INDUCTION OF NEURONAL APOPTOSIS INHIBITORY PROTEIN
EXPRESSION IN RESPONSE TO ANDROGEN DEPRIVATION BY NF-κB
IN PROSTATE CANCER CELLS

by

HELEN HOI-LUN CHIU

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

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

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Pathology)

THE UNIVERSITY OF BRITISH COLUMBIA

October 2007

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ABSTRACT

Androgen deprivation therapy is an efficacious treatment for advanced prostate cancer (CaP) by inducing apoptosis of prostate cells. Despite the initial effectiveness of this systemic therapy, the cancer will inevitably recur and progress to an androgen-independent stage. The molecular mechanism by which some CaP cells may bypass the cell death induced by androgen deprivation is unclear. Emerging studies have highlighted the role of the inhibitor of apoptosis protein (IAP) family members in conferring an enhanced ability of malignant cells to survive in conditions normally resulting in cell death. Therefore, we explored levels of expression of these anti-apoptotic proteins in CaP cells in response to androgen deprivation. Levels of neuronal apoptosis inhibitory protein (NAIP) mRNA were significantly increased in response to castration of hosts. The increase in NAIP mRNA levels in response to androgen deprivation was further confirmed in an in vitro system. Nuclear factor (NF)-κB, for which constitutive activity has been implicated in CaP, is suspected to play a role in the expression of IAPs. Using a NF-κB luciferase reporter construct, we demonstrated that the transcriptional activity of NF-κB was inhibited by androgen. In vitro, nuclear localization of NF-κB correlated with the DNA-binding activity of NF-κB as determined by electrophoretic mobility shift assay in human CaP cell lines with different androgen requirement and androgen receptor status. However, in vivo, the DNA-binding activity of NF-κB was independent from its protein levels in the nucleus. Importantly, elevated expression of NAIP correlated to the increased DNA-binding activity of NF-κB in vivo in response to castration of the hosts. To determine if the transcription of NAIP was directly regulated by NF-κB, subsequent characterization of three κB-like sites in the regulatory regions of the NAIP locus led us
to confirm the physiological relevance of the κB-like site within the second intron of the gene locus using chromatin immunoprecipitation assay. Our observations suggest that transcription of NAIP may be regulated by NF-κB via regulatory element(s) in the NAIP locus in response to androgen deprivation. Thus, this study underlines a plausible mechanism by which some CaP cells may acquire the ability to resist apoptosis in androgen-deprived conditions.
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<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>avidin: biotinylated enzyme complex</td>
</tr>
<tr>
<td>ADT</td>
<td>androgen deprivation therapy</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
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<tr>
<td>ARE</td>
<td>androgen response element</td>
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<tr>
<td>Bcl-2</td>
<td>B cell lymphoma-2</td>
</tr>
<tr>
<td>BIR</td>
<td>baculoviral IAP repeat</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>Brn-2</td>
<td>brain-2</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CARD</td>
<td>caspase recruitment domain</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>DRE</td>
<td>digital rectal examination</td>
</tr>
<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DU145</td>
<td>brain-derived metastatic prostate cancer cell line</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>HSP</td>
<td>heat-shock protein</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IkB</td>
<td>inhibitor of κB</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB kinase</td>
</tr>
<tr>
<td>LNCaP</td>
<td>lymph-node carcinoma of the prostate cell line</td>
</tr>
<tr>
<td>NAIP</td>
<td>neuronal apoptosis inhibitory protein</td>
</tr>
<tr>
<td>NCoR</td>
<td>nuclear receptor co-repressor</td>
</tr>
<tr>
<td>NEMO</td>
<td>nuclear factor-κB essential modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>NOD</td>
<td>nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>NOD-SCID</td>
<td>non-obese diabetes-severe combined immunodeficient</td>
</tr>
<tr>
<td>PAX-2</td>
<td>DNA paired box-2</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC3</td>
<td>bone metastatic prostate cancer cell line</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PIA</td>
<td>proliferative inflammatory atrophy</td>
</tr>
<tr>
<td>PIN</td>
<td>prostatic intra-epithelial neoplasia</td>
</tr>
<tr>
<td>PSA</td>
<td>prostate-specific antigen</td>
</tr>
<tr>
<td>qPCR</td>
<td>real-time quantitative PCR</td>
</tr>
<tr>
<td>RING finger</td>
<td>really interesting new gene zinc-finger</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RPMI</td>
<td>medium developed at Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription PCR</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>s.c.</td>
<td>subcutaneously</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SFM</td>
<td>serum-free medium</td>
</tr>
<tr>
<td>SMA</td>
<td>spinal muscular atrophy</td>
</tr>
<tr>
<td>SRC-1</td>
<td>steroid receptor co-activator 1</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>TNM</td>
<td>tumour-node-metastasis</td>
</tr>
<tr>
<td>UGE</td>
<td>urogenital sinus epithelium</td>
</tr>
<tr>
<td>UGM</td>
<td>urogenital sinus mesenchyme</td>
</tr>
<tr>
<td>UGS</td>
<td>urogenital sinus</td>
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ACKNOWLEDGEMENTS

There are many people I wish to thank for their support throughout the process of my research thesis. Firstly, I would like to thank my supervisor, Dr. Marianne Sadar, for giving me the invaluable opportunity to pursue my studies in her laboratory. Her patient guidance, constant support and continual encouragement have been paramount to my studies. Thanks to Rina Mawji who provided countless advices and shared her extensive knowledge about laboratory work. Thanks to Jean Wang and Gang Wang who contributed to the technical work of this thesis. My gratitude to all the members and alumni of the Prostate Group of the Genome Sciences Centre (Theresa L’Heureux, Teresa Tam, Terence Yung, Tammy Romanuik, Dr. Steven Quayle, Dr. Richard Sobel, Nasrin Mawji, Mary Flores, Dr. Katie Meehan, Dr. Jae Kyung Myung, Heidi Hare, Dr. Barbara Comuzzi, Angela Beckett) for their assistance, insightful discussions, constructive feedbacks and moral support. It has been a pleasure working with such an incredible research team. Thank you to my committee members, Dr. Peggy Olive, Dr. Cheryl Helgason and Dr. David Walker for their insights and critical review of my thesis. My sincere thanks to Dr. Jaclyn Hung and Joanna Ip for opening the doors of cancer research to me at the B.C. Cancer Research Centre and the constant encouragement. Special thanks to a number of family members and friends who have given me much help and support during the challenging times in the course of this research experience. I am particularly indebted to my parents, Maria and David, and my younger sister, Vivian, for their unwavering love and support.

Words cannot fully express how thankful I am. From the bottom of my heart, thank you all!
DEDICATION

To my parents
CO-AUTHORSHIP STATEMENT

CHAPTER 2

The experiments were designed, conducted and analyzed by myself, Helen Chiu, and my supervisor, Dr. Marianne Sadar. Additionally, Jean Wang co-authored and performed the experiments involving the animal work of the LNCaP hollow fibre model and the LNCaP xenograft model and immunohistochemistry described in the chapter. Dr. Marianne Sadar and I were responsible for the remainder of the manuscript preparation.
1 INTRODUCTION

1.1 PROSTATE CANCER

1.1.1 Epidemiology and the challenge

Prostate cancer is the most common cancer affecting 0.8% of the male population and the third leading cause of cancer death in Canadian men (1). According to the Canadian Cancer Statistics, it is estimated that 22,300 men will be diagnosed with prostate cancer and 4,300 men will die from the disease in 2007 (1). In the United States where prostate cancer is the second cancer killer in men, an estimated 218,890 new cases and 27,050 deaths will occur in 2007 due to the cancer (2). Intriguingly, about 30% of men over the age of 50 who were not clinically diagnosed with prostate cancer had histological evidence of prostate cancer based on autopsy studies (3). Hence, most men who have prostate cancer will likely die with the cancer instead of dying from the disease.

Prostate cancer primarily affects the elderly worldwide with three-quarters of cases occurring in men aged 65 years and older (4). The incidence and mortality of prostate cancer is the highest among African-American men (5,6). The disease prevails as a major male health problem in Western countries, whereas the incidence and mortality associated with the disease is intermediate among the European and South American countries and lowest in the Asian population (4). The risk factors contributing to the ethnic and geographic differences have yet to be identified and confirmed. Approximately 10% of prostate cancer is associated with family history while most cases appear to be sporadic, and little is known about the aetiology of prostate cancer (7). Deciphering the molecular abnormalities in prostate cancer remains a very complex issue due to the heterogeneous and multifocal nature of the tumours (8,9).
1.1.2 The prostate

Structure and function. The prostate is a tubuloalveolar exocrine gland of the male reproductive system that is posterior to the rectum and superior to the urinary bladder. It is roughly the shape and size of a walnut. The gland is penetrated by the urethra and the ejaculatory ducts. The prostate is defined into three major histologically distinctive glandular zones: peripheral (70—75 %), central (20—25 %) and transitional (5—10 %) zones (Fig. 1.1) (10,11). The main function of the adult prostate gland is to store and secrete a large number of compounds such as lipids, acid phosphatase, citric acid, kallikrein proteases and other proteolytic enzymes. The alkaline serous white fluid constitutes a major fraction of the seminal fluid that liquefies and nourishes sperms. The formation, synthesis and release of prostatic secretions are regulated by androgens, primarily dihydrotestosterone (DHT), the more potent form of testosterone. Mature prostatic duct is composed of distinct cell types: luminal secretory (columnar) epithelial cells, basal epithelial cells, stromal smooth muscle cells, some rare neuroendocrine cells and prostatic stem cell candidates (Fig. 1.2) (12). These cells express unique patterns of differentiation markers. For examples, the most common prostatic epithelial cells, the luminal secretory cells, express cytokeratin 8 and 18, the basal epithelial cells express markers such as cytokeratin 19, p63 and glutathione-S-transferase (GST)-pi, whereas the scarce neuroendocrine cells display chromogranin A and secrete products such as serotonin and neurophysin (13-16).

Physiological development. The prostate begins to develop from the endodermal urogenital sinus (UGS) derived from the cloacae of the hindgut in human foetus. UGS consists of urogenital sinus epithelium (UGE) and the embryonic connective tissue,
Figure 1.1. Zonal anatomy of the prostate. The prostate is divided into several anatomic regions. The regions with defined architecture are designated relative to the urethra as the reference point. Adapted from De Marzo et al., 2007 (17).
Figure 1.2. Morphology of the prostatic duct. A diagram of a cross-section of a prostatic duct illustrating the cell types present. The cell types found in the prostatic duct include luminal secretary epithelial cells, basal epithelial cells, stromal smooth muscle cells, neuroendocrine cells and stem cell candidates. A list of commonly used differentiation markers is labelled for each cell type. Adapted from Marker et al., 2003 (12)
urogenital sinus mesenchyme (UGM). During normal development, the process of
differentiation and growth is tightly controlled and highly orchestrated by androgens and
various growth factors. Mesenchymal-epithelial interactions are critical in prostatic
development as interactions between UGE and UGM are required for the prostate to
develop from UGS in the presence of androgens. Specifically, the UGM is responsible
for the fate of the prostatic epithelium, formation and growth of epithelial buds, ductal
morphogenesis, differentiation of secretory epithelium and expression of specific
secretory proteins (reviewed in (18)). In turn, the differentiation of the UGM to
surrounding smooth muscles of the epithelium is governed by the paracrine signals from
UGE (19,20). The development of the prostate is completed by the end of puberty. The
highly differentiated adult prostate is normally maintained at a growth-quiescent state. It
is postulated that perturbations of the homeostatic interactions between epithelium and
stromal smooth muscles leads to prostatic pathologies such as prostatic carcinoma (21).
This hypothesis is supported by the observation that dysplastic epithelial sites in an \textit{in
vivo} prostatic carcinogenesis model are associated with dedifferentiation of adjacent
stromal smooth muscle cells towards fibroblastic characteristics (22). Nevertheless,
further decipherment in the molecular mechanisms involved in the stromal-epithelial
interactions contributing to the carcinogenesis of prostate cancer is keenly awaited.

\textit{Androgen signalling}. Androgen signalling plays a pivotal role in the normal
physiological development, growth and function of the prostate. The majority of
androgens are secreted by Leydig cells of the testes, while a minor amount is synthesized
by the adrenal cortex of the adrenal gland in men. The cytoplasmic enzyme 5a-reductase,
primarily isoform type II, synthesizes DHT from circulating testosterone (23).
The effect of androgens is mediated through the steroid hormone receptor, androgen receptor (AR). The gene encoding AR is located on the X chromosome at Xq11-12 (24). AR is a multi-domain protein containing an N-terminal domain that is involved in transcriptional activation, a DNA binding domain with two zinc finger motifs, a hinge region and a C-terminal ligand-binding domain (Fig. 1.3). The AR modulates androgen-regulated gene transcription (Fig. 1.4). In its inactive state, AR is mostly found in the cytoplasm stabilized by heat-shock proteins (HSP) (25). HSP-bound AR is inactive and cannot bind to regulatory elements on target genes. Androgen-binding induces dissociation from heat shock proteins, hyperphosphorylation, conformational changes and dimerization of the receptor (25-27). DHT has an affinity for the AR as much as 10 times higher than testosterone (28). Upon activation, the AR homodimers translocate to the nucleus where they bind to androgen response elements (ARE) in the androgen-regulated gene loci (29,30). Nevertheless, it is important to note that the naturally occurring AREs are generally unique from each other and distinct from the consensus ARE, 5'-GGA/TACANNNTGTCT-3' (where N = any nucleotide), as determined by Roche et al. (31) using in vitro biochemical assays. As a part of the transcriptional machineries, AR modulates the expression of target genes in conjunction with co-activators, such as steroid receptor co-activator 1 (SRC-1) (32), and co-repressors, such as nuclear receptor co-repressor (NCoR) (33).

