ABSTRACT

As the most frequently diagnosed cancer in North American man, prostate cancer can progress to the androgen independent stage after initial response to androgen ablation therapy. The molecular mechanisms involved in the hormonal progression of prostate cancer are not completely understood. Here, we analyze changes in the transcriptome of prostate cancer cells at different stages of progression to reveal potential mechanisms.

Applying Affymetrix GeneChip technology, we identified the transcriptomes in response to stimulation of androgen and PKA pathways in human prostate cancer cells. In addition to PSA, other common target genes were identified. Genes differentially expressed in response to androgen and stimulation of the PKA pathway in vitro were also differentially expressed during hormonal progression in vivo.

Upon androgen stimulation, androgen receptor binds to a functional androgen response element within the promoter region of SESN1, a p53 targeted gene, and represses its expression. The expression of SESN1 was induced by castration in LNCaP xenografts, but the expression was eventually suppressed again in the androgen independent stage of prostate cancer. Knockdown of SESN1 promoted the proliferation of prostate cancer cells.

Expression patterns of androgen-regulated genes in androgen independent tumours were revealed to be more similar to that from before castration than to the tumors under androgen ablation. The β-catenin, a potent coactivator of the androgen receptor, and Wnt pathway was deregulated in androgen-independent tumours. There was increased nuclear colocalization and interaction of androgen receptor and β-catenin with hormonal progression of prostate cancer.

This study provides insight into hormonal effects on prostate cancer and possible pathways involved in the development of androgen independent disease, as well as potential therapeutic targets.
# TABLE OF CONTENTS

Abstract ........................................................................................................... ii
Table of Contents ............................................................................................ iii
List of Tables ................................................................................................... vii
List of Figures .................................................................................................. viii
List of Abbreviations ........................................................................................ x
Acknowledgements .......................................................................................... xii
Dedication ......................................................................................................... xiii
Co-authorship Statement ................................................................................... xiv

## 1 Introduction ............................................................................................ 1

1.1 Prostate cancer .......................................................................................... 1
  1.1.1 Epidemiology of prostate cancer ......................................................... 1
  1.1.2 Diagnosis of prostate cancer ............................................................... 1
  1.1.3 Androgen deprivation therapy and the hormonal progression of prostate cancer 2

1.2 Androgen receptor .................................................................................... 4
  1.2.1 The structure of AR ............................................................................ 4
  1.2.2 Ligand dependent activation of the AR .............................................. 4
  1.2.3 AR in carcinogenesis of prostate cancer ........................................... 6
  1.2.4 β-Catenin as a co-activator of AR .................................................... 6
  1.2.5 Ligand-independent activation of AR by protein kinase A pathway ...... 8

1.3 In vivo models for studying the hormonal progression of prostate cancer ....... 9

1.4 Summary and research objectives ............................................................. 10
  1.4.1 Hypothesis ........................................................................................ 11
  1.4.2 Objectives ......................................................................................... 11
  1.4.3 Thesis overview ............................................................................... 11

1.5 References ................................................................................................ 15

## 2 Identification of genes targeted by the androgen and PKA signaling pathways in prostate cancer cells ............................................................. 22

2.1 Introduction .............................................................................................. 22
3.2.8 immunohistochemistry.................................................................56
3.3 Results........................................................................................................56
  3.3.1 SESN1 T2 is the predominant transcript suppressed by androgen........56
  3.3.2 SESN1 protein level is negatively regulated by androgen.....................57
  3.3.3 AR is required to repress SESN1 promoter activity...............................57
  3.3.4 Mapping the activity of SESN1-T2 promoter.......................................58
  3.3.5 AR interacts with the SESN1 promoter upon androgen stimulation........58
  3.3.6 Knockdown of SESN1 promoted the proliferation of CaP cells..............59
  3.3.7 Expression of SESN1 is initially induced by castration, but later suppressed when prostate cancer is androgen independent........................................59
3.4 Discussion...............................................................................................60
3.5 References..............................................................................................70

4 Crosstalk between the androgen receptor and β-Catenin in castrate resistant prostate cancer.................................................................74
  4.1 Introduction...............................................................................................74
  4.2 Materials and methods............................................................................75
    4.2.1 Hollow Fiber and subcutaneous xenograft models of prostate cancer....75
    4.2.2 RNA isolation and microarray analysis.............................................75
    4.2.3 Expression profile analysis.............................................................75
    4.2.4 Quantitative RT–PCR (qRT–PCR)...................................................76
    4.2.5 Whole-cell lysates and western blot analyses...................................77
    4.2.6 Immunoprecipitation.......................................................................77
    4.2.7 Double immunofluorescent microscopy...........................................77
  4.3 Results......................................................................................................78
    4.3.1 The LNCaP hollow fiber model correlated with clinical hormonal progression of prostate cancer.................................................................78
    4.3.2 Global gene expression profiles of hormonal progression of prostate cancer.................................................................79
    4.3.3 Reactivation of the AR pathway in castrate resistant prostate cancer....80
    4.3.4 Increased expression of AR and β-catenin in androgen-independent prostate cancer.......................................................................................80
4.3.5 Expression profile of members of the Wnt pathway in androgen-independent prostate cancer

4.3.6 Increased colocalization of AR and β-catenin in androgen-independent prostate cancer

4.3.7 Increased interaction between AR and β-catenin in androgen-independent prostate cancer

4.4 Discussion

4.5 References

5 Summary and future directions

5.1 Crosstalk between the AR and PKA pathways

5.2 Androgen suppresses the expression of SESN1

5.3 Crosstalk between the AR and β-catenin and the reactivation of AR in androgen-independent prostate cancer

5.4 Conclusions

5.5 References

6. Appendix

6.1 Animal care certificate

6.2 Biohazard approval certificate
LIST OF TABLES

Chapter 2
Table 2.1 Primers for gene expression using semi-Q-RT-PCR..........................38
Table 2.2 Expression of genes that significantly increased in LNCaP cells in response to R1881 and FSK.................................................................39
Table 2.3 Genes significantly differentially expressed in LNCaP cells in opposite directions in response to R1881 and FSK........................................40
Table 2.4 Expression of genes that significantly decreased in LNCaP cells in response to R1881 and FSK.................................................................40

Chapter 4
Table 4.1 Primers used in the Q-RT-PCR.........................................................88
Table 4.2 Genes significantly expressed in clinical samples of androgen-independent prostate cancer with p-value<0.001 .........................................................89
LIST OF FIGURES

Chapter 1
Figure 1.1 Hollow fiber model of prostate cancer

Chapter 2
Figure 2.1 Cluster analyses of genes differentially expressed across all treatments
Figure 2.2 Genes differentially expressed in response to androgen and FSK
Figure 2.3 Common genes differentially expressed in response to androgen and FSK
Figure 2.4 AR is required for changes in expression of some genes in response to FSK
Figure 2.5 In vivo expression of genes identified in vitro to be differentially expressed in response to androgen and FSK during hormonal progression in the LNCaP hollow fiber model

Chapter 3
Figure 3.1 The AR signaling pathway negatively regulates SESN1 expression in prostate cancer cells
Figure 3.2 Induction of SESN1 promoter activity by p53 is inhibited by AR
Figure 3.3 AR negatively regulates SESN1 promoter activity
Figure 3.4 AR binds to the SESN1 promoter upon androgen stimulation
Figure 3.5 Knockdown of SESN1 promoted the proliferation of prostate cancer cells
Figure 3.6 Expression of SESN1 in the hormonal progression of CaP

Chapter 4
Figure 4.1 The LNCaP hollow fiber model mimics hormonal progression of clinical prostate cancer
Figure 4.2 Genes differentially expressed during the hormonal progression of prostate cancer in the hollow fiber model
Figure 4.3 Cluster analysis of the status of the androgen pathway in hormonal progression in the hollow fiber model
Figure 4.4 Expression of AR, β-catenin, and regulators of β-catenin in the hollow fiber model
Figure 4.5 Co-localization and endogenous interaction of AR and β-catenin in xenografts during hormonal progression in castrated mice........................................96

Figure 4.6 Crosstalk between β-catenin and the AR in castrate resistant prostate cancer.97
LIST OF ABBREVIATIONS

ADT androgen deprivation therapy
AF activation function
AICaP androgen independent prostate cancer
ANOVA analysis of variance
AR androgen receptor
ARA70 AR associated coregulator 70
ARE androgen response element
cAMP cyclic adenosine monophosphate
CaP prostate cancer
CCND1 cyclin D1
CDC6 cell division cycle 6
ChIP chromatin immunoprecipitation
CK casein kinase
CREB cAMP responsive element binding protein
CSNK casein kinase
CTNNBIP1 catenin, beta interacting protein 1
DBD DNA-binding domain
DHT dihydrotestosterone
DMEM Dulbecco’s Modified Eagle’s Medium
DRE digital rectal examination
EIF2B5 eukaryotic translation initiation factor 2B, subunit 5
ELL2 elongation factor, RNA polymerase II, 2
FBS fetal bovine serum
FKBP5 FK506 binding protein 5
FSK forskolin
GAPDH glyceraldehyde-3-phosphate dehydrogenase
GSK3β glycogen synthase kinase 3 beta
HMGCR 3-hydroxy-3-methylglutaryl-Coenzyme A reductase
IHC immunohistochemistry
INSIG1 insulin induced gene 1
ITGB5 integrin, beta 5
KLK kallikrein
LBD ligand-binding domain
LEF lymphoid enhancer-binding factor
LNCaP lymph node carcinoma of the prostate cell line
LYN Yamaguchi sarcoma viral related oncogene homolog
MAPK mitogen activated protein kinase
MMP16 matrix metallopeptidase 16
NAV1 neuron navigator 1
NCoR  nuclear receptor co-repressor
NFkB  nuclear factor of kappa light polypeptide gene enhancer in B-cells
NKX3-1 NK3 homeobox 1
NLS   nuclear localization signal
NTD   N-terminal domain
NUMA1 nuclear mitotic apparatus protein 1
OSR2  odd-skipped related 2
PAGE  polyacrylamide gel electrophoresis
PAR1  Prader-Willi/Angelman region-1
PBS   phosphate buffered saline
PCR   polymerase chain reaction
PKA   protein kinase A
PLCB4 phospholipase C, beta 4
PSA   prostate-specific antigen
qPCR  real-time quantitative PCR
RHOU  ras homolog gene family, member U
RPMI medium developed at Roswell Park Memorial Institute
RT-PCR reverse transcription PCR
SCID  Severe Combined Immunodeficiency
SDS   sodium dodecyl sulfate
SESN1 sestrin 1
siRNA small interferon RNA
SMRT  silencing mediator for retinoic acid and thyroid hormone receptor
SOCS2 suppressor of cytokine signaling 2
SPCS3 signal peptidase complex subunit 3
SRC-1 steroid receptor co-activator 1
TCF   a transcription factor
TP53  tumor protein p53
WCL   whole cell lysate
Wnt   wingless-type
YES1  Yamaguchi sarcoma viral oncogene homolog 1
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DEDICATION

To my daughter: Vickie YY Wang

&

my wife: Ren Yuan
CO-AUTHORSHIP STATEMENT

The experiments were designed, conducted and analyzed by myself, Gang Wang, and my supervisor, Dr. Marianne Sadar. However, a number of people also contributed to the work. Dr Steven Jones and Dr Marco Marra helped conceive and design the experiments in Chapter 2. Additionally, Jun Wang performed the experiments involving the animal work of the LNCaP hollow fibre model and the LNCaP xenograft model and immunohistochemistry in all the chapters. Dr. Marianne Sadar and I were responsible for writing the manuscripts.
1 Introduction

1.1 Prostate cancer

1.1.1 Epidemiology of prostate cancer

Prostate cancer (CaP) is the most common cancer and the third leading cause of cancer death in Canadian men [1]. The causes of CaP are unknown and thought to involve interactions among genetic, dietary, hormonal, and lifestyle factors. The established risk factors for CaP include old age, positive family history, and African-American race or ethnicity. Other potentially modifiable risk factors of CaP include smoking, alcohol, diet and nutrition [2].

1.1.2 Diagnosis of prostate cancer

Early detection of CaP is achieved by screening methods such as the digital rectal examination (DRE) and the serum prostate-specific antigen (PSA) test [3]. PSA is a 33 kDa serine protease synthesized in the prostate luminal epithelial cells and secreted into the lumen of prostate ducts to liquefy the seminal coagulum and release the spermatozoa [4]. The PSA gene is androgen-regulated, as evidenced by the androgen responsive elements (AREs) in its promoter as well as enhancer region [5-7]. It has been the principle serum biomarker for CaP since early 1990s, when its serum levels were shown to correlate well with increasing clinical stage and tumor volume [2, 8, 9]. Serum PSA levels are also influenced by other prostate disease conditions, such as benign prostate hyperplasia, prostatitis, age and race [10]. As an androgen-regulated gene, serum PSA levels rapidly decline after androgen ablation therapy and the subsequent rise in levels of PSA are indicative of progression to androgen-independence. Therefore, although not specific, PSA is still used as a sensitive marker for screening and diagnosis, and also as a surrogate end point in monitoring the treatment of hormone-refractory prostate cancer [11].

Definitive diagnosis of CaP can only be established based on biopsy results, usually performed using a transperineal approach with a needle. The systematic sextant biopsy of the prostate under transrectal ultrasound guidance was introduced less than twenty years ago and revolutionized the detection of CaP, as well as provide information
about tumor grade, volume assessment and local staging [12]. After diagnosis, CaP is generally graded using the Gleason grade system. According to the histological pattern, the pathologist assigns a grade to the most predominant foci, and a second grade to the next most predominant foci. The two grades are added together to get a Gleason score ranging from 2 to 10 [13]. For example, if the most predominant foci was grade 3, and the next most predominant foci was grade 4, the Gleason score would be 3+4 = 7. Low scores from 2 to 4 indicate more differentiated lesions, while higher scores from 8 to 10 indicate poorly differentiated lesions and the scores ranging from 5 to 7 are intermediate. The Gleason score has been recently updated to meet the needs of contemporary surgical pathology practice [14]. The same as other tumor types, the tumor-node-metastasis (TNM) system was also issued over 15 years ago to stage the CaP [15]. The stage T indicates a primary tumor with local extension confined to the prostate gland (stage T1-2) or invasive beyond the prostatic capsule (stage T3-4). Stage N/M indicate the metastasis to local lymph nodes (stage N1-3) or to distant organs (stage M1). Comprehensive consideration based on the tumor grade and stage, age and general health of the patient is needed to assess the appropriate treatment for the patient.

1.1.3 Androgen deprivation therapy and the hormonal progression of prostate cancer

The treatment of CaP is principally based on the stage of the disease, also considering the grade, the age and life expectancy of the patient. For localized CaP, radical prostatectomy [16, 17] and external-beam radiotherapy [18] are generally applied. More recently, novel strategies such as brachytherapy (reviewed in [19]) and cryotherapy (reviewed in [20]) have provided options with more specific targeting and less side effects for early stage CaP.

For those patients with metastasis, or advanced CaP, the only effective systemic treatment is androgen deprivation therapy (ADT). This therapy is based on the recognition of the role of androgens, including testosterone and its more potent metabolite dihydrotestosterone (DHT), in the development of the prostate gland as well as the etiology and progression of prostate carcinogenesis. The seminal work of Huggins et al. to demonstrate the beneficial effects of ADT for advanced CaP, laid the foundation
for the current standard approach of androgen blockade through surgical and/or medical castration [21]. Interventions aimed at various levels of the endocrine hypothalamic–pituitary–testicular–adrenal axis via disruption of the androgen–androgen receptor (AR)–DNA signaling pathways have proven to be useful ADT strategies for CaP. There are several approaches for ADT that include: (1) gonadatropin-releasing hormone (GnRH) agonist/antagonist (eg, Leuprolide, Goserelin and Abarelix) which down-regulate or directly inhibit GnRH receptors; (2) nonsteroidal AR antagonists (e.g., flutamide, bicalutamide, nilutamide); (3) steroidal agents with mixed actions (e.g., cyproterone acetate); (4) adrenal androgen inhibitors (eg, ketoconazole, hydrocortisone); (5) 5α-reductase inhibitors (finasteride) which inhibit DHT biosynthesis; and (6) natural antiandrogens/antiestrogens, soy isoflavones, and mammalian lignans that inhibit 5α-reductase and aromatase.

Despite initial response to ADT, disease progression to an androgen-independent state occurs in the majority of patients [22]. Relapsed disease after primary treatment with ADT is referred to as hormone-refractory or androgen independent (AICaP) or castration-resistant disease. Although intermittent ADT, recently introduced, can prolong the time to evolution of androgen independence (reviewed in [23]), AICaP is still the end point for most patients and the molecular mechanisms underlying hormonal progression remain unknown. General theories of clonal selection and adaptation that attempted to explain this progression have been replaced largely by molecular concepts relating to the AR.

Several theories have been advanced that point to the AR as a probable factor involved in hormonal progression of CaP and include the following: 1) the AR is expressed in the nuclei of the majority of hormone refractory tumors which suggests the transactivation of AR in this stage of disease [24-26]; 2) mutations in the AR with clinical relevance can result in hypersensitivity to low castrate levels of androgens or activating of AR by steroids other than androgens [27]; 3) amplification of the AR gene has been detected in 20% to 30% of androgen independent tumors [28]; 4) increased expression of the AR was consistently shared amongst multiple model systems [29]; 5) AR target genes such as PSA are re-expressed in androgen-independent disease [30-33]; 6) the timing and sequence of use of the family of anti-androgens may prolong the time to
androgen independence [34, 35]; 7) ligand-independent activation of the AR has been shown to occur in CaP cells [36-40]; 8) the AR has been shown to be necessary for the proliferation of androgen independent CaP cells [41]; and 9) the low levels of androgen remaining in clinical tissues from castrated men [42] are sufficient to mediate biological activity [43]. Therefore, although the hormonally progressed CaP is androgen independent, it may still be AR dependent.

1.2. Androgen Receptor

1.2.1. The structure of AR

The AR is part of a family of steroid receptors that also includes estrogen, progesterone, glucocorticoid and mineralocorticoid receptors. The gene for the AR is located on the X chromosome (q11-12) and consists of eight exons that encode a protein of 919 amino acids in length, depending on the number of amino acids in the polymorphic repeat regions, with four structurally and functionally distinct domains. The ligand-binding domain (LBD) at the C-terminus of the receptor consists of amino acids 676-919 and is the region where androgens and anti-androgens bind. The DNA-binding domain (DBD) consists of amino acids 559-624 and is essential for the binding of the receptor to androgen responsive elements (AREs) in the genome associated with target genes. Between the LBD and DBD, there is a hinge region containing a nuclear localization signal (NLS) at amino acids 608–625. Finally, the N-terminal domain (NTD), amino acids 1-558, contains the activation function (AF) 1 region involved in interaction with the transcriptional machinery (reviewed in [44]).

1.2.2. Ligand dependent activation of the AR

The process of ligand-induced transformation of the AR is not completely understood, although it is known that the unliganded AR exists predominantly in the cytoplasm in an unfolded state [45]. Upon ligand binding: 1) heat-shock proteins are dissociated; 2) post-translational modification of the AR occurs (e.g. phosphorylation and acetylation); 3) the AR conformation changes including N/C terminal interaction for the exposure of the NLS in the hinge region; 4) the AR translocates to the nucleus; 5) the AR
forms homodimers; 6) the AR binds to AREs on DNA; and 7) recruitment of coactivators and release of corepressors (summarized in [44]).

There are at least 169 proteins that have been classified as potential AR coregulators [46]. Some well studied AR co-regulators include CBP [47], SRC-1 [48], SRC-3 [49] and β-catenin [50]. Co-regulators can modify transcriptional function of the AR via the following mechanisms: 1) influence the ligand selectivity of the receptor; 2) modulate the appropriate folding of AR or facilitate N/C terminal interaction; 3) direct modification of histones; 4) recruitment of chromatin-modifying complexes; and 5) recruitment of the basal transcriptional machinery (reviewed in [51]). Given the fact that AR is generally expressed in prostate tumors and their metastases [24], it is conceivable that hormonal progression of CaP is not only due to the mutation and amplification of AR itself, but is also a consequence of aberrant regulation of AR activity by its coregulators.

