Androgen Controlled Regulatory Systems in Prostate Cancer

-Potential new therapeutic targets and prognostic markers

Peter Hammarsten

Umeå University
2008
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- Potential new therapeutic targets and prognostic markers

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Cover illustration: A schematic drawing of the prostate with adjacent tissues.

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To Magdalena and Ellen
ABSTRACT

BACKGROUND: Prostate cancer is by far the most common cancer among Swedish men. Some patients have an aggressive lethal disease, but the majority of affected men have long expected survival. Unfortunately, the diagnostic tools available are insufficient in predicting disease aggressiveness. Novel prognostic markers are therefore urgently needed. Furthermore, metastatic prostate cancer is generally treated with castration, but the long-term effects are insufficient. Additional studies are therefore needed to explore how the effects of this therapy can be enhanced. Prostate growth and regression is beside testosterone controlled by locally produced regulators. Vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) are two of the major regulators in the normal prostate and in prostate tumours.

MATERIALS AND METHODS: VEGF and EGFR were explored in the prostate, by treating rats with either anti-VEGF or anti-EGFR treatment during castration and testosterone-stimulated prostate growth. Rats with implanted androgen-independent prostate tumours were treated with an inhibitor of both VEGF receptor-2 (VEGFR-2) and EGFR. Stereological techniques, immunohistochemistry, western blotting and quantitative real-time PCR were used to evaluate these experiments. Furthermore, prostate tissue from untreated prostate cancer patients was used to retrospectively explore the expression of phosphorylated-EGFR (pEGFR) in relation to outcome.

RESULTS: Anti-VEGF treatment during testosterone-stimulated prostate growth, inhibited vascular and prostate growth. Anti-EGFR treatment during castration and testosterone-stimulated prostate growth resulted in enhanced castration effects and inhibited prostate growth. Anti-vascular treatment of androgen-independent prostate cancer with an inhibitor of VEGFR-2 and EGFR, that targets the normal and tumour vasculature, enhanced the effects of castration. Low immunoreactivity for pEGFR in prostate epithelial cells, both in the tumour and also in the surrounding non-malignant tissue, was associated with good prognosis.

CONCLUSIONS: Anti-vascular treatment, with an inhibitor of VEGFR-2 and EGFR, in combination with castration could be an effective way to treat androgen-insensitive prostate tumours. VEGF and EGFR signalling are necessary components in testosterone-stimulated prostate growth. Phosphorylation of EGFR could be a useful prognostic marker for prostate cancer patients. Tumours may affect the surrounding non-malignant tissue and pEGFR immunoreactivity in the morphologically normal prostate tissue can be used to retrieve prognostic information.
This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

   *Ingela Franck Lissbrant and Peter Hammarsten have contributed equally to this work.


**POPULÄRVETENSKAPLIG SAMMANFATTNING**

**Bakgrund:** Prostatacancer är den vanligaste cancerformen bland män i Sverige. Varje år får ungefär 10 000 män diagnosen prostatacancer och av dem kommer ca 2 500 att dö av sjukdomen. En del patienter har alltså en aggressiv dödlig sjukdom, medan andra har en långsamt växande tumör som inte kommer att orsaka några större problem. Tyvärr finns det idag inte tillräckligt bra diagnostik för att avgöra vilka som verkligen behöver behandling och vilka som klarar sig lika bra utan. Detta leder till att vissa patienter överbehandlas med terapier som kan ge allvarliga biverkningar och att andra män som verkligen behöver intensiva terapier inte får sådan eller får den för sent.


**Material och metoder:** Olika modellsystem användes för att studera hur kastrationsbehandlingen fungerar och hur den kan förstärkas. Vävnadsprover från prostatatumörer användes för att studera förekomsten av ett aktiverat protein kallat "EGFR", som stimulerar tumörväxt. Tumörprover från patienter med prostatacancer användes för att hitta nya metoder att bättre kunna förutsäga tumörernas aggressivitet.

**Resultat:** Studierna visar att blodkärl är viktiga för prostatatumörers tillväxt och att mycket av kastrationsbehandlingens effekter orsakas av att blodkärlen skrumpnar i tumören och dess omgivning. Vidare konstaterades att proteiinerna "VEGF" och "EGFR" är viktiga för prostatatumörers tillväxt. Behandling som angrepp blodkärlen i tumören och dess omgivning samt proteiinet "EGFR", visade sig förstärka effekten av kastration. Dessutom fann vi att den aktiverade formen av "EGFR" i tumören men också i den normala vävnaden runt tumören, kan ge information om vilka män som behöver behandling. Den kan också ge information om vilka som kanske kan lämnas utan behandling, eftersom deras tumörer inte är särskilt aggressiva.

**Slutsatser:** Kombinationsbehandling som angriper både blodkärlen och "EGFR" kan vara ett effektivt sätt att behandle patienter med spridd prostatacancer. Den aktiverade formen av "EGFR" är ett tecken på tumörens aggressivitet, både när den mäts i tumören men även i den friska vävnaden runt tumören. Den kan förutsäga om tumören är aggressiv och då behövs intensiv behandling. Den kan också tala om vilka tumörer som är långsamt växande med god prognos.
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<td>--------------</td>
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<tr>
<td>AR</td>
<td>Androgen Receptor</td>
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<tr>
<td>βC</td>
<td>Betacellulin</td>
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<tr>
<td>BPH</td>
<td>Benign Prostatic Hyperplasia</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
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<td>DHT</td>
<td>Dihydrotestosterone</td>
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<td>DLP</td>
<td>Dorsolateral Prostate</td>
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<td>DRE</td>
<td>Digital Rectal Examination</td>
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<td>EFS</td>
<td>Event-Free Survival</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>EGFR</td>
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<td>ER</td>
<td>Epiregulin</td>
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<td>HB-EGF</td>
<td>Heparin-Binding Epidermal Growth Factor</td>
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<td>HER</td>
<td>Human Epidermal Growth Factor Receptor</td>
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<td>HRPC</td>
<td>Hormone Refractory Prostate Cancer</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>pAKT</td>
<td>Phosphorylated Activated Protein Kinase</td>
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<td>PBS</td>
<td>Phosphate Buffer Saline</td>
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<tr>
<td>PBS-T</td>
<td>Phosphate Buffer Saline Tween</td>
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<td>pEGFR</td>
<td>Phosphorylated Epidermal Growth Factor Receptor</td>
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<td>P-EFS</td>
<td>Probability of Event-Free Survival</td>
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<td>PSA</td>
<td>Prostate Specific Antigen</td>
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<td>RET</td>
<td>REarranged during Transfection</td>
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<td>RT-PCR</td>
<td>Real Time-Polymerase Chain Reaction</td>
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<td>SDS</td>
<td>Sodeum Dodecyl Sulfate</td>
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<td>SD</td>
<td>Standard Deviation</td>
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<td>SE</td>
<td>Standard Error</td>
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<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
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<td>TGF-α</td>
<td>Transforming Growth Factor-α</td>
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<td>TINT</td>
<td>Tumour Indicating Normal Tissue</td>
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<td>TMA</td>
<td>Tissue Micro Array</td>
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<td>Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labelling</td>
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<td>TYR</td>
<td>Tyrosine</td>
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<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
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INTRODUCTION

The Prostate

Prostate anatomy and physiology

The human prostate is a chestnut shaped exocrine gland, which surrounds the urethra just below the urinary bladder in males. It is enclosed by a fibrous capsule and a tubuloalveolar gland that empty into the urethra. In humans the prostate can be divided into three different zones (central, transitional and peripheral), where the urethra is the reference point. Most prostate tumours arise from the peripheral zone. In rodents the prostate is divided into several different lobes (anterior, ventral, lateral and dorsal), which have different morphology and physiology. The human prostate is composed of ducts lined with basal epithelial cells, secretory luminal epithelial cells and neuroendocrine cells, surrounded by a fibromuscular stroma. A basement membrane separates the epithelial cells from the fibromuscular stroma. The fibromuscular stroma is composed of smooth muscle cells (SMC), fibroblasts, mast cells, macrophages, all embedded in a collagenous matrix together with nerves, lymphatic and blood vessels. In the mouse prostate the fibromuscular stroma is largely absent and instead a thin border of fibromuscular cells surrounds the glands. Secretion from the gland is believed to be involved in male fertility by enhancing sperm motility and survival. Although the prostate is involved in fertility, it is not required for reproduction. The most known secreted protein is prostate specific antigen (PSA), which is a serine protease and produced by luminal epithelial cells. Normally PSA is secreted into the ductal lumina, transported to the urethra and removed by ejaculation. During conditions where the basal epithelial cell layer and the basal membrane are disrupted as in prostate cancer, inflammation and benign prostate hyperplasia (BPH) PSA may leak into the surrounding fibromuscular stroma and vasculature. Thereby PSA can be elevated in the blood circulation. Seminal vesicles and the prostate gland together produce most of the ejaculate.

Prostate tumour morphology and pathophysiology

Prostate tumours consist of malignant cells that form more or less differentiated glandular structures and a tumour stroma. Basal epithelial cells are absent in prostate tumours and the tumour vasculature partly lacks periendothelial cells (vascular SMCs and pericytes). The tumour stroma differs from the normal stroma in terms of composition and the expression of growth factors, cytokines, angiogenic factors and proteolytic enzymes. It consists of fibroblasts, myofibroblasts, endothelial cells, pericytes and inflammatory cells.
INTRODUCTION

Prostate and tumour growth control

Castration is the most important treatment of prostate cancer patients with metastasis. Charles Higgins was awarded the Nobel Prize 1966 for the discovery that castration led to involution of the prostate gland 15,16. Unfortunately, the exact mechanisms behind the castration response were not shown and are still under investigation. Studies of prostate growth control are important, as the long-term effect of castration treatment is insufficient 17. New therapy targets for prostate cancer are needed and particularly treatment that could enhance and prolong the effects of castration.

The prostate is androgen regulated and thereby dependent on androgens for development, growth, and maintenance of size and function 18,19. Testosterone is the main androgen in males and produced by Leydig cells in the testis. Production of testosterone is stimulated by the hypothalamus through luteinising hormone releasing hormone (LHRH) which activates the pituitary gland to produce luteinising hormone (LH), which in turn stimulates the Leydig cells. There is also a negative feedback loop where testosterone inhibits the release of LHRH. Testosterone is converted in the prostate by 5-alpha-reductase to dihydrotestosterone (DHT), which is a more potent androgen 20. Both testosterone and DHT can bind the androgen receptor (AR), and the activated AR is translocated from the cytoplasm to the nucleus where it activates gene transcription.

