From DEPARTMENT OF MEDICINE, HUDDINGE AND
DEPARTMENT OF MOLECULAR MEDICINE AND
SURGERY
Karolinska Institutet, Stockholm, Sweden

THE ROLE OF ANDROGEN IN PROSTATE CANCER CELLS INVASION

Yin-Choy Chuan

Karolinska Institutet

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不要同情自己！同情自己是卑劣懦夫的勾当。

挪威的森林 –**村上春树**

Don’t feel sorry for yourself. Only arseholes do that!

From ‘Norwegian Wood’ – Haruki Murakami

To Mr. Hau Chin Tiong and family

ABSTRACT

Prostate cancer (PCa) is the most commonly diagnosed cancer in men in developed countries. Androgen deprivation therapy (ADT) is the mainstay treatment for patient diagnosed with PCa. However, the cancer will eventually relapse with metastatic cancer and become resistant to the treatment. Since mortality in PCa is due to our inability to manage metastatic cancer, there is a great need for a better understanding of the cancer progression to develop novel therapeutic principles. As reflected by the therapeutic effects of (ADT) for PCa, it has become clear that intact androgen receptor signaling is necessary for the development of the disease. Previous works in our group have focused on studies of androgen regulated genes in a systematic and high-throughput manner using microarray technology. In those studies, several androgen-regulated genes were identified, some of which could be important in the regulation of PCa cell invasion.

The aim of this thesis was to characterize a set of androgen-regulated genes in PCa and investigate the role of androgen on PCa cell invasion and the mode of actions of those selected genes in the invasion process.

In this thesis, we demonstrate that androgen stimulation of the androgen sensitive PCa cell line, LNCaP-FGC up-regulate those selected genes at both mRNA and protein levels. For the first time, we also demonstrated that androgen induces the invasiveness of LNCaP-FGC through matrigel. This process was mediated by Ezrin, one of our selected genes. Androgen treatment phosphorylates ezrin in Thr-567 and Tyr-353 in a sequential manner and can also induce ezrin gene expression. The phosphorylation event was mediated by protein kinase C alpha and Src tyrosine kinase, respectively. Androgen furthermore induces the translocation of both protein kinase C alpha and ezrin to the cell membrane and their association. Inhibition of ezrin using short interference RNA or the overexpression of T567A and Y353F-ezrin mutants significantly reduces androgen-induced Matrigel invasion but does not affect cell proliferation or cell adhesion.

We also demonstrate that androgens also increase gene expression of ezrin in LNCaP-FGC cell, through a mechanism involving the transcription factor c-Myc. Our finding that c-Myc binds to an E-Box in Ezrin promoter region leads us to suggest that androgen induction of ezrin is indirect and mediated by c-Myc. In addition, androgen treatment prolonged the half-life of c-Myc protein in LNCaP-FGC. We propose that this effect is achieved through changes in Ezrin phosphorylation, which leads to activation of downstream Akt and downregulation of GSK-3β signaling resulting in the inhibition of the degradation of c-Myc protein. Ezrin protein expression and cell invasion in two other androgen insensitive cell lines, LNCaP-R and PC3 are also depend on c-Myc.

In conclusion, we have shown that androgen can induce PCa cell invasion that mediated through two distinct proteins c-Myc and ezrin. Ezrin also acts as a key modulator of c-Myc induced tumorigenesis in PCa cells

LIST OF PUBLICATIONS

I. See-Tong Pang, Amilcar Flores-Morales, Lambert Sköög, **Yin-Choy Chuan**, Gunnar Norstedt, Åke Pousette
Regulation of matrix metalloproteinases 13 expression by androgen in prostate cancer
Oncology Reports, 2004, 11:1187-92

II. **Yin Choy Chuan***, See-Tong Pang*, Anders Bergh, Gunnar Norstedt, Åke Pousette
Androgens induce CD-9 in human prostate tissue.
International Journal of Andrology, 2005, 28:291-6

III. **Yin-Choy Chuan**, See-Tong Pang, Angel Cedazo-Minguez, Gunnar Norstedt, Åke Pousette, Amilcar Flores-Morales
Androgen induction of prostate cancer cells invasion is mediated by ezrin.
Journal of Biological Chemistry, 2006, 281:29938-48

IV. **Yin-Choy Chuan#**, Diego Iglesias-Gato, Leandro Fernandez-Perez, Gunnar Norstedt, Åke Pousette, Amilcar Flores-Morales
Ezrin mediates c-Myc actions in prostate cancer cells invasion
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* Shared authorship
\# Corresponding author

CONTENTS

LIST OF ABBREVIATIONS

ADT	Androgen deprivative therapy
AR	Androgen receptor
ARE	Androgen receptor response element
ARG	Androgen-regulated gene
BPH	Benign prostatic hyperplasia
CGH	Comparative genomic hybridization
CNA	Copy number alteration
DHT	Dihydrotestosterone
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ET-1	Endothelin-1
ETAR	Endothelin A receptor
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
GDI	GDP dissociation inhibitor
GSK-3β	Glycogen synthase kinase 3β
HGPIN	High grade prostatic intraepithelial neoplasia
HRPC	Hormone refractory prostate cancer
HSP90	Heat shock protein 90
IFG	Insulin-like growth factor
IL-6	Interleukin-6
KGF	Karotinocyte growth factor
LBD	Ligand binding domain
LCM	Laser Capture Microdissection
LHRH	Luteinizing hormone-releasing hormone
MAPK	Mitogen activated protein kinase
MLC	Myosin light-chain
MLCK	Myosin light-chain kinase
MMP	Matrix metalloproteinase
NCOA	Nuclear receptor coactivator
PCa	Prostate cancer
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide kinase-3
PIA	Proliferative inflammatory atropy
PIN	Prostatic intraepithelial neoplasia
PIP2	Phosphatidylinositol-4,5-bisphosphate
PKCα	Protein kinase Cα
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
PTHrP	Parathyroid hormone-related protein
ROCK	Rho-associated kinase
SCID	Severe Combined Immunodeficiency
SDF-1	Stroma derived factor-1
siRNA	small interfering RNA
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitor of metalloproteinase
TRAMP	Transgenic adenocarcinoma mouse model
VEGF	vascular endothelial growth factor
WASP	Wiscott-Aldrich Syndrome Protein

1 THESIS SUMMARY – MAIN SECTION

1 INTRODUCTION

The Prostate is an endocrine, walnut-sized gland that is located directly beneath the bladder, surrounding the upper part of the urethra. The Prostate gland is part of the male reproductive system and its main function is to produce seminal fluid, the milky substance that transports sperm. Prostate epithelial cells also produce a large number of compounds like prostate specific antigen, spermine and acid phosphatases. Testosterone stimulates the growth and function of the prostate during puberty, as well as the production of prostatic fluid for semen (1).

Prostate disorders, especially prostate cancer (PCa), draw large attention from the medical research community. Prostate cancer is a malignant tumor that usually initiate in the outer part of the prostate and is the most common form of male cancer in developed countries. More than 200000 men in USA were diagnosed with PCa in 2007, accounting for 9% (27,050) of all cancer deaths in men. In Sweden, approximately 10000 men are diagnosed with PCa each year (2), being the main cause of cancer related death in the country. PCa is very common, an estimated 40% of all men older than 70 years have prostate tumors but most are asymptomatic (3). In most cases, PCa grows very slowly and the majority of patients can live for years without any clinical problem (4,5).

The incidence of prostate cancer has dramatically increased in the last ten years, due to the implementation of PSA testing for patients with low urinary tract syndrome and asymptomatic patients (6). PSA testing has also resulted in a significant downward trend in tumor staging at the time of diagnosis and an increased number of patients treated with curative intent by prostatectomy and/or radiation therapy. On the other hand, PSA screening remains controversial because it lacks specificity since PSA is elevated in other common prostate diseases such as benign prostatic hyperplasia (BPH). Accordingly, a definitive PCa diagnosis usually involves analysis of tissue biopsies. There is no definitive proof that PSA testing decreases PCa mortality and a major concern is that it may detect a significant number of clinically insignificant cancers. Recent studies suggest that PSA screening gives an over-diagnosis rate of 30-50% of (7-9). So, the current challenge of PCa diagnostics is to distinguish the men carrying the disease that may be cured with treatment, from those men that do not require treatment.

A number of treatment alternatives exist with curative potential in the case of locally advanced PCa, in the form of prostatectomy, radiation and androgen ablation therapy. On the other hand, PCa patients with evidence of distant metastasis have a very poor prognosis and no curative treatment exists. Androgen ablation provides temporary relief, inducing remission of 80-90% of patients with metastatic cancer, unfortunately after two to three years, hormone resistant tumors ensue, metastasis soon develops and

most patients die within two years. Since mortality in PCa is due to our inability to manage metastatic cancer, there is a great need for a better understanding of PCa progression and ultimately a need for novel therapeutically principles.

PCa has a complex disease etiology and is influenced by age, hormone factors, environmental factors and multiple pre-disposition genes. In addition to this, the initiation and progression of PCa seems to be related to defined set acquired genetic events including deletions, duplications and translocations. How these genetic events results in tumor formation is a matter of intense research. As reflected by the therapeutic effects of androgen-ablation therapy for PCa, it has become clear that intact androgen receptor signaling is also necessary for the development of the disease. Previous work in our group has focused in the study of androgen regulated genes in a systematical and high-throughput manner using microarray technology. In this thesis we have tried to characterize some these genes and to investigate their importance for the progression and invasion capacity of PCa cells.

1.1 PROSTATE DISEASES

There are three main health problems related to the prostate gland: prostatitis, benign prostatic hyperplasia (BPH), and PCa. Prostatitis can occur in men of all ages, while BPH and PCa are conditions of mainly older men. BPH is presented as prostatic enlargement, causing obstructive and irritative lower urinary tract symptoms. It is caused by overgrowth of the prostatic stroma and epithelial cells. These developments can lead to infection and inflammation in the prostate gland. Unlike PCa, BPH tends to affect all elderly men regardless of their racial background and geographical location (10,11).

Inflammation, which is related to exposure to infectious agents and/or ingestion of carcinogens are postulated to directly injure the prostate epithelium, resulting in the histological lesions known as Proliferative Inflammatory Atrophy (PIA), followed by excessive epithelial cell proliferation/regeneration, and to give rise to Prostatic Intraepithelial Neoplasia (PIN) lesions (12-15). PIN is considered as a premalignant stage of PCa (16-18). It does not affect serum PSA level and the lesions do not invade the basal membrane of the prostate gland.

PCa is a malignant tumor of the prostate gland that grows uncontrollably. Unlike BPH and PIN, the excessive cell growth does not remain localized within the gland, and eventually tumor cells invade other parts of the body. In the early stage of PCa, there are relatively few signs and symptoms. The most common presenting features of PCa arise from urinary tract obstruction due to enlargement of prostate gland (19). Bladder outflow obstruction often develops abruptly, leading to nocturia and urgency, frequency and hesitancy of micturition. These symptoms are also seen in benign cases of prostatic enlargement. An acute onset of impotence also raises the possibility of the presence of PCa. Most advanced PCa will become highly aggressive and invasive, and they will reach a clinical stage associated with an increased incidence of skeletal metastases as

the disease progresses. When distant metastases of PCa develop, bone pain is the common presenting feature. Prostate tumor growth is androgen dependent. Therefore, androgen ablation is the mainstay therapy of progressive PCa, causing regression of most prostate tumors. However, the initial response to the therapy is almost always followed by a relapse to an unresponsive, hormone refractory stage, which is incurable.

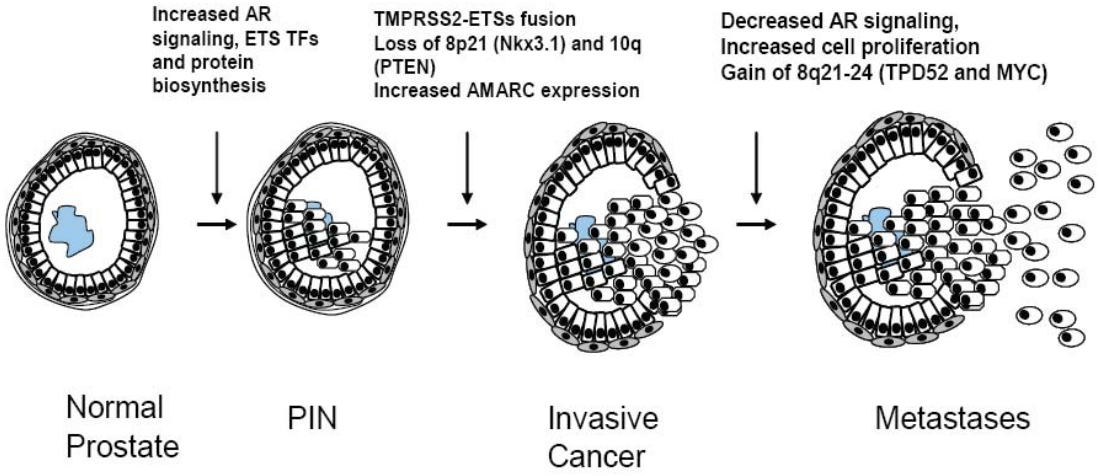

Figure 1. Pathway for human prostate cancer progression. Stages of progression are correlated with loss/gain of specific chromosome regions, dysregulation of androgen receptor signaling and the activity of growth factors, candidate tumor suppressor genes and oncogenes.

