GENE EXPRESSION IN PROSTATE CANCER

by

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B.Sc., The University of British Columbia, 2003

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

OCTOBER 2008

ABSTRACT

Development and maintenance of the prostate is dependent on androgens and the androgen receptor. The androgen pathway continues to be important in prostate cancer. Here, we evaluated the transcriptome of prostate cancer cells in response to androgen using Long Serial Analysis of Gene Expression (LongSAGE) libraries. We identified 35 genes with novel associations to androgen signalling and validated 24 of these genes using quantitative real time-polymerase chain reaction (qRT-PCR). These genes were: *ARL6IP5, BLVRB, C19orf48, C1orf122, C6orf66, CAMK2N1, CCNI, DERA, ERRFI1, GLUL, GOLPH3, HM13, HSP90B1, MANEA, NANS, NIPSNAP3A, SLC41A1, SOD1, SVIP, TAOK3, TCP1, TMEM66, USP33,* and *VTA1.* The physiological relevance of these expression trends was evaluated *in vivo* using the LNCaP Hollow Fibre model.

There is no cure for castration-recurrent prostate cancer (CRPC), and the mechanisms underlying the disease are not known. To address this problem, we assayed the transcriptome of LNCaP human prostate cancer cells as they progress to castration-recurrence *in vivo* using replicate LongSAGE libraries. We identified 96 novel genes consistently differentially expressed in CRPC. The expression profiles support a role for the transcriptional activity of the androgen receptor genes (*CCNH, CUEDC2, FLNA,* and *PSMA7*), steroid synthesis and metabolism genes (*DHCR24, DHRS7, ELOVL5, HSD17B4*, and *OPRK1*), neuroendocrine cell genes (*ENO2, MAOA, OPRK1, S100A10,* and *TRPM8*), and proliferation genes (*GAS5, GNB2L1, MT-ND3, NKX3-1, PCGEM1, PTGFR, STEAP1,* and *TMEM30A*) in castration-recurrence.

Screening for prostate cancer using serum levels of prostate-specific antigen has resulted in the over-treatment of indolent disease. Novel diagnostic and prognostic markers for prostate cancer are required. To address this need, the levels of 27 transcripts were investigated with qRT-PCR. Expression of POP3 (100 kb from EST CFI40309) was prostate-specific, with restricted expression of *ADAM2,* POP1 (50 kb from AK000023), POP4 (truncated *TMEFF2*), POP10 (intron of *ADAM2*), *ELOVL5, RAMP1*, and *SPON2. ELOVL5, NGFRAP1,* POP5 (intron of *NCAM2*), POP8 (intron of *EFNA5*), *RAMP1, SPON2,* and *TMEM66* were differentially expressed between laser microdissected tumour and normal clinical samples of prostatic tissue.

These studies suggest that *ADAM2, ELOVL5*, POP1, POP3, POP4, POP10, *RAMP1*, and *SPON2* may be good candidates for biomarkers of prostate cancer.

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LIST OF ABBREVIATIONS

ACPP	prostate acid phosphatase
ACTH	adrenocorticotropic hormone
ADAM2	ADAM metallopeptidase domain 2
Akt	protein kinase B
AMD1	adenosylmethionine decarboxylase 1
AQP3	aquaporin 3
AR	androgen receptor
AREs	androgen response elements
ARF1	ADP-ribosylation factor 1
ARL6IP5	ADP-ribosylation like factor-6 interacting protein 5
AS	androgen-sensitive
BAD	BCLXL/BCL2 associated death promoter
BAX	BCL2-associated X protein
BCL-2	B-cell CLL/lymphoma 2
BCL2L1	BCL2-like 1
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
BTG1	anti-proliferative b-cell translocation gene 1
CAK	cyclin-dependent activating kinase
CAMK2	calcium/calmodulin-dependent kinase II
CAMK2N1	calcium/calmodulin-dependent protein kinase II inhibitor 1
CCND1	cyclin D1
CCNH	cyclin H
CCT2	chaperonin containing TCP1 subunit 2
CD151	CD151 molecule
CD44	CD44 molecule
CDKN1A	cyclin-dependent kinase inhibitor 1A
CDKN1B	cyclin-dependent kinase inhibitor 1B
CDKN2A	cyclin-dependent kinase inhibitor 2A
cDNA	complementary deoxyribonucleic acid
CHGA	chromogranin A
CHGB	chromogranin B

ChIP-seq	chromatin immunoprecipitation-sequence
CI	confidence interval
CR	castration-recurrent
CRIB	cdc42/Rac interacting and binding
CRPC	castration-recurrent prostate cancer
CT	computed tomography
CUEDC2	CUE-domain-containing-2
Cx	castration
DHCR24	24-dehydrocholesterol reductase
DHRS7	dehydrogenase/reductase SDR-family member 7
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
DRE	digital rectal exam
ds	double stranded
DSTN	destrin (actin depolymerizing factor)
EASE	expression analysis systematic explorer
EBRT	external beam radiation therapy
EFNA5	ephrin-A5
EGF	epidermal growth factor
ELOVL5	elongation of long chain fatty acids family member 5
ENO2	gamma neuronal enolase 2
ERAD	endoplasmic reticulum associated degradation
ERK	extracellular signal-regulated kinase
ERRF11	ERBB receptor feedback inhibitor
EZH2	enhancer of zeste homolog 2
FBS	fetal bovine serum
FFPE	formalin-fixed and paraffin-embedded
FGFRL1	fibroblast growth factor receptor like 1
FHIT	fragile histidine triad gene
FISH	fluorescence in situ hybridization
FLNA	filamin A
GAPDH	glyceraldehyde-3-phosphate

GAS5	growth arrest specific 5 on chromosome 1
GLO1	glyoxalase I
GLUL	glutamate-ammonia ligase
GNB2L1	guanine nucleotide binding protein beta polypeptide 2 like 1
GO	Gene Ontology
GOLPH3	golgi phosphoprotein 3
GRB10	growth factor receptor bound protein 10
GSTP-1	glutathione-S-transferase P1
HES6	hairy and enhancer of split 6
HGF	hepatocyte growth factor
HGNC	HUGO gene nomenclature committee
HM13	Histocompatibility (minor) 13
HMGB2	high mobility group box 2
HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
HN1	hematological and neurological expressed 1
HPA	hypothalamus-pituitary-adrenal
HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)
HSD174	hydroxysteroid (17-beta) dehydrogenase 4
HSD17B3	hydroxysteroid (17-beta) dehydrogenase 3
HSD17B5	hydroxysteroid (17-beta) dehydrogenase 5
HSP90B1	heat shock protein 90 kDa beta member 1
IGF-1	insulin-like growth factor
IL6	interleukin 6
JNK/SAPK	c-Jun N-terminal kinase/stress-activated protein kinase
kb	kilobases
KEGG	Kyoto encyclopedia of genes and genomes
KLK3	kallikrein 3 = PSA
KPNB1	karyopherin/importin beta 1
LCM	laser capture microdissection
LDT	linker-derived tag
LHRH	leutinizing hormone releasing hormone
LongSAGE	long serial analysis of gene expression

MANEA	mannosidase, endo alpha
MAOA	monoamine oxidase A
MAPK	mitogen activated protein kinase
MARCKSL1	MARCKS-like 1
MDK	midkine
MGC	mammalian gene collection
MGMT	O-6-methylguanine-DNA methyltransferase
MNE	mean normalized expression
MRI	magnetic resonance imaging
MT-ND3	NADH ubiquinone oxidoreductase chain 3
NANS	n-acetylneuraminic acid synthase
NCAM2	neural cell adhesion molecule 2
NCOA1	nuclear receptor coactivator 1
NCOA2	nuclear receptor coactivator 2
NFkB	nuclear factor kappa B
NGFRAP1	nerve growth factor receptor associated protein 1
NIPSNAP3A	nipsnap homologue 3A
NKX3-1	NK3 homeobox 1
NTS	neurotensin
ODC1	ornithine decarboxylase 1
OPRK1	opioid receptor kappa 1
OR	odds ratio
PC	prostate cancer
PCGEM1	prostate specific non-coding gene
PCOTH	prostate collagen triple helix
PCR	polymerase-chain reaction
PGK1	phosphoglycerate kinase 1
PI3K	phosphoinositide-3-kinase
PIA	proliferative inflammatory atrophy
PIK3CD	phosphoinositide-3-kinase catalytic delta polypeptide
PIN	prostatic intra-epithelial neoplasia
PKA	protein kinase A

POP1	transcript 100 kb from mRNA AK000023
POP2	transcript 4 kb from mRNA AL832227
POP3	transcript 50 kb from EST CFI40309
POP4	transcript from the intron of TMEFF2
POP5	transcript from the intron of NCAM2; accession DO668384
POP6	transcript from the intron of FHIT
POP7	transcript from the intron of TNFAIP8
POP8	transcript from the intron of EFNA5
POP9	transcript from the intron of DSTN
POP10	transcript from the intron of ADAM2; accession DO668396
POP11	transcript 87 kb from EST BG194644
POP12	transcript from the intron of EST BQ226050
PPP2CB	protein phosphatase 2 catalytic subunit beta isoform
PPP2R1A	protein phosphatase 2 regulatory subunit A alpha
Pre-Cx	pre-castration
PROM1	prominin 1
PSA	prostate-specifc antigen = KLK3
PSCA	prostate stem cell antigen
PSMA	prostate-specifc membrane antigen
PSMA7	proteasome macropain subunit alpha type 7
PTEN	phosphatase and tensin homolog
PTGFR	prostaglandin F receptor
PTHrP	parathyroid hormone-related protein
QF	quality factor
qRT-PCR	quantitative real-time polymerase chain reaction
R1881	methyltrienolone; synthetic androgen
RAD	responsive to androgen-deprivation
RAMP1	receptor (calicitonin) activity modifying protein 1
RB1	retinoblastoma 1
RefSeq	reference sequence
RNA	ribonucleic acid
RT	reverse transcriptase

s.c.	subcutaneous
S100A10	S100 calcium binding protein A10
SAGE	serial analysis of gene expression
SBDS	Shwachman-Bodian-Diamond syndrome
SD	standard deviation
SDHA	succinate dehydrogenase complex subunit A, flavoprotein
SH3	Src-homology-3
shortSAGE	short serial analysis of gene expression
SLC25A4	solute carrier family 25 member 4
SLC25A6	solute carrier family 25 member 6
SLC41A1	solute carrier family 41, member 1
SOD1	superoxide dismutase 1
SPON2	spondin 2
SQLE	squalene epoxidase
Src	v-src sarcoma
ss	single stranded
SSH	suppressive subtractive hybridization
STEAP1	six transmembrane epithelial antigen of the prostate 1
SVIP	small VCP/p97-interacting protein
TAOK3	tao kinase 3
TCP1	T-complex 1
TMEFF2	transmembrane protein with EGF-like and two follistatin-like domains 2
TMEM30A	cell cycle control protein 50A
TMEM66	transmembrane protein 66
TNFAIP8	tumor necrosis factor, alpha-induced protein 8
TNM	tumour-node-metastasis
TP53	tumour protein p53
TRPM8	transient receptor potential cation channel subfamily M member 8
VTA1	vps20-associated 1
Wnt	Wingless
YWHAQ	tyrosine 3 monooxygenase/tryptophan 5 monooxygenase activation protein theta polypeptide

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Marianne Sadar, for being an inspiring mentor and role model. Her guidance, support, and dedication have been instrumental to my growth as a young scientist. To her, I will always be grateful. Members of Marianne's laboratory and department have also been instrumental to my education; providing excellent technical help, feedback, and discussions that have contributed to the success of this thesis. In particular, I would like to acknowledge some current and past laboratory and department members: Nasrin (Rina) Mawji, Dr. Richard Sobel, Dr. Steven Quayle, Dr. Mohammad Qadir, Jas Khattra, Yongjun Zhao, Jean Wang, Theresa L'Heureux, Iran Travakoli, Gang Wang, Helen Chiu, Janice Wortman, Dr. Joanne Johnson, Ainsley Clement, Dr. Simon Haile, Dr. Jae Kyung Myung, Dr. Katie Meehan, Dr. Barbara Comuzzi, and Heidi Hare. I would also like to thank: my Supervisory Committee, Dr. Wan Lam, Dr. Marco Marra, Dr. Robert Holt, and Dr. Cheryl Wellington for critical assessments, helpful comments, and advice; and my Comprehensive Exam Committee, Dr. Wan Lam, Dr. Yuzhuo Wang, Dr. Marcel Bally, and Dr. Mladen Korbelik, for admitting me to candidacy.

My thesis would not be possible without the love and support of my friends and family. I appreciate my friends Elise LaRue, Kristy Garbutt, Julie Boylan, Marlo Small, and Tanya Dayman for their patience during this busy time of my life. I would like to thank my sisters, Crystal and Juliane Romanuik, for calling and visiting often throughout my studies. My grandparents, Sophie and Andy Nicholson, the late Peter Moleschi, and the late Ann and John Romanuik, were my inspiration to practice cancer research. I am indebted to my parents, Colleen and Dayle Romanuik, for always believing in me and encouraging me in my pursuits. Last, but not least, I would like to acknowledge my financé, Ryan Giraud, for the laughter and balance in my life.

DEDICATION

To my grandparents:

Sophie and Andy Nicholson

&

In memory of:

Peter Moleschi, & Ann and John Romanuik

CO-AUTHORSHIP STATEMENT

The experiments described within this thesis were conceived, designed, conducted, and analyzed by me, Tammy L. Romanuik, and Dr. Marianne D. Sadar. All manuscripts were written by me and Dr. Marianne D. Sadar. A number of additional people contributed to the work in each of the chapters as outlined below.

CHAPTER II

Mr. Gang Wang generated the total RNA for the construction of the SAGE libraries. Dr. Marco Marra provided support for the SAGE library construction with sequencing by Dr. Robert A. Holt. Dr. Steven J.M. Jones aided in the analysis of data. Technical assistance was provided by Jean Wang (animal experiments), and Angelique Schnerch (whole library mappings).

CHAPTER III

Dr. Marco Marra was responsible for SAGE library construction and sequencing. Olena Morozova (tag clustering) and Allen Delaney (library clustering) aided in bioinformatic analysis. Technical assistance was provided by Jean Wang (animal experiments).

CHAPTER IV

Dr. Takeshi Ueda provided the clinical samples with medical history and Dr. Thomas Thomson, performed the pathology review. Technical assistance was contributed by Theresa L'Heureux and Iran Travakoli (laser microdissection), Dr. Margaret Sutcliffe (pathology) and Lorena Barclay (tissue sections). Statistical support was provided by Dr. Nhu Le.

CHAPTER I

INTRODUCTION

1.1 THE NORMAL AND DISEASED PROSTATE

1.1.1 Mortality, morbidity, and risk factors of prostate cancer

Prostate cancer is the most common malignancy in Canadian men, and the third leading cause of cancer death[1]. In Canada, the lifetime probability of developing prostate cancer is one-in-seven, while the lifetime probability of dying from prostate cancer is one-in-twenty-seven[1]. In 2008, it is estimated that 24,700 Canadian men will be diagnosed with prostate cancer, and another 4,300 will die from this disease[1]. Worldwide, there were 679,000 new cases of prostate cancer in 2002[2]. Prostate cancer is a disease of the aged. The majority of new cases are diagnosed in men aged 60-69 (39%), while the majority of deaths by prostate cancer occur in men aged greater than 80 years (53%; estimated statistics for 2008)[1]. In the United States, the incidence of prostate cancer in African Americans is 70% higher than in Caucasians, and Caucasians have higher morbidity rates than Asians[2]. In contrast, autopsy studies indicate that the prevalence of prostate cancer is relatively uniform among men from different countries and races[3]. In addition to age and race, family history and diet (intake of fat, vitamin D) are also risk factors for prostate cancer[3].

1.1.2 Normal prostate

Function and anatomy

The prostate is a walnut-sized gland located just below the bladder, adjacent to the rectum, and flanked by the seminal vesicles. It surrounds the urethra and acts as a sphincter to regulate the release of urine from the bladder[4]. However, the primary function of the prostate is to produce and store secretions. Constituents of prostate secretions include: acid phosphatase, albumin, cholesterol, fibrinolytic enzymes, plasminogen activator, spermidine, and proteolytic enzymes such as prostate-specific antigen (PSA)[5-7]. The smooth muscle cells of the prostate contract to propel the expulsion of semen during ejaculation[7].

Prostate secretions are released into the lumen of prostatic ducts by luminal secretory epithelial cells. The ducts of the prostate are comprised of luminal secretory epithelial cells and rare

neuroendocrine cells. These cells are located along the basal epithelial cells and basal lamina, and are surrounded by stromal smooth muscle cells[8]. Markers for these cell types include cytokeratins 8 and 18 (luminal epithelial cells), chromogranin A and serotonin (neuroendocrine cells), keratin 5 and p63 (basal epithelial cells), and smooth muscle actin (stromal cells)[8, 9]. Stromal smooth muscle cells and luminal epithelial cells express the androgen receptor[10].

Regulation by the androgen pathway

The development and maintenance of the prostate are regulated by androgens[11], such as testosterone, and the more potent androgen dihydrotestosterone (DHT). The majority of androgens are produced by the testes (95%), while the remaining 5% are synthesized by the adrenal glands[12]. Without testicular androgens, it is believed the concentration of circulating adrenal androgens is insufficient for prostate growth and survival[13]. Androgens are derivatives of cholesterol and easily pass through the lipid-rich bilayer of the plasma membrane and bind to intracellular androgen receptor (AR).

The AR protein is 110 kDa (919 amino acids). It contains a carboxy-terminal ligand binding domain, amino-terminal transactivation domain, and central deoxyribonucleic acid (DNA) binding domain in the hinge region complete with a nuclear localization signal. The AR gene is located on the X chromosome q11-12 with 8 coding exons spanning 2.7 kb[14].

In the cytosol, the ligand binding domain of the AR is accessible to androgen due to the chaperone activity of heat-shock proteins[15]. Heat shock proteins also prevent pre-mature nuclear localization of the AR[16], and degradation of the AR by the proteasome[17]. Upon binding ligand, AR becomes phosphorylated, changes conformation, and is released from the heat-shock proteins[16]. AR homo-dimerizes in a head-to-tail configuration[18], homes to the nucleus[19] via its nuclear localization signal, and binds to androgen response elements (AREs) of DNA[20]. The AR dimer interacts with a palindrome DNA sequence nGnACnnnnnGTnCn, where 'n' is any nucleotide[21-26]. Once bound to DNA, the AR recruits co-activators (e.g., Tip60)[27] or co-repressors (e.g., NCoR)[28], to regulate gene expression. Direct or indirect targets of the androgen signalling axis (i.e., pathway) have functions in cell growth[29], survival[13], differentiation[11], and secretion[30]. However, the complete spectrum of genomic targets of the androgen-axis, (i.e.,

genes regulated by androgens), still remain to be discovered. Identification of the genes whose expressions are regulated by androgens is required to elucidate the mechanisms underlying the growth and survival of normal and cancer cells.

1.1.3 Cytopathology and pathophysiology

PIA

Focal atrophy is a term used to describe the reduced cytoplasm of secretory epithelial cells. Simple or post-atrophic hyperplasia is described as proliferative inflammatory atrophy (PIA) if it is accompanied by inflammation (lymphocyte penetration) and proliferation (Ki67 staining)[31]. Some groups have observed a transition from PIA to adenocarcinoma[32], and PIA to high grade prostatic intra-epithelial neoplasia[33], suggesting that PIA may be a precursor lesion to prostate cancer.

PIN

Prostatic intra-epithelial neoplasia (PIN) is suggested by Bostwick to be a precursor lesion of prostate cancer[34]. PIN is closely associated with prostate cancer and featured by the proliferation of secretory epithelial cells[34]. This contrasts with normal prostatic ducts and acini that contain single layers of secretory epithelial cells. Furthermore, the secretory epithelial cells of PIN exhibit enlarged nuclei and nucleolus[34]. PIN is described as either high grade or low grade. In low grade PIN, the basal cell layer is intact, there is some cell stratification, but a small nucleolus. In contrast, high grade PIN has a *partially* disrupted basal cell layer, cell stratification, and a prominent nucleolus [35].

Prostate cancer

Prostate cancer is a disease of the luminal epithelial cells. Prostate cancer is completely devoid of basal epithelial cells as indicated by the absence of p63 staining, thereby distinguishing it from PIN[34-36]. Prostate cancer is invasive and has the ability to metastasize to other tissues, while PIN does not. In prostate cancer, cell morphology and tissue architecture are altered (see **Section 1.2.6 Gleason grading**). Prostate cancer cells are slow growing. Therefore, at the time of detection, it is likely the patient has been living with prostate cancer for many years (e.g., 15-20

years). The implementation of PSA screening in 1986 (see **Section 1.2.2 PSA**) has resulted in most prostate cancer being detected while it is small and localized[37]. Of the men whose cancer metastasizes, as much as 83% of cases involve bone[38]. Palliative treatment for metastatic prostate cancer includes androgen-deprivation therapy. However, eventually metastatic prostate cancer becomes fatal.

1.2 DETECTION, DIAGNOSIS, AND MONITORING OF PROSTATE CANCER

1.2.1 DRE

Most prostate cancer is detected using a combination of the digital rectal exam (DRE) and PSA testing[39]. DRE involves manual palpitation of the prostate through the walls of the rectum to gauge the size, firmness, and shape of the prostate. This is possible due to the proximal placement of the prostate to the rectum. One limitation to DRE is that the physician can only feel the surface of the prostate that faces the rectum, although the majority of prostate cancers do arise in this region[4]. Moreover, the test is subjective to the physician performing it. Approximately 25-50% of the cases of prostate cancer detected by DRE are no longer localized[39]. When used alone, DRE testing has not reduced mortality from prostate cancer[40].

1.2.2 PSA

Prostate-specific antigen (PSA), also known as human kallikrein 3 (*KLK3*), is a member of the kallikrein family of genes[41]. All fifteen kallikrein genes are located in a cluster on chromosome 19q133-4. The expression of PSA is restricted to the prostate[42] and to humans. PSA gene expression is regulated by androgens[43-45] with at least three androgen response elements located in the promoter and enhancer regions[46]. The PSA gene was first cloned in 1989[47].

PSA protein is a serine protease[5] synthesized by the prostate that functions to liquefy seminal fluid[6]. The concentration of PSA in semen is between 0.5 and 2 mg/mL[48]. In healthy men, a small fraction of this PSA leaks from the prostate into the bloodstream. However, with prostate adenocarcinoma there is a breakdown of the basement membrane, loss of the basal cell layer, lack of cellular polarity, and collapsed architecture of prostatic ducts, which results in significantly more PSA released into the bloodstream[49]. Serum PSA levels exceeding 4 ng/mL

are suggestive of cancer and warrant further investigation and referral to an urologist. Since 1986 when PSA was first approved for detection of prostate cancer, prostate cancers are now detected at an earlier stage while they are still small and localized. However, with PSA screening many clinically insignificant cancers that will not impact the mortality of the patient are also detected[50]. Better prognostic markers are needed to distinguish those cancers requiring radical treatment from indolent disease. PSA is moderately specific (93%) and poorly sensitive (24%) as a biomarker for prostate cancer detection[51]. In addition to carcinoma of the prostate, PSA is expressed in normal prostate tissue and benign prostatic hypertrophy, and levels of circulating PSA are affected by age, race, and body mass[49]. Levels of serum PSA correlate with tumour volume[52] and are used to monitor prostate cancer response to therapy. However, exceptions have been reported where PSA and response to therapy can be discordant[49, 53, 54]. Novel biomarkers for early and late stage prostate cancer are also needed.

1.2.3 Biopsy

When prostate cancer is suspected, a needle biopsy is used to confirm and aid diagnoses. The number of biopsies taken from patients can vary between six to fourteen. While greater than six biopsies do increase detection rates, studies show that prognosis is not affected by the number of biopsies, and therefore the clinical management of prostate cancer remains unchanged[55]. Pathology is used in conjunction with other clinical tests to determine the aggressiveness of the disease and clinical stage of prostate cancer (see **Sections 1.2.5 TNM staging** and **1.2.6 Gleason grading**). Biopsy is often coupled with a transrectal ultrasound to guide biopsy needles.

1.2.4 Other clinical tests

A computed tomography (CT) scan or magnetic resonance imaging (MRI) may be used to identify and monitor metastatic lesions in high risk patients. These radiographic imaging systems are not sensitive enough to detect metastases in asymptomatic patients[56]. CT entails the construction of a digital three dimensional image assembled from multiple X-rays. The majority (~91%) of prostate cancer metastases to the bone are osteoblastic (bone forming)[57]; CT scans are useful in visualising these bone lesions. In contrast to CT, MRI does not use ionizing radiation and provides better contrast for soft tissues, making it particularly well suited to visualizing tumours[58].

1.2.5 TNM staging

The Tumour-Node-Metastasis (TNM) system for staging cancers is used to describe the spread of the disease. The most recent guidelines for TNM staging were published in 2002 by Greene [59] and summarized by Chang[60] as it pertains to prostate cancer. Briefly, the 'T' refers to the extent of the primary tumour in the prostate, and if applicable, also the invasion of the seminal vesicles or other nearby structures; the 'N' describes the involvement of the tumour within the lymph nodes; and the 'M' describes the extent or location of distant metastases such as bone or non-regional lymph nodes. The detailed definition of the TNM staging system is presented in **Table 1.1**[59].

Importantly, the TNM staging system has been divided into clinical and pathologic classification. Clinical staging refers to the staging that was determined before therapeutic intervention and is based on results obtained from DRE, transrectal ultrasound, and Gleason grading information of biopsy tissue. Serum PSA levels and imaging results (if available) may also be incorporated into determining the clinical stage of prostate cancer. Clinical staging does not change even if subsequent pathologic staging information is conflicting. Pathologic staging is based on the histological information obtained from tissue removed surgically, that can include assessment of regional lymph node, bladder, and rectal involvement[60].

1.2.6 Gleason grading

The Gleason grading system was first described in 1966[61], and modified in 1967, 1974, and 1977[62-64]. The Gleason grading system is used by pathologists to describe the degree of differentiation of prostate tissue retrieved with biopsy, prostatectomy, or occasionally by transurethral resection of the prostate. Five Gleason patterns (grades) describe the tissue architecture, with pattern five representing the least structured tissue. The sum of the two most prevalent Gleason grades is referred to as the Gleason score. The definitions of Gleason patterns have transformed somewhat over the years to accommodate modern advances in prostate cancer diagnosis[65]. In 2005 the International Society of Urological Pathology came to a consensus on an improved Gleason grading system[65, 66]. The Gleason grading system as it is used today is depicted and summarized in **Figure 1.1**[66].

The Gleason score is prognostic[61] and strongly associated with aggressive prostate cancer. When combined with information of TNM stage and pre-treatment levels of serum PSA, Gleason score can segregate patients who are at low, intermediate, and high risk of PSA failure following first-line treatment for localized cancer. Low risk patients have Gleason scores ≤ 6 and a TNM stage T1c/T2a with a PSA level ≤ 10 ng/mL; intermediate risk patients have Gleason scores of 7 and a TNM stage $\leq$ T2b with a PSA level >10 ng/mL, but ≤ 20 ng/mL; high risk patients have Gleason scores ≥ 8 or a TNM stage T2c or a PSA level > 20 ng/mL[67].

1.3 TREATMENT OF PROSTATE CANCER

1.3.1 Localized

Patients with prostate cancer localized within the prostatic capsule are eligible for active surveillance, radical prostatectomy, or radiotherapy. Active surveillance may be a good choice for men with a life expectancy less than 10 years (those over the age of 65) and prostate cancer that is low in Gleason grade and volume. In active surveillance the progression of the cancer is closely monitored using tests such as the DRE, measurement of serum PSA levels, and biopsy sampling. Within eight years, 34% of men who choose active surveillance will develop metastatic disease[68].

Radical prostatectomy is the surgical removal of the entire prostate and sufficient nearby tissue to ensure negative surgical margins. In 1887, McGill of the Leeds General Infirmary was reportedly the first surgeon to completely remove a prostate for the treatment of prostatic disease[69]. Today, radical prostatectomies are performed either at retropubic or perineal incision sites, openly or laproscopically, and with or without the use of a robot[70]. Possible complications of surgical intervention include infection, incontinence, and impotence[71]. Radical prostatectomy is an excellent treatment for prostate cancer and only 15% of men may experience biochemical recurrence at eight years following surgery (defined as rising PSA levels)[72].

Brachytherapy is a treatment option for low risk patients that involves the implantation of radioactive pellets (iodine^{125} or palladium^{103}) into the prostate. With the help of CT images and transrectal ultrasound, the radioactive seeds are guided into place. Brachytherapy is considered

comparable to prostatectomy at treating prostate cancer. However, with brachytherapy only 7% of men experience biochemical recurrence at eight years of follow-up[73].

External beam radiation therapy (EBRT) differs from brachytherapy in that the radiation is administered externally. To minimize the damage to surrounding normal tissue, typically lower doses of radiation are used compared with brachytherapy. Neoadjuvant therapy with chemotherapeutic agents or androgen-deprivation (more on this below) has been proposed to improve the cytotoxicity of EBRT on prostate cancer cells[74].

1.3.2 Metastatic

There is no cure for metastatic prostate cancer, although palliative treatment is available in the form of androgen-deprivation therapy. In the late 19th century it was observed that orchiectomy (removal of the testes) causes atrophy of the prostate gland in dogs[75]. The castration of men for treatment of an enlarged prostate soon followed[76], but it wasn't until the mid 20th century that Huggins and Hodges showed that the growth and survival of the prostate requires androgens, the male sex hormones produced by the testes[13].

Several treatment options constitute androgen-deprivation therapy. In addition to orchiectomy, available drugs for hormone manipulation include antiandrogens (e.g., flutamide, bicalutamide, and nilutamide), 5'alpha-reductase inhibitors (e.g., finasteride), leutinizing hormone releasing hormone agonists (e.g., leuprolide, goserelin, and buserelin), and ketoconazole[71]. An alternative approach to androgen-deprivation is intermittent androgen suppression which involves cycling androgen-deprivation therapy between breaks in treatment. Following androgen-deprivation, restoration of physiological androgen levels cause tumour cells to differentiate[77]. It is assumed that this treatment regime may delay prostate cancer progression.

1.4 CASTRATION-RECURRENCE

The majority (~85%) of the men who receive androgen-deprivation therapy initially exhibit a positive response because their tumours are androgen-stimulated[71]. Unfortunately, all men who receive androgen-deprivation therapy display biochemical recurrence within ~18 months[78], and

succumb to the disease within another ~18 months[78-80]. The mechanisms underlying progression to castration-recurrence are unknown; however, there is evidence to suggest that the AR is still active in this stage of the disease[81]. Proposed mechanisms underlying castration-recurrent prostate cancer that involve the AR include AR hypersensitivity to low androgen concentrations due to gene amplification[82, 83], changes in AR co-regulators[84, 85], intraprostatic *de novo* synthesis of androgen[86] or metabolism of AR ligands from residual adrenal androgens[87, 88], AR promiscuity of ligand specificity due to mutations[89], and activation of the AR by alternative signalling initiated by growth factors, cytokines, or kinases (does not require the ligand binding domain)[90-92].

Levels of serum PSA typically correlate with tumour volume during progression[52]. However, this association is less reliable in castration-recurrent prostate cancer[49]. Therefore, there is a need to identify novel diagnostic and prognostic biomarkers of castration-recurrent prostate cancer.

1.5 MODELS OF PROSTATE CANCER

1.5.1 Cell lines

Tissue culture provides the flexibility to test conditions that would be challenging to achieve *in vivo*, and the ability to have strict control of variables and systematically isolate factors to test a hypothesis. Primary cultures of prostate cancer cells have proven difficult to maintain partly due to their slow rate of proliferation. The establishment of the first prostate cancer cell lines was in the late 1970s and early 1980s from prostate cancer metastases[93-95]. These cell lines, LNCaP, PC-3, and DU145, remain the most commonly used prostate cancer cell lines[96].

LNCaP cells were isolated from lymph node metastases from a 50-year-old Caucasian man with prostate cancer [97]. LNCaP cells express the AR and PSA[43], and are sensitive to androgens[93]. DU145 cells were isolated from a brain metastasis of a 69-year-old Caucasian man with prostate cancer and lymphocytic leukemia[95], while PC-3 cells were isolated from a lumbar metastasis of a 69-year-old Caucasian man. Unlike LNCaP cells, both DU145 and PC-3 cells are androgen-insensitive because they do not express *AR* and do not respond to androgens[94-96]. Other prostate cancer cell lines are reviewed by Sobel and Sadar[96, 98].

1.5.2 In vivo models

In vivo models are a powerful tool to investigate prostate cancer under physiological conditions. The Balb/C Nu/Nu mouse is the most common background used to host human xenografts[99]. Rejection of the foreign human xenograft is curbed because these mice are immuno-compromised; lacking a thymus due to a mutation in the *Foxn1* gene[100]. Balb/C Nu/Nu mice have been used to create a xenograft model with the LNCaP cell line facilitated by addition of a reconstituted basement membrane product, Matrigel[101]. LNCaP cells form tumours at the site of injection, but rarely metastasize[96]. Intriguingly, the LNCaP xenograft model progresses to castration-recurrence in castrated hosts, mimicking the hormonal progression of prostate cancer observed in patients[102]. Hormonal progression may be monitored with serum PSA testing from tail vein blood samples, and levels correlate with tumour volume in this model[102]. Due to significant vascularization, one problematic feature of the LNCaP xenograft model is that it is not possible to separate the tumour tissue from the host tissue sufficiently for down-stream molecular analysis. As a solution to this contamination problem, the LNCaP Hollow Fibre model was developed[103].

In the LNCaP Hollow Fibre model, LNCaP cells suspended in media and Matrigel are injected into hollow fibres, sealed by heat on each end of the fibre, and implanted subcutaneously onto the back of immuno-compromised mice. This compartmentalization, using the fibre walls as a barrier, physically separates the prostate cancer cells from the host cells. Importantly, the diffusion of proteins, metabolites, oxygen, and other factors into or out of the fibre is not impeded. Unlike the LNCaP xenograft model, the LNCaP Hollow Fibre model can be used to harvest pure populations of prostate cancer cells suitable for molecular analysis (**Chapters II** and **III**) from the same host mouse over a time-course experiment. The retrieval of hollow fibres involves only minor surgery thereby allowing retrieval of serial sets of samples. The LNCaP Hollow Fibre model mimics hormonal progression of prostate cancer, as measured by serum PSA levels[103] and facilitates molecular analysis of samples during different stages of progression.

1.5.3 Human tissue

Sources

Cell lines and *in vivo* models play important roles in basic research. However, samples isolated from human tissue represent the ultimate setting for investigation. For the study of localized, androgen-sensitive prostate cancer, human prostate samples retrieved at the time of prostatectomy are ideal. Both normal tissue and tumour tissue may be retrieved from the same patient. However, localized prostate cancer is often multifocal[104] and normal tissue adjacent to tumour tissue may be altered compared to normal cells from a benign prostate.

For the study of advanced, metastatic, and/or castration-recurrent prostate cancer, human prostate samples are not readily available. Treatment offered to patients with these stages of cancer is limited to palliative radiation or androgen-deprivation therapy. Treatment options do not include surgery because it would be ineffective at treating disseminated disease. Therefore, ethically it would be inappropriate to subject patients to surgery that is not of benefit to them. Moreover, the majority of metastasic prostate cancer is located in the bone. Samples of late-stage prostate cancer are generally from locally advanced disease obtained from rapid autopsy[105].

Preservation

Human tissue obtained from patients at surgery or rapid autopsy will quickly decay and become unsuitable for laboratory research without sufficient preservation. The most abundant source of preserved tissue is formalin-fixed and paraffin-embedded (FFPE). These specimens are located in tissue repositories of hospitals for archival purposes. Because the tissues are stored for several years, they are accompanied by detailed clinical information including patient outcomes. Due to the abundance of samples and the wealth of patient information, these tissues are ideal for retrospective molecular studies[106]. One limitation to using archival FFPE tissue for molecular studies is the difficulty in obtaining patient consent. Much success has been achieved with the immunohistochemical analysis of FFPE tissues, and some success with DNA based assays (*in situ* hybridization and sodium bisulfite/hydroquinone DNA modification and polymerase chain reaction (PCR) with methylation specific primers). In contrast, RNA analysis using FFPE tissue has been challenging because ribonucleic acid (RNA) is sheared by the mechanical stresses of paraffin embedding, and formalin fixation causes RNA methylol modifications and cross-links

protein amino groups to RNA nucleotides[107]. For these reasons, frozen tissue is optimal for tissue preservation and isolation of RNA if transcript analysis is desired. Regrettably, there are very few tissue repositories that keep archival tissue at sub zero temperatures. Therefore, only prospective studies that anticipate analysis would be impeded by the FFPE preservation method, set-aside fresh tissue for freezing. It is easier to obtain consent in prospective studies, however, it may take up to 10 years to obtain follow-up medical history of patients.

Laser microdissection

Prostate cancer is heterogeneous[38]. Often times tissue collected at the time of prostatectomy contains both normal tissue and multifocal carcinoma[104]. These foci may have arisen from independent tumours or represent the spread of the primary tumour[108]. Laser microdissection is a powerful method to isolate the cells of interest from a mosaic of other cell types, and is an essential step that precedes molecular analysis that is sensitive to contamination. While laser microdissection of prostate cancer tissue will yield samples that primarily contain neoplastic luminal epithelial cells, adjacent normal laser microdissected tissue samples will be a mixture of luminal and basal epithelial cells.

Early microdissection techniques were imprecise and were only suitable for the basic manipulation of large specimens. They could not be utilized for the separation of specific cell types. As the technique evolved, manual dissection tools such as micromanipulators[109] allowed for greater control of dissections, but were impractical, tedious, and inefficient. In the early 1970s, researchers began experimenting with lasers as a means to isolate cells. It was not until 1996 that modern day 'laser capture microdissection (LCM)' was first described by Emmert-Buck *et al*[110] as developed by the National Institute of Health. Briefly, cell(s) of interest may be selected for and cut out with a laser and isolated using a thermosensitive film. Upon heating with the laser, the film will adhere to cells adjacent to it allowing selected cells to be physically separated from unselected tissue. Upon reconstitution in a DNA, RNA, or protein buffer, selected cells gently dissociate from the film and can be manipulated as desired[110]. An improvement on LCM is available from Molecular Machines & Industries. In this improvement, cells are captured onto the lids of Eppendorf tubes via a membrane intermediate. This feature

eliminates the step of transferring the film with adherent cells to a tube for further manipulation, and thus reduces the probability of introducing contamination.

1.6 GENE EXPRESSION

1.6.1 Gene expression analyses of prostate cancer

Biomarkers and therapeutic targets of prostate cancer have been identified by differential gene expression analyses by comparing samples that represent: 1) tumour versus normal tissue[111-130]; 2) high versus low Gleason grade[131-133]; 3) progressive versus latent cancer[133, 134]; 4) metastatic versus localized cancer[121, 135-138]; and 5) castration-recurrent versus androgen-sensitive [38, 121, 130, 132-134, 137, 139-160]. Select genes representing biomarkers or therapeutic targets of prostate cancer are presented in **Table 1.2**.

Genes regulated by androgens have also been identified by differential gene expression analyses of prostate cancer cells[43, 86, 161-177]. Select androgen responsive genes are presented in **Table 1.2**.

The *TMPRSS2*-ETS family of gene fusions, for example, were discovered in prostate cancer[178] using cancer outlier profile analysis to identify over-expressed genes from a subset of microarray studies of the Oncomine database[179]. Fusions between the androgen-regulated gene *TMPRSS2* and the ETS gene family of transcription factors result in the androgen regulation of transcription factors[178]. Several lines of evidence suggest that the *TMPRSS2*-ETS family of gene fusions and/or accompanying deletions are associated with clinicopathological indicators[180] such as biochemical progression following prostatectomy[181, 182], and metastatic hormone refractory prostate cancer[183, 184]. The *TMPRSS2*-ETS fusions are not specific to prostate cancer, however, as they are also found in PIN lesions. Therefore, TMPRSS2-ETS fusions are an early event in the development of prostate cancer.

