Invasive and Metastatic Properties of Advanced Prostate Cancer

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2009

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Front cover: A normal mouse prostatic gland invaded with tumor cells, in DAPI staining.

ISBN 978-91-628-7761-3

Printed by Intellecta Infolog AB, Västra Frölunda, Sweden

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ABSTRACT

Prostate cancer is initially androgen-dependent (AD) and therefore androgen deprivation therapy (ADT) is generally used to treat advanced prostate cancer. However, the long-term treatment effects are insufficient and over time an androgen-independent (AI) tumor relapses, which is generally highly aggressive and metastatic. Treatment regimens in the AI stage are only palliative and median patient survival is less than a year. Therefore, new treatment concepts are urgently needed. The purpose of this thesis was to investigate molecular and cellular characteristics of advanced prostate cancer. The specific focus was on characteristics related to invasivity and metastatic ability in the AI stage. An experimental model system comprising of an AD and an AI prostate cancer cell line was used for *in vitro* studies in cell culture and *in vivo* studies in immunodeficient mice. In addition, samples from prostate cancer patients were included in the studies and evaluated by immunohistochemical analyses. Studies performed using the experimental model showed that transition into androgen-independency was associated with several prometastatic alterations, including increased migration and tumor cell invasivity into blood vessels. Further, the AI tumors displayed elevated levels of N-cadherin, matrix metalloproteinase 9 (MMP-9) and membrane type-1(MT1)-MMP and decreased expression of the tumor suppressor E-cadherin compared to the AD tumors. Further studies demonstrated that intraprostatic AI tumors were suppressed when grown in intact mice compared to castrated mice, probably by androgen-regulated factors secreted from the prostatic stromal cells. In addition, the proinvasive factor N-cadherin was increased by androgen deprivation in experimental AI tumors and in samples from human prostate cancer. Similarly, N-cadherin was increased in specimens from AI prostate tumors compared to early non-treated tumors and was associated with Gleason score and metastasis. Finally, the results show that the lymphangiogenic factor vascular endothelial growth factor C (VEGF-C) and its receptor VEGFR-3 were elevated in primary tumors from patients with regional lymph node metastases compared to patients without lymph node metastases. In summary, this thesis shows that androgen deprivation and the subsequent development of AI tumors are associated with several prometastatic alterations in the prostate cancer cells. The results also suggest that AI tumors do not thrive in the prostatic environment and supports previous observations of frequent progression of AI prostate cancer as metastases in patients. Moreover, the results indicate a possible role for VEGF-C and N-cadherin in promoting dissemination of tumor cells to distant sites. Thus, N-cadherin and VEGF-C might be potential therapeutic targets for future anti-metastatic treatment for advanced prostate cancer.

Key words: Prostate cancer; Androgen-independent; Castration-resistant; Metastasis, Invasion; Lymphangiogenesis; Cell adhesion; N-cadherin; VEGF-C; MRI

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. Jennbacken K., Vallbo C., Wang W., Damber JE.
Expression of vascular endothelial growth factor C (VEGF-C) and VEGF receptor-3 in human prostate cancer is associated with regional lymph node metastasis. *The Prostate. 2005 Oct 1;65(2):110-116*

II. Jennbacken K., Gustavsson H., Welén K., Vallbo C., Damber JE.
Prostate cancer progression into androgen independency is associated with alterations in cell adhesion and invasivity. *The Prostate. 2006 Nov 1;66(15):1631-1640*

III. Jennbacken K., Gustavsson H., Tešan T., Horn M., Vallbo C., Welén K., Damber JE.
The prostatic environment suppresses growth of androgen-independent prostate cancer xenografts: An effect influenced by testosterone. *The Prostate 2009. In press*

IV. Jennbacken K., Tešan T., Wang W., Gustavsson H., Damber JE., Welén K.
N-cadherin increases after androgen deprivation and is associated with metastasis in prostate cancer. *In manuscript*

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ABBREVIATIONS

ADAMTS1	ADAM metallopeptidase with thrombospondin type 1, motif 1
AD	Androgen-dependent
ADT	Androgen deprivation therapy
AI	Androgen-independent
AR	Androgen receptor
ARE	Androgen response element
BPH	Benign prostatic hyperplasia
CAF	Carcinoma associated fibroblast
CAM	Cell adhesion molecule
DHT	Dihydrotestosterone
DLP	Dorsolateral prostate
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial mesenchymal transition
FBS	Fetal bovine serum
FBS-DCC	Fetal bovine serum - dextran charcoal treated
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GnRH	Gonadotropin releasing hormone
IGF	Insulin-like growth factor
KGF	Keratinocyte growth factor
LH	Luteinizing hormone
LNCaP	Lymph node carcinoma of the prostate
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MT1-MMP	Membrane type 1-matrix metalloproteinase
MVD	Microvessel density
NE	Neuroendocrine
NRCAM	Neuronal cell adhesion molecule
PCDH20	Protocadherin 20
PDGF	Placental derived growth factor
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate specific antigen
RGS2	Regulator of G-protein signaling 2, 24 kDa
RT-PCR	Reverse transcriptase polymerase chain reaction
SCID	Severe combined immunodeficiency
SEM	Standard error of the mean
TGF-β	Transforming growth factor β
TURP	Transurethral resection of the prostate
VEGF-C	Vascular endothelial growth factor C
VEGFR-3	Vascular endothelial growth factor receptor 3
ZEB1	Zinc finger E-box binding homeobox 1

INTRODUCTION

The normal prostate gland

Anatomy and physiology

The prostate gland is a small, rounded organ with a diameter of approximately 4 cm. It is positioned immediately below the urinary bladder, where it encircles the proximal portion of the urethra. The prostate consists of glands, smooth muscles and connective tissue and is enclosed by a fibrous capsule-like structure. The glandular ducts open up into urethra. The human prostate can be divided into three distinct zones; the peripheral zone, the transitional zone and the central zone.[1] The peripheral zone is the largest and the most common origin of prostate cancer.[2] Studies of prostate cancer are often performed in rodent models. In contrast to humans, the rodent prostate gland consists of lobes; the anterior lobe, the dorsal and lateral lobe (collectively referred to as the dorsolateral lobe) and the ventral lobe.[3,4] There is no clear analogy between the lobular structure of the rodent prostate and the zonal architecture of the human prostate. However, studies indicate that the dorsolateral lobe is most similar to the human peripheral zone.[4]

The prostatic glands produce a weakly alkaline secretion that contributes to about 30% of the semen. The secretion contains protein and ions and function as a liquefying agent that assists in sperm motility and its alkalinity protects the sperm in their passage through the acidic environment of the female vagina. The secretion is ejected into the urethra by peristaltic contractions of the muscular wall. The serine protease prostate specific antigen (PSA), also known as kallikrein III is perhaps one of the most well known secreted protein from the prostate.[5] The prostate gland remains relatively small throughout childhood and begins to grow at puberty under the stimulus of testosterone. It reaches an almost stationary size by the age of about 20 years and remains mostly at this size up to the age of about 50 years. At that time, the prostate may start growing again, which sometimes leads to a state called benign prostatic hyperplasia (BPH).

Morphology

The glandular ducts are lined by a prostatic epithelium where three distinct cell types can be distinguished; luminal cells, basal cells and neuroendocrine cells (fig.1). The predominant cell type is the secretory luminal cell. Luminal cells are

terminally differentiated and characterised by the expression of the androgen receptor (AR).[6] They produce PSA and prostatic acid phosphatase (PAP) and they are dependent on androgens for survival.[7] The basal cells are relatively undifferentiated and they express low levels of AR[8] but are not dependent on androgens for survival.[6,7] They lack secretory function and expression of PSA. Their function is not fully understood but it is believed that a subset of the basal cells function as stem cells in the prostate.[9] It has been suggested that the androgen-independent (AI) prostate stem cells give rise to a population of androgen responsive transit amplifying cells that in turn can amplify the number of luminal cells.[9,10] The characteristics of the transit amplifying cells are proposed to be intermediate between basal cells and luminal cells. Finally, the third prostatic epithelial cell type is the neuroendocrine cell, which are terminally differentiated and androgen-insensitive cells dispersed throughout the basal cell layer. They contain serotonin and thyroid-stimulating hormone that support the growth of the luminal cells.[11] The stroma is composed of smooth muscle cells, endothelial cells, nerves, fibroblasts, dendritic cells and infiltrating immune cells. The fibroblastic stromal cells express AR and are androgen responsive.[12-14] They produce growth factors for the epithelial cells in an androgen-dependent (AD) manner[15] and the crosstalk between the stroma and epithelium is an important regulator of prostate growth and differentiation.[16]

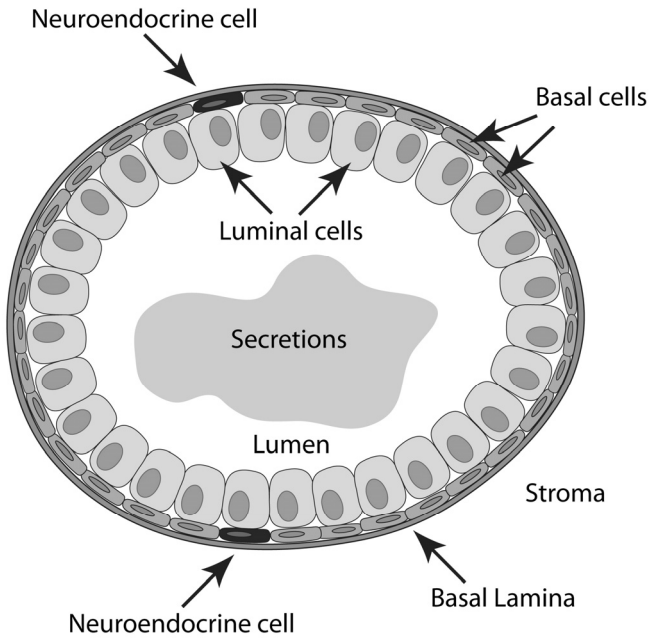

Figure 1: Schematic illustration of the different cell types within the epithelium of a human prostate gland; luminal cells, basal cells and neuroendocrine cells.

Regulation of the prostate gland

Development and growth of the prostate gland is highly dependent on androgens. The production of androgens is regulated from hypothalamus by secretion of gonadotropin-releasing hormone (GnRH), which acts on the pituitary gland. The pituitary responds with secretion of luteinizing hormone (LH), which thereafter induces the secretion of testosterone from the Leydig cells of the testis. In addition, the hypothalamus release corticotropin-releasing hormone (CRH) that induces the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH influences the adrenal glands to produce testosterone and other weak androgens, for example adrenostenediol. Of the circulating testosterone, 95% originates from the testis and the remaining 5% originates from the adrenal glands (fig. 2).

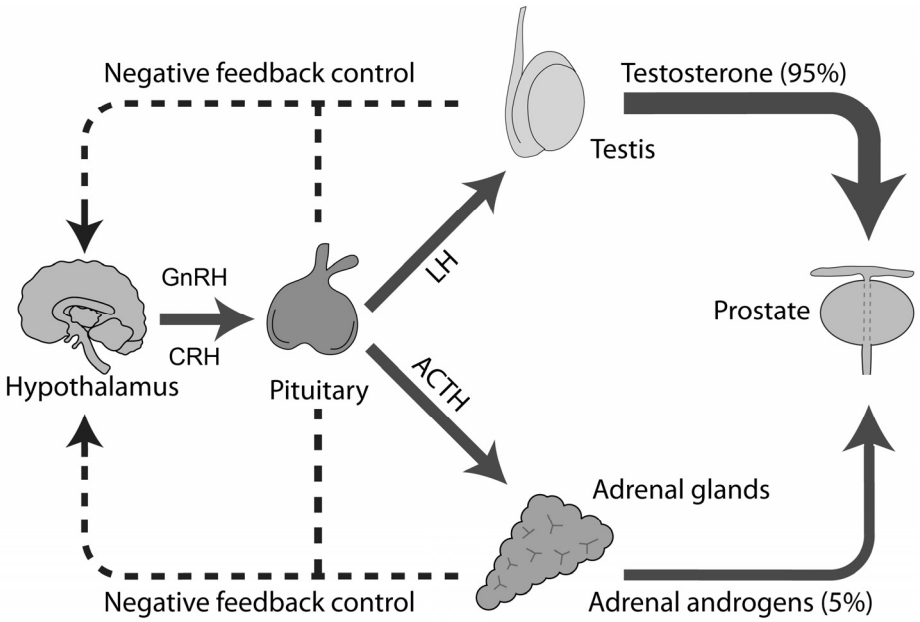

Figure 2: The production of testosterone is under the superior control of the hypothalamus and the pituitary gland. The hypothalamus secretes GnRH and CRH that influences the pituitary to produce LH and ACTH, respectively. LH influences the testis to produce testosterone and ACTH regulates the production of testosterone and other weak androgens from the adrenal glands. The majority of the testosterone originates from the testis. GnRH = gonadotropin-releasing hormone; CRH = corticotropin-releasing hormone; LH = luteinizing hormone; ACTH = adrenocorticotropic hormone.

Circulating testosterone diffuses into the epithelial and stromal cells of the prostate where it is converted by the enzyme 5α-reductase into dihydrotestosterone (DHT). Both testosterone and DHT can bind the AR, but DHT has a stronger binding affinity and is more potent.[17] Ligand-free AR in the cytosol is bound to heat-shock proteins (Hsp-70 and Hsp-90) that stabilize the receptor and protects it from degradation. Androgen binding to the receptor induces a conformational change that results in dissociation of the Hsp proteins. Two AR with bound ligand then form a homodimer that is stabilized by phosphorylation and transported into the

nucleus. Inside the nucleus the complex binds to target genes termed androgen response elements (AREs) and initiates transcription of genes regulating growth, differentiation and survival.

Effects of androgen deprivation

The normal prostate gland needs androgens for survival. Androgen withdrawal results in loss of secretory function, decreased cell proliferation and a rapid reduction in glandular size,[18] which is caused by a widespread apoptosis among the epithelial cells.[14,19] It was for a long time assumed that castration-induced epithelial cell death was mediated by decreased AR signalling in the epithelial cells. However, recent studies indicate that it is in fact the stroma that regulates the major effects observed in the epithelium. Mice that expressed stromal AR but not epithelial AR responded similarly to androgen withdrawal as mice expressing AR in both stromal and epithelial cells. Mice that lacked AR in the stromal cells did not respond to castration at all.[19] In addition, the prostate epithelial cell death is preceded by a major reduction in blood flow[20,21] and by apoptosis of the endothelial cells.[22] Similarly, testosterone administration results in the complete regeneration of the prostate gland, which is preceded by an increase in blood flow and regrowth of the vasculature.[20,23] Castration-induced prostate involution is therefore partly caused by insufficient blood flow.

Prostate cancer

General background

Prostate cancer is one of the major health issues in the Western countries and for no other cancer form the incidence increases so quickly. Most likely, the increasing number of diagnosed cases originates from a frequent use of PSA as a diagnostic tool. Prostate cancer is the most frequently diagnosed cancer form in men in Sweden and approximately 9000 new cases are discovered each year. Prostate cancer also accounts for the most common cancer related death among men in Sweden, and each year about 2300 men die of the disease (Swedish Cancer Registry 2007).

The cause of prostate cancer is not known. Its occurrence is strongly related to age, and the majority of the patients are between 60 and 70 years when diagnosed.[24] Prostate cancer is rarely diagnosed before the age of 50 years. There are large

geographic variations in the incidence, and prostate cancer is more common in Europe and in USA than in Asia, which points to the importance of lifestyle and environmental factors. It has been suggested that isoflavonoids, which are constituents of soy, have a protective effect against prostate cancer, which could explain the low incidence in Asia. It is also generally considered that lycopenes have a protective effect against development of prostate cancer. In contrast, high energy intake, high body mass index (BMI) as well as the metabolic syndrome are considered risk factors for prostate cancer.[25] There is also an ongoing discussion if prostatic inflammation may contribute to the development and progression of prostate cancer. Some epidemiological studies have shown a significant association between prostatitis and prostate cancer[26] while others have failed to demonstrate an association.[27] Hereditary factors account for a relatively small fraction (5-7%) of the cases. In 1996, the first susceptible prostate cancer gene was discovered and it was named Hereditary prostate cancer 1 (HPC1). However, studies have shown that there are only a small fraction of the hereditary cases that are caused by HPC1 and hereditary at prostate cancer is probably complex.[25]

Prostate carcinogenesis

The cellular origin of prostate cancer is still controversial. It has been proposed that luminal cells are responsible for the tumor-initiating capacity, due to the fact that most prostate cancers display luminal characteristics. However, there is now increasing evidence that prostate cancer arises from the undifferentiated stem cells that are present in the prostate.[9] Since the stem cells do not express AR and are independent of androgens, their presence is specifically interesting for the development of AI disease (see below).

