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ORTHOTOPIC PC-3 TUMOR XENOGRAFTS IN STUDIES ON PROSTATE CANCER GROWTH AND METASTASIS

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To Oskari and Aaro

ABSTRACT

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Prostate cancer is generally a slowly developing disease. However, some cancers develop into an aggressive, metastasic and consequently life-threatening state. The mechanisms of prostate cancer spread are still mainly unidentified but hormones and growth factors are known to been involved. The forming of new blood vessels *i.e.* angiogenesis is crucial for tumor growth. Blood vessels and lymphatic vessels are also prominent routes for metastasis. Both angiogenic and lymphangiogenic factors are overexpressed in prostate cancer. We established an *in vivo* model to study the factors effecting human prostate cancer growth and metastasis. Tumors were produced by the orthotopic inoculation of PC-3 prostate cancer cells into the prostates of immunodeficient mice. Like human prostate tumors, these tumors metastasized to prostate-draining lymph nodes. Treatment of the mice with the bisphosphonate alendronate known to decrease prostate cancer cell invasion *in vitro* inhibited metastasis and decreased tumor growth. Decreased tumor growth was associated with decreased angiogenesis and increased apoptosis of tumor cells. To elucidate the role of angiogenesis in prostate cancer progression, we studied the growth of orthotopic PC-3 tumors overexpressing fibroblast growth factor b (FGF8b) known to be expressed in human prostate cancer. FGF8b increased tumor growth and angiogenesis, which were both associated with a characteristic gene expression pattern. To study the role of lymphangiogenesis, we produced orthotopic PC-3 tumors overexpressing vascular endothelial growth factor C (VEGF-C). Blocking of VEGF-C receptor (VEGFR3) completely inhibited lymph node metastasis whereas overexpression of VEGF-C increased tumor growth and angiogenesis. VEGF-C also increased lung metastases but, surprisingly, decreased spread to lymph nodes. This suggests that the expanded vascular network was primarily used as a route for tumor spreading. Finally, the functionality of the capillary network in subcutaneous FGF8b-overexpressing PC-3 tumors was compared to that of tumors overexpressing VEGF. Both tumors showed angiogenic morphology and grew faster than control tumors. However, FGF8b tumors were hypoxic and their perfusion and oxygenation was poor compared with VEGF tumors. This suggests that the growth advantage of FGF8b tumors is more likely due to stimulated proliferation than effective angiogenesis. In conclusion, these results show that orthotopic prostate tumors provide a useful model to explore the mechanisms of prostate cancer growth and metastasis.

Keywords: angiogenesis, FGF, hypoxia, lymphangiogenesis, metastasis, orthotopic, prostate cancer, VEGF

YHTEENVETO

ORTOTOOPPISET PC-3 –KASVAIMET ETURAUHASSYÖVÄN KASVUN JA LEVIÄMISEN TUTKIMISESSA

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Eturauhassyöpä kehittyy yleensä hitaasti. Jotkut syövistä muuttuvat kuitenkin aggressiivisiksi ja etäpesäkkeitä muodostaviksi, jolloin kyseessä on hengenvaarallinen sairaus. Eturauhassyövän leviämisen mekanismit tunnetaan huonosti, mutta hormonien ja useiden kasvutekijöiden tiedetään vaikuttavan niihin. Verisuonten uudismuodostus eli angiogeneesi on välttämätön syövän kasvulle. Lisäksi veri- ja imusuonet ovat tärkeitä etäpesäkkeiden muodostumisreittejä. Eturauhassyöpäsolut tuottavat runsaasti sekä angiogeenisiä että lymfangiogeenisiä (imusuonia uudismuodostavia) kasvutekijöitä. Kehitimme tässä väitöskirjatyössä mallin, jonka avulla ihmisen eturauhassyöpää voidaan tutkia immunopuutteisissa hiirissä. Tuotimme kasvaimet istuttamalla PC-3-soluja ortotooppisesti hiirien eturauhasiin, joissa ne muodostivat paikallisiin imusolmukkeisiin etäpesäkkeitä lähettäviä kasvaimia. Kun hiirille annosteltiin syöpäsolujen invaasiota estävää alendronaattia, kasvainten ja etäpesäkkeiden kasvu hidastui. Tämä johtui todennäköisesti kasvaimen vähentyneestä angiogeneesistä ja lisääntyneestä apoptoosista. Ihmisen eturauhassyöpäsolujen tiedetään tuottavan fibroblastikasvutekijä 8b:tä (FGF8b). Istutimme hiiriin ortotooppisesti PC-3 soluja, jotka yli-ilmensivät FGF8b:tä. FGF8b lisäsi kasvainten angiogeneesiä ja kasvua kontrolliin verrattuna. Lisäksi FGF8b lisäsi solunjakaantumiseen, angiogeneesiin ja etäpesäkkeiden muodostumiseen liittyvien geenien ilmentymistä. Tutkimme lymfangiogeenisiä vaikutuksia samankaltaisella ortotooppisella kasvainmallilla, jossa PC-3 solut yli-ilmensivät endoteelikasvutekijä C:tä (VEGF-C). Kun estimme VEGF-C:n reseptorin (VEGFR3) toiminnan, etäpesäkkeiden muodostuminen estyi kokonaan. Myös VEGF-C:n ylituotanto lisäsi kasvainten kasvua ja verisuonitusta. Samalla kun kasvainperäinen VEGF-C lisäsi etäpesäkkeiden muodostumista keuhkoihin, se vähensi yllättäen niiden muodostumista imusolmukkeisiin. Tämä viittaa siihen, että etäpesäkkeet levisivät pääasiallisesti kasvainten tiheän kapillaariverkoston kautta. Vertasimme FGF8b:tä yli-ilmentävien ihonalaisten kasvaimien ominaisuuksia VEGF:ää yli-ilmentäviin kasvaimiin. Kasvaimet, jotka tuottivat FGF8b:tä tai VEGF:ää kasvoivat kontrollikasvaimia nopeammin, mutta FGF8b-kasvaimet olivat huonosti hapetettuja eli hypoksisia. Tämä viittaa siihen, että FGF8b:n aikaansaama kasvuetu johtuu pääasiassa sen solunjakautumista kiihdyttävistä eikä sen angiogeneesiä lisäävistä ominaisuuksista. Nämä tulokset osoittavat, että ortotooppinen prostatasyöpämalli on käyttökelpoinen työkalu eturauhassyövän kasvun ja etäpesäkkeiden muodostumisen mekanismien tutkimiseen.

Avainsanat: angiogeneesi, eturauhassyöpä, etäpesäke, FGF, hypoksia, lymfangiogeneesi, ortotooppinen, VEGF

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ABBREVIATIONS

AdlacZ-Ig Recombinant adenovirus expressing β-galactosidase
AdVEGFR3-Ig Recombinant adenovirus expressing VEGFR3-Ig fusion protein
AR Androgen receptor
BPH Benign prostatic hyperplasia
CDKN1B Cyclin-dependent kinase inhibitor 1B
CT Computed tomography
CYP17 Cytochrome P450 enzyme
DHT Dihydrotestosterone
DKK-1 Dickkopf homolog 1
DMEM Dulbecco's Modified Eagle's Medium
EF5 Pentafluoropropyl-acetamide
EGF Epidermal growth factor
ER Estrogen receptor
ERK Extracellular signal-regulated kinases
FAZA Fluoroazomycinarabino-furanoside
FBS Fetal bovine serum
FDG Fluorodeoxyglucose
FDG-6-P FDG-6-phosphate
FETNIM Fluoroerythronitroimidazole
FGF Fibroblast growth factor
FGFR Fibroblast growth factor receptor
FMISO Fluoromisonimidatzole
GLUT Glucose transporter
GO Gene ontology
GSTP Glutathione transferase
GTP Guanosine triphosphate
H&E Hematoxylin-eosin
HG-PIN High grade PIN
HIF Hypoxia inducible factor
HPC Hereditary prostate cancer
HRPC Hormone resistant prostate cancer
IGF Insulin-like growth factor
IPA Ingenuity pathway analysis
ISEL *In situ* end labeling
KO Knock-out
LG-PIN Low grade PIN
MAPK Mitogen activated protein kinase
MEK Mitogen activated protein kinase
MMP Matrix metalloproteinase
MRI Magnetic resonance imaging
NO Nitric oxide
OPN Osteopontin, also known as **SPP1**
PBS Phosphate buffered saline
PC-3 Prostate cancer cell line
PDGF Platelet-derived growth factor
PET Positron emission tomography
PI3-K Phosphatidyl inositol 3-kinase
PI Propidium iodide
PIA Proliferative inflammatory atrophy
PIGF Placental growth factor
PIN Prostatic intraepithelial neoplasia
pO_2 Oxygen partial pressure
PSA Prostate-specific antigen
PTEN Phosphatase and tensin homologue
pVHL von Hippel-Lindau factor protein
RNASEL Ribonuclease L
RTK Receptor tyrosine kinase
RT-PCR Reverse transcription polymerase chain reaction
RT-qPCR Quantitative, reverse transcription, real-time polymerase chain reaction
s.c. Subcutaneous
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPECT Single photon emission computed tomography
SPARC Osteonectin
SPP1 Secreted phosphoprotein 1, also known as osteopontin
sVEGFR Soluble VEGFR
svVEGF Snake venom VEGF
T/B Tumor/Blood
TFsvVEGF *Trimeresurus flavoviridis* snake venom VEGF
TGF Transforming growth factor
TRAMP Transgenic adenocarcinoma mouse prostate
VEGF Vascular endothelial growth factor
VEGF-B-F Vascular endothelial growth factor B-F
VEGFR VEGF receptor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numbers I-IV:

I Johanna M. Tuomela, Maija P. Valta, H. Kalervo Väänänen and Pirkko L. Härkönen: Alendronate decreases orthotopic PC-3 prostate tumor growth and metastasis to prostate-draining lymph nodes in nude mice. BMC Cancer 2008 28;8:81.

II Johanna Tuomela, Maija Valta, Jani Seppänen, Kati Tarkkonen, H. Kalervo Väänänen and Pirkko Härkönen: Overexpression of vascular endothelial growth factor C increases growth and alters the metastatic pattern of orthotopic PC-3 prostate tumors. BMC Cancer 2009 12:9:362.

III Maija P.Valta, Johanna Tuomela, Heikki Vuorikoski, Niina Loponen, Riina-Minna Väänänen, Kim Pettersson, H. Kalervo Väänänen, and Pirkko L. Härkönen: FGF-8b induces growth and rich vascularisation in an orthotopic PC-3 model of prostate cancer. J Cellular Biochemistry 2009 1;107(4):769-84.

IV Johanna Tuomela, Tove J. Grönroos, Maija P. Valta, Jouko Sandholm, Aleksi Schrey, Jani Seppänen, Päivi Marjamäki, Sarita Forsback, Ilpo Kinnunen, Olof Solin, Heikki Minn and Pirkko L. Härkönen: Fibroblast growth factor 8b (FGF-8b) induces aberrant vasculature and poor oxygenation in fast growing PC-3 prostate tumour xenografts. Submitted.

1. INTRODUCTION

The prostate is a male secretory organ, which normally produces 20% of the seminal fluid. During ejaculation, the prostate secretes citric acid, prostaglandins, spermine and other basic molecules into the urethra to neutralize seminal fluid and to keep the sperm mobile. Finally, it protects seminal fluid from the acidic secretions of the vagina. The prostate typically enlarges with age. Benign prostatic hyperplasia BPH represents an inescapable phenomenon for the aging population. As well, lower urinary tract symptoms (LUTS) are associated with aging. The lifetime risk of prostate cancer is approximately 10% for men in Western countries. Progression of prostate cancer is a multistep process and its mechanisms are poorly understood. Currently, there is no curative treatment for the metastatic disease.

Androgens are essential for the maintenance of the normal morphology and function of the prostate. Androgens control the growth and secretions of the prostate gland, but they also have a central role in the development of BPH and prostate cancer. The effects of androgens are mediated via paracrine and autocrine molecules, which include growth factors and their receptors. Important counterparts in prostate cancer progression are angiogenic growth factors, which induce the sprouting of new capillaries to ensure the availability of oxygen, glucose and nutrients for the rapidly growing tumors (Ferrara 2005). Angiogenesis is necessary for normal development and tissue repair as well as for pathological conditions such as tumor growth (Folkman 1971). Tumor metastasis is also dependent on angiogenesis. Tumor angiogenesis is often uncontrolled resulting in chaotic and heterogeneous vascularization, which may lead to metabolic changes and decreased O_2 and glucose concentrations. Consequently, hypoxia is a common feature of solid tumors. As early as 1909, hypoxic tumors were reported to be more resistant to radiotherapy (Schwarz 1909) and are known to be more resistant to chemotherapeutic drugs as reviewed by Teicher (Teicher 2009). Hypoxia also affects the aggressiveness of tumors and therefore an increasing interest has developed in hypoxia imaging, in order to recognize hypoxic tumors from well-oxygenated ones (Pennacchietti et al. 2003, Jokilehto et al. 2006).

Vascular endothelial growth factor (VEGF) is essential for vasculogenesis and angiogenesis. VEGF stimulates endothelial cell proliferation, migration and the formation of capillaries. It also increases vascular permability and causes vasodilation and therefore plays an important role in tumorigenesis. A lymphangiogenic member of the VEGF family, VEGF-C, also stimulates endothelial cells and induces angiogenesis through VEGFR2 (Joukov et al. 1996, Cao et al. 2003, Bauer et al. 2005). The lymphangiogenic effects of VEGF-C are mediated through VEGFR3 (Joukov et al. 1996) and are crucial in embryogenesis (Kärkkäinen et al. 2004, Kukk et al. 1996). VEGF-C is overexpressed in several human cancers and its expression correlates with the increased density of lymphatic vessels, lymphatic invasion and metastasis in lymph nodes (Kurebayashi et al. 1999, Nakamura et al. 2005, He, Kärpänen & Alitalo 2004, Choi et al. 2005).

Fibroblast growth factors (FGFs) play an important role in the development of hormonal cancers (Dorkin et al. 1999, Marsh et al. 1999, Valve et al. 2001). In healthy adults, low expression of FGF8 is found mainly in reproductive organs (Marsh et al. 1999, Valve et al. 1997) but during embryogenesis FGF8 has an important role in gastrulation, development of the nervous system, the heart, skeleton and limbs (Crossley, Martin 1995, Mahmood et al. 1995, Sun et al. 1999). FGF8b has found to be the most malignant of the four isoforms found in humans (MacArthur et al. 1995b).

The present study was undertaken to investigate the regulation of prostate cancer growth and metastasis in a relevant environment using an orthotopic prostate tumor model. Such a model allows the study of the epithelial-stromal-cancer cell crosstalk, which is important in the signaling of both VEGFs and FGFs. Furthermore, the orthotopic tumor model was used in studies to analyze the effects of the nitrogen-containing bisphosphonate alendronate on prostate tumor growth and metastasis. All tumors and metastases were evaluated by histomorphometrical and immunohistochemical methods. The gene expression changes associated with ectopic overexpression of FGF8b in PC-3 cells and orthotopic tumors were studied using microarray analysis. In addition, the effects of VEGF and FGF8b on the tumor microenvironment were studied using autoradiography, partial oxygen pressure measurements, flow cytometry and by measuring the uptake of [^{18}F]-labeled markers.

2. REVIEW OF THE LITERATURE

2.1. Prostate cancer

2.1.1. Ethnic and dietary factors in prostate cancer

Every year adenocarcinoma of the prostate is diagnosed in 500,000 men worldwide (Grönberg 2003). In developed countries, prostate cancer is the most frequently diagnosed cancer and after lung cancer the second most frequent cause of cancer-related death among males (Damber, Aus 2008, Jemal et al. 2008). The incidence of prostate cancer is different in every continent and varies between ethnic populations (Bray et al. 2002). A part of this variation can be explained by differences in the quality of health care systems, cancer registration and the different age distributions, but also true differences in the incidence exist. According to the Finnish Cancer Registry, Finnish men have the highest risk of prostate cancer in Europe (95.3 new cases per 100,000 men). The number of new diagnoses in Finland was 4,198 during 2007. In the same year, the number of deaths due to prostate cancer was 792. The highest cancer incidence rate in the world is among African-American men in the USA (Henderson et al. 1988, Mucci, Spiegelman 2008) and the lowest among Chinese men in Asia (Wilt, Thompson 2006). These rates also parallel other prostate disorders, such as BPH, PIN (Prostatic Intraepithelial Neoplasia) and prostatic inflammation *i.e.* prostatitis (Dennis, Lynch & Torner 2002). The cause of the variation is unknown and appears to be affected by several factors, such as diet, environment, endocrine factors or genetics (Parkin, Bray & Devesa 2001). Approximately 30-50% of prostate cancer patients have evidence of metastatic disease, which causes high morbidity and mortality. In advanced prostate cancer, 25-42% of the patients have metastases in para-aortic, prostate draining lymph nodes (Bader et al. 2003) and 60-90% have metastases in bone (Jacobs 1983).

There are encouraging studies that suggest that prostate cancer can be prevented by modifications of lifestyle (for a review, see Jayachandran, Freedland 2008). Avoiding red meat, fat and excessive consumption of dairy products reduced the risk of prostate cancer (Parkin, Bray & Devesa 2001). Inadequate uptake of vitamin D is considered to be a risk factor for prostate cancer (Mucci, Spiegelman 2008). Increasing the uptake of antioxidants and flavonoids and especially selenium, lycopene, vitamin E, rye brans and soy (Jayachandran, Freedland 2008, Bylund et al. 2005, Smit et al. 2007) have also been shown to have a beneficial impact on prostate cancer prevention and even on the disease progression. However, the latest results from the SELECT study have been disappointing showing no effects of antioxidants on prostate cancer (Gaziano et al. 2009, Lippman et al. 2009).

2.1.2. Somatic gene defects and hereditary factors in prostate cancer

The malignant changes in human prostate cancer cells include both genetic and epigenetic alterations. During prostate cancer progression, epigenetic changes arise

earlier than genetic defects (Nelson, De Marzo & Yegnasubramanian 2009). The most common genetic defects are chromosomal translocations leading to the fusion between the androgen-regulated gene (TMPRSS2, Transmembrane serine protease 2) and genes from the ETS (E-twenty six) family (most commonly the ERG, ETS Related Gene), which result in defects in androgen signaling and in a malignant phenotype (Tomlins et al. 2005, Cerveira et al. 2006). Whether epigenetic changes promote the appearance of TMPRSS2-ETS family fusion transcripts, or collaborate with fusion transcript expression in the pathogenesis of prostate cancer, has not been established. Recent data show that the appearance of the fusion gene is relevant in certain stages of prostate cancer but disappears later (Hermans et al. 2006, Saramäki et al. 2008). Unlike many other cancers, only a small percentage of prostate cancers are inherited. There are a few chromosomal loci which are associated with hereditary prostate cancer (Elo, Visakorpi 2001, Langeberg, Isaacs & Stanford 2007). These include 1q24-25 (HPC1 locus) with mutations in the endoribonuclease RNase L gene (RNASEL) and 17p11 the 3' processing endoribonuclease HPC2/ELAC2 (Rökman et al. 2001, Rökman et al. 2001, Rökman et al. 2002, Tavtigian et al. 2001, Nupponen et al. 2004). Chromosome 8 is often a target for genetic alterations in prostate cancer, with loss in 8p and gain in 8q. The Myc oncogene and PSCA (prostatic stem cell antigen) are both located at chromosome 8 (8q24.1 and 8q24.2, respectively). Both are amplified in prostate cancer, especially in poorly differentiated tumors and metastatic disease (Nupponen et al. 1998). The macrophage scavenger receptor 1 gene (MSRI), which is considered as a risk gene of prostate cancer is also located in chromosome 8 (8p22) (Nupponen et al. 2004, Nupponen et al. 2004, Xu et al. 2002).

Two major types of genetic alterations occur in the androgen receptor (AR) in hormone-resistant prostate cancer (HRPC). There are both somatic mutations and genomic amplifications (Dong 2006). The AR gene has been shown to be amplified in 80% of hormone-refractory cancers (Trapman, Brinkmann 1996, Linja et al. 2001). Amplification and overexpression of AR has been shown to be associated with advanced disease (Newmark et al. 1992, Veldscholte et al. 1990, Wallen et al. 1999, Zhao et al. 2000, Koivisto et al. 1999). Overexpression of AR is linked to gene amplification but it may also occur independently (Koivisto et al. 1999). Androgen withdrawal as a treatment of prostate cancer overcomes the disease for some time but it has been suggested that the AR gene amplificates which may eventually cause androgen hypersensitivity (Palmberg et al. 2000).

PTEN (phosphatase and tensin homologue) is a tumor suppressor gene, which normally inhibits the PI3K-Akt (phosphatidylinositol 3'kinase-protein kinase B) signaling pathway and affects cell-cycle progression and cell survival. In prostate cancer, the PTEN gene is often mutated or lost (Porkka, Visakorpi 2004, Wang, Parsons & Ittmann 1998). Reduced levels of PTEN have been shown to correlate with the grade and stage of advanced disease (McMenamin et al. 1999, Li et al. 1997).

NKX3.1 functions as a homeobox gene and is essential in organogenesis as well as maintaining normal prostate epithelium (Bhatia-Gaur et al. 1999, Bowen et al. 2000).

NKX3.1 is a prostate-specific androgen-regulated gene, which has frequently been deleted in early prostate cancer (He et al. 1997, Asatiani et al. 2005, Gelmann, Bowen & Bubendorf 2003). NKX3.1 plays distinct roles in the initiation and progression of PIN lesions. NKX3.1 is a candidate prostate tumor suppressor gene that demonstrates haploinsufficiency. According to studies on transgenic mice, disruption of one or both copies of the murine NKX3.1 gene leads to the development of epithelial hyperplasia and PIN. This appears to be a consequence of delayed exit from the cell cycle by differentiating prostate luminal epithelial cells in NKX3.1 mutant mice. Gene expression profiling has provided additional insight into the basis of haploinsufficiency in NKX3.1 mutant mice. A reduction in NKX3.1 dosage leads to dramatic alterations in the expression of a subset of genes by altering the probability of a target gene existing in the "on" or "off" state (Kim et al. 2002).

CDKN1B (Cyclin-dependent kinase inhibitor 1B) is located at chromosome 12p12-13. It codes protein p27 that acts as a cyclin-independent kinase inhibitor (De Marzo et al. 1998). In high-grade PIN (HG-PIN) and prostate cancer, CDKN1B may be inactivated or lost, which correlates with poor prognosis. Inactivation of CDKN1B is also correlated with loss of PTEN expression (Cote et al. 1998, Halvorsen, Haukaas & Akslen 2003) and increased proliferation (Halvorsen, Haukaas & Akslen 2003).