1.2 DIAGNOSIS OF PCA

Prostatic-specific antigen (PSA) is one of the few molecular markers routinely used for detection and monitoring PCa response to treatment. It is a serine protease which is believed to have a physiological role in liquefying the seminal fluid (20). Transcription of PSA is governed by androgens, restricting high-level production to the prostatic epithelial cells. The normal prostate architecture keeps PSA tightly confined and only a minute proportion leaks into the circulatory system during normal conditions, however in patients with PCa and BPH serum levels of PSA increased. The increased blood levels of PSA in men with cancer cannot be explained by increased PSA expression. During the development of PCa, PSA expression may actually decrease slightly (21). Although there is no experimental data on the mechanism of the increased PSA release, it is believed to result from the disruption of prostate architecture seen in prostate tumors, such as disordering of the basement membrane and loss of the basal cell layer, ductal lumen architecture and epithelial cell polarity.

In the United States, PSA was approved by FDA as a marker to monitor patients treated for PCa as well as a diagnostic marker. It has led to an increased incidence of PCa and a decrease of the proportion of metastatic or locally advanced cancer at diagnosis (6). In the context of detecting PCa, an increase of the PSA level in blood prompts

recommendation to undergo prostate biopsy, with PSA of 4µg/l being the traditional threshold level. Studies show that men with PSA levels above this threshold have cancer detection rates of 27-44% (22-24). However, this threshold is frequently criticized because numerous studies have reported that PCa is not rare in men with PSA values less than 4ng/ml (25). In addition, approximately another 20% of patients undergo primary treatment of PCa still develop metastasis as a result of dissemination of cancer cells outside of prostate gland prior to diagnosis (26). To address this issue, The Prostate Cancer Convention Trial (PCCT) has developed a calculating formula to use PSA and other risk factors to provide a continuous estimate of cancer risk, rather than using them as a binary positive or negative test result (27).

The severity of PCa is measured by both its grade and stage. Categorizing of PCa takes into account the cancer aggressiveness, or Gleason grade which is based on the histological pattern of the cells (as compared to normal cells) and its stage based on its overall size, and whether it has spread into other areas (28). When PSA levels in blood of a suspected patient is elevated, 6-12 needle biopsies are taken for pathological evaluation. The Gleason score system accounts for the five distinct patterns by which tumor cells tend to differentiate as they change from normal cells. The score runs from 1-5, where 1 corresponds to cells that are nearly normal and 5 represent poorly differentiated tumor. The biopsy sample is assigned one Gleason grade corresponding to the most common pattern, and a second Gleason grade to the next most common pattern. The two grades are added, and the Gleason score is determined. This score tends to predict the aggressiveness of the disease. Cancer staging is used to determine the extent of PCa progression. The methods used for staging are Magnetic Resonance Imaging (MRI), Digital Rectal Exam (DRE) and Bone scintography. Treatment of PCa is highly depends on the stage of tumor spreads, health and age of patients as well as potential side effects of treatment.

1.3 THE PCA ETIOLOGY

The etiology of PCa is far from understood. When it comes to the risk of developing PCa, there are three factors that play a key role, these are age, family history and race. There is also strong evidence that the environmental factors, particularly our diet have a major impact in PCa development.

PCa is a disease of the old, being rarely diagnosed in men before the age of 50. The incidence increases rapidly after the age of 50 and more than 70% are diagnosed in men aged 65 and above (29,30). Family history of PCa may account for 5-20% of all PCa cases (31,32). Men with a single relative with a history of PCa are twice as likely to develop the disease (33). The incidence of PCa varies widely among races and geographical area, with by far the highest rate seen in the USA and Canada. Data from Surveillance Epidemiology and End Results (SEER) registries (covered 10% of the USA population) indicate that PCa incidence is highest among African-American with as high as 250/100 000 (http://seer.cancer.gov/). Mainland China has the lowest PCa

incidence (2.4/100 000) based on a population study in Shanghai (34). The incidence of PCa in Sweden is 197/100 000 (35).

Epidemiologic studies suggest that a western diet rich in red meat, calcium, milk and dietary products may increase the cancer risk (36-38). In contrast, relatively high dietary intake of soybean products (39), cruciferous vegetables (40) and antioxidant molecules such as selenium, carotenoids, lycopenes, Green tea and vitamin D and E may protect against PCa (41-47). An interesting support for the relevance of environmental factors to PCa comes from migration studies; immigrants who move from low-risk countries in Asia to high-risk countries, such as the USA, have an increased incidence of PCa in successive generations (48,49). However, the incidence of PCa in Asian-American remained low compared to Caucasian-American. This suggests that Asian-American retain at least one genetic or lifestyle characteristic that protect them from the risk of PCa (48). The link between obesity and the risk of PCa remains unclear. Research has shown that PSA in obese men can be low despite the presence of the disease; potentially this can lead to a delay in diagnosis and treatment, and an increased risk of dying from PCa (50).

In combination with age, family history and race, genetic factors may probably contribute to PCa development. The consistent finding of genetic susceptibility to PCa suggests that there are germline sequence variants that predispose individuals who carry them to the disease (51-53). Two recent genome-wide association studies of PCa identified PCa risk variants at the chromosome locus 8q24 (54,55). The overall statistical evidence showed a strong association of the single nucleotide polymorphisms (SNPs) and the risk of PCa. The 8q24 is the most frequently gained chromosomal region in PCa that harbored oncogene MYC. It is possible that the risk variants identified may modify c-MYC regulation by predisposing to genomic instability or altering long-range regulation of expression. Linkage analyses based on genome-wide scans have also mapped susceptibility loci for PCa to other chromosomes (53,56,57). Identification of genetic variants that predict PCa incidence could have an impact on cancer screening, diagnosis and treatment. These genetic variants could also lead to the discovery of new tumor suppressor and oncogenes.

1.4 TREATMENT OF PCA

There is no standard treatment for PCa patients. Typically, men with disease in the early stage of cancer that is confined to the prostate gland will undergo active surveillance, prostatectomy or radiotherapy. In advanced PCa, androgen deprivation therapy (ADT) is the mainstay systematic therapy. The common types of ADT are orchiectomy, LHRH agonist or androgen receptor antagonists. Although all these therapeutic options are effective in controlling PCa growth, the loss of testosterone confers significant side effects in nearly all men (58,59). Besides the conventional ADT, several pharmaceutical agents that work primarily by controlling androgen actions are in phase III prostate cancer clinical trials, for example 5-α reductase inhibitor finasteride (60) and dutasteride (61), HSP90 inhibitor 17-AAG (62) and the

selective estrogen receptor modulator agonist toremifene (63). A clinical trial testing the use of finasteride as chemoprevention in PCa has failed to show significant results (60,64).

The understanding that PCa development occurs as a result of interactions between genes and environment, has led to the development of several additional chemoprevention strategies targeting DNA damage, proliferation of premalignant cells and inflammation (65,66). For instance, studies have shown that an imbalance of reactive oxygen species in cells results in intracellular damage in PIN and PCa cells (67,68). Antioxidant molecules including Selenium, lypocene, Vitamin E and D which are believed to prevent DNA damage by oxygen free radicals are in clinical trials. In addition, 3-HMG-CoA reductase inhibitor (Statins), a commonly used cholesterol-lowering drug has shown potential when it comes to reducing PCa risk and progression (69,70). Celecoxib, a selective inhibitor of cyclooxygenase-2 has shown to have a promising effect on PCa (71,72). All of these strategies target separate biological endpoints and synergistic effects are likely to occur. Multi-agent combinations may become important in future chemoprevention regimes.

When the ADT treatment fails and the prostate tumor relapses or there is already metastasis at the time of diagnosis, therapy is problematic. At this point, the therapeutic options are limited to treatment such as bisphosphonates (73) and mitoxantrone in combination with prednisone (74) to reduce skeletal complication and pain. All these drugs are palliative and do not increase survival. Despite the advances in research on PCa, few molecular mechanisms have been proposed to explain the development of hormone refractory PCa (HRPC) (75,76) which can serve as potential therapeutic targets for HRPC. Although there is no curable treatment for metastasis and HRPC, the disease progression can be delayed and the survival prolonged for PCa patients. Recent studies showed that a combination treatment with prednisone and docetaxel significantly prolonged the survival of men with metastatic HRPC compared to mitoxantrone and prednisone (77-79). The optimal sequences and timing of treatment are undergoing evaluation. Phase III studies combining docetaxel with agents that target bone (Astrasentan) (80), tumor vasculature (thalidomide) (81) and the vitamin D receptor (Calcitriol) (82) for HRPC treatment are ongoing.

1.5 ANDROGEN RECEPTOR SIGNALING AND PCA

The circulating androgen, testosterone is primarily secreted by Leydig cells in the testis. Most of the circulating testosterone is bound to albumin and sex-hormone binding globulin, with a small fraction freely dissolved in serum. When testosterone enters into prostate cells, it is converted into dihydrotestosterone (DHT) by 5-α reductase. DHT is a more potent androgen and has five times higher binding affinity to the androgen receptor (AR) compared to testosterone. When bound to androgens, the AR undergoes a conformational change that leads to dissociation from heat shock protein (HSP). Subsequently, the AR dimerizes, translocates into the nucleus and binds to androgen response elements (AREs) in the promoter regions of target genes, resulting in the

concomitant recruitment of co-regulatory proteins, the formation of active transcription complex and transcription of androgen regulated genes.

The AR is required for the development, growth and secretory function of the prostate gland. The action of AR during prostate gland development is complex and involves interactions with stroma-epithelial cells (83). In normal stroma cells, AR activates the secretion of growth peptides known as andromedins (84,85) that will initiate cell proliferation and survival signals in epithelial cells through specific plasma membrane receptors. On the other hand, the ligand-occupied AR in prostatic epithelial cells functions as growth suppressor to inhibit andromedin stimulation and induce epithelial differentiation. Many of the gene targets of androgens in stroma cell have yet to be identified.

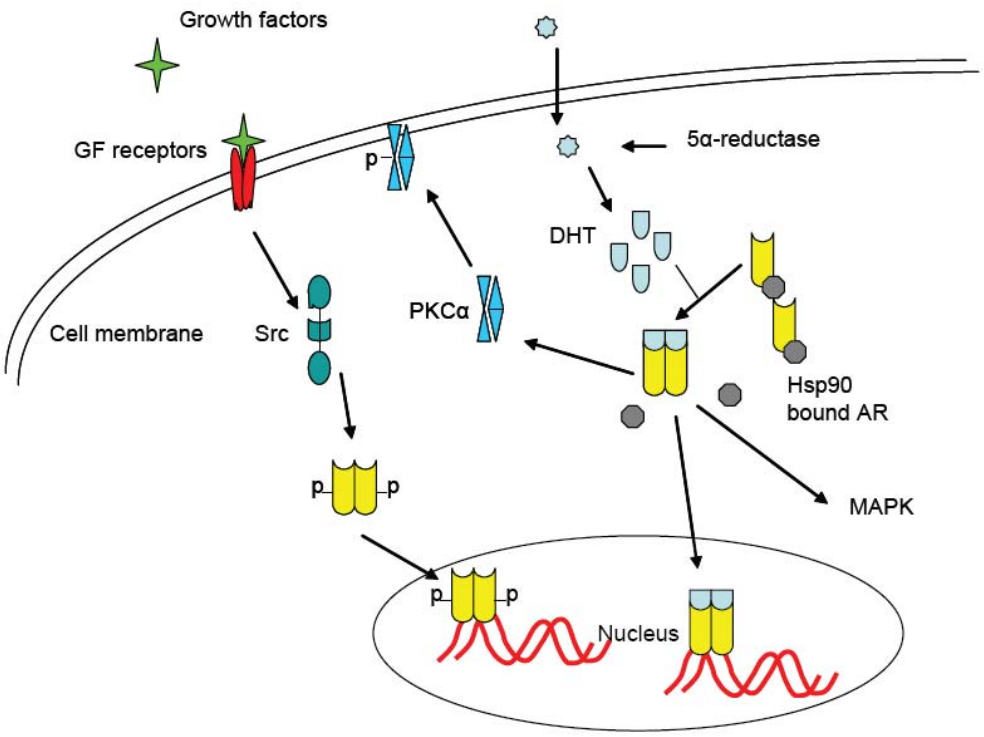

Figure 2. Androgen receptor signalng in target cells. Circulating testosterone is converted to DHT inside the cell. DHT is a high affinity ligand of the AR. Ligand binding induces the receptor to dissociate from HSP90 and translocate to the nucleus where it binds androgen response elements in gene promoters and regulate transcription. The AR has numerous non-genomic actions such as the activation of PKCα. In addition, ligand independent activation can be triggered by AR phosphorylation, as a result of growth factor or cytokine stimulation.

AR signaling is known to be necessary for the development of PCa, as PCa cells require AR activity for growth and survival (86).The exact role of AR in PCa initiation

is unknown. Immunohistochemistry shows that AR is present in primary PCa, metastasis and HRPC regardless of tumor stages (87). The incidence of AR mRNA amplification in primary tumor is relatively low (1-2%), but was reported to be amplified in 20-30% of HRPC patients (88-91). Only a small percentage of men with HRPC loose AR expression (92). These results suggest that AR amplification is involved in development of HRPC. Although there is no evidence that correlates AR expression to prognosis or duration of response to ADT treatment (93), PCa patients harboring AR amplication had longer response duration to ADT and a longer medium survival after development of HRPC compared to patients without AR amplification (94,95).

AR point mutations can result in altered ligand specificity. The first mutated AR was cloned from the LNCaP human prostate cancer cell line. This mutation at codon 877 on the ligand binding domain altered the receptor conformation and allowed the receptor to respond to a broad spectrum of steroid hormones such as estrodiol, progesterone and cyproterone acetate, as well as the antiandrogens flutamide and nilutamide (96,97). The McGill Androgen Receptor Gene Mutations Database has catalogued the tumor-derived mutants and the analysis revealed that most of the mutations were located within the LBD (exons 4-8) (98). The majority of the AR mutations that altered the receptor specificity can be clustered in three areas; codons 701-720 results in AR activation by other steroid hormones (99-101), codons 874-910 that results in broadened ligand specificity including anti-androgens (102,103) and codons 670-678 that result in higher responsiveness to DHT (104). Another mutation at Gln640-Ser produces a constitutively active receptor without ligand stimulation (105).

