From the **Department of Biosciences and Nutrition**
Karolinska Institutet, Stockholm, Sweden

ROLE OF ESTROGEN RECEPTOR BETA IN MOUSE PROSTATE AND BLADDER WITH REFERENCES TO HUMAN DISEASES

Otabek Imamov

Karolinska Institutet

Stockholm 2007

All previously published papers were reproduced with permission from the authors.

Додажонимга ва Абуламга

ABSTRACT

After the discovery of estrogen receptor beta (ERβ) in 1995, it became clear that in many "non-classical" estrogen-responsive organs, were in fact direct targets for estrogen. One of these organs is prostate gland, where ERβ is abundant in epithelium. Creation of a mouse with inactivated ERβ (ERβ-/- mouse) was indispensable for dissecting the role of ERβ in different organs. Studies in this thesis focus on the ventral prostate and urinary bladder of ERβ-/- mice.

Our studies with the ventral prostate showed that ERβ has an antiproliferative and pro-differentiative role in epithelium and in ERβ-/- mice there is accumulation of incompletely differentiated so-called intermediate cells. Comparison of the protein expression profiles between ERβ-/- mice and their wild-type littermates showed dysregulation of several proteins, associated with differentiation. One of the proteins which was over-expressed is the serine protease inhibitor (SPINK). The human counterpart of SPINK is tumor associated trypsin inhibitor (TATI), a known tumor marker for prostate adenocarcinoma. In paraffin embedded section of human prostate, we found that in cancers with low differentiation grade (high Gleason score), ERβ is downregulated with concomitant upregulation of TATI.

Unlike the prostate, in the urinary bladder ERβ is expressed in the basal cell layer not in the differentiated epithelium and the epithelium of the urinary bladder is fully differentiated. Despite this, in female ERβ-/- mice there are changes resembling human interstitial cystitis. We found that the most likely cause of urothelial destruction is massive infiltration of urothelium with macrophages and γδT-cells. We concluded that altered ERβ signaling in the immune system is the cause of urothelial destruction.

One of the nagging issues in the study of ERβ has been the identity of the natural ligand for this receptor in the prostate. There is evidence that the most abundant estrogenic steroid in the prostate is 5α-androstane-3β, 17β-diol (3β-Adiol), a metabolite of 5α-dihydrotestosterone (DHT). We were puzzled over this because blockers of the conversion of testosterone (T) to DHT, 5α-reductase inhibitors, are used in the treatment of BPH and have been tested for prevention of prostate cancer. We speculated that 5α-reductase inhibitors would lead to a less well differentiated prostatic epithelium. We tested this idea in mice and found that blocking 5α-reductase type 2 (SRD5A2) results in altered differentiation of mouse ventral prostate epithelium, similar to what is seen in ERβ-/- mice. This alteration could be prevented by treatment with ERβ specific agonist, DPN.

The studies in this thesis lead to the conclusion that ERβ-selective modulators could be of benefit in the treatment and/or prevention of prostate cancer and interstitial cystitis.

LIST OF PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals

I. **Imamov O**, Morani A, Shim GJ, Omoto Y, Thulin-Andersson C, Warner M, Gustafsson JÅ (2004). Estrogen receptor beta regulates epithelial cellular differentiation in the mouse ventral prostate. Proc Natl Acad Sci U S A. 2004 Jun 22;101(25):9375-80.

II. Schwend T, **Imamov O**, Williams C, Ruppert T, Waage L, Bergerheim U, Oschepkov B, Margaret Warner and Jan-Åke Gustafsson (2007). Serine protease inhibitor Kazal type 3, a marker for poorly differentiated prostatic epithelium in ER beta -/- mice and in prostate cancer. Cancer Research (in revision, CAN-07-2184)

III. **Imamov O**, Morani A, Lewandowski S, Schwend T, Svechnikov K, Warner M, Söder O and Gustafsson JÅ (2007). Blocking of 5 alpha-reductase type 2 alters differentiation of prostatic epithelium due to inhibition of ER beta signaling. Submitted.

IV. **Imamov O**, Yakimchuk K, Morani A, Schwend T, Wada-Hiraike O, Razumov S, Warner M, and Gustafsson JÅ (2007) Estrogen receptor beta deficient female mice develop a bladder phenotype resembling humaninterstitial cystitis Proc Natl Acad Sci U S A. 2007 Jun 5;104(23):9806-9

CONTENTS

LIST OF ABBREVIATIONS

ERβ	Estrogen receptor beta
3β Adiol	5α-androstane-3β, 17β-diol
3βHSD7	3β-hydroxysteroid dehydrogenase type 7
DBD	DNA binding domain
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
E2	17β-Estradiol
ERα	Estrogen receptor alpha
ERE	Estrogen response element
LBD	Ligand-binding domain
LHRH	Luteinizing hormone releasing hormone
CYP7B	Cytochrome P450 7B
SERM	Selective estrogen receptor modulator
SPINK	Serine protease inhibitor
T	Testosterone
TATI	Tumor associated trypsin inhibitor
CHT	Combined hormonal treatment
MAB	Maximal androgen blockade
PIN	Prostate intraepithelial neoplasia

1 INTRODUCTION

1.1 HISTORICAL PERSPECTIVE

In 1941, Huggins et al, in a paper describing the beneficial effect of endocrine treatment for locally advanced prostate adenocarcinoma, established a basis for the hormonal treatment of prostate cancer (Huggins and Hodges 1941; Huggins, Scott et al. 1941; Huggins, Stevens et al. 1941). In retrospective studies published in 1946 and 1950, Nesbit analyzed 1818 prostate cancer cases investigating the outcomes of various treatment strategies (Nesbit and Plumb 1946; Nesbit and Baum 1950). He found that combination of the non-steroidal estrogen, diethylstilbestrol (DES), with bilateral orchiectomy was the best treatment option for the patients with locally advanced disease. The hypothetical mechanism of action of estrogens was speculated to be suppression of testosterone synthesis through negative feedback on the hypotalamo-pituitary axis. Interestingly, the idea of Huggins that there could be a direct estrogenic influence on the prostatic epithelium was ignored. Of possible significance in this context was the finding that the synthetic estrogen Chlorotrianisene, introduced into the clinic in 1951, was efficient despite the fact that it did not lower testosterone levels to castrated values (Baba, Janetschek et al. 1982).

The first evidence of cardiovascular side effects of DES treatment came with the results of Veterans´ Administration Cooperative Urological Research Group (VACURG) in 1967 (1967). The study was conducted in a randomized fashion, comparing 5 mg DES with placebo and included more than 2000 cases. One of the major conclusions of the study was that DES at a dose of 5 mg/day

caused an extremely high cardiovascular mortality rate. As clinicians became aware of the cardiovascular complications accompanying the use of DES, its use in the treatment of prostate cancer began to decline and two alternatives were introduced to the clinic. Canadian endocrinologist, Fernand Labrie, was and still is a strong proponent of the use of LHRH agonists. He introduced them into clinical treatment of prostate cancer and revolutionized the field. Then came the first non-steroidal antiandrogen, flutamide, marking the beginning of the era of a combined hormonal treatment (CHT) also known as maximal androgen blockade (MAB). In terms of median survival, the method was superior to LHRH agonist monotherapy (Labrie, Dupont et al. 1986) and soon got recognition in the urological world. Estrogens were relegated to a small niche in the urological armamentarium.

1.2 DISCOVERY OF ESTROGEN RECEPTORS

While pharmaceutical interest was very focused on androgens and anti-androgens in the treatment of prostate cancer, basic science was trying to clarify the actions of estrogen in the prostate. The idea that estradiol exerted its effects through a receptor was established in 1962 when Elwood Jensen and colleagues identified ERα in uterine cytosol (Jensen and Jacobson 1962). Identification was based on the high affinity of ERα for 17β-estradiol (E$_2$) and this was only possible when highly radioactive [^{3}H] E2 was synthesized. More than 20 years after its discovery, ERα was cloned with the help of very specific antibodies raised in the Jensen laboratory (Greene, Nolan et al. 1980; Walter, Green et al. 1985; Green, Walter et al. 1986). ERα turned out to be a member of

the nuclear receptor super gene family, the first member of which, the glucocorticoid receptor, had been cloned in 1984 (Miesfeld, Okret et al. 1984). There followed quite rapidly the cloning of the other members of this super family, including androgen, progesterone, mineralocorticoid, vitamin D and vitamin A receptors (Conneely, Sullivan et al. 1986; Jeltsch, Krozowski et al. 1986; Arriza, Weinberger et al. 1987; Petkovich, Brand et al. 1987; Baker, McDonnell et al. 1988; Brand, Petkovich et al. 1988; Trapman, Klaassen et al. 1988). With cross hybridization techniques, using the conserved DNA-binding domain as a hybridization probe, a surprising number of novel members of this family was discovered and for some of these receptors no known ligand has yet been discovered. In 1995, during a search for nuclear receptors in the prostate, the second estrogen receptor, ERβ, was discovered (Kuiper, Enmark et al. 1996).

The structural architecture of ERα and ERβ is typical for all other members of the NR family, namely an N-terminal region (A/B domain), containing constitutively active transactivation region (AF-1); a DNA-binding domain (DBD, C domain), which contains the P-box, a short motif responsible for DNA-binding specificity and involved in dimerization of ER; a D domain that behaves as a flexible hinge between the C and E domains, and contains the nuclear localization signal (NLS); and an E domain. This is the ligand-binding domain (LBD), whose secondary structure of 12 α-helices is responsible for ligand binding. Comparisons of the protein structure of the two ERs showed 96% identity in DBD and 59% homology in LBD (Kuiper, Enmark et al. 1996). Hypothetically that meant that both ERs would bind to the same response

elements on the DNA, but the ligands, activating receptors, could be different. The N-terminal region, containing AF-1 and the C-terminus with AF-2 was even less conserved. This would predict different co-regulator binding properties between two receptors (Fig. 1).
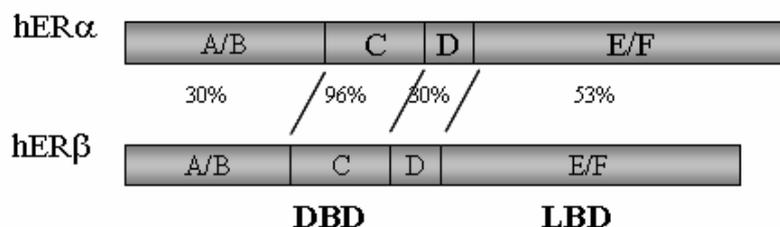
Figure 1. Comparison of human ERα and ERβ. **DBD** – DNA binding domain. **LBD** – ligand binding domain

Although all of the details of the signaling through ER have not been worked out, the following is a simplified scheme as to what happens: ERs are expressed in the cells of a target tissue in an inactive form in complexes with chaperones. Once the E_2 binds to ER, one of the chaperones, HSP-90, dissociates from the complex and ERs are released; ER monomers then associate to form dimers and the dimers bind to specific regions of DNA, attracting co-modulator proteins and influencing the transcription of different target genes.