Interestingly, androgen-independent prostate cancer cells frequently retain the expression of AR. Specifically, AR has been implicated in androgen-independent prostate cancer as a result of hypersensitive AR due to gene amplification and/or mutations, constitutive activity of AR by non-androgenic molecules, constitutively active AR co-
Figure 1.3. **Functional domains of AR.** Androgen receptor (AR) is a multi-domain transcription factor that consists of an N-terminal domain (NTD) that is involved in transcriptional activation, a DNA binding domain (DBD) with two zinc finger motifs, a hinge region and a C-terminal ligand-binding domain (LBD).
Figure 1.4. Mechanism of androgen-dependent AR signalling. Before binding to the AR, Testosterone (T) is transported and converted into dihydrotestosterone (DHT) by 5α-reductase in the cytoplasm. Binding of ligand promotes the release of AR from the heat-shock proteins (HSP) and phosphorylation of AR. Subsequently, the activated AR dimerizes, translocates into the nucleus, binds to androgen response element in the target gene locus and modulates the transcription of the target gene together with co-activators, such as steroid receptor co-activator 1 (SRC-1).
regulators or crosstalk of AR signalling with other signalling pathways (i.e. growth factors) (extensively reviewed in (34,35)). These observations strongly suggest that AR plays a significant role in prostate cancer progression to androgen independence. They also imply that therapy targeting any aspect of the mechanisms of AR action could treat both androgen-dependent and androgen-independent prostate cancer effectively.

1.1.3 Detection and diagnosis

Detection and diagnosis of malignant cells that are clinically significant and require treatments is a constant challenge in the clinical setting. The digital rectal examination (DRE) combined with a blood test for serum prostate specific antigen (PSA) levels are the standard methods of detection for prostate cancer.

*Digital rectal examination.* Prostate cancer typically arises in the peripheral zone (36). Since this part of the prostate lies in close proximity with the rectum, physicians may perform DRE by inserting a gloved finger inside the rectum to examine the part of the prostate that is closest to the rectal wall for any abnormal lump or nodule in the region. However, such an abnormality may or may not indicate the presence of prostate cancer. Moreover, for many cases, the DRE does not reveal abnormality that the doctor can palpate, because a tumour may be too small to be palpable or may locate in a part of the prostate that is inaccessible from the rectal area. Furthermore, DRE is not standardized and highly variable among physicians (37,38). Thus, a relatively more sensitive blood test for PSA is commonly employed in concert with DRE.

*PSA Test.* PSA is the principle serum biomarker for prostate cancer since the early 1990s. The 33 kDa serine protease, first described in 1971 and purified in 1979, is normally secreted by the luminal epithelial cells of the prostate (39-41). The expression
of this human kallikrein family member located on chromosome 19 is regulated by AR (42). The primary function of PSA is to liquefy seminal coagulum in ejaculate through its proteolytic function (39). PSA measurement reflects prostate cancer risk, with the risk and aggressiveness of cancer increasing with the PSA level in the blood serum (43-45). After confirming its usefulness in a multi-institutional study, total PSA with a threshold of 4.0 ng/ml as the upper limit of “normal” is commonly adopted thereafter (46). Using PSA cut-off of 4.0 ng/ml, two groups (47,48) found that most prostate cancer patients with localized disease could have been diagnosed about 5 years earlier than their clinical diagnosis without PSA testing. Subsequently, PSA testing in conjunction with other diagnostic procedures was approved by the United States Food and Drug Administration for early detection of prostate cancer in 1994 (49).

Prostatic biopsy and Gleason grade. Definitive diagnosis of prostate cancer requires a biopsy. A biopsy is an invasive procedure of removing prostate tissue with a needle. Multiple biopsies may be performed to remove multiple samples from different parts of the prostate. To enhance specificity and increase detection rate, additional biopsies may be taken as guided by transrectal ultrasound of the prostate (50). As a pathologist examines the prostate tissue from a biopsy for histological abnormalities, the Gleason score is assigned to help predict the behaviour of the cancer if it is indeed present (51). Grade ranges from 1 (the least aggressive) to 5 (the most aggressive histological pattern) are assigned to each of the two most prevalent foci in a biopsy (Fig. 1.5). The grades are added together to give a Gleason score. The scored histological patterns correlate with the clinical outcome of the patients: the higher the score, the higher the likelihood for the cancer to progress and spread (51,52).
Staging. In the diagnosis of prostate cancer, the cancer is staged according to the tumour-node-metastasis (TNM) system established in 1992 (53). The TNM system describes the extent of the cancer. Stage T1 to T4 is designated to the primary tumour confined in the prostatic capsule and neighbouring seminal vesicles and bladder. Stage N is assigned to cancer metastasized to regional lymph nodes, whereas stage M is attributed to metastasis at distant sites, such as non-regional lymph nodes and bone. Together with other considerations, such as tumour grade, family history of prostate cancer and age of the patients, the staging allows the physicians to advise the appropriate treatment options to the patients.

1.1.4 Natural history

Prostatic intra-epithelial neoplasia. The natural history of prostate cancer is reflected in its malignant potential, the extent to which the malignancy contributes to the progression and its response to treatment (Fig. 1.6). Early prostate cancer usually has no symptom and the carcinogenesis of prostate cancer remains ambiguous. Morphologically, prostatic epithelial lesion with disruption of the basal layer of acini appears to be a good indicator of early invasion. Based on the observations of the morphologic features in radical prostatectomy specimens, McNeal and Bostwick (54) proposed in 1986 that intraductal dysplasia or prostatic intra-epithelial neoplasia (PIN) is the precursor lesion that precedes invasive carcinoma. Specifically, early invasion of prostate cancer occurs frequently in association with foci of increasing grades of PIN with the loss of basal cell layer integrity (55).

Proliferative inflammatory atrophy. More recently, it is postulated that prostate cancer may be driven by chronic inflammation in the prostate due to continuous injuries
Figure 1.5. *Gleason grading system.* The Gleason grading is a system for classifying prostate cancer tissue by assigning a number (1-5) to the differentiation of the cells in the specimen when examined under a microscope. Grade 1: Circumscribed nodule of closely packed but separate, uniform, rounded to oval, medium-sized acini (larger glands than grade 3). Grade 2: Like grade 1, fairly circumscribed, yet at the edge of the tumour nodule, there may be minimal infiltration. Glands are more loosely aggregated and not quite as uniform as grade 1. Some glands infiltrate into surrounding stroma. Grade 3: Discrete glandular units; typically smaller glands than seen in grade 1 or 2; infiltrates in stroma; prominent variation in size and shape; smoothly circumscribed small cribriform nodules of tumour. Grade 4: Fused acinar glands; disruption and loss of gland units. Poorly differentiated. Grade 5: Total disruption and loss of gland units. No glandular differentiation, composed of solid sheets, cords, or single cells. Source: Epstein et al, 2006 (52)
Normal prostate

PIA

Low-grade PIN

High-grade PIN

Localized CaP

Metastatic CaP

Androgen-independent CaP

Figure 1.6. The progression of prostate cancer. The normal prostate progresses through a series of morphological and molecular changes forming proliferative inflammatory atrophy (PIA) and prostatic intra-epithelial lesion (PIN) followed by carcinoma of the prostate (CaP). Eventually, malignant cells gain the ability to metastasize to distant sites, such as lymph nodes and bone. The final stage of CaP occurs when cancer cells no longer require androgen to grow and survive. See text for the detailed discussion on the natural history of prostate cancer.
and insults in the prostate (17). The hypothesis stems from the earlier observations that prostatic focal atrophy, termed proliferative inflammatory atrophy (PIA), exhibited an increased expression of the proliferation marker, Ki-67, GST-pi and Bcl-2, indicating enhanced proliferation, stress-induced response and reduced apoptosis at the regenerative lesions respectively (56). The group further demonstrated that high-grade PIN, but not carcinoma, merged with PIA (57). These findings suggest that PIA may give rise to prostate cancer directly or indirectly via high-grade PIN. Like prostate cancer, both high-grade PIN and PIA are found predominantly in the peripheral zone (58-63). Whether PIA and PIN are causative in the evolution of prostate cancer remains a debateable topic; moreover, the exact mechanism by which PIA and PIN contribute to tumourigenesis awaits further investigation that may involve development of animal models which mimic the progression.

**Invasive and metastatic carcinoma.** Accumulating somatic alterations and genetic instability may enable the malignant prostate tumours to undergo transforming proliferation and dedifferentiation and progress to invasive carcinoma (64,65). A pooled analysis from six non-randomized studies and a population-based study demonstrate that men with poorly differentiated prostate cancer had approximately 10-times higher risk of dying from the disease than those who had well-differentiated tumours with favourable clinical outcomes (66,67). Locally advanced prostate cancer refers to the disease that has started to invade nearby organs, such as the seminal vesicles and bladder without evidence of distant metastasis. At the terminally advanced stage, prostate cancer tends to metastasize to the lymph node and the bone of the patient. Mortality from prostate cancer is directly related to metastasis. Better understanding of the underlying molecular
mechanisms involved in the progression of prostate cancer will not only enable early
detection and prevention but also improve the prognosis coupled with appropriate
treatments for the patients.

1.1.5 Treatment options

The decision on appropriate treatment strategy should be based on factors such as
the staging of the tumour, the extent of the disease, the patient’s life expectancy and
requirements for the quality of life by weighing the benefits against the side effects of the
therapeutic options. In most cases, prostate cancer is a chronic disease with slow
progression; consequently, watchful waiting by monitoring the disease regularly without
undergoing any immediate therapy may be considered for men with a low risk of dying
from the cancer in their life-time to avoid harmful side effects of treatments.

Localized therapies. Local prostate cancer is conventionally treated with radical
prostatectomy and external-beam radiotherapy. Newer treatments include brachytherapy
(reviewed in (68)) by implanting radioactive seeds into the prostate and cryotherapy
(reviewed in (69)) by using pressurized gas-driven probes to destroy cancer cells with
rapid freezing and thawing. Total PSA and its kinetic variations, such as PSA velocity
and PSA doubling time (i.e. time to double the PSA level), may be used to monitor the
effect of therapy. Failure of therapy is characterized by a biochemical recurrence based
on a rising PSA level. For men undergoing local therapy, the serum PSA level should
become undetectable after treatment. Thus, a subsequent increase in PSA is usually the
earliest sign of cancer progression. Following radical prostatectomy for organ-confined
prostate cancer, about 15% of men will develop biochemical failure that precedes clinical
evidence of metastatic disease at a median of 8 years followed by a median of 5 years to
death (70). Androgen deprivation therapy (ADT) may be employed as neoadjuvant or adjuvant treatment in combination with all the treatment options described thus far to downsize the tumours and enable complete eradication of the cancer (reviewed in (71-73)). For instance, neoadjuvant androgen ablation prior to radiotherapy significantly minimized the neighbouring gastrointestinal and genitourinary tissue from exposure to high-dose radiation by reducing the field size (74).

Systemic therapies. For the management of metastatic disease, the role of ADT is well-established since its inception more than 65 years ago by Huggins and Hodges (75). A population study based on the Surveillance, Epidemiology and End Results (SEER)- and Medicare-linked database with 6,098 men 65 years and older concludes that the median survival for men with metastatic cancer was 26 months after androgen deprivation therapy, approximately 13 months more than those who were untreated (76). ADT may be performed by surgical castration with orchiectomy to remove testicles or chemical castration with drugs that suppress testosterone production or its effects. Bilateral orchiectomy is permanent and irreversible. Medical castration may be achieved with luteinizing hormone-releasing hormone agonists, steroidal and non-steroidal antiandrogens and inhibitors of steroid synthesis such as ketoconazole that inhibits cytochrome P450 enzymes in adrenal cells (77). Both orchiectomy and luteinizing hormone-releasing hormone agonists have an adverse effect on bone mass causing osteoporosis as a result of long-term androgen deprivation (78-80).

The rationale for ADT is based on the dependency of prostate cells on androgens to grow and survive. The series of events immediately following castration are well-characterized in rat and collectively contribute to the programmed cell death of the
prostate cells (reviewed in (81); Fig. 1.7). Briefly, upon castration, the serum testosterone levels rapidly decline and a dramatic response in prostate cells frequently occurs within 24 hours after castration. At the molecular level, castration induces changes in the expression of a wide range of genes in addition to androgen-responsive genes, giving rise to the systemic biochemical and morphological changes in the hosts. ADT may thereby upset the intricate cellular pathways in the prostate cells by affecting the expression of a wide catalogue of genes involved in AR-dependent and AR-independent prostate cancer progression (82). As an early event after castration, the fragmentation of genomic DNA is catalyzed by calcium magnesium-dependent endonuclease activity (83). Castration also induces apoptosis and degeneration of capillaries and constriction of larger blood vessels in the prostate (84). Hence, with the dramatic changes in gene expression, induction of genomic instability and reduction of blood flow, the homeostasis in prostate cells is disrupted by ADT in a highly complex manner. Eventually, the responses to ADT will lead to the rapid apoptotic involution of prostatic tissue due to a major irreversible loss of prostatic epithelial cells (85).