The increased transcriptional activity of the steroid receptors are also concomitant with enhanced phosphorylation of these receptors [52-54]. It has been a long time since the first evidence of the phosphorylation of several serine residues in AR following ligand binding was illustrated. This protects the AR from proteolytic degradation and stabilizes AR homodimers [55]. In addition to stabilization of AR, there is emerging evidence for modification of AR transcriptional activity through phosphorylation (reviewed in [56]). The AR contains a number of putative phosphorylation sites for serine-proline-directed kinase, DNA-dependent kinase, protein kinase C, casein kinase I and II, PKA, MAPK, Akt, calmodulin kinase II, and tyrosine kinases. The identified phosphorylation sites include Ser-16, Ser-81, Ser-94, Ser-213, Ser-256, Ser-308, Ser-424, Ser-515, Ser-650, and Ser-791, all of which were confirmed by mutagenesis, peptide mapping or mass spectrometry [57-63]. However, the correlation between AR transcriptional activity and the status of specific phosphosites is far from being fully understood and is even found to be controversial among different studies. These discrepancies may be due to tissue/cell specificity and differing methods applied in various laboratories.
1.2.3 AR in carcinogenesis of prostate cancer

A functional AR is necessary for the development of the prostate and CaP. The AR is not only highly expressed in different stages of CaP but is also associated with the hormonal progression and lower recurrence-free survival [25, 64, 65]. However, the mechanisms behind the contribution of AR to carcinogenesis and its important target genes in the development and progression of CaP are not fully understood.

Among the androgen target genes, PSA is the best characterized and several AREs have been identified within the promoter and enhancer region of its genome [5-7]. Other androgen regulated genes include prostatic acid phosphatase [66], kallikrein 2 [67], cyclin dependent kinases 2 and 4 (CDK2 and CDK4) [68] and NKX3.1 [69]. There is evidence that the AR controls the expression of genes involved in cell proliferation and survival in CaP via transcriptional regulation (reviewed in [46]). It will take exquisite molecular experiments with precise gene knockout or knockin models before we are able to precisely define the most important AR-regulated genes for the prostate and CaP progression.

CaP is associated with a series of gain-of-function mutations in oncogenes such as Ras and activation of Bcl-2 [70, 71]. On the other hand, loss of function in tumor suppressor genes can contribute to CaP progression. An example of an androgen regulated tumor suppressor gene is maspin. Its expression sensitizes CaP cells to doxazosin-induced apoptosis [72] and inhibits angiogenesis and tumor growth in a model of CaP bone metastasis [73]. p53 regulates the expression of maspin by binding directly to the p53 consensus-binding site present in the maspin promoter [74]. Expression of maspin is also regulated by a negative hormonal responsive element recognized by the AR [75] and its expression is induced by androgen ablation [76]. In this thesis study, we revealed another p53 target gene that is regulated by AR. This gene is SESN1 and is a potential tumor suppressor.

1.2.4. β-catenin as a co-activator of AR

An important AR coregulator is β-catenin which has two distinct functions in cadherin-based cell adhesion as well as mediating the Wnt-signaling pathway [77, 78]. The first function is through association with the cytoplasmic region of E-cadherin at the
cell membrane [79]. This is where most of the β-catenin is localized, because cytoplasmic β-catenin is constitutively targeted for the phosphorylation of serine and threonine residues in the N-terminal domain by CK1ε and GSK3β which leads to ubiquitination [80]. Wnt ligands that bind to Frizzled receptors inhibits CK1ε and GSK3β, which result in an accumulation of β-catenin in the nucleus where it mediates transcriptional activity of Tcf/Lef family of transcription factors [81, 82]. The crosstalk between Wnt and AR pathways takes place at several levels: 1) Wnt ligands such as Wnt3a promotes the ligand dependent/independent activation of AR [83]; 2) β-catenin binds to and acts as a transcription co-activator of AR [50, 84-87]; 3) GSK3β is a negative regulator of AR-mediated transcription [88-90]; 4) competition between AR and Tcf/Lef for nuclear β-catenin [91]; and 5) the Tcf/Lef target gene cyclin D1 can bind to the AR-NTD as a transcription co-repressor [92-94].

Conformational changes of the AR upon ligand binding provide the structural basis for the recruitment of cofactor proteins and the transcriptional machinery [95]. The interaction between the AR and many of the coregulators is believed to be mediated through an activation function 2 (AF2) region in the LBD of the AR and the so-called nuclear receptor box “LxxLL” motif in the nuclear-receptor-interacting domain (NID) of coregulators [96]. Although β-catenin also contains LxxLL motifs, the interaction between AR and β-catenin is mediated by the LxxLL binding motifs within LBD of AR and the β-catenin armadillo repeats 1-6 (especially repeats 5 and 6) [85, 86].

Transcriptional coregulators are believed to serve a critical role in promoting a more active AR during hormonal progression of CaP. β-catenin has been reported to promote the oncogenicity of the AR. Specifically, it increases AR-mediated gene activation in the presence of not only DHT, but also 17β-estradiol, as well as the weaker adrenal androgen, androstenedione, a steroid that remains present in chemically castrated patients [50]. β-catenin also coactivates mutant forms of AR that are clinically relevant [97]. Therefore, β-catenin could lower the concentration requirement of androgen, change the ligand specificity of AR and act as a coactivator for both wild-type and mutated AR in the hormonal progression of CaP. However, there is still no direct evidence for the in vivo interaction of endogenous β-catenin and the AR.
1.2.5. Ligand-independent activation of AR by protein kinase A pathway

Phosphorylation of steroid receptors and/or co-regulators is associated with increased transcriptional activity of the receptor upon ligand binding [52-54]. This also applies to the AR [98]. The AR contains a number of putative phosphorylation sites for serine-proline-directed kinase, DNA-dependent kinase, protein kinase C, casein kinase I and II, protein kinase A (PKA), MAPK, Akt, calmodulin kinase II, and tyrosine kinases. Cross-talk between the AR and multiple protein kinase pathways has been reviewed elsewhere [44]. Of particular interest is the PKA signal transduction pathway.

The cAMP-PKA pathway is one of the most common and versatile signaling pathways in eukaryotic cells and is involved in the regulation of cellular functions in almost all tissues in mammals. Various extracellular signals converge on this signal pathway through ligand binding to G protein-coupled receptors. Therefore the cAMP-PKA pathway is tightly regulated at several levels and is involved in regulating diverse cellular processes such as cell cycle, proliferation, differentiation microtubule dynamics, chromatin condensation and decondensation, nuclear envelope disassembly and reassembly, as well as intracellular transport mechanisms and ion fluxes (reviewed in [99]). Activation of the PKA pathway results in neuroendocrine differentiation in LNCaP cells [100, 101] which is implicated in androgen independent CaP. Cross-talk between the AR and PKA signal transduction pathways has been studied in androgen-depleted human CaP cells maintained in culture [37, 38, 102-104]. These studies have shown that anti-androgens can block PKA induction of PSA mRNA [38] and androgen-responsive reporters [37, 38, 102]. Treatment of transfected cells with a cAMP analog or coexpression of the catalytic subunit of PKA inhibits the binding of an AR corepressor, silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) to AR [105]. Further evidence supporting ligand-independent activation of the AR through the PKA pathway includes increased AR-ARE complex formation with nuclear extracts from cells exposed to activators of PKA [38]. These data suggest activation of the AR via the PKA pathway.

Here, I compared the transcriptomes of CaP cells in response to activation of PKA and AR to examine subsets of genes regulated by these two pathways. Characterization of
some of the target genes aided delineation of the status of AR and PKA pathways during the hormonal progression of CaP.

1.3. \textit{In vivo} models for studying the hormonal progression of prostate cancer

For many years, research on hormonal progression of CaP has been hindered by the very limited availability of experimental model systems. The key features of an ideal model of CaP include: 1) of human origin; 2) reproducible; 3) secrete PSA; 4) initially androgen sensitive; 5) progress to an androgen independent state; 6) reasonable time frame for prevention, diagnostic and therapeutic studies; and 7) facilitate studies on the relationship between phenotype and genotype (modified from [106, 107]). A good model should offer the possibility to compare gene expression profile that will provide insight into the pathways involved in the transition of CaP through its various phenotypic stages. There are several human CaP xenograft models that have been employed for the study of hormonal progression of CaP [108-111].

To study the correlation between phenotype and genotype in the progression of CaP to androgen independence, homogeneous populations of cancer cells are required for meaningful results. Harvested CaP xenografts contain considerable and variable contamination with host tissue preventing direct use of these tumors for DNA, RNA, and protein extraction. To circumvent these problems our lab developed a hollow fiber model that allows CaP cells to grow \textit{in vivo} while restricting the infiltration of contaminating host cells [112] (Fig 1.1). The fibers contain pores (Mr 500,000 molecular weight cutoff) that allow oxygen and essential factors to enter, and waste products and secreted factors to leave the fiber. When CaP cells are grown in this manner, they still progress to androgen independence after castration of the host as indicated by serum PSA, PSA mRNA and proliferation studies [112]. Given the optimal range of cell density, this model can provide predicted pattern of hormonal progression of CaP [112]. While xenograft models require sacrificing different animals for each time-point along hormonal progression, the hollow fiber model allows harvesting of matched \textit{in vivo} CaP cells from the same animals at each time-point throughout the course of the experiment. This model is ideal for investigating the escape from androgen regulation by providing a source of uncontaminated RNA and protein to investigate the molecular changes.
involved in the progression of CaP to androgen independence. The potential limitation of this model is that the tumor cells growing in the hollow fiber can not escape from the fiber and migrate to distant sites to form metastases. Furthermore, angiogenesis and stromal interaction are restricted. Therefore, although oxygen, nutrition and other factors can freely pass into the fiber while secreted factors from the tumor leave the fiber to enter the circulation, there is no direct contact between the tumor cells and in situ stroma.

Essentially, the true significance of new and refined models will only be revealed in the “post-genome” era as we strive to correlate relationships between gene structure and function of the genetic programs expressed during CaP development and progression [106]. Although some of the xenograft models contain key features of hormonal progression of CaP, there is still no direct genomic evidence to support any of these models’ similarity to the actual clinical scenarios. In this thesis study, we compared a model of CaP progression to clinical samples to provide the first genomic evidence for the physiological relevance of this model to the clinical scenario.

1.4. Summary and research objectives

The only effective systemic therapy available for metastatic CaP is ADT. The inability of ADT to completely and permanently eliminate all CaP cell populations is manifested by the predictable pattern of initial response and relapse with the ultimate progression to androgen independence. The molecular mechanisms involved in the development of AICaP are unknown. Evidence has suggested that AR is still an important factor in the hormonal progression of CaP. As a transcription factor, once activated, AR would bind to AREs located in the promoter and enhancer regions of specific target genes and serve to activate or to repress transcription of genes involved in morphogenesis and differentiation of prostate gland as well as the development and progression of CaP. How androgen regulates the expression of target genes, especially androgen suppressed genes, remains to be elucidated. The mechanism(s) by which AR is activated in the absence of androgen during hormonal progression of CaP are not fully understood, although the crosstalk between AR and protein kinase pathways such as the PKA pathway and the involvement of β-catenin has been proposed. There are several xenograft models, including the hollow fiber model, available for studying of the
hormonal progression of CaP while none of these has been validated at the genomic level compared to human cancer tissues.

1.4.1 Hypothesis

The underlying hypothesis driving this thesis research is that crosstalk between AR and other signaling pathways facilitates the reactivation of AR in the absence of testicular androgen, which leads to the deregulation of androgen-regulated genes resulting in hormonal progression of CaP.

1.4.2 Objectives

The objective of this thesis research was to characterize the molecular signatures of hormonal progression and the mechanisms for the reactivation of AR in the androgen independent stage of CaP. This objective would be achieved through three specific aims. In **Aim 1**, we would characterize the transcriptional program of CaP cells responsive to androgen and stimulation of PKA activity. This work will provide the evidence of crosstalk between AR and the PKA pathway. **Aim 2** would characterize the transcriptional regulation of SESN1, a potential tumor suppressor gene, by AR. This androgen suppressed gene may be a potential marker as well as a novel therapeutic target for the progression of CaP. In **Aim 3**, we would perform the Affymetrix Genechip analysis to characterize the transcriptional signature of AICaP in the CaP hollow fiber model and further study the crosstalk between AR and β-catenin during the progression of CaP.

1.4.3 Thesis overview

The remainder of this thesis describes in detail the experiments I performed to address these specific aims.

In **Chapter 2**, the transcriptomes associated with the androgen and PKA pathways and common transcriptional targets in response to stimulation of both pathways were identified in human CaP cells using Affymetrix GeneChip technology (Human Genome U133 plus2). Statistically significant changes in the levels of 858 genes in response to androgen and 303 genes in response to activation of the PKA pathway were determined using GeneSpring software. Expression of a subset of these genes (22) that
were transcriptional targets for the androgen and/or PKA pathways were validated by RT-PCR and western blot analyses. Application of small interfering RNAs to the AR revealed that in addition to PSA (also called KLK3), levels of expression of KLK2 and SESN1 were regulated by AR activated by both the androgen and PKA signaling pathways. SESN1 was identified as a gene repressed by activated AR. These results provide a broad view of the effects of the androgen and PKA signaling pathways on the transcriptional program of CaP cells and indicate that only a limited number of genes are targeted by cross-talk between AR and PKA pathways.

In Chapter 3, we further investigated the mechanisms through which SESN1 was negatively regulated by androgen and examined a potential role of SESN1 in CaP progression. First, we showed that the SESN1 transcript-2 (T2) was the predominant transcript suppressed by androgen, which was also confirmed at the protein level. Using different SESN1-T2 promoter/reporter constructs, we identified that p53 induces the transcription of SESN1 and that the AR can repress the p53-induced transcription in CaP cells. Moreover, the data from chromatin immunoprecipitation (ChIP) assay showed that AR associated with an ARE located within the SESN1 promoter region upon androgen stimulation. Castration induced the expression of SESN1 expression in LNCaP cell xenografts, but the expression was eventually suppressed again in androgen independent stage of CaP. Finally, knockdown of SESN1 promoted the proliferation of CaP cells. This study provides a novel insight of AR function and increased the understanding of potential crosstalk between AR and p53 pathways and its involvement in CaP progression. As a potential tumor suppressor, SESN1 would be a novel prognostic marker for CaP, especially androgen independent CaP as well as a promising therapeutic target for this disease.

The work in Chapter 4 investigated genes differentially expressed during the hormonal progression of CaP. Applying the Affymetrix Genechip analysis, we confirmed the genomic similarity of the hollow fiber CaP model to the clinical samples and identified over five thousand genes with differential expression during the hormonal progression of CaP in this model. We clustered the fiber model samples according to the expression profile of the androgen-regulated genes previously identified, to investigate the status of the AR pathway during the hormonal progression of CaP. In spite of the
similar hormone environment as the androgen ablation samples, the AICaP samples had an expression profile similar to androgen dependent samples before castration. This is the first genomic evidence for the reactivation of the AR signal transduction pathway in AICaP. Pathway based characterization of gene expression profile also indicated the activation of the Wnt pathway and the dissociation of β-catenin from the cell membrane. Downstream Tcf/Lef transcription factors of the Wnt pathway were not activated. Instead, together with the increased expression of AR and β-catenin, there was increased nuclear colocalization and interaction of AR and β-catenin in AICaP. Together, this is the first evidence of aberrant activation of the AR through the Wnt/β-catenin signaling pathway during the progression of CaP to the androgen-independent stage.

Finally, Chapter 5 summarizes all these experiments and discusses the relevance of these findings to CaP research. This chapter also discusses current work and proposes future experiments to build on the results described in this thesis.
Figure 1.1: Hollow fiber model of prostate cancer. Histology of LNCaP cells grown inside of hollow fibers in vivo. Longitudinal sections of fixed tissue stained with H&E.
1.5 References


20


Identification of genes targeted by the androgen and PKA signaling pathways in prostate cancer cells

2.1 Introduction

Prostate cancer is a major health problem in Western countries. It is the most frequently diagnosed cancer among men and the second leading cause of male cancer death [1]. The androgenic hormones, testosterone and dihydrotestosterone exert their cellular effects by binding with the AR, a member of the family of intracellular steroid hormone receptors that function as ligand-dependent transcription factors. Ligand-activated AR, complexed with coactivator proteins and general transcription factors, bind to cis-acting AREs located in the promoter and enhancer regions to activate or repress the transcription of specific target genes. Androgen-regulated genes are involved in morphogenesis and differentiation of the prostate gland as well as the development and progression of prostate diseases such as adenocarcinoma of the prostate [2-4]. The recognition that normal and neoplastic prostate epithelial cells depend on circulating androgens for their continued survival and growth led to the development of effective endocrine-based therapy for prostate carcinoma [5]. Most prostate cancers initially respond to androgen-ablation therapy. However, these tumors eventually become androgen-independent (hormone refractory) and grow despite androgen ablation. The possible mechanisms of the development of hormone refractory prostate cancer can be divided into two pathways - those involving AR and those that bypass the receptor. These pathways are not mutually exclusive and frequently coexist in hormone refractory prostate cancer [6]. Some possible pathways involving AR-mediated survival of prostate cancer include amplification or mutations of the receptor, alteration of coactivators, deregulation of growth factors or cytokines and cross-talk with other pathways such as MAPK and PKA pathways [7].

The cAMP-PKA pathway is one of the most common and versatile signal transduction pathway in eukaryotic cells and is involved in regulation of cellular functions in almost all tissues in mammals. Various extracellular signals converge on this
signaling pathway through ligand binding to G protein-coupled receptors. The cAMP-PKA pathway is tightly regulated at several levels and itself is involved in the regulation of diverse cellular processes such as cell cycle, proliferation and differentiation and regulation of microtubule dynamics, chromatin condensation and decondensation, nuclear envelope disassembly and reassembly, as well as regulation of intracellular transport mechanisms and ion fluxes [8]. Neuroendocrine differentiation of prostate cancer cells is implicated in androgen independent prostate cancer and can be induced in LNCaP cells by activation of the PKA pathway [9, 10]. Cross-talk between the AR and PKA signal transduction pathways occurs in androgen-depleted human prostate cancer cells maintained in culture [11]. These studies have shown that anti-androgens can block PKA induction of PSA mRNA [12] and androgen-responsive reporters [12-14]. Activation of the PKA pathway inhibits the binding of the co-repressor SMRT to AR [15]. These data suggest activation of AR via the PKA pathway.

In the present study, we applied Affymetrix GeneChip (Human Genome U133 plus2) analysis to characterize the global program of transcription that reflects the cellular response to R1881, a synthetic androgen, and forskolin (FSK), an activator of adenylyl cyclase to synthesize cAMP which in turn stimulates PKA activity. Through the analysis of gene expression profiles in response to both of these two treatments we were able to identify common targets of cross-talk between the androgen and PKA pathways.

