Cancer related gene expression in the human prostate zones

Genexpressie van kanker gerelateerde genen in de humane prostaatzones

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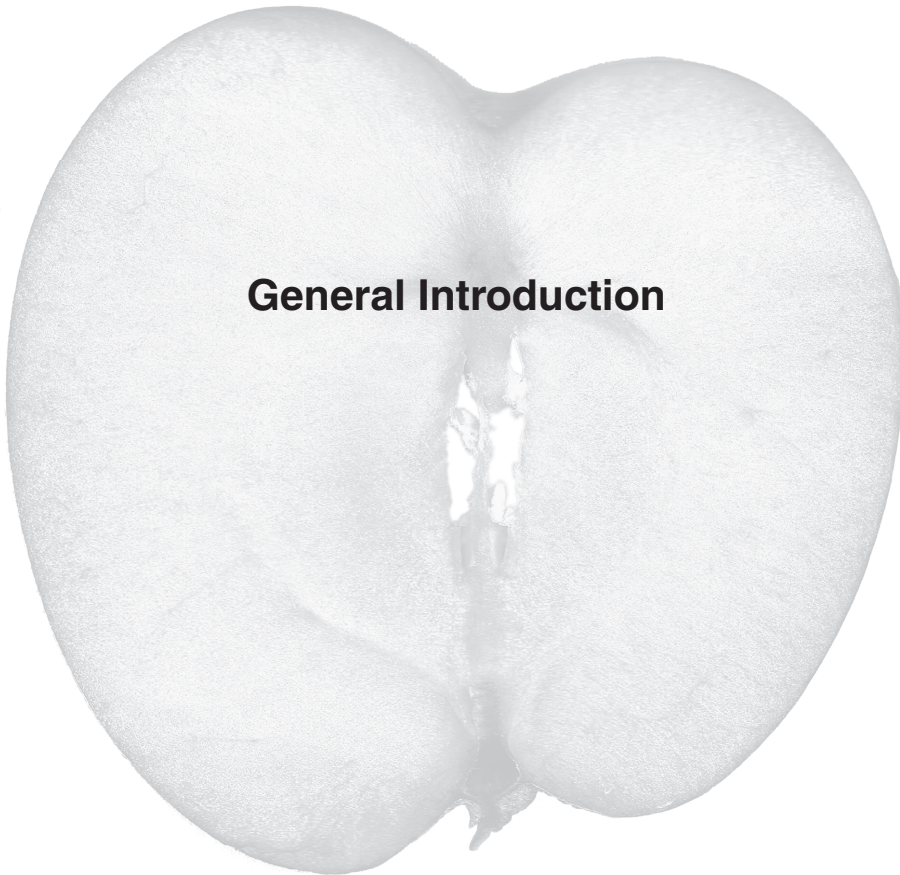

General Introduction

The normal prostate

The prostate is the largest accessory gland of the male reproductive system. (Figure 1) The healthy adult prostate is about the size of a chestnut and conical in shape. In general, it measures 20 ml in volume, though it can become five or six time that size with increasing age. The prostate is shaped like an inverted pyramid and lies between the bladder and the pelvic floor (1). The prostate supplies about 30% of the volume of the seminal fluid. The normal prostate is composed of epithelial glands and stroma. These glands represent the terminal tubular portion of long tubulo-alveolar glands that radiate from the urethra. The glands are lined by two cell layers: an outer low cuboidal layer and an inner layer of tall columnar mucin-secreting epithelium. Half of the volume of the prostate is occupied by the fibromuscular stroma between the glands.

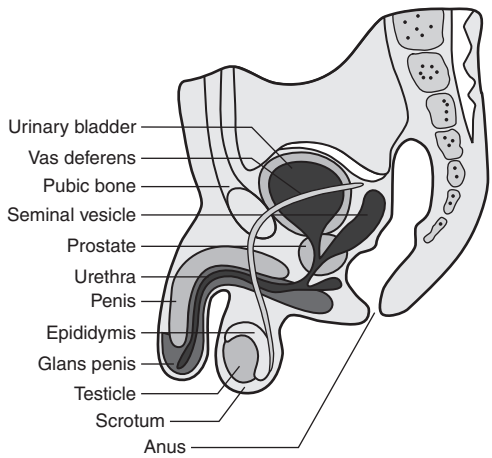

Figure 1. Male reproductive system

The prostate zones

The prostate consist of several zones, the peripheral zone, the central zone and the transition zone (Figure 2). Prostate cancer mainly occurs in peripheral zone, whereas benign prostatic hyperplasia (BPH) merely occurs in transition zone. BPH is a benign enlargement of the prostate. The concept of zonal anatomy was first described by McNeal (2,3).

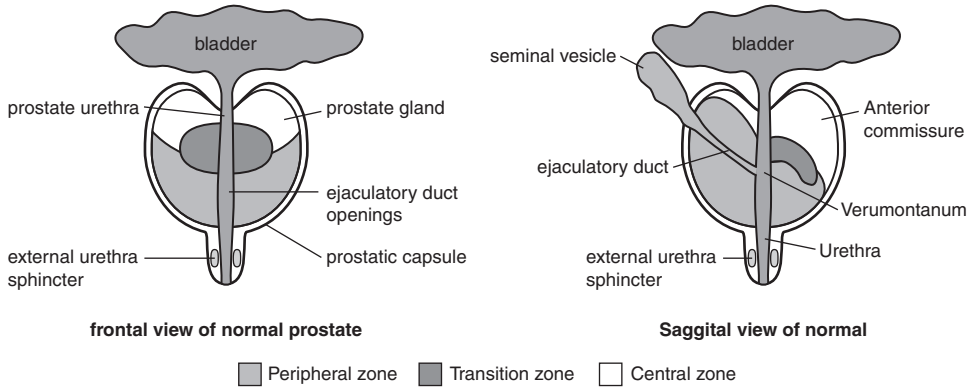

Figure 2. The human prostate zones

The peripheral zone

The peripheral zone forms about 70% of the glandular portion of the prostate and is located at the dorsal and dorso-lateral side of the prostate. Most prostate cancers arise from this zone. In order to establish whether a patient has a diagnosis of prostate cancer, transrectal ultrasound guided biopsies of the prostate are taken mostly from peripheral zone.

The central zone

The central zone represents about 25% of the prostate gland. It is wedge shaped and has its broad base just below the bladder. The ejaculatory duct runs in the middle of the central zone.

The transition zone

The transition zone, which is located peri-urethrally and more centrally in the prostate, forms about 5% of the normal prostate. This part of the prostate can grow extensively in elderly men with BPH (benign prostatic hyperplasia) (1). BPH is a benign prostate enlargement and occurs almost exclusively in the transition zone.

Less than 30% of prostate cancers consist of transition zone tumors, they have lower biochemical recurrence rates and are less malignant than the tumors originating in the peripheral zone (4,5)

Hormonal environment of the prostate gland

In the adult, prostate gland size is preserved through a homeostatic balance between the process of cell renewal and cell death. This balance is, among others, regulated by hormones secreted by the endocrine system, mainly androgens,

of which testosterone is the major circulating form.

The hormonal environment of the prostate gland is largely dependent upon the part of the endocrine system that involves the hypothalamic-pituitary-testicular axis.
The hypothalamus, initiates a series of events that leads to the secretion of testosterone (Figure 3). Luteinising hormone-releasing hormone (LHRH), and corticotrophin-releasing hormone (CRH), that acts on the pituitary gland to release additional hormones, are locally released by the hypothalamus.

The hormones secreted by the pituitary gland are luteinising hormone (LH), follicle stimulating hormone (FSH) and adrenocorticotrophin (ACTH). These hormones have their effects on the testes and adrenal glands when entering the circulation. Upon this stimulation, the Leydig cells of the testes then start producing testosterone.

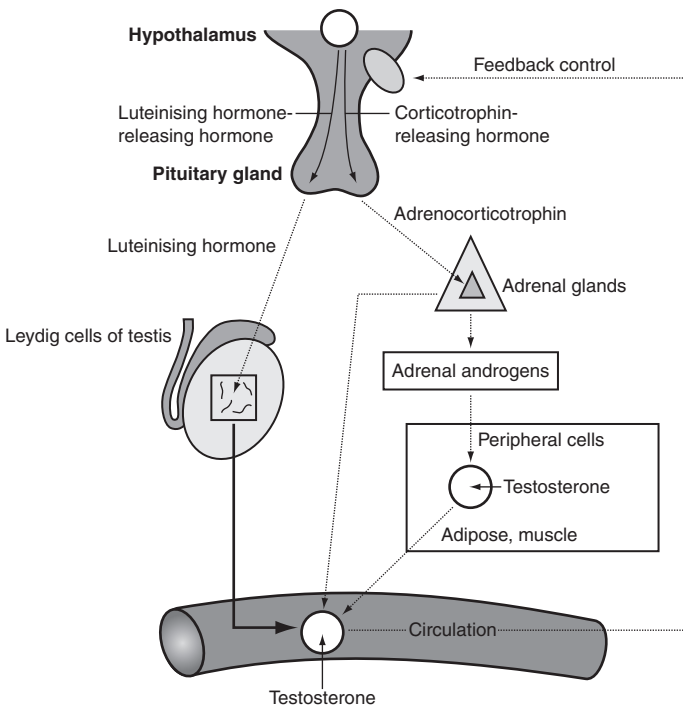

Figure 3. Men who are 5 alpha-reductase deficient are unable to convert testosterone into DHT and these men don't develop a prostate.

In the prostate, testosterone is metabolised to dihydrotestosterone (DHT) by the enzyme 5 alpha-reductase. DHT binds to the androgen receptor (AR) within the

glandular cells. Inside the cell nucleus, the AR-DHT complex targets specific DNA sequences known as androgen response elements (AREs). These AREs activate cell functions, including growth and proliferation, which play a major role in prostate cancer.

Prostatic Hyperplasia

BPH is a benign enlargement of the prostate and is a common condition among aging men. BPH is rarely life threatening when treated. Several types of BPH can be distinguished, which vary from stromal, fibromuscular, muscular fibroadenomatous to fibromyoadenomatous nodules occurring almost exclusively within the transition-zone of the prostate (4). Possibly 25% of men have some degree of hyperplasia at fifty years of age and approximately 80% of 70-80 year old men have histological evidence of BPH (6). However, in only about 10% of these cases will BPH be symptomatic and severe enough to require surgical or medical therapy.

Available drugs like finasteride and episteride, which act to inhibit the enzyme 5-alpha reductase, block the growth-promoting androgenic effect and diminishes prostatic enlargement (7). Another class of drugs used to treat BPH are the alpha adrenergic blockers, which cause relaxation of smooth muscle in prostate and help to relieve obstruction (8). Drug therapy must be continued to remain effective.

Histologically, nodular prostatic hyperplasia consists of nodules of ducts and intervening stroma. Most of the hyperplasia is composed of glandular proliferation, but the stroma is also increased, and in some cases may predominate (9). The ducts may be more variably in size. Nodular hyperplasia is not a precursor to carcinoma (10).

Prostatic Adenocarcinoma

Although with lower prevalence than benign disease, adenocarcinoma of the prostate is common. It is the most frequent non-skin malignancy in elderly men. Prostatic adenocarcinoma is rare before the age of 50, but autopsy studies of unrelated cases have found prostatic adenocarcinoma in approximately 70% of men in their 60s (11). Some of these carcinomas are small and clinically insignificant and do not pose a threat to life or health, however, prostatic adenocarcinoma is second only to lung carcinoma as a cause for tumor-related deaths among males (12).

Men with a higher likelihood of developing a prostate cancer (in the U.S.) include those of older age, African American heritage, and family history of this disease.

Those with an affected first-degree relative have double the risk (13).

Prostate cancers may be detected by digital rectal examination, by ultrasonography (transrectal ultrasound), or by screening with a blood test for prostate specific antigen, PSA (13-15). Rectal examinations are poor methods to reliably detect all prostate cancers, particularly the small tumors. PSA, on the other hand is reasonably sensitive (80%), though often with false positives with respect to cancer diagnosis (16).

PSA is a glycoprotein produced almost exclusively in the epithelium of the prostate gland. The PSA serum level in older men is usually less than 3 ng/mL (normal ranges vary depending upon which assay is used). A mildly increased PSA (3 to 10 ng/ml) in a patient with a very large prostate can be due to nodular hyperplasia, or to prostatitis, rather than carcinoma. A rising PSA (more than 0.75 ng/mL per year) is suspicious for prostatic carcinoma, even if the PSA is in the normal range. Transrectal needle biopsy, often guided by ultrasound, is needed to confirm the diagnosis after suspicion of prostate cancer. Incidental carcinomas can be found in prostate tissue from transurethral resections for nodular hyperplasia or cysto-prostatectomies or Millen (16).

Cytological characteristics of adenocarcinoma include enlarged round, hyperchromatic nuclei that have a single prominent nucleolus. Less differentiated carcinomas have fused glands called cribriform glands, in addition to solid nests or sheets of tumor cells, and many tumors have two or more of these patterns. Prostatic adenocarcinomas almost always arise in the peripheral zone of the prostate and are often multifocal (17).

Prostatic adenocarcinomas are usually graded according to the Gleason grading system based on the pattern of growth. There are 5 grades (from 1 to 5) based upon the architectural patterns, in which adenocarcinomas of the prostate are given two grades, based on the most abundant grade and second most abundant grade. These two grades are added to get a final score ranging from 2 to 10. The stage is established by the size and location of the cancer, whether it has invaded the prostatic capsule or seminal vesicle, and whether it has metastasized (18). The grade and the stage correlate well with each other and with the prognosis. The prognosis of prostatic adenocarcinoma varies broadly with tumor stage and grade. In general, cancers with a Gleason score of <6 are low grade and not aggressive. Advanced prostatic adenocarcinomas typically cause urinary obstruction, metastasize to regional (pelvic) lymph nodes and to the bones, causing blastic metastases in most cases. Metastases to the lungs and

liver are seen in a minority of cases (19).

Epithelial to mesenchymal transition

Epithelial to mesenchymal transition (EMT) is an interconversion between epithelial and mesenchymal states, in which polarized epithelial cells can obtain mesenchymal characteristics that resembles those of fully differentiated fibroblasts or myofibroblasts (20). These fibroblastic cells are capable of locomotion and induce cellular changes including alteration of epithelial cell-cell and cell-matrix adhesion contacts of their active cytoskeleton. In addition, molecular programs which are able to both degrade and integrate the extracellular matrix (ECM) are activated by these fibroblasts. These capabilities in matrix remodelling enable motile transitioning cells to invade through basement membranes and continue migration in ECM, a process that is often identified in tumor invasiveness (21). The EMT program has been identified to trigger a variety of tissue remodelling events during development, including formation of the secondary oral palate, in which differentiated epithelial cells undergo an EMT and so become integrated into the mesenchymal compartment, thereby completing the program of palatogenesis (22). Interestingly, Foxf2 knockout mice have a defect in secondary oral palate formation, suggesting a role for Foxf2 in EMT (Chapter 5). Secondary palate formation occurs in relatively well-differentiated epithelial cells that are destined to become defined mesenchymal cell types. This raises the possibility that EMTs may also be induced under certain physiological or pathological conditions in adult tissues, including tumor invasion and metastasis processes. EMT related alterations occur in coordination with other cellular programs, such as cell survival and proliferation. Examples of genes involved in the EMT program are transcription factors Twist, Snail, Slug and ZEB2, all able to induce EMTs in epithelial cells. In EMT, loss of E-cadherin is consistently reported in both development and tumor metastasis, whereas mesenchymal makers as N-cadherin, vimentin and fibronectin are often up-regulated. Furthermore, exposure of TGFβ1 in human mammary epithelial cells, also lead to induction of an EMT (23). Evidence of self-renewing and stem-like cells within tumors was provided by studies in neoplastic cells (24). These cells have been called cancer stem cells or tumor initiating cells and are suggested to behave in a similar way as mesenchymal-appearing cells, which have just undergone EMT (23). Understanding the changes that occur in the microenvironment of prostate cells undergoing EMT, will be critical to understand how primary prostate tumor cells convert to metastatic cells.

Forkhead transcription factors

Forkhead / winged helix proteins belong to a large family of evolutionary conserved 110 amino acid DNA binding proteins (25,26). It has been more than a decade since discovery of the Drosphila transcription factor forkhead and subsequent identification of the mamalian orthologues of the forkhead DNA binding domain (27). Forkhead genes encode a subgroup of helix-turn-helix class of proteins. The arrangement of loops connecting the β strands that flank one of the three α helices, gives rise to a butterfly like appearance, hence the name "winged-helix" transcription factors (28). It is a relatively invariant structure, with most amino acids being conserved between family members. This has made it difficult to understand the molecular mechanisms underlying the sequence specificity of the DNA-binding domains. All Forkhead factors can bind DNA, the functional effect of this can be either the activation or the inhibition of gene transcription. In contrast to the DNA-binding domains, there is almost no sequence homology between the transactivation or repression domains of members of the forkhead family, and little is known about their interactions with the transcriptional machinery. The forkhead family has been implicated in a variety of cellular processes and they play a role in embryonic development and also in disease.

Evolution

Forkhead genes have thus far been found in opisthokont organisms (animals + fungi), including several species of ascomycetic fungi and a wide variety of metazoans. Their absence in the Arabidopsis genome and failure to identify forkhead genes in any protist, support the idea that this gene family is found exclusively in animals and fungi (29). Forkhead genes contribute in the regulation of many developmental and metabolic processes. A number of forkhead proteins are involved in several morphogenetic processes during embryonic development, and their meaning is indicated by correlation between forkhead number and anatomical complexity of the animals (30). A model for Fox cluster evolution has been visualized by expression in the bilateria. This model depicts the evolution of the FoxL1/FoxC/FoxF/FoxQ1 genes and their expression domains (Figure 4). Organization of Fox genes in five extant taxa were shown at the tips of the tree. Colour coding indicated the expression of FoxC, FoxF and FoxL1 in the mesoderm, and FoxQ in the endoderm. This model predicts that a four-gene cluster has evolved by the base of the bilaterians, and was co-ordinately expressed in mesendodermal derivatives. Some gene loss is also inferred, including one FoxQ1 and one FoxL1 gene in humans (following block duplication of the cluster), and FoxQ1 in the lineage leading to insects.

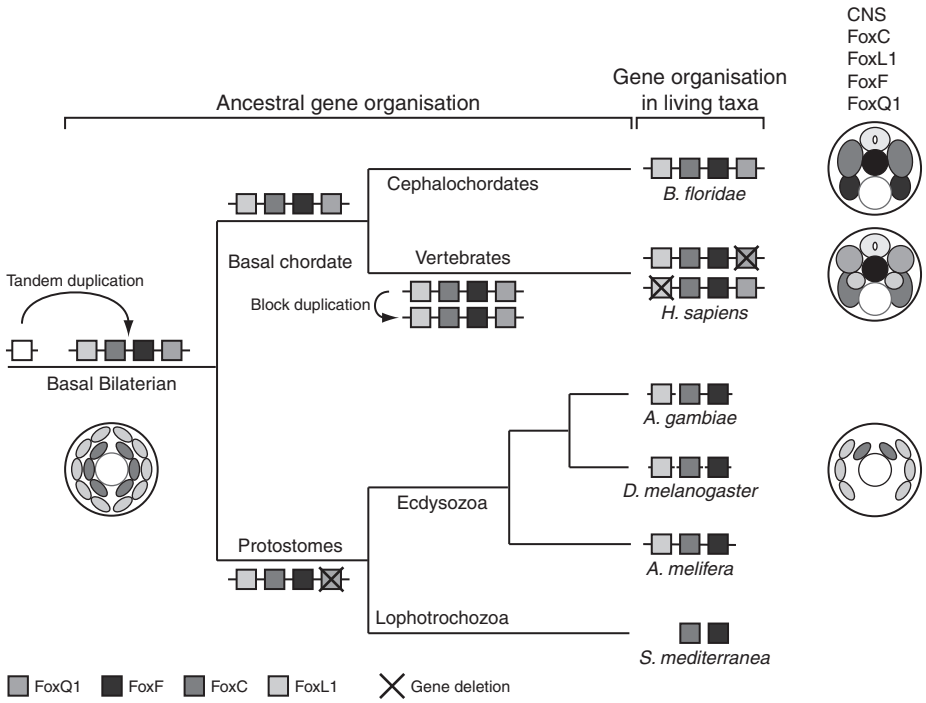

Figure 4. A model for the evolution of FoxF, FoxC, FoxL1 and FoxQ1 genes and their expression domains (31). The tree shows a basic phylogeny of the bilateria. Organization of Fox genes in six existing taxa is shown at the tips of the tree, and to the right of this are illustrative representations of embryos in transverse section with dorsal to the top. The model predicts that a four-gene cluster had evolved by the base of the bilaterians and was expressed in endo-mesodermal derivatives. Some gene loss is also inferred, including one FoxQ1 and one FoxL1 gene in humans (following block duplication of the cluster), and FoxQ1 in the lineage leading to protostomes.