The repetitive DNA triplet sequences, CAG and GCC are found in Exon 1 of the AR gene. One of these repeats, CAG has been implicated in the development of PCa. One study has correlated the CAG repeat number with incidence of PCa in different ethnic groups. Asian men with the longest repeat number had the lowest PCa incidence compared to African-American who generally has short CAG repeats (106). This might be explained by an *in vitro* study that showed that short CAG repeat AR protein is more stable and active (107).

Another possible mechanism that regulates AR activity in PCa is the modulation of transcription co-regulatory proteins. Elevated level of AR expression in HRPC was correlated to the levels of coactivator proteins NCOA1 and NCOA2 (108). These changes are associated with increased AR activation even at physiological level of adrenal-derived androgens. This finding suggests that overexpression of coactivators in HRPC could facilitate AR transactivation and enhance the response to low levels of androgens and result in a growth advantage for PCa cells. Ligand independent activation of AR through alteration of growth factors like insulin growth factor-1 (IGF-1), kerotinocyte growth factor (KGF) and epidermal growth factor (EGF) and cytokines such as Interleukin-6 in the absence of androgens has been implicated (109). IGF-1 was the most potent factor tested and induced a fivefold rise in PSA secretion in LNCaP (110). These growth factors are ligands for receptor tyrosine kinases and initiate

intracellular signaling cascades. The mechanism by which ligand independent AR activation occurs is poorly understood.

1.6 ESTROGEN RECEPTOR SIGNALING AND PCA

There are two isoforms of estrogen receptor, α and β. ERβ is highly expressed in prostatic epithelium in adulthood (111), while ERα is predominantly expressed in adult prostatic stroma and to a lesser extent in the adult prostatic epithelium. The natural role of estrogens during prostatic development is unclear, it has been proposed that excessive estrogenization during prostate development may contribute to the high incidence of BHP and prostate carcinoma observed in the aging male population (112). However, available epidemiological evidence does not support associations between baseline plasma estrogen levels or estrogen-related polymorphisms and prostate cancer (113,114).

Knockout of both ERα and ERβ in mice showed basically normal prostatic development and growth, with the exception of ERβ knockout mice who exhibit basal epithelial cell hyperplasia and reduced apoptosis (115). Dynamic changes in ERβ expression have been observed in PCa progression. Most reports on ERβ concur that levels decline in localized PCa with increasing grade from PIN through high Gleason score (116,117). This loss of ERβ expression in organ confined PCa has been shown to be epigenetically regulated by progressive hypermethylation of ERβ promoter causing transcriptional silencing (118). In support of ERβ as a putative tumor suppressor, a recent study using adenoviral vectors found that expression of ERβ in PCa cell lines inhibited growth and invasiveness. These results suggest that estrogen action through ERβ may play an important role in prostate carcinogenesis and loss of ERβ in higher grade tumors permits proliferation and eventually metastasis (119). Interestingly, both synthetic antiestrogen (toremifene) and natural phytoestrogen (genistein) prevent development of PCa in the transgenic adenocarcinoma mouse prostate (TRAMP) mouse model possibly by acting as ERβ agonists (120,121).

In contrary to this concept, studies on PCa clinical samples revealed the remerges of ERβ expression in metastatic PCa to varying degree (122,123). The ERβ promoter analysis found complete hypomethylation of the CpG islands in the 5' flanking region in metastatic PCa which permits high ERβ gene expression at metastatic sites. It is currently unclear whether ERβ has as anti-proliferative role in PCa or promotes metastasis and growth at distant metastatic sites. However, this suggests that metastatic PCa cells are targets of estrogen action and this may thus be a potential therapeutic target for PCa treatment.

1.7 TUMOR MICROENVIRONMENT AND PCA PROGRESSION

Multiple genetically unstable cell populations with diverse karyotypes, growth rate and ability to invade and produce metastasis is a feature of cancer progression. Metastasis is

a highly inefficient pathological process with multiple steps. As originally proposed in the "Soil and seed" theory by Paget in 1889, the metastases outcome depends on the intrinsic properties of cancer cells and their interaction with the environment. Several biological processes are driven by tumor microenvironment interactions. Therefore, understanding tumor environment is essential in order to understand the metastatic process. Alteration of the matrix environment surrounding the cancer cells leads to coordinated and stable changes of important cell function like polarity, secretion, migration and invasion (124,125). Cancer cells can also undergo morphological epithelial-mesenchymal transition (EMT) to acquire altered behavior like increased motility, leading to the migration and invasion of cancer cells (126,127). It has also been reported that cancer cells can mimic the gene expression profile of cells in a new microenvironment (128), thereby facilitating growth at metastatic sites. Understanding the molecular mechanisms of tumor-microenvironment interaction could lead to future new therapeutic targets for PCa treatment.

1.7.1 Prostate Microenvironment and Epithelial-Stroma Interaction

Prostate epithelia are composed of a cohesive sheet of polarized cells in an apical-basal orientation in relation to an underlying basement membrane. The surrounding supportive stroma cells are embedded within interstitial extracellular matrix (ECM). The stroma cells are responsible for depositing ECM, regulating epithelial differentiation, regulating inflammation, maintaining ECM homeostasis by secreting matrix metalloproteinases (MMPs), the formation of basement membrane and directing stroma-epithelial interaction through the secretion of growth factors. In normal prostate gland development, stroma cells direct prostate epithelial cell growth and development in an androgen dependent manner, and that functional differentiation of prostate epithelium requires androgen-driven processes in both epithelial and stroma (129,130).

Genetic and cell biology studies indicate that tumor growth is not just determined by malignant cancer cells, but also by the tumor stroma, in addition to inflammatory cells and angiogenesis (131-133). In PCa, epithelial-stroma interactions that modulate AR co-regulator recruitment and AR functions are altered in the stroma microenvironment (129,134,135). In comparison to normal prostate stroma, the tumor stroma is also different in terms of growth factors, cytokines and proteolytic enzyme expression (134,135), and it is commonly referred to as 'activated stroma' (136). Growth factor communication is often bidirectional between stroma and epithelial and it is coordinated with other signaling cascades. Elevated secretion of several growth factors and cytokines in prostate such as VEGF, IGF-1, IL-6, TGF-β, EGF are reported during PCa progression which can regulate androgen receptor signaling in epithelial tumor cells (137). These soluble growth factors have been shown to be involved in ligand independent activation of AR via MAPK, STAT3, Akt and Src pathways. Additives or synergistic interactions between AR and growth factors can culminate positive feedback cycles and facilitate aggressive local cancer growth and invasion or metastatic spread to distal organs.

The epithelial and surrounding stroma cells have distinct morphology which is reflected in the characteristic genes each cell type expresses. Epithelial cells express distinct junction protein E-Cadherin, whereas stroma cells express N-Cadherin and mesenchymal specific vimentin (138). During EMT, a process that determines cell increased expression of vimentin and N-Cadherin but decreased expression of E-cadherin has been documented. Gene expression analysis of PCa tumors using microarray also showed increased expression of stroma signature genes (139,140). This is correlated with increased cell migration, invasion and motility of PCa cells (141). A number of growth factors such as EGF, TGFα/β, FGF as well as solid matrix collagen have been shown to induce EMT associated phenotypic changes (127,142). The cancer cells with mesenchymal phenotype can secrete matrix metalloproteinases and become more responsive to the induction of growth factors and cytokines in the surrounding tumor environment and acquire increased malignant potential.

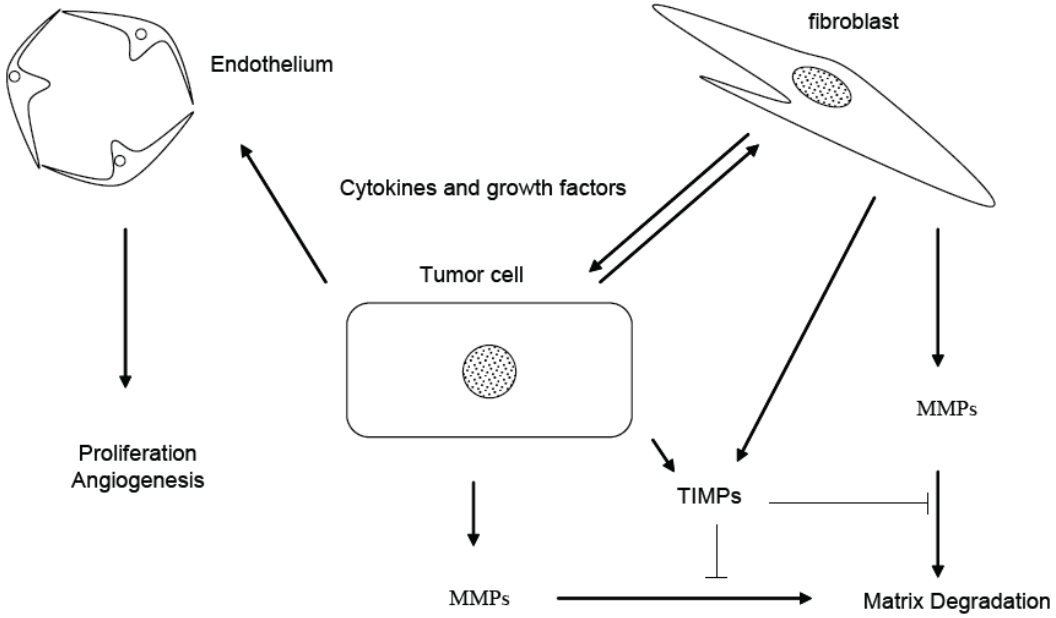

Figure 3. Epithelial-stroma interaction in prostate tumor microenvironment leading to increased cell invasion. During cancer progression, tumor and stromal cells interact through paracrine acting growth factors and cytokines. This results in changes in the composition of the extracellular matrix, with secretion of metalloproteinases (MMPs) and its inhibitors (TIMPs). Depending on the balance of these two factors and the presence of chemoattractants that promote motility, tumor cells can become invasive. Endothelial cells are also subjects to the actions of paracrine factors and participate in cell invasion by promoting angiogenesis.

Activated stroma in PCa tumorigenesis has another important morphological and biochemical transition that involves stroma fibroblast cells. The myofibroblast transition that fibroblast undergoes is a common feature in wound healing and tumorigenesis (143,144). The signals that mediate the transition are not fully understood. Cancer cells can induce the process by releasing TGFβ and PDGF, in the

cancer microenvironment (144,145). The cancer associated myofibroblasts probably also promote PCa progression through specific communication with cancer cells. Evidence from the observation that co-inoculation of normal stroma fibroblast either of embryonic origin or from mammary glands with prostate tumor cells *in vivo* inhibited their growth (146,147). In contrast, cancer associated or immortalized fibroblasts have been shown to promote tumor cells growth (148,149). The growth stimulatory of cancer associated fibroblasts could result from the transactivation of normal stroma to activated stroma in tumor microenvironment.

1.7.2 BONE MICROENVIRONMENT AND PCA METASTASIS

Most patients with advanced PCa will experience complications from bone metastasis that are incurable. PCa bone metastases are usually osteoblastic in nature with characteristic deposition of unstructured woven bone, and it is clear that bone resorption and formation are dysregulated. Clinical evidences show that both osteoblastic and osteolytic processes contribute to PCa metastatic phenotype (150-152).

After PCa cells have intravasculate, migration into the bone marrow is likely driven by chemo-attractants derived from bone such as stroma-derived factor-1 (SDF-1 or CXCL12). The SDF-1 receptor CXCR4 expressed is increased in metastatic PCa cells, while neutralizing antibodies to SDF-1 decrease the metastatic load and proliferation of PCa cell lines (153,154) Bone is constantly being remodeled by balancing osteoblastic and osteoclastic activity. This balance is disrupted by the presence of PCa cells, probably through ECM or growth factors secreted by cancer cells to facilitate the formation of a microenvironment for PCa cell growth. Several growth factors, some of which are produced by PCa cells, are implicated in the osteoblastic activity either directly or indirectly. Growth factors such as bone morphogenetic proteins (BMPs), TGFβ1 and 2, IGFs, PDGF and Wnt have direct effects on osteoblastic function, whereas VEGF has an indirect effect by modifying the bone microenvironment. All these factors regulate osteoblast function by activating signaling pathways involved in regulation of osteoblast proliferation and differentiation (154). PSA could also induce PCa bone metastasis by cleaving parathyroid hormone-related protein (PTHrP), an endocrine hormone that stimulates bone resorption. Degradation of PTHrP by PSA can therefore decrease bone resorption and modify bone homeostasis and microenvironment (155,156). However, a contradictive result showed that forced expression of PTHrP in PCa cells resulted in greater tumor progression in bone (157). The proposed explanation is that NH3 terminal fragments of PTHrP cleaved by PSA could stimulate the new bone formation by activating endothelin A receptor (ET_AR).