Soon after ERα was found, virtually all tissues in the body were screened for its presence. As predicted, ERα was found in the mammary gland, uterus, placenta, liver, central nervous system, cardiovascular system and bones. These tissues expressed high levels of ERα and responded to E_2 by increasing the transcription of certain estrogen-controlled genes. In other tissues, including prostate, testis, gall bladder, skin, lymphatic and

4

hematopoietic systems, which do respond to estrogen, ERα expression was not detectable (Ciocca and Roig 1995). This is how the classification of target tissues into "classical target tissues" and "non-classical target tissues" began.

The prostate gland was classified as a non-classical E2-target tissue. ERα was found in the prostate, but it was localized exclusively in the stromal part of the gland (Bashirelahi, Kneussl et al. 1979; Chaisiri and Pierrepoint 1980; Kozak, Bartsch et al. 1982; Swaneck, Alvarez et al. 1982). This finding led to the concept that all direct influences of estrogen on the prostate are mediated by stromal ER and possibly via growth factor signaling pathways (Prins, Birch et al. 2001; Prins, Birch et al. 2001). The dogma of an obligatory role for stroma in the estrogen actions in the prostate had to be revised after ERβ was discovered. Not only was ERβ cloned from the rodent prostate, it is abundantly expressed in prostatic epithelium. Interestingly, ERβ is expressed in both the epithelium and stroma of the human prostate.

1.3 CREATION OF KNOCKOUT MOUSE MODELS

Very early after its discovery, it was found that ERβ does not elicit the classical estrogen actions in the uterus and pituitary. To many endocrinologists this simply meant that ERβ was a vestigial receptor. However, to other investigators, this led to the idea that ERβ-selective agonists could act on the prostate and breast without having the worst side effects of E2 i.e., chemical castration and uterine cancer. Study of ER knockout mice was to prove very fruitful in the quest of the functions of ERβ.

Silencing of a gene of interest with unknown function by creating genetically modified mice became a classical method in modern science (Doetschman, Gregg et al. 1987; Thomas and Capecchi 1987). A mouse with inactivated ERα (ERα-/-) was created in 1993 (Lubahn, Moyer et al. 1993). It was reported that male ERα-/- mice have altered spermatogenesis with reduced fertility, but that the prostate was not affected morphologically (Eddy, Washburn et al. 1996). ERβ-/- mice were generated in 1998 (Krege, Hodgin et al. 1998). Interestingly, in ERβ-/- mice at 2-3 months of age, the prostates were morphologically normal. However, as mice aged, the ventral prostate developed foci of epithelial hyperplasia. Our laboratory published several papers describing mouse prostatic epithelial hyperplasia in the absence of ERβ signaling (Krege, Hodgin et al. 1998; Weihua, Makela et al. 2001; Imamov, Morani et al. 2004). The incidence of this phenotype increased with age, very seldom seen in 6-month-old mice, it could be found in almost every mouse at the age of 24 months. These lesions were reminiscent of a well-recognized morphological precursor of prostate cancer in humans, namely prostatic intraepithelial neoplasia (PIN) (Bostwick and Brawer 1987). However, we have never seen cellular atypia in PIN-like lesions in mice and never detected any signs of *carcinoma in situ* (CIS). Mouse hyperplastic lesions showed only mild tissue atypia, similar to low-grade PIN (LG PIN) in humans. This phenotypical feature of ERβ-/- mice became one of the corner stones in the ERβ research. The two other large labs studying ERβ-/- mice saw no abnormalities in the prostate and called our findings a "cutting

artifact" (Krege, Hodgin et al. 1998; Dupont, Krust et al. 2000; Weihua, Makela et al. 2001).

At about the same time, another knockout mouse model useful to study estrogen signaling became available. In 1998, Evan Simpson's group reported the creation of aromatase knockout mouse (ArKO)(Fisher, Graves et al. 1998). Aromatase (P450arom) is the enzyme that catalyzes metabolic transformation of C19 steroids into estrogens. ArKO mice cannot produce estrogens but do express both ERs. The prostates of these mice were reported to be enlarged and hyperplastic (Jarred, McPherson et al. 2003). Thus by the end of 90s, researchers in the estrogen field had three mouse knockout models to use for the dissection of ERα and ERβ function. These studies are ongoing and our knowledge base is growing everyday with new data coming from different laboratories. However, the accumulated data on estrogen signaling is already at the stage when new estrogen receptor modulating agents are close to being introduced into the clinics.

1.4 TESTOSTERONE-5α-DIHYDROTESTOSTERONE-5α-ANDROSTANE-3β, 17β-DIOL (3βADIOL) PATHWAY

The reduction of T to DHT is a very well characterized biochemical reaction, discovered by Bruchovsky and Wilson (Bruchovsky and Wilson 1968). The reaction is catalyzed by the enzymes, 5α-reductase type I and II. The type II enzyme is in fact used as a target for pharmacological therapy of BPH. Blocking 5α-reductase in the prostate is considered beneficial, since DHT is a more potent agonist for AR than T. Finasteride (Proscar, MSD)

and dutasteride (Avodart, GSK), widely used in the clinical practice, are 5α-reductase inhibitors.

For many years DHT was thought to be essential for the development of the prostate. However upon inactivation of both 5α-reductases, mice have a completely functional prostate (Mahendroo, Cala et al. 2001). If it is not essential for development of sex accessory tissue, what is the role of DHT in the body? Our lab suggested that an important function of DHT is that it is a precursor of the second estrogen in the body, 5α-androstane-3β, 17β-diol, 3β-Adiol (Weihua, Lathe et al. 2002). Figure 2 outlines the T-DHT pathway. As is clear from the figure, DHT can be metabolized to two stereoisomers: 5α-androstane-3α, 17β-diol and 5α-androstane-3β, 17β-diol.
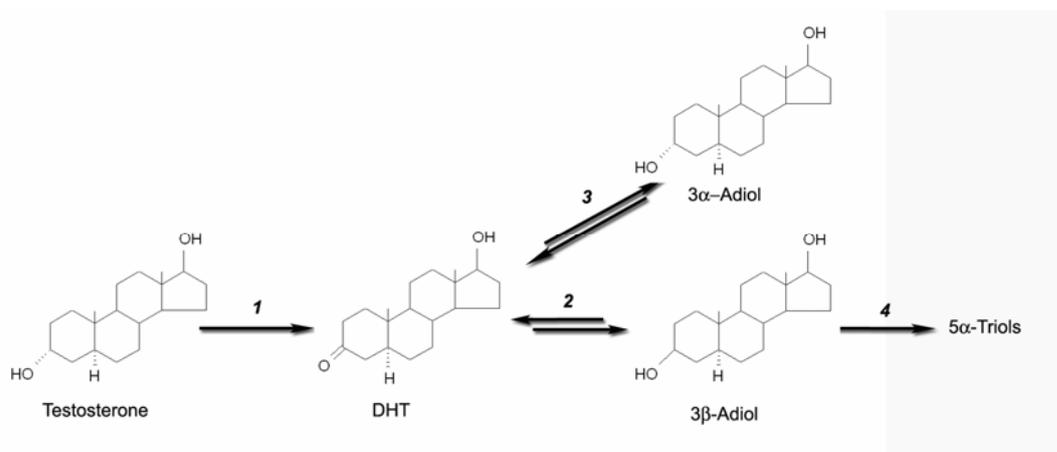
Figure 2. Testostosterone metabolic pathway in prostate. **1** - 5α reductase type II (SRD5A2). **2**- 3β-hydroxysteroid dehydrogenase / Δ^5, Δ^4-isomerase. **3**- 3α-hydroxysteroid dehydrogenase. 4- 3β-Adiol hydroxylase (P4507B1)

This biochemical reaction is reversible and catalyzed by 3α- and 3β-hydroxysteroid dehydrogenases (3α-HSD and 3β-HSD). 5α-Androstane-3α, 17β-diol is an androgenic steroid, possibly serving as a depot for DHT. 5α-

androstane-3β, 17β-diol (3β-Adiol) is an estrogenic steroid, capable of activating both ERα and ERβ. Moreover, the concentration of 3β-Adiol in the prostate is higher than of E2, making it a perfect candidate to be a natural intracrine hormone for ERβ (Weihua, Lathe et al. 2002) (Weihua, Lathe et al. 2002). 3β-Adiol undergoes further transformation to inactive triols, a reaction catalyzed by 3β-Adiol hydroxylase (CYP7B1).

1.5 ESTROGEN RECEPTORS AND PROSTATIC EPITHELIUM
1.5.1 ERα

The tragic outcome of the use of diethylstilbestrol (DES) in pregnant women is one of the dramas in the modern history of medicine. DES is a synthetic non-steroidal estrogen that was prescribed to about 4 million women in the USA between 1938 and 1971 to prevent miscarriages (reviewed in (Schrager and Potter 2004)). The drug was ineffective in preventing miscarriages but *in utero* DES exposure caused vaginal adenocarcinoma and cervical cancer in the daughters of the women taking medication (1976). The sons of DES-treated mothers are also reported to have higher incidence of genital abnormalities, testicular cancer (Henderson, Benton et al. 1976; Docimo, Silver et al. 2000) and squamous metaplasia in prostate (Driscoll and Taylor 1980). This process of a hormonal programming of a developing organ predisposing it to the changes in adulthood is called imprinting.

In order to understand the toxicity of DES in human fetuses, it was quite natural to test the effects of DES in mice. Administration of DES during the neonatal period resulted in prostate enlargement and increased risk of dysplasia in adulthood (vom Saal, Timms et al. 1997; Strauss, Makela et al.

1998; Prins, Birch et al. 2001). Similar effects have been reported for rats, undergoing *in utero* estrogenization (Prinsac, Birch et al. 2001). The question which of the ERs mediates these effects was open until the definitive study from the group of Korach was published (Prins, Birch et al. 2001). It was shown that ERα-/- mouse prostates are resistant to prenatal estrogenization while ERβ-/- and wild type (Wt) are equally sensitive. At this point, it became clear that negative effects of prenatal estrogenization are mediated by ERα. Since ERα is localized in the prostatic stroma, it was concluded that prostatic epithelium receives signals from stromal ERα through some growth factor signaling pathways.

In 2005 we showed (Omoto, Imamov et al. 2005) that ERα is abundantly expressed in the epithelium of the developing prostate so it is not necessary to invoke a mechanism involving growth factors from the stroma. During the specific time frame of 2nd to 4th weeks of postnatal life, ERα and not ERβ is predominantly expressed in the prostatic epithelium. This transient expression coincides with high proliferative activity and branching morphogenesis of the prostatic epithelium. Around the 4th week of postnatal life, marking the end of proliferation and beginning of differentiation and functional activation of epithelium, ERα is switched off and ERβ becomes dominant. Even with the naked eye, it is obvious that once removed from its site at the base of the bladder, the ERα -/- prostate has an overall appearance quite different from wild-type (Wt) prostates. The gland does not maintain its shape but tends to spread out as though the structure is weak. The reason for this apparently fragile structure became clear when the gland was examined

under a microscope. Overall, the ductal system of the ERα -/- mouse ventral prostate is composed of two main very long primary ducts with no branching at the bifurcation of the two ducts. These observations suggest a role for ERα in branching morphogenesis of the prostate.

