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# THE EFFECTS OF FIBROBLAST GROWTH FACTOR 8b ON REPRODUCTIVE ORGANS AND PROSTATE TUMORIGENESIS

by

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# ABSTRACT

Fibroblast growth factors (FGFs) are involved in the development and homeostasis of the prostate and other reproductive organs. FGF signaling is altered in prostate cancer. Fibroblast growth factor 8 (FGF8) is a mitogenic growth factor and its expression is elevated in prostate cancer and in premalignant prostatic intraepithelial neoplasia (PIN) lesions. FGF8b is the most transforming isoform of FGF8. Experimental models show that FGF8b promotes several phases of prostate tumorigenesis - including cancer initiation, tumor growth, angiogenesis, invasion and development of bone metastasis. The mechanisms activated by FGF8b in the prostate are unclear.

In the present study, to examine the tumorigenic effects of FGF8b on the prostate and other FGF8b expressing organs, an FGF8b transgenic (TG) mouse model was generated. The effect of estrogen receptor *beta* (ERβ) deficiency on FGF8b-induced prostate tumorigenesis was studied by breeding FGF8b-TG mice with ERβ knockout mice ($BERKO_{FVB}$).

Overexpression of FGF8b caused progressive histological and morphological changes in the prostate, epididymis and testis of FGF8b-TG-mice. In the prostate, hyperplastic, preneoplastic and neoplastic changes, including mouse PIN (mPIN) lesions, adenocarcinomas, sarcomas and carcinosarcomas were present in the epithelium and stroma. In the epididymis, a highly cancer-resistant tissue, the epithelium contained dysplasias and the stroma had neoplasias and hyperplasias with atypical cells. Besides similar histological changes in the prostate and epididymis, overexpression of FGF8b induced similar changes in the expression of genes such as osteopontin (*Spp1*), connective tissue growth factor (*Ctgf*) and FGF receptors (*Fgfr*s) in these two tissues. In the testes of the FGF8b-TG mice, the seminiferous epithelium was frequently degenerative and the number of spermatids was decreased. A portion of the FGF8b-TG male mice was infertile. Deficiency of ERβ did not accelerate prostate tumorigenesis in the FGF8b-TG mice, but increased significantly the frequency of mucinous metaplasia and slightly the frequency of inflammation in the prostate. This suggests putative differentiation promoting and anti-inflammatory roles for ERβ.

In summary, these results underscore the importance of FGF signaling in male reproductive organs and provide novel evidence for a role of FGF8b in stromal activation and prostate tumorigenesis.

**KEY WORDS**: epididymis, epithelium, estrogen receptor β, fibroblast growth factor 8, prostate, stroma, transgenic mouse, tumorigenesis.

Teresa Elo

FIBROBLASTIKASVUTEKIJÄ 8b:n VAIKUTUKSET LISÄÄNTYMIS-ELIMISSÄ JA ETURAUHASSYÖVÄN SYNNYSSÄ

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# TIIVISTELMÄ

Fibroblastikasvutekijät (FGF) osallistuvat eturauhasen ja muiden lisääntymiselinten kehityksen säätelyyn ja homeostaasin ylläpitoon. Eturauhassyövässä FGF-signalointi on muuttunut. Fibroblastikasvutekijä 8 (FGF8) on mitogeeninen kasvutekijä, jonka määrä on lisääntynyt eturauhassyövässä ja eturauhassyövän esiasteissa, PIN- (*prostatic intraepithelial neoplasia*) muutoksissa. FGF8b on FGF8:n isomuoto, jolla on kyky muuntaa kohdesolut pahanlaatuisiksi. Kokeellisten mallien perusteella FGF8b osallistuu useisiin eturauhassyövän kehityksen vaiheisiin, kuten syövän syntyyn, kasvaimen kasvuun, verisuonituksen muodostumiseen, syövän tunkeutumiseen ympäröivään kudokseen ja luustoetäpesäkkeiden kehittymiseen. FGF8b:n vaikutusmekanismit eturauhasessa ovat huonosti tunnettuja.

Tässä tutkimuksessa kehitettiin FGF8b-siirtogeeninen (FGF8b-TG) hiirimalli, jonka avulla tutkittiin FGF8b:n vaikutuksia eturauhasessa ja muissa siirtogeeniä ilmentävissä kudoksissa. Lisäksi tutkittiin estrogeeni reseptori β:n (ERβ) puutoksen vaikutusta pahanlaatuisten muutosten kehittymiseen FGF8b-TG-hiirissä risteyttämällä FGF8b-TG-hiiret ERβ-poistogeenisten ($BERKO_{FVB}$) hiirten kanssa.

FGF8b aiheutti eteneviä muutoksia FGF8b-TG-hiirten eturauhasessa, lisäkiveksissä ja kiveksissä. Eturauhasen epiteelissä ja stroomassa todettiin hyperplastisia, preneoplastisia ja pahanlaatuisia muutoksia, kuten PIN-muutoksia, adenokarsinoomaa, sarkoomaa ja karsinosarkoomaa. Lisäkiveksessä, joka tunnetaan syöpäresistenttinä kudoksena, kehittyi dysplastisia muutoksia epiteeliin. Lisäkiveksen stroomaan kehittyi pahanlaatuisia muutoksia sekä hyperplasiaa, jossa solut olivat atyyppisia. Eturauhasen ja lisäkiveksen muutokset FGF8b-TG-hiirissä olivat samankaltaisia sekä histologialtaan että geenien, kuten osteopontiinin (*Spp1*), tukikudoksen kasvutekijän (*Ctgf)* ja FGF-reseptorien (*Fgfr*s) ilmentymisen suhteen. Useiden FGF8b-TG-hiirten kiveksissä siemenepiteeli oli degeneratiivista ja osa koirashiiristä oli lisääntymiskyvyttömiä. ERβ:n puutos ei nopeuttanut eturauhassyövän kehittymistä FGF8b-TG-hiirissä, mutta lisäsi merkittävästi musinoosin metaplasian määrää sekä hieman inflammaation määrää, mikä puoltaa käsitystä ERβ:n toiminnasta erilaistumista edistävänä ja tulehdusta estävänä tekijänä eturauhasessa.

Tulokset korostavat FGF-signaloinnin merkitystä lisääntymiselimissä ja osoittavat FGF8b:n aiheuttavan strooman aktivaatiota ja siihen liittyvää eturauhassyöpää.

**AVAINSANAT**: estrogeenireseptori β, epiteeli, eturauhanen, eturauhassyöpä, fibroblastikasvutekijä 8, lisäkives, siirtogeeninen hiiri, strooma.

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# ABBREVIATIONS

| | |
|---|---|
| ADT | Androgen deprivation therapy |
| AP | Anterior prostate |
| AR | Androgen receptor |
| $ARR_2PB$ | Probasin promoter |
| BERKO | Estrogen Receptor Beta Knockout |
| BPH | Benign Prostatic Hyperplasia |
| cDNA | Complementary DNA |
| Cre | Cyclization recombinase |
| CSC | Cancer stem cells |
| CTGF | Connective tissue growth factor |
| DLP | Dorsolateral prostate |
| EMT | Epithelial-to mesenchymal transition |
| ER | Estrogen receptor |
| ERKO | Estrogen receptor alpha KO |
| ERα | Estrogen receptor alpha (ESR1) |
| ERβ | Estrogen receptor beta (ESR2) |
| FGF | Fibroblast growth factor |
| FGFR | Fibroblast growth factor receptor |
| FHF | FGF homologous factor |
| FVB/N | Laboratory mouse strain |
| GEM | Genetically engineered Mouse |
| GF | Growth factor |
| HGPIN | High grade prostatic intraepithelial neoplasia |
| HRPC | Hormone resistant prostate cancer |
| HSPGs | Heparan sulfate proteoglycans |
| hzBERKO | Heterozygous BERKO |
| iFGFR1 | Inducible FGFR1 |
| IHC | Immunohistochemical |
| IS | Initial segment |
| KO | Knockout |
| LADY | Probasin-SV40 T-antigen TG mouse model |
| LGPIN | Low grade prostatic intraepithelial neoplasia |
| LNCaP | Prostate cancer cell line |
| mPIN | Mouse PIN |
| PC-3 | Prostate cancer cell line |
| PCR | Polymerase chain reaction |
| PIN | Prostatic intraepithelial neoplasia |
| PLCγ | Phospholipase C gamma |
| PSA | Prostate specific antigen |
| PTEN | Phosphatase and tensin homologue in chromosome 10 |
| qRT-PCR | Quantitative RT-PCR |
| RTF | Rete testis fluid |
| RTK | Receptor tyrosine kinases |
| RT-PCR | Reverse transcriptase PCR |
| S115 | Mouse breast cancer cell line |
| SEF | Similar expression to FGFs |
| SNPs | Single nucleotide polymorphisms |
| SPP1 | Secreted phosphoprotein 1, also known as osteopontin |
| Spry | Sprouty |
| SV40 | Simian virus 40 |
| T | Testosterone |
| TG | Transgenic |
| TGFβ | Transforming growth factor beta |
| TRAMP | Transgenic adenocarcinoma of the mouse prostate |
| TKI | Tyrosine kinase inhibitors |
| UGE | Urogenital sinus epithelium |
| UGM | Urogenital sinus mesenchyme |
| UGS | Urogenital sinus |
| VP | Ventral prostate |
| WT | Wild type |

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-III.

I Teresa D. Elo, Eeva M. Valve, Jani A. Seppänen, Heikki J. Vuorikoski, Sari I. Mäkelä, Matti Poutanen, Paula M. Kujala and Pirkko L. Härkönen (2010): Stromal activation associated with development of prostate cancer in prostate-targeted fibroblast growth factor 8b transgenic mice, *Neoplasia* 12(11), 915-927.

II Teresa Elo, Petra Sipilä, Eeva Valve, Paula Kujala, Jorma Toppari, Matti Poutanen and Pirkko Härkönen (2012): Fibroblast growth factor 8b causes progressive stromal and epithelial changes in the epididymis and degeneration of the seminiferous epithelium in the testis of transgenic mice, *Biology of Reproduction*, 86(5):157, 1-12.

III Teresa Elo, Lan Yu, Eeva Valve, Sari Mäkelä and Pirkko Härkönen: Deficiency of estrogen receptor beta modulates epithelial differentiation but does not accelerate prostate tumorigenesis in FGF8b transgenic mice. (*Manuscript)*

# 1. INTRODUCTION

The prostate is a male accessory reproductive gland, which produces secretions in the seminal fluid. Androgens control the differentiation, growth and function of the prostate and are involved in the development of common prostatic diseases, such as benign prostatic hyperplasia (BPH) and prostate cancer. Other steroid hormones, such as estrogens also regulate prostatic differentiation and growth and are implicated in the development of prostatic diseases (Cunha *et al.,* 2004, Kawashima & Nakatani, 2012).