Nomenclature

The nomenclature of forkhead transcription factors was revised in 2000 (32). Fox (for “Forkhead box”) is used as root symbol and officially states that the same name is utilized for orthologous genes in different species and reflects phylogenetic relationships by including a letter that indicates subfamily. Within a subfamily, each gene is identified by a number (e.g.FOXF2), the typography follows the conventions used in other species (FOXF2 in Homo sapiens, Foxf2 in Mus musculus and FoxF2 in all others) and proteins are distinguished from genes by the use of roman type (e.g. FoxF2). New and old name and other useful information concerning nomenclature can be found on http://biology.pomona.edu/fox/. The most commonly used synonyms for human, mouse and rat forkhead genes are listed in table 1.

Table 1. The more commonly used synonyms of human, mouse, and rat *Fox* names

FoxA1	HNF3
FoxA2	HNF3
FoxA3	HNF3
FoxB1	Fkh5
FoxB2	Fkh4
FoxC1	FREAC-3, FKHL7, Mf1, Fkh1
FoxC2	Mfh1
FoxD1	FREAC-4, BF2
FoxD2	FREAC-9, Mf2
FoxD3	HFH2, Genesis
FoxD4	FREAC-5, Fkh2, HFH-6
FoxE1	FKHL15, TTF2
FoxE2	HFKH4
FoxE3	FREAC-8
FoxF1	FREAC-1, HFH-8
FoxF2	FREAC-2, Lun
FoxG1	BF-1
FoxH1	FAST1
FoxI1	FREAC-6, HFH-3, Fkh10
FoxJ1	HFH4
FoxK1	ILF, MNF
FoxL1	FREAC-7, Fkh6
FoxM1	Trident, HFH-11, INS1
FoxN1	Whn
FoxN2	HTLF
FoxO1	FKHR
FoxO3	FKHRL1
FoxO4	AFX1
FoxP1	QRF1
FoxQ1	HFH-1, HFH1L

Scope of this thesis

Prostate cancer (PC) and benign prostatic hyperplasia (BPH) are two common prostate diseases originating in the prostate peripheral and transition zone. When this project started, the molecular basis for the differences between the prostate zones was unknown. We hypothesized that the predisposition of prostate cancer in peripheral zone and BPH in the transition zone originates from preexisting molecular differences in the normal zones. The gene expression profiling studies that had been conducted on PC and BPH mainly focused on the expressing profiles associated with the diseases and not on the confinement of the diseases to the different prostate zones. In Chapter 2 of this thesis we investigate gene expression of the human prostate zones.

Two important genes that emerged from the zonal expression data were FOXF1 and FOXF2. We hypothesize that these transcription factors are imperative in clarifying prostate diseases. Not much was known about the general expression of forkhead transcription factors in the prostate. Chapter 3 describes the analysis of 12 forkhead transcription factors in normal prostate, prostate cancer, prostate metastases, androgen-dependent and androgen-independent xenografts and several prostate cell lines. In this analysis we observed that FOXF2 was of interest

because it was lower expressed in prostate cancer compared to normal prostate and higher expressed in transition zone compared to peripheral zone.

In Chapter 4, we studied the FOXF2 pathway in the human prostate stroma. To accomplish this, siRNA studies directed against FOXF2 were performed to identify the target genes of FOXF2 by microarray analyses. After exploring FOXF2 gene expression and FOXF2 regulated pathways in human adult prostate in Chapter 4, we explored the potential role for Foxf2 in prostate development. We investigated the phenotypic changes in the urogenital sinus (UGS) of Foxf2 knockout and wild-type mice. Chapter 5 will discuss the effect of Foxf2 knockdown in UGS anatomy. Chapter 6 discusses the results of this thesis and future perspectives.

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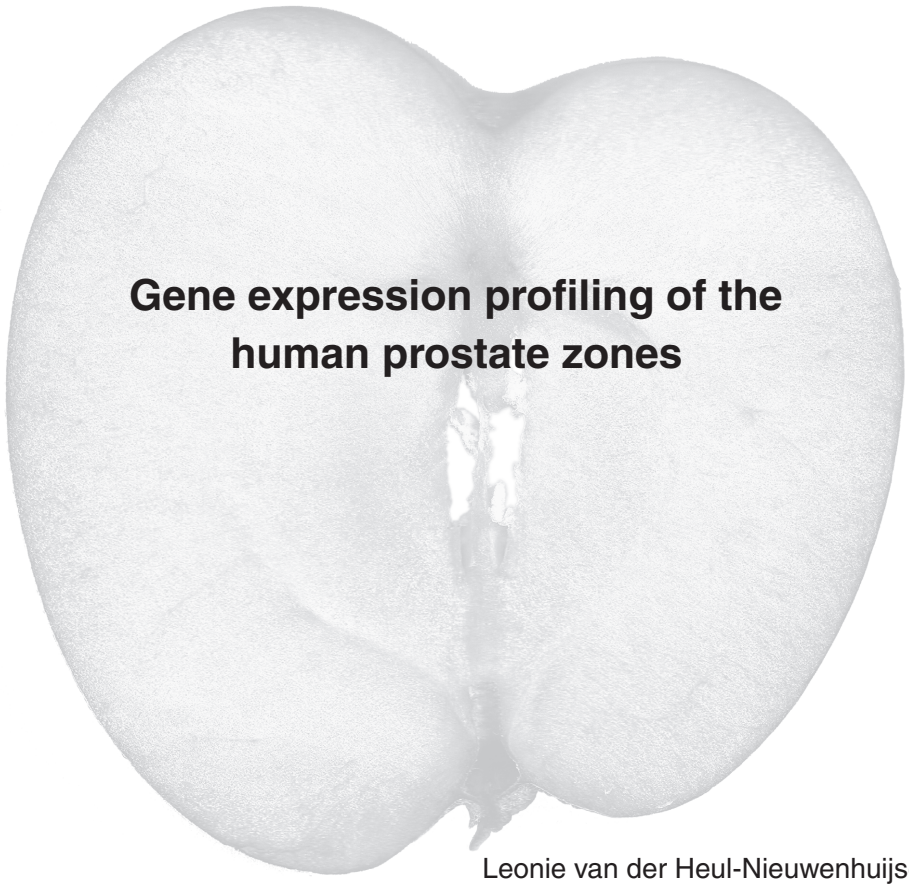
Chapter

2



Gene expression profiling of the human prostate zones

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BJU Int 2006: 98(4); 886-97

Abstract

Objective. The prostate consists of several zones; the peripheral zone, the transition zone and the central zone. Aggressive tumors predominantly occur in the peripheral zone, whereas benign prostatic hyperplasia (BPH) occurs almost exclusively in the transition zone. To understand the basis of these prostate diseases and their confinement to a specific zone, gene expression patterns in the unaffected peripheral and transition prostate zones were investigated.

Methods. Expression profiling of both prostate zones was performed by microarray analyses. RT-PCR of the top 18 genes confirmed the microarray analyses. RT-PCR performed with common cell type markers indicated that the differential expression between the two zones was not caused by an unequal distribution of different cell types. Primary stromal (PrSC) and epithelial prostate cells (PrEC) were used to study cell type expression in the 12 highest differentially expressed zonal-specific genes.

Results. 346 genes were identified that were preferentially expressed in the transition or peripheral zone. Few of the transition zone-specific genes, including ASPA, FLJ10970 and COCH were also observed to be stroma-specific. Database comparisons with other microarray studies showed that gene expression profiles of prostate cancer and BPH correlate with the expression profiles of the peripheral and transition zone, respectively.

Conclusion. Our data indicate that gene expression differs between peripheral and transition zone and we speculate that stromal-epithelial interactions could be responsible for the distinct zonal localization of prostate diseases.

Introduction

Prostate cancer (PC) and benign prostatic hyperplasia (BPH) are two common prostate diseases (1). BPH is a benign enlargement of the prostate and it is estimated that up to 80% of 70-80 year old men have histological evidence of BPH (2). When treated, BPH is rarely life threatening. Several types of BP fibromyoadenomatous nodules occurring almost exclusively within the transition zone of the prostate (3). Prostate cancer is the most frequently diagnosed cancer in Western countries and is the second leading cause of cancer deaths in these parts of the world. The majority of prostate cancers occur in the peripheral zone, which is located at the dorsal and dorso-lateral side of the prostate. Less than 30% of prostate cancers consist of transition zone tumors, they have lower bio-

chemical recurrence rates and are less malignant than the tumors originating in the peripheral zone (4). BPH, on the other hand, occurs almost exclusively in the transition zone, which is located peri-urethrally and more centrally in the prostate. BPH can be an overgrowth or hyperplasia of both stromal and epithelial compartments and does not involve prostatic intraepithelial neoplasia (PIN). PC evolves from PIN and/or other atypically proliferation lesions (5). The molecular basis for the differences between the prostate zones is unknown. Given that the diseases have a strong preference for a specific zone, we assume that this predisposition originates from preexisting molecular differences in the normal zones. Up till now, several gene expression profiling studies have been conducted on PC and BPH, reviewed by Nelson et al. (6). These studies however, focused on the expressing profiles associated with the diseases and not on the confinement of the diseases to the different prostate zones.

Morphologically, pathologists consider the transition zone of the prostate similar to the peripheral zone (7). Other studies also showed that the transition zone and the peripheral zone are morphologically much alike. The zones of the prostate gland consist of a complex environment of both stromal and epithelial cells. The complexity of the interaction between both these cell types has been the focus of exploration in the past decades, and remains an intensively studied field of research (8). Therefore it is very important to take cell type distribution and cell type characteristics into consideration when studying the prostate zones. Cunha et al. (9) demonstrated that stromal cells have the ability to modulate the differentiation pattern of normal prostatic epithelium. This association between the two cell types is thought to be critical for normal tissue development and maintenance as well as the origin and progression of disease processes like PC and BPH.

The present study was performed using oligo microarray analyses to identify the differences in gene expression between the normal peripheral and transition zone. Expression of the differentially expressed genes was analyzed in stromal and epithelial primary cells. Database comparisons with other microarray studies were performed to correlate gene expression profiles of cancer and BPH with the expression profiles of the normal peripheral and transition zone. Our results indicate that the molecular variations in gene expression between the two zones could be responsible for the origin of distinct zonal diseases.

Materials & Methods

Prostate samples

The prostate specimens were obtained from men who suffered from prostate cancer and who underwent a radical prostatectomy at the Department of Urology, Erasmus MC. (More clinical and demographical information see table 1).

Patient	Nationality	Data of surgery	Type of surgery	[PSA] ng/ml B.S.	[PSA] ng/ml A.S.	Extra treatments
P3	Dutch	02-05-2002	Radical prostatectomy	9.1	<0.1	no
P5	Dutch	02-07-2002	Radical prostatectomy	9.6	<0.1	no
P10	Dutch	04-09-2002	Radical prostatectomy	5.1	<0.1	no
P11	Dutch	17-07-2002	Total cystectomy (incl prostate)	2.5	NA	Chemo-therapy B.S.
P14	Dutch	29-10-2002	Radical prostatectomy	11.0	<0.1	no
P16	Dutch	21-10-2002	Radical prostatectomy	6.0	<0.1	no
P18	Dutch	12-11-2002	Radical prostatectomy	15.0	1.7	no
P19	Dutch	12-11-2002	Radical prostatectomy	6.1	<0.1	no
P27	Dutch	24-07-2003	Radical prostatectomy	22.0	<0.1	Radiation therapy A.S.

Table 1. Clinical and demographical information on investigated patients B.S.= before surgery, A.S.= after surgery

The experimental protocols were approved by the Erasmus MC Medical Ethics Committee according to the Medical Research Involving Human Subjects Act. Immediately after surgery, the prostate was examined by a pathologist, who provided a 0.5-cm thick transverse section of the prostate. Immediately thereafter, the samples were snap-frozen in liquid N_2-cooled iso-pentane and stored at –80°C. Each sample was examined histologically using hematoxylin and eosin-stained cryostat sections. The frozen sections were independently scored by two pathologists for presence of PC and BPH and to affirm zonal origin. Frozen sections from both zones without any evidence for BPH or PC were used as a template to isolate the normal tissue. Only when both pathologists confirmed the zonal specific tissue independent from each other, it was accepted as proper tissue. The pathologist scored the number of nuclei of stomal and epithelial cells by microscopy. This analysis was semi-quantitative and performed by two independent specialists.

Microarrays

The oligoarrays were produced at the Erasmus Center for Biomics and contained 18,584 oligos, representing approximately 15,000 different human genes (Unigene Clusters). The oligo libraries that were spotted on aminosilane slides are comprehensive oligo collections designed by Compugen and manufactured by Sigma-Genosys. The oligos were printed by a Virtek Chipwriter Professional arrayer (Virtek Vision International, Waterloo, Canada). Control spots included landmarks, spotting buffer, alien oligos (SpotReport Alien Oligo Array, La Jolla, Stratagene), poly d[A]40-60, salmon sperm DNA, and human COT-1 DNA.

RNA isolation, amplification, amino-allyl labeling and array hybridization

RNA was isolated from the prostate samples by homogenization with a cell disrupter (PCU, Kinematica GmbH, Luzern, Switzerland) in RNAbee reagent (Teltest Inc, Friendswood, USA). Chloroform was added and centrifuged at 12,000g. The water phase was extracted and precipitated with isopropanol. The RNA was washed with 75% ethanol and dissolved in RNAse free water. Afterwards, the RNA was treated with DNA-free (Ambion, Huntingdon, United Kingdom) for DNA digestion. Five μg of total RNA was used for a T7-based linear mRNA amplification protocol essentially as described by Van Gelder et al. (10).

An amino-allyl group was incorporated in the aRNA during the amplification step. Afterwards, this amino-allyl group was coupled to an N-hydroxysuccinimide modified Cy3 or Cy5 dye (Amersham, NJ, USA). The aRNA from the transition zone was labeled with Cy3 (green) and the aRNA from the peripheral zone of the same patient was labeled with Cy5 (red) and hybridized to the same array. The experiments were performed in dye swap duplicate. The microarray glass slides were pre-hybridized in a 5xSSC, 0.1% SDS, 10% BSA solution for 1 hour at 45°C. Thereafter, the slides were washed with water (2x10 minutes) and isopropanol (5 minutes) and centrifuged for 4 minutes at 1,000 rpm to dry. Probe solutions were hybridized on the array overnight at 45°C in a TECAN HS4800 hybridization station (TECAN Benelux B.V.). During hybridization the solution was continuously moving. Afterwards, the arrays were automatically washed in the TECAN with 2xSSC/0.05% SDS (at 42°C), 1xSSC and 0.2xSSC (at RT) and dried under a stream of N_2-gas. The arrays were scanned in a ScanArray Express HT (PerkinElmer Nederland B.V.). Spot intensities were quantified by Imagene (BioDiscovery Inc. El Segundo, USA). Spots signals were normalized per median subarray. The programs Cluster and TreeView were used for hierarchical clustering of arrays and genes and visualization. Data were clustered by correlation uncentered, average linkage. Comparisons with other microarray databases were performed using SRS7 (Lion Bioscience AG, Heidelberg, Germany). Significance analysis of microarrays (SAM) was performed. Statistical

analysis of the database comparisons were performed according to Bonferroni statistical test.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The reverse transciptase reaction was performed with 1 μg RNA from the prostate samples with oligo-T12 primer and pre-incubated for 10 minutes at 70°C. First Strand buffer, DTT, dNTPs and RNAsin were added and incubated for 2 minutes at 37°C. The RT reaction was initiated by MMLV-RT and incubated for 1 hour at 37°C. After this, the reaction was kept 10 minutes at 90°C and immediately thereafter frozen.

PCR products were run through 1% agarose gels and scanned with Imago, compact Imagine systems (B&L systems, Maarssen, the Netherlands). The PCR was performed with 30 cycles (except for the GAPDH primers, which were performed with 25 cycles) and an annealing temperature of 60°C for all primers (Table 2). PCR reaction mixtures contained 2.5 μl 0.5 U/μl Taq polymerase, 5 μl 10 x PCR buffer, 3 μl 25 mM $MgCl_2$ (Promega Benelux b.v., the Netherlands), 1 μl 10 mM dNTPs, 3 μl 100 ng/μl forward and reversed primers (Invitrogen, Breda, the Netherlands) and 1 μl cDNA. The gel bands were imaged and quantified using ImageQuant (Molecular Dynamics, USA). All experiments were repeated at least three times. Gene expression data were calculated as ratios between the peripheral and transition zone. The data were corrected for GAPDH expression and transferred into a ^{2}log ratio and illustrated in TreeView.

PrSC and PrEC cell culture

Primary human prostate epithelial cells (PrEC) and primary human prostate stromal cells (PrSC) were purchased and cultured according to manufacturer's guidelines (Clonetics Human and Animal Cell Systems, Cambrex Bio Science Walkersvill, Inc). RNA was isolated from the PrSC and PrEC by lysing cells in RNAbee reagent. Chloroform was added and centrifuged at 12,000g. The water phase was extracted and precipitated with isopropanol. The RNA was washed with 75% ethanol and dissolved in RNAse free water. The PrEC and PrSC were extracted from whole prostate samples without separation of the zones (information provided by Clonetics Inc.). Passages 5 and 7 were used for RNA isolation. To minimize selection and adaptation of cells, only early passages 5 and 7 were used for isolation and the cells were passaged when they were 70% confluent to keep the variation smallest.

Table 2. Primer sequences for all primers used in the RT-PCR experiments.
Primers were designed to be, if possible, intron spanning and human-specific (Y, yes; N, no).
All primers were chosen within 1000 nt from poly-A-tail.