Despite initial effectiveness in eliminating non-cancerous and cancerous prostate cells, the major disadvantage of ADT is that eventually some prostate cancer cells will become resistant to the loss of androgen. The androgen-independent prostate cancer cells will begin to proliferate and grow despite the absence of androgen as reflected in increasing PSA levels subsequent to treatment. Relapsed disease after primary treatment with surgery or hormonal therapy is referred to as hormone-escaped, therapy-resistant, hormone-refractory, recurrent or androgen-independent; the latter term will be used in this thesis.
Figure 1.7. The activation of the apoptotic pathway in response to androgen withdrawal. A schematic summary of the biochemical and morphological events in prostate cells following androgen withdrawal. While the epigenetic reprogramming of cells is reversible at the initiation of the cascade, prolonged androgen deprivation results in the irreversible steps leading to induced apoptotic death of the prostate cells. See text for details. ODC, ornithine decarboxylase; CDK-2, cyclin-dependent kinase-2; TFG, transforming growth factor; TRPM-2, testosterone-repressed prostate message-2; PARP, poly-(ADP-ribose) polymerase. Source: Denmeade et al., 1996 (81)
It appears that androgen ablation fails to initiate the programmed cell death pathway in a subset of androgen-independent cells. Possible mechanisms for this failure could involve increased expression of genes associated with enhanced cellular survival or decreased expression of genes that are involved in triggering apoptosis. Evidences with the expression of Bcl-2, an anti-apoptotic oncoprotein, correlate with disease state, particularly with an augmented expression at the androgen-independent state (86,87). Moreover, Lin et al. (88) demonstrated in the classical prostate cancer cell line model, LNCaP, that the anti-apoptotic ability of Bcl-2 is required for the progression from an androgen-dependent to an androgen-independent state. Nevertheless, it is unclear how the increased expression occurs and if it is an absolute requirement for the progression of prostate cancer. Furthermore, multiple mechanisms may be involved in conferring anti-apoptotic ability to prostate cancer cells and enhance their survival to progress to androgen-independence.

Intermittent ADT was introduced recently aiming to delay androgen-independent outgrowth, improve the patients’ quality of life and minimize the long-term complications associated with androgen deprivation such as osteoporosis (reviewed in (89)). Briefly, with constant monitoring of PSA levels, the modified ADT involves cyclic administration of androgen deprivation by chemical castration until a clinical response is demonstrated and followed by an off-therapy interval until symptoms recurred. Intermittent ADT is considered experimental with anticipation of the results from long-term randomized clinical trials to determine whether it will produce any survival advantage (90).

Androgen-independent disease causes death. Unfortunately, besides palliation
with chemotherapy, there is no known cure for androgen-independent prostate cancer, and the cellular mechanisms associated with the hormonal progression remain elusive. Nevertheless, like many other cancers, the current strategic trend is geared towards identifying specific cellular targets involved, so that novel non-surgical, non-invasive, personalized adjuvant treatment options may be developed for the disease.

1.2 MODELS FOR STUDYING THE HORMONAL PROGRESSION OF PROSTATE CANCER

*In vitro models.* A variety of human prostate cell lines have been derived from primary tissue sources, and clonal derivatives of previously established lines have been developed for investigating various aspects of prostate cancer using *in vitro* and *in vivo* model systems (91,92). To better understand the effect of androgen on the progression of prostate cancer, *in vitro* human prostate cancer cell lines with different androgen requirements and origins in the disease state may be employed selectively for independent experiments. LNCaP is the classic prostate cancer cell line derived from a supraclavicular lymph node of a patient whose prostate cancer was exhibiting androgen-independent growth and this cell line retains PSA expression and androgen sensitivity (93). LNCaP cells express a mutated AR (T877A) that is functional but can be activated by not only androgens but also other steroids, such as progesterone, estradiol and anti-androgens (94). Androgens increase the levels of PSA mRNA, and attenuate the levels of prostatic acid phosphatase mRNA, another major prostate-specific protein (95). In contrast to LNCaP cells, DU145 cells and PC3 cells are two widely used androgen-insensitive cell lines that do not express AR or PSA. DU145 cells (96) were derived from a brain metastasis from an untreated prostate cancer patient with a history of leukemia,
whereas PC3 cells (97) were established from bone metastasis of a patient with androgen-independent prostate cancer.

*In vivo models.* Murine models that mimic the progressive biochemical features of prostate cancer have been developed using human prostate cancer lines to facilitate our understanding of the hormonal progression of prostate cancer at the molecular level. Xenograft models are commonly employed to investigate various aspects of the hormonal progression of prostate cancer. Prostate cancer cell suspensions consisting of LNCaP cells can be grafted subcutaneously or intraprostatically in male nude mice or male severe combined immunodeficient (SCID) mice to generate tumours (98,99). To stimulate androgen deprivation, surgical castration is performed on the hosts. In parallel to the clinical representation, LNCaP xenograft mimics the hormonal progression from androgen-sensitive to androgen-independence as monitored by the host’s serum PSA level that correlates with tumour volume (100). Tumours may be harvested from the host at various stages of the hormonal progression to androgen independence. Interestingly, LNCaP xenografted tumours were not accompanied by reduction in tumour volume upon castration (100). DU145 cells and PC3 cells can also be used in xenografts; in fact, these poorly differentiated cells exhibit a higher malignant potential than LNCaP cells in xenografts.

In addition to xenografts developed from human prostate cancer cell lines, several xenograft models have been developed from primary human prostate tumour tissues from prostate cancer patients grafted subcutaneously in nude or SCID mice in the past years (101-105). Similar to LNCaP, CWR22 tumour expressed a mutated AR (H874Y) that can be activated by adrenal androgen dehydroepiandrosterone, estradiol, progesterone and
the antiandrogen hydroxyflutamide (106). Like LNCaP xenografts, some of these
xenografts of primary tumours, such as CWR22 (107,108), PC-346 (102) LuCaP 23
(104), LAPC-4 (103), LuCaP 35 (105) are androgen-sensitive with PSA expression,
respond to castration to varying extents and relapse in an androgen-independent manner,
though generally at a slower rate and are more challenging to establish initially as
compared to the LNCaP xenografts. All xenograft models allow stromal-
microenvironment interactions which are suggested to play a critical role in the
pathogenesis and progression of prostate cancer (109). However, tumour cells from
xenograft systems are highly vascularised and thus infiltrated with host cells, impeding
them from providing pure samples for subsequent molecular analyses.

To circumvent the contamination by host cells associated with xenograft models,
the \textit{in vivo} LNCaP hollow fibre model was developed by Sadar et al. (110). This model
enables retrieval of "pure" populations of prostate cancer cells devoid of host cells during
various stages of the hormonal progression from androgen-sensitive to androgen-
independence upon castration as monitored by serum PSA levels in male
immunocompromised mice. Briefly, suspensions of LNCaP cells in matrigel are loaded
into porous polyvinylidene difluoride hollow fibres with a molecular weight cut-off of
500 kDa (i.e. approximately 20 nm in pore size) that permit efficient exchange of soluble
factors and provide attachment support. The ends of the fibre are sealed prior to
subcutaneous implantation of the fibres into the back of the mice. Androgen deprivation
is achieved by surgical castration of the hosts. Subsequent to castration, the prostate
cancer cells will progress in a manner that involves an initial regression in levels of
serum PSA prior to an upsurge of serum PSA in the host. The animals bearing the hollow
fibres of LNCaP cells are generally healthy as indicated by weight and behaviour. While xenograft models require sacrificing different animals for each time-point along the hormonal progression, the hollow fibre model is ideal for obtaining matched in vivo prostate cancer cells from the same animals at each time-point throughout the course of the experiment, as packages of prostate cancer cells in hollow fibres may be retrieved from the same hosts at the desired time-points. Both the LNCaP xenograft model and LNCaP hollow fibre model mirror the clinical progression of prostate cancer in response to androgen ablation therapy, and both in vivo models permit the use of serum PSA to monitor hormonal progression.

Potentials of these models are yet to be realized. Unexplored manipulation of the host, such as medical castration, may be performed in these in vivo models to investigate other aspects in the hormonal progression to androgen-independence. To investigate the molecular mechanisms underlying the hormonal progression of prostate cancer, the hollow fibre model could also be optimized to provide suitable in vivo sources for various biochemical applications, such as co-immunoprecipitation and chromatin immunoprecipitation assays. In this study, a protocol has been established to isolate biologically active nuclear extracts from the LNCaP hollow fibre model for DNA-binding assays to investigate changes in transcriptional activity in response to androgen deprivation by surgical castration of the host.

1.3 INHIBITOR OF APOPTOSIS PROTEINS

The newly identified inhibitors of apoptosis proteins (IAPs) family plays a role of apoptotic resistance in prostate cancer. IAPs protect cells from apoptosis induced from a variety of stimuli primarily by direct interactions with caspases, a class of cystein-
aspartyl proteases that are fundamental for most of the properties of apoptotic cell death, though other mechanisms have also been described. Homologues of IAPs have been identified from a variety of life forms from viruses to vertebrates. The first mammalian IAP, neuronal apoptosis inhibitory protein (NAIP/BIRC1) was identified by positional cloning in an effort to determine the genetic defect in spinal muscular atrophy by positional cloning (111). Deletions in the gene encoding NAIP are associated with the severity of the disease due to the loss of neuroprotective activity in suppressing apoptosis (112). Subsequently, seven human IAPs identified so far include c-IAP1 (BIRC2/HIAP2), c-IAP2 (BIRC3/HIAP1) (113), XIAP (BIRC4) (112), survivin (BIRC5) (114), apollon (BIRC6) (115), livin (BIRC7/KIAP/ML-IAP) (116) and IAP-like protein-2 (BIRC8/ILP-2) (117). All family members are characterized by one or more 70-80 cysteine- and histidine-rich baculoviral IAP repeat (BIR) domains, consisting of core variable sequence C(X)2C(X)6W(X)3D(X)5H(X)6C (X = any amino acid) (Fig. 1.8). The BIR domains, which directly interact with caspases and other proteins, are central to the ability of IAP to block apoptosis.

As the only known intrinsic regulators of the caspase cascade that modulate the activity of both initiator and effector caspases, IAPs can block apoptotic cell death triggered via the intrinsic or the extrinsic death pathways through caspase inhibition, among other mechanisms (Fig. 1.9) (118). Most IAPs have demonstrated direct binding to and inhibited activated caspase-3 and capase-7, the effector caspases of both the intrinsic and extrinsic pathways (119). Additionally, all IAPs, except survivin, directly interact with and inhibit caspase-9, the initiator caspase of the intrinsic pathway (119,120). It seems to be a general rule that the first two BIR domains are associated with
Figure 1.8. Domain structure of the mammalian IAP family. IAP members typically contain one to three N-terminal baculovirus IAP repeat (BIR) domains. RING finger, really interesting new gene zinc-finger; CARD, caspase recruitment domain; NOD, nucleotide-binding oligomerization domain; LRR, leucine-rich repeats; UBC domain, ubiquitin-conjugating domain. Adapted from Hunter et al, 2007 (118).
Figure 1.9. Intrinsic and extrinsic cell death pathways. The intrinsic pathway triggered by various forms of stress results in the release of mitochondrial proteins, such as cytochrome c. In the cytoplasm, cytochrome c interacts with the apoptotic protease activating factor 1 (Apaf-1) and ATP to form the apoptosome with procaspase-9. The apoptosome binds and activates caspase-9 which then recruits the effector caspases, such as caspase-3 and caspase-7. The extrinsic pathway involves binding of death signals, such as tumour necrosis factor (TNF) and Fas ligand, to their corresponding cell surface death receptors, such as TNF receptor and Fas receptor. Recruitment of adaptor proteins to the intracellular death domains of the death receptors leads to procaspase-8 activation and subsequent activation of effector caspases. Activation of the effector caspases leads to a series of proteolytic events which result in the disintegration of the cell. IAPs can effectively inhibit the initiator and effector caspases and other components involved in both intrinsic and extrinsic pathways. Adapted from Hunter et al., 2007 (118)
direct binding and inhibition of caspase-3 and caspase-7, whereas the third BIR domain is attributed to the inhibition of caspase-9 (121-126). Certain IAPs, such as XIAP, c-IAP1, c-IAP2 and livin, also possess RING domains with E3 ubiquitin ligase activity to target their substrates for ubiquitinylation and subsequent proteosomal degradation (Fig. 1.8) (127). Additional functional domains, such as caspase recruitment domain (CARD) and nucleotide-binding oligomerization domain (NOD), confer uniqueness to individual IAP members with distinct structures and functions that are yet to be confirmed (Fig. 1.8).

With their pivotal roles in regulating apoptosis, it is not surprising that the deregulation of IAPs is associated with various malignancies, suggesting the idea that IAPs may facilitate escape of cancer cells from apoptosis (extensively reviewed in (118)). Emerging studies on the expression and relevance of IAPs highlight their roles in the prognosis of prostate cancer. Elevated expression of c-IAP1, c-IAP2, XIAP and survivin has been shown by immunochemistry and immunoblotting in a transgenic mouse model of prostate cancer and prostatectomy specimens from cancer patients (128). Furthermore, the increased IAP expression appears to be an early event in the development of prostate cancer (128). Specifically, survivin is associated with resistance to cell death in response to anti-androgen therapy and chemotherapy in prostate cancer cells, as a double negative survivin mutant tends to sensitize the cells to death induced by flutamide and paclitaxel in vitro and in vivo respectively (129,130). Similarly, a pre-clinical study demonstrated that an antisense oligonucleotide directed towards XIAP in the prostate cancer xenograft model appears to lower the apoptotic threshold to taxanes in a dose-dependent manner (131). Hence, elucidation of the mechanism by which IAPs protect prostate cancer cells from dying by induced cell death will be invaluable for the development of novel
therapeutic strategies to prevent the progression to androgen-independence.

