UMEÅ UNIVERSITY MEDICAL DISSERTATIONS
New Series No. 1293 ISSN 0346-6612 ISBN 978-91-7264-862-3

Targeting the Prostate Tumor Microenvironment and Vasculature

The role of Castration, Tumor-Associated Macrophages and Pigment Epithelium-Derived Factor

Sofia Halin

Department of Medical Biosciences, Pathology
Umeå University
SE-901 87 Umeå

Responsible publisher under Swedish law : the Dean of the Medical Faculty

Copyright © Sofia Halin

ISBN 978-91-7264-862-3

ISSN 0346-6612, New Series No.1293

Cover: Copenhagen rat

E-version available at http://umu.diva-portal.org

Printed by Print & Media

Umeå, Sweden, 2009

”Det kan ju gå, det är ju inte omöjligt”

- *Anders Bergh*

ABSTRACT

BACKGROUND: Prostate cancer is the most common cancer among Swedish men. For patients with metastatic prostate cancer the standard therapy is castration, a treatment that initially provides symptomatic relief but unfortunately is not curative. New therapeutic targets for advanced prostate cancer are therefore needed. Prostate cancers are composed of tumor epithelial cells as well as many non-epithelial cells such as cancer associated fibroblasts, blood vessels and inflammatory cells. Many components of the tumor microenvironment such as tumor associated macrophages and angiogenesis have been shown to stimulate tumor progression. This thesis aims to explore mechanisms by which the local environment influences prostate tumor growth and how such mechanisms could be targeted for treatment.

MATERIALS AND METHODS: We have used animal models of prostate cancer, *in vitro* cell culture systems and clinical materials from untreated prostate cancer patients with long follow up. Experiments were evaluated with stereological techniques, immunohistochemistry, western blotting, quantitative real-time PCR, PCR arrays and laser micro dissection.

RESULTS: We found that the presence of a tumor induces adaptive changes in the surrounding non-malignant prostate tissue, and that androgen receptor negative prostate tumor cells respond to castration treatment with temporarily reduced growth when surrounded by normal castration-responsive prostate tissue. Further, we show that macrophages are important for prostate tumor growth and angiogenesis in the tumor and in the surrounding non-malignant tissue. In addition, the angiogenesis inhibitor Pigment epithelium-derived factor (PEDF) was found to be down-regulated in metastatic rat and human prostate tumors. Over-expression of PEDF inhibited experimental prostate tumor growth, angiogenesis and metastatic growth and stimulated macrophage tumor infiltration and lymphangiogenesis. PEDF was found to be down-regulated by the prostate microenvironment and tumor necrosis factor (TNF) α.

CONCLUSIONS: Our studies indicate that not only the nearby tumor microenvironment but also the surrounding non-malignant prostate tissue are important for prostate tumor growth. Both the tumor and the surrounding non-malignant prostate were characterized by increased angiogenesis and inflammatory cell infiltration. Targeting the surrounding prostate tissue with castration, targeting tumor associated macrophages, or targeting the vasculature directly using inhibitors like PEDF were all shown to repress prostate tumor growth and could prove beneficial for patients with advanced prostate cancer.

CONTENTS

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. **Halin S**, Hammarsten P, Wikström P and Bergh A. Androgen-insensitive prostate cancer cells transiently respond to castration treatment when growing in an androgen-dependent prostate environment. *The Prostate* 2007; 67: 370-377.

II. **Halin S**, Häggström Rudolfsson S, van Rooijen N and Bergh A. Extratumoral macrophages promote tumor and vascular growth in an orthotopic rat prostate tumor model. *Neoplasia* 2009;11:177-186.

III. **Halin S**, Wikström P, Häggström Rudolfsson S, Stattin P, Doll JA, Crawford SE, and Bergh A. Decreased Pigment epithelium-derived factor is associated with metastatic phenotype in human and rat prostate tumors. *Cancer Research* 2004; 64:5664-5671.

IV. **Halin S**, Häggström Rudolfsson S, Doll JA, Crawford SE, Wikström P and Bergh A. Pigment epithelium-derived factor stimulates tumor macrophage recruitment and is down-regulated by the prostate tumor microenvironment. *Manuscript.*

ABBREVATIONS

AR	Androgen Receptor
BrdU	Bromodeoxyuridine
CAFs	Cancer Associated Fibroblasts
CSF-1	Colony Stimulating Factor 1
DHT	Dihydrotestosterone
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EPC	Endothelial Progenitor Cell
FGF2	Fibroblast Growth Factor 2
GS	Gleason Score
HIF-1	Hypoxia Inducible Factor 1
IL-10	Interleukin 10
IL1β	Interleukin 1β
IL-6	Interleukin 6
iNOS	Inducible Nitric Oxide Synthase
LH	Luteinizing Hormone
MCP-1/CCL2	Monocyte Chemoattractant Protein 1
MMPs	Matrix MetalloProteinases
NFκB	Nuclear Factor κ B
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PEDF	Pigment Epithelium-Derived Factor
PSA	Prostate Specific Antigen
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
TAMs	Tumor Associated Macrophages
TGFβ	Transforming Growth Factor β
TNFα	Tumor Necrosis Factor α
UGM	UroGenital sinus Mesenchyme
VEGF	Vascular Endothelial Growth Factor
VP	Ventral Prostate
Wt	Wildtype

INTRODUCTION

The importance of new prognostic markers and therapies for prostate cancer

It is believed that all men will eventually develop prostate cancer if they only live long enough. As many as 50 % of elderly men are expected to have prostate cancer but the majority will never be aware of the disease [1]. This makes prostate cancer the most common cancer in men in Sweden. Using current diagnostic procedures prostate cancer will be detected in roughly 9 000 Swedish men annually and about 25% of them will die from their disease (Swedish Cancer Registry). Prostate tumors are often multifocal and heterogeneous and the natural history of these tumors can vary from indolent to aggressive and metastasizing. So the question is not whether the patient *has* prostate cancer or not, but instead *what types* of prostate cancers he has. The most important factors to determine prostate cancer prognosis are tumor stage, histological grade and the levels of prostate specific antigen (PSA) [2]. When combining these factors, patients can either be divided into low, intermediate or high-risk patients. The majority of newly diagnosed cases have intermediate values where the ability to predict tumor behavior is limited.

New prognostic markers for this group of patients are needed.

For localized prostate cancer the patient can choose between radical surgery, radiation therapy or "watchful waiting" where the patient is followed over time and only treated if the tumor progresses [3]. Side effects of treatments can be considerable, for example incontinence and impotence, and therapies should therefore only be given to those who will benefit from them. Although these therapies are intended to cure, about 30 % of treated patients are not cured [4]. For advanced and metastatic prostate cancer there is no cure. Palliative hormonal therapy is the standard treatment and aims to decrease the stimulatory effects of androgens on the prostatic tumor cells. Initially this provides symptomatic relief but most tumors will sooner or later grow independently of circulating androgens and relapse to a more aggressive phenotype that ultimately kills the patient.

New therapeutic targets for prostate cancer are urgently needed.

The Prostate

Prostate anatomy, morphology and function

The prostate is a gland about the size and shape of a walnut. It is located in front of the rectum, just below the urinary bladder and surrounds the urethra. The prostate has a tree-like structure of glandular ducts composed of an epithelial parenchyma embedded within a stroma tissue matrix.

The mature prostatic epithelium has three main cell types - basal, secretory luminal and neuroendocrine. The basal cells rest on the basement membrane and probably include stem cells that form the proliferative compartment of the prostate epithelium. These cells give rise to the transitional cells that in turn give rise to basal cells, differentiated luminal cells and the neuroendocrine cells [5-7]. The luminal cells represent the major cell type and synthesize and secrete proteins of the seminal plasma, including PSA and prostatic acid phosphatase that together with fluid from the seminal vesicles forms most of the ejaculate. The secretory proteins are important for sperm motility and survival [8]. Although fertility is impaired in the absence of a prostate, the prostate is not required for reproduction.

The prostatic epithelium is surrounded by a stroma containing smooth muscle cells, fibroblasts, myofibroblasts, nerves, blood vessels, lymphatics and infiltrating immune cells. The prostate stroma not only physically supports the glandular epithelium but also contributes with important paracrine signals.

The human prostate is divided into three anatomical zones: the peripheral zone, the transitional zone and the central zone [9]. The peripheral zone comprises the majority of the gland, approximately 65%, and the majority of cancers are believed to originate at this site [10]. In rats, the prostate is composed of four well-defined lobes: the ventral (VP), dorsal, lateral and anterior prostate [11]. Histologically, the rodent prostate has a much higher ratio of epithelium to stroma, and the smooth muscle layer is limited to thin sheaths surrounding the glands [12, 13].

Prostate growth control

The prostate develops from the embryonic urogenital sinus, which is composed of urogenital sinus epithelium and urogenital sinus mesenchyme (UGM). Prostatic development is dependent on both epithelial-mesenchymal interactions and androgenic (male) steroids [14, 15]. The prostate fully develops after puberty under the influence of increased levels of circulating androgens.

INTRODUCTION

Testosterone is the main circulating androgen, and is synthesized by the Leydig cells of the testis under control of luteinizing hormone (LH) secreted by the pituitary gland. LH secretion is in turn regulated by gonadotropin-releasing hormone from the hypothalamus. At this level testosterone has a negative feedback effect to maintain the circulating levels of testosterone within normal levels. A small portion of androgens are also produced in the adrenal glands [16]. In the prostate, testosterone is rapidly metabolized by 5α-reductase to the more potent dihydrotestosterone (DHT) [17, 18]. DHT then interacts with nuclear androgen receptors (AR) and along with several co-activator proteins this complex regulates the expression of androgen-related genes [19]. ARs in rat prostate are expressed by luminal epithelial cells, stromal cells, and periendothelial cells [20, 21], but generally not by endothelial cells [21]. Stem cells are AR negative and the basal cells rarely express AR [22].

The prostate is dependent on androgens for growth, function and maintenance of tissue architecture. When the supply of androgens is depleted or when androgen action is blocked, by castration or anti-androgenic therapies respectively, the prostate luminal epithelial cells will undergo apoptosis [23-26]. The loss of epithelial cells consequently contributes to the shrinkage or regression of the prostatic tissue following castration [27, 28]. Both stromal cells and epithelial basal cells are, however, maintained during androgen ablation [29, 30].

It has, until recently, been generally perceived that this loss of cells is a direct effect of androgen withdrawal on the epithelial cells. However, several studies have shown that AR in the stroma could indirectly mediate the effects observed in the epithelium through paracrine mechanisms. When AR positive wild-type (wt) UGM (representing prostate stroma) is combined with AR-negative non-malignant epithelium, and grown as tissue recombinants *in vivo*, it results in prostatic development [31]. ARs in prostate stromal cells are therefore considered critical for prostate development. To further show the importance of ARs in the stroma, tissue recombinants combined of wt-UGM and AR-negative epithelium or wt-UGM and AR-positive epithelium were compared after castration. Androgen withdrawal induced apoptosis in AR deficient epithelial cells to the same extent as epithelial cells containing AR, indicating that castration effects in the epithelium are regulated by the stroma [32]. Epithelial ARs are therefore not required for castration induced apoptosis. AR in the mature epithelium is found to mainly maintain differentiation and suppress proliferation of these cells [33, 34].

Observations also suggest that the vasculature could be an additional target for androgen action in the prostate. Vascular disruption to the prostate leads to tissue regression and epithelial apoptosis similar to the regression response seen after androgen withdrawal [35]. Based on that and similar studies the effects of androgens on blood flow to the prostate gland was examined in rodents. Indeed, blood flow to the prostate was shown to be drastically reduced shortly after surgical castration [36, 37]. This reduction in prostate blood flow can be explained by an increase in endothelial apoptosis and vasoconstriction of larger vessels [36, 38, 39]. The apoptotic peak of endothelial cells was reached at 24 h after castration, while maximal epithelial cell apoptosis was not reached until 72 h after castration [39]. This suggests that the rat prostatic vascular system is a primary target for androgen action and that prostate regression after castration is an indirect result of the early effects of androgen withdrawal on the vasculature. Epithelial apoptosis is thus a consequence of a hypoxic environment associated with the lack of sufficient blood flow [40, 41].

The mechanisms behind androgenic control of the prostate vasculature are not fully understood. One explanation could be that androgens normally stimulate the production of critical angiogenic factors in AR-positive prostate epithelium and stromal cells. In the absence of androgens the production of these factors is lost leading to subsequent endothelial death. Testosterone has for instance been shown to regulate the production of important angiogenesis factors like vascular endothelial growth factor (VEGF) [42, 43]. However, recent studies have also shown that ARs are expressed on some vascular smooth muscle cells and endothelial cells suggesting that androgens possibly influence the vasculature directly [20, 44]. The functions of ARs on endothelial or other vascular cells are nevertheless unknown.

Androgen supplementation to castrated rats stimulates prostate regrowth. Endothelial cells are the first cells that proliferate and normalization of the vasculature volumes and blood flow occurs prior to prostate epithelial regrowth [45]. Also in this process paracrine angiogenic signaling from AR-positive cells are important. This was illustrated when VEGF was inhibited with concomitant testosterone replacement therapy. Here endothelial apoptosis was increased which also resulted in reduced organ regrowth [46].

In summary, castration induced prostate regression could be the result of several parallel mechanisms; 1) direct effects of androgen-withdrawal on the epithelial cell, 2) altered paracrine signaling from AR-positive stromal cells and 3) tissue hypoxia caused by reduced blood flow (Fig 1).

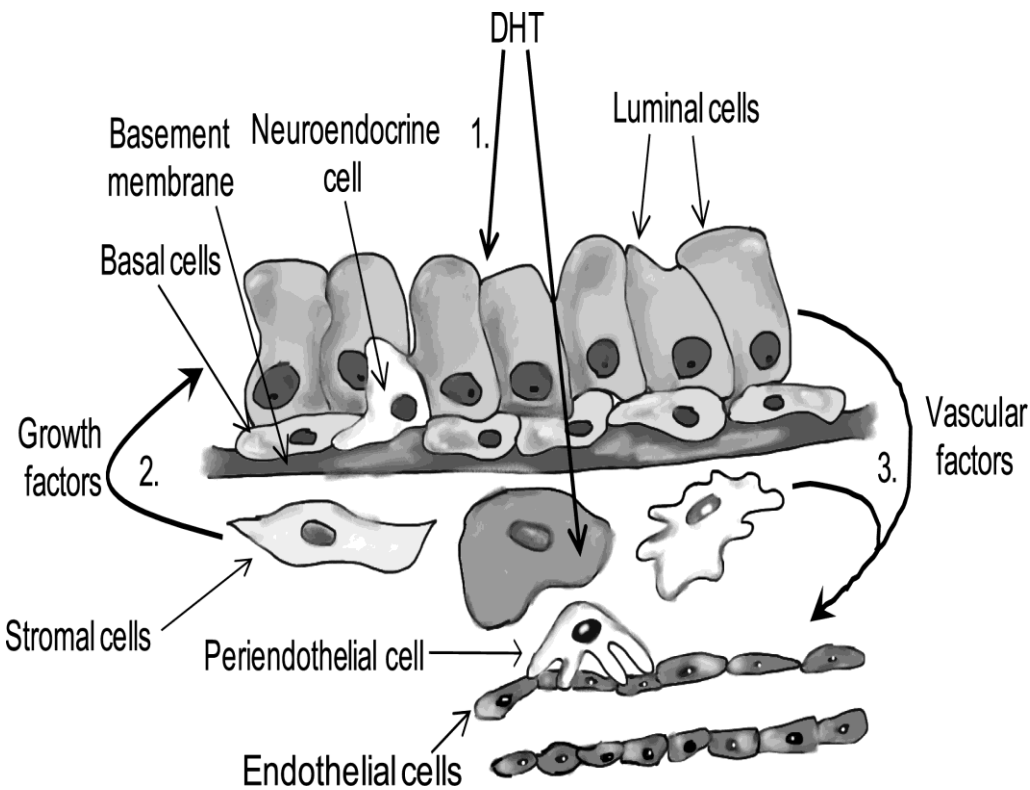

Figure 1. Schematic illustration of normal prostate growth control

Dihydrotestosterone (DHT) stimulates androgen receptors present in both epithelial and stromal cells. Castration reduces the levels of DHT and induces prostate epithelial apoptosis. This could be due to; (1) direct effects of androgen-withdrawal on the epithelial cells, (2) loss of paracrine signaling from androgen responsive stromal cells that normally support the epithelium or (3) loss of vascular factors that normally support the vasculature, resulting in hypoxia and decreased blood flow causing subsequent epithelial death.