Primer name	Primer sequence	Intron	Human
		Spanning	Specific
SLC14A1 forward	5' GGAGGAATGTTCATGGC 3'	Y	N
SLC14A1 reverse	5' AGACCCTGGAAGTTAGCTG 3'		
THBS4 forward	5' CAGCACAGACAACTGCC 3'	Y	N
THBS4 reverse	5' GCCAGGATCACTGTTCAT 3'		
ASPA forward	5' ACTCGTTCCATAGCCAAGT 3'	Y	N
ASPA reverse	5' TCGTCTTCCCATCAAGAG 3'		
FLJ10970 forward	5' TCACCCTGGTGAGACTGT 3'	Y	N
FLJ10970 reverse	5' GGCCAAAGATGGAGATAATA 3'		
GIG2 forward	5' ATGGGACTTTGAGAAGAGG 3'	N	N
GIG2 reverse	5' GCCATCTCACTGTTCACAT 3'		
ADM forward	5' CCAGGACATGAAGGGTG 3'	Y	Y
ADM reverse	5' GCTGATCTTGCTCCTGG 3'		
TFPI2 forward	5' GGCTGCAAGTGAGTGTG 3'	Y	Y
TFPI2 reverse	5' CAATCCTCCCTGCTAACA 3'		
BST2 forward	5' AGTACTACCCCAGCTCCC 3'	Y	N
BST2 reverse	5' ACCCGCTCAGAACTGAT 3'		
Hepsin forward	5' ACCAAAGTCAGTGACTTCCG 3'	Y	Y
Hepsin reverse	5' ACAGGAGTCCCAGACAGCAG 3'		
EGR1 forward	5' CCTGCACCCTTGTACAGTGTC 3'	N	Y
EGR1 reverse	5' CATGTCAAGCCATCAGCAAG 3'		
GAPDH forward	5' ACGGGAAGCTTGTCATCA 3'	Y	Y
GAPDH reverse	5' ATGGCATGGACTGTGGTC 3'		
PLAB forward	5' CCTGCAGTCCGGATACT 3'	Y	Y
PLAB reverse	5' CCGAGAGATACGCAGGT 3'		
TARP forward	5' TCACAATGGATCCCAAAGAC 3'	Y	Y
TARP reverse	5' TTGAGGAGCAGGAGGAGG 3'		
Collagen forward	5' CCTCCTGGAGAGAATGGT 3'	Y	N
Collagen reverse	5' CCTATAGCTCCTGGCAAA 3'		
Fibronectin forward	5' GTACATGCTTCGGTCAGG 3'	Y	Y
Fibronectin reverse	5' GGTCTGTAAAGGTTGGCA 3'		
Myosin forward	5' CTGTCCTCTATGGCAATGA 3'	Y	Y
Myosin reverse	5' TCTAGCAGCACTTCCCTC 3'		
VWF forward	5' AGCACCATCTACCCTGTG 3'	Y	Y
VWF reverse	5' CCTTCATGCAGAACGTAAG 3'		
Keratin 8 forward	5' CTCCAGGCTGAGATTGAG 3'	N	N
Keratin 8 reverse	5' TGACGTTCATCAGCTCCT 3'		
Chromogranin A forward	5' TCCAGGTCCGAGGCTACC 3'	Y	Y
Chromogranin A reverse	5' GCCTTGTCTCCTACTCCTGACT 3'		
Enolase 2 forward	5' GACTGAGGACACATTCATTGC 3'	Y	Y
Enolase 2 reverse	5' CCTGATCTGTGATAGTTCACC 3'		
Calcitonin forward	5' GGACTATGTGCAGATGAAGGC 3'	Y	Y
Calcitonin reverse	5' CCTCATGTTAGCATGCCCC 3'		
CD4 forward	5' AGCGGATGTCTCAGATCA 3'	Y	N
CD4 reverse	5' TGCTACATTCATCTGGTCC 3'		

Statistical analyses

Statistical analysis for comparison of the RT-PCR data (Figure 1b and 2) was performed using a paired t-test. Also the results illustrated in Figure 3 were analyzed by a paired t-test. The Bonferroni statistical test was applied for analyses of database comparisons (Figure 4 and 5). SAM analysis was performed on the microarray data. 338 genes were found to be significantly different between the prostate zones with a false positive rate of 4.2. This means that 4.2 genes of the 338 significant genes are expected to be false positive.

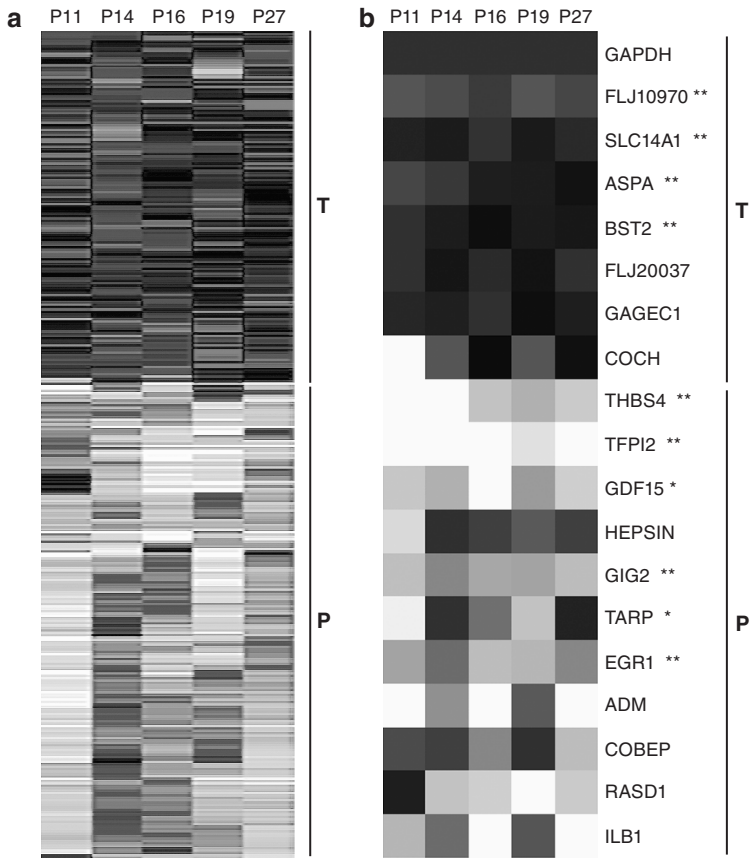


Figure 1. a) Hierarchical cluster analysis of gene expression of the prostate zones. Overview of 346 differentially expressed genes, of which 199 are higher expressed in the peripheral (P, red) and 147 are higher expressed in the transition zone (T, green). Rows represent individual genes represented as log2 ratio P/T and columns represent the averages of the dye swaps of 5 individuals (p11-p27).
b) RT-PCR analysis of 18 genes, selected from the 346 genes, was performed to confirm the microarray data. The RT-PCR data are represented as 2log expression ratio of peripheral (P) zone versus transition (T) zone. The values are corrected for the internal control GAPDH.
* $p<0.05$ and ** $p<0.01$.

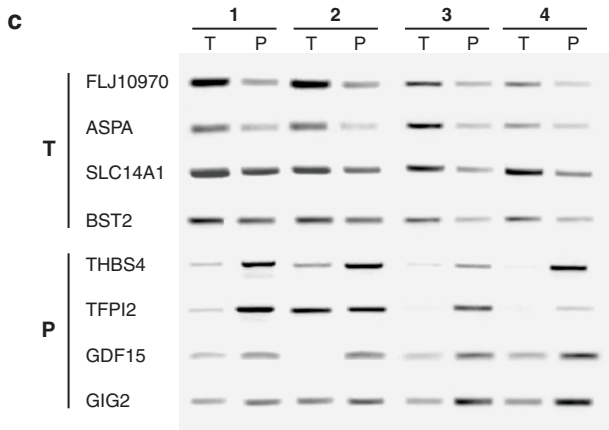


Figure 1c) RT-PCR of 8 differentially expressed genes on the prostate zones from 4 additional subjects (P3, P5, P10 and P18) to validate the microarray data.

Results

Gene expression profiling of the prostate zones.

To survey the molecular variation between the transition and peripheral zone we performed microarray analyses. In each experiment, we compared gene expression of the peripheral and transition zone from the same subject, in total of 5 men. The experiments were performed in dye swap duplicate (10 microarrays). 346 differentially expressed genes were selected [ratio peripheral/transition $\geq {}^{2}\log |0.5|$ and present (unflagged in ≥ 7 of the 10 arrays)]. When the gene was present in 7/10 arrays with positive ratio, it means that 7 of the 10 times the gene is higher expressed in peripheral zone than in transition zone. 199 genes were higher expressed in the peripheral zone and 147 genes were higher expressed in the transition zone. Statistical significance analysis of microarrays (SAM) confirmed differential expression of 185 genes of the 346 genes, with a false positive rate of 4.2. To visualize the relationship between the samples and most differentially expressed genes we performed an unsupervised hierarchical clustering (Figure 1a). The genes that were higher expressed in the transition zone are shown in green and the genes that were higher expressed in the peripheral zone are shown in red. The top 18 of these differentially expressed genes, 7 from transition and 11 from peripheral zone, were chosen to verify the microarray data with RT-PCR. The expression data from the RT-PCR confirmed the microarray profiles and 10 genes were expressed significantly different ($P< 0.05$) between the two zones (Figure 1b).

To further substantiate reproducibility of the microarray data, expression of the prostate zones of 4 more subjects (P3,P5,P10 and P18) were analyzed by

RT-PCR with 4 differentially expressed genes from the transition and the periperal zone (Figure 1c). This resulted in independent confirmation of the microarray data and the RT-PCR experiments of the first set of samples.

Cell Type distribution in the two prostate zones.

Next, we investigated whether the differential gene expression in the two zones is caused by differences in cell type distribution. The frozen sections of the 9 investigated subjects, were scored for quantity of stroma and epithelium in each zone by a pathologist. The average proportion of stromal and epithelial cells was approximately 50:50 and this equal distribution was identical in the peripheral and transition zone fragments studied. RT-PCR was performed with known markers for the most common cell types for 7 subjects. Both zones were analyzed for expression of stromal markers (fibronectin, myosin and collagen), epithelial markers (keratin 5, keratin 8, prostate specific antigen), neuro-endocrine markers (enolase 2, chromogranin A, calcitonin), endothelial marker (von Willebrand factor) and a lymphocyte infiltration marker (CD4) (8,11). Keratin 5 is a specific basal cell marker and keratin 8 is a luminal epithelium marker. There was no significant differential expression of these cell type markers between the two prostate zones (Figure 2). The identical distribution of stroma and epithelium in the frozen sections, and the equal expression of cell type markers in the RT-PCR, suggest that the quantity of cells is not different between the two zones. Instead, we would argue that the qualitative differences in cell type characteristics form the basis of the observed zonal-specific gene expression.

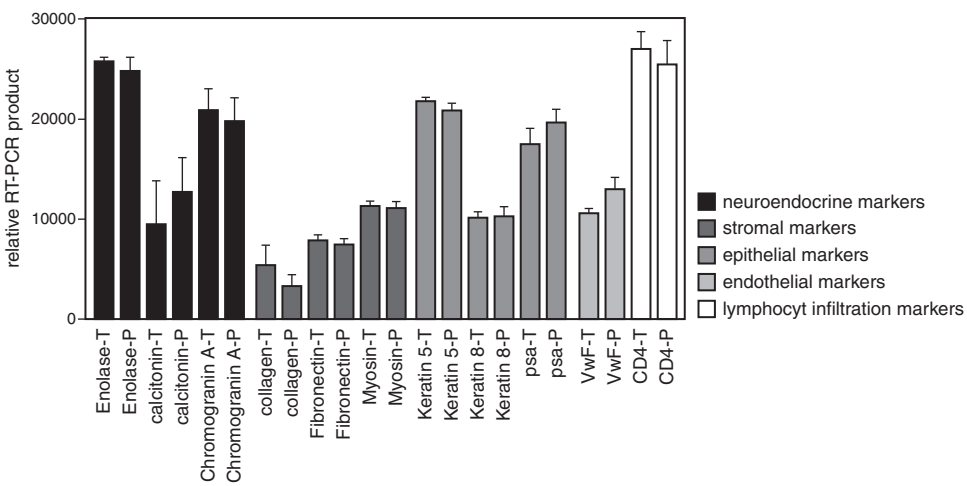


Figure 2. RT-PCR analyses of common cell type markers to investigate cell type distribution between the peripheral and transition zone. The averaged relative expression ratio (± SEM) from 7 subjects of at least 3 independent RT-PCR experiments is shown. The RT-PCR data were quantified by ImageQuant. A paired t-test was performed showing no significant differ-

ences in the distribution of the common cell type markers between the two prostate zones. P=peripheral zone and T=transition zone.

Differential expression of genes from the prostate zones in PrSC and PrEC.

To determine whether the zonal differences can be ascribed to stroma or epithelial characteristics, we studied gene expression of zonal-preferential genes in primary human prostate stromal (PrSC) and primary human epithelial prostate cells (PrEC). The cell type and purity of these primary cells were verified using two stromal markers (fibronectin, myosin) and two epithelial markers (keratin 5, keratin 8)(Figure 3a).

Several of the differentially expressed genes in the transition zone are preferentially expressed in PrSC (Figure 3b). The hypothetical protein FLJ10970, COCH and ASPA are stroma-specific, with a significant difference of $p<0.05$. SLC14A1 and FLJ20037 have a more equal expression distribution and BST2 is preferentially expressed in epithelial cells ($p<0.05$). The genes that are higher expressed in the peripheral zone (TFPI2, ADM, GDF15 and GIG2) are expressed in both stromal and epithelial primary cells. THBS4 was preferentially expressed in stroma.

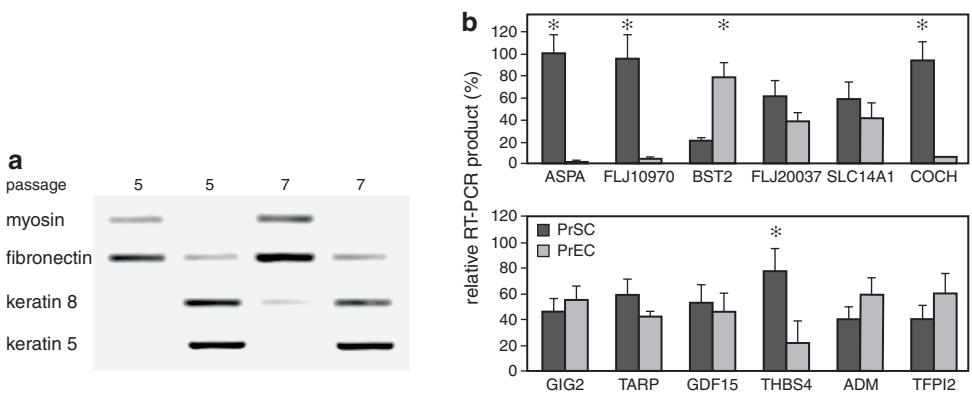
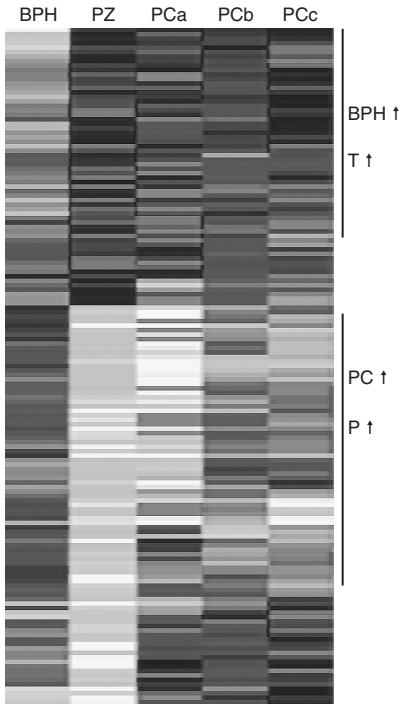


Figure 3. a) Verification of expression analysis of stromal and epithelial markers in prostate stromal PrSC and epithelial PrEC primary cells. RT-PCR analyses of 4 markers were performed to verify the purity of primary cells PrSC and PrEC for passages 5 and 7.
b) RT-PCR analysis of differentially expressed genes from the prostate zones in PrSC and PrEC. 6 genes that were higher expressed in transition zone and 6 genes that were higher expressed in peripheral zone were analyzed by RT-PCR in PrSC and PrEC. The values are averaged percentages of relative gene expression (quantative expression of PrSC/PrEC with respect to the total expression x100) from at least 6 RT-PCR experiments (± SEM).

Differential gene expression in prostate cancer and BPH compared to the differentially expressed genes in normal transition and peripheral zone.
We addressed the question whether the differentially expressed genes from the prostate zones are also differentially expressed in the prostate diseases. The 346 genes differentially expressed in the prostate zones were compared to the datasets of Singh et al. (Pca), Dhanasekaran et al. (PCb), and Lapointe et al (PCc) (Figure 4) (12,13,14). From each of these datasets, we calculated the average expression of each gene of primary PC or BPH samples and compared them to the averaged expression of the zonal-preferential genes. Figure 4 illustrates that the genes higher expressed in the transition zone tend to overlap with the genes that are higher expressed in BPH ($p=0.056$) and genes lower expressed in prostate cancer. Almost all genes that are higher expressed in PC are higher expressed in the peripheral zone ($p<0.0001$).

Differential gene expression in stroma and PC as compared to zonal differentially expressed genes
To determine whether there is an expression correlation between the genes associated with stroma and zonal-preferentially expressed genes, we compared our dataset to the data of Stuart et al. (15). In their study, microarray analyses were performed on 88 prostate specimens and gene expression was correlated to relative content of tumor and stroma. First, we compared the differentially expressed genes from the prostate zones with the genes associated with stroma as defined by Stuart et al. Second, we examined whether genes classified as tumor-associated, overlapped with the differentially expressed genes from the prostate zones. The comparisons between the datasets were performed via unsupervised hierarchical clustering and the results are visualized in TreeView (Figure 5). The genes that were higher expressed in the transition zone show a significant overlap ($p<0.0001$) with the genes associated with stroma from Stuart et al. Furthermore, the genes that were higher expressed in the peripheral zone overlap with the differentially expressed genes in tumor tissue ($p<0.0001$).



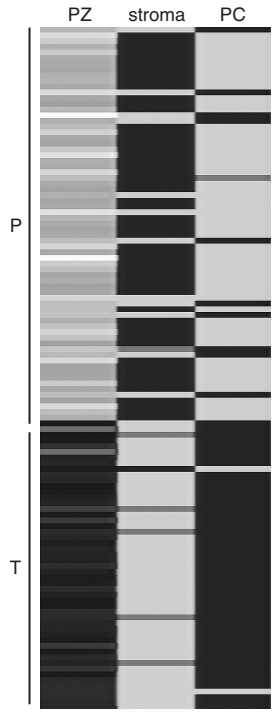


Figure 4 (left). Comparison between the differentially expressed genes in the prostate zones and genes that were higher expressed in PC or BPH. Zonal-specific gene expression (prostate zones, PZ) is represented as the average value from 10 microarrays (dye-swap experiments of 5 subjects) for each gene. Next to this column, the columns PCa; PC databases from Singh et al (13), PCb; PC database from Dhanasekaran et al (14), PCc; PC database from Lapointe et al (15), and BPH (14) are presented. The genes were unsupervised clustered.

Figure 5 (right). Comparison of our dataset to the data of Stuart et al. (16). Rows represent 346 differentially expressed genes and columns represent the average of 10 microarrays (dye-swap experiments of 5 subjects; prostate zones, PZ; P, peripheral zone; T, transition zone), stroma-associated genes (stroma) and tumor-associated genes (PC) as defined by Stuart et al. (16). The genes were unsupervised clustered.

Discussion

Our analyses were conducted to identify differences in gene expression between the transition and peripheral zone of the prostate. Since the prostate diseases PC and BPH are preferentially present in the peripheral and transition zone respectively, it is our hypothesis that the disease restriction can be explained by differences in gene expression profiles between these two zones. Only areas of the prostate zones with no suspicion or evidence of histological hyperplasia

or neoplasia were used for our analyses. Nevertheless, we cannot fully exclude that in these normal prostate tissue fragments of elderly men, imperceptible changes towards BPH or prostate cancer are present. However, in our opinion this tissue is of highest interest for our analyses, since at the age of 50-60 years, first signs of hyperplasia and neoplasia become apparent suggesting that this is the prostatic milieu supporting initiation and outgrowth of abnormalities. So far, most studies focused on gene expression profiling of prostate diseases and investigated the molecular variation of PC and BPH compared to normal tissue (6). Little attention has been given to the molecular bases of the prostate zones as possible explanation for the distinct occurrence of the prostate diseases. Here, we demonstrated that there is differential expression between the two normal prostate zones using microarray analyses. Statistical evaluation using SAM, substantiated differential expression of most selected genes. In addition, RT-PCR of a selected set of genes confirmed differential expression in the patient samples tested by microarray analyses and in four additional subjects.
So far, there is only one study in which gene expression of different prostate zones was taken into account. Stamey et al. analyzed gene expression in the different prostate zones investigating which zonal tissue was most suitable as a control for differential gene expression analysis in prostate cancer (16). The gene expression profiles of PC were strongly affected by the zonal location of control tissue, which indicates the distinction between the normal prostate zones. Although it was not their purpose to investigate the genes that distinguish the normal peripheral from the normal transition zone, we still could extract zonal specific genes from their studies. We identified an overlap of 12 genes of the 18 extracted genes between the two datasets (Table 3). This strongly supports the reliability of the data, considering that these independent studies were performed using two different microarray platforms and different patient samples.