1.5.2 ERβ

ERβ-/- mice, despite showing signs of hyperplasia with aging, still have functionally active prostate glands. Studies from our group demonstrated that in the absence of ERβ, proliferation of prostatic epithelium is increased and apoptosis is suppressed. We have postulated that the general function of ERβ in the prostate is repression of proliferation. Our observations are supported by reports from other groups, testing this hypothesis in cell lines (Cheng, Lee et al. 2004).

Based on our observations of altered terminal differentiation of mammary gland epithelium in the absence of ERβ signaling (Forster, Makela et al. 2002), we hypothesized that ERβ plays a general role in the regulation of epithelial differentiation. Epithelial cells in the prostate form a continuum of cells in different stages of differentiation. The continuum can be roughly divided into three groups or stages of differentiation: basal cells, intermediate cells and luminal cells. Intermediate cell group is also known as transiently proliferating/amplifying pool of cells, because of the capability of these cells for rapid proliferation. TP/A group of cells is also divided into basal intermediate and luminal intermediate cells (Fig. 3).
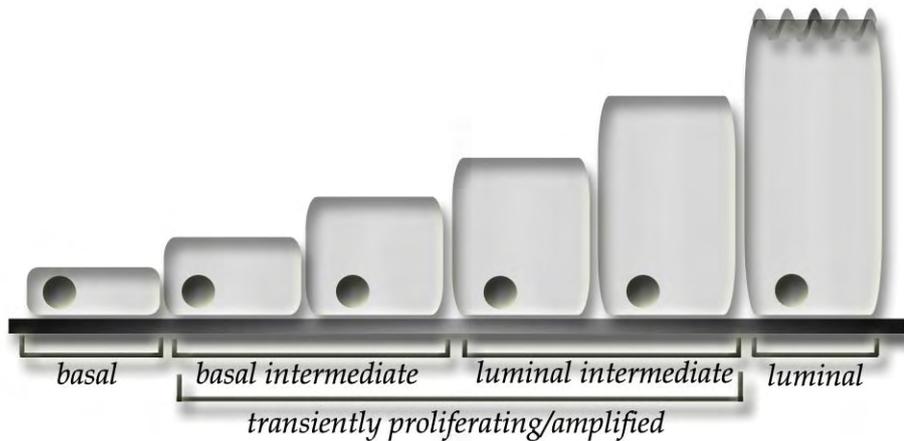
Figure 3. Prostatic epithelium differentiation continuum

These cellular pools are characterized by a specific protein expression profile that makes it possible to differentiate them one from the other. This protein expression pattern is also known as a cytokeratin profile (Isaacs and Coffey 1989; Bonkhoff, Stein et al. 1994; Wang, Hayward et al. 2001) . Basal cells are localized in the basal cellular layer, attached to the basement membrane. These cells express AR and are believed to include a pool of prostatic stem cells (English, Drago et al. 1985; Hayward, Brody et al. 1996). Basal cells are androgen sensitive, but independent of androgens for survival. Upon stimulation with androgen, they undergo a slow division process that can be symmetrical – giving rise to two similar basal cells - or asymmetrical, when one of daughter cells is entering the differentiation process. Intermediate cells (also known as transiently proliferating/amplified cells, TP/A) represent an in-between stage of prostatic epithelial differentiation. These cells are androgen sensitive but also dependent upon androgens for survival. Upon androgen stimulation, they proliferate, but upon androgen

withdrawal, they die. The luminal cell pool consists of highly specialized secretory cells, located at the luminal side of the duct. These cells produce components of prostatic secretion and eventually die by shredding off into the lumen. Luminal cells are androgen sensitive and dependent, but since they are highly specialized, they have lost the ability to proliferate. Androgen stimulates secretory activity of these cells.

Studies in our group showed that, in the absence of ERβ signaling, mouse prostatic epithelial differentiation is altered, resulting in the accumulation of cells in TP/A stage, most probably belonging to the basal intermediate group (Imamov, Morani et al. 2004). These cells are capable of rapid proliferation upon androgen stimulation.

We used cytokeratin profiles to characterize the cellular composition of ERβ-/- prostates. We found that the differentiation pattern in the absence of ERβ signaling was altered in that the ratio between the three cell pools is shifted towards cells in the TP/A stage. That means that there are fewer cells possessing luminal and basal phenotype, and more cells in the intermediate pool capable of rapid proliferation process, hence the increased proliferation rate in ERβ-/- mouse prostates.

1.6   PROSTATE CANCER PREVENTION TRIAL FROM ERβ POINT OF VIEW

The results of the Prostate Cancer Prevention Trial (PCPT) were published in 2003. The PCPT was a multicenter prospective double-blinded study of Finasteride (Proscar MSD) as a preventive agent for prostate cancer (Thompson, Goodman et al. 2003; Pitts 2004; Rubin and Kantoff 2004). The

rationale for the study was that a 5α-reductase inhibitor, could reduce the incidence of prostate cancer by decreasing intraprostatic levels of DHT. More than 18000 healthy volunteers aged 55 or above were randomized into two arms: finasteride 5 mg daily and placebo. After 7 years of treatment, as anticipated, the incidence of prostate cancer in finasteride arm was reduced (18.4% vs. 24.8% in the placebo arm). However, there was a big surprise when the histology of the cancers was examined. In the finasteride-treated group the incidence of poorly-differentiated, aggressive Gleason 7-10 tumors was 67% higher (finasteride, 280/757, 37%, vs. placebo 237/1068, 22.2%; $P < 0.001$). Despite the reduction in cancer risk in the finasteride arm, the increased incidence in Gleason score above 7 is unacceptable to most urologists since it is a strong indication of very poor survival.

The authors of the original paper provided several possible explanations for the phenomenon. First, they attributed the higher incidence of aggressive tumors in the finasteride arm to likely "treatment effect"; clonal selection of tumors more sensitive to low androgen environment; or selective killing of the low-grade tumors. There were several editorials questioning the interpretation of the results. One problem in particular is the fact that treatment with finasteride changes prostate morphology (Civantos, Soloway et al. 1996). It is thought that finasteride treatment causes similar morphological alterations as seen in LHRH treated prostates (Rubin and Kantoff 2003; Rubin and Kantoff 2004; Rubin, Allory et al. 2005). Thus, according to some authors, the Gleason grading system is not applicable, since essentially non-malignant morphological alterations can lead to over grading. Some analysts suggest that

14

since the same number of high Gleason tumors were detected in placebo group, it is the number of low-Gleason tumors that is changed after finasteride treatment, suggesting that finasteride is only effective preventing low-Gleason tumors (Andriole, Bostwick et al. 2005).

We contributed to the discussion of the PCPT with our own view of what happened after 7 years of blocking 5α-reductase. It is our hypothesis that higher incidence of poorly differentiated tumors in finasteride-treated arm of the PCPT is caused by lack of the natural ERβ ligand, 3β-Adiol (Imamov, Lopatkin et al. 2004). From the clinical point of view, blocking 5α-reductase activity and altering the conversion of T to DHT has obvious benefits that have been discussed elsewhere. However, one unforseen casuality lies downstream of such biochemical intervention. It is 3β-Adiol which, as a natural ligand for ERβ, is prodifferentiative. We think that finasteride should not be given as monotherapy, but should be given in combination with ERβ agonists. Interestingly, even before PCPT, a small study reported on 52 men with PSA higher than 4 ng/ml, but no morphological evidence of prostate cancer. After randomization into active and placebo groups and 12 months´ treatment, it showed significantly higher cancer incidence in finasteride group, while the incidence of PIN was not different (Cote, Skinner et al. 1998). A second report from the PCPT study retracts the evidence that the Gleason scores were indeed higher in the finasteride arm of the study (Etzioni, Howlader et al. 2005) and this has led to further debate about the design and interpretation of the study. The debate about the PCPT results is still ongoing.

At the moment, a study of a new, dual 5α-reductase inhibitor, Dutasteride is being performed (Andriole, Bostwick et al. 2004). The study, named Reduction by Dutasteride of Prostate Cancer Events (REDUCE), will analyze not only Gleason score, but the cancer aggressiveness by using specific markers for aggressive growth. However, one has to keep in mind that Dutasteride due to its 45-fold higher potency compared to finasteride and the ability to inhibit both isoforms of 5α-reductase, type I and II, would result in much lower serum and intraprostatic concentrations of DHT. These observations would suggest that the amount of 3β-Adiol, available for ERβ activation would be even less than under finasteride treatment, possibly resulting in more prominent changes in prostatic morphology. Phytoestrogens seem to be good candidates for combination with finasteride. New synthetic compounds, capable to activate ERβ without affecting ERα are on their way to the clinics (Neubauer, McNulty et al. 2003). One should expect the appearance of a new class of prodifferentiative agents, perhaps useful for cancer prevention, as neo-adjuvant therapy and in combined treatment of prostate cancer.

1.7   ERS AND PROSTATIC STROMA

It is known that an imbalance in the ratio between estrogen and androgen is involved in the pathogenesis of BPH. Estrogenic activity in the prostate is the sum of the actions of both E2 and 3β-Adiol (Weihua, Lathe et al. 2002) as well as the responses of ERα and ERβ. At the same time, intraprostatic levels of E2 are very low compared to 3β-Adiol (Weihua, Lathe et al. 2002).

DHT and 3βAdiol are both synthesized in the stroma where 5α-reductase type II (Luo, Dunn et al. 2003), and 17β HSD type 7 are primarily located. 3β-Adiol can activate both ERβ and ERα (Kuiper, Lemmen et al. 1998). At present, the clinical rationale for the use of 5α-reductase inhibitors in treatment of BPH is that stromal AR is responsible for stromal overgrowth and indeed, administration of 5α-reductase inhibitors, effectively lead to prostatic involution. There is one puzzling fact, which does not fit with the explanation that 5α-reductase inhibitors are efficient in BPH by reducing androgens in aging prostate. The fact is that that estrogen/androgen ratio in the aging prostate is shifted in favor of estrogen, because of the decline in androgen (Schatzl, Brossner et al. 2000).

We propose an alternative explanation for the beneficial use of 5α-reductase inhibition in the treatment of BPH. It involves ERα, 3βAdiol and CYP7B1. It is as follows: DHT is converted into 3βAdiol in the stroma where it activates ERα. The estrogenicity of 3βAdiol is regulated by cellular level of CYP 7B1. If CYP7B1 levels are low, ERα is activated and stromal growth ensues. We think that 5α-reductase inhibitors work because, by reducing DHT, they also decrease the level of stromal 3βAdiol.

This role of 5α-reductase inhibition helps us to solve the problem of why it is that the incidence of prostate cancer increases as the T levels decline with age and why androgen replacement therapy does not increase BPH or development of prostate cancer. Interestingly, a recently performed large cohort study showed inverse relationship between serum levels of T and

cancer aggressiveness (Massengill, Sun et al. 2003). The same phenomenon was reported for circulating androgen bioactivity (Raivio, Santti et al. 2003).

There is one other important player to be considered if we are to understand estrogen action in the prostate. This player is ERβcx. ERβcx does not bind to E2 or to 3βAdiol but if expressed in the same cell as ERα it acts as a dominant repressor of this receptor (Moore, McKee et al. 1998; Ogawa, Inoue et al. 1998). If ERβcx is expressed in stromal cells with ERα, stromal growth should be repressed. Questions about cellular localization and regulation of the expression levels of ERβcx and CYP7B1 need to be answered for an appropriate understanding of estrogen signaling in prostate disease.

