THE ROLE OF THE ANDROGEN RECEPTOR PATHWAY IN PROSTATE CANCER PROGRESSION

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De rol van de androgeenreceptor cascade in de progressie van prostaatkanker

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CONTENTS

LIST OF ABBREVIATIONS

ACSL3	acyl-CoA synthetase long-chain family member 3
AD	androgen-dependent
AI	androgen-independent
AR	androgen receptor
BCL2	B-cell CLL/lymphoma 2
BPH	benign prostatic hyperplasia
CGH	comparative genomic hybridization
DHT	dihydrotestosterone
DKK3	dickkopf homolog 3
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
ENDOD1	endonuclease domain containing 1
ERG	v-ets erythroblastosis virus E26 oncogene homolog (avian)
ETV1	ets variant 1
FCS	fetal calf serum
FGF	fibroblast growth factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HGF	hepatocyte growth factor
HNPC	hormone-naïve prostate cancer
HRPC	hormone-refractory prostate cancer
IGF1	Insulin-like growth factor 1
IL6	interleukin 6 (interferon, beta 2)
JAK/STAT	Janus kinase/ signal transducer and activator of transcription 1
KGF	keratinocyte growth factor
MAPK	mitogen-activated protein kinase
MCCC2	methylcrotonoyl-Coenzyme A carboxylase 2
MTT	3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazoliumbromide
OH-flutamide	hydroxyflutamide
PAP	prostate acid phosphatase
PBGD	porphobilinogen deaminase
PI3K	phosphoinositide-3-kinase
PIN	prostatic intraepithelial neoplasia
PCa	prostate cancer
PCR	polymerase chain reaction
PSA	prostate specific antigen
PTEN	phosphatase and tensin homolog
R1881	17-methyltrienolone
RNA	ribonucleic acid
TMPRSS2	transmembrane protease, serine 2
TP53	tumor protein p53
TURP	transurethral resection of the prostate
TWIST1	twist homolog 1
VAV3	vav 3 guanine nucleotide exchange factor
Wt	wild-type

Chapter 1

General Introduction and Scope of the Thesis

1.1. Introduction

Prostate adenocarcinoma is the most frequently diagnosed non-cutaneous male malignancy in the Western countries. In The Netherlands, 9516 men were diagnosed with prostate cancer in 2006, of which 1 out of 4 may die as a result of the disease [1]. If one considers the frequency of latent cancers estimated from autopsy studies, the numbers can be as high as 30% of men in their sixties, indicating that the prostate epithelial cells are particularly susceptible to malignant transformation [2]. Nevertheless, only a fraction of these latent tumors will eventually manifest itself clinically. At present, diagnosis of the disease at an early stage is essential for the successful eradication of the cancer, as no curative treatment exists for advanced metastasized tumors. However, the early detection of prostate tumors carries the risk of over-treating cancers that pose no threat to the patient, aggravated by the fact that these treatments often have severe side effects. Present challenges in prostate cancer research are the identification of prognostic markers that, at an early stage, can distinguish the indolent tumors from those that will progress and become life threatening, and the development of targeted therapies for advanced disease. To achieve this, a thorough knowledge of the intrinsic mechanisms for prostate cancer onset and progression is essential.

1.2. Normal prostate

The prostate is an exocrine male reproductive organ whose function is to produce prostatic fluid, rich in sugars, proteins and enzymes that make up 30% of the semen and regulate the liquidity of the ejaculate and the motility of the sperm. The normal human prostate is about 3 cm in diameter, has the shape of a chestnut and is located immediately bellow the bladder neck, surrounding the urethra. It can be divided into three anatomic regions: the transition zone, consisting of two pear-shaped lobes located peri-urethrally; the central zone, located behind the proximal prostatic urethra, surrounding the ejaculatory ducts; and the peripheral zone, which contains more than 70% of the normal prostate gland and envelopes the previous zones (Fig. 1) [3]. The peripheral and the transition zones have similar glandular structures composed of small, round acini embedded in a loose stroma with randomly oriented smooth muscle fibers. However, the transitional zone has a more compact stroma and is the site of origin of benign prostatic hyperplasia (BPH), whereas the peripheral zone is more prone to malignant transformation. The central zone has a different embryonic origin than the other two zones, and unlike those, it has large irregular acini with extensive intraluminal folds and a dense stromal compartment. The acini and ducts of the glandular tissue are lined by a double layer of epithelial cells [4]. The luminal secretory cells of the inner layer have a cuboidal shape and produce a variety of proteins and enzymes, such as prostate acid phosphatase (PAP) and prostate specific antigen (PSA), which are released into the seminal fluid [5]. A continuous outer layer of flattened non-secretory basal cells separates the epithelium from the basement membrane. Neuroendocrine cells can also be found, scattered throughout the glands, mostly in or just above the basal cell layer. Although the exact function of the basal cell compartment is not clear, it is thought to harbor a stem cell population responsible for the renewal of the basal, luminal and the neuroendocrine cells [6,7]. Other investigators argue that basal cells have ultrastructural features of active differentiated cells and produce enzymes capable of converting

adrenal steroid precursors into active androgens, suggesting a regulatory role on the maintenance of the luminal compartment [8,9]. But while the biological function of the basal cell layer is uncertain, its value in the diagnosis of prostate cancer is unquestionable, as the progressive loss of the basal epithelial cells is a histological marker for malignant transformation of the gland [10,11].

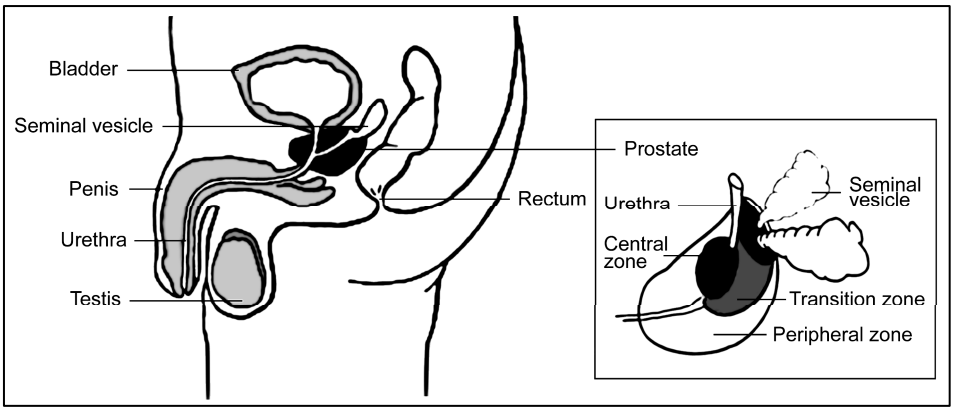

Figure 1 – Schematic representation of the prostate anatomy. The prostate is a chestnut shaped gland located immediately bellow the bladder neck, and can be divided into three anatomic regions: the transitional zone, the central zone and the peripheral zone.

1.3. Prostate cancer development

The origin of prostate cancer is still the subject of intense research. In 1996, Bonkhoff *et al.* suggested that the basal cells, being the compartment with the highest proliferative activity, were the most likely to suffer malignant transformation [12]. The authors proposed the stem cell model for prostate cancer pathogenesis, which postulates the existence of an androgen-independent stem cell population within the basal cell compartment. This stem cell population would be the source of prostate cancer, the proliferating cells then partly differentiating into secretory luminal cells and neuroendocrine cells, giving rise to all the cell lineages observed in prostate tumors [12,13]. The alternative hypothesis is that the luminal epithelial cells are the origin of prostate cancer. This hypothesis is based on the observations that (i) the basal cell compartment is absent in prostate adenocarcinomas [10,11]; (ii) unlike basal cells, most prostate tumor cells express androgen-receptor (AR), PAP and PSA [14]; and (iii) under certain conditions, luminal cells are also capable of self-renewal [15].

The oncogenic events that underlie prostate cancer development are matter of extensive investigation. It has been estimated that the accumulation of roughly a half-dozen independent genetic aberrations are necessary for the gradual progression from benign gland to malignant tumors [16,17]. Prostate cancer progression can be divided in five stages: pre-malignant, locally confined, infiltrating, metastatic and hormone-refractory disease. High-grade prostatic intraepithelial neoplasia (PIN) is widely assumed as the precursor lesion of locally confined prostate cancer. Much evidence

corroborates this theory: (i) PIN can be found in men early in their fifties, thus preceding the onset of cancer; (ii) its incidence increases with age; (iii) like prostate tumors, it is preferentially located in the peripheral zone of the prostate and, (iv) it is often found in close proximity with invasive carcinoma [18]. Furthermore, high-grade PIN shows many morphological and genetic aberrations found in locally confined disease. High-grade PIN is characterized by a cellular proliferation within the prostatic ducts and acini, with these cells presenting a large nucleus, prominent nucleoli and increased cytoplasmatic density. The basal cell layer may be disrupted and fragmented, but the benign architecture of the gland is still preserved [18]. Organ-confined prostate cancer is characterized by the complete absence of the basal cell layer, with the tumor growth invading the adjacent stroma but without penetrating through the prostatic capsule. In locally advanced cancer, the tumor penetrates the capsule and infiltrates surrounding tissues, such as the seminal vesicles, rectum or the bladder neck [19]. Tumors that transgress the prostatic boundaries are more aggressive and likely to progress into metastatic disease [20]. Besides local and distant lymph-nodes, the most common sites of prostate cancer metastasis are the bone, lung and liver [21]. At this stage, patients are offered systemic hormonal-therapy, but despite an initial improvement, these tumors eventually become resistant to therapy and recur as hormone-refractory disease. Hormonal-therapy and the mechanisms of hormone-refractory growth will be discussed bellow.

1.4. Prostate cancer diagnosis

Until 15 years ago prostate cancer was frequently detected at an advanced stage of the disease, when the patient presented himself to the physician with lower urinary tract symptoms, micturation problems and/or metastasis related complains. At that time, palpation via digital rectal examination (DRE) was the only method to examine the prostatic gland. Nowadays, DRE by itself is considered to be a rather ineffective tool as it is limited to bigger palpable tumors, which border the rectal wall. The introduction of transrectal ultrasonografy (TRUS) has allowed for the visualization of the entire prostate gland and for the detection of impalpable tumors. However, by far not all prostatic adenocarcinomas are visible by ultrasonografy and this technique is mainly used for measuring the volume of the prostate and for guiding needle biopsies [22]. In the mid 1980s, a test changed the course of prostate cancer history: the measurement of serum concentration of PSA [23,24]. PSA is a protein produced almost exclusively by the luminal epithelial cells of the prostate. Aberrations in the glandular architecture at the tumor site leads to leakage of PSA, which eventually enters the blood stream through diffusion. The serum PSA test allows for the detection of prostate tumors at very early stages, and since its introduction it has resulted in a dramatic increase in the documented incidence of prostate adenocarcinoma worldwide. In addition to its use in the detection of prostate cancer, the PSA test is also used in the follow up of tumor recurrences in patients who underwent therapy. However, increased serum concentrations of PSA are not specific for prostate cancer. Basically, any abnormality of the prostate gland, such as benign prostatic hyperplasia (BPH), infection/inflammation (prostatitis), as well as ageing, can lead to an increase in PSA. Due to the lack of specificity of PSA screening, many patients undergo unnecessary biopsies or treatment

for indolent tumors [24,25,26]. Furthermore, the reference serum PSA cutoff of 4 ng/ml fails to detect about 20% of high-grade prostate tumors, but lowering this cutoff to 3 ng/ml could result in a negative biopsy rates as high as 70% to 80%. In 2005, the Prostate Cancer Prevention Trial concluded that there is no PSA cutoff that retains simultaneously high sensitivity and specificity [27]. In an attempt to enhance the diagnostic and prognostic potential of PSA, multiple adaptations of the PSA test have been developed. These include measurements of PSA density (PSA level divided by the volume of the prostate), PSA velocity (rate at which PSA increases over time) and free/total PSA (percentage of free PSA in relation to PSA bound to serum proteins). Finally, to differentiate cancer from other prostate disorders, PSA testing must be combined with physical examination [28]. Upon an increased PSA value and/or suspicious DRE, the final diagnosis will always be established by histological examination of needle biopsies.

Due to the limitations of PSA tests and to the invasive nature of the needle biopsy procedure, the development of novel diagnostic and prognostic markers for prostate cancer has become an urgent and challenging enterprise. Recently, α-methylacyl-CoA racemase (AMACR), kallikrein 2 (KLK2), annexin 3, hepsin, prostate cancer antigen 3 (PCA3/DD3), early prostate cancer antigen (ECPA), TMPRSS2:ERG/ETV1 gene fusions, urokinase plasminogen activator and receptor (uPA/uPAR), among others, have emerged as candidate biomarkers [28,29]. Independent large-scale trials still have to prove whether or which of these markers can be applied clinically in the detection and management of prostate cancer.

1.5. Prostate cancer hormonal therapy

The detection of prostate cancer at early stages of the disease allows for a curative treatment of these patients. There are three main options for organ-confined disease: active surveillance, radical prostatectomy and radiotherapy. When choosing the treatment modality, the clinician must take into account the life expectancy of the patient, the characteristics of the tumor and the side effects of the treatment on the quality of life of the patient. In this perspective, an eighty-year old man, with an indolent tumor and limited life expectancy, is preferentially directed into a clinical surveillance program, whereas a sixty-year old patient with clinically localized disease is more likely to receive curative therapy.

Unfortunately, if regional or distant metastasis are present, the cancer is no longer curable and only palliative treatment may be offered [23]. Since, in 1941, Charles Huggins and his team first discovered that castration inhibited the growth of prostate tumors, androgen ablation became the mainstay therapy for advanced disease [30]. Hormonal therapy for prostate cancer consists of the surgical or pharmacological castration of the patient, by bilateral orchidectomy or administration of LHRH agonists, respectively, both leading to a decrease in testosterone production [31]. However, these approaches do not inhibit the production of adrenal androgens or *de novo* steroidogenesis by the tumor cells themselves. The novel drug abiraterone is a inhibitor of CYP17A1, a key enzyme in the biosynthesis of androgens [32]. In phase I and II clinical trials, abiraterone was capable of reducing testosterone production bellow castration levels and showed strong antitumor activity, which is now being evaluated in

phase III trials. Antiandrogens supplementation is often given to prevent an initial testosterone flare, associated with the commencement of LHRH therapy [33]. Furthermore, antiandrogens are also used to block the effect of adrenal androgens, which in the absence of testosterone may play a role in activating the androgen receptor [33]. Most common antiandrogens used in the treatment of advanced prostate cancer are flutamide, bicalutamide, nilutamide and cyproterone acetate [33,34]. Unfortunately, although the great majority of the patients will respond well to the hormonal therapy, these tumors will eventually recur as hormone-refractory disease within 1-3 years [35,36]. This condition has a very poor prognosis with a median survival time of 5-10 months. Recently, a new generation of antiandrogenic drugs, RD162 and MDV3100, showed promising results in second-line treatment of castration-resistant tumors and are entering phase III trials [37].

1.6. The AR pathway in normal prostate homeostasis

Androgens are also known as the male sex hormones, as they play a central role in the development of the male phenotype during fetal life and puberty, and control male fertility and sexual behavior in adults. Testosterone and 5α-dihydrotestosterone (DHT) are essential for the normal development and maintenance of the prostate. Prostate organogenesis initiates at around 10 weeks of gestation and is strictly dependent on androgens, as the outgrowth of the prostatic buds from the urogenital sinus and differentiation of prostatic epithelium will not occur in the absence of androgens. During puberty, testosterone production by the Leydig cells of the testis is switched on, driving the final growth and maturation of the adult prostate. In adult life, androgens remain essential for the maintenance of prostate structure and function, while androgen ablation leads to prostate involution and preferential loss of luminal epithelial cells [38,39].

The actions of androgens are mediated by the AR, a transcription factor of the steroid receptor family. The AR gene is located on the X chromosome at q11.2-q12, spans almost 100 Kb and contains 8 exons that encode a protein with an apparent molecular mass of 110-114 kDa. The structure of the androgen receptor, which is conserved over the other members of the steroid receptors family, is typically divided in three functional domains (Fig. 2) [40,41,42]. The N-terminal domain (NTD), the largest domain of the AR, is encoded by exon 1 and contains the transactivation function of the receptor. The DNA-binding domain (DBD) is responsible for the sequence specific interaction of the receptor with DNA. It is encoded by exons 2 and 3 and also contains sequences involved in AR homodimerization. The ligand-binding domain (LBD), which is encoded by exons 4 to 8, contains the androgen binding function and also plays a role in receptor dimerization [43]. Curiously, truncation of the LBD results in a constitutively active receptor [44]. The DBD and the LBD are linked by a stretch of about 50 amino acids, called the hinge-region. The nuclear localization signal (NLS) that targets the nuclear translocation of the receptor is located in this region.

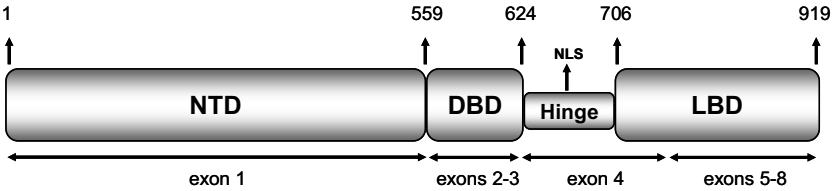

Figure 2 - Schematic representation of the androgen receptor structure. The AR is composed of three functional domains: the N-terminal domain (NTD), the DNA-binding domain (DBD) and the ligand-binding domain (LBD). A nuclear localization signal (NLS), located in the hinge region, targets nuclear translocation upon hormone binding. Amino acid and exon numbering are indicated above and under, respectively.

The AR is highly expressed in the luminal layer of glandular epithelia of male accessory sex organs, breast and skin, and surrounding smooth muscle, fibroblasts and accepted model for the intracellular action mesenchimal stromal cells. Other AR expressing tissues include skeletal muscle, vascular endothelium, brain, bone and hair follicles [45]. Consequently, these are also the tissues primarily affected by alterations in AR signaling, which associate with impairments in male virilisation and fertility, spinal and bulbal muscular atrophy, osteoporosis, acne, hair loss (alopecia) and cardiovascular disease [46]. The currently accepted model for the intracellular actions of androgens in the prostate is depicted in Figure 3.

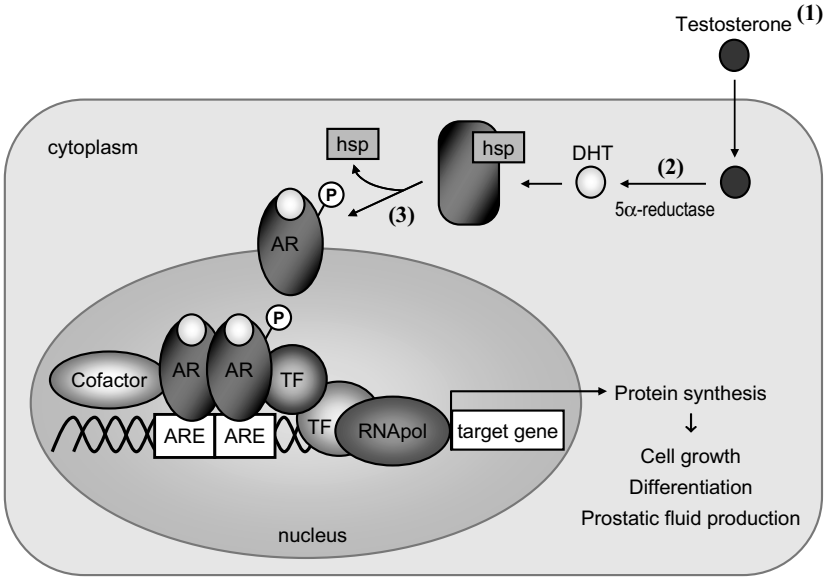

Figure 3 - The AR signaling pathway in the prostate. Sites of therapeutical intervention practiced currently are marked with numbers: (1) chemical/surgical castration can be used to reduce the levels of circulating testosterone; (2) conversion of testosterone to its fully active metabolite DHT can be targeted with inhibitors of 5α-reductase enzyme; (3) AR antagonists can be used to compete with DHT and testosterone for binding to the AR. TF: transcription factors. RNA pol: RNA polymerase; ARE: androgen response elements; hsp: heat-shock proteins.

Testosterone is synthesized in the testis under the control of luteinizing hormone (LH) from the pituitary. It enters the target cells by diffusion and, in the cytoplasm of some cell types, is converted by 5α-reductases to the more potent metabolite DHT. Upon AR binding, the receptor will undergo a conformational change, dissociate from chaperone heat-shock proteins and translocate to the nucleus. The active form of the receptor then binds as a homodimer to specific Androgen Response Elements (AREs) in the promotors/enhancers of target genes. Here, the AR interacts with specific nuclear cofactors, including co-activators or co-repressors, and the general transcription machinery, regulating expression of genes involved in the proliferation, differentiation and maintenance of the prostate [43,47,48]. The expression pattern of androgen-regulated genes is cell-type specific and changes with the developmental stage. During embryonic development androgens induce the expression of growth factors in mesenchymal stroma cells, which in turn indirectly stimulate the proliferation of prostatic epithelial cells that do not yet express the AR. In the adult prostate, secretory luminal cells, as well as the stromal cells, do express the AR, but at this stage the role of androgens is no longer to promote growth but rather to maintain the structure, differentiation state and secretory function of the gland [38]. In adult prostatic luminal cells, androgens regulate the expression of genes involved in the production and secretion of the components of prostatic fluid. These include genes encoding secreted proteins, such as PAP and PSA, but also genes involved in the metabolism of proteins, steroids, lipids and polyamines [49]. Furthermore, androgens inhibit epithelial cell death, since castration results in prostatic involution and nearly complete loss of these cells through apoptosis. Besides their role in normal prostate development and homeostasis, androgens may also fuel the growth of prostate tumors. The first indication that prostate adenocarcinomas were androgen-dependent came from Huggins *et al.* over six decades ago [30]. In a revolutionary experiment, the authors showed that prostate tumors shrank upon castration, setting the basis for hormonal therapy.

1.7. The AR pathway in prostate cancer development and progression

Only in atypical situations will androgens stimulate proliferation of adult prostatic epithelial cells, for instance during regeneration after castration/injury or in the case of malignant transformed cells. The fact that androgens stimulate the proliferation of prostate cancer epithelial cells has puzzled investigators for decades. The recent discovery that over 60% of the prostate tumors harbor a fusion between the TMPRSS2 gene and an ETS gene family member has shed light into this enigma [50]. TMPRSS2 is an androgen-regulated gene expressed in normal and malignant prostate epithelial cells, whereas ETS family members are transcription factors that regulate expression of growth and development related genes. ETS factors are normally not expressed in prostate epithelial cells, but fusions with 5' untranslated region of the TMPRSS2 gene provide androgen-responsive promotor/enhancer elements that drive robust expression of these potential oncogenes. In fact, the ETS factor most frequently fused in prostate cancer is the ERG oncogene, which is also involved in chromosomal translocations in Ewing sarcoma, myeloid leukemia and cervical carcinoma [51,52,53]. Up to date, fusions with ERG, ETV1, ETV4 and ETV5 factors have been detected in prostate cancer but TMPRSS2:ERG gene fusions are by far the most common, perhaps because both

genes are located in the same orientation within the same chromosomal locus (21q22.3) [54,55]. Following the discovery of TMPRSS2, additional 5′ fusion partners have been identified, including SLC45A3, ACSL3, HERV-K_22q11.3, HNRPA2B1, C15orf21, KLK2, CANT1, FLJ35294 and DDX5, most of which are prostate-specific and androgen-regulated [55,56,57]. These findings suggest that deregulation of ETS oncogenes through fusions with androgen-responsive genes may be a possible explanation for the androgen-regulated growth of prostate adenocarcinomas. However, the biological role of aberrant ETS factors expression is still controversial, since prostate specific expression of ERG or ETV1 in mice induced PIN but not cancer [56,58]. Furthermore, *in vitro* overexpression of these proto-oncogenes did not affect proliferation but promoted cell migration and invasion. Finally, recent studies have shown that aberrant ERG expression cooperated with PTEN loss to promote progression from PIN to invasive adenocarcinomas, setting another piece in this puzzle [59,60,61].

Androgen ablation therapy of patients with disseminated disease offers no more than a temporary tumor remission and relieve of the symptoms, as the cancer will eventually become resistant and relapse. Theoretically, androgen-dependent prostate cancer cells have two possible mechanisms for surviving and growing under androgen-depleted conditions: to sensitize the AR pathway to the low androgen concentrations or to bypass the AR pathway by invoking alternative survival and growth pathways (Fig. 4) [62].

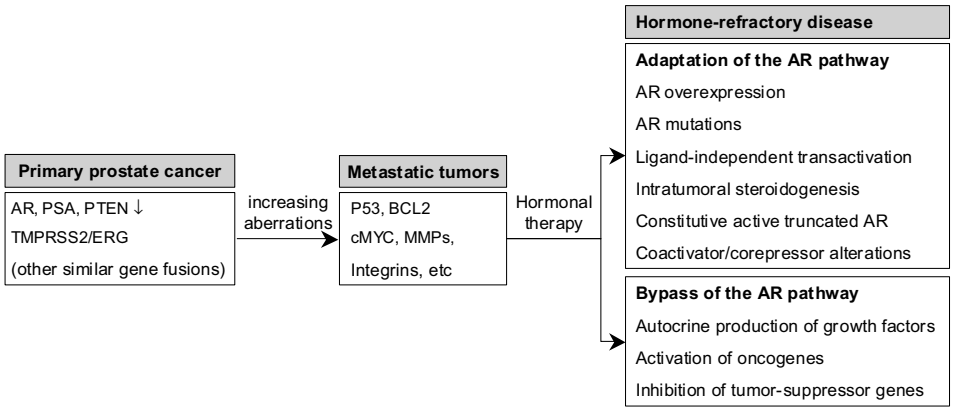

Figure 4 - The AR pathway in prostate cancer progression. Two strategies to overcome hormonal therapy: to adapt the AR pathway or to invoke alternative survival and growth pathways

Immunohistochemical analysis of prostate biopsies showed that the great majority of the androgen-independent cancers express the AR, often at higher levels than in primary tumors [63,64,65]. This observation suggests that the AR pathway may still be functionally active in hormone-refractory disease. AR gene amplification is observed approximately 30% of the hormone-refractory tumors and has been proposed to confer a growth advantage under androgen ablation conditions [65,66,67]. Other

modifications of the AR pathway that may induce hormone-refractory growth include activating AR mutations, intratumoral steroidogenesis, ligand-independent activation by cross-talk with other signaling pathway, constitutively active AR isoforms and alterations in AR co-regulators [62,68,69]. Many AR mutations have been discovered in prostate cancer that alter ligand specificity and receptor activity, the most widely known and intensely studied being the T877A mutation [70]. This mutation results in a threonine to alanine substitution in the ligand-binding domain of the AR, enabling receptor binding and activation by numerous non-androgenic compounds, including AR antagonists such as flutamide or cyproterone acetate [71]. This and similar AR mutations are relatively rare in hormone-naïve disease, but its frequency is much increased in recurrent tumors after hormonal therapy [72,73,74]. Also, two mutations (E231G and K580R) were recently identified that lead to increased AR basal activity and by itself were enough to produce prostate cancer in transgenic mouse models, implicating the AR as a potential oncogene [75,76]. A growing list of AR mutations is available in the AR Mutation Database (http://ww2.mcgill.ca/androgendb/) [77]. Recent reports suggest that prostate tumors may be able to synthesize androgens *de novo* and/or to convert adrenal steroids into testosterone and DHT [78,79]. These studies showed an increased expression of steroidogenic enzymes, associated with elevated intratumoral androgen levels, in hormone-refractory metastasis. In this manner, local androgen production by prostate cancer cells may permit tumors to circumvent the depletion of circulating androgens. Cumulative evidence shows that the AR may also be activated in the absence of androgenic ligands, by cross-talk with Ras/MAPK, PI3/AKT and JAK/STAT pathways [80,81]. Activation of these pathways by numerous signaling factors, such as IL6, IGF1, KGF or EGF, can induce AR phosphorylation, consequent homodimerization, nuclear translocation and stimulation of AR target genes (Fig. 5A) [82]. Another potential mechanism for maintaining the AR pathway in the absence of ligand is the expression of constitutively active AR isoforms, lacking the C-terminal LBD domain. These constitutively active isoforms may arise from alternative splicing variants, truncating mutations or proteolytic cleavage, all of which have been recently detected in hormone-refractory samples [83,84,85]. The control of AR function also involves interaction of the receptor with a number of co-factors that regulate AR transcriptional activity. Alterations in ARA55, ARA70, SRC1 or TIF2 co-activators, as well as NCOR1 and NCOR2 co-repressors, among others, have been described in hormone-refractory disease [86,87]. However, these reports are often conflicting and further studies are necessary to elucidate the precise role of AR coregulators in prostate cancer progression. The mechanisms of AR pathway adaptation during prostate cancer progression will be further discussed in Chapters 3 and 4.

As an alternative to adapt the AR pathway, tumor cells may become truly androgen-independent, escaping the demand for androgens and an active receptor. To effectively bypass the AR pathway, cancer epithelial cells must be able to survive the apoptotic signals triggered by androgen ablation and invoke alternative growth pathways. This may be achieved by autocrine production of growth factors or its receptors, by activation of oncogenes and/or by deactivation of tumor suppressor genes.

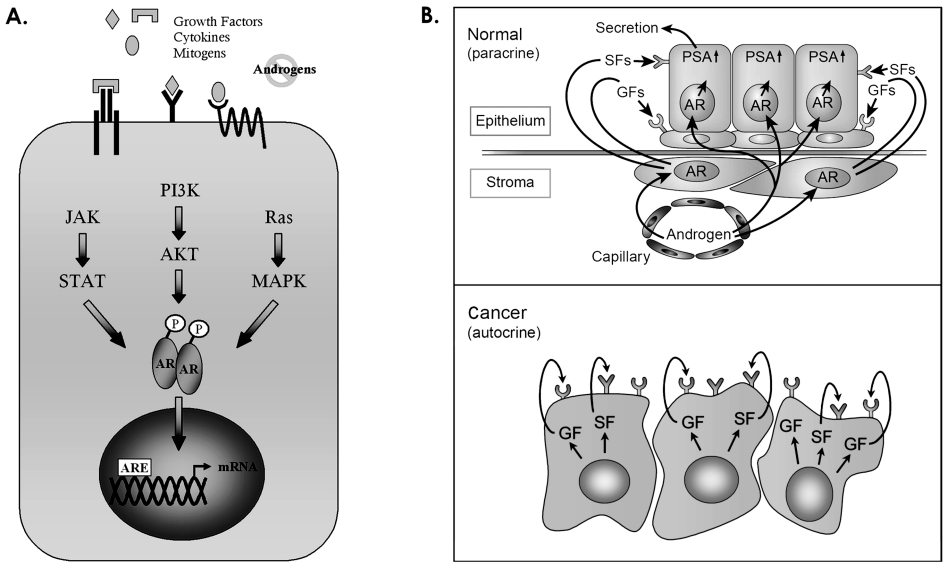

Figure 5 – Mechanisms for hormone-refractory growth. **(A)** Ligand-independent AR activation via cross-talk with intracellular signaling pathways. **(B)** Bypass of the AR pathway by autocrine production of survival (SF) and growth factors (GF).

Programmed cell death (apoptosis) and cell division are part of cell renewal and differentiation in benign cells. But the balance between these two processes is shifted during prostate malignancy (and other cancers) towards cell growth and proliferation. The cell cycle regulators MYC, RB and EZH2, as well as key regulators of apoptosis, such as TP53, PTEN (pro-apoptotic) or BCL2 (anti-apoptotic), have all been reported to be deregulated in prostate cancer [88,89]. Survival and growth of prostate epithelial cells are also regulated in paracrine manner by soluble factors secreted by stromal cells in response to androgens, including IGF1, KGF, FGF family members, EGF, HGF, TGFB, IL6, among others [90]. A switch to autocrine production of these growth factors by epithelial cells may be a potential mechanism to circumvent AR signaling (Fig. 5B). Chapter 5 of this thesis will address the various means to bypass the AR pathway.

1.8. Model systems in prostate cancer investigation

To investigate the mechanisms whereby prostate cancer develops and progresses, identify disease markers and potential therapeutical targets or to test novel drugs, the use of prostate cancer model systems is essential for basic research. The ideal model would reproduce the characteristics of the different stages of prostate cancer in humans, from initiation to metastasis, including hormone-refractory disease. But in practice, this ideal model system does not exist and researchers must meticulously choose, from the available human and animal derived systems, the model that best suits the particular question addressed. Animal model systems include transgenic and knockout mice,

hormonally/carcinogenically induced prostate cancer in rats and spontaneous prostate cancer in dogs. These model systems have the advantages of allowing the study of the initiation events in prostate carcinogenesis and the testing of (dietary) prevention programs. Eventually, the results of animal models must be extrapolated and validated in human derived systems, such as mouse xenografts or *in vitro* cell lines. In the xenograft models, human prostate tissue is transplanted and propagated in immune-deficient mice. These systems allow the maintenance of human prostate tumors *in vivo*, being particularly suitable for the study of tumor progression, biomarker identification and the testing of novel therapies. The major limitation of human model systems (xenografts and cell lines) is that these are derived from established prostate tumors and, therefore, cannot replicate prostate cancer initiation. *In vitro* cell line cultures offer unlimited sample amounts and extra experimental flexibility, being used for long-term hormonal manipulations and in the basal research of oncogenes, tumor suppressor genes and signaling pathways. Due to the technical difficulties in establishing permanent *in vitro* cultures of human prostate carcinomas, the number of cell lines available is very limited and the vast majority of the *in vitro* studies are based on the three cell lines and its derivatives: LNCaP, PC3 and DU145. Although undoubtedly valuable, these "classical" cell lines have serious limitations: the LNCaP cell line used as prototype for androgen-responsive disease, expresses a mutated (T877A) AR, showing altered hormone response properties; whereas PC3 and DU145, unlike most prostate tumors, do not express AR or PSA [71,91]. Therefore, in our laboratory added efforts were set into the generation of xenograft and *in vitro* cell lines representing different disease stages, among which the PC346 model used in this manuscript. A review of currently available human prostate cancer cell lines and xenografts is presented in Chapter 2.

1.9. Scope of the thesis

Activation of the AR by androgens is responsible for the normal development and maintenance of the prostate but also sustains its malignant outgrowth. Whether the AR pathway is still driving prostate cancer survival and growth in patients under androgen blockade is a clinically relevant issue. With this project we seek for a better understanding of the involvement of the AR pathway in the progression of androgen-dependent prostate cancer into androgen-independence. It is our hypothesis that androgen-dependent tumors have two possible strategies for growing in a low-androgen environment: to sensitize the AR pathway to the lowered androgen concentrations and/or to bypass the AR pathway by invoking alternative survival and growth pathways.

To assess our hypothesis we made use of an *in vitro* panel of human prostate cancer cell lines, the PC346 progression model. PC346C, PC346DCC, PC346Flu1 and PC346Flu2 are a panel of prostate cancer cell lines derived from the transurethral resection of the prostate of a non-progressive patient. The PC346C cell line was directly produced from the PC346P xenograft. The other mentioned cell lines were derived from the parental PC346C by long-term culture on androgen-depleted medium (PC346DCC) or in androgen-depleted medium supplemented with hydroxyflutamide (PC346Flu1 and PC346Flu2). Chapter 2 summarizes the establishment and characterization of the

PC346 progression model and compares it to other available human prostate cancer cell lines and xenografts.

The first aim of this study was to assess whether the *in vitro* long-term hormone depletion would select for modifications in the AR pathway and how these modifications could affect cell growth and androgen response. To characterize the AR status, cell growth and hormone-responsiveness of the PC346 cell lines, the AR was sequenced and quantified, its activity determined by reporter assays, and hormone-responsiveness was assessed by growth assays upon stimulation with androgens and antiandrogens. This work is described in Chapter 3, where we address how changes in the AR pathway may result in an adaptive advantage for progression of prostate tumors. Furthermore, we show that the cell lines developed for this project are a representative model system to investigate the role of AR signaling in prostate cancer progression.

In Chapter 4 we focus on the hypothesis that prostate cancer cells achieve androgen-independence by adapting their AR pathway to the androgen-depleted conditions. We used microarray technology to compare the expression pattern of androgen-regulated genes between the androgen-responsive and androgen-independent PC346 cell lines, in an attempt to: (i) establish the gene expression program regulated by the AR in these cell lines; (ii) establish whether the AR is still functional in the androgen-independent sublines; (iii) identify the mechanism(s) by which the AR pathway may be adjusted to the low androgen/high antiandrogen levels; (iv) identify androgen-regulated genes that could possibly be used in the diagnosis/prognosis of prostate cancer or as a therapeutic target.

The final aim of this project was to explore whether the AR pathway was still active in the androgen-independent cells under androgen-deprived conditions and to identify putative alternative growth/survival pathways. To accomplish this, the androgen-independent sublines, cultured in their respective androgen-deprived selection medium, were compared with the parental androgen-responsive PC346C (supplemented with androgens), by using expression microarrays (Chapter 5). By further comparing the differentially expressed genes in the AI sublines versus the androgen-responsive PC346C, with the androgen-regulated genes established in Chapter 4, we intend to: (i) assess whether the AR pathway is “ON” or “OFF” in the AI sublines; (ii) identify putative alternative growth/survival pathways; (iii) select and validate a set of non-androgen-regulated genes that could drive prostate cancer growth. To guide the selection of these genes, the microarray data produced in this study was linked to an assortment of publicly available gene expression databases on human prostate tumors, xenografts and cell lines, using the SRS platform [92]. This allowed access to a comprehensive collection of data leading to crucial clues on how the AR pathway evolves and how putative oncogenes and tumor suppressors are regulated in prostate cancer progression.

1.10. References

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Chapter 2

The Human PC346 Xenograft and Cell Line Panel: a Model System for Prostate Cancer Progression

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ABSTRACT

Objective: Prostate cancer (PCa) model systems that reflect the different disease stages are essential for studying the development and progression of PCa and for testing new treatment modalities. This review summarizes the establishment and characterization of the PC346 progression model, and compares it to other human PCa cell lines and xenografts available.

Methods: The PC346 model was derived from the transurethral resection of a primary prostate tumor. Tumor samples were subcutaneously implanted into athymic mice, resulting in the development of a series of xenografts, from which *in vitro* cell cultures were established.

Results: The PC346 panel includes sublines with hormone-response characteristics that range from androgen-sensitive to androgen-independent growth. *In vivo* and *in vitro* selection of androgen-sensitive lines under androgen-depleted conditions replicated the clinically relevant relapse phenomenon, and resulted in a series of modifications in the androgen-receptor (AR) pathway: AR mutation, overexpression and downregulation.

Conclusions: The PC346 panel reproduces many biological characteristics of the different phases of clinical PCa and the most common AR modifications observed in hormone-refractory tumors, being a valuable addition to the limited collection of model systems currently available.

INTRODUCTION

The understanding of prostate cancer (PCa) basic biology and the development and testing of novel therapies require the use of suitable model systems. An ideal model system should mimic the characteristics of different stages of prostate cancer in humans, from cancer initiation, progression, hormone-refractory disease and metastasis. There are different types of PCa models: animal systems, such as transgenic and knockout mice, hormonally/ carcinogenically induced PCa in rats and spontaneous dog models; or systems derived from (human) prostate tissue, such as xenografts and *in vitro* cell lines [1]. This review will focus on the latter, xenografts and *in vitro* culture models established from human prostate tumors, in particular the development and characterization of the PC346 model.

Xenografts are models in which human prostate tissue is transplanted into an animal, generally an immune-deficient mouse. In this way, human prostate tumors can be propagated *in vivo* for indefinite periods of time, allowing the study of tumor progression under different experimental conditions and the testing of novel therapies. Although xenografts resemble more closely the *in vivo* situation of the patient, *in vitro* cultures offer more experimental flexibility, a higher control over environmental variables and unlimited sample amounts. On the other hand, *in vitro* cell lines lack the interaction with the prostate environment and miss the effect of stromal/epithelial interactions and vascularization. These model systems are useful and complementary for defining the role of hormones in PCa progression and the signaling pathways involved in hormone-refractory cell proliferation. A wide variety of *in vivo* and *in vitro* models are necessary to represent the different characteristics and progression of human PC. Unfortunately, due to the poor growth of human prostate tissue *in vitro* and *in vivo*, the number of xenografts and cell lines available is limited. Furthermore, many of the available systems were established from metastatic lesions or represent the advanced-stage hormone-refractory state of the disease, whereas models for the androgen-dependent state are rare [2, 3]. Until the mid nineties, researchers were restricted to the “classical” *in vitro* PC-3, DU145 and LNCaP, but extensive efforts during the past decade have lead to a considerable increase in the collection of xenografts and cell lines available (Table 1). Traditionally, PCa xenografts have been grafted and propagated subcutaneously. Recently, Wang and associates showed that subrenal grafting of human prostate tissue resulted in more efficient tumor-take and short-term recovery than subcutaneous grafting, probably due to the high degree of vascularity of the site [4]. Besides the higher tumor-take rate, subrenal grafting resulted in better histopathological differentiation and could also be used with benign and low-grade tumor tissues, which are generally very difficult to grow subcutaneously. Although the authors have proved that the prostate tissue could be efficiently grafted, maintained and recovered from the subrenal compartment, they have not addressed whether these xenografts could be serially transplanted as permanent lines. Furthermore, the subrenal site has a limited xenograft carrying capacity, and the grafting surgery and follow up of tumor growth are technically complex. Despite the need for further optimization, subrenal grafting is a promising technique that will hopefully help boost the development of novel xenograft models.

Table 1 - Overview of established xenografts and *in vitro* culture models for human prostate cancer.

Name	Year	Origin	Androgen response	AR expression	PSA expression	AR sequence
Prostate Cancer Xenografts						
PC-EW [5]	1984	Lymph node	AD	Yes	Yes	Wt
PC82 [6]	1980	Primary	AD	Yes	Yes	Wt
PC133 [3]	1981	Bone	AI	No	No	n/a
PC135 [3]	1982	Primary	AI	No	No	n/a
PC295 [7]	1996	Lymph node	AD	Yes	Yes	Wt
PC310 [7]	1996	Primary	AD	Yes	Yes	Wt
PC324 [7]	1996	Primary	AI	No	No	n/a
PC339 [7]	1996	Primary	AI	No	No	n/a
PC374 [7]	1996	Skin	AI	No	No	n/a
PC346P* [7]	1996	Primary	AS	Yes	Yes	Wt
PC346B [3]	2000	Primary	AS	Yes	Yes	Wt
CWR21 [8]	1993	Primary	n/a	n/a	n/a	n/a
CWR22 [8, 9]	1993	Primary	AD	Yes	Yes	H874Y
CWR31 [8]	1993	Primary	n/a	n/a	n/a	n/a
CWR91 [8]	1993	Primary	n/a	n/a	n/a	n/a
LuCaP 23.1 [10]	1996	Lymph node	AS	Yes	Yes	n/a
LuCaP 23.8 [10]	1996	Lymph node	AS	Yes	Yes	n/a
LuCaP 23.12 [10]	1996	Liver	AS	Yes	Yes	n/a
LuCaP 35 [11]	2003	Lymph node	AS	Yes	Yes	Wt
LuCaP 41[12-14]	1999	Primary	AS	Yes	Yes	n/a
LuCaP 49 [15]	2002	Lymph node, small cell carcinoma	AI	No	No	n/a
LuCaP 58 [12-14]	1999	Lymph node	AS	Yes	Yes	n/a
LuCaP 69 [12-14]	1999	Bowel	n/a	Yes	Yes	Wt
LuCaP 70 [12-14]	1999	Liver	n/a	Yes	Yes	n/a
LuCaP 73 [12-14]	1999	Pelvis	AI/AS	Yes	Yes	n/a
LAPC-3 [16]	1997	Primary	AI	Yes	Yes	Wt
LAPC-4 [16]	1997	Lymph node	AD	Yes	Yes	Wt
LAPC-9 [17]	1999	Bone	AS	Yes	Yes	Wt
MDA PCa-31 [1]	1998	Liver	n/a	Yes	Yes	n/a
MDA PCa-40 [1]	1998	Liver	n/a	No	No	n/a
MDA PCa-43 [1]	1998	Adrenal	n/a	Yes	Yes	n/a
MDA PCa-44 [1]	1998	Skin	n/a	No	No	n/a
TEN12 [18]	2004	Primary	AD/AS	Yes	Yes	Wt
BM18 [19]	2005	Bone	AD	Yes	Yes	n/a
Prostate Cancer *In Vitro* Cell Lines						
PC-3 [20]	1978	Bone	AI	No	No	n/a
DU 145 [21]	1978	Brain	AI	No	No	n/a
LNCaP [22]	1980	Lymph node	AD/AS	Yes	Yes	T877A
1013L [23]	1980	Primary, transitional cell carcinoma	AI	No	No	n/a
UM-SCP-1 [24]	1984	Primary, squamous cell carcinoma	AI	No	No	n/a
ARCaP [25]	1996	Ascites	AI/AS	Yes	Yes	Wt
MDA PCa 1**	1996	Ascites	AI	No	No	n/a
MDA PCa 2a [26]	1997	Bone	AI/AS	Yes	Yes	L701H, T877A
MDA PCa 2b [26]	1997	Bone	AI/AS	Yes	Yes	L701H, T877A
LAPC-4 [16]	1997	Lymph node	AD	Yes	Yes	Wt
22Rv1 [27]	1999	Primary, CWR22R xenograft	AI/AS	Yes	No	H874Y, 2xExon3
PC346C [3]	2000	Primary, PC346P xenograft	AS	Yes	Yes	Wt
PSK-1 [28]	2000	Primary, small cell carcinoma	AI	No	No	n/a
CWR-R1 [29]	2001	Primary, CWR22R xenograft	AI/AS	Yes	No	H874Y
DuCaP [11]	2001	Dura mater	AI/AS	Yes	Yes	Wt
VCaP [30]	2001	Vertebra	AI/AS	Yes	Yes	Wt

This table includes a list of original xenografts and *in vitro* cultures, but not their derivative sublines, artificially immortalized lines or lines previously reported as cross-contaminants. AD: androgen-dependent; AI: androgen-independent; AS: androgen-sensitive; Wt: *wil- type* sequence; n/a: not available. *The PC346P xenograft has also been previously referred to as PC346. ** MDA PCa 1 was derived from the same patient as the ARCaP cell line. The LuCaP series has been extended with xenografts LuCaP 77, 81, 86.2, 92.1, 93, 96, 105 and 115 [31]. To date, no detailed information has been published on the origin, androgen responsiveness and AR status of these lines, which therefore were not included in this table.

PC346 is a progression model designed to study the mechanisms of androgen-independent outgrowth, which inevitably follows endocrine therapy of patients. The PC346 panel consists of five xenografts and six cell lines, which represent different phases of clinical PC, from androgen-responsive growth, through tumor relapse, to androgen independence. In this article we will review the establishment and characterization of the complete PC346 panel, including the most recent additions.

Origin of he PC346 tumor model

A Caucasian 68 years old male was diagnosed with bladder cancer and advanced prostate adenocarcinoma, presenting 17 ng/ml serum PSA. He had negative scans for both bone and lymph node metastasis (T4N0M0), and underwent four weeks treatment with cyproterone acetate prior to a transurethral resection (TUR) of the prostate. The tumor was dissected from surrounding tissue and small pieces of solid tumor tissue (approximately 30 mm^3) were subcutaneously implanted in both shoulders of male athymic NMRI nude mice (Naval Medical Research Institute, Bethesda, Maryland, USA). Two samples of the TUR material were propagated in parallel, giving rise to two distinct lines, PC346P and PC346B, from which a panel of five xenografts and six *in vitro* cell cultures was established (Fig. 1). Histologically, the primary patient material (Gleason score 9) and resulting xenografts show a low degree of differentiation, with lack of glandular structures (Fig. 2A).

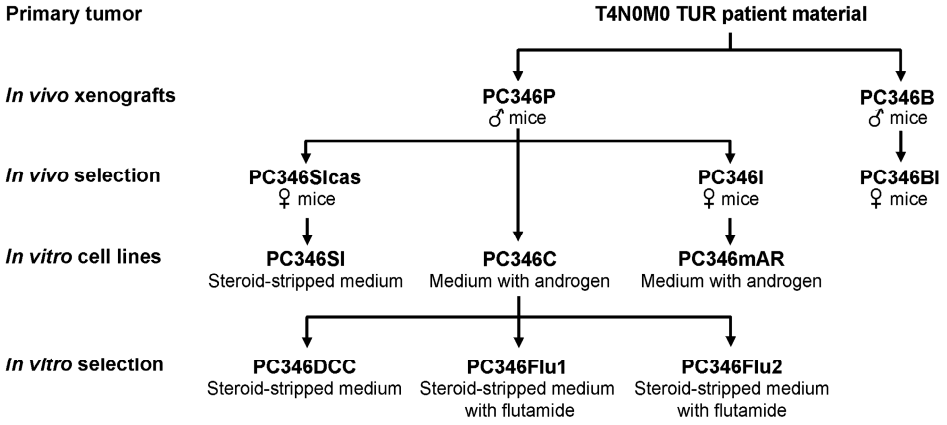


Figure 1 - Development of the PC346 human prostate cancer progression model

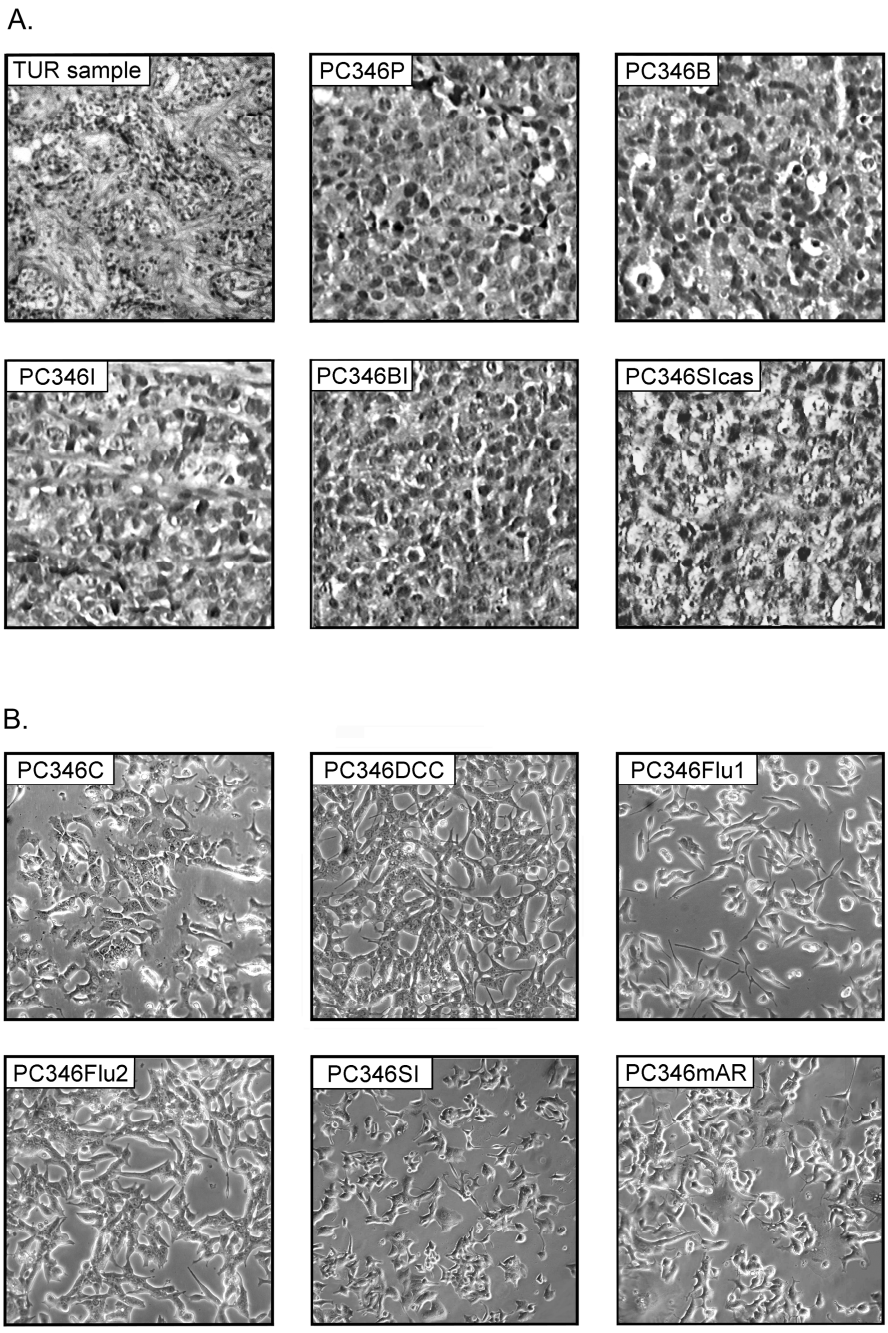


Figure 2 - Morphological characteristics of PC346 xenografts and cell lines. **(A)** Hematoxylin-eosin stained sections from formaldehyde-fixed, paraffin-embedded patient TUR material and derived xenografts (original amplification 100x). **(B)** Phase contrast microscopic photographs of the *in vitro* cell cultures (200x).

Establishment and growth characteristics of PC346 xenografts

The parental PC346P and PC346B tumors show similar growth characteristics in intact male mice: a high (85-90%) take rate of the transplants, 3-8 weeks lag-phase and an average doubling-time of about 10 days (Table 2). Both these xenografts are being propagated in intact male NMRI nude mice for over 50 passages and, to date, tumor growth characteristics have remained constant. These xenografts were originally identified as androgen-dependent, since tumor transplantation into female or castrated male mice rarely resulted in tumor development [7]. The tumors that did develop had a prolonged lag-phase and a 2-4 fold reduction in growth rate (Table 2). Furthermore, roughly half of these tumors eventually regressed and disappeared within a few months, and the remaining tumors would not survive subsequent re-transplantations in female or castrated males. This posed great difficulties to the development of androgen-independent (AI) sublines, and called for a more elaborate selection approach.

The AI sublines PC346I and PC346BI were derived from PC346P and PC346B, respectively, by initially transplanting these tumors in female mice supplemented with testosterone (through a subcutaneous silastic implant [32]). After 50 days, once the tumors were established and growing, the testosterone implant was removed and some tumors showed a regression followed by regrowth. These regrowing tumors were subsequently passaged into non-supplemented female mice. Only two of these recurrent tumors were able to grow in female mice: the PC346I and PC346BI xenografts (Fig. 1). PC346I and PC346BI tumors grow equally well in female and male mice, showing AI growth characteristics with no significant response to castration (Table 2).

For the androgen-sensitive PC346P and PC346B xenografts, androgen ablation of established tumors (300-800 mm^3) yielded considerably different results from those of castration prior to transplantation. Castration of mice bearing PC346P tumors, resulted in variable responses, including tumor regression (in one third of the cases), tumor retardation followed by growth relapse, and continued growth (Fig. 3A). Despite this high variation, the growth rate of PC346P is significantly decreased upon castration (Fig. 3B). Interestingly, established PC346B tumors, did not significantly respond to castration of the tumor-bearing mice (Fig. 3B). These results indicate that both PC346P and PC346B are androgen-responsive but not strictly dependent on androgens for growth. The fact that PC346P and PC346B, two xenografts derived from the same tumor, respond in a different way to castration, suggests heterogeneity in the original patient material. Indeed, tumor heterogeneity is not a novel finding nor is it exclusive to prostate carcinomas [33].

In1981 Isaacs *et al.* hypothesized that prostate tumors contained a mixture of cells with different androgen sensitivities, and that the transition to androgen-independence involved the “clonal expansion” of AI cells already present prior to the androgen ablation treatment [34]. This hypothesis is supported by Craft *et al.*, who used serial dilution and fluctuation analysis of the LAPC-9 xenograft model to show that AI cells were already present in the original tumor at a frequency of about 1 per 10^5-10^6 androgen-dependent cells [17]. Moreover, the fact that prostate carcinomas are often multifocal, with two or more histologically but also genetically distinct tumors within the prostate, further corroborates the multiclonal character of prostate carcinomas [35-37]. An alternative hypothesis for the transition to the AI state is that PCa cells adapt to the

altered hormonal environment by changing gene expression. In reality, the transition to androgen-independence is a complex process that probably involves both clonal selection, and adaptative upregulation of growth promoting or downregulation of apoptotic genes [38]. The diverse assortment of PC346 lines provides a valid model system to help elucidate this phenomenon.