1.4 NUCLEAR FACTOR-KB

The nuclear factor (NF)-κB is a family of ubiquitously expressed dimeric transcription factors that modulate a multitude of immune and inflammatory responses as well as cell proliferation, differentiation, apoptosis, adhesion, survival and oncogenesis (reviewed in (132,133)). The mammalian NF-κB family is comprised of five members: p65/RelA, RelB, c-Rel, p50/p105/NF-κB1, p52/p100/NF-κB2 (reviewed in (133)). These proteins share a Rel homology domain that mediates DNA-binding, dimerization and specific interactions with inhibitor of κB (IκB) (Fig. 1.10) (134-136). Dormant NF-κB is bound by IκB and is retained primarily in the cytoplasm (137). A variety of stimuli, such as cytokines (e.g. tumour necrosis factor-α (TNF-α), interleukins), viral infection and intracellular stresses, activate NF-κB mainly through the canonical pathway that involves the phosphorylation of the IκB kinase (IKK) complex, which consists of the catalytic subunits (IKK-α and IKK-β) and the regulatory component (IKK-γ/ NEMO), and subsequent ubiquitylation and proteosomal degradation of IκB (Fig. 1.11) (138-140). An alternative signalling pathway of NF-κB involves the activation of p52/RelB dimers as a result of the processing of the p100 precursor protein, which mostly associates with RelB as a heterodimer in the cytoplasm (Fig. 1.10) (141). The liberated NF-κB dimers are then activated to translocate to the nucleus and bind to regulatory κB elements of a diverse array of target genes (142,143). Different NF-κB dimers exhibit different binding affinities for the κB site, bearing the consensus sequence 5’-GGGRNNYYCC-3’ [R = purine, N = any base, Y = pyrimidine] (144).

Constitutive activation of NF-κB has been attributed to the aggressiveness of
Figure 1.10. Domain structure of the mammalian NF-κB family. All NF-κB members contain an N-terminal Rel homology domain (RHD) that is important for DNA-binding, dimerization and nuclear localization. p65, RelB and c-Rel have C-terminal transactivation domains (TAD). Additionally, RelB has a leucine-zipper domain (LZD). p100 and p105 contain glycine-rich regions (GRR) and C-terminal domains with ankyrin repeats which block DNA-binding in the native protein. Adapted from Hayden and Ghosh, 2004 (133).
Figure 1.11. NF-κB signal transduction pathways. In the classical pathway, NF-κB dimers, such as p65/p50 heterodimers, are maintained in the cytoplasm by interaction with inhibitor of κB (IκB). Ligand-activation of cell surface receptors leads to activation of the IκB kinase (IKK) complex containing the catalytic subunits, IKK-α and IKK-β, and the regulatory subunit, IKK-γ. IKK complex then phosphorylates IκB at two serine residues, leading to the phosphorylation, ubiquitinylation and proteosomal degradation of the inhibitors. Dissociation from IκB allows the NF-κB dimers to enter the nucleus to modulate the transcription of target genes. The alternative pathway involves activation of an IKK complex that contains two IKK-α subunits. The IKK complex phosphorylates two serine residues on p100, leading to its partial processing at the C-terminal. The resulting p52/RelB heterodimer then translocates to regulate the transcription of target genes. The two pathways are involved in different cellular functions by modulating the expression of distinct sets of genes. Adapted from Karin and Greten, 2005 (145).
prostate cancer. In a recent immunohistochemical evaluation of prostatectomy specimens, elevated NF-κB immunoreactivity directly relates to the tumour stage, tumour grade and biochemical relapse (146,147). Crosstalk has been described between NF-κB and AR signalling pathways. Mutual antagonistic effects of NF-κB and AR were demonstrated in transient transfections of NF-κB and AR in COS-1 cells (148). Furthermore, the p65 subunit was able to inhibit AR-mediated transactivation at the PSA promoter in LNCaP cells (149). In contrast, Suh et al. (150) demonstrated that transient transfection of AR in the AR-negative PC3 cells and DU145 cells induced NF-κB-dependent transcriptional activity, even though the stimulation was blunted in the presence of ligand. It also appears that the survival signalling of androgen may be attributed to increased NF-κB DNA-binding activity in response to treatment with ligand (151). Although different experimental conditions may contribute to these contradictory data, the pleomorphic relationship between NF-κB and AR remains to be clarified.

Importantly, the oncogenic role of NF-κB lies in its ability to modulate anti-apoptotic gene targets (132). The anti-apoptotic activity of NF-κB in prostate cancer cells has been attributed to its ability to transcriptionally up-regulate Bcl-2 expression through binding to a site in the promoter region of the gene (152). Bcl-2 appears to confer resistance to apoptosis in prostate cancer cells from androgen ablation and radiotherapy (153-155). Moreover, the expression of Bcl-2 is required for the hormonal progression of prostate cancer cells to androgen-independence (88). More recently, the expression of IAPs have also been attributed to the anti-apoptotic activity of NF-κB. NF-κB signalling induces the expression of c-IAP1, c-IAP2 and XIAP (156). The expression of c-IAP2 and survivin are confirmed to be directly regulated by NF-κB through binding on the
regulatory elements of the genes (157,158). Consistently, IKK inhibitor inhibited c-IAP2 expression and sensitized prostate cancer cells to apoptosis induced by TNF-α (159). In turn, the TNF-α-induced transcriptional activity of NF-κB can be regulated by c-IAP2 involving its C-terminal RING domain in a possible positive feedback mechanism (160). As the anti-apoptotic role of NF-κB in the progression of prostate cancer is being further discerned, insights into the mechanisms will enable intervention to breakdown the NF-κB-dependent anti-apoptotic barrier.

1.5 RESEARCH OBJECTIVES AND HYPOTHESIS

1.5.1 Objectives and hypothesis

Androgen deprivation therapy by medical or surgical castration is the cornerstone treatment for advanced prostate cancer. The rationale for this systemic therapy is that prostate cancers cell will die in response to decreased circulating androgen. Nevertheless, despite the initial effectiveness of this treatment, the patients will inevitably succumb as the cancer recurs in an androgen-independent manner. The mechanism(s) by which prostate cancer cells escape the apoptotic fate in androgen-deprived conditions are poorly understood. Recent findings suggest that IAPs and constitutive NF-κB enable cancer cells to resist induced apoptosis. Furthermore, the transcription of some IAPs can be directly regulated by NF-κB, a transcription factor which constitutive activity has been implicated in prostate cancer. It is the goals of this thesis to investigate the expression of IAPs in prostate cancer cells in response to androgen deprivation and to determine if the anti-apoptotic role of NF-κB is involved in the expression of IAPs through transcriptional regulation. The hypothesis of this thesis is that the expression of IAPs involves the transcriptional regulation by NF-κB in response to androgen deprivation in prostate
cancer cells.

1.5.2 Specific aims

1. To identify which IAPs are increasingly expressed in response to castration by employing the *in vivo* LNCaP hollow fibre model. This work will identify IAPs that may be responsible for enhancing the anti-apoptotic ability in some prostate cancer cells in response to androgen deprivation and determine a candidate IAP for further study on its transcriptional regulation.

2. To determine the effect on NF-κB activity in response to castration by employing the *in vivo* LNCaP hollow fibre model. This work will highlight the effect of androgen ablation on NF-κB activity.

3. To investigate the effect of androgen on the expression of the candidate IAP and NF-κB transcriptional activity *in vitro*. This aim will confirm if the expression of the candidate IAP and NF-κB transcriptional activity are similarly affected by androgen.

4. To decipher the molecular mechanism by which the expression of the candidate IAP may be regulated by NF-κB. Work towards this aim will provide insight into a potential mechanism by which prostate cancer cells may bypass apoptosis in the loss of androgen.
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2 INDUCTION OF NEURONAL APOPTOSIS INHIBITORY PROTEIN EXPRESSION IN RESPONSE TO ANDROGEN DEPRIVATION BY NF-κB IN PROSTATE CANCER CELLS*

2.1 INTRODUCTION

Androgen deprivation with medical or surgical castration is an effective systemic approach for the treatment of prostate cancer (CaP). This therapy is based on the dependency of prostate cells on androgen to grow and survive. Thus, the effectiveness of androgen deprivation lies in its ability to induce cell death in prostate cells (1). Unfortunately, despite the initial responsiveness to androgen ablation, the cancer will only regress transiently before it eventually recurs and progresses to androgen-independence. The molecular mechanism by which CaP cells survive under the androgen-depleted conditions remains unclear. Specifically, different factors may render some cancer cells less prone to the cell death induced by androgen deprivation.

A family of proteins termed inhibitors of apoptosis proteins (IAPs), characterized by the presence of one or more baculoviral IAP repeat domains, is capable of rescuing cells destined for death via the caspase cascade (reviewed by (2)). To date, eight human IAPs have been identified. These are the neuronal apoptosis inhibitory protein (NAIP/BIRC1), c-IAP1 (BIRC2/HIAP2), c-IAP2 (BIRC3/HIAP1), XIAP (BIRC4), survivin (BIRC5), apollon (BIRC6), livin (BIRC7/KIAP/ML-IAP) and IAP-like protein-2 (BIRC8/ILP-2) (3-9). IAPs primarily function by restraining the activity of the caspase family. For instance, NAIP was shown to directly inhibit the cell death effector proteases, caspase-3 and caspase-7 (10) and associate with the initiator caspase, caspase-9 (11).

* A manuscript version of this chapter has been submitted. Chiu, HHL, Wang, J., Sadar MD. Induction of neuronal apoptosis inhibitory protein expression in response to androgen deprivation by NF-κB in prostate cancer cells. Submitted.
IAPs also modulate the survival of cells through other determined as well as unexplored means. Thus, it is not surprising that many have discovered deregulation of IAPs in various malignancies (reviewed in (12)). Elevated expression of IAPs was evident as an early event in the pathogenesis of CaP (13). Emerging studies also demonstrate the role of IAPs in conferring drug-resistance in CaP cells (14-16). Recently, the cytoprotective properties of IAPs have been shown to be associated with the nuclear factor (NF)-κB signalling pathway (reviewed by (12,17)), and c-IAP1, c-IAP2, XIAP and survivin are confirmed NF-κB targets (18-21).

The ubiquitously expressed NF-κB family includes p65/RelA, RelB, c-Rel, p50/p105/NF-κB1 and p52/p100/NF-κB2. Homodimers and heterodimers comprising these subunits regulate a multitude of genes and proteins that are involved in survival and programmed cell death among a variety of other critical biological functions (reviewed in (22)). Although other non-canonical pathways have been described, the activation of NF-κB typically involved phosphorylation of the inhibitor of κB (IκB), associated with the NF-κB factors, by IκB kinase complex. Subsequent ubiquitylation and proteosomal degradation of IκB allows the nuclear translocation, DNA-binding and transcriptional regulation of the transcription factor on target genes by binding to the κB sites in the gene loci. The NF-κB signalling pathway is strongly associated with the development and progression of CaP as well as other malignancies (reviewed in (23-25)). Constitutively active NF-κB, p65/p50 heterodimer, has been implicated in several studies to have a crucial role in the resistance to apoptosis in CaP cells and in the disease progression (26-29). The most common approach for NF-κB to antagonize apoptosis is by modulating the expression of anti-apoptotic genes at the level of transcription (30). To this end, IAPs
might play a key role in resistance to apoptosis in CaP cells via NF-κB signalling.

Here we evaluated the expression of IAPs in CaP in response to androgen deprivation both in vivo and in vitro and detected statistically significant increases in \textit{NAIP} expression that correlated with increased NF-κB DNA-binding. We report for the first time functional cis-regulatory elements of NF-κB on the \textit{NAIP} locus. These findings underlie a novel mechanism by which some CaP cells may acquire enhanced anti-apoptotic properties, thereby promoting the progression to an androgen-independent state.

2.2 MATERIALS AND METHODS

2.2.1 Animals, cell culture and reagents

Male athymic nude mice and male NOD-SCID mice were obtained from Taconic Farms (Germantown, NY, USA) and the Animal Research Centre of the B.C. Cancer Agency (Vancouver, B.C. Canada) respectively. All procedures on the mice were performed in compliance with regulations on the care and use of experimental animals under the Animal Care Certificates issued by the University of British Columbia (Vancouver, BC, Canada). Human CaP cell line, LNCaP, was routinely maintained in RPMI 1640 supplemented with 5 % (v/v) FBS (HyClone, Logan, UT, USA). PC3 and DU145, the other human CaP cell lines used in this study, were cultured in DMEM with 4500 mg D-glucose/L supplemented with 5 % and 10 % FBS respectively. All cell lines were maintained at 37 °C in 5 % CO₂ in a humidified incubator. All cell culture media were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. LNCaP cells were provided by L.W.K. Chung (Emory University School of Medicine, Atlanta, GA, USA), whereas PC3 cells and DU145 cells were obtained from American Type
Culture Collection (Rockville, MD, USA). Cell culture media and antibiotics were purchased from StemCell Technologies (Vancouver, BC, Canada). Dihydrotestosterone (DHT) and synthetic androgen, R1881 (Perkin-Elmer, Woodbridge, ON, Canada), were reconstituted in ethanol (vehicle). Human recombinant tumour necrosis factor-α (TNF-α) (Roche Diagnostics) was used in the presence of 1 mg/ml BSA. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

2.2.2 LNCaP hollow fibre model and castration

The experimental procedure involved in the LNCaP hollow fibre model was performed in male athymic nude mice as described previously (31). Briefly, subconfluent cultures of LNCaP cells in RPMI 1640 supplemented with 10 % FBS were loaded into polyvinylidene difluoride hollow fibres (Spectrum Laboratories, Laguna Hills, CA, USA) using an 18-gauge needle at a seeding density of $3 \times 10^7$ cells/ml with BD Matrigel™ Basement Membrane Matrix (Becton Dickinson Biosciences, San Jose, CA, USA). The fibres were cut into about 2-cm pieces, the ends were heat-sealed and the fibres were implanted into the nude mice that were 6-8 weeks of age. A total of 20-24 fibres were implanted in bundles at different regions subcutaneously (s.c.) at the back of each animal. Serum PSA was monitored weekly after implantation. Seven days after implantation of the fibres, castration of mice was performed by making a small incision in the scrotum to excise each testicle after ligation of the cord. To close the incision, surgical suture was used. A group of 5 mice were left intact from any major surgical procedure. For two other groups of 5 procedural control mice, a mock castration was performed by making the incision without removal of the testicles or a testosterone pellet (2.5 mg; Innovative Research of America, Sarasota, FL, USA) was added s.c. to the back of the mice anterior
to the fibres upon castration. An equal number of fibres from each animal was retrieved at each of the indicated time points. LNCaP cells within the fibres were harvested in ice cold PBS and subjected to total RNA isolation or subcellular protein extraction. Except for collection of blood samples, mice were anesthetised with isofluorane (Abbott Laboratories, Montreal, QB, Canada) administered by a vaporizer before any invasive procedure.