GSTPs (Glutathione S transferases) are enzymes that protect prostate cells against exposure to environmental carcinogenesis and oxidative stress. GSTP1 protein is absent in over 90% of prostate cancers (Katoh et al. 2008, Nakayama et al. 2004) and also 70% of HG-PIN lesions (Nakayama et al. 2004). The genetic aberration is also found in 6% of PIA (proliferative inflammatory atrophy) but it is not detected in benign prostatic hyperplasia or normal prostatic tissue (Nakayama et al. 2003). Its transcription is inhibited by the hypermethylation of CpG island sequences in the GSTP gene (Brooks et al. 1998, Lee et al. 1994).

2.1.3. Natural history of prostate cancer

Prostatatic diseases are heterogeneous both in clinical behavior and morphology (Miller, Torkko 2001). The most significant benign condition of the prostate is benign prostatic hyperplasia (BPH). BPH often occurs in the transition zone of the prostate, while prostate cancer usually occurs in the peripheral zone (McNeal 1984). Other common conditions of the prostate are acute or chronic inflammation (Nickel, Nyberg & Hennenfent 1999). Prevalence of chronic nonbacterial prostatitis is from 2% to 16%. The main symptoms are chronic pelvic pain and voiding dysfunctions, which have an impact on quality of life (Nickel et al. 2005). The etiology of prostatic inflammation usually remains unknown (Mehik et al. 2000). Chronic inflammation may induce an increased growth of fibromuscular tissue and cause BPH. However, the concept that inflammation can promote prostate cancer is still highly speculative (Sciarra et al. 2008). PIN is characterized as a growth pattern of prostate epithelial cells. It has been considered as a putative precursor of prostate cancer due to its

physical and temporal association with carcinoma. In low-grade PIN (LG-PIN), the epithelial cells grow in multiple layers projecting into the lumen of the gland, but the basal cell layer is still intact. In high grade PIN (HG-PIN) the basal layer has been described as discontinued (Cheng et al. 1998). HG-PIN resembles differentiated adenocarcinoma, and therefore, they are difficult to separate from small cancer lesions. The majority of prostate cancers are adenocarcinomas. The molecular mechanisms behind invasive prostate cancer and its connections to other transformations in the prostate are still largely unknown.

The rate of latent carcinomas found in autopsies from patients who died due to other reasons than cancer increases by age (Damber, Aus 2008, Sakr et al. 1994). Microscopic lesions are found 70-80% of men over 80 years. At diagnosis, prostate cancer is most often restricted to the prostate. Prostate cancer can be screened by means of the PSA (prostate specific antigen) test but the actual cancer diagnosis is based on examination of a histological or cytological biopsy sample. The most common method to predict the behaviour of adenocarcinoma of the prostate is the Gleason score (Gleason, Mellinger 1974). In this grading system the samples are histologically assessed according to the morphology and organization of the prostatic glands and distributed from 2 to 10, where 2 represents the least aggressive form. The Gleason score is the sum of the two most common characteristics of tumor histology (grades 1-5). Clinically, the stage of prostate cancer is also displayed by the WHO's TNM (tumor, node, metastasis) classification system, where TX-T4 represents tumor occurrence and size, NX-N1 means metastasis in regional lymph nodes and MX-M1 metastasis in distant organs.

2.1.4. Treatment of prostate cancer

Gleason scoring of prostate cancer is used in clinical decision-making about the prostate cancer treatment mode (Egevad et al. 2002). The localized stage is the most common stage of prostate cancer. Early stage prostate cancer can be successfully treated with surgical prostatectomy, radiotherapy or it may be left under watchful waiting. Only 10% of prostate cancers develop to clinical disease and a recommendation is that if a man has a life expectancy of less than 10 years and if the tumor is localized within the prostate, careful monitoring is the first choice of treatment (Damber, Aus 2008). Younger men with poorly differentiated prostate cancers are treated more intensively (Johansson et al. 2004). Clinicians have to estimate the impact of the treatment, and sometimes even very old men benefit from treatment. However, it has been demonstrated that radical prostatectomy is the only effective treatment apart from watchful waiting. However, 17 men have to be operated to avoid one relapse (Damber, Aus 2008, Wong et al. 2006).

If the tumor cells have invaded outside the prostatic capsule, androgen ablation therapy can be used to prevent tumor growth and further spread. Androgen ablation therapy has been used for over 60 years (Huggins 1967, Huggins, Hodges 2002) and it

is often combined with radiotherapy to relieve pain in advanced disease. Testicular androgen can be removed by orchiectomy, inhibition of pituitary secretion of luteinazing hormone (LH) or follicle stimulating hormone (FSH), or administration of estrogens to reduce the secretion of gonadotropin-releasing hormone (GnRH) of the hypothalamus. The effects of androgens in the prostate can also be blocked using antiandrogens. Most (70-80%) patients treated with hormone therapy get relief to their symptoms. However, side effects and toxic effects are common, although mild compared with other cancer therapies (Damber, Aus 2008).

The effects of hormone therapy may last number of years and improve the quality of life and survival of prostate cancer patients. Eventually, metastatic prostate cancer develops to the final, hormone-independent stage (Isaacs 1999), for which there is no cure available and only palliative therapy can be given. Bone pain may be relieved using bisphosphonate treatment. Until recently, chemotherapy has not been considered as an effective treatment against prostate cancer but recent studies of docetaxel alone or combined with estramustine improved survival of patients with hormone-independent cancer (Fizazi et al. 2007, Petrylak 2006).

Moreover, new drugs such as inhibitors of angiogenesis, and signal transductors, or vaccines against prostate cancer are under investigation. Recently, promising effects have been gained using chemoprevention of prostate cancer by means of 5α-reductase inhibitor (Arena 2008, Musquera et al. 2008). Abiraterone acetate therapy based on new CYP17 inhibitor also showed promising results in clinical trials (Attard et al. 2009, Attard et al. 2008, Yap et al. 2008). Cancer stem cells have received much attention as a possible new drug target (Maitland, Collins 2008, Kasper 2008). Thus, the results are very interesting, although targeted therapy options have not proven to have significant effects on survival.

2.2. Regulation of prostate cancer growth

2.2.1. Hormone responsiveness and hormone regulation

Development and growth of the prostate gland as well as prostate carcinogenesis are dependent on androgens (Huggins 1967). During development, the primary target for androgens is the urogenital mesenchyme, which directs budding and branching of the epithelium through paracrine mediators (Sugimura, Cunha & Donjacour 1986). Conversely, the developing epithelium induces differentiation and the organization of the stromal components, so the interaction between the epithelium and mesenchyme is bidirectional (Cunha 2008). The secretion of testosterone is regulated with a negative feedback loop. The production is regulated by the hypothalamus, which secretes GnRH. GnRH induces LH secretion of the pituitary gland, which thereafter gives a signal to Leydig cells in the testis to synthesize testosterone. A small portion of androgens originates from the adrenal cortex. In the prostate, testosterone is converted to dihydrotestosterone (DHT) by 5α-reductase entzyme. Both DHT and testosterone bind and activate AR but DHT binds with higher

affinity. AR is expressed both in the mesenchymal and epithelial cells of the prostate. Ligand-binding to AR induces conformational changes in the AR-chaperone complex. AR is dissociated from the chaperone proteins and transported to the nucleus, where it binds the androgen responsive element (ARE) of DNA and initiates transcription (Rau et al. 2005). Androgens increase the proliferation of androgen-dependent prostate cancer cells. However, mutations in AR may lead to androgen independent prostate cancer, where mutant AR may respond to a very low concentration of androgens (Culig et al. 2003, Palmberg et al. 2000).

The developing prostate is also sensitive to other hormones, including estrogens. Estrogens were previously used in the treatment of prostate cancer, until less cardiovascularly toxic compounds were found. Furthermore, the effects of exogenous estrogen treatment are still controversial. Interestingly, exposure to low doses of estrogen during gestation in the mouse has been reported to increase adult prostate weight and androgen receptor levels, as well as significantly increasing prostatic glandular budding (vom Saal et al. 1997). On the other hand, higher doses of estrogen resulted in a permanent suppression of prostate growth, a reduced response to androgens, and an induction of epithelial hyperplasia in adulthood (Härkönen, Mäkelä 2004). It has been shown that reduced responsiveness to androgens is related to a decrease in AR expression and may be associated with lower serum androgen levels. These data suggested that the effects of estrogens mediate changes in androgen levels via suppression of the hypothalamic-pituitary-gonadal axis (Jarred et al. 2000). In addition, estrogens also have direct antiandrogenic effects on the prostate (Martikainen et al. 1987). Estrogen acts through receptors (ER) α and β. ERα gene is expressed in prostate cancer cells suggesting that estrogen may have a role in prostate carcinogenesis (Schulze, Barrack 1987). ERβ is expressed in prostate epithelium (Kuiper et al. 1997), where it may exert an antiprolifrative and immunoprotective role in the prostate perhaps limiting tissue damage or modulating expression of stimulus for immune cell filtration (for review, see Prins, Korach 2007). Differential expression of estrogen receptors, alfa and beta and the ability to metabolize estrogens may be critical in defining how various estrogenic or anestrogenic compounds modulate prostate carcinognesis.

2.2.2. Growth factor and cytokine regulation

The critical role played by stroma-epithelium crosstalk in carcinogenesis and progression of prostate cancer has been increasingly recognized. These interactions are mediated by a variety of paracrine factors secreted by cancer cells and/or stromal cells (Niu, Xia 2009). The major prostatic growth factor families include the FGF family (Kwabi-Addo, Ozen & Ittmann 2004), the VEGF family (Nicholson, Theodorescu 2004), transforming growth factor-β (TGF-β) (Zhu, Kyprianou 2005), the insulin-like growth factor (IGF) family (Gennigens, Menetrier-Caux & Droz 2006) and the epidermal growth factor (EGF) family (Mimeault, Pommery & Henichart 2003).

2.2.2.1. Fibroblast growth factors and their receptors

Fibroblast growth factors (FGFs) comprise a large family of signaling molecules with various functions in development as well as in adult physiology (Ornitz, Itoh 2001, Eswarakumar, Lax & Schlessinger 2005). FGFs have also been associated with a wide variety of malignancies, including prostate cancer. The human FGF gene family consists of at least 23 different genes that share sequence similarity. FGFs mediate their cellular function through binding to and activating the family of high-affinity tyrosine kinase receptors (FGFRs). Currently, five distinct FGFRs have been identified (FGFR1 to FGFR5), although most FGF actions are mediated through receptors 1-4 (Powers, McLeskey & Wellstein 2000, Sleeman et al. 2001, Schlessinger et al. 2000). The tyrosine kinase receptors are transmembrane proteins, consisting of an extracellural ligand-binding domain with three immunoglobulin-like domains (Schlessinger et al. 2000) and an intracellular tyrosine kinase domain (Johnson, Williams 1993, Hunter et al. 2007). FGFRs 1-3 undergo alternative splicing with two alternative exons (IIIb and IIIc) (Ornitz et al. 1996), where isoform b is found in epithelial cells and isoform c predominantly in mesenchymal tissue (Orr-Urtreger et al. 1993). FGF binding to heparin-sulphate proteoglycans induces FGFR dimerization and phosphorylation of receptor subunits, which leads to biological responses (Powers, McLeskey & Wellstein 2000). FGFs also bind to heparin and heparin-sulphate proteoglycans with low-affinity without binding to the receptor but do not transmit biological signals through them alone (Powers, McLeskey & Wellstein 2000).

2.2.2.2. Fibroblast growth factors in the prostate

FGF and androgen signaling are important in prostatic development. Prostatic growth and development are regulated by the action of androgens in the mesenchyme. FGF7 and FGF10 have been shown to mediate the effects of androgens and to be crucial factors in normal prostate development (Cronauer et al. 2003, Lu et al. 1999, Pu et al. 2007).

In healthy human adults, prostate epithelial cells express low levels of FGF6 (Ropiquet et al. 2000), FGF9 (Jin et al. 2004), and FGF17 (Polnaszek et al. 2004). Using RT-qPCR, FGF1, FGF5, FGF8 and FGF13 have also been detected (Valve et al. 2001, Kwabi-Addo, Ozen & Ittmann 2004). Prostate stromal cells express FGF2, FGF7 and FGF9 and also small quantities of FGF10 (Ropiquet et al. 2000, Kwabi-Addo, Ozen & Ittmann 2004, Giri, Ropiquet & Ittmann 1999). Normal human prostate stromal cells express FGFR2 IIIc and epithelial cells express FGFR1 IIIc, FGFR2 IIIb, FGFR3 IIIb and FGFR4 (Cotton et al. 2008). In the normal prostate, homeostasis is maintained mainly by crosstalk of stromal FGF7 and FGF10 and epithelial FGFR2 IIb or between epithelial FGF9 and stromal FGF3. Changes in expression of FGFs and their receptors lead to disruption of homeostasis and may be associated with prostatic

lesions (Cotton, O'Bryan & Hinton 2008). FGF1 (acidic FGF), FGF2 (basic FGF), FGF6, FGF8 and FGF17 are all expressed at increased levels in prostate cancer as paracrine and/or autocrine growth factors to prostate cancer cells (Valve et al. 2001, Kwabi-Addo, Ozen & Ittmann 2004, Presta et al. 2005, Heer et al. 2004). An isoform switch from FGFR2 IIIc to FGFR2 IIIb may also lead to malignant growth (Kwabi-Addo et al. 2001).

2.2.2.3. Fibroblast growth factor 8 in development and in prostate cancer

FGF8 was originally cloned from the Shionogi mouse mammary tumor-derived SC-3 cell line as an androgen-induced growth factor (AIGF) (Tanaka et al. 1998). FGF8 is essential in development, especially in the gastrulation, somitogenesis and morphogenesis of the nervous system, pharyngeal tissue, cardiac structures, limbs, kidney and male reproductive tissue, including the prostate. Its expression in adult tissues is limited to steroid hormone-targeted tissues like reproductive tissue and the genitourinary tract. Various immunohistochemical, RNA and *in situ* hybridization analyses of clinical samples have demonstrated increased expression of FGF8 in prostate cancer epithelium but none or little in normal prostate (Dorkin et al. 1999, Valve et al. 2001, Tanaka et al. 1998).

Alternative splicing of the human FGF8 gene allows transcription of four different isoforms designated FGF8a, FGF8b, FGF8e and FGF8f (Ghosh et al. 1996), and of these, FGF8b has been proposed to be the major isoform expressed in prostate cancer. The expression of FGF8b correlates with the stage and grade of the disease (Gnanapragasam et al. 2003).

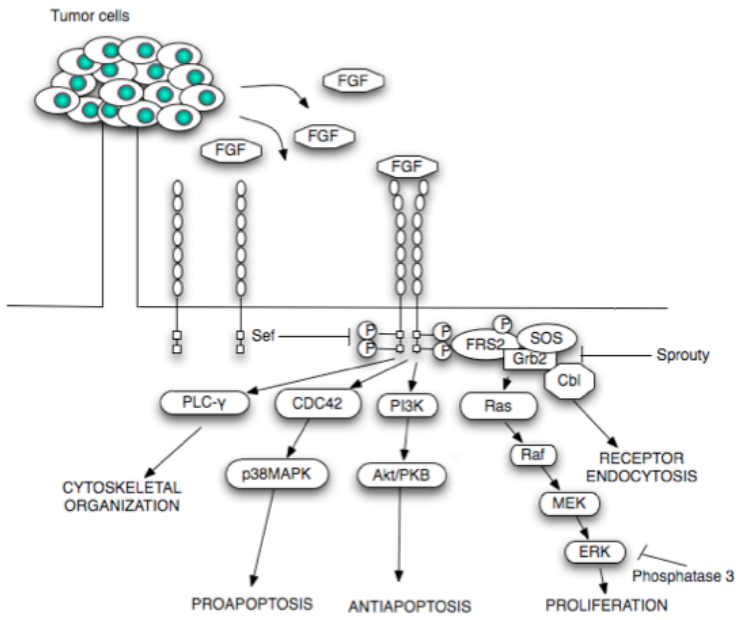
It has been suggested that FGF8 is regulated by androgens in prostate cancer (Gnanapragasam et al. 2003, Wang et al. 1999). The regulation occurs at the level of FGF8 promoter, where androgen regulated elements (ARE) have been found (Gnanapragasam et al. 2002). However, there is also expression of FGF8 in androgen independent prostate cancer (Dorkin et al. 1999). Studies carried out *in vitro* and *in vivo* in experimental models have shown that FGF8 increases the growth, invasion and tumorigenity of prostate cancer (Rudra-Ganguly et al. 1998, Song et al. 2000) and that FGF8 targeted to prostate epithelium causes PIN lesions in transgenic mice (Song et al. 2002). Recent studies by Valta and coworkers have shown that FGF8 is involved in prostate cancer bone metastasis (Valta et al. 2008), possibly via regulation of osteoblast differentiation (Valta et al. 2008, Valta et al. 2006), and angiogenesis (Mattila et al. 2001, Aragon-Ching, Dahut 2008), which is a critical step in prostate cancer growth.

2.2.2.4. Fibroblast growth factor 8 signaling in cancer

Prostate cancer cells express FGF receptors 1-4 at different frequencies. Expression of receptors 1 and 4 is associated with prostate cancer (Kwabi-Addo, Ozen & Ittmann

2004, Freeman et al. 2003, Wang et al. 2004, Wang, Stockton & Ittmann 2004), while the role of receptor 2 is controversial (Valve et al. 2001). The role of FGFR3 in prostate cancer is largely unknown (Valve et al. 2001, Hernandez et al. 2009). FGF receptor activation is downstream of multiple signal transduction pathways, including phospholipase Cγ, PI-3K, mitogen activated protein kinase (MAPK) and signal transducers and activators of transcription (STAT) pathways, which all have important roles in prostate cancer progression (Mohammadi et al. 2005, Dvorak, Dvorakova & Hampl 2006) (Figure 1). Little is known about the regulation of FGF signal transduction. Syndecan 1 protein is a heparin-sulfate proteoglycan, which modulates FGFR activity. Syndecan 1 has been shown to be significantly overexpressed in prostate cancer cells and associated with decreased survival (Zellweger et al. 2003). However, recent studies have demonstrated that endogenous inhibitors of FGFs regulate their signaling. Several of these proteins have been shown to decrease the biological activities of FGFs and their effects in cancer progression. One of these proteins, Sef (Similar expression to FGFs), is downregulated in prostate cancer (Darby et al. 2006). Sef inhibits tyrosine phosphorylation of FGFR1 and FGFR2. Sprouty proteins negatively regulate FGF signaling by inhibiting Ras-MAPK pathway. Expression levels of Sprouty 1 and 4 mRNA are decreased in many cancers (Kwabi-Addo, Ozen & Ittmann 2004, Wang et al. 2006). MAPK phosphatase 3 also inhibits the MAPK pathway by dephosphorylating ERK2. As a result of downregulation of FGF inhibitors, signal transduction is increased, which induces enhanced proliferation, resistance to cell death, increased motility and invasiveness, increased angiogenesis, enhanced metastasis, resistance to chemotherapy and radiation and androgen-independency, all of which enhance tumor progression and aggressiveness (Kwabi-Addo, Ozen & Ittmann 2004).

Figure 1. FGF signaling pathways. (Modified from Dailey et al. 2005).



2.2.3. Tumor angiogenesis and lymphangiogenesis

2.2.3.1. Mechanisms of tumor angiogenesis

Initiation of angiogenesis (angiogenic switch) is dependent on the balance between angiogenic and anti-angiogenic factors. Angiogenesis is generally a result of endothelial cell stimulation by different cytokines or mitogenic growth factors, such as VEGFs, angiopoietins, TGF-α and ß, platetet derived growth factor (PDGF), tumor necrosis factor α (TNF-α), interleukins, chemokines and FGFs (Presta et al. 2005, Hanahan, Folkman 1996). Their production is low in normal cells but upregulated in cancer. Angiogenic stimulation causes production of proteases, which assists the endothelial cells to invade the surrounding tissue, proliferate and finally form new vessels. Different anti-angiogenic factors include thrompospondin, endostatin, tumstatin or vasostatin (Ferrara 2005). The angiogenic - anti-angiogenic balance can be also affected by external factors such as oncogenes, tumor suppressor genes, hormone metabolites or, most importantly, hypoxia (Hanahan, Folkman 1996, Patiar, Harris 2006, Bergers, Benjamin 2003, Nyberg, Xie & Kalluri 2005).

Tumor vessels may sprout from pre-existing vessels (angiogenesis) (Folkman 1971, Patan, Munn & Jain 1996) or may be formed by circulating endothelial precursors (vasculogenesis) (Lyden et al. 2001). Vasculogenesis is the main method of vessel formation during embryogenesis but after birth its role is less significant. Recently, postnatal vasculogenesis has been shown to be involved in tumor vasculature or the revascularization process following trauma, e.g., after cardiac ischemia (Rafii 2002).

Tumor growth and survival are dependent on the ability of the tumor cells to induce and maintain neovascularization in tumor tissue. However, tumors have often aberrant vasculature (Brown, Wilson 2004). Tumor vessels may also be mosaic vessels, where tumor cells intravasate into the vessel lumen and remain temporarily confined in the vessel walls with endothelial cells (Mollica, Jain & Netti 2003, Folkman 2001, Carmeliet 2005, Chang et al. 2000, Auguste et al. 2005). It has been suggested that blood-conducting channels could exist without endothelial cells in certain tumors. This feature is called vascular mimicry, where such channels are formed by tumor cells (Auguste et al. 2005, Shirakawa et al. 2002). Abnormal vascularization leads to regions with low oxygen concentrations, *i.e.* hypoxia, which is an important feature of cancer (Folkman 1971, Brown, Wilson 2004).

2.2.3.2. Mechanims of lymphangiogenesis

The peripheral lymphatic vasculature has developed through fusion of mesenchymal lymphatic spaces, including mesodermal lymphatic progenitor cells that develop separately from blood vessels (Sabin 1902, Huntington and McClure 1908). Lymphatic endothelial cells develop from venous endothelial progenitor cells (Sleeman et al. 2001). The lymphatic vascular system has no intrinsic pumping

mechanism; therefore the drainage is mostly passive. The lymphatic system drains excess fluid from the extracellular spaces to blind-ended lymphatic capillaries and returns it to the blood vascular system. Lymphatic vessels are found in almost all tissues, exept bone, cartilage, bone marrow, neural tissue and placenta. The structure of lymphatic vessels conforms closely to that of vessels of the venous system. However, the lymphatic capillaries are more permeable, thin-walled and lack basement membrane or it is rudimentary, and there are no pericytes. The lymphatic capillaries drain to lymph nodes, where antigen presentation and clonal expansion of B cells takes place.