Prostate cancer

General background

The cause of prostate cancer is still not known. Ageing is the single most significant risk factor for developing prostate cancer and the median patient age at diagnosis is 75 years [47]. Although the incidence rate has increased, probably due to the introduction of PSA testing, the mortality rate is almost unchanged [2]. The incidence and mortality for prostate cancer varies in different regions around the world. The environment and diets are suggested to explain this difference [48]. Prostate cancer is a multifocal and heterogeneous disease and several tumors can be found in the prostate within an individual patient at diagnosis [49]. Recently, it was discovered that a significant part of all prostate cancers overexpress an oncogene, E twenty-six (ETS) transcription factor (usually ERG) [50]. The most common mechanism of overexpression was by fusion of ERG to the androgen regulated transmembrane protease serine 2 gene, TMPRSS2 [50, 51]. The function of the TMPRSS2-ERG fusion gene is mainly believed to drive transition to invasive prostate cancer [51].

Diagnosis and prognosis of prostate cancer

Most prostate cancers are today diagnosed on the basis of PSA testing, followed by rectal palpation and transrectal ultrasound together with sampling of biopsies that are examined histologically. PSA is measured in the blood and assess the risk of having prostate cancer. It is considered normal to have a PSA value between 0 and 3 ng/ml and a PSA value > 10 ng/ml indicates a substantial risk of having prostate cancer. The majority of patients have a PSA value between 3 and 10 ng/ml, which also could be caused by other conditions besides prostate cancer, like inflammation or benign hyperplasia.

Biopsies from the prostate are taken from patients with elevated PSA levels. Usually, 6-12 small biopsies are taken with a needle through the rectal wall. Although ultrasound is used as guidance, the exact locations of the tumors are not always evident and therefore biopsies may sample unrepresentative tissue. If a biopsy contains cancer, it is scored according to the Gleason system [52]. The most common and the second most common area are scored on a differentiation scale ranging from 1 to 5, where 5 represents the lowest differentiated and most aggressive tumor pattern. The sum of the most common grades gives the tumor its Gleason score (GS) (2-10). GS is a strong predictor of prostate cancer outcome in

low (≤5) and high (8-10) scored tumors [53, 54]. However, most patients have a GS 6-7 which give poor prognostic indication for the individual patient [53].

Advanced prostate cancer is characterized by spreading outside the prostate and the most common sites for prostate cancer metastasis are to lymph nodes and bone. Different methods are used, for instance PSA level in serum, bone scintography, CT and MRI, to determine how advanced the tumor is and thereby also decide what type of treatment the patient should be given.

Treatments of prostate cancer

For localized disease the patient can be cured by radical prostatectomy or radiotherapy. If the life expectancy of the patient is short and the prostate tumor is at an early stage it is common that the patient is only subjected to watchful waiting [2]. Younger patients are usually recommended to have treatment with curative intent. Even though treatment is subjected at early stages of the disease up to 30 % of the patients relapse after prostatectomy [4]. This suggests that shedding of tumor cells is an early event in prostate cancer progression. This is supported by studies showing that over 70 % of the patients have prostate tumor cells in their bone marrow already before surgery [4].

For advanced and metastatic prostate cancer the option is palliative therapy in the form of surgical or chemical castration. Castration lowers circulating androgen levels and in most patients this will lead to a reduced tumor burden and symptomatic relief. The beneficial effects of androgen deprivation was first described by Huggins et al. already in 1941 [27, 55], findings that later awarded him the Nobel Prize in 1966. Initially, castration reduces proliferation and increases apoptosis in prostate tumor cells [26, 56]. However, the exact mechanism behind the initial response to castration is unknown. It is assumed that the castration response is primarily a direct effect of androgen withdrawal from the tumor epithelial cells, but just like in normal prostate, indirect effects from prostate stroma and vasculature are also likely.

Although the treatment can hinder tumor progression for some time the tumor eventually relapses and grows in an apparently androgen-independent manner [56, 57]. Recent studies do however indicate that androgens can be synthesized locally in prostate tumors from circulating adrenal steroids or cholesterol and thereby maintain androgen receptor signaling despite low levels of circulating androgens [58-60]. This indicates that many prostate tumors still maintain their AR signaling after

castration and this stage of the disease, previously referred to as androgen-independent, is now called castration resistant prostate cancer. Treatment of patients with castration resistant prostate cancer with the cytotoxic drug docetaxel in combination with prednisone has been shown to give an overall survival benefit [61, 62].

Many novel and more potent agents targeting the AR signaling pathways are currently being tested in clinical trials for treatment of castration resistant prostate cancer [63]. However, this is the terminal stage for prostate cancer that ultimately leads to death of the patients. New treatment options and ways to improve and sustain the beneficial effects of androgen ablations are therefore urgent.

Tumor microenvironment

Tumors are highly complex tissues of neoplastic cells and stromal cell compartments that together with extra cellular matrix (ECM) create the complexity of the tumor microenvironment.

The tumor stromal cells are distinct from the normal mesenchymal tissues in both composition and their gene expression profile [64]. In addition, cancer-reactive stromal cells, such as inflammatory cells (lymphocytes, macrophages and mast cells), vascular cells (endothelial, pericytes and smooth muscle cells) and fibroblasts, actively support tumor growth and play an important role in the initiation and progression of prostate and other cancers [65-70].

Alterations in the prostate tumor stroma could possibly be used as prognostic markers. For instance, prostate cancer patients with profound alterations in tumor stroma morphology have a poor clinical outcome [71, 72]. Also, loss of AR expression in the prostate tumor stroma and in the normal prostate stroma surrounding the tumor was shown to be associated with increased GS, metastasis, poor outcome and a poor response to castration therapy [73]. Other alterations, such as angiogenesis [74] and accumulation of macrophages [75], in the prostate tumor stroma have also been linked to prostate cancer prognosis.

Activated fibroblasts in tumors are referred to as cancer-associated fibroblasts (CAFs) and are a key component of the tumor stroma. CAFs can stimulate tumor progression and metastasis by secreting growth factors like transforming growth factor β (TGFβ), promote angiogenesis, and stimulate infiltration of immune cells [66, 76].

Tumor associated macrophages (TAMs) are one type of immune cells that infiltrate tumors. TAMs represent a major component of the tumor stroma and are present in almost all solid tumors [68]

Tumor-associated macrophages

Although activated macrophages may have anti-tumor activity, TAMs have also been shown to promote tumor initiation and progression. Clinical evidence show that increased intratumoral macrophage density correlates with poor prognosis in most tumor types [77, 78]. Lissbrant et al. showed that macrophage density correlated with tumor angiogenesis and shorter survival in prostate cancer [75]. However, contrasting results has also been reported [79]. Two distinct polarization states have been described for macrophages: the M1 and M2 macrophage. The M1 phenotype is proinflammatory and has tumoricidal activity whereas M2 macrophages, in contrast, promote angiogenesis, growth and metastasis. TAMs are generally described as M2 macrophages. However, macrophages show a high degree of plasticity during tumor development and progression and mixed M1 and M2 phenotypes have also been described [77] and this could explain some of the conflicting results.

TAMs derive from monocytes circulating in the blood and enter tumors in all stages of tumor progression [68, 78]. Many factors produced by tumors such as colony-stimulating factor 1 (CSF-1), monocyte chemoattractant protein-1 (MCP-1/CCL2), VEGF, platelet derived growth factor (PDGF) and TGFβ are chemotactic for monocytes [80]. At the tumor site, monocytes/macrophages then interact with the tumor and stromal cells to make an environment rich in chemoattractants and growth factors. In this setting, TAMs are able to promote tumor progression in several parallel ways (Fig.2).

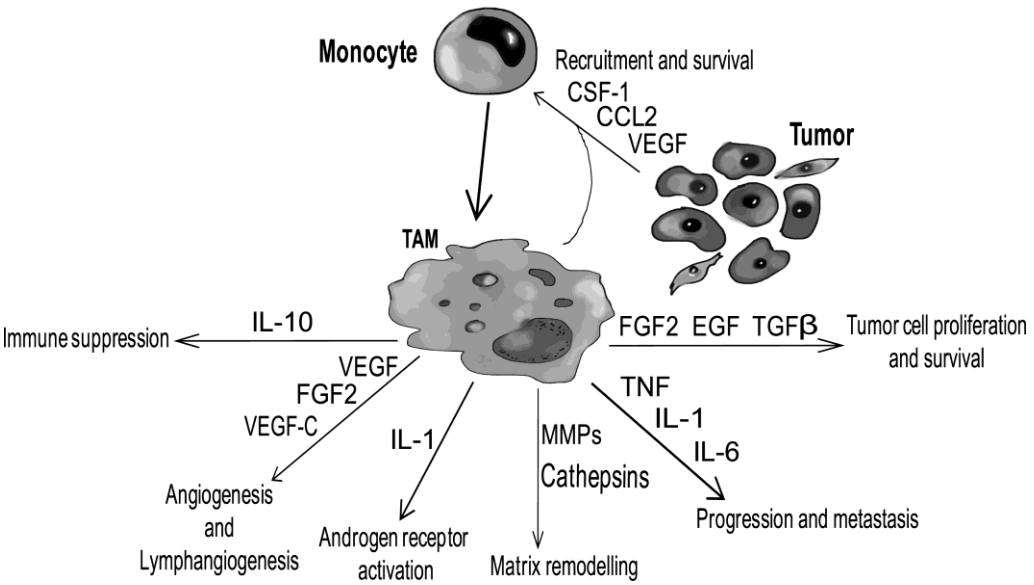

Figure 2. Pro-tumoral functions of tumor-associated macrophages (TAM).

TAMs produce growth factors like fibroblast growth factor 2 (FGF2), epidermal growth factor (EGF) and TGFβ that stimulate tumor cells directly [81]. Inflammatory cytokines secreted by TAMs, such as tumor necrosis factor α (TNFα), interleukin 6 (IL-6) and interleukin 1β (IL1β), can increase tumor invasiveness and metastasis [82]. In addition, IL1β converts androgen receptor modulators from being inhibitory to stimulatory, connecting TAMs to castration resistant prostate cancer [83]. Moreover, macrophages are often found at sites of basement-membrane breakdown and at the invasive front of tumors where they release matrix-degrading enzymes like matrix metalloproteinases (MMPs) [84] and cathepsins [85, 86] and thereby enhance tumor invasion. Further, TAMs and tumor cells release interleukin 10 (IL-10) and other immunosuppressive cytokines that suppress anti-tumor immune responses [80]. TAMs also promote tumor angiogenesis and lymphangiogenesis (see below) by secreting for instance TNFα, VEGF, FGF2 and VEGF-C [78].

Angiogenesis

Normal tissue function depends on blood vessels that provide oxygen, nutrients and remove metabolic waste. All blood vessels contain a layer of endothelial cells that are associated with pericytes and/or smooth muscle cells.

In the embryo blood vessels form by vasculogenesis; that is, when endothelial cell precursors, or angioblasts, assemble into a primitive vascular network. This vascular labyrinth then expands and remodels in the process of angiogenesis [87, 88].

Sprouting angiogenesis is the most extensively studied mechanism and involves several sequential steps. In response to angiogenic factors the blood vessel dilates and the endothelium is destabilized and activated. Next, local activated proteases like MMPs degrade the basement membrane and the ECM surrounding the endothelial cells. This is followed by endothelial cell invasion, proliferation and migration into solid sprouts connecting neighboring vessels. Finally, a vessel lumen is formed and the vessel is stabilized by covering pericytes [89].

Physiological angiogenesis is tightly controlled and occurs in adult life during female reproductive cycles, wound healing and possibly also in male reproductive organs [74]. In contrast, uncontrolled angiogenesis can contribute to pathological conditions such as neoplastic growth.

Tumor angiogenesis

In 1971, Folkman proposed the concept that tumors are unable to grow beyond a certain size unless they are able to recruit blood vessels from the existing vasculature. He proposed that tumor cells secrete angiogenic factors that stimulate vascular growth and that inhibiting this process could be used as an anti-cancer treatment [90]. The ideas were not accepted by the research community at first and it was not until years later the first tumor-derived angiogenic factors, FGF2 and VEGF, were discovered [91, 92]. In the last decade, angiogenesis has been extensively studied and today there are several anti-angiogenic therapies in clinical trials and also clinically approved drugs [88, 93-95].

Tumors are able to grow without a vasculature to a size no more than 1-2 mm in diameter [96, 97]. In this avascular phase the tumor is nourished by diffusion of oxygen and nutrients provided by nearby blood vessels. In order to exceed this dormant stage, tumors switch to an angiogenic phenotype and attract blood vessels from the surrounding stroma. The transition from the avascular dormant stage to

the angiogenic phase is often referred to as the "angiogenic switch". The tumor achieves this by secreting pro-angiogenic factors and/or by suppressing anti-angiogenic factors (Table 1) resulting in new vessel formation and exponential tumor growth [98].

Table 1. Example of angiogenic and anti-angiogenic factors

Angiogenesis stimulators	Angiogenesis inhibitors
Vascular endothelial growth factor (VEGF)-A, -B, -C, -D	Thrombospondin (TSP) -1, -2
Epidermal growth factor (EGF)	Endostatin
Fibroblast growth factor (FGF) 2	Angiostatin
Transforming growth factor (TGF) β	Pigment epithelium-derived factor (PEDF)
Platelet-derived growth factor (PDGF)	Interferon γ
Angiopoietin (Ang)-1, -2	Tumstatin
Tumor necrosis factor (TNF) α	Arresten
Interleukin (IL)-8	Maspin

Important inducers of angiogenesis in tumors are hypoxia and genetic mutations. Hypoxia results in increased levels of the transcription factor hypoxia inducible factor-1 (HIF-1), that is rapidly degraded under normal oxygen levels. HIF-1 drives the transcription of several genes important for angiogenesis, including VEGF.
The tumor vasculature is structurally abnormal with highly irregular and tortuous vessels, they are leaky, and often lack periendothelial cells and have blind ends. This results in highly variable blood flow and hypoxic areas within the tumor [99].

These newly formed vessels also provide a route for cancer cells to disseminate and metastasize. Further, for metastatic cells to grow at the distant site, they need to induce angiogenesis [100].

Mechanisms for tumor vascularization

Although sprouting angiogenesis is the most described mechanism for tumor vascularization, other mechanisms are also possible (Fig. 3). Another variant of angiogenesis is intussusceptive angiogenesis. In this type of vessel formation the preexisting vessels split into two new vessels. Endothelial cells of opposite walls make contact by which a "bridge" is formed. A pillar of pericytes and myofibroblasts invade and cover the newly shaped wall. These pillars then grow in

size and the endothelial cells retract and two separate vessels are formed [101, 102]. This type of angiogenesis can also be observed in smaller arteries and veins [103]. New vessels can also grow through the recruitment of endothelial progenitor cells (EPCs) that are circulating in the blood, although the quantitative importance of this is under debate [104]. Chemoattractants secreted by the tumor are sensed by EPCs that arrest, migrate, incorporate and differentiate into mature endothelial cells [105]. Already existing vessels can also promote tumor growth without evoking an angiogenic response. In this process which is called co-option, the tumor cells grow along and surround the pre-existing vessels. Small dormant micrometastasis probably co-opt with host vessels and are thereby able to survive [106]. Tumor cells are also able to dedifferentiate to an endothelial phenotype and make tube-like structures. This "vascular mimicry" of tumor cells as endothelial cells occurs mainly in aggressive tumors [107].