Prostate cancer is a multifocal and heterogeneous disease, where several tumor locations are found within the prostate at the time of diagnosis.[28] In general, prostate tumors are considered to be slowly growing.[29] It is estimated that about half of the elderly male population have an insignificant, latent prostate cancer but the vast majority of them will never suffer from the disease. One problem today is to identify progression markers to distinguish indolent tumors from those tumors that will progress rapidly and cause the death of the patient.

The progression of prostate epithelial cells into a malignant phenotype is a multistep process (fig. 3). In many men, a precursor stadium to prostate cancer can be found, so called prostatic intraepithelial neoplasia (PIN). These premalignant lesions can develop into malignant tumors, which in the beginning are localized within the prostate. Additional genetic alterations result in development of a locally invasive tumor, which could break through the prostatic border and invade

surrounding tissue. Prostate cancer preferentially forms metastases to the bones and to the lymph nodes. As the normal prostate, prostate cancer is initially AD for growth and survival. This feature makes it possible to treat prostate cancer with androgen deprivation therapy (ADT). AD tumors respond to castration in a similar way as the normal prostate tissue, i.e. a reduction in blood flow and apoptosis of endothelial cells[30] and of epithelial tumor cells,[31,32] which altogether results in reduced tumor burden. However, the initial androgen-dependency is generally lost during tumor progression and after a certain time an AI tumor relapses. AI tumors (or hormone-refractory tumors) are highly aggressive and metastatic and the patient survival is generally less than a year.

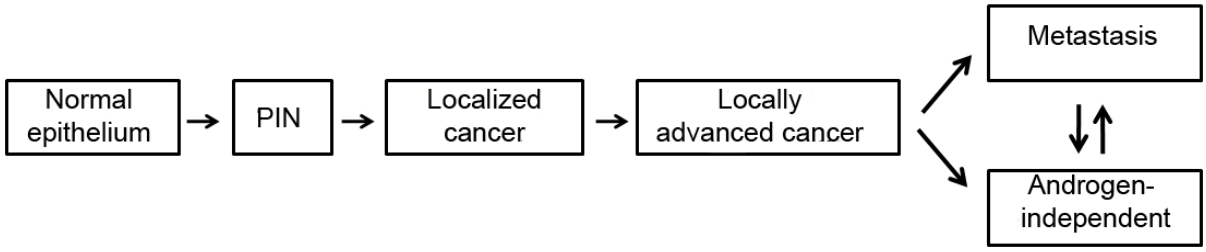

Figure 3: The progression of prostate cancer is a multistep process.
(PIN = prostatic intraepithelial neoplasia).

Diagnosis and pathology

The common method to diagnose prostate cancer is through rectal palpation or transrectal ultrasound together with core biopsies from the prostate. In addition, the PSA test is commonly used to assess the risk for prostate cancer. Normal prostate tissue prevents PSA to reach the blood. However, in the diseased prostate (i.e. prostate cancer and prostatitis), the basement membrane is leaky, resulting in increased blood levels of PSA. A normal PSA value is in the range of 0-3 ng/ml. However, many men over 50 years have a PSA value between 3 and 10 ng/ml, which could be due to prostate cancer, but more often this is due to BPH. Unfortunately, the PSA test has low specificity and sensitivity and therefore it is not possible due to PSA to differentiate between low and high malignancies. If the PSA value is above 100 ng/ml it is generally an indication of widespread metastatic disease.[25] After confirmed prostate cancer diagnosis, additional investigations with regard to metastases are performed, which include radionuclide bone scans to detect possible metastatic lesions in the bone. In addition, in relation with prostatectomy, regional lymphadenoectomy could be performed to investigate for presence of lymph node metastases with subsequent histology. Prostate tumors are usually classified according to the TNM system, which makes it possible to study prostate tumor stage over time as well as prognosis for the individual patient (table I).

Table I: TNM classification of prostate tumors

T – Primary tumor	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically unapparent, neither palpable nor visible by imaging
T1a	Tumor incidental histologic finding in 5% or less of tissue resected
T1b	Tumor incidental histologic finding in more than 5% of tissue resected
T1c	Tumor identified by needle biopsy
T2	Tumor confined within the prostate
T2a	Tumor involves half of a lobe or less
T2b	Tumor involves more than half of a lobe but not both lobes
T2c	Tumor involves both lobes
T3	Tumor extends through the prostatic capsule
T3a	Unilateral extracapsular extension
T3b	Bilateral extracapsular extension
T3c	Tumor invades seminal vesicles
T4	Tumor is fixed or invades adjacent structures other than the seminal vesicles: bladder neck, external sphincter, rectum, levator muscles, or pelvic wall
N – Regional lymph nodes	
NX	Regional lymph node cannot be assessed
N0	No regional lymph node metastases
N1	Metastases in regional lymph nodes
M – Distant metastases	
MX	Distant metastases cannot be assessed
M0	No distant metastases
M1	Distant metastases

The most common way to obtain tissue specimens from the prostate is with needle biopsies through the rectal wall. There is no standard direction on how many biopsies that should be sampled and how they should be taken. However, usually between 6 and 12 biopsies are taken. The Gleason score[33] is the most commonly used histological grading system for prostate cancer and it correlates with tumor progression.[34,35] This method was established in the 1960s and is based on the growth pattern of the tumor. The Gleason score is the sum of the most common and the most aggressive growth patterns that are graded from 1 to 5, with 1 being the least aggressive and 5 the most aggressive.

Treatment of prostate cancer

Treatment of localized prostate cancer

Localized prostate cancer is the most commonly diagnosed cancer stage. The choice of treatments is active surveillance, radical prostatectomy or radiotherapy and is based on the patient's life expectancy and grade of malignancy. The recommendation is that men with a short life expectancy who have early stage prostate cancer should be followed by surveillance as first choice.[36] Younger patients with longer life expectancy or patients with more poorly differentiated tumors are offered curative treatment. The most common curative treatment in Sweden is removal of the prostate gland with radical prostatectomy. Another curative treatment option for localized prostate cancer is radiotherapy.[36]

Treatment of locally advanced prostate cancer

In patients with extracapsular tumor extension, prostatectomy is not the first option, since there can be difficulties to remove the whole tumor. Treatment recommendations for locally advanced prostate cancer are instead a combination of hormonal therapy and dose-escalating radiotherapy.[37] Another option is hormonal treatment in the form of anti-androgens or castration therapy (see below).

Treatment of metastatic prostate cancer

Metastatic prostate cancer is treated by ADT. Already in 1941 Huggins and coworkers performed their pioneering work on hormonal treatment of advanced prostate cancer,[38] which was later awarded with the Nobel Prize. Testicular androgens can be eliminated by surgical or medical castration. Medical castration includes treatment with GnRH analogs that exerts its pharmacological action through downregulation of the GnRH receptors present on pituitary gland. This results in inhibition of LH and the subsequent testosterone secretions from the Leydig cells of the testis. GnRH agonists decrease serum testosterone to castration levels after 3-4 weeks. This is preceded by a transient increase in serum testosterone, known as the flare period, which could cause worse symptoms for the patient.[36,39] To avoid the flare period, GnRH antagonists that have direct actions on the receptors, have recently been developed.[40] Chemical castration can also be obtained by administration of estrogens, which results in decreased secretion of LH from the hypothalamus via a negative feedback loop. In addition, anti-androgens, such as bicalutamide, can be used to block the androgenic effects. Anti-androgens have peripheral actions by directly binding and blocking the AR in the prostate cancer cells.[36,39]

About 80% of the patients will have symptomatic relief after ADT.[36] Although endocrine therapy is palliative and not biologically curative, this treatment could

contribute to the decline in mortality rates by delaying death from prostate cancer long enough for the patient to die of unrelated causes. From animal experiments and clinical trials it has been shown that early initiation of endocrine therapy is beneficial, at least in more aggressive cancers, and improves survival.[41,42] Intermittent androgen ablation is a treatment modality that has been introduced into the clinic[43] but there are no relevant endpoints yet, therefore this should so far be considered as experimental.

Treatment of AI prostate cancer

Treatment of AI prostate cancer is only palliative and median patient survival is less than a year. Despite the AI nature of the tumor, it is of importance to continue with ADT in this stage of disease.[44] In addition, a second-line treatment with anti-androgens can be beneficial for the patient. Treatment with the cytotoxic drug docetaxel has been introduced in the clinic and it has been shown to improve median survival with a few months.[45,46] Prednisone is also used in the treatment of AI prostate cancer and it often results in improved well-being. Development of new treatment strategies for the AI stage of prostate cancer is of importance and is urgently needed. There are several drugs that are in early clinical trials.[47] However, there are no conclusive results yet and the role of these drugs for treatment of prostate cancer will be proven in the future.

Mechanisms of development of AI prostate cancer

ADT results in a temporarily relief for patients with advanced prostate cancer, but will eventually trigger the development of an AI prostate tumor. AI prostate cancer is highly aggressive and metastatic and is a major challenge for clinicians. The factors that trigger development of AI prostate cancer are currently not known. Neither is the point when the molecular alterations that promote AI prostate cancer occur. Results from an early study suggested that untreated metastatic tumors already contain the alterations needed for recurrence to occur during the pressure of ADT.[48] However, later studies instead support the theory that ADT trigger the molecular alterations that result in development of an AI prostate cancer.[49,50] Recurrent prostate tumors often reexpress AR target genes and AR is observed at high frequency in these tumors.[51-53] In addition, AI tumors respond to additional hormonal manipulations, such as anti-androgens.[54] These data suggest that most of the recurrent tumors are neither AI nor hormone-refractory, because they continue to depend on the AR signaling axis for growth. A more accurate name for AI/hormone refractory prostate cancer is therefore “castration-resistant” prostate cancer. Transition into androgen-independency is a complex process and despite a lot of effort in resolving this issue, the detailed molecular mechanisms remain

unknown. Theories to the development of AI prostate cancer includes several AR related mechanisms but also mechanisms not related to the AR.

AR related mechanisms

Much attention has been paid to the AR in the development of AI prostate cancer. Several mechanisms that serve to activate the AR in absence of androgens have been described (fig. 4).

1) Amplification of the AR gene could be one possible mechanism that facilitates proliferation of prostate cancer cells in low concentrations of androgens. Visakorpi and colleagues reported that AR amplifications are increased during ADT,[55] which has later been confirmed by others.[52,56] Amplifications have also been observed in bone metastases[57] suggesting the involvement of AR in the progression of the disease.

2) Point mutations in the steroid binding domain of the AR are another mechanism that has been proposed to be of importance in development of AI prostate cancer. Mutations result in a promiscuous receptor that allows non-specific binding of ligands such as estrogens and non-steroidal anti-androgens. The frequency of AR mutations increases in the AI stage[58,59] and has also been observed in lymph node and bone metastases.[60] Veldscholte et al was the first to describe that the LNCaP cell line harbor a AR mutation in the ligand-binding domain, thus allowing it to be activated by other ligands than androgens.[61]

3) Hypersensitivity of the AR is another mechanism that can drive the proliferation of the prostate cancer cells under low androgen concentrations. Gregory et al have reported that this mechanism includes increased stabilization and increased nuclear transportation of the AR.[62,63]

4) Coactivators interact with steroid receptors and enhance their ligand-dependent transactivation. Some examples of co-activators that have been reported to be upregulated in AI prostate cancer are androgen receptor associated protein-70 (Ara70), steroid-receptor coactivator-1 (SRC-1), transcriptional intermediary factor-2 (TIF-2) and receptor-associated co-activator-3 (RAC3).[62,64,65]

5) Androgen deprivation can also result in activation of intracellular signaling transduction pathways that can drive the proliferation of the prostate cancer cells instead of androgens. These pathways can facilitate activation of the AR in absence of androgens or the other alternative is that the AR is bypassed altogether. It has for instance been demonstrated that AR can be

activated by insulin-like growth factor 1 (IGF-1), the keratinocyte growth factor (KGF) and the epidermal growth factor (EGF).[66]

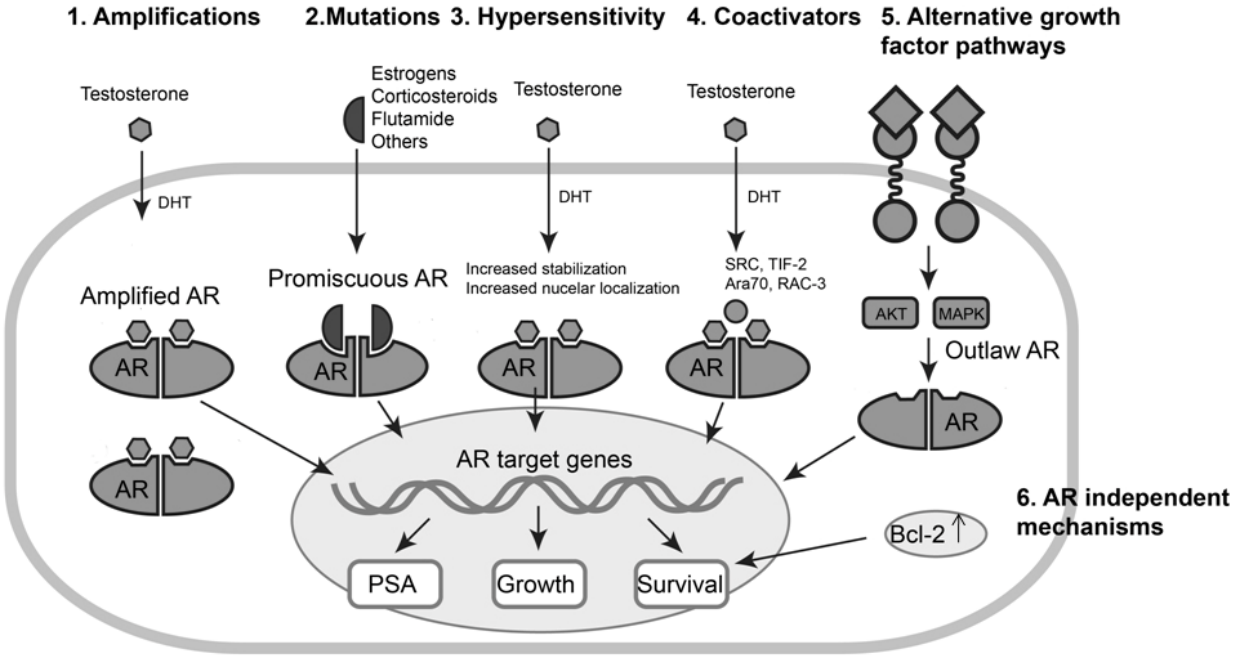

Figure 4: Mechanisms that have been suggested to be responsible for development of AI prostate cancer. Theories include several AR related mechanisms (1-5) but also mechanisms not related to the AR (6). AR = androgen receptor; DHT = dihydrotestosterone; PSA = prostate specific antigen.

Other mechanisms

6) Development of androgen-independency can also be explained by the upregulation of the anti-apoptotic protein Bcl-2 (fig. 4). Bcl-2 is not normally expressed by the secretory prostate epithelial cells.[67] However, PIN lesions frequently express Bcl-2 and increased levels are observed in AI specimens.[67] Correspondingly, Bcl-2 is upregulated after castration in experimental models of prostate cancer[68] and inhibition of Bcl-2 resulted in delayed progression to androgen-independency.[69] Androgen deprivation therefore seems to induce signals that results in bypassing of the apoptotic response.