Androgens regulate prostate epithelial growth and regression through the stroma, by inducing production of paracrine growth factors, so called andromedins. In TA cells
stromal factors regulate survival and proliferation, and in AR-positive luminal epithelial cells these stromal factors only regulate survival. Many androgenic effects on the prostate epithelium do not require epithelial AR, but instead are elicited by the paracrine action of AR-positive stromal cells. This has been shown in prostatic tissue recombinants composed of AR-negative epithelium and AR-positive stroma, where hormonal ablation of hosts induce apoptosis and decrease proliferation virtually identical to prostatic tissue recombinants containing wild-type epithelium. Moreover, this castration-induced prostatic epithelial apoptosis and decreased proliferation was blocked by testosterone in both wild-type and AR-negative prostatic tissue recombinants 24,33-35.

Androgen-AR signalling in luminal epithelial cells inhibits proliferation and maintains the functional differentiation status of the luminal epithelial cells. This has been shown in conditional knock-outs that lacked the AR in luminal epithelial cells, where the AR was deleted at onset of puberty 36,37. Additionally, androgen stimulation of luminal epithelial cells induces the production of paracrine growth factors that affect the vasculature and the stroma 38,39. However, androgen-dependent prostate cancer cells transplanted to AR-negative male mice have demonstrated that androgen-AR signalling in prostate cancer cells gains the ability to promote proliferation and possible also survival, and still keeps the ability to promote differentiation 40. This shows that transformation to a malignant phenotype involves a shift from a paracrine mode to an autocrine mode, where AR signalling in the tumour cells directly activates the production of autocrine growth factors 40. This shift in prostate cancer cells can be explained by gene fusions and the AR-Skp2 pathway. Recurrent gene fusions have been found in a majority of prostate cancers, where regulatory elements controlled by androgens are fused to oncogenic transcription factors, leading to the overexpression of oncogenes 41. A recent study has shown that the Skp2 protein, which is stimulated by androgens and promotes proliferation, is expressed in androgen-dependent prostate cancer cells 42.

The prostate vasculature has a key role in regulating growth and regression of the prostate epithelium 43. Hormonal ablation induced involution of the ventral prostate in rats is preceded by a decreased blood flow 29,44,45, endothelial cell apoptosis 46 and local tissue hypoxia 47. This shows that subsequent epithelial cell apoptosis and prostate involution, is partly caused by insufficient blood flow 29,44. Testosterone-stimulated regrowth of the prostate epithelium in the ventral prostate in castrated rats is preceded by an increase in blood flow 44, endothelial cell proliferation and regrowth of the vasculature 48. These observations show that vascular changes may be of major importance for castration-induced involution and testosterone stimulated regrowth of the prostate. The stroma and luminal epithelial cells regulate vascular growth and regression in the prostate, by producing paracrine growth factors 27,38,39,49-52. Androgens also regulate the prostate vasculature by direct effects on periendothelial cells and endothelial cells 27,30.
Figure 1. Schematic illustration of normal prostate growth control. Prostate epithelial growth and regression is regulated by direct effects of androgens on luminal epithelial cells, paracrine signalling from the luminal epithelial cells, paracrine signalling from the stroma, and by the vasculature. The luminal epithelial cells, smooth muscle cells (SMCs) and periendothelial cells (vascular SMCs and pericytes) express the androgen receptor (AR +). The vascular endothelial growth factor (VEGF) is secreted from luminal epithelial cells and binds to VEGF receptor-2 (VEGFR-2) on endothelial cells. Stromal cells secrete the epidermal growth factor receptor (EGFR) ligands, which mainly have direct effects on the luminal epithelial cells and possibly also basal epithelial cells.

Interestingly and considerably less studied, the non-malignant prostate tissues that surround prostate tumours regulate their growth and regression. For example, all blood and lymph vessels supplying and draining a tumour pass through the surrounding non-malignant tissue and must be continuously adapted to increasing demands. A recent study in rats, with orthotopically transplanted androgen-insensitive tumours, has shown that castration reduces vascular density in the non-malignant tissue that surround prostate tumours and this in turn was accompanied by increased tumour cell hypoxia and tumour cell death. Thus, the environment makes an androgen-sensitive tumour androgen-dependent.
In conclusion, prostate epithelial growth and regression is regulated by: direct effects of androgens on luminal epithelial cells, paracrine signalling from the luminal epithelial cells, paracrine signalling from the stroma, and by the vasculature (Figure 1). Additionally, in androgen-dependent prostate cancer cells, androgen-AR signalling gains the ability to promote proliferation and possible also to inhibit apoptosis by autocrine signalling. The morphologically normal tissue surrounding prostate tumours may regulate tumour growth (Figure 2).
Prostate Growth Factors

Introduction of growth factors

As previously described stromal cells, epithelial cells and periendothelial cells produce growth factors in the prostate under the influence of androgens. These androgen controlled and locally produced growth factors can either promote or inhibit prostate growth and regulate differentiation of epithelial cells, through autocrine and paracrine signalling. In prostate tumours several of these growth factors are altered. The major growth factor families in the prostate include among others the fibroblast growth factor (FGF) family, the transforming growth factor-β (TGF-β) family, the insulin-like growth factor (IGF) family, the epidermal growth factor receptor (EGFR) family and the vascular endothelial growth factor (VEGF) family.

There are many FGF members, such as FGF2, 8, 9, 10 and keratinocyte growth factor (KGF/FGF7), which are produced by the stroma. Prostate epithelial cells express multiple FGF receptors (FGFR), FGFR-1 and FGFR-2 are localised on basal epithelial cells, FGFR-4 on luminal epithelial cells, and FGFR-3 is also expressed on the epithelium. There are several studies indicating that the FGF family plays a role in prostate cancer progression. Recent studies have shown that overexpression of FGF10 by the prostate stroma or a constitutive active FGFR-1 on prostate basal epithelial cells is sufficient to induce prostate cancer in mouse models.

TGFβ exist in three different isoforms (TGFβ-1,-2 and -3) and the TGFβ receptors-1 (TGFβR-1) and -2 mediate ligand signalling. They are potent inhibitors of epithelial growth and migration, but promote stromal cell proliferation and migration. TGFβ production is enhanced in prostate cancer. During prostate cancer progression the cancer cells loose their sensitivity to TGFβ1:s growth inhibitory effects. High expressions of TGFβ1, loss of TGFβR or loss of TGFβ sensitivity are all poor prognostic factors. Importantly, a study has shown that a knock-out of the TGFβR-2 gene in prostate fibroblasts results in prostate intraepithelial neoplasia.

IGF1 is produced mainly by prostate stromal cells and the IGF receptor-1 (IGFR-1) is expressed on prostate basal and luminal epithelial cells. Receptor activation stimulates proliferation, survival and inhibits apoptosis in prostate cancer cells. Androgens stimulate stroma IGF1 synthesis and the production of IGF1 increases in prostate cancer cells.
**The Epidermal Growth Factor Receptor**

*Introduction of EGFR*

In addition to androgens, prostate growth and function are controlled by locally secreted autocrine and paracrine regulators, as stated previously. One of these regulators is the epidermal growth factor receptor (EGFR) and its ligands \(^{50,51,70,71}\). EGFR is a 170-kDa tyrosine kinase transmembrane glycoprotein expressed in normal tissues in many organs and different types of tumours \(^{72-74}\). In the prostate, EGFR is expressed mainly in epithelial cells \(^{39,75}\). Most of the EGFR ligands; amphiregulin, betacellulin (\(\beta\)C), EGF, heparin-binding EGF (HB-EGF) and transforming growth factor \(\alpha\) (TGF-\(\alpha\)) are produced in the rodent prostate, with the exception for epiregulin (ER) which has not been detected \(^{75}\). Several of the ligands are produced in the prostate stroma and some of them in the epithelium \(^{39,52}\). The EGFR is part of a family of four closely related transmembrane receptors: EGFR (HER-1 [human epidermal growth factor receptor -1], erbB-1); HER-2 (erbB-2/neu); HER-3 (erbB-3); and HER-4 (erbB-4) (Figure 3A) \(^{76}\).

HER-2 has some homology with EGFR and no ligand has been identified for HER-2, instead it can form heterodimeric complexes with EGFR when EGFR binds its

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**Figure 3.** A: The EGFR system with ligands. B: Schematic representation of the EGFR with the ligand binding site (L), tyrosine kinase portion (TK), transmembrane portion (located between L and TK), and nine tyrosine residues (Y) on the intracellular portion.
ligands \textsuperscript{73,77}. The other two receptors, HER-3 and HER-4, bind several ligands including the neuregulins (NRG) and ER \textsuperscript{74}. This receptor family are expressed in prostate epithelial cells \textsuperscript{78} and increased expressions of these receptors have all been described in prostate cancer \textsuperscript{79-83}.

**EGFR activation and function**

The binding of a ligand to EGFR results in its dimerization, it forms homodimeric complexes or heterodimeric complexes with other receptors of the erbB family (usually with HER-2). This leads to phosphorylation of nine different tyrosine (TYR) residues, via either receptor tyrosine kinase autophosphorylation or via Src-dependent phosphorylation (at TYR845 and TYR1101) (Figure 3B). The phosphotyrosine residues are recognized by several adaptor molecules possessing src sequence homology (SH2/SH3) \textsuperscript{73,74,77}. This, in turn, activates intracellular signalling cascades, including the ras/MAP kinase, phosphatidylinositol-3'-OH (PI3) kinase, and signal transducer and activator of transcription (STAT)-3 signal transduction pathways \textsuperscript{73}. Activation of EGFR results in proliferation, differentiation, secretion, migration, angiogenesis and inhibition of apoptosis \textsuperscript{70,72,84,85}.

EGFR activation in different tissues and tumours results in cellular responses in both epithelial and endothelial cells \textsuperscript{70,72}. Inhibition of the EGFR in a variety of EGFR-expressing tumour cells reduces basic fibroblast growth factor (bFGF), interleukin 8 (IL-8), TGF-\(\alpha\) and VEGF expression, and thereby also angiogenesis. This suggests that the anti-angiogenic effect caused by EGFR inhibition is indirect, but in some models EGFR have also been detected on blood vessels, indicating a possible direct effect on angiogenesis \textsuperscript{72}.

