ROLE OF PROLACTIN IN THE PROSTATE GLAND

STUDIES IN TRANSGENIC MOUSE MODELS

JON KINDBLOM
ABSTRACT

Role of prolactin in the prostate gland - Studies in transgenic models

Jon Kindblom

Department of Physiology, The Sahlgrenska Academy, Göteborg University, Sweden

The aim of the studies in this thesis was to investigate the role of prolactin (PRL) in normal and pathophysiological conditions of the prostate gland using three different genetically modified mouse models. Benign and malignant disorders of the prostate are highly prevalent in aging men. Although an indispensable role of androgens in prostate biology is undisputed, the molecular mechanisms underlying benign prostatic hyperplasia (BPH) and prostate cancer remain largely uncharacterized. PRL is one of the non-androgenic hormones and growth factors that have been implicated in the etiology of these disorders. Both the PRL ligand and its receptors are normally expressed in human and rodent prostate.

General overexpression of a rat PRL transgene (Mt-PRL) resulted in hyperprolactinemia and a dramatic enlargement of the murine prostate gland in older transgenic males with a concomitant elevation of circulating testosterone. The prostatic hyperplasia was primarily stromal with some focal epithelial dysplasia. Prevalence and degree of prostate enlargement did not differ in transgenic lines exhibiting different serum PRL levels (range 15-250 ng/ml) and androgen levels showed no correlation with prostate size in individual animals. Castration and testosterone resubstitution studies demonstrated that the hyperplastic prostate phenotype was not dependent on elevated androgen levels. Prolonged exogenous androgen administration in wildtype males did not significantly affect prostate size or histological appearance. Ductal morphogenesis was increased in Mt-PRL prostate, either due to direct PRL stimulation or indirectly via an altered androgenic status. A prostate-specific PRL transgenic mouse (Pb-PRL) was generated using the minimal probasin promoter. Expression of the transgene was restricted to the prostate and detectable from 4 weeks of age. The Pb-PRL males developed a dramatic prostatic hyperplasia while maintaining normal circulating androgen levels. Furthermore, an increased stromal cell androgen receptor immunoreactivity was demonstrated in both Mt-PRL and Pb-PRL prostate as compared to both normal and testosterone-treated wildtype controls. PRL receptor deficiency (PRLR -/-) resulted in significant loss of epithelial cells and increased postcastrational regression in the dorsal prostate lobe. A decreased formation of premalignant changes and a complete lack of tumor induction in PRLR -/- males crossed to the C3/Tag transgenic prostate cancer model strongly suggest a role for PRL in prostate carcinogenesis.

In conclusion, increased levels of PRL have significant stimulatory effects on prostate ductal development and lead to hyperplastic growth in the adult gland, independent of elevations in circulating androgen levels. In contrast, loss of PRLR function has only subtle essential effects on prostate development whereas a role for PRL in prostate carcinogenesis is demonstrated.
LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Wennbo H, Kindblom J, Isaksson OGP and Törnell J.
Transgenic mice overexpressing the prolactin gene develop dramatic enlargement of the prostate gland.

II. Kindblom J, Dillner K, Ling C, Törnell J and Wennbo H
Progressive prostate hyperplasia in adult prolactin transgenic mice is not dependent on elevated serum androgen levels.

III. Kindblom J, Dillner K, Sahlin L, Robertson F, Ormandy CJ, Törnell J and Wennbo H.
Prostate hyperplasia in a transgenic mouse with prostate-specific expression of prolactin.
*Endocrinology* 2003; in press.

Prostate Development and Carcinogenesis in Prolactin Receptor Knockout Mice.
Accepted for publication, *Endocrinology* (2003).
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<th>Description</th>
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<tr>
<td>-/-</td>
<td>homozygous gene-deficiency</td>
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<tr>
<td>+/-</td>
<td>heterozygous gene-deficiency</td>
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<tr>
<td>aa</td>
<td>amino acids</td>
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<td>AP</td>
<td>anterior prostate lobe</td>
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<tr>
<td>AR</td>
<td>androgen receptor</td>
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<tr>
<td>BAC</td>
<td>bacterial artificial chromosomes</td>
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<tr>
<td>bGH</td>
<td>bovine growth hormone</td>
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<td>BPH</td>
<td>benign prostatic hyperplasia</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CAIS</td>
<td>complete androgen insensitivity syndrome</td>
</tr>
<tr>
<td>CIS</td>
<td>cytokine inducible SH2-domain-containing protein</td>
</tr>
<tr>
<td>CZ</td>
<td>central zone</td>
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<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
</tr>
<tr>
<td>DLP</td>
<td>dorsolateral prostate</td>
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<td>DP</td>
<td>dorsal prostate lobe</td>
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<tr>
<td>E</td>
<td>exon</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<td>estrogen receptor</td>
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<td>FSH</td>
<td>follicle stimulating hormone</td>
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<td>GH</td>
<td>growth hormone</td>
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<td>GHR</td>
<td>growth hormone receptor</td>
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<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
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<td>human prolactin</td>
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<tr>
<td>I-</td>
<td>intermediate</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
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<td>interleukines</td>
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<tr>
<td>JAK</td>
<td>janus kinase</td>
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<tr>
<td>kb</td>
<td>kilo base pair(s)</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>L-</td>
<td>long</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
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<td>LHRH</td>
<td>luteinizing hormone releasing hormone</td>
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<td>LP</td>
<td>lateral prostate lobe</td>
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<tr>
<td>MAP</td>
<td>mitogen activated protein</td>
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<td>mitogen activated protein kinase</td>
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<td>m-AAT</td>
<td>mitochondrial aspartate aminotransferase</td>
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<td>Mt-1</td>
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<td>promoter</td>
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<td>prostate cancer</td>
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<tr>
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<td>polymerase chain reaction</td>
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<td>prolactin inhibiting factors</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>PL</td>
<td>placental lactogen</td>
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<td>PRL</td>
<td>prolactin</td>
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<td>PRLR</td>
<td>prolactin receptor</td>
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<td>PSA</td>
<td>prostate specific antigen</td>
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<td>PZ</td>
<td>peripheral zone</td>
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<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
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<td>rPRL</td>
<td>rat prolactin</td>
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<tr>
<td>RT</td>
<td>reverse transcription</td>
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<tr>
<td>S-</td>
<td>short</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology region 2</td>
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<tr>
<td>SH3</td>
<td>Src homology region 3</td>
</tr>
<tr>
<td>SHBG</td>
<td>steroid hormone-binding globulin</td>
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<tr>
<td>SOCS</td>
<td>suppressors of cytokine signaling</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
</tr>
<tr>
<td>SV</td>
<td>seminal vesicles</td>
</tr>
<tr>
<td>TZ</td>
<td>transitional zone</td>
</tr>
<tr>
<td>UGS</td>
<td>urogenital sinus</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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<tr>
<td>VP</td>
<td>ventral prostate lobe</td>
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INTRODUCTION
Prostate disease in the form of both benign hyperplasia and malignancy is an increasingly common clinical problem in the aging western male population. Although efforts to gain insight into the ethiology of these conditions have increased during the past decade, a detailed understanding of the pathophysiological processes involved is still lacking. Prostate research has traditionally focused on the action of androgens in the gland. Androgens are undeniably essential for normal prostate development and function (1). However, a growing body of work has suggested a vital role for non-androgenic hormones and growth factors in the induction of prostatic disease (2). This thesis focuses on the role of prolactin as one such non-androgenic hormone/growth factor involved in regulation of prostate growth and development. The results presented add to our basic knowledge of prostate development and growth and possibly has implications for future treatment of human prostate disorders.

THE PROSTATE GLAND
General physiology and function of the prostate gland
The prostate gland is an exocrine gland found in all mammals. It secretes enzymes, amines, lipids and metal ions, essential for the normal function of the spermatozoa. Accumulation and secretion of extraordinarily high levels of citrate is one of the principal functions of the prostate gland of humans and other animals (3). The presence of the prostate is universal in mammals; when compared among species the prostate is recognized by variations in its anatomy, biochemistry and pathology. The mature mammalian prostate is a glandular organ consisting of epithelial and stromal cell types that are hormonally regulated. The epithelium comprises a single layer of polarized columnar epithelial cells, together with basal cells and neuroendocrine cells. The epithelial cells provide secretions that empty through ducts into the urethra to form a major component of the seminal plasma of the ejaculate. The surrounding stromal compartment includes fibroblasts and smooth muscle cells, in addition to neuronal, lymphatic and vascular components. One notable functional difference between murine and human prostate is the presence of prostate specific antigen (PSA) expression in humans. PSA is an androgen-regulated serine protease produced by both prostate epithelial cells and prostate cancer and is the most commonly used serum marker for prostate cancer. It is also widely used to monitor responses to therapy. Genes related to human PSA have been detected in several nonhuman primate species, but not in other mammalian species, including mouse, rat, dog, rabbit, pig and cow (4).
Prostate development

In all mammals, androgen formed in the developing testes is responsible for the aspects of male development in which the Wolffian ducts, urogenital sinus and urogenital tubercle are transformed into the epididymis/vas deferens, prostate and penis. It is also well known that androgens and mesenchymal-epithelial interactions are required for the formation and growth of the prostate. Development of glandular organs such as the prostate involves the process of branching morphogenesis. The developing prostate lobes begin as an epithelial bud that invades the surrounding mesenchyme, projecting dividing epithelial cords or tubes away from the site of initiation. Growth of the prostatic ductal network during the prepubertal period is considered nonuniform, with ductal growth being highest in the distal region, at the ductal tips, and much lower in the proximal region closest to the urethra (5, 6).

In rodents, the critical time period for ductal budding and the ensuing process of ductal growth and branching commences at day 15 of gestation and concludes approximately 4 to 5 weeks postpartum (7-9). Work by Sugimura et al has demonstrated that during the first 15 days after birth, over 75% of tips and branch-points of the adult gland are formed in the ventral lobe, and a majority of ductal tips and branch-points are also formed in the dorsolateral prostate (7). Consequently, ductal structure morphogenesis is nearly completed in the presence of very low testosterone levels and the increase in prostatic wet weight is only modest. Neonatal castration studies have demonstrated that neonatal prostatic ductal morphogenesis is sensitive to, but does not require, chronic androgen stimulation (10). At puberty, the murine testosterone levels rise significantly, and prostatic wet weight and DNA content increase more rapidly. This is in contrast to the situation in the human male, in which prostate morphogenesis occurs entirely during the fetal period, with ductal development primarily occurring in the first half of gestation (11).

Prostate anatomy and structure in humans and rodents

The glandular structure of the prostate is common to most species, including human and mouse, although there are significant anatomical and structural differences between the human and murine prostate gland, which is commonly multilobular in rodents and alobular in humans. In the following paragraphs a more detailed description of the human and rodent prostate anatomy and structure is presented.
The murine prostate

In contrast to human anatomy, the rodent prostate shows a lobular anatomy, where organized and encapsulated individual lobes that arise from the urogenital sinus are located in specific positions around the urethra, but not completely circumscribing it (see Fig. 1B). In mouse, the prostate can be divided into anatomically distinct lobe pairs, which are not encased by abundant stroma and a capsule into a single gland as in the human prostate. The individual lobes are defined, according to their position relative to the bladder, as the ventral, dorsal, lateral and anterior (also known as the coagulating gland) prostate lobes (VP, DP, LP, AP). The DP and LP are often grouped together as the dorsolateral prostate (DLP). All lobes are responsive to estrogens and to androgens, but to varying degrees; the VP is more sensitive to androgens and the AP more sensitive to estrogens (12, 13). The proportion between prostate epithelial and stromal compartments differs between species, in adult rodents the epithelial to stromal ratio is approximately 5 to 1. In contrast, normal prostate of human and other primates demonstrate approximately equal numbers of stromal and epithelial cells (14, 15). The VP has no clear homologous counterpart in the prostate of higher animals, whereas the LP and DP are considered to be similar to the prostate structure seen in higher animals and human, for review see (16, 17). Although the ducts of the individual lobes are similar, there are characteristic cross-sectional patterns of ductal branching specific to each lobe. The VP and LP lobes attach to the urethra by two or three main ducts that show extensive “oak tree” branching, whereas the DP lobe demonstrates multiple main urethral ducts with less extensive “palm tree” branching morphology (7). The ductal system also shows regional variation in morphology and functional activity (18). A division of the rodent ductal system of each lobe into regional segments, defined as proximal, intermediate and distal with respect to the urethra is often used (19). Any comparative analysis of epithelial cell function and morphology in mouse prostate must therefore take lobular and regional orientation into consideration.

The individual glands making up each lobe of the mouse prostate are surrounded by an very thin stroma, composed of only a few layers of spindle cells interspersed amongst collagen fibers. The immediate periductal stroma is surrounded by loose connective tissue, imparting a lobular architecture to the prostate, without the abundant intervening dense collagenous stroma surrounding the glands of adjacent "lobules" in the human prostate. In addition, nerve bundles which are seen within the prostate stroma (interior to the capsule) in the posterolateral aspects of the human gland, are not observed within the thin rim of mouse stroma. Rather, thick nerve bundles are typically
located in the peristromal loose connective tissue, often in sections of the DLP.

The mouse DP is composed of branching ducts and glands lined by simple columnar and occasionally slightly stratified and tufted columnar epithelium. The moderate degree of infolding is intermediate between the AP and the flatter luminal borders of the LP and VP. The secretory cells have lightly eosinophilic granular cytoplasm, and the central to basally located small uniform nuclei contain inconspicuous or small nucleoli. Gland lumens contain homogenous eosinophilic secretions.