7) Prostate tumors are extremely heterogeneous and are probably comprised of tumor cells with varying degree of androgen-sensitivity and responsiveness. One theory to the development of AI disease is the clonal expansion theory.[9] Coffey and Isaacs have suggested that androgen-independence is due to the existence of AI prostate cancer stem cells in the original population of prostate cancer cells.[70] Androgen deprivation would result in depletion of the

AD cells but promote disease progression by activating normally quiescent cancer stem cells that would repopulate the tumor with AI cells. Craft et al provided further support of the clonal expansion theory and showed that AI cells account for 1 in 10^5 cells even before initiation of androgen ablation.[71]

Interactions between cancer cells and stroma

Earlier studies by Cunha and coworkers have revealed that the normal prostatic stromal cells control the differentiation and development of the normal epithelial cells and prostate gland.[72] In a similar way, there exists a continuous communication between prostate tumor cells and stromal cells. Several biological experiments have provided profound evidence that stromal cells play an important role in prostate cancer initiation and progression. When non-tumorigenic prostate epithelial cell lines were combined with carcinoma-associated fibroblasts (CAFs) and implanted as xenografts in immunodeficient mice, tumors were established. Tumors were not formed when epithelial cells were injected alone,[73-75] showing the importance of CAFs in tumor initiation and progression.

The reciprocal interactions between stroma and cancer cells are not only promoting growth of the cancer cells. The cancer cells also have a profound influence on the stromal cells and the stroma in the vicinity of the tumor is often referred to as "reactive stroma". Experiments addressing the issue of differences between CAFs and normal stroma have shown a dramatic difference in tumor forming capacity of non-tumorigenic epithelial cells mixed with cancer stroma or normal stroma.[75] The CAFs promoted a rapid tumor development and progression, while normal stromal cells restricted tumor formation. Therefore, during the course of tumor development there are profound changes in the stroma, helping the neighbouring cancer cells to survive, proliferate and ultimately forming metastases.[76]

Although androgens are important for maintenance of the normal prostate, they are not alone responsible for the regulation. There are several growth factors identified that promote interactions and communications between the stromal and epithelial compartments. The major prostatic growth factor families include fibroblast growth factor (FGF) family, transforming growth factor-β (TGF-β) family, IGF family and EGF family. During the course of prostate tumorigenesis many of the normal prostate growth factors are altered.[77] Exactly how the reactive stromal cells influence and regulate the tumorigenic process are not well defined. It is most possible that the interactions between the stromal cells and cancer cells differ depending on tumor stage. Furthermore, because there are phenotypic differences between AD and AI prostate cancer cells it would be interesting to reveal differences in their respective communications with the stromal cells.

Invasion and metastasis

The metastatic process

Primary tumors impair normal tissue function. However, they are only responsible for about 10% of the deaths from cancer. The remaining 90% of the cancer deaths are caused by metastatic disease.[78] Metastases create a great chaos throughout the body and therefore they are the most dangerous manifestations of the cancer process. For unknown reasons, certain types of tumors never metastasize, while others have a high probability to do so. For instance, prostate cancer has a high propensity to form metastases to the regional lymph nodes and to the bones.

There are a number of sequential steps that a cancer cell must overcome to succeed in the establishment at a new site (fig. 5).[79,80] The first step is the detachment of single cancer cells from the primary tumor, which involves alterations in the cell adhesion profile. To gain access to the vessels for further transportation, the tumor cells must break through the basement membrane and path their way through the tissue. The invasive properties of tumor cells enable them to move through the vessel wall and enter into the circulation, a process called intravasation. Once in the circulation, individual cancer cells may travel with the blood or lymph flow to distant sites. However, the blood represents a hostile environment for metastasizing tumor cells. Hydrodynamic shear forces are present in the blood, which may tear the cancer cells apart.[78] Experimental studies have shown that survival is greatly enhanced if the tumor cells can attract platelets to escort them through the rapid blood flow.[81] In addition, the most common way for invading cancer cells to move through the tissue is as a unit with other cancer cells, thus enhancing their survival in the circulation.[78] Further, once the tumor cells enter the circulation they will lose their anchorage to the underlying stroma. Like normal cells, cancer cells may continue to depend on solid substrates for survival. Many cells will therefore rapidly die as a result of anoikis (apoptosis that is triggered by detachment from a solid substrate).[82]

The next step for the metastasizing tumor cells that have managed to survive in the circulation is to invade the new organ by adhering to the vessel wall and penetrate into the surrounding tissue, a process called extravasation. At this point, tumor cells use two alternative options. Either they can start proliferate inside the vessel, creating a small tumor that pushes on the vessel wall and forces the endothelial cells to separate so the tumor can pass. The other option is to invade the endothelial wall directly and start proliferating at the new tissue.[78] The last step, called colonization, is perhaps the most challenging. Many metastasizing tumor cells will die once they have arrived at the new site, while others will remain dormant for many years.[83] In general, the number of micrometastases in a cancer patient vastly

increases those that will eventually expand in size.[78] To be able to expand, the cancer cell must initiate the formation of new blood vessels, a process called angiogenesis. Oxygen and nutrients can only diffuse 1 mm in distance and therefore newly formed metastatic foci cannot grow over a size of 1-2 mm^3 without initiating angiogenesis.[84]

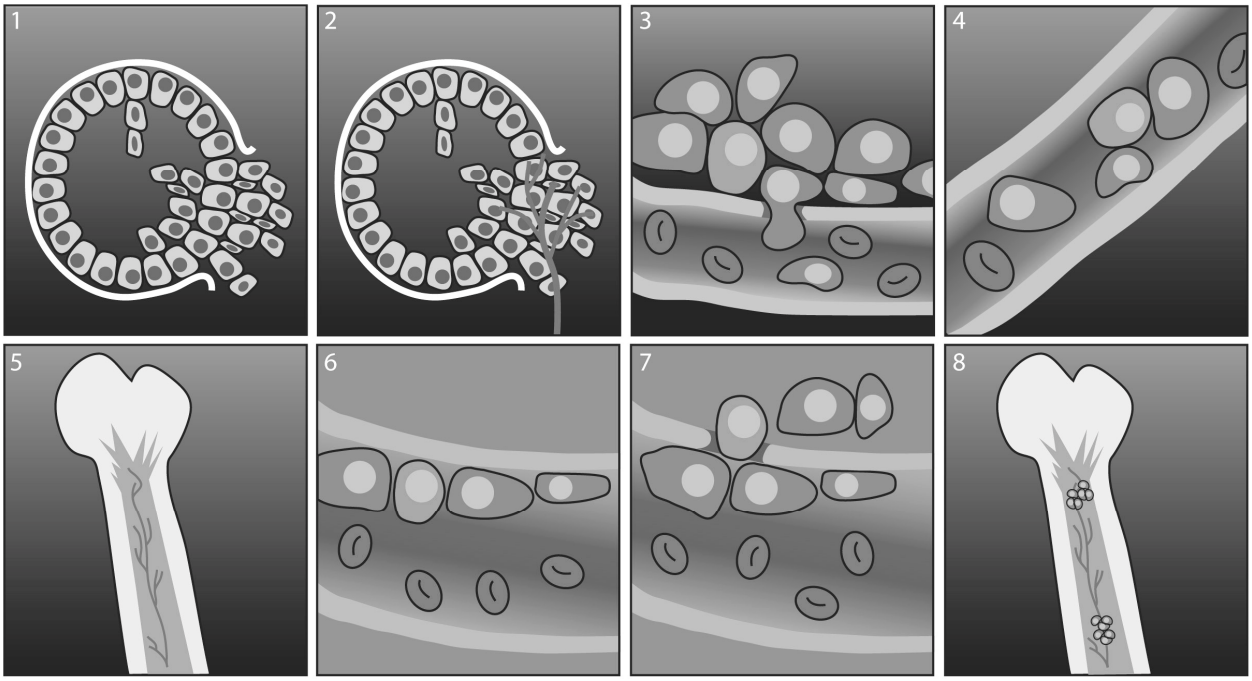

Figure 5: The metastatic cascade consists of several interrelated, sequential steps. 1) Tumorigenesis. 2) Angiogenesis. 3) Detachment of tumor cells, invasion through the tissue and intravasation into vessels. 4) Transport through the circulation. Tumor cells must survive the hydrodynamic shear forces that are present in the circulation. Survival is greatly enhanced if the tumor cells form aggregates with each other or with platelets. 5) Arrival at the new site. For prostate cancer this is often the bone. 6) Tumor cells adhere to the vessel wall. 7) Extravasation into the new organ parenchyma. 8) Colonization, initiation of angiogenesis and subsequent proliferation. Experimental studies have shown that the last step is the rate-limiting.

Overall, metastasis is an inefficient process. Of the several millions of tumor cells that are seeded into the circulation it is only a small fraction that successfully complete all the steps in the metastatic cascade.[85,86] Experimental studies have lead to the conclusion that the initial steps in the metastatic cascade are completed easily for most tumor cells.[87,88] However, the last step involving the colonization and initiation of angiogenesis at the new site is the rate-limiting. It has been reported that three days after intraportally injection of melanoma tumor cells, 83% of the cells had extravasated but only 2% of the cells formed micrometastases.

Furthermore, 0.02% of the cells persisted and developed into lethal metastases.[88] Similar results have been obtained by others.[89]

In order to acquire motility and invasiveness, the prostate cancer cells must shed many of their epithelial traits and undergo drastic alterations; the epithelial-mesenchymal transition (EMT). EMT involves changes in cell morphology and gene expression pattern, resulting in gain of mesenchymal characteristics. These alterations results in extended and elongated cancer cells, allowing for increased migratory capacity.[90] In addition, there are major changes in the gene expression profile during EMT. Expression of E-cadherin and cytokeratins are repressed and instead mesenchymal markers, such as N-cadherin, vimentin and fibronectin, are induced.[78]

The seed and soil theory

It has long been recognized that different cancer types show an organ-specific pattern of metastasis. For instance, prostate cancer preferentially forms metastases in the bones and metastases from breast cancer are often detected in the brain, lungs, bone and liver. Already in 1889, Stephen Paget published a paper that described the seed and soil theory.[91] He had noticed that certain types of tumor cells (the seed) had a high propensity to form metastases in specific organs (the soil) and he proposed that this was due to the compatibility between the seed and the soil. This idea was challenged in 1920s by James Ewing, who suggested that the patterns of blood flow were the primary reason for organ-specific metastasis.[79] Today we know that both models are valid. Autopsy studies and experimental animal studies support the concept that both blood flow and compatibility factors contribute to metastatic spread to various organs.[79] Together, these studies show that the blood flow determine the initial fate of the tumor cells and decide in which organ the tumor cells will end up after they have left the primary tumor. However, after the cancer cells have arrested in an organ, their ability to grow there is dependent on molecular interactions between the cancer cells and the new environment. In addition, the new organ must be able to support the cancer cells with the proper growth factors. Because chemokines and their receptors are involved in the homing of lymphocytes and hematopoietic cells to specific organs, it could be reasoned that also cancer cells use chemokines to home to specific organs. In an elegant study by Muller and coworkers it was shown that breast cancer cells express the chemokine receptors that match the chemokines that are expressed in the organs where these cells end up as metastases.[92]

Tumor dormancy

It has been observed that metastatic relapse of a tumor can occur decades after removal of the primary tumor.[93] It has therefore been hypothesized that primary tumors shed metastatic cells to the circulation in an early phase of the disease. These scattered tumor cells can persist in an inactive state for many years, called tumor dormancy.[83] Which mechanisms that awake the cancer cells are at present unknown. Studies that have modeled tumor dormancy mathematically indicate that continuous slow growth is unlikely. Instead a model favoring discontinuous growth with periods of quiescence is more likely.[94,95] Judah Folkman and colleagues could show the existence of preangiogenic metastatic foci in the metastatic niche. In these preangiogenic metastases the proliferation was counter balanced by apoptosis, resulting in no net increase in tumor volume. When these foci gained the ability to vascularize, tumor dormancy ceased and cancer cells started to proliferate.[96] Another possible contributor to tumor dormancy is presence of solitary cancer cells at the metastatic site.[97] Many cells that arrive in the secondary site fail to initiate cell division but remain as quiescent cancer cells. It has been shown that recovered solitary mammary carcinoma cells from liver tissue, retained their tumorigenic capacity when re-injected into the mammary fat pad of mice.[97] These experiments show that despite their apparent dormancy at the secondary site, the cells are still active. A better understanding of tumor dormancy and the molecular factors that contribute to the subsequent initiation of cell division is important to be able to treat metastatic disease.

Lymphangiogenesis and metastasis

The lymphatic system

The involvement of the lymphatic system in the metastatic process has been intensively investigated over the last years. Many carcinomas, including prostate cancer, metastasize to sentinel lymph nodes via the lymphatic vessels. Therefore, sign of lymph node metastases is often used as cancer staging in the clinical setting. Presence of cancer cells in the lymph nodes are considered as an adverse prognostic advent in many carcinomas.[98] Thus, removal of the regional lymph nodes is often standard procedures, resulting in improved patient survival.

Lymphatic vessels resemble blood vessels, but are generally thinner. The lymphatic endothelium has poorly developed junctions and large interendothelial gaps, which results in a relatively free import of interstitial fluid. The lymphatic capillaries also lack a continuous basement membrane and are devoid of pericytes. These properties make lymphatic vessels susceptible for invasion by tumor cells. Traditionally, the lymphatic system has been considered to be passively involved in

the metastatic process. However, studies have demonstrated formation of new lymphatic vessels in tumors, so called lymphangiogenesis,[99-101] which is evidence of lymphatic vessel activation in cancer. The detailed molecular mechanisms behind lymphangiogenesis have been poorly understood but have improved after the identification of specific lymphatic growth factors and lymphatic endothelial markers. The specific lymphatic endothelial markers include lyve-1,[102] podoplanin[103] and prox-1.[104]

Vascular endothelial growth factor C (VEGF-C)

The first lymphangiogenic growth factor was isolated in 1996 from human prostatic carcinoma cells.[105] It was found to belong to the group of vascular endothelial growth factors (VEGFs) and it was named VEGF-C. Later studies identified another family member, VEGF-D[106] that also stimulated growth of lymphatic vessels. VEGF-C and VEGF-D are glycosylated, secretory proteins and by means of proteolytical processing several forms are generated. They mediate their effects on lymphatic vessels through the VEGF receptor 3 (VEGFR-3). The expression of VEGFR-3 was originally thought to be restricted to lymphatic endothelial cells[107] but further studies revealed that VEGFR-3 was also expressed by a small subset of blood vessels.[108,109] By proteolytic processing, VEGF-C and VEGF-D gain the ability to bind to the VEGFR-2 that is expressed on blood vessels. Thus, they can have actions on blood vascular endothelial cells and induce angiogenesis.[110] VEGF-C is definitively one of the main lymphangiogenic growth factor but since its discovery others have been identified, including PDGF-BB,[111] FGF-2[112] and the angiopoietin-1 (Ang-1) and -2 (Ang-2).[113]

The expression of VEGF-C mRNA is upregulated by different factors, including PDGF, EGF, TGF-β and also by serum.[114] In addition, it has been demonstrated that androgen deprivation induces expression of VEGF-C in prostate cancer cells.[115] In contrast, VEGF-C is not regulated by hypoxia, RAS oncoprotein or mutant p53, which are potent inducers of VEGF expression.[114] In normal human adult tissue, VEGF-C is expressed most prominently in the heart, placenta, ovary and in the small intestine.[105] Macrophages are another important source of VEGF-C.[116]

VEGF-C and its role in lymphatic metastasis

Today, there is strong evidence that lymphangiogenesis does occur in the presence of VEGF-C (fig. 6). VEGF-C can induce hyperplasia of preexisting lymphatic vessels[117] and can also stimulate the proliferation of newly formed lymphatic vessels.[118] Overexpression of VEGF-C in transgenic and xenograft models of human cancer induced lymphangiogenesis around the tumors and enhanced metastatic spread of cancer cells to regional lymph nodes.[119-122]

VEGF-C is upregulated in many types of human malignancies and is also related to the appearance of lymph node metastases.[123-126] Previous to our publication (paper I), Tsurusaki et al reported on elevated mRNA levels in tumors from patients with lymph node metastases in comparison to tumors from patients without lymph node metastases.[127] Similarly, expression of VEGFR-3 by lymphatic endothelial cells has been shown to be associated with lymph node metastasis in prostate cancer.[128]

