Mechanisms of androgen-independent Prostate Cancer Progression

WHICH WAY TO GO?

Mechanismen van progressie van androgeen-onafhankelijke prostaatkanker

LANGS WELKE WEG?

Proefschrift

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General Introduction

General Introduction

1.1 Cancer and prostate

Finding a cure for cancer is a quest many life-scientists are on. Whether a cure for all types of cancer ever can be found, is doubtful. More probable, little pieces of knowledge will form a more complete view of cancer and will provide us with new targets for intervention. Cancer development is complex and treatment has to aim at inhibiting many facets of cancerous growth and spread.

In this chapter, the complexity of prostate cancer development, at the molecular level, is described. Furthermore, the use of conventional and modern techniques for discovery of new targets for intervention is discussed. One of the potential targets (REPS2) and its function in cellular processes is described in more detail.

1.1.1 *Prostate development*

To describe the development of prostate cancer, I will first discuss normal development of the prostate. The prostate originates from the embryonic urogenital sinus, consisting of mesenchyme and epithelium. Interactions between mesenchyme (stroma in the adult prostate) and epithelium are necessary for prostate development, and only in the presence of androgens prostatic development occurs (Marker et al., 2003). During development of the prostate, androgens act on androgen receptor (AR)-expressing mesenchymal cells. The mesenchymal cells induce epithelial duct formation and differentiation into secretory epithelial cells. In turn, epithelial cells stimulate mesenchymal cells to differentiate into smooth muscle cells. Although epithelial development seems to be dependent only on androgen action in the mesenchyme, AR expression in the epithelium is obligatory for proper secretory function in adulthood (Cunha et al., 2003). The indirect regulation of epithelial growth and differentiation suggests the presence of paracrine, androgen-induced mediators, that are secreted by the mesenchyme and act on epithelial cells. Likely candidates are soluble growth factors and many are suggested to play a role. Fibroblast growth factors, FGF7 and FGF10, have been implicated (Thomson, 2001), as well as epidermal growth factor (EGF), and transforming growth factors TGFα, TGFβ1 and TGFβ3 (Haughney et al., 1998). During adulthood, maintenance and homeostasis of the prostate gland need to be controlled, and this also requires functional interactions between stroma and epithelium. The smooth muscle cells of the stroma maintain epithelial structure and function, and the epithelial cells maintain smooth muscle differentiation. Androgens are necessary for these interactions; if androgens are removed, the prostate regresses by apoptotic cell death (Isaacs et al., 1994).

During prostate carcinogenesis, stromal-epithelial interactions become disrupted. This disruption could occur through loss of normal homeostatic control by genetic changes in epithelial or smooth muscle cells or both (Hayward et al., 1997). Abnormal secretion of androgen-induced or constitutively expressed factors by epithelial cells, abnormal stimulation of epithelial proliferation by stroma, abnormal expression of growth factors and growth factor receptors, and changes in the extracellular matrix, all

are suggested mechanisms that may contribute to carcinogenesis of the prostate (Cunha et al., 2003).

1.1.2 *Prostate cancer*

For the western male population, the lifetime risk to die from prostate cancer is estimated to be 3% (World Cancer Report 2003, WHO). As many as 33.3% of men over 80 are estimated to have undetected prostate cancer foci (Sanchez-Chapado et al., 2003). However, since prostate cancer growth is slow, most men will die from other causes (Ruijter et al., 1999). The major problem of prostate cancer is that detection of the early treatable stages is difficult and treatment of late stages is only palliative. Thus far, the most powerful diagnostic tool for prostate cancer is detection of a rising level of prostate-specific antigen (PSA) in the serum. The serum concentration of this protein correlates reasonably with tumour stage and volume (Noldus et al., 1998; Polascik et al., 1999). However, despite the widespread use of PSA screening, one-third of new cases has already developed locally advanced disease at detection (Catalona et al., 1993; Johansson et al., 1997). Initially, treatment of prostate cancer consists of local therapies like prostatectomy and radiation, or a "watchful waiting" strategy is chosen, pending stage of disease and age of the patient. Although about two-third of patients are cured after local therapy, in the remaining one-third the cancer had already spread beyond the surgical margins. These tumours cannot be removed and will eventually develop into advanced metastatic disease (Barry et al., 2001; Ward et al., 2003).

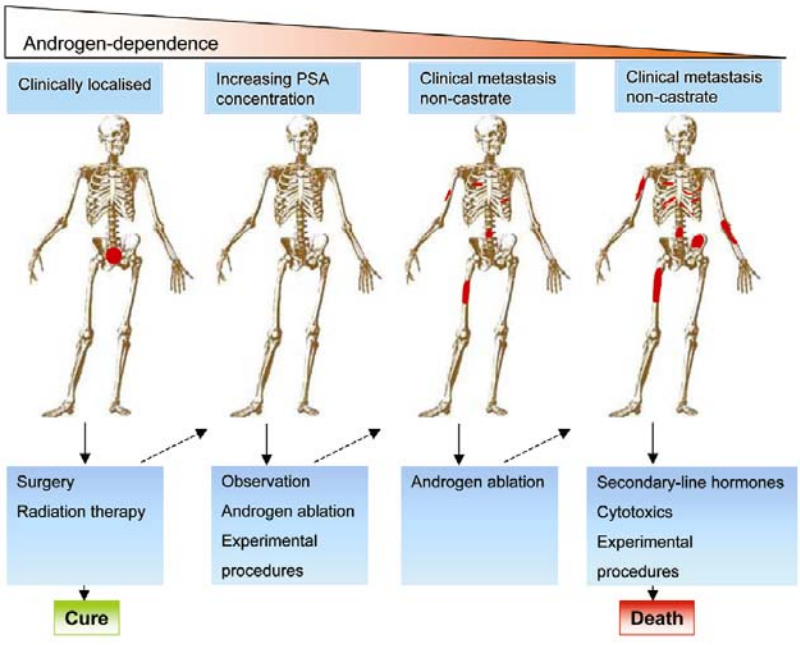

Figure 1.1. **Different stages of prostate cancer and the accompanying treatment options**
Treatment of prostate cancer is only curative when the cancer is restricted to the prostate or its direct vicinity. During progression of prostate cancer towards metastatic disease (spread mainly to the bone), dependence on androgens for growth decreases and treatment options become limited.

Patients with metastatic prostate cancer receive androgen ablation therapy, which is based on the dependence of prostate cells on androgens for growth and survival. In 80% of cases, androgen ablation reduces tumour size initially, but a portion of the tumour, consisting of androgen-independent cells, remains (Goodin et al., 2002). These androgen-independent cells are thought to originate from androgen-sensitive cells that undergo adaptation, or are intrinsically present and selected for during androgen ablation (Patterson et al., 2002). Either way, the importance of biochemical and molecular mechanisms involved in appearance of androgen-independent cells during prostate cancer progression is generally recognised and will be discussed further.

1.2 Androgen and growth factor signalling

Androgen-induced growth and survival is required for proliferation and maintenance of human prostate cells. Prostate cancer development into the advanced stages at some point usually involves a transition from androgen-dependent to androgen-independent growth, and consequently these cells then use other signalling pathways for proliferation and survival. These alternative pathways may still involve androgen receptor signalling or are truly androgen receptor-independent.

1.2.1 *Androgen signalling cascade*

Androgens are necessary for development, maintenance and functional differentiation of the prostate (Arnold and Isaacs, 2002; Cunha et al., 1987). Androgens bind to the androgen receptor (AR), an intracellular receptor that becomes activated upon ligand-binding. After androgen binding the receptor undergoes conformational changes, leading to dimerization and translocation to the nucleus. The activated nuclear AR can bind to specific sequences in promoter regions of androgen-regulated genes and modulate transcription (Balk, 2002; Gelmann, 2002). Regulation of androgen-induced or -repressed genes involves recruitment of several co-activators, co-repressors, transcriptions factors, and other nuclear proteins (Brinkmann et al., 1999). Androgen-regulated genes are involved in many cellular processes in the prostate: cell cycle control, DNA repair, lipid metabolism, signal transduction, and more (DePrimo et al., 2002).

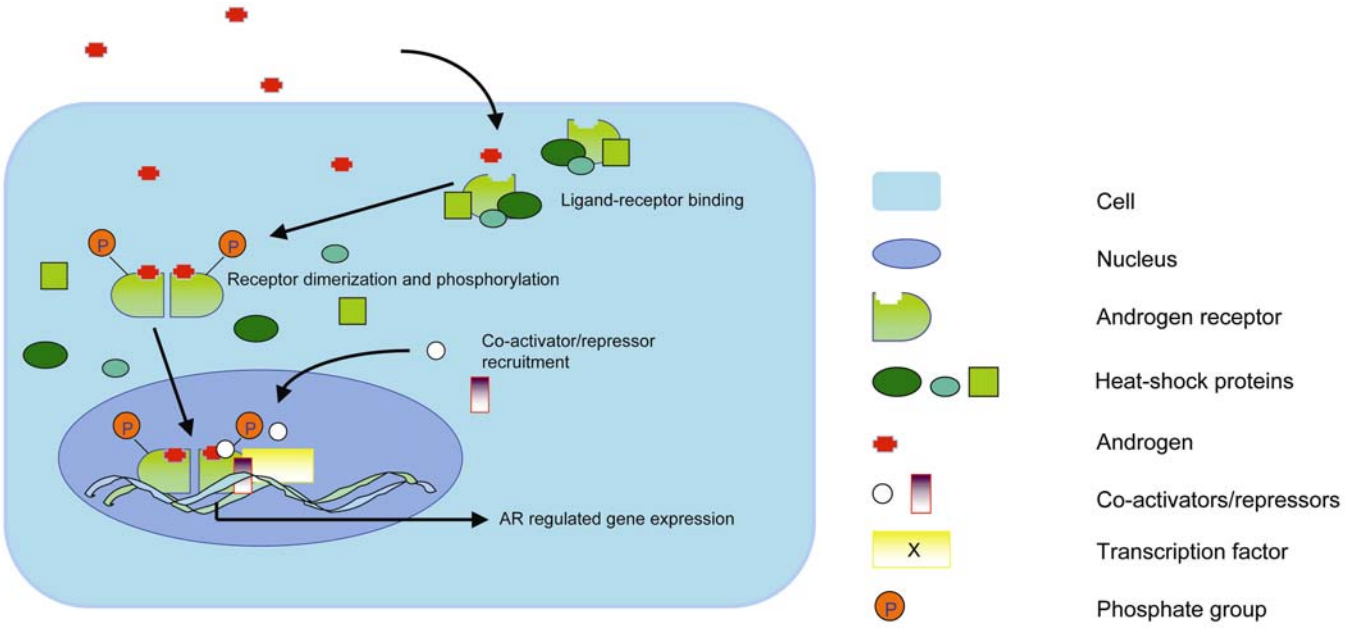

Figure 1.2. **AR activation and transcription modulation**
The AR is activated when ligand enters the cell and binds the receptor. A conformational change dissociates the androgen receptor from its accompanying chaperone proteins. The receptor becomes phosphorylated, homo-dimerizes and translocates to the nucleus. In the nucleus, the receptor modulates transcription after recruitment of several co-activators, co-repressors, transcription factors and other DNA modifying proteins.

1.2.2 *AR involvement in prostate cancer*

There are two important questions with respect to androgen signalling and carcinogenesis of the prostate:

- What is the role of the AR in development and progression of prostate cancer?
- Does androgen ablation therapy contribute to development of androgen-independent prostate cancer?

AR signalling has been target for treatment of advanced prostate cancer since Huggins and Hodges (1941) found that prostate cancer regresses when androgens are removed from the circulation by surgical removal of the main source of androgen, the testes (Huggins and Hodges, 2002). Blocking the AR signalling cascade leads to induction of apoptosis in the prostate and consequently regression of the tumour (Kyprianou et al., 1990). Although 80% of patients respond to androgen ablation, the response is temporary and tumour cells gain the ability to grow in an androgen-deprived environment (Crawford, 1992). The cause or causes of development of androgen-independence has been subject of study for many years. Since most advanced tumours still contain AR, loss of expression is not likely a cause of therapy resistance (Culig et al., 1993; Hobisch et al., 1995). Suggested mechanisms implicated in development of androgen-independence include AR-activating mutations, AR amplification, altered expression and activity of AR co-activators or co-repressors or crosstalk with activated peptide growth factor receptor signalling pathways (Balk, 2002; Shaffer and Scher, 2003).

AR mutations that change the sensitivity of the AR for androgens or other steroid hormones have been found, but clinical relevance has not been proven. Mutation rates vary from 0% to 50% in androgen-independent tumour samples (Evans et al., 1996; Suzuki et al., 1993; Taplin et al., 1995; Tilley et al., 1996). Another way to compensate for a low level of circulating androgens is AR gene amplification. Visakorpi et al. (Visakorpi et al., 1995) and Koivisto et al. (Koivisto et al., 1997; Visakorpi et al., 1995)) found AR gene amplification in 30% of recurrent prostate cancers. In these studies, AR gene amplification correlated with increased expression of AR protein. Since AR gene amplification is found only in patients who had received ablation therapy, it was suggested that androgen withdrawal selects for cells that are more sensitive to low levels of circulating androgens (Trapman and Brinkmann, 1996). Next to androgens, other factors like peptide growth factors can also activate the AR and compensate in that manner for a low level of androgens (Ruijter et al., 1999). The following paragraphs will describe functions of peptide growth factors in normal prostate function and in prostate cancer. Furthermore, crosstalk between androgens and growth factor signalling pathways will be discussed.

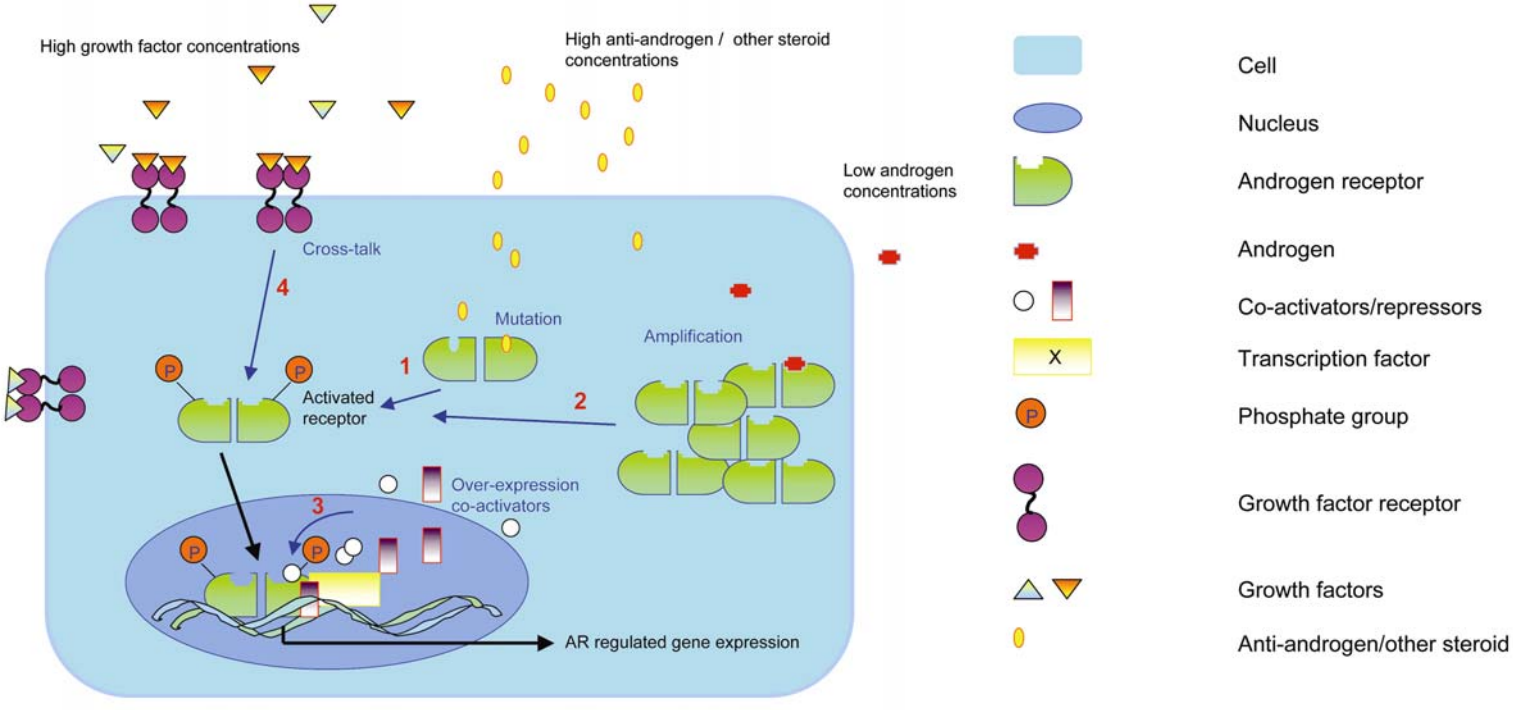

Figure 1.3. **Possible mechanisms of AR activation in an androgen-deprived environment**

1. AR activating mutations enable the receptor to bind other ligands, e.g. anti-androgens, and to become activated.
2. AR amplification/over-expression. More receptor molecules sensitise the cell for a low concentration of androgen.
3. Over-expression of co-activators augments transcriptional activity of the AR in the presence of ligand.
4. Crosstalk with other signal transduction pathways activates the AR via other mechanisms then ligand-binding.

1.2.3 *Growth factor involvement in prostate cancer*

In normal prostate, growth factor pathways are regulated in a paracrine manner: stroma cells produce growth factors for which epithelial cells posses receptors (Russell et al., 1998). EGF, for example, is produced by stroma cells under the influence of androgens (Hiramatsu et al., 1988; Nishi et al., 1996) and binds the EGF receptor on epithelial cells. The epithelial cells depend on these factors for their growth and differentiation. However, in advanced stages of prostate cancer, when androgen level is low as a result of androgen ablation, epithelial cells may start to produce their own growth factors and become independent of androgen receptor activity (Cunha, 1994; Wong and Wang, 2000). Moreover, the tumour cells do no longer need the prostate environment and can metastasise outside the organ. Not only expression of peptide growth factors is altered in advanced prostate cancer, their receptors are also subject to changes (Djakiew, 2000). The best studied peptide growth factor-signalling pathway is that of EGF and its receptor.

The EGF receptor is activated by a number of ligands, including EGF and TGF-α (Carpenter and Cohen, 1990; Massague, 1990). These two peptide growth factors are structurally related and, as they use the same receptor, their functions overlap. Both EGF and TGF-α switch from a paracrine to an autocrine regulation of EGF receptor activation during progression of prostate cancer and their expression becomes elevated (Ching et al., 1993; Fowler et al., 1988; Glynne-Jones et al., 1996; Harper et al., 1993). EGF receptor expression also increases and has been correlated with increasing malignancy (Di Lorenzo et al., 2002).

Although patient studies are inconclusive, androgen ablation has been shown to increase EGF receptor expression, while in normal prostate the receptor is down-regulated by androgens (St-Arnaud et al., 1988; Traish and Wotiz, 1987). Interestingly EGF has been described to stimulate not only growth of cancer cells, but also the invasive capacity of prostate cancer cells is enhanced (Jarrard et al., 1994; Rajan et al., 1996). Other peptide growth factors implicated in prostate cancer development and progression include FGFs, IGFs (IGF1 and IGF2), NGF, PDGF, VEGF and TGF-β (Djakiew, 2000). In this thesis, the focus will be on EGF signalling and the involvement of this pathway in advanced prostate cancer.

1.2.4 *EGF signalling cascade*

The EGF transmembrane receptor is a member of a protein tyrosine kinase subfamily consisting of EGFR/ERBB, HER2/ERBB2, HER3/ERBB3, and HER4/ERBB4 (Burgess et al., 2003; Roskoski, 2004; Ullrich and Schlessinger, 1990). After ligand binding, the EGF receptor forms a homodimer or dimerizes with one of the other members of the family (Wells, 1999). Dimerization activates the intrinsic kinase activity of the cytoplasmic tail of the receptor, which induces phosphorylation of the other member of the receptor pair and thus forms docking sites for cytoplasmic adaptor molecules (Yarden, 2001). Subsequently, these activated adaptors activate members of the RAS family. Dependent on cellular context, many effectors and pathways are known to be activated by the EGF receptor via RAS proteins. They include the mitogen-activated protein kinase (MAPK), the phosphoinositide 3-phosphate lipid kinase pathway (PI3Kinase pathway), guanine nucleotide exchange

factors for the RAC1, RALA and RHO small GTPases, and many more (Pruitt and Der, 2001; Wells, 1999).

The activated MAPK (MEK) phosphorylates an extracellular (signal-) regulated kinase (ERK), which translocates to the nucleus. In the nucleus, ERKs activate transcription factors which induce transcription of genes that can play a role in proliferation, differentiation and migration (Garbay et al., 2000).

The PI3Kinase pathway facilitates the conversion of phosphatidylinositol 4,5-phosphate (PIP2) to phosphatidylinositol 3,4,5-phosphate (PIP3) which promotes activation of an AKT/PKB survival pathway. An important inhibitor of this pathway is the tumor suppressor PTEN (phosphatase and tensin homolog). PTEN is lost or mutated in many cancers, including prostate cancer (Li et al., 1997; Vlietstra et al., 1998; Wang et al., 1998). Loss of PTEN expression constitutively activates the PI-3Kinase pathway by phosphorylation of AKT, resulting in loss of susceptibility to induction of apoptosis by cytotoxic therapy or androgen-ablation (del Peso et al., 1997; Kulik et al., 1997). It has been shown that in advanced stages of prostate cancer significantly more AKT is phosphorylated, compared to the earlier stages (Malik et al., 2002). Furthermore, androgen-ablation has been reported to increase the activity of PI3K and AKT (Murillo et al., 2001). Therefore, the PI3Kinase pathway seems to be essential for survival of prostate cancer cells in the absence of androgens (Lin et al., 1999). Since EGF activates the PI3Kinase pathway, autocrine activation of the EGF receptor may be a significant factor adding to the progression of prostate cancer from androgen-dependent to androgen-independent growth.

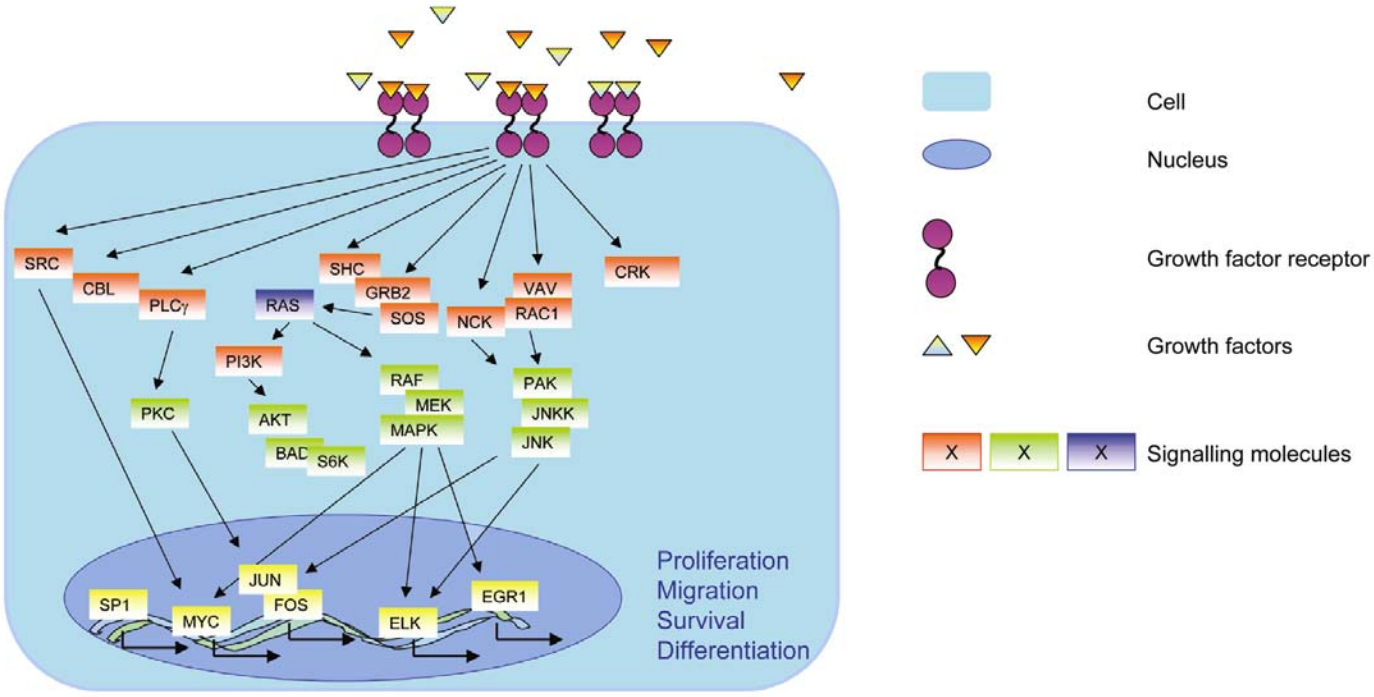

Figure 1.4. EGF receptor signalling cascade
The EGF receptor is activated by ligand binding. Consequently, depending on cellular context, diverse, partly overlapping signal transduction pathways are activated that lead to different cellular responses.

1.2.5 *Crosstalk investigations*

To investigate possible roles that growth factors and androgens play in prostate cancer development, prostate cancer cell lines and animal models are helpful tools. An androgen-dependent and an androgen-independent cell line are essential to study the mechanisms of progression towards androgen-independence *in vitro*. One truly well-characterised androgen-dependent human cell line available is the LNCaP cell line (lymph node carcinoma of the prostate) (Horoszewicz et al., 1980). This line contains a functional AR which harbours a mutation that broadens its ligand specificity. This mutated AR can be activated by oestrogens, progestins and antiandrogens (Grigoryev et al., 2000; Veldscholte et al., 1990). Many androgen-independent LNCaP sub-lines have been established (van Steenbrugge et al., 1989; Wu et al., 1994), providing suitable tools for studying progression of human prostate cancer. Another cell line with AR expression is CWR-R1, which is derived from the recurrent human prostate cancer xenograft CWR22 and is androgen-responsive (Gregory et al., 2001). CWR-R1 also contains a mutated AR that is different from the LNCaP mutation, but changes its characteristics similarly to the LNCaP mutation (Tan et al., 1997). Most prostate cancer cell lines are derived from metastatic lesions and are androgen-independent. PC3 (Kaighn et al., 1979) and DU145 (Mickey et al., 1977) are well known and widely used androgen-independent cell lines. These lines do not express the AR and yield poorly differentiated tumours when injected into nude mice (Kaighn et al., 1979; Mickey et al., 1977; Tilley et al., 1990).

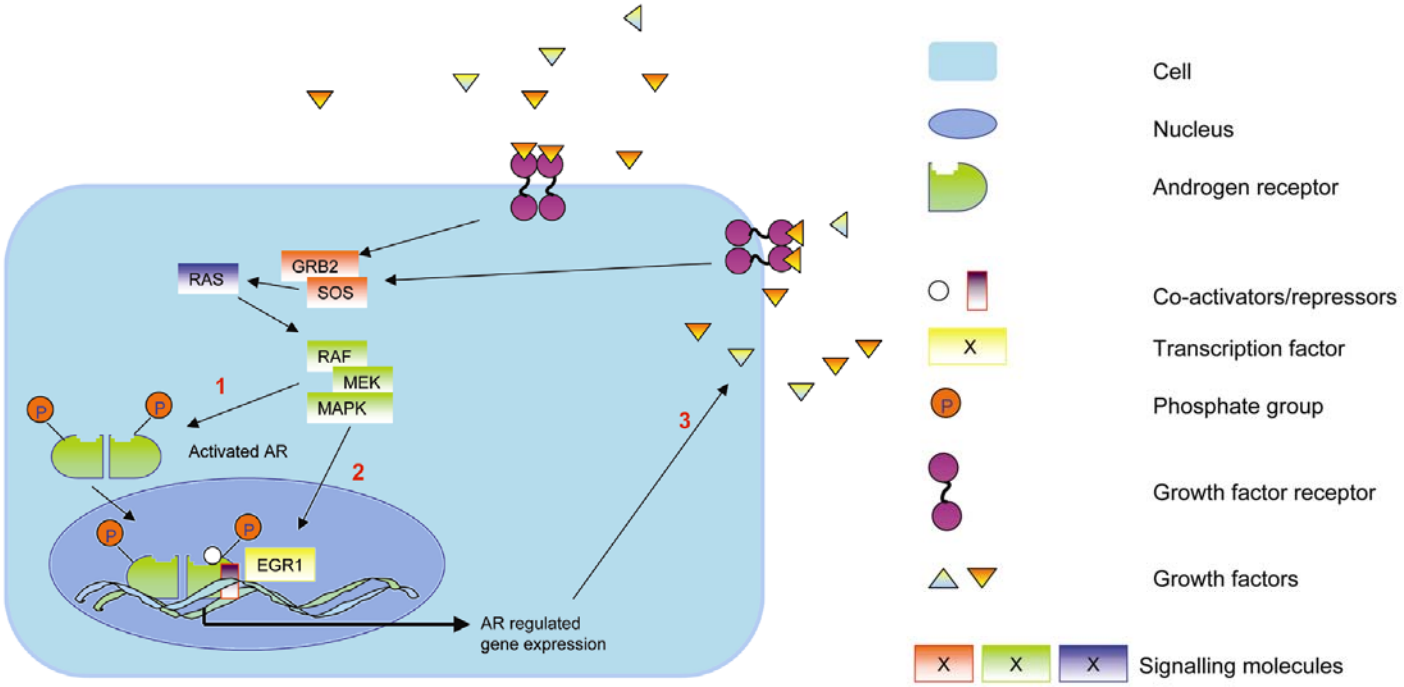

Figure 1.5. **Possible crosstalk mechanisms**
1. Activation of AR via phosphorylation by the MAPKinase pathway
2. Regulation of AR-dependent transcription via activation or increased expression of specific transcription factors
3. AR-mediated regulation of peptide growth factor expression and/or receptors

Normal prostate and early prostate cancer cells are dependent on androgens, but also on growth factors for homeostasis and growth (Russell et al., 1998). When prostate cancer cells are stimulated with androgens or EGF in vitro, they start to proliferate. Interestingly, when the cells are treated with EGF and androgens simultaneously, proliferation is stimulated synergistically, indicating that the AR and EGF receptor pathways influence each other (Guo et al., 2000; Schuurmans et al., 1988a). It is possible that the AR directly influences EGF receptor signalling, but a mechanism where components of the EGF receptor signalling pathway activate the AR is also feasible (Grossmann et al., 2001)

Reports on direct action of androgens on EGF signalling show that androgens stimulate expression of EGF by stromal cells in normal prostate, but not in prostate cancer where EGF is expressed in an autocrine manner in epithelial cells (Russell et al., 1998; Sherwood and Lee, 1995). In agreement with this, expression of EGF was observed in the prostate cancer cell line LNCaP, but no increase in EGF expression by androgen treatment could be shown (Connolly and Rose, 1990). Androgens have also been reported to stimulate expression of EGF receptor (Schuurmans et al., 1988b), but data are conflicting because there are also many reports indicating increased EGF receptor expression after androgen ablation (Di Lorenzo et al., 2002; Djakiew, 2000; Fiorelli et al., 1991). Despite these conflicting data, a detailed analysis of the changing pattern of expression of the EGF receptor and its ligands during progression of prostate cancer did indicate that a switch from paracrine to autocrine EGF signalling can play a role in the autonomous growth of androgen-independent prostate cancer cells (Scher et al., 1995).

Effects of peptide growth factors on AR activity have also been reported. For example, EGF has been described to decrease AR expression (Mizokami et al., 1992). A more indirect effect of EGF on AR activation has been suggested by several other groups. Craft et al. (1999) and Cullig et al. (1994) reported that EGF simulates AR trans-activation in the absence of androgens, but in the presence of AR (Craft et al., 1999; Culig et al., 1994). Gregory et al. (2003) showed that androgen-activated AR is required for EGF activation of AR trans-activation. This EGF-mediated AR activation is partly achieved via the MAPK pathway, since specific EGF receptor inhibitors or MAPK inhibitors can diminish the effect (Gregory et al., 2003).

Induction of proliferation, next to AR trans-activation, has also been reported to result from crosstalk between EGF receptor- and AR-regulated pathways (Torring et al., 2003; Ye et al., 1999), although others report divergent stimulation of proliferation (Bell et al., 2003; Guo et al., 2000; Jones et al., 2001). Despite these investigations, the interaction between AR- and EGF receptor-mediated pathways is not fully understood, but many observations point towards an important role for both signalling pathways in regulation of prostate cancer growth. Changes in prostate cancer cells that activate these signalling pathways can give rise to a more aggressive phenotype. Proteins involved in androgen- or EGF-signalling that are either up- or down-regulated or mutated during progression of prostate cancer are therefore possible targets for intervention. Identification and functional studies of such proteins can explain their effect on progression of disease.

1.3 REPS2 and progression of prostate cancer

Thus far, it is difficult to predict which prostate tumours will progress, and which will not. Therefore, reliable markers are needed to distinguish between relatively harmless well treatable tumours that will not progress and metastasise, and aggressive life-threatening tumours. Treatment of the former tumour type would not be necessary, whereas surgical removal of the latter should be performed as soon as possible. To determine proteins that are up- or down-regulated during progression of prostate cancer we used a technique called differential display. This technique is based on differences in mRNA content between two cell types. The flow chart of the technique is illustrated in Figure 1.6.