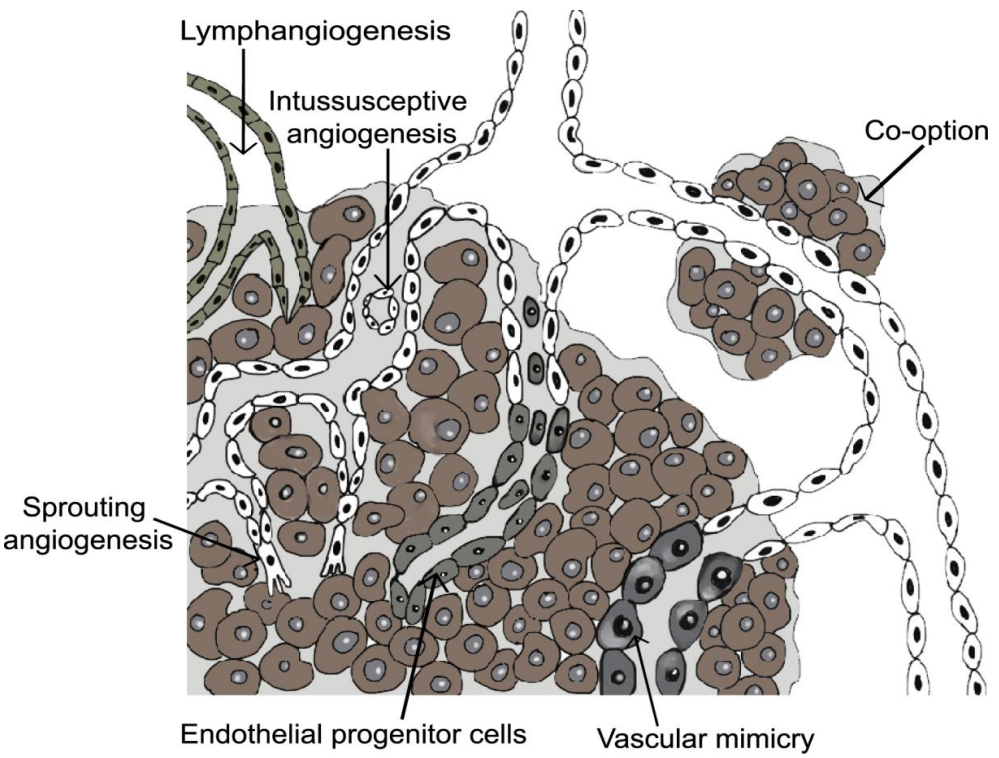

Figure 3. Different mechanisms of tumor vascularization

Lymphangiogenesis

The lymphatic system is a network of capillaries that drains most of the organs. It is a one way transport system that drains fluids and lymphocytes and returns it to the circulation. The involvement of the lymphatic system and lymphangiogenesis in the metastatic process has been intensively investigated over the past years. Lymph node metastases are seen in many types of cancer, including prostate cancer, and via the lymphatics cancer cells are able to spread to other organs [108]. Important lymphangiogenic factors include VEGF-C and VEGF-D that bind to the receptor VEGFR-3 on lymph endothelial cell and thereby stimulate lymphangiogenesis [108].

Pigment Epithelium-Derived Factor

Pigment epithelium-derived factor (PEDF) is a 50 kDa secreted glycoprotein that was first described in the 1980s after being identified and isolated from conditioned media of primary human fetal retinal pigment epithelial cells [109, 110]. Its gene (Serpinf1) has been mapped to human chromosome 17p13 [111]. PEDF belongs to the non-inhibitory serine protease inhibitor family [112] and is expressed in almost all human tissues including the prostate [113]. The role of PEDF in the human body is still unclear and sometimes contradictory. PEDF has been described as having multiple biological properties such as neuroprotective, anti-angiogenic, and anti-tumoral [114]. It has also been implicated in both pro- and anti-inflammatory processes [115-119]. The mechanisms by which PEDF perform its pleiotropic activities are still largely unknown, but different PEDF receptors which trigger divergent intracellular signals have been suggested.

Antitumor effects of PEDF

The anti-tumor potential of PEDF is based on its multiple effects and involves anti-angiogenesis, tumor cell differentiation and tumor cell apoptosis.

Anti-Angiogenesis

PEDF has been shown to be one of the most potent endogenous inhibitors of angiogenesis, being more than twice as potent as angiostatin and seven times more potent than endostatin [120]. Dawson et al. showed that PEDF prevented endothelial cell migration alone or in the presence of potent angiogenic inducers including VEGF, PDGF, IL-8 and FGF2 [120].

The anti-angiogenic effects of PEDF have been associated with apoptosis in activated endothelial cells. Endothelial cells stimulated by VEGF or FGF2 expose the receptor Fas on their surface. PEDF on the other hand, is able to induce the expression of endothelial Fas ligand (FasL). When expressed simultaneously Fas and FasL are able to induce apoptosis in endothelial cells, and subsequently inhibit angiogenesis [121]. Moreover, PEDF has been shown to disrupt the balance between pro- and anti-angiogenic factors by down-regulating VEGF and MMP9 and up-regulating thrombospondin 1 in gliomas [122, 123]. Another mechanism for PEDF on endothelial cells was demonstrated by Cai and collaborators, involving the activation of γ-secretase-dependent cleavage of the C terminus of VEGF-receptor 1, which consequently inhibits VEGF-receptor 2 induced angiogenesis [124].

PEDF does not seem to be essential for viability since PEDF knockout mice are born alive and healthy [125]. Interestingly, PEDF depletion during prostate development results in hyperplasia and increased vascular density of the organ [125]. Increased microvasculature was also noted in the retina, kidney and pancreas [125]. These findings show that PEDF could play an important role in the maintenance of a proper angiogenic balance at least in some organs including the prostate. PEDF's role as an important angiogenesis inhibitor in normal tissues implies that its loss could be involved also in tumor vascularization.

During the last decade an increasing number of studies have shown that PEDF have tumor inhibitory effects. Decreased PEDF expression is linked to increased metastases and poor prognosis in pancreatic cancer[126], breast cancer [127] and gliomas [128] indicating the significant role of PEDF in the development of many types of cancer. This prompted further investigations into the effects of over-expressing PEDF in various tumors. Forced expression, silencing or treatment with recombinant PEDF has shown that PEDF is a potent tumor suppressor in many tumor types [122, 125, 129-140].

The effects of PEDF on primary tumor growth and progression could be explained only from inhibition of tumor angiogenesis, however, other mechanisms are also possible and described below.

Tumor cell differentiation and direct tumor suppression

PEDF have neurotrophic and neuroprotective activities and in tumors of neuronal origin, PEDF promotes differentiation. In retinoblastoma cells, PEDF causes differentiation manifested by neurite-like extensions [110]. Crawford et al. showed

that intratumoral injection of recombinant PEDF to neuroblastomas resulted in a less malignant phenotype, with induced expression of neurofilament, a marker of neural-cell differentiation [131]. PEDF epitopes have also been shown to induce neuroendocrine differentiation of prostate PC-3 tumors with consequential tumor suppression [132].

The nuclear factor-κB (NFκB) pathway has been implicated in the neurotrophic and neuroprotective signals. In cerebellar granule cells, this pathway has been shown to be regulated by PEDF [141]. PEDF activates NFκB by inducing the phosphorylation and degradation of its inhibitor IkB. This leads to activation and nuclear translocation of NFκB and consequently the transcription of anti-apoptotic and neuroprotective genes [141]. NFκB has also been shown to control the formation of neuroendocrine differentiation in prostate cancer cells [142].

In addition, PEDF triggers apoptosis in some tumor cell lines directly [122, 129, 133, 143]. The mechanisms behind induced tumor cell apoptosis are not known but are thought to be similar to that observed in endothelial cells. In cultured prostate tumor cells, PEDF induced apoptosis was also shown to be augmented by hypoxia [125].

PEDF has also been shown to reduce the migratory and invasive potential of melanoma and glioma cells [122, 133].

PEDF regulation

PEDF is generally down-regulated in prostate cancer and prostate cancer cell lines [125]. The underlying mechanism behind the regulation is unknown but hypoxia down-regulates PEDF protein in prostate stromal cells and cancer cell lines *in vitro* [125]. PEDF can also be down-regulated by androgens in prostate stromal cells and is up-regulated by castration in rat ventral prostate and in some human prostate tumors [125]. If this increase in PEDF levels is important for the vascular regression seen after castration in rats (see above) is still unknown.

AIMS

General Aims

The idea that cancer cells interact with its microenvironment is not new. Already in 1889, Paget published his seed and soil hypothesis, suggesting that certain tumor cells (the seed) have specific affinity for certain organs (the soil) and that metastasis only occurred when these two were compatible [144]. Another finding already in 1863 was that of Virchow, who observed the connection between inflammation and cancer [145]. During the last decade the tumor microenvironment has received more attention and many studies now support both these ideas showing the importance of the tumor microenvironment for tumorigenesis.

The general aim of this thesis was to target different compartments of the prostate tumor microenvironment to provide potential new therapies for prostate cancer.

Specific Aims

- To study how an androgen-independent prostate tumor and the normal androgen-dependent prostate tissue interact in response to castration therapy using an orthotopic rat tumor model

- To study the importance of tumor associated macrophages for prostate tumor growth and angiogenesis, in an orthotopic rat tumor model

- To examine PEDF expression during prostate tumor progression in rat and human prostate tumors

- To study the role of PEDF over-expression for prostate tumor growth, angiogenesis and metastasis in an orthotopic rat tumor model.

MATERIALS AND METHODS

Animals and Treatments

Adult male Copenhagen rats were used for *in vivo* experiments. All animal work was approved by the local ethical committee for animal research.

The Dunning Tumors

The Dunning prostate tumor system consists of transplantable rat prostatic adenocarcinomas that differ in their histology, growth rate, androgen sensitivity, and metastatic potential [146]. All sublines derive from a spontaneous tumor in the dorso-lateral prostate of a 22-month old Copenhagen rat. The original tumor was found in the beginning of the 1960s by Dr. W.F. Dunning, who named it Dunning R3327 [147]. Following serial passages, that tumor gave rise to the well differentiated and androgen sensitive R3327-H and PAP tumors. These tumors are transplantable as small tumor pieces on Copenhagen rats. During passage of the Dunning H subline *in vivo*, several other sublines with different characteristics have emerged (Table 2) [146, 148, 149]. All of these sublines have been established as *in vitro* cell lines, which can be injected back to Copenhagen rats to give tumors *in vivo*.

Table 2. Characteristics of the Dunning tumor sublines used in this thesis

Tumor subline[146, 148]	Histology	Androgen sensitivity	Tumor doubling time (days)	Metastatic capacity
R3327 PAP	WD	AS	12 ± 2	Low
AT-1	A	AI	2.2 ± 0.3	Low
AT-2	A	AI	2.4 ± 0.2	Low to moderate
AT-3	A	AI	1.9 ± 0.3	High
MatLyLu	A	AI	1.7 ± 0.3	High

WD = well differentiated and A = anaplastic

AS = androgen sensitive and AI = androgen insensitive

Low: <5%, moderate: >5% and <20%, and high metastatic capacity: >75% of subcutaneously transplanted rats develop distant metastases

In vitro cell lines (paper I-IV)

AT-1, AT-2, AT-3 and MatLyLu where grown in RPMI 1640 with 10 % fetal calf serum, 0.2 % Na-Bic, 50 µg/ml gentamycin and 250 nM dexamethasone in 37° C and 5 % CO_2, according to the manufacturer's instructions. Before inoculation the cells were grown to about 75 % confluence, trypsinized, counted in a Burker chamber and diluted in RPMI to the appropriate concentration.

Subcutaneous implantation (paper III)

Due to the different characteristics of the Dunning tumors they were chosen to represent different tumor grades. Small pieces (about 1 mm^3) or tumor cells ($2x10^6$) were inoculated subcutaneously (s.c) on the flank of approximately 10 week old male Copenhagen rats (Charles River, Germany). When the tumor had reached a size of about 1-2 cm in diameter, the animals were sacrificed and the tumors removed and frozen in liquid nitrogen before further analysis.

Orthotopic implantation and tissue preparation (paper I, II, IV)

For experimental and morphological studies, the tumor cells were injected into the prostate (VP) of immunocompetent and syngenic rats. This setting somewhat resembles the tumor-stromal interactions and microenvironment seen in prostate cancer patients.

During anesthesia, an incision was made in the lower abdomen to expose the VP lobes. AT-1 ($2x10^3$) or transfected MatLyLu ($1x10^4$) cells in a volume of 50 µl were carefully injected into one lobe of the VP using a Hamilton syringe. The different experiments were initiated as described below.

For morphological analysis the animals were first sedated and perfusion fixed in 4 % paraformaldehyde and the tissues of interest were removed, weighed and fixed by immersion for another 24 hours before embedded in paraffin for histological examinations. Transfected MatLyLu tumors (paper IV) and clodronate treated AT-1 tumors (paper II) and controls were directly immersion-fixed in 4 % paraformaldehyde after sacrifice. One hour before sacrifice the animals were injected intra peritoneally (i.p) with bromodeoxyuridine (BrdU, Sigma-Aldrich) to mark proliferating cells. At the same time, intended animals were injected i.p with the tissue hypoxia marker pimonidazole (Hypoxyprobe, Chemicon) which stains tissues with pO_2 less than 10 mmHg. For RNA and protein analysis (paper II and

IV), animals were killed and the tissues removed, frozen in liquid nitrogen and stored at -80° C before further analysis.

Experimental procedures

Castration (paper I)

For castration studies fast growing, androgen insensitive, anaplastic and low metastatic AT-1 tumor cells were used. All animals were injected orthotopically with the same amount of tumor cells and further divided into two weight-matched groups. The first group served as controls and rats were sacrificed at day 7, 10 and 14 after tumor cell injection. Seven days after tumor cell injection, the second group was castrated by scrotal incision. These animals were sacrificed 3 (day 10) and 7 days (day 14) later.

Clodronate-Liposomes (paper II)

Circulating phagocytic cells were eliminated by i.p injections of dichloromethylene-bisphosphonate (clodronate) liposomes. Clodronate was a gift from Roche Diagnostics GmbH (Mannheim, Germany) and was incorporated into liposomes by van Rooijen et al [150]. Phagocytic cells ingest and digest the liposomes. This is followed by intracellular release and accumulation of clodronate that at a certain intracellular concentration induces apoptosis of the phagocytic cells [151]. We administered clodronate-liposomes (1 ml/100g body weight) every second day starting 4 days before AT-1 tumor cell injection. As controls, equal amounts of phosphate-buffered saline (PBS) injections were used.

PEDF over-expression in vivo (paper IV)

To study the effects and functions of PEDF on rat prostate tumor cells, MatLyLu cells transfected with a plasmid vector containing human PEDF cDNA or control vector (see below) was injected to the VP as described. Rats were sacrificed 7 and 23 days post tumor cell injection.

TNFα stimulation in vivo (paper IV)

To study the effects of TNFα on PEDF expression *in vivo*, recombinant rat TNFα (Sigma-Aldrich, total 1 µg/animal) or control solution (PBS) were injected into non-tumor bearing VP of Copenhagen rats. Six hours later the animals were killed and the VP was obtained.

Cell Culture

Hypoxia treatment (paper II)

To test the effects of hypoxia on the transcription of angiogenic factors and cytokines, AT-1 cells were grown in complete medium and incubated in a hypoxic incubator (1 % O_2, 5 % CO_2, 94 % N_2, Billups-Rothenberg) or in normoxia (21 % O_2, 5 % CO_2, 74 % N_2) for 6 and 24 hours at 37°C.

Conditioned medium (paper III and, IV)

Cell lines (AT-1, AT-2, AT-3 and MatLyLu) were grown in recommended media (see above) to approximately 70-80 % confluence, carefully rinsed in PBS, incubated in serum-free medium (RPMI 1640, 0.2 % Na-Bic and 250 nM dexamethasone) for 4 hours, and carefully washed again. Fresh serum-free medium was added, and the cells were incubated for 48 h in 37°C, 5 % CO_2. Before PEDF protein analysis or purification, the medium was collected and centrifuged to remove cell debris. The conditioned medium was then further concentrated and dialyzed against PBS using centrifugal filters with a 10 kDa cutoff (Millipore), according to the manufacturer's instructions. The BCA protein assay reagent kit (Pierce Chemical Co.) was used to determine protein concentration.