The ligand for ET_AR, endothelin-1 (ET-1) is a mitogenic factor for osteoblastic expressed in mice and humans (158,159). Plasma ET-1 concentration are significantly increased in metastatic PCa patients, and ET_AR antagonist (atrasentan) reduced skeletal morbidity in men with advanced PCa, indicating the importance of ET-1 in PCa bone metastasis (160,161). Gene expression microarray analysis on calvaria treated with ET-

1 identified several downstream targets of ET-1 in osteoblast, with possible role in osteoblast function included secreted factors (IL-6, Wnt5a, TIMP3, RANKL) and secreted inhibitor of Wnt signaling (DKK1). *In vitro* study has showed that androgens stimulated PCa cell growth via stroma cells, specifically by activating Wnt signaling in osteoblasts. Thus, inhibition of Dkk1 expression by ET-1 could relieve negative regulation of osteoblast activity (162) and facilitate tumor cell growth in bone. Another set of experiments showed that co-culture of PCa cell lines with osteoblasts induced the osteoblasts to express RUNX2 and osteocalcin (163). The interaction induces proliferation of both osteoblast and PCa cells, specifically by activating Wnt signaling via androgen receptor signaling (164).

1.8 GENETIC AND MOLECULAR CHANGES ASSOCIATED TO PCA PROGRESSION

PSA levels in serum and Gleason histological examination of prostate gland can predict the likelihood of occurrence of PCa in clinic. On the other hand, these diagnostic methods fail to explain why tumors with identical clinical and histological factors have remarkably different clinical outcome. The development of human cancers is associated to accumulation of genetic and molecular alterations in cancer cells. Extensive characterization of DNA copy number alteration (CNA) in PCa have revealed recurrent losses in locations known to harbor potential tumor suppressor genes such as 13q (RB1), 10p (PTEN), 16q (CDH1), 5p (APC) and 18q (SMAD4) (65,165), as well as recurrent gains at 8q (Myc).

PCa can be manifested with multiple tumors, each composed of heterogeneous lesions with distinct pathologic, histologic and biologic and genetic variation. These variations make profiling of genetic changes associated with PCa progression difficult. The use of grossly dissected PCa tumor specimens in studies to document genetic changes, lack the specificity to determine the key different between histologic subtypes of PCa. These analyses cannot answer whether PCa progresses through a linear disease model or whether the distinct molecular and genetic changes that are evident in advanced tumors arise independently.

Two of the most recent systematic studies of CNA in PCa identified distinct molecular subtypes of PCa in relation to clinical outcome and histological stages of PCa progression (166,167). Both studies have used microarray-based CGH analysis with sub-megabase resolution. Although different microarray platforms were used, the two studies detected, the common recurrent aberrations in relation to PCa described above. Loss at 8p11-p23 (peak lose at 8p21; NKX3.1) and an intrachromosomal rearrangement resulting in the expression of the TMPRSS2-ERG fusion were frequently exhibited in more aggressive PCa tumors compared to less aggressive ones. The fusion product of TMPRSS2, a prostate specific androgen-regulated gene with ERG, member of ETS family of transcription factors leads to the overexpression of an androgen-responsive oncoprotein. Evidence suggests that the TMPRSS2-ERG gene fusion is related to the invasive phenotype of higher tumor stage of PCa (168,169). This

gene fusion could act as a PCa marker to improve detection and predict prognosis in combination with other potential PCa biomarkers like AMACR (170), which was identified by expression profiling.

c-Myc locus (8q24) amplification is the most common genetic event associated to metastatic progression in PCa. Upregulation of MYC expression can drive cell-cycle progression and has been linked to genomic stability (171). Forced overexpression of c-Myc in mouse prostate and in human normal epithelial cells resulted in tumor transformation with an invasive phenotype. Studies on Nkx3.1 and Pten mutant mice have also revealed the important tumor suppressor role of both genes in PCa and in progression to HRPC (172,173).

Several other genetic alterations have being related to aggressiveness and metastasis of PCa, although they occur with less frequency. Additional efforts are needed to identify the relevant PCa related genes located in these chromosomal regions. Because of the treatment protocols currently used, there is a lot of difficulty to obtain matched androgen sensitive and HRPC samples from the same patients. Therefore, the genetic and molecular changes related to PCa progression to hormone refractory stage in human tumors are not well understood.

1.9 SIGNALING PATHWAY AND AR SIGNALING IN PCA INVASION AND METASTASIS

Cancer cell invasion is a complex process essential for metastasis. To increase invasiveness, the tumor cells undergo EMT, which requires drastic reprogramming on genetic and physiological levels. This leads to alterations in cell-cell and cell-ECM adhesion, reorganization of actin-cytoskeleton and finally increased cell motility. The tumor cells use similar migration mechanism to those that occur in normal cells during physiological processes such as wound healing and embryonic morphogenesis. The key cell signaling components involved in this process are integrins, focal adhesion and Rho-GTPases (174). For cells to move in 3D environment such as matrigel and tissues, an additional proteolytic component is added for the remodeling of ECM.

Integrins are cell surface receptors that will form αβ heterodimers for cell adhesion to ECM proteins (175). They can function as co-receptors of growth factor receptors, allowing effective transduction of signals from the basal cell surface to the cytoplasm and nucleus and vice versa. Thus, integrins are involved in the regulation of prostate epithelial growth and oncogenesis through multiple pathways and networks. The ligand specificities rely on both subunits of a given αβ heterodimer. Depending on cell types and ECM composition, focal adhesion and migration can be regulated by different integrins (176). When cells formed contact with surrounding matrix, integrins come into contact with ECM ligands and cluster in the cell membrane (177,178). Clustered integrins recruit adaptor and signaling proteins and assembly focal adhesion complex via their intracellular domains. This focal adhesion complex is composed of cytoskeletal proteins such as talin, α-actinin, vinculin and paxilin as well as focal

adhesion kinase (FAK) (179,180). Activated FAK then recruit regulatory molecules such as Src and Fyn to the focal adhesion site, resulting in the phosphorylation of paxilin. These proteins contain SH2, SH3 and proline-rich domains that can bind adaptor proteins and recruit actin-binding protein (vinculin, paxilin and α-actinin) and regulatory proteins (PI3K and Rho-GTPases family) to focal contact. Expression of dominant negative Rho-GTPases has been shown to interfere with these processes (181).

Insight into integrin expression and ECM composition has come from immunohistochemical analysis of PCa tissues (182,183). The ECM composition of prostatic tumor gland is altered compared to normal gland. Specifically, laminin 5 and collagen VII are lost in cancer, but laminin 10/11 and collagen IV are retained. This directly correlates with the loss of laminin 5 binding integrins, α6β4, and the reduced expression of α3β1 in cancer cells. The changes in ECM and integrin composition are likely to be important for PCa metastatic progression. The role of AR in integrin signaling in PCa is poorly understood. Progress in this area has been hindered by the lack of *in vivo* immunohistochemical data in metastatic tissues and most studies rely on analysis in tissue cultures. Metastatic, AR negative PC3 cells express integrin β4, which is suppressed by forced expression of AR in the cells, in parallel to decreased invasion activity (184,185). This may indicate that reduced androgen/androgen receptor signaling in high grade PCa (140) could be behind enhanced expression and activation of integrins (186-188).

Over 80% of PCa metastases are found in the bone, which has collagen I as primary ECM component. Integrins α2β1 is primarily responsible for adhesion to collagen and its' found to be highly expressed in PC3 cells compared to less metastatic cell lines. Treatment of PC3 with bone derived TGF-β1 increases α2β1 expression as well as adhesion and spreading (189). Thus, signaling through collagen/TGF-β 1 in the bone environment may favor metastatic growth in part by increasing integrin engagement. How signaling through TGFβ1 and α2β1 impacts AR function in the metastatic cell is unknown.

Integrin expression and function can also be modulated by transmembrane 4 protein superfamily members such as CD81/KAI1, which has been identified as a possible metastasis suppressor in PCa (190). CD81 exerts its effect by limiting the distribution and association of integrins and growth factor receptors on the cell surface (191,192). Another study also demonstrated that expression in PCa of a related protein, CD9 seems to be inversely correlated with PCa progression (193).

Before and during focal adhesion development in the migrating cell, actin filament will assemble and elongate through the action of crosslinking proteins such as myosin II and α-actinin. Rho family GTPases, including Rho, Rac and Cdc42 have been found to mediate all of the above processes in various ways. To form protrusion of migration

cell leading edge, growing actin filaments will connect to adaptor protein to push the

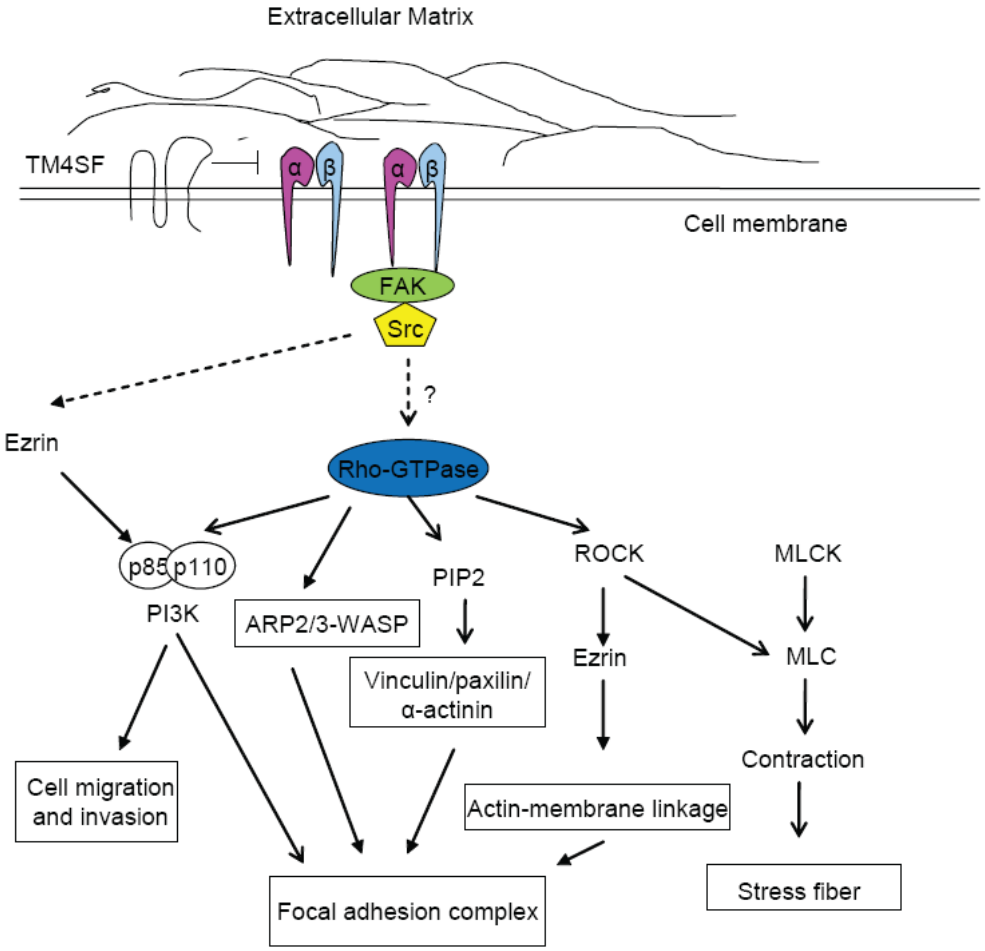

Figure 4. Integrin signaling in focal adhesion formation and cell migration. Integrins interact with extracellular matrix proteins and assemble signaling complex upon activation of the focal adhesion kinase (FAK). Numerous signaling molecules are activated downstream FAK, including ezrin, PI3kinase, the RhoGTPase family, among others, which leads to the remodeling of actin cytosqueleton, resulting in cell migration, formation of focal adhesion complexes and contraction of stress fibers.

cell membrane to an outward direction. This is achieved when actin polymerizes by coupling to the actin-nucleating ARP2/3 complex, which then binds to the multifunctional adaptor protein, Wiscott-Aldrich syndrome protein (WASP). The ARP2/3/WASP complex connects to the inner leaflet of plasma membrane via phopholipid PI(4,5)P2. This process requires activation of Rho, Rac and Cdc42 by PIP2 to activate WASP in order to induce filopodia ruffling and extension. The contraction of actin filament is controlled by myosin II. The Ca^{2+} and calmodulin-dependent myosin light-chain kinase (MLCK) phosphorylates the myosin light chain (MLC), which activates myosin II. This is counteracted by dephosphorylation via the MLC phosphatase. Rho regulates the process predominantly through its downstream effector, the Rho-associated serine/threonine kinase. The actin-filament contraction promotes the shortening of the migrating cell body and generates inward tension towards focal adhesion points. Another important protein that is involved in the actin assembly process is linker protein ezrin. Most of the ezrin in a resting cell exists in a dormant

state with masked C-terminal and N-terminal domains. Once activated through interaction with lipid PIP2 and/or as a substrate of tyrosine and serine/threonine kinases, ezrin will associate with α-actinin and induce the formation of cell surface structure that promotes cell migration. In support of the role of ezrin in cell migration process, it has been showed that an activated ezrin form appeared to be involved indirectly in the activation of Rho through the sequestration of Rho-GDI (GDP-dissociation inhibitors) complex and results in the release of free Rho that associates with the membrane and initiates downstream events.

Many studies have shown that up-regulation of Rho-GTPases signaling in tumors is linked to increase invasion and metastatic potential (194,195). It has been shown that Rho-GTPases can activate AR in a ligand independent manner (196), but the role of Rho-GTPases on AR and PCa progression is unknown. In this thesis, we demonstrated that activation of the ezrin protein, a target of Rho-GTPase kinase is mediated by AR signaling and is suppressed by ROCK. We have also observed activation of RhoA in PCa cell by androgen stimulation. Both androgen and ezrin were found to induce PI3K signaling (197,198) which can potentially leads to RhoA activation. So, RhoA may act downstream of ezrin and PI3K/Akt signaling in PCa and induce PCa invasion and metastases. Further studies are needed to elucidate androgen receptor and Rho-GTPases signaling in PCa progression.