2.2 Materials and Methods

2.2.1 Cell culture and general methods

LNCaP cells and 22Rv1 cells were obtained from the American Type Culture Collection (Bethesda, MD) and maintained in RPMI media (StemCell Technologies, Vancouver, BC) with 10% fetal bovine serum (StemCell Technologies, Vancouver, BC). Before treatments, 1.2 × 10^6 cells with passage numbers from 35 to 45 were seeded in Falcon (Becton Dickinson Laboratories, Franklin Lakes, NJ) 10cm culture dishes and 24 hours afterwards, starved by 48 h culture in serum-free media. After serum deprivation, cells were treated for 16 hours with either synthetic androgen 10 nM R1881 (NEN Life Science Products Inc. Boston, MA) or ethanol carrier alone (0.000385% v/v), 1µM FSK (Sigma-Aldrich Canada Ltd. Oakville, ON) or DMSO carrier alone (0.01% v/v).
2.2.2 Whole cell lysate and immunoblotting assays

Whole cell lysate (WCL) was isolated using ReadyPrep protein extraction kits (BioRad, CA). Samples were stored at -80°C until use. Protein quantitation was performed using a modified Bradford assay kit (BioRad, CA). WCL from each sample were electrophoresed on an 8.5% or 12% SDS-PAGE gel. Briefly, 20µg of protein was mixed with appropriate volumes of 2× SDS-PAGE loading buffer (0.5 M Tris pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue, 0.05% DTT), boiled for 5 minutes and electrophoresed at 150 V. Proteins were transferred to Immobilon-P (Millipore, Billerica, MA) for 60 min at 100V. Membranes were washed in phosphate buffered saline (PBS). Western blot analyses were performed using Odyssey infrared imaging system (LI-COR, Lincoln, NE) according to the manufacturer's protocol. The antibodies used in these studies were obtained from various suppliers: AR-441 (Santa Cruz), PSA (clone ER-PR8 from DAKO) and β-actin (Abcam).

2.2.3 siRNA Assay

Double-stranded siRNAs were purchased from Dharmacon Research (Lafayette, CO). A 21-nucleotide double-stranded siRNA duplex generated against the amino terminus of the AR at nucleotides 293–312 (5'-aagcccatcgtagaggcccca-3') as reported before [16] will be referred to in the following as the AR-siRNA (AR1). A single control nucleotide double-stranded siRNA duplex, referred to as the control siRNA (ARc), was generated to the inverted sequence of AR at nucleotides 293–313 (5'-accccggagatgctacccgaa-3') and functioned as a nonspecific control siRNA for the RNAi experiments where indicated. As previously described, on day 0, LNCaP cells were seeded at an initial density of 1.5 x 10^6 cells into Falcon 10cm culture dishes and incubated for 24 h in medium A (phenol red-free RPMI 1640 medium supplemented with 10% FBS lacking antibiotics). On day 1, the cells were starved with phenol red-free RPMI 1640 medium, and transfected with control or AR-siRNAs at a final concentration of 100nM using the Oligofectamine reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. The cells were incubated with siRNAs for 48 h. The guidelines for siRNA silencing were followed as detailed in the instructions.
2.2.4 RNA isolation and microarray analysis

Total RNA from 3 independent experiments was extracted from cells using Trizol (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. RNA samples from cells were analyzed by Affymetrix Genechip microarray. The syntheses of cDNA and biotinylated cRNA were performed according to the protocols provided by the manufacturer (Affymetrix, Santa Clara, CA). Biotinylated fragmented cRNA probes were hybridized to the HGU133 plus2 Genechips (Affymetrix), which contained probe sets for over 50,000 known transcripts and expressed sequence tags (ESTs). Hybridization was performed at 45°C for 16 h in a hybridization oven (Affymetrix). The Genechips were then automatically washed and stained with streptavidin–phycoerythrin conjugate in an Affymetrix Genechip Fluidics Station. Fluorescence intensities were scanned with a GeneArray Scanner (Affymetrix). Hybridizations were carried out in triplicate independently for each condition.

2.2.5 Expression profile analysis

Comparative analysis between expression profiles for Affymetrix experiments was carried out using GeneSpring™ software version 7 (Silicon Genetics, CA). The "Cross gene error model for deviation from 1.0" was active. Gene expression data was normalized in two ways: "per chip normalization" and "per gene normalization". For "per chip normalization", all expression data on a chip is normalized to the 50th percentile of all values on that chip. For "per gene normalization", the data for a given gene is normalized to the median expression level of that gene across all samples. The data sets were then assigned to two groups for either experiment (R1881 and Ethanol, FSK and DMSO). The expression profiles from 3 independent experiments were compared using Student two-sample t-test (parametric test, assume variances equal) to identify genes that were differentially expressed between the two groups. For sample clustering, standard correlation was applied to measure the similarity of the expression pattern between different samples.
2.2.6 Semi-Quantitative RT-PCR

RT-PCR was performed applying SuperScript™ one-step RT-PCR with Platinum Taq kits (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. The primers for each of the genes refer to Table 2.1. Briefly, 0.5µg of total RNA was applied in a 10µl reaction with both the primers of each genes and GAPDH. The reaction mixture was incubated at 50°C for 30 min, denatured at 94°C for 2min prior to 28 to 30 cycles of: 94°C for 15s, 56°C for 30s and 72°C for 1min and 5min at 72°C for final extension. The PCR products were electrophoresed on a 1.3% agarose gel in the presence of ethidium bromide (0.001% v/v). The images were scanned using EagleEye II camera (Stratagene, La Jolla, CA). The density of the bands was measured using ImageQuant Version 5.2 software (Amersham Biosciences, UK). The ratio of genes of interest to GAPDH was used to evaluate the fold change in levels of the difference between the two groups.

2.2.7 Hollow fiber model of prostate cancer

Male athymic nude mice, 6–8 weeks of age, were obtained from the breeding program at the Joint Animal Facility of the British Columbia Cancer Agency. Hollow fiber model of prostate cancer was performed as described previously [17]. Briefly, LNCaP cells were suspended in RPMI plus 20% FBS, and loaded into the Polyvinylidene difluoride hollow fibers (Laguna Hills, CA) with the aid of an 18-gauge needle at a seeding density of 3 x 10^7 cells/ml. The fibers were sectioned into about 2 cm pieces, heat sealed, and implanted s.c. in nude mice. A total of 24 fibers were implanted in each animal, in bundles of eight fibers at three different regions in the animal. Castration of mice was performed by making a small incision in the scrotum to excise each testicle after ligation of the cord. Surgical suture was used to close the incision. At each time point, 8 fibers from the same mouse were harvested and total RNA was isolated from LNCaP cells grown inside the fibers using Trizol (Invitrogen Life Technologies, Carlsbad, CA).
2.3 Results

2.3.1 A genome-wide view of androgen and FSK

In the absence of androgen, PSA gene expression is induced by activation of the PKA pathway with FSK by a mechanism dependent on a functional AR [12]. Whether AR is the direct target of PKA pathway or PKA activates AR indirectly through phosphorylation of AR co-activators is still unclear. To identify other genes targeted by crosstalk between the androgen and PKA pathways, experiments were undertaken to characterize the genome-wide expression profiles for androgen and FSK. LNCaP human prostate cancer cells were employed because these cells express endogenous functional AR and PSA [18, 19]. LNCaP cells were exposed for 16 h to FSK (1 µM) or R1881 (10 nM) since this time and these concentrations have been previously shown to be optimal for the maximum induction of PSA mRNA [12]. Total RNA was extracted from the biological triplicate samples and analyzed using Affymetrix Genechips (Human Genome U133plus2 GeneChip) to determine the global gene expression patterns. The Human Genome U133plus2 GeneChip contains greater than 54,000 probe sets and can be used to analyze the expression level of more than 47,000 transcripts and variants, including approximately 38,500 well-characterized human genes.

Significance analysis of microarrays (SAM) [20] was applied to identify genes differentially expressed across the different conditions. Using a one-way ANOVA (p<0.01), 1,435 probe sets were determined to be differentially expressed across the various conditions. A hierarchical two-dimensional clustering algorithm based on similarity of expression patterns was applied to these 1,435 probe sets (Fig 2.1, represented on each row) and experimental samples (represented on each column). As expected each triplicate was clustered together within each of the experimental groups indicating a high level of reproducibility between the biological replicates.

2.3.2 Changes in gene expression in response to androgen

Genes differentially expressed in LNCaP cells upon exposure to R1881 or FSK were identified as those cases where the level of transcript changed significantly from levels in the relevant controls. A p-value of ≤ 0.01 was used to identify genes that were differentially expressed by at least 1.3-fold with androgen stimulation. These criteria
revealed a total of 858 genes as significantly different between the mean expression values for R1881-treated versus control samples. Many of these genes have been previously reported to be regulated by androgen in normal prostate or prostate cancer. Examples of these include the well-characterized androgen-regulated genes such as PSA (KLK3), KLK2 and FKBP5 which were induced 6.4-fold ($p=0.0017$), 21.6-fold ($p=4.08E-05$), and 86.5-fold ($p=0.0007$), respectively. A number of genes not previously reported to be androgen-regulated were also detected and include PKIB (-6.7-fold, $p=0.002$), SESN1 (-2-fold, $p=0.006$), Dlc2 (2.1-fold, $p=0.006$) and RHOU (21.34-fold, $p=1.63E-05$). While expression of the majority (784) of these genes increased with androgen stimulation, 74 genes decreased in expression and include SESN1 (-2-fold, $p=0.006$), OSR2 (-3.36-fold, $p=0.002$), MMP16 (-4.67-fold, $p=0.003$), and PKIB (-6.7-fold, $p=0.002$) (Fig 2.2A).

### 2.3.3 Changes in gene expression in response to FSK

Generally there was a relatively lower fold-change in gene expression achieved by FSK as compared to R1881. A total of 303 genes were detected as significantly different with a $p$-value $\leq 0.05$ and the fold-change of at least 1.3 with FSK stimulation as compared to control. Expression of the majority of these genes, 204, was increased with FSK stimulation while expression of 99 genes was decreased (Fig 2.2B). PSA (KLK3) was induced by FSK treatment by 2-fold ($p=0.012$), consistent with previous studies in this cell line [12]. This time point of 16 h was optimal to achieve maximum levels of PSA transcript induced by both FSK and androgen (R1881). Other genes may have differing kinetics which would have to be examined empirically plus FSK effects may be transient as opposed to R1881 which has a relatively long half-life.

### 2.3.4 Validation of Affymetrix data by semi-quantitative RT-PCR

To confirm that gene expression changes observed were truly representative of the original samples, we selected several genes that showed distinctly different expression in response to either or both of R1881 and FSK for semi-Q-RT-PCR analysis. Consistent with the GeneChip data, semi-Q-RT-PCR confirmed increased expression of FKBP5, RHOU, ELL2, PTPRM, ORM and SOCS2 and decreased expression of PKIB and OSR2 in response to R1881 when normalized to the housekeeping gene GAPDH.
Similarly increased levels of CDC6, EIF2B5, ITGB5, NCOA6IP, and decreased levels of NUMA1 transcripts were confirmed by RT-PCR using RNA isolated from cells treated with FSK (Fig. 2.2D).

### 2.3.5 Changes in gene expression in response to both androgen and FSK

Forty-six probe sets representing 32 genes were significantly changed at least 1.3-fold in response to both androgen and FSK. Applying the Venn diagram, expression of 22 genes was increased by both androgen and FSK (Fig 2.3A). Table 2.2 provides a list of the common genes that increased in expression by both treatments which include PSA (KLK3) and KLK2. Eight genes were differentially expressed by R1881 and FSK in opposite directions (Table 2.3). Expression of SESN1 and NAV1 were the only genes to be decreased in response to both R1881 and FSK (Table 2.4).

Semi-Q-RT-PCR was employed to validate changes in expression of some of these genes including: those increased in response to both androgen and FSK such as PSA (KLK3) (4.45-fold induction in response to R1881, p=0.0065; 2.13-fold induction in response to FSK, p=0.024), HMGCR, INSIG1, KLK2, Dlc2, and FLJ22649; those decreased in response to both androgen and FSK such as SESN1; and genes differentially expressed in response to androgen and FSK in opposite directions such as MMP16 and MGC61716. In LNCaP cells, these genes showed changes in gene expression by semi-Q-RT-PCR that were consistent with the Affymetrix data (Fig 2.3B).

To explore whether differential expression of these genes in response to androgen and FSK was consistent in another androgen responsive human prostate cancer cell line, semi-Q-RT-PCR was employed using RNA isolated from 22Rv1 cells that express the AR [21, 22]. 22Rv1 cells showed similar trends in changes in expression of the PSA (6.18-fold induction in response to R1881, p=3.8E-05; 1.75-fold induction in response to FSK, p=0.005), as well as HMGCR, INSIG1, KLK2, and Dlc2 genes as compared to LNCaP cells (Fig 2.3C). However, there were some exceptions. Expression of SESN1 and MMP16 did not change in response to androgen or FSK (Fig 2.3C). FLJ22649 and MGC61716 transcripts could not be detected in 22Rv1 cells after 40 cycles of PCR (data not shown).
2.3.6 AR is required for changes in gene expression in response to FSK

To clarify whether changes in gene expression in response to both R1881 and FSK were dependent upon the presence of a functional AR, AR-siRNA was used to inhibit the expression of AR. LNCaP cells were transfected for 48 hours with AR-siRNA (AR1) or nonspecific control siRNA (ARc) before R1881 or FSK treatment. After 16 hours of treatment, whole cell lysate (WCL) and total RNA were harvested to investigate the levels of protein and mRNA of AR and the commonly regulated genes. In the control transfection with scramble siRNA (ARc), R1881 increased levels of AR protein (Fig 2.4A). This was consistent with ligand stabilizing the AR protein to increase levels as previously reported [23, 24]. FSK did not significantly alter the levels of AR protein ($p=0.091$). Sixty-four hours after transfection of the AR-siRNA, the level of AR protein was reduced significantly in cells treated with vehicle, R1881 and FSK ($p=7.7E-07$, 0.0089 and 0.0028 respectively). Similarly, the induction of PSA protein by R1881 (4.5-fold, $p=0.013$) and FSK (2.1-fold, $p=0.048$) was completely blocked by transfection of cells with AR-siRNA ($p=0.014$ and 0.003 respectively) (Fig. 2.4 B).

Application of semi-Q RT-PCR detected increased expression of KLK2, PSA, HMGCR and INSIG1 in the presence of scramble siRNA (ARc) (Fig 2.4E). AR-siRNA blocked the baseline expression of KLK2 (Fig 2.4C), PSA (Fig 2.4D) and HMGCR (Fig. 2.4E) ($p=0.014$, 0.007 and 0.0045 respectively), but not INSIG1 ($p=0.176$) (Fig 2.4F). In cells treated with R1881, AR-siRNA blocked the induced expression of KLK2, PSA, HMGCR as well as INSIG1 ($p=0.002$, 0.026, 0.006 and 0.01 respectively). In cells treated with FSK, AR-siRNA blocked the induced expression of KLK2 and PSA ($p=0.007$ and 0.035), but not HMGCR and INSIG1 ($p=0.821$ and 0.97). Thus, AR-siRNA blocks the FSK-induced expression of KLK2 and PSA, consistent with a role for the AR in this mechanism. However, induction of expression of INSIG1 and HMGCR by FSK was independent of the AR. Expression of SESN1 was repressed by both treatments (Fig 2.4G). In the presence of scramble siRNA (ARc), R1881 and FSK both inhibit the expression of SESN1 ($p=0.002$ and 4.86E-05 respectively), consistent with the results from the microarray data and RT-PCR validation. Application of AR-siRNA (AR1) attenuated the reduced expression of SESN1 by R1881 or FSK ($p=0.01$ and 0.02). This
implies that SESN1 is a gene that is repressed by activated AR. Although, AR-siRNA also blocked basal levels of expression of SESN1 ($p=0.00013$).

2.3.7 *In vivo* expression of genes differentially expressed in response to androgen and FSK

To investigate the role of the genes differentially expressed in response to androgen and FSK in the hormonal progression prostate cancer, we applied the LNCaP hollow fiber model. This *in vivo* model uses hollow fibers to provide a reproducible means of obtaining "pure" populations of LNCaP cells free of host cell contamination during different stages of progression to androgen independence for molecular analysis requiring RNA and protein extracts [17]. Total RNA was isolated from samples harvested from LNCaP cells grown in the implanted hollow fibers in Nude mice at the time points of pre-castration (intact), 10 days after castration and 52 days after castration when the prostate cancer progressed to the stage of androgen independence which was indicated by increased expression of PSA. Semi-Q-PCR was performed to detect changes in expression of the genes identified *in vitro* in response to androgen and/or FSK. After castration, expression of androgen-stimulated genes such as PSA (KLK3), KLK2, Dlc2, ELL2, HMGCR, INSIG1, RHOU and SOCS2 was decreased compared to intact levels, while the expression of androgen-suppressed genes such as MMP16 and SESN1 was increased (Fig 2.5). Interestingly, expression of genes that responded *in vitro* to FSK, such as CDC6, ITGB5 and NUMA1 also changed in response to castration while NCOA6IP, another gene that responded *in vitro* to FSK, showed no change during hormonal progression. As prostate cancer progressed to the androgen independent stage, the expression of PSA (KLK3), KLK2, ELL2, RHOU, SOCS2, MMP16, CDC6, and NUMA1 returned to the levels detected before castration. The expression of Dlc2, HMGCR, INSIG1 and ITGB5 remained decreased while SESN1 remained elevated in androgen-independent cells at 52 days after castration as compared to intact levels. PKIB, which decreased *in vitro* in response to androgen, showed no significant change during hormonal progression.
2.4 Discussion

The androgen signaling pathway plays an important role in the prostate. Activation of the PKA pathway has also been shown to play a role in prostate biology and pathology. These two pathways cross-talk through the AR in androgen deprived prostate cancer cells [12]. The present studies investigated genes commonly regulated by androgen and the PKA pathways in prostate cancer cells and revealed the following. 1) Androgen stimulated changes in expression of 858 genes. The majority (784) showed increased expression with only 74 genes showing decreased expression relative to controls. Genes not previously reported to be differentially expressed in response to androgen were also identified. 2) Activation of the PKA pathway by FSK altered the expression of 303 genes of which 204 showed increased expression while 99 showed decreased expression relative to controls. This provides the first expression profile of genes altered by activation of the PKA pathway in prostate cancer cells. 3) Only 32 genes were commonly differentially expressed by stimulation of both pathways. 4) Application of AR-siRNA revealed that the induction of members of the kallikrien family and repression of SESN1 by androgen and FSK required functional AR. 5) Genes differentially expressed in response to androgen and stimulation of the PKA pathway in vitro were also differentially expressed during hormonal progression in vivo.

2.4.1 Genes differentially expressed in response to androgen

Androgen-mediated gene expression involves binding of hormone with the AR protein, resulting in its nuclear translocation and interaction with AREs in regulatory regions of specific genes [25]. Binding to AREs in the promoter and enhancer of genes facilitates interactions with the general transcriptional machinery leading to gene transcription [25]. Androgens play an important role in secretion, growth and maintenance of function of the prostate. In this study, expression of 858 genes was significantly altered in LNCaP cells by exposure to androgen including known androgen-regulated genes such as PSA (KLK3), KLK2, FKBP5, ELL2, SOCS2, PTPRM, ORM and OSR2 [4, 26-31]. Previous studies using cDNA microarrays revealed changes in expression of 146 to 517 genes in LNCaP cells in response to androgen [4, 27].
Genes not previously reported to change in expression in response to androgen include RHOU and PKIB. RHOU (WRCH1) encodes a CDC42-related GTPase belonging to the RHO family and is a WNT target genes [32]. Over-expression of RHOU induces the same effects as over-expression of Wnt1 in the regulation of cell morphology, cytoskeletal organization, and cell proliferation [32]. Thus, up-regulation of RHOU by androgen and in androgen-independence may contribute to increased proliferation of prostate cancer cells. PKIB, a member of the cAMP-dependent protein kinase inhibitor family, is an example of a gene whose expression was decreased with androgen. The gene product of PKIB may interact with the catalytic subunit of cAMP-dependent protein kinase to act as a competitive inhibitor [33]. Decreased expression of PKIB by androgen may indirectly activate PKA. However, in vivo there was no significant change in gene expression of PKIB in response to castration or hormonal progression to androgen independence at the time points examined.