In Western countries, prostate cancer is the most common cancer in males (Ferlay *et al.,* 2007, Siegel *et al.,* 2012). Prostate cancer is a heterogeneous disease and the molecular mechanisms underlying the disease are still poorly understood. Genetic factors have a major contribution to prostate cancer susceptibility (Giovannucci *et al.,* 2007, Lichtenstein *et al.,* 2000), but as the disease is polygenic, the effects of single genetic polymorphisms are often minor and differ among patients (Nakagawa *et al.,* 2012). Environmental factors, such as diet and lifestyle, may contribute to the disease susceptibility, but none of these factors are causative or predictive in prostate cancer (Schultz *et al.,* 2011). At the early phase, prostate cancer can be treated by surgery and by hormonal therapy. The advanced, metastasized prostate cancer becomes insensitive to hormonal therapy and is incurable. Studies investigating the molecular biology of prostate cancer are required to enable development of methods for predicting disease outcome at its early stages and to improve treatments to prevent the development of hormone refractory prostate cancer (HRPC) and to reduce side-effects (Damber & Aus, 2008).

The epididymis is a male accessory reproductive organ, which has functions in sperm maturation and storage that contribute to fertility. The epididymis is known for its high cancer resistance, and neoplasias of epididymal origin are extremely rare (Ganem *et al.,* 1998). The mechanisms underlying the epididymal cancer-resistence remain to be solved.

Fibroblast growth factors (FGFs) form a large family of peptide growth factors (GFs) that have important roles in embryonic development, wound healing and adult tissue homeostasis. FGF-signaling is deregulated in many types of human cancers and this can contribute to deregulated growth and promote cancer progression by several mechanisms (Turner & Grose, 2010). FGF8 is a mitogenic GF, whose expression is upregulated in hormonal cancers (Mattila & Härkönen 2007) including prostate cancer (Dorkin *et al.,* 1999b, Gnanapragasam *et al.,* 2003, Leung *et al.,* 1996, Tanaka *et al.,* 1998, Valve *et al.,* 2001) and also in premalignant prostatic intraepithelial neoplasia (PIN) lesions (Valve *et al.,* 2001). FGF8b is a FGF8 isoform with highest transforming potential (MacArthur *et al.,* 1995a). The presence of FGF8 in human PIN lesions (Valve *et al.,* 2001) and the results of a previous genetically engineered mouse (GEM) model (Song *et al.,* 2002) suggest that FGF8 is involved in the initiation of prostate tumorigenesis. FGF8 is also expressed in the HRPC (Dorkin *et al.,* 1999a) and in bone metastasis of prostate cancer (Valta *et al.,* 2008) and *in vitro* and *in vivo* experiments using prostate cancer cell lines implicate that FGF8 can promote several phases of

cancer progression such as tumor growth (Song *et al.,* 2000, Valta *et al.,* 2009), angiogenesis (Tuomela *et al.,* 2010) and the development of bone metastasis (Valta *et al.,* 2008).

In the present study, a FGF8b transgenic (TG) mouse model was generated to examine the effects and mechanisms induced by overexpression of FGF8b *in vivo* in the prostate. Besides the prostate, expression of the transgene and alterations contributing to fertility were found in the epididymis and testis of the FGF8b-TG-mice and these were also investigated. In addition, the effect of abrogation of estrogen receptor β (ERβ), a potential tumor suppressor, on prostate tumorigenesis in the FGF8b-TG mice was studied by breeding the FGF8b-TG mice with ERβ knockout (BERKO) mice.

# 2. REVIEW OF THE LITERATURE

## 2.1 THE PROSTATE

### 2.1.1 Structure and development of the prostate

The prostate is the largest male accessory reproductive gland and it exists only in mammals. The human prostate is sized similarly to a walnut and lies between the base of the urinary bladder and rectum. The human prostate completely surrounds the proximal urethra (Figure 1A). It is a single lobed, compact gland that can be anatomically divided to three glandular zones: central (CZ); peripheral (PZ); and transitional zones (TZ). In addition, there is a large, nonglandular region called anterior fibromuscular stroma (AFS) (McNeal, 1968, Roy-Burman *et al.,* 2004). The prostate anatomy of rodents, which are often used as experimental models, differs from that of humans (Figure 1B). The rodent prostate is composed of four pairs of lobes: the ventral (VP); anterior (AP); lateral (LP); and dorsal prostate (DP), which incompletely surround the urethra. LP and DP are often referred to as the dorsolateral prostate (DLP) due to a common ductal system. The rodent DP is considered analogous to the posterolaterally localized PZ of human prostate, from which, most of the prostate cancers arise (Roy-Burman *et al.,* 2004, Shappell *et al.,* 2004). Histologically, the prostate is composed of epithelium and stroma. The epithelium forms the glandular acini, which are surrounded by the stroma. In both human and rodent prostates, the epithelium contains luminal, basal and neuroendocrine cells, and the prostate stroma constitutes mainly of smooth muscle and fibroblastic cells**.** The luminal cells are the secretory cells of the prostate, whereas the basal cells, which locate near the basement membrane, are the dividing progenitor cells that give rise to other epithelial cell types. Neuroendocrine cells present the smallest proportion of prostatic cell types. The ratio of different cell types in the prostate is different between rodents and humans. In the rodent prostate, the relative amount of basal and neuroendocrine cells is smaller than in humans and the basal cells do not form a continuous layer around the secretory cells as in human. The proportion of stroma is significantly smaller in the rodent prostate (Roy-Burman *et al.,* 2004, Shappell *et al.,* 2004).

The development of prostate begins prenatally from the endoderm-derived urogenital sinus (UGS) as the urogenital sinus epithelium (UGE) protrudes buds to the surrounding urogenital sinus mesenchyme (UGM). The development continues pre- and postnatally by elongating and branching of the buds and differentiation of epithelial and stromal cells – processes, which are completed during puberty. Development of the prostate is dependent on androgens and mesenchymal-epithelial interactions (Cunha *et al.,* 2004, Cunha, 2008). Pioneering studies by Cunha (1972), using tissue recombinant techniques, showed that the UGM directs the differentiation of the UGE during prostate development.

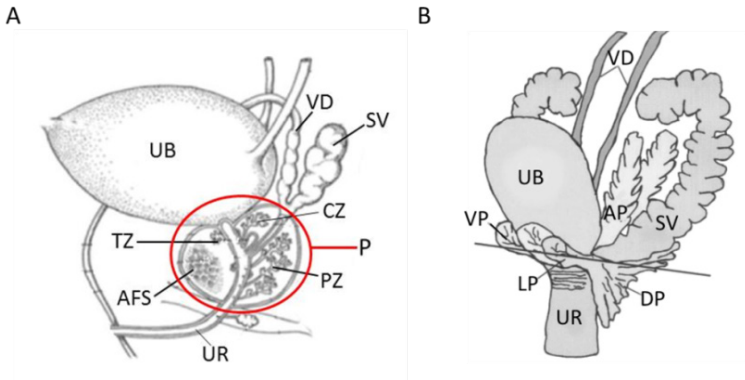


**Figure 1.** Comparative anatomy of human (A) and mouse (B) prostate and associated structures. Human prostate (P) is single lobed and composed of zones: TZ, transitional zone; CZ, central zone; PZ, periferal zone and AFS, anterior fibromuscular stroma. Mouse prostate is composed of four pairs of lobes: VP, ventral prostate; LP, lateral prostate; DP, dorsal prostate and AP, anterior prostate. UB, Urinary bladder; UR Urethra; SV, Seminal vesicle; VD, Vas deferens. (Modified from Timms *et al.,* 2011 and Shappell *et al.,* 2004).

The effects of androgen to UGE differentiation during the embryonic development are mediated through androgen receptors (ARs) expressed exclusively in the UGM. Mesenchymally expressed, paracrine-acting molecules, named andromedins, may mediate the effects of androgens to the UGE (Thomson, 2008). By definition, an andromedin should be androgen-regulated and its inhibition should lead to androgen insensitivity and impairment of prostate development. Several genes expressed in the prostate mesenchyme, including members of the FGF family, have been suggested to be andromedins, but none of these molecules has fulfilled all the criteria of an andromedin (Thomson, 2008). However, during prostate development, the signaling between the epithelium and mesenchyme is reciprocal and signals from the UGE are required for differentiation of UGM to smooth muscle (Cunha *et al.,* 1992). In the female rodent embryos, the development of prostate can be induced by androgen. In adult women, the Skene's glands (urethral glands) may be analogous to the male prostate, but this issue remains controversial (Thomson, 2008).

### 2.1.2 Function of the prostate

The main function of the prostate is to produce secretions in the seminal fluid. These secretions are produced by the luminal epithelial cells. Prostatic fluid makes up about 20 percent of the ejaculate volume and it is rich in proteins. It contains proteases such as prostate specific antigen (PSA), other kallikreins, prostatic acid phosphatase (PAP), and several proteins associated with immunity or sperm activation and survival (Lee *et al.,* 1986). Prostatic fluid contains high levels of zinc, which plays a role in sperm motility and has antimicrobial functions (Kelleher *et al.,* 2011). The function of PSA is to liquefy seminal fluid by degrading semenogelin. Proteins secreted by the mouse prostate differ from those secreted by the human prostate and, for example, PSA is not expressed in the mouse. However, prostatic-secretory protein 94 (PSP94), Zinc-a2-

glycoprotein (ZAG) and heat-shock proteins are expressed in both human and mouse prostates (Fujimoto *et al.,* 2006).

### 2.1.3 Hormone regulation of the prostate

#### *2.1.3.1 The general mechanisms of sex steroid hormone action*

Streroid hormones, such as androgens, estrogens and progesterone exert their effects in the target cells through binding to specific receptors. Steroid hormone receptors, including AR and estrogen receptors (ERs), are members of the nuclear receptor superfamily and their basic structure is similar (Beato & Klug, 2000). They consist of five distinct functional domains referred to as transactional (A/B) domain, DNA binding (C) domain, a hinge region (D domain), ligand-binding (E) domain and the F domain of unknown function. In the cells, steroid hormone receptors reside in the cytoplasm in an inactive form, bound by a multiprotein inhibitory complex. Binding of the receptor's specific ligand activates the receptors, which can then form dimers, bind to specific DNA sequences and function as transcription factors. New evidence has challenged this concept as AR and ERs can also be activated in a ligand-independent manner by intracellular signaling pathways activated by GFs. In addition, a subpopulation of steroid hormone receptors, which reside in the cytoplasm and plasma membrane can mediate non-genomic actions, for example, by rapid activation of signaling pathways without activating transcription (Beato & Klug, 2000, Prins & Korach, 2008).