After copious attempts, we recently produced a hormone-refractory subline of PC346P by direct transplantation into female mice: the PC346SIcas xenograft. In female mice, this xenograft has a 75% tumor-take rate, 4-10 weeks lag-phase and a doubling-time of 10 days. As with PC346I and PC346BI, tumor-take and growth rates of PC346SIcas were not significantly different between castrated and sham-castrated male mice, indicating that the subline is androgen-independent (Table 2).

Table 2 – Tumor development of PC346 xenografts in male, castrated male and female mice.

	Males			Castrated males			Females		
	TT	LP	TD	TT	LP	TD	TT	LP	TD
PC346P	89 ± 4%	25-45	8.1 ± 0.5	16 ± 9%	37-79	32.0 ± 7.7	10 ± 5%	67-116	19.5 ± 3.0
PC346B	87 ± 5%	19-62	12.1 ± 2.6	8 ± 5%	30-52	56.4 ± 22.0	10 ± 6%	56-122	25.8 ± 11.5
PC346I	83 ± 7%	23-35	10.5 ± 1.4	81 ± 7%	29-55	8.5 ± 0.8	71 ± 7%	30-49	10.1 ± 1.0
PC346BI	86 ± 8%	19-32	11.8 ± 1.6	80 ± 12%	19-35	11.2 ± 2.1	82 ± 7%	18-52	11.0 ± 0.8
PC346SI	93 ± 3%	30-51	10.6 ± 1.7	90 ± 3%	28-58	12.4 ± 0.8	75 ± 8%	34-69	10.1 ± 1.7

TT, tumor take, percentage of tumors that develop after transplantation. LP, lag phase: time (days) till tumor volume reaches 100 mm^3. TD, tumor-doubling time.

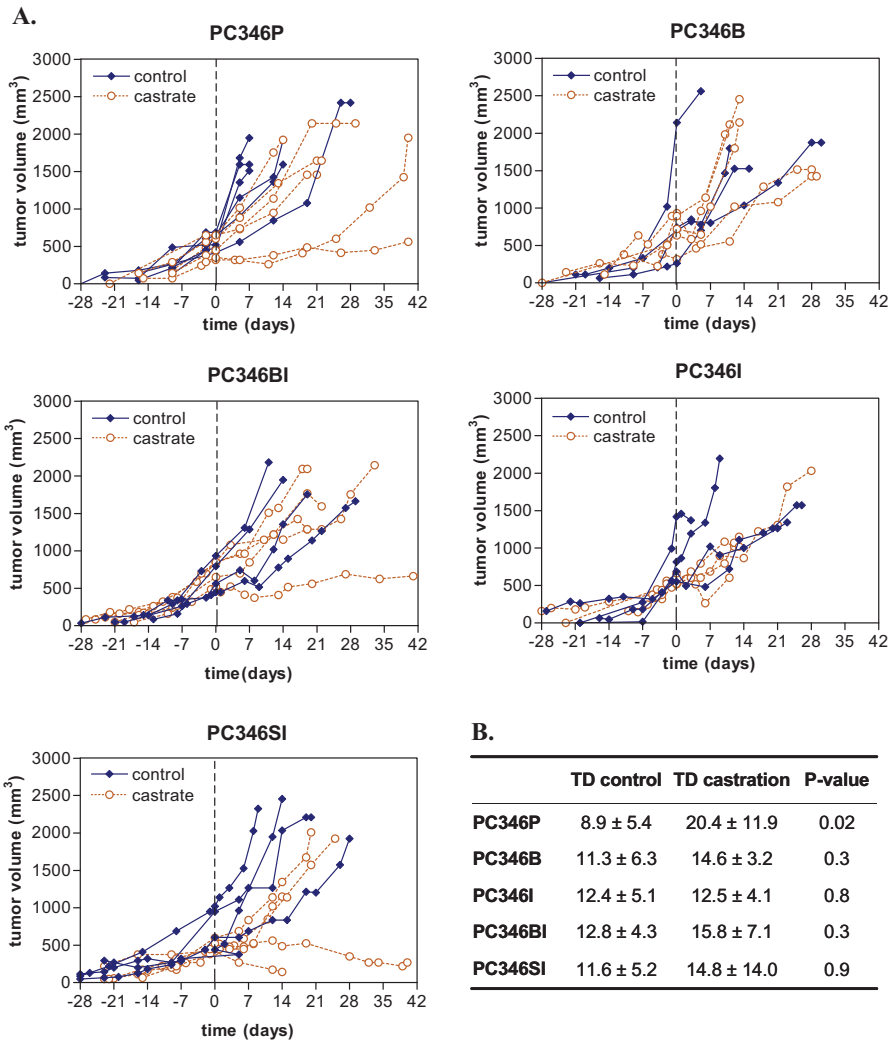
Establishment and growth characteristics of PC346 in vitro cell lines

Efforts to generate *in vitro* cultures from the various PC346 xenografts culminated in the development of PC346C, PC346mAR and PC346SI, from the PC346P, PC346I and PC346SIcas xenografts, respectively (Fig. 1). Unfortunately, until now we have been unable to establish permanent *in vitro* lines from PC346B and PC346BI. The PC346C cell line, like its parental xenograft PC346P, is androgen-responsive: it grows slowly in steroid-stripped medium, is 2-3 fold stimulated by the synthetic androgen R1881 and is not stimulated by the antiandrogen hydroxyflutamide (Fig. 4).

To mimic *in vitro* the endocrine therapy applied to patients with advanced metastatic PC, we continuously cultured (>2 years) PC346C in androgen-depleted conditions, producing the PC346DCC, PC346Flu1 and PC346Flu2 sublines [39]. PC346DCC was derived from passage 45 of PC346C by culturing in steroid-stripped medium. The flutamide-resistant PC346Flu1 and PC346Flu2 sublines were produced from passage 45 and 75 of PC346C, respectively, by culturing in steroid-stripped medium supplemented with 1 μM of hydroxyflutamide. The resulting AI sublines not only show distinct morphologic features (Fig. 2B), but also different hormone response properties. PC346DCC grows well in the absence of androgens and is unresponsive to either R1881 or hydroxyflutamide (Fig. 4). PC346Flu1 grows optimally in steroid-stripped medium or in medium supplemented with hydroxyflutamide, being inhibited by physiologic concentrations of androgens. PC346Flu2 and PC346mAR share similar hormone responses. These lines are stimulated by both R1881 and hydroxyflutamide,

raising the question whether the AR is mutated. Finally, the PC346SI is, like PC346DCC, unresponsive to hormonal stimulation.

A.



B.

	TD control	TD castration	P-value
PC346P	8.9 ± 5.4	20.4 ± 11.9	0.02
PC346B	11.3 ± 6.3	14.6 ± 3.2	0.3
PC346I	12.4 ± 5.1	12.5 ± 4.1	0.8
PC346BI	12.8 ± 4.3	15.8 ± 7.1	0.3
PC346SI	11.6 ± 5.2	14.8 ± 14.0	0.9

Figure 3 - Response of PC346 xenografts to castration. Tumors were subcutaneously implanted into the shoulders of 6-8 weeks old male NMRI nu/nu mice, and tumor volume was measured weekly with calipers. **(A)** tumor growth in castrated male mice (--o--), compared to control sham-operated animals (–♦–). Mice were castrated when tumor volume reached 300-800 mm^3; vertical, dashed line at t0 indicates the time point of castration. Tumor volume is plotted for each individual animal. **(B)** effect of castration on tumor-doubling time (TD) of xenografts. The growth rates were determined by linear regression of the tumor volume (in semi-logarithmic scale) over time, and the TD calculated from the 1/slope of tumor volume rise; p-values were determined by Mann-Whitney statistical test.

Status of the AR pathway in the PC346 panel

The progression of human prostate cancer during endocrine therapy is characterized by an inevitable transition from androgen responsiveness towards androgen independence. Androgen regulation through testosterone and dihydrotestosterone is mediated by the androgen receptor (AR), a member of the steroid receptor family and a key transcription factor for genes involved in the proliferation, maintenance and differentiation of the prostate [40]. AR expression is present in the vast majority of primary prostate tumors, hormone-refractory cancers and metastasis [41, 42], and the AR pathway may still be actively implicated in the growth of androgen-independent cancer cells.

The PC346 panel of xenografts and *in vitro* cell lines constitutes an excellent tool to further study the role of the AR in the progression to AI PC. To assess the status of the AR pathway in the different lines, we quantified the AR and the AR-target gene PSA by western blotting. Additionally, exons 2-8 of the AR gene were checked for mutations by SSCP analysis and sequencing.

Western blot analysis revealed that, despite the very low levels of AR protein in PC346SIcas, PC346DCC and PC346mAR, all xenografts and cell lines expressed the receptor (Fig. 5). The PC346I xenograft and PC346Flu1 cell line overexpress the receptor in comparison to their respective androgen-responsive parental lines, PC346P and PC346C. Conversely, PC346BI and PC346DCC have downregulated AR expression upon selection under androgen-depleted conditions. PSA protein was also detected in all lines, although at extremely low levels in PC346DCC. The cellular localization of AR protein was assessed by immunocytochemistry. AR expression in PC346C, PC346Flu1, PC346Flu2 and PC346mAR cells, cultured in their respective selection medium, was localized in the nucleus. PC346DCC showed cytoplasmic AR expression, which was extremely low but above background staining of the negative control (no primary antibody). As observed in the western blot, PC346Flu1 showed increased AR expression compared to the other *in vitro* lines. PC346SI revealed a heterogeneous expression pattern, for the most part localized in the cytoplasm, but some positive nuclei were also present. In the androgen-sensitive xenografts PC346P and PC346B, propagated in intact male mice, the AR was mainly localized in the nucleus. In the androgen-independent xenografts PC346I and PC346BI, which were propagated in castrated mice, a stronger cytoplasmic staining was observed, together with some positive nuclei (Fig. 6). When the cell lines and xenografts were grown in the presence of androgens, nuclear staining was always predominant, also for the AR positive AI lines (data not shown).

Sequence analysis of the AR gene revealed a *wild-type* receptor in PC346P, PC346B, PC346BI and PC346SIcas xenografts, and in PC346C, PC346Flu1 and PC346SI cell lines. A Thr to Ala substitution at codon 877 in the steroid-binding domain was found in the PC346I xenograft and in two cell lines: PC346mAR and PC346Flu2. A novel mutation was detected in codon 311 of PC346DCC AR, resulting in Lys to Arg substitution [39]. However, AR reporter assays show no evidence of altered transactivation properties of this mutated receptor, which responds to R1881 and hydroxyflutamide like the *wild-type* AR (wtAR).

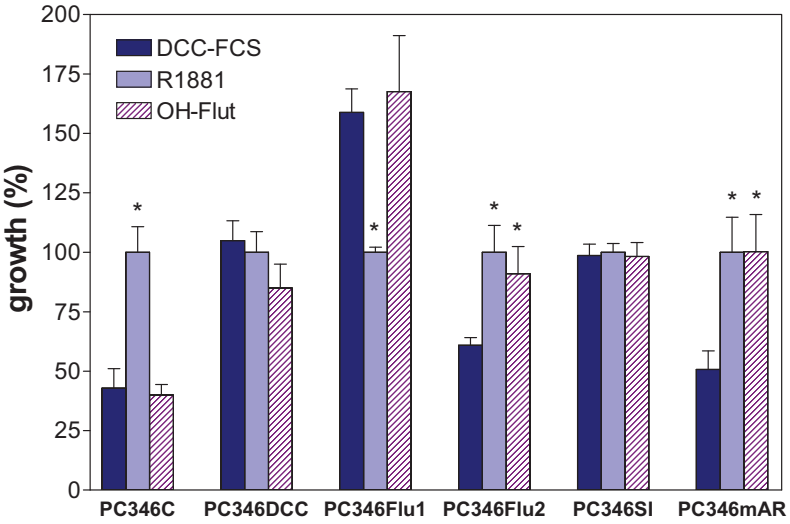


Figure 4 - Hormone sensitivity of PC346 in vitro cell lines. Cell growth was assessed after 10 days stimulation with 0.1 nM R1881 (R1881), 1 μM hydroxyflutamide (OH-Flut) or vehicle (DCC-FCS), using a MTT assay described previously [39]. Growth in the presence of 0.1 nM R1881 was set at 100 % and the results are expressed as mean + SEM of at least three independent experiments. *p < 0.05 by non-parametric Mann-Whitney test.

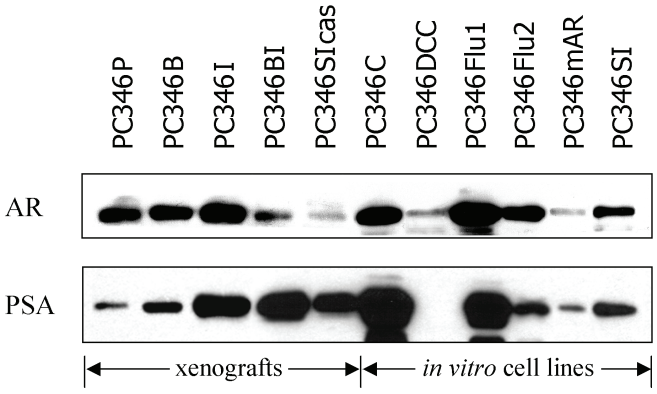


Figure 5 - Western blotting analysis of AR and PSA protein expression. Western blotting was performed on 10 μg total protein extract, using rabbit polyclonal antibodies sp197 anti-human AR [67] and anti-human prostate-specific antigen (DakoCytomation BV, The Netherlands), as described previously [68]. For the protein isolation, the androgen-independent PC346I, PC346BI, and PC346SIcas xenografts were propagated in castrated male mice, while the parental androgen-sensitive lines PC346P and PC346B were propagated in intact males. Each *in vitro* cell line was cultured in its respective selection medium: PC346C and PC346mAR on medium supplemented 0.1 nM of R1881; PC346DCC and PC346SI on steroid-stripped medium; PC346Flu1 and PC346Flu2 on steroid-stripped medium supplemented with 1 μM OH-flutamide.

Theoretically, androgen-dependent PCa cells have two possible strategies to evade androgen ablation and/or antiandrogen therapy: to adapt the AR pathway, or to invoke an alternative survival/growth pathway [43, 44]. Our observations with the PC346 AI sublines suggest that the AR pathway may still be important and functionally active in most hormone-refractory cancers. Indeed, other groups have observed in patient material increased AR levels in relapsed tumors in comparison to primary tumors [45, 46], while AR gene amplification has been reported in up to 30% of recurrent cancers [13, 47, 48]. It has been proposed that higher receptor levels could lead to sensitization to residual androgen concentrations and broadened ligand-specificity [49] or to constitutive ligand-independent activation [50]. Recently, Han *et al.* reported a somatic AR mutation (E251G), which is able to convert the receptor into a potent oncogene sufficient to cause invasive and metastatic PCa in transgenic mice [51]. This new data suggests that the AR may be a proto-oncogene and that alteration of the normal AR signaling can facilitate transformation and progression. Additionally, AR point mutations that alter the activity and binding-specificity of the receptor have been clinically associated with disease progression [52, 53]. The T877A mutation was the first AR mutation described in PC. It was first reported in the LNCaP cell line and is known to widen ligand-specificity of the receptor to estrogens, progestins, adrenal androgens and even to synthetic antiandrogens such as flutamide or cyproterone acetate [54]. The detection of the T877A mutation in the PC346mAR and PC346Flu2 cell lines was consistent with the results of the *in vitro* proliferation assay, which showed growth stimulation in presence of either R1881 or hydroxyflutamide. It is striking that the same mutation has been found in three lines of the panel: PC346I, PC346mAR and PC346Flu2. PC346mAR was established *in vitro* from the PC346I xenograft, which explains the presence of the mutation in the cell line. Codon 877 of the AR gene is in fact a known mutation “hotspot” in hormone-refractory PC, justifying the idea that the T877A mutation may have arisen independently in PC346I and PC346Flu2 [55-57]. To further investigate the origin of the T877A mutation in the PC346 model, we performed allele-specific oligo (ASO) hybridization, a sensitive technique capable of identifying 1 mutated cell out of 99 cells with *wild-type* AR. ASO blotting did not detect the T877A mutation in the patient’s TUR material nor in any other xenograft but PC346I, where it became detectable after 2 subsequent passages in female mice. The fact that the mutation is not present in the other AI xenografts and that the original PC346P has retained its hormone response properties, suggests that it may have occurred spontaneously, being selected for by propagation in female mice. Nevertheless, we cannot exclude that cells with mutated AR were already present in the patient, representing less than 1% of an originally heterogeneous cell population. To assess the stability of the *in vitro* cultures and exclude that the AR mutations arose due to genomic instability, these were previously analyzed using Genome-wide Comparative Genomic Hybridization (CGH) microarrays [39]. We found few chromosomal abnormalities, most of them present in every cell line, indicating that they originated prior to the development of the sublines. Only four chromosomal modifications detected in the androgen-independent sublines were not present in the parental PC346C, loss of chromosome Y and gain of 18 in PC346Flu2, loss of 8p in PC356DCC and gain of 11 in PC346mAR, which confirms the relative genomic stability of the sublines [39].

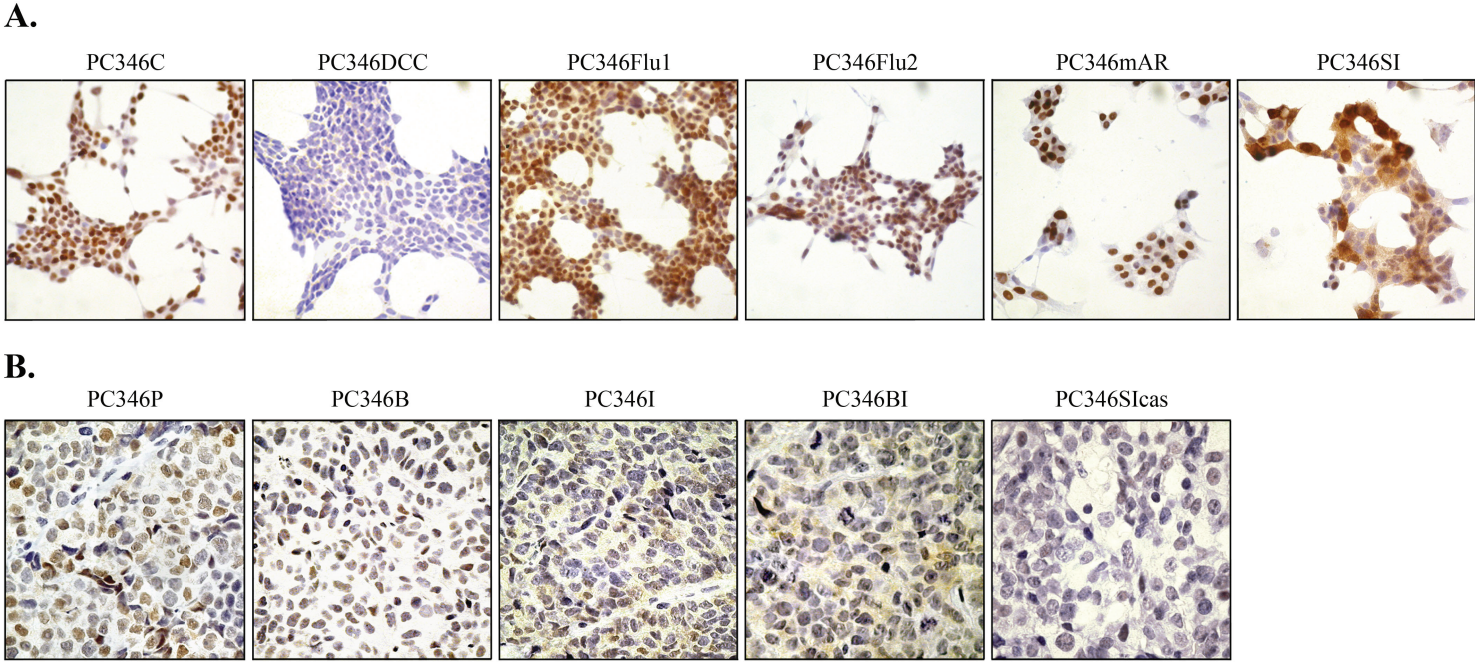


Figure 6 - Androgen receptor immunocytochemistry of the PC346 *in vitro* cell lines **(A)** and xenografts **(B)**. Each line was cultured under the selection conditions described in figure 5. Specimens were incubated with anti-AR monoclonal primary antibodies AR441 (NeoMarkers) for the xenografts and F39.4.1 [69] for the *in vitro* lines, stained with diaminobenzidin (DAB) and counter-stained with hematoxylin.

The PC346BI, PC346SI and PC346DCC express low levels of AR and PSA, and are unresponsive to hormonal manipulations, suggesting that they may have bypassed the AR pathway. The mechanisms for bypassing the AR pathway are not yet fully known, but may involve autocrine production of growth-stimulatory factors [58], activation of oncogenes [59] and/or downregulation of apoptotic signals [60, 61].

The androgen-responsive characteristics and the status of the AR pathway in the PC346 panel are summarized in Table 3.

Table 3 – Overview of the PC346 panel: AR status, PSA expression and androgen-responsiveness

	AR status		PSA expression	Hormone response
	Expression	Sequence (exons 2-8)		
PC346P xeno	+	wt	+	++
PC346B xeno	+	wt	+	++
PC346I xeno	++	T877A	++	-
PC346BI xeno	-/+	wt	++	-
PC346SIcas xeno	-/+	wt	+	-
PC346C cells	+	wt[a]	++	++
PC346DCC cells	-/+	K311R[a]	-	-
PC346Flu1 cells	++	wt[a]	++	+[b]
PC346Flu2 cells	+	T877A[a]	+	+
PC346mAR cells	-/+	T877A	-/+	+
PC346SI cells	+	wt	+	-

[a] For these cell lines, exon 1 of the AR gene was previously analysed by automatic sequencing [39].
[b] Proliferation of PC346Flu1 is inhibited by androgens at physiologic concentrations (Fig. 4).

Comparison to other xenograft and cell line model systems for human prostate cancer

PC346P and PC346B are among the very few PCa xenografts exhibiting androgen-sensitive growth characteristics and wtAR expression. Although these tumors rarely develop in female and castrated male mice, castration of mice bearing established PC346P tumors resulted in variable responses, including tumor regression, tumor retardation followed by growth relapse, or continued growth. Interestingly, established PC346B tumors did not significantly respond to castration. This is in contrast to the strictly androgen-dependent xenografts PC82, PC295 and PC310 previously established in our laboratory [3, 7, 62], which do not develop in females or castrated males and show complete regression after castration of tumor-bearing animals without tumor relapse.

The LAPC-4 [16] and LuCaP-23 [10] xenograft models have been reported to show a response to castration very similar to our results with PC346P xenograft. In contrast to PC346P, LuCaP-23 tumors seem less dependent on androgens, since tumors do develop and grow in castrated mice. CWR22 [9] and LAPC-9 [17] also show recurrent growth after androgen ablation, but with a considerably longer (3-6 months) time to relapse. The LAPC and LuCaP models were derived from metastatic lesions of

patients with hormone-refractory disease, while CWR22 expresses the rare H874Y mutant AR [63], therefore raising the question whether these are representative models for the hormone-naïve stage of PC. In contrast, PC346P is derived from a primary tumor of a non-progressive patient and is androgen-sensitive, although with a heterogeneous response to castration.

The number of *in vitro* PCa cell line models is limited, and most of them represent the late-stage hormone-refractory phenotype. Until recently, the majority of the *in vitro* studies were based on the "classical" LNCaP, PC3 and Du145 cell lines. Although most prostate tumors are AR-positive, PC3 [20] and Du145 [21] show little or no AR and PSA expression [64]. Also, the androgen-responsive LNCaP cell line, often used as a model for androgen-dependent disease, has altered hormone-response properties due to the expression of a mutated AR [65]. Over the past decade, a few novel PCa cell lines have become available, among which ARCaP, VCaP, DuCaP, LAPC-4, MDA PCa 2a/b and CWR22Rv1 [66]. Of all these cell lines, the lymph node derived LAPC-4 is the only one that presents truly androgen-dependent characteristics [66]. Therefore, the androgen-sensitive PC346C cell line, derived from a primary tumor and expressing *wild-type* AR, is a unique and valuable contribution to the available *in vitro* models.

CONCLUSIONS

The PC346 model consists of a panel of six cell lines and five xenografts that recapitulates the relapse phenomenon observed in patients upon endocrine therapy: after an initial response to androgen ablation, tumors ultimately resume growth in an androgen-independent manner. By *in vivo* and *in vitro* hormonal manipulations of the androgen-sensitive PC346P, PC346B and PC34C, we established a set of AI sublines, which reproduce the most common AR modifications observed in hormone-refractory disease: downregulation, overexpression and mutation. This unique panel of xenografts and cell lines constitutes a valuable PCa progression model for basic and translational research.

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Chapter 3

Androgen Receptor Modifications in Prostate Cancer Cells Upon Long-Term Androgen Ablation and Antiandrogen Treatment

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ABSTRACT

To study the mechanisms whereby androgen-dependent tumors relapse in patients undergoing androgen blockade, we developed a novel progression model for prostate cancer (PCa). The PC346C cell line, established from a transurethral resection of a primary tumor, expresses *wild-type* (*wt*) androgen-receptor (AR) and secretes prostate-specific antigen (PSA). Optimal proliferation of PC346C requires androgens and is inhibited by the antiandrogen hydroxyflutamide. Orthotopic injection in the dorsal-lateral prostate of castrated athymic nude mice did not produce tumors, whereas fast tumor growth occurred in sham-operated males. Three androgen-independent sublines were derived from PC346C upon long-term *in vitro* androgen deprivation: PC346DCC, PC346Flu1 and PC346Flu2. PC346DCC exhibited androgen-insensitive growth, which was not inhibited by flutamide. AR and PSA were detected at very low levels, coinciding with a background AR activity in a reporter assay, which suggests that these cells have bypassed the AR pathway. PC346Flu1 and PC346Flu2 were derived by culture in steroid-stripped medium supplemented with hydroxyflutamide. PC346Flu1 strongly up-regulated AR expression and showed 10-fold higher AR activation than the parental PC346C. PC346Flu1 proliferation was inhibited *in vitro* by R1881 at concentrations ≥ 0.1nM, consistent with a slower tumor growth rate in intact males than in castrated mice. PC346Flu2 carries the well-known T877A AR mutation, causing the receptor to become activated by diverse nonandrogenic ligands including hydroxyflutamide. Array-based Comparative Genomic Hybridization revealed little change between the various PC346 lines. The common alterations include gain of chromosomes 1, 7 and 8q and loss of 13q, which are frequently found in prostate cancer. In conclusion, by *in vitro* hormone manipulations of a unique androgen-dependent cell line expressing *wt*AR, we successfully reproduced common AR modifications observed in hormone-refractory prostate cancer: downregulation, overexpression and mutation.

INTRODUCTION

The androgens testosterone and dihydrotestosterone (DHT) are essential for the normal development and maintenance of the prostate but also sustain its malignant outgrowth. The actions of androgens are mediated by the androgen-receptor (AR), a member of the steroid receptor family and a chief regulatory transcription factor for genes involved in the proliferation and differentiation of the prostate [1]. As prostate cancer (PCa) development is initially dependent on androgens, androgen ablation, often supplemented with antiandrogens, is the mainstay therapy for metastasized disease [2]. Despite the initial success of this therapy, in most cases the cancer will relapse within 1-3 years as an incurable hormone-refractory condition.

The mechanisms whereby androgen-dependent tumors can survive and grow in patients under androgen blockade are not yet fully understood. Theoretically, androgen-dependent PCa cells have two possible strategies for growing in a low-androgen environment: to sensitize the AR pathway for high activity under low androgen concentrations or to bypass the AR pathway by invoking alternative survival and growth pathways [3,4].

Experimental observations suggest that the AR pathway may still be functionally active in hormone-refractory cancer. Immunohistochemical staining of tumor biopsies showed that the AR is expressed in most of the prostate cancers, often at higher levels in relapsed tumors than in primary tumors [5,6]. AR gene amplification has been reported in up to 30% of recurrent tumors [7,8,9]. How AR upregulation might confer a growth advantage under androgen ablation conditions is still not fully understood. In a recent study, Chen *et al* showed that increased levels of AR were sufficient to induce hormone-refractory growth in otherwise hormone-sensitive LNCaP and LAPC4 cells, which was in turn dependent on a functional ligand-binding domain [10]. It has been proposed that higher receptor levels could lead to sensitization for residual androgen concentrations and broadened ligand-specificity [10] or to constitutive ligand-independent activation [11]. Additionally, AR point mutations that alter the activity and binding specificity of the receptor have been clinically associated with disease progression. The first AR mutation described in PCa was the T877A mutation present in the LNCaP cell line, which widens ligand-specificity to estrogens, progestins, adrenal androgens and even to synthetic antiandrogens such as flutamide or cyproterone acetate [12]. Since then, AR sequence analysis has been carried out in numerous clinical samples, and a growing list of reported mutations can be found in the AR Mutation Database (http://ww2.mcgill.ca/androgendb/) [13]. Several findings suggest that the AR can also be activated in a ligand-independent fashion by cross-talk with polypeptide growth factor or cytokine signal transduction pathways [14]. Finally, alterations in the expression of various AR coregulators have been found in PCa cells and account for an additional mechanism to modulate AR activity [15,16,17].

All mechanisms discussed above depend on a functional AR pathway. Alternatively, androgen-dependent PCa cells may become truly androgen-independent, bypassing the need for the presence of androgens or the expression of the receptor. To effectively bypass the AR pathway, cells must be able to survive the apoptotic signal normally induced by androgen ablation and invoke alternative growth pathways. This hypothesis is supported by the high frequency of alterations in the anti-apoptotic Bcl-2

oncogene and in the pro-apoptotic PTEN tumor-suppressor gene in androgen-independent PCa [18,19,20,21,22]. Furthermore, several growth factors, which are normally regulated in a paracrine manner by nondiseased prostate stromal cells, are found to be upregulated in hormone-refractory cancer, concomitant with a switch to autocrine production by epithelial cancer cells [23].

To investigate the mechanisms regulating the proliferation of malignant cells in hormone-refractory disease, we developed a novel progression model for prostate cancer. The PC346C cell line was intentionally established from a primary tumor to represent the situation in hormone-naïve patients. It expresses wild-type AR and shows androgen-dependent growth characteristics. From PC346C, three androgen-independent sublines were derived by long-term culture in steroid-stripped medium either alone, PC346DCC, or supplemented with the antiandrogen flutamide, PC346Flu1 and PC346Flu2. These androgen-independent sublines show distinctive growth and hormone-sensitivity properties, providing an interesting model system to study the role of the AR and other pathways in androgen-independent growth.

MATERIAL AND METHODS

Reagents and cell lines

The basis culture medium used in the maintenance of PC346 cell lines consisted of DMEM-F12 (Cambrex BioWhitaker, Belgium) medium supplemented with 2% foetal calf serum (FCS; PAN Biotech GmbH, Aidenbach, Germany), 1% insulin-transferrin-selenium (Gibco BRL), 0.01% bovine serum albumin (Boehringer Mannheim, Germany), 10 ng/ml epidermal growth factor (Sigma-Aldrich), penicillin/streptomycin antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin; BioWhitaker, Belgium); plus the following additions: 100 ng/ml fibronectin (Harbor Bio-Products, Tebu-bio, The Netherlands), 20 μg/ml fetuine (ICN Biomedicals, The Netherlands), 50 ng/ml choleratoxin, 0.1 mM phosphoethanolamine, 0.6 ng/ml triiodothyronine and 500 ng/ml dexametason (all from Sigma). For the MTT assays and transfections a simplified version of this medium was used, containing dextran-coated charcoal (DCC) treated FCS and without the additions.

The parental PC346C cell line was derived from the transurethral resection (TUR) of the prostate of a patient, which had negative scans for both bone and lymph node metastasis, and underwent four weeks treatment with Androcur® prior to the operation. This prostatic carcinoma specimen was initially implanted subcutaneously in male NMRI nu/nu mice (Taconic, M&B, Denmark), yielding the androgen-dependent PC346 xenograft [24,25]. The permanent in vitro PC346C cell line was subsequently established from the twelfth mouse passage of the PC346 xenograft. Cells were grown in T25 Primaria^{TM} tissue culture flasks (BD Biosciences Benelux N.V, The Netherlands) at 37^{o}C under 5% CO2 humidified atmosphere, in the medium described above supplemented with 0.1 nM of R1881 (NEN, Boston MA, USA).

To mimic *in vitro* the endocrine therapy applied to patients with advanced metastatic PCa, we continuously cultured (>2 years) PC346C in androgen deprived conditions, producing the androgen independent sublines: PC346DCC, PC346Flu1 and PC346Flu2. PC346DCC was derived from passage 45 of PC346C by culturing in DCC-stripped medium. The flutamide resistant PC346Flu1 and PC346Flu2 sublines were

produced from passage 45 and 75 of PC346C, respectively, by culturing in DCC-stripped medium supplemented with 1 μM of hydroxyflutamide (OH-flutamide, Schering-Plough Research Institute, New Jersey, USA).

Growth assays

To determine the effects of androgens and antiandrogens on cell proliferation, we performed a growth assay based on the enzymatic reduction of the tetrazolium salt MTT (3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide; AppliChem, Belgium) by metabolically active cells. Cell growth was determined as described previously [26], with a few modifications. Briefly, 10^4 cells were seeded per well of 96-well microtiter plates, in 100 μl of steroid-stripped medium (without additions). Cells were allowed to attach for 24 h, after which 8 wells were assayed immediately to establish t_0. To the remaining wells, 100 μl of culture medium was added either alone or supplemented with increasing concentrations of R1881 (ranging from 1 pM to 1 nM) or OH-flutamide (0.1 nM to 10 μM); cells were then incubated for 7 or 10 days. At the end of the incubations, 30 μl (15 μl for t_0) of 5 mg/ml MTT was added to each well and cells were further incubated for 4 h at 37°C. After carefully removing the medium, we added to each well 100 μl buffered DMSO (8 volumes of DMSO for 1 volume of buffer 0.1 M glycine, 0.1 M NaCl, pH 10.5), and determined the absorbance at 570 nm with a BIO-RAD 550 microplate reader. Wells containing medium alone served as blanks. The results were expressed as mean ± SEM of at least three independent experiments, each with 8 replicate wells per cell line per hormonal condition.

Sequencing and FISH analysis of AR gene

Genomic DNA from the parental and derivative cell lines was isolated using the Puregene Genomic DNA Isolation kit (Gentra Systems, Minneapolis, USA), according to the manufacturer's protocol. Exons 1 to 8 of the AR were amplified by PCR in 12 portions using primers described previously [27], and automatically sequenced with ABI Prism Genetic Analyzer (Applied Biosystems). AR gene copy number was analyzed by fluorescence *in situ* hybridization (FISH) as described by Linja *et al* [7].

AR and PSA immunoblots

Cells were lyzed in RIPA buffer (100 mM Tris-HCl pH 7.4, 5 mM EDTA, 1% Triton X-100, 1% desoxycholate, 0.1% SDS) with protease inhibitors. Protein concentrations were determined by a Bradford assay (BioRad) and 10 μg of protein was loaded in sample buffer on a 30% SDS-polyacrylamide gel as described previously [28]. Gels were blotted onto a nitrocellulose membrane and the proteins of interest were visualized by chemiluminescense using the F39.4.1 anti-AR [29] or ER-Pr8 anti-PSA [30] mouse monoclonal antibodies and goat-anti-mouse secondary antibody conjugated with horseradish peroxidase (DakoCytomation BV, The Netherlands).

Quantitative TaqMan real-time PCR

Total RNA from two different passages of each cell line was isolated with RNAzol B reagent (Campro Scientific, Veenendaal, The Netherlands) according to the manufacturer's protocol. The RNA samples were further purified through RNeasy

columns (Qiagen), and the quality of the RNA was checked by 1 % agarose gel electrophoresis.

cDNA was synthesized from 2 μg total RNA: the samples were initially incubated with 10 ng/μl Oligo(dT)$_{12-18}$ primer (Invitrogen) for 10 min at 70°C, in 22 μl volume and chilled on ice. 8 μl 5x first-strand buffer (Invitrogen), 4 μl 100 mM DTT, 2 μl 10 mM dNTP mix, 20 U RNasin ribonuclease inhibitor (Promega) and 400 U MMLV-reverse transcriptase (Invitrogen) were then added, followed by 1h incubation at 37°C. Reverse transcriptase was inactivated by heating to 95°C for 10 min, and samples were stored at -20°C. Additionally, 0.5 μg RNA of each PC346 cell lines was pooled to produce the standard cDNA to be used in the calibration curve.

TaqMan real-time PCR analysis was performed in duplicate for each sample / standard on a 96-well Optical Reaction Plate with Barcode (Applied Biosystems), using an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). Amplification was performed in 40 μl reaction mixture, containing 1x Taqman buffer A (Applied Biosystems), 4 mM $MgCl_2$, 250 μM dNTPs mix, 2 ng/μl of each primer, 5 pmol FAM-labeled probe (Eurogentec, Belgium), 1U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 8 μl of cDNA sample /standard. Primers and probes: AR forward, 5'-CATCAAGGAACTCGATCGT-3'; AR reverse, 5'-GAACTGATGCAGCTCTCTC-3'; AR probe, 5'-ACATCCTGCTCAAGACGCTCCT-3'; PSA forward, 5'-CCCTCAGAAGGT GACCA-3'; PSA reverse, 5'-ACCACCTTGGTGTACAGG-3'; PSA probe, 5'-TATCAC GTCATGGGGCAGTG-3'; RNA polymerase II forward, 5'-GATCGGAAGCACATGACT-3'; RNA polymerase II reverse, 5'-CTGTGGCAAGTGCATGTA-3'; RNA polymerase II probe, 5'-AAGATGCAAGAGGAGGAAGAGGT-3'. Gene specificity of the primers and probes was checked by BLAST queries and intron-spanning primers were chosen to avoid amplification of contaminating DNA. GAPDH primers and probe were purchased as kit Human GAPD (GAPDH) Endogenous Control (VIC probe) from Applied Biosystems. For each gene, a standard curve was constructed from serial dilutions of the reverse-transcribed PC346 RNA pool, which was then used to determine the quantity of target message from the threshold cycle (Ct) value. Two housekeeping genes were used as endogenous controls: RNA polymerase II and GAPDH. AR and PSA quantities for each sample were normalized against the average of the two internal controls. The results are presented for each cell line as the average relative quantity of the two passages analyzed ± SEM.

AR transactivation assays

PC346C and PC346Flu1 cells were seeded in 24-well plates at a density of 1 x 10^5 cells/well. To bring AR activity down to basal levels before the transfection, cells were androgen-starved in charcoal-stripped serum for 2 days. For the transfections, we added per well: 30 μl serum-free minimum medium containing 1 μl Fugene 6 (Boehringer Mannheim, Germany), 0.125 μg pEF4-LacZ control plasmid (Invitrogen BV, The Netherlands) and 0.125 μg $(ARE)_2$-TATA-Luc reporter plasmid [31] (previously referred to as ARE_2-E1b-luciferase), in a final volume of 500 μl. After overnight incubation, cells were stimulated for 24 h with 1 nM R1881, 1 μM OH-flutamide or 1 nM R1881 + 1 μM OH-flutamide. The next morning, cells were lysed in Reporter Lysis Buffer (Promega Benelux BV, The Netherlands) and the luciferase and galactosidase activities were measured as described previously [32]. The assay was performed in triplicate, each

experiment with 4 replicate wells per cell line per hormonal condition. Luciferase activity was normalized to the β-galactosidase control and the results are presented as the luciferase activity relative to PC346C basal activity in steroid-stripped medium.

Mouse tumor take assays

Orthotopic injections were performed on 6-8 weeks old male athymic nude NMRI nu/nu mice (Taconic, M&B, Denmark) as described previously [33], with some minor modifications. Briefly, cells in the exponential growth phase were harvested by trypsination, resuspended in PBS and 10^6 cells (in 20 μl) were injected into one of the dorsal-lateral lobes of the prostate. Bilateral castration was performed one week prior to tumor cell injection using standard surgical techniques. Tumor growth was measured weekly by transrectal ultrasonography as described previously [34]. Tumor volume was calculated from formula $V = (\pi/6)(d_1 \times d_2)^{3/2}$, with d_1 and d_2 being two perpendicular tumor diameters. Mice were sacrificed when the tumor size reached 1500 mm^3 or when showing signs of suffering from tumor burden. At the end of the experiment blood samples were collected for plasma PSA determination, as previously described [34]. The experiment was performed in two parts: first PC346Flu1 was tested and compared to the parental PC346C (10 castrated and 10 sham-operated mice were used per cell line); secondly, PC346DCC and PC346Flu2 were tested together with another group of the parental line (8 castrated and 8 sham-operated mice per cell line). Since there was no significant difference between the two PC346C groups, these results were analyzed together. All experiments were performed in agreement with The Netherlands' Experiments on Animals Act (1977) and the European Convention for Protection of Vertebrate Animals used for Experimental Purposes (Strasbourg, 18 March 1986). Approval was obtained from the Experimental Animal Committee (DEC). Tumor doubling times (TD) were determined by linear regression of 2Log-transformed tumor volume over time, using the previously described SAS procedure PROC MIXED (version 8.2, SAS Institute Inc., Cary, NC, USA) [35]. Doubling times were calculated from the 1/slope of tumor volume rise.

Genome-wide Comparative Genomic Hybridization (CGH) Arrays

For these experiments we used 1 Mb spaced genome-wide BAC arrays, containing a total of 3659 BAC clones robotically spotted in triplicate onto CMT-GAPS-coated slides (Ultra Gaps, Corning, Schiphol-Rijk, The Netherlands). These arrays were produced in the Department of Human Genetics, University Medical Center Nijmegen, The Netherlands, as described previously [36]. Probe labeling, array hybridization, image processing and data analysis were performed as previously described [36]. Briefly, 500 ng of the cell lines genomic DNA was labeled by random priming with Cy3-dUTP (Amersham Biosciences), combined with 500 ng Cy5-dUTP-labeled reference DNA and hybridized onto the genome-wide BAC arrays using a GeneTAC Hybridization Station (Genomic Solutions, Cambridgshire, UK). After scanning, Cy3 to Cy5 ratios were determined, $\log_2$-transformed and normalized per array subgrid by Lowess regression with the software package SAS version 8.0 (SAS Institute). Thresholds for copy number gain and loss were set at $\log_2$ Cy3/Cy5 ratio of +0.3 and -0.3, respectively.

RESULTS

Growth characteristics and hormone sensitivity of the PC364 cell line panel

The parental PC346C cell line showed androgen-responsive growth characteristics. PC346C proliferation was optimal in the presence of 0.1 nM of R1881 (Fig. 1A), which stimulated growth about 5-fold after 10 days incubation (Fig. 1B). This growth-stimulatory effect of R1881 was inhibited by 1 μM OH-flutamide. In steroid-stripped (DCC) medium, the proliferation of PC346C was clearly impaired but cells could still survive and slowly divide (Fig. 1A). Long-term culture (>2 years) of the androgen-responsive PC346C cell line under androgen-deprived conditions resulted in the PC346DCC subline. PC346DCC exhibited true androgen-independent characteristics, with rapid proliferation in steroid-stripped medium and unresponsiveness to either R1881 or OH-flutamide. The flutamide-resistant PC346Flu1 and PC346Flu2 sublines were derived from PC346C by culturing in androgen-depleted medium supplemented with 1 μM OH-flutamide. PC346Flu1 proliferated optimally under low androgen concentration, and growth inhibition was observed at R1881 concentrations ≥ 0.1 nM ($P=0.05$), the optimal growth concentration for the parental line (Fig. 1B). Like PC346C, PC346Flu2 showed 2-fold growth stimulation with 0.1 nM R1881, but proliferated faster than the parental cell line in androgen-depleted medium. PC346Flu2 cells were not inhibited by OH-flutamide, which instead seemed to act as a growth agonist ($P=0.05$).

Sequencing and FISH analysis of the AR gene

AR sequencing revealed a *wild-type* AR in the parental PC346C and the PC346Flu1 subline. AR mutations were detected in both PC346DCC and PC346Flu2 sublines. In PC346DCC the K311R AR mutation was found, which has not been previously described in the literature. The T877A mutation was present in the PC346Flu2 subline. This mutation, which was originally reported for the LNCaP cell line, is well known for broadening AR specificity to diverse nonandrogenic steroids such as estrogens, progestins, adrenal androgens, and even the synthetic antiandrogen flutamide [12]. FISH analysis revealed that PC346C and all derivative sublines contain one copy of AR gene (data not shown).

AR and Prostate-Specific Antigen (PSA) expression

To assess the status of the AR pathway, expression of AR and the androgen-regulated PSA were analyzed by western blot and quantitative TaqMan RT-PCR. Western blot analysis showed that in PC346DCC the AR was downregulated to very low levels, concomitant with a decreased PSA expression (Fig. 2A). In contrast, AR protein expression in PC346Flu1 was upregulated by about 4-fold relative to the parental PC346C. PC346Flu2 showed AR protein levels identical to those observed in PC346C, although mRNA quantity seemed to be reduced (Fig. 2B). All cell lines expressed the prostate differentiation marker PSA when cultured in their respective selection medium. The highest PSA level was found for the androgen-dependent PC346C but it must be noted that its growth medium contained 0.1 nM R1881, in contrast to the selection medium for PC346Flu1 and PC346Flu2, which was supplemented with OH-flutamide. In general, TaqMan RT-PCR quantification confirmed the findings from the western blot analysis, revealing upregulation of PC346Flu1 AR mRNA and very low levels of AR and PSA mRNA in PC346DCC.

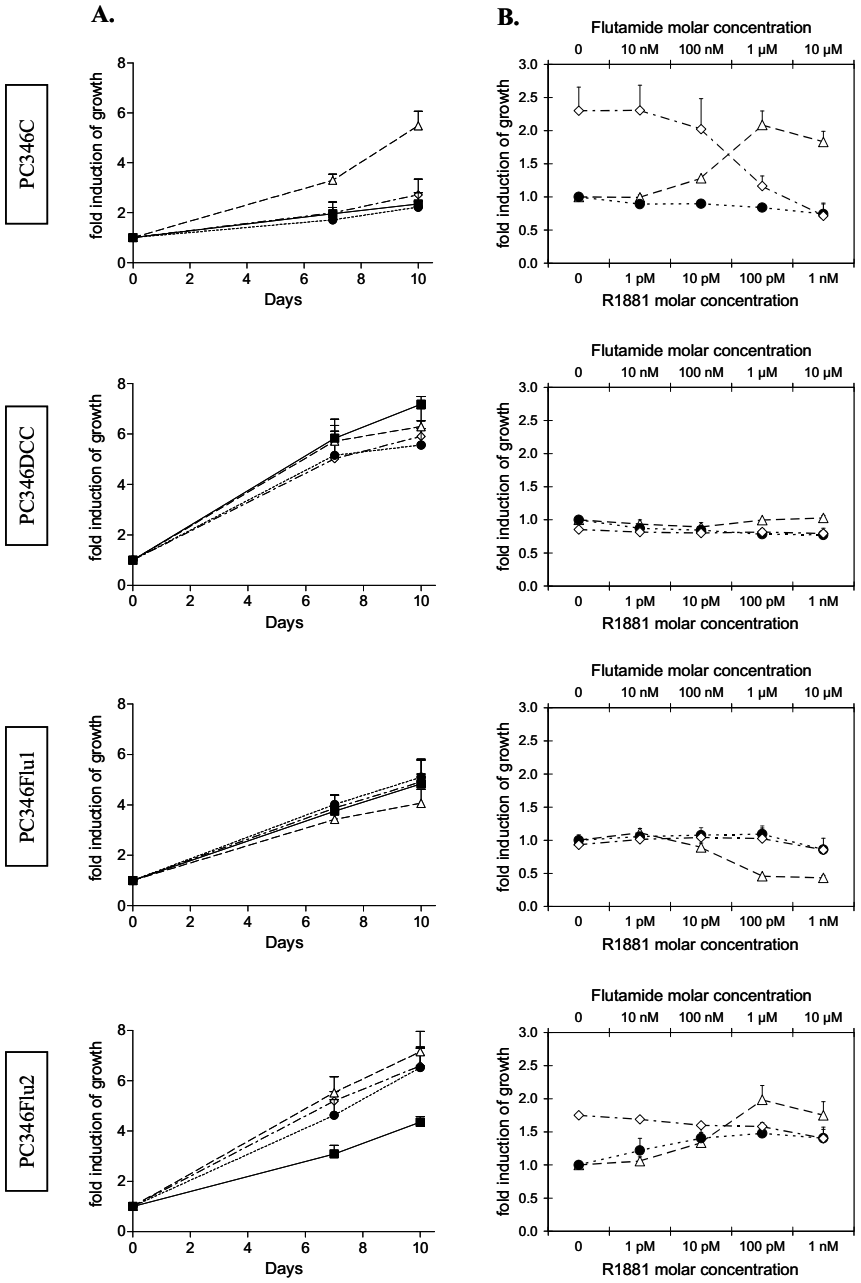
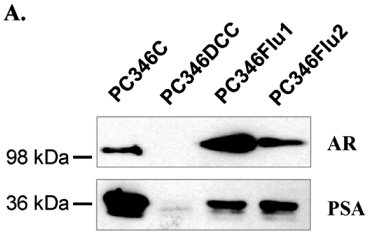


Figure 1 - Growth characteristics and hormone sensitivity of PC346 cell lines. **A**, PC346C, PC346DCC, PC346Flu1, PC346Flu2 cells were incubated in charcoal stripped-medium (■) supplemented with 0.1 nM R1881 (Δ), 1 µM OH-flutamide (●) or 1 µM OH-flutamide + 0.1 nM R1881 (0.01 nM for PC346Flu1; ◊). Cell number was measured by MTT assay on days 0, 7 and 10. Results are presented as fold increase in cell number relative to day 0. **B**, Cells were incubated for 10 days with increasing concentrations of R1881 (Δ; lower x-axis), OH-flutamide (●; upper x-axis)

or 0.1 nM R1881 with increasing concentrations of OH-flutamide (◊). Cell number was measured by MTT assay and results are presented as fold increase in cell number relative to no hormone addition. Values (±SEM) represent the mean of at least three independent experiments each carried out in 8 replicates.

A.



B.

	AR mRNA quantity	PSA mRNA quantity
PC346C	1.000 ± 0.191	1.960 ± 0. 094
PC346DCC	0.053 ± 0.016	0.029 ± 0. 018
PC346Flu1	2.924 ± 0.661	0.935 ± 0. 222
PC346Flu2	0.553 ± 0.277	1.176 ± 0. 015

Figure 2 - Expression of AR and PSA in the parental and derivative cell lines. Each cell line was cultured in its respective selection medium: PC346C on medium supplemented with 2 % FCS and 0.1 nM of R1881, PC346DCC on medium supplemented with 2 % steroid-stripped FCS, PC346Flu1 and PC346Flu2 on medium supplemented with 2 % steroid-stripped FCS and 1 mM OH-flutamide. **A,** protein expression analyzed by western blotting. **B,** mRNA quantity determined by TaqMan real-time RT-PCR. AR and PSA quantities were normalized using the average of two internal controls: RNA polymerase II and GAPDH, and set at 1 for the parental PC346C. The relative values are presented as the mean of two different passages ± SEM.

AR reporter transactivation studies

To determine whether the changes in cell proliferation in response to androgens and antiandrogens were concomitant with changes in AR pathway activity, transcriptional activity of the endogenous receptor was evaluated in a transactivation assay, using the androgen-responsive $(ARE)_2$-TATA minimum promoter linked to a luciferase reporter (Fig. 3). AR reporter activity in the parental PC346C was induced 12-fold by 1 nM R1881 but not by OH-flutamide. The PC346DCC subline showed background levels of AR reporter activity that remained unaffected by either hormone or antiandrogen. PC346Flu1, which expresses high levels of the AR protein, showed 50-fold induction with 1 nM R1881, a response that could not be effectively repressed by 1 µM OH-flutamide. Flutamide, however, did not activate the AR reporter in this subline.

Animal studies

To extend the *in vivo* characterization in an *in vivo* setting, the parental PC346C and derivative sublines were injected orthotopically into male athymic nude mice and tumor growth was monitored weekly by transrectal ultrasonography (Fig. 4). Tumor take and tumor doubling time (TD) were compared in non-castrated versus castrated mice between the different cell lines (Table 1). In accordance to the *in vitro* studies, also in the *in vivo* assay, PC346C showed androgen-dependent characteristics. Although no tumor developed in castrated animals, tumor-growth in sham-operated males occurred in 17 out of the 18 animals tested, with doubling time (TD) of 6.7 days. The PC346DCC subline, which exhibited hormone-unresponsive proliferation *in vitro*, produced tumors in both castrated and sham-operated mice. However, tumor growth was slower and more variable in castrated mice than in sham-operated mice (TD 16.3 vs. 7.6 days, P = 0.06).

In contrast, PC346Flu1 tumors grew faster in castrated animals than in androgenically intact males (TD 6.7 vs. 13.0 days, P < 0.0001). These results are in agreement with the growth-inhibitory effect of androgens on this subline initially revealed by the *in vitro* assay. Like that of PC346C, *in vivo* tumor growth of PC346Flu2 was also greatly affected by castration of the animals, with only 3 out of 8 castrated mice developing tumors bigger than 100 mm^3.

At the end of the experiments, serum samples were taken from each animal to quantify the PSA produced by the orthotopic xenografts. PSA levels were the highest in the mice bearing the androgen-sensitive PC346C and PC346Flu2 tumors, and very low in castrated mice and mice bearing the androgen-unresponsive PC346DCC (Table 1). It is noteworthy that, in androgenically intact mice, PC346Flu1 expresses considerably less PSA than the parental line, despite showing an augmented response to hormone in the *in vitro* transactivation assay.

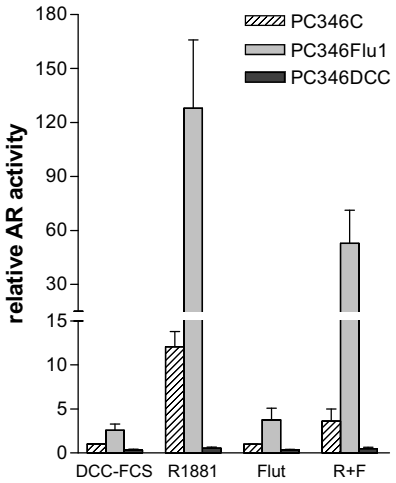


Figure 3 - Transactivation of the $(ARE)_2$-TATA-Luc reporter in PC346C, PC346Flu1 and PC346DCC. Cells were transiently transfected with the $(ARE)_2$-TATA-Luc reporter and pEF_4LacZ for normalization, and stimulated for 24h with either steroid-striped medium alone (DCC-FCS) or supplemented with 1 nM R1881 (R1881), 1μM OH-flutamide (Flut) or 1 nM R1881 + 1μM OH-flutamide (R+F). AR activity is presented as the normalized luciferase activity relative to the basal activity of parental PC346C in steroid-striped medium (DCC-FCS), which was set at 1.

Genome-wide Comparative Genomic Hybridization (CGH) Arrays

Array CGH was performed to assess the genomic stability of the cell lines and to determine whether major chromosomal alterations were associated with the development of androgen independence. PC346 and derivative sublines appeared to be stable presenting few chromosomal abnormalities. All cell lines showed amplifications of chromosomes 1, 7, 8q and 20, and deletion of 13q and of a small region in chromosome 10q around the PTEN locus (Fig. 5). Only three major chromosomal modifications were found in the androgen-independent sublines: PC346Flu2 showed amplification of chromosome 18 and deletion of Y, whereas PC346DCC presented a deletion in 8p.