**EGFR in cancer**

The EGFR has been established as a cellular oncogene and over one-third of all solid tumours express EGFR. In many tumours, EGFR expression may act as a prognostic marker, which predicts poor cancer specific survival and more advanced disease stage. Many tumourigenic processes have been shown to be mediated by EGFR signal transduction pathways, which include cell survival, cell cycle progression, angiogenesis, tumour cell invasion and spread of metastases \textsuperscript{73}. In contrast to the carefully regulated physiological EGFR signalling which occurs in the normal prostate, there are several studies showing that aberrant EGFR signalling is present during prostate cancer \textsuperscript{73,74,86}. A mutated form of EGFR, called EGFRvIII, is frequently present in prostate cancer \textsuperscript{87,88}. It lacks 267 amino acids from its extracellular domain, which results in the loss of a large proportion of the ligand-binding domain. EGFRvIII is a constitutively active form of EGFR and over-expressed in human prostate cancer, at the same time the expression of the wild type EGFR is decreased \textsuperscript{79-83,88}. As EGFRvIII is not detected by antibodies against wild type EGFR, this could contribute to reported discrepancies in EGFR expression in prostate cancer tissue. EGFRvIII expression in prostate cancer is significantly
INTRODUCTION

associated with shorter time to biochemical relapse, decreased time to death from biochemical relapse and decreased overall survival. In prostate cancer there is a switch in production of the dominant stimulatory ligand EGF, to TGF-α as the dominant ligand. EGF and TGF-α have different effects on the expression levels of EGFR in prostate cancer cell lines, because TGF-α decreases the degradation of EGFR. Thus, with the same number of EGFR:s, this switch in ligand production results in prolonged signalling, as may occur in prostate cancer. In the normal prostate, stromal cells produce EGF and TGF-α, while epithelial cells express the EGFR. However, prostate tumour epithelial cells co-express both ligands and the EGFR. This shows that tumour epithelial cells gains an autocrine loop in prostate cancer and loss the stromal paracrine modulation of EGFR function. A new roll for the EGFR has recently been discovered in cancer, where EGFR physically associates with and stabilises the sodium/glucose cotransporter (SGLT1) to promote glucose uptake into cancer cells. This function does not require EGFR kinase activity. Thus, EGFR has also a kinase-independent role in promoting metabolic homeostasis in cancer cells.

High levels of immunostaining for EGFR in prostate tumours is associated with high Gleason grade, advanced tumour stage, and high risk for prostate-specific antigen recurrence. EGFR signalling plays a important role during the progression to castration-resistant and metastatic prostate cancer. Anti-EGFR treatment prevents growth of castration-resistant prostate cancer. Immunohistochemistry studies of the activated form of EGFR in non-small cell lung cancer (NSCLC) patients have proven that phosphorylation of EGFR at tyrosine residue 845 is a prognostic marker in NSCLC patients. Furthermore studies in invasive breast cancer patients have shown that high phosphorylated epidermal growth factor receptor (pEGFR) expression is significantly associated with poor survival, and related to angiogenesis and invasiveness. Phosphorylation of EGFR at TYR845 by c-Src is involved in the regulation of receptor function and prostate tumour progression. C-Src is a non-receptor protein kinase responsible for signal transduction during differentiation, adhesion and migration. It has a role in the development of prostate cancer and during androgen-independent growth.

**EGFR and androgens**

During castration-induced involution and androgen-induced regrowth of the prostate, the expression of EGFR increases. Androgens seem to regulate EGFR expression levels in the prostate. The EGFR ligands have also been proven to be regulated by androgens, such as EGF and TGF-α. Furthermore, EGFR activation by EGF induces growth of the ventral prostate in newborn rats and EGF can inhibit castration induced prostate regression in normal rats. This implies that EGFR may be an important modulator of prostate growth and regression. In addition, androgen has been shown to up-regulate EGFR expression and activity in human prostate tumour cells.
On the other hand, EGFR has been suggested to be involved in the mechanisms underlying the development of androgen-independent prostate cancer \[118-120\]. A recent report has indicated that EGF can induce tyrosine phosphorylation of the androgen receptor and thereby activation of the receptor. EGF and its downstream tyrosine kinases, which are elevated during hormone-ablation therapy, can induce tyrosine phosphorylation of the androgen receptor and could be important for prostate tumour growth under androgen-depleted conditions \[120\]. There is also evidence that EGF directly can activate the androgen receptor in the absence of androgens \[118\].

The Vascular Endothelial Growth Factor

Introduction of VEGF

VEGF is one of the most well-characterized angiogenesis factors. Gene expression of VEGF is activated by the transcriptional complex hypoxia-inducible factor in response to hypoxia and results in enhanced blood flow \[121\]. Cloning of VEGF in 1989 \[122,123\] was a major milestone in understanding of angiogenesis and tumour angiogenesis. VEGF is the most prominent factor responsible for induction of angiogenesis, which induces endothelial cell proliferation, differentiation, migration, tube formation and vessel assembly \[124\].

The VEGF family consists of five glycoproteins referred to as VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PIGF) \[125,126\]. The best characterized and most important form in tumour tissues is VEGF, which have four alternative splicing isoforms \[127,128\]. VEGF\[165\] is the predominant isoforms and is commonly overexpressed in a variety of human solid tumours. The VEGF ligands bind to and activate three different tyrosine kinase receptors: vascular endothelial growth factor receptor 1 (VEGFR-1, FLT-1), VEGFR-2 (KDR) and VEGFR-3 (FLT-4), the first two of which bind to VEGF (Figure 4) \[127\]. VEGFR-2 is primarily expressed on endothelial cells and the key mediator of VEGF-induced angiogenesis, which include stimulation of endothelial cell proliferation and differentiation \[129\]. VEGFR-1 is also expressed on endothelial cells and on several other types of cells, such as macrophages \[127\]. This receptor has a 10-fold higher binding affinity to VEGF, but exerts less activation of intracellular signalling intermediates than VEGFR-2 \[130\]. This means that VEGFR-1 can function as a negative regulator of angiogenesis, by binding VEGF and preventing its binding to VEGFR-2 \[131\]. VEGFR-3 preferentially binds VEGF-C and VEGF-D, and is expressed mainly on lymphatic endothelial cells but also on vascular endothelial cells \[132\]. VEGFR-3 is involved in cardiovascular development and remodelling of primary vascular networks during embryogenesis, and has a crucial role in post-natal lymphangiogenesis \[133,134\]. The neuropilins (NP-1 and NP-2) act as co-receptors for VEGFR, as they increase the binding affinity of VEGF ligands to VEGFR (Figure 4). It have been suggested the neuropilins can
signal independent from VEGFR, but the role of VEGF activated neuropilin signalling is not fully elucidated \(^\text{127}\).

**Figure 4.** VEGF family members and receptors.

**VEGF in cancer**

Strong evidence has shown that VEGF is involved in the prostate cancer growth process \(^\text{135}\). VEGF stimulates tumour angiogenesis and blood flow; by stimulating endothelial cell proliferation, survival, migration and invasion; increasing permeability of existing vessels; and enhances chemotaxis and homing of bone marrow-derived vascular precursor cells (endothelial cells and pericytes) \(^\text{136,137}\). In addition, VEGF also has other functions besides stimulating angiogenesis which includes autocrine effects on tumour cell function (survival, migration, invasion), immune suppression, and homing of bone marrow progenitors to prepare for metastasis \(^\text{138}\).

In the prostate, VEGF is expressed in epithelial cells and expression is regulated by androgens, both in the normal prostate and in prostate cancer \(^\text{38,139-143}\). In a rat model with prostate cancer castration reduced VEGF expression and microvessel density in the tumour tissue \(^\text{141}\). Androgen deprivation in a human prostate cancer cell line, LNCaP, decreased VEGF mRNA expression \(^\text{143}\). Furthermore, increased microvessel density is associated with VEGF expression \(^\text{142}\).
**New Blood Vessel Formation**

*Introduction of new blood vessel formation*

Blood vessels are compassed of endothelial cells formed as tubes and outside of the endothelium there are SMCs and pericytes. Normal tissue function depends on blood vessels that provide with oxygen, nutrients and remove metabolic waste products. Understanding how blood vessels form has become a principal objective the last decade. In the early 1970's Dr Judah Folkman and colleges presented evidence that tumours are dependent on blood vessels to grow beyond 1 mm³, each tumour must then be able to induce new blood vessel formation from existing endothelial cells (the angiogenesis process). This lead to the hypothesis that tumour growth could be inhibited by anti-angiogenic therapy, which in turn have lead to extensive research on the angiogenesis field. Today there are several anti-angiogenic agents in clinical trials and also clinically approved drugs.

**Vasculogenesis**

Blood vessels in the embryo form through vasculogenesis, where undifferentiated precursor cells (angioblasts) differentiate to endothelial cells that assemble into a

*Figure 5. Schematic illustration of new blood vessel formation.*
primitive vascular network. Angioblasts may migrate extensively before in situ differentiation and plexus formation. VEGF, VEGFR-2 and basic fibroblast growth factor (bFGF) influence angioblast differentiation. Molecules mediating interactions between endothelial cells and matrix macromolecules, fibronectin or matrix receptors (α5 integrin), also affect vasculogenesis. It was once believed that endothelial precursors only exist during embryonic life. However, endothelial precursor cells have been identified in bone marrow and in peripheral blood in adults. Granulocyte-monocyte colony-stimulating factor (GM-CSF), bFGF, VEGF and IGF-1 stimulate endothelial precursor differentiation and mobilisation. Such endothelial precursors home to angiogenic sites and are involved in new blood vessel formation in adults. This may be a target for future therapy.

Angiogenesis

Angiogenesis is defined as the development of new capillaries from endothelial cells in existing blood vessels, which occurs in embryonic development and postnatal life. New vessels in the adult arise mainly through angiogenesis, although vasculogenesis also may occur. Physiological angiogenesis is tightly regulated during adult life and occurs during the female reproductive cycle, wound healing, tissue repair, and as shown by researchers from Umeå also in the male reproductive organs. In contrast, angiogenesis also contribute to pathological processes such as rheumatoid arthritis, psoriasis, diabetic retinopathy and cancer. The angiogenesis process is regulated by angiogenesis stimulators and inhibitors that act directly or indirectly on endothelial cells. These factors either stimulate or inhibit survival, proliferation, differentiation or migration. In tissues where the endothelial cells are quiescent there are a balance of angiogenesis stimulators and inhibitors. However, when stimulators are up regulated or inhibitors are down regulated, there is an angiogenic “switch” and the angiogenesis process starts.

It begins with an enlargement of the parent vessel which then “sprouts”, or is divided by pillars of periendothelial cells (“intussusception”) or by transendothelial cells (“bridging”) which then split into individual capillaries. Sprouting angiogenesis begins with proteolytic breakdown of the basal membrane and extra cellular matrix. The endothelial cells then start to proliferate and migrate into a new tube structure. At the end of the process, despite if it occurs by sprouting, intussusception or bridging, there is a maturation phase. During this phase periendothelial cells enclose and stabilise the vessel by inhibiting endothelial proliferation and migration, and stimulate the production of extracellular matrix.