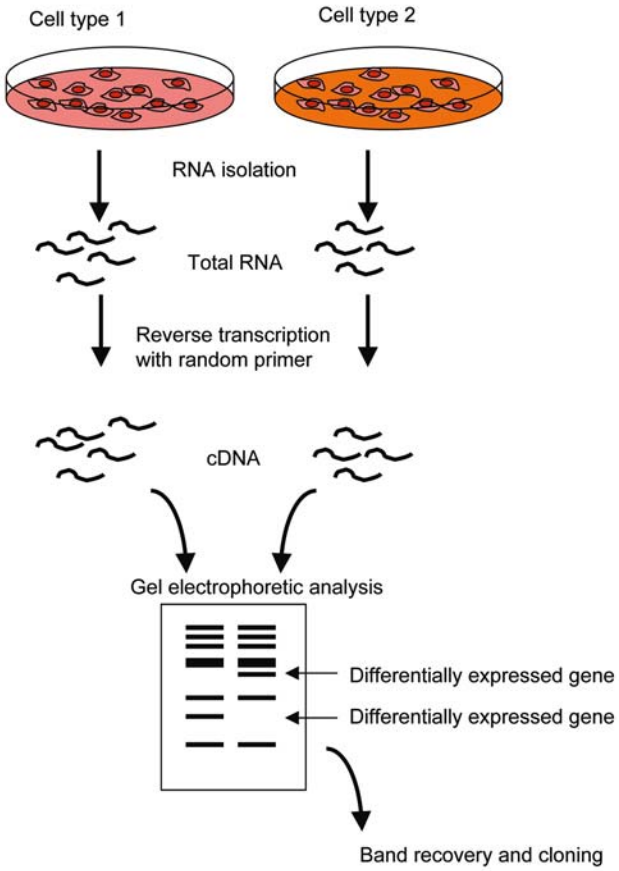

Figure 1.6. **Flow-chart of differential display analysis**
Two samples of RNA are isolated, e.g. one from cell type 1 and the other from cell type 2. After a reverse transcription procedure, where RNA is reverse transcribed into cDNA the two samples are analysed by electrophoresis. Differentially expressed cDNA bands are slices out and the sequences determined.

REPS2 was identified in our laboratory, by differential display, from a 4 kb transcript down-regulated in androgen–independent compared to androgen-dependent prostate cancer cell lines and xenografts (Chang et al., 1997). The full-length cDNA of the transcript contains an open reading frame encoding a protein of 659 amino acid residues. The sequence of this clone revealed a 99% homology to the protein POB1 (REPS2), and 60-80% homology to the functional domains of the human REPS1

protein. The REPS2 protein contains an EPS homology (EH) domain, a putative EH domain, a coiled-coil region and two proline-rich motifs (Ikeda et al., 1998; Koshiba et al., 1999).

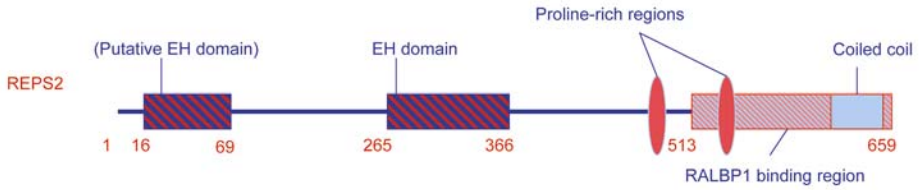

Figure 1.7. **Functional domains of REPS2**
Linear map of REPS2. The position of EH domains, proline-rich regions, RALBP1 binding region and the coiled coil is indicated.

These functional domains indicate a possible function of the protein.

- Via the coiled-coil region, REPS2 binds RALBP1, a protein involved in inactivation of the small GTPases RAC1 and CDC42.
- Via its two proline-rich regions REPS2 interacts with the growth factor receptor adaptor protein GRB2 (Ikeda et al., 1998).
- The EH-domain of REPS2 was found to bind directly to EPN1 (Epsin1) and EPS15 (Morinaka et al., 1999; Nakashima et al., 1999). EPS15 also contains EH domains and is involved in endocytosis of activated tyrosine kinase receptors (Santolini et al., 1999).

1.3.1 *Clathrin-mediated endocytosis of EGF receptor*

Many proteins with an EH-domain have been identified and shown to be involved in endocytosis (Santolini et al., 1999). Endocytosis of activated tyrosine kinase receptors is mediated through formation of clathrin-coated pits and leads to receptor degradation or receptor recycling (Sorkin and Waters, 1993). Clathrin forms a triskelion of heavy and light chains, which organize together in cage-like structures. The whole process of clathrin coat formation is tightly regulated and involves a whole array of accessory proteins (Brodin et al., 2000). The main factor in the clathrin coat formation process is the adaptor-related protein complex 2, AP-2. This complex consists of four subunits, two large (α and β) and two small subunits (σ2 and μ2), and associates with clathrin and the other mediators of endocytosis. Clathrin interacts with the β subunit of AP-2 (Ahle and Ungewickell, 1989), and EPS15, EPN1, amphiphysin, AP180, and auxilin interact with both the α-appendage and the β subunit (Owen et al., 2000; Pearse et al., 2000). Binding of activated EGF receptor to AP-2 initiates formation of the clathrin coated lattice, recruitment of accessory proteins and subsequent internalisation of the receptor (Santolini et al., 1999). EPS15 was shown to be involved in formation of the growing rim of the clathrin coat (Tebar et al., 1996) and to be essential for endocytosis of EGF receptors (Carbone et al., 1997).

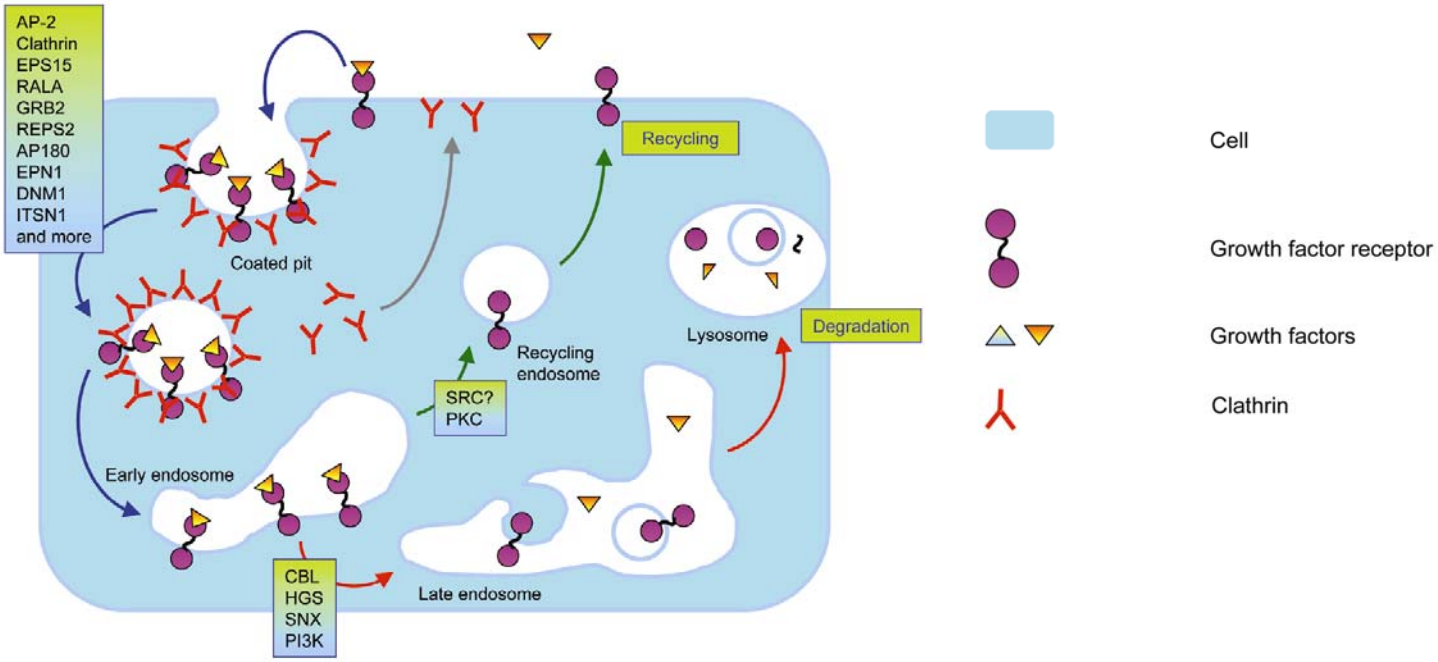

Figure 1.8. Clathrin-mediated endocytosis
After ligand binding, growth factor receptors are sequestered in clathrin-coated pits. This process is tightly regulated by many proteins like EPS15, EPN1, AP180, and many more. The pit is pinched off and becomes an early endosome. The receptor can then be either recycled back to the membrane, via a recycling endosome, or degraded via late endosomes and finally the lysosome.

1.3.2 *Endocytosis, growth factor signalling, REPS2 and prostate cancer*

Expression of REPS2, a binding partner of EPS15 and EPN1, is lost during prostate cancer progression. REPS2 also binds to GRB2 and RALBP1, which are also involved in internalisation (Jiang et al., 2003; Jullien-Flores et al., 2000; Martinu et al., 2002), and deletion mutants of REPS2 have been shown to inhibit endocytosis of EGF and insulin (Nakashima et al., 1999). These observations point towards involvement of REPS2 in receptor internalisation and to involvement of receptor internalisation in prostate cancer development.

Internalisation of activated EGF receptor does not only lead to down-regulation of receptors and attenuation of signalling, but has also been implicated as essential for signal-transduction itself. For example, the adaptor protein GRB2 was found to be associated with activated EGF receptor and activated RAS proteins in the endosomes (Jiang and Sorkin, 2002). The subcellular localisation of activated growth factor receptors seems to determine which signal transduction pathways are activated and when (Burke et al., 2001). The specificity of these so-called signalling endosomes was shown by the fact that inhibition of endocytosis of EGF or insulin receptors by a dominant interfering mutant of dynamin results in inhibition of activation of the protein kinases ERK1 and ERK2, and in an increase of phospholipase Cγ activity (Ceresa and Schmid, 2000; Vieira et al., 1996).

It appears that endocytosis is an essential component of efficient and accurate signalling of growth factor receptors. Furthermore, trafficking and signalling have been suggested to control each other in a feedback manner (Clague and Urbe, 2001). Many molecules involved in receptor endocytosis regulation are also able to activate EGF

signal transduction pathways. GRB2, RALBP1, EPS8, HGS, RAB5a and CBL are such molecules (Clague and Urbe, 2001; Di Fiore and Scita, 2002; McPherson et al., 2001; Urbe et al., 2000). REPS2 potentially possesses these qualities as well: involvement in endocytosis via EPN1 and EPS15, and involvement in EGF-signalling via RALBP1 and GRB2. Furthermore, expression of REPS2 is lost during prostate cancer progression and abnormal expression of endocytic proteins has been implicated in tumour development and mitogenic signalling (Di Fiore and Gill, 1999; Floyd and De Camilli, 1998).

Investigations performed to elucidate possible functions of REPS2 in signalling and internalisation of growth factor receptors, together with its possible involvement in prostate cancer progression, are described in Chapters 2 and 3.

1.4 Microarray analysis

Recently, microarray analysis, a new tool to analyse differences in mRNA expression between two conditions, was developed. The technique is bases on specific RNA expression in a certain sample. RNA expression profiles of, for example, healthy tissue and cancer tissue, or treated and untreated cell lines are measured and compared. This method allows comparison of expression levels of potentially all genes in the whole genome between different conditions in one single experiment. The flow chart of the technique is illustrated in Figure 1.9. In Chapters 4 and 5, this technique is used to determine the genetic differences between androgen-dependent and androgen-independent cells. Furthermore, crosstalk between androgen and growth factor receptor signalling pathways in prostate cancer progression is investigated.

A well-performed microarray experiment leaves researchers with an enormous amount of data. Therefore, computerized methods have been developed for analysis. The software developed is diverse and new programs emerge almost every day. Since analysis of data is the key to reliable results, the principles will be discussed here.

A microarray data analysis can be divided in 4 steps:

1. Processing the raw expression data to acquire background and foreground signals of both channels and checking the quality of each spot.
2. Normalizing the acquired red/green ratios in order to adjust for variation caused by other factors then differential expression.
3. Pattern determination to find interesting groups of genes to analyse further.
4. Annotation, to determine the function of selected genes and their possible relations.

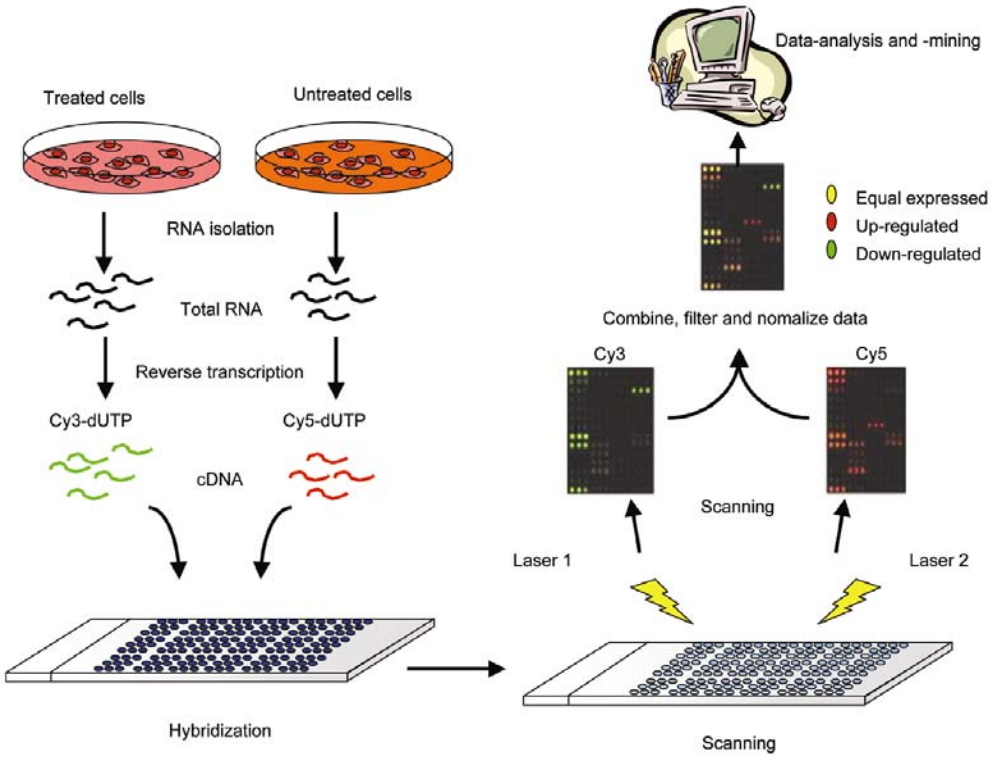

Figure 1.9. **Flow-chart of microarray analysis**
Two samples of RNA are isolated, e.g. one from treated and the other from untreated cells. After a reverse transcription procedure, where RNA is reverse transcribed into cDNA and the different dyes, Cy3 and Cy5, are incorporated, the two samples are hybridised to the microarray slide. After appropriate washing, the slides are then scanned and the generated data can be analysed after combining the two channels and normalization of the acquired signal intensities (Russo et al., 2003; Schulze and Downward, 2001).

Step 1. Processing of the data comprises determination of foreground and background intensities of the Cy5 and Cy3 signals. The background signal is subtracted from the foreground signal, which results in a corrected value for either the Cy5 or Cy3 signal of that spot. Background signals can be determined by several methods, either by defining the local background near the spots, or using a non-linear filter called the morphological opening, which determines the background at the actual spot location (Smyth et al., 2003). It is common practice to present expression data as a log2-differential expression ratio (experimental signal/control signal). Many methods to determine spot quality have also been developed. The main purpose is to be able to flag unreliable spots, e.g. if the foreground signal is lower then the background signal or if the spot has a strange shape or distribution of pixel intensities.

Step 2. Normalization corrects for distorted expression ratios caused by Cy5/Cy3 bias, differences in printing tips, handling procedure, etc. The Cy5/Cy3 bias is caused by differences in labelling efficiency and scanning properties of the two dyes. The intensities of the Cy3 dye are often higher. Global normalization is widely used and correction is based on a constant difference between Cy5 and Cy3 signals that is

subtracted from the ratio. Generally, all genes on the array are used for normalization, based on the assumption that the summation of the expression ratios between control and experimental sample is close to zero. This holds true for a test where the difference between experimental and control samples is small and the differentially expressed genes are distributed symmetrically. The use of so-called housekeeping genes, which have constant expression levels between samples, is another method for normalization, but the definition of these genes might be a problem (Yang et al., 2001). Global normalization, although widely used, is not always the best method. This correction method is simplified, while more sophisticated ways of correction, based on location, spot intensity, and background values have been developed and proven to be valid (Kim et al., 2002).

Step 3. When reliable ratios are acquired, the data can be used to start to unravel the biological meaning behind the experiment. The first step is to organize the list of primary data, often consisting of expression ratios for tens-of-thousands of genes in a large number of experiments. A natural way to analyse and visualize the data is cluster analysis. Clustering involves grouping together genes that behave similar in time or between different conditions, and therefore have similar expression patterns. The method is based on maximizing the intra-class similarity and minimizing the inter-class similarity. Next to organizing and reordering the gene list, clustering software is designed to visualize the results in a typical manner. Genes are depicted with a colour, which reflects the expression ratio, qualitatively and quantitatively. Furthermore, the relationships between genes is represented by a tree, the distance between the branches correlates with the distance or similarity between genes. Genes with similar expression patterns, and ideally similar function, are therefore clustered together, have similar colour and are adjacent in the tree (Eisen et al., 1998).

Many clustering methods and software exists, based on all kinds of classifiers, hierarchical clustering, k-means clustering, graph-theoretic clustering or self-organizing maps, etc. (Quackenbush, 2001; Raychaudhuri et al., 2001). To obtain reliable results, the use of a combination of several clustering methods might be ideal (Kaminski and Friedman, 2002). In the end, cluster analysis is a tool to provide biologists with visual graphics instead of large lists and tables, to be able to determine what genes to focus on in further research.

Step 4. To obtain meaningful biological results, the function of the selected groups of genes has to be determined. Again, many tools for annotation of large sets of genes have been developed. These are based either on gene ontology like GenMAPP and MaPPFinder (Dahlquist et al., 2002; Doniger et al., 2003) or on co-appearance in literature like PubGene (Jenssen et al., 2001). Development of such tools is still in progress and needs integrated knowledge of different professionals. Computer technology, biological information, statistic tools, and laboratory experience have to be combined to engage the challenge of microarray analysis fully.

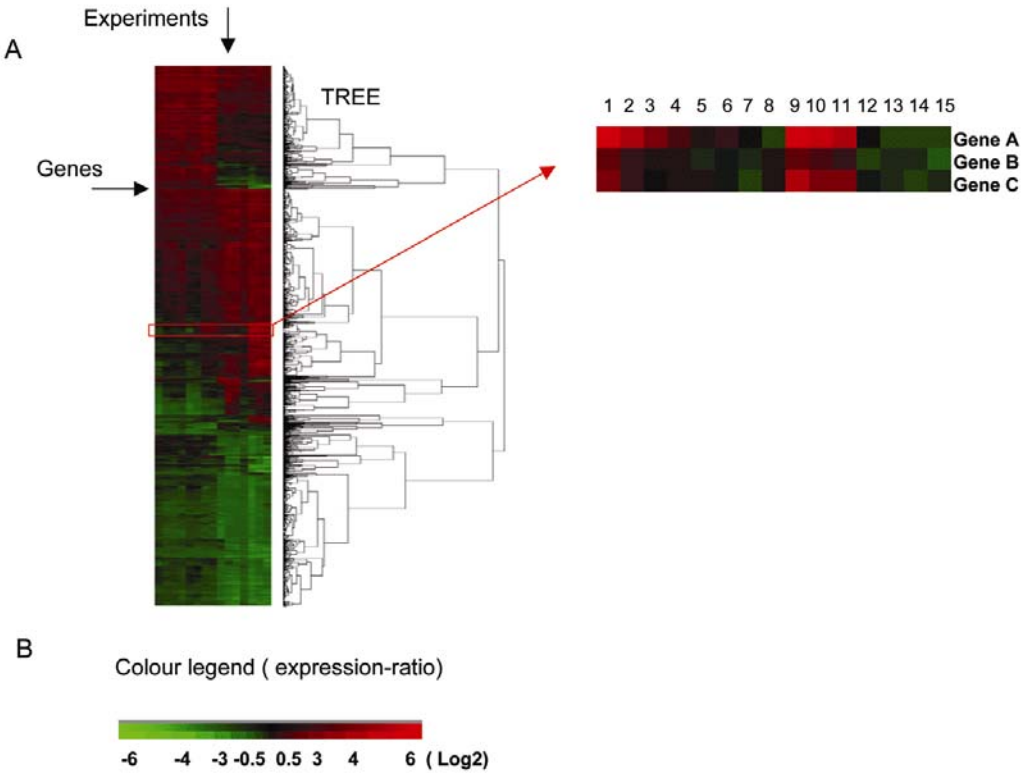

Figure 1.10. **Hierarchical cluster analysis**
A. Example of a generated cluster diagram. The columns represent the different experiments, and the rows represent the different genes.
B. Colour legend. The colour reflects the expression ratio, qualitatively and quantitatively.

1.5 Aims and outline of this thesis

Prostate cancer progression involves a switch from androgen-dependence to androgen-independence. Genetic differences between these states have been subject of research for many years. New tools have been developed to determine differentially expressed genes, but eventually the function of these genes and their functional interconnection determines the value for therapy. Genes with a differential expression pattern can be used for diagnostic means and for intervention. The protein REPS2 was found with a conventional technique, differential display, as a transcript for which expression was lost in androgen-independent prostate cell lines and xenografts (Chang et al., 1997). Concerning this protein we asked the following question, which is addressed in Chapters 2 and 3.

1. *Via which mechanisms does loss of expression of the endocytosis protein REPS2 contribute to an androgen-independent phenotype of prostate cancer cells?*

The involvement of growth factors like EGF in prostate cancer progression is evident (Russell et al., 1998). Since REPS2 is involved in EGF signalling and also implicated in prostate cancer progression, the impact of this signalling molecule on invasion and proliferation of androgen-dependent prostate cancer cells was investigated. Furthermore, possible crosstalk between androgen- and growth factor signalling pathways in these cells was studied. Proliferation and invasion were examined, both biologically and with microarray analysis of androgen-dependent

prostate cancer cells treated with androgens or EGF, addressing the following questions (Chapter 4):

2. *Is it possible to identify genes, activated by androgens or EGF, that induce proliferation and invasion of prostate cancer cells?*
3. *Do EGF- and androgen-signalling overlap in prostate cancer cells and if so, to what extent?*

We were also interested in the mechanisms that control androgen-independent proliferation of prostate cancer cells. Therefore, microarray analysis was performed on androgen-dependent and androgen-independent prostate cancer cells treated with androgens or EGF. Using the generated dataset, the following questions were dealt with (Chapter 5):

4. *What are the differences between androgen-dependent and androgen-independent cells in gene-expression and hormonal control of gene-regulation?*
5. *Can expression analysis provide insight in how androgen-independent cells proliferate without addition of androgens or EGF?*

In **Chapter 2** the differential expression of REPS2 between androgen-dependent and androgen-independent prostate cancer cells, and the effect of the REPS2 protein on cell growth and signalling is described.

In the experiments described in **Chapter 3** the involvement of endocytosis of EGF receptor in prostate cancer progression and the effect of REPS2 on that process is investigated.

In **Chapter 4** the effect of androgen and EGF on proliferation and invasion of prostate cancer cells is described. Additionally, microarray analysis of EGF-treated prostate cancer cells was performed and compared to publicly available microarray datasets of androgen-treated prostate cancer cells and prostate cancer patient samples.

In **Chapter 5** microarray analysis is described of androgen-dependent and androgen-independent LNCaP cells treated with androgens or EGF. The differences between these cell lines in gene expression and gene regulation is investigated in the context of regulation of cell proliferation.

The results of these investigations, in the context of the above questions, are discussed in **Chapter 6**.

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REPS2/POB1 is down-regulated during human prostate cancer progression and inhibits growth factor signalling in prostate cancer cells

REPS2/POB1 is down-regulated during human prostate cancer progression and inhibits growth factor signalling in prostate cancer cells

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The nucleotide sequences reported in this paper have been submitted to GenBank under accession number AF511533 (REPS2a) and AF512951 (REPS2b).

Abstract

During progression of prostate cancer, cellular changes occur, leading to a transition from androgen-dependent to androgen-independent growth. One aspect of this transition is a switch from androgens to growth factors, like epidermal growth factor (EGF), as primary regulators of proliferation. We examined the involvement of REPS2/POB1 in this process. REPS2/POB1 is an EH domain-containing protein, reported to be involved in signalling via RALBP1 and to play a role in endocytosis of EGF receptors. Furthermore, the protein is relatively highly expressed in androgen-dependent as compared to androgen-independent human prostate cancer cell lines and xenografts.

Next to the known REPS2/POB1 protein, an open reading frame encoding REPS2/POB1, with 139 additional amino acid residues at the NH_2-terminus, was cloned and found to be expressed in prostate cancer cells. Overexpression, by transient transfection, of both forms of REPS2/POB1 in prostate cancer cell lines, induced apoptosis within 48 hours. At shorter time intervals after transfection, signalling towards a TPA response element (TRE) luciferase reporter was found to be inhibited. From these experiments, it is concluded that REPS2/POB1, through its influence on the RAL signalling pathway, is involved in growth factor signalling. Decreased expression of REPS2/POB1 during progression of prostate cancer may therefore result in loss of control of growth factor signalling and consequently in loss of control of cell proliferation.

Introduction

The transition from androgen-dependent to androgen-independent growth during prostate cancer progression is of great concern, since androgen-independent tumours are incurable. One possible mechanism underlying this transition is a switch from androgens to growth factors as primary regulators of prostate cancer cell proliferation. Fibroblast growth factors (FGFs), insulin-like growth factors (IGFs) and epidermal growth factor (EGF) are examples of autocrine factors which stimulate growth of advanced, androgen-independent, prostate cancer (Russell et al., 1998). For EGF, binding of the ligand to the receptor induces cell proliferation by activating well-characterised signal transduction pathways that involve phosphorylation and activation of mitogen-activated protein kinases (MAPKs). The activation of the GTP-ase RAS is one of the initial steps in EGF signalling, and once RAS is activated, it can facilitate activation of the serine/threonine kinase RAF, PI3-kinase and RALA-GEF, a guanine nucleotide exchange factor which activates the small GTP-ase RALA (reviewed by (Pruitt and Der, 2001). Activated RALA binds to RALBP1, which exhibits GAP (GTPase activating protein) activity towards CDC42 and RAC1 (Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995).

REPS2/POB1 is a protein partner of RALBP1, contains a single EH domain, two SH3 binding sites and several other functional regions (Ikeda et al., 1998). REPS2/POB1 has been implicated in endocytosis and signal transduction (Ikeda et al., 1998; Kariya et al., 2000; Nakashima et al., 1999) and is currently evaluated for its putative role in prostate cancer progression.

Endogenous expression of REPS2/POB1 in prostate cancer cell lines

In a previous study, reduced mRNA expression of REPS2/POB1 was found in the androgen-independent human prostate cancer cell lines PC3, DU145 and LNCaP-LNO, as compared to the expression level in the androgen-dependent LNCaP-FGC cell line. In addition, using a panel of human prostate cancer xenografts, REPS2/POB1 expression was also found to be markedly reduced in androgen-independent prostate cancer xenografts (Chang et al., 1997).

Using a hypothalamus cDNA library, a 1987 bp cDNA clone was obtained containing an open reading frame encoding a protein of 659 amino acid residues. This protein consists of the published coding region of REPS2/POB1 (accession number AF010233) minus one glutamine at position 43, and 139 additional amino acid residues at the NH_2-terminal end (Figure 2.1A). All reported studies so far, however, made use of the shorter 521 amino acid REPS2/POB1 protein. We examined the possible translation of the extended REPS2/POB1 protein in human cells using a specific REPS2/POB1 antibody and were able to detect 78 kDa and 58 kDa protein bands in lysates from prostate cancer cell lines (Figure 2.1B). These results indicate that, both the 659 amino acid residue, and the 521 amino acid residue REPS2/POB1 protein are expressed in these cell lines.

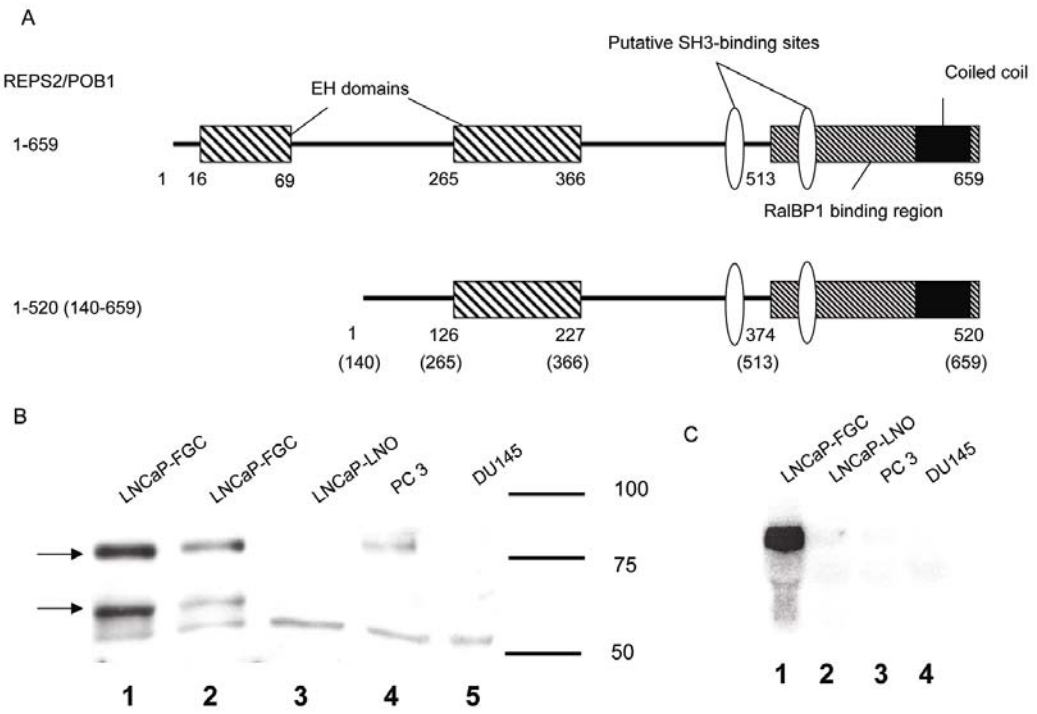

Figure 2.1. **Analysis of endogenous REPS2/POB1 expression in prostate cancer cell lines**
The LNCaP-FGC (passage number 22-30) and LNCaP-LNO (passage number 78-100) cell lines, were kindly provided to us by Dr. J.S. Horoszewicz (Buffalo, NY). The LNCaP-FGC cell line is identical to the one which can be obtained from the American Type Culture Collection (Rockville, MD). LNCaP-FGC, LNCaP-LNO, PC3 and DU145 cells were maintained at 37°C in a 5% CO_2 atmosphere in RPMI 1640 plus 5% v/v fetal calf serum (FCS) or serum depleted of steroids by dextran-coated-charcoal (DCC) treatment (van Steenbrugge et al., 1991). The LNCaP-LNO cell line has been derived from an early passage (6^{th}) of the parental LNCaP-FGC cell line (Horoszewicz et al., 1980).(**A**) Linear map of REPS2/POB1 (1-659) and REPS2/POB1 (140-659), indicating the relative location of EH domains, proline-rich regions, RALBP1 binding region, and predicted coiled coil. (Adapted from Ikeda et al. 1998) (**B**) Western blot analysis of lysates of LNCaP-FGC passage numbers 26 and 28 (*lanes 1 and 2*), LNCaP-LNO (*lane 3*), PC3 (*lane 4*) and DU 145 (*lane 5*) after 7% SDS-PAGE, showed REPS2/POB1 down-regulation in androgen-independent prostate cancer cell lines. The antiserum was generated by immunising rabbits with two REPS2/POB1 peptides; peptide 1: amino acid residues 446-461 and peptide 2: amino acid residues 566-580, and then affinity-purified. The arrows point at the two REPS2/POB1 protein forms. (**C**) Northern blot analysis of REPS2/POB1 mRNA expression in prostate cancer cell lines. Total RNA of LNCaP-FGC (*lane 1*), LNCaP-LNO (*lane 2*), PC3 (*lane 3*) and DU 145 (*lane 4*), was hybridized with a REPS2/POB1 cDNA probe.

An other argument in favour of the co-existence of a longer REPS2/POB1 protein, is the observation that the translation start sequence, proposed by Kozak (Kozak, 1987), is more consistent with the sequence around the more 5' ATG, described herein, then the translation start sequence of REPS2/POB1 described by Ikeda et al. (1998) (Figure 2.2A). Furthermore, the additional 139 NH_2-terminal amino acid residues form a putative second EH domain (Figure 2.2B) and this amino acid sequence exhibits high homology with the mouse Reps1 and human REPS1 proteins (72% identity). Whether both REPS2/POB1 products are formed by alternative first ATG usage or alternative pre-mRNA splicing, is currently unknown. We observed a good correlation between mRNA and protein expression of REPS2/POB1 in DU145, PC3, LNCaP-FGC and LNCaP-LNO cells. The LNCaP-FGC cells were found to express REPS2/POB1 mRNA and protein at relatively high levels, whereas LNCaP-LNO and DU145 cells do not express

detectable levels of REPS2/POB1 mRNA and protein. The PC3 cells show low expression of REPS2/POB1 protein and no detectable expression of REPS2/POB1 mRNA (Figure 2.1B and 2.1C).