1.6.2 Methods to evaluate gene expression

PCR

In 1983 one of the most influential biochemical and molecular biology techniques of the 20th century was conceived, polymerase chain reaction (PCR). The first report of PCR was published in *Science* in 1985[185], for which Kary Mullis won the 1993 Nobel Prize in Chemistry. In PCR, double-stranded (ds)DNA is melted to single stands (ss) to generate templates for synthesis. Next, primers complementary to the target sequence anneal to the template. Finally, DNA synthesis is extended along the length of the template. This three-step process is repeated for several cycles. PCR amplifies DNA exponentially when there are excess reagents. In this exponential phase there is a direct relationship between the number of amplicons at any given PCR cycle and the amount of the starting templates from the first PCR cycle. As the reaction proceeds to completion, reagent starvation causes the relationship between the concentration of DNA and the cycle number to become linear and eventually plateau. The protocol for PCR can be modified and used to quantitate gene expression. Quantitative real-time (qRT)-PCR[186-190] is very sensitive at detecting differences in transcript expression because it monitors product amplification throughout all phases of PCR, including the exponential phase. qRT-PCR methodologies differ by their detection chemistries. The two most popular methods of qRT-PCR are described here.

Sybr green is a cyanine dye that fluoresces when it binds the minor groove of dsDNA. The fluorescence is proportional to the concentration of DNA and has a broad dynamic range that spans over 6 orders of magnitude[191]. Sybr green is used for the detection of amplicons in qRT-PCR[188]. The main feature of sybr green qRT-PCR is that it can detect products amplified from any primer set and even non-specific products. In contrast to the sybr green method, TaqMan qRT-PCR[189, 190] detects only the transcripts of interest. The specificity lies in the probe, which is complementary to the transcript at a location between the forward and reverse primers. The probe is equipped with a 5' fluorophore (e.g., FAM or TET) and 3'quencher (e.g., BHQ-1). When the fluorophore and quencher are in close proximity, the quencher absorbs the fluorescence of the fluorophore, preventing detection by the qRT-PCR machine. However, when DNA is amplified, the probe is broken apart by the 5' nuclease activity of Taq DNA polymerase. When the fluorophore and quencher are released, the emission spectra from the fluorophore is no longer extinguished. Therefore, the amount of fluorescence is proportional to the number of

specific PCR products. Advantages to this method include the specificity of detecting only transcripts of interest, as well as the possibility to multiplex reactions in single wells when unique fluorophores are used.

As described, qRT-PCR is an excellent method for quantitation of transcript expression. However, for whole transcriptome expression studies, high through-put platforms like complementary (c)DNA[192] or oligonucleotide microarrays[193] are more suitable.

Miroarrays

Microarrays consist of hundreds to thousands of probes that represent individual annotated or predicted transcripts. On a cDNA array, the cDNA probes are spotted onto a solid surface such as glass. This is in contrast to oligonucleotide arrays in which the probes are synthesized directly on the chip using photolithography (e.g., Affymetrix Gene Chip)[193]. Typically, cDNA samples to be compared for relative transcript expression on cDNA arrays are labelled with different fluorophores (e.g., Cy5 or Cy3), mixed together, and co-hybridized to fixed probes on the slide. The degree of hybridization (and fluorescence) is related to the amount of transcript in the sample. Importantly, differential changes in gene expression that are identified with this method are relative. Oligonucleotide arrays are different, in that expression is normalized to standards to generate absolute expression values that may be compared across studies. One drawback to both cDNA and oligonucleotide microarrays is that they require *a priori* knowledge of the sequences of transcripts to design the probes. Therefore, the study is limited to the probes contained on the chip.

Subtractive Hybridization

Suppressive subtractive hybridization (SSH)[194] is a technique that enriches for rare differentially expressed transcripts using the principles of suppressive PCR. SSH does not require *a priori* knowledge of transcript sequences. The method consists of two steps, the normalization step that equalizes the abundance of cDNAs within the test population, and the subtraction step that eliminates the common sequences between the test and the control populations. One limitation of SSH is that the technique is not quantitative.

SAGE

Serial Analysis of Gene Expression (SAGE)[195, 196] offers advantages over the other methods described thus far for evaluating transcript levels. Compared to qRT-PCR, SAGE is more high-throughput, permitting the analysis of potentially all the polyadenylated transcripts of the transcriptome. The transcriptome is the set of all messenger RNA (mRNA) molecules, or transcripts, produced in one or many cells. In contrast to cDNA microarrays, SAGE transcript expression information is in absolute units (i.e., tag counts), so it may be compared to other experiments performed at a different time and in a different laboratory. Dissimilarly to oligonucleotide microarrays, SAGE does not require *a priori* knowledge of transcript sequence information, and is not limited to a certain number of transcripts. Finally, unlike SSH, SAGE provides information regarding the degree of differential expression between transcripts.

SAGE is based on the concept that a short nucleotide sequence called a tag is almost always sufficient to map to the transcriptome. Furthermore, the number of times a tag is observed is related to the expression level of the transcript it represents. Briefly, the steps involved in the method of SAGE are: 1) restriction enzyme digestion of cDNA by the Nla III anchoring enzyme to generate CATG overhangs; 2) separation of the sample into two equal parts, and ligation of cDNA to unique adapters that bind complementary to the Nla III cut-site; 3) restriction enzyme digestion of cDNA by the Bsm I[195] (or Mme I in LongSAGE)[196] tagging enzyme that recognizes and binds to a sequence in the adapters and cuts the cDNA 14 (i.e., in shortSAGE)[195] or 21 (i.e., in LongSAGE)[196] basepairs downstream to create tags; 4) the two unique adapter-bound tag species are mixed and ligated together to create ditags; 5) ditags are amplified by PCR with primers specific to the unique adapters; 6) ditags are released from the adapters by restriction enzyme digestion with Nla III anchoring enzyme; 7) ditags are concatenated into a long chain for cloning into a bacterial vector and propagation; 8) clones are sequenced; 9) tags are counted; and lastly 10) the tag sequences are mapped to the transcriptome to reveal their identity. The two methods of SAGE mentioned above are called short[195] and long[196] in reference to the length of the tag that is generated. The longer the tag length, the greater the probability the tag will map unambiguously to the transcriptome. This is an advantage over shorter tags, because ambiguous mappings are non-informative.

1.7 RESEARCH HYPOTHESIS AND OBJECTIVES

1.7.1 Background summary

Prostate cancer is the most common malignancy in Canadian men, and the third leading cause of cancer death. Androgens are important for the development and maintenance of the prostate gland and continue to play a central role in all stages of prostate cancer. The complete spectrum of genomic targets of androgen signalling have yet to be elucidated and will aid in the understanding of the mechanisms involved in prostate biology and pathology. The androgen-regulated gene PSA, is used as a biomarker for the screening and monitoring of prostate cancer. PSA testing has resulted in the detection and over-treatment of clinically insignificant disease. New prognostic markers are urgently needed to delineate which cancers will progress to incurable late-stage cancer, known as castration-recurrent prostate cancer. Gene expression signatures of prognostic markers may be enriched in castration-recurrence, thereby providing justification to search for them in this context. The mechanisms underlying progression of prostate cancer to castration-recurrence are unknown. Gene expression profiling will yield support for or against the proposed models of castration-recurrent prostate cancer.

1.7.2 Hypothesis and objectives

The over-arching hypothesis is that the application of LongSAGE will catalogue gene expression signatures that are indicative of the mechanisms underlying the growth and progression of prostate cancer, and reveal potential biomarkers of prostate cancer. The objectives of this thesis were to determine the regulation of the transcriptome by the androgen-axis in prostate cancer, identify the gene expression profile associated with *in vivo* progression of prostate cancer to castration-recurrence, and delineate the expression characteristics of novel biomarkers of prostate cancer. These objectives were met with the following **Specific aims**:

1. Utilize LongSAGE to identify transcripts differentially expressed in LNCaP human prostate cancer cells maintained *in vitro* and treated with, or without androgen. Use qRT-PCR to validate the expression trends in the *in vivo* LNCaP Hollow Fibre model following androgen-deprivation. This aim will reveal genomic targets of the androgen signalling axis (**Chapter II**).

2. Employ LongSAGE to identify transcripts differentially expressed in the *in vivo* LNCaP Hollow Fibre model during hormonal progression to castration-recurrent prostate cancer. This aim will reveal gene expression signatures representative of castration-recurrence, and substantiate proposed models of castration-recurrent prostate cancer (**Chapter III**).

3. Evaluate gene expression profiles of candidate biomarkers of castration-recurrent prostate cancer using qRT-PCR to determine their regulation by androgen, their specificity to the prostate and cancer, and levels of expression in clinical samples obtained by prostatectomy. This work will characterize candidate genes for their potential to be biomarkers of prostate cancer (**Chapter IV**).

Table 1.1 Definition of TNM

Primary Tumor (T)	
Clinical	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically inapparent tumor neither palpable nor visible by imaging
T1a	Tumor incidental histologic finding in 5% or less of tissue resected
T1b	Tumor incidental histologic finding in more than 5% of tissue resected
T1c	Tumor identified by needle biopsy (e.g., because of elevated PSA)
T2	Tumor confined within prostate*
T2a	Tumor involves one-half of one lobe or less
T2b	Tumor involves more than one-half of one lobe but not both lobes
T2c	Tumor involves both lobes
T3	Tumor extends through the prostate capsule**
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles: bladder neck, external sphinchter, rectum, levator, muscles, and/or pelvic wall

*Note: Tumor found in one or both lobes by needle biopsy, but not palpable or reliably visible by imaging, is classified as T1c.
**Note: Invasion into the prostatic apex or into (but not beyond) the prostatic capsule is classified not as T3 but as T2.

Pathologic (pT)	
pT2*	Organ confined
pT2a	Unilateral, involving one-half of one lobe or less
pT2b	Unilateral, involving more than one-half of one lobe but not both lobes
pT2c	Bilateral disease
pT3	Extraprostatic extension
pT3a	Extraprostatic extension**
pT3b	Seminal vesicle invasion
pT4	Invasion of bladder, rectum

*Note: There is no pathologic T1 classification.
**Note: Positive surgical margin should be indicated by an R1 descriptor (residual microscropic disease).

Regional Lymph Nodes (N)	
Clinical	
NX	Regional lymph nodes were not assessed
N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node(s)
Pathologic	
pNX	Regional nodes not sampled
pN0	No positive regional nodes
pN1	Metastases in regional node(s)

Distant Metastasis (M)*	
MX	Distant metastasis cannot be assessed (not evaluated by any modality)
M0	No distant metastasis
M1	Distant metastasis
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

*Note: When more than one site of metastasis is present, the most advanced category is used. pM1c is most advanced.

Abbreviations: TNM, tumor-node-metastasis; PSA, prostate-specific antigen.

Table 1.2 Androgen-regulated genes and biomarkers or therapeutic targets of prostate cancer identified by gene expression analyses

Gene	Classification	Reference(s)
	Therapeutic Targets	
AKR1C3	Gene expression signature is different between castration-recurrent and androgen-stimulated PC	142
AR	"	142, 144, 159
FGFR1	"	152
IL6	"	143
MMP9	"	142
NKX3.1	"	142, 144, 155, 159
NR4A1	"	144
PIK3CD	"	140
PPP3CA	"	140, 153
TMEFF2	"	153, 155
GSK-3 beta	Gene expression signature is different between metastatic and localized PC	121
NR4A1	"	137
CTSD	Gene expression signature is different between early and late stage PC	134
HSD17B4	Gene expression signature is different between high and low Gleason grade	131
HSD17B4	Gene expression signature is different between tumour and normal	114, 120, 127
	Biomarkers	
KLK3	Prognostic: predicts response to therapy	49
Hepsin	Prognostic: predicts relapse	113, 124
PIM-1	"	124
AMACR	Prognostic: predicts progression to castration-recurrent PC	121, 139, 155
AZGP1	"	133
Chromogranin A	"	130, 132
MUC1	"	133, 156
TMPRSS2-ETS	"	178, 181, 183, 184
TRPM8	"	142, 144, 150
EZH2	Prognostic: predicts progression to metastatic PC	121, 135
KLK3	Monitoring: is indicative of response to therapy	49
PSMA	Monitoring: is indicative of metastases	138
AMACR	Diagnostic: differentiates between tumour and normal	114, 116, 117, 119, 127, 131, 148
GSTP-1	"	128, 129
KLK3	"	49
PCA3	"	123
	Androgen-regulated genes	
ACPP	"	43
B2M	"	171
CAMK2	"	166
FKBP5	"	163, 170
KLK3	"	43
NKX3.1	"	174
PDEF	"	175
PMEPA1	"	176
PSMA	"	177
TMPRSS2	"	173

" = same as above

PC = prostate cancer

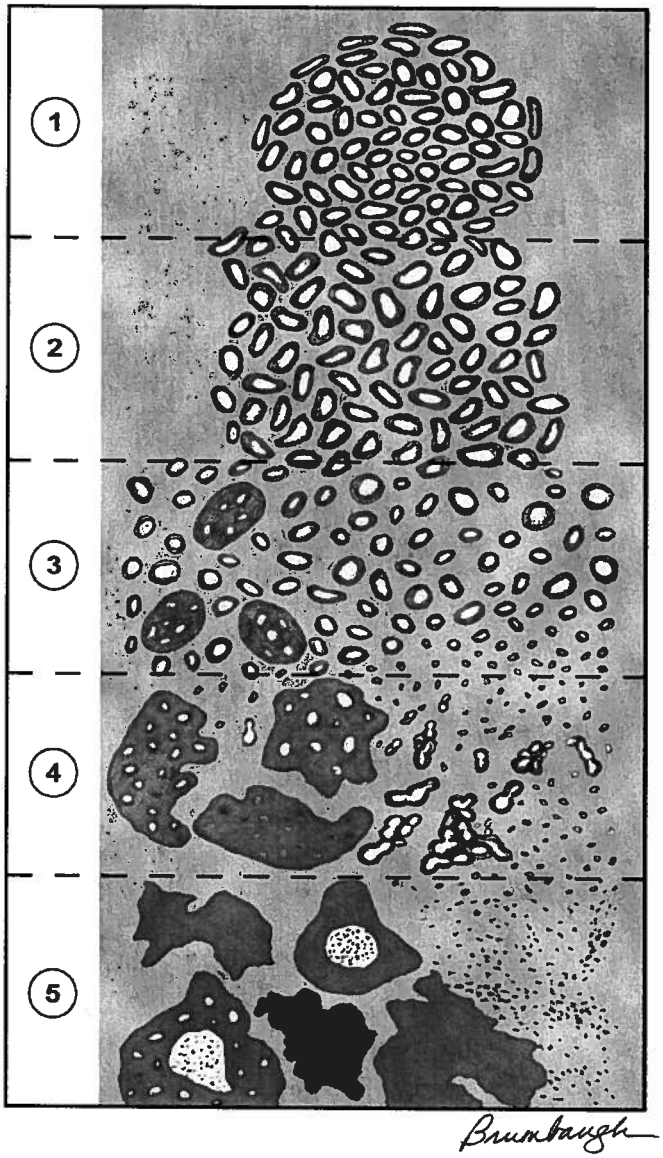

Figure 1.1 Modified Gleason grading system. Pattern 1: Circumscribed nodule of closely packed but separate, uniform, rounded to oval, medium-sized acini (larger glands than pattern 3). Pattern 2: Like pattern 1, fairly circumscribed, yet at the edge of the tumor nodule, there may be minimal infiltration. Glands are more loosely arranged and not quite as unifrom as Gleason pattern 1. Pattern 3: Discrete glandular units; typically smaller glands than seen in Gleason pattern 1 or 2; infiltrates in and among nonneoplastic prostate acini; marked variation in size and shape; smoothly circumscribed small cribriform nodules of tumor. Pattern 4: Fused microacinar glands; ill-defined glands with poorly formed glandular lumina; large cribriform glands; cribriform glands with an irregular border; hypernephromatoid. Pattern 5: Essentially no glandular differentiation, composed of solid sheets, cords, or single cells; comedocarcinoma with central necrosis surrounded by papillary, cribriform, or solid masses.
Used with the permission of Lippincott Williams & Wilkins (Wolters Kluwer Health).
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CHAPTER II

REGULATION OF THE TRANSCRIPTOME BY THE ANDROGEN-AXIS IN PROSTATE CANCER*

2.1 INTRODUCTION

Androgens mediate their effect through the androgen receptor (AR) and together they play integral roles in the development and maintenance of the prostate. In the absence of a functional androgen-axis during development, the prostate will fail to form[1]. The size of the prostate increases with the elevation of levels of androgens in males during puberty[2]. Androgens promote proliferation, differentiation, and survival of prostate cells[1] and are also associated with prostate carcinogenesis. Men that have used excess androgens in the form of anabolic steroids have a higher incidence of prostate cancer[3-5]. Association of prostate cancer with levels of androgens has also been reported in rodents[6, 7]. Reduction of androgen in humans or dogs before puberty by castration is associated with decreased incidence of prostate cancer[8, 9]. Castration of adult males causes apoptosis of prostatic epithelium, involution and reduction of the prostate[10-12]. Thus the prostate gland is an androgen-dependent organ where androgens are the predominant mitogenic stimulus[13]. The dependency of the prostate epithelium on androgens provides the underlying rationale for treating prostate cancer with chemical or surgical castration (androgen-deprivation)[14].

The AR is a ligand-activated transcription factor[15] that regulates transcription of genes that contain androgen response elements (AREs) in the upstream or downstream regulatory regions of the promoter and/or enhancer. Kallikrein 3 (*KLK3*) is an example of a gene that contains numerous functional AREs that the AR interacts with to increase transcription in response to androgens[16-19]. KLK3, also known as prostate-specific antigen (PSA), is the main tumour marker for prostate cancer and has been used clinically for 15 years[20]. Serum levels of PSA correlate with tumour volume[21]. However, as a screening and monitoring tool for prostate cancer, serum PSA levels are subject to false positives and false negatives[20].

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* A version of this chapter will be submitted for publication. Romanuik, TL., Wang, G., Holt, RA., Jones, SJM., Marra, MA., Sadar, MD. Regulation of the transcriptome by the androgen-axis in prostate cancer. *In preparation.*

Identification of the genes that change in expression in response to androgen in prostate cells is essential for the understanding of androgen-dependency of the normal prostate and the proliferation, survival, and hormonal progression of prostate cancer. Here, we apply Long Serial Analysis of Gene Expression (LongSAGE)[22] to create transcript libraries of prostate cancer cells maintained in the presence or absence of androgen. We describe 24 genes never before identified or validated to alter expression in response to androgen treatment. These genes were: *ARL6IP5, BLVRB, C19orf48, C1orf122, C6orf66, CAMK2N1, CCNI, DERA, ERRFI1, GLUL, GOLPH3, HM13, HSP90B1, MANEA, NANS, NIPSNAP3A, SLC41A1, SOD1, SVIP, TAOK3, TCP1, TMEM66, USP33,* and *VTA1.* Statistically significant changes in expression of *ARL6IP5, CAMK2N1, ERRFI1, HSP90B1,* and *TAOK3* in response to reduced levels of circulating androgens were measured using *in vivo* samples.

2.2 MATERIALS AND METHODS

2.2.1 Cell culture

LNCaP human prostate cancer cells (American Type Culture Collection, Bethesda, MD, USA) were maintained in RPMI-1640 media (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 10% v/v fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 units/ mL penicillin and 100 units/mL streptomycin (antibiotics; Invitrogen, Burlington, ON, Canada). DU145 and PC-3 human prostate cancer cells were maintained in DMEM (Stem Cell Technologies) supplemented with 10% v/v FBS and 5% v/v FBS, respectively with antibiotics. All cells were maintained at 37°C with 5% CO_2.

2.2.2 Long serial analysis of gene expression

RNA sample generation

1 x 10^6 LNCaP cells were seeded in 10 cm-diameter dishes. The next day, cells were serum-starved for 48 hours and then treated for 16 hours with 10 nM synthetic androgen R1881 (also known as methyltrienolone; PerkinElmer, Woodbridge, ON, Canada), or solvent (vehicle) control, ethanol (final concentration 2.85 x 10^{-4} %). Total RNA was extracted using TRIZOL Reagent (Invitrogen) following the manufacturer's instructions. RNA quality and quantity were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada) and RNA 6000 Nano LabChip kit (Caliper Technologies, Hopkinton, MA, USA).

LongSAGE library production

LongSAGE[22] libraries were constructed with 5 μg of total RNA using the Invitrogen I-SAGE Long kit and protocol with alterations as previously published[23]. Briefly, double-stranded cDNA was synthesized from total RNA and digested with Nla III. The sample was split in half and linkers type I and II were added and ligated to Nla III overhangs. An Mme I digestion resulted in 17-21 base-pair (bp) LongSAGE tags. The tags with unique linkers were combined and ligated together to form ditags. Ditags (131 bp) were amplified with primers designed to recognize sequences within linkers type I and II using PCR. This scale-up PCR was performed in 48 wells of a 96 well plate (50 μL/well) using a $1/20^{th}$ dilution of template cDNA and 25 and 27 cycles of PCR (R1881 and vehicle LongSAGE library, respectively). Following an Nla III digestion to remove the linkers, the 36 bp ditags were concatenized. Concatemers sized 1300-1700 bp were digested with Nla III (1 minute) to increase the efficiency of cloning into pZErO-1 vectors. Cloned concatemers were transformed into One Shot TOP10 Electrocompetent *Escherichia coli* and colonies were picked with the Q-Pix robot (Genetix) and cultured in 2x Yeast-Tryptone media with 50 μg/mL zeocin and 7.5% (v/v) glycerol.

Sequencing

Glycerol stocks of transformed bacteria were used to inoculate larger cultures for alkaline lysis plasmid preparation[24]. Plasmid preparations were separated by agarose gel electrophoresis and visualized by ultraviolet light and sybr green. $1/24^{th}$ BigDye v3.1 terminator cycle sequencing reactions were performed with tetrad thermal cyclers (BioRad, Waltham, MA, USA) and visualized with capillary DNA sequencers, models 3700 and 3730xl (Applied Biosystems, Foster City, CA, USA). Each library was sequenced to a depth of ~ 100,000 LongSAGE tags. Flanking vector sequences were removed and the LongSAGE tags were extracted from each sequence read. On average, 34 and 38 LongSAGE tags were sequenced in each read (R1881 and vehicle libraries, respectively). Sequence data were filtered for non-recombinant clones.

Gene expression analysis

LongSAGE expression data was analyzed with DiscoverySpace 3.2.4 and 4.01 software[25] (http://www.bcgsc.ca/bioinfo/software/discoveryspace/). Duplicate ditags (identical copies of a ditag) and singletons (tags counted only once) were retained for analysis. Sequence data were

filtered for bad tags (tags with one N-base call) and linker-derived tags (artifact tags). Only LongSAGE tags with a sequence quality factor (QF) greater than 95% were included in analysis[23]. Where indicated, a clustering algorithm was used to amalgamate 1-off tags (tags one bp incorrect from a complete map to a transcript) with likely 'parent' tags to improve the mapping capability of LongSAGE tags by apparently reducing PCR/sequencing errors[23]. This clustering algorithm altered the number of tag types (i.e., species) without changing the total number of tags. In instances where clustering was used, the 95% QF cutoff was not. To filter data for candidate transcript validation, a p-value cutoff ($p \leq 0.001$) was employed according to the Audic and Claverie test statistic[26]. The Audic and Claverie statistical method was used to identify differentially expressed tags between LongSAGE libraries because the method takes into account the sizes of the libraries and tag counts. LongSAGE tags that mapped ambiguously to more than one gene, and tags that differed by less than 2-fold were excluded from the candidate list. LongSAGE tags were mapped to reference sequence (RefSeq; May 30th, 2005) and Ensembl Gene (v31.35d), unless otherwise stated.

2.2.3 Quantitative real-time polymerase chain reaction

qRT-PCR was performed on TRIZOL-extracted RNA from LNCaP (10% serum or ± R1881), DU145 (10% serum) and PC-3 (5% serum) cells maintained *in vitro*, and LNCaP cells maintained in the *in vivo* Hollow Fibre model[27] (see below). Contaminating genomic DNA was removed from *in vitro* RNA samples using DNA-free or TURBO DNA-free (Ambion, Austin, TX, USA). Input RNA (1 μg) was reverse transcribed with SuperScript III First Strand Synthesis kit (Invitrogen). A 10 μL qRT-PCR reaction included 1 μl of template cDNA (0.1 μL for limited LNCaP Hollow Fibre samples), 1x Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) and 0.3 μM each of forward and reverse intron-spanning primers that produce products between 85-115 bp in size (see **Table 2.1** for primer sequences). qRT-PCR reactions were cycled as follows in a 7900HT Sequence Detection System (Applied Biosystems): 50 °C for 2 min, 95 °C for 2 min, (95 °C for 0.5 min, 55-56 °C for 0.3-0.5 min, and 72 °C for 0.5 min) for 40-45 cycles, 95 °C for 0.25 min, 60 °C for 0.25 min, and 95 °C for 0.25 min. All qRT-PCR reactions were performed in technical triplicates for each of at least three biological replicates. cDNAs (from different conditions) and genes [target and reference (glyceraldehyde-3-phosphate, *GAPDH*)] to be directly compared were assayed in the same instrument run. No-template reactions (negative controls) were run for each gene to ensure that DNA had not contaminated

the qRT-PCR reactions. Only qRT-PCR data with single-peak dissociation curves were included in this analysis. Efficiency checks were performed for each primer pair in each cell line. PCR products were sequenced to verify the identity of quantified transcripts. The two-tailed, two-sample Student's T-tests were performed to identify significant differences in transcript expression. The F-test was used to identify unequal variance among samples to be compared.

2.2.4 LNCaP Hollow Fibre model

Animals

Five-week-old male athymic BALB/c Nude mice were obtained from Taconic Farms (Hudson, NY, United States of America) and kept in the British Columbia Cancer Research Centre (Vancouver, BC, Canada). Mice were maintained on a Harlan/Teklad irradiated diet with a constant supply of autoclaved water and housed in cages (three animals/cage) at 21°C± 3°C with light/dark cycling (light between 6 AM and 6 PM). All animal experiments were performed according to a protocol approved by the Committee on Animal Care of the University of British Columbia.

Hollow fibre model

Polyvinylidene difluoride hollow fibres (M_r 500,000 molecular weight cutoff; 1-mm internal diameter; Spectrum Laboratories, Rancho Dominguez, CA, USA) were prepared and implanted as previously described[27]. Briefly, LNCaP human prostate cancer cells (3×10^7 cells) at passage 47 (provided by Dr. L.W.K. Chung at the Emory University School of Medicine, Atlanta, GA, USA) were injected into hollow fibres. The fibres were sealed and subcutaneously (s.c.) implanted into mice. Seven days post fibre implantation (day zero), mice were either castrated or left intact as controls. Blood was drawn via the tail vein each week to measure serum KLK3 levels to monitor the response to castration. Serum KLK3 levels were determined by enzymatic immunoassay kit (Abbott Laboratories, Abbott Park, IL, USA). Bundles of fibres were removed at day zero (Pre-Cx; four fibres) and day 10 (Cx; four fibres). Total RNA was isolated immediately from cells harvested from the fibres. Compromised fibres that were contaminated with mouse cells, as indicated by an infiltration of red blood cells that was determined by visual inspection, were not used in this study.

2.3 RESULTS

2.3.1 Summary of LongSAGE libraries

LongSAGE was employed to obtain quantitative gene expression profiles of human prostate cancer cells treated with or without synthetic androgen R1881. LNCaP human prostate cancer cells were chosen as the model cell line for evaluating androgen signalling because they express a functional AR, respond to androgens, they can be grown *in vitro* as a monolayer or *in vivo* as a xenograft or in the Hollow Fiber model[27-29]. LNCaP cells have been used extensively in prostate cancer research. The time of 16 hours for treatment and concentration of R1881 (10 nM) are optimal for induction of *KLK3*[30].

LongSAGE libraries were sequenced to a total of 121,760 (R1881) and 103,391 (vehicle) tags (**Table 2.2**). The libraries were filtered on several levels to leave only useful tags for analysis. First, bad tags were removed if they contained at least one N-base call in the LongSAGE tag sequence. Notably, when bad tags were filtered the percentages of duplicate ditags in the R1881 and vehicle LongSAGE libraries were 6% and 5%, respectively. Early SAGE studies suggest duplicate ditags likely represent polymerase chain reaction (PCR) artifacts due to the low probability the same two tags will ligate together to form ditags[31]. However, with deep LongSAGE library sequencing and highly expressed transcripts, this random probability is greater[32]. A recent study[33] suggests that discarding duplicate ditags in LongSAGE analysis may introduce bias affecting the fold differences in tag expression between libraries for all tags observed at a frequency >(113-224)/100,000. Therefore, to avoid introducing this bias we opted to retain duplicate ditags. PHRED software was used to call bases for the sequencing of the LongSAGE tags[34, 35]. PHRED has a small, but significant error rate in base-calls. To ascertain which tags potentially contained these erroneous base-calls, we calculated a tag sequence quality factor (QF) and probability[23]. The second line of filtering removed LongSAGE tags with probabilities less than 0.95 (QF < 95%). Linkers of known sequence were introduced into SAGE libraries as primers for amplifying ditags prior to concatenation[31]. These linker sequences were designed so they do not map to the human genome. At a low frequency, linkers ligate to themselves creating linker-derived tags (LDTs). These LDTs do not represent transcripts and are removed from the LongSAGE libraries. After filtering, there were 97,981 total useful tags representing 23,828 tag sequences in the R1881 LongSAGE library, and 85,861 total useful tags representing 24,592 tag sequences in the vehicle LongSAGE library. Due to redundancy in the

expressed sequences, the combined number of useful tag types in the R1881 and vehicle LongSAGE libraries was 38,574. The remainder of the data analysis in this manuscript was carried out using this filtered data.

2.3.2 Tag frequency and transcript abundance

Tag frequency spanned over three orders of magnitude corresponding to transcript abundance of 5 to 8,746 copies per cell (based on minimum and maximum observed tag counts of 1 and 1714; see **Table 2.3** legend for explanation of calculations). The distribution of LongSAGE tag frequencies per 100,000 tags revealed the majority (64 and 67%) of tag types in each LongSAGE library (R1881 and vehicle, respectively) were singletons (tags counted only once). This result was consistent with other published SAGE libraries reporting 66% singletons[36]. Singletons can represent very low abundance transcripts ($\leq$ 5 transcript copies per cell) or PCR/sequencing errors. Estimates indicate that less than 17% of LongSAGE tags in a library contain PCR/sequencing errors[37]. Coincidently, 17% of the total tags in the R1881 and vehicle LongSAGE libraries roughly equal the number of singletons in each LongSAGE library (**Table 2.3**). Although initial estimates suggest 6.8-10% of shortSAGE tags contain PCR/sequencing errors, more recent experimental evidence suggests the actual error rate is much lower ($\leq$ 2%)[38]. This implies that an error rate of 17% may also be an overestimate for LongSAGE tags. Tag types counted 2-4 times per 100,000 tags (10-20 transcript copies per cell) and 5-9 times per 100,000 tags (25-45 transcript copies per cell) were the second and third most common groups of tag types, respectively. Generally, high frequency tags were less common. The majority of total tags in each LongSAGE library were derived from a few tag types detected between 10-99 times per 100,000 tags (50-495 transcript copies per cell).

2.3.3 Mapping distribution of LongSAGE tags

When mapped tags (v38 Ensembl) were clustered to amalgamate 1-off tags (see Materials and Methods, Gene Expression Analysis for a description) and tags that mapped ambiguously were removed, the tag types in the R1881 and vehicle LongSAGE libraries represented 7,484 genes and 7,441 genes, respectively (**Table 2.4**). Tag types that mapped ambiguously constituted 13% (R1881 and vehicle), while 36% (R1881) and 35% (vehicle) of tag types did not map to the genome (**Table 2.4**). Due to the fact that these tags were clustered, the majority of the tags that

did not map to the genome probably represent true unannotated transcripts rather than PCR/sequencing errors. Approximately 28% of tags in each LongSAGE library mapped to the opposite strand of known genes. These LongSAGE tags either represent transcription from previously undescribed coding regions or true antisense transcripts. Each LongSAGE library contained tags representing transcripts from 32% of the genes in the Ensembl gene database. This percentage is indicative of the depth of coverage of the transcriptome achieved with LongSAGE. Alternatively, this percentage indicates that one third of known Ensembl genes were expressed in LNCaP cells under these experimental conditions. This percentage is substantial when considering tag types from the Mouse Atlas Project (8.55 million total LongSAGE tags generated from 72 libraries of mouse development) mapped to 57% of the Ensembl transcript database[23]. Approximately 63% (R1881) and 61% (vehicle) of the genes that mapped to Ensembl's database were associated with more than one tag type to suggest that most gene expression was represented by transcript variants which is consistent with previous observations[23]. When the mapped LongSAGE tags (Reference Sequence -RefSeq- May 18, 2006) were clustered to amalgamate 1-off tags and tags that mapped ambiguously were removed, 53% of tags mapped solely to known exons, 9% solely to known introns (novel transcript variants), and 38% to intergenic regions (novel genes or transcript variants).

The two most abundant tag types in the LongSAGE libraries were shared by both libraries. The first highly abundant LongSAGE tag mapped to human mitochondrial NADH ubiquinone oxidoreductase chain 4. The protein product of this gene transfers electrons from NADH to ubiquinone to generate adenosine triphosphate as metabolic energy. Using the Ensembl database, the second most abundant LongSAGE tag mapped to a non-coding gene of human mitochondria. In contrast to the higher abundance classes, the lower abundance classes were enriched for LongSAGE tags that mapped to genes with functions in regulating transcription (**Table 2.3**). This is particularly significant because the percentages of LongSAGE tags that mapped to the genome in the lower abundance class were reduced compared to the higher abundance classes (**Table 2.3**). Together this implies that the number of tags that map to genes with a function in transcription may be underestimated, as low abundance tags may be underrepresented.

2.3.4 Differential gene expression

Venn analysis identified that 36% and 38% of tag types were exclusive to the R1881 and vehicle LongSAGE libraries, respectively (**Figure 2.1**). The unique expression of tag types indicates differential expression depending upon androgen stimulation. Unfortunately, the biological relevance of this differential expression was complicated by the fact that 85% (R1881) and 88% (vehicle) of these exclusive LongSAGE tags were singletons. Consistent with our observation that low abundance tags did not map as readily to the genome, exclusive tags also did not map as readily as tags shared between both libraries. Only 17% and 15% of tags exclusive to R1881 and vehicle LongSAGE libraries, respectively, mapped unambiguously sense to RefSeq, in contrast to 39% of shared tags.

A scatter plot illustrates observed tag counts in LongSAGE libraries relative to the confidence intervals (CIs; 95%, 99%, and 99.9%) of respective p-values ($p \leq 0.05$, 0.01, and 0.001) by Audic and Claverie statistics[26] (**Figure 2.2**). A significant number (891) of tags were differentially expressed ($p \leq 0.05$) between the two LongSAGE libraries (**Figure 2.2** and **Table 2.5**) even though these 891 tags represented a minority (2%) of all tag types. LongSAGE tags statistically ($p \leq 0.001$) differentially represented between the libraries were enriched in the higher abundance classes compared to the lower abundance classes (**Table 2.3**). Additionally, 90% of the LongSAGE tags were statistically ($p \leq 0.001$) differentially represented between the libraries with $\geq$ 2-fold differences, compared to only 17% of tags with p-values greater than 0.001 ($p > 0.001$).

A stringent p-value cutoff ($p \leq 0.001$) was employed prior to validation of changes in expression of a gene in response to androgen. LongSAGE tags that were differentially expressed, but mapped ambiguously to more than one gene, and/or differed by less than 2-fold between the treatment groups, were excluded from analysis. Application of these criteria reduced the LongSAGE tags from 131 to 93. These 93 tags represented 87 genes. Analysis of differentially expressed LongSAGE tags revealed that 54 LongSAGE tags that mapped to 52 genes were previously known to change in expression in the direction observed in response to androgen in prostate cancer cells. Of these, the expression of 41 genes increased as expected, including the well-known androgen-regulated gene, *KLK3* (**Table 2.6**). The expression of 11 genes decreased

in response to androgen, and were consistent with previous reports (**Table 2.7**). Genes previously not reported to alter expression in response to androgen in prostate cancer cells were represented by 39 LongSAGE tags. These tags represented the expression of 20 genes that were increased, excluding non-coding and intergenic regions, (**Table 2.8**), and expression of 15 genes that were decreased (**Table 2.9**) in response to androgen. The 93 tags were represented by 87 genes because one tag did not map (**Table 2.8**) and two tags mapped to intergenic regions of human mitochondrial genome (**Tables 2.8** and **2.9**). Three genes were represented twice in the tables (*CAMK2N1*, *PPAP2A,* and *SORD*). One gene, *KRT8*, was categorized in both the known and not previously known categories due to the sense of the mapping (**Tables 2.6** and **2.9**).

2.3.5 Validation of changes in gene expression in response to androgen

Quantitative real time-polymerase chain reaction (qRT-PCR) was used to validate changes in gene expression in response to androgen of 39 (13 known; 26 novel) of the 87 total genes identified by LongSAGE. Of the 35 genes previously not reported to change expression in response to androgens in prostate cancer cells, only 26 were quantified by qRT-PCR, because technical limitations and gaps in the transcriptome databases prevented the analysis of 9 genes. That is, specific qRT-PCR primers could not be designed due to repetition in the genome, or because the tag mapped to an unannotated transcript variant. There were 24 of the 26 (92%) novel genes that displayed statistically significant differential expression in response to androgen as measured by qRT-PCR (**Figure 2.3A**). *BLVRB, C19orf48, C1orf122, ERRFI1, GLUL, GOLPH3, HM13, HSP90B1, NANS, SLC41A1, TAOK3, TCP1, TMEM66,* and *USP33* all increased levels of expression in response to androgen, while *ARL6IP5, C6orf66, CAMK2N1, CCNI, DERA, MANEA, NIPSNAP3A, SOD1, SVIP,* and *VTA1* decreased in response to androgen (**Figure 2.3A**). Under the experimental conditions and primers used, we did not measure statistically significant changes in expression of *PRNPIP* and *CAPNS1*. A false discovery rate (FDR)[39] of 29% was expected of the LongSAGE data based on the Audic and Claverie p-value $\leq$ 0.001. This FDR represents the anticipated percentage of type I errors (i.e., false positives). We observed only 2/26 (8%) false positives, suggesting that the other filter parameters (e.g., $\geq$ 2-fold difference in expression level) may have the increased the chances of validation by qRT-PCR. Moreover, the expression trends for all 13 genes known to change expression in response to androgen in prostate cancer cells correlated between the LongSAGE and qRT-PCR data. *ADAMTS1, CENPN, CREB3L4, FKBP5, KLK3, LRIG1, NCAPD3, PAK1IP1,* and *RHOU* all

increased levels of expression in response to androgen while *CXCR7, NTS, PRKACB,* and *ST7* decreased in response to androgen (**Figure 2.3B**).

2.3.6 Cell-type specificity of gene expression

To determine if expression of candidate genes was unique to LNCaP cells, we assayed for constitutive levels of expression of 18 known and novel candidate genes in prostate cancer cell lines DU145[40] and PC-3[41] using qRT-PCR (**Figure 2.4**). Genes chosen included those that both increased (*ADAMTS1, CAPNS1, CENPN, CREB3L4, ERRFI1, FKBP5, HSP90B1, KLK3, LRIG1, NCAPD3, PAK1IP1,* and *TAOK3)* and decreased expression in response to androgen (*ARL6IP5, CAMK2N1, CCNI, CXCR7, PRKACB* and *ST7*). No obvious trends were observed depending on whether expression of the genes increased, or decreased, in response to androgen. All genes tested, except *ERRFI1*, were expressed at a lower level in PC-3 and DU145 cells relative to LNCaP cells. This suggests that the majority of genes that alter levels of expression in response to androgen were enriched in LNCaP cells relative to PC-3 and DU145 cells. These data are consistent with both DU145 and PC3 cells being androgen-insensitive and lacking a functional AR[40, 41].