2.4.2 Genes differentially expressed in response to FSK

FSK activates adenylyl cyclase to synthesize cAMP which binds to the R subunits of the inactive holoenzyme (PKA). The catalytic subunits then dissociate and become active. The free catalytic subunit can phosphorylate serine and threonine residues; its entrance into the cell nucleus and subsequent phosphorylation of transcription factors - for example, CREB, CREM, NF-kB and nuclear receptors - forms the basis of PKA regulation of transcriptional activation [34]. However, FSK can also interact with other proteins such as glucose transporters which may influence gene expression [35]. This is the first report of the impact of FSK on the transcriptome of prostate cancer cells. A total of 303 genes were detected as differentially expressed in response to FSK. Some of these genes are involved in cell division, migration and transcriptional regulation.

Activation of the PKA pathway by FSK or other compounds can increase proliferation, alter cell morphology, and induce neuroendocrine differentiation of prostate cancer cells [9, 10, 36-38]. Consistent with these observations, activation of the PKA pathway resulted in altered expression of genes involved in cell division such as increased CDC6 and decreased NUMA. Transcription of CDC6 is regulated in response to mitogenic signals through transcriptional control mechanism involving E2F proteins.
Cdc6 is expressed in proliferating but not quiescent mammalian cells and thought to play a fundamental role in controlling initiation of DNA replication [40]. Consistent with this role, here Cdc6 transcript was reduced in vivo with castration when proliferation decreases and then levels increased in androgen independence when proliferation resumes. Interestingly, when compared to benign tissue the levels of CDC6 are decreased in PC3 prostate cancer cells that are devoid of both AR and p53 [41]. Nuclear mitotic apparatus protein (NUMA) is a mitotic centrosomal component that is essential for the organization and stabilization of spindle poles [42]. NUMA responds to external signals such as hormones that induce cell division [43]. Here, NUMA1 transcript increased in vivo in response to castration as compared to intact and androgen independent levels. Trends in expression of these two genes were consistent with suggestion of decreased activation of the PKA pathway with castration, followed by increased PKA activity with androgen independence.

2.4.3 Genes differentially expressed in response to both androgen and FSK

The Human Genome U133plus2 GeneChip contains oligonucleotides for 38,500 genes. Here we detected changes in expression of 858 and 303 genes in response to androgen and FSK, respectively, with 32 overlapping genes. This number is much greater than the expected overlap of genes by random chance calculated to be 6.75 (858*303/38,500).

Genes differentially expressed in response to both treatments but not involving AR as a common mechanism include those involved in cholesterol metabolism such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) [44] and insulin induced gene 1 (INSIG1) which both decreased in response to castration; dynein light chain 2 (Dlc2) which may play a role in apoptosis [45] and decreased in response to castration; FLJ22649, also known as SPCS3 (signal peptidase complex subunit 3 homolog) which cleaves the signal sequence of the precursor proteins to release the mature form to the extracellular space [46].

MMP16 and MGC61716 are examples of genes whose expression was significantly altered by both treatments but in opposite directions (i.e., MMP16 was repressed by androgen and increased by FSK). MMP16 belongs to the matrix
metalloproteinase (MMP) family which is involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, tissue remodeling, as well as in disease processes, such as arthritis and metastasis [47]. Decreased MMP16 expression by androgen can promote cell-cell aggregation and clone formation of prostate cancer cells. On the contrary, in the absence of androgens such as would be encountered with androgen ablation therapy, increased expression of MMP16 by stimulation of PKA may result in the breakdown of extracellular matrix and facilitate the metastasis of prostate cancer. Here indeed expression of MMP16 increased in response to castration as predicted. MGC61716 (MTERFD2) plays a central role in the control of mitochondrial rRNA and mRNA synthesis in mammalian mitochondria [48]. MGC61716 represents a gene that increases in expression in response to androgen but decreases in response to FSK.

To date the only gene that has been reported to be induced by both androgen and activation of the PKA pathway in prostate cancer cells by an AR-dependent mechanism is PSA (KLK3) [12]. Here we show for the first time that KLK2 was also induced by both androgen and FSK through a mechanism dependent on the AR. Considering that there is over 75% homology between the 6.5 kb upstream regulatory sequences of KLK2 and PSA, it is perhaps not surprising that these two genes have much the same expression patterns. KLK3 and KLK2 have both been used for the detection of prostate cancer [49] and hormonal progression.

While most genes show an increase in expression in response to androgen through a mechanism involving the AR, only a few genes have been shown to be repressed. These include Maspin [50, 51], a gene regulated by p53 [52]. Here we identify another p53 regulated gene, SESN1, to be repressed in response to both androgen and FSK by a mechanism that at least in part requires a functional AR. Interestingly, in 22Rv1 cells that contain reduced and mutated p53 [53], SESN1 was not repressed by androgen or FSK. The relationship between p53 and the androgen axis is complicated since p53 interacts with the AR to disrupt its N-terminal to C-terminal interaction thereby inhibiting DNA-binding activity [54]. Androgen also inhibits the expression [55] and nuclear accumulation of p53 [56]. Withdrawal of androgen or application of an anti-androgen can increase p53 expression and activity [57, 58]. While this may explain the high basal
expression of SESN1 as shown here in the absence of androgen, a mechanism as to why AR-siRNA reduced these levels remains elusive and the subject of further investigation ongoing in our lab. Induction of SESN1 follows similar trends to other known p53-regulated genes with increases occurring with cell cycle arrest [59]. Thus decreased proliferation of prostate cancer cells in response to castration would be expected to be associated with increased levels of SESN1 as observed here.

2.4.4 In vivo expression of genes differentially expressed in response to androgen and FSK during hormonal progression

PSA is an example of an androgen-regulated gene with several well-characterized AREs to which the AR binds to initiate transcription [60-62]. The re-expression of PSA suggests that the AR plays an important role in androgen independent disease. Evidence supporting the role of AR in hormonal progression of prostate cancer was recently summarized [7]. Consistent with these findings, in the present study, expression of androgen-stimulated genes such as PSA, KLK2, ELL2, RHOU and SOCS2 was reduced initially after castration with subsequent increased levels in the androgen independent stage. Expression of androgen-suppressed genes such as MMP16 increased initially with castration with subsequent decreased levels in the androgen independent stage while the expression of PKIB did not change. The expression of some other androgen-stimulated genes such as Dlc2, HMGCR and INSIG1 was reduced initially after castration but did not increase to the original levels in the androgen independent stage. These results indicate the reactivation of the androgen-response pathway in the absence of androgens, but this reactivation may be incomplete.

Expression of genes responsive to FSK also changed with castration. These genes include CDC6, ITGB5 and NUMA1. Regulation of PKA pathway by castration may involve both non-genomic and genomic effects of androgen. Testosterone is capable of elevating cAMP levels by binding to sex hormone binding globulin associated with its receptor [63] which would result in increased PKA activity. In addition, or possibly alternatively, here we identify that androgen decreased expression of PKIB, which is a competitive inhibitor of PKA. This would provide a novel mechanism for androgen to indirectly activate the PKA pathway. However, the expression of PKIB responded to
androgen in 16 hours \textit{in vitro} (Fig 2.2A/C) while there was no change 10 days after castration \textit{in vivo} (Fig 2.5). One interpretation of these data could suggest transient regulation of PKIB by androgen. In the absent of androgen, such as in patients receiving hormone ablation therapy for prostate cancer, the PKA pathway may contribute to androgen independent activation of AR. With androgen independence there was re-expression of the FSK-stimulated gene, CDC6, and decreased expression of FSK-suppressed gene, NUMA1 (Fig 2.5). These findings suggest the activation of PKA pathway in the progression of prostate cancer to the androgen independent stage.

\textbf{2.4.5 Summary}

In summary, a global view at the level of transcription reveals the cellular response of androgen responsive prostate cancer cells to stimulation of the androgen and PKA pathways and crosstalk between these pathways. Aberrant activation of the AR through the PKA signaling pathway may play a role in the progression of prostate cancer to the androgen-independent state. Further investigation of the mechanism involved in activation of the AR by cross-talk with the PKA pathway may lead to viable therapies for androgen independent prostate cancer. The development of new tumor models to investigate activation of the AR and regulation of cell cycle and apoptosis might yield results relevant to understanding of hormone refractory prostate cancer.
Table 2.1 Primers for gene expression using semi-Q-RT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Exons</th>
<th>PCR product (bp)</th>
<th>Forward primer (5' to 3')</th>
<th>Reverse primer (5' to 3')</th>
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</thead>
<tbody>
<tr>
<td>CDC6</td>
<td>4-8</td>
<td>488</td>
<td>CTTAAGCGCGATCTGCAAG</td>
<td>GAACGTGACAGATTGTGACAG</td>
</tr>
<tr>
<td>Dlc2</td>
<td>1-3</td>
<td>454</td>
<td>TCCGTTGAAAGTCACACCC</td>
<td>TTGGTTTGTCTGTCGACGA</td>
</tr>
<tr>
<td>EIF2B5</td>
<td>3-6</td>
<td>480</td>
<td>GGAGATGTCCCTCCGTGATGT</td>
<td>TAGTTGAGTATGACCTGTTG</td>
</tr>
<tr>
<td>ELL2</td>
<td>5-8</td>
<td>478</td>
<td>AGTGCACATCGGGAAGGCA</td>
<td>CAAAAGCTGTTTCTGAGGAG</td>
</tr>
<tr>
<td>FKBP5</td>
<td>8-11</td>
<td>436</td>
<td>GCTGTGGTTGAGAGTGTGTT</td>
<td>AAGCAAGCAAAGAGAAATGA</td>
</tr>
<tr>
<td>FLJ22649</td>
<td>1-6</td>
<td>499</td>
<td>AACTCACTTTCTGACCTTCTG</td>
<td>TCTGGAAATGGAACAGATACG</td>
</tr>
<tr>
<td>GAPDH</td>
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<td>353</td>
<td>CCGAGCCCACTGTGCTCAG</td>
<td>CCCAGCCTTCTGCATGCTG</td>
</tr>
<tr>
<td>HMGR</td>
<td>11-13</td>
<td>590</td>
<td>TGGTGACACAGGAACTGAA</td>
<td>AGTCTGTCAATGACCTGCT</td>
</tr>
<tr>
<td>INSIG1</td>
<td>5-6</td>
<td>440</td>
<td>ATTTTTTCAGAGGGGGTCA</td>
<td>CTGGGGTTGATGTTTAGGTG</td>
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<tr>
<td>ITGB5</td>
<td>4-7</td>
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<td>GAGGCTCACAGCAACTTCC</td>
<td>AATTTCCACGTTGTTCCCA</td>
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<td>KLK2</td>
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<td>420</td>
<td>GGGGCCTGAGGATGAGAGAG</td>
<td>GCACAAACTCCTGCTGGGGGGGT</td>
</tr>
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<td>KLK3 (PSA)</td>
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<td>521</td>
<td>GGCACTGGCTGTAGTTGAGC</td>
<td>CACCCGACGCTGTTTGTG</td>
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<tr>
<td>MGE81716</td>
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<td>592</td>
<td>TCGATGTTTCCTCCCTTGAC</td>
<td>TTGAGGATGATGTCTGGA</td>
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<tr>
<td>MMP1</td>
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<td>NCOA6IP</td>
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<td>GCACGTGGCTCGATGACGA</td>
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<td>GCAAGAAGGGAAGAAGAGG</td>
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<td>OSR2</td>
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<td>CAGATCAAGAGTTTTCGCTG</td>
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<td>PKIB</td>
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<td>RHOU</td>
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<td>SESN1</td>
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<td>ATCGGACTGTGAAATCAGT</td>
<td>GTCCAAATGCGCTGCTAA</td>
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<tr>
<td>SOCS2</td>
<td>2-3</td>
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<td>GTCGGTCTATCTTTGGACAT</td>
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Table 2.2 Expression of genes that significantly increased in LNCaP cells in response to R1881 and FSK

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<th>Symbol</th>
<th>Affymetrix ID</th>
<th>Fold Change in R1881</th>
<th>P-value in R1881</th>
<th>Fold Change in FSK</th>
<th>P-value in FSK</th>
<th>Description</th>
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<td>1.32</td>
<td>0.025</td>
<td>Acyl-CoA synthetase-long-chain family member 3</td>
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<td>C20orf155</td>
<td>223978_s_at</td>
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<td>0.003867</td>
<td></td>
<td></td>
<td>chromosome 20 open reading frame 155</td>
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<td></td>
<td>225324_at</td>
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<td>0.00778</td>
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</tr>
<tr>
<td></td>
<td>232118_at</td>
<td>1.77</td>
<td>0.04597</td>
<td></td>
<td></td>
<td></td>
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<td>CAMKK2</td>
<td>210787_s_at</td>
<td>6.65</td>
<td>0.00112</td>
<td>1.50</td>
<td>0.0171</td>
<td>Calcium/calmodulin-dependent protein kinase kinase 2, beta</td>
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<tr>
<td>Dla2</td>
<td>229106_at</td>
<td>2.10</td>
<td>0.006807</td>
<td>1.52</td>
<td>0.0253</td>
<td>dynein light chain 2</td>
</tr>
<tr>
<td>EG1</td>
<td>225159_s_at</td>
<td>4.44</td>
<td>0.00328</td>
<td>1.32</td>
<td>0.00731</td>
<td>Endothelium-derived gene 1</td>
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<tr>
<td>EVI5</td>
<td>209717_s_at</td>
<td>2.04</td>
<td>0.00479</td>
<td>1.32</td>
<td>0.0426</td>
<td>ecotropic viral integration site 5</td>
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<tr>
<td>FADS1</td>
<td>208662_s_at</td>
<td>2.67</td>
<td>0.00156</td>
<td>1.34</td>
<td>0.0452</td>
<td>601065683F1 Nih_MGC_19_Homo sapiens cDNA clone IMAGE:34529255 5' mRNA sequence,</td>
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<tr>
<td>FLJ22649</td>
<td>222753_s_at</td>
<td>3.77</td>
<td>0.00872</td>
<td>1.80</td>
<td>0.0111</td>
<td>hypothetical protein FLJ22649 similar to signal peptidase SPC2223</td>
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<tr>
<td>GNAI3</td>
<td>201179_s_at</td>
<td>2.11</td>
<td>0.0044</td>
<td>1.64</td>
<td>0.0452</td>
<td>Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3</td>
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<td></td>
<td>201181_at</td>
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<td></td>
<td></td>
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<tr>
<td>HMCR</td>
<td>202540_s_at</td>
<td>3.59</td>
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<td>1.56</td>
<td>0.0256</td>
<td>3-hydroxy-3-methylglutaryl-Coenzyme A reductase</td>
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<td>INSRG1</td>
<td>201625_s_at</td>
<td>3.83</td>
<td>0.00134</td>
<td>1.71</td>
<td>0.0035</td>
<td>insulin induced gene 1</td>
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<td></td>
<td>201626_s_at</td>
<td>4.58</td>
<td>0.0023</td>
<td>1.68</td>
<td>0.0157</td>
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<tr>
<td>KIAA0690</td>
<td>216913_s_at</td>
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<td>0.00851</td>
<td>1.53</td>
<td>0.00579</td>
<td>KIAA0690</td>
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<td>KLK2</td>
<td>209654_s_at</td>
<td>31.36</td>
<td>0.00022</td>
<td>1.92</td>
<td>0.00744</td>
<td>kallikrein 2, prostatic</td>
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<tr>
<td></td>
<td>210339_s_at</td>
<td>21.60</td>
<td>4.08E-05</td>
<td>1.95</td>
<td>0.0085</td>
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</tr>
<tr>
<td>KLK3</td>
<td>204582_s_at</td>
<td>8.42</td>
<td>0.00172</td>
<td>2.06</td>
<td>0.0118</td>
<td>Kallikrein 3, (prostate specific antigen)</td>
</tr>
<tr>
<td></td>
<td>204583_s_at</td>
<td>5.38</td>
<td>0.00405</td>
<td>2.03</td>
<td>0.0194</td>
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<tr>
<td>MAP7</td>
<td>215471_s_at</td>
<td>1.93</td>
<td>0.00846</td>
<td>1.35</td>
<td>0.00947</td>
<td>Microtubule-associated protein 7</td>
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<td>NGLY1</td>
<td>220742_s_at</td>
<td>1.96</td>
<td>0.00498</td>
<td>1.31</td>
<td>0.0274</td>
<td>N-glycanase 1</td>
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<tr>
<td>SMP1</td>
<td>217768_s_at</td>
<td>1.62</td>
<td>0.00744</td>
<td>1.45</td>
<td>0.0228</td>
<td>Small membrane protein 1</td>
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<tr>
<td>TRG0</td>
<td>211144_s_at</td>
<td>11.44</td>
<td>0.0057</td>
<td></td>
<td></td>
<td>T-cell receptor (V-J-C) precursor; Human T-cell receptor gamma chain VJCI-CII-CIII region mRNA, complete cds</td>
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<tr>
<td></td>
<td>209613_s_at</td>
<td></td>
<td>3.18</td>
<td>0.0046</td>
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<tr>
<td>UBE2J1</td>
<td>217623_s_at</td>
<td>6.16</td>
<td>0.00374</td>
<td>1.46</td>
<td>0.0439</td>
<td>Ubiquitin-conjugating enzyme E2, J1 (UBCS homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>221644_s_at</td>
<td>2.87</td>
<td>0.00696</td>
<td>1.54</td>
<td>0.00339</td>
<td>Transcribed sequence with moderate similarity to protein sp:399150 (H.sapiens)</td>
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<tr>
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<td>228559_at</td>
<td>23.68</td>
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<td>0.00227</td>
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### Table 2.3 Genes significantly differentially expressed in LNCaP cells in opposite direction in response to R1881 and FSK

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Affymetrix ID</th>
<th>Fold Change in R1881</th>
<th>P-value in R1881</th>
<th>Fold Change in FSK</th>
<th>P-value in FSK</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>APPBP2</td>
<td>202530_at</td>
<td>4.58</td>
<td>0.00218</td>
<td>-1.43</td>
<td>0.013</td>
<td>amyloid beta precursor protein (cytoplasmic tail) binding protein 2</td>
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<td>MAF</td>
<td>209348_s_at</td>
<td>21.29</td>
<td>0.000168</td>
<td>-1.35</td>
<td>0.0149</td>
<td>V-maf murine erythroblastic leukemia viral oncogene homolog (avian)</td>
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<tr>
<td>MGC61716</td>
<td>226486_at</td>
<td>2.30</td>
<td>0.000992</td>
<td>-1.64</td>
<td>0.0254</td>
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<td>MJD</td>
<td>205415_s_at</td>
<td>1.67</td>
<td>0.00499</td>
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<td></td>
<td>Machado-Joseph disease (spinocerebellar ataxia 3, olivopontocerebellar ataxia 3, autosomal dominant, ataxin 3)</td>
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<tr>
<td>233162_x_at</td>
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<td>MMP16</td>
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<td>0.0223</td>
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### Table 2.4 Expression of genes that significantly decreased in LNCaP cells in response to R1881 and FSK