#### *2.1.3.2 The role of androgens*

The development, normal growth and maintenance of the prostate in adulthood are dependent on androgens. Androgens regulate the differentiation of secretory cells and the expression of genes encoding for major secretory proteins of prostate (Hayward & Cunha, 2000, Wilson, 2011). Testosterone (T), the main circulating androgene, is produced by the testicular Leydig cells, which are under the control of pituitary gland-synthetized luteinizing hormone (LH). Circulating androgens, in turn, cause a negative feedback on the hypothalamus-pituitary axis, regulating the secretion of GnRH and LH. In the stromal and basal epithelial cells of prostate, T is converted by type 2 5-α-reductase to a more potent androgen, 5-α-dihydrotestosterone (DHT) (Steers, 2001). In contrast to the embryonic prostate, in which AR is expressed almost exclusively in the mesenchymal cells, in the mature prostate, both the epithelial and the stromal cells express AR (Pelletier *et al.,* 2000). This enables both direct and indirect effects of androgens on the mature prostate epithelium (Figure 2). Generally, the effects of androgens in the prostate are pro-proliferative and anti-apoptotic. Androgen deprivation by castration or by other means induces apoptosis and atrophy in the prostate (Wilson, 2011). Although many of the effects of androgens to the epithelium, such as proliferation and cytodifferentiation are mediated by stromally expressed AR, studies reveal that postpubertally, epithelial AR expression regulates the expression of secretory proteins (Donjacour & Cunha, 1993, Prins & Birch, 1995). A recent

knockout (KO) mouse model showed that ARs expressed by luminal epithelial cells control the proliferation of basal cells (Simanainen *et al.,* 2007).

#### *2.1.3.3 The role of estrogens*

In males, estrogens are synthesized by the adrenal cortex. Estrogens can also be converted from T by the aromatase enzyme, which is expressed in the adipose tissue, and in the stromal cells of the prostate (Ellem *et al.,* 2004) (Figure 2). Estrogens can have both direct and indirect effects in the prostate. Direct effects are mediated by the estrogen receptors ERα (ESR1) and ERβ (ESR2), which are expressed in the prostate. Indirectly, estrogens function by causing a negative feedback on the hypothalamus-pituitary gland axis, which leads to suppression of T synthesis. Estrogens can stimulate the release of prolactin (PRL) from the pituitary, which has effects in the prostate (Harkonen & Makela, 2004, Prins & Korach, 2008).

ERα and ERβ are encoded by separate genes (Kuiper *et al.,* 1996, Mosselman *et al.,* 1996, Walter *et al.,* 1985) but their structure is highly homologous. The greatest dissimilarity between the two ERs is in the A/B domain in which they have only 24% homology and this may account for many of the functional differences found between the two receptors, such as the interactions with co-activators and proteins (Prins & Korach, 2008). Although ERα and ERβ bind the endogenous estradiols (estradiol, estrone and estriol) with similar affinities (Kuiper *et al.,* 1997), there are differences in their binding affinities for other endogenous steroids and phytoestrogens (Harris *et al.,* 2003, Kuiper *et al.,* 1998).

In the adult prostate, ERα is expressed mainly in the stromal cells and ERβ mainly in the epithelial cells (Makela *et al.,* 2000, Schulze & Claus, 1990) (Figure 2). There is increasing evidence that activation of ERα and β has opposite effects on the proliferation of prostatic cells. ERα mediates the proliferative and ERβ the anti-proliferative effects of estrogens (Ellem & Risbridger, 2009, Kawashima & Nakatani, 2012, Prins & Korach, 2008). Both ERα and ERβ are expressed in the developing human and rodent prostate epithelium and stroma with the varying site and intensity of the expression during the development (Shapiro *et al.,* 2005). The roles of ERs in the prostate development have been investigated by several KO mouse models. While the initial studies analyzing the conventional ERα KO (ERKO) mice did not find any prostate phenotype (Eddy *et al.,* 1996, Lubahn *et al.,* 1993), more recent studies using the cell type specific ERKO mice revealed that ERα expressed by the prostatic stromal cells is required for normal prostatic branching morphogenesis and for normal proliferation and differentiation of the stromal cells (Chen *et al.,* 2009). The results further suggested that stromally expressed ERα regulates the morphogenesis of the epithelium via paracrine mechanisms through secretion of insulin-like growth factor (IGF1) and FGF10 (Chen *et al.,* 2012b). The reports on the phenotype of ERβ KO (BERKO) prostates are controversial, some supporting a role for ERβ in differentiation of prostate epithelial cells (Imamov *et al.,* 2004) whereas others do not (Antal *et al.,* 2008, Dupont *et al.,* 2000). Exposure to increased levels of natural or synthetic estrogens during *in utero* development or in adulthood induces the development of

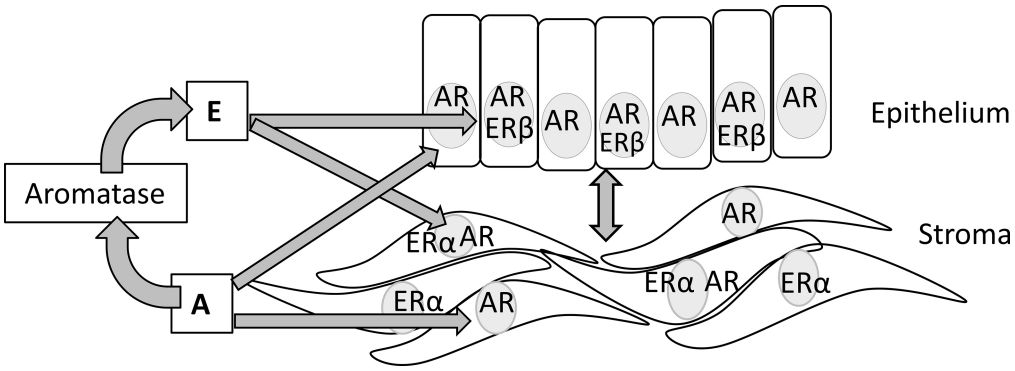


**Figure 2.** The effects of androgens (A) and estrogens (E) in the prostate. A is converted to E by the aromatase enzyme in the prostate stroma. In the adult prostate, E and A can have direct effects in both prostatic epithelium and stroma because their receptors are expressed in both compartments. ERβ is mainly expressed in the epithelium, ERα in the stroma and the effects mediated by the two ERs are different. Interaction between epithelium and stroma enables the paracrine effects of E and A.

squamous metaplasia in human and rodent prostate (Driscoll & Taylor, 1980, Pylkkanen *et al.,* 1993, Sugimura *et al.,* 1988). Estrogen-induced development of prostatic squamous metaplasia is mediated by ERα (Couse & Korach, 2004) expressed in the epithelial cells of the prostate (Chen *et al.,* 2012a).

Animal studies have provided evidence that exposure to estrogens can contribute to aberrant and malignant growth of prostate (Prins & Korach, 2008). Due to decreased levels of serum T, an increased amount of adipose tissue and decreased levels of prostatic DHT there is a significant rise both in the serum E2/T ratio and in the intraprostatic estrogens/androgens ratio in the aging men (Shibata *et al.,* 2000, Vermeulen *et al.,* 2002). This relative rise in the estradiol levels can contribute to development of BPH, inflammation and prostate cancer in aging men.

### 2.1.4 Benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH) is characterized by progressive hyperplasia of both the epithelium and the stroma of the prostate and it can lead to formation of nodules, which can compress the urethra. BPH is usually found in the TZ and in the periurethral area of the prostate. BPH is an extremely common, age-related disease, which can be histologically defined in approximately 20% of 40 year-old, in 70% of 60-year-old and in 90% of 80-year-old men (Kumar *et al.,* 2010, Untergasser *et al.,* 2005). Approximately one fourth of the men, who have histologically defined BPH have clinical symptoms caused by the increased resistance to urinary flow (Kumar *et al.,* 2010). Androgens are required for the development of BPH, but estrogens may also be involved (Kawashima & Nakatani, 2012). However, the molecular mechanisms underlying the development of BPH are complex and still unknown (Schauer & Rowley, 2011, Untergasser *et al.,* 2005). Naturally, BPH occurs in humans, chimpanzees and dogs, but not in rodents, which complicates the experimental modeling the disease (Mahapokai *et al.,* 2000). Presence of reactive stroma and

prostatic inflammation are suggested as etiological factors in BPH, because both are frequently found in BPH prostates. Inflammatory cells and activated myofibroblasts can secrete cytokines and GFs, which could promote the growth of the epithelium and stroma (Schauer & Rowley, 2011, Wang *et al.,* 2008b). BPH can be medically treated by decreasing prostatic smooth muscle tone by α-blockers, by inhibitors of 5-α-reductase and by transurethral resection of the prostate. Several new therapies such as focused ultrasound and laser therapy exist (Kumar *et al.,* 2010).

### 2.1.5 Prostate Cancer

Prostate cancer is the most common malignant disease in men in the Western countries and the second or third leading cause of cancer related deaths in the US and Europe, respectively (Ferlay *et al.,* 2007, Siegel *et al.,* 2012). Typically, prostate cancer is a disease occurring in men over 50 years of age. Based on autopsies, the incidence of histological prostate cancer is high – occurring in 20 percent of men in their 50's and in 40-70 percent of men between 70-80 years of age (Haas *et al.,* 2008, Kumar *et al.,* 2010).

In the prostate cancer*, i.e.* in the adenocarcinoma of the prostate, the epithelial cells of the prostate become malignant. In approximately 70 percent of cases, prostate cancer arises from the PZ. Histologically, prostate cancer is characterized by acini that are smaller and more crowded than in the benign prostate. In contrast to the normal prostate, the basal cell layer is typically absent or discontinous in the cancer. The nuclei of the cancer cells are enlarged and often contain several nucleoli (Kumar *et al.,* 2010).

Localized prostate cancer is usually asymptomatic, more advanced cancer can cause urinary symptoms and back pain if the disease has metastasized to vertebrate. Clinical diagnosis of prostate cancer includes digital rectal examination, transrectal ultrasonography and examination of histopathology from needle biopsies (Damber & Aus, 2008, Kumar *et al.,* 2010). In prostatic diseases, PSA leaks from the prostate to the circulation. Serum PSA levels are used for detection and monitoring of prostate cancer. Measurement of serum PSA levels from middle-aged men is a powerful method for predicting prostate cancer risk, but there is a problem in overdiagnosis, because PSA is not prostate cancer specific (Damber & Aus, 2008, Ulmert *et al.,* 2009). In addition, prostate cancer is a heterogenic disease and some of the cancers are not prone to develop advanced disease but rather remain indolent. Currently, there is no way to predict which of the early stage prostate cancers will progress and which will remain as an indolent disease and this poses a main challenge for clinicians (Damber & Aus, 2008).

Prostate cancer is treated by surgery, external and interstitial radiation therapy and hormonal manipulations. In old men with early stage disease, a strategy of monitoring disease progression called "watchful waiting" is applied. In the localized prostate cancer, the most common treatment is the radical prostatectomy. The prognosis following this surgery depends on the pathologic stage and grade of the disease. Advanced disease is treated by androgen deprivation therapy (ADT)

accomplished by means of orchiectomy or by chemical castration by luteinizing hormone releasing hormone (LHRH) agonists. ADT typically induces disease remission, but after some time the tumors develop resistance to anti-androgens, which will lead to rapid progression of the tumors and incurable form of the disease (Damber & Aus, 2008, Kumar *et al.,* 2010).