Tumour angiogenesis

Tumours induce angiogenesis to be able to grow beyond 1 mm³, the tumour “switches” from the prevascular to the vascular phase. Tumour blood vessels also provide a route for tumour cells to metastasise and they differ in many ways.
from the normal vasculature. These vessels are highly irregular and complex, they are leaky, have discontinuous lining of endothelial cells and basement membrane, often lack periendothelial cells, and have arterio-venous shunts and blind ends. The tumour endothelial cells have a much higher proliferation rate, altered production of genes and altered surface markers compared to normal endothelial cells. This results in a highly variable blood flow and hypoxic areas in the tumour. It is also possible that endothelial precursors are recruited to the site of tumour angiogenesis.

<table>
<thead>
<tr>
<th>Angiogenesis stimulators</th>
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<td>Transforming growth factor (TNF-α)</td>
<td>Interferon-β (INFβ), INFγ</td>
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<td>Troponin-1</td>
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<tr>
<td>Hepatocyte growth factor (HGF)</td>
<td>Anti-thrombin III</td>
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Prostate Cancer

Introduction of prostate cancer

Prostate cancer is one of the most common cancers and the second leading cause of cancer death in men in Western industrialized countries. In Sweden, prostate cancer is the most common cause of cancer death and around 10 000 men are diagnosed with the disease every year, and approximately 2 500 of these men will die of their cancer (Swedish Cancer Registry 2004). Prostate cancer is mainly a disease of the elderly and the median age at diagnosis is 75 years. The incidence for prostate cancer differs largely between different regions around the world, and the environment and lifestyle could be important factors that may explain this difference. In addition, the genetic background may also have some effects on the disease risk. It’s a multifocal disease, where approximately three different tumours are usually found within an individual patient at diagnosis. At diagnosis the disease is classified as local or advanced, and at advanced disease the cancer has spread beyond the prostate capsule.

Some of the prostate cancer patients have rapidly progressing lethal disease, but the majority of the patients have a long expected survival. Although curative treatments may reduce risk of progression and prostate cancer mortality, these treatments have adverse effects including erectile dysfunction and incontinence.
INTRODUCTION

and a large proportion of the patients would have survived their prostate cancer even without treatment. There are several prognostic tools available for prostate cancer. The Gleason score system is the strongest predictor of prostate cancer outcome, especially for low (≤5) and high (8 - 10) Gleason score tumours. Unfortunately, more than 70% of the localised tumours are graded as Gleason score 6 or 7 on diagnostic biopsies and these patients have highly variable and largely unpredictable outcomes. Furthermore, there is no imaging method available that could detect prostate cancer and therefore biopsies may not sample the most aggressive tumour or sample only non-malignant prostate tissue. Biopsies that contain prostate tumours are therefore often undergraded, as only a very small proportion (about 1/1000) of the prostate gland is sampled. Therefore additional methods to guide biopsies and novel prognostic markers are urgently needed.

Diagnosis of prostate cancer

Today, more patients with prostate cancer are being diagnosed in early stages of the disease compared to 10 years ago and the incidence has been increasing over the last few decades, due to increased PSA-measurements. The PSA test is commonly used to assess the risk for having prostate cancer. It is considered normal to have a PSA value within the range 0 to 3 ng/ml in the blood. A PSA value within the range 3-10 ng/ml indicate that the patient should receive closer follow up, where measuring free versus bound PSA may differ prostate cancer from benign hyperplasia (BPH). If the PSA value is >10 ng/ml there is a substantial risk that the patient may have prostate cancer. Unfortunately, the PSA test has low specificity and sensitivity. A majority of the patients also have a PSA value with in the 3-10 ng/ml range, which could be caused by other conditions besides prostate cancer, such as inflammation or BPH. In addition, studies have shown that countries with active PSA screening have similar mortality in prostate cancer compared to those without screening.

If the PSA level together with clinical data indicates that the patient may have prostate cancer, 6-12 needle biopsies are taken from the prostate with or without ultrasound guidance. As men with low PSA are generally not biopsied and men with high PSA are subjected to multiple biopsy sessions until a tumour is found, the value of the PSA test is difficult to evaluate. However, ultrasound is not a good imaging tool for detecting prostate cancer and therefore makes the biopsies unrepresentative. The biopsies are evaluated to see if prostate cancer is present.

Prognosis of prostate cancer

If a biopsy contains prostate cancer, the differentiation status of the biopsy is scored according to the Gleason score system. It is the strongest prognostic tool available today for prostate cancer. The most common and the second most common area are scored on a differentiation scale ranging from 1 to 5, where 5 is the lowest
differentiated tumour. The majority of the prostate cancer patients have Gleason score 6 or 7 (3+3, 3+4 or 4+3).

The methods used to determine whether the prostate cancer is local (not spread outside the fibrous capsule), locally advanced (spread outside the fibrous capsule but no metastases) or advanced (metastatic disease); include radio nuclide bone scan, magnetic resonance imaging (MRI) and digital rectal examination (DRE).

Treatment of prostate cancer

Treatment of prostate cancer varies depending on age, prognostic group, presence of co-morbidity and if the tumour is local, locally advanced or advanced. Local prostate cancer is treated with radical prostatectomy, irradiation, anti-androgens or “watchful waiting” [184,185]. Watchful waiting means that patients only receive symptomatic treatment. In advanced prostate cancer, where the tumour has metastasised preferentially to the bone or lymph nodes, treatments are chemical or surgical castration [15]. Castration therapy is a palliative treatment and lowers the androgen levels, which reduces tumour size and pain associated with bone metastases. Unfortunately, the tumour will relapse within a few years and become hormone refractory, where tumour cells grow although castration treatment and leads to death of the patient [17,119]. Hormone refractory prostate cancer (HRPC) is treated with a combination of docetaxel and prednisone, which gives an overall survival benefit.

A more accurate name for HRPC is “castration-resistant prostate cancer”. After castration treatment, testosterone is still present at low levels in the blood. HRPC cells are believed to be supersensitive to androgen, due to there ability to grow although low levels of testosterone [119,186]. In addition, there are enzymes present in HRPC cells that can convert adrenal steroids to testosterone and the levels of testosterone in the prostate and metastases are then not decreased as in the blood. This may be explained by the up regulation of genes that convert adrenal steroids to testosterone [187,188].

Tyrosine kinase inhibitors

Inhibition of ligand-induced tyrosine kinase activity is an attractive therapeutic target and several drugs are being developed for this purpose, so called tyrosine kinase inhibitors.

Gefitinib (Iressa®, ZD1839 from AstraZeneca) is a highly selective EGFR tyrosine kinase inhibitor, which have good inhibition effect against various cancer cells in vivo, alone and combined with other cytotoxic drugs [189,190]. It inhibits EGFR tyrosine activity with 100-fold greater selectivity over other tyrosine kinases, and is anti-angiogenic and reduces cell proliferation in tumours expressing EGFR [72,189,191]. ZD6474 (AstraZeneca) is a tyrosine kinase inhibitor that inhibits VEGFR-2, EGFR and RET (REarranged during Transfection). It inhibits VEGFR-2 signalling in endothelial cells and thereby also tumour angiogenesis, consequently ZD6474 has
demonstrated anti-tumour effects in many different human tumour xenografts. The EGFR inhibitory effect is mainly on tumour cell growth and survival 192.

**Soluble chimeric VEGF-receptor protein**

VEGF bioactivity can be inhibited by treatment with a soluble chimeric VEGF-receptor protein flt(1-3)IgG, which in known to neutralize VEGF activity in various types of tissues 193-195. Flt(1-3)IgG has been used in a rat model of hormonally induced ovulation, which resulted in almost complete suppression of corpora lutea angiogenesis. No effect was observed on the pre-existing ovarian vasculature 194. Treatment with flt(1-3)IgG in endochondral bone formation, resulted also in almost complete suppression of blood vessel invasion 195.
INTRODUCTION

AIMS

General aim
Every year 10 000 Swedish men are diagnosed with prostate cancer and it’s the most common cause of cancer death in Sweden. However, the majority of these men have a clinically insignificant prostate cancer that will not need to be treated or have a curable prostate cancer that is local, but approximately 25% of these men will die from their prostate cancer. In other words, it is important to find markers, which could predict which patients have tumours with aggressive invasive and metastatic potential. Then patients with favourable outcome would not need morbidity associated treatments, whereas patients with a high risk of early metastasis or death would receive more intensive treatment.

In 1966 Charles Higgins was awarded the Nobel Prize for the discovery that castration lead to the shrinkage of the prostate. However, the mechanism behind this treatment is still not fully understood. Castration treatment is purely palliative and the only treatment available for metastatic prostate cancer. It lowers androgen levels, which dramatically reduces tumour growth and size. Unfortunately, the tumour will relapse within a few years and leads to death of the patient. In addition to androgens, prostate growth and function are controlled by locally secreted autocrine and paracrine regulators. These factors need to be further explored as they may prove to be novel targets in effective therapies against prostate cancer.

Specific aims

- To study the roll of VEGF and angiogenesis in testosterone stimulated prostate growth.

- To explore the roll of EGFR during prostate tissue growth and regression.

- To examine if anti-vascular treatment with an inhibitor of VEGFR2 and EGFR, in an orthotopic model of androgen-independent prostate cancer, could enhance castration treatment.

- To examine if expression of pEGFR in non-malignant and malignant prostate tissue is a potential prognostic marker for outcome in prostate cancer patients.
MATERIALS AND METHODS

Patients

*Materials from transurethral resection of the prostate (paper IV)*

Between 1970s’ and early 1990s’, specimens were obtained from patients who underwent transurethral resection of the prostate (TURP) at the hospital in Västerås, Sweden, due to obstructive voiding problems, and where subsequent histological analysis showed presence of prostate cancer. At that time, serum prostate-specific antigen (PSA) was not yet used for diagnostics in Sweden. Tissue specimens were formalin-fixed, paraffin-embedded and graded according to the Gleason system. Radio nuclide bone scan was performed shortly after diagnosis for detection of metastases. Patients had not received any anti-cancer therapy prior to TURP. The study includes 303 patients, of which 259 patients were followed with watchful waiting after TURP. At symptoms from metastases patients received palliative treatment with androgen ablation and in a few cases radiation therapy or oestrogen therapy, according to therapy traditions in Sweden during that time period. In addition, we also analysed 44 patients that were treated with palliative treatment immediately after diagnosis. From specimens collected, tissue micro arrays (TMA) were constructed using a Beecher Instrument (Sun Prairie, WI, USA). TMA:s contained 5-8 samples of tumour tissue (cores with a diameter of 0.6 mm) and 4 samples of non-malignant tissue from each patient. This patient material has been described in more detail previously. This study was approved by the local Research Ethics Committee in Umeå, Sweden.

Animals and Treatments

Animals used in studies were housed in a controlled environment, and food and water were provided ad libitum. The experiments were approved by the local animal ethical committee in Umeå, Sweden.