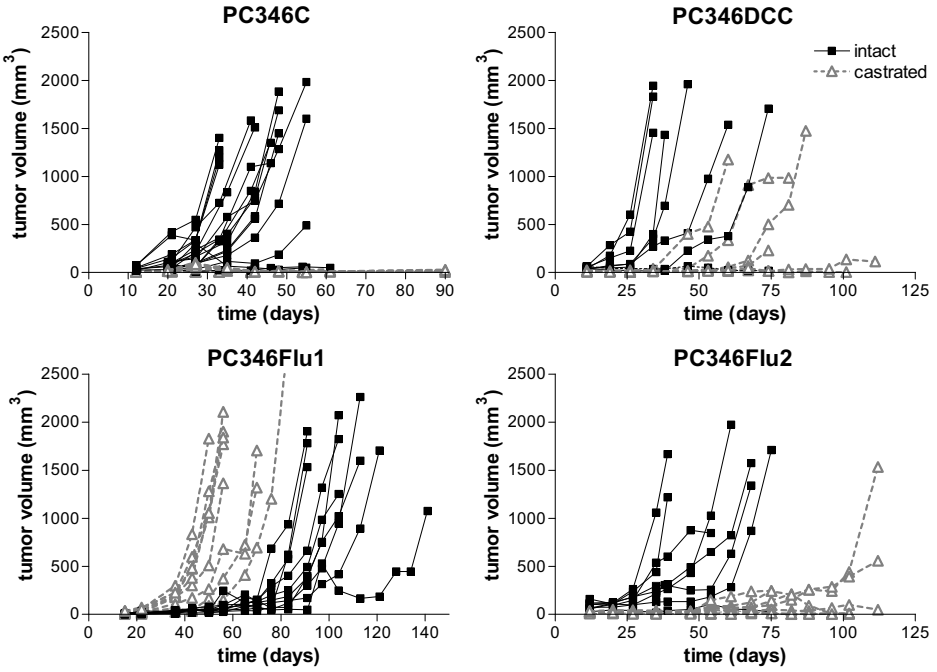


Figure 4 - In vivo growth of the parental and derivative cell lines in castrated versus androgenically intact male mice. 1 million cells were injected orthotopically in the dorsal-lateral lobe of the prostate of NMRI nu/nu mice and tumor growth was monitored weekly by transurethral ultrasonography. Graphical representation of tumor growth in sham-operated males –■– compared to growth in animals castrated 1 week prior to injection --△--. Each line represents one animal.

Table 1 - Tumor doubling time (TD) and PSA secretion in intact versus castrated mice

	Intact males		Castrated males		P-value TD Intact vs. Castrated
	TD (days)	PSA index (ng/ml per g)	TD (days)	PSA index (ng/ml per g)	
PC346C	6.7 (5.6 - 8.3)	459.8 ± 136.4	–	–	<0.0001
PC346DCC	7.6 (5.8 - 11.3)	58.8 ± 29.4	16.3 (10.0 - 44.9)	*b.d.*	0.0600
PC346Flu1	13.0 (9.3 - 21.3)	169.6 ± 139.6	6.7 (5.4 - 8.8)	70.5 ± 5.6	0.0096
PC346Flu2	12.4 (8.4 - 23.7)	342.3 ± 145.4	*58.2* *	65.6 ± 5.3	<0.0001

Tumor doubling times (TD), 95% confidence intervals (in parentheses below TD) and p-values were calculated using the SAS procedure Proc Mixed [35]. Tumor PSA expression was determined in mouse serum from tumor-takers, and is presented as ng PSA / ml serum per g of tumor (PSA index). Since PC346C castrated mice did not develop tumors, TD and PSA index were not determined for this group. * Calculation of the TD was not statistically reliable since only 3 animals developed tumors. *b.d.,* below detection.

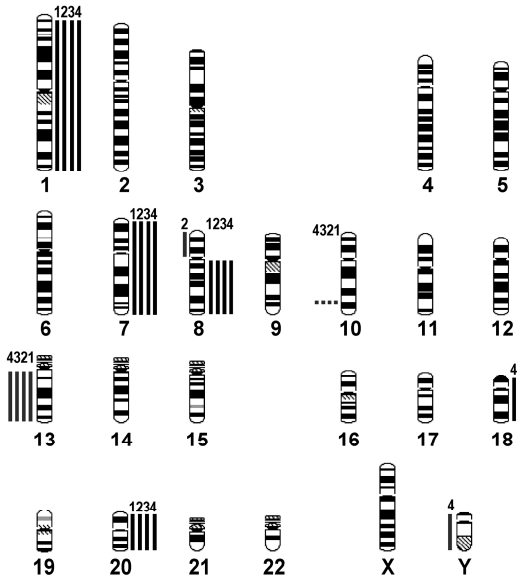


Figure 5 - Chromosomal alterations identified by genome-wide CGH Arrays. A black bar to the right of the chromosome indicates amplification, whereas a grey bar to the left indicates loss of a chromosomal region. The numbers above the lines represent the cell lines: PC324C (1), PC346DCC (2), PC346Flu1 (3) and PC346Flu2 (4).

DISCUSSION

Theoretically, androgen-dependent prostate cancer (Pca) cells have two possible strategies to evade androgen ablation and/or antiandrogen therapy: to adapt the AR pathway, or to invoke an alternative survival/growth pathway. In practice however, these strategies are not mutually exclusive and PCa cells probably use a balance of both to their advantage. We show here that long-term *in vitro* hormone depletion can reproduce the most common AR modifications observed in hormone-refractory prostate cancer patients: PC346Flu1 and PC346Flu2 have adapted their AR pathway, by AR overexpression or mutation, respectively, whereas PC346DCC seems to have bypassed the AR pathway (Fig. 6).

Human prostate carcinomas are among the most difficult cell types from which to establish permanent *in vitro* cultures, which has hampered the development of cell line models representative of the different stages of this disease. Until recently, the majority of the *in vitro* studies were based on a limited number of cell lines: LNCaP, PC3 and DU145. Although most prostate tumors are AR-positive, the “classical” PC3 [37] and Du145 [38] cells show little or no AR and PSA expression [39]. Also, the androgen-

responsive LNCaP cell line, often used as a model for early androgen-responsive disease, has altered hormone-responsive properties due to the expression of a mutated AR [40]. Over the past decade, a few novel PCa cell lines have become available, among which the ARCaP, VCaP, DuCaP, LAPC-4, MDA PCa 2a/b and CWR22Rv1 [41]. However, all of these cell lines but the CWR22Rv1 are derived from metastatic lesions and LAPC-4 is the only that presents truly androgen-dependent characteristics [41]. Our new PC346C cell line is unique in the combination of androgen-responsive growth, *wild-type* AR expression, PSA secretion and primary tumor origin, providing a valuable model system for the androgen-dependent stages of the disease. We used this cell line to replicate the clinical progression of PCa in patients under endocrine therapy, producing a panel of androgen-independent sublines: PC346DCC, PC346Flu1 and PC346Flu2.

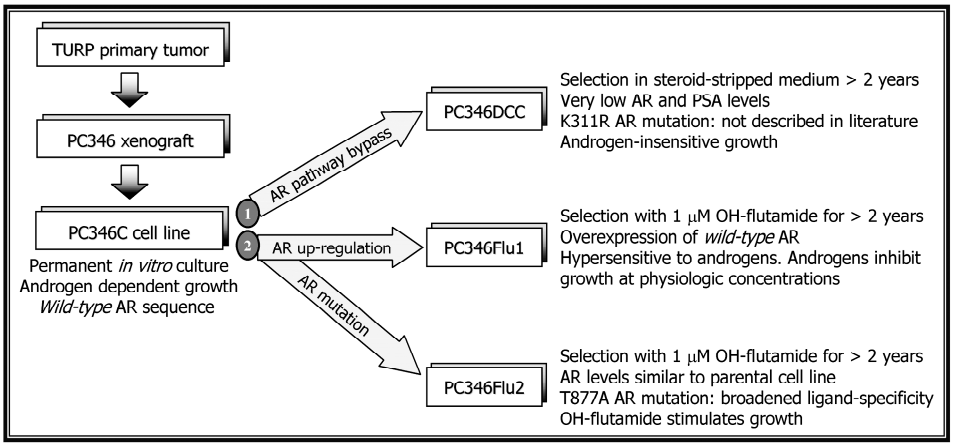


Figure 6 – Overview of the PC346 cell line panel. Prostate cancer cells have two possible strategies to grow in a low-androgen environment: (1) to activate alternative growth stimulatory pathways bypassing the demand for an active AR (PC346DCC), or (2) to sensitize the AR pathway to the low androgen concentrations, for example, by AR up-regulation (PC346Flu1) or AR mutations (PC346Flu2). These strategies are not mutually exclusive and PCa cells probably use a balance of both to survive and grow under androgen-ablation treatment.

PC346DCC exhibits *in vitro* a fast androgen-independent growth, which is unresponsive to both R1881 and OH-flutamide. AR and PSA were found to be very low expressed both at the mRNA and protein levels, but no deletion of the AR gene was detected by FISH analysis. We also found no evidence of AR promoter methylation by either bisulfite sequencing or methylation-specific PCR (data not shown). This is in contrast to the methylation of the AR promoter in DU145 and suggesting that the AR in PC346DCC was downregulated at the transcriptional level rather than epigenetically [42]. A novel mutation was detected in codon 311 of the AR, resulting in Lys to Arg substitution. The effect of this mutation on AR trans-activation is unknown and difficult to assess from the reporter assay due to the very low levels of endogenous receptor. Nevertheless, AR reporter activity was at background levels and was not induced by

R1881, excluding the K311R substitution as a potent activating AR mutation. These data suggest that PC346DCC has bypassed the AR pathway, and we are currently investigating the mechanisms regulating its proliferation. Interestingly, the orthotopic tumor growth assay appears to contradict the *in vitro* results. It seems that PC346DCC proliferation *in vivo* may be affected by castration. A possible explanation for this apparent contradiction could be that the effect of castration on PC346DCC cells is an indirect effect on the host prostate environment. Androgens stimulate prostate stromal cells to produce growth factors that regulate the proliferation of epithelial cells [43]. Recent studies suggest that androgens are also essential for the survival of vascular endothelial cells in androgen-dependent tissues [44,45] and stimulate regrowth of the prostatic vasculature in castrated rats [46]. Taken together, castration does not affect only the cancer epithelial cells, but the whole prostate environment by reducing the blood flow and disrupting stromal-epithelial interactions.

The PC346Flu1 subline expresses high androgen-receptor levels and in an AR reporter assay, it showed a 10-fold stronger response to androgens than the parental PC346C. FISH analysis revealed no AR genomic amplification and other mechanisms such as transcriptional control or protein stabilization may be involved in increasing AR protein levels in this cell line. Although Chen *et al* previously suggested that increased AR levels conferred agonistic activity to AR antagonists [10], we did not observe AR stimulation by OH-flutamide in the PC346Flu1 line. Interestingly, PC346Flu1 proliferation is inhibited by androgens at concentrations that are normally stimulatory for PC346C. This paradoxal growth inhibitory effect by androgens has been previously reported for various LNCaP variants similarly subjected to long-term culture in the absence of androgens [47,48,49]. All three studies reported an overexpression of the AR protein and, for the two variants LNCaP-abl and LNCaP-104R, the hormone inhibitory effect was preceded at early passages by a hypersensitivity to low levels of androgens. Kokontis *et al* also observed that androgen inhibited LNCaP-104R proliferation by cell-cycle arrest and not by induction of apoptosis, and that this effect could be reverted by subsequent continuous passage in androgen-containing medium [50]. This androgenic repression phenomenon could be a possible mechanism underlying withdrawal responses observed in some patients upon discontinuation of the endocrine treatment. In this context, intermittent hormone therapy, in cycles blocking growth and restoring androgen-regulation, may be an interesting treatment option to retard tumor progression.

The PC346Flu2 subline expresses a mutated AR with altered ligand-binding properties, such that OH-flutamide, the antiandrogen upon which this subline was selected, is converted into an agonist [12]. This type of AR point mutations, that broaden the ligand-specificity of the receptor to antiandrogens, have also been proposed as a possible explanation for the antiandrogen withdrawal syndrome [51]. These data support that AR activating mutations may confer growth advantage upon androgen-ablation therapy to a subset of prostate tumors, particularly in patients treated with antiandrogens.

The karyotype of two variants of the parental PC346C has been previously reported [52]. The PC346C variant described in our study exhibited a near-tetraploid chromosome complement and few structural abnormalities, which were homogenously present in almost every cell: 92~99 (4n), XXYY, +1, +5, +7, +7, del(8)(p11.1), +i(8)(q10),

-13, -13, +19, +20, -22. Our array CGH data confirmed most of the alterations reported in the previous karyotype, but did not detect a significant amplification of chromosomes 5 and 19 and deletion of 22. The sensitivity of the array CGH technology may not be high enough to detect single copy amplifications or deletions in a tetraploid background. Most of the chromosomal abnormalities detected by CGH were present in all the cell lines of the panel, showing that these originated in the parental PC346C prior to the development of the sublines. Amplification of 8q and deletion of 10q and 13q are among the most common genetic alterations in prostate cancer [53]. Several candidate genes have been previously identified in these locations: the c-MYC oncogene in 8q, the PTEN tumor-suppressor gene in 10q, RB1 and BRCA2 in 13q [54]. Gain of chromosomes 1, 7 and 20 are also frequently detected in prostate tumor specimens and cell lines [55]. Only three major chromosomal modifications were detected in the androgen-independent sublines, which were not present in the parental PC346C, confirming the relative genomic stability of the cell lines. The loss of chromosome Y in PC346Flu2 and 8p in PC356DCC are well-known aberrations that have been associated with prostate cancer progression [53]. This suggests that the long-term androgen ablation selected not only for AR mutations but also for chromosomal alterations that benefit tumor growth, and therefore that the sublines are derived from PC346C by clonal selection.

In conclusion, the PC346 cell line panel mimics many biological characteristics of different stages of *in vivo* prostate tumors. It comprises properties ranging from androgen-dependent growth to fast androgen-independent growth characteristic of advanced progressive disease, while reproducing the most common AR modifications observed in hormone-refractory disease. It therefore provides a valuable model system to investigate the mechanisms that dictate androgen-independent growth, with emphasis to the role of AR signaling in prostate cancer progression.

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Chapter 4

Gene Expression Profile of the Androgen Receptor Pathway in Androgen-Responsive versus Hormone-Refractory PC346 Prostate Cancer Cells

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ABSTRACT

Background: Prostate cells are dependent on androgens for their survival and growth. This characteristic is maintained in (early) prostate cancer, where the androgen-regulated prostate-specific antigen (PSA) is used in the diagnosis and follow-up of the disease, and has been exploited in the management of metastatic disease using androgen ablation therapy.

Objective: To characterize the role of the androgen-receptor (AR) pathway in prostate cancer progression and identify potential diagnostic/prognostic disease markers.

Methods: Microarray analysis was used to establish the androgen-regulated gene expression profile of PC346 cell lines. The resulting AR-target gene signature was linked to multiple prostate cancer databases, from *in vitro* cell lines, xenografts and patient-derived samples, published previously. ENDOD1, MCCC2 and ACSL3 were selected as potential disease markers for RT-PCR quantification in a distinct set of human prostate specimens.

Results: A total of 107 transcripts were differentially-expressed upon stimulation of PC346 cells with the synthetic androgen R1881 or the antiandrogen flutamide. Among these were well-known AR-target genes such as TMPRSS2, KLK2, and FKBP5, but also novel genes, including MAFB, KLF9, NFIB and EHF. Gene ontology annotations clustering revealed enrichment towards genes involved in transcription regulation, signal transduction, regulation of cell cycle and apoptotic processes, and cellular differentiation. Additionally, R1881 induced the expression of genes related to the metabolism of proteins, carbohydrates and lipids that contribute to the production and secretion of prostatic fluid. These distinct functional clusters were differentially modulated during prostate cancer progression. A considerable fraction of AR pathway genes involved in differentiation and secretory function of the prostate were up-regulated in primary prostate cancer but repressed in metastasis. Conversely, genes implicated in cell survival, proliferation, cytoskeletal remodeling and adhesion, were overexpressed in metastasis. Ultimately, three candidate genes were selected as potential disease markers: MCCC2 was overexpressed in low-grade prostate cancer, whereas ENDOD1 and ACSL3 were down-regulated in high-grade tumors and metastatic samples.

Conclusions: The AR pathway is adapted in advanced and hormone-refractory prostate cancer, through selective modulation of differentiation, proliferation and invasion functions. These findings may have implications in the current therapy for metastatic prostate tumors and in the development of prostate cancer markers.

INTRODUCTION

Prostate cancer is the most frequently diagnosed non-cutaneous malignancy in men and the second leading cause of cancer deaths in the western countries [1]. Prostate cancer is a highly heterogeneous condition, exhibiting a wide range of biological and clinical manifestations. While some patients develop an asymptomatic disease course and rather die with the cancer than from the cancer, others present with a more aggressive and/or more advanced disease at the time of diagnosis [2]. When the tumor is confined to the prostate, it can be efficiently treated by radical surgery and/or radiation therapy, but once the tumor has disseminated, systemic therapy is required. Since prostate cells, normal or malignant, require androgens for their survival, the golden standard for the treatment of evasive prostate tumors is androgen ablation through chemical or surgical castration, which may be combined with the administration of androgen receptor (AR) antagonists [2]. Most patients will benefit from this therapy, the tumors may shrink and the symptoms ameliorate. Unfortunately, the cancer will eventually recur as a fatal hormone-refractory condition, for which at the moment no curative treatment exists [3,4]. A pertinent question is how prostate cancer cells that are initially dependent on androgens for survival can resume growth in an androgen-deprived environment. One possibility is that prostate cancer cells achieve this by adapting their AR pathway to the low androgen/high antiandrogen levels. On the other hand, cancer cells may activate alternative growth pathways, while shutting down tumor suppressors and apoptotic signals [5,6].

In the present study, we focused on the role of the AR pathway in prostate cancer progression. The expression pattern of androgen-regulated genes in androgen-responsive and castration-resistant cell lines was established, with the goal to: (i) determine whether the AR pathway is still functionally active in hormone-refractory disease; (ii) identify the mechanism(s) by which the AR pathway may be adjusted to the low androgen/high antiandrogen levels; (iii) identify androgen-regulated genes that could potentially be used in the diagnosis/prognosis of prostate cancer or as a therapeutic target. For this purpose, we used microarray technology to characterize the transcriptional program activated by the synthetic androgen R1881 and the antiandrogen hydroxyflutamide. As model system we used the PC346 cell lines (Table 1): the androgen-responsive PC346C parental cell line and its hormone-refractory derivative sublines PC346DCC, PC346Flu1 and PC346Flu2 [7]. These castration-resistant sublines reproduce common AR modifications observed in hormone-refractory disease: AR down-regulation (PC346DCC), AR mutation (PC346Flu2) and AR over-expression (PC346Flu1), making it a unique and valuable model for this study.

Table 1 – Characteristics of the PC346 cell line panel: AR status and hormone response

	PC346C	PC346DCC	PC346Flu1	PC346Flu2
AR status	wt AR	AR low	AR high	T877A AR
PSA expression	+ +	-	+ +	+ +
Growth on steroid-stripped medium	-/+	+ +	+ +	+
Growth with 0.1 nM R1881	+ +	+ +	+	+ +
Growth with 1 µM OH-flutamide	-/+	+ +	+ +	+ +

MATERIALS AND METHODS

Reagents and cell lines

The basic culture medium used in the maintenance of PC346 cell lines consisted of DMEM-F12 medium (Cambrex BioWhitaker, Belgium) supplemented with 2% fetal calf serum (FCS; PAN Biotech GmbH, Aidenbach, Germany), 1% insulin-transferrin-selenium (Gibco BRL), 0.01% bovine serum albumin (Boehringer Mannheim, Germany), 10 ng/ml epidermal growth factor (Sigma-Aldrich), penicillin/streptomycin antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin; BioWhitaker, Belgium); plus the following additions: 100 ng/ml fibronectin (Harbor Bio-Products, Tebu-bio, The Netherlands), 20 mg/ml fetuine (ICN Biomedicals, The Netherlands), 50 ng/ml choleratoxin, 0.1 mM phosphoethanolamine, 0.6 ng/ml triiodothyronine and 500 ng/ml dexametason (all from Sigma). PC346C cells were maintained in culture in the complete medium mentioned above, supplemented with 0.1 nM 17-methyltrienolone (R1881; NEN, Boston MA, USA). PC346DCC selection medium was supplemented as described above, but depleted from androgens by using dextran-coated charcoal (DCC) treated FCS. PC346Flu1 and PC346Flu2 culture medium was also androgen depleted by using 2% DCC-FCS, and supplemented with 1 μM of hydroxyflutamide (OH-flutamide, Schering-Plough Research Institute, New Jersey, USA). For the hormone stimulations, a simplified version of the culture medium was used, containing 2% DCC- FCS without the above mentioned additions (minimal medium). Cells were grown in T25 Primaria^{TM} tissue culture flasks (BD Biosciences Benelux N.V, The Netherlands) at 37^{o}C under 5% CO_2 humidified atmosphere.

Hormone stimulations and expression microarray analysis

Cells were seeded in their respective selection medium to reach ~50% confluency and allowed to attach overnight. The next day, medium was replaced with 2% DCC-FCS in minimal medium and cells were starved for 48h, to bring AR activity to basal levels before the hormone stimulations. Subsequently, cells were stimulated with either vehicle, 1 nM R1881 or 1 μM OH-flutamide for 4, 8 or 16h. After stimulations, cells were rinsed twice with PBS and stored at -20^{o}C until RNA isolation. Total RNA was isolated with RNAzol B reagent (Campro Scientific, Veenendaal, The Netherlands) and further purified through RNeasy columns (Qiagen) with on-column DNA digestion, according to the manufacturer's protocol. RNA quality was checked on 1% agarose gel.

Cy3- or Cy5-labelled RNA probes were produced by incorporating amino-allyl UTP during RNA amplification, followed by coupling to N-hydroxysuccinimide modified dye. Briefly, 3 μg RNA was used for a T7-based linear mRNA amplification protocol, described previously [8]. Amino-allyl UTP, plus equal amount of unmodified rUTP, was incorporated into aRNA with T7 Megascript Kit (all from Ambion), according to manufacturer's protocol. Amplified RNA was purified and concentrated using Microcon-YM 30 columns (Amicon®) to rinse three times with 300 μl RNAse-free water. Finally, 2 μg aminoallyl-modified RNA, in a maximum of 3.33 μl of RNAse-free water, was incubated with 1.66 μl sodium bicarbonate buffer (0.3 M, pH 9) and 5 μl Cy3- or Cy5-dye (CyScribe Post-Labeling Kit, Amersham, NJ, USA), for 1h in the dark at room temperature. Reaction was stopped with 5 μl 4 M hydroxylamine HCl (Sigma), contra-labelled probes were combined and purified/concentrated using Microcon-YM 30

columns. Probe was collected in 5-15 µl final volume and resuspended in 80 µl Ambion hybridization buffer number 1.

For the microarray we used double-dye oligoarrays representing about 15,000 human genes, on which labelled hormone-stimulated RNA was cohybridized with its contra-labelled time-matched vehicle (ethanol) control. Two microarrays were performed per condition: in one experiment the stimulated samples were labeled with Cy3 and the unstimulated reference with Cy5, in the other experiment in vice-versa (dye-swap); this was done to exclude dye-preferential binding to oligonucleotides on the microarray. In addition, two independent cell passages were used for each of these experiments, to account for the biological variability.

The oligoarrays used in this study were produced at the Erasmus Center for Biomics. Briefly, a human 18,584 oligonucleotides library (Compugen, Sigma-Genosys) was spotted on aminosilane slides using a Virtek Chipwriter Professional arrayer (Virtek Vision International, Waterloo, Canada). Control spots included landmarks, spotting buffer, alien oligonucleotides (SpotReport Alien Oligo Array, La Jolla, Stratagene), poly d[A]40-60, salmon sperm DNA, and human COT-1 DNA. Before the hybridization, microarray slides were prehybridized in 5x SSC, 0.05% SDS, 4% BSA solution for 30 min at 45°C, washed twice with RNAse-free water for 2 min, rinsed with isopropanol and spin-dried for 3 min at 1500 g. Microarray hybridizations were performed overnight at 45°C, with continuous agitation, in a HS4800 Hybridization Station (Tecan Benelux BV). Finally, the arrays were washed automatically in the Hybridization Station using: 2x SSC/0.05% SDS (at 45°C), 1x SSC and 0.2x SSC (at room temperature), and dried under a stream of N2, before scanning.

Data extraction and analysis

Arrays were scanned in a ScanArray Express HT scanner (Perkin Elmer, Nederland BV) and spot intensities were quantified using Imagene software (Bio Discovery Inc, El Sequndo, CA, USA). To balance Cy3 and Cy5 spot intensities, Loewess normalization per subarray was performed using limma-package (http://bioinf.wehi.edu.au/limma/) from Bioconductor (http://www.bioconductor.org) [9,10]. To scale between arrays, the global median intensity per array was set at 1000. Dye intensities below 200 were then thresholded at 200, to minimize noise and make fold-change on the low-intensity range more robust against outliers. Spots with intensities below the threshold (200) for both Cy3 and Cy5 channels, in more than 50% (>3/6) of the arrays for each time-course, were excluded from the analysis. Sample to vehicle-control ratios were then calculated and 2log transformed. Spots that showed opposite effects for the dye-swap/biological replicates were excluded from further analysis; effects were called opposite if the mean 2log ratio for the three time-points tested were $\geq 0,5$ for one dye and below $\leq -0,5$ for the dye-swap. Following normalization and all the above-mentioned quality controls, the 2log intensity ratios from both replicates were averaged for each time point. This data was stored in SRS7 (Sequence Retrieval System version 7, Lion Bioscience AG, Heidenberg, Germany), which was also used for the comparisons with other previously published/publicly available databases [11].

Hierarchical clustering and data visualization was performed using Cluster and TreeView programs (Eisen Labs: http://rama.lbl.gov), respectively. Significance Analysis of Microarrays (SAM; http://www-stat.stanford.edu/~tibs/SAM) was used to determine

which genes were statistically different between stimulated samples and non-stimulated references. Gene ontology clustering was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID: http://david.abcc.ncifcrf.gov) [12,13]. The pathway and functional analyses were generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com).

cDNA synthesis and RT- PCR analysis

Total RNA was isolated as described above and cDNA was synthesized using MMLV-reverse transcriptase kit and Oligo(dT)$_{12-18}$ primer (Invitrogen), according to manufacturer's protocol. cDNA samples were stored at -20°C. The PCR reaction mixtures for the semi-quantitative microarray validation PCRs contained 1.25 µL of 0.5 U/µL Taq polymerase, 2.5 µL 10x PCR buffer, 1.5 µL 25 mM $MgCl_2$ (Promega Benelux b.v., the Netherlands), 1 µL 10 mM dNTPs, 1.5 µL 100 ng/µL forward and reversed primers (Invitrogen, Breda, the Netherlands) and 5 µL cDNA (previously diluted 1:20), in a final volume of 25 µl. Primers were designed using the computer program Oligo Primer Analysis Software version 6.22 (Molecular Biology Insights Inc, USA). Gene specificity was checked by BLAST and, whenever possible, intron-spanning primers were chosen to avoid amplification of contaminating DNA. RT-PCRs were performed in a 3 step cycling program consisting: of an initial denaturation step at 94^{o}C for 5 min, followed by N cycles of: 94^{o}C - 45 sec, Tannealing^{o}C - 45 sec, 72^{o}C - 45 sec; and a final 10 min extension step at 72^{o}C. Primer sequences, annealing temperatures (Tannealing) and number of cycles (N) for each PCR are summarized in Table 2. Experiments were repeated twice, using distinct cell passages. The household gene GAPDH was used for normalization.

For the quantitative PCR analysis, normal and tumor samples from patients were obtained from the frozen tissue bank of the Erasmus Medical Center (Rotterdam, the Netherlands). The specimens were collected between 1984 and 2001. The experimental protocols were approved by the Erasmus MC Medical Ethics Committee according to the Medical Research Involving Human Subjects Act. Additional information about these specimens was provided previously [14]. TaqMan real-time PCR analysis was performed in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), using AmpliTaq Gold DNA polymerase (Applied Biosystems), according to manufacturer's specifications. Validated primers and probes from TaqMan Gene Expression Assays (Applied Biosystems) were used for quantification of ACSL3 (Hs01071247_m1), MCCC2 (Hs00223257_m1), ENDOD1 (Hs00826684_m1) and GAPDH (Hs99999905_m1), according to the PCR settings provided by Applied Biosystems. PBGD was quantified using 0.33 µM of primers forward: 5'-CAT GTC TGG TAA CGG CAA TG-3' and reverse: 5'-GTA CGA GGC TTT CAA TGT TG-3' primers, in Power SybrGreen PCR Master mix (Applied Biosystems), according to thermocycling protocol recommended by the manufacturer. Transcript quantities for each sample were normalized against the average of two endogenous references and relative to a calibrator. The two housekeeping genes used as endogenous references were PBGD and GAPDH; a mixture of cDNAs from prostate carcinoma xenografts was used as the calibrator. Graphs and statistics were performed with GraphPad Prism (version 3.0). P-values < 0.05 were considered significant.

Table 2 - Primer sequences, annealing temperature and number of cycles for RT-PCR

Gene	Forward primer	Reverse primer	$T_{annealing}$ (°C)	Cycles N
AR	CTA CCA GCT CAC CAA GCT CCT G	ACA AGG CAC TGC AGA GGA GTA G	60	30
PSA	TTG TTA CAT TGA CAG GAG ACA CAC T	CTC AGG TCT GAG AAG TTA AGG TCA G	60	30
KLK2	AGA TGA AGA CTC CAG CCA T	GAT ACC TTG AAG CAC ACC A	60	30
PART1	GAG CCA GCC AAT CAC TT	AGC AGC ACT CAG GCG T	58	35
TPD52	TTT CAA TGT GTT GGA AAC TGT AA	TAG AAT ACC TTG GCC TCT ATG C	58	30
GPR88	CCA AGG CGT CTC TTT AAG T	ATG GCA ACT CAT ACT GGT G	60	30
FKBP5	GAA TAC ACC AAA GCT GTT GA	CTC TTC CTT GGC ATC CT	58	30
TMPRSS2	CCT CTG GTC ACT TCG AAG AAC	GTA AAA CGA CGT CAA GGA CG	60	35
STEAP1	AGA AGA TGC CTG GAT TGA	CTT CTT CCT CAA GCA TGG	58	30
ID3	GGA GCT TTT GCC ACT GAC TC	GCT CCT GAG CAC CAG GTT TA	60	30
TRIB1	ATG GGA CTT TGA GAA GAG G	GCC ATC TCA CTG TTC ACA T	60	35
GAPDH	ATG GCA TGG ACT GTG GTC	ACG GGA AGC TTG TCA TCA	58	25

Cycling program used was 94°C: 5min; (94°C: 45 sec/ Tannealing°C: 45 sec/ 72°C: 45 sec) x N cycles; 72°C: 10 min

RESULTS

Gene expression pattern of PC346 cells treated with R1881 and hydroxyflutamide

To characterize the expression profile of androgen receptor target genes in prostate cancer cells, we used expression microarray analysis on the PC346 cell line panel incubated with the androgen analogue R1881 or the antiandrogen OH-flutamide. The PC346 model system is composed of four cell lines: the androgen-sensitive PC346C and three hormone-refractory sublines, derived from the parental PC346C by long-term androgen ablation (PC346DCC), supplemented with the antiandrogen OH-flutamide (PC346Flu1 and PC346Flu2). All these sublines exhibit different properties with respect to AR status and responsiveness (summarized in Table 1) [7].

For the expression analysis we stimulated the cells with 1 nM R1881 or 1 μM OH-flutamide for 4, 8 or 16h and cohybridized the labeled RNA with its time-matched vehicle (ethanol) control. Two microarrays were performed per condition, using two independent cell passages in dye-swap, to account for the biological variability and potential dye-preferential effects. Early time-points were chosen in order to enrich for primary AR targets, and minimize indirect secondary targets.

The two replicates per time-point were averaged and a total of 107 differentially-expressed transcripts were selected to constitute the AR pathway signature: 74 up-regulated and 33 down-regulated by R1881 and/or OH-flutamide (Tables 3 to 6). Spots were considered to be differentially-expressed if the absolute 2log ratio ≥ 0.5 (ratio ≥ 1.42 or ≤ 0.71) for all three time-points, for at least one cell type. Significance Analysis of Microarrays (SAM) was used to determine which genes were statistically different between stimulated samples and non-stimulated references. In total, there were 253 SAM significant genes, with a false discovery rate (FDR) set at 0.05 (Supplementary Tables S1 to S4). From our 107 signature transcripts, considered differentially-expressed according to the above-mentioned selection criteria, 77 were statistically significant by SAM. Although being clearly regulated (absolute 2log ratio > 0.5 for all 3 time-points), the remaining 30 (28%) transcripts of our AR-target signature were not SAM significant, which was due to a large variation across the 3 time-points. Since temporal regulation was observed for such few transcripts, possibly because of the narrow time frame chosen, no analysis was performed on the dynamics of gene-expression variation across time. Therefore, the expression ratios presented in the tables and figure 4 are from the average of all three time-points per condition. Finally, the fact that a considerable number of SAM significant transcripts were not included in our AR-regulated signature was due to our choice to set the 2log ratio threshold at 0.5.

The androgen-sensitive PC346C subline responded to the R1881 stimulation with increased expression of 18 genes, while 2 were down-regulated. Among these are some well-known AR regulated genes, such as KLK2, STEAP1, TMPRSS2 and FKBP5. The hormone-refractory sublines showed distinct responses to R1881 and OH-flutamide. PC346Flu1, which expresses 4-fold higher AR levels than the parental cell line, showed a “super-activation” of the AR pathway by R1881, not only in the magnitude of the gene expression but also in the number of regulated genes (20 androgen-regulated genes in the parental PC346C versus 91 in PC346Flu1). Conversely, the PC346DCC subline, which expresses residual levels of AR protein, showed no detectable changes in gene expression after the hormone treatments. Neither PC346C,

PC346DCC nor PC346Flu1 showed significant alterations in the transcriptional profile in response to OH-flutamide. In contrast, PC346Flu2 cells, which express the T877A mutated AR, responded to both R1881 and this antiandrogen, although the response to the latter was weaker (14 genes up-regulated by R1881 versus 8 up-regulated by OH-flutamide; Tables 3 and 5, respectively).

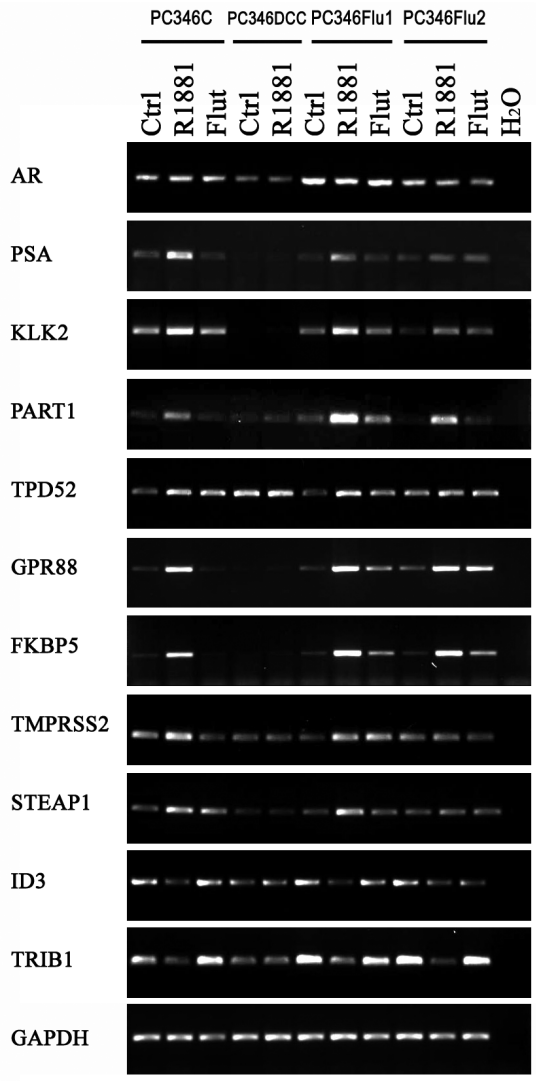


Figure 1 - Validation of the microarray results. Reverse-transcriptase PCR analysis of a set of 10 androgen-regulated genes in PC346 cell lines. Cells were stimulated with 1 nM R1881 (R1881), 1 μM OH-flutamide (Flut) or vehicle control (Ctrl) for 16h. AR expression is also shown here, although the receptor was not androgen-regulated in the microarray analysis. ID3 and TRIB1 were shown to be androgen-repressed in the microarray assays, whereas the other genes were up-regulated by androgens. GAPDH was used as endogenous reference.

Table 3 - List of genes up-regulated by R1881

GenBank ID	HUGO_Symbol	HUGO_Name	Cytoband	Cell line	2log ratio	SAM q-value
NM_018674	ACCN4	amiloride-sensitive cation channel 4 pituitary	2q35	PC346C	0.74	0.000
NM_004457	ACSL3	acyl-CoA synthetase long-chain family member 3	2q34-q35	PC346Flu1	2.04	0.000
NM_014109	ATAD2	ATPase family AAA domain containing 2	8q24.13	PC346Flu1	0.83	0.000
AK027213	BBS10	Bardet-Biedl syndrome 10	12q21.2	PC346Flu1	0.77	0.000
NM_020235	BBX	bobby sox homolog Drosophila	3q13.1	PC346Flu1	0.76	0.007
AK024850	C2orf31	chromosome 2 open reading frame 31	2q34	PC346Flu1	1.32	0.000
NM_006079	CITED2	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2	6q23.3	PC346Flu1	0.96	0.000
AK026498	CYP2U1	cytochrome P450 family 2 subfamily U polypeptide 1	4q25	PC346Flu1	1.50	0.000
NM_012062	DNM1L	dynamin 1-like	12p11.21	PC346Flu1	1.36	0.000
NM_018456	EAF2	ELL associated factor 2	3q13.33	PC346Flu1	2.28	0.000
AK026517	EHF	ets homologous factor	11p12	PC346Flu1	0.62	0.000
AK022827	EIF2C3	eukaryotic translation initiation factor 2C 3	1p34.3	PC346C	0.55	0.056
AK022827	EIF2C3	eukaryotic translation initiation factor 2C 3	1p34.3	PC346Flu1	1.03	0.000
NM_012081	ELL2	elongation factor RNA polymerase II 2	5q15	PC346Flu1	1.75	0.000
NM_012081	ELL2	elongation factor RNA polymerase II 2	5q15	PC346Flu2	1.04	1.093
AF111849	ELOVL5	ELOVL family member 5 elongation of long chain fatty acids FEN1/Elo2	6p21.1-p12.1	PC346Flu1	1.46	0.000
AB020637	ENDOD1	endonuclease domain containing 1	11q21	PC346Flu1	1.26	0.000
AB020637	ENDOD1	endonuclease domain containing 1	11q21	PC346Flu2	1.11	1.093
NM_019018	FAM105A	family with sequence similarity 105 member A	5p15.2	PC346Flu1	1.04	0.000
AK024648	FAM107B	family with sequence similarity 107 member B	10p13	PC346Flu1	0.76	0.007
AL137343	FAM84A	family with sequence similarity 84 member A	2p24.3	PC346Flu1	1.14	0.000
NM_004117	FKBP5	FK506 binding protein 5	6p21.3-21.2	PC346C	1.88	0.000
NM_004117	FKBP5	FK506 binding protein 5	6p21.3-21.2	PC346Flu1	4.21	0.000
NM_004117	FKBP5	FK506 binding protein 5	6p21.3-21.2	PC346Flu2	1.66	1.093
AK024715	FLJ21062 *	Hypothetical protein FLJ21062 *	7q21.13	PC346Flu1	1.41	0.000
NM_020474	GALNT1	UDP-N-acetyl-alpha-D-galactosamine:polypeptide	18q12.1	PC346Flu1	0.79	0.000
NM_005271	GLUD1	glutamate dehydrogenase 1	10q23.3	PC346Flu1	1.11	0.000
NM_002069	GNAI1	guanine nucleotide binding protein G protein alpha inhibiting activity polypeptide 1	7q21	PC346Flu1	0.94	0.000
AB042410	GPR88	G protein-coupled receptor 88	1p21.3	PC346C	1.35	0.000
AB042410	GPR88	G protein-coupled receptor 88	1p21.3	PC346Flu1	3.04	0.000
AB042410	GPR88	G protein-coupled receptor 88	1p21.3	PC346Flu2	2.56	0.125

NM_001530	HIF1A	hypoxia-inducible factor 1 alpha subunit basic helix-loop-helix transcription factor	14q21-q24	PC346Flu1	1.16	0.000
NM_003543	HIST1H4H	histone cluster 1 H4h	6p21.3	PC346Flu1	1.72	0.000
M60721	HLX	H2.0-like homeobox	1q41-q42.1	PC346Flu1	0.73	0.000
M60721	HLX	H2.0-like homeobox	1q41-q42.1	PC346Flu2	0.63	1.093
NM_014642	IQCB1	IQ motif containing B1	3q13.33	PC346Flu1	0.81	0.139
NM_002241	KCNJ10	potassium inwardly-rectifying channel subfamily J member 10	1q22-q23	PC346C	0.67	0.056
AL137384	KIAA1109	KIAA1109	4q27	PC346C	0.63	0.027
NM_001206	KLF9	Kruppel-like factor 9	9q13	PC346Flu1	0.83	0.000
AF188747	KLK2	kallikrein-related peptidase 2	19q13.41	PC346C	0.79	0.000
AF188747	KLK2	kallikrein-related peptidase 2	19q13.41	PC346Flu1	1.12	0.000
AF188747	KLK2	kallikrein-related peptidase 2	19q13.41	PC346Flu2	1.03	1.093
AK026375	LOC93622 *	Hypothetical protein BC006130 *	4p16.1	PC346Flu1	1.02	0.000
NM_005461	MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B avian	20q11.2-q13.1	PC346Flu1	1.06	0.000
NM_003010	MAP2K4	mitogen-activated protein kinase kinase 4	17p11.2	PC346Flu1	0.78	0.000
AB050049	MCCC2	methylcrotonoyl-Coenzyme A carboxylase 2 beta	5q12-q13	PC346Flu1	1.05	0.000
AK021627	MORC4	MORC family CW-type zinc finger 4	Xq22.3	PC346Flu1	1.24	0.000
AF142409	MS4A6A	membrane-spanning 4-domains subfamily A member 6A	11q12.1	PC346Flu1	0.76	0.450
NM_005956	MTHFD1	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1	14q24	PC346C	0.61	0.000
NM_016498	MTP18 *	Mitochondrial protein 18 kDa *	22q	PC346C	0.64	0.000
NM_000662	NAT1	N-acetyltransferase 1 arylamine N-acetyltransferase	8p23.1-p21.3	PC346Flu1	1.68	0.000
AF039944	NDRG1	N-myc downstream regulated gene 1	8q24.3	PC346Flu1	1.63	0.000
NM_006096	NDRG1	N-myc downstream regulated gene 1	8q24.3	PC346Flu1	2.60	0.000
NM_005596	NFIB	nuclear factor I/B	9p24.1	PC346Flu1	0.77	0.077
NM_020529	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha	14q13	PC346C	0.74	0.000
NM_020529	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha	14q13	PC346Flu1	2.39	0.000
NM_020529	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha	14q13	PC346Flu2	0.65	1.093
NM_016590	PART1 *	prostate androgen-regulated transcript 1 *	5q12.1	PC346Flu1	1.45	0.000
NM_006810	PDIA5	protein disulfide isomerase family A member 5	3q21.1	PC346C	0.50	0.000
NM_006810	PDIA5	protein disulfide isomerase family A member 5	3q21.1	PC346Flu1	1.42	0.000
NM_016166	PIAS1	protein inhibitor of activated STAT 1	15q	PC346Flu1	1.31	0.000
AF070670	PPM1A	protein phosphatase 1A formerly 2C magnesium-dependent alpha isoform	14q23.1	PC346Flu1	0.72	0.000
NM_004156	PPP2CB	Protein phosphatase 2 formerly 2A catalytic subunit beta isoform	8p12-p11.2	PC346Flu1	0.94	0.000
NM_002923	RGS2	regulator of G-protein signaling 2 24kDa	1q31	PC346Flu2	0.73	0.000
D16875	RHOB	ras homolog gene family member B	2p24	PC346Flu1	1.61	0.000

AK001478	RHOU	ras homolog gene family member U	1q42.11-q42.3	PC346C	0.77	0.000
AK001478	RHOU	ras homolog gene family member U	1q42.11-q42.3	PC346Flu1	2.29	0.000
AK001478	RHOU	ras homolog gene family member U	1q42.11-q42.3	PC346Flu2	1.05	0.000
AB051826	RHOU	ras homolog gene family member U	1q42.11-q42.3	PC346Flu1	2.54	0.000
NM_005627	SGK1	serum/glucocorticoid regulated kinase 1	6q23	PC346Flu1	0.85	0.000
AB040914	SHROOM3	shroom family member 3	4q21.1	PC346Flu1	1.01	0.012
NM_004595	SMS	spermine synthase	Xp22.1	PC346Flu1	1.14	0.000
NM_003082	SNAPC1	small nuclear RNA activating complex polypeptide 1 43kDa	14q22	PC346Flu1	0.62	0.166
NM_003104	SORD *	sorbitol dehydrogenase *	15q15.3	PC346Flu1	1.20	0.000
NM_012449	STEAP1	six transmembrane epithelial antigen of the prostate 1	7q21	PC346C	0.79	0.000
NM_012449	STEAP1	six transmembrane epithelial antigen of the prostate 1	7q21	PC346Flu1	1.69	0.000
NM_012449	STEAP1	six transmembrane epithelial antigen of the prostate 1	7q21	PC346Flu2	0.97	1.091
AK026813	STEAP2	six transmembrane epithelial antigen of the prostate 2	7q21	PC346Flu1	1.07	0.000
AK026813	STEAP2	six transmembrane epithelial antigen of the prostate 2	7q21	PC346Flu2	0.74	0.000
NM_005656	TMPRSS2	Transmembrane protease serine 2	21q22.3	PC346C	0.59	0.117
NM_005079	TPD52	tumor protein D52	8q21	PC346Flu1	1.22	0.000
NM_005079	TPD52	tumor protein D52	8q21	PC346Flu2	0.69	0.208
AF294628	TWSG1	twisted gastrulation homolog 1 Drosophila	18p11.3	PC346Flu1	0.99	0.000
NM_003115	UAP1	UDP-N-acteylglucosamine pyrophosphorylase 1	1q23.3	PC346Flu1	0.96	0.000
NM_003359	UGDH	UDP-glucose dehydrogenase	4p15.1	PC346C	0.58	0.098
NM_003359	UGDH	UDP-glucose dehydrogenase	4p15.1	PC346Flu1	1.91	0.000
AK001647	USP40	ubiquitin specific peptidase 40	2q37.1	PC346C	0.68	0.000
AB020676	WWC1	WW and C2 domain containing 1	5q34	PC346Flu1	0.74	0.000
AK022814	ZBTB10	zinc finger and BTB domain containing 10	8q13-q21.1	PC346Flu1	1.34	0.000
AK022814	ZBTB10	zinc finger and BTB domain containing 10	8q13-q21.1	PC346Flu2	0.54	1.093
NM_006006	ZBTB16	zinc finger and BTB domain containing 16	11q23.1	PC346C	0.88	0.000
NM_006006	ZBTB16	zinc finger and BTB domain containing 16	11q23.1	PC346Flu1	1.55	0.000
NM_006006	ZBTB16	zinc finger and BTB domain containing 16	11q23.1	PC346Flu2	1.55	1.093
AF025771	ZNF189	zinc finger protein 189	9q22-q31	PC346Flu1	1.50	0.000
AK026383	NDRG1	cDNA clone *		PC346Flu1	2.05	0.000
AL157445		cDNA clone *		PC346Flu1	1.02	0.007
D17210		cDNA clone *		PC346Flu1	0.97	0.000

* no approved HUGO symbol / name exist for this entry. If present, gene symbol / name from the UNIGENE database are given in alternative.

Table 4 - List of genes down-regulated by R1881

GenBank ID	HUGO_Symbol	HUGO_Name	Cytoband	Cell line	2log ratio	SAM q-value
NM_005688	ABCC5	ATP-binding cassette sub-family C CFTR/MRP member 5	3q27	PC346Flu1	-0.68	0.282
AK026288	ATHL1	ATH1 acid trehalase-like 1 yeast	11p15.5	PC346Flu1	-0.96	0.008
NM_012342	BAMBI	BMP and activin membrane-bound inhibitor homolog Xenopus laevis	10p12.3-p11.2	PC346Flu1	-0.98	0.000
NM_001197	BIK	BCL2-interacting killer apoptosis-inducing	22q13.31	PC346Flu1	-0.82	0.112
AF075110	C14orf4	chromosome 14 open reading frame 4	14q24.3	PC346Flu1	-1.29	0.000
NM_017766	CASZ1	castor zinc finger 1	1p36.22	PC346Flu1	-0.98	0.112
NM_001305	CLDN4	claudin 4	7q11.23	PC346Flu1	-0.63	0.282
AK024378	FAM131A	family with sequence similarity 131 member A	3q27.1	PC346Flu1	-0.71	0.052
NM_004480	FUT8	fucosyltransferase 8 alpha 1 6 fucosyltransferase	14q24.3	PC346Flu1	-1.01	0.316
NM_002165	ID1	inhibitor of DNA binding 1 dominant negative helix-loop-helix protein	20q11	PC346Flu1	-0.81	0.018
NM_002165	ID1	inhibitor of DNA binding 1 dominant negative helix-loop-helix protein	20q11	PC346Flu2	-1.07	1.087
X69111	ID3	inhibitor of DNA binding 3 dominant negative helix-loop-helix protein	1p36.13-p36.12	PC346Flu1	-1.25	0.000
NM_006769	LMO4	LIM domain only 4	1p22.3	PC346Flu1	-0.82	0.000
NM_017572	MKNK2	MAP kinase interacting serine/threonine kinase 2	19p13.3	PC346Flu1	-0.68	0.088
NM_005377	MYCL2	v-myc myelocytomatosis viral oncogene homolog 2 (avian)	Xq22-q23	PC346C	-0.97	0.116
NM_006312	NCOR2	nuclear receptor co-repressor 2	12q24	PC346Flu1	-0.57	0.052
U90907	PIK3R3	phosphoinositide-3-kinase regulatory subunit 3 p55 gamma	1p34.1	PC346Flu1	-1.01	0.008
AF113132	PSAT1	phosphoserine aminotransferase 1	9q21.2	PC346Flu1	-0.78	0.000
NM_004577	PSPH	phosphoserine phosphatase	7p15.2-p15.1	PC346Flu1	-0.85	0.041
NM_015923	SLC3A2	solute carrier family 3, member 2	11q13	PC346Flu1	-0.61	0.263
NM_003943	STBD1	starch binding domain 1	4q24-q25	PC346Flu1	-0.79	0.022
NM_003714	STC2	stanniocalcin 2	5q35.1	PC346Flu1	-0.63	0.088
AK000401	TANC1	tetratricopeptide repeat ankyrin repeat and coiled-coil containing 1	2q24.2	PC346Flu2	-0.72	1.087
AL133074	TP53INP1	tumor protein p53 inducible nuclear protein 1	8q22	PC346Flu1	-1.25	0.000
NM_003287	TPD52L1	tumor protein D52-like 1	6q22-q23	PC346Flu1	-0.71	0.402
AF205437	TRIB1	tribbles homolog 1 Drosophila	8q24.13	PC346Flu1	-1.63	0.000
U55055		Oral cancer candidate gene mRNA clone		PC346Flu2	-1.01	1.087
NM_018588		hypothetical protein *		PC346C	-0.64	0.194
NM_018588		hypothetical protein *		PC346Flu1	-0.55	0.422
AK022971		cDNA clone *		PC346Flu1	-0.66	0.450
AK022971		cDNA clone *		PC346Flu2	-0.71	1.087

* no approved HUGO symbol / name exist for this entry. If present, gene symbol / name from the UNIGENE database are given in alternative.

Table 5 - List of genes up-regulated by hydroxyflutamide

GenBank ID	HUGO_Symbol	HUGO_Name	Cytoband	Cell line	2log ratio	SAM q-value
AB020637	ENDOD1	endonuclease domain containing 1	11q21	PC346Flu2	0.51	0.158
NM_004117	FKBP5	FK506 binding protein 5	6p21.3-21.2	PC346Flu2	0.57	0.226
AB042410	GPR88	G protein-coupled receptor 88	1p21.3	PC346Flu2	1.57	0.000
NM_002923	RGS2	regulator of G-protein signaling 2 24kDa	1q31	PC346Flu2	0.57	0.000
AK026813	STEAP2	six transmembrane epithelial antigen of the prostate 2	7q21	PC346Flu2	0.61	0.118
AB020676	WWC1	WW and C2 domain containing 1	5q34	PC346Flu2	0.52	0.158
D17099		cDNA clone *		PC346Flu2	0.90	0.926
AL049966		cDNA clone *		PC346Flu2	0.92	0.926

* no approved HUGO symbol / name exist for this entry. If present, gene symbol / name from the UNIGENE database are given in alternative.

Table 6 - List of genes down-regulated by hydroxyflutamide

GenBank ID	HUGO_Symbol	HUGO_name	Cytoband	Cell line	2log ratio	SAM q-value
NM_002165	ID1	inhibitor of DNA binding 1 dominant negative helix-loop-helix protein	20q11	PC346Flu2	-0.58	0.301
NM_002241	KCNJ10	potassium inwardly-rectifying channel subfamily J member 10	1q22-q23	PC346Flu2	-0.84	0.301
NM_006854	KDELR2	KDEL Lys-Asp-Glu-Leu endoplasmic reticulum protein retention receptor 2	7p22.1	PC346C	-0.78	1.118
AB028451	NCOR1	nuclear receptor co-repressor 1	17p11.2	PC346Flu2	-1.00	0.301
NM_001269	RCC1	regulator of chromosome condensation 1	1p36.1	PC346Flu2	-0.70	0.301
NM_000370	TTPA	tocopherol alpha transfer protein	8q13.1-q13.3	PC346Flu2	-0.83	0.301

* no approved HUGO symbol / name exist for this entry. If present, gene symbol / name from the UNIGENE database are given in alternative.

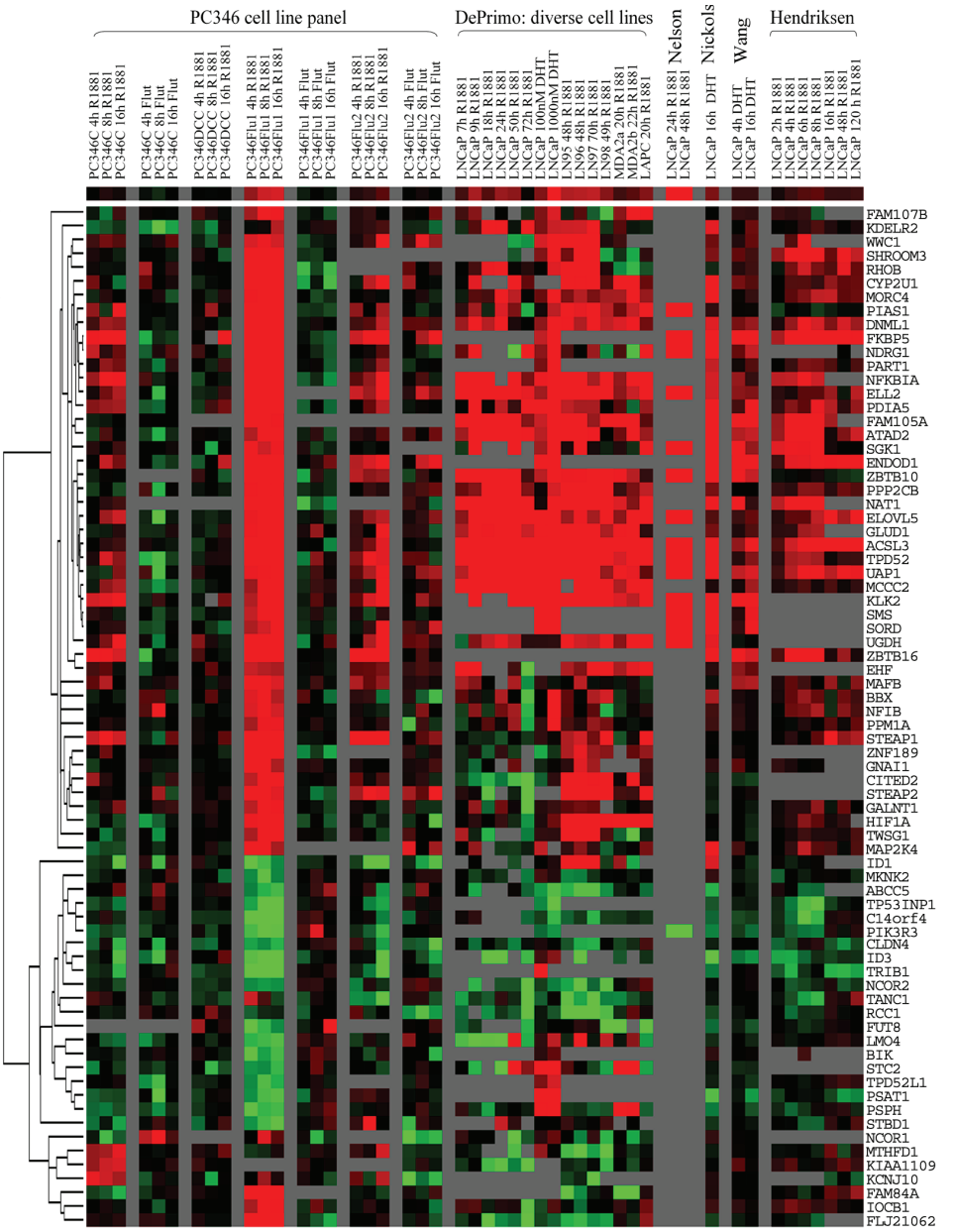


Figure 2 - Expression profile of androgen-responsive genes in PC346 cells linked to publicly available databases on AR transcriptional regulation. On the left side, PC346C, PC346Flu1 and PC34Flu2 were exposed to 1 nM R1881 or 1 μM OH-flutamide for 4, 8 and 16h, whereas PC346DCC was stimulated with 1 nM R1881 only. On the right side, our gene signature was assessed in the databases from DePrimo et al., Nelson et al., Nickols et al., Wang et al. and Hendriksen et al. (see table 7 for database details). Heat-map is presented for the 2log expression ratio between hormone-treated samples and respective time-matched vehicle controls. Red and green colors represent induction and repression, respectively, whereas black indicates no regulation. Grey squares indicate missing data due to low expression, poor data quality or absence of probes for the respective transcript in the array platform used for the study.