#### *2.1.5.1 Aetiology and origin of prostate cancer*

Both genetic and environmental factors contribute to the risk of prostate cancer. The familial history of prostate cancer is the strongest single risk factor of developing prostate cancer (Giovannucci *et al.,* 2007). Men with one first degree relative with prostate cancer have over twice the risk of developing the disease compared to men with no family history (Zeegers *et al.,* 2003). Extensive twin studies show that the heritability, *i.e.* the proportion of cancer risk explained by the genetic factors, of prostate cancer is 42 percent, which is higher than in other common cancers (Lichtenstein *et al.,* 2000). There are some rare inherited mutations that have high influence to prostate cancer risk, such as the mutations in BRCA1, BRCA2 or HOXB13 but most of the genetic variations associated with prostate cancer risk are supposed to have only minor effects on the disease risk (Bambury & Gallagher, 2012). Genome-wide association analysis (GWAS) have found more than 40 single nucleotide polymorphisms (SNPs) associated with prostate cancer risk, which mainly locate in the intergenic or intronic noncoding areas of the genome, suggesting that these areas are involved in the regulation of genes (Nakagawa *et al.,* 2012). However, each of these SNPs has only a modest contribution to the disease susceptibility - the odds ratios (ORs) of specific SNPs vary between 1.02-1.86. Especially the genomic area of 8q24 is of interest, because it contains several SNPs associated with prostate cancer susceptibility. However, no genes have been defined in this area and the biological significance remains to be elucidated (Nakagawa *et al.,* 2012).

The incidence of prostate cancer is elevated markedly in the countries with a high standard of living compared to countries with low standards of living**.** In contrast, the mortality rates due to prostate cancer are higher in the less developed regions of the world (Center *et al.,* 2012). The contribution of ethnic origin and nationality to prostate cancer risk is well known, but the exact mechanisms behind the differential risk are not known. The incidence of prostate cancer is highest in African Americans, intermediate in the Caucasians and lowest in the Asian men (Danley *et al.,* 1995). This suggests a major contribution of genes to disease susceptibility. High prostate cancer incidence among African Americans compared to low incidence in native Africans may be the result of admixturing genes of African Americans with Caucasians of European descent during the centuries (Gunderson *et al.,* 2011). However, the fact that the cancer risk of the immigrants rises after migration from low-incidence region to high incidence region (Cook *et al.,* 1999) supports the importance of environmental factors, such as dietary factors, in prostate cancer risk. High intake of fats, meat, dairy products and calcium seem to raise prostate cancer risk, whereas diets rich in vegetables, phytoestrogens and lycopene seem to have protective effects. Protective roles for vitamin A, C, D and E and for selenium are also implicated. However, despite several

studies, none of the dietary factors has convincingly proven to be causative or preventive in prostate cancer (Schultz *et al.,* 2011).

Prostate cancer, like other cancers, is the result of a critical combination of several somatic mutations and epigenetic changes which develop slowly. There is convincing experimental evidence suggesting a subpopulation of basal cells as the origin of tumor initiating cancer stem cells (CSC) in prostate cancer (Goldstein *et al.,* 2010, Maitland *et al.,* 2011, Oldridge *et al.,* 2012). However, the cell type origin of prostate cancer still remains controversial, since studies on mouse prostate support CSCs to reside among the luminal epithelial cells (Wang *et al.,* 2009). Therefore, there is currently no consensus to claim a single cell type to be the origin of prostate cancer (De Marzo *et al.,* 2010, Oldridge *et al.,* 2012).

#### *2.1.5.2 Progression of prostate cancer*

A presumable premalignant change preceding prostate cancer is the prostatic intraepithelial neoplasia (PIN) (Bostwick & Qian, 2004, McNeal & Bostwick, 1986). Cytologically, the changes in PIN resemble those seen in the prostate cancer, but in PIN the glands are typically larger and contain papillary infoldings. Histologically, PIN can be divided to low and high grade PIN (LGPIN and HGPIN). Despite the lack of direct evidence for development of prostate cancer from PIN, the spatial and temporal association of PIN lesions with prostate cancer supports this association (Bostwick & Qian, 2004). Furthermore, many of the molecular and genetic changes found in prostate cancers are also found in HGPIN but not in normal prostate (Sakr & Partin, 2001). For example, the TMPRSS-ETS fusions including the TMPRSS2-ERG fusion, which result from chromosomal translocations and are frequently found in aggressive clinical prostate cancers (Tomlins *et al.,* 2005) are found also in 20 percent of PIN lesions (Cerveira *et al.,* 2006). Another lesion suggested to be a precursor of prostate cancer is proliferative inflammatory atrophy (PIA) (De Marzo *et al.,* 1999) but the evidence of its association with prostate cancer is insufficient (Woenckhaus & Fenic, 2008).

In the early stage of the prostate cancer, the disease is localized and malignant cells are found only in the prostate. More advanced prostate cancer can invade to contiguous organs, such as the seminal vesicle, bladder neck and rectum or metastasize to lymph nodes and bones (Kumar *et al.,* 2010). Evaluation of cancer grade and stage are important in planning the treatment and giving the prognosis. The Gleason system, which is based on the evaluation of glandular differentiation status of the tumor, is commonly used for grading of prostate cancer (Gleason & Mellinger, 1974). In addition, the stage of the cancer is evaluated using the TNM system that is based on the extent of the primary tumor and the site of the metastasis (Kumar *et al.,* 2010).

#### *2.1.5.3 Hormone regulation of prostate cancer*

Androgens and activation of AR are required in the development and progression of prostate cancer (Huggins & Hodges, 2002, Isaacs, 1994). In the adult prostate, ARs are expressed both in the luminal epithelial cells and the stroma (Pelletier *et al.,* 2000). In

the early stage of prostate cancer, androgens promote the growth and survival of the cancer cells and tumors respond to androgen deprivation therapy (ADT). In the advanced prostate cancer, the cells become resistant to anti-androgens and grow independently of ADT (Rove *et al.,* 2012). This stage is called hormone refractory or castrate resistant prostate cancer (HRPC, CRPC, respectively).

Despite the development of hormone therapy resistance, prostate cancer cells continue to express AR and the expression of AR is increased in HRPC compared to nontreated prostate cancers (Linja *et al.,* 2001, Trapman & Brinkmann, 1996). In about 30 percent of the patients, the increased expression of AR is a result of amplification of the AR gene, which results in increased sensitivity to androgens (Linja & Visakorpi, 2004). Other somatic mutations found in the AR gene may modify the ligand-dependency of AR, such that factors other than androgens can activate the receptor (Linja *et al.,* 2001). ADT selects for the AR amplification and development of androgen hypersensitivity (Palmberg *et al.,* 2000). HRPC tumors can develop other compensatory mechanisms to adapt to ADT, such as increased intratumoral androgen synthesis (Locke *et al.,* 2008, Stanbrough *et al.,* 2006). It is notable that if the prostate cancer stem cells (CSCs) reside in the basal compartment and are AR-negative, the ADT does not target CSC but actually provides their selection and thus enables the tumor to grow again (Oldridge *et al.,* 2012).

Besides the central role of androgens and AR signaling in prostate cancer, estrogens have a significant contribution to prostate tumorigenesis (Bonkhoff & Berges, 2009, Ellem & Risbridger, 2009, Harkonen & Makela, 2004, Hartman *et al.,* 2012, Kawashima & Nakatani, 2012, Prins & Korach, 2008). In mice, neonatal exposure to excessive estrogens causes "imprinting" of the prostatic tissue and leads to increased incidence of prostatic hyperplasia and PIN in the adulthood (Pylkkanen *et al.,* 1993). Similar effect of neonatal exposure to maternal diethylstilbestrol (DES), a synthetic form of estrogen, has been suggested in humans. Furthermore, in adult rodents, development of PIN and prostatic adenocarcinoma can be induced by long term treatment with estradiol (E2) and T (Noble, 1977, Ricke *et al.,* 2008). Studies with GEM models have provided evidence that, in general, ERα is responsible for the pro-proliferative and tumorigenic effects of estrogens, whereas ERβ mediates the beneficial anti-proliferative and anti-inflammatory effects of estrogen in the prostate (Ellem & Risbridger, 2009, Hartman *et al.,* 2012, Kawashima & Nakatani, 2012). For example, prostatic dysplasia induced by neonatal estrogen exposure is mediated by ERα (Prins *et al.,* 2001). Moreover, treatment with E2 and T induces the development of PIN in BERKO mice in the same way as in the WT mice, but not in ERKO mice (Ricke *et al.,* 2008). However, the results on the importance of ERβ as a tumor suppressor are conflicting, because some studies found that BERKO prostates were hyperplastic and had defects in the epithelial cell differentiation (Imamov *et al.,* 2004, Weihua *et al.,* 2001) but other studies did not uncover any phenotype in the BERKO prostates (Antal *et al.,* 2008, Dupont *et al.,* 2000).

In line with suggested tumor-promoting functions of ERα, Bonkhoff *et al.* (1999) showed that the expression of ERα is gradually increased during human prostate cancer progression. However, opposing results on ERα expression in human

prostate tumorigenesis also exist (Li *et al.,* 2000, Leav *et al.,* 2001). In support of the tumor suppressive role of ERβ, it is expressed in high percentage of normal prostate luminal epithelial cells, but the expression is decreased in the hyperplastic foci, PIN and in carcinomas (Fixemer *et al.,* 2003, Horvath *et al.,* 2001, Leav *et al.,* 2001, Muthusamy *et al.,* 2011, Pasquali *et al.,* 2001) However, results are conflicting as some studies have found that increased expression of ERβ occurs during prostate cancer progression and in metastasis (Fixemer *et al.,* 2003, Horvath *et al.,* 2001, Leav *et al.,* 2001, Torlakovic *et al.,* 2002, Walton *et al.,* 2009). Some of the discrepancy in the results of the different studies may be explained by the inability of some of the techniques that are used to distinguish between the isoforms of the human ERβ, especially hEβ1, hERβ2 and hERβ5 (Moore *et al.,* 1998). A recent study showed that, in contrast to tumor- and metastasis suppressive properties of hERβ1 (Mak *et al.,* 2010), hERβ2 and hERβ5 can promote prostate cancer cell migration and invasion and associate with poor prognosis (Leung *et al.,* 2010). In line with this, hERβ1 and hERβ2 have opposite effects on the regulation of genes associated with prostate cancer cell proliferation and metastasis (Dey *et al.,* 2012). Interestingly, the TMPRSS2-ERG-fusion gene frequently found in prostate cancer is estrogen regulated and transcriptionally induced by ERα agonist and suppressed by ERβ agonist (Setlur *et al.,* 2008).

#### *2.1.5.4 Growth factor regulation of prostate cancer*

The growth of the epithelium-derived malignant cells is likely to be stimulated by stromal derived paracrine GFs, such as transforming growth factor *beta* (TGFβ), platelet derived growth factor (PDGF), FGF, epidermal growth factor (EGF), hepatocyte growth factor (HGF) and IGF1 (Berry *et al.,* 2008). In the prostate cancer, the emergence of androgen independent stage and progression of the disease is associated with the increased or dysregulated expression of GFs and their receptors (Rau *et al.,* 2005, Reddy *et al.,* 2006, Reynolds & Kyprianou, 2006). One method to escape the normal growth control of the stroma is to sustain self-sufficiency of GFs by increased expression of GFs or GFRs by cancer cells. In the absence of androgen, AR can be activated by GF pathways. Conversely, ARs can activate several GFs, and there is a complex interplay between GF and AR signaling pathways that is deregulated in prostate cancer encouraging the survival and invasion of prostate cancer cells (Berry *et al.,* 2008, Zhu & Kyprianou, 2008).