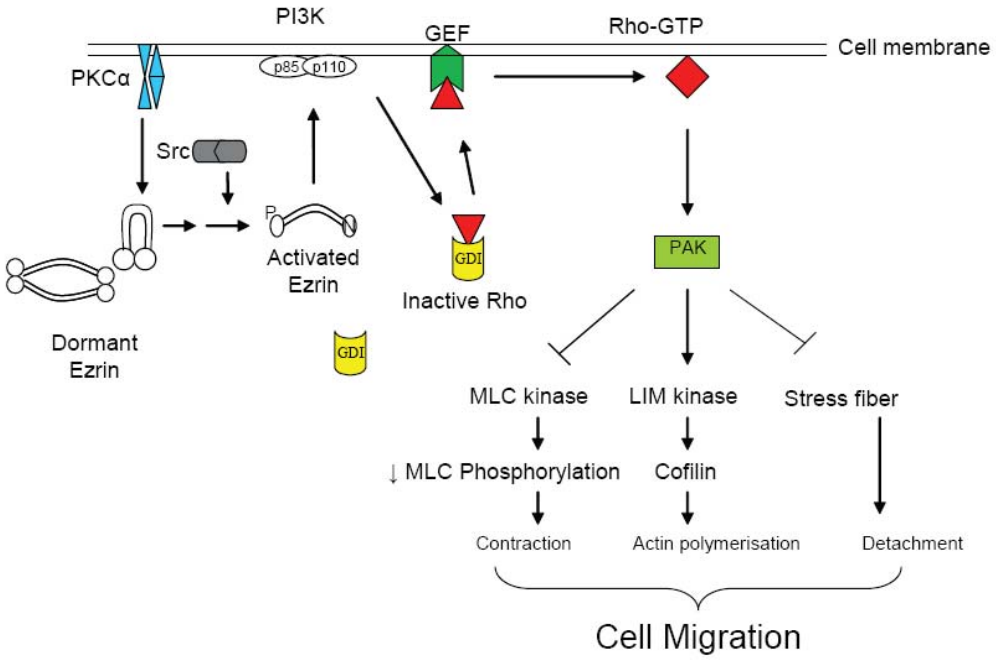

Figure 5. Signaling in cell migration. Ezrin can exist in two forms, an inactive intra- or intermolecular complex and an active form where it is bound to the membrane and F-actin. Activation of ezrin requires the phosphorylation on Thr567 by PKCα and possibly other kinases. In its actin bound form, ezrin can be further phosphorylated on tyrosine residues by Src and other tyrosine kinases. These phosphorylation sites provide docking points for the assembly of signaling complexes leading to the activation of multiple signaling pathways including PI3 kinase and RhoGTPases, which results in changes in cell migration and invasion.

The movement of migrating cell in 3D environments such as tissues requires proteolytic remodeling of the ECM. The matrix metalloproteinases (MMPs) protein family members play an important role in ECM remodeling and cell invasion. The MMPs are zinc dependent endopeptidases that play multiple roles in biology of ECM, such as release of cryptic fragments and neo-epitopes from ECM macromolecules, release of growth factors and modification of the cell-ECM interface (199). All these processes are involved in cancer invasion and metastasis. The activity of MMPs can be inhibited by its inhibitor, Tissue Inhibitor of Metalloproteinases (TIMPs). The expression and interaction of MMPs and TIMPs appear to be involved in invasion and metastases capacity of various cancers (200). Tissue expression and serum level studies on PCa patient tumor samples showed correlation of MMP-1, MMP-2 and MMP-3 to PCa progression and metastasis (201-204). In this thesis, we observed that androgen treatment of LNCaP-FGC cell line induced expression and activation of MMP-13. This suggests androgen receptor signaling might act as an important player in ECM remodeling and PCa progression.

A general model for androgen actions on regulating signaling pathways in PCa invasion and metastasis cannot be suggested at this moment. Microarray studies on different stages of PCa tumors revealed a reduction of AR signaling in PCa progression. Although AR signaling was showed to regulate the activity of several kinase pathways in PCa, those pathways became more active when PCa progressed from androgen sensitive to hormone refractory. The cause of this may lie in the enhancement of other growth factor receptors and integrin signaling during oncogenic transformation on PCa progression. Also, we cannot neglect the potential ligand independent AR function in PCa cell-microenvironment interaction. Development of better *in vivo* and *in vitro* PCa models will help to elucidate the molecular mechanisms behind AR actions on invasion and metastatic PCa.

1.10 MODELS TO STUDY AR SIGNALING ON PCA PROGRESSION

1.10.1 PCa Cell Lines

Most PCa cell lines used today are derived from human PCa patients. Three most commonly used and well characterized PCa models are LNCaP-FGC, DU145 and PC3. LNCaP was derived from lymph node metastatic PCa, DU145 was derived from brain metastases and PC3 was isolated from patient bone lesion. The other less commonly used and characterized PCa cell lines are CWR22rv1, LuCaP 23, LuCaP 35, MDA PCa 2a, VCaP, LAPC-4, PC-82, PreC and RWPE. The original source and general properties of these cell lines have recently been reviewed (205-207).

LNCaP-FGC is the most commonly used cell line to study androgen actions in PCa. However, this line is less aggressive and not a good model to study cell invasion and metastases *in vivo*. The line has undergone experimental selection to produce sublines with variable metastatic capability and responses to androgen (208-210). One of the

sublines, C4-2 was found to be androgen independent in proliferation, highly tumorigenic and metastases to bone when injected into SCID mice (211). Although it formed osteoblastic bone metastases (which happened to 90% of bone metastases in men diagnosed with bone metastases PCa), it is limited by the long time frame between injection of the cancer cells and the appearance of detactable osseous metastases (210) compared to PC3 cells. PC3 is a highly invasive androgen independent PCa line that does not express AR and forms metastatic osteolystic lesion in bone when injected into SCID mice (212). Several PC3 sublines have been generated toward propagating more aggressively metastatic variants or lines that metastasize to specific tissues. When AR expression was forced in PC3 cells, they became less invasive, indicating an alternative AR function in suppressing PCa progression (213). In general, the different sublines generated from LNCaP and PC3 are not fully genetically and phenotypically characterized. One should be cautious when interpreting the data generated from those sublines.

Model	Source	AR status/Androgen dependent	Notes
LNCaP	Lymph node metastases	Mutated AR/Androgen dependent	Not tumorigenic
C4	Clone of LNCaP	AR+/Androgen dependent	Not tumorigenic in castrated host
C4-2	Clone of C4	AR+/Androgen independent	Tumorigenic and metastases to bone
C4-2B	Clone of C4, developed in bone	AR+/Androgen independent	Tumorigenic and metastases to bone
MDA-PCa-2a	Bone metastases	Mutated AR/Androgen dependent	Not tumorigenic in castrated host
CWR22rv1	Xenograft	WT AR/Androgen dependent	Not tumorigenic in castrated host
LAPC-4	Xenograft	AR/Androgen dependent	Tumorigenic and response to androgen ablation, developed androgen independent tumor
VCaP	Vertebral metastases	AR/Androgen dependent	Tumorigenic in castrated host and developed androgen independent tumor

Table 1. Human PCa cell lines for AR signaling study

PCa is not a disease of a single cell type and the cancer development involves complex epithelial-stroma interaction. LNCaP cells require the addition of matrix collagen formulations such as Matrigel for efficient tumor growth in mice. Mixing of LNCaP cells with bone derived stroma, followed by injection into SCID mice to study

epithelial-stroma interaction in bone metastases has been performed (209,214). Generation of a larger set of prostate stroma cell lines is needed to better study the cell-cell interaction during PCa invasion.

1.10.2 Murine Models

The initial attempts to find animal systems to study PCa were hindered by the fact that spontaneous PCa is rare in rodent and the cross species differences in anatomy and morphology of human and rodent prostate gland is vast. Despite these concerns, rodents model involve for xenograft studies, hormone treatments as well as, transgenic and knockout rodents have been actively pursued as alternative models for PCa invasion and metastases studies.

SCID mice, which lack the ability to make T and B lymphocytes, are routinely used as a model to study PCa. Xenograft tumors in SCID mice have been used to study the mechanisms involved in the transition to the androgen refractory state (215,216) and bone metastases (212,217).

In the prostate reconstitution model in mice, the urigenital sinus epithelial and mesenchymal cells are implanted under the renal capsule. Prostate like structures are evident after 90 days. This methodology allows the study of selective genes of interest in the progression of PCa by using retroviral–mediated infection prior to implantation. This model has been used to study roles of c-Myc, Ras and p53 on PCa transformation and progression (218,219).

Hormone inducible Nobel rats and mice were initially developed to study the alteration of androgen and estrogen ratios in PCa progression (220-222). Subcutaneous implantation of capsules containing large doses of testosterone and estrogen results in highly reproducible progression of prostatic epithelial hyperplasia to invasive carcinoma. A recent report indicate that similar treatment causes prostate hyperplasia in C57BL/6, CD-1, or C57BL/6xJ129 mice (223) opening the possibility of studying PCa in a large variety of pre-existing knockout mice models.

The identification of prostate specific promoter elements has facilitated the development of mouse models for PCa. One of the most widely used transgenic mice, known as TRAMP (transgenic adenocarcinoma mouse model) model contains a minimal probasin promoter driving the expression of SV40 large T and small t tumor antigens (224,225). This mouse displays high grade PIN and/or PCa within 12 weeks of age and develop metastases by 30 weeks primarily to the lungs and lymph nodes, and develops androgen refractory PCa after androgen depletion (226,227). The TRAMP model has an extremely rapid pace of PCa progression from PIN to invasive carcinoma. Another transgenic model which only expresses SV40 large T antigen, known as LADY mice was generated and it has a less aggressive disease progression (228). Androgen depletion of these transgenic mice results in temporary regression, followed by the formation of poorly differentiated carcinoma. It can also serve as a model to

study the transition to androgen refractory state. Another transgenic model of interest is the one overexpressing c-myc in prostate epithelium. These animals develop PCa, which progress to metastatic stage (219). Interestingly, the degree of response to c-myc overexpression in mouse prostatic epithelial is dose related, with increased expression giving rise to more severe phenotypes (229,230). These findings suggest the possibility that c-myc is sufficient to drive normal prostate epithelial to metastasis tumor in the absence of any secondary changes, which consistent with report of Myc gene amplification in human PCa (231).

Relatively few knockout mouse models have been reported to display prostatic epithelial defects. The generation of loss of function of Nkx3.1;PTEN compound mice offer a good model to study PCa development. Both Nkx3.1 and PTEN are tumor suppressor genes that are commonly silenced in PCa (232,233). This Nkx3.1;PTEN mutant mice display an increased incidence of HGPIN or early carcinoma lesions, and eventually invasive carcinoma and lymph nodes metastases (173,234). Interestingly, in this model prolonged exposure to reduced levels of androgen accelerates prostate cancer progression (172). Recently, the pes-ARKO-TRAMP mice model, where the AR allele is inactivated only in prostatic epithelium was created. Loss of AR epithelial function in the TRAMP background caused reduced differentiation and hyperproliferative prostate epithelial cells. Restoration of androgen/AR actions in pes-ARKO-TRAMP mice significantly reduced epithelial proliferation (130). This study suggests a role of epithelial AR in maintaining prostate homeostasis. This new model offers a good opportunity to gain insight to androgen/AR functions in PCa progression.

Although much progress has been made in developing PCa models over the last years, it is notable that no current model can recapitulate all features of PCa initiation and progression in humans. Several well-characterized gain-of-function as well as loss-of-function models display features of PIN and early carcinoma, but do not progress to frequent invasive carcinoma and metastatic disease. The TRAMP and LADY PCa evolve rapidly to form invasive tumors that have neuroendocrine features but its relevance for human pathology in unclear. Importantly, none of the available models demonstrate highly reproducible metastasis to bone, a characteristic feature of human prostate cancer.

1.11 GENE EXPRESSION PROFILE IN PCA PROGRESSION

Clinical application of microarray based gene expression profiles has been accepted in breast cancer and lymphoma management. Gene expression profiling studies in PCa have also attempted to identify specific gene profiles that differentiate normal from cancer tissues, correlate to Gleason score, predict aggressive tumor subtypes and recurrences (235-238). Although those studies confirmed different expression profiles between primary and metastatic/aggressive PCa, there are inconsistencies and generally poor concordance between studies. Most of these problems could be attributed to small sample size and inclusion of grossly dissected tissues. High Gleason grade tumors often appear as small groups of malignant cells separated by prostate stroma and

normal epithelial cells making the samples very heterogeneous. Moreover, a large variability exists between samples with the same samples Gleason-sum scores (238). For studies focusing on recurrence events, additional limitations are caused by variations in defining recurrence and by the small number of samples that yield profile data. All these factors make comparison and identification of robust signatures for PCa predictions from multiple studies difficult.

Despite these limitations several novel findings have being described. Comparison of expression profiles identified far fewer differentially expressed transcripts between PIN and localized PCa compared to PIN and BPH. This suggests that the key transitions in PCa progression may occur after the development of PIN precursor lesions. Transition from PIN to localized PCa seems to be associated with a marked overexpression of ETS family member protein (239,240). The overexpression of ETS family members ERG, ETV1 and ETV4 in PIN through fusion with the androgen-regulated gene TMPRSS2 and the increase in protein biosynthesis suggest a link between AR signaling, ETS transcription and protein biosynthesis in early PCa development.