In tumors, lymphangiogenesis occurs mainly in the peritumoral area, which enables tumor cell dissemination to the lymphatic vessels. VEGF-C is overexpressed in various human cancers including breast cancer (Kurebayashi et al. 1999, Nakamura et al. 2005, Choi et al. 2005) and prostate cancer (Tsurusaki et al. 1999, Zeng et al. 2004, Li et al. 2004, Jennbacken et al. 2005). In experimental tumors, overexpression of VEGF-C induces peritumoral lymphangiogenesis and tumor cell invasion to the lymphatic vessels (Skobe et al. 2001, Mandriota et al. 2001, Mattila et al. 2002, Brakenhielm et al. 2007). In contrast, other studies have revealed that intratumoral lymphatic vessels may not be functional or that the peripheral lymphatics may not function properly, suggesting that role of lymphangiogenesis is not crucial in primary tumor cell dissemination (Padera et al. 2002, Wong et al. 2005). The reason for the unfunctional intratumoral lymphatic vessels is mainly the increased interstitial pressure, which is caused by a chaotic structure of leaky blood vessels (Tammela et al. 2005).

2.2.3.3. Vascular endothelial growth factor

In adults, angiogenesis occurs only in special circumstances such as during wound healing or during the female reproductive cycle. VEGF is the main angiogenic molecule involved in both physiologic and pathologic angiogenesis (Senger et al. 1983). It is essential for *de novo* vasculogenesis, formation of new vessels and sprouting of new capillaries from pre-existing ones (Connolly et al. 1989, Ferrara et al. 1996). VEGF stimulates endothelial cell proliferation, migration, capillary-like tube formation, survival and it also increases the permeability of the formed vessels (Leung et al. 1989, Senger et al. 1994). It also causes vasodilation through the induction of the endothelial nitric oxide (NO) synthase and the increase of NO production (Auguste et al. 2005, Hood et al. 1998). VEGF belongs to the gene family comprising of dimeric glycoproteins, which bind and activate VEGF receptor tyrosine kinases: VEGF, VEGF-B, VEGF-C, VEGF-D, VEGF-E and VEGF-F and placenta growth factor (PlGF) (Tammela et al. 2005). VEGF was originally characterized as a vascular permeability factor (VPF) (Senger et al. 1983). VEGF binds to receptors VEGFR1 and VEGFR2, neuropilin-1 and neuropilin-2 (de Vries et al. 1992, Quinn et al. 1993, Kolodkin et al. 1997, Chen et al. 1997). VEGF mostly binds to its receptors in

endothelial cells but it also has interactions with other cells such as HSCs (haemotopoietic stem cells), monocytes, osteoblasts and neurons (Ferrara, Gerber & LeCouter 2003, Gerber et al. 2002, Sun, Guo 2005). Six isoforms of VEGF (with different number of amino acids, which are marked with a number after VEGF) are produced by alternative splicing. $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ are the major isoforms secreted by most cells types. $VEGF_{145}$, $VEGF_{183}$ and $VEGF_{206}$ are uncommon (Robinson, Stringer 2001).

VEGF is highly expressed in most cancers (Hanahan, Folkman 1996, Bergers, Benjamin 2003, Wegiel et al. 2008). In prostate cancer, VEGF expression is regulated by androgens (Jain et al. 1998, Burchardt et al. 2000, Stewart et al. 2001), and recent studies suggest a correlation between angiogenesis and biological aggressiveness with a parallel increase in Gleason grade (Mao et al. 2008). A high tissue level of VEGF is associated with increased angiogenesis and predicts poor prognosis (Stewart et al. 2001). Serum VEGF concentrations fall after prostatectomy, and patients with metastasic prostate cancer have a higher serum level for VEGF compared with the normal population (George et al. 2004). Thereby, serum VEGF concentrations correlate with cancer progression and survival. However, the usefulness of VEGF as a diagnostic and a prognostic marker along with PSA is currently under investigation (Delongchamps, Peyromaure & Dinh-Xuan 2006).

VEGF gene expression is regulated by several stimuli, such as growth factors (PDGF, TGF, some FGFs, EGF and IGF-1), cytokines, p53 mutation, estrogens, androgens, TSH (thyroid stimulating hormone), tumor promoters and NO (Robinson, Stringer 2001, Finkenzeller et al. 1992, Goldman et al. 1993, Frank et al. 1995, Soh et al. 1996, Cohen et al. 1996). Hypoxia, mediated by hypoxia inducible factors (HIF1 and HIF2) is an important upregulator of VEGF (Shweiki et al. 1992, Forsythe et al. 1996, Ema et al. 1997, Semenza 2003). Upregulation of VEGF is associated with specific transforming events including mutations in Ras and Wnt signaling (Zhang, Gaspard & Chung 2001). VEGF is regulated at the level of mRNA stability. The 5'- and 3'-untranslated regions of the VEGF gene confer increased mRNA stability during hypoxia. Two proteins are involved in mRNA stabilization: HuR (AU-rich element binding protein) (Levy et al. 1998) and PAIP2 (polyadenylated binding protein-interacting protein) (Onesto et al. 2004). VEGF expression can also be regulated at the translational level, which ensures that sufficient levels of VEGF are produced even under unfavorable conditions (Yoo, Mulkeen & Cha 2006).

2.2.3.4. Vascular endothelial growth factor C

VEGF-C is a crucial paracrine factor that induces lymphangiogenesis. It was first found from human prostate cancer cells (PC-3) as a ligand for the angiogenic receptor (VEGFR2) (Joukov et al. 1996) and the lymphangiogenic receptor (VEGFR3) (Joukov et al. 1996, Lee et al. 1996). It is produced as a single 58 kD intracellular propeptide, the N-terminal and C-terminal ends are proteolytically cleaved to generate the 31 kD

and 29 kD polypeptides that bind to VEGFR3 with high affinity and VEGFR2 with low affinity (Joukov et al. 1997). Additional processing by plasmin and other proteases results in a 21 kD fully processed VEGF-C. The unprocessed 58 kD VEGF-C is also able to bind to VEGFR2 and VEGFR3 with lower affinity and can act as an antagonist to fully processed VEGF-C (Joukov et al. 1997).

In vitro, VEGF-C is a mitogenic growth factor and it induces endothelial cell survival and migration (Joukov et al. 1996, Bauer et al. 2005, Cao et al. 1998). VEGF-C induces angiogenesis *in vivo* through VEGFR2 (Joukov et al. 1996, Cao et al. 1998, Laakkonen et al. 2007, Tammela et al. 2008). The lymphangiogenic effects of VEGF-C are mediated through VEGFR3. During embryogenesis, VEGF-C is co-expressed with its receptors mainly in the regions of lymphangiogenesis and developing mesenterium (Kärkkäinen et al. 2004, Kukk et al. 1996, Lymboussaki et al. 1999). Overexpression of VEGF-C in a transgenic mouse model leads to lymphatic vessel hyperplasia in skin with no effects on blood vasculature (Jeltsch et al. 1997). VEGF-C -/- mice die prenatally at day E10.5 due to fluid accumulation in tissues (Dumont et al. 1998). Although vasculogenesis and angiogenesis took place in these embryos, the large vessels were disorganized. The heterozygous VEGF-C +/- mice survive but they have severe lymphedema associated with hypoplasia of dermal lymphatic vessels, which indicates that development of lymphatic vasculature is concentration-dependend and both alleles are needed in normal development (Kärkkäinen et al. 2004). Without VEGF-C, endothelial cells cannot form sprouting lymphatic vessels. In VEGF-C -/- mice, the sprouting can be rescued by VEGF-C or VEGF-D but not with VEGF, which indicates that the lymphangiogenic effects of VEGF-C are mediated through VEGFR3 (Kärkkäinen et al. 2004).

VEGF-C and VEGFR3 are upregulated in vascular endothelial cells in pathological conditions such as solid tumors. The expression of VEGF-C has been shown to correlate with lymph node metastasis (He et al. 2004 Kurebayashi et al. 1999, Nakamura et al. 2005b, Choi et al. 2005). Tumor cells that overexpress VEGF-C induce peritumoral lymphangiogenesis and tumor cell invasion to lymphatic vessels in animal models (Mattila et al. 2002, Skobe et al. 2001, Mandriota et al. 2001). The effects of VEGF-C can be blocked by VEGFR3 inactivation (He et al. 2002, Lin et al. 2005, Pytowski et al. 2005). A proteolytically processed form of VEGF-C is also strongly angiogenic (Joukov et al. 1996, Laakkonen et al. 2007, Tammela et al. 2008).

VEGF-C mRNA transcription is induced in endothelial cells in response to proinflammatory cytokines (interleukin-1β) (Ristimäki et al. 1998). The regulation of transcription by these cytokines indicates that VEGF-C could regulate lymphatic vessel function during inflammation. The VEGF-C gene promoter contains NF-κB binding sites. The transcription factor NF-κB is a key regulator in carcinogenesis (Lin, Kain, 2003). It is constitutively activated in human prostate adenocarcinomas and correlates with disease progression (Shukla et al., 2004). The presence of these

binding sites suggests that NF-κB may be involved in inducing VEGF-C mRNA (Chilov et al. 1997).

2.2.3.5. Other vascular endothelial growth factors

Placental growth factor (PlGF) has three isoforms: PlGF-1, PlGF-2 and PlGF-3. It was originally found in a human placental cDNA library (Maglione et al. 1991, Cao et al. 1997). It binds to VEGFR1 and neuropilin-1 (Migdal et al. 1998) and cross talks with VEGFR2 (Autiero et al. 2003). PlGF forms homodimers and heterodimers with VEGF (Kurz et al. 1998). PlGF has both angiogenic and arteriogenic potential. It is expressed in endothelial cells, trophoblasts and some human tumors. It has an important role in placental development (Tseng et al. 2006). Recently, it has been found to be associated with recurrence, metastasis and the survival of cancer patients. Mice lacking PlGF gene are viable and fertile. However, they have a defect in retinal vessel formation and reduced postnatal angiogenesis (Carmeliet et al. 2001).

The angiogenic growth factor VEGF-B, is expressed as two isoforms in humans (Olofsson et al. 1996). Its actions are mediated through VEGFR1 and neuropilin-1 (Olofsson et al. 1998). During development, it is found in the developing myocardium (Lagercrantz et al. 1998). In adults, it is expressed in most tissues, the highest expression levels being detected in heart and skeletal muscle (Li et al. 2008). VEGF-B has been shown to regulate the plasminogen activity of endothelial cells and recently it has also been shown to function as a survival factor for neural cells (Olofsson et al. 1998). Seemingly, its functions are not crucial since VEGF-B -/- mice survive with a slightly reduced myocardial size, dysfunction in coronary veins and arterial conduction defect (Sun et al. 2004, Aase et al. 2001, Bellomo et al. 2000). The molecular mechanisms that regulate VEGF-B expression remain poorly understood.

VEGF-D is the closest relative of VEGF-C. It is mitogenic in endothelial cells, angiogenic and lymphangiogenic *in vivo,* and binds to VEGFR2 and VEGFR3 (Achen et al. 1998, Stacker et al. 1999). VEGF-D is present in most adult tissues and it is most abundant in the lung. Like VEGF-C, VEGF-D increases lymphangiogenesis and metastasis in the lymph nodes, at least in some experimental tumors (Von Marschall et al. 2005). VEGF-D -/- mice are healthy and fertile with a functional lymphatic system (Baldwin et al. 2005). Mice overexpressing VEGF-D develop a hyperplastic vascular network, similar to VEGF-C overexpressing mice, indicating a role in lymphangiogenesis (Jeltsch et al. 1997, Veikkola et al. 2001). In humans, VEGF-D has been shown to have correlations with prognosis for lymphatic invasion and survival of cancer patients. It induces the formation of lymphatic vessels within tumors and promotes metastasis (He, Kärpänen & Alitalo 2004, Stacker et al. 2001).

Homologues of VEGF-E (also called the Orf-virus-VEGF) have been identified in the genome of the parapoxvirus Orf (Lyttle et al. 1994). The activities of VEGF-E are similar to VEGF (Lyttle et al. 1994, Shibuya 2003). There is a group of proteins from different Orf virus strains and the name VEGF-E is used a collective term for them all

(Meyer et al. 1999). All variants bind and activate VEGFR2 but not VEGFR1 or VEGFR3 (Ogawa et al. 1998). VEGF-E seems to be as effective inducer of endothelial cell proliferation as $VEGF_{165}$, even through most VEGF-Es lack the heparin-binding basic domain. It is a potential pro-angiogenic therapy target since K14 driven VEGF-E transgenic mice (Kiba et al. 2003) have shown a significant increase in angiogenesis in subcutaneous tissue without clear side effects compared with VEGF overexpressing mice (Larcher et al. 1998, Xia et al. 2003).

The most recently found members of the VEGF family are VEGF-Fs (Suto et al. 2005) including svVEGF from *Bothrops insularis* (golden lancehead snake) and *TF*svVEGF from *Trimeresurus flavoviridis* (yellow-green tree viper). VEGF-F consists of VEGF related proteins of hypotensive factor (HP), increasing capillary permeability protein (ICPP) and vammin from vipers. VEGF-F has a structure homology with $VEGF_{165}$. TFsvVEGF binds to VEGFR1 and svVEGF binds to VEGFR2 (Yamazaki et al. 2005b). The snakes use VEGF-Fs in addition to VEGF. It seems that VEGF-Fs may contribute to the enhancement of toxicity in envenomation, but they seem to have individual characteristics according to the classification of the host snake (Yamazaki et al. 2009). VEGF-F has a heparin-binding site and it has been shown to exhibit specific blockage activity against $VEGF_{165}$ both *in vitro* and *in vivo* (Yamazaki et al. 2005a).

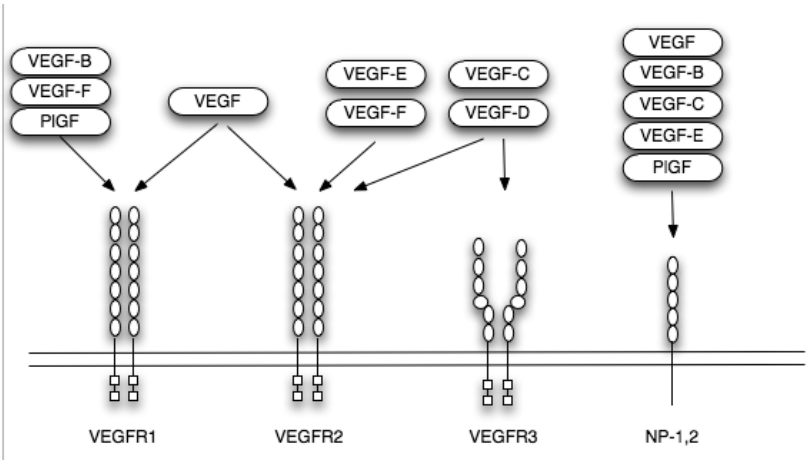
2.2.3.6. Vascular endothelial growth factor receptors

To date, three VEGF receptors have been found. They are receptor tyrosine kinases with several immunoglobulin-like loops in the extracellular domain and split tyrosine kinase domains in the intracellular side (Figure 2). They are specific to endothelial cells and have structural and functional similarities to PDGF (placental-derived growth factor) receptors. VEGFR1 and VEGFR2 are angiogenic receptors that very specifically bind different VEGFs. VEGF, VEGF-B, PlGF and *TF*svVEGF bind to VEGFR1 (de Vries et al. 1992, Olofsson et al. 1998, Park et al. 1994), while VEGF, VEGF-C, VEGF-D, VEGF-E and svVEGF bind to VEGFR2 (Joukov et al. 1996, Quinn et al. 1993, Achen et al. 1998, Ogawa et al. 1998, Shibuya 2006a). VEGFR3 is a lymphangiogenic receptor and binds VEGF-C and VEGF-D (Joukov et al. 1996).

VEGFR1 (also called FLT-1) is composed of seven extracellular immunoglobulin-like domains, a single transmembrane region and an intracellular tyrosine kinase domain (Shibuya et al. 1989). VEGFR1 is expressed in endothelial cells and also other cell types such as macrophages, monocytes, osteoblasts, placental trophoblasts, renal mesengial cells and haematopoitetic stem cells (Zachary, Gliki 2001). VEGFR1 gene expression is upregulated by hypoxia (Gerber et al. 1997). VEGFR1 promotes angiogenesis (Fong et al. 1995) and cell migration (Lyden et al. 2001, Barleon et al. 1996, Clauss et al. 1996). VEGFR1 is a negative regulator of angiogenesis during embryonic development, but plays a role as an angiogenic factor under pathological conditions (Shibuya 2006b). The release of a soluble component of VEGFR1

(sVEGFR1) has been shown to inhibit VEGF-induced endothelial cell proliferation with high affinity (Kendall, Wang & Thomas 1996). The sVEGFR1 is expressed in many solid tumors and may act as an antagonist to VEGF or PlGF (Lamszus et al. 2003).

Figure 2. Receptor binding specifity of different VEGFs. (Modified by Saaristo et al. 2000).



VEGFR2 also called KDR in human (Terman et al. 1991) and Flk-1 in mouse (Matthews et al. 1991, Millauer et al. 1993) is expressed in endothelial cells and also in neural cells, osteoblasts, megakaryocytes and haematopoietic stem cells (Ferrara, Gerber & LeCouter 2003). The major structure of VEGFR2 is similar to VEGFR1 with seven extracellular immunoglobulin-like domains, transmembrane domain and an intracellular tyrosine kinase domain. VEGFR2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF and it promotes tubular elongation (Ortega, Hutchings & Plouet 1999, Shinkaruk et al. 2003). The survival effect and vascular permeability are mediated through the PI3-K/Akt pathway, migration through the p38MAPK pathway and endothelial cell proliferation through the MEK-ERK pathway (Gerber et al. 2002). Recently, a soluble form of VEGFR2 (sVEGFR2) has been found from mouse and human plasma (Ebos et al. 2004). Similar to the soluble form of VEGFR1 (sVEGFR1), sVEGFR2 may have regulatory consequences with respect to VEGF-mediated angiogenesis (Szentirmai et al. 2008). However, the physiological and pathological roles of sVEGFR2 are still uncharacterized (Ebos et al. 2004).

VEGFR3 (also called Flt-4) (Aprelikova et al. 1992) is a receptor for lymphangiogenic growth factors VEGF-C and VEGF-D (Joukov et al. 1996, Achen et

al. 1998). Unlike VEGFR1 and VEGFR2, VEGFR3 has only six immunoglobulin-like domains (Pajusola et al. 1994). VEGFR3 is modified by means of alternative splicing and the two splice variants differ in the C-terminal region (Hughes 2001). The VEGF-C/VEGFR3 pathway is crucial for the development of lymphatic vasculature as well as lymphangiogenesis in wound healing, cardiovascular development and remodeling the embryonal vascular network (Paavonen et al. 2000). VEGFR3 is present in all endothelia during embryogenesis, but in adults it becomes restricted to lymphatic endothelium and certain fenestrated blood vascular endothelial cells. VEGFR3 is upregulated in blood vascular endothelial cells in pathological conditions like vascular tumors and the periphery of solid tumors (Jennbacken et al. 2005, Partanen, Alitalo & Miettinen 1999). VEGFR3 is widely distributed in vascular tumors as a marker of lymphatic endothelial cell differentation (Partanen et al. 1999).

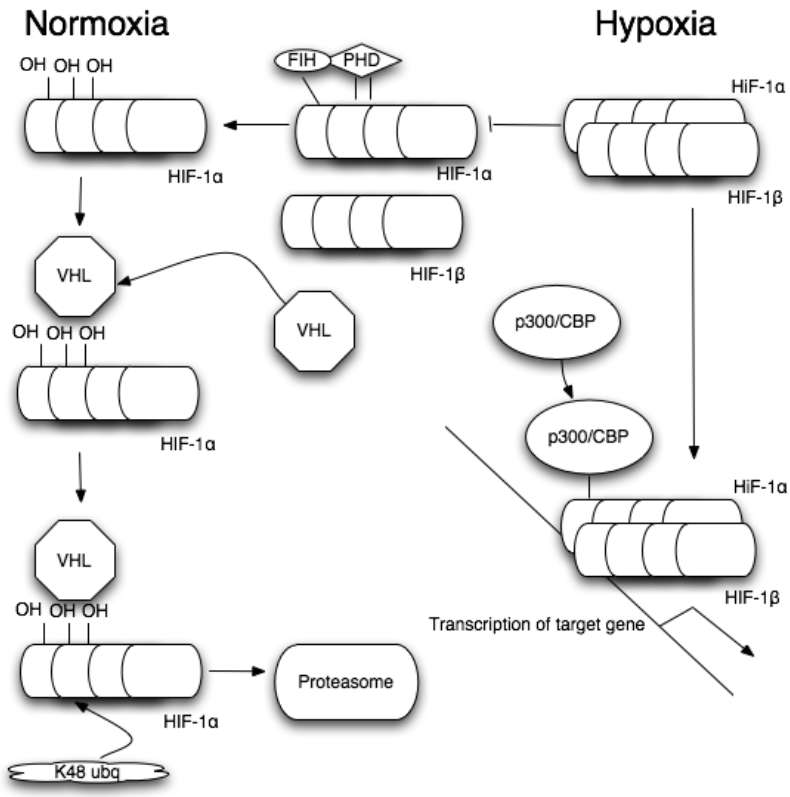
$VEGF_{165}$, PlGF, VEGF-B and VEGF-E also bind to neuronal cell surface receptor neuropilin-1 (Soker et al. 1998, Bagri, Tessier-Lavigne 2002). Neuropilin-2 binds to $VEGF_{165}$, PlGF and VEGF-C. During embryonic development, neuropilins are expressed in the nervous, cardiovascular and skeletal systems (Kitsukawa et al. 1995, Miao et al. 2000). In adults, they are expressed in endothelial cells, widely in tumor cells, lungs, heart, kidneys, pancreas, osteoblasts and bone marrow cells (Soker et al. 1998, Tordjman et al. 1999). Neuropilins enhance angiogenesis and lymphangiogenesis by means of forming heterodimers with VEGFR1 and VEGFR2 (Klagsbrun, Takashima & Mamluk 2002).