Like androgens, estrogens can regulate the expression of GFs and other cancer progression promoting factors in the stroma and affect prostate tumorigenesis in a paracrine manner. For example, induction of ERα induces the expression of TGFβ and matrix metalloproteinase 2 (MMP2) in the prostate stroma (Yu *et al.,* 2011a). There is evidence for GF regulation by estrogens during prostate development and ERα can downregulate the expression of FGF10 in the developing prostate mesenchyme (Huang *et al.,* 2005). Ligand independent activation of ERs by GF signaling pathways have been described in breast cancer (Coutts & Murphy, 1998) and they may be important also in HRPC.

#### *2.1.5.5 Role of stroma in prostate cancer*

Although prostate adenocarcinoma is a malignancy of the epithelium-derived cells, the importance of the stromal microenvironment in tumorigenesis and tumor progression in prostate cancer and in other types of cancers has been recognized increasingly during the last decade (Barron & Rowley, 2012, Chung *et al.,* 2005, Cunha *et al.,* 2003, Hanahan & Weinberg, 2011, Niu & Xia, 2009). The cellular composition of the stroma present in prostate cancer is different from the normal fibromuscular stroma of prostate and is referred to as the "reactive stroma." The reactive stroma resembles wound repair stroma and is characterized by the presence of activated fibroblasts called myofibroblasts or cancer associated fibroblasts (CAFs), which express increased amounts of extracellular matrix (ECM) components such as collagen, tenascin and proteases (Barron & Rowley, 2012, Tuxhorn *et al.,* 2002a). These cells replace the smooth muscle cells abundant in the normal prostate stroma. There is also increased number of inflammatory cells and capillaries in the tumor-surrounding stroma, which can promote development and progression of prostate cancer (De Marzo *et al.,* 2007). The cells of the reactive stroma secrete GFs and cytokines, which promote cancer cell survival and induce angiogenesis and neurogenesis such as TGFβ, FGFs, and connective tissue growth factor (CTGF) (Dakhova *et al.,* 2009, Tuxhorn *et al.,* 2002b, Yang *et al.,* 2005, Yang *et al.,* 2008). Tissue recombinant experiments show that CAFs are able to induce malignant transformation of epithelial cells, derived from BPH (Hayward *et al.,* 2001). Cancer cells, in turn, secrete factors that modulate the reactive stroma. Especially, TGFβ signaling has a central role in the regulation of reactive stroma (Barron *et al.,* 2010, Franco *et al.,* 2011, Gerdes *et al.,* 2004, Tuxhorn *et al.,* 2002a, Tuxhorn *et al.,* 2002c) even though the general role of TGFβ in cancer is complicated because it has both cancer suppressive and promoting functions (Bierie & Moses, 2006).

The formation of reactive stroma is an early event in prostate tumorigenesis, which initiates already during PIN formation (Tuxhorn *et al.,* 2001, Tuxhorn *et al.,* 2002a). Changes in the epithelial cell integrity in PIN but also in other prostatic diseases can induce the formation of the reactive stroma (Barron & Rowley, 2012). The reactive stroma co-evolves with neoplastic epithelium during tumor progression. Despite the usual genetic stability of the normal stroma, genetic and epigenetic changes are present in the cells of reactive stroma (Hanson *et al.,* 2006, Macintosh *et al.,* 1998, Rodriguez-Canales *et al.,* 2007). The origin of CAFs in the reactive stroma remains unknown but tissue resident cell types, such as fibroblasts, vasculature-derived pericytes and smooth muscle cells, and cells of bone marrow-origin are possible candidates (Barron & Rowley, 2012). The role of AR in the regulation of reactive stroma is not fully understood. There is evidence from recent studies that AR, expressed in portion of fibroblasts, can stimulate the expression of stromal GFs which, in turn, stimulate the proliferation of the prostate cancer cells (Tanner *et al.,* 2011). The function of AR is required for myodifferention of prostate fibroblasts (Gerdes *et al.,* 2004). Because of their potential importance already in the early steps of the tumorigenesis but also in the development of metastasis (Li *et al.,* 2012), the reactive stroma and the epithelium-stromal interactions have become important targets for

developing therapies for prostate cancer (Chung *et al.,* 2003, Chung *et al.,* 2005, Karlou *et al.,* 2010). Figure 3 presents a summary of the interactions between epithelium and stroma in the normal and diseased prostate.

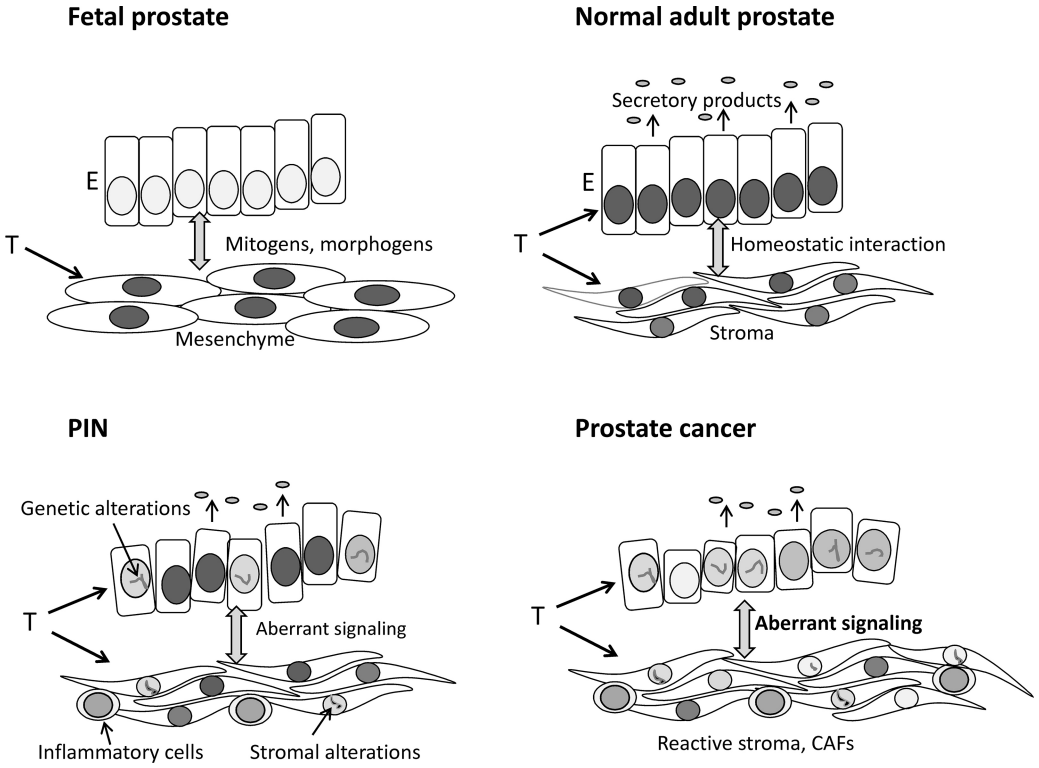


**Figure 3.** Interactions between epithelium (E) and mesenchyme/stroma in the developing prostate, adult prostate and during prostate tumorigenesis. During development (top left), ARs are expressed in the mesenchyme and activated by low levels of androgens (T), which induce epithelial proliferation and differentiation in a paracrine way. Reciprocally, factors secreted by the epithelium induce the differentiation of the mesenchyme to smooth muscle. In the adult prostate (top right), high levels of T act through ARs expressed in both compartments. Paracrine two-directional signaling between the compartments is important in maintaining homeostasis. In the premalignant changes in the prostate (PIN) (bottom left), genetic alterations in the epithelium cause changes in the signaling between the compartments and induce the development stromal alterations leading to formation of reactive stroma. In prostate cancer (bottom right), altered signaling leads to a vicious cycle, which drives progressive dedifferentiation and proliferation (Modified from Cunha *et al.,* 2003 and Barron *et al.,* 2012).

## 2.2 THE EPIDIDYMIS

### 2.2.1 Structure and development of the epididymis

The epididymis is a tubular organ, which is located adjacent to the testis and connects the efferent ducts (EDs) to the vas deferens. The epidimis consists of a single highly convoluted duct (Belleannee *et al.,* 2012, Cornwall, 2009). The epididymis is divided into three anatomical regions, which are from the proximal to distal end of the organ: caput; corpus; and cauda (Figure 4). Due to its distinctive histology and gene expression pattern, the most proximal part of the caput is considered as a separate region in many species, referred to as initial segment (IS). Unlike rodents, the human epididymis does not contain IS. The epididymal regions are further divided to altogether 10 separate lobes lined by connective tissue septae (Belleannee *et al.,* 2012, Cornwall, 2009).

Throughout its whole length, the epididymal duct is lined by pseudostratified epithelium. The diameter of the duct and the luminar space increases and the epithelial cell height reduces from proximal to distal end. The epididymal epithelium is surrounded by the stroma/interstitium composed of smooth muscle and fibroblastic cells and blood vessels. Several types of cells with specific functions exist in the epididymal epithelium. Secretion of specific proteins and absorption of fluid and molecules by the epithelium are required to generate a favorable environment for sperm maturation in the epididymal lumen. The most common cell type of the epithelium are the principal cells, which have stereocilia on their apical surface and are capable of absorbing and secreting fluid and proteins. The basal cells, which reside in the base of the epithelium, adjacent to the basement membrane, are the second most abundant cell type. Apical and narrow cells are both present mainly in the apical border

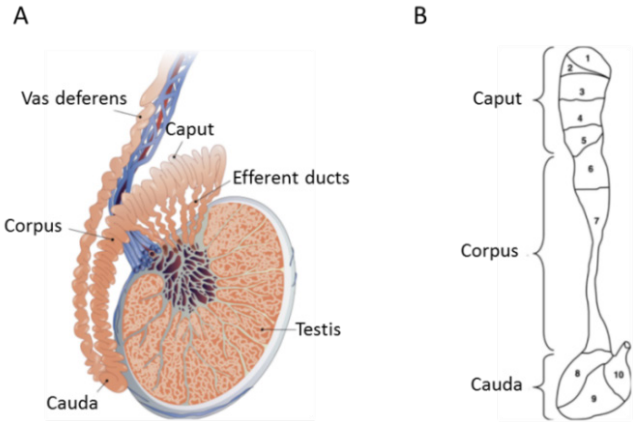


**Figure 4.** The anatomy of the epididymis. Structure and position of human epididymis, which is composed of three regions: caput; corpus; and cauda (A). The segmental structure of mouse epididymis (B). Segment 1 is also known as the initial segment (Modified from Belleannee *et al.,* 2012 and Johnston *et al* 2005).

of the IS. They have both specific functions in the endocytosis, a process in which molecules and fluid are actively taken inside the cell. Clear cells are found in other epididymal regions, except the IS, and they function in the endocytosis of specific proteins and regulation of luminal fluid pH. Halo cells, sparcely located along the whole epididymal epithelium, are lymphocytes and monocytes responsible for the immune defense of the epididymis (Belleannee *et al.,* 2012, Cornwall, 2009).