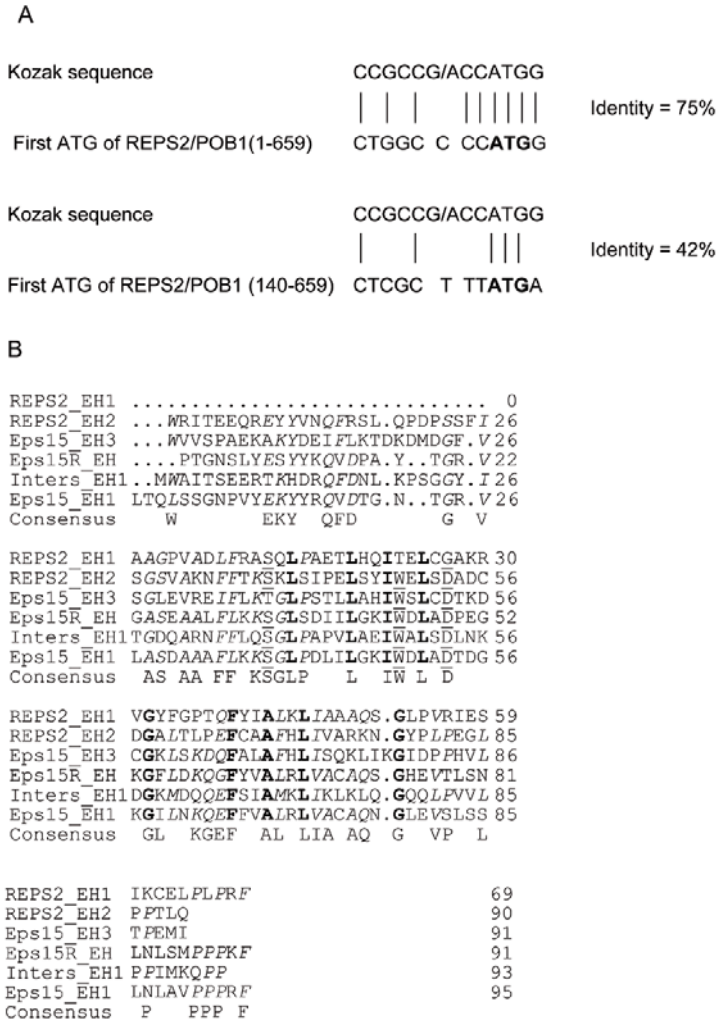

Figure 2.2. **Sequence properties of REPS2/POB1 cDNA**
(**A**) Comparison of translation start site of REPS2/POB1 and REPS2/POB1(140-659) with the start site proposed by Kozak (Kozak, 1987). (**B**) Alignment of several EH domains. REPS2_EH1 is the EH domain found in the first 139 amino acid residues of REPS2/POB1. Also presented are: REPS2_EH2: REPS2/POB1 (275-364); Eps15_EH3: EPS15 (218-313); Eps15R_EH: EPS15R (13-103); Inters_EH1: Intersectin2 (28-118) and Eps15_EH1: EPS15 (16-103). Amino acids shared between more than 50% of the EH domains are in italics, between more than 75% are underlined and 100% alignment is shown in bold. All sequences are from human. Sequences were aligned using the DNAMAN program.

Role of REPS2/POB1 in signal transduction: apoptosis

To determine the cellular localisation of REPS2/POB1 in prostate cancer cells, LNCaP-FGC and LNCaP-LNO cells were transiently transfected with cDNA encoding REPS2/POB1 or REPS2/POB1(140-659), fused to GFP. Cells were analysed with fluorescence microscopy at different time points after transfection. It was observed that both variants of REPS2/POB1 mainly reside in the cytosol and not in the nucleus (Figure 2.3A). Subsequently, the cells were subjected to prolonged expression of GFP-tagged REPS2/POB1, REPS2/POB1(140-659) or part of the androgen receptor (AR), to

examine the effect of REPS2/POB1 and REPS2/POB1(140-659) on prostate cancer cell growth. The tagged AR was used to evaluate non-specific effects of the transfection procedure. REPS2/POB1 was found to induce programmed cell death. Transfected cells which showed DNA fragmentation and cell membrane damage, as observed using Hoechst and propidium iodide staining were considered to be apoptotic (Figure 2.3B-D). REPS2/POB1(140-659) or REPS2/POB1 induced apoptosis in 45% of transfected cells already within 48 hours and up to 60% after 96 hours (Fig 2.3E). Based on these observations, a regulating role for REPS2/POB1 in a signalling pathway that controls apoptosis was hypothesised.

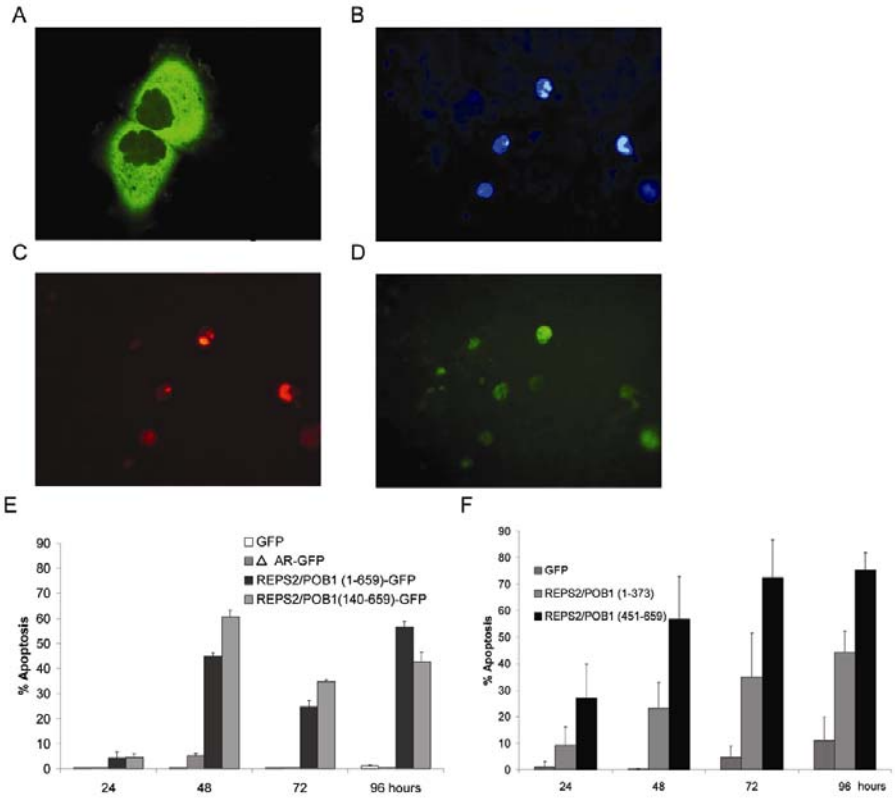

Figure 2.3. **Induction of apoptosis after transfection with REPS2/POB1-GFP**
REPS2/POB1 and REPS2/POB1(140-659) cDNA was obtained from a human brain, thalamus 5'-stretch plus cDNA library (Clontech) and cloned into the pEGFP-N_2 vector (Clontech) between the Xho1 and Kpn1 sites. Deletion mutant REPS2/POB1 (1-373) and REPS2/POB1 (451-659) were synthesised by PCR and ligated in pEGFP-N_2. pEGFP-ΔAR consists of a HindIII fragment of the cDNA encoding the NH_2-terminal part of the androgen receptor (AR), cloned into pEGFP-N_2. LNCaP-FGC cells transfected with cDNA encoding REPS2/POB1-EGFP, ΔAR-EGFP or EGFP alone (1μg/ml medium) were analysed by fluorescence microscopy. (**A**) For localisation of the REPS2/POB1-EGFP product, LNCaP cells transfected with pEGFP-RESP2/POB1 were studied using confocal laser scanning microscopy. LNCaP-FGC cells transfected with REPS2/POB1-EGFP show cytoplasmic localisation of REPS2/POB1-EGFP. (Magnification: 100x). Analysis after 72 hours of transfection with REPS2/POB1-EGFP with fluorescence microscopy. (**B**) Hoechst 33342 staining, (**C**) Propidium iodide staining (**D**) REPS2/POB1-EGFP expression. (Magnification: 40x). (**E**) Cells with positive Hoechst and propidium iodide fluorescence were quantified as a percentage of EGFP positive cells at different time points after transfection. (**F**) Apoptosis induction by deletion mutant constructs of REPS2/POB1 fused to GFP quantified as a percentage of EGFP positive cells, at different time points after transfection. The results shown are the mean ± SD of three independent experiments; each experiment included two independent culture plates.

We investigated whether the role which REPS2/POB1 seems to play in induction of apoptosis can be explained when the function of one of its binding partners, RALBP1, is implicated. When we transfected LNCaP cells with a deletion mutant of REPS2/POB1 lacking the RALBP1 binding domain (1-373), only 30-40% of the transfected cells became apoptotic (72-96 hours), while a mutant containing only the RALBP1 binding domain of REPS2/POB1 (451-659), induced apoptosis in 40-75% of the transfected cells (Figure 2.3F). Since the expression levels of the two proteins were similar, these observations suggest that the RALBP1 binding properties of REPS2/POB1 are at least partly responsible for apoptosis induction in these cells.

RALBP1 is a protein with GAP (GTPase activating protein) activity towards RAC1 and CDC42 (Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995). Activated RAC1 and CDC42 have been implicated in signalling towards cell survival (Joneson and Bar-Sagi, 1999; Osada et al., 1999) and, because of its GAP activity, RALBP1 is capable of inhibiting these functions. The GAP activity of RALBP1, in turn, can be inhibited by activated RALA, which translocates RALPB1 to the cell membrane and physically away from RAC1 and CDC42 (Matsubara et al., 1997). The presence of REPS2/POB1, as a cytosolic protein, may in this respect function to counteract activated RALA, maintaining a number of RALBP1 molecules in the cytosol and thus available to inactivate RAC1 and CDC42. Overexpression of REPS2/POB1 may result in a strong inhibition of RAC1 and CDC42 signalling, which may consequently result in the observed induction of apoptosis.

The deletion mutant that lacks the RALBP1 binding domain also induces apoptosis, but less efficient, suggesting that the two EH domains of REPS2/POB1 may also play a role. The C-terminal EH domain of REPS2/POB1 binds to EPS15 and Epsin and is involved in internalisation of growth factor receptors (Nakashima et al., 1999). Disruption of endocytosis might lead to improper signal transduction and consequently to cell death. For example, it has been reported that inhibition of endocytosis of EGF receptors or insulin receptors by a dominant interfering mutant of dynamin results in inhibition of the activation of the protein kinases ERK1 and ERK2, emphasising the importance of signalling during and after endocytosis.

Role of REPS2/POB1 in signal transduction: inhibition of signalling

It has been described that RAC1 and CDC42 are involved in activation of JUN (Coso et al., 1995) and heterodimers of JUN and FOS activate, among others, genes controlled by promoter sequences containing a TRE (TPA response element) (Angel et al., 1987). A phorbolester responsive reporter system (5xTRE luciferase reporter) was used to investigate the effect of transient REPS2/POB1 expression in the human prostate cancer cell line PC3. The TRE reporter can be activated by several growth factors (including EGF), serum and synthetic compounds like phorbol esters. Since PC3 cells are not very sensitive to EGF (Janssen et al., 1995), 15% v/v fetal calf serum was used to activate transcription of the luciferase reporter gene. It was observed that luciferase transcription was increased approximately 8-12 fold over control expression by addition of serum. Furthermore, short term transfection (24 hours) with increasing amounts of REPS2/POB1 cDNA/well inhibited serum-induced activation of the TRE reporter. Using 10, 30 and 100 ng REPS2/POB1 cDNA/well, a 46%, 80% and 90% reduction in luciferase activity was observed, respectively, compared to mock-transfected cells (Figure 2.4). This reduction in luciferase activity was not a result of REPS2/POB1-induced apoptosis and a decreasing cell number, because apoptosis is not observed at 24 hours after transfection. Furthermore, in a parallel transfection assay, where a RSV-luciferase reporter was used, no significant inhibition of luciferase activity was observed with increasing amounts of REPS2/POB1 cDNA. These results indicate

that overexpression of REPS2/POB1 exerts a marked inhibitory effect on serum-induced signalling to a TRE reporter.

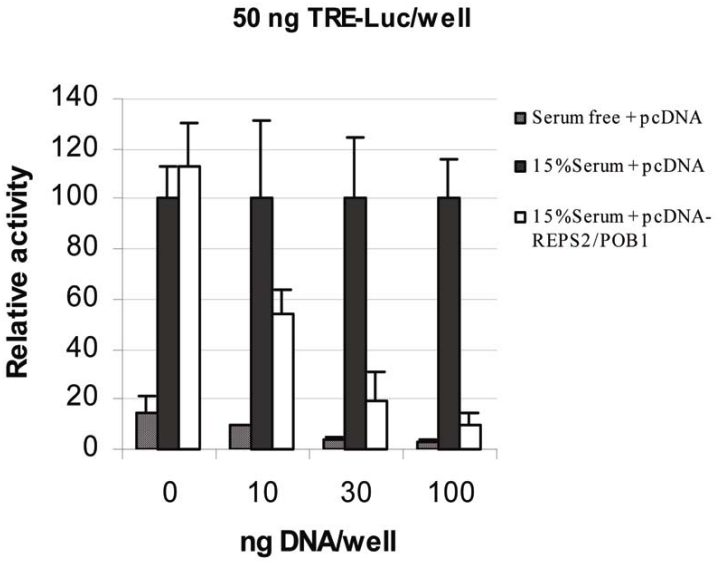

Figure 2.4. **Effect of REPS2/POB1 on TRE-Luc activation in PC3 cells**
pcDNA3.1-REPS2/POB1 was constructed from pEGFP-RESP2/POB1. REPS2/POB1 cDNA was cloned into pcDNA3.1 (Invitrogen). between the Nhe1 and Kpn1 sites. 5xTRE-TATA-Luc was constructed from 5xTRE-TATA-CAT6 (a gift from Dr. A. Cato, Karlsruhe, Germany). PC3 cells were cotransfected with 50ng TRE-Luc plasmid DNA, in combination with 0, 10, 30 or 100 ng of psDNA3.1-REPS2/POB1 or the empty pcDNA3.1 vector. Stimulation with 15% FCS was started immediately after the start of transfection, and luciferase activity was determined at 24 hours with the Topcount NXT^{TM} microplate luminescence counter (Packard Bioscience BV; Meriden, CT, USA). The total amount of DNA in each transfection was normalised using pTZ19U plasmid (US biochemical). The results are expressed relative to the level of luciferase activity in cells cotransfected with the empty vector and the TRE-Luc vector after stimulation with serum. The mean ± SD of an experiment performed in triplicate is shown. Similar results were observed in two independent experiments.

In advanced prostate cancer cells, the expression status of REPS2/POB1 does not represent overexpression, but rather loss of expression. Consequently, the inhibiting activity of RALBP1 on RAC1 and CDC42 may be compromised, resulting in loss of control of cell survival and consequently mitogenic signalling.

It is concluded that loss of REPS2/POB1 expression, which occurs during progression of prostate cancer, results in dysregulation of growth factor signalling. This can be the result of loss of the interaction of REPS2/POB1 with RALBP1 or the result of loss of function of REPS2/POB1 during growth factor receptor internalisation. Furthermore, it is also possible that both mechanisms simultaneously play a role in REPS2/POB1-induced dysregulated growth factor signalling. Since RALBP1, like REPS2/POB1, has also been reported to be involved in endocytosis (Jullien-Flores et al., 2000), effects of REPS2/POB1 on endocytosis, via binding to RALBP1, and then on signal transduction, can not be excluded. Furthermore, it has been described that dysregulated endocytosis can lead to mitogenic signalling, and that mutation or abnormal expression of endocytic proteins is implicated in tumour development (Di Fiore and Gill, 1999; Floyd and De Camilli, 1998). Further research utilising stable inducible cell lines expressing REPS2/POB1 at a more physiological level, will be used to unravel the precise role of REPS2/POB1 in signalling and endocytosis.

Taken all data together, there is evidence to suggest that loss of REPS2/POB1 expression, during progression of prostate cancer, results in loss of control of cell

growth signalling. This can be a direct effect through RALBP1 or an indirect effect via a function in growth factor internalisation. Further investigations of the role of REPS2/POB1 in control of prostate cancer progression are important to improve our understanding of androgen-independent tumour growth.

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EGF signalling in prostate cancer cell lines is inhibited by a high expression level of the endocytosis protein REPS2

EGF signalling in prostate cancer cell lines is inhibited by a high expression level of the endocytosis protein REPS2

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Abstract

In advanced prostate cancer, cellular changes occur leading to a transition from androgen-dependent to androgen-independent growth. During this transition, proliferation of androgen-dependent prostate cancer cells becomes more and more dependent on growth factors, like the epidermal growth factor (EGF). Endocytosis of growth factor receptors, one of the mechanisms that controls growth factor signalling, was observed to be markedly changed in advanced metastatic prostate cancer.

Internalisation and signalling of EGF receptors was examined in different prostate cancer cell lines, in relation to the expression level of the endocytosis-related REPS2 gene. It was observed that a high level of REPS2 correlates with reduced EGF-internalisation. To investigate this more thoroughly, prostate cancer cells with inducible REPS2 expression were generated. Using these cells, it was found that REPS2 induction indeed results in reduction of EGF internalisation. Furthermore, when EGF receptor signalling was evaluated, by examination of mRNA expression for several EGF-responsive genes (EGF receptor, EGR-1, FOS, JUN), it was observed that induced expression of REPS2 exerts an inhibiting effect on this signalling.

From these experiments, it is concluded that increased REPS2 expression negatively affects EGF receptor internalisation and subsequent signalling. Therefore, decreased REPS2 expression during prostate cancer progression, observed in earlier work, may result in enhanced EGF receptor expression and signalling, which could add to the androgen-independent state of advanced prostate cancer.

Introduction

Treatment of prostate cancer involves surgery of the affected region of the gland and treatment of advanced prostate cancer focuses on inhibition of growth inducing signals, mainly by androgen ablation. However, given enough time, the tumour is suggested to switch from androgens to growth factors as primary regulators of proliferation. This transition leads to therapy-resistance of the cancer cells and eventually will result in recurrence of the disease (Russell et al., 1998).

Epidermal growth factor (EGF) and transforming growth factor alpha are potent mitogens involved in autocrine regulation of proliferation, angiogenesis and metastatic spread of advanced prostate cancer (Scher et al., 1995). The EGF receptor can bind many ligands, among which EGF and TGF-α (Jones et al., 1999). Upon ligand-binding the receptor dimerizes and is activated by autophosphorylation of tyrosine residues in the intracellular tail of the receptor. Subsequent activation of well-characterized signal transduction pathways (MAPK) results in proliferation and survival of tumour cells (Gullick and Srinivasan, 1998; Yarden, 2001).

During prostate cancer progression, the EGF receptor expression level is regulated by transcriptional and post-transcriptional mechanisms, including up-regulation of receptor mRNA and down-regulation of EGF receptor protein by EGF. Intracellular trafficking of the EGF receptor was suggested to cause the overall degradation of the EGF receptor protein in prostate cancer cells (Seth et al., 1999). This trafficking involves endocytosis of receptor-ligand complexes in clathrin-coated pits and subsequent degradation of these complexes in endosomes. Furthermore, in contrast to degradation, active signalling complexes are also newly formed during endocytosis (Di Fiore and Gill, 1999; Waterman and Yarden, 2001). Endocytosis is reported to be an essential component of efficient and accurate signalling of growth factor receptors (Ceresa and Schmid, 2000).

Dhanasekaran et al. (2001) published a large set of cDNA array analyses of a commercial pool of normal prostate tissues compared to normal human prostate, prostate hyperplasia, prostate cancer, and prostate cancer metastasis samples. In Table 3.1 we have summarized a selection of genes from the prostate cancer expression data set, for which it is known from literature that these genes are involved in growth factor receptor endocytosis. Of these 15 genes, 8 showed a differential expression between the normal prostates and the four investigated metastasis group in the Dhanasekaran data-set. Interestingly, except for amphiphysin, these endocytosis-related genes were down-regulated during metastasis, while in non-metastatic prostate cancer samples some genes were down-regulated while others were not regulated compared to expression in normal prostate. These data indicate that the process of endocytosis is subject to changes during development of metastatic prostate cancer, and suggests a role for altered endocytosis in metastasis.

Table 3.1. **Functions of endocytosis-related genes and their expression in metastatic prostate cancer tissues as compared to normal prostate tissues.**

Protein name	Function in endocytosis	Expression in metastasis versus normal
AP-2	Coat protein; recruitment of other proteins	Down
Endophilin	Invagination of the coated membrane	Down
CALM	Clathrin coat assembly	Down
Amphiphysin	Fission of clathrin coated pits	Up
RAB5a	Regulation of trafficking	Down
Dynamin 2	Fission of clathrin coated pits	Down
Intersectin 1	Fission of clathrin coated pits	Down
Caveolin 2	Caveolar exit	Down
NUMB	Clathrin coat assembly (possible)	No change
Neuronal SHC	Routing of activated receptors	No change
Synaptotagmin	Clathrin coat assembly	No change
Clathrin L	Coat protein	No change
RALBp1	Clathrin coat assembly	No change
EPS15	Clathrin coat assembly	No change
Clathrin H	Coat protein	No change

Genes were selected from cDNA array analyses, which were made available to the public by Dhanasekaran et al., 2001. Genes were considered to be up- or down-regulated when the fold change was at least 1.4.

Several proteins control endocytosis and subsequent intracellular trafficking. One of the essential regulatory proteins is EPS15 (Epidermal growth factor receptor pathway substrate 15). EPS15 binds to AP2 (adaptor protein complex 2) at the growing rim of the invaginating pit, and interacts with other endocytic proteins such as Epsin, Intersectin, and POB1 or REPS2 (Waterman and Yarden, 2001). The REPS2 protein contains a coiled-coil region, two proline-rich motifs and an EH domain (EPS15 homology domain). The two proline rich regions of REPS2 have been shown to interact with the growth factor receptor adaptor protein GRB2 (Ikeda et al., 1998) and the EH-domain of REPS2 was found to bind directly to Epsin and EPS15 (Kariya et al., 2000). Interestingly, GRB2 has also been identified as a regulator of EGF receptor endocytosis (Martinu et al., 2002). Based on these observations and the finding that expression of deletion mutants of REPS2 inhibits internalisation of EGF and insulin, REPS2 was suggested to be involved in growth factor receptor signalling and regulation of endocytosis (Nakashima et al., 1999)

In a previous study, reduced expression of REPS2 was found in androgen-independent human prostate cancer cell lines and xenografts, as compared to a relatively high expression level in androgen-dependent cells and xenografts (Chang et al., 1997). Furthermore, over-expression of REPS2 in prostate cancer cells inhibits growth factor signalling, and results in induction of apoptosis (Oosterhoff et al., 2003).

To begin to understand if and how changes in the process of endocytosis might influence development of metastatic prostate cancer, the present investigation was focused on internalisation and signalling of the EGF receptor in relation to the expression level of REPS2. It was observed that REPS2 inhibits EGF signal transduction, possibly by reducing the rate of EGF receptor endocytosis. These data

provide further support for the hypothesis that loss of REPS2 expression during prostate cancer progression directly impacts on growth factor signalling. This stimulation of growth factor signalling can contribute to maintenance of androgen-independent growth.

Material and Methods

Materials and cell culture

Cell culture flasks and plastic disposables were obtained from Fisher Scientific (Houston, TX, USA). RPMI 1640 and culture chemicals were purchased from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Foetal calf serum (FCS) was from Greiner (Frickenhausen, Germany). Restriction and modifying enzymes were obtained from Roche (Indianapolis, IN, USA). Tetramethylrhodamine-conjugated EGF (EGF-rhodamine) was purchased from Molecular Probes (Eugene, OR, USA). ^{125}I-EGF was obtained from Amersham Biosciences Corp (Piscataway, NJ, USA). Receptor grade EGF from mouse submaxillary glands was provided by Sigma (Saint Louis, MI, USA). Anti-EGF receptor antibody (1005) and anti-phosphorylated tyrosine antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-REPS2 polyclonal and immunoaffinity purified antibody was manufactured as described (Oosterhoff et al., 2003). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were from Sigma (St. Louis, MI, USA). ECL detection reagents were from Perkin Elmer (Wellesley, MA, USA). Cells were maintained as described (Oosterhoff et al., 2003).

Plasmid constructions

REPS2-GFP was subcloned as described (Oosterhoff et al., 2003). For stable inducible transfection we used the ecdyson-inducible expression kit (Invitrogen, Carlsbad, CA, USA). REPS2 cDNA was cloned into the pIND/Hygro vector, using Kpn I and Xba I restriction sites.

^{125}I-EGF binding and internalisation assay

Prostate cancer cells (DU145, PC3, PC3-R2 and LNCaP-FGC), were grown subconfluent (90%) in 6 wells plates. Cells were serum starved overnight and washed in binding buffer (RPMI, supplemented with 20mM HEPES pH7.4, and 0.1% BSA). Cells were incubated with excess EGF, by adding mixtures containing 5 ng/ml ^{125}I-EGF (specific activity: 3 – 4.5 MBq/μg) and 10ng/ml unlabeled EGF, for 60 minutes at 4°C. Cells were then either transferred to 37°C, for 10 or 45 minutes, or directly washed twice with ice-cold phosphate-buffered saline, to establish binding at 0 min. To remove membrane-bound ^{125}I-EGF the cells were washed twice with ice-cold acid buffer (25mM NaOH/ acetic acid, pH 3.8 and 150mM NaCl) and subsequently with PBS. Cells were lysed in 1N NaOH and radioactivity was determined by counting in a γ-counter (Cobra Quatum, Perkin Elmer, Boston, MA, USA). To determine bound EGF to the membrane, cells were washed with PBS after incubation at 4°C and lysed immediately.

EGF-rhodamine binding and internalisation assay

PC3 cells were grown on glass coverslips coated with 0.1% w/v gelatine, and were transfected with pEGFP-REPS2 using FuGENE, or were used untransfected. The cells were treated with 40ng/ml EGF-rhodamine in RPMI with 25mM Hepes for 60 min on ice (4ºC), transferred to 37°C and further incubated for 0, 5 or 10 min. Subsequently, the

cells were fixed with 3% v/v paraformaldehyde and embedded in Vectashield mounting medium (Vector laboratories Inc.; Burlingame, CA, USA). An Axioplan 2 fluorescence microscope (Carl-Zeiss; Thornwood, NY, USA) was used for analysis. Digital pictures were taken with a Coolsnap-pro colour camera (Roper Scientific, BV; Trenton, NJ, USA).

Western blotting and Northern blotting

Western blot analysis was essentially performed as described (Blok et al., 2003).Total RNA extraction and Northern blotting was performed as described (Chang et al., 1997). Ethidium bromide staining was used to verify equal loading of total RNA.

Results

EGF receptor internalisation and expression in androgen-dependent and androgen-independent prostate cancer cell lines

Internalisation of the EGF receptor plays a major role in controlling EGF signalling, and EGF signalling may play a role in development of advanced androgen-independent prostate cancer. To investigate EGF receptor internalisation, we studied the uptake of ^{125}I-EGF and expression of the EGF receptor in the androgen-dependent cell line LNCaP, in comparison to the androgen-independent cell lines DU145 and PC3.

It was observed that androgen-dependent LNCaP cells internalise significantly less ^{125}I-EGF, compared to androgen-independent PC3 and DU145 cells (Figure 3.1A). Furthermore, it was also shown that at the start of the experiment, LNCaP cells bound significantly less ^{125}I-EGF than the other cell lines, indicating a lower EGF receptor level in LNCaP cells than in PC3 and DU145 cells (Figure 3.1A, indicated by the open symbols).

To verify whether decreased binding of ^{125}I-EGF to LNCaP cells (Figure 3.1A, open symbols) was the result of decreased EGF receptor expression, Western blotting was performed. It was observed that expression of EGF receptor protein in LNCaP cells at time point zero was indeed much lower compared to the expression level in PC3 and DU145 cells (Figure 3.1B). The non-specific protein band, observed in cell lysates of LNCaP cells, just above the EGF receptor protein band, is an unknown protein, which is only observed in LNCaP cells. Furthermore, when the three cell lines were cultured in the presence of EGF, a rapid (15–45 min) transient increase in EGF receptor expression, as observed in PC3 and DU145 cells, was not observed in LNCaP cells. Interestingly, a gradual decrease in EGF receptor expression at later time points, between 1 and 48h, which is known to be caused by degradation after internalisation of the activated receptor, could be observed in all three cell lines (Figure 3.1B). The faint extra protein band, appearing in PC3 cells after 2 hours of EGF-treatment, seems to increase with longer incubation periods. It is possible that this protein band represents the dephosphorylated EGF receptor, because when phosphorylation was assessed upon EGF stimulation with an anti-phospho tyrosine antibody, we observed an immediate increase of phophorylated forms of the receptor, which decreased after 45-60 minutes of EGF-treatment (Figure 3.3C).

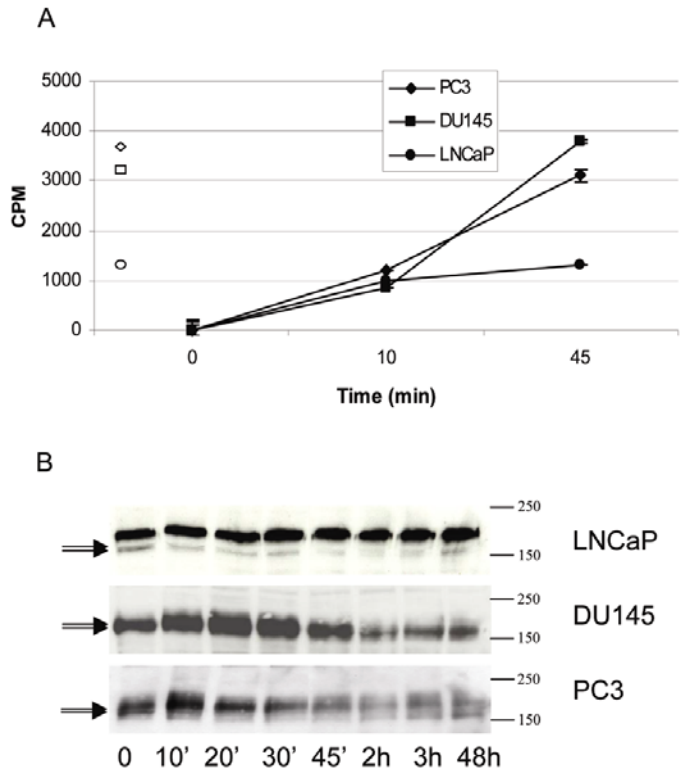

Figure 3.1. **Internalisation and expression of EGF receptor in prostate cancer cell lines.**
A. Internalisation of ^{125}I-EGF was measured in LNCaP, PC3 and DU145 cells at 0, 10 or 45 minutes after start of internalisation. After incubation cells were subjected to acid wash and lyses to estimate the amount of internalised EGF. The results shown are the mean ± SD of two experiments performed in duplicate; each experiment included two independent culture plates. Results are presented as counts per minute (cpm), corrected for cell number and background binding. The open circle, square and diamond represent total ^{125}I-EGF binding to the cell surface at the start of the experiment.
B. LNCaP, PC3 and DU145 cells were incubated with 20ng/ml EGF for the indicated times. The amount of EGF receptor was determined by Western blot analysis with an anti-EGF receptor antibody. The arrows indicate the position of the 170kDa EGF receptor proteins. Equal amounts of protein were loaded on each lane, as measured using Bradfords reagent, and verified after electrophoresis by Ponceau S staining.

Involvement of REPS2 in EGF receptor internalisation

REPS2 expression is significantly higher in androgen-dependent prostate cancer cell lines (LNCaP) and xenografts, compared to androgen-independent cell lines (PC3 and DU145) and xenografts (Chang et al., 1997). To determine whether changed expression of REPS2 would affect EGF receptor endocytosis, we transiently transfected PC3 cells with a construct encoding GFP-tagged REPS2 and studied endocytosis of rhodamine-labelled EGF by fluorescent microscopy, after 24 hours. Cells expressing the REPS2-GFP fusion protein bound less ligand and were deficient in EGF-rhodamine internalisation (Figure 3.2). These results suggest that over-expression of full-length REPS2 has an inhibiting effect on EGF receptor endocytosis and may therefore possibly affect subsequent signal transduction. In the above experiment REPS2 was expressed as a fusion protein, and the expression level was possibly very high. High expression of REPS2 has been shown to induce apoptosis (Oosterhoff et al., 2003). Therefore, a more physiological approach was taken next. Using the androgen-independent cell line

PC3, which expresses a low level of REPS2 (Oosterhoff et al., 2003), a PC3 subline with stable integration of a construct that allows inducible REPS2 expression (PC3-R2) was generated. For this gene construct, induction occurs because the activated ecdyson receptor dimerizes to the RXR receptor, which turns on expression of the REPS2 gene. When these cells were treated with Muristerone A (a ligand for the ecdyson receptor) for 24 hours, a higher expression level of the REPS2 protein was detected on Western blot (Figure 3.3A). Moreover, this expression level could be further enhanced using the synthetic RXR ligand LG268 (kindly provided by Ligand Pharmaceuticals) (Saez et al., 2000) (Figure 3.3A).

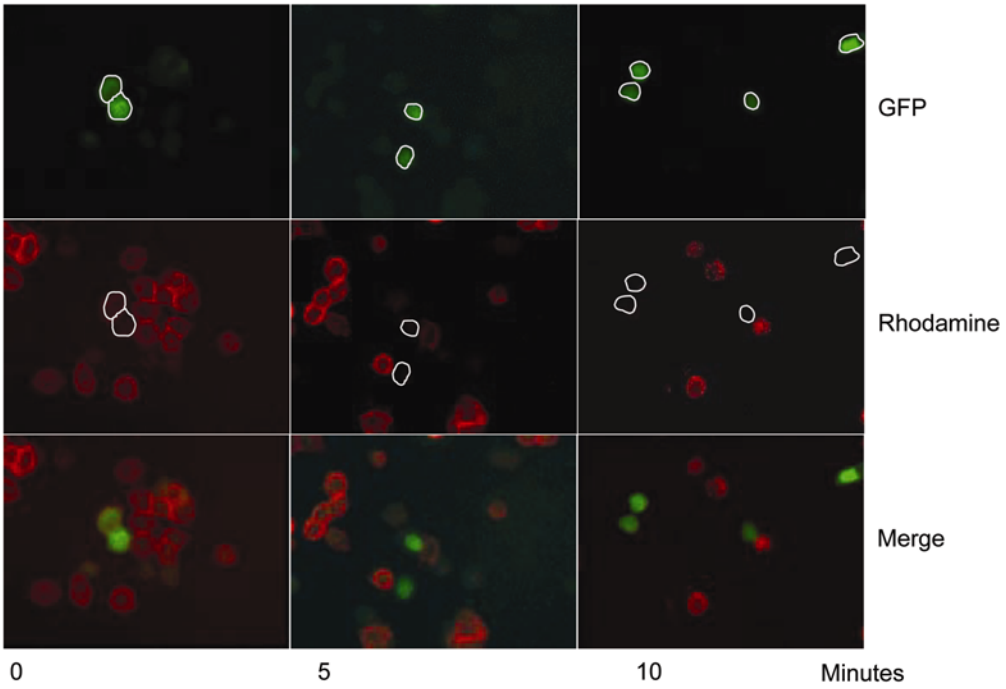

Figure 3.2. **EGF binding and internalisation in cells expressing REPS2-GFP**
PC3 cells were transfected with a construct encoding REPS2-GFP. After 24 hours, the transfected cells were incubated with EGF-rhodamine (40ng/ml) for 0, 5 and 10 minutes at 37°C. Cells were fixed and analysed by fluorescence microscopy. The upper panel visualizes REPS2-GFP transfected cells, and the middle panel shows EGF-rhodamine within the same frame and the lower panel shows the merged channels. Cells expressing REPS2-GFP are indicated (magnification: 40x).