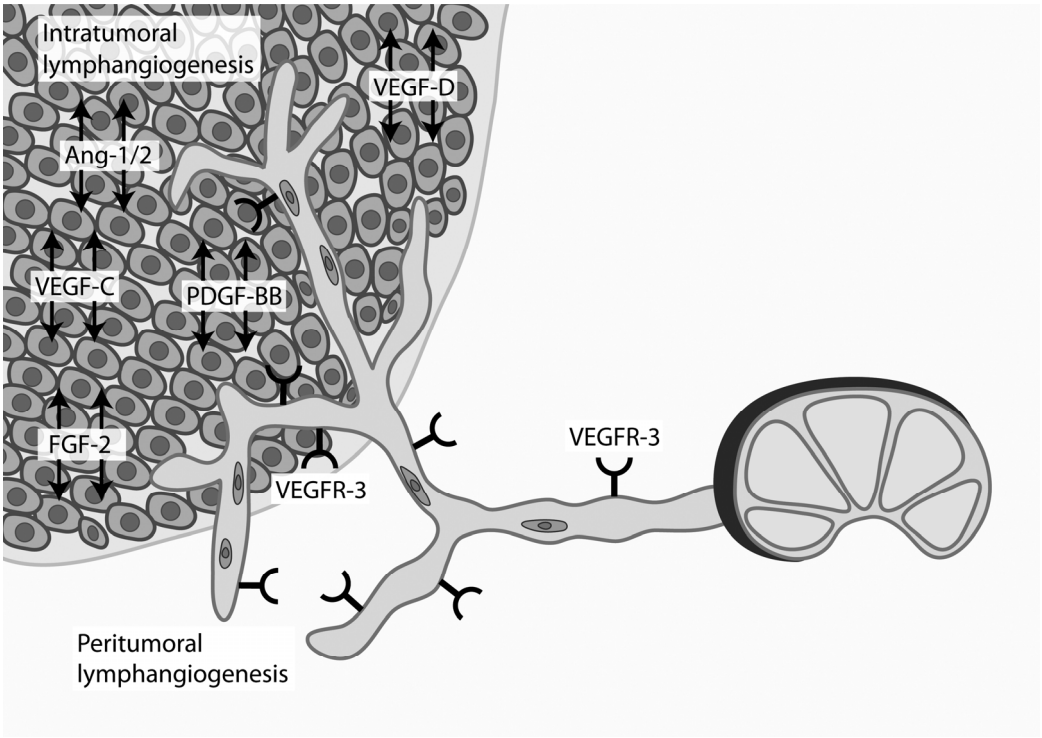

Figure 6: Lymphangiogenic growth factors (VEGF-C, VEGF-D, PDGF-BB, Ang-1, Ang-2, FGF-2) are secreted from the tumor cells and induce the formation of new lymphatic vessels. VEGF-C/D is the most studied lymphangiogenic growth factors and they mediate their effects via VEGFR-3 that is expressed on the lymphatic endothelial cells. Increased lymphatic vessel density in tumors is associated with presence of regional lymph node metastases. Both peritumoral and intratumoral lymphatic vessels have been observed in tumors but there are doubts if the intratumoral lymphatic vessels are functional.

More recently, is has been observed that lymphangiogenesis not only occur in the vicinity of the primary tumor but also in the sentinel lymph nodes.[129,130] Notably, lymph node lymphangiogenesis was observed even before the arrival of tumor cells, which indicate that the primary tumor starts to prepare the metastatic site prior to the dissemination of tumor cells.[131,132] Although metastasis to distant organs most likely occurs via the hematogeneous system, it might require the initial spread of tumor cells to the regional lymph nodes, from where the cancer cells can spread further. This is supported by a study where metastasis to distant organs was not observed without simultaneous lymph node metastasis.[129] Correspondingly,

human lymph nodes infiltrated with metastatic melanoma cells also exhibited lymphatic vessel growth, indicating that lymph node lymphangiogenesis can be a feature of human cancer.[133]

Cell adhesion molecules in cancer

Cell adhesion molecules (CAMs)

Cell adhesion molecules (CAMs) are present between cells and between cells and the extracellular matrix (ECM) and they constitute key components that maintain the normal structure, integrity and function of cells and tissues.[134] Epithelial cells connect to each other and to the ECM by adherens junction, desmosomes and cell-matrix adhesion complexes (fig. 7). The adherens junctions, consisting of the cadherin class of CAM, connect to the cytoskeletal actin filaments, which creates an adhesion belt around the cell. The desmosomes on the other hand, connect adjacent cells via the intermediate filaments. Cell-cell adhesion is mainly mediated via the cadherins and cell-matrix adhesion is mainly mediated via the integrins. In addition, there are also adhesive proteins that are part of the tight junctions. Tight junction proteins maintain the polarity of epithelial cells by separating the apical side from the basolateral side of the cells. During cancer development, there are major rearrangements and alterations in the CAMs, resulting in increased tumor cell motility.

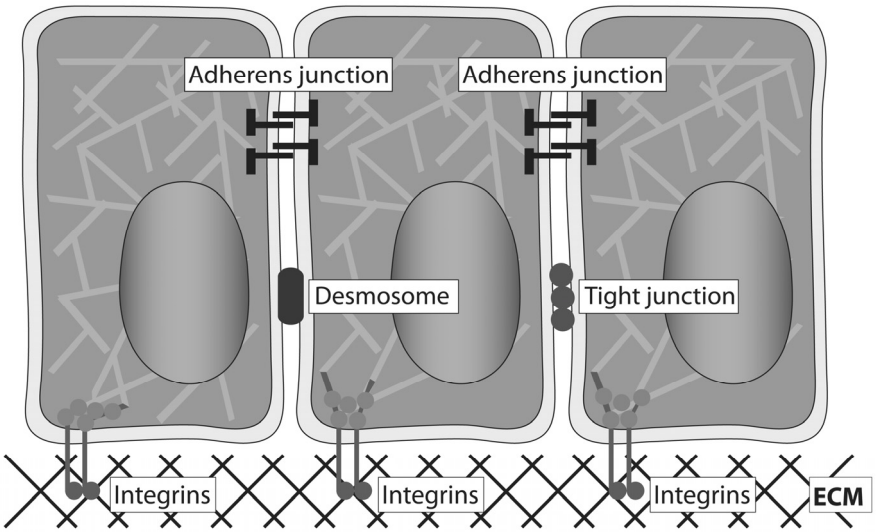

Figure 7: Epithelial cells are connected to each other via adherens junctions and desmosomes. The adherens junctions provide cell-cell adhesion via the cadherins, which is connected to the actin cytoskeleton that form an adhesion belt around the cell. The desmosomes connect to the intermediate filaments. Tight junctions separate the apical side from the basolateral side, keeping the epithelial cells in a polarized state. Cell-matrix adhesion is mainly mediated via integrins. ECM = extracellular matrix.

Cadherins

Structure and function

The cadherins are a superfamily of CAMs that mediate adhesions between cells in the presence of extracellular calcium. Different cadherins are differentially expressed during embryonic development, indicating distinct functions in cell adhesion. The cadherins are composed of a large extracellular domain that binds with homophilic bonds to cadherins on neighboring cells, resulting in stable forces between adjacent cells. Cadherins also have one transmembrane segment and a highly conserved intracellular domain. The cytoplasmic part of the cadherins is anchored to the actin cytoskeleton via a second group of proteins called the catenins.[134] The linkage to the cytoskeleton is crucial for the cell adhesion function of the cadherins. There are three major catenins; α-, β-, and γ-catenin that regulate the function of the cadherins.[135] The most extensively studied cadherins are the three classical cadherins E-cadherin, N-cadherin and P-cadherin.

E-cadherin and cancer

E-cadherin maintains the integrity and polarity of normal epithelial tissue. Over the last years, several investigations on the functional role of E-cadherin have been performed. Inhibition of the function of E-cadherin with blocking antibodies resulted in disruption of cell contacts *in vitro* and induction of a more motile phenotype.[136-138] Conversely, forced expression of E-cadherin in cancer cells impaired the invasive capacity.[137,139] These results clearly demonstrated a critical role for E-cadherin in tumor invasion. Furthermore, using the Rip1Tag2 transgenic model of pancreatic cancer, Perl et al could establish a casual role between loss of E-cadherin and transition from adenoma to carcinoma.[140] Therefore, loss of E-cadherin results in disruption of the tight contacts that exist between adjacent epithelial cells allowing single metastatic cells to escape from the primary tumor. In most cases, loss of E-cadherin is due to transcriptional repression by hypermethylation or chromatin rearrangements in the promoter region.[141-144] Recent reports have also highlighted the role of snail, slug, twist and ZEB1 in silencing of the E-cadherin gene.[145-148] These transcription factors act as repressors of E-cadherin gene expression, and thus inducing the metastatic phenotype. In addition, loss of E-cadherin can also be a consequence of mutations of the E-cadherin gene[149] or aberrant tyrosine phosphorylation of E-cadherin and associated proteins by receptor tyrosine kinases,[150,151] which result in disruption of E-cadherin mediated adhesion.

Reduction in E-cadherin not only results in decreased cell adhesiveness that is important for the first step in the metastatic cascade. It also affects signaling transduction pathways that can influence later steps in the metastatic cascade.[152,153] A critical intracellular event resulting from loss of E-cadherin is the accumulation of free β-catenin in the cytosol. Besides for being an important component of the

cadherin complex, β-catenin also function as a transcription factor and participates in Wnt-mediated signaling transduction.[153] Non-sequestered, free β-catenin is rapidly phosphorylated by glycogen synthase kinase 3β (GSK-3β) in the adenomatous polyposis coli (APC)/GSK-3β/axin complex and subsequently degraded by the ubiquitin-proteasome pathway. If the tumor suppressor APC is non-functional or if GSK-3β activity is blocked by activated Wnt-signaling, β-catenin accumulates at high levels in the cytosol. It is thereafter translocated to the nucleus, where it binds to members of the TCF/LEF transcription factors and modulates the transcription of target genes, including c-myc, cyclin D1, fibronectin and matrilysin.[154-157] There are also studies demonstrating that β-catenin can bind AR in prostate cancer cells and thus regulate transcription of androgen-regulated genes.[158,159]

E-cadherin has been extensively studied in prostate cancer. E-cadherin expression is inversely related to prostate tumor grade[160-165] and to adverse clinicopathological features.[165] E-cadherin is reduced in high grade tumors with positive surgical margins[166] and is downregulated in lymph node metastases [167] and bone metastases[168] from prostate cancer. Interestingly, there are also studies that have demonstrated the re-expression of E-cadherin in metastatic cancer cells,[169,170] where the primary tumor was E-cadherin negative. This might imply that loss of E-cadherin is a transient event that can be influenced by the surrounding environment. Cumulative evidence suggest that E-cadherin act as a broad suppressor of growth and invasion of epithelial cancer and its functional elimination in tumors represents a key step in the acquisition of the invasive and metastatic phenotype.

The cadherin switch

The loss of E-cadherin in cancer is most often accompanied by gain of another CAM, namely N-cadherin. This "cadherin switching" plays an essential role for the metastatic process. In normal tissue, N-cadherin is extensively expressed in the nervous system but can also be found in the vascular system and in the myocardium.[171,172] Although N-cadherin forms the typical homophilic interactions to other N-cadherin expressing cells, heterotypic interactions to other molecules have also been described.[173] Experimental studies demonstrate that N-cadherin induces a scattered cell morphology and increases the motility of tumor cells.[174-177] In addition, N-cadherin promotes invasiveness and metastasis in several animal models of cancer.[174,178]

The most possible mechanism by which N-cadherin renders tumor cells more motile is through the homophilic adhesion to other cells. Firstly, N-cadherin mediates a dynamic cell adhesion[179] resulting in weaker interactions between adjacent cells than E-cadherin.[180] Thus, it allows the dissociation of single cells from the primary tumor. Secondly, homophilic interactions between tumor cells

and N-cadherin expressing tissue, such as the stroma and vasculature facilitate the transit through the tissue and survival of tumor cells in distant organs. It has for instance been demonstrated that N-cadherin promoted the transmigration of melanoma cells through the vasculature.[181,182] It is largely unknown how N-cadherin participates in the dynamic remodeling of the cytoskeleton that is required for cell motility. Studies have demonstrated that the invasive capacity of N-cadherin is in part due to a functional interaction with the FGF receptor 1 (FGFR-1). The interaction between FGFR-1 and N-cadherin causes sustained signaling of the MAPK-ERK signaling pathway, which ultimately leads to increased transcriptional activation of the proinvasive enzyme matrix metalloproteinase 9 (MMP-9).[183] In addition to the promigratory role, it has been demonstrated that N-cadherin promotes survival of carcinoma cells, through inactivation of the pro-apoptotic molecule Bad and activation of the anti-apoptotic Bcl-2.[175,184] It has also been reported that N-cadherin can be involved in angiogenesis. The extracellular domain of N-cadherin can be cleaved by plasmin resulting in a soluble fragment of 90 kDa. This fragment stimulates angiogenesis and has been shown to be elevated in serum of prostate cancer patients.[185,186] It has been suggested that N-cadherin expression is dominant over E-cadherin expression because experimental studies have shown that the proinvasive action of N-cadherin persisted even in the presence of E-cadherin.[174,187]

In prostate cancer, the cadherin switch has been observed in more aggressive prostate cancer cell lines.[177,188] In addition, N-cadherin has been observed in poorly differentiated areas of prostate cancer and its expression correlated to Gleason score.[188-190] It has also been demonstrated that expression of N-cadherin was associated with seminal vesicle invasion, pelvic lymph node infiltration and shorter time to clinical recurrence and skeletal metastasis.[165]

Integrins

The integrins are a diverse family of CAMs that are expressed in all cell types and they play a crucial role in cell survival, proliferation, migration, gene expression and activation of growth factor receptors. They mediate interactions between cells and the ECM by serving as receptors for various molecules, such as fibronectin, vitronectin, collagen and laminin. The integrins are heterodimers and are formed by a non-covalently link between one α-subunit and one β-subunit. At this time, there are 18 α-subunits and 9 β-subunits known to be expressed, which can assembly into 24 different integrin heterodimers with distinct functions.[191] The functional specificity is determined by each pairing and the cell type that expresses the integrin. Each integrin generally consists of a large extracellular domain, a transmembrane part and a short cytoplasmic tail, which are involved in the signaling system of the cell. They possess the ability to use bidirectional signaling. In response to stimuli received from the interior of the cell the integrins can

regulate the adhesiveness towards ECM; called inside-out signaling. In addition, in response to ligand binding to the outside they can transduce signals from the extracellular domain to the inside of the cell; called outside-in signaling.[192,193]
During prostate cancer development and progression there are major changes in the expression profile of the integrins, both in their levels and distribution.[194] These changes enable tumor cells to convert from a stationary to a mobile phenotype. Several reports show a deregulated integrin expression in the progression of prostate cancer, where most α and β subunits are decreased.[191,194] Some integrin subunits, such as α2 are also elevated in metastatic lesions in comparison to the primary tumors, indicating a role in facilitating tumor cell migration and metastasis.[195] Bone is a frequently involved metastatic site in prostate cancer and several studies have implicated the importance of the subunit αv in combination with β3 for the attachment and survival of prostate cancer cells in the bones.[196,197] αvβ3 is upregulated in prostate cancer[198] and prostate cancer cells isolated from bone metastases express αvβ3 integrin receptors. αvβ3 could also promote extravasation of tumor cells by mediating the interaction to the blood vascular endothelium.[199]

Role of MMPs for metastasis

MMPs have long been recognized as important players for the metastatic process. The MMPs comprise a family of zinc-dependent endopeptidases that are synthesized as inactive zymogens and usually become activated outside the cell by means of other MMPs or by serine proteinases. The MMPs can cleave almost all components of the extracellular matrix that constraint the movement of the tumor cells. Through the controlled cleavage of the ECM components the MMPs assist cancer cells in the invasive process by creating a passage for the tumor cells to nearby vessels. In addition, the modulation of the ECM results in release of sequestered growth factors, which further stimulates the invasive process. Moreover, the MMPs may activate latent forms of growth factors and proenzymes through proteolytic cleavage.[200] Notably, in many cases the MMPs are found to be synthesized by recruited stromal cells, such as fibroblasts, macrophages and mast cells, rather than by the tumor cells themselves.[201] Several soluble factors that are secreted from the cancer cells have been shown to induce the expression of MMPs from fibroblasts *in vitro*, for example EMMPRIN (extracellular matrix metalloproteinase inducer).[202] The activity of the MMPs is tightly regulated by endogenous inhibitors, including the tissue inhibitors of metalloproteinases (TIMPs) and α2-macroglobulin.[200]

The contribution of the MMPs to tumor progression has been shown in several animal models, where overexpression results in a more invasive phenotype. Conversely, blocking the function of the MMPs leads to reduced tumor

aggressiveness.[203] Clinical data also strongly support a role for the MMPs in progression of human cancer. The levels of MMPs are upregulated in nearly every human cancer, which correlates with advanced tumor stage, increased invasion and metastasis and shortened patient survival.[200] Due to MMPs broad involvement in the metastatic process, pharmaceutical inhibitors have been tested in clinical trials but so far without success, [204] most probably due to the complexity of the MMPs.