Transfection (paper IV)

The human PEDF cDNA cloned into a pCEP4 vector (Invitrogen) was kindly provided by Dr. Susan Crawford at NorthShore University, Evanston. The pCEP4 vector is an episomal mammalian expression vector that uses cytomegalovirus immediate early promoter for high level transcription of the inserted gene. pCEP4 also carries the hygromycin B resistance gene for stable selection in transfected cells. Before transfection the lethal hygromycin B concentration for MatLyLu cells was determined to 400 µg/ml. MatLyLu cells were then transfected with PEDF vector or empty control vector using Lipofectamine (Invitrogen), according to protocol. Hygromycin B resistant clones were chosen and the expression of PEDF was confirmed by western blot.

Endothelial migration assay (paper IV)

To test if PEDF protein from the transfected MatLyLu cells was biologically active, PEDF was purified from conditioned media and tested in an *in vitro* angiogenesis assay. PEDF cDNA was engineered to encode a COOH-terminal

hexahistidine tag [120]. Recombinant PEDF could therefore be purified on a HisTrap HP column according to the manufacturer's instructions (Novagen). The eluted sample was dialyzed against PBS using a dialysis cassette with 10 kDa cutoff (Pierce). Purification was determined by Coomassie stained SDS polyacrylamide gels.

HUVEC endothelial cells (Cascade Biologics, Paisley, UK) were grown in Medium 200 (Cascade Biologics) supplemented with Low serum growth supplement (Cascade Biologics) and migration was studied in modified Boyden chambers. Serum-free medium with 0.1 % bovine serum albumin containing test substances were placed in the lower chambers and covered with a collagen 1 (Cohesion) coated chemotaxis membrane (Neuroprobe). Approximately 10 000 cells, resuspended in serum-free media, were seeded in the top wells. After incubation for 6 hours, 37°C, the filters were stained and cells that had not passed through the membrane were removed and the remaining cells were counted under a light microscope.

Viability assay (paper IV)

Viability of the transfected MatLyLu cells, was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Roche Diagnostics). PEDF transfected cells (MatLyLu-PEDF) or control cells (MatLyLu-CON) (10 000 cells/well) were seeded in 100 µl in a 96 well plate and incubated for 72 hours. After that, 10 µl of MTT labeling reagent was added to each well and incubated for four hours. Viable cells metabolize MTT to purple formazan crystals. Then 100 µl of solubilization solution was added into each well and incubated over night to solubilize the crystals. The resulting colored solution was quantified by measuring absorbance at 550 nm subtracted with the reference wavelength at 650 nm.

TNFα stimulation in vitro (paper IV)

The effect of TNFα on PEDF mRNA expression *in vitro* was tested. AT-1 cells (5 x 10^5) were seeded in 1 ml complete medium in a 12 well plate and allowed to settle over night. The cells were carefully washed in PBS, incubated in serum-free media for 4 hours, and washed again. Rat recombinant TNFα (Sigma-Aldrich) diluted in serum-free media was then added to the cells (1, 10, 100 ng/ml, 3 wells per concentration) and incubated for 18 hours. Serum-free media was used as

controls (3 wells). RNA was then prepared as described (see below). The results were confirmed in two independent experiments.

Patient Material (paper III)

From the 1970s' to 1980s' samples were collected at the hospital in Västerås, Sweden, from patients who underwent transurethral resection due to voiding problems. Samples were formalin-fixed, paraffin-embedded and subsequent histological examination showed cancer that was graded according to the Gleason system [54]. The presence of metastasis and local tumor stage were also determined. The patients had not received any anti-cancer treatment prior to diagnosis and were left untreated until symptoms occurred. At that time-point, the patients were subjected to palliative treatment with androgen deprivation or radiotherapy [152]. From these specimens, 26 tumors with GS 8-10 were selected to include two groups: (M0) no presence of bone metastases at diagnosis and survival > 7 years; and (M1) presence of bone metastases and survival ≤ 5 years.

Protein Analyses

Protein preparation (paper III and IV)

Small pieces from the subcutaneous frozen Dunning tumors were quickly detached and homogenized using a Micro Dismembrator (B.Braun Biotech International GmbH) at 2000 rpm for 45 seconds. Frozen sections of the orthotopic AT-1 tumors were examined prior to homogenization to localize the tumors. Small pieces were then carefully dissected out and homogenized as described. The homogenized tissues were added into lysis buffer containing 0.5 % NP-40, 0.5 % NaDOC, 0.1 % SDS, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM NaF and Complete protease inhibitor (Boehringer). Samples were mixed and incubated on ice for 30 minutes followed by centrifugation at 20 000 x g in 4° C for 30 min, and the supernatants were isolated. Protein from cells was extracted using the same lysis buffer followed by centrifugation (20 000 x g, 4° C, 30 min). The BCA protein assay reagent kit (Pierce Chemical Co.) was used to determine protein concentrations.

Western Blot (paper III and IV)

Samples were reduced and separated by electrophoresis on 7.5-10 % SDS-polyacrylamide gels and transferred to polyvinyldufluoride membranes. The membranes were blocked in 5 % milk and incubated with primary antibodies for

PEDF (the antibody for rat PEDF was provided by Susan Crawford at NorthShore University, Evanston [120] and the antibody for human PEDF was from Chemicon). After washing, membranes were incubated with appropriate secondary antibodies and proteins were detected using enhanced chemiluminescence (Amersham Biosciences). Coomassie-stained gels confirmed equal loading and molecular size standards (BioRad Laboratories AB) were included as controls.

Immunohistochemistry and morphology (paper I-IV)

Antibodies

Sections were stained using primary antibodies against caspase-3 (Cell Signaling Technology) for apoptosis, Ki67 (Dako) and BrdU (Dako) for proliferation, factor VIII and endoglin (Dako) for blood vessels, CD68 (AdB Serotec) for monocytes/macrophages, iNOS (Abcam) for cytotoxic macrophages, LYVE-1 (Abcam) for lymphatic vessels and Hypoxyprobe (Chemicon) for hypoxia.

Other antibodies used for immunohistochemistry were AR (Upstate Lake Placid), smooth muscle actin (Dako), IL1β (R&D systems), MMP9 (Santa Cruz Biotechnology), PEDF [120], PEDF (Chemicon), VEGF (Santa Cruz Biotechnology), TNFα (AdB Serotec) and Synaptophysin (Dako).

Stereology

Sections were first stained with the respective antibodies (factor VIII, CD68, LYVE-1, iNOS) by immunohistochemistry and the volume densities (percentage of tissue volume occupied by the defined tissue compartment) were evaluated by a point counting method as described by Weibel [153]. Using a 121 point square lattice, mounted in the eye-piece of a light microscope, the numbers of intersections falling on each tissue compartment were counted in randomly chosen fields. The volume densities of tumor and normal prostate tissue in the VP were determined in the same way on hematoxylin-eosin stained sections by counting the number of grid intersection falling on the respective compartment. Tumor weight was then estimated by multiplying tumor density with VP weight.

In a different method, used to determine the percentage of stained cells (caspase-3, Ki67, BrdU), the fractions were assessed in 500-1000 cells. Apoptosis was also identified using a terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay according to protocol (Roche) and the percentage of apoptotic cells was determined in 2000 cells of each tumor.

In human tissues Ki67, factor VIII and endoglin were counted in three hotspot fields for each tumor. PEDF immunoreactive score was determined by multiplying an estimate of the fraction immunoreactive cells (0-1) with an estimate of the staining intensity (0-2).

RNA Analyses

Laser Micro dissection (paper III)

Human tissue samples were mounted on Histogene laser capture microdissection slides (Arcturus), deparaffinized, rehydrated and stained with Arcturus staining solution. Sections were dehydrated by increasing the percentage of ethanol according to protocol (Arcturus). A laser capture microscope (PixCell II, Arcturus) was used to isolate tumor epithelial cells on special caps (HS CapSure, Arcturus). RNA was prepared as described by Specht et al. [154]. Briefly, cells were lysed in lysis buffer containing 10 mM Tris/HCL (pH 8.0), 0.1 mM EDTA (pH 8.0), 2 % SDS (pH 7.3) and 500 µg/ml proteinase K (Sigma) over night at 60°C, followed by heat inactivation. RNA was then precipitated and washed and resuspended in RNase-free water. Fivehundred microdissected shots of each sample were used for cDNA synthesis.

RNA preparation (paper II-IV)

RNA, from rat tissues and cells, was extracted using the TRIzol method according to protocol. Briefly, homogenized tissues or cells were added in 0.8-1 ml Trizol and incubated. Chloroform was then added, the sample vortexed vigorously, incubated in room temperature for 3 min, and centrifuged. The RNA containing upper phase was isolated and RNA was precipitated using isopropanol. The RNA was washed with 70 % ethanol and dissolved in RNase-free water. The concentrations were quantified spectrophotometrically at 260 nm (DU 640 Spectrophotometer, Beckman Coulter) or using a nanodrop (Thermo scientific). RNA integrity was verified by ethidium bromide staining of 28 S and 18 S rRNA after agarose gel electrophoresis.

cDNA synthesis (paper II-IV)

RNA (500 ng) was reverse transcribed using Superscript II (Invitrogen) in a 10-µl reaction according to protocol. Briefly, total RNA was mixed and incubated with 2.5 µM random hexamers (Applied Biosystems) and 5 mM dNTPs at 65°C for 5 min. The samples were quickly chilled on ice and first-strand buffer, 0.1 M DTT,

20 U RNasin (Promega) and 100 U of Superscript II were added. cDNA synthesis was initiated with 10 min at 25°C followed by 50 min at 42°C and inactivation at 70°C for 15 min. For PCR-array studies, total RNA from individual animals in each group was pooled together and DNase-treated (Sigma-Aldrich) to remove contaminating DNA. For cells, total RNA from 3-4 different cell batches were pooled together and DNase-treated the same way. RT^2 profiler PCR array first strand kit C-02 (SABiosciences) was used to synthesize cDNA of 1 µg total RNA according to protocol.

Quantitative Real-Time RT-PCR and PCR-Array analyses (paper II-IV)

Quantifications of mRNA levels were performed by real-time reverse transcribed-PCR using the LightCycler SYBR Green I technology (Roche). Reactions were performed according to protocol (Roche), using optimized primers for each factor studied. Melting curve analysis was used to confirm specificity and negative controls were run in parallel. Data were analyzed using LightCycler analysis software 3.5.3 (Roche).

RT^2 Profiler PCR arrays, rat angiogenesis and rat chemokines (SABiosciences), were performed according to the manufacturer's instructions using the ABI Prism 7900 HT instrument (Applied Biosystems) and ABI prism 7900 SDS software 2.1. The data was then analyzed with PCR array data analysis template downloaded from the superarray website (www.sabiosciences.com) and normalized to the expression levels of housekeeping genes.

Statistics

The Student's t-test or Mann-Whitney U test was used for comparison between groups. Linear correlation coefficient or Spearman's rho test was used for correlation studies. A P value < 0.05 was considered significant. Statistical analyses were performed using the statistical software Statistica 6.0 or the latest version of SPSS.

RESULTS AND DISCUSSION

Paper I

Summary

Cell death among epithelial cells and involution of normal prostate glands after castration seem to be directed primarily through effects in the prostate stroma and vasculature [32, 36, 37, 39]. In this study, we therefore investigated how AR negative tumor cells responded to castration when surrounded by normal androgen-dependent prostate tissue and vasculature.

Castration effects on the tumor cells mediated by the surrounding non-malignant prostate and vasculature

A small number of AR negative AT-1 tumor cells were injected into one of the VP lobes and the effects of castration were examined. We found that AT-1 tumor cells responded to castration with transiently retarded tumor growth. This was mainly due to increased tumor epithelial cell apoptosis since proliferation was unaffected. Vascular density inside the tumor was unaffected by castration, suggesting that the tumor vessels were insensitive to androgen deprivation. As expected, the vascular density in the VP lobes decreased after castration, suggesting decreased blood flow to the organ. In line with this the fraction of hypoxic epithelial cells was increased both in the VP and in the tumor. Tumor cell apoptosis was therefore likely caused by increased hypoxia due to decreased blood supply from the surrounding VP.

Effects on the non-malignant prostate mediated by the tumor

The vascular density in the VP lobes surrounding the growing tumor increased with time, suggesting that the tumor stimulated its blood supply through the surrounding non-malignant tissue. The tumor apparently forces the surrounding tissue to cooperate.

Although vascular response in the VP was similar to previous studies in non-tumor containing VP [20], the weight of the VP lobe containing the tumor was unaffected early after castration but was decreased at the later time point. The reason for this is unknown but contrary to previous results, proliferation or at least BrdU labeling was increased early after castration in the VP, suggesting that the tumor was to some extent able to modify the normal castration response. After castration the non-malignant epithelial cells close to the tumor still had active AR signaling in the nucleus. This suggests that the tumor changes AR regulation in the adjacent non-

malignant prostate tissue and may explain the lack of reduced epithelial cell proliferation and weight seen in the VP early after castration.

Castration effects on non-malignant prostate epithelium surrounded by prostate tumor cells

The tumor cells sometimes grew between the basement membrane and the covering smooth muscle cells and thereby surrounded normal glands with AR negative cells. We were therefore able to study how normal epithelial cells respond to castration when surrounded by an androgen-independent tissue. We found that these epithelial cells responded to castration with increased apoptosis with about the same magnitude as the tumor, which was significantly lower compared to that in normal glands outside the tumor. This suggests that castration response in prostate epithelial cells is highly dependent on responses in the most adjacent cells.

Discussion

Why does an androgen-independent prostate tumor respond to castration when growing inside the prostate?

The rat ventral prostate is one of the most androgen sensitive tissues known and it normally responds to castration with decreased blood flow and subsequent glandular involution. A prostate tumor growing in this environment is likely dependent on support from its surroundings and changes in the surrounding tissue will therefore influence the tumor. What type of prostate cells that are most important for the castration response are however unknown. Normal epithelium surrounded by prostate tumor cells responded to castration although it had lost its contact with the androgen dependent stroma. This suggests that altered paracrine signaling from prostate stromal cells is not the only explanation for the castration response in the normal epithelium. However, as the response was of lower magnitude the prostate stroma probably plays an important role. Direct effects of androgen withdrawal could also explain the response in the non-malignant epithelial cells inside the tumor. The tumor cells however could not respond directly as they lacked the androgen receptor. The response in the tumor and in the normal epithelium inside the tumor was therefore likely caused by effects on the prostate vasculature and blood flow.

Castration therapies are mainly used as palliative treatment for metastases and generally not for tumors only localized to the prostate. Surprisingly, nothing is known about if and how the surrounding non-malignant tissue responds to

castration in metastases. As effects in the tissue surrounding the tumor are of importance for the castration response in primary tumors, further studies are needed to examine the castration response in metastases stroma and surrounding tissues. In order to improve the efficacy of castration treatment increased understanding of how and why metastases respond to castration is of major importance.

Is androgen ablation a safe treatment for localized tumors?

Also poorly differentiated tumors responded to castration when localized in the prostate suggesting that androgen ablation could be used as a treatment of aggressive and AR negative tumors in the prostate. The initial response to castration with increased tumor apoptosis, probably due to hypoxia in the tumor, suggests that the treatment is effective during this phase. Tumor progression was however only stopped temporarily showing that the tumor cells quickly adapted to grow in hypoxia. As hypoxia has shown to select for more aggressive tumors [41, 155-157] castration treatment that fails to kill all the tumor cells could therefore potentially make the remaining tumor cells more aggressive. New treatments that target hypoxia resistant tumor cells are needed to extend the initial effects of castration.

How does the tumor influence the surrounding non-malignant tissue?

The tumor affects not only non-malignant cells in the nearby tumor stroma but also influences the surrounding organ. Vascularity increased with time in the non-malignant tissue outside the tumor. This suggests that the tumor secreted angiogenesis promoting signals to secure increased blood supply through its surroundings. The tumor also delayed but not inhibited the castration response in the neighboring non-malignant epithelial cells. The reason for this is unknown but could possibly be due to growth factors secreted by the tumor that alter AR signaling and response to therapy.