Table 7 - Description of the androgen-regulation and prostate cancer databases linked via SRS

First Author (date)	Reference	Query
DePrimo (2002) [46]	LNCaP, LN95, LN96, LN97, LN98, LAPC, MDA2a, MDA2b cell lines	1nM R1881, 10nM DHT, 100nM DHT and 1000nM DHT different time points from 7 to 72h
Hendriksen (2006) [28]	LNCaP	1nM R1881 time-course from 2h to 120h
Nelson (2002) [47]	LNCaP	24h and 48h 1nM R1881
Nickols (2007) [48]	LNCaP	16h 1nM DHT
Wang (2007) [49]	LNCaP	4h and 16h 100nM DHT
Best (2005) [50]	10 hormone-naive prostate cancers	10 hormone-refractory primary prostate tumors
Chandran (2007) [51]	64 primary prostate tumor samples	24 hormone-refractory metastatic samples from 4 patients
Lapointe (2004) [52]	41 benign prostate tissue adjacent to cancer	62 primary prostate tumor samples 9 lymph node metastasis
Singh (2002) [53]	50 benign prostate tissue adjacent to cancer	52 primary prostate tumor samples, of which 8 recurred after radical prostatectomy and 13 remained relapse-free (>4 years)
Tamura (2007) [54]	10 hormone-naive prostate cancers	18 hormone-refractory primary and metastatic tumor samples
Varambally (2005) [55]	4 benign prostate tissues	5 clinically localized prostate cancers 5 metastatic samples
Yu (2007) [16]	60 benign prostate tissue adjacent to cancer 23 disease free donor prostate tissue	62 primary prostate tumors 24 metastatic samples from 4 patients

Validation of the microarray data

The microarray data was validated by two approaches: an experimental approach using RT-PCR, and a bioinformatics approach linking our gene signature to a set of publicly available databases on androgen response. We selected 10 androgen-regulated genes to be further validated by RT-PCR: PSA, KLK2, PART1, TPD52, GPR88, FKBP5, TMPRSS2, STEAP1, ID3 and TRIB1. It is worth noting that our microarray analysis did not detect regulation of PSA expression in response to the hormonal treatments, but since this is a prominent AR target gene, it was included in the RT-PCR analysis. The AR and GAPDH genes were also included in the RT-PCR assay, to confirm the AR status of the cell lines and for normalization purposes, respectively. Consistent to previous knowledge on the PC346 cell panel, RT-PCR showed decreased expression of AR in PC346DCC and over-expression in PC346Flu1. The AR mRNA itself was not regulated by hormonal treatment. RT-PCR analysis further confirmed the differential expression of all selected genes in the same direction predicted by the microarray analysis (Fig. 1). Furthermore, the RT-PCR also showed a stronger effect of the hormone-treatment on the PC346Flu1 cell line, in contrast to the almost absent induction of PC346DCC cells, when compared to the parental PC346C, for most genes analysed. As observed in the microarray assay, PC346Flu2 showed equivalent responses to R1881 and OH-flutamide for many regulated genes (Fig. 1, Tables 3-6).

Recently, a series of studies have been published that analyzed gene expression in response to androgens stimulation in cell lines and xenografts (Table 7). Of the 107 transcripts in our signature, 73 were present in at least 3 of the 5 databases and were included for further analysis. More than 90% of the linked genes overlapped with previously reported androgen-regulated targets. Genes with the strongest inductions in our present work also showed consistently high inductions in multiple previous reports, suggesting that the products of these genes may play a basic role in the biological function of the prostate (Fig. 2). Using our unique cell line panel, we were able to identify novel androgen-responsive genes such as MAFB, KLF9, NFIB, STBD1, BIK or HLX.

Biologic processes coordinated by the AR pathway

The androgen-regulated signature genes were classified according to Gene Ontology (GO) Biological Processes using the Database for Annotation, Visualization and Integrated Discovery (DAVID: http://david.abcc.ncifcrf.gov) [12,13].

Consistent with the physiological roles of androgens, this approach revealed that the AR target genes selected in the present study operate in the regulation of transcription and intracellular signaling pathways, the metabolism of proteins, lipids and carbohydrates, and the regulation of cell proliferation and differentiation (Figure 3A). The largest category includes genes encoding for transcription factors and transcription regulators, such as NFIB, KLF9, HIF1A, MAFB, EHF, NCOR1, NCOR2, PIAS1 and several zinc finger proteins (ZNF189, ZBTB10, ZBTB16 and CASZ1). This was followed by genes involved in intracellular signal transduction, including the G protein-coupled receptors pathway (GPR88, RGS2, GNAI1), small GTPases of the Ras family (RHOB, RHOU), mitogen-activated protein kinase cascade (MAP2K4, MKNK2, TRIB1) and other protein kinases/phosphatases (PPM1A, PPP2CB, PIK3R3, SGK1). Other AR responsive genes have an effect on cellular proliferation through regulation of cell cycle and apoptotic processes (e.g. RCC1, BBX, BIK, TP53INP1). Concomitant with the role of

androgens on prostate development and maturation, another major cluster included genes involved in cellular differentiation, such as TPD52, TWSG1, NDRG1, ID1 and ID3. Finally, androgen induced the metabolism of proteins, carbohydrates and lipids that contribute to the production and secretion of prostatic fluid. R1881 target genes included MTHFD1, PSPH, PSAT1 and MCCC2, encoding enzymes in the metabolism of methionine, serine and leucine amino acids, respectively. Furthermore, up-regulation of the translation initiation factor EIF2C3 potentially promotes peptide synthesis. Moreover, genes participating in protein folding (PDIAS5, FKBP5), glycosylation (FUT8, GALNT1) and trafficking (DMN1L, KDELR2) were also regulated by R1881. Apart from proteins and amino acids, prostatic fluid is also rich in lipids, polyamines, sorbitol and several metal ions. Indeed, R1881 also stimulated expression of ACSL3 and ELOVL5, which participate in the elongation of fatty-acids, spermine synthase (SMS), part of the polyamine synthetic pathway, sorbitol dehydrogenase (SORD), secreted by the prostate into the seminal fluid, and the ion channels ACCN4 and KCNJ10.

Table 8 – Summary of significantly enriched Gene Ontology (GO) categories

Annotation Cluster 1	Enrichment Score: 2.43	Count	P_Value	Gene List
organic acid metabolic process		10	0.0025	ACSL3, ELOVL5, PSPH,
amine metabolic process		9	0.0022	SMS, UGDH, GLUD1, MTHFD1,
amino acid metabolic process		7	0.0046	PPP2CB, MCCC2, PSAT1
Annotation Cluster 2	Enrichment Score: 2.34	Count	P_Value	Gene List
apoptosis		12	0.0019	DNM1L, PPP2CB, ELL2, BIK,
programmed cell death		12	0.0021	RHOB, MTP18, TPD52L1, TP53INP1,
cell development		12	0.049	SGK1, NFKBIA, ID3, ZBTB16
Annotation Cluster 3	Enrichment Score: 2.27	Count	P_Value	Gene List
developmental process		27	0.0088	FUT8, MAFB, MTP18, TPD52L1, CITED2,
cell differentiation		19	0.0042	TTPA, SHROOM3, EHF, NDRG1, TP53INP1, SGK1, BIK, RHOB, ZBTB16, UGDH, IQCB1, ID1, ID3, ELL2, DNM1L, LMO4, HLX, KLF9, PPP2CB, TPD52, TWSG1, NFKBIA
Annotation Cluster 4	Enrichment Score: 2.12	Count	P_Value	Gene List
regulation of developmental process		7	0.002	MAFB, PPP2CB,
regulation of cell differentiation		6	0.0013	TWSG1, NFKBIA, ZBTB16,
hemopoiesis		5	0.016	SHROOM3, IQCB1
Annotation Cluster 5	Enrichment Score: 1.69	Count	P_Value	Gene List
negative regulation of metabolic process		7	0.023	PPP2CB, ZNF189, NCOR1,
negative regulation of transcription		6	0.018	NCOR2, ID1, ID3, ZBTB16
Annotation Cluster 6	Enrichment Score: 1.49	Count	P_Value	Gene List
regulation of cellular process		33	0.014	TP3INP1, SNAPC1, TPD52L1, ZBTB16,
regulation of gene expression		23	0.017	NFKBIA, ZBTB10, LMO4, NFIB, PIAS1,
regulation of transcription		22	0.017	MKNK2, EHF, NCOR1, NCOR2, MAFB, KLF9, BIK, RHOB, BBX, ID1, ID3, HLX, ZNF18, TWSG1, IQCB1, PPM1A, RGS2, CITED2, PPP2CB, HIF1A, CASZ1, RCC1

To automate the functional classification, quantify the degree of enrichment of each cluster and select statistically significant functional categories we used the DAVID Functional Annotation Clustering tool. This tool identified 6 statistically significant Annotation Clusters, which associated with the metabolism of organic acids (lipids and amino acids), apoptosis, cell differentiation, developmental processes, regulation of transcription and regulation of cellular processes (Table 8).

The involvement of androgen-regulated genes in pathological conditions was further investigated by using the Ingenuity database (Ingenuity® Systems, www.ingenuity.com). The strongest associations were found for cancer, reproductive system, dermatological and cardiovascular diseases (Fig. 3B).

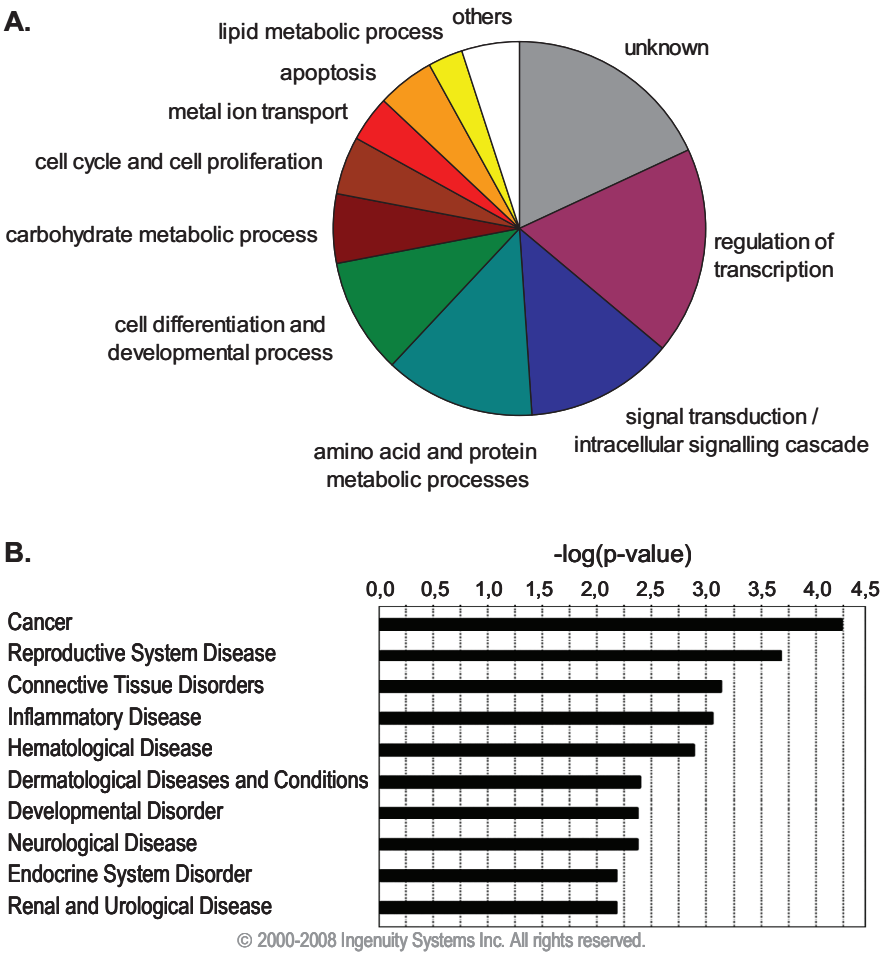
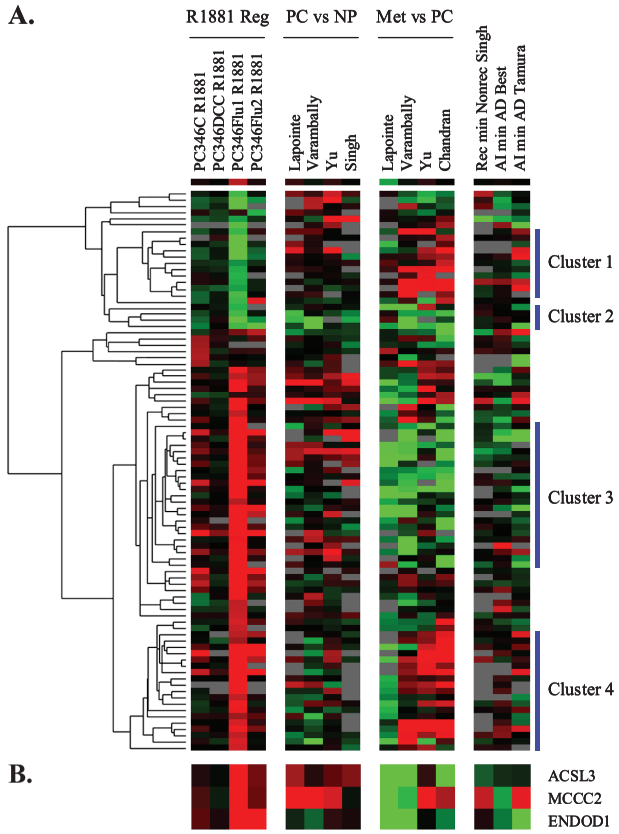


Figure 3 - Biological processes regulated by the selected androgen-target genes. **(A)** Pie-graph representing genes categorized according to most prominent biological function. Gene ontology annotations were extracted using DAVID [12,13]. **(B)** Involvement of the AR pathway genes in disease determined using Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com).

The AR pathway in prostate cancer development and progression

To investigate how the AR pathway is modulated during the development and progression of prostate cancer we linked our androgen-regulated gene signature to seven independent prostate cancer microarray databases. These studies included specimens of "normal prostate" and prostate tumors from diverse disease stages, whose main characteristics are summarized in Table 7. A total of 89 hormone-responsive genes were present in at least 4 of the 7 databases, and were selected for further analysis. In Figure 4, we show the hierarchical clustering of the R1881-responsive genes (first block of 4 columns), next to primary cancer versus normal prostate (second block), metastasis versus primary cancer (third block), and finally recurrent versus non-recurrent and hormone-refractory versus hormone-naïve disease (fourth block). The clustering analysis revealed four major gene groups: R1881-repressed and up-regulated during progression to metastatic disease (Cluster 1), R1881-repressed and down-regulated during progression (Cluster 2), R1881-induced and down-regulated during progression (Cluster 3), R1881-induced and up-regulated during progression (Cluster 4). About one third of the R1881-regulated genes was differentially-expressed between primary tumors and normal prostate in at least two databases. To this group contributed mainly R1881-induced genes that showed up-regulation in prostate cancer. These are genes that play a role in the production of prostatic fluid and in secretory function of the prostate, including SORD, ACSL3, ELOVL5, FKBP5, PDIA5, GLUD1 and UAP1. However, when comparing metastatic cancer to primary tumors, 23 of the R1881-induced genes were down-regulated (Fig. 4, Cluster 3), while 11 androgen-repressed genes were up-regulated (Fig. 4, Cluster 1). In total, these two clusters made up a considerable fraction (40%) of the androgen-responsive genes, and their expression pattern in metastasis suggests that the AR pathway is selectively down-regulated at this stage of the disease. In contrast, another group of R1881-stimulated genes showed increased expression in metastasis compared to primary tumors (Fig. 4, Cluster 4). This cluster is enriched for genes involved in survival/cellular proliferation (MAFB, ELL2, TPD52, EHF, HIF1A, HLX and SGK) and cell remodeling/adhesion (RHOU, SHROOM3, MORC4, TWSG1). Conversely, a group of R1881-repressed genes down-regulated in metastasis included genes involved in cellular differentiation and development (ID1, ID3, LMO4 and TPD52L1) (Fig. 4, Cluster 2). Finally, we assessed the activation state of the AR pathway in recurrent and in hormone-refractory disease. The collection of datasets in this category is limited to three non-concordant databases: Best *et al.* and Tamura *et al.* compared hormone-naïve with hormone-refractory samples, Singh *et al.* evaluated biochemical recurrence following radical prostatectomy. Therefore, the overlap between the three databases was modest. Nevertheless, the general trend is the same as for the progression of primary cancer to metastatic disease: genes down-regulated in metastasis tend to be down-regulated in recurrent versus non-recurrent and/or hormone-refractory versus homone-naïve disease, and vice-versa. These results suggest that the common mechanisms may govern the progression to different states of prostate cancer disease.



Cluster 1	Cluster 3	Cluster 4
PSAT1	RHOB	HLX
FUT8	KLK2	SNAPC1
C14orf4	SORD	ELL2
TP53INP1	PART1	GPR88
PIK3R3	PDIA5	TPD52
NCOR2	ACSL3	SHROOM3
MKNK2	ELOVL5	RHOU
FAM131A	CYP2U1	MORC4
ABCC5	GLUD1	HIST1H4H
STC2	STEAP1	FAM105A
PSPH	EAF2	FAM107B
	TWSG1	HIF1A
Cluster 2	PPM1A	PPP2CB
TPD52L1	ENDOD1	EHF
LMNO4	STEAP2	ATAD2
ID3	NAT1	MAFB
ID1	PIAS1	BBX
	FKBP5	SGK
	GNAI1	IQCB1
	FLJ21062	
	UGDH	
	C2orf31	
	ZNF189	

Figure 4 - Expression of androgen-responsive genes in prostate cancer samples from patients. **(A)** Heat-map representation of publicly available expression data from human prostate cancer using the 107-gene signature (see table 7 for database details). **(B)** Androgen-responsive genes selected for further quantitative PCR analysis. Color scheme as described for figure 2.

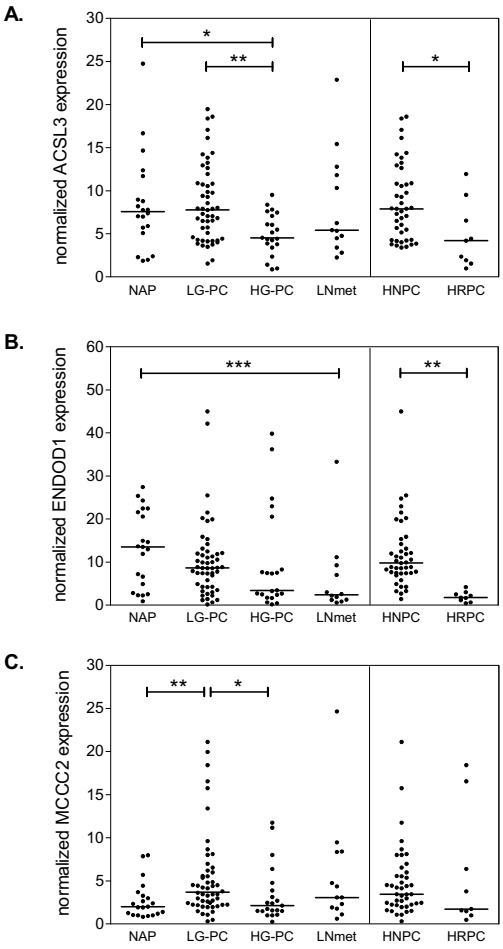


Figure 5 - Quantitative RT-PCR analysis of four androgen-responsive genes ACSL3, ENDOD1 and MCCC2 in a distinct set of prostate samples. NAP: normal adjacent prostate; LG-PC: low-grade prostate cancer, including Gleason score from 5-7; HG-PC: high-grade prostate cancer, including Gleason score from 8-10; LNmet: lymph-node metastasis; HNPC: hormone-naïve prostate cancer (primary location); HRPC: hormone-refractory prostate cancer (primary location); (*) p-value ≤ 0.05 and (**) p-value ≤ 0.005 using Mann-Whitney two-tailed test; (***) p-value ≤ 0.05 with Post linear-trend test.

AR target genes as markers for disease diagnosis and prognosis

The last objective of this study was to identify genes that could possibly be used as tools in the diagnosis of prostate cancer or in predicting the course of disease. We selected three R1881-regulated genes to be analyzed by quantitative PCR on normal prostate and prostate carcinoma specimens obtained at our institute: ACSL3, MCCC2 and ENDOD1. These candidates were selected based on their strong androgen-induction, potential pathological function but, most importantly, on the fact that their expression was confirmed to be altered across multiple prostate cancer databases analyzed (Fig.

4B). In this sense, ACSL3 seems to be slightly up-regulated in primary prostate tumors and strongly repressed in metastatic cancer. Furthermore, fusion of the ACSL3 gene to the ETS family member ETV1 has recently been reported, making it an interesting gene for follow-up [41]. MCCC2 was strongly up-regulated in primary cancer, although its expression in metastasis and hormone-refractory disease varies in the different databases (Fig. 4B). Finally, ENDOD1 was one of the strongest R1881-induced genes in our microarray profile, and showed decreased expression in metastasis and hormone-refractory tumors, suggesting a possible role in disease progression (Fig. 4B).

Quantitative PCR analysis included 21 samples of benign prostate tissue (adjacent to cancer), 73 primary prostate tumors and 13 lymph node metastasis. The primary tumors consist of 52 low-grade samples, 21 samples from late-stage poorly-differentiated tumors and 9 hormone-refractory specimens, obtained from patients operated by radical prostatectomy or transurethral resection of the prostate (TURP). ACSL3 expression was significantly decreased during progression from low-grade to high-grade tumors ($P=0.005$; Fig. 5A). ENDOD1 exhibited a stepwise down-regulation during disease progression ($P<0.05$ for Post linear-trend test), which is consistent with the results from the prostate cancer databases (Fig. 5B). Finally, MCCC2 was up-regulated in well-differentiated tumors ($P<0.005$), but its expression decreased during progression to high-grade cancer ($P<0.05$; Fig. 5C). This biphasic expression of MCCC2 during prostate cancer progression might explain the variation observed across the different databases mentioned above. Furthermore, expression of all three candidate genes was decreased during progression to hormone-refractory disease, although the trend for MCCC2 was not statistically significant.

DISCUSSION

In order to design better diagnostic and prognostic tools for prostate cancer and to develop more efficient therapies for late stage disease, it is essential to methodically understand the processes by which this disease develops and progresses. In this sense the AR pathway is of great interest for clinicians, researchers and pharmaceutical industry as it plays a crucial role in prostate malignancy. To investigate whether the AR pathway is functionally active in hormone-refractory prostate disease, we started by establishing the expression program of AR target genes in PC346 cell lines stimulated with R1881 or hydroxyflutamide.

R1881 stimulation of the androgen-sensitive PC346C subline resulted in differential expression of 20 genes, including the well-known AR target genes TMPRSS2, KLK2 and TPD52. Consistent with the expression of *wild-type* AR, OH-flutamide did not mediate transcription of AR-target genes in PC346C cells.

The castration-resistant PC346DCC subline, which expresses very low levels of the receptor, showed to be insensitive to R1881 stimulation. These results suggest that the AR pathway is not essential for the growth of PC346DCC cells. How these cells have bypassed the AR pathway, surviving and proliferating in the absence of androgens, is still not clear. Hypothetically, it could have been achieved through activation of oncogenes or alternative growth pathways, and/or repression of tumor suppressors.

Conversely, the cell line overexpressing the AR, PC346Flu1, showed a "super-activation" of the pathway, not only in the number of regulated genes but also in the strength of this regulation. This reveals two important aspects: (i) although these cells have been cultured in the absence of androgens for longer than two years, the AR is still functional and can be activated by the presence of its ligand; (ii) the AR seems to be hyper-sensitive, likely due to the high levels of the receptor, which may be sufficient to support cell growth under the hormone-depleted conditions. Interestingly, PC346Flu1 proliferation is inhibited by physiological concentrations of androgens, both *in vivo* and *in vitro*, suggesting that AR "super-activation" is unfavorable for cell growth, possibly by inducing cellular differentiation [7]. This is in line with a previous report, which showed that prostate epithelial cells tolerate a narrow-range of AR expression and activity, by undergoing apoptosis in the absence of AR expression and cell cycle arrest upon AR hyper-stimulation [15].

In PC346Flu2 subline, carrying a mutated receptor, transcription of AR-target genes was regulated by both R1881 and OH-flutamide, although the stimulatory effect of the latter was weaker. This is in agreement with the agonistic action of OH-flutamide on the T877A mutated AR in promoting rather than inhibiting the growth of PC34Flu2 cells.

In general, from these analyses we can conclude that, in the majority of hormone-refractory prostate cancer cells subjected to long-term androgen ablation, the AR pathway is modified and still able to respond to stimuli. Furthermore, it is worth noting how the AR transcription patterns of the three hormone-refractory sublines reflected their respective AR modifications and growth characteristics.

To investigate the biologic processes coordinated by the AR target genes we used DAVID and Ingenuity tools to extract and cluster Gene Ontology Annotations. Consistent with the physiological roles of androgens in prostate development and maturation, the selected gene-signature is enriched for functions in transcription regulation, intracellular signal transduction, differentiation and regulation of cell proliferation and cell death. Further functions are associated with the metabolism of proteins, lipids and carbohydrates, which can be related to the production and secretion of prostatic fluid. Pathway analysis using Ingenuity showed strong association of the androgen-regulated genes to pathological conditions as cancer, reproductive system, dermatological and cardiovascular diseases (Fig. 3 and Table 8).

Next we evaluated the role of the AR pathway in prostate cancer development and how it is modulated during cancer progression by linking our androgen-regulated gene signature to seven previously published microarray databases on clinical tumor samples. Together, these databases comprise 178 "normal prostate" samples and 331 malignant specimens, including metastasis, recurrent tumors and hormone-refractory samples (Table 7). It is worth noting that the definition of "normal prostate" is not the same across the different studies. While most authors used benign tissue adjacent to the tumor as the "normal" reference, Yu *et al.* used normal prostatic epithelia from individuals without evidence of prostatic disease [16]. They showed that the expression profile of prostate cells was not only altered within the tumor itself, but alterations were also detected in apparently benign tissue around the borders of the tumor. This so-called field-effect has been reported in various other studies, and it is believed to be more evident the closer the distance to the tumor [17,18,19]. Disparity in the sampling of

the “normal prostate” reference may certainly contribute to the variation seen between the diverse studies, together with differences in study design, microarray platforms, and most importantly, in the characteristics of the tumors included.

In summary, our AR-response profiling revealed that a considerable fraction of AR pathway genes were up-regulated in primary prostate cancer compared to normal prostate and down-regulated in metastasis. Further inspection of this gene cluster showed enrichment for genes involved in differentiation and secretory function of the prostate, functions which are redundant, if not detrimental, in progressive disease (Fig.4, Cluster 3). On the other hand, the cluster of androgen-regulated genes over-expressed in metastasis is enriched for genes involved in cell survival, proliferation, cytoskeletal remodelling and adhesion, all crucial functions in tumor progression and invasion (Fig. 4, Cluster 4).

It is generally accepted that the AR pathway accounts for the tumor growth in most prostate cancer patients even under hormonal ablation therapy. This hypothesis is supported by numerous reports that the AR itself is expressed in the majority of prostate cancers and often amplified in metastasis and hormone-refractory tumors [20,21,22,23,24]. Chen *et al.* has shown recently that AR overexpression is the most common modification following androgen ablation treatment, and is sufficient to confer hormone-refractory growth [25]. Furthermore, clinical tumor relapse is determined by PSA recurrence, which may give the impression that the AR pathway has become again fully functional. However, the results of our present study showed a selective down-regulation of AR target genes, questioning the over-simplistic view of the AR pathway as the driving force for prostate cancer growth and proliferation. In fact, the raise in serum PSA levels during relapse rather reflects the expansion of the tumor burden than increased AR activity in the tumor tissue self [26]. Indeed, Sterbis *et al*. reported that increased risk of biochemical recurrence was associated with low expression of tissue PSA mRNA [27]. Furthermore, the authors observed that serum PSA levels did not correlate with tissue mRNA expression, which was decreased in malignant compared to benign prostate epithelial cells [27]. By using distinct cell lines to establish the androgen-response signature and expanding the patient-derived database sets, our results corroborate previous observations from Hendriksen *et al.,* which used the androgen-response expression profile from LNCaP cells to interrogate a set of prostate cancer xenografts and Lapointe’s patient-derived samples [28]. Shortly thereafter, with distinct bioinformatics approaches, two other studies confirmed an attenuated androgen signaling signature in high-grade and metastatic prostate cancer, indicating that down-regulation of the AR pathway, although controversial, is not likely to be an artifact [29,30].

The mechanisms for this selective modulation of the AR pathway during prostate cancer progression are yet undefined, but we speculate that it may be dictated by an imbalance in AR co-regulators and/or interactions with other signaling pathways. Indeed, alterations in several AR co-activators and co-repressors have been previously detected in prostate cancer and, in particular, in hormone-refractory disease [31,32]. Furthermore, crosstalk between the AR and other growth factor pathways has been shown to activate AR signaling and selectively regulate a fraction of the AR transcriptional program, in response to IGF and EGF [33,34].

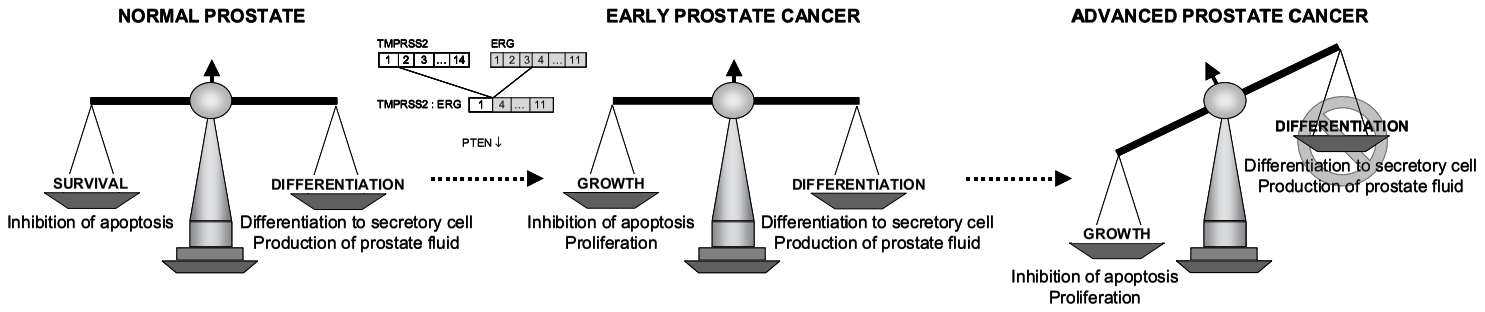


Figure 6 - Proposed model for prostate cancer progression. In normal prostate epithelial cells the AR maintains the balance between survival and differentiation. Cooperation between TMPRSS2:ERG and PTEN loss, is a potential mechanism suggested to drive the transition from prostatic intraepithelial neoplasia (PIN) to prostate adenocarcinoma. These aberrations are early events in prostate cancer development and are present in a large fraction of tumors. Initiation of prostate cancer is marked by a switch from androgen-dependent survival to androgen-responsive proliferation. As cancer progresses, the balance is tilted towards tumor growth, while genes involved in prostate differentiation and secretory function are selectively repressed and genes promoting proliferation are up-regulated. In advanced disease, this mechanism eventually culminates in poorly-differentiated fast-growing tumors

To accommodate these novel insights into our current knowledge of prostate cancer disease, we propose the following model for the development and progression of prostate tumors (Fig. 6): in the normal prostate the AR maintains prostate homeostasis and secretory functions through a delicate balance between cell survival and differentiation. A yet unknown trigger leads to a switch from androgen-dependent survival to androgen-stimulated cellular proliferation. Recent findings implicate gene fusions between androgen-regulated genes and ETS transcription factor family members in this process. The TMPRSS2-ERG fusion is the most frequent rearrangement, being detected in up to 50% of prostate tumors [35]. The androgen-responsive promotor region of the TMPRSS2 gene drives robust expression of ERG, an oncogene that is also frequently involved in chromosomal translocations in Ewing sarcoma, myeloid leukemia and cervical carcinoma [36,37,38]. Up to date, multiple other ETS family members and 5′ fusion partners have been identified in related rearrangements in prostate cancer [39,40,41,42]. However, the biological role of ETS fusions in prostate cancer development is still controversial, since ERG and ETV1 by themselves, do not seem to be tumorigenic [40,43]. Recent evidence suggests that ERG overexpression cooperates with PTEN loss in the progression from PIN to prostate adenocarcinoma [44,45]. It is worth noting that PC346 cells do not carry the TMPRSS2-ERG or TMPRSS2-ETV1 fusions, nor show increased expression of these oncogenes (unpublished data). Therefore, it remains unclear which mechanism may drive androgen-sensitive growth of PC346C cells. Nevertheless, it is still possible that other less common fusions partners that we did not test yet may be involved. We hypothesize that at early stages, when tumors are well differentiated, expression of prostate-specific genes and genes involved in the production/secretion of prostatic fluid is maintained or even increased due to the growth of the epithelial cell compartment. As tumors progress and become more aggressive, genes involved in prostate differentiation and secretory function are selectively repressed, while genes promoting proliferation are up-regulated. This mechanism will eventually culminate in a fast-growing, poorly-differentiated late-stage disease (Fig. 6).

In order to identify androgen-regulated genes that could possibly be used in the diagnosis/prognosis of prostate cancer, we selected from our 107-gene signature three androgen-regulated genes: MCCC2, ENDOD1 and ACSL3. Quantitative PCR analysis showed increased MCCC2 expression in early-stage, well-differentiated tumors, while ENDOD1 and ACSL3, were decreased in late-stage tumors and metastasis. To assess the prognostic value of these genes we compared primary prostate cancer that eventually developed distant metastasis after radical surgery with the non-recurrent tumors, but saw no significant differences (data not shown). Ultimately, the large inter-individual variation resulted in a poor separation between the diverse disease stages, even when the differences in expression were statistically significant. This limits the applicability of MCCC2, ENDOD1 or ACSL3 as independent diagnostic markers, by preventing the setting of an expression cutoff with both high specificity and sensitivity. However, the performance of these candidates may be improved in combination with other disease markers, such as PSA or ETS gene fusions, which has yet to be evaluated in the diagnosis and prognosis of prostate cancer. Finally, the down-regulation of all three candidate genes in hormone-refractory compared to hormone-

naïve disease is in agreement with an attenuation of the AR pathway, providing important clues on the mechanisms of prostate cancer progression.

CONCLUSIONS

The gene expression profile of the AR pathway in the three hormone-refractory sublines reflected their respective AR modifications: AR down-regulation correlated with deficient activation of AR-target genes; high-levels of AR resulted in more differentially-expressed genes and stronger regulation upon R1881 stimulation; finally, the T877A mutated AR responded to both R1881 and flutamide.

The AR pathway is very versatile, being involved in many biological processes including cellular proliferation, regulation of apoptosis and differentiation. The balance between these different functions dictates the homeostasis of the prostatic gland. The results of the present study suggest that the AR pathway is selectively modulated during prostate cancer progression, leading to repression of genes involved in cellular differentiation and up-regulation of anti-apoptotic and proliferation genes. Future studies are ongoing to elucidate the mechanisms of this selective adaptation of the AR signaling and how it may be targeted in prostate cancer therapy.

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SUPPLEMENTARY DATA

Table S1 - List of SAM significant genes (q-value < 0.05) for PC346C cell line

GenBank ID	HUGO_Symbol	HUGO_Name	Stimuly	2log ratio	SAM q-value
NM_004117	FKBP5	FK506 binding protein 5	R1881	1.88	0.000
AB042410	GPR88	G protein-coupled receptor 88	R1881	1.35	0.000
AB035266	NRXN2	neurexin 2	R1881	0.91	0.048
NM_006006	ZBTB16	zinc finger and BTB domain containing 16	R1881	0.88	0.000
AF188747	KLK2	kallikrein-related peptidase 2	R1881	0.79	0.000
NM_012449	STEAP1	six transmembrane epithelial antigen of the prostate 1	R1881	0.79	0.000
AK001478	RHOU	ras homolog gene family member U	R1881	0.77	0.000
NM_020529	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells	R1881	0.74	0.000
NM_018674	ACCN4	amiloride-sensitive cation channel 4 pituitary	R1881	0.74	0.000
AL109698	ZNF710	zinc finger protein 710	R1881	0.71	0.000
AK001647	USP40	ubiquitin specific peptidase 40	R1881	0.68	0.000
AL360260	METRN	meteorin glial cell differentiation regulator	R1881	0.68	0.000
NM_004231	ATP6V1F	ATPase H transporting lysosomal 14kDa V1 subunit F	R1881	0.66	0.027
NM_017802	HEATR2	HEAT repeat containing 2	R1881	0.64	0.000
NM_016498	MTP18 *	Mitochondrial protein 18 kDa *	R1881	0.64	0.000
AL137384	KIAA1109	KIAA1109	R1881	0.63	0.027
NM_014606	HERC3	hect domain and RLD 3	R1881	0.62	0.048
Z21967	POU6F1	POU class 6 homeobox 1	R1881	0.62	0.039
NM_012099	CD3EAP	CD3e molecule epsilon associated protein	R1881	0.61	0.000
NM_005956	MTHFD1	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1	R1881	0.61	0.000
U36561	FUS	fusion involved in t 12 16 in malignant liposarcoma	R1881	0.60	0.027
AK024535	STAT3	signal transducer and activator of transcription 3 acute-phase	R1881	0.59	0.000
NM_006624	ZMYND11	zinc finger MYND domain containing 11	R1881	0.59	0.027
Y12018	PSEN1	presenilin 1 Alzheimer disease 3	R1881	0.59	0.027
NM_005081	ZNF142	zinc finger protein 142	R1881	0.58	0.048
AK022564	C14orf159	chromosome 14 open reading frame 159	R1881	0.57	0.000
NM_014848	SV2B	synaptic vesicle glycoprotein 2B	R1881	0.55	0.000
NM_003738	PTCH2	patched homolog 2 Drosophila	R1881	0.55	0.039
AJ002787	PRKCB1	protein kinase C beta 1	R1881	0.54	0.000
NM_012164	FBXW2	F-box and WD repeat domain containing 2	R1881	0.54	0.039
NM_006992	LRRC23	leucine rich repeat containing 23	R1881	0.53	0.000
NM_017772	TBC1D22B	TBC1 domain family member 22B	R1881	0.51	0.048
NM_006810	PDIA5	protein disulfide isomerase family A member 5	R1881	0.50	0.000

NM_015865	SLC14A1	solute carrier family 14 urea transporter member 1 Kidd blood group	R1881	0.50	0.048
NM_006869	CENTA1	centaurin alpha 1	R1881	0.49	0.000
NM_002493	NDUFB6	NADH dehydrogenase ubiquinone 1 beta subcomplex 6 17kDa	R1881	0.48	0.048
AB033023	YEATS2	YEATS domain containing 2	R1881	0.46	0.048
AK021498		hypothetical protein FLJ36031 *	R1881	0.44	0.027
AK001829		Hypothetical gene *	R1881	0.42	0.027
NM_000844	GRM7	glutamate receptor metabotropic 7	R1881	0.42	0.000
NM_000855	GUCY1A2	guanylate cyclase 1 soluble alpha 2	R1881	0.35	0.048
NM_001741	CALCA	calcitonin/calcitonin-related polypeptide alpha	R1881	0.26	0.027
AF159093		Homo sapiens endogenous retrovirus RAN1 polymerase-like protein (pol)	R1881	0.21	0.048
NM_016318	P2RX2	purinergic receptor P2X ligand-gated ion channel 2	Flutamide	-0.39	0.000

* no approved HUGO symbol / name exist for this entry. If present, gene symbol / name from the UNIGENE database are given in alternative.

Table S2 - List of SAM significant genes (q-value < 0.05) for PC346DCC cell line

GenBank ID	HUGO_Symbol	HUGO_Name	Stimuly	2log ratio	SAM q-value
NM_013284	POLM	polymerase DNA directed mu|polymerase DNA directed mu	R1881	0.56	0.000
NM_006685	SMR3B	submaxillary gland androgen regulated protein 3 homolog B mouse	R1881	0.44	0.000

* no approved HUGO symbol / name exist for this entry. If present, gene symbol / name from the UNIGENE database are given in alternative.

Table S3 - List of SAM significant genes (q-value < 0.05) for PC346Flu1 cell line

GenBank ID	HUGO_Symbol	HUGO_Name	Stimuly	2log ratio	SAM q-value
NM_004117	FKBP5	FK506 binding protein 5	R1881	4.21	0.000
AB042410	GPR88	G protein-coupled receptor 88	R1881	3.04	0.000
NM_006096	NDRG1	N-myc downstream regulated gene 1	R1881	2.60	0.000
AB051826	RHOU	ras homolog gene family member U	R1881	2.54	0.000
NM_020529	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells	R1881	2.39	0.000
AK001478	RHOU	ras homolog gene family member U	R1881	2.29	0.000
NM_018456	EAF2	ELL associated factor 2	R1881	2.28	0.000
AK026383		cDNA clone *	R1881	2.05	0.000
NM_004457	ACSL3	acyl-CoA synthetase long-chain family member 3	R1881	2.04	0.000
NM_003359	UGDH	UDP-glucose dehydrogenase	R1881	1.91	0.000

NM_012081	ELL2	elongation factor RNA polymerase II 2	R1881	1.75	0.000
NM_003543	HIST1H4H	histone cluster 1 H4h	R1881	1.72	0.000
NM_012449	STEAP1	six transmembrane epithelial antigen of the prostate 1	R1881	1.69	0.000
NM_000662	NAT1	N-acetyltransferase 1 arylamine N-acetyltransferase	R1881	1.68	0.000
AF039944	NDRG1	N-myc downstream regulated gene 1	R1881	1.63	0.000
D16875	RHOB	ras homolog gene family member B	R1881	1.61	0.000
NM_006006	ZBTB16	zinc finger and BTB domain containing 16	R1881	1.55	0.000
AK026498	CYP2U1	cytochrome P450 family 2 subfamily U polypeptide 1	R1881	1.50	0.000
AF025771	ZNF189	zinc finger protein 189	R1881	1.50	0.000
AF111849	ELOVL5	ELOVL family member 5 elongation of long chain fatty acids FEN1 Elo2	R1881	1.46	0.000
NM_016590	PART1 *	Prostate androgen-regulated transcript 1 *	R1881	1.45	0.000
NM_006810	PDIA5	protein disulfide isomerase family A member 5	R1881	1.42	0.000
AK024715	FLJ21062 *	Hypothetical protein FLJ21062 *	R1881	1.41	0.000
NM_012062	DNM1L	dynamin 1-like	R1881	1.36	0.000
AK022814	ZBTB10	zinc finger and BTB domain containing 10	R1881	1.34	0.000
AK024850	C2orf31	chromosome 2 open reading frame 31	R1881	1.32	0.000
NM_016166	PIAS1	protein inhibitor of activated STAT 1	R1881	1.31	0.000
AB020637	ENDOD1	endonuclease domain containing 1	R1881	1.26	0.000
AK021627	MORC4	MORC family CW-type zinc finger 4	R1881	1.24	0.000
NM_005079	TPD52	tumor protein D52	R1881	1.22	0.000
NM_003104	SORD *	Sorbitol dehydrogenase *	R1881	1.20	0.000
NM_001530	HIF1A	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	R1881	1.16	0.000
AL137343	FAM84A	family with sequence similarity 84 member A	R1881	1.14	0.000
NM_004595	SMS	spermine synthase	R1881	1.14	0.000
AF188747	KLK2	kallikrein-related peptidase 2	R1881	1.12	0.000
NM_005271	GLUD1	glutamate dehydrogenase 1	R1881	1.11	0.000
AK026813	STEAP2	six transmembrane epithelial antigen of the prostate 2	R1881	1.07	0.000
NM_005461	MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B avian	R1881	1.06	0.000
NM_016050	MRPL11	mitochondrial ribosomal protein L11	R1881	1.05	0.000
AB050049	MCCC2	methylcrotonoyl-Coenzyme A carboxylase 2 beta	R1881	1.05	0.000
NM_019018	FAM105A	family with sequence similarity 105 member A	R1881	1.04	0.000
AK022827	EIF2C3	eukaryotic translation initiation factor 2C 3	R1881	1.03	0.000
AK026375	LOC93622 *	Hypothetical protein BC006130 *	R1881	1.02	0.000
AL157445		cDNA clone *	R1881	1.02	0.007
AB040914	SHROOM3	shroom family member 3	R1881	1.01	0.012
AK024941	DNAJC3	DnaJ Hsp40 homolog subfamily C member 3	R1881	0.99	0.041
AF294628	TWSG1	twisted gastrulation homolog 1 Drosophila	R1881	0.99	0.000
D17210		cDNA clone *	R1881	0.97	0.000
NM_006079	CITED2	Cbp p300-interacting transactivator with Glu Asp-rich carboxy-terminal domain 2	R1881	0.96	0.000
NM_003115	UAP1	UDP-N-acteylglucosamine pyrophosphorylase 1	R1881	0.96	0.000
NM_004156	PPP2CB	Protein phosphatase 2 formerly 2A catalytic subunit beta isoform	R1881	0.94	0.000

NM_002069	GNAI1	Guanine nucleotide binding protein G protein alpha inhibiting activity polipeptide 1	R1881	0.94	0.000
NM_004434	EML1	echinoderm microtubule associated protein like 1	R1881	0.94	0.012
NM_007107	SSR3	signal sequence receptor gamma translocon-associated protein gamma	R1881	0.92	0.005
AK000691	SLC22A23	solute carrier family 22 member 23	R1881	0.90	0.012
NM_014269	ADAM29	ADAM metallopeptidase domain 29	R1881	0.89	0.007
NM_006788	RALBP1	ralA binding protein 1	R1881	0.87	0.028
NM_005627	SGK1	serum/glucocorticoid regulated kinase 1	R1881	0.85	0.000
NM_006697	MTMR11	myotubularin related protein 11	R1881	0.85	0.005
AK025240	ZNRF2	zinc and ring finger 2	R1881	0.84	0.005
NM_001206	KLF9	Kruppel-like factor 9	R1881	0.83	0.000
NM_014109	ATAD2	ATPase family AAA domain containing 2	R1881	0.83	0.000
NM_012152	EDG7	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 7	R1881	0.83	0.000
AK025119	C3orf58	chromosome 3 open reading frame 58	R1881	0.82	0.000
AL080209	CREB3L2	cAMP responsive element binding protein 3-like 2	R1881	0.81	0.022
AL049998	PIK3C2A	phosphoinositide-3-kinase class 2 alpha polypeptide	R1881	0.81	0.000
NM_014174	THYN1	thymocyte nuclear protein 1	R1881	0.81	0.005
NM_001655	ARCN1	archain 1	R1881	0.80	0.005
NM_020474	GALNT1	UDP-N-acetyl-alpha-D-galactosamine: N-acetylgalactosaminyltransferase 1	R1881	0.79	0.000
NM_006343	MERTK	c-mer proto-oncogene tyrosine kinase	R1881	0.79	0.000
NM_003010	MAP2K4	mitogen-activated protein kinase kinase 4	R1881	0.78	0.000
AK027213	BBS10	Bardet-Biedl syndrome 10	R1881	0.77	0.000
M14200	DBI	diazepam binding inhibitor	R1881	0.77	0.028
NM_020188	C16orf61	chromosome 16 open reading frame 61	R1881	0.76	0.005
AK024648	FAM107B	family with sequence similarity 107 member B	R1881	0.76	0.007
NM_020235	BBX	bobby sox homolog Drosophila	R1881	0.76	0.007
AB029018	PDZRN3	PDZ domain containing RING finger 3	R1881	0.75	0.033
AB020676	WWC1	WW and C2 domain containing 1	R1881	0.74	0.000
NM_018037	RALGPS2	Ral GEF with PH domain and SH3 binding motif 2	R1881	0.74	0.007
NM_006516	SLC2A1	solute carrier family 2 facilitated glucose transporter member 1	R1881	0.74	0.005
M60721	HLX	H2.0-like homeobox	R1881	0.73	0.000
NM_000849	GSTM3	glutathione S-transferase M3 brain	R1881	0.73	0.007
AF070670	PPM1A	protein phosphatase 1A formerly 2C magnesium-dependent alpha isoform	R1881	0.72	0.000
M27544	IGF1	insulin-like growth factor 1 somatomedin C	R1881	0.70	0.012
NM_005606	LGMN	legumain	R1881	0.69	0.007
AF052174	BBX	bobby sox homolog Drosophila	R1881	0.69	0.018
NM_018067	MAP7D1	MAP7 domain containing 1	R1881	0.66	0.018
NM_012132	CLDN8	claudin 8	R1881	0.65	0.000
NM_007212	RNF2	ring finger protein 2	R1881	0.65	0.012
NM_013257	SGK3	serum/glucocorticoid regulated kinase family member 3	R1881	0.64	0.000
AK026762	CBLL1	Cas-Br-M murine ecotropic retroviral transforming sequence-like 1	R1881	0.64	0.012
AB020633	FRYL	FRY-like	R1881	0.63	0.018

NM_006323	SEC24B	SEC24 related gene family member B S. cerevisiae	R1881	0.63	0.000
AL080078	TMEM30B	transmembrane protein 30B	R1881	0.63	0.007
AK000122	IQCK	IQ motif containing K	R1881	0.63	0.005
AK027250	HOOK1	hook homolog 1 Drosophila	R1881	0.63	0.012
NM_014384	ACAD8	acyl-Coenzyme A dehydrogenase family member 8	R1881	0.62	0.007
NM_018116	MSTO1	misato homolog 1 Drosophila	R1881	0.62	0.033
AB037765	TXNDC16	thioredoxin domain containing 16	R1881	0.62	0.005
AK026517	EHF	ets homologous factor	R1881	0.62	0.000
NM_019844	SLCO1B3	solute carrier organic anion transporter family member 1B3	R1881	0.61	0.000
NM_014822	SEC24D	SEC24 related gene family member D S. cerevisiae	R1881	0.61	0.007
AL117666	LRIG1	leucine-rich repeats and immunoglobulin-like domains 1	R1881	0.61	0.000
AB032991	NDFIP2	Nedd4 family interacting protein 2	R1881	0.61	0.028
AB018314	PPP1R13B	protein phosphatase 1 regulatory inhibitor subunit 13B	R1881	0.60	0.012
NM_006885	ZFHX3	zinc finger homeobox 3	R1881	0.60	0.018
NM_003389	CORO2A	coronin actin binding protein 2A	R1881	0.60	0.028
AB033040	RNF150	ring finger protein 150	R1881	0.59	0.041
NM_004039	ANXA2	annexin A2	R1881	0.59	0.028
NM_006380	APPBP2	amyloid beta precursor protein cytoplasmic tail binding protein 2	R1881	0.59	0.012
NM_000860	HPGD	hydroxyprostaglandin dehydrogenase 15- NAD	R1881	0.59	0.012
NM_003243	TGFBR3	transforming growth factor beta receptor III	R1881	0.59	0.012
NM_002539	ODC1	ornithine decarboxylase 1	R1881	0.59	0.041
NM_005746	PBEF1	pre-B-cell colony enhancing factor 1	R1881	0.58	0.018
AK001822	ARRDC1	arrestin domain containing 1	R1881	0.58	0.005
AL049969	PDLIM5	PDZ and LIM domain 5	R1881	0.56	0.012
NM_003489	NRIP1	nuclear receptor interacting protein 1	R1881	0.56	0.007
L27479	C9orf61	chromosome 9 open reading frame 61	R1881	0.54	0.000
NM_020233	C17orf48	chromosome 17 open reading frame 48	R1881	0.54	0.005
AK000028		Hypothetical LOC90024 *	R1881	0.54	0.007
NM_018844	BCAP29	B-cell receptor-associated protein 29	R1881	0.52	0.012
NM_003144	SSR1	signal sequence receptor alpha translocon-associated protein alpha	R1881	0.52	0.041
NM_003711	PPAP2A	phosphatidic acid phosphatase type 2A	R1881	0.52	0.041
Y09836		3 UTR of hypothetical protein ORF1 *	R1881	0.52	0.028
AK022884	SGEF *	Src homology 3 domain-containing guanine nucleotide exchange factor *	R1881	0.52	0.012
NM_019891	ERO1LB	ERO1-like beta S. cerevisiae	R1881	0.51	0.005
NM_002245	KCNK1	potassium channel subfamily K member 1	R1881	0.51	0.033
NM_013255	MKLN1	muskelin 1 intracellular mediator containing kelch motifs	R1881	0.51	0.012
NM_007229	PACSIN2	protein kinase C and casein kinase substrate in neurons 2	R1881	0.51	0.007
AF147388	ADAM10	ADAM metallopeptidase domain 10	R1881	0.51	0.041
NM_004315	ASAH1	N-acylsphingosine amidohydrolase acid ceramidase 1	R1881	0.50	0.028
AF052107	MBOAT2	membrane bound O-acyltransferase domain containing 2	R1881	0.49	0.033
AK000776	ROR1	receptor tyrosine kinase-like orphan receptor 1	R1881	0.49	0.012

AF303889	ROPN1	ropporin rhophilin associated protein 1	R1881	0.48	0.012
NM_006117	PECI	peroxisomal D3 D2-enoyl-CoA isomerase	R1881	0.48	0.018
NM_017530	ZNF821	zinc finger protein 821	R1881	0.48	0.041
NM_001695	ATP6V1C1	ATPase H transporting lysosomal 42kDa V1 subunit C1	R1881	0.48	0.041
NM_002210	ITGAV	integrin alpha V vitronectin receptor alpha polypeptide antigen CD51	R1881	0.48	0.000
NM_018455	CENPN	centromere protein N	R1881	0.47	0.022
NM_004040	RHOB	ras homolog gene family member B	R1881	0.46	0.000
S71611		cDNA clone *	R1881	0.46	0.012
NM_006705	GADD45G	growth arrest and DNA-damage-inducible gamma	R1881	0.45	0.012
NM_005853	IRX5	iroquois homeobox 5	R1881	0.45	0.022
AK023844	GRHL2	grainyhead-like 2 Drosophila	R1881	0.45	0.007
AB040936	KIAA1503 *	Similar to KIAA1503 protein *	R1881	0.45	0.033
NM_002553	ORC5L	origin recognition complex subunit 5-like yeast	R1881	0.44	0.005
AK023249	PLEKHF2	Pleckstrin homology domain containing family F with FYVE domain member	R1881	0.43	0.012
NM_004578	HRES1	HTLV-1 related endogenous sequence	R1881	0.43	0.012
NM_004226	STK17B	serine/threonine kinase 17b	R1881	0.42	0.005
NM_004267	CHST2	carbohydrate N-acetylglucosamine-6-O sulfotransferase 2	R1881	0.42	0.005
NM_017992	EDEM3	ER degradation enhancer, mannosidase alpha-like 3	R1881	0.41	0.033
AF294278	PRDM16	PR domain containing 16	R1881	0.40	0.007
NM_002736	PRKAR2B	protein kinase cAMP-dependent regulatory type II beta	R1881	0.40	0.033
AF038174	C9orf91	chromosome 9 open reading frame 91	R1881	0.40	0.012
AL049471	ARID5B	AT rich interactive domain 5B MRF1-like	R1881	0.39	0.033
AF124819	TM9SF3	transmembrane 9 superfamily member 3	R1881	0.39	0.022
NM_014584	ERO1L	ERO1-like S. cerevisiae	R1881	0.37	0.033
NM_005648	TCEB1	transcription elongation factor B SIII polypeptide 1 15kDa elongin C	R1881	0.37	0.033
NM_001609	ACADSB	acyl-Coenzyme A dehydrogenase short/branched chain	R1881	0.37	0.033
NM_017694	FLJ20160 *	FLJ20160 protein *	R1881	0.35	0.022
NM_000514	GDNF	glial cell derived neurotrophic factor	R1881	0.35	0.012
AJ250475	EPDR1	ependymin related protein 1 zebrafish	R1881	0.35	0.012
NM_020182	TMEPAI	transmembrane prostate androgen induced RNA	R1881	0.34	0.012
AK025772	RABL3	RAB member of RAS oncogene family-like 3	R1881	0.34	0.028
NM_014666	CLINT1	clathrin interactor 1	R1881	0.33	0.022
NM_005300	GPR34	G protein-coupled receptor 34	R1881	0.33	0.018
AK026239	FAM118A	family with sequence similarity 118 member A	R1881	0.32	0.012
NM_001284	AP3S1	adaptor-related protein complex 3 sigma 1 subunit	R1881	0.32	0.041
NM_018366	CNO	cappuccino homolog mouse	R1881	0.32	0.041
NM_013332	HIG2 *	Hypoxia-inducible protein 2 *	R1881	0.31	0.033
NM_002806	PSMC6	proteasome prosome macropain 26S subunit ATPase 6	R1881	0.31	0.033
AF075098	LTBP1	latent transforming growth factor beta binding protein 1	R1881	0.31	0.022
AK024586	TNC	tenascin C hexabrachion	R1881	0.29	0.033
NM_014664	N4BP1 *	Nedd4 binding protein 1 *	R1881	0.29	0.028

AK022331	FAM152A	family with sequence similarity 152 member A	R1881	0.28	0.012
NM_000446	PON1	paraoxonase 1	R1881	0.27	0.012
U79249	CCDC52	coiled-coil domain containing 52	R1881	0.27	0.012
NM_000815	GABRD	gamma-aminobutyric acid GABA A receptor delta	R1881	0.26	0.028
NM_016262	TUBE1	tubulin epsilon 1	R1881	0.26	0.018
NM_000068	CACNA1A	calcium channel voltage-dependent P/Q type alpha 1A subunit	R1881	0.25	0.007
NM_012318	LETM1	leucine zipper-EF-hand containing transmembrane protein 1	R1881	0.25	0.022
AK027235	CRTAP	cartilage associated protein	R1881	0.25	0.012
NM_004147	DRG1	developmentally regulated GTP binding protein 1	R1881	0.24	0.033
AF116709		Homo sapiens PRO2605 mRNA, complete cds *	R1881	0.24	0.033
S75763	FUS	fusion involved in t 12 16 in malignant liposarcoma	R1881	0.22	0.012
NM_017617	NOTCH1	Notch homolog 1 translocation-associated Drosophila	R1881	0.22	0.028
AK027219		CDNA FLJ23566 fis clone LNG10880 *	R1881	0.21	0.000
AF070620		cDNA clone *	R1881	0.21	0.012
AF085985		Full length insert cDNA clone YU03C05 *	R1881	0.20	0.033
NM_006891	CRYGD	crystallin gamma D	R1881	0.18	0.033
AF264717	MTMR4	myotubularin related protein 4	R1881	0.18	0.041
AF147337		cDNA clone *	R1881	-0.41	0.022
AF075080		Full length insert cDNA YQ80D07 *	R1881	-0.43	0.022
NM_017791	FLVCR2	feline leukemia virus subgroup C cellular receptor family member 2	R1881	-0.46	0.041
NM_014459	PCDH17	protocadherin 17	R1881	-0.47	0.000
AB046768	EPB41L5	erythrocyte membrane protein band 4.1 like 5	R1881	-0.51	0.008
NM_018103	LRRC8D	leucine rich repeat containing 8 family member D	R1881	-0.53	0.000
NM_016641	MIR16 *	Membrane interacting protein of RGS16 *	R1881	-0.54	0.018
NM_015556	SIPA1L1	signal-induced proliferation-associated 1 like 1	R1881	-0.54	0.033
NM_006113	VAV3	vav 3 guanine nucleotide exchange factor	R1881	-0.55	0.041
NM_001913	CUX1	cut-like homeobox 1	R1881	-0.55	0.041
NM_004760	STK17A	serine/threonine kinase 17a	R1881	-0.56	0.018
NM_000675	ADORA2A	adenosine A2a receptor	R1881	-0.65	0.033
NM_003897	IER3	immediate early response 3	R1881	-0.67	0.013
NM_003311	PHLDA2	pleckstrin homology-like domain family A member 2	R1881	-0.77	0.041
AF113132	PSAT1	phosphoserine aminotransferase 1	R1881	-0.78	0.000
NM_003943	STBD1	starch binding domain 1	R1881	-0.79	0.022
NM_002165	ID1	inhibitor of DNA binding 1 dominant negative helix-loop-helix protein	R1881	-0.81	0.018
NM_006769	LMO4	LIM domain only 4	R1881	-0.82	0.000
NM_012446	SSBP2	single-stranded DNA binding protein 2	R1881	-0.84	0.018
NM_004577	PSPH	phosphoserine phosphatase	R1881	-0.85	0.041
D28449	ID3	inhibitor of DNA binding 3 dominant negative helix-loop-helix protein	R1881	-0.88	0.000
AK026945	TRIB3	tribbles homolog 3 Drosophila	R1881	-0.92	0.028
AK026288	ATHL1	ATH1 acid trehalase-like 1 yeast	R1881	-0.96	0.008
NM_012342	BAMBI	BMP and activin membrane-bound inhibitor homolog Xenopus laevis	R1881	-0.98	0.000

U90907	PIK3R3	phosphoinositide-3-kinase regulatory subunit 3 p55 gamma	R1881	-1.01	0.008
NM_004864	GDF15	growth differentiation factor 15	R1881	-1.10	0.033
AL133074	TP53INP1	tumor protein p53 inducible nuclear protein 1	R1881	-1.25	0.000
X69111	ID3	inhibitor of DNA binding 3 dominant negative helix-loop-helix protein	R1881	-1.25	0.000
AF075110	C14orf4	chromosome 14 open reading frame 4	R1881	-1.29	0.000
NM_004316	ASCL1	achaete-scute complex homolog 1 Drosophila	R1881	-1.42	0.018
AF205437	TRIB1	tribbles homolog 1 Drosophila	R1881	-1.63	0.000

* no approved HUGO symbol / name exist for this entry. If present, gene symbol / name from the UNIGENE database are given in alternative.