2.3.7 In vivo changes in gene expression in response to androgen-deprivation

The LNCaP Hollow Fibre model combined with qRT-PCR was employed to capture *in vivo* gene expression representative of physiological levels and castrated levels of androgen (**Figure 2.5**). We expected that the genes that had increased levels of expression *in vitro* in response to androgens, would decrease expression *in vivo* in response to castration (androgen-deprivation). Conversely, we expected that the genes that had decreased levels of expression *in vitro* in response to androgens, would increase expression *in vivo* in response to castration. These *in vivo* results would be consistent with androgen-responsiveness of the candidate genes. Of the candidate genes examined, 13 of 16 genes showed significant changes in gene expression in response to androgen-deprivation (**Figure 2.5**). As anticipated, expression of *ARL6IP5, CAMK2N1, CXCR7,* and *ST7* increased, while *CENPN, CREB3L4, ERRFI1, FKBP5, KLK3, LRIG1, NCAPD3, PAK1IP1,* and *TAOK3* decreased levels of expression in response to castration. No significant changes in gene expression *in vivo* was measured for *ADAMTS1, HSP90B1, or PRKACB*, suggesting that *in vivo*, other factors may influence their expression.

Alternatively, the expression kinetics of each specific gene and half-life of its transcript may vary considerably. The time of harvesting samples and measuring changes in expression of genes in response to androgen-deprivation was at 10 days *in vivo* compared to 16 hr *in vitro* in response to addition of androgens (10 nM R1881). Different levels of androgen may also have profound effects on proliferation and differentiation. Physiological levels of androgen in male Nude mice may be considerably lower than the levels used *in vitro*. Androgen at 10 nM inhibits proliferation of LNCaP cells *in vitro* while 0.1 nM is optimal for proliferation[42].

2.4 DISCUSSION

Androgens are essential for the growth, development and maintenance of the prostate. The importance of androgens and AR continues throughout most stages of prostate cancer and provides a therapeutic pathway for clinical intervention. Androgen-deprivation and drugs that block the transcriptional activity of the AR provide treatments for locally advanced and metastatic prostate cancer. Unfortunately, these forms of therapy are not curative and the malignancy will progress to a stage that no longer responds to androgen-deprivation therapies. Identification of genes whose expression changes in response to androgen and androgen-deprivation in prostate cancer will reveal the genes and pathways involved in the proliferation, survival and potentially hormonal progression of this disease.

Here, we created LongSAGE libraries to obtain quantitative gene expression profiles of LNCaP human prostate cancer cells treated with, or without, androgen and revealed the following: 1) 33,385 tag types in the R1881 LongSAGE library and 31,764 tag types in the vehicle LongSAGE library; 2) the majority (64% to 67%) of tag types in each LongSAGE library were singletons which may represent very low abundance transcripts ($\leq$ 5 transcript copies per cell); 3); when mapped tags were clustered and ambiguous mappings were removed, the tag types in the R1881 and vehicle LongSAGE libraries represented 7,484 genes and 7,441 genes, respectively; 4) 53% of tags mapped solely to known exons, 9% solely to known introns (novel transcript variants), and 38% to intergenic regions (novel genes or transcript variants); 5) the most highly abundant LongSAGE tag mapped to human mitochondrial NADH ubiquinone oxidoreductase chain 4 involved in metabolic energy; 6) the lower abundance classes were enriched for genes with functions in regulating transcription; 7) 87 genes were differentially

expressed by two-fold ($p \leq 0.001$) in response to androgen representing 0.34% of the total tag types (131 differentially expressed tag types / 38,574 total tag types); 8) novel androgen regulated genes (direct or indirect) identified and validated were *ARL6IP5, BLVRB, C19orf48, C1orf122, C6orf66, CAMK2N1, CCNI, DERA, ERRFI1, GLUL, GOLPH3, HM13, HSP90B1, MANEA, NANS, NIPSNAP3A, SLC41A1, SOD1, SVIP, TAOK3, TCP1, TMEM66, USP33,* and *VTA1*; 9) expression of *ADAMTS1, ARL6IP5, CAMK2N1, CAPNS1, CENPN, CREB3L4, CCNI, CXCR7, FKBP5, HSP90B1, KLK3, LRIG1, NCAPD3, PAK1IP1, PRKACB, ST7,* and *TAOK3* was increased in LNCaP cells compared to prostate cancer cells lacking a functional AR; and 10) significant differences in levels of expression of *ARL6IP5, CAMK2N1, CENPN, CREB3L4, CXCR7, ERRFI1, FKBP5, KLK3, LRIG1, NCAPD3, PAK1IP1, ST7,* and *TAOK3* were measured *in vivo* in response to androgen-deprivation.

We identified 87 genes with statistical significant differences in levels of expression, with 35 genes identified here for the first time as showing changes in expression in response to androgen in prostate cancer cells. Expression trends were validated for 26 of these 35 genes using qRT-PCR. These studies confirmed that levels of expression of 24 genes (*ARL6IP5, BLVRB, C19orf48, C1orf122, C6orf66, CAMK2N1, CCNI, DERA, ERRFI1, GLUL, GOLPH3, HM13, HSP90B1, MANEA, NANS, NIPSNAP3A, SLC41A1, SOD1, SVIP, TAOK3, TCP1, TMEM66, USP33,* and *VTA1*) respond to androgen in prostate cancer cells. The products of these genes are involved in amino acid and protein synthesis, cofactor transport, protein trafficking, response to oxidative stress, as well as signalling pathways that regulate gene expression, proliferation, apoptosis, and differentiation.

Androgen alters the expression of genes whose protein products may affect local glutamine concentrations in prostate cancer cells. Glutamine is the most common amino acid present in mammalian blood[43]. This amino acid is involved in a variety of cellular processes such as metabolism, apoptosis, proliferation, and protein synthesis/degradation[44]. Glutamate-ammonia ligase (*GLUL*) and solute carrier family 41, member 1 (*SLC41A1*) transcripts were increased in response to androgen. A function of GLUL is to metabolize glutamine from glutamate and ammonia[45]. The activity of GLUL is dependent on binding to divalent cations such as Mg^{2+}. Interestingly, SLC41A1 is a putative membrane protein that mediates preferential Mg^{2+} uptake in epithelial cells[46]. Expression of ADP-ribosylation like factor-6 interacting protein 5 (*ARL6IP5*)

was decreased in response to androgen. Inferred by homology to a rat protein, it probably inhibits glutamate uptake into cells[47, 48]. Taken together, these results suggest that androgen signalling promotes the synthesis of glutamine.

In response to androgen, there were alterations in expression of genes whose protein products function in protein folding, alteration, degradation, and transport. For example, androgen increased levels of expression of T-complex 1 gene (*TCP1*), which is an essential molecular chaperone that resides in the cytosol and aids in the folding of cytoskeletal and cell cycle proteins[49]. TCP1 has been shown to be over-expressed in human colorectal cancer[50]. Histocompatibility (minor) 13 (HM13), n-acetylneuraminic acid synthase (NANS), and mannosidase, endo alpha (MANEA), function to alter protein structure. HM13 is an endoplasmic reticulum membrane protein that proteolyzes signal peptides and generates epitopes recognizable by the immune system[51], and NANS catalyses the synthesis of sialic acid to generate glycoproteins[52]. While expression of genes for these proteins was increased by androgen, expression of *MANEA* was decreased. MANEA is a golgi apparatus membrane protein thought to hydrolyse the glucosyl unit from glycosylated mannosidase[53]. Androgen lowered gene expression of small VCP/p97-interacting protein (*SVIP*), which inhibits endoplasmic reticulum associated degradation (ERAD). Thus, with decreased SVIP there should be increased ERAD which degrades misfolded or unfolded proteins to prevent accumulation of aberrant proteins[54] and is consistent with increased protein synthesis in prostate epithelial cells in response to androgen. Androgen also increased expression of golgi phosphoprotein 3 (*GOLPH3*), a membrane protein of the golgi stack that is suggested to regulate protein trafficking[55] and decreased expression of vps20-associated 1 (*VTA1*) and nipsnap homologue 3A (*NIPSNAP3A*) that have roles in multivesicular body sorting[56] and vesicular transport (inferred by homology to a *Caenorhabditis elegans* protein)[57, 58], respectively. The main molecular chaperone of the endoplasmic reticulum is heat shock protein 90 kDa beta member 1 (HSP90B1)[59]. In this study, expression of *HSP90B1* was increased *in vitro*, but we did not detect change in expression *in vivo* in response to androgen. The low 1.6-fold change in expression of *HSP90B1* in response to androgen *in vitro*, may be insufficient to overcome the biological variation *in vivo*. HSP90B1 mediates folding, assembly, and secretion of proteins[60]. It can protect cells from apoptosis[61], present malfolded proteins to the proteosome[59], and aid in antigen presentation via MHC class I molecules[59]. Expression of *HSP90B1* is induced by metabolic stress [glucose starvation,

estrogen, and interleukin 6 (IL-6)][59]. Overall, these results indicate the androgen signalling axis regulates protein production and transport.

Androgens regulate gene expression of proteins involved in signal transduction pathways. Tao Kinase 3 (TAOK3) is a member of the sterile 20-family of kinases[62]. These kinases are often involved in mitogen activated protein kinase (MAPK) pathways, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and extracellular signal-regulated kinase (ERK). JNK/SAPK and ERK pathways are usually activated in response to stress signals and pro-inflammatory cytokines, respectively. Signalling through JNK/SAPK and ERK pathways result in mammalian cellular responses such as proliferation, differentiation and/or apoptosis[63]. Whether TAOK3 is a positive or negative regulator of MAPK pathways is controversial[64, 65]. In our study, there was an association between relatively high levels of *TAOK3* gene expression and androgen in prostate cancer cells both *in vitro* and *in vivo*.

ERBB receptor feedback inhibitor 1 (*ERRFI1*) gene expression was increased in response to androgen which is consistent with reports of this gene being induced by growth factors, stress, and hormones[66]. *ERRFI1* gene expression in AR-negative DU145 and PC-3 cells was not significantly different, but displayed a trend of increased *ERRFI1* expression compared to AR-positive LNCaP cells. The levels of expression of *ERRFI1* had a trend that correlated to doubling time of the cell lines, with PC3 cells doubling faster than DU145 which is quicker than LNCaP[67]. These results suggest that levels of expression of *ERRFI1* may be due to increased proliferation, and not AR. Immediate, early response *ERRFI1* gene encodes a non-kinase adaptor protein containing a cdc42/Rac interacting and binding (CRIB) domain, Src-homology-3 (SH3) domain binding motif, and a 14-3-3 protein binding motif[66]. The biological significance of the SH3 and 14-3-3 binding motifs have yet to be determined. However, the CRIB domain of ERRFI1 has been shown to negatively regulate Cdc42, epidermal growth factor (EGF) receptor, and hepatocyte growth factor (HGF) signalling, while positively regulating nuclear factor kappa B (NFκB) signalling[66]. Each of these pathways has an effect on MAPK signalling.

CAMK2N1 is an inhibitor to calcium/calmodulin-dependent kinase II (CAMK2)[68]. CAMK2 is a well characterized ubiquitously and highly expressed protein involved in a plethora of cellular

processes. The action of CAMK2 is most studied in neurons, where CAMK2 is thought to be involved in gene expression, cell signalling, ion-channel function, cytoskeletal interactions and morphology[69]. Interestingly, *CAMK2* gene expression has been shown to increase in response to androgens[70]. Therefore, the down-regulation of gene expression of its inhibitor, CAMK2N1, by androgens, as shown here, could result in increased CAMK2 activity in the presence of androgens. *CAMK2N1* gene expression *in vivo* increased following castration of the hosts. Interestingly, we detected two LongSAGE tags for *CAMK2N1* each of which were decreased in response to androgen, but each with distinct levels and fold-change. These two tags probably represent alternative splicing of *CAMK2N1* with each transcript variant differentially regulated.

Androgen elicits oxidative stress in LNCaP prostate cancer cells[71]. However, it is not yet clear if this occurs simply as a byproduct of induction of proliferation, or a more direct relationship between AR and expression of genes involved in the regulation of oxidative stress. Here, we provide support for the latter. Superoxide dismutase 1 (SOD1) is an enzyme capable of converting free superoxide radicals to molecular oxygen or hydrogen peroxide[72] and protecting cells from oxidative damage. *In vitro SOD1* expression was decreased by androgens. Interestingly, expression of *SOD1* is lower in prostatic intraepithelial neoplasia (PIN) and prostate cancer relative to benign prostatic tissue[73], suggesting that defense against superoxide radicals is compromised. These clinical data support the suggestion that dietary supplements of antioxidants may aid in the prevention of prostate cancer[74].

2.5 CONCLUSION

Delineation of the molecular basis of androgen action in the prostate requires identification of genes and pathways. Here, we report 24 genes that alter levels of expression in response to androgen in prostate cancer cells that are involved in protein synthesis and trafficking, response to oxidative stress, transcription, proliferation, apoptosis, and differentiation. These genes are potentially critical for the function and maintenance of the prostate and represent targets for clinical intervention.

Table 2.1 Primer sequences and amplification product sizes for candidate transcripts

Gene	RefSeq Access. No.	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size	Exons[1]
ADAMTS1	NM_006988	ACTGCAAGGCGTAGGACAG	CCACAAGCATGGTTTCCAC	92	1-2
ARL6IP5	NM_006407	CATGTTTGGAGGAGTCATGG	GAGGTTCCGAAGTCTCAACG	91	2-3
BLVRB	NM_000713	GAAGTACGTGGCTGTGATGC	CCAGGTCATGTTTGGAGATG	113	4-5
C1orf122	NM_198446	AGCTCCTGGACACCATCG	GCTCCAGGTTTGGCTGAGAC	103	2-3
C19orf48	NM_199249	AAGGGCCTGACCATCACTC	ACGCCTAGGCAGGAAACAG	96	1-2
C6orf66	NM_014165	AAAGATGAAAAGCTGCTGTCG	CTGAATTCCTTCGGCTCTTG	113	2-3
CAMK2N1	NM_018584	TGCAGGACACCAACAACTTC	GCACGTCATCAATCCTATCATC	114	1-2
CAPNS1	NM_001003962	AGATGGCACTGGACAAATCC	TCCTATAGCAAGGCAGTGAGG	106	10-11
CCNI	NM_006835	TCATTCCTGATTGGCTTTCTC	GAAAGGTGATGTGCCACAAG	103	6-7
CENPN	NM_018455	ATACACCGCTTCTGGGTCAG	TGCAAGCTTTCTTCATTTCG	99	6-7
CREB3L4	NM_130898	TTCCAGAGTCGACCAGAAGC	TGTTACGTCCTTGTGGGTCA	87	9-10
CXCR7	NM_020311	CCCGGAGGTCATTTGATTG	GCTGATGTCCGAGAAGTTCC	87	1-2
DERA	NM_015954	AGTGGCTGAAGCCAGAACTC	AAGCTGCATATCTTCCAGTCAC	99	8-9
ERRF11	NM_018948	CCGATAACCATGGCCTACAG	ATTCATCGGAGAGATTTGG	87	3-4
FKBP5	NM_004117	CGCAGGATATACGCCAACAT	GAAGTCTTCTTGCCCATTGC	86	11-12
GAPDH	NM_002046	CTGACTTCAACAGCGACACC	TGCTGTAGCCAAATTCGTTG	114	8-9
GLUL	NM_002065	TGCCATACCAACTTCAGCAC	TGCCGCTTGCTTAGTTTCTC	89	6-7
GOLPH3	NM_022130	CTCCAGAAACGGTCCAGAAC	CCACCAGGTTTTTAGCTAATCG	114	3-4
HM13	NM_178580	GGCCAAGGGAGAAGTGACAG	ATGCCTCTGTTCCCTCTTTG	95	10-11
HSP90B1	NM_003299	GCATCTGATTACCTTGAATTGG	TGGGCTCCTCAACAGTTTC	115	6-7
KLK3	NM_001648	CCAAGTTCATGCTGTGTGCT	CCCATGACGTGATACCTTGA	111	4-5
LRIG1	NM_015541	GACGGCTGTGAAGAAAAAGC	CTGTGGAGTCCGGGTGATAC	92	18-19
MANEA	NM_024641	TAGCAATCGAGATGATCAAAAC	AAGAGCATTGCCAGTCTTCG	109	4-5
NANS	NM_018946	CGGTCAGTGCGTCTTGTG	ATTTTCACTTTGGCCACCAC	113	5-6
NCAPD3	NM_015261	GGGCGCTTCTTACTCTCCTC	GGGTGAGAATTTTTCTTTCTTGG	98	16-17
NIPSNAP3A	NM_015469	CCATGAGGATCCCAGAGTTG	TCAGTGGTGAAAACGATGTAGG	101	5-6
NTS	NM_006183	CCACAAAATCTGTCACAGCAG	CCTTTCCATTTTTGTCATTTCC	89	3-4
PAK1IP1	NM_017906	CGTGTCTTGGAGTGTGGCTA	AGGCTCCTTTTTGCCAATTT	113	9-10
PRKACB	NM_182948	GCCACGACAGATTGGATTG	AATTGCTGGTATCTCCAGAGC	89	9-10
PRNPIP	NM_024066	CCTCAGCCTGCAACACATAG	AAGCCTCGATAGGCGAGTG	92	6-7
RHOU	NM_021205	CCCGTGAGACTCCAACTCTG	TGAAGCAGAGCAGGAAGATG	100	2-3
SLC41A1	NM_173854	GCACACCACCCTCACACTC	TCCAGTCTGCGATGTACAGG	89	10-11
SOD1	NM_000454	CCCAGGTTAACCCAGAACG	ACCCCTGCTTGTTTGTTGTC	88	4-5
ST7	NM_018412	CGGAACTTATGGGGGTCTTC	ACAGACTGGATGGGAGGATG	102	14-15
SVIP	NM_148893	AGGGTTCTCAAGCTGTCGTC	TGCAAGCTTTGCTCTTTTCTC	101	1-2
TAOK3	NM_016281	CGCAGAGCACACCTTGAG	CGCTCTTGCCTTTCCAATAG	98	20-21
TCP1	NM_030752	TGTGGCCGATGTGTCTATTG	ACCTTTGCCCAAGTCATCTG	109	11-12
TMEM66	NM_016127	GGGCAGCTATTCGGTATGTTC	TGCATCCAGTGTTTGACTCC	110	5-6
USP33	NM_201624	AAATGTGGTAATGTGATGCTTAGG	GGTCGCAGGATAACTTCAGG	113	23-24
VTA1	NM_016485	CGCACTTTTCAATACAATTTCC	CATCTTCATACTGCAAAGCACTG	110	10-11

[1] Exons according to Ensembl

Table 2.2 Composition of LongSAGE libraries

			Library	R1881	Vehicle
			Unfiltered Total Tags	121,760	103,391
			No. of Bad Tags	528	383
Minus Bad Tags			Total Tags	121,232	103,008
			Tag Types	33,385	31,764
			No. of Duplicate Ditags	6,763	5,193
			% of Duplicate Ditags	5.579	5.041
			Average QF[r] of Tags	89.64	89.67
			No. of Tags QF<95%	22,816	17,095
	QF ≥ 95%		Total Tags	98,416	85,913
			Tag Types	23,830	24,594
			Total Tags Combined	184,329	
			Tag Types Combined	38,576	
			No. of LDTs[s] Type I	219	34
			No. of LDTs Type II	216	18
		Minus LDTs	Total Tags	97,981	85,861
			Tag Types	23,828	24,592
			Total Tags Combined	183,842	
			Tag Types Combined	38,574	

r QF, Quality Factor
s LDTs, Linker-derived Tags

Table 2.3 Characteristics of LongSAGE tag frequency distribution

Tag Frequency & Abundance							
	Tag Count per 100,000[t]	≤1	2-4	5-9	10-99	100-999	≥1,000
	Transcript Copies per Cell[u]	≤5	10-20	25-45	50-495	500-4,995	≥5,000
	% Transcript Abundance in Cell[v]	≤0.001	0.002-0.004	0.005-0.009	0.01-0.099	0.1-0.999	≥1
R1881	Total Tags	15,141	13,985	11,055	32,800	21,971	3,029
	Tag Types	15,141	5,464	1,703	1,417	101	2
Vehicle	Total Tags	16,562	10,229	11,633	26,466	18,453	2,518
	Tag Types	16,562	4,427	2,195	1,313	93	2
% of Tags that Map to Transcription Factors[α, z, δ]		9.14	8.94	7.95	6.0	0	0
% of Tags that Map[χ,β,δ]		29.40	57.82	76.22	83.1	85	100[γ]
% of Tags Significantly Differentially Expressed[ε, a, δ]		0.4	1.45	16.17	25.38	58.12	100

t Tag count per 100,000 = (observed tag count/total tags in the library) x 100,000
u Transcript copies per cell[w] = (observed tag count/total tags in the library) x 500,000
v % Transcript abundance in cell[w] = (transcript copies per cell/500,000) x 100%
w Calculation based on ~500,000 transcripts in a cell (Hastie and Bishop 1976)
α % of tags that map as transcription factors =
(no. of genes with "transcription regulation acivity"/no. of genes with unambiguous sense mappings and GO terms) x 100%
z Mapped unambigously sense to RefSeq and subjected to Gene Ontology (GO) analysis
δ Tag types from each tag frequency class of R1881 and vehicle LongSAGE libraries were combined
χ % of tags that map = (no. of genes with sense mappings/combined total tag types) x 100%
β Mapped sense (incl. ambiguous) to RefSeq
γ One tag was mapped sense using Ensembl gene
ε % of tags significantly differentially expressed =
(no. of significantly differently expressed tag types in class/combined total tag types in class) x 100%
a Statistics according to the Audic and Claverie test statistic ($p \leq 0.001$)

Table 2.4 LongSAGE tag mappings [x]

Library	No. of Tag Types that Mapped Unambiguously to (Genes)	No. of Tag Types that Mapped Ambiguously	No. of Tag Types that Did Not Map	Total No. of Tag Types (Clustered).[y]
R1881	14,587 (7,484)	3,754	10,215	28,556
Vehicle	13,626 (7,441)	3,286	9,066	25,978

[x] Ensembl gene (v38) was used for mapping

[y] Clustering amalgamated 1-off tags with likely 'parent' tags to correct for PCR/sequencing errors
Clustering altered the number of tag types without changing the total number of tags in the libraries

Table 2.5 Number of tag types found to be significantly differentially expressed between R1881 and vehicle libraries [a]

Direction of Change	$p \leq 0.001$	$p \leq 0.01$	$p \leq 0.05$
Up Regulated	83	196	455
Down Regulated	48	120	436
Total	131	316	891
% of All Tag Types	0.34%	0.82%	2.31%

a Statistics according to the Audic and Claverie test statistic

Table 2.6 LongSAGE tags corresponding to genes known to increase expression in response to androgen in LNCaP cells[a,n]

LongSAGE Tag Sequence	Tags/100,000 [d,t] Vehicle	R1881	Fold [c,d] Change	RefSeq/Ensembl Access. No.	HGNC Gene Symbol	Description[φ]
GTGACAAGTGACAGAGT	1	19	20	NM_007011	*ABHD2*	Abhydrolase domain containing 2, transcript variant 1
ACGTCACCATTTTTAAC	1	24	20	NM_004457	*ACSL3*	Acyl-CoA synthetase long-chain family member 3, transcript variant 1
TACTTTATAAGTATTGG	14	59	4.2	NM_006988	*ADAMTS1**	ADAM metallopeptidase with thrombospondin type 1 motif, 1
TAGCTCTATGGGGGGAG	35	75	2.1	NM_000701	*ATP1A1*	ATPase, Na+/K+ transporting, alpha 1 polypeptide, transcript variant 1
GTTGTGGTTAATCTGGT	48	109	2.3	NM_004048	*B2M*	Beta-2-microglobulin
ACTTAAGGAACTTATCT	14	42	3.0	NM_015415	*BRP44*	Brain protein 44
AAAGGAAAATAAAAATT	3	27	9	NM_018455	*CENPN**	Centromeric protein N
CTGTGATGTGACTCCTG	5	30	6	NM_030806	*C1orf21*	Chromosome 1 open reading frame 21
CAGATGAGATGTGAGCT	5	33	7	NM_130898	*CREB3L4**	cAMP responsive element binding protein 3-like-4
TGTTTATCCTAAACTGA	21	115	5.5	NM_020548	*DBI*	Diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)
TCCCCGTGGCTGTGGGG	106	356	3.36	NM_014762	*DHCR24*	24-dehydrocholesterol reductase
GAAATTAGGGAAGCCTT	9	34	4	NM_015036	*ENDOD1*	Endonuclease domain containing 1
AGATCCTACTTAGTATG	16	51	3.2	NM_004462	*FDFT1*	Farnesyl-diphosphate farnesyltransferase 1
GTTCCAGTGAGGCCAAG	3	50	20	NM_004117	*FKBP5**	FK506 binding protein 5
ACCTAGCCACTGCTGGG	1	24	20	NM_002247	*KCNMA1*	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1, transcript variant 2
GGATGGGGATGAAGTAA	50	366	7.3	NM_001648	*KLK3**	Kallikrein 3, (prostate-specific antigen), transcript variant 1
CCTCCAGCTACAAAACA	35	223	6.4	NM_002273	*KRT8*	Keratin 8
TAAAATATTGAAGTGTC	ND[b]	42	40	NM_015541	*LRIG1**	Leucine-rich repeats and immunoglobulin-like domains 1
TCCCTGAGCACCATTGC	ND	35	40	NM_015261	*NCAPD3**	Non-SMC condensin complex subunit D3
GGACTTTCCTTCCCTCT	1	72	70	NM_006096	*NDRG1*	N-myc downstream regulated gene 1
TTTAGGTAAACGAAAGC	19	56	2.9	NM_014445	N/A[q]	Stress-associated ER protein 1
AGGTTTTGCCTCATTCC	13	38	2.9	ENSG00000196930	N/A[q]	Similar to Vesicle-associated membrane protein-associated protein A mRNA
ATGCAGCCATATGGAAG	20	208	10	NM_002539	*ODC1*	Ornithine decarboxylase 1
GCCAAGGGGCCAGCTGC	17	45	2.6	NM_002541	*OGDH*	Oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide), nuclear gene encoding mitochondrial protein, transcript variant 1
TAATTTTTACTTTGTAC	5	39	8	NM_017906	*PAK1IP1**	PAK1 interacting protein 1
TATGTAATATGCTTTCT	27	164	6.1	NM_003711	*PPAP2A*	Phosphatidic acid phosphatase type 2A, transcript variant 1
AAACACCAACAACTGGG	5	31	6	NM_003711	*PPAP2A*	Phosphatidic acid phosphatase type 2A isoforms 1 and 2
GTGTTTACGTGATCCAC	1	18	20	NM_004578	*RAB4A*	RAB4A, member RAS oncogene family
TATGTATAAATGGACCT	ND	16	20	NM_021205	*RHOU**	Ras homolog gene family, member U
TTTGAAATGAGGTCTGT	14	48	3.4	NM_002970	*SAT*	Spermidine/spermine N1-acetyltransferase
GCAACAGCAATAGGATT	3	22	7	NM_014302	*SEC61G*	Sec61 gamma subunit, transcript variant 1
GCGCTGGAGTGAGATGG	59	126	2.1	NM_031287	*SF3B5*	Splicing factor 3B, subunit 5, 10kDa
GGATTTGAACATATGAA	ND	13	10	NM_033102	*SLC45A3*	Solute carrier family 45, member 3
ACCTTGTGCCCGATTCT	47	238	5.1	NM_003104	*SORD*	Sorbitol dehydrogenase

Table 2.6 continued

LongSAGE Tag Sequence	Tags/100,000 Vehicle	Tags/100,000 R1881	Fold Change	RefSeq/Ensembl Access. No.	HGNC Approved Gene Symbol	Description
AAAATCTGCCACTCAGG	ND	12	10	NM_003104	*SORD*	Sorbitol dehydrogenase
GTGCAGGGAGACATCTG	3	55	20	NM_012391	*SPDEF*	SAM pointed domain containing ets transcription factor
TTAAGGGATGATGGCTT	ND	12	10	NM_024636	*STEAP4*	STEAP family member 4
TACTACAGCTATATTTG	16	52	3.3	NM_016192	*TMEFF2*	Transmembrane protein with EGF-like and 2 follistatin-like domains 2
TGATGTCTGGTCTGAAT	1	17	20	NM_020182	*TMEPAI*	Transmembrane, prostate androgen induced RNA, transcript variant 1
CAAATAAATTATGCGAT	5	64	10	NM_005656	*TMPRSS2*	Transmembrane protease, serine 2
TGAAAAGCTTAATAAAT	7	28	4	NM_005079	*TPD52*	Tumor protein D52, transcript variant 3
TTAAAGATTTAGACACC	10	36	3.6	ENSG00000140416	*TPM1*	Tropomyosin 1 apha chain
TTCTCTACACAATTGTA	6	36	6	NM_006022	*TSC22D1*	TSC22 domain family, member 1, transcript variant 1

a Statistics according to the Audic and Claverie test statistic ($p \leq 0.001$)
b ND, not detected
c ND tags were assigned a value of 1 when calculating fold change
d Appropriate significant figures are displayed
n Ambiguously mapped tags and tags with a fold change less than 2-fold have been excluded from the table
q N/A = there is no HGNC approved gene symbol for this tag
t Tag count per 100,000 = (observed tag count/total tags in the library) x 100,000
φ In cases where a tag mapped to >1 transcript variant of the same gene the RefSeq accession number for transcript variant 1 was displayed
* Gene further characterized in this paper

Table 2.7 LongSAGE tags corresponding to genes known to decrease expression in response to androgen in LNCaP cells[a,n]

LongSAGE Tag Sequence	Tags/100,000[d,t] Vehicle	R1881	Fold Change[c,d,j]	RefSeq/Ensembl Access. No.	HGNC Gene Symbol	Description[φ]
CAAAAGCTTATTCTTGT	29	3	-10	NM_016613	*C4orf18*	Chromosome 4 open reading frame 18, transcript variant 2
TCACACAGTGCCTGTCG	19	1	-20	NM_020311	*CXCR7**	Chemokine orphan receptor 1
ACAAACCCCCACCCCAG	41	7	-6	NM_013330	*NME7*	Non-metastatic cells 7, protein expressed in, transcript variant 1, Nucleoside diphosphate kinase
AATCTCTCAATTATAGG	34	9	-4	NM_006183	*NTS**	Neurotensin
ATCAACTGGAGGCTCAG	15	ND[b]	-20	NM_005013	*NUCB2*	Nucleobindin 2
CCAAAATTAGGAAAAAC	15	1	-20	NM_002577	*PAK2*	p21 (CDKN1A)-activated kinase 2[k]
TTACGTTTGGGAAAAAT	19	2	-9	NM_032971	*PCDH11Y*	Protocadherin 11 Y-linked, transcript variant a[k]
TGACTTTGGTGCCGTTA	12	ND	-10	NM_003629	*PIK3R3*	Phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma)
AGCAAATATGTCAAGGG	47	16	-2.9	NM_182948	*PRKACB**	Protein kinase, cAMP-dependent, catalytic, beta, transcript variant 1
GACTATTCCATATTAAA	27	1	-30	NM_018412	*ST7**	Suppression of tumorigenicity 7, transcript variant A
GAGGGTTTTAAATGGAG	79	9	-9	NM_001077	*UGT2B17*	UDP glucuronosyltransferase 2 family, polypeptide B17

a Statistics according to the Audic and Claverie test statistic ($p \leq 0.001$)
b ND, not detected
c ND tags were assigned a value of 1 when calculating fold change
d Appropriate significant figures are displayed
j Negative fold change value indicates down-regulation in response to R1881
k Tag has a single base pair permutation, insertion, or deletion with respect to gene
n Ambiguously mapped tags and tags with a fold change less than 2-fold have been excluded from table
t Tag count per 100,000 = (observed tag count/total tags in the library) x 100,000
φ In cases where a tag mapped to >1 transcript variant of the same gene the RefSeq accession number for transcript variant 1 was displayed
* Gene further characterized in this paper

Table 2.8 LongSAGE tags corresponding to genes not previously reported to increase expression in response to androgen in LNCaP cells[a,n]

LongSAGE Tag Sequence	Tags/100,000[d,t] Vehicle	R1881	Fold Change[c,d]	RefSeq/Ensembl Access. No.	HGNC Gene Symbol	Description[φ]
TCTTTATTAGAAAAAAA	ND[b]	16	20	NM_014265	*ADAM28*	ADAM metallopeptidase domain 28, transcript variant 1[k]
AGGAGCAAAGGAAGGGG	51	107	2.1	NM_000713	*BLVRB**	Biliverdin reductase B (flavin reductase (NADPH))
TTTTGGGGGCTTTTAGC	16	44	2.8	NM_198446	*C1orf122**	Chromosome 1 open reading frame 122
GGGCCCCAAAGCACTGC	22	69	3.1	NM_199249	*C19orf48**	Chromosome 19 open reading frame 48
CCCCAGTTGCTGATCTC	24	60	2.5	NM_001003962	*CAPNS1**	Calpain, small subunit 1, transcript variant 2
CTTAAGAAAAATGCACT	1	23	20	NM_018948	*ERRFI1**	ERBB receptor feedback inhibitor 1
TACAGTATGTTCAAAGT	13	52	4.0	NM_002065	*GLUL**	Glutamate-ammonia ligase (glutamine synthetase), transcript variant 1[g,i]
TTAATAGTGGGGCTTTC	10	39	3.9	NM_022130	*GOLPH3**	Golgi phosphoprotein 3 (coat protein)
GCCAGGGCGGGCCACTG	ND	16	20	NM_178580	*HM13**	Histocompatibility (minor) 13, transcript variant 2[e]
GAGGAAGAAGAAGCAGC	ND	14	10	NM_003299	*HSP90B1**	Heat shock protein 90kDa beta (Grp94), member 1
GGCAAGGGGGGTCCCCA	1	20	20	NM_002273	*KRT8*	Keratin 8[m]
ACTCCAAAAAAAAAAAAA	41	81	2.0	XM_376154	N/A[q]	Similar to 40S ribosomal protein S15 (RIG protein), transcript variant 1
GGGTTGGCTTGAAACCA	6	30	5	ENSG00000210151	N/A	Non-coding predicted mitochondrial gene[m]
GAGAGCTCCCGTGAGTG	72	122	1.7	NC_001807[p]	N/A	Intergenic region of mitochondrial genome
TCGGACGTACATCGTTA	40	223	5.6	No map	N/A	N/A
GCAAAAAAATCAAGTCT	22	66	3.0	NM_018946	*NANS**	N-acetylneuraminic acid phosphate synthase (sialic acid synthase)
TCTTTTAGCCAATTCAG	2	36	20	NM_006167	*NKX3-1*	NK3 transcription factor related, locus1[m]
TACTTTTGGCCTGGCTG	6	35	6	NM_173854	*SLC41A1**	Solute carrier family 41, member 1
GAGAGCCTCAGAATGGG	5	26	5	NM_016281	*TAOK3**	TAO kinase 3
GAAGTTATGAAGATGCT	41	106	2.6	NM_030752	*TCP1**	T-complex protein 1, transcript variant 1
CAGTTCTCTGTGAAATC	40	93	2.3	NM_016127	*TMEM66**	Transmembrane protein 66
ATGGCTTTGTTTTGGTT	ND	14	10	NM_201624	*USP33**	Ubiquitin specific protease 33, transcript variant 2

a Statistics according to the Audic and Claverie test statistic ($p \leq 0.001$)
b ND, not detected
c ND tags were assigned a value of 1 when calculating fold change
d Appropriate significant figures are displayed
e Gene family, but not this family member, previously described to change expression in response to androgens
g Protein known to change expression in reponse to androgens
h Gene known to change expression in response to androgens, but in the opposite direction as reported here
i Gene known to change expression in response to androgens in cells other than prostate
k Tag has a single base pair mutation , insertion , or deletion with respect to gene map
m Tag maps to the strand opposite of the gene
n Ambiguously mapped tags and tags with a fold change less than 2-fold have been excluded from the table
p NC_001807, refers to the complete genome of mitochondria in humans
All mitochondrial genes in the RefSeq database are assigned the same accession number by NCBI
q N/A, there is no HGNC approved gene symbol or description for this tag
t Tag count per 100,000 = (observed tag count/total tags in the library) x 100,000
φ In cases where a tag mapped to >1 transcript variant of the same gene the RefSeq accession number for transcript variant 1 was displayed
* Gene further characterized in this paper

Table 2.9 LongSAGE tags corresponding to genes not previously reported to decrease expression in response to androgen in LNCaP cells[a,n]

LongSAGE Tag Sequence	Tags/100,000[d,t] Vehicle	R1881	Fold[c,d,j] Change	RefSeq/Ensembl Access. No.	HGNC Gene Symbol	Description[φ]
GTCTAGAATCTGTACCC	29	8	-4	NM_006407	*ARL6IP5**	ADP-ribosylation-like factor 6 interacting protein 5
TCAAGAGCCGAAGGAAT	12	ND[b]	-10	NM_014165	*C6orf66**	Chromosome 6 open reading frame 66
GTATTTGCAAAAATGCC	118	24	-4.9	NM_018584	*CAMK2N1**	Calcium/calmodulin-dependent protein kinase II inhibitor 1
AAAAGAGAAAGCACTTT	30	5	-6	NM_018584	*CAMK2N1**	Calcium/calmodulin-dependent protein kinase II inhibitor 1
TTATAACTGAATTTAGT	51	11	-4.6	NM_006835	*CCNI**	Cyclin I[i,h]
GCCAGGAGAAGGGACAG	34	7	-5	NP_775809	*CNBD1*	N/A[m]
TGGTACTCATTTCAGGC	12	ND	-10	NM_015954	*DERA**	2-deoxyribose-5-phosphate aldolase homolog
AATCATAATGGATTCTT	16	ND	-20	NM_024641	*MANEA**	Mannosidase, endo-alpha
CTAAGACTTCACCAGCC	19	2	-10	ENSG00000210082	N/A[q]	Non-coding predicted mitochondrial rRNA gene[k]
CATTTGGTATTTTCGTC	30	8	-4	NC_001807[p]	N/A	Intergenic region of mitochondrial genome
GTATTTCAGTGTCTGTC	33	9	-4	NM_015469	*NIPSNAP3A**	Nipsnap homolog 3A
GTGTGTGGTGCCCCCAG	23	5	-5	NM_024066	*PRNPIP**	Prion protein interacting protein
GTGTTAACCAGCTAAAG	122	60	-2.0	NM_002948	*RPL15*	Ribosomal protein L15
GCACAAGAAGATTAAAA	58	25	-2.3	NR_002746	*SNORD47*	Small nucleolar RNA, C/D box 47 on chromosome 1
AAAAAGCAGATGACTTG	77	37	-2.1	NM_000454	*SOD1**	Superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))
GTTTGGTTATAAATTCT	26	3	-10	NM_148893	SVIP*	Hypothetical protein DKFZp313A2432, transcript variant 1
TATTAGAGAATGAAAAG	17	2	-9	NM_016485	*VTA1**	VPS20-associated 1 homologue

a Statistics according to the Audic and Claverie test statistic ($p \leq 0.001$)
b ND, not detected
c ND tags were assigned a value of 1 when calculating fold change
d Appropriate significant figures are displayed
h Gene known to change expression in response to androgens, but in the opposite direction as reported here
i Gene known to change expression in response to androgens in cells other than prostate
j Negative fold change value indicates down-regulation by R1881
k Tag has a single base pair permutation, insertion, or deletion with respect to gene
m Tag maps to the strand opposite of the gene
n Ambiguously mapped tags and tags with a fold change less than 2-fold have been excluded from the table
p NC_001807 refers to the complete genome of mitochondria in humans
All mitochondrial genes in the RefSeq database are assigned the same accession number by NCBI
q N/A = there is no HGNC approved gene symbol for this tag
t Tag count per 100,000 = (observed tag count/total tags in the library) x 100,000
φIn cases where a tag mapped to >1 transcript variant of the same gene the RefSeq accession number for transcript variant 1 was displayed
* Gene further characterized in this paper

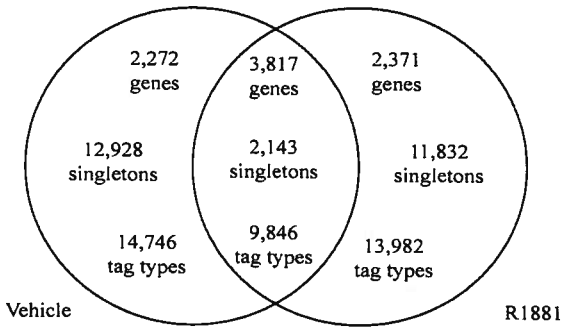

Figure 2.1 Relationship between LongSAGE library compositions. This Venn Diagram shows the tag types and genes exclusive to, and shared by each LongSAGE library, R1881 and vehicle. Tags were mapped unambiguously sense to RefSeq transcripts and redundant mappings were removed. Singletons are tags counted only once in each library, but may be common to both libraries.