Changes in the tumor adjacent non-malignant tissue could possibly be used for diagnosis and/or prognosis of prostate tumors. Prostate biopsies only account for a small fraction of the prostate and therefore often sample non-malignant tissue although a tumor is present. Alterations in the non-malignant tissue could be exploited to indicate the presence of a tumor and perhaps also its aggressiveness. Low levels of phosphorylated epidermal growth factor receptor in non-malignant prostate glands adjacent to the tumor have for instance shown to be associated with a more favorable outcome (Hammarsten et al., unpublished results). Other studies

from our group also show that loss of AR expression in the normal prostate stroma was associated with poor outcome [73]. Angiogenesis and accumulation of macrophages in the prostate tumor stroma have also been linked to poorer prognosis [74, 75]. Our study suggests that angiogenesis is increased and macrophages (paper II) also accumulate in the prostate tissue further away from the tumor and this could possibly give additional diagnostic/prognostic information.

Paper II

Summary

In this paper we studied the importance of tumor associated macrophages for orthotopic AT-1 tumor growth and angiogenesis and in addition studied the expression of angiogenic factors and cytokines in the tumor.

To understand the mechanisms behind the increased vascularity in the AT-1 tumor model (paper I), we first analyzed endothelial cell proliferation inside the tumor (intratumoral) and in the surrounding normal VP (extratumoral). Intratumoral endothelial cell proliferation was particularly high suggesting active angiogenesis in tumors. In the extratumoral tissue, the fraction of proliferating endothelial cells correlated with extratumoral vascular density, showing that new vessels were formed at this site. Proliferation of endothelial cells both in the tumor and in the VP correlated with tumor size.

To investigate what factors the AT-1 tumor expressed that could stimulate angiogenesis we used a rat angiogenesis PCR array. We compared expression in the orthotopic tumor *in vivo* with expression in sham injected normal VP. The results showed that the tumor produced numerous angiogenic factors that could explain the increased vascularity. Many of these factors, for instance CCL2, IL1β and IL-6, are associated with inflammation. As TAMs are known to stimulate tumor progression and angiogenesis we therefore examined macrophage content in the AT-1 tumor model.

The tumor contained a high number of CD68 (a marker for monocytes/macrophages) positive cells, especially in the invasive zone. In the extratumoral tissue the number of macrophages increased with time and tumor size and also here the highest number was found close to the tumor border. The average intratumoral macrophage density, however, inversely correlated to both tumor size and intratumoral vascular density. Conversely, the average extratumoral macrophage density, although it was much lower compared to the macrophage

density inside the tumor, highly correlated with tumor size, extratumoral vascular proliferation and extratumoral vascular density. Double staining of macrophages and proliferation showed that macrophages often were in close contact with proliferating endothelial cells in capillaries but also with proliferating vascular smooth muscle cells in arteriols and venules. As macrophages and monocytes show high plasticity, different functions at different tumor locations are possible [77, 78, 80]. The different types of monocytes/macrophages present in this study are however unknown but macrophages in the extratumoral tissue could possibly be of particular importance.

To further determine the importance of infiltrating monocytes/macrophages for AT-1 prostate tumor growth, circulating monocytes were reduced using Clodronate containing liposomes. In clodronate-liposome treated rats, macrophage density was decreased both in the tumor and in adjacent normal prostate tissue, but complete depletion was not seen. Although the reduction in macrophage content was modest, we observed a significant reduction in tumor growth. Angiogenesis was inhibited both intra- and extratumorally. In addition, suppressed proliferation of smooth muscle cells in larger vessels was noted. This suggests that monocyte infiltration is important for prostate tumor growth and for growth of micro-vessels as well as larger blood vessels.

To examine if some of the angiogenic factors that were up-regulated in the AT-1 tumor could be produced by macrophages, we compared the AT-1 tumor cells *in vitro* with the orthotopic tumors using the same array. Several factors showed increased expression levels *in vivo* and could thus be expressed by host cells. MMP9 and IL1β expressions were also verified with immunohistochemistry by intense staining of macrophages. IL1β has been shown to convert AR modulators from being inhibitory to stimulatory and thereby alter AR regulation [83]. Macrophage secretion of IL1β could thus be one explanation for the altered response to castration in the normal non-malignant tissue seen in paper I.

In addition, we also studied the expression of chemokines in the orthotopic tumor and found that important factors for monocyte recruitment, like CCL2 and CSF-1, were up-regulated compared to normal prostate tissue.

In summary, we suggest that monocytes/macrophages are important for prostate tumor growth and angiogenesis in the tumor and particularly in the surrounding non-malignant tissue. In addition, macrophages seem important for the expansion of larger arteriols and venules, thereby increasing the blood flow to and from the

newly formed capillaries inside the tumor. Furthermore, the tumor expressed several angiogenic factors that could stimulate vascular growth both in the tumor and in its nearby surrounding. As some of these factors most likely are produced by macrophages, decreasing macrophages could be used as an antivascular/antitumor treatment.

Discussion

Methodological considerations using clodronate-liposome for macrophage depletion

To reduce the infiltration of monocytes to the orthotopic tumor we used clodronate-liposomes injected intraperitoneally. Phagocytic cells engulf the liposomes and clodronate is released inside the cells with consequent apoptosis. Circulating monocytes have been shown to be depleted [158] but as new monocytes constantly enter the circulation from the bone marrow a continuous supply of clodronate-liposomes is needed. We therefore administered clodronate-liposomes before and during tumor growth to reduce the monocyte infiltration to the tumor. As controls we chose to inject PBS instead of PBS-liposomes, as they can alter normal macrophage function when digested although they are not toxic to the macrophages [150]. Free clodronate released by apoptotic monocytes have a short half-life in circulation and direct effects on the tumors are therefore unlikely [150].

Could macrophage depletion be used as a treatment for prostate cancer?

In this study the treatment was started even before the tumor cells were implanted. The effects were therefore most likely on tumor establishment. How reduction of monocytes would affect an already established prostate tumor is not known. Macrophages have been shown to be particularly important for the angiogenic switch in other tumors [69, 159, 160]. Inhibition of vascular growth was also one of the main effects seen in our study. Macrophage suppressing therapies could therefore possibly be used as adjuvant treatment inhibiting angiogenesis and establishment of metastases.

Treatments that target TAMs could be designed in several ways. Directly target monocytes as with clodronate-liposomes could be one option, but as monocytes and macrophages are important cells of the innate immune system this could possibly lead to unwanted side effects. Another possibility is to indirectly target important chemokines attracting monocytes to the tumor site. Inhibitions of CSF-1 and CCL2 have for instance shown to reduce monocyte/macrophage infiltration to

experimental tumors [161-164]. CSFR1 antagonist are also available [69]. However, simultaneous inhibition of several factors would probably be needed as many factors are known to stimulate monocyte recruitment. As monocytes show high plasticity a third possible way would be to maneuver them into a cytotoxic and tumor suppressing state.

Paper III

Summary

Another way to inhibit angiogenesis is by increasing levels of endogenous angiogenesis inhibitors. PEDF is a potent angiogenesis inhibitor [120] of particular importance in the prostate [125] and in this paper we characterized and examined the relationship between PEDF and prostate tumor progression.

Here we used subcutaneous Dunning tumors with different characteristics (see materials and methods) and found that PEDF was highly expressed in low metastatic tumors (PAP and AT-1) compared to moderately (AT-2) and highly metastatic tumors (AT-3 and MatLyLu). The differences in PEDF expression was found both at the protein and the mRNA levels, *in vitro* and *in vivo*, suggesting that down-regulation occurred at the transcriptional level. Both AT-1 and MatLyLu are anaplastic, fast growing and androgen-insensitive tumors that mainly differ in their ability to metastasize. Decreased PEDF in the Dunning tumor model was therefore primarily associated with an increased metastatic potential. However, MatLyLu tumors had significantly higher vascular density and proliferation compared to the AT-1 tumors, and PEDF levels inversely correlated to these two parameters. PEDF could therefore possibly repress metastatic spread by decreasing angiogenesis and/or tumor cell proliferation.

Before further analyses on the functional role of PEDF in prostate tumors, we examined whether a similar pattern existed also in human prostate tumors. To simulate the relationship between AT-1 and MatLyLu, tissues from patients with poorly differentiated tumors (GS 8-10) of similar sizes were divided into two groups. The first group (M1) represented the typical characteristics for poorly differentiated prostate tumors, with the presence of bone metastasis at diagnosis and survival ≤ 5 years after diagnosis. The second group (M0) was somewhat more unusual since no presence of bone metastasis was found at diagnosis and subsequently they also had a more favorable outcome with survival > 7 years after diagnosis. The M0 group had higher PEDF mRNA and protein levels compared to

the M1 group. An inverse correlation for PEDF mRNA was also found with vascularity and proliferation.

In summary, this paper suggests that decreased PEDF is associated with a metastatic phenotype in human and rat prostate tumors.

Discussion

At the time of publication, little was known about the function of PEDF in different tumors. Later on, an inverse correlation between PEDF levels, grade and metastatic potential has however been obtained for several tumors types including breast [127], lung [165], pancreatic carcinoma [126], glioma [128], melanoma [137] and lymphangiomas [166]. Moreover, PEDF have shown both tumor and metastasis suppressing activities in several experimental tumor model systems (see introduction for more details). Inhibition of angiogenesis, increased tumor cell apoptosis, decreased tumor cell invasion and tumor cell differentiation have been suggested explanations for PEDF tumor inhibition. PEDF has also been shown to have contrary effects in neuronal tissues, where it promotes survival and protects the cells from apoptosis. The mechanisms behind PEDF's divergent effects are not fully understood and not examined thoroughly in tumors.

The reason for decreased PEDF expression in tumors is also unknown. Loss of tumor suppressors could be due to genetic or epigenetic alterations but microenvironmental factors could also be of importance, e.g. hypoxia and androgens have been shown to suppress PEDF protein expression in prostate tumor and stromal cells [125]. In line with this, PEDF immunostaining showed less intensity in hypoxic parts of the AT-1 tumors and in human tumors still containing PEDF the staining was heterogeneous.

The function and regulation of PEDF in prostate tumors was therefore further analyzed in paper IV.

Paper IV

Summary

In this study we extended our findings regarding PEDF and examined effects of PEDF over-expression on prostate tumor growth and metastasis. For this the metastatic and highly aggressive MatLyLu tumor cells that expressed low levels of PEDF (paper III) were transfected with a plasmid vector containing human PEDF cDNA.

We found that PEDF over-expression, as expected, inhibited orthotopic rat prostate tumor growth, angiogenesis and suppressed the growth of metastases. Surprisingly, histological examinations also showed that PEDF over-expression stimulated lymphangiogenesis and macrophage infiltration to the tumors. PEDF transfected MatLyLu tumors contained more inducible nitric oxide synthase (iNOS) positive cytotoxic macrophages compared to controls suggesting that PEDF may stimulate anti-tumor immunity.

In addition, we studied prostate environmental regulation of PEDF in rat prostate tumors. For this we used AT-1 tumor cells that expressed high levels of PEDF mRNA and protein both *in vitro* and subcutaneously (paper III). When these tumor cells were injected into the VP, PEDF mRNA and protein expressions were decreased suggesting that some factor in the prostate microenvironment inhibited PEDF expression. We could show that one such factor could be TNFα, since TNFα down-regulated PEDF both *in vitro* and *in vivo*. TNFα was expressed by inflammatory cells, mainly macrophages, in the ortothopic AT-1 tumors suggesting that macrophages could repress PEDF transcription and thereby probably facilitate tumor angiogenesis and metastatic spread.

Discussion

Methodological considerations

Transfection of MatLyLu cells resulted in high PEDF protein levels *in vitro* when using Hygromycin B to select for clones containing the plasmid. *In vivo*, unfortunately, PEDF expression was gradually lost. This was probably due to insufficient replication of the plasmid in rat cells. For stable transfection other plasmids or vectors are needed.

In this study human PEDF was expressed in rat prostate tumor cells. Streck et al. showed that human protein was more effective in reducing human neuroblastoma xenografts compared to murine neuroblastomas suggesting a difference in efficacy [138]. However, murine PEDF was not more effective than human PEDF for treating murine cancer [138]. Ek et al. transfected human and rat sarcoma cells to over-express human PEDF and showed similar results in both tumor types [140].

Is PEDF a prostate tumor suppressor?

This and several other studies (see introduction for details) show that PEDF can arrest tumor growth, angiogenesis and metastasis. PEDF is also lost during tumor progression in many tumors. This suggests that PEDF treatment could be used as a

new anti-angiogenesis/anti-tumor treatment. An advantage with endogenous angiogenic inhibitors like PEDF is that they are able to inhibit several angiogenesis inducers simultaneously compared to more specific drugs. This is important as tumors express multiple angiogenesis stimulators as shown in paper II. PEDF has been shown to be one of the most potent endogenous angiogenesis inhibitors [120] and is therefore promising as an anti-angiogenesis treatment.

Studies showing pronounced and long term effects of PEDF tumor treatment used stable transfections where PEDF was expressed in all tumor cells already at tumor establishment and during the entire study [133, 134]. Transient over-expression, like in this study, demonstrated slower growth kinetics and decreased metastatic growth only during the time when PEDF could be detected [135, 136]. Then, when PEDF expression declined, the tumors grew in the same pace as controls implying that the expression needs to be sustained in order to achieve long term effects. No study so far has shown that PEDF can shrink tumors. PEDF could therefore possibly be used as a treatment to inhibit tumor progression, to enhance the effects of other treatments or as an adjuvant to postpone tumor relapse.

Here we show that both macrophages and lymphangiogenesis increased by PEDF over-expression, characteristics known to facilitate tumor growth. Although PEDF over-expression increased cytotoxic macrophage numbers, this was only a minor fraction of all macrophages in the tumor. Increased macrophage tumor infiltration during PEDF treatment could therefore finally make the tumors more aggressive.

PEDF regulation

Our study together with the study by Doll et al. [125] showed that the microenvironment down-regulated PEDF in prostate tumors. AT-1 cells injected into the prostate lost almost all PEDF protein expression after 10 days. Similar experiments with AT-1 tumor cells injected subcutaneously did not decrease PEDF levels, showing that regulation of PEDF was context dependent (results not shown).

TNFα secreted by macrophages and other stromal cells in the prostate microenvironment could be one explanation for decreased PEDF mRNA in tumor cells. As PEDF stimulated macrophage accumulation, macrophages may down-regulate PEDF as a negative feed-back loop. Hypoxia in tumors could then further suppress PEDF protein expression. In tumors where the PEDF gene is not completely silenced, inhibition of TNFα might increase the expression of PEDF and possibly delay tumor progression.

CONCLUSIONS

- AR negative prostate tumor cells responds to castration when growing in the androgen dependent prostate environment

- Tumor adjacent non-malignant tissue has increased vascularity and inflammatory cell infiltration, and a delayed response to castration

- Tumor associated macrophages are important for prostate tumor growth and angiogenesis both in the tumor and in the surrounding non-malignant tissue

- Both the tumor epithelial and stromal cells express several angiogenic factors and chemokines that can stimulate angiogenesis and monocyte recruitment

- Decreased PEDF levels are associated with a metastatic and more vascularized phenotype in human and rat prostate tumors

- PEDF over-expression represses rat prostate tumor growth, angiogenesis and growth of metastasis. In addition PEDF over-expression stimulates cytotoxic macrophage tumor infiltration and lymphangiogenesis

- PEDF is down-regulated by the prostate tumor microenvironment and TNFα.

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Tumor epithelial cells as well as resident and infiltrating non-epithelial cells together establish the molecular composition of the tumor microenvironment. Many of these microenvironmental factors will probably have opposing roles in tumor progression either by promoting or inhibiting this process. If the effects are pro- or anti-tumoral are probably context-dependent. The interactions between different cells in the tumor microenvironment are bidirectional and can each change the phenotype of the other. Often, microenvironmental interactions will lead to vicious cycles that promote tumor progression. Modern cancer therapy has therefore shifted from exclusively targeting tumor epithelial cells to targeting also cells in the adjacent microenvironment as well as their interactions.