1.8   ESTROGEN IMPRINTING OF PROSTATE

We have already touched the subject of estrogen imprinting of the prostate and the role of ERα in this phenomenon. Here we would like to discuss the role of ERβ in this fascinating phenomenon. The term "estrogen imprinting" was first suggested by Rajfer and Coffey in their hallmark publication in 1978 (Rajfer and Coffey 1978).

In the 1980's the McLachlan laboratory pioneered the use of the DES in mice for investigating the mechanism of the human DES syndrome. Permanent alterations occur in the male and female genital tract when mice are exposed to DES at certain critical times during development. In male mice, prenatal exposure to DES is associated with poor semen quality, prostatic disease, cryptorchidism, testicular neoplasia, feminization of the seminal vesicles and stromal inflammation (Newbold, Pentecost et al. 1989;

Pylkkanen, Santti et al. 1991; Beckman, Newbold et al. 1994). Imprinting by DES in the developing prostate is absolutely dependent on the presence of ERα, and ERα-/- mice are resistant to imprinting by DES (Prins, Birch et al. 2001). Several distinct critical periods or "windows in time" when estrogen influences tissue morphogenesis have been observed for many organs. For the uterus, vagina, prostate and lung, one critical period when estrogen influences morphogenesis is between embryonic days 9-16. There is a second critical period between postnatal days 1-6 when the prostate and the skeleton are imprinted (Migliaccio, Newbold et al. 1995). In the developing mammary gland, on the other hand, estrogen influences morphogenesis at puberty. Sato et al have provided evidence that DES-induced abnormalities of reproductive organs are associated with altered expression levels of DNA-methyltransferases and DNA methylation (Sato, Fukata et al. 2006).

The studies of estrogen imprinting have always focused on a negative aspect of inappropriate estrogen administration *in utero*. The question of positive imprinting of estrogen is less well addressed. Since mice do not spontaneously develop prostate cancer, it is difficult in this species to study protection against cancer that might occur during *in utero* treatment. In the human population, such protection probably exists, even though only indirect evidence on the matter is available. Soy phytoestrogens in Asian style diets are considered the key factor in low incidence of prostate cancer in certain countries. Phytoestrogen-containing products were known to be healthy long before the discovery of ERs. Population studies of the influence of Western style diet on incidence of prostate cancer provided evidence that

Asian food does protect against prostate cancer. Clearly, it is not only the beneficial properties of Asian style diet, but also unhealthy properties of Western style diet that have to be taken into consideration. Soybean isoflavones suppress the development of invasive carcinomas of the rat prostate/seminal vesicles (Onozawa, Kawamori et al. 1999) and genistein and daidzein possess anti-cancer effects at relatively early stages of prostate cancer development (Kato, Takahashi et al. 2000); (Jarred, Keikha et al. 2002). Moreover, according to population studies, based on careful retrospective analysis of Japanese and Chinese migrants, the protective effect of Asian style diet lasts in the first and also in a second generation of emigrants, despite their changed life-style (Cook, Goldoft et al. 1999). Interesting evidence came from the analysis of Multiethnic Cohort Study (MEC), performed between 1993-1996, which involved 215,000 cases. After careful statistical analysis of the obtained incidence data, the authors postulate the existence of "residual effects of exposures during childhood", which had an important influence on natural history of hormone dependent cancers, including prostate cancer in adulthood .

Thus, we can hypothetically distinguish two types of imprinting: a negative imprinting, well documented in DES administration studies; and positive imprinting probably lying behind the Asian anti-cancer protection. Following this logic, one can hypothesize that the two types of imprinting are mediated independently by the two estrogen receptors ERα and ERβ and that they occur at distinct windows in time during development. Although we have clear indications as to when these windows occur in rodents, nothing is

20

known about them in human development. It is possible that activation of ERα signaling during a certain timeframe in childhood would predispose for prostate cancer, while activation of ERβ at some other time point would offer protection.

Positive imprinting by soy phytoestrogens, mediated by ERβ, occurs perhaps also during specific periods of ERβ activity. Since some phytoestrogens, like genistein, show higher affinity to ERβ than to ERα (Miller, Collini et al. 2003; Kolonel, Altshuler et al. 2004), one can speculate that, throughout prenatal development, the plasma concentration of such phytoestrogens is not enough to stimulate ERα, and just enough to stimulate ERβ. However, it is very difficult to speculate over the cellular substrate of positive imprinting.

Our speculations on negative and positive imprinting do, of course, require future research to dissect the exact mechanisms of this phenomenon. Our division of imprinting into positive and negative is also quite subjective. The DES catastrophe caused a great number of diseases and Asian style diet does protect against prostate cancer. However, imprinting is a polyorganic event, and protection against prostate cancer can be associated with negative influence on other organs and systems. Although ERβ signaling can be used as a target for medical treatment of prostatic diseases, it is too early to consider the concept of positive imprinting as a pharmacological strategy in cancer prevention.

1.9 ERβ AND URINARY BLADDER

Out interest in the role of ERβ in the bladder stems from the fact that ERβ is expressed in bladder urothelium and that the debilitating disease, interstitial cystitis (IC), is influenced by estrogen. We speculated that ERβ could be involved in the differentiation of the urothelium. The predominant ER in the bladder is ERβ with very little ERα expression (Kuiper, Lemmen et al. 1998; Miller, Collini et al. 2003). Reduced ERβ expression levels have been reported in the bladders of rats with chemically induced cystitis (Saunders, Maguire et al. 1997).

The etiology of IC is unknown and available treatment options are limited and mostly palliative. IC is almost exclusively a disease of young women and symptoms tend to worsen premenopausally or during ovulation. Several pieces of evidence indicate that IC, chronic pelvic pain and abacterial prostatitis might share the same pathogenetic mechanism. It has been reported that up to 70% of men with chronic abacterial prostatitis have cystoscopic signs of IC (Taylor and Al-Azzawi 2000; Acar, Cayan et al. 2006). There is a published hypothesis, which links prostatitis, IC, chronic pelvic pain and urethral syndrome. In this report the author postulates that these diseases share "dysfunctional urinary epithelium and potassium recycling" impairment (Miller, Rothman et al. 1995). Furthermore, all these diseases have been reported to respond to sitosterols and phytoestrogens, such as quercetin and genistein (Bouic, Etsebeth et al. 1996; Berger, Miller et al. 1998; Parsons, Greene et al. 2005). At the same time, as mentioned above,

phytoestrogens, like genistein are better ligands for ERβ than ERα (Shoskes, Zeitlin et al. 1999; Miller, Collini et al. 2003; Sun, Chen et al. 2007).

When we observed that female ERβ-/- mice develop a bladder phenotype resembling IC, we formulated a hypothesis that ERβ might function as a differentiation factor for bladder urothelium, and failure to differentiate might be involved in the pathogenesis of IC.

The study described in Paper IV, aimed to test this hypothesis, revealed completely different role of ERβ in IC.

1.10 ESTROGENS AND THE IMMUNE SYSTEM

In the immune system the overall effect of E2 is determined by a balance between ERα and ERβ signaling. In three different animal models: ERα-/-, ERβ-/- and the aromatase knockout (Ar-/-) mice, loss of each component in the estrogen signaling pathway, produces a distinct immune phenotype with development of autoimmune nephritis in ERα-/- mice (Miller, Collini et al. 2003), myeloid leukemia in ERβ-/- mice (Kuiper, Enmark et al. 1996) and Sjogren's syndrome in Ar-/- mice (Shim, Kis et al. 2004). There is a clear role for ERβ in regulating the differentiation of pluripotent hematopoietic progenitor cells and all female mice over one year of age develop lymphoma with splenomegaly, enlarged lymph nodes and liver tumors. Surprisingly, the males have a normal immune system. Sex differences in the immune system are also clearly evident in humans. Autoimmune diseases affect women primarily, with some occurring 10 times more frequently in women than in men (Shim, Wang et al. 2003). Estrogen

has contradictory effects in autoimmune diseases (Shim, Warner et al. 2004).

It worsens lupus erythematosus but ameliorates rheumatoid arthritis

1.11 SELECTIVE ESTROGEN RECEPTOR MODULATORS

One of the pioneers of chemotherapy, Paul Ehrlich, was the first to suggest that a good medication should influence the parasite without affecting the host. The concept of a medication, designed to be receptor-specific became a rule of a thumb in designing new pharmacological agents.

As is clear now, there are two major ERs expressed in different tissues like breast and prostate. Moreover, these receptors are often expressed in the same tissue and oppose each other´s actions while the same ligands can modulate the activity of both of them. In cell lines (Beeson 1994) and in some tissues (Strom, Hartman et al. 2004; Imamov, Shim et al. 2005), E2 in the presence of ERα elicits proliferation but, in the presence of ERβ, it inhibits proliferation, providing the perfect example of opposite effects caused by the same hormone. Likewise, the prostate has epithelial/stromal ERβ and stromal ERα. According to our studies, activation of ERβ can be beneficial in treatment of prostate cancer and possibly cancer prevention.

Initially, as alluded to above, the main mechanism of estrogen in the treatment of prostate cancer was believed to be through the hypothalamo-pituitary-gonadal axis with subsequent inhibition of T synthesis. However, several estrogenic compounds are shown to act independently of this pathway. Many hypothetical mechanisms have been described in the literature, including disruption of apoptotic regulators (Weihua, Makela et al. 2001), depolymerization of microtubules (Forster, Makela et al. 2002),

inhibition of DNA synthesis (Rafi, Rosen et al. 2000), induction of apoptosis (Dahllof, Billstrom et al. 1993) and interruption of cell-cycle (Kuwajerwala, Cifuentes et al. 2002). Interestingly, all of these effects have been attributed to ERβ and not ERα signaling. Moreover, all the above-mentioned effects, seen in the experimental settings, can be a part of the same signaling mechanism downstream of ERβ. This concept might rationalize the ongoing search for selective estrogen receptor modulators (SERMs) for the treatment of prostatic diseases.

The standard test for an estrogen, stimulation of growth of the uterus is, of course, still a good test for an ERα agonist but there is no single good test for an ERβ agonist. In fact, there may not be such a thing as a single good ERβ agonist. What is emerging is an array of ERβ-selective agonists, each with a specific profile of genes, which they influence (Qadan, Perez-Stable et al. 2001). Although we know what is a consensus ERE, most estrogen responsive genes do not contain perfect consensus sequences and the transcriptional activity of ERα or ERβ on such sequences is influenced by the chemical structure of the estrogenic ligand. Hall and Korach (Kumar, Garcia et al. 2001) have evaluated the activities of ERα and ERβ on four different EREs (vitellogenin A2, human pS2, lactoferrin and complement 3) in the presence of E2, phytoestrogens and xenoestrogens. In terms of transactivation by ERα and ERβ, the vitellogenin and lactoferrin promoters were not discriminatory. The pS2 and complement 3 were most responsive to ERβ. In addition, the transcriptional activity of either receptor on any promoter varied with the ligand. Another factor influencing selectivity of ER ligands is that the

influence of estrogen receptors on transcription is not confined to EREs. Estrogen receptors modulate transcription at AP-1 and Sp1 sites and interact with the NFkB pathway (Merchenthaler, Hoffman et al. 2005). The action of the two receptors at these sites can be opposite to each other but this depends on cellular context and it is not possible to predict how ERα and ERβ will influence transcription at these sites. Selective ERα and ERβ ligands have already been developed which have actions on selective target tissues and even selective target genes (Hall and Korach 2002).