The expression level of REPS2 in the inducible cell line (PC3-R2) is comparable to the endogenous level in LNCaP cells (Figure 3.3A). Furthermore we have not observed or detected loss of cells during 6 days of REPS2-induction, which indicates that there is no significant apoptosis-induction in cells with a moderate expression level of REPS2. Therefore, these PC3-R2 cells were used for further investigations into the role of REPS2 in EGF receptor internalisation and signalling, in relation to the development of androgen-independent prostate cancer.

When EGF receptor internalisation was measured 48 hours after induction of full-length REPS2 in PC3-R2 cells, an approximate 35% reduction in internalisation of ^{125}I-EGF was observed (Figure 3.3B). This indicates that REPS2 induction exerts an effect on internalisation of the EGF receptor, and/or that REPS2 induction affects EGF receptor expression. When total binding of ^{125}I-EGF at the start of the experiment was measured, it was observed that in REPS2-induced PC3-R2 cells only 15% less ^{125}I-EGF

was bound, compared to non-induced PC3-R2 cells (Figure 3.3B, indicated by the open symbols). This result indicates that increased expression of REPS2 does not clearly negatively affect EGF receptor expression. To verify this, Western blotting was performed. In the absence of any EGF-stimulation, EGF receptor level in induced and non-induced PC3-R2 cells was not found to be markedly changed (Figure 3.3C: 0 min.).

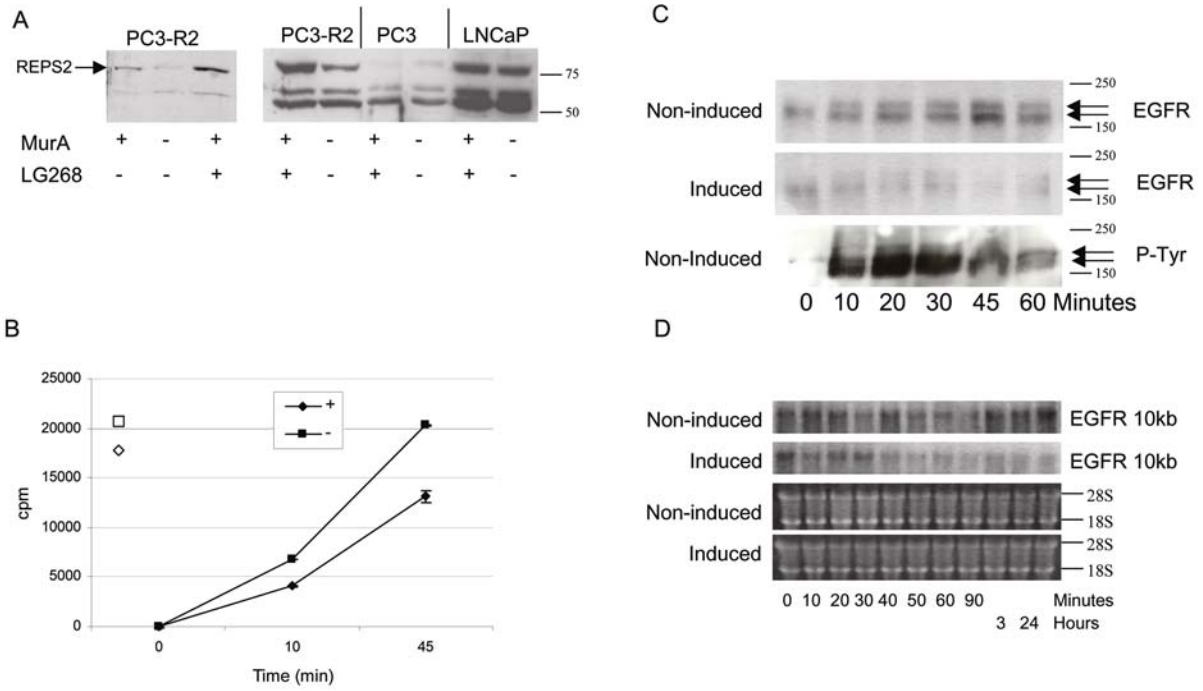

Figure 3.3. **Internalisation and expression of EGF receptor in REPS2 inducible PC3-R2 cells**

A. Induction of REPS2 expression in PC3-R2 cells. PC3-R2 cells were incubated with Muristerone A (MurA; 2.5 μg/ml) alone, or in combination with LG268 (100nM) for 24 hours. As a control non-induced cells are shown. In a separate experiment, the expression level of REPS2 in PC3-R2 cells is compared to the endogenous REPS2 levels in PC3 and LNCaP cells. Expression level of REPS2 was determined by Western blot analysis using purified anti-REPS2 polyclonal antibody (Oosterhoff et al., 2003). Equal amounts of protein were loaded on each lane of the gel, as measured using Bradfords reagent, and verified after electrophoresis by Ponceau S staining.

B. Internalisation of ^{125}I-EGF in PC3-R2 cells with (black diamond) or without (black square) induction of REPS2 expression. The open diamond and square represent total ^{125}I-EGF binding to the cell surface at the start of the experiment. The results shown are the mean ± SD of two experiments with duplicate incubation on two independent culture plates. Results are corrected for background binding.

C. Expression level of EGF receptor in PC3-R2 cells, with (induced) or without (non-induced) induction of REPS2. PC3-R2 cells were incubated with 20ng/ml EGF for 0, 10, 20, 30, 45 and 60 minutes. The amount of EGF receptor was determined by Western blot analysis with an anti-EGF receptor antibody. Anti-phosphorylated tyrosine antibody was used to show fast and transient increase of phosphorylated-tyrosine at the same height as the EGF receptor after addition of 20 ng/ml EGF. Equal amounts of protein were loaded on each lane of the gel, as measured using Bradfords reagent, and verified after electrophoresis by Ponceau S staining. The result is representative for an experiment performed in triplo.

D. Northern blot analysis of PC3-R2 cells, with or without MurA/LG268 induction of REPS2 expression: PC3-R2 cells were incubated with 20ng/ml EGF for 0, 10, 20, 30, 40, 50, 60, 90 and 180 minutes and 24 hours and total RNA was isolated. Expression of the EGF receptor mRNA was determined using a specific cDNA probe. Equal amounts of RNA were loaded on each lane of the gel, as calculated using OD260 measurements, and verified after electrophoresis by ethidium bromide staining. The result is representative for an experiment performed in duplo.

It was also observed that in non-REPS2-induced PC3-R2 cells expression of EGF receptor was initially increased upon culture in the presence of EGF, while in REPS2-induced PC3-R2 cells EGF stimulation results in a reduction of EGF receptor expression (Figure 3.3C). Interestingly, these results are very comparable to the data presented in Figure 3.1B for the EGF-stimulated parental PC3 and DU145 cells (both expressing a low level of REPS2) in comparison to LNCaP cells (expressing a high level of REPS2). In order to verify that indeed the EGF receptor was detected on Western blot, phosphorylation at tyrosine residues was measured (for the non-induced situation). Because phosphorylation of the EGF receptor itself is an abundant event after EGF stimulation, indeed a fast and transient increase in phosphorylated tyrosine was observed at the same height as the EGF receptor (Figure 3.3C). To elucidate whether inhibition of EGF receptor up-regulation by EGF occurs at the mRNA level, Northern blotting was performed. It was observed that induced PC3-R2 cells do not show an increased EGF receptor mRNA level as is seen in non-induced cells upon stimulation with EGF (Figure 3.3D). These data seem to indicate that induction of expression of REPS2 in a cell line, which normally expresses only a low level of REPS2, negatively influences EGF-induced EGF receptor expression. Since mRNA and protein expression of the EGF receptor gene (in the absence of EGF stimulation) was not affected by induction of REPS2 expression (Figure 3.3C and Figure 3.3D, 0 min.) and REPS2 decreases EGF receptor internalisation (Figure 3.3B), a possible mechanism could be that decreased endocytosis results in decreased EGF-signalling to the nucleus. This leaves the question whether REPS2 induction would also affect expression of other EGF responsive genes.

Involvement of REPS2 in EGF receptor signalling

Since the rate of internalisation of EGF receptor is markedly reduced by REPS2 induction in PC3-R2 cells, it was investigated whether REPS2 induction would inhibit EGF receptor signalling and consequently would have an effect on regulation of mRNA expression of a number of EGF-responsive genes. Therefore, the expression of the early response genes EGR1, FOS, and JUN, was investigated by Northern blotting. As shown in Figure 3.4A, EGR1 expression was transiently up-regulated by EGF, in both induced and non-induced PC3-R2 cells. However, the EGR1 mRNA level was already decreasing after 40 minutes in the induced cells, whereas in non-induced cells up-regulation sustained up to 60 minutes after addition of EGF (Figure 3.4A). For FOS mRNA expression a very similar observation was made (Figure 3.4B). Interestingly, EGF did not regulate the JUN mRNA level, but overall expression of JUN mRNA in the induced cells was reduced (Figure 3.4C). As a control for equal loading ethidium bromide staining is shown (Figure 3.4D).

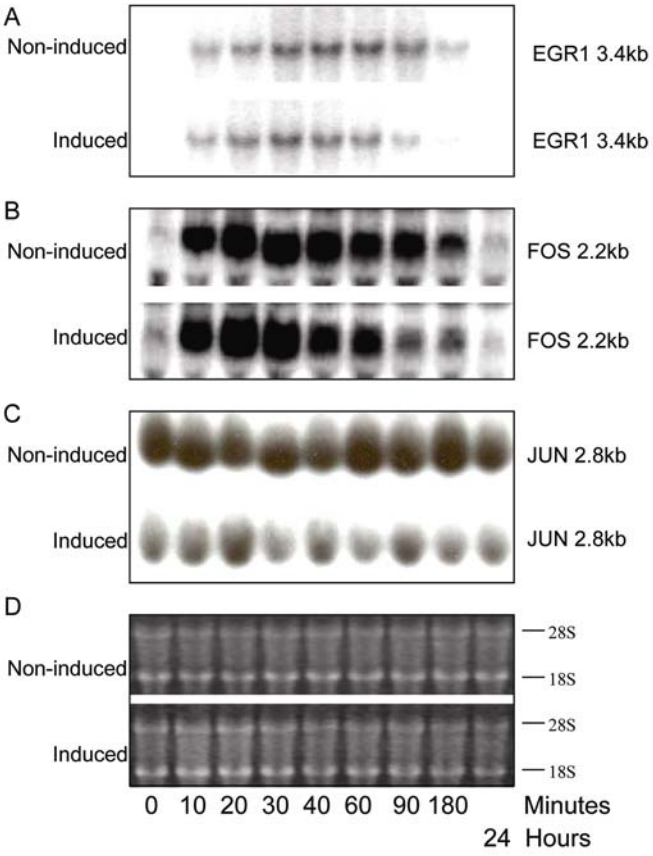

Figure 3.4. **Northern blot analysis of PC3-R2 cells, with or without MurA/LG268 induction of REPS2 expression**
PC3-R2 cells were incubated with 20ng/ml EGF for the indicated times and total RNA was isolated. Expression of the early response genes (A) EGR1, (B) FOS, and (C) JUN was determined using specific cDNA probes. Equal amounts of RNA were loaded on each lane of the gel, as calculated using OD260 measurements, and verified after electrophoresis by ethidium bromide staining (D). The results are representative for an experiment performed in duplo.

Discussion

During progression of prostate cancer, growth factors become important regulators of cell proliferation (Russell et al., 1998), and growth factor receptor internalisation and consequent degradation, control the strength and duration of growth factor-induced signalling (He et al., 2003). Using cDNA array data from literature (Dhanasekaran et al., 2001) we observed that several genes involved in growth factor internalisation are down-regulated in metastatic prostate cancer.

In the present experiments, endocytosis and binding of ^{125}I-EGF was studied in a number of androgen-dependent and androgen-independent prostate cancer cell lines. The finding that the binding of EGF is relatively high in androgen-independent compared to androgen-dependent cell lines, is in agreement with a pronounced role for growth factor signalling during hormone-independent prostate cancer growth. In addition, we found increased expression of EGF receptors in androgen-independent cell lines, which explains the increased EGF binding to these cells. It was suggested

previously that EGF-induced endocytosis causes EGF receptor protein degradation in prostate cancer cells (Seth et al., 1999) as was shown in B82 Mouse L cells (Wiley et al., 1991) and HeLa cells (Vieira et al., 1996). Our experiments show that prostate cancer cells internalise 100% of the initially bound ligand within 45 minutes, which can explain the observed EGF receptor protein down-regulation after EGF administration.

Parallel to increased EGF receptor signalling in androgen-independent cell lines, in earlier work we found that expression of the endocytosis-related and growth factor signalling-involved gene REPS2 was markedly down-regulated (Chang et al., 1997; Oosterhoff et al., 2003). In the present experiments the involvement of this protein was investigated in relation to the observed changes in EGF signalling during androgen-independent prostate cancer growth. When we induced REPS2 expression (using an inducible vector system) back to a physiological level in androgen-independent PC3 cells (PC3-R2 cells), total endocytosis activity was decreased by approximately 35%. This reduction in ^{125}I-EGF internalisation may be caused either by a decreased EGF receptor expression after EGF-stimulation, or by a direct effect of REPS2 on endocytosis. The fact that in the non-REPS2-induced situation the amount of ^{125}I-EGF, internalised after 45 minutes, does not exceed the initial amount bound to the cells at time point 0 minutes, indicates that no extra ^{125}I-EGF is bound in that time frame. During the experiment only the initially bound EGF is internalised and at time point 0 minutes the expression levels of the EGF receptor are similar between REPS2-induced and non-induced cells. Therefore, the decrease in ^{125}I-EGF endocytosis is not due to the decreased EGF receptor expression after EGF-stimulation. Considering the functional domains of REPS2 and the function of its binding partners, a direct role for REPS2 in endocytosis-regulation seems more likely.

The mechanism through which REPS2 reduces ligand-dependent internalisation can be explained when we take a number of REPS2 binding partners into account. REPS2 has been shown to bind to RALBP1, EPA15, Epsin, and indirectly to AP-2 (Kariya et al., 2000). These proteins complex together and are involved in building the clathrin lattice, a dynamic process of association and disassociation of endocytosis-related proteins (Pearse et al., 2000). We hypothesise that REPS2 can disturb the dynamics of interactions required for an effective assembly of clathrin-coated pits, by recruiting and trapping its binding partners RALBP1, EPS15, Epsin and AP-2 in a complex, which would inhibit propagation of endocytosis. Additionally, GRB2 has recently been implicated in endocytosis regulation (Jiang et al., 2003; Martinu et al., 2002) and since REPS2 was reported to bind to GRB2 (Ikeda et al., 1998), this might be another candidate involved in endocytosis disruption by REPS2.

Internalisation of activated EGF receptor is necessary for proper signalling (Ceresa and Schmid, 2000) and several effector proteins involved in signal transduction have been found to be present in early endosomes (Burke and Wiley, 1999; Jiang and Sorkin, 2002; Oksvold et al., 2000; Oksvold et al., 2001). One question, which then remained, was whether REPS2-induced changes would indeed translate into changes in EGF receptor signalling at the level of expression of EGF-responsive genes. Northern blot analysis showed that EGF receptor, EGR1, FOS and JUN mRNAs were either less induced by EGF or had lower expression levels in REPS2 expressing cells compared to control cells. Although the effect of REPS2 on EGF-induced expression is not severe, the fact that it is found on more then one occasion and for four EGF-responsive genes, indicates that expression of REPS2 influences EGF receptor signalling in a fairly comprehensive manner.

Considering that induction of REPS2 expression seems to results in reduced EGF receptor endocytosis, that EGF-regulated gene expression is influenced by induction of REPS2 expression (present results) and that over-expression of REPS2 in prostate cancer cells inhibits growth factor signalling (Oosterhoff et al., 2003), a significant role for REPS2 in regulation of EGF signalling seems likely. Reduced REPS2 expression during prostate cancer progression may therefore result in increased EGF signalling and consequently contribute to carcinogenic properties.

Down-regulation of REPS2 expression most likely is not the only factor involved in increased EGF signalling during advanced prostate cancer development. As presented herein, published expression data from different stages of prostate cancer progression were evaluated and eight out of fifteen genes involved in endocytosis were found to be differentially expressed during prostate cancer progression. This result indicates that the process of endocytosis most likely is dysregulated during prostate cancer development. Interestingly, it has been described that dysregulated endocytosis can lead to mitogenic signalling. Furthermore, mutation or abnormal expression of endocytic proteins has been implicated in tumour development (Di Fiore and Gill, 1999; Floyd and De Camilli, 1998). The present results indicate that further investigations into the role of endocytosis during prostate cancer progression will improve our understanding of androgen-independent tumour growth.

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Cluster-analysis of androgen- and EGF-regulated genes in relation to proliferation and invasion of prostate cancer cells

Cluster-analysis of androgen- and EGF-regulated genes in relation to proliferation and invasion of prostate cancer cells

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Abstract

Progression of prostate cancer includes molecular and cellular changes leading to a transition from androgen-dependent to androgen-independent growth. The initial androgen-dependent prostate cancer cells possibly switch from proliferation induced by androgens to proliferation that is regulated by growth factors, like epidermal growth factor (EGF). EGF and androgens both induce proliferation and invasion in prostate cancer cells. With microarray-analysis, contribution of, and overlap between androgen-regulated and EGF-regulated genes involved in proliferation and invasion of LNCaP cells, was evaluated. Proliferation-related transcripts showed overlap between androgen- and EGF-regulated genes. However, the majority of genes involved in invasion did not show such overlap.

Upon comparison of the androgen-regulated genes in LNCaP cells with RNA-expression data from prostate samples (normal, hyperplasia, cancer, metastases), it was observed that a around 50% of these androgen-regulated genes, also present in the prostate cancer dataset, were differentially expressed in metastatic prostate cancer samples as compared to normal prostate. For EGF-regulated genes, this was less clear, although many EGF-regulated invasion-related genes were found to be differentially expressed in metastatic prostate cancer as compared to normal prostate.

These results indicate that EGF and androgens use partly the same pathways to control cell proliferation and different pathways to control cell invasion in LNCaP cells. Furthermore, the present data support the hypothesis that, although to a lesser extent, the EGF-pathway, next to the androgen receptor-pathway, is an important factor in the series of events that control prostate cancer progression.

Introduction

Androgen ablation therapy for advanced prostate cancer, initially reduces tumor growth effectively in over 80% of cases, but usually some cells escape from treatment and in due time will lead to appearance of an androgen-independent tumor (Crawford, 1992). A possible mechanism causing prostate cancer cells to become androgen-independent involves a switch from steroid hormones to polypeptide growth factors as primary regulators of growth. Fibroblast growth factors, insulin-like growth factors, transforming growth factors and epidermal growth factor (EGF) are autocrine factors that are able to stimulate growth of advanced, androgen-independent, prostate cancer (Djakiew, 2000; Russell et al., 1998). Another aspect of androgen-independent prostate cancer progression is metastatic outgrowth to other organs like pelvic lymph nodes and bone, which is observed in a high percentage of cases. Interestingly, metastatic properties of prostate cancer cells can be modulated by androgens (Liao et al., 2003), but also by some of the growth factors which are potentially involved in the transition from androgen-dependent to androgen-independent prostate cancer growth (Russell et al., 1998). In particular EGF has been shown to induce both proliferation and invasion of prostate cancer cells (Jarrard et al., 1994; Unlu and Leake, 2003).

The present study has focused on EGF signal transduction, whereby ligand-receptor binding induces cell proliferation and invasion by activating well-characterized signal transduction pathways that involve phosphorylation and activation of mitogen-activated protein kinases (MAPKs). The activation of GTP-ases of the RAS family is one of the initial steps in this signal transduction cascade. Once a RAS protein is activated, it can facilitate activation of the serine/threonine kinase RAF1, PI3-kinase and RAL-GEF (guanine nucleotide exchange factor), which activate the small GTP-ase RALA (Pruitt and Der, 2001). Activation of many of these signalling molecules induces expression of genes through activation of a number of different transcription factors.

The androgen receptor (AR) also acts as a transcription factor and can be activated by MAPK (Craft et al., 1999; Culig et al., 1994). Therefore, there is the possibility of overlap and synergy between signalling by androgens and EGF in prostate cancer progression. To study transcriptional regulation by EGF in prostate cancer cells we used a comprehensive cDNA array for analysis of the expression pattern of a human prostate cancer cell line (LNCaP) treated with EGF. To analyse the possible crosstalk between signalling between androgens and EGF, we also compared our results with expression profiles of LNCaP cells treated with androgens, available from the public domain (http://www.cpdr.org/LNCaP/GeneChip/). Furthermore, androgen- and EGF-regulated genes were compared to a set of genes differentially expressed in prostate cancer progression (Dhanasekaran et al., 2001). It was observed that many genes involved in proliferation and invasion were regulated by androgens and/or EGF, and many of these genes could be implicated in metastatic prostate cancer progression. The present results indicate that in therapies for advanced prostate cancer, both androgen receptor-signalling and EGF-signalling should be taken into consideration.

Materials and Methods

Materials and chemicals

Cell culture flasks and plastic disposables were obtained from Fisher Scientific (Houston, TX, USA). RPMI 1640 and culture chemicals were purchased from GIBCO

Invitrogen Corporation (Carlsbad, CA, USA). Fetal calf serum (FCS) was from Greiner (Frickenhausen, Germany).

Cell culture and EGF treatment

The LNCaP cells (passage number 22-30), were kindly provided to us by Dr. J.S. Horoszewicz (Buffalo, NY). The LNCaP cell line is identical to the one that can be obtained from the American Type Culture Collection (Rockville, MD). LNCaP cells were maintained at 37°C in a humidified incubator containing 5% CO_2/95% air in RPMI 1640 supplemented with 200 IU/ml penicillin, 200 μg/ml streptomycin and 5% v/v fetal calf serum (FCS). Before EGF treatment, LNCaP cells were cultured for 48 h in medium containing 5% v/v dextran coated charcoal-treated FCS (DCC-FCS) and then treated with additional added EGF (20 ng/ml) for various periods of time. Media were refreshed every two days. Total RNA was extracted by lysing the cells with 3 M lithium chloride/6 M urea (Chang et al., 1997).

DNA microarray hybridisations and data analysis

DNA microarrays consisting of amplified cDNAs from Incyte's Human LifeSeq® Foundation printed on glass slides were used in these studies (Agilent, Palo Alto, CA, USA). Each array contained cDNAs representing over 14,000 unique human genes and expressed sequence tags (ESTs). For each hybridisation, 13 μg of total RNA was used in a direct labelling protocol. The RNAs of the different time points were all hybridised on separate arrays, with RNA of untreated cells as reference sample. Labelling, hybridisation, scanning procedures and data compiling was performed by ServiceXS, Leiden, The Netherlands (http://www.servicexs.com/), with Agilents G2566AA Feature Extraction Software. The compiled experimental data were further analysed using Microsofts Excel program.

Spots of which the mean signal corrected for the mean background was less than 3 times higher than the standard deviation (SD) of the background were flagged and excluded from analysis. This calculation excluded spots with low intensity, compared to the background intensity, as well as spots with low spot quality, determined by SD of the background. Additionally, spots of which the mean signal was less then two-fold higher than the mean background were also excluded from analysis.

Gene annotation was determined with OMIM™ (Online Mendelian Inheritance in Man™) and LocusLink (NCBI) at http://www.ncbi.nlm.nih.gov/. Hierarchical and K-means clustering was performed at http://ep.ebi.ac.uk/EP/EPCLUST/.

Growth studies

LNCaP cells were maintained in RPMI 1640 medium containing 5% FCS. One day prior to plating, the cells were transferred to medium containing 5% DCC-FCS. The cells were passaged to 24-well plates, at 5000 cells per well and treated with epidermal growth factor (EGF) for the indicated lengths of time at a concentration of 20 ng/ml. Media were refreshed every two days. 16 h prior to harvest, the cells were incubated with 9.25 KBq ^{3}H-thymidine (specific activity 46 Ci/mMol). Subsequently, the cells were washed twice in PBS and lysed in 1ml NaOH for 1 h at 37°C. ^{3}H-Thymidine incorporation was measured in 0.5 ml lysate diluted in 4.5 ml scintillation fluid using a 2500TR scintillation analyser (Packard Bioscience BV, Groningen, The Netherlands). Standard deviations were calculated from quadruplicate incubations within one representative experiment.

Invasion assay

After culture in RPMI containing 5% FCS, LNCaP cells were detached with trypsin and seeded in the upper well of a modified Boyden chamber (Transwell, 6.5 mm inserts, 8 µm pores; Corning Costar, Cambridge, MA, USA) at $5x10^4$ cells/well in 100µl medium, with or without 20 ng/ml EGF and/or 0.1 nM R1881. In the lower well, 600 µl 5% DCC-FCS-supplemented medium with or without EGF and/or R1881 was loaded. After 72 hours of culturing, the number of cells that had migrated through the filter into the lower well were counted in triplicate. EGF treatment was also varied in time. Cells were cultured in a modified Boyden chamber for 6 days with or without added EGF as indicated in the legends to the figures. Media were refreshed every two days. The experiments were performed in triplicate, and were repeated 3 times.

Results

Androgen and EGF stimulate growth and invasion of LNCaP cells

Both normal prostate cells as well as prostate cancer cells depend, during most stages of oncogenic progression, on androgens for growth and maintenance. The presently used LNCaP cell line is a human prostate cancer cell line which is derived from a lymph node metastasis (Horoszewicz et al., 1980). Not only are LNCaP cells dependent on androgens for their growth, but they also respond to EGF (Guo et al., 2000; Schuurmans et al., 1991). To verify that the LNCaP cell line used in our laboratory showed the same growth characteristics as described in literature, the growth-response to either androgens or EGF was determined by ^{3}H-thymidine incorporation assay.

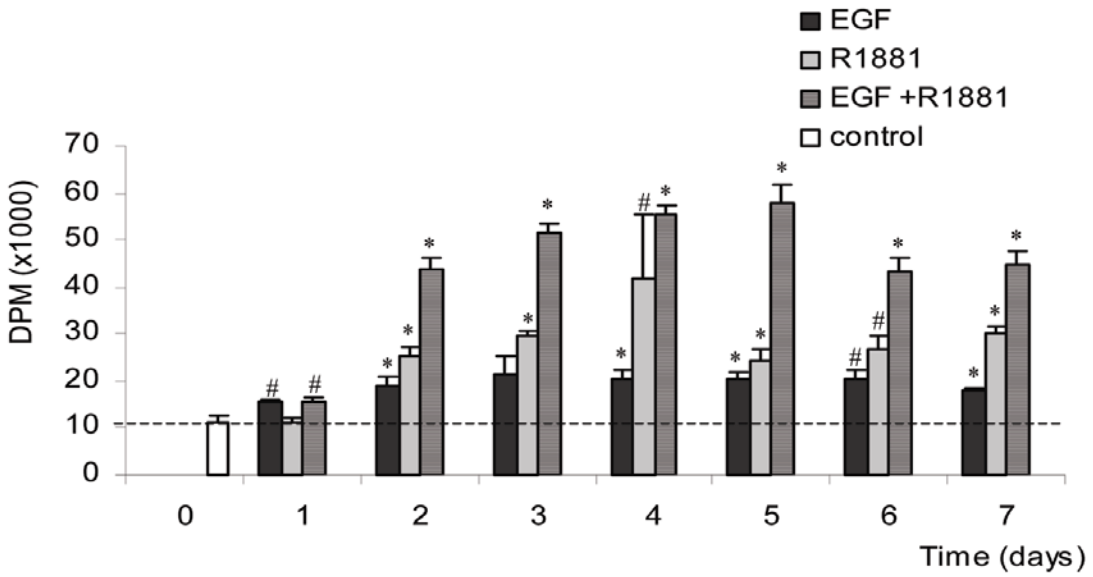

Figure 4.1. **Proliferation induction by EGF and/or R1881 treatment of LNCaP cells**

LNCaP cells were treated for the indicated times with 20 ng/ml EGF and/or 0.1 nM R1881. Media were refreshed every two days. Proliferation rates after 7 days of culturing were determined measuring ^{3}H-thymidine incorporation during the last 16 hours. Differences between control and treated samples were statistically significant with a paired-samples t test (* $P \leq 0.008$, # $P \leq 0.055$).

EGF (20 ng/ml) was found to induce cell growth in LNCaP cells within 2 days and this effect reached maximum stimulation after 3 days (Figure 4.1). Stimulation of cell growth by the synthetic androgen R1881 (0.1 nM) was observed after 2 days and reached its

maximum after 4 days of treatment. Administration of both EGF and R1881 together had an additive effect on cell growth (Figure 4.1). This indicates that androgens and growth factors utilize different signalling pathways, as was suggested previously (Guo et al., 2000). Because LNCaP cells respond to EGF and R1881, these cells provide us with a model to study molecular mechanisms involved in stimulation of prostate cancer cell growth by EGF compared to stimulation of growth by androgens.

An important risk during prostate cancer progression, next to androgen-independent growth, is migration and invasion of prostate cancer cells to nearby and more distant tissues and organs. EGF has been reported to stimulate invasion of prostate cancer cells in vitro (Jarrard et al., 1994). To confirm stimulation of invasion of LNCaP cells by EGF, we studied the invasive capacity of LNCaP cells after addition of EGF, using the Boyden chamber micro-invasion assay. As expected, an increase in invasion after EGF treatment was observed (Figure 4.2A). Interestingly, when cells were incubated only the last day in medium containing added EGF we measured a 4-fold increase in invasion over the control situation (Figure 4.2A; 5-/1+).

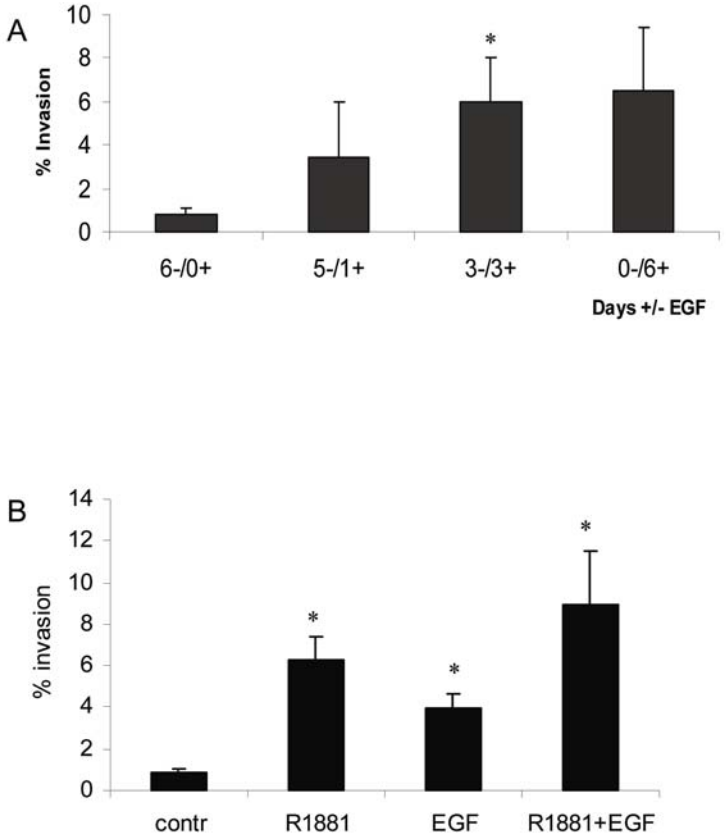

Figure 4.2. **Invasion stimulation by EGF and/or R1881 treatment of LNCaP cells**

A. LNCaP cells were treated with 20 ng/ml EGF for 1, 3 or 6 days, when EGF was added after 5, 3 or 0 days of culture respectively (* $P < 0.04$). Media were refreshed every two days. Invasion was determined by counting the cells that had passed across a Boyden chamber membrane. Results are presented as % of the originally seeded cell number.

B. LNCaP cells were treated with 20 ng/ml EGF and/or 0.1 nM R1881 for 3 days, and the number of cells that had migrated across the membrane was counted and presented as % of the originally seeded cell number. (* $P < 0.03$)

When the cells were cultured for a total of 3 days (Fig 2A; 3-/3+) or 6 days (Fig 2A; 0-/6+) in the presence of EGF, only a 2-fold increase in invasion was observed between days 1 and 3, and no further increase between days 3 and 6. Proliferation of migrated cells in the lower well may contribute to this effect, but it is also possible that stimulation of invasion is initiated during the first 24 hours in the presence of added EGF, and needs more time to take effect.

To address possible crosstalk between R1881 and EGF stimulated pathways, the effect of R1881 on invasion of LNCaP cells was also studied. LNCaP cells were treated with R1881 (0.1 nM) and/or EGF (20 ng/ml) for 3 days, and thereafter invasion was assessed. Both hormones were found to stimulate the cells to migrate through the transwell. Furthermore an additive effect was observed when both hormones were administered together (Figure 4.2B).

Androgen- and EGF-regulated genes involved in stimulation of growth and invasion of LNCaP cells

LNCaP cells were passaged, grown on medium containing 5% charcoal-stripped serum for 2 days and subsequently stimulated with EGF (20 ng/ml) for 0, 1.5, 24, 72 and 144 hours. Total RNA was isolated and hybridised to a micro-array containing approximately 14,000 human cDNAs (Agilent). The data were normalized as indicated in Materials and Methods.

Expression levels changed more than two-fold in response to EGF stimulation in 380 (2.7%) of the transcripts after 1.5 hours, in 1544 (10.8%) after 24 hours, in 95 (0.7%) after 72 hours and in 166 (1.1%) after 144 hours of EGF treatment. To check whether the generated expression profiles were relevant, we examined the regulation of 9 previously reported EGF-regulated genes in prostate cancer cells.