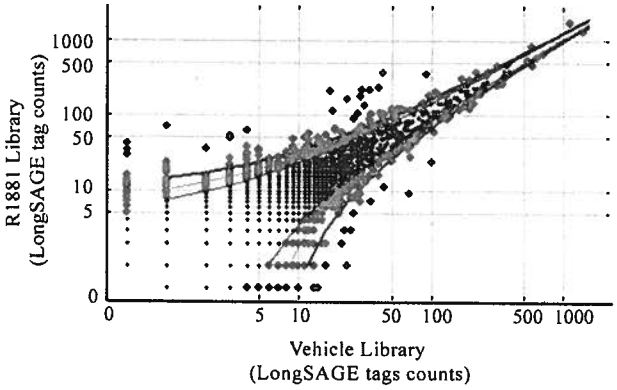

Figure 2.2 Confidence intervals highlight expressed tag types with non-linear relationships between LongSAGE libraries. Scatter plot dots represent tag types and their placement on the axis indicates the frequency of observation in either of the LongSAGE libraries. Tag types that fall outside the confidence interval (CI) lines are statistically significantly differentially expressed (Audic and Claverie test statistic); outer line, 99.9% CI; middle line, 99% CI; and inner line, 95% CI.

Figure 2.3 Androgen regulation of genes as measured by qRT-PCR. ***A*** Candidate genes not previously implicated to change expression in response to androgens in prostate cancer cells: *ARL6IP5, BLVRB, C19orf48, C1orf122, C6orf66, CAMK2N1, CAPNS1, CCNI, DERA, ERRFI1, GLUL, GOLPH3, HM13, HSP90B1, MANEA, NANS, NIPSNAP3A, PRNPIP, SLC41A1, SOD1, SVIP, TAOK3, TCP1, TMEM66, USP33,* and *VTA1*; and ***B*** Genes known to change levels of expression in response to androgens: *ADAMTS1, CENPN, CREB3L4, CXCR7, FKBP5, KLK3, LRIG1, NCAPD3, NTS, PAK1IP1, PRKACB, RHOU,* and *ST7.* LNCaP cells were treated for 16 hours prior to harvesting RNA, and analysing mRNA levels by qRT-PCR. Fold-change was calculated by normalizing the mean normalized expression (MNE) of transcripts in R1881-treated cells to the mock vehicle-treated cells. In doing this, the vehicle treatment fold-change became one and standard deviation (SD) zero. Error bars represent ± SD for biological sextuplets. [*] Asterisk indicates significant differential gene expression according to the Two-Sample Student's T-test ($p \leq 0.05$) for unequal variance.

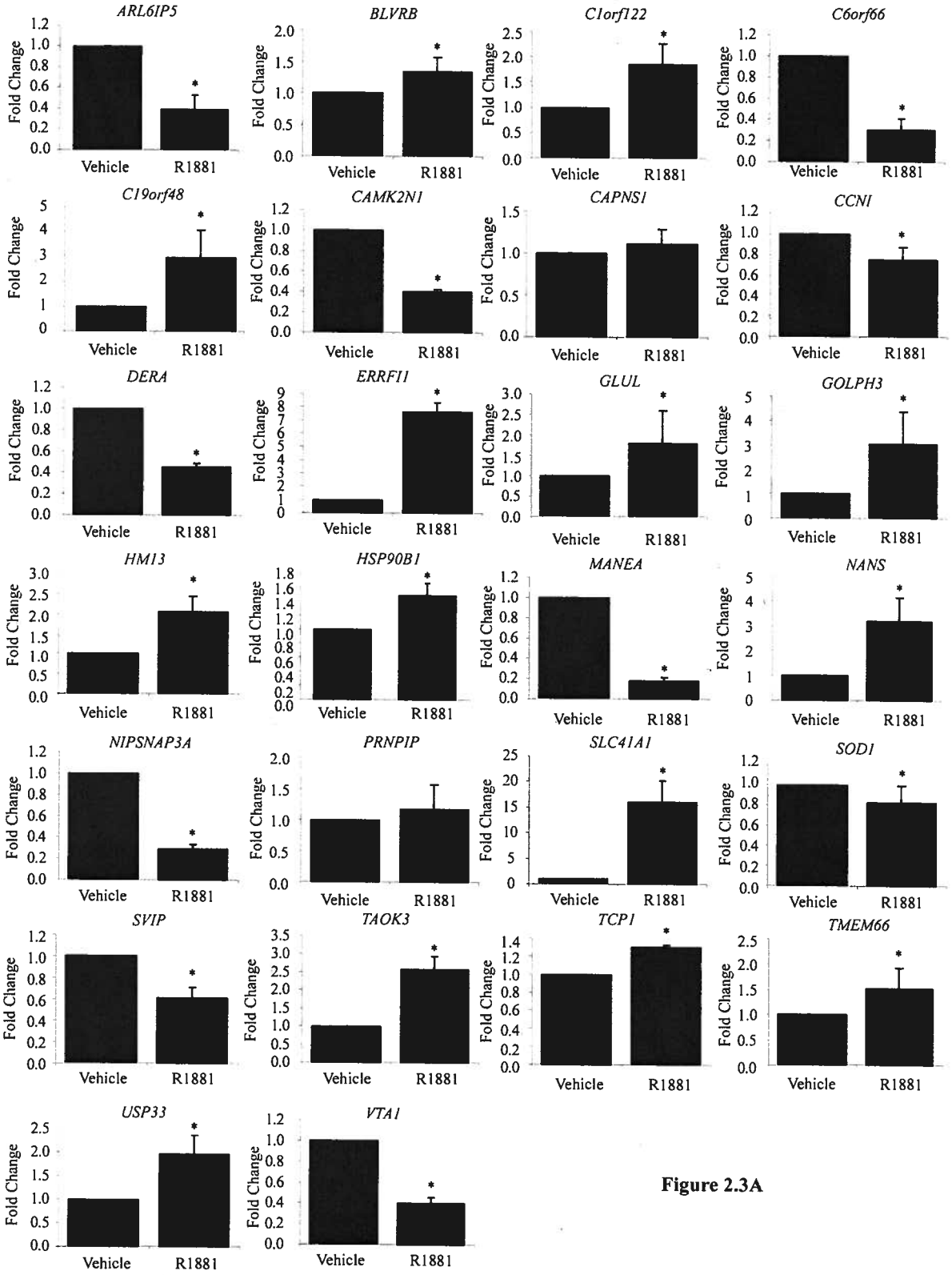
ARL6IP5
BLVRB
C1orf122
C6orf66
C19orf48
CAMK2N1
CAPNS1
CCNI
DERA
ERRFI1
GLUL
GOLPH3
HM13
HSP90B1
MANEA
NANS
NIPSNAP3A
PRNPIP
SLC41A1
SOD1
SVIP
TAOK3
TCP1
TMEM66
USP33
VTA1
Fold Change
Vehicle
R1881

Figure 2.3A

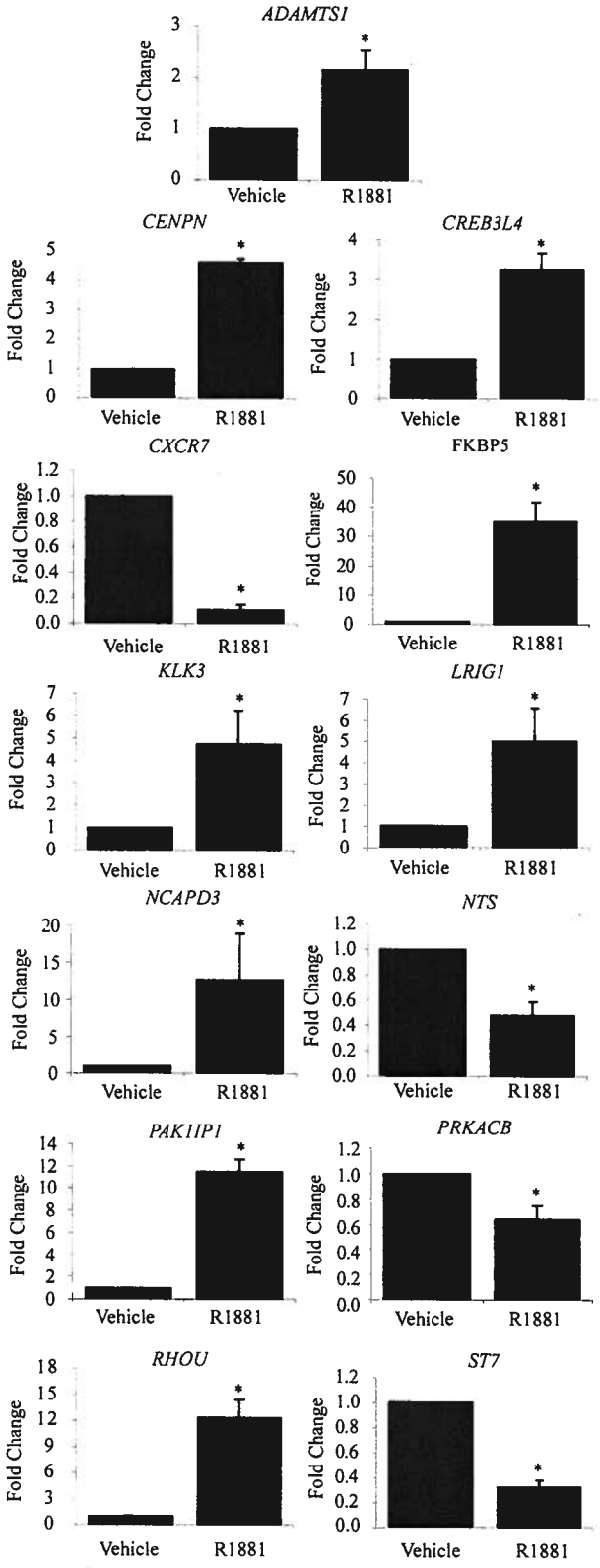
ADAMTS1
CENPN
CREB3L4
CXCR7
FKBP5
KLK3
LRIG1
NCAPD3
NTS
PAK1IP1
PRKACB
RHOU
ST7
Fold Change
Vehicle
R1881

Figure 2.3B

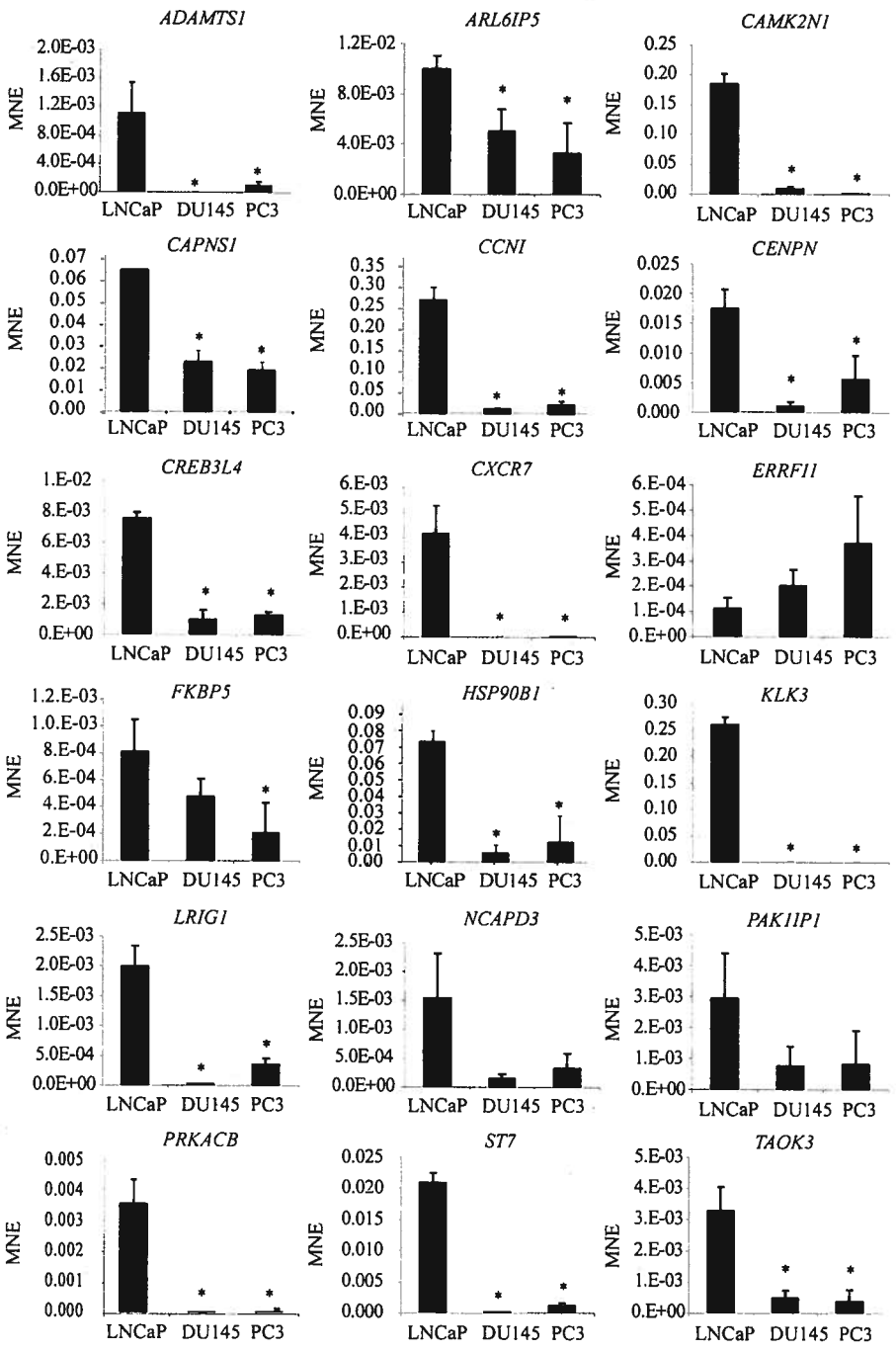

Figure 2.4 Differential expression of candidate genes in LNCaP, DU145, and PC-3 cells. LNCaP, DU145, and PC-3 cells were analyzed by qRT-PCR using probes for *ADAMTS1, ARL6IP5, CAMK2N1, CAPNS1, CCNI, CENPN, CREB3L4, CXCR7, ERRFI1, FKBP5, HSLP90B1, KLK3, LRIG1, NCAPD3, PAK1IP1, PRKACB, ST7,* and *TAOK3*. Error bars represent ± SD for biological triplicates. [*] Asterisks indicate the significant differential gene expression in each cell line compared to LNCaP cells according to the Two-Sample Student's T-test ($p \leq 0.05$) for equal (unpaired) or unequal variance as determined appropriate with the F-test

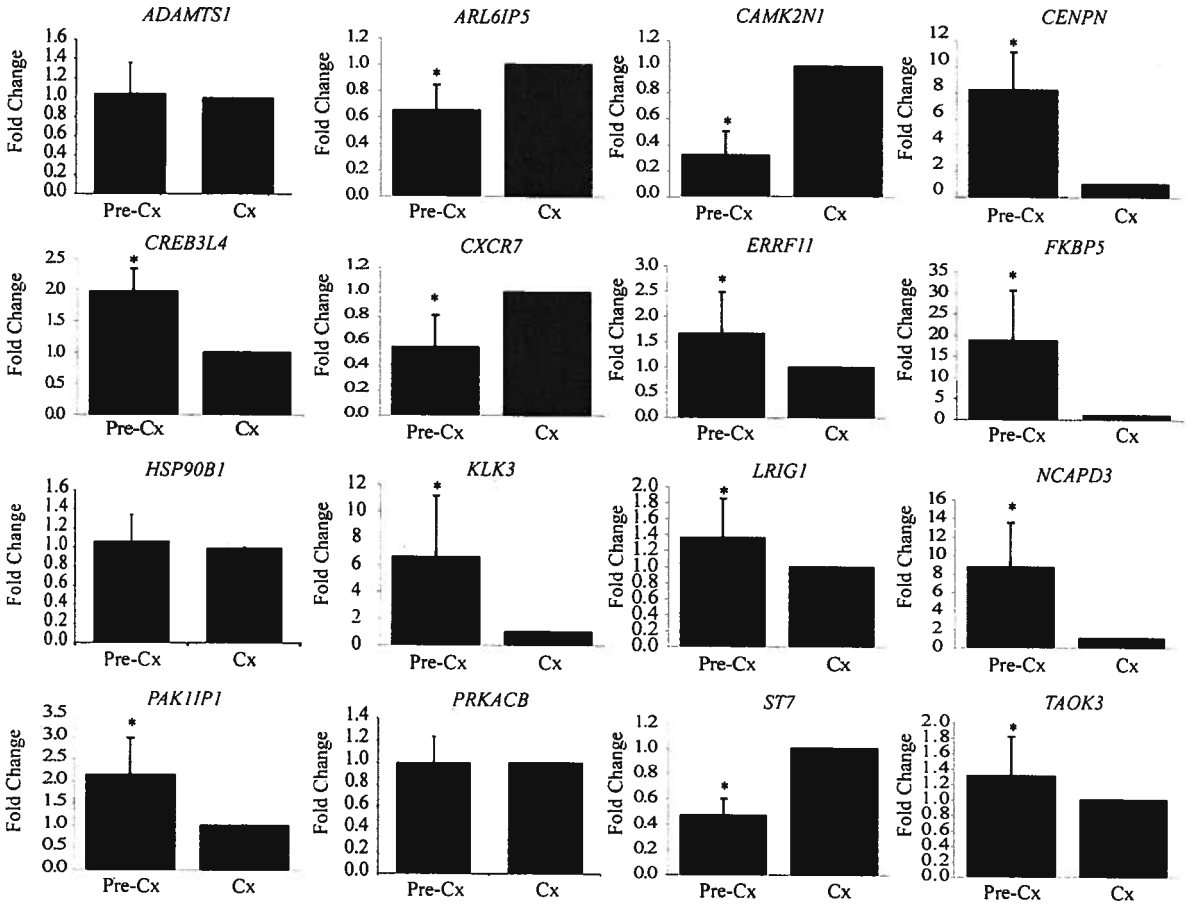

Figure 2.5 Androgen regulation of genes in the *in vivo* Hollow Fibre model of prostate cancer. LNCaP cells from the Hollow Fibre model were analyzed by qRT-PCR using probes for *ADAMTS1, ARL6IP5, CAMK2N1, CENPN, CREB3L4, CXCR7, ERRFI1, FKBP5, HSP90B1, KLK3, LRIG1, NCAPD3, PAK1IP1, PRKACB, ST7,* and *TAOK3*. Cx, castrated mice, 10 days post castration, n = 12; Pre-Cx, pre-castration, day 0 of castration, n = 15. Exception: *LRIG1* gene expression in Cx samples was represented by 11 mice. Fold-change was calculated by normalizing the mean normalized expression (MNE) of transcripts in the Pre-Cx sample to the castrate sample. In doing this, the Cx sample fold-change became one and standard deviation (SD) zero. Error bars represent ± SD. [*] Asterisks indicate the significant differential gene expression with respect to Cx according to the Two-Sample Student's T-test ($p \leq 0.05$) for unequal variance.

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CHAPTER III

GENE EXPRESSION ASSOCIATED WITH IN VIVO PROGRESSION TO CASTRATION-RECURRENT PROSTATE CANCER*

3.1 INTRODUCTION

Systemic androgen-deprivation therapy by orchiectomy or agonists of gonadotropic releasing hormone are routinely used to treat men with metastatic prostate cancer to reduce tumour burden and pain[1]. This therapy is based on the dependency of prostate cells for androgens to grow and survive[2]. The inability of androgen-deprivation therapy to completely and effectively eliminate all metastatic prostate cancer cell populations is manifested by a predictable and inevitable relapse, referred to as castration-recurrent prostate cancer (CRPC)[3]. CRPC is non-responsive to most conventional cancer therapies and fatal to the patient within 16-18 months of onset[4-6].

The mechanisms underlying progression to CRPC are unknown. However, there are several models to explain its development. One such model indicates the involvement of the androgen signalling pathway[3, 7-9]. Key to this pathway is the androgen receptor (AR) which is a steroid hormone receptor and transcription factor[10].The AR mediates androgen-regulated gene transcription[11]. The AR is found inactive and stabilized by heat-shock proteins in the cytoplasm of prostate cells[12]. Upon binding of androgen, the phosphorylation status and conformation of the AR changes, thereby presumably releasing the heat-shock proteins[12]. The AR dimerizes[13], translocates to the nucleus[14], binds to androgen response elements (AREs) of DNA[15], and recruits co-factors to regulate gene expression[16]. The resulting changes in gene expression promote proliferation[17], survival[2], differentiation[18], and secretion[19].

Mechanisms of progression to CRPC that involve or utilize the androgen signalling pathway include: hypersensitivity due to *AR* gene amplification[20, 21]; changes in AR co-regulators [e.g., nuclear receptor coactivator 1 (*NCOA1*) and nuclear receptor coactivator 2 (*NCOA2*)][22, 23]; intraprostatic *de novo* synthesis of androgen [i.e, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (*HMGCS1*), and squalene epoxidase (*SQLE*)][24] or metabolism of AR ligands from

* A version of this chapter will be submitted for publication. Romanuik, TL., Morozova, O., Delaney, A., Marra, MA., Sadar, MD. Gene expression associated with *in vivo* progression to castration-recurrent prostate cancer. *In preparation.*

residual adrenal androgens [e.g., hydroxysteroid (17-beta) dehydrogenase 3 (*HSD17B3*) and hydroxysteroid (17-beta) dehydrogenase 5 (*HSD17B5*)][25, 26]; AR promiscuity of ligand specificity due to mutations[27]; and ligand-independent activation of AR by growth factors [protein kinase A (PKA), interleukin 6 (IL6), and epidermal growth factor (EGF)][28-30]. Activation of the AR can be determined by assaying for the expression of target genes [e.g., prostate-specific antigen (PSA; gene also known as kallikrein 3; *KLK3*)[11], prostate acid phosphatase (*ACPP*)[31], and NK3 homeobox 1 (*NKX3-1*)[32]].

The neuroendocrine model of CRPC entails the transdifferentiation of normal luminal secretory epithelial prostate cells into neuroendocrine-like prostate cancer cells[33]. Transdifferentiation is encouraged by androgen-deprivation therapy, as the length of treatment time in patients correlates with the prevalence of neuroendocrine-like prostate cancer cells[34, 35]. Moreover, LNCaP adenocarcinoma of the prostate cells transdifferentiate into neuroendocrine-like cells following: androgen-deprivation *in vivo*[36]; or treatment with the catalytic subunit of PKA[37], IL6[38], or EGF[39]. In contrast to normal neuroendocrine prostate cells, neuroendocrine-like prostate cancer cells do not express basal cell markers[33], are associated with metastasis and death[40, 41], and over-express the B-cell CLL/lymphoma 2 (*BCL-2*) anti-apoptotic oncogene[42]. Neuroendocrine-like prostate cancer cells do not express *AR* or *KLK3*, but do express neuroendocrine markers gamma neuronal enolase 2 (*ENO2*), neurotensin (*NTS*) chromogranin A (*CHGA*), and chromogranin B (*CHGB*)[33, 43]. Neuroendocrine-like prostate cancer cells secrete hormones serotonin, parathyroid hormone-related protein (PTHrP), and bombesin[33, 43].

A stem cell model for CRPC has been proposed[44]. Stem cells have an extensive capacity for self-renewal due to the signalling of Hedgehog, Notch, and Wingless (Wnt) pathways[45]. The tumour suppressor genes phosphatase and tensin homolog (*PTEN*) and tumour protein p53 (*TP53*) have also been implicated in stem cell self-renewal[45]. Self renewal makes stem cells an attractive candidate for the origin of prostate cancer. Support for the presence of adult prostate stem cells is evidenced by the regeneration of the prostate following replacement of androgens in castrated rodents[46]. Prostate stem cells may be selected for following androgen-deprivation. Recently, gene expression profiling has revealed putative markers of prostate cancer stem cells such as prostate stem cell antigen (*PSCA*), anti-proliferative b-cell translocation gene 1 (*BTG1*),

IL6, and hydroxyprostaglandin dehydrogenase 15-(NAD) (*HPGD*)[47]. Prostate cancer stem cells may express CD44 molecule (*CD44*) and prominin 1 (*PROMI*)[48], but not *AR* or *KLK3*[49].

The final suggested model of CRPC involves an imbalance between cell growth and cell death in the prostate[8]. Both processes may occur at the cellular level to potentially result in a net proliferation to increase tumour burden. Genes involved in the regulation of the cell cycle [e.g., cyclin D1 (*CCND1*), cyclin-dependent kinase inhibitor 1A (*CDKN1A*), cyclin-dependent kinase inhibitor 1B (*CDKN1B*), retinoblastoma 1 (*RB1*)] and cell survival/death [*BCL2*, BCL2-associated X protein (*BAX*), BCL2-like 1 (*BCL2L1*), and TP53] are prognostic factors for prostate cancer[50, 51].

Here, we describe long serial analysis of gene expression (LongSAGE) libraries[52, 53] made from RNA sampled from biological replicates of the *in vivo* LNCaP Hollow Fibre model of prostate cancer as it progresses to the castration-recurrent stage. Gene expression signatures that were consistent among the replicate libraries were applied to the models of CRPC to determine which of the suggested mechanisms underly progression.

3.2 MATERIALS AND METHODS

3.2.1 Cell culture. LNCaP human prostate cancer cells (provided by Dr. L.W.K. Chung at the Emory University School of Medicine, Atlanta, GA, USA) were maintained in RPMI-1640 media (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 5% v/v fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 units/ mL penicillin and 100 units/mL streptomycin (Invitrogen, Burlington, ON, Canada). Cells were maintained at 37°C with 5% CO_2.

3.2.2 Animals. Six-week-old male athymic BALB/c Nude mice were obtained from Taconic Farms (Hudson, NY, United States of America) and kept in the British Columbia Cancer Research Centre animal facilities (Vancouver, BC, Canada). Mice were maintained on a Harlan/Teklad irradiated diet with a constant supply of autoclaved water and housed in cages (three animals/cage) at 21°C ± 3°C with light/dark cycling (light between 6 AM and 6 PM). All

animal experiments were performed according to a protocol approved by the Committee on Animal Care of the University of British Columbia.

3.2.3 In vivo LNCaP Hollow Fibre model. LNCaP cells (3×10^7 at passage 43) suspended in media (50% v/v) and extracellular matrix (Matrigel; 50% v/v; BD Biosciences, Mississauga, ON Canada) were injected into Polyvinylidene difluoride hollow fibres (M_r 500,000 molecular weight cutoff; 1-mm internal diameter; Spectrum Laboratories, Rancho Dominguez, CA, USA). The fibres were heat-sealed prior to subcutaneous (s.c.) implantation into mice. Seven days post fibre implantation, mice were castrated to reduce levels of circulating androgens. Blood was drawn from the tail vein each week to measure levels of serum PSA to monitor the response to androgen-deprivation therapy and onset of castration-recurrence. Serum PSA levels were determined by enzymatic immunoassay kit (Abbott Laboratories, Abbott Park, IL, USA). Fibres were removed on three separate occasions representing different stages of hormonal progression that were androgen-sensitive (AS), responsive to androgen-deprivation (RAD), and castration-recurrent (CR). Samples were retrieved immediately prior to castration, as well as 10 and 72 days post-surgical castration.

3.2.4 RNA sample generation, processing, and quality control. Total RNA was isolated immediately from cells harvested from the *in vivo* Hollow Fibre model using TRIZOL Reagent (Invitrogen) following the manufacturer's instructions. Genomic DNA was removed from RNA samples with DNaseI (Invitrogen). RNA quality and quantity were assessed by the Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada) and RNA 6000 Nano LabChip kit (Caliper Technologies, Hopkinton, MA, USA).

3.2.5 Quantitative real-time polymerase chain reaction. To confirm that the transcriptomes of the samples reflect the different stages of prostate cancer progression (AS, RAD, and CR), *KLK3* mRNA expression trends were measured by quantitative real time-polymerase chain reaction (qRT-PCR) prior to LongSAGE library construction. Input RNA (0.5 μg) was reverse transcribed to cDNA with SuperScript III First Strand Synthesis kit (Invitrogen). A 10 μL reaction included 1 μl of template cDNA, 1x Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) and 0.3 μM each of forward and reverse intron-spanning primers (*KLK3,*

F': 5'-CCAAGTTCATGCTGTGTGCT-3' and R:' 5'-CCCATGACGTGATACCTTGA-3'; glyceraldehyde-3-phosphate (*GAPDH),* F': 5'-CTGACTTCAACAGCGACACC-3' and R:' 5'-TGCTGTAGCCAAATTCGTTG-3') that produce products 111 and 114 bp in size, respectively. Reactions were cycled as follows in a 7900HT Sequence Detection System (Applied Biosystems): 50 °C for 2 min, 95 °C for 2 min, (95 °C for 0.5 min, 55 °C for 0.3-0.5 min, and 72 °C for 0.5 min) for 40-45 cycles, 95 °C for 0.25 min, 60 °C for 0.25 min, and 95 °C for 0.25 min. Reactions for biological replicates were performed in technical triplicates. cDNAs (from different time-points along progression) and genes (target-*KLK3* and reference-*GAPDH*) to be directly compared were assayed in the same instrument run. Reactions without template (negative controls) were run for each gene to ensure that DNA had not contaminated the reactions. Only data with single-peak dissociation curves were included in this analysis. Efficiency checks were performed for each primer pair in each cell line. Products were sequenced to verify the identity of quantified transcripts.

3.2.6 LongSAGE library production and sequencing. RNA from the hollow fibres of three mice (biological replicates) representing different stages of prostate cancer progression (AS, RAD, and CR) were used to make a total of nine LongSAGE libraries. LongSAGE[53] libraries were constructed and sequenced at the Genome Sciences Centre, British Columbia Cancer Agency. Five micrograms of starting total RNA was used in conjunction with the Invitrogen I-SAGE Long kit and protocol, with alterations as previously published[54].

3.2.7 Gene expression analysis. LongSAGE expression data was analyzed with DiscoverySpace 4.01 software[55] (http://www.bcgsc.ca/bioinfo/software/discoveryspace/). Sequence data were filtered for bad tags (tags with one N-base call) and linker-derived tags (artifact tags). Only LongSAGE tags with a sequence quality factor (QF) greater than 95% were included in analysis.

The phylogenetic tree was constructed with a distance metric of 1-r (where "r" equals the Pearson correlation coefficient). The Pearson correlation is a measurement of similarity used to correlate variables (e.g., LongSAGE libraries). Here, it was used as a similarity measurement because it is not sensitive to scaling or differences in average expression level. Correlations

were computed (including tag counts of zero) using the Regress program of the Stat package written by Ron Perlman, and the tree was optimized using the Fitch program[56] in the Phylip package[57]. Graphics were produced from the tree files using the program TreeView[58].

Tag clustering analysis was performed using the Poisson distribution-based K-means clustering algorithm. The K-means algorithm clusters tags based on count into 'K' partitions, with the minimum intracluster variance. PoissonC was developed specifically for the analysis of SAGE data[59]. The java implementation of the algorithm was kindly provided by Dr. Li Cai (Rutgers University, NJ, USA). An optimal value for K (K=10) was determined as described[60].

3.3 RESULTS

3.3.1 LongSAGE library construction and composition

RNA isolated from the LNCaP Hollow Fibre model was obtained from at least three different mice (13N, 15N, and 13R; biological replicates) at three stages of cancer progression that were androgen-sensitive (AS), responsive to androgen-deprivation (RAD), and castration-recurrent (CR). To confirm that the samples represented unique disease-states, we determined the levels of *KLK3* mRNA, a biomarker that correlates with progression, using quantitative real time-polymerase chain reaction (qRT-PCR; **Figure 3.1**). As expected, *KLK3* mRNA levels dropped in the stage of cancer progression that was RAD versus AS (58%, 49%, and 37%), and rose in the stage of cancer progression that was CR versus RAD (229%, 349%, and 264%) for mice 13R, 15N, and 13N, respectively (**Figure 3.1**). Therefore, we constructed nine LongSAGE libraries, one for each stage and replicate.

Each LongSAGE library was sequenced to 310,072 - 339,864 tags for a combined total of 2,931,124 tags that were filtered prior to analysis (**Table 3.1**). First, 'bad tags' were removed because they contain at least one N-base-call in the LongSAGE tag sequence. The sequencing of the LongSAGE libraries was base-called using PHRED software[61, 62]. Tag sequence-quality factor (QF) and probability were calculated to ascertain which tags contain erroneous base-calls[63]. The second line of filtering removed LongSAGE tags with probabilities less than 0.95 (QF < 95%). Linkers were introduced into SAGE libraries as known sequences utilized to

amplify ditags prior to concatenation[52]. At a low frequency, linkers ligate to themselves creating linker-derived tags (LDTs). These LDTs do not represent transcripts and were removed from the LongSAGE libraries. A total of 2,305,589 useful tags represented by 263,197 tag types remained after filtering. Data analysis was carried out on this filtered data.

3.3.2 LongSAGE library and tag clustering

The LongSAGE libraries were hierarchically clustered and displayed as a phylogenetic tree. In most cases, LongSAGE libraries made from the same disease stage (AS, RAD, or CR) clustered together more closely than LongSAGE libraries made from the same biological replicate (mice 13N, 15N, or 13R; **Figure 3.3**). This suggests the captured transcriptomes were representative of disease stage with minimal influence from biological variation.

Identification of groups of genes that behave similarly during progression of prostate cancer was conducted through K-means clustering of tags using the PoissonC algorithm[59]. For each biological replicate (mice 13N, 15N, or 13R), all tag types were clustered that had a combined count greater than ten in the three libraries representing disease stages (AS, RAD, and CR) and mapped unambiguously sense to a transcript in reference sequence (RefSeq; February 28th, 2008)[64] using DiscoverySpace4 software[55]. By plotting within cluster dispersion (i.e., intracluster variance) against a range of clusters, K (**Figure 3.3**), we determined that ten clusters best embodied the expression patterns present in each biological replicate. This was decided based on the inflection point in the graph (**Figure 3.3**). K-means clustering was performed over 100 iterations, so that tags would be grouped in clusters determined to fit best most often. The most common clusters are displayed in **Figure 3.4**. In three instances, there were similar clusters in only two of the three biological replicates. Consequently, changes in gene expression during progression were represented in 11 patterns (**Figure 3.4**). Differences among expression patterns for each biological replicate may be explained by biological variation, the probability of sampling a given LongSAGE tag, and/or imperfections in K-means clustering (e.g, variance may not be a good measure of cluster scatter).

3.3.3 Gene ontology enrichment analysis

We conducted Gene Ontology (GO)[65] enrichment analysis using Expression Analysis Systematic Explorer (EASE)[66] software to determine whether specific GO annotations were over-represented in the K-means clusters. Enrichment was defined by the EASE score (p-value ≤ 0.05) generated during comparison to all the other clusters in the biological replicate. This analysis was done for each biological replicate (mice 13N, 15N, or 13R).

To enable visual differences between the 11 expression trends, the clusters were amalgamated into five major trends: 1) up during progression; 2) down during progression; 3) constant during progression; 4) expression peak at the stage of cancer progression that was RAD; and 5) expression valley at the stage of cancer progression that was RAD (**Figure 3.4**). To be consistent, the GO enrichment data was combined into five major trends which resulted in redundancy in GO terms. To simplify the GO enrichment data, similar terms were pooled into representative categories. Categorical gene ontology enrichments of the five major expression trends are shown in **Figure 3.5**. These data indicate that steroid binding, heat shock protein activity, de-phosphorylation activity, and glycolysis all decreased in the stage that was RAD, but increased again in the stage that was CR. Interestingly, steroid hormone receptor activity continues to increase throughout progression. Both of these expression trends were observed for genes with GO terms for transcription factor activity or secretion. The GO categories for genes with kinase activity and signal transduction displayed expression trends with peaks and valleys at the stage that was RAD. The levels of expression of genes involved in cell adhesion rose in the stage that was RAD, but dropped again in the stage that was CR.

Altogether, genes with functional categories that were enriched in expression trends revealed that the AR signalling pathway was perturbed during progression of prostate cancer to castration-recurrence (**Figure 3.5**). For example, GO terms steroid binding, steroid hormone receptor activity, heat shock protein activity, chaperone activity, and kinase activity could represent the cytoplasmic events of AR signalling. GO terms transcription factor activity, regulation of transcription, transcription corepression activity, and transcription co-activator activity could represent the nuclear events of AR signalling. AR-mediated gene transcription may result in splicing and protein translation, to regulate general cellular processes such as

proliferation (and related nucleotide synthesis, DNA replication, oxidative phosphorylation, oxioreductase activity, and glycolysis), secretion, and differentiation.

It should be noted, however, that both positive and negative regulators were represented in the GO enriched categories (**Figure 3.5**). Therefore, a more detailed analysis was required to determine if the pathways represented by the GO-enriched categories were promoted or inhibited during progression to CRPC. Moreover, many of the GO enrichments that were consistent with changes in the AR signalling pathway were generic, and could be applied to the other models of CRPC.

3.3.4 Consistent differential gene expression associated with progression of prostate cancer

Pair-wise comparisons were made between LongSAGE libraries representing the transcriptomes of different stages (AS, RAD, and CR) of prostate cancer progression from the same biological replicate (mice 13N, 15N, or 13R). Among all three biological replicates, the number of consistent significant differentially expressed tag types were determined using the Audic and Claverie test statistic[67] at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ (**Table 3.2**). The tags represented in **Table 3.2** were included only if the associated expression trend was common among all three biological replicates. The Audic and Claverie statistical method is well-suited for LongSAGE data, because the method takes into account the sizes of the libraries and tag counts. Tag types were counted multiple times if they were over, or under-represented in more than one comparison. The number of tag types differentially expressed decreased by 231 - 267% as the stringency of the p-value increased from $p \leq 0.05$ to 0.001.

Tag types consistently differentially expressed in pair-wise comparisons were mapped to RefSeq (March 4th, 2008). Tags that mapped anti-sense to genes, or mapped ambiguously to more than one gene were not included in the functional analysis. GO, Kyoto Encyclopedia of Genes and Genomes (KEGG; v45.0)[68] pathway, and SwissProt (v13.0)[69] keyword annotation enrichment analyses were conducted using EASE (v1.21; March 11th, 2008) and FatiGO (v3; March 11th, 2008)[70] (**Table 3.3**). This functional analysis revealed that the expression of genes involved in signalling increased during progression, but the expression of genes involved in

protein synthesis decreased during progression. Cell communication increased in the stage that was RAD but leveled off in the stage that was CR. Carbohydrate, lipid and amino acid synthesis was steady in the RAD stage but increased in the CR stage. Lastly, glycolysis decreased in the RAD stage, but was re-expressed in the CR stage (**Table 3.3**).

Tag types differentially expressed between the RAD and CR stages of prostate cancer were of particular interest (**Table 3.4**). This is because these tags potentially represent markers for CRPC and/or are involved in the mechanisms of progression to CRPC. These 193 tag types (**Table 3.2**) were mapped to databases RefSeq (July 9th, 2007), Mammalian Gene Collection (MGC; July 9th, 2007)[71], or Ensembl Transcript or genome (v45.36d)[72]. Only 135 of the 193 tag types were relevant (**Table 3.4**) with 48 tag types that mapped ambiguously to more than one location in the *Homo Sapiens* transcriptome/genome, and another 10 tag types that mapped to *Mus musculus* transcriptome/genome. *Mus musculus* mappings may be an indication of minor contamination of the *in vivo* LNCaP Hollow Fibre model samples with host (mouse) RNA. These 135 tag types represented 114 candidate genes with 7 tag types that did not map to the genome, 5 tag types that mapped to unannotated genomic locations, and 9 genes that were associated with more than one tag type. **Table 3.4** shows the LongSAGE tag sequences and tag counts per million tags in all nine libraries. Tags were sorted into groups based on expression trends. These trends are visually represented in the 'trend legend' for interpretation. Mapping information was provided where available.