*Anti-VEGF treatment during castration (paper I)*

Adult male C57 Black mice (Taconic, Mölegård, Denmark) were anesthetized and castrated via the scrotal route. Intact animals were used as controls. After 7 days, the castrated animals received each day a subcutaneous (s.c.) injection of long-acting testosterone esters (Sustanon, 10 mg/kg/day; donated by Organon, Oss, The Netherlands) and an intraperitoneal (i.p.) injection of vehicle (IgG 10 mg/kg/day, or 80 μl PBS/day) or flt(1-3)IgG (10 mg/kg/day; a soluble VEGF receptor that neutralizes VEGF bioactivity; Genentech, CA, USA) for 4 days. After 11 days after
castration, the animals were injected with Bromodeoxyuridine (BrdU, 50 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO, USA), in order to label proliferating cells. BrdU is a thymidine analogue which is incorporated into DNA in the S-phase of the cell cycle. One hour later the animals were anesthetized and perfusion fixed in Bouins solution. The prostate lobes were subsequently dissected out and weighed and then fixed by immersion for 24 hours in the same fixative, dehydrated and embedded in paraffin. For further details regarding anti-VEGF treatment during castration see paper I.

Anti-EGFR treatment (paper II)

Adult old male Sprague Dawley rats (B&K, Stockholm, Sweden) were anesthetized and castrated via the scrotal route. Effect of Gefitinib (an inhibitor of EGFR signalling) on castration induced prostate involution was studied by dividing the animals into three groups; intact animals, animals castrated and treated with Gefitinib (150 mg/kg/day, per os [p.o.]; donated by AstraZeneca Södertälje, Sweden) for 3 days, and animals castrated and treated with vehicle (0.85% NaCl with MES buffer, pH 5.7) p.o. for 3 days. Effect of Gefitinib on testosterone stimulated prostate growth in castrated animals was studied by using three groups; animals castrated 7 days earlier, animals castrated 10 days earlier and treated with testosterone esters (Sustanon, 10 mg/kg/day, s.c.) and Gefitinib (150 mg/kg/day) from day 7 to 10 after castration, animals castrated 10 days earlier and treated with testosterone esters (10 mg/kg/day) and vehicle from day 7 to 10 after castration. One hour prior to sacrifice, the animals were injected with BrdU (50 mg/kg, i.p.). One hour later the animals were anesthetized and fixed by vascular perfusion with buffered formalin solution and the ventral prostate lobes were removed, weighted, post fixed in the same fixative and embedded in paraffin.

EGFR expression was studied in the ventral prostate during castration induced prostate involution by using 4 groups; intact animals, animals castrated for 1 day, 3 days and 7 days. EGFR expression was studied in the ventral prostate during testosterone stimulated prostate growth in castrated animals by using 3 groups; animals castrated 8 days, 9 days and 10 days earlier and treated with testosterone esters (10 mg/kg/day) from day 7 to 10 after castration. At sacrifice, the prostates were frozen in liquid nitrogen to prevent RNA degradation and later used for RT-PCR or Western blot. For further details regarding anti-EGFR treatment during castration and testosterone replacement see paper II.

Androgen-independent prostate tumour model (paper III)

To construct an animal model of androgen-independent prostate cancer we used the Dunning rat AT-1 prostate cancer cell line (donated from J Isaacs, Johns Hopkins Oncology Center, Baltimore, USA) that is transplantable. An orthotopic implantation of AT-1 cells was made into the ventral prostate of normal immunocompetent rats. These AT-1 prostate cancer cells are androgen-insensitive,
poorly differentiated, fast growing, low metastatic, and were grown in culture as previously described. Adult male Copenhagen rats (Charles River, Germany) were anesthetized and an incision was made in the lower abdomen to expose the ventral prostate lobes. Then 2000 AT-1 cells in 50 μl RPMI were injected into one lobe of the ventral prostate. The contralateral ventral prostate lobe and dorsolateral prostate (DLP) served as controls and were not injected. For further details regarding the androgen-insensitive prostate tumour model see paper III.

**Combined anti-VEGFR-2 and anti-EGFR treatment (paper III)**

Rats were divided into five weight-matched groups. The first group was castrated via scrotal incision on day 7 after tumour cell injection and sacrificed 3 days later. The second group was also castrated as the first group, and in addition treated with ZD6474 (50 mg/kg, p.o.; an inhibitor of VEGFR-2, EGFR and RET signalling; donated by AstraZeneca) daily from day 7 and sacrificed 3 days later. The third group was only treated with ZD6474 for 3 days from day 7 and onwards and sacrificed at day 10. The fourth and fifth groups were left untreated and sacrificed at day 7 and day 10, respectively. One hour prior to sacrifice of the animals were injected with BrdU (50 mg/kg, i.p.) and with pimonidazole (Hypoxprobe, 100 mg/kg, i.p.; Chemicon, Temecula, CA, USA), which marks hypoxic tissue. Animals were anesthetized and perfusion fixed in paraformaldehyde. The ventral prostate, DLP, kidneys, liver, and lungs were removed and weighed. Perfusion fixated tissues were fixed by immersion for another 24 hours, dehydrated and paraffin-embedded. For further details regarding combined anti-VEGFR-2 and anti-EGFR treatment during castration see paper III.

**In Vitro Studies**

**Cell culture of AT-1 tumour cells (paper III)**

AT-1 tumour cells were grown in RPMI with 10% fetal calf serum (FCS), 50 μg/ml gentamycin, 2.5 μM dexametasone and 0.2% NaBic in 37°C, 5% CO₂. Cells were detached from the cell culture vessel with trypsin, and counted with a Bürker chamber and diluted in RPMI to appropriate volume. For further details regarding cell culture of AT-1 tumour cells see paper III.

**Dose-dependent growth inhibition of AT-1 tumour cells (paper III)**

AT-1 tumour cells were harvested and plated in a volume of 100 μl at 5000 cells/well in microtitre plates. These cells were cultured until cell growth was exponential and then ZD6474 was added to the media to inhibit cell growth. ZD6474 was diluted in cell culture media to achieve different working concentrations ranging from 0 to 30 μM, and each working concentration had 0.3% DMSO. Plates were incubated at 37°C for 72h. To quantify the cytotoxic effect of
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ZD6474, a fluorometric microculture cytotoxicity assay (FMCA) was used. It is based on the measurement of fluorescence generated from cellular hydrolysis of fluorescein diacetate (FDA) to fluorescein and fluorescence is linearly related to cell number. FDA (Sigma-Aldrich) was added to each well and plates were incubated, followed by fluorescence determination. For further details regarding dose-dependent growth inhibition of AT-1 tumour cells see paper III.

Morphologic Analysis

Apoptosis (paper I-III)
Apoptotic cells were identified in Meyer’s hematoxylin (Sigma-Aldrich) stained sections, by using standard morphological criteria, by TUNEL staining (In situ cell detection kit POD, Roche Diagnostics, Mannheim, Germany; paper I-II), or by active caspase-3 (Cell Signal Technology, MA, USA; paper III) immunohistochemistry staining. The number of TUNEL-labelled epithelial cells were measured in 1000 non-malignant (paper I, III), 1500 non-malignant (paper I), 500 non-malignant (paper II) and 1000 malignant (paper III) epithelial cells in each tissue sample. The number of TUNEL-labelled endothelial cells per blood profile was measured, and 200 (paper I) and 100 (paper II) vessel profiles per tissue sample were examined. For further details regarding morphologic analysis of apoptosis see paper I-III.

Cell proliferation (paper I-III)
Proliferating cells were immunostained with a monoclonal antibody against BrdU (Dako, Stockholm, Sweden) using biotinylated goat anti-mouse IgG and a peroxidase-labeled ABC reagent (Vector laboratories, Burlingame, CA, USA). The number of BrdU positive epithelial cells were measured in 500 non-malignant (paper I-II) and 500 malignant (paper III) epithelial cells in each tissue sample. The number of BrdU positive endothelial cells per blood profile were measured, and 200 (paper I) and 100 (paper II) vessel profiles per tissue sample were examined. For further details regarding morphologic analysis of cell proliferation see paper I-III.

Stereology (paper I-III)
Sections of the ventral prostate were stained with hematoxylin and eosin (Sigma Diagnostics, St. Louis, MO, USA) and by immunohistochemistry, and examined in a light microscope equipped with a square lattice (121 points) in the eye-piece. Volume densities (percentage of tissue volume occupied by the defined tissue compartment) were determined in hematoxylin and eosin, and immunohistochemistry stained paraffin sections from the ventral prostate using point counting morphometry as described by Weibel 201 i.e. counting the number of
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grid intersections falling on the measured tissue compartment and reference space in randomly chosen areas.

In paper II, volume densities (percentage of tissue volume occupied by the defined tissue compartment) of stroma, glandular lumen, glandular epithelium and blood vessels (blood vessel lumina + blood vessel walls) were determined at 400 X magnification. In paper I, volume densities of stroma, glandular lumen, and glandular epithelium were assessed at 100 X magnification, and of blood vessel lumina at 400 X magnification by counting hits falling on vascular lumina and on stroma. In paper III, volume density of blood vessels immunostained with antibodies against factor VIII-related antigen (Dako, Stockholm, Sweden), volume densities of tumour and normal prostate tissue in the ventral prostate (using multiple sections through different parts of the lobe), as well as the volume densities of necrotic tumour tissue and hypoxic tumour tissue (immunostained for hypoxyprobe; Chemicon), were determined.

In paper I and II, the total weight (=volume) of the different components of the ventral prostate was determined by multiplying the total lobe weight by the volume density of the respective component. In paper III, total tumour and normal prostate weight as well as the weight of viable tumour tissue (total tumour weight - tumour necrosis weight) were then estimated by multiplying the volume density with prostate weight (paper III). In these calculations we assume that the specific gravity of prostate tissue is 1.048 and that changes in tissue volume during fixation and tissue processing influence all groups in the same way. For further details regarding stereologic analysis see paper I-III.