Table 4.1. **Transcription regulation of 9 genes in prostate cancer cells after EGF-treatment, according to literature and the present analysis using cDNA arrays**

Gene	Up- or down-regulation in EGF-treated cells		Time period of regulation	Present cDNA array analysis (Time in hours)			
				1.5	24	72	144
EGR1	Up (Tsai et al., 2000)	(epithelial cells)	Transient & early	-2.7x	+2.3x	-1x	+1.1x
TIEG	Up (Blok et al., 1995)	(LNCaP)	Transient & early	+12.6x	+2.1x	-1.1x	flagged
FOS	Up (Blok et al., 1995)	(LNCaP)	Transient & early	flagged	+5.8x	-1.3x	flagged
JUN	Up (Blok et al., 1995)	(LNCaP)	Transient & early	flagged	+5.2x	flagged	flagged
CCND1 (Cyclin D1)	Up (Perry et al., 1998))	(LNCaP)	Transient & early	+2.4x	+3.2x	+1.5x	+1.5x
CDKN1B ($p27^{Kip1}$)	Down (Ye et al., 1999)	(MDA Pca cells)	After 24 hours	-1.1x	-2.5x	+1.0x	-1.6x
Kallikrein 3	Down (Henttu & Vihko, 1993)	(LNCaP)	Up to 5 days	+4.3x	-1.8x	+1.4x	-1.3x
Androgen receptor	Down (Mizokami et al., 1992)	(LNCaP)	After 12-48 hours	+1.8x	-1.8x	+1.2x	-2.2x
UDP glycosyltransferase 2, B17	Down (Guillemette et al., 1997)	(LNCaP)	After 8 days	-1.9x	-5.3x	-1.2x-	-2.3x

Spots of which the mean signal corrected for the mean background was less than 3 times higher than the standard deviation (SD) of the background were flagged. Fold change is indicated with – when down-regulated and + when up-regulated. (Blok et al., 1995; Guillemette et al., 1997; Henttu and Vihko, 1993; Mizokami et al., 1992; Perry et al., 1998; Tsai et al., 2000; Ye et al., 1999)

The transient and early genes, EGR1, TIEG, FOS, JUN and CCND1 showed an increased expression on the micro-array until 24 hours. Of the genes that were reported to be down-regulated, only UDP-glycosyltransferase responded clearly in accordance with literature. CDKN1B ($P27^{KIP1}$), kallikrein 3, and the androgen receptor (AR) were reported to be down-regulated by EGF, but this was not always the case for all micro-array data (Table 4.1).

Both EGF and R1881 induce cell growth (Figure 4.1) and invasion (Figure 4.2) in LNCaP cells. EGF and androgens may exert such effects by regulating the same genes and/or different genes. To determine the correlation between EGF-regulated genes and R1881-regulated genes we searched literature for sources of androgen-regulated genes in LNCaP cells. DePrimo et al. (2002) published a transcriptional analysis of R1881-regulated genes in LNCaP cells (DePrimo et al., 2002). Xu et al. (2001) and Clegg et al. (2002) prepared expression profiles of androgen responsive transcripts in LNCaP cells by serial analysis of gene expression (SAGE) (Clegg et al., 2002; Xu et al., 2001). Additionally, Segawa et al. (2002) evaluated androgen regulated genes in LNCaP cells using Affimetrix oligonucleotide arrays (Segawa et al., 2002). Segawa et al. (2002) used the concentration of R1881 (0.1 nM) that has been found to induce maximum growth (Schuurmans et al., 1988a; Taneja et al., 2001), which is also the concentration used in the present experiments. Therefore, the data set of Segawa et al. (2002) was compared to the presently identified EGF-regulated genes. The time-series used in the two experiments were different: in the EGF-experiment, array-analysis was performed after 1.5, 24, 72 and 144 hours, while for the R1881-experiment, 1, 6, 12 and 24 hours of incubation was chosen. However, since the number of genes that was regulated by EGF only after either 72 or 144 hours was low (10 genes), this was assessed not to be a problem.

K-means clustering is a method that recognizes and visualizes regulatory patterns in sets of genes. To do so, the data are partitioned in a user defined K number of groups around the mean of all data-points. To optimally visualize the results, K-means clustering with 4 clusters was performed on genes regulated at least 2-fold in 1 data-point by both EGF and R1881 (Figure 4.3). 224 genes were found to be regulated by EGF and R1881, 44% in the same direction (co-regulated, either up or down) and 56% in the opposite direction (contra-regulated, either up/down or down/up). Clusters 3 and 4 show contra-regulated genes, cluster 2 contains genes that are co-regulated (mainly up-regulated) by both EGF and R1881, and cluster 1 contains a more diverse group with low expression levels (Fig 4.3A).

Using OMIM™ (Online Mendelian Inheritance in Man™) and LocusLink (NCBI), genes involved in invasion (including cell motility and cell adhesion; n=37) and genes involved in proliferation (including growth, apoptosis or oncogenes; n=48) were identified, and distribution of these genes in the earlier defined four clusters was indicated (Figure 4.3B). It was observed that clusters 3 and 4 contain a high percentage of genes involved in invasion, while genes involved in proliferation were mainly found in clusters 2 and 3 (Fig 4.3B). Interestingly, most genes involved in proliferation were present in cluster 2, containing genes that are mainly up-regulated by EGF and R1881, which indicates that the effect of EGF and androgens on cell growth is partly through stimulation of transcription of the same genes.

Since the majority of genes (68%) involved in invasion were contra-regulated (Figure 4.3A; clusters 3 and 4), regulation of invasion by EGF and R1881 seems to result mainly from stimulation or repression of different genes.

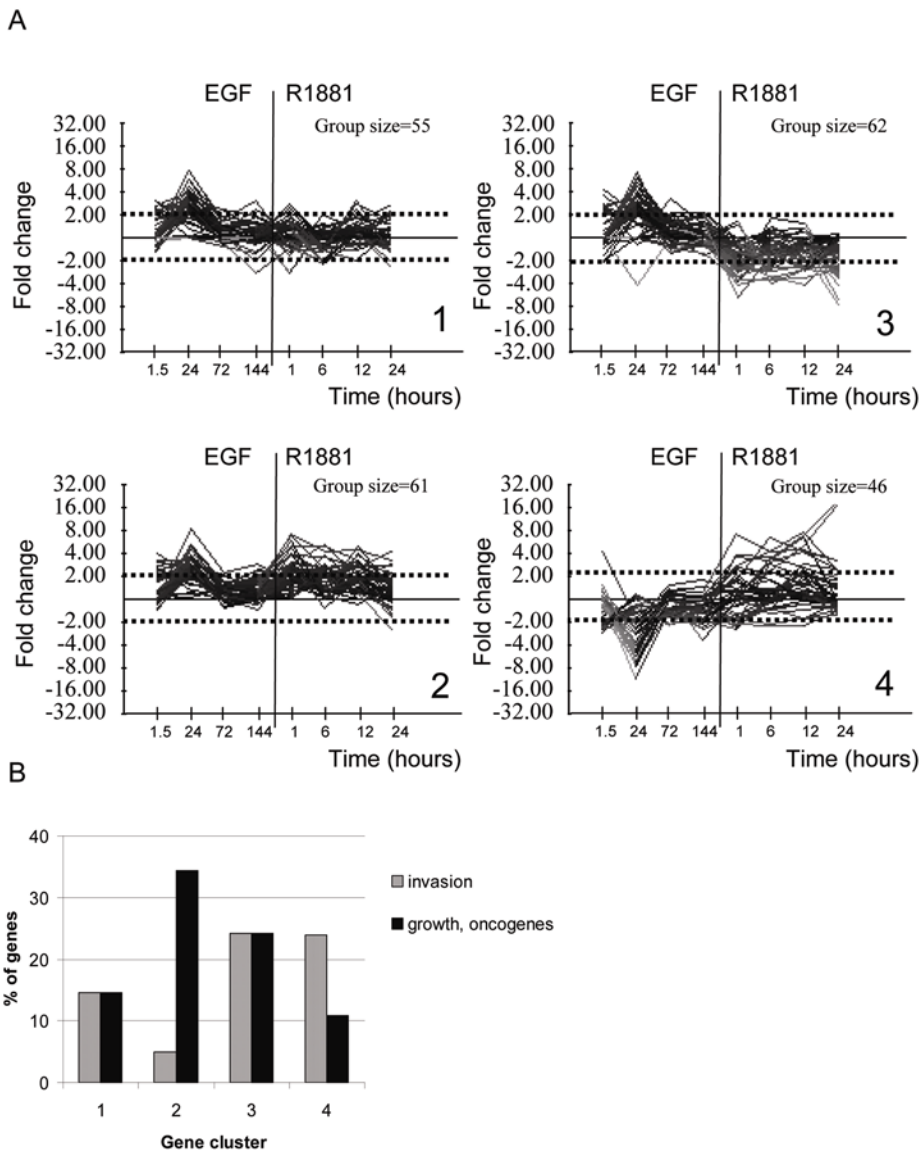

Figure 4.3. **K-means clustering analysis compares EGF- or R1881- induced gene expression in LNCaP cells**

A. K-means clustering, using Euclidian distance measurements, of EGF-regulated transcripts and R1881-regulated transcripts (Segawa et al., 2002). K=4, cut-off is shown at 2-fold change.

B. Genes involved in invasion or proliferation (growth, oncogenes) as % of total number of genes present in cluster 1, 2, 3 or 4.

Evaluation of a possible involvement of R1881- and EGF-regulated genes in progression of prostate cancer

Both androgen- and EGF-signalling play a role in prostate cancer progression. Therefore, the present cell line data on EGF and the additional cell line data on androgens obtained from literature, were compared to a large gene expression data set containing normal prostate samples, next to benign hyperplasia, prostate cancer and hormone-refractory prostate cancer metastatic samples (Dhanasekaran et al., 2001).

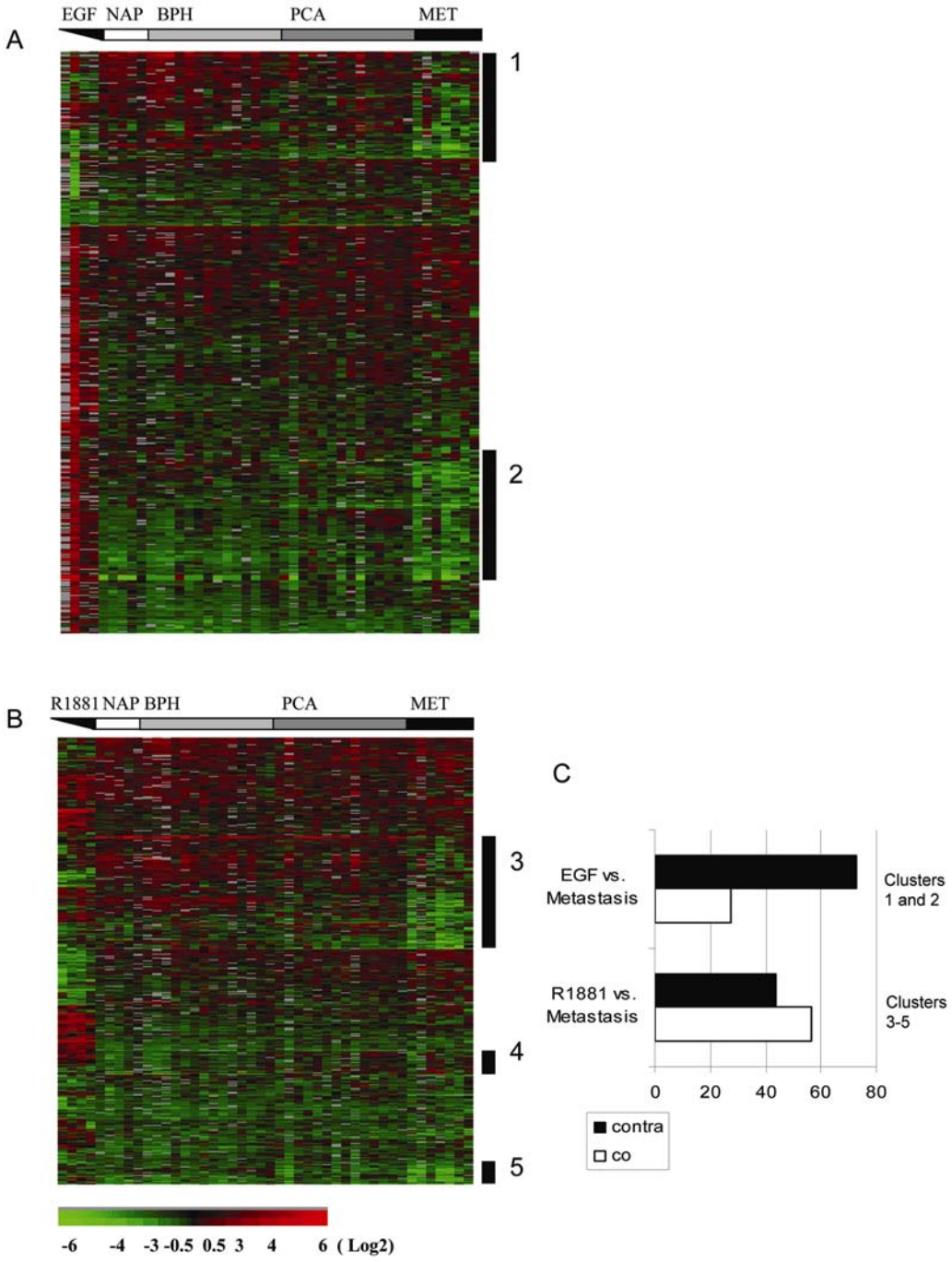

Figure 4.4. **Hierarchical cluster analysis, to compare gene expression in LNCaP cells and different prostate samples**

This analysis uses correlation based distance measurements and complete linkage of gene expression in prostate cancer samples (Dhanasekaran et al., 2001) compared with genes regulated by either EGF or R1881 (Segawa et al., 2002) in LNCaP cells. NAP: normal adjacent prostate, BPH: benign prostate hyperplasia, PCA: prostate cancer, MET: metastasis. Each row represents a single transcript, each column an experiment and red and green represent up- and down-regulation respectively (see scale).

A. Clusters 1 and 2: compared with genes regulated by EGF in LNCaP cells

B. Clusters 3, 4 and 5: compared with genes regulated by R1881 in LNCaP cells (Segawa et al., 2002).

C. Genes (clusters 1-5 of Figure 4A and B) regulated by EGF or R1881 presented as % of the number of genes co-regulated or contra-regulated compared to their expression in metastasis (MET).

First, hierarchical clustering of genes up- or down-regulated at least two-fold in one time point in our EGF dataset and also present in the prostate expression dataset was performed. Results are shown as cluster diagrams, each row represents a single transcript, each column an experiment and red and green represent up- and down-regulation respectively. As shown in Figure 4.4, two metastasis restricted clusters were generated (Figure 4.4A; clusters 1 and 2).

These gene clusters were identified as metastasis restricted, based on different expression levels in the metastasis group as compared to the other prostate cancer samples. Genes in cluster 1, e.g. are mainly down-regulated (green) in the metastasis samples as compared to the other prostate samples (red). A similar evaluation was performed for genes that were up- or down-regulated by androgens at least two-fold in one time point in the dataset from Segawa et al. (2002) and present in the prostate cancer expression data set from Dhanasekaran et al., (2001). As shown in Figure 4.4B, three other clusters could now be identified. Expression levels of genes present in clusters 3 and 5 (and 1 and 2) were metastasis restricted, while expression levels of genes present in cluster 4 were generally prostate cancer specific.

Genes in clusters 1 and 2 were all down-regulated in prostate cancer metastasis as compared to their expression level in normal and cancer samples, but only 27% of these genes were also down-regulated by EGF (Figure 4.4C; upper open bar). This suggests that many EGF target genes are not important in metastatic progression. In contrast, from the genes present in clusters 3, 4 or 5, 57% was co-regulated by androgens (Figure 4.4C, lower open bar), indicating that androgen target genes play a much more significant role in hormone–refractory metastasis.

To this point, the present results show that clusters that discriminate metastatic prostate cancers from other prostate samples can be found within EGF- or androgen-regulated genes. However, since we are interested in crosstalk between androgen- and EGF-signalling pathways, cluster analysis on genes regulated by both EGF and R1881 and present in the Dhanasekaran et al. (2001) dataset was performed. The overlap between the used datasets involves only 83 genes and is too small to draw conclusions about the importance of crosstalk between EGF and androgen signalling for prostate cancer progression.

Of these 83 genes, 39 genes showed differential expression in metastasis samples as compared to normal and BPH samples (Fig 4.5; clusters 6, 7 and 8). Cluster 6 contains genes that are differentially expressed in prostate cancer and metastasis, while clusters 7 and 8 are differentially expressed only in metastasis. In the three indicated clusters, a weak correlation was observed between EGF and androgen target genes and their expression in prostate cancer progression: 16 genes (34%) are regulated in the same direction by EGF, androgens and in prostate cancer progression (Table 4.2).

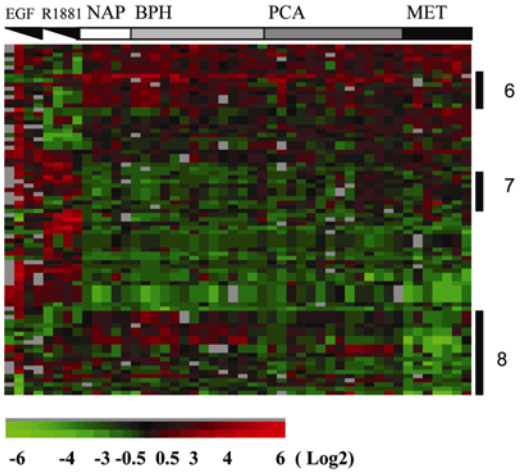

Figure 4.5. **Hierarchical cluster analysis, to compare gene expression in LNCaP cells and different prostate samples**
This analysis uses correlation based distance measurements and complete linkage, of gene expression in prostate cancer samples compared with genes regulated by EGF and R1881 in LNCaP cells. . Each row represents a single transcript, each column an experiment and red and green represent up- and down-regulation respectively (see scale).
Clusters 6, 7 and 8 are differentially expressed in metastasis (MET) or prostate cancer (PCA) compared to normal prostate (NAP) and benign hyperplasia (BPH) and regulated by EGF and R1881.

Table 4.2 **Genes that are regulated in the same direction by EGF, androgens and in prostate cancer metastasis**

Genes **down**-regulated by EGF, androgens and in metastasis	
NAME	FUNCTION
Activated leucocyte cell adhesion molecule	Cell adhesion
Transcription elongation factor A (SII)-like 1	Negative regulation of transcription
RAB6, member RAS oncogene family (2x)	Intracellular protein transport
Ste20-related serine/threonine kinase	Apoptosis; actin stress fiber dissolution
High-mobility group protein 17-like 3	Enhanced transcription
Phospholipase A2, group IIA	Membrane lipid catabolism

Genes **up**-regulated by EGF, androgens and in metastasis	
NAME	FUNCTION
Similar to rat HREV107 (2x)	Unknown
Solute carrier family 4, anion exchanger, member 2	Oxidative stress
Envoplakin	Cytoskeleton
Calcium (ca2+) homeostasis endoplasmic reticulum protein	Calcium homeostasis
Galactose-4-epimerase	Carbohydrate metabolism
SREBP CLEAVAGE-ACTIVATING PROTEIN	Lipid metabolism
Translocase of outer mitochondrial membrane 34 (2x)	Mitochondrial translocation

Name and function of genes that are regulated in the same direction by EGF, androgens and in prostate cancer metastasis. Whether transcripts are present twice (2x) on the microarray is indicated.

Discussion

In the present experiments, administration of both EGF and R1881 simultaneously was found to have an additive effect on cell growth and invasion of LNCaP cells. This indicates that androgens and growth factors utilize divergent signalling pathways in these cells, as was suggested previously by Guo et al. (Guo et al., 2000). However, there are also several reports on crosstalk between androgen- and EGF-signalling in the prostate. Schuurmans et al. (1988b) reported that R1881 induces EGF receptor expression, while Mizokami et al. (1992) found that EGF down-regulates androgen receptor (AR) mRNA expression in LNCaP cells (Mizokami et al., 1992; Schuurmans et al., 1988b). Furthermore, there is also evidence that EGF stimulates AR activity via the MAPK pathway (Craft et al., 1999; Culig et al., 1994), but proliferation of LNCaP cells induced by androgens was not found to be mediated by activation of MAPK (ERK1/2) (Bell et al., 2003). Another possible level at which crosstalk between androgen- and EGF-signalling can take effect is by regulation of the same genes. For example, both androgens and EGF inhibit expression of CDKN1B and stimulate expression of CDK2 (Ye et al., 1999), thus inducing proliferation. Although many observations do point towards an important role for both signalling pathways in regulation of prostate cancer growth, the interaction between AR- and EGF receptor-mediated pathways is still not fully understood. Since we found additive stimulation of both invasion and cell proliferation by androgens and EGF, crosstalk between these hormones was investigated further with microarray-analysis.

Upon comparison of the present cDNA array data on EGF stimulation of LNCaP cells with data from literature on R1881 stimulation of the same cell line (Segawa et al., 2002), a considerable overlap in regulation of genes involved in proliferation was observed. This indicates that some crosstalk between EGF- and androgen-signalling exists at this level. For regulation of genes involved in invasion, however, this was much less evident; EGF and R1881 regulated only 32% of the invasion-related genes in the same direction, up or down. It seems logical that androgen- or EGF-regulation of these proliferation- or invasion-involved genes results in changed proliferation or invasion, but individual genes, or groups of genes should be studied in detail in order to confirm their involvement.

When we compared genes differentially expressed in hormone refractory metastasis samples (as compared to normal or tumor samples of prostate cancer patients) with the EGF- and R1881-regulated genes, we found that 43% of the genes regulated by EGF and 41% of the genes regulated by R1881 were metastasis-specific. These figures are high, but only a quarter of the EGF-regulated genes are co-regulated when compared to their expression in metastasis, while for R1881-regulated genes this is more than 50%. These data suggest that in hormone-refractory metastasis, the EGF signalling pathway might be hardly activated, while the androgen receptor signalling pathway still seems to play a significant role.

The androgen receptor-signalling pathway is still largely active in androgen-independent prostate cancer cells, as has been suggested before. For example, it has been observed that some genes that are differentially expressed in hormone refractory xenografts compared to androgen-dependent xenografts, are still androgen-responsive (Amler et al., 2000; Mousses et al., 2002). However, whether activation of the AR in an androgen-deprived environment is achieved through autocrine stimulation by growth factors, as has been suggested by others (Feldman and Feldman, 2001; Grossmann et al., 2001), can not be extracted from the present data. A possible way to investigate this, would be to compare androgenic signalling in hormone-dependent prostate cancer cells

with EGF signalling in androgen-independent prostate cancer cells. These investigations are currently being conducted.

Recently, it has been reported that stimulation of AR transactivation in advanced prostate cancer cells by EGF requires an already partly activated AR (Gregory et al., 2003). Since we used EGF to stimulate LNCaP cells in the absence of added androgens, and the AR is therefore expected to be largely or completely inactive in these cells, activation of AR-regulated pathways and co-regulated genes should not be found. In correspondence with this, it was observed that most EGF- and R1881-regulated genes were contra-regulated, either in LNCaP cells or in metastasis, although 43% was still co-regulated.

A major draw back of comparison of two microarray datasets of different experiments, is loss of data due to the use of different chips. The overlap between androgen-regulated genes from Segawa et al., 2002, and the microarray used for the EGF experiment is only 40%. Furthermore, when the EGF- and androgen-regulated sets of genes were compared with the set of hormone refractory prostate metastasis-specific genes, the overlap between the three datasets was found to be very low (n = 83), so that no relevant conclusions could be drawn.

In summary, LNCaP cells are stimulated by EGF and androgens to proliferate and to migrate. Addition of both these hormones together showed additive effects. Analysis of gene expression profiles indicates that additive proliferation induction is partly achieved by induction of the same genes, while stimulation of invasion by EGF and androgen is mainly achieved via different signalling pathways. Additionally, gene-expression analyses indicated that androgen target genes are still activated in hormone-refractory metastatic prostate cancer samples and therefore offer potential targets for intervention. For EGF-regulated genes, this was less clear, although many of the genes, which were found to be regulated by EGF and differentially expressed in metastatic prostate cancer, are thought to be involved in invasion.

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Expression profiling of androgen-dependent and -independent LNCaP cells : EGF versus androgen signalling

Expression profiling of androgen-dependent and -independent LNCaP cells : EGF versus androgen signalling

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Abstract

Prostate cancer development often includes a shift from androgen-dependent towards androgen-independent growth. It is hypothesized that, during this transition, growth factors like the epidermal growth factor (EGF) gain importance as activators of cell proliferation. To study the differences between androgen-dependent and androgen-independent cells, androgen- and EGF-regulation of growth and gene-expression was analysed in the androgen-dependent human prostate cancer cell line LNCaP-FGC (FGC) and its androgen-independent derivative line LNCaP-LNO (LNO).

It was observed that androgen-dependent FGC cells require exposure to either androgens or EGF to proliferate. This is in contrast to androgen-independent LNO cells that showed significant proliferation in medium depleted of androgens and growth factors. Gene expression data were obtained for the androgen-dependent FGC and androgen-independent LNO cells cultured in the presence or absence of androgens (synthetic R1881) or EGF for different time periods. Expression profiling showed that many cell cycle genes including a number of androgen- and EGF-regulated genes were constitutively activated in androgen-independent LNO cells. Furthermore, the overlap between changes in gene expression activated by androgen and EGF receptor signalling pathways was found to be very high (75%). These results partly explain why androgen-independent LNO cells can proliferate in the absence of androgenic stimulation.

Introduction

Molecular mechanisms involved in the transition from androgen-dependent to androgen-independent prostate tumour growth have been subject of research for many years. Candidate mechanisms involving the androgen receptor (AR) have been suggested, including mutations that activate AR transcriptional activity, over-expression of co-activators of the AR, and crosstalk with other growth signalling pathways (Balk, 2002; Shaffer and Scher, 2003). The focus of the present study is on androgen crosstalk with the epidermal growth factor (EGF), next to changes in gene-expression and gene-regulation, during progression of prostate cancer.

Peptide growth factors are, next to androgens, essential for prostate growth and maintenance of prostate function (Russell et al., 1998). In normal prostate, peptide growth factors act in a paracrine manner: stroma cells produce growth factors which act on epithelial cells (Hayward et al., 1997). In prostate cancer, production of growth factors switches to an autocrine mode, where epithelial cells become stimulated to produce their own growth factors (Wong and Wang, 2000). In addition, growth factor signalling during prostate cancer progression is enhanced because the expression levels of several important growth factor receptors increase.

Djakiew (2000) and Russel (1998) extensively reviewed the involvement of many growth factors and their receptors in prostate cancer (Djakiew, 2000; Russell et al., 1998). The EGF receptor has been reported to be expressed at higher level in malignant prostate tissue, compared to normal prostate (Di Lorenzo et al., 2002; Scher et al., 1995). However, other investigators reported no change or even a decrease in EGF receptor expression (Robertson et al., 1994; Turkeri et al., 1994). Possible regulation of EGF and its receptor by androgens is also subject of discussion. In normal prostate, androgens stimulate expression of EGF in stromal cells (Hiramatsu et al., 1988; Nishi et al., 1996), but in prostate cancer cells this is not observed (Connolly and Rose, 1990; Russell et al., 1998). Furthermore, it is not clear whether androgenic control of EGF receptor expression involves increased or decreased expression (Di Lorenzo et al., 2002; Djakiew, 2000; Fiorelli et al., 1991; Schuurmans et al., 1988). Still, a detailed analysis of the changing pattern of expression of EGF and the EGF receptor during progression of prostate cancer did indicate that a switch from paracrine to autocrine EGF signalling can play a role in autonomous growth of androgen-independent prostate cancer cells (Scher et al., 1995).

The intracellular domain of the EGF receptor is a tyrosine kinase, which, upon ligand binding of the receptor, becomes activated to form a docking site for several cytoplasmic adaptor proteins (Wells, 1999; Yarden, 2001). These adaptors sequester proteins that stimulate different kinase cascades such as the MAP kinase cascade. As a result, newly phosphorylated proteins may directly affect cell growth, differentiation and migration, independent of transcriptional regulation, or may activate transcription factors, that induce expression of genes which play a role in these processes (Garbay et al., 2000; Yarden, 2001). The AR is a transcription factor, which is activated by androgens and then translocates to the nucleus where the receptor-ligand complex regulates transcription of many genes involved in growth and maintenance of the normal prostate (Balk, 2002; Gelmann, 2002). In androgen-independent prostate cancer, the AR has been suggested to be activated by other substances then androgens (Grossmann et al., 2001). Since activation of the AR may involve phosphorylation (Brinkmann et al., 1999; Gelmann, 2002) and the EGF signalling

pathway activates MAP kinases, EGF-induced and androgen-independent activation of the AR seems a possibility. In fact, activation of the AR via MAP kinases by EGF, and also through HER2, another EGF receptor, has been reported (Culig et al., 1994; Gregory et al., 2003; Yeh et al., 1999).

The human prostate cancer cell line LNCaP-FGC (FGC) is a model for androgen-dependent growth. In the current study we show that this cell line indeed depends on androgen or EGF action for its growth. An important derivative of this cell line is the androgen-independent LNCaP-LNO (LNO) cell line, which was developed from cultures of an early passage of LNCaP-FGC (Chang et al., 1997; Horoszewicz et al., 1980; van Steenbrugge et al., 1991). Using microarray-analysis, these androgen-dependent FGC and androgen-independent LNO cell lines were investigated, to establish the differences between these cell lines on gene-expression and gene-regulation. Furthermore to determine to what extent androgenic- and EGF-signalling become intertwined during the transition from androgen-dependent to androgen-independent prostate cancer growth. Constitutive activation of cell cycle genes in androgen-independent cells was observed, next to an increase of overlap between androgen- and EGF-induced genes.

Materials and Methods

Materials and chemicals

Cell culture flasks and plastic disposables were obtained from Fisher Scientific (Houston, TX, USA). RPMI 1640 and culture chemicals were purchased from GIBCO Invitrogen Corporation (Carlsbad, CA, USA). Fetal calf serum (FCS) was from Greiner (Frickenhausen, Germany), EGF from SIGMA-Aldrich Chemie BV (St. Louis, MI). R1881 (methyltrienolone) was purchased from NEN (Boston, MA), AG1478 from Calbiochem (San Diego, CA). Bicalutamide (ICI176.334, casodex) was from AstraZeneca (Macclesfield, UK), and was dissolved freshly in ethanol prior to use.

Cell culture and treatment

The LNCaP-FGC cells (FGC, passage number 22-30) were kindly provided to us by Dr. J.S. Horoszewicz (Buffalo, NY). Identical cells can be obtained from the American Type Culture Collection (Rockville, MD). FGC cells were maintained in RPMI 1640 supplemented with 200 IU/ml penicillin, 200 μg/ml streptomycin and 5% v/v fetal calf serum (FCS) at 37°C in a humidified incubator containing 5% CO_2/95% air. The LNO cell line has been derived from an early passage (6^{th}) of the parental FGC cell line (Horoszewicz et al., 1980). These cells were grown under the same conditions as their parental cells, except that the serum used was DCC-FCS, depleted of steroids by dextran-coated-charcoal treatment using 0.1% dextran and 1% charcoal (van Steenbrugge et al., 1991).

Before EGF or androgen treatment, LNCaP cells were cultured for 48 h culture in medium containing 5% v/v DCC-FCS and then treated with additional added EGF (20 ng/ml) or R1881 (0.1 nM) for various periods. Total RNA was extracted by lysing the cells with 3 M lithium chloride/6 M urea (Chang et al., 1997).

Micro-array analysis

DNA microarrays consisting of amplified cDNAs from Incyte's Human LifeSeq® Foundation printed on glass slides were used in these studies (Agilent, Palo Alto, CA).

Each array contained over 14,000 unique human genes and expressed sequence tags (ESTs). For each hybridization, 13 μg of total RNA was used in a direct labeling protocol. The RNAs of the different time points were all hybridized on separate arrays, with RNA of untreated cells as reference sample. Labeling, hybridization, scanning procedures and data compiling was performed by ServiceXS, Leiden, The Netherlands (http://www.servicexs.com/), with Agilents G2566AA Feature Extraction Software. The compiled experimental data were further analysed using Microsoft's Excel program and Rosetta Resolver Gene Expression Data Analysis System (Rosetta Inpharmatics LLC). Gene annotations were done with GO-MAPPER, a locally developed annotation tool (Smid and Dorssers, 2004). Cluster-analysis was performed with Expression Profile data CLUSTering and analysis (EPCLUST) at http://ep.ebi.ac.uk/EP/EPCLUST/.

Cell growth study

FGC and LNO cells were grown for three days in DCC-FCS and then plated in 24 well plates ($4{\cdot}10^4$ cells/ml). Three days after plating, test media were administered with 20ng/ml EGF, 0.1 nM R1881, 3 mM Casodex and 300 nM AG1478. Another three days later, these media were refreshed, and after 6 days of incubation cells were lysed in 1 M NaOH, and OD 260 was determined.

Results

Androgen-dependent (FGC) and androgen-independent (LNO) human LNCaP prostate cancer cell lines were treated with the synthetic androgen R1881 (0.1 nM) or EGF (20 ng/ml) for 0, 1.5, 12, 24 or 72 hours in medium depleted of steroids (5% v/v DCC-FCS). After treatment, total RNA was isolated and gene expression was measured using comprehensive oligoarrays. Using the Rosetta Resolver Gene Expression Data Analysis System (Rosetta Inpharmatics LLC) and the Microsoft®Excel program (Microsoft Corporation), up- or down-regulated genes were identified. Genes with a p-value of ≤ 0.05 and a changed expression of at least 2-fold in at least one data point, were defined as regulated. Because the oligoarray data obtained with RNA isolated after 12h of treatment of FGC cells with EGF was of poor quality, inconsistent data from this array were left out from the analysis.

Differences in gene expression between androgen-dependent and androgen-independent LNCaP cell lines under non-hormone-stimulated conditions

Androgen-dependent FGC and androgen-independent LNO cell lines originate from the same patient-sample and the same genetic background (van Steenbrugge et al., 1991). These cell lines represent a model, where FGC cells need androgens or growth factors or both to proliferate (are androgen-dependent), whereas LNO cells have progressed and proliferate without further hormonal substitutions (are androgen-independent). This cellular difference leads to at least two questions. First, since androgen-independent LNO cells do not require added androgens or EGF for growth, to what extent are the associated signal transduction pathways constitutively activated? Second, do differentially expressed cell cycle genes play a role in androgen-independent growth of LNO cells?