Table S4 - List of SAM significant genes (q-value < 0.05) for PC346Flu2 cell line

GenBank ID	HUGO_Symbol	HUGO_Name	Stimuly	2log ratio	SAM q-value
AB042410	GPR88	G protein-coupled receptor 88	Flutamide	1.57	0.000
AK001478	RHOU	ras homolog gene family member U	R1881	1.05	0.000
AK026813	STEAP2	six transmembrane epithelial antigen of the prostate 2	R1881	0.74	0.000
NM_002923	RGS2	regulator of G-protein signaling 2 24kDa	R1881	0.73	0.000
NM_002923	RGS2	regulator of G-protein signaling 2 24kDa	Flutamide	0.57	0.000
NM_003676	DEGS	degenerative spermatocyte homolog 1 lipid desaturase Drosophila	Flutamide	0.67	0.000
NM_019844	SLCO1B3	solute carrier organic anion transporter family member 1B3	Flutamide	0.63	0.000
NM_001206	KLF9	Kruppel-like factor 9	Flutamide	0.47	0.000
AK002088	UBE2E3	ubiquitin-conjugating enzyme E2E 3 UBC4/5 homolog yeast	Flutamide	0.40	0.000

* no approved HUGO symbol / name exist for this entry. If present, gene symbol / name from the UNIGENE database are given in alternative.

Chapter 5

Bypass Mechanisms of the Androgen Receptor Pathway in PC346 Hormone-Refractory Prostate Cancer Cells

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ABSTRACT

Background: Prostate cancer is at its onset dependent on androgens for survival and growth, making androgen ablation/blockade the cornerstone treatment for invasive tumors. However, despite initial remission, the cancer will inevitably recur as hormone-refractory disease.

Objective: The aim of this study is to investigate how androgen-dependent prostate cancer cells eventually survive and resume growth under androgen-deprived and antiandrogen supplemented conditions, with focus on genes particularly involved in the bypass of the androgen-receptor (AR) pathway.

Methods: For this purpose, microarray technology was used to analyze the differential expression pattern between androgen-responsive and hormone-refractory prostate cancer cell lines. As model system we used the androgen-responsive PC346C cells and its derivative castration-resistant sublines: PC346DCC (very low AR levels), PC346Flu1 (AR overexpressed) and PC346Flu2 (T877A mutated AR). VAV3, TWIST1 and DKK3 were selected for further quantification by specific real-time PCR, in an independent set of prostate cancer samples.

Results: Microarray analysis revealed 487 transcripts differentially expressed between the androgen-responsive and the castration-resistant cell lines. Most of these genes were common to all three hormone-refractory sublines and only a minority (~5%) was AR-regulated target genes. Pathway analysis revealed enrichment in functions involving cellular movement, cellular growth/proliferation and cell death, as well as association with cancer and reproductive system disease. PC346DCC presented the highest number (276) of differentially expressed genes and a significant down-regulation of AR target genes (p-value 10^{-7}), supporting the hypothesis that this subline bypassed the AR pathway. Among these, VAV3 and TWIST1 oncogenes were overexpressed, whereas the expression of the tumor-suppressor DKK3 was repressed. Subsequent validation in human prostate tumor samples confirmed deregulation of these genes during prostate cancer progression.

Conclusions: The large overlap of differentially-expressed genes between all castration-resistant sublines suggests an ultimate convergence to similar growth enhancing adaptations, despite the different AR modifications. PC346Flu1 and PC346Flu2 acquired this hormone-refractory growth by adaptation of the AR pathway, whereas PC346DCC activated alternative survival/growth pathways through down-regulation of the apoptosis-promoting DKK3 gene and overexpression of TWIST1 and VAV3 proto-oncogenes. Furthermore, these genes were deregulated in clinical samples of prostate tumors and lymph-node metastasis, making them potential disease markers and interesting targets for novel therapeutical approaches.

INTRODUCTION

Prostate cancer is the second leading cause of male cancer deaths in the Western countries and an increasing problem in those adopting Western lifestyle and diet [1]. Advances in screening and diagnosis have allowed the detection of tumors at earlier stages, when curative therapy is still feasible [2]. For late stage disseminated disease however, current therapies are merely palliative and no curative treatment exists. Since the growth of prostate tumors is originally androgen-dependent, metastatic cancers are generally treated with androgen ablation therapy, with or without antiandrogen supplementation [2]. The vast majority of these patients show a significant (12-18 months) clinical regression, but the cancer eventually escapes androgen suppression and recurs as incurable hormone-refractory condition [3,4]. To survive and resume growth in an androgen-deprived environment prostate cancer cells must either adapt the AR pathway to the androgen-depleted conditions or invoke alternative survival and growth pathways [5]. Much experimental evidence exists to support both mechanisms, which are not necessarily mutually exclusive. AR expression was shown to be maintained in the majority of patients that underwent hormonal therapy and showed recurrence of disease, suggesting a role of the AR also in late stage disease [6,7]. Moreover, the AR gene is amplified and/or overexpressed in about 30% of the hormone-refractory tumors, and it has been proposed this could sensitize the receptor for the residual androgen concentrations present under hormonal therapies [8,9,10]. Furthermore, several AR mutations, resulting in increased activity or broadened ligand-specificity to alternative steroids and antiandrogens, have been associated with disease progression [11,12]. Other modifications of the AR pathway that may induce hormone-refractory growth include intratumoral steroidogenesis, ligand-independent activation by cross-talk with other signaling pathways, alterations in the balance of AR co-regulators or expression of constitutively active truncated AR isoforms [5,13,14]. Interestingly, recent work from others and us has revealed that the AR pathway may be selectively attenuated in advanced/metastatic disease [15,16,17]. Since the AR pathway is also involved in the processes of cellular differentiation and prostate maturation, it is tempting to suggest that prostate cancer cells may eventually gain growth advantage by inhibiting the AR induced differentiation. Prompted by these results, we focused the present study on alternative survival and growth pathways, which are independent of androgen-receptor activation. To effectively bypass the AR pathway, cancer epithelial cells must be able to survive the apoptotic signals triggered by hormonal therapies and invoke alternative growth pathways. Autocrine production of growth factors or its receptors, activation of oncogenes and inhibition of tumor-supressor genes are all possible mechanisms for bypassing the AR pathway. Consistent with this hypothesis, paracrine growth factors that are normally secreted by prostate stroma cells, such as EGF, IGFI, HGF, KGF or IL6, are found to be overexpression in hormone-refractory cancer in association with a switch to autocrine production by cancer epithelial cells [18]. In addition to being potential mitogens, mounting evidence indicates that these growth hormones are also able to cross-talk with the AR signaling pathway, leading to expression of AR target genes in the absence of androgens [19]. Therefore, it still has to be established whether the autocrine production of these growth factors represents an

adaptation or a true bypass of the AR signaling pathway. Alterations in the anti-apoptotic BCL2 oncogene and in the pro-apoptotic P53 and PTEN tumor-suppressor genes have also been found in prostate cancer [20,21]. However, these events mostly occur earlier in prostate progression, making them less likely candidates for the switch to hormone-refractory growth in late stage disease. Nevertheless, by inhibiting prostate cancer cell death and shifting the balance towards cellular proliferation, genes involved in the regulation of apoptosis may also play a role in hormone-refractory growth.

To explore the mechanism(s) by which androgen-dependent prostate cancer cells become resistant to hormonal therapy, we used microarray technology to interrogate the differences in gene expression between androgen-responsive and castration-resistant cell lines. As model system we used the androgen-responsive PC346C cell line and its hormone-refractory sublines PC346DCC, PC346Flu1 and PC346Flu2. These sublines were derived from the parental PC346C by long-term androgen ablation (PC346DCC), supplemented with the antiandrogen hydroxyflutamide (PC346Flu1 and PC346Flu2). Previous studies revealed distinct AR modifications in all three castration-resistant sublines, which corresponded to diverse mechanism of hormone-refractory growth. Whereas PC346DCC, expressing very low levels of AR and PSA, showed evidence of bypassing of the AR pathway, PC346Flu1 exhibited 4-fold AR up-regulation and PC346Flu2 was shown to carry the T877A AR mutation. Both PC346Flu1 and PC346Flu2 sublines replicate the progression to hormone-refractory disease through adaptations of the AR pathway. Therefore, we focused on the PC346DCC subline to select for genes particularly involved in the bypass of the AR pathway. In addition to providing novel insights into the mechanisms of prostate cancer progression, the genes identified here may prove useful as prognostic markers and potential targets for novel therapeutical approaches.

MATERIALS AND METHODS

Reagents and cell lines

The basic culture medium used in the maintenance of PC346 cell lines consisted of DMEM-F12 medium (Cambrex BioWhitaker, Belgium) supplemented with 2% fetal calf serum (FCS; PAN Biotech GmbH, Aidenbach, Germany), 1% insulin-transferrin-selenium (Gibco BRL), 0.01% bovine serum albumin (Boehringer Mannheim, Germany), 10 ng/ml epidermal growth factor (Sigma-Aldrich), penicillin/streptomycin antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin; BioWhitaker, Belgium); plus the following additions: 100 ng/ml fibronectin (Harbor Bio-Products, Tebu-bio, The Netherlands), 20 mg/ml fetuine (ICN Biomedicals, The Netherlands), 50 ng/ml choleratoxin, 0.1 mM phosphoethanolamine, 0.6 ng/ml triiodothyronine and 500 ng/ml dexametason (all from Sigma). PC346C cells were maintained in culture in the complete medium described above, supplemented with 0.1 nM 17-methyltrienolone (R1881; NEN, Boston MA, USA). PC346DCC selection medium was supplemented as described above, but depleted from androgens by using dextran-coated charcoal (DCC) treated FCS. PC346Flu1 and PC346Flu2 culture medium was also androgen depleted by using 2% DCC-FCS, and supplemented with 1 μM of hydroxyflutamide (OH-flutamide, Schering-Plough Research Institute, New Jersey, USA).

Cells were grown in T25 Primaria™ tissue culture flasks (BD Biosciences Benelux N.V, The Netherlands) at 37°C under 5% CO_2 humidified atmosphere.

Expression microarray analysis

Cells were seeded in their respective selection medium to reach ~50% confluency and allowed to grow for 2 days. Then, cells were rinsed twice with PBS and stored at -20°C until RNA isolation. Total RNA was isolated with RNAzol B reagent (Campro Scientific, Veenendaal, The Netherlands) and further purified through RNeasy columns (Qiagen) with on-column DNA digestion, according to the manufacturer's protocol. RNA quality was checked on 1% agarose gel.

Cy3- or Cy5-labelled RNA probes were produced by incorporating amino-allyl UTP during RNA amplification, followed by coupling to N-hydroxysuccinimide modified dye. Briefly, 3 μg RNA was used for a T7-based linear mRNA amplification protocol, described previously [22]. Amino-allyl UTP, plus equal amount of unmodified rUTP, was incorporated into aRNA with T7 Megascript Kit (all from Ambion), according to manufacturer's protocol. Amplified RNA was purified and concentrated using Microcon-YM 30 columns (Amicon®) to rinse three times with 300 μl RNAse-free water. Finally, 2 μg aminoallyl-modified RNA, in a maximum of 3.33 μl of RNAse-free water, was incubated with 1.66 μl sodium bicarbonate buffer (0.3 M, pH 9) and 5 μl Cy3- or Cy5-dye (CyScribe Post-Labeling Kit, Amersham, NJ, USA), for 1h in the dark at room temperature. Reaction was stopped with 5 μl 4 M hydroxylamine HCl (Sigma), contra-labelled probes were combined and purified/concentrated using Microcon-YM 30. Probe was collected in 5-15 μl final volume and resuspended in 80 μl Ambion hybridization buffer number 1.

For the microarray we used double-dye oligoarrays representing about 15,000 human genes, on which labelled RNA from the each hormone-refractory subline was cohybridized with contra-labelled PC346C. Four microarrays were performed per condition, using two distinct cell passages in dye-swap. This was done to account for the biological variability and to exclude dye-preferential binding to oligonucleotides on the microarray. The oligoarrays used in this study were produced at the Erasmus Center for Biomics. Briefly, a human 18,584 oligonucleotides library (Compugen, Sigma-Genosys) was spotted on aminosilane slides using a Virtek Chipwriter Professional arrayer (Virtek Vision International, Waterloo, Canada). Control spots included landmarks, spotting buffer, alien oligonucleotides (SpotReport Alien Oligo Array, La Jolla, Stratagene), poly d[A]40-60, salmon sperm DNA, and human COT-1 DNA. Before the hybridization, microarray slides were prehybridized in 5x SSC, 0.05% SDS, 4% BSA solution for 30 min at 45°C, washed twice with RNAse-free water for 2 min, rinsed with isopropanol and spin-dried for 3 min at 1500g. Microarray hybridizations were performed overnight at 45°C, with continuous agitation, in a HS4800 Hybridization Station (Tecan Benelux BV). Finally, the arrays were washed automatically in the Hybridization Station using: 2x SSC/0.05% SDS (at 45°C), 1x SSC and 0.2x SSC (at room temperature), and dried under a stream of N2, before scanning.

Data extraction and analysis

Arrays were scanned in a ScanArray Express HT scanner (Perkin Elmer, Nederland BV) and spot intensities were quantified using Imagene software (Bio Discovery Inc, El

Sequndo, CA, USA). To balance Cy3 and Cy5 spot intensities, Loewess normalization per subarray was performed using limma-package (http://bioinf.wehi.edu.au/limma/) from Bioconductor (http://www.bioconductor.org) [23,24]. To scale between arrays, the global median intensity per array was set at 1000. Dye intensities below 200 were then thresholded at 200, to minimize noise and make fold-change on the low-intensity range more robust against outliers. Spots with intensities below the threshold (200) for both Cy3 and Cy5 channels in more than 2 of the 4 arrays performed per subline were excluded from the analysis. Sample to reference ratios were then calculated and 2log transformed. Spots that showed opposite effects for the dye-swap/biological replicates were excluded from further analysis; effects were called opposite if the mean 2log ratio for the per subline were $\geq$ 0,5 for one dye and below $\leq$-0,5 for the dye-swap. Following normalization and all the above-mentioned quality controls, the 2log intensity ratios were averaged for the replicates of each subline. This data was stored in SRS7 (Sequence Retrieval System version 7, Lion Bioscience AG, Heidenberg, Germany), which was also used for the comparisons with other previously published/publicly available databases [25]. Hierarchical clustering and data visualization was performed using Cluster and TreeView programs (Eisen Labs: http://rama.lbl.gov). Significance Analysis of Microarrays (SAM; http://www-stat.stanford.edu/~tibs/SAM) was used to determine which genes were statistically different between stimulated samples and non-stimulated references. Gene ontology clustering was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID: http://david.abcc.ncifcrf.gov) [26,27]. The pathway and functional analyses were generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com).

cDNA synthesis and RT- PCR analysis

Normal and tumor specimens from patients used for quantitative real-time RT-PCR analysis were obtained from the frozen tissue bank of the Erasmus Medical Center (Rotterdam, the Netherlands). The specimens were collected between 1984 and 2001. The experimental protocols were approved by the Erasmus MC Medical Ethics Committee according to the Medical Research Involving Human Subjects Act. Additional information about these specimens was provided previously [28]. Total RNA was isolated as described above and cDNA was synthesized using MMLV-reverse transcriptase kit and Oligo(dT)$_{12\text{-}18}$ primer (Invitrogen), according to manufacturer's protocol. cDNA samples were stored at -20°C. TaqMan real-time PCR analysis was performed in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), using AmpliTaq Gold DNA polymerase (Applied Biosystems), according to manufacturer's specifications. Validated primers and probes from TaqMan Gene Expression Assays (Applied Biosystems) were used for quantification of VAV3 (Hs00916821_m1), TWIST1 (Hs00361186_m1), DKK3 (Hs00951307_m1) and GAPDH (Hs99999905_m1), according to the PCR settings provided by Applied Biosystems. PBGD was quantified using 0.33 μM of primers forward: CATGTCTGGTAACGGCAATG and reverse: GTACGAGGCTTTCAATGTTG primers, in Power SybrGreen PCR Master Mix (Applied Biosystems), according to thermocycling protocol recommended by the manufacturer. Transcript quantities for each sample were normalized against the average of two endogenous references and relative to a calibrator. The two housekeeping genes used as endogenous references were PBGD and GAPDH; a

mixture of cDNAs from prostate carcinoma xenografts was used as the calibrator. Graphs and statistics were performed with GraphPad Prism (version 3.0). P-values < 0.05 were considered significant.

RESULTS

Microarray profile of differentially expressed genes between the androgen-responsive PC346C and its hormone-refractory sublines PC346DCC, PC346Flu1 and PC346Flu2

Expression array analysis was performed to explore whether the AR pathway is still active in the hormone-refractory cells under androgen-deprived conditions and identify putative alternative growth/survival pathways. Each of the hormone-refractory sublines were cultured in their respective selection medium (steroid-stripped medium for PC346DCC, supplemented with 1 μM OH-Flutamide for PC346Flu1 and Flu2) and hybridized on the microarrays, together with the parental androgen-responsive PC346C (cultured in complete medium supplemented with 0.1 nM R1881). To account for the biological variability and dye-preferential binding to oligonucleotides on the microarray, four arrays were performed per condition, using two independent cell passages in dye-swap. Variation in expression pattern was analysed per hormone-refractory subline, and spots were considered to be differentially expressed if the absolute 2log ratio ≥ 0.5 (ratio ≥ 1.42 or ≤ 0.71) for at least three of the 4 arrays and for the average of all 4 arrays. According to these criteria, there were a total of 487 differentially regulated transcripts in the castration-resistant sublines compared to androgen-sensitive PC346C, most of which were overlapping all three refractory sublines (Fig. 1). With 276 differentially regulated transcripts, PC346DCC showed the strongest divergence from the parental line, whereas PC346Flu2 revealed the least alterations (127 transcripts). Significance Analysis of Microarrays (SAM) was used to determine statistical significance of the selected genes and, at a 5% false discovery rate, 392 of the 487 (80%) selected reached statistical significance. A list of the top 100 differentially expressed genes, respective expression ratios and statistical analysis is presented in Table 1. Interestingly, a considerable proportion (64/487) of the differentially regulated genes clustered at distinct genomic locations on chromosomes 4, 5, 6, 8, 11 and 18 (Table 2). A comprehensive list of all the regulated genes per cell line is provided in Supplementary Tables S1 to S3.

AR pathway is down-regulated in PC346DCC

To investigate the activation state of the AR pathway in the PC346DCC, PC346Flu1 and PC346Flu2, the SRS database was used to link and compare our present data with a previously established androgen-response gene signature (Fig. 2A). The androgen-response signature of the PC346 cell lines consisted of 114 AR target genes, which are listed in Supplementary Table S4. Of the 487 differentially-regulated transcripts in the castration-resistant sublines, only 27 were AR target genes (<6%), indicating that other genes and pathways are also involved in hormone-refractory proliferation of these sublines. Furthermore, AR target genes were down-regulated in PC346DCC (p-value = 10^{-7}), whereas their expression in PC346Flu1 and PC346Flu2 was not significantly affected (Fig. 2B). However, although not statistically significant for the AR pathway as a

whole, PC346Flu1 and PC346Flu2 did show lower induction of some androgen-responsive genes such as KLK2, STEAP1, STEAP2 and EHF.

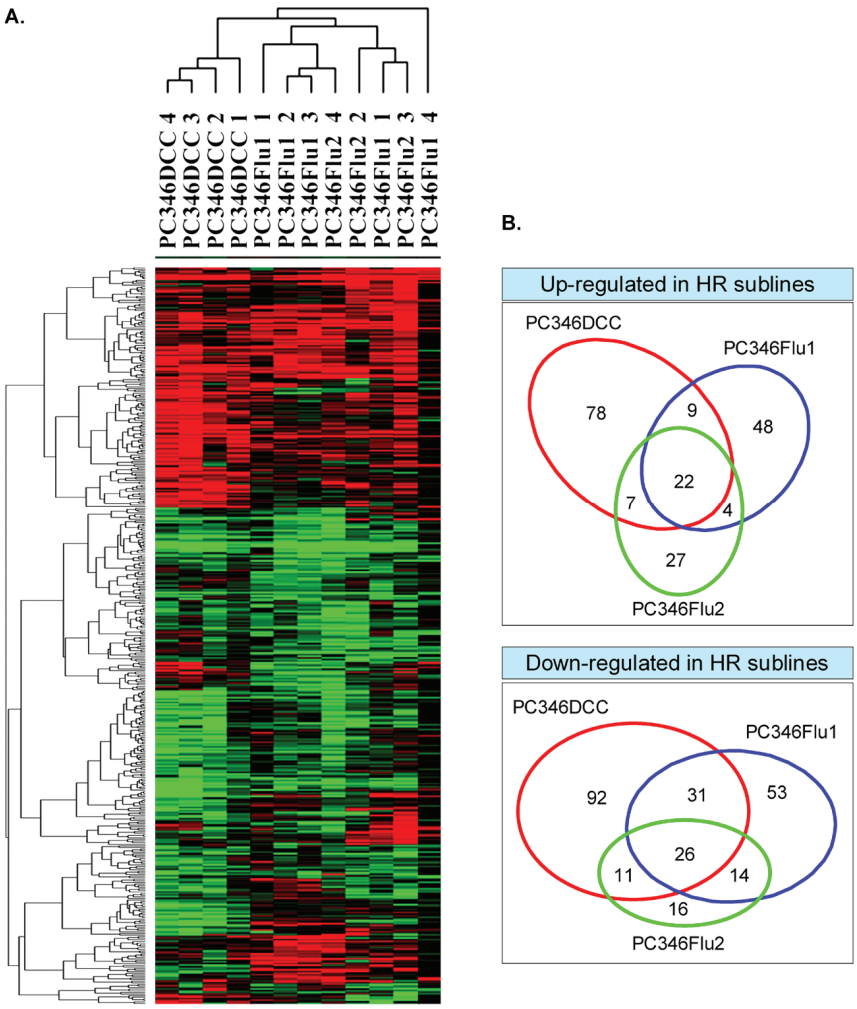

Figure 1 - Differentially-expressed genes in PC346DCC, PC346Flu1 and PC346Flu2 sublines compared to the androgen-responsive PC346C. PC346C was cultured in complete medium with 0.1 nM R1881, whereas the hormone-refractory sublines were culture in dextran-coated charcoal stripped medium (PC346DCC), supplemented with 1 M of the antiandrogen hydroxyflutamide (PC346Flu1 and PC346Flu2). A) Heat-map representation: red and green colors represent up-regulation and down-regulation, respectively, whereas black indicates no difference between sublines and parental PC234C cells. Grey squares indicate missing data, either due to low expression levels, poor data quality or absence of probes for the respective transcript in the array platform used for the study. B) Venn-diagram of the number of regulated genes in the different sublines.

Table 1 - Top 100 differentially expressed genes, expression ratios and SAM q-values

GenBank_Acc	Cytoband	HUGO_symbol	HUGO_Gene Name	PC346DCC		PC346Flu1		PC346Flu2	
				2log ratio	qvalue	2log ratio	qvalue	2log ratio	qvalue
NM_004982	12p11.23	KCNJ8	potassium inwardly-rectifying channel subfamily J member 8	2.7	0.000	1.2	0.000	1.9	0.000
NM_000790	7p11	DDC	dopa decarboxylase aromatic L-amino acid decarboxylase	1.9	0.005	0.1	0.253	2.4	0.218
NM_013452	Xp22	VCX	variable charge X-linked	2.3	0.000	1.6	0.015	1.7	0.049
NM_014269	4q34	ADAM29	ADAM metallopeptidase domain 29	1.5	0.000	1.4	0.011	2.2	0.000
NM_004750	19p12	CRLF1	cytokine receptor-like factor 1	2.1	0.000				
NM_006113	1p13.3	VAV3	vav 3 guanine nucleotide exchange factor	2.0	0.000	0.2	0.031	0.4	0.218
NM_003226	21q22.3	TFF3	trefoil factor 3 intestinal	1.9	0.000	-0.5	0.211	1.5	0.049
NM_012463	12q24.31	ATP6V0A2	ATPase H transporting lysosomal V0 subunit a2	0.8	0.028	1.2	0.051	1.7	0.000
AB037810	1q42.2	SIPA1L2	signal-induced proliferation-associated 1 like 2	0.9	0.046	1.5	0.000	1.7	0.049
NM_001823	14q32	CKB	creatine kinase brain	1.7	0.000	1.5	0.015	1.6	0.000
NM_006332	19p13.1	IFI30	interferon gamma-inducible protein 30	1.7	0.000	1.1	0.025	0.7	0.218
NM_001072	2q37	UGT1A1	UDP glucuronosyltransferase 1 family polypeptide A1	0.2	0.380	1.7	0.015	-0.1	0.714
NM_016084	17p11.2	RASD1	RAS dexamethasone-induced 1	1.6	0.000	1.2	0.000	0.5	0.218
NM_005794	14q11.2	DHRS2	dehydrogenase/reductase SDR family member 2	1.4	0.003	1.5	0.071	1.2	0.051
U58096	Yp11.2	TSPY1	testis specific protein Y-linked 1	1.0	0.037	1.4	0.000		
NM_013253	11p15.2	DKK3	dickkopf homolog 3 Xenopus laevis	-0.6	0.005	1.4	0.000	0.7	0.218
NM_005804	19p13.12	DDX39	DEAD Asp-Glu-Ala-Asp box polypeptide 39	1.4	0.000	0.3	0.253	0.8	0.194
NM_006721	10q22	ADK	adenosine kinase	1.4	0.000	0.1	0.422	0.7	0.194
AK026892	22q13.31	CERK	ceramide kinase	1.4	0.000	0.0	0.321	0.5	0.218
AL049949	10q22.3	C10orf56	chromosome 10 open reading frame 56	1.3	0.000	0.5	0.048	0.4	0.218
NM_016639	16p13.3	TNFRSF12A	tumor necrosis factor receptor superfamily member 12A	0.6	0.028	1.3	0.000	0.5	0.218
NM_001902	1p31.1	CTH	cystathionase cystathionine gamma-lyase	1.3	0.020	0.5	0.044	0.7	0.194
S67154	3q28	EIF4A2	eukaryotic translation initiation factor 4A isoform 2	0.1	0.589	1.3	0.021	-0.5	0.101
NM_003516	1q21.2	HIST2H2AA3	histone cluster 2 H2aa3	-0.1	0.645	1.2	0.000	0.8	0.194
NM_003712	19p13	PPAP2C	phosphatidic acid phosphatase type 2C	1.2	0.000	0.9	0.044	1.2	0.000
Y09836		ORF*	3 UTR of hypothetical protein ORF1*	0.7	0.046	1.0	0.011	1.2	0.101
L07383	3p21.3	RPSA	ribosomal protein SA	0.1	0.513	1.2	0.071		
NM_005311	7p12-p11.2	GRB10	growth factor receptor-bound protein 10	1.1	0.028	0.7	0.008	0.5	0.218
Y11158	16p13.3	SNORA10	small nucleolar RNA H/ACA box 10	-0.1	0.513	1.1	0.000	0.5	0.218
AF086251	11q21	SESN3	sestrin 3	0.1	0.534	1.1	0.048	1.1	0.049
NM_014214	18p11.2	IMPA2	inositol myo -1 or 4 -monophosphatase 2	0.3	0.242	0.0	0.515	1.1	0.000
NM_003234	3q29	TFRC	transferrin receptor p90 CD71	1.1	0.000	0.8	0.025	0.4	0.218
U90878	10q22-q26.3	PDLIM1	PDZ and LIM domain 1 elfin	1.0	0.000	0.4	0.135	0.4	0.287
NM_003243	1p33-p32	TGFBR3	transforming growth factor beta receptor III	1.0	0.000	0.1	0.287	0.5	0.236
NM_014061	Xp11.22	MAGEH1	melanoma antigen family H 1	1.0	0.020	-0.5	0.090	0.0	0.725

NM_002795	17q12	PSMB3	proteasome prosome macropain subunit beta type 3	0.5	0.113	0.6	0.063	1.0	0.000
NM_001814	11q14.2	CTSC	cathepsin C	1.0	0.000				
NM_016192	2q32.3	TMEFF2	transmembrane protein with EGF-like and two follistatin-like domains 2	0.0	0.645	0.3	0.115	1.0	0.049
AF190900	1q32.1	KLHL12	kelch-like 12 Drosophila	0.2	0.466	0.7	0.000	1.0	0.194
NM_001327	Xq28	CTAG1A	cancer/testis antigen 1A	0.6	0.046	0.9	0.000	0.6	0.194
NM_004282	6p12.3-p11.2	BAG2	BCL2-associated athanogene 2	0.9	0.020	0.4	0.044	0.7	0.194
NM_003524	6p21.3	HIST1H2BH	histone cluster 1 H2bh	0.2	0.380	0.9	0.000	0.2	0.384
NM_018303	6p25.3	EXOC2	exocyst complex component 2	0.9	0.000	0.2	0.253	0.2	0.636
AK021498	7q22.3	FLJ36031*	Hypothetical protein FLJ36031*	0.9	0.028	-0.1	0.321	0.1	0.755
AF200348	2p25	PXDN	peroxidasin homolog Drosophila	-1.2	0.003	-1.3	0.000	0.9	0.064
AL157449	17q21.33	PPP1R9B	protein phosphatase 1 regulatory inhibitor subunit 9B	0.9	0.007	0.9	0.000	0.6	0.218
NM_001262	1p32	CDKN2C	cyclin-dependent kinase inhibitor 2C p18 inhibits CDK4	0.5	0.211	0.1	0.459	0.9	0.194
NM_001831	8p21-p12	CLU	clusterin	0.1	0.534	0.9	0.000	0.7	0.049
NM_003311	11p15.5	PHLDA2	pleckstrin homology-like domain family A member 2	0.0	0.624	0.9	0.011	0.8	0.194
NM_000213	17q25	ITGB4	integrin beta 4	0.9	0.086	-1.3	0.000	0.1	0.777
NM_002165	20q11	ID1	inhibitor of DNA binding 1 dominant negative helix-loop-helix protein	-1.2	0.018			-0.6	0.500
AK024495	11p15.5	LRRC56	leucine rich repeat containing 56	-1.2	0.000	0.2	0.321	0.5	0.218
NM_017860	1q21.2	C1orf56	chromosome 1 open reading frame 56	-0.5	0.037	-1.2	0.000	-0.6	0.053
NM_004457	2q34-q35	ACSL3	acyl-CoA synthetase long-chain family member 3	-1.2	0.009	-0.7	0.025	-0.3	0.101
NM_005544	2q36	IRS1	insulin receptor substrate 1	-1.2	0.020	-0.1	0.394	0.0	0.714
D80010	2p25.1	LPIN1	lipin 1	-1.2	0.000	-1.1	0.015	-0.9	0.053
NM_005045	7q22	RELN	reelin	-1.2	0.003	-0.6	0.101	-0.2	0.714
NM_007173	11q14.1	PRSS23	protease serine 23	-1.2	0.018	-0.9	0.021	-0.9	0.101
NM_003255	17q25	TIMP2	TIMP metallopeptidase inhibitor 2	0.6	0.028	-1.3	0.031	-0.2	0.678
AF200348	2p25	PXDN	peroxidasin homolog Drosophila	-1.2	0.003	-1.3	0.000	0.9	0.064
NM_007011	15q26.1	ABHD2	abhydrolase domain containing 2	-1.5	0.000	-1.1	0.032	0.1	0.573
AF271070	12q13.11	SLC38A1	solute carrier family 38 member 1	-1.3	0.000	-0.7	0.044	-0.2	0.678
L29496	Xq11.2-q12	AR	androgen receptor	-1.3	0.018	0.9	0.000	-0.3	0.194
NM_019005	7p22-p21	FLJ20323*	Hypothetical protein FLJ20323*	-0.9	0.000	-1.3	0.000	-0.6	0.134
AK026517	11p12	EHF	ets homologous factor	-0.7	0.028	-1.3	0.000	-0.6	0.309
NM_000213	17q25	ITGB4	integrin beta 4	0.9	0.086	-1.3	0.000	0.1	0.777
NM_001311	14q32.33	CRIP1	cysteine-rich protein 1 intestinal	-1.4	0.018	0.4	0.101	-0.9	0.115
AL050367	10p13	C10orf38	chromosome 10 open reading frame 38	0.1	0.380	-1.4	0.000	-0.6	0.084
AL122055	6q21	CDC2L6	cell division cycle 2-like 6 CDK8-like	-1.4	0.000	-1.3	0.000	-1.2	0.000
NM_005864	14q11.2-q12	EFS	embryonal Fyn-associated substrate	-1.4	0.009	0.4	0.135	-0.6	0.064
NM_002151	19q11-q13.2	HPN	hepsin transmembrane protease serine 1	-1.0	0.000	-0.9	0.000	-1.4	0.053
NM_016598	3p21.31	ZDHHC3	zinc finger DHHC-type containing 3	-1.4	0.003	-1.1	0.000	-0.9	0.053
NM_005510	6p21.3	DOM3Z	dom-3 homolog Z C. elegans	0.5	0.211	0.1	0.422	-1.4	0.053
NM_000944	4q21-q24	PPP3CA	protein phosphatase 3 formerly 2B catalytic subunit alpha isoform	-1.4	0.028	-1.2	0.000	-1.0	0.084

AF252283	13q21	KLHL1	kelch-like 1 Drosophila	-1.5	0.000	0.5	0.115	0.1	0.738
NM_003278	3p22-p21.3	CLEC3B	C-type lectin domain family 3 member B	-1.5	0.005	-1.3	0.000	-1.3	0.084
AK024917	1p22	DDAH1	dimethylarginine dimethylaminohydrolase 1	-0.3	0.380	-1.5	0.000	0.1	0.760
AK000216	3p21.31	FLJ20209*	Hypothetical protein FLJ20209*	-1.5	0.000	-0.7	0.115	-0.7	0.084
AK000028	4q24	LOC90024*	Hypothetical LOC90024*	-1.5	0.000	-0.7	0.000	-0.5	0.120
AL049963	4q22-q24	SLC39A8	solute carrier family 39 zinc transporter member 8	-1.5	0.000	-1.4	0.000	-0.9	0.053
X75684	3q21-q25	TM4SF1	transmembrane 4 L six family member 1	0.6	0.211	-1.5	0.000	0.5	0.194
X15667		GPRP*	Glutathione peroxidase-related protein GPRP*	-1.6	0.022	0.2	0.185	-0.8	0.053
NM_006006	11q23.1	ZBTB16	zinc finger and BTB domain containing 16	-1.6	0.003	0.1	0.444	0.3	0.328
NM_012116	19q13.2	CBLC	Cas-Br-M murine ecotropic retroviral transforming sequence c	-1.6	0.000	-1.2	0.000	-0.4	0.218
NM_006167	8p21	NKX3-1	NK3 homeobox 1	-1.6	0.003	-0.1	0.459	-0.3	0.413
AK026331	2q35	CHPF*	Chondroitin polymerizing factor*	-1.7	0.000	-0.8	0.048	-0.2	0.678
AB020637	11q21	ENDOD1	endonuclease domain containing 1	-1.7	0.000	-0.4	0.160	0.2	0.678
M26663	19q13.41	KLK3	kallikrein-related peptidase 3	-2.0	0.009	0.0	0.207	-0.4	0.392
NM_001359	8q21.3	DECR1	2 4-dienoyl CoA reductase 1 mitochondrial	-1.9	0.000	-1.1	0.000	-1.6	0.053
NM_014333	11q23.2	CADM1	cell adhesion molecule 1	-1.0	0.000	-1.9	0.000	-0.1	0.725
NM_004117	6p21.3-21.2	FKBP5	FK506 binding protein 5	-2.0	0.000	0.1	0.459	0.4	0.218
AB020968	6q22.2	MARCKS	myristoylated alanine-rich protein kinase C substrate	-1.6	0.000	-2.0	0.000	-1.3	0.053
NM_003307	21q22.3	TRPM2	transient receptor potential cation channel subfamily M member 2	-2.1	0.000	0.0	0.536	-0.1	0.725
AK026813	7q21	STEAP2	six transmembrane epithelial antigen of the prostate 2	-2.3	0.000	-0.8	0.135	-0.8	0.176
NM_012449	7q21	STEAP1	six transmembrane epithelial antigen of the prostate 1	-2.3	0.000	-0.9	0.036	-0.5	0.236
AF188747	19q13.41	KLK2	kallikrein-related peptidase 2	-2.3	0.000	-0.7	0.063	-0.8	0.053
AB042410	1p21.3	GPR88	G protein-coupled receptor 88	-2.4	0.000	-0.3	0.394	0.8	0.194
NM_014380	Xq22.2	NGFRAP1	nerve growth factor receptor TNFRSF16 associated protein 1	-0.2	0.513	-2.5	0.000	-2.7	0.000
AF216077	9q32	COL27A1	collagen type XXVII alpha 1	-2.1	0.000	-2.8	0.000	-1.7	0.053
NM_017935	4q24	BANK1	B-cell scaffold protein with ankyrin repeats 1	-2.9	0.000	-1.8	0.000	-0.8	0.053

* no approved HUGO symbol/name exist for this entry. If present, gene symbol/name from the UNIGENE database are given in alternative.
2log_ratio>0 indicates overexpression in the hormone-refractory subline, compared to parental PC346C. 2log_ratio<0 indicates down-regulation in the hormone-refractory subline

Table 2 - Chromosomal clustering of the differentially expressed transcripts

Cell line	#genes regulated	up/down regulated	Cytoband	#genes cytoband*	Bonferroni p-value
PC346DCC	7	down	4q21-24	56	0.003
PC346DCC	9	down	5q11-23	130	0.028
PC346DCC	6	down	6q14-23	65	0.026
PC346DCC	8	down	8p11-22	74	0.003
PC346DCC	10	down	8q11-24	131	0.006
PC346Flu1	6	down	11p11-15	92	0.068
PC346Flu2	14	up	18	109	<0.0001

p-values determined by Fisher's exact test with Bonferroni correction for multiple testing
* number of genes located in the cytoband that are expressed by the indicated cell line

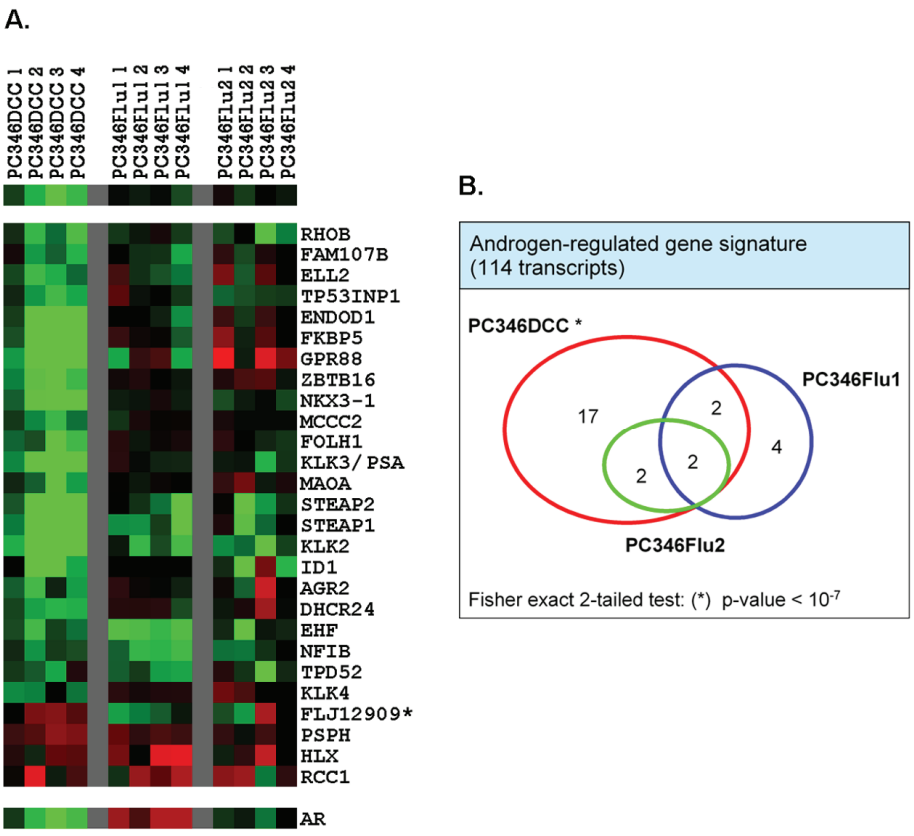

Figure 2 - Activation state of the AR pathway in the PC346DCC, PC346Flu1 and PC346Flu2. Differentially-expressed genes in PC346 hormone-refractory sublines versus parental PC346C were linked to a previously established androgen-response gene signature (see Materials and Methods section). **(A)** Heat-map representation of androgen-responsive genes deregulated in any of the PC346 hormone-refractory sublines. Color scheme as described in Fig. 1. **(B)** Venn-diagram and respective statistics.

Gene ontology and pathway analysis identifies cancer signature

The selected 487-gene signature was classified according to Gene Ontology (GO) Biological Processes using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [26,27]. Annotation clustering analysis showed enrichment in categories involved in organ development, reproductive system differentiation, cellular growth, differentiation and apoptosis (Table 3). Ingenuity Pathway Analysis was used to identify enrichment in “diseases and disorders”, “molecular and cellular functions”, and to search for intrinsic pathways/networks within the selected gene sets (www.ingenuity.com). Cancer and reproductive system disease were ranked in the top 3 of “diseases and disorders”, which logically confirmed the enrichment of genes associated with prostate cancer, such as hepsin, clusterin, vitamin D receptor, trefoil factor 3, tumor protein D52, the androgen-receptor self and several of its target genes (Fig. 3A and 3B, respectively). Furthermore, we used Network analysis to screen the 276-gene signature of PC346DCC for potential alternative growth pathways that could be involved in bypassing the AR signaling. Interestingly, signaling via growth-hormone receptor (GHR), insulin receptor (INSR) and epithermal growth factor receptor was among the top 10 Networks (score = 20) showing deregulation in PC346DCC (Fig. 3C).

Integrative analysis reveals genes deregulated in prostate cancer progression

To identify genes modulated in prostate cancer that could explain hormone-refractory growth through bypass of the AR pathway, we linked the 276-gene signature from PC346DCC with data from seven prostate cancer microarray studies published previously [16,29,30,31,32,33,34] (Table 4). Only genes present in at least 5/7 databases (209 genes) and deregulated in at least 3/7 (111 genes) were included for further analysis. Hierarchical clustering performed on the signature genes (first column), next to primary cancer vs. normal prostate (second column), metastatic cancer vs. primary cancer (third column), and finally hormone-refractory vs. hormone-naïve disease (fourth column), is shown in Fig. 4. Approximately 30% of the genes differentially expressed in PC346DCC were found to be consistently deregulated in metastatic prostate cancer compared to organ-confined disease.

TWIST1, DKK3 and VAV3 as markers for disease diagnosis and prognosis

Based on their recognized pathological functions and consistent deregulation in multiple prostate cancer databases, TWIST1, VAV3 and DKK3 were selected for their potential role in the bypass of the AR pathway. In this manner, TWIST1 and VAV3 are putative oncogenes involved in growth hormone signaling, as revealed by Ingenuity Pathway Analysis (Fig. 3C). On the other hand, DKK3 is a tumor suppressor, showing strong down-regulation in the datasets from Chandran *et al.*, Lapointe *et al.* and Varambally *et al.* (Fig. 4C). Whereas TWIST1 showed consistent up-regulation in primary and metastatic prostate cancer datasets from Varambally *et al.*, Yu *et al.*, Lapointe *et al.* and Chandran *et al.*, VAV3 was down-regulated in primary tumors followed by up-regulation in metastasis (Fig. 4C).

Table 3 - Summary of the Gene Ontology (GO) biological processes significantly enriched in the hormone-refractory gene signature

		count	P value
Annotation Cluster 1	**Enrichment Score: 4.42**		
	multicellular organismal development	86	1.8E-05
	anatomical structure development	78	7.7E-05
	system development	68	3.9E-05
Annotation Cluster 2	**Enrichment Score: 4.24**		
	cell differentiation	69	7.7E-05
	cell development	53	3.1E-05
Annotation Cluster 3	**Enrichment Score: 4.09**		
	apoptosis	38	4.1E-05
	programmed cell death	38	5.0E-05
Annotation Cluster 4	**Enrichment Score: 2.68**		
	regulation of phosphorylation	8	1.7E-03
	regulation of phosphate metabolic process	8	2.3E-03
Annotation Cluster 5	**Enrichment Score: 2.67**		
	amino acid and derivative metabolic process	21	5.8E-04
	amine metabolic process	21	6.0E-03
	nitrogen compound metabolic process	22	6.7E-03
Annotation Cluster 6	**Enrichment Score: 2.15**		
	regulation of biological process	134	3.0E-03
	regulation of cellular process	124	1.4E-02
	regulation of gene expression	63	6.7E-02
Annotation Cluster 7	**Enrichment Score: 2.15**		
	neurogenesis	16	7.0E-03
	neuron differentiation	14	6.2E-03
Annotation Cluster 8	**Enrichment Score: 2.03**		
	cellular lipid metabolic process	29	3.8E-03
	lipid metabolic process	28	1.6E-03
Annotation Cluster 9	**Enrichment Score: 1.82**		
	DNA packaging	16	8.9E-03
	establishment and/or maintenance of chromatin architecture	15	1.7E-02
	chromosome organization and biogenesis	17	2.3E-02
	chromatin assembly or disassembly	9	2.1E-02
	nucleosome assembly	7	1.8E-02
Annotation Cluster 10	**Enrichment Score: 1.70**		
	development of primary sexual characteristics	7	2.0E-02
	sex differentiation	6	1.8E-02
	reproductive developmental process	6	2.2E-02
	gonad development	5	4.9E-02
Annotation Cluster 11	**Enrichment Score: 1.42**		
	growth	12	8.3E-02
	regulation of cell size	11	1.8E-02
	cell growth	10	3.7E-02

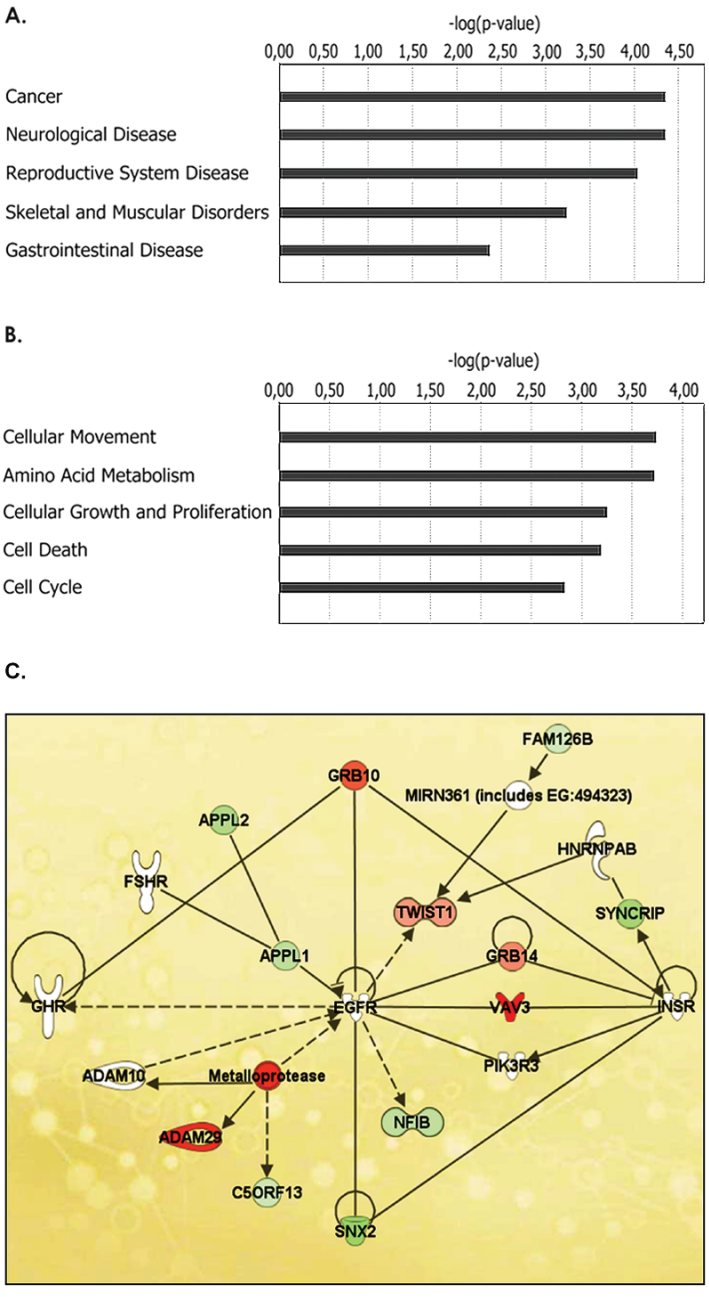

Figure 3 - Biological processes deregulated in the hormone-refractory sublines. Top 5 biological functions enriched in the castration-resistant sublines: **(A)** diseases and disorders, **(B)** molecular and cellular functions. (C) Example of Network analysis for PC346DCC showing deregulation of hormone and growth-factor receptor signaling: up-regulated genes are represented in red and repressed genes in green. Analysis was performed using Ingenuity Pathway Analysis software (www.ingenuity.com).