We cross-referenced these 114 candidate genes with 28 papers that report global gene expression analyses on tissue samples from men with 'castration-recurrent', 'androgen independent,' 'hormone refractory,' 'androgen-ablation resistant,' 'relapsed,' or 'recurrent' prostate cancer, or animal models of castration-recurrence[73-100]. The candidate genes were identified with HUGO Gene Nomenclature Committee (HGNC)[101] approved gene names, aliases, descriptions, and accession numbers. The gene expression trends of 18 genes of 114 genes were previously associated with CRPC. These genes were: *ACPP, ADAM2, AMACR, AMD1, ASAH1, DHCR24, FLNA, KLK3, KPNB1, PLA2G2A, RPL13A, RPL35A, RPL37A, RPL39, RPLP2, RPS20, STEAP2,* and *TACC* (**Table 3.4**). To our knowledge, the gene

expression trends of the remaining 96 genes have never before been associated with CRPC (**Tables 3.4** and **3.5**).

A literature search helped to gauge the potential of these 96 genes to be novel biomarkers or therapeutic targets of CRPC. The results of this literature search are presented in **Table 3.5**. We found 31 genes that encode for protein products that are known, or predicted, to be plasma membrane bound or secreted extracellularly (Bioinformatic Harvester; May 14th, 2008)[102]. These genes were: *ABHD2, AQP3, B2M, C19orf48, CD151, CXCR7, DHRS7, ELOVL5, ENDOD1, ENO2, FGFRL1, GNB2L1, GRB10, HLA-B, MARCKSL1, MDK, NAT14, NELF, OPRK1, OR51E2, PLCB4, PTGFR, RAMP1, S100A10, SPON2, STEAP1, TFPI, TMEM30A, TMEM66, TRPM8,* and *VPS13B*. Secretion of a protein could facilitate detection of the putative biomarkers in blood, urine, or biopsy sample. Twenty of the candidate genes are known to alter their levels of expression in response to androgen. These genes were: *B2M, BTG1, C19orf48, CAMK2N1, CXCR7, EEF1A2, ELOVL5, ENDOD1, HSD17B4, MAOA, MDK, NKX3-1, ODC1, P4HA1, PCGEM1, PGK1, SELENBP1, TMEM66, TPD52,* and *TRPM8*[24, 54, 103-114]. Genes regulated by androgen may be helpful in determining the activation status of AR in CRPC. Enriched expression of a protein in prostate tissue could be indicative of whether a tumour is of prostatic origin. Five of these 96 genes are known to be over-represented in prostate tissue[108, 115-117]. These genes were: *NKX3-1, PCGEM1, SPON2, STEAP1,* and *TPD52.* Twenty genes (*ABHD2, BNIP3, EEF1A2, GALNT3, GLO1, HSD17B4, MARCKSL1, MDK, ODC1, OR51E2, PCGEM1,* PCOTH, *PP2CB, RPS18, SELENBP1, SLC25A4, SLC25A6, STEAP1, TPD52,* and *TRPM8*) have known associations to prostate cancer[88, 118-134]. Six genes (*C1orf80, CAMK2N1, GLO1, MAOA, PGK1,* and *SNX3)* have been linked to high Gleason grade[89, 135, 136], and eight genes (*B2M, CD151, COMT, GALNT3, ODC1, PCGEM1,* PCOTH, and *TPD52*) have been implicated in the 'progression' of prostate cancer[89], and 15 more genes (*CD151, CXCR7, DHRS7, GNB2L1, HES6, HN1, NKX3-1, PGK1, PIK3CD, RPL11, RPS11, SF3A2, TK1, TPD52,* and *VPS13B*) in the metastasis of prostate cancer[137, 138].

3.4 DISCUSSION

Genes that change levels of expression during hormonal progression may be indicative of the mechanisms involved in CRPC. Large-scale gene expression analyses have been performed with tissue samples from men with advanced prostate cancer[73-89], and animal or xenograft models of CRPC[90-100]. Here we provide the most comprehensive gene expression analysis to date of prostate cancer with approximately 3 million tags sequenced using *in vivo* samples at various stages of hormonal progression.

The LNCaP Hollow Fibre model[139] mimics the hormonal progression observed clinically in response to host castration as measured by levels of expression of PSA[139, 140]. The stages of progression include: AS, RAD, and CR. The LNCaP Hollow Fibre model overcomes some of the limitations problematic in other studies, such as host contamination of prostate cancer cells. LNCaP human prostate cancer cells were grown in hollow fibres that were implanted subcutaneously into immunocompromised mice. The fibres are impervious to the movement of cells into or out of the fibre, but porous to proteins and metabolites[141]. The fibres permit the isolation of pure populations of prostate cancer cells. Moreover, the progression of cells to CRPC may be followed within the same host mouse over time, because the retrieval of a subset of fibres entails only minor surgery[141]. The power to evaluate progression within the same mouse minimizes biological variation. Furthermore, the model involves the growth of a human cell line *in vivo* to potentially minimize heterogeneity in the sample.

Deeply sequenced LongSAGE libraries[52, 53] were made using RNA sampled from the *in vivo* LNCaP Hollow Fibre model of prostate cancer as it progresses from the AS to CR stage. We used this LNCaP atlas to identify changes in gene expression that may provide clues of underlying mechanisms resulting in CRPC. Suggested models of CRPC involve: the AR[3]; steroid synthesis and metabolism[9]; neuroendocrine prostate cancer cells[43]; prostate cancer stem cells[44]; and/or an imbalance of cell growth and cell death[8].

3.4.1 Support for or against the proposed models of castration-recurrent prostate cancer

Androgen receptor (AR)

Transcriptional activity of AR

The AR is suspected to continue to play an important role in the hormonal progression of prostate cancer. The AR is a ligand-activated transcription factor with its activity altered by changes in its level of expression or by interactions with other proteins. Here, we identified changes in expression of 5 genes that are known, or suspected, to impact the transcriptional activity of the AR in CRPC versus RAD. Cyclin H (*CCNH*), and proteasome macropain subunit alpha type 7 (*PSMA7*) displayed increased levels of expression, while CUE-domain-containing-2 (*CUEDC2*), filamin A (*FLNA*), and high mobility group box 2 (*HMGB2*) displayed decreased levels of expression. The expression trends of *CCNH, CUEDC2, FLNA,* and *PSMA7* in CRPC may result in increased AR signalling through mechanisms involving protein-protein interactions or altering levels of expression of AR. CCNH protein is a component of the cyclin-dependent activating kinase (CAK). CAK interacts with the AR and increases its transcriptional activity [142]. Over-expression of the proteosome subunit PSMA7 promotes AR transactivation of a PSA-luciferase reporter[143]. A fragment of the protein product of *FLNA* negatively regulates transcription by AR through a physical interaction with the hinge region[144]. CUEDC2 protein promotes the degradation of progesterone and estrogen receptors[145]. These steroid receptors are highly related to the AR, indicating a possible role for CUEDC2 in AR degradation. Thus, decreased expression of *FLNA* or *CUEDC2* could result in increased activity of the AR. Decreased expression of *HMGB2* in CRPC is predicted to decrease expression of at least a subset of androgen-regulated genes that contain palindromic AREs[146]. Here, genes known to be regulated by androgen were enriched in expression trend categories with a peak or valley at the RAD stage of prostate cancer progression. Specifically, 8 of the 13 tags (62%) exhibiting these expression trends 'E', 'F', 'J', 'K', or 'L' represented known androgen-regulated genes, in contrast to only 22 of the remaining 122 tags (18%; **Tables 3.4** and **3.5**). Overall, this data supports increased AR activity in CRPC, which is consistent with re-expression of androgen-regulated genes as previously reported[99].

Steroid synthesis and metabolism

In addition to changes in expression of AR or interacting proteins altering the transcriptional activity of the AR, recent suggestion of sufficient levels of residual androgen in CR prostate tissue provides support for an active ligand-bound receptor[147]. The AR may become re-activated in CRPC due to the presence of androgen that may be synthesized by the prostate *de novo*[9] or through the conversion of adrenal androgens[24-26]. In a phase I clinical trial, abiraterone acetate reduced levels of testosterone and PSA in the serum of patients with CRPC[148]. Abiraterone acetate inhibits *CYP17* to prevent the synthesis of steroids. Together, these data support continued AR signalling in CRPC . Here, the expression of 5 genes known to function in steroid synthesis or metabolism were significantly differentially expressed in CRPC versus RAD. Of these genes, 24-dehydrocholesterol reductase (*DHCR24*), dehydrogenase/reductase SDR-family member 7 (*DHRS7*), elongation of long chain fatty acids family member 5 (*ELOVL5*), hydroxysteroid (17-beta) dehydrogenase 4 (*HSD17B4*), and opioid receptor kappa 1 (*OPRK1*) displayed increased levels of expression. The expression trends of *DHCR24, DHRS7, ELOVL5, HSD17B4*, and *OPRK1* in CRPC may result in increased steroid synthesis or metabolism.

Steroid synthesis and metabolism is controlled by the hypothalamus-pituitary-adrenal (HPA) axis. Leutinizing hormone releasing hormone (LHRH) is secreted by the LHRH cells of the hypothalamus. LHRH stimulation causes the pituitary gland to release leutinizing hormone and adrenocorticotropic hormone (ACTH), which acts on the testes and adrenal gland to produce androgens (testosterone and androstenedione, respectively). *OPRK1* gene product functions in the HPA axis. The OPRK1 agonist, U50488H, stimulates the release of ACTH from the pituitary gland in rhesus monkeys. This action is blocked by the OPRK1-specific antagonist, norbinaltorphimine. It should be noted that mice do not produce the enzyme necessary for the synthesis of adrenal steroids[149], and so castrated mice presumably would not produce androstenedione upon stimulation of OPRK1 and release of ACTH.

Cholesterol is the precursor of steroid hormones. The enzyme DHCR24 converts desmosterol to cholesterol in the final step of cholesterol synthesis[150]. DHRS7 and RoDH-like 3-alpha hydroxysteroid dehydrogenase are retinoid pathway enzymes and SDR family members[151]. In addition to its role as a retinol dehydrogenase, RoDH-like 3-alpha hydroxysteroid

dehydrogenase converts 3-alpha androstanediol (a weak androgen) to 5-alpha dihydrotestosterone (a potent androgen)[152]. Although a role for DHRS7 in androgen metabolism has not been established, it is possible this enzyme may also exhibit promiscuous substrate specificity. The androgen metabolic pathway enzyme HSD17B4 converts testosterone (a relatively potent androgen) to androstenedione (a relatively weak androgen)[152]. ELOVL5 protein functions in fatty acid synthesis, and may be important in the synthesis of the male effect pheromone in goats[153, 154]. Overall, increased levels of expression of *DHCR24, DHRS7, ELOVL5, HSD17B4*, and *OPRK1* may be indicative of the influence of adrenal androgens, or the local synthesis of androgen, to reactivate the AR to promote the progression of prostate cancer in the absence of testicular androgens.

Neuroendocrine

Androgen-deprivation induces neuroendocrine differentiation of prostate cancer. Here, the expression of 8 genes that are associated with neuroendocrine cells were significantly differentially expressed in CRPC versus RAD. Of these genes, *ENO2* (see above), *OPRK1* (see above), S100 calcium binding protein A10 (*S100A10*), and transient receptor potential cation channel subfamily M member 8 (*TRPM8*) displayed increased levels of expression, and hairy and enhancer of split 6 (*HES6*), karyopherin/importin beta 1 (*KPNB1*), monoamine oxidase A (*MAOA*), and receptor (calicitonin) activity modifying protein 1 (*RAMP1*) displayed decreased levels of expression.

The expression trends of *ENO2, MAOA, OPRK1, S100A10,* and *TRPM8* in CRPC may be indicative of neuroendocrine differentiation. The protein product of *ENO2* is a marker for neuroendocrine differentiation in prostate cells[43]. Neuroendocrine-like prostate cancer cells secrete hormones such as serotonin and neurotensin[43]. S100A10 protein mediates transport of serotonin receptors (5-HT_{1B}) to the plasma membrane[155]. MAOA is a mitochondrial enzyme that inactivates the neurotransmitter serotonin[156]. Down-regulation of *MAOA* in CRPC may render the cells sensitive to serotonin signalling. TRPM8 is a membrane channel protein that regulates the secretion of neurotensin in the neuroendocrine pancreatic tumour cell line, BON[157]. Finally, the role of *OPRK1* in promoting ACTH release (see steroid synthesis and metabolism section above) also supports this neuroendocrine model[158]. However, some inconsistencies were

observed here. Decreased expression of *KPNB1, RAMP1,* and *HES6* in CRPC is contrary to the neuroendocrine model of prostate cancer. PTHrP is expressed by prostatic neuroendocrine cells[159]. PTHrP possesses both paracrine and intracrine signalling properties. Intracrine PTHrP signalling involves shuttling between the cytoplasm and the nucleus[160]. *KPNB1* gene encodes an intracellular transport receptor for PTHrP that is important for intracrine signalling[161]. Moreover, RAMP1 forms a receptor for calcitonin gene-related peptide, a neuroendocrine hormone[43], with calcitonin receptor like receptor[162]. Finally, *HES6* expression is a marker of the neuroendocrine phenotype in the prostate cancer models CR2 TAg[163] and Cre LoxP *TP53* (PE -/-), *RB1* (PE -/-)[164]. Overall, however, there was more support for, than against, the neuroendocrine model of CRPC. Increased expression of genes associated with neuroendocrine differentiation in response to androgen deprivation *in vivo* is consistent with neuroendocrine differentiation of LNCaP xenografts in castrated mice[36].

Stem cell

The protein products of 6 genes that have been associated with stem cells were significantly differentially expressed in CRPC versus RAD. Of these genes, aquaporin 3 (*AQP3*), *BTG1* (see above), and spondin 2 (*SPON2*) displayed increased levels of expression, and CD151 molecule (*CD151*), *HES6* (see above), and hematological and neurological expressed 1 (*HN1*) displayed decreased levels of expression. The expression trends of *AQP3, BTG1,* and *SPON2* support a role for stem cells in CRPC. *AQP3* is a marker for progenitor cells of the fetal airway[165]. Expression of the *BTG1* gene is altered in putative prostate cancer stem cells[47]. SPON2 is a secreted activator of Wnt[166]. Wnt signalling is thought to be important for self-renewal in intestinal stem cells[45]. However, the expression trends of *CD151, HES6,* and *HN1* do not support a role for stem cells in CRPC. Recently, *HN1* has been identified as a nerve regeneration-associated gene in newts and mice[167, 168]. HES6 protein is an effector of Notch[169]. The Notch signalling pathway is important for the self-renewal of human mammary stem cells. *CD151* is expressed on transient amplifying epithelial cells in human adult airway tissue[170]. Taken together, it remains unclear if the stem cell model of CRPC was represented in the LNCaP Hollow Fiber model. It should be noted, however, that the existence of prostate cancer stem cells is controversial[171]. Further studies are necessary to confirm the markers of this population, if it indeed exists.

Proliferation and survival

The protein products of 31 genes that are associated with cell growth, cell cycle arrest, cell death, and/or survival were significantly differentially expressed in CRPC versus RAD. Of these genes, adenosylmethionine decarboxylase 1 (*AMD1*), BCL2/adenovirus E1B 19kDa interacting protein 3 (*BNIP3*), *BTG1*(see above), calcium/calmodulin dependent protein kinase 2 inhibitor 1 (*CAMK2N1*), fibroblast growth factor receptor like 1 (*FGFRL1*), glyoxalase I (*GLO1*), NADH-ubiquinone oxidoreductase chain 3 (*MT-ND3*), nerve growth factor receptor associated protein 1 (*NGFRAP1*), *NKX3-1* (see above), prostate specific non-coding gene (*PCGEM1*), protein phosphatase 2 catalytic subunit beta isoform (*PPP2CB*), prostaglandin F receptor (*PTGFR*), *S100A10* (see above), solute carrier family 25 member 4 (*SLC25A4*), six transmembrane epithelial antigen of the prostate 1 (*STEAP1*), cell cycle control protein 50A (*TMEM30A*), transmembrane protein 66 (*TMEM66*), *TRPM8* (see above), and *WDR45L* (see above) displayed increased levels of expression. In contrast, chaperonin containing TCP1 subunit 2 (*CCT2*), growth arrest specific 5 on chromosome 1 (*GAS5*), guanine nucleotide binding protein beta polypeptide 2 like 1 (*GNB2L1*), growth factor receptor bound protein 10 (*GRB10*), MARCKS-like 1 (*MARCKSL1*), midkine (*MDK*), ornithine decarboxylase 1 (*ODC1*), prostate collagen triple helix (PCOTH), phosphoinositide-3-kinase catalytic delta polypeptide (*PIK3CD*), protein phosphatase 2 regulatory subunit A alpha (*PPP2R1A*), solute carrier family 25 member 6 (*SLC25A6*), and tyrosine 3 monooxygenase/tryptophan 5 monooxygenase activation protein (theta polypeptide; *YWHAQ*) displayed decreased levels of expression.

Proliferation

The gene expression trends of *GAS5, GNB2L1, MT-ND3, NKX3-1, PCGEM1, PTGFR, STEAP1,* and *TMEM30A* were in agreement with the presence of proliferating cells in CRPC. *GAS5* is a small nucleolar RNA that is required for growth arrest in T-cells[172]. The expression of *GNB2L1* mRNA was decreased in CRPC compared to RAD. The *GNB2L1* gene encodes the RACK1 adapter protein. Over-expression of *GNB2L1* in NIH3T3 cells inhibits insulin-like growth factor (IGF-1)-mediated proliferation and promotes cell adhesion[173]. Moreover, over-expression of *GNB2L1* in MCF7 cells negatively regulates IGF-I-induced activation/phosphorylation of protein kinase B (Akt), reducing cell survival following etoposide treatment[174]. Over-expression of *GNB2L1* suppresses v-src sarcoma (Src) activity, and delays

progression through the cell cycle in colon cancer cells[175]. It is conceivable that reduced levels of expression of *GNB2L1* may increase the proliferation and survival of cells. *MT-ND3* is a human-encoded mitochondrial gene important for oxidative phosphorylation. Expression of *MT-ND3* is indicative of metabolically active cells. *NKX3-1* is a tumour suppressor. *NKX3-1* mutant mice develop PIN-like lesions[176], and over-expression of *NKX3-1* results in reduced proliferation and enhanced apoptosis[177]. NKX3-1 protein antagonizes Akt and stabilizes TP53[177]. Expression of *NKX3-1* mRNA *in vitro* is significantly reduced in the castration-recurrent C4-2 prostate cancer cell line, compared to androgen-sensitive LNCaP prostate cancer cell line[93]. Interestingly, we observed that a transcript anti-sense to *NKX3-1* was highly expressed in the stages of cancer progression that were AS and CR, but not RAD. Anti-sense transcription may hinder gene expression from the opposing strand[178], and therefore, represents a novel mechanism by which *NKX3-1* expression may be silenced. *PCGEM1* mRNA was highly expressed in CRPC versus RAD. *PCGEM1* is a prostate-specific, non-coding transcript that promotes cell proliferation and colony formation in LNCaP cells[179]. *PCGEM1* also rescues LNCaP cells from doxorubicin-induced apoptosis. Reduced expression of *TP53* and *CDKN1A* are observed in *PCGEM1*-over-expressing cells[180]. Together with prostaglandin D2, PTGFR enhances the proliferation of prostate cancer cells via the phosphoinositide-3-kinase (PI3K)/Akt pathway[181]. *TMEM30A* were in agreement with proliferating cells in CRPC. *TMEM30A* is the human homolog of yeast *Cdc50p*. Cdc50p is required for the localization of Bni1p, a protein important for microtubule regulation in asymmetric cell division of budding yeast[182]. STEAP1 protein functions in cellular communication and promotes the growth of LAPC9 prostate tumours[183]. However, some inconsistencies include the expression trends of *BTG1, FGFRL1,* and PCOTH and that may be associated with non-cycling cells. Decreased proliferation is associated with increased expression of *BTG1*[184] and *FGFRL1*[185]. Based upon the increased proliferation of LNCaP cells with increased expression of PCOTH, the decreased expression illustrated herein would be expected to be associated with reduced growth[186]. Overall, there was more support at the transcriptome level for proliferation than not, which was consistent with increased proliferation in the LNCaP Hollow Fiber model[139].

Cell survival

Gene expression trends of *GLO1, S100A10, TRPM8,* and *PI3KCD* suggest cell survival pathways are active following androgen-deprivation and/or in CRPC. Expression of *GLO1, S100A10,* and *TRPM8* were elevated in the stage of cancer progression that was CR versus RAD. GLO1 promotes resistance to apoptosis in response to etoposide and adriamycin in leukemia cells[187], and S100A10 binds Bcl-xL/Bcl-2 associated death promoter (BAD) and inhibits its induction of apoptosis in rodent ovarian cells[188]. TRPM8 is required for the survival of LNCaP cells[189] possibly through regulation of intracellular calcium stores[190], which function to promote growth arrest and apoptosis in prostate cancer cells[191]. LNCaP cells decrease proliferation in response to androgen-deprivation. The mechanism is suggested to involve constitutively active PI3K/Akt pathway due to the deletion of the *PTEN* tumour suppressor[192, 193]. The PI3K/Akt pathway is the predominant survival pathway in LNCaP cells[194]. The PI3K/Akt pathway inhibitors, wortmannin or LY294002, cause apoptosis of LNCaP cells under conditions of androgen-deprivation[194]. The expression of *PI3KCD* was highest in the RAD stage of prostate cancer and was consistent with an active PI3K/Akt pathway following androgen-deprivation. While expression trends for these genes support the observed increased tumour burden observed in CRCP, gene expression trends of *CAMK2N1, CCT2, MDK, TMEM66,* and *YWHAQ* may oppose such suggestion. The levels of *CAMK2N1* and *TMEM66* mRNA were increased in CRPC versus RAD. Over-expression of *TMEM66* in human embryonic kidney cells promotes apoptosis[195], and CAMK2N1 protein inhibits the signalling of CAMK2, a kinase that supports cell survival of prostate cells[196]. In contrast, the levels of *CCT2, MDK,* and *YWHAQ* mRNA were decreased in CRPC versus RAD. CCT2 is a protein chaperone indispensable for cell survival[197]. MDK is a cytokine that participates in cell growth, survival, angiogenesis, migration, and transformation[198]. siRNA knock down of *MDK* gene expression in LNCaP cells results in enhanced tumour necrosis factor alpha-induced cell death[199]. In contrast to earlier reports in which *MDK* gene and protein expression was determined to be higher in late stage cancer[94, 199], we observed a drop in the levels of *MDK* mRNA in CRPC versus RAD. *MDK* expression is negatively regulated by androgen[96]. Therefore, the decreased levels of *MDK* mRNA in CRPC may suggest that the AR is reactivated in castration-recurrence. The relatively low level of expression of *YWHAQ* mRNA in CRPC was not consistent with cell survival. YWHAQ binds BAD and prevents it from inducing apoptosis in rodent ovarian cells[188].

YWHAQ also binds and negatively regulates pro-apoptotic Bax protein[200]. Taken together, these data neither agree nor disagree with the activation of survival pathways in CRPC.

Other

The significance of the gene expression trends of *AMD1, BNIP3, GRB10, MARCKSL1, NGRAP1, ODC1, PPP2CB, PPP2R1A, SLC25A4, SLC25A6,* and *WDR45L* that function in cell growth or cell death/survival were not straightforward. For example, *BNIP3* and *WDR45L,* both relatively highly expressed in CRPC versus RAD, may be associated with autophagy. BNIP3 promotes autophagy in response to hypoxia[201], and the WDR45L-related protein, WIPI-49, co-localizes with the autophagic marker LC3 following amino acid depletion in autophagosomes[202]. Autophagy, or "self-eating" is the process of digesting cellular components in response to nutrient deprivation. Autophagy has two possible outcomes. Breakdown of cellular components may provide temporary "food" for a nutrient-deprived cell and promote cell survival. Alternatively, if nutrient-deprivation persists, self-eating may lead to cell death[203]. It is not known if BNIP3 or putative WDR45L-associated autophagy results in cell survival or death. Levels of expression of *NGFRAP1* were increased in CRPC versus RAD. The protein product of *NGFRAP1* interacts with p75 (NTR). Together they process caspase 2 and caspase 3 to active forms, and promote apoptosis in 293T cells[204]. NGFRAP1 requires p75 (NTR) to induce apoptosis. However, LNCaP cells do not express p75 (NTR), and so it is not clear if apoptosis would occur in this cell type[205]. Studies on *NGFRAP1* in the prostate are lacking. The levels of expression of *MARCKSL1* and *GRB10* were relatively low in CRPC versus RAD. Over-expression of *MARCKSL1* results in increased proliferation of retinal cells, but has no effect on NIH3T3 cells[206]. Moreover, the adapter protein GRB10, may positively or negatively regulate insulin-like growth factor signalling, depending on the presence of the milieu of GRB10-interacting proteins[207, 208]. These data indicate that the effects of expression of *MARCKSL1* and *GRB10* are cell-type specific.

Pairs of genes (*AMD1* and *ODC1, PPP2CB* and *PPP2R1A,* and *SLC25A4* and *SLC25A6*) from the same pathways, exhibit differential expression trends in CRPC versus RAD, and yield conflicting information regarding the proliferative and/or survival status of CRPC. ODC1 and ADM1 are rate limiting enzymes of the polyamine pathway. In the prostate, ADM1 catalyzes

the conversion of s-adenosylmethionine to decarboxylated s-adenosylmethionine, and ODC1 catalyzes the conversion of ornithine to putrescine. Spermidine synthase catalyzes the reaction between putrescine and an acetyl group from decarboxylated s-adenosylmethionine to create spermidine. Upon the addition of another acteyl group from decarboxylated s-adenosylmethionine, spermidine is converted to spermine by spermine synthase. The significance of polyamines in prostate cancer is not clear[209] as spermidine and putrescine promote proliferation, and spermine is associated with differentiation[210, 211]. ODC1 protein over-expression has been observed in human prostate cancer specimens, however, the degree of over-expression lessens in CR tissue[212]. This is consistent with our observation that *ODC1* mRNA levels progressively decreased during progression to castration-recurrence. The significance of decreasing levels of *ODC1* mRNA and increasing levels of *AMD1* mRNA in CRPC is not known. However, simultaneous reduced expression of *ODC1* and *ADM1* in prostate cancer cells results in reduced proliferation[213]. *PPP2CB* and *PPP2R1A* encode isoforms of the catalytic and regulatory subunits of protein phosphatase 2 (PPP2), respectively[214]. The contradictory expression of these subunits may reflect the dual nature of PPP2. PPP2 is essential for cell survival, but emerging evidence suggests it may also be a tumour suppressor[215]. SLC25A4 and SLC25A6 are highly related ADP/ATP carriers of the mitochondrial membrane. These proteins are key players in oxidative phosphorylation, but they also participate in the formation of the mitochondrial transition pore, a complex required for the release of cytochrome C and the induction of apoptosis[216]. The opposite regulation of *SLC25A4* and *SLC25A6* in CRPC may represent the antagonizing roles of mitochondria in cell growth and cell death.

Overall, genes involved in cell growth and cell death pathways were altered in CRPC. Increased tumour burden may develop from a small tip in the balance when cell growth outweighs cell death. Unfortunately, the contributing weight of each gene is not known, making predictions difficult based on gene expression alone of whether proliferation and survival were represented more than cell death in this model of CRPC. It should be noted that LNCaP cells are androgen-sensitive and do not undergo apoptosis in the absence of androgens. The proliferation of these cells tend to decrease in androgen-deprived conditions, but eventually with progression begin to grow again mimicking clinical CRPC. Similarly, increased proliferation is observed in CR samples from the LNCaP Hollow Fibre model[139].

3.5 CONCLUSION

Here, we describe the LNCaP atlas, a compilation of LongSAGE libraries that catalogue the transcriptome of human prostate cancer cells as they progress to CRPC *in vivo*. Using the LNCaP atlas, we identified differential expression of 96 genes that were associated with castration-recurrence *in vivo*. These genes were characterized for their potential to be therapeutic targets of CRPC. Moreover, changes in gene expression profiles were identified that support a role for the AR, steroid synthesis and metabolism, neuroendocrine cells, and increased proliferation in castration-recurrence. The gene expression trends neither supported nor discounted a role for stem cells, or an imbalance of cell death in CRPC.

Table 3.1 Composition of LongSAGE libraries

		Library	S1885	S1886	S1887	S1888	S1889	S1890	S1891	S1892	S1893
		Mouse-Condition	13N-AS*	13N-RAD†	13N-CR‡	15N-AS	15N-RAD	15N-CR	13R-AS	13R-RAD	13R-CR
		Unfiltered Total Tags	310,516	318,102	339,864	338,210	310,072	326,870	337,546	314,440	335,504
		No. of Bad Tags	955	1,010	1,083	1,097	983	737	900	744	832
Minus Bad Tags		Total Tags	309,561	317,092	338,781	337,113	309,089	326,133	336,646	313,696	334,672
		Tag Types	79,201	96,973	99,730	81,850	84,499	88,249	79,859	91,438	90,675
		No. of Duplicate Ditags	19,761	12,220	12,678	21,973	17,471	12,836	24,552	12,786	13,127
		% of Duplicate Ditags	6.38	3.85	3.74	6.52	5.65	3.94	7.29	4.08	3.92
		Average QF§ of Tags	0.85	0.88	0.87	0.86	0.89	0.88	0.88	0.80	0.87
		No. of Tags QF<0.95%	63,057	62,872	71,576	68,993	54,627	54,470	68,981	101,215	69,647
	Q ≥0.95	Total Tags	246,504	254,220	267,205	268,120	254,462	271,663	267,665	212,481	265,025
		Tag Types	52,033	67,542	66,748	52,606	59,374	64,985	53,715	54,682	64,837
		Total Tags Combined					2,307,345				
		Tag Types Combined					263,199				
		No. of LDTs|| Type I	124	72	174	179	84	186	164	118	301
		No. of LDTs Type II	19	9	54	56	33	40	60	24	59
	Minus LDTs	Total Tags	246,361	254,139	266,977	267,885	254,345	271,437	267,441	212,339	264,665
		Tag Types	52,031	67,540	66,746	52,604	59,372	64,983	53,713	54,680	64,835
		Total Tags Combined					2,305,589				
		Tag Types Combined					263,197				

* AS, Androgen-sensitive
† RAD, Responsive to androgen-deprivation
‡ CR, Castration-recurrent
§ QF, Quality Factor
|| LDTs, Linker-derived Tags

Table 3.2 Number of tag types consistently and significantly differentially expressed among all three biological replicates and between conditions*

Comparison	Change	$p \leq 0.001$	$p \leq 0.01$	$p \leq 0.05$
AS† vs. RAD‡	Up in RAD	21	44	83
	Down in RAD	68	105	149
	Total	89	149	232
RAD vs. CR§	Up in CR	24	45	89
	Down in CR	46	59	104
	Total	70	104	193
AS vs. CR	Up in CR	111	167	294
	Down in CR	127	168	256
	Total	238	335	550

* Statistics according to the Audic and Claverie test statistic
† AS, Androgen-sensitive
‡ RAD, Responsive to androgen-deprivation
§ CR, Castration-recurrent

Table 3.3 Top five enrichments of functional categories of tags consistently and significantly differentially expressed among all three biological replicates and between stages of prostate cancer*

Top 5 GO† categories	P-value‡	Top 5 KEGG§ annotations	P-value‖	Top 5 SwissProt annotations	P-value‖
AS vs. RAD: Up in RAD ¶					
Cell communication	2.E-02	Stilbene, coumarine and lignin biosynthesis	1.E-02	Antioxidant	7.E-04
Extracellular	2.E-02	Butanoate metabolism	2.E-02	Cell adhesion	5.E-03
Extracellular matrix	2.E-02	2,4-Dichlorobenzoate degradation	2.E-02	Signal	6.E-03
Synaptic vesicle transport	3.E-02	Cell adhesion molecules (CAMs)	2.E-02	Fertilization	7.E-03
Synapse	4.E-02	Alkaloid biosynthesis II	5.E-02	Amyotrophic lateral sclerosis	7.E-03
AS vs. RAD: Down in RAD					
Glycolysis	3.E-05	Glycolysis / Gluconeogenesis	3.E-05	Glycolysis	3.E-07
Glucose catabolism	1.E-04	Ribosome	2.E-03	Pyrrolidone carboxylic acid	8.E-05
Hexose catabolism	1.E-04	Carbon fixation	3.E-03	Pyridoxal phosphate	2.E-04
Hexose metabolism	2.E-04	Fructose and mannose metabolism	2.E-02	Gluconeogenesis	3.E-04
Monosaccharide catabolism	2.E-04	Urea cycle and metabolism of amino groups	3.E-02	Coiled coil	5.E-03
RAD vs. CR: Up in CR					
Acid phosphatase activity	4.E-02	gamma-Hexachlorocyclohexane degradation	5.E-03	Lyase	2.E-03
Lyase activity**	7.E-02	Glycolysis / Gluconeogenesis	3.E-02	Immune response	5.E-03
Carbohydrate metabolism**	9.E-02	O-Glycan biosynthesis	5.E-02	Signal	6.E-03
Extracellular**	1.E-01	Ether lipid metabolism**	6.E-02	Glycolysis	7.E-03
Catabolism**	1.E-01	Phenylalanine, tyrosine and tryptophan biosynthesis **	6.E-02	Progressive external ophthalmoplegia	1.E-02
RAD vs. CR: Down in CR					
Cytosolic ribosome	2.E-09	Ribosome	2.E-11	Ribosomal protein	6.E-10
Large ribosomal subunit	1.E-07	Urea cycle and metabolism of amino groups	1.E-02	Ribonucleoprotein	3.E-08
Cytosol	2.E-07	Arginine and proline metabolism	4.E-02	Acetylation	1.E-05
Cytosolic large ribosomal subunit	2.E-07	Type II diabetes mellitus**	1.E-01	Elongation factor	1.E-03
Protein biosynthesis	2.E-07	Phenylalanine metabolism**	1.E-01	rRNA-binding	2.E-03
AS vs. CR: Up in CR					
Synapse	4.E-03	Butanoate metabolism	2.E-03	Glycoprotein	2.E-03
Extracellular	5.E-03	Ascorbate and aldarate metabolism	2.E-02	Vitamin C	7.E-03
Transition metal ion binding	7.E-03	Phenylalanine metabolism	2.E-02	Lipoprotein	1.E-02
Metal ion binding	2.E-02	Linoleic acid metabolism	2.E-02	Signal	1.E-02
Extracellular matrix	2.E-02	gamma-Hexachlorocyclohexane degradation	2.E-02	Heparin-binding	1.E-02
AS vs. CR: Down in CR					
Cytosolic ribosome	4.E-12	Ribosome	2.E-09	Acetylation	2.E-07
Biosynthesis	7.E-11	Carbon fixation	9.E-04	Ribosomal protein	1.E-06
Macromolecule biosynthesis	2.E-10	Glycolysis / Gluconeogenesis	3.E-03	Glycolysis	7.E-05
Protein biosynthesis	1.E-08	Glycosphingolipid biosynthesis - lactoseries	4.E-02	Ribonucleoprotein	8.E-05
Eukaryotic 43S preinitiation complex	2.E-08	Glutamate metabolism**	8.E-02	Protein biosynthesis	1.E-04

* Statistics according to the Audic and Claverie test statistic ($p \leq 0.05$)
† GO, Gene Ontology
‡ P-value represents the raw EASE (Expression Analysis Systematic Explorer) score
§ KEGG, Kyoto Encyclopedia of Genes and Genomes
‖ Unadjusted p-value was computed using FatiGO
¶ AS, androgen-sensitive; RAD, responsive to androgen-deprivation; CR, castration-recurrent
** Not statistically significant ($p \geq 0.05$)

Table 3.4 Gene expression trends of LongSAGE tags that consistently and significantly altered expression in CR prostate cancer*

	13N			15N			13R					
Tag Sequence	AS§ S1885	RADII S1886	CR¶ S1887	AS S1888	RAD S1889	CR S1890	AS S1891	RAD S1892	CR S1893	Trend‡	Gene**	Accession§§
TCTAGAGAACACTGTGC	12†	79	382	7	67	136	7	52	200	A	*ACPP* ‡‡	NM_001099
TAATTTTTCTAAGGTGT	101	311	648	119	397	895	120	546	918	A	*C1ORF80*	ENSG00000186063
TGAGAGAGGCCAGAACA	8	39	150	4	39	144	7	33	95	A	N/A	Genomic
CTCATAAGGAAAGGTTA	637	952	1680	653	1170	1540	688	1620	1930	A	*RNF208*	BC090061
GATTTCTATTTGTTTTT	89	169	446	116	208	339	86	311	555	A	*SERINC5*	ENSG00000164300
GTTGGGAAGACGTCACC	426	571	742	273	417	741	262	363	495	A	*STEAP1*	NM_012449
GAGGATCACTTGAGGCC	191	299	449	134	189	589	187	203	314	B	*AMACR* ‡‡	BC009471
TTGTTGATTGAAAATTT	219	197	528	273	197	479	232	391	586	B	*AMD1* ‡‡	NM_001634
TTTGCTTTTGTTTTGTT	53	16	169	34	51	129	7	28	72	B	*AQP3*	NM_004925
GTTCGACTGCCCACCAG	45	28	101	52	47	122	34	42	106	B	*ASAH1* ††	NM_177924
TAATAAACAGGTTTTTA	426	232	648	332	315	700	138	250	491	B	*ASAH1* ‡‡	NM_177924
TCACAGCTGTGAAGATC	85	110	277	161	71	258	310	438	945	B	*BTG1*	NM_001731
AAAAGAGAAAGCACTTT	24	75	199	19	35	85	15	90	552	B	*CAMK2N1*	NM_018584
CAAAACAGGCAGCTGGT	4	71	169	15	83	162	37	75	268	B	*CAMK2N1* ††	NM_018584
AGGAGGAAGAATGGACT	33	59	187	49	67	247	26	42	223	B	*CCNH*	NM_001239
TTTTAAAAATATAAAAT	89	83	243	97	130	269	64	170	382	B	*COMT*	NM_000754
GAATGAAATAAAAAATA	134	252	626	209	240	357	116	160	272	B	*DHRS7*	NM_016029
AAAGTGCATCCTTTCCC	118	146	318	153	220	394	288	231	646	B	*FGFRL1*	NM_001004356
AAACTGAATAAGGAGAA	24	51	236	19	51	438	19	146	283	B	*GALNT3*	NM_004482
TTTAAGGAAACATTTGA	4	4	75	4	4	81	0	0	57	B	*GALNT3* ††	NM_004482
CCAACCGTGCTTGTACT	191	327	521	202	279	534	172	363	510	B	*GLO1*	NM_006708
GAGGGCCGGTGACATCT	300	378	1170	321	476	1230	254	447	1030	B	*H2AFJ*	NM_177925
TATCATTATTTTTACAA	57	63	161	67	63	181	75	94	181	B	*HSD17B4*	NM_000414
AATGCACTTATGTTTGC	16	8	64	22	16	77	19	28	98	B	N/A	No map
ACCTTCGCAGGGGAGAG	0	0	19	0	4	41	0	5	34	B	N/A	Genomic
ATAACCTGAAAGGAAAG	0	16	56	7	4	74	0	28	87	B	N/A	No map
GTGATGTGCACCTGTTG	0	0	38	4	0	30	0	5	45	B	N/A	No map
GTTTGGAGGTACTAAAG	20	43	94	34	87	169	34	90	234	B	N/A	Genomic
TTTTCAAAAATTGGAAA	0	35	180	7	4	59	0	19	61	B	N/A	No map
GAAAAATTTAAAGCTAA	394	397	569	433	598	788	853	862	1060	B	*NGFRAP1*	NM_206917
CAAATTCAGGGAGCACA	0	4	139	4	16	228	0	14	136	B	*OPRK1*	NM_000912
CTATTGTCTGAACTTGA	0	8	109	0	12	70	0	9	227	B	*OR51E2*	BC020768
ATGCTAATTATGGCAAT	4	12	75	4	8	74	0	5	57	B	*PCGEM1*	NR_002769
CAGAAAGCATCCCTCAC	4	43	195	0	16	111	7	33	264	B	*PLA2G2A* ‡‡	NM_000300
TAATTTTAGTGCTTTGA	16	75	154	37	59	162	4	57	132	B	*PTGFR*	NM_000959
TTGTTTGTAAATAGAAT	0	12	94	0	4	162	0	14	72	B	*QKI*	NM_206853
TAAACACTGTAAAATCC	0	4	75	0	4	66	0	0	42	B	*QKI* ††	NM_206853
AGCAGATCAGGACACTT	20	35	112	15	16	140	15	42	98	B	*S100A10*	NM_002966
CTGCCATAACTTAGATT	37	55	161	93	63	192	56	99	264	B	*SBDS*	NM_016038
TGGCTGAGTTTATTTTT	20	24	79	41	8	96	4	42	147	B	*SFRS2B*	NM_032102
GAAGATTAATGAGGGAA	126	142	277	108	130	402	101	188	325	B	*SNX3*	NM_003795
ATGGTACTAAATGTTTT	16	47	124	37	28	88	11	19	76	B	*SPIRE1*	NM_020148
TATATATTAAGTAGCCG	45	39	101	45	75	133	41	75	178	B	*STEAP2* ‡‡	NM_152999
CAACAATATATGCTTTA	24	32	82	75	32	136	26	99	212	B	*STEAP2* ††	NM_152999
TTTCATTGCCTGAATAA	24	43	150	34	59	114	22	61	178	B	*TACC1* ‡‡	NM_006283
TTGGCCAGTCTGCTTTC	8	16	67	4	4	77	0	5	38	B	*TMEM30A*	ENSG00000112697
ATATCACTTCTTCTAGA	12	4	26	7	4	26	0	52	140	C	*ADAM2* ‡‡	NM_001464
ATGTGTGTTGTATTTTA	812	338	768	1010	315	1020	269	702	865	C	*BNIP3*	NM_004052
CCACGTTCCACAGTTGC	601	291	599	530	346	700	381	339	559	C	*ENO2*	NM_001975
CTGATCTGTGTTTCCTC	16	0	26	0	4	41	19	0	34	C	*HLA-B*	BC013187
AGCCCTACAAACAACTA	382	441	596	508	456	619	400	631	1010	C	*MT-ND3*	ENSG00000198840
ATATTTTCTTTGTGGAA	20	12	90	7	0	48	4	0	23	C	N/A	No map
CAAGCATCCCCGTTCCA	2400	2130	2440	2730	1720	2250	1020	2010	2340	C	N/A	ENSG00000211459
GTTGTAAAATAAACTTT	118	83	172	228	87	247	112	203	378	C	N/A	Genomic
TTGGATTTCCAAAGCAG	12	0	19	0	0	33	0	0	26	C	N/A	Genomic
TCTTTTAGCCAATTCAG	138	181	420	381	326	468	389	334	457	C	*NKX3-1* ††	NM_006167
TGATTGCCCTTTCATAT	73	39	86	86	39	107	108	99	181	C	*P4HA1*	NM_000917
GTAACAAGCTCTGGTAT	28	16	56	49	24	66	11	19	72	C	*PJA2*	NM_014819