In contrast to the prostate, the epididymis, along with most parts of the urogenital organs, is of mesodermal origin and develops from the Wolffian duct (Joseph *et al.,* 2009). In mice, the intermediate mesoderm gives rise to mesonephric tubules and the Wolffian duct at the embryonic day 10 (E10). Most of the epididymis and the vas deferens derive from the cranial pole of the Wolffian duct, whereas the efferent ducts (EDs) and the IS develop from the mesonephric tubules. The common mesonephric origin of the kidney and the epididymis probably explains certain similarities between these organs such as the presence of ionic gradients. During later embryonic development, T production induces the epididymal duct to become convoluted and elongated (Joseph *et al.,* 2009). Epithelial-mesenchymal interactions are important in the development of epididymis. Mesenchymal factors such as inhibin A are required for proper coiling of the duct (Tomaszewski *et al.,* 2007). After birth, the epididymal cells proliferate at a slow rate until the beginning of the puberty. Terminal differentiation of the epididymal cell types occurs during puberty and it is influenced by T and appearance of spermatozoa (Kirchhoff, 1999).

### 2.2.2 Function of the epididymis

In addition to serving as a transport route for the spermatozoa, the absorption of fluid, supporting sperm maturation and acting as a storage location for sperm are the main functions for the epididymis (Cornwall, 2009). The epididymis is characterized by segment specific functions and gene expression profiles. Most of the fluid entering from the rete testis is absorbed in the efferent ducts and in the IS, which results in the concentration of the luminal compounds and spermatozoa (Mann et al 1981). The proximal epididymis (caput and IS) is, metabolically, the most active region of the epididymis and is responsible for most of the protein secretion (Cornwall, 2009, Orgebin-Crist, 1998). The capacity of spermatozoa to fertilize the egg increases upon their transit through the epididymis. Especially the proximal part of the epididymis plays an important role in sperm maturation (Jones, 1999). Spermatozoa undergo several morphological, biochemical and physiological changes during their transit through the epididymis that associate with their maturation (Hinrichsen & Blaquier, 1980, Yeung *et al.,* 1997). The maturation of the spermatozoa in the epididymis is caused by the interactions with the luminal proteins secreted by the principal cells and by the changes in the pH and ion concentration along the duct. However, the exact molecular and biochemical mechanism responsible for sperm maturation are not known (Cornwall, 2009). The cauda epididymis is less active in the protein secretion, but it serves as storage of sperm. In the cauda, the mature spermatozoa can be stored in a functional but metabolically quiescent stage for several weeks (Acott & Carr, 1984, Jones, 1999). The low intraluminar pH in the cauda enables the storage in an immotile

stage (Acott & Carr, 1984). In addition to serving as a maturation and reservoir place for the spermatozoa, protection of spermatozoa from pathogens and from the "host" immune system are also important functions of the epididymis (Cornwall, 2009).

### 2.2.3 Epididymal diseases

The most common disease in the epididymis is inflammation *i.e.* epididymitis. It is commonly caused by sexually transmitted pathogens such as *Neisseria gonorrhoeae* and *Chlamydia trachomatis* or by enterobacteria such as *Esterichia coli.* Chronic epididymitis can lead to reduced fertility or infertility (Haidl *et al.,* 2008). Interestingly, tumors and adenocarcinomas originating from the epididymis are extremely uncommon (Ganem *et al.,* 1998). In line with this, the epithelial cells of the epididymis seem to be highly resistant to tumorigenesis also in the transgenic (TG) mouse models. The mechanisms behind the epididymal tumor-resistance remain to be elucidated (Yeung *et al.,* 2012).

### 2.2.4 Hormone and growth factor regulation of epididymis

Androgens, especially DHT, are the main hormonal regulators of the epididymal function. Androgens enter the epididymis both via the circulation and via the rete testis fluid and in which their concentration is high (Belleannee *et al.,* 2012). ARs are expressed throughout the whole length of the epididymal epithelium (Zhou *et al.,* 2001). Castrations and efferent duct ligation (EDL) experiments in rodents demonstrate that especially the proximal part of the epididymis *i.e.* the initial segment, is highly dependent on the androgens and other factors in the rete testis fluid (Fan & Robaire, 1998). Many of the genes expressed in the epididymis are androgen regulated and results from microarray analysis show that the highest number of androgen regulated genes is found in the caput epididymis (Chauvin & Griswold, 2004, Sipila *et al.,* 2006). Interestingly, the disruption of AR expression in the caput epididymis in site specific KO (ARKO) mice lead to epididymal obstruction and infertility (Krutskikh *et al.,* 2011, O'Hara *et al.,* 2011). In addition to androgens, estrogens and retinoids are required for the normal function of the epididymis. There is a high concentration of estrogens in the caput epididymal fluid and ERα and ERβ are expressed in the epididymis (Zhou *et al.,* 2002). The importance of the estrogen signaling for male fertility is highlighted by the phenotype of ERKO male mice. In these mice, the loss of ERα function leads to impaired fluid absorption in the IS and EDs resulting in swollen EDs, dilated rete testis and infertility (Zhou *et al.,* 2002).

As androgen treatment is not sufficient to rescue epithelial cells from apoptosis triggered by the EDL in the IS of epididymis, rete testis fluid contains other testis-derived lumicrine factors, which are required for the maintenance of epithelial cells in the IS. Expression of several genes in the IS are regulated by such lumicrine factors, but these factors remain unknown. However, there is evidence that members of the FGF family (discussed in detail in chapter 2.3.4.2 of this thesis) or spermatozoa-derived factors could serve as lumicrine regulators of the epididymis (Belleannee *et al.,* 2012).

## 2.3 FIBROBLAST GROWTH FACTORS (FGFS)

Mammalian FGFs constitute a large family of polypeptide GFs, which have diverse biological functions in embryonic development and in adult tissue homeostasis and wound repair. FGFs are nominated as FGF1-FGF23, but as mouse FGF15 and human FGF19 are orthologues, the total number of FGFs is 22 in both species (Beenken & Mohammadi, 2009, Itoh & Ornitz, 2011, Ornitz & Itoh, 2001). FGF11-FGF14 are currently rather referred to as FGF-homologous factors (FHFs) than true members of the FGF family, because despite their structural homology to FGFs, they do not bind to or activate FGF receptors (FGFRs) (Olsen *et al.,* 2003). Therefore, the actual number of mammalian FGFs can be considered 18.

The first FGFs, FGF1 and FGF2, were purified in the 1970's from bovine pituitary gland and brain as mitogenic factors for cultured fibroblasts (Gospodarowicz *et al.,* 1975, Gospodarowicz *et al.,* 1978). FGFs exist in multicellular animals but not in unicellular organisms (Itoh & Ornitz, 2011).

### 2.3.1 Structure and function of FGFs

Vertebrate FGFs vary in size from 17 to 34 kDa. All FGFs and FHFs have a homologous core region showing 16-65 percent sequence identity. This core region consists of 120-130 amino acids ordered into 12 β-strands. Specific areas in the core region are responsible for binding to FGFRs and heparan sulfate glycoaminoglygans (HSGAG). The core region is flanked by divergent amino- and carboxytermini, that account for specific biological properties of the different FGFs (Beenken & Mohammadi, 2009, Eswarakumar *et al.,* 2005, Ornitz & Itoh, 2001).

According to the genome sequence analyses, the FGF gene family has expanded by large-scale genome duplications in two phases during the evolution. The first phase of these duplications took place during early metazoan evolution and resulted in the generation of ancestor genes of the seven FGF subfamilies (FGF13-like, FGF4-like, FGF5-like, FGF-8-like, FGF9-like, FGF10-like and FGF15/19-like). Another large-scale genome duplication event took place in the early evolution of the vertebrates and expanded each of the FGF subfamilies to contain 3-4 members (Itoh & Ornitz, 2008, Itoh & Ornitz, 2011).

Based on their mechanisms of action, FGFs can be classified to paracrine, endocrine and intracrine factors (Itoh & Ornitz, 2011). Most FGFs (FGF1-10, FGF16-18, FGF20 and FGF22) are paracrine GFs that are secreted from the cell and mediate their effects through binding FGFRs on the surface of neighboring cells. Most paracrine FGFs contain an N-terminal signaling peptide that is cleaved as the protein is secreted. Unlike others, FGF9,-16 and -20 have uncleavable bipartite hydrophobic sequences required for their secretion. Moreover, FGF1 and FGF2 lack the signal peptide and are not secreted by the classical endoplasmic reticulum-Golgi pathway (Beenken & Mohammadi, 2009, Itoh & Ornitz, 2011). They can, however, be secreted by a non-vesicular unconventional mechanism (Mignatti *et al.,* 1992, Mohan *et al.,* 2010, Nickel, 2011) or be released from damaged cells. Although classified as

paracrine FGFs, there is evidence showing that FGF1-3 can be directly translocated to nucleus and act also as intracrine factors (Antoine *et al.,* 1997, Chlebova *et al.,* 2009). Interestingly, recent studies provide evidence that FGF10 and FGF8 can be internalized by the cell and translocated to nuclei (Kosman *et al.,* 2007, Suzuki *et al.,* 2012). All paracrine acting FGFs have a heparin binding site and after secretion, they readily bind to heparin sulphate proteoglycans (HSPGs) that are present in the ECM and on the cell surface. HSPGs can serve as an extracellular storage reserve of FGFs, simultaneously limiting their diffusion further. Binding to heparin or heparan sulphate coactivator are required for the formation of stable interaction with FGFR (Schlessinger *et al.,* 2000). Paracrine FGFs act as differentiation and growth promoting factors in embryonic development. KO mice for different paracrine FGFs display a variety of developmental defects, phenotypes varying from early embryonic lethal to wide range of malformations of many organ systems reflecting the diverse functions of FGFs. Some FGF KO mice represent normal (FGF1 KO) or mild phenotypes such as abnormally long hair (FGF5 KO) (Beenken & Mohammadi, 2009, Itoh & Ornitz, 2011).

Endocrine or hormone-like FGFs (FGF15/19,- 21 and -23) function through binding to FGFRs. Interaction of endocrine FGFs with the FGFRs requires transmembrane proteins called α- and β-Klotho (Itoh, 2010). Endocrine FGFs contain a signal sequence but their heparin binding site is structurally different from that of paracrine acting FGFs. This results in lower heparin binding affinity, which enables them to function in an endocrine manner throughout the body. Endocrine FGFs regulate bile acid, lipid, phosphate and vitamin D metabolisms. KO mice for endocrine FGFs show phenotypes with impaired lipid, glucose, bile acid or vitamin D metabolism (Beenken & Mohammadi, 2009, Itoh, 2010).

Intracrine FGFs (FGF11-14, FHFs) lack the signal peptide required for secretion and function as intracellular molecules independently of FGFRs. Intracrine FGFs regulate the electrical excitability of neurons and other cell types (Goldfarb, 2005). Evolutionarily, the intracrine FGFs (FHFs) present probable ancestors of the FGF family (Itoh & Ornitz, 2008, Itoh & Ornitz, 2011).

