SAARA LEHMUSVAARA

Traditional and Novel Treatments for Prostate Cancer

Discoveries at the molecular level

ACADEMIC DISSERTATION
To be presented, with the permission of
the board of Institute of Biomedical Technology of the University of Tampere,
for public discussion in the Jarmo Visakorpi Auditorium,
of the Arvo Building, Lääkärinkatu 1, Tampere,
on June 28th, 2012, at 12 o'clock.

UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION
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www.uta.fi/taju
http://granum.uta.fi

Cover design by
Mikko Reinikka

Acta Universitatis Tamperensis 1745
ISBN 978-951-44-8844-3 (print)
ISSN-L 1455-1616
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 1217
ISBN 978-951-44-8845-0 (pdf)
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2012

Yhteenveto

Eturauhassyövän tehokas hoito ei ole ongelmatonta. Vaikka suurin osa eturauhassyövistä voidaan hoitaa leikkaus- tai sädehoidolla, silti eturauhassyöpä aiheuttaa teollisuusmaiden miehillä toiseksi eniten syöpäkuolemia. Syöpäkuolemien suuri määrä johtuu eturauhassyövän yleisyydestä – se on miesten yleisin syöpä – sekä tehokkaan hoidon puuttumisesta etäpesäkkeitä lähettävään eli metastasoivaan eturauhassyöpään.

Eturauhassyövän tappava muoto on aggressiivinen ja metastasoiva. Tätä muotoa vastaan on tehokkain endokriininen hoito, vaikka silläkään ei voi parantaa potilasta. Metastasoitunut eturauhassyöpä kehittää resistenssin hoitoa vastaan keskimäärin 18–24 kuukaudessa. Hoitoresistenttiyden syntymisen jälkeen potilaan odotettavissa oleva elinaika on enää keskimäärin 20 kuukautta. Intensiivisestä tutkimuksesta huolimatta resistenssin syntymiseen johtavat molekyylitason tapahtumat ovat yhä osittain epäselviä. Kun ymmärrämme paremmin näitä molekyylitason tapahtumia, voimme kehittää tehokkaampia hoitomenetelmiä eturauhassyöpään ja parantaa potilaiden selviytymistä.

Tämän väitöskirjatutkimuksen tarkoituksena oli määrittää kahden yleisesti eturauhassyövän hoidossa käytetyn endokriinisen hoidon molekyylitason vaikutuksia eturauhassyöpäkudoksessa. Hyödynsimme harvinaista kliinistä materiaalia 28 eturauhassyöpäpotilaasta, jotka olivat saaneet endokriinistä hoitoa neoadjuvantisti ennen eturauhasen leikkaushoitoa. Tästä materiaalista määritimme kaikkien tunnettujen proteiinia koodaavien geenien ja yli 700 mikroRNA-geenin ilmentymistasot cDNA-mikrosirutekniikalla. Lisäksi määritimme geenien syöpäkudosspesifiset ilmentymistasot heterogeenisistä näytteistä hyödyntämällä Bayesin kaavaan perustuvaa *in silico* -mallinnusmenetelmää.

Harvinaisen potilasaineiston ansiosta pääsimme tutkimaan kudosnäytteistä ilmiöitä, joita aiemmin on tutkittu pääasiassa soluviljelyolosuhteissa tai koe-eläinmalleissa. Vaikka kliinisellä tasolla tutkimamme endokriinisten hoitojen, GnRH-agonistihoidon ja antiandrogeenihoidon, välillä ei havaita eroja, geenien

ilmentymisen tasolla erot olivat huomattavat. Määritimme myös eturauhassyövän yleisimmän geenifuusion, *TMPRSS2:ERG*-fuusion, yleisyyden näytemateriaalissamme ja tutkimme, miten endokriininen hoito vaikuttaa fuusion säätelemien geenien ilmentymiseen syöpäkudoksessa. Hoitamattomassa kontrolliryhmässä geenifuusion huomattiin lisäävän solujen kasvuun vaikuttavien geenien ilmentymistä. Endokriininen hoito näytti kuitenkin hiljentävän näiden geenien ilmentymistä ja täten vähentävän eroja fuusiopositiivisten ja fuusionegatiivisten syöpien välillä.

Lisäksi tutkimuksessa karakterisoimme hoitoihin voimakkaimmin vaikuttavien geenien suoraa riippuvuutta androgeenireseptorivälitteisestä säätelystä sekä niiden syöpäkudosspesifistä ilmentymistä. Löysimme useita mikroRNA-geenejä ja kaksi proteiinia koodaavaa geeniä (*NEDD4L* ja *TPD52*), jotka osoittautuivat lupaaviksi hoidon tehokkuuden ja resistenttiyden synnyn indikaattoreiksi. Näiden geenien todellinen käyttökelpoisuus biomarkkereina selviää kuitenkin vasta lisätutkimuksilla.

Lopuksi tehostimme virusvälitteistä geeninsiirtoa eturauhassyöpä- ja muihin syöpäsoluihin uusilla kationisilla peptideillä ja muilla pienillä kationisilla molekyyleillä. Mikäli geeninsiirto saataisiin kyllin tehokkaaksi, virusvälitteisellä geeniterapialla voitaisiin mahdollisesti hoitaa eturauhassyöpää sekä muita syöpiä. Tutkimuksessa havaitsimme kuitenkin, etteivät kationiset peptidit olleet pieniä kationisia molekyylejä tehokkaampia soluviljelykokeissa. Tarvitsemme siis vielä lisätutkimuksia löytääksemme kyllin tehokkaan geeninsiirtomenetelmän syövän geeniterapiaan.

Tämä väitöskirjatutkimus antaa arvokasta uutta tietoa eturauhassyövän molekyylitason ilmiöistä ja osaltaan edesauttaa tehokkaampien hoitomuotojen kehittämistä.

Contents

List of original publications

This thesis is based on the following original articles, which are referred by the corresponding Roman numerals.

I Lehmusvaara S, Erkkilä T, Urbanucci A, Waltering K, Seppälä J, Larjo A, Tuominen VJ, Isola J, Kujala P, Lähdesmäki H, Kaipia A, Tammela TLJ, and Visakorpi T (2012): Chemical castration and antiandrogens induce differential gene expression in prostate cancer. J Pathol (published online, DOI 10.1002/path.4027)

II Lehmusvaara S, Erkkilä T, Urbanucci A, Jalava SE, Seppälä J, Kaipia A, Kujala P, Lähdesmäki H, Tammela TLJ, and Visakorpi T (2012): Goserelin and bicalutamide treatments alter the expression of microRNAs in the prostate. Prostate (in press)

III Lehmusvaara S, Rautsi O, Hakkarainen T, and Wahlfors J. (2006): Utility of cell-permeable peptides for enhancement of virus-mediated gene transfer to human tumor cells. Biotechniques 40; 573-574, 576.

Abbreviations

Ad	Adenovirus
AICDA	Activation induced cytidine deaminase
AIF	Apoptosis inducing factor
AKR1C3	Aldo-keto reductase family 1, member C3
AKT	V-akt murine thymoma viral oncogene homolog 1
ALD	Adrenoleukodystrophy
AMACR	Alpha-methylacyl-CoA racemase
Antp	Antennapedia homeodomain
APC	Antigen presenting cell
AR	Androgen receptor
ARBS	Androgen receptor binding site
ARE	Androgen responsive element
ChIP	Chromatin immunoprecipitation
CLL	Chronic lymphocytic leukemia
CPP	Cell penetrating peptide
CRAd	Conditionally replicative adenovirus
CRPC	Castration resistant prostate cancer
CTC	Circulating tumor cell
CYP51	Cytochrome P450 C51
CYP17	Cytochrome P450 C17
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DNA-PKc	DNA-dependent protein kinase complex
EIF3SH	Eukaryotic translation initiation factor 3, subunit H
EML4	Echinoderm microtubule-associated protein-like 4
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
FDA	Food and Drug Administration

FC	Fold change
FDHT	18F-fluoro-5α-dihydrotestosterone
FDR	False discovery rate
FISH	Fluorescence *in situ* hybridization
FSH	Follicle-stimulating hormone
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GnRH	Gonadotropin releasing-hormone
GR	Glucocorticoid receptor
HDAC	Histone deacetylase
HSP	Heat shock protein
IHC	Immunohistochemistry
LBD	Ligand binding domain
LH	Luteinizing hormone
LTR	Long terminal repeat
MAB	Maximum androgen blockage
MET	Mesenchymal-to-epithelial transition
miRNA	MicroRNA
MOI	Multiplicity of infection
NCOA2	Nuclear receptor coactivator 2
NCOR1	Nuclear receptor corepressor 1
NCOR2	Nuclear receptor corepressor 2
NKX3-1	NK3 homeobox 1
NSCLC	Non-small-cell lung cancer
NTD	N-terminal domain
PAP	Prostatic acid phosphatase (also called ACPP)
PARP1	Poly (ADP-ribose) polymerase 1
PBMC	Peripheral blood mononuclear cells
PDCD4	Programmed cell death 4
PI3K	phosphoinositide 3-kinase
PIN	Prostatic intraepithelial neoplasia
PLAU	Plasminogen activator
PR	Progesterone receptor
PSA	Prostate specific antigen
RB1	Retinoblastoma 1

RNP	Ribonucleoprotein
seq	Sequencing
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SRD5A3	Steroid 5α-reductase 3
TOP2B	Topoisomerase II beta
TPD52	Tumor protein D52
TSS	Transcription start site
VEGF	Vascular endothelial growth factor
VSV-G	Vesicular stomatitis virus glycoprotein

Abstract

The efficient treatment of prostate cancer faces several challenges. Although an overwhelming majority of all prostate cancers can be efficiently treated by radical prostatectomy or radiation therapy, prostate cancer remains one of the main cancer killers in the Western world. The mortality rate is high for two reasons: prostate cancer has the most frequent occurrence of all cancers affecting men, and effective treatments against metastatic prostate cancer do not exist.

The lethal form of prostate cancer is aggressive and metastasizes to other tissues. For this form, the most efficient treatment is endocrine therapy. However, this therapy is not curative. Resistance against treatment develops in an average of 18-24 months, and after resistance has developed, the mean overall survival time of patients with metastatic prostate cancer is only 20 months. Despite intensive studies, the molecular mechanisms leading to resistance to endocrine therapy remain obscure. The understanding of these molecular mechanisms will enhance the development of more efficient treatment methods and will improve the survival of prostate cancer patients.

This study aimed to determine the molecular consequences of the two most commonly used endocrine therapies for prostate cancer. We utilized rare clinical material from 28 prostate cancer patients who had undergone neoadjuvant endocrine therapy and analyzed the expression levels of all known protein-coding genes and over 700 miRNAs from the prostate cancer samples by microarray. Furthermore, we determined the cancer-specific gene expression levels from heterogeneous prostate tissue samples using an *in silico* Bayesian modeling tool.

The rare clinical material enabled us to study events that have previously been studied using mainly *in vitro* or animal models. We detected great differences in transcriptome levels between the two endocrine therapies, GnRH agonist and antiandrogens, despite their similar clinical outcomes. In addition, we determined the frequency of the most common fusion gene in prostate cancer, *TMPRSS2:ERG*, from the samples and determined how the endocrine treatment affected the ERG-regulated

genes. In non-treated patients, the *TMPRSS2:ERG* fusion enhanced the expression of proliferation-related genes. Interestingly, the endocrine therapies reduced the expression of these genes and diminished the differences between fusion-positive and fusion-negative samples.

In addition, we characterized possible androgen receptor dependent regulation and cancer specificity of the most differently expressed genes after endocrine therapy. Several miRNAs and two protein-coding genes (*NEDD4L* and *TPD52*) showed their potential as prognostic biomarkers for the formation of treatment resistance. However, more studies are needed to explore their potency fully.

The last part of the study explored the capacity of novel polycationic peptides to enhance the transduction of viral gene transfer vectors into prostate and other cancer cells. With optimized transduction efficiency, viral gene therapy could be used as a novel treatment method for prostate and other cancers. However, our study revealed that polycationic peptides were not more efficient than polybrene and protamine sulphate, which are the small cationic compounds traditionally used in *in vitro* cell culture models. Thus, more studies with different approaches are needed to obtain clinically sufficient gene transfer efficiency with the current viral vectors.

This study provides valuable, novel information regarding the transcriptional events in prostate cancer and can assist in the development of more effective treatment methods for prostate cancer.

1. Introduction

Compared to other common cancer types, prostate cancer has several unique characteristics. For example, only little is known about the causes and risk factors of prostate cancer. Age, ethnicity and family history increase the risk of prostate cancer, whereas the effects of certain diets, amount of exercise, sexual activity, overweight or smoking have not been noted to significantly affect prostate cancer appearance (Damber and Aus, 2008). Lack of androgens is known to protect men from prostate cancer; however, controversially, a high amount of androgens does not increase the risk compared to a low amount of androgens (Isaacs, 1994).

Treatment of prostate cancer has been problematic in many cases. Unlike with breast cancer, for example, genetic markers that would predict the aggressiveness of prostate cancer or could provide guidance for optimal treatment do not exist. Although localized prostate cancer can be treated highly effectively with radical prostatectomy or radiation therapy, prostate cancer remains the second highest cause of cancer deaths in men in Finland and other parts of Western world. A reason for this is the lack of effective treatment methods against aggressive and metastatic prostate cancer. Prostate cancer is a highly hormone-sensitive malignancy; therefore, hormone ablation therapy is an exceedingly effective treatment, even for advanced prostate cancer. Unfortunately, hormonal therapy is never curative, and on average, metastasized cancer progresses to castration resistant prostate cancer (CRPC) in 18-24 months (Seruga et al., 2011).

A better understanding of the molecular mechanisms behind prostate cancer initiation, progression, and the formation of resistance to treatments can lead to the development of novel and more effective treatment methods. Completely new approaches, such as immune and gene therapy, may also provide future benefits.

2. Review of the literature

2.1 Prostate cancer

Prostate cancer is a disease of elderly men, as the median age of diagnosis is 71 (National Cancer Institute, Surveillance, Epidemiology and End Results (SEER) Program, USA, http://seer.cancer.gov/publications/prostate/inc_mort.pdf). In the developed countries, the lifetime risk of getting prostate cancer is 16%, meaning that every sixth man will be diagnosed with prostate cancer (Jemal et al., 2010). Approximately 650 000 new cases are diagnosed and 136 000 men die from prostate cancer annually in the developed countries (Jemal et al., 2011). In Finland in 2010, 4712 new cases were diagnosed and 847 men died from prostate cancer (Finnish Cancer Registry, www.cancerregistry.fi). While incidence has increased in all developed countries, mortality has remained the same or has decreased slightly during the last decade (Jemal et al., 2010). Approximately 90% of newly diagnosed prostate cancers are localized or regional, for which the 5-year relative survival approaches 100% (Jemal et al., 2010). However, for the patients whose cancer has metastasized at the time of diagnosis, the 5-year survival rate is only 30%.

2.1.1 Diagnosis of prostate cancer

If prostate cancer is suspected, such as due to elevated serum prostate specific antigen (PSA) levels, a patient normally undergoes digital rectal examination, from which local staging (T staging) of prostate cancer is assessed. Further, a biopsy sample from the prostate confirms the presence or absence of prostate cancer. According to the biopsy sample, the pathological stage and Gleason score are defined (Epstein, 2010). The diagnosis of prostate cancer is always based on histological examination, and the treatment decision is based on the level of PSA, T stage and pathological findings. If the cancer is suspected to be metastatic, magnetic

resonance imaging and bone scans can be used to identify the possible metastasis (Heidenreich et al., 2011; van der Kwast et al., 2003).

2.1.2 Challenges in the treatment of prostate cancer

2.1.2.1 Localized prostate cancer

Studies of prostate specimens from healthy men show that 30% of men in their 30s' and 50% of men in their 50s' harbor asymptomatic foci of prostate cancer (Sakr et al., 1994; Shen and Abate-Shen, 2010). These findings indicate that the development of prostate cancer is a slow process, and many prostate cancers never progress to clinically detectable disease. Today, due to the widely used PSA testing, almost one-half of diagnosed prostate cancers pose a low risk of progression over 15-20 years (Klotz, 2010). The great majority of these cancers never develop to life-threatening disease, but some of them do (Albertsen et al., 2005; Lu-Yao et al., 2009). At present, it remains clinically challenging to avoid overtreatment and to distinguish patients that need treatment from those who do not.

2.1.2.2 Metastasized prostate cancer

Approximately 35% of all prostate cancer patients will experience a rise in PSA levels in 10 years after radical prostatectomy, and with a median time of eight years, 34% of those will develop metastatic disease (Pound et al., 1999). The most frequent site of metastases from prostate cancer is bone (up to 90% of the cases), the second is lung (45-53% of the cases) and the third is liver (25-30% of the cases) (Bubendorf et al., 2000). The lung and liver are rarely the sole metastatic sites (Saitoh et al., 1984). While other solid cancers that metastasize into bone usually form osteolytic (bone-lysing) lesions, prostate cancer metastasis typically form osteoblastic (bone-forming) lesions (Ibrahim et al., 2010; Logothetis and Lin, 2005).

To form a metastasis, numerous changes need to occur in a tumor cell. It is known that the process known as epithelial-to-mesenchymal transition (EMT) is required in cancer cells, and several pathways, such as the phosphoinositide 3-kinase/ v-akt murine thymoma viral oncogene homolog 1 (PI3K/Akt) and Wnt/β-

catenin pathways are involved (reviewed by Ibrahim et al., 2010; Jin et al., 2011). However, a more detailed picture is still lacking. In addition, the question of why prostate cancer cells bind to human bone marrow endothelial cells with higher affinity than to other endothelial cells remains unanswered. It has been shown that both osteoclasts and prostate cancer cells express certain integrins and chemoattractive factors, and this expression may help prostate cancer cells to migrate to the bone matrix (reviewed by Ibrahim et al., 2010; Jin et al., 2011), but a more detailed picture is needed. The understanding of the molecular mechanisms behind metastasis formation and bone tropism in prostate cancers is another challenge in the treatment of prostate cancer.

2.1.2.3 Castration resistant prostate cancer

Prostate cancer is a highly androgen dependent disease, and therefore, androgen ablation therapy either with physical or chemical castration, is an effective treatment against prostate cancer (Palmberg et al., 1999). However, the treatment is not curative, and CRPC is formed when cancer cells are adapted to low levels of androgens and continue growing. From a molecular perspective this translates to reactivated androgen receptor (AR) signaling and crosstalk between AR and functional oncogenic survival pathways (Seruga et al., 2011). In clinics, activated AR signaling can be detected from increased PSA levels (Mottet et al., 2011). In metastatic prostate cancer, castration resistance appears after a median time of 18-24 months (Damber and Aus, 2008). After the formation of CRPC, the mean overall survival time is only 20 months (Halabi et al., 2009).

At present, CRPC has no cure; therefore, determining the molecular mechanisms leading to treatment resistance and developing novel treatment methods remain major clinical challenges.

2.2 Molecular mechanisms of prostate cancer

Prostate cancer is a heterogeneous group of diseases; every tumor has its own characteristics. However, certain genetic rearrangements or alterations in gene expression are more common than others. For example, the fusion of ETS

transcription factors to other genes, which are most commonly androgen regulated, can be found in approximately every second cancers (Tomlins et al., 2005). The more advanced and aggressive the tumor is, the more genetic and epigenetic alterations and expression changes exist. In CRPC, chromosome 8q gain has been identified in 70-90% of the cases, and loss of 8p has been found in approximately 70% of the cases (Nupponen et al., 1998; Visakorpi et al., 1995b). Moreover, the loss of the phosphatase and tensin homolog (*PTEN*) and the amplification and mutation of *AR* are commonly identified (Taylor et al., 2010; Uzoh et al., 2009; Visakorpi et al., 1995a). The mutation frequency, instead, is low, 0.9 nt per megabase, in prostate cancer (Berger et al., 2011). Several oncogenes that are commonly mutated in other cancers, such as *KRAS* and *BRAF,* are rarely mutated in prostate cancer (Taylor et al., 2010).

In the following sections, genes and pathways are presented that, according to current knowledge, are most crucial in the development of prostate cancer.

2.2.1 Altered AR signaling

Androgen receptor is a member of the steroid hormone receptor family and a ligand-activated nuclear transcription factor (Evans, 1988). The *AR* gene is located on the X chromosome at area q11-12. Unligated AR protein resides primarily in the cytoplasm, where it is bound to heat shock proteins (HSPs). HSPs stabilize the tertiary structure of AR in a conformation that permits androgen binding. Ligand binding results in the dimerization of AR and in the release from HSPs followed by translocation into the nucleus. In the nucleus, AR binds to certain areas of the genome, where it regulates the transcription of its target genes together with its coactivators and corepressors. (Reviewed by Taplin, 2007).

Several microarray studies have detected AR target genes in prostate cancer cells. AR transcription factors have been found to directly or indirectly regulate 1.5-4.3% (100-350) of genes expressed in prostate cancer cells (Dehm and Tindall, 2006). AR regulates several different functions of prostate cells including protein synthesis and secretion, polyamine synthesis, lipogenesis, cell cycle regulation, cell survival and apoptosis (Dehm and Tindall, 2006). AR protein is present in all stages of prostate cancer, but the AR expression level has no apparent correlation with prognosis or

response to androgen ablation therapy (Taplin, 2007). Despite intensive research, the role of AR in prostate cancer initiation is not fully known.

The expression of *AR* is essential for prostate cancer development and it also stays active in the castration resistant disease stage. Only a small portion of CRPC truly lacks the expression of *AR*. In those rare cases, the inhibition possibly occurs through the hypermethylation of the *AR* gene promoter (Kinoshita et al., 2000; Linja and Visakorpi, 2004). In CRPC, cells have adapted to lower amounts of androgens by different mechanisms. These include amplification, overexpression and mutations of the *AR* gene (Chen et al., 2004; Taplin et al., 1999; Visakorpi et al., 1995a; Waltering et al., 2009), altered expression levels of the *AR* coregulators and corepressors (Gregory et al., 2001), the ligand-independent activity of AR splice variants (Dehm et al., 2008; Hu et al., 2009; Sun et al., 2010) and the expression of steroidogenic enzymes enabling intracrine testosterone production (Locke et al., 2008; Montgomery et al., 2008b) These mechanisms are presented in Figure 1 and in more detail in chapters 2.2.1.1 - 2.2.1.4.

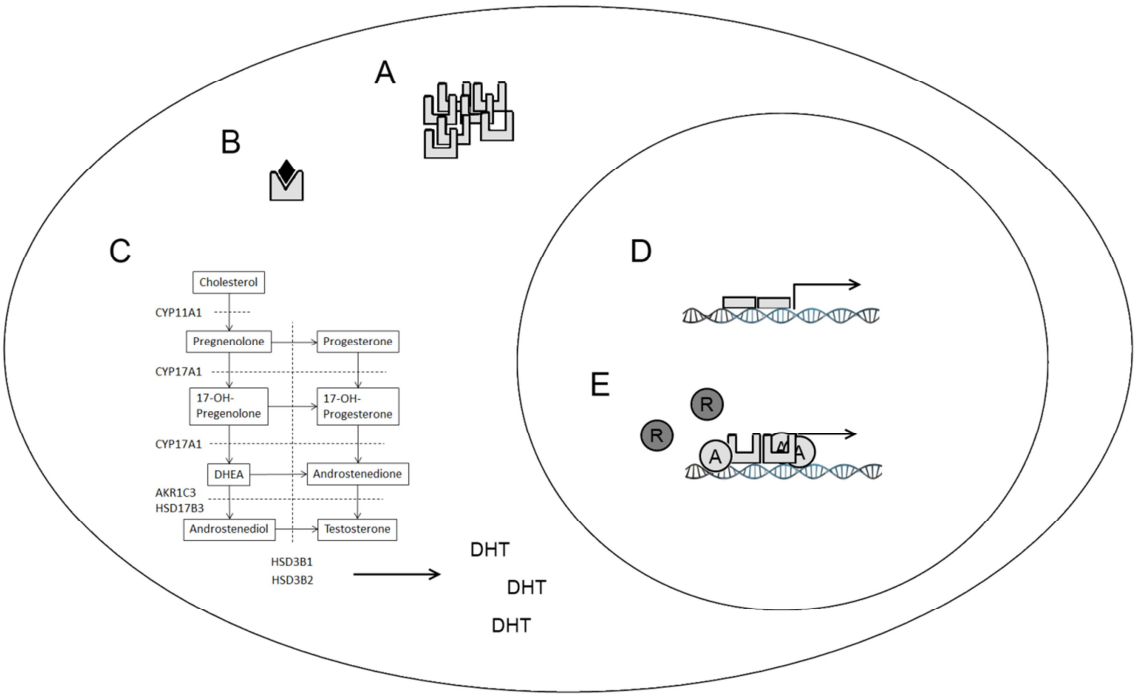

Fig. 1. *Castration resistant prostate cancer cells can adapt to low levels of androgens by several different mechanisms, including androgen receptor (AR) amplification* ***(A)****, mutations that allow AR activation by other ligands than dihydrotestosterone (DHT)* ***(B)****, intratumoral steroidogenesis that can produce DHT from dehydroepiandrosterone (DHEA) or from cholesterol* ***(C)****, alternative splicing of AR that allows ligand-independent AR activation* ***(D)*** *and alterations in AR co-activator and co-repressor levels* ***(E)****.*

2.2.1.1 Overexpression, amplification and mutations of AR

AR has an essential role in castration resistant tumor progression. The role of AR has been revealed in the study of Chen et al. (2004) with hormone sensitive and hormone refractory xenograft pairs. The study demonstrated that the increased expression of *AR* was the only genetic change found consistently in the castration resistant xenografts compared to hormone naïve xenografts. Moreover, overexpression of *AR* accelerated tumor growth *in vivo,* and silencing of AR with short interfering RNA (siRNA) reduced tumor growth. The hormone refractory growth also remained ligand dependent, and higher AR levels were able to convert antagonistic suppression of antiandrogens into agonistic activation.

Amplification of the *AR* gene has been shown to occur in approximately 30% of CRPCs (Visakorpi et al., 1995a). As expected, amplification leads to increased AR expression and thus it sensitizes the cancer cells to lower amounts of androgens (Linja et al., 2001; Waltering et al., 2009).

Mutations of the AR gene are notably rare in untreated prostate cancer but have been found in approximately 10-30% of CRPCs (Linja and Visakorpi, 2004). The use of AR antagonists, particularly flutamide, is associated with a higher frequency of mutations compared to physical castration (Taplin et al., 1999; Taplin et al., 2003). The majority of mutations cluster in three areas of the AR gene, where they can broaden ligand specificity to other steroid hormones or antiandrogens or can sensitize the receptor to dihydrotestosterone (DHT) (Taplin, 2007).

2.2.1.2 Expression of steroidogenic enzymes

Over 90% of circulating testosterone is produced in the testes. The rest is produced mainly in the adrenal glands. In addition to small amounts of testosterone, adrenal glands produce high amounts of dehydroepiandrosterone (DHEA), which is a weak androgen (Cai and Balk, 2011).

It has been demonstrated that prostate cells can take up DHEA and convert it to androstenedione and later to testosterone (Fig. 2) (Cai and Balk, 2011; Labrie et al., 2000). The key enzyme for converting androstenedione to testosterone is aldo-keto reductase family 1 member C3 (AKR1C3) (Cai and Balk, 2011; Lin et al., 1997). It has been suggested that the conversion of abundant adrenal DHEA to testosterone in

prostate cancer cells would enable cancer cells to adapt to low levels of androgens after castration. Indeed, gene expression studies have identified increased expression of *AKR1C3* and steroid 5α-reductase 1 (*SRD5A1)* enzymes that mediate testosterone and DHT synthesis (Fig. 2) (Hofland et al., 2010; Montgomery et al., 2008a; Stanbrough et al., 2006). This result would also explain why the levels of testosterone and DHT remain relatively high in tumor cells despite the very low levels in circulation after castration.

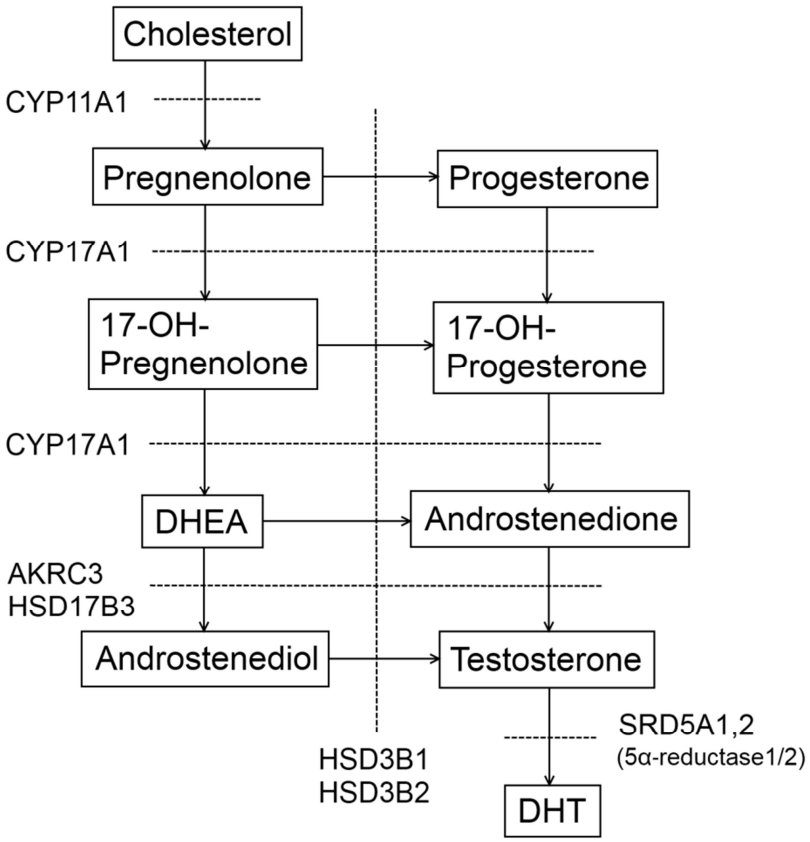

Fig. 2. *The main metabolic steps of the androgen biosynthesis pathway from cholesterol to DHT. Products formed during biosynthesis are represented in the boxes. Dashed lines represent the active positions of the enzymes that are involved in the biosynthesis pathway.*

Another mechanism for cancer cells to adapt low levels of androgens was suggested some years ago, when it was demonstrated that many enzymes involved in steroidogenesis from cholesterol (Fig. 2) were upregulated during endocrine therapy in castration resistant metastases or in an LNCaP cell line model (Holzbeierlein et al., 2004; Locke et al., 2008; Montgomery et al., 2008a). If this mechanism is accurate, intratumoral *de novo* androgen synthesis would also make cancer cells

independent from adrenal androgen production. However, some studies have been unable to detect enzymatic activity related to intratumoral steroidogenesis from prostate cancer or CRPC tissue samples (Hofland et al., 2010), leaving the role of *de novo* androgen synthesis in the development of CRPC unclear.

2.2.1.3 Splice variants of AR

AR splice variants were first found in the 22Rv1 cell line, from where Dehm et al. (2008) identified two truncated versions of AR that lacked the C-terminal end after exon 2 or 3 but contained a cryptic exon 2b. The truncated isoforms lacked a ligand-binding domain and were thus constitutively active and promoted androgen-independent proliferation in 22Rv1 cells. Further studies have revealed several novel splice variants, including variants that lack the entire C-terminal end after the second or third exon (Guo et al., 2009; Hu et al., 2009; Watson et al., 2010), and variants that skip exons 4,5,6 or 7 but contain an exon 8 (Sun et al., 2010; Watson et al., 2010). All of these splice variants lack a ligand-binding domain; therefore, they are proposed to contribute to the ligand-independent activity of AR and to the development of CRPC.