The LP has flatter luminal edges, with only sparse infoldings, with the abundant luminal space containing more particulate eosinophilic secretions. The epithelium is cuboidal to short columnar, with more clear to lightly granular cytoplasm and small uniform basally located nuclei. The VP also has flatter luminal edges and only focal epithelial tufting or infoldings. The abundant luminal spaces contain homogenous pale serous secretions. The nuclei are small, uniform and are typically basally located, with inconspicuous or small nucleoli. The mouse AP lies adjacent to the seminal vesicles (SV), along its curving length. Histologically, the AP demonstrates a more papillary and cribriform growth pattern compared to the other lobes, with cuboidal to columnar epithelial cells, typically containing central nuclei with inconspicuous to small nucleoli, and eosinophilic granular cytoplasm.

The gland lumens contain abundant slightly eosinophilic secretions. The architecture of the AP glands and the relationship to the Wolffian duct derived seminal vesicle is considered reminiscent of the human central zone.

Fig. 1. Structural anatomy of human (A) and murine (B) prostate demonstrating important differences in lobular composition. Illustration generously provided by Dr. M. Shen, Robert Wood Johnson Medical School.
The human prostate

There are of course similarities between the mouse and human prostate which support the use of mouse models for study of basic prostate function and development as well as for identifying key events in development and progression of prostate pathophysiology. However, important differences between the prostate in the two species exist, including the gross and micro anatomy, which may impact on basic aspects of pathologic analysis in mouse models and the application of the models in prostate cancer research. Despite the apparent recognition of distinct lobes in the developing human prostate, these are not easily recognizable in the adult prostate, which does not possess a defined external lobation. Nevertheless, in anatomical nomenclature the proposed terminology recognizes the following regions: basis, apex, right, left and middle lobes and isthmus (20). A clinically more useful terminology has been proposed by McNeal (21, 22), dividing the prostate into four separate zones based on morphology and named using the urethra as a central anatomic reference point; the anterior fibromuscular stroma that occupies up to one-third of the prostate volume in the normal prostate and contains minimal glandular tissue, the central zone (CZ), the periurethral transition zone (TZ) and the peripheral zone (PZ) (see Fig. 1A). This zonal terminology by McNeal has gradually gained a wide acceptance.

The PZ contains the majority of the glandular tissue in the normal prostate; approximately 75 % in prostates without BPH, and represents the most frequent site of prostate carcinoma origin (23). The PZ is also the predominant site for the occurrence of the prostatic cancer precursor lesions or prostatic intraepithelial neoplasia (PIN). The PZ is located predominantly in the posterior and lateral aspects of the gland, extends to the apex and variably anteriorly, and surrounds the CZ towards the base. The TZ is composed of lobules of glands with shorter ducts compared to those reaching out to the PZ and is often separated from the PZ by an indistinct band of collagenous tissue, which becomes more pronounced as the TZ is expanded by BPH. In the young, post-pubertal adult, architectural and histological differences in the glands of the TZ and the PZ are not well defined, but the TZ is the main site of BPH in the human prostate, characterized by nodules of glandular and stromal hyperplasia in addition to diffuse non-nodular enlargement (23).
PROSTATE PATHOPHYSIOLOGY

Benign and malignant disorders of the prostate gland are among the most common diseases affecting aging males in the industrialized countries. With demographic changes indicating a further aging of the population, the prevalence can be expected to increase further. Despite continuous research efforts over the last decades, a detailed understanding of the etiology behind prostate disease has not yet been reached.

Benign Prostatic Hyperplasia (BPH)

BPH is one of the most prevalent age-dependent diseases of adult males. Prevalence of histological BPH is reported at 50% by age 60 and reaches approximately 90% by age 80 years or older (24). Around half of these cases will also meet a clinical definition of BPH with a varying degree of symptoms presented. BPH is a non-malignant enlargement of the prostate gland due to both epithelial and stromal hyperplasia that in time produces an inward transmission of pressure on the urethra and an increased resistance to urine flow (25). BPH frequently leads to lower urinary tract symptoms, these primarily include voiding problems, frequency, nocturia, urgency, urge incontinence and stress incontinence. In many cases, patient life quality is significantly affected and medication or surgical intervention deemed appropriate. Indications for treatment of BPH are thus mainly associated with decreased patient life-quality. Nonetheless, a subset of BPH patients require surgical intervention due to more severe indications such as renal insufficiency, chronic retention or infection. A detailed understanding of the underlying mechanisms behind the age related changes leading to such a high frequency of prostate hyperplasia is lacking.

Androgens are clearly required for development of BPH and reduction of androgenic effects through 5-alpha reductase inhibitors is utilized in the pharmacotherapy of BPH. Treatment with 5-alpha reductase inhibitors rapidly reduces DHT serum levels up to 70% and over time results in an average decrease in prostate volume of 20-30% (26). The most reported side effects include diminished libido and impotence. In addition, alpha-1 adrenoreceptor antagonists are increasingly used, either given in combination with a 5-alpha reductase inhibitor or separately (27). The mechanism of action for the antagonists is primarily thought to be a decrease in smooth muscle cell contractility in the bladder neck and prostatic urethra leading to improved urinary flow. Traditional surgical techniques such as transurethral resection of the prostate are still appropriate for some patients, although with improved medical treatments available the number of men undergoing surgery is most likely declining (28).
Prostate cancer

Prostate carcinoma (PCa) is the most common malignant disease diagnosed in Sweden and presently comprises close to one third of all male cancer incidence. Incidence numbers have steadily increased over that past decades, presumably owing in large part to earlier detection and an aging population. PCa is also the leading cause of cancer-related death in Swedish males. This pattern is also seen in other industrialized countries of the northern hemisphere. Large international and interethnic differences are evident for both incidence and mortality of PCa. In global terms, PCa is the third most common cancer men and predicted to be the most common male cancer by the year 2015 (29). Incidence numbers are highest in Sweden, North America, Australia and France, ranging from around 50 to 135 cases per 100,000 person-years, versus less than 5 per 100,000 in low-incidence regions such as south-eastern Asia (30). However, the vast majority of men harbouring pathologic evidence of prostate cancer are not clinically diagnosed with the disease. The indolent nature of most prostate cancers explains why it is far more common to die with prostate cancer than as a direct result of the disease.

Ethiology of prostate cancer

PCa is an extraordinarily heterogeneous disease with a variety of prognostic factors influencing the ultimate outcome for the patient. The most established risk factors involved in initiation and development of PCa include ageing, race, dietary or other environmental factors and a family history of prostate disease. Aging is considered the most prominent risk factor, with approx 75% of all cases diagnosed in men between 50-70 years of age. There is also strong circumstantial evidence that androgens are implicated in the pathogenesis of PCa, yet there has so far been no conclusive evidence, despite numerous studies, that levels of circulating testosterone in individuals developing prostate cancer are higher than in controls. (31-35) However, one recent metaanalysis of previously published studies on hormonal predictors of risk for PCa did indicate that men with serum testosterone in the upper quartile of the population distribution have an approximately two-fold higher risk of developing prostate cancer (36).

During the last few years, much effort has gone into determining molecular genetic mechanisms involved in the development of prostate malignancy. Hereditary prostate cancer is a subtype of familial prostate cancer in which the susceptibility gene is inherited in a Mendelian fashion (37). Epidemiological studies indicate that dominantly inherited susceptibility genes with high
penetrance cause nearly 10% of all prostate cancer cases and as much as 40% of early onset disease (<55 years of age) (37, 38). As much as a 10-fold increase in life-time risk of developing prostate cancer is consequently reported in men with multiple first-degree relatives affected. Family-based linkage analysis have led to the identification of 7 different prostate cancer susceptibility loci, located on several chromosomes (39). Despite efforts, no major susceptibility gene for prostate cancer has yet been identified. The possible existence of multiple prostate cancer genes may explain why there has been only limited confirmatory evidence of linkage for currently known highly penetrant susceptibility loci or specific genes.

Diagnosis and treatment

The initial diagnostic instruments for detecting prostate carcinoma include primarily rectal examination and analysis of prostate specific antigen (PSA) in the serum. The diagnosis can then be verified by prostatic biopsies allowing histological grading and if needed further diagnostic steps are taken to correctly stage the malignant disease (40). Treatment of prostate cancer relies on multiple strategies depending on the grading and staging of the malignant disease. In the case of locally confined malignant disease, curative treatment in the form of radical resection of the prostate or radiation therapy is possible. Almost half of all prostate cancers are localised at the time of diagnosis. Non-localised cancer of the prostate is at present considered incurable, but endocrine therapy can significantly prolong survival and alleviate symptoms. Androgen deprival has been the treatment of choice for advanced prostate cancer over the past 50 years. Testicular production of testosterone is prevented by surgical orchiectomy or pharmacological treatment with a luteinizing hormone-releasing hormone (LHRH) agonist. In addition, a nonsteroidal or steroidal antiandrogen is often given to block the action of adrenal androgens. A recent metaanalysis comparing monotherapy (orchiectomy or LHRH agonist) and combined androgen blockade in advanced prostate cancer showed no increase in 2-year overall survival and only a modest difference in overall survival rate at 5 years with combined androgen blockade (41).

Animal models for prostate studies

In addition to the human male, spontaneous prostate disease is only described in other primates and possibly dogs (42). In rodents, no evidence of naturally occurring prostate carcinoma or any significant prostatic hyperplasia has been reported. Natural rodent models for studies of prostate disease are therefore unavailable. However, both prostate cancer and benign hypertrophy or
hyperplasia can be induced in the rodent prostate through genetic modulation or chemical induction and several such models have been established. The advent of transgenic techniques in mice have put increasing focus on the mouse as a model organism for in vivo studies aiming at understanding gene function and by this gain insights into human pathophysiological conditions. With the mouse genome project recently completed, the possibilities for direct comparison with the corresponding human genes exist.

Transgenic models for prostate tumor studies
The lack of spontaneous development of prostate malignancy in all species except human and other primates. Several transgenic mouse models have recently been established for use in prostate cancer studies, for complete reviews see (43, 44). The purpose of utilizing these animal models is to identify specific molecular changes in early malignant disease. As the mouse does not spontaneously develop prostate malignancy, different transgenic strategies for *in vivo* tumor induction have been developed. Such transgenes are preferably under the control of a prostate-specific promoter region such as probasin or C3, capable of directing expression to prostate epithelial cells.

There are two general classes of transgenic models of prostate cancer. The first consists of models resulting from enforced expression of SV40 early genes. These models include the TRAMP model, utilizing the minimal rat probasin promoter to express the SV40 early genes (T and t antigens; Tag) (45). In addition a number of transgenic lines use the long probasin promoter to express large T antigen have been established. This models displays progressive disease ranging from epithelial hyperplasia or PIN to adenocarcinoma and development of metastases (46). Also in this class of models are the C3(1)-Tag mice which were used for the carcinogenesis studies presented in paper IV of this thesis. The C3(1)-Tag transgenic animals develop progressive prostate cancer with metastatic capabilities. Use of the 5’ C3 region and not the complete C3 gene leads to a partial loss of promoter specificity in this model. Consequently, mammary gland expression of the transgene gives rise to mammary adenocarcinoma in the female (47). Two additional models, Cryptdin-2-T and Gg-SV40 T, also develop progressive prostate cancer although the promoters used to drive SV40 large T antigen expression in these cases are not inherently prostate specific.
The second general class of transgenic models for prostate studies utilizes the promoters mentioned above to express various “natural” molecules that have previously been suggested to play a role in development of prostate cancer. The list is extensive but includes c-myc, Bcl-2 and dominant negative TGFβ. It is interesting to note that in the majority of these models, only a relatively mild phenotype, primarily epithelial hyperplasia or low-grade PIN, is observed and often these phenotypes do not arise until the mice are of advanced age. One such example is the AR transgenic mouse, expressing increased levels of AR protein specifically in prostate secretory epithelium (48). Older AR transgenic mice developed focal areas of intraepithelial neoplasia, resembling human high-grade PIN, but no further malignancy was observed. No reports of any tumorigenic effects of exogenously added androgens in these models are available. A certain resistance to malignant transformation in the murine prostate compared to humans is thus strongly suggested.
PROLACTIN

The lactogenic effects of pituitary extract was first shown in pseudopregnant rabbits in 1928 (49). The previously unknown lactogenic substance was then purified from sheep pituitaries and named prolactin (PRL) in the early thirties. Due to difficulties in extraction and concentration procedures and lack of suitable bioassays, it took until the 1970s before human PRL could be successfully isolated and purified (50). The human PRL gene and corresponding cDNA was then cloned in the 1980s (51, 52). The PRL gene was subsequently cloned in a number of species and is now known to be present in all vertebrates (53). Prolactin (PRL) has classically been regarded as a purely pituitary-derived peptide hormone but over the last decade expression of the PRL gene has also been demonstrated in several extrapituitary tissues (54). The majority of circulating PRL comes from lactotroph secretion, whereas extrapituitary PRL is considered to act in a paracrine or autocrine fashion.

The PRL gene, variants and structure

PRL is a member of the growth hormone (GH) gene family, comprising of GH, variant-GH (GH-V), PRL and placental lactogens (PLs). All these genes are believed to have evolved from a common ancestral gene several hundred million years ago and are now divided in two different branches, the GH and the PRL branch. The gene structures of GH and PRL are rather similar and both contain 5 exons. The PRL gene is 4-5 times longer than the GH gene due to longer intron sequences (55). They show similarities in both amino acid sequence and to some extent biological function. The PRL gene is present in all vertebrates and, with the exception of fish, all PRLs so far identified consist of 197-199 amino acids (aa) and contain six cysteins forming three intermolecular disulphide bonds. In the rat, the rPRL gene is located on chromosome 17, approx 10 kb long, composed of 5 exons and 4 introns. The human PRL (hPRL) gene is approximately 10 kb long, located on chromosome 6 and contains an additional exon, E1a, at the 5´-end (52). The E1a exon is only transcribed in extrapituitary sites, generating a 134 bp longer transcript differing only in the 5´-untranslated region (UTR), compared to the pituitary transcripts (54). Prolactin gene transcription is regulated by two independent promoter regions. The proximal 5,000-bp region directs pituitary-specific expression, while a more upstream promoter region controls extrapituitary expression (56).