### 2.3.2 FGF receptors (FGFRs)

All FGFs (except intracrine FGFs) mediate their cellular responses by binding to and activating FGFRs, designated FGFR1-FGFR4. HSPGs binding to both FGF and FGFR stabilize this mutual complex (Beenken & Mohammadi, 2009). A fifth related receptor, FGFR5 (FGFRL1) with a FGF-binding ability exists, but it does not contain a tyrosine kinase domain and may act as a negative regulator of FGF signaling (Wiedemann & Trueb, 2000).

FGFR1-4s are receptor tyrosine kinases (RTKs) composed of an extracellular ligand-binding domain, a transmembrane domain and a cytoplasmic domain containing the tyrosine kinase domain and regulatory sequences. The extracellular domain of FGFRs is responsible for ligand binding and consists of three immunoglobulin (Ig) like domains, designated D1-D3. In the linker region between D1 and D2, there is a stretch

of seven to eight acidic residues designated the "acid box." In the D2 domain, there is a conserved positively charged region that functions as a binding site for heparin. D2 and D3 domains are responsible for ligand binding, whereas D1 and acidic box have autoinhibitory functions (Eswarakumar *et al.,* 2005).

Binding of FGF and HSGAG to FGFR results in formation of a symmetrical dimer containing two FGF-FGFR-HSPG–complexes (Schlessinger *et al.,* 2000), which induces transphosphorylation of tyrosine residues in the intracellular domain of the receptor. Subsequently, the phosphorylated tyrosines of FGFR are bound by intracellular signal transduction molecules - FRS2, PLCγ and SRC family members (Figure 5). The docking protein FRS2α, which is phosphorylated following FGFR activation, is a key component in the FGF signaling. Its phosphorylation leads to activation of both the RAS-MAPK-pathway and PI-3K-pathway. Activation of FGFRs also induces hydrolysis of phosphatidylinositol (PI), release of intracellular calcium and activation of PLCγ-pathway. As the end-point, the activated signaling pathways regulate the expression of target genes and modulate cellular functions such as proliferation and differentiation (RAS-MAPK-pathway), cell survival (PI3K-pathway) and cell morphology/migration (PLCγ-pathway) (Dailey *et al.,* 2005, Dorey & Amaya, 2010).

One characteristic of FGFRs is that several receptor isoforms are generated by alternative splicing of FGFR transcripts. Functionally, the most important alternative splicing site exists in the third immunoglobulin like domain (D3) of the FGFRs1-3, in which alternative splicing of exons 8 and 9 creates the so-called "IIIb- and IIIc-isoforms" (also called b- and c-isoforms, respectively), that differ in their ligand binding specificities (Table 1). Similar alternative splicing does not exist in FGFR4, making the number of FGFRs with distinct ligand binding specificities seven (Eswarakumar *et al.,* 2005). Generally, the IIIb-isoforms are expressed in the epithelial and the IIIc-isoforms in the mesenchymal/stromal compartments of the organs (Orr-Urtreger *et al.,* 1993). Moreover, distinct FGFs can usually activate either the IIIb or the IIIc-isoforms of the receptors (Zhang *et al.,* 2006) (Table 1). Usually the epithelially secreted FGFs activate the mesenchymally/stromally expressed receptors and vice versa, making the directional signaling from epithelium to mesenchyme and from mesenchyme to epithelium possible. However, FGF1 makes an exception because it can activate both the IIIb and the IIIc-isoforms of all FGFRs and it is therefore called "the universal FGF ligand." Several FGF ligands can bind and activate the same FGFRs, meaning that there is a lot of redundancy in the FGF-system (Eswarakumar *et al.,* 2005).

The importance and the distinct biological roles of different FGFRs and their isoforms during the development are represented by variable phenotypes of FGFR KO mice (Coumoul & Deng, 2003, Eswarakumar *et al.,* 2005). For example, the FGFR1 and FGFR1IIIc KO mice are both embryonic lethal due to gastrulation defect, whereas the FGFR1IIIb KO mouse appears phenotypically normal. In humans, mutations in FGFRs cause multiple forms of skeletal dwarfisms and craniosynostosis syndromes (Marie *et al.,* 2005). On the other hand, dysregulated FGFR signaling is involved in several types of cancers (Turner & Grose, 2010).

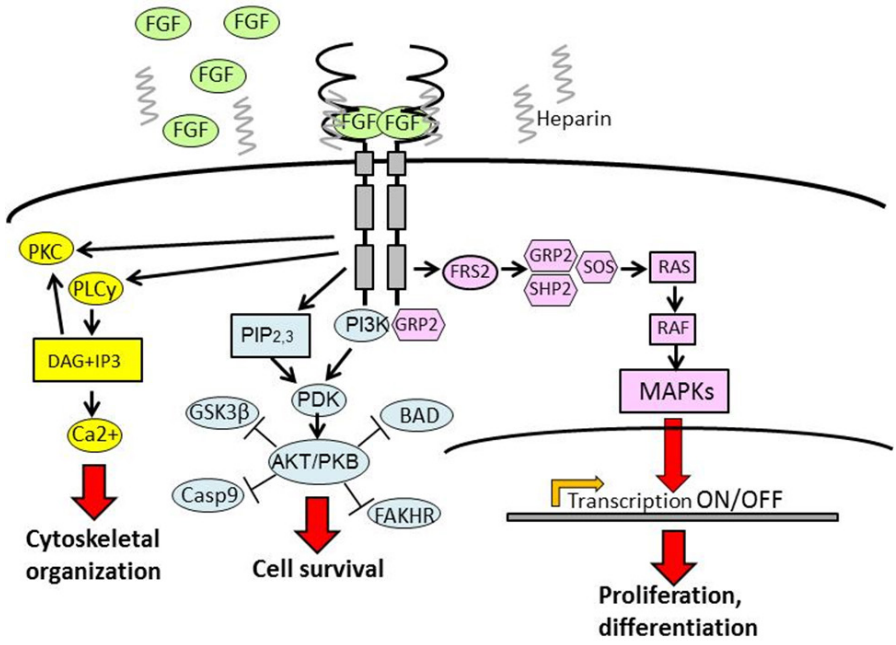


**Figure 5.** Simplified model of transduction pathways and cellular responses activated by FGFRs. (Modified from Dailey *et al.*, 2005 and Acevedo *et al.*, 2009).

**Table 1.** Binding specificities of FGFs to FGFRs. FGF11 subfamily (FGF11,12,13,14) is excluded because they do not activate FGFRs. (Modified from Ornitz *et al.,* 1996 and Zhang *et al.,* 2006).

| **FGF subfamily** | **Members** | **FGFRs activated** |
|---|---|---|
| FGF1 subfamily | FGF1<br>FGF2 | FGFR 1b, 1c, 2b, 2c, 3b, 3c, 4<br>FGFR 1c, 3c, > 2c, 1b, 4 |
| FGF4 subfamily | FGF4, FGF5, FGF6 | FGFR 1c, 2c > 3c, 4 |
| FGF7 subfamily | FGF3, FGF7, FGF10, FGF22 | FGFR 2b > 1b |
| FGF8 subfamily | FGF8, FGF17, FGF18 | FGFR 3c > 4 > 2c > 1c >>3b |
| FGF9 subfamily | FGF19, FGF21,FGF23 | FGFR 3c >2c >1c > 3b >> 4 |
| FGF19 subfamily | FGF19, FGF21, FGF23 | FGFR 1c, 2c, 3c, 4 (weak activity) |

### 2.3.3 Biological response and regulation of FGF signaling

In most cell types, activation of FGFRs results in increased proliferation and migration. However, in some cell types, such as chondrocytes, FGFs can induce cell cycle arrest and promote differentiation (Dailey *et al.,* 2003). However, FGF-signaling inhibits differentiation of other cell types, such as osteoblasts (Mansukhani *et al.,* 2000). Response to FGF signaling can also vary in the same cell type depending on the differentiation stage. For example, FGF signaling induces proliferation of immature osteoblasts, but increases apoptosis in differentiated osteoblasts (Mansukhani *et al.,* 2000) and chondrocytes (Sahni *et al.,* 2001). Taken together, the cellular responses to FGF signaling can vary enormously depending on the cell type and differentiation stage (Dailey *et al.,* 2005).

The present data suggest that any FGF ligand binding to an identical FGFR will produce a similar biological response. Although activation of different FGFRs triggers the same signaling pathways, the amplitude of the signal is dependent on the receptor type (Dailey *et al.,* 2005). Experiments show that the mitogenic signal in response to identical ligands is higher in cells expressing FGFR1 or -2 compared to cells expressing FGFR3 or -4 (Ornitz *et al.,* 1996). These differences in the responses among FGFRs are due to the differences in the activity of their intracellular tyrosine kinase domains (Lin *et al.,* 1998, Ornitz *et al.,* 1996, Raffioni *et al.,* 1999). However, the variable cellular and biological responses triggered by FGFs cannot only be explained by straightforward receptor or signal transduction pathway activation. The cellular context dependent interplay among different signaling networks is regulated by multiple factors such as the presence or abundance of specific signal tranducing molecules, negative and positive regulators, transcription factors and these finally determine the biological response to FGF signaling (Dailey *et al.,* 2005, Turner & Grose, 2010).

As FGF signaling controls several important cellular functions and deregulated signaling can cause developmental abnormalities or cancer, signaling must be tightly regulated. This regulation can take place at several levels of signal transduction pathway. Negative regulators of FGFs signaling, which belong to FGF synexpression group include Sprouty proteins (Spry1-4), SEFs (similar expression with FGFs) and MAPK3 phosphatase (DUSP6) (Thisse & Thisse, 2005). These proteins are regulated by FGF signaling and create negative feedback loops by inhibiting FGF signaling. Sprys function by binding to Grb2 and/or binding to Raf, thus inhibiting the MAPK-pathway but also signaling by PLCγ. SEF can directly inhibit FGFRs or inhibit ERK-signaling by preventing ERK phosphorylation by MEK. MKP3 is a phosphatase that negatively regulates FGF signaling by dephosphorylating activated ERK (Thisse & Thisse, 2005).

## 2.3.4 FGF signaling in male reproductive organs

### *2.3.4.1 FGF signaling in the testis*

Male sex determination during early embryonic development is dependent on FGF signaling mediated by FGF9. Targeted deletion of FGF9 in the male (XY) mouse embryos leads to failure in Sertoli cell differentiation and to a complete male-to female sex reversal (Colvin *et al.,* 2001). In the fetal testis, FGF2 functions as a survival factor for Sertoli cells and it is also mitogenic for gonocytes (Van Dissel-Emiliani *et al.,* 1996). Several other FGFs, such as FGF1-5, FGF7-8 and FGF10 are expressed in the fetal, immature and/or adult rat testis (Cancilla *et al.,* 2000). Studies show that different FGFs and FGFRs have distinct expression patterns during different phases of testicular maturation and during specific stages of spermatogenesis (Cancilla & Risbridger, 1998, Cancilla *et al.,* 2000). FGF8 is expressed in fetal gonocytes of mouse and rat (Cancilla *et al.,* 2000, Valve *et al.,* 1997). Although FGF8 mRNA is detected in the adult testis (Schmitt *et al.,* 1996, Wu *et al.,* 1997), the cellular location is unclear. However, the presence of FGF8 in the RTF suggests that it is secreted by the seminiferous epithelium (Kirby *et al.,* 2003).