To exclude that the differential gene expression is due to a different cell type distribution, we quantified the amount of stromal and epithelial cells in frozen sections of both prostate zones. The average proportion of stromal and epithelial cells was 50:50 and this distribution was the same in both prostate zones. In addition, we performed RT-PCR analyses with markers for most common cell types and showed that there were no significant differences between the two zones. We speculate that the difference in gene expression is not caused by a difference in quantity of cells, but by a qualitative distinction in cell type characteristics. Pathologists consider the morphology of the prostate zones alike and also Laczkó et al. (8) showed for several cell type markers that there were no significant differences between the peripheral and transition zone. An indication that qualitative cell type characteristics play an important role is the discovery

of 3 genes (ASPA, FLJ10970 and COCH), which were higher expressed in the transition zone and were also exclusively expressed in stromal cells. Also the comparison with the study from Stuart et al (15) indicates a contribution of cell type characteristics when it revealed a significant overlap between genes higher expressed in the transition zone and genes associated with stroma. The interaction between stroma and epithelium is crucial in the progression of prostate

GenBank_Acc	Genes present on both arrays	Log2 ratio P/T
Peripheral zone		
NM_001935	DPP4	0.390481646
NM_000700	ANXA1	0.016763477
NM_004476	FOLH1	0.864793013
NM_004864	GDF15	1.709116833
NM_006475	POSTN	0.845773593
NM_005069	SIM2	flagged
NM_000166	GJB1	flagged
Transition zone		
NM_003239	TGFB3	-1.257835567
NM_002899	RBP1	-1.07198958
NM_003304	TRPC1	-0.359069209
NM_006159	NELL2	-2.413583788
NM_001321	CSRP2	-0.511974608
NM_000049	ASPA	-1.052217602
NM_006307	SRPX	-0.978049822
NM_005278	GPM6B	-0.748098383
NM_001928	DF	-1.360622651
M62402	IGFBP6	-0.730010298
NM_001146	ANGPT1	flagged

Table 3. Comparison of 346 differentially expressed genes from the prostate zones and the dataset from Stamey et al. (16). We could extract 40 genes from the dataset by Stamey et al. (Tables 3 and 4 (16)) that were differentially expressed between the normal prostate zones, of which 12 genes were higher expressed in the peripheral and 28 genes were higher expressed transition zone. 11 of the 28 genes from the transition zone were present on our oligoarrays, of which 9 were higher expressed in the transition zone in both datasets. 7 of the genes from the peripheral zone were present on our oligoarrays, of which 3 were higher expressed in the peripheral zone in both datasets. The table shows the 18 genes present on both arrays and the genes that overlap between the two studies. P, peripheral zone; T, transition zone.

cancer and BPH (9,11,17). Our data show that cell characteristics of stroma and epithelium and with that, the interactions between stroma and epithelium, may already be different in the two normal zones. Therefore, we could speculate

that these zonal differences in stromal-epithelial interactions form the basis of susceptibility of the distinct prostate zonal diseases.
There is a prominent overlap between the genes that were higher expressed in the normal peripheral zone and the genes that were reported to be higher expressed in PC (12-14). Dhanasekaran et al., Lapointe et al., and Singh et al. studied gene expression profiles of prostate confined PC as compared to gene expression in the normal prostate tissue. The comparison to our data revealed a set of genes that are higher expressed in normal peripheral tissue and PC in all datasets. Also the tumor-associated genes from the dataset from Stuart et al. overlapped with the genes higher expressed in the peripheral compared to transition zone. This significant overlap can be interpreted in two ways. First, the gene expression signature of the normal peripheral zone remains dominantly present in PC and simply displays its origin. Second however, the unique expression profile within the peripheral zone could demonstrate a zonal-specific microenvironment in which malignant rather than benign growth is supported. This implies that the genes that are up-regulated in prostate cancer are already highly expressed in the peripheral zone. In other words, this set of genes might be involved in origin and progression of PC.

Two of the genes that were higher expressed in the peripheral zone were GDF15 and TARP. Growth differentiation factor 15 (GDF15 also known as PLAB, PDF, MIC-1) is a growth factor and member of the bone morphogenetic protein family. Relatively little is known about the specific function of GDF15, but a number of factors link it to epithelial tumors. Liu et al (18) demonstrated that GDF15 secretion was markedly increased in various PC cell lines and suggest a role for GDF15 in tumor dissemination. Also several others demonstrated that GDF15 is higher expressed in PC than in normal tissue (19,20). Furthermore GDF15 is strongly induced by p53 and it is up-regulated by androgens (21). TARP is another example of a gene that is higher expressed in the peripheral zone and PC. Like GDF15, TARP is up-regulated by androgens (22). Expression of TARP in PC3 cells resulted in a more rapid growth rate and differential gene expression in these prostate cancer cells (23). The higher expression of GDF15 and TARP in the normal peripheral zone and their potential role in PC, would support the hypothesis of the presence of a predisposing “cancer susceptibility signature” in the peripheral zone.

Since the malignant transformation of the transition zone is infrequent, the distinct expression profile within this zone might have a protective function with specific genes and pathways involved in anti-tumor processes. Two important development-regulating pathways of which several genes were found higher

expressed in the transition zone, include the Wnt and forkhead pathways. Wnt secreted proteins are essential mediators of cell-cell signaling during development and play a role in cell fate control and homeostasis. Importantly, deregulation of the Wnt pathway has been associated with cancer development and progression (24). Wissmann et al. (25) studied RNA expression levels of 40 genes of the Wnt pathway in primary prostate carcinomas and observed various genes to be up- and down-regulated in certain prostate cancers. These data further substantiated our notion of a complex role of tumor progression and tumor suppression for the Wnt pathway, which is not yet fully comprehended. Wnt proteins and beta-catenin play a role as mediators of epithelial-mesenchymal interactions in various organs. We observed higher expression of several genes of the Wnt pathway in the transition zone of the prostate and hypothesize that this pathway might contribute, via stromal-epithelial interactions, to the distinction of the benign and malignant disease development in the transition and peripheral zone. Next to the Wnt pathway, we observed that several members of the forkhead gene family were higher expressed in the transition zone. The forkhead genes encode a family of transcription factors, which are regulators of development. During embryogenesis, many tissues and cell populations express particular forkhead proteins (26). Forkhead family member FOXO3a can induce a delay in the G_2-M phase of the cell cycle, in response to stress stimuli during which FOXO3a contributes to repair of damaged DNA. It was also hypothesized that under high levels of stress it might induce a program of cell death (27). Alvarez et al. (28) demonstrated that forkhead transcription factors have a function in the control of mitotic gene expression, conserved from yeast to mammals. Both studies and more, indicate a protective role of the forkhead genes in the process of oncogenic transformation. Interestingly, two of the forkhead genes that were higher expressed in the transition zone, FOXF1 and FOXF2, were also higher expressed in mesenchyme compared to epithelium during embryogenesis, and may have functions in epithelial-mesenchymal cross-talk (29). The identification of zonal-specific expression of genes of the Wnt and forkhead pathways, strengthens our concept that stromal-epithelial interactions are important in the dissimilarity of the prostate zones. In addition, both these pathways may contribute to the development of benign disease and protection against frequent malignant transformation of the transition zone of the prostate.

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Chapter

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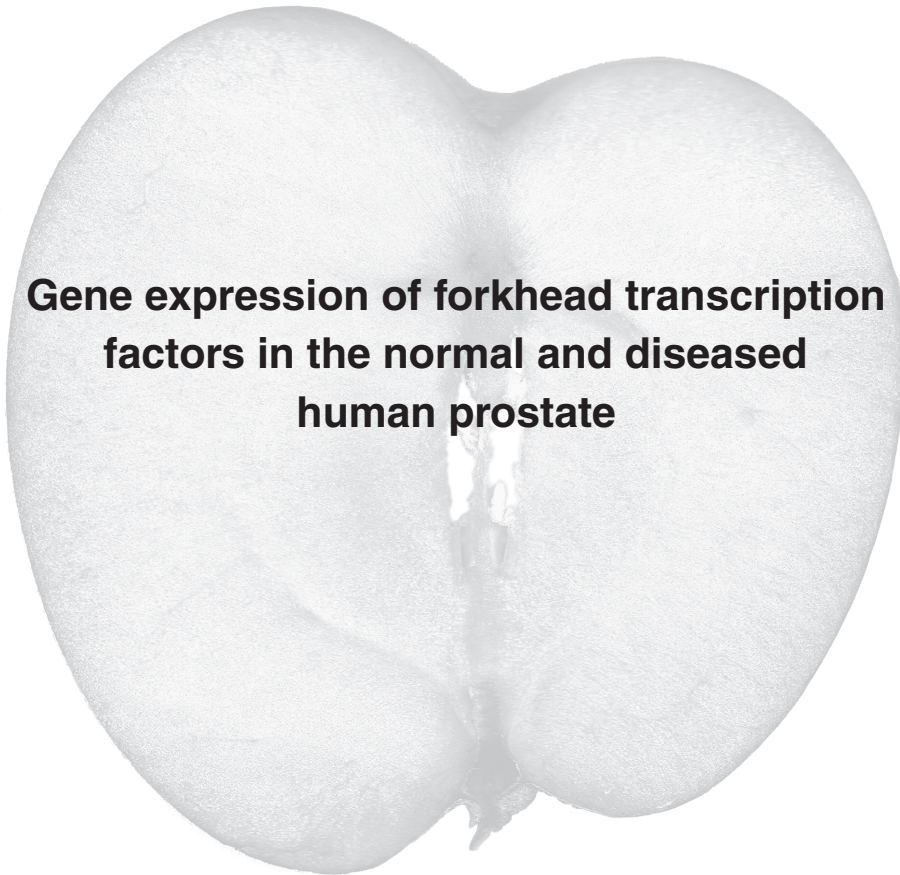

Gene expression of forkhead transcription factors in the normal and diseased human prostate

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Abstract

Objective. Since the first forkhead transcription factor (Fox) was identified, its family members have been implicated in a variety of cellular processes including embryonic development and disease. Here, we focus on the unexplored expression of forkhead transcription factors in normal prostate and prostate diseases.

Material and Methods. We analyzed a set of 12 different forkhead transcription factors by quantitative RT-PCR in the prostate zones, prostate cancer, lymph node metastases, benign prostatic hyperplasia (BPH), xenografts and several prostate cell lines.

Results. Striking differences between the expression of various forkhead family members were observed. Most prominent were the high expression of FOXF1 and FOXF2 in the normal prostate transition zone and BPH and their decreased expression in prostate cancer. Interestingly, although the FOXF genes are stroma-specific, some of the androgen-independent prostate cancer xenografts uniquely express these two genes. FOXD1 and FOXD2 were higher expressed in prostate cancer and lymph node metastases. FOXA1 and FOXC1 have an opposite expression pattern with respect to androgen-dependent growth of prostate cancer cell lines and xenografts.

Conclusions. Various members of the Fox family are differentially expressed in the zones of the normal prostate and in benign and malignant outgrowths. The expression profiles of FOXF1 and FOXF2 genes suggest a role in epithelial to mesenchymal transition (EMT), while FOXA1 and FOXC1 expression is linked to androgen-associated growth status of cancer.

Introduction

It has been more than a decade since discovery of the Drosophila transcription factor forkhead and subsequent identification of the mammalian orthologues of the forkhead DNA binding domain (1). Forkhead genes encode a subgroup of helix-turn-helix class of proteins. The arrangement of loops connecting the β strands that flank one of the three α helices, gives rise to a butterfly like appearance, hence the name "winged-helix" transcription factors (2). It is a relatively invariant structure, with most amino acids being conserved between family members. This has made it difficult to understand the molecular mechanisms underlying the sequence specificity of the DNA-binding domains. All forkhead factors can bind DNA and the functional effect of this can be either the activation or the inhibition of gene transcription. In contrast to the DNA-binding domains, there is almost no sequence homology between the transactivation or repression domains of members of the forkhead family, and little is known about their interactions with the transcriptional machinery (3). The forkhead family has been implicated in a variety of cellular processes and they play an important role in embryonic development and disease. Several forkhead factors, like FOXP3, FOXN1, FOXJ1 and members of the FOXO subfamily have crucial roles in various aspects of the immune system (4,5). Lehmann et al. (6) suggested the importance of forkhead factors in disease and development and speculated that soon regulators and downstream target genes of forkhead transcription factors will be discovered to explain a range of human diseases.

Little is known about the role of the forkhead family in the developing and adult prostate. Immunohistochemical localization of Foxa1 and Foxa2 revealed epithelial nuclear staining of both members in the developing mouse prostate, but only Foxa1 in the adult mouse prostate (7,8). Foxa1 is essential for full prostate ductal morphogenesis as was demonstrated using Foxa1-deficient mice (8). During prostate cancer progression, FOXA1 remains highly expressed in the cancer epithelium, while FOXA2 is turned on in neuroendocrine small cell-type carcinomas (9). A direct link between prostate cancer progression and Foxm1 was established by the observation that transgenic Foxm1 mice, crossbred with the TRAMP and LADY prostate cancer mouse models, had accelerated development and growth of prostatic tumors (10).

The role of various Fox genes in prostate cancer progression might be explained by their interaction with the androgen receptor (AR) pathway. The AR is a nuclear receptor that is activated upon testosterone or dihydrotestosterone binding and generally signals growth of prostate cancer cells (11). Besides the above mentioned FOXA1 and FOXA2, also FOXG1, FOXH1, FOXO1 and FOXO3 affect the AR cascade. The general theme is that these Fox proteins (all except FOXO3)

repress AR activity by directly binding the AR protein. Takayama et al. support this idea and show that FOXP1 is an androgen-responsive transcription factor that negatively regulates AR signaling in prostate cancer cells (12).
In our previous study, it was found that forkhead transcription factors are differentially expressed between the prostate zones (13). FOXF1 and FOXF2 are higher expressed in the transition zone as compared to the peripheral zone. This observation initiated our interest in the potential role of Fox genes in prostate disease development since benign prostatic hyperplasia (BPH) and prostate cancer preferentially occur in the transition and peripheral zone, respectively. In order to identify which forkhead genes are expressed in the prostate and relate their expression to prostate diseases and cell types, we investigated expression of 12 different forkhead transcription factors by real time RT-PCR.

Materials & Methods

Samples and cell lines

The prostate specimens were obtained from patients who underwent a radical prostatectomy at the Department of Urology, Erasmus MC. The experimental protocols were approved by the Erasmus MC Medical Ethics Committee according to the Medical Research Involving Human Subjects Act. Prostate samples include normal tissue from the transition and peripheral zones and benign prostatic hyperplasia (BPH) from a special protocol described previously (13). Prostate cancer samples from the prostate and from lymph node metastases (Table. 1) were obtained from the frozen tissue bank of the Erasmus Medical Center as described by Hendriksen et al. (14). The prostate samples used by different groups (15-19) in Figure 1 were all macrodissected. Macrodissection generally implies that immediately after surgery the pathologist macroscopically isolates different prostate samples. Presence and amount of cancer cells and other cell types are then evaluated using histology. One of the issues that need to be considered is that it is impossible to macrodissect prostate cancer samples without normal epithelium or stroma contaminating the sample. You would need to microdissect the samples to enrich for cancer tissue and separate cancer cells from normal epithelium and the stromal compartment.

However, since the stroma has been indicated to play a major role in cancer initiation and progression, it is of great importance to study prostate cancer cells in context with their surrounding stroma. In most gene expression studies including our own, macrodissected prostate cancer tissue was used to examine expression profiles of the whole tumor tissue including cancer cells and their

Table 1. Clinical characterization and follow up of patients

	normal prostate	organ-confined prostate cancer, no metastases	organ-confined prostate cancer, developed metastases
No. patients, unique	17	36	10
age at treatment, median (range)	61 (54-72)	61.5 (49-73)	64 (49-70)
Gleason score, n			
6		23	4
7		9	2
8-10		2	4
PSA at diagnosis (ng/ml) median (range)		9.2 (0.3-181)	32 (0.5-64.3)
epithelium in tissue (average %) (range)	68 (60-80)	80 (60-100)	81 (70-90)
cancer in tissue (average %) (range)	0	85 (70-100)	90 (70-100)

supporting environment such as fibroblasts, smooth muscle cells, endothelium, and more. In this study we demonstrate that the stroma-specific genes FOXF2 and FOXF1 are implicated in human prostate disease and if these samples were cancer-cell enriched by microdissection, we would not have found this association. It is essential to realize that if one wants to study gene expression changes in cancer development, to take all different cell types present in the tumor into consideration. Types of arrays used by different groups: Singh et al. (15) and Stuart et al. (16) used Affymetrix U95Av2 arrays for their experiments, Lapointe et al. (17) used cDNA array containing 26260 probes, Varambally et al. (18) used Affymetrix U133 2.0 arrays and Yu et al. used Affymetrix HG_U95A,B,C (19). Primary human prostate epithelial cells (PrEC) and primary human prostate stromal cells (PrSC) were purchased and cultured according to manufacturer's guidelines (Clonetics Human and Animal Cell Systems, Cambrex Bio Science Walkersvill, Inc., USA). To minimize adaptation and variation of cells, only early passages 5 and 7 were used for RNA isolation when they were 70% confluent. The LNCaP, PC3 and DU145 prostate cancer cell line was maintained in RPMI 1640 with 5% FCS and penicillin/streptomycin (Invitrogen, Merelbeke, Belgium). Before R1881 treatment, LNCaP cells were androgen deprived for 72 hours in medium containing 5% dextran-filtered, charcoal-stripped FCS with a medium replacement after 36 hours. After androgen deprivation, the medium was supplemented for 2, 4, 6, or 8 hours with 1 nmol/L R1881 or ethanol vehicle. The basic culture medium used in the maintenance of PC346 cell lines consisted of DMEM-F12 (Cambrex BioWhittaker, Verviers, Belgium) supplemented with 2% FCS (PAN Biotech, Aidenbach, Germany), 1% insulin-transferrin-selenium (GIBCO BRL, Gaithersburg, MD), 0.01% BSA (Boehringer-Mannheim, Mannheim, Germany), 10 ng/ml epidermal

growth factor (Sigma-Aldrich, Milan, Italy) and penicillin/streptomycin antibiotics (100 U/ml penicillin, 100 g/ml streptomycin; Cambrex BioWhittaker) plus the following additions: 100 ng/ml fibronectin (Harbor Bio-Products, Tebu-bio, the Netherlands), 20 g/ml fetuine (ICN Biomedicals, Zoetermeer, The Netherlands), 50 ng/ml cholera toxin, 0.1 mM phosphoethanolamine, 0.6 ng/ml triiodothyronine and 500 ng/ml dexamethasone (all from Sigma-Aldrich).