Table 3.4 continued

	13N			15N			13R					
	AS	RAD	CR	AS	RAD	CR	AS	RAD	CR			
Tag Sequence	S1885	S1886	S1887	S1888	S1889	S1890	S1891	S1892	S1893	Trend	Gene	Accession
ACAGTGCTTGCATCCTA	85	75	139	108	98	203	101	118	196	C	*PPP2CB*	NM_004156
AGGCGAGATCAATCCCT	57	39	101	37	24	122	131	66	268	C	*PSMA7*	NM_002792
TATTTTGTATTTATTTT	73	59	180	93	51	111	22	94	253	C	*SLC25A4*	NM_001151
TTATGGATCTCTCTGCG	1050	1260	1820	1140	1300	2260	1990	1010	1530	C	*SPON2*	NM_012445
CAGTTCTCTGTGAAATC	767	515	1060	855	503	914	467	608	1200	C	*TMEM66*	NM_016127
AAATAAATAATGGAGGA	138	59	255	82	118	284	165	90	159	C	*TRPM8*	NM_024080
ATGTTTAATTTTGCACA	61	87	154	157	59	195	217	85	344	C	*WDR45L*	NM_019613
GGGCCCCAAAGCACTGC	861	543	1180	1020	657	1590	1240	739	937	E	*C19orf48*	NM_199249
TCCCCGTGGCTGTGGGG	1670	1390	2290	1740	1410	1720	3370	970	1180	E	*DHCR24* ‡‡	BC004375
GCATCTGTTTACATTTA	487	201	345	444	208	468	684	226	423	E	*ELOVL5*	NM_021814
GAAATTAGGGAAGCCTT	317	153	311	310	181	542	359	193	298	E	*ENDOD1*	XM_290546
GGATGGGGATGAAGTAA	2780	1160	4780	2950	1350	3620	2930	1230	1890	E	*KLK3* ‡‡	NM_001648
TGAAAAGCTTAATAAAT	313	142	322	474	181	332	273	179	314	E	*TPD52*	NM_001025252
GTTGTGGTTAATCTGGT	1770	634	1270	1800	806	1190	2480	659	960	F	*B2M*	NM_004048
GAAACAAGATGAAATTC	4380	1170	2260	5300	1110	2720	3750	2220	2830	F	*PGK1*	NM_000291
AGCACCTCCAGCTGTAC	2150	1130	648	2060	1560	939	1560	1200	722	G	*EEF2*	NM_001961
GCACAAGAAGATTAAAA	536	228	124	762	425	195	838	278	174	G	*GAS5*	NR_002578
CCGCTGCGTGAGGGCAG	451	169	56	429	197	44	516	94	0	G	*HES6*	NM_018645
GCCCAGGTCACCCACCC	585	55	4	519	79	7	456	66	0	G	*LOC644844*	XM_927939
ATGCAGCCATATGGAAG	2650	386	82	2470	216	129	1210	259	98	G	*ODC1*	NM_002539
CGCTGGTTCCAGCAGAA	1420	811	479	1250	959	553	800	589	374	G	*RPL11*	NM_000975
AAGACAGTGGCTGGCGG	2650	1730	1220	2460	1860	1350	2120	1630	1270	G	*RPL37A* ‡‡	NM_000998
TTCTTGTGGCGCTTCTC	925	543	217	1030	708	273	1130	419	306	G	*RPS11* ††	NM_001015
GGTGAGACACTCCAGTA	463	252	165	485	346	192	363	245	159	G	*SLC25A6*	NM_001636
AGGTTTTGCCTCATTCC	982	515	281	1200	491	243	688	782	166	H	*ABHD2*	NM_007011
TGAAGGAGCCGTCTCCA	317	272	187	392	295	199	366	259	140	H	*ATP5G2*	NM_001002031
CTCAGCAGATCCAAGAG	191	185	67	254	232	66	142	231	79	H	*C17orf45*	NM_152350
CTGTGACACAGCTTGCC	308	397	172	209	307	125	295	226	110	H	*CCT2*	NM_006431
TCTGCACCTCCGCTTGC	495	606	277	426	570	276	366	471	204	H	*EEF1A2*	NM_001958
GCCCAAGGACCCCCTGC	114	114	38	138	98	41	101	42	4	H	*FLNA* ‡‡	NM_001456
TTATGGGATCTCAACGA	564	425	180	642	452	317	430	490	253	H	*GNB2L1*	NM_006098
TCTGCAAAGGAGAAGTC	81	102	38	105	87	26	165	80	30	H	*HMGB2*	NM_002129
CTTGTGAACTGCACAAC	268	228	124	231	177	103	273	160	57	H	*HN1*	NM_016185
TCTGAAGTTTGCCCCAG	313	291	150	254	299	155	187	226	72	H	*MAOA*	NM_000240
TTAATTGATAGAATAAA	483	350	199	422	287	103	273	235	83	H	*MAOA*	NM_000240
GGCAGCCAGAGCTCCAA	1200	1260	420	1050	672	350	681	819	23	H	*MARCKSL1*	NM_023009
CCCTGCCTTGTCCCTCT	353	240	112	310	263	107	176	193	102	H	*MDK*	NM_001012334
CTGTGGATGTGTCCCCC	649	476	169	459	389	214	430	297	117	H	N/A	No map
CTCCTCACCTGTATTTT	1120	771	262	1220	979	313	666	730	261	H	*RPL13A* ‡‡	NM_012423
GCAGCCATCCGCAGGGC	1980	1770	809	2300	1730	928	2150	1570	1020	H	*RPL28*	NM_000991
GGATTTGGCCTTTTTGA	3470	2070	1370	4170	2910	1540	2800	2870	2500	H	*RPLP2* ‡‡	NM_001004
TCTGTACACCTGTCCCC	2320	1670	850	1930	1880	825	2130	1490	1120	H	*RPS11*	NM_001015
GCTTTTAAGGATACCGG	1510	1050	626	1860	1120	593	1550	1550	960	H	*RPS20* ‡‡	NM_001023
CCCCAGCCAGTCCCCAC	921	519	281	788	664	357	1100	438	291	H	*RPS3*	NM_001005
CCCCCAATGCTGAGGCC	89	138	26	90	94	30	90	80	30	H	*SF3A2*	NM_007165
GCCGCCATCTCCGAGAG	195	102	30	168	118	55	172	108	30	H	*TKT*	NM_001064
GGCCATCTCTTCCTCAG	349	307	202	317	346	173	277	254	121	H	*YWHAQ*	NM_006826
AGGCTGTGTTCCTCCGT	16	39	11	34	67	22	26	38	8	I	*ACY1*	NM_000666
TGCCTCTGCGGGGCAGG	446	649	427	399	664	424	501	462	317	I	*CD151*	NM_004357
GGCACAGTAAAGGTGGC	175	216	142	332	350	173	456	316	204	I	*CUEDC2*	NM_024040
TCACACAGTGCCTGTCG	49	71	7	30	47	15	34	66	4	I	*CXCR7*	NM_001047841
TGTGAGGGAAGCTGCTT	53	87	15	67	102	52	52	90	42	I	*FKBP10*	BC016467
TGCTTTGCTTCATTCTG	28	63	26	22	79	26	49	118	61	I	*GRB10*	NM_005311
GTACTGTATGCTTGCCA	170	212	82	134	153	88	123	188	113	I	*KPNB1* ‡‡	NM_002265
GTGGCAGTGGCCAGTTG	106	193	97	123	173	96	94	137	76	I	N/A	ENSG00000138744
GGGGAGCCCCGGGCCCG	61	63	26	30	51	18	34	57	0	I	*NAT14*	NM_020378
TGTTCAGGACCCTCCCT	28	67	26	60	63	26	60	28	0	I	*NELF*	NM_015537
TTTTCCTGGGGATCCTC	41	130	15	37	87	33	56	104	45	I	PGOTH	NM_001014442

Table 3.4 continued

	13N			15N			13R					
	AS	RAD	CR	AS	RAD	CR	AS	RAD	CR			
Tag Sequence	S1885	S1886	S1887	S1888	S1889	S1890	S1891	S1892	S1893	Trend	Gene	Accession
GAAACCCGGTAGTCTAG	41	75	4	37	75	26	52	151	30	I	*PLCB4*	NM_000933
GTCTGACCCCAGGCCCC	126	205	82	119	193	103	157	179	38	I	*PPP2R1A*	NM_014225
GGCCCGAGTTACTTTTC	231	150	75	161	232	136	142	160	45	I	*RPL35A* ††	NM_000996
GTTCGTGCCAAATTCCG	881	696	390	1100	712	523	497	782	461	I	*RPL35A* ‡‡	NM_000996
TTACCATATCAAGCTGA	877	535	311	1130	598	405	636	791	578	I	*RPL39* ‡‡	NM_001000
GCTGCAGCACAAGCGGC	268	244	127	45	216	125	157	71	11	I	*RPS18* ††	NM_022551
AGCTCTTGGAGGCACCA	203	319	206	142	421	243	269	259	162	I	*SELENBP1*	NM_003944
TGCTGGTGTGTAAGGGG	69	102	45	82	87	37	105	75	30	I	*SH3BP5L*	NM_030645
GAGAGTAACAGGCCTGC	191	150	71	112	181	111	108	165	64	I	*SYNC1*	NM_030786
CTGAAAACCACTCAAAC	394	508	225	306	547	236	310	381	200	I	*TFPI*	NM_006287
TAAAAAAGGTTTCATCC	183	248	127	86	130	66	142	268	87	I	*TFPI*	NM_006287
CTCCCTCCTCTCCTACC	28	32	4	30	39	7	71	24	0	I	*TK1*	NM_003258
CATTTTCTAATTTTGTG	544	744	236	407	771	181	288	664	185	J	N/A	No map
TGATTTCACTTCCACTC	3480	5260	3910	3700	6110	3590	3040	5960	2600	K	*MT-CO3*	ENSG00000198938
TTTCTGTCTGGGGAAGG	130	236	82	123	201	111	101	188	113	K	*PIK3CD*	NM_005026
GCCGCTACTTCAGGAGC	256	370	199	224	330	169	142	316	38	K	*RAMP1*	NM_005855
ATGGTTACACTTTTGGT	93	161	94	75	208	118	60	226	95	K	*UTX*	NM_021140
CACTACTCACCAGACGC	2820	3900	3020	2740	4290	2440	2620	3120	1260	K	*VPS13B* ††	ENSG00000132549
CTAAGACTTCACCAGTC	7120	11000	9730	6390	10900	8330	3610	8870	7850	L	N/A	ENSG00000210082

* Statistics according to the Audic and Claverie test statistic ($p \leq 0.05$)

† Tag count per 1 million = (observed tag count/total tags in the library) x 1,000,000

‡ Trends are descibed from A to L in the trend legend below. For some genes the trend is indistinguishable between two possibilties. In addition to p-value considerations, significantly different trends were also required to display uniform directions of change in each biological replicate.

§ AS, Androgen-sensitive

‖ RAD, Responsive to androgen-deprivation

¶ CR, Castration-recurrent

** Genes were represented by HGNC approved nomenclature when available. Non-HGNC gene names were not italicized.

†† Tag maps antisense to gene

‡‡ Gene is known to display this expression trend in castration-recurrence

§§ Accession numbers were displayed following the priority (where available): RefSeq>Mammalian Gene Collection>Ensembl Gene If the tag mapped to more than one transcript variant of the same gene, the accession number of the lowest numerical transcript variant was displayed.

Trend Legend:

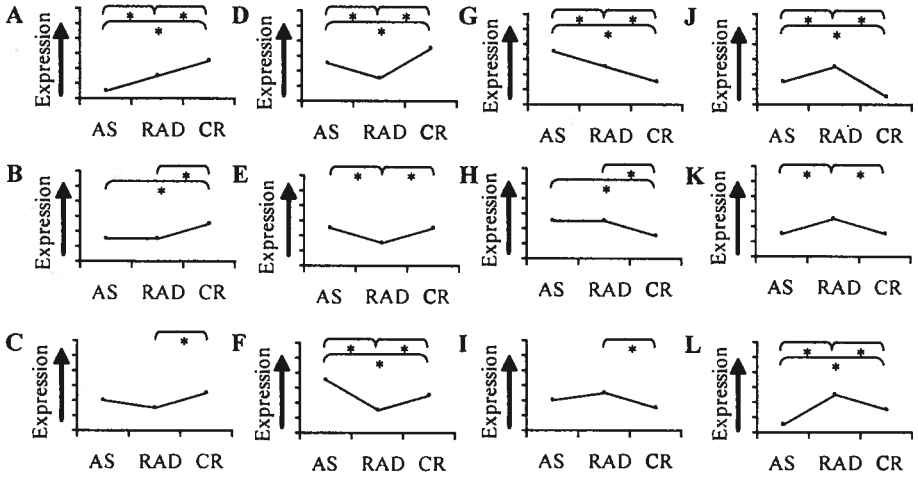

Table 3.5 Characteristics of genes with novel association to castration-recurrence *in vivo*

Gene*	S or PM†	Reg. by A‡	Spec. to P§	Associated with CaP‖	GG¶	Prog.**	Mets††	CR‡‡
ABHD2	PM	-	-	Y↑	-	-	-	-
ACY1	-	-	-	-	-	-	-	-
AQP3	PM	-	-	-	-	-	-	-
ATP5G2	-	-	-	-	-	-	-	-
B2M	S&PM	Y↑	-	-	-	Y↑	-	Y↓
BNIP3	-	-	-	Y↓	-	-	-	-
BTG1	-	Y↓	-	-	-	-	-	-
C17orf45	-	-	-	-	-	-	-	-
C19orf48	S	Y↑	-	-	-	-	-	-
C1orf80	-	-	-	-	Y↑	-	-	-
CAMK2N1	-	Y↓	-	-	Y↑	-	-	-
CCNH	-	-	-	-	-	-	-	-
CCT2	-	-	-	-	-	-	-	-
CD151	PM	-	-	-	-	Y↑	Y↑	-
COMT	-	-	-	-	-	Y↓	-	-
CUEDC2	-	-	-	-	-	-	-	-
CXCR7	PM	Y↓	-	-	-	-	Y↑	Y↑
DHRS7	PM	-	-	-	-	-	Y↓	-
EEF1A2	-	Y↑	-	Y↑	-	-	-	-
EEF2	-	-	-	-	-	-	-	-
ELOVL5	PM	Y	-	-	-	-	-	-
ENDOD1	S	Y↑	-	-	-	-	-	-
ENO2	PM	-	-	-	-	-	-	-
ENSG00000210082	-	-	-	-	-	-	-	-
ENSG00000211459	-	-	-	-	-	-	-	-
FGFRL1	PM	-	-	-	-	-	-	-
FKBP10	-	-	-	-	-	-	-	-
GALNT3	-	-	-	Y↑	-	Y↓	-	-
GAS5	-	-	-	-	-	-	-	-
GLO1	-	-	-	Y↑	Y↑	-	-	-
GNB2L1	PM	-	-	-	-	-	Y↑	-
GRB10	PM	-	-	-	-	-	-	-
H2AFJ	-	-	-	-	-	-	-	-
HES6	-	-	-	-	-	-	Y↑	Y↑
HLA-B	PM	-	-	-	-	-	-	-
HMGB2	-	-	-	-	-	-	-	Y↑
HN1	-	-	-	-	-	-	Y↑	-
HSD17B4	-	Y↑	-	Y↑	-	-	-	-
LOC644844	-	-	-	-	-	-	-	-
MAOA	-	Y	-	-	Y↑	-	-	-
MARCKSL1	PM	-	-	Y↑	-	-	-	-
MDK	S&PM	Y↓	-	Y↑	-	-	-	Y↑
MT-CO3	-	-	-	-	-	-	-	-
MT-ND3	-	-	-	-	-	-	-	-
NAAA	-	-	-	-	-	-	-	Y↑
NAT14	PM	-	-	-	-	-	-	-
NELF	PM	-	-	-	-	-	-	-
NGFRAP1	-	-	-	-	-	-	-	-

Gene	S or PM	Reg. by A	Spec. to P	Associated with CaP	GG	Prog.	Mets	CR
NKX3-1	-	Y↑	Y	-	-	-	Y	-
ODC1	-	Y↑	-	Y↑	-	Y↓	-	Y↑
OPRK1	PM	-	-	-	-	-	-	-
OR51E2	PM	-	-	Y↑	-	-	-	-
P4HA1	-	Y	-	-	-	-	-	-
PCGEM1	-	Y↑	Y	Y↑	-	Y↑	-	-
PCOTH	-	-	Y	Y↑	-	Y↑	-	-
PGK1	-	Y↑	-	-	Y↑	-	Y↑	↓§§ -
PIK3CD	-	-	-	-	-	-	Y↑	Y↑
PJA2	-	-	-	-	-	-	-	-
PLCB4	PM	-	-	-	-	-	-	-
PPP2CB	-	-	-	Y↓	-	-	-	-
PPP2R1A	-	-	-	-	-	-	-	-
PSMA7	-	-	-	-	-	-	-	-
PTGFR	PM	-	-	-	-	-	-	-
QKI	-	-	-	-	-	-	-	-
RAMP1	PM	-	-	-	-	-	-	-
RNF208	-	-	-	-	-	-	-	-
RPL11	-	-	-	-	-	-	Y↓	-
RPL28	-	-	-	-	-	-	-	-
RPS11	-	-	-	-	-	-	Y↓	-
RPS18	-	-	-	Y↑	-	-	-	-
RPS3	-	-	-	-	-	-	-	-
S100A10	PM	-	-	-	-	-	-	-
SBDS	-	-	-	-	-	-	-	-
SELENBP1	-	Y↓	-	Y↓	-	-	-	-
SERINC5	-	-	-	-	-	-	-	-
SF3A2	-	-	-	-	-	-	Y↑	-
SFRS2B	-	-	-	-	-	-	-	-
SH3BP5L	-	-	-	-	-	-	-	-
SLC25A4	-	-	-	Y↑	-	-	-	-
SLC25A6	-	-	-	Y↑	-	-	-	-
SNX3	-	-	-	-	Y↑	-	-	-
SPIRE1	-	-	-	-	-	-	-	-
SPON2	S	-	Y	-	-	-	-	-
STEAP1	PM	-	Y	Y↑	-	-	-	-
SYNC1	-	-	-	-	-	-	-	-
TFPI	S	-	-	-	-	-	-	-
TK1	-	-	-	-	-	-	Y↑	-
TKT	-	-	-	-	-	-	-	-
TMEM30A	S&PM	-	-	-	-	-	-	-
TMEM66	S&PM	Y↑	-	-	-	-	-	-
TPD52	-	Y↑	Y	Y↑	-	Y↑	Y↓	-
TRPM8	PM	Y↑	-	Y↑	-	-	-	Y↓
UTX	-	-	-	-	-	-	-	-
VPS13B	PM	-	-	-	-	-	Y↑	-
WDR45L	-	-	-	-	-	-	-	-
YWHAQ	-	-	-	-	-	-	-	-

* Genes were represented by HGNC approved nomenclature when available. Non-HGNC gene names were not italicized

† S or PM, gene product is thought to be secreted (S) or localize to the plasma membrane (PM)

‡ Reg. by A, gene expression changes in response to androgen in prostate cells

§ Spec. to P, gene expression is specific to- or enriched in- prostate tissue compared to other tissues

‖ CaP, gene is differentially expressed in prostate cancer compared to normal, benign prostatic hyperplasia, or prostatic intraepithelial neoplasia

¶ GG, gene is differentially expressed in higher Gleason grade tissue versus lower Gleason grade tissue

** Prog., gene expression correlates with late-stage prostate cancer or is a risk factor that predicts progression

†† Mets, gene expresion is associated with prostate cancer metastsis in human samples or *in vivo* models

‡‡ CR, gene is associated with castration-recurrent prostate cancer in human tissue or *in vivo* models, but exhibits an opposite trend of this report

§§ Y, yes; ↑, high gene expression; ↓, low gene expression

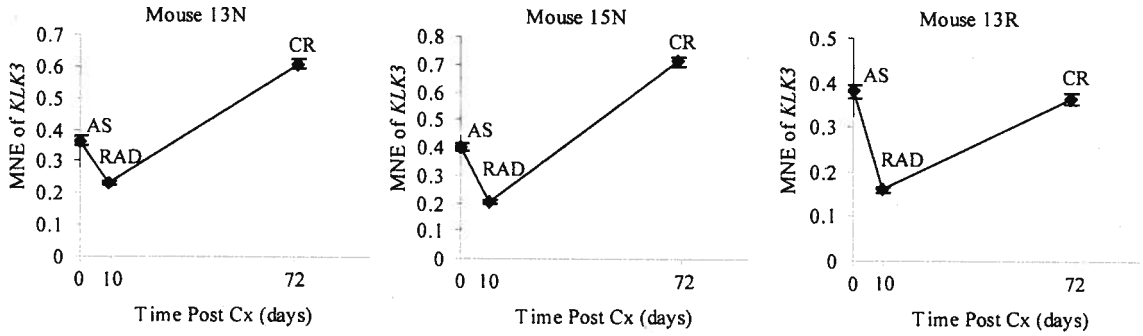

Figure 3.1 qRT-PCR analysis of *KLK3* gene expression during hormonal progression of prostate cancer to castration-recurrence. RNA samples were retrieved from the *in vivo* LNCaP Hollow Fibre model at different stages of cancer progression that were: AS, androgen-sensitive, day zero (just prior to surgical castration and 7 days post-fibre implantation); RAD, responsive to androgen-deprivation, 10 days post-surgical castration; and CR, castration-recurrent, 72 days post-surgical castration. MNE, mean normalized expression, calculated by normalization to glyceraldehyde-3-phosphate (*GAPDH*). Error bars represent ± standard deviation of technical triplicates. Each mouse represents one biological replicate.

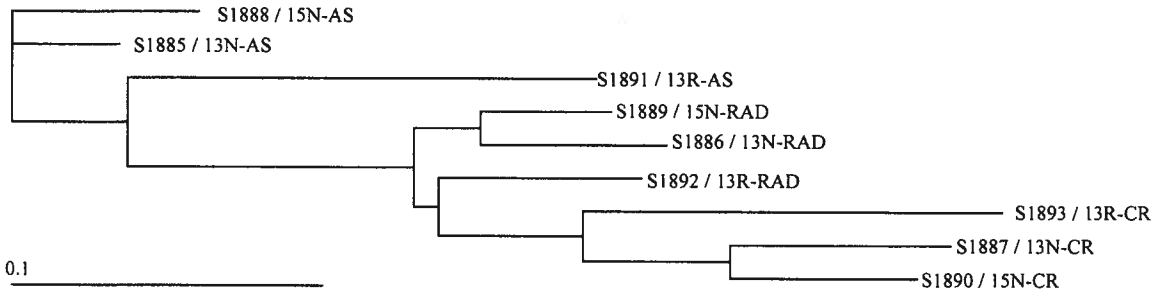

Figure 3.2 Clustering of the nine LongSAGE libraries in a hierarchical tree. The tree was generated using a Pearson correlation-based hierarchical clustering method and visualized with TreeView. LongSAGE libraries constructed from similar stages of prostate cancer progression (AS, androgen-sensitive; RAD, responsive to androgen-deprivation; and CR, castration-recurrent) cluster together. 13N, 15N, and 13R indicate the identity of each animal.

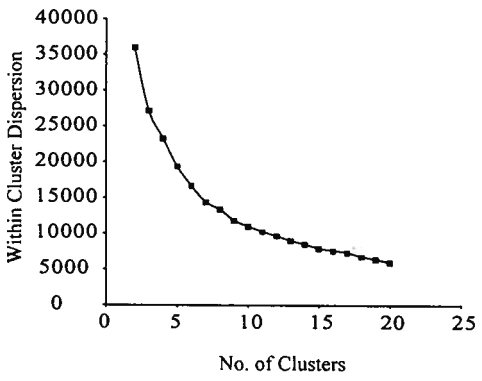

Figure 3.3 Ten K-means clusters are optimal to describe the expression trends present during progression of prostate cancer to castration-recurrence. K-means clustering was conducted over a range of K (number of clusters) from K=2 to K=20 and the within-cluster dispersion was computed for each clustering run and plotted against K. The within-cluster dispersion declined with the addition of clusters and this decline was most pronounced at K=10. The graph of within-cluster dispersion versus K shown here is for mouse 13N, but the results were similar for mice 15N and 13R.

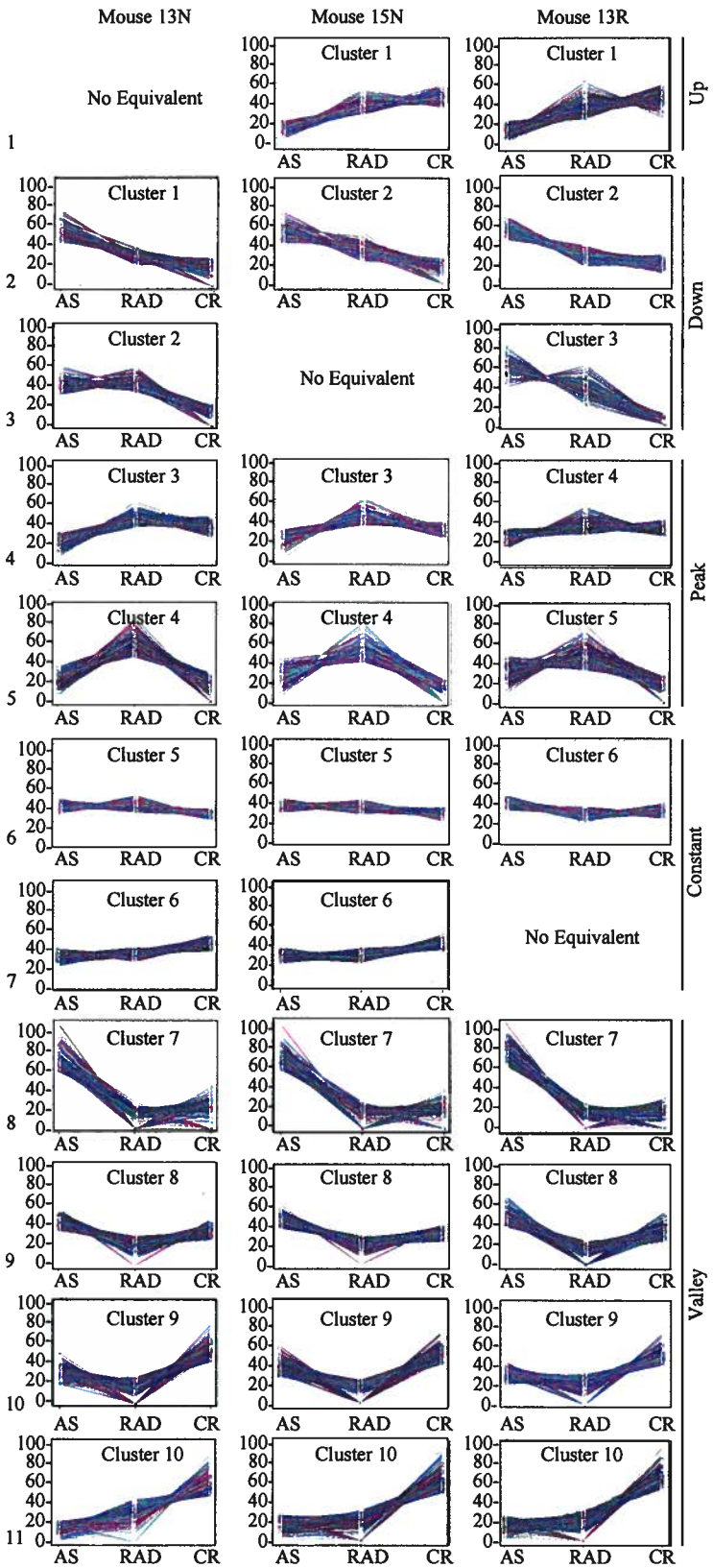

Figure 3.4 K-means clustering of tag types with similar expression trends. PoissonC with K=10 (where K = number of clusters) was conducted over 100 iterations separately for each biological replicate (mice 13N, 15N, and 13R) and the results from the iterations were combined into consensus clusters shown here. Plotted on the x-axes are the long serial analysis of gene expression (LongSAGE) libraries representing different stages of prostate progression: AS, androgen-sensitive; RAD, responsive to androgen-deprivation; and CR, castration-recurrent. Plotted on the y-axes are the relative expression levels of each tag type, represented as a percentage of the total tag count (for a particular tag type) in all three LongSAGE libraries. Different colours represent different tag types. Each of the ten clusters for each biological replicate are labeled as such. 'No equivalent' indicates that a similar expression trend was not observed in the indicated biological replicate. Eleven expression patterns are evident in total and are labeled on the left. K-means clusters were amalgamated into five major expression trends: 'up' during progression, 'down' during progression, 'constant' during progression, expression 'peak' in the RAD stage, and expression 'valley' in RAD stage.

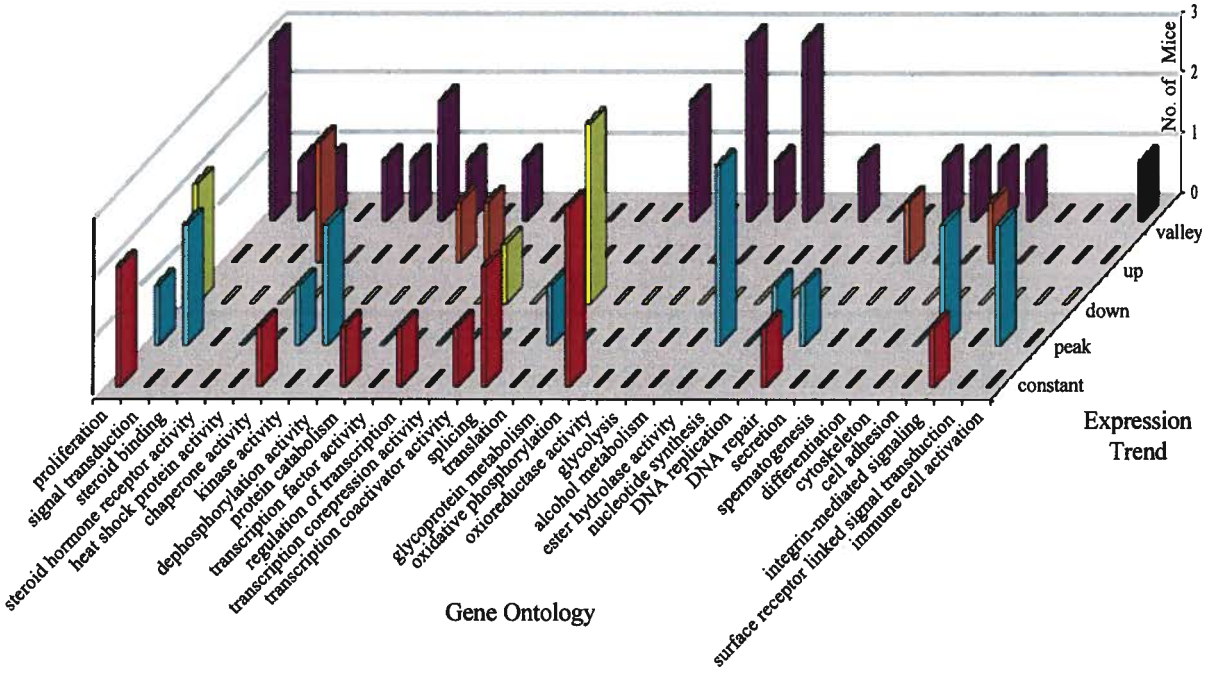

Figure 3.5 Gene ontology enrichments of the five major expression trends. Plotted on the x-axis are Gene Ontology (GO) categories enriched in one or more of the five major expression trends. On the z-axis the five major expression trends are: 'up' during progression, 'down' during progression, 'constant' during progression, expression 'peak' in the RAD stage, and expression 'valley' in RAD stage. The y-axis displays the number of biological replicates (number of mice: 1, 2, or 3) exhibiting enrichment. The latter allows one to gauge the magnitude of the GO enrichment and confidence.

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CHAPTER IV

EXPRESSION CHARACTERISTICS OF NOVEL BIOMARKERS OF PROSTATE CANCER*

4.1 INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer, and the third leading cause of cancer death in Canadian men[1]. Twenty-seven per 100,000 deaths in men were from prostate cancer in Canada in 2004[2]. This number is approximately 5-times that of Japan at 5.5 per 100,000 deaths. In the USA, 86 men die from prostate cancer each day. However, these numbers only represent the 2.5 to 3% of men who die from the disease from the 10% of men over 50 years who will have clinical progression. Autopsy studies indicate that 30% of men over the age of 50 have malignant cells in their prostate[3]. The European Study of Screening and the Prostate Cancer Prevention Trial indicate that screening for prostate cancer elevates the incidence rate[4] with increases in the ratio of incidence to mortality from 2.5:1 to 17:1. This suggests that a substantial proportion of men with clinically insignificant disease are being over-treated. In other words, their disease will never cause morbidity or mortality. The current treatments for organ-confined malignancy are brachytherapy[5], external beam radiation[6], or radical prostatectomy[7]. These forms of therapy can produce significant morbidity such as incontinence and impotence and are not effective for disease that has spread outside the prostatic capsule. Only palliative therapy is available for disseminated disease which requires reducing levels of testosterone (androgen) and/or using antiandrogens[8, 9]. Thus, there is an urgent need for selective intervention to spare those men from receiving unnecessary treatment, but still provide radical curative treatment to those men who will develop clinically significant disease. Currently there are no prognostic tools that can distinguish aggressive tumours from latent tumours.

Prostate-specific antigen (PSA) has been utilized as a serum biomarker to monitor and screen for prostate cancer since 1986 and 1994, respectively[10]. A recommendation for biopsy has been set at an arbitrary serum PSA level of 4 ng/mL. However, at this threshold, PSA is moderately specific and poorly sensitive as a biomarker for detection of prostate cancer. Specificity is defined as the percentage of men without prostate cancer who have serum PSA levels under the

* A version of this chapter has been submitted for publication. Romanuik, TL., Ueda, T., Le, N., Thomson, T., Sadar, MD. Expression characteristics of novel biomarkers of prostate cancer. *Submitted.*

4 ng/mL threshold[10]. For PSA, this percentage is 93%[11]. In addition to carcinoma of the prostate, PSA is expressed in normal prostate tissue, prostatitis, and benign prostatic hyperplasia, and levels of circulating PSA are affected by age, racial background, physical activity, and body mass[12]. Digital rectal exam, transrectal ultasound, and prostate biopsy may also cause increases in serum PSA levels[13]. In contrast to specificity, sensitivity is defined as the percentage of men with prostate cancer who have serum PSA levels over the 4 ng/mL threshold[10]. For PSA, this percentage is 24%[11]. Furthermore, 27% of men with borderline serum PSA levels (3.1-4 ng/mL) have detectable prostate cancer by biopsy[14].

Serum PSA levels correlate with the degree of dissemination[15, 16] and aggressiveness[16] of prostate cancer. For example, serum PSA levels >10 ng/mL are associated with a high pathological stage (odds ratio (OR) 1.7) and high Gleason sum (i.e., 7-10; OR 1.9), respectively, compared to PSA levels < 4 ng/mL[16]. Following radical prostatectomy or brachytherapy, 7-15% of prostate cancers will exhibit biochemical recurrence at eight years of follow-up as defined by rising PSA levels[17, 18]. However, approximately one percent of prostate cancer patients will develop metastases following first-line therapy concomitant with serum PSA levels $\leq$ 2ng/mL[19]. Therefore, measurement of serum PSA levels is inadequate for monitoring progression for a small subset of patients.

Patients receiving androgen-deprivation therapy for disseminated disease will relapse and their disease will progress to the terminal, castration-recurrent prostate cancer for which there is no effective treatment[20-22]. Initial response to androgen-deprivation therapy is measured by PSA nadir. PSA nadir is prognostic of the time it takes to reach castration-resistant prostate cancer and death[23]. However, it is unknown whether pre-treatment serum PSA levels can predict response to androgen-deprivation therapy. Therefore, there is a great need for novel prognostic markers of castration-recurrent prostate cancer.

These limitations of PSA emphasize the need for new biomarkers to accurately detect, monitor, and predict the aggressiveness of prostate cancer. In particular, biomarkers that are prognostic and/or signify the propensity to rapidly develop advanced disease are required. Such biomarkers may stem from gene expression studies using *in vivo* models of advanced prostate cancer. Here,

we characterize the expression of genes and novel non-coding transcripts that were previously identified by Long Serial Analysis of Gene Expression (LongSAGE)[24] and Subtractive Hybridization[25] technologies using samples from the *in vivo* LNCaP Hollow Fibre model[26]. This model allows the analyses of gene expression at various stages of hormonal progression in castrated hosts. LongSAGE can theoretically sample nearly all the transcripts present in a transcriptome[27]. Both LongSAGE and Subtractive Hybridization technologies can be used to discover unannotated transcripts. We chose 27 differentially expressed transcripts for further investigation to resolve the feasibility of their clinical utility in the diagnosis, prognosis, imaging, or treatment of prostate cancer. The transcripts were chosen based on their novelty and/or the ability of their gene product to be secreted or expressed on the cell surface.