Table 4 - Description of prostate cancer databases linked via SRS

First Author	Reference	Samples
Best (2005) [29]	10 hormone-naive prostate cancers	10 hormone-refractory primary prostate tumors
Chandran (2007) [30]	64 primary prostate tumor samples	24 hormone-refractory metastatic samples from 4 patients
Lapointe (2004) [31]	41 benign prostate tissue adjacent to cancer	62 primary prostate tumor samples
		9 lymph node metastasis
Tamura (2007) [32]	10 hormone-naive prostate cancers	18 hormone-refractory primary and metastatic tumor samples
Tomlins (2007) [16]	15 benign epithelial tissue adjacent to cancer	30 primary prostate tumor samples
		3 hormone-naive and 17 hormone-refractory metastasis
Varambally (2005) [33]	5 benign prostate tissues	5 clinically localized prostate cancers
		5 hormone-refractory metastatic samples
Yu (2004) [34]	60 benign prostate tissue adjacent to cancer	62 primary prostate tumors
	23 disease free donor prostate tissue	24 hormone-refractory metastasis

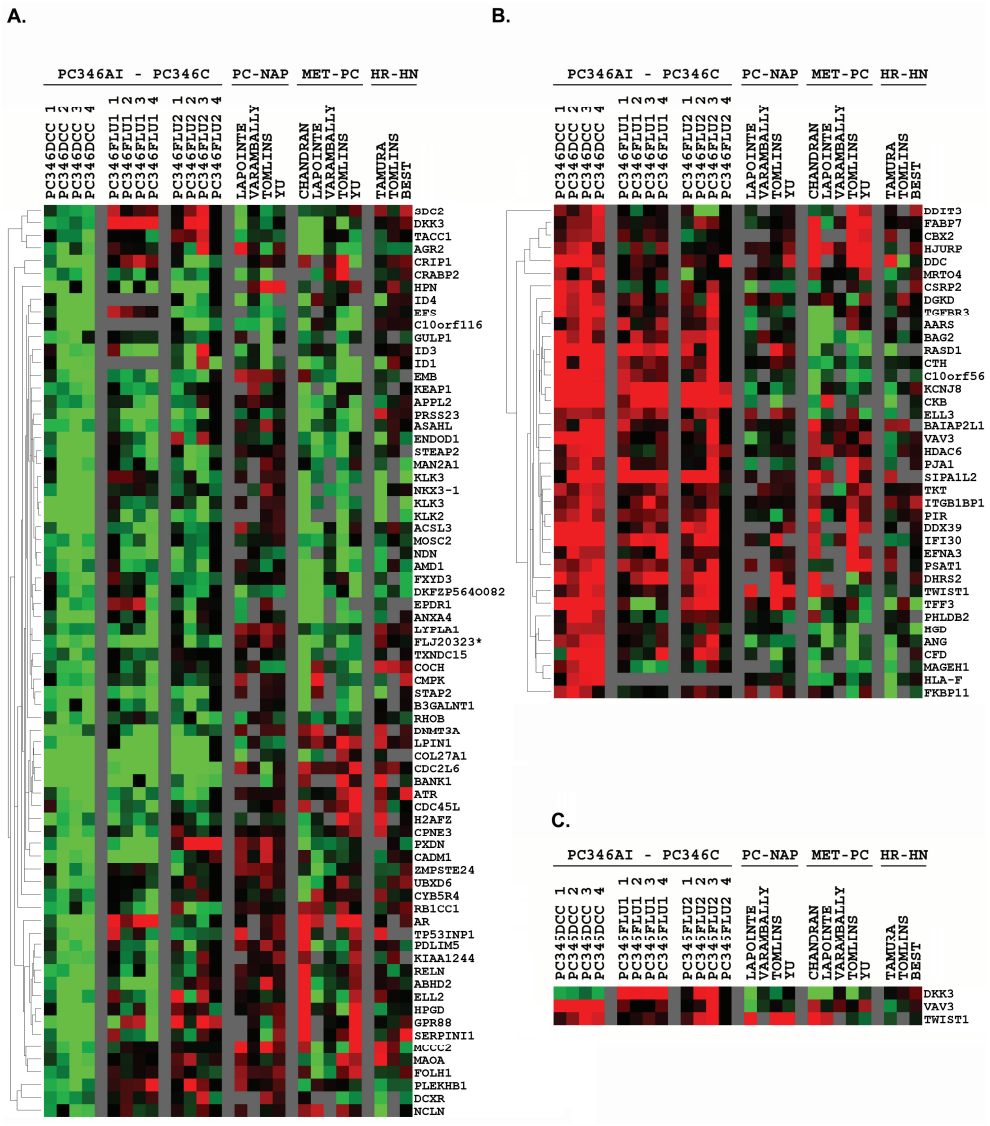

Figure 4 - Expression of the androgen-independent PC346DCC signature genes in prostate cancer samples from patient tumors. The 276-gene signature from PC346DCC was linked to data from 7 prostate cancer microarray databases of primary (Lapointe, Varambally, Tomlins, Yu), metastatic (Chandran, Lapointe, Varambally, Tomlins, Yu) and hormone-refractory tumors (Tamura, Tomlins and Best). Only genes present in at least 5/7 databases (209 genes) and deregulated in at least 3/7 (111 genes) were included in the analysis. Heat-map representation of **(A)** 72 overexpressed and **(B)** 39 repressed genes in PC346DCC. (C) Deregulated genes selected for further qPCR analysis. Color scheme as described in Fig.1. Grey squares indicate missing data, either due to low expression levels, poor data quality or absence of probes for the respective transcript in the array platform used for the study. PC-NAP: prostate cancer minus normal adjacent prostate; MET-PC: metastasis minus primary prostate tumors; HR-HN: hormone-refractory minus hormone-naïve tumors.

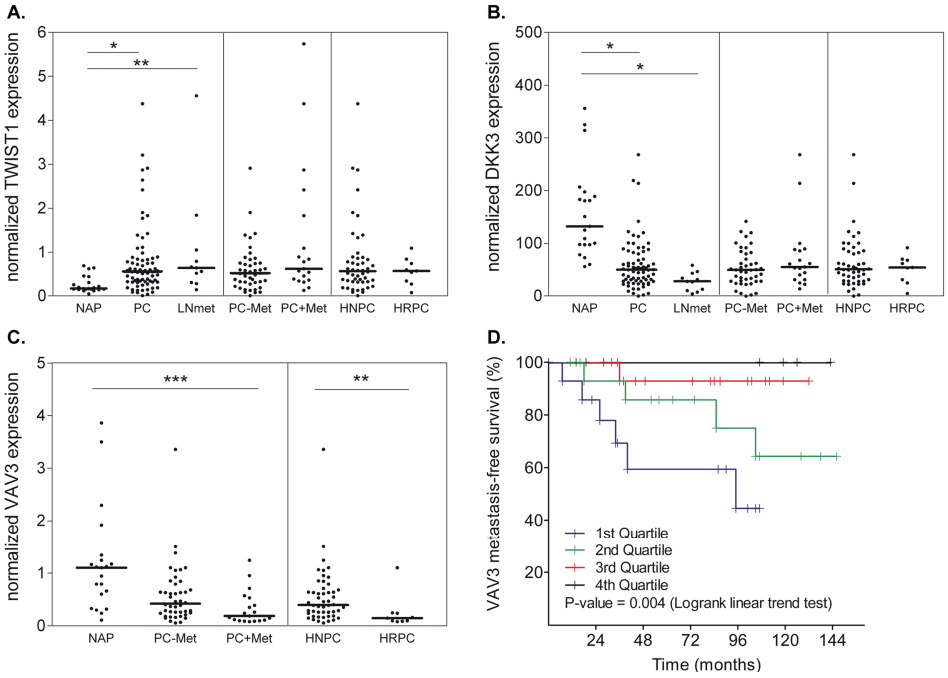

Figure 5 - Quantitative RT-PCR analysis of TWIST1, DKK3 and VAV3 in an independent set of prostate samples. Prostate tumor samples were obtained by radical prostatectomy or transurethral resection of the prostate of patients being operated at Erasmus MC clinic. This panel contains 21 benign prostate tissue samples and 74 adenocarcinomas at different disease stages. **(A)** TWIST1; **(B)** DKK3; **(C)** VAV3 expression in prostate samples; **(D)** VAV3 metastasis-free survival analysis. NAP: normal adjacent prostate; PC: primary prostate cancer; LNmet: lymph-node metastasis; PC-Met: non-progressive organ-confine prostate cancer; PC+Met: primary tumor from progressive prostate cancer that either had or developed metastasis during subsequent follow-up; HN: hormone-naïve; HR: hormone-refractory; (*) p-value ≤ 0.0001 and (**) p-value ≤ 0.005 using Mann-Whitney two-tailed test. (***) p-value ≤ 0.0001 with Post linear-trend test.

Quantitative PCR analysis was performed on an independent set of prostate samples, obtained by radical prostatectomy or transurethral resection of the prostate of patients being operated at Erasmus MC clinic. This panel contained 21 benign prostate tissue samples and 74 adenocarcinomas at different disease stages. Quantitative PCR analysis showed up-regulation of TWIST1 in primary prostate cancer samples and lymph-node metastasis (P-value = 0.0001 and 0.002, respectively). No difference was observed between hormone-refractory (HRPC) and hormone-naïve tumors (HNPC) (Fig. 5A). DKK3 expression was significantly decreased in prostate cancer and lymph-node metastasis (P-value ≤ 0.0001), although no difference was observed during progression from organ-confined to metastatic or hormone-refractory disease (Fig. 5B). VAV3 expression decreased gradually during prostate cancer progression, with the lowest levels observed in metastatic prostate tumors (P-value = 0.0001 for Post linear-trend test) and hormone-refractory samples (P-value = 0.005 for HNPC vs. HRPC; Fig. 5C).

Lymph-node samples were removed from the VAV3 analysis in Fig. 5C, because VAV3 expression was highly increased in normal lymph-node compared to normal prostate tissues (data not shown). In these settings, the presence of remnants of normal lymph-node tissue can lead to over-estimation of the real VAV3 quantity in lymph-node metastasis. Kaplan-Meier analysis showed a direct correlation between VAV3 expression and metastasis-free survival (P-value = 0.004 for Logrank trend test; Fig. 5D).

DISCUSSION

In the present study we used microarray analysis to identify differences in the gene expression pattern of the androgen-responsive PC346C cell line and its castration-resistant sublines: PC346DCC, PC346Flu1 and PC346Flu2. This analysis detected 487 transcripts differentially regulated in the hormone-refractory cells versus the parental PC346C. Many of these were common to all three hormone-refractory sublines, despite the different AR pathway modifications, suggesting similar growth enhancing adaptations (Fig. 1). These shared genes could be divided in four main categories: regulation of cell cycle progression and proliferation (ex. HPN, NDN, ATR, ABL2, DHRS2), development and cellular differentiation (CLEC3B, KCNJ8, ADAM29, DNMT3A), fatty acid and steroid metabolism (LPIN1, DECR1, ACSL3) and intracellular signaling transduction (PPAP2C, PPP3CA, PRSS23, GRB10, SIPA1L2). Interestingly, 40 of the genes down-regulated in PC346DCC clustered in just four genomic locations: 4q21-24 (7 genes), 5q11-23 (9 genes), 6q14-23 (6 genes) and chr8 (18 genes). This is more than would be expected by chance. Also 6 genes down-regulated in PC346Flu1 and 14 up-regulated genes in PC346Flu2 clustered at 11q11-15 and chr18, respectively (Table 2). Duplication of chromosome 18 and loss of 8p has been previously reported in PC346Flu2 and PC346DCC, respectively, and may explain the clustering observed at these loci [35]. However, no evidence of chromosomal amplifications or deletions was detected at the other loci mentioned. A possible explanation is that epigenetic mechanisms, such as promotor methylation or histone modifications, may be involved in the transcriptional regulation of these large chromosomal regions [36]. Indeed, expression of DNA methyltransferase DNMT3A and histone deacetylase HDAC6 was altered in PC346DCC, supporting this hypothesis. Nevertheless, losses at 4q, 5q, 6q, 8p and 11p have been frequently reported in prostate cancer specimens and these loci are suspected of harbouring potential tumor suppressor genes [37,38].

Previously, we have shown that PC346DCC, PC346Flu1 and PC346Flu2 display different AR modifications that resulted in distinct mechanisms of hormone-refractory growth [35]. PC346DCC revealed very low levels of the AR and it's target gene prostate specific antigen (PSA), and was insensitive to androgen stimulation in growth assays, promotor transactivation assays and expression microarray profiling (submitted data)[35]. These results suggest that the AR pathway has been bypassed and is not essential for the growth of PC346DCC cells. The present study further substantiates this hypothesis by showing a strong down-regulation of AR target genes compared to the parental PC346C (Fig. 2). PC346Flu1, on the other hand, expresses

high levels of the AR and previously showed a “super-activation” of this receptor in response to androgens, both in transactivation assays as in expression microarray analysis. Interestingly, the proliferation of this subline is inhibited by physiological concentration of androgens, and is optimal in the absence of this ligand. A possible explanation for this growth suppressive effect is that the “super-activation” of the AR by androgens in PC346Flu1 may be tilting the balance towards cellular differentiation [39,40]. It is worth noting how few AR target genes are deregulated in PC346Flu1 versus the parental PC346C (Fig. 2). This suggests that the AR pathway remained active in the PC346Flu1 cells cultured in androgen-depleted medium supplemented with AR antagonist hydroxyflutamide. The few AR target genes that were differentially expressed in PC346Flu1 include EHF, NFIB and HLX, which are involved in development and differentiation processes. In this context, the EHF transcription factor (also known as ESE-3) is particularly interesting, as its expression is specific for epithelial cells and it is involved in glandular differentiation [41,42]. It has recently been shown that EHF is frequently down-regulated in prostate tumor specimens and silenced in PC3 and DU145 prostate cancer cell lines by promotor methylation at evolutionary conserved CpG sites [43]. Re-expression of EHF in these cells inhibited clonogenic survival and induced apoptosis [43]. EHF is one of the strongest repressed genes in PC346Flu1 being up-regulated upon androgen stimulation, which makes it a good candidate for the growth suppressive effect of androgens on this cell line. The third hormone-refractory subline studied here, PC346Flu2, carries the T877A AR mutation, well known for causing broadened receptor activation by non-androgenic ligands, including flutamide [44,45]. Consistent with the presence of this “promiscuous” AR, the growth of PC346Flu2, as well as expression of AR target genes, are stimulated by both the synthetic androgen R1881 and the antiandrogen hydroxyflutamide (submitted data) [35]. In PC346Flu2, the presence of the T877A AR mutation allows for the maintenance of AR activity in the selection medium supplemented with 1 μM hydroxyflutamide. This is further substantiated in the present study by the observation that PC346Flu2 is the least divergent of the hormone-refractory sublines, with no more than 127 differentially regulated transcripts compared to the parental PC346C (Fig. 1).

Both PC346Flu1 and PC346Flu2 sublines have an active AR and acquired hormone-refractory proliferation through adaptations of the AR pathway. Therefore, to study alternative survival and growth pathways independent of the AR we further focused on the expression profile of PC346DCC cells. The Ingenuity Pathway Analysis program was used to screen the 276-gene signature of PC346DCC for potential gene Networks that could be involved in bypassing the AR signaling. Among the top 10 Networks, emerged the signaling pathway via growth-hormone receptor (GHR), insulin receptor (INSR) and epidermal growth factor receptor (EGFR) as a potential candidate (Fig. 3C). These receptors are not themselves deregulated in PC346DCC but several partners of this signaling pathway were, including VAV3 and TWIST1. VAV3 and TWIST1 are potential oncogenes, and while VAV3 can interact with and cross-activate signaling via hormone and growth receptors, expression of TWIST1 is in turn stimulated by EGF and IGF1 [46,47,48,49]. Both VAV3 and TWIST1 are overexpressed in PC346DCC cells, and survey of 7 previous microarray studies revealed a consistent up-regulation in metastatic patient material (Fig. 4C). Another interesting candidate gene for

the bypass of the AR pathway was the DKK3 tumor suppressor, which was down-regulated in PC346DCC and multiple databases of primary and metastatic tumors (Fig. 4C). While compelling evidence links these genes to prostate cancer pathogenesis, it is not known whether TWIST1, VAV3 or the tumor suppressor DKK3 may have a functional role in developing resistance to hormonal therapy.

TWIST1 is a helix-loop-helix transcription factor, regulator of embryonic morphogenesis, and highly expressed in many types of human cancer [50]. The role of TWIST1 as a potential oncogene was first suggested through a functional screen for cDNAs that could counteract the pro-apoptotic effects of the MYC oncogene. In that study, TWIST1 expression bypassed P53-induced growth arrest and promoted colony formation, consistent with a potential role as oncoprotein [51]. Yang *et al.* showed that suppression of TWIST1 expression in highly metastatic mammary carcinoma cells specifically inhibited their ability to metastasise to the lung, while ectopic expression resulted in activation of mesenchymal markers and induction of cell motility [52]. Previous studies also implicated TWIST1 in the development and progression of prostate cancer, showing that its expression was up-regulated in prostate adenocarcinomas and correlated with Gleason grading and increased metastatic potential [53,54,55]. Furthermore, inactivation of TWIST1, through small interfering RNA, induced growth arrest and suppressed migration and invasion abilities in androgen-independent prostate cancer cell lines DU145 and PC3 [54,56]. We quantified TWIST1 expression in a panel of patient derived material, comprising 21 normal prostate samples (adjacent to cancer), 74 primary prostate tumors, of which 9 hormone-refractory samples, and 13 lymph-node metastasis. Among the primary tumors are 59 samples of early organ-confined disease, 9 samples of invasive tumors that eventually developed metastasis during follow-up and 6 tumors with lymph-node and/or distant metastasis at the time of operation. Quantitative PCR confirmed overexpression of TWIST1 in primary prostate cancer samples and lymph-node metastasis (Fig. 5A). In this patient cohort, TWIST1 expression could not predict progression, as it did not differ between non-progressive organ-confined tumors and primary cancers that eventually developed metastasis. Furthermore, TWIST1 expression was not increased in hormone-refractory tumors when compared to hormone-naïve samples, suggesting that TWIST1 overexpression alone may not enough to confer hormone-refractory growth. However, since TWIST1 is strongly up-regulated in prostate cancer samples it may still be useful as a cancer marker or therapeutical target.

VAV3 is a member of the VAV family of oncoproteins, GTPase guanine nucleotide exchange factors that regulate receptor protein tyrosine kinases. It can be activated upon engagement of growth factor receptors, such as EGFR, PDGFR, INSR or IGF1R, and in turn activate downstream PLC and PI3K signaling pathways [48,49,57]. Previous studies have implicated VAV3 in the pathogenesis of the prostate: (i) VAV3 expression has been detected in the prostate, at increased levels in cancer cells [58]; (ii) it has been shown to interact with the AR pathway, stimulating ligand-independent cell growth in LNCAP-hormone-refractory cells [58,59,60]; and (iii) targeting of constitutively active VAV3 expression to the prostate induced prostate cancer in mice [61]. Surprisingly, in our patient derived samples, VAV3 expression decreased gradually

during prostate cancer progression, with the lowest levels in metastatic and hormone-refractory samples (Fig. 5C). Furthermore, Kaplan-Meier analysis showed a direct correlation between VAV3 expression levels and metastasis-free survival (Fig. 5D). These results encourage the use of VAV3 as a potential prognosis marker in prostate cancer, and provide a possible mechanism for the bypass of AR pathway in hormone-refractory tumors. However, the decrease of VAV3 expression in prostate cancer progression was unexpected, considering the function of VAV3 as a potential oncogene. In fact, VAV3 has three transcript variants, the full length VAV3, the VAV3 beta isoform and the truncated VAV3.1 variant, which has no guanine nucleotide exchange activity (GEF) due to lack of N-terminal domains [48,57,62]. The truncated transcript is expressed in many tissues and is the major variant in the prostate [48,62]. Because the effect of VAV3 on cell division and AR activation is dependent on GEF activity, this variant is not oncogenic and has most likely a different function than the full-length protein. It was proposed that this VAV3.1 variant may function as a dominant negative of other VAV family members [48,62]. In this context, a decrease in the VAV3.1 variant could actually result in increased activity of oncogenic VAV proteins. Clearly, it is essential to characterize the different VAV3 variants in the prostate and evaluate how the balance of these is affected during prostate cancer progression, before one can consider its use in the clinic. These results exemplify the limitation of large-scale expression profiling assays that rely on a single probe per gene. Ultimately, to investigate gene expression in the context of human disease, it may not be enough to quantify the major known transcript but one may need to consider the different isoforms and how these variants interact with each other [63,64]. In the near future we expect to be able to answer how different splice variants from the same gene (including VAV3) can relate to prostate cancer, using exon microarray analysis of the patient tumor material.

DKK3 is part of an evolutionary conserved gene family encoding secreted proteins, which play an important role in vertebrate embryonic development as antagonists of Wnt/beta-catenin signaling. DKKs are further implicated in bone formation and bone disease, Alzheimer's and cancer [65]. DKK3 was proposed to function as a tumor suppressor since it was found to be down-regulated in a number of malignancies including kidney, bladder, lung, pancreas and prostate cancer [66]. Reduced DKK3 expression may, at least in part, be explained by promotor methylation, which has been detected in various cancers, including over 65% of prostate tumors [67]. Additional reports showed consistent reduction of DKK3 expression in prostate adenocarcinomas, particularly those with a high Gleason grade [68,69,70]. Moreover, small interfering RNA-mediated down-regulation of DKK3 enhanced cell cycle progression and disrupted three-dimensional acinar morphogenesis in RWPE-1 prostate epithelial cells [68]. Conversely, ectopic expression of DKK3 resulted in decreased proliferation, inhibited colony formation and induced apoptosis of LNCaP, PC3 and DU145 cell lines [67,68,69]. In our patient samples, DKK3 expression decreased in prostate cancer and lymph-node metastasis, but no difference was observed in hormone-refractory samples (Fig. 5B). As for TWIST1, DKK3 might be useful as a cancer marker, but could not predict tumor progression, nor explain recurrence after hormonal therapy. Interestingly, injection of an adenovirus vector carrying DKK3 showed a dramatic anti-tumor effect in a

xenograft human prostate cancer model, inhibiting tumor growth and lymph node metastasis and prolonging mice survival [69,71]. Such results encourage the development of therapies targeting DKK3 in advanced metastatic disease.

CONCLUSIONS

The present study showed that alterations in the AR pathway are common events in prostate cancer progression to hormone-refractory disease. While AR overexpression in PC346Flu1 and mutation in PC346Flu2 allowed the maintenance of AR activity under androgen ablation and antiandrogen treatment, PC346DCC acquired complete independence from AR signaling by activating alternative survival pathways. In PC346DCC, activation of VAV3 and TWIST1 oncogenes and down-regulation of DKK3 tumor suppressor may constitute a possible mechanism for bypassing the AR pathway. The fact that TWIST1 and DKK3 expression was deregulated in both hormone-refractory and hormone-naïve patient samples, suggests that these alterations occur earlier in prostate cancer progression and do not act alone in inducing hormone-refractory growth. Indeed, both VAV3 and TWIST1 are known to interact with growth factor signaling, which could be the effector mechanism in stimulating cellular proliferation, whereas DKK3 down-regulation may promote survival through inhibition of apoptosis. These results grant further investigations on the use of VAV3, TWIST1 and DKK3 as prostate cancer markers and in the development of targeted therapies for advanced disease.

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SUPPLEMENTARY DATA

Table S1 - Genes differentially expressed in PC346DCC vs PC346C expression ratios and SAM q-values

GenBank ID	Cytoband	Symbol	HUGO Gene Name	PC346DCC		PC346Flu1		PC346Flu2	
				2log ratio	qvalue	2log ratio	qvalue	2log ratio	qvalue
NM_017935	4q24	BANK1	B-cell scaffold protein with ankyrin repeats 1	-2.9	0.000	-1.8	0.000	-0.8	0.053
AB042410	1p21.3	GPR88	G protein-coupled receptor 88	-2.4	0.000	-0.3	0.394	0.8	0.194
S75755	19q13.41	KLK3	kallikrein-related peptidase 3	-2.3	0.000	-0.3	0.160	-0.4	0.531
AF188747	19q13.41	KLK2	kallikrein-related peptidase 2	-2.3	0.000	-0.7	0.063	-0.8	0.053
NM_012449	7q21	STEAP1	six transmembrane epithelial antigen of the prostate 1	-2.3	0.000	-0.9	0.036	-0.5	0.236
AK026813	7q21	STEAP2	six transmembrane epithelial antigen of the prostate 2	-2.3	0.000	-0.8	0.135	-0.8	0.176
NM_003307	21q22.3	TRPM2	transient receptor potential cation channel, subfamily M, member 2	-2.1	0.000	0.0	0.536	-0.1	0.725
AF216077	9q32	COL27A1	collagen type XXVII alpha 1	-2.1	0.000	-2.8	0.000	-1.7	0.053
NM_004117	6p21.3-21.2	FKBP5	FK506 binding protein 5	-2.0	0.000	0.1	0.459	0.4	0.218
NM_001359	8q21.3	DECR1	2 4-dienoyl CoA reductase 1 mitochondrial	-1.9	0.000	-1.1	0.000	-1.6	0.053
M26663	19q13.41	KLK3	kallikrein-related peptidase 3	-1.8	0.018	0.2	0.253	-0.4	0.252
AB020637	11q21	ENDOD1	endonuclease domain containing 1	-1.7	0.000	-0.4	0.160	0.2	0.678
AK026331	2q35	CHPF*	Chondroitin polymerizing factor*	-1.7	0.000	-0.8	0.048	-0.2	0.678
AK000554	15q26.1	ABHD2	abhydrolase domain containing 2	-1.7	0.000	-1.5	0.000	-0.2	0.657
NM_012116	19q13.2	CBLC	Cas-Br-M murine ecotropic retroviral transforming sequence c	-1.6	0.000	-1.2	0.000	-0.4	0.218
NM_006167	8p21	NKX3-1	NK3 homeobox 1	-1.6	0.003	-0.1	0.459	-0.3	0.413
NM_006006	11q23.1	ZBTB16	zinc finger and BTB domain containing 16	-1.6	0.003	0.1	0.444	0.3	0.328
AB020968	6q22.2	MARCKS	myristoylated alanine-rich protein kinase C substrate	-1.6	0.000	-2.0	0.000	-1.3	0.053
X15667	14q24.1	GPRP*	Glutathione peroxidase-related protein GPRP*	-1.6	0.022	0.2	0.185	-0.8	0.053
AL049963	4q22-q24	SLC39A8	solute carrier family 39 zinc transporter member 8	-1.5	0.000	-1.4	0.000	-0.9	0.053
AK000028	4q24	LOC90024*	Hypothetical LOC90024*	-1.5	0.000	-0.7	0.000	-0.5	0.120
AK000216	3p21.31	FLJ20209*	Hypothetical protein FLJ20209*	-1.5	0.000	-0.7	0.115	-0.7	0.084
AL109729	15q26.1	ABHD2	abhydrolase domain containing 2	-1.5	0.000	-1.0	0.044	0.2	0.531
NM_003278	3p22-21.3	CLEC3B	C-type lectin domain family 3 member B	-1.5	0.005	-1.3	0.000	-1.3	0.084
AF252283	13q21	KLHL1	kelch-like 1 Drosophila	-1.5	0.000	0.5	0.115	0.1	0.738
NM_000944	4q21-q24	PPP3CA	protein phosphatase 3 formerly 2B catalytic subunit alpha isoform	-1.4	0.028	-1.2	0.000	-1.0	0.084
AL122055	6q21	CDC2L6	cell division cycle 2-like 6 CDK8-like	-1.4	0.000	-1.3	0.000	-1.2	0.000
NM_016598	3p21.31	ZDHHC3	zinc finger DHHC-type containing 3	-1.4	0.003	-1.1	0.000	-0.9	0.053
NM_005864	14q11.2-q12	EFS	embryonal Fyn-associated substrate	-1.4	0.009	0.4	0.135	-0.6	0.064
NM_001311	14q32.33	CRIP1	cysteine-rich protein 1 intestinal	-1.4	0.018	0.4	0.101	-0.9	0.115
L29496	Xq11.2-q12	AR	androgen receptor dihydrotestosterone receptor	-1.3	0.018	0.9	0.000	-0.3	0.194

AF271070	12q13.11	SLC38A1	solute carrier family 38 member 1	-1.3	0.000	-0.7	0.044	-0.2	0.678
NM_007011	15q26.1	ABHD2	abhydrolase domain containing 2	-1.3	0.000	-0.8	0.051	0.3	0.531
NM_007173	11q14.1	PRSS23	protease serine 23	-1.2	0.018	-0.9	0.021	-0.9	0.101
AF200348	2p25	PXDN	peroxidasin homolog Drosophila	-1.2	0.003	-1.3	0.000	0.9	0.064
NM_005045	7q22	RELN	reelin	-1.2	0.003	-0.6	0.101	-0.2	0.714
D80010	2p25.1	LPIN1	lipin 1	-1.2	0.000	-1.1	0.015	-0.9	0.053
NM_004457	2q34-q35	ACSL3	acyl-CoA synthetase long-chain family member 3	-1.2	0.009	-0.7	0.025	-0.3	0.101
NM_005544	2q36	IRS1	insulin receptor substrate 1	-1.2	0.020	-0.1	0.394	0.0	0.714
AK024495	11p15.5	LRRC56	leucine rich repeat containing 56	-1.2	0.000	0.2	0.321	0.5	0.218
NM_002165	20q11	ID1	inhibitor of DNA binding 1 dominant negative helix-loop-helix protein	-1.2	0.018			-0.6	0.500
NM_001546	6p22-p21	ID4	inhibitor of DNA binding 4 dominant negative helix-loop-helix protein	-1.1	0.014			-0.7	0.272
NM_006829	10q23.2	C10orf116	chromosome 10 open reading frame 116	-1.1	0.009			-0.9	0.084
NM_004315	8p22-p21.3	ASAH1	N-acylsphingosine amidohydrolase acid ceramidase 1	-1.1	0.000	-0.6	0.135		
M83822	4q31.23-31.3	LRBA	LPS-responsive vesicle trafficking beach and anchor containing	-1.1	0.003	-0.6	0.044	-0.6	0.101
NM_019844	12p12	SLCO1B3	solute carrier organic anion transporter family member 1B3	-1.1	0.028	-0.2	0.394	0.6	0.194
AL137506	5q12.1	ELOVL7	ELOVL family member 7 elongation of long chain fatty acids yeast	-1.1	0.022	-0.3	0.229	0.1	0.786
NM_001153	2p13	ANXA4	annexin A4	-1.1	0.003	-0.6	0.160	-0.2	0.556
NM_003711	5q11	PPAP2A	phosphatidic acid phosphatase type 2A	-1.1	0.018	-0.8	0.015	0.0	0.789
NM_000860	4q34-q35	HPGD	hydroxyprostaglandin dehydrogenase 15- NAD	-1.1	0.022	-0.3	0.253	0.1	0.785
NM_002487	15q11.2-q12	NDN	necdin homolog mouse	-1.1	0.000	-0.8	0.063	-0.5	0.101
NM_014932	3q26.31	NLGN1	neuroligin 1	-1.0	0.022	-0.7	0.051	-0.6	0.089
NM_001758	11q13	CCND1	cyclin D1	-1.0	0.000	-0.9	0.048	-1.4	0.000
NM_002151	19q11-q13.2	HPN	hepsin transmembrane protease serine 1	-1.0	0.000	-0.9	0.000	-1.4	0.053
NM_005025	3q26.1	SERPINI1	serpin peptidase inhibitor clade I neuroserpin member 1	-1.0	0.000	-0.4	0.229	-0.1	0.714
NM_014333	11q23.2	CADM1	cell adhesion molecule 1	-1.0	0.000	-1.9	0.000	-0.1	0.725
NM_015415	1q24	BRP44	brain protein 44	-1.0	0.009	-0.5	0.090	-0.7	0.089
NM_007043	12q21.1	KRR1	KRR1 small subunit SSU processome component homolog yeast	-1.0	0.022	-0.2	0.515	-0.7	0.134
AK026295		FLJ22642 *	CDNA FLJ22642 fis clone HSI06970*	-1.0	0.018	-0.6	0.080	-0.2	0.714
NM_017720	19p13.3	STAP2	signal transducing adaptor family member 2	-1.0	0.000	-1.1	0.000	-0.8	0.053
AK024215	3q25.32	MFSD1	major facilitator superfamily domain containing 1	-1.0	0.009	-0.2	0.287	0.0	0.725
NM_004476	11p11.2	FOLH1	folate hydrolase prostate-specific membrane antigen 1	-1.0	0.014	0.1	0.394	-0.1	0.725
NM_001634	6q21-q22	AMD1	adenosylmethionine decarboxylase 1	-1.0	0.028	-0.4	0.135	-0.6	0.084
NM_001184	3q22-q24	ATR	ataxia telangiectasia and Rad3 related	-1.0	0.020	-0.6	0.048	-0.7	0.064
NM_000240	Xp11.4-11.3	MAOA	monoamine oxidase A	-1.0	0.043	0.0	0.535	0.3	0.413
NM_018050	12p13.2	MANSC1	MANSC domain containing 1	-0.9	0.000	-1.1	0.011		
X69111	1p36.13-36.12	ID3	inhibitor of DNA binding 3 dominant negative helix-loop-helix protein	-0.9	0.055	-1.0	0.101	-0.3	0.714
D86976	19p13.3	HMHA1	histocompatibility minor HA-1	-0.9	0.009	-0.4	0.090	0.1	0.636
AY007729	8p21.2	C8orf58	chromosome 8 open reading frame 58	-0.9	0.022	-0.3	0.229	0.3	0.309

AK024677	4q21.1	ASAHL	N-acylsphingosine amidohydrolase acid ceramidase -like	-0.9	0.009	-0.8	0.044	-0.3	0.531
NM_005095	1p32-p34	ZMYM4	zinc finger MYM-type 4	-0.9	0.000	-0.2	0.459	-0.6	0.115
NM_019005	7p22-p21	FLJ20323*	Hypothetical protein FLJ20323*	-0.9	0.000	-1.3	0.000	-0.6	0.134
AF095844	2p24.3-q24.3	IFIH1	interferon induced with helicase C domain 1	-0.9	0.028	-0.5	0.185	-0.5	0.194
U52054	5q11.1	EMB	embigin homolog mouse	-0.9	0.022	-0.7	0.042	-0.5	0.150
AF261758	1p33-p31.1	DHCR24	24-dehydrocholesterol reductase	-0.9	0.003	0.1	0.459	0.3	0.500
D16875	2p24	RHOB	ras homolog gene family member B	-0.9	0.020	-0.2	0.253	-0.7	0.064
AL049969	4q22	PDLIM5	PDZ and LIM domain 5	-0.8	0.022	-0.5	0.160	-0.3	0.691
U89715	11q13.5-q14.1	PLEKHB1	pleckstrin homology domain containing family B evectins member 1	-0.8	0.014	0.5	0.115	0.4	0.218
AL133074	8q22	TP53INP1	tumor protein p53 inducible nuclear protein 1	-0.8	0.018	0.0	0.515	-0.4	0.064
D42039	15q13	MESDC2	mesoderm development candidate 2	-0.8	0.022	-0.5	0.071	-0.2	0.691
AB032962	10p12.1	GPR158	G protein-coupled receptor 158	-0.8	0.018			0.0	0.789
NM_002372	5q21-q22	MAN2A1	mannosidase alpha class 2A member 1	-0.8	0.022	-0.3	0.185	-0.6	0.064
NM_012081	5q15	ELL2	elongation factor RNA polymerase II 2	-0.8	0.009	-0.3	0.229	0.3	0.236
NM_001878	1q21.3	CRABP2	cellular retinoic acid binding protein 2	-0.8	0.014	-0.4	0.115	-0.4	0.084
NM_018844	7q22-q31	BCAP29	B-cell receptor-associated protein 29	-0.8	0.046	-0.4	0.185	-0.1	0.714
AF067972	2p23	DNMT3A	DNA cytosine-5- -methyltransferase 3 alpha	-0.8	0.005	-0.7	0.063	-0.5	0.101
NM_004086	14q12-q13	COCH	coagulation factor C homolog cochlin Limulus polyphemus	-0.8	0.014	-0.5	0.135	-0.1	0.725
NM_005671	8p12-p11.2	UBXD6	UBX domain containing 6	-0.8	0.009	-0.1	0.527	0.1	0.748
U90904	15q11.2	NIPA2	non imprinted in Prader-Willi/Angelman syndrome 2	-0.8	0.022	0.0	0.527	-0.1	0.714
AK000745	8q22.1	MTDH	metadherin	-0.8	0.020	-0.4	0.160	-0.1	0.725
NM_017898	1q41	MOSC2	MOCO sulphurase C-terminal domain containing 2	-0.8	0.003	-0.5	0.115	-0.2	0.218
D28450	4q24	H2AFZ	H2A histone family member Z	-0.8	0.003	-0.6	0.044	-0.3	0.531
NM_003100	5q23	SNX2	sorting nexin 2	-0.8	0.022	-0.3	0.101	-0.4	0.134
AL050082	8q21	WWP1	WW domain containing E3 ubiquitin protein ligase 1	-0.8	0.009	-0.4	0.160	-0.3	0.150
NM_002103	19q13.3	GYS1	glycogen synthase 1 muscle	-0.8	0.003	0.2	0.287	-0.1	0.714
AB050049	5q12-q13	MCCC2	methylcrotonoyl-Coenzyme A carboxylase 2 beta	-0.7	0.018	0.0	0.532	0.0	0.789
NM_004540	21q21.1	NCAM2	neural cell adhesion molecule 2	-0.7	0.009	0.0	0.321	0.0	0.781
AB033070	6q23.3	KIAA1244	KIAA1244	-0.7	0.009	-0.4	0.051	-0.1	0.725
NM_012445	4p16.3	SPON2	spondin 2 extracellular matrix protein	-0.7	0.028	0.5	0.211	0.4	0.218
NM_003909	8q21.3	CPNE3	copine III	-0.7	0.028	-0.5	0.101	0.0	0.725
NM_012289	19p13.2	KEAP1	kelch-like ECH-associated protein 1	-0.7	0.003	-0.5	0.101	-0.2	0.714
NM_001777	3q13.1-q13.2	CD47*	CD47 antigen Rh-related antigen integrin-associated signal transducer*	-0.7	0.022	0.1	0.459	0.0	0.725
NM_017423	4q31.1	GALNT7	UDP-N-acetyl-alpha-D-galactosamine: N-acetylgalactosaminyltransferase 7	-0.7	0.028	-0.5	0.115	-0.1	0.691
NM_006408	7p21.3	AGR2	anterior gradient homolog 2 Xenopus laevis	-0.7	0.028	0.0	0.485	0.2	0.236
NM_004549	11q14.1	NDUFC2	NADH dehydrogenase ubiquinone 1 subcomplex unknown 2 14.5kDa	-0.7	0.022	0.2	0.287	-0.7	0.000

U84526	22q11.21	CDC45L	CDC45 cell division cycle 45-like S. cerevisiae	-0.7	0.070	-0.6	0.044	-0.2	0.272
AF147408	1p36.13	PLEKHM2	pleckstrin homology domain containing family M with RUN domain member 2	-0.7	0.046	-0.5	0.160	-0.2	0.356
AF161429	4q12	FIP1L1	FIP1 like 1 S. cerevisiae	-0.7	0.022	-0.2	0.135	0.1	0.636
NM_016315	2q32.3-q33	GULP1	GULP engulfment adaptor PTB domain containing 1	-0.7	0.028	-0.1	0.211	-0.2	0.218
NM_002715	5q23-q31	PPP2CA	protein phosphatase 2 formerly 2A catalytic subunit alpha isoform	-0.7	0.037	-0.3	0.160	-0.4	0.089
NM_015916	10p	FAM26B	family with sequence similarity 26 member B	-0.7	0.000	-0.4	0.051	-0.4	0.150
NM_000465	2q34-q35	BARD1	BRCA1 associated RING domain 1	-0.7	0.046	-0.1	0.444	0.1	0.783
NM_014074			PRO0529 protein	-0.7	0.037	-0.4	0.115	-0.3	0.531
AK025766	12q24.31	BRI3BP	BRI3 binding protein	-0.7	0.014	-0.6	0.025	-0.1	0.714
NM_006372	6q14-q15	SYNCRIP	synaptotagmin binding cytoplasmic RNA interacting protein	-0.7	0.046	0.1	0.444	-0.5	0.053
AJ250475	7p14.1	EPDR1	ependymin related protein 1 zebrafish	-0.7	0.055	0.3	0.358	-0.3	0.588
NM_003781	3q25	B3GALNT1	beta-1 3-N-acetylgalactosaminyltransferase 1 globoside blood group	-0.7	0.000	-0.6	0.090	-0.5	0.084
NM_005857	1p34	ZMPSTE24	zinc metallopeptidase STE24 homolog S. cerevisiae	-0.7	0.070	0.0	0.524	-0.3	0.636
AK023519			cDNA FLJ13457	-0.7	0.018	-0.4	0.115	-0.2	0.691
AK023844	8q22.3	GRHL2	grainyhead-like 2 Drosophila	-0.6	0.022	-0.3	0.253	-0.3	0.084
M73547	5q22-q23	REEP5	receptor accessory protein 5	-0.6	0.037	0.0	0.515	-0.1	0.714
AK023339	8q22.1	C8orf38	chromosome 8 open reading frame 38	-0.6	0.000	0.0	0.527	-0.1	0.714
NM_005971	19q13.11-13.12	FXYD3	FXYD domain containing ion transport regulator 3	-0.6	0.055	-0.1	0.422	-0.5	0.053
NM_015584	17q11.2	POLDIP2	polymerase DNA-directed delta interacting protein 2	-0.6	0.037	0.2	0.253	-0.1	0.725
NM_002620	4q12-q21	PF4V1	platelet factor 4 variant 1	-0.6	0.340	0.4	0.287	-0.3	0.714
NM_018279	12q21.1	TMEM19	transmembrane protein 19	-0.6	0.055	-0.2	0.321	0.4	0.471
AK023269	2p23.1	LCLAT1	lysocardiolipin acyltransferase 1	-0.6	0.043	-0.5	0.135	-0.3	0.714
AF070569	17p13.3	C17orf91	chromosome 17 open reading frame 91	-0.6	0.037	0.0	0.527	-0.2	0.252
NM_001505	7p22	GPER	G protein-coupled estrogen receptor 1	-0.6	0.070	0.2	0.358	-0.1	0.714
AK024648	10p13	FAM107B	family with sequence similarity 107 member B	-0.6	0.070	-0.5	0.185	0.0	0.725
AK026278	5q31.1	TXNDC15	thioredoxin domain containing 15	-0.6	0.022	-0.3	0.135	0.0	0.731
AK025245	12q22	KITLG	KIT ligand	-0.6	0.037	-0.6	0.135	-0.3	0.309
AL133063	20q13.33	C20orf158*	Chromosome 20 open reading frame 158*	-0.6	0.003	-0.1	0.485	-0.3	0.101
AK000445	12q13.3	HOXC9	homeobox C9	-0.6	0.020	-0.2	0.394	-0.4	0.252
NM_018171	12q24.1	APPL2	adaptor protein phosphotyrosine interaction PH domain and leucine zipper 2	-0.6	0.022	-0.5	0.101	-0.2	0.714
AL365369	19p13.3	NCLN	nicalin homolog zebrafish	-0.6	0.000	0.0	0.532	0.1	0.774
NM_014781	8p22-q21.13	RB1CC1	RB1-inducible coiled-coil 1	-0.6	0.037	-0.6	0.115	-0.6	0.328
AL049964	2	PS1TP4*	HBV preS1-transactivated protein 4*	-0.6	0.020	-0.8	0.044	-0.4	0.356
NM_013253	11p15.2	DKK3	dickkopf homolog 3 Xenopus laevis	-0.6	0.005	1.4	0.000	0.7	0.218
D63477	8q24.22	EFR3A	EFR3 homolog A S. cerevisiae	-0.6	0.037	-0.2	0.321	-0.1	0.725
AF216292	9q33-q34.1	HSPA5	heat shock 70kDa protein 5 glucose-regulated protein 78kDa	-0.6	0.009	0.0	0.515	-0.1	0.714

NM_004917	19q13.41	KLK4	kallikrein-related peptidase 4	-0.6	0.000	0.2	0.160	0.3	0.309
NM_016230	6p	CYB5R4	cytochrome b5 reductase 4	-0.6	0.020	-0.3	0.253	0.0	0.725
NM_016308	1p32	CMPK1	cytidine monophosphate (UMP-CMP) kinase 1, cytosolic	-0.6	0.046	-0.4	0.115	-0.2	0.531
NM_016286	17q25.3	DCXR	dicarbonyl/L-xylulose reductase	-0.6	0.009	0.1	0.358	0.0	0.789
NM_002600	1p31	PDE4B	phosphodiesterase 4B cAMP-specific phosphodiesterase E4 dunce homolog	-0.6	0.009	-0.6	0.090	-0.2	0.617
AK025205	4q13.3-q21.3	DKFZP564O0823	DKFZP564O0823 protein*	-0.5	0.037	-0.3	0.101	-0.3	0.272
AF250226	12q12-q13	ADCY6	adenylate cyclase 6	-0.5	0.005	-0.2	0.321	-0.1	0.691
NM_017947	18q12	MOCOS	molybdenum cofactor sulfurase	-0.5	0.055	-0.6	0.071	0.7	0.194
NM_020390	3q26.2	EIF5A2	eukaryotic translation initiation factor 5A2	-0.5	0.136	0.0	0.287	0.0	0.714
NM_006283	8p11	TACC1	transforming acidic coiled-coil containing protein 1	-0.5	0.022	-0.2	0.422	0.4	0.218
NM_005662	8p11.2	VDAC3	voltage-dependent anion channel 3	-0.5	0.028	0.1	0.444	-0.1	0.714
NM_001431	6q23	EPB41L2	erythrocyte membrane protein band 4.1-like 2	-0.5	0.018	0.1	0.253	0.1	0.738
NM_014158	Xq24	C1GALT1C1	C1GALT1-specific chaperone 1	-0.5	0.046	0.0	0.530	-0.1	0.657
NM_007114	3p21-p12	TMF1	TATA element modulatory factor 1	-0.5	0.157	-0.1	0.422	-0.3	0.500
NM_006330	8q11.23	LYPLA1	lysophospholipase I	-0.5	0.028	-0.6	0.090	-0.2	0.531
J04621	8q22-q23	SDC2	syndecan 2	-0.5	0.028	0.4	0.135	0.4	0.218
NM_020239	1q21.2	CDC42SE1	CDC42 small effector 1	-0.5	0.022	-0.2	0.160	-0.1	0.714
AK027200	15q25.2	WDR73	WD repeat domain 73	-0.5	0.028	0.2	0.253	-0.2	0.272
AL079292	5q11.2	DHX29	DEAH Asp-Glu-Ala-His box polypeptide 29	-0.5	0.211	-0.2	0.160	-0.2	0.531
AK024732	6q13	OGFRL1	opioid growth factor receptor-like 1	0.5	0.211	-0.1	0.444	0.3	0.218
AK022971		FLJ12909*	CDNA FLJ12909 fis clone NT2RP2004400*	0.5	0.113	-0.6	0.051	-0.1	0.725
NM_002795	17q12	PSMB3	proteasome prosome macropain subunit beta type 3	0.5	0.113	0.6	0.063	1.0	0.000
NM_001446	6q22-q23	FABP7	fatty acid binding protein 7 brain	0.5	0.086	0.1	0.394	0.2	0.500
NM_013240	21q21.3	N6AMT1	N-6 adenine-specific DNA methyltransferase 1 putative	0.5	0.018	0.3	0.101		
NM_004083	12q13.1-q13.2	DDIT3	DNA-damage-inducible transcript 3	0.5	0.055	-0.1	0.287	-0.6	0.150
X07695	12q12-q13	KRT4	keratin 4	0.6	0.157	0.1	0.358	0.2	0.471
NM_017446	21q21.3	MRPL39	mitochondrial ribosomal protein L39	0.6	0.113	0.2	0.358	-0.5	0.588
NM_014505	12q	KCNMB4	potassium large conductance calcium-activated channel subfamily M beta member 4	0.6	0.007	0.1	0.422		
NM_001605	16q22	AARS	alanyl-tRNA synthetase	0.6	0.100	0.4	0.185	0.3	0.356
NM_017707	1p36.12	DDEFL1	development and differentiation enhancing factor-like 1	0.6	0.007	0.2	0.358	0.0	0.731
NM_000187	3q21-q23	HGD	homogentisate 1 2-dioxygenase homogentisate oxidase	0.6	0.037	0.1	0.321	0.0	0.731
AL157459	17q25.3	CBX2	chromobox homolog 2 Pc class homolog Drosophila	0.6	0.028	0.2	0.253	-0.2	0.714
NM_005737	2q37.1	ARL4C	ADP-ribosylation factor-like 4C	0.6	0.020	0.1	0.473	0.4	0.287
NM_016183	1p36.13	MRTO4	mRNA turnover 4 homolog S. cerevisiae	0.6	0.113	0.1	0.253	-0.3	0.134
NM_006620	6q23-q24	HBS1L	HBS1-like S. cerevisiae	0.6	0.293	0.0	0.485	0.1	0.789
AK026290	15q15.3	ELL3	elongation factor RNA polymerase II-like 3	0.6	0.000	0.6	0.011	0.4	0.218
NM_004952	1q21-q22	EFNA3	ephrin-A3	0.6	0.055	0.3	0.321	0.2	0.236

M61872	7q11.21	ZNF92	zinc finger protein 92	0.6	0.007	-0.1	0.422		
NM_016594	12q13.12	FKBP11	FK506 binding protein 11 19 kDa	0.6	0.070	-0.1	0.394	0.2	0.218
NM_000903	16q22.1	NQO1	NAD P H dehydrogenase quinone 1	0.6	0.242	0.5	0.185	0.2	0.236
AF181999	10p12.2	PTF1A	pancreas specific transcription factor, 1a	0.6	0.293	-1.4	0.000	0.0	0.748
AF263547	1p32-p31	DAB1	disabled homolog 1 (Drosophila)	0.6	0.046	0.5	0.090	0.4	0.218
NM_004577	7p15.2-p15.1	PSPH	phosphoserine phosphatase	0.6	0.014	0.4	0.038	0.2	0.617
NM_013370	16q23.3	OSGIN1	oxidative stress induced growth inhibitor 1	0.6	0.000	0.5	0.101	-0.1	0.725
AF045229	10q25	RGS10	regulator of G-protein signaling 10	0.6	0.003	-0.2	0.185	0.2	0.617
NM_017966	11q12.2	VPS37C	vacuolar protein sorting 37 homolog C (S. cerevisiae)	0.6	0.007	-0.1	0.473	0.0	0.783
NM_018410	2q37.1	HJURP	Holliday junction recognition protein	0.6	0.037	0.1	0.444	0.0	0.789
NM_014811	9q34.3	KIAA0649	KIAA0649	0.6	0.000	0.1	0.394	0.1	0.771
AK021892	Xq13.1	PJA1	praja 1	0.6	0.028	0.4	0.160	0.3	0.384
NM_017661	15q21.3	ZNF280D	zinc finger protein 280D	0.6	0.014	0.1	0.444	0.7	0.218
AK022847	20q11.23	ACTR5	ARP5 actin-related protein 5 homolog yeast	0.6	0.043	0.3	0.229	0.1	0.500
NM_016835	17q21.1	MAPT	microtubule-associated protein tau	0.6	0.293	-0.2	0.358	-0.5	0.194
AK023807	2q12.1	ANAPC1	anaphase promoting complex subunit 1	0.7	0.136	0.2	0.253	0.1	0.725
Y11177	7p21.2	TWIST1	twist homolog 1 acrocephalosyndactyly 3 Saethre-Chotzen syndrome Drosophila	0.7	0.043	0.1	0.185	0.5	0.194
NM_001468	Xp11.23	GAGE1	G antigen 1	0.7	0.003	0.4	0.211	0.6	0.000
NM_017705	15q23	PAQR5	progestin and adipoQ receptor family member V	0.7	0.000	0.4	0.025	0.4	0.176
Y09836	5q13	MAP1B	microtubule-associated protein 1B	0.7	0.046	1.0	0.011	1.2	0.101
AB028949	1p36.21	KIAA1026*	KIAA1026 protein*	0.7	0.020	-0.2	0.090	0.3	0.556
NM_016115	2p16-p14	ASB3	ankyrin repeat and SOCS box-containing 3	0.7	0.018	0.1	0.473	0.2	0.678
NM_013275	16q24.3	ANKRD11	ankyrin repeat domain 11	0.7	0.179	0.3	0.115	0.2	0.413
D17222	3p14.3	TKT	transketolase Wernicke-Korsakoff syndrome	0.7	0.007	0.3	0.135	0.2	0.556
AK025974	11q12.2	CCDC86	coiled-coil domain containing 86	0.7	0.014	0.2	0.229	0.1	0.774
NM_015369	16p13	TP53TG3*	TP53TG3 protein*	0.7	0.007				
AF034102	11q13	SLC29A2	solute carrier family 29 nucleoside transporters member 2	0.7	0.007	0.7	0.038	0.7	0.089
NM_007158	1p22	CSDE1	cold shock domain containing E1 RNA-binding	0.7	0.157	0.7	0.031	0.9	0.194
AB040967	11p15.4	OSBPL5	oxysterol binding protein-like 5	0.7	0.086			0.1	0.471
NM_000476	9q34.1	AK1	adenylate kinase 1	0.7	0.022	0.4	0.115	0.2	0.272
Z69892			MRNA clone ICRFp507I1077*	0.7	0.005	0.3	0.185	0.0	0.789
NM_003213	12p13.2-13.3	TEAD4	TEA domain family member 4	0.7	0.014	0.2	0.287	0.2	0.218
NM_004763	2p25.2	ITGB1BP1	integrin beta 1 binding protein 1	0.7	0.022	0.5	0.160	0.2	0.356
D16935	9q21.2	PSAT1	phosphoserine aminotransferase 1	0.7	0.007	0.5	0.031	0.5	0.218
NM_018842	7q21.3-22.1	BAIAP2L1	BAI1-associated protein 2-like 1	0.7	0.113	0.3	0.229	0.4	0.218
NM_001145	14q11.1	ANG	angiogenin ribonuclease RNase A family 5	0.8	0.000	0.1	0.358	0.4	0.218
NM_005631	7q32.3	SMO	smoothened homolog Drosophila	0.8	0.018	0.3	0.160	0.7	0.089
NM_001928	19p13.3	CFD	complement factor D adipsin	0.8	0.157	0.3	0.321	0.4	0.272

NM_012463	12q24.31	ATP6V0A2	ATPase H transporting lysosomal V0 subunit a2	0.8	0.028	1.2	0.051	1.7	0.000
NM_018950	6p21.3	HLA-F	major histocompatibility complex class I F	0.8	0.014				
U35875	14q32.1-32.2	BDKRB2	bradykinin receptor B2	0.8	0.007	0.2	0.321	0.3	0.218
AK023343	14q11.2	RNASE7	ribonuclease RNase A family 7	0.8	0.037	0.4	0.160	0.4	0.218
NM_012168	1p36.22	FBXO2	F-box protein 2	0.8	0.000	0.0	0.459	0.1	0.731
NM_006044	Xp11.23	HDAC6	histone deacetylase 6	0.8	0.020	0.1	0.444	0.3	0.328
NM_003662	Xp22.2	PIR	pirin iron-binding nuclear protein	0.8	0.007	0.5	0.021	0.5	0.194
NM_004490	2q22-q24	GRB14	growth factor receptor-bound protein 14	0.8	0.100	0.3	0.253	0.5	0.218
AK022845	9q34.13-34.3	C9orf58	chromosome 9 open reading frame 58	0.8	0.014	0.2	0.287	0.2	0.657
AL137663	3q13.2	PHLDB2	pleckstrin homology-like domain family B member 2	0.8	0.043	-0.2	0.135	0.3	0.176
AF086471	11p15.1	SPTY2D1	SPT2 Suppressor of Ty domain containing 1 S. cerevisiae	0.8	0.100	-0.1	0.515	0.0	0.731
AK026812	1q32.3	LOC91548*	Hypothetical protein LOC91548*	0.8	0.020	-0.1	0.473	0.1	0.748
NM_003680	1p35.1	YARS	tyrosyl-tRNA synthetase	0.8	0.014	0.1	0.358	0.4	0.236
NM_001321	12q21.1	CSRP2	cysteine and glycine-rich protein 2	0.8	0.014	-0.3	0.135	0.3	0.328
D17169				0.8	0.070	-0.1	0.506	0.2	0.755
NM_000309	1q22	PPOX	protoporphyrinogen oxidase	0.8	0.020	0.2	0.321	0.0	0.725
NM_014683	17p11.2	ULK2	unc-51-like kinase 2 C. elegans	0.8	0.000	0.3	0.090	-0.7	0.053
NM_000376	12q12-14	VDR	vitamin D 1 25- dihydroxyvitamin D3 receptor	0.9	0.037	0.2	0.321	0.0	0.781
NM_013401	11q12-q13.1	RAB3IL1	RAB3A interacting protein rabin3 -like 1	0.9	0.000	-0.2	0.253	-0.1	0.714
AL157449	17q21.33	PPP1R9B	protein phosphatase 1 regulatory inhibitor subunit 9B	0.9	0.007	0.9	0.000	0.6	0.218
AF086393	10q26.11	NANOS1	nanos homolog 1 Drosophila	0.9	0.037	0.2	0.321	0.0	0.471
AK025627	18q11.2	CABLES1	Cdk5 and Abl enzyme substrate 1	0.9	0.018	0.2	0.229	0.5	0.218
AF088076		cDNA *	Full length insert cDNA clone ZE01A04*	0.9	0.003	-0.2	0.253	0.0	0.789
NM_003648	2q37.1	DGKD	diacylglycerol kinase delta 130kDa	0.9	0.000	0.3	0.185	0.3	0.617
AK023159	5q33.3	LSM11	LSM11 U7 small nuclear RNA associated	0.9	0.003	0.1	0.422	0.5	0.218
AF086924	4p16.1	PPP2R2C	protein phosphatase 2 formerly 2A regulatory subunit B gamma isoform	0.9	0.000			0.0	0.725
AK023178	5p15.33	EXOC3	exocyst complex component 3	0.9	0.003	0.2	0.185	0.2	0.617
NM_014883	4q22.1	FAM13A1	family with sequence similarity 13 member A1	0.9	0.003	0.1	0.287	0.0	0.678
AB037810	1q42.2	SIPA1L2	signal-induced proliferation-associated 1 like 2	0.9	0.046	1.5	0.000	1.7	0.049
NM_018303	6p25.3	EXOC2	exocyst complex component 2	0.9	0.000	0.2	0.253	0.2	0.636
NM_004282	6p12.3-p11.2	BAG2	BCL2-associated athanogene 2	0.9	0.020	0.4	0.044	0.7	0.194
Y11161	17p13.1	EIF4A1	eukaryotic translation initiation factor 4A, isoform 1	0.9	0.003	0.9	0.038	0.4	0.218
AK021498	7q22.3	FLJ36031*	Hypothetical protein FLJ36031*	0.9	0.028	-0.1	0.321	0.1	0.755
U17632				1.0	0.003	1.4	0.008	1.1	0.194
NM_001814	11q14.1-q14.3	CTSC	cathepsin C	1.0	0.000				
NM_014061	Xp11.22	MAGEH1	melanoma antigen family H 1	1.0	0.020	-0.5	0.090	0.0	0.725
NM_003243	1p33-p32	TGFBR3	transforming growth factor beta receptor III	1.0	0.000	0.1	0.287	0.5	0.236
U90878	10q22-q26.3	PDLIM1	PDZ and LIM domain 1 elfin	1.0	0.000	0.4	0.135	0.4	0.287

U58096	Yp11.2	TSPY1	testis specific protein Y-linked 1	1.0	0.037	1.4	0.000		
NM_003234	3q29	TFRC	transferrin receptor p90 CD71	1.1	0.000	0.8	0.025	0.4	0.218
AB049629				1.1	0.000	0.0	0.515	0.3	0.588
NM_005311	7p12-p11.2	GRB10	growth factor receptor-bound protein 10	1.1	0.028	0.7	0.008	0.5	0.218
NM_003712	19p13	PPAP2C	phosphatidic acid phosphatase type 2C	1.2	0.000	0.9	0.044	1.2	0.000
L36587				1.3	0.000	0.2	0.358	-0.2	0.714
NM_001902	1p31.1	CTH	cystathionase cystathionine gamma-lyase	1.3	0.020	0.5	0.044	0.7	0.194
AL049949	10q22.3	C10orf56	chromosome 10 open reading frame 56	1.3	0.000	0.5	0.048	0.4	0.218
AK026892	22q13.31	CERK	ceramide kinase	1.4	0.000	0.0	0.321	0.5	0.218
NM_006721	10q22	ADK	adenosine kinase	1.4	0.000	0.1	0.422	0.7	0.194
NM_005804	19p13.12	DDX39	DEAD Asp-Glu-Ala-Asp box polypeptide 39	1.4	0.000	0.3	0.253	0.8	0.194
NM_005794	14q11.2	DHRS2	dehydrogenase/reductase SDR family member 2	1.4	0.003	1.5	0.071	1.2	0.051
NM_014269	4q34	ADAM29	ADAM metallopeptidase domain 29	1.5	0.000	1.4	0.011	2.2	0.000
NM_016084	17p11.2	RASD1	RAS dexamethasone-induced 1	1.6	0.000	1.2	0.000	0.5	0.218
NM_006332	19p13.1	IFI30	interferon gamma-inducible protein 30	1.7	0.000	1.1	0.025	0.7	0.218
NM_001823	14q32	CKB	creatine kinase brain	1.7	0.000	1.5	0.015	1.6	0.000
NM_000790	7p11	DDC	dopa decarboxylase aromatic L-amino acid decarboxylase	1.9	0.005	0.1	0.253	2.4	0.218
NM_003226	21q22.3	TFF3	trefoil factor 3 intestinal	1.9	0.000	-0.5	0.211	1.5	0.049
NM_006113	1p13.3	VAV3	vav 3 guanine nucleotide exchange factor	2.0	0.000	0.2	0.031	0.4	0.218
NM_004750	19p12	CRLF1	cytokine receptor-like factor 1	2.1	0.000				
NM_013452	Xp22	VCX	variable charge X-linked	2.3	0.000	1.6	0.015	1.7	0.049
NM_004982	12p11.23	KCNJ8	potassium inwardly-rectifying channel subfamily J member 8	2.7	0.000	1.2	0.000	1.9	0.000

* no approved HUGO symbol / name exist for this entry. If present, gene symbol / name from the UNIGENE database are given in alternative. 2log_ratio>0 indicates over-expression in the androgen-independent subline, compared to parental PC346C. 2log_ratio<0 indicates down-regulation in the androgen-independent subline.