To assess constitutive differences between the two cell lines, all non-hormone-stimulated data from androgen-dependent FGC cells were compared to all non-

hormone-stimulated data from androgen-independent LNO cells. In practice, this implies that the average of all Cy5-data for over 17,000 genes on the arrays were compared for each cell line. Transcripts for which the SD was higher then 25% of the average were excluded from this analysis. To verify the quality of collected data, to be able to compare the cell lines, several tests were conducted. The average Cy5 signals in FGC cells and in LNO cells showed a normal distribution, and signal intensities of positive and negative controls on the arrays were no more that 1.4-fold different. Furthermore, known differentially expressed genes like TRPS1 and REPS2 (Chang et al., 1997), TMEFF2 (Gery et al., 2002), vimentin (Singh et al., 2003), CDC2 (Kallakury et al., 1999), IGFBP2 (Bubendorf et al., 1999) and the AR (Balk, 2002) were indeed detected as differentially expressed between the two cell lines (not shown).

It was observed that 1350 genes out of 6702 expressed genes that met the restrictions were differentially expressed between the two non-hormone-stimulated cell lines; in androgen-independent LNO cells 848 transcripts were expressed higher and 502 transcripts were expressed lower, as compared to androgen-dependent FGC cells (Figure 5.1). Because LNO cells proliferate when cultured under non-hormone-stimulated circumstances and the androgen-dependent FGC cells are then largely inhibited in growth, it is expected that this growth difference will be reflected in the profiles of gene expression.

To determine whether androgen- or EGF-regulated pathways are constitutively activated, potential androgen or EGF target genes were compared with the cohort of differentially expressed genes in the non-hormone stimulated situation. Androgen or EGF target genes were defined as genes with a changed expression of at least 2-fold in at least one data point after EGF or androgen exposure of FGC cells. Approximately 21% (181 out of 848) of the genes with a 2-fold higher expression in LNO cells (Figure 5.1A, grey area) were found to be up-regulated by R1881, or EGF, or both in FGC cells. Of the genes with a 2-fold lower expression in LNO cells compared to FGC (Figure 5.1A, white area) approximately 14% (70 out of 502) were also down-regulated by R1881 or EGF or both in FGC cells (Figure 5.1A). It is suggested that approximately 20% of the identified differentially expressed genes are differentially expressed because androgen- and EGF-signalling is constitutively activated in LNO cells (Figure 5.1A). Therefore, the capacity of androgen-independent cells to proliferate without androgenic stimulation, might be caused in part by constitutive activation of androgen- and EGF-signalling.

Next, to assess a possible role of differentially expressed cell cycle genes, the known functions of all genes that were differentially expressed between non-hormone-stimulated cell lines were determined using the annotation tool GO-MAPPER that was developed by Smid and Dorssers (2004) (Smid and Dorssers, 2004). This program categorizes genes into the different levels of the gene-ontology hierarchy (http://www.geneontology.org/doc/GO.doc.html). From the annotated genes (606 could be annotated) that were higher expressed in LNO cells, 31% were categorized in "cell growth and maintenance" (of which 15% in "cell proliferation"), 59% in "metabolism" and 10% in "cell communication". Genes with a 2-fold lower expression in LNO cells were also categorized, and the result displayed more genes in "cell communication" (26%) and less in "cell proliferation" (5%).

In total 105 "proliferation-annotated" genes (Figure 5.1B) were differentially expressed between androgen-dependent FGC and androgen-independent LNO cell lines. Interestingly, 29 of the differentially expressed genes appeared to be androgen-

or EGF-regulated (Figure 5.1B). These results further strengthen the hypothesis that androgen-independent cells may partly regulate proliferation by constitutive activation of androgen- or EGF-signalling pathways or both.

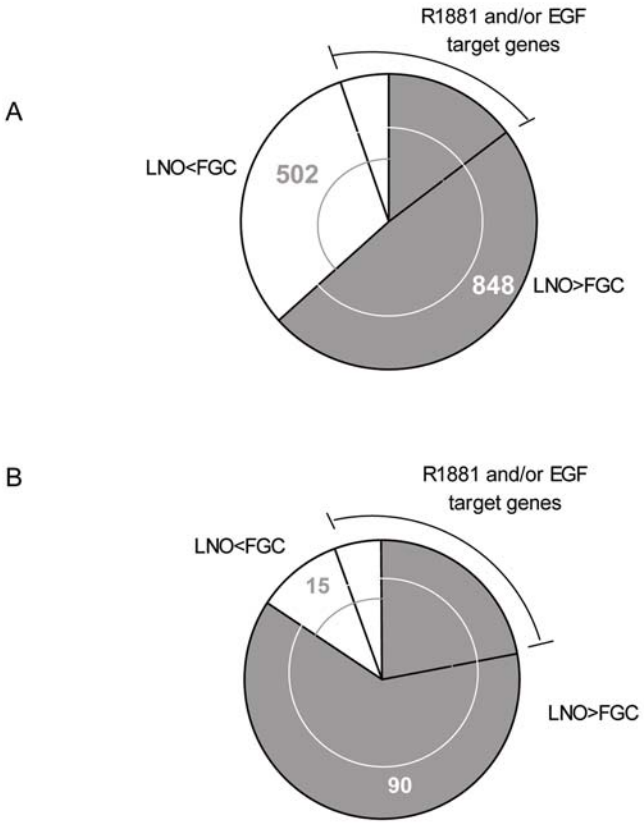

Figure 5.1. **Differential gene expression between androgen-dependent (FGC) and androgen-independent (LNO) LNCaP cell lines**
A. Number of genes differentially expressed between androgen-independent (LNO) and androgen-dependent (FGC) cells. Androgen (R1881) and EGF target genes are indicated within these differential genes. 848 genes are at least 2-fold higher expressed in LNO cells as compared to FGC cells. 502 genes are at least 2-fold lower expressed in LNO cells as compared to FGC cells.
B. Number of differentially expressed genes between LNO and FGC cells involved in proliferation. Androgen (R1881) and EGF target genes are indicated within these proliferation genes. 90 proliferation-involved genes are at least 2-fold higher expressed in LNO cells as compared to FGC cells. 15 proliferation-involved genes are at least 2-fold lower expressed in LNO cells as compared to FGC cells.
The filled segments represent transcripts that are higher expressed in LNO compared to FGC cells (LNO>FGC), and the open segments represent transcripts that are lower expressed in LNO cells compared to FGC cells (LNO<FGC).

Androgen- and EGF-regulated genes in androgen-dependent and androgen-independent LNCaP cell lines

Androgen-dependent FGC cells need steroid hormones or growth factors to proliferate, whereas androgen-independent LNO cells proliferate through endogenous stimulation of growth. Comparison and analysis of androgen- and EGF-regulated genes in both cell lines provides information to what extent signal transduction pathways

converge to stimulate growth in androgen-dependent FGC cells and androgen-independent LNO cells.

It was observed that there is much overlap between androgen- and EGF-regulated genes in both cell lines. In androgen-dependent FGC cells, at least 40% of the genes that are regulated by R1881 are also regulated by EGF (Figure 5.2; Table 5.1), indicating that R1881 and EGF stimulate partly the same cellular processes. Most (97%) of these overlapping genes are regulated in the same direction, either up or down (Table 5.1). In LNO cells the overlap between R1881 and EGF up-regulated genes is much larger, although the absolute numbers are in the same range as for FGC cells (Figure 5.2; Table 5.1). Almost 75% of androgen up-regulated genes in LNO cells are also EGF up-regulated in these cells. This may indicate that in androgen-independent LNO cells EGF receptor signalling has made androgenic signalling partly redundant. Therefore, instead of androgens, locally produced EGF may act as a primary mitogen for LNO cells.

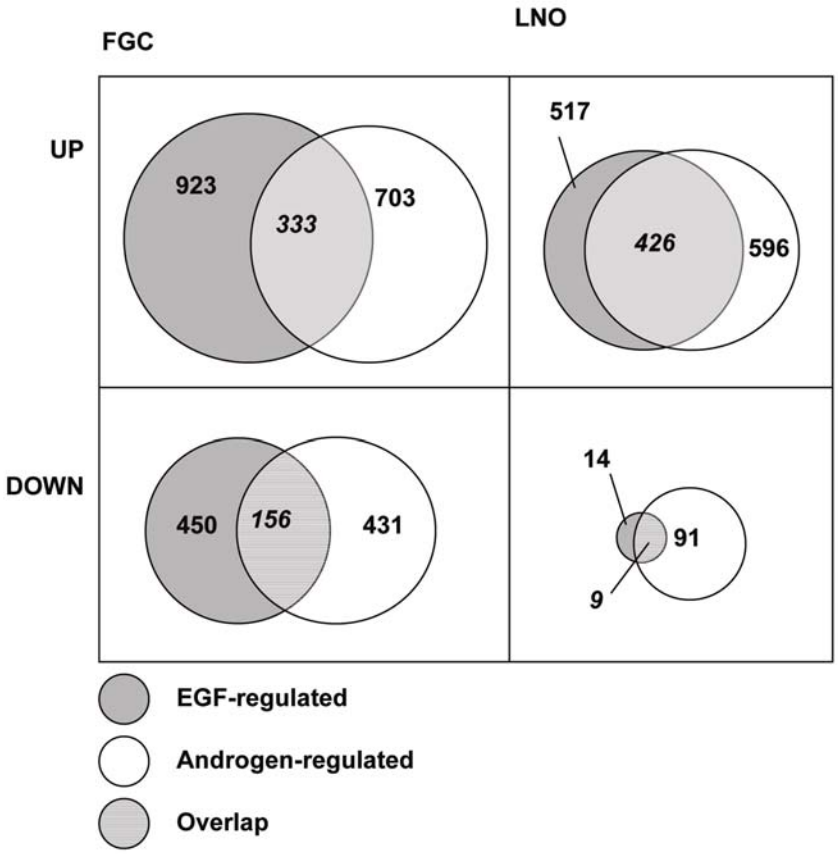

Figure 5.2. **Number of identified androgen- and EGF-regulated genes in androgen-dependent FGC and androgen-independent LNO cell lines**
The number of androgen-regulated transcripts is presented in white and EGF-regulated genes in grey. The number of genes regulated by both are shown in the overlapping fragments and are indicated in italics. The upper two pictures show up-regulated genes, the lower two down-regulated genes. Genes regulated in FGC cells are on the left, genes regulated in LNO cells are on the right.

Table 5.1. **Number of genes up- or down-regulated by R1881 or EGF or both in FGC or LNO cells or both.**

		up	up	up	up	down	down	down	down
		FGC R1881	FGC EGF	LNO R1881	LNO EGF	FGC R1881	FGC EGF	LNO R1881	LNO EGF
up	FGC R1881	703	333	235	210				
up	FGC EGF		923	381	379	11			
up	LNO R1881			596	426	1	1		
up	LNO EGF				517	1	0	0	
down	FGC R1881					431	156	23	5
down	FGC EGF	8					450	28	8
down	LNO R1881	1	4					91	9
down	LNO EGF	1	0	0					14

The number of genes regulated at least two-fold with a p-value ≤ 0.05 in at least one time point by androgen (R1881) or EGF or both, in FGC cells or LNO cells or both.

To assess this hypothesis in a biological setting, proliferation assays were performed. FGC and LNO cells were cultured in the presence or absence of R1881, EGF, and/or specific inhibitors of AR action (Bicalutamide) or EGF receptor signalling (AG1478) for 6 days. Bicalutamide is an anti-androgen that inhibits the transcriptional activity of the AR in LNCaP cells (Veldscholte et al., 1992). AG1478 is a highly specific inhibitor of the EGF receptor that attenuates EGF signalling in LNCaP cells (Guo et al., 2000). It was observed that androgen-dependent FGC cells showed a very low proliferation rate during the time frame of the experiment in the non-hormone-stimulated situation, while the androgen-independent LNO cells showed a 6-fold increase in cell growth. Furthermore, upon culture of FGC cells in the presence of R1881 or EGF, cell growth was increased by 3-fold or 2.2-fold, respectively. When both compounds were added together, an additive effect was observed (4.4-fold), indicating indeed separate pathway usage by androgens and EGF in the androgen-dependent FGC cell line. In contrast to these observations, R1881 had only a minor effect on growth of LNO cells (Figure 5.3; 1.4-fold on average), while EGF supplementation, alone or in combination with R1881, had no effect on growth of these cells.

If stimulation of growth by the two hormones (either separate or combined) was restrained by specific inhibitors (Bicalutamide or AG1478), it was observed that these compounds act as expected in androgen-dependent FGC cells. In androgen-independent LNO cells, however, the antiandrogen Bicalutamide inhibits only androgen signalling, while hardly no effect was observed of the EGF receptor inhibitor AG1478 (Figure 5.3). So, despite the fact that many proliferation-annotated genes are regulated by androgens and EGF and are higher expressed in the androgen-independent LNO cell line, inhibition of EGF signalling at receptor level hardly affects growth of this cell line. Therefore, it is hypothesized that, in androgen-independent LNO cells, parts of the proliferation machinery are constitutively activated through mutations, deletions or amplifications of key genes acting downstream from the androgen or EGF receptors, or through autonomous activation of other signalling pathways.

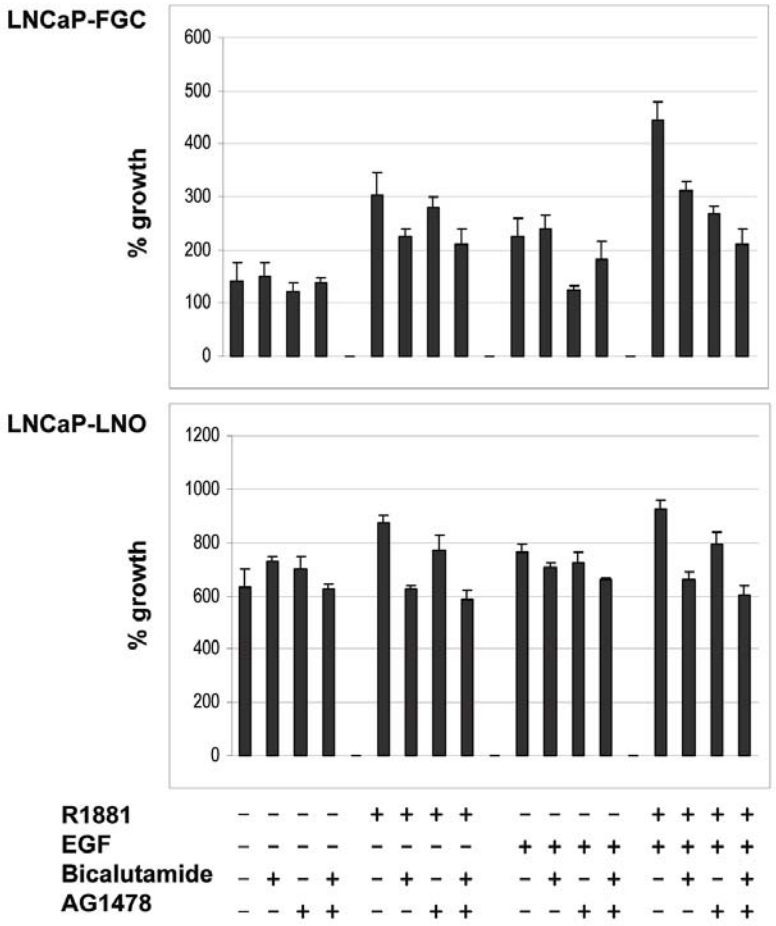

Figure 5.3. **Regulation of growth of androgen-dependent FGC and androgen-independent LNO cells by androgen (R1881), EGF, Bicalutamide and AG1478**
Cells were incubated with 20ng/ml EGF, 0.1 nM R1881, 3 mM Bicalutamide and 300 nM AG1478 in the indicated combinations. After 6 days cells were harvested in 1N NaOH and OD260 values were measured in diluted samples. Growth is expressed as a percentage of the OD260 value at the start of the incubations. The experiments were conducted in 24 well plates; each measurement is the average of four wells +/- SD. Results shown are representative of an experiment performed three times. All control incubations were performed in the presence of vehicle (0.1% ethanol)

Interestingly, in the prostate cancer cell lines described herein, we found 56 genes involved in PI3Kinase pathway signalling to be differentially expressed between the two lines in the non-hormone-stimulated situation. Of these 56 differential genes, 7 were androgen- or EGF-regulated. Another 29 genes were androgen- or EGF-regulated, but not differentially expressed (Figure 5.4). The PI3Kinase pathway is involved in prostate cancer progression and is essential for survival of LNCaP cells in an androgen-deprived environment (Lin et al., 1999). Therefore, regulation and expression differences of the PI3Kinase pathway between FGC and LNO cells were assessed by screening phosphorylated AKT kinase expression in these cells after stimulation by EGF or androgen. It was observed that AKT kinase was highly phosphorylated in both cell lines in the present non-hormone stimulated situation, but expression of phosphorylated AKT in FGC was twice as high as its expression in LNO. Addition of either R1881 or EGF had no effect on activation or expression levels of AKT kinase in both cell lines (data not shown). This indicates that the PI3Kinase pathway is constitutively activated in both cell lines, and is therefore not responsible for the

proliferative advantage of androgen-independent prostate cancer cells over androgen-dependent prostate cancer cells.

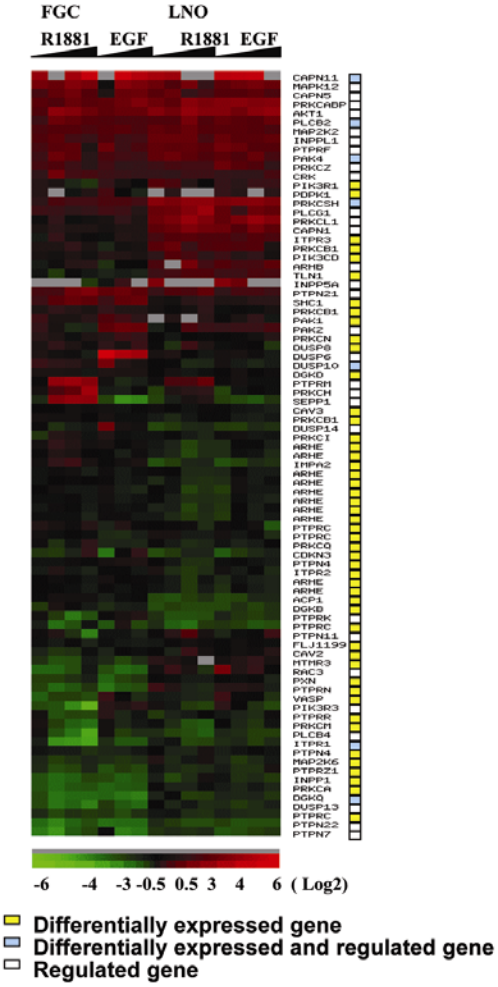

Figure 5.4. **Transcriptional regulation of genes involved in PI3Kinase signalling**
Hierarchical clustering with uncentered distance measure, of all PI3Kinase-involved transcripts that are regulated either by androgen (R1881) or EGF at least two-fold and in at least one time point, or are differentially expressed between FGC and LNO. Whether the transcripts are regulated (white boxes), differentially expressed (yellow boxes) or both (blue boxes), is indicated on the right. Samples analysed are represented by columns, and shown from left to right are: FGC cells, treated with R1881 (0.1 nM) for 1.5, 12, 24 or 72 hours; FGC cells treated with EGF (20 ng/ml) for 1.5, 24 or 72 hours; LNO cells treated with R1881 (0.1 nM) for 1.5, 12, 24 or 72 hours; LNO cells treated with EGF (20 ng/ml) for 1.5, 12, 24 or 72 hours. Each row represents a single transcript and red and green represent up- and down-regulation, respectively (see scale).

Hierarchical clustering of genes, differentially regulated between androgen-dependent and androgen-independent LNCaP cell lines

So far, differences between androgen-dependent FGC and androgen-independent LNO cell lines in a non-hormone-stimulated situation, and the overlap between androgen and EGF signalling for these two cell lines, was analysed. As a subsequent step, we aimed to identify regulated genes that may contribute to functional differences between the two cell lines.

First, all genes that were regulated in at least one of the arrays were analysed using hierarchical clustering at http://ep.ebi.ac.uk/EP/EPCLUST/ (Figure 5.5). Some highly androgen- or EGF-regulated genes and their positions in the clusters are depicted (Figure 5.5). The black bars to the left of the figure indicate clusters for which gene regulation in LNO cells is clearly different from gene regulation in FGC cells. Because we are interested in these gene clusters, ANOVA analysis was used to calculate which regulated genes were indeed differentially regulated between FGC and LNO cells.

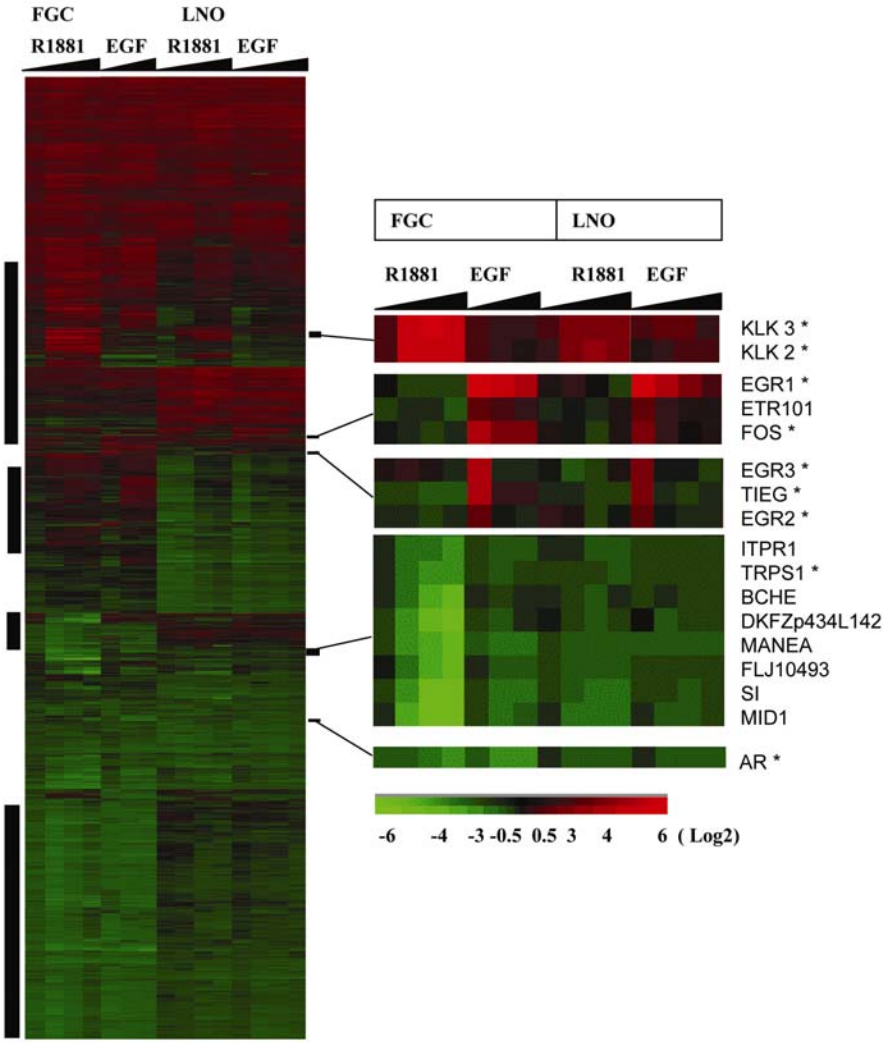

Figure 5.5. **Transcriptional program activated by R1881 or EGF in FGC or LNO cells**
Hierarchical clustering with uncentered distance measure, of all transcripts that are regulated either by androgen (R1881) or EGF at least two-fold and in at least one time point. Samples analysed are represented by columns, and shown from left to right are: FGC cells treated with R1881 (0.1 nM) for 1.5, 12, 24 or 72 hours; FGC cells treated with EGF (20 ng/ml) for 1.5, 24 or 72 hours; LNO cells treated with R1881 (0.1 nM) for 1.5, 12, 24 or 72 hours; LNO cells treated with EGF (20 ng/ml) for 1.5, 12, 24 or 72 hours. Each row represents a single transcript, and red and green represent up- and down-regulation respectively (see scale). Black bars on the left indicate regions with differential gene expression signatures. Enlarged sections on the right indicate representative androgen- or EGF-regulated genes. Genes indicated with an asterisk have previously been reported as androgen- or EGF-regulated genes.

Using the Rosetta Resolver Gene Expression Data Analysis System, 1138 genes were identified which were significantly differentially regulated between FGC and LNO (at least 2-fold and at least in one time point, by either EGF or androgen; p-value<0.05) (Figure 5.6A). Within this dataset, two distinct clusters were formed that were either up-regulated in androgen-independent LNO cells and not regulated in androgen-dependent FGC cells (cluster A) or vice versa (cluster B). All transcripts, and transcripts specifically present in the two clusters were annotated using GO-MAPPER (Smid and Dorssers, 2004) to a cellular function as indicated in Figure 5.6B. Interestingly, it was observed that cluster B contains more transcripts involved in proliferation and transport and less transcripts involved in transcription and communication as compared to cluster A.

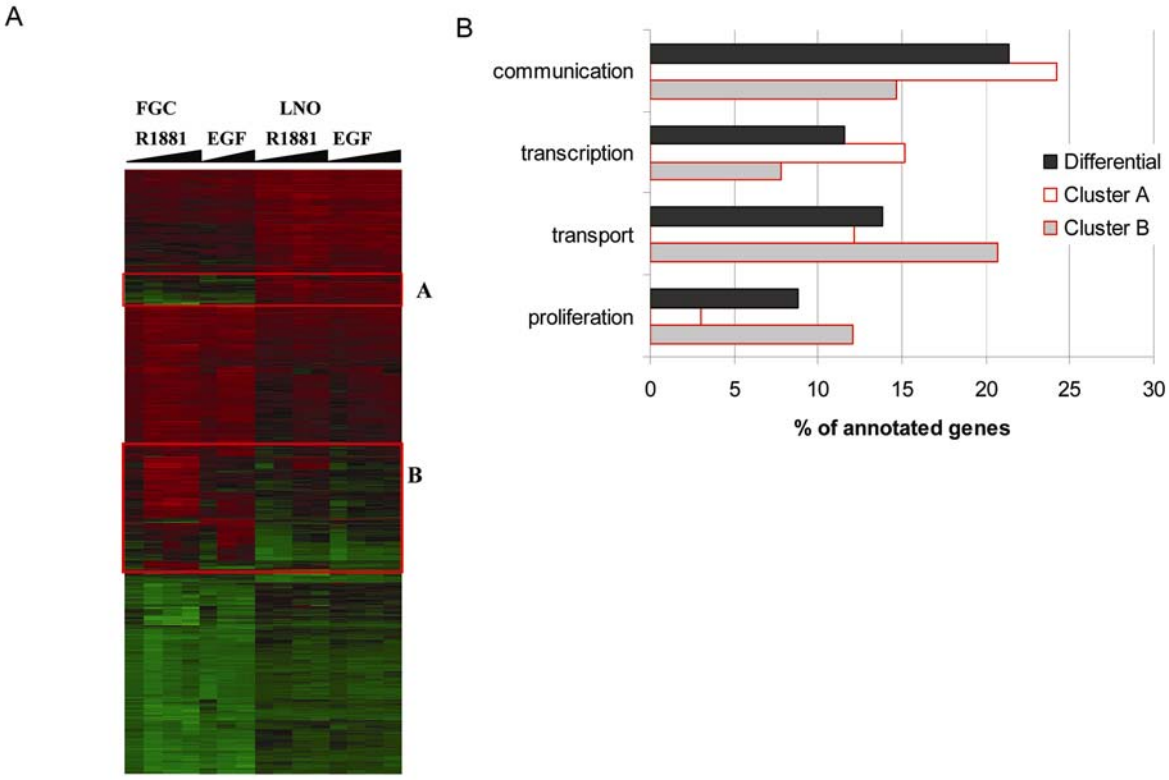

Figure 5.6. **Differential transcription activation by androgen (R1881) or EGF in FGC or LNO cells**

A. Hierarchical clustering with uncentered distance measure, of all transcripts that are either regulated by androgen (R1881) or EGF at least two-fold in at least one time point and are differentially regulated between FGC and LNO as determined by Anova analysis with Rossetta Resolver, P<0.05. Indicated cluster A contains transcripts that are up-regulated in LNO cells but not regulated in FGC cells by androgen and/or EGF. Cluster B contains transcripts that are not regulated in LNO cells but up-regulated in FGC cells by androgen and/or EGF.

B. Annotation of all genes differentially regulated or present in cluster A or B as % of annotated genes in these groups. Annotation was performed using GO-MAPPER (Smid and Dorssers, 2004).

This suggests that cluster B contains genes that play a role in cell cycle control. To verify this, genes that were identified by Whitfield et al. (2002), which showed periodic variation during the cell cycle in HeLa cells (Whitfield et al., 2002) were compared to

the EGF- or androgen-regulated genes from Figure 5.6A. 202 genes were found to overlap between the Whitfield data set (containing 1134 genes) and our 1138 regulated genes (Figure 5.7). Of these 202 overlapping genes, 77 were present within cluster B. This number is remarkably high and indicates that cluster B indeed contains a subgroup of genes that may play a role in cell cycle control. Furthermore, since regulation by androgens and EGF of genes included in cluster B is very distinct between FGC and LNO cell lines, it is hypothesized that at least parts of the mechanisms that control the cell cycle are differentially regulated in androgen-dependent cells as compared to androgen-independent cells.

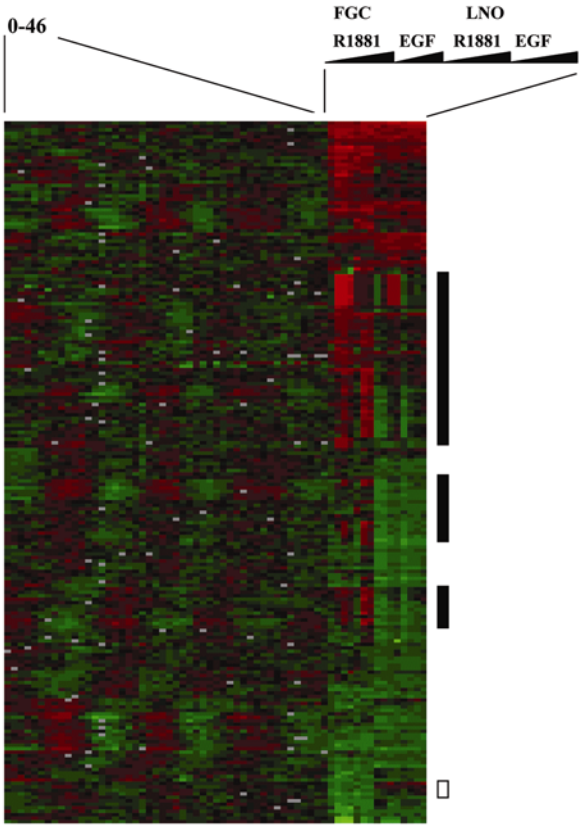

Figure 5.7. **Analysis of transcripts periodically expressed in the cell cycle and regulated by androgen or EGF in FGC or LNO**
Hierarchical clustering with uncentered distance measure, of transcripts present in the Thy-Thy3 (0-46 hours) cell cycle experiment described by Whitfield et al. (Whitfield et al., 2002) and the present LNCaP experiment. Samples analysed are represented by columns, and shown from left to right are: HeLa cells, sampled each hour until 46 hours after synchronized release from cell cycle arrest; FGC cells treated with R1881 (0.1 nM) for 1.5, 12, 24 or 72 hours; FGC cells treated with EGF (20 ng/ml) for 1.5, 24 or 72 hours; LNO cells treated with R1881 (0.1 nM) for 1.5, 12, 24 or 72 hours; LNO cells treated with EGF (20 ng/ml) for 1.5, 12, 24 or 72 hours. The regions indicated by black bars contain genes that are up-regulated in FGC and not regulated in LNO cells. The white bar indicates a small region representing genes for which this regulation is in a reverse manner.

Discussion

Androgen-independent prostate cancer growth is the endpoint of tumour development, from normal growth of prostate cells that is induced and regulated by androgens to aberrant growth that is induced and maintained by other factors and

mechanisms. These factors and mechanisms include sensitization of the androgen receptor and activation of other signalling pathways, like cytokine and growth factor signalling or oncogenic signalling (Balk, 2002; Grossmann et al., 2001).

The human prostate cancer cell line LNCaP-FGC (FGC) is a model for androgen-dependent growth. In the current investigation, we show that this cell line depends on either androgen or EGF for its growth. In contrast, growth of the LNCaP-LNO (LNO) cell line, an androgen-independent sub-line of FGC is not dependent on androgen or EGF, and influenced only to a minor extent by exposure to these hormonal factors.

As a model for transition from androgen-dependent to androgen-independent prostate cancer growth, the above-mentioned two cell lines were explored. To identify differences between androgen-dependent and -independent growth, gene expression profiling was performed. Expression of both androgen and EGF receptors was approximately twice as high in FGC as compared to LNO cells, as determined by Western blotting (not shown). Because many genes were activated by androgen and EGF in the androgen-independent LNO cell line, it was concluded that receptor expression levels were sufficient to generate a response. Various analyses of the results were executed yielding information about differential signal transduction pathway usage by the two cell lines.

Using the averages of the intensities of the reference samples, that represented a non-hormone-stimulated situation, a group of genes was defined that is differentially expressed between the androgen-dependent and the androgen-independent cell lines. This group includes a large number of androgen- and EGF-regulated and/or proliferation-related genes. Possibly, constitutive activation of these specific clusters of genes in the androgen-independent cell line adds to the autonomous growth behavior. The observations that the androgen-independent cells grew well on steroid hormone-free medium, and that addition of R1881 or EGF, or addition of an anti-androgen or EGF receptor-inhibitor, had little effect on this growth, further indicated the presence of a constitutive activated proliferation pathway. Furthermore, it is interesting to note that in LNO cells, the absolute number of EGF-regulated genes involved in proliferation is remarkably lower as compared to the number in FGC cells (29 in LNO, 67 in FGC).

Upon analysis of genes that were differentially regulated by R1881 or EGF or both, it was observed that the overlap between EGF and R1881 activated genes is approximately 75%. In the androgen-dependent FGC cells, this number is much lower, since only 40% of EGF and androgen activated genes overlap. Whether the EGF receptor pathway and the AR pathway in androgen-independent LNO cells indeed show more crosstalk then in FGC, could not be confirmed, since LNO cells hardly respond to specific anti-androgen (Bicalutamide) and EGF receptor signal inhibitor (AG1478). This last observation, however, could indicate that the proliferation machinery is constitutively activated through mutations, deletions or amplifications of key regulatory genes acting down-stream from the androgen or EGF receptors, or through activation of other signalling pathways.