To study the AR signaling in PCa progression, Hendriksen et al. selected 200 androgen regulated genes from LNCaP and/or xenografts, and used this gene expression signature for profiling PCa xenografts and patient derived samples (241). The results showed an increase of AR signaling activity in well differentiated PCa compared to normal prostate. However, AR signaling, as defined by the gene signature, is down-regulated in correlation to the ability to metastasize. The authors hypothesized that localized cancer cells became more aggressive by selective down-regulation of androgen regulated genes that inhibit proliferation and induce differentiation. When Tomlins et al. profiled 101 specific cell populations isolated from 44 individual tumors using laser-capture microdissection (LCM), they found a similar pattern of increased expression of androgen regulated genes in PIN and decreased expression in high grade and metastatic PCa (140). Comparison of the expression profile of hormone refractory metastatic cancer with hormone sensitive metastatic cancer generated from other studies shared the same findings; showing a reduction of androgen-induced genes in hormone refractory metastatic PCa while the expression of genes involved in proliferation were increased (139,242,243).

In summary, marked differences in AR actions occur in normal prostate and low grade carcinomas in comparison to metastatic tumors. Reduced expression of androgen-regulated genes, as defined in a few cell lines, should not be interpreted as an inactivation of the AR. A more likely explanation is that AR transcriptional actions are influenced by context. PCa progression is associated with activation of several signaling pathways, including the increased activity of tyrosine kinases in addition to multiple genetic alterations that can result in altered activity and specificity of the AR. Understanding this phenomena is of marked importance because most metastatic tumors in humans express the AR and respond to androgen ablation therapy. Additional gene expression studies that specifically measure the transcriptional actions of androgens in metastatic tumors may help to better interpret the differences in

expression profiles between advanced and localized PCa. In any case, it seems that AR independent pathways can drive PCa metastatic progression and opportunities exist for the identification of novel molecular targets outside the group of androgen regulated genes.

2 AIM OF THE STUDY

The overall aim of the present thesis was to evaluate the role of androgens on PCa cells invasion. Studies were designed to characterize novel androgen-regulated genes and their involvement in PCa cells invasion. The studies were performed in PCa cell lines and PCa patient samples.

The specific aims were:

- To characterize the expression of a set of androgen-regulated genes in PCa
- To investigate the role of androgen in PCa cell invasion
- To investigate the mode of actions of c-Myc and ezrin on PCa cell invasion

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 LNCaP-FGC, LNCaP-R and PC3 Cell Lines

In paper I, II, III and IV, a prostate cancer cell line, LNCaP-FGC, was used as a model to study androgen actions in prostate cancer. LNCaP-FGC is an androgen-sensitive human prostate adenocarcinoma cell line derived from the left supraclavicular lymph node metastasis of a 50-year-old Caucasian male in 1977. LNCaP-FGC cells carry a T877A mutation on the androgen receptor gene. This gain-of-function mutation in the AR allows it to bind to and be activated by other ligands like estrogen and progesterone. The androgen resistant LNCaP-r, is a subline of LNCaP-FGC (208). These cells possess many features of its parental line but its proliferation is androgen independent. LNCaP-R, like LNCaP-FGC express AR and PSA but has shorter doubling time and tends to grow more aggressively. The PC-3 cell line was initiated from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old male Caucasian. It does not express AR and has a greatly reduced dependence upon serum for growth when compared to normal prostatic epithelial cells. PC-3 cells do not proliferate in response to exogenously added androgens, glucocorticoids, or epidermal or fibroblast growth factors. LNCaP-FGC and PC-3 cells have been widely used for prostate cancer research.

3.1.2 Human Tumor Samples

In paper I, fine needle aspiration biopsy materials from 9 patients with suspected prostatic carcinoma were used. Seven of the selected specimens were characterized as prostatic carcinoma and two were classified as BPH.

In paper II, a total of 9 paraffin embedded prostate cancer tissue sections of different Gleason grades and tissue from the prostate of one patient on androgen-ablation therapy prior to prostatectomy were selected from the tissue bank of the Department of Pathology, Umeå University. Tissue microarray (TMA) slides were also obtained from the Cooperative Human Tissue Network and the Tissue Array Research Program (TARP) of the National Cancer Institutes of Health, Bethesda, MD, USA. The array (T-PR-1C) contains a total of 79 prostatic samples and 72 matched controls. The prostate tumor samples included primary tumors with Gleason grade 3-5 and high grade tumors with Gleason score 6-8.

In paper III, 3 prostate cancer patient samples were used. The patients were diagnosed and received radical prostatectomy. All patients were subjected to 1-2 weeks of neoadjuvant hormone therapy with cyproterone acetate prior to the prostatectomy.

3.2 METHODS

In all papers used in this thesis, routine methods such as Western Blot, Real-time PCR and Immunohistochemistry were widely used and are fully described in the papers.

3.2.1 Matrigel Invasion Assay

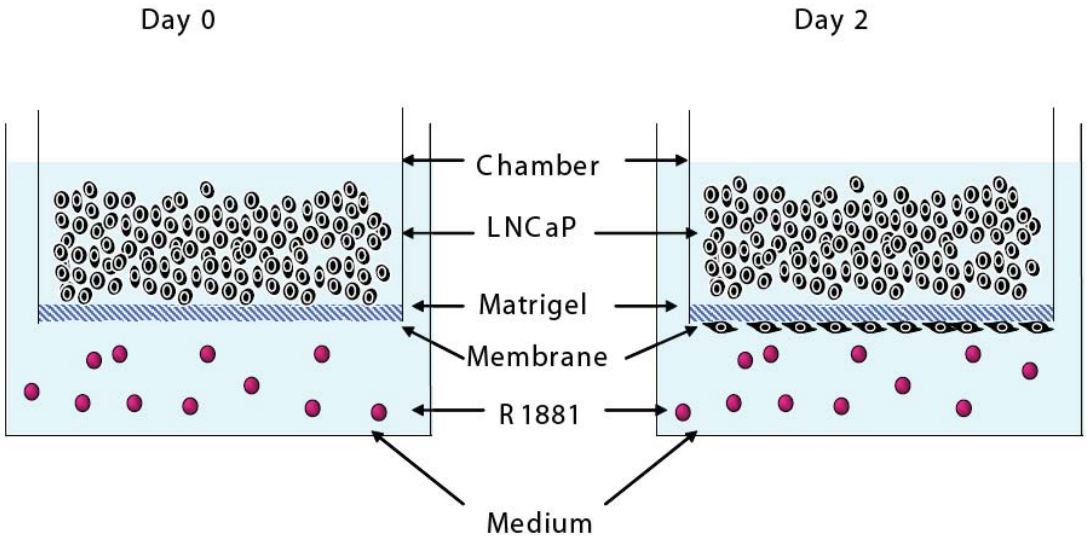

Figure 6. Matrigel Invasion Assay. Cells are seeded on top of a filter covered with matrigel and are allowed to invade in response to a stimulant such as synthetic androgen R1881.

For a malignant tumor cell to metastasize to other tissues, the ability to invade and penetrate the surrounding extracellular matrix barrier is crucial. Matrigel invasion assay is an *in vitro* cell invasion assay developed to determine the invasive property of malignant cells. Matrigel is a commercially available extract of solubilized basement membrane components derived from the Engelbreth-Holm-Swarm mouse sarcoma cell line. It is the most commonly used matrix barrier in this assay. To setup the Matrigel invasion assay we employed a Transwell or Boyden chamber containing 6.5 mm polycarbonated filter (with 8 μM pore size). Briefly, the Matrigel was evenly dispensed onto the porous filters and allowed to solidify. The cells were seeded in the upper chamber. The lower chamber contained the same medium as the upper chamber plus the addition of a chemo-attractant. In this case, androgen was added into the lower chamber to determine hormone-induced invasion. The cells in the chambers were incubated at 37ºC in an incubator for the indicated time. Cells that invaded through the matrix and attached to the membrane were fixed, stained with crystal violet solution and counted under a light microscope.

3.2.2 Soft Agar Colony Formation Assay

Anchorage-independent growth is one of the hallmarks of malignant transformation of cancer cells. The soft agar colony formation assay is a method used to monitor anchorage-independent growth of cells on semisolid culture media. The semisolid agar prevents cell movement and allows the progeny clonal of individual cells to stay together and form colonies. It is considered the most accurate and stringent *in vitro* assay for detecting the malignant transformation of cells. Briefly, the assay was performed in 6cm plates. A bottom layer of enriched supporting media agar was poured, and allowed to solidify at room temperature. It was followed by a layer containing a low concentration of agar mixed with a specified number of cells. Androgen receptor antagonist, bicalutamide was added into both layers to study androgen dependency. The plates were placed in the incubator and after two weeks, colonies were stained with iodonitrotetrazolium chloride and counted.

3.2.3 Chromatin Immunoprecipitation

ChIP is a method used to determine protein DNA binding activity in living cells or tissues. The principle underpinning this method is that formaldehyde can react with primary amines located on amino acids and the bases on DNA molecules to form a covalently crosslinked complex between the protein and its interacting DNA. Following formaldehyde fixation, the cells are lysed and extracts are sonicated to shear the DNA to a length of 0.5-1kb. An antibody targeting a protein of interest is then used to immunoprecipitate the protein:DNA complex. The DNA protein crosslinks are reversed by heating and the protein released is digested with proteinase K. The DNA is then purified and identified by PCR using specific primers to the suspected binding region. In this thesis ChIP was used to demonstrate the binding of c-Myc to the ezrin gene promoter region in response to androgen treatment.

3.2.4 Luciferase Activity Assay

The luciferase reporter assay has been widely used to study eukaryotic gene expression and cell physiology. The applications include receptor activity, mRNA processing and transcription factors. The pGL2 luciferase reporter vectors are designed for quantitative analysis of factors that potentially regulate mammalian gene expression. These vectors carry the coding region of wildtype firefly (Photinus pyralis) luciferase. In our study, the luciferase activity of pGL2 vectors was driven by a fragment of human ezrin gene promoter region predicted to contain a c-myc binding site. After the vector was transfected into cultured cells, the luciferase activity was measured using a luminometer. The light intensity reflects the transcriptional activity of the inserted ezrin promoter region in relation to specific experimental condition, i.e., androgen stimulation in our study. A vector expressing β-galactosidase gene was cotransfected with pGL2 and a β-galactosidase assay was performed to normalize the luciferase activity.

3.2.5 Pulse-Chase Experiment

Pulse-chase experiment is a method to determine the half-life of a specific protein. In cells, proteins are constantly being synthesized and degraded. To label newly synthesized protein, a "pulse" of radioactive ^{35}S methionine is added into the cell culture. The radioactive methionine is incorporated into newly synthesized proteins and makes them radioactive. After the labeling, the unincorporated radioactive methionine is removed and replaced with a non-radioactive (cold) version. Cells are lysed at selected time points and the protein of interest is immunoprecipitated using a specific antibody. The immunoprecipitated material is separated by SDS-PAGE and the amount of labeled protein is visualized by autoradiography. In this thesis pulse-chase experiments were used to examine androgen regulation of c-myc stability.

4 RESULTS

4.1 IDENTIFICATION OF ANDROGEN-REGULATED GENES POTENTIALLY INVOLVED IN CELL INVASION

Androgens act through the AR to regulate the proliferation, apoptosis, invasion and metastasis of PCa cells. When the cells are treated with androgens, the hormone diffuses through the cell membrane and binds to the androgen receptor. Ligand activated AR then translocates to the cell nucleus and binds to androgen response elements in the promoter region of a regulated gene, recruits other proteins to form a transcription complex and subsequently regulates the gene expression.

In previous microarray based gene expression studies, we have identified several androgen-regulated genes, some of which could be important in the regulation of PCa cell invasion and cancer progression. In paper I, II and III, we investigated if matrix metalloproteinase 13 (MMP-13), CD-9 and ezrin are androgen regulated in PCa using LNCaP-FGC, an androgen sensitive PCa cell line as a model. Real time quantitative PCR was used to analyze MMP-13, CD9 and ezrin mRNA and the mode of regulation by androgens. Our results showed that all of the selected genes were upregulated by androgen treatment and that the effect was inhibited by the AR antagonist, bicalutamide. Using the protein synthesis inhibitor, cyclohexamide, we found that induction of MMP-13 mRNA was not inhibited in LNCaP-FGC cells suggesting a direct effect of androgen on the MMP-13 gene. On the other hand, androgen actions on ezrin mRNA expression seemed to be a secondary effect since a *de novo* synthesized protein was needed to induce its expression. Changes in protein levels after androgen treatment for CD9 and ezrin correlated well to variations in mRNA levels.

Interestingly, western blot analysis showed that androgen treatment of LNCaP-FGC results in activation of the MMP-13 protein. MMP-13 is a secreted 452 residue protein which is released from cells as an inactive zymogen and activated extracellularly by removal of its propeptide. Cleavage of the 84 residue propeptide can be catalysed by other MMPs such as MMP-2 and MMP-14, or by factors like plasmin (244). Activated MMP-13 takes part in the activation of proMMP-9 into MMP-9. The mechanism of androgen activation of the MMP-13 enzyme is unclear.

4.2 DIFFERENTIALLY EXPRESSED GENES IN ASSOCIATION WITH PCA PROGRESSION

Deregulation of androgen controlled genes is common during PCa progression. Immunohistochemistry was performed on PCa patient biopsies, paraffin sections from tumors and tissue microarray slides to study protein expression levels in relation to PCa progression.

In our study, no obvious difference in expression of MMP-13 protein level was detected between BPH and cancer cells obtained by fine needle biopsies, neither was any obvious correlation to malignancy grading. We cannot conclude that MMP13 has prognostic or diagnostic value in PCa because the samples size used was too small to make a general conclusion. Interestingly, the BPH and cancer cells showed focal and intense cytoplasmic staining. MMP-13, also called collagenase III has high activity towards collagens (245), it may therefore play a role in ECM turnover and contribute to cell invasion. Following our publication, an independent report has shown higher plasma concentration of MMP-13 in PCa patients with metastases compared to patients with organ-confined carcinoma and control group samples. After ADT, the plasma MMP-13 level decreased markedly (246). These results taken together with our findings of androgen regulation suggest that MMP-13 in PCa could be a diagnostic marker for PCa. In other tumor types, the presence of MMP-13 is associated with highly invasive lesions of the tumors, suggesting that this protein may contribute to cancer progression (247,248).