Table S2 - Genes differentially expressed in PC346Flu1 vs PC346C expression ratios and SAM q-values

GenBank ID	Cytoband	Symbol	HUGO Gene Name	PC346DCC		PC346Flu1		PC346Flu2	
				2log ratio	qvalue	2log ratio	qvalue	2log ratio	qvalue
AF216077	9q32	COL27A1	collagen type XXVII alpha 1	-2.1	0.000	-2.8	0.000	-1.7	0.053
NM_014380	Xq22.2	NGFRAP1	nerve growth factor receptor TNFRSF16 associated protein 1	-0.2	0.513	-2.5	0.000	-2.7	0.000
AB020968	6q22.2	MARCKS	myristoylated alanine-rich protein kinase C substrate	-1.6	0.000	-2.0	0.000	-1.3	0.053
NM_014333	11q23.2	CADM1	cell adhesion molecule 1	-1.0	0.000	-1.9	0.000	-0.1	0.725
NM_017935	4q24	BANK1	B-cell scaffold protein with ankyrin repeats 1	-2.9	0.000	-1.8	0.000	-0.8	0.053

X75684	3q21-q25	TM4SF1	transmembrane 4 L six family member 1	0.6	0.211	-1.5	0.000	0.5	0.194
AK024917	1p22	DDAH1	dimethylarginine dimethylaminohydrolase 1	-0.3	0.380	-1.5	0.000	0.1	0.760
AK000554	15q26.1	ABHD2	abhydrolase domain containing 2	-1.7	0.000	-1.5	0.000	-0.2	0.657
AL050367	10p13	C10orf38	chromosome 10 open reading frame 38	0.1	0.380	-1.4	0.000	-0.6	0.084
AL049963	4q22-q24	SLC39A8	solute carrier family 39 zinc transporter member 8	-1.5	0.000	-1.4	0.000	-0.9	0.053
AF181999	10p12.2	PTF1A	pancreas specific transcription factor, 1a	0.6	0.293	-1.4	0.000	0.0	0.748
NM_000213	17q25	ITGB4	integrin beta 4	0.9	0.086	-1.3	0.000	0.1	0.777
AK026517	11p12	EHF	ets homologous factor	-0.7	0.028	-1.3	0.000	-0.6	0.309
NM_019005	7p22-p21	FLJ20323*	Hypothetical protein FLJ20323*	-0.9	0.000	-1.3	0.000	-0.6	0.134
NM_003278	3p22-p21.3	CLEC3B	C-type lectin domain family 3 member B	-1.5	0.005	-1.3	0.000	-1.3	0.084
AF200348	2p25	PXDN	peroxidasin homolog Drosophila	-1.2	0.003	-1.3	0.000	0.9	0.064
AL122055	6q21	CDC2L6	cell division cycle 2-like 6 CDK8-like	-1.4	0.000	-1.3	0.000	-1.2	0.000
NM_003255	17q25	TIMP2	TIMP metallopeptidase inhibitor 2	0.6	0.028	-1.3	0.031	-0.2	0.678
NM_012116	19q13.2	CBLC	Cas-Br-M murine ecotropic retroviral transforming sequence c	-1.6	0.000	-1.2	0.000	-0.4	0.218
NM_017860	1q21.2	C1orf56	chromosome 1 open reading frame 56	-0.5	0.037	-1.2	0.000	-0.6	0.053
NM_000944	4q21-q24	PPP3CA	protein phosphatase 3 formerly 2B catalytic subunit alpha isoform	-1.4	0.028	-1.2	0.000	-1.0	0.084
D80010	2p25.1	LPIN1	lipin 1	-1.2	0.000	-1.1	0.015	-0.9	0.053
NM_016598	3p21.31	ZDHHC3	zinc finger DHHC-type containing 3	-1.4	0.003	-1.1	0.000	-0.9	0.053
NM_018050	12p13.2	MANSC1	MANSC domain containing 1	-0.9	0.000	-1.1	0.011		
NM_005709	11p15.1-p14	USH1C	Usher syndrome 1C autosomal recessive severe	-0.5	0.136	-1.1	0.000	-0.9	0.053
D28449	1p36.13-p36.12	ID3	inhibitor of DNA binding 3 dominant negative helix-loop-helix protein	-0.8	0.070	-1.1	0.042	-0.3	0.714
NM_001359	8q21.3	DECR1	2 4-dienoyl CoA reductase 1 mitochondrial	-1.9	0.000	-1.1	0.000	-1.6	0.053
NM_003688	Xp11.4	CASK	calcium/calmodulin-dependent serine protein kinase MAGUK family	0.0	0.617	-1.1	0.042	0.5	0.218
NM_017720	19p13.3	STAP2	signal transducing adaptor family member 2	-1.0	0.000	-1.1	0.000	-0.8	0.053
AK026654	2q31.1	CYBRD1	cytochrome b reductase 1	-0.1	0.638	-1.0	0.000	-0.1	0.714
AK023221	6q12-q13	SMAP1	stromal membrane-associated GTPase-activating protein 1	0.1	0.466	-1.0	0.000	0.1	0.617
NM_018843	7q21.12	SLC25A40	solute carrier family 25 member 40	0.1	0.438	-1.0	0.008	-0.2	0.636
NM_005596	9p24.1	NFIB	nuclear factor I/B	-0.5	0.046	-1.0	0.000	-0.4	0.120
AL109729	15q26.1	ABHD2	abhydrolase domain containing 2	-1.5	0.000	-1.0	0.044	0.2	0.531
X69111	1p36.13-p36.12	ID3	inhibitor of DNA binding 3 dominant negative helix-loop-helix protein	-0.9	0.055	-1.0	0.101	-0.3	0.714
NM_002414	Xp22.32	CD99	CD99 molecule	0.0	0.624	-1.0	0.042	-0.6	0.000
NM_012449	7q21	STEAP1	six transmembrane epithelial antigen of the prostate 1	-2.3	0.000	-0.9	0.036	-0.5	0.236
AB023182	12p11.23	STK38L	serine/threonine kinase 38 like	-0.2	0.534	-0.9	0.044	-0.4	0.272
NM_004772	5q22.1	C5orf13	chromosome 5 open reading frame 13	-0.4	0.380	-0.9	0.015	-0.5	0.120
AF085880	5q31.1	C5orf24	chromosome 5 open reading frame 24	-0.5	0.028	-0.9	0.048	-0.6	0.089
NM_007173	11q14.1	PRSS23	protease serine 23	-1.2	0.018	-0.9	0.021	-0.9	0.101
NM_001758				-1.0	0.000	-0.9	0.048	-1.4	0.000
NM_017992	1q24-q25	EDEM3	ER degradation enhancer, mannosidase alpha-like 3	-0.5	0.028	-0.9	0.042	-0.2	0.588

AJ277587	18p11.21	SPIRE1	spire homolog 1 Drosophila	-0.2	0.493	-0.9	0.036	0.4	0.218
NM_002607	7p22	PDGFA*	Platelet-derived growth factor alpha polypeptide*	-0.4	0.018	-0.9	0.071	-0.2	0.120
NM_016613	4q32.1	C4orf18	chromosome 4 open reading frame 18	-0.7	0.037	-0.9	0.063	-0.4	0.101
AL133555	20q13.31	C20orf108	chromosome 20 open reading frame 108	-0.7	0.046	-0.9	0.031	-0.7	0.084
NM_003711	5q11	PPAP2A	phosphatidic acid phosphatase type 2A	-1.1	0.018	-0.8	0.015	0.0	0.789
NM_002487	15q11.2-q12	NDN	necdin homolog mouse	-1.1	0.000	-0.8	0.063	-0.5	0.101
NM_002412	10q26	MGMT	O-6-methylguanine-DNA methyltransferase	0.0	0.624	-0.8	0.008	0.0	0.731
AK002197	12p11.22	KLHDC5	kelch domain containing 5	-0.2	0.513	-0.8	0.015	-0.2	0.714
NM_007011	15q26.1	ABHD2	abhydrolase domain containing 2	-1.3	0.000	-0.8	0.051	0.3	0.531
AK026062	10p12.31	DNAJC1	DnaJ Hsp40 homolog subfamily C member 1	-0.3	0.100	-0.8	0.000	-0.5	0.089
NM_012096	3p21.1-p14.3	APPL1	adaptor protein phosphotyrosine interaction PH domain and leucine zipper containing 1	-0.5	0.100	-0.8	0.115	-0.3	0.218
AK026331	2q35	CHPF*	Chondroitin polymerizing factor*	-1.7	0.000	-0.8	0.048	-0.2	0.678
NM_002391	11p11.2	MDK*	Midkine neurite growth-promoting factor 2*	0.0	0.645	-0.8	0.051	0.7	0.218
AK024175	5p15.33	PDCD6	programmed cell death 6	-0.2	0.513	-0.8	0.044	-0.1	0.725
AK025697	3q29	FBXO45	F-box protein 45	-0.3	0.380	-0.8	0.000	-0.5	0.252
AL049964		PS1TP4	HBV preS1-transactivated protein 4*	-0.6	0.020	-0.8	0.044	-0.4	0.356
AL117661	10p15.1	TAF3	TAF3 RNA polymerase II TATA box binding protein TBP -associated factor 140kDa	-0.1	0.534	-0.8	0.000	-0.4	0.089
NM_015973	11q13.2	GAL	galanin prepropeptide	0.6	0.179	-0.8	0.000	0.1	0.738
AF295378	3q13	MAGEF1	melanoma antigen family F 1	-0.7	0.100	-0.8	0.000	-0.4	0.089
AK024677	4q21.1	ASAHL	N-acylsphingosine amidohydrolase acid ceramidase -like	-0.9	0.009	-0.8	0.044	-0.3	0.531
AJ011910			MRNA sequence IMAGE clone 193253*	0.3	0.340	-0.8	0.025	-0.1	0.725
AF131846	2p13	GFPT1	glutamine-fructose-6-phosphate transaminase 1	0.3	0.242	-0.7	0.000	-0.1	0.714
NM_018132	6p12.3	CENPQ	centromere protein Q	-0.3	0.211	-0.7	0.044	-0.5	0.176
NM_012211	15q23	ITGA11	integrin alpha 11	-0.2	0.493	-0.7	0.011	-0.4	0.471
NM_002156	2q33.1	HSPD1	heat shock 60kDa protein 1 chaperonin	0.0	0.617	-0.7	0.011	-0.1	0.714
NM_001873	4q32.3	CPE	carboxypeptidase E	-0.4	0.022	-0.7	0.044	-0.5	0.053
AB033034	12q23.3	GNPTAB	N-acetylglucosamine-1-phosphate transferase alpha and beta subunits	-0.5	0.037	-0.7	0.005	-0.7	0.064
AL049365	9q34.11	FAM102A	family with sequence similarity 102, member A	-0.2	0.513	-0.7	0.051	-0.5	0.084
AJ227869	Xp11.21	ZXDB	zinc finger X-linked duplicated B	-0.4	0.340	-0.7	0.048	-0.7	0.384
AY007149	1p36.13-q41	CEP350	centrosomal protein 350kDa	-0.1	0.589	-0.7	0.051	-0.5	0.309
AL049265	5q11	IL6ST	interleukin 6 signal transducer gp130 oncostatin M receptor	-0.6	0.070	-0.7	0.080	-0.9	0.053
AK000028	4q24	FLJ20021*	Hypothetical LOC90024*	-1.5	0.000	-0.7	0.000	-0.5	0.120
AF067972	2p23	DNMT3A	DNA cytosine-5- -methyltransferase 3 alpha	-0.8	0.005	-0.7	0.063	-0.5	0.101
NM_005079	8q21	TPD52	tumor protein D52	-0.4	0.293	-0.7	0.025	-0.6	0.442
AF271070	12q13.11	SLC38A1	solute carrier family 38 member 1	-1.3	0.000	-0.7	0.044	-0.2	0.678
NM_002822	12q12	TWF1	twinfilin actin-binding protein homolog 1 Drosophila	-0.2	0.534	-0.7	0.044	-0.2	0.678

NM_017606	8p21.1	ZNF395	zinc finger protein 395	-0.3	0.380	-0.7	0.000	-0.4	0.588
AB019565	1p35	IFI6	interferon, alpha-inducible protein 6	-0.1	0.631	-0.7	0.063	0.0	0.731
AL355720	21q22.3	AGPAT3	1-acylglycerol-3-phosphate O-acyltransferase 3	-0.1	0.513	-0.7	0.000	-0.2	0.500
NM_020317	1p36.13-p35.1	C1orf63	chromosome 1 open reading frame 63	-0.3	0.513	-0.7	0.051	0.0	0.725
NM_014141	7q35-q36	CNTNAP2	contactin associated protein-like 2	-0.3	0.293	-0.7	0.025	-0.2	0.556
U95646	15q21-q22	PPIB	peptidylprolyl isomerase B (cyclophilin B)	-0.1	0.573	-0.7	0.036	-0.5	0.115
AK021818	11q21-q22.1	DYNC2H1	dynein cytoplasmic 2 heavy chain 1	-0.4	0.070	-0.7	0.042	-0.5	0.101
NM_005496	3q26.1	SMC4	structural maintenance of chromosomes 4	-0.4	0.136	-0.7	0.048	-0.6	0.120
NM_001733	12p13	C1R	complement component 1 r subcomponent	0.0	0.589	-0.7	0.042	-0.3	0.328
AB033007	5q35.1	ERGIC1	endoplasmic reticulum-golgi intermediate compartment ERGIC 1	-0.3	0.157	-0.7	0.042	-0.1	0.725
NM_001089	16p13.3	ABCA3	ATP-binding cassette sub-family A ABC1 member 3	-0.5	0.070	-0.7	0.071	-0.4	0.194
AK023395	4q12	FIP1L1	FIP1 like 1 S. cerevisiae	0.1	0.573	-0.6	0.015	-0.3	0.500
AL137300	Xp22.33	DHRSX*	Dehydrogenase reductase SDR family X-linked*	-0.2	0.412	-0.6	0.015	-0.4	0.101
NM_018465	9p24.1	C9orf46	chromosome 9 open reading frame 46	0.0	0.645	-0.6	0.048	0.0	0.725
AK025133	1q32.1	NUCKS1	nuclear casein kinase and cyclin-dependent kinase substrate 1	0.0	0.605	-0.6	0.054	-0.1	0.725
AF301222	11p14.1	CCDC34	coiled-coil domain containing 34	-0.3	0.380	-0.6	0.044	0.3	0.500
NM_004183	11q13	BEST1	bestrophin 1	-0.1	0.629	-0.6	0.042	-0.2	0.678
AK023397	3p21.31	FYCO1	FYVE and coiled-coil domain containing 1	-0.2	0.558	-0.6	0.042	-0.5	0.089
NM_007212	1q25.3	RNF2	ring finger protein 2	0.1	0.558	-0.6	0.021	-0.2	0.657
NM_003851	1q24	CREG1	cellular repressor of E1A-stimulated genes 1	0.2	0.340	-0.6	0.011	-0.2	0.714
AL049229	3q29	SENP5	SUMO1/sentrin specific peptidase 5	-0.2	0.466	-0.6	0.048	-0.1	0.714
AK025766	12q24.31	BRI3BP	BRI3 binding protein	-0.7	0.014	-0.6	0.025	-0.1	0.714
NM_002561	17p13.3	P2RX5	purinergic receptor P2X ligand-gated ion channel 5	-0.4	0.380	-0.6	0.054	0.0	0.789
M83822	4q31.23-q31.3	LRBA	LPS-responsive vesicle trafficking beach and anchor containing	-1.1	0.003	-0.6	0.044	-0.6	0.101
AK001843	2q33.1	FAM126B	family with sequence similarity 126 member B	-0.1	0.573	-0.6	0.101	-0.7	0.064
AK022971			CDNA FLJ12909 fis clone NT2RP2004400*	0.5	0.113	-0.6	0.051	-0.1	0.725
AK021958	15q22.2	NARG2	NMDA receptor regulated 2	0.1	0.558	-0.6	0.015	-0.3	0.588
NM_001498	6p12	GCLC	glutamate-cysteine ligase catalytic subunit	-0.2	0.534	-0.6	0.044	-0.2	0.691
NM_018155	3q23	SLC25A36	solute carrier family 25 member 36	0.3	0.380	-0.6	0.021	-0.2	0.309
AF007128	12p13.33	FBXL14	F-box and leucine-rich repeat protein 14	-0.2	0.438	-0.6	0.115	-0.3	0.531
U03886	Xp22.3	PNPLA4	patatin-like phospholipase domain containing 4	0.3	0.293	-0.6	0.025	0.4	0.218
AK001036	5q31	SMAD5	SMAD family member 5	-0.3	0.211	-0.6	0.071	-0.4	0.101
NM_002600	1p31	PDE4B	phosphodiesterase 4B cAMP-specific phosphodiesterase E4 dunce homolog Drosophila	-0.6	0.009	-0.6	0.090	-0.2	0.617
NM_006287	2q31-q32.1	TFPI	tissue factor pathway inhibitor lipoprotein-associated coagulation inhibitor	-0.1	0.629	-0.6	0.011	-0.2	0.714
AL117612	8q23	MAL2	mal T-cell differentiation protein 2	-0.3	0.136	-0.6	0.063	-0.3	0.218
AK023269	2p23.1	LCLAT1	lysocardiolipin acyltransferase 1	-0.6	0.043	-0.5	0.135	-0.3	0.714
AF272357	9q34.3	NPDC1	neural proliferation differentiation and control 1	-0.5	0.070	-0.5	0.090	-0.4	0.176

D70836	19p12	ZNF714	zinc finger protein 714	0.3	0.242	-0.5	0.115	-0.4	0.272
D14678	6p21.3	KIFC1	kinesin family member C1	0.0	0.589	-0.5	0.031	-0.4	0.064
NM_002948	3p24.2	RPL15	ribosomal protein L15	-0.3	0.380	-0.5	0.015	-0.4	0.084
AF131814	1p36.12-p35.1	NPAL3	NIPA-like domain containing 3	-0.4	0.113	-0.5	0.135	-0.5	0.101
NM_003676	1q42.12	DEGS1	degenerative spermatocyte homolog 1 lipid desaturase Drosophila	0.8	0.020	-0.5	0.160	-0.1	0.725
NM_018660	8p21.1	ZNF395	zinc finger protein 395	-0.5	0.022	-0.5	0.005	-0.2	0.691
NM_004864	19p13.1-13.2	GDF15	growth differentiation factor 15	-0.4	0.513	-0.5	0.160	0.7	0.194
AL049998	11p15.5-p14	PIK3C2A	phosphoinositide-3-kinase class 2 alpha polypeptide	-0.4	0.157	-0.5	0.160	-0.6	0.328
AL133057	7q22.1	LRWD1	leucine-rich repeats and WD repeat domain containing 1	0.1	0.412	0.5	0.054	0.5	0.194
NM_001998	3p25.1	FBLN2	fibulin 2			0.5	0.287		
NM_001513	14q24.3	GSTZ1	glutathione transferase zeta 1 maleylacetoacetate isomerase	0.1	0.493	0.5	0.080	0.2	0.413
NM_017745	Xp11.4	BCOR	BCL6 co-repressor	0.1	0.573	0.5	0.051	0.2	0.471
M27544	12q22-q23	IGF1	insulin-like growth factor 1 somatomedin C	-0.4	0.028	0.5	0.080	0.0	0.789
NM_000269	17q21.3	NME1	non-metastatic cells 1 protein NM23A expressed in	-0.2	0.493	0.5	0.090	0.1	0.471
AK024100	9q34.11	PTGES2	prostaglandin E synthase 2	0.0	0.605	0.5	0.044	0.2	0.531
AK023459	10p13	C10orf97	chromosome 10 open reading frame 97	-0.3	0.513	0.5	0.031	0.0	0.725
NM_001269	1p36.1	RCC1	regulator of chromosome condensation 1	0.4	0.293	0.6	0.160	0.3	0.531
NM_014846	8p22	KIAA0196	KIAA0196	0.6	0.022	0.6	0.044	0.5	0.194
S81522	2q33-q34	EEF1B1	eukaryotic translation elongation factor 1 beta 1	0.1	0.589	0.6	0.071	0.5	0.328
NM_005246	5q21	FER	fer fps/fes related tyrosine kinase phosphoprotein NCP94	0.6	0.037	0.6	0.000	0.1	0.748
NM_017870	11q12.2	TMEM132A	transmembrane protein 132A	0.3	0.211	0.6	0.071	0.3	0.442
NM_015940	14q24.3	COQ6	coenzyme Q6 homolog, monooxygenase (S. cerevisiae)	0.3	0.242	0.6	0.000	0.1	0.766
NM_000908	5p14-p13	NPR3	atrionatriuretic peptide receptor C guanylate cyclase C			0.6	0.011		
D50914	8q24.3	BOP1	block of proliferation 1	-0.4	0.000	0.6	0.015	0.2	0.218
NM_006432	14q24.3	NPC2	Niemann-Pick disease type C2	-0.1	0.534	0.6	0.031	-0.1	0.714
NM_014501	19q13.43	UBE2S	ubiquitin-conjugating enzyme E2S	0.5	0.086	0.6	0.080	0.6	0.064
NM_007314	1q24-q25	ABL2	v-abl Abelson murine leukemia viral oncogene homolog 2 arg Abelson-related gene	0.7	0.037	0.6	0.054	0.5	0.218
NM_006516	1p35-p31.3	SLC2A1	solute carrier family 2 facilitated glucose transporter member 1	0.1	0.558	0.6	0.063	0.1	0.774
NM_003900	5q35	SQSTM1	sequestosome 1	0.1	0.380	0.6	0.063	0.2	0.218
AK026290	15q15.3	ELL3	elongation factor RNA polymerase II-like 3	0.6	0.000	0.6	0.011	0.4	0.218
NM_014731	20p13	ProSAPiP1*	ProSAPiP1 protein*	-0.5	0.157	0.6	0.115	-0.4	0.120
NM_001757	21q22.13	CBR1	carbonyl reductase 1	0.4	0.242	0.6	0.135	0.3	0.531
NM_002795	17q12	PSMB3	proteasome prosome macropain subunit beta type 3	0.5	0.113	0.6	0.063	1.0	0.000
NM_000190	11q23.3	HMBS	hydroxymethylbilane synthase	0.1	0.513	0.6	0.071	0.3	0.194
M96843	3p21.1	ID2B	inhibitor of DNA binding 2B dominant negative helix-loop-helix protein	0.0	0.624	0.6	0.031	0.0	0.789
NM_004804	2q11.2	CIAO1	cytosolic iron-sulfur protein assembly 1 homolog S. cerevisiae	0.5	0.242	0.6	0.038	0.2	0.309
Y11162	19p13	SNORA68	small nucleolar RNA H/ACA box 68	0.4	0.211	0.7	0.031	0.1	0.218
AK023856	2p16.1	LOC339803*	Hypothetical protein LOC339803*	0.2	0.293	0.7	0.021	0.5	0.194

AF034102	11q13	SLC29A2	solute carrier family 29 nucleoside transporters member 2	0.7	0.007	0.7	0.038	0.7	0.089
Y11159	9q34	RPL12	ribosomal protein L12			0.7	0.031		
U87169		STYX	serine/threonine/tyrosine interacting protein	0.2	0.340	0.7	0.054	0.4	0.218
D83782	3p21.31	SCAP	SREBF chaperone	0.2	0.412	0.7	0.054	0.2	0.617
NM_004184	14q32.31	WARS	tryptophanyl-tRNA synthetase	0.1	0.412	0.7	0.063	0.1	0.471
AK024665	11q13	MUS81	MUS81 endonuclease homolog S. cerevisiae	0.5	0.070	0.7	0.011	0.6	0.218
NM_005311	7p12-p11.2	GRB10	growth factor receptor-bound protein 10	1.1	0.028	0.7	0.008	0.5	0.218
NM_016539	19p13.3	SIRT6	sirtuin silent mating type information regulation 2 homolog 6 S. cerevisiae	0.2	0.293	0.7	0.025	0.3	0.413
NM_002455	1q21	MTX1	metaxin 1	0.5	0.046	0.7	0.036	0.3	0.328
AF190900	1q32.1	KLHL12	kelch-like 12 Drosophila	0.2	0.466	0.7	0.000	1.0	0.194
NM_000713	19q13.1-q13.2	BLVRB	biliverdin reductase B flavin reductase NADPH	-0.1	0.589	0.7	0.008	0.1	0.789
NM_004039	15q21-q22	ANXA2	annexin A2	-0.3	0.136	0.7	0.080	0.1	0.785
NM_007158	1p22	CSDE1	cold shock domain containing E1 RNA-binding	0.7	0.157	0.7	0.031	0.9	0.194
AK026298	15q21.1	C15orf48	chromosome 15 open reading frame 48			0.7	0.015		
NM_007111	13q34	TFDP1	transcription factor Dp-1	0.7	0.018	0.8	0.048	0.5	0.218
AL050024	7p21.1	SOSTDC1	sclerostin domain containing 1	-0.1	0.493	0.8	0.038	0.1	0.755
NM_001916	8q24.3	CYC1	cytochrome c-1	0.0	0.573	0.8	0.063	0.1	0.356
NM_020411	Xp11.22-p11.21	XAGE1	X antigen family member 1	0.3	0.003	0.8	0.038		
NM_003234	3q29	TFRC	transferrin receptor p90 CD71	1.1	0.000	0.8	0.025	0.4	0.218
M60721	1q41-q42.1	HLX	H2.0-like homeobox	0.3	0.293	0.8	0.063	0.3	0.218
L29496	Xq11.2-q12	AR	androgen receptor dihydrotestosterone receptor	-1.3	0.018	0.9	0.000	-0.3	0.194
NM_003523	6p21.3	HIST1H2BE	histone cluster 1 H2be	0.5	0.113	0.9	0.021	0.4	0.218
AL133572	12q24.31	FBXL10	F-box and leucine-rich repeat protein 10	0.5	0.086	0.9	0.005	1.1	0.049
AJ223352	6p21.33	HIST1H2BK	histone cluster 1 H2bk	0.2	0.340	0.9	0.025	0.3	0.356
NM_000849	1p13.3	GSTM3	glutathione S-transferase M3 brain	0.6	0.086	0.9	0.000	0.9	0.176
Y11161	17p13.1	EIF4A1	eukaryotic translation initiation factor 4A, isoform 1	0.9	0.003	0.9	0.038	0.4	0.218
NM_001831	8p21-p12	CLU	clusterin	0.1	0.534	0.9	0.000	0.7	0.049
NM_003311	11p15.5	PHLDA2	pleckstrin homology-like domain family A member 2	0.0	0.624	0.9	0.011	0.8	0.194
AL157449	17q21.33	PPP1R9B	protein phosphatase 1 regulatory inhibitor subunit 9B	0.9	0.007	0.9	0.000	0.6	0.218
NM_003712	19p13	PPAP2C	phosphatidic acid phosphatase type 2C	1.2	0.000	0.9	0.044	1.2	0.000
NM_001327	Xq28	CTAG1A	cancer/testis antigen 1A	0.6	0.046	0.9	0.000	0.6	0.194
NM_003524	6p21.3	HIST1H2BH	histone cluster 1 H2bh	0.2	0.380	0.9	0.000	0.2	0.384
NM_003525	6p21.3	HIST1H2BI	histone cluster 1, H2bi	0.3	0.293	1.0	0.008	0.1	0.789
Y09836	5q13	MAP1B	microtubule-associated protein 1B	0.7	0.046	1.0	0.011	1.2	0.101
AF086251	11q21	SESN3	sestrin 3	0.1	0.534	1.1	0.048	1.1	0.049
NM_006332	19p13.1	IFI30	interferon gamma-inducible protein 30	1.7	0.000	1.1	0.025	0.7	0.218
Y11158	16p13.3	SNORA10	small nucleolar RNA H/ACA box 10	-0.1	0.513	1.1	0.000	0.5	0.218
L07383	3p21.3	RPSA	ribosomal protein SA	0.1	0.513	1.2	0.071		

NM_016084	17p11.2	RASD1	RAS dexamethasone-induced 1	1.6	0.000	1.2	0.000	0.5	0.218
NM_012463	12q24.31	ATP6V0A2	ATPase H transporting lysosomal V0 subunit a2	0.8	0.028	1.2	0.051	1.7	0.000
NM_004982	12p11.23	KCNJ8	potassium inwardly-rectifying channel subfamily J member 8	2.7	0.000	1.2	0.000	1.9	0.000
NM_003516	1q21.2	HIST2H2AA3	histone cluster 2 H2aa3	-0.1	0.645	1.2	0.000	0.8	0.194
NM_016639	16p13.3	TNFRSF12A	tumor necrosis factor receptor superfamily member 12A	0.6	0.028	1.3	0.000	0.5	0.218
S67154	3q28	EIF4A2	eukaryotic translation initiation factor 4A isoform 2	0.1	0.589	1.3	0.021	-0.5	0.101
NM_014269	4q34	ADAM29	ADAM metallopeptidase domain 29	1.5	0.000	1.4	0.011	2.2	0.000
U17632				1.0	0.003	1.4	0.008	1.1	0.194
U58096	Yp11.2	TSPY1	testis specific protein Y-linked 1	1.0	0.037	1.4	0.000		
NM_013253	11p15.2	DKK3	dickkopf homolog 3 Xenopus laevis	-0.6	0.005	1.4	0.000	0.7	0.218
NM_001823	14q32	CKB	creatine kinase brain	1.7	0.000	1.5	0.015	1.6	0.000
NM_005794	14q11.2	DHRS2	dehydrogenase/reductase SDR family member 2	1.4	0.003	1.5	0.071	1.2	0.051
AB037810	1q42.2	SIPA1L2	signal-induced proliferation-associated 1 like 2	0.9	0.046	1.5	0.000	1.7	0.049
NM_013452	Xp22	VCX	variable charge X-linked	2.3	0.000	1.6	0.015	1.7	0.049
NM_001072	2q37	UGT1A1	UDP glucuronosyltransferase 1 family polypeptide A1	0.2	0.380	1.7	0.015	-0.1	0.714

* no approved HUGO symbol / name exist for this entry. If present, gene symbol / name from the UNIGENE database are given in alternative.
2log_ratio>0 indicates over-expression in the androgen-independent subline, compared to parental PC346C. 2log_ratio<0 indicates down-regulation in the androgen-independent subline.

Table S3 - Genes differentially expressed in PC346Flu2 vs PC346C expression ratios and SAM q-values

GenBank ID	Cytoband	Symbol	HUGO Gene Name	PC346DCC		PC346Flu1		PC346Flu2	
				2log ratio	qvalue	2log ratio	qvalue	2log ratio	qvalue
NM_014380	Xq22.2	NGFRAP1	nerve growth factor receptor TNFRSF16 associated protein 1	-0.2	0.513	-2.5	0.000	-2.7	0.000
AF216077	9q32	COL27A1	collagen type XXVII alpha 1	-2.1	0.000	-2.8	0.000	-1.7	0.053
NM_001359	8q21.3	DECR1	2 4-dienoyl CoA reductase 1 mitochondrial	-1.9	0.000	-1.1	0.000	-1.6	0.053
NM_001758				-1.0	0.000	-0.9	0.048	-1.4	0.000
NM_005510	6p21.3	DOM3Z	dom-3 homolog Z C. elegans	0.5	0.211	0.1	0.422	-1.4	0.053
NM_002151	19q11-q13.2	HPN	hepsin transmembrane protease serine 1	-1.0	0.000	-0.9	0.000	-1.4	0.053
X04236	6p12.1	RN7SK	RNA, 7SK small nuclear	-0.2	0.513	-0.6	0.054	-1.4	0.115
AB020968	6q22.2	MARCKS	myristoylated alanine-rich protein kinase C substrate	-1.6	0.000	-2.0	0.000	-1.3	0.053
NM_003278	3p22-p21.3	CLEC3B	C-type lectin domain family 3 member B	-1.5	0.005	-1.3	0.000	-1.3	0.084
AL122055	6q21	CDC2L6	cell division cycle 2-like 6 CDK8-like	-1.4	0.000	-1.3	0.000	-1.2	0.000
NM_016306	3q27.3	DNAJB11	DnaJ Hsp40 homolog subfamily B member 11	-0.4	0.046	-0.2	0.287	-1.1	0.089
AK000933	1q42-q44	KMO	kynurenine 3-monooxygenase kynurenine 3-hydroxylase	-0.4	0.179	0.0	0.527	-1.0	0.064
NM_015544	17q11.2	TMEM98	transmembrane protein 98	-0.1	0.534	-0.3	0.211	-1.0	0.053

NM_007173	11q14.1	PRSS23	protease serine 23	-1.2	0.018	-0.9	0.021	-0.9	0.101
D70833	19p12	ZNF208	zinc finger protein 208	0.0	0.645	-0.7	0.044	-0.9	0.089
D80010	2p25.1	LPIN1	lipin 1	-1.2	0.000	-1.1	0.015	-0.9	0.053
NM_006829	10q23.2	C10orf116	chromosome 10 open reading frame 116	-1.1	0.009			-0.9	0.084
NM_005709	11p15.1-p14	USH1C	Usher syndrome 1C autosomal recessive severe	-0.5	0.136	-1.1	0.000	-0.9	0.053
NM_016598	3p21.31	ZDHHC3	zinc finger DHHC-type containing 3	-1.4	0.003	-1.1	0.000	-0.9	0.053
AL049963	4q22-q24	SLC39A8	solute carrier family 39 zinc transporter member 8	-1.5	0.000	-1.4	0.000	-0.9	0.053
AL049265	5q11	IL6ST	interleukin 6 signal transducer gp130 oncostatin M receptor	-0.6	0.070	-0.7	0.080	-0.9	0.053
NM_017935	4q24	BANK1	B-cell scaffold protein with ankyrin repeats 1	-2.9	0.000	-1.8	0.000	-0.8	0.053
NM_002245	1q42-q43	KCNK1	potassium channel subfamily K member 1	-0.2	0.534	-0.5	0.101	-0.8	0.053
AF188747	19q13.41	KLK2	kallikrein-related peptidase 2	-2.3	0.000	-0.7	0.063	-0.8	0.053
NM_002252	2p24	KCNS3	potassium voltage-gated channel delayed-rectifier subfamily S member 3	0.2	0.438	-0.6	0.080	-0.8	0.053
NM_003489	21q11.2	NRIP1	nuclear receptor interacting protein 1	-0.2	0.513	-0.6	0.063	-0.8	0.053
NM_017720	19p13.3	STAP2	signal transducing adaptor family member 2	-1.0	0.000	-1.1	0.000	-0.8	0.053
AF076617	Xp22.32	SLC25A6	solute carrier family 25 mitochondrial carrier adenine nucleotide translocator 6	0.0	0.638	-0.5	0.025	-0.8	0.000
X15667				-1.6	0.022	0.2	0.185	-0.8	0.053
NM_013309	15q11.2-21.3	SLC30A4	solute carrier family 30 zinc transporter member 4	-0.6	0.022	0.1	0.444	-0.8	0.053
NM_001417	12q13.13	EIF4B	eukaryotic translation initiation factor 4B	0.0	0.645	-0.1	0.473	-0.8	0.064
NM_001184	3q22-q24	ATR	ataxia telangiectasia and Rad3 related	-1.0	0.020	-0.6	0.048	-0.7	0.064
NM_014683	17p11.2	ULK2	unc-51-like kinase 2 C. elegans	0.8	0.000	0.3	0.090	-0.7	0.053
AK024018	19q13.32	GEMIN7	gem nuclear organelle associated protein 7	0.3	0.380	-0.3	0.160	-0.7	0.000
NM_004549	11q14.1	NDUFC2	NADH dehydrogenase ubiquinone 1 subcomplex unknown 2 14.5kDa	-0.7	0.022	0.2	0.287	-0.7	0.000
AK001843	2q33.1	FAM126B	family with sequence similarity 126 member B	-0.1	0.573	-0.6	0.101	-0.7	0.064
NM_012384	20q13.33	GMEB2	glucocorticoid modulatory element binding protein 2	0.2	0.340	0.0	0.517	-0.7	0.053
AY008271	4q22-q23	SMARCAD1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily a containing DEAD/H box 1	-0.3	0.113	-0.2	0.287	-0.7	0.084
NM_015415	1q24	BRP44	brain protein 44	-1.0	0.009	-0.5	0.090	-0.7	0.089
AB033034	12q23.3	GNPTAB	N-acetylglucosamine-1-phosphate transferase alpha and beta subunits	-0.5	0.037	-0.7	0.005	-0.7	0.064
D70835	19p12	ZNF675	zinc finger protein 675	0.1	0.493	-0.7	0.080	-0.7	0.194
L38937	17q21.2	FAM134C	family with sequence similarity 134 member C	-0.4	0.242	-0.3	0.211	-0.6	0.053
NM_001173	14q12	ARHGAP5	Rho GTPase activating protein 5	-0.2	0.513	-0.5	0.101	-0.6	0.053
AF100640				-0.4	0.242	-0.3	0.160	-0.6	0.089
M83822	4q31.23-31.3	LRBA	LPS-responsive vesicle trafficking beach and anchor containing	-1.1	0.003	-0.6	0.044	-0.6	0.101
NM_003541	6p22-p21.3	HIST1H4K	histone cluster 1 H4k	-0.5	0.293	0.0	0.533	-0.6	0.101
NM_014936	6p21.1	ENPP4	ectonucleotide pyrophosphatase/phosphodiesterase 4 putative function	-0.7	0.046	-0.4	0.160	-0.6	0.053
NM_005864	14q11.2-q12	EFS	embryonal Fyn-associated substrate	-1.4	0.009	0.4	0.135	-0.6	0.064
M32219	5q14.2	RPS23	ribosomal protein S23	-0.2	0.534	-0.3	0.115	-0.6	0.115
AL049998	11p15.5-p14	PIK3C2A	phosphoinositide-3-kinase class 2 alpha polypeptide	-0.4	0.157	-0.5	0.160	-0.6	0.328

NM_016171	2q35-q36	PTMA	prothymosin, alpha	0.1	0.573	0.1	0.444	-0.6	0.150
AK024800	20q13.2-13.3	NFATC2	nuclear factor of activated T-cells cytoplasmic calcineurin-dependent 2	-0.1	0.558	-0.4	0.101	-0.6	0.176
AF024698	19q13.43	ZNF583	zinc finger protein 583	-0.1	0.573	-0.4	0.090	-0.6	0.150
AJ276095	6p21.33-21.1	DEF6	differentially expressed in FDCP 6 homolog mouse	-0.6	0.014	-0.2	0.287	-0.6	0.053
AF085838				-0.1	0.589	0.1	0.444	-0.6	0.084
NM_002414	Xp22.32	CD99	CD99 molecule	0.0	0.624	-1.0	0.042	-0.6	0.000
NM_002165	20q11	ID1	inhibitor of DNA binding 1 dominant negative helix-loop-helix protein	-1.2	0.018			-0.6	0.500
NM_014781	8p22-q21.13	RB1CC1	RB1-inducible coiled-coil 1	-0.6	0.037	-0.6	0.115	-0.6	0.328
NM_001190	19q13	BCAT2	branched chain aminotransferase 2 mitochondrial	0.1	0.558	0.0	0.524	-0.5	0.053
NM_001873	4q32.3	CPE	carboxypeptidase E	-0.4	0.022	-0.7	0.044	-0.5	0.053
NM_017948	9q22.31	NOL8	nucleolar protein 8	-0.2	0.558	-0.3	0.160	-0.5	0.053
NM_006294	8q22	UQCRB	ubiquinol-cytochrome c reductase binding protein	-0.4	0.242	-0.1	0.517	-0.5	0.120
AF086462	10q22.3	C10orf58	chromosome 10 open reading frame 58	0.1	0.605	-0.3	0.287	-0.5	0.101
NM_005646	1q42.3	TARBP1	TAR HIV-1 RNA binding protein 1	-0.2	0.466	-0.1	0.473	-0.5	0.120
NM_001958	20q13.3	EEF1A2	eukaryotic translation elongation factor 1 alpha 2	-0.1	0.558	0.3	0.211	-0.5	0.134
NM_018123	1q31	ASPM	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	-0.4	0.100	0.1	0.422	-0.5	0.101
U52054	5q11.1	EMB	embigin homolog mouse	-0.9	0.022	-0.7	0.042	-0.5	0.150
NM_001197	22q13.31	BIK	BCL2-interacting killer apoptosis-inducing	-0.1	0.573	-0.5	0.115	-0.5	0.053
NM_004510	2q37.1	SP110	SP110 nuclear body protein	0.2	0.340	-0.2	0.185	-0.5	0.471
AF086535	1p34.3	C1orf102	chromosome 1 open reading frame 102	-0.2	0.009	0.0	0.422	0.5	0.218
NM_018255	18q12.2	ELP2	elongation protein 2 homolog S. cerevisiae	0.1	0.645	0.0	0.358	0.5	0.194
AF086001				0.5	0.070	0.0	0.524	0.5	0.089
NM_001494	10p15	GDI2	GDP dissociation inhibitor 2	0.1	0.412	-0.1	0.473	0.5	0.037
AK001829	18q23			0.0	0.605	-0.2	0.185	0.6	0.176
NM_004046	18q12-q21	ATP5A1	ATP synthase H transporting mitochondrial F1 complex alpha subunit 1	0.3	0.380	0.0	0.515	0.6	0.194
NM_004287	17q21	GOSR2	golgi SNAP receptor complex member 2	0.2	0.380	0.5	0.031	0.6	0.194
NM_015681	17p11.2	B9D1	B9 protein domain 1	0.2	0.293	0.3	0.160	0.6	0.194
NM_001660	3p21.2-p21.1	ARF4	ADP-ribosylation factor 4	0.1	0.573	-0.3	0.287	0.6	0.218
NM_016086	7q11.23	STYXL1	serine/threonine/tyrosine interacting-like 1	0.6	0.100	0.2	0.071	0.6	0.194
NM_001468	Xp11.23	GAGE1	G antigen 1	0.7	0.003	0.4	0.211	0.6	0.000
NM_019844	12p12	SLCO1B3	solute carrier organic anion transporter family member 1B3	-1.1	0.028	-0.2	0.394	0.6	0.194
AK026854	9p13.2	WDR32	WD repeat domain 32	0.3	0.086	0.1	0.211	0.6	0.194
NM_016215	9q34.3	EGFL7	EGF-like-domain multiple 7	0.6	0.242	-0.1	0.135	0.6	0.194
NM_014913	18q23	ADNP2	ADNP homeobox 2	0.1	0.293	0.1	0.287	0.6	0.218
AF151810	11q13	STARD10	StAR-related lipid transfer START domain containing 10	0.6	0.070	0.2	0.358	0.6	0.194
NM_002391	11p11.2	MDK*		0.0	0.645	-0.8	0.051	0.7	0.218
NM_016097	18q12	IER3IP1	immediate early response 3 interacting protein 1	0.1	0.513	-0.3	0.211	0.7	0.218
NM_004786	18q21.2	TXNL1	thioredoxin-like 1	0.1	0.493	-0.1	0.321	0.7	0.049

NM_017947	18q12	MOCOS	molybdenum cofactor sulfurase	-0.5	0.055	-0.6	0.071	0.7	0.194
NM_005151	18p11.32	USP14	ubiquitin specific peptidase 14 tRNA-guanine transglycosylase	-0.2	0.493	-0.1	0.321	0.7	0.089
NM_014302	7p11.2	SEC61G	Sec61 gamma subunit	0.2	0.438	0.2	0.287	0.7	0.176
NM_018570				0.4	0.211	0.5	0.063	0.7	0.150
AK000543	18q12.2	C18orf10	chromosome 18 open reading frame 10	0.1	0.466	0.0	0.515	0.7	0.194
NM_000104	2p21	CYP1B1	cytochrome P450 family 1 subfamily B polypeptide 1	-0.6	0.293	0.0	0.515	0.7	0.218
NM_004282	6p12.3-p11.2	BAG2	BCL2-associated athanogene 2	0.9	0.020	0.4	0.044	0.7	0.194
NM_006993	10q24.31	NPM3	nucleophosmin/nucleoplasmin 3	0.6	0.157	0.1	0.485	0.7	0.150
NM_005631	7q32.3	SMO	smoothened homolog Drosophila	0.8	0.018	0.3	0.160	0.7	0.089
NM_001831	8p21-p12	CLU	clusterin	0.1	0.534	0.9	0.000	0.7	0.049
NM_001075	4q13.2	UGT2B10	UDP glucuronosyltransferase 2 family polypeptide B10	0.0	0.589	0.2	0.211	0.7	0.194
AK001079	18q11.1	OSBPL1A	oxysterol binding protein-like 1A	0.5	0.022	0.0	0.394	0.7	0.194
NM_004864	19p13.1-13.2	GDF15	growth differentiation factor 15	-0.4	0.513	-0.5	0.160	0.7	0.194
M22538	18p11.31-11.2	NDUFV2	NADH dehydrogenase ubiquinone flavoprotein 2 24kDa	-0.1	0.573	-0.1	0.444	0.7	0.047
NM_002867	1p32-p31	RAB3B	RAB3B member RAS oncogene family	-0.3	0.000	0.1	0.090	0.8	0.194
AB037735	18p11.23	ARHGAP28	Rho GTPase activating protein 28					0.8	0.218
AK002107	1p32-p31	RAB3B	RAB3B member RAS oncogene family	-0.4	0.018	0.2	0.287	0.8	0.194
NM_005178	19q13.1-q13.2	BCL3	B-cell CLL/lymphoma 3	-0.4	0.046	-0.3	0.229	0.8	0.194
NM_005804	19p13.12	DDX39	DEAD Asp-Glu-Ala-Asp box polypeptide 39	1.4	0.000	0.3	0.253	0.8	0.194
AB042410	1p21.3	GPR88	G protein-coupled receptor 88	-2.4	0.000	-0.3	0.394	0.8	0.194
NM_003516	1q21.2	HIST2H2AA3	histone cluster 2 H2aa3	-0.1	0.645	1.2	0.000	0.8	0.194
NM_000985	18q21	RPL17	ribosomal protein L17	0.4	0.043	0.2	0.185	0.8	0.000
NM_000849	1p13.3	GSTM3	glutathione S-transferase M3 brain	0.6	0.086	0.9	0.000	0.9	0.176
NM_005909	5q13	MAP1B	microtubule-associated protein 1B	0.3	0.380	0.1	0.394	0.9	0.194
NM_001262	1p32	CDKN2C	cyclin-dependent kinase inhibitor 2C p18 inhibits CDK4	0.5	0.211	0.1	0.459	0.9	0.194
AF200348	2p25	PXDN	peroxidasin homolog Drosophila	-1.2	0.003	-1.3	0.000	0.9	0.064
AF190900	1q32.1	KLHL12	kelch-like 12 Drosophila	0.2	0.466	0.7	0.000	1.0	0.194
NM_016192	2q32.3	TMEFF2	transmembrane protein with EGF-like and two follistatin-like domains 2	0.0	0.645	0.3	0.115	1.0	0.049
NM_002795	17q12	PSMB3	proteasome prosome macropain subunit beta type 3	0.5	0.113	0.6	0.063	1.0	0.000
AL133572	12q24.31	FBXL10	F-box and leucine-rich repeat protein 10	0.5	0.086	0.9	0.005	1.1	0.049
NM_014214	18p11.2	IMPA2	inositol myo -1 or 4 -monophosphatase 2	0.3	0.242	0.0	0.515	1.1	0.000
AF086251	11q21	SESN3	sestrin 3	0.1	0.534	1.1	0.048	1.1	0.049
NM_005794	14q11.2	DHRS2	dehydrogenase/reductase SDR family member 2	1.4	0.003	1.5	0.071	1.2	0.051
Y09836	5q13	MAP1B	microtubule-associated protein 1B	0.7	0.046	1.0	0.011	1.2	0.101
NM_003712	19p13	PPAP2C	phosphatidic acid phosphatase type 2C	1.2	0.000	0.9	0.044	1.2	0.000
NM_003226	21q22.3	TFF3	trefoil factor 3 intestinal	1.9	0.000	-0.5	0.211	1.5	0.049
NM_001823	14q32	CKB	creatine kinase brain	1.7	0.000	1.5	0.015	1.6	0.000
AB037810	1q42.2	SIPA1L2	signal-induced proliferation-associated 1 like 2	0.9	0.046	1.5	0.000	1.7	0.049

NM_013452	Xp22	VCX	variable charge X-linked	2.3	0.000	1.6	0.015	1.7	0.049
NM_012463	12q24.31	ATP6V0A2	ATPase H transporting lysosomal V0 subunit a2	0.8	0.028	1.2	0.051	1.7	0.000
NM_004982	12p11.23	KCNJ8	potassium inwardly-rectifying channel subfamily J member 8	2.7	0.000	1.2	0.000	1.9	0.000
NM_014269	4q34	ADAM29	ADAM metallopeptidase domain 29	1.5	0.000	1.4	0.011	2.2	0.000

* no approved HUGO symbol / name exist for this entry. If present, gene symbol / name from the UNIGENE database are given in alternative.
2log_ratio>0 indicates over-expression in the androgen-independent subline, compared to parental PC346C. 2log_ratio<0 indicates down-regulation in the androgen-independent subline.