Scoring of pEGFR staining (paper IV)

The immunoreactivity of pEGFR was evaluated without any knowledge of patient data. The pEGFR immunoreactivity was assessed by a score that combined staining intensity and distribution. Tumour epithelial cells, luminal epithelial cells and basal epithelial cells were assessed separately. Staining intensity and distribution for pEGFR was graded as 0 (no staining), 1 (predominantly unstained with smaller stained areas), 2 (stained and unstained areas are about equally large), 3 (predominantly stained tissue with smaller unstained areas), 4 (all epithelial cells are moderately stained) and 5 (all epithelial cells are strongly stained). The pEGFR staining score are the mean values of 5-8 graded samples of tumour tissue or 4 graded samples of non-malignant tissue. For further details regarding scoring of pEGFR see paper IV.
RNA Analysis

**RNA preparation (paper II-III)**
Total RNA was prepared from the rat ventral prostate (paper II) and Dunning rat AT-1 prostate cancer cells (paper III) by the TRIzol extraction method (Invitrogen, Stockholm, Sweden), according to the manufacturer's instructions. Ventral prostates and cells were homogenised with a micro dismembrator (B. Braun Biotech International GmbH, Melsungen, Germany) at 2000 rpm for 45 seconds, and chloroform and 0.8 ml Trizol was added to the homogenate. It was vortexed and incubated at room temperature for 3 minutes, and then centrifuged. The aqueous phase was isolated and isopropyl alcohol was used to precipitate the RNA. The RNA was washed with 70% ethanol and dissolved in sterile DEPC-treated water. The RNA concentrations were measured spectrophotometrically and the integrity of the RNA was assessed on a 1% agarose gel by ethidium bromide staining of 18 S and 28 S ribosomal RNA.

**cDNA preparation (paper II-III)**
Total RNA was reversed transcribed using random hexamers (Applied Biosystems, Sundbyberg, Sweden) and Superscript II reverse transcriptase (Invitrogen). All cDNA reactions were run in duplicates and post-diluted in nuclease free water. Five hundred ng total RNA was mixed with random hexamers and dNTP’s. The samples were incubated at 65°C for 5 minutes and then chilled on ice. First-strand buffer (0.1 M DTT and 20U RNAsin; Promega Madison, WI, USA) and superscript II were added. The cDNA synthesis was started with 25°C for 10 minutes followed by 42°C for 50 minutes, and ends with an inactivation step at 70°C for 15 minutes.

**Real-time quantitative PCR (paper II-III)**
Quantification of EGFR (paper II-III) and VEGFR-2 (paper III) mRNA was performed by using real-time quantitative PCR with the Light Cycler SYBR Green I technology (Roche Molecular Biochemical, Bromma, Sweden). The PCR reactions were done with 0.5 μM primers (see indicted papers for primer pair design), 3 mM MgCl₂, enzyme mix (nucleotides, Taq DNA polymerase, sybr green I dye and buffer) and cDNA in a total volume of 10 μl (paper II) and 20 μl (paper III). To confirm amplification specificity, the PCR products were subjected to a melting curve analysis and run on a 2% agarose gels. Each experimental sample was run in duplicates, and negative and positive controls were always run in parallel. The quantification data were analyzed with the LightCycler analysis Software 3.5.3 (Roche Diagnostics, Bromma, Sweden). The relative values of EGFR and VEGFR-2 mRNA in the samples are calculated from a standard curve obtained by amplification of five-fold serial dilution of reversed transcribed total RNA from a reference sample. The mRNA values are presented as percent in comparison to the reference group.
MATERIALS AND METHODS

Protein Analysis (paper II-III)

Protein from cells were extracted by using lysis buffer (0.5% NP-40, 0.5% Na DOC, 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 cocktail [Boehringer Mannheim, Germany]; paper III). Frozen ventral prostates were first homogenised by a Micro Dismembrator at 2000 rpm for 45 seconds (paper II). Then the homogenates were added to an adequate amount of lysis buffer to extract proteins and sample were mixed and incubated on ice for 30 minutes (paper II). Centrifugation was performed at 20 000 x g in 4°C for 30 minutes and the supernatants were isolated. The concentration determined by the BCA protein assay reagent kit (Pierce Chemical Co., IL, USA).

Western blot analysis (paper II-III)

In Western blot analysis, protein samples were electrophoresed on 7.5% SDS-polyacrylamide gels under reducing conditions and fractionated proteins were electrophoretically transferred onto Hybond-P PVDF membranes (Amersham Biosciences, Uppsala, Sweden). Membranes were blocked in 2% BSA (Bovine serum albumin; Sigma-Aldrich; paper II) or 5% dry milk (paper III), 0.05% Tween-20 in PBS (PBS-T) prior to incubation with sheep polyclonal antibody against EGFR (Abcam, Cambridge, UK; paper II-III), mouse monoclonal antibody against VEGFR-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; paper III) or rabbit polyclonal antibody against actin (Sigma-Aldrich). After incubation with peroxidase-conjugated secondary antibodies (Amersham Biosciences) proteins were detected using enhanced chemiluminescence detection system (Amersham Biosciences). Molecular sizes of protein bands were determined by parallel electrophoresis of molecular weight markers (Bio-Rad Laboratories AB, Sundbyberg, Sweden).

Immunohistochemistry (paper I, II, IV)

In paper I, mouse paraffin-embedded ventral prostates were stained for androgen receptor, VEGF and VEGFR-2. Sections were incubated with primary polyclonal rabbit antibodies against the androgen receptor (PG 21; Upstate, Lace Placid, NY, USA), VEGF (sc 507; Santa Cruz Biotechnology), and a rat monoclonal antibody against VEGFR-2 (sc 1147; Santa Cruz Biotechnology). The sections were washed, incubated with the secondary antibodies, and processed using a Vectastain Elite ABC kit (Vector) or a Histostain-plus kit (Zymed, San Fransisco, CA, USA) and immunoreactions were visualized by an AEC+ kit (Dako, Stockholm, Sweden). For further details regarding staining of mouse ventral prostate see paper I.
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In paper II, rat paraffin-embedded ventral prostates were stained for the EGFR and pEGFR at TYR845. Sections were incubated with primary polyclonal rabbit antibodies against EGFR (sc-03; Santa Cruz Biotechnology) and pEGFR (pEGFR TYR845; Cell Signalling, Danvers, MA, USA). Sections were washed, incubated with the secondary antibodies, and immunoreactions were visualized using the Vectastain Elite ABC kit (Vector) with DAB (Dako, Stockholm, Sweden) as chromogen. For further details regarding staining of rat ventral prostate see paper II.

In paper IV, human paraffin-embedded prostate tissues were stained for pEGFR at TYR845. Sections were incubated with a primary polyclonal rabbit antibody against pEGFR. Sections were washed, incubated with the secondary antibodies, and immunoreactions were enhanced by a catalysed signal amplification system (Dako, Corporation, Carpinteria, CA, USA) and staining was completed by incubation with DAB. For further details regarding staining of human prostate tissue see paper IV.

Statistics (paper I-IV)

Bivariate correlations were calculated using the Spearman’s rank correlation test. Correlations between nominal variables and continuous variables were analysed using the Kendall’s tau b correlation test. Patients included in survival analysis with the Kaplan-Meier and Cox regression were followed with watchful waiting. The duration of event-free survival (EFS) is defined as the time from TURP until the date of prostate cancer death, death of other causes, or if no death occurred, until the date of last follow-up. Differences in outcome between groups in the Kaplan-Meier analysis were tested with the log-rank test. The prognostic relevance of pEGFR immunoreactivity was examined by Cox regression analysis alone and together with Gleason score. Groups were compared with the Mann-Whitney U-test (paper I, II, IV) and the student t-test (paper III). Means and medians are presented ± standard deviation (SD) and probability of event-free survival (P-EFS) is presented ± standard error (SE). Quadratic regression was used for curve estimation in the FMCA. The level of statistical significance was defined as $P<0.05$ (two-sided). Statistical analysis was performed using the SPSS 14.0.0 software for Windows (SPSS Inc., Chicago, IL, USA; paper I-IV) and the statistical software Statistica 6.0 (StatSoft, Tulsa, OK, USA; paper III).
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Paper I
This study have investigated whether inhibition of VEGF with flt(1-3)IgG, which neutralizes VEGF bioactivity \(^{194,195,202}\), is able to inhibit testosterone-stimulated prostate vascular and glandular growth in castrated mice.

Treatment of castrated mice with testosterone and vehicle (IgG or PBS, gave similar results) resulted in an increase in ventral prostate lobe weight. Testosterone increased endothelial cell proliferation and vascular volume. It also increased epithelial cell proliferation and epithelial volume, and decreased epithelial cells apoptosis. All these effects of testosterone, except the induced increase in epithelial cell proliferation and differentiation, were markedly attenuated in testosterone and flt(1-3)IgG treated castrated mice.

We observed that anti-VEGF treatment inhibited the testosterone-induced increase in endothelial cell proliferation and vascular volume, and enhanced the decrease in endothelial cell apoptosis in the ventral prostate. These observations suggest that VEGF could be an important angiogenesis factor in the prostate, as it mediates testosterone-stimulated growth of prostate vasculature. However, prostate vasculature growth was not fully inhibited by anti-VEGF treatment, which indicates that other angiogenesis factors are involved.

Anti-VEGF treatment in this study also reduced testosterone-stimulated growth of the prostate epithelium. This effect could be secondary to the inhibition of prostate vascular growth, as previously observed during anti-VEGF treatment of corpus luteum growth \(^{194}\). Insufficient blood supply is known to increased apoptosis \(^{203-206}\). This suggests that the increased apoptosis of prostate epithelial cells and reduced tissue growth in the anti-VEGF treated group may be caused by insufficient blood supply. Furthermore, we observed that the testosterone-stimulated increase in epithelial cell proliferation and epithelial differentiation was unaffected by anti-VEGF treatment. This shows that anti-VEGF treatment does not affect the proliferative and differentiating effect, only with the anti-apoptotic effect of androgens in prostate epithelial cells \(^{207,208}\).

In this study the androgen receptors were localized mainly in prostate epithelial cells and in some stromal cells, and not in endothelial cells. This may suggest that the effects of testosterone on prostate endothelial cells are indirect. However, other studies have shown that the androgen receptor is present also in rodent periendothelial cells and human endothelial cells in the prostate \(^{27,30}\). In the testosterone treated and castrated animals, VEGF expression was present in the prostate epithelial cells and VEGFR-2 expression was observed in blood vessels. VEGFR-2 mediates most of the biological effects of VEGF, which includes...
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stimulation of endothelial cell proliferation and differentiation \textsuperscript{129,209}. We could not detect VEGFR-2 in the normal prostate epithelium and this suggests that the effect observed on epithelial cell apoptosis after anti-VEGF treatment is probably indirect.

This study demonstrated that testosterone stimulates the production of VEGF in the prostate epithelium and that VEGF is an important regulator of prostate vascular and glandular growth. Thus, VEGF is necessary for normal prostate growth. In addition, other studies have observed that anti-VEGF treatment can inhibit growth of experimental prostate cancers \textsuperscript{210-213}. In conclusion, VEGF play a central role in regulating prostate tissue growth in normal and malignant prostate tissue. For a more detailed results and discussion together with tables and figures, see paper I.