All VEGF receptors are expressed during embryonic development (Kaipainen et al. 1993). Inactivation of any of the receptors leads to death because of disorganized blood or lymphatic vessels (Dumont et al. 1998, Fong et al. 1995, Shalaby et al. 1995).

2.2.4. Tumor hypoxia

All animals of the size of one millimeter or larger need a more effective system than diffusion to carry oxygen to their cells. Similarly, the cells in healthy tissues and in tumors need a functional capillary network; otherwise, the area suffers from hypoxia. Hypoxia is common in pathological conditions such as heart disease, stroke, wounds and cancer. Normal oxygen concentration in tissues is approximately 40 mmHg; however, the term hypoxia is relevant and dependent on the metabolic activity of the tissue. Oxygen concentration under 10 mmHg is usually considered hypoxic, and tumor tissue is often hypoxic. Hypoxia can be *chronic*, when diffusion of oxygen cannot reach rapidly growing tumor mass (Gray et al. 1953) or is can be *acute* caused by temporarily blocked vessel or transient perfusion of plasma only (Brown 1979).

Figure 3. Regulation of HIF. HIF-1 is a dimer of HIF-1α and HIF-1 ß subunits. Under normoxia, HIF-1 is degraded by ubiquitination of pVHL protein complex. The interaction between pVHL and HIF-1 subunit is regulated by hydroxylation of HIF-1. Under hypoxic conditions, hydroxylation and ubiquination of HIF-1 are suppressed and HIF-1 is overexpressed. In normoxia, HIF-1 is hydroxylated and interaction with the p300/CBP co-activator and hypoxia responsive element is inhibited.



Inadequate oxygen supply can influence cellular functions. There is evidence that tumor cells may become more aggressive in hypoxic conditions (Pennacchietti et al. 2003, Jokilehto et al. 2006, Sutherland et al. 1996). Hypoxia-inducible genes are involved in several pathways, such as angiogenesis, apoptosis, immortalization, proliferation, metabolism and migration (Semenza, 2003). Hypoxia is sensed by the MAP kinase pathway, and the biological consequences are mediated mainly by HIFs, which are transcription factors that can activate angiogenesis, anaerobic glycolysis or glucose transport. HIF-1 is a dimer of HIF-1α and HIF-1ß subunits (Wang, Semenza 1995). Both are constitutively expressed in various cell types. Regulation of HIFs is based on balance between formation and degradation of HIF-1α (Figure 3). Under normal oxygenation conditions, HIF-1α is poorly detected, since it is degraded rapidly

by ubiquitination of a complex containing pVHL (von Hippel-Lindau) tumor suppressor protein. The interaction between pVHL and HIF-1α subunit is regulated by hydroxylation of a proline residue of HIF-1α, which requires oxygen and iron for its activity (Maxwell et al. 1999, Cockman et al. 2000, Ohh et al. 2000, Kamura et al. 2000, Tanimoto et al. 2000, Masson, Ratcliffe 2003). The enzyme required for pVHL binding to HIF-1α and which acts as an oxygen sensor for the cells is HIF- prolyl-hydroxylase (HIF-PH) (Jaakkola et al. 2001). Under hypoxic conditions, HIF-1α is overexpressed as a result of suppressed prolyl hydroxylation and ubiquitination of HIF-1α and degradation is decreased (Jaakkola et al. 2001, Ivan et al. 2001).

In normoxia, the asparagine residue in the C-terminal transactivation domain of HIF-1α is oxygen-dependently hydroxylated and interaction with the p300/CBP [CREB (cAMP-response-element)-binding protein] co-activator and HRE (hypoxia response element) is inhibited (Wang, Semenza 1995). Thus, hypoxia allows both stabilization and transactivation of HIF-1 α and promotes transcription of hypoxia-inducible genes.

It has been shown that in mitochondria, there are also other mechanisms for oxygen sensing such as flavoprotein oxidoreductase, which recognizes when the flavin cofactor is in an oxidized or reduced state (Ward 2008). The ability to adapt according the availability of oxygen concentrations provides a critical advantage to all O_2-dependent life. All these responses help the tissue to survive low oxygen concentrations.

2.2.4.1. Non-invasive imaging of hypoxia

The effectiveness of radiation therapy is directly dependent on the O_2 concentration (Schwartz, 1909, Gray et al. 1953). Hypoxia decreases the proliferation of tumor cells, which lowers the efficacy of the most chemotherapeutic drugs as they are targeted against dividing cells (Brown, Wilson 2004, Brown, Giaccia 1998). Perfusion of drugs into target tissue is also less efficient. Tumors with identical clinical status can vary greatly in the extent of hypoxia. Therefore, in order to select appropriate therapy, it is important to identify the hypoxic status of each tumor and metastasis (Vaupel, Harrison 2004). Today, hypoxia can be detected by means of immunohistochemistry, using antibodies against pimonidatzole, HIF-1α, GLUT1 or VEGF but no clinically feasible and non-invasive methods are available for assessment of tumor hypoxia.

Lately, a great interest in non-invasive imaging of solid tumors using radiolabeled markers has arisen (Chapman et al. 1998). Several ^{18}F-labeled 2-nitroimidazole-based compounds have been synthesized to be used with positron emission tomography (PET) as hypoxia markers (Lim, Berridge 1993, Yang et al. 1995, Tewson 1997, Dolbier et al. 2001). The reductive metabolism of these compounds leads to their activation and subsequent formation of covalent bonds with cellular macromolecules under hypoxic conditions. Thus, these compounds tend to accumulate in sites of

hypoxia, which renders them suitable for imaging (Varghese, Gulyas & Mohindra 1976). A hypoxia marker 2-(2-nitro-1[*H*]-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl)-acetamide (EF5) has been shown to predict radiotherapy resistance in individual rodent tumors (Evans et al. 1996). Furthermore, pharmacological properties of the compound have been well documented and recently EF5 has been successfully labeled with ^{18}F (Dolbier et al. 2001). ^{18}F-labeled EF5 ([^{18}F]EF5) has some advantages over previously reported ^{18}F-labeled hypoxia markers including fluoromisonidazole ([^{18}F]FMISO), (Grierson et al. 1989), fluoroazomycinarabinofuranoside ([^{18}F]FAZA) (Parliament et al. 1992, Groshar et al. 1993, Sorger et al. 2003), fluoroerythronitroimidazole ([^{18}F]FETNIM) (Yang et al. 1995) and fluoroetanidazole ([^{18}F]FETA) (Tewson 1997). EF5 is known to have uniform biodistribution in rodents (Laughlin et al. 1996) due to the high lipophility (octanol-water partition coefficient 5.7) of the compound. Furthermore, the compound is stable *in vivo, i.e.* no drug metabolites have been detected in the plasma or urine of the experimental animals (Dolbier et al. 2001, Minn H et al. 2008, Koch 2002). Unlabeled EF5 has been used as a hypoxia marker in patients with good correspondence to other hypoxic markers (Evans et al. 2000, Kaanders et al. 2002). [^{18}F]EF5 is still under clinical evaluation (Minn H et al. 2008).

2.2.5. Prostate cancer metastasis

2.2.5.1. Mechanisms of metastasis

Normal cells do not survive when they become detached from their original site. Therefore, outstanding changes must take place before the cancer cells can escape from the primary tumor and re-establish growth at distant locations. According to Hanahan and Weinberg (Hanahan, Weinberg 2000), malignant human tumors share six common alterations in cell physiology: self-sufficiency in growth signals, limitless proliferation potential, insensitivity to growth–inhibitory and apoptotic signals, sustained angiogenesis, invasion and metastasis. It is common that cancer cells escape from tumors via blood and lymphatic vessels. Although metastases are common in advanced cancer, the phenomenon is inefficient in terms of the number of cells that successfully move to a new site. However, a small number of cells survive and form metastases, but they may be dormant for long times before causing clinical problems (Wikman, Vessella & Pantel 2008). Growth at the new site also requires the formation of new vessels *i.e*. angiogenesis (Folkman 1971, Radisky, Hagios & Bissell 2001).

Cancers of epithelial origin, such as prostatic adenocarcinoma, must first break contact with their neighbors, traverse the basement membrane and migrate through stroma before they reach vessels that can carry them to distant sites (Nguyen, Massague 2007). Usually, the transport happens through blood or lymphatic vessels. A common route of metastasis is lymphatic drainage from where tumor cells are either trapped to their first-pass organs, regional lymph nodes, or transported to the blood stream through the thoracic duct and the superior vena cava or through lymph nodes themselves, which allow a wider dissemination of tumor cells (Fisher, Fisher 1966,

Fisher, Fisher 1970). The first-pass organs are also important in the angiogenic route, which results in the liver becoming a common site of metastasis (Arya et al. 2006). Different cancers metastasize to different sites. Anatomical factors and the direction of blood flow are behind the phenomenon (Batson 1940, Stevens, Ewing 1928) but also other mechanisms attract tumor cells to specific sites. According to Paget's "seed and soil" theory "seeds" are tumor cells and "soil" is the target tissue with appropriate growth factors, which could be recognized by tumor cell receptors (Paget 1889).

Degradation of extracellular matrix is performed by proteinases (matrix metalloproteinases, serine proteinases, cystein proteinases or aspartyl proteinases). Matrix metalloproteinases (MMPs) are required for invasion. MMPs are able to catalyze degradation of type IV, V, VII, IX and X collagen and other substrates in the basement membrane and extracellular matrix, *e.g*. fibronectin and elastin. MMP-2 is particularly associated with invasiveness and is considered as a marker of poor prognosis (Talvensaari-Mattila et al. 1998). Both MMP-2 and MMP-9 are overexpressed in advanced prostate cancer (Wegiel et al. 2008). In order to antagonize MMP, there are two types of inhibitors: polypeptide tissue inhibitors of matrix metalloproteinases (TIMPs) and synthetic drugs that chelate the Zn^{2+}, which is required for MMP activity. The best-known compound of that group is Marimastat, which is a broad-spectrum inhibitor for all metalloproteinases. It inhibits capillary sprout and blocks tumor growth *in vivo* and it is also being tested in clinical trials (Phases I, II and III) against ovarian, lung, pancreatic and gastric cancers. However, it is not effective against advanced cancer. Bisphosphonates do not affect secretion of MMPs, but they have been shown to inhibit their gelatinolytic activity (Boissier et al. 2000, Teronen et al. 1999).

2.2.5.2. Bone metastasis

Bone metastasis is a common feature of advanced prostate cancers with poor prognosis and no treatment options. Even though the current treatments may be effective in suppressing tumor growth and relieving tumor associated bone pain, they do not provide long-term remission from the disease. Tumor growth in skeletal sites is the main cause of morbidity in advanced prostate cancer and requires new treatment approaches.

Establishment of a successful metastasis at a secondary location requires a fertile soil at the metastatic site, as first suggested by Paget (1889). In prostate cancer, several factors enhance metastatic growth in bone. Prostate cancer cells are osteomimetic. After entering the bone marrow, the cancer cells have high adhesion to osteoblasts and they gain properties similar to bone cells (Koeneman, Yeung & Chung 1999). It has been shown that in bone marrow, prostate cancer cells loose some of their epithelial properties and instead achieve mesenchymal features. For example, they switch from E-cadherin to N-cadherin, loose their polarity and cell-cell interactions, increase their motility, and express mesenchymal even bone related

markers, such as collagen, osteocalcin, vimentin and fibronectin (Gravdal et al. 2007) and cell signaling genes (Knerr et al. 2004). This phenomenon is called epithelial-mesenchymal transition. Epithelial-mesenchymal transition is an important phenomenon in embryonic development, when epithelial cells migrate to distant sites. In cancer, it is a feature of an invasive phenotype (Hugo et al. 2007, Mattila, Härkönen 2007).

Bone metastases of prostate cancer are mainly osteosclerotic (osteoblastic, bone forming) or mixed lesions with both osteosclerotic and osteolytic (osteoclastic, bone resorbing) activity. The formation of the lesions requires interaction between tumor cells and the bone microenvironment. It has been shown that in the presence of prostate cancer cells, bone matrix formation as well as degradation is stimulated. Prostate cancer cells produce factors that perturb the bone microenvironment in ways that affect the normal functional balance between osteoblast and osteoclast activities, resulting in osteosclerotic metastases (Logothetis, Lin 2005). Prostate cancer cell express several factors that are needed in normal bone function such as BMPs, TGFb FGFs and VEGF (Autzen et al. 1998, Harris et al. 1994, Matuo et al. 1987, Ferrer et al. 1997, Dai et al. 2004). It has also been hypothesized that the cancer induced bone degradation releases cytokines and growth factors from the bone matrix, which in turn stimulates proliferation of prostate cancer cells. This interaction is called vicious cycle (Logothetis, Lin 2005, Guise et al. 2006, Choueiri et al. 2006).

Androgen ablation therapy is an effective treatment form against prostate cancer but it decreases bone mineral density as a side-effect. Advanced prostate cancer develops bone metastasis, which causes bone pain. There is no cure for bone metastasis but palliative drugs, including bisphosphonates, can be used to relieve symptoms (Kanis et al. 1996, McCloskey et al. 2001, Reginster et al. 2005, Brown, Guise 2007). Bisphosphonates are an effective option in osteoporosis treatment, also in males (Kawahara et al. 2008).

2.3. Bisphosphonates

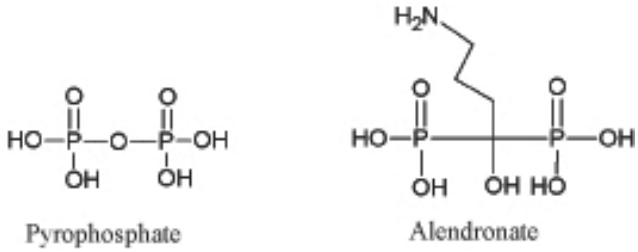
Bisphosphonates are synthetic analogs of the naturally occurring pyrophosphate. These widely used drugs exhibit peculiar pharmacokinetics. As highly water-soluble compounds, they are poorly absorbed from the GI-tract. These compounds bind bone hydroxyapatite eagerily and after parental administration they are quickly adsorbed to the calcified bone matrix. The rapid accumulation to bone keeps the drug concentrations in the blood low. Studies carried out by Fournier and coworkers (Fournier et al. 2002) have revealed, however, that at least in rats, some bisphosphonates including clodronate, zoledronate and ibandronate transiently accumulate in the prostate rather than in several other non-calcified tissues before being trapped by bone. Prostatic levels of bisphosphonates reached a peak at 30-60 minutes after administration of the drug and subsequently declined. The mechanism of

bisphosphonate accumulation in some tumors is not understood. The tumor-calcification, necrosis, or activated macrophages might have a role in the uptake process (Caraglia et al. 2006, Wakchoure et al. 2006, Merrell et al. 2007).

The nitrogen-containing bisphosphonate alendronate inhibits PC-3 prostate cancer cell adhesion to extracellular matrix proteins *in vitro* at low, non-toxic concentrations (Boissier et al. 2000, van der Pluijm et al. 1996, Boissier et al. 1997). In addition, alendronate reduces the invasion and migration of PC-3 cells in a dose dependent manner (Virtanen et al. 2002). Promising results have also been reported in animal models. In an *in vivo* ovarian cancer model, alendronate inhibited intraperitoneal dissemination of cancer cells (Hashimoto et al. 2005). Alendronate has been shown to have inhibitory effects on osteoclast generation and maturation as well as on osteoclast activity (Oades et al. 2003). Although bone metastases of prostate cancer are usually more osteosclerotic than osteolytic, there are indications that bisphosphonates such as zoledronate relieve bone pain and inhibit fractures (Parker 2004, Parker 2005, Smith et al. 2007, Saad 2008). Preferential prostatic accumulation and beneficial effects in bone increase the possibilities of exploiting bisphosphonates as adjuvant therapy in prostate cancer treatment, at least in the subgroups with severe bone pain (Parker 2005).

Bisphosphonates are administered orally or intravenously and are usually well tolerated. Recently, however, the new side effect, osteonecrosis of the jaws has been described in association with bisphosphonate use. Osteonecrosis of the jaws is induced by the new generation bisphosphonates, such as zoledronate, pamidronate or alendronate (Cheng et al. 2005). The condition is similar to the historical entity of mandibular necrosis in jaws, which was a severe disease of workmen in the phosphorus industry. Later, in the 20th century before improved protection, osteonecrosis in jaws occurred among workers of the missile industry due to white phosphorous and in the watch industry due to luminescence (Cheng et al. 2005, Gomez Font, Martinez Garcia & Olmos Martinez 2008). The occurrence of osteonecrosis of the jaws after treatment of bisphosphonates is low (0.1%). However, if one or more of the risk factors (such as invasive dental operations or smoking) are present, the risk might be as high as 10% (Gebara, Moubayed 2009).

Figure 4. Chemical structure of pyrophosphate and alendronate.



2.3.1. Mechanisms of action of bisphosphonates

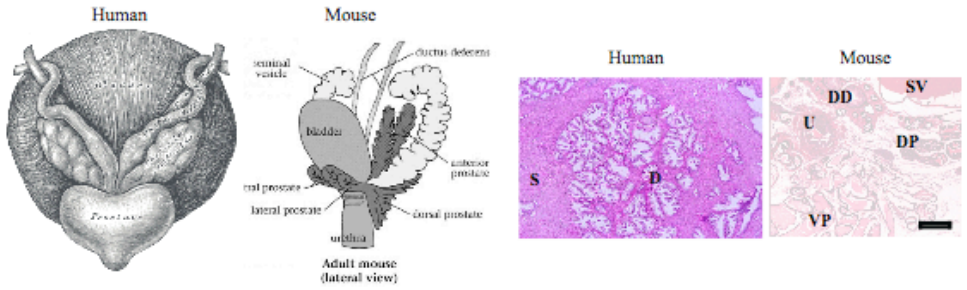
Bisphosphonates are pyrophosphate analogs (Figure 4). They are divided into two groups, pyrophosphate-resembling bisphosphonates and nitrogen-containing bisphosphonates depending of the structure of the side chain (Gomez Font, Martinez Garcia & Olmos Martinez 2008). The inhibitory effects of nitrogen-containing bisphosphonates are mediated mainly via the mevalonate pathway (Boissier et al. 1997, Virtanen et al. 2002, Oades et al. 2003). The mechanisms that have been recognized are based on their ability to impair post-translational prenylation of Ras, Rac and Rho (Caraglia et al. 2006, Virtanen et al. 2002). Nitrogen-containing bisphosphonates have been found to target farnesylpyrophosphate and/or geranylgeranylpyrophosphate synthetase, which leads to decreased generation of farnesyl diphosphate and geranylgeranyl diphosphate, respectively. These intermediates are needed for post-translational prenylation of small GTP-binding proteins (Ras, Rac and Rho), which are essential for many cellular functions such as proliferation, survival and invasion. It is probable that interference with prenylation reactions is involved in many effects of nitrogen-containing bisphosphonates such as decreased angiogenesis and cell migration as well as increased apoptosis (Caraglia et al. 2006). However, the precise mode of action of nitrogen-containing bisphosphonates is not understood. The study of Dunford et al. revealed that nitrogen containing bisphosphonates, risedronate and zoledronate, actually increased the activity of unprenylated GTP-binding proteins, which indicates that nitrogen containing bisphosphonates can give conflicting signals (Dunford et al. 2006). Both nitrogen-containing bisphosphonates and pyrophosphate-resembling bisphosphonates have also been shown to increase resistance to the apoptotic and growth inhibitory effects in some cancer cell lines. These effects are associated with activation of the p38 mitogen activated protein kinase pathway and may support cell survival and promote proliferation. Interestingly, the p38-mediated effects do not appear to depend on the inhibition of the mevalonate pathway (Merrell et al. 2007).

Recent reports have shown that nitrogen-containing bisphosphonates can induce apoptosis and/or cause growth arrest to the G2/M phase in the same way as pyrophosphate-resembling bisphosphonates by production of an endogenous ATP analog (Mönkkönen et al. 2006). The newer nitrogen-containing bisphosphonates can directly inhibit proliferation (Oades et al. 2003, Wakchoure et al. 2006). However, these growth inhibitory effects can be overcome with excess geranylgeranyl pyrophosphate, which links this mechanism back to the bisphosphonate inhibition of the mevalonate pathway. It seems that the cellular outcome is dependent on the balance of the proapoptotic and antiapoptotic signals. It is therefore possible that completing of nitrogen-containings bisphosphonate therapy with e.g. p38 inhibitor could shift this balance to the most favorable outcome.

2.4. Animal models to study prostate cancer

Prostate cancer can be studied in a limited manner using cell or tissue cultures. However, *in vivo* models are needed to investigate issues related to tumor forming and metastasis. Prostate cancer is the most common cancer in Western males but it occurs rarely in other animal species and only few experimental models of spontaneous prostate cancer exist. An appropriate animal model mimics the key features of human prostate adenocarcinoma, such as response to androgens, relatively slow growth and metastases (Bostwick, Ramnani & Qian 2000). The optimal model should also have similar embryological origin, biological behavior, histology, biochemical properties and molecular and genetic characteristics as human disease. In addition, they should be easy to maintain in large numbers and have stable features. Tumors should be easily and successfully implanted or induced (Geldof, Rao 1990, Pollard 1998, Wang, Wong 1998, Bosland 1999).

Figure 5. Anatomy of human and rodent prostate. Human prostate is a nut-shaped gland surrounding the urethra. Also mouse prostate surrounds urethra (U) but has several lobes: ventral lobe (VP), dorsal lobe (DP and lateral lobe. Anterior prostate *i.e.* coagulating gland is not considered as a part of prostate, instead it is included to sexual glands as well as seminal vesicles (SV). Prostate consists of secreting ducts (D) and stroma (S). In humans, the proportion of stroma is larger compared with mouse prostate. In mouse prostate, the dorsolateral lobe has common features with the human prostate. DD = Ductus deferens (Modified from Wikimedia Commons and tvmouse).



There are differences in the macroscopical anatomy and histology of mouse and human prostate glands. Both have similar epithelial cell types and the prostates are composed of glands and ducts although in different ratios (Sugimura, Cunha & Donjacour 1986, Roy-Burman et al. 2004) (Figure 5). Mouse models provide the possibility of environmental and genetic manipulation, and hereby several groups have reported prostate tumors in various transgenic mouse lines, which may be used to study prostate cancer.

There are several good models to study prostate cancer, but not one that would be ideal. Each model has its strengths and weaknesses, meaning the researcher has to consider the most suitable model to test the hypothesis.