The first studies have demonstrated that at least some of the splice variants are enriched in castration resistant xenograft models and tissue samples (Dehm et al., 2008; Hu et al., 2009), and they are upregulated during the prostate cancer progression (Guo et al., 2009). Moreover, both Hu et al. (2009) and Guo et al. (2009) reported that high expression levels of the AR-V7/AR3 splice variant predicted tumor recurrence following radical prostatectomy. However, a recent study demonstrated that the expression levels of AR splice variants are actually only 0.1-2.5% of that of the full-length AR in patient samples and xenografts (Watson et al., 2010). In addition, AR splice variants require full-length AR to activate AR target genes and the activity of splice variants can be blocked by inactivating full length AR with antiandrogen MDV3100 or siRNAs. Controversially, another study that was published almost simultaneously reported a splice variant in which exons 5, 6 and 7 are deleted (Sun et al., 2010). The study indicated that this variant was resistant to the antiandrogen flutamide, functioned together with full-length AR and enabled the growth of castration resistant tumor xenografts. Thus, to clarify the exact

role of the AR splice variants in the progression of prostate cancer, more studies are needed.

2.2.1.4 Altered expression of AR coregulators

Today, over 160 different proteins have been identified as putative AR coregulators (Heemers and Tindall, 2007). These coregulators have different functions: e.g., NCOA1, 2 and 3 (aka SRC1, 2 and 3), p300, Tip60, KDM1A (aka LSD1) and JMJD2C regulate histone acetylation and methylation; SUMO1, 2 and 3, and PIAS1, 3 and x function in a sumoylation pathway; and BRCA1 and 2 are involved in DNA repair.

The roles of coregulators at different stages of prostate cancer progression have been studied widely, but results have been partly contradictory. In the systematic study by Linja et al. (2004), the expression of 16 AR coregulators were evaluated; the expression levels of only *PIAS1* and *NCOA1* (*SRC1*) were significantly altered in CRPC compared to untreated prostate cancer. However, another study by Mäki et al. (2006), identified only mild elevation in the expression levels of *NCOA1* in CRPC compared to untreated prostate cancer. In addition, *BAG1*, another AR coactivator, was amplified in a subset of CRPCs (Mäki et al., 2007). BAG1 isoform L is the most capable of interacting with AR and the same study showed it to be overexpressed in CRPC.

Enzymes that repress the transcriptional activation by binding to AR are called AR corepressors. Corepressors compete for the same sites in AR as do many coactivators; thus, the expression levels of corepressors can determine the activity of AR signaling in prostate cancer cells (Chmelar et al., 2007). Indeed, mutation and/or downregulation of nuclear receptor corepressors 1 and 2 (*NCOR1* and *NCOR2*) have been demonstrated in many primary and metastasized cancers (Taylor et al., 2010).

Taken together, an enormous number of AR coregulators have been identified, but their exact roles in cancer progression and formation of castration resistance remains poorly defined. Due to the heterogenic nature of prostate cancer, the expression levels of coregulators vary from one data set to another. Thus, perhaps digging deeper into the protein interactions and signaling pathways would truly clarify the function of AR coregulators in prostate cancer. Ultimately, AR

coregulators contribute to AR signaling and prostate cancer, and thus they remain promising targets for novel drugs.

2.2.2 *TMPRSS2:ERG* fusion

Until recently, recurrent gene fusions formed by genomic rearrangements were known to characterize some leukemias, lymphomas or sarcomas, but not common epithelial tumors (Mitelman et al., 2007). However, in 2005, Tomlins et al., (2005) identified a fusion of AR-regulated gene *TMPRSS2* with a well-known oncogene *ERG* in approximately every second prostate cancer. The *TMPRSS2:ERG* fusion most commonly combines the first exon of *TMPRSS2* with the fourth exon of *ERG*, which results in the expression of *ERG* being driven by the AR regulatory elements of the *TMPRSS2* promoter and enhancer (Tomlins et al., 2005) (Fig. 3).

The *TMPRSS2:ERG* fusion has been shown to occur in approximately 50% of prostate carcinomas and 15-20% of prostatic intraepithelial neoplasia (PIN) lesions, but never in benign prostate glands (Cerveira et al., 2006; Mosquera et al., 2008; Shen and Abate-Shen, 2010; Tomlins et al., 2009).

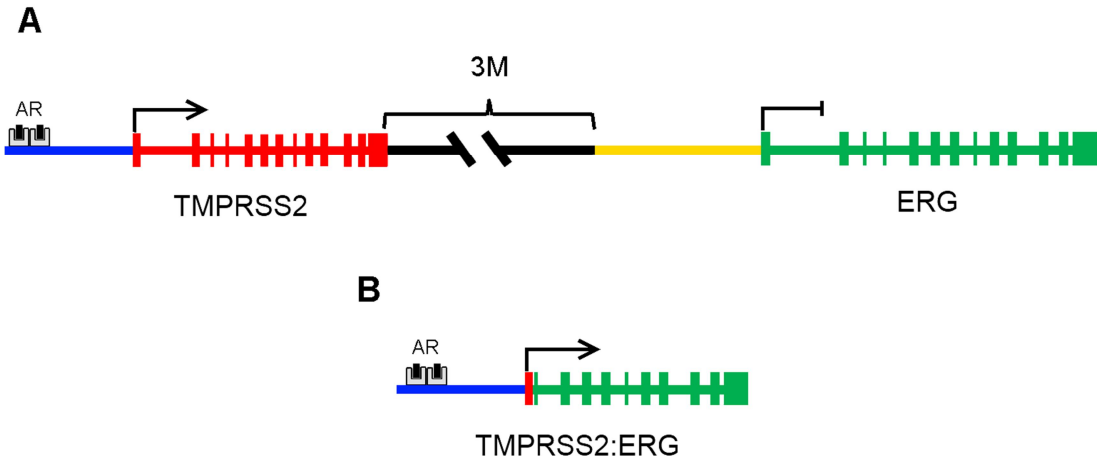

Fig. 3. (A) *Normal genomic arrangement. TMPRSS2 and ERG genes are located in chromosome 21 and are separated by a distance of approximately 3 megabases. In prostate cells the expression of TMPRSS2 is activated by androgen receptor (AR) regulation and the expression of ERG remains inactivated.* ***(B)*** *The TMPRSS2:ERG gene fusion. In the most common rearrangement, the gene regulation area and the first exon of TMPRSS2 are fused with the fourth exon of ERG. In consequence, ERG becomes AR regulated and its expression is activated. Blue and yellow colors indicate the gene regulation areas, and black indicates the genomic area between TMPRSS2 and ERG.*

2.2.2.1 Role of the TMPRSS2:ERG fusion in prostate cancer development

Over 90% of prostate cancers that overexpress *ERG* harbor the *TMPRSS2:ERG* fusion (Tomlins et al., 2005). Functional studies of ERG overexpression in several different *in vitro* models have demonstrated that the *TMPRSS2:ERG* gene fusion induces migration and invasion without increasing cellular proliferation (Klezovitch et al., 2008; Tomlins et al., 2008; Tomlins et al., 2009). In two independent studies, transgenic mice overexpressing ERG under the androgen inducible probasin promoter developed mouse PIN but not malignant cancer (Klezovitch et al., 2008; Tomlins et al., 2008). This result indicates an important causal role for the *TMPRSS2:ERG* fusion in the transformation of prostate epithelium and also demonstrates the weakness of ERG as an independent oncogene in prostate cancer. Later studies revealed that mice with haploinsufficient *Pten* or *Pten* knockdown together with probasin-induced overexpression of *ERG* remarkably accelerated the progression of high-grade PIN to prostatic adenocarcinoma (Carver et al., 2009; Zong et al., 2009). However, King et al. (2009) were unable to detect the formation of PIN in mice overexpressing the *TMPRSS2:ERG* fusion alone, and crossing *TMPRSS2:ERG* mice with $Pten^{+/-}$ mice disclosed the clear formation of PIN but not more malignant types of tissue reformation. The different outcomes between the studies can be at least partly explained by different interpretations of mouse prostate histology and the use of different mouse strains (King et al., 2009). Another study revealed that the combined overexpression of *AR* and *ERG* in murine prostate leads to the formation of poorly differentiated and invasive adenocarcinoma (Zong et al., 2009). Taken together, these studies indicate that ERG may have a role both in the transformation of prostate epithelium to neoplasia and in the progression of prostate tumorigenesis, but malfunction of *AR*, *PTEN* and possibly some other genes are required.

Recent mass spectrometric analysis and immunoprecipitation assays of ERG revealed DNA-dependent protein kinase complex (DNA-PKcs), its interacting subunits ku70 and ku80, and poly (ADP-ribose) polymerase 1 (PARP1) as proteins that interact with ERG (Brenner et al., 2011). The study further determined that DNA-PKcs and PARP1 are required for ERG-induced gene activation, cell invasion and metastasis. Interestingly, the clinically used PARP1 inhibitor Olaparib significantly reduced the growth of *TMPRSS2:ERG* fusion-positive but not fusion-

negative xenografts in mice. Because most transcription factors, including ERG, are difficult to target therapeutically, the inhibition of essential interacting proteins such as PARP1 could present drug development targets for fusion-positive cancers.

2.2.2.2 Fusion formation

What is the mechanism for how gene fusions occur? The fact that the *TMPRSS2:ERG* gene fusion is formed in every second prostate cancer raises the assumption that the fusion may be developed systematically. Indeed, evidence from fluorescence *in situ* hybridization (FISH) assays suggest that in LNCaP cells and in the prostate epithelium PrEC cells the colocalization of *TMPRSS2* and *ERG* is induced by DHT stimulation (Lin et al., 2009; Mani et al., 2009). In addition, a combined treatment of LNCaP cells with DHT and irradiation, or with chemotherapeutic drugs that cause genotoxic stress, induced the formation of the *TMPRSS2:ERG* fusion transcripts (Lin et al., 2009; Mani et al., 2009). Lin et al. (2009) suggested that several enzymes, such as Gadd45 and activation induced cytidine deaminase (AICDA), are also involved in the DHT and genotoxic stress induced DNA double stranded breaks. Moreover, Haffner et al. (2010) revealed that topoisomerase II beta (TOP2B) binds to the activated AR and induces DNA double strand breaks, which can lead to the *TMPRSS2:ERG* fusion without exogenous genotoxic stress. Taken together, recent evidence from *in vitro* studies suggest that the formation of the *TMPRSS2:ERG* fusion can be AR-mediated together with other enzymes (Haffner et al., 2010; Lin et al., 2009; Mani et al., 2009). Still, whether the AR-mediated genomic instability leads specifically to the formation of the *TMPRSS2:ERG* fusion with unknown mechanisms or whether the gene fusions are generally common in prostate tissue remains unclear.

2.2.2.3 Differential gene expression in fusion-positive prostate cancers

Several gene expression profiling studies have identified expression signatures that distinguish fusion-positive and fusion-negative prostate cancers (Iljin et al., 2006; Setlur et al., 2008; Tomlins et al., 2008; Yu et al., 2010). Iljin et al. (2006) identified a strong association with epigenetic reprogramming and especially the high

expression of *HDAC1* in fusion-positive tumors. However, in the study of Setlur et al. (2008), the fusion signature was associated with estrogen receptor (ER) signaling. In addition, Tomlins et al. (2008) identified several target genes for ERG, including metalloproteinases MMP3, MMP9 and ADAM19, and urokinase plasminogen activator (PLAU), that all have been implicated in cell invasion. Moreover, ERG overexpression has recently been revealed to affect AR signaling remarkably (Yu et al., 2010, see next chapter).

2.2.2.4 The TMPRSS2:ERG fusion and AR

Formation of the *TMPRSS2:ERG* fusion deletes the original promoter of *ERG* and places *ERG* under AR-dependent regulation (Fig. 3). ERG is a common transcription factor that has been shown to bind close to the transcription start site (TSS) of 68% of the protein coding genes in the VCaP cell line (Yu et al., 2010). In a recent study, Yu et al. (2010) used chromatin immunoprecipitation (ChIP) – sequencing (seq) analysis of the VCaP cell line to show that over 90% of the genes that harbor AR binding also have an ERG binding site. Moreover, 44% of the sites overlapped physically. In functional studies the authors discovered that the overexpression of *ERG* leads to reduced expression of *AR* and many AR target genes, thereby disrupting androgen signaling. In localized prostate tumor samples, the study revealed a negative correlation between *AR* and *ERG* expression. Interestingly, in a metastatic state the expression levels of both *ERG* and *AR* were high, which indicates the loss of the inhibitory effect (Yu et al., 2010). In addition, as mentioned above (see chapter 2.2.2.1), combined overexpression of *AR* and *ERG* in mice led to the formation of advanced prostate cancer (Zong et al., 2009).

2.2.2.5 Clinical significance of the TMPRSS2:ERG fusion

The association of the *TMPRSS2:ERG* fusion with the clinical parameters is partly contradictory. In most studies, the fusion has not demonstrated a statistically significant association with either Gleason grade or tumor stage (Gopalan et al., 2009; Hermans et al., 2009; Lapointe et al., 2007; Nam et al., 2007; Saramäki et al., 2008). In addition, the association of the *TMPRSS2:ERG* fusion with prognosis has

varied widely. Several studies have indicated an association with poor prognosis (Attard et al., 2008; Demichelis et al., 2007; Nam et al., 2007; Wang et al., 2006), some with good prognosis (Hermans et al., 2009; Petrovics et al., 2005; Saramäki et al., 2008), and number of studies have not identified any association (Gopalan et al., 2009; Lapointe et al., 2007; Leinonen et al., 2010). To a certain extent, this variation can be explained by size and differences in patient material or by methodological differences. In addition, the expression of different fusion transcripts (Boormans et al., 2011; Hermans et al., 2009) may affect the prognosis. During the fusion transformation, the chromosomal area between *TMPRSS2* and *ERG* can be either deleted or translocated, and Attard et al. (2008) revealed a significant association between deletion and poor prognosis. However, Leinonen et al. (2010) could not find a similar association.

2.2.3 Other crucial genes, pathways and genomic regions

2.2.3.1 PTEN and the PI3K/Akt pathway

PTEN is a tumor suppressor gene that is frequently mutated or deleted in many cancers, including prostate cancer (Salmena et al., 2008). The loss of *PTEN* activates the PI3K/Akt pathway, which promotes cancer cell proliferation and survival (Uzoh et al., 2009). Loss of *PTEN* is also associated with a higher Gleason score and progression to aggressive and castration resistant disease (Pourmand et al., 2007; Reid et al., 2010a; Shen and Abate-Shen, 2010), but it does not predict prognosis (Pourmand et al., 2007). A copy number loss of *PTEN* can occur as an early event in prostate carcinogenesis, but it is more common in a metastatic and castration resistant phase (Shen and Abate-Shen, 2010; Taylor et al., 2010). A recent study has disclosed that 40% of primary and 100% of metastatic cancers harbor significant deregulations or mutations in one or more steps of the PI3K pathway (Taylor et al., 2010).

The association of *PTEN* loss to the formation of castration resistant and aggressive metastatic disease in mouse models has hinted that PTEN may directly interact with AR (Mulholland et al., 2006). However, the detailed mechanism remained unknown until two recent studies demonstrated that AR signaling and the

PI3K pathway regulate each other equally (Carver et al., 2011; Mulholland et al., 2011). AR inhibition activates Akt signaling, and similarly, PI3K pathway inhibition activates AR signaling. The inhibition of either one activates the other and thus maintains tumor cell survival. Interestingly, co-inhibition of both pathways caused remarkable cancer regression in *Pten*$^{-/-}$ prostate cancer model and xenografts (Carver et al., 2011).

Many of the prostate cancers with loss of *PTEN*, also harbor an *ERG* rearrangement, but cancers with an *ERG* rearrangement are not enriched by loss of *PTEN* (Carver et al., 2009; King et al., 2009). As demonstrated in mouse studies, PTEN and ERG appear to have synergistic effects for the development of prostate cancer (Carver et al., 2009; King et al., 2009; Zong et al., 2009, see chapter 2.2.2.1). Interestingly, however, an analysis of 308 prostate cancer patient samples indicated that the presence of a *PTEN* loss and the absence of the *TMPRSS2:ERG* fusion predicts a worse prognosis than the presence of both a *PTEN* loss and the *TMPRSS2:ERG* fusion (Reid et al., 2010a). As expected, the absence of both a *PTEN* loss and the *TMPRSS2*:ERG fusion predicts better prognosis.

2.2.3.2 NKX3-1

NK3 homeobox 1 (*NKX3-1*) contains many highly interesting features. For example, the chromosomal region 8p21.2 where *NKX3-1* is located, is one of the most commonly deleted regions in prostate cancer. Up to 85% of prostate adenocarcinomas and 60% of PINs harbor loss-of-heterozygosity in that area. However, mutations in the *NKX3-1* gene locus are rare, and the expression of the other alleles remains at hardly detectable levels (Abdulkadir, 2005; Shen and Abate-Shen, 2010). In addition, studies in knock-out mice have demonstrated that a deletion in one or both alleles of *NKX3-1* causes the formation of PIN but not adenocarcinoma. Moreover, a study by Lei et al., (2006) linked *PTEN*, *NKX3-1* and *AR* expression together by revealing that both PTEN and AR positively regulate *NKX3-1*, and NKX3-1 negatively regulates AR. Furthermore, these researchers showed that *PTEN* loss reduces the *NKX3-1* expression level, which increases AR activation and leads to tumor initiation. Taken together, these studies suggest that NKX3-1 functions as a haploinsufficient tumor suppressor during the early

pathogenesis of prostate cancer (Abdulkadir, 2005; Shen and Abate-Shen, 2010; Tomlins et al., 2006).

2.2.3.3 EZH2

EZH2, a member of the polycomb group family of transcriptional repressors, is upregulated in a majority of metastatic prostate tumors and regulates cell proliferation *in vivo* (Varambally et al., 2002). *EZH2* overexpression leads to the epigenetic silencing of developmental regulators and tumor suppressor genes and it also predicts poor clinical outcome (Yu et al., 2007). In addition, a recent study indicated the role of the *TMPRSS2:ERG* fusion in the regulation of EZH2 signaling (Yu et al., 2010). ChIP-seq analysis revealed that ERG binds to the promoter area of *EZH2* and a number or *EZH2* target genes. Overexpression of *ERG* in LNCaP cells initiated increased *EZH2* expression and decreased expression of *EZH2* target genes, thereby enhancing EZH2-mediated epigenetic silencing.

2.2.3.4 Retinoblastoma

Retinoblastoma (*RB1*) is a well-known tumor suppressor, that prevents tumorigenesis by suppressing cell cycle progression in several tissues (Burkhart 2008). A recent study by Sharma et al. (2010) demonstrated that the *RB1* copy number loss was overrepresented in CRPC compared to hormone naïve prostate cancer and was associated with poor clinical outcome. Moreover, retinoblastoma has been shown to control the regulation of AR expression via E2F transcription factor 1. Thus the loss of *RB1* is linked to the expression of *AR* and it may initiate the formation of CRPC.

2.2.3.5 Gain of chromosome area 8q24

Chromosome arm 8q is the most common area of genetic gains in prostate cancer, and from the 8q arm, area 24 is the most commonly amplified (Nupponen et al., 1998; Taylor et al., 2010; Visakorpi et al., 1995b). It has been indicated that the amplification of 8q24 is more common in advanced and metastasized prostate cancer

than in local cancers (Jenkins et al., 1997). Additionally, amplification of 8q24 predicts poor prognosis (Sato et al., 1999).

The well known oncogene *MYC* is located in chromosomal area 8q24. The oncogenic properties of *MYC* in prostate cancer have been confirmed in a transgenic mouse model expressing *MYC* in prostate (Ellwood-Yen et al., 2003). Those mice developed murine PIN followed by invasive adenocarcinoma. However, the amplification of *MYC* does not necessarily correlate with overexpression of MYC protein (Nupponen et al., 1999; Savinainen et al., 2004).

Perhaps a more promising target gene for 8q24 gain is *EIF3SH* (eukaryotic translation initiation factor 3, subunit H, also known as *EIF3S3* or *eIF3-p40*). The amplification of *EIF2SH* has been shown to correlate with pathological tumor stage and is highest in CRPC (up to 50% of the samples) (Saramäki et al., 2001). Moreover, the expression of *EIF3SH* was shown to be higher in prostate cancer compared to BPH, and the overexpression of *EIF3SH* increased proliferation *in vitro* (Savinainen et al., 2004; Savinainen et al., 2006).

Instead of only 8q24, larger areas, even the entire 8q arm, are often gained in prostate cancer; thus other target genes in the region might be important. Other potential candidates from the 8q arm could be Elongin C (Porkka et al., 2002), tumor protein D52 (*TPD52*) (Wang et al., 2004) and nuclear receptor coactivator 2 (*NCOA2*) (Taylor et al., 2010).

2.2.4 Alterations in microRNA expression

MicroRNAs (miRNAs) are small,~ 22 nucleotide long, endogenous RNAs that mediate post-transcriptional regulation by pairing with the mRNAs of protein-coding genes to direct their repression (Guo et al., 2010). At present, almost 1 900 miRNAs have been identified to operate in humans (Feb 2012, www.miRBase.com) and they are predicted to control the activity of approximately 30% of all protein coding genes (Filipowicz et al., 2008). Initially, miRNAs were thought to repress only protein output, but mRNA-array experiments and ribosome profiling have shown that destabilization and degradation of target mRNAs are the predominant reasons for reduced protein levels (Guo et al., 2010).

Approximately 40% of the miRNAs are located within either intronic or exonic areas of protein coding genes; the remainder of miRNAs are located in intergenic regions. If a miRNA is located within a protein coding gene, the expression of the gene affects the expression of the miRNA. Approximately 30% of miRNAs are grouped into clusters (Catto et al., 2011). All miRNAs in one cluster are thought to be regulated in the same manner at the same time.

2.2.4.1 Biogenesis of microRNAs

MiRNAs are processed from precursor molecules called pri-miRNAs. Certain miRNAs are clustered close to each other in genome; in those cases the pri-miRNA can contain sequences of several miRNAs. Pri-miRNAs fold into hairpin structures that complex with Drosha-DGCR8 which processes them into ~ 70-nucleotide hairpins known as pre-miRNAs. Next, exportin 5 transports pre-miRNAs to the cytoplasm where the Dicer-TRBP complex cleaves them to~22 bp miRNA duplexes. One strand is subsequently selected to function as mature miRNA, while the other strand is degraded. Mature miRNAs function as components of ribonucleoprotein (RNP) complexes called miRNPs or miRNA-induced silencing complexes called miRISCs. As parts of these complexes, miRNAs bind to the miRNA binding sites of mRNA molecules. These binding sites correspond with miRNA nucleotides 2-8, which represent the "seed region" of the miRNA. With few exceptions, miRNA binding sites lie in the 3'UTR of the mRNA and are often present in multiple copies. (Reviewed by Filipowicz et al., 2008).

2.2.4.2 Cancer related microRNAs

After the discovery of the tumor suppressor role of miR-15a and miR-16-1 in chronic lymphocytic leukemia (CLL) in 2002 (Calin et al., 2002), several other tumor suppressor and oncogenic miRNAs have been identified for most human malignancies. For example, chromosome regions of let-7 miRNA family members were found to be deleted in multiple malignancies, including lung, breast, urothelial, ovarian and cervical cancers (Croce, 2009). In addition, the miR-17-92 cluster was

the first found to be overexpressed in B-cell lymphoma (He et al., 2005) and later in many different tumors (Volinia et al., 2006).

MiRNAs have been found to mediate several cancer-related pathways, including proliferation, apoptosis, angiogenesis and EMT (Croce, 2009; Gregory et al., 2008). Because a single miRNA can have dozens or hundreds of targets, it can inhibit tumorigenicity in one tissue type and induce it in other types (Croce, 2009). For example, miR-221 and miR-222 target the KIT oncogene in erythroblastic leukemia (Felli et al., 2005) but repress important tumor suppressors PTEN, p27, p57 and TIMP3 in several solid tumors (Croce, 2009) .

Moreover, miRNAs can have very essential refinement functions during a tumor progression. For example, the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) was first determined to inhibit an EMT by targeting E-cadherin transcriptional repressors ZEB1 and SIP1 (Gregory et al., 2008). However, a later study revealed that overexpression of the miR-200 family promotes metastatic colonization (Korpal et al., 2011). The authors suggested that in the process of metastatic formation, the miR-200 family first needs to be silenced to form the invasive tumor type by EMT, but for successful colonization, tumor cells require opposite mesenchymal-to-epithelial transition (MET), and for that purpose reactivation of the miR-200 family is necessary.

Oncogenic miR-21 is upregulated in several cancers and regulates cell proliferation, invasion, migration and antiapoptotic mechanisms (Krichevsky and Gabriely, 2009; Volinia et al., 2006). Several target genes are identified for miR-21, including PDCD4 (programmed cell death 4). Other important cancer related miRNAs include miR-17-92/miR-106-25 clusters, which modulate TGFβ signaling (Petrocca et al., 2008; Volinia et al., 2006).

Some miRNAs are also regulated by oncogenes. For example, MYC and MYCN, (another member of MYC family of oncogenes) have been shown to activate miR-9 (Ma et al., 2010). The same study also reveals that miR-9 regulates E-cadherin and increases cell motility and invasiveness.

2.2.4.3 Prostate cancer related microRNAs

At present, several dozens of miRNAs have been shown to be differentially regulated in prostate cancer compared to benign prostate tissue. These miRNAs often regulate the key pathways necessary for carcinogenesis (Catto et al., 2011). For example, miR-20a from the miR-17-92 cluster, miR-25 and miR-205 target the E2F1 transcription factors that are involved in apoptosis avoidance. Moreover, the tumor suppressor miRNAs miR-15a and miR-16-1 inhibit carcinogenic cell proliferation by targeting cell cycle gene CCND1 (Bonci et al., 2008). In addition, miR-99a, -99b and -100 target growth regulatory kinase mTOR in addition to chromatin-remodeling factors SMARCA5 and SMARCD1 (Sun et al., 2011).

Moreover, miR-203 has been shown to be downregulated in metastatic prostate cancer; functional studies using mouse models indicated that miR-203 re-expression decreased metastatic lesions *in vivo*. In addition, miR-203 re-expression decreased cell growth, increased apoptosis and induced cell cycle arrest and MET *in vitro* (Saini et al., 2011). In the target analysis, the authors identified prometastatic genes, including Survivin, ZEB2, Bmi1 and Smad4 as direct targets of miR-203.

A well-known tumor suppressor, p53, regulates several miRNAs, which is also the case in prostate cancer tissues. Among p53 regulated miRNAs is the putative tumor suppressor miR-145, which has been shown to target BNIP3, a transcriptional repressor of apoptosis inducing factor (AIF) in prostate cancer (Chen et al., 2010). Overexpression of miR-145 in PC-3 and DU-145 cell lines reduced cell growth and increased apoptosis (Chen et al., 2010; Zaman et al., 2010). Other interesting p53 regulated miRNAs include miR-34a, which has been shown to be underexpressed in CD44+ cells, which are putative cancer stem cells (Liu et al., 2011). The enforced overexpression of miR-34a also negatively regulates the tumor initiating capacity of the same cells. Moreover, intravenous injection of miR-34a oligonucleotides in an orthotopic mouse model reduced PC-3 tumor burden and diminished lung metastasis in LAPC9 tumors (Liu et al., 2011).

2.2.4.4 Androgen regulated microRNAs and microRNAs that regulate AR

The AR transcription factor regulates the expression of hundreds of genes, including some miRNA genes. Several miRNAs are defined to be directly or indirectly

androgen regulated in cell line models and xenografts (Ribas et al., 2009; Shi et al., 2011; Waltering et al., 2011). ChIP-seq-data have confirmed the AR binding close to miR-125b-2, miR-21, miR-32 and miR-148a genes (Jalava et al., 2012; Ribas et al., 2009; Shi et al., 2011). Overexpression of miR-21 has also been revealed to enhance tumor growth in an LNCaP xenograft model, even after castration (Ribas et al., 2009). Certain other miRNAs, like miR-141 are upregulated after DHT exposure or AR overexpression in an LNCaP cell line model (Waltering et al., 2011), although the direct AR mediated regulation remains unclear.

As it has been estimated that miRNAs regulate 30% of all protein coding genes and because AR is an important transcription factor regulating hundreds of genes, it is quite probable that miRNAs regulate AR. Indeed, a recent paper from Östling et al. (2011) validated 13 miRNAs that bound to the 3'UTR of AR. From those miRNAs, the expression levels of miR-34a and miR-34c negatively correlated with AR levels in prostate cancer tissue samples.

2.2.4.5 MicroRNAs as prognostic markers in prostate cancer

MiRNA expression profiling has been established in several different cancer types (Lu et al., 2005; Volinia et al., 2006), including prostate (Ambs et al., 2008; Martens-Uzunova et al., 2011; Porkka et al., 2007; Schaefer et al., 2010; Wang et al., 2009). In general, miRNA expression is downregulated in cancer tissue compared to normal tissue, although certain miRNAs are upregulated (Lu et al., 2005; Ozen et al., 2008). From prostate cancer tissue samples, differential miRNA expression profiles have been detected in prostate cancer compared to BPH or normal prostate (Ambs et al., 2008; Martens-Uzunova et al., 2011; Porkka et al., 2007), in aggressive phenotype (Gleason grade $\geq$ 8) compared to nonaggressive phenotype (Gleason grade $\leq$ 5) (Wang et al., 2009), in lymph node metastasis compared to local prostate cancer (Martens-Uzunova et al., 2011) and in hormone naïve compared to castration resistant tumor samples (Porkka et al., 2007). The differential expression levels of miRNAs in prostate cancer tissue indicate their potential as novel diagnostic and prognostic markers. Indeed, Schaefer et al. (2010) were able to discriminate tumor samples from normal samples at 72% accuracy with one miRNA (miR-205), and at 82% accuracy with a combination of several miRNAs. Moreover, the authors

proposed that miR-96 functions as a prognostic marker, as patients with high miR-96 expression had also higher risk for biomedical recurrence. In addition, Martens-Uzunova et al. (2011) disclosed that according to the miRNA expression profile, organ-confined prostate cancer samples could be divided into two groups, where one of the groups was strongly associated with poor outcome.

2.3 Traditional treatments for prostate cancer

At present, the great majority of prostate cancers are treated with traditional methods, using radical prostatectomy or radiation for localized disease and endocrine therapy for metastasized disease. The wide introduction of PSA testing in the early 1990s' has increased the number of patients with diagnosed localized, well-differentiated cancers that may not need immediate treatment. With traditional treatments, positive treatment response can be achieved in >90% of prostate cancer patients.

In the following sections, the effects of PSA testing and traditional treatment methods are presented in more detail.

2.3.1 PSA testing and the utility of PSA screening

PSA testing was first introduced to aid the determination of disease progression in patients with prostate cancer, but the use was soon expanded to include prostate cancer detection (Ferro et al., 1987; Leman and Getzenberg, 2009). The utility of PSA screening to prevent the incidence of death from prostate cancer has been discussed since the initiation of PSA testing. In 2009, the results from two large screening trials from the USA and Europe were reported (Andriole et al., 2009; Schröder et al., 2009). Altogether, over 250 000 men took part in the studies. After 7-10 years of follow-up in a study by Andriole et al. (2009), the rate of death from prostate cancer did not differ significantly between the screening and control groups. The study of Schröder et al. (2009), instead, detected a 20% reduction in the death in the screening group compared to the control group. However, the authors noted that 1410 men would need to be screened and 48 men would need to be treated to

prevent one death from prostate cancer. Thus, PSA screening would lead to a high risk of overdiagnosis and overtreatment.

2.3.2 Active surveillance

Due to the increased PSA testing today, almost 50% of diagnosed cancers are small and well-differentiated which pose a low risk of progression over 15-20 years (Table 1). For those patients, active surveillance is an option. Active surveillance patients may initially remain untreated but are followed and treated if progression occurs during follow-up (Heidenreich et al., 2011; Klotz, 2010). The follow-up includes regular PSA tests every 3-6 months and biopsies every 3 to 5 years. Progression criteria include PSA doubling in less than 3 years, PSA progression to > 10 ng/ml, or Gleason grade progression to ≥ 7 (Heidenreich et al., 2011; Klotz, 2010).