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<tr>
<th>Symbol</th>
<th>Affymetrix ID</th>
<th>Fold Change in R1881</th>
<th>P-value in R1881</th>
<th>Fold Change in FSK</th>
<th>P-value in FSK</th>
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<td>SESN1</td>
<td>218346_s_at</td>
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<td>0.00589</td>
<td>-1.32</td>
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<td>Sestrin 1</td>
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40
Figure 2.1: Cluster analysis of genes differentially expressed across all treatments. Unsupervised clustering analysis of all the 12 samples according to the 1,435 genes designated significantly changed across all conditions by standard correlation using the GeneSpring microarray analysis software. The genes are represented by each row and the experimental samples are represented on each column (in triplicates).
Figure 2.2: Alterations of gene expression induced by R1881 and FSK. (A) Androgen regulated genes ($p \leq 0.01$ and fold change $\geq 1.3$) and, (B), FSK regulated genes ($p \leq 0.05$ and fold change $\geq 1.3$). The left panel showed the hierarchical two-dimensional clustering algorithm with the genes represented by each row and the experimental samples represented on each column. The right panel indicates genes that were validated by RT-PCR for R1881 (C), and FSK (D). Cells were treated with R1881 (10nM) or ethanol carrier alone, FSK (1µM) or DMSO carrier alone for 16h.
Figure 2.3: Genes commonly regulated by R1881 and FSK. (A) Venn diagrams showing the number of genes significantly up-regulated, down-regulated, or overlapping by R1881 and FSK treatments in LNCaP cells. (B/C) Validation of common changes in gene expression in response to R1881 and FSK in LNCaP cells (B) and 22Rv1 cells (C) by semi-quantitative RT-PCR. Numbers beside gel represent the fold-change measured by the Genechip in LNCaP cells. Total RNA (0.5µg) was applied in a 10µl reaction with the primers of each gene and GAPDH.
Figure 2.4: Androgen receptor is required for PKA induced expression of some genes. Western blot analyses of AR protein (A) and PSA protein (B) in LNCaP cells transfected with scrambled (ARc) or AR-siRNA (AR1) and then treated with R1881 or FSK for 16 h. β-actin serves as a loading control. Semi-quantitative RT-PCR analyses of transcript levels of KLK2 (C), PSA (D), HMGCR (E), INSIG1 (F) and SESN1 (G) in LNCaP cells transfected with scrambled (ARc) or AR-siRNA (AR1) and then treated with R1881 or FSK for 16 h. GAPDH serves as a loading control. The ratio of genes of interest to GAPDH was used to evaluate the difference between the two groups. The mean and standard deviation of three independent replicates are shown. Significant differences between each condition (p-value) were determined by a Student t-test.
Figure 2.5: Expression of androgen and PKA regulated genes in the hollow fiber prostate cancer model. Total RNA samples were harvested from the LNCaP cells grown in the implanted hollow fibers in nude mice at the time points of pre-castration (d0), 10 days (d10) and 52 days (d52) after castration. Semi-Q-PCR was performed in a 10µl reaction with the primers of each gene and GAPDH. The ratio of genes of interest to GAPDH was used to evaluate the difference among the time points. Y-axis showed the fold change related to the expression level on d0. The mean and standard deviation of three independent replicates are shown. Significant differences between each condition (p-value) were determined by a Student t-test. *, p<0.05; **, p<0.01.
2.5 References

3 Androgen Receptor Negatively Regulates the Expression of SESN1, a Potential Tumor Suppressor

3.1 Introduction:

Androgens are essential for differentiation and function of the prostate as well as for proliferation and survival of prostate cells. Androgens mediate their effects by binding to the AR. The AR is a transcription factor that binds to cis-acting AREs located in the promoter and enhancer regions of specific target genes and serves to activate or to repress transcription of genes involved in differentiation, function, morphology, proliferation, and survival of prostate cells [1-3]. Androgen ablation decreases proliferation and causes apoptosis of prostate epithelial cells with subsequent involution of the prostate and thereby provides an effective treatment for prostate cancer (CaP). Few genes with bona fide AREs have been identified that alter proliferation of prostate cells in response to androgen. These include maspin [4], NK3 homeobox 1 (NKX3-1) [5], protein kinases C delta (PKCdelta) [6], insulin-like growth factor binding protein 3 (IGFBP3) [7], prader-willi/angelman region-1 (PAR1) [8], prostate stem cell antigen (PSCA) [9], c-Met [10], and p21 [11].

There are a number of studies reporting the mechanisms of up-regulation of the target genes (such as PSA, a CaP tumor marker) by AR in response to androgen [12-14]. However, for those genes which are silenced by androgen, the mechanisms are still unknown. SESN1 (protein name Sestrin-1), a target gene of p53 tumor suppressor [15], is an example of one of the few androgen suppressed genes that requires the presence of AR for decreased expression in response to androgen [16]. SESN1 maps to chromosome 6q21 and the protein is believed to be a regulator of cellular growth and belongs to the GADD family of growth arrest and DNA damage-inducible genes. There are three transcript isoforms of SESN1 of which SESN1-T1 is not induced by p53, whereas both SESN1-T2 and SESN1-T3 are highly induced by p53 [15]. The second intron of SESN1 gene has a confirmed p53 binding site [15].

We hypothesize that activated AR inhibits the expression of SESN1 through direct binding to the SESN1 promoter and/or crosstalk with p53. This hypothesis is based

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on the facts that: 1) decreased SESN1 expression in response to androgen is dependent upon AR [16]; 2) SESN1 is a p53 target gene with a functional p53-responsive element in its regulatory region [15]; 3) decreased expression of SESN1 results in growth stimulation that is independent of the p53 pathway [15]; 4) SESN1 promoter region contains a putative ARE; 5) AR inhibits p53 activity and nuclear accumulation [17]; and 6) bicalutamide, an anti-androgen, enhances the activation of p53-mediated transcription [18].

In this study, we provide multiple lines of evidence to show that the AR negatively regulates the transcription of SESN1-T2 through direct binding to a functional ARE in the promoter region of SESN1 and knockdown of SESN1 expression increased the proliferation of CaP cells. Our results provide a novel mechanism of androgen action on the proliferation of prostate cells and could signify a therapeutic strategy for the treatment of CaP.

3.2 Materials and Methods

3.2.1 Cell culture and general methods

LNCaP and PC3 cells were obtained from the American Type Culture Collection (Bethesda, MD, USA) and maintained in RPMI media (StemCell Technologies, Vancouver, BC, Canada) supplemented with 10% fetal bovine serum (FBS) (StemCell Technologies) or Dulbecco's Modified Eagle's Medium (DMEM) media (StemCell Technologies) supplemented with 5% FBS, respectively. LNCaP cells (1.2 x 10^6), passage numbers 40 to 45, were seeded in Falcon 10 cm culture dishes (Becton Dickinson Laboratories, Franklin Lakes, NJ, USA) for 24 h prior to adding fresh serum-free media for an additional 48 h. After serum deprivation, cells were treated with either synthetic androgen, R1881 (NEN Life Science Products Inc., Boston, MA, USA), or ethanol vehicle alone (0.000285% v/v).

3.2.2 Western Blot Analysis

Whole-cell lysate (WCL) was isolated using ReadyPrep protein extraction kits (BioRad, CA, USA). Samples were stored at -80°C until use. Protein quantitation was completed using BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). WCL from
each sample were electrophoresed on an 10% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE). Proteins were transferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA) for 60 min at 100 V. Membranes were washed in phosphate-buffered saline. Western blot analyses were performed using ECL western blotting kit (Amersham Life Science, Piscataway, NJ, USA) according to the manufacturer's protocol. The antibodies used in these studies were obtained from various suppliers: SESN1 (Novus Biologicals Inc, Littleton, CO, USA), AR-441 (Santa Cruz, Santa Cruz, CA, USA), and β-actin (Abcam, Cambridge, MA, USA).

3.2.3 Quantitative RT–PCR (qRT–PCR)

Oligo-d(T)-primed total RNAs (0.5 µg per sample) were reverse-transcribed with SuperScript III (Invitrogen Life Technologies, Carlsbad, CA, USA). An appropriate dilution of cDNA and gene-specific primers were combined with SYBR Green Supermix (Invitrogen) and amplified in the ABI 7900 real-time PCR machine (Applied Biosystems, Foster City, CA, USA). All qPCR reactions were performed in triplicate. Ct (threshold cycle number) and expression values with standard deviations were calculated in Excel. Primer sequences for real-time PCRs were as follows: SESN1-T1: 5-AGGTGTCCCTTCAAAGATGTG-3 (forward) and 5-TGGTCCCTGTCTCCTAGTGTCGTC-3 (reverse), SESN1-T2: 5-GCGACCAGGACGAGGAAC-3 (forward) and 5-ACCTCCACTTGGAGGATCT-3 (reverse), SESN1-T3: 5-GCCATCCCTTCTCCTGAATTTG-3 (forward) and 5-GAACCTTTGAAGCAGACACC-3 (forward) and 5-AGCTCTGTTGCGTTGTCGTC-3 (reverse), GAPDH: 5-CTGACTTCAACAGCGACACC-3 (forward) and 5-TGCTCTGACTCAGCCCATCC-3 (reverse). Real-time amplification was performed with initial denaturation at 95°C for 2 min, followed by 40 cycles of two-step amplification (95°C for 15 sec, 55°C for 30 sec).

3.2.4 SESN1 Promoter Plasmid Constructs and Luciferase Assay

SESN1 5’ flanking DNA was obtained by PCR-mediated amplification of human genomic DNA using oligonucleotide primers corresponding to the SESN1 gene T2 transcript. The sequences for primers were 5-GGTACCTCCTGTTCTGTCGCCATAACT-3 (-969), 5-GGTACCGCTGCTGTTGTTTGGTTCTAGG-3 (-356), 5-GGTACCGACAGTGCA
CGCTCACCTC-3 (-262), 5-GGTACCATTAGCTGTTCGTGGGGAAC-3 (-211), and 5-GGTACCCAGCGCCTCAGTCAC-3 (+18). DNA fragments corresponding to -969/+18, -356/+18, -262/+18, -211/+18 of the SESN1-T2 5’ flanking region were inserted into the KpnI site of pGL3 basic (Promega, Madison, WI, USA), which contains firefly luciferase as a reporter gene. pGL3 basic containing the DNA fragments of the SESN1-T2 5’-flanking DNA was referred to as pSESN1-969, pSESN1-356, pSESN1-262 and pSESN1-211 respectively. OmicsLink™ p53 Expression Clone was purchased from GeneCopoeia (Germantown, MD USA, Catalog No.: EX-B0105-M01). The SESN1-T2-ARE-del promoter reporter was constructed applying the Quickchange II site directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using primers: 5-GACAGTGCACGCTCAAGAATTAGCTGTTCG-3 and 5-CGAACAGCTAATTCTGAGCGTGCACTGTC-3.

LNCaP cells (1.2 × 10^5) were plated on 6-well plates and incubated in RPMI 1640 with 10% FBS for 1 day, resulting in 50-60% confluence. Plasmid DNA was mixed with 5 µl of Lipofectin agent (Invitrogen) and incubated for 15 min at room temperature before being added to the wells. Medium was replaced after 24 h by RPMI 1640 with or without R1881 (i.e. serum-free media). PC3 cells (1.0 × 10^5) were plated on 6-well plates and incubated in DMEM with 5% FBS for 1 day, resulting in 50-60% confluence. Plasmid DNA was mixed with 4 µl of Fugene6 transfection reagent (Roche Diagnostics, Laval, Quebec, Canada) and incubated for 15 min at room temperature. The total amount of plasmid DNA used was normalized to 3 µg/well by the addition of empty plasmid. Six hours after transfection, cells were treated with R1881 or ethanol as a control and were incubated for another 24 h before collected using cell passive lysis buffer (Promega). Luciferase activities were measured using a commercial kit from Promega according to the manufacturer's protocol and activities were normalized to protein as previously described [19]. Luciferase activities were expressed as relative luminescent units/mg/min of protein. All transfection experiments were carried out in triplicate and repeated three times.
3.2.5 Chromatin immunoprecipitation (ChIP)

ChIP was modified from that of Narayanan et al. [20]. LNCaP cells (3.5 × 10^6) were plated in 15-cm dishes. After 24 h, the cells were serum starved for 48 h before treatment with 10 nM R1881 or vehicle (ethanol) for the indicated times. The proteins were cross-linked with 1 % formaldehyde for 10 min at 37°C. The cells were washed with cold PBS once, scraped in 1 ml of PBS with 1x Complete™ EDTA-free protease inhibitor (Roche Diagnostics), pelleted and resuspended in SDS lysis buffer (1 % SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1], 1x protease inhibitor). After lysis on ice for 10 min, the cell extract was sonicated (Sonicator 3000 Ultrasonic Liquid Processor, Misonix, Farmingdale, NY, USA) 10 times for 30 s each with an output level 1 in an ice water bath. The average length of the sheared DNA fragments was 600-800 bp as monitored by agarose gel electrophoresis. The sonicated sample was pelleted at 13,000 rpm for 5 min at 4°C. The supernatant was diluted 10-fold with ChIP dilution buffer [0.22 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl, 1× protease inhibitor]. Diluted supernatant (100 µl) was reserved as input. After pre-clearing with 50 µl nProtein A-Sepharose™ beads (Amersham Biosciences) in TE (1:1) with 2 µg of sheared salmon sperm DNA for 30 min at 4°C, the remaining proteins were incubated with 5 µg of anti-AR antibody (AR441; Santa Cruz Biotechnologies) or mouse IgG overnight at 4°C. The antibody-protein-DNA complex was precipitated by incubating with 100 µl of 1:1 nProtein A-Sepharose™ beads for 2 h at 4°C. The beads were pelleted for 30 s at 4°C and washed once each sequentially with low-salt wash buffer [0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl] and TE. The protein-DNA complex was eluted from the beads with 50 µl elution buffer (1 % SDS, 0.1 M NaHCO3) two times at room temperature. The cross-linking of the DNA protein complex was reversed by incubating at 65°C for 6 h. The DNA was recovered and purified using the QIAquick® PCR Purification Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer’s instructions. The AR binding region was amplified using Q-PCR. The primers were as follows: 5-AGATTCCCTGTCCCTGAAG-3 and 5-GTTCCCCACGAAACAGCTAAT-3. The thermal cycling conditions were 50 °C for 2 min, 95 °C for 2 min, followed by 45 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72
°C. Percentage input was calculated by dividing the arbitrary Q-PCR numbers obtained from each sample by that of the input.

3.2.6 Cell proliferation assay

LNCaP cells (1x10^4) were plated in 96-well Falcon Primaria tissue culture plates in phenol red-free and antibiotic-free RPMI containing 0.5% FBS in a final volume of 0.1 ml. The cells were incubated at 37°C for 24 h before siRNA transfection. On day 1, the cells were transfected with siCONTROL Non-Targeting siRNA control or ON-TARGETplus SMARTpool SESN1-siRNAs (Dharmacon, Chicago, IL, USA) at a final concentration of 100nM using the Oligofectamine reagent (Invitrogen) according to the manufacturer’s instructions. The cells were incubated with siRNAs for another 12 h. The guidelines for siRNA silencing were followed as detailed in the instructions provided at the Dharmacon Research website (http://www.dharmacon.com/). On day 2, the cells were treated with 0.1nM R1881 or ethanol as a control for 24, 48 and 72 hours. Cell proliferation was assessed by incubating with 20 ul of BrdU for 2 h at 37°C. 150 ul of media were removed and the plates were dried down in a Hybrid oven at 60°C for 2 h. The cells were stored at 4°C until ELISA was performed. The protocol from the manufacturer of the kit was followed (Roche Diagnostics, Laval, Quebec, Canada) and ELISA data was acquired at 30 min after addition of the substrate.

3.2.7 Xenografts

Male SCID mice, 6-8 weeks of age, were obtained from the breeding program at the Animal Research Centre of the British Columbia Cancer Agency. All procedures were performed in compliance with regulations on the humane use and care of laboratory animals under an appropriate animal license issued by the University of British Columbia (Vancouver, BC, Canada). Mice were subcutaneously inoculated with LNCaP cells suspended in 75 µl of RPMI 1640 (5% FBS) with 75 µl of Matrigel (Becton Dickinson Laboratories). Tumors were measured weekly and their volumes calculated by the formula LxWxHx0.5236. Castration was performed by making a small incision in the scrotum to remove each testicle following ligation of the cord. At different tumor stages, the grafts were harvested, measured, and fixed for immunohistochemical analyses.
3.2.8 Immunohistochemistry

Xenografts were fixed in 10% neutral buffered formalin, processed through alcohols, and embedded in paraffin. Tissue sections (5 µm) were blocked in immunohistochemistry solution (Immunovision Technologies, Brisbane, CA), immunostained with anti-SESN1 (Novus) or anti-PSA (C-19, Santa Cruz, Santa Cruz, CA, USA) antibodies, and protein bands detected with the ABC kit (Vector Laboratories, Burlingame, CA). Following incubation, sections were washed, incubated with either a biotinylated anti-rabbit or anti-mouse antibody (Vector Laboratories), and then incubated with avidin-biotin complex (Vector Laboratories). The samples were visualized using 3,3’-diaminobenzidine in PBS and 3% H$_2$O$_2$. All sections used for immunohistochemistry were lightly counterstained with 5% (w/v) Harris hematoxylin.

3.3 Results

3.3.1 SESN1-T2 is the predominant transcript suppressed by androgen

Levels of SESN1 mRNA are decreased by androgen stimulation of prostate cancer cells [16]. However, the SESN1 gene has three transcript isoforms (T1/T2/T3) that differ in the transcription start site and the first exon [15]. To characterize which of the three transcripts were expressed and regulated by androgen in LNCaP cells, we designed PCR primers for detection of each of the three transcript isoforms by application of Q-PCR. SESN1 transcript T2 was most abundant and also significantly suppressed by stimulation with androgen (Fig. 3.1A). Androgen inhibited expression of SESN1-T2 transcript in a dose-dependent manner (Fig. 3.1B). Levels of SESN1-T2 transcript were gradually reduced from 0.1 nM R1881 (p<0.05). The level of SESN1-T2 transcript was reduced by approximately 2.5-fold at 10 nM R1881, compared to levels in samples without R1881 (p<0.001). Expression of prostate-specific antigen (PSA) is induced by androgen in LNCaP cells [12] and was measured as a positive control for androgen stimulation using aliquots of the same RNA. A temporal study of the kinetics of repression of SESN1-T2 transcript revealed that after 16 hours of treatment with androgen, the level of mRNA was reduced to 40% of the initial level (Fig 3.1C).
3.3.2 SESN1-T2 protein level is negatively regulated by androgen

To assess the levels of Sestrin-1 protein in response to androgen stimulation, whole cell lysates were analysed by western blot using a mouse anti-Sestrin-1 antibody immunized to the C-terminus, a common sequence for all three Sestrin-1 isoforms. No bands were detected at the expected MW for the proteins encoded by T1 (68 kDa) and T3 (48 kDa). One protein band of approximately 55 kDa was detected and corresponded to the expected MW for the protein product of SESN1-T2. The protein band at 55 kDa was significantly decreased in response to stimulation with R1881 (10 nM) after 16 hours (Fig 3.1D). Thus, levels of protein followed the same trend as the levels of SESN1 T2 transcript in response to physiological levels of androgen [21].

3.3.3 AR is required to repress SESN1 promoter activity

SESN1 is a p53-regulated gene and a p53-binding site has been identified in the SESN1 promoter [15]. LNCaP cells express both AR and wild-type p53 [22]. To determine if the AR was required for decreased expression of SESN1 T2 transcript, we employed PC3 prostate cancer cells. These cells do not express functional AR or p53 proteins, thereby allowing assessment of the contribution of both transcription factors in the regulation of transcription of SESN1. PC3 cells were cotransfected with expression vectors for p53 and/or AR protein along with the SESN1-T2-969 luciferase reporter. Consistent with previous reports of no induction of SESN1 in cells lacking p53 protein expression [15], activity of the SESN1-T2 promoter was very low in the absence of p53 expression (Fig 3.2). Although this region of the SESN1-T2 promoter has not been previously reported to contain a known p53-binding site, there was over 30-fold induction of promoter activity in cells transfected with p53 (p<0.001). Induction of SESN1 promoter activity by p53 was reduced by expression of full length AR (p<0.01). Addition of R1881 with AR had a synergistic effect on suppression of the p53-induced SESN1-T2 promoter activity (p<0.05). Thus, AR repressed the induction of SESN1 promoter activity by p53.