2.4.1. Xenograft models to study prostate cancer

The term *xenograft* means inoculation of tumor cell suspension or implantation of tissue pieces from *foreign* species. Usually human material and immunodeficient mice are used. If the transplantable material is from the *same* species, it is called an *allograft*. Tumor transplants from genetically nearly identical individuals *e.g.* material from the same inbred mouse strain produce syngeneic tumors, and no immunocompromised animals are needed. Knowledge of the genomic profile, such as PTEN mutation, is an important tool for selecting an appropriate preclinical model, which mimics a specific disease stage. Xenogratfs allow the production of an adequate number of tumors at the same time, they are easy to maintain and the studies are repeatable.

The subcutaneous model is the most commonly used xenograft model (Sharkey, Fogh 1979). It is an appropriate model to study tumor growth and morphology with however, certain limitations. In subcutaneous tumors, the blood and lymphatic vasculature is often incompletely formed, which has to be taken into consideration when comparing the treatments. Tumor spread and metastasis formation is also restricted. In order to mimic the metastatic disease, where cancer cells have escaped from the primary tumor to the circulation, cells can be inoculated into the blood stream either via the tail vein (Shevrin, Gorny & Kukreja 1989) or via the left cardiac ventricle (Wu et al. 1998). These metastasis models are very suitable for studying bone metastases, which are important metastastic sites for prostate cancer, but very difficult to reach with other xenograft models. Tumor cell -bone interactions can be studied using intratibial inoculation, where tumor cells are inoculated to bone marrow cavity of mouse tibia (Corey et al. 2002, Valta et al. 2008, Wakchoure et al. 2009). However, using this model, several steps of metastasis cascade remain unstudied.

When tissue-specific targeted therapies are studied, the inoculation environment becomes crucial. Orthotopic human tumor xenografts provide the most relevant stromal environment to study prostate tumor growth, invasion and metastasis. In the orthotopic prostate tumor model, the tumor cells are inoculated into the prostate of immunodeficient mice. The cells form primary tumors in the prostate interacting with the mouse prostate stroma (Stephenson et al. 1992, Vieweg et al. 1994, Waters, Janovitz & Chan 1995, Rembrink et al. 1997). Orthotopic prostate tumor forms metastases to prostate-draining lymph nodes or to distant sites such as the lungs depending oo the characteristics of the inoculated cell line (Stephenson et al. 1992).

The widely used xenograft models of prostate cancer are androgen-independent PC-3 and DU-145 cell lines and androgen-dependent LNCaP cells (van Bokhoven et al. 2003, van Weerden, Bangma & de Wit 2009). PC-3 and DU-145 lack the expression of AR as well as secretion of PSA, which both are characteristic of hormone-responsive prostate cancer (HRPC). LNCaP cells are more close to HRPC expressing AR and PSA but have limited tumorigenity. LNCaP response to antiandrogens is aberrant due to a point mutation in a ligand-binding domain of the

AR, whereupon they are also sensitive to other hormones such as progesterone and estrogen (Veldscholte et al. 1990, Veldscholte et al. 1992).

There is also a panel of transplantable xenografts, which have not been able to grown as cell culture. These xenografts have interesting characteristics that mimic human disease (van Weerden et al. 2009). Implanting of them into the prostate is demanding, since the implanted piece should be small enough not to be necrotic and simultaneously it should contain tumor cells instead of only stroma in order to produce prostatic tumors. The benefit of the tumor implants is that they do not disseminate to the bloodstream or to lymphatic draining artificially (too quickly) and so resemble human disease. The tissue architecture with tumor-stroma interaction can also support tumor growth and improve the occurrence of metastasis (An et al. 1998).

2.4.2. Transgenic mouse models to study prostate cancer

Advances in knowledge of molecular biology of human malignancies have led to a variety of transgenic models for prostate cancer. In these models, the heterologous genes are targeted to the prostate epithelium. The most commonly used promoter elements are rat probasin-gene (PB), the rat C3 prostate steroid-binding protein gene, the human prostate specific antigen (PSA) gene, mouse mammary tumor virus (MMTV) long terminal repeat (LTR), and the mouse cryptin gene (Green et al. 1998, Bosland 1996, Bhatia-Gaur et al. 1999, Garabedian, Humphrey & Gordon 1998). Genetically engineered mice overexpress a transgene (oncogene or point mutation), knock-in gene of genetic point mutation or either complete or conditional (cre-lox system) knock-out gene in their prostate (Carver, Pandolfi 2006, Van Dyke, Jacks 2002, Adams, Cory 1991). Transgenic models are useful for studying therapeutic strategies, genetic characteristics, biological behavior, biochemical properties, histology, and cancer progression. However, transgenic models are not ideal for testing hormone dependent cancer, since most of the models are androgen-independent.

The SV40 small and large T-antigens are widely used in transgenic lines. These viral antigens are potent inactivators of tumor-suppressor proteins and their expression in the prostate epithelium enhances the development of neoplasia in the prostate (Gingrich et al. 1996, Kasper et al. 1998). The TRAMP (transgenic adenocarcinoma mouse prostate) model was developed in 1995 and has been used extensively as a prostate cancer model over the past decade (Greenberg et al. 1995). TRAMP mice provide a suitable animal model to study chemopreventive and therapeutic targets for prostate cancer (Gingrich et al. 1996). TRAMP mice express PB-SV40 T antigen in the ventral and dorsal prostate and develop adenocarcinoma in the dorso-lateral prostate at the age of 12 weeks. The tumors metastasize to para-aortic lymph nodes and lungs. Occasionally, metastases are also found in bone or in other distant sites (Gingrich et al. 1996). These mice provide a good experimental model to study primary neoplasia of the prostate. However, metastases develop at a relatively slow

frequency (Roy-Burman et al. 2004). Shirai and colleagues have developed another SV-40 Tag -targeted probasin promoter rodent model using Sprague-Dawley rat (Asamoto et al. 2001). Rat prostates are more suitable for protein analysis due to their bigger size, but the weakness of the model is that cancer develops mainly in the ventral prostate (Harper et al. 2009).

The tumor suppressor gene PTEN is mutated in a variety of human malignancies (Steck et al. 1997). One allele of PTEN is lost approximately in 70% of human prostate cancers, and homozygous inactivation of PTEN is associated with advanced disease. In order to define the importance of PTEN level in prostate cancer progression, several transgenic models ($PTEN^{+/-}$, PTEN hypomorphic and PTEN conditional knock-out) have been created (Wang et al. 2003, Simpson, Parsons 2001, Gray et al. 1998, Di Cristofano et al. 1998, Trotman et al. 2003). Loss of one allele of PTEN is associated with the development of HG-PIN with incomplete penetrance after a long latency. In the hypomorphic model (30% reduction of PTEN), invasive prostatic adenocarcinoma develops at incomplete penetrance. Complete loss of PTEN results in the development of invasive prostate cancer with complete penetrance after a long latency (6 months) (Carver, Pandolfi 2006, Trotman et al. 2003). Conditional loss of PTEN also results an invasive prostate cancer (Wang et al. 2003). These tumors metastasize to the lymph nodes and lung, which was not detected in other transgenic PTEN models.

An increased copy number of the c-Myc gene is common in human cancers. Overexpression of c-Myc has been used in transgenic mouse lines (Ellwood-Yen et al. 2003). In these mice, PIN-lesions develop at the age of 2 to 4 months and invasive prostate tumors, which metastasize to the regional lymph nodes and to the lung, in 6 to 12 months of age. Overexpression of c-Myc leads to a gene profile similar to human c-Myc-positive prostate cancer including disappearance of NKX3.1 during tumorigenesis (He et al. 1997) and co-expression of Pim-1 kinase, which is shown to co-operate with c-Myc in human prostate cancer progression (van Lohuizen et al. 1989, van Lohuizen et al. 1991).

2.4.3. Hormonally or chemically induced prostate cancer models

Rats are mainly used to produce hormonally or chemically induced prostate tumors, since mice are resistant to induction of prostatic tumors by chemical carcinogens. The induction of prostate cancer is to some extent dependent on the used strain. Lobind Wistar as well as ACI/seg rats have increased incidence of spontaneous prostate neoplasia, probably due to high circulating concentration of testosterone (Blouin, Basle & Chappard 2005). These spontaneous adenocarcinomas develop at a very old age (24-33 months) (Ward et al. 1980). Noble rats have a very low incidence of spontaneous prostatic carcinoma. Prostate and seminal vesicle adenocarcinomas can be induced in Noble rats with testosterone/estradiol (Noble 1977, Bernoulli et al. 2007, Bernoulli et al. 2008b, Bernoulli et al. 2008a, Yatkin et al. 2009) or MNU(1-

methyl-1-nitrosurea)/testosterone (Bosland 1999, McCormick et al. 1998) combinations. When Lobound Wistar rats are treated similarly, the incidence of tumors increases and the age of occurrence lowers (Pollard 1998, Pollard 1992). Other genotoxic chemical compounds used to induce prostatic carcinomas are N-nitrosobis (2-oxoprobyl) amine (BOP), 3,2'-dimethyl-4-amino-biphenyl (DMAB) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine (PhIP) (Shirai et al. 2000).

3. AIMS OF THE PRESENT STUDY

The purpose of this study was to establish and investigate appropriate tumor models to study prostate cancer growth and metastasis *in vivo*.

The specific aims were:

1. to establish an orthotopic model of prostate cancer
2. to use the model in studies on effects of bisphosphonate alendronate on prostate tumor growth and metastasis
3. to study the role of angiogenesis and lymphangiogenesis in the growth and invasion of orthotopic prostate tumors
4. to characterize the microenvironment of angiogenic PC-3 tumors expressing FGF8b and VEGF

4. MATERIALS AND METHODS

4.1. REAGENTS (I-IV)

Dulbecco's (DMEM) cell culture medium, FBS and antibiotics were purchased from Gibco BRL (Paisley, Scotland, UK). L-glutamine was purchased from Fluka (Buchs, Switzerland). Trypsin/EDTA and PBS were from Biochrom AG, Germany, and ß-actin was from Sigma. The enhanced chemiluminescence detection system (ECL) and heparin-sepharose were from Amersham Pharmacia Biotech (Uppsala, Sweden). Colored size markers for Western blotting were from BioRad (Hercules, CA). Trizol reagent was from Invitrogen (Carlsbad, CA). The RNAlater and RNeasy Mini Kit were from Qiagen (Germany) and PAXgene solution from PreAnalytics (Switzerland). The high Capacity cDNA Archive Kit was purchased from Applied Biosystems (Foster City, CA, USA). Alendronate was obtained from Merck & Co., Inc., (Whitehouse Station, NJ, USA). Green Dye 33022 for cell suspension, used in the orthotopic inoculation was from Roberts Oy, Turku, Finland. Pepsin was from Merck, Darmstadt, Germany. A mouse-on-mouse kit and Vectastain ABC reagent for immunohistochemistry were from Vector Laboratories (Burlingame, CA). All plastic culture dishes and plates were from Nunc (Roskilde, Denmark). Temgesic® was from Schering-Plough (Brussels, Belgium) and Isoflurane was from Baxter Oy (Helsinki, Finland).

4.2. ANTIBODIES (I-IV)

The antibodies used in Western Blotting were goat polyclonal anti-human VEGF antibody and mouse monoclonal anti-FGF8b neutralising antibody, both R&D systems (Minneapolis, MN), and goat polyclonal anti-human VEGF-C were from Abcam (UK) and goat polyclonal FGFR2 antibody C-17 was from Santa Cruz Biotechnology (CA, USA). Horseradish peroxidase-labeled antigoat IgG from DAKO (Denmark) or horseradish peroxidase-labeled anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc, USA) was used as a secondary antibody.

The antibodies used in immunohistochemistry were rat anti-CD34 from Santa Cruz (USA), CD31 rat anti-mouse IgG from PharMingen (A Becton Dickinson Company, CA, USA), goat polyclonal VEGFR2-antibody from R&D (UK), VEGFR3-antibody was obtained as a gift from Prof. Kari Alitalo, (University of Helsinki, Finland, Kubo et al. 2002), mouse monoclonal anti-Ki67 was from Novocastra Laboratories Ltd. (Newcastle upon Tyne, UK), rabbit polyclonal anti-Caspase 3 was from Labvision (CA, USA), mouse monoclonal anti-HIF-1 was from BD Biosciences (CA, USA), rabbit anti-human GLUT1 was from Alphadiagnostics (TX, USA), and mouse monoclonal anti-LYVE-1, which was obtained as a gift from Dr. Jackson (MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, Jackson et al. 2001). The secondary antibodies used in immunohistochemistry were biotin-labeled rabbit anti-mouse from DAKO (Denmark A/S, Glostrup, Denmark) or

Vectastain (CA, USA). The Klenow FragEL™ DNA Fragmentation Detection Kit was purchased from Calbiochem, Germany.

4.3. CELL CULTURE AND MOLECULAR BIOLOGICAL METHODS (I-IV)

4.3.1. Cell culture (I-IV)

The human androgen independent prostate cancer cell line PC-3 was obtained from the American Tissue Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented (10%) with heat-inactivated calf serum and 2 mM L-glutamine. In order to determine proliferation, the cells were plated at a density of 40×10^3 cells/well in 24-well plates. Cell numbers were counted using a Coulter Counter (Coulter, Harpenden, UK) according to the manufacturer's instructions.

4.3.2. Transfection of PC-3 cells with VEGF, VEGF-C and FGF8b (II-IV)

The expression vector pcDNA3.1 (Invitrogen, CA, USA) containing the human VEGF, VEGF-C (both provided by Prof. Kari Alitalo, University of Helsinki, Finland) or FGF8b (provided by Dr. P. Roy-Burman, Keck School of Medicine, University of Southern California, CA) cDNA in an EcoRI site and empty pcDNA3.1 vector (mock) were used. Plasmids (2 μg/well on a six-well plate) were transfected into PC-3 cells using LipofectAMINE (Invitrogen, CA, U.S.A) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were transferred to selection medium containing neomycin (500 μg G418/mL) for 3 weeks. Isolated colonies were cloned. Cell clones were characterized by Northern and Western blotting methods and one PC-3/VEGF clone, one PC-3/VEGF-C clone, two PC-3/FGF8b clones (1 and 15) and one PC-3/mock clone were used in the *in vivo* studies.

4.3.3. RNA analysis (I-IV)

The cells were scraped from the dishes, and the tumors were homogenized by Ultra-Turrax (Rose Scientific Ltd., Canada). Total RNA was extracted from PC-3 cells using either the guanidinium isothiocyanate method (Chomczynski, Sacchi 1987) or Trizol reagent. For microarray analyses, RNA was further purified by means of RNeasy Mini Kit and DNAsel (both Qiagen Germany) according to the manufacturer's instructions. The quality of RNA was detected by spectrophotometry on formaldehyde gels or by Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Northern blotting, RT-PCR and Southern blotting were performed as previously described (Ruohola et al. 1995).

4.3.4. Western blotting (I-IV)

Serum-free DMEM conditioned by PC-3 cell clones for two days was harvested from the cultures. The conditioned medium was centrifuged to remove cell debris. Heparin-sepharose-bound proteins conditioned by 3×10^6 cells from tumors were extracted by 5 min incubation in Laemmli sample buffer at 95°C and separated by 10% SDS-PAGE gel electrophoresis. After transfer to nitrocellulose membranes (Bio-Rad), proteins were detected using a 1:500 dilution of VEGF, VEGF-C, FGF8b or FGFR2 antibodies. A secondary antibody, horseradish peroxidase-labeled anti-goat or anti-rabbit IgG was used at 1:2000 dilution. Protein bands were visualized using the ECL chemiluminescence detection system.

VEGF-C protein concentrations from cell lysates and 24 h conditioned media of cell clones were further quantified by ELISA (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. Data were expressed as VEGF-C protein concentration (ng/mL) corresponding to 10^6 cells.

4.3.5. Zymography (III)

Samples of the media conditioned by PC-3, PC-3/FGF8b or PC-3/mock cells were lyophilized to correspond to 200,000 cells. Proteins were separated under non-reducing conditions in 10% SDS-PAGE gel electrophoresis containing gelatin (1 mg/ml). After electrophoresis, gels were washed three times with 50 mM Tris-HCl, pH 7.5, 2.5% Triton X-100 to remove SDS. Gelatinolytic activity was developed by incubation in 50 mM Tris-HCl, pH 7.5 containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ for 72 hours at 37°C. Gels were fixed for 1 hour with acetic acid:methanol:water (7:50:43, v/v/v) and stained with 100 mL of 0.002% Coomassie Blue G250. Gelatinolytic activities of MMP-2 and MMP-9 were observed as transparent bands on dark blue-stained gel. Conditioned medium from HT-1080 human fibrosarcoma cells was used as a positive control for the production of MMP-2 (62 kDa), proMMP-2 (72 kDa) and proMMP-9 (92 kDa) (Ruohola et al. 2001).

4.4. TUMOR MODELS

4.4.1. Animals (I-IV)

Eight week old male athymic Balb/C nu/nu mice (Harlan, Netherlands) were maintained in a pathogen-free environment, one animal per cage under controlled environmental conditions. The animal experiments were carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and the Statute 1076/85 § and 1360/90 of The Animal Protection Law in Finland and EU Directive 86/609. The experimental procedures were reviewed by the local Ethics Committee on Animal Experimentation in the University of Turku and approved by the local Provincial State Office of Western Finland.

4.4.2. Subcutaneous tumors (II-IV)

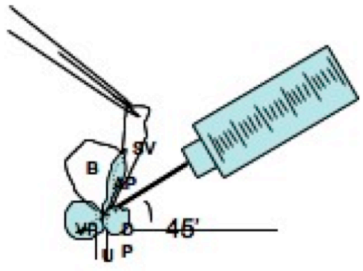
Parental human prostate carcinoma (PC-3) tumor cells or PC-3 cells transfected with VEGF-C (PC-3/VEGF-C), VEGF (PC-3/VEGF), FGF8b (PC-3/FGF8b) or a control vector (PC-3/mock) were inoculated subcutaneously (5×10^6 cells in 100 µL PBS) into the back of the neck of athymic nude mouse. Animal welfare was monitored daily for clinical signs and animals were weighed once a week. The tumors were allowed to grow for four to six weeks.

4.4.3. Orthotopic prostate tumors (I-III)

At near confluence, the PC-3 cells were harvested and suspended in a sterile dye solution consisting of phosphate-buffered saline with green food color (0.5 µg/ml; 33022, Roberts Oy).

Half an hour before inoculation of tumor cells, an analgesic drug was injected subcutaneously. The mice were anesthetized and placed in a supine position under a sterile cover. An incision was made 3 mm above the pubic symphysis and the bladder and seminal vesicles were carefully lifted to expose the dorsal prostate. Then, 5×10^5 cells with green dye were slowly inoculated into the ventral prostate through the dorsal prostate at a 45° angle (Figure 5). The inoculation was made close to the midline, avoiding the urethra. The success of inoculation could be verified by means of the green color added to the cell suspension. Therefore, if leakage into the peritoneal cavity or urethra/bladder was observed the mice were not included in the experiment. Inoculation was performed with a 30 G needle attached to a 25 µL glass syringe (both Hamilton Bonaduz, Switzerland). After inoculation, the abdominal muscle layer was closed with a 4-0 absorbable suture (Bondek plus, polyglycolic acid-coated suture, Genzyme, Germany) and the skin was closed with a 4-0 nonabsorbable suture (monofilament, polyamid suture, Genzyme, Germany).

Figure 6. Orthotopic inoculation. Seminal vesicles (SV) were carefully lifted to expose prostate. Orthotopic inoculation was performed to ventral prostate (VP) through dorsal prosate avoiding urethra (U). AP = Anterior prostate, B = Bladder.



The welfare of the mice was monitored daily and the mice were weighed once a week. Tumors were allowed to grow for four weeks. The appropriate study period and inoculated cell number were determined according to a previous experiment, where two cell numbers (5×10^5 and 1×10^6) and time periods (4 and 8 weeks) were used (unpublished data of author).

4.4.4. Treatment of mice with alendronate (I)

In order to study the effects of alendronate on prostate tumor growth and invasion, mice were randomized according to weight into 2 groups (*n*=15-16 mice/group). Mice in the alendronate group were daily treated with alendronate (0.01 mg/mouse/d s.c.) in 100 µL PBS. Mice in the control group were injected daily with 100 µL PBS. Alendronate or control treatment was started on the day of the orthotopic inoculation of 5×10^5 PC-3 cells. Mice were sacrificed 4 weeks after inoculation.

4.4.5. Blocking the VEGFR3 using AdVEGFR3-Ig (II)

Recombinant adenoviruses (a gift from Professor Seppo Ylä-Herttuala, Kuopio, Finland) expressing the VEGFR3-Ig fusion protein (AdVEGFR-3Ig) (Zeng et al. 2006) or ß-galactosidase (AdLacZ) were administred via the tail vein 5 minutes before PC-3 or PC-3/VEGF-C cell inoculation. The dose used in this experiment was 1.2 x 10^8 pfu in 50 µL (Isaka et al. 2004).

4.4.6. In vivo *measurements (II-IV)*

4.4.6.1. Volume of the subcutaneous tumors (II-IV)

Tumor measurements were performed twice a week and the tumor volume was counted according to the following formula: $V=(\pi/6)(d_1 x d_2)^{3/2}$, where d_1 and d_2 are perpendicular tumor diameters (Wärri et al. 1993).

4.4.6.2. Radiography (II-III)

The bones of orthotopically inoculated mice were subjected to X-ray analysis by means of a faxitron MX-20 DC-2 X-ray cabinet (30 kVp, 6s, Faxitron X-ray Corp., Il, USA). The mice were radiographed two weeks and four weeks after inoculation of the tumor cells.

4.4.6.3. Oxygen partial pressure (pO_2) measurement (IV)

In order to measure the pO_2 values in the tumors, the Licox® Revoxode CC1.2 polarographic probe supplied with a probe-specific microchip allowing automatic calibration was used. The probe was inserted into the subcutaneous tumor tissue by advancing it retrogradely along an insertion needle catheter lumen, which was then

removed. Tissue temperature was measured with a needle probe and temperature-adjusted pO_2 (mmHg) was graphically displayed and stored digitally. The whole length of the oxygen-sensitive part of the probe was at least 2 mm inside the tumor throughout the measurements to prevent contamination from the room-air pO_2. The duration of the pO_2 measurement was sufficient to establish a stable pO_2 level, which was then registered and stored. Three tumors from PC-3/FGF8b, PC-3/VEGF and PC-3/mock groups were measured over a time period of 20 min. The gluteus muscle of the experimental animal served as a control site after measurements to verify the proper function of the Licox® probe.