Genes previously identified by LongSAGE and examined here are ADAM metallopeptidase domain 2 (*ADAM2*), calcium/calmodulin-dependent protein kinase II inhibitor 1 (*CAMK2N1*), 24-dehydrocholesterol reductase (*DHCR24*), elongation of long chain fatty acids family member 5 (*ELOVL5*), glyoxalase 1 (*GLO1*), MARCKS-like 1 (*MARCKSL1*), nerve growth factor receptor associated protein 1 (*NGFRAP1*), phosphoglycerate kinase 1 (*PGK1*), proteasome macropain subunit alpha type 7 (*PSMA7*), receptor activity modifying protein 1 (*RAMP1*), Shwachman-Bodian-Diamond syndrome (*SBDS*), spondin 2 (*SPON2*), transmembrane protein 30A (*TMEM30A*), transmembrane protein 66 (*TMEM66*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein theta polypeptide (*YWHAQ*). Subtractive Hybridization is particularly well suited for the identification of differentially expressed low abundance transcripts[28]. The twelve novel transcripts identified as differentially expressed in the LNCaP Hollow Fibre model using Subtractive Hybridization generally have low protein-coding potential and poor conservation across species[25]. These transcripts are referred to as POP 1 through 12: POP1, transcript 100 kilobases (kb) from mRNA AK000023; POP2, transcript 4 kb from mRNA AL832227; POP3, transcript 50 kb from EST CFI40309; POP4, transcript from the intron of transmembrane protein with EGF-like and two follistatin-like domains 2 (*TMEFF2*); POP5, transcript from the intron of neural cell adhesion molecule 2 (*NCAM2*; accession DO668384); POP6, transcript from the intron of fragile histidine triad gene (*FHIT*); POP7, transcript from the intron of tumor necrosis factor, alpha-induced protein 8 (*TNFAIP8*); POP8, transcript from the intron of ephrin-A5 (*EFNA5*); POP9, transcript from the intron of actin depolymerizing factor destrin (*DSTN*); POP10, transcript from the intron of

ADAM2 (accession DO668396); POP11, transcript 87 kb from EST BG194644; and POP12, transcript from the intron of EST BQ226050[25]. The expression of these 27 transcripts was measured in a variety of cell types and tissues, including laser microdissected human prostatectomy samples, to characterize these genes and assess their feasibility as potential biomarkers for prostate cancer.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture

Cell lines were maintained in RPMI-1640 media (LNCaP, 22Rv1, and COS1 cells), DMEM media (PC-3, DU145, and RKO cells), or MEM media (MG63, CV1, HEPG, and MCF7 cells). Cells were obtained from the American Type Culture Collection, Bethesda, MD, USA, with the exceptions of RKO, COS1, and MCF7, which were kindly provided by I. Tai (Genome Sciences Centre, BC Cancer Agency (BCCA)), J. Vielkind (Cancer Endocrinology, BCCA), and M. Bally (Advanced Therapeutics, BCCA), respectively. All media (Stem Cell Technologies, Vancouver, BC, Canada) was supplemented with 100 units/mL penicillin and 100 units/mL streptomycin (Invitrogen, Burlington, ON, Canada), as well as 10% v/v fetal bovine serum (FBS; HyClone, Logan, UT, USA) with the exception of PC-3 and 22Rv1 cells which only received 5% v/v FBS. Cells were maintained at 37°C with 5% CO_2. For androgen treatments, LNCaP cells (1×10^6) were seeded in 10 cm diameter dishes. The next day, cells were serum-starved for 48 hours and then treated for 16 hours with 10 nM synthetic androgen R1881 (PerkinElmer, Woodbridge, ON, Canada), or vehicle control, ethanol (final concentration 2.85×10^{-4} %) in serum-free media.

4.2.2 Clinical samples

Frozen prostate specimens from 84 patients who had undergone radical prostatectomies were received by our laboratory in OCT compound. Informed consent was obtained from each patient participating in the study according to guidelines set forth by the UBC BCCA Research Ethics Board. Prostatectomy specimens were accompanied by information including the age of the patient, prior treatment history, serum PSA levels prior to surgery, and TNM clinical and pathological stage. This information is summarized in **Table 4.1** for all samples used in this paper. Any patient who had received pre-surgical hormone ablation treatment was excluded.

Tissue blocks were sectioned (8μm) onto membrane slides and fixed in 70% ethanol. H&E slides were made every 10-15 membrane sections for pathology review and used as guidance for laser microdissection. Selected membrane slides were stained with hematoxylin, dehydrated, and subsequently laser microdissected for epithelial cells by μCut MMI AG (MMI Molecular Machines & Industries AG, Glattbrugg, Switzerland) (**Figure 4.1**).

4.2.3 RNA preparation for gene expression analysis

Total RNA from cell lines was harvested using TRIZOL Reagent (Invitrogen) following the manufacturer's instructions. Total RNA from normal human tissue (adrenal gland, bone marrow, brain (cerebellum), brain (whole), fetal brain, fetal liver, heart, kidney, liver, lung, placenta, prostate, salivary gland, skeletal muscle, testis, thymus, thyroid gland, trachea, uterus, and spinal cord) was obtained commercially from Clontech (Mountain View, CA, USA). Total RNA from sections of human prostate tissue was extracted using the RNA Easy Micro Kit (Qiagen, Mississauga, ON, Canada) and concentrated by speed vacuum centrifugation (SPD IIIV Speed Vac, Thermo Electron Corporation).

Contaminating genomic DNA was removed from RNA samples by TURBO DNA-free (Ambion Inc., Austin, TX, USA) or DNase I from the RNA Easy Micro Kit. RNA quality and quantity was assessed using the NanoDrop ND-1000 (NanoDrop Technologies Inc, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada) with RNA 6000 Nano LabChip kit (Caliper Technologies, Hopkinton, MA, USA). RNA of poor quality (RIN < 2.8) and insufficient quantity (< 531 ng) was not used in this study.

4.2.4 Relative quantitation of gene expression

Input RNA was reverse transcribed with SuperScript III First Strand Synthesis kit (Invitrogen). For most RNA samples, a quantity of 0.5 μg was used in the reverse transcriptase (RT) reaction, but for limited sample quantities, such as those from the laser microdissected prostate tissue, 0.1μg or 0.05 μg of RNA was used. A 10 μL qRT-PCR reaction consisted of 1μl of template cDNA, 1x TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and 0.9 μM each of forward and reverse primers and 0.25 μM of TaqMan probe (FAM-BHQ-1

or TET-BHQ-1; Integrated DNA Technologies Inc., San Diego, CA, USA) that produce specific PCR products ranging in size between 85-235 bp (see **Table 4.2** for primer and probe sequences). qRT-PCR reactions were cycled as follows in a 7900HT Sequence Detection System (Applied Biosystems): 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 0.25 min followed by 60 °C for 1 min. All qRT-PCR reactions were performed using technical triplicates. cDNAs (from different conditions/patients) and genes (target and reference) to be directly compared were assayed in the same instrument run. Glyceraldehyde-3-phosphate (*GAPDH*) was used as a reference gene for all experiments except the androgen regulation experiment in which succinate dehydrogenase complex, subunit A, flavoprotein (*SDHA*) was used instead. These reference genes were chosen for their stability across samples and their level of expression. Reactions without template were run for each gene to ensure that DNA had not contaminated the qRT-PCR reactions. Efficiency checks were performed for each primer pair.

4.2.5 Statistical analysis

To identify significant changes in gene expression in response to androgen we used the Two-Sample Student's T-test for unequal variance. Non-parametric methods were employed with data that was sampled from non-normal distributions. For gene expression analysis on RNA from laser microdissected prostatic tissue, the Spearman's correlation test was used to identify associations to patient age or PSA levels, and the Kruskal Wallis test was used to identify significant differences between gene expression in normal and tumour tissue, or TNM stages of cancer. A p-value cut-off of 0.05 was employed for all tests.

4.3 RESULTS

4.3.1 Tissue-specificity of gene expression

To characterize tissue-specific expression of genes compared to carcinoma of the prostate, we employed Taq Man[29] quantitative real time-polymerase chain reaction (qRT-PCR) using RNA isolated from five human prostate cancer cells (LNCaP, MDA-PCa-2B, 22Rv1, PC-3, and DU145) and non-prostate human cancer cell lines that included: MG63, osteosarcoma cells; RKO, colon carcinoma cells; HEPG, hepatocellular carcinoma cells; MCF7, mammary adenocarcinoma cells; and large T-antigen transformed, and normal monkey kidney cells (COS1, and CV1, respectively). Gene expression was displayed relative to the levels of

expression of each transcript in LNCaP cells. Cells were maintained in tissue culture under individualized conditions for optimal growth to gauge constitutive levels of gene expression.

Expression of genes *ADAM2* and *POP11* were relatively specific for LNCaP cells (**Figure 4.2**). *ADAM2* and *POP10* (intron of *ADAM2*) showed differences in expression in HEPG cells suggesting tissue-specific expression of splice variants of *ADAM2*. *MARCKSL1, POP1, POP2, POP3, POP4, POP5, POP12*, and *SPON2* were enriched in human prostate cancer cell lines compared to all other human cancer cell lines tested (**Figure 4.2**). Genes with these enriched expression trends have the potential to be markers for prostate cancer, while ubiquitously expressed genes may be general cancer markers, provided there is differential expression between cancer and normal cells.

To address tissue-specific expression of the 27 genes in benign tissues, we measured levels of transcripts in 20 human tissue samples (adrenal gland, bone marrow, cerebellum, whole brain, fetal brain, fetal liver, heart, kidney, liver, lung, placenta, prostate, salivary gland, skeletal muscle, testis, thymus, thyroid gland, trachea, uterus, and spinal cord). Gene expression was displayed relative to the levels in normal human prostate tissue. POP3 was the only transcript to exhibit exclusive expression in normal prostate tissue (**Figure 4.3**), suggesting it is a prostate-specific gene. This was consistent with expression of POP3 predominantly in LNCaP, MDA-PCa-2B, and 22RV1 cells that express androgen receptor and low expression in all other cell lines examined (**Figure 4.2**). Some genes were expressed at a level on par with that of the normal prostate in the adrenal gland (*ELOVL5*) and testis (*ELOVL5* and POP1; **Figure 4.3**). Interestingly, both adrenal glands and testes produce androgens that are essential for regulating the growth of the prostate[30]. POP1 had relatively specific expression in prostate cancer cell lines with similar expression patterns to POP3, while *ELOVL5* had broad expression across most cell lines (**Figure 4.2**). *ADAM2* and POP10 (intron of *ADAM2*) showed similar expression patterns in prostate, placenta, testis with the exception of expression of only *ADAM2* in thymus tissue (**Figure 4.3**). This data supports the tissue-specific expression of splice variants of *ADAM2*. POP4 (splice variant of *TMEFF2*) was expressed in the prostate, brain, and prostate cancer cells only expressing the androgen receptor. Both *RAMP1* and *SPON2* had relatively restricted expression in prostate and uterine tissues (**Figure 4.3**). Notably, men do not have

uterine or placental tissue. *SPON2* had expression relatively specific for prostate cancer cell lines (LNCaP and MDA-PCa-2B) while *RAMP1* was also highly expressed in MG63 osteosarcoma cells (**Figure 4.2**). Together, these data suggest that *ADAM2, ELOVL5,* POP1, POP3, POP4, POP10, *RAMP1,* and *SPON2* have relatively restricted expression patterns in adult male prostate.

4.3.2 Androgen regulation of gene expression

The androgen signalling axis plays an important role in the growth, survival, and differentiation of the prostate[31-33]. Treatment for locally advanced and metastatic prostate cancer includes androgen-deprivation therapy. Thus, it is essential to determine if levels of expression of any of the 27 genes are altered by androgen. To do this, levels of expression of these genes were assessed in prostate cancer cells with androgen receptor (LNCaP, MDA-PCa-2B, and 22Rv1) and without a functional androgen receptor (PC-3 and DU145)[34-38]. Expression of *ADAM2, CAMK2N1, DHCR24, MARCKSL1, NGFRAP1,* POP1, POP3, POP4, POP5, POP7, POP8, POP10, POP11, *SPON2,* and *TMEM66* transcripts were enriched in prostate cancer cell lines with a functional androgen axis (compare levels in LNCaP, MDA-PCa-2B and 22Rv1 to PC3 and DU145 cells in **Figure 4.2**). Although MCF7 mammary carcinoma cells express the androgen receptor at low levels[39], activation of the endogenous androgen signalling axis has not been documented[40]. With this potential lack of androgen signalling, expression of only *ADAM2, MARCKSL1,* POP1, POP3, POP4, POP5, POP10, POP11, and *SPON2* were not obviously elevated in MCF7 cells (**Figure 4.2**). Of these genes, expression of *ADAM2,* POP1, POP3, POP10 and *SPON2* were generally restricted to the prostate.

Differential expression of these 27 genes in response to androgen was also measured in LNCaP cells treated with 10 nM of synthetic androgen R1881 for 16 hours. Expression of 11 genes (*DHCR24, ELOVL5, GLO1, PGK1*, POP4, POP6, POP7, POP8, *SPON2, TMEM66,* and *YWHAQ)* increased, while significant decreases in expression of 5 genes *(ADAM2, CAMK2N1,* POP5, POP10, and POP11) were detected (**Figure 4.4**). Androgen regulation of genes *MARCKSL1, NGFRAP1,* POP1, POP2, POP3, POP9, POP12, *PSMA7, RAMP1, SBDS,* and *TMEM30A* were not detected (**Figure 4.4**). Although expression of *MARCKSL1, NGFRAP1,* POP1, and POP3 were elevated in prostate cancer cells with endogenous androgen receptor

compared to those cells without a functional receptor, no evidence supports that these genes are regulated by androgen. Enhanced expression of *ELOVL5, GLO1, PGK1*, POP6, and *YWHAQ* in response to androgen, while lacking enrichment in prostate cancer cells with endogenous androgen receptor compared to those without a functional receptor, suggests that these genes may be regulated by non-specific downstream effects of androgens such as changes in proliferation, metabolism, and/or differentiation.

4.3.3 Characterization of gene expression in prostate cancer

To determine if levels of any of the 27 transcripts were altered in prostate cancer compared to benign prostate epithelial cells, total RNA was isolated from 28 laser microdissected samples of prostate obtained by radical prostatectomy from Japanese prostate cancer patients. Laser microdissection was employed because prostate cancer is typically a heterogeneous disease[41] with multiple foci[42]. These studies revealed that levels of expression of *RAMP1* and *SPON2* were significantly increased, while levels of expression of *ELOVL5, NGFRAP1,* POP5, POP8, and *TMEM66* were significantly decreased in malignant compared to normal epithelial prostate cells (**Figure 4.5**; the Kruskal Wallis test, $p \leq 0.05$). *MARCKSL1*, POP2 and POP10 were borderline significantly increased ($p<0.1$), while *PSMA7* was borderline decreased (**Figure 4.5**). Borderline significant changes in expression of genes indicate that analysis using a larger sample size may be required to achieve statistical significance. No significant differences in expression were measured between malignant compared to normal epithelial prostate cells for *ADAM2, CAMK2N1, DHCR24, GLO1, PGK1,* POP1, POP3, POP4, POP6, POP7, POP9, POP11, POP12, *SBDS, TMEM30A,* and *YWHAQ* (**Figure 4.5**).

To determine if the levels of expression of genes in tumour tissue samples correlated to patient age, PSA level[10], and stage of the disease (Tumour-Node-Metastasis, TNM)[43], only levels of each transcript in the tumour samples were utilized in statistical analyses. No association between the expression of any of the candidate genes and the age of the patient was detected using Spearman's correlation ($p \leq 0.05$). Only borderline significance was obtained between TNM stage of prostate cancer (clinical or pathological) and the expression for *RAMP1* ($p = 0.07$) and POP12 ($p = 0.09$) as assessed with the Kruskal Wallis test ($p \leq 0.05$). Additional patient samples are also required to test associations in transcript levels with Gleason grade or

score[44]. However, expression of *PGK1* and POP9 were independently positively correlated with high serum PSA levels (**Figure 4.6**; Spearman's correlation, $p \leq 0.05$), with borderline significance for *RAMP1* ($p = 0.07$) and POP8 ($p = 0.08$). Men with high levels of serum PSA at the time of first line therapy (e.g., prostatectomy) have a greater risk of prostate cancer recurrence[10]. The expression of POP9, *PGK1,* and possibly POP8 and *RAMP1,* may be prognostic due to a correlation with serum PSA levels prior to surgery.

4.4 DISCUSSION

Gene expression studies have been performed using prostate tissue in attempts to identify prognostic markers. The first major report was from the University of Michigan where 10k cDNA arrays were used to probe more than 50 normal and neoplastic prostate specimens. This study identified hepsin and *PIM-1* to be over-represented in prostate cancer. The results were also validated at the protein level using 700 clinical samples[45]. Despite over-expression in prostate cancer, surprisingly reduced or absent levels in cancer were associated with increased risk of relapse after prostatectomy[45]. Thus, the expression profiles of hepsin and *PIM-1* will be difficult to interpret and apply for clinical decisions, thereby emphasizing the need to identify and characterize better markers for prostate cancer.

Here, genes and novel non-coding transcripts previously identified to be differentially expressed in an *in vivo* model of hormonal progression of prostate cancer were characterized and revealed the following: 1) prostate-specific expression of POP3 and restricted tissue expression of *ADAM2*, POP1, POP4, POP10, *ELOVL5, RAMP1*, and *SPON2*; 2) changes in expression of *ADAM2, CAMK2N1, DHCR24, ELOVL5, GLO1, PGK1*, POP4, POP5, POP6, POP7, POP8, POP10, POP11, *SPON2, TMEM66,* and *YWHAQ,* in response to androgen; 3) differential levels of expression of *ELOVL5, NGFRAP1,* POP5, POP8, *RAMP1, SPON2, TMEM66* and possibly *MARCKSL1,* POP2, POP10, and *PSMA7* between clinical samples of normal and malignant prostate tissue; and 4) correlation with clinical parameters and levels of *PGK1,* POP9 and possibly POP8, POP12, and *RAMP1* transcripts. Due to the broad expression of *MARCKSL1, NGFRAP1, PGK1,* POP2, POP5, POP8, POP9, POP12, *PSMA7,* and *TMEM66* across many tissues, these genes or transcripts have limited application as biomarkers for prostate cancer. A summary of results is presented in **Table 4.3**.

ELOVL5 was broadly expressed across most cell lines examined, yet showed restricted expression in benign tissues to the prostate, adrenal, and testis. Enhanced levels of expression of *ELOVL5* in cell lines could be interpreted to be associated with malignancy. Yet, when comparing levels of expression in malignant versus benign prostate epithelial cells, levels were significantly decreased in the tumours. Notably, all of the candidate transcripts in this paper were identified in an *in vivo* model for castration-recurrent prostate cancer, and not from primary tumours. Therefore, differential expression between tumour and normal tissue from prostatectomy patients was not necessarily expected. Curiously, levels of expression increased in response to androgen. This result is confirmed by a previous publication[46]. ELOVL5 protein functions in fatty acid synthesis. Importantly, lipogenesis is important for the synthesis of androgen precursors. Recently, local production of androgen has been implicated in castration-recurrent prostate cancer[47].

Levels of POP1 and POP3 transcripts did not change in response to androgen, yet were detected specifically in prostate cancer cell lines that expressed androgen receptor. These transcripts were not expressed in prostate cancer cells that did not express androgen receptor or by any of the other cell lines tested. In addition to the prostate, POP1 was also expressed in the testis, while POP3 expression was specific for the prostate, both benign and tumour. No differences in levels of expression of POP1 and POP3 were measured in benign or malignant epithelial cells from clinical samples. It should be noted that although tissue samples were laser microdissected, basal cells are likely present in the normal samples. By definition of prostate cancer, the tumour samples would not contain basal cells. The contribution of the basal cells to the expression trends observed here remain to be determined. For these reasons, differential expression between primary tumours and normal tissue may not be observed. POP1 and POP3 represent non-coding transcripts for mRNA AK000023 (POP1) and transcript 50 kb from EST CFI40309 (POP3)[25]. Non-coding transcripts display a diverse array of functions including the regulation of expression of other genes. Sense non-coding transcripts can silence gene expression by recruiting chromatin remodelling complexes that methylate and deacetylate histones of specific genomic sequences such as *XIST*. Alternatively, intergenic non-coding transcripts may promote the expression of the surrounding gene by recruiting chromatin remodelling complexes that demethylate and acetylate histones in the wake of RNA polymerase II (e.g., *XITE* transcript action on the *TSIX* gene). Moreover, steric hindrance of sense transcription via antisense

transcription, and formation of RNA hybrids between non-coding transcripts and target transcripts may also lead to transcriptional suppression. Double stranded RNAs may result in RNA interference, RNA masking, RNA hyperediting, and degradation[48]. These non-coding POP transcripts are not considered to be microRNAs because their sequences range between 155-231 bp in length. Thus, further investigation of POP1 and POP3 non-coding transcripts in prostate biology and pathology is warranted based upon their prostate-specific expression and potential to regulate gene expression.

POP4 had restricted expression to the prostate and brain, and was detected only in prostate cancer cells that expressed androgen receptor. Increased levels of POP4 transcript were measured in response to androgen, but no differences were measured between normal and malignant prostate epithelial cells. POP4 is protein-coding for a truncated isoform of the transmembrane protein TMEFF2[49]. The truncation of TMEFF2 eliminates the transmembrane domain, creating a secreted isoform[49]. Full-length TMEFF2 protein has been associated with castration-recurrent prostate cancer[50], and is currently being targeted by antibodies for the treatment of metastatic prostate cancer[51, 52]. Since POP4 is a secreted form of TMEFF2 that is not elevated in prostate cancer compared to normal prostate, it is unclear if serum levels would provide any additional clinical information to serum PSA.

POP10 (intron of *ADAM2*) expression was restricted to the prostate, testis, and placenta. *ADAM2* exhibited the same restricted expression, but was additionally detected in the thymus. Curiously, although POP10 was borderline significantly increased in clinical samples of prostate cancer, while *ADAM2* was not, expression of POP10 and *ADAM2* were relatively restricted to LNCaP cells with decreased levels measured in the other prostate cancer cell lines tested. Levels of POP10 (and *ADAM2*) were decreased in response to androgen. Together, these data suggest that these two variant transcripts may share common androgen-response element(s) in the regulatory regions to modulate transcription. However, tissue specificity was observed for these two transcripts which support additional mechanisms to regulate transcription or mRNA processing.

Expression of *RAMP1* was significantly higher in tumour compared to normal prostate tissue from prostatectomy patients, indicating it has the potential to be detected at tumour sites. Consistent with the restricted expression of *RAMP1* in the prostate and uterus, *RAMP1* was expressed in all prostate cancer cell lines examined, except DU145. Of the non-prostate cell lines examined, *RAMP1* was only expressed in MG63 cells. The protein product of *RAMP1* is expressed on the plasma membrane, indicating it has the potential to be detected at tumour sites. Since no changes in expression were detected in response to androgens, this protein could potentially be used to monitor and/or image metastatic prostate cancer in patients regardless of whether they are receiving androgen-deprivation therapies. An example of this can be drawn from prostate-specific membrane antigen (PSMA) that is expressed on the plasma membrane[53]. PSMA is a prostate-specific biomarker[54] used clinically for detection of recurrent prostate tumours and locate metastases[55]. A radiolabelled antibody, ^{111}In-capromab or Prostascinct, binds to PSMA at the site of soft-tissue metastatic prostate cancer, and is visualized by an immunoscintigraphy scan[53]. Detection of metastatic cancer using imaging, has the advantage of being minimally invasive. Moreover, it can be performed repeatedly for the monitoring of disease.

Expression of *SPON2* increased in response to androgen, was elevated in tumour versus normal prostate, and was restricted to the prostate and uterus. However, unlike RAMP1 expression trends, *SPON2* was not expressed broadly across all prostate cancer cell lines with detection only in LNCaP and MDA-PCa-2B cells. These data may imply that expression of *SPON2* may not be uniformly expressed by all prostate cancer and limited to only a subset of cancers. *SPON2* gene expression is reported to be variable among non-laser microdissected samples of prostate cancer obtained by radical prostatectomy[56]. Prostate-specific expression of *SPON2* has also been suggested from studies that interrogated publicly available SAGE databases[56]. However, some discrepancies exist in the literature for whether expression of *SPON2* is elevated in tumour versus normal prostate tissue. No significant differences in levels of *SPON2* protein among primary tumour, lymph node metastases, bone metastasis, or locally recurrent tumours in castration-recurrent patients were detected using formalin-fixed, paraffin-embedded tissue[57]. In contrast, levels of *SPON2* transcript is significantly increased in castration-recurrent prostate cancer from the LNCaP Hollow Fibre model[24]. Whether these discrepancies stem from sample preparation or methods of detection, remains to be determined. Application of radiolabelled

antibodies (^{86}Y-19G9) to SPON2 protein has been proposed for the detection of prostate cancer with successfully imaging of LNCaP xenografts using positron emission tomography (PET)[57]. Surprisingly, despite the fact SPON2 is a secreted protein, it was detected in close proximity to the tumour; perhaps because it weakly associates with plasma membranes[57]. Despite this sequestration in tumours, elevated levels of SPON2 protein may be detectable in the serum of prostate cancer patients. Similar to PSA, expression of *SPON2* is prostate-specific. However, SPON2 is potentially superior to PSA as a serum marker because of its elevated expression in malignancy compared to normal cells as shown here. Recently a sandwich enzyme-linked immunosorbent assay for SPON2 protein was used for the diagnosis and early detection of ovarian tumours[58].

Numerous markers of prostate cancer have been described as androgen-regulated. For example, fusions between the un-translated region of the androgen-regulated gene *TMPRSS2* and the ETS gene family (whose protein products are transcription factors) result in the deleterious misregulation of groups of genes in response to hormone[59]. *TMPRSS2*-ETS family of gene fusions are associated with biochemical progression following prostatectomy[60, 61] and metastatic, castration-recurrent prostate cancer[62, 63]. Measurement of *TMPRSS2*-ETS transcript highlights that some biomarkers of prostate cancer may only be detected at the level of DNA or RNA. Unfortunately, genomic and transcript detection has thus far been restricted to use with biopsy tissue, which is invasive and inconvenient to sample[64]. Recent studies have shown that tumour cells of metastatic, castration-recurrent prostate cancer are shed into the circulation and can be isolated and interrogated by DNA or RNA molecular analysis[65-67]. Genomic amplification of the androgen receptor has been detected by fluorescence *in situ* hybridization (FISH) in circulating tumour cells of patients with castration-recurrent prostate cancer[65]. This approach to biomarker detection may potentially be applied to non-coding POPs. It should be noted that the levels of POP transcripts were very low (~12 qRT-PCR cycle thresholds higher than glyceraldehyde-3-phosphate (GAPDH); data not shown), indicating that an assay more sensitive than FISH may be required for their detection. Reverse transcriptase-PCR analysis has been successfully applied to samples of circulating tumour cells from men with metastatic prostate cancer[67]. It is conceivable that the assay could be adapted for qRT-PCR for improved sensitivity.

Circulating tumour cells may also be used as a source for detection of protein expression[65]. The androgen-affected transcripts *ADAM2, ELOVL5*, and *TMEM66* were enriched in castration-recurrent prostate cancer[24], and code for plasma membrane proteins. Their over-expression may be detectable via immunohistochemistry of fixed circulating tumour cells based on the previous success of a similar study[65].

Detection of non-coding POPs is not restricted to blood samples. In fact, exfoliated tumour cells are also present in urine. Recently, detection of the non-coding transcript *PCA3* in whole urine has been used to improve predictions of prostate biopsy outcome using the PROGENSA PCA3 assay[68, 69]. This assay is to be used in conjunction with *PSA*, as *PCA3* mRNA levels are normalized to *PSA* mRNA levels in the urine. Due to an association with pre-treatment PSA levels, the development of urine assays for POP9, and possibly POP8, might yield useful prognostic information when applied to clinical samples.

Typically an assay for decreased gene expression is more challenging to design than for increased gene expression, because high quality controls are needed to interpret the results. Gene expression may be assessed by measuring the levels of expression of transcripts, or by testing for epigenetic markings such as methylation and acetylation at promoters. The advantage of evaluating epigenetics as opposed to transcript levels, is that a positive result can be obtained for decreased gene expression. For example, gene silencing of glutathione-S-transferase P1 (*GSTP-1*) due to promoter methylation is present in >90% of prostate cancer, but infrequent in benign tissue. Promoter methylation has been detected using quantitative methyl specific PCR in urine sediments of prostate cancer patients with a specificity of 98% and sensitivity of 78%[70]. Interestingly, the gene *EFNA5* is a target for promoter methylation and gene silencing in non-Hodgkin's lymphoma[71]. The POP8 transcript is expressed from an intron of *EFNA5*, and the expression of POP8 was lower in tumour compared to normal prostate tissue. These data suggest that the promoter of POP8 may also be silenced due to promoter methylation and may be a target for epigenetic assays.

Biomarkers often perform better when they are part of a panel of genes. For example, the promoter methylation of *GSTP-1* indicates prostate cancer with a specificity of 100% and

sensitivity of 87% when used in combination with cyclin-dependent kinase 2A (*CDKN2A*), ADP-ribosylation factor 1 (*ARF1*), and o-6-methylguanine-DNA methyl transferase (*MGMT*)[72]. This is a significant improvement over *GSTP-1* alone. Moreover, high gene expression of enhancer of zeste homolog 2 (*EZH2*) indicates aggressive disease and poor survival for prostate cancer patients[73]. EZH2 protein is responsible for the transcriptional silencing of numerous genes, via recruitment of the histone deacetylase, in metastatic prostate cancer. However, gene silencing is insufficient to account for all gene expression changes that are expected to occur during the progression of prostate cancer[74]. The combination of gene expression profiles of *PCA3*, prostein, and transient receptor potential cation channel subfamily M member 8 (*TRPM8*), worked in concert with *EZH2* to provide additional prognostic power in a study of 106 patients with matched prostatectomy samples[75]. Although the differentially expressed genes *NGFRAP1*, POP5, POP8, and *TMEM66* in tumour versus normal prostate samples were not also prostate-specific, when used in combination with prostate restricted genes *ADAM2*, POP1, POP3, POP4, POP10, *ELOVL5, RAMP1*, and *SPON2* in a biomarker assay, the panel of genes may be clinically useful.

4.5 CONCLUSION

In summary, 27 potential biomarkers of prostate cancer were characterized for prostate-specific expression, regulation by androgen, and expression in clinical samples of prostate cancer. POP3 was prostate-specific with restricted expression of *ADAM2,* POP1, POP4, POP10, *ELOVL5, RAMP1,* and *SPON2.* The expression of *ADAM2, CAMK2N1, DHCR24, ELOVL5, GLO1, PGK1*, POP4, POP5, POP6, POP7, POP8, POP10, POP11, *SPON2, TMEM66,* and *YWHAQ* genes changed in response to androgen. *ELOVL5, NGFRAP1,* POP5, POP8, *RAMP1, SPON2,* and *TMEM66* were significantly differentially expressed between laser microdissected tumour and normal prostatic tissue, and *PGK1* and POP9 were positively associated with pre-prostatectomy serum PSA levels. Together, these studies suggest that *ADAM2, ELOVL5,* POP1, POP3, POP4, POP10, *RAMP1,* and *SPON2* may be good candidates for biomarkers of prostate cancer.

Table 4.1 Information on the samples used for laser microdissection and gene expression analysis, and the patient's they were taken from

Sample No.*	Patient ID †	Age (years)	PSA ‡ (ng/mL)	Stage §		Normal or Tumor ‖	Gleason Grade ¶	Gleason Sum **
				Clinical	Pathological			
1	A	59	9.7	N/A††	2b	N	N/A	N/A
2	"‡‡	"	"	"	"	T	3+4	7
3	B	64	19.0	1c	3b	N	N/A	N/A
4	"	"	"	"	"	T	4+4	8
5	C	71	24.2	2b	2b	N	N/A	N/A
6	"	"	"	"	"	T	3+4	7
7	D	68	9.5	2a	3b	N	N/A	N/A
8	"	"	"	"	"	T	5+4	9
9	E	64	19.1	2a	3b	T	3+4	7
10	F	71	5.5	2b	2b	N	N/A	N/A
11	G	69	25.1	1c	N/A	N	N/A	N/A
12	"	"	"	"	"	N	N/A	N/A
13	"	"	"	"	"	T	4+3	7
14	H	67	6.4	2a	2a	N	N/A	N/A
15	"	"	"	"	"	T	4+4	8
16	I	64	7.7	2a	2a	N	N/A	N/A
17	J	70	29.9	2b	3b	T	3+3	6
18	K	62	10.0	2a	2b	N	N/A	N/A
19	L	63	15.6	2a	2b	T	3+4	7
20	M	74	5.2	2b	2b	N	N/A	N/A
21	N	70	14.1	2b	3b	T	4+3	7
22	O	N/A	N/A	N/A	N/A	T	3+4	7
23	"	"	"	"	"	N	N/A	N/A
24	"	"	"	"	"	T	3+5	8
25	P	74	5.7	2a	2a	N	N/A	N/A
26	Q	69	8.0	2b	3b	N	N/A	N/A
27	R	68	22.2	2a	3a	N	N/A	N/A
28	S	73	20.3	3a	3b	T	4+4	8

* Sample No., sample number labeled '1 'to '28'
† Patient ID, patient identification labeled 'A' to 'S'
‡ PSA, prostate-specific antigen serum levels upon diagnosis
§ Stage, Tumor Node Metastses (TNM) staging system
‖ N (Normal), normal prostate tissue; T (Tumor), tumor prostate tissue
¶ Gleason Grade, grading system to describe degree of differentiation of tumor tissue cells. Gleason grading was applied to the slide of tissue used for laser microdissection by a trained pathologist
** Gleason Sum, cummulative score of the two most prominent Gleason Grades present on the slide of tissue
†† N/A, not applicable or not available
‡‡ ", same as above

Table 4.2 Primer and probe sequences for qRT-PCR of candidate transcripts

Gene	Forward Primer (5'-3')	Probe (5'-3')	Reverse Primer (5'-3')
ADAM2	TGGTGAAAGTTAATTTCCAAAGG	ATTCAAGCGATGAGCAACCT	TCATGGCATCTCTGTTGTCC
CAMK2N1	TGCAGGACACCAACAACTTC	AGCAAGCGGGTTGTTATTGA	GCACGTCATCAATCCTATCATC
DHCR24	GAGGCAGCTGGAGAAGTTTG	TGCTGTATGCCGACTGCTAC	CTTGTGGTACAAGGAGCCATC
ELOVL5	GTTTGTCGTCAGTCCCTTCC	CGTCCATACCTCTGGTGGAA	TGGTCTGGATGATTGTCAGC
GAPDH	CTGACTTCAACAGCGACACC	CGACCACTTTGTCAAGCTCA	TGCTGTAGCCAAATTCGTTG
GLO1	AAAGGTTTGAAGAACTGGGAGTC	AAGGCCTGGCATTTATTCAA	TTCAATCCAGTAGCCATCAGG
MARCKSL1	GCAGCCAGAGCTCCAAGG	CCAACGGCCAGGAGAATG	AAGTCTCCATTGCTTTTCACG
NGFRAP1	GTCACTCGCGTCTGGCTAC	AAAGCGGAGCAGGTCTGC	GCCGCGGAGACACTTAGC
PGK1	GAAGGGAAGGGAAAAGATGC	CGAGCCAGCCAAAATAGAAG	GACATCCCCTAGCTTGGAAAG
POP1*	AAGCTCTTGCTAGGCATGTAGG	CCTGGACAGCCCATTCTTTA	TTTGGGTAGACATTTCCCC
POP2	GGAGGATCAACAGCAGCATT	CAACTGTGCTCCATTGACGT	GGTATCATTGAGGCTGGGTG
POP3	TATGGTGTGCCATTTCTGGA	CCGTTTGCATCTCTGAGTGA	GTGGAACAAAATCCCCTCCT
POP4	CCCTTGTGCAAATGGGTTA	TCATTTATGATAGCCACACATGA	TTGTTCCCTTCACTCTTTTGTTC
POP5	TTTGGAAAGGTGAGCCTCTG	CATTGTTTGGGCAGGAGAGT	AAAGAAGTGGACGTGGCAC
POP6	TTTAAGTGGTTCAGCACACAAAAC	CAAAAGGATGACCTTGGGAA	TGATGACTTCCTTGTGTTTAACAAA
POP7	TTGGTTTCTGGACCCTTTTG	AAAGCTTGAGGGTGGTGATG	CAGAAGAGCAGGGTGGGTAG
POP8	TTTCGGTTCCTTTCCTCTTC	CCCACATTCCATTTCAAACA	ATTCCTTTATGGCTTGAAGGGT
POP9	CCTGTTTCCCAGTCACACCT	TTAACAATTCCCAAGCACCC	ATTTGTCTTCCACCACAGGC
POP10	TTGCTAGGGAAAAGCAGCAT	TTCTTCACCAAACTCTCTAAAACAGA	GAATCATAAGGCAGCCTCCTT
POP11	GTTCGCTCTTGGCTTTGAAC	TTCCCTGTCCCCTAACTCCT	TTTGCCTTTTGCAGAATGTG
POP12	TGTGACAAAATGGGAGGACA	GCTTGTTTGAGTTGCAAGCA	CAGAAAAGTGTATGGCAGGGA
PSMA7	CGTCAAGAAGGGCTCGAC	AAGAAGTCAGTGGCCAAACTG	CGCACTGTTCTTTCATCCTG
RAMP1	CCTCACCCAGTTCCAGGTAG	CAGGACCATCAGGAGCTACA	CATGTGCCAGGTGCAGTC
SBDS	CGCCTGCTACAAAAACAAGG	CGTGGAAAAAGACCTCGAT	CAAACACTGAGTGGGTCTGC
SDHA	ACCAGGTCACACACTGTTGC	ACATGGAGGAGGACAACTGG	CCTGTGGTGTCGTAGAAATGC
SPON2	CCCAGCAGGGACAATGAG	TGTAGACAGCGCCTCAGTTC	CACAGTCCCCAGGACGAC
TMEM30A	GGATGTGACACCTTGCTTTTG	CCATTAACTTCACACTGGAAAAG	ACGTAACGACGATGGTTTTG
TMEM66	GGGCAGCTATTCGGTATGTTC	CGAAAACCAGAACTGCATCA	TGCATCCAGTGTTTGACTCC
YWHAQ	CTGAGATCCATCTGCACCAC	AGCCAATGCAACTAATCCAGA	ACCGGAAGTAATCACCCTTC

* Genes were represented by HGNC approved nomenclature when available. Non-HGNC gene names were not italicized.