The in vivo growing xenografts PC82, PC133, PC135, PC295, PC310, PC324, PC329, PC339, PC346, PC346I, PC346B, PC346BI, PC374 and PC374F were propagated by serial transplantation on male nude mice as described (20,21). PC82, PC295, PC310, and PC329, derived from primary tumors or local metastases, are androgen-dependent. PC133, PC324, PC339, PC346, PC346I, PC346B, PC346BI, PC374 and PC374F are derived from distant metastases or local progressive disease and are androgen-independent (PC133, PC324 and PC339) or androgen-sensitive (PC346 and PC374). PC135 is androgen independent and is derived from a lymph node metastasis.

RNA isolation and quantitative real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA from prostate specimens and cell lines was isolated using RNAbee reagent as described by the manufacturer (Tel-Test, Inc. Friendswood, TX, USA). The reverse transciptase reaction was performed with 1 µg RNA from the samples with oligo-T12 primer and pre-incubated for 10 minutes at 70°C. First Strand buffer, DTT, dNTPs and RNAsin were added and incubated for 2 minutes at 37°C. The RT reaction was initiated by MMLV-RT and incubated for 1 hour at 37°C. After this, the reaction was kept 10 minutes at 90°C and immediately thereafter frozen.

Quantitative real-time RT-PCR analysis was done with an ABI Prism 7700 Sequence Detection System using AmpliTaq Gold according to the manufacturer's specifications (Applied Biosystems, Foster City, CA). The probes and primers for Taqman Gene Expression Assays were purchased by Applied Biosystems. The amount of target gene expressed was normalized to an endogenous reference and relative to a calibrator. The endogenous reference was glyceraldehyde-3-phosphate dehydrogenase; a mixture of cDNAs of the prostate carcinoma xenografts was used as the calibrator. The following primers were used: FOXA1, Hs00270129_m1; FOXC1, Hs00559473_s1; FOXJ2, Hs00218236_m1; FOXK2, Hs00189612_m1; FOXN1, Hs00186096_m1; FOXM1, Hs00153543_m1; FOXF1, Hs00230962_m1; FOXF2, Hs00230963_m1; FOXP1, Hs00415004_m1; FOXD1, Hs00270117_s1; FOXD2, Hs00270129_s1; GAPDH, Hs99999905_m1.

Statistical analysis for comparison of the RT-PCR data was performed using a paired t-test.

Results

Twelve different forkhead transcription factors (Fox) were analyzed by quantitative RT-PCR analyses in prostate cancer cell lines, xenografts, normal prostate tissues, BPH and prostate cancer tissue. Fox genes were selected based on differential gene expression in various microarray studies and published literature mentioned above with respect to cancer and AR interaction (Figure 1) (15-19).

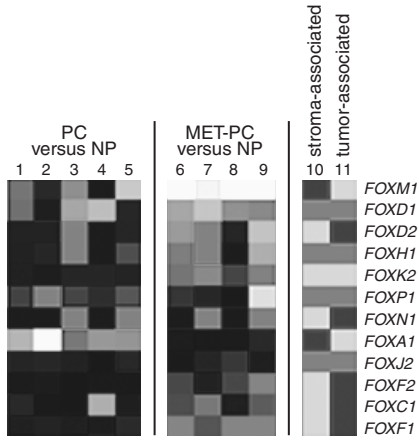


Figure 1. In silico expression analysis of several Forkhead transcription factors in microarray databases related to normal prostate and prostate diseases. Difference in expression in prostate cancer (PC) and metastasized prostate cancer (Met-PC) as compared to normal prostate tissue was based on microarray studies by Singh et al. (15) column 2, Lapointe et al., (17) columns 3 and 7, Varambally et al. (18) columns 4 and 8, Yu et al. (19) columns 5 and 9. Columns 1 and 6 are averages of these studies of PC versus normal and Met-PC versus normal, respectively. Expression ratios between PC versus normal prostate (NP) and MET-PC versus NP are shown in different intensity colours. Gene expression was ordered by Excel sorting from highest to lowest expression and illustrated by Treeview. Red indicates higher expression in PC or MET-PC as compared to NP. Green indicates the opposite. Stroma and tumor-associated expression of Fox genes are represented by columns 10 and 11 and based on a study by Stuart et al. (16). Red indicates a significant positive correlation and green a significant negative correlation between gene expression and amount of stroma or tumor.

Fox gene expression in prostate cancer cell lines and xenografts

The expression of the 12 Fox genes was investigated in different prostate cancer cell lines including the androgen receptor positive LNCaP and PC346C, and the androgen-independent PC3 and DU145 (Figure 2a). In addition, expression was determined in our panel of prostate cancer xenografts, consisting of androgen-dependent, sensitive and independent growing tumors (22,23). Since the prostate cancer xenografts contain mouse contaminants such as stroma, endothe-

lium and blood cells, each quantitative RT-PCR Fox gene assay was checked for cross-reactivity with RNA extracted from mouse sarcoma tissue. None of the RT-PCRs gave detectable signals on mouse tissues within the first 35 PCR cycles, showing human-specific gene expression detection (data not shown). In Figure 2a, the strict androgen-dependent PC82, PC295, PC310 and PC329 were compared to the AR negative PC133, PC135, PC324 and PC339. Some of the Fox genes have a xenograft or cell line-specific expression pattern, explaining the outsized error bars. FOXC1 is notably expressed in all androgen-independent xenografts, but extremely high in PC135. Also FOXN1 is mostly undetectable, except for high expression in DU145 and PC135. FOXP1 is a unique outlier with high expression in PC295 (more information on individual cell lines and xenografts in supplementary Table 1).

One of the most striking observations in the comparison between androgen-dependence and independence is the inverse expression pattern of FOXA1 and FOXC1. While FOXA1 is higher expressed in androgen-dependent cell lines and xenografts, FOXC1 reveals an opposite association (Figure 2b). Also expression of FOXF1 and FOXF2 stands out by the unique expression in some androgen-independent xenografts (Figure 2c).

Whether any of the Fox genes is directly androgen regulated was determined by gene expression analysis of LNCaP cells cultured for 8 hours in the absence or presence of 1 nM R1881. None of the 12 Fox genes showed a significant difference upon androgen treatment, which is in agreement with published expression microarray data that so far, did not identify Fox genes as potential AR targets in LNCaP cells (24). In contrast, Takayama et al. recently published that FOXP1 is an androgen-responsive transcription factor that negatively regulates AR signaling in prostate cancer cells (12).

Fox gene expression in primary prostate epithelial and stromal cells

Primary human prostate stromal cells (PrSC) and primary human prostate epithelial cells (PrEC) were used to study the expression of Fox genes in normal prostate stroma and epithelium (Figure 3). FOXF1 and FOXF2 were highly expressed in PrSC, but not in PrEC, which confirms previous findings that these genes are highly stroma-specific (25). FOXH1, FOXA1 and FOXP1 were not expressed, while the other Fox genes tested, show low expression with no or minor differences between PrSC and PrEC.

	Origin	Androgen response	Androgen Receptor expression	FOXF2	FOXF1	FOXA1	FOXC1	FOXH1	FOXM1	FOXN1	FOXP1	FOXJ2	FOXD1	FOXD2	FOXK2
Cell lines															
LNCaP	Lymph node	AD	Yes	0.00	0.00	0.69	0.03	0.28	0.05	0.01	0.36	0.51	0.12	0.16	0.14
PC346C	Primary PC346P xenograft	AD	Yes	0.00	0.00	1.40	0.07	0.92	0.48	0.23	0.00	1.46	0.05	1.06	0.25
PC3	Bone	AI	No	0.00	0.00	0.07	0.56	0.10	0.34	0.00	0.00	0.38	1.42	0.54	0.21
DU145	Brain	AI	No	0.01	0.00	0.07	1.61	0.00	0.62	2.23	0.00	0.59	1.28	0.49	0.28
Xenografts															
PC82	Primary	AD	Yes	0.00	0.00	0.50	0.04	0.00	0.11	0.00	0.11	0.28	0.17	0.78	0.26
PC295	Lymph node	AD	Yes	0.01	0.00	1.72	0.07	0.78	1.06	0.00	3.56	1.17	0.83	1.17	0.78
PC310	Primary	AD	Yes	0.04	0.00	1.01	0.08	0.30	0.25	0.00	0.20	1.27	1.06	0.25	0.12
PC329	Primary	AD	Yes	0.00	0.01	0.79	0.03	0.06	0.03	0.00	0.26	0.74	0.03	0.04	0.98
PC133	Bone	AI	No	3.05	0.02	0.18	0.22	0.35	0.63	0.23	0.21	0.86	2.60	0.95	0.70
PC135	Primary	AI	No	0.00	0.00	0.07	10.81	0.62	0.22	6.22	0.01	0.65	0.78	2.49	0.46
PC324	Primary	AI	No	0.13	2.46	0.27	2.78	0.08	0.70	0.00	0.00	0.43	2.26	3.48	1.13
PC339	Primary	AI	No	0.00	0.00	0.00	0.39	0.06	0.06	0.00	0.07	0.12	0.26	0.46	0.28

Supplementary Table 1. Ratio data real-time PCR (Expression/GAPDH). Gene expression of 12 different forkhead transcription factors in androgen-dependent cell lines (LNCaP and PC346C), androgen-independent cell lines (PC3 and DU145), androgen-dependent xenografts (PC82, PC295, PC310 and PC329) and androgen-independent xenografts (PC133, PC135, PC324 and PC339). LNCaP data shown are averages of two independent studies. Origin of cell lines and xenografts, Androgen responsiveness (AD: androgen dependent, AI: Androgen independent) and androgen receptor (AR) expression are indicated.

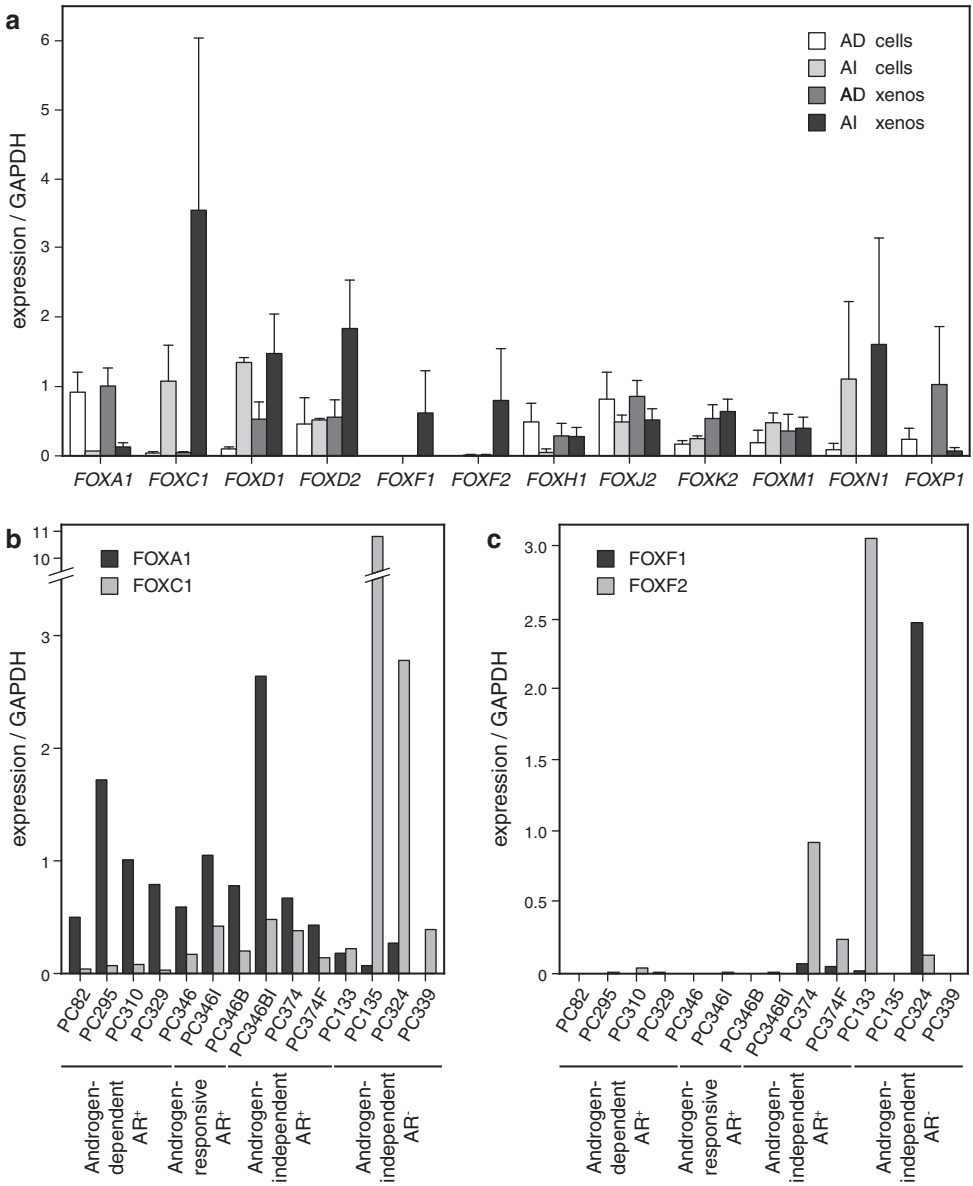


Figure 2. a) Expression analysis of Fox genes in various prostate cancer androgen-dependent (AD cells) and independent (AI cells) cell lines and xenografts (AD xenos and AI xenos). Expression of Fox genes was investigated by quantitative RT-PCR analyses of RNA extracted from androgen-dependent cell lines (LNCaP and PC346c), androgen-independent cell lines (DU145 and PC3), androgen-dependent xenografts (PC82, PC295, PC310 and PC329) and androgen-independent xenografts (PC133, PC135, PC324 and PC339). Data are represented as averaged ratio of Fox gene expression/GAPDH expression (±SEM).
b) Gene expression analyses of FOXA1 and FOXC1 in human prostate cancer xenografts; androgen-dependent, AR positive (AR+); androgen-responsive, AR+; androgen-independent,

AR+ and androgen-independent, AR negative (AR-). Expression is represented as ratio of FOXA1 or FOXC1 gene expression/GAPDH expression. **c)** Gene expression analyses of FOXF1 and FOXF2 by quantitative RT-PCR in several types of human prostate cancer xenografts. Expression is represented as ratio of FOXF gene expression/GAPDH expression.

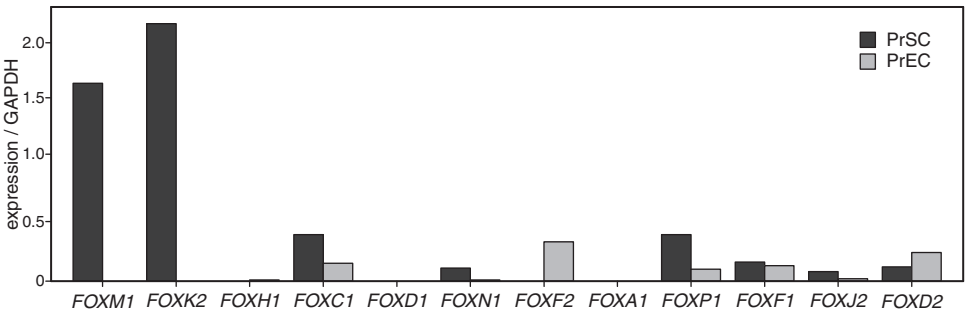


Figure 3. Gene expression analyses of several Fox genes in human prostate primary stromal (PrSC) and epithelial cells (PrEC). Expression is represented as ratio of Fox gene expression /GAPDH expression.

Fox gene expression in normal prostate and prostatic diseases

In order to identify differential expression of the selected Fox genes between the normal prostate, benign prostatic hyperplasia (BPH) and prostate cancer (PC), RNA was extracted from the normal prostate transition zone (TZ) and peripheral zone (PZ) of 7 radical prostatectomy samples and from 5 samples of BPH, 10 samples of organ-confined PC and 6 PC lymph node metastases (LN) (Figure 4).

Expression of FOXH1 and FOXM1 was in all cases very low and not significantly different between the various groups (data not shown).

The quantitative RT-PCR confirmed our previous finding that FOXF1 and FOXF2 are higher expressed in the TZ as compared to the PZ (12). FOXA1, FOXC1, FOXJ2 and FOXK2 were equally expressed between the prostate zones. FOXP1 was higher expressed in PZ, while FOXD1 and FOXD2 were expressed at a very low level in the normal prostate.

With respect to prostate diseases, most Fox genes are somewhat higher expressed in BPH as compared to the expression in normal TZ in which the benign enlargement originates (FOXC1, FOXP1, FOXJ2 and FOXK2) (Figure 4). Both FOXF1 and FOXF2 remain highly expressed in BPH but not different from TZ. The PC malignant growths, revealed a more differential pattern. FOXD1 and FOXD2 are up-regulated in PC and LN as compared to gene expression in the normal PZ. On the other hand, FOXF1 and FOXF2 show a consistent down regulation upon progression of the malignant disease.

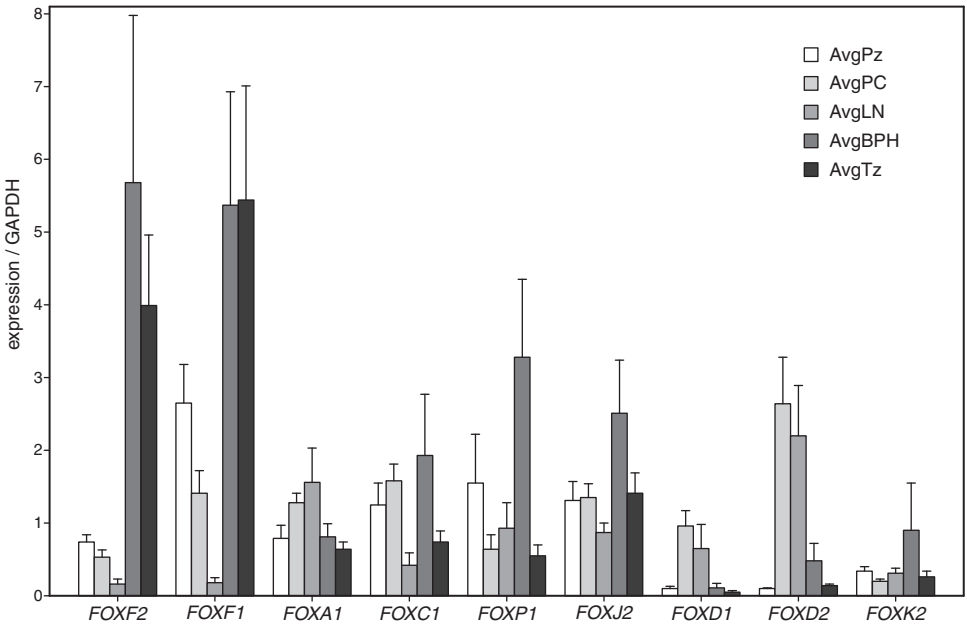


Figure 4. Gene expression of several Fox genes in prostate cancer (PC), benign prostatic hyperplasia (BPH), lymph node metastasis (LN), peripheral zone (PZ) and transition zone (TZ).

Predictive value of Fox gene expression in radical prostatectomy samples

Since the expression pattern of FOXF1 and FOXF2 in PZ, PC and LN are opposite from FOXD1, we investigated if any of these genes or their combination could predict whether local cancer after radical surgery recurs (Figure 5). FOXF1 and FOXF2 were significantly higher expressed in normal prostate (n=17) as compared to local prostate cancer (n=46) confirming the data shown in Figure 4. Also the higher expression of FOXD1 in PC as compared to normal prostate was substantiated. However, there was no expression difference of these three Fox genes between men that did (n=10) or did not (n=36) develop metastases after radical prostatectomy.