2 AIMS OF THE THESIS

As I started out, the antiproliferative role ERβ in the prostate had already been deduced from the morphological differences between ERβ-/- mice and their wt littermates, i.e., foci of epithelial cellular proliferation specifically in ERβ-/- mice. Furthermore, the endogenous ligand for ERβ in the prostate had already been identified as 3β-Adiol. As a urologist I was interested in the antiproliferative role of ERβ in the human prostate and was very curious as to whether ERβ could influence bladder epithelium and have a role in interstitial cystitis. In addition, if 3β-Adiol was the ERβ ligand in the prostate, I was bothered by the fact that treatment with finasteride blocks the synthesis of the important ligand. My aims can be summarized as follows:

- Further characterize the role of ERβ in prostatic epithelium of mice (Paper I)

- Search for a homology in the ERβ functions between rodent and human prostate (Paper II)

- Characterize specific changes of prostatic epithelium when the synthesis of 3β-Adiol is blocked (Paper III)

- Investigate the possible role of ERβ in the pathogenesis of specific changes of mouse female bladder urothelium, resembling human interstitial cystitis (Paper IV)

3 NOTES ON METHODOLOGY

3.1 LABORATORY ANIMALS

Animals were used in accordance with the guidelines for care and use of experimental animals issued by Stockholm's Södra Djurförsöksetiska Nämnd. Mice were fed a standard diet with *ad libitum* access to water. Knockout and wt mice were bred from heterozygous pairs. Genotyping, using PCR, was performed on DNA isolated from the tails of 2-week-old mice as described elsewhere.

ERβ-/- mice were originally created by insertion of a selection marker for neomycin resistance into the coding region for ERβ. We are fully aware of possible problems associated with interpretation of data obtained from such mice. Expression of genes in the neighborhood can be affected by the insertion. Strain of mice and the genetic background is very important in understanding the phenotype of knockout animals and we used the backcross 10th generation to minimize genetic instability issues. The biggest problem though is the fact that deletion of the protein occurs in every cell of the body and the protein is absent during the development. Surviving mice have compensated for this loss by activation of different physiologic pathways.

We tried to use extensive controls and a common sense to minimize the problems with interpretation of data.

3.2 IMMUNOHISTOCHEMISTRY

We used paraffin embedded tissues, fixed with 4% paraformaldehyde buffered solution. The method itself has a certain limitations, being subjective by nature. However we never used intensity of the staining as comparative criteria, instead the absence or the presence of a signal or calculation of positive

cells were applied during our studies. In order to control the Immunohistochemical stainings, we used negative controls (no primary antibody), positive controls (a tissue of known immunoreactivity) and in some cases, preadsorbed antibodies.

3.3   WESTERN BLOTTING

In most of the cases we used a standard protocol for Western blot detection by SDS-gel separation. However during protein extraction we seldom used a tissue homogenate, instead we separated the samples to membrane, nuclear and cytosolic fractions.

3.4   ISOTOPE-CODED PROTEIN LABELING (ICPL) IN COMBINATION WITH LC-ESI-MS/MS

The biggest advantage of the method is that it allowed direct comparison of protein expression in a mixture of two samples. Since the isotope labeling of samples is done in the very beginning, and the samples are mixed and treated as one, one can minimize the amount of errors that can be introduced by the digestion and analysis.

One big question about the identification of proteins is the threshold of Mascot score change that can be considered as regulation. In our studies, we first measured the standard deviation of score introduced by the method within one sample, which happened to be 10% and then used 2SD change as criteria of true change.

4 RESULTS AND DISCUSSION

4.1 PAPER I

In order to obtain a deeper insight into the role of ERβ in mouse prostatic epithelium, we tried to characterize its involvement in the three major growth regulatory processes: proliferation, differentiation and apoptosis. It turned out that the lack of ERβ does not only cause an increase in proliferation rate, as was shown before, but also suppresses apoptosis and alters differentiation of prostatic epithelium.

ERβ-/- and wt littermate male mice were injected with bromdeoxyuridine (BrdU) and sacrificed after 48 hours. We began by testing the reported proliferation suppression by ERβ and observed that in the absence of ERβ, the number of cell nuclei which incorporated BrdU was almost 3.5 fold higher in ERβ-/- mice than in wt littermates. Next we used the TUNEL assay to study apoptosis. We saw that apoptosis is markedly suppressed in ERβ-/- VPs, and that antiapoptotic protein bcl-2 is upregulated. In fact, bcl-2 is known to be an estrogen regulated protein, so it was natural to speculate that it is regulated by ERβ in mouse ventral prostate.

The most fascinating finding though came when we stained VP sections for p63 – a known marker for the basal epithelial cells. Not only was the number of positive cells increased, but the position of p63-expressing cells was also affected. We found p63 positive cells in the luminal compartment of VP epithelium (Fig 4).
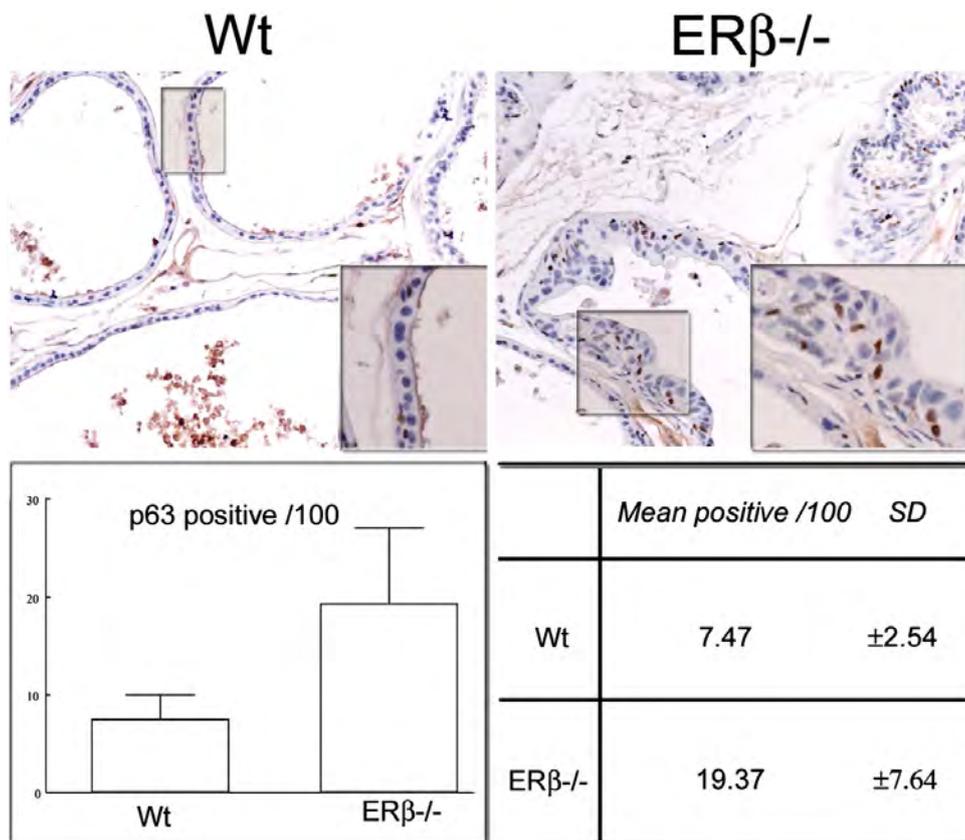
Figure 4. Immunohistochemical detection of p63 in wt and ERβ-/- ventral prostate. Positive nuclei counted per 100 revealed statistically significant difference (plotted). In the table, the number means positive per 100.

The next question concerned the cellular composition of the prostatic epithelium in the absence of ERβ signaling. We approached this question by using fluorescent activated cell sorting of isolated prostatic epithelial cells from wt and ERβ-/- mice. It turned out that cells positive for Ck19, a basal-intermediate cell marker, accumulate in the prostate when ERβ is knocked out. This means that ERβ-/- prostate has more cells expressing AR that are sensitive and dependent on androgen stimulation and capable of rapid proliferation upon androgen stimulation. ERβ therefore seems to be responsible for terminal differentiation of prostatic epithelium.

In this study we provided evidence that ERβ is a prodifferentiative factor for prostatic epithelium.

4.2   PAPER II

One of the integral questions of my studies was whether phenotypical changes in ERβ-/- mice have a homology in humans. In other words, can we use the knowledge obtained using mice and extrapolate it to humans. We tried to address this question in paper II.

We started with a comparison of the proteins expressed in wt and ERβ-/- mice. We used isotope-coded protein labeling (ICPL) in combination with LC-ESI-MS/MS to make the comparison. This method allowed direct comparison of proteins in wt and ERβ-/- prostates. Among numerous proteins that were changed in ERβ-/- mouse prostates, we found over-expression of one that has been described as a tumor marker for humans, namely serine protease inhibitor Katzla type III (SPINK), known also as Tumor Associated Trypsin Inhibitor (TATI).

We confirmed our results with a series of western blots and checked whether expression of TATI is correlated with the expression of ERβ in samples of human prostate cancer of various differentiation grade. Our study showed that TATI is negatively correlated with ERβ expression and positively with Gleason score (Fig. 5).

This study gave us confidence that the function of ERβ in rodent prostatic epithelium is homologous to human prostatic epithelium and that ERβ expression is downregulated in poorly differentiated prostatic adenocarcinomas.
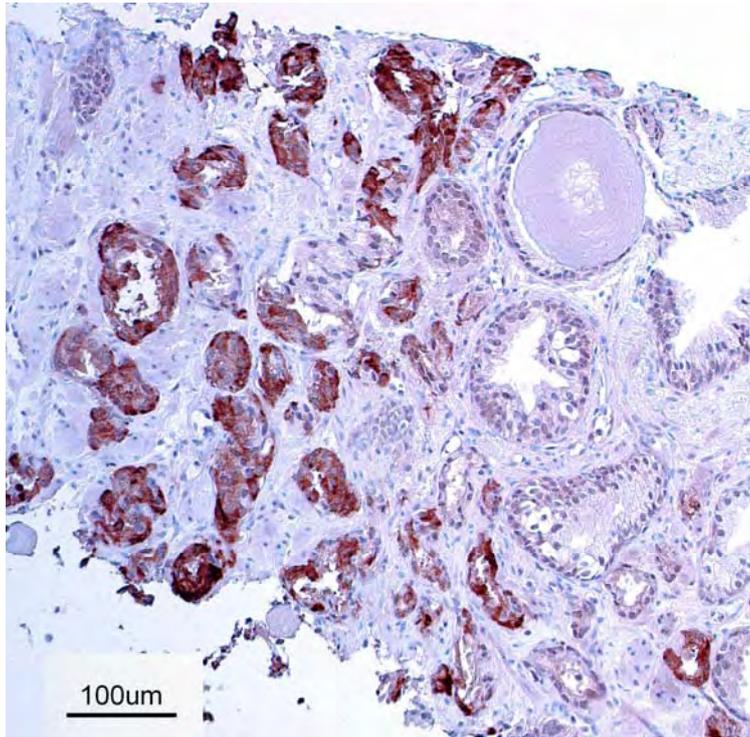
Figure 5. Expression of TATI in cancerous and normal parts of human prostatic epithelium

4.3 PAPER III

In this study, we tested our hypothesis that long-term blocking of 5α-reductase type II results in inhibition of production of 3β-Adiol and reduced function of ERβ.