Paper II

Recent studies have indicated that EGFR mediate important regulatory signals in the normal and malignant prostate tissue \textsuperscript{72,189,191,214}, but the functional importance of EGFR signalling is unclear. In this study, we have located EGFR immunoreactivity both to basal and luminal epithelial cells in the rat ventral prostate. Immunoreactivity was also observed in some blood vessels, but other stroma components had low immunoreactivity. In addition, weak immunoreactivity for pEGFR was exclusively located to the prostate epithelium. These observations suggest that the main target for EGFR ligands seems to be the prostate epithelium and possibly also blood vessels. We examined if the EGFR levels in the prostate were regulated by testosterone. Both castration alone and testosterone treatment in castrated rats increased EGFR mRNA levels, EGFR protein levels and immunoreactivity of pEGFR. This suggests that EGFR could play an important regulatory role during involution and testosterone-stimulated prostate growth. We therefore inhibited EGFR with Gefitinib, a tyrosine kinase inhibitor of EGFR, during castration-induced involution and testosterone-stimulated prostate growth.

Anti-EGFR treatment for 3 days during castration did not decrease ventral prostate weight further. However, epithelial and blood vessels weights were significantly reduced compared to castration alone. This group also had decreased epithelial and endothelial cell proliferation, and increased epithelial cell apoptosis compared to castration alone. In addition, daily treatment with anti-EGFR treatment for 7 and 14 days decreased ventral prostate weight further compared to castration alone 7 and 14 days after castration. Thus, anti-EGFR treatment during castration-induced involution of the ventral prostate enhances the effects of castration, by increasing epithelial and vascular regression.

The enhanced increase in epithelial cell apoptosis and decrease in proliferation after combined anti-EGFR and castration treatment, indicate that castration induced increase in EGFR expression and signalling could be a physiological way to
counteract the effects of androgen withdrawal. In addition, castration has been shown to increase the levels of several EGFR ligands such as amphiregulin and TGF-α in the normal and malignant prostate tissue \(^{113,214}\). Several of the EGFR ligands that mediate anti-apoptotic and proliferative signals to the prostate epithelium (amphiregulin, βC, EGF, HB-EGF) are produced in the stroma \(^{52}\). This suggests that EGFR ligands produced in the stroma may counteract the effects of castration in the prostate epithelium. EGFR expression has been shown to be increased in malignant compared to non-malignant prostate tissue \(^{215}\). With this in mind and that EGFR signalling is increased during castration, it seems rational that anti-EGFR treatment could be used to enhance the effects of castration in prostate tumours. A recent study has shown that anti-EGFR treatment act in synergy with castration to inhibit growth of androgen-independent prostate cancer xenografts \(^{214}\). In this study, we observed that anti-EGFR treatment enhances the vascular regression during castration. As the immunoreactivity of EGFR and pEGFR are low in prostate blood vessels, it is more likely that EGFR signalling affect blood vessels indirectly by stimulating the production of angiogenesis factors. Anti-EGFR treatment could then affect the prostate vasculature indirectly by decreasing the production of angiogenesis factors like bFGF, IL-8, TGF-α and VEGF in the prostate epithelium \(^{72}\).

Castrated animals were also treated with anti-EGFR treatment during testosterone-stimulated prostate growth and this inhibited, but did not fully prevent testosterone-stimulated prostate growth. This resulted in an inhibited increase in ventral prostate weight, epithelial weight and blood vessel weight compared to the testosterone and vehicle-treated group. These inhibitory effects in the anti-EGFR treated-group were also accompanied by an inhibited increase in epithelial and endothelial cell proliferation, and an enhanced epithelial cell apoptosis. Thus, anti-EGFR treatment inhibits testosterone-stimulated ventral prostate growth by inhibiting vascular and epithelial regrowth, although the animals received as high dose of testosterone esters as 10 mg/kg/day.

In conclusion, our results in this study and previous results suggest that anti-EGFR treatment (targeting mainly the epithelium; paper II) and anti-VEGF treatment (targeting mainly the vasculature; paper I) may act in synergy to inhibit normal prostate and prostate tumour growth. For a more detailed results and discussion together with tables and figures, see paper II.

**Paper III**

We have developed an animal model, where androgen-independent AT-1 rat prostate cancer cells are grown orthotopically within the normal prostate in immunocompetent rats \(^{53}\). This model have been used to show that castration causes apoptosis and hypoxia among AR negative AT-1 prostate tumour cells, by reducing
blood flow in the surrounding non-malignant prostate tissue. The present study showed that a drug (ZD6474; inhibits VEGFR-2, EGFR and RET) that targets the vasculature in the tumour and the surrounding non-malignant prostate tissue could partly mimic and enhance the effects of castration.

Castration of the orthotopic model of androgen-independent prostate cancer significantly decreased the viable tumour weight (total tumour weight – tumour necrosis weight) compared to intact controls. This suggests that the androgen-independent AT-1 tumour cells respond to castration when growing in an androgen-dependent environment. Castration caused significantly decreased vascular density in the non-malignant prostate tissue surrounding the tumour, but no vascular response was seen in the tumour. This decrease in vascular density may have caused the observed increase in tumour hypoxia. Furthermore, apoptosis was significantly increased after castration in the tumour, the surrounding non-malignant prostate tissue (ipsilateral ventral prostate lobe) and the contralateral ventral prostate lobe (lobe without tumour cells).

Treatment of the orthotopic model with ZD6474 reduced the tumour weight compared to intact controls. However, the viable tumour weight was not significantly increased compared to 3 days earlier. Vascular densities were decreased by ZD6474 treatment in the tumour and in the surrounding non-malignant prostate tissue compared to intact controls, but the tumour hypoxia was not increased. This treatment also caused increased tumour cells apoptosis, but tumour cell proliferation was unaffected. However, the vascular density in the contralateral ventral prostate lobe was not affected by ZD6474 treatment.

Treatment with ZD6474 decreased vascular density in the tumour and in the surrounding non-malignant prostate tissue. These results suggest that blood vessels in the tumour and normal prostate are dependent on signalling through VEGFR-2, which are present on prostate blood vessels. ZD6474 may also affect AT-1 tumour cells directly by inhibiting VEGFR-2 and EGFR, which are expressed on AT-1 tumour cells. Results in this study from reverse transcription-PCR and western blotting showed that AT-1 tumour cells express both VEGFR-2 and EGFR. However, the IC$_{50}$ values for ZD6474 to inhibit proliferation of AT-1 tumour cells (paper III) and proliferation of endothelial cells, and the peak plasma concentration at doses examined (50mg/kg; unpublished observations) suggested that ZD6474 acts mainly by vascular effects in vivo.

The effects of ZD6474 treatment were compared with castration treatment and both of these treatments had similar inhibitory effect on tumour growth (similar tumour weight inhibition), although some differences were observed. ZD6474 decreased vascular densities more in the tumour and tumour cell proliferation was higher in ZD6474-treated animals, compared to castrated animals. Tumour cells apoptosis,
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hypoxia and necrosis were similar in both groups. The major difference between these two treatments groups were the response in the non-malignant prostate tissue surrounding the tumour. Castration resulted as expected in prostate epithelial and vascular involution, whereas ZD6474 had almost no effect on the non-malignant tissue, except a decrease in vascular density in the surrounding non-malignant prostate tissue.

In this orthotopic model, castration only targets the vasculature in the non-malignant prostate tissue surrounding the tumour \(^{44,53}\). The tumour vasculature was unaffected after castration, because AT-1 cells secrete angiogenic factors like VEGF independent of androgens \(^{219}\). On the other hand, ZD6474 targets the tumour vasculature and the non-malignant prostate vasculature directly by inhibiting the effects of locally produced EGFR ligands and VEGF.

The most pronounced anti-tumour effect was seen when ZD6474 treatment and castration were combined, probably because these two treatments work through different mechanisms (as described above). Combined treatment was significantly more effective in increasing tumour cell apoptosis, tumour cell hypoxia, and tumour cell necrosis, and in decreasing tumour vascular density compared to ZD6474 treatment or castration alone. However, combined treatment did not reduce tumour weight compared to ZD6474 treatment or castration alone, possibly because of the short (3 days) treatment period. In conclusion, this study suggests that combined treatment with castration and ZD6474 could be an effective way to treat androgen-independent prostate tumours. For a more detailed results and discussion together with tables and figures, see paper III.

**Paper IV**

Studies have shown that high tumour EGFR expression are significantly associated with high Gleason grade, advanced tumour stage, and high risk for prostate-specific antigen recurrence \(^{98-102}\). To our knowledge, pEGFR immunoreactivity has previously not been studied in prostate cancer, but studies in non-small cell lung cancer and breast cancer patients have shown that high immunoreactivity of pEGFR in tumours are associated with poor outcome \(^{108-110}\).

Patients with high tumour or high non-malignant luminal pEGFR had significantly shorter cancer specific survival than patients with low tumour or low non-malignant luminal pEGFR. In addition, high tumour or high non-malignant luminal pEGFR in patients with Gleason score 6 or 7 and in patients with Gleason score 6 had significantly shorter cancer specific survival than those with low tumour or low non-malignant luminal pEGFR in the subgroups. Multivariate Cox regression analyses which included Gleason score showed that tumour pEGFR and non-


malignant luminal pEGFR were independent prognostic markers from Gleason score. Thus, high immunoreactivity of pEGFR at TYR845 in prostate epithelial cells, both in the tumour and adjacent non-malignant tissue, was significantly associated with shorter cancer-specific survival for prostate cancer patients. Phosphorylation of EGFR at TYR845 is mediated by c-Src, which is involved in the regulation of the receptor function and prostate tumour progression. Aberrant c-Src activity promotes prostate cancer progression and androgen-independent growth. Furthermore, pEGFR immunoreactivity could independently predict prostate cancer outcome in a subgroup of patients with GS 6–7 tumours and in a subgroup of patients with GS 6 tumours. The results in this study indicate that pEGFR may possible become a useful prognostic marker for prostate cancer patients, when validated in other patient cohorts. This marker could also be used to select patients which could benefit from anti-EGFR treatment or anti-Src treatment.

The immunoreactivity of pEGFR in prostate tumour epithelial cells and non-malignant epithelial cells were correlated with each other, Gleason score, metastasis, tumour size, and tumour cell proliferation. Tumour pEGFR immunoreactivity was also correlated with tumour vascular density. Patients with metastases had significantly higher expression for tumour and non-malignant epithelial pEGFR compared to patients without metastases.

Cut-offs for pEGFR was obtained on the basis of ROC curve analysis and the cut-offs used in the survival analyses were 2.78 (high tumour pEGFR $\geq 2.78$) and 2.88 (high non-malignant epithelial EGFR $\geq 2.88$) for tumour and non-malignant epithelial cell staining, respectively. The cut-off could however also be set to 3 with a similar ability to separate cases with different prognosis. Thus, prostate cancer patients in which most of or all the tumour or non-malignant epithelial cells are immunoreactive for pEGFR have poor survival. This fact makes pEGFR a practically useful marker, as it can rather easily be scored in a microscope.