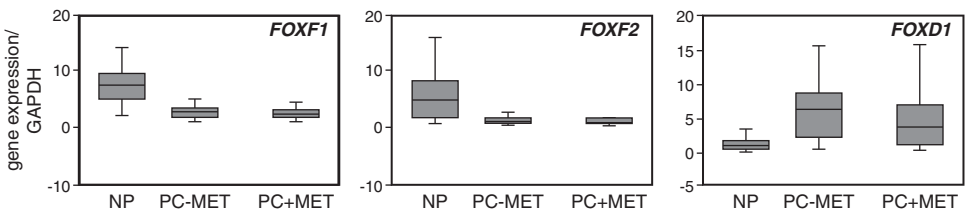


Figure 5. Expression comparison of FOXF1, FOXF2, FOXD1 in normal prostate (NP) and organ-confined prostate cancer in patients that did (PC+MET) or did not (PC-MET) develop

metastases after radical prostatectomy. FOXF1 and FOXF2 are significantly higher expressed in NP compared to PC+/-MET. FOXD1 is highest expressed in PC+MET compared to NP and is also higher expressed in PC-MET compared to NP. In each comparison NP is significantly different from PC + and – MET ($P<0.05$).

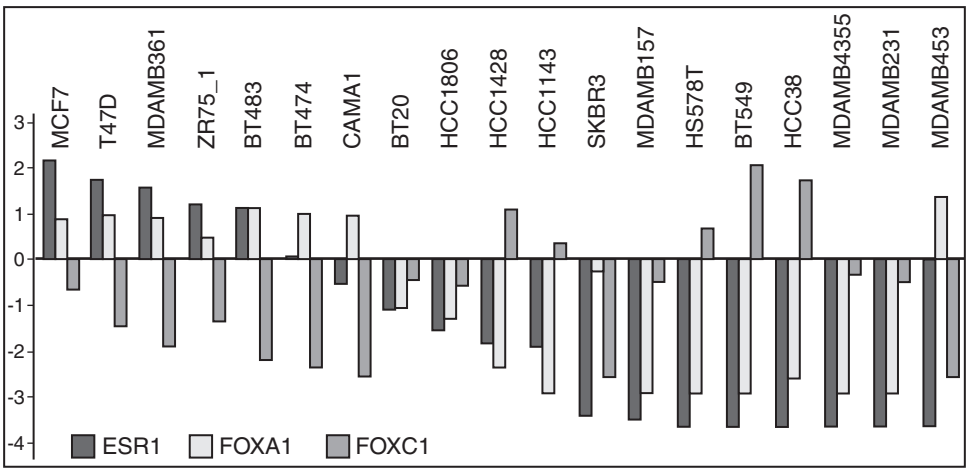
Discussion

The aim of this study was to get a better understanding of the expression and potential roles of forkhead transcription factors in the healthy and diseased prostate. Although the importance of Fox genes in development and disease is well established, their potential role in prostate development and outgrowths is poorly studied (4,5,26). Twelve different Fox genes were selected and gene expression was studied by quantitative RT-PCR analyses in various prostate tissues.
Several of the selected Fox genes have a clear differential expression pattern in the comparisons presented. FOXF1 and FOXF2 were selected based on their association to stroma, lower expression in prostate cancer (Figure 1) and their significant higher expression in TZ as compared to PZ and (13). We confirmed that expression is decreased in prostate cancer andprostate cancer metastases and showed that FOXF expression is stroma-specific. Taken together, it appears that FOXF1 and FOXF2 are involved in regulating normal prostate homeostasis through the stromal compartment, particularly in the TZ. This role for FOXF genes was already suggested for mice in murine gut development and during mouse and rat embryogenesis (27). Like for mouse gut development, we suggest that these genes are involved in stromal-epithelial interaction. Importantly, the FOXF genes might also play a role in epithelial-mesenchymal transition (EMT) since the stroma-specific FOXF genes are expressed uniquely in several late stage androgen-independent xenografts.

FOXD1 is higher expressed in prostate cancer and lymph node metastases as compared to normal prostate. We investigated whether this opposite expression pattern of FOXF1/FOXF2 and FOXD1 would be different between primary tumors that will or will not form metastatic disease after radical prostatectomy with intention to cure. A more extensive quantitative RT-PCR analysis clarified that their expression had no prognostic value.

It is suggested that FOXA proteins play a role in development and progression of prostatic adenocarcinoma (9). Average FOXA1 expression was higher in peripheral zone, prostate cancer and lymph node metastases as compared to BPH and transition zone. Due to considerable variability between the patient samples, this difference did not reach statistical significance. We observed an inverse

expression pattern of FOXA1 and FOXC1 between androgen-dependence and independence. FOXA1 is higher expressed in AR positive, androgen-dependent cell lines and xenografts, while FOXC1 reveals an opposite expression pattern. We extracted data from Bild et al. (28) and identified the same inverse expression pattern of FOXA1 and FOXC1 in estrogen receptor positive versus negative breast cancer cell lines (supplementary Figure 1). In agreement, FOXA1 was shown to be a prognostic marker for luminal subtype breast cancers by Thorat et al. (29). Also our data indicates a potential role for FOXA1 and FOXC1 as markers for androgen-dependent growth of prostate cancer.



Supplementary figure 1. Expression of estrogen receptor alpha (ESR1), FOXA1 and FOXC1 in breast cancer cell lines. Affymetrix gene expression data was retrieved from Bild et al (28) and it was normalized and mean centered for each of the genes. Cell lines were ordered from highest to lowest ESR1 expression. FOXA1 is always high expressed in ESR1 positive cell lines and poorly expressed in ESR1 negative cells. FOXC1 reveals an opposite expression pattern. As shown in Figure 2b, this expression pattern is highly similar to the expression correlation of the AR and FOXA1/FOXC1 in prostate cancer xenografts.

This inventory of the expression of 12 Fox genes in several prostate tissues further provided evidence for a role of forkhead transcription factors in prostate maintenance and abnormal outgrowths. FOXF genes and FOXD1 are differentially expressed between normal and cancer, while FOXA1 and FOXC1 expression changes during PC progression, indicating their involvement in regulating the balance between normal and diseased tissue.

Acknowledgements

We thank prof. dr. Theo van der Kwast and dr. Arno van Leenders for their histopathological examination of the prostate samples and dr. Wytske van Weerden for providing the prostate cancer xenograft samples.

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Chapter

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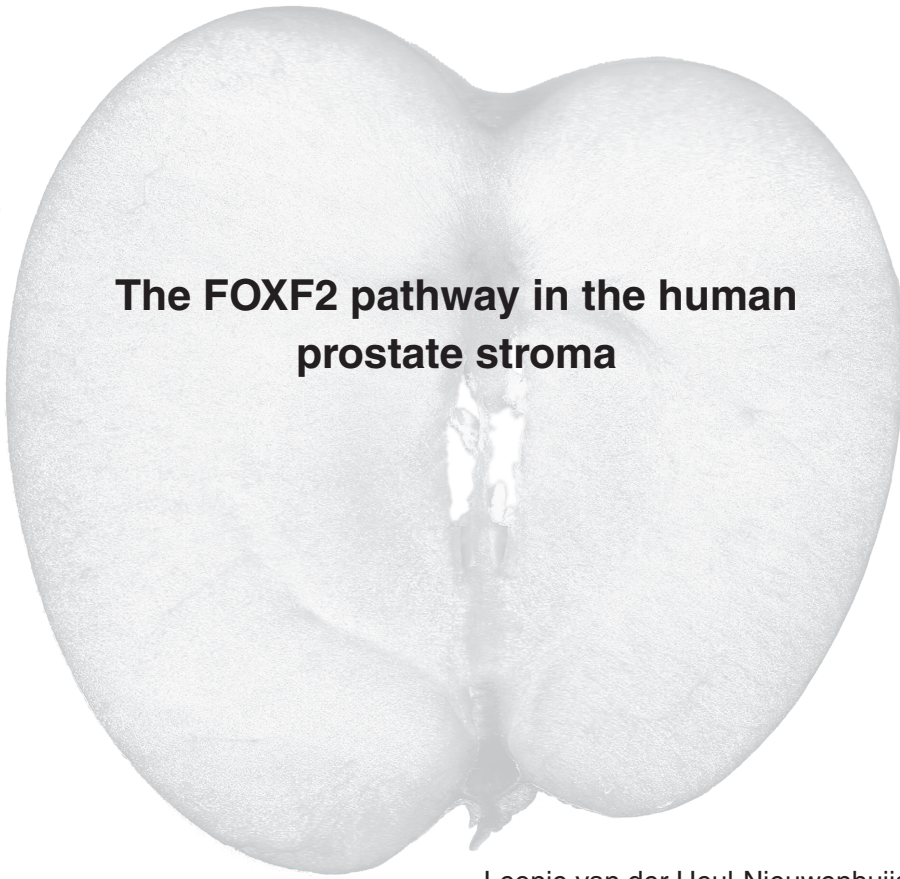

The FOXF2 pathway in the human prostate stroma

Leonie van der Heul-Nieuwenhuijsen
Natasja Dits
Wilfred Van IJcken
Don de Lange
Guido Jenster

Submitted

Abstract

Background. FOXF2 is a member of the large family of forkhead transcription factors and its expression pattern suggests a role in prostate cancer development. FOXF2 expression is stroma-specific and higher expressed in the prostate transition zone than the prostate peripheral zone. Moreover, expression of FOXF2 is decreased in prostate cancer.

Methods. To identify the genes and pathways regulated by FOXF2, we compared microarray expression profiles of primary prostate stroma cells (PrSC) treated with control or siRNA directed against FOXF2.

Results. From our microarray analyses, we selected 190 differentially expressed genes, of which 104 genes were higher expressed in PrSC cells treated with FOXF2 siRNA and 86 were higher expressed in PRSC cells treated with negative control siRNA. Eight of the strongest differentially expressed genes were validated by RT-PCR. Genes down-regulated by FOXF2 included MT1E, MT1F, PDGFA, ITGB1 and PSG7 and genes up-regulated by FOXF2 included WASF2, BAMBI and CXCL12. Ingenuity pathway analysis showed several pathways significantly regulated by FOXF2, including PPAR signaling, PDGF signaling and ECM signaling. GSEA analysis revealed that FOXF2 up-regulated genes were down-regulated in the same PrSC cells treated with TGFβ3.

Conclusions. The distinct expression pattern of FOXF2 in the prostate, its effect on expression of extracellular matrix signaling, and its opposing role in the TGFβ3 pathway, suggests a role for FOXF2 in prostate homeostasis and stroma-epithelial interactions.

Chapter

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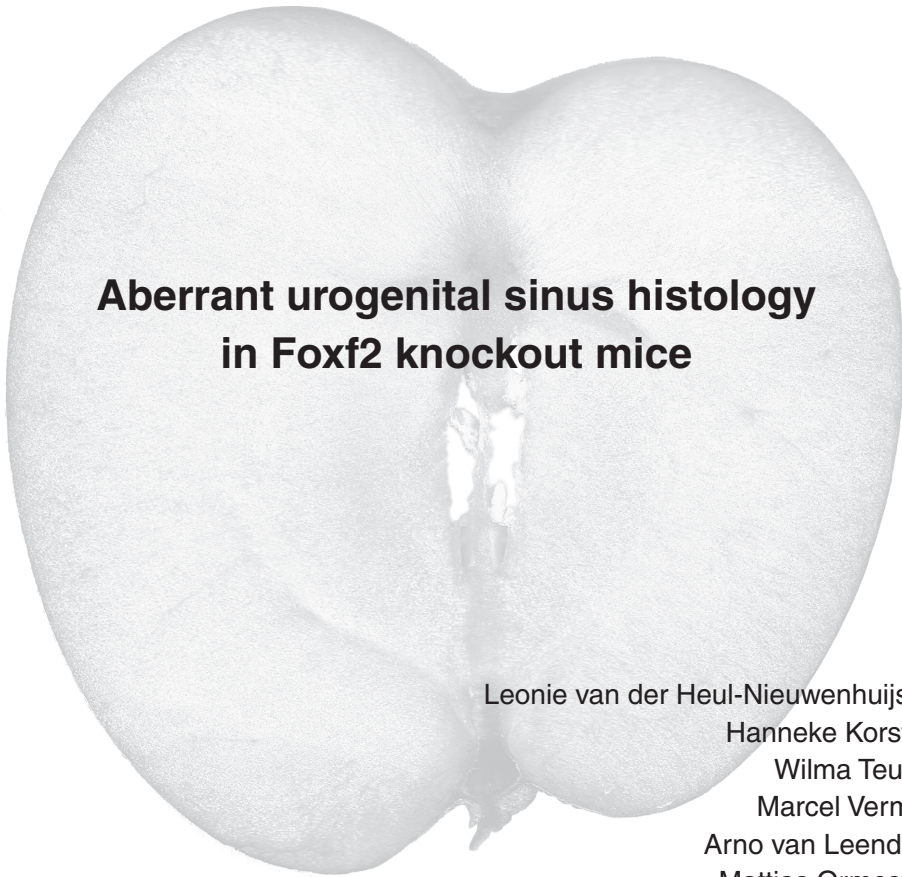

Aberrant urogenital sinus histology in Foxf2 knockout mice

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Manuscript in preparation

Abstract

Foxf2 knockout mice give rise to a gene specific phenotype in which the embryos develop to term and die within hours after birth due to a cleft in the secondary palate preventing proper feeding and breathing. After exploring FOXF2 gene expression and FOXF2 regulated pathways in human adult prostate in previous studies, the potential role of Foxf2 in prostate development was investigated. To accomplish this, histology of the urogenital sinus (UGS) of wild-type mice and Foxf2 knockout mice was compared. Overall, no significant differences in origin of UGS, like urethra and prostatic bud formation, were observed. However, at cellular level considerable differences were observed between the wild-type mice and Foxf2 knockouts. The stromal compartment in Foxf2 knockouts showed less extracellular eosinofilic matrix, which indicates a role for FOXF2 in attachment of proteins to the cell-matrix. Furthermore, the extracellular matrix seemed disordered and also the epithelial cells of the prostate ducts appeared less well organized. These findings are in concurrence with our recent microarray data on human prostate target genes of FOXF2, in which we established that various extracellular matrix (ECM) proteins are regulated by FOXF2. The data are also in agreement with the abnormal gut phenotype, in which extracellular matrix, particularly collagens, were severely reduced in Foxf2 mutant intestine, which caused epithelial depolarization and tissue disintegration. Apparently, Foxf2 is not involved in prostate formation, but might control stromal-epithelial interactions.

Introduction

Evidence accumulates that carcinogenesis often involves deregulation of pathways that are important in development (1). Understanding how the mammalian prostate develops and functions will contribute to a better understanding of prostate cancer. In human prostate, differential gene expression was discovered between the prostate zones, which indicates, that predestination of prostate diseases originates in the prostate zones (2). FOXF2 was highly expressed in the transition zone of the prostate, stroma specific and it was higher expressed in normal prostate compared to prostate cancer. FOXF2 was also considered to be involved in epithelial to mesenchymal transition since it was highly expressed in androgen-independent xenografts (3). These findings suggest an important role for FOXF2 in stroma-epithelial cross-talk. After exploring FOXF2 gene expression and FOXF2 regulated pathways in human adult prostate in previous studies, we wanted to identify the potential role for Foxf2 in prostate development. An important tool to study the effect of loss of a gene of interest in organ development is the knockout mouse model. We made use of Foxf2 knockout

mice, which give rise to a gene specific phenotype, in which the Foxf2 mutants develop to term and die within hours after birth due to a cleft in the secondary palate preventing proper feeding and breathing (4). Furthermore, Foxf2 mutants suffer from gastrointestional defects. Extracellular matrix, especially collagens, was severely reduced in Foxf$^{-/-}$ intestine, which caused epithelial depolarization and tissue disintegration. Ormestad et al. concluded that Foxf proteins are mesenchymal factors that control epithelial proliferation and survival and link hedgehog to Bmp and Wnt signaling (5). Aitola et al. suggested that Foxf2 is expressed in mesenchyme of tissues where stromal-epithelial interaction is needed for morphogenesis and cell differentiation (6). Foxf2 is highly expressed in urinary tract and is expressed adjacent to the transitional epithelium (7). The prostatic buds begin to appear from urogenital epithelium at approximately E17 and develop and branch within the urogenital mesenchyme (8). We studied the urogenital sinus (UGS) of E18.5 embryos of Foxf2$^{-/-}$ and wild-type mice, in particular the prostatic ducts and neighboring mesenchyme. To investigate whether changes in expression of Foxf2 in prostate development is associated with FOXF2 regulated genes in men; we performed immunohistochemical staining of metallothioneins and laminins.

Material & Methods

Immunohistochemistry

Wild-type and Foxf2$^{-/-}$ E18.5 embryos were obtained from Dr. P. Carlsson, Lundberg lab, Gothenburg, Sweden and stored in formalin (4). Tissues for immunohistochemistry were fixed in freshly prepared buffered 4% formalin for approximately 16 h at room temperature, dehydrated and embedded in paraffin. The UGS was isolated from the mouse embryo before paraffin sections were made. Sections were cut at 4 µm and attached to AAS-coated slides. Antibodies used for immunostaining were directed against Laminin (M0638, DAKO), Metalothionein (M0639, DAKO). Microwave treatment for antigen unmasking was applied by boiling in 10 mM sodium citrate (pH 6.0) for 15 minutes and cooling at room temperature for 30 minutes. Primary antibodies were incubated overnight at 4°C, and incubation with biotin labeled secondary antibodies was performed for 1 h at room temperature (Goat Anti-Mouse-biotin (E0433, 1:400, DAKO) or Rabbit Anti-Goat biotin (SC-2774, 1:400, Santa Cruz). Immunoreactivity was visualized by streptavidin-peroxidase incubation (HK320-UK, 1:50, BioGenex, San Ramon, CA).

Results

Histological analysis of the UGS in Foxf2 knockout and wild-type mice

The UGS was investigated in Foxf2 knockout mice to study the phenotypic changes. H&E stained paraffin sections of 5 different 18,5E embryos were microscopically analyzed in wild-type mice versus Foxf2 mutants. Prostatic buds arise from the urethra and are surrounded by stroma. Phenotypic differences were found between the Foxf2$^{-/-}$ and wild-type mice (Figure 1). The stroma compartment in Foxf2 knockouts show less extracellular eosinofilic matrix. The stroma appears chaotic and gaps between the cells dominate the area (a). Cells surrounding the epithelial ducts detached in several Foxf2 mutants (b) and several parts of the epithelial compartment, like the prostate ducts (c), appeared less organized than in wild-type mice.

Laminin expression in the UGS of wild-type and Foxf2$^{-/-}$ mice

Laminin consists of a large family of proteins which are components of extracellular matrix. The main non-collagenous component of the basal lamina is laminin, which forms the sheets of protein that form the substrate for of all internal organs. Laminin proteins are important because they form sheets that holds overall body structures together (9). Previously, it was observed that FOXF2 regulates expression of several ECM proteins, including lama1 and lamc2, which were down-regulated by FOXF2. For this reason laminin was analyzed by immunohistochemistry in the UGS. We observed that knockdown of Foxf2 resulted in distorted ECM. Laminin appears to be higher expressed in the cytoplasm of the stromal compartment of Foxf2$^{-/-}$ mice compared to wild-type mice (Figure 2).