A potential candidate pathway to induce proliferation in androgen-independent cells is the PI3Kinase pathway. It has previously been reported that, in LNCaP cells, the PI3Kinase pathway is constitutively active (Pfeil et al., 2004) and essential for survival in an androgen-deprived environment (Lin et al., 1999). It was observed that androgen-ablation increased the activity of PI3Kinase and AKT during establishment of androgen-independent sublines of LNCaP, and that addition of androgens blocked that effect (Murillo et al., 2001). It should also be mentioned, however that other reports

do not described such a difference in expression of PI3Kinase and AKT after long-term androgen ablation of FGC cells (Pfeil et al., 2004).

In the present study, it was observed that 56 PI3Kinase-annotated genes were differentially expressed between FGC and LNO cells and 36 PI3Kinase-annotated genes were androgen- or EGF-regulated (of which 7 genes are both differentially expressed and hormone-regulated). However, when we measured activity of PI3Kinase by measuring phosphorylated AKT, constitutive activation of the PI3Kinase pathway in both cell lines was found, with the highest activity in FGC and no effect of either androgen or EGF addition. Constitutive active PI3Kinase could be an important determinant which prevents FGC cells from dying during androgen ablation, but the lower activity of the PI3Kinase pathway in LNO cells cannot explain the proliferative advantage of the androgen-independent cells. Therefore other proliferation-inducing pathways must be activated in these cells.

Cluster-analysis of regulated genes resulted in formation of groups of genes that showed differential regulation between FGC and LNO cells. Two groups were extremely different, either up-regulated by EGF and/or androgens in LNO and not regulated by EGF and/or androgens in FGC, or vice versa. A remarkably high number of genes involved in proliferation was found in the group that was up-regulated in FGC and not regulated in LNO cells. Furthermore, when compared to previously identified genes regulated during the cell cycle in HeLa cells (Whitfield et al., 2002), 77 cyclic genes were found to be up-regulated in FGC and not regulated in LNO. Up-regulation of these cell cycle regulated genes specifically in FGC could indicate that this asynchronous cell population contains many cycling cells after addition of androgens and/or EGF. If this is the case, then LNO cells should express many cell cycle regulated genes without addition of androgens or EGF, since these cells grow well under those circumstances. Indeed 138 genes from the Whitfield data-set were higher expressed in LNO as compared to FGC in the non-hormone-stimulated situation. Furthermore, 33 out of these 138 transcripts were implicated in cancer, which also indicates that LNO cells possess a phenotype associated with proliferative tumour cells. Recently an interesting comparison was made between LNCaP cells inhibited in growth by methylseleninic acid (MSA) and periodically expressed cell cycle genes (Whitfield et al., 2002). Most cell cycle-involved genes (74%) were found to be down-regulated in LNCaP cells that were inhibited in growth (Zhao et al., 2004). We found 60% of the cell cycle regulated genes to be up-regulated in LNCaP-FGC cells that were stimulated in growth by either R1881 or EGF.

Validation of differentially expressed or regulated genes will be required. However, several genes previously reported and shown to be differentially expressed between FGC and LNO cells were confirmed with these microarray experiments. B-cell translocation gene 1 as well as REPS2 and TRPS1 were determined by RNA differential display (25) and also by these microarray-analyses to be differentially expressed between FGC and LNO cells. Furthermore, EGF- or androgen-regulated genes like EGR1, TIEG, FOS, PSA and AR were previously reported (40-42) and confirmed here (Fig. 5).

In summary, it is suggested that androgen-independent LNO cells can proliferate in the absence of androgenic stimulation, because: first, many cell cycle genes including a number of androgen- and EGF-regulated genes are constitutively activated; second, the overlap between androgen receptor signalling and EGF receptor signalling

pathways is very high (80%); third, other unknown signal transduction pathways have been activated and induce proliferation.

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General Discussion

General Discussion

Prostate cancer in its early stages is an endocrine-controlled cancer, but, while it progresses, the cancer switches from androgen-dependent to -independent growth. The organ and early cancerous lesions that develop from it both need androgens for development, maintenance and growth. However, when the cancer progresses, it survives and grows without androgens. A significant portion of prostate cancer research focuses on mechanisms behind the transition from androgen-dependent to androgen-independent growth, to eventually find possible targets for intervention (Chapter 1). The experiments described in this thesis focus on two aspects of androgen-independent prostate cancer growth:

1. The protein REPS2, which is lost from androgen-independent prostate cancer and plays a role in EGF receptor signalling (Chapters 2 and 3)
2. EGF receptor signalling as a potential alternative proliferation- and invasion-inducing pathway during androgen-independent prostate cancer growth (Chapters 4 and 5).

6.1 REPS2

REPS2 was originally identified by our laboratory as a protein encoded by a transcript with high expression in androgen-dependent and low expression in androgen-independent human prostate cancer cell lines and xenografts (Chang et al., 1997). As indicated in Table 1, others confirmed this finding when comparing gene expression in normal prostate with that in advanced prostate cancer. Because of the potential role of REPS2 in development of androgen-independent prostate cancer, for the experiments described in Chapters 2 and 3 the following question was posed*:*

- *Via which mechanisms does loss of expression of the endocytosis protein REPS2 contribute to an androgen-independent phenotype of prostate cancer cells?*

6.1.1 *REPS2 induces apoptosis*

As described in Chapter 2, overexpression of REPS2 induces apoptosis of prostate cancer cells and inhibits transcription from an EGF responsive reporter construct. It was hypothesized that REPS2 acts via binding to RALBP1 and consequently via inactivation of RAC1 and CDC42 signalling. RALBP1 functions mainly as a CDC42 Gap protein (Cantor et al., 1995). CDC42 has been implicated in cell cycle control via stimulation of E2F-mediated transcription, possibly by inducing Cyclin D1 expression (Gjoerup et al., 1998). CDC42-induced expression of Cyclin E and Cyclin A (Chou et al., 2003; Philips et al., 2000) has also been described, linking CDC42 activity directly to progression through the G1 phase of the cell cycle. Inhibition of CDC42, by RALBP1 via overexpression of REPS2, could therefore lead to inhibition of the cell cycle.

Whether increased REPS2 expression indeed leads to inhibition of the cell cycle or has a more general inhibiting effect on cell survival, however, remains to be elucidated. Identification of the p65 subunit of the cell survival protein complex NF-κB as a binding partner of REPS2 raised a new possible explanation for the observed

induction of apoptosis by REPS2 (Penninkhof et al., 2004). In a series of experiments, Penninkhof et al. (2004) described specific binding of p65 to the EH domain of REPS2, but did not find a physiologic response. In our report Oosterhoff et al. (2003) (Chapter 2) we described induction of cell death in cells transiently expressing REPS2-GFP (Oosterhoff et al., 2003). However, this effect was only seen in cells with a fairly high expression of RESP2-GFP. Since over-expression of proteins can lead to artificial effects, a stable cell line with a moderate, more physiological level of REPS2-expression was generated (described in Chapter 3). Upon testing these cells, increased apoptosis could not be detected. Therefore, we measured proliferation, but no significant decrease in cell number by induced REPS2 expression was observed. Apparently, more subtle ways of measuring cellular changes are required for observations on possible effects of physiological changes in the expression level of REPS2.

6.1.2 *REPS2 inhibits growth factor signalling*

Transient transfection with wild-type REPS2 showed decreased transcription from an EGF-responsive reporter-construct (Chapter 2). The experiments described in Chapter 3 were performed with a physiological level of REPS2 expression and also showed decreased EGF signalling. The mechanism, hypothesized in Chapter 3, concerns REPS2-induced inhibition of EGF signalling through inhibition of EGF receptor endocytosis. Interestingly, EGF receptor endocytosis was shown to be inhibited by both over-expression of REPS2-GFP (Chapter 2) and moderate expression of wild-type REPS2 (Chapter 3).

Again, the ability of REPS2 to bind RALBP1 and consequently to inactivate CDC42 could explain the observed effect. Both proteins have been described to be involved in growth factor endocytosis: RALBP1 as part of a complex with AP-2 (Jullien-Flores et al., 2000), and CDC42 by binding to ACK2, a kinase which, when activated, binds to the clathrin heavy chain (Erickson and Cerione, 2001). Additionally, Epsin and Eps15, two endocytosis-involved binding partners of REPS2 (Morinaka et al., 1999; Torrisi et al., 1999), could also play a role in inhibition of endocytosis by REPS2.

From the experiments using a physiological expression level of REPS2, it was also clear that the observed effects of REPS2 induction were reproducible but not very pronounced. It was therefore of interest to find that the expression of many other proteins involved in endocytosis becomes down-regulated during development of prostate cancer metastasis (Dhanasekaran et al., 2001).

6.1.3 *REPS2 as target gene in prostate cancer progression*

Taken together, results regarding apoptosis induction of REPS2 in prostate cancer cells should be interpreted with caution, but the effect on growth factor signalling and growth factor receptor endocytosis seem to be of importance. Loss of REPS2 expression in androgen-independent prostate cancer and consequently loss of control over growth factor signalling, either directly or via inhibition of endocytosis of activated receptors, is hypothesized to lead to a more malignant phenotype.

Differential expression of genes, for example between androgen-dependent and androgen-independent prostate cancer, is nowadays determined by the use of microarrays. A practical data mining tool that enables researchers to efficiently quarry

genes and datasets of publicly available cancer microarray studies, is the Oncomine Infrastructure (Rhodes et al., 2004). Using this tool, all microarray data available to the public domain from normal human prostate or prostate cancer were examined for the expression of REPS2. The results are summarized in Table 6.1.

Table 6.1: **Mean expression level of REPS2 in different prostate cancer samples as investigated by the indicated first authors**

Study Name	Sample 1	Sample 2	Mean 1	Mean 2	P Value
LaTulippe (2002)	Normal Prostate (n=3)	Prostate Cancer (n=23)	-0.14	0.09	0.27
LaTulippe (2002)	Prostate Cancer (n=23)	Metastatic Prostate Cancer (n=9)	0.09	0.02	0.81
LuoJH (2002)	Normal Prostate (n=15)	Prostate Cancer (n=15)	-0.07	0.20	0.01*
Singh (2002)	No PSA recurrence at 3 years (n=31)	PSA Recurrence at 3 years (n=8)	-0.02	-0.06	0.86
Singh (2002)	Nontumor Prostate (n=48)	Prostate Cancer (n=52)	-0.08	-0.01	0.46
Singh (2002)	Prostate Cancer: Gleason 6 (n=15)	Prostate Cancer: Gleason 8,9 (n=4)	0.05	-0.43	0.02*
Welsh (2001)	Normal Prostate (n=9)	Prostate Cancer (n=25)	-0.84	0.03	0.00*
Welsh (2001)	Prostate Cancer: Gleason 6 (n=8)	Prostate Cancer: Gleason 8,9 (n=7)	-0.05	-0.09	0.84

Mean expression levels of REPS2 in the indicated prostate samples. Gleason scores indicate stages of prostate cancer, high scores correlate with more advanced stages. The gene is considered to be up-regulated (red) if the mean expression level of sample 1 is lower then the mean expression level of sample 2, and down-regulated (green) if the mean expression level of sample 1 is higher then the mean expression level of sample 2. The p value is a measure of significance of the difference between the two tissue samples provided by the Oncomine website (*p value≤ 0.02). For more information on data calculation: www.oncomine.org and (Rhodes et al., 2004).

Compared to normal prostate tissue, REPS2 expression is up-regulated (red) in prostate cancer samples. However, REPS2 expression is down-regulated (green) in prostate cancer with an aggressive, most likely androgen-independent, phenotype. These results, although not all significant, correspond with our data on REPS2 expression in androgen-independent cells as compared to androgen-dependent cells, described in Chapter 2.

One report (Henshall et al., 2003), not mentioned in the table, studied 72 prostate cancer samples and determined the correlation between REPS2 gene expression and PSA relapse of the patient. PSA relapse is generally accepted as indicative for relapse of the disease after surgery or during hormone treatment. Decreased expression of REPS2 was found to be strongly correlated with relapse of the PSA level measured in blood of the patients (Henshall et al., 2003). It was calculated that patients with a lower expression of REPS2 have an approximate 6-fold increased risk of relapse, which indicates that REPS2 may serve as a prognostic indicator.

6.2 EGF receptor signalling in androgen-dependent and –independent prostate cancer cells

To determine in more detail which signalling pathways are affected in prostate cancer progression, microarray analysis were performed. Because the EGF pathway has been described to be involved in prostate cancer progression (Russell et al., 1998), in Chapter 4 the following questions were asked:

- *Is it possible to identify genes, activated by androgens or EGF, that induce proliferation and invasion of prostrate cancer cells?*
- *Do EGF- and androgen-signalling overlap in prostate cancer cells and if so, to what extent?*

6.2.1 *Regulation of proliferation and migration*

Androgen-independent prostate cancer cells use different mechanisms to grow under circumstances where androgen availability is low. One possible mechanism for prostate cancer cells to overcome androgen ablation is to switch from androgens to growth factors, like EGF, for regulation of growth (Djakiew, 2000; Russell et al., 1998). Besides changed growth characteristics, advanced prostate cancer also displays a high metastatic potential: of all prostate cancer patients, approximately one third develop metastasic lesions outside the prostate, mainly in the pelvic bone. It is thought that both androgens and EGF stimulate the metastatic potential of prostate cancer.

In the androgen-dependent prostate cancer cell line LNCaP proliferation and invasion are both enhanced by androgens (Liao et al., 2003; Schuurmans et al., 1991) and EGF (Jarrard et al., 1994; Unlu and Leake, 2003). Because we were interested in the transcriptional aspects of these processes in prostate cancer, this cell line was used to study the regulatory effects of androgens and EGF on genes involved in modulation of proliferation and invasion.

Significant effects of androgens and EGF on proliferation and migration rates of LNCaP cells are shown in Chapter 4. Using microarray analysis on LNCaP cells treated with EGF (our own data), and microarray data of LNCaP cells treated with R1881 (Segawa et al., 2002), the genetic response in LNCaP cells to androgens and EGF was examined. Of the genes that were regulated by androgens (Segawa et al., 2002) or EGF, approximately 25% was found to be involved in cell growth and maintenance, and almost half of these genes was classified to be involved in proliferation regulation. Interestingly, we found the same numbers for both androgen- and EGF-regulated genes. For genes involved in invasion, the numbers were much smaller: only 1.5-3% was annotated as involved in cell motility and another 3% was involved in cell adhesion. It seems logical that androgen- or EGF-regulation of transcription of these particular genes result in an increase in proliferation and invasion, but individual genes or groups of genes should be studied in detail in to confirm their involvement.

As described in Chapter 4, possible crosstalk between androgen- and EGF-signalling was also analysed. We found more than 50% overlap between androgen- and EGF-regulated genes involved in proliferation, and approximately 30% for genes involved in invasion, and hypothesised that EGF and androgens use partly the same pathways to control growth and for the most part separated pathways to control invasion.

Since the comparisons between androgen- and EGF-regulated genes described in Chapter 4 are performed with data partly generated by others (Segawa et al., 2002),

the limited overlap in both data sets is a problem. Of all androgen-regulated genes present in the Segawa dataset, only 40% was also present on our cDNA array. The problem of limited overlap between datasets was also encountered when the impact of the androgen- and EGF-pathways in prostate cancer progression was examined. Microarray analysis of patient material was compared with the microarray data on androgen- or EGF-treated LNCaP cells. Only 83 genes were represented in the prostate cancer dataset (Dhanasekaran et al., 2001) and also regulated by androgens and EGF. The number of genes regulated by androgens and EGF and specifically expressed in metastatic prostate cancer was only 39, and of these genes only 16 were regulated in the same direction. From this last experiment we learned that the number of genes was too small to draw any conclusions

Thus, comparing our data with published data has its limitations. Therefore, we generated new data on androgen- and EGF-induced genes in an androgen-dependent and an androgen-independent prostate cancer cell line (Chapter 5). Noteworthy, the two cell lines were related, the same person performed all the experiments at the same time, and gene regulation was analysed in 4 time-points, for each variable measured. These experiments and their results are described in Chapter 5 and will be discussed below.

6.2.2 *Gene regulation in androgen-dependent versus androgen-independent prostate cancer cells*

Transition from androgen-dependent to androgen-independent proliferation of prostate cancer cells is the focal point of this thesis. In Chapter 5, using the androgen-dependent LNCaP-FGC cell line and its androgen-independent derivate LNCaP-LNO, the following questions were raised:

- *What are the differences between androgen-dependent and androgen-independent cells in gene-expression and hormonal control of gene-regulation?*
- *Can expression analysis provide insight in how androgen-independent cells proliferate without addition of androgens or EGF?*

First, the expression level of ~17,000 genes in the non-hormone-stimulated situation was compared. Of ~1,300 genes the difference in expression between androgen-dependent and -independent cells was more then 2-fold. The reliability of these results was verified by statistical tests and by confirmation of expression of known differentially expressed genes. Functional annotation of these genes was determined and many genes involved in cell cycle control were found to be up-regulated in androgen-independent cells as compared to androgen-dependent cells. Furthermore, 20% of differentially expressed genes involved in proliferation were also androgen or EGF target genes. These results seem to indicate that androgen-independent cells have become independent because a portion of androgen- or EGF-regulated genes is constitutively activated. Upon inhibiting signalling at the level of the androgen receptor or the EGF receptor, it was observed that constitutive growth of the androgen-independent cells could not be impeded. This means that androgen-independent growth can be the result of mutations, deletions or amplifications of key genes acting downstream from the androgen or EGF receptors, or androgen-independent growth can be the result of autonomous activation of other signalling pathways.

Second, androgen- and EGF-regulation of gene expression as well as overlap between regulated genes in androgen-dependent and androgen-independent cells was evaluated. Crosstalk between androgen- and growth factor signalling pathways is hypothesised to be involved in prostate cancer progression (Balk, 2002; Shaffer and Scher, 2003). Overlap between androgen- and EGF-induced genes could give insight in the extent and nature of this crosstalk. Although, one should bear in mind that induction of gene expression is an endpoint of signalling and not necessarily is achieved via the same pathways. In androgen-independent cells a larger (75% versus 47%) overlap was found between androgen- and EGF-regulated genes than in androgen-dependent cells. More overlap could translate into less dependence on androgens for regulation of cellular processes like proliferation and maintenance. The genes that show overlap in androgen-independent cells and not in androgen-dependent cells are mainly involved in signal transduction and metabolism, indicating that for these processes, downstream of the androgen and EGF receptors, signalling is clearly intertwined.

6.3 Concluding remarks

In this thesis we aimed to discuss molecular and cellular mechanisms involved in androgen-independent prostate cancer growth.

In Chapters 2 and 3, it was observed that loss of expression of the endocytosis related gene REPS2 in prostate cancer cells enhances EGF signalling. Furthermore, research by others indicated that loss of REPS2 in prostate cancer results in an approximate 6-fold increased risk of relapse of the disease, indicating that REPS2 has the potential to become a valuable prognostic tool during advanced disease. It was also observed that expression of many other endocytosis related genes is changed in advanced prostate cancer. Therefore, altered endocytosis could stimulate growth factor signalling and may add to the hormone-independent nature of advanced prostate cancer.

In Chapters 4 and 5, other mechanisms putatively involved in androgen-independent prostate cancer growth were analysed. It is suggested that androgen-independent cells can proliferate in the absence of androgenic stimulation because: first, many cell cycle genes including a number of androgen- and EGF-regulated genes are constitutively activated; second, the overlap between androgen receptor signalling and EGF receptor signalling pathways is very high (75%); third, other unknown signal transduction pathways have been activated and induce proliferation.

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Summary

Summary

Prostate cancer has a high incidence in the western world. Early detection of the disease is crucial for successful management, since late stages of the disease cannot be cured. Unfortunately, however, conventional detection through rising PSA levels is already too late in one-third of the cases. These patients have metastatic disease, which can only be treated temporarily by androgen ablation therapy. The main problem of metastatic prostate cancer is a transition from initially androgen-dependent growth to androgen-independent growth. Androgen-independence of prostate cancer cells implies resistance to androgen ablation therapy, eventually leading to death of the patient.

Next to androgens, many other growth and differentiation inducing factors play a role during development and homeostasis of the prostate and during progression of prostate cancer. Peptide growth factors like EGF, TGF-α, FGF, IGF, NGF, PDGF, VEGF, and TGF-β have all been hypothesized to be involved in prostate cancer growth. The proposed mechanism of androgen-independent prostate cancer progression is through stimulation of proliferation via these factors as compensation for lack of proliferation stimulation through androgens. Furthermore, crosstalk between androgen signalling and growth factor signalling seems to play a role in prostate cancer growth. Growth factors are reported to activate androgen receptors and androgens to induce growth factor and growth factor receptor expression.

In this thesis we focussed mainly on EGF signalling. First, because REPS2, a protein potentially involved in androgen-independent prostate cancer, acts through affecting EGF signalling, and second, because gene profiling indicated that EGF and androgen signalling seem to intertwine in androgen-independent prostate cancer.

We postulated and addressed the following questions in this thesis:

- *Via which mechanisms does loss of expression of the endocytosis protein REPS2 contribute to an androgen-independent phenotype of prostate cancer cells?*
- *Is it possible to identify genes, activated by androgens or EGF, that induce proliferation and invasion of prostate cancer cells?*
- *Do EGF- and androgen-signalling overlap in prostate cancer cells and if so, to what extent?*
- *What are the differences between androgen-dependent and androgen-independent cells in gene-expression and hormonal control of gene-regulation?*
- *Can expression analysis provide insight in how androgen-independent cells proliferate without addition of androgens or EGF?*

In **Chapter 1**, an overview of prostate (cancer) development, androgen signalling, growth factor signalling, and growth factor receptor endocytosis is given, as well as a description of the microarray technique and analysis.

In **Chapters 2 and 3** the function of REPS2 was investigated. The endocytotic protein REPS2 was found in our laboratory as a protein encoded by a transcript that was down-regulated in androgen-independent as compared to androgen-dependent prostate cancer cells. Considering the functional domains this protein possesses, we tried to identify the function of REPS2 in relation to growth factor endocytosis and EGF

signalling during progression of prostate cancer. Therefore, REPS2 was transfected in prostate cancer cells. As described in **Chapter 2**, an increase in apoptosis (programmed cell death) was found when the protein was expressed at a high level. Furthermore, we measured an inhibition of transcriptional activity of a TRE-luciferase reporter, which indicates that REPS2 inhibits EGF-signalling. Since these studies were performed with transiently transfected cells, which could lead to artificial results, we analysed EGF receptor endocytosis and signalling in a stably transfected cell line with inducible REPS2 expression at a physiological level (described in **Chapter 3)**. Using this androgen-independent prostate cancer cell line we found inhibition of EGF endocytosis by increased REPS2 expression. Additionally, an increased level of REPS2 were shown to inhibit signalling via the EGF receptor. In conclusion we hypothesise that loss of REPS2 in androgen-independent prostate cancer cells contributes to an androgen-independent phenotype by loss of control of EGF-endocytosis and therefore loss of control of EGF-signal transduction.

EGF signalling and its possible involvement in prostate cancer progression was investigated further in **Chapters 4 and 5**. Here we describe microarray experiments performed with the prostate cancer cell line LNCaP. First, we investigated induction of proliferation and migration of prostate cancer cells by EGF and androgens. Additional effects of EGF and androgens on these cellular processes were found when both hormones were administrated together. Using microarray analysis, we tried to identify genes that would account for the effects. A comparison was made between EGF-induced genes and R1881-induced genes in LNCaP cells. The latter study, used for this comparison, was performed by Segawa et al., 2002. Little overlap between EGF-induced and R1881-induced genes was found for invasion-involved genes, but most genes involved in proliferation were regulated by both hormones. We concluded that EGF and R1881 partly use the same pathways to control proliferation.

Additionally, we compared these two datasets with data on gene expression in a series of prostate cancer samples, provided by Dhanasekaran et al., 2001. We found a large portion of R1881-regulated genes (also present in the prostate dataset) to be metastasis specific, indicating that in these androgen-independent tumour samples the androgen-signalling pathway is still active. For EGF-regulated genes this was less clear. A problem we encountered during these analyses was loss of data-points due to comparison of different microarray chips. Therefore we performed new microarray experiments, with androgen-dependent and androgen-independent LNCaP cells treated with EGF or R1881; these experiments and analyses are outlined in **Chapter 5**.

We focussed mainly on the differences between androgen-dependent and androgen-independent gene expression and regulation. A fairly large group of genes was found to be *expressed* differentially between the androgen-dependent and –independent cell lines in the non-hormone stimulated situation. Annotation of these differentially expressed genes showed that many were involved in cell cycle regulation, and some of these genes were found to be EGF- or androgen-target genes.

Next we analysed the overlap between EGF- and R1881-induced genes. It was observed that the overlap between EGF- and androgen-signalling was increased from 40% in androgen-dependent to 75% in androgen-independent LNCaP cells. Furthermore, upon analysing genes which were regulated by EGF or R1881, but differentially between androgen-dependent and androgen-independent cells, it was shown that in androgen-dependent cells many proliferation involved genes are up-

regulated by EGF or R1881, whereas in androgen-independent cells this is not the case. These results were confirmed by proliferation studies with EGF and R1881 in both cell lines.

Finally, we postulate, as a possible answer to the question how androgen-independent cells grow without additional EGF or androgen, that this may be effectuated because 1) proliferation-involved genes are stimulated, 2) proliferation-stimulating pathways are constitutively active, and 3) EGF-signalling largely overlaps with androgen-signalling; all of this reducing the need for androgens as the principal regulator of growth. The present findings are discussed in **Chapter 6**.

Samenvatting

Samenvatting

Prostaatkanker komt veel voor in de westerse wereld. Detectie in een vroeg stadium is cruciaal voor een succesvolle behandeling, omdat vergevorderde stadia ongeneeslijk zijn. Helaas is detectie door middel van stijgende PSA-waarden (prostaat specifiek antigeen) in éénderde van de gevallen al te laat. Deze patiënten hebben uitgezaaide prostaatkanker, wat alleen tijdelijk behandeld kan worden door androgeen-depletietherapie. Het belangrijkste probleem bij uitgezaaide prostaatkanker is dat er een overgang optreedt van androgeen-afhankelijkheid van de prostaatkanker- cellen naar androgeen-onafhankelijkheid. Androgeen-onafhankelijkheid van prostaatkanker-cellen betekent resistentie tegen androgeen-depletietherapie, wat uiteindelijk de dood van de patiënt tot gevolg heeft.

Naast androgenen spelen vele andere groei- en differentiatie-stimulerende stoffen een rol gedurende de ontwikkeling en homeostase van de prostaat en gedurende de ontwikkeling van prostaatkanker. Groeifactoren zoals EGF, TGF-α, FGF, IGF, NGF, PDGF, VEGF en TGF-β zijn allemaal mogelijk betrokken bij de groei van prostaatkanker. Een mogelijk mechanisme voor het ontstaan van androgeen-onafhankelijke prostaatkanker is stimulatie van groei door deze stoffen in plaats van androgenen. Verder is het mogelijk dat samenspraak tussen door groeifactoren gestimuleerde signaaltransductie en androgeen-signaaltransductie een rol speelt in prostaatkankergroei. Uit publicaties blijkt dat groeifactoren de androgeen-receptoren kunnen activeren, en dat androgenen de expressie van groeifactoren en van groeifactor-receptoren kunnen stimuleren.

In het onderzoek beschreven in dit proefschrift hebben we ons voornamelijk bezig gehouden met de EGF- signaaltransductie. Ten eerste, omdat REPS2, een eiwit dat mogelijk betrokken is bij androgeen-onafhankelijke prostaatkanker, EGF-signaaltransductie beïnvloedt, en ten tweede omdat uit genen-expressie profielen bleek dat EGF-signaaltransductie verweven lijkt te worden met aspecten van androgeen-signaaltransductie in androgeen-onafhankelijke prostaatkanker.

Op basis van het voorgaande komen in dit proefschrift de volgende vragen aan de orde:

- *Door welk mechanisme kan afname van REPS2-expressie bijdragen aan het androgeen-onafhankelijke fenotype van prostaatkanker-cellen?*
- *Is het mogelijk om door EGF of androgenen geactiveerde genen te identificeren die groei en invasie van prostaatkanker-cellen stimuleren?*
- *Overlappen EGF- en* androgeen-signaaltransductie *in prostaatkanker-cellen en zo ja, in hoeverre?*
- *Zijn genexpressie en genregulatie anders in androgeen-afhankelijke dan in androgeen-onafhankelijke cellen?*
- *Kan de analyse van expressiepatronen inzicht geven in hoe androgeen-onafhankelijke cellen kunnen groeien zonder toevoeging van EGF en androgenen?*

In **Hoofdstuk 1** is naast een overzicht van prostaat(kanker)-ontwikkeling, androgeen-signaaltransductie, groeifactor-signaaltransductie, en groeifactor-receptor endocytose, een beschrijving gegeven van de microarray-techniek en -analyse.

De **Hoofdstukken 2 en 3** beschrijven het onderzoek naar de functie van REPS2. Het endocytose-eiwit REPS2 is gevonden in ons laboratorium op basis van een transcript dat lager tot expressie komt in androgeen-onafhankelijke cellen vergeleken met androgeen-afhankelijke cellen. Gelet op de functionele domeinen die dit eiwit heeft, hebben we geprobeerd de functie van REPS2 te achterhalen in relatie tot groeifactor-endocytose en EGF-signaaltransductie gedurende de progressie van prostaatkanker. REPS2 werd daarom artificieel tot expressie gebracht in prostaatkanker-cellen door transiënte transfectie. Zoals beschreven in H**oofdstuk 2**, werd een toename in apoptose (geprogrammeerde cel-dood) gevonden als het eiwit in hoge concentraties tot expressie werd gebracht. Verder werd een remmend effect gevonden op de transcriptionele activiteit van een TRE-reporter, wat aangeeft dat REPS2 de EGF-signaaltransductie remt. Omdat deze studies uitgevoerd werden met transiënte getransfecteerde cellen, wat mogelijk tot artificiële resultaten kan leiden, analyseerden we EGF-receptorendocytose en signaaltransductie met een stabiele getransfecteerde cellijn met induceerbare REPS2–expressie in fysiologische concentratie (beschreven in **Hoofdstuk 3**). Gebruik makend van deze androgeen-onafhankelijke prostaatkanker cellijn vonden we een remming van EGF-endocytose door een verhoogde REPS2-expressie. Bovendien werd aangetoond dat verhoogde concentratie van REPS2, via de EGF-receptor, signaaltransductie remt. De resultaten leiden tot de hypothese dat afname van REPS2-expressie in androgeen-onafhankelijke prostaatkanker-cellen bijdraagt aan de ontwikkeling van een androgeen-onafhankelijk fenotype door verlies van controle op EGF-endocytose en daardoor op EGF-signaaltransductie.

Het onderzoek naar EGF-signaaltransductie en de mogelijke betrokkenheid ervan in prostaatkanker progressie wordt verder uiteengezet in de **Hoofdstukken 4 en 5**. Hierin staan de microarray-experimenten beschreven die uitgevoerd zijn met de prostaatkanker-cellijn LNCaP. Ten eerste is in prostaatkanker-cellen de inductie van groei en invasie door EGF en androgenen onderzocht. Als beide hormonen tegelijkertijd werden toegevoegd, worden additionele effecten van EGF en androgenen gevonden op deze cellulaire processen. Met behulp van microarray-analyse werd geprobeerd om genen te vinden die deze effecten kunnen veroorzaken. Genen die worden geïnduceerd in LNCaP cellen door EGF en androgenen werden met elkaar vergeleken. Voor de androgeen-geïnduceerde genen werd een studie gebruikt die was uitgevoerd door Segawa et al. (2002). Er werd weinig overlap gevonden tussen EGF-geïnduceerde en androgeen-geïnduceerde genen die betrokken zijn bij invasie, maar voor genen die betrokken zijn bij proliferatie is de overlap groot. We concludeerden daaruit dat EGF en androgenen gedeeltelijk dezelfde signaaltransductie-routes gebruiken om proliferatie te stimuleren.

Daarnaast zijn deze twee datasets vergeleken met genexpressie data van een serie prostaatkanker-monsters, geplubiceerd door Dhanasekaran et al. (2001). Een groot deel van de androgeen-gereguleerde genen (die ook voorkwamen in de prostaat-dataset) was metastase-specifiek, wat suggereert dat in deze androgeen-onafhankelijke tumormonsters de androgeen-signaaltransductieroute nog wel actief is. Deze correlatie is minder duidelijk voor EGF-gereguleerde genen. Tijdens deze analyses stuitten we op het probleem, dat in de vergelijking van verschillende microarray experimenten data verloren gaat omdat verschillende microarray-chips zijn gebruikt. Daarom hebben we nieuwe microarray-experimenten uitgevoerd met

androgeen-afhankelijke en androgeen-onafhankelijke prostaatkanker-cellen, behandeld met EGF of androgenen. Deze experimenten en analyses staan beschreven in **Hoofdstuk 5**.

In dat hoofdstuk focussen we voornamelijk op de verschillen tussen androgeen-afhankelijke en androgeen-onafhankelijke genregulatie en genexpressie. Een redelijk grote groep genen komt anders tot expressie in androgeen-afhankelijke dan in androgeen-onafhankelijke prostaatkanker cellijnen in ongestimuleerde omstandigheden. Veel van deze genen zijn betrokken bij regulatie van de celcyclus en een aantal hiervan blijkt EGF- of androgeen-targetgen te zijn.

Vervolgens werd de overlap tussen EGF- of androgeen-gereguleerde genen geanalyseerd. De overlap tussen EGF- en androgeen-signaaltransductie blijkt te zijn toegenomen van 40% in androgeen-afhankelijke naar 75% in androgeen-onafhankelijke prostaatkanker-cellen. Verder blijkt dat veel genen betrokken bij proliferatie opgereguleerd worden door EGF en androgenen in androgeen-afhankelijke, maar niet in androgeen-onafhankelijke cellen. Proliferatie-studies met EGF en androgenen in beide cellijnen bevestigden deze resultaten.

Tenslotte komen we tot het volgende mogelijke antwoord op de vraag hoe androgeen-onafhankelijke cellen kunnen groeien zonder extra toegevoegde EGF of androgenen: de noodzaak voor aanwezigheid van androgenen als voornaamste groei-stimulatoren wordt gereduceerd, omdat

1) genen betrokken bij proliferatie al geactiveerd zijn
2) signaaltransductie-routes constitutief geactiveerd zijn
3) EGF-signaaltransductie voor een groot deel overlapt met androgeen-signaaltransductie.