Table 4.3 Review of expression trends of candidate genes

Gene	Ex. in CRPC vs. RAD*	Ex. in RAD vs. AS †	En. in AR + CaP cells ‡	Reg. by A §	S or PM Prot. ‖	En. in Human ¶	En. in CaP Cell Lines**	En. in Normal Prostate ††	Assoc. w/ PSA ‡‡	Ex. in Tumor vs. Normal §§
ADAM2	↑	NS	Y ‖‖	↓	PM	Y	Y	Y	N	NS
CAMK2N1	↑	↑	Y	↓	N	N	N	N	N	NS
DHCR24	↑¶¶	↓	Y	↑¶¶	N	Y	N	N	N	NS
ELOVL5	↑	↓	N	↑¶¶	PM	N	N	Y	N	↓
GLO1	↑	NS	N	↑	N	N	N	N	N	NS
MARCKSL1	↓	NS	Y	N	PM	Y	Y	N	N	NS(↑¶¶)
NGFRAP1	↑	NS	Y	N	N	N	N	N	N	↓
PGK1	↑	↓	N	↑¶¶	N	N	N	N	Y+	NS
POP1***	↑	UD	Y	N	NA	Y	Y	Y	N	NS
POP2	↑	UD	N	N	NA	Y	Y	N	N	NS
POP3	↑	UD	Y	N	NA	Y	Y	Y	N	NS
POP4	↑	UD	Y	↑	NA	Y	Y	Y	N	NS
POP5	↑	UD	Y	↓	NA	Y	Y	N	N	↓
POP6	↑	UD	N	↑	NA	Y	N	N	N	NS
POP7	↑	UD	Y	↑	NA	Y	N	N	N	NS
POP8	↑	UD	Y	↑	NA	Y	N	N	N	↓
POP9	↑	UD	N	N	NA	N	N	N	Y+	NS
POP10	↑	UD	Y	↓	NA	Y	N	Y	N	NS
POP11	↑	UD	Y	↓	NA	Y	Y	N	N	NS
POP12	↑	UD	N	N	NA	Y	Y	N	N	NS
PSMA7	↑	NS	N	N	N	N	N	N	N	NS
RAMP1	↓	↑	N	N	PM	Y	N	Y	N	↑
SBDS	↑	NS	N	N	N	Y	N	N	N	NS
SPON2	↑	NS	Y	↑	S	Y	Y	Y¶¶	N	↑
TMEM30A	↑	NS	N	N	S&PM	Y	N	N	N	NS
TMEM66	↑	NS	Y	↑	S&PM	N	N	N	N	↓
YWHAQ	↓	NS	N	↑	N	N	N	N	N	NS

* Ex. in CRPC vs. RAD, Gene expression in castration-recurrent prostate cancer versus responsive to androgen-deprivation in the LNCaP Hollow Fiber model

† Ex. in RAD vs. AS, Gene expression in the stage of prostate cancer that is responsive to androgen-deprivation versus androgen-sensitive in the LNCaP Hollow Fiber model

‡ En. in AR+ CaP cells, Gene expression is enriched in androgen receptor positive prostate cancer cells versus androgen receptor negative prostate cancer cells

§ Reg. by A, Expression of gene is regulated by androgen; Arrow indicates direction of regulation with androgen

‖ S or PM Prot., Gene product is a secreted (S) or plasma membrane (PM) protein

¶ En. in Human; Gene expression is enriched in human cell lines versus monkey kidney cells

** En. in CaP Cell Lines, Gene expression is enriched in prostate cancer cell lines versus cell lines of cancer from other organ sites

†† En. in Normal Prostate, Gene expression is enriched in normal human prostate tissue versus other normal human tissues

‡‡ Assoc. w/ PSA, Gene expression in laser microdissected sample is associated with serum prostate-specific antigen levels measured from the patient at the time of diagnosis of prostate cancer; '+' indicates a positive association

§§ Ex. in Tumor vs. Normal, Gene expression in tumor tissue versus normal prostate tissue

‖‖ Y, yes; N, no; NS, no significant difference; NA, not applicable; UD, undetermined; ↑, higher expression; ↓, lower expression

¶¶ Known

*** Genes were represented by HGNC approved nomenclature when available. Non-HGNC gene names were not italicized.

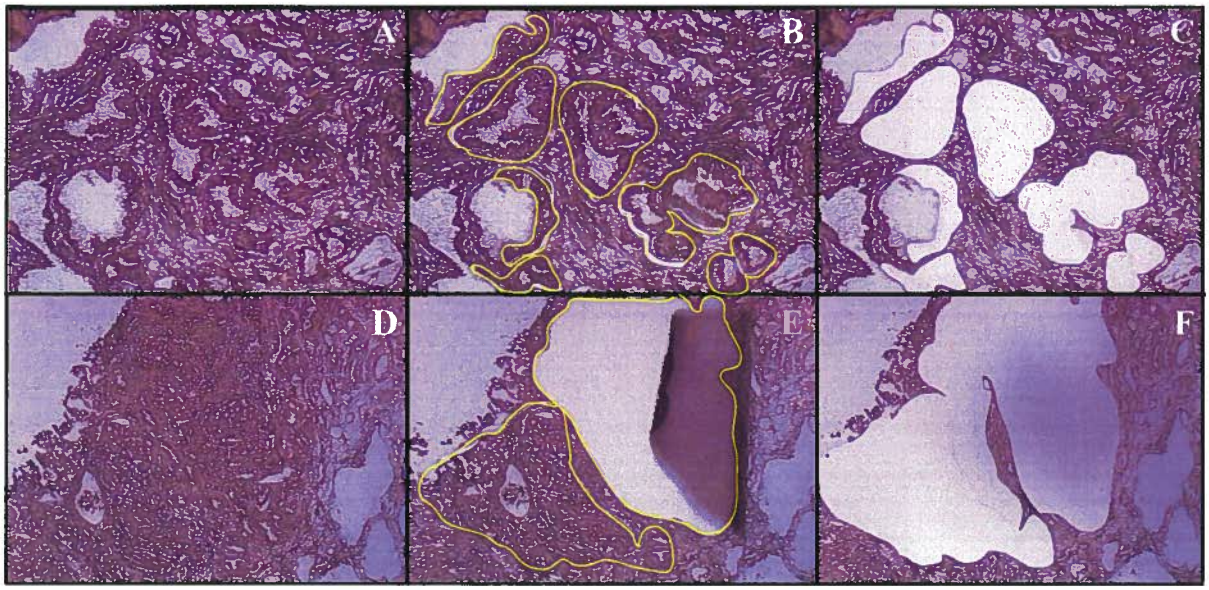

Figure 4.1 Laser microdissection of normal and tumour prostate tissue. Selected prostate epithelial cells were cut at 20x magnification using laser power and collected onto adhesive caps. Images show tissue prior to cutting (**A** and **D**), post-cutting (location of cut is highlighted yellow; **B** and **E**), and remaining post capture on an adhesive cap (**C** and **F**). Images **A-C** represent normal tissue, while images **D-F** represent tumour tissue.

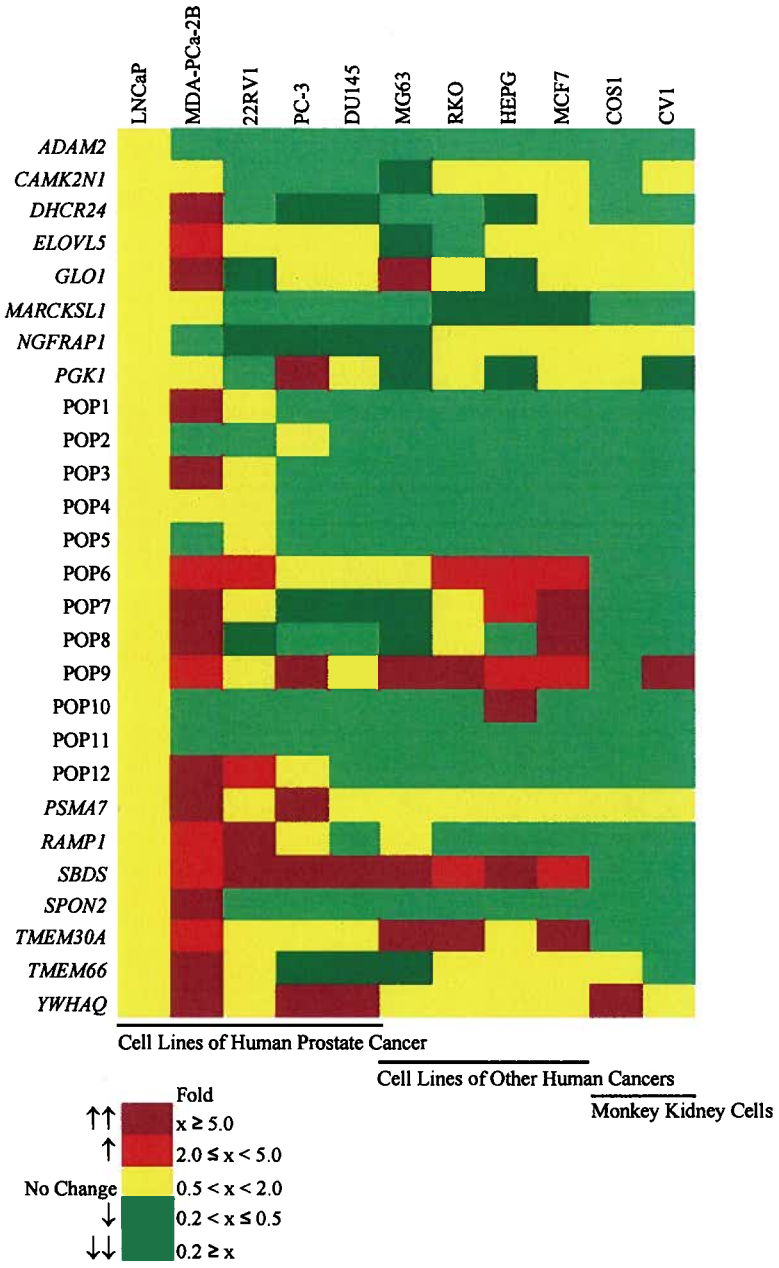

Figure 4.2 Specificity of gene expression for human prostate cancer. RNA was isolated from LNCaP, MDA-PCa-2B, 22Rv1, PC-3, DU145, MG63, RKO, HEPG, MCF7, COS1, and CV1 cells and analyzed by qRT-PCR using primers and probes for *ADAM2, CAMK2N1, DHCR24, ELOVL5, GLO1, MARCKSL1, NGFRAP1, PGK1*, POP1, POP2, POP3, POP4, POP5, POP6, POP7, POP8, POP9, POP10, POP11, POP12, *PSMA7, RAMP1, SBDS, SPON2, TMEM30A, TMEM66,* and *YWHAQ*. Heat map indicates the average degree of fold-change in gene expression relative to LNCaP cells of three biological replicates. Non-HGNC gene names were not italicized.

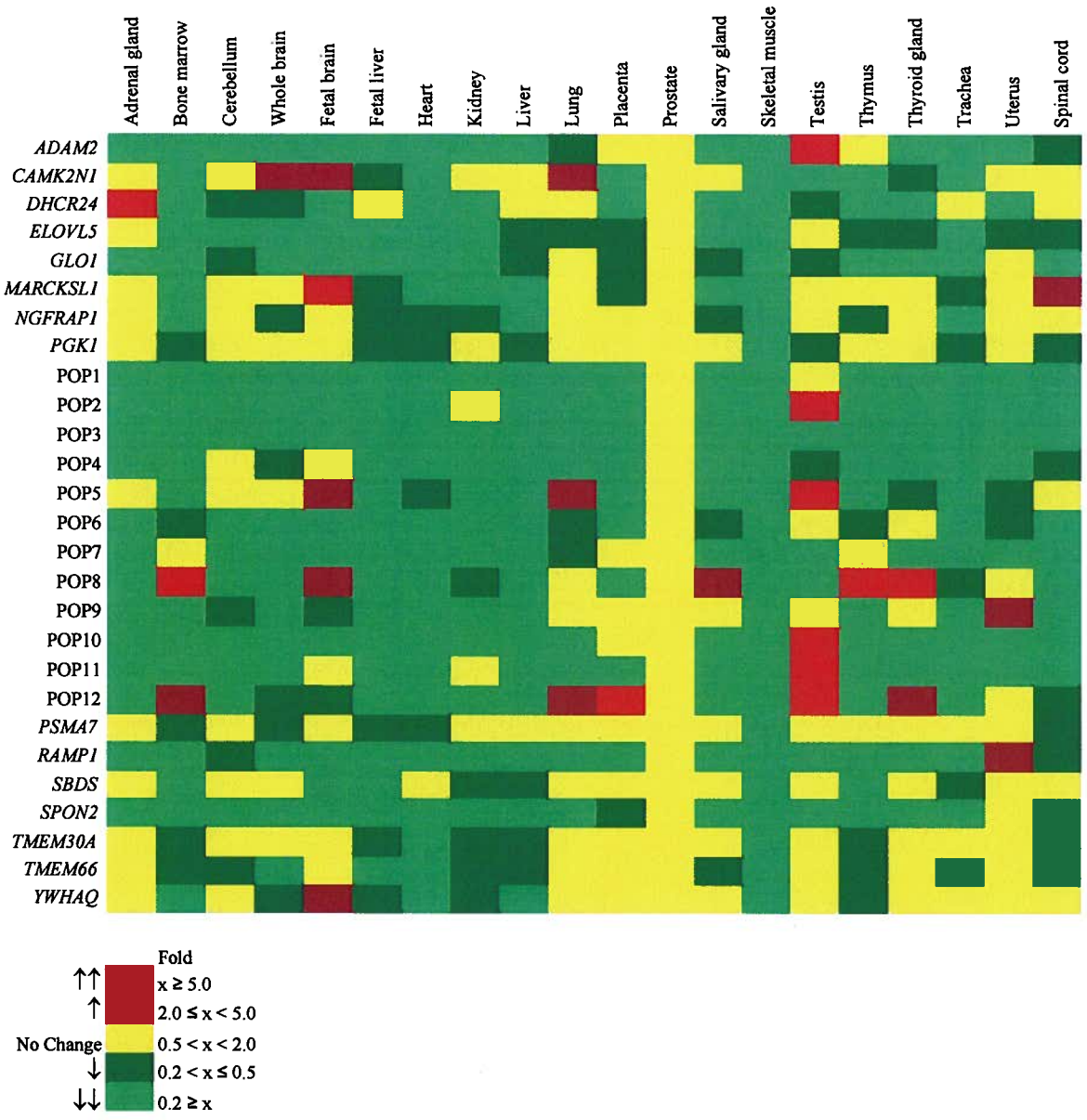

Figure 4.3 Specificity of gene expression for normal prostate tissue. RNA was obtained commercially from normal human tissues (adrenal gland, bone marrow, cerebellum, whole brain, fetal brain, fetal liver, heart, kidney, liver, lung, placenta, prostate, salivary gland, skeletal muscle, testis, thymus, thyroid gland, trachea, uterus, and spinal cord) and analyzed by qRT-PCR using primers and probes for *ADAM2, CAMK2N1, DHCR24, ELOVL5, GLO1, MARCKSL1, NGFRAP1, PGK1*, POP1, POP2, POP3, POP4, POP5, POP6, POP7, POP8, POP9, POP10, POP11, POP12, *PSMA7, RAMP1, SBDS, SPON2, TMEM30A, TMEM66,* and *YWHAQ*. Heat map indicates the degree of fold-change in gene expression relative to prostate tissue. Non-HGNC gene names were not italicized.

Figure 4.4 Regulation of gene expression by androgen. RNA was harvested from LNCaP cells that were treated with R1881 and analyzed by qRT-PCR. Candidate biomarkers assayed for gene regulation by androgen include: *ADAM2, CAMK2N1, DHCR24, ELOVL5, GLO1, MARCKSL1, NGFRAP1, PGK1*, POP1, POP2, POP3, POP4, POP5, POP6, POP7, POP8, POP9, POP10, POP11, POP12, *PSMA7, RAMP1, SBDS, SPON2, TMEM30A, TMEM66,* and *YWHAQ*. Fold-change was calculated by normalizing the mean normalized expression (MNE) of transcripts in R1881-treated cells to the mock vehicle-treated cells. In doing this, the vehicle treatment fold-change became one and standard deviation (SD) zero. Error bars represent ± SD for six biological replicates. [*] Asterisk indicates significant differential gene expression according to the Two-Sample Student's T-test ($p \leq 0.05$) for unequal variance. Non-HGNC gene names were not italicized.

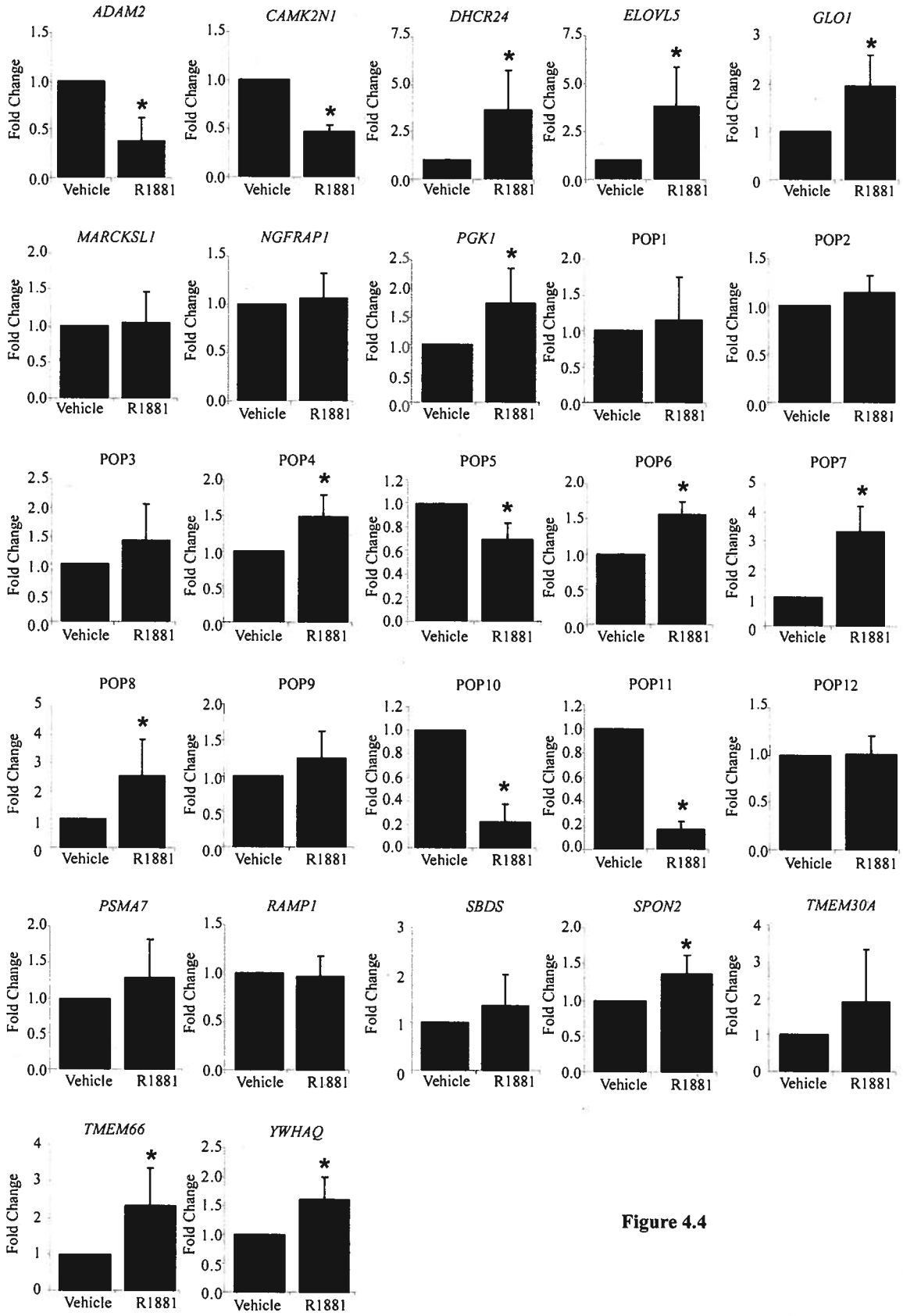
ADAM2
CAMK2N1
DHCR24
ELOVL5
GLO1
MARCKSL1
NGFRAP1
PGK1
POP1
POP2
POP3
POP4
POP5
POP6
POP7
POP8
POP9
POP10
POP11
POP12
PSMA7
RAMP1
SBDS
SPON2
TMEM30A
TMEM66
YWHAQ
Fold Change
Vehicle
R1881

Figure 4.4

Figure 4.5 Candidate biomarkers are differentially expressed between normal and tumour prostate. RNA was isolated from laser microdissected normal and tumour human prostate tissue (n=19; one of each randomly selected from each patient with available tissue) and analyzed by qRT-PCR using primers and probes for *ADAM2, CAMK2N1, DHCR24, ELOVL5, GLO1, MARCKSL1, NGFRAP1, PGK1*, POP1, POP2, POP3, POP4, POP5, POP6, POP7, POP8, POP9, POP10, POP11, POP12, *PSMA7, RAMP1, SBDS, SPON2, TMEM30A, TMEM66,* and *YWHAQ*. Plotted on the y-axis is the mean normalized expression (MNE) of candidate genes against the x-axis of normal and tumour tissue. A p-value cut-off of $p \leq 0.05$ (Kruskal Wallis test) was used to determine statistically significant differential gene expression. Box and Whisker plots display the median MNE (bolded line), the first and third quartile range (the box), minimum and maximum non-outlier values (whiskers), and outliers (open circles). Non-HGNC gene names were not italicized.

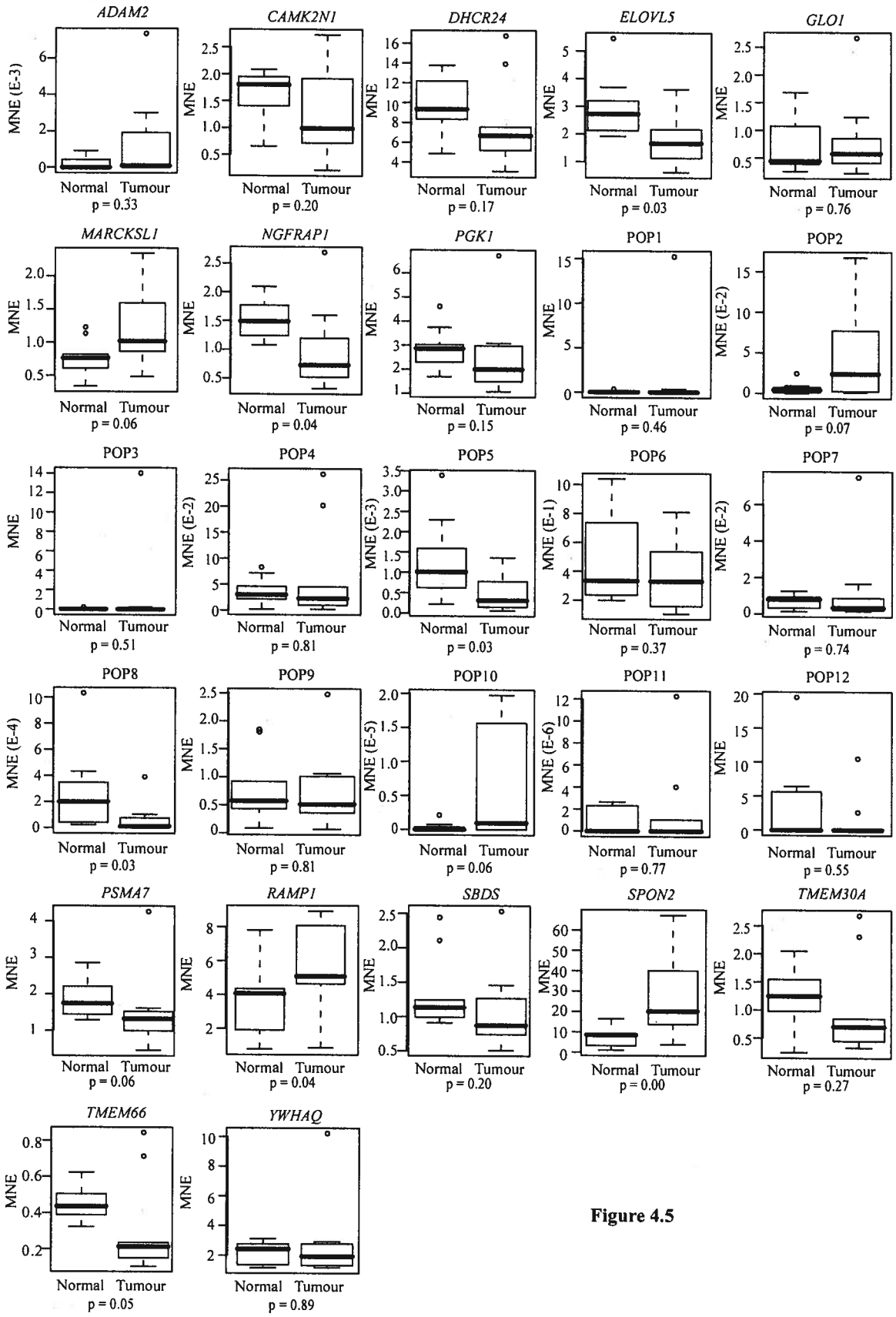
ADAM2
MNE (E-3)
Normal Tumour
p = 0.33
CAMK2N1
MNE
Normal Tumour
p = 0.20
DHCR24
MNE
Normal Tumour
p = 0.17
ELOVL5
MNE
Normal Tumour
p = 0.03
GLO1
MNE
Normal Tumour
p = 0.76
MARCKSL1
MNE
Normal Tumour
p = 0.06
NGFRAP1
MNE
Normal Tumour
p = 0.04
PGK1
MNE
Normal Tumour
p = 0.15
POP1
MNE
Normal Tumour
p = 0.46
POP2
MNE (E-2)
Normal Tumour
p = 0.07
POP3
MNE
Normal Tumour
p = 0.51
POP4
MNE (E-2)
Normal Tumour
p = 0.81
POP5
MNE (E-3)
Normal Tumour
p = 0.03
POP6
MNE (E-1)
Normal Tumour
p = 0.37
POP7
MNE (E-2)
Normal Tumour
p = 0.74
POP8
MNE (E-4)
Normal Tumour
p = 0.03
POP9
MNE
Normal Tumour
p = 0.81
POP10
MNE (E-5)
Normal Tumour
p = 0.06
POP11
MNE (E-6)
Normal Tumour
p = 0.77
POP12
MNE
Normal Tumour
p = 0.55
PSMA7
MNE
Normal Tumour
p = 0.06
RAMP1
MNE
Normal Tumour
p = 0.04
SBDS
MNE
Normal Tumour
p = 0.20
SPON2
MNE
Normal Tumour
p = 0.00
TMEM30A
MNE
Normal Tumour
p = 0.27
TMEM66
MNE
Normal Tumour
p = 0.05
YWHAQ
MNE
Normal Tumour
p = 0.89

Figure 4.5

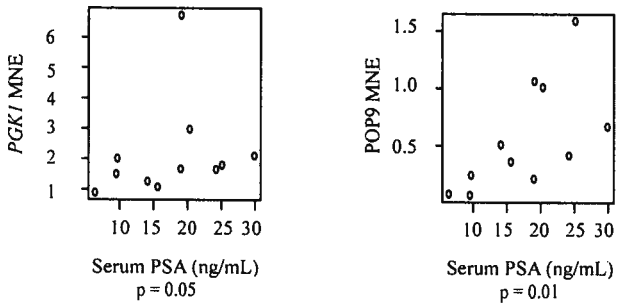

Figure 4.6 Transcript expression in tumour tissue correlate with circulating levels of serum PSA in the patient. RNA was isolated from laser microdissected human prostate tumour tissue (n=11; one randomly selected from each patient with available tissue and PSA information) and analyzed by qRT-PCR. Plotted against serum PSA levels of the patient, mean normalized expression (MNE) of *PGK1* or POP9 in tumour tissue were statistically significantly associated according to Spearman's correlation test ($p \leq 0.05$).
Non-HGNC gene names were not italicized.

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CHAPTER V

CONCLUSION AND RECOMMENDATIONS FOR FUTURE WORK

5.1 CONCLUSION AND FUTURE DIRECTIONS

The over-arching hypothesis of this thesis was that the application of Long Serial Analysis of Gene Expression (LongSAGE) would catalogue gene expression signatures that are indicative of the mechanisms underlying the growth and progression of prostate cancer, and reveal potential biomarkers of prostate cancer. To address this hypothesis, we determined the regulation of the transcriptome by the androgen-axis in prostate cancer because the androgen pathway is important in prostate cancer and provides a means for clinical intervention. Next, we identified the gene expression profile associated with *in vivo* progression of prostate cancer to castration-recurrence because there is no cure for castration-recurrent prostate cancer (CRPC), and the mechanisms underlying the disease are not known. Finally, we determined the expression characteristics of novel biomarkers of prostate cancer because screening for prostate cancer using serum levels of prostate-specific antigen has resulted in the over-treatment of indolent disease. Therefore, novel diagnostic and prognostic markers for prostate cancer are needed.

In **Chapter II**, we evaluated the transcriptome of prostate cancer cells in response to androgen using deep sequencing of LongSAGE libraries. There were 131 tags (87 genes) that displayed statistically significant ($p \leq 0.001$) differences in expression in response to androgen. Many of the genes identified by LongSAGE (35/87) have not been previously reported to change expression in the direction or sense observed. The expression trends of 24 novel genes were validated using quantitative real time-polymerase chain reaction (qRT-PCR). These genes were: *ARL6IP5, BLVRB, C19orf48, C1orf122, C6orf66, CAMK2N1, CCNI, DERA, ERRFI1, GLUL, GOLPH3, HM13, HSP90B1, MANEA, NANS, NIPSNAP3A, SLC41A1, SOD1, SVIP, TAOK3, TCP1, TMEM66, USP33,* and *VTA1*. The physiological relevance of these expression trends was evaluated *in vivo* using the LNCaP Hollow Fibre model. Novel androgen-responsive genes identified here participate in protein synthesis and trafficking, response to oxidative stress, transcription, proliferation, apoptosis, and differentiation. These processes may represent the molecular mechanisms of androgen-dependency of the prostate. Genes that participate in these pathways may be targets for therapies or biomarkers of prostate cancer.

A limitation to the study is the inability to determine whether genes are direct or indirect targets of androgen receptor. Androgens (i.e., 0.1 nM R1881) can stimulate proliferation. The effects of proliferation and androgen may be indistinguishable. Here, we used 10 nM R1881 to stimulate the cells. At this physiological concentration, LNCaP proliferation is minimal[1]. Therefore, the changes to the transcriptome likely represent the effects of androgen and not proliferation. However, the contribution of other transcription factors cannot be discounted. Future work could include chromatin immuno-precipitation sequence (ChIP-seq) analysis. ChIP-seq is a combination of ChIP[2] and next generation sequencing[3, 4]. ChIP-seq is a method for evaluating the binding sites of a transcription factor in the genome. With antibodies specific for the androgen receptor, genomic DNA that interacts with the transcription factor may be enriched and sequenced. Once the genomic sequences are mapped back to the genome, sites of androgen receptor binding may be identified. ChIP-seq has been successfully applied to interferon gamma-stimulated HeLa cells to evaluate STAT1 binding[5]. By cross-referencing ChIP-seq and LongSAGE data, true androgen-regulated genes may be separated from down-stream signalling events.

In **Chapter III**, we assayed the transcriptome of LNCaP human prostate cancer cells as they progress to castration-recurrence *in vivo* using replicate LongSAGE libraries. We refer to these libraries as the LNCaP atlas. We identified 96 novel genes consistently differentially expressed in CRPC. We characterized these genes for their potential to be new therapeutic targets or biomarkers of CRPC, and found that 31 genes have protein products that are either secreted or are located at the plasma membrane, 20 genes changed expression in response to androgen, and 5 genes have enriched expression in the prostate. Furthermore, expression of 20, 6, 8, and 15 genes have previously been linked to prostate cancer, Gleason grade, progression, and metastasis, respectively. The expression profiles of castration-recurrence neither supported nor discounted a role for stem cells genes (*AQP3*, *BTG1*, *CD151*, *HES6*, *HN1*, and *SPON2*), or cell survival genes (*AMD1*, *BNIP3*, *CAMK2N1*, *CCT2*, *GLO1*, *GRB10*, *MARCKSL1*, *MDK*, *NGFRAP1*, *ODC1*, *PIK3CD*, *PPP2CB*, *PPP2R1A*, *S100A10*, *SLC25A4*, *SLC25A6*, *TMEM66*, *TRPM8*, *WDR45L*, and *YWHAQ*) in CRPC. However, the expression profiles of castration-recurrence support a role for the transcriptional activity of the androgen receptor genes (*CCNH, CUEDC2, FLNA,* and *PSMA7*), steroid synthesis and metabolism genes (*DHCR24, DHRS7, ELOVL5, HSD17B4*, and *OPRK1*), neuroendocrine cell genes (*ENO2, MAOA, OPRK1, S100A10,* and *TRPM8*), and

proliferation genes (*GAS5, GNB2L1, MT-ND3, NKX3-1, PCGEM1, PTGFR, STEAP1,* and *TMEM30A*) in castration-recurrence.

LongSAGE libraries were constructed and sequenced to generate replicate gene expression profiles representative of three stages of prostate cancer progression: androgen-sensitive (AS), responsive to androgen deprivation (RAD), and castration-recurrent (CR). In this study, we focused on gene expression that was different between RAD and CR stages. However, the data may be analysed from other angles. These LongSAGE libraries will be submitted to the publicly accessible database gene expression omnibus[6]. Researchers may download the complete libraries (referred to as the LNCaP atlas) to test their hypotheses. We envision researchers querying the LNCaP atlas for their gene-of-interest to determine its level of expression during different stages of prostate cancer progression. In our laboratory, it would be of interest to cross-reference the genes that were identified as differentially expressed between the AS and RAD stages of cancer progression with the androgen-regulated genes that were identified in **Chapter II**. For those androgen-responsive genes not validated in the LNCaP Hollow Fibre model, this *in vivo* LongSAGE data would be an excellent resource to determine the *in vivo* relevance of gene regulation. Also of interest to us, would be to compare the gene expression of the AS and CR stages. Although we found support for the model that the androgen receptor is reactivated in castration-recurrence, we also identified evidence of a neuroendocrine and proliferative phenotype. Therefore, by comparing gene expression in the stages of prostate cancer that are AS and CR, one may be able to enrich for genes representing non-androgen receptor-mediated mechanisms of CRPC.

In **Chapter IV**, we determined the levels of expression of 27 novel biomarkers of prostate cancer and included several that encode for plasma membrane proteins (*ADAM2, ELOVL5, MARCKSL1, RAMP1, TMEM30A*, and *TMEM66*), secreted proteins [*SPON2, TMEM30A, TMEM66,* and truncated *TMEFF2* (referred to as POP4)], intracellular proteins (*CAMK2N1, DHCR24, GLO1, NGFRAP1, PGK1, PSMA7, SBDS,* and *YWHAQ*), as well as non-coding transcripts referred to as POP 1 (transcript 100 kilobases (kb) from mRNA AK000023), POP2 (transcript 4 kb from mRNA AL832227), POP3 (transcript 50 kb from EST CFI40309), POP5 (transcript from the intron of *NCAM2*, accession DO668384), POP6 (transcript from the intron of *FHIT*), POP7 (transcript from the intron of *TNFAIP8*), POP8 (transcript from the intron of

EFNA5), POP9 (transcript from the intron of *DSTN*), POP10 (transcript from the intron of *ADAM2*, accession DO668396), POP 11 (transcript 87 kb from EST BG194644), and POP12 (transcript from the intron of EST BQ226050). Expression of POP3 was prostate-specific, with restricted expression of *ADAM2,* POP1, POP4, POP10, *ELOVL5, RAMP1*, and *SPON2*. The expression of *ADAM2, CAMK2N1, DHCR24, ELOVL5, GLO1, PGK1*, POP4, POP5, POP6, POP7, POP8, POP10, POP11, *SPON2, TMEM66,* and *YWHAQ* changed in response to androgen. *ELOVL5, NGFRAP1,* POP5, POP8, *RAMP1, SPON2,* and *TMEM66* were significantly differentially expressed between laser microdissected tumour and normal clinical samples of prostatic tissue, and *PGK1* and POP9 were positively associated with pre-prostatectomy serum PSA levels. These results suggest that *ADAM2, ELOVL5*, POP1, POP3, POP4, POP10, *RAMP1*, and *SPON2* may be good candidates for biomarkers of prostate cancer.

The next step to characterizing these genes as potential biomarkers of prostate cancer would be to correlate gene expression with prognosis. Future studies will include greater numbers of laser microdissected tumour and normal prostatic tissue. The 28 patient tissue specimens used here were insufficient to identify an association between levels of gene expression and Gleason grade, currently the mainstay prognostic tool used in the clinic. If prognostic significance is achieved with any or several of the candidate biomarkers, then pre-clinical validation of a PROSTAChip will follow. Biomarkers often perform better as part of a panel of genes (e.g., *GSTP-1* and *EZHZ*)[7-10]. Therefore, the miniature design of the PROSTAChip, a microarray containing probes that correspond to the candidate biomarkers, is ideal for limited sample volume, such as that retrieved at biopsy. If the PROSTAChip is shown to perform better at identifying subtypes of cancers with the propensity to progress to advanced disease, then the PROSTAChip has a significant potential for application in the clinic.

The overall contribution of this thesis to the field of prostate cancer research is the identification and characterization of potential biomarkers and therapeutic targets of prostate cancer.

5.2 REFERENCES

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APPENDIX I

Ethics certificates

The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: **H07-0047**

INVESTIGATOR OR COURSE DIRECTOR: **Sadar, Marianne**

DEPARTMENT: **Medicine**

PROJECT OR COURSE TITLE: **Genomic and proteomic analysis of androgen independent prostate cancer**

APPROVAL DATE: **08-04-11**

APPROVED CONTAINMENT LEVEL: 2

FUNDING AGENCY: **National Institutes of Health**

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Wesbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

[redacted]

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 FAX: 604-822-5093

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A05-1794

Investigator or Course Director: Marianne Sadar

Department: Medicine, Department of

Animals:

Mice Male athymic Nude mice, BALB/c Strain 180

Start Date: November 1, 2005 **Approval Date:** January 4, 2008

Funding Sources:

Funding Agency: National Institutes of Health
Funding Title: Genomic and proteomic analysis of androgen independent prostate cancer

Funding Agency: Health Canada
Funding Title: Proteomics associated with the progression of prostate cancer to androgen-independence.

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093

University of British Columbia - British Columbia Cancer Agency
Research Ethics Board (UBC BCCA REB)

UBC BCCA Research Ethics Board
Fairmont Medical Building (6th Floor)
614 - 750 West Broadway
Vancouver, BC V5Z 1H5
Tel: (604) 877-6284 Fax: (604) 708-2132
E-mail: reb@bccancer.bc.ca
Website: http://www.bccancer.bc.ca > Research Ethics
RISe: http://rise.ubc.ca

Certificate of Expedited Approval: Annual Renewal

PRINCIPAL INVESTIGATOR:	INSTITUTION / DEPARTMENT:	REB NUMBER:
Marianne Sadar	BCCA/Genome Sciences Center (BCCA)	H05-60099

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:
N/A
Other locations where the research will be conducted:
N/A

PRINCIPAL INVESTIGATOR FOR EACH ADDITIONAL PARTICIPATING BCCA CENTRE:
N/A

SPONSORING AGENCIES AND COORDINATING GROUPS:
Canadian Institutes of Health Research (CIHR)

PROJECT TITLE:
Development Of Custom Array For The Prognosis Of Prostate Cancer

APPROVAL DATE: June 12, 2008
EXPIRY DATE OF THIS APPROVAL: June 12, 2009
PAA#: H05-60099-A003

CERTIFICATION:

1. The membership of the UBC BCCA REB complies with the membership requirements for research ethics boards defined in Division 5 of the Food and Drug Regulations of Canada.
2. The UBC BCCA REB carries out its functions in a manner fully consistent with Good Clinical Practices.
3. The UBC BCCA REB has reviewed and approved the research project named on this Certificate of Approval including any associated consent form and taken the action noted above. This research project is to be conducted by the provincial investigator named above. This review and the associated minutes of the UBC BCCA REB have been documented electronically and in writing.

The UBC BCCA Research Ethics Board has reviewed the documentation for the above named project. The research study as presented in documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC BCCA REB.

[redacted]

Chair **First Vice-Chair** **Second Vice-Chair**

If you have any questions, please call:
Bonnie Shields, Manager, BCCA Research Ethics Board: 604-877-6284 or e-mail: reb@bccancer.bc.ca
Dr. George Browman, Chair: 604-877-6284 or e-mail: gbrowman@bccancer.bc.ca
Dr. Joseph Connors, First Vice-Chair: 604-877-6000-ext. 2746 or e-mail: jconnors@bccancer.bc.ca
Dr. Lynne Nakashima, Second Vice-Chair: 604-707-5989 or e-mail: lnakas@bccancer.bc.ca