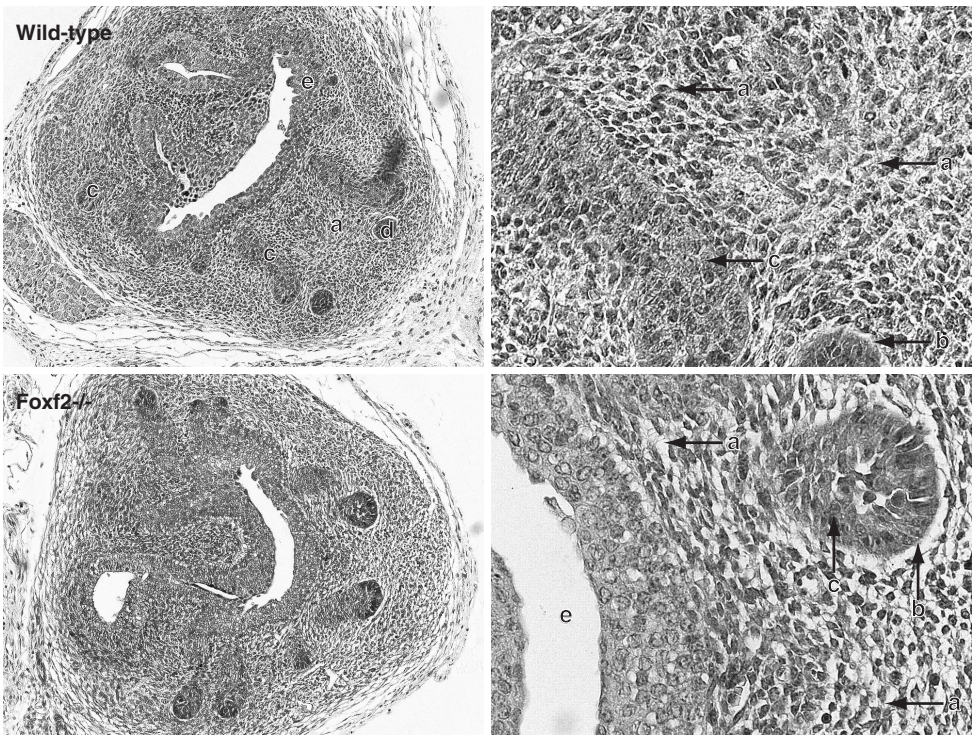

Figure 1. Phenotypic differences of the urogenital sinus in Foxf2 mutant compared to wild-type mice. Arrows indicate: a stroma, b cell layer around epithelial prostate ducts, c prostate ducts, d seminal vesicles, e urethra. Left 100x and right 400x magnification.

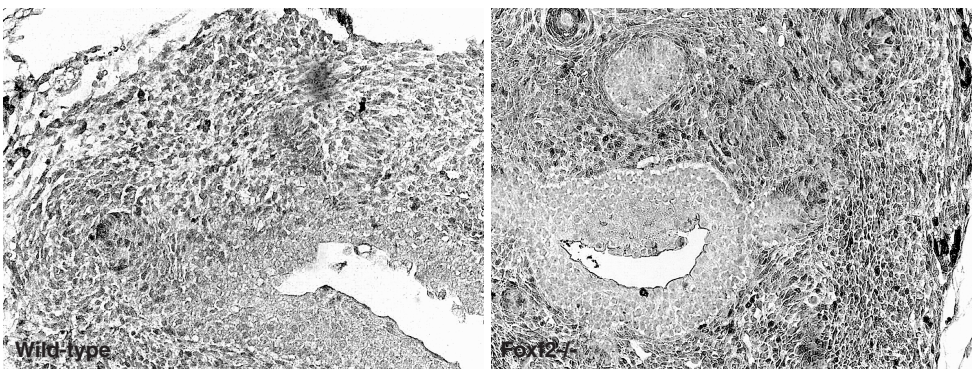

Figure 2. Laminin expression in Foxf2 mutant and wild-type mice. Expression of laminin in the stromal compartment around the prostatic ducts is higher in Foxf2$^{-/-}$ mice compared to wild-type mice. Magnification 200x

Metallothionein expression in the UGS of wild-type and Foxf2$^{-/-}$ mice

Metallothioneins (MTs) have been reported to be differentially expressed in tumors and a specific role for MTs in prostate oncogenesis was suggested by Moussa et al (10,11). Within the metallothionein gene locus on chromosome 16 MT1E, MT2a, MT1F, MT1G, MT1H, MT1X were down-regulated by FOXF2 in human prostate stromal cells (chapter 3). To investigate their protein expression in prostate histology, we analyzed metallothionein in 2 different Foxf2$^{-/-}$ and wild-type mice. Metallothioneins are higher expressed in the prostate ducts, as compared to the surrounding mesenchyme, of Foxf2$^{-/-}$ mutants compared to wild-type mice (Figure 3). This is in concordance with the previous findings in adult prostate.

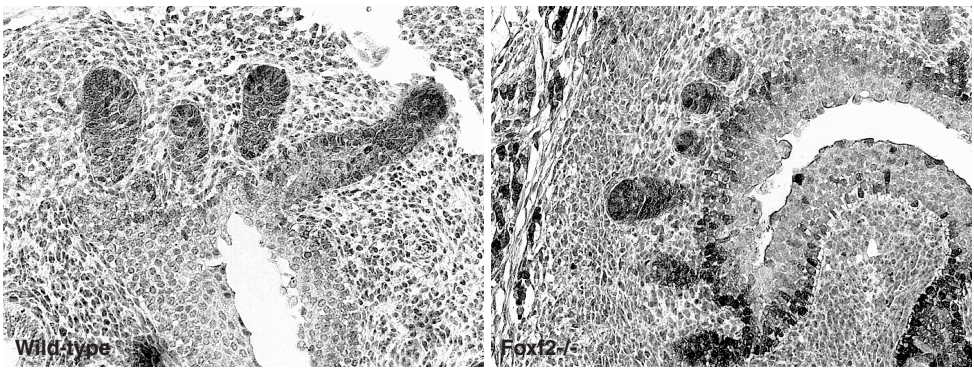

Figure 3. Metallothionein expression in Foxf2 mutant and wild-type mice. Expression of metallothionein in the prostatic ducts is higher in Foxf2$^{-/-}$ mice compared to wild-type mice. Magnification 200x

Discussion

The aim of this study was to get a better understanding of the role of forkhead transcription factor Foxf2 in prostate development. In our previous work we have shown that FOXF2 is higher expressed in the transition zone compared to the peripheral zone of the adult prostate and that it is mainly expressed in prostate stroma (2). In addition, FOXF2 expression was decreased in prostate cancer compared to normal prostate (3). Its high expression in androgen independent xenografts suggested a role in epithelial tot mesenchymal transition. Altogether, it appears that FOXF2 is involved in regulating normal prostate homeostasis through the stromal compartment, particularly in the transition zone. This role for Foxf genes was already suggested in murine gut development and in mouse and rat embryogenesis (5,7). In order to identify a potential role for Foxf2 in prostate development, we investigated the UGS of Foxf2$^{-/-}$ and wild-type mice. H&E stained

paraffin sections of wild-type mice versus Foxf2 mutants were microscopically analyzed and important differences between the wild-type mice and Foxf2 mutants were observed. Less extracellular eosinofilic matrix was observed in the stromal compartment of Foxf2 knockouts. The stroma appears chaotic and there were more gaps between the cells than in wild-type mice. The epithelial cells of the ducts appeared disorganized. Foxf2 seems to modulate cell-matrix attachment and formation of adhesion structures. Furthermore, protein expression of metallothioneins and laminins was analyzed and we showed higher expression of both proteins in Foxf2 mutants compared to wild-type mice. Ormestad et al. investigated the role of Foxf2 in gut development and found pour adhesion between cells, in which epithelial, mesenchyme and two muscular layers were separated from each other. The basal lamina and basement membrane were both indistinct and frequently replaced by gaps of extracellular space and ECM staining was reduced. In fibroblast cell studies it was shown that inhibition of Foxf target genes lead to loss of collagen synthesis and suggested a role for Foxf proteins in ECM production by fibroblasts (7). This is in concurrence with our data and so it is probable that also in prostate development Foxf2 plays a similarly important role in ECM modeling.

In the future, creation of conditional Foxf2 knockouts in various adult mouse models would be very informative to further elucidate the function of Foxf2. In conclusion, the current findings in UGS are in concurrence with the data of our recent microarray studies were we investigated human target genes of FOXF2 in the prostate, in which we established that ECM proteins are regulated by FOXF2.

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Chapter

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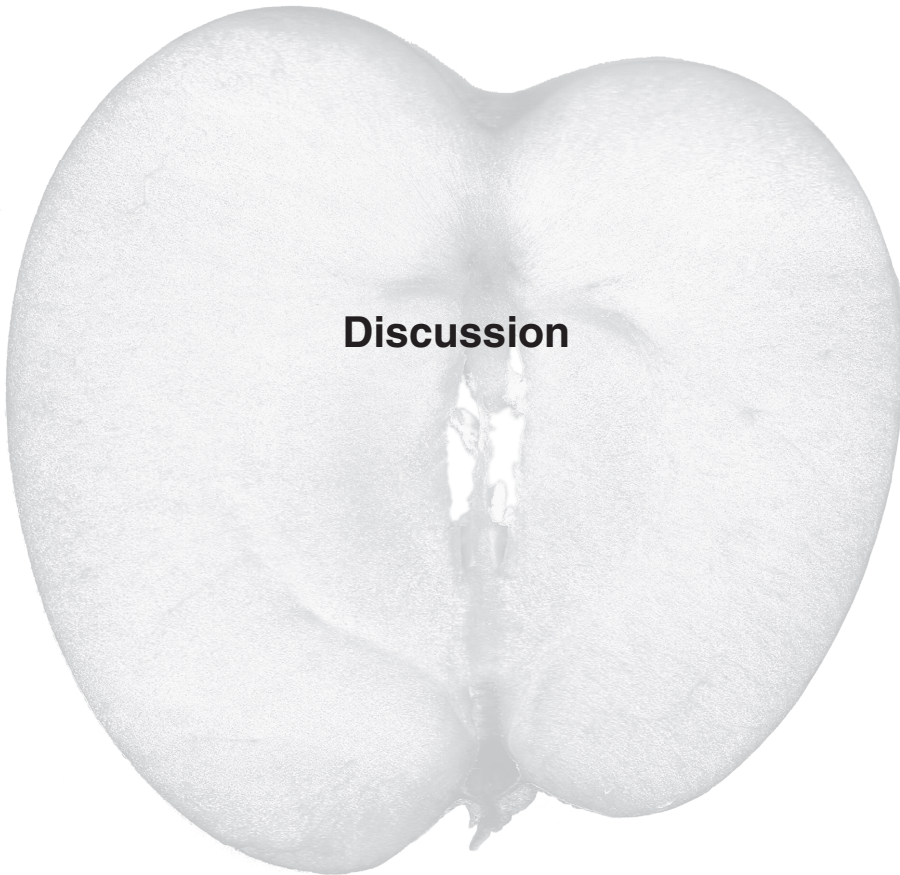

Discussion

Origin of prostate diseases explained by differential gene expression between the zones

The prostate consist of several zones in which different diseases arise. Prostate cancer mainly occurs in the peripheral zone whereas BPH only appears in the transition zone. We investigated gene expression of the human prostate zones to explore why these different diseases originate from distinct zones. To date, most studies focused on gene expression profiling of prostate diseases and investigated the molecular variation of PC and BPH compared to normal tissue (1). Little attention has been given to the molecular bases of the prostate zones as possible explanation for the distinct occurrence of the prostate diseases. Using microarray technology, we demonstrated that there is differential expression between the two normal prostate zones (2). Evidence suggests that the difference in gene expression is not caused by a difference in quantity of certain cells, but by a qualitative distinction in cell type characteristics. This was demonstrated in Chapter 2, where frozen prostate sections of 9 patients were scored for quantity of stromal and epithelial cells. The average proportion of stromal and epithelial cells was approximately 50:50 and this equal distribution was alike in both transition and peripheral zone. Additionally, both zones were analyzed for expression of several stromal, epithelial, endothelial, neuro-endocrine and lymphocyte infiltration markers. There was no significant differential gene expression of these cell type markers between the prostate zones.

Since quality of cell types is important in the prostate zone, it is important to study the interaction between stroma and epithelium, given that it is crucial in the progression of prostate cancer and BPH (3-5). Our data supports the view that cell characteristics of stroma and epithelium and with that, the interactions between stroma and epithelium, may already be different in the two normal zones. The major network that Ingenuity pathway analysis linked to the prostate zonal-preferential genes was cell-to-cell signaling and interaction. Consequently, we speculate that differences in stromal-epithelial interactions between the prostate zones form the basis of susceptibility of the distinct prostate zonal diseases.

Gene expression of the family of forkhead transcription factors in the prostate

Forkhead transcription factors FOXF1 and FOXF2 were selected from microarray data for further study because they were higher expressed in the transition zone compared to peripheral zone. Not much was known about forkhead transcription factor expression (Fox) in the prostate and thus, to comprehend the potential role of Fox proteins in general, we first explored the expression of 12 different Fox genes in different prostate tissues, including normal prostate, primary prostate cancer, androgen-dependent and independent xenografts and several

cell lines (6). Most of the 12 Fox genes are not markedly expressed in prostate, but FOXF1, FOXF2, FOXD1, FOXC1 and FOXA1 are distinctly expressed in the prostate (Chapter 3). FOXF2 is our main focus, because we discovered that its expression was significantly down-regulated in prostate cancer and proved to be stroma-specific. This gave FOXF2 a potential role as anti-tumor protein since it was highly expressed in the transition zone compared to the tumor-prone peripheral zone. Interestingly, its expression was elevated in androgen-independent xenografts, which also suggests a role for FOXF2 in epithelial to mesenchymal transition (EMT). In this thesis, we focused on PC rather than BPH, however, it would be interesting to further elucidate the role of FOXF2 in BPH, since we observed high expression of this gene in this disease, however, this can also be explained by the simple fact that BPH arises in the transition zone (6). Possibly, it can explain the benign characteristics of this transition zonal disease compared to the malignant qualities of PC in peripheral zone, where FOXF2 is lower expressed.

Exploration of potential target genes of FOXF2

To investigate the functional relevance of FOXF2, we elucidated its target genes and functional pathways (chapter 4). To achieve this, siRNA experiments were performed and evaluated by microarray analyses, in which we compared PrSC cells treated with negative control siRNA and siRNA directed against FOXF2. Several pathways, i.e. PDGF signaling, PPAR signaling, PI3K/AKT signaling, integrin signaling, VEGF signaling and actin cytoskeleton signaling were found in the microarray studies. FOXF2 was discovered to regulate extracellular matrix (ECM)) and cytoskeletal proteins, which pinpointed a role for the FOXF2 pathway to cellular assembly and organization. Enhanced production and activation of extracellular matrix proteins has been described in prostate carcinoma (7) and the following sections will discuss the role and involvement of FOXF2 in ECM modeling together with other ECM-associated proteins to further unravel the molecular basis of prostate diseases.

FOXF2 and its role in extracellular matrix remodeling

Molecular insights into signaling pathways and regulators of ECM proteins in the prostate and prostate cancer are beginning to emerge. Matrix metalloproteinases (MMPs) are Zn(2+) binding endopeptidases that break down a variety of components of the ECM. These proteins are regulated in normal and pathologic tissue remodeling processes, such as wound healing, angiogenesis and tumor invasion. The latter is of great importance since MMPs are involved in prostate cancer matrix invasion and metastasis (7). In microarray analyses, several components and regulators of the ECM were found to be down-regulated by FOXF2. One of

the genes down-regulated by FOXF2 was MMP1. Furthermore, tissue inhibitor of metalloproteinase 3 (TIMP3) was found to be up-regulated by FOXF2, which is reported as inhibitor of MMPs. The balance between MMPs and TIMPs regulates remodeling of the extracellular matrix (ECM) and FOXF2 seems to play a role in adapting the ECM in this balance (8). It is of importance to discuss ECM signaling and its relation to FOXF2 in combination with prostate cancer.
The balance between MMPs and TIMPs regulates remodeling of the ECM (9). A scheme from Biocarta (www.biocarta.com) illustrates an example of ECM signaling (Figure 1). We propose that FOXF2 could initiate a similar signaling cascade since we found several related genes in our microarray analysis. We found TIMP3 up-regulated by FOXF2, which inhibits MMP2 in the scheme, but is able to suppress several other MMPs as well (8).

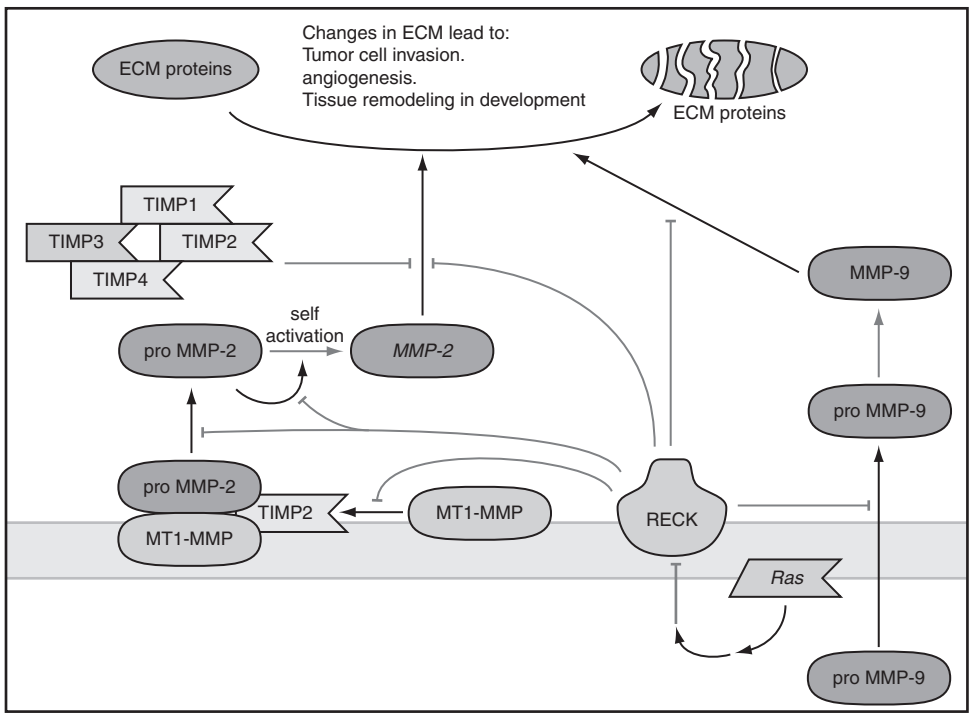

Figure 1. Regulation of extracellular matrix proteins

CXCL12, a target gene of FOXF2

CXCL12 was highly up-regulated by FOXF2 in the prostate stromal cells. Since CXCL12 is implicated in promoting prostate cancer metastasis it might link the FOXF2 pathway to cancer. To address this, we analyzed the expression of this gene in normal prostate, primary prostate tumors and prostate cancer metastases

in a large cohort of prostate cancer patients (Chapter 4). CXCL12 was higher expressed in normal prostate versus prostate cancer and it was elevated in PC lymph node metastases. CXCL12 is thought to advance prostate cancer progression in later stages (10). CXCL12 is described to regulate several MMPs in different prostate cancer cell lines, which potentiate tissue invasion and penetration of basement membranes by metastatic prostate cancer cells (11,12). In normal prostate environment, CXCL12 is higher expressed than in primary prostate tumors, where it possibly regulates prostate stroma homeostasis by regulating ECM remodeling. In later stages of prostate cancer, CXCL12 might switch into a tumor promoter and, next to FOXF2, play a role in EMT. It appears that these two proteins have an inhibitory role in normal stroma, but in later stages, are able to obtain the ability to interconverse epithelial cells into cells with mesenchymal characteristics.