Several clinical follow-up studies have represented the safety and effectiveness of active surveillance. Although the median follow-up time of these studies is still short, the results look promising. At the 10-year time point, no prostate cancer related deaths in men on active surveillance have occurred. Approximately 30-50% of the patients on active surveillance were eventually treated, and there was no difference in the mortality rate compared to patients treated at the time of diagnosis. (Reviewed by Klotz, 2010).

2.3.3 Radical prostatectomy

Radical prostatectomy is the most routinely used treatment for localized low- or intermediate-risk prostate cancers (Table 1) (Heidenreich et al., 2011). Today, cancer-specific survival rates after radical prostatectomy are good: 95%, 90% and 79% for 5, 10 and 15 -years follow-up, respectively. However, the use of radical prostatectomy is recommended only for selected patients with high-risk prostate cancer (e.g., low-volume localized cancer). Neoadjuvant endocrine therapy prior to radical prostatectomy has resulted in no improvements for overall or disease-free survival (Kumar et al., 2006; Shelley et al., 2009). The significance of an adjuvant endocrine therapy following radical prostatectomy has been controversial, and no clear benefit has been indicated (Heidenreich et al., 2011; Kumar et al., 2006). For

the patients with positive surgical margins or high-risk (T3) prostate cancer, immediate postoperative radiotherapy has been shown to improve progression free survival in 5-year follow-up studies significantly (Bolla et al., 2005; Heidenreich et al., 2011; Wiegel et al., 2009).

***Table 1**. Prostate cancer risk groups.*

Risk	PSA[a] (ng/ml)	Gleason score	Clinical stage[b]
Low	< 10	< 7	T1, T2a
Intermediate	10-20	7	T2b
High	> 20	> 7	T2c, T3

[a]PSA, prostate specific antigen. [b]Clinical stages; T1, Clinically unapparent tumor, not palpable or visible by imaging; T2, Tumor confined within prostate; T2a, Tumor involves 50% or less of one lobe; T2b, Tumor involves > 50% of one lobe but not both lobes, T2c, Tumor involves both lobes; T3 Tumor extends through the prostate capsule.

2.3.4 Radiation therapy

Currently, radiation therapy and radical prostatectomy are equal alternatives for the treatment of localized prostate cancer. Comparative clinical trials have failed to disclose any superiority towards either radiation therapy or radical prostatectomy, although large randomized clinical trials with long follow-ups are still lacking (Kupelian et al., 2002; Welz et al., 2008).

External beam radiation therapy is the most commonly used radiation treatment method. Today, doses of ≥74 Gy are recommended for low-risk prostate cancer and 76-81 Gy for intermediate-risk disease (Heidenreich et al., 2011; Peeters et al., 2006).

Transperineal brachytherapy, in which radioactive “seeds” are injected directly into a tumor, has been shown to be a safe and effective treatment for low-risk prostate cancer, and this therapy represents an equivalent alternative to external beam radiation therapy and radical prostatectomy (Heidenreich et al., 2011).

Unlike radical prostatectomy, concomitant and adjuvant endocrine therapy combined with radiation therapy has been shown to be beneficial, especially for high-risk prostate cancer patients (Bolla et al., 2005; D'Amico et al., 2008). Thus,

concomitant and adjuvant endocrine therapy is the mandatory treatment after radiation therapy for high-risk prostate cancer patients (Heidenreich et al., 2011).

2.3.5 Endocrine therapy

More than 70 years ago, Huggins and Hodges first observed the relationship between testosterone and prostate cancer progression (Huggins and Hodges, 1941). These researchers also documented the clinical benefits of physical castration or estrogen injections for patients with advanced prostate cancer. Today, chemical castration with endocrine therapy has mainly replaced physical castration as a treatment method for prostate cancer. Endocrine therapy is used when cancer relapses after primary treatment (radical prostatectomy or radiation therapy) or if cancer is detected in an advanced or metastatic stage (Mottet et al., 2011). Approximately 35% of men will experience biochemical recurrence within 10 years following radical prostatectomy (Boorjian et al., 2011; Pound et al., 1999; Roehl et al., 2004), and 18% of all men will develop metastatic disease by 15 years after the surgery (Pound et al., 1999). Endocrine therapy is highly effective in the sense that 95% of patients respond initially to the treatment (Palmberg et al., 1999), but at the same time, it is highly ineffective in the sense that endocrine therapy is never curative. At present, several different endocrine treatment options are available.

2.3.5.1 GnRH agonist therapy

Gonadotropin releasing-hormone (GnRH) is a small hormone that is synthesized in the neuronal cells of the hypothalamus. From there, GnRH it is transported to the pituitary gland. In the pituitary gland, GnRH binds to its receptor, GnRHR, on the surfaces of gonadotrope cells. The binding stimulates the synthesis and release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Further in the pathway, LH activates the synthesis of testosterone from the testes, which subsequently inhibits the production of GnRH (Fig. 4a).

From this perspective, it was a total surprise for Auclair et al. (1977) in the late 1970's, when they tested a newly developed GnRH agonist that greatly enhances the effects of GnRH, and noticed opposite effects from what they were expecting

(Auclair et al., 1977; Labrie et al., 2005). Originally, GnRH agonist was developed as a treatment for infertility, but in experiments with rats it contradictory decreased the amount of testis and prostate and reduced the amount of testosterone. Soon it came clear that GnRH is secreted from the hypothalamus in an episodic manner, which prevents receptor desensitization and is mandatory for the continuous production of LH and androgens. If the GnRH receptor is over-activated with an abundant amount of GnRH agonist, it causes the inhibition of its function and a reduced amount of bioactive LH (Labrie et al., 2005; Tammela, 2004) (Fig. 4b and 5).

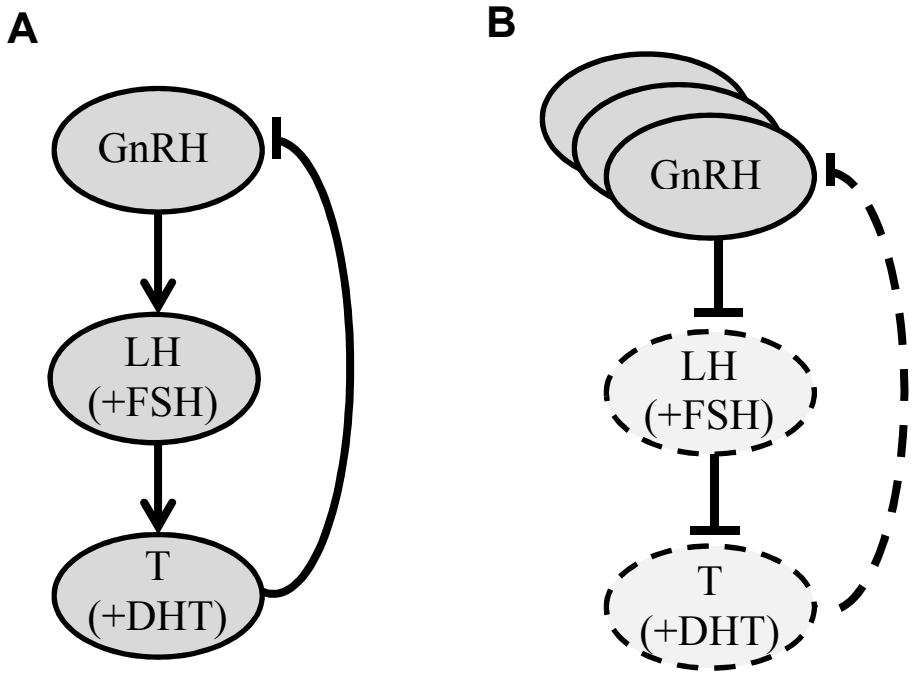

Fig. 4. *The effect of a GnRH agonist.* ***(A)*** *Normally, the secretion of GnRH positively regulates the production and secretion of LH (and FSH), which further regulates the production of testosterone. Testosterone subsequently negatively regulates the production of GnRH.* ***(B)*** *The continuous administration of GnRH agonist causes GnRH receptor desensitization, which inhibits the release of bioactive LH and the production of testosterone. GnRH, gonadotropin releasing-hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; T, testosterone; DHT, dihydrotestosterone.*

This discovery quickly led to the development of the GnRH agonist therapy for prostate cancer. Today, 95% of patients on GnRH agonist therapy achieve stable testosterone suppression into castration levels (<50 ng/ml) in 1-3 weeks (Seidenfeld et al., 2000). Several randomized trials have demonstrated the equivalency of GnRH agonist therapy and surgical castration in treatment response, survival rate, disease

progression and time to treatment failure (Moreau et al., 2006; Parmar et al., 1985). GnRH agonist therapy is also psychologically a more accepted treatment method compared to physical castration. All of these advantages have led GnRH agonist therapy to become the most commonly used hormonal treatment of prostate cancer (Heidenreich et al., 2008).

2.3.5.2 Antiandrogens

Androgens, such as testosterone and its more active form of DHT, activate AR, which regulates the expression of hundreds of genes, including many genes that are involved in the cell cycle and proliferation during prostate cancer progression (Dehm and Tindall, 2006). Antiandrogens competitively prevent the binding of testosterone and DHT to AR and thus inhibit its activation (Reid et al., 1999). Hence, the amount of testosterone in the circulation remains unchanged; only the binding of testosterone to its receptor is prevented (Tammela, 2004) (Fig. 5).

Antiandrogen treatment was originally designed for combination therapy with castration. However, recent studies have disclosed similar survival rates for non-hormonal antiandrogen monotherapy and chemical or physical castration for patients with locally advanced cancer (Iversen et al., 2000; Tyrrell et al., 1998). In addition, antiandrogen treatment caused less and milder adverse effects and higher quality of life compared to GnRH agonist therapy (Iversen et al., 2000). However, in metastasized cancers castration has achieved a survival benefit of 6 weeks compared to antiandrogen treatment (Tyrrell et al., 1998). Thus, non-steroidal antiandrogen treatment is recommended as an alternative to castration for patients with locally advanced prostate cancer (Anderson, 2003; Heidenreich et al., 2008).

2.3.5.3 Maximum androgen blockage

The testis is the main organ for testosterone production. In addition, the adrenal glands produce approximately about 5% of the total amount of testosterone. The adrenal glands also produce DHEA, its sulfate DHEA-S, and some androstenedione (4-dione). These inactive precursor steroids can be converted to testosterone in the

peripheral tissues, including the prostate, and they present approximately 40-50% of the total amount of androgens (Labrie et al., 2005; Tammela, 2004) (Fig. 5).

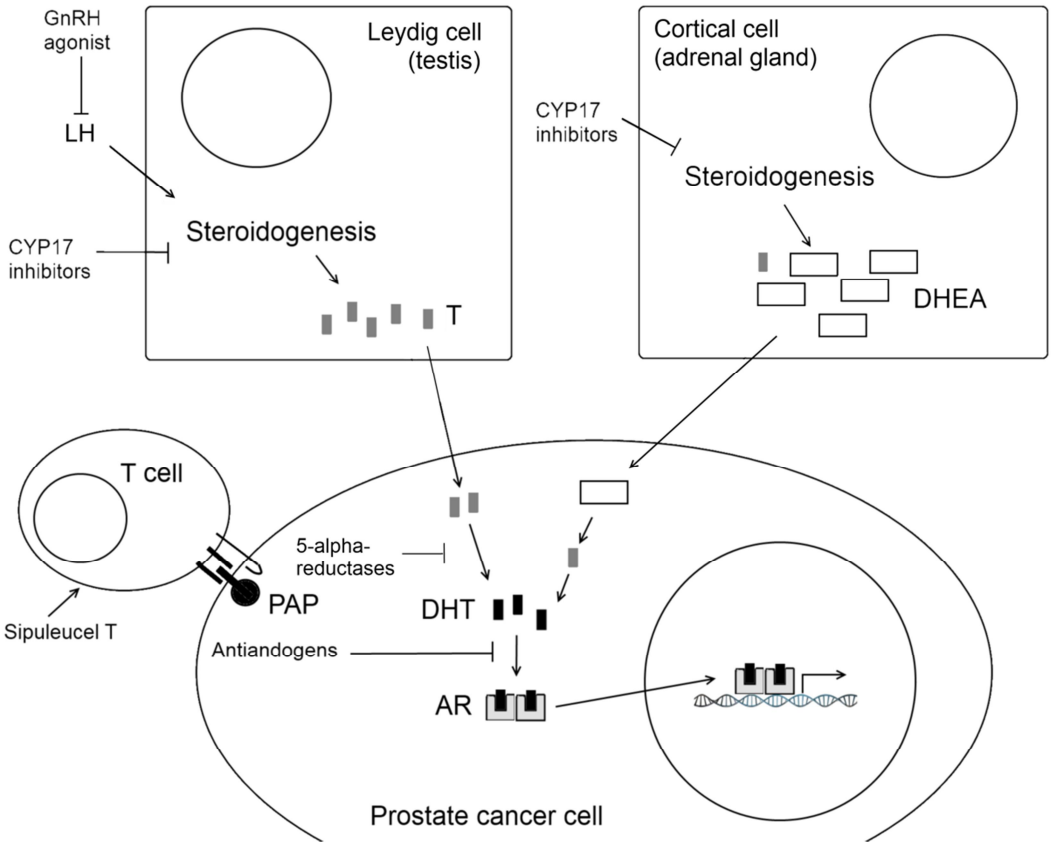

Fig. 5. *Synthesis of testosterone (T) and molecular mechanisms of current treatment methods for prostate cancer. T is produced mainly in the testes by the steroidogenesis pathway. A small amount of T is also produced in the adrenal glands. Dehydroepiandrosterone (DHEA) is the main secretary steroidal product from the adrenal glands. DHEA can be converted to T and further to DHT in prostate cells and possibly also in prostate cancer cells. In prostate cancer cells, T is converted to DHT, which then activates AR. Active AR is transferred to the nucleus, where it binds to AR binding sites and regulates the expression of its target genes. Chemical castration (e.g., GnRH agonist) inhibits T synthesis in the testes, while CYP17 inhibitors (e.g., abiraterone) inhibit steroidogenesis both in the testes and in the adrenal glands. 5α-reductases inhibit the conversion of T to DHT, and antiandrogens (e.g., bicalutamide or MDV3100) prevent DHT binding to AR. Effective AR inhibition leads to growth arrest and apoptosis of prostate cancer cells. Sipuleucel-T "educates" T-cells to recognize prostatic acid phosphatase (PAP) antigen and activates the immune system to destroy PAP-presenting cells. Radical prostatectomy and radiation methods aim to remove or destroy all cancerous tissue, and taxanes cause apoptosis by disturbing mitosis (not shown in the figure).*

Maximum androgen blockage (MAB) aims to inhibit both the androgen production from the testes using GnRH agonist and the effects of adrenal androgens using antiandrogens in combination. Several randomized clinical trials have been set up to measure the efficacy of MAB compared to androgen suppression by GnRH agonist or orchiectomy alone (Prostate Cancer Trialists' Collaborative Group, 2000; Eisenberger et al., 1998; Samson et al., 2002). Latest meta-analysis of the randomized trials disclosed a moderate, but statistically significant difference in the 5-year survival rate as a benefit of MAB (Samson et al., 2002). The authors also noted that more severe adverse effects and reduced quality of life were observed with patients undergoing MAB treatment (Mottet et al., 2011; Samson et al., 2002).

2.4 Novel treatments for prostate cancer

Although traditional treatments are highly effective for the great majority of all prostate cancer patients, treatments for metastasized and endocrine treatment resistant prostate cancers have long been only palliative. Docetaxel was the first drug that demonstrated survival benefit against CRPC in 2004. During the last two years, four other novel compounds have improved the survival of CRPC patients in large phase III clinical trials.

In the following sections, recent clinically approved drugs and other potential novel compounds are presented in more detail.

2.4.1 Chemoprevention of prostate cancer

The prevention of prostate cancer incidence is appealing for several reasons. First, prostate cancer has a high frequency, and second, prostate cancer is a disease of elderly men, and even a delay in carcinogenesis could result in reduced incidence. At the moment, two large phase III clinical trials aiming to prevent prostate cancer incidence are completed: the Prostate Cancer Prevention Trial (PCPT) with finasteride (Thompson et al., 2003) and Reduction by Dutasteride of Prostate Cancer Events (REDUCE) with dutasteride (Andriole et al., 2010).

Finasteride and dutasteride are 5α-reductase inhibitors. The 5α-reductases are enzymes that convert testosterone to DHT, which has a greater affinity for AR (Fig.

2 and 5). Three isoforms of 5α-reductase are encoded, but only types 1 and 2 are known to contribute to testosterone conversion (Nacusi and Tindall, 2011). Finasteride inhibits only 5α-reductase type 2, whereas dutasteride inhibits both types 1 and 2.

In clinical phase III trials, both finasteride and dutasteride were shown to reduce the incidence of prostate cancer compared to placebo groups: 18.4% vs. 24.4% for finasteride and 19.9% vs. 25.1% for dutasteride (Andriole et al., 2010; Thompson et al., 2003). Surprisingly, both studies also revealed more high-grade tumors in treatment groups compared to the placebo group. Moreover, sexual side effects were more common, but urinary symptoms were less common after both treatments compared to the controls. Although statistical distortion towards high-grade tumors in treatment groups can be an artifact, the U.S. Food and Drug Administration (FDA) has not approved the use of 5α-reductase inhibitors to prevent prostate cancer (Nacusi and Tindall, 2011, Theoret et al., 2011).

In a recent clinical trial, the efficiency of dutasteride in preventing prostate cancer progression in low-risk prostate cancer patients was evaluated (Fleshner et al., 2012). During three years follow-up, the risk of pathological or therapeutic progression (the primary endpoint of the trial) was reduced in patients given dutasteride compared with placebo (37% of 144 men in the dutasteride arm vs. 48% of 145 men in the control arm, p=0.009). However, the three years of follow-up is a notably short period and long-term outcomes remain unknown.

2.4.2 Taxanes

The year 2004 was a keystone for novel treatment methods for prostate cancer, when for the first time, survival benefit was achieved for CRPC patients in two large phase III clinical trials. The drug was docetaxel combined with estramustine (Petrylak et al., 2004) or prednisone (Tannock et al., 2004), and it provided an increased overall survival of approximately three months (Berthold et al., 2008). In addition, progression free survival was increased, PSA was reduced more that 50% and improved quality of life was obtained in a notable percentage of patients.

Taxanes function as anti-mitotic compounds. Taxanes bind to β-tubulin and thus suppress spindle-microtubule dynamics and disrupt mitosis, causing cell cycle arrest

and apoptosis (Kavallaris, 2010; Petrylak, 2006). Moreover, both docetaxel and paclitaxel, another taxane, have been shown to inhibit transcriptional activity and the nuclear translocation of AR (Zhu et al., 2010). This inhibition may enhance the efficiency of taxanes in the treatment of prostate cancer.

Unfortunately, docetaxel treatment is not curative, and most men treated with docetaxel experience progression of their disease within one year from the start of treatment (Tannock et al., 2004). Remarkably, patients with docetaxel-resistant CRPC have achieved an average survival benefit of 2.5 months in a phase III clinical trial with cabazitaxel compared to the palliative drug mitoxantrone (de Bono et al., 2010).

2.4.3 CYP17 inhibitors

While GnRH agonist therapy efficiently diminishes testosterone production from the testes, adrenal glands still produce small amounts of testosterone. In addition, adrenal glands produce large amounts of DHEA, a weak androgen, which can be further synthesized to testosterone. The synthesis may also occur in prostate cancer cells (Fig. 1, 2 and 5) (Cai and Balk, 2011). Indeed, intratumoral androgens at concentrations capable of activating AR have been detected from xenograft models of castrated mice and tissue samples of CRPC patients (Locke et al., 2008; Montgomery et al., 2008b).

Ketoconazole is an antifungal agent that has been found to suppress testicular and adrenal androgen production in some men. This discovery raised the possibility of using ketoconazole in prostate cancer therapy (Small et al., 2004). Ketoconazole inhibits mainly cytochrome P450 C51 (CYP51), which normally converts lanosterol to cholesterol, and weakly inhibits cytochrome P450 C17 (CYP17), the key enzyme in steroid biosynthesis (Attard et al., 2011; Small et al., 2004). In 2004, a phase III clinical trial revealed modest anti-tumor activity for ketoconazole in CRPC patients: a PSA decrease of >50% was observed in 27% of patients who where treated with ketoconazole together with antiandrogen withdrawal compared to 11% of patients when treated with antiandrogen withdrawal alone. However, no difference in overall survival was observed (Small et al., 2004).

A year later, a better-targeted CYP17 inhibitor, abiraterone acetate, was introduced in a phase I clinical trial (Attard et al., 2005). Earlier, abiraterone had already reduced testosterone plasma concentrations and the weights of ventral prostates in mice (Barrie et al., 1994). The first phase I clinical trial affirmed safety and activity of the drug (Attard et al., 2005). In a phase II study, the anti-tumor activity of abiraterone was verified: a PSA decline of >50% was observed in 51% of CRPC patients, and circulating tumor cell (CTC) counts declined from five or greater to less than five in 41% of patients (Reid et al., 2010b). The promising results led to a recently published phase III clinical trial (de Bono et al., 2011). In this trial abiraterone acetate (1 000 mg) was administered together with 5 mg of prednisone twice daily for CPRC patients who had previously received docetaxel. The placebo group received only prednisone. The primary end point, overall survival, increased almost 4 months in the abiraterone acetate-prednisone group compared to the placebo group, and all secondary end points used, including progression-free survival and PSA response rate, favored the treatment group (de Bono et al., 2011). These promising results gave strong evidence to the hypothesis that continued androgen signaling contributes to disease progression, and blocking adrenal and intratumoral androgen synthesis by inhibiting CYP17 can produce tumor responses in CRPC patients even after docetaxel treatment.

2.4.4 Second-generation antiandrogens

MDV3100 is the most promising compound of so-called second-generation antiandrogens. MDV3100 has been shown to bind to AR with 5- to 8-fold greater affinity than the first-generation antiandrogen bicalutamide (Tran et al., 2009) (Fig. 5). MDV3100 also impairs nuclear translocation, DNA binding and coactivator recruitment of AR, and inhibited growth in the AR-overexpressing VCaP cells. *In vivo* experiments have revealed that MDV3100 shrinks the LNCaP xenograft tumors in castrated mice (Tran et al., 2009). A first phase I/II clinical trial was established with 140 patients with metastatic, progressive castration resistant prostate cancer (Scher et al., 2010). In the study, PET imaging confirmed the 20-100% decrease in 18F-fluoro-5α-dihydrotestosterone (FDHT) binding after administration of MDV3100. In addition, treatment with MDV3100 decreased serum PSA levels by

>50% in 56% of patients, and was associated with tumor regression and stable disease in soft tissue and in bone. Dosages up to 240 mg per day were well tolerated. The phase III trial with MDV3100 (160 mg/d) is ongoing. According to the latest press release, the MDV3100 treatment arm achieved an overall survival advantage of 4,8 months compared to the placebo arm in the phase III trial; the study was stopped early, and MDV3100 was also offered to the patients in the placebo arm (http://investors.medivation.com/releasedetail.cfm?ReleaseID=620500 8.2.2012).

Certain other promising second-generation androgens such as ARN-509, are also under investigation. ARN-509 has shown even greater efficacy than MDV3100 in a clinically valid murine xenograft model of human CRPC, and it is currently in early-phase clinical trials (Clegg et al., 2012; Yap et al., 2011).

One highly interesting novel AR inhibitor is EPI-001, a small molecule that binds to the AF-1 region in the N-terminal domain (NTD) of AR (Sadar, 2011). Currently, all antiandrogens, including MDV3100, inactivate AR through its C-terminal ligand binding domain (LBD). Additionally, chemical or surgical castration functions through the LDB by reducing the amount of ligand. However, it is well-known that in CRPC, the androgen receptor LBD is activated, either by adrenal androgens, *de novo* synthesis of androgens, or additional ligands whose binding is possible through mutations in the LBD. Constitutively active AR splice variants are also lacking LBD, not NTD (see chapter 2.2.1.3). *In vitro* studies revealed that EPI-001 inhibited protein-protein interactions with AR and reduced AR interactions with androgen response elements on target genes without attenuating the transcription activities of other steroid receptors such as the progesterone receptor (PR) or the glucocorticoid receptor (GR) (Andersen et al., 2010). Moreover, EPI-001 reduced the growth of androgen dependent LNCaP cells without affecting the growth of AR independent PC-3 or DU-145 prostate cancer cells. This treatment also reduced the volume of subcutaneous LNCaP xenograft tumors from both intact and castrated mice.

2.4.5 Immunotherapeutic cancer vaccines

Sipuleucel-T (Provenge) is the first therapeutic cancer vaccine approved by the FDA. Sipuleucel-T is designed to target prostatic acid phosphatase (PAP, also known as ACPP), a tissue antigen expressed by prostate cancer cells (Cheever and

Higano, 2011). The vaccine is elegantly produced *ex vivo* by the patient's own peripheral blood mononuclear cells (PBMCs), including antigen presenting cells (APCs) and T-cells. PBMCs are exposed to a recombinant fusion protein composed of PAP and granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF activates APCs that further process and present the PAP to T-cells. After approximately 40 hours of incubation of PBMCs with the PAP-GM-CSF fusion protein, T-cells have obtained the capacity to recognize and kill PAP-positive cells (Fig. 5), and the PBMC pool, which is the Sipuleucel-T vaccine, is returned to patient's circulation (Cheever and Higano, 2011; Goldman and DeFrancesco, 2009). Patients will undergo this process three times every two weeks, and each dose contains progressively more activated APCs and PAP-specific T-cells.

The efficacy of Sipuleucel-T against CRPC has been studied in three double-blind, placebo-controlled, multicenter, individual, phase III studies with asymptomatic metastasized CRPC (Higano et al., 2009; Kantoff et al., 2010a). There were no remarkable differences in the median time to disease progression in any of the studies, but the overall survival was 3.3 - 4.5 months longer in the treatment arms compared to the control arms. Adverse effects, including chills, fever and headache, were mild in the majority of cases (Cheever and Higano, 2011; Kantoff et al., 2010a), occurring within one day of vaccine infusion and most resolved within two days. Surprisingly, antibodies against PAP were detected in only 28.5% of the patients, and T-cell proliferation responses to PAP were observed in only 27.3% of the patients in the Sipuleucel-T arm in a phase III trial (Kantoff et al., 2010a). Still, Sipuleucel-T has been shown to prolong patient survival without detectable immune responses.

After the third phase III study in April 2010, the FDA approved Sipuleucel-T as the first therapeutic vaccine against CRPC. Thus, it became the next systemic approach after docetaxel and cabazitaxel, that can prolong survival in CRPC patients.

In addition to Sipuleucel-T, other cancer vaccines against prostate cancer are also under investigation. PROSTAVAC-VF is a Poxvirus based PSA-targeted recombinant vaccine that has shown promising efficacy in a randomized phase II clinical trial for metastatic CRPC patients (Kantoff et al., 2010b). Similar to the Sipuleucel-T trials, no detectable antibody responses to PSA occurred, and there were no differences in progression free survival between the treatment and control

arms. Still, the median overall survival of patients in the treatment arm was 8.5 months longer than that in the control arm (25.1 vs 16.6 months). A larger phase III trial is planned to confirm the results.

2.4.6 Alpharadin

Alpharadin is a radioisotope containing an α-particle emitting nuclide. Its efficiency was recently assessed in a randomized, placebo-controlled phase III trial in over 900 patients with symptomatic CRPC with bone metastases. Alpharadin targets bone metastases with extremely short range high-energy α-radiation that spares bone marrow and, therefore, limits toxic effects. Overall survival, the primary end point of the study was significantly increased from 11.2 months for those receiving placebo to 14.0 months for patients in the alpharadin arm (p= 0.002). Similar to the phase III study for abiraterone, the phase III study for alpharadin was stopped early, and treatment with alpharadin was offered to the patients on the placebo arm (Saylor et al., 2011; Yap et al., 2011).

2.4.7 Other novel compounds

Several novel compounds for the targeted therapy of CRPC are in different phases of clinical trials. Several have returned encouraging preliminary results, whereas others have failed. For example, a phase II trial of the clusterin inhibitor OGX-011 initiated a promising decline in PSA of ≥ 50% in 58% of patients and increased progression free and overall survival (Chi et al., 2010). Moreover, the vascular endothelial growth factor (VEGF) inhibitor bevacizumab exhibited encouraging results in phase II clinical trials, with a PSA decline of ≥ 50% in 75-90% of patients (Ning et al., 2010; Picus et al., 2011) and long overall survival time (Ning et al., 2010), but failed to demonstrate survival benefits in a phase III trial. Thus, the efficiency of novel compounds cannot be approved before the survival benefit is confirmed in large randomized phase III clinical trials.

Figure 6 summarizes the current treatment options for prostate cancer patients.

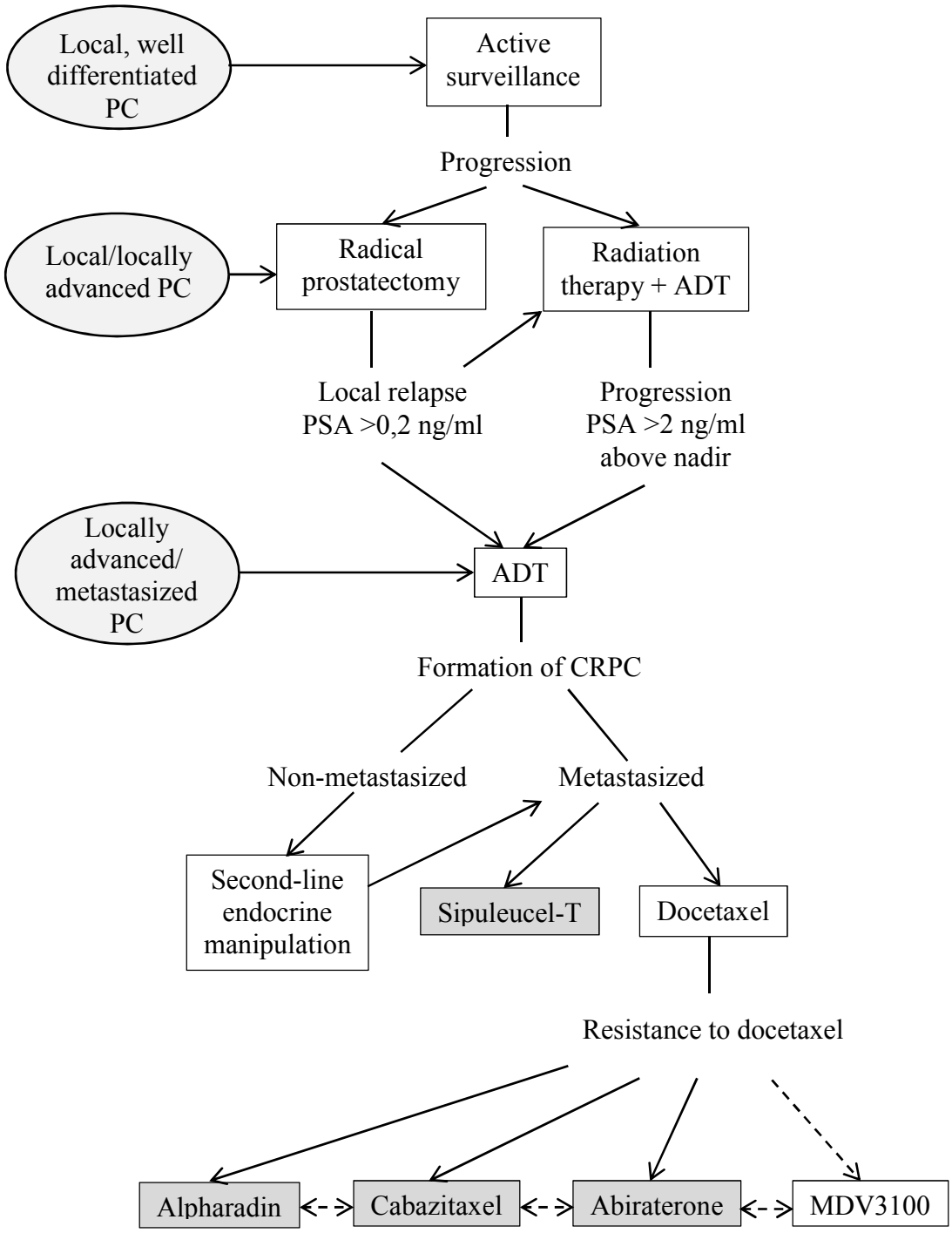

Fig. 6. *The most common treatment "path" for prostate cancer patients. Treatment possibilities depend on the cancer stage at the time of diagnosis and the aggressiveness of the cancer. Treatments in grey boxes (Sipuleucel-T, Alpharadin, Cabazitaxel and Abiraterone) have been clinically approved by the FDA in 2010 or 2011. The results from phase III clinical trial of MDV3100 have not been published. The most optimal order of novel treatments after docetaxel resistance has not been assessed. PC, prostate cancer; PSA, prostate specific antigen; ADT, androgen deprivation therapy; CRPC castration resistant prostate cancer.*

2.4.8 Gene therapy

Gene therapy means transferring genetic material into a cell with curative intent (Verma and Weitzman, 2005). However, inefficient transfer of genetic materia remains the major obstacle in gene therapy and several different methods have been developed to overcome this hindrance. Virus-based vector systems have been widely studied and used due to their natural capacity to transfer genetic material into cells. In addition, non-viral gene transfer vectors are under investigations and in clinical trials.