In paper II, we reported that CD9 protein level in PCa was not related to primary Gleason score in neither tissue microarray nor paraffin embedded samples. In prostate tissue from one patient under androgen ablation therapy no staining was observed in luminal epithelial cells, indicating that CD9 expression in human epithelial cells is androgen sensitive; this is in line with the results obtained in LNCaP-FGC. An independent study, has found CD9 expression to have an inverse correlation with PCa progression and metastasis. The study confirmed our finding of reduced or absent CD9 staining in tumor samples from patients undergoing ADT (193).

An interesting study in progressive cervical carcinomas reported down-regulation of CD9, but re-expression at the sites of vascular space invasion, indicating tumor cells might use CD9 for cell migration and invasion into vasculature (249). CD9 forms complexes with certain integrin subunits depending on cell types. Integrins play important roles in the interaction between cells with different types of ECM (175). Therefore, some of the effects of androgen on cytoskeletal proteins that result in attenuated adhesion and/or matrigel invasion of PCa cells may be associated to CD9 regulation of integrin signaling. Two other binding partners of CD9 from the IgG superfamily, EWI-2 and EWI-F, have been found to associate with actin-linked ezrin-radixin-meosin proteins (250). The function of one ERM family protein, ezrin was related to androgen induced PCa cells invasion in this thesis. So, it is possible that CD9, integrin and ERM may act together in a complex to regulate cell motility, polarity and invasion in PCa cells.

In paper III, ezrin levels were examined by immunohistochemistry in the prostate tissue of three patients obtained from prostate biopsies before and after radical prostatectomy. Although proper statistic analysis was not possible due to the small number of samples, our data suggests that ezrin expression is under androgen control in human prostate. Previous studies in our group have reported strong ezrin staining in HGPIN compared to benign prostate epithelial and cancer cells. This led us to hypothesize that ezrin

expression in precancerous lesions may be important for tumorigenesis (251). However, the sample sizes were too small to provide statistical support to our conclusion. Subsequently, a follow up study reported that ezrin expression correlated with PCa Gleason score and seminal vesicle invasion (252). These data support a role for ezrin in the biology of human PCa progression.

4.3 ANDROGEN REGULATION OF PCA CELL INVASION

Androgen signaling plays an important role in prostate gland homeostasis and cancer development. But, the effect on PCa cell migration and invasion through ECM is largely unknown. In paper III and IV, cell experiments were carried out to investigate androgen effects on PCa cell invasion through matrigel. In the study published in paper III, we observed that synthetic testosterone, R1881 stimulated LNCaP-FGC cell invasion through matrigel. This effect was blocked by androgen receptor antagonist, bicalutamide. As the regulation of cell motility by ezrin is well documented, we investigated the effect of ezrin on androgen induced PCa cell invasion by using siRNA knockdown and overexpression of ezrin in PCa cells. According to our results, the effects of androgen on cell invasion are mediated by ezrin in PCa cells. However, forced expression of wildtype ezrin did not induce PCa cell invasion in the absence of androgen. Overexpression of two dominant negative forms of ezrin mutants (mutation on two phosphorylation sites of ezrin, Thr567 and Tyr353 amino acid residues) blocked the androgen effects on cell invasion. Western blot analysis showed that R1881 treatment caused phosphorylation on both Thr567 and Tyr353 residues on ezrin protein. Phosphorylation of ezin protein by androgen is a sequential effect where initial Thr567 phosphorylation is followed by phosphorylation in Tyr353. These results demonstrate that androgen stimulates the expression as well as phosphorylation of ezrin, which leads to increased invasiveness.

After the publication of paper III, another group reported that Thr567 function is the result of a rapid phosphorylation turnover. The Thr567 phosphorylated ezrin is tightly associated with F-actin while the dephosphorylated form associates with the membrane. The rapid changes of Thr567 phosphorylation may help to maintain a dynamic relationship between ezrin and its binding partners on membrane and actin filaments (253). The effect of the rapid phosphorylation turnover of Thr567 on Tyr353 phosphorylation remains unknown.

In paper IV, we continued to explore androgen receptor signaling that influence the PCa cell invasion in relation to ezrin protein. c-Myc was reported to be an androgen regulated gene in PCa cell (254) and its expression is correlated to PCa progression and metastasis (140,166). We hypothesized that androgen action on ezrin expression in PCa is mediated through the c-Myc protein. In this study, we demonstrated that c-Myc is an androgen regulated gene on both mRNA and protein levels. The effect of androgen in PCa cell invasion requires c-Myc expression.

Western blot analysis of protein extracts from PCa cells transfected with either c-Myc siRNA or a wildtype c-Myc expression plasmid showed ezrin protein expression correlated with c-Myc protein level. This indicates that the regulation of ezrin expression by androgen could be mediated through c-Myc. When we searched for transcription factor binding sites on the proximal promoter region of the ezrin gene, we found a potential c-Myc binding site, an E-Box. However, no AR response element was evident. We next cloned the ezrin promoter region harboring the E-Box into the pGL2 plasmid and studied the activity after R1881 stimulation. We demonstrated that the ezrin promoter region activity is regulated by R1881 and also by c-Myc. ChIP experiments also showed physical binding of c-Myc to the ezrin promoter under R1881 stimulation. Our findings indicate that androgen regulated ezrin transcription is indeed mediated via c-Myc.

These two papers demonstrate that androgen stimulates ezrin expression and phosphorylation in PCa cells. Activated ezrin will induce PCa cell invasion through matrigel. The androgen effect on ezrin expression is indirect and mediated through the transcription factor c-Myc.

4.4 PATHWAYS THAT REGULATE PCA CELL INVASION

In paper III, we demonstrated that ezrin needs to be activated by phosphorylation by kinases in order to induce PCa cell invasion through matrigel. Several signaling pathways have been linked to ezrin controlled cell invasion and migration in different cell types. But, there was no evidence that related these kinase pathways to androgen induced invasion triggered by ezrin. We therefore decided to explore which kinases are involved in androgen induced ezrin phosphorylation. When we used pharmacological inhibitors to inhibit kinases that had been reported to phosphorylate ezrin Thr567 *in vitro* (255,256), we found that only the inhibition of PKCα and PIP2 blocked androgen induced ezrin phosphorylation on the Thr567 and Tyr353 residues, and led to a dramatic diminishment of androgen induced invasion. In contrast, inhibition of Rho-activated kinase, ROCK gave opposite results.
Tyr353 phosphorylated ezrin was reported to have an important role by interacting with p85, the regulatory subunit of PI3 kinase, resulting in activation of the downstream protein kinase Akt (197). When we treated LNCaP-FGC cell with PI3K inhibitor, LY294002, cell invasiveness was blocked but no effect was observed on androgen induced ezrin phosphorylation. This finding is in line with previous reports that PI3K act downstream of ezrin. If the tyrosine kinase directly phosphorylates Tyr353 is unclear but it has been reported to be dependent on Src kinase activity. Accordingly, treatment of LNCaP-FGC with Src kinase inhibitor, PP2 resulted in inhibition of androgen effects on both matrigel invasion and Tyr353 phosphorylation.

The results from paper III indicate that PKCα and PIP2 are involved in androgen induced Thr567 phosphorylation, whereas Tyr353 phosphorylation is Src kinase dependent. PI3K/Akt signaling is located downstream of ezrin in androgen induced PCa cell invasion through matrigel.

4.5 ANDROGEN NON-GENOMIC ACTIONS IN PCA CELL INVASION

In addition to transcriptions that function in the nucleus as described in the introduction, recent evidence suggest that androgen can exert rapid, non-genomic effects on cellular processes through modulation of discrete signal transduction pathways like protein kinase A, protein kinase C and MAPK (257). AR has been found to interact with the intracellular tyrosine kinase, Src and trigger its activation. For this effect AR can function cooperatively with ERα or ERβ (258,259). Androgens can also stimulate second messenger cascades through at least one membrane receptor, which is typically not blocked by AR antagonists (260).

Androgen receptor antagonist, bicalutamide has been widely used to study androgen receptor function both *in vivo* and *in vitro*. In all papers included in this thesis, the drug was used to study effects of androgen receptor inhibition. However, the bicalutamide treatment of LNCaP-FGC cells resulted in effects that we did not postulate. In paper III, we demonstrated that although bicalutamide treatment blocked androgen induced PCa cell invasion, the drug itself had a positive effect in promoting cell invasion. We also observed induction of PKCα translocation to plasma membrane by bicalutamide in the presence and absence of androgen. The most likely explanation for this observation is that these constitute non-genomic actions of the AR that could be activated by AR bicalutamide binding. Previous studies have shown that bicalutamide could switch from antagonist to agonist of the AR during long term androgen ablation (261,262). Further studies are needed to address whether the mechanisms unveiled in LNCaP-FGC could be translated into possible side effects of bicalutamide based AR blockage.

4.6 THE INTERPLAY OF C-MYC AND EZRIN IN PCA CELL INVASION

In paper IV, we demonstrated that besides regulating the expression of c-Myc, androgen stimulation can also stabilize and prolong c-Myc protein half life in PCa cells. Western blot analysis revealed significantly lower c-Myc protein levels in PCa cells expressing ezrin mutants compared to control cells. Overexpression of c-Myc induced the proliferation and tumorigenic capacity of PCa cells and the effect of c-Myc was blocked by co-transfection of dominant negative ezrin mutants. From these data, we hypothesized that activated ezrin plays an important role in androgen induced c-Myc protein stabilization.

The Ubiquitin/Proteasome degradation of c-Myc is mediated through GSK-3β mediated phosphorylation of the Thr58 residue in c-Myc. GSK-3β kinase signaling is inhibited by the PI3K/Akt pathway. When LNCaP-FGC cells were treated with

androgen, we observed increased PI3K activity (by measuring Akt Ser473 phosphorylation) followed by down-regulation of GSK-3β signaling (measured as phosphorylated Thr216 GSK-3β). Pharmacological inhibitors of PI3K (LY294002) and Akt (SH-6) both inhibited androgen induced PCa cell invasion and expression of c-Myc and ezrin protein. When we inhibited GSK-3β activity using Lithium chloride, the c-Myc degradation in the absence of androgen was significantly delayed. It was previously shown that Tyr353 phosphorylated ezrin binding to the p85 regulatory subunit of PI3K leads to activation of Akt. We tested if expression of dominant negative ezrin mutants could alter the levels of Ser473 Akt and Thr216 GSK-3β in PCa cells. By western blot, we confirmed down-regulation of Akt and up-regulation of GSK-3β signaling in PCa expressing ezrin mutants under androgen stimulation.

Additional evidence was provided by overexpression of the PI3K/Akt antagonist, PTEN, in PCa cells. LNCaP-FGC does not express functional PTEN protein. Our data demonstrated that transient overexpression of PTEN cause a down-regulation of c-Myc and ezrin expression in PCa cells and subsequent reduction of cell invasion, proliferation and tumorigenesis.

The data presented in paper IV describes a novel mechanism regulating PCa cell invasion that involves the interplay between ezrin and c-Myc. Androgens induce ezrin expression via c-Myc. Phosphorylation of ezrin by androgen actions leads to activation of PI3K/Akt signaling and deactivation of GSK-3β. These effects prevent the degradation and promote the synthesis of c-Myc. Taken together, this positive feedback loop involving c-Myc and ezrin influences tumorigenic properties of PCa cells.

5 DISCUSSION

The ability of tumor cells to form metastases is the major cause of PCa related cancer death. Metastases formation is a multiple steps process that begins with cancer cells that migrate and invade neighboring connective tissues, the lymphatic system and blood vessels. The signaling pathways that are involved in cell migration and invasion have been extensively examined. PCa cells have the unique feature that proliferation, survival and metastases are controlled by androgens through the AR signaling pathway. However, how the AR regulates PCa cell invasion remains poorly understood.

As mentioned in the introduction, cell invasion in 3D and tissues involve the coordination of multiple steps that are controlled by different pathways. To induce PCa cell invasion, AR signaling has to regulate these pathways either directly or indirectly. Previous gene expression profile analysis on prostate gland and PCa cell lines performed by our group identified a set of androgen-regulated genes (ARG) that might be involved in PCa cell invasion. This set of ARG provides a platform to further study the complex mechanisms involved in androgen actions for this phenomenon. In this thesis, we have demonstrated that androgen stimulation of hormone sensitive PCa cells induce the expression of a number of genes that lead to tumor cell invasion. The ARG included in this study are matrix metalloproteinase 13 (MMP-13, ECM degradation and remodeling), CD9 (integrin signaling) and ezrin (actin-membrane linker protein).

In this thesis, we have demonstrated that androgen treatment of hormone sensitive PCa cells induce expression of MMP-13, CD9 and ezrin at both mRNA and protein levels. Our results show that androgen regulated gene expression of invasion-related genes can be either direct or indirect. For instance, induction of ezrin mRNA in our study was found to be inhibited by the protein synthesis inhibitor cyclohexamide. This suggests that an androgen-induced, *de novo* synthesized protein is needed to induce ezrin expression. One of the factors that may be responsible for androgen-induced ezrin expression is c-myc. The oncogene c-myc was found to be induced by androgens and to bind the proximal promoter region of the ezrin gene regulating its transcription.