MMP-2 and MMP-9 are of specific interest for the metastatic process, since they are known to degrade collagen IV that is an important constituent of the basement membrane. In prostate cancer, MMP-2 and MMP-9 are elevated and the expressions are related to advanced non-organ confined disease.[205,206] Another MMP that has been strongly implicated in the metastatic process is the membrane-type 1 (MT1)-MMP. This protease promotes invasion and metastasis of experimental prostate cancer[207] and is highly expressed in human prostate cancer bone metastases.[208]

AIMS OF THE THESIS

General Purpose

Prostate cancer is the most common type of cancer among men in Sweden and there are approximately 9000 new cases each year. Many of these men will have an insignificant cancer, which will not harm them. However, approximately 25% of the tumors are aggressive and metastasize to lymph nodes and to the bones. Initially, ADT is successful in reducing tumor volume, but over time, a highly metastatic AI tumor relapses, which will eventually cause the death of the patient. To improve treatment for patients with AI prostate cancer, further studies of molecular and cellular characteristics in this stage are needed. The general purpose of the present thesis was to investigate invasive and metastatic properties of advanced prostate cancer, with a special emphasis on the AI stage. With the following background the specific aims were:

Specific aims

- To investigate the expression pattern of the lymphangiogenic factor VEGF-C and its receptor VEGFR-3 in human prostate cancer and the possible association of these factors to lymph node metastasis.

- To evaluate if LNCaP-19 is a relevant model for studies on the invasive properties of AI prostate cancer.

- To investigate molecular and cellular alterations that are specifically related to transition into androgen-independency, with emphasis on characteristics related to the invasive and metastatic process.

- To develop a technique to follow intraprostatic tumor growth with magnetic resonance imaging (MRI) in mice.

- To investigate how different microenvironments and androgen levels influence the growth of AD and AI prostate tumors.

MATERIALS AND METHODS

Patient material

Formalin-fixed and paraffin-embedded tissues were obtained from patients with prostate cancer from the Department of Urology, Sahlgrenska University Hospital, Gothenburg, Sweden. The studies were conducted with ethical approval of the local research ethical committee.

Materials obtained by prostatectomy (paper I)

Prostate cancer specimens were obtained from 22 patients by open prostatectomy, based on the assumption that the patients had localized prostate cancer. As routine, regional lymph nodes were resected and judged for presence of metastases. The patients had not received preoperative neoadjuvant hormonal therapy. Eleven patients were positive for metastases in regional lymph nodes and eleven had no known metastases. Mean Gleason score for patients without and with lymph node metastases was 6.6 (range 6-7) and 7.6 (range 7-9) respectively.

Materials obtained by transurethral resection of the prostate (TURP) (paper IV)

Specimens were obtained from 53 patients that underwent transurethral resection of the prostate (TURP). 28 patients had TURP-diagnosed untreated prostate cancer in stage T1b and 25 patients had recurrent castration-resistant prostate cancer after ADT. T1b tumors are clinically unapparent tumors that are not palpable and where the tumor finding involves more than 5% of the resected tissue. In the hormone naïve T1b group mean age was 77 years (range 60-90) and mean Gleason score was 6.6 (range 5-9). In the castration-resistant group, mean age was 79 years (range 65-88) and mean Gleason score was 9.1 (range 7-10).

Materials obtained through prostate biopsies (paper IV)

Prostate biopsies from 11 patients were obtained sequentially during prostate cancer progression and PSA was measured in serum at the same time points. Biopsy 1: sampled at the time of prostate cancer diagnosis prior to ADT. Biopsy 2: sampled approximately three months after initiation of ADT. Biopsy 3: sampled when the tumor relapsed as indicated by a rise in PSA or when the patient had symptomatic progression. Mean Gleason scores in the groups were 7.4, 7.5 and 8.2 respectively. Mean age at the time of diagnosis was 73 years (range 63-81).

Cell lines and cell culture

The human prostate cancer cell line LNCaP was obtained from American Type Culture Collection (Manassas, VA, USA). LNCaP is an AD cell line once established from a lymph node metastasis from a castration-resistant patient. LNCaP expresses the AR and secretes PSA and is one of the most common AD cell lines used in prostate cancer research. LNCaP has a mutated AR that can bind androgens in addition to other steroid hormones.[61]

To be able to study transition into androgen-independency we developed an AI cell line, LNCaP-19, from the LNCaP cells.[209] LNCaP-19 was developed in vitro by culturing the normal LNCaP cells in medium containing steroid-depleted serum. LNCaP-19 expresses the AR, although at lower levels than LNCaP, and has an increased angiogenesis compared to its parental cell line. It establishes tumors in castrated animal, indicating true AI growth. In paper IV, we also included the AI cell line PC-3. PC-3 was established from a human prostate cancer bone metastases[210] and PC-3 is a dedifferentiated and highly aggressive cell line that lacks AR and expression of PSA.

Cells were routinely maintained in RPMI 1640 (PAA Laboratories, Linz, Austria), modified according to the manufacturer's protocol and supplemented with antibiotics and 10% fetal bovine serum (FBS) (Gibco^{TM}, Invitrogen LTd., Paisley, UK) for LNCaP and PC-3 and 10% dextran-charcoal treated FBS (DCC-FBS)(steroid-depleted) for LNCaP-19. Cells were tested and found free of mycoplasma.

Animals and tumor cell implantation

Male athymic BALB/c nude mice were used for subcutaneous and orthotopic implantations of tumor cells (paper II-IV). BALB/c nude mice are immunodeficient and lack T-lymphocytes, which thereby allows establishment of human tumors. For the metastasis experiment (paper IV) we used SCID CB17 mice. SCID mice are more immune compromised than BALB/c nude mice and they lack both T- and B-lymphocytes. Tumor cells are therefore more tumorigenic and have a higher propensity to establish metastases after implantation in SCID mice. Mice were at least 7 weeks old when the experiments started. Mice turn sexually mature at this age and this is therefore a common starting point for studies on prostate cancer. The use of animals was approved by the animal ethical committee in Gothenburg.

Subcutaneous implantation of cells (paper II-IV)

Two million tumor cells, suspended in equal volumes of culture medium and matrigel (BD Bioscience, Bedford, MA, USA), were inoculated subcutaneously in the flank of the mice. Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, which is a tumor that is rich in ECM proteins. The major components are laminin, collagen IV, heparan sulphate proteoglycans, entactin and nidogen and various growth factors are also present. The use of Matrigel facilitates the establishment of human cancer cell lines as xenografts in mice. Prior to cell injection, castration or sham-operation was performed via a scrotal incision under anaesthesia. Tumors were measured with a caliper and the formula (length x width^2 x 0.5) was used to calculate tumor volume. The experiment was ended after a certain time point or when the tumors reached a volume of maximum 1300 mm^3.

Orthotopic implantation of cells (paper III and IV)

Mice were anesthesized and a transversal incision was performed in the lower abdomen. In experiments involving castrated mice, castration was performed prior to cell injection via the abdominal incision. One million cells suspended in matrigel (BD Bioscience) were injected into the dorsolateral lobe of the prostate using a 30-gauge needle. The abdominal incision was closed with interrupted sutures. The dorsolateral lobe was chosen because of its resemblances with the peripheral zone in humans, which is the most common origin of human prostate cancer. The experiments were ended after 9 weeks. The metastasis experiment in SCID mice was ended after 15 weeks. Mice were examined for metastases and primary tumors and macroscopic lymph node metastasis were collected.

Treatment with testosterone (paper III)

Three weeks after orthotopic LNCaP-19 tumor cell implantation, castrated mice were divided into two groups with comparable tumor volumes (mean tumor volume = 62 mm^3). One group received testosterone, 10 mg/kg (Sustanon® 250, Organon, Oss, The Netherlands), with subcutaneous injections every other day. Treatment proceeded until week 9 when the experiment was concluded. Castrated and intact mice were included as controls and received vehicle during the same time period.

Magnetic resonance imaging (MRI)

In paper III, the technique of MRI was applied to monitor orthotopic tumor growth over time in the mice. We used a 7.05 T Bruker BioSpec MR system (Bruker BioSpin, Ettlingen, Germany) equipped with a 72 mm volume coil for transmission of radiofrequency for MRI. A linear 4-element phased array coil with 16 mm circular elements served as a receive coil. Twenty coronal slices through the normal prostate/prostate tumor were acquired with a multi-slice, multi-echo sequence. The scan time was 13 minutes per data set. Regions of interest were defined manually in each slice using the ParaVision program. Calculation of tissue volumes was performed as the sum of the area of the slices multiplied by the slice thickness.

In vitro studies

Anchorage-independent growth (paper II)

Anchorage-independent growth is a hallmark of malignant tumor cells. Thus, cancer cells can grow and survive without attaching themselves to the underlying matrix. It has been suggested that cell-cell adhesion molecules or integrins are involved in anchorage-independent growth. In this study, anchorage-independent growth of the cancer cells was assessed by colony formation in soft agar. Fifty thousand cancer cells in serum free medium were suspended with an equal volume of 2% low-melt agarose (A-9045, Sigma-Aldrich, St Louis, MO) and seeded in Petri dishes, pre-coated with 1% standard agarose (162-0100, Bio-Rad Laboratories, CA). Colony formation ability was investigated in triplicates in culture medium containing 10% DCC-FBS and in culture medium containing 10% DCC-FBS plus 0.1 nM R1881 (NEN Life Science Products, Inc., Boston, MA). Colonies, defined as cell clusters consisting of more than 10 cells, were counted after 28 days under a light microscope. The experiment was repeated three times.

Cell migration (paper II)

Ability to migrate is another property that is essential for cancer cells to be able to invade surrounding tissue and metastasize. Migration assays were performed using Transwell polycarbonate membranes with 12 μm pore size (3403, Corning, Life Sciences, Acton, MA). Eighty-five thousand cancer cells in culture medium containing 1% DCC-FBS were seeded in the inserts in duplicates. Culture medium containing 10% DCC-FBS was added to the lower chambers and used as chemoattractant. To investigate possible androgenic effects on migration, wells with addition of 0.1 nM R1881 in both upper and lower chamber were also included. Cells were incubated for 24 hours. Migrating cells on the lower side of

the membrane were fixed in methanol and stained with hematoxylin. Cells were counted in 10 objective fields, under a light microscope, at 200x magnification. The experiment was repeated three times.

Cell adhesion assay (paper II)

Fibronectin and vitronectin are two of the most common components of the ECM. Migrating cancer cells form transient attachments to these structures when they move through the tissue. The adhesion is often mediated via integrins, which are expressed on the surface of the tumor cells. Cell culture plates with 96 wells, were coated with fibronectin (10 µg/ml) or vitronectin (3 µg/ml) (Sigma-Aldrich, St Louis, MO). Unspecific binding was blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO). Cancer cells were seeded in triplicates in serum free culture medium. To investigate transient attachments cells were only allowed to adhere for two hours, which would simulate tumor cell arrival at a new site or migration through the tissue. Adherent cells were fixed with 95% ethanol and stained with crystal violet. Dye was extracted with Triton-100 over night and the absorbance was read at 550 nm. Background levels of cell adhesion were determined in wells coated with BSA alone and these values were subtracted from values obtained for vitronectin and fibronectin. The experiment was repeated three times.

Cell culture for RNA preparation (paper II and IV)

For RNA analyses, cells were cultured in 10% DCC-FBS, without or with addition of the synthetic androgen R1881 in different concentrations (0.1 nM and 1 nM). After a certain time (see specific papers), cells were trypsinized and RNA was prepared (see below).

RNA analyses

RNA preparation (paper II-IV)

Total RNA from cells and tumor tissue was prepared with TRIzol solution (paper II) (Invitrogen Ltd.) or by using the RNeasy mini kit (paper IV) (Qiagen Gmbh, Hilden, Germany) according to the manufacturer's recommendations.

Preparation of RNA for the microarray analysis (paper III) was performed by combining the TRIzol method with the RNeasy mini kit. The combination of these methods results in low degradation of the RNA and usually results in very pure

RNA of good quality that can be used for microarrays. Another advantage is that the RNA does not need to be DNase treated, which is not recommended when the RNA will be used for microarray experiments. Tumor tissues were homogenized in TRIzol reagent according to the manufacturer`s protocol. Chloroform was added and after a centrifugation step, the aqueous phase was collected and transferred onto a mini prep column from the RNeasy mini kit and the subsequent steps were according to Qiagen's protocol. RNA quality and concentration were measured using a bioanalyzer (Agilent 2100, Agilent Technologies Inc., Santa Clara, CA, USA) and nanodrop (Thermo Scientific NanoDrop, Saveen Werner, Limhamn, Sweden) respectively.

cDNA synthesis (paper II and IV)

Total RNA was reversely transcribed by the M-MLV enzyme into cDNA by using oligo dT (paper II) or random hexamers (IV). 18S was used as an endogenous control in the real-time reactions in paper IV and therefore random hexamers were used because these primers convert both messenger RNA (mRNA) and ribosomal RNA (rRNA) into cDNA (oligo dT does not). For further details refer to the specific papers.

Polymerase chain reaction (PCR) (paper II and IV)

In paper II, PCR reactions were performed on a conventional PCR apparatus. For the PCR reactions, cDNA was mixed with PCR buffer (1X), $MgCl_2$ (1.5 mM), deoxynucleotides (200 μM of each), primers (0.4 μM of each primer) and Taq polymerase (1.67 U). For specific details regarding primer sequences, number of cycles and annealing temperatures see paper II. GAPDH was used as an internal control. PCR products were electrophorezed on a 2% agarose gel containing ethidium bromide. Primers were purchased from CyberGene AB (Huddinge, Sweden) and other chemicals were from Promega (Madison, WI, USA).

In paper IV, real-time PCR was performed with the ABI Prism 7500 Fast Sequence Detector (Applied Biosystems, Applera Corporation, Foster City, CA, USA). PCR primers and TaqMan MGB probes were purchased as TaqMan Gene expression Assays (Applied Biosystems). PCR parameters were according to the manufacturer's protocol and the ΔΔCt method was used for relative mRNA quantification. PCR reactions for target genes and 18S endogenous control were performed in duplicates for all samples and repeated twice.

Microarray experiment and data analysis (paper III)

Gene expression in the LNCaP-19 tumors was evaluated using the Human Gene 1.0 ST from Affymetrix Inc. (Santa Clara, CA, USA) according to the manufacturer's protocol. Three groups of LNCaP-19 tumors were included as follows: 1: subcutaneous tumors from intact mice (sc intact), 2: orthotopic tumors from intact mice (ort intact) and 3: orthotopic tumors from castrated mice (ort castrated). Two comparisons were made; 1) "ort intact" versus "sc intact" and 2) "ort intact" versus "ort castrated".

Data was normalized using the robust multi-array analysis (RMA) method. Probes that displayed signal intensity below 50 in all samples were removed from further analysis. Data was log 2 transformed and a SAM analysis was performed to identify differentially expressed genes between groups. The false discovery rate was set at 5% and a fold change of two or above was considered to be a change in gene expression. Gene ontology analysis was performed on differentially expressed genes using the Database for Annotation, Visualization and Integrated Discover (DAVID) tools (http://david.abcc.ncifcrf.gov/).