2.4.8.1 Non-viral gene therapy

Genetic material can be transferred into cells with several innovative methods. So called nanomedicine has been developed mainly to enhance the delivery of small drug molecules into target cells. However, certain nanoparticles, such as polymeric micelles, have been successfully used to transfer even siRNA molecules into cells (Schleef et al., 2010). Other gene transfer methods include cationic lipids that surround the transgene and increase the permeability lipid bilayer. This system has been commonly used in *in vitro* applications with low toxicity. However, i*n vivo* toxicity may occur, and transfection efficiency is limited in tissues other that skin. In addition to lipids, other imaginative applications have been developed to physically enhance gene delivery (Schleef et al., 2010). These treatments include gene gun or particle bombardment, where DNA is precipitated on small gold particles and then shot into the cells using pressurized gas. These gas methods have been used especially for DNA vaccines to improve the poor transfection efficiency of plain needle injection, and data from the first clinical trials are promising (Frelin et al., 2010; Schleef et al., 2010).

In general, non-viral gene therapy is cost effective and relatively safe, but suffers from poor gene transfer efficiency and difficulties reaching target tissues other than the skin (Koynova and Tenchov, 2011; Schleef et al., 2010).

Cell penetrating peptides

Cell penetrating peptides (CPPs), also known as cell permeable peptides or protein transduction domains, fall into the category of non-viral gene delivery methods.

CPPs are small, cationic peptides that facilitate the rapid translocation of their cargo molecules across cell membranes (Järver et al., 2010). The most commonly used CPPs are extracted from the Tat-protein of HIV-1 (Frankel and Pabo, 1988) and the antennapedia homeodomain (Antp) of *Drosophila melanogaster* (Joliot et al., 1991). The mechanism of cell entry is still under some debate, but currently the endocytosis-mediated uptake route has been accepted by most, although direct penetration may also occur (Vives et al., 2008). The cargo repertoire of CPPs is wide. Therapeutic peptides or proteins are easy to attach to CPPs, but CPPs are also used to enhance the transfer of plasmids, short hairpin RNAs (shRNAs), nanoparticles or viral vectors through cell membrane (Gratton et al., 2003; Järver et al., 2010).

The efficiency of CPPs has been demonstrated in several *in vitro* studies, and some efficacy has been reported also *in vivo* (Järver et al., 2010). At present, the lack of cell specificity is the major limitation of the therapeutic use of CPPs, although possible toxicity and rapid degradation in circulation are also challenges that remain to be solved (Vives et al., 2008).

2.4.8.2 Viral gene therapy

Viruses are naturally effective vectors for transferring genetic material into cells. Different virus types have their advantages and disadvantages, and thus, several different viral vectors are in use and are being investigated to achieve optimal gene transfer conditions. Pathogenic, wild type viruses have been modified in several ways to reduce their pathogenesis, increase safety, and expand or modify their natural tropism.

Two commonly used virus vectors and their use in therapy applications are presented here.

Lentiviruses

Lentiviruses belong to the retrovirus family, which consist of enveloped RNA viruses that normally integrate into the host cell's genome. Lentiviral gene transfer vectors are most commonly developed from the HIV-1 virus (Verma and Weitzman, 2005). Today, so-called third-generation lentiviruses are in general use. In those

vectors, all virulent genes have been removed; only the factors that are required for viral particle production, infection and integration are left (Singer and Verma, 2008). Moreover, a deletion in the 3' long terminal repeat (LTR) prevents viral genomic RNA production from the integrated provirus, and enables transcriptional targeting (Miyoshi et al., 1998). Pseudotyping the vector with vesicular stomatitis virus glycoprotein (VSV-G) broadens its tropism to most human cells (Naldini, 1998). Unlike most other retroviral vectors, lentiviruses are able to transduce non-dividing cells such as stem cells (Naldini, 1998).

Third generation lentiviruses are normally produced by co-transfection of four plasmids into cells. One plasmid contains the elements required for packing the transgene into viral particles, the transgene itself and the elements for efficient transgene expression. Three other helper plasmids contain the elements to encode structural and envelope proteins for viral particle production. These elements are not packed into the viral vector and thus, the vectors are replication-incompetent and safe (Singer and Verma, 2008).

After the unfortunate gene therapy trial for treating X-linked severe combined immunodeficiency, where four patients developed leukemia that was caused by murine retroviral vector integration close to the *LMO2* oncogene (Hacein-Bey-Abina et al., 2003; Hacein-Bey-Abina et al., 2010), great efforts have been made to confirm the safety of integrating virus vectors. The first step for safety was the development of nonintegrating lentiviral vectors that effectively transduced both dividing and non-dividing cells *in vivo* (Yanez-Munoz et al., 2006), but recently more focus has been invested in determining common lentivirus insertion sites (Biffi et al., 2011; Naldini, 2011), and in generating site-specific integration vectors (Lombardo et al., 2011).

In the last few years, the development of lentiviral vectors has evolved to the first clinical trials. Because lentiviral vectors integrate into the genome and result in stable transgene expression, their use is optimal for long term transgene expression such as to cure genetic diseases. Indeed, lentiviral vectors have demonstrated their safety and efficacy, such as in the clinical trial of two patients that suffered X-linked adrenoleukodystrophy (ALD) (Cartier et al., 2009). In this trial, CD34+ leukocytes were collected and transduced *ex vivo* with the lentiviral vector carrying the ATP-binding cassette transporter *ABCD1*. As a result, up to 14% of granulocytes, monocytes, and T- and B- lymphocytes expressed the ALD protein encoded by

ABCD1, and the patients achieved clear therapeutic benefits comparable with commonly used hematopoietic stem cell transplantation. Phase I/II trials with a larger patient cohort are ongoing (Naldini, 2011). Recently, promising results were also obtained in the cancer therapy field when a small number of T-cells ($1.5x10^5$ cells/kg) from a chronic CLL patient were transduced *ex vivo* with a lentiviral vector expressing the B-cell antigen CD19 (Porter et al., 2011). Re-infused transgenic T-cells expanded at least 1000-fold and caused tumor lysis syndrome, loss of B-cells and complete remission from CLL that lasted at least 10 months.

Adenoviruses

Unlike lentiviruses, adenoviruses (Ads) are non-enveloped viruses with a double stranded DNA genome coding over 50 proteins (Verma and Weitzman, 2005). Viral DNA does not integrate into the genome. Adenovirus is the most commonly used viral vector in gene therapy clinical trials (Gene Therapy Clinical Trials Worldwide, http://www.abedia.com/wiley/vectors.php). The most commonly used adenoviruses are serotypes 5 and 2. In the first-generation Ad vectors, the early-phase genes from E1 and E3 areas have been deleted and a transgene is added to E1 area. Normally, E1A activates the expression of other Ad transcription units, and therefore, the deletion causes replication deficiency. First-generation Ads can be produced in large amounts in cell lines where E1 genes are stably expressed. In second-generation Ads, the E2 and E4 transcription units are deleted or mutated to reduce immunogenicity and increase the possible transgene insertion size. Additionally, third-generation "gutless" Ads, in which all viral genes except the packaging signal have been removed, have been developed. This approach enlarges the cloning capacity and potentially reduces the immunogenicity but demands the use of a wild type helper Ad for propagation, thus generating problems with vector purification during production. (Reviewed by Verma and Weitzman, 2005).

Early cancer gene therapy trials have revealed the limited transduction efficiency of traditional, replication deficient viral vectors, and thus, replication-selective viruses have been designed to overcome such hindrances (Yamamoto and Curiel, 2010). So-called oncolytic Ads or conditionally replicative Ads (CRAds) have been developed to replicate specifically in tumor cells.

Wild-type adenovirus can infect non-proliferating cells, but for replication the virus needs to activate the cell cycle through p53 or Rb binding. Because cancer

cells are proliferating, the deletion of those Ad genes that regulate cellular proliferation inhibits viral replication in normal cells but not in cancer cells (Alemany et al., 2000). Another mechanism is to place the E1A gene under the control of a cancer- or tissue-specific promoter (e.g., PSA). Unfortunately these promoters are not completely specific, and the promoter leakage remains a safety obstacle (Alemany et al., 2000).

The replication of an oncolytic Ad *per se* destroys tumor cells, but oncolysis can also activate the immune system, revealing hidden tumor antigens and thus targeting the immune response against the tumor (Pesonen et al., 2011). Oncolytic Ads can also be "armed" with immune-stimulatory cytokines or chemokines, such as GM-CSF. Highly promising results were obtained from 20 patients with advanced metastasized tumors that were treated with a single intratumoral injection of an oncolytic Ad containing a GM-CSF transgene (Cerullo et al., 2010). Two patients experienced complete extermination of all measurable tumors, one had a 52% reduction in tumor volume and five experienced disease stabilization that lasted 98-490 days. Interestingly, no detectable difference in response was observed between injected and non-injected metastasized tumors.

2.4.8.3 Gene therapy against prostate cancer

Prostate cancer has several features that make it an attractive target for cancer gene therapy. First, the prostate gland is not a life-sustaining organ; therefore, separation between normal and cancerous prostate tissue is unnecessary. Second, several prostate specific promoters have been identified, allowing for the targeting of transgene expression. Third, intraprostatic administration of gene therapy vectors is possible by transurethral, transperineal or transrectal approaches. Fourth, evaluation and imaging of the prostate is relatively simple, and secreted PSA can be used as a surrogate marker for cancer progression or treatment efficiency. (Reviewed by Lu, 2009)

At present, adenovirus is the most commonly used virus vector for prostate cancer gene therapy trials, but retrovirus and vaccinia viruses have also been tested (Lu, 2009). Several gene therapy strategies have been investigated to treat both local and metastasized prostate cancer. To date, the great majority of clinical trials have been

phase I or II, and they have demonstrated the safety of gene therapy, though efficacy has remained modest (Freytag et al., 2007b). Adenoviral gene therapy trials against locally recurrent prostate cancer have generally resulted in a short-term reduction in PSA levels in a percentage of patients (Freytag et al., 2007b). However, in one trial the treatment of oncolytic Ad led to increased PSA doubling time from a mean of 17 to 31 months (Freytag et al., 2007c). Moreover, another study showed that Ad treatment combined with radiotherapy caused significantly fewer positive post-treatment biopsies compared to radiotherapy alone (Freytag et al., 2007a). The effect was provocative, especially in patients falling into the intermediate-risk group; there, 0 of 12 patients had a positive biopsy, which was clearly better than expected (>30%).

Thus far, the only effective administration route for viral vectors has been intratumoral because the patients' own antibodies inhibit viral vectors in the blood stream (Freytag et al., 2007b). Therefore, the treatment of metastatic disease remains challenging, though it is not hopeless. Currently, immunotherapy or vaccine-based strategies have shown the most promising results (see chapter 2.4.5), and the cancer gene therapy field is strongly focused on immunotherapeutic applications.

3. Aims of the study

The general aims of the study were to determine the effects of endocrine treatment on transcriptome level and to study the effects of the *TMPRSS2:ERG* gene fusion on gene expression in prostate cancer with or without endocrine treatment. In addition, an aim of the study was to identify means to enhance virus-mediated gene delivery to tumor cells, including prostate cancer.

The specific aims were:

1) To detect the effects and differences of two endocrine treatments on the prostate cancer transcriptome.
2) To determine the synergistic effects of the *TMPRSS2:ERG* gene fusion and endocrine therapy on gene expression levels in prostate cancer.
3) To investigate the effects of endocrine treatment and the *TMPRSS2:ERG* gene fusion on microRNA expression patterns in prostate cancer.
4) To investigate the utility of cationic peptides for enhancing viral gene transfer to prostate cancer and other cancer cells.

4. Materials and Methods

4.1 Clinical samples (I, II)

In these studies, we used two different clinical sample sets. The first sample set was obtained from a randomized clinical trial, in which 28 men with localized prostate cancer received neoadjuvant endocrine treatment (bicalutamide or goserelin acetate) or no treatment (control group) for three months. After neoadjuvant therapy, patients underwent radical prostatectomy.

The second sample set contained 15 freshly frozen BPHs, 27 prostatectomy specimens and 15 transurethral resection specimens from CRPCs. Both sample sets were obtained from Tampere University Hospital (Tampere, Finland). Table 2 presents the two sample sets in more detail.

The Ethics Committee of Tampere University Hospital approved the use of the clinical material, and written informed consent was obtained from the patients.

Table 2. Sample sets used in this study. Table is adapted from original article I.

	Number of samples	Subgroups	% of cancer in tissue
1st sample set "neoadjuvant trial"	28	Non-treated control (11 samples), bicalutamide 150 mg per day (9), goserelin 3.6 mg every 4 weeks (8)	0-85%
2nd sample set "independent"	57	BPH (15), PC (27), CRPC (15)	> 80%

BPH, benign prostate hyperplasia; PC, prostate cancer; CRPC, castration resistant prostate cancer.

4.2 Expression profiling (I, II)

Microarray hybridizations were performed from the clinical sample set of 28 neoadjuvant treated patients from samples that contained variable amounts of tumor tissue (Table 2).

For the mRNA expression profiling of > 25,000 annotated genes, microarray hybridizations were performed at the Finnish Microarray Center at the Turku Center for Biotechnology (Turku, Finland). Total RNA (300 ng) was amplified with the Illumina RNA TotalPrep Amplification kit (Ambion, Austin, Texas), and cRNA was hybridized to an Illumina HumanHT-12 Expression BeadChip version 3 according to the manufacturer's instructions. Microarrays were scanned with the Illumina BeadArray Reader, BeadScan software version 3.5.

For the microRNA expression profiling human microRNA V2 microarray chips (Agilent Technologies, Santa Clara, CA) were used. The V2 array contained probe sets for 723 human microRNAs based on Sanger miRBase v 10.1. Microarray hybridizations were performed according to the manufacturer's instructions. First, total RNA (100 ng) was labeled with Cyanine 3-pCp and hybridized using the microRNA Labeling Reagent and Hybridization Kit (Agilent Technologies), followed by incubation at 55°C for 20 hours. Microarrays were scanned with an Agilent Microarray scanner BA and Agilent Feature Extraction software (V. 10.7.1.1) was used for data extraction.

4.3 *In silico* data analysis (I, II)

Because the amount of cancer tissue in the neoadjuvant trial specimens varied from 0 to 85%, DSection, an *in silico* Bayesian modeling tool, was established to predict cancer specific expression (Erkkilä et al., 2010). Briefly, the amount of neoplastic cancer cells, benign epithelia cells and stromal cells from specimens were assessed by hematoxylin and eosin staining and possessed with ImageJ 1.41N (NIH, USA) image analysis software. Utilizing this information and microarray data from heterogeneous samples, DSection analysis calculated the expression value for each microarray probe for each tissue type and for each treatment group. The expression

values of mRNA and microRNA probes were combined to obtain sufficient statistical power for microRNA expression analysis.

4.4 Microdissection and qRT-PCR (I)

Eleven freshly frozen slides from the neoadjuvant trial were stained with HistoGene Staining Solution (Arcturus Bioscience Inc, California, USA). Laser capture microdissection (Arcturus, Veritas) was established to separate cancerous and stromal tissue compartments. RNA from microdissected samples was extracted with a PicoPure RNA Isolation Kit (Arcturus Bioscience Inc) according to manufacturer's instructions, followed by cDNA translation.

For qRT-PCR, Maxima SYBR Green/Rox qPCR Master Mix (Fermentas) and a CFX96 Real-Time System apparatus (Bio-Rad) were used. *β-actin* was used as a reference gene. Annealing was performed at 60°C.

4.5 Fluorescence *in situ* hybridization (I, II)

Three-color fluorescence *in situ* hybridization (FISH) was performed on tissue microarray (TMA) slides from the neoadjuvant trial material as previously described (Saramäki et al., 2008). Briefly, a locus-specific bacterial artificial chromosome probe for *ERG* (RP11-164E1) was labeled with digoxigenin-dUTP (Roche Applied Science), a probe for *TMPRSS2* (RP11-814F13) was labeled with Alexa Fluor 594-dUTP (Molecular Probes/Invitrogen) and a probe for the region between the two (RP11-367P1) was labeled with biotin-dUTP (Roche Applied Science), followed by hybridization. Slides were stained with anti–digoxigenin-FITC (Vector Laboratories) and streptavidin-Pacific Blue (Invitrogen) and then counterstained with an anti-fade solution (Vectashield, Vector Laboratories) containing 0.1 mmol/l 4′,6-diamidino-2-phenylindole. To analyze the images, Image-Pro Plus 6.1 software (Media Cybernetics Inc.) was used.

4.6 Immunohistochemistry (I, II)

Polyclonal rabbit antibodies against ERG (EPR3864 Epitomics, Burlingame, CA) were used for immunohistochemical (IHC) staining. TMA sections from a neoadjuvant trial were deparaffinized, and antigen was retrieved by autoclave cooking in 5 mM Tris-HCl - 1 mM EDTA (pH 9) at +121˚C for 2 min. A power-Vision+TM Poly-HRP IHC Detection Kit (ImmunoVision Technologies Corporation, Brisbane, CA) was used for antibody visualization. Sections were counterstained with hematoxylin. The ERG staining was scored as positive or negative. Staining intensity was measured from only the cancerous areas in a blinded fashion. Cancerous areas were defined with a cocktail of three antibodies: two mouse monoclonal antibodies against the basal cell layer (p63 diluted 1:200, LabVision Fremont, CA, and HMW keratin, clone 34beta12, diluted 1:100, LabVision, Fremont CA) and a rabbit monoclonal antibody against AMACR (clone 13H4, Dako, Copenhagen, Denmark), as described by Tolonen et al. (2011).

4.7 Gene ontology and clustering analysis (I, II)

To determine the gene ontologies, we used the web-based integrated data mining system WebGestalt (Zhang 2005). An unsupervised hierarchical clustering analysis was performed with the GeneSpring Analysis platform version GX 11 (Agilent Technologies, CA) for all microRNAs with expression over threshold (239 microRNAs) and for all mRNA genes with >1.5-fold differential expression between the subgroups. Average linkages between subgroups were used and similarities were measured with the Euclidean method.

4.8 Cell lines (III)

PC-3 (ATCC CCL-136), MG-63 (ATCC CRL-1427), SKOV3.ip1 (a kind gift from David T. Curiel, University of Alabama at Birmingham, AL, USA), Hey (a kind gift from Judy Wolf, Andersson Cancer Center, TX, USA) and COS-7 (a kind gift from Marika Ruponen, University of Kuopio, Finland) were used for transduction

experiments, and 293T (ATCC CRL-11268) and 293 (ATCC CRL-1573) cells were used for viral production. All cell lines were cultured at +37°C under 5% CO_2. PC-3 and Hey –cells were grown in RPMI 1640 medium, and others were grown in DMEM (Dulbecco's modified Eagle medium, BioWhittaker, Cambrex, USA). Both media contained 10% fetal bovine serum (FBS, EuroClone, Great Britain), 2 nM L-glutamine and 50 µg/ml gentamicin (BioWhittaker).

4.9 Viral vectors and transduction enhancers (III)

Both adeno- and lentiviral vectors were produced in a biosafety level 2-3 laboratory. An Ad vector AdTK-GFP was the first-generation E1/E3-deleted replication-deficient vector with a thymidine kinase (TK) –green fluorescent protein (GFP) fusion transgene, as described in more detail by Pellinen et al. (2004). WOX-TK-GFP was the second generation lentivector containing the same fusion transgene as the Ad vector. The production is described in more detail by Meriläinen et al. (2005). For transduction, a multiplicity of infection (MOI) of 1 was used for both vector types, and transduction efficiency (percent of GFP positive cells) was determined with a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA).

To enhance transduction, the polycations polybrene (8 µg/ml, Sigma-Aldrich) and protamine sulfate (5 µg/ml, Sigma-Aldrich) were used. In addition, cationic peptides derived from the HIV-1 TAT-protein (TAT1, YGRKKRRQRRR and TAT2, GRKKRRQRRRPPQ) or *Drosophila melanogaster* Antennapedia homeodomain (Antp, RQIKIWFQNRRMKWKK) (Inbio Ltd., Tallinn, Estonia) were used in 0.5 mM concentrations as described by Gratton et al. (2003).

4.10 Statistical analysis (I, II, III)

A chi-square test was used to analyze the significant differences between treatment groups in IHC staining (**I**). Differences in the gene expression in sample groups from the second sample set were measured by one-way ANOVA with a Bonferroni multiple comparison test (**I**). Fisher's exact test was used to measure the significant enrichment of ERG and AR in certain gene sets (**I, II**). One-way ANOVA with

Dunnett's post hoc test for multiple comparisons was used to detect the differences in viral transductions (**III**).

5. Results

Endocrine treatment is the standard treatment for advanced prostate cancer and this therapy has been used for decades. Still, the molecular consequences of the treatment are poorly known. In this study, we utilized rare clinical material from neoadjuvant treated prostate cancer patients, and determined genome wide gene expression differences between non-endocrine treated and two endocrine treated patient groups. We also determined the *TMPRSS2:ERG* fusion status from the samples and detected the effects of the fusion in non-treated and endocrine treated samples.

Viral gene therapy is a potential treatment application against CRPC and other aggressive cancers, but the transduction efficiency often remains too low. In this study, we determined the potency of cell-permeable peptides for enhancing viral transduction efficiency.

5.1 Validation of predictive expression values from DSection analysis (I)

Because the amount of cancer tissue in samples varied between 0% and 85%, we utilized an *in silico* Bayesian modeling tool, DSection (Erkkilä et al., 2010), to predict cancer cell specific expression of mRNAs and microRNAs. To confirm the predicted expression values from DSection, we laser-capture microdissected cancer and stromal compartments from 11 tissue samples, extracted RNA and ran the qPCR analyses for 10 differently expressed genes. Strikingly, 9 of the 10 genes exhibited similar expression profiles in DSection and qPCR analysis, both in cancer and stromal tissue.

5.2 Bicalutamide and goserelin treatments initiate differential mRNA and microRNA expression profiles (I, II)

The antiandrogen bicalutamide and the GnRH agonist goserelin are both commonly used endocrine treatments for prostate cancer. Here, we detected how these two treatments affect mRNA and microRNA expression profiles in cancer tissue. To determine the most differently expressed mRNAs, we used a fold change (FC) >2 and a false discovery rate (FDR) <0.01 as thresholds. Because the expression differences of microRNAs are naturally smaller than mRNAs, we used FC >1.4 and FDR <0.05 as thresholds for microRNAs.

We detected great differences in expression profiles between bicalutamide and goserelin treatments in both mRNA and microRNA expression levels (Fig. 7). Altogether, only 16% and 19% of the most differentially expressed mRNAs and microRNAs, respectively, were common for both treatments.

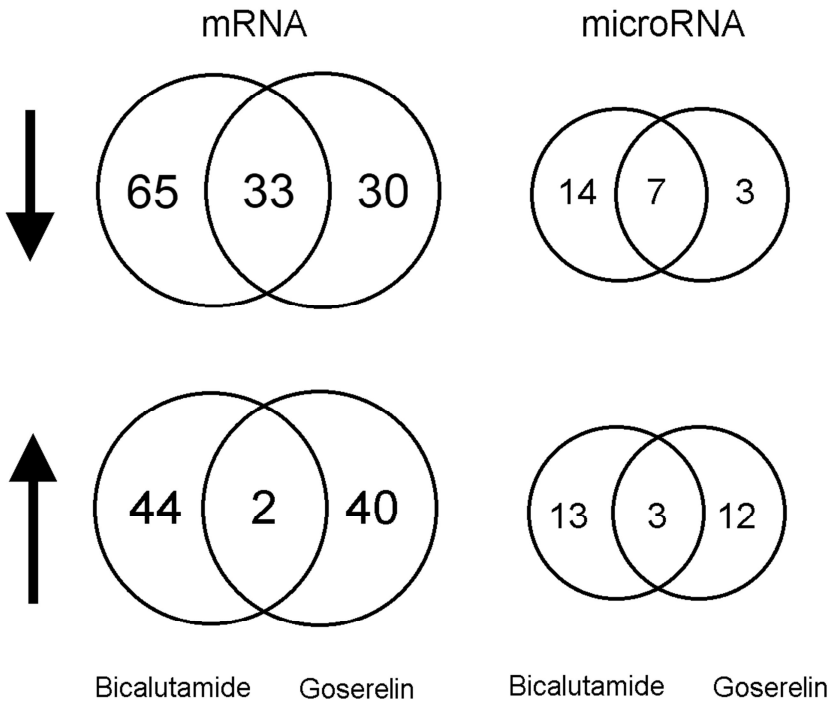

Fig. 7. *Number of up- and down-regulated (solid arrows pointing up and down, respectively) mRNAs and microRNAs after bicalutamide and goserelin treatments compared to non-treated control. FC >2, FDR <0.01 for mRNAs and FC >1.4, FDR <0.05 for microRNAs. Figure is modified from original articles I and II.*

5.2.1 Androgen regulation and relevance to prostate cancer

Bicalutamide and goserelin treatments inhibit the activity of AR in cancer tissue. Therefore, we investigated whether mRNAs and microRNAs with reduced expression levels after treatment were directly androgen regulated.

First, we utilized our recent data from the DHT stimulated LNCaP-based cell line models and the VCaP cell line (Waltering et al., 2009). We detected over 2-fold upregulation in 30-38% of those mRNAs for which expression was reduced after the treatments. In addition, from those microRNAs which expression was reduced after endocrine treatments, miR-141 and miR-32 were over 1.5-fold upregulated in the LNCaP and VCaP cell lines treated with DHT (Waltering et al., 2011).

By utilizing recent ChIP-seq data from the same LNCaP model (Urbanucci et al., 2011) and from the VCaP cell line (Yu et al., 2010), we determined AR binding sites (ARBSs) from which AR can potentially regulate the closest genes. To confirm the active ARBS further, we utilized histone methylation data from two independent datasets (Yu et al., 2010, He et al., 2010). Approximately one-half of the mRNAs with reduced expression after endocrine treatment contained ARBS closest to their transcription start site (TSS). In addition, four microRNAs (miR-32, miR-30d, miR-30b and miR-17) had ARBS closest to them, closest to the cluster where they are located, or closest to the protein coding gene that regulates the microRNA expression.

Altogether, we detected 24 mRNAs and one microRNA (miR-32) that fulfilled the criteria of reduced expression after endocrine treatment, ARBS close to the TSS and DHT induction in cell culture models (Table 3). These transcripts are the most probably expressed from directly AR-regulated genes in prostate cancer tissue.

Furthermore, we wanted to assess the possible relevance of the most differently expressed genes to prostate cancer. To analyze the differentially expressed mRNAs, we utilized expression data from 13 separate microarray studies of prostate cancer presented in the Oncomine database (www.Oncomine.org, accessed May 2011). With a threshold p-value of 0.01 and FC>1.5, we determined that 41-61% of the genes with the most reduced expression after the treatments were upregulated in prostate cancer compared to BPH at least in one of the 13 studies.

Moreover, we identified differentially expressed microRNAs in prostate cancer from two publicly available sample sets. One contained 102 samples from normal

Table 3. Genes with reduced expression after endocrine treatments. Table is modified from original articles I and II.

Gene name	>2-fold reduced expression[a]	DHT regulation *in vitro*[b]	ARBS Urbanucci et al.[c]	ARBS Massie et al.[c]	H3K4me1 and ARBS overlap[d]	NSD score and ARBS overlap[d]
FKBP5	both	LNCaP and VCaP	+	+	1	1
TMEFF2	both	LNCaP	+	-	-	1
FAM110B	both	LNCaP	+	+	2	1
NEDD4L	bic	LNCaP	+	+	2	1
MME	both	VCaP	+	+	1	1
TMPRSS2	bic	LNCaP and VCaP	+	+	1	1
MBOAT2	bic	LNCaP and VCaP	+	+	-	-
CLDN8	bic	LNCaP and VCaP	-	+	1	1
TBC1D8	bic	LNCaP and VCaP	+	+	-	2
HOMER2	bic	LNCaP and VCaP	+	-	1	1
DHCR24	bic	LNCaP and VCaP	+	+	1	1
KLK4	bic	LNCaP	-	+	1	-
RAB3B	bic	LNCaP	+	+	-	2
BRP44	bic	LNCaP	+	+	2	-
C1orf116	bic	LNCaP	+	+	2	-
TPD52	bic	VCaP	+	+	2	1
RDH10	both	LNCaP and VCaP	-	+	-	1
KCNN2	both	LNCaP	+	+	1	1
LCP1	bic	LNCaP	+	+	1	2
PMEPA1	bic	LNCaP	+	+	2	2
KHDRBS3	gos	VCaP	-	+	1	-
LAMA3	gos	LNCaP	+	+	1	1
ATAD2	gos	LNCaP	+	+	-	-
hsa-miR-32	bic	LNCaP	+	N/A	N/A	N/A

[a] Reduced expression after bicalutamide (bic) or goserelin (gos) or both treatments. Fold change >2, false discovery rate <0.01. [b] Genes were considered as dihydrotestosterone (DHT) regulated if they were upregulated at least 2-fold in the cell lines after 4 or 24 hours of DHT treatment. [c] Genes are considered to contain ARBS if the gene is located closest to the ARBS according to the data of Urbanucci et al.(2011), and if the ARBS is found from the area of 25kb from TSS according to the data of Massie et al. (2011). [d] H3K4me1 data are from Yu et al. (2010) and NSD scores are calculated as in the study of He et al. (2010). Numbers indicate the number of studies (Urbanucci et al. or Massie et al.), where ARBS overlap with methylation peak.

adjacent prostate and normal lymph node and their cancerous counterparts (Martens-Uzunova 2011), and the other contained 54 BPH, normal prostate cancer or CRPC samples altogether (Jalava et al., 2012). From the 24 microRNAs with >1.4-fold reduced expression, 14 were upregulated in prostate cancer or CRPC compared to BPH or normal prostate.

5.2.2 Potential biomarkers

Novel prognostic markers for prostate cancer are urgently needed. We hypothesized that if AR-regulated genes whose expression is reduced following endocrine therapy are reactivated in a castration resistant disease stage, they could function as prognostic markers for the formation of CRPC.

To test our hypothesis, we selected four AR-regulated genes that were downregulated after the endocrine treatments. We detected the expression levels of the genes from an independent set of BPH, primary prostate cancers and CRPC samples by qPCR. Two of the genes, *TPD52* and *NEDD4L* were indeed significantly upregulated in both untreated prostate cancer and CRPC samples.