The puzzling results of Prostate Cancer Prevention Trial, led us to think that the increase in the number of poorly differentiated (high Gleason) tumors in the Finasteride-treated arm of the trial might be explained by the loss of the pro-differentiative steroid, 3β-Adiol. In order to test our hypothesis, we studied both mice and the human non-malignant prostate epithelial cell line, BPH-1.

Wt mice were treated with Finasteride, combination of Finasteride and ERβ-specific agonist DPN and vehicle control for 60 days. Mice were then sacrificed and used to check differentiation profile of prostatic epithelium. We found that blocking 5α-reductase type II by Finasteride resulted in similar

changes in expression of differentiation markers as seen in ERβ-/- mice, i.e. altered terminal differentiation with accumulation of p63 positive cells. Moreover, adding DPN to the treatment helped reverse the differentiation failure. We also measured 3β-Adiol concentration in the prostate using radioimmunoassay (RIA) and observed a decrease of concentration of 3β-Adiol upon Finasteride treatment.

In order to check whether specific changes observed in mice are due to inhibition of DHT synthesis in the prostate, we used BPH-1 cell line which is known not to express AR and hence not sensitive to changes in DHT levels. The BPH-1 cell line is known to express ERβ. We confirmed ERβ expression in these cells. Upon treatment of BPH-1 cells with finasteride, 3β-Adiol concentration in cell media was reduced and the pattern of cytokeratin expression was altered as it was in the *in vivo* experiment. Upon addition of DPN, the changes in cytokeratin profile could be prevented.

We concluded that blocking 5α-reductase type II resulted in reduction in 3β-Adiol concentration and changes in prostate epithelium homologous to ERβ-/- VP phenotype. This study provided evidence that use of finasteride leads to inhibition of ERβ signaling and to a more poorly differentiated prostate.

4.4   PAPER IV

This paper addressed a question whether ERβ can be involved in pathogenesis of interstitial cystitis.

From clinical experience and scattered evidence in the literature it is known that phytoestrogenic compounds can be helpful in treatment of

interstitial cystitis. We postulated that since ERβ is expressed in the bladder epithelium, it might be involved in the pathogenesis or interstitial cystitis. We thought in the beginning of the study that ERβ might play a similar role in the bladder urothelium as in prostatic epithelium, i.e. prodifferentiative. We expected that a decrease in ERβ activity would lead to altered differentiation, resulting in alteration of synthesis of glycosaminoglycans (GAG) – a protective layer of high-molecular weight sugar molecules attached to the protein core.

We began by checking the phenotype of ERβ-/- female mouse bladders and observed changes in epithelium, resembling interstitial cystitis in humans. These changes became apparent after puberty and became progressively worse thereafter. To our surprise, on the basis of cytokeratin expression, there was no alteration in the differentiation of the urothelium (Fig.6).
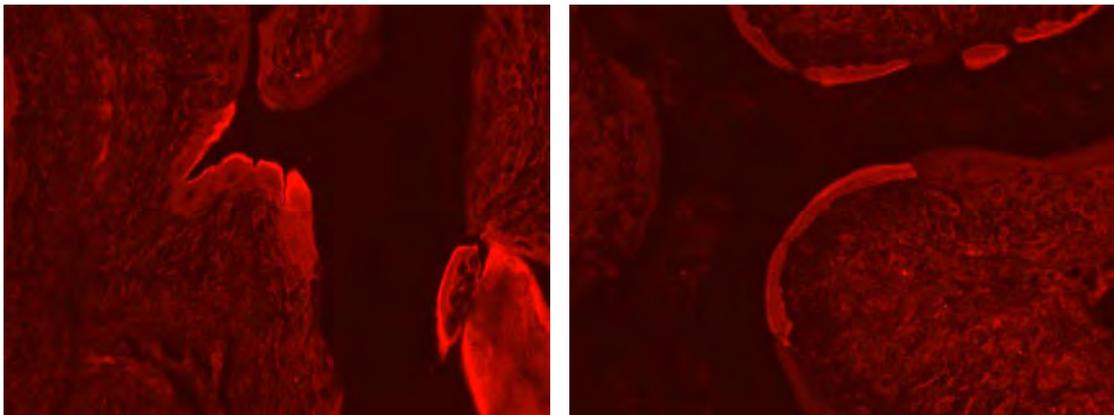
Figure 6. Immunohistochemical detection of cytokeratin 20 in ERβ-/- (A) and wt (B) female bladders of 4 months old mice.

In patients with interstitial cystitis the concentration of GAG in the urine is reduced. Surprisingly, the GAG content in the urine of ERβ-/- female mice was

increased. At the same time, as occurs in patients with cystitis, permeability of bladder wall for sodium fluorescein was increased in ERβ-/- mice.

We concluded that ERβ-/- female mice show an IC-like phenomenon in the bladder that is not dependent on differentiation of epithelium, and therefore could not be explained by our hypothesis.

Further investigation showed that the mechanism underlying destruction of urothelium was a marked increase in infiltration of immune cells into the bladder. These cells appeared even before the manifestation of the phenotype. Immunohistochemical staining with markers for immune cells showed that just before the destruction of epithelium there is an increased infiltration of lamina propria by macrophages, followed by accumulation of γδT cells in the areas of epithelial destruction. Interestingly, γδT cells have been reported to be accumulated in the bladder urothelium of interstitial cystitis patients, providing additional evidence for the homology of the phenotype seen in our mice with human disease (Fig. 7).
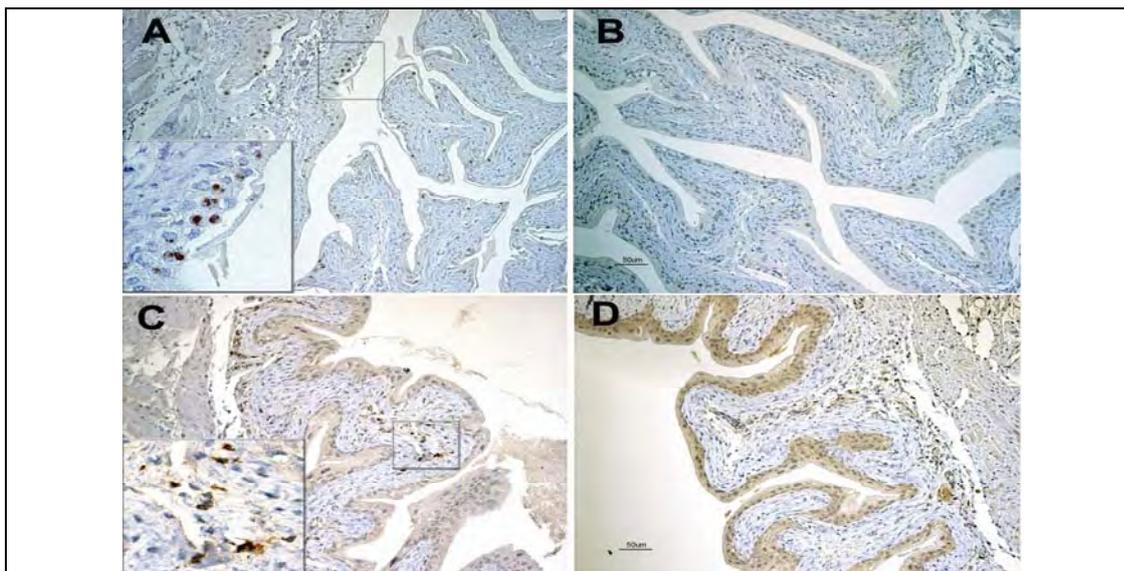
Figure 7. T cells and macrophage infiltration of ERβ-/- female bladders. Infiltration of urothelium by γδT cells in ERβ-/- (A) and wt (B) mice. Bladder sections from 7-month-old ERβ-/- (A) and wt mice (B) were stained with γδ

TCR and sections from 3.5-month-old ERβ-/- (C) and wt mice (D) were stained with F4/80 antibodies and counterstained with hematoxylin. (Magnifications: A–D, x20; Insets, x60.)

We concluded that ERβ-/- female mice do develop bladder phenotype resembling interstitial cystitis in humans, however local expression of ERβ is not likely to play a role in this phenomenon. More likely it is suppression of ERβ signaling in immune system that can be involved in the pathogenesis of interstitial cystitis.

5 FUTURE PROSPECTIVES

The studies in this thesis provided some insights into the functional role of ERβ in prostate and bladder. However, the work is far from being finished. The history of ERβ research is very short and the role of this receptor as a target for pharmacological interventions is only emerging.

There are some aspects of prostate research that I think would be worth following. Most exciting would be to try to understand the role of ERβ and ERα in the development of prostatic gland, i.e. to test the hypothesis of "positive" and negative imprinting.

Moreover it is still not quite clear at what stages of prostate cancer ERβ agonists would be of benefit. I suppose ERβ agonists could be of benefit for the patients with PSA levels in so-called grey zone (4-7 ng/ml) with pathological signs of PIN lesions. In this case the indication for ERβ agonists would perhaps not be the treatment but prevention of prostate cancer. The other interesting possibility could be use of ERβ agonists in a neo-adjuvant setting, i.e. pre and perioperatively. Perhaps, bringing a tumor to a more differentiated and less invasive state could help preventing positive margins and local recurrence.

One of the biggest questions of bringing ERβ agonists to the clinical practice would be designing the medications so that they can be not only ER specific, but perhaps tissue or even cell-type specific.

Interestingly, we still do not know to what extent suppression of ERβ in the immune system influences the phenotypical changes in ERβ-/- prostates. Our studies with bladder phenotype clearly show that changes in ERβ expression in the immune system has major influence on urogenital organs. In

fact, since ERβ is expressed in bone marrow and in most of the immune cells, it is natural to speculate that ERβ agonists would affect natural history of cancer not only via direct effects on epithelial cells, but also through infiltrating immune cells.

ACKNOWLEDGEMENTS

Our laboratory assistants: Annamarie Witte, and Christina Thulin-Andersson. Christina, kommer du ihåg den gamla Otabek som inte kunde ett ord på svenska? Jag kommer att sakna dig och våra småprat!

Prof. Lotta Wikström for helping with Swedish medical license exams.

My collaborators from Danderyd Sjukhus, Prof. Ulf Bergerheim and Dr. Linda Waage. Linda, I am not going to miss you, since I am sure we will meet very soon in the frontiers of clinical work! Good luck with your research projects!

People from Medical Nutrition that helped me all along: Ylva Ekendahl, Lena Magnell, Monica Ahlberg and Marie Franzen.