The hPRL cDNA is composed of 914 nucleotides and encodes a 227 aa pre-hormone, including a signal peptide of 28 aa. The mature hPRL thus contains 199 aa and the molecular mass of mature PRL is approx 23 kDa. PRL is an all-α-helix protein containing ~50% α-helices, with the remaining
protein folding into non-organized loop structures. Although the tertiary structure has not been determined, PRL is predicted to adopt the four-helix bundle folding described for the GHs (53, 57).

Control of PRL synthesis and secretion

The synthesis and secretion of PRL by lactotrophic cells in the anterior pituitary gland is subjected to multiple regulators. These can broadly be classified as endocrine, paracrine, juxtacrine or autocrine, depending on their respective origin. The secretory activity of the lactotrophs reflects a balance between local and distant inhibitory and releasing factors. In the absence of target gland hormones to provide feedback control over the lactotrophs, PRL also to some extent auto regulates its own release (58). In the hypothalamus, PRL interacts with the dopaminergic systems. Dopamine has long been attributed a dominant role as an inhibitor of Prl secretion by acting itself as the main Prl inhibiting factor (PIF) (59). Dopamine binds to type-2 dopamine receptors that are functionally linked to membrane channels and G proteins, thereby suppressing the high intrinsic secretory activity of the pituitary lactotrophs. In addition to inhibition of PRL release by controlling calcium fluxes, dopamine activates several interacting intracellular signaling pathways and suppresses PRL gene expression and lactotroph proliferation.

Pituitary PRL acts via a classic endocrine pathway. It is secreted in a pulsatile fashion, displaying a circadian rhythm with a maximum during sleep. It is secreted into the circulation and transported to peripheral sites where it acts on target cells via specific receptors located on the plasma membrane. Pituitary PRL expression is controlled by a proximal promoter, which requires the Pit-1 transcription factor for trans-activation (60). The promoter is divided into a proximal region and a distal enhancer, both of which are necessary for optimal pituitary-specific expression. The pituitary-type promoter is regulated primarily by dopamine. Thus, PRL homeostasis should be viewed in the context of a fine balance between the action of dopamine as an inhibitor and the many hypothalamic, systemic, and local factors acting as stimulators. Among these are thyroid releasing hormone (TRH), estrogens, neuropeptides and some additional growth factors. In spite of the similarity of the mature proteins, PRL is differentially regulated in pituitary and extrapituitary sites.

In humans, the synthesis of extrapituitary PRL is driven by a proximal promoter, located 5.8 kb upstream of the pituitary-specific start site (61). This promoter is silenced in the pituitary, does not bind Pit-1 and is not affected by dopamine or estrogens. Exon 1a, serving as the alternative transcriptional start site, is spliced into exon 1b, yielding an identical coding region to the pituitary
transcript, except for a longer 5'-untranslated region. The alternative upstream promoter contains binding sites for several transcription factors but its regulation is still poorly understood.

THE PRL RECEPTOR (PRLR)

PRLR gene and primary structure

The gene encoding human PRLR is located on chromosome 5 and contains at least 10 exons, with an overall length exceeding 100 kb. In several species, including rat, mouse and human, multiple isoforms of membrane-bound PRLR resulting from alternative splicing of the primary transcript have been identified. This is in contrast to the PRL ligand gene, for which a single transcript encodes a unique mature protein. The PRLR isoforms differ in the length and composition of their intracellular domain, the cytoplasmic tail. They are referred to as long (L-), intermediate (I-) or short (S-) PRLR with respect to their size. In human, one long, one intermediate and two short isoforms have been identified (reviewed in) (53). In rat, all three isoforms are present, whereas, in mice, one long and three short isoforms have been identified (62, 63). In addition, soluble isoforms have also been identified, indicating a PRL binding protein. Regardless of post-transcriptional splicing events, the extracellular ligand-binding domain is identical in all isoforms.

The type 1 cytokine receptor superfamily

The PRLRs and the GHR are both non-kinase receptors whose activation of signaling pathways requires participation of receptor-associated kinases, such as Janus kinases or Src kinases. Signal transduction by these receptors mainly involves the JAK/Stat pathway (64). The PRL and GH receptors share a homology in their extracellular regions, characterized by the conserved cysteine residues and the tryptophan-serine-x-tryptophan-serine motif, they are therefore classified to the type 1 cytokine receptor superfamily (65-67). This includes, in addition to PRLR and GHR, several interleukins (IL), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), Oncostatin M (OM), erythropoietin (TPO), gp 130 and the obesity factor leptin. Although apparently genetically unrelated, all the class I cytokine family receptors contain stretches of highly conserved amino acids, both in the extracellular and intracellular domains.
Distribution and regulation of PRLR expression

PRL binding sites have been identified in almost every tissue and cell type of adult mammals and they are generally considered widely distributed in other vertebrates (53). Fairly recent studies have also demonstrated wide expression of PRLR in fetal development of both human and rat. In rat, mRNA encoding the two isoforms of the receptor was expressed widely in tissues derived from all three germ layers (68). In the human fetus, PRLRs are expressed in a diverse range of tissues in the human fetus by 7.5 weeks of gestation. The widespread expression in tissues of different origin and the significant changes in the distribution of receptors within a single tissue during ontogeny indicate a role for the lactogenic hormones in tissue differentiation and organ development early in gestation.

Fig.2. Illustration of prolactin receptor activation. Interaction of the PRL binding site 1 with the membrane-proximal D2 extracellular (EC) domain of a PRLR molecule (step 1) induces interaction of binding site 2 of the PRL ligand with a second PRLR (step 2). Upon receptor dimerization, the Box1-associated tyrosine kinases (Jak2) of the intracellular domain (IC) will transphosphorylate each other (step 2) and subsequently phosphorylate (P) the tyrosine residues (Y) of the PRLR itself (step 3). Note that activation of the short form of the PRLR does not result in Tyr phosphorylation of the receptor itself. (Adapted from Freeman et al. (56) Copyright © 2000 the American Physiological Society)
PROLACTIN SIGNAL TRANSDUCTION
The diversity of proposed PRL functions has been correlated to the nearly ubiquitous expression of the different PRLR isoforms and the presence of distinct intracellular signaling pathways (53). The first step in the mechanism of action of PRL is the binding of PRL ligand to the cell surface receptor, PRLR. The ligand binds in a two-step process in which site 1 on the PRL ligand molecule binds to one receptor molecule, after which a second receptor molecule binds to site 2 on the hormone, forming a homodimer consisting of one molecule of PRL and two receptor molecules (53) (these steps are illustrated in Fig.2.). The cytoplasmic tail of the PRLR lacks any intrinsic enzymatic activity, including kinase activity, as do all cytokine receptors identified so far (67). Signal transduction by these receptors therefore depends upon association with tyrosine kinases called Janus kinases (JAKs), which link ligand binding to tyrosine phosphorylation of both the receptor itself and signaling proteins recruited to the receptor complex (64, 69).

The Jak/Stat pathway
All cytokines work in combination with one or several JAKs to transmit the hormonal signal within the cell. In 1994, as previously demonstrated for the GHR, JAK2 was identified as the primary JAK kinase associated with the PRLR (70-72). JAK2 is not induced by ligand binding, but rather constitutively associated with the PRLR (73). Ligand-induced receptor dimerization brings two JAK2 molecules close, allowing for JAK activation through transphosphorylation. Activated JAK2 then phosphorylates specific tyrosine residues on the PRLR, important for recruitment of downstream transducer molecules. Dimerization of the receptor upon ligand binding thus induces rapid tyrosine phosphorylation and activation of the JAK kinase followed by phosphorylation of the receptor. Generation of JAK2-deficient mice has demonstrated that lack of functional JAK2 causes an embryonic lethality due to the absence of definitive erythropoiesis. Additional studies have shown that Jak2 plays a critical, nonredundant role in the function specific cytokines receptors, including erythropoietin, thrombopoietin, IL-3, granulocyte/macrophage-CSF and IFNgamma receptors (74).

The major pathway of downstream cytokine receptor signaling following JAK2 activation involves members of the Stat family, signal transducers and activators of transcription, a group of latent cytoplasmic proteins first discovered in the early 1990s and to date including eight members (75). STATs are transcription factors that mediate cytokine and growth factor induced signals that culminate in various biological responses, including proliferation and differentiation. STAT1,
STAT3, STAT5a and STAT5b are the central transducer molecules of signaling pathways initiated by PRLR activation (56, 76). The general mode of STAT activation was discovered in the 1990s and has since been reviewed extensively in the literature (77, 78). Following ligand-induced receptor dimerization, the receptor undergoes tyrosine phosphorylation by the associated JAK. The receptor phosphotyrosines interact with the SH2 domain of a STAT, making it part of the receptor/JAK complex. The STATs are subsequently phosphorylated by the activated JAKs on a positionally conserved C-terminal tyrosine residue. This obligatory phosphorylation allows the formation of homo- or hetero-dimers of STAT proteins which dissociate from the complex and translocate to the nucleus where it activates specific DNA promoter elements of cytokine target genes (78).

Targeted gene disruptions of STAT5a and STAT5b in mice have confirmed these molecules as the major transducers of PRL signaling in both prostate and mammary gland. The phenotypes in these mice are closely related to those of the PRL and PRLR gene knockout mouse models, mainly emphasizing the irreplaceable role of PRL in reproduction and mammopoiesis. In addition to STAT5a and STAT5b, STAT3 and STAT1 have also been shown to be activated by PRL in cells of myeloid, lymphoid, and mammary origin (79, 80). STAT5a, which is strongly activated in response to PRL, is the principal and obligate mediator of mammopoietic and lactogenic PRLR signaling. Lack of STAT5a signaling, as evidenced in STAT5a-deficient mice, leads to incomplete mammary lobuloalveolar outgrowth during first pregnancy and failure to lactate after parturition (81). This phenotype could however be partially overcome by a combination of consecutive pregnancies and suckling stimulations, indicating a capacity to activate alternative signaling pathways that could restore development and function of mammary epithelium (82). Deletion of both STAT5a/b in gene targeted mice results in the loss of remaining prolactin functions in mice, namely corpus luteum development and the development of female fertility. In these regards, the STAT5a/b deficient phenotype is identical to that observed in PRLR deficient mice and is consistent with the concept that all the physiological functions of prolactin rely on the ability of the PRLR to activate STAT5a/b. The demand for STAT5a/b functionality also applies to the effects of growth hormone receptor (GHR) activation (78). PRL signaling in rat prostate tissue is primarily transduced via STAT5a and STAT5b, likely supporting the viability of prostate epithelial cells during long-term androgen deprivation (83). In the prostate, studies in STAT5a deficient mice have provided evidence for a direct role of STAT5a in the maintenance of normal tissue architecture and function of the mouse prostate (84). Lack of STAT5a function results in a distinct prostatic phenotype characterized by an increased occurrence of cyst formation with disorganization and detachment of prostate epithelial
cells. In addition to PRL, other polypeptide factors known to activate STAT5 include insulin-like growth factor I (IGF1), epidermal growth factor (EGF) and interleukin-6 (IL-6).

**Additional PRLR signaling pathways and negative regulatory control systems**

The JAK-STAT cascade is considered the most important signaling pathway used by cytokine receptors and existing data clearly indicate that JAK2 activity, as induced by PRLR dimerization, is necessary for PRL action (85). However, in addition to the JAK-STAT pathway, a cytokine receptor complex can simultaneously operate multiple signal-transduction pathways which usually express contradictory properties. Such other proximally activated signaling cascades also likely contribute to PRL-induced gene expression. Members of the PRLR-associated tyrosine kinases of the Src family have been shown to be activated by PRL (69), possibly independent of JAK2 activation. In addition, the Ras/Raf/MAP kinase pathway is also activated by PRL and may be involved in the proliferative effects of the hormone (86). Considerable convergence and crosstalk between the JAK-STAT and MAPK pathways has also been demonstrated (87, 88).

Internalization of the PRL ligand/receptor complex upon ligand binding is widely reported, and nuclear translocation of PRL ligand after receptor has been demonstrated. The functional relevance of this has remained uncertain. Interestingly, it was recently demonstrated that the intranuclear PRL ligand interacts with cyclophilin B, a protein of the immunophilin family (89). The intranuclear prolactin/cyclophilin B complex then acts as a transcriptional inducer by interacting directly with STAT5, resulting in the removal of the STAT-repressor protein inhibitor of activated STAT 3 (PIAS3), thereby enhancing STAT5 DNA-binding activity and prolactin-induced, STAT5-mediated gene expression (90). These findings demonstrate mechanistically how an intranuclear polypeptide hormone can potentiate its own signal, and perhaps contribute to its own specificity.