To determine the potency of microRNAs as progression markers, we utilized a set of 99 primary prostate cancer samples with patient histories (Taylor et al., 2010). First we focused on to the microRNAs that had reduced expression levels after endocrine treatments and were upregulated in primary prostate cancer and/or CRPC. From this group we determined that the high expression levels of five microRNAs (miR-141, miR-30d, miR-210, miR-375 and miR-130b) predicted significantly poorer progression free survival. Next, we focused on the group of microRNAs with increased expression after the endocrine treatments and downregulated expression in primary prostate cancer and/or CRPC. From this group, we determined that low expression of four microRNAs (miR-204, miR-125b, miR-100 and miR-135a) predicted significantly poorer progression free survival.

5.3 Effect of the *TMPRSS2:ERG* fusion on hormone naïve and endocrine treated prostate cancers (I, II)

To assess the effects of the *TMPRSS2:ERG* fusion on hormone naïve and endocrine treated patients, we determined fusion status from the neoadjuvant endocrine trial samples with FISH and IHC assays. Approximately 60% of the samples were fusion positive. Due to the small amount of samples, we combined the bicalutamide treated and goserelin treated samples as one endocrine treated group. Thus we created four subgroups: the fusion-positive (F+) cases and the fusion-negative (F-) cases in the control group, and the F+ cases and the F- cases in the treatment group.

We first determined how the fusion affects mRNA and microRNA expression levels in hormone naïve prostate cancers. From protein coding genes, we detected 869 genes with increased expression and 67 genes with decreased expression in the F+ cases compared to the F- cases (FC>1.6). With microRNAs, however, the expression differences were not as clear. In the F+ cases compared to the F- cases in the control group (FC>1.4) 23 microRNAs had increased expression, and 24 microRNAs had decreased expression.

Subsequently, we determined how endocrine treatment affects the F+ and F- cases. In the F- cases the treatment reduced the expression of only 69 protein coding genes, but strikingly, the expression levels of 601 protein coding genes were reduced in the F+ cases. Thus, the expression of ERG brings a significant amount of additional genes under AR regulation. Again, expression differences were not similarly remarkable with microRNAs.

We performed an unsupervised hierarchical clustering analysis for all mRNA genes with >1.5-fold differential expression between the subgroups and for all microRNAs with expression over threshold (239 microRNAs). Interestingly, we detected that the four subgroups clustered in the same manner with mRNAs and microRNAs (Fig. 8). Both clustering analyses showed that the F+ cases in the control group clustered separately into one arm and other subgroups into another arm. Thus, endocrine treatment seems to diminish the differences between the F+ and F- cases.

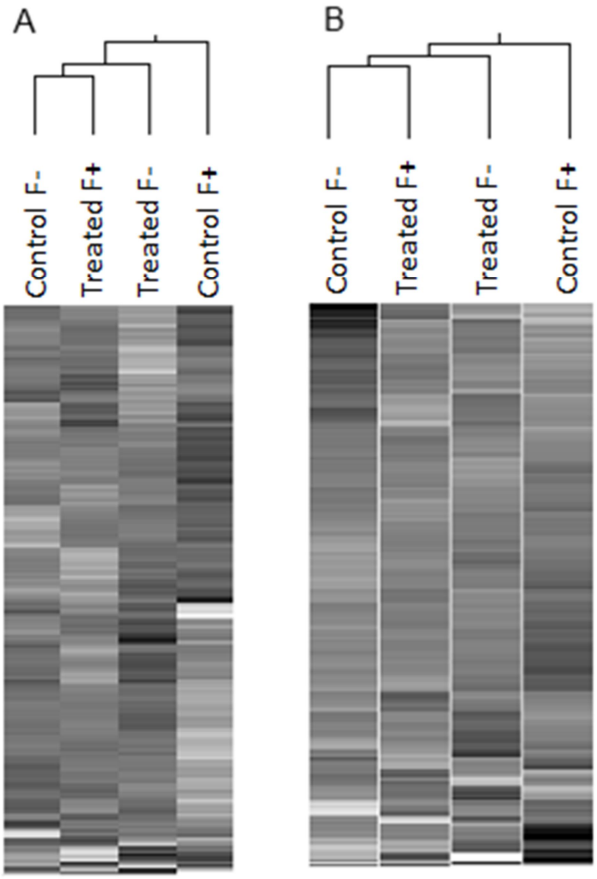

Fig. 8. *Unsupervised clustering analyses of mRNAs* ***(A)*** *and microRNAs* ***(B)****. Both clustering analyses show similar distribution of the treatment groups. F-, fusion-negative; F+, fusion-positive. Figure is modified from original article II.*

5.3.1 AR and ERG binding sites in *TMPRSS2:ERG* positive samples

To assess possible direct AR and ERG regulation of the most differently expressed genes in the F+ cases, we utilized publicly available ChIP-seq data from the *TMPRSS2:ERG* fusion-positive VCaP cells (Yu et al., 2010). By reanalyzing the data we determined the ERG binding sites for 68% and 41% of all protein coding genes and microRNAs, respectively. The AR binding sites were fewer, 25% for all protein coding genes and 20% for all microRNA genes. Over 90% of the genes that had an AR binding site also contained an ERG binding site.

Interestingly, we detected significant enrichment of binding sites in certain groups of genes. First, in the group of 869 genes that had increased expression in the F+ cases compared to the F- cases in hormone naïve tumors, 85% of the genes contained an ERG binding site and 36% had an AR binding site (p=3.3e-25 and p=

1.8e-25, respectively, Fisher's exact test). Second, a similar enrichment was detected in the group of 601 genes with decreased expression in the F+ cases after the endocrine treatment, where 86% of the genes contained an ERG binding site, and 40% contained an AR binding site (p=4.4e-21 and p= 6.2e-27, respectively). With microRNAs, the statistically significant enrichment of ERG binding sites was not detected, most probably due to the small number of microRNAs. AR binding sites were enriched in a group of microRNAs with increased expression after the endocrine treatment in the F+ cases (64% $p<0.04$).

Taken together, AR binding seems to be enriched in the same group of genes that are ERG regulated, and endocrine treatment reduces most of the expression from the genes that are both AR and ERG regulated.

5.3.2 Ontology analysis

To determine the identities of the 869 genes with increased expression in the F+ cases compared to F- cases in hormone naïve tumors and the 601 genes with reduced expression in F+ cases after the endocrine treatment, we performed an ontology analysis for the groups. Interestingly, all significantly enriched ontology groups in both groups were related to the proliferation: e.g., "cell cycle", "M phase" and "mitosis" (a cut-off of >7 genes and an adjusted $p<1.0e-4$) (Fig. 9).

5.4 Cationic peptides and small compounds enhance viral gene transfer in prostate cancer and other cancer cells (III)

Viral gene therapy is a promising novel treatment method for CRPC and other cancer types that lack curative treatment. The major hurdle of truly effective therapy is still insufficient gene transfer efficiency. Small cationic compounds can be used to enhance viral attachment to a cell membrane and thus enhance transduction. In this study, we determined weather cationic CPPs would boost viral transduction more efficiently than other cationic compounds.

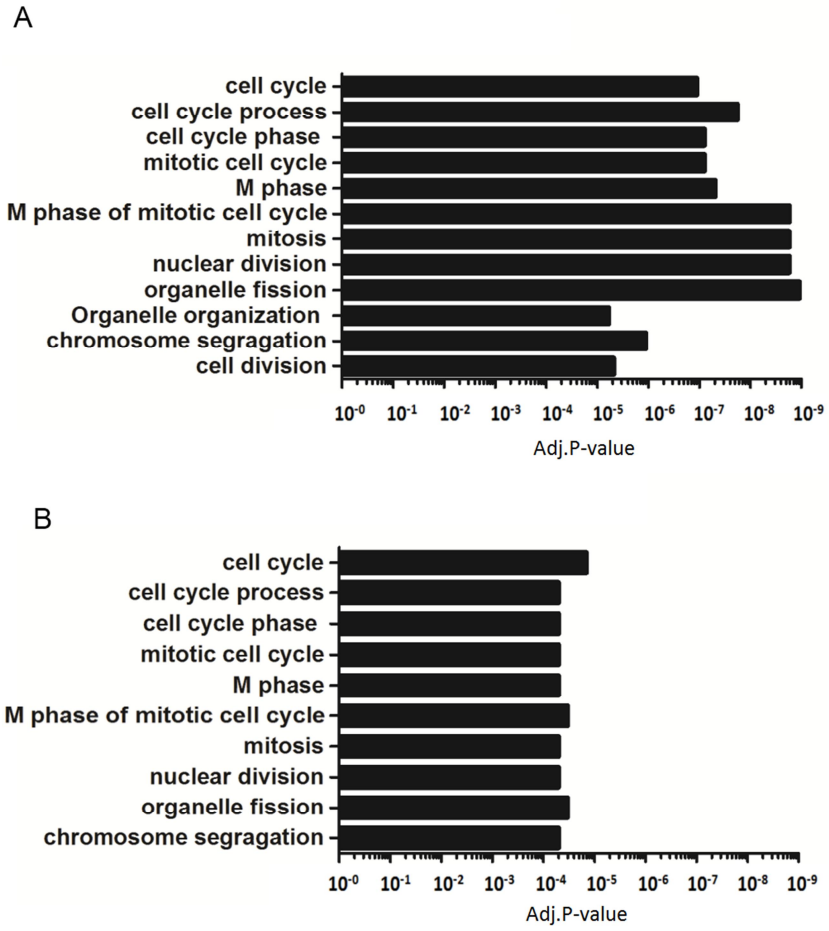

Fig. 9. *Significantly enriched ontologies.* ***(A)*** *The group of 869 genes with increased expression in fusion-positive cases compared to fusion-negative cases in hormone naïve prostate cancer.* ***(B)*** *The group of 601 genes with decreased expression after endocrine treatment in fusion-positive cases. All ontologies appeared to be related to cell proliferation. Figure is adapted from original article I.*

We transduced different cancer cells lines with adeno- or lentiviral vectors combined with several different cationic compounds. Cationic CPPs enhanced both adenoviral and lentiviral gene transfers to all cancer cell lines. Antp was a more efficient enhancer than were TAT-peptides. However, the small compounds polybrene and protamine sulfate increased efficiency similarly to or even more so than CPPs (Fig. 10).

In conclusion, in studies performed *in vitro* cationic peptides and small compounds are useful tools to enhance viral transduction efficiency. However, due to better efficiency and smaller cost, polybrene and protamine sulfate are potentially better enhancers than are cationic peptides.

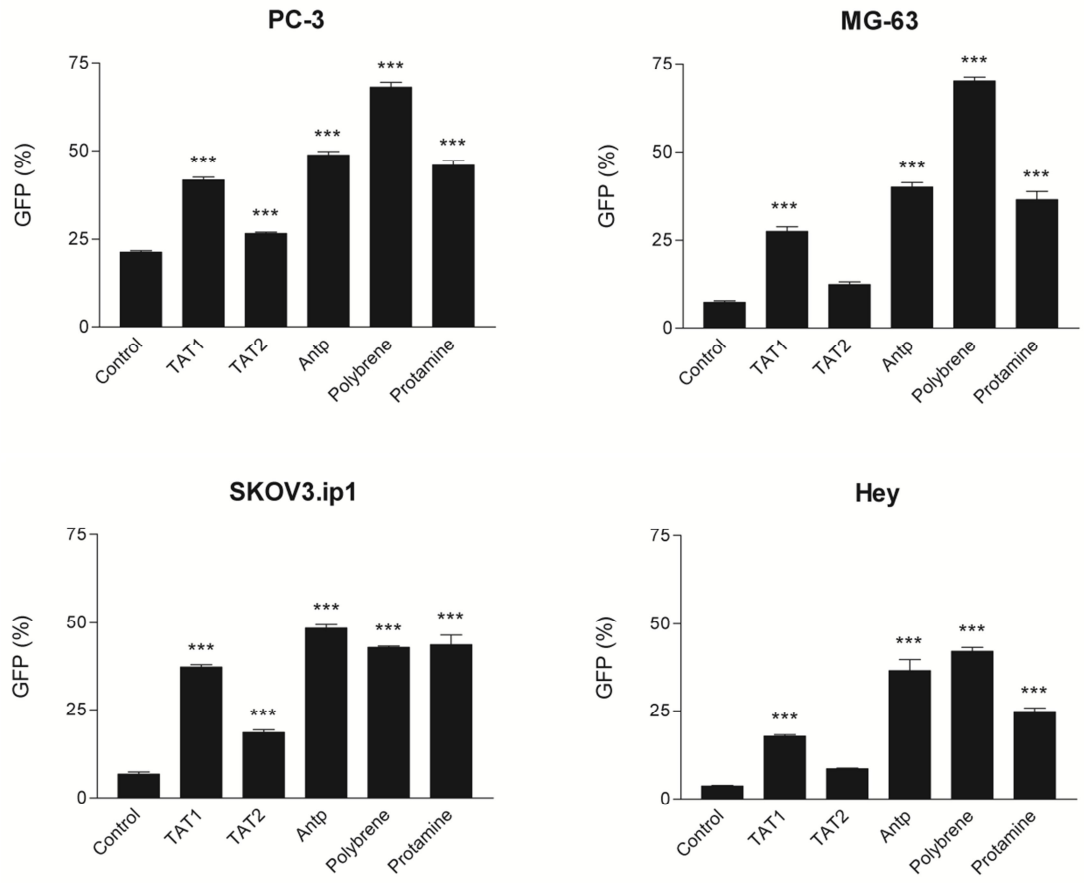

Fig. 10. *Percentage of adenoviral transduced cells (GFP-positive) using different cationic compounds and peptides in prostate (PC-3), osteosarcoma (MG-63) and ovarian (SKOV3.ip1 and Hey) cancer cell lines. Similar results were also obtained with lentivirus. Figure is adapted from original article III.*

6. Discussion

In this study, we determined the molecular differences between two commonly used endocrine treatments and verified how the *TMPRSS2:ERG* fusion affects the treatment response in terms of transcriptome levels. We also detected AR- and ERG-regulated genes from these rare, neoadjuvant treated tissue samples. Moreover, we enhanced viral-mediated gene transfer with cationic peptides and small compounds to determine their potency as a novel treatment methods against prostate and other cancers.

6.1 Differential expression after bicalutamide and goserelin treatments

We detected clear differences in mRNA and microRNA expression levels between bicalutamide and goserelin treatments. The GnRH agonist goserelin reduces androgen secretion from the testes, whereas the antiandrogen bicalutamide prevents DHT binding to AR. Both therapies reduce the activity of AR, but as our study shows, molecular consequences differ remarkably.

Mostaghel et al. (2007) and Holzbeierlein et al. (2004) have also previously measured gene expression levels after medical castration. When we compared the most differentially expressed genes, few genes were common between our, Mostaghel's and Holzbeierlein's studies. The lack of common differentially expressed genes could be explained by the use of different drugs or combinations of drugs in the three studies. Naturally, different sample sets and data analysis may also contribute to the differences observed in these studies. Taken together, these results show that different drugs may have remarkably different effects in transcriptome level, although the clinical outcome remains the same.

6.2 Potential biomarkers

Novel prognostic markers for prostate cancer are urgently needed. In this study, we determined microRNA genes and protein coding genes as potential prognostic markers. We showed that the expression of *NEDD4L* and *TPD52* were decreased after bicalutamide treatment and increased again in the castration resistant disease stage. In addition, we determined that the expression of miR-125b was increased after both endocrine treatments and decreased in CRPC compared to BPH. Moreover, the low expression of miR-125b predicted faster progression.

The ubiquitin protein ligase NEDD4L is a known regulator of epithelial sodium channels. NEDD4L has also been shown to ubiquitinate TGFβ-activated Smad2 and Smad3 proteins (Gao et al., 2009), epithelial growth factor receptor substrate 15 (EPS15) (Woelk et al., 2006) and prostate transmembrane protein PMEPA1 (Xu et al., 2003). The NEDD4L pathway appears to be highly androgen regulated; in addition to PMEPA1, SGK, the activator of NEDD4L, is also AR-regulated (Shanmugam et al., 2007), and Smad3, the target of NEDD4L, has been shown to be an AR corepressor that acts via direct interaction with AR (Hayes et al., 2001).

Tumor protein D52 (TPD52) is known to be overexpressed in prostate cancer and in several other cancers, including breast and ovarian cancers (Rubin et al., 2004). Androgens have been shown to enhance the expression of TPD52, and an androgen responsive element (ARE) has been found 600 bp upstream of the gene TSS (Nelson et al., 2002; Rubin et al., 2004). The higher expression of TPD52 in castration resistant metastases compared to benign controls has also been shown (Wang et al., 2007).

We also identified several microRNAs that could potentially function as prognostic biomarkers. One of these microRNAs was miR-125b, which has been confirmed to be downregulated in prostate cancer compared to benign prostate tissue in several studies by different microarray platforms, deep-sequencing and qRT-PCR (Ozen et al., 2008; Schaefer et al., 2010; Sun et al., 2011). miR-125b has also been shown to be androgen regulated in LNCaP cells, and the AR binding site close to the microRNA has been defined with ChIP-qPCR (Shi et al., 2011). The same study also suggested that miR-125b inhibits the apoptosis regulator Bak1 and thus functions as an oncogene.

Although these molecules behaved as potential progression markers, our preliminary results need to be validated with larger clinical sample sets and the expression data should be compared with overall prognosis and patient survival before final conclusions are made.

6.3 The *TMPRSS2:ERG* fusion

6.3.1 ERG-regulated genes

ERG is a transcription factor that has been shown to bind to the promoter area of 68% of the known genes in a VCaP cell line (Yu et al., 2010). The *TMPRSS2:ERG* fusion brings the expression of *ERG* under AR regulation. In this study, endocrine treatment reduced the expression of 601 genes in the F+ cases and only 69 genes in the F- cases. Decreased activity of AR reduces the activity of ERG in the F+ cases, and thus, those 601 genes are supposedly directly ERG-regulated. Indeed, ERG binding site analysis from the VCaP cell line showed that 86% of these genes harbor ERG binding sites. The ontology analysis of these 601 genes revealed significant enrichment in cell proliferation related ontologies.

Recently, Yu et al. (2010) reported that AR and ERG chromatin binding profiles overlap. Additionally, they reported that ERG may reduce AR activity, and in the presence of androgens, the knock-down of ERG enhances the expression of AR-regulated genes. We approached the relationship of ERG and AR by determining the effects of reduced AR activity by endocrine therapy to the F+ and F- samples. Interestingly, our data propose more synergistic roles for AR and ERG. Here we determined that co-activity of AR and ERG enhanced the expression of their target genes, as in the F+ cases in the control group. Moreover, reducing the AR activity by endocrine therapy reduced the expression of 601 genes in the F+ cases. This equaled 8.7 times more genes than in the F- cases. Thus, we propose that the *TMPRSS2:ERG* fusion brings notably more genes under AR regulation.

Similar to the expression of mRNAs, the expression of microRNAs was also greatly determined by the *TMPRSS2:ERG* gene fusion. In addition, a fundamental difference appeared in the quantity and quality of the microRNAs that responded to

the endocrine treatment. The difference was dependent on the fusion status of the sample.

6.3.2 Endocrine therapy reduces the differences between the F+ and F- cases

Clustering analysis of mRNAs and microRNAs revealed that the F+ and F- cases clustered into different arms before treatment but into the same arm after treatment. Moreover, endocrine treatment reduced the expression of the most differently expressed genes between the F+ and F- cases in the non-treated control group. Thus, endocrine treatment diminishes the differences between the F+ and F- cases, and may explain why F+ and F- patients achieve similar recurrence free survival rates after endocrine treatment (Leinonen et al., 2010).

6.4 The efficiency of cell-permeable peptides

To determine the efficacy of CPPs for boosting viral gene transfer, we transduced different cancer cell lines with lenti- and adenoviral vectors complexed with cationic compounds. Despite several studies, it still remains unclear whether CPPs penetrate into cells passively or via active endocytosis (Vives et al., 2008). Our study suggests that the efficacy of CPPs is simply dependent on their positive charge, as cationic polybrene and protamine sulfate were equally good or even better transduction enhancers than CPPs. It is likely that both CPPs and cationic small compounds function as cationic bridges between anionic viral membranes or capsids and anionic cell membranes, thus increasing the physical interaction of viruses and cells. Our results do not support the hypothesis of active endocytosis as the intake mechanism, although it may play a role with smaller cargo compounds.

Cationic CPPs did enhance transduction efficiency of both viral vectors, but to a lesser extent than was obtained by Gratton et al. (2003) and was obtained with polybrene or protamine sulfate in our study. Possibly due to poor cost-effectiveness, the CPP-virus combination has not been used in further gene therapy applications. Instead, Tat and Antp peptides have been fused to the ectodomain of the coxsackievirus-adenovirus receptor (CAR) (Kuhnel et al., 2004). By incubating the

Adenovirus vectors with CAR-Tat or CAR-Antp fusion proteins, Kuhnel et al. (2004) were able to facilitate Ad transduction to CAR-negative cells more efficiently than by incubating viruses with Tat peptide alone. Moreover, Han et al. (2007) fused Tat-peptide directly to the adenoviral fiber knobs and enhanced Ad transduction to both high- and low-CAR containing cells *in vitro* and *in vivo*.

Although gene therapy applications are still far from clinical success, the potential is high. Oncolytic adenoviruses are the most promising vector types for cancer gene therapy, although the expression of the adenoviral receptor, CAR, is commonly low in CRPC. Thus, widening the tropism of Ad vectors (e.g., by adding Tat peptide to its fiber knob) may be a useful application for gene therapy against CRPC.

6.5 The future of prostate cancer treatment

Treatment options for CRPC have increased remarkably in the last two years. In 2010-2011, four new treatment methods, namely, cabazitaxel, abiraterone, Sipuleucel-T and alpharadin proved their efficacy in phase III clinical trials and achieved FDA approval as novel treatment methods against CRPC. In addition, the results from a phase III clinical trial of MDV3100 are expected to be published during the year 2012, and two dozen novel agents are in phase III clinical trials against metastatic CRPC (Seruga et al., 2011). Although most of them will fail to show any efficacy, several may prove their potency and could increase patient survival time.

Ahead of several novel treatment methods races the question of optimal order of drug administration. Sipuleucel-T has demonstrated its efficiency in chemotherapy naïve CRPC, while other compounds have been studied only in patients with docetaxel resistant CRPC. In which order should the novel drugs be administered to obtain the most effective results? Here we once again confront the lack of biomarkers. Analytically validated and clinically qualified biomarkers can help in choosing the right treatment that would most benefit the patients (Yap et al., 2011). This study here presents some new potential biomarkers for further validation.

It is also likely that the most efficient treatments can be achieved by combinatorial regiments. Combination can be chosen either “vertically” such that

drugs act along the same pathway, or "horizontally" such that drugs target parallel malfunctioning pathways (Yap et al., 2011). Naturally, to obtain the most effective results, this requires information regarding malfunctioning pathways in each individual - in other words, personalized medicine.

6.5.1 Personalized medicine

Personalized medicine can be defined as a treatment based on the molecular characteristics of an individual patient (Wistuba et al., 2011). Practically, this approach requires molecular characterization of a tumor from each patient to select the most optimal treatment method (Wistuba et al., 2011). There are several novel technologies available for the molecular characterization of a tumor; of these, deep-sequencing is perhaps the most promising.

Several successful stories from targeted therapies already exist. For example, a systematic genome-wide screening of potential oncogenes led to the discovery of a single mutation point in *BRAF*, which is a member of RAS–RAF–pathway and a well-known oncogene (Davies et al., 2002). The mutation was shown to occur with high frequency in malignant melanoma and with lower frequency in several other cancers. This finding led to the development of a mutation-specific BRAF inhibitor which showed exceptional treatment response in patients with BRAF mutated advanced metastatic melanoma (Flaherty et al., 2010).

Another example is a finding from 2007, when the echinoderm microtubule-associated protein-like 4 (*EML4*) gene was shown to be fused with the anaplastic lymphoma kinase (*ALK*) gene in 6.7% of non-small-cell lung cancer (NSCLC) patients (Soda et al., 2007). Although the percentage of patients is small, an ALK inhibitor has demonstrated excellent efficiency and results in increased survival with the subset of NSCLC patients harboring the *EML4-ALK* fusion in the first clinical trials (Kwak et al., 2010; Shaw et al., 2011).

For the most effective treatment choice, reliable biomarkers are essential. Most biomarkers are used to detect only a single genetic mutation, amplification or translocation (Wistuba et al., 2011). Recently, however, some multigene biomarkers, - so called "gene signatures" - have been accepted for clinical use. The most

commonly used are the 70-gene MammaPrint and the 16-gene OncotypeDX test for the prognosis of early-stage breast cancer (Majewski and Bernards, 2011).

Prostate cancer, like any other cancer, is a group of genetically heterogeneous diseases. Molecular differences that cannot be revealed by histological analysis can vary greatly from one tumor to another. This kind of differences were also observed in this study, which revealed significant differences in gene expression between the *TMPRSS2:ERG* fusion-positive and fusion-negative samples. A growing body of evidence shows that molecular alterations can affect treatment response. For example, the *TMPRSS2:ERG* fusion has been associated with higher PSA decline in abiraterone treated patients (Attard et al., 2009). The clinically used PARP1 inhibitor Olaparib significantly reduced the growth of *TMPRSS2:ERG* fusion-positive but not fusion-negative xenografts in mice (Brenner et al., 2011). In an *in vivo* *Pten*$^{-/-}$ prostate cancer model, co-inhibition of the AR and PI3K pathways by MDV3100 and BEZ235 caused remarkable cancer regression compared to single-pathway therapies (Carver et al., 2011).

The lack of patient grouping may well have been a reason why some promising novel compounds have failed to show statistically significant treatment results in large clinical trials. If a drug functions only for patients with a certain relatively rare genetic alteration, those effects can be lost in a large group of patients lacking the alteration. The variation between patients regarding treatment response is clear. For example, in phase III clinical trials of docetaxel only half of the men experienced >50% decline in serum PSA and one-quarter experienced improvements in quality of life (Petrylak et al., 2004; Tannock et al., 2004). Docetaxel, cabazitaxel, sipuleucel-T and abiraterone are all novel drugs that increase the *mean* overall survival by approximately three months each. This result means that a portion of patients obtain a substantially longer-lasting response while a large portion of patients do not experience improvement of their condition. By better understanding the molecular consequences of current and novel drugs, we could offer the most effective ones for individual patients, which could lead to better treatment response, reduced side effects of poorly functioning drugs and cost savings.

The use of the PSA test has dramatically increased prostate cancer diagnosis, but has had little effect on mortality rates (Schröder et al., 2009). The natural history of prostate cancer is highly variable and difficult to predict. Gleason score, tumor stage, margin status and PSA concentration are used to predict disease outcome; at best,

they can achieve 75-85% accuracy (Han et al., 2003). Recently, however, Cuzick et al. (2011) showed that the signatures of 31 genes related to cell cycle progression more accurately predicted biochemical recurrence and death from prostate cancer than Gleason score, PSA concentration or pathological stage.

In prostate cancer, molecular based groupings of patients have not yet been used in clinical trials due to a lack of molecular biomarkers. Hopefully, in the near future, this deficiency will change.

6.5.2 The future of gene therapy

The concept of gene therapy is ambitious, but unfortunately the efficiency of gene therapy applications in clinical trials in general has remained modest. However, the promising results from individual patients (e.g., Cerullo et al., 2010; Freytag et al., 2007c), help maintain hope. Perhaps personalized medicine and the genome wide analysis of patients and their malfunctioning organs will explain the variation in the efficiency between patients. The most optimal gene therapy application could then be selected individually.

Naturally, improvements in viral vectors and treatment strategies are also needed. At the moment, immunological approaches hold the greatest promise, due to the success of Sipuleucel-T and PROSTAVAC-VF in clinical trials. Boosting or "guiding" the immune system of a patient to destroy malignant cells, may have sound like science fictions couple of years ago, but this strategy has now proven its efficiency in large, randomized phase III clinical trials. Perhaps some other applications that now sound fully unrealistic will become normal treatment methods after one or two decades.

6.6 Conclusions

In this study, we utilized rare clinical material and studied the effects of two commonly used endocrine treatments on the prostate cancer transcriptome. We have identified 24 direct target genes of AR and 601 genes that become AR targets via the *TMPRSS2:ERG* fusion. We have also identified the effect of endocrine therapies and the *TMPRSS2:ERG* fusion on the expression of microRNAs. In addition, we have

evaluated the potency of cationic compounds for enhancing viral gene transfer to prostate cancer and other cancer cell lines.

This study brings novel information regarding the influence of traditional endocrine treatments in the transcriptome of prostate cancer cells, and presents possibilities for enhancing novel gene therapy methods for the efficient treatment of prostate cancer. Increased understanding of the molecular biology of prostate cancer has finally led to the approval of more efficient novel drugs against CRPC. In the near future, we hope to increase the survival of CRPC patients such that the life-threatening character of this disease disappears.

7. Acknowledgements

This doctoral research was initiated in the University of Eastern Finland (former University of Kuopio), A.I. Virtanen Institute for Molecular Sciences, in docent Jarmo Wahlfors' (PhD) research group in 2004, and continued in the University of Tampere, Institute of Biomedical Technology in Prof. Tapio Visakorpi's (MD, PhD) group from 2007- 2012. The Graduate School of Molecular Medicine of A.I. Virtanen Institute and the Tampere Graduate Program in Biomedicine and Biotechnology are acknowledged for providing both financial support and possibilities to proceed with my studies in various useful courses.

I am sincerely thankful for both of my excellent supervisors, Jarmo Wahlfors and Tapio Visakorpi, for their guidance and support over these years.

I am especially grateful for Jarmo's endless interest in my projects from the very beginning until the end, and the inspiration and enthusiasm that he instilled within me on desperate days. I am also forever thankful for his extremely valuable advices and encouragement during the later phase of my thesis. Without his support, this thesis would still be in process.

I owe the most sincere gratitude to Tapio for the endless trust that he has shown on me, regardless of whether my projects were successful. His habit of keeping his office door open and the easy-to-access atmosphere whenever he is present is highly appreciated. I am also truly grateful to him for providing the project with valuable patient material. That saved my thesis.

I am deeply thankful for all of my coworkers from Kuopio and Tampere. The years in Kuopio were immemorial, thanks to a great JW-group: Riikka Pellinen (PhD), Tiina Wahlfors (PhD), Ann-Marie Määttä (PhD), Outi Rautsi (PhD), Anna Ketola (MD), Katja Häkkinen (MSc), Tuula Salonen and Marko Björn (BSc). I especially want to thank Riikka for excellent guidance and support in Kuopio and also during my years in Tampere, and Katja for deep friendship and many, many crazy moments that we shared during the time in Kuopio. You are diamonds.

I am also grateful for all the past and present members of Tapsalab, who are too many to mention by name. My special gratitude belongs to Sanni Jalva (MSc) for introductions to many techniques used in the lab. I am also grateful for Alfonso Urbanucci (PhD), Kati Waltering (PhD) and Outi Saramäki (PhD) for sharing many memorable moments outside of the lab. After-work -pints and midnight text messages were sometimes extremely valuable. I also want to thank our skillful technicians Maritta Vakkuri and Päivi Martikainen, for their invaluable assistance in the lab and their great company during lunch breaks.

My warmest appreciation goes to all of my co-authors, who gave true depth to my articles. I especially want to thank the talented "TUT guys", Prof. Harri Lähdesämäki (D.Tech), Timo Erkkilä (MSc Tech), Antti Larjo (MSc Tech) and Janne Seppälä, who turned the impossible into the possible and obtained so much interesting data from our sample material. I truly enjoyed our collaboration. I also want to thank Prof. Teuvo Tammela (MD, PhD), my co-author and thesis committee member, for always bringing a clinical perspective to basic research. It has been appreciated.

My thesis revisers, docent Pipsa Saharinen (PhD) and professor Kimmo Taari (MD, PhD) are sincerely acknowledged for their valuable comments and feedback that improved my thesis.

I am also truly thankful to those 30 men who selflessly volunteered to the clinical trial and provided highly valuable patient samples for research.

I am also deeply thankful for my dear parents for their limitless support. I am thankful to mom especially for regular phone calls and endless interest in my life. For dad: I am deeply thankful to you for being a man in my life, and always present when I need you. I love you both.