Scientific and lunch gang of mednut: Tove Berg, Pernilla Karlsson, Ming Chen, Waffa Osman, Erik Hedman, Ylva Rosengarten, Hanna Sagelius, Eva Schmidt!

Rikard Hofslagare, not only an IT-wizard but a cool person to talk to! Going to miss our discussions on modern history and WWII!

Finally the people who actually make the world of mednut go round, Gunnel Almberger and Lars Nybom!

And last, but not the least, C57Bl mice (Wt, ERKO, BERKO and ArKO). Thank you for sacrificing your lives and teaching us more about prostate physiology.

Oh, of course, thanks also to the Swedish Cancer Society for financing my work!

6 REFERENCES

.
(1967). "Treatment and survival of patients with cancer of the prostate. The Veterans Administration Co-operative Urological Research Group." <u>Surg Gynecol Obstet</u> **124**(5): 1011-7.

(1976). "Exposure in utero to diethylstilbestrol and related synthetic hormones. Association with vaginal and cervical cancers and other abnormalities." <u>Jama</u> **236**(10): 1107-9.

Acar, D., S. Cayan, et al. (2006). "The effect of tamoxifen on bladder functions and histology, and the role of estrogen receptor beta in a rat chemical cystitis model." <u>Neurourol Urodyn</u>.

Andriole, G., D. Bostwick, et al. (2004). "Chemoprevention of prostate cancer in men at high risk: rationale and design of the reduction by dutasteride of prostate cancer events (REDUCE) trial." <u>J Urol</u> **172**(4 Pt 1): 1314-7.

Andriole, G., D. Bostwick, et al. (2005). "The effects of 5alpha-reductase inhibitors on the natural history, detection and grading of prostate cancer: current state of knowledge." <u>J Urol</u> **174**(6): 2098-104.

Arriza, J. L., C. Weinberger, et al. (1987). "Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor." <u>Science</u> **237**(4812): 268-75.

Baba, S., G. Janetschek, et al. (1982). "The effects of chlorotrianisene (Tace) of kinetics of 3H-testosterone metabolism in patients with carcinoma of the prostate." <u>Br J Urol</u> **54**(4): 393-8.

Baker, A. R., D. P. McDonnell, et al. (1988). "Cloning and expression of full-length cDNA encoding human vitamin D receptor." <u>Proc Natl Acad Sci U S A</u> **85**(10): 3294-8.

Bashirelahi, N., E. S. Kneussl, et al. (1979). "Measurement and characterization of estrogen receptors in the human prostate." <u>Prog Clin Biol Res</u> **33**: 65-84.

Beckman, W. C., Jr., R. R. Newbold, et al. (1994). "Molecular feminization of mouse seminal vesicle by prenatal exposure to diethylstilbestrol: altered expression of messenger RNA." <u>Journal of Urology</u> **151**(5): 1370-8.

Beeson, P. B. (1994). "Age and sex associations of 40 autoimmune diseases." <u>Am J Med</u> **96**(5): 457-62.

Berger, R. E., J. E. Miller, et al. (1998). "Bladder petechiae after cystoscopy and hydrodistension in men diagnosed with prostate pain." <u>J Urol</u> **159**(1): 83-5.

Bonkhoff, H., U. Stein, et al. (1994). "Multidirectional differentiation in the normal, hyperplastic, and neoplastic human prostate: simultaneous demonstration of cell-specific epithelial markers." <u>Hum Pathol</u> **25**(1): 42-6.

Bostwick, D. G. and M. K. Brawer (1987). "Prostatic intra-epithelial neoplasia and early invasion in prostate cancer." <u>Cancer</u> **59**(4): 788-94.

Bouic, P. J., S. Etsebeth, et al. (1996). "beta-Sitosterol and beta-sitosterol glucoside stimulate human peripheral blood lymphocyte proliferation: implications for their use as an immunomodulatory vitamin combination." <u>Int J Immunopharmacol</u> **18**(12): 693-700.

Brand, N., M. Petkovich, et al. (1988). "Identification of a second human retinoic acid receptor." <u>Nature</u> **332**(6167): 850-3.

Bruchovsky, N. and J. D. Wilson (1968). "The conversion of testosterone to 5-alpha-androstan-17-beta-ol-3-one by rat prostate in vivo and in vitro." <u>J Biol Chem</u> **243**(8): 2012-21.

Chaisiri, N. and C. G. Pierrepoint (1980). "Examination of the distribution of oestrogen receptor between the stromal and epithelial compartments of the canine prostate." <u>Prostate</u> **1**(3): 357-66.

Cheng, J., E. J. Lee, et al. (2004). "Expression of estrogen receptor beta in prostate carcinoma cells inhibits invasion and proliferation and triggers apoptosis." <u>FEBS Lett</u> **566**(1-3): 169-72.

Ciocca, D. R. and L. M. Roig (1995). "Estrogen receptors in human nontarget tissues: biological and clinical implications." <u>Endocr Rev</u> **16**(1): 35-62.

Civantos, F., M. S. Soloway, et al. (1996). "Histopathological effects of androgen deprivation in prostatic cancer." Semin Urol Oncol **14**(2 Suppl 2): 22-31.

Conneely, O. M., W. P. Sullivan, et al. (1986). "Molecular cloning of the chicken progesterone receptor." Science **233**(4765): 767-70.

Cook, L. S., M. Goldoft, et al. (1999). "Incidence of adenocarcinoma of the prostate in Asian immigrants to the United States and their descendants." J Urol **161**(1): 152-5.

Cote, R. J., E. C. Skinner, et al. (1998). "The effect of finasteride on the prostate gland in men with elevated serum prostate-specific antigen levels." Br J Cancer **78**(3): 413-8.

Dahllof, B., A. Billstrom, et al. (1993). "Estramustine depolymerizes microtubules by binding to tubulin." Cancer Res **53**(19): 4573-81.

Docimo, S. G., R. I. Silver, et al. (2000). "The undescended testicle: diagnosis and management." Am Fam Physician **62**(9): 2037-44, 2047-8.

Doetschman, T., R. G. Gregg, et al. (1987). "Targetted correction of a mutant HPRT gene in mouse embryonic stem cells." Nature **330**(6148): 576-8.

Driscoll, S. G. and S. H. Taylor (1980). "Effects of prenatal maternal estrogen on the male urogenital system." Obstet Gynecol **56**(5): 537-42.

Dupont, S., A. Krust, et al. (2000). "Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes." Development **127**(19): 4277-91.

Eddy, E. M., T. F. Washburn, et al. (1996). "Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility." Endocrinology **137**(11): 4796-805.

English, H. F., J. R. Drago, et al. (1985). "Cellular response to androgen depletion and repletion in the rat ventral prostate: autoradiography and morphometric analysis." Prostate **7**(1): 41-51.

Etzioni, R. D., N. Howlader, et al. (2005). "Long-term effects of finasteride on prostate specific antigen levels: results from the prostate cancer prevention trial." J Urol **174**(3): 877-81.

Fisher, C. R., K. H. Graves, et al. (1998). "Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene 10.1073/pnas.95.12.6965." PNAS **95**(12): 6965-6970.

Forster, C., S. Makela, et al. (2002). "Involvement of estrogen receptor beta in terminal differentiation of mammary gland epithelium." Proc Natl Acad Sci U S A **99**(24): 15578-83.

Green, S., P. Walter, et al. (1986). "Cloning of the human oestrogen receptor cDNA." J Steroid Biochem **24**(1): 77-83.

Greene, G. L., C. Nolan, et al. (1980). "Monoclonal antibodies to human estrogen receptor." Proc Natl Acad Sci U S A **77**(9): 5115-9.

Hall, J. M. and K. S. Korach (2002). "Analysis of the molecular mechanisms of human estrogen receptors alpha and beta reveals differential specificity in target promoter regulation by xenoestrogens." J Biol Chem **277**(46): 44455-61.

Hayward, S. W., J. R. Brody, et al. (1996). "An edgewise look at basal epithelial cells: three-dimensional views of the rat prostate, mammary gland and salivary gland." Differentiation **60**(4): 219-27.

Henderson, B. E., B. Benton, et al. (1976). "Urogenital tract abnormalities in sons of women treated with diethylstilbestrol." Pediatrics **58**(4): 505-7.

Huggins, C. and C. V. Hodges (1941). "Studies on prostatic cancer; effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of prostate." Cancer Research **1**: 293.

Huggins, C., W. W. Scott, et al. (1941). "Studies on prostatic cancer III. The effect of fever, of desoxycorticosterone and of estrogen on clinical patients with metastatic carcinoma of the prostate." J Urol **46**: 997.

Huggins, C., R. E. Stevens, Jr., et al. (1941). "Studies on prostatic cancer; effects of castration on advanced carcinoma of prostate gland." Arch Surg **43**: 209.

Imamov, O., N. A. Lopatkin, et al. (2004). "Estrogen receptor beta in prostate cancer." N Engl J Med **351**(26): 2773-4.

Imamov, O., A. Morani, et al. (2004). "Estrogen receptor beta regulates epithelial cellular differentiation in the mouse ventral prostate." Proc Natl Acad Sci U S A **101**(25): 9375-80.

Imamov, O., G. J. Shim, et al. (2005). "Estrogen receptor beta in health and disease." Biol Reprod **73**(5): 866-71.

Isaacs, J. T. and D. S. Coffey (1989). "Etiology and disease process of benign prostatic hyperplasia." Prostate Suppl **2**: 33-50.

Jarred, R. A., M. Keikha, et al. (2002). "Induction of apoptosis in low to moderate-grade human prostate carcinoma by red clover-derived dietary isoflavones." Cancer Epidemiol Biomarkers Prev **11**(12): 1689-96.

Jarred, R. A., S. J. McPherson, et al. (2003). "Anti-androgenic action by red clover-derived dietary isoflavones reduces non-malignant prostate enlargement in aromatase knockout (ArKo) mice." Prostate **56**(1): 54-64.

Jeltsch, J. M., Z. Krozowski, et al. (1986). "Cloning of the chicken progesterone receptor." Proc Natl Acad Sci U S A **83**(15): 5424-8.

Jensen, E. V. and H. I. Jacobson (1962). "Basic guides to the mechanism of estrogen action." Recent Prog Horm Res **18**: 387-414.

Kato, K., S. Takahashi, et al. (2000). "Suppressive effects of dietary genistin and daidzin on rat prostate carcinogenesis." Jpn J Cancer Res **91**(8): 786-91.

Kolonel, L. N., D. Altshuler, et al. (2004). "The multiethnic cohort study: exploring genes, lifestyle and cancer risk." Nat Rev Cancer **4**(7): 519-27.

Kozak, I., W. Bartsch, et al. (1982). "Nuclei of stroma: site of highest estrogen concentration in human benign prostatic hyperplasia." Prostate **3**(5): 433-8.

Krege, J. H., J. B. Hodgin, et al. (1998). "Generation and reproductive phenotypes of mice lacking estrogen receptor beta." Proc Natl Acad Sci U S A **95**(26): 15677-82.

Kuiper, G. G., E. Enmark, et al. (1996). "Cloning of a novel receptor expressed in rat prostate and ovary." Proc Natl Acad Sci U S A **93**(12): 5925-30.