Table S4 - List of androgen-receptor target genes

GenBank ID	Symbol	HUGO Name
		R1881 up-regulated genes
NM_018674	ACCN4	amiloride-sensitive cation channel 4 pituitary
NM_004457	ACSL3	acyl-CoA synthetase long-chain family member 3
NM_006408	AGR2	anterior gradient homolog 2 (Xenopus laevis)
NM_014109	ATAD2	ATPase family AAA domain containing 2
AK027213	BBS10	Bardet-Biedl syndrome 10
NM_020235	BBX	bobby sox homolog Drosophila
AK024850	C2orf31	chromosome 2 open reading frame 31
NM_006079	CITED2	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2
AK026498	CYP2U1	cytochrome P450 family 2 subfamily U polypeptide 1
NM_014762	DHCR24	24-dehydrocholesterol reductase
NM_012062	DNM1L	dynamin 1-like
NM_018456	EAF2	ELL associated factor 2
AK026517	EHF	ets homologous factor
AK022827	EIF2C3	eukaryotic translation initiation factor 2C 3
NM_012081	ELL2	elongation factor RNA polymerase II 2
AF111849	ELOVL5	ELOVL family member 5 elongation of long chain fatty acids FEN1/Elo2
AB020637	ENDOD1	endonuclease domain containing 1
NM_019018	FAM105A	family with sequence similarity 105 member A

AK024648	FAM107B	family with sequence similarity 107 member B
AL137343	FAM84A	family with sequence similarity 84 member A
NM_004117	FKBP5	FK506 binding protein 5
AK024715	FLJ21062 *	Hypothetical protein FLJ21062 *
NM_004476	FOLH1	folate hydrolase (prostate-specific membrane antigen) 1
NM_020474	GALNT1	UDP-N-acetyl-alpha-D-galactosamine:polypeptide
NM_005271	GLUD1	glutamate dehydrogenase 1
NM_002069	GNAI1	guanine nucleotide binding protein G protein alpha inhibiting activity polypeptide 1
AB042410	GPR88	G protein-coupled receptor 88
NM_001530	HIF1A	hypoxia-inducible factor 1 alpha subunit basic helix-loop-helix transcription factor
NM_003543	HIST1H4H	histone cluster 1 H4h
M60721	HLX	H2.0-like homeobox
NM_014642	IQCB1	IQ motif containing B1
NM_002241	KCNJ10	potassium inwardly-rectifying channel subfamily J member 10
AL137384	KIAA1109	KIAA1109
NM_001206	KLF9	Kruppel-like factor 9
AF188747	KLK2	kallikrein-related peptidase 2
NM_001648	KLK3	kallikrein-related peptidase 3
NM_004917	KLK4	kallikrein-related peptidase 4
AK026375	LOC93622 *	Hypothetical protein BC006130 *
NM_005461	MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B avian
NM_000240	MAOA	monoamine oxidase A
NM_003010	MAP2K4	mitogen-activated protein kinase kinase 4
AB050049	MCCC2	methylcrotonoyl-Coenzyme A carboxylase 2 beta
AK021627	MORC4	MORC family CW-type zinc finger 4
AF142409	MS4A6A	membrane-spanning 4-domains subfamily A member 6A
NM_005956	MTHFD1	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1
NM_016498	MTP18 *	Mitochondrial protein 18 kDa *
NM_000662	NAT1	N-acetyltransferase 1 arylamine N-acetyltransferase

NM_006167	NKX3-1	NK3 homeobox 1
AF039944	NDRG1	N-myc downstream regulated gene 1
NM_006096	NDRG1	N-myc downstream regulated gene 1
NM_005596	NFIB	nuclear factor I/B
NM_020529	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
NM_016590	PART1 *	prostate androgen-regulated transcript 1 *
NM_006810	PDIA5	protein disulfide isomerase family A member 5
NM_016166	PIAS1	protein inhibitor of activated STAT 1
AF070670	PPM1A	protein phosphatase 1A formerly 2C magnesium-dependent alpha isoform
NM_004156	PPP2CB	Protein phosphatase 2 formerly 2A catalytic subunit beta isoform
NM_002923	RGS2	regulator of G-protein signaling 2 24kDa
D16875	RHOB	ras homolog gene family member B
AK001478	RHOU	ras homolog gene family member U
AB051826	RHOU	ras homolog gene family member U
NM_005627	SGK1	serum/glucocorticoid regulated kinase 1
AB040914	SHROOM3	shroom family member 3
NM_004595	SMS	spermine synthase
NM_003082	SNAPC1	small nuclear RNA activating complex polypeptide 1 43kDa
NM_003104	SORD *	sorbitol dehydrogenase *
NM_012449	STEAP1	six transmembrane epithelial antigen of the prostate 1
AK026813	STEAP2	six transmembrane epithelial antigen of the prostate 2
NM_005656	TMPRSS2	Transmembrane protease serine 2
NM_005079	TPD52	tumor protein D52
AF294628	TWSG1	twisted gastrulation homolog 1 Drosophila
NM_003115	UAP1	UDP-N-acteylglucosamine pyrophosphorylase 1
NM_003359	UGDH	UDP-glucose dehydrogenase
AK001647	USP40	ubiquitin specific peptidase 40
AB020676	WWC1	WW and C2 domain containing 1
AK022814	ZBTB10	zinc finger and BTB domain containing 10

NM_006006	ZBTB16	zinc finger and BTB domain containing 16
AF025771	ZNF189	zinc finger protein 189
AK026383	NDRG1	N-myc downstream regulated 1
AL157445		cDNA clone *
D17210		cDNA clone *
		R1881 down-regulated genes
NM_005688	ABCC5	ATP-binding cassette sub-family C CFTR/MRP member 5
AK026288	ATHL1	ATH1 acid trehalase-like 1 yeast
NM_012342	BAMBI	BMP and activin membrane-bound inhibitor homolog Xenopus laevis
NM_001197	BIK	BCL2-interacting killer apoptosis-inducing
AF075110	C14orf4	chromosome 14 open reading frame 4
NM_017766	CASZ1	castor zinc finger 1
NM_001305	CLDN4	claudin 4
AK024378	FAM131A	family with sequence similarity 131 member A
NM_004480	FUT8	fucosyltransferase 8 alpha 1 6 fucosyltransferase
NM_002165	ID1	inhibitor of DNA binding 1 dominant negative helix-loop-helix protein
X69111	ID3	inhibitor of DNA binding 3 dominant negative helix-loop-helix protein
NM_006769	LMO4	LIM domain only 4
NM_017572	MKNK2	MAP kinase interacting serine/threonine kinase 2
NM_005377	MYCL2	v-myc myelocytomatosis viral oncogene homolog 2 (avian)
NM_006312	NCOR2	nuclear receptor co-repressor 2
U90907	PIK3R3	phosphoinositide-3-kinase regulatory subunit 3 p55 gamma
AF113132	PSAT1	phosphoserine aminotransferase 1
NM_004577	PSPH	phosphoserine phosphatase
NM_015923	SLC3A2	solute carrier family 3, member 2
NM_003943	STBD1	starch binding domain 1
NM_003714	STC2	stanniocalcin 2
AK000401	TANC1	tetratricopeptide repeat ankyrin repeat and coiled-coil containing 1
AL133074	TP53INP1	tumor protein p53 inducible nuclear protein 1

NM_003287	TPD52L1	tumor protein D52-like 1
AF205437	TRIB1	tribbles homolog 1 Drosophila
U55055		Oral cancer candidate gene mRNA clone
NM_018588		hypothetical protein *
AK022971		cDNA clone *
		Flutamide up-regulated genes
AB020637	ENDOD1	endonuclease domain containing 1
NM_004117	FKBP5	FK506 binding protein 5
AB042410	GPR88	G protein-coupled receptor 88
NM_002923	RGS2	regulator of G-protein signaling 2 24kDa
AK026813	STEAP2	six transmembrane epithelial antigen of the prostate 2
AB020676	WWC1	WW and C2 domain containing 1
D17099		cDNA clone *
AL049966		cDNA clone *
		Flutamide down-regulated genes
NM_002165	ID1	inhibitor of DNA binding 1 dominant negative helix-loop-helix protein
NM_002241	KCNJ10	potassium inwardly-rectifying channel subfamily J member 10
NM_006854	KDELR2	KDEL Lys-Asp-Glu-Leu endoplasmic reticulum protein retention receptor 2
AB028451	NCOR1	nuclear receptor co-repressor 1
NM_001269	RCC1	regulator of chromosome condensation 1
NM_000370	TTPA	tocopherol alpha transfer protein

* no approved HUGO symbol / name exist for this entry. This gene list includes the 107 transcripts of the PC346 cell lines AR-regulated signature, previously characterized in Chapter 4, plus 7 well-validated AR targets that failed to be detected by the referred microarray analysis: AGR2, DHCR24, FOLH1, KLK3 (PSA), KLK4, MAOA and NKX3-1.

Chapter 6

General Discussion

Introduction

Currently, prostate cancer detected at an early stage can be successfully eradicated by radical prostatectomy or radiotherapy, but no curative treatment exists for metastatic disease. Since the discovery that androgens promote the growth of prostate tumors, androgen ablation therapy, often combined with androgen receptor (AR) antagonists, is used to treat disseminated disease [1,2]. Unfortunately, this treatment is merely palliative as the cancer will eventually become resistant to hormonal therapy and recur [3,4]. Therefore, the development of prognostic markers and targeted therapies for advanced disease are urgent topics in prostate cancer research. To achieve this, a thorough understanding of the intrinsic mechanisms governing prostate cancer survival, proliferation and progression to hormone-refractory stage is crucial. Since the androgen receptor pathway is essential for prostate development, homeostasis and malignant outgrowth, a main goal of this study was to address the role of this pathway in the proliferation of hormone-resistant cells. Simultaneously, we were interested in identifying genes and pathways that besides the AR could also promote survival and growth of androgen-independent cells. In this process, the last aim was to identify genes and pathways involved in prostate cancer progression and potential disease markers.

AR pathway modifications during progression to hormone-refractory disease

To investigate the mechanisms of prostate cancer progression and identify potential disease markers the choice of a suitable model system is critical. During the course of this research, we used the PC346 cell line panel, a progression model especially established to study the mechanisms of hormone-refractory outgrowth that inevitably follows endocrine therapy of prostate cancer patients. The parental PC346C cell line was unique in the combination of primary tumor origin, *wild-type* AR expression, PSA secretion and androgen-responsive growth, replicating the hormone-naive stage of prostate cancer. To mimic the mono and combined hormonal therapies offered to prostate cancer patients, the PC346C cells were cultured, for a minimum period of 2 years, in steroid-stripped medium either alone or supplemented with the AR antagonist flutamide. In this manner, three hormone-resistant sublines PC346DCC (monotherapy), PC346Flu1 and PC346Flu2 (combined-therapy) were established. The AR pathway status of all these cell lines was fully characterized using *in vitro* and *in vivo* hormone-stimulated growth assays, AR sequencing, AR and PSA quantification and AR reporter assays. Afterwards, microarray analysis on the PC346 cell line panel stimulated with the androgen analogue R1881 or the anti-androgen OH-Flutamide was performed to characterize the expression profile of AR target genes in prostate cancer cells.

While PC346C expresses a *wild-type* AR, requires androgens for optimal proliferation and is inhibited by the antiandrogen flutamide, the three hormone-resistant sublines acquired a variety of AR modifications: overexpression, mutation and down-regulation. Two main conclusions can already be drawn from the development of these cell lines and the xenograft model system described in Chapter 2: (i) AR modifications are frequent events following long-term hormonal manipulations and (ii) the AR pathway seems to play an important role in prostate cancer progression, since AR adaptations associated with hormone-refractory growth. In addition, by reproducing common AR modifications observed in hormone-refractory disease, the *in vitro* PC346 cell lines provide a valuable model system for the study of prostate cancer progression.

AR gene amplification is probably the most common AR modification in prostate cancer, being detected in up to 30% of hormone-refractory [5,6,7]. It is still not known how AR overexpression can activate the receptor under androgen-depleted conditions. Hypothetically, two mechanisms are possible: increased sensitivity to residual androgen levels or constitutive ligand-independent activation. AR overexpression is represented in our panel by the PC346Flu1 cell line. Although FISH analysis did not show genomic AR amplification in PC346Flu1, elevated AR mRNA levels were measured by RT-PCR, indicating increased transcriptional activity or RNA stability as probable modes of AR up-regulation. PC346Flu1 expressed a *wild-type* AR sequence but expression levels of the receptor were 4-fold higher than in the parental PC346C. This AR overexpression coincided with a 10-fold higher AR reporter activity and a "super-activation" of AR target genes in the microarray analysis, in response to the synthetic androgen R1881. Some authors have proposed that increased AR levels not only sensitised the receptor to residual androgen concentrations but also conferred agonistic activity to AR antagonists [8,9]. However, PC346Flu1 proliferation was optimal in the absence of androgens and was unaffected by flutamide supplementation. The lack of agonistic activity of flutamide on PC346Flu1 cells was further confirmed in the AR reporter assays and expression microarray analysis. Likewise, Konkontis *et al.* also failed to replicate the antagonist to agonist conversion in hormone-refractory LNCaP-104R cells, which express 15-fold more AR protein that respective androgen-sensitive parental LNCaP-104S cells. Furthermore, whereas proliferation of LNCaP-104S cells was inhibited by the AR antagonist casodex the growth of LNCaP-104R cells was not [10,11]. These results suggest that the proliferation of PC346Flu1 cells, as well as LNCaP-104R, is not dependent on residual androgens, but is maintained by constitutive AR activation resistant to AR antagonists. This view is supported by findings from Dehm *et al.*, which, by introducing disabling mutations in the ligand-binding domain, showed that ligand binding was not necessary for constitutive AR activation in C4-2 cells [12]. The authors also observed increased transactivation activity of the AR N-terminal domain in these cells, compared to parental LNCaP. Similar processes could be playing a role in constitutive AR activation in PC346Flu1 cells. All together, AR overexpression may lead to different mechanisms of activation, depending on the background of the cells, the type or the duration of the androgen-depletion treatment.

Curiously, growth of PC346Flu1 was inhibited by physiologic concentrations of androgens both *in vitro* and *in vivo*. This paradoxal growth inhibitory effect of androgens was previously reported in various hormone-resistant LNCaP sublines with similar AR overexpression, being preceded at earlier passages by hypersensitivity to low levels of androgens [9,10,11,13]. A possible explanation for this phenomenon resides on the Janus-faced functions of the AR, on one hand promoting survival and proliferation, on the other hand inducing cellular differentiation and maturation [14]. In this context, super-activation of the AR may tilt the balance towards terminal differentiation, thus hampering cell growth [15].

The EHF transcription factor is an AR up-regulated gene that was strongly repressed in PC346Flu1 in flutamide supplemented selection medium, when compared to the R1881 supplemented parental PC346C. EHF is a good candidate for the growth suppressive effect of androgens on PC346Flu1 subline as: (i) it is epithelial cell specific, (ii) involved in glandular differentiation, (iii) frequently down-regulated in prostate tumor

specimens and (iv) ectopic expression in prostate cancer cells inhibited clonogenic survival and induced apoptosis [16,17,18,19]. Whether EHF indeed suppresses PC346Flu1 growth in the presence of androgens still has to be established with future RNAi knockdown experiments. Previous reports showed that this growth suppressive effect of androgens was not due to increased apoptosis, but to G1 cell cycle arrest, accompanied by an increase in cdk inhibitors $p21^{Waf/Cip1}$ and $p27^{Kip}$ [10,15]. The authors suggested that androgen-induced down-regulation of c-Myc oncogene levels or up-regulation of the tumor suppressor GADD45γ could take part in this process [11,20]. The mechanism by which androgens regulate c-Myc or GADD45γ levels in those cells, or whether EHF plays a role in this process, are as yet undefined and deserve future attention.

A crucial question remains: is the AR pathway indeed required for PC346Flu1 growth or is the overexpressed AR just a bystander, possibly only relevant in the initial response to androgen-deprivation? The fact that expression of AR target genes was not significantly decreased in PC346Flu1, compared to parental PC346C, suggests that AR pathway may still be maintained in the absence of androgens. Previous AR knockdown experiments, using RNA interference technology, have shown induction of apoptosis and growth inhibition, not only in hormone-dependent cells but also, in multiple castration-resistant cells lines and xenografts [21,22,23,24,25,26]. Although similar experiments are necessary to conclusively relate the AR to the proliferation of PC346Flu1, the results above confirm the importance of the AR pathway in hormone-refractory growth.

Another hormone-resistant subline investigated in our study, PC346Flu2, was growth stimulated by both R1881 and the antiandrogen hydroxyflutamide. AR sequencing revealed the T877A mutation, well-known for its ability to broaden the AR activity to diverse non-androgenic ligands including flutamide [27,28]. Thus, the PC346Flu2 cell line replicates another common AR modification (activating mutation) detected in about 10% of hormone-refractory tumors. The detection of the T877A AR mutation also coincided with a similar gene expression response to both R1881 and flutamide in the microarray analysis, although the stimulatory effect of the latter was weaker. As expected, when directly compared to the parental PC346C, PC346Flu2 was the least divergent subline, which is in agreement with the hypothesis that the growth of both these cell lines is regulated by ligand-activated AR pathway. However, basal growth of PC346Flu2 in the absence of R1881 or flutamide is retarded but still 2-fold faster than the parental PC346C, indicating additional mechanisms may be involved in either activating the AR or alternative survival pathways. The control of AR function involves interaction with a number of co-factors that regulate AR transcriptional activity. Alterations in AR co-regulators, such as SRC1, TIF2, NCOR1 or NCOR2, have been described in hormone-refractory disease [29,30]. In fact, nuclear receptor interacting protein 1 (NRIP1), a repressor of AR transactivation [31,32], has been found to be down-regulated in PC346Flu2, and could represent a possible mechanism to adapt AR signaling to residual androgens levels in the medium. Another possibility is the production of androgens by the cancer cells themselves [33,34]. For our PC346 cell lines this is an improbable mechanism, as no hint of steroidogenic enzymes modulation was detected in the expression microarray analysis. As for PC346C, the AR pathway

seems to play an active role in PC346Flu2 proliferation, but only AR knockdown experiments can unequivocally show if it is also driving the basal growth of unstimulated PC346Flu2 cells.

In contrast, the PC346DCC cell line expresses very little AR and PSA and showed background levels of AR reporter activity, which was not up-regulated by R1881. This cell line exhibited fast growth that was insensitive to androgen depletion or antiandrogen treatment. Curiously, a novel mutation was detected in codon 311 of PC346DCC AR, which resulted in a Lys to Arg substitution. However, AR reporter assays showed no evidence of altered transactivation properties of this mutated receptor, which had a similar response as the wild-type receptor to R1881 and hydroxyflutamide (unpublished data). Furthermore, in a microarray analysis, PC346DCC did not show differences in gene expression in response to R1881 stimulation. Altogether, these results suggest that PC346DCC has bypassed the AR pathway and that alternative growth pathways guide survival and proliferation of these cells. Expression profiling of PC346DCC confirmed down-regulation of AR-target genes and three candidates were chosen for their potential involvement in AR pathway bypass: VAV3, TWIST1 and DKK3. These candidates were selected based on the consistent deregulation across the multiple prostate cancer metastasis databases analysed and potential pathological function. In this manner, VAV3 and TWIST1, found to be overexpressed in PC346DCC cells and metastasis datasets, are potential oncogenes, whereas the down-regulated DKK3 is a putative tumor suppressor.

VAV3 is a GTPase guanine nucleotide exchange factor that mediates receptor protein tyrosine kinase signaling [35,36]. It is activated by growth factor receptors, such as EGFR, PDGFR, IR and IGF1R, and interacts with respective downstream signaling molecules including Shc, Gbr2, PLC and PI3K [35,36,37]. VAV3 has been previously implicated in prostate cancer, where it has been detected in increased amounts and may cross-talk with the AR pathway, stimulating hormone-refractory cell growth [38,39,40]. Furthermore, a mouse model targeting constitutively active VAV3 expression to the prostate induced prostate cancer and inflammation, suggesting a direct role of VAV3 in prostate cancer development [41]. Surprisingly, in our prostate cancer patient cDNA panel, VAV3 expression was down-regulated during prostate cancer progression and in hormone-refractory samples. A possible explanation for this unexpected finding could lie on the multiple VAV3 splice variants. There are 3 major transcripts of VAV3: the full-length VAV3 alpha, the super-active VAV3 beta, and the short VAV3.1 variant [35,36,42]. The quantitative-PCR probes used in our study target the last 2 of the 27 exons in VAV3 gene, and it is possible that they capture preferentially the short C-terminal VAV3.1 transcript. This short VAV3.1 variant lacks guanine nucleotide exchange activity due to the truncation of N-terminal domains. It is expressed in many tissues and is the major variant in the prostate [36,42]. Due to the lack of the activation domains the VAV3.1 variant is not oncogenic and it has been proposed to functions as a dominant negative of other VAV family members [36,42]. In this context, a decrease in the VAV3.1 variant could actually result in increased activity of oncogenic VAV proteins. Therefore, it is essential to characterize the different VAV3 variants during prostate cancer progression. At the moment, a genome-wide exon microarray analysis is ongoing on our prostate tissue panel used in the quantitative-PCR assays. Among the other

genes, VAV3 is well-covered by the microarrays used in this study, which will help us identify the major transcript variants in the prostate and how their balance is affected in the different stages of prostate cancer.

TWIST1 is a helix-loop-helix transcription factor, highly expressed in many types of human cancer, and involved in the processes of cancer cell survival, proliferation and migration [43,44,45,46,47]. In particular, TWIST1 appears to regulate metastasis by promoting an epithelial-mesenchymal transition through down-regulation of E-cadherin [48,49]. Recently, increased TWIST1 levels were also detected in prostate tumors, correlating with Gleason-grade and metastasis [48,49]. The function of TWIST1 in prostate cancer progression was further supported by the suppressive effect of TWIST1 inactivation on migration and invasion abilities of hormone-refractory prostate cancer cells [49]. All together, these results made it a good candidate for the bypass of the AR pathway in PC346DCC cells. Analysis of TWIST1 expression in our prostate tumor panel confirmed up-regulation in prostate cancer and metastasis, but the expression levels did not correlate with Gleason-score or progression to hormone-refractory disease. Basically, our results indicate that increased TWIST1 expression by itself is not a representative mechanism of hormone-refractory growth in clinical samples. However, like VAV3, TWIST1 was also shown to crosstalk with growth receptor signaling pathways, such as EGF and IGF1, suggesting that activation of these alternative pathways could be the effector mechanism in the progression to androgen independence [50,51]. Alternative survival and growth pathways bypassing the AR will be discussed in a separate section bellow.

The last candidate gene analysed, DKK3, belongs to the evolutionary conserved Dickkopf gene family, implicated in the modulation of Wnt/beta-catenin signaling [52]. DKKs play an important role in vertebrate development, bone formation, Alzheimer's disease and cancer. Down-regulation of DKK3 is frequently detected in multiple human cancers, including prostate tumors, which has been associated with promotor methylation [53,54,55]. In the prostate, DKK3 seems to play a role in acinar differentiation and malignant growth, by inducing apoptosis and inhibits prostate cancer cell proliferation [56,57]. In our human prostate cancer panel, DKK3 expression was significantly decreased in primary prostate tumors and metastasis, but did not associate with tumor progression nor hormone-refractory disease. These results suggest that DKK3 down-regulation is probably an early event in prostate carcinogenesis. In conclusion, activation of oncogenes and tumor suppressor down-regulation may be driving PC346DCC survival and proliferation, but TWIST1 and DKK3 by themselves do not appear to represent common mechanisms of hormone-refractory growth in clinical samples.

To follow alterations in the AR pathway during the development and progression of prostate cancer, the AR-regulated gene signature from PC346 cells was linked to seven microarray databases of normal prostate, primary tumor and metastatic cancer material (Chapter 4). One cluster consisted of AR up-regulated genes that were overexpressed in metastases and were involved in cell survival, proliferation, cytoskeletal remodeling and adhesion, all crucial functions in tumor progression and invasion. However, the major cluster consisted of AR up-regulated genes, which were often overexpressed in primary tumors but down-regulated in metastasis. This cluster is

enriched for genes involved in differentiation and secretory function of the prostate, functions that are redundant and disadvantageous for a rapidly propagating tumor.

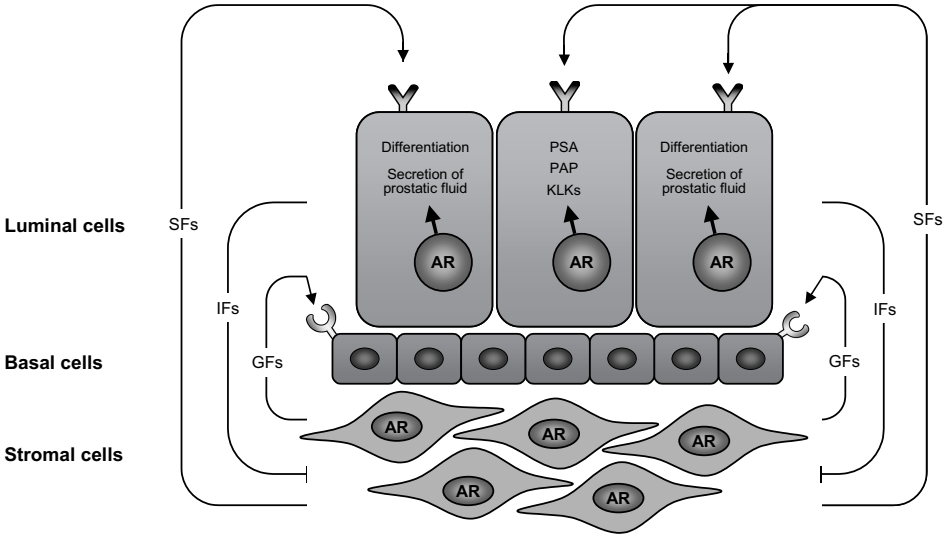

Figure 1 - Mechanism of AR function on different cell types in the healthy prostate. AR is expressed in prostate stromal cells and luminal epithelial cells, but not in basal cells. The basal cell compartment contains a self-renewal population responsible for restoring aged or injured cells, which will acquire AR expression as they commit to the luminal cell lineage. AR expression will further drive differentiation of luminal cells and maintain their function in the production and secretion of prostatic fluid components, including prostate specific antigen (PSA), prostate acid phosphatase (PAP) and several kallikreins (KLKs). Furthermore, androgens indirectly promote proliferation of basal cells through regulation of paracrine growth factors (GFs) expression by stromal cells. Stromal cells also produce survival factors (SFs) that inhibit apoptosis of luminal epithelial cells. To prevent the epithelial cells from proliferating indefinitely in the presence of androgens, a negative feedback signal, from the luminal to the stromal cells, regulates the production of stromal growth factors. (IFs): inhibitory factors.

The view of the AR pathway as a driving force for cancer growth and proliferation, may be over-simplistic as mounting evidence suggests that this pathway may have different functions in different stages of the disease. Previously, Hendriksen *et al.* also observed a partial down-regulation of the AR pathway in advanced disease, by using the androgen-response program from LNCaP cells to interrogate a set of prostate cancer xenografts and Lapointe's patient derived samples [58]. Two subsequent studies, using distinct bioinformatics approaches, further confirmed an attenuated androgen signaling signature in high-grade and metastatic prostate cancer [59,60]. Finally, Sterbis *et al.* showed that decreased tissue PSA mRNA was associated with increased risk of recurrence after radical prostatectomy [61]. This is not the case for PSA levels in serum, which can be explained by the fact that serum levels reflect the increasing tumor burden during progression, whereas tissue levels denote alterations in the tumor cell

microenvironment. Therefore, attenuation of the AR pathway in advanced stages of prostate cancer was observed in multiple studies and may reflect a real mechanism for progression rather than an artifact of the *in vitro* system used in the present study. This attenuation of the AR pathway in advanced stages of prostate cancer may reflect tumor dedifferentiation, but whether it is a cause or the result still has to be clarified.

Two recent studies support opposing roles of the AR in prostate cancer cells by showing that: (i) knockdown of the AR in epithelial hormone-refractory CWR22rv1 cells increased invasion in bone lesion assays and *in vivo* mouse models; (ii) whereas restoration of AR in AR-negative PC3 cells decreased invasion; (iii) mice lacking prostate epithelial AR have increased apoptosis in secretory luminal cells but increased proliferation of basal-intermediate cells; (iv) AR knockdown in both prostate stroma and epithelium of ARKO-TRAMP mouse models resulted in smaller primary prostate tumors than *wild-type* TRAMP littermates; (v) whereas targeted AR knockdown limited to prostate epithelial cells resulted in increased tumor growth compared to *wild-type* TRAMP mice [62,63]. Based on these results, the authors suggested that the AR might function both as tumor suppressor and proliferator in prostate cancer, depending on the cell type targeted. Thus, the AR may promote differentiation of luminal secretory cells, suppress growth of basal-intermediate cells and inhibit metastasis but, simultaneously, promote survival and proliferation of prostate tumor epithelium by regulating paracrine stromal factors (Fig. 1) [64].

Alternative survival and growth pathways in hormone-refractory prostate cancer

In addition to the androgen receptor, other pathways have been shown to stimulate prostate cancer growth. For example, EGF, IGF1, HGF, KGF, FGFs, IL6 and/or respective receptors are often overexpressed in prostate cancer, in particular in hormone-refractory samples [64]. These growth factors are normally secreted by stromal cells, some under androgen regulation, binding to receptors expressed on neighbouring epithelial cells. However, in hormone-refractory cells a shift has been reported from paracrine secretion to autocrine expression of these growth factors by epithelial cells [64]. Deregulation of growth factor pathways was further substantiated by the present study, where we observed variation not on the expression level of growth factors and receptors, but of multiple other members of these signaling cascades, among which the oncogenes VAV3 and TWIST1. The effect of peptide growth factors/cytokines on androgen independent proliferation of prostate cancer cells is mainly mediated via the Ras/MAPK, PI3K/AKT and STAT3 signaling cascades [65,66]. These signaling pathways may directly activate transcription of genes involved in cell survival, proliferation and migration, but may also indirectly activate the AR pathway in a ligand-independent manner (Fig. 2). In the absence of androgens, MAPK and AKT kinases may induce AR phosphorylation and activation, whereas STAT3 can bind ligand-free AR and facilitate its translocation to the nucleus. Both these mechanisms ultimately result in ligand-independent expression of AR target genes [65,66]. Thus cytokines and peptide growth factors may function as alternative survival/growth pathways, for example in the subgroup of AR negative prostate tumors, and/or as an adaptation of AR pathway by preserving AR activity under androgen ablation conditions. Recent reports have implicated Src, a member of the Src-family kinases, in the proliferation of hormone-refractory tumors. Src kinase was found to be overexpressed in prostate cancer, where

Src inhibitors decreased proliferation and invasion of cell lines and xenografts [67,68]. Src-family kinases are nonreceptor protein tyrosine kinases responsible for signal transduction in many cellular and oncogenic processes. Src kinases are activated upon binding to cell surface receptors, such as G-protein coupled receptors, growth factor receptors and integrins, or other intracellular nonreceptor protein kinases [67,69]. In turn, activated Src kinases signal via the MAPK, PI3K, STAT3 and FAK pathways (Fig. 2). Src kinases are also activators of VAV3 and TWIST1, either directly by phosphorylating and releasing inhibitory tyrosine Y173 of VAV3, or indirectly by activating STAT3, a transcription activator for TWIST1 [70,71]. These results link the Src pathway to growth factor pathways and to our candidate genes selected for hormone-refractory progression. Previous studies have shown that decreased AR activity in hormone-refractory disease correlated with increased Src activity, and that Src activation induced androgen-independent growth [60]. These results reveal the Src pathway as a potential therapeutical target in prostate cancer and justify future clinical trials with Src inhibitors in hormone-refractory disease.

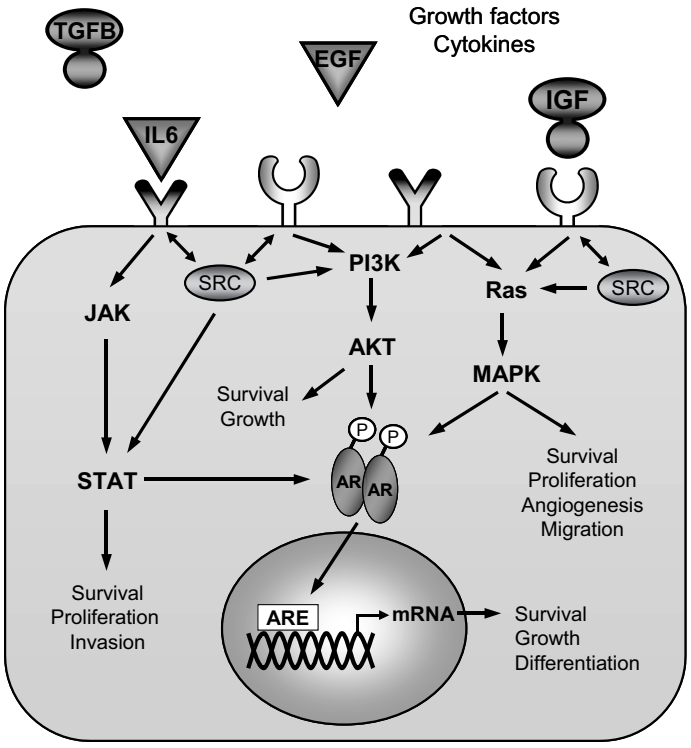

Figure 2 - AR pathway crosstalk. Growth factors and cytokines binding to membrane receptors on prostate cancer cells activate, among others, Ras/MAPK, PI3K/AKT and JAK/STAT signaling cascades. Intracellular kinases may then phosphorilate and activate the AR, leading to androgen-independent transcription of AR target genes. Furthermore, these pathways by themselves are also involved in survival, growth and invasion processes.

Hypothetical mechanism of prostate cancer progression

The results of the present study, combined with our current knowledge of prostate cancer disease, can be summarized in the mechanistic model proposed in Fig. 3. In this model, the AR pathway plays a crucial role in the normal prostate and initial stages of prostate malignancy. However, the AR has dual cellular functions, dependent on the cell type and context on which it is activated: on one hand it promotes survival and proliferation, on the other hand it induces cellular differentiation and maturation. In healthy settings, the AR maintains prostate function through a delicate balance between cell survival and differentiation. Malignant outgrowth is triggered by the switch from androgen-dependent survival to androgen-stimulated cellular proliferation. Possible candidates for initiating this process are gene fusions between androgen-regulated genes and ETS transcription factor family members [72,73,74]. The most frequent of these rearrangements is the TMPRSS2-ERG fusion, which can be detected in as much as the half of prostate tumors [75]. So far, multiple ETS family members (ETV1, ETV4, ETV5, ELK4 or FLI1) and 5′ fusion partners (SLC45A3, ACSL3, HERV-K_22q11.3, HNRPA2B1, C15orf21, KLK2, CANT1, FLJ35294 and DDX5) have also been identified [72,73,74,76]. However, ectopic overexpression of ERG or ETV1 in prostate epithelial cells RPWE and PrEC had no effect on cell proliferation, anchorage-independent growth nor did it cause cell transformation. Instead, it made the epithelial cells invasive through Matrigel assays. Conversely, ERG or ETV1 knockdown in VCaP and LNCaP cells, respectively, significantly inhibited invasion without affecting proliferation [73,77]. Furthermore, prostate specific expression of ERG or ETV1 in mice induced prostatic intraepithelial neoplasia (PIN) but it did not progress into carcinoma [73,77]. In contrast, ETS translocations are not frequently found in association with PIN lesions in human prostate [78]. These results indicate that ERG or ETV1 overexpression alone is not sufficient to initiate neither prostate neoplasia nor human PIN. Tomlins *et al.* proposed that preceding genetic lesions deregulate cellular proliferation resulting in PIN, whereas ETS fusions drive the transition to carcinoma [79]. Recently, two independent transgenic mouse models revealed a cooperation between TMPRSS2:ERG and PTEN loss in the transition from prostatic intraepithelial neoplasia (PIN) to prostate adenocarcinoma [80,81]. In addition, these studies have shown that PTEN loss/down-regulation and ETS gene fusions co-occur in early disease, thus confirming that this mechanism may also be involved in prostate cancer initiation in humans. Neither TMPRSS2-ERG nor TMPRSS2-ETV1 fusions have been detected in PC346 cell lines, and it is unclear which mechanism drives androgen-sensitive growth of PC346C cells. Nevertheless, it is still possible that other less common fusions partners that we have not tested are involved.

At early stages, when tumors are well differentiated, AR signaling simultaneously stimulates tumor growth and maintains expression of genes involved in prostate function and secretion of prostatic fluid. As cancer progresses, the AR pathway is attenuated due to down-regulation of AR target genes involved in prostate cell differentiation and maturation. Hormonal therapy also plays a role in this AR pathway attenuation, which will eventually culminate in fast-growing, poorly-differentiated tumors. Nevertheless, AR knockdown experiments suggest that the AR pathway may remain vital for most hormone-refractory cells, as it induced apoptosis and inhibited growth of multiple castration-resistant cell lines and xenografts [21,22,23,24,25,26]. Upon

hormone therapy, androgen-refractory cells may resume growth by adaptations of the AR pathway and/or activation of alternative growth pathways (Fig. 3) [82]. Our cell line model represents two of these AR modifications: AR mutation (PC346Flu2) and AR overexpression (PC346Flu1), as well as AR pathway bypass through activation of oncogenes and tumor suppressor down-regulation (PC346DCC). During the preparation of this manuscript, a novel mechanism for androgen-receptor adaptation was discovered, implicating constitutively active truncated AR variants in hormone-refractory growth. These variants may be produced either by alternative splicing or by proteolytic cleavage mediated by calpain [83,84,85]. In either case, truncated AR, lacking the C-terminal LBD domain, is produced that exhibits constitutive activity independent of ligand. We have not yet investigated whether such truncated variants are present in the PC346 panel, and the possibility remains that such phenomenon could be responsible for PC346Flu1 or PC346Flu2 proliferation in the absence of androgens.

Prostate cancer progression markers

The last goal of this study was to identify markers for the diagnosis and/or prognosis of prostate cancer, or with potential value for targeted therapies. Currently, the most widely used cancer marker is prostate specific antigen (PSA). The serum PSA test is used not only in the detection of prostate cancer, but also in the follow up of tumor recurrences after therapy [86,87]. However, the PSA test is neither specific, as serum PSA levels may also be increased by other prostatic diseases, such as benign prostatic hyperplasia (BPH) or prostatitis, nor can it predict tumor progression [87,88,89].

In the present study, following expression microarray analysis, 6 candidate genes were selected for real-time PCR quantification in a distinct set of prostate cancer samples. This selection included 3 androgen-regulated genes, ACSL3, ENDOD1, MCCC2, and 3 genes unaffected by androgens, VAV3, TWIST1 and DKK3. All these genes showed deregulation in prostate cancer, but their diagnostic/prognostic value was limited due to high inter-individual variation and poor separation across the different disease states. The best separation between normal prostate tissue and prostate cancer samples was seen for the expression of the tumour suppressor DKK3, which showed a sensitivity of 63% and specificity of 95% (data not shown). Furthermore, DKK3 is a secreted protein, which facilitates quantification in serum, urine or prostatic fluid. But its down-regulation in cancer makes it technically less suitable as a diagnosis tool. On the other hand, VAV3 could be an interesting prognostic marker as its expression decreased stepwise during progression to invasive prostate cancer and hormone-refractory disease. Furthermore, low VAV3 expression associated with decreased metastasis-free survival. These were unexpected findings as VAV3 belongs to a family of oncoproteins and showed increased expression levels in metastatic prostate tumors across multiple public microarray databases. A possible explanation for this discrepancy was described above and could lie on the fact that VAV3 has multiple splice variants. Therefore, before VAV3 may be considered as a prognostic tool, one must first clarify the reason for such discrepancies and how the different splice variants contribute to prostate cancer progression. In conclusion, an optimal diagnostic marker must be simultaneously sensitive, specific for prostate cancer and detectable by non-invasive methods. On the other hand, an optimal prognostic marker should, at an earlier disease stage, be able to distinguish the indolent from the life threatening tumors, or to predict

the response to therapy. Of the six candidates analysed in the present study, DKK3 and VAV3 had the most potential as disease markers, but further optimisation and validation will be necessary to develop reproducible and robust assays. In addition, all the tested candidate genes showed deregulated expression patterns in prostate malignancy, thus providing important clues on the mechanisms of prostate cancer development and progression.

Therapeutical implications

Several findings made during the course of this study may have repercussions in our understanding of prostate cancer progression and in the way we treat hormone-refractory disease. In this sense, we showed that:

(i) AR modifications occurred in the vast majority of castration-resistant cell lines and xenografts subjected to androgen ablation and/or antiandrogen treatment. Selective pressure from hormonal treatment may enrich for AR amplification and AR mutations that are activated by non-androgenic compounds, such as antiandrogen flutamide (Chapters 2 and 3). These results suggest that the AR pathway plays an important role in prostate cancer proliferation but current hormonal therapy is not capable of efficiently shutting down AR signaling. The failure of these therapies is not caused only by the selection of activating AR mutations. Constitutively active AR variants originating from alternative splicing or proteolytic cleavage, ligand-independent AR activation by crosstalk with other intracellular signaling cascades, local androgens production by prostate tumor cells and alterations in AR co-regulators may all activate the AR under hormone blockade therapy (Fig. 3) [82]. Recently, new generation antiandrogens, MDV3100 and RD162, have been produced for the treatment of advanced prostate tumors. These new compounds induced significant responses in 40% of the castration-resistant patients, reaching 50% decline in PSA, and are now entering a phase III trial. But whether these effects can be maintained longer than the reported 3 months is still unknown [90]. Both MDV3100 and RD162 target the ligand-binding domain of the AR, and may not be efficient in blocking constitutive ligand-independent AR activity. Therefore, compounds capable of targeting the AR transactivation or DNA-binding domains might prove more efficient in blocking AR activity and deserve more attention in drug development.

(ii) Increased AR levels may result in a hyper-responsive receptor, active in the absence of ligand and/or sensitive to residual androgen levels. AR overexpression in PC346Flu1 cell line resulted in growth inhibition in the presence of physiological concentrations of ligand (Chapters 2 and 3). This androgenic repression phenomenon could be a consequence of AR induced cellular differentiation and a possible mechanism underlying hormonal therapy withdrawal responses observed in some patients.

(iii) AR pathway is attenuated in advanced metastatic prostate cancer, which may reflect the hormonal therapy given to disseminating tumors. This down-regulation is selective and includes genes involved in development and cellular differentiation, of which expression is less favourable during accelerated growth and invasion (Chapter 4). These results and the observed growth inhibitory effect of androgens on hyper-reactive hormone-refractory cells suggest intermittent hormone therapy, in cycles blocking growth and restoring androgen-regulation, as a potential treatment option to retard

tumor progression. The feasibility of intermittent hormone therapy is under investigation in multiple clinical trials [91]. Although final data from phase III trials are still due, in the past years over a dozen phase II trials, including more than 2000 patients, have evaluated the efficacy and safety of this treatment modality. Despite beneficial effects on quality of life and reduced morbidity, preliminary results show no evidence supporting a delay of the progression to hormone-refractory disease. Nevertheless, this does not exclude the possibility that intermittent therapy may delay progression of specific subgroups of tumors, in particular those exhibiting AR amplification/up-regulation. Furthermore, it is also possible that the study protocols still need further optimization, for example on the type of hormonal therapy offered, when to commence the treatment and the duration of the on- and off-periods in each cycle. Since cell line and xenograft studies show that the growth inhibitory effects are reached at higher androgen concentrations than those that stimulate growth, it is also important to optimize the extent of testosterone recovery in the off-treatment periods.

(iv) Activation of cytokine and peptide growth factors pathways is a possible mechanism of androgen-independent growth (Chapter 5). These pathways signal via Src kinase, MAPK, PI3K and STAT3 cascades, among others, which in turn may directly induce survival, proliferation and invasion processes, or phosphorylate and activate the AR or its coregulators (Fig. 2). Additionally, Src kinase is a key regulator of healthy bone turnover and appears to be involved in prostate cancer metastasis to the bone [92,93,94]. In this sense, Src, MAPK, PI3K or STAT3 inhibitors have the potential to target simultaneously both AR and alternative growth pathways, thus providing an interesting therapeutical approach for hormone-refractory prostate cancer. Inhibitors targeting these pathways, such as AZD6244 (ras/MAPK), AZD0530 (Src), perifosine (PI3K/AKT) or INCB18424 (JAK/STAT), are currently being tested in clinical trials on a variety of tumors [67,95,96,97]. The available data on prostate cancer trials is disappointing. A phase II trial of the Src inhibitor AZD0530 in patients with advanced hormone-refractory disease showed limited clinical efficacy [98]. Also, the AKT inhibitor perifosine had modest clinical activity in biochemically recurrent prostate cancer [96]. The same is true for Iressa (gefitinib), a selective tyrosine kinase inhibitor specific for epidermal growth factor receptor [99]. Nevertheless, most of these studies included a small number of patients with late hormone-refractory disease, and it remains to be tested how these drugs perform in earlier disease stages or together with androgen ablation therapy. Novel compounds with enhanced *in vitro* and *in vivo* potency are currently under development, which, in combination with hormonal therapies, may improve the treatment of advanced prostate cancer.

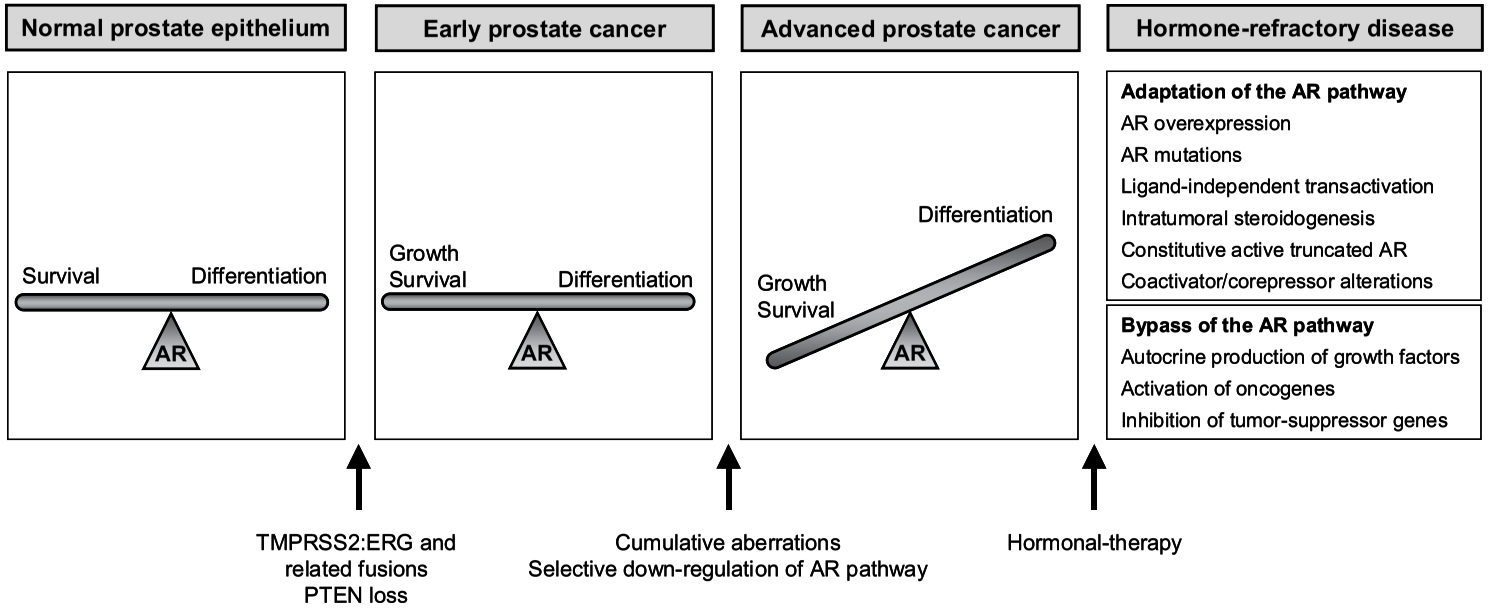

Figure 3 - Mechanistic model of prostate cancer progression. In normal prostate epithelium there is equilibrium between survival and differentiation functions of the AR. The switch from androgen-dependent survival to androgen-responsive proliferation marks the development of prostate cancer. Cooperation between TMPRSS2:ERG (or related fusions) and PTEN loss is a possible initiating event in prostate tumorigenesis. In advanced stages, the AR pathway is selectively modulated and the balance is tilted towards tumor growth. Hormonal-therapy is offered to patients with advanced invasive disease, but the tumors will eventually become resistant to androgen ablation/blockade by either adapting or bypassing the AR pathway.

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SUMMARY

SAMENVATTING

SUMMARY

Prostate cancer is the most diagnosed male cancer in the western society. In most of the cases it has a benevolent course and these patients rather die with the prostate tumor than from the disease. However, once the tumor has escaped the prostate and metastasized, no curative treatment is available and palliative hormonal-therapy is often offered. The aim of the present study was to address the following critical questions in this field of research: (i) How do we distinguish the benevolent tumors from those with an aggressive outcome? (ii) What is the role of the AR pathway in prostate cancer progression? (iii) What other survival and growth pathways are involved in hormone-refractory proliferation?

Basic and translational prostate cancer research relies on diverse model systems to investigate disease mechanisms, identify diagnostic/prognostic markers and test novel therapies. In **Chapter 2** we reviewed *in vivo* and *in vitro* models for human prostate cancer, with particular focus on the PC346 panel used throughout this study. Thanks to a unique combination of primary tumor origin, *wild-type* AR expression, PSA secretion and androgen-responsive growth, the PC346C cell line is a predilect model to replicate hormone-naïve prostate cancer. Three hormone-refractory sublines were derived *in vitro* from the androgen-responsive PC346C, upon long-term hormonal manipulations: PC346DCC (androgen ablation), PC346Flu1 and PC346Flu2 (supplemented with antiandrogen flutamide).

In **Chapter 3** we further characterized the AR status and hormone response of these castration-resistant sublines, compared to the parental PC346C cells. The PC346DCC subline was shown to express background levels of AR and PSA and was insensitive to hormonal stimulations, suggesting it may have bypassed the AR pathway. In turn, PC346Flu1 overexpressed a *wild-type* AR, being hypersensitive to and growth inhibited by R1881. Finally, the T877A AR mutation was identified in PC346Flu2. This mutation is known to promote proliferation in the presence of non-androgenic ligands, including the antiandrogen flutamide, and explains the stimulatory effect of both R1881 and hydroxyflutamide on PC346Flu2 cells. Herein, we showed that long-term hormonal manipulations selected for AR modifications that guide hormone-refractory growth.

The role of the AR pathway in prostate cancer progression was addressed in **Chapter 4**. To begin with, the AR-regulated gene signature of PC346 cell lines was established using expression microarray analysis. Consistent with the physiological role of the AR pathway in prostate development, maturation and function, the androgen-regulated genes identified in PC346 cells are involved in the processes of transcription regulation, intracellular signal transduction, differentiation and regulation of cell proliferation and cell death. Furthermore, R1881 also induced genes associated with the metabolism of proteins, carbohydrates and lipids that contribute to the production and secretion of prostatic fluid. Subsequently, this AR target signature was linked to previous prostate cancer microarray studies, to explore how the AR pathway is modulated during the disease development and progression. This approach showed a consistent attenuation of the AR pathway in late stage metastatic disease, which appeared to be selective for genes involved in cellular differentiation and maintenance of secretory function. These findings, together with the growth inhibitory effect of androgens on

PC3436Flu1, question the current over-simplistic view of the AR pathway as the mechanism of prostate cancer proliferation.

Besides the AR, alternative survival and growth pathways were investigated, by profiling differential gene expression between hormone-naive PC346C cells and derivative hormone-refractory sublines (Chapter 5). Deregulation of growth factor signaling was detected in PC346DCC, the subline believed to have bypassed AR pathway. In addition, the tumor suppressor DKK3, and the VAV3 and TWIST1 oncogenes were evaluated for their role in prostate cancer progression and as potential prognostic markers.

Finally, in Chapter 6 we summarize the findings of this study, integrating with the current knowledge in the field, to propose a mechanistic model for prostate cancer progression and potential therapeutical strategies for hormone-refractory disease.

SAMENVATTING

Prostaatkanker is de meest voorkomende vorm van kanker bij mannen in de westerse samenleving. In de meeste gevallen is sprake van een gunstig ziekteverloop en overlijden deze patiënten niet als gevolg van de prostaattumor. Wanneer de tumor is uitgezaaid is er geen genezende behandeling mogelijk, en wordt palliatieve hormonale therapie vaak aangeboden. Het doel van de huidige studie is meer inzicht te krijgen in enkele voor dit onderzoeksgebied essentiële vraagstellingen: (i) Hoe identificeren we de tumoren met een gunstig verloop van tumoren met een agressief ziekteverloop? (ii) Wat is de rol van de androgeenreceptor (AR) cascade in de progressie van prostaatkanker? (iii) Welke andere overlevings- en groeicascades zijn betrokken bij hormoon-refractaire proliferatie?

Basaal en translationeel prostaatkanker onderzoek heeft verschillende modelsystemen nodig voor het bestuderen van de ziektemechanismen, het zoeken naar diagnostische en prognostische merkstoffen en de ontwikkeling van nieuwe behandelingen. In **Hoofdstuk 2** geven we een overzicht van *in vivo* en *in vitro* modellen voor humane prostaatkanker, met de focus op het PC346 panel gebruikt in onze studie. De PC346C cellijn is afkomstig van een tumor uit de prostaat, vertoont *wild-type* AR expressie en prostaat specifiek antigen (PSA) secretie, en is afhankelijk van androgeen voor optimale groei. Dankzij deze unieke combinatie is PC346C een bijzonder geschikt model om de hormoon-gevoelige fase van prostaatkanker te vertegenwoordigen. Drie androgeen-onafhankelijke sublijnen zijn afgeleid van de androgeen-gevoelige PC346C cellen, na langdurige *in vitro* hormoonbehandelingen: PC346DCC (androgeen ablatie), PC346Flu1 and PC346Flu2 (aangevuld met het antiandrogeen flutamide).

In **Hoofdstuk 3** hebben we de status van de AR cascade en de hormoon-gevoeligheid van deze androgeen-onafhankelijke cellijnen gekarakteriseerd en vergelijken met de oorspronkelijke PC346C cellen. De drie gegenereerde PC346 sublijnen hebben allen een uniek fenotype. De PC346DCC brengt vrijwel geen AR of PSA meer tot expressie en groeit androgeen-onafhankelijk, een aanwijzing dat de AR cascade mogelijk is omzeild. Daarentegen, vertoont PC346Flu1 over-expressie van *wild-type* AR en is supergevoellig geworden voor androgenen. Ten slotte, werd de T877A AR mutatie in PC346Flu2 cellen aangetroffen. Deze gemuteerde AR wordt ook geactiveerd door antiandrogenen, zoals het antiandrogeen flutamide, en verklaart de groei stimulerende uitwerking van zowel R1881 als flutamide op PC346Flu2 cellen. Hierbij hebben we aangetoond dat langedurige hormonale behandelingen selecteerde voor AR afwijkingen die leiden tot androgeen-onafhankelijke groei.

De rol van de AR cascade in progressie van prostaatkanker wordt in **Hoofdstuk 4** beschreven. Om te beginnen, is het AR-gereguleerd genexpressie profiel van PC346 cellijnen vastgesteld door middel van de microarray technologie. De androgeen-gereguleerde genen geïdentificeerd in PC346 cellen weerspiegelen de fysiologische rol van de AR cascade in de ontwikkeling, rijping en functie van de prostaat. Het merendeel van deze genen is betrokken bij de processen van transcriptie regulatie, intracellulaire signaal transductie, differentiatie en regulering van celproliferatie en celdood. Bovendien, induceert R1881 ook genen betrokken bij het metabolisme van eiwitten, koolhydraten en vetten die bijdragen aan de productie en secretie van de prostaatvloeistof. Vervolgens, is deze AR-gereguleerde signatuur gekoppeld aan eerder

gepubliceerde prostaatkanker microarray studies, om na te gaan hoe de AR cascade wordt gemoduleerd tijdens de ontwikkeling en progressie van deze ziekte. Deze aanpak liet een consistente afname van de AR cascade activiteit zien bij gemetastaseerde ziekte. Deze afname leek selectief voor genen die betrokken zijn bij cellulaire differentiatie en het onderhoud van de secretiefunctie. Deze bevindingen, samen met het groeiremmende effect van androgenen op PC3436Flu1, trekken het huidige oversimplistische inzicht van de AR cascade als het mechanisme van prostaatkanker proliferatie in twijfel.

Behalve de AR, zijn alternatieve overlevings- en groeicascades onderzocht door de differentiële genexpressie tussen hormoon-naïeve PC346C cellen en hormoon-refractaire sublijnen te analyseren (**Hoofdstuk 5**). Deregulering van groeifactorcascades werd ontdekt in PC346DCC, de sublijn die de AR cascade omzeilde. Verder, zijn het tumorsuppressorgen DKK3 en het oncogenen VAV3 en TWIST1 beoordeeld op hun rol in de progressie van prostaatkanker en als potentiële prognostische merkstoffen.

Tenslotte, worden in **Hoofdstuk 6** de bevindingen van deze studie besproken en geïntegreerd in de huidige kennis op dit gebied. Hierbij, stellen we een mechanistisch model voor prostaatkanker progressie en potentiële therapeutische strategieën voor.

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