A negative regulatory control of cytokine signaling in order to avoid over stimulation also exists. Among the negative regulators identified, the suppressors of cytokine signaling, or SOCS, have received much attention in the past few years. The SOCS family currently includes 8 members, SOCS-1 to -7 and CIS (cytokine-inducible SH2-domain containing protein). Expression of the SOCS is rapidly induced by cytokines. It has been demonstrated that early expression of SOCS genes (SOCS-1 and SOCS-3) effectively switches off PRL-signaling and that the later expressed SOCS-2 gene can restore the sensitivity of cells to PRL, partly by suppressing the SOCS-1 inhibitory effect (91).
Fig. 3. Illustration of PRL signal transduction pathways. Following ligand-receptor interaction and tyrosine phosphorylation of Jak2 and the PRLR itself (described in Fig. 2.), the receptor phosphotyrosines interact with the SH2 domain of a STAT molecule, making it part of the receptor/JAK complex. The STAT is subsequently Tyr phosphorylated by the activated JAKs. This obligatory phosphorylation allows the formation of homo- or hetero-dimers of STAT proteins which dissociate from the complex and translocate to the nucleus where it activates specific DNA promoter elements of cytokine target genes. The short form of PRLR is not Tyr phosphorylated, but activated Jak2 can serve as docking site for STAT1. The mitogen-activated protein kinase (MAPK) cascade: PRLR activation also induces the MAPK cascade. Phosphotyrosine residues of the activated L-PRLR isoform serve as docking sites for adapter proteins (Shc/Grb2/SOS) connecting the receptor to the Ras/Raf/MAPK cascade. Proposed down regulators of cytokine signaling have been shown to inhibit Jak kinases (SOCS) or compete with STATs for phosphotyrosine docking sites on the PRLR (CIS) (From Freeman et al. (56) Copyright © 2000 the American Physiological Society)
GENERAL PROLACTIN PHYSIOLOGY

PRL was originally isolated and purified from the pituitary gland due to its mammopoietic and lactogenic properties in rabbits (49). It was also demonstrated to promote the formation and action of the corpus luteum. Since then, more than 300 specific functions have been attributed to PRL in a number of different species (53). PRL is thus reported to affect more physiological processes than all other pituitary hormones combined (53). These classically include regulation of mammary gland development, initiation and maintenance of lactation, behavioral modification, immune modulation and osmoregulation. On a cellular level, PRL exerts mitogenic, morphogenic and secretory activities. This broad range of effects has led to the concept of a dual function of PRL, as a circulating hormone and a cytokine (54). The generation of both PRL ligand and receptor deficient mice has mainly emphasized the irreplaceable role of PRL in lactational and reproductive function (92). Homozygous females of both models are infertile and show total lack of lobuloalveolar development whereas heterozygous PRLR-deficient females alone show failure of lactation attributable to greatly reduced mammary gland development after their first, but not subsequent, pregnancies (93, 94).

PROLACTIN IN HUMAN PATHOPHYSIOLOGY

The clinical effects of hyperprolactinemia are well known. Causes of hyperprolactinemia are numerous, but the PRL secreting tumors of the anterior pituitary gland are perhaps most recognized. Other alterations in neuroendocrine control mechanisms regulating secretion are also known, usually resulting in modestly elevated PRL levels. Pharmacotherapy interfering with generation and action of dopamine is one cause of PRL alterations. In contrast, isolated PRL deficiency is only sporadically reported, resulting mainly in alactogenesis, loss of milk production, following delivery. In the following paragraph, prolactinomas are used to illustrate the clinical effects of hyperprolactinemia.

Prolactinomas

Physicians have long been aware of the existence of prolactinomas, these are tumors of lactotroph cells in the anterior pituitary that hyper-secrete PRL. Pituitary tumors have an annual incidence of approximately 25 per million head of population and around half of these are prolactin producing. Mixed tumors expressing both PRL and growth hormone also occur, these are derived from mammosomatotrophic cells. Prolactinomas appear both in the form of microadenomas and as macroadenomas. The total incidence is higher in females (approx. 3:1), in which microadenomas are more frequent, whereas males typically present with macroadenomas (95). The most frequent
features of significant hyperprolactinemia in both males and females are hypogonadal symptoms, headache and galactorrhea (95). Hypogonadal symptoms include a decreased libido, oligo- or amenorrhea, infertility, and erectile dysfunction in males. In addition, the presence of an expansive sellar mass (macroadenomas) can result in visual defects, severe headache and hypopituitarism. The standard primary treatment is pharmacotherapy using dopamine agonists, such as bromocriptine. Surgical removal of the tumor is also considered in some cases, most often due to intolerance of medicine or in tumors not responding to dopamine agonists (96).

**PROLACTIN ACTION IN THE PROSTATE GLAND**

PRL-mediated effects in the prostate are well described and supported by both in vivo models and in vitro work on cells and organ cultures. In the late 1960s and early 1970s, the first reports on growth promoting effects of PRL on the accessory sexual glands in several species, including rodents, were published (97-99). Most of the described PRL prostatic effects have been demonstrated in normoandrogenic, intact, animals. However, several reports also indicate androgen-independent effects of PRL (100, 101). To date, around 600 publications detailing various aspects of PRL action in the prostate gland have been published. In the following paragraphs, a more detailed account of known PRL effects in the prostate is presented.

**Proliferative effects**

There is ample evidence that PRL exerts a trophic effect on malignant prostate cells in vitro. PRL induced effects on proliferation in androgen-insensitive human prostate cell lines, such as DU145 and PC3, have been reported (102). In human BPH organ cultures, PRL can significantly increase the cell proliferation rate (103). Dihydrotestosterone, oestrogen and progesterone have also been reported to exert weaker proliferating effects than PRL. Associated with the growth-promoting effect of PRL is its effect on ornithine decarboxylase (ODC) in the lateral prostate of rat. ODC is a rate-limiting enzyme in polyamine biosynthesis, and polyamines have been classified as growth mediators due to their effects on somatic DNA- and RNA-synthesis in somatic cells (104, 105).

In vivo models have demonstrated enhanced growth of rodent prostate lobes after pituitary grafting under the renal capsule (106), or local grafting to a specific lobe (107, 108). In rat, anterior pituitary grafting to the lateral prostate lobe results in significant growth, specifically in the lateral lobe, compared to controls (107). These findings indicate a local direct effect of PRL on the lateral prostate lobe, independent of circulating androgen levels. In mice, implantation of a single anterior
pittuitary into the ventral prostate of intact mice resulted in a significant increase in weight and the area occupied by the ventral prostate. Prostate growth was associated with the elevation of circulating PRL. In addition, hyperplastic lesions were also noted in the grafted prostate lobes of these animals (108).

Hyperprolactinemia has also been reported to induce prostatic dysplasia in vivo. Noble rats, treated with testosterone and estradiol-17β2 for a prolonged time period, develop dorsolateral lobe dysplasia, a pre-neoplastic lesion. In these rats, the dysplasia is mediated via estradiol-induced hyperprolactinemia, as evidenced by effective inhibition of dysplastic evolution through bromocriptin co-treatment (109). In rat, a transient increase in PRL secretion prior to puberty can result in lateral prostate inflammation. Prepubertal exposure to compounds that increase PRL secretion also increase the incidence of lateral prostate inflammation in the adult rat (110). An additional study reports that early lactational exposure to atrazine, a toxic agent that suppresses suckling-induced PRL release in the nursing female, results in subsequent prostatitis in the male offspring. In these mice, lack of lactational exposure to PRL (postnatal day 1-9) leads to impaired tuberoinfundibular neuronal growth and as a consequence prepubertal PRL levels become elevated. This results in higher incidence and severity of lateral prostate inflammation in the offspring, evident at 120 days of age (111).

**Regulation of apoptosis**

The unique prostatic cellular phenotypes are induced and maintained by interaction between epithelium and adjacent stroma through intimate intercellular signaling pathways. Maintenance of cell and tissue homeostasis is dependent upon the dynamic balance of cell proliferation, differentiation, and apoptosis through interactions between cells and their microenvironment (112). A reduced rate of apoptosis is considered involved in the etiology of benign prostate hyperplasia (BPH) in the human prostate gland (113) with several studies demonstrating a decreased apoptotic rate in hyperplastic prostate (114, 115). The concept of PRL regulation of target tissue size by controlling not only proliferative activity but also programmed cell death is relatively new. PRL has been reported to suppress apoptosis in several target tissues, including hematopoietic cells, prostate and mammary gland, but also to induce cell death in the corpus luteum (116). Concerning the prostate gland, an in vitro study by Ahonen et al. has demonstrated that PRL can significantly inhibit apoptosis in androgen deprived dorsal and lateral rat prostate cultures, as assessed by nuclear morphology and
in situ DNA fragmentation analysis (117). This indicates a possible physiological role for PRL as a survival factor for prostate epithelium. In earlier in vivo work, a significant delay of castration-induced regression of the lateral rat prostate has been noted in pituitary graft bearing animals (100, 118, 119). In addition, these studies indicated that androgen receptors (AR) did not mediate PRL actions on the prostate gland, as evidenced by the failure of flutamide to inhibit the delay in prostatic regression. These results also revealed a lobe-specific response to PRL in the androgen-deprived prostate. Taken together, these observations suggest that in addition to known trophic actions in target tissues, PRL may regulate cell population density by prolonging survival through anti-apoptotic mechanisms.

**PRL in prostate metabolism**

A major function of the prostate gland is to accumulate and secrete extraordinarily high levels of citrate. In addition to citrate, the normal and BPH prostate also accumulate the highest levels of zinc in the body. Zinc is thought to help to extend the functional life span of the ejaculated spermatozoa and spermatozoal defects are frequently observed in zinc-deficient rodents (120). These specialized metabolic processes are the result of unique metabolic capabilities of the secretory epithelial cells. Interestingly, in prostate cancer the capability for citrate production is lost and the ability for high zinc accumulation is diminished (121).

In rat, PRL has been shown to androgen-independently stimulate citrate production exclusively in the lateral lobe of the prostate (104). The stimulatory effects of PRL on the citrate level have also been confirmed in monkey (122). In vitro studies in prostate epithelial cells and organ cultures, obtained from pig and rat lateral lobe, as well as in the human cancer cell lines LNCaP and PC-3, demonstrated that the increased citrate level was due to androgen-independent PRL-induced transcription of the precursor of mitochondrial aspartate aminotransferase (m-AAT) (123, 124). M-AAT is the key enzyme in the metabolic pathway of prostate citrate production. This transcription has been shown to be mediated via the protein kinase C (PKC) pathway (124, 125). Furthermore, in vitro studies of rat lateral prostate epithelial cells has reported that PRL stimulates the biosynthesis of pyruvate dehydrogenase, an enzyme involved in the supply of acetyl-CoA for the citrate synthesis. Studies of mitochondrial (m)-aconitase, the key enzyme in the citrate oxidation, have provided direct evidence that PRL, androgen-independently, regulates the m-aconitase gene in citrate-producing mammalian cells, including human (126). Earlier in vivo and in vitro studies of rat and pig prostate demonstrated that the regulation of m-aconitase is cell-specific and can be stimulatory, as in the case of rat ventral prostate, or inhibitory, as in rat lateral prostate. In non-citrate producing cells, including
rat dorsal prostate, the hormone has none of these effects (127-129). In vivo and in vitro studies of rat have revealed that the accumulation of zinc in the prostate also is regulated by PRL, independently of androgens. PRL increases both cellular and mitochondrial zinc levels of citrate-producing lateral prostate cells, decreases the zinc levels in ventral prostate cells and has no effect on dorsal prostate cells (130). Since high level of zinc has an inhibitory effect on m-aconitase and thereby citrate oxidation, this provides an additional way of regulating the citrate level.

**Auto/paracrine action of PRL in the prostate gland**
The presence of both PRL ligand and receptors in both human and rodent prostate gland is well documented (131-135). The possibility for regulation of PRLR expression by its own ligand has been reported in several tissues, including the prostate gland. In rat prostate, both testosterone and estrogen has been shown to regulate the level of the long PRL receptor mRNAs in a tissue-specific manner (134). Increased PRLR expression levels have been reported in dysplastic lesions, whereas in lower grade carcinomas the receptor expression levels approximated those found in normal prostatic epithelium (132). Results from this study suggest that PRL may participate in early neoplastic transformation of the gland. Furthermore, elevated tissue levels of PRL in latent moderately or poorly differentiated type prostatic carcinoma have been reported (136).
The expression of PRL ligand in rat dorsal and lateral prostate was found to be androgen dependent in vivo, in castrated and in testosterone-treated castrated rats, as well as in vitro in organ cultures (135). These results could indicate a role for PRL as an autocrine/paracrine growth factor, regulated by androgen and mediator of androgenic downstream effects in the rat prostate. Recently recognized, the existence of crosstalk between the signal transduction systems of steroid hormones and peptide hormones/growth factors provides a mechanism for locally produced growth factor influence on AR activation (137, 138). In the progression of prostate cancer to an androgen-independent state, local growth factors, such as PRL, may prove instrumental in regulation of cell growth.
ANDROGEN ACTION IN THE PROSTATE GLAND

The prostate gland depends on androgens for its development and maintenance of its structural and functional integrity. The necessity of androgen action is illustrated by the minimal or absent development of the prostate gland caused by congenital AR dysfunction or deficiency of 5α-reductase in human males (139). Integrity of the prostate is also dependent on androgen in other mammals. Following castration, the rodent prostate undergoes rapid involution as a result of programmed cell death, or apoptosis, in glandular epithelium and endothelium. (140) Within 2-3 weeks following castration, a majority of glandular epithelial cells are lost in both human and rodent castrates (141).