### 2.2.3 LNCaP xenograft model

LNCaP cells suspended in RPMI 1640 with 5 % (v/v) FBS and 50 % (v/v) Growth Factor-Reduced Matrigel™ were injected and inoculated via 27-gauge needle s.c. into the backs of male NOD-SCID mice, 6-8 weeks old. Tumour volume and serum PSA were monitored weekly after inoculation. The tumours were measured with callipers and their volumes were calculated by the formula: length × width × height × 0.5236. When each tumour averaged ≈ 100 mm³ in volume, the animal was castrated in the same manner as described for the hollow fibre model. Mice were anesthetised with isofluorane administered by a vaporizer before castration. At the time points indicated, mice were sacrificed using CO₂ gas, and the tumours were excised and prepared for immunohistochemistry.

### 2.2.4 Serum PSA levels

Blood samples were obtained from mice weekly subsequent to implantation of fibres (i.e. hollow fibre model) or inoculation (i.e. xenografts) by a small incision in the dorsal tail vein using a sterile scalpel, and 50 μl was collected in a hematocrit capillary tube. The IMx® Total PSA Assay (Abbott Laboratories), an enzymatic immunoassay kit, was used to determine the serum PSA levels according to the manufacturer’s protocol.
The mouse sera were diluted 10 times in diluent to perform the assay. For the hollow fibre model, the serum levels were normalized to the number of fibres in the mice at the time of the assay.

### 2.2.5 Immunohistochemistry

Xenograft tumours were fixed in formalin and embedded in paraffin blocks. Tissue sections (5 µm thick) were deparaffinised and rehydrated in deionized water. These sections were pretreated for antigen retrieval by heating in microwave and applying Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA, USA) and soaked in 3 % H₂O₂ to block endogenous peroxidase activity. After washing with water and PBS, the samples were blocked in Clear Vision™ Immunohistochemistry (IHC) Blocking Solution (ImmunoVision Technologies, Brisbane, CA, USA). The slides were immunostained with anti-p65 antibody (C-20; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at 1:100 in PBS overnight at 4 °C. As negative controls, rabbit immunoglobulin (Vector Laboratories, Burlingame, CA, USA) was used to replace the primary antibody. The VECTASTAIN® ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used for detection. Peroxidase activity was localized with 3,3'-diaminobenzidin, and the sections were counterstained with hematoxyline before dehydration and mounting. The slides were examined using a Zeiss AxioPlan 2 Microscope (Carl Zeiss, Toronto, ON, Canada) and images were taken. The same sections of the same animals were stripped and reprobed with anti-human NAIP antibody (R&D Systems, Minneapolis, MN, USA) at 1:50 in PBS following the same procedure.

### 2.2.6 In-vitro androgen deprivation

LNCaP cells (1.5 x 10⁶) were plated in 10-cm dishes. After 24 h, the media was
replaced with RPMI 1640 supplemented with 5 % charcoal-stripped bovine serum and 10 nM DHT. After 20 h of culture in the presence of DHT, the cells were washed with serum-free medium (SFM) to remove residual DHT. For *in-vitro* androgen deprivation, the media was replaced with fresh SFM and the cells were cultured in the androgen-deprived environment for another 27 h before harvesting. Control cells were maintained in SFM supplemented with 10 nM DHT after washing. Cells were harvested and total RNA was isolated.

### 2.2.7 RNA extraction and RT-PCR

Total RNA was extracted from cells using Trizol® Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription (RT-PCR) and real-time quantitative PCR (qPCR) were performed separately. Poly(A)$^+$ RNA was reverse transcribed using oligo(dT) and the SuperScript™ III First-Strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. cDNA was diluted 10 times after reverse transcription. Subsequent qPCRs were performed using 1 μl of the diluted cDNA as template.

### 2.2.8 Real-time quantitative PCR analysis

qPCR was performed in triplicates for each biological sample with Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen) according to the manufacturer’s instructions using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). As listed in Table 2.1, primers were designed to generate a PCR products of <200 bp. 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 15 s at 72 °C. Levels of expression were normalized to the *glyceraldehyde-3-phosphate*
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Primers</th>
<th>Antisense Primers</th>
<th>Size</th>
</tr>
</thead>
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<tr>
<td>c-IAP1</td>
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</tr>
<tr>
<td>XIAP</td>
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<td>110</td>
</tr>
<tr>
<td>Survivin</td>
<td>TCCGGTTGGCGCTTTCTT</td>
<td>TCTCTTATTTGTTGCTTTTGC</td>
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</table>

Table 2.1. Primers for gene expression analyses using qPCR. The common name of each gene, sequences of sense and antisense in 5' to 3' orientation and size of the specific product size (in base pairs) are shown. The sequences of c-IAP1 primers and survivin primers were obtained from Primer Bank (http://pga.mgh.harvard.edu/primerbank/) and Real Time PCR Primer and Probe Database (http://medgen.ugent.be/rtprimerdb/) respectively.
dehydrogenase (GAPDH) housekeeping gene.

### 2.2.9 Subcellular protein extracts preparation

LNCaP cells were plated at $3.5 \times 10^6$ per 15-cm dish. After 24 h, the media was replaced with SFM. After 24 h, the cells were treated with SFM supplemented with BSA (1 mg/ml) in the presence or absence of TNF-α (10 ng/ml), an inducer of NF-κB activity, and harvested after 15 min in ice cold PBS. PC3 cells and DU145 cells were plated at $3 \times 10^6$ per 15-cm dish and harvested after 24 h in ice cold PBS. The protein extraction protocol was modified from that of Andrews and Faller (32). Briefly, cells were allowed to swell and lysed in 1 ml hypotonic buffer containing 10 mM KCl, 10 mM HEPES [pH 7.9], 0.5 % (v/v) NP-40, 1.5 mM MgCl$_2$, 0.5 mM DTT, 1× Complete™ EDTA-free protease inhibitor (Roche Diagnostics), 1× phosphatase inhibitor cocktails 1 & 2 (Sigma-Aldrich, St. Louis, MO, USA) for 10 min followed by vigorous vortexing for 10 s. Cells were pelleted for 30 s. The pellet was resuspended and washed in 1 ml of fresh hypotonic buffer and centrifuged for 30 s. The supernatant (i.e. cytosolic fraction) was removed to a fresh 1.5 ml tube and frozen at -80 °C. The pellet was resuspended in 100 μl high salt buffer containing 420 mM NaCl, 20 mM HEPES [pH 7.9], 25 % (v/v) glycerol, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM DTT, 1× protease inhibitor, 1× phosphatase inhibitor cocktails 1 & 2, and the suspension was vigorously shaken on ice for 30 min with occasional vortexing. After centrifugation for 5 min, the supernatant (i.e. nuclear fraction) was removed and stored at -80 °C. The protein concentration of the extracts was quantified by Bradford assay using protein assay dye reagent (Bio-Rad, Hercules, CA, USA) as measured at 595 nm using the VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). The proteins were subjected to Western blot analysis and
2.2.10 Western blot analysis

For the detection of the subunits of NF-κB, p65 and p50, nuclear extracts (20 μg proteins) were denatured in 2× SDS-PAGE loading buffer and boiled for 5 min prior to being separated by electrophoresis through 10 % SDS-PAGE gel and transferred onto Hybond™-C Extra nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). After blocking with Odyssey® blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) in PBS (1:1) for 1 h, membranes were probed with anti-p65 (C-20) and anti-p50 (H-119) antibodies from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) at 1:1,000 and monoclonal anti-β-actin antibody (ab8226) from Abcam (Cambridge, MA, USA) at 1:1,000 in Odyssey® blocking buffer in PBS (1:1) with 0.1% Tween® 20. Subsequent to incubation with primary antibodies overnight at 4 °C, the blots were incubated with appropriate IRDye® secondary antibodies (LI-COR Biosciences) at 1:10,000 and visualized with Odyssey® Infrared Imaging System (LI-COR Biosciences).

For the detection of NAIP, cytosolic extracts (60 μg proteins) were denatured in 4× SDS-PAGE loading buffer and boiled for 5 min prior to being separated by electrophoresis through 9 % SDS-PAGE gel and transferred onto Immobilon™ PVDF membrane (Millipore, Billerica, MA, USA) overnight at 4 °C. After blocking with 5 % dry skim milk (w/v) TBS blotto with 0.1% Tween® 20, membranes were probed with anti-human NAIP antibody (ab25968) from Abcam at 1:500. Subsequent to incubation with primary antibodies in blotto overnight at 4 °C, the blots were incubated with appropriate secondary horseradish peroxidase-conjugated antibody (Santa Cruz) at 1:5,000 and developed with SuperSignal® West Femto Maximum Sensitivity Substrate.
(Pierce, Rockford, IL). The membranes were stripped with Restore™ Western Blot Stripping Buffer according to the manufacturer's instructions and reprobed with monoclonal anti-β-actin antibody (ab8226) from Abcam.

2.2.11 Electrophoretic mobility shift assay

Double-stranded oligonucleotides (22 base pairs), NF-κB consensus oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3'), NF-κB mutant oligonucleotide (5'-AGTTGAGGGCGACTTTCCCAGGC-3'), κB-like-1 consensus oligonucleotide (5'-ATTCAGGGGGGTACAGTGCAT-3'), κB-like-1 mutant oligonucleotide (5'-ATTCAGGGGGTACAGTGCAT-3'), κB-like-2 consensus oligonucleotide (5'-AGGATGGGGGATTTACAGTCAT-3'), κB-like-2 mutant oligonucleotide (5'-AGGATGGGCGATTTACAGTCAT-3'), κB-like-3 consensus oligonucleotide (5'-ATAGAAGGGTAGTTTCCCAGGCT-3'), κB-like-3 mutant oligonucleotide (5'-ATAGAAGGGTACTTCCCAGGCT-3'), were radiolabeled and used for EMSA. The NF-κB consensus and mutant oligonucleotides were obtained from Santa Cruz Biotechnologies. Briefly, the oligonucleotides were annealed (for all custom-designed oligonucleotides) and labelled with Redivue™ adenosine 5'-[γ-32P] using T4 polynucleotide kinase (Invitrogen) at 37 °C for 45 min. After labelling, the oligonucleotides were purified with ProbeQuant™ G-50 Micro Columns (Amersham Biosciences) prior to use in EMSAs.

For DNA-binding, nuclear extracts (5-10 μg proteins) were incubated in a final volume of 25-30 μl of 10 mM HEPES (pH 7.9), 80 mM NaCl, 10 % (v/v) glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1 μg poly(dI-dC) (Amersham Biosciences) with the 32P-labeled oligonucleotides for 30 min at room temperature. For supershift assays, the
nuclear extracts were preincubated with 2 μg of anti-p65 antibody (AX) or anti-p50 antibody (C-19X) from Santa Cruz Biotechnologies for 30 min at room temperature. For competition binding assays, the nuclear extracts were preincubated with 250-fold excess unlabeled oligonucleotides for 30 min at room temperature. The protein-DNA complexes were resolved in a non-denaturing 4.5% polyacrylamide gel containing 2.5% glycerol and 0.5× TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA) at room temperature. The gels were dried, exposed on Phosphor screen (Molecular Dynamics, Sunnyvale, CA, USA) and subjected to autoradiography using STORM™ 860 PhosphorImager (Molecular Dynamics). The density of the shifted band that corresponds to a protein-DNA complex was analyzed using ImageQuant® 5.2.

2.2.12 NF-κB luciferase reporter activity assay

LNCaP cells were plated at 3 × 10⁵ cells per well in six-well plates. After 24 h, a NF-κB luciferase reporter vector (Panomics, Fremont, CA, USA) containing six tandem copies of the consensus κB site were transiently transfected into the cells at 3 μg per well using Lipofectin reagent (Invitrogen). After 24 h, cells were treated as indicated in figure legends for an additional 24 h. The cells were then harvested in 1× Passive Lysis Buffer (Promega, Madison, WI, USA) and frozen at -80 °C. Prior to measurement of luciferase activity, the lysates were thawed and the debris was spun down at 12,500 rpm for 5 min at 4 °C. Luciferase activity in the cell lysates (i.e. supernatant) collected was measured using the Luciferase Assay Reagent (Promega, Madison, WI, USA) as detected by a multifunctional microplate reader, Safire²™ (Tecan, Grödig, Austria). The luciferase activity was normalized to the protein concentration in each well as determined using Bradford assay.
2.2.13 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was modified from that of Narayanan et al. (33). LNCaP cells were plated at $3.5 \times 10^6$ in 15-cm dish. After 24 h, the media was replaced with SFM. After 24 h, the cells were treated with SFM supplemented with BSA (1 mg/ml) in the presence or absence of TNF-α (10 ng/ml) for 30 min. 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 1× 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], 1× protease inhibitor). After lysis on ice for 10 min, the cell extract was sonicated (Sonicator 3000 Ultrasonic Liquid Processor, Misonix, Farmingdale, NY, USA) in an ice water bath ten times for 30 s each with an output level of 1. The average length of the sheared DNA fragments was 200-800 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). 100 µl was reserved as input. After preclearing 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-p65 antibody (C-20; Santa Cruz Biotechnologies) or rabbit 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) according to the manufacturer's instructions. The promoter and intronic regions of IκB-α and NAIP were amplified using the primers in Table 2.2 using qPCR. 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 from dividing the arbitrary qPCR numbers obtained by each sample by that of the input.