Protein analyses

Protein preparation (paper II and IV)

Total protein was prepared by homogenization in lysis buffer consisting of PBS (pH 7.5), 0.15 M NaCl, 0.25% Tween 20 and protease inhibitors (1X, Complete™ Mini, Roche Diagnostics, Mannheim, Germany). Samples were sonicated, centrifuged and the supernatants were collected. Protein concentrations were measured using the BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer's protocol.

Western blot (paper II and IV)

Fifty microgram of reduced protein was loaded on a 4-12% Bis-Tris gradient gel and electrophoresis was performed using MOPS (Invitrogen, Carlsbad, CA, USA) as running buffer. Fractionated proteins were electroblotted onto a polyvinyldifluoride (PVDF) membrane (Hybond-P, GE Healthcare, Buckinghamshire, England). Membranes were blocked in 5% dry milk and subsequently incubated with primary antibodies over night at 4 °C. Details regarding primary antibodies and concentrations can be found in the specific papers. Membranes were incubated with a peroxidase labelled secondary antibody and immunoreactions were detected using the ECL™ Western blotting detection

system (GE Healthcare). As an internal control, actin (1/5000, A2066, Sigma-Aldrich, St Louis, MO, USA) was used. The positive controls used can be found in the specific papers.

Immunohistochemistry (paper I-IV)

Tissue sections were deparaffinized and rehydrated and sections were heated in antigen unmasking solution (Vector Laboratories, Inc., Burlingame, CA, USA) for antigen retrieval. Endogenous peroxidase was quenched in 0.3% hydrogen peroxide in methanol. Immunohistochemistry was performed using the Vectastain ABC kit (Vector Laboratories, Inc.). Sections were incubated with primary antibodies over night at 4 ºC. For antibodies and concentrations see the specific papers. The biotin streptavidin complex was visualized by using Nova Red substrate (paper I) or DAB (paper II-IV) and sections were counterstained with Mayer's hematoxylin and mounted. Negative controls were performed by omitting primary antibodies.

Evaluation of immunostaining (paper I-IV)

The VEGF-C, VEGFR-3 (paper I) and E-cadherin staining (paper IV) were evaluated by means of a semi-quantitative scoring system for staining intensity and proportion of positive cells. Intensity and proportion of positive cells were judged between 0 and 3 and the total score for each slide were then obtained by multiplying these scores. The intensity was scored as 0 = no detectable signal; 1 = weak staining; 2 = moderate staining; 3 = strong staining. The proportion of positive cells were scored as 0 = no positive cells; 1 = <1/3 positive cells; 2 = 1/3-2/3 positive cells; 3 = >2/3 positive cells. For the N-cadherin staining (paper IV), there was no large variation in staining intensity between tumors and therefore intensity was not taken into consideration. Proportion of positive cells was scored as 0 = no positive cells; 1 = <25% positive cells; 2 = 25-50% positive cells; 3 = 50-75% positive cells; 4 = >75% positive cells. Sections were evaluated in a blinded fashion at 200 x magnification.

Microvessel density (MVD) was evaluated with CD34 immunostaining. In paper III, blood vessels were counted at 200 x magnification in five representative fields in each tumor section. In paper II, number of vessels containing tumor cells was counted at 400 x magnification in 10 randomly chosen fields in each section. Percent invasivity was defined as number of vessels containing tumor cells divided with total number of vessels with a visible lumen. A blood vessel was defined as any immunostained endothelial cell or endothelial cell cluster, separated from adjacent vessels, according to Weidner et al.[211]

Statistical analyses

The Mann-Whitney U test was used to analyze differences between independent groups. The correlations between the N-cadherin/VEGF-C/VEGFR-3 score and the Gleason score in the tumors were analyzed by using the Spearman's rank correlation test. Comparisons of proportion of positive N-cadherin cases between the hormone naïve and castration-resistant group and between tumors from patients with metastases and without metastases were analyzed with Fishers chi-square test. Wilcoxon signed rank test was used to analyze differences between dependent groups, i.e. differences in N-cadherin and E-cadherin staining between biopsies collected from prostate cancer patients during tumor progression. A P-value <0.05 was considered statistically significant. Statistical analysis was performed with the statistical program SPSS for Windows.

RESULTS AND COMMENTS

Paper I

Recent studies have indicated that the lymphangiogenic growth factor VEGF-C promotes lymph node metastasis.[120-122] In this work, we investigated the expression patterns of VEGF-C and its receptor VEGFR-3 in human prostate cancer specimens and their possible association with lymph node metastasis.

Expression of VEGF-C and VEGFR-3 was evaluated with immunohistochemistry by using a semi-quantitative scoring system, combining the staining intensity and proportion of positive cells. Benign prostate epithelial cells were either negative or showed a weak, scattered staining pattern for VEGF-C. In contrast, VEGF-C was expressed by a majority of the tumor cells but with different intensities. Tumors from patients with lymph node metastases had a significantly higher score than tumors from patients without metastases. Spearman's rank correlation test showed no significant correlation between the VEGF-C score and the Gleason score. Our results were in accordance with a previous report by Tsurusaki et al, showing elevated mRNA levels of VEGF-C in lymph node positive prostate tumors.[127]

In addition, VEGFR-3 was expressed by lymphatic endothelial cells and some blood vessel endothelial cells and, interestingly, also in the cytoplasma of the malignant epithelial cells. In accordance with the VEGF-C results, VEGFR-3 expression was higher in lymph node positive tumors compared to lymph node negative tumors. Spearman's rank correlation test showed a significant correlation between the VEGFR-3 score and the Gleason score. Previous to our publication, Li et al also reported of cytoplasmic expression of VEGFR-3,[212] which suggests that the receptor might be involved in regulation of the tumor cells directly. Besides stimulating lymphangiogenesis, VEGF-C could therefore also possibly play a role in promoting tumor cell proliferation and subsequent invasion.

The elevated expression of VEGF-C and VEGFR-3 in prostate cancer could be a reason for metastasis to regional lymph nodes and these factors could thereby be potential targets for anti-metastatic treatment of prostate cancer.

Paper II

Development of AI tumors is the main reason for death of prostate cancer and bone metastases are frequently detected at this stage. To develop new treatment strategies, molecular and cellular alterations that are associated with transition into androgen-independency and metastasis need to be clarified. To achieve this goal, well-defined animal models are essential. Our group has developed the AI cell line LNCaP-19, from the AD cell line LNCaP.[209] The aim of the present work was to further characterize LNCaP-19 and to identify molecular and cellular alterations that are specifically related to the transition into androgen-independency, with focus on events related to the invasive and metastatic process.

Transition into androgen-independency was associated with ability to grow without anchor in softagar and increased migratory capacity, which both are properties that promote establishment of metastatic foci. LNCaP-19 also displayed an increased cell adhesion to vitronectin and fibronectin, two major components of ECM and plasma. Migrating tumor cells form transient attachments to the matrix, often via integrins, and the results herein therefore indicate elevated levels of some integrin or other adhesive molecule in the LNCaP-19 cell line. However, no major differences in the integrin expression profiles between the cell lines were established. The only integrin subunit that was differentially expressed was the collagen binding integrin α2, which was elevated in LNCaP-19. Interestingly, a cadherin switch was identified in the LNCaP/LNCaP-19 tumor model. The tumor suppressor E-cadherin was downregulated in LNCaP-19 tumors compared to the LNCaP tumors. Instead, expression of the proinvasive factor N-cadherin was observed in LNCaP-19. Furthermore, LNCaP-19 displayed increased levels of MMP-9 and MT1-MMP compared to LNCaP. By modulating the ECM, these proteases make space for migrating tumor cells, allowing them to reach their final metastatic destination. Finally, we investigated if the molecular alterations had an effect on the actual capacity for the LNCaP-19 cells to invade blood vessels and reach the circulatory system. As expected, we found a higher proportion of blood vessels filled with tumor cells in the LNCaP-19 tumors than in the LNCaP tumors, showing that the alterations had a profound effect on the invasive potential of the AI tumors.

From the results of this study we conclude that LNCaP-19 is a relevant model for studies on AI prostate cancer and invasion. This model could be valuable for evaluating new treatment strategies for AI prostate cancer. This study also demonstrates that transition into androgen-independency is directly associated with several prometastatic alterations.

Paper III

We were interested in studying the growth behavior of AD and AI prostate tumors in different microenvironments and at different androgen levels. The LNCaP and LNCaP-19 cell lines were used as a model system and tumor cells were implanted orthotopically or subcutaneously in intact and castrated immunodeficient mice. One purpose was also to evaluate MRI for monitoring orthotopic tumor growth in real-time in the mice.

Firstly, we investigated tumor growth in intact mice. We found that growth of the AI LNCaP-19 was suppressed in the prostatic microenvironment compared to the subcutaneous site. In contrast, the opposite was observed for the AD LNCaP. LNCaP grew rapidly at the prostatic site, which corresponds to the local growth of an AD primary tumor. These results imply that the prostatic environment influences AD and AI tumor cells differently. The retarded growth of AI tumors in the prostate corresponds to the clinical observations of the behavior of human prostate cancer. Local relapse of an AI tumors is relatively rare while deaths from metastatic disease are more common.[213] We therefore suggest that AI tumors preferentially grow at other sites than the prostate.

Next, we investigated the androgenic effects on the LNCaP-19 tumor growth in different settings. Interestingly, castration of the mice did not affect ectopic LNCaP-19 tumors but resulted in increased tumor growth in the prostatic environment. In addition, testosterone treatment of castrated mice with orthotopic LNCaP-19 tumors reversed the accelerated tumor growth. From these results it can be suggested that the local prostatic microenvironment, including the prostatic stromal cells, was responsible for the suppression of the AI LNCaP-19 tumors in the prostate. In contrast to the LNCaP-19 tumors, castration of mice with orthotopic AD LNCaP tumors resulted in impaired tumor development.

Development and growth of the orthotopic tumors were easily followed over time with MRI. The tumor could be distinguished from the surrounding organs at all stages during the course of tumor progression. Tumors as small as 10 mm^3 could be distinguished from the normal prostate and irregular shapes could be determined with a high degree of precision.

To reveal factors that are responsible for the retarded growth of AI tumors in the prostate, a microarray experiment was performed. Three groups of LNCaP-19 tumors were included as follows: 1: subcutaneous tumors from intact mice (sc intact), 2: orthotopic tumors from intact mice (ort intact) and 3: orthotopic tumors from castrated mice (ort castrated). Two comparisons were made; 1) “ort intact” versus “sc intact” and 2) “ort intact” versus “ort castrated”. We identified a set of genes that are the most plausible candidates for causing the slowly growing phenotype of LNCaP-19. Upregulated genes in the repressed tumors growing in

intact mice included ADAM metallopeptidase with thrombospondin type 1, motif 1 (ADAMTS1), protocadherin 20 (PCDH20) and regulator of G-protein signaling 2, 24 kDa (RGS2). ADAMTS1 is a multifunctional protein that inhibits angiogenesis,[214] probably by sequestering VEGF[215] and release of anti-angiogenic peptides from thrombospondin[216] and ADAMTS1 has been shown to be downregulated in human prostate cancer.[217] PCDH20 was recently identified as a tumor suppressor in non-small-cell lung cancer[218] and RGS2 has been shown to inhibit AI activation of the AR.[219] Downregulated genes in the repressed intraprostatic LNCaP-19 tumors included N-cadherin and neuronal cell adhesion molecule (NRCAM). N-cadherin and NRCAM are both cell adhesion molecules with a neuronal origin that facilitate migration and metastasis of tumor cells.[165,179,190,220-222]

From this study, we conclude that prostatic stromal cells were responsible for the growth inhibition of orthotopic AI tumors. This is mediated through induction of genes inhibiting angiogenesis and tumor growth and suppression of genes promoting cell adhesion and metastasis. Furthermore, this study indicates that AI tumors do not thrive in the intact prostatic environment and supports previous clinical observations of frequent progression of prostate cancer as metastases in patients with castration-resistant disease. We also conclude that MRI is a useful method for following orthotopic tumor growth in real-time in the mice.

Paper IV

N-cadherin has been implicated as an important prometastatic factor and its expression is elevated in several human malignancies.[223] Most prostate cancer patients treated by ADT show an initial response, but the treatment effect is temporary and is often followed by regrowth of an aggressive and highly metastatic tumor. This study was conducted to investigate if N-cadherin is influenced by androgen deprivation and if its expression is associated with metastasis in prostate cancer.

The results showed that expression of N-cadherin increased in absence of androgens in the AI LNCaP-19 tumor model *in vitro* and *in vivo*. In the AI PC-3 cell line, N-cadherin was present but androgen deprivation did not result in enhanced N-cadherin expression. One possible explanation for the discrepant results is that LNCaP-19 expresses the AR and PC-3 does not, which might reflect the involvement of the AR in the regulation of N-cadherin. Increased levels of N-cadherin are often accompanied by a concomitant decrease in E-cadherin and therefore, we investigated if E-cadherin decreased in absence of androgens in LNCaP-19. In subcutaneous LNCaP-19 tumors and in cultures *in vitro*, androgen deprivation did not alter the E-cadherin levels. In contrast, castration reduced E-

cadherin levels in the orthotopic LNCaP-19 tumors. These results show that E- and N-cadherin are not directly associated with each other. It also shows the importance of the normal prostate tissue in controlling the expression of E-cadherin. We suggest that the intact prostate induces the E-cadherin expression, thereby keeping the differentiation of the AI tumors. We also investigated if increased N-cadherin was associated with EMT or neuroendocrine (NE) differentiation in the animal model. However, no clear associations could be established.

Next, the N-cadherin findings were validated in human prostate cancer specimens. Accordingly, N-cadherin was more frequently observed in castration-resistant tumors compared to early, non-treated T1b tumors. There was also a significant correlation between the N-cadherin score and the Gleason score. Interestingly, when the castration-resistant group was divided in two based on the metastasis status of the patients, tumors from patients with established metastases had an increased N-cadherin score compared to patients without known metastases. To further assess the direct influence of castration on N-cadherin in human samples, its expression was investigated in a biopsy material taken sequentially during prostate cancer progression. The main finding was that N-cadherin increased in the prostate tumors already three months after initiation of ADT and its expression was sustained at the same level in the relapsed AI tumors. In contrast, we found no differences in E-cadherin staining in the biopsy material.

From the results of this study it might be suggested that ADT directly influences the prostate cancer cells to acquire properties that make them more motile and invasive at the relapsed castration-resistant tumor stage.

GENERAL DISCUSSION

One of the greatest challenges in the prostate cancer research field is to find ways to treat advanced prostate cancer. Today, locally advanced and metastatic prostate cancer is treated by ADT, which only offer a temporarily relief for the patients. Unfortunately, an AI prostate tumor eventually relapses. At present, there are few effective second-line treatments that can be initiated in the castration-resistant stage, and the response to these treatments is also highly variable. There is therefore an urgent need for new treatment options at this stage of disease. To be able to develop new therapeutic strategies for these patients, increased knowledge about the biology of AI tumors are clearly needed.

Characteristics of human AI prostate cancer

One common feature of AI tumors is the presence of an activated AR, demonstrated by nuclear staining and expression of several androgen-regulated genes.[51-53] Recent results indicate that despite castrate levels of testosterone in serum, intratumoral levels of androgens remain relatively high in castration-resistant tumors. Intratumoral androgen levels have been reported to be increased in bone metastases from human castration-resistant tumors compared to untreated primary tumors[224] and tumor progression of the LNCaP model after castration was associated with elevated intratumoral androgen levels.[225] These observations indicate that prostate cancer cells can synthesize their own androgens. Accordingly, it was demonstrated that elevated intratumoral androgen levels were associated with increased levels of genes involved in the androgenic part of the steroid metabolism.[224-226] Currently, there is a CYP17 inhibitor, abiraterone, in clinical trials for castration-resistant prostate cancer,[227] which so far has shown promising results. In addition, AI tumors display an increased angiogenesis in comparison to AD tumors[217,228] and anti-angiogenic therapy could be another promising approach to target AI prostate cancer. Another feature of AI tumors is NE differentiation. NE transdifferentiation is induced in response to androgen deprivation[229,230] and is observed in bone metastatic lesions from prostate cancer patients[231] and is significantly associated with death from prostate cancer.[232] Recently, gene expression profiling of human AI tumors have been performed by some research groups. Unfortunately, there are no conclusive results regarding the characteristics of AI tumors from the different studies.[233-235] As described in the introduction, prostate cancer, and especially AI tumors, is extremely heterogeneous, and even within the same patient, the phenotype of different metastatic deposits can vary considerably.[231] This fact, together with methodological aspects, is probably a

reason for lack of agreement between different studies. The heterogeneity of AI tumors really comprises a major challenge in the development of new therapeutic concepts.