My dearest thanks go to my beloved common-law husband and father of our unborn child, Kalle, who has made my life more stable and fulfilled with (bad) humor. I am sorry that everyday life in science is quite boring, but thanks for continuing to ask.

Finally, my deepest gratitude is dedicated to the greatest teacher I have ever had, lama Ole Nydahl, who returned my enthusiasm for molecular biology and motivation into research, and encouraged me to continue my career in science. His example is the greatest inspiration one can ever imagine. I am also forever grateful

for all of the friends on a way, with whom I have shared so many shiny moments fulfilled with laughter and joy.

This study was financially supported by the National Technology Agency of Finland (TEKES), the Kuopio University Foundation, the Pirkanmaa Cultural Foundation, the Reino Lahtikari Foundation, the Sigrid Juselius Foundation, the Medical Research Fund of Tampere University Hospital, AstraZeneca, which provided a non-restricted grant, and the Scientific Foundation of the city of Tampere.

Tampere, May 2012

Saara Lehmusvaara

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9. Original communications

Journal of Pathology
J Pathol (2012)
Published online in Wiley Online Library
(wileyonlinelibrary.com) DOI: 10.1002/path.4027

ORIGINAL PAPER

Chemical castration and anti-androgens induce differential gene expression in prostate cancer

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Abstract

Endocrine therapy by castration or anti-androgens is the gold standard treatment for advanced prostate cancer. Although it has been used for decades, the molecular consequences of androgen deprivation are incompletely known and biomarkers of its resistance are lacking. In this study, we studied the molecular mechanisms of hormonal therapy by comparing the effect of bicalutamide (anti-androgen), goserelin (GnRH agonist) and no therapy, followed by radical prostatectomy. For this purpose, 28 men were randomly assigned to treatment groups. Freshly frozen specimens were used for gene expression profiling for all known protein-coding genes. An *in silico* Bayesian modelling tool was used to assess cancer-specific gene expression from heterogeneous tissue specimens. The expression of 128 genes was > two-fold reduced by the treatments. Only 16% of the altered genes were common in both treatment groups. Of the 128 genes, only 24 were directly androgen-regulated genes, according to re-analysis of previous data on gene expression, androgen receptor-binding sites and histone modifications in prostate cancer cell line models. The tumours containing *TMPRSS2–ERG* fusion showed higher gene expression of genes related to proliferation compared to the fusion-negative tumours in untreated cases. Interestingly, endocrine therapy reduced the expression of one-half of these genes and thus diminished the differences between the fusion-positive and -negative samples. This study reports the significantly different effects of an anti-androgen and a GnRH agonist on gene expression in prostate cancer cells. *TMPRSS2-ERG* fusion seems to bring many proliferation-related genes under androgen regulation.

Keywords: neoplasia; prostatic; neoadjuvant; endocrine therapy; LHRH; *TMPRSS2–ERG*

Received 8 December 2011; Revised 4 February 2012; Accepted 9 March 2012

No conflicts of interest were declared.

Introduction

Prostate cancer is a highly hormone-sensitive malignancy [1]. More than 70 years ago, Huggins and Hodges [2] observed the relationship between testosterone and prostate cancer progression; they also documented the clinical benefits of castration or oestrogen injections in patients with advanced prostate cancer. Thirty years later, the discovery of testosterone reduction in male rats with gonadotropin-releasing hormone (GnRH) treatment [3] quickly led to the development of GnRH agonist therapy for prostate cancer [4]. Several randomized trials have demonstrated the equivalency of chemical and surgical castration on treatment response of prostate cancer [5,6].

Anti-androgen treatment was originally designed to be used in combination with castration. Later, similar survival rates for non-steroidal anti-androgen monotherapy and castration have been reported in patients with locally advanced cancer [7,8]. In metastasized cancers, however, castration is superior to anti-androgen treatment [7]. Therefore, non-steroidal anti-androgen treatment can be used as an alternative to castration for patients with locally advanced prostate cancer [9,10].

Although 95% of patients initially respond to endocrine treatment [11], practically all cancers eventually become resistant to it. Mechanisms such as amplification and over-expression of the androgen receptor (*AR*) gene, mutations of the *AR* leading to promiscuous ligand usage, altered expression of the *AR* coregulators, truncated AR splice variants and the expression of steroidogenic enzymes enabling intracrine testosterone

production have been suggested to mediate the development of castration-resistant prostate cancer (CRPC) [12].

The AR is a transcription factor that regulates the expression of hundreds of genes. However, only one bona fide common AR target gene important in prostate cancer has been identified. The *TMPRSS2–ERG* fusion gene has been identified in up to one-half of all prostate cancers [13]. Despite several studies, the functional mechanism of ERG in prostate cancer progression remains unclear. Recently, Yu *et al* [14] reported that chromatin binding of the ERG and AR transcription factors overlaps and that ERG disrupts AR signalling.

The treatment of CRPC remains a major clinical problem. The mean overall survival after disease progression is only approximately 20 months [15]. However, new potential treatments for CRPC have recently emerged. In addition to docetaxel and cabazitaxel, a novel anti-androgen, MDV3100, and a CYP17 inhibitor, abiraterone, have also demonstrated efficacy for the treatment of CRPC [16,17]. Therefore, the ability to predict treatment response to initial endocrine therapy is important to assess whether additional treatments should be initiated. A decline in prostate-specific antigen (PSA) after endocrine therapy has been reported to predict treatment response [11]. Still, improved biomarkers that can be evaluated at the time of diagnosis are needed.

Despite the wide clinical use of GnRH agonists and anti-androgens, their effects at the transcriptome level are poorly studied. To the best of our knowledge, direct comparisons of the effects of these two treatment modalities on gene expression have not been published. In this study, we utilized rare clinical specimens from neoadjuvant endocrine-treated patients and examined the gene expression patterns induced by the GnRH agonist, goserelin, and a non-steroidal anti-androgen, bicalutamide. In addition, we evaluated the effects of the *TMPRSS2–ERG* fusion on the expression profiles induced by the endocrine treatments.

Methods

Clinical samples

A randomized clinical trial comparing the neoadjuvant GnRH analogue and an anti-androgen was conducted at the Tampere University Hospital in Finland between 2004 and 2006. Twenty-eight men with localized prostate cancer were randomized into three groups: no treatment (11 men); anti-androgen (bicalutamide, 150 mg/day administered orally for 12 weeks, 9 men); or GnRH agonist (goserelin acetate, 3.6 mg administered by subcutaneous injection every 4 weeks for 12 weeks, 8 men) (see Supporting information, Table S1; clinicopathological characteristics of the cases are found in Table S2). After neoadjuvant endocrine treatment (or no treatment), patients underwent a radical prostatectomy. Fresh specimens from prostatectomies were embedded in Tissue-Tek® (Sakura, Alphen aan den Rijn, The Netherlands) and frozen in liquid nitrogen. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Adjacent sections before and after RNA extraction site were also cut and stained with haematoxylin and eosin (H&E). The stained slides were scanned and visualized with a virtual microscope system [18] and the amounts of cancer, benign epithelium and stroma in the specimens were assessed. A tissue microarray (TMA) was constructed from formalin-fixed, paraffin-embedded prostatectomy specimens.

The second set of samples (see Supporting information, Table S1) was obtained from the Tampere University Hospital (Tampere, Finland). The specimens were confirmed to contain $> 70\%$ of malignant or non-malignant epithelial cells using H&E-stained slides. Total RNA was extracted from the frozen sections with Trizol (Invitrogen), and first-strand cDNA synthesis was performed using SuperScript III reverse transcriptase (Invitrogen) and random primers (Fermentas, Glen Burnie, MA, USA).

The use of clinical material in this study was approved by the Ethical Committee of the Tampere University Hospital. Written informed consent was obtained from the patients.

Expression profiling

Microarray hybridization was performed in the Finnish Microarray Centre at the Turku Centre for Biotechnology, Turku, Finland. First, 300 ng RNA was amplified using the Illumina RNA TotalPrep Amplification kit (Ambion, Austin, TX, USA), followed by cRNA hybridization with Illumina's Human HT-12 Expression BeadChip, v 3 (targeting $> 25\,000$ annotated genes), according to the manufacturer's instructions. Finally, microarray chips were scanned with the Illumina BeadArray Reader, BeadScan software v 3.5. (submitted to Array Express, Ref ID E-MEXP-3081).

In silico data analysis

Because the amount of cancer in the prostatectomy specimens varied (0–85%), an *in silico* Bayesian modelling tool was used to predict the gene expression in different tissue compartments [19]. Briefly, using the percentages of different cell types for each sample, DSection analysis calculated the expression values for each probe in benign epithelia, stroma and malignant cells. Before DSection analysis, the samples were normalized. First, the probes with a value of < 100 in all of the samples were excluded. Second, the average and standard deviation (SD) of the probes in every sample were calculated separately and defined to 0 and 1, respectively. DSection analysis calculated the average expression values of the individual genes for each treatment group and each tissue compartment. Therefore,

rather than assessing the differences between individuals, the expression differences between treatment groups were quantified.

Microdissection

Freshly frozen slides from the prostatectomy specimens were stained with HistoGene Staining Solution (Arcturus Bioscience, CA, USA). Both the cancerous and stromal tissue compartments were obtained by laser capture microdissection (Arcturus, Veritas). RNA from the microdissected samples was extracted with the PicoPure RNA Isolation Kit (Arcturus Bioscience), according to the manufacturer's instructions, and followed by first-strand cDNA synthesis as described above.

qRT–PCR

For qRT–PCR analysis, the Maxima SYBR Green/Rox qPCR Master Mix (Fermentas) and CFX96 Real-Time System apparatus (Bio-Rad) were used. Primer sequences are listed in Table S3 (see Supporting information). All annealing steps occurred at 60 °C, and β-*actin* was used as a reference gene.

Immunohistochemistry

Immunostainings were performed using polyclonal rabbit antibodies (Sigma-Aldrich, St. Louis, MO, USA) against TMEFF2 (HPA026553, diluted 1 : 200), TPD52 (HPA028427, diluted 1 : 8000) and NEDD4L (HPA024618, diluted 1 : 20). Polyclonal rabbit antibody against ERG (EPR3864, diluted 1 : 100; Epitomics, Burlingame, CA, USA) was used. The TMA sections were deparaffinized, followed by antigen retrieval in 5 mM Tris–HCl:1 mM EDTA, pH 9, at 121 °C for 2 min in an autoclave. Bound antibody was visualized with the Power-Vision$^+$ Poly-HRP IHC Detection Kit (ImmunoVision Technologies, Brisbane, CA, USA). The sections were counterstained with haematoxylin and the staining was scored on a scale of 0–3 (0, no staining; 1, weak staining; 2, moderate staining; and 3, high-intensity staining) for TMEFF2, TPD42 and NEDD4L. ERG staining was scored as positive or negative. Staining intensity was measured only from the cancerous areas in a blinded fashion by one of the authors (SL). Cancerous areas were confirmed with a mixture of two mouse monoclonal antibodies against the basal cell layer (p63, diluted 1 : 200; and HMW keratin, clone 34β12, diluted 1 : 100; both from LabVision, Fremont, CA, USA) and a rabbit monoclonal antibody against AMACR (clone 13H4; Dako, Copenhagen, Denmark), as described [20].

Fluorescence *in situ* hybridization

Three-colour fluorescence *in situ* hybridization (FISH) was carried out on the TMA slides as previously described [21]. Probes for *ERG* (RP11-164E1), *TMPRSS2* (RP11-814F13) and the region in between (RP11-367P1) were used.

Ontology analysis

The web-based integrated data-mining system WebGestalt [22] was used to determine the gene ontologies.

Statistical analyses

The Benjamini–Hochberg method, one-way ANOVAs with Bonferroni's multiple comparison, χ^2 and Fishers' exact tests were used for statistical analyses.

Results

To assess gene expression specifically in cancer cells from heterogeneous tissue samples, we used an *in silico* Bayesian modelling tool, DSection [19], to analyse microarray data. This tool allows the simultaneous estimation of gene expression in three tissue compartments (cancer, epithelium and stroma) and in the three experimental groups (control, goserelin and bicalutamide). To validate the modelling tool, we first assessed the differential expression in cancer tissue and between non-treated control and treatment groups with and without DSection. Without DSection, the samples were first categorized into three groups, depending on their cancer tissue content: low (0–9%), moderate (23–49%) and high (74–85%). We then compared the results with the DSection model. In all the groups, the genes were ranked based on their fold change (FC) between the treatment and control. We observed clear dissimilarity between the ranked lists of the lowest purity group (0–9%) and the DSection model (9% of genes in common), whereas the group with 74–85% purity were 71% similar with the DSection model. Therefore, DSection seems to reliably estimate cancer tissue-specific expression in heterogeneous tissue specimens.

To further validate DSection, we microdissected tissue specimens and extracted RNA from both the cancer and stromal compartments of 11 samples (three or four from each group) and measured the expression of 10 genes by qRT–PCR. Only one of 10 genes (*MAOA*) showed differences between the DSection prediction and qRT–PCR (Figure 1).

To determine similarities and differences between the bicalutamide and goserelin treatment groups, we compared gene expression profiles induced by the two treatments in cancer tissues. Altogether, 128 genes had > two-fold reduced expression [false discovery rate (FDR) < 0.01], among which 33 genes were common to both treatment groups (Figure 2a; see also Supporting information, Table S4). Among 86 genes that showed increased expression, two genes were common (Figure 2b; see also Supporting information, Table S5). Overall, only 16% of the most differently expressed genes were common to both treatments.

Given that both bicalutamide and goserelin treatments aim to inhibit AR signalling, we focused on the genes with reduced expression after the treatments. To

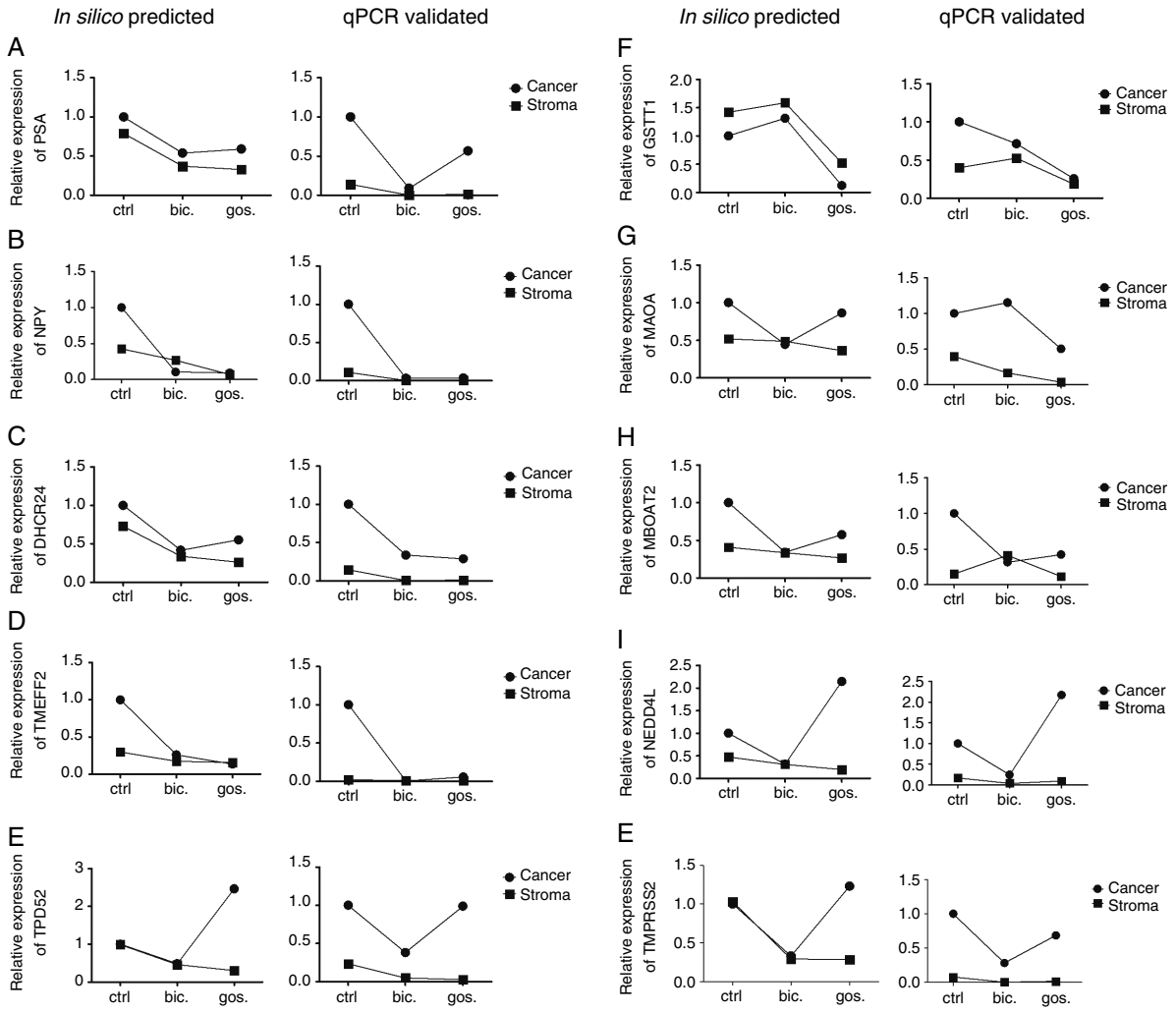

Figure 1. Validation of DSection *in silico* Bayesian model prediction. *In silico*-predicted and qRT–PCR-assayed gene expression differences between non-treated control, bicalutamide and goserelin treatments of 10 genes: *PSA* (A); *NPY* (B); *DHCR24* (C); *TMEFF2* (D); *TPD52* (E); *GSTT1* (F); *MAOA* (G); *MBOAT2* (H); *NEDD4L* (I); and TMPRSS2 (J). The expression of cancer tissue in control sample was normalized to 1. Ctrl, control; bic, bicalutamide; gos, goserelin.

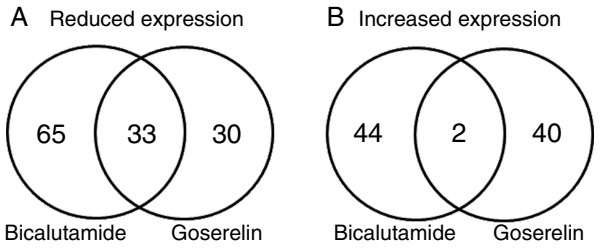

Figure 2. Number of differentially expressed genes after bicalutamide or goserelin treatments (FC > 2; FDR < 0.01). (A) Genes with reduced expression compared to non-treated control group. (B) Genes with increased expression compared to non-treated control group.

examine which of the genes with reduced expression are direct AR targets, we first utilized our previously published data on DHT-stimulated genes in an AR over-expressing LNCaP-based cell line model and in a VCaP cell line [23]. From 128 genes, 45 (35%) were > two-fold up-reguated in cell lines (see Supporting information Table S4). In addition, we used two independent chromatin immunoprecipitation (ChIP)-sequencing (seq)-derived AR binding sites (ARBSs) data in an LNCaP-based cell line model [24,25]. ARBS data overlapped > 60% between the two studies (see Supporting information, Table S4). To confirm the active ARBSs, we utilized histone methylation data from two independent datasets [14,26]. The other dataset contained ChIP-seq data of the monomethylated H4K3, and the other dataset utilized the changes in H3K4me2 signal resulting nucleosome stabilization–destabilization (NSD) score for the nucleosomes. Both histone modifications have been shown to be associated with active enhancer areas [26,27]. Approximately half of the 128 genes contained a potentially active enhancer area with ARBS (see Supporting information, Table S4). Taken together, from the 128 genes that had > two-fold reduced expression after the endocrine treatment, 24 genes were induced by DHT stimulation in the cell line models, showed ARBSs close to the TSS according to the two independent studies or, alternatively, showed ARBS close to the TSS according to one of the studies and contained

Table 1. Genes whose expression was > two-fold reduced after bicalutamide and/or goserelin treatments, were > two-fold induced by DHT treatment, in cell culture models, had ARBS, and potentially active enhancer

Gene name	FC ctrl vs bic	FDR ctrl vs bic	FC ctrl vs gos	FDR ctrl vs gos
FKBP5	4.0	1.9E-05	2.8	0.0004
TMEFF2	3.8	0.008	7.2	0.001
FAM110B	3.5	0.0003	2.1	0.02
NEDD4L	3.2	8.5E-05	0.5	0.001
MME	3.1	1.9E-05	4.8	1.9E-06
TMPRSS2	3.0	0.0007	0.8	0.2
MBOAT2	2.9	5.8E-07	1.7	0.0005
CLDN8	2.7	0.003	1.7	0.1
TBC1D8	2.6	5.6E-07	1.4	0.005
HOMER2	2.5	5.7E-05	1.3	0.2
DHCR24	2.4	0.0003	1.8	0.01
KLK4	2.4	0.006	1.2	0.5
RAB3B	2.3	0.0007	1.2	0.3
BRP44	2.2	0.002	1.4	0.1
C1orf116	2.1	0.003	1.5	0.1
TPD52	2.1	0.0001	0.4	2.9E-05
RDH10	2.0	0.006	2.2	0.007
KCNN2	2.0	0.001	2.4	0.0006
LCP1	2.0	0.0001	1.1	0.5
PMEPA1	2.0	0.001	0.8	0.1
KHDRBS3	2.0	0.001	2.6	0.0002
LAMA3	1.8	0.0009	2.5	5.5E-05
ATAD2	1.7	0.002	2.1	0.0003

For more details, see main text and Table S4 (see Supporting information). FC, fold change; FDR, false discovery rate; bic, bicalutamide; gos, goserelin. Genes that are indicated in bold type are > two-fold reduced after both endocrine treatments.

potentially active enhancer (Table 1; see also Supporting information, Table S4). The differential expression of six of the 24 genes was confirmed with qRT–PCR (Figure 1).

Next, we studied the expression of three of these directly androgen-regulated genes at the protein level. We immunostained the trial specimens with antibodies against TMEFF2, TPD52 and NEDD4L and observed abundant staining in all of the samples with the TPD52 and NEDD4L antibodies, which clearly illustrates high expression at the protein level in prostate cancer. However, significant differences between the treatment groups could not be assessed (Figure 3a, b). Instead, TMEFF2 staining was significantly weaker in both the bicalutamide- and goserelin-treated samples compared to control samples ($p < 0.0001$, χ^2 test; Figure 3c, d).

Further, we determined whether the genes with reduced expression after the endocrine treatment would be reactivated in CRPC. We measured the expression levels of four selected genes, *TMEFF2, DHCR24, TPD52* and *NEDD4L*, in an independent set of benign prostate hyperplasia (BPH), previously untreated prostate cancers (prostatectomy specimens) and CRPC samples, using qRT–PCR (Figure 3e–h). We found that *DHCR24, TPD52* and *NEDD4L* were significantly over-expressed in the prostate cancer samples when compared to the BPH samples and that *TMEFF2* had a trend towards significance ($p < 0.0001$). The expression of *TPD52* and *NEDD4L* was also significantly increased in the CRPC samples compared to the BPH samples ($p < 0.001$ and < 0.05, respectively), and *DHCR24* and *TMEFF2* had a trend towards significance.

Finally, we assayed *TMPRSS2-ERG* fusion in the trial samples by using FISH and IHC (see Supporting information, Figure S1). Of the 25 samples containing enough cancerous area for the assay, 15 samples (60%) were positive for the fusion gene (see Supporting information, Table S6). Due to the small number of cases, we combined the bicalutamide and goserelin groups into one endocrine-treated sample group.

First, we evaluated role of ERG and AR in the control and endocrine treated groups. In the control group, we detected substantially higher expression levels of 869 genes in the fusion-positive (F^+) cases compared to the fusion-negative (F^-) cases (Figure 4a; see also Supporting information, Table S7). In contrast, the treatment reduced the expression of 601 genes in the F^+ cases but only 69 genes in the F^- cases ($p < 0.0001$, χ^2 test, Figure 4b; see also Supporting information, Table S8). Interestingly, one-half (430) of the genes that were up-regulated in the F^+ cases versus F^- cases in the control group were common to those that were down-regulated after the treatment in the F^+ cases (Figure 4c).

To assess whether the differentially expressed genes are direct targets of ERG and AR, we utilized ChIP-seq data from the *TMPRSS2–ERG* fusion-positive VCaP cell line published by Yu *et al* [14]. We re-analysed the data and determined the genes closest to the ERG binding sites (ERGBSs) and ARBSs. On average, 68% and 25% of all genes in the genome possess an ERGBS and ARBS, respectively, and, as previously shown [14], > 90% of the genes with an ARBS are also targets of ERG. The most significant enrichment in ERGBSs occurred in a group of 869 genes that

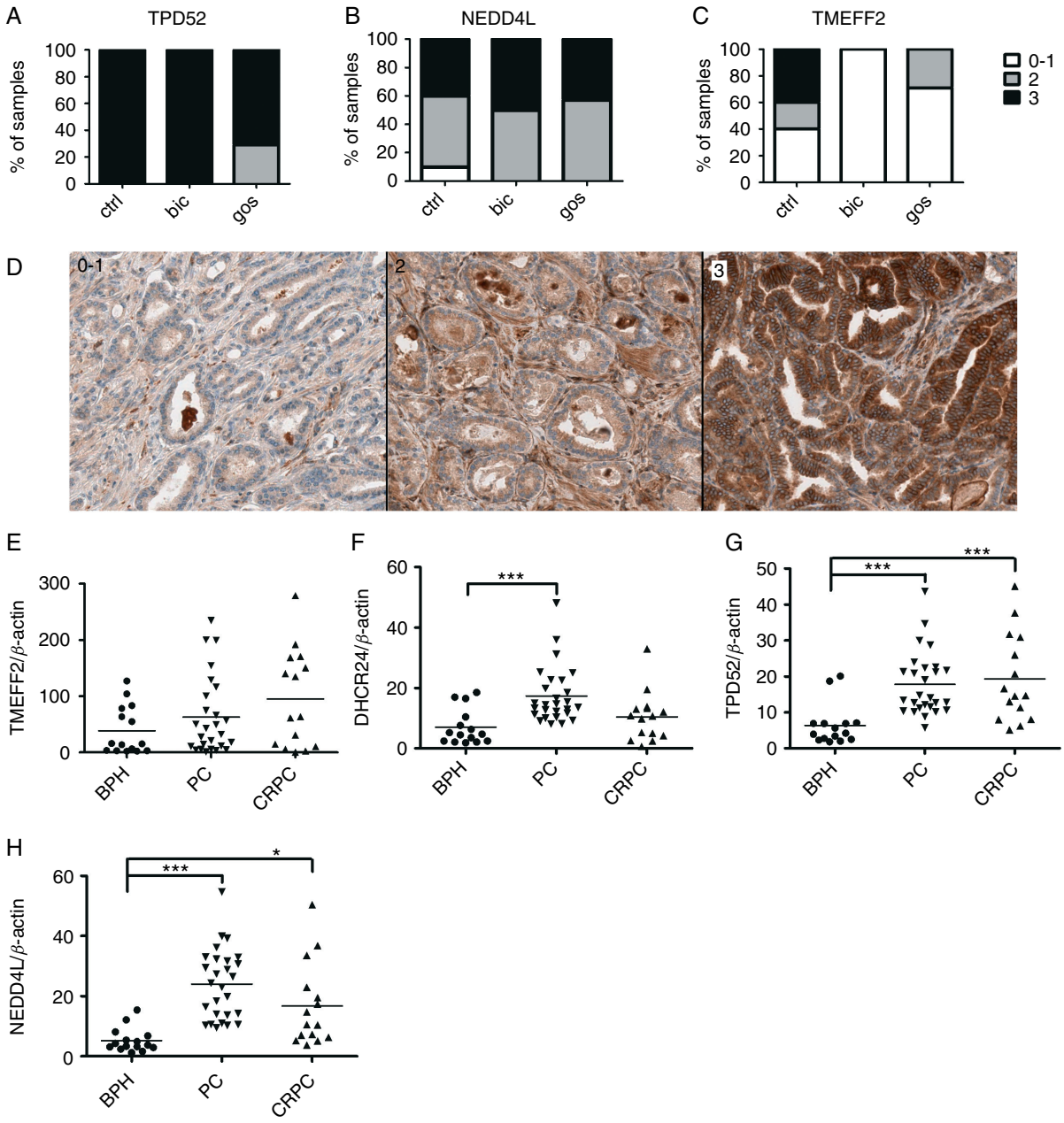

Figure 3. Immunohistochemical staining of the trial material with antibody against TPD52 (A), NEDD4L (B) and TMEFF2 (C); bic, bicalutamide; gos, goserelin. (D) Representative images of TMEFF2 staining; 0–1, no or low staining; 2, moderate staining; 3, high-intensity staining. Staining intensity was measured only from cancerous areas, which were confirmed with AMACR, p63 and keratin HMW triple staining. Differences between treatment groups (non-treated control, bicalutamide and goserelin) were significant with TMEFF2 antibody ($p < 0.0001$, χ^2 test) but not with TPD52 of NEDD4L. (E–I) Gene expression levels of four candidate genes, *TMEFF2* (E), *DHCR24* (F), *TPD52* (G) and *NEDD4L* (H), in benign prostate hyperplasia (BPH), hormone-naive prostate cancer (PC) and castration-resistant prostate cancer (CRPC), measured with qRT–PCR; $^*p < 0.05$, $^{***}p < 0.001$, one-way ANOVA with Bonferroni's multiple comparison test.

showed increased gene expression in the F^+ cases compared to the F^- cases in the control group (85% harboured an ERGBS; $p = 3.3e\text{-}25$, Fishers' exact test; Figure 4a). Similarly, in a group of the 601 genes that showed decreased expression in the F^+ cases after the treatment 86% harboured an ERGBS ($p = 4.4e\text{-}21$; Figure 4b). In addition, the same groups of genes had also significant ARBSs enrichment: 36% of the 869 genes and 40% of the 601 genes harboured an ARBS, ($p = 1.8e\text{-}25$ and $p = 6.2e\text{-}27$, respectively; Figure 4a, b).