2.2.14 Statistical analysis

Data are presented as the mean ± standard deviation (SD). To assess statistical significance of differences, unpaired Student's t-test was used for statistical analysis, except for the analyses of experiments using the in vivo hollow fibre samples where 2-way Analysis of Variance (ANOVA) was applied. P-value ≤ 0.05 was considered significant as indicated by asterisks.

2.3 RESULTS

2.3.1 IAP genes are differentially expressed in vivo in response to castration

To assess the expression of the IAPs genes, NAIP, c-IAP₁, XIAP and survivin, in response to androgen deprivation, the in vivo LNCaP hollow fibre model was applied. The model provides human CaP cells that are free from contamination with host cells by maintaining the cells in hollow fibres implanted subcutaneously in nude mice (31). LNCaP cells are the best-characterized CaP cell line that secretes prostate-specific antigen (PSA), the biomarker for CaP as well as other prostatic diseases, and responds, at
<table>
<thead>
<tr>
<th>Binding Site</th>
<th>Primers</th>
<th>Size</th>
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<tr>
<td>lκB-α</td>
<td>GACGACCCAATTCAATCG</td>
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<td>NAIP_κB-like-3</td>
<td>GAGCTGTGATTGTGCCATTG</td>
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</tr>
</tbody>
</table>

Table 2.2 Primers used in the ChIP assay. qPCR assays were established for the κB-like sites on *NAIP*. The κB-like sites in the promoter and the second intron of *NAIP*, the sequences of the sense and antisense in 5' to 3' orientation and the size of the specific amplified product (in base pairs) are shown. The primer pair for the NF-κB binding site on lκB-α (34) was included as a positive control.
least initially, to androgen deprivation. Serum PSA levels were monitored weekly subsequent to implantation of hollow fibres. As shown in Fig. 2.1A, the serum PSA levels of hosts dropped by an average of 57% by 10 days after castration. Total RNA was isolated from LNCaP cells retrieved before castration (i.e. 7 days after implantation) and 10 days after castration (i.e. 17 days after implantation). Levels of IAP mRNA, \( NAIP, c-LAP1, XIAP \) and \( survivin \), were assessed using qPCR (Fig. 2.1B). In parallel to its serum level, \( PSA \) was down-regulated in response to castration as expected, consistent with clinical representation (35,36). The levels of mRNA for the IAPs, except \( survivin \), were increased in response to castration. However, only the above 2-fold increase in \( NAIP \) expression was statistically significant. Thus, androgen deprivation by castration increases the levels of \( NAIP \) mRNA in CaP cells \textit{in vivo}.

### 2.3.2 Androgen alters the levels of NAIP mRNA and the transcriptional activity of NF-\( \kappa \)B in prostate cancer cells

To test whether the increased levels of \( NAIP \) mRNA measured from \textit{in vivo} samples were due to decreased androgen, we isolated total RNA from \textit{in vitro} LNCaP cells that were subjected to 28 h of androgen deprivation subsequent to a 24-h pre-treatment in 10 nM DHT. The expression of \( PSA \) (i.e. as a control) and \( NAIP \) were evaluated using qPCR (Fig. 2.2A). The levels of \( PSA \) mRNA were decreased as expected while levels of \( NAIP \) mRNA were increased in the cells subjected to androgen deprivation as compared to cells without androgen deprivation. These results are consistent with the \textit{in vivo} expression of \( NAIP \) in response to castration, and the data suggest that androgen deprivation increases the expression of \( NAIP \) in CaP cells.

Previous studies demonstrate crosstalk between the androgen receptor (AR) and
Figure 2.1. Differential expression levels of IAP genes in response to castration in the LNCaP hollow fibre model. (A) Effects of castration on nude mice bearing LNCaP cells were monitored by measuring serum PSA levels. The serum PSA levels were normalized to the number of hollow fibres remaining in the hosts. Pre-castrate levels of PSA were in the range of 14-18 ng/ml and set at 100% for each host. The bars represent the mean percentages of the pre-castrate serum PSA levels ± SD (n = 4) on 7 d after implantation (pre-Cx) and 10 d after castration (post-Cx). (B) Total RNA was isolated from the in vivo LNCaP hollow fibre models 7 d after implantation (pre-Cx) and 10 d after castration (post-Cx). qPCR was performed with primers specific for each gene. The expression levels of each gene were normalized to the mRNA levels of GAPDH in each biological replicate. Data are calculated as mean fold change relative to the pre-castrate levels (set as 1-fold) from three different animals (n = 3). PSA, an androgen-regulated gene, was assessed as a positive control. The levels of significance between cells obtained from each time point were determined by 2-way ANOVA: * P ≤ 0.05, ** P ≤ 0.01.
Figure 2.2. Androgen inhibits expression of NAIP and the transcriptional activity of NF-κB. (A) LNCaP cells were maintained in 10 nM DHT for 24 h prior to separating them into two groups (n = 3 for each group). While one group was maintained in 10 nM DHT (no AD), the other group was replenished with SFM devoid of androgen (AD). After 27 h of culture, total RNA was isolated from the cells and the expression levels of PSA and NAIP were assessed using qPCR. Levels of mRNA for these genes were normalized to levels of GAPDH mRNA in each replicate. PSA mRNA was included as a positive control. (B) LNCaP cells were transfected with the NF-κB luciferase reporter gene construct (3 μg). The cells were treated with 10 ng/ml TNF-α, 10 nM R1881 or SFM with vehicle (i.e. BSA for TNF-α treatment and ethanol for R1881 treatment). After 24 h of culture, the cells were harvested and luciferase activities were measured. Luciferase activities were normalized to the protein levels in each well. AD, androgen deprivation. The means ± SD of triplicates are shown. The levels of significance between cells subjected to different treatments were determined by Student’s t-test: * P ≤ 0.05, ** P ≤ 0.01. Representative results of multiple experiments are shown.
NF-κB in an androgen-responsive fashion (37,38). To confirm if androgen has a direct effect on NF-κB in our model, the transcriptional activity of NF-κB was evaluated through the use of a luciferase reporter construct with six tandem κB sites in the promoter region. The reporter was transfected into LNCaP cells which were then treated with 10 nM R1881, a synthetic androgen. Treatment of cells with TNF-α was used as a positive control to induce NF-κB transcriptional activity. As shown in Fig. 2.2B, the treatment of LNCaP cells with R1881 for 24 h resulted in a 27% reduction in NF-κB activity as compared to the vehicle control. Thus, androgen inhibits the transcriptional activity of NF-κB.

2.3.3 Expression and DNA-binding activity of NF-κB in prostate cancer cells maintained in vitro

To further investigate the influence of androgen and AR on NF-κB, the levels of nuclear NF-κB protein and the DNA-binding activity of NF-κB were examined using human CaP cell lines with different androgen requirements and AR status. Unlike LNCaP cells, PC3 cells and DU145 cells are androgen-insensitive and lack a functional AR. Nuclear extracts obtained from unstimulated LNCaP cells, TNF-α-stimulated LNCaP cells, PC3 cells and DU145 cells were subjected to Western blot analysis to determine relative protein levels of the NF-κB subunits, p65 and p50. These nuclear extracts were also used to determine the DNA-binding activity of NF-κB using EMSA. Nuclear extract from TNF-α-stimulated LNCaP cells were included as a positive control. The specificity of the shifted bands corresponding to the different NF-κB-DNA complexes was confirmed by supershift assay in the presence of anti-p65 or -p50 antibody or by competition assay in the presence of excess non-labelled consensus or mutant
oligonucleotide probes. Different CaP cell lines exhibited varying levels of NF-κB proteins in nuclear extracts (Fig. 2.3A). As shown in Fig. 2.3B, the differential levels of p65 and p50 proteins corresponded with differential NF-κB-DNA-binding activity in the nuclear proteins of in vitro CaP cells. Specifically, the androgen-insensitive DU145 cells exhibited the highest nuclear levels of protein and DNA-binding activity of NF-κB amongst the unstimulated CaP cells. Interestingly, the nuclear levels of protein and DNA-binding activity of NF-κB in unstimulated cells were DU145 > PC3 > LNCaP. This observation implies that DNA-binding activity of NF-κB is related to the nuclear localization and levels of the NF-κB subunits present in the nuclear proteins. Moreover, as expected, these results also suggest that the nuclear levels of protein and DNA-binding activity of NF-κB may involve multiple mechanisms in addition to androgen requirement and AR status in vitro.

2.3.4 Expression of NAIP and NF-κB in vivo in response to castration

Correlation between expression of NAIP and p65, the subunit of NF-κB with a transactivation domain, in response to androgen deprivation was examined by IHC using LNCaP xenografts harvested from hosts before castration and 10 days after castration. As shown in Fig. 2.4, the levels of p65 protein increased slightly while the levels of NAIP protein increased much more noticeably in response to castration of the host. Specifically, the increases of NAIP protein localized near the periphery of the tumour tissue near blood vessels, whereas the increase of p65 protein was relatively homogeneous within the tumour tissue. However, the heterogeneity of NAIP staining may be attributed to macrophages residing in these tissues (39) and was thus further examined using the hollow fibre model that restricts infiltration of cells as described below. Immunostaining
Figure 2.3. Differential expression and DNA-binding activity of NF-κB in CaP cells.

Nuclear extracts were prepared from untreated LNCaP cells, TNF-α-stimulated LNCaP cells (i.e. positive control), PC3 cells and DU145 cells. (A) Levels of NF-κB protein in the nucleus were measured using Western blot analysis with anti-p65, anti-p50 and anti-β-actin (as loading control) antibodies and nuclear extracts (20 μg proteins) from LNCaP, PC3 and DU145 cells. (B) DNA-binding activity of NF-κB was assessed using EMSA. EMSAs were performed by incubation of the nuclear extracts (10 μg proteins) of CaP cell lines with 32P-labelled oligonucleotide probe containing a consensus NF-κB DNA-binding motif. To ensure specificity, nuclear extracts were preincubated with antibodies (Ab) for p65 or p50 or an excess of non-labelled consensus (cNF-κB) or mutant (mNF-κB) NF-κB oligonucleotides. SS, supershifted antibody-protein-DNA complex. Representative results of multiple experiments are shown.
Figure 2.4. Levels of NAIP and NF-κB protein in LNCaP tumours before and after castration of the hosts. LNCaP xenograft tumour tissues were obtained from mice sacrificed when the tumour averaged \( \approx 100 \text{ mm}^3 \) in volume before castration (pre-Cx) and 10 days after castration (post-Cx). Sections of tumour tissues were immunostained (diaminobenzidine with hematoxylin counterstaining) for NAIP and the p65 subunit of NF-κB. The box within each image is a cropped region from the upper right-hand corner of the image to enable a closer view of the expression levels. Tumour tissues from the same LNCaP xenografts were incubated with secondary antibody only prior to staining with hematoxylin as a control. Magnification, 400×.
with secondary anti-rabbit antibody alone (Fig. 2.4) indicated that the positive staining was not due to non-specificity of secondary antibodies or the IHC procedures.

The expression of NAIP and NF-κB were also examined in the subcellular extracts of pure population of CaP cells obtained from the LNCaP hollow fibre model as described earlier using Western blot analysis. On the one hand, levels of NAIP protein at approximately 160-kDa were consistently elevated in three hosts subsequent to castration (Fig. 2.5A). The protein levels correlated with the transcript levels of NAIP in CaP cells devoid of macrophages harvested from the hollow fibre model. On the other hand, the protein levels of the NF-κB subunits, p65 and p50, remain constant in the nucleus upon castration of the hosts (Fig. 2.5B). Three groups of procedural control mice (i.e. intact, mock castration, castration with the addition of testosterone pellet) were maintained throughout the same length of time as the hollow fibre model experiment with castrated mice. Like the castrated mice, serum PSA levels were monitored weekly subsequent to implantation (Fig. 2.6A) and the protein levels of NF-κB were determined (Fig. 2.6B-D). The results obtained from the procedural control mice confirmed that the results obtained from the castrated mice were not due to artefacts from the invasive surgical procedures performed on the hosts, but in fact due to the castration and reduction of androgen.