A mechanism whereby AR signaling could interfere with the invasion process is through regulation of kinases and phosphatases with a role in cell invasion. In addition to the classical paradigm of AR as a transcriptional activator, evidences suggest that the AR is able to activate the MAP kinase pathway through a mechanism independent of its transcriptional activity. These effects of androgen are proposed to occur via a membrane-associated and/or cytoplasmic receptor (257). The AR and other steroid hormone receptors were found to interact with the intracellular tyrosine kinase c-Src and trigger its activation (258,259). Activated c-Src was shown to regulate ezrin tyrosine phosphorylation and thereby influence its functions (263,264). Other non-genomic androgen actions include the ability to rapidly modulate the activity of ion channels resulting in changes in intracellular calcium levels (260,265). Here we also

demonstrated that androgen stimulation on LNCaP-FGC cells could activate PKCα, because this effect is shared by bicalutamide it is likely to be a newly described non-genomic effect of the AR. The mechanisms behind these actions are still unclear but it seems to be a key step in ezrin-mediated PCa cell invasion.

Cancer cell invasion through a surrounding connective tissue barrier is highly dependent on the interplay between adhesion and proteolytic activities. The deposition of proteases such as members of the MMP family is essential in tissue remodeling, which favors tumor cell invasion. The ECM and its degradation products created by MMP proteolysis could signal cancer cells through their cell surface receptor integrins and affect cell behavior. Integrins may also have more complex roles by coordinating their actions with MMPs and serine proteases, which together may increase cancer cell invasion (143,266). Our findings demonstrate that androgen stimulation of LNCaP-FGC induces the expression of MMP-13 as well as several integrin subunits (267) our unpublished observations). Besides regulating the expression, androgen also activates MMP-13 by inducing unknown extracellular signaling. Interestingly, other studies have showed MMPs secreted by stroma and macrophage cells at the tumor invasive front have a major role in ECM remodeling and promote the invasion of tumor cells (209,268). The role and importance of MMP-13 in PCa invasion by androgen stimulation is unclear and further studies are needed to elucidate the significance for androgen induced PCa invasion. However, we cannot rule out the possibility that MMPs secreted from the tumor microenvironment may have a large impact on PCa invasion since LNCaP-FGC show a low invasive capacity *in vitro*.

In this thesis, we demonstrated that androgen induced PCa cell invasion is mediated through ezrin protein activity. Ezrin is a molecular linker that connects cell surface protein to the actin cytoskeleton (269,270). This interaction provides an intracellular scaffold for the formation of specialized membrane domains that facilitate signal transduction through a number of growth factor receptor and adhesion molecules (271). Studies have identified some of the binding partners of ezrin, including the hyaluronan receptor CD44 (272), Fes kinase (273), p85 regulatory unit of PI3K (197), PIP2 (274) and adaptor protein EBP50 (275). Ezrin can regulate cell survival, adhesion, migration and invasion, phenomena that are important for tumor development (276,277). Notably, ezrin upregulation is also observed in various metastatic tumors in addition to PCa, suggesting a common selective pressure for ezrin expression in cancer metastasis.

Elimination of ezrin by siRNA or production of dominant negative ezrin mutants blocked the invasion capability of both androgen sensitive and androgen independent PCa cells. This suggests that this protein is a key regulator of cancer cell invasion. Microarray analysis on gene expression profile in PCa tumors failed to detect the regulation of this gene during cancer progression (140,238). One immunohistochemistry study on PCa tumors has correlated the protein expression to Gleason score (252). This indicates that measuring ezrin mRNA and total protein expression in PCa may not be a good marker for cancer progression and metastasis. Because of the importance of ezrin phosphorylation for its activity, phosphorylated

ezrin could be a better prognostic or diagnostic marker as compared to total ezrin. There are at least four phosphorylation sites identified on the ezrin protein and each have a distinct function. Further studies are needed to identify the most useful posttranslational modification of ezrin in its potential role as diagnostic marker.

Upstream Ezrin

Kinase	Amino acid	Effect	Reference
PKCα	Thr567	Cell motility	(255)
PKCι	Thr567	Microvilli formation	(278)
Rho	Thr567	Activate ezrin	(279)
Src	Tyr145, Tyr477	Cell adhesion	(264), (280)
PIP_2	Thr567	Apical localization	(256)
PKA	Ser66	Apical-membrane Cytoskeleton remodeling	(281)

Downstream Ezrin

Kinase	Effect	Reference
Fes Kinase	Cell scattering	(273)
Cdc42	Cell migration, filopodia formation	(282), (283)
MAPK	Enhance microvillus length	(284)
Src	Cell motility and invasion	(264)
FAK	Cell-matrix adhesion	(285)
ROCK	Fibroblast transformation	(286)
PI3K	Cell survival	(197)

Table 2. Ezrin and Kinase signaling. Upper panel listed kinases that have reported to phosphorylate ezrin protein at various phosphorylation sites. Lower panel listed kinases that activate by ezrin protein.

The c-Myc is a powerful regulator of cell growth, differentiation, proliferation and apoptosis. The key roles of c-Myc in PCa metastatic progression have been demonstrated in mouse models. Prostate specific overexpression of c-Myc results in immortalisation of normal human prostatic epithelium and drives it to a metastatic tumor (219,287). In this thesis, we also demonstrated a function of c-Myc to regulate PCa cell invasion through ezrin. Given its potent effects on cell fate, it is not surprising that cells have evolved complex networks for ensuring proper c-Myc expression levels. The expression of c-Myc can be regulated at different levels like transcriptionally, post-transcriptionally (c-Myc mRNA stability and translation) or post-translationally (protein stability). Deregulation of c-Myc occurs in most human cancers (277,288,289). The c-Myc protein stability is controlled by two phosphorylation sites, threonine 58 and serine 62 located in the N-terminus of c-Myc which are well conserved across species and in c-Myc family members (290,291). Serine 62 is a target of Extracellular receptor kinase (Erk) and Threonine 58 is targeted by GSK-3β (292,293). Analysis of the effects

of the phosphorylation revealed that Serine 62 phosphorylation will stabilize c-Myc whereas Threonine 58 will destabilize the protein. In this thesis, we demonstrated that c-Myc is very unstable in PCa cells in the absent of androgen stimulation. Except the classical genomic role in regulating the c-Myc mRNA expression, androgen stimulation also stabilizes the protein. We observed that the androgen action on c-Myc stability is achieved through phosphorylation/activation of ezrin protein, subsequently leads to activation of PI3K/Akt and down-regulates GSK-3β signaling. However, it is not clear if the regulation is through post-transcriptional or post-translational level. Further studies are needed to address this issue.

The papers presented in this thesis are trying to elucidate the role of androgen on PCa cells invasion. We described a novel role of androgen in PCa cell invasion through matrigel that involved the interplay between c-Myc, ezrin and several kinase pathways. A schematic diagram summarizing the working model is shown in figure 7. Androgen induced PCa cell invasion could also be influenced by extracellular signaling from surrounding tumor microenvironment. For instance, metalloproteinases degradation of ECM can activate PCa cell integrin signaling, which is regulated by transmembrane 4 family proteins. During cancer progression to HRPC, genetic and molecular changes in tumor cells combined with active tumor-stroma interaction can lead to dysregulation of this complex cell invasion network. Indeed, dysregulation of c-Myc, ezrin and CD9 (discussed in previous sections) are observed in PCa progression. In addition, several growth factors signaling can also contribute to the improper activation of AR signaling and kinases during PCa progression. Most of the molecular and genetic mechanisms remain unknown. Therefore, a detailed understanding of deregulation of AR signaling and cell invasion process is necessary for better control of the disease. This thesis is merely a small step in the understanding of PCa cell invasion process.

6 GENERAL CONCLUSION AND FUTURE OUTLOOK

In this thesis, we have characterized expression of androgen-regulated genes in particular ezrin, MMP-13 and CD9. Furthermore, we have for the first time demonstrated that androgen induces PCa cell invasion through the activation of two distinct protein molecules, c-Myc and ezrin. In addition, androgen induced invasion involve the activation of several important cell signaling pathways, which are not exclusive to androgens and can be modulated by extracellular signals from surrounding tumor microenvironment. Therefore, the pathways here described can also be at use in HRPCa under the control of cytokines and growth factors or triggered by intracellular chages derived from aquired genetic events associated to progression. The study has led to a hypothetical model for androgen induced cell invasion as presented in Figure 7.

It is important to obtain a detailed understanding of signaling pathways as exemplified on diagram above. A mechanistic insight of cell invasion is important not only from a principal biological perspective but also for finding potential targets to future anticancer drug development. Future work along these lines may provide candidate targets for drug development. The Figure depicts tested drug targets e.g. the androgen receptor and integrins, targets under clinicl evaluation such as Src and PKCa inhibitors and also untested drug targets. We believe it is important to further explore molecular action of ezrin and the stability and turnover aspects of c-myc expression, in this respect.

Ezrin is expressed in epithelial cells and contains several phosphorylation sites where each has its distinct functions. Our understanding is that ezrin is a mediator of PCa cell invasion for both androgen sensitive and insensitive cells. The main control of this process seems to be exerted at the level of ezrin phosphorylation, by highly dedicated kinase pathways. Our findings that a novel function of activated ezrin protein appears to involve the regulation of c-Myc protein stability makes ezrin a thinkable drug target in cases of advanced and metastasic PCa. Specific pharmacological drugs that inhibit different kinase pathways have been developed. However, toxicity and side effects of such drugs on normal tissues can be problematic. It is of course a difficult task to process new drug target and one needs to take into consideration many aspects related e.g to specificity and suitability for drug developments. Furthermore, the exact mechanisms of how ezrin positions itself is the cross road of multiple pathways regulating cell invasion is still largely unknown. A deeper understanding of these mechanisms may lead to the development of more specific drugs for PCa treatment.

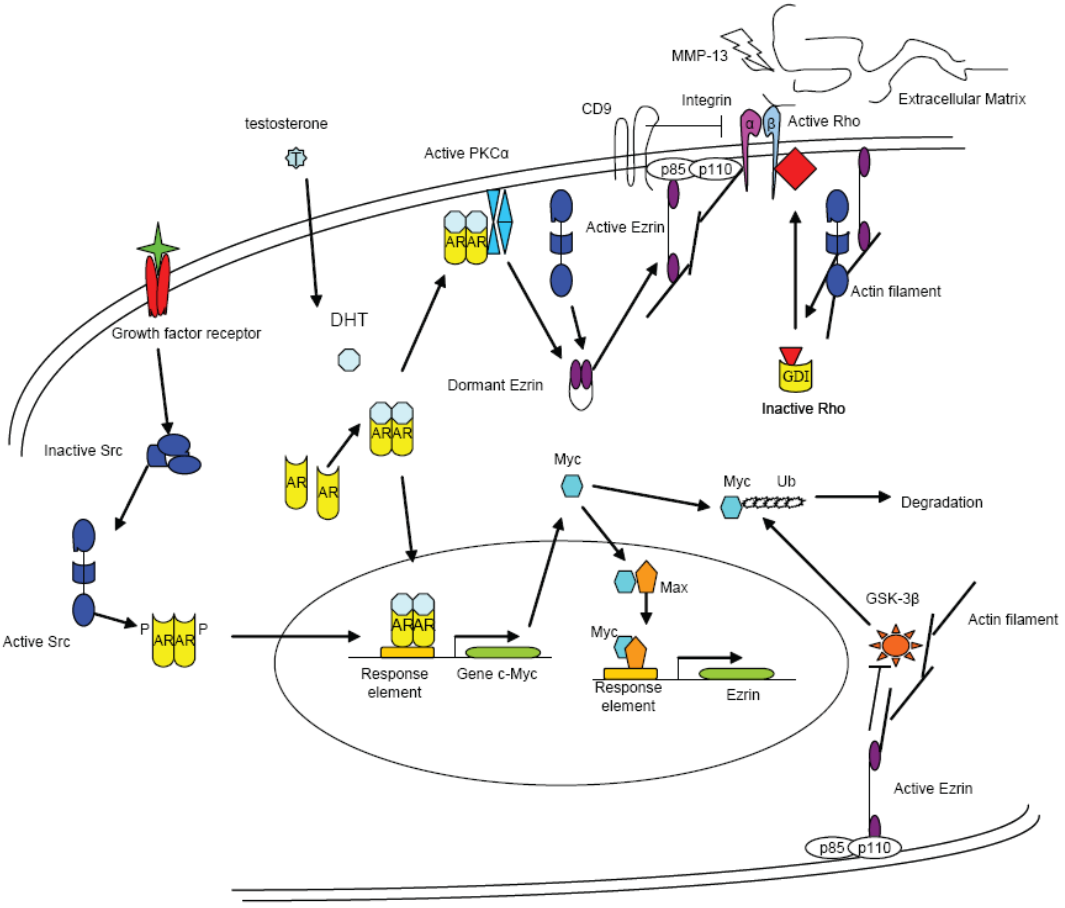

Figure 7. Schematic diagram of our hypothetical model for androgen induced PCa cell invasion involving the activation of ezrin and c-myc. For explanations see text.

Although AR signaling seems to be decreasing during PCa metastatic development, the expression of ezrin and c-Myc were found to be increased upon PCa progression. This suggests that activation of other signals evoked by e.g. growth factors secreted by surrounding tumor microenvironment, can take part in regulating cell invasion. A combined treatment that targets several pathways with lower toxicity and side effects may serve as future paradigm for advanced PCa treatment. Our studies have only covered androgen regulated genes with an invasion-related function, representings only a small step in the understanding of PCa cell invasion. The ultimate goal is to gain sufficient insights into cell invasion to be able to build models for PCa cell invasion that can be used to identify more specific drugs target to interfere with the process. In the future, such models may become sufficiently advanced to comprise the dynamics of signaling, the variability of tumor stages and the complexity of different cell types.

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