3.3.4 Mapping the activity of SESN1-T2 promoter

To identify the region of the SESN1 promoter that was sensitive to repression by androgen, we employed deletions constructs of the first 1000 bp of the SESN1 T2
promoter that was highly inducible by p53, as shown above. LNCaP cells, that express both AR and wild-type p53, were transfected with various truncated regions of the SESN1 T2 promoter. Reporter constructs with the proximal 1000-bp promoter fragment (pGL3-SESN1-969) and the 350 bp fragment (pGL3-SESN1-356) had similar activities that were inhibited by androgen in LNCaP cells (Fig 3.3). The pGL3-SESN1-262 reporter had relatively higher activity and was also inhibited by androgen. No repression of SESN1 promoter activity by androgen was detected for the 211 bp fragment (pGL3-SESN1-T2-211). Thus, differential activity of the SESN1 T2 promoter by androgen required the sequence between -262 and -211 bp. Sequence analysis of the ~27 kb SESN1 genomic region applying the CONSITE program (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite), a web-based tool for finding cis-regulatory elements in genomic sequences, revealed a potential AR binding site, SESN1-ARE, between bp -230 to -216 of the T2 promoter with a sequence of AGCCCAttcCGTACC. Therefore, we deleted bp -247 to -214 to create pGL3-SESN1-T2-ARE-del which was not inhibited by androgen. Taken together, these data suggest that bp region -247 to -214 was required for repression of SESN1 promoter activity by androgen.

3.3.5 AR interacts with the SESN1 promoter upon androgen stimulation

To further explore the regulatory mechanisms by which androgen represses SESN1 expression, we examined whether androgen affects SESN1 transcription through the binding of AR to the potential ARE between bp -230 to -216 of the T2 promoter. This region was consistent with the results obtained from deletion construct experiments showing loss of androgen repression with removal of bp -247 to -214 of the promoter. ChIP assay with the AR antibody was employed using LNCaP cells treated with 10 nM R1881. Applying Q-PCR with primers spanning this region, we showed that AR interacts at the potential ARE as early as 0.5 h after R1881 stimulation and was still detectable at 8 h (Fig 3.4). These results support that the AR interacts with the promoter region of SESN1-T2 to form DNA-protein complexes to repress expression of SESN1 in response to androgen.
3.3.6 Knockdown of SESN1 promoted the proliferation of CaP cells

SESN1 is a p53 target gene and its expression is increased by growth arrest [15], thereby leading to the suggestion that SESN1 is a potential tumor suppressor gene. Therefore, the impact of SESN1 deficiency on proliferation of CaP cells was determined. For these studies, strategies to assess the impact of transient SESN1 depletion were employed by transfection of LNCaP cells with SESN1-siRNA or control-siRNA. ON-TARGETplus SMARTpool siRNA is composed of three sets siRNA sequences which can increase the silencing efficiency, lower the concentration required and decrease the off-target effect. Twelve hours post-transfection, cells were treated for 24, 48 or 72 h with optimal concentration of androgen to promote proliferation (0.1nM R1881) [23]. Levels of SESN1 T2 mRNA and protein were reduced by transfection of SESN1-siRNA for the duration of the experiment (Fig 3.5A). Androgen (0.1 nM) significantly increased proliferation of LNCaP cells as measured by BrdU incorporation across all times examined (Fig 3.5B). Since this concentration of androgen suppresses SESN1 expression (Fig 3.1B), we anticipated that SESN1-siRNA would not have a large effect on proliferation in the presence of androgen. Indeed, only a small decrease in proliferation was measured between androgen and androgen with SESN1-siRNA treatment (Fig 3.5B). Greatest effects on knocking down SESN1 expression with siRNA was observed in the absence of androgen as expected when endogenous SESN1 T2 would normally be elevated. In the absence of androgen, proliferation of LNCaP cells was increased with transfection of SESN1-siRNA compared to cells transfected with control-siRNA (p<0.01 on day 1, p<0.001 on days 2 and 3). Proliferation was the highest in cells treated with androgen and SESN1-siRNA. Consistent with the suggestion that SESN1 is a tumor suppressor, reduced expression of SESN1 increases proliferation of prostate cancer cells. Together these data provide a potential mechanism of how androgen increases the proliferation of prostate cells through regulation of SESN1 expression.

3.3.7 Expression of Sestrin-1 is initially induced by castration, but later suppressed when prostate cancer is androgen independent

The role of androgens in the regulation of Sestrin-1 expression, as well as the expression pattern of Sestrin-1 in the hormonal progression of CaP, was next examined in
To do this, expression of Sesrin1 was measured by immunohistochemistry in LNCaP xenografts harvested from intact (non-castrated, AD), castrated (androgen ablation, AA), and castrated mice with rising PSAsignifying androgen-independent disease (AI, also called castration-resistant). Immunohistochemistry for PSA in adjacent sections was included as a control. Weak expression of Sestrin-1 correlated with strong expression of PSA in LNCaP xenografts harvested from non-castrated hosts (Fig. 3.6). Sestrin-1 is a nuclear protein [15]. Most of the expression of Sestrin-1 was in the cytoplasm in AD and AI stages while in the AA stage there was some nuclear staining and high expression (Fig 3.6) As expected, based on cell culture showing elevated Sestrin-1 expression in the absence of androgen, xenografts from castrated animals had increased expression of Sestrin-1 when the expression of PSA decreased. Importantly, in the AI stage of CaP, as indicated by the re-expression of PSA, the level of Sestrin-1 was suppressed and comparable to levels measured in the presence of androgen. The expression of Sestrin-1 was inversely correlated to expression of PSA\textit{ in vivo} and its expression was consistent with the suggestion of re-activation of the AR in castrate-resistant disease. These data are also consistent with Sestrin-1 being a potential tumor suppressor by showing increased expression during the temporary reduced growth with castration followed by decreased expression when the disease becomes AI and begins to grow again in the absence of testicular androgens.

3.4 Discussion

AR is suspected to be an important player involved in the AI stage of CaP [19, 24-38]. It is thought to promote tumor growth through AR-mediated transcription of growth stimulating genes regardless of the presence of testicular androgen. Alternatively, AR can potentially repress transcription of tumor suppressor genes as shown here. Tumors can arise from the net effect of both activation of oncogenes and inactivation of tumor suppressor genes. It is therefore critical to identify genes that are induced, as well as suppressed, by AR-dependent mechanisms.

Transcription of SESN1 is regulated by p53. Levels of SESN1 mRNA is decreased by androgen in prostate cancer cells [16]. The present studies investigated the mechanisms by which SESN1 was negatively regulated by androgen and revealed the
following: (1) SESN1 T2 was the predominant transcript suppressed by androgen; (2) levels of SESN1 protein were decreased by androgen; (3) androgen inhibited SESN1-T2 promoter activity by a mechanism that was dependent on the AR; (4) AR interacted with the SESN1 promoter upon androgen stimulation; (5) SESN1-ARE was required for androgen to inhibit SESN1-T2 promoter activity; (6) knockdown of SESN1 increased proliferation of CaP cells; and (7) \textit{in vivo}, expression of Sestrin-1 was initially induced by castration, but later was suppressed when prostate cancer became androgen-independent.

The \textit{SESN1} gene has mutually exclusive splicing of exons 1, 2 and 3 to 4 that is associated with the expression of three alternative transcript isoforms that predict the encoding proteins to have differing N-terminal domains \cite{15}. Tumor suppressor protein p53 selectively induces only two of the three transcripts. Transcript SESN1-T1 (+exon 1) is not induced by p53, whereas both SESN1-T2 (+exon 2) and SESN1-T3 (+exon 3) are highly induced by p53 \cite{15}. Here, the SESN1-T2 transcript was shown to be predominantly expressed in LNCaP prostate cancer cells and its expression was regulated by androgen. At the protein level, T2 was the only SESN1 isoform detected and it too was suppressed by androgen. Levels of SESN1-T2 protein followed the similar trend as the levels of SESN1 T2 transcript in response to androgen. The protein level of SESN1-T2 decreased only after 16h of treatment most likely due to the stability of the protein. These data provide the first line of evidence of differential levels of expression of SESN1 transcripts in response to androgen in CaP cells that express AR.

Nuclear hormone receptors can activate or repress the transcription of their target genes \cite{39}. Upon ligand-binding, the AR would bind to \textit{cis}-acting AREs located in the promoter and/or enhancer regions of specific target genes and serve to activate or to repress transcription \cite{1-3}. Application of a reporter gene construct of the SESN1-T2 promoter in PC3 prostate cancer cells that do not express AR, confirmed that AR was required to block transcription of SESN1-T2. Deletion mapping of the SESN1-T2 promoter revealed that the region between bp -247 and -214 of the promoter was required for inhibition by androgen. Within this region, at bp -230 to -216 of the SESN1-T2 promoter there was a predicted ARE with the sequence AGCCCAttcCGTACC. AR was found to physically interact within this region as shown by ChIP assays. These data suggest that AR represses the expression of SESN1-T2 by interaction with a functional
ARE at bp -230 to -216 in the SESN1-T2 promoter. Alternatively, AR could interact with p53 bound to a p53 site in this region to repress transcription. This is currently being examined.

Indirect interaction between p53 and AR pathways to decrease AR transcription has been previously reported [40, 41]. Expression of SESN1 is induced by p53 through a p53-binding site located in the second intron of SESN1 [15]. Here, we suggest that a functional p53-binding site is present in the SESN1-T2 promoter (-969 to +18) based upon the strong induction of SESN1-T2-969 luciferase activity with forced expression of p53 in cells that lack endogenous p53. AR can inhibit p53 activity and nuclear accumulation [17] while anti-androgens can enhance the activation of p53-mediated transcription [18]. Expression of AR significantly repressed SESN1-T2-969 promoter activity induced by p53 regardless of androgen, although R1881 afforded some additional suppression. These results are consistent with AR antagonizing p53-mediated induction of SESN1 expression. Another similar example is the p53 target gene Maspin which also has an ARE (GTACTCtgaTCTCC) (from bp -399 to -386) [4] and two p53 binding sites: GAACATGTTGGAGGCCTTTTG (from bp -224 to -204) and GGACAAGCTGCAAGGCTTGAGT (from bp -153 to -129) in its promoter region [42].

Although the mechanisms of hormonal progression of CaP are currently unclear, it is believed that androgen-independent tumor cells are still dependent on AR [43]. Multiple lines of evidence have shown that the AR may be transcriptionally reactivated in androgen-independent CaP by crosstalk with other pathways or by residual low concentrations of androgen [19, 35, 44-50]. Consistent with re-activation of the AR in androgen-independent tumors, SESN1 expression was re-suppressed in the androgen independent stage of the xenograft CaP model. SESN1 is a potential tumor suppressor gene located at 6q12. Consistent with this gene being a potential tumor suppressor, there is frequent loss of chromosome 6q in prostate cancer [51, 52]. The loss of 6q is suggested to be a late event that is found in 39% of prostate cancer metastases or a 5-fold increased risk of loss of heterozygosity (LOH) in non-organ confined prostate cancer compared to organ confined disease [51-53]. Loss of 6q also had a higher frequency (40%) in recurrent CaP as compared with 22% in primary CaP samples [51]. Thus decreased levels
of expression of SESN1 in androgen-independent tumors would potentially correlate to increased tumor growth. Here, we show that knockdown of SESN1 by siRNA significantly increased the proliferation of LNCaP cells. Taken together, these data imply that loss of SESN1 by either LOH or through AR-mediated repression may play a role in androgen independent tumor growth.
Figure 3.1. AR signaling pathway negatively regulates SESN1 expression in prostate cancer cells. A, SESN1 transcript isoforms expressed in LNCaP cells and differential inhibition by R1881. LNCaP cells treated for 16 h in the presence or absence of 10 nM R1881. Oligo-d(T)-primed total RNAs (0.5 µg per sample) were reverse-transcribed followed by real-time PCR for SESN1 individual transcripts expression. Levels of SESN1 mRNA were normalized to levels of GAPDH mRNA. B, R1881 dose-dependent inhibition of levels of SESN1-T2 transcript. Total RNA was isolated from LNCaP cells in treated with concentrations of R1881 ranging from 0.01 to 100 nM as indicated and analyzed by Q-PCR for levels of SESN1-T2 and PSA mRNAs. Related expressions are calculated compared to the vehicle control (ethanol). C, Time course study of R1881 inhibition of levels of SESN1-T2 transcript. Total RNA that was isolated from LNCaP cells treated with 10 nM R1881 from 1 h to 37 h as indicated were analyzed for levels of SESN1-T2 mRNA by Q-PCR. Related expressions are calculated compared to the control (before treatment). D, Time course study of R1881 inhibition of levels of SESN1 protein in LNCaP cells. Cells were treated with R1881 (10 nM) or Ethanol (Eth) as a control for 3h, 8h and 16h before the whole cell lysates were harvested and analyzed by western blot using antibodies to SESN1 (SESN1) and β-actin (loading control). 20 µg of protein were loaded in each lane. (T1/T2/T3: transcript 1/2/3; *: p<0.05, **: p<0.01, ***: p<0.001.)
Figure 3.2. Induction of SESN1 promoter activity by p53 is inhibited by AR. PC3 cells were transfected with pSESN1-T2-968 (2.5 ug/well), an expression plasmid for p53 (0.25 ug/well), and an expression vector for AR (0.25 ug/well) and treated with or without R1881 (10 nM) for 24 hours before harvesting and measuring luciferase activities. Bars represent the mean relative luminescent units/min/mg of protein ± S.D of 3 separate experiments, each determined with triplicate dishes. (*: p<0.05, **: p<0.01, ***: p<0.001.)
Figure 3.3. AR negatively regulates SESN1 promoter activity. *Left*, schematic representation of SESN1-T2 promoter-deletion constructs. *Right*, relative luciferase activities of SESN1-T2 promoter-deletion constructs. SESN1-T2 promoter-deletion constructs (3 μg/well) were transiently transfected into LNCaP cells. Twenty four hours after transfection, cells were treated with R1881 (10 nM) for an additional 48 h before harvesting and measuring luciferase activities. Bars represent the mean relative luminescent units/min/mg of protein ± S.D of 3 separate experiments, each determined with triplicate dishes. (**: p<0.01, ***: p<0.001.)
Figure 3.4. AR binds to the SESN1 promoter upon androgen stimulation. LNCaP cells were treated with 10 nM R1881 or ethanol for 0, 0.5, 3, or 8 h as indicated. DNA was isolated from 3.5x10^6 cells for each group and immunoprecipitated with an antibody against the AR or nonspecific mouse IgG as a negative control. Using immunoprecipitated DNA as a template, the putative AR binding region at -234 to -214 of the SESN1-T2 proximal promoter was amplified by Q-PCR. Q-PCR using unimmunoprecipitated (input) DNA was performed to monitor amplification efficiency of the primer pair. Percentage input was calculated from dividing the arbitrary Q-PCR numbers obtained by each sample by that of the input. Related binding was calculated from dividing the percentage input of each sample to that of the ethanol treated control sample. Bars represent the mean relative binding ± S.D of 3 separate experiments, each determined with triplicate dishes. (*: p<0.05.)
Figure 3.5. Knockdown of SESN1 promoted the proliferation of prostate cancer cells. A, Knockdown of expression of SESN1-T2 by siRNA. LNCaP cells were transfected with control-siRNA or SESN1-siRNA. Total RNA and protein lysates were analyzed for expression of SESN1-T2 transcript by Q-PCR (top panel) or for protein by western blot analysis with the anti-SESN1 antibody or an anti-β-actin antibody (bottom panel) respectively. Top panel, bars represent the mean fold-change of SESN1-T2 transcript normalized to β-actin over control levels ± S.D. from three separate experiments. B, Proliferation assay of LNCaP cells with SESN1 knockdown in presence or absence of androgen. LNCaP cells were incubated with siRNAs for 12 h before treatment with 0.1nM R1881 or ethanol (Eth) as a control for additional 0, 24, 48 and 72 h. The cell proliferation was assessed by incubating with 20ul of BrdU for 2 h at 37°C. 150 µl of media were removed and the plates were dried down in a Hybrid oven at 60°C for 2 h. The Roche BrdU proliferation kit was applied and ELISA data was acquired at 30 min post substrate addition. The proliferation rates were represented by the reading of BrdU incorporation of each sample. (*: p<0.05, **: p<0.01, ***: p<0.001. Error bars: standard error from six replicates.)
Figure 3.6. Expression of Sestrin-1 in the hormonal progression of CaP. LNCaP subcutaneous xenografts were established in SCID mice. Eight weeks after tumor cell injection, half of the mice were castrated. At different tumor stages, the grafts were harvested, measured, and fixed for immunohistochemical analyses. Tumor tissues were stained with PSA (A, C and E), or Sestrin1 (B, D and F) antibody. AD: androgen dependent from intact mice immediately prior castration; AA: androgen ablation at the PSA nadir at 1 weeks after castration; AI: androgen independent when serum PSA became elevated over the nadir at 5 weeks after castration. (Insets, high magnification images)
3.5 References


Crosstalk between the Androgen Receptor and β-Catenin in Androgen-Independent Prostate Cancer

4.1 Introduction

Androgen ablation is currently the most effective systematic therapy available for prostate cancer patients with metastatic disease. However, this therapy is palliative and after an initial response to androgen ablation, most tumors eventually begin to grow in the absence of testicular androgens to form androgen independent disease (also called hormonal refractory or castration-resistant) [1]. Molecular mechanisms underlying hormonal progression to the androgen independent stage remains unknown. One mechanism suspected to play a role is related to the transcriptional activity of the androgen receptor (AR). The AR is a ligand-dependent transcription factor that is a member of the steroid receptor family. Ligand-activated AR, complexed with coactivator proteins and general transcription factors, bind to androgen response elements (AREs) located in the promoter and enhancer regions to activate or repress the transcription of specific target genes suspected to be involved in proliferation.

One important coregulator of the AR is β-catenin which interacts with the AR in response to androgen to increase the transcriptional activity of the AR in cells maintained as a monolayer [2, 3]. Levels of β-catenin and AR are both increased in androgen-independent prostate cancer. Mutated forms of β-catenin which can result in a stabilized protein have also been detected in prostate cancer [4, 5]. β-catenin has dual functions that involve both cell adhesion and signal transduction in response to Wnt ligands [6, 7]. The cellular localization of β-catenin is important for its function. Consistent with its role in cell adhesion, β-catenin is predominantly detected at the cell membrane where it interacts with E-cadherin and α-catenin [8]. In response to Wnt ligands, β-catenin can also be detected in the nucleus and cytoplasm where it can interact with TCF/LEF transcription factors to initiate transcription of target genes such as c-myc and cyclin D1 [9-11]. β-catenin activity is regulated by phosphorylation followed by degradation. This process involves interaction with GSK3β, axin, and adenomatous polyposis coli (APC). Phosphorylation of β-catenin by CK1ε and GSK3β on serine and threonine residues target it for degradation by the ubiquitin proteasome pathway [12]. Degradation of β-catenin is inhibited by

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Wnt signaling which results in elevation of levels of β-catenin in the nucleus and interaction with Tcf/Lef transcription factors to regulate expression of target genes [9, 13].

Crosstalk between Wnt and AR pathways occurs at several levels: 1) Wnt ligands can transactivate the AR [14]; 2) β-catenin interacts with the AR in nuclei to increase its transcriptional activity as measured by androgen-induced reporter gene constructs [2, 3, 15-17]; 3) GSK3β negatively regulates AR-mediated transcription in nuclei [18-20]; 4) competition for β-catenin can occur between AR and Tcf/Lef [21]; and 5) the Tcf/Lef target gene, cyclin D1, can interact with the AR in nuclei to inhibit AR transcriptional activity [22-24].

In the present study, we applied Affymetrix GeneChip (Human Genome U133 plus 2) combined with the LNCaP xenograft and Hollow Fiber models to identify global changes in gene expression associated with hormonal progression of prostate cancer to androgen independence. One pathway identified to be activated in androgen independent samples was the Wnt/β-catenin signaling pathway. Colocalization and interaction of AR and β-catenin were detected for the first time in vivo in androgen-independent tumors, but surprisingly not from tumors harvested from non-castrated mice. These data suggest a role for β-catenin interaction with the AR in the progression of prostate cancer to the terminal androgen-independent stage.

4.2 Materials and Methods

4.2.1 Hollow Fiber and subcutaneous xenograft models of prostate cancer

The LNCaP Hollow Fiber model of prostate cancer was performed as described previously [25, 26]. Subcutaneous xenografts were prepared in male SCID mice inoculated with LNCaP cells suspended in 75 µl of RPMI 1640 (5% FBS) with 75 µl of Matrigel (Becton Dickinson Labware) in the flank region using a 27 gauge needle. Tumor volumes of xenografts were measured weekly and calculated by the formula LxWxHx0.5236. Serum PSA levels were measured weekly as reported previously [25, 26].