4.4.7. Characterization of the tumors

4.4.7.1. Histology (I-IV)

The subcutaneous or orthotopic prostate tumors were exposed by removing the overlaying skin and the volume was measured according to the method of Janik and coworkers (Janik, Briand & Hartmann 1975) ($\pi/6 \times d_1 \times d_2 \times d_3$). The prostate lobes, selected internal organs (lungs, kidneys, adrenal glands, liver and spleen), regional para-aortic lymph nodes (iliac and sacral), distant lymph nodes (inguinal, sciatic, axillary and brachial), hind limbs and vertebrae were macroscopically examined for the occurrence of malignancies and immersed in 4% neutral-buffered formalin. Bone samples were decalcified in 10% EDTA-solution for two weeks before further processing. All samples were embedded in paraffin and 5 µm sections were stained with hematoxylin and eosin (H&E) using standard techniques. The relative area of metastases in the prostate-draining lymph nodes was determined from H&E stained sections with an analysis software for histomorphometry (ImageJ, 1.37v, Wayne Rasband, National Institutes of Health, USA or AxioVision 4.3 software, Carl Zeiss GmBH, Germany).

4.4.7.2. Immunohistochemistry (I-IV)

Tumor tissue sections were cut on silane coated glass slides. Paraffin-embedded sections were deparaffinized and rehydrated. The endogenous peroxidase activity of paraffin-embedded and frozen sections was blocked by incubation in 3% peroxide in methanol. In order to minimize non-specific binding, the slices were pre-incubated in the normal goat or horse serum. Then, the sections were immunostained with CD34, CD31, m-LYVE-1, VEGFR2, VEGFR3, Ki67 or GLUT1 antibodies. The biotin-labeled rabbit anti-mouse was used as a secondary antibody. A mouse-on-mouse kit was used with Ki67 antibody staining to inhibit non-specific staining of anti-mouse secondary antibody. Immunoperoxidase staining was performed using an ABC kit (Vector laboratories, California, USA). Slides were stained with diaminobenzidine (DAB) and counterstained with Meyer's hematoxylin. The ISEL method (*in situ* end labeling, Calbiochem, Germany), which detects DNA fragmentation, was used to

detect apoptotic cells. Negative controls (sections of every sample stained without the primary antibody) were used to verify the specificity of staining.

Three representative microscope fields inside each tumor were analyzed (Leica DMRB, Leica Microsystems, Heerburg, Germany); the images were taken with a digital camera (Leica DC 300F, Leica Microsystems, Heerburg, Germany). The length of the CD34, CD31, m-LYVE-1, VEGFR2 and VEGFR3-positive vessels was counted from the three most representative fields of each tumor by drawing lines following stained vessels and measuring the length of lines using ImageJ or AxioVision softwares (ImageJ, 1.37v, Wayne Rasband, National Institutes of Health, USA, and AxioVision 4.3 software, Carl Zeiss GmBH, Germany, respectively). The relative number of Ki67- and GLUT1-positive cells was counted on three non-overlapping sections at 500 μm intervals. Altogether, 1.000 cells/tumor were counted. The relative area of necrosis (number of necrotic squares from one representative whole tumor section) was determined using a 10 × 10 ocular grid and presented as percentage. The results were blind-tested by two independent analysts comparing study versus control.

4.4.7.3. Analysis of metastases by RT-qPCR (III)

Total RNA from serum, lung, liver and bone marrow samples was extracted using an RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions, and a known amount of internal standard molecule (Nurmi, Lilja & Ylikoski 2000) was added to each sample as RNA after cell lysis. RNA was transcribed into cDNA with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). PCR analyses were performed according to previously described protocols (Väänänen et al. 2008). Briefly, quantitative RT-qPCR assays, with real-time detection based on time-resolved fluorescence, were used to determine both FGF8b mRNA and internal standard RNA levels and original FGF8b expression levels were calculated based on these results. The external standard curve consisted of three points between 1.3×10^2 and 1.3×10^5 molecules per milliliter and standards were prepared as previously described (Väänänen et al. 2008).

4.4.7.4. Tumor perfusion analysis (IV)

The perfusion marker Hoechst 33342 (15 mg/kg) was intravenously injected into subcutaneous tumors-bearing nude mice 2 minutes before sacrifice. Frozen, 8 μm tumor tissue sections were fixed in 4% paraformaldehyde for 1 h at 4ºC. Images were acquired from frozen sections using a Zeiss AxioVert 200M fluorescence microscope (Carl Zeiss GmbH, Germany).

4.4.7.5. Flow cytometry (IV)

Subcutaneous tumors were collected in ice cold PBS supplemented with pepsin (0.5%, pH 1.4) immediately after sacrifice of the mice. Tumor tissue was

homogenized with Ultra-Turrax (Rose Scientific Ltd., Canada) and incubated at 37°C for 1 h. The suspension was collected and filtered prior to analysis. For cell cycle analysis, tumor cell homogenate was incubated in hypotonic buffer (1% Triton X-100, 0.05 mg/mL propidium iodide, PI, in PBS) for 20 min at 4°C. The cell cycle phases (G0/G1, S and G2/M) and the fraction of apoptotic cells were calculated with ModFit cell cycle modeling software (Verity Software House, Inc., ME, USA) For cell enumeration, cells were analyzed using TrueCount tubes (Becton Dickinson, MD, USA). Briefly, the amount of cells in the tube was calculated by comparing the previously known amount of beads in the tube with cell numbers in a known volume. All flow cytometry was performed using a FACSCalibur (Becton Dickinson) flow cytometer and CellQuestPro software (Becton Dickinson).

4.4.8. Evaluation of a hypoxia marker [^{18}F]EF5 (IV)

4.4.8.1. Synthesis of [^{18}F]EF5 and [^{18}F]FDG (IV)

^{18}F-EF5 (^{18}F-2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)-acetamide) was synthesized from 2-(2-nitro-1H-imidazol-1-yl)-N-(2,3,3-trifluoroallyl)-acetamide using high specific radioactivity ^{18}F-F_2 as the labeling reagent (Bergman, Solin 1997). The specific radioactivity of [^{18}F]EF5, decay corrected to the end of synthesis, exceeded 3.7 GBq/μmol. Radiochemical purity was higher than 98.5 % in every production. [^{18}F]FDG was synthesized from mannosyl triflate using a nucleophilic method. Radiochemical purity exceeded 95% and specific radioactivity was approximately 74 GBq/μmol at the end of synthesis.

4.4.8.2. [^{18}F]EF5 and [^{18}F]FDG uptake in tumors (IV)

[^{18}F]EF5 and [^{18}F]FDG were intravenously injected (5.6 ± 1.1 and 6.6 ± 1.4 MBq, respectively) into separate subcutaneous tumor-bearing mice, and allowed to distribute 120 minutes before sacrifice. Plasma glucose levels were measured (Analox GM9, Analox Instruments Ltd, London, UK) from blood samples obtained by cardiac puncture. Blood, tumor and muscle tissues were rapidly removed, counted for ^{18}F-radioactivity in a well counter (3" x 3" NaI (TI) crystal, Bicron 3MW3/3P, Bicron Inc., Newbury, Ohio; USA) and weighed. The uptake of ^{18}F-radioactivity in tissues was calculated as percentage of injected dose per gram tissue weight taking into account the background from the counter measurements and the decay of radioactivity. Tumor-to-blood uptake ratios (T/B ratios) were then calculated for both [^{18}F]EF5 and [^{18}F]FDG.

4.4.8.3. Intratumoral distribution of ^{18}F-radioactivity (IV)

The intratumoral biodistribution of [^{18}F]EF5 in tumors was studied using digital autoradiography (Fujifilm BAS 5000, Fuji Photo Film Co. Ltd., Tokyo, Japan). After sacrifice, tumors were removed, rapidly frozen in dry ice/isopentane and cut with a

cryomicrotome into 20 μm sections. Muscle tissue samples were frozen and cut similarly in order to have a reference for radioactivity uptake. Tumor sections were then exposed on an imaging plate (Fuji BAS TR2025, Fuji Photo Film Co., Japan) for 4.0 ± 0.5 hours. The spatial distribution of radioactivity from tumor and muscle sections was recorded autoradiographically with a phosphoimager (Fujifilm BAS-5000, Fuji Photo Film Co. Ltd., Tokyo, Japan). The dynamic linear range of this system is four decades, and the resolution (i.e. pixel size) of the images is 25 × 25 μm.

4.4.8.4. Analysis of glucose metabolites (IV)

The amount of 2-[^{18}F]fluoro-2-deoxyglucose-6-phosphate ([^{18}F]FDG-6-P) was determined from tumor homogenates from animals injected with [^{18}F]FDG by the use of radioHPLC (Merck Hitachi).

4.4.9. Microarray analysis (III)

4.4.9.1. Microarray hybridization (III)

In the first array Agilent Human 1A (V2) oligo microarray containing 22,575 genes was used for studying gene expression differences between PC-3/FGF8b and PC-3/mock cells. Total RNA was used for cDNA synthesis, and *in vitro* transcription was carried out using an Agilent Low RNA input fluorescent linear amplification kit (Agilent Technologies). The fluorescent complementary RNA was used for oligo microarray hybridization at the Biomedical Biochip Center (Helsinki, Finland). In the second array, Agilent Mouse two-color cDNA microarray containing 9,607 features was used for studying gene expression differences between PC-3/mock and PC-3/FGF8b orthotopic prostate tumors. Total RNA was used for cDNA synthesis, and *in vitro* transcription was carried out using an Agilent Direct labeling kit (Agilent Technologies). Two biological replicates of PC-3/FGF8b tumors and 3 biological replicates of PC-3/mock tumors were hybridized to chips. Both PC-3/FGF8b samples were hybridized with each PC-3/mock sample so that there were 6 hybridizations altogether.

4.4.9.2. Microarray data analysis (III)

Gene Spring 7.2 software (Agilent Technologies) was used for analysis of the first array data of cells. To be included in the analysis, a gene had to have a *t*-test *p*-value of < 0.05 in all inspected conditions. Further a criterion of a more than 2-fold difference between test and control expression values was set. After normalization, a total of 19,925 genes passed the quality filtering, 43 genes were found to be significantly deviated and more than 2-fold upregulated and 126 genes more than 2-fold downregulated when comparing PC-3/FGF8b *vs.* PC-3/mock samples. For analyzing the function of these up- and downregulated genes, PubMed and GeneSpring gene annotation update tools were first used to annotate the genome as

fully as possible by using GenBank Accession numbers, UniGene Cluster Numbers, Locus Link gene symbols (NCBI, NIH), and GO (Gene Ontology) terms. Ingenuity Pathways Analysis (IPA, Ingenuity Systems) was also used for analysis of the functional networks of the genome.

R software (Development core team 2005) was used for analysis of the second array data of tumors. After normalization, altogether 55 genes were found to be more than 2-fold upregulated and 6 genes were found to be more than 2-fold downregulated when comparing PC-3/FGF8b *vs.* PC-3/mock tumors. Ingenuity Pathways Analysis and the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis et al. 2003) were used to analyze the data from Agilent mouse microarrays.

4.4.9.3. Quantitative RT-PCR analysis of the differentially regulated genes (III)

Two µg of total RNA was used to make cDNA via reverse transcriptase (M-MLV, Promega Corp., Madison WI, USA). cDNA was amplified by DyNAzyme II DNA Polymerase (Finnzymes, Finland) and Eppendorf Mastercycler Gradient (Eppendorf AG, Hamburg, Germany) and diluted. cDNA was used in a probe-based qPCR method, ProbeLibrary (ProbeLibrary, Roche Diagnostics GmbH, Roche Applied Science, Switzerland). Primers were made by Oligomer (Helsinki, Finland) and the human Universal Probelibrary set was from Roche. ProbeLibrary qPCR was performed with an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA) using the ABgene PCR ROX master mix (ABgene, Epsom, UK) at the Turku Centre for Biotechnology (Turku, Finland). Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to normalize the data.

4.5. STATISTICAL ANALYSES

Except for the microarray data, statistical analyses were carried out using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, California USA) based on the distribution of the data (normal or nonparametric) and sample size. The normality of distribution was tested by means of Shapiro–Wilks' *W*-test. The statistical significances of differences were tested by means of independent *t*-test, the non-parametric Mann–Whitney *U*-test or Kruskal-Wallis test with Dunn's post or Tukey's test. A *p*-value less that 0.05 was considered statistically significant.

5. RESULTS AND DISCUSSION

5.1. ALENDRONATE AND PROSTATE CANCER

The orthotopic prostate tumor model was first used to study the effects of an amino-bisphosphonate, alendronate, on prostate tumors.

5.1.1. The effects of alendronate on occurrence of orthotopic PC-3 tumors and tumor metastases

Androgen ablation therapy is the main treatment for metastatic prostate cancer (Huggins 1967, Denmeade, Isaacs 2002). Androgen ablation therapy is an effective treatment for prostate cancer but it has negative effects on bone homeostasis. Several studies have reported that androgen ablation therapy increases osteoporosis. With a history of androgen ablation therapy, 14% of men had osteoporotic hip fractures compared with 1% of prostate cancer patients without androgen ablation therapy (Smith et al. 2007, Daniell 1997). Alendronate (Fosamax®, Merck. & Co. Whitehouse Station NJ) has been approved as treatment of osteoporosis in men as it increases bone mineral density and decreases vertebral fractures by inhibiting osteoclast generation and maturation (Oades et al. 2003, Oades 2003). Bisphosphonate treatment can also be used to inhibit bone destruction or at least relieve bone pain of patients with bone metastasis (Parker 2004, Parker 2005, Saad 2008, Saad et al. 2002, Saad et al. 2004).

The effects of alendronate on prostate tumors or other soft tissue had not been demonstrated previously but *in vitro* data from our research group showed its inhibitory effects on migration and invasion on PC-3 cells (Virtanen et al. 2002). This prompted us to study the *in vivo* growth and invasion of orthotopic prostate tumors modeling prostate cancer. In the present study, the mice were treated by daily injections of either alendronate (0.05 mg/kg/day) or PBS (control) s.c. Treatments were started concomitantly with orthotopic inoculation of 5×10^5 PC-3 cells and continued for 4 weeks. Tumor growth in the prostate was observed in most of the nude mice. At 4 weeks, tumor occurrence was 73% in the alendronate-treated group and 81% in the PBS-treated group, which was predicted on the basis of the preliminary experiment with different cell numbers and experimental periods. Results of the present study demonstrate that alendronate is able to inhibit prostate tumor growth. The prostate volume (including tumor) was significantly smaller in the alendronate group than in the control group ($p < 0.05$). A major effect of alendronate, in addition to being associated with a decreased tumor size, was a decreased volume of lymph node metastases. Histomorphometric quantification of the relative area of metastases showed that the proportion of tumor area in the iliac and sacral lymph nodes was significantly smaller in the alendronate group than in the control group ($p < 0.001$). However, the difference in occurrence of metastases between the groups was not statistically significant. Four weeks after inoculation of PC-3 cells, metastases were found in 64% of prostate draining iliac and sacral lymph nodes in the group that received alendronate. In the mice treated with PBS, metastasis in the iliac and sacral

nodes was found in 54% of the animals. Lymph node metastases develop via the lymphatic pathway. Staining of orthotopic tumors with a mouse-LYVE-1 antibody specific for lymphatic endothelium showed a rich network of lymphatic vessels. This may be due to high expression of VEGF-C in PC-3 cells. In the tumors grown for 4 weeks, staining was observed only in the peripheral area of the tumors and in the peritumoral area. No differences were detected in the density of mouse-LYVE-1 - stained lymphatic capillaries between alendronate-treated and control groups.

5.1.2. Effects of alendronate on morphology, angiogenesis, and apoptosis of orthotopic PC-3 tumors

No changes in morphology were observed in histological (H&E) staining of the prostate tumors in alendronate *vs*. control groups. To assess the characteristics of tumors in more detail, immunohistochemical stainings were performed. The relative number of apoptotic cells was determined by means of the ISEL method. Apoptosis was increased by 40% in alendronate-treated tumors in comparison to control tumors ($p < 0.05$). This is in agreement with the results of other studies in which *in vitro* cultures (Fromigue, Lagneaux & Body 2000, Jagdev et al. 2001) and *in vivo* tumor models (Zhou et al. 2005) have been used. Relatively high concentrations of bisphosphonates have usually been needed to trigger apoptosis *in vitro,* but *in vivo* bisphosphonate treatments with doses and schedules comparable to those used in clinical treatments have been reported to induce apoptosis of tumor cells in several models as regards both bone (Padalecki, Guise 2002) and visceral metastases (Hashimoto et al. 2005, Hashimoto et al. 2005, Wakchoure et al. 2006, Hiraga et al. 2004, Yamagishi et al. 2004, Ory et al. 2005). This suggests that in addition to direct cellular effects, other mechanisms may also contribute to decreased cancer cell survival *in vivo*.

Another effect that may contribute to decreased tumor size and tumor cell survival in alendronate-treated mice is inhibition of angiogenesis. The density of blood capillaries in orthotopic PC-3 tumors was identified by using anti-CD34 antibody, specific for endothelial cells. The capillary density was significantly lower in the alendronate-treated group than in the control group ($p < 0.001$). This observation is also in accordance with earlier reports on the capacity of various bisphosphonates to inhibit angiogenesis *in vitro* and *in vivo* in the context of some physiological and pathological conditions. Decreased angiogenesis associated with bisphosphonate treatment has been related to modulation of endothelial cell proliferation, adhesion and migration, and apoptosis (Salven et al. 1998, Ledoux et al. 2006). Zoledronate as well as alendronate have been found to inhibit the secretion and activity of MMP-2 and MMP-9 by tumor cells and tumor-infiltrating macrophages in mouse models of cervical (Giraudo, Inoue & Hanahan 2004) and prostate cancer (Stearns 1998). In addition, both minodronate and zoledronate affect VEGF interaction with VEGF receptors and signaling in endothelial cells (Hashimoto et al. 2005, Wakchoure et al. 2006). In our prostate tumors, alendronate facilitated apoptosis and inhibited

angiogenesis, the latter leading to restricted nutrition and oxygenation, thereby affecting tumor growth. There was, however, no statistically significant increase in the proportion of necrotic areas in alendronate-treated tumors. Decreased angiogenesis is also associated with decreased metastatic potential (Kimbro, Simons 2006) and in our study, could contribute as such to the decreased metastatic growth in lymph nodes.

The inhibitory effects of nitrogen-containing bisphosphonates are largely mediated via the mevalonate pathway by impairing the post-translational prenylation of small GTP-binding proteins (Ras, Rac and Rho) (Caraglia et al. 2006, Virtanen et al. 2002). Nitrogen-containing bisphosphonates have been found to target farnesylpyrophosphate and/or geranylgeranylpyrophosphate synthetase, which leads to decreased generation of farnesyl diphosphate and geranylgeranyl diphosphate. These intermediates are needed for post-translational prenylation of Ras, Rac and Rho, which are essential for many cellular functions such as proliferation, survival and invasiveness. It is probable that interference with prenylation reactions is involved in many effects of nitrogen-containing bisphosphonates such as decreased angiogenesis and cell migration as well as increased apoptosis (Caraglia et al. 2006). Recent reports have shown evidence that newer nitrogen-containing bisphosphonates can directly induce apoptosis and/or cause growth arrest in the G2/M phase in the same way as pyrophosphate-resembling bisphosphonates by production of an endogenous ATP analog (Mönkkönen et al. 2006). However, these growth inhibitory effects can be overcome with excess geranylgeranyl pyrophosphate, which links this mechanism to the bisphosphonate inhibition of the mevalonate pathway.

In conclusion, alendronate treatment of osteoporotic men also has other beneficial indications; alendronate inhibits tumor progression by increasing apoptosis and decreasing angiogenesis, which may help to prevent prostate cancer progression and tumor cell growth in distant sites.

5.2. VEGF-C INCREASES GROWTH OF PROSTATE TUMORS AND ALTERS METASTASIS PATTERN

A specific drug target for prostate cancer is the blocking of the signaling of cancer progression-affecting growth factors such as VEGFs. The orthotopic prostate tumor model was next used to study the effects of VEGF-C on prostate tumors and metastasis by blocking the receptor of VEGF-C (VEGFR3) and secondly, by ectopic overexpression of VEGF-C in tumors.

5.2.1. Blocking the VEGFR3 of mice bearing orthotopic PC-3 tumors

The expression of VEGF-C and its receptor VEGFR3 has been shown to correlate with clinical markers in prostate cancer (Zeng et al. 2004, Li et al. 2004). However, it is not clear whether VEGF-C is only a marker of progressed disease or if it also facilitates lymph node metastasis. Experimental models suggest that VEGF-C is

involved in tumor lymphangiogenesis and is often upregulated in metastatic tumors. On the other hand, Padera and coworkers (Padera et al. 2002) and Wong and coworkers (Wong et al. 2005) have shown that lymph node metastasis may develop even without increased lymphangiogenesis.

Our previous studies have shown that orthotopic PC-3 prostate tumors metastasize to prostate-draining lymph nodes via the lymphatic vessels. In order to study the role of VEGF-C and VEGFR3 in that pathway, VEGFR3 was blocked using recombinant adenoviruses expressing the VEGFR3-Ig fusion protein (Ad-VEGFR-3Ig) (He et al. 2005). ß-galactosidase (Ad-LacZ-Ig) -expressing fusion proteins were used as controls. Ad-VEGFR3-Ig or Ad-LacZ-Ig was administered via the tail vein 5 minutes before orthotopic PC-3 cell inoculation. Tumors were allowed to grow for 4 weeks and the effects of VEGFR3-Ig on tumor growth, morphology, lymphangiogenesis, angiogenesis and metastasis on the iliac and sacral lymph nodes and lungs were studied. Tumor occurrence in the prostate was 15/22 (68%) and 21/23 (91%) in VEGFR3-Ig and lacZ-Ig groups, respectively. The mean size of PC-3 tumors was significantly smaller in mice injected with VEGFR3-Ig than in lacZ-Ig -injected control mice (77 ± 30 mm^3 *vs*. 120 ± 71 mm^3, respectively, $p < 0.05$).

VEGFR3-Ig treatment significantly decreased the density of lymphatic vessels as well as blood vessels in orthotopic PC-3 tumors ($p < 0.05$ in both). The histological analysis of prostate-draining lymph nodes showed that VEGFR3-Ig treatment effectively inhibited lymph node metastasis. Tumor metastases were found in 19% of the lacZ-Ig injected tumor-bearing mice but in none in the VEGFR3-Ig mice. As expected, no metastasis was found in the lungs. These results are in line with previous studies (He et al. 2005, Burton et al. 2008) suggesting that VEGF-C signaling via VEGFR3 has a major role in lymph node metastasis. The roles of angiogenic and lymphangiogenic growth factors are partly overlapping. The blockade of VEGFR3 also decreased blood vessel density. VEGFR3 has been shown to contribute to formation of blood capillaries in different tumor models (Laakkonen et al. 2007), which may explain the effects on angiogenesis as well as the decreased sizes of orthotopic PC-3 tumors.