Patients with castration-resistant prostate cancer most often have disseminated disease and not local tumor growth. Thus, these patients are not operated on a regular basis and tumor samples from the AI stage are therefore difficult to obtain, but collection of tissue has started to improve after the development of various rapid autopsy programs. Due to difficulties in obtaining human AI samples, experimental animal models of prostate cancer have been applied in the research. Reliable and well-characterized model systems of prostate cancer are of outmost importance to achieve the challenge in finding cure also for the AI stage of prostate cancer.

Animal models of prostate cancer

Prostate cancer only arises spontaneously in humans and in dogs and more rarely in rodents. Thus, spontaneous models of prostate cancer are few. Instead, there are some inducible prostate cancer models available in certain rat strains, where tumors can be induced by administration of chemicals or testosterone.[236-238] Other existing prostate cancer models include transgenic models and xenograft models. The transgenic models, for example the TRAMP (transgenic adenocarcinoma of the mouse prostate) model, have the benefit of modeling the whole spectrum of the disease as it occurs, with progression from premalignant lesions through localized tumors to disseminated disease.[239] Xenograft models, as we used in our studies, provide the opportunity to study human prostate cancer in an *in vivo* context in rodents. They also allow the assessment of reciprocal stromal-tumor cell interactions and metastasis. Over the years, several human prostate cancer cell lines have been established and used for inoculation in immune compromised mice. The classical human prostate cancer cell lines used in laboratories worldwide includes PC-3,[210] DU-145[240] and LNCaP.[241] Different research groups have developed these cell lines further into several sublines with different androgen responsiveness and metastatic capacity. For instance, AI sublines have been developed *in vitro* by culturing AD LNCaP cells in absence of androgens, which was how LNCaP-19 was established.[209] AI cell lines have also been developed *in vivo* by growth in castrated mice.[242] In addition, various sublines have been established from metastatic tissue in mice, which generate cell lines with increased propensity to form metastases.[243] There are many factors to take into consideration when choosing cell line for implantation in mice. It is critical to understand the basic characteristics of the cell line and how relevant it is for the human disease. For instance, many human prostate cancer cell lines lack expression of AR and PSA, which is commonly expressed in human prostate cancer. It is also important to use

to correct cell line/model for the scientific problem that is being studied and different animal models can mimic various aspects of clinical prostate cancer. One drawback of the xenograft models is the use of immunodeficient mice, which leaves out the immune component that is of importance in tumor biology. Another drawback is that xenograft tumors grow rapidly and that not all tumor stages can be reproduced. Tumor cells are most often implanted at the subcutaneous site but it might be more relevant to implant the tumor cells in the correct anatomical site from where the cells origin, i.e. orthotopic implantation. A major problem with the existent xenograft models of prostate cancer is the rare formation of bone metastases. The underlying cause of this is not known. Because of this, special systems have been devised to study the growth of prostate cancer cells in bone, and they include injections of tumor cells directly into the bone marrow of tibia or femur.

MR as an imaging tool for monitoring tumor growth in mice

An orthotopic tumor growth resembles the features of human prostate cancer more closely than subcutaneous models and is therefore preferable in many aspects. However, to be able to follow tumor growth, as well as the effects of treatment over time, sacrificing animals at different time points is usually needed. Thus, large cohorts of animals have to be included in longitudinal studies and each animal provides data for one time point only. In paper III we applied a non-invasive technique by using MRI to follow the growth of orthotopic tumors in real-time. This technique allows fast, precise and repetitive measurements of the tumor at different time points in the same animal. The use of MRI has a wide application in experimental studies of prostate cancer in animal models, including discrimination between normal prostate tissue and tumor[244,245] and for determining intratumoral vasculature and hypoxic areas.[246,247]

Transition into androgen-independency is associated with prometastatic properties

The model system that comprises LNCaP and LNCaP-19 resembles human prostate cancer progression into androgen-independency in several ways. LNCaP-19 displays increased angiogenesis, secretes PSA and has the capacity to grow under androgen deprived conditions.[209] An advantage with the model system is that differences between the cell lines reflect tumor progression into androgen-independency and are not due to heterogeneity between samples that are a common problem when using human materials. From the work presented in paper II, we

conclude that many invasive and metastatic traits are acquired during transition into androgen-independency. These included enhanced migratory capacity, ability to grow without anchor and increased adhesion to the ECM proteins fibronectin and vitronectin. In addition, transition into androgen-independency resulted in induction of the invasive promoting factors N-cadherin, MMP-9 and MT1-MMP and downregulation of the tumor suppressor E-cadherin in the LNCaP-19 cells, which are itself intriguing with regard to the fact that ADT is the only available choice of treatment for patients with advanced prostate cancer. The induction of metastatic traits in the LNCaP-19 cell line ultimately resulted in increased potential to invade tumor blood vessels (fig. 8). There is limited knowledge on the molecular basis for the invasion tumor cells into blood vessels. The maturation of tumor blood vessel is often limited, leaving vessels destabilized with poor pericyte coverage and this phenotype might facilitate tumor cell invasion. We[248] and others[249] have recently shown that the degree of pericytes coverage influences tumor cell invasion into blood vessels. In the LNCaP-19 tumors, we observed that blood vessels with less pericyte coverage were more often invaded by tumor cells.[248]

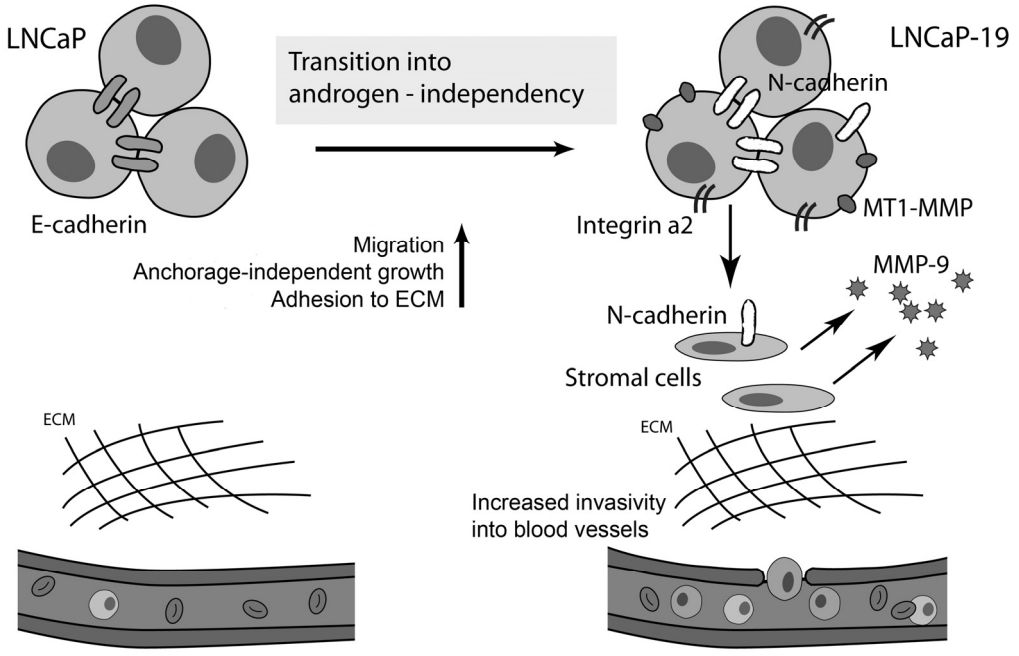

Figure 8: Transition of the AD LNCaP into AI LNCaP-19 involves several proinvasive alterations. LNCaP-19 cells displayed increased anchorage-independent growth, migratory capacity and adhesion to the ECM components vitronectin and fibronectin in comparison to the LNCaP cells. The LNCaP cells express the tumor suppressor E-cadherin on the cell surface, which could prevent detachment between adjacent cells and subsequent invasion into blood vessels. On the other hand, the LNCaP-19 cells express the proinvasive factors N-cadherin, MT1-MMP and integrin α2 on their cell surface and induce the expression of MMP-9 from the surrounding stromal cells. Together, the changes induced at transition into androgen-independency resulted in enhanced invasivity of the LNCaP-19 cells into blood vessels.

Results from paper II show that LNCaP-19 has the ability to complete the first steps in the metastatic cascade, i.e. detachment from surrounding tumor cells, migration through the tissue and intravasation into blood vessels to seed tumor cells into the circulation. In addition, recently obtained data also demonstrate that LNCaP-19 can complete the later steps of the metastatic cascade, which includes extravasation and colonization at the new site. The last step is the most challenging for the tumor cells and it has also been demonstrated that this is the rate-limiting step in the formation of metastases.[87,88] After orthotopic implantation LNCaP-19 forms metastases to primary and renal lymph nodes and also to the lungs and kidneys. After intracardiac administration of tumor cells, metastases to the spine were observed. In addition, LNCaP-19 grows in the bone after intratibial injection and the established tumors showed an osteoblastic response. One important finding of our study is that AI tumor cells are more invasive than AD tumor cells, which suggests a direct link between induction of invasive and metastatic properties and androgen deprivation and subsequent development of an AI tumor. Exactly how the invasive program is activated remains to be determined.

AI tumors are suppressed in the prostatic microenvironment

Interactions between stromal cells and tumor cells are of great importance in the progression of prostate cancer.[72] The stromal cells influence the tumor cells and therefore varying tumor physiology and tumor properties can be observed depending on the site of growth.[250,251] The results from paper III indicated that AI tumor cells did not thrive in the prostatic microenvironment. The observation is in accordance with clinical experience, where AI tumors rarely relapse locally in the prostate but rather as metastases.[213] Our observation is also supported by experimental studies in mice from other groups. Decreased tumor growth at the orthotopic site compared to ectopic sites has recently been reported for an experimental AI prostate cancer model.[252] Moreover, in the TRAMP model, castration of the mice promoted progression of the AI tumors at distant sites and to a lesser extent locally in the prostate.[253] It has also been shown that the ability of the AI C4-2 tumors to metastasize to bone were higher when injected subcutaneously compared to orthotopically, which also illustrates the inhibitory role of the prostatic environment.[254]

Castration results in a rapid regression of the normal epithelial cells and affects the secretion of androgen-regulated factors from the stromal cells. In paper III, inoculation of LNCaP-19 cells in the prostate of a castrated host resulted in increased tumor growth and it could therefore be suggested that the prostatic microenvironment secrets testosterone regulated factors that influence the growth of AI tumors negatively. The prostate is a quiescent organ where the stromal cells

play a vital role in controlling the epithelial cells, either in a positive or negative direction. It has been shown that the normal prostatic stroma responds to androgen treatment by restraining the proliferation of the epithelial cells.[255] In addition, it has been shown that when the rat prostate Dunning tumors where grafted in presence of normal stromal mesenchyme it resulted in inhibition of tumor growth and induction of a more differentiated morphology.[256,257] These studies illustrate that the normal prostatic environment controlled the tumor and could overcome the defective genetic alterations, resulting in tumors with less aggressive potential. Interestingly, the suppressed tumor growth of LNCaP-19 in the intact prostate (paper III) was accompanied with increased expression of E-cadherin (paper IV) when compared to E-cadherin expression in the fast growing tumors from castrated mice. Expression of E-cadherin is observed in differentiated epithelial tissues and thus, the results suggest that an intact prostatic environment keeps the AI tumors in a higher differentiation stage, resulting in a more controlled tumor growth.

The suppressed tumor growth of LNCaP-19 in the intact prostate was most likely due to an upregulation of angiogenesis inhibitors and tumor suppressors, including ADAMTS1,[214,217] PCDH20[218] and RGS2.[219] In addition, the prostatic microenvironment repressed genes that are known to be involved in cell adhesion and metastasis, which included N-cadherin[165,179,190] and NRCAM.[220-222] Interestingly, many genes related to differentiation and migration of neuronal cells were suppressed by the prostatic tissue in the slowly growing LNCaP-19 tumors. Why a neuronal-like phenotype promotes and benefit tumor growth is presently not known. Various factors have been isolated from prostatic stromal cells that are likely to influence the growth of tumors.[15,77,258] However, the factors that were responsible for the effects in this study remains to be identified.

Prometastatic alterations induced by ADT

In our studies, we could show that N-cadherin was increased after androgen deprivation (paper IV) and subsequent transition into androgen-independency (paper II). N-cadherin was induced in response to androgen deprivation both in experimental and human prostate cancer. Based on our results from paper IV it is reasonable to believe that the AR plays a role in the suppression of N-cadherin, since no effects were seen in the AI cell line without AR. An ARE has been identified in intron 1 of the N-cadherin gene[259] and it is possible that the repressed N-cadherin gene expression is attributed to a direct inhibition by binding of activated AR to this site. However, the ARE might not be involved in the regulation of N-cadherin, and there is also a possibility that the regulation is indirect through another androgen regulated factor. Similarly to N-cadherin, VEGF-C is also induced by androgen deprivation and recently it was shown that expression of VEGF-C was inhibited by NKX3.1 that is an androgen-regulated gene.[260]

Androgen deprivation therefore results in decreased NKX3.1 and increased VEGF-C expression. Exactly how AR and androgens regulate N-cadherin are at present unknown and further studies are needed to be able to address this issue.

In paper IV, we observed an upregulation of N-cadherin in human tumor samples already three months after initiation of ADT. At the same time, the PSA value had reached nadir in the majority of the patients and the tumor was in a regressed state. These findings might therefore indicate that elevation of N-cadherin was not due to transition into the AI stage, but was rather a direct consequence of androgen deprivation. However, another possibility is that despite the low PSA values, transition into androgen-independency could already have occurred and therefore increased N-cadherin could be one of the factors initiating the metastatic phenotype acquired by AI tumors. In the literature, N-cadherin is mostly described as a factor that promotes migration and metastasis of tumor cells. However, N-cadherin has also been reported to be involved in survival of tumor cells, via induction of anti-apoptotic pathways.[175,184] Consequently, induction of N-cadherin after ADT can promote a survival advantage for tumor cells at low androgen levels. With these results in hand it is tempting to speculate that ADT induces molecular alterations in the tumor cells that will give the tumor cells an advantage in forming metastases in the relapsed AI stage. Correspondingly, there are other reports in the literature supporting the possibility that androgen deprivation could induce the metastatic machinery in the cells. Nestin, which is an intermediate filament protein that has a role in migration and metastasis of tumor cells, are induced in response to androgen withdrawal.[261] Similarly, expression of VEGF-C[115,262] and MMP-9[263] is upregulated in the absence of androgens.

ADT is standard treatment for patients with locally advanced or metastatic prostate cancer. The benefits for the patients are indisputable and it has been suggested that ADT should be initiated in an early phase of the disease.[41,42] In addition, clinical findings demonstrate that it is of importance to continue with ADT also in patients with relapsed AI tumors.[44] However, conflicting data are reported in the literature, regarding the effects of androgen deprivation on the cellular level. Disruption of the androgen signaling pathway by ADT may results in deregulation of the cell control, which could contribute to the carcinogenic process. Early initiation of ADT might therefore speed the development of castration-resistant disease. In addition, it has been suggested that treatment with anti-androgens such as bicalutamide would induce alterations in the prostatic environment that promote emergence of AI tumors.[264] In support of our findings, studies have shown that an intact androgen signaling pathway in prostate tumor cells decreased invasion and metastasis in animal models.[265,266] In contrast, there are also reports showing that testosterone signaling via AR promotes invasion of prostate tumor cells *in vitro*.[267] Because of the contradicting data in the literature, further studies emphasizing the molecular effects of androgen deprivation on prostate cancer cells are clearly needed.