Only a few studies have been done where prognostic markers were observed in the normal tissue surrounding a prostate tumour. It’s important to define changes in the non-malignant tissue adjacent to tumours, because these changes could be of diagnostic importance and diagnostic biopsies often sample only non-malignant prostatic tissue even when tumours are present. Immunoreactivity of pEGFR could possibly be used to classify prostate cancer patients with negative biopsies (no tumour tissue present) according to their need for close follow-up. Tumours may secret EGFR ligands that cause high pEGFR in the surrounding non-malignant tissue. A recent study in experimental animals, have shown that the tumours increase the vascular density of the surrounding non-malignant prostate tissue and this indicates that the tumour stimulates the blood supply in their surrounding. Furthermore, pEGFR levels in the non-malignant tissue are
positively correlated with tumour size (paper IV), and factors like PTEN, p27 and Ki-67 are altered in normal glands close to prostate tumours 221. These results indicate that non-malignant tissue adjacent to prostate tumours cannot be classified as normal 53,180,220-222. Therefore, we suggest that it could be called “Tumour Indicating Normal Tissue” (TINT) and further studies are needed to define TINT. In conclusion, changes in TINT influence outcome and when more closely defined could be a potential new target for therapy. For a more detailed results and discussion together with tables and figures, see paper IV.
CONCLUSIONS

- Testosterone stimulates vascular growth in the rat ventral prostate lobe indirectly by increasing epithelial VEGF synthesis and VEGF is a necessary component in testosterone-stimulated prostate growth.

- Increased EGFR signalling after castration mediates stimulatory effects that balance castration-induced prostate regression and EGFR signalling is a necessary component in testosterone-stimulated prostate growth.

- Anti-vascular treatment with an inhibitor of VEGFR2 and EGFR, that targets the vasculature in the androgen-insensitive prostate tumour and the surrounding non-malignant prostate tissue, partly mimics and even enhances the effects of castration.

- Treatment with an inhibitor of VEGFR2 and EGFR in combination with castration treatment could be a particularly effective way to treat androgen-insensitive prostate tumours.

- Low pEGFR immunoreactivity in malignant and non-malignant prostate tissue is significantly associated to good prognosis in prostate cancer patients, and pEGFR levels may provide information about which patients with Gleason 6 and 7 tumours that will survive their cancer even without treatment.

- Tumours may influence the surrounding non-malignant tissue and pEGFR immunoreactivity in the morphologically normal prostate tissue can be used to retrieve prognostic information.
It is important to understand the androgen controlled regulatory systems in the normal prostate and in prostate cancer. Increased knowledge in this field will probably give rise to new therapeutic targets and prognostic markers for prostate cancer patients.

One of the biggest challenges in prostate cancer research is to find a way to cure patients with metastatic prostate cancer. Today, treatment of metastatic prostate cancer is castration therapy, which only temporarily prevents the disease from killing the patient. Unfortunately, prostate metastases will start to grow independent of circulating testosterone and become castration-resistant, \(^{17,119}\). The exact mechanism behind castration therapy, which causes the normal prostate and hopefully also prostate tumours (primary tumours and metastases) to regress or temporarily inhibit further tumour growth, is however incompletely understood \(^{15,16}\). When trying to understand the effects of castration, or rather the lack of effect in castration-resistant prostate tumours, it is useful to start by elucidating how castration works in the normal prostate. With this knowledge at hand, it may be possible to boost the local regulatory mechanisms responsible for castration–induced tissue regression. It is also possible to more exactly combine and time castration treatment with other therapies like radiotherapy and chemotherapy, in order to prolong life or even cure the patient.

We have studied prostate growth control by investigating the role of VEGF and EGFR in the prostate. Both are normally expressed in the prostate and regulated by testosterone \(^{38,113,114}\), but in castration–resistant prostate cancer they may be more or less independent of androgens \(^{105-107,219}\). Our studies have shown that EGFR synthesis and activation are regulated by testosterone. Furthermore, we showed that EGFR is a necessary component in testosterone-stimulated prostate growth. EGFR signalling after castration seems to be a stimulatory counteracting system during prostate regression and this signalling is active weeks after castration. These results show that EGFR is important in prostate growth control and therefore anti-EGFR treatment could possibly be used to inhibit normal prostate growth, for example in BPH. EGFR should be further studied in prostate tumours, by investigating if anti-EGFR treatment in combination with castration could enhance the effects of castration. When treating patients with anti-EGFR therapies, it could be useful to select patients for this therapy by evaluating their immunoreactivity of pEGFR in the tumour and non-malignant prostate tissue. Castration leads to involution of the prostate, but the prostate do not disappear and at certain size the involution stops \(^{15,16}\). Obviously growth factor systems, such as the EGFR system, are active after castration although there are low levels of androgen in the blood. These results
demonstrated that the EGFR system should be further investigated in the normal prostate and prostate tumours. It is not fully elucidated how EGFR and its ligands are regulated by androgens, where they are synthesised in the prostate and how the system functions in prostate tumours. EGFR ligands, such as TGF\(\alpha\) and amphiregulin, are up regulated by testosterone in the normal prostate and prostate tumours \(^{113,214}\). Studies have also indicated that TGF\(\alpha\) is the dominant EGFR ligand in prostate cancer \(^{74}\) and HER2 may be a prognostic marker for prostate cancer patients \(^{89,223}\). It also important to study the EGFR system in metastases and bone tissue, as it is not the primary tumour that kills the patients but the metastases.

We have showed that normal prostate tissue growth is angiogenesis dependent and that VEGF is a necessary component in testosterone-stimulated prostate growth. Furthermore, we have demonstrated that anti-VEGF treatment during testosterone-stimulated prostate growth resulted in inhibited vascular growth and increased epithelial cell apoptosis, but epithelial cell proliferation was unaffected. One can speculate about what would happen if these animals were treated for a long time with anti-VEGF or other anti-angiogenic treatment during testosterone-stimulated prostate growth. The proliferation of prostate epithelial cells was rather high and was unaffected by the anti-angiogenic treatment, possibly the animals could develop dysplasia or even cancer depending on the treatment duration. Never the less, our finding clearly suggests that prostate tissue growth can be inhibited by anti-angiogenic treatment. This effect could possibly be used to treat both BPH and prostate cancer. However, more studies are needed to elucidate the relative importance of VEGF and other angiogenic factors in prostate cancer, both in primary tumours and metastases. Probably more factors are involved that are important in prostate tumour angiogenesis, for example the EGFR.

In cancer research, tumours are often the focus of interest but a few studies including our studies have shown that tumours may influence the surrounding normal tissue \(^{53,180,220,221}\). The non-malignant tissue could then provide prognostic markers for prostate cancer. Tumours may for example secrete factors that influence the “soil” by increasing the blood vessel density and thereby increase the blood flow through the surrounding tissue \(^{53,221}\). Adaptations in the surrounding tissue can thus serve both as targets for therapy and diagnostic markers. In line with this, phosphorylated activated protein kinase (pAKT) immunoreactivity in non-malignant prostate tissue has shown to be a prognostic marker for prostate cancer outcome \(^{220,221}\). Experimental tumours alter the morphology of the surrounding normal prostate tissue \(^{53}\), and factors like PTEN, p27 and Ki-67 are altered in normal glands close to prostate tumours \(^{221}\). Additional studies are therefore needed to explore how tumour affects the surrounding normal tissue.
In an orthotopic animal model of androgen-independent prostate cancer we demonstrated that combined inhibition of VEGFR-2 and EGFR with ZD6474, which targets the vasculature in the tumour and surrounding non-malignant tissue, could partly mimic and enhance the effects of castration. These results show that the vasculature in the surrounding non-malignant tissue is a target for therapy and could possibly be widely used in anti-tumour therapy. This treatment could affect androgen-insensitive tumours growing in an androgen-dependent environment. Probably the EGFR inhibition targeted mainly the non-malignant prostate epithelium and possibly also the prostate tumour cells. The VEGFR-2 inhibition probably targeted mainly the vasculature in the tumour and surrounding non-malignant tissue. These results suggest that combined treatment with ZD6474 and castration could be used to enhance the castration effects on human androgen-independent prostate tumours. The time-frame is important and probably the best effect is achieved by combining castration and ZD6474, and treatment with ZD6474 added later will probably not achieve the same effect. However, additional studies are needed to elucidate how metastases and the surrounding bone tissue are affected by this treatment. Animal models with bone metastases that relapse will be good model systems to study combined treatment with castration, anti-VEGFR-2 and anti-EGFR treatment.

One of the other big challenges in prostate cancer research is to find new prognostic markers. Today, the majority of the localised tumours are graded as GS 6 or 7 and these patients have variable and unpredictable outcome \cite{175}. Thus, it is difficult to treat these patients and many will be over treated with crippling therapies, and this in turn will lead to under-treatment of those that need intensive therapy. There is no imaging method available that detects prostate cancer and therefore biopsies often sample only non-malignant prostate tissue, also when a tumour is present \cite{180}. Furthermore, biopsies that contain prostate tumours are often undergraded because only 1/1000 of the prostate is sampled.

We have shown that low pEGFR immunoreactivity in prostate tumours and non-malignant prostate tissue is significantly associated with good prognosis for prostate cancer patients. This marker is even useful for patients with Gleason score 6 and 7 tumours. However, this prognostic marker should be verified in other cohorts where patients were followed with watchful waiting and other treatments, before it can be used in the clinic. Studies should also be made where the importance of pEGFR levels are clarified, probably the levels of pEGFR give some advantage for prostate tumours as the prognosis are affected by the pEGFR immunoreactivity. As EGFR is phosphorylated at TYR845 and this is a c-Src dependent phosphorylation site, it is possible that Src-inhibitors could reduce the relative risk associated with high pEGFR immunoreactivity in prostate cancer patients. The EGFR system is important in prostate cancer and therefore additional prognostic markers could possibly be
found in this system. It is also important to elucidate the EGFR ligands that cause the high pEGFR levels during prostate cancer and then use them as therapeutic targets.

The fact that tumours may influence the surrounding non-malignant tissue may be of major diagnostic (and therapeutic, see above) importance. Prostate biopsies generally contain non-malignant tissue and this tissue could be used to identify high-risk patients, where additional biopsies are required or early treatment is needed. The morphologically normal tissue surrounding a prostate tumour can not be classified as normal \textsuperscript{180,220,221} and we suggest that it could instead be called “Tumour Indicating Normal Tissue” (TINT). Additional studies are needed to define TINT, which could be used in prostate cancer diagnosis, prognosis and in finding potential new targets for therapy.
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