However, testosterone is not the major androgen responsible for growth of the prostate. Testosterone is converted in target cells to dihydrotestosterone (DHT) by the 5α-reductase enzyme which is expressed in two isoforms. In the prostate type 2 5α-reductase is the isoform primarily responsible for DHT formation (142). It has been demonstrated that stromal cells express both isoforms, whereas epithelial cells preferentially express the less active type 1 isoform (142, 143). Testosterone and DHT both bind to the androgen receptor, but yet exert biologically distinct effects. These differences are considered due to kinetic differences of binding of androgens to the receptor. DHT binds with a greater affinity to the androgen receptor than does testosterone. This results in potential differences in function of the hormone response element, DNA activation, and subsequent messenger RNA production. In addition, studies using differential display gene arrays have revealed fundamental differences in signal transduction pathways depending on which hormone binds to the androgen receptor (144). AR is a member of the steroid hormone receptor family of genes. Like the other members of this family of transcription factors, the exons of the AR gene code for functionally distinct regions of the protein similar to the modular structure of other steroid hormone receptor genes. The AR genomic organization is conserved throughout mammalian evolution. As the AR gene is located on the X chromosome it is single-copy in males, allowing for the phenotypic manifestation of mutations without the influence of a wild-type co-dominant allele. More spontaneous mutations of human AR have been identified than of any other gene, partly because AR is not essential to the formation of a viable human organism. Complete loss of AR function in genetic males (XY) results in the complete androgen insensitivity syndrome (CAIS). The main phenotypic characteristics of individuals with CAIS are, female external genitalia, a short, blind ending vagina, the absence of Wolffian duct derived structures, the absence of a prostate, development of gynecomastia and the absence of pubic and axillary hair.
ESTROGEN ACTION IN THE PROSTATE GLAND

A role for estrogens have long been implicated in prostate physiology and pathophysiology. The expression of both known estrogen receptor subtypes in adult human and rodent prostate is now well established, with expression of ERα described primarily in a subset of stromal cells and ERβ restricted to the ductal epithelium (145-147). While the newly discovered ER beta shares many of the functional characteristics of ER alpha, the molecular mechanisms regulating the transcriptional activity of ER beta may be distinct from those of ER alpha. In human prostate, the growth effects of estrogens during fetal development are mediated primarily by ERβ, which can be immunodetected in the nuclei of nearly 100% of epithelial and in the majority of stromal cells throughout gestation (148). Interestingly, the growing incidence of BPH with increasing age coincides with a shift in the androgen/estrogen ratio in favour of estrogens, not restricted to serum hormone values, but also seen in the prostate itself (149, 150).

Exogenous estrogen administration in adult rodents leads to squamous metaplasia (SQM) of the anterior prostate lobe (13, 151). SQM is considered an abnormal form of epithelial differentiation described in several organs. The term metaplasia signifies a reversible change where one adult cell type is replaced by another adult cell type. Recent work has established that both initiation and progression of the prostatic squamous metaplasia is mediated by the stromal ERα receptor. A hierarchy of estrogen responsiveness in the three prostatic lobes has been revealed in male mice, with the anterior lobe being the most responsive, the dorsolateral lobe less responsive, and the ventral lobe the least responsive (13). Tissue recombinant studies using epithelium and stroma from wildtype and transgenic mice lacking a functional ERα (αERKO) or ERβ (βERKO) have demonstrated that the development of SQM is mediated through stromal ERα. (152, 153). Neonatal exposure of rodents to high doses of estrogen is known to permanently imprint the growth and function of the prostate and predispose the gland to hyperplasia and severe dysplasia analogous to prostatic intraepithelial neoplasia with aging (152). Estrogen imprinting, also referred to as developmental estrogenization, can be achieved by administration of single or multiple estrogen doses at day 1-5 post partum. Estrogen effects on pituitary lactotrophs are well known and several studies suggest that neonatal estrogen treatment can induce long-term alterations in pituitary synthesis and release of PRL (154-156). It is thus quite possible that the prostate effects of estrogen imprinting are in fact partly PRL-mediated.
The recent generation and characterisation of the various estrogen modulated mouse models (αERKO, βERKO, αβERKO and ArKO) has provided new insights regarding the role of estrogens in prostate growth and development (157). Furthermore, a distinct phenotype of focal epithelial hyperplasia in the VP has been reported in aging mice lacking functional ERβ (βERKO) (158, 159), while no apparent prostate pathology or enlargement has yet been reported in αERKO or αβERKO (157). These findings are indicative of an antiproliferative role for epithelial ERβ and also suggest that an unbalanced stromal ERα action could contribute to the phenotype observed. The ArKO (aromatase knockout) mouse model, lacking endogenous estrogen production due to a non-functional aromatase enzyme, also bears interesting resemblance to the Pb-PRL transgenic prostate phenotype. In the ArKO mouse, the combined effects of estrogen absence and elevated androgen and PRL levels result in a moderate prostate enlargement with hyperplasia evident in all lobes and tissue compartments (160). Furthermore, an associated upregulation of epithelial AR was demonstrated in the ArKO mouse and has been suggested to contribute to the observed phenotype. In the absence of endogenous estrogen (ArKO) or ERs (αERKO and βERKO), prostate development occurs normally, suggesting that intact estrogen signaling is not essential for the initiation of neonatal prostate growth.
INTERACTIONS BETWEEN PRL AND ANDROGEN/ESTROGEN

Interactions between PRL and androgens have long been recognized (98, 161, 162). Prolactin-mediated augmentation of androgenic in vivo effects on the prostate gland have been described in mice. A proposed mechanism for PRL-induced increase in testicular androgen synthesis is the upregulation of LH receptors on Leydig cells responsible for androgen production. (163, 164) In humans, elevated serum levels of PRL have been shown to increase both prostatic uptake and metabolism of testosterone (165). PRL serum levels have also been shown to correlate to AR content in the benign human prostate (166). Conversely, an increase in androgens can negatively affect pituitary PRL release.

Estrogens are known to stimulate growth of pituitary lactotrophs (167, 168) and also to promote PRL release resulting in elevated PRL levels systemically (169, 170). Estrogens affect PRL release by acting directly on the lactotrophs or indirectly on the hypothalamic dopaminergic system as well as on a variety of PRL secretagogues of hypothalamic or pituitary origin. Conversely, PRL is able to stimulate expression of both ERα and ERβ in corpus luteum and decidua during pregnancy (171-173). In addition to this, PRL has been shown to stimulate E2 binding activity or mRNA levels in the mammary gland (174), and liver (175). In the prostate, effects of estrogen treatment appear to be in part mediated by increased PRL levels (176), something that is further demonstrated in the dysplastic prostate model of estrogen-treated Noble rats previously discussed (109).
AIMS OF THIS THESIS

A role for PRL in both normal prostate growth and function and in pathophysiological conditions of the prostate has long been suggested. The aims of this thesis were to investigate the effects of hyperprolactinemia or increased prostatic PRL expression in the development of abnormal growth in the mouse prostate gland. Secondly, we aimed to assess the importance of alterations in androgen status for the transgenic phenotypes and to characterize ductal development and mature morphology in PRL transgenic and PRLR deficient mouse prostates.

The specific aims of this thesis were:

I. To study the effect of prolonged hyperprolactinemia on mouse prostate (paper I)

II. To investigate the role of elevated androgens in development of prostate hyperplasia in adult PRL transgenic and wildtype mice (paper II)

III. To study the PRL effect on androgen receptor distribution in the prostate (paper II and III)

IV. To examine the prostatic effects of prostate-specific overexpression of the PRL gene under normal testosterone levels (paper III)

V. To assess PRL effects on ductal development in the neonatal and postpubertal prostate (paper III)

VI. To study the consequences of PRLR deficiency in the murine prostate gland (paper IV)

VII. To examine the effect of loss of PRLRs in premalignant and tumorigenic processes in the prostate (paper IV)
METHODOLOGICAL CONSIDERATIONS

GENETICALLY ENGINEERED ANIMALS
The advent of transgenic techniques to facilitate transfer of a gene to a model organism has allowed us to study and understand the function of a specific gene. A transgenic organism is identified by the integration of an extra or exogenous fragment of DNA into its genome. The most common research animal species to be used in transgenic work are the nematode worm, the fruit fly and the mouse. The first successful method for integration of foreign genomic material described in mice was the viral transfer presented by Jaenisch et al. in 1976 (177). Development of the zygote microinjection technique by Thomas et al. in 1980 has proved even more instrumental in modern genomic research. (178) This study demonstrated that microinjection of foreign DNA into the pronucleus of a fertilized egg (a zygote) could result in genomic integration and subsequent expression of foreign DNA in the animal. Palmiter et al. reported the generation of a growth hormone transgenic mouse by microinjection technique in 1982 (179). Thousands of transgenic mouse models have since been established, presenting the research community with invaluable tools for investigating the functional in vivo role of target genes. The microinjection technique is schematically presented in Fig.4.

Transgenic construct design
If aiming to over express a certain gene product, one needs to consider the following aspects. Is the gene structure and sequence known and available? In what tissue or cell type is the gene to be over expressed? How is the transgenic expression to be detected and discriminated from endogenous expression?

For successful expression of proteins in bacteria it is sufficient to use a cDNA construct. This is in contrast to expression in mammals where intron sequences can considerably affect expression levels. It has been demonstrated that the first intron is particularly significant for transgenic expression and lack of intron sequences in a transgenic construct results in decreased expression levels (180, 181). Gene constructs in which the protein-encoding DNA sequences are contained within a genomic segment (comprising most or all of the natural introns of the corresponding gene) are thus shown to be expressed more efficiently than their intronless counterparts. For this reason, a genomic DNA construct is preferred. However, this is not always possible due either to the size of the gene or to the complete gene structure being unidentified. Constructs larger than 30 kb have proved difficult to use in ordinary plasmid vector work. An alternative approach in both these cases is a cDNA
construct. In addition, bacterial artificial chromosomes (BACs) and P1-derived artificial chromosomes (PACs) offer transfer of large fragments of cloned genomic DNA into the host genome. BACs/PACs have proved very important in functional studies through transfection because of their large size and stability (182).

**MICROINJECTION TECHNIQUE**

Fig. 4. Schematic presentation of the steps involved in the common DNA microinjection technique (used in generation of the Mt-PRL and Pb-PRL transgenic mice used in paper I-III). Illustration appears courtesy of Dr. H Wennbo.
Choice of promoter

Every mammalian gene includes a promoter region, deciding the spatial (where) and temporal (when) expression pattern of the gene in question. The promoter region chosen for a transgenic construct will therefore decide which cells will express the transgene and also influence the temporal expression pattern. The vast range of known gene promoters allows for both cell specific or non-specific expression patterns. Regulation of expression levels can also be achieved by exogenous induction methods, such as oral administration of heavy metals for induction of metallothionein promoters (183).

If a general expression of the transgene is required, the metallothionein (Mt) promoter used in the Mt-PRL transgene construct in this thesis (paper I-II) is a good choice. The metallothionein gene is expressed in most tissues of the animal and expression is furthermore initiated during early embryonic stages (ref). As mentioned, Mt-promoter driven expression of a transgene can be further increased by the addition of zinc to the drinking water, but the Mt-promoter is not considered completely silent under any normal physiological conditions. Cell specific expression is also achievable by a number of promoters, such as the whey acidic protein (WAP) promoter for mammary epithelium and the probasin (Pb) promoter for prostate epithelium used in this thesis (paper III-V) (184, 185). By combining inducible systems with a cell specific promoter an inducible cell specific transgene expression can be obtained.

Integration site

The integration of the transgene in the genome is considered a random event and the number of copies inserted can not be regulated. In the vast majority of zygote injections the integration will occur at a single position on the chromosome. As a consequence, the resulting animal will be hemizygous for the integrated transgene. It is considered preferable to generate more than one line of transgenic animals expressing the transgene at an acceptable level as this allows for comparisons to rule out a phenotype owing to a heterozygous mutation introduced by the integration of the transgene. Homozygous transgenic animals resulting from the mating of two heterozygous animals should be used with caution. The reason being that in these animals a part of the genome has potentially been homozygously destroyed. They may be used for breeding, but the consequences of the introduced mutation are difficult to predict and homozygous transgenic animals are therefore not particularly suited for experimental use.
Preparation of the microinjection DNA solution

Purity and concentration of the DNA solution to be microinjected is of utmost importance. If the DNA solution is not absolutely particle free, injection into the pronucleus of the zygote will be exceedingly difficult. The purification methods include use of commercially available columns as well as classical isotochoforese and electro elution. The desired DNA concentration range is between 2 and 10 µg/ml. Higher concentrations of DNA are toxic to the zygote and lower concentrations rapidly decrease the probability of successful integration taking place. In addition, if the injected volume of DNA solution is too large the zygote survival rate quickly decreases, regardless of DNA concentration.

Identification of transgenic animals and transgene expression

The identification of transgenic animals is a multi-step process. The founder animals are first identified at the DNA level. Lines of transgenic animals are then generated from founders and expression of the transgene is characterized at RNA or protein level. Verification of transgenic expression in the desired tissues denotes the successful generation of a new transgenic animal. Founder animals are analyzed at the DNA level. Typically, a tail biopsy is taken at 2 weeks of age and DNA is prepared from this. The transgene is then identified either by Southern blot hybridization, or PCR using one primer located in the promoter and the other in the structure gene of the construct. Southern blot is more time consuming but results are generally considered more reliable than those obtained by PCR. Southern blot verification of founder animals is therefore preferred, while PCR is commonly used for initial screening of founders and the subsequent genotyping of founder offspring. Expression of the transgene should be analyzed in the transgenic offspring generated from the founder animals. When cDNA constructs are used it is important to design the construct in such a way as to make transgenic mRNA expression detectable and have interference from the contaminating cDNA construct. This can be achieved by including intron sequences in the construct allowing discrimination between DNA and processed mRNA (intron-free). If the transgene contains an endogenous gene (for the purpose of overexpression), one must also be able to discriminate between mRNA expression and protein production of the transgene and the endogenous gene. Sometimes this is not feasible and detection methods must then be sufficiently sensitive to detect even small differences in expression levels in transgenic animals compared to levels in the controls.
Zygote injection technique

The most efficient technique for generating transgenic mice is zygote injection. It involves injecting foreign DNA into a fertilized egg, or zygote, and then transferring the egg for further development in a pseudopregnant mother (see Fig 4.). The transgenic animal born is termed a founder. It is then bred to obtain more animals with the same DNA insertion. The new DNA normally integrates into the genome by a random, nonhomologous recombination event. One or multiple copies of the DNA may integrate at one site in the genome. Factors deciding the efficiency of microinjections are primarily; construct DNA concentration, construct DNA size and DNA form (supercoiled vs. linear with a variety of different ends), as well as the site of injection in the fertilized egg (male pronucleus, female pronucleus, or cytoplasm) and finally buffer composition. The optimal conditions for integration have proved to entail injection of a few hundred linear molecules into the male pronucleus of fertilized one-cell eggs. Under these conditions about 25% of the mice that develop inherit one or more copies of the microinjected DNA fragment (186).

