexpressing GAK enhanced the rate of average tumour volume growth to a degree that was highly significant. Furthermore, DOX significantly inhibited tumour growth in the DEST+DOX group compared to the DEST-DOX group. Overall these results validate the previous observations made in the first and second xenograft, and provide some new insight into the role GAK may play during progression to AI.

These studies provide substantial evidence to suggest that GAK does not coregulate AR as initially believed. However, it does not preclude the possibility that GAK may play a substantial role in progression to AI. Most recent studies have demonstrated that GAK appears to be involved in clathrin coated vesicle endocytosis, a cellular process which is critical to all aspects of cell development. Of particular interest is a recent study conducted by Lee, whereby GAK conditional knockout mice were generated [233]. Conventional GAK knockout mice deemed to be embryonically lethal, therefore conditional knockout mice were generated, and these mice were subsequently mated to mice expressing Cre recombinase under the control of a few key tissue specific promoters. Deletion of GAK from the brain, liver, and skin caused mice to die shortly after birth. Furthermore, using a tamoxifen-inducible promoter system, deletion of GAK in adult mice results in fatality. Ultimately this appears to be a direct result of a complete blockage of clathrin coated vesicle endocytosis due to a lack of clathrin coated pit formation. Despite the fact that GAK does not coregulate AR or enhance progression to AI through AR dependent mechanisms, it is not impossible to suggest that any disregulation in GAK production may result in an enhanced rate of progression towards AI through an alternative mechanism. Certainly the results presented here seem to

suggest that overexpression of GAK, while not androgen regulated, does appear to enhance tumour take and tumour volume growth rates.

Future studies involving the generation of a DOX inducible GAK knockdown LNCaP cell line would indeed be beneficial in terms of verifying GAK's role in progression to AI. Certainly as mentioned previously, GAK knockdown generates lethal results during development; however whether or not its role is critical during progression is an obvious next step. The results presented here lay the groundwork for future studies of this nature, and the results of which may possibly even lead to a potential mechanism by which progression to AI can be manipulated and possibly even prevented.

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