This thesis presents some of the interactions between prostate tumor cells and its microenvironment and also introduces the more remote surrounding non-malignant prostate tissue as an essential component. The tumor cells are apparently able to influence the non-malignant prostate tissue and in addition the surrounding environment can affect the tumor cells. When targeting these interactions and the vasculature in particular, orthotopic prostate tumor growth was inhibited.

Castration-induced epithelial cell death in the normal prostate is, partly, mediated by acute vascular regression with accompanying hypoxia (see introduction for details). We now showed that castration effects in primary tumors could be mediated by effects in the surrounding normal prostate tissue and vasculature. In our study, the tumor vasculature in an AR negative tumor was unresponsive to castration treatment but other studies have shown a castration induced significant reduction of tumor vessel density in AR positive tumors [167, 168]. Androgen ablation may therefore be considered as an anti-angiogenic treatment causing acute hypoxia and tumor cell death. We however showed that tumor epithelial cell apoptosis was less prominent than in epithelial cells in the non-malignant tissue and that castration only temporarily inhibited tumor growth. Hypoxia has been shown to promote tumor progression by selecting for apoptosis resistance, genetic alterations, and increased metastatic capacity [41, 155-157, 169, 170]. In addition, hypoxic tumor cells are usually resistant to radio- and chemotherapy [171] impairing treatment responses. Castration therapy inducing hypoxia but failing to kill all the tumor cells might therefore promote tumor progression and in the long run make things worse.

The effects of castration therapy therefore need to be enhanced. As one example of this, we have shown that castration treatment of the orthotopic tumor model combined with the tyrosine kinase inhibitor ZD6474 had pronounced anti-tumor effects by targeting both tumor vessels (ZD6474) and normal prostate vessels (castration) [172]. The use of hypoxia activated cytotoxins [173] or anaerobic bacteria [174] homing to hypoxic tumors promoting tumor cell death could also prove to be useful in combination with androgen ablation. Tirapazamine [175], a drug that selectively kills hypoxic cells, used simultaneously as castration also showed additive effects (Johansson, unpublished results). Combination of castration and PEDF treatment could prove to be efficient since hypoxia augmented prostate tumor apoptosis induced by PEDF [125]. Long-term effects of castration are less clear. Localized tumors examined months after initial anti-androgen treatment showed improved oxygenation in about half of the patients [176]. The reason for this is unknown but could possibly be due to "vascular normalization" as hypothesized for other anti-angiogenic treatments [99], or by decreased oxygen consumption [41]. Increased blood flow may enhance drug uptake and radiation therapy and at that time point these treatments may work in synergy with castration. This time frame however needs to be determined.

Castration treatment is primarily used for treatment of patients with metastatic prostate cancer. The exact mechanisms by which castration therapy works in primary tumors and particularly in metastasis are poorly understood. As our results show that castration effects in primary tumors are at least in part mediated by effects in the surrounding normal prostate tissue and vasculature, the next step will be to study the importance of the tumor microenvironment and the surrounding tissues also in metastases. Especially as metastases have a different stroma and are surrounded by completely different tissues. One of the biggest challenges in prostate cancer research is to find ways to improve the treatments of patients with metastatic disease. In order to enhance and prolong the effects of castration we probably need to target tumor epithelial cells, the different tumor stromal cells and the surrounding normal tissue simultaneously as effective as possible.

The role of macrophages in metastatic prostate cancer is not fully established. Human prostate bone metastasis contained considerably more macrophages than primary tumors (our own unpublished observations). In breast cancer, TAMs have been shown to increase tumor cell migration, invasion and intravasation [69]. TAMs were shown to be in close contact with both endothelial cells and tumor cells and thereby facilitate tumor cells entering the blood stream [177]. In our study we also

saw that TAMs had close contact with blood vessel and produced MMP9 known to enhance tumor invasion [178, 179].

Macrophages have also been shown to produce EGF and thereby stimulate tumor cell migration. The tumor cells in return secrete CSF-1 inducing a paracrine loop [177, 180]. In our study EGF mRNA expression was however decreased suggesting that this particular type of interaction may not be present in the largely non-metastatic AT-1 tumor model.

Thousands of tumor cells can be shed to the circulation every day and probably already early in tumor progression, but only a few of these will produce clinically detectable metastasis [4, 100]. The roles of macrophages in the later steps of the metastatic cascade are less defined. As our and other studies show that macrophages are important for tumor establishment and angiogenesis in primary tumors they likely play this role in metastases as well. Whether macrophages are actively involved in angiogenesis in metastasis are unknown and needs to be further investigated. In breast cancer, TAMs have been shown to respond to and produce estrogens [181, 182]. If local macrophages in metastasis are able to contribute to the production of androgens and thereby take part in castration resistance is unknown and also needs to be further investigated. If so, castration or AR blockage together with macrophage depletion could prove beneficial. TNFα has been shown by several studies to promote metastasis and macrophages are the most potent producers of this factor [69, 183]. One way that TNFα increases metastasis could be by repressing PEDF expression. Inhibition of monocyte recruitment or using TNFα inhibitors [183] to tumors might increase the levels of PEDF and thereby inhibit angiogenesis and tumor progression.

PEDF appears as a promising anti-tumor therapy targeting the tumor in multiple ways, i.e. anti-angiogenic, induction of tumor apoptosis and tumor cell differentiation [184, 185], and as shown in our work by possibly increasing anti-tumor immunity. However, PEDFs role in macrophage recruitment and inflammation needs to be further examined to enhance possible anti-tumor immunity effects while reducing possible stimulatory effects.

The tumor microenvironment is complex with numerous different interactions and signaling cascades that together with tumor heterogeneity makes it difficult to design future cancer therapy drugs. Which are the most important interactions for tumor progression and metastatic growth? It also raises the question of whether changes in the tumor stroma and cancer cells are fully reversible, or if there is a

point of no return. We have studied a few interactions in the prostate tumor microenvironment that are components of an immense system. This is important to realize as tumors often escape and relapse after good initial response to a specific treatment. If one growth factor is blocked there are multiple other factors present eager to play a leading role.

This was exemplified in our work showing that the tumors expressed multiple angiogenesis inducers simultaneously. Therefore, treatments unable to kill all the tumors cells could in the long run select for more aggressive tumors expressing alternative growth stimulating factors. Personalized treatments with several targets in combination aiming at the tumor cells, the nearby tumor microenvironment, and the surrounding tissue are probably needed to eradicate or at least persistently suppress advanced and metastatic tumors.

For future cancer treatments, selection of patients and the right combination of treatments given in the most effective order and at the best suitable time point will probably be needed to improve the life of patients with metastatic prostate cancer.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Prostatacancer är den vanligaste maligna tumörsjukdomen hos svenska män. Varje år diagnostiseras ungefär 9 000 män med prostatacancer och ca 25 % av dem kommer att dö av sjukdomen. En del patienter har en snabbväxande tumör och dödlig sjukdom, medan andra har en långsamt växande tumör som inte kommer att orsaka några större problem. När tumörerna är små och lokaliserade till prostatan kan de botas genom operation eller strålning. Därför är det viktigt att tumörerna upptäcks tidigt och behandlas. Problemet med detta är att behandlingen inte skiljer på vilken typ av prostatacancer patienter har. Detta leder till att vissa patienter överbehandlas med terapier som kan ge allvarliga biverkningar som följd medan de som skulle behöva kraftfullare behandling inte får sådan eller får den för sent.

Har tumören spridit sig och börjat växa i andra organ, vanligast skelett, finns ingen bot. I dessa fall kastreras mannen dvs. att man på olika sätt avlägsnar manligt könshormon eftersom könshormoner kan stimulera metastaserna att växa. Detta bromsar och lindrar sjudomen en tid men förr eller senare återkommer tumören som slutligen dödar patienten.

Därför behövs bra metoder att förutsäga tumörernas aggressivitet, och nya effektiva behandlingar för spridd prostatacancer.

Prostatacancer uppstår när körtelceller i prostatan okontrollerat börjar tillväxa och invadera omgivande vävnad. Tumörcellerna är beroende av sin omgivning för att kunna växa och sprida sig, t ex måste tumören stimulera inväxt av blodkärl för att tillgodose behovet av näring och syre. Tumörcellerna bildar tillsammans med andra stödjeceller därmed en komplex miljö som gynnar tumörtillväxten.

Vi har framförallt använt oss av djurmodeller med prostatacancer för att undersöka om vi genom att påverka prostatatumörens omgivning, i synnerhet blodkärl, kan bromsa tumören.

Studierna visar att tumören förändrar den friska vävnaden runt omkring sig. Bland annat ansamlas immunceller, det bildas fler blodkärl och vävnaden svarar annorlunda på kastrering. Vi visar också att kastrering har stora effekter på den normala prostatavävnaden som omger tumören, framförallt på blodkärlen, och att detta kan bromsa en tumör som normalt inte svarar på behandlingen. Dessutom visar vi att en typ av immunceller, makrofager, är viktiga för tumörens tillväxt. När antalet makrofager i och omkring tumören minskades hade tumören färre blodkärl och växte sämre. Vi undersökte även ett protein, Pigment epithelium-derived factor

(PEDF), som bromsar tillväxten av nya blodkärl. Vi fann att metastaserande prostatatumörer hos både patienter och i djurmodeller hade låga nivåer av PEDF. När PEDF återinfördes till dessa tumörceller fick tumören färre blodkärl, sämre tillväxt och mer långsamtväxande metastaser. Vi såg också att PEDF stimulerade tillströmningen av makrofager in till tumören, framförallt en sort som kan döda tumörceller. Vi visar också att normal prostatavävnad som omger tumören kan vara en orsak till de låga nivåerna av PEDF i vissa tumörer.

Våra studier visar att prostatatumörers tillväxt kan bromsas genom att påverka celler i tumörens omgivning, något som skulle kunna användas för att förstärka och effektivisera behandlingen av patienter med spridd prostatacancer. Vi visar även att tumören förändrar den omgivande friska vävnaden, förändringar som ska undersökas vidare för att eventuellt hitta nya diagnos- och prognosmarkörer.

ACKNOWLEDGEMENTS

Så till den viktigaste sidan där jag får chansen att tacka alla som har hjälpt mig med den här avhandlingen och som har supportat mig under mina år som doktorand.

Ett stort **TACK** till:

Anders Bergh, min utmärkta handledare och mentor! Tack för all uppmuntran, för att du trott på mig, för att du gett mig friheten att utvecklas och testa mina idéer, för att du tar dig tid och är så otroligt kunnig och entusiastisk.

Stina Rudolfsson, min biträdande handledare och vän! Tack för allt stöd och all hjälp med allt från att hålla fast möss ☺ till att lära mig labbet. Din positiva karisma gör att det är så roligt att jobba med dig! Tack också för att du bjöd in mig i bokklubben så att jag läser lite annat än forskning .

Pernilla Wikström, min biträdande handledare och förebild! Tack för att du har motiverat mig genom alla år och alltid tagit dig tid för alla mina frågor. Du är beundransvärt begåvad och ambitiös och jag ser verkligen upp till dig.

Sigrid ”Siggan” Kilter, vår eminenta råttkirurg! Ingen utmaning är för svår för dig! Tack för att du alltid ställer upp och för att det är så trevligt att jobba med dig. Det gäller bara att ha en frukt eller lite fika redo när blodsockret faller hos oss båda ☺. Jag längtar tills jag får komma tillbaka och jobba med dig.

Elisabeth Dahlberg, Birgitta Ekblom och Pernilla Andersson, våra fantastiska färgningsexperter! Utan er hjälp hade det här aldrig gått så jag är oerhört tacksam. Tack även till **Ulla-Stina Spetz** som hjälpte mig i början av min tid som doktorand. **Åsa Skytt**, du hjälpte mig på många sätt både i labbet och som en vän. Jag saknar dig så mycket! Kan fortfarande inte förstå att du är borta.

Thanks to my co-authors and collaborators **Jennifer Doll** and **Susan Crawford** for your help, ideas and comments on the PEDF work. Thanks also to my other co-authors **Pär Stattin** and **Nico van Rooijen.**

Alla medlemmar eller före detta medlemmar i Bergh-gruppen. **Anna**, jag saknar dig och din energi, hoppas våra vägar möts igen. **Peter**, min medförfattare och trevliga rumskamrat. **Lotta**, är vårdcentralen verkligen roligare än att forska? **Nina**, vår allvetande och googlande guru. **Emma**, som tålmodigt satt långt in på lunchen och visade mig ”mappar” som jag inte förstod ett smack av! **Hani**, hoppas du kommer att trivas i gruppen.

ACKNOWLEDGEMENTS

Ulrika och **Karin** för att det är så trevligt att dela rum med er. Har varit supertrist att sitta helt själv hela hösten.

Karin Bodén, **Terry Persson** och **Åsa Lundsten**, stort tack för all hjälp över åren! Ni är toppen! **Tack också till alla på institutionen** för att ni bidrar till en mycket trevlig arbetsplats.

Anna, Emelie och **Patricia**, studenter som på olika sätt bidragit till mina projekt även om allt inte tog sig in i den här boken. Tack så mycket!

Till alla **vänner och släktingar**, det är en rikedom att ni finns. Speciellt tack till **Kerstin** min ”storasyster” för att du finns där för mig och min familj. Tack också till **Kicki** och **Ullis**, jag räknar in er i närmaste familjen, vi träffas alltför sällan. **Jessica**, trots att vi inte hörs så ofta nu för tiden är du den samma fortsätt med det!

Till mina **svärföräldrar Arne** och **Iréne** för att ni är så bra farmor och farfar till mina barn.

Mamma och **Pappa,** tack för allt ni gjort och gör för mig, är så glad att ni är nära både fysiskt och själsligt. Ni är bäst! **Daniel**, min begåvade och snälla lillebror. Tack för allt och tack för hjälpen med omslaget. **Malin** och lilla grynet i magen, snart är jag faster!

Mitt livs stora kärlek och bästa vän, **Svenne**, för allt jag inte kan beskriva med ord. Du är inte bara den smartaste person jag känner utan även den snällaste. Från mitt hjärta, tack! Våra små mirakel, **Nathalie** och **Nova-Lisa**, för att ni inspirerar och lär mig så mycket och skänker mig så mycket glädje.

REFERENCES

1. Sakr WA, Grignon DJ, Crissman JD, et al. High grade prostatic intraepithelial neoplasia (HGPIN) and prostatic adenocarcinoma between the ages of 20-69: an autopsy study of 249 cases. In vivo. May-Jun 1994;8(3):439-443.

2. Damber JE, Aus G. Prostate cancer. *Lancet.* May 17 2008;371(9625):1710-1721.

3. Bhatnagar V, Kaplan RM. Treatment options for prostate cancer: evaluating the evidence. *Am Fam Physician.* May 15 2005;71(10):1915-1922.

4. Morgan TM, Lange PH, Vessella RL. Detection and characterization of circulating and disseminated prostate cancer cells. *Front Biosci.* 2007;12:3000-3009.

5. Isaacs JT, Coffey DS. Etiology and disease process of benign prostatic hyperplasia. *Prostate Suppl.* 1989;2:33-50.

6. Collins AT, Habib FK, Maitland NJ, Neal DE. Identification and isolation of human prostate epithelial stem cells based on alpha(2)beta(1)-integrin expression. *J Cell Sci.* Nov 2001;114(Pt 21):3865-3872.

7. van Leenders GJ, Schalken JA. Stem cell differentiation within the human prostate epithelium: implications for prostate carcinogenesis. *BJU Int.* Sep 2001;88 Suppl 2:35-42; discussion 49-50.

8. Arienti G, Carlini E, Nicolucci A, Cosmi EV, Santi F, Palmerini CA. The motility of human spermatozoa as influenced by prostasomes at various pH levels. *Biol Cell.* Jan 1999;91(1):51-54.

9. McNeal JE. Normal histology of the prostate. *Am J Surg Pathol.* Aug 1988;12(8):619-633.

10. McNeal JE. Regional morphology and pathology of the prostate. *Am J Clin Pathol.* Mar 1968;49(3):347-357.

11. Jesik CJ, Holland JM, Lee C. An anatomic and histologic study of the rat prostate. *Prostate.* 1982;3(1):81-97.

12. Cunha GR, Hayward SW, Dahiya R, Foster BA. Smooth muscle-epithelial interactions in normal and neoplastic prostatic development. *Acta Anat (Basel).* 1996;155(1):63-72.