5.2.2. The effects of ectopic VEGF-C on growth of PC-3 tumors

Lymphangiogenic growth factor (VEGF-C) was originally cloned from PC-3 cells (Joukov et al. 1996). To create a stably VEGF-C overexpressing cell line, PC-3 cells were transfected with the pcDNA3.1 vector containing the full coding sequence for VEGF-C (Joukov et al. 1996). One clone expressing VEGF-C mRNA and protein at a high level (PC-3/VEGF-C) was selected for tumor experiments. Both ELISA analysis and Western blot analysis showed overexpression in the PC-3/VEGF-C clone compared with PC-3/mock. Westrn blot analyses revealed that mainly the partially prosessed (29/31 kD) VEGF-C polypeptides were expressed.

First, the tumorigenity of the PC-3/VEGF-C and PC-3/mock cells was tested by inoculating them subcutaneously into the nude mice (n= 8 and 12, respectively). Ectopic expression of VEGF-C increased subcutaneous tumor growth ($p < 0.001$ at all time points between 2 to 4 weeks). At 4 weeks, when the experiment was terminated, the PC-3/mock tumors were small (47 ± 49 mm^3) while the PC-3/VEGF-C tumors were 24 times larger (1132 ± 1132 mm^3).

Then, the PC-3/VEGF-C and PC-3/mock cells were inoculated orthotopically into prostate of the nude mice. The occurrence of tumors was 29/33 (88%) in the PC-3/VEGF-C group and 24/28 (86%) in the PC-3/mock group and they were measured only at the end of the experiment. As in the subcutaneous tumors, the orthotopic tumors overexpressing VEGF-C were markedly bigger (400 ± 281 mm^3 *vs*. 48 ± 19 mm^3, $p < 0.001$). This is in line with clinical data, which shows a correlation between VEGF-C expression and cancer progression (Tsurusaki et al. 1999, Jennbacken et al. 2005).

5.2.3. The effects of VEGF-C on vascularization of the orthotopic PC-3 tumors

The morphology of VEGF-C-expressing tumors differed greatly from that of PC-3/mock tumors. The tumors showed a rich network of capillary-like structures in H&E staining, whereas PC-3/mock tumors were homogenous and solid with only a small number of blood vessels. Blood capillaries were identified by immunohistochemical staining for CD34 and VEGFR2. The length of capillaries was calculated from the three representative fields of tumors. The density of blood capillaries was significantly higher in VEGF-C overexpressing tumors compared with PC-3/mock tumors ($p < 0.001$). The density of VEGFR2 positive vessels was also higher in the PC-3/VEGF-C tumors in comparison to PC-3/mock tumors ($p < 0.05$). This could be explained by the contribution of VEGFR3 to angiogenesis in addition to promoting lymphatic endothelial growth and vessel formation. Another mechanism could be the ability of VEGF-C to bind and activate VEGFR2 that is primarily expressed on blood capillary endothelium (Joukov et al 1997). PC-3 cells transfected with VEGF-C gene expressed and secreted almost exclusively the proteolytically processed VEGF-C form that also binds VEGFR2, although with lower affinity (Joukov et al. 1997).

In VEGF-C overexpressing orthotopic PC-3 tumors and in PC-3/mock tumors, lymphatic vessels were located mainly in the tumor periphery and only few were found within the tumor tissue. Surprisingly, no difference was detected between PC-3/VEGF-C and PC-3/mock tumors. The distribution of lymphatic vessels was also studied using an antibody against VEGFR3. Positive staining was seen mainly in tumor periphery, and again no statistically significant difference in distribution of VEGFR3 positive vessels was detected between PC-3/VEGF-C *vs*. PC-3/mock or PC-3(VEGFR3-Ig) *vs.* PC-3(lacZ-Ig), using anti-VEGFR3 antibody. In addition to LYVE-1 and VEGFR3, there are also other markers to stain lymphatic vessels. These include

Prox-1 and podoplanin (Sleeman, Schmid & Thiele 2009). It has been reported that none of these markers is exclusively or homogenously expressed on tumor lymphatic vessels. VEGFR3 is expressed on tumor-associated angiogenic blood vessels, particularly in tip-cells (Tammela et al. 2008, Valtola et al. 1999), and also in tumor cells (Su et al. 2007). Lyve-1 is expressed in tumor-associated macrophages (Schledzewski et al. 2006, Maruyama et al. 2005). Podoplanin is expressed in tumor cells and also in tumor-associated fibroblasts (Schacht et al. 2005, Kawase et al. 2008). Prox-1 is a most strongly espressed in lymphatic vessels (Kawai et al. 2008). These observations mean that combination of markers is needed in order to reliably identify tumor lymphatic vessels (Sleeman, Schmid & Thiele 2009). Several studies have reported that tumor centers lack functional lymphatic vessels (Padera et al. 2002, Wong et al. 2005) or that it is the peripheral lymphatic capillaries but not those within the tumors that mediate tumor cell spread to lymph nodes (Wong et al. 2005). It has also been claimed that increased tumor pressure within the tumors may compress the thin-walled lymphatic capillaries and compromise their function (Padera et al. 2002). In our experiments, it seems possible that the highly expanded network of blood vessels may have caused pressure that might have impaired or prevented the growth of lymphatic capillaries in the tumor tissue and, consequently, metastasis to the lymph nodes.

5.2.4. The effects of VEGF-C on orthotopic PC-3 tumor metastasis

The occurrence of lymph node metastases four weeks after orthotopic inoculation of PC-3/VEGF-C and PC-3/mock cells was studied by histological and immunohistochemical staining and histomorphometry of the lymph nodes and the lungs. Surprisingly, metastases were found in only 21% of regional iliac and sacral lymph nodes in the mice bearing PC-3/VEGF-C tumors compared with 58% of those with PC-3/mock tumors ($p < 0.01$). Histomorphometric analysis of lymph nodes did not show significant differences in the relative metastatic tumor area in the iliac and sacral lymph nodes between the groups 4 weeks after inoculation of tumor cells. Interestingly, while a very high expression of VEGF-C in orthotopic PC-3 tumors was associated with decreased lymph node metastasis, the rate of lung metastasis was clearly increased. The occurrence of lung metastases was 48% in mice inoculated with PC-3/VEGF-C cells and 8% of the control mice ($p < 0.001$). It is possible that lung metastases arose via hematogenous pathways, facilitated by the extensive blood capillary network in these tumors. Another possibility is that the tumor cells that spread via lymphatic vessels passed lymph nodes and metastasized from lymph nodes to distant sites, which might explain the decreased lymph node metastasis. Hirakawa and coworkers (Hirakawa et al. 2007) have recently reported that VEGF-C can facilitate tumor cell metastasis to distant sites by modulating lymph node vascularization and function. If this was the case in our PC-3/VEGF-C tumor-bearing mice, the remaining reasonably dense network of peritumoral lymphatic vessels would have been able to mediate spread of tumor cells via the lymph nodes to the lung.

5.2.5. The effects of VEGFR3-Ig fusion protein on orthotopic PC-3/VEGF-C tumors

A group of mice inoculated with PC-3/VEGF-C cells was also treated with VEGFR3-Ig fusion protein or lacZ-Ig to study whether blockage of the VEGFR3 pathway is able to oppose the effects of VEGF-C overexpression on tumor growth, on lymphatic and blood vessel networks and on tumor metastasis. As in parental PC-3 tumors treated with VEGFR3-Ig, the density of lymphatic capillaries was also inhibited in VEGFR3-Ig *vs*. lacZ-Ig -treated PC-3/VEGF-C prostate tumors whereas no statistically significant effect was observed in blood capillary density. This suggests that the formation of new lymphatic capillaries in PC-3 tumors is dependent on VEGFR3 function even in the presence of massive angiogenesis. VEGFR3-Ig treatment did not affect CD34-positive blood capillary density, nor did it have an influence on tumor size, which also suggests that increased angiogenesis was mediated by VEGFR2 pathways.

5.3. FGF8b INCREASES GROWTH OF PROSTATE TUMORS AND INDUCES ANGIOGENESIS

A proportion of cancers progress to advanced disease causing morbidity and mortality. Prostate cancer has a very heterogeneous nature with different features at different stages. In the third part of this study, we established a tumor model that overexpressed FGF8b in order to investigate the role of this factor, which is frequently expressed by cancers, in prostate tumor growth, metastasis, morphology and genetic alterations behind these phenomena.

5.3.1. The effects of FGF8b on proliferation of PC-3 cells, expression of FGF receptors and MMP production

FGF8b-transfected PC-3 cell clones 1 (highest FGF8b mRNA and protein expression) and 15 (moderate FGF8b mRNA and protein expression) were compared with two empty vector-transfected PC-3 cell clones (PC-3/mock). To investigate clonal variation, both clones were tested for their properties *in vitro* and their ability *in vivo* to produce orthotopic tumors in nude mice, and the results were found to be similar. The PC-3/FGF8b clone 1 and PC-3/mock clone 1 were chosen for the following experiments.

Transfection with FGF8b increased the growth of the cells on average by 1.5-fold and induced an elongated morphology in comparison with PC-3/mock cells. FGF8b signals through the FGF receptor splice forms FGFR1IIIc, FGFR2IIIc, FGFR3IIIc and FGFR4 (Ornitz et al. 1996, MacArthur et al. 1995a, Olsen et al. 2006). We studied the expression of mRNAs for FGF8b receptors in PC-3 cells and in FGF8b- or mock-transfected PC-3 cell clones by means of RT-PCR. The cells expressed the isoforms FGFR1IIIc, FGFR3IIIc and FGFR4, but FGFR2IIIc expression was not detected in any of the cell clones. Next, RT-PCR was used to study the expression of receptors for

FGF8b in tumors. Even though the PC-3 cells did not show expression of FGFR2IIIc *in vitro*, the expression was found both in FGF8b-transfected and mock-transfected tumors *in vivo*. Expression of FGFR1IIIc, 3IIIc and FGFR1 remained unaltered. Considering the lack of FGFR2 mRNA and protein expression in PC-3 parental and transfected cells, it is probable that the signal in the RT-PCR of tumor tissue was derived from the surrounding normal prostate infiltrated by tumor tissue or from stromal components rather than from tumor cells. More sensitive methods such as in situ hybridization would be needed to specify the origin of FGFR2.

Endothelial cell stimulation for angiogenesis is generally the result of an interplay between different cytokines and growth factors, such as VEGFs, FGFs, TGFs, PDGF, angiopoietins, interleukins and chemokines (Presta et al. 2005, Hanahan, Folkman 1996). Endothelial stimulation causes production of proteases, which assist the endothelial cells to invade the surrounding tissue, proliferate and finally form new vessels. Our results show that FGF8b increased MMP-9 production in PC-3 cells. These findings are in line with previous ones showing that FGF-8b induces prostate cancer cell proliferation (Heer et al. 2004, Song et al. 2000) and invasion (Song et al. 2000), as well as MMP production in prostate and breast cancer cells (Ruohola et al. 2001, Udayakumar, Nagle & Bowden 2004). In addition, the observed induction of MMP-9 may play a role in prostate cancer-associated angiogenesis as well as the initial phases of the metastasizing process (Kaplan, Rafii & Lyden 2006). These findings strongly support the idea that FGF-8 acts as a regulator of angiogenesis in prostate cancer. These changes are likely to take place via autocrine mechanisms through the activation of FGFRs specific for FGF8b (Presta et al. 2005).

5.3.2. The effects of FGF8b on growth, morphology and metastasis in PC-3 tumors

Subcutaneous PC-3/FGF8b tumors were statistically significantly bigger than PC-3/mock tumors as early as 3 weeks after inoculation of cancer cells ($p < 0.001$) and the difference continued to increase throughout the 6-week study period. In orthotopic tumors, the difference was significant only at 8 weeks after orthotopic inoculation (PC-3/FGF8b *vs.* PC-3/mock, $p < 0.01$). The proliferation rate of the tumors was determined using immunostaining for Ki67. It showed an increased number of proliferating cells in orthotopic PC-3/FGF8b tumors compared with PC-3/mock tumors ($p < 0.05$).

Interestingly, macroscopic and microscopic analysis revealed strikingly increased vessel-like formation in PC-3/FGF8b tumors compared with PC-3/mock tumors. Histological examination revealed that PC-3/FGF8b tumors contained significantly more capillaries or capillary-like structures than PC-3/mock tumors. However, these capillary-like structures were wide and distorted containing large cisternal spaces filled with blood and very loose tissue surrounded by extracellular fluid. According to the endothelial-specific CD31 staining of the tumors, capillary density was clearly

increased in the FGF8b-transfected tumors ($p < 0.001$), although the staining result with another endothelial marker CD34 did not reach statistical significance. There were also some necrotic areas. All this indicates that blood flow in the newly formed tumors might be abnormal. These features are also very common in clinical tumor samples, where tumor vessels are often highly disorganised and leaky (Brown, Wilson 2004). Even in highly vascularized tumors, the structure and function of the capillary network is aberrant, leading to chaotic and heterogeneous tumor vascularization (Brown, Wilson 2004). It seems that part of the tumor vessels are mosaic vessels, formed by tumor cells together with endothelial cells (Chang et al. 2000). These vessels are often blind-ended and leaky leading to non-functional perfusion of tumors.

Metastasis to prostate draining lymph nodes was slightly increased in the PC-3/FGF8b tumors compared with PC-3/mock but the difference was not statistically significant. The histomorphometric analysis of the relative area of metastasis did not reach statistical significance. Interestingly, metastases were found in the lungs of two mice bearing PC-3/FGF8b tumors but none in those with PC-3/mock tumors. No metastases were found in other organs by histological examination. However, when using a RT-qPCR we found an increased amount of FGF8b mRNA in the lungs of mice that had been orthotopically inoculated with PC-3/FGF8b cells compared with PC-3/mock ($p < 0.05$, the number of mice was 3 in both groups). A similar trend was seen in the serum, liver and bone marrow of mice that had been orthotopically inoculated with PC-3/FGF8b cells compared with PC-3/mock, but these differences did not reach statistical significance. However, these results suggest an increased hematogenic spread of the PC-3/FGF8b cancer cells.

5.3.3. FGF8b induces changes in gene expression profiles in PC-3 cells and tumors

We used the DNA microarray technique to study the possible mechanisms behind FGF8b-induced growth, angiogenic morphology and spread of the tumors.

First, human oligoarrays were used to compare FGF8b and PC-3/mock cells. This analysis revealed 43 significantly deviated and at least two-fold upregulated and 126 at least two-fold downregulated genes. GeneSpring Gene Ontology (GO) tools were used to categorize these differentially expressed genes according to biological function. The predominantly overrepresented GO groups associated with FGF8-induced genes in PC-3 cells were those pertaining to cell-cell signaling, response to external stimulus and development. The statistically most enriched GO category for genes downregulated by FGF8 in PC-3 cells was energy metabolism.

As an additional tool for investigating the biological pathways represented by the differentially expressed genes, Ingenuity Pathway Analysis (IPA) was used. Two statistically significant networks associated with the FGF8-induced genes in PC-3 cells were identified. The first was associated with embryonal development, cellular

growth and proliferation and cellular movement. The genes of other network were categorized as regulation of gene expression, cancer and DNA replication, recombination and repair. Six statistically significant networks were associated with the FGF8-repressed genes. Three were associated with cell death, one with energy production and nucleic acid metabolism, one with cell-to cell signaling and one to cancer and cell cycle.

Secondly, the mouse microarray analysis was performed in order to primarily study the effects of FGF8 on host-derived compartments in orthotopic PC-3 prostate tumors. These analyses revealed 55 significantly at least two-fold upregulated and six at least two-fold downregulated genes. As in the microarray analysis of FGF8b *vs*. mock cells, development and response to external stimulus were among the statistically overrepresented GO biological function categories in the FGF8-induced genes in tumors. Using IPA, we found several upregulated genes, which were related to cell-to-cell signaling and interaction and cellular growth and proliferation. Other IPA categories among the FGF8-induced genes in the prostate tumors included post-translational modification, cell death, organism injury and abnormalities, hematological system development and function, inflammatory disease and tissue morphology. No statistically significant GO groups or IPA networks could be generated for the downregulated genes in the array of tumor samples.

5.3.3.1. Changes in the gene expression profile associated with angiogenesis and metastasis

After birth, angiogenesis takes place only in special circumstances such as wound healing and reproduction, and in pathological conditions like cancer. FGFs and VEGFs are involved in angiogenesis by autocrine and paracrine manners (Presta et al. 2005). There is evidence that FGFs and VEGFs require each other and their receptors in angiogenesis (Presta et al. 2005). In line with the angiogenic-like morphology of the FGF8b-transfected tumors, microarray analyses and RT-qPCR revealed FGF-8b induction of several transcripts with tumor growth and angiogenic potential, such as dimethylarginine dimethylaminohydrolase 2 (DDAH2), midkine (MDK), osteonectin (SPARC), and Src homology 2 domain-containing transforming protein 1 (SHC1) (Kostourou et al. 2003, Pucci-Minafra et al. 2008, Jendraschak, Sage 1996, Stoica et al. 2002, Saucier et al. 2004). In an analysis of the orthotopic tumors studied here, we found upregulation of angiogenic small inducible cytokine A12 (CCL2) (Salcedo et al. 2000) and small inducible cytokine A11/small chemokine (C-C motif) ligand 11 (CCL11) (Salcedo et al. 2000). The transcripts that are principally induced by the mouse stromal environment explain the differences in the expression patterns between the PC-3 cells and tumors. The communication between prostatic stromal cells to prostatic epithelial cells is very important in the normal function of the prostate as well as in the development of prostate cancer (Cunha et al. 2003, Chung et al. 1989, Olumi et al. 1999).

Osteonectin and osteopontin (OPN/SPP1) were induced by FGF8b in the PC-3 cells and tumors. Osteonectin has been previously shown to promote prostate cancer metastasis to bone (De et al. 2003). Our finding of osteopontin induction in the FGF8-transfected orthotopic tumors is in line with a recent study by Khohavirdi, which showed that osteopontin is upregulated by FGF8 in mouse prostate epithelium (Khodavirdi et al. 2006). Osteopontin has also been shown to be associated with prostate cancer bone metastasis (Hotte et al. 2002) and to be upregulated by FGFR1 in prostate cancer (Freeman et al. 2003). The array of orthotopic tumors also revealed FGF8-repression of dickkopf-1 (DKK-1), an inhibitor of Wnts. DKK-1 has been shown to block Wnt-mediated osteoblastic activity in PC-3 cells in an *in vivo* mouse model (Hall et al. 2005). These findings are of special interest, keeping in mind the finding that FGF-8 is involved in prostate cancer bone metastasis (Valta et al. 2008), possibly via regulation of osteoblastic differentiation (Valta et al. 2006). It is thus possible that FGF-8-caused differences in the transcription of SPARC, OPN and DKK-1 could enhance the formation of sclerotic lesions associated with bone metastases.

5.4. THE EFFECTS OF FGF8b ON TUMOR MICROENVIRONMENT

To date, PSA is the only marker for prostate cancer in clinical use. The diverse nature of prostate cancer hampers the prediction of clinical outcome and more information of molecular mechanisms behind prostate cancer progression is needed (Fidler, Kim & Langley 2007). Studying tumor microenvironment using markers of hypoxia and glucose metabolism may help to answer some of these questions. Aim of the final part of the thesis was to further evaluate the effects of FGF8b on tumor microenvironment and, on the other hand, evaluate the usefulness of [^{18}F]EF5 as a hypoxia marker.

5.4.1. The effects of FGF8b on growth and morphology of s.c. PC-3 tumors

PC-3 cells were stably transfected with the expression vector pcDNA3.1 containing human FGF8b or VEGF cDNA in an EcoRI site. Empty vector-transfected PC-3 cells were used as a control (mock).

The tumorigenity of PC-3/FGF8b, PC-3/VEGF and PC-3/mock cells was studied by inoculating them to the back of the neck of nude mice. Mock-transfected cells were able to produce only small tumors (749 ± 19 mm^3), whereas PC-3/VEGF and PC-3/FGF8b tumors grew larger (4036 ± 51 and 3436 ± 41 mm^3, respectively, $p < 0.001$) during the 6-week observation period.

Under macroscopical examination, both PC-3/FGF8b and PC-3/VEGF tumors showed an overall angiogenic morphology. They were reddish in color and many of the PC-3/FGF8b tumors were fragile and haemorrhagic (as with the orthotopic PC-

3/FGF8b tumors, described in work III). The morphology of the PC-3/mock tumors was solid and homogenous. Morphological examination of the hematoxylin-eosin (H&E) -stained sections showed a rich capillary network and large sinusoid-like vessels, especially in the PC-3/VEGF tumors. Capillaries in the PC-3/FGF8b tumors were more aberrant, containing large cisternal spaces filled with blood. The number of capillaries was modest in the PC-3/mock tumors. Furthermore, vascularization was verified using immunohistochemical staining for the endothelial marker CD31. Using this staining, we discovered a significantly increased ($p < 0.001$) capillary density in both PC-3/FGF8b and PC-3/VEGF tumors, as compared with those found in the PC-3/mock tumors. As discussed earlier, angiogenic-like tumors may have a chaotic and heterogenous vascularization (Brown, Wilson 2004). In this way, the microenvironmental homeostasis in tumors is disrupted and metabolic changes, such as oxygen and glucose gradients, develop at microregional levels (Vaupel, Harrison 2004).

5.4.2. The effects of FGF8b on proliferation in s.c PC-3 tumors

Cell cycle phases were determined from PC-3/FGF8b, PC-3/VEGF and PC-3/mock tumor homogenates using flow cytometry. The homogenates were incubated in hypotonic solution and the DNA content of the PI-labeled cells was measured using FACSCalibur flow cytometer (Becton Dickinson). Data was acquired using CellQuest Pro (Becton Dickinson) and the cell cycle phase analysis was performed using ModFit LT (Verity Software House, Inc.). The data show that the highest number of proliferative cells (S and G2/M phases) was found in PC-3/VEGF tumors. The results of proliferative cells were confirmed using immunohistochemical stainings against Ki67. Ki67-positivity was found in 26% of the PC-3/FGF8b, 33% of the PC-3/VEGF and 10% of the PC-3/mock tumor cells.