2.3.5 DNA-binding activity of NF-κB in prostate cancer cells in vivo

To study the effect of castration on DNA-binding activity of NF-κB, nuclear extracts isolated from the LNCaP hollow fibre model before castration and after castration were subjected to EMSA (Fig. 2.7A). The specificity of the shifted bands corresponding to the different NF-κB-DNA complexes was confirmed by supershift assay in the presence of anti-p65 or -p50 antibody or competition assay in the presence of
Figure 2.5. Levels of NAIP and NF-κB protein in LNCaP cells from the LNCaP hollow fibre model before and after castration of the hosts. (A) Cytosolic extracts (60 μg proteins) from LNCaP cells harvested from the hollow fibre model after implantation (pre-Cx) and 10 d after castration (post-Cx) were subjected to Western blot analysis with anti-NAIP antibody. Bands at 160 kDa corresponded to NAIP protein. The membrane was stripped and reprobed with anti-β-actin as a loading control. (B) The nuclear extracts (20 μg protein) from LNCaP cells harvested from the hollow fibre model were also investigated in Western blot analysis (Odyssey® detection method) with anti-p65, anti-p50 and anti-β-actin (as a loading control) antibodies. Solid bars mark protein extracts from the same animal before and after castration (n = 3). All images are representative of biological replicates and multiple experiments.
Figure 2.6. Levels of NAIP and NF-κB protein in LNCaP cells from the LNCaP hollow fibre model in procedural control mice. (A) Serum PSA levels were monitored in the LNCaP hollow fibre model. The serum PSA levels were normalized to the number of hollow fibres remaining in the hosts. Pre-castrate levels of PSA were in the range of 14-18 ng/ml. The solid bars represent the mean percentages of the pre-castrate serum PSA levels ± SD (n = 4) on 7 d and 17 d after implantation. Intact, no surgical procedures; Mock Cx, surgery but no removal of gonads; Cx + T, castration and immediate testosterone replacement. Mock castration or castration was performed on day 7. The levels of significance between cells obtained from each time point were determined by 2-way ANOVA: * P ≤ 0.05. Nuclear extracts were prepared from LNCaP cells harvested from the procedural control mice at the same time points used for castrated mice, 7d and 17 d after implantation of the hollow fibres. (B) Intact, no major surgical procedure was performed on the mice throughout the experiment after implantation of the fibres. (C) Mock Cx, a small incision in the scrotum was made without removal of the testicles; (D) Cx + T, a testosterone pellet was added to each mouse upon castration on 7 d after implantation. Levels of NF-κB in the nuclear extracts (20 μg proteins) were measured by Western blot analysis using anti-p65, anti-p50 and anti-β-actin (as loading control) antibodies. All images are representative of biological replicates.
Figure 2.7. Increased DNA-binding activity of NF-κB in response to castration of the hosts. (A) EMSAs were performed by incubating nuclear extracts (5 μg protein) obtained from the in vivo LNCaP hollow fibre models with 32P-labelled oligonucleotide probe containing a consensus NF-κB DNA-binding motif. Controls for binding specificity include nuclear extracts of in vitro untreated LNCaP cells and TNF-α-stimulated LNCaP cells that were preincubated with antibodies (Ab) for p65 or p50 or an excess of non-labelled consensus (cNF-κB) or mutant (mNF-κB) NF-κB oligonucleotides. SS, supershifted antibody-protein-DNA complex. (B) EMSAs were performed using matched samples (pre-Cx and post-Cx) from 3 different animals (n = 3). The bands corresponding to the NF-κB-DNA complex in EMSAs were quantified using densitometry. The solid bars represent the mean fold-change of the biological triplicates of post-Cx as compared with the pre-Cx levels (set as 1-fold). The levels of significance between cells obtained from each time point were determined by 2-way ANOVA: * P ≤ 0.05.
excess non-labelled consensus or mutant oligonucleotide probes. The densities of the bands corresponding to the NF-κB-DNA complexes were quantified and expressed as average fold-induction relative to the pre-castrate levels from biological triplicates (Fig. 2.7B). Intriguingly, despite similar nuclear expression of the NF-κB subunits (Fig. 2.5B), NF-κB DNA-binding activity was elevated significantly in response to castration. Importantly, the changes in DNA-binding activity correlated with the expression of NAIP at the mRNA and protein levels (Fig. 2.1B & 2.5A). Nuclear extracts from procedural control mice obtained at different times (described earlier) had similar NF-κB DNA-binding activity as shown using EMSA (Fig. 2.8A-C), thereby eliminating the possibility that the results with castrated mice were due to artefacts from surgical procedures. Thus, castration and the reduction of androgen increase NF-κB DNA-binding activity.

### 2.3.6 Binding of NF-κB in the NAIP locus

NF-κB may promote anti-apoptotic properties via transcriptional regulation of anti-apoptotic genes. In fact, some members of the IAP family, *c-IAP2* (40) and *survivin* (18), are direct binding targets of NF-κB. Recent studies suggest that the expression of *NAIP* may be directly regulated by NF-κB (41,42), but biological confirmation has not been shown. Here we observed that the increase in NAIP expression correlated with NF-κB-DNA-binding activity in response to androgen deprivation. Based upon this correlation, we sought to determine a potential role for NF-κB in the transcriptional regulation of *NAIP*. The human *NAIP* locus [GeneBank Accession No. U19251] (43) on chromosome 5 was examined for putative κB site, GGGRNNYYCC [R = purine, N = any base, Y = pyrimidine] using ConSite [http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/] (44) with 80 % cut-off. As shown in Table 2.3, initial screening
Figure 2.8. DNA-binding activity of NF-κB in procedural control mice. Nuclear extracts were prepared from LNCaP cells of the in vivo hollow fibre implanted in procedural control mice at the same time points used for castrated mice, 7 d and 17 d after implantation of the hollow fibres. (a) Intact, no surgical procedure was performed on the mice throughout the experiment; (b) Mock Cx, a small incision in the scrotum was made without removal of the testicles on 7 d after implantation; (c) Cx + Testosterone, a testosterone pellet was added to each mouse upon castration on 7 d after implantation. EMSAs were performed by incubating the nuclear extracts (5 μg proteins) with 32P-labelled oligonucleotide probe containing a consensus NF-κB DNA-binding motif. To ensure binding specificity, nuclear proteins of in vitro untreated LNCaP cells and TNF-α-treated LNCaP cells were preincubated with antibodies (Ab) for p65 or p50 or an excess of non-labelled consensus (cNF-κB) or mutant (mNF-κB) NF-κB oligonucleotides. SS, supershifted antibody-protein-DNA complex. EMSAs were performed using in vivo samples from biological triplicates. All images are representative of biological replicates.
Table 2.3. NF-κB-binding sites in the *NAIP* promoter and second intron. The sequences and location of κB-like sites in the promoter and second intron of *NAIP*. Identity indicates the number of nucleotides which are identical to the 10 nucleotides of the NF-κB consensus sequence. Coordinates determined from the sequence are based on GeneBank Accession No. U19251 (43). R = any purine, N = any nucleotide, Y = any pyrimidine.

<table>
<thead>
<tr>
<th>Site</th>
<th>Coordinates</th>
<th>Sequence (5'—3')</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consensus</td>
<td></td>
<td>GGGRNYYYCC</td>
<td></td>
</tr>
<tr>
<td>κB-oligo</td>
<td></td>
<td>GGGACTTTCC</td>
<td></td>
</tr>
<tr>
<td>κB-like-1</td>
<td>-1520 to -1511</td>
<td>GGGGATTAC</td>
<td>9/10</td>
</tr>
<tr>
<td>κB-like-2</td>
<td>-241 to -232</td>
<td>GGGGCTATCC</td>
<td>9/10</td>
</tr>
<tr>
<td>κB-like-3</td>
<td>498 to 507bp</td>
<td>GGTAATTTCC</td>
<td>9/10</td>
</tr>
<tr>
<td></td>
<td>5' to exon 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
with ConSite revealed two κB-like sites in the promoter region and one κB-like site within the second intron of NAIP that are highly homologous to the consensus κB site. To test NF-κB binding on these κB-like sequences, EMSA was employed using custom oligonucleotide probes containing the κB-like sites and nuclear extracts from in vitro and in vivo (i.e. from the hollow fibre model) LNCaP cells (Fig. 2.9A-C). Mutant oligonucleotide probe with a mutated base pair for each custom probe was used in competition assay to confirm the specificity of the binding sequence in the NF-κB-DNA complexes. NF-κB from nuclear extracts demonstrated enhanced DNA-binding activity on all κB-like sites (Fig. 2.9A-C) similar to that achieved with the consensus κB sites (Fig. 2.7A). NF-κB complexes binding on these κB-like sites suggested that the expression of NAIP may be transcriptionally regulated by NF-κB binding to these sites located within the promoter and intronic regions in the gene locus.

To validate the physiological relevance of NF-κB binding on these κB-like sites in the promoter and intronic regions of NAIP, ChIP assays were performed on the κB-like sites in LNCaP cells stimulated with TNF-α. Subsequent to cross-linking of protein-DNA complexes and sonication of the nuclear proteins, immunoprecipitations were performed using an antibody specific for p65. Primers specific for the κB-like sites on the promoter and intronic regions of NAIP were used to amplify immunoprecipitated DNA using qPCR. Immunoprecipitation of sonicated nuclear extracts with rabbit IgG was performed in place of anti-p65 antibody as controls for no antibody. As shown in Fig. 2.9D, the recruitment of p65 was modestly enhanced on all κB-like sites in the NAIP locus in response to TNF-α as compared with the recruitment in the vehicle control. Only the recruitment on κB-like-3 site located within the second intronic region demonstrated
Figure 2.9. NF-κB is recruited to the regulatory elements in the NAIP locus. Nuclear proteins from untreated LNCaP cells, TNF-α-treated LNCaP cells and the LNCaP cells obtained from the in vivo hollow fibre models at the time points of pre-castration (pre-Cx) and 10 d after castration (post-Cx) were used to perform EMSA using $^{32}$P-labelled oligonucleotide probes containing (A) κB-like-1 site, (B) κB-like-2 site and (C) κB-like-3 site in the NAIP locus. EMSA was performed using in vivo samples from biological triplicates; all images are representative of the replicates. (D) LNCaP cells were treated with 10 ng/ml TNF-α (or BSA only as the vehicle control) for 30 min and used in ChIP assay with rabbit IgG (i.e. no antibody control) or anti-p65 antibody as described in Materials and Methods. Eluted DNA fragments were then purified and used for qPCR with primers designed to amplify the IκB-α enhancer (i.e. positive control) or the κB-like sites in the promoter and second intron of NAIP locus. The percentage input of each sample was averaged from triplicates. The levels of significance between cells subjected to different treatments were determined by Student’s $t$-test: * $P \leq 0.05$, ** $P \leq 0.01$. 
statistically significant increase in physical association.

2.4 DISCUSSION

Despite much effort in investigating the hormonal progression of CaP in the past decades, the underlying molecular mechanism remains elusive. It is postulated that androgen deprivation may lead to new outgrowth of cells with distinct molecular properties that are resistant to apoptosis (45). In the current study, we have profiled the expression of IAPs, which are known for their anti-apoptotic functions, in response to androgen deprivation in the in vivo hollow fibre model, and we have investigated the possible role of NAIP in the survival of CaP cells in androgen-deprived conditions as a direct regulatory target of NF-κB.

Accumulating evidence from recent studies supports the role of IAPs in CaP as anti-apoptotic regulators of caspase (reviewed in (46)). Here we identified that one of these IAP genes, NAIP, was significantly up-regulated in the in vivo LNCaP hollow fibre model in response to castration of the host. Unexpectedly, the expression of survivin was significantly down-regulated and the expression of c-IAPl and XIAP were not significantly altered. c-IAPl and XIAP are known to be transcriptionally regulated by NF-κB (19,20), and thus the lack of statistical significance may be due to the subtle changes in NF-κB activity demonstrated at the designated time points during the hormonal progression or differences in the kinetics of individual genes. Alternatively, the expression of these genes and survivin might require factor(s) which facilitate the NF-κB signalling and the transcriptional regulation may vary depending on the cellular context and experimental conditions. Collecting in vivo samples from additional time-points after castration may help to address these unknowns by providing a comprehensive profile of
IAPs expression and NF-κB activity during the hormonal progression to androgen independence. Here, only *NAIP* was consistently and significantly differentially expressed in response to androgen ablation of the hosts with the increase in transcript levels corresponding to the protein expression in response to androgen deprivation.

NAIP is the founding member of the human IAPs identified. Its deficiency, as a result of deletions in a gene region, has been primarily associated with the most severe phenotypes of a hereditary neurodegenerative disorder, spinal muscular atrophy (SMA), due to the loss of its neuroprotective activity in motor neurons in the spinal cord (9). Expression of NAIP in tissues that are not exclusively neuronal and not directly associated with SMA suggests functions beyond its neuronal context (39,47). NAIP protects mammalian cells from apoptosis induced by a variety of stimuli (7). As a caspase regulator, native NAIP inhibits caspase-3 and caspase-7, the effector caspases, and associates with caspase-9, the initiator caspase, in the presence of ATP (10,11). The presence of a central nucleotide binding oligomerization domain and a carboxyl-terminal leucine-rich repeat domain might enable NAIP to promote additional cytoprotection and other functions uniquely from other IAP members (11). However, little is known about the regulatory events of NAIP.

In this study, we observed a direct link between NAIP expression and NF-κB DNA-binding activity in response to androgen deprivation. The upregulation of *NAIP* gene expression and its dependence on androgen was validated by *in vitro* experiments controlling for the presence of androgens. Androgen deprivation increased levels of NAIP mRNA, and the complementary experiment showed that the presence of androgen inhibited NF-κB activity. The reduction in NF-κB transcriptional activity in the presence
of androgen as demonstrated by the NF-κB luciferase reporter assay suggests that the differential NF-κB binding activity evident from in vivo LNCaP cells corresponded to the androgen levels in the microenvironment of the CaP cells. This means that when a host bearing CaP cells is castrated, an increase in NF-κB DNA-binding activity should be observed in response to castration. Previously reported crosstalk between AR and NF-κB may suggest the mechanism by which this occurs. AR and p65 were shown to mutually repress the transactivation activity of each protein (37,38). Consistent with these studies, here, transfecting a NF-κB luciferase reporter construct similar to that used by Palvimo et al. (38) in LNCaP cells with endogenous AR yielded similar inhibition in the presence of androgen.