Finally, we performed an ontology analysis for the most interesting groups of genes. Those were the group of 869 genes with increased expression in the F^+ cases compared to the F^- cases in a control group and the group of 601 genes with decreased expression in the F^+ cases after the endocrine treatment (Figure 4a, b). Same groups also had the most significant enrichment of ERGBS and ARBS, as mentioned above. Interestingly, we detected that the significantly enriched ontologies in both groups were strongly related to proliferation, eg 'cell cycle', 'M phase' and 'mitosis'

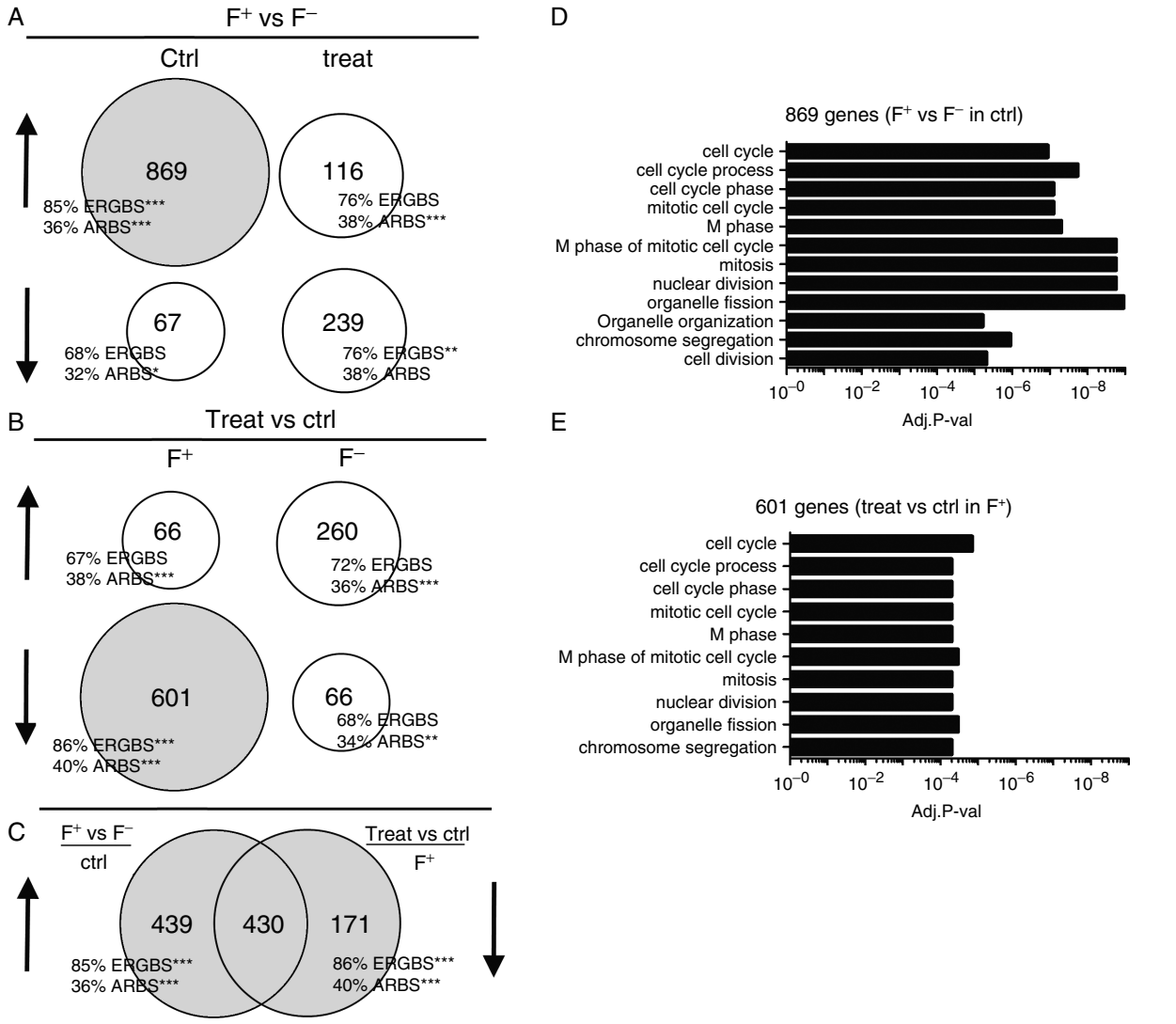

Figure 4. Number of differentially expressed genes and their ontologies according to *TMPRSS2–ERG* fusion and endocrine treatment status. (A) Genes with increased and decreased expression in the fusion-positive (F^+) versus fusion-negative (F^-) cases in control (crtl) and endocrine-treated (treat) groups. (B) Genes with increased and decreased expression after the endocrine treatment in the F^+ and F^- cases. (C) Half (430) of those genes with increased expression in the F^+ cases in the control group (left circle, altogether 869 genes) had reduced expression levels after the endocrine treatment (right circle, altogether 601 genes). Fold change > 1.6. Asterisks represent the statistical significance of AR and ERG binding site enrichment: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Fisher's exact test. Binding frequency in the whole genome was 68% for ERG and 25% for AR. Binding sites were retrieved from the publicly available VCaP cell line ChIP-seq datasets [14]. (D–E) Significantly enriched gene ontologies of biological processes. Ontologies were determined from the group of 869 genes with increased expression in F^+ cases in the control group (D) and from the group of 601 genes with reduced expression after endocrine treatments in F^+ cases (E), with the limits: > seven genes/ontology group and adjusted *p* value (adj.P-val.) < 0.0001; calculated with Fisher's exact test and Benjamini–Hochberg multiple test.

(Figure 4d, e; cut-off > 7 genes and an adjusted $p < 0.0001$).

Discussion

In this study, we took advantage of rare clinical specimens from neoadjuvant endocrine-treated patients and studied the differences between the two most commonly used endocrine treatments, the GnRH agonist goserelin and the anti-androgen bicalutamide. Surprisingly, the two endocrine treatments appeared to regulate different genes. A chronic GnRH agonist administration leads to down-regulation of its receptor and subsequently to reduction of androgen secretion to castrate levels. The anti-androgens, in contrast, bind directly to the AR ligand binding domain and compete with DHT or testosterone binding. However, both treatments aim to inactivate the AR signalling pathway. Despite similar clinical outcome, the findings here indicate that the molecular responses induced by GnRH agonists and anti-androgens are at least partially different.

Mostaghel *et al* [28] previously measured gene expression levels after medical castration, using the GnRH antagonist acyline in healthy volunteers. In addition, Holzbeierlein *et al* [29] characterized the gene

expression changes after combined androgen blockade with goserelin and flutamide for 3 months in patients with localized prostate cancer. When we compared the most differentially expressed genes, few genes were common to this study and Mostaghel's and Holzbeierlein's studies. The lack of common differentially expressed genes could be explained by the use of different drugs or combinations of drugs in the three studies. In addition, different microarray platforms, normalization methods, choices of arbitrary threshold values and statistical analyses may contribute to the differences observed in these studies.

From 128 genes with > two-fold reduced expression, only 24 had ARBS closest to their TSS and were induced by DHT > two-fold in AR-dependent cell line models. These 24 genes are most probably directly androgen-regulated, while the reduced expression of other 104 genes may be due to the secondary effects. It is noteworthy that the samples from the trial have been collected after 3 months of therapy, which may explain a large proportion of secondary effects. This time point also excludes early androgen-responsive genes and reveals only long-term AR targets. Furthermore, the ARBSs, H3K4 methylation data and gene expression data following DHT stimulation are derived from prostate cancer cell line models. Thus, it is possible that some of these 104 genes could be direct AR targets in prostate tissue.

H3K4 momomethylation has been shown to be associated with transcription binding at enhancers and demethylation with both TSS and enhancers [27,30]. In this study, we utilized the histone methylation data of two independent studies to identify potential enhancer areas [14,26]. Although potential enhancer areas were found, they do not necessarily regulate the expression of the closest gene, and therefore the possibility of false positives and false negatives is present. Only reporter and chromatin conformation assays could reliably identify the correct target genes for enhancer areas. Thus, to reliably detect AR target genes, we combined the methylation data with ARBS- and DHT-induced expression data.

Because endocrine treatment is not curative and the disease eventually relapses, we investigated the genes that are down-regulated after the endocrine treatment and reactivated in the castration-resistant stage. Such genes could be potential biomarkers for response to hormonal therapy. We detected a trend of increased *TMEFF2* and *DHCR24* expression levels and a statistically significant over-expression of *TPD52* and *NEDD4L* in CRPC cases compared to cases of BPH. The expression levels of *TPD52, DHCR24* and *TMEFF2* have previously been shown to be androgen-regulated [31–33]. In addition, *NEDD4L* has been implicated in AR signalling [34,35]. Moreover, the expression levels of *TPD52* and *TMEFF2* have been demonstrated to be increased in prostate cancer, especially in CRPC [32,36,37].

We also assessed the influence of *TMPRSS2–ERG* fusion on gene expression in both untreated and endocrine-treated cases. It appears that ERG sensitizes cells to the endocrine therapy, because 8.7-times more genes were down-regulated after endocrine treatment in the F^+ cases compared to the F^- cases (601 versus 69 genes). Both ERGBSs and ARBSs were significantly enriched in the regions near the down-regulated genes. Notably, the majority of the down-regulated genes are the same genes that showed increased expression in the F^+ cases compared to the F^- cases in the control group. Therefore, the endocrine treatment mainly affected the genes that were highly expressed in the F^+ cases and diminished the differences between the F^+ and F^- tumours.

It has been shown that ERG expression is increased by androgens in the VCaP cell line [14]. This can also be seen in our data, with increased expression of putative ERG target genes in the F^+ cases of the hormone-naïve control group. In our data, endocrine treatment reduced the expression of 601 genes in the F^+ cases but only 69 genes in the F^- cases. These 601 genes are most probably direct targets of ERG, because the androgen-dependent nature of TMPRSS2 expression renders the genes under control of fused ERG. Indeed, 86% of these genes had ERGBS closest to their TSS. Interestingly, the ontology analysis revealed that these genes are strongly involved in cell cycle and mitosis. Thus, it seems that the fusion brings many proliferation-associated genes under androgen regulation.

Previously it has been shown that ETS transcription factor binding sites were often close to ARBSs [38]. Recently, Yu *et al* [14] also reported that AR and ERG chromatin binding profiles overlap. Additionally, Yu *et al* reported that ERG may reduce AR activity and, in the presence of androgens, the knock-down of ERG enhances the expression of AR-regulated genes. However, our data suggest more synergistic roles for AR and ERG; co-activity of these transcription factors enhances the expression of their target genes, as in the F^+ cases in the control group, and reduction of androgens in the treatment group reduces the expression of ERG target genes in the F^+ cases.

Several other studies have also identified differences in the gene expression between *TMPRSS2–ERG* fusion-positive and -negative prostate cancers [39–41]. Similar to our study, they have identified more genes with increased expression than reduced expression in the fusion-positive compared to the fusion-negative cases [39,40]. Approximately 30–50% of genes with increased expression in the fusion-positive cases reported in these studies were also increased > 1.3-fold in this study.

Sample heterogeneity is a major problem in microarray studies, especially in diseases such as prostate cancer, where the cancerous areas are small and often surrounded by normal cells. In this study, we used the *in silico* probabilistic analysis tool, DSection, to artificially isolate the cancerous areas. We detected a 71% overlap when we compared the gene expression profile of the DSection model to the gene expression

profile of samples with a high percentage of cancerous area (high-cancer). Interestingly, *TPD52*, one of the most differentially expressed genes, was part of the group of genes that DSection predicted to be the most differentially expressed. However, TPD52 expression was not different, according to the expression profile of the samples with a high cancer compartment. The qRT–PCR from the microdissected samples confirmed the differential expression of TPD52. This example shows that the DSection prediction model can produce reliable expression data that overcome the heterogeneity of the prostate cancer tissue specimens.

In conclusion, we utilized this rare clinical material from neoadjuvant-treated PC patients and found a clear difference in the gene expression levels induced by an anti-androgen and a GnRH agonist. This indicates different cellular consequences of these two forms of androgen deprivation. In addition, we showed that the endocrine treatments induce different gene expression changes in PC, depending on *TMPRSS2–ERG* fusion. Many of the treatment-responsive genes in the fusion-positive cases were related to proliferation. The weakness of the study was the low number of cases. Thus, it is vital that the findings should be validated in larger samples, although unlikely in similar trial settings.

Acknowledgment

We wish to thank Ms Mariitta Vakkuri, Ms Päivi Martikainen, Ms Anni Järvinen, Ms Salla Kolmihaara and Ms Anne Luuri for their skilled technical assistance. Grant support was received from the Academy of Finland, the Cancer Society of Finland, the Reino Lahtikari Foundation, the Sigrid Juselius Foundation, competitive research funding from Pirkanmaa Hospital District and a non-restricted grant from AstraZeneca.

Author contributions

AK, PM and TT guided and carried out the clinical trial; JI established dual a-colour three-antibody immunostaining; VT developed the virtual microscopy system to facilitate IHC analysis; AU and KW carried out the cell line experiments; TE and HL developed the DSection analysis and carried out bioinformatic analysis; JS analysed AR and ERG binding site data; AL analysed the histone methylation data; TV planned the experiments and guided the work; and SL analysed data and carried out experiments. The manuscript was written by SL and TV with the assistance of all other authors.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Figure S1. Example images from FISH and IHC assays of *TMPRSS2–ERG* fusion.

Table S1. More detailed information of the two clinical sample sets that were used in this study.

Table S2. Pathological characterization of patients involved in the endocrine trial.

Table S3. Sequences of qRT–PCR primers that were used to validate the bioinformatic analyses.

Table S4. More detailed list of genes with reduced expression after bicalutamide and/or goserelin treatments. Their expression after androgen induction in prostate cancer cell lines is indicated, as well as the AR binding sites close to their TSS.

Table S5. List of genes with increased expression after bicalutamide and/or goserelin treatments.

Table S6. Distribution of the *TMPRSS2–ERG* fusion-positive and -negative samples in the treatment and control groups.

Table S7. List of genes with increased expression in the F^+ compared to the F^- cases in the control and treatment groups. AR and ERG binding sites in VCaP cell line are also indicated for each gene.

Table S8. List of genes with decreased expression after the endocrine treatment in the F^+ and F^- cases. Similarly to Table S6, AR and ERG binding sites in the VCaP cell line are indicated for these genes.

Goserelin and Bicalutamide Treatments Alter the Expression of MicroRNAs in the Prostate

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BACKGROUND. Although endocrine therapy has been used for decades, its influence on the expression of microRNAs (miRNAs) in clinical tissue specimens has not been analyzed. Moreover, the effects of the *TMPRSS2:ERG* fusion on the expression of miRNAs in hormone naïve and endocrine-treated prostate cancers are poorly understood.

METHODS. We used clinical material from a neoadjuvant trial consisting of 28 men treated with goserelin (n = 8), bicalutamide (n = 9), or no treatment (n = 11) for 3 months prior to radical prostatectomy. Freshly frozen specimens were used for microarray analysis of 723 human miRNAs. Specific miRNA expression in cancer, benign epithelium and stromal tissue compartments was predicted with an in silico Bayesian modeling tool.

RESULTS. The expression of 52, 44, and 34 miRNAs was affected >1.4-fold by the endocrine treatment in the cancer, non-malignant epithelium, and stromal compartments, respectively. Of the 52 miRNAs, only 10 were equally affected by the two treatment modalities in the cancer compartment. Twenty-six of the 52 genes (50%) showed AR binding sites in their proximity in either VCaP or LNCaP cell lines. Forty-seven miRNAs were differentially expressed in *TMPRSS2:ERG* fusion positive compared with fusion negative cases. Endocrine treatment reduced the differences between fusion positive and negative cases.

CONCLUSIONS. Goserelin treatment and bicalutamide treatment mostly affected the expression of different miRNAs. The effect clearly varied in different tissue compartments. *TMPRSS2:ERG* fusion positive and negative cases showed differential expression of miRNAs, and the difference was diminished by androgen ablation. *Prostate* © 2012 Wiley Periodicals, Inc.

KEY WORDS: **prostatic carcinoma; TMPRSS2:ERG; endocrine therapy; androgen ablation; LHRH agonist**

Additional supporting information may be found in the online version of this article.

Grant sponsor: Academy of Finland; Grant sponsor: Cancer Society of Finland; Grant sponsor: Reino Lahtikari Foundation; Grant sponsor: Sigrid Juselius Foundation; Grant sponsor: Tampere University Hospital; Grant sponsor: AstraZeneca.

The authors declare no conflict of interest.

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Received 21 February 2012; Accepted 14 May 2012
DOI 10.1002/pros.22545
Published online in Wiley Online Library (wileyonlinelibrary.com).

INTRODUCTION

Endocrine therapy is the gold standard treatment for advanced prostate cancer. Today, Gonadotropin-releasing hormone (GnRH) agonist therapy is the most common endocrine treatment modality [1,2]. Non-steroidal antiandrogens were originally designed for combined androgen blockade, but further studies have disclosed similar survival rates with antiandrogen monotherapy and castration in locally advanced prostate cancer [3,4]. Both GnRH agonist and antiandrogen therapies reduce the activity of androgen receptor (AR)-mediated signaling and therefore reduce the growth of prostatic tumors [5]. However, the mechanisms differ; while antiandrogens function as AR antagonists and reduce the binding of 5α-dehydrotestosterone (DHT) to AR, GnRH agonist inactivates AR by reducing the serum levels of testosterone.

Endocrine therapy is an effective but not curative treatment. On average, 18–24 months after the initiation of endocrine treatment, metastatic prostate cancer relapses and continues to grow as castration resistant prostate cancer (CRPC) [6]. The mean overall survival after disease progression is approximately 20 months [7]. The mechanism by which prostate cancer transforms to its lethal form is not completely known. However, the notion that CRPC maintains functional and active AR signaling is now commonly accepted [8,9].

The *TMPRSS2:ERG* fusion gene is a well-known AR target gene and up to 50% of PCs harbor such fusion [10]. Studies with transgenic mice have shown that ERG overexpression alone causes mouse prostatic intraepithelial neoplasia (PIN) but not malignancy [11,12]. However, further studies have suggested that the loss of *Pten* together with the overexpression of *ERG* can accelerate the progression of high-grade PIN to prostatic adenocarcinoma in mice [13,14]. Moreover, combined overexpression of *AR* and *ERG* in the murine prostate leads to the formation of poorly differentiated and invasive adenocarcinoma [14]. Thus, *TMPRSS2:ERG*, in combination with other genetic changes, has been shown to have a role in both the initiation and progression of the disease, but a more detailed understanding of the function of the fusion in prostate cancer is needed.

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate the expression of protein-coding genes primarily by destabilizing the target mRNAs or inhibiting translation [15]. Today, 1,424 miRNAs have been identified (www.mirbase.org, Sept 2011), and each miRNAs can regulate several, even hundreds, of protein-coding genes. Genes encoding miRNAs are located in the genome either alone or in clusters, within protein-coding genes or in intergenic regions [16,17].

miRNAs have been predicted to be able to control the activity of 30% of protein-coding genes [18]. Because miRNAs are strongly involved in regulation and development, the altered miRNA expression in human malignancies is not surprising [19]. Today, several dozen miRNAs have been shown to be differentially expressed in prostate cancer [20]. Several miRNAs, such as miR-125b, miR-21, and miR-32, have also been shown to be directly androgen-regulated in cell line models and xenografts [21–24].

Little is known regarding how endocrine therapy affects the expression of miRNAs in vivo. Moreover, the effect of the *TMPRSS2:ERG* gene fusion on miRNA expression is unclear. In this study, we utilized rare clinical material from neoadjuvant endocrine-treated patients to measure the miRNA expression upon treatment with GnRH agonist or antiandrogens, in different prostatic tissue compartments, and in *TMPRSS2:ERG* fusion positive and negative cases.

MATERIALS AND METHODS

Clinical Samples

A randomized clinical trial to investigate the effects of neoadjuvant GnRH analog and antiandrogen in patients undergoing radical prostatectomy was conducted in the Tampere University Hospital, Finland. The sample set has previously been described in detail [25]. Briefly, 28 men with localized prostate cancer were randomized into three equal groups: no treatment, antiandrogen or GnRH agonist for 3 months following radical prostatectomy. Total RNA from fresh specimens was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Adjacent sections before and after the sections used for RNA extraction were also cut and stained with hematoxylin and eosin. The amount of cancer, benign epithelium, and stroma in specimens was assessed from the slides. Tissue microarray (TMA) was constructed from paraffin-embedded formalin-fixed (FFPE) prostatectomy specimens.

The use of clinical material in this study was approved by the Ethical Committee of the Tampere University Hospital. Written informed consent was obtained from the patients.

Expression Profiling

Microarray analysis of miRNA expression was performed using human miRNA V2 microarray chips (Agilent Technologies, Santa Clara, CA). Each array contained probe sets for 723 human miRNAs

according to Sanger miRBase v 10.1. The microarrays were performed according to the manufacturer's instructions. Briefly, 100 ng of total RNA was labeled with Cyanine 3-pCp and hybridized using the miRNA Labeling Reagent and Hybridization Kit (Agilent Technologies). Hybridization was performed at 55°C for 20 hr. The microarrays were scanned with Agilent's Microarray scanner BA, and the data were extracted using Agilent's Feature Extraction software (V. 10.7.1.1). The data were submitted to ArrayExpress, ref ID: E-MEXP-3530.

In Silico Data Analysis

Because the amount of cancer in the prostatectomy specimens varied from 0% to 85%, an in silico Bayesian modeling tool was used to estimate the miRNA expression in different tissue compartments [26]. Briefly, DSection analysis was used to calculate the expression value for each probe in benign epithelial, stromal, and malignant cells by utilizing the information regarding the percentage of different cell types in the sample assessed as described above. To obtain greater statistical power in the calculations, the differential expression of mRNAs from the same samples were included to the analysis [25]. Before DSection analysis, the samples were normalized. First, all of the probes yielding a value of 100 or less in all of the samples were filtered away, leaving 180 miRNAs for further analysis. Second, the average and standard deviation of the probes in every sample were calculated separately and defined as 0 and 1, respectively. DSection analysis calculated the average expression values of the individual miRNAs for each treatment group and each tissue compartment. Thus, rather than assessing the differences between individuals, the expression differences between treatment groups were instead quantified. The accuracy of DSection has previously been validated with qRT-PCR analysis of microdissected samples [25].

Cell Culturing

LNCaP cells (ATCC, Rockville, MD) were cultured in normal cell culturing conditions with and without 10 μM bicalutamide (AstraZeneca, London, UK) for 2 days followed by RNA extraction with Tri-reagent (Thermo Fisher Scientific, Waltham, MA).

qRT-PCR

cDNA synthesis for PSA was performed with AMV reverse transcriptase (Finnzymes, Vantaa, Finland) and for miRNAs by using TaqMan microRNA assay (Applied Biosystems, Foster City, CA) according to manufacturers' instructions. Expression of PSA and three miRNAs was measured with the Maxima SYBR Green/Rox qPCR Master Mix (Fermentas, Burlington, Canada) or TaqMan miRNA assay, respectively, by using CFX96 Real Time System apparatus (Bio-Rad Laboratories, Hercules, CA). The expression of miRNAs was normalized to the total amount of RNA.

Fluorescence In Situ Hybridization and Immunohistochemistry

The *TMPRSS2:ERG* fusion and *ERG* expression was determined by fluorescence in situ hybridization (FISH) and immunostaining as previously described [25].

Statistical Analysis

Unsupervised hierarchical clustering was performed for all 180 miRNAs that yielded a value of 100 or higher in any of the samples. The baseline of the values was transformed to the median of all miRNAs. Average linkages between miRNAs and subgroups were used, and similarities were measured with the Euclidean method. The Benjamini-Hochberg method was used to measure false discovery rates (FDRs) for miRNA expression values. Fisher's exact test was used to calculate the binding site enrichment. The Mantel–Cox test was established to calculate the differences in survival times.

RESULTS

Expression of miRNAs in Different Tissue Compartments in the Prostate

We utilized DSection software to assess the expression of miRNAs in cancer, non-malignant epithelium (referred to as simply epithelium), and stromal compartments. We compared the expression differences of different compartments in a non-treated control group and bicalutamide-treated and goserelin-treated groups. In the control group, we detected 14 miRNAs with >1.4-fold higher expression and three miRNAs with >1.4-fold lower expression in cancer tissue compared with epithelium and stoma (FDR <0.05, Table I). The expression of 11 of the 14 of these ''cancer-specific'' miRNAs was >1.4-fold lower after bicalutamide and/or goserelin treatment in the cancer tissue. In addition, the expression of two of the three miRNAs with lower expression in cancer tissue compared with epithelium and stoma increased after endocrine treatment.

By comparing the effect of endocrine therapy in cancer, epithelial and stromal compartments, we detected that the treatment had the greatest impact on

TABLE I. MicroRNAs That Are >1.4-Fold Differently Expressed in Cancer Tissue Compared to Benign Epithelium and Stroma Tissues (FDR <0.05)

	Epithelium versus cancer		Stroma versus cancer		Expression after treatment
MicroRNA	FC	FDR	FC	FDR	
Increased expression in cancer tissue					
hsa-miR-9	13.1	7.1E−07	4.9	5.0E−05	↓[a]
hsa-miR-9*	9.2	2.0E−06	3.8	0.0002	↓
hsa-miR-7	2.1	0.01	2.9	0.001	↑[b]
hsa-miR-210	2.1	0.0006	2.3	0.0003	↓
hsa-miR-30d	2.0	0.0001	2.2	0.00006	↓
hsa-miR-429	1.8	0.009	1.9	0.01	↓
hsa-miR-32	1.7	0.03	3.2	0.0002	↓
hsa-miR-149	1.6	0.02	2.0	0.003	↓
hsa-miR-130b	1.6	0.002	1.8	0.0005	↓
hsa-miR-200a	1.5	0.05	1.6	0.04	↓
hsa-miR-425	1.5	0.0006	1.5	0.001	—
hsa-miR-30b	1.5	0.001	1.6	0.0003	↓
hsa-miR-200b	1.5	0.04	1.5	0.05	↓
hsa-miR-663	1.4	0.05	1.6	0.02	↑
Decreased expression in cancer tissue					
hsa-miR-1	2.0	0.009	1.8	0.03	↑
hsa-miR-204	1.6	0.005	2.3	0.00008	↑
hsa-miR-370	1.4	0.03	1.4	0.03	—

[a]Expression is >1.4-fold reduced after the endocrine treatment.
[b]Expression is >1.4-fold increased after the endocrine treatment.

the cancer tissue. In the cancer tissue, the expression of 52 miRNAs was altered >1.4-fold (FDR <0.05) after bicalutamide and/or goserelin treatment. The treatment had a milder effect in stroma, where 34 miRNAs presented altered expression. As expected, endocrine treatments affected mostly different miRNAs in cancer, epithelium and stromal tissues (Fig. 1, Supplementary Table S1). Only five miRNAs were commonly differentially expressed in all of the tissues. The expression patterns also varied greatly between benign epithelium and cancer; the expression of only 10 miRNAs was >1.4-fold altered in both tissues after endocrine treatment (Fig. 1).

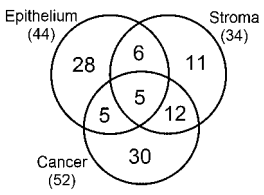

Fig. 1. Venn diagram of miRNAs that are >1.4-fold differentially expressed (FDR <0.05) after bicalutamide and/or goserelin treatment in benign epithelium, stroma, or cancer tissues in human prostate.

Expression of miRNA Expression of microRNAs After Bicalutamide and Goserelin Treatment in Cancer

Here, we more closely evaluated the differentially expressed miRNAs after bicalutamide or goserelin treatment compared to the non-treated group, specifically in the cancer tissue. Of 52 differentially expressed miRNAs, the expression of 24 miRNAs was reduced after endocrine treatment. Fourteen of those 24 miRNAs were downregulated only after bicalutamide treatment, three only after goserelin treatment and seven after both treatments (Fig. 2a,b, Table II). In addition, the expression of 28 miRNAs increased after endocrine treatment. Thirteen of these miRNAs were upregulated only after bicalutamide, and 12 were upregulated only after goserelin. Three miRNAs were upregulated after both endocrine treatments.

To confirm that the expression changes of miRNAs were truly drug dependent, we treated androgen dependent LNCaP cells with 10 μM bicalutamide for 2 days and measured the expression of miR-32, miR-141, and miR-17 by using qRT-PCR. Expression level of PSA was used as a control for the treatment efficacy. As in the trial material, the expression of the three miRNAs was downregulated after bicalutamide treatment (Fig. 2c–f).

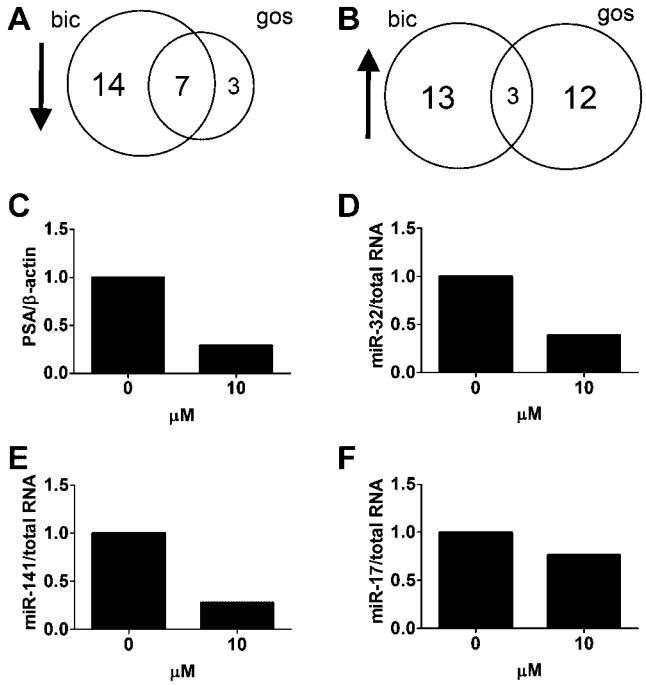

Fig. 2. The most differentially expressed miRNAs after bicalutamide and goserelin treatment compared with the non-treated control group. **A**: miRNAs with decreased expression. **B**: miRNAs with increased expression. Fold change >1.4, False discovery rate <0.05. Bic, bicalutamide; gos, goserelin. **C–F**: Relative expression according to qRT-PCR of PSA (C), miR-32 (D), miR-141 (E), and miR-17 (F) after bicalutamide treatment (10 μM, 2 days) compared to no treatment in LNCaP cell line. Results are average of two biological replicates.

Next, we assessed whether these 52 differentially expressed miRNAs are direct targets of AR. For this purpose, we used our recent chromatin immunoprecipitation (ChIP)—sequencing (seq) derived AR binding sites (ARBSs) data in an LNCaP-based cell line model [27]. In addition, we utilized ChIP-seq data from the VCaP cell line published by Yu et al. [28]. We used several criteria to assess ARBS for each miRNA. First, the ARBS was considered to regulate intergenic miRNA genes if the miRNA was closer to the binding site than any other gene. Second, ARBS close to clusters of intergenic miRNAs was considered to regulate the expression of the entire cluster. Third, for intragenic miRNAs or clusters of miRNAs the ARBS located close to the transcription starting site of the protein-coding gene was considered to regulate the miRNAs located within the gene locus. miRNAs or clusters of miRNAs located within a protein-coding gene, but in the reverse DNA strand were considered intergenic miRNAs. Finally, if the miRNAs had homologues (e.g., the mature form was expressed in several genomic areas), the miRNA was considered to be AR regulated if ARBS was found close to any of the miRNA homologues. With these criteria, we detected ARBSs for 32% and 9% of all miRNAs in VCaP and LNCaP, respectively. The binding sites found in LNCaP were mostly also present in VCaP. From the most differently expressed miRNAs in our data set, 26 miRNAs (50%) presented ARBS (Table II). Nine of these miRNAs (17%) had ARBS in both VCaP and LNCaP. The rest had ARBSs only in VCaP.

Next, we assessed how many of those 52 differentially expressed miRNAs were transcriptionally altered in prostate cancer. We utilized the recent data regarding differentially expressed miRNAs between normal prostate tissue and prostate cancer from two different sample sets that we have previously published [24,29]. One sample set contained 102 freshly frozen clinical samples from normal adjacent prostate and normal lymph node and their cancerous counterparts [29], and the other set contained 54 benign prostate hyperplasia (BPH), prostate cancer and CRPC samples [24]. Among the 24 miRNAs with reduced expression after endocrine treatment, 12 were upregulated in prostate cancer compared with normal tissue or BPH (Table II). In addition, five miRNAs, miR-32, miR-17, miR-30b, miR-130b and miR-203, were upregulated in CRPC compared with BPH. miR-32, miR-17 and miR-130b were upregulated in both prostate cancer and CRPC compared with adjacent normal tissue or BPH. Among 28 miRNAs with increased expression, seven were downregulated in prostate cancer compared with normal tissue or BPH, and nine miRNAs were downregulated in CRPC compared with BPH (Table II).

We also used the expression data from 99 primary prostate cancer samples with follow-up data from Taylor et al. [30] to determine the feasibility of using the miRNAs as prognostic markers. Among the miRNAs with decreased expression after endocrine treatment, we found that high expression of miR-30d, miR-141, miR-375, miR-210, or miR-130b indicated significantly worse progression-free survival (Supplementary Fig. S1a–e). All of these miRNAs were also upregulated in prostate cancer compared with BPH or adjacent normal prostate according to our studies (Table II). In addition, among the miRNAs with increased expression after endocrine treatment, low expression of miR-204, miR-135a, miR-125b, or miR-100 indicated significantly worse progression-free survival (Supplementary Fig. S1f–i).