5.4.3. Perfusion and oxygenation of s.c. PC-3 tumors

The labeling intensity of fluorescent perfusion marker Hoechst 33342 shows differences in the tumor perfusion (Pugachev et al. 2005). The labeling was strongest in the PC-3/VEGF tumors, where a signal was detected in both intratumoral and peritumoral areas. The labeling intensity was modest in the PC-3/FGF8b and PC-3/mock tumors, which indicates poor perfusion and accordingly hypoxia in the PC-3/FGF8b and PC-3/mock tumors. Until recently, the only way to study tumor hypoxia was through the use of the Eppendorf® microelectrode probes. These probes are placed into tumor tissue to measure partial oxygen pressure, (Vaupel et al. 1991), where approximately 200 pO_2 values from different locations within the tumor may be obtained using several tracks during measurement. We studied intratumoral oxygenation using a Licox® Revoxode CC1.2 polarographic probe. In contrast to the Eppendorf® method, the polarographic needle electrode (Licox® system) gives proper information of tumor oxygenation in one larger selected area. According to the

measurements, recordings concerning VEGF tumors showed the highest pO_2 level, whereas PC-3/FGF8b and PC-3/mock tumors were severely hypoxic. The mean pO_2 value for the PC-3/VEGF tumors was 17.4 ± 2.2 mmHg, which was much higher than the values detected from the PC-3/FGF8b and in the PC-3/mock tumors (2.3 ± 0.6 mmHg, and 1.3 ± 0.3 mmHg, respectively) ($p < 0.001$). These results further suggest that the increased vasculature detected in the FGF8 over-expressing tumors did not result in increased oxygenation.

5.4.4. Hypoxia of s.c. PC-3 tumors

Hypoxia is a common feature of prostate cancer. Hypoxic tumor cells are three-fold more resistant to radiation therapy and chemotherapy compared with the well-oxygenated tumor cells (Brown, Wilson 2004, Grönroos, Minn 2007, Minn et al. 2008). Furthermore, there is evidence that hypoxic tumor cells might select a pathway for a malignant phenotype (Pennacchietti et al. 2003, Jokilehto et al. 2006). Non-invasive imaging of hypoxia is thus needed to distinguish the radiotherapy responsive tumors from radioresistant tumors (Grönroos, Minn 2007). The accumulation of [^{18}F]EF5 in the present study, expressed as a T/B ratio, was significantly lower ($p < 0.05$) in PC-3/VEGF tumors compared with PC-3/FGF8b and PC-3/mock tumors, whereas no significant difference in T/B (tumor-to blood) ratio was seen between PC-3/FGF8b and PC-3/mock tumors. Intratumoral distribution of [^{18}F]EF5 was studied using digital autoradiography. The intratumoral distribution of ^{18}F-radioactivity was at most tenfold higher in hot spot areas when compared with low-radioactivity background areas inside the tumor. In order to confirm the distribution of the [^{18}F]EF5 to hypoxic areas, the tumor samples were stained using known hypoxic markers glucose transporter (GLUT1) and the hypoxia-inducible transcription factor (HIF1). The GLUT1 and HIF1, which are expressed in human prostate tumors, showed co-localization with [^{18}F]EF5 in our experimental xenografts, as expected and confirmed [^{18}F]EF5 as a suitable hypoxia marker.

5.4.5. Glucose metabolism of s.c. PC-3 tumors

A common feature of invasive cancers is altered glucose metabolism, known as aerobic glycolysis (Gatenby, Gillies 2004). The molecular mechanisms leading to an upregulated aerobic glycolysis in tumors are not well known, but an elevated expression of GLUTs, such as GLUT1, is commonly seen in tumor cells (Mellanen et al. 1994). The GLUT gene family is one of the many families known to be regulated by HIF-1, which is expressed under hypoxic conditions (Harris 2002). In order to evaluate the metabolic activity of FGF8b- and VEGF-expressing tumours, we measured the uptake of ^{18}F-labeled glucose analog fluoro deoxy glucose [^{18}F]FDG. The intratumoral distribution of [^{18}F]FDG determined by digital autoradiography showed uniformly distributed uptake in tumours. We found a significantly lower ($p < 0.05$) T/B uptake ratio of [^{18}F]FDG in FGF8b tumours (7.4 ± 0.7) compared with that seen in VEGF and mock tumours (14.3 ± 8.9 and 17.8 ± 9.3, respectively).

Since morphological studies indicated a lower number of cells in FGF8b tumours, which would affect the relative level of [^{18}F]FDG uptake, we evaluated cellular density in the different tumour models by flow cytometry. Enumeration analysis showed that indeed FGF8b tumours contained fewer cells/cm^3 than VEGF and mock tumours ($p = 0.06$). Even though this decrease did not reach statistical significance, the same result was obtained by counting nuclei per field from H&E stained sections. When the [^{18}F]FDG uptake data was normalised to cell number, the differences between FGF8b and mock tumours disappeared, and according to the normalised data, the uptake was smallest in VEGF tumours. Immunohistochemical staining of GLUT1 correlated with the uptake of [^{18}F]FDG, showing a trend toward a lower expression pattern of GLUT1 in VEGF tumours compared with the others ($p < 0.05$). Hypoxic tumors often compensate hypoxia by increasing the glucose uptake (Gatenby, Gillies 2004).

The uptaken [^{18}F]FDG is converted to [^{18}F]FDG-6-phosphate ([^{18}F]FDG-6-P), and the usual assumption is that [^{18}F]FDG-6-P is not a substrate for subsequent enzymatic reactions and that tumor hot spots reflect trapping of [^{18}F]FDG-6-P. We measured the amount of formed [^{18}F]FDG-6-P by radioHPLC and as assumed, it was the major form found and there were no differences between the tumor groups. The mean plasma glucose level varied from 7.9 to 10.6 mmol/L and did not differ significantly between the three tumor groups.

Our study revealed that all three tumor models have different profiles considering hypoxia, perfusion and metabolism. The PC-3/mock tumors represent a hypoxic tumor and mimic therefore well human prostate tumors. Hypoxic tumors often compensate the poor oxygen status with increased glucose consumption. The PC-3/VEGF tumors are well oxygenated and their glucose consumption is not increased compared with control tissue. The PC-3/FGF8b tumors have features of aggressive phenotype but the molecular mechanism of chaotic increase in angiogenesis is not revealed. The tumors are highly hypoxic and use the increased levels of glucose. Althought the capillary network is poor, glucose can be supplied to a fast growing tumor.

Our study confirms earlier studies showing that the number of capillaries does not reveal the condition of oxygen supply (Komar et al. 2008). Oxygenation status is an extremely important prognostic indicator for therapy resistance. Our results suggest that oxygenation status can vary in tumors, which show a highly vascular morphology. This might suggest that the blood flow in PC-3/VEGF tumors is more effective in comparison to PC-3/FGF8b and PC-3/mock tumors. Our studies also support earlier evidence that hypoxia and accelerated glycolysis are common but independent phenomena of the malignant phenotype of tumors (Gatenby, Gillies 2004). Acute hypoxia can stimulate glycolysis in normal tissues and some tumors, but it is not the primary cause of glycolysis in individual tumors in all tumor types (Gatenby, Gillies 2004).

5.4.6. Hypoxia imaging

2-nitro-imidazole-based compounds bind to cellular macromolecules under hypoxic conditions and are therefore used as hypoxic markers. The development of labeled nitro-imidazole derivatives for non-invasive PET or SPECT imaging has been a great challenge in recent decades (Chapman 1979). The first clinical hypoxia studies were performed using [^{18}F]FMISO to quantify the hypoxia from lung, head and neck and prostate cancer patients (Rasey et al. 1996). [^{18}F]FMISO had limitations such as slow accumulation to tumors, low background ratio and high amount of metabolites, and therefore it failed to gain wide clinical acceptance. However, other ^{18}F-labeled nitro-imidazole compounds have been developed to meet the challenge. Evaluation of [^{18}F]FETNIM showed high and heterogenous uptake in head and neck tumors. However, the high hydrophilicity of [^{18}F]FETNIM led to early accumulation in tumors, which was an unfavorable phenomenon for radionuclear therapy (Lehtiö et al. 2001). Unfortunately, the high hydrophilicity was also detected in [^{18}F]FAZA (Souvatzoglou et al. 2007). In this thesis study, a new fluorine-labeled nitro-imidazole compound, EF5 was used. EF5 is a lipophilic compound and it has better pharmacokinetic properties than other nitro-imidazole compounds (Minn et al. 2008). Clinical evaluation of [^{18}F]EF5 is still ongoing, but the results of our *in vivo* studies show that the uptake of [^{18}F]EF5 is high and distribution is heterogeneous compared with muscle background. This is supported by our findings of [^{18}F]EF5 hotspots inside the tumor with the non-hypoxic tumor border.

5.5. GENERAL DISCUSSION

5.5.1. Orthotopic prostate tumor model

The subcutaneous model is the most commonly used xenograft model (Sharkey, Fogh 1979). However, subcutaneously-growing human tumors in immunodeficient mice, do not sufficiently represent clinical cancer, especially with regard to metastasis and drug sensitivity (Hoffman, 1999). The orthotopic environment with an appropriate blood and lymphatic vasculature is important when treatments of prostate cancer are evaluated. The first aim of this study was to further develop a model to study prostate cancer *in vivo*. We started with the orthotopic prostate tumor model of Stephenson and coworkers (Stephenson et al. 1992). After some modifications, such as improving the surgical technique, anesthesia and analgesics, and adding dye to the inoculated cell suspension, the improved model was adequate for *in vivo* studies. The green dye in the cell suspension confirmed the success of the inoculation. If artificial spread of cell suspension was detected, the mouse was excluded from the experiment and only true metastases were evaluated in histological analyses. The prostate tumor growth and metastasis to prostate draining, iliac and sacral, lymph nodes were time and dose (cell number) dependent. Results were repeatable, and tested using PC-3 and DU-145 cell lines (unpublished data from author). The PC-3 cell line was chosen for the *in vivo*

studies of this thesis because of they were easier to transfect compared with DU-145 cells.

First, we used the orthotopic PC-3 tumor model, with parental PC-3 cells, to study the effects of alendronate. As discussed in Chapter 1.1, earlier studies of our laboratory have shown that alendronate has inhibitory effects on the migration and invasion of PC-3 cells *in vitro* (Virtanen et al. 2002). Using the orthotopic prostate tumor model, we were also able to demonstrate these findings *in vivo*. We found differences between the alendronate group and the control group in tumor growth and metastasis, and gained more information of the mechanisms by performing immunohistochemical stainings of the tumors. These results demonstrate that the orthotopic prostate tumor model may be a potential preclinical instrument in drug development.

As in human disease, metastases of orthotopic prostate tumors develop in prostate-draining lymph nodes or at distant sites such as the lungs depending on the characteristics of the inoculated cell line (Stephenson et al. 1992). Orthotopic PC-3 tumors metastasize to iliac and sacral lymph nodes but rarely to other sites. Unlike human advanced prostate cancer, the orthotopic prostate tumors very rarely metastasize to bone. The growth of orthotopic prostate tumors is relatively fast. This has both advantages and disadvantages. Fast growth allows large-scale screening of compounds before clinical trials, which is an important feature in drug development. However, human prostate cancer is a slowly and gradually progressing disease, and using xenografts, only one stage at the time can be studied. It is shown that prostate cancer cells such as PC-3 cells are very avidly directed to bone, when inoculated intracardiacly (unpublished data of author). These findings suggest that the number of circulating tumor cells limits the development of bone metastasis, which therefore needs more time to form.

Another disadvantage of the orthotopic inoculation model is the difficulty of monitoring tumor growth over time. One possibility to follow the tumor growth in individual mouse is to use a micro-imaging technique with transrectal ultrasonography (Kraaij et al. 2002) or magnetic resonance imaging (MRI) (Degrassi et al. 2007, Jennbacken et al. 2009). Prostate cancer cells can be transfected with luciferase or fluorescent proteins, which allows follow-up using *in vivo* luminescence imaging (Jantscheff et al. 2009) or *in vivo* fluorescence imaging (Yang et al. 2006, Hoffman 2002), respectively. However, both imaging methods have limitations. The signal from luciferase expressing orthotopic prostate tumor is so strong that it covers signal from prostate-draining lymph nodes. Therefore, it is impossible to differentiate the signal from the tumor and metastases. A drawback in fluorescence imaging is its too weak depth resolution, especially using green fluorescence proteins. Today, new stronger red and near infra-red proteins such as *cherry, tomato, plum* (Wang et al. 2004) and more recently *katushka* (Shcherbo et al. 2007), are available, and they have shown promising results. Physiological processes in prostate cancer can be followed

using single photon emission computed tomography (SPECT) or positron emission tomography (PET) combined with CT. All imaging methods discussed here require rather expensive equipment and trained personnel to use them.

The majority of published experiments are performed with PC-3, DU-145 or LNCaP cells (van Bokhoven et al. 2003, van Weerden, Bangma & de Wit 2009). Such a limited number of cell lines restrain the knowledge of prostate cancer progression since these models represent only a few types of advanced prostate cancer. The development of new permanent cell lines for prostate cancer was difficult until the 1990s. Today prostate cancer xenografts PC-346C, 22Rv1, CWR-R1, DuCaP, LAPC-4, MDA Pca1, MDA Pca2a, MDA Pca2b and VCaP have been caryotyped and compared in an extensive study by van Bokhoven and coworkers (van Bokhoven et al. 2003, van Weerden, Bangma & de Wit 2009). Results showed that xenografts had different characteristics concerning for example AR, androgen responsiveness and PSA secretion. This allows more options in comparison to traditional models in selecting different gene expression profiles that mimic specific disease stages or subgroups of patients (van Weerden, Bangma & de Wit 2009, Marques et al. 2005).

We took a different approach to the problem of the limited features of prostate cancer cell lines. We aimed to develop new cell lines by manipulating the PC-3 cells to overexpress VEGF, VEGF-C and FGF8b in order to mimic features of human prostate cancer. The manipulations changed the behaviour of tumors. All growth factors increased tumor growth and VEGF-C as well as FGF8b induced changes in metastatic capacity. However, none of these cell lines were able to produce bone metastases, when inoculated orthotopically into the prostate. The results of our previous studies show that PC-3/FGF8b cells increase tumor growth in bone, when inoculated intratibially (Valta et al. 2008). In future studies, study periods as well as the number of inoculated cells need adjustment. Possibly double-transfection with two or more factors can also be considered. However, transfection of PC-3 cells with several constructs encoding different proteins (growth factors, growth factor receptors, other mediators or regulators) might help in the analysis of multifactorial tumor regulation. Altogether, the advanced imaging and analysis of the growth and spreading of orthotopic tumors will provide a powerful tool to study regulation and therapeutic response of prostate cancer.

5.5.2. The role of angiogenesis and lymphangiogenesis in prostate cancer

VEGF-mediated angiogenesis plays an important role in prostate tumor growth and metastasis (Folkman 1971, Siddiqui, Klotz 2009). VEGF receptors are overexpressed in prostate cancer and plasma levels of VEGF are raised in metastatic disease compared with localized disease or healthy controls. Anti-angiogenic and anti-vascular therapies are under investication in order to reverse the tumor progression from malignant pro-angiogenic state to quiescent non-angiogenic state (Naumov et al. 2008). In our study, the treatment of orthotopic PC-3 tumor bearing mice with

alendronate, deacresed the vascular density and concequently to some extend inhibited tumor growth. However, the therapeutic benefit of antiangiogenic therapy is complex and probably involves several mechanisms. The overexpression of angiogenic gowth factors VEGF, FGF8b and VEGF-C in our experimental tumors showed increased vascular density and accelerated tumor growth and metastasis. We successfully inhibited tumor metastasis by blocking the lymphangiogenic receptor of growth factor VEGF-C. The blockade decreased tumor growth but the tumor was not totally atrophied, and the angiogenic receptor of the VEGF-C as well as other factors that are overexpressed in tumors were still functional. It seems that the functions of different growth factors are partly overlapping and they can also affect via different receptors. Therefore, antiangiogenic therapies against single growth factor or receptor are not effective enough alone but have shown promising results in combination with drugs that use other mechanisms.

Bevacizumab (Avastin^{TM}, Genentech/Roche) is a monoclonal antibody that binds to VEGF and prevents angiogenesis. Phase II trial by Cancer and Leukemia Group B (CALCB 90006) have shown sustained decline of PSA in 65% of the prostate cancer patients. There is ongoing Phase III trial of combination with Bevacizumab and docetaxel, where first results demonstrate that the therapy is active and well tolerated. The investigation is still continuing.

Thalidomide, a notorious drug, which was originally developed to treat morning sickness, is currently FDA approved for use in leprosy. It has immunomodulatory, anti-inflammatory and anti-angiogenic properties (Sonpavde, Hutson 2006). Phase II ramdomized trial compared the efficacy of docetaxel and docetaxel in combination with thalidomide in 75 prostate cancer patients. The results showed decline of PSA in 53% of the patients of the combined group compared with 37% in docetaxel alone (Dahut et al. 2004). Analogues of thalidomide (revlimid and actimid) as well as VEGF-inhibitor sofranib are under Phase II study. Angiostatin and endostatin have been tested preclinically in animal models.

The structure and the main function of lymphatic vessels were revealed already in the beginning of the last century. However, the lymphangiogenic growth factors and their receptors were not found until the past decade. The most importantly, VEGFR3 was identified as a first specific lymphatic marker (Joukov et al. 1996, Lee et al. 1996). It was found to affect proliferation, migration and survival of the lymphatic endothelial cells. VEGF-C and VEGF-D were later descriped as specific inducers of lymphangiogenesis (Joukov et al. 1996). Even if several factors are regocnized, the contribution of the lymphatic system in tumor metastasis is not obvious. The paracrine and autocrine factors in tumor microenvironment are affecting tumor cell spread. These factors as well as anatomy of the lymphatic vessels are partly overlapping with blood vessels. Consequently, tumor cells can spread between these two systems.

There is strong evidence that lymphatic vessels promote lymph node metastasis of prostate cancer. VEGFR3 is found to be overexpressed in tumors that formed lymph node metastasis. Our studies showed that by inhibiting the function of VEGFR3, metastasis of prostatic tumors could be prevented. The role of VEGF-C is interesting. It is shown to increase lymph node metastasis but on the other hand, there is no correlation between expression of VEGF-C and Gleason score. This indicates that VEGF-C is not promoting tumor dediffertiation but instead providing a path for tumor cell escape from primary tumor. When we overexpressed the VEGF-C in orthotopic PC-3 tumors, the rate of metastasis to lungs was increased, and the lymph node metastasis was decreased. This shows again that effects of growth factors and their receptor are complicated and parallel, and inhibition should be targeted to several pathways to effectively prevent tumor cell spread. In our study, the overexpression of VEGF-C was not increasing the number of peritumoral lymphatic vessels. This finding can be explained with fast growing experimental prostate tumors, which may induce high pressure in tissue preventing the lymphatic vessel dissemination inside the tumor mass and flatten the existing lymphatic vessels as nonfunctional. The orthotopic prostate tumor model is useful instrument in studying prostate cancer but prostate xenografts differ from the slowly growing clinical disease.

5.5.3. The role of hypoxia in prostate cancer

Hypoxia is progressively emerging, common feature of prostate tumors associated with poor prognosis. There is a correlation between tumor hypoxia and Gleason grade, and there is also evidence that hypoxia has a role in disease progression (Tomlins et al. 2007). Hypoxia may participate in prostate cancer progression through its interaction with DNA repair mechanisms and induction of genetic instability (Marignol et al. 2008). The resistance to apoptosis is central for the development of cancer and may hamper the therapy. The fluctuations in oxygenation have been shown to lead increased expression of Bcl-2 family survival factors, which suppresses apoptosis through Pim-1 and Akt (Aho et al. 2004). Our studies showed a positive correlation between FGF8b expression and hypoxia, and a negative correlation between FGF8b and partial oxygen pressure in tumor tissue, even if the FGF8b tumors grew fast and had increased number of proliferative cells. This indicates FGF8b as a marker of poor prognosis.

The understanding of the mechanisms behind tumor metastasis is crucial for development of targeted therapies for preventing the spread of the tumor cells. Our studies have revealed members of FGF and VEGF-family growth factors and their receptor as potential drug targets for prevention of prostate cancer metastasis. The interplay between cellular responces to cytokines, growth factors and oxidative stress is known to been related to survival of cancer cells. The manipulation of one factor will influence to the others, triggering adaptation and resistance. The combination of hypoxia or angiogenesis targeting therapy with routine treatment may be efficient strategy for management of prostate cancer.

6. CONCLUSIONS

The purpose of the present study was to establish an orthotopic xenograft model to study the growth and metastasis of prostate cancer. On the basis of the experiments, the following conclusions can be drawn.

1. The modified orthotopic inoculation model mimics prostate cancer by forming tumors in the prostate and uses angiogenic or lymphangiogenic routes to metastasize. It is suitable for studies on the regulation of prostate cancer growth and spread and testing of new drug targets and anti-cancer therapies.
2. The treatment of orthotopic prostate tumor-bearing mice with bisphosphonate alendronate was associated with strongly inhibited angiogenesis and an increased number of apoptotic cells in tumors, which may explain decreased tumor growth. The decreased lymph node metastasis was in accordance with previous *in vitro* results of alendronate inhibition of PC-3 prostate cancer cell invasion. This suggests that alendronate, which is widely used in the prevention of osteoporosis and treatment of other resorbtive bone diseases, also has antitumoral effects.
3. Ectopic expression of FGF8b and VEGF-C increases the growth and angiogenesis of orthotopic prostate tumors. FGF8b slightly increased the metastasis to prostate-draining lymph nodes but participation of other factors may be needed for high metastasic behaviour. Blocking of the receptor (VEGFR3) for lymphangiogenic growth factor (VEGF-C) inhibited lymph node metastasis as expected. Surprisingly, overexpression of VEGF-C increased lung metastasis and decreased metastasis to the lymph nodes, which suggests that under these conditions, the angiogenic route of metastasis is chosen. These data demonstrate that metastasizing is a multistep process with several pathways, which need to be inhibited to obtain an effective blockade of prostate cancer metastasis.
4. Characterization of the tumor microenvironment, especially tumor hypoxia is important. Our results showed that the tumors overexpressing the angiogenic FGF8b differed from the tumors overexpressing the angiogenic VEGF in perfusion and oxygenation. In contrast to the well-oxygenated VEGF tumors, FGF8b tumors presented a unique hypoxia and glucose consumption status suggesting the FGF8b as a marker of malignant phenotype.

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