FOXF2 and its role in regulating metallothioneins

Metallothioneins (MTs) have been reported to be differentially expressed in tumors and a specific role for MTs in prostate oncogenesis was suggested by Moussa et al (13,14). We found that FOXF2 siRNA treatment resulted in up-regulation of most MTs, like MT1E, MT2a, MT1F, MT1G, MT1H and MT1X within the metallothionein gene locus on chromosome 16. MTs are a family of low molecular weight, heavy metal-binding proteins characterized by a high cysteine content and lack of aromatic amino acids (15). In mammals, the cysteine residues are absolutely conserved and serve to coordinate heavy metal atoms such as zinc, cadmium, and copper via mercaptide linkages. These qualities provide protection against toxic metals and oxidative stress, and by controlling the cellular zink levels, it is offering a protective role in apoptosis. In carcinogenesis, high MT expression was linked to increased cell proliferation and less apoptotic cells (16). The tumor stroma may react to tumor growth and aggressiveness by the expression of MTs and consequently develops a resistance to apoptosis. The process of carcinogenesis is related to a failure in immune response against cancer cells that enables tumor progression and dissemination. This cancer-specific suppression is crucial for tumor survival. The evaluation of MT expression in cancer and its stroma seems to correlate with the level of immune system inhibition. To this point, many genes of the MT and MMP families were found to be down-regulated by FOXF2. Given that FOXF2 is higher expressed in the transition zone, in which prostate tumors are infrequent and less aggressive as compared to peripheral zone tumors, we suggest that FOXF2 controls normal prostate homeostasis, among others by regulating MT and MMP function.

FOXF2 and TGFβ opposing pathways?

Analysis by Gene Set Enrichment Analysis (GSEA) revealed that a significant number of FOXF2 up-regulated genes were down-regulated when the same PrSC cells were treated with TGFβ3 (17), indicating that FOXF2 and TGFβ3 are opposing pathways. Current investigations demonstrate that TGFβ plays key roles in prostate carcinogenesis by regulating tumor initiation, progression and metastasis through a diverse repertoire of tumor-cell-autonomous and host-tumor interactions (18). It can operate as both suppressor and activator. TGFβ signaling in stromal fibroblasts can suppress tumorigenesis in adjacent epithelium whereas it can promote invasion and metastasis during later stages of carcinoma progression. TGFβ-mediated regulation is highly complex and plays a variety of roles in numerous networks (19). TGFβ was described to be involved in EMT in cancer development and progression and it stimulates these phenomena, among others, via MMP-dependent mechanisms (20-22). MMP28 is able to induce EMT and cell invasion through TGFβ dependent mechanism in lung carcinoma cell and in normal mammary epithelial cells TGFβ was reported to be an inducer of EMT as well (23,24). Furthermore, TGFβ is involved in disintegration of cell-cell adhesions, cytoskeletal remodeling and cell-matrix adhesion (25-31). The latter feature involves remodeling of the cell contact with basal lamina and engages activation of matrix metalloproteinases, MMP2 and MMP9, which are regulated by TGFβ. The basal lamina is a specialized ECM structure, composed of collagen type IV, laminin and nidogen. Increased synthesis of MMPs in response to TGFβ leads to degradation of collagen type IV. As will be discussed below, several of these proteins are also regulated by Foxf2 in prostate development and thus again suggest a connection between these two pathways. In addition, in GSEA analyses, a significant correlation was identified between genes up-regulated by both FOXF2 and TGFβ3. Since both FOXF2 and TGFβ are implicated in EMT it may be that they share these common pathways to initiate EMT.

FOXF2 and stroma-epithelial interactions

An important manifestation in prostate tumor progression is the presence of reactive stroma. This is the reactive connective tissue located in most malignant tumors that surrounds the infiltrating tumor cells. It mainly consists of activated myofibroblastic cells and an altered extracellular matrix (ECM) (34). The stromal changes are the result of interactions during tumor progression between malignant cells, ECM, and the fibroblasts that normally populate the connective tissue, and involve also inflammatory cells and blood vessels. In breast carcinoma, paracrine factors such as platelet derived growth factor (PDGF) (35) and TGFβ (36,37) secreted by the tumor cells, could initiate formation of reactive stroma. In view of the fact that we found FOXF2 to be a stromal transcription

factor regulating, among others, ECM proteins and PDGFA, and is associated with TGFβ, it is possible that FOXF2 is involved in controlling reactive stroma formation in transition zone.

The role of Foxf2 in prostate development

Comprehension of mammalian prostate development and function will result in a better understanding of prostate cancer. FOXF2 is higher expressed in the transition zone, its expression is stroma specific and it is higher expressed in normal prostate compared to prostate cancer (2). In addition, a role for FOXF2 was suggested in EMT (6). After exploring FOXF2 gene expression and FOXF2 regulated pathways in human adult prostate in previous studies (chapter 4), we wanted to identify the potential role for Foxf2 in prostate development. The preliminary role of Foxf2 was investigated in mouse prostate development and we observed that the stroma compartment in Foxf2 knockouts showed less extracellular eosinofilic matrix, suggesting a role for Foxf2 in attachment of proteins to the cell matrix. Furthermore, the stroma appeared disorganized and epithelial cells in the prostate ducts also look disordered in Foxf2 knockout mice. Metallothionein staining showed higher protein expression in the urogenital sinus of $Foxf2^{-/-}$ knockout compared to wild-type mice. Another interesting phenomenon is that $Foxf2^{-/-}$ knockout mice give rise to a phenotype, in which the Foxf2 mutants develop a cleft in the secondary palate. An important EMT event in development is formation of the secondary oral palate, in which differentiated epithelial cells undergo an EMT and so become integrated into the mesenchymal compartment, thereby completing the program of palatogenesis (32). It appears that these $Foxf2^{-/-}$ mice are unable to undergo this last stage of EMT because they are lacking Foxf2. This strengthens the idea that in adult life FOXF2 is involved in EMT since it is known that highly conserved EMT programs are implicated in tumor progression (33). This is in concurrence with the results we found in human prostate cells and once more indicates a very important role for FOXF2 in regulating prostate stroma homeostasis and EMT.

FOXF2, a role in epithelial to mesenchymal transition?

Carcinoma invasion of epithelial cancers usually is accompanied by the loss of an epithelial phenotype and acquisition of a fibroblastic or mesenchymal phenotype, referred to as epithelial tot mesenchymal transition (EMT). EMT is a crucial step in conversion of early stage to invasive cancer. MMPs are correlated to poor prognosis and linked to EMT in various diseases (38-41). Since FOXF2 is highly expressed in androgen-independent xenografts and is suggested to regulate several ECM proteins, it appears to be involved in EMT. So, this important transition step from early primary tumors to invasive and metastatic tumors, might be

regulated by FOXF2. Supporting this, as described above, is the fact that Foxf2 knockout mice have a defective formation of the secondary cleft palate, which indicates that Foxf2 is involved in this EMT event during prostate development. All members of the TGFβ super family have been reported as major induction signals of EMT, although the precise signaling pathways may differ during various EMT events. It could well be that in EMT events in the prostate, FOXF2, next to TGFβ, is involved in this signaling, as they share a significant overlap between genes up-regulated in TGFβ3 treated PrSC cells. Interestingly, TGFβ3 knockout mice present a cleft palate phenotype, similar to the phenotype in Foxf2 knockout mice, and it has been described that TGFβ3 originally has a role in initiating EMT in secondary palate fusion (32).
In the majority of human carcinoma cell population, loss of E-cadherin is common in cells that have previously passed EMT. Various EMT-inducing transcription factors, including Snail, Slug, Twist1, FOXC2, ZEB and Goosecoid have been recognized in tumor invasion and metastasis in a variety of tissues. FOXF2 can possibly be such a transcription factor for EMT in the prostate. Next to EMT, earlier mentioned transcription factors have also been described in cancer stem cells (CSCs) (42). It has been suggested that these CSCs are responsible for driving tumor growth and metastasis. It was demonstrated that mammary stem cells, with special $CD44^{high}/CD24^{low}$ antigenic phenotype which characterized these CSCs, express EMT markers (43).

Future perspectives

The results described in this thesis revealed that FOXF2 plays an important role in the prostate. Exploration of conditional Foxf2 knockouts in various adult mice PC models would be very informative to further elucidate the function of Foxf2. We hypothesize that knockout of Foxf2 in, for instance Pten knockout mice will lead to earlier tumor initiation and progression. Furthermore, we speculated that FOXF2 is involved in EMT. We analyzed gene expression of FOXF2 in a small amount of prostate cancer lymph node metastasis samples, but saw no elevated expression. However, it is necessary to determine the level of gene expression of FOXF2 in many more samples. If we can find elevated gene expression of FOXF2 in lymph node metastases it would strengthen its role in EMT. Since we studied the role of FOXF2 in stroma cells, the next step would be to analyze its role in epithelial prostate cancer cells by overexpressing FOXF2 in these cells. This will depict regulation of pathways in both stroma and epithelium and so further completes understanding of the role of FOXF2 in prostate cancer progression. Additionally, we could analyze EMT markers in these epithelial cells and evaluate these cells for presence of $CD44^{+}/\alpha_2\beta_1{}^{+}/CD133^{+}$ cancer stem cell phenotype in the prostate. Besides gene expression analyses, we attempted to investigate

FOXF2 protein expression by western blot and immunohistochemistry analyses. It is essential to determine expression and localization of FOXF2 in tissues and cell lines at the protein level and we have put a lot of effort into this. Unfortunately, we were unsuccessful in reliably establishing these techniques with commercial available antibodies for visualizing these FOX proteins. For future experiments, serious investments into development of FOXF2 antibodies have to be made. In this thesis we focused on PC rather than BPH. We observed somewhat higher expression of FOXF2 in BPH than in transition zone. It would be very informative to study the role of FOXF2 in BPH in clinical prostate samples and demonstrate its possible involvement in the development of this disease.

In conclusion, FOXF2 has a unique stroma-specific expression in developing and adult prostate tissues. From mouse knockout experiments it appears that Foxf2 is vital for normal tissue development. FOXF2 seems to have an inhibitory role in normal stroma, where it is thought to regulate prostate homeostasis in transition zone via several ECM proteins, CXCL12, TGFβ and MTs. This suppressive role of FOXF2 might explain the predisposition of PC and BPH. However, next to these tumor inhibiting qualities, in later stages, FOXF2 appears able to obtain the ability to interconverse epithelial cells into cells with mesenchymal characteristics. This possible capacity of controlling EMT by FOXF2 may involve CXCL12 and TGFβ. Next to actively participating in tumor growth and progression, the stroma seems to have tumor suppressive qualities as well.

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Summary

Prostate cancer is second only to lung cancer as a cause for tumor-related deaths among men. Approximately 80% of 80 year old men suffer from prostate cancer, however not all these carcinomas pose a threat to life or health. The prostate consist of several zones, the transition, peripheral and central zone. Prostate cancer mainly occurs in the peripheral zone whereas benign prostatic hyperplasia (BPH) only occurs in the transition zone. BPH is a benign enlargement of the prostate and a common condition among aging men. We hypothesized that underlying gene expression will explain the confinement of these diseases to the transition and peripheral zone. In Chapter 1, gene expression profiling of the human prostate zones was performed by microarray analyses. 346 genes were differentially expressed between the prostate zones, which means that 346 genes were higher or lower expressed in one of the two zones. Few of the transition zone-specific genes, including FOXF1, FOXF2, COCH, ASPA and FLJ10970, were also observed to be stroma-specific and were not expressed in epithelial cells. This suggests that stroma-epithelial communication is different between the prostate zones. The forkhead transcription factor family was analyzed in the prostate to investigate its role in prostate and prostate disease. In Chapter 3, we investigated gene expression of 12 different forkhead transcription factors. Gene expression was analyzed in the prostate zones, prostate cancer, lymph node metastases, BPH, xenografts and several prostate cell lines. Expression of FOXA1, FOXC1, FOXD1, FOXF1 and FOXF2 was higher expressed in certain tissues. Markedly, FOXA1 and FOXC1 appear to have opposing function, where FOXA1 is mainly expressed in androgen dependent cell lines and xenografts, while FOXC1 is primarily expressed in androgen independent cell lines and xenografts. FOXD1 was higher expressed in prostate cancer compared to normal prostate, while FOXF1 and FOXF2 were lower expressed in prostate cancer compared to normal tissue.

Since FOXF2 is lower expressed in prostate cancer and higher in transition zone, is stroma-specific and linked to epithelial to mesenchymal transition (EMT) in prostate cancer xenografts, we further investigate its role in prostate function. Chapter 4 describes the target genes of FOXF2 that we identified by comparing microarray expression profiles of primary prostate stroma cells (PrSC) treated with control or siRNA directed against FOXF2. We found that extracellular matrix proteins, metallothioneins and CXCL12 were regulated by FOXF2. Furthermore, TGFβ seems to play an important role next to FOXF2. FOXF2 appears to control normal prostate homeostasis by regulating these proteins. The fact that prostate cancer occurs less frequent in the transition than the peripheral zone could be explained by the fact that FOXF2 regulates homeostasis more prominent in this

zone compared to the peripheral zone. In this respect, FOXF2 appears to have a tumor suppressive function.

In Chapter 5 we investigated prostate development in Foxf2 knockout mice. We compared the urogenital sinus in wild-type mice and Foxf2 knockout mice. In Foxf2 knockouts the stromal compartment showed less eosinofilic extracellular matrix, which indicates a role for Foxf2 in attachment of proteins to the cell-matrix. Proteins like laminin and metallothionein, which have been discussed in chapter 4, are higher expressed in Foxf2 knockout mice compared to wild-type. This confirms the idea that Foxf2 is involved in stroma regulation. In Chapter 6, the findings of these studies are discussed with a focus on prostate zones, prostate diseases and the potential role of FOXF2.

Samenvatting

Prostaatkanker is, na longkanker, de meest frequente doodsoorzaak aan kanker in de Westerse wereld. Bijna 80% van mannen van 80 jaar heeft prostaatkanker, echter lang niet al deze mannen zullen aan prostaatkanker overlijden.
De prostaat bestaat uit verschillende zones, de transitie, de perifere en de centrale zone. Prostaatkanker wordt voornamelijk gevonden in de perifere zone, terwijl benigne prostaat hyperplasie (BPH) exclusief te vinden is in de transitie zone. BPH is een goedaardige zwelling van de prostaat die lijdt tot plasklachten bij oudere mannen. Omdat prostaatkanker en BPH preferentieel gelokaliseerd zijn in een bepaald deel van de prostaat, hebben wij als hypothese gesteld, dat onderliggende genexpressie van de verschillende prostaatzones de voorkeurslocatie van de ziektes kan verklaren. In Hoofdstuk 2 zijn genexpressie profielen bepaald van de perifere en transitie zone met behulp van microarray analyses. 346 genen kwamen differentieel tot expressie, wat betekent dat 346 genen hoger of lager tot expressie komen in een van de twee zones. Een aantal van de genen die hoger tot expressie komen in de transitie zone zoals FOXF1, FOXF2, COCH, ASPA en FLJ10970, bleken preferentieel in prostaat stroma cellen aan te staan en niet tot expressie te komen in prostaat epitheliale cellen. Dit suggereert dat de communicatie tussen het stroma en epitheel anders is tussen de twee zones. De familie van forkhead transcriptie factoren werd in de prostaat onderzocht om een idee te krijgen welke leden van deze familie een mogelijke rol zouden kunnen spelen in prostaat en prostaatziekte ontwikkeling.

In Hoofdstuk 3 wordt de genexpressie van 12 verschillende forkhead transcriptie factoren in de prostaat besproken. Deze expressie werd getest in verschillende weefsels, zoals normaal prostaatweefsel, prostaatkanker, prostaatkanker lymfeklier metastasen, prostaat xenografts en verschillende prostaat cellijnen. We zagen dat expressie van FOXA1, FOXC1, FOXD1, FOXF1 en FOXF2 hoger tot expressie kwamen in bepaalde weefsels. Een opvallende observatie is dat FOXA1 en FOXC1 een tegengestelde functie lijken te hebben, waarbij FOXA1 juist hoger tot expressie komt in androgeen afhankelijke cellijnen en xenografts, terwijl FOXC1 juist in androgeen onafhankelijke cellijnen en xenografts hoger tot expressie komt. FOXD1 kwam hoger tot expressie in prostaatkanker vergeleken met normaal weefsel, terwijl FOXF1 and FOXF2 juist lager tot expressie kwamen in prostaatkanker.

Omdat FOXF2 lager tot expressie komt in prostaatkanker en juist hoger in de transitie zone, het een stroma-specifiek eiwit is en in prostaatkanker xenografts met epitheel-mesenchymale transitie (EMT) geassocieerd werd, wilden we meer

leren over de rol van FOXF2 in de prostaat. In Hoofdstuk 4 beschrijven we de targetgenen van FOXF2, die we gevonden hebben door FOXF2 in primaire prostaat stroma cellen uit te zetten, door middel van siRNA studies. Met behulp van microarray analyses konden we achterhalen welke genen gereguleerd worden door dit eiwit. We vonden dat extracellulaire matrix eiwitten, metallothionein eiwitten en CXCL12 gereguleerd worden door FOXF2. Ook TGFβ lijkt een belangrijke rol te spelen naast FOXF2. De belangrijke regulerende rol van het stroma in de prostaat wordt door deze bevindingen beduidend verstrekt en het lijkt erop dat FOXF2 de kwaliteit bezit om het stroma in evenwicht te houden. Dat er in de transitie zone, waarin FOXF2 hoger tot expressie komt dan in de perifere zone, minder prostaatkanker voorkomt, zou kunnen worden verklaard door het feit dat FOXF2 de homeostase hier beter kan regelen dan in de perifere zone. In dat opzicht lijkt FOXF2 dus een tumorremmende functie te hebben.

In Hoofdstuk 5 onderzochten we de ontwikkelende prostaat in Foxf2 knockout muizen. Dit is gedaan door de urogenitale sinus te vergelijken in normale muizen met Foxf2 knockout muizen. In Foxf2 knockout muizen is de stuctuur van de extracellulaire matrix aangedaan, het stroma is veranderd en eiwitten als metallothionein en laminin, die we ook in Hoofdstuk 4 besproken hebben, komen hoger tot expressie in Foxf2 knockout muizen vergeleken met normale muizen. Ook hier is dus een duidelijk indicatie dat FOXF2 een rol speelt in stroma regulatie. In Hoofdstuk 6 worden de bevindingen van deze studies bediscussieerd, waarin de prostaatzones en bijbehorende ziektes en de rol van FOXF2 in de prostaat centraal staan.

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Ik hou van je!

Leonie

Curriculum vitae

Leonie van der Heul-Nieuwenhuijsen is geboren te Dordrecht op 30 juni 1978. In 1996 is zij geslaagd voor het Atheneum diploma aan het Titus Brandsma College te Dordrecht.
Aansluitend begon zij met de opleiding Biologie aan de Universiteit van Utrecht waar zij in juni 2002 haar doctoraal diploma behaalde. Tijdens deze opleiding behaalde zij tevens haar propedeuse van de lerarenopleiding Biologie aan de Hogeschool te Rotterdam (HR&O).
Als onderdeel van de studie Biologie liep zij twee afstudeerstages, waaronder de eerste bij het RITOX, onderdeel van de Universiteit van Utrecht en de tweede bij het Unilever Health Institute(UHI), onderdeel van het Unilever research centrum te Vlaardingen.
In juli 2002 begon zij haar promotieonderzoek aan de Erasmus Universiteit Rotterdam, op de afdeling Urologie, waarvan de resultaten beschreven staan in dit proefschrift. Het onderzoek naar kanker gerelateerde genexpressie in de humane prostaatzones werd begeleid door dr.ir. G.W.Jenster en Prof.C.H.Bangma.
Vanaf januari 2008 heeft zij als postdoc gewerkt bij de afdeling Celbiologie van het Erasmus MC, onder begeleiding van dr. W. de Laat (hij is met zijn groep per 01-09-2008 naar het Hubrecht Laboratorium te Utrecht verhuisd).
In november 2008 is zij als postdoc begonnen bij Prof. H.A.Drexhage, op de unit autoimmuunziekten van de afdeling Immunologie van het Erasmus MC. Momenteel werkt zij mee aan het Europese project Moodinflame, waarbij onder andere, afwijkende monocyt expressieprofielen in patiënten met psychiatrische aandoeningen worden onderzocht.

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I want to walk with you
On a cloudy day
Come away with me
And I'll never stop loving you

Norah Jones