Association of miRNA Expression With the *TMPRSS2:ERG* Fusion

Next, we evaluated how the *TMPRSS2:ERG* fusion affects the expression of miRNAs with and without

TABLE II. Top Differentially Expressed miRNAs After Bicalutamide or Goserelin Treatments

MicroRNA	FC[a] bic versus ctrl	FDR[b] bic versus ctrl	FC gos versus ctrl	FDR gos versus ctrl	ARBS[c]	PC versus BPH/normal[d]	CRPC versus BPH[d]
Decreased after bicalutamide treatment							
hsa-miR-144	2.8	0.006	0.8	0.4			
hsa-miR-32	2.3	0.009	1.3	0.2	Both	Up	Up
hsa-miR-30d	2.0	0.0003	1.1	0.1	Both	Up	
hsa-miR-363	1.9	0.0003	0.9	0.5		Up	
hsa-miR-663	1.9	0.01	0.5	0.009		Up	
hsa-miR-141	1.9	0.003	0.8	0.4		Up	
hsa-miR-631	1.7	0.05	0.8	0.3			
hsa-miR-375	1.7	0.02	0.6	0.2		Up	
hsa-miR-629*	1.7	0.03	0.9	0.5	VCaP		
hsa-miR-345	1.6	0.04	0.9	0.4			
hsa-miR-557	1.5	0.03	0.8	0.1	VCaP		
hsa-miR-106a	1.5	0.003	1.3	0.008			
hsa-miR-17	1.5	0.0005	1.2	0.004	Both	Up	Up
hsa-miR-30b	1.4	0.002	0.8	0.5	Both		Up
Decreased after goserelin treatment							
hsa-miR-130b	1.4	0.05	1.6	0.006	VCaP	Up	Up
hsa-miR-203	1.3	0.2	1.5	0.04		Up	
hsa-miR-301a	1.1	0.4	1.4	0.03			
Commonly decreased after both treatments							
hsa-miR-9	6.1	0.00006	7.5	0.00003			
hsa-miR-9*	4.1	0.0005	5.7	0.0001		Up	
hsa-miR-429	1.7	0.03	2.6	0.0016		Up	
hsa-miR-210	2.3	0.002	1.7	0.02		Up	
hsa-miR-149	1.9	0.01	1.6	0.03	VCaP	Down	
hsa-miR-200a	1.6	0.04	2.1	0.005			
hsa-miR-200b	1.4	0.05	2.3	0.0009		Up	
Increased after bicalutamide treatment							
hsa-miR-424	2.1	0.007	0.8	0.2			
hsa-miR-204	2.0	0.0005	1.3	0.2	VCaP	Down	
hsa-miR-381	1.9	0.002	0.9	0.3	VCaP		Down
hsa-miR-218	1.9	0.00006	0.9	0.3	VCaP	Down	
hsa-miR-1	1.9	0.04	1.1	0.5		Down	
hsa-miR-551b	1.9	0.04	1.2	0.4	VCaP	Down	
hsa-miR-376a	1.6	0.001	1.0	0.5	VCaP		Down
hsa-miR-376c	1.6	0.002	1.1	0.6	VCaP		Down
hsa-miR-152	1.6	0.0002	0.9	0.2			Down
hsa-miR-377	1.5	0.003	0.9	0.2	VCaP		Down
hsa-miR-23b	1.5	0.005	1.0	0.1	VCaP	Down	

(*Continued*)

TABLE II. (*Continued*)

MicroRNA	FC[a] bic versus ctrl	FDR[b] bic versus ctrl	FC gos versus ctrl	FDR gos versus ctrl	ARBS[c]	PC versus BPH/normal[d]	CRPC versus BPH[d]
hsa-let-7e	1.5	0.001	1.0	0.3		Down	
hsa-miR-143*	1.4	0.02	0.7	0.04	VCaP	Down	Down
Increased after goserelin treatment							
hsa-miR-7	0.7	0.2	3.0	0.006		Up	Up
hsa-miR-135a	0.9	0.5	2.6	0.002	Both		
hsa-miR-148a	1.2	0.1	2.3	0.0006	VCaP		Up
hsa-miR-1229	0.9	0.5	2.1	0.007			
hsa-miR-663	0.5	0.01	2.1	0.009		Up	
hsa-miR-10b	1.3	0.05	1.9	0.007			
hsa-miR-638	1.1	0.5	1.7	0.08	VCaP		
hsa-miR-134	0.9	0.4	1.6	0.05	VCaP		
hsa-let-7c	1.3	0.02	1.5	0.004	Both	No	
hsa-miR-128	1.2	0.02	1.5	0.01			
hsa-miR-601	0.8	0.2	1.5	0.04	VCaP		
hsa-miR-887	0.9	0.4	1.4	0.02	Both		
Commonly increased after both treatments							
hsa-miR-99a	1.7	0.0003	2.3	0.0003	Both		Down
hsa-miR-125b	1.9	0.0001	2.0	0.0002	Both		Down
hsa-miR-100	1.9	0.00005	1.7	0.0004			Down

bic, bicalutamide; gos, goserelin.
[a]Fold change (FC) >1.4.
[b]False discovery rate (FDR) <0.05.
[c]Androgen binding sites (ARBSs) was determined utilizing ChIP-Seq data from LNCaP [27] and VCaP [28] cell lines. ''Both'' indicates the existing binding site in both cell lines.
[d]Up- or down-regulated expression in prostate cancer (PC) or castration resistant prostate cancer (CRPC) has been determined according to Refs. [24,29].

endocrine therapy. Using fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) assays, we detected 60% of the samples as fusion positive [25]. Because of the low number of samples, we combined the bicalutamide and goserelin treatment groups into one endocrine treatment group. Thus, we had four subgroups: fusion positive (F+) and fusion negative (F−) cases in the control group and F+ and F− cases in the treatment group.

First, we compared the F+ cases to the F− cases in the control group and detected 23 and 24 miRNAs with decreased and increased expression, respectively (FC > 1.4, Supplementary Table S2). Next, we determined how the endocrine treatment affects the F+ and F− cases. We found that endocrine treatment enhanced the expression of miRNAs more in the F− cases than in the F+ cases and decreased the expression more in the F+ cases than in the F− cases (Fig. 3, Supplementary Table S3). We evaluated ARBS and ERG binding sites for these miRNAs with the same criteria as above and determined that ARBSs were significantly enriched in the group of miRNAs with increased expression in the F+ cases after treatment ($P < 0.05$, Fig. 3a).

Among the 23 miRNAs with increased expression in the F+ cases compared with the F− cases in the control group, 11 were common with the group of 16 miRNAs with decreased expression after treatment in the F+ cases (Fig. 4a). Similarly, 6 of 18 miRNAs with decreased expression in the F+ cases compared with the F− cases in the control group were common with the 11 miRNAs whose expression was increased by treatment in the F+ cases (Fig. 4b). To further study the differential expression in different subgroups, we performed an unsupervised hierarchical clustering analysis for all of the miRNAs in the four subgroups (Fig. 4c). We found that the F+ cases in the control group clustered separately into one arm while the three other subgroups were more similar. Thus, endocrine therapy alters the miRNA expression profile of the F+ cases toward the expression profile of the F− cases. To further confirm this phenomenon, we focused on the expression values of the 17 miRNAs that were differentially expressed in the F+ cases compared with the F− cases in the control group and that were affected by treatment in the F+ group (shown in Fig. 4a,b). We found that the expression difference in the miRNAs in the control groups was diminished by the treatment (Fig. 4d,e).

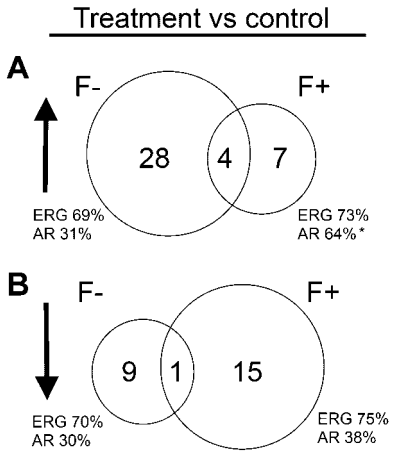

Fig. 3. The most differentially expressed miRNAs after endocrine treatment in fusion negative (F−) and fusion positive (F+) cases. AR and ERG binding sites were measured from the data in [28]. The average number of binding sites was 41% for ERG and 19.5% for AR (**A**) miRNAs with increased expression. **B**: miRNAs with decreased expression. Fold change >1.4. *, enrichment *P*-value <0.05, calculated with Fisher's exact test.

DISCUSSION

By assessing of the amount of tissue compartment per sample and using the sophisticated Bayesian modeling tool DSection, we were able to determine the tissue-specific expression of each miRNA in all three treatment groups. The great majority of cancer-specific miRNAs in the control group were affected by endocrine treatment, indicating treatment response at the miRNA level. Endocrine treatment seemed to have the weakest effect on stromal tissue and the strongest effect on cancer tissue. The difference between benign and malignant epithelium was surprisingly high. This difference suggests that AR signaling is different in malignant and benign epithelial cells. Indeed, the direct effect of androgens in normal epithelia has previously been shown to be differentiation whereas in malignant cells androgens cause proliferation [31].

We showed that the expression of miRNAs in cancer was clearly different after endocrine treatments. Several previous studies have also shown that miRNAs are regulated by androgens [21–23]. Therefore, the expression of several miRNAs was predicted to be reduced after endocrine treatment. Our data also demonstrate several miRNAs with increased expression after endocrine treatment. In general, the expression of miRNAs has been shown to be reduced during the progression of prostate and other cancers [29,32]. Thus, the relatively high number of miRNAs with increased expression after endocrine treatment can indicate treatment response, reduced cancerous characteristics, greater apoptosis or a reduced proliferation rate.

Surprisingly, a significant difference was observed in the miRNA expression profiles after bicalutamide and goserelin treatments. Among the 19 miRNAs

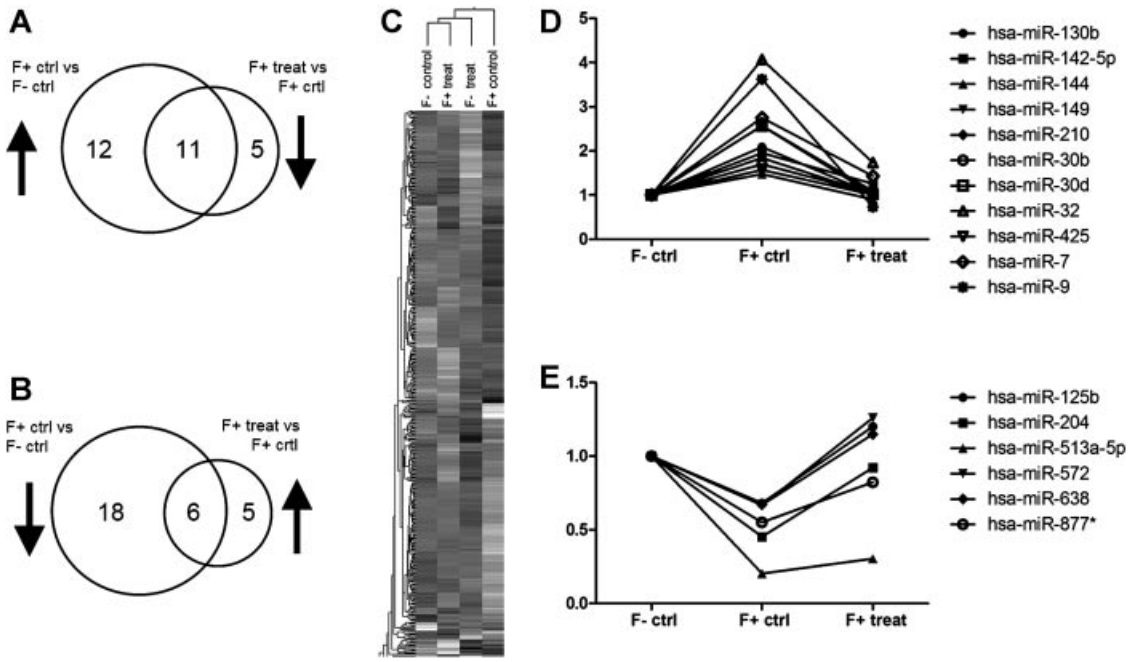

Fig. 4. Differentially expressed miRNAs in TMPRSS2:ERG fusion positive (F+) and negative (F−) cased in the control group (ctrl) and endocrine-treated group (treat). **A**: miRNAs with the most increased expression in F+ cases compared with F− cases in the control group (left circle) and the most increased expression in the treatment group compared with the control group in F+ cases (right circle). **B**: miRNAs with the most decreased expression in F+ cases compared with F− cases in the control group (left circle) and the most increased expression in the treatment group compared with the control group in F+ cases (right circle). **C**: Unsupervised hierarchical clustering of four subgroups: fusion positive (F+) and fusion negative (F−) cases in the control group and F+ and F− cases in the treatment group. The white color indicates decreased expression, and the black color indicates increased expression. **D**: Expression profile of 11 miRNAs that are common to both groups in (A). The expression of all of these miRNAs is increased in the F+ cases in the control group compared with the F− cases in the control group or the F+ cases in the treatment group. **E**: Expression profile of six miRNAs that are common to both groups in (B). The expression of all of these miRNAs is decreased in the F+ cases in the control group compared with the F− cases in the control group or the F+ cases in the treatment group.

with decreased expression, six were common to both treatments, and among the 23 miRNAs with increased expression, only three were common to both treatments. Goserelin affects the androgen production from the testis whereas bicalutamide prevents DHT binding to the AR. Our previous study has also shown that the mRNA expression varies substantially after these treatments [25]. Thus, although both treatment modalities affect the androgen signaling pathway, their effects on gene expression in prostate tissue seem to be quite different.

As previously shown, the expression of miR-141, miR-30d, miR-210, miR-375, and miR-130b was upregulated in prostate cancer compared with BPH or normal prostate tissue [24,29]. We found that these miRNAs were downregulated after the endocrine treatment. According to the reanalyzed data from Taylor et al. [30], worse progression-free survival was predicted when these miRNAs were expressed at high levels. In addition, we detected ARBSs for miR-30d and miR-130b, which indicates a possible direct regulation of the miRNAs by AR. Moreover, miR-30d and miR-130b were highly expressed in cancer tissue compared with epithelium or stroma. Altogether, these data suggest that AR-regulated miR-30d and miR-130b may be oncogenic and could be used as prognostic markers for prostate cancer. However, additional functional studies are needed to fully explore the roles of miR-30d and miR-130b in prostate cancer. Recent studies have revealed tumorigenic properties of miR-30d in several cancer cell lines and in vivo breast cancer xenografts and have suggested that p53 may be its target gene [33,34]. Moreover, miR-130b has shown oncogenic properties in human T-cell leukemic blood cells by inhibiting the expression of tumor protein 53-induced nuclear protein 1 (*TP53INP1*) [35] and in gastric cancer by silencing the tumor suppressor *RUNX3* [36].

In addition to miR-130b, we showed the reduced expression after the endocrine treatments of some other potentially oncogenic miRNAs, such as miR-32, miR-17, and miR-30b. As Table I shows, the

expression of these miRNAs is increased in CRPC. We have recently shown that miR-32 has oncogenic properties by reducing apoptosis [24], whereas miR-17 is a member of well-known oncogenic miR-17/92 cluster [37,38]. Recently, miR-30b/30d cluster have been proposed to enhance metastasis in human melanoma by targeting GalNAc transferase GALNT7 [39]. Taken together, we demonstrated, here, that endocrine treatment reduces the expression of several oncogenic miRNAs and their re-expression might indicate lack of treatment response and emergence of CRPC.

Among the 15 miRNAs whose expression decreased after treatment in the *TMPRSS2:ERG* fusion positive cases, 11 contained an ERG binding site and five lacked an AR binding site. Thus, these five miRNAs most likely have become AR regulated through the *TMPRSS2:ERG* fusion. Interestingly, three of these five miRNAs (miR-106a, miR-363, and miR-20b) belong to the miR-106-363 cluster, which has been shown to harbor oncogenic properties [40]. Moreover, the fourth miRNA in this group, miR-210, has been recognized to be strongly hypoxia induced, and its expression is elevated in several cancers [41,42]. In addition, high expression of miR-210 is associated with poor prognosis in a variety of cancers, including prostate cancer, as illustrated by the data in the Taylor et al. [30] study. Our analyses also revealed higher expression of miR-210 in cancer tissue compared with non-malignant epithelium or stroma. The fifth miRNA, miR-7, has been shown to downregulate epidermal growth factor receptor signaling and thus inhibit growth in several cancers [43,44]. However, the role of miR-7 in prostate cancer remains unknown.

Unsupervised hierarchical clustering showed that the fusion positive cases in the treatment group clustered close to the fusion negative cases. This analysis demonstates that when the activity of AR is reduced by endocrine therapy, the expression of ERG is also reduced, which leads to the conversion of the expression profile of fusion positive cases in a endocrine-treated group towards the fusion negative profiles. These data could explain why the endocrine-treated fusion positive and negative patients show similar progression-free survival [45].

The *TMPRSS2:ERG* fusion causes overexpression of the *ERG* transcription factor. ERG has been shown to bind to the proximal promoter area of 68% of all genes in the VCaP cell line [28]. The ERG binding sites have also been shown to overlap with ARBSs. Approximately 90% of AR-regulated genes are also ERG-regulated. Yu et al. [28] have shown that ERG represses the expression of androgen-induced genes in localized prostate cancers. In contrast, our previous data on the mRNA expression suggested a more synergistic role for AR and ERG [25]. The findings here further emphasize such synergy.

In this study, we have identified several miRNAs that are affected by endocrine therapy and that may have a role in prostate cancer tumorigenesis. We have demonstrated that both the type of treatment and the *TMPRSS2:ERG* fusion greatly affect the expression of miRNAs.

ACKNOWLEDGMENTS

We wish to thank Ms. Mariitta Vakkuri, Ms. Päivi Martikainen, Ms. Salla Kolmihaara, and Ms. Anne Luuri for their skilled technical assistance. Grant support was received from the Academy of Finland, Cancer Society of Finland, Reino Lahtikari Foundation, Sigrid Juselius Foundation, Competitive Research Funding of Tampere University Hospital Finnish Doctoral Programme in Computational Sciences, and a non-restricted grant was received from AstraZeneca.

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Utility of cell-permeable peptides for enhancement of virus-mediated gene transfer to human tumor cells

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BioTechniques 40:573-576 (May 2006)
doi 10.2144/000112152

Cell-permeable peptides can penetrate the cell membrane and become internalized either alone or coupled to other molecules. Their value has been recognized especially in vaccination and gene therapy studies (for a review, see Reference 1). Gratton et al. reported recently about the use of these peptides in enhancement of virus-mediated gene delivery in vitro and in vivo (2). They showed that polybasic peptides derived from *Drosophila* Antennapedia homeodomain (Antp) or human immunodeficiency virus type 1 (HIV-1) transactivator protein (TAT) improved adenoviral and retroviral transduction in cultured monkey COS-7 cells, bovine aortic endothelial cells, and human umbilical vascular endothelial cells, as well as in mouse arteries, muscle, and skin in vivo when precomplexed with viral vector particles. Based on their results, Gratton et al. (2) suggested that highly positively charged peptides can enhance the transduction by concentrating viral particles to the cell surface and by improving receptor-dependent uptake mechanisms.

Insufficient transduction efficiency is still considered the major problem in gene therapy research. Because high gene transfer rate is particularly important in most cancer gene therapy approaches and Gratton et al. (2) did not test their concept in human tumor cells, we conducted a series of experiments to verify the utility of cell-permeable peptide-complexed virus vectors in cancer gene therapy. In addition to the Antp peptide (RQIKIWFQNRRMKWKK), we used two versions of the HIV-1 TAT peptide: TAT1 (YGRKKRRQRRR, used in most earlier studies) (1) and TAT2 (GRKKRRQRRRPPQ, presumably used by Gratton et al.) (2). Furthermore, two polycationic compounds, polybrene (hexadimethrine bromide) and protamine sulfate, were used to identify the contribution of a plain electrostatic effect (i.e., the reduction of the electrostatic repulsion between negatively charged viral particles and cell membranes). Polybrene has been known to enhance retroviral infection since the late 1960s (3). It is nowadays a commonly used enhancer of retroviral transduction, and its mechanism of action (4,5) and positive effect to adenoviral gene transfer (6) have been elucidated. Protamine sulfate has been designated as a more clinically relevant alternative for polybrene in retroviral gene therapy (7), and its utility in adenoviral gene transfer has been acknowledged (8).

The cell-penetrating peptides were incubated with a serotype 5, E1/E3-deleted adenovirus vector AdTK-GFP (9) and a second generation VSV-G pseudotyped lentivirus vector WOX-TK-GFP (10) as described in the original report (2). Polybrene (8 µg/mL) and protamine sulfate (5 µg/mL) were added to virus dilutions, and the resulting complexes were then used for transduction of one monkey kidney fibroblast cell line (COS-7) and four different human cancer cell lines representing ovarian carcinoma (SKOV3.ip1, HEY), prostate carcinoma (PC-3), and osteosarcoma (MG-63). The human tumor cell lines were selected due to their known features as targets for viral gene transfer. All of these cell lines were moderate or poor targets for lentiviral and/or adenoviral vectors (9,11), and transduction of these cells would apparently benefit from peptide-mediated enhancement. The results, indicated as proportion of green fluorescent protein (GFP) positive cells, were determined by flow cytometry, and a one-way analysis of variance with Dunnett's post hoc test for multiple comparisons was used for statistical analysis.

To verify the results obtained by Gratton and coworkers (2), we examined the peptide- and polycation-mediated enhancement of viral gene transfer efficiency in COS-7 cells (Figure 1). Analysis of TK-GFP positive

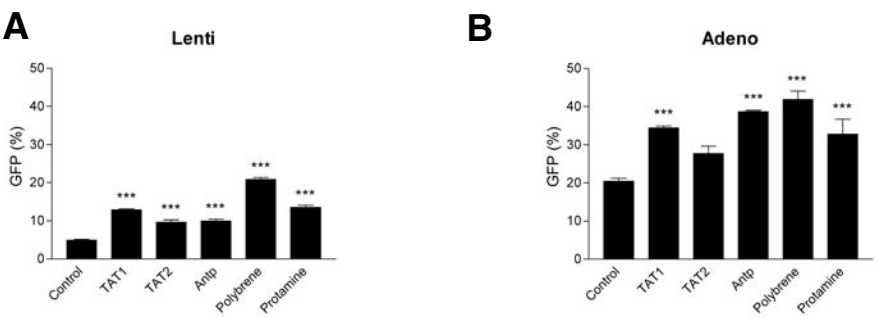

Figure 1. Transduction rates of COS-7 cells using the peptide- or polycation-assisted lentiviral (A) or adenoviral (B) gene transfer. The two human immunodeficiency virus type 1 (HIV-1) TAT-derived peptides (TAT1, TAT2) and *Drosophila* Antennapedia homeodomain-derived peptide (Antp) were purchased from Inbio Ltd. [purity: high-performance liquid chromatography (HPLC) grade, >95%; Tallinn, Estonia]. The peptides (0.5 mM) were complexed with lentivirus and adenovirus vectors as described by Gratton et al. (2). The polycations used were polybrene (8 µg/mL; Sigma-Aldrich Chemie GmbH, Munich, Germany) and protamine sulfate (5 µg/mL; Sigma-Aldrich Chemie). Multiplicity of infection 1 of both vector types was used in transductions, and the results [percent of green fluorescent protein (GFP) positive cells] were obtained with flow cytometry (FACSCalibur™; Becton Dickinson, San Jose, CA, USA) 2–4 days later. The bars are means of 3 different experiments ± SEM. One-way analysis of variance with Dunnett's post hoc test for multiple comparisons (GraphPad Prism 3.0; GraphPad Software, San Diego, CA, USA) was used for statistical analysis. ***, $P < 0.001$ as compared with the control group (transduction with no additive compounds).

cells by flow cytometry 2 days (adenovirus) or 4 days (lentivirus) posttransduction confirmed that the Antp peptide can significantly improve adenoviral and lentiviral gene transfer ($P < 0.001$). However, this effect was not as impressive as observed earlier (2) (i.e., from 10% with adenovirus vector alone to almost 95% when complexed with the Antp peptide). In our experiments, the Antp peptide doubled the gene transfer efficiency of both vector types, and a similar effect was obtained with the TAT-derived peptides. Interestingly, polybrene and protamine were able to boost the gene delivery with both vector types equally well (protamine) or even better (polybrene) than any of the peptides. It is difficult to determine, why Gratton and colleagues obtained higher enhancement of adenoviral gene delivery with Antp peptide even though their peptide concentration was similar to that used in our experiment. It is possible that the COS-7 cell populations in two different laboratories may not be completely identical or the quality of the adenovirus and peptide preparations may play a role. Furthermore, there may be minor differences in the complex formation or transduction protocols, resulting in variation in the peptide-mediated enhancement. Taken together, our results with COS-7 cells confirm the enhancement observed by Gratton et al. (2), but also point out that depending on the conditions and materials used, the degree of gene delivery enhancement is likely to vary from laboratory to laboratory.

To test the utility of peptide-mediated enhancement in human tumor cell lines, SKOV3.ip1, HEY, PC-3, and MG-63 cells were transduced identically as COS-7 cells. As shown in Figure 2, all the peptides and polycations were able to boost viral gene delivery into human tumor cells similarly as shown with COS-7 monkey fibroblasts. The results regarding the tumor cell lines can be summarized as follows: (*i*) peptides enhanced significantly both lentiviral and adenoviral gene transfer ($P < 0.001$), and in all cases the order of enhancement was Antp ≥ TAT1 > TAT2; (*ii*) peptides could not significantly improve the poor lentiviral transduction of MG-63 cells; (*iii*) polycations were efficient transduction enhancers with both vector types ($P < 0.001$), except in two cases (lentivirus complexed with protamine sulfate, in PC-3 and MG-63 cells); and (*iv*) polybrene turned out to be a better enhancer than any of the peptides, and in most cases the protamine sulfate effect was also comparable to that of the best peptides.

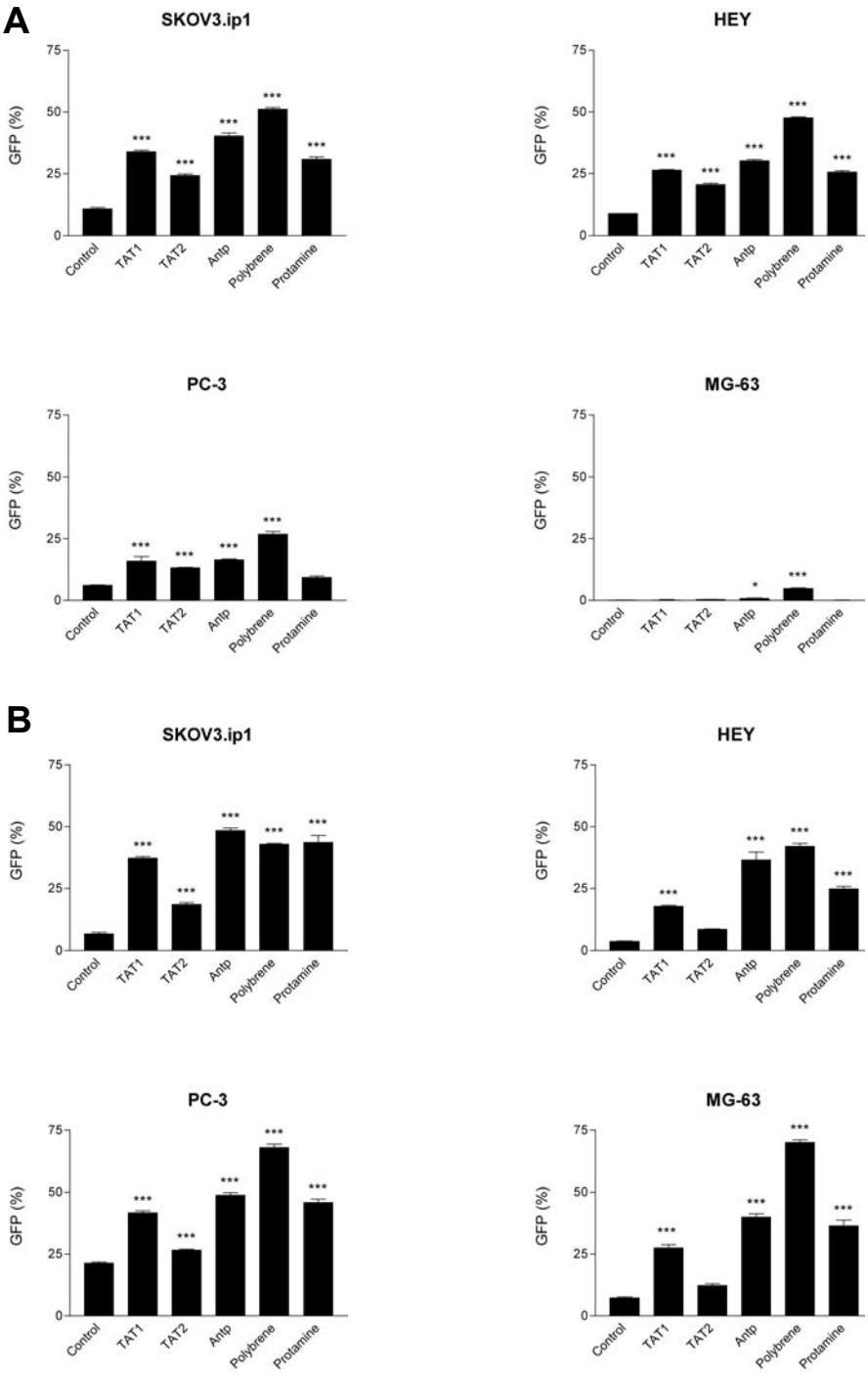

Figure 2. Transduction rates of four human cancer cell lines using peptide- or polycation-assisted lentiviral (A) or adenoviral (B) gene transfer. Identical reagents and experimental conditions as indicated in the legend to the Figure 1 were used. ***, $P < 0.001$, and *, $P < 0.05$ as compared with the control group (transduction with no additive compounds). GFP, green fluorescent protein; TAT1 and TAT2, two human immunodeficiency virus type 1 (HIV-1) TAT-derived peptides; Antp, *Drosophila* Antennapedia homeodomain-derived peptide.

Taken together, our results demonstrate that the transduction rate of adenovirus and lentivirus vectors can be significantly boosted with TAT and Antp cell permeable peptides, but similar or better results can be obtained with commonly used polycations. The effect of each peptide or polycation turned out to be surprisingly similar in all the studied cell lines, suggesting that the effect was due to simple electrostatic interactions and was not dependent on, for example, the protein composition of the target cell plasma membrane. However, the enhancing effect appeared to be dependent on the peptide sequence; TAT1 and TAT2 peptides displayed different degrees of enhancement, especially with adenovirus vector, although their net charges were identical. In our studies, we used only one peptide concentration (0.5 mM) that was also chosen by the authors of the original paper (2). It can be speculated that this peptide concentration was already toxic, and lower concentrations could have yielded better results. This was not the case however, since we observed practically no enhancement of gene transfer with 0.1 mM peptide concentration (results not shown). Furthermore, 0.5 mM peptides did not induce any notable cytotoxicity in any of the cell lines (as judged by microscopical examination 48 h posttransduction, results not shown). Higher than 0.5 mM concentrations could theoretically improve the effect, but these concentrations would become very expensive, especially when compared with the polycations. The cost per transduction with the peptides is approximately 500 times higher than with polybrene and protamine (as determined on the basis of prices in Finland in 2005). Even though polybrene yielded better enhancement than protamine sulfate, one has to bear in mind that polybrene may have toxic side effects, and it is not a clinically approved molecule. Thus, considering the in vitro results presented in this report, the clinical utility of the tested molecules and their costs, it is apparent that protamine sulfate has the best risk/benefit ratio compared with any of the peptides or polybrene.

ACKNOWLEDGMENTS

This work was supported by The Academy of Finland grant no. 48590 to J.W. Inbio Ltd. (Tallinn, Estonia) is gratefully acknowledged for their kind cooperation regarding the accelerated peptide delivery.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Received 16 January 2006; accepted 13 February 2006.

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