In vitro CaP cell lines demonstrated distinctive nuclear levels and DNA-binding activity of NF-κB that was independent of AR status. These results were consistent with previous reports that nuclear localization of the subunits, p65 and p50, was responsible for the corresponding NF-κB activity associated with the disease progression and androgen-responsiveness of CaP cells (48-51). Curiously, nuclear levels of NF-κB did not correlate to its binding activity when using extracts prepared from samples maintained in vivo in response to castration of the hosts. Thus, NF-κB activity in vivo may be modulated by post-translational modification of the NF-κB subunits, such as phosphorylation and acetylation (reviewed in (52)). Nevertheless, while results from the in vivo LNCaP hollow fibre model suggest that the NAIP expression and NF-κB activity responded to changes in androgen status, results using AR-negative cells suggest that it is important to consider the cellular context when studying the effect of androgen deprivation in CaP cells. Moreover, it would be unreliable to study the effect of androgen
deprivation employing an *in vitro* strategy alone. To this extent, how the AR status would affect NF-κB activity remains a challenging concept to be interpreted since studies which suggested crosstalk between AR and NF-κB signalling pathways were only conducted *in vitro*.

Our observations of elevated levels of NAIP that correlated with the NF-κB binding activity in *in vivo* CaP cells during hormonal progression are consistent with the results of Poma et al. (41) which showed that the novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin, decreased mRNA levels of NAIP in human hepatic cancer cells. They also agree with the findings of Notarbartolo et al. (42) that the multi-drug-resistant leukemia cells with abundant NAIP expression exhibited constitutive activation of NF-κB. These data suggest that NAIP may be transcriptionally regulated by NF-κB. Here, the application of EMSA revealed NF-κB DNA-binding to three previously uncharacterized NF-κB regulatory binding elements in the *NAIP* promoter (about 1.5 kb and 200 bp upstream of transcriptional start site) and intronic (between exon 2 and exon 3) regions. However, ChIP experiments validated increased NF-κB binding *in situ* on only one of these sites. This was the regulatory element in the second intron. Although these ChIP experiments did not show statistically significant increase in NF-κB DNA-binding on the other κB-like sites in the promoter region, they may still be functional κB-like sites merely requiring optimization and/or a different set of experimental conditions. The increase in NF-κB binding on the κB-like sites as demonstrated by ChIP is minimal as compared to the binding on the IκB-α enhancer upon TNF-α stimulation. This is not surprising as *NAIP* expression may be regulated by multiple factors. Previous studies demonstrate that PAX2 (53), a developmental transcription factor and Brn-2 (54), a POU
domain transcription factor may regulate the transcription of \textit{NAIP} via direct binding on putative regulatory elements in the gene locus. However, since the binding of those transcription factors to their respective putative regulatory elements on the \textit{NAIP} locus was only demonstrated in EMSA, an \textit{in vitro} assay, the binding \textit{in situ} has yet to be confirmed using method by which physiological conditions, i.e. chromatin structure, are considered. Yet another possibility is that there are differences in cofactors involved in the transcriptional complexes under different conditions. Furthermore, putative regulatory elements of other transcription factors are yet to be validated (54). Alternatively, NF-κB activity induced by androgen-deprivation may be different from that induced by TNF-α. Together, these findings demonstrate that NF-κB can regulate the transcription of the \textit{NAIP} gene through cis-regulatory elements that resemble the NF-κB consensus binding motif. Intriguingly, the κB-like-3 site which demonstrated the significant increase in binding upon TNF-α stimulation lies 5’ upstream and in close proximity to the constitutive transcription start site within the non-long-terminal-repeat promoter as identified by Romanish et al. (47). The resulting transcript will yield the same protein product as the commonly-cited transcript described by Chen et al. (43). All in all, our observations demonstrate that NAIP may join c-IAP1, c-IAP2 and survivin as NF-κB-regulated IAPs via transcriptional regulation on regulatory elements in the gene locus.

2.5 CONCLUSION AND SIGNIFICANCE

In summary, the current study identified a direct link between the expression of NAIP and NF-κB activity \textit{in vitro} and \textit{in vivo} in response to androgen deprivation in CaP cells and characterized three functional cis-regulatory elements of \textit{NAIP} in the promoter and intronic regions. The elevated expression of NAIP in androgen-depleted conditions,
at least in part, is mediated through NF-κB regulation on the κB-like sites in the gene locus. Our results, coupled with other groups' observations, suggest that NAIP may enhance survival of CaP cells by allowing them to bypass the apoptotic fate in response to androgen deprivation. The enhanced survival may enable the CaP cells to progress to the terminal stage with androgen-independent phenotypes. This mechanism may involve clonal selection for a subpopulation of pro-survival CaP cells that may prevail by increased NF-κB DNA-binding activity in response to androgen withdrawal. Thus, although androgen ablation remains the most effective means to reduce the tumour burden of androgen-dependent disease, our findings reveal a potential mechanism by which the disease may progress to the androgen independent stage. It is hopeful that elucidation of the molecular pro-survival mechanism mediated by NF-κB will translate into improved management of CaP through modulating the anti-apoptotic activity. Further investigation on how NAIP and other IAPs are regulated and expressed is warranted and could lead to development of novel intervention to prevent CaP progression.
2.6 REFERENCES


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3. CONCLUDING CHAPTER

3.1 PERSPECTIVES AND FUTURE DIRECTIONS

Androgen deprivation is an effective systemic treatment for prostate cancer. Normally, androgen deprivation results in apoptosis of prostate cells. The molecular mechanism by which a subpopulation of prostate cancer cells escapes cell death normally achievable by androgen deprivation is unclear. In this thesis, the IAP family was explored in prostate cancer cells which do not undergo apoptosis in spite of androgen deprivation. The levels of NAIP mRNA were significantly increased in vivo in response to castration of hosts as well as in vitro in response to androgen deprivation. Increased levels of NAIP mRNA corresponded to increased DNA-binding activity of NF-kB in vivo. Androgens inhibited the transcriptional activity of NF-kB which is postulated to play a role in the expression of IAPs. NF-kB was shown to physically associate to previously uncharacterized kB-like sites in the NAIP locus. The physiological relevance of NF-kB interaction with one of the putative kB-like sites within the second intron of the NAIP gene locus was validated using the ChIP assay. Hence, the findings of the work in this thesis suggest that NF-kB may transcriptionally regulate NAIP in response to androgen deprivation in prostate cancer cells. These data point to a molecular mechanism by which IAPs may contribute to the loss of apoptosis of prostate cancer cells under androgen-deprived conditions.

To establish the role of IAPs in the progression of prostate cancer, much work still needs to be done. Potential future directions of investigation may include characterization of 1) the anti-apoptotic role of NAIP and other IAPs in response to androgen deprivation; 2) the undefined roles of IAPs; 3) the transcriptional regulation by...
NF-kB and other candidate transcription factors in response to androgen withdrawal; 4) IAPs and their transcriptional regulation at different stages of prostate cancer progression.

To confirm the role of NAIP in conferring prostate cells with enhanced resistance to apoptosis induced by androgen deprivation, functional experiments are required. If NAIP plays a major role in reducing apoptosis of prostate cancer cells in response to androgen deprivation, then overexpressing NAIP using expression vector constructs in an androgen-dependent xenograft model should result in the loss of tumour regression in response to castration of hosts when compared to animals with control vectors. Alternatively, antisense oligonucleotide and RNA interference (RNAi) strategies specifically designed against endogenous NAIP transcripts may be employed to block the expression of NAIP in the xenograft model. Such experiments would be expected to yield data showing an increase in sensitivity to androgen deprivation as compared to injection of control oligonucleotides or RNAi. For these overexpression and knockdown studies, CWR22, a serially transplantable prostate cancer xenograft may be an ideal model to use as the tumour regresses upon androgen withdrawal and relapses to an androgen-independent stage subsequent to biochemical recurrence (1). A similar in vitro and in vivo approach described in the work of this thesis and discussed above may also be applied to explore the anti-apoptotic role of other IAPs, such as livin and apollon, which are relatively understudied in the context of prostate cancer. Alternatively, flow cytometry may be applied to assess the apoptotic property of cells that highly express IAPs using specific antibodies for the IAPs and caspases to confirm the effect of IAPs on the specific IAP targets in the apoptotic pathways.

The roles of IAPs may appear to be redundant as endogenous caspase inhibitors.
However, the presence of functional domains, such as CARD and NOD, in addition to the BIR domains suggests that IAPs may possess unique characteristics in enhancing the survival of cancer cells. For example, NOD is commonly associated with sensing apoptosis, innate immunity and inflammatory responses (2), but recently this functional domain in NAIP was shown to be involved in the ATP-dependent interaction with caspase-9 (3). An alternative approach to study the role of IAPs in prostate cancer could involve the characterization of the unexplored functions of IAPs and their variants. One general way to investigate this aspect is by studying the functional effects, such as apoptosis and proliferation, with mutated IAPs containing part of or completely devoid of the functional domain of interest. Intriguingly, c-IAP2 was suggested to be involved in regulating NF-κB in a positive feedback loop (4). Hence, it will be of interest to determine if other IAPs can similarly contribute to a positive feedback mechanism. This could be explored by manipulating levels of IAPs by overexpression or knockdown of IAPs and measuring the effects on the activity of NF-κB followed by elucidating the underlying mechanism leading to such effects.

NF-κB stands out as a good candidate for a diverse scope of investigation in the progression of prostate cancer due to its multifaceted roles in the cell. These roles include resistance to apoptosis, cytokine production and proliferation. As described in Chapter 2, the NF-κB DNA-binding activity was increased, whereas the nuclear levels of NF-κB subunits were unchanged after androgen ablation in hosts. While lack of correlation between the NF-κB DNA-binding activity and nuclear levels of the subunits may be due to subtle differences that are undetectable by Western blot analyses, the results also suggest that the activity of NF-κB may be altered without inducing the translocation of
the subunits to the nucleus. Specifically, post-translational modifications or cooperative interactions with modulators may allow NF-kB to selectively regulate the transcription at distinct target regulatory elements (5). Nuclear modifications, such as phosphorylation and acetylation, of p65 have been described to modulate the NF-kB activity depending on the cellular context (6). It is critical to determine the triggers and outcomes of the post-translational modifications of NF-kB on the malignant effect conferred by this multifunctional transcription factor, and data on these understudied aspects in the context of prostate cancer are keenly awaited. Besides NF-kB, other transcription factors may also regulate the transcription of IAPs in response to androgen deprivation in prostate cancer cells. Two examples are PAX2 (7) and Brn-2 (8) which have been suggested to regulate the transcription of NAIP. However, expression and activities of these transcription factors has not been examined for correlation with expression of NAIP in the context of hormonal manipulation in prostate cancer cells. Investigation of the regulation of transcription of other IAP genes by NF-kB or by other suspected transcription factors in prostate cancer in response to androgen deprivation could involve a similar in vitro and in vivo approach as outlined in this thesis. To determine the mechanism of regulation of transcription of the IAP family, the possibility of performing in vivo ChIP assays using the hollow fibre model could be explored. Global mapping of binding sites of NF-kB and other candidate transcription factors during the hormonal progression of prostate cancer using high throughput ChIP technologies, such as ChIP with DNA microarray analysis and ChIP with DNA sequencing (9,10), may provide a comprehensive profile of the differential transcriptional regulation during various stages of prostate cancer progression. Validation of the differential bindings should accompany these studies by
regular ChIP assays with conventional PCR or qPCR of the target regulatory regions. However, this is a biased approach and cannot identify unexpected proteins binding to the regulatory regions. Thus, alternative approaches such as in \textit{vivo} DNA footprinting could be used to identify differentially occupied DNA sequences on the regulatory region of genes during hormonal progression.

Currently, the underlying molecular mechanisms involved in the loss of apoptosis in response to androgen ablation therapy and hormonal progression of prostate cancer are not fully understood. Therefore, it will also be of interest to investigate the anti-apoptotic role of IAPs using \textit{in vivo} samples obtained from prostate cancer xenografts and LNCaP hollow fibre model at multiple time-points leading to androgen-independence as characterized by a rising titre of serum PSA in castrated hosts. Finally, to exploit the possibility of targeting IAP, more clinical studies will be required to determine the prevalence of individual IAPs at different clinical stages of prostate cancer as well as neoplastic lesions, i.e. PIN, since the number of similar studies is currently limited. An understanding of the expression profile of individual IAPs in clinical samples with advanced methods such as laser capture microdissection and microarray analyses will provide new insights and comprehensive profile on the molecular mechanisms involved in the initiation and progression of prostate cancer. All in all, data gathered from these proposed studies could lead to the development of novel strategies to induce cell death in selectively targeted tumour cells. The knowledge gained could potentially have prognostic value or could be translated into therapeutic solutions for the effective clinical management of prostate cancer.
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4. APPENDIX

4.1 ANIMAL CARE CERTIFICATES

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A03-0260

Investigator or Course Director: Marianne Sadar

Department: Endocrinology & Metabolism

Animals: Mice 120

Start Date: September 15, 2003 Approval Date: October 25, 2006

Funding Sources:

Funding Agency: US Army
Funding Title: Development of a Potential Therapy for Prostate Cancer Based Upon the Androgen Receptor

Funding Agency: US Department of Defense
Funding Title: Novel Approaches for Blocking Activation of the Androgen Receptor

Funding Agency: US Department of Defense
Funding Title: Development of a Potential Therapy for Prostate Cancer Based Upon the Androgen Receptor

Unfunded title: N/A

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

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

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

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

Animal Care Certificate

Application Number: A05-1794
Investigator or Course Director: Marianne Sadar
Department: Medicine, Department of
Animals Approved: Mice Male athymic Nude mice, BALB/c Strain 180
Start Date: November 1, 2005 Approval Date: January 6, 2006
Funding Sources:

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

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

Unfunded title: N/A

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

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

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

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

The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: H07-0047
INVESTIGATOR OR COURSE DIRECTOR: Sadar, Marianne
DEPARTMENT: Medicine
PROJECT OR COURSE TITLE: Genomic and proteomic analysis of androgen independent prostate cancer
APPROVAL DATE: 07-03-05
APPROVED CONTAINMENT LEVEL: 2
FUNDING AGENCY: National Institutes of Health

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

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

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

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