It has earlier been reported that N-cadherin is increased in poorly differentiated prostate cancer and that its expression correlates to Gleason score.[188-190]. In addition, in a recent study it was shown that N-cadherin expression in untreated prostate tumors was associated with pelvic lymph node infiltration and shorter time to skeletal metastases.[165] Whether the increased N-cadherin observed after initiation of ADT in paper IV really leads to a poor prognosis and if it has a meaning for the propensity for tumor cells to form metastases remains to be clarified. However, a possible clue could be that patients with established metastases more frequently expressed N-cadherin. Increased levels of N-cadherin in poorly differentiated and castration-resistant prostate cancer specimens open the possibility to use N-cadherin antagonists as second-line therapy for castration-resistant prostate cancer. At present, an anti-N-cadherin peptide, ADH-1 (Exherin™) is being evaluated in experimental animal models of cancer and in clinical trials against N-cadherin expressing tumors.[268-271] This peptide disrupts N-cadherin cell adhesion, thereby preventing the adhesion to stromal components and neighboring tumor cells. The main effect of the disrupted N-cadherin adhesion is believed to work through increased apoptosis. Targeted therapy using an N-cadherin antagonist in combination with chemotherapy or other targeted therapies could be a novel approach also for treating metastatic and castration-resistant prostate cancer.

The role of the lymphatic system for metastasis

The blood circulatory system is clearly important in the dissemination of tumor cells. The contribution of the lymphatic system to the spread of tumor cells is however less obvious. There are certainly a variety of factors that influence if a tumor cell will spread via the blood or lymphatic system. In addition, the lymphatic vessels converge and empty its lymph into the venous part of the hematogeneous system. Consequently, there are interconnections between the two systems and therefore tumor cells can spread from one to the other. Most likely, dissemination of tumor cells in the two systems occurs in parallel.

Tumor lymphangiogenesis has been intensively investigated during recent years. Today, there is great evidence that lymphatic vessels promote lymph node metastasis and presence of lymph node metastases is considered a poor prognostic sign in many types of cancer, including prostate cancer.[98] In paper I, we could show that one of the main lymphangiogenic growth factors, VEGF-C, was more prominently expressed in prostate tumors from patients with presence of regional lymph node metastases than in tumors from patients without lymph node metastases. Our results are confirmed by studies from other investigators.[127,272] We and others[272] could not establish a correlation between VEGF-C and Gleason score, which indicates that VEGF-C is not directly involved in promoting tumor

progression to a more dedifferentiated stage. Rather, it seems that VEGF-C contributes to tumor progression by proving a path for tumor cell escape via the lymphatic network. Corresponding to the VEGF-C results, the receptor VEGFR-3 was also elevated in the lymph node positive tumors. We demonstrated presence of VEGFR-3 in the cytoplasma of the tumor cells, which was in accordance with a previous study published by Li et al.[212] Possibly, the VEGFR-3 could promote tumor growth via an autocrine regulatory loop, but further studies are needed to reveal the significance of this finding. There are other reports that demonstrate expression of VEGF receptors in both cancer cell lines and solid human tumors.[273-279] In the prostate, VEGFR-1 and VEGFR-2 have been found to be expressed by malignant epithelial cells and the proliferation of the tumor cells could be driven by VEGF.[280,281] In addition, it has been demonstrated that by inhibiting VEGFR-1,2 and 3 expression from the tumor cells it resulted in decreased migratory capacity of the tumor cells and enhanced sensitivity to chemotherapy. The cytoplasmic receptors might therefore be involved in the survival of the tumor cells.[282]Although the function of tumor cell derived VEGF receptors are not completely understood, the concomitant expression of ligand and receptor suggests that the receptors mediate biological effects in an autocrine fashion. However, most reports regarding VEGFR-3 involves its expression by lymphatic endothelial cells, which is associated with presence of lymph node metastases in several types of cancer, including prostate cancer.[128,283,284]

In paper I, it would have been relevant to study the lymphatic vessel density in the tumors but at the time we published the article, specific antibodies for any of the lymphatic markers lyve-1, podoplanin or prox-1, were not available. Other reports regarding lymphatic vessel density in prostate cancer have proven that presence of peritumoral lymphatic vessels and tumor cell invasion into peritumoral lymphatic vessels were associated with lymph node metastases.[99,285,286] Because of the high physical pressure inside tumors there have been doubts if lymphatic vessels could penetrate into expanding primary tumors[287,288] However, presence of functional intratumoral lymphatic vessels in the centre of the tumor has been reported by other groups.[289] Thus, the issue whether intratumoral lymphatic vessels are functional is controversial and needs further investigations.

The lymphatic system may play an active role in the metastatic process by directly facilitating recruitment of cancer cells into the vessels. However, exactly how this is accomplished is not known but several theories have been proposed. The simplest explanation for the metastasis enhancing effects of VEGF-C would be that by increasing number of lymphatic vessels, it results in increased surface contact area between invading cancer cells and the lymphatic vessels. In addition, there is experimental evidence that lymphatic endothelial cells secrete chemokines that could attract invading tumor cells to the vessels.[92,290] There is no direct experimental evidence that interactions between the tumor cells and the lymphatic

endothelium are required for entry into the lymphatic vessels. However, studies suggest that the mannose receptor 1 (MR-1) and common lymphatic endothelial and vascular endothelial receptor 1 (CLEVER-1) might be important mediators of cancer cell adhesion to the lymphatic endothelium.[291] Another possible explanation could be that VEGF-C alters the lymphatic vessel permeability, thereby making the vessels more prone to promote metastases. To address the question how VEGF-C increases lymphatic dissemination of tumor cells, Hoshida et al used intravital microscopy to image the lymphangiogenic process and entry of tumor cells into the lymphatic vessels. From these studies they concluded that VEGF-C induced hyperplasia of the lymphatic vessels, thereby increasing the surface contact area for the invading tumor cells. In addition, VEGF-C increased the lymphatic flow rate, which enhanced the delivery of tumor cells to the lymph nodes and also increased the establishment of metastases.[292]

There have been attempts to block lymphatic dissemination of tumor cells by introducing soluble VEGFR-3 in animal models of cancer.[121,293,294] These experiments resulted in decreased lymphatic vessels around the tumor and in addition, suppression of lymph node metastases. In support of this, it has been shown that glucocorticoids, which have some benefits in the treatment of hormone-refractory patients, suppress expression of VEGF-C and also tumor lymphangiogenesis.[295] Blocking the VEGF-C/VEGFR-3 signaling axis therefore seem to be one reasonable approach to combat the metastatic spread of tumor cells. Collectively, targeted therapy directed against VEGF-C and the formation of lymphatic vessels in the tumor may interfere with the initial steps of the lymphatic dissemination of tumor cells.

CONCLUSIONS

- VEGF-C and VEGFR-3 are elevated in lymph node positive prostate tumors. Thus, they could play an important role in promoting metastasis to regional lymph nodes. Blocking the VEGF-C signaling axis may therefore be a potential new treatment strategy for inhibiting dissemination of tumor cells via the lymphatic system.

- Transition into androgen-independency is associated with induction of several proinvasive and metastatic properties.

- The model system with LNCaP-19 mimics AI human prostate cancer in many aspects and is therefore a relevant model for studies on AI prostate cancer and invasion.

- AI prostate cancer cells are growth inhibited in the prostatic environment, probably by androgen-regulated factors that originate from the prostatic stromal cells. This finding corresponds with the clinical observation that human castration-resistant prostate cancer mainly progress as metastases and not locally in the prostate.

- MRI is a convenient imaging tool for monitoring growth of orthotopic prostate tumors in mice.

- ADT induces expression of the proinvasive factor N-cadherin in prostate tumor cells. This might indicate that androgen deprivation induces molecular alterations in the cancer cells, which could promote the invasive and metastatic phenotype observed in patients with castration-resistant prostate cancer.

- Expression of N-cadherin increases with tumor grade and is also associated with metastasis in human prostate cancer. N-cadherin could be a possible therapeutic target for treatment of prostate cancer.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Prostatacancer är den vanligaste cancerformen bland män i Sverige och varje år diagnostiseras ca 9000 nya fall. Det är även den cancerform som orsakar flest dödsfall hos svenska män, och årligen dör ca 2300 män av prostatacancer i Sverige.

De flesta prostatatumörer växer långsamt, men det finns också de tumörer som är mer aggressiva. Det manliga könshormonet testosteron reglerar till en början tillväxten av en prostatatumör och därför säger man att tumören är androgen-beroende. Tillväxten av prostatacancer kan därför bromsas genom att avlägsna testosteronet i kroppen. Detta görs genom kastration. Behandlingen fungerar till en början väl och leder till en minskad tumörstorlek. Dock är denna behandling inte botande och i regel återkommer cancern efter något år. I detta stadium regleras tumören inte längre av testosteron och den benämns därför som androgen-oberoende. Androgen-oberoende tumörer är aggressiva och ofta bildas metastaser (eller så kallade dottertumörer) i lymfkörtlar och i skelettet. I dagsläget finns ingen tillfredsställande behandling i detta skede av sjukdomen och prognosen är dålig. För att kunna utveckla nya behandlingsstrategier för androgen-oberoende prostatacancer krävs ytterligare kunskap om egenskaperna hos dessa tumörer. Detta avhandlingsarbete har fokuserat på att kartlägga egenskaper hos androgen-oberoende prostatacancer, med fokus på de egenskaper som är förknippade med att tumören kan sprida sig till andra organ och bilda metastaser.

I studierna användes ett experimentellt modellsystem med androgen-beroende och androgen-oberoende celler från prostatacancer. Cellernas egenskaper studerades i cellodlingssystem samt i försöksdjur. Diverse molekylärbiologiska metoder användes för att utvärdera resultaten. För att konfirmera resultaten från djurstudierna inkluderades även material från patienter med prostatacancer.

Inledningsvis användes modellsystemet för att studera vilka förändringar som sker i tumörcellerna när de övergår från att vara androgen-beroende till att vara androgen-oberoende. Ett flertal förändringar i de androgen-oberoende tumörcellerna identifierades, däribland en ökad rörlighet och en ökad förmåga att invadera blodkärl i tumörer. Ytterligare studier påvisade en ökning av proteiner som främjar bildningen av metastaser (N-cadherin, MMP-9 och MT1-MMP) och en minskning av proteiner som hämmar tumörtillväxt (E-cadherin), i androgen-oberoende cancerceller.

I nästa studie undersöktes hur olika typer av tumörer påverkades av den omgivande miljön i prostatan. Resultaten visade att androgen-oberoende tumörceller hämmades då de växte i prostata på försöksdjur. Detta indikerar att normal prostata

utsöndrar olika substanser som påverkar tumörcellerna i modellen så att de växer långsammare. Resultaten från denna studie överensstämmer med det kliniska förloppet hos många patienter. Hos patienter med androgen-oberoende prostatacancer sker tillväxten främst i form av metastaser i skelettet och inte av den ursprungliga tumören i prostata.

I de inledande studierna identifierades N-cadherin som en intressant faktor som börjar produceras när tumörcellerna blir androgen-oberoende. N-cadherin är ett protein som främjar bildningen av metastaser. I de efterföljande studierna undersöktes hur N-cadherin påverkades av kastrationsbehandling. Huvudfyndet var att N-cadherin ökade i tumörerna efter att testosteronet hade avlägsnats, både i djurmodeller och i prov från prostatacancerpatienter. Förekomst av N-cadherin i prostatacancerproverna var även förknippat med metastaser i skelettet. Denna studie visar att kastrationsbehandlingen leder till att N-cadherin börjat produceras från cancercellerna. En blockering av N-cadherin skulle därför kunna komplettera kastrationsbehandlingen och därigenom förbättra effekterna av denna.

Prostatacancer sprider sig ofta till lymfkörtlarna och därför undersöktes vilken betydelse det lymfatiska systemet har för denna process. VEGF-C är ett protein som främjar tillväxten av lymfkärl. Fler lymfkärl runt tumören kan i sin tur öka risken för att cancercellerna ska använda sig av dessa för att sprida sig vidare i kroppen. I studien analyserades förekomst av VEGF-C i prostatacancerprov från patienter. Resultaten visade att nivån av VEGF-C var högre i de tumörer som hade spridit sig till närliggande lymfkörtlar jämfört med de tumörer som inte hade spridit sig till lymfkörtlarna. En möjlig väg att hämma spridningen av tumörceller i kroppen via lymfkärlen skulle därför kunna vara att blockera VEGF-C.

Sammantaget visar detta avhandlingsarbete att utvecklingen av androgen-oberoende prostatacancer är associerat med ett flertal förändringar i tumörcellerna som främjar metastasering. Hämning av N-cadherin och VEGF-C skulle kunna vara möjliga framtida angreppspunkter för behandling av prostatacancer.

ACKNOWLEDGEMENTS

Firstly, my warmest and most sincere gratitude to **Professor Jan-Erik Damber**, **PhD Karin Welén** and **PhD Christina Vallbo**. I will forever be thankful to all of you for making this possible.

Jan-Erik, my supervisor, who has guided me in the scientific research field of prostate cancer. Your clinical expertise and ability to interpret results in a wider perspective have been invaluable. Thank you for always being so supportive and encouraging. Also, you have a talent for cheering up when it feels difficult and problematic.

Thank You **Karin**, my outstanding co-supervisor, for always being nearby and taking your time for all my concerns and questions. Thanks for constructive ideas, great enthusiasm and for discussions. This could not have been done without you. I hope we will continue to make great science and laugh together!

I am also very thankful to **Tina**, my co-supervisor during my first years as a PhD student, for taking me under your wings and introducing me in the lab world and for always being so helpful. Also thanks for all nice chats and for lending me different things.

I would also like to thank the other members of the Damber group:

Heléne, my friend and PhD companion since the first day. I am so thankful to the way you have contributed to this thesis with your many ideas, criticism and constructive feedback. Thanks for always lending a helping hand and head when I need it. We have been a perfect team during these years and I will really miss our cooperation! Also, thanks for our many nice and very adventurous horse riding tours. **Tajana**, my other great PhD companion. Special thanks for always being so kind and always taken your time whatever the issue – open caps, solving problems, planning experiments, buying chocolate and for being my personnel shopper. In addition, your critical reading and constructive rearrangements of manuscripts have been invaluable. Thanks for Barca and for making most of my days at work hilarious, I will miss working with you too! **Wanzhong**, my very much appreciated prostate cancer college during these years. Thanks for being the expert I needed in the world of immunohistochemistry and for your never ending enthusiasm when trying to teach me about prostate morphology and Gleason score. Also, thanks for a good time during these years. **Anita**, for taken care of my mice, for all your never

ending sectioning and for making my work so much easier. Special Thanks to **Lilian**, for introducing me at EBM and how to handle research animals. Your experience and operation skills have really been invaluable. **Gunilla**, for always being very helpful and friendly, whatever the request. You are the best secretary around. **Karina Jernsand,** former student in our group**,** for help with laboratory work. I really appreciated it! I also want to wish good luck to our new group member **Anna**.

Thanks to **Michael Horn** at the Center for Mouse Physiology and Bioimaging for running the MRI very smoothly and the Microarray Resource Centre at Lund University for help with microarray lab work.

In addition, I want to thank all my former colleagues at KK Speclab and present colleagues at Lundberg Laboratory for Cancer Research for creating a pleasant atmosphere, for your assistance and all nice parties, lunches and coffee breaks! Special thanks to **Ulric** for expert help with the really nice illustrations in my thesis. I also like to thank **Mattias** for excellent "coaching" and comments on the text and **Tom** for a nice and fun collaboration in our left hand project.

THANK YOU!

Elin and Anna-Stina, two of my closest friends from the youth. You are the best of friends and I promise I will call you more often once this is over!

All my wonderful **Friends**. Thank you for always being there for me. I am so glad I have you all.

Tack **Morfar** för att du alltid är intresserad av hur det går för mig och vad jag forskar om.

Mum and **Dad**. Thank you for everything you have done for me. You are always so supportive and believe in me and always helping out whatever the issue. I really appreciate it. You are the best parents I can wish for! My best "Lilla Syster Yster **Anna**" for always being there when there is trouble and problems. I am so glad I have you!

Min **Göran**, for endless support, for scheduling my work hours and for all distraction! You are the best and I love you very much!

We also wish to acknowledge grant support from the Swedish Cancer Society, Sahlgrenska University Hospital, The Swedish Johanniterhjälpen, Prostatacancerförbundet, The Swedish Medical Society, Research Foundations of af Jochnick, C. & S. Hagströmer, M. & B. Gustavsson, G. Nilsson, A. Gabrielsson, M. Bergvall, Å. Wiberg, H. Fries and P. Falk.

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