Alle resultaten worden bediscussieerd in **Hoofdstuk 6**.

Dankwoord

Dankwoord

De afgelopen 5 (!) jaar zaten vol ups en downs. Mede door mijn geweldige collega's, vrienden en familie heb ik mijn promotie tot een goed einde gebracht. Ik wil graag van de gelegenheid gebruik maken om een paar hier speciaal in het zonnetje te zetten.

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Michiel, ik kan niet zonder jou

List of publications

Josien K. Oosterhoff, Fred Penninkhof, Albert O. Brinkmann, J. Anton Grootegoed, Leen J.Blok.
REPS2/POB1 is downregulated during human prostate cancer progression and inhibits growth factor signalling in prostate cancer cells.
Oncogene. 2003 May 15;22(19):2920-5.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12771942.

Josien K. Oosterhoff, Liesbeth C. Kühne, J. Anton Grootegoed, Leen J.Blok
EGF signalling in prostate cancer cell lines is inhibited by a high expression level of the endocytosis protein REPS2.
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Josien K. Oosterhoff, Liesbeth C. Kühne, J. Anton Grootegoed, Leen J. Blok.
Cluster-analysis of androgen- and EGF-regulated genes in relation to proliferation and invasion of prostate cancer cells.
Submitted for publication.

Josien K. Oosterhoff, J. Anton Grootegoed, Leen J. Blok.
Expression profiling of androgen-dependent and –independent LNCaP cells; EGF versus androgen signalling.
Submitted for publication.

Curriculum vitae

Josien Oosterhoff werd geboren op 27 januari 1975 in Leeuwarden. In 1993 haalde ze haar VWO diploma aan de Gereformeerde Scholengemeenschap Amersfoort. Vanaf 1993 tot 1998 studeerde ze Biologie in Wageningen. Ze specialiseerde zich in de moleculaire biologie door achtereenvolgens onderzoek te doen naar de functie van het ORF699 van het *Autographa californica* nuclear polyhedrosis virus o.l.v. Prof.dr. R. Goldbach (Virolgie, WUR, Wageningen), naar optimale kweekcondities voor karper-bloedcellen o.l.v. Prof.dr. W. van Muiswinkel (Celbiologie en Immunologie, WUR, Wageningen) en naar de rol van IL-4 en IL-5 bij astma o.l.v. Prof. W. Sewell (St. Vincents Hospital, Sydney, Australië). In september 1999 begon ze aan het in dit proefschrift beschreven onderzoek naar de mechanismen van progressie van androgeen-onafhankelijke prostaatkanker o.l.v. Prof.dr. J.A. Grootegoed en Dr.ir. L.J. Blok.

Abbreviations

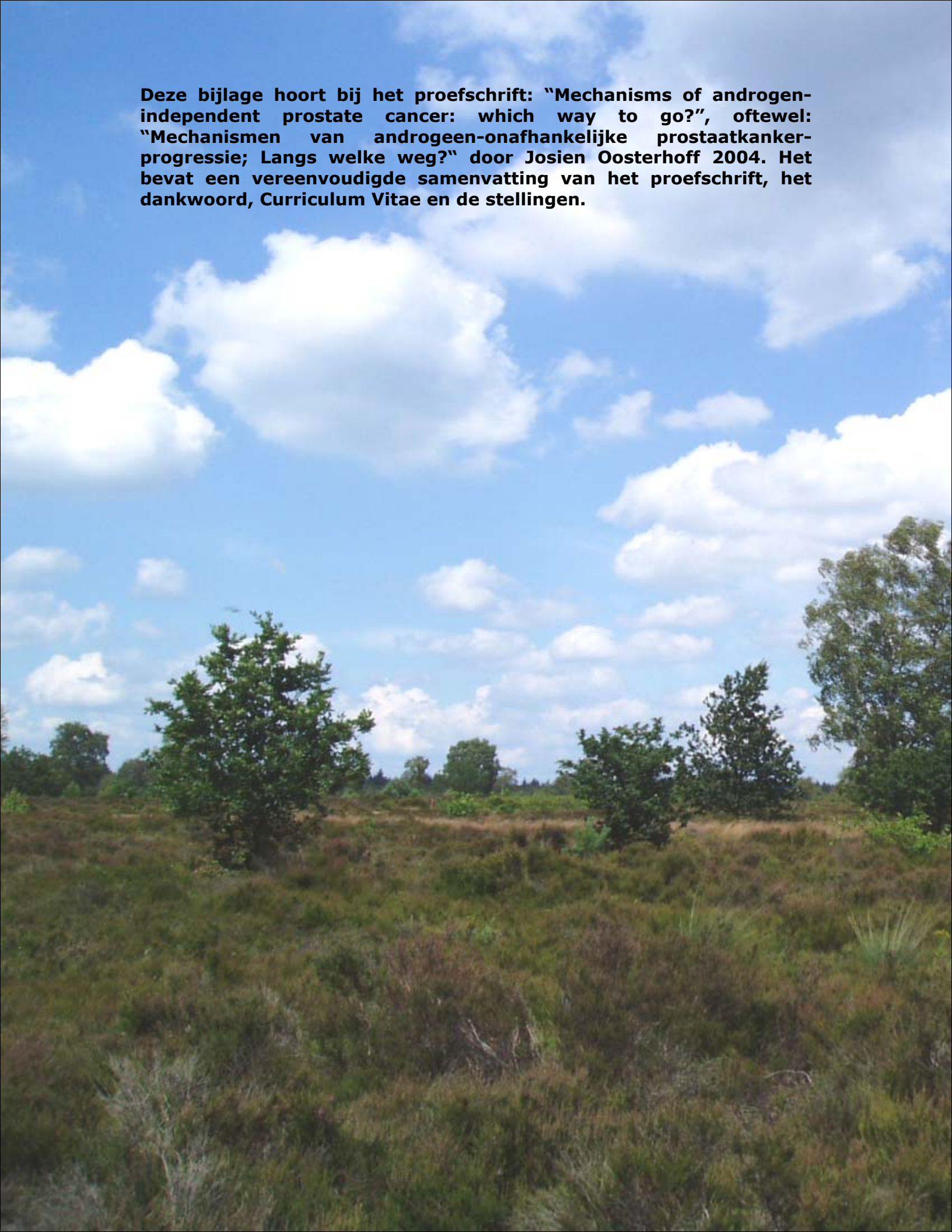
ACK2	Activated CDC42-associated tyrosine kinase 2
AP-2	Adaptor-related protein complex 2
AR	Androgen receptor
BSA	Bovine serum albumin
CALM	Calmodulin 1
CBL	Cas-Br-M (murine) ecotropic retroviral transforming sequence
CDC2	Cell division cycle 2
CDC42	Cell division cycle 42
CWR22	Case Western Reserve22
DCC	Dextran-coated charcoal treated
DNA	Deoxyribonucleic acid
e.g.	For example (*exempli gratia*)
EGF	Epidermal growth factor
EGR-1	Early growth response 1
EH	EPS homology
EPN1	Epsin 1
EPS	Epidermal growth factor receptor pathway substrate
ERK	extracellular (signal-) regulated kinase
FCS	Foetal calf serum
FGC	Fast growing colony
FGF	Fibroblast growth factor
FOS	v-Fos FBJ murine steosarcoma viral oncogene homolog
GAP	GTPase-activating protein
GEF	Guanine exchange factor
GFP	Green fluorescent protein
GRB2	Growth factor receptor-bound protein 2
GTP	Guanine triphosphate
HGF/SF	Hepatocyte growth factor/scatter factor
HGS	Hepatocyte growth factor-regulated tyrosine kinase substrate
IGF	Insulin-like growth factor
IGFBP2	Insulin-like growth factor binding protein 2
JUN	v-Jun sarcoma virus 17 oncogene homolog
kDa	Kilo Dalton
LNCaP	Lymph node carcinoma of human prostate
LNO	Lymph node original
MAPK	Mitogen-activated protein kinase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PC3	Prostatic carcinoma 3
PI3Kinase	Phosphoinositide-3-kinase
POB1	Partner of RALBP1
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
RAC1	Ras-related C3 botulinum toxin substrate 1
RALA	v-Ral simian leukemia viral oncogene homolog A (Ras related)
RALBP1	RALA binding protein 1
REPS2	RALBP1 associated EPS domain containing 2
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute 1640
RXR	Retinoid X receptor
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SH3	SRC homology 3
SHC	SRC homology 2 domain containing
TGF-α	Transforming growth factor-α
TMEFF2	Transmembrane protein with EGF-like and two follistatin-like domains
TRE	TPA responsive element
TRPS1	Trichorhinophalangeal syndrome I

WHICH WAY TO GO?

Josien Oosterhoff

Mechanisms of androgen-independent prostate cancer progression

Deze bijlage hoort bij het proefschrift: "Mechanisms of androgen-independent prostate cancer: which way to go?", oftewel: "Mechanismen van androgeen-onafhankelijke prostaatkanker-progressie; Langs welke weg?" door Josien Oosterhoff 2004. Het bevat een vereenvoudigde samenvatting van het proefschrift, het dankwoord, Curriculum Vitae en de stellingen.



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Mechanisms of androgen-independent Prostate Cancer Progression

WHICH WAY TO GO?

Mechanismen van progressie van androgeen-onafhankelijke prostaatkanker

LANGS WELKE WEG?

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof.dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
donderdag 21 oktober 2004 om 16.00 uur

door

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geboren te Leeuwarden

Samenvatting

Inleiding

Prostaatkanker komt veel voor in de westerse wereld. Het is cruciaal voor een succesvolle behandeling dat de kanker in een vroeg stadium wordt gedetecteerd, omdat vergevorderde prostaatkanker ongeneeselijk is. Helaas wordt bij éénderde van de gevallen de ziekte te laat ontdekt en is er sprake van uitgezaaide prostaatkanker. Deze patiënten worden dan, naast dat hun prostaat verwijderd wordt, behandeld met een zogenaamde androgeen-depletietherapie. Deze therapie is erop gebaseerd dat de prostaatcellen en ook prostaatkanker-cellen androgenen (mannelijke hormonen, zoals testosteron) nodig hebben om te overleven. Als de androgenen dus kunstmatig laag worden gehouden door androgeen-depletietherapie gaan die cellen dood. Helaas kunnen prostaatkanker-cellen na een bepaalde periode zonder androgenen ook overleven en worden ze resistent tegen de androgeen-depletietherapie, wat uiteindelijk de dood van de patiënt tot gevolg heeft. Deze omslag van androgeen-afhankelijkheid naar androgeen-onafhankelijkheid is het hoofdonderwerp van mijn onderzoek.

Naast androgenen zijn er ook nog vele andere stoffen die een rol spelen tijdens het normale functioneren van de prostaat en het ontstaan van prostaatkanker. Een groep van deze stoffen zijn de groeifactoren. Van deze stoffen wordt verondersteld dat ze de groei van androgeen-onafhankelijke prostaatkankercellen stimuleren als vervanging van de androgenen. Het zou ook kunnen dat deze stoffen niet als vervanging van androgenen gaan werken, maar meer gaan samenwerken met androgenen om zo het tekort aan androgenen te compenseren.

In dit proefschrift wordt voornamelijk het onderzoek beschreven naar de invloed van EGF (epidermale groeifactor) op de progressie van prostaatkanker. Ten eerste omdat REPS2, een eiwit wat mogelijk betrokken is bij androgeen-onafhankelijke prostaatkanker, invloed heeft op de EGF-werking. Ten tweede omdat uit experimenten bleek dat androgenen en EGF meer lijken te gaan samenwerken in androgeen-onafhankelijke prostaatkanker, vergeleken met androgeen-afhankelijke prostaatkanker.

Het onderzoek

Op basis van het voorgaande werd aan de hand van de volgende vragen het onderzoek opgebouwd:

1. Door welk mechanisme kan afname van REPS2-expressie[1] bijdragen aan de androgeen-onafhankelijkheid van prostaatkankercellen?
2. Is het mogelijk om genen te identificeren die door EGF of androgenen groei en invasie[2] van prostaatkankercellen stimuleren?
3. Werken EGF en androgenen samen en zo ja, in hoeverre?
4. Verschillen androgeen-afhankeljke en androgeen-onafhankelijke cellen van elkaar op genetisch niveau?

5. Kan de analyse van gen-expressiepatronen inzicht geven in hoe androgeen-onafhankelijke cellen kunnen groeien zonder EGF en androgenen?

In **Hoofdstuk 1** is een overzicht gegeven van de prostaat(kanker)-ontwikkeling, het werkingsmechanisme van androgenen en groeifactoren en een beschrijving van de microarray-techniek en –analyse. Deze techniek wordt vooral gebruikt voor het onderzoek beschreven in de hoofstukken 4 en 5. Simpel gezegd is het doel ervan om in één experiment het totale expressiepatroon, dus de activiteit van alle genen, van bijvoorbeeld androgeen-afhankelijke cellen te bepalen.

De **Hoofdstukken 2 en 3** beschrijven het onderzoek naar de functie van REPS2. In ons laboratorium is het eiwit REPS2 gevonden als een eiwit dat lager tot expressie komt in androgeen-onafhankelijke cellen vergeleken met androgeen-afhankelijke cellen. Gelet op de functionele domeinen die dit eiwit heeft, hebben we geprobeerd de functie van REPS2 te achterhalen in relatie tot groeifactor-internalisatie[3] en het werkingsmechanisme van EGF gedurende de progressie van prostaatkanker. Met behulp van moleculaire technieken werd REPS2 artificieel tot expressie gebracht in prostaatkanker-cellen. Zoals beschreven in **Hoofdstuk 2** werd een toename in apoptose (geprogrameerde cel-dood) gevonden in cellen die veel REPS2 tot expressie brachten. Verder werd een remmend effect gevonden op het EGF werkingsmechanisme. Omdat de technieken die we voor deze experimenten gebruikten tot artificiële resultaten kunnen leiden hebben we vervolgens cellen gemaakt waarin we REPS2 gecontroleerd tot expressie kunnen brengen (beschreven in **Hoofdstuk 3**). Met behulp van deze cellen konden we een remming van EGF-internalisatie aantonen als we de concentratie van REP2 verhoogden. Bovendien werd weer een remmend effect gevonden op het EGF werkingsmechanisme. De resultaten leiden tot de hypothese dat afname van REPS2-expressie tijdens de ontwikkeling van androgeen-onafhankelijke prostaatkanker bijdraagt aan het androgeen-onafhankelijke karakter, door verlies van controle op EGF-internalisatie en daardoor op het EGF werkingsmechanisme.

Het onderzoek naar het EGF werkingsmechanisme en de mogelijke betrokkenheid ervan in prostaatkanker-progressie wordt verder uiteengezet in de **Hoofdstukken 4 en 5**. Hierin staan de microarray-experimenten beschreven die uitgevoerd zijn met de prostaatkankercellen. Ten eerste is de stimulatie van groei en invasie door androgenen en EGF onderzocht. Met behulp van microarray-analyse werd geprobeerd om genen te vinden die deze effecten kunnen veroorzaken. Genen die worden geactiveerd door EGF of door androgenen werden met elkaar vergeleken. Voor de androgeen-geactiveerde genen werd een studie uitgevoerd door Segawa et al., 2002 gebruikt. Er werd niet veel samenwerking gevonden tussen EGF en androgenen wat betreft stimulatie van invasie, maar voor groei werd wel veel samenwerking gevonden.
Daarnaast hebben we de EGF en de androgeen dataset vergeleken met genexpressie data van een serie prostaatkanker-monsters, gepubliceerd

door Dhanasekaran et al., 2001. Een groot deel van de androgeen-gereguleerde genen (die ook voorkwamen in de prostaatkankerset) was specifiek voor monsters van uitgezaaide tumoren, wat suggereert dat in deze androgeen-onafhankelijke tumoren het androgeen werkingsmechanisme nog wel actief is. Deze correlatie is minder duidelijk voor EGF-gereguleerde genen. Tijdens deze analyses stuiten we op het probleem dat door verschillende microarray-experimenten met elkaar te vergelijken data verloren gaat omdat er verschillende microarrays zijn gebruikt met verschillende genen erop. Daarom hebben we nieuwe microarray-experimenten uitgevoerd met zowel androgeen-afhankelijke als androgeen-onafhankelijke cellen, behandeld met EGF of androgenen. Deze experimenten en analyses zijn beschreven in **Hoofdstuk 5**.

In dat hoofdstuk focussen we voornamelijk op de verschillen tussen androgeen-afhankelijke en androgeen-onafhankelijke genregulatie en genexpressie. Een redelijk grote groep genen komt anders tot expressie in androgeen-afhankelijke dan in androgeen-onafhankelijke prostaatkanker cellijnen in ongestimuleerde omstandigheden. Veel van deze genen zijn betrokken bij regulatie van de celcyclus en een aantal hiervan blijkt EGF- of androgeen-targetgen te zijn.

Vervolgens werd de overlap tussen EGF- of androgeen-gereguleerde genen geanalyseerd. De overlap tussen EGF- en androgeen-signaaltransductie blijkt te zijn toegenomen van 40% in androgeen-afhankelijke naar 75% in androgeen-onafhankelijke prostaatkanker-cellen. Verder blijky dat veel genen betrokken bij proliferatie opgereguleerd worden door EGF en androgenen in androgeen-afhankelijke, maar niet in androgeen-onafhankelijke cellen. Proliferatie-studies met EGF en androgenen in beide cellijnen bevestigden deze resultaten.

Tenslotte komen we tot het volgende mogelijke antwoord op de vraag hoe androgeen-onafhankelijke cellen kunnen groeien zonder extra toegevoegde EGF of androgenen: de noodzaak voor aanwezigheid van androgenen als voornaamste groei-stimulatoren wordt gereduceerd, omdat 1) genen betrokken bij proliferatie al gestimuleerd worden, 2) signaaltransductie-routes constitutief geactiveerd zijn en 3) omdat EGF-signaaltransductie voor een groot deel overlapt met androgeen-signaaltransductie. Alle resultaten worden bediscussieerd in **Hoofdstuk 6.**

Voetnoten

[1] Gen-expressie houdt in dat een gen (een stukje DNA dat informatie bevat voor de vorming van een specifiek eiwit) geactiveerd wordt, en dat het bewuste eiwit gemaakt wordt.

[2] Invasie betekend dat een cel zijn oorspronkelijke positie verlaat en zich beweegt naar een andere plaats en zich daar vastzet.

[3] Groeifactor-internalisatie is het proces waarbij door vervormingen van de celwand groeifactoren van buiten de cel naar binnen de cel worden getransporteerd.

Dankwoord

De afgelopen 5 (!) jaar zaten vol ups en downs. Mede door mijn geweldige collega's, vrienden en familie heb ik mijn promotie tot een goed einde gebracht. Ik wil graag van de gelegenheid gebruik maken om een paar hier speciaal in het zonnetje te zetten.

Ten eerste mijn promotor en copromotor Anton Grootegoed en Leen Blok.
Anton, hartelijk bedankt voor het kritisch doorlezen van al mijn schrijfwerk door de jaren heen, zonder jouw commentaar was de inhoud van dit proefschrift veel minder leesbaar geworden.
Leen, je was en bent geweldig! Een betere begeleider kan een AIO zich niet wensen. Je enthousiasme, peptalks, lees-, correctie- en denkwerk waren onmisbaar. De mogelijkheid om op elk moment van de dag binnen te komen walsen met een al dan niet blijde boodschap vond ik erg bijzonder. En niet te vergeten je beroemde RNA isolaties, al blijven we twisten over de handigheid van je methode, het levert de mooiste resultaten op. Dank je wel!

De leden van leescommissie: Prof.dr. Jan Trapman, Dr. Ruud Delwel en Dr. Paul van Bergen en Henegouwen, bedankt voor het kritische commentaar op dit proefschrift. Paul, jouw wil ik speciaal nog bedanken voor je praktische hulp bij het endocytose-onderzoek.

Dan natuurlijk mijn collega's van (tegenwoordig) V&O heel erg bedankt voor alle gezelligheid tijdens lunches, koffiepauzes, taarteetfestijnen, borrels en (in een grijs verleden) peukpauzes. En niet te vergeten de "oude" 5e, het voorgaande geldt ook voor julllie! Voor mij blijft het vroegere E&V toch altijd een beetje mijn echte werkplek. Met een stel van jullie heb ik na het werk vaak genoten van etentjes, film etc.. Leve de VPRO-dag! En dan natuurlijk het meidenclubje: Mila, Arzu, Eline, Evelyne, Susanne, Liesbeth, Jenny en Marja (in willekeurige volgorde en hopelijk zonder iemand te vergeten), de bijzonder gezellige eetavondjes waren altijd weer een succes en een heerlijke gelegenheid om lekker te kankeren en te relativeren. Als dames waren we zowiezo superieur, toch Susanne en Eline? Hoezee voor "onze" kamer!
Maar ook door praktische tips en hulp in en rond het lab hebben ik veel aan jullie gehad, bedankt. In dit kader wil ik graag speciaal Liesbeth bedanken voor haar hulp bij het het vaak moeizame REPS2-onderzoek, heel erg veel dank!
De mensen van het JNI die deelnamen aan het prostaatcluster, bedankt voor de nuttige discussies en besprekingen, het was prettig om zo nu en dan vanuit andere invalshoeken het

prostaatkankerprobleem te bekijken. Met name wil ik Guido noemen, bedankt voor je enthousiaste bijdrages.
Ook wil ik graag Piet van Vliet noemen, je hebt als student hard gewerkt aan het REPS2-verhaal, ondanks dat je resultaten niet in het proefschrift terecht zijn gekomen, bedankt voor je inzet.

Een speciaal plekje voor Marieke. Als huisgenoot, nee eerder al, als studiegenoot en down-under, stonden onze neuzen dezelfde kant op. Dankzij jou ben ik überhaupt aan dit avontuur begonnen en ook dankzij jou ben ik er tot het eind mee doorgegaan, wat jij kan kan ik ook! Sinds afgelopen jaar zitten we wat verder van elkaar af, maar ondanks dat blijf je belangrijk voor me. Bedankt voor je support en je handige lijstje.

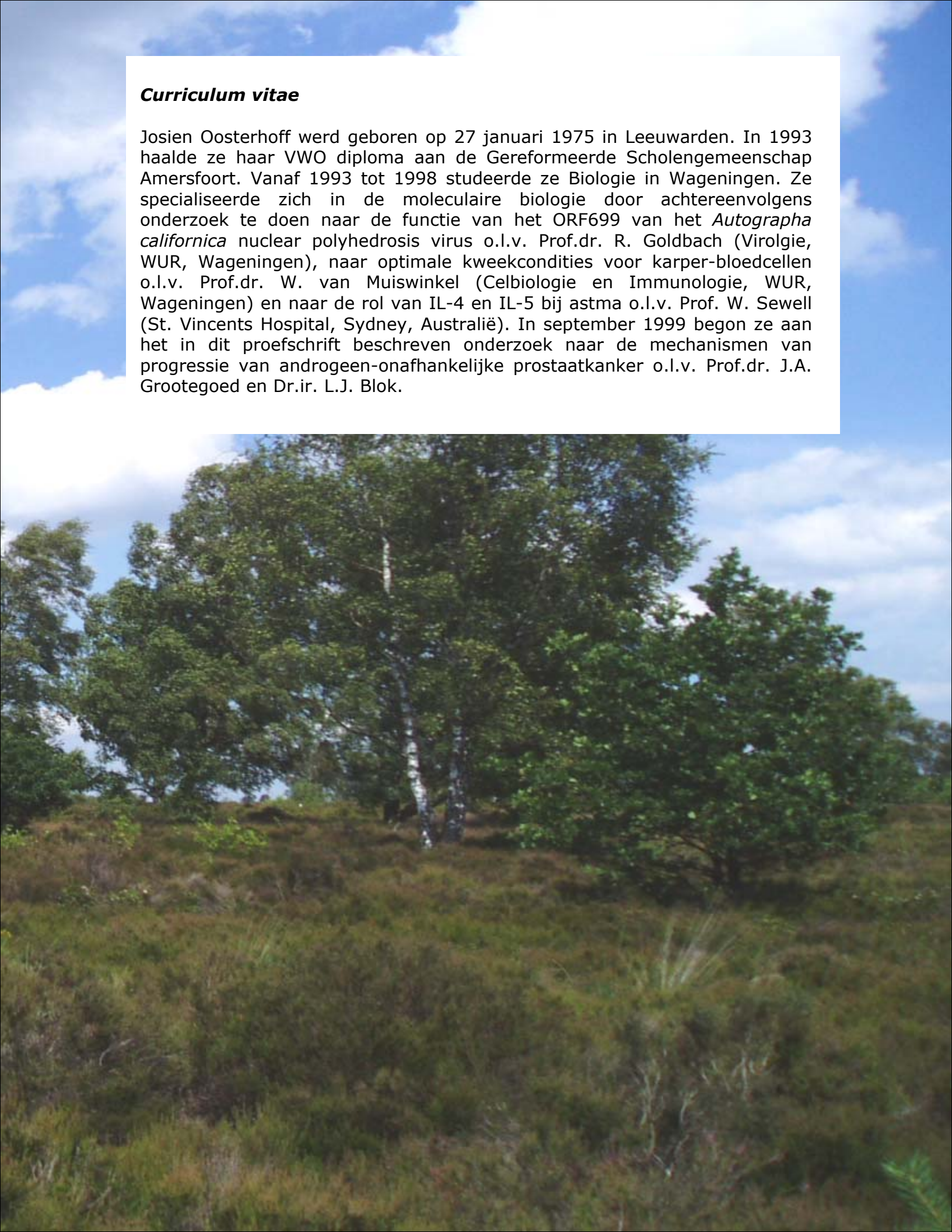
Mijn paranimfen Anne en Anneke, heel erg bedankt dat jullie die speciale rol willen vervullen. Ik vind het super dat er zulke geweldige Wageningse meiden in Rotterdam wonen! Laten we blijven borrelen, dansen en ouwehoeren tot we bejaard zijn!

Lieve vrienden en vriendinnen, heel erg bedankt dat jullie er zijn en waren om me steeds weer op tijd te herinneren aan wat echt belangrijk is in het leven. Bier en feestjes! (geintje).

En dan mijn familie voor jullie talent om me al dan niet op vriendelijke wijze te stimuleren door te gaan en niet op de laatste plaats voor jullie gebeden, dank jullie wel. Ik hoop dat jullie een beetje trots op me zijn.
Mijn tweede familie (sinds 19 mei 2004), dank jullie wel voor de interesse in mijn onderzoek al was het misschien niet altijd even makkelijk te volgen of interessant.

Lief, heel veel dank voor je eeuwig nodige steun, je gebed en je vertrouwen in mij. Ook je wil om te begrijpen waar ik mee bezig was en je hulp met het maken van een goed draaiende cd-rom waren onmisbaar.

Michiel, ik kan niet zonder jou



Curriculum vitae

Josien Oosterhoff werd geboren op 27 januari 1975 in Leeuwarden. In 1993 haalde ze haar VWO diploma aan de Gereformeerde Scholengemeenschap Amersfoort. Vanaf 1993 tot 1998 studeerde ze Biologie in Wageningen. Ze specialiseerde zich in de moleculaire biologie door achtereenvolgens onderzoek te doen naar de functie van het ORF699 van het *Autographa californica* nuclear polyhedrosis virus o.l.v. Prof.dr. R. Goldbach (Virolgie, WUR, Wageningen), naar optimale kweekcondities voor karper-bloedcellen o.l.v. Prof.dr. W. van Muiswinkel (Celbiologie en Immunologie, WUR, Wageningen) en naar de rol van IL-4 en IL-5 bij astma o.l.v. Prof. W. Sewell (St. Vincents Hospital, Sydney, Australië). In september 1999 begon ze aan het in dit proefschrift beschreven onderzoek naar de mechanismen van progressie van androgeen-onafhankelijke prostaatkanker o.l.v. Prof.dr. J.A. Grootegoed en Dr.ir. L.J. Blok.

Stellingen

1. Activatie van EGF-signaaltransductie en verhoogde expressie van REPS2 zijn tegengesteld gecorreleerd.
   Dit proefschrift

2. Het expressieniveau van REPS2 heeft voorspellende waarde voor het verloop van prostaatkanker.
   S.M. Henshall et al., 2003; Dit proefschrift

3. Gebruik van standaard DNA-microarrays is aan te bevelen als men datasets van verschillende genomics-onderzoekers goed wil vergelijken.
   Dit proefschrift

4. Het verschil tussen androgeen-afhankelijke en -onafhankelijke prostaatkanker-cellen is voornamelijk de lagere expressie niveaus van EGF- en androgeen-target genen.
   Dit proefschrift

5. Microarray-analyse is gericht op de expressie van een groot aantal genen en de keuze voor één gen voor vervolgonderzoek is daarom niet logisch.

6. Terwijl men over het algemeen niet in een hokje gestopt wil worden, is dat vaak wel een wens van patiënten en artsen en tevens een belangrijk doel van het genomics onderzoek.

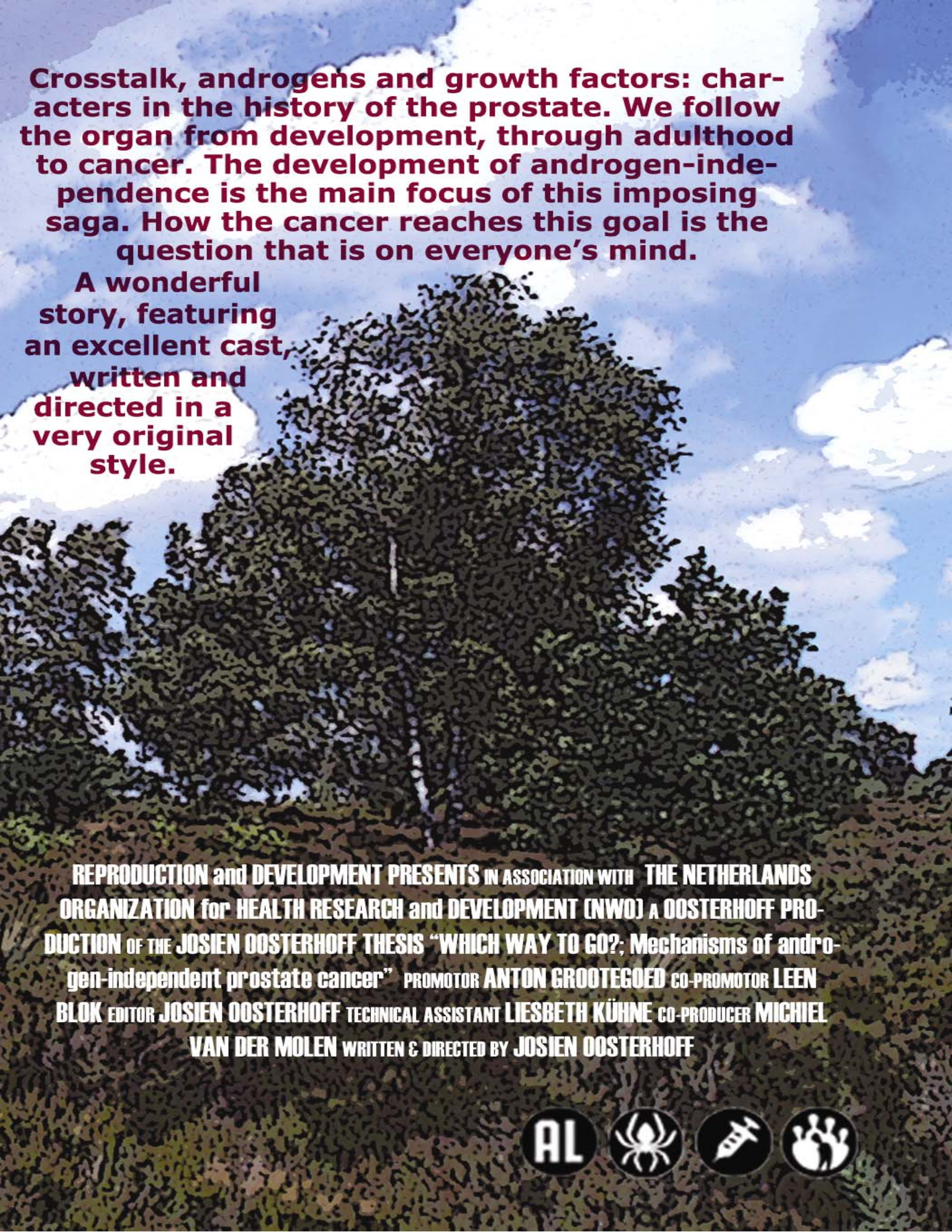
7. De prostaat is alleen door een lage androgeen-concentratie tijdens de ontwikkeling van de vrouwelijke foetus een strikt mannelijk orgaan.
   C.J. Wolf et al., 2001

8. Groeiremming door androgenen in androgeen-onafhankelijke cellijnen is geen fysiologische respons.
   J.E. Fowler & W.F. Whitmore, 1982

9. Hoe verder signaaltransductie-onderzoek vordert, hoe moeilijker signaaltransductie-routes zijn te volgen.

10. "Drukken" van een proefschrift op cd-rom verlaagt de kosten maar niet het aantal geïnteresseerde lezers.

11. Hoe dichter je bij je werk woont, hoe makkelijker je er afstand van kan nemen.


Crosstalk, androgens and growth factors: characters in the history of the prostate. We follow the organ from development, through adulthood to cancer. The development of androgen-independence is the main focus of this imposing saga. How the cancer reaches this goal is the question that is on everyone's mind.
A wonderful story, featuring an excellent cast, written and directed in a very original style.
REPRODUCTION and DEVELOPMENT PRESENTS IN ASSOCIATION WITH THE NETHERLANDS ORGANIZATION for HEALTH RESEARCH and DEVELOPMENT (NWO) A OOSTERHOFF PRODUCTION OF THE JOSIEN OOSTERHOFF THESIS "WHICH WAY TO GO?; Mechanisms of androgen-independent prostate cancer" PROMOTOR ANTON GROOTEGOED CO-PROMOTOR LEEN BLOK EDITOR JOSIEN OOSTERHOFF TECHNICAL ASSISTANT LIESBETH KÜHNE CO-PRODUCER MICHIEL VAN DER MOLEN WRITTEN & DIRECTED BY JOSIEN OOSTERHOFF