4.2.2 RNA isolation and microarray analysis

RNA was isolated and analyzed using microarrays as described previously [26]. Briefly, total RNA was extracted from cells using Trizol (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. RNA samples from cells were analyzed by Affymetrix Genechip microarray. The synthesizes of cDNA and biotinylated cRNA were performed according
to the protocols provided by the manufacturer (Affymetrix, Santa Clara, CA). Biotinylated fragmented cRNA probes were hybridized to the HGU133 plus2 Genechips (Affymetrix). Hybridization was performed at 45°C for 16h in a hybridization oven (Affymetrix). The Genechips were automatically washed and stained with streptavidin–phycoerythrin conjugate in an Affymetrix Genechip Fluidics Station. Fluorescence intensities were scanned with a GeneArray Scanner (Affymetrix). Hybridizations were carried out independently for each condition using 3 biological replicates.

4.2.3 Expression profile analysis

Comparative analyses between expression profiles for Affymetrix experiments were carried out using GeneSpring™ software version 7.2 (Silicon Genetics, CA, USA). GeneSpring™ software is an analytical workbench enabling scientists to visualize and manipulate gene expression data. Experimental data from microarrays, Affymetrix chips, SAGE, or any technique that associates numbers with genes can easily be imported for rigorous analysis. The expression profiles from three animals were compared using two-way ANOVA to identify genes that were differentially expressed across the three groups. The dataset, GEO GDS1390, for clinical samples of androgen-dependent and androgen-independent prostate cancer was downloaded from PUBMED and student’s T-test was applied to identify the differentially expressed genes with p-values lower than 0.001. For sample clustering, standard correlation was applied to measure the similarity of the expression pattern between different samples. Class prediction was performed to calculate the similarity of the samples from the LNCaP hollow fiber model to the clinical samples of androgen-independent prostate cancer.

4.2.4 Quantitative RT–PCR (qRT–PCR)

Oligo-d(T)-primed total RNAs (0.5 µg per sample) were reverse-transcribed with SuperScript III (Invitrogen Life Technologies, Carlsbad, CA, USA). An appropriate dilution of cDNA and gene-specific primers were combined with SYBR Green Supermix (Invitrogen) and amplified in ABI 7900 real-time PCR machine (Applied Biosystems, Foster City, CA, USA). All qPCR reactions were performed in triplicate. The threshold cycle number (Ct) and expression values with standard deviations were calculated in Excel. Primer sequences for real-time PCRs are listed in Table 4.1. Real-time amplification was performed with initial denaturation at 95°C for 2 min, followed by 40 cycles of two-step amplification (95°C for 15 sec, 55°C for 30 sec).
4.2.5 Whole-cell lysates and western blot analyses

Protein from whole-cell lysate was isolated using Trizol according to the manufacturer's protocol. Samples were stored at -80°C until use. The antibodies used in these studies were obtained from various suppliers: AR (PG21, Upstate Biotechnology, Lake Placid, NY, USA); PSA (C-19, Santa Cruz, Santa Cruz, CA, USA); β-catenin (Cell Signaling, Danvers, MA, USA); phospho-Y142 β-catenin (Abcam, Cambridge, MA, USA); and β-actin (Abcam).

4.2.6 Immunoprecipitation

Protein was extracted from LNCaP cells or xenograft tumor samples using Triton X-100 lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris HCl). The cell lysates of the xenografts were stripped of albumin by using Montage Albumin Deplete Kit (Millipore, Billerica, MA, USA). A total of 250 µg of cell lysate per immunoprecipitation were used to immunoprecipitate AR using anti-AR antibody (PG21, Upstate Biotechnology, Lake Placid, NY, USA) or β-catenin using an anti-β-catenin antibody (Cell Signaling, Danvers, MA, USA) with µMACS protein G Microbeads (Miltenyi Biotec, Auburn, CA, USA). Proteins were detected by western blot analysis using anti-AR and anti-β-catenin antibodies.

4.2.7 Double immunofluorescent microscopy

Tissue sections (5 µm) were blocked in immunohistochemistry solution (Immunovision Technologies, Brisbane, CA) and immunostained with anti-AR antibody (AR441, Santa Cruz, CA, USA) (1:10) at 4º C overnight, then anti-β-catenin antibody (Cell Signaling, Danvers, MA, USA) (1:20) at 4º C overnight. For immunofluorescence staining, sections were incubated with fluorescein isothiocyanate (FITC)-labelled anti-rabbit IgG for β-catenin and tetramethylrhodamine isothiocyanate (TRITC)-labeled anti-mouse IgG for AR (both 1:200) for 30 min. The immunofluorescence staining of proteins was detected using a Zeiss Axioplan-2 Fluorescence Microscope (Zeiss, Toronto, ON, Canada).
4.3 Results

4.3.1 The LNCaP hollow fiber model correlates to clinical hormonal progression of prostate cancer

The LNCaP hollow fiber model provides a reproducible method to obtain human prostate cancer cells that are free from host contamination from in vivo studies [25]. A strength of this model is that it allows analyses of matched samples from the same animal at different time points due to the noninvasive procedures to harvest the fibers. Here we apply this model to obtain protein and RNA from LNCaP cells harvested from castrated mice during the different stages of progression to assess changes in gene expression and identify pathways associated with androgen independent growth. Consistent with previous reports, LNCaP cells maintained in hollow fibers progressed to androgen independence after castration of the host as indicated by levels of serum PSA (Fig 4.1A). The removal of fibers from the animals caused further reduction in serum PSA due to the removal of tumor cells. Serum PSA levels are correlated to tumor burden [27]. At least a three-fold increase in serum PSA over the nadir level was still observed in spite of removal of fibers to indicate androgen-independent re-expression of PSA at 31 days after castration. Analyses of levels of PSA mRNA (Fig 4.1B) and PSA protein (Fig 4.1C) from LNCaP cells harvested from fibers immediately prior to castration (intact or androgen dependent, AD), after castration at day 10 (androgen ablation or AA), or 31 days after castration when serum PSA becomes elevated again (androgen-independent or AI) were consistent with hormonal progression in response to castration. PSA mRNA and protein were reduced by approximately 80% in response to castration (AA) as compared to levels from samples harvested from intact mice (AD). Levels of expression of PSA mRNA and protein returned to pre-castrate levels in androgen-independent samples (compare levels in AD to AI in Figs 4.1B&C). These samples were next used for Affymetrix analysis of changes in the transcriptome during hormonal progression and expression profiles compared to Affymetrix data available for clinical samples of prostate cancer.

To confirm the similarity of the androgen-independent samples harvested from the hollow fiber model to clinical samples, we screened a group of the most significantly differentially expressed genes in the clinical samples composed of both androgen-dependent and androgen-independent prostate cancers [28]. There were 79 genes identified to be significantly
differentially expressed between androgen-independent versus androgen-dependent clinical samples with the p-value lower than 0.001 (Table 4.2). A hierarchical two-dimensional clustering algorithm based on similarity of expression patterns was applied to these 79 genes. Comparison of expression profiles demonstrated a tendency to subgroup the androgen-independent samples from the fiber model together with the clinical androgen-independent prostate cancers, while the androgen-dependent from the fiber model clustered together with the clinical androgen-dependent prostate cancers (Fig. 4.1D). The class prediction analysis revealed the androgen ablation and androgen-independent samples from the fiber model have positive similarity scores to the clinical androgen-independent prostate cancers, but androgen-independent samples have significant more similarity scores than the androgen ablation samples (p<0.05) (Fig. 4.1E). These data provide evidence that the LNCaP hollow fiber model is a relevant model to study hormonal progression of prostate cancer.

4.3.2 Global gene expression profiles of hormonal progression of prostate cancer

After confirmation of similarity between the hollow fiber model and the clinical samples at the transcriptome level, significance analysis of microarrays [29] was applied to identify genes differentially expressed across the different stages of hormonal progression. Using a two-way analysis of variance, 5667 genes were measured as differentially expressed with p-values lower than 0.05 across the various time points (Fig. 4.2). These 5667 genes were further clustered by standard correlation according to the expression pattern during the hormonal progression to androgen-independence Cluster A (represented by CTNNBIP, a regulator of β-catenin) included 814 genes whose expression increased in androgen-independence. Cluster B (represented by OSR2, β-catenin (CTNNB1), and AR) included 1143 genes whose expression increased after castration, and further increased or remained elevated in androgen-independence. Cluster C (represented by MMP16 and Wnt5A) included 489 genes whose expression increased after castration, but decreased in androgen-independence. Cluster D (represented by androgen regulated genes Dlc2 and FKBP5, TP53, GSK3B, LEF1 and TCF3) included 1540 genes whose expression decreased in response to castration and remained decreased in androgen-independence. Cluster E (represented by CSNK2B, CSNK1E, CCND1, MYC, and PLCB4) included 1226 genes whose expression decreased only when the tumor progressed to androgen-independence. Cluster F (represented by androgen regulated genes KLK3, KLK2, ELL2, SOCS2
and RHOU) included 455 genes whose expression decreased in response to castration, but increased in androgen-independence.

4.3.3 Reactivation of the AR pathway in androgen independent prostate cancer

Hormonal progression of prostate cancer has been suggested to be at least partially due to the reactivation of AR after androgen ablation therapy [30]. To characterize the status of the AR pathway in androgen-independent prostate cancer, a hierarchical two-dimensional clustering algorithm that was based on similarity of expression patterns was applied to 1092 genes previously identified to be differentially expressed in response to androgen in LNCaP cells maintained in cell culture [26]. The genes are represented on each row (Fig 4.3) and experimental samples are represented on each column. Comparison of expression profiles demonstrated a strong tendency to subgroup the samples with respect to the stage of the tumors. This suggested that distinct and consistent differences in the levels of transcript occurred during hormonal progression. Of particular interest, hierarchical cluster analysis revealed that the expression patterns of androgen-regulated genes in androgen-independent samples were more similar to that of the androgen-dependent than to androgen ablation samples. These data are consistent with re-expression of some androgen-regulated genes in androgen-independent prostate cancer xenografts obtained from castrated hosts as previously observed [31].

4.3.4 Increased expression of AR and β-catenin in androgen-independent prostate cancer

Genes differentially expressed in androgen-independent prostate cancer represented many biological categories; however, several of these genes have functional attributes that could contribute to castration-resistant growth. The most notable were the marked increase in levels of mRNA for AR (p=0.04) (Fig 4.4A) and β-catenin (p=2.45E-05) (Fig 4.4B) in the androgen-independent samples measured by both Affymetrix and Q-PCR. Western blot analysis confirmed increased levels of AR (Fig 4.4C) and β-catenin (Fig 4.4D) proteins in androgen-independent prostate cancer samples. These data are consistent with clinical data describing elevated expression of AR and β-catenin in hormone refractory prostate cancer.

Cytoplasmic or nuclear localization of β-catenin is required to mediate Wnt signaling. For β-catenin to accumulate in the cytoplasm, it requires phosphorylation on its tyrosine residue (Y142) to decrease interaction with α-catenin [12, 32, 33]. Therefore levels of phosphorylated β-catenin were measured during the different stages of hormonal progression by western blot
analyses with an antibody against phospho-Y142 of β-catenin. No phosphorylation of Y142 could be detected in samples from intact mice (Fig 4.4E). Androgen ablation increased phosphorylation of Y142-β-catenin to detectable levels which were further increased with androgen-independence. These data imply that the cellular localization of β-catenin may be altered with hormonal progression of prostate cancer to the cytoplasmic or nuclear form to mediate signal transduction.

4.3.5 Expression profile of members of the Wnt pathway in androgen independent prostate cancer

β-catenin is the mediator of Wnt signal transduction pathway and its activity is regulated by many Wnt pathway members. Wnt pathway members were enriched (78 out of 180) in the list of differentially expressed gene during hormonal progression (5667 out of 55023 for all genes in the Affymetrix Genechip). To investigate the status of the Wnt pathway in the hormonal progression of prostate cancer, we validated changes in the expression of some members of Wnt pathway during the prostate cancer progression as first indicated by Affymetrix data. Consistent with increased levels of Y142 phospho-β-catenin in androgen-independent prostate cancer, expression of tyrosine kinase-associated YES1 and LYN that phosphorylate β-catenin [33] were increased with androgen independence (Fig. 4.4F). The β-catenin activator WNT2B was also significantly increased with androgen independence. Consistent with activation of the β-catenin pathway in androgen independent samples, the expression of β-catenin inhibitors such as CSNK2B, CSNK1E, GSK3B, TP53, WNT5A and PLCB4 were decreased (Fig. 4.4F). The β-catenin downstream transcription factors, TCF3 and LEF, and expression of their target genes, MYC and CCND1, were significantly decreased, while expression of the transcription inhibitor, CTNNBIP1, was increased in androgen-independent prostate cancer. Together these data are consistent with the hypothesis of an increased cytoplasmic pool of β-catenin without increased activity of its downstream transcription factors (TCF and LEF).

4.3.6 Increased colocalization of AR and β-catenin in androgen-independent prostate cancer

Activation of the Wnt pathway should induce the accumulation of β-catenin in cytoplasm which would be expected to facilitate the activity of downstream transcription factors. While we expect to see increased cytoplasmic or nuclear accumulation of β-catenin based upon elevated
levels of phospho-Y142-β-catenin, we in fact measured a decrease in its downstream transcription factors and target genes in androgen independent prostate cancer. One possible mechanism for why no concomitant increases in TCF/LEF target genes were measured may involve competition for β-catenin with the AR [21]. To further explore for possible crosstalk between β-catenin and AR in androgen-independent prostate cancer, we investigated the colocalization of AR and β-catenin by fluorescent microscopy using xenografts harvested before and after castration. Tissue sections were prepared from xenografts harvested the day of castration, at the PSA nadir, and when samples were androgen-independent and stained using antibodies against AR and β-catenin. The staining pattern for AR in xenografts harvested from non-castrated mice was strongly nuclear as expected, while β-catenin staining was predominately at cell borders (Fig. 4.5A). After castration, the AR was diffuse in the cytoplasm with little detected within the nucleus, while β-catenin was still mainly at the cell borders with little cytoplasm diffusion and low colocalization with AR. Androgen-independent xenografts from castrated mice, showed nuclear localization of β-catenin in cells that predominately co-localized with AR (arrowheads). Levels of β-catenin at cell borders were not observed to change in different stages of prostate cancer, whereas cytoplasmic levels increased with progression. These data suggest that colocalization of AR and β-catenin only occurs in vivo in androgen-independent prostate cancer and contradictory to in vitro data, not in the presence of androgen in non-castrated mice.

4.3.7 Increased interaction between AR and β-catenin in androgen-independent prostate cancer

In cell culture, β-catenin interacts with the AR and acts as a coactivator to increase the transcriptional activity of the AR in response to androgen [2, 3, 15-17]. To date all studies have examined these interactions using cells maintained as a monolayer with no in vivo reports. Since β-catenin interacts with molecules involved in cell adherence, it would seem that cells maintained in a monolayer may not provide the optimal model to predict what may occur in a tumor or spheroid structure. Thus here we provide the first in vivo study of interaction between AR and β-catenin. In addition this is also the first study to attempt to show changes in this interaction during hormonal progression; something that cannot be accurately mimicked in vitro. To do this, endogenous complexes of AR and β-catenin were co-immunoprecipitated from
LNCaP xenografts at various stages of hormonal progression. Xenografts were harvested at the day of castration (intact mice), at the PSA nadir (castrated), and when androgen-independent, and proteins immunoprecipitated using antibodies directed to β-catenin or AR. The immunoprecipitated complexes were probed by western blot analyses. Consistent with the lack of colocalization in the presence of androgen in non-castrated mice (Fig 4.5A), no interaction between AR and β-catenin was detected in the presence of androgen (Fig 4.5B). Importantly these data showed increased levels of endogenous AR/β-catenin complex from in vivo samples harvested from androgen-independent prostate cancer, compared to androgen-dependent and androgen ablation tissues (Fig. 4.5B). These data are in agreement with the colocalization studies shown in Fig 4.5A and together they suggest that AR interacts with β-catenin in androgen-independent prostate cancer.

4.4 Discussion

The Wnt/β-catenin pathway contributes to prostate biology and pathology. The AR is suspected to play an important role in androgen-independent prostate cancer. These two pathways may cross-talk in androgen-independent prostate cancer. The present studies investigated genes differentially expressed during the hormonal progression of prostate cancer and revealed the following: 1) the LNCaP hollow fiber model correlated with clinical hormonal progression of prostate cancer; 2) hormonal progression of prostate cancer in the hollow fiber model was associated with differential expression of 5,667 genes; 3) the expression pattern of androgen-regulated genes in androgen-independent prostate cancer was more similar to that obtained before castration compared to the tumors receiving androgen ablation at PSA nadir; 4) expression of AR and β-catenin were increased in androgen-independent prostate cancer; 5) nuclear colocalization and protein-protein interaction between the endogenous AR and endogenous β-catenin were increased in vivo in androgen-independent prostate cancer; and 6) deregulation of the Wnt pathway in androgen-independent prostate cancer led to dissociation of β-catenin from the cell membrane but not to activation of its downstream Tcf/Lef transcription factors.
4.4.1 The LNCaP hollow fiber model correlates to clinical hormonal progression of prostate cancer

A number of animal models are available to investigate the mechanisms underlying the development and pathogenesis of the prostate cancer. We developed an *in vivo* model that encompasses the use of hollow fibers to obtain tumor cells that were free from contamination with host cells and to allow harvesting of multiple samples from an individual mouse at different stages of progression [25]. LNCaP cells seeded in fibers that were subcutaneously implanted into the mice provided measurable levels of serum PSA that decreased by 90% to a nadir after castration, and subsequently serum PSA increased within 4-5 weeks after castration signifying progression to androgen-independence (Fig. 4.1A). Data from this fiber model for both serum PSA and levels of PSA mRNA and protein were consistent with those data obtained with the LNCaP xenograft model [34]. Condition clustering and the class prediction analysis provided support that the hollow fiber model mimics clinical samples at the level of global transcription during hormonal progression (Fig. 4.1D). These data provide the first evidence of similarity between the LNCaP hollow fiber model to the clinical scenario at the transcriptome level for hormonal progression.

4.4.2 Reactivation of AR pathway in androgen-independent prostate cancer

PSA is an example of an androgen-regulated gene that contains several well-characterized androgen response elements (AREs) in the promoter and enhancer regions to which the AR binds to initiate transcription [35-37]. The re-expression of PSA suggests that the AR plays a role in androgen-independent disease. Affymetrix Genechip analysis identified changes in expression of 1,092 genes in response to androgen stimulation in LNCaP cells [26]. Here we clustered the fiber model samples according to the expression profile of these androgen-regulated genes to investigate the status of AR pathway during the hormonal progression of prostate cancer. Surprisingly, in spite of the similar androgen environment as the androgen ablation samples, the androgen-independent prostate cancer samples shared a more common expression profile of androgen-regulated genes with the androgen-dependent samples before castration (Fig. 4.3). Specific examples include the expression of androgen-regulated genes such as KLK3, KLK2, ELL2, SOCS2, and RHOU that were re-expressed in androgen-independent prostate cancer. Expression of genes that are known to be suppressed by androgen, such as
MMP16 [26], were re-suppressed in androgen-independent prostate cancer (Fig 4.2). Together these data suggest reactivation of the AR in androgen-independent disease as previously suggested [38-40]. One potential mechanism for activation of the AR in the absence of testicular androgen may involve changes in protein-protein interactions with the AR and coregulators in response to alternative signal transduction pathways [30].