Kuiper, G. G., J. G. Lemmen, et al. (1998). "Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta." Endocrinology **139**(10): 4252-63.

Kumar, A. P., G. E. Garcia, et al. (2001). "2-methoxyestradiol blocks cell-cycle progression at G(2)/M phase and inhibits growth of human prostate cancer cells." Mol Carcinog **31**(3): 111-24.

Kuwajerwala, N., E. Cifuentes, et al. (2002). "Resveratrol induces prostate cancer cell entry into s phase and inhibits DNA synthesis." Cancer Res **62**(9): 2488-92.

Labrie, F., A. Dupont, et al. (1986). "Advantages of the combination therapy in previously untreated and treated patients with advanced prostate cancer." J Steroid Biochem **25**(5B): 877-83.

Lubahn, D. B., J. S. Moyer, et al. (1993). "Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene." Proc Natl Acad Sci U S A **90**(23): 11162-6.

Luo, J., T. A. Dunn, et al. (2003). "Decreased gene expression of steroid 5 alpha-reductase 2 in human prostate cancer: implications for finasteride therapy of prostate carcinoma." Prostate **57**(2): 134-9.

Mahendroo, M. S., K. M. Cala, et al. (2001). "Unexpected virilization in male mice lacking steroid 5 alpha-reductase enzymes." Endocrinology **142**(11): 4652-62.

Massengill, J. C., L. Sun, et al. (2003). "Pretreatment total testosterone level predicts pathological stage in patients with localized prostate cancer treated with radical prostatectomy." J Urol **169**(5): 1670-5.

Merchenthaler, I., G. E. Hoffman, et al. (2005). "Estrogen and estrogen receptor-{beta} (ER{beta})-selective ligands induce galanin expression within gonadotropin hormone-releasing hormone-immunoreactive neurons in the female rat brain." Endocrinology **146**(6): 2760-5.

Miesfeld, R., S. Okret, et al. (1984). "Characterization of a steroid hormone receptor gene and mRNA in wild-type and mutant cells." Nature **312**(5996): 779-81.

Migliaccio, S., R. R. Newbold, et al. (1995). "Alterations in estrogen levels during development affects the skeleton: use of an animal model." Environmental Health Perspectives **103 Suppl 7**: 95-7.

Miller, C. P., M. D. Collini, et al. (2003). "Constrained phytoestrogens and analogues as ERbeta selective ligands." Bioorg Med Chem Lett **13**(14): 2399-403.

44

Miller, J. L., I. Rothman, et al. (1995). "Prostatodynia and interstitial cystitis: one and the same?" Urology **45**(4): 587-90.

Moore, J. T., D. D. McKee, et al. (1998). "Cloning and characterization of human estrogen receptor beta isoforms." Biochem Biophys Res Commun **247**(1): 75-8.

Nesbit, R. M. and W. C. Baum (1950). "Endocrine control of prostatic carcinoma, clinical and statistical survey of 1,818 cases." J.A.M.A. **143**: 1317.

Nesbit, R. M. and R. T. Plumb (1946). "Prostatic carcinoma, follow-up on 795 patients trated prior to endocrine era and comparison of survival rates between these and patients treated by endocrine therapy." Surgery **20**(263).

Neubauer, B. L., A. M. McNulty, et al. (2003). "The selective estrogen receptor modulator trioxifene (LY133314) inhibits metastasis and extends survival in the PAIII rat prostatic carcinoma model." Cancer Res **63**(18): 6056-62.

Newbold, R. R., B. T. Pentecost, et al. (1989). "Female gene expression in the seminal vesicle of mice after prenatal exposure to diethylstilbestrol." Endocrinology **124**(5): 2568-76.

Ogawa, S., S. Inoue, et al. (1998). "Molecular cloning and characterization of human estrogen receptor betacx: a potential inhibitor ofestrogen action in human." Nucleic Acids Res **26**(15): 3505-12.

Omoto, Y., O. Imamov, et al. (2005). "Estrogen receptor alpha and imprinting of the neonatal mouse ventral prostate by estrogen." Proc Natl Acad Sci U S A **102**(5): 1484-9.

Onozawa, M., T. Kawamori, et al. (1999). "Effects of a soybean isoflavone mixture on carcinogenesis in prostate and seminal vesicles of F344 rats." Jpn J Cancer Res **90**(4): 393-8.

Paech, K., P. Webb, et al. (1997). "Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites." Science **277**(5331): 1508-10.

Parsons, C. L., R. A. Greene, et al. (2005). "Abnormal urinary potassium metabolism in patients with interstitial cystitis." J Urol **173**(4): 1182-5.

Petkovich, M., N. J. Brand, et al. (1987). "A human retinoic acid receptor which belongs to the family of nuclear receptors." Nature **330**(6147): 444-50.

Pitts, W. R., Jr. (2004). "The clinical implications of the Prostate Cancer Prevention Trial (PCPT)." BJU Int **93**(7): 1120-1.

Prins, G. S., L. Birch, et al. (2001). "Estrogen imprinting of the developing prostate gland is mediated through stromal estrogen receptor alpha: studies with alphaERKO and betaERKO mice." Cancer Research **61**(16): 6089-97.

Prins, G. S., L. Birch, et al. (2001). "Estrogen imprinting of the developing prostate gland is mediated through stromal estrogen receptor alpha: studies with alphaERKO and betaERKO mice." Cancer Res **61**(16): 6089-97.

Prins, G. S., L. Birch, et al. (2001). "Influence of neonatal estrogens on rat prostate development." Reprod Fertil Dev **13**(4): 241-52.

Prinsac, G. S., L. Birch, et al. (2001). "Influence of neonatal estrogens on rat prostate development." Reprod Fertil Dev **13**(4): 241-52.

Pylkkanen, L., R. Santti, et al. (1991). "Regional differences in the prostate of the neonatally estrogenized mouse." Prostate **18**(2): 117-29.

Qadan, L. R., C. M. Perez-Stable, et al. (2001). "2-Methoxyestradiol induces G2/M arrest and apoptosis in prostate cancer." Biochem Biophys Res Commun **285**(5): 1259-66.

Rafi, M. M., R. T. Rosen, et al. (2000). "Modulation of bcl-2 and cytotoxicity by licochalcone-A, a novel estrogenic flavonoid." Anticancer Res **20**(4): 2653-8.

Raivio, T., H. Santti, et al. (2003). "Reduced circulating androgen bioactivity in patients with prostate cancer." Prostate **55**(3): 194-8.

Rajfer, J. and D. S. Coffey (1978). "Sex steroid imprinting of the immature prostate. Long-term effects." Invest Urol **16**(3): 186-90.

Rubin, M. A., Y. Allory, et al. (2005). "Effects of long-term finasteride treatment on prostate cancer morphology and clinical outcome." Urology **66**(5): 930-4.

Rubin, M. A. and P. W. Kantoff (2003). "Prevention of prostate cancer with finasteride." N Engl J Med **349**(16): 1569-72; author reply 1569-72.

Rubin, M. A. and P. W. Kantoff (2004). "Effect of finasteride on risk of prostate cancer: how little we really know." J Cell Biochem **91**(3): 478-82.

Sato, K., H. Fukata, et al. (2006). "Neonatal exposure to diethylstilbestrol alters the expression of DNA methyltransferases and methylation of genomic DNA in the epididymis of mice." Endocr J **53**(3): 331-7.

Saunders, P. T., S. M. Maguire, et al. (1997). "Expression of oestrogen receptor beta (ER beta) in multiple rat tissues visualised by immunohistochemistry." J Endocrinol **154**(3): R13-6.

Schatzl, G., C. Brossner, et al. (2000). "Endocrine status in elderly men with lower urinary tract symptoms: correlation of age, hormonal status, and lower urinary tract function. The Prostate Study Group of the Austrian Society of Urology." Urology **55**(3): 397-402.

Schrager, S. and B. E. Potter (2004). "Diethylstilbestrol exposure." Am Fam Physician **69**(10): 2395-400.

Shim, G. J., L. L. Kis, et al. (2004). "Autoimmune glomerulonephritis with spontaneous formation of splenic germinal centers in mice lacking the estrogen receptor alpha gene." Proc Natl Acad Sci U S A **101**(6): 1720-4.

Shim, G. J., L. Wang, et al. (2003). "Disruption of the estrogen receptor beta gene in mice causes myeloproliferative disease resembling chronic myeloid leukemia with lymphoid blast crisis." Proc Natl Acad Sci U S A **100**(11): 6694-9.

Shim, G. J., M. Warner, et al. (2004). "Aromatase-deficient mice spontaneously develop a lymphoproliferative autoimmune disease resembling Sjogren's syndrome." Proc Natl Acad Sci U S A **101**(34): 12628-33.

Shoskes, D. A., S. I. Zeitlin, et al. (1999). "Quercetin in men with category III chronic prostatitis: a preliminary prospective, double-blind, placebo-controlled trial." Urology **54**(6): 960-3.

Strauss, L., S. Makela, et al. (1998). "Genistein exerts estrogen-like effects in male mouse reproductive tract." Mol Cell Endocrinol **144**(1-2): 83-93.

Strom, A., J. Hartman, et al. (2004). "Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D." Proc Natl Acad Sci U S A **101**(6): 1566-71.

Sun, Y., M. Chen, et al. (2007). "EGF and HB-EGF modulate inward potassium current in human bladder urothelial cells from normal and interstitial cystitis patients." Am J Physiol Cell Physiol **292**(1): C106-14.

Swaneck, G. E., J. M. Alvarez, et al. (1982). "Multiple species of estrogen binding sites in the nuclear fraction of the rat prostate." Biochem Biophys Res Commun **106**(4): 1441-7.

Taylor, A. H. and F. Al-Azzawi (2000). "Immunolocalisation of oestrogen receptor beta in human tissues." J Mol Endocrinol **24**(1): 145-55.

Thomas, K. R. and M. R. Capecchi (1987). "Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells." Cell **51**(3): 503-12.

Thompson, I. M., P. J. Goodman, et al. (2003). "The influence of finasteride on the development of prostate cancer." N Engl J Med **349**(3): 215-24.

Trapman, J., P. Klaassen, et al. (1988). "Cloning, structure and expression of a cDNA encoding the human androgen receptor." Biochem Biophys Res Commun **153**(1): 241-8.

Walter, P., S. Green, et al. (1985). "Cloning of the human estrogen receptor cDNA." Proc Natl Acad Sci U S A **82**(23): 7889-93.

Wang, Y., S. Hayward, et al. (2001). "Cell differentiation lineage in the prostate." Differentiation **68**(4-5): 270-9.

Weihua, Z., R. Lathe, et al. (2002). "An endocrine pathway in the prostate, ERbeta, AR, 5alpha-androstane-3beta,17beta-diol, and CYP7B1, regulates prostate growth." Proc Natl Acad Sci U S A **99**(21): 13589-94.

Weihua, Z., S. Makela, et al. (2001). "A role for estrogen receptor beta in the regulation of growth of the ventral prostate." Proc Natl Acad Sci U S A **98**(11): 6330-5.

vom Saal, F. S., B. G. Timms, et al. (1997). "Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses." Proc Natl Acad Sci U S A **94**(5): 2056-61.