Gene deletion strategies

The establishment of gene targeting (a.k.a. gene knockout, homologous recombination, ES-cell technique) by Thomas and Capecchi in 1987 (187) has proved to be of equal importance. The use of homologous recombination in embryonic stem (ES) cells made interaction with the mouse genome at a specific position possible, thus permitting the mutation of specific genes. A wide array of knockout models have now been established and further technical improvements have made both temporal and spatial gene deletion possible. These accomplishments have given unique insights into the specific biological properties and functions of specific genes and furthermore provided valuable models for many human pathological conditions.

Briefly, the technique makes it possible to replace endogenous genes with genetically modified target vectors. ES cells are transfected with the targeting vector and are then screened for recombinatory events by methods of Southern blot or PCR. Following identification of targeted ES cells, these are injected into blastocysts, giving rise to an animal which is a mix of the two cell types, a so called chimera. As ES cells are totipotent, they can differentiate into any cell type. If the targeted ES cells have entered the germ line of the chimeric mouse, the genetic alteration will be passed on to the next generation.
GENE EXPRESSION PROFILING

Functional genomics is the study of gene function through the parallel expression measurements of genomes, most commonly using the technologies of DNA micro arrays, and serial analysis of gene expression. Micro array usage in drug discovery is expanding, and its applications include basic research and target discovery, biomarker determination, pharmacology, toxicogenomics, target selectivity, development of prognostic tests and disease-subclass determination.

Micro array analysis

The micro array technology is based on the classic RNA-DNA hybridization technique. A micro array consists of a glass slide with probes attached to it. The probes are synthesized directly on the glass substrate and millions of copies of each probe are located within a discrete area on the array. 16 different oligomeric probes (25-mer) cover each gene included in the array. Each probe is synthesized to match the target gene perfectly (PM probe). Each PM probe has a companion oligomer identical to the PM probe except for a central single base difference (mismatch or MM probe). The MM probe serves as a control for hybridization specificity and allows for quantification and subtraction of signals caused by non-specific cross-hybridization (Affymetrix Micro Array Suite User Guide V.4.0). Total RNA is prepared and converted into cDNA, which is then in vitro transcribed to generate the cRNA used in chip hybridization. An alternative micro array approach is based on the use of cDNA clones, amplified by PCR and spotted on glass plates. Advantages include relative cost-effectiveness once the cDNA clones are obtained and also having control and test cDNA hybridized simultaneously to the same microarray using different fluorescent molecules. The potential lack of specificity and difficulty to discriminate between closely related genes are considered the major limitations in cDNA arrays. In paper IV, we utilized the Affymetrix GeneChip system to measure the expression of approximately 12 000 genes, allowing for phenotype comparison between PRLR deficient (PRLR⁻/⁻) male mice and wildtype (PRLR⁺/⁺) controls. The microarray used represents approximately 6000 known genes and 6000 uncharacterized expressed sequence tags, or ESTs. RNA from 5-8 prostate lobes of each genotype was pooled in equimolar ratios prior to probe preparation, chip hybridization (Affymetrix U74A/U74Av2) and analysis (Affymetrix MicroArray Suite 4 and 5). The experiment was replicated three times for the ventral lobe and twice for the dorsal lobe. Genes were identified which showed consistent change in expression level across the replicates, the significance of which was tested using Student’s paired T-test. Alterations in expression level were confirmed by real-time RT-PCR.
ANIMAL PROSTATE TUMOR MODELS

Several transgenic mouse models have recently been established for use in prostate cancer studies. As the mouse does not spontaneously develop prostate malignancy, different transgenic strategies for in vivo tumor induction have been developed. These include use of the tumorigenic large T antigen, usually under the control of a prostate-specific promoter region such as probasin or C3. These oncogenic models provide us with a working in vivo system in which to study tumor induction, progression and metastasis mechanisms on an molecular level. Clearly, all comparisons to human carcinogenesis must be made with caution. In addition to the underlying genetic differences there is also the aspect that transgenic tumor formation is induced using an agent not naturally occurring in humans who spontaneously develop these cancer forms. Nevertheless, much fundamental knowledge concerning basic cancer biology has been extracted from such models and they will certainly continue to provide a vital tool in cancer research.

The C3/ T(AG) transgenic mouse model

A transgenic mouse model for prostate and mammary cancer containing a recombinant gene expressing the early region of simian virus 40 (SV40) large tumor antigen (TAg) under the regulatory control of the rat prostatic steroid binding protein C3(1) gene. Male C3/T(AG) transgenic mice develop prostatic hyperplasia in early life that progresses to adenoma or adenocarcinoma in the majority of animals surviving to longer than 7 months of age (47). Prostate cancer metastases to lung have also been observed. Female C3(1)/TAG transgenic mice develop mammary adenocarcinomas with metastatic capabilities.
RESULTS AND COMMENTS

PAPER I

**PRL transgenic mice develop dramatic prostate enlargement**

The ethiology of benign prostate disease remains largely unclear, despite many years of experimental and clinical efforts to understand the underlying mechanisms. The importance of androgens for prostate development, growth and function is unquestionable. Androgen involvement in prostate pathophysiology is also well established. However, other hormones and growth factors are by now also strongly implicated to be involved in abnormal prostate growth. Among these, PRL has the proven ability to act on both normal and abnormal prostatic cells. In order to study the prostatic effects of prolonged hyperprolactinemia, transgenic mice overexpressing the rat PRL gene were generated and studied. A plasmid construct employing the metallothionein-1 gene promoter to drive expression of the rat PRL gene was made and used to generate the PRL transgenic mice by standard microinjection procedure (see methodological considerations). Two lines of PRL transgenic mice and a separate founder were generated and included in the study. The two lines exhibited serum PRL levels of approximately 15 ng/ml and 250 ng/ml respectively, while the separate founder had S-PRL levels of 100 ng/ml. All of the Mt-PRL mice developed dramatic enlargement of the prostate gland compared to age-matched controls at 10-15 months of age. The ventral prostate was on average 9 times larger (wet weight) than controls and the dorsolateral lobes were 20 times larger (wet weight) than controls. Total DNA content in the dorsolateral lobes was increased 4.7 times (155+/−34 µg DNA/prostate lobe vs. 33+/−5 µg DNA/prostate lobe in controls) and in the ventral lobes 4.2 times (96+11µg DNA/prostate lobe vs. 23+5µg DNA/prostate lobe in controls, P<0.01).

Histologically, all lobes of the prostate gland in the transgenic mice showed focal hyperplasia and glands distended from secretion. The amount of interductal stroma was also dramatically increased. In individual older animals dysplastic features, such as prominent nucleoli, could be seen but no malignant tumor formation was observed. Furthermore, the seminal vesicles of Mt-PRL transgenic animals were massively enlarged (1,22+0,17gr. vs. 0,17+0,03gr., Wennbo, unpubl. data). In order to determine if PRL could exert direct effect on the prostate gland the expression of the rPRL transgene, the PRLR and the endogenous mPRL was analyzed by RT-PCR. Specific mRNA for the
rPRL transgene was detected in both dorsolateral and ventral prostatelobes in all transgenic lines. Additionally, in normal and transgenic animals the expression of PRLR and endogenous mPRL was detected in all prostate lobe types. Furthermore no correlation between rPRL serum levels and prostate weight could be found in the transgenic animals.

The serum levels of testosterone were increased around 3-fold in the PRL transgenic animals compared to controls (21.0±3.0 nmol/L vs. 6.2±2.9 nmol/L in controls). However, testosterone levels of individual transgenic animals could not be correlated to prostate weight in either the dorsolateral nor the ventral lobe. Serum IGF-I levels were moderately elevated, around 30%, in PRL transgenic males. In order to clarify the potential role of elevated IGF-I levels in the PRL transgenic animals, mice overexpressing the bovine GH gene were included in the study. The bGH transgenic males displayed elevated IGF-I levels (495 ng/mL vs. 415 ng/mL in PRL transgenic and 317 ng/mL in controls). Testosterone levels were not significantly increased compared to controls. The dorsolateral prostate weight of bGH transgenic males was only moderately increased (1,6 times larger than controls, uncorrected for the 1,4 times increase in body weight) and the ventral lobe weights were unchanged compared with normal mice. This indicated that the effect of PRL was not primarily mediated through elevated plasma IGF-I levels. The present study suggests that PRL is an important factor in the development of prostate hyperplasia acting directly on the prostate gland or via increased plasma levels of testosterone.

**PAPER II**

**Elevated levels of circulating androgens are not required for development of prostate hyperplasia in adult PRL transgenic mice**

Transgenic mice overexpressing the rat prolactin (PRL) gene under control of the metallothionein-1 promoter (Mt-1) develop a dramatic prostatic enlargement. In addition to circulating levels of transgenic rPRL, these animals also display significantly elevated serum testosterone levels. We therefore aimed to elucidate the role of circulating androgen levels in the promotion of abnormal prostate growth in the adult PRL transgenic mouse prostate.

Prostate weight, gross morphology, histology and androgen-receptor distribution patterns were analyzed in castrated and testosterone-substituted adult PRL transgenic and age matched wild-type males. Castrations were performed at 12 weeks-of-age and slow-release testosterone or placebo
pellets were subcutaneously implanted perioperatively. Separate groups of transgenic and control animals received either 7.5 mg (T7.5) or 30 (T30) mg of testosterone or placebo pellets. The lower dose (T7.5) was selected to give normophysiological testosterone levels and produced testosterone levels that did not differ significantly from those in wildtype controls. The higher dose (T30) was successfully selected to give wildtype males testosterone levels comparable to those seen in PRL transgenic males. 8 weeks after pellet implantation, male transgenic and control mice were euthanized by heart puncture under general anesthesia and serum was collected. The urogenital tract was removed en bloc, and the individual prostate lobes (anterior/dorsolateral/ventral) were carefully dissected and separated. Results showed that progressive prostatic hyperplasia in adult PRL transgenic males was not affected by substitution to serum testosterone levels corresponding to wildtype. Immunohistochemical studies revealed a significantly increased proportion of AR-positive epithelial cells in all lobes of the PRL transgenic prostate versus wild-type. Stromal AR positivity was also noted more frequently in PRL transgenic males. Changes AR distribution in transgenic prostate were not affected by castration and resubstitution to normal androgen levels. The present study demonstrates that progressive prostate hyperplasia in adult PRL transgenic mice is not dependent on elevated serum androgen levels. In addition, our results suggest that prolonged hyperprolactinemia results in changes in prostate epithelial and stromal cell androgen receptor distribution. This could unquestionably result in an increased androgen sensitivity in the prostate gland, thereby influence the observed phenotype.

**Prolonged androgen treatment has no significant effect on prostate growth in wildtype adult mice**

Previous findings in rodents regarding prolonged androgen treatment and prostate growth are conflicting. Both unaffected prostate size and induction of hyperplasia has been observed after prolonged testosterone treatment in rats (188, 189). In order to determine the long-term effects of elevated circulating androgen levels on the prostate gland of control male mice, a separate group of 12-week-old WT littermates (C57BL/6JxCBA-strain) were sham-operated and subcutaneously implanted with 30 mg of testosterone slow-releasing pellet (T30). After 8 weeks of treatment, prostates were dissected and serum samples obtained. On average, these animals displayed a fourfold increase in serum testosterone levels compared with controls (Table II). The testosterone levels of T-treated controls did not significantly differ from levels found in untreated Mt-PRL transgenic males (16.88±2.24 and 23.68±3.43 respectively, P=0.66). Prostate wet weight in testosterone-treated WT
did not significantly differ from that in untreated WT males, either as separate dorsolateral and ventral lobe weights nor as total organ weight. Histological appearance of the prostate lobes was not different from that observed in untreated controls. Epithelial cell AR-positivity was increased by androgen treatment whereas stromal AR content was unaffected. This would suggest that the hyperplastic phenotype is not primarily mediated via androgen stimulation of the prostatic epithelium. These findings establish that prolonged androgen stimulation of young adult male mice has no significant effects on prostate growth or histological appearance. Data further support the conclusions drawn from the results in castrated and androgen substituted Mt-PRL males, indicating that the hyperplastic process in Mt-PRL transgenic prostate is not dependent on an elevated state of circulating androgens.