13. Flickinger CJ. The fine structure of the interstitial tissue of the rat prostate. *Am J Anat.* May 1972;134(1):107-125.

14. Marker PC, Donjacour AA, Dahiya R, Cunha GR. Hormonal, cellular, and molecular control of prostatic development. *Dev Biol.* Jan 15 2003;253(2):165-174.

15. Cunha GR. Mesenchymal-epithelial interactions: past, present, and future. *Differentiation.* Jul 2008;76(6):578-586.

16. Labrie F, Belanger A, Dupont A, Luu-The V, Simard J, Labrie C. Science behind total androgen blockade: from gene to combination therapy. *Clin Invest Med.* Dec 1993;16(6):475-492.

17. Coffey DS, Isaacs JT. Control of prostate growth. *Urology.* Mar 1981;17(Suppl 3):17-24.

18. Krieg M, Weisser H, Tunn S. Potential activities of androgen metabolizing enzymes in human prostate. *J Steroid Biochem Mol Biol.* Jun 1995;53(1-6):395-400.

19. Shang Y, Myers M, Brown M. Formation of the androgen receptor transcription complex. *Mol Cell.* Mar 2002;9(3):601-610.

20. Johansson A, Rudolfsson SH, Wikstrom P, Bergh A. Altered levels of angiopoietin 1 and tie 2 are associated with androgen-regulated vascular regression and growth in the ventral prostate in adult mice and rats. *Endocrinology.* Aug 2005;146(8):3463-3470.

21. Prins GS, Birch L, Greene GL. Androgen receptor localization in different cell types of the adult rat prostate. *Endocrinology.* Dec 1991;129(6):3187-3199.

22. Bonkhoff H, Remberger K. Differentiation pathways and histogenetic aspects of normal and abnormal prostatic growth: a stem cell model. *Prostate.* Feb 1996;28(2):98-106.

23. English HF, Drago JR, Santen RJ. Cellular response to androgen depletion and repletion in the rat ventral prostate: autoradiography and morphometric analysis. *Prostate.* 1985;7(1):41-51.

24. Kerr JF, Searle J. Deletion of cells by apoptosis during castration-induced involution of the rat prostate. *Virchows Arch B Cell Pathol.* Jun 25 1973;13(2):87-102.

25. Kyprianou N, Isaacs JT. Activation of programmed cell death in the rat ventral prostate after castration. *Endocrinology.* Feb 1988;122(2):552-562.

26. Ohlson N, Wikstrom P, Stattin P, Bergh A. Cell proliferation and apoptosis in prostate tumors and adjacent non-malignant prostate tissue in patients at different time-points after castration treatment. *Prostate.* Mar 1 2005;62(4):307-315.

27. Huggins C, Hodges CV. Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941. *J Urol.* Jul 2002;168(1):9-12.

28. Lee C. Physiology of castration-induced regression in rat prostate. *Prog Clin Biol Res.* 1981;75A:145-159.

29. Berry PA, Maitland NJ, Collins AT. Androgen receptor signalling in prostate: effects of stromal factors on normal and cancer stem cells. *Mol Cell Endocrinol.* Jun 25 2008;288(1-2):30-37.

30. Isaacs JT. Role of androgens in prostatic cancer. *Vitam Horm.* 1994;49:433-502.

31. Cunha GR, Lung B. The possible influence of temporal factors in androgenic responsiveness of urogenital tissue recombinants from wild-type and androgen-insensitive (Tfm) mice. *J Exp Zool.* Aug 1978;205(2):181-193.

32. Kurita T, Wang YZ, Donjacour AA, et al. Paracrine regulation of apoptosis by steroid hormones in the male and female reproductive system. *Cell Death Differ.* Feb 2001;8(2):192-200.

33. Simanainen U, Allan CM, Lim P, et al. Disruption of prostate epithelial androgen receptor impedes prostate lobe-specific growth and function. *Endocrinology.* May 2007;148(5):2264-2272.

34. Wu CT, Altuwaijri S, Ricke WA, et al. Increased prostate cell proliferation and loss of cell differentiation in mice lacking prostate epithelial androgen receptor. *Proc Natl Acad Sci U S A.* Jul 31 2007;104(31):12679-12684.

35. Lekas E, Engstrand C, Bergh A, Damber JE. Transient ischemia induces apoptosis in the ventral prostate of the rat. *Urol Res.* Jun 1999;27(3):174-179.

36. Lekas E, Johansson M, Widmark A, Bergh A, Damber JE. Decrement of blood flow precedes the involution of the ventral prostate in the rat after castration. *Urol Res.* 1997;25(5):309-314.

37. Shabsigh A, Chang DT, Heitjan DF, et al. Rapid reduction in blood flow to the rat ventral prostate gland after castration: preliminary evidence that androgens influence prostate size by regulating blood flow to the prostate gland and prostatic endothelial cell survival. *Prostate.* Aug 1 1998;36(3):201-206.

38. Hayek OR, Shabsigh A, Kaplan SA, et al. Castration induces acute vasoconstriction of blood vessels in the rat prostate concomitant with a reduction of prostatic nitric oxide synthase activity. *J Urol.* Oct 1999;162(4):1527-1531.

39. Shabisgh A, Tanji N, D'Agati V, et al. Early effects of castration on the vascular system of the rat ventral prostate gland. *Endocrinology.* Apr 1999;140(4):1920-1926.

40. Shabsigh A, Ghafar MA, de la Taille A, et al. Biomarker analysis demonstrates a hypoxic environment in the castrated rat ventral prostate gland. *J Cell Biochem.* 2001;81(3):437-444.

41. Rudolfsson SH, Bergh A. Hypoxia drives prostate tumour progression and impairs the effectiveness of therapy, but can also promote cell death and serve as a therapeutic target. *Expert Opin Ther Targets.* Feb 2009;13(2):219-225.

42. Haggstrom S, Lissbrant IF, Bergh A, Damber JE. Testosterone induces vascular endothelial growth factor synthesis in the ventral prostate in castrated rats. *J Urol.* May 1999;161(5):1620-1625.

43. Joseph IB, Nelson JB, Denmeade SR, Isaacs JT. Androgens regulate vascular endothelial growth factor content in normal and malignant prostatic tissue. *Clin Cancer Res.* Dec 1997;3(12 Pt 1):2507-2511.

44. Godoy A, Watts A, Sotomayor P, et al. Androgen receptor is causally involved in the homeostasis of the human prostate endothelial cell. *Endocrinology.* Jun 2008;149(6):2959-2969.

45. Franck-Lissbrant I, Haggstrom S, Damber JE, Bergh A. Testosterone stimulates angiogenesis and vascular regrowth in the ventral prostate in castrated adult rats. *Endocrinology.* Feb 1998;139(2):451-456.

46. Lissbrant IF, Hammarsten P, Lissbrant E, Ferrara N, Rudolfsson SH, Bergh A. Neutralizing VEGF bioactivity with a soluble chimeric VEGF-receptor protein flt(1-3)IgG inhibits testosterone-stimulated prostate growth in castrated mice. *Prostate.* Jan 1 2004;58(1):57-65.

47. Aus G, Robinson D, Rosell J, Sandblom G, Varenhorst E. Survival in prostate carcinoma--outcomes from a prospective, population-based cohort of 8887 men with up to 15 years of follow-up: results from three countries in the population-

based National Prostate Cancer Registry of Sweden. *Cancer.* Mar 1 2005;103(5):943-951.

48. Kolonel LN, Altshuler D, Henderson BE. The multiethnic cohort study: exploring genes, lifestyle and cancer risk. *Nat Rev Cancer.* Jul 2004;4(7):519-527.

49. Meiers I, Waters DJ, Bostwick DG. Preoperative prediction of multifocal prostate cancer and application of focal therapy: review 2007. *Urology.* Dec 2007;70(6 Suppl):3-8.

50. Tomlins SA, Rhodes DR, Perner S, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science.* Oct 28 2005;310(5748):644-648.

51. Tomlins SA, Bjartell A, Chinnaiyan AM, et al. ETS gene fusions in prostate cancer: from discovery to daily clinical practice. *Eur Urol.* Aug 2009;56(2):275-286.

52. Gleason DF, Mellinger GT. Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. *J Urol.* Jan 1974;111(1):58-64.

53. Andren O, Fall K, Franzen L, Andersson SO, Johansson JE, Rubin MA. How well does the Gleason score predict prostate cancer death? A 20-year followup of a population based cohort in Sweden. *J Urol.* Apr 2006;175(4):1337-1340.

54. Egevad L, Granfors T, Karlberg L, Bergh A, Stattin P. Prognostic value of the Gleason score in prostate cancer. *BJU Int.* Apr 2002;89(6):538-542.

55. Huggins C. Endocrine Control of Prostatic Cancer. *Science.* Jun 18 1943;97(2529):541-544.

56. Litvinov IV, De Marzo AM, Isaacs JT. Is the Achilles' heel for prostate cancer therapy a gain of function in androgen receptor signaling? *J Clin Endocrinol Metab.* Jul 2003;88(7):2972-2982.

57. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer.* Oct 2001;1(1):34-45.

58. Montgomery RB, Mostaghel EA, Vessella R, et al. Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth. *Cancer Res.* Jun 1 2008;68(11):4447-4454.

59. Holzbeierlein J, Lal P, LaTulippe E, et al. Gene expression analysis of human prostate carcinoma during hormonal therapy identifies androgen-responsive genes and mechanisms of therapy resistance. *Am J Pathol.* Jan 2004;164(1):217-227.

60. Stanbrough M, Bubley GJ, Ross K, et al. Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. *Cancer Res.* Mar 1 2006;66(5):2815-2825.

61. Petrylak DP, Tangen CM, Hussain MH, et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med.* Oct 7 2004;351(15):1513-1520.

62. Tannock IF, de Wit R, Berry WR, et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med.* Oct 7 2004;351(15):1502-1512.

63. Chen Y, Sawyers CL, Scher HI. Targeting the androgen receptor pathway in prostate cancer. *Curr Opin Pharmacol.* Aug 2008;8(4):440-448.

64. Dakhova O, Ozen M, Creighton CJ, et al. Global gene expression analysis of reactive stroma in prostate cancer. *Clin Cancer Res.* Jun 15 2009;15(12):3979-3989.

65. Orimo A, Weinberg RA. Stromal fibroblasts in cancer: a novel tumor-promoting cell type. *Cell Cycle.* Aug 2006;5(15):1597-1601.

66. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer.* May 2006;6(5):392-401.

67. Alberti C. Prostate cancer progression and surrounding microenvironment. *Int J Biol Markers.* Apr-Jun 2006;21(2):88-95.

68. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature.* Jul 24 2008;454(7203):436-444.

69. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer.* Apr 2009;9(4):239-252.

70. Chung LW, Baseman A, Assikis V, Zhau HE. Molecular insights into prostate cancer progression: the missing link of tumor microenvironment. *J Urol.* Jan 2005;173(1):10-20.

71. Ayala G, Tuxhorn JA, Wheeler TM, et al. Reactive stroma as a predictor of biochemical-free recurrence in prostate cancer. *Clin Cancer Res.* Oct 15 2003;9(13):4792-4801.

72. Yanagisawa N, Li R, Rowley D, et al. Stromogenic prostatic carcinoma pattern (carcinomas with reactive stromal grade 3) in needle biopsies predicts biochemical recurrence-free survival in patients after radical prostatectomy. *Hum Pathol.* Nov 2007;38(11):1611-1620.

73. Wikstrom P, Marusic J, Stattin P, Bergh A. Low stroma androgen receptor level in normal and tumor prostate tissue is related to poor outcome in prostate cancer patients. *Prostate.* Jun 1 2009;69(8):799-809.

74. Lissbrant IF, Lissbrant E, Damber JE, Bergh A. Blood vessels are regulators of growth, diagnostic markers and therapeutic targets in prostate cancer. *Scand J Urol Nephrol.* Dec 2001;35(6):437-452.

75. Lissbrant IF, Stattin P, Wikstrom P, Damber JE, Egevad L, Bergh A. Tumor associated macrophages in human prostate cancer: relation to clinicopathological variables and survival. *Int J Oncol.* Sep 2000;17(3):445-451.

76. Ostman A, Augsten M. Cancer-associated fibroblasts and tumor growth--bystanders turning into key players. *Curr Opin Genet Dev.* Feb 2009;19(1):67-73.

77. Biswas SK, Sica A, Lewis CE. Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms. *J Immunol.* Feb 15 2008;180(4):2011-2017.

78. Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. *Cancer Res.* Jan 15 2006;66(2):605-612.

79. Shimura S, Yang G, Ebara S, Wheeler TM, Frolov A, Thompson TC. Reduced infiltration of tumor-associated macrophages in human prostate cancer: association with cancer progression. *Cancer Res.* Oct 15 2000;60(20):5857-5861.

80. Allavena P, Sica A, Solinas G, Porta C, Mantovani A. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Crit Rev Oncol Hematol.* Apr 2008;66(1):1-9.

81. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer.* Jan 2004;4(1):71-78.

82. Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell.* Mar 2005;7(3):211-217.

83. Zhu P, Baek SH, Bourk EM, et al. Macrophage/cancer cell interactions mediate hormone resistance by a nuclear receptor derepression pathway. *Cell.* Feb 10 2006;124(3):615-629.

84. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell.* Jan 27 2006;124(2):263-266.

85. Gocheva V, Joyce JA. Cysteine cathepsins and the cutting edge of cancer invasion. *Cell Cycle.* Jan 1 2007;6(1):60-64.

86. Lindahl C, Simonsson M, Bergh A, et al. Increased levels of macrophage-secreted cathepsin S during prostate cancer progression in TRAMP mice and patients. *Cancer Genomics Proteomics.* May-Jun 2009;6(3):149-159.

87. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med.* Apr 2000;6(4):389-395.

88. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature.* Dec 15 2005;438(7070):932-936.

89. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol.* Jun 2007;8(6):464-478.

90. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med.* Nov 18 1971;285(21):1182-1186.

91. Folkman J, Klagsbrun M, Sasse J, Wadzinski M, Ingber D, Vlodavsky I. A heparin-binding angiogenic protein--basic fibroblast growth factor--is stored within basement membrane. *Am J Pathol.* Feb 1988;130(2):393-400.

92. Rosenthal RA, Megyesi JF, Henzel WJ, Ferrara N, Folkman J. Conditioned medium from mouse sarcoma 180 cells contains vascular endothelial growth factor. *Growth Factors.* 1990;4(1):53-59.

93. Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer.* Oct 2002;2(10):727-739.

94. Folkman J. Angiogenesis. *Annu Rev Med.* 2006;57:1-18.

95. Folkman J. Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov.* Apr 2007;6(4):273-286.

96. Brem S, Brem H, Folkman J, Finkelstein D, Patz A. Prolonged tumor dormancy by prevention of neovascularization in the vitreous. *Cancer Res.* Aug 1976;36(8):2807-2812.

97. Holmgren L, O'Reilly MS, Folkman J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat Med.* Feb 1995;1(2):149-153.

98. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell.* Aug 9 1996;86(3):353-364.

99. Fukumura D, Jain RK. Tumor microvasculature and microenvironment: targets for anti-angiogenesis and normalization. *Microvasc Res.* Sep-Nov 2007;74(2-3):72-84.

100. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer.* Aug 2002;2(8):563-572.

101. Hillen F, Griffioen AW. Tumour vascularization: sprouting angiogenesis and beyond. *Cancer Metastasis Rev.* Dec 2007;26(3-4):489-502.

102. Patan S, Munn LL, Jain RK. Intussusceptive microvascular growth in a human colon adenocarcinoma xenograft: a novel mechanism of tumor angiogenesis. *Microvasc Res.* Mar 1996;51(2):260-272.

103. Djonov VG, Kurz H, Burri PH. Optimality in the developing vascular system: branching remodeling by means of intussusception as an efficient adaptation mechanism. *Dev Dyn.* Aug 2002;224(4):391-402.

104. Ahn GO, Brown JM. Role of endothelial progenitors and other bone marrow-derived cells in the development of the tumor vasculature. *Angiogenesis.* 2009;12(2):159-164.