β-catenin has been reported to be a relatively specific coactivator of the AR with only the vitamin D receptor of the steroid receptor family also showing some interaction [3]. These studies are based upon over-expression of β-catenin to increase the transcriptional activity of the AR measured by reporter gene constructs in response to androgen [2, 3]. Chromatin immunoprecipitation assays detected β-catenin recruitment to the PSA promoter [41]. In yeast cells exposed to dihydrotestosterone, repeat 6 of the armadillo repeats of β-catenin interacts with the AR ligand-binding domain [3]. Interaction between β-catenin and the AR is reported to be dependent on androgen due to enhanced interaction upon the addition of ligand [2, 3]. These previous reports are based upon studies using cells maintained in culture as a monolayer which may not reflect in vivo conditions especially when considering the importance of adhesion molecules in modulating the function of β-catenin. Curiously we did not detect interaction of β-catenin with the AR in vivo in xenografts harvested from non-castrated mice. Instead we detected interaction between β-catenin and AR with hormonal progression in castrated mice (Fig. 4.5B), which was consistent with colocalization studies (Fig. 4.5A). These data are the first to show in vivo interactions between the AR and β-catenin and highlight potential discrepancies when extrapolating from solely in vitro experiments. The importance of validating endogenous complexes in in vivo physiological or pathological conditions is based upon: 1) protein-protein interactions are dependent upon concentrations of the proteins as well as the levels of stimulation, and thus overexpression of a protein by transfection may lead to false-positives; 2) overexpression or forced expression of proteins may cause aberrant cellular localization and/or inappropriate timing of expression if the expression of proteins are “normally” dependent upon the cell cycle phase; 3) protein modifications required for interactions may vary in different cells or under different cellular conditions; 4) cell cultures cannot substitute the physiological milieu of in vivo conditions nor can cell cultures mimic accurately the hormonal progression that occurs in vivo in response to castration of the host; and 5) the three-dimensional architecture of the xenograft and potential effects on E-cadherin and other cell adhesion molecules may not be
accurately represented using cells maintained in monolayer. These points are especially important when investigating protein-protein interactions of the AR activated by non-androgenic pathways involving signal transduction pathways that may be not be accurately mimicked in vitro.

E-cadherin mediates cell-cell contact and regulates the levels of β-catenin localized in the cytoplasm with overexpression of E-cadherin causing redistribution of β-catenin to the cell membrane to reduce cytoplasmic and nuclear pools [3, 42]. Loss of E-cadherin in androgen-independent prostate cancer leads to increased levels of β-catenin in the cytoplasm [43-45]. Consistent with our results shown here, expressions of both AR and β-catenin have been reported to be elevated in androgen-independent prostate cancer [46, 47]. Potential mechanisms of how β-catenin is able to enhance the transcriptional activity of the AR include facilitating the movement of the AR to the nucleus in response to androgen [15], modifying ligand requirement of the AR to utilize androstenedione and estradiol as agonists [2], and interactions with GRIP1, CARM1, p300 and FHL2 (reviewed in [48]).

4.4.3 Activity of Wnt/ β-catenin pathway in androgen-independent prostate cancer

The Wnt pathway and its interaction with AR have been suspected to play important roles in prostate cancer [48-50]. In Fig 4.6 we illustrate changes in the expression levels of activators of β-catenin (Wnt2b, Yes1) that were increased (enlarged font size) while the inhibitors of β-catenin (Gsk3β, Ck1, Ck2, p53, Wnt5a and Plcβ) were decreased (reduced font size) in androgen-independent prostate cancer related to the androgen-dependent and/or androgen ablation samples as determined in these studies. These changes in expression would predict that β-catenin would dissociate from the cell membrane to facilitate its downstream transcription factors. However, expression of Tcf3 and Lef were also decreased. Competition for available β-catenin between Tcf3/Lef and AR has been suggested [21]. Increased levels of β-catenin combined with decreased Tcf3/Lef would provide more β-catenin protein to be recruited by AR. Together these data suggest an increased pool of β-catenin would be available for potential interaction with other transcription factors such as AR. Other players in Wnt pathway may influence the transactivation of AR. These include GSK-3β [18-20] and cyclin D1 [22-24] that interact with the AR to repress AR activity. Decreased expression of GSK-3β and cyclin D1 as
shown in this study could contribute the reactivation of AR pathway in androgen-independent prostate cancer.

Together these data provide a working model for a potential mechanism of progression to androgen-independent prostate cancer that involves aberrant expression of members of the Wnt pathway to promote the interaction between β-catenin and AR and thereby increase the transactivation of the AR to initiate transcription of genes normally regulated by androgen. Aberrant activation of the AR through the Wnt/β-catenin signaling pathway may play a role in the progression of prostate cancer to the androgen-independent state. Development of inhibitors that block protein-protein interactions between the AR and β-catenin may lead to viable therapies for this terminal stage of the disease.
Table 4.1  Primers used in the Q-RT-PCR

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Table 4.2  genes significantly expressed in androgen independent prostate cancer clinical samples with p-value<0.001

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Fig. 4.1. The LNCaP hollow fiber model mimics hormonal progression of clinical prostate cancer. A, Levels of serum PSA in at least three mice implanted with hollow fibers containing LNCaP cells after castration. B, Levels of PSA mRNA measured by Affymetrix (indicated by the line) and Q-PCR (columns) using total RNA harvested from hollow fibers from animals at day 0, day 10 and day 31. Fold-change is based upon levels of PSA mRNA normalized to levels of GAPDH mRNA relative to levels of samples collected at day 0 (AD) and set at 1-fold. Samples are matched from the same animal at the different time points. AD signifies total RNA samples collected from animals immediately prior to castration at day 0 during growth in the presence of...
androgen. AA signifies RNA samples collected from animals 10 days after castration during androgen ablation. AI represents RNA samples collected at day 31 after castration when PSA becomes re-expressed signifying androgen-independent growth. NE is the normalized expression. C, Western blot analysis of levels of PSA protein normalized to β-actin protein using whole cell lysates collected at the same time points as described above. Lower panel shows densitometry analysis of PSA protein normalized to β-actin using matched samples from at least 3 different mice and set at 1-fold using the samples at day 0 (AD). (D) Unsupervised clustering analysis of Affymetrix data using RNA samples collected from the hollow fiber model (n=9) with the clinical samples of prostate cancer (n=20) according to the 79 genes identified to be most significantly differentially expressed in androgen-independent versus androgen-dependent clinical samples, by standard correlation. The genes are represented by each row and the experimental samples are represented on each column. 10R/11L/12R are the identities of the 3 mice bearing fibers containing LNCaP cells. The suffix (AD, AA, or AI) represent when the sample was collected from the animal as described above. The red box highlights the cluster for AI samples (clinical and from the fiber model). E, Class prediction analysis to calculate the similarity of the fiber model samples to the clinical androgen-independent prostate cancer samples according to the expression pattern of the same group of genes depending of stage of hormonal progression (e.g, AD, AA, AI). Y-axis represents the margin scores to the androgen-independent clinical samples. Bars represent the mean ± standard deviation (SD).
Fig. 4.2. Genes differentially expressed during the hormonal progression of prostate cancer in the hollow fiber model. Unsupervised clustering analyses of the 5667 genes according to their expression pattern during hormonal progression by standard correlation using the GeneSpring microarray analysis software. The genes are represented by each row and the experimental samples are represented on each column (in biological triplicates). Two-way ANOVA analysis of genes differentially expressed across all stages of progression (p≤0.05). The arrows represent the expression patterns of each cluster during hormonal progression from AD, to AA with castration, and finally AI. For example, expression of CTNNBIP1 does not change from AD to AA, but increases when AI.
**Fig. 4.3. Cluster analysis of the status of the androgen pathway in hormonal progression in the hollow fiber model.** Unsupervised clustering analysis of samples harvested from the hollow fiber model (n=9) according to the 1092 genes identified to be androgen-regulated in a previous report [26], by standard correlation using the GeneSpring microarray analysis software. The genes are represented by each row and the experimental samples are represented on each column. 10R/11L/12R are the identities of the 3 mice bearing fibers containing LNCaP cells. The red box highlights the clustering of AD samples with AI samples.
Fig. 4.4. Expression of AR, β-catenin, and regulators of β-catenin in the hollow fiber model. 
A, Levels of AR mRNA measured by Affymetrix (indicated by the line) and Q-PCR (columns) using total RNA harvested from the hollow fibers from animals at day 0, day 10 and day 31. Fold-change is based upon levels of AR mRNA normalized to levels of GAPDH mRNA relative to levels of samples collected at day 0 (AD) and set at 1-fold. B, Levels of β-catenin mRNA in the fiber model measured by Affymetrix (line plot) and Q-PCR (column plot). C, Western blot analysis of levels of AR protein normalized to β-actin protein using whole cell lysates collected at the same time points as described above. Lower panel shows densitometry analysis of AR protein normalized to β-actin using matched samples from at least 3 different mice and set at 1-fold using the samples at day 0 (AD). D, Western blot analysis of levels of β-catenin protein using the same samples as described in C. E, Levels of Y142-phospho-β-catenin protein in these same samples. F, Levels of mRNAs of genes known to be involved in regulating β-catenin in samples harvested from the hollow fiber model measured by Affymetrix (line plot) and Q-PCR (column plot). AD, androgen dependent; AA, androgen ablation; AI androgen independent; left Y-axis is the fold-change detected by Q-PCR; right Y-axis is the normalized expression (N.E.) for Affymetrix data; the bars represent the mean ± SD.
Fig. 4.5. Co-localization and endogenous interaction of AR and β-catenin in xenografts during hormonal progression in castrated mice. A, LNCaP xenografts were harvested from intact mice (AD); from mice 7 days after castration (Cx) at the PSA nadir; and when serum PSA indicated androgen independence (AI) in castrated mice. Sections of xenografts were stained with antibodies against β-catenin (fluorescein isothiocyanate-labelled, green) together with antibodies against AR (tetramethylrhodamine isothiocyanate-labelled, red). Nuclear colocalization between AR and β-catenin (orange staining) is indicated by the white arrow. B, Co-immunoprecipitation of the endogenous complex of AR and β-catenin from xenografts harvested as described in A. Immunoprecipitations were performed using both anti-AR antibody (left) and anti-β-catenin antibody (right). Immune complexes were probed by western blot analyses as indicated in the figure.
Fig. 4.6. Crosstalk between β-catenin and the AR in androgen-independent prostate cancer.
The schematic shows differentially expression of members of the Wnt pathway in androgen-independent prostate cancer and the impact on AR transcriptional activity. See the text for a description.
4.5 References


5 Summary and future directions

The molecular mechanisms that contribute to the progression of CaP are not completely understood. The AR appears to play a significant role in this process. However, it is not apparent how the AR is activated in the absence of testicular androgens. Additionally, new markers of disease progression are required that distinguish disease subtypes associated with different prognoses and therapeutic responses. In this thesis, I have described the experiments that I performed to identify and characterize genes associated with the hormonal progression of CaP.

5.1 Crosstalk of AR and PKA pathway

The androgen-signaling pathway plays an important role in the prostate. Activation of the PKA pathway has also been shown to play a role in prostate biology and pathology. These two pathways cross-talk through the AR in androgen-deprived prostate cancer cells [1]. In this study, a global view at the level of transcription revealed the cellular response of androgen-responsive prostate cancer cells to stimulation of the androgen and PKA pathways and cross-talk between these pathways. This study provided the first expression profile of genes altered by activation of the PKA pathway in prostate cancer cells. Application of AR-siRNA revealed that the induction of members of the kallikrein family and repression of SESN1 by androgen and FSK required a functional AR. Genes differentially expressed in response to androgen and stimulation of the PKA pathway in vitro were also differentially expressed during hormonal progression in vivo.

Cross-talk between the AR and PKA signal transduction pathways has been studied in androgen-depleted human prostate cancer cells maintained in culture [1-5]. These studies have shown that anti-androgens can block PKA induction of PSA mRNA [1], androgen-responsive reporters [1-3], and increase AR-ARE complex formation in nuclear extracts from cells exposed to activators of PKA [1]. Combined with our current findings, it is conceivable that the PKA pathway is responsible for at least a subset of AICaP. The status of the PKA pathway in biopsy samples from patients could be evaluated by applying the transcriptional signature of CaP cells in response to PKA. This would confirm the involvement of the PKA pathway in the hormonal progression of CaP,
identify the proportion of patients with PKA-induced activation of AR and provide the basis of specific therapies.

To date, there are no studies that examine the \textit{in vivo} effects of activation of the PKA pathway on phosphorylation of the AR using appropriate levels of FSK. Although Gioeli et al. found increased phosphorylation of the AR at Ser-650 in response to 50 µM FSK, a PKA activator [6], this concentration of FSK inhibits activation of the AR in non-transfected cells, while FSK at a concentration of 1 µM concentration is optimal to induce PSA mRNA, nuclear translocation, activation of the AR and its DNA-binding activity [1, 7]. Therefore, it is still unknown as to whether the AR is a direct target of phosphorylation by PKA or an indirect target through phosphorylation of its interacting proteins. Further studies should be performed to examine the phosphorylation status of AR in response to appropriate concentrations of compounds and the changes in protein-protein interaction in response to stimulation of the PKA pathway. After characterizing the effects of PKA in AICaP on phosphorylation of the AR and its interacting proteins, viable therapies could be designed to specifically inhibit the activation of this pathway, block the interaction of the responsible protein or kinase with the AR or the specific PKA responsive phosphorylation site(s) of the AR.

5.2 Androgen suppresses the expression of SESN1

Androgen is suggested to promote tumor growth in androgen-responsive cells, through AR-mediated regulation of transcription of growth-stimulating genes [8]. Alternatively, we propose androgen, via activation of the AR, can repress the transcription of tumor-suppressor genes. Tumors can arise from the net effect of both activation of oncogenes and inactivation of tumor suppressor genes. It is therefore critical to identify both positive and negative regulators of tumor growth. Our finding that the AR represses the expression of SESN1 could shed new light on the proliferation of prostate cells in response to androgen. Androgen regulates the transcription of SESN1 in prostate cancer. We demonstrated that the AR binds to the promoter of the SESN1 gene after androgen stimulation to repress SESN1 promoter activity. \textit{In vivo}, the protein level of Sestrin-1 is in the opposite direction of PSA. Therefore, SESN1 could be a novel prognostic marker to monitor the progression of CaP in response to androgen ablation.
We anticipate levels of SESN1 to become elevated upon androgen ablation with levels declining due to the onset of androgen independent disease to increase proliferation. Our data demonstrates that the AR represses SESN1 transcription and suggests a direct regulatory role for SESN1. SESN1 is a member of the growth arrest and DNA damage-induced genes (GADD) family [9]. Expression of SESN1 mRNA is induced in response to serum starvation, and repressed by addition of serum to starved cells [9]. Whereas growth arrest is associated with induction of SESN1, growth stimulation is associated with repression of SESN1 expression, which is consistent with our experiment. Therefore, it is conceivable that inhibition of SESN1 expression through the reactivation of AR or via other mechanisms during hormonal progression might directly contribute to androgen independence and more aggressive phenotypes of CaP.

Before SESN1 could be applied as a novel therapeutic target, further functional studies should be done. Knock-out or knock-in animal models could be generated to address the global effect of SESN1 in vivo. For more mechanistic studies, proteomics can be applied to identify the proteins that interact with Sestrin-1. Smaller peptides of Sestrin-1 could be cloned and tested to identify the functional domains of this protein.

From a therapeutic point of view, reexpression of SESN1 in prostate tumors may offer hope for reversing the tumour phenotype. Here, we highlight the importance of the AR in the hormonal progression of CaP and provide the rational for therapeutic studies directed toward blocking the AR. Re-expression of SESN1 may be achieved by targeting both activation and repression modes. For primary tumors, it is likely that the activation is partially impaired, but the repression function is intact. Although, it will probably be difficult to restore transcription of SESN1 through activation of p53, it may be more feasible to block the repression mediated by the AR binding to the ARE. Treating tumors with ligands that block the binding of AR to the SESN1-ARE or with reagents that compete strongly for binding to the AR are possible methods of blocking ARE-mediated repression. Our discovery of AR-mediated repression of SESN1 offers another opportunity to increase the expression of SESN1 in prostate cancer, which may in turn reduce the aggressiveness of this disease. Furthermore, after mapping the functional domains of Sestrin-1, smaller peptides or molecules that mimic the function of Sestrin-1
can also be applied. Restoration of the expression or function of Sestrin-1 in cancer cells by the methods mentioned above could be a novel avenue for the treatment of CaP.

5.3 Crosstalk of AR and β-catenin and the reactivation of AR in the AICaP

The androgen-signaling pathway is important in the development of CaP. Re-expression of androgen-regulated genes, such as PSA, suggests that the AR also plays an important role in androgen-independent disease. Other evidence supporting the AR as a probable factor in the hormonal progression of prostate cancer has been reviewed recently [10]. In Chapter 2 of this thesis, application of Affymetrix Genechip analysis aided identification about nine hundred genes with their expression regulated by androgen [11]. In Chapter 4, we first confirmed the genomic similarity of the hollow fiber model of CaP to clinical samples and further clustered the fiber model samples according to the expression profile of these androgen-regulated genes to investigate the status of the AR pathway during the hormonal progression of CaP. The most striking finding is that the status of the AR pathway in the AICaP samples is more similar to that of the pre-castration samples than that of samples taken after castration. Consistent with this finding is the re-expression of androgen-regulated genes such as KLK3, KLK2, ELL2, SOCS2, and RHOU, as well as re-suppression of the androgen-suppressed gene MMP16 [11] in AICaP. These data support the reactivation of the AR in androgen-independent disease as previously suggested [12-14]. Re-activation of the AR in the absence of testicular androgens involves changes in multiple signal transduction pathways which have been reviewed recently [10]. Deregulation of these pathways may lead to altered protein-protein interactions between the AR and its coregulators.

Deregulation of the Wnt pathway and β-catenin has been shown to play a role in prostate biology and pathology. These two pathways may cross-talk through the AR in androgen-independent prostate cancer cells. The studies in Chapter 4 of this thesis investigated genes differentially expressed during the hormonal progression of CaP and indicated that deregulation of the Wnt pathway leads to the dissociation of β-catenin from the cell membrane but not to its downstream Tcf/Lef transcription factors. In addition to the up-regulation of both AR and β-catenin, there was increased nuclear colocalization and interaction of AR with β-catenin in AICaP. This is the first evidence of the
involvement of β-catenin in CaP in vivo. Importantly, we showed increased interaction between these two proteins in the androgen independent stage. ChIP experiments should be performed to measure recruitment of β-catenin with AR to the AREs of androgen-regulated genes, such as PSA. The mechanism of the shift in function and localization of β-catenin requires further study. From a therapeutic point of view, based on the mechanisms of the crosstalk between these two pathways, we should be able to block the activation of the AR through interaction with the Wnt/β-catenin pathway. The interventions can theoretically involve: 1) knockdown the expression of β-catenin; 2) highly expressing small peptides containing the tyrosine residues of β-catenin which competitively inhibit the phosphorylation of β-catenin at these sites and lead to its degradation; or 3) highly expressing the armadillo repeats of β-catenin or LBD of AR containing LxxLL motifs to competitively block the interaction between them; or 4) screen small molecules which can competitively or permanently block the interaction of AR and β-catenin.

5.4 Conclusions

In conclusion, identification of the transcriptional profile of CaP cells in response to androgen and stimulation of PKA revealed common genes targeted by these pathways. Deregulation of AR and Wnt/β-catenin pathways and cross-talk between the AR and PKA pathways were identified with CaP progression. Reactivation of the AR through the PKA and/or Wnt/β-catenin signaling pathway may be responsible for the progression of CaP to the androgen-independent state in a subset of patients. AR repressed the transcription of SESN1 to suggest a regulatory role for the AR as a transcriptional repressor of this tumor suppressor gene in CaP cells. Inhibition of SESN1 expression through the reactivation of AR or other mechanisms during the hormonal progression might directly contribute to androgen-independence and more aggressive phenotypes of CaP.
5.5 References


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
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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.

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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

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