**PAPER III**

**Prostate-specific expression of a PRL transgene leads to significant prostate hyperplasia**

The Mt-PRL transgenic model provided a tool for studying the effects of a prolonged hyperprolactinemic state on the prostate, with a concomitant increase in serum androgen levels. However, the specific role of an increased local PRL expression in the absence of known (e.g. s-testosterone) and unknown systemic alterations in relation to the hyperplastic phenotype remained largely uncharacterized. With the aim of generating prostate-specific overexpression of a PRL transgene, a construct was generated in which the prostate-specific rat Pb promoter element was employed to drive expression of the rPRL gene. Microinjections were performed and transgenic lines were established. Male mice expressing the transgene (Pb-PRL) developed significant hyperplasia of both the dorsolateral and ventral prostate lobes evident by 10 weeks of age and differences in glandular size (wet weight) compared to controls progressed throughout adulthood. RT-PCR showed expression of the Pb-PRL transgene from around 4 weeks after birth and was restricted to the dorsolateral and ventral prostate lobes. Serum androgen levels were unaltered compared to controls throughout the animal life span. Histological characteristics included a dramatically increased cellularity of the stromal compartment, ductal dilation due to secretion and focal areas of glandular hyperplasia. Furthermore, immunohistochemical analysis revealed a significant increase in stromal cell distribution of AR and ERα. In contrast, distribution of ERβ was nearly uniform in both Pb-PRL transgenic and wildtype prostate. Transgenic rPRL was detectable at low levels in the circulation of older transgenic animals, associated with the continuing increase in prostate size. In summary the
Pb-PRL transgenic represents a new model for the study of local PRL effects on the prostate. Most significantly, the development of Pb-PRL hyperplasia occurs mainly postpubertally and in a setting of normal androgen levels, thereby resembling the situation in the adult human prostate.

**Comparative analysis of prostate ductal structure and cellular composition**

In order to reveal possible phenotypic differences in ductal architecture due to different onset of transgenic rPRL expression, micro dissection technique was used to examine branching morphogenesis of individual lobes in Mt-PRL and Pb-PRL transgenic prostate. Analysis was performed at 12 weeks of age. Quantification was made by counting primary urethral ducts and terminal ductal tips allowing calculation of ductal branch-point numbers. This analysis defined the distinctive ductal branching patterns of each lobe. In 12-week-old Pb-PRL prostate no statistically significant difference was detected in the number of branch points per duct and the number of ductal tips present in each lobe compared to wildtype controls. However, marked ductal dilation and elongation was seen in the Pb-PRL from an early age and complete micro dissection was not achievable in animals over 20 weeks of age due to the formation of a densely fibrous interductal stroma that abrogated its normally high susceptibility to collagenase. In contrast, counting of ducts and tips in Mt-PRL ventral and lateral prostate lobes at the same age demonstrated a significant increase, with approximately a doubling in the number of branching points and terminal tips compared to wild type, while the number of main urethral ducts remained unchanged. In the Mt-PRL dorsal lobe, the number of ducts emanating from the urethra showed a non-significant increase (145% of control, p=0.561) with significantly increased number of branch points and tips. The ducts were also elongated and more dilated compared to controls. In prostate lobes of older Mt-PRL animals, micro dissection was also prevented by formation of a densely fibrous stroma.

To further distinguish differences in prostate phenotype, analysis of relative tissue compartments was performed and comparisons made between Pb-PRL, Mt-PRL and control animals aged 16-20 weeks. Quantification was undertaken by measurement of tissue areas by manual tracing of epithelium, interductal stroma and lumen using calibrated image analysis software. Cell nuclei in these areas were also counted manually and area density (nuclei/0.1mm sq.) was calculated. In Mt-PRL animals the ducts were grossly distended; the luminal area increased (VP 143% p<0.05, DP 192% p<0.01, LP 168% p=0.013 of control), with a flattened epithelium resulting in reduction of both epithelial area (VP 46%, DP 26%, LP 59% of control, p<0.01) and epithelial cell density. Area
density was also significantly increased in the interductal stroma of Mt-PRL transgenic dorsal and lateral lobes, whereas the ventral lobe stroma exhibited a non-significant increase (151% of control, \( p=0.1746 \)). Pb-PRL transgenic tissue component analysis likewise revealed a significant increase in cellular density of the interductal stroma compartment in ventral, dorsal and lateral lobes. In contrast, a significant reduction in epithelial area (50% of control, \( p<0.05 \)) and cellular density was evident only in the ventral lobe. Increased luminal area was also seen in the ventral and dorsal lobes (VP 167%, DP 143% of control, \( p<0.05 \)). Calculation of the stroma to epithelium cellular ratio (SER) thus showed a distinct stromal shift in both transgenic models compared to normal mouse prostate. In wildtype controls the lobe-specific SER varied between 1:2.5 and 1:10, whereas, in all lobes of Mt-PRL and Pb-PRL transgenic prostate stromal and epithelial cells were present in approximately equal numbers.

**PAPER IV**

**Prostate development and gene expression profiling in PRLR deficient mice**

The generation of a PRL receptor deficient mouse model (PRLR\(^{-/-}\)), by Ormandy et al. (94) provided a new tool for assessing PRL dependent effects on the prostate gland. In an effort to identify any essential developmental roles of PRL in the prostate, we investigated prostate development and prostate gene expression profiles in PRLR\(^{-/-}\) animals. Histological analysis revealed a small increase in dorsolateral and ventral prostate weights, but no change was seen in the weight of the anterior prostate in PRLR\(^{-/-}\) animals. The dorsal, but not ventral or lateral lobes showed a small but significant (-12%, \( p<0.05 \)) loss of epithelial cells. Microdissection was used to examine branching morphogenesis of individual prostate lobes and was quantified by counting urethral ducts and ductal tips and branch points. This analysis clearly defined the distinctive ductal branching patterns of each lobe. The ventral and lateral prostate lobes were attached to the urethra by two or three main ducts which showed extensive 'oak tree' branching morphology, while the dorsal prostate consisted of many ducts attached to the urethra which showed less extensive 'palm tree' branching morphology. There was no difference between PRLR\(^{+/+}\) and PRLR\(^{-/-}\) animals in the number of ducts, the number of branch points per duct, or the number of ductal tips present in each lobe. The more simply branched coagulating gland also showed no differences between genotypes. Oligonucleotide microarrays were then used to identify any essential transcriptional roles of prolactin. A small set of genes involved in sperm/oocyte interaction and copulatory plug formation with significantly
decreased expression was identified. Infertility or reduced fertility was apparent in the PRLR-/- males. The results from our micro arrays were validated using real-time RT-PCR. These findings establish a subtle but essential role for PRL in prostate development and reproductive function.

SV40T-induced prostate carcinogenesis in PRLR deficient prostate

PRL involvement in tumor formation or progressive malignant disease of the prostate has been indicated. To address this question we investigated SV40T-induced prostate carcinogenesis in PRLR-/- males. Crossbreeding strategies were used to obtain the required mixed genotypes. By mating 10 PRLR-/- males with 10 homozygous C3-SV40T females, and then mating the resultant females with the PRLR-/- males we produced animals that were heterozygous or wild type for C3-SV40T and PRLR+/+ or PRLR-/-+. Control PRLR+/+ animals were produced by the use of 10 PRLR+/+ in an identical but separate scheme to ensure similar genetic diversity between groups. Studies revealed that the area of SV40T-induced prostate intraepithelial neoplasia (PIN) was reduced by 28% in the ventral lobe but not the dorsal lobe. Furthermore, no tumors were seen in 20 homozygous (PRLR-/-) knockout animals compared to 1/11 detected in wildtype and 4/21 found in heterozygous (PRLR+/+) animals. These findings establish a possible role for functioning prolactin signaling in the induction of neoplastic changes in the prostate.
GENERAL DISCUSSION

Long-term exposure to elevated serum PRL levels leads to prostate hyperplasia in the Mt-PRL transgenic mouse

The prostate hyperplasia evident in 100% of the Mt-PRL transgenic males confirms the growth promoting effects of prolonged PRL stimulation on the prostate gland (paper I). Interestingly, the lack of correlation between circulating transgenic rPRL levels and the degree of prostate enlargement in individual transgenic animals suggests that the confirmed local expression of the transgene in the prostate may be more important than the general overexpression resulting in hyperprolactinemia. An important characteristic of the Mt-PRL transgenic phenotype is the elevated serum testosterone levels. In our studies, these were on approximately 3 times higher than those of wildtype controls with significant variations seen in individual transgenic males (3.7-34 nmol/L). Some transgenic animals demonstrated testosterone levels below those seen in wildtype controls and no correlation with degree of prostate enlargement seen in individual transgenic animals.

Reports of proliferative effects of PRL on the prostate date back to the 1960s and 1970s(106, 190). In vivo studies have been performed primarily in rodents, often using pituitary grafts to induce hyperprolactinemia over short time periods (106, 108, 191). Similar results have been obtained by grafting pituitary glands directly to the prostate (107). Additional studies have confirmed growth promoting effects of PRL while others have indicated androgen-independent PRL effects as demonstrated by a delay in the rate of castration-induced prostatic regression (100, 118, 119). Most previous studies have focused on short-term effects of PRL treatment in contrast to our Mt-PRL model of prostatic hyperplasia which focuses on the long-term effects of chronic hyperprolactinemia.

The role of PRL in the human prostate is less clear. No clear correlation between serum PRL levels and risk of developing BPH or prostate cancer in men has been established. Furthermore, there is no clear evidence that basal PRL serum levels change with age (192), although some studies indicate that both basal and TRH-stimulated PRL secretion may be augmented in a subset of aged men (193, 194). Interestingly, some clinical studies have shown that there are increased levels of PRL in the prostatic tissue of patients with BPH (195) and prostate cancer (136). Other studies have shown
decreased PRL serum levels following TURP (196-198), suggesting loss of local PRL production or a prostatic influence on pituitary PRL secretion. Involvement of the prostate in the feedback regulation of serum PRL have also been suggested in rodents (199).

The PRL effect on the adult prostate is independent of elevated serum androgen levels
An important species difference in human and rodent responses to hyperprolactinemia is the reported effects on circulating androgen levels. In humans, hyperprolactinemia is associated with lowered serum testosterone levels. In rodents, PRL does not clearly possess similar effects, and serum androgen levels in previously presented hyperprolactinemic rodent models have mostly been reported as unchanged (200). As described, our Mt-PRL transgenic males exhibited elevated mean serum testosterone levels, although no individual correlation to the degree of prostate enlargement existed. Expression of the transgene was also demonstrated locally in the testes and the androgen elevation seen in Mt-PRL could be attributed to the known direct-stimulating effects of PRL on LH receptor expression and testosterone production in the Leydig cells (201-203).

In order to investigate the effects of androgenic influence on the prostate in Mt-PRL transgenic animals, we designed a castration and testosterone resubstitution study using age-matched Mt-PRL transgenic and wildtype male mice (paper II). The aim was to normalize circulating testosterone levels in young adult transgenic males for a prolonged time period (8 weeks). Earlier reports using pituitary grafts have shown a proliferative effect of PRL regardless of androgen status (106, 191). There is also evidence, from rat models, that increased PRL levels delay the castration-induced prostatic regression (118). These previous findings correspond with our result of continued abnormal prostate growth in the transgenic mice during the eight week period when serum testosterone was reduced to wildtype levels (paper II).

Prolonged androgen treatment does not significantly affect prostate growth and morphology in the wildtype mouse
Although the role of androgens in human BPH is debated, they indisputably play a permissive role in its development as BPH does not occur in castrated men. A permissive action of androgens for the hyperplastic Mt-PRL phenotype was also demonstrated in our castration animal studies, demonstrating loss of hyperplastic phenotype and comparable postcastrational regressive changes in transgenic and control prostate (paper II). The effects of long-term androgen stimulation on human
prostate are ambiguous. In elderly men, prostate cellular hyperplasia occurs frequently despite decreases in testicular androgen production and peripheral levels of androgen reaching the prostate. Testicular endocrine function declines steadily with age. By 75 years of age, mean plasma testosterone levels are reported to be approximately 65% of those found in young males (204). The decrease in bio-active (non SHBG-bound) testosterone levels is even more pronounced (205). This is probably partly due to an increased SHBG binding capacity associated with age (206-208). In contrast, prolonged testosterone administration in young adult wildtype males had no significant effect on prostate growth or histological appearance.

**Local overexpression of a PRL transgene in the developed mouse prostate leads to marked and primarily stromal hyperplasia**

The recent discovery of PRL ligand expression in the prostate has focused attention on local PRL effects that may act in an auto/paracrine fashion. To study the effects of local increase in PRL activity under normal testosterone levels, we generated a transgenic model that expressed the rat PRL gene under the control of the prostate-specific Pb minimal promoter. As shown, the Pb-PRL males developed significant prostatic hyperplasia with a primarily stromal phenotype. The transgene is not active in the neonatal stage of prostate ductal development since the probasin promoter expression is highly androgen-dependent. Ductal development is initiated around embryonic day 15 and is essentially completed by 4-5 weeks postpartum (7). We could not detect transgenic expression before 4 weeks of age. Thus, the Pb-PRL hyperplastic change is initiated in prostates with a normally developed ductal structure. The Pb-PRL males exhibited normal serum androgen levels throughout their lifespan, further supporting our conclusion that elevated circulating androgen levels are not responsible for the hyperplastic phenotype. The stromal hyperplasia is of special interest since it is similar to most cases of human BPH, where normally over 75% of the total volume is of a stromal nature (209, 210). A changed balance between proliferative and apoptotic activity in the aging prostate has been proposed as a mechanism for BPH induction and progression. Reduced apoptotic activity in hyperplastic stroma of the prostate could explain some of this imbalance (211). In additional studies not included in this thesis, we have identified reduced apoptotic activity in both Mt-PRL and Pb-PRL transgenic prostates. We have observed apoptosis-related differential gene expression (212) and using immunohistochemical apoptosis markers (caspase-3 and single stranded-DNA antibodies) in situ differences were detected (Dillner K. et al., submitted). The hyperplastic prostates of our transgenic models thus share interesting histological and molecular characteristics
with human BPH.