105. Rafii S, Lyden D, Benezra R, Hattori K, Heissig B. Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? *Nat Rev Cancer.* Nov 2002;2(11):826-835.

106. Holash J, Maisonpierre PC, Compton D, et al. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science.* Jun 18 1999;284(5422):1994-1998.

107. Maniotis AJ, Folberg R, Hess A, et al. Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am J Pathol.* Sep 1999;155(3):739-752.

108. Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. *Nature.* Dec 15 2005;438(7070):946-953.

109. Tombran-Tink J, Johnson LV. Neuronal differentiation of retinoblastoma cells induced by medium conditioned by human RPE cells. *Invest Ophthalmol Vis Sci.* Aug 1989;30(8):1700-1707.

110. Tombran-Tink J, Chader GG, Johnson LV. PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. *Exp Eye Res.* Sep 1991;53(3):411-414.

111. Goliath R, Tombran-Tink J, Rodriquez IR, Chader G, Ramesar R, Greenberg J. The gene for PEDF, a retinal growth factor is a prime candidate for retinitis pigmentosa and is tightly linked to the RP13 locus on chromosome 17p13.3. *Mol Vis.* Jun 19 1996;2:5.

112. Becerra SP, Sagasti A, Spinella P, Notario V. Pigment epithelium-derived factor behaves like a noninhibitory serpin. Neurotrophic activity does not require the serpin reactive loop. *J Biol Chem.* Oct 27 1995;270(43):25992-25999.

113. Tombran-Tink J, Mazuruk K, Rodriguez IR, et al. Organization, evolutionary conservation, expression and unusual Alu density of the human gene for pigment epithelium-derived factor, a unique neurotrophic serpin. *Mol Vis.* Nov 4 1996;2:11.

114. Filleur S, Nelius T, de Riese W, Kennedy RC. Characterization of PEDF: a multi-functional serpin family protein. *J Cell Biochem.* Apr 1 2009;106(5):769-775.

115. Sanagi T, Yabe T, Yamada H. The regulation of pro-inflammatory gene expression induced by pigment epithelium-derived factor in rat cultured microglial cells. *Neurosci Lett.* May 20-27 2005;380(1-2):105-110.

116. Takanohashi A, Yabe T, Schwartz JP. Pigment epithelium-derived factor induces the production of chemokines by rat microglia. *Glia.* Sep 2005;51(4):266-278.

117. Wang JJ, Zhang SX, Mott R, et al. Anti-inflammatory effects of pigment epithelium-derived factor in diabetic nephropathy. *Am J Physiol Renal Physiol.* May 2008;294(5):F1166-1173.

118. Zamiri P, Masli S, Streilein JW, Taylor AW. Pigment epithelial growth factor suppresses inflammation by modulating macrophage activation. *Invest Ophthalmol Vis Sci.* Sep 2006;47(9):3912-3918.

119. Zhang SX, Wang JJ, Gao G, Shao C, Mott R, Ma JX. Pigment epithelium-derived factor (PEDF) is an endogenous antiinflammatory factor. *FASEB J.* Feb 2006;20(2):323-325.

120. Dawson DW, Volpert OV, Gillis P, et al. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. *Science.* Jul 9 1999;285(5425):245-248.

121. Volpert OV, Zaichuk T, Zhou W, et al. Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor. *Nat Med.* Apr 2002;8(4):349-357.

122. Guan M, Pang CP, Yam HF, Cheung KF, Liu WW, Lu Y. Inhibition of glioma invasion by overexpression of pigment epithelium-derived factor. *Cancer Gene Ther.* May 2004;11(5):325-332.

123. Zhang T, Guan M, Xu C, Chen Y, Lu Y. Pigment epithelium-derived factor inhibits glioma cell growth in vitro and in vivo. *Life Sci.* Sep 29 2007;81(16):1256-1263.

124. Cai J, Jiang WG, Grant MB, Boulton M. Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1. *J Biol Chem.* Feb 10 2006;281(6):3604-3613.

125. Doll JA, Stellmach VM, Bouck NP, et al. Pigment epithelium-derived factor regulates the vasculature and mass of the prostate and pancreas. *Nat Med.* Jun 2003;9(6):774-780.

126. Uehara H, Miyamoto M, Kato K, et al. Expression of pigment epithelium-derived factor decreases liver metastasis and correlates with favorable prognosis for patients with ductal pancreatic adenocarcinoma. *Cancer Res.* May 15 2004;64(10):3533-3537.

127. Cai J, Parr C, Watkins G, Jiang WG, Boulton M. Decreased pigment epithelium-derived factor expression in human breast cancer progression. *Clin Cancer Res.* Jun 1 2006;12(11 Pt 1):3510-3517.

128. Guan M, Yam HF, Su B, et al. Loss of pigment epithelium derived factor expression in glioma progression. *J Clin Pathol.* Apr 2003;56(4):277-282.

129. Abe R, Shimizu T, Yamagishi S, et al. Overexpression of pigment epithelium-derived factor decreases angiogenesis and inhibits the growth of human malignant melanoma cells in vivo. *Am J Pathol.* Apr 2004;164(4):1225-1232.

130. Abramson LP, Stellmach V, Doll JA, Cornwell M, Arensman RM, Crawford SE. Wilms' tumor growth is suppressed by antiangiogenic pigment epithelium-derived factor in a xenograft model. *J Pediatr Surg.* Mar 2003;38(3):336-342; discussion 336-342.

131. Crawford SE, Stellmach V, Ranalli M, et al. Pigment epithelium-derived factor (PEDF) in neuroblastoma: a multifunctional mediator of Schwann cell antitumor activity. *J Cell Sci.* Dec 2001;114(Pt 24):4421-4428.

132. Filleur S, Volz K, Nelius T, et al. Two functional epitopes of pigment epithelial-derived factor block angiogenesis and induce differentiation in prostate cancer. *Cancer Res.* Jun 15 2005;65(12):5144-5152.

133. Garcia M, Fernandez-Garcia NI, Rivas V, et al. Inhibition of xenografted human melanoma growth and prevention of metastasis development by dual antiangiogenic/antitumor activities of pigment epithelium-derived factor. *Cancer Res.* Aug 15 2004;64(16):5632-5642.

134. Hase R, Miyamoto M, Uehara H, et al. Pigment epithelium-derived factor gene therapy inhibits human pancreatic cancer in mice. *Clin Cancer Res.* Dec 15 2005;11(24 Pt 1):8737-8744.

135. Mahtabifard A, Merritt RE, Yamada RE, Crystal RG, Korst RJ. In vivo gene transfer of pigment epithelium-derived factor inhibits tumor growth in syngeneic murine models of thoracic malignancies. *J Thorac Cardiovasc Surg.* Jul 2003;126(1):28-38.

136. Matsumoto K, Ishikawa H, Nishimura D, Hamasaki K, Nakao K, Eguchi K. Antiangiogenic property of pigment epithelium-derived factor in hepatocellular carcinoma. *Hepatology.* Jul 2004;40(1):252-259.

137. Orgaz JL, Ladhani O, Hoek KS, et al. 'Loss of pigment epithelium-derived factor enables migration, invasion and metastatic spread of human melanoma'. *Oncogene.* Sep 21 2009.

138. Streck CJ, Zhang Y, Zhou J, Ng C, Nathwani AC, Davidoff AM. Adeno-associated virus vector-mediated delivery of pigment epithelium-derived factor restricts neuroblastoma angiogenesis and growth. *J Pediatr Surg.* Jan 2005;40(1):236-243.

139. Wang L, Schmitz V, Perez-Mediavilla A, Izal I, Prieto J, Qian C. Suppression of angiogenesis and tumor growth by adenoviral-mediated gene transfer of pigment epithelium-derived factor. *Mol Ther.* Jul 2003;8(1):72-79.

140. Ek ET, Dass CR, Contreras KG, Choong PF. Pigment epithelium-derived factor overexpression inhibits orthotopic osteosarcoma growth, angiogenesis and metastasis. *Cancer Gene Ther.* Jul 2007;14(7):616-626.

141. Yabe T, Wilson D, Schwartz JP. NFkappaB activation is required for the neuroprotective effects of pigment epithelium-derived factor (PEDF) on cerebellar granule neurons. *J Biol Chem.* Nov 16 2001;276(46):43313-43319.

142. Smith ND, Schulze-Hoepfner FT, Veliceasa D, et al. Pigment epithelium-derived factor and interleukin-6 control prostate neuroendocrine differentiation via feed-forward mechanism. *J Urol.* Jun 2008;179(6):2427-2434.

143. Takenaka K, Yamagishi S, Jinnouchi Y, Nakamura K, Matsui T, Imaizumi T. Pigment epithelium-derived factor (PEDF)-induced apoptosis and inhibition of vascular endothelial growth factor (VEGF) expression in MG63 human osteosarcoma cells. *Life Sci.* Nov 4 2005;77(25):3231-3241.

144. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer.* Jun 2003;3(6):453-458.

145. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet.* Feb 17 2001;357(9255):539-545.

146. Isaacs JT, Isaacs WB, Feitz WF, Scheres J. Establishment and characterization of seven Dunning rat prostatic cancer cell lines and their use in developing methods for predicting metastatic abilities of prostatic cancers. *Prostate.* 1986;9(3):261-281.

147. Dunning WF. Prostate Cancer in the Rat. *Natl Cancer Inst Monogr.* Oct 1963;12:351-369.

148. Isaacs JT. Development and characteristics of the available animal model systems for the study of prostatic cancer. *Prog Clin Biol Res.* 1987;239:513-576.

149. Smolev JK, Heston WD, Scott WW, Coffey DS. Characterization of the Dunning R3327H prostatic adenocarcinoma: an appropriate animal model for prostatic cancer. *Cancer Treat Rep.* Mar-Apr 1977;61(2):273-287.

150. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods.* Sep 14 1994;174(1-2):83-93.

151. van Rooijen N, Sanders A, van den Berg TK. Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. *J Immunol Methods.* Jun 14 1996;193(1):93-99.

152. Stattin P, Bergh A, Karlberg L, Tavelin B, Damber JE. Long-term outcome of conservative therapy in men presenting with voiding symptoms and prostate cancer. *Eur Urol.* 1997;32(4):404-409.

153. Weibel ER, ed. *"Stereological methods": Practical methods for biological morphometry*. London: Academic Press; 1979; No. 1.

154. Specht K, Richter T, Muller U, Walch A, Werner M, Hofler H. Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. *Am J Pathol.* Feb 2001;158(2):419-429.

155. Ghafar MA, Anastasiadis AG, Chen MW, et al. Acute hypoxia increases the aggressive characteristics and survival properties of prostate cancer cells. *Prostate.* Jan 1 2003;54(1):58-67.

156. Butterworth KT, McCarthy HO, Devlin A, et al. Hypoxia selects for androgen independent LNCaP cells with a more malignant geno- and phenotype. *Int J Cancer.* Aug 15 2008;123(4):760-768.

157. Alqawi O, Wang HP, Espiritu M, Singh G. Chronic hypoxia promotes an aggressive phenotype in rat prostate cancer cells. *Free Radic Res.* Jul 2007;41(7):788-797.

158. Sunderkotter C, Nikolic T, Dillon MJ, et al. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol.* Apr 1 2004;172(7):4410-4417.

159. Lin EY, Li JF, Gnatovskiy L, et al. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res.* Dec 1 2006;66(23):11238-11246.

160. Lin EY, Pollard JW. Tumor-associated macrophages press the angiogenic switch in breast cancer. *Cancer Res.* Jun 1 2007;67(11):5064-5066.

161. Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med.* Mar 19 2001;193(6):727-740.

162. Aharinejad S, Abraham D, Paulus P, et al. Colony-stimulating factor-1 antisense treatment suppresses growth of human tumor xenografts in mice. *Cancer Res.* Sep 15 2002;62(18):5317-5324.

163. Loberg RD, Ying C, Craig M, et al. Targeting CCL2 with systemic delivery of neutralizing antibodies induces prostate cancer tumor regression in vivo. *Cancer Res.* Oct 1 2007;67(19):9417-9424.

164. Loberg RD, Ying C, Craig M, Yan L, Snyder LA, Pienta KJ. CCL2 as an important mediator of prostate cancer growth in vivo through the regulation of macrophage infiltration. *Neoplasia.* Jul 2007;9(7):556-562.

165. Zhang L, Chen J, Ke Y, Mansel RE, Jiang WG. Expression of pigment epithelial derived factor is reduced in non-small cell lung cancer and is linked to clinical outcome. *Int J Mol Med.* May 2006;17(5):937-944.

166. Sidle DM, Maddalozzo J, Meier JD, Cornwell M, Stellmach V, Crawford SE. Altered pigment epithelium-derived factor and vascular endothelial growth factor levels in lymphangioma pathogenesis and clinical recurrence. *Arch Otolaryngol Head Neck Surg.* Nov 2005;131(11):990-995.

167. Matsushima H, Goto T, Hosaka Y, Kitamura T, Kawabe K. Correlation between proliferation, apoptosis, and angiogenesis in prostate carcinoma and their relation to androgen ablation. *Cancer.* Apr 15 1999;85(8):1822-1827.

168. Montironi R, Bartels PH, Thompson D, Diamanti L, Prete E. Androgen-deprived prostate adenocarcinoma: evaluation of treatment-related changes versus no distinctive treatment effect with a Bayesian belief network. A methodological approach. *Eur Urol.* 1996;30(3):307-315.

169. Harris AL. Hypoxia--a key regulatory factor in tumour growth. *Nat Rev Cancer.* Jan 2002;2(1):38-47.

170. Subarsky P, Hill RP. The hypoxic tumour microenvironment and metastatic progression. *Clin Exp Metastasis.* 2003;20(3):237-250.

171. Marignol L, Coffey M, Lawler M, Hollywood D. Hypoxia in prostate cancer: a powerful shield against tumour destruction? *Cancer Treat Rev.* Jun 2008;34(4):313-327.

172. Hammarsten P, Halin S, Wikstom P, Henriksson R, Rudolfsson SH, Bergh A. Inhibitory effects of castration in an orthotopic model of androgen-independent prostate cancer can be mimicked and enhanced by angiogenesis inhibition. *Clin Cancer Res.* Dec 15 2006;12(24):7431-7436.

173. Ahn GO, Brown M. Targeting tumors with hypoxia-activated cytotoxins. *Front Biosci.* 2007;12:3483-3501.

174. Wei MQ, Mengesha A, Good D, Anne J. Bacterial targeted tumour therapy-dawn of a new era. *Cancer Lett.* Jan 18 2008;259(1):16-27.

175. Marcu L, Olver I. Tirapazamine: from bench to clinical trials. *Curr Clin Pharmacol.* Jan 2006;1(1):71-79.

176. Milosevic M, Chung P, Parker C, et al. Androgen withdrawal in patients reduces prostate cancer hypoxia: implications for disease progression and radiation response. *Cancer Res.* Jul 1 2007;67(13):6022-6025.

177. Wyckoff JB, Wang Y, Lin EY, et al. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res.* Mar 15 2007;67(6):2649-2656.

178. Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell.* Oct 27 2000;103(3):481-490.

179. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer.* Mar 2002;2(3):161-174.

180. Wyckoff J, Wang W, Lin EY, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res.* Oct 1 2004;64(19):7022-7029.

181. Mor G, Yue W, Santen RJ, et al. Macrophages, estrogen and the microenvironment of breast cancer. *J Steroid Biochem Mol Biol.* Dec 1998;67(5-6):403-411.

182. Mor G, Sapi E, Abrahams VM, et al. Interaction of the estrogen receptors with the Fas ligand promoter in human monocytes. *J Immunol.* Jan 1 2003;170(1):114-122.

183. Balkwill F. TNF-alpha in promotion and progression of cancer. *Cancer Metastasis Rev.* Sep 2006;25(3):409-416.

184. Ek ET, Dass CR, Choong PF. PEDF: a potential molecular therapeutic target with multiple anti-cancer activities. *Trends Mol Med.* Oct 2006;12(10):497-502.

185. Fernandez-Garcia NI, Volpert OV, Jimenez B. Pigment epithelium-derived factor as a multifunctional antitumor factor. *J Mol Med.* Jan 2007;85(1):15-22.