**Neonatal or prepubertal overexpression of PRL promotes prostatic ductal morphogenesis**

The morphology of the ductal branching in the murine prostate gland has been studied extensively (7-9). As presented, ductal formation is initiated around embryonic day 15 and considered essentially completed 35 days postpartum. Prostate lobe microdissection techniques have been developed and found to effectively demonstrate changes in ductal structures (7), reflecting exposure to developmentally active factors. As presented in paper III, expression of a PRL transgene (Mt-PRL) during the essential period of ductal morphogenesis results in a significant increase in ductal branching. Approximately a doubling in the number of distal tips and branching points was noted in all prostate lobes of young adult Mt-PRL transgenic prostate compared to littermate wildtype controls. The Mt-1 gene in mice is known to be abundantly expressed from mid-gestation (213) and continues to be expressed throughout the period of ductal formation. Prior use of the Mt-1 promoter in transgenic models has also demonstrated neonatal expression. In contrast, the Pb-PRL transgene was not expressed before 4 weeks postpartum due to the androgen-dependence of the probasin promoter. Consequently, the prostate of Pb-PRL transgenic males did not demonstrate any significant changes in ductal morphogenesis compared with wildtype littermates. Possible changes in neonatal androgen sensitivity induced by expression of the Mt-PRL transgene could obviously account for part of this phenotype. However, neonatal castration experiments by Donjacour et al have demonstrated that significant branching morphogenesis occurs in the absence of androgens(10). Furthermore, androgen replacement following neonatal castration results in precocious ductal formation, but final numbers of ductal tips and branchpoints do not exceed those seen in adult control males (10).

**Evidence of increased androgen sensitivity in the PRL transgenic phenotypes**

The proposed imbalance in cell death and cell proliferation that leads to age-dependent prostatic hyperplasia is possibly related to an increased sensitivity of the prostate to androgens. Several modes of PRL influence on prostatic androgen sensitivity have been proposed earlier, including upregulation of both ARs (214) and the 5α-reductase enzyme responsible for conversion of testosterone to DHT (215). In a human BPH study, cytosolic and nuclear AR content was shown to be proportional to plasma PRL levels (166). A decreased T/DHT ratio, due to both decrease in plasma testosterone levels and possibly an increase in DHT levels, may be involved in the development of BPH in elderly men (216, 217) and in the prostate, PRL is proposedly capable of up
regulating the 5α-reductase gene, thereby contributing to the increase in DHT availability.

Furthermore, we found an increased occurrence of AR in the prostatic stroma of both our PRL transgenic models compared to controls. In Mt-PRL, this change was unaffected by reduction of serum testosterone to wildtype levels. In addition, there was no increase in AR immunoreactivity of the stromal cells in androgen treated wildtype males. These findings indicate a direct effect of PRL, likely to increase the local responsiveness to androgen stimulation. As the conversion of testosterone to the more active androgen DHT primarily occurs in the stroma due to the strong stromal presence of the type 2 5α-reductase isoform, an increased effect of available androgens in the transgenic prostates may therefore be expected. This may further contribute to the predominantly stromal phenotype observed in PRL transgenic prostates.

**PRL gene overexpression induces a distinct shift in the stroma to epithelium ratio**

Both Mt-PRL and Pb-PRL prostates were characterized by a marked expansion of the stromal compartment. The stromal hypercellularity seen in all prostatic lobes resulted in a marked increase in the stroma to epithelium ratio (SER) which is often used to describe prostatic morphology. These changes correspond well to the morphology of human prostate in which the stromal component is much more pronounced than in rodents. Histological characterizations of non-hyperplastic and hyperplastic human prostate have recently confirmed that both BPH and non-BPH prostate tissues are comprised of approximately 80% stroma (210, 218). Thus, it is not clear how the histological composition contributes to the pathophysiology of clinical symptoms associated with BPH, although an increased SER is seen in symptomatic versus asymptomatic BPH (209). The interesting theory by McNeal proposing that BPH represents a “reawakening” of the embryonic and inductive potential of prostatic stroma (219) still requires confirmation.

**Subtle essential effects of PRL in normal mouse prostate**

The generation of PRLR deficient mice, PRLR−/−, allowed specific studies aimed at determining the role of PRL in normal prostate development and maintenance (paper IV). Although subtle, the loss of PRLR signaling in PRLR−/− males resulted in significant changes in prostate tissue composition, with a partial loss of epithelial content in the dorsal lobe. Histologically, no major differences compared to controls were noted. However, prostate lobe wet weights were slightly increased, possibly due to increased ductal secretion. Ductal development, as assessed by micro dissection, was also unaltered in PRLR−/− prostate, indicating that PRL does not play an essential role in neonatal
prostate development. The subtle changes observed in PRLR⁻/⁻ are in sharp contrast to the significant alterations in ductal formation and histological composition induced by prolonged PRL overexpression.

PRL influence on male fertility was also assessed in the PRLR⁻/⁻ males. A subset of PRLR deficient males (~10%) proved completely infertile. In fertile animals the mating behaviour appeared normal but a significant latency to first pregnancy was seen in PRLR⁻/⁻ animals and overall they had only 40% probability of producing a first pregnancy compared to PRLR⁺/+ animals. Interestingly these animals show a return to full fertility following the first pregnancy, possibly due to subsequent post-partum matings. In the PRL transgenic males, fertility has not yet been extensively studied, but approximately 30-50% of Mt-PRL males fail to reproduce successfully (unpubl. data), whereas Pb-PRL males display full fertility. The difference between the general and local PRL transgenic models would indicate that the reduced fertility is due to systemic effects of increased PRL, such as modified sexual behaviour or testicular effects, rather than a disturbance due to the prostatic alterations.

**Possible role of PRL in promoting prostatic carcinogenesis and premalignant change**

Crossbreeding of PRLR deficient mice and the prostate tumor transgenic mice, C3(T)Ag, resulted in a reduction of premalignant changes (PIN) in the ventral prostate lobe and a complete lack of prostate tumor induction in homozygous (PRLR⁻/⁻) receptor-deficient males (paper IV). Although this study included limited numbers of animals, the results are still clearly suggestive of a role for PRLR signaling in malignant transformation. Evidence of PRL involvement in prostate cancer has generated some interest over the past decades. Clinical reports have indicated both a poorer prognosis in prostate cancer patients displaying elevated PRL levels (220) and improved clinical outcome with reduction of PRL in addition to androgen deprival (221). Further, a recent study demonstrated an experimental survival effect of PRL on the androgen-independent human prostate cancer cell line PC3, through inhibition of apoptosis (222).

There are also indications of PRL involvement in the development of breast cancer. Animal tumor model studies have demonstrated the potentially beneficial effects of antagonizing PRL action. Administration of a monoclonal PRLR antibody, PrR-7A, was shown to block formation of mammary carcinoma and reduce intraductal hyperplasia in a carcinogen-induced mouse model (223). Recently, the PRL antagonist hPRL-G129R was found to inhibit human breast cancer cell proliferation via induction of apoptosis (224, 225). These results correspond with previous results reported by our group on mammary tumor formation in Mt-PRL transgenic female mice (226),
showing that activation of the PRLR is sufficient for induction of mammary carcinomas in mice.

**Final remarks**

In summary, the work presented in this thesis increase our understanding of the long-term growth promoting effects of PRL on the prostate gland. The hyperplastic growth was found to be independent of elevations in circulating testosterone levels, although normophysiological testosterone levels have an expected permissive role for development of the phenotype. Furthermore, the prostatic effects of prostate-specific expression of a PRL transgene without any noticeable systemic effects, emphasize the need for a better understanding of the existing local PRL expression in human prostate. The primarily stromal phenotype shares interesting properties with human BPH and further characterization of key molecular events in the hyperplastic process could provide significant information. In addition, the reduced malignant potential indicated in prostates of PRLR deficient mice indicate that development of specific PRL receptor antagonists will provide us with a new tool not only vital in assessing local PRL effects but possibly also useful in a clinical setting.
CONCLUSIONS

1/ General (Mt-PRL) and local (Pb-PRL) overexpression of the PRL gene leads to dramatic prostatic hyperplasia with a predominantly stromal component.

2/ Elevated circulating androgen levels are not required for the progression of prostate hyperplasia in adult Mt-PRL and Pb-PRL transgenic male mice.

3/ Prolonged androgen treatment in adult wildtype mice does not significantly affect prostate size or morphology.

4/ PRL increases stromal androgen receptor content in the prostate (independently of elevations in circulating androgens).

5/ PRL stimulates ductal morphogenesis in the developing neonatal murine prostate either directly or via alterations in androgen status and/or local sensitivity.

6/ PRLR deficiency results in limited lobe-specific epithelial loss and increased postcastrational regress in the murine prostate.

7/ A role for PRL in prostate carcinogenesis is strongly suggested.
FUTURE PERSPECTIVES

The results presented in this thesis have contributed to our understanding of PRL effects on the prostate. The potential influence of this and other non-androgenic hormones and growth factors on both normal prostate development/function and not least in pathophysiological conditions clearly needs to be considered. This is emphasized by the lack of a basic understanding of the processes involved in the ethiology of both BPH and prostate cancer despite decades of mainly androgen-related research efforts. Below are listed two of our current or planned activities aimed at furthering the knowledge of PRL-related effects in basic prostate biology and pathophysiology.

1. Identification of key molecular events in the induction and progression of prostatic hyperplasia seen in our PRL transgenic models using global gene profiling techniques, such as cDNA micro arrays, followed by validation of differentially expressed transcripts on a protein level.

2. Elucidation of the role of PRL in induction and progression of malignant disease in the prostate through further studies using the genetically modified mice, including both the PRL ligand overexpressing transgenes and the PRLR-deficient mice presented in this thesis.
ACKNOWLEDGEMENTS

Research is very much a team effort, and the completion of this work would not have been possible without all my gifted collaborators. In addition, all my other colleagues at the endocrine division and elsewhere have contributed in making this an enjoyable experience. I would especially like to express my gratitude to;

Jan Törnell, my supervisor, for recruiting me to the transgenic group and then sharing your knowledge and analytical mind to the great benefit of the work done over these years.

Håkan Wennbo, my co-supervisor, for your never-failing support, enthusiasm and constructive approach to the scientific work. Also, I am grateful for the culinary masterpieces often served at your dinner table.

Olle Isaksson, my co-supervisor, for scientific encouragement, enthusiastic support and for always providing the resources needed to carry out the work.

Karin Dillner, my dear collaborator, for all your help, enthusiastically sharing the daily ups and downs of experimental science and for the great teamwork that has really benefited us both. I hope it will continue in the future.

My co-author and antipodean mentor, Chris Ormandy, for enthusiastically sharing your expertise in this area and thereby making much of the following work possible. I hope we can continue this collaboration in the years to come.

Charlotte Ling, my co-author, for fruitful collaboration and for being such a terrific person, always spreading good vibes in the workplace.

My other coauthors; Ruijin Shao, Lena Sahlin, Fiona Robertson, Matthew Naylor, Jessica Harris, Samantha Oakes, Paul Kelly and Jeff Green for pleasant collaboration. Special thanks to Maud Petterson, Britt Masironi and Karin Karlsson for excellent technical assistance in this work.

Vincent Goffin and Sophie Bernichtein in Paris, for involving us in such an interesting collaboration.

My friends and colleagues in the Knockout Invest team; Ola Brusehed for great comradery and enjoyable conversations in the cubicle over the years, Fredrik Frick for being 100% positive and for sharing my interest in anything “hi-tech”. Bob Olsson, Mohammad Bohlooly-Y and Daniel Lindén for all the laughs in and outside of the lab.

Former members of the transgenic group; Maria Gebreh-Medin, Klara Sjögren, Käre Hultén and Jonas Sandstedt for good companionship during my first years in the group. Special thanks to Mia Umerus for all the excellent technical assistance.

The seniors at the endocrine lab, Håkan Billig, Jan Oscarsson and Staffan Edén for your general support and for creating a stimulating and academic environment for your PhD students.
All other past and present friends and colleagues at the endocrine lab (dept. of physiology) including; Emil Egecioglu, Linda Carlsson, Joakim Larsson, Anders Friberg, Louise Svensson, Emilia Markström, Caroline Améen, Anna Ljungberg, Masoumeh Jalouli, Ulrika Edvardsson, Lars Hedin, Katarina Rask, Karin Sundfeldt, Eva Svensson, Bodil Svanberg. Special thanks also to Lena Olofsson and Gunnel Larsson, for all your help with practical matters at the department during this time.

All friends and colleagues at RCEM; including Claes Ohlsson, Jan-Olov Jansson, Ville Wallenius, Stanko Skrtic, Kristina Wallenius, Marie Lindberg, Sophia Moverare, Åsa Tivesten. Special thanks to Ulla-Britt Libera, for all your helpful assistance in practical matters.

My father, for sharing with me your passion for the medical profession and scientific work in particular. My mother, for your loving encouragement and support of any endeavor I may undertake. Frida, my sister, for being truly loving and generous. Pål, my brother, for constantly teaching me to look at the world just a little differently. Jeanne, for your support and for giving me very constructive advice in the writing of this thesis. Göran, for always being so helpful and welcoming.

Åke and Anne-Marie, my dear in-laws, for your constant support and genuine interest in what we do.

Filippa, my little miracle, for just being you and making everything worthwhile.

Jenny, my LOVE, for leading the way and for being a truly remarkable person.

This thesis was supported by grants from the Göteborg Medical Society, Swedish Cancer Society, The Medical Faculty at Göteborg University, Sahlgrenska University hospital, Assar Gabrielsson foundation and AstraZeneca R&D.
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