In the adult testis, each FGFR is expressed in a distinct stage of spermatogenesis. In addition, FGFRs are expressed in the Leydig cells, peritubular cells and Sertoli cells (Cancilla & Risbridger, 1998). All FGFR variants, except FGFR2IIIb and FGFR3IIIb are expressed in the developing and adult testis providing suitable binding partners for the FGF ligands present (Cancilla *et al.,* 2000). Moreover, FGFR1 and 4 are expressed in the tails of elongating spermatids suggesting a role in sperm tail development or function (Cancilla & Risbridger, 1998). Interestingly, a dominant negative FGFR1 TG mouse model shows that decreased FGFR1-signaling during spermiogenesis leads to decreased sperm production and reduced ability of sperm to undergo capacitation, which an essential biochemical process for sperm to achieve the ability to fertilize the egg (Cotton *et al.,* 2006).

Altogether, the discrete cell type and developmental phase specific expression patterns of different FGFs and FGFRs in the testis suggest that FGF signaling is involved in regulating the proliferation and differentiation of specific cell types in the testis. Moreover, FGFs can regulate spermatogenesis and sperm function and contribute to the development of testicular cancers (Cotton *et al.,* 2008).

### *2.3.4.2 FGF signaling in the epididymis*

The requirement of FGF signaling for the normal development of the epididymis was recently demonstrated using KO mice, which lacked FGF8 expression in the mesoderm (T-Cre; $FGF8^{flox/\Delta 2,3}$) (Kitagaki *et al.,* 2011). These mice manifested premature degeneration of Wolffian ducts and failed to develop cranial mesonephric tubules resulting in the absence or incomplete development of male accessory reproductive tissues - the epididymis, vas deferens and efferent ductules. During the development of male reproductive tract, FGF8 signals through FGFR1 and/or FGFR2. A more severe phenotype in the reproductive tissues of FGFR1/2 KO mice than in FGF8 KO mice

suggests that other FGF ligands such as FGF4 or FGF10 are also involved in the process (Kitagaki *et al.,* 2011). Involvement of FGF signaling in the development of Wolffian duct derivates is supported by the results from other GEM models. TG mice overexpressing FGF3 have profound abnormalities in the Wollfian duct derivates including the epididymis (Chua *et al.,* 2002). The MKP3 (Dusp6) KO mice, which are supposed to have disrupted FGF signaling, also display structurally abnormal and enlarged caput and corpus epididymides (Xu *et al.,* 2010).

In the adult mouse epididymis, the majority of known FGFs (excluding FGF4 subfamily members) and all FGFRs (including the IIIb and IIIc isoforms of FGFR1-3) are expressed at low levels (Fon Tacer *et al.,* 2010). Different FGFs and FGFRs seem to have distinctive expression patterns in the epididymal segments. For example, FGFR1 is expressed at moderate levels in all the epidymal segments whereas high levels of FGFR2 are restricted to cauda epididymis (Johnston *et al.,* 2005, Tomsig & Turner, 2006). In addition to epididymally expressed FGFs, FGFs secreted by testis such as FGF2, FGF4 and FGF8, and transiting to epididymis via rete testis fluid (RTF) can act as lumicrine factors regulating the growth of the epididymis (Kirby *et al.,* 2003). Lumicrine regulation by testicular GFs is essential especially for the maintenance of the normal initial segment, which in turn, is required for male fertility. The epithelial cells of the initial segment express FGFRIII1c (Kirby *et al.,* 2003).

#### *2.3.4.3 FGF signaling in the prostate*

Several FGFs and FGFRs are expressed in the developing and adult prostate. During the induction of prostate development, mesenchymally-secreted FGF7 and FGF10 could function as andromedins that mediate the effects of androgen from the UGM to urogenital UGE (Thomson, 2008). The studies on androgen regulation of FGF7 and 10 are controversial. Initial *in vitro* studies showed androgen regulation of FGF7 and FGF10 in prostatic stromal cells (Lu *et al.,* 1999, Yan *et al.,* 1992) whereas subsequent studies exploring mouse tissues and *in vitro* organ cultures concluded that FGF7 is not directly regulated by androgens *in vivo* but FGF10 may be (Pu *et al.,* 2007, Thomson *et al.,* 1997, Thomson & Cunha, 1999). Moreover, FGF7-KO mice develop normal prostates (Guo *et al.,* 1996) whereas FGF10-KO mice develop only rudimentary prostatic buds. This indicates that mesenchymally secreted FGF10 has an important role in prostate development (Donjacour *et al.,* 2003). However, in the cultures of UGS, FGF10 alone was not sufficient to induce the prostatic budding, but the presence of androgens was also required (Donjacour *et al.,* 2003). KO mouse models show that FGFR2IIIb and the signaling molecule FRS2α are also required for normal prostate development (Kuslak *et al.,* 2007, Lin *et al.,* 2007, Zhang *et al.,* 2008). Organ culture experiments demonstrate that FGF10 activates MAPK-ERK-pathway in the UGS during prostate bud induction and treatment with FGFR inhibitor PD173074 blocks all the androgen induced changes including proliferation, prostatic bud formation and changes in the gene expression (Kuslak & Marker, 2007). The role FGF10 in prostatic morphogenesis is undeniable but the exact mechanisms of function and interaction with androgens remain unclear.

In the adult prostate, the expression of FGFs and FGFRs is partitioned into epithelium and stroma and directional and reciprocal communication between these compartments is considered to be important in maintaining tissue homeostasis (Cotton *et al.,* 2008, Kwabi-Addo *et al.,* 2004) (Figure 6). Studies using ELISA and immunohistochemical stainings show that normal human prostate stroma expresses significant quantities of FGF2, FGF9 and FGF7 (Giri *et al.,* 1999a), whereas FGF10 is present at low levels (Ropiquet *et al.,* 2000b). In the prostatic epithelium, FGF17 is expressed at low levels by the luminal cells (Polnaszek *et al.,* 2004) and FGF6 by the basal cells (Ropiquet *et al.,* 2000a). In addition, expression of FGF1, FGF5, FGF8 and FGF13 are detected in normal human prostate by RT-PCR but not by other less sensitive methods (Ghosh *et al.,* 1996, Kwabi-Addo *et al.,* 2004, Valve *et al.,* 2001). In the adult mouse prostate the FGFs expressed are mostly the same as in human, but additionally mRNAs for FGF11, FGF12, FGF16, FGF18 and FGF22 are present (Fon Tacer *et al.,* 2010, Foster *et al.,* 1999).

Based on the immunohistochemical staining, FGFR1 and -2 are expressed in the basal epithelial cells but not by the luminal cells of human prostate (Giri *et al.,* 1999a, Hamaguchi *et al.,* 1995). According to the results of receptor isoform-specific RT-PCRs gained from human prostate specimens and primary cultures of prostate epithelial and stromal cells, prostate epithelial cells express FGFR2IIIb, FGFR3IIIb and FGFR1IIIc, whereas stromal cells express FGFR1IIIc, FGFR2IIIc and FGFR3IIIc (Ittman & Mansukhani, 1997, Kwabi-Addo *et al.,* 2001). FGFR4 is expressed mainly in the luminal epithelial cells of the normal prostate and rarely also in the stromal cells (Wang *et al.,* 2004). Adult mouse prostate have been reported to express all the FGFRs, except FGFR3IIIc (Fon Tacer *et al.,* 2010) or FGFR4 (Foster *et al.,* 1999).

Homeostasis in the adult prostate is considered to be maintained by reciprocal communication between the epithelium and stroma (Figure 6). Based on *in vitro* and *in vivo* rodent models, this two-way communication can be mediated by stromal FGF7/FGF10 signaling through epithelial FGFR2IIIb and epithelial FGF9 through stromal FGFR3IIIc (Jin *et al.,* 2004, Lu *et al.,* 1999, Schmitt *et al.,* 1996). In case of FGF7 and FGF10, androgen regulation may be involved.

## 2.3.5 FGF signaling in prostate tumorigenesis

As directional FGF signaling is important in development and maintaining the tissue homeostasis in the adult prostate and several FGFs have mitogenic/transforming abilities, it is not suprising that FGF signaling system is a subject of extensive research on the prostate cancer field. In fact, several lines of evidence from both mouse and human studies show that deregulated FGF signaling is involved in prostate tumorigenesis (Kwabi-Addo *et al.,* 2004).

### *2.3.5.1 FGFs in prostate tumorigenesis*

Studies on human prostate tissue specimens show that the expression of several FGFs, such as FGF1 (Dorkin *et al.,* 1999b) FGF2 (Cronauer *et al.,* 1997, Dorkin *et al.,* 1999b), FGF6 (Ropiquet *et al.,* 2000a), FGF8 (Dorkin *et al.,* 1999b, Gnanapragasam *et*

*al.,* 2003, Leung *et al.,* 1996, Tanaka *et al.,* 1998, Valve *et al.,* 2001), FGF17 (Heer *et al.,* 2004) and FGF19 (Feng *et al.,* 2013) is upregulated in prostate cancers (Figure 6). Expression of FGF1, FGF6 and FGF8 is increased also in the premalignant PIN lesions (Dorkin *et al.,* 1999b, Ropiquet *et al.,* 2000a, Valve *et al.,* 2001). Furthermore, increased levels of FGF1, FGF2 and FGF8 associate with the advancing stage or grade of prostate cancer (Dorkin *et al.,* 1999a, Dorkin *et al.,* 1999b, Murphy *et al.,* 2009).

The roles of FGF2 and FGF8 in prostate tumorigenesis have been widely studied. Some studies have found FGF2 expression in the stroma of prostate cancer specimens (Giri *et al.,* 1999a), whereas others investigating more advanced prostate cancers observed FGF2 expression in the prostate cancer cells (Cronauer *et al.,* 1997, Giri *et al.,* 1999a). Moreover, levels of FGF2 are increased in the serum samples of prostate cancer patients as well as in metastatic prostate cancer derived PC-3 and DU145 cell lines (Cronauer *et al.,* 1997). FGF2 may act as a paracrine, stroma-derived factor in localized prostate cancers and change to an autocrine-acting factor during cancer progression (Kwabi-Addo *et al.,* 2004). The role of FGF2 as a cancer promoting factor is also supported by the results from the "transgenic adenocarcinoma in mouse prostate" (TRAMP) model, in which FGF2 is upregulated during cancer progression to a poorly differentiated phase (Huss *et al.,* 2003). Furthermore, crossing the TRAMP-mice with the FGF2-KO mice resulted in a significant decrease in the occurrence of metastatic cancers and in increased survival of mice that were hetero- or homozygous for the FGF2 KO-allele (Polnaszek *et al.,* 2003). It has been suggested that especially the high molecular weight forms (22 and 25 kDa) of FGF2, which preferentially localize to nucleus are important in prostate tumorigenesis, since they are expressed in TRAMP tumors (Huss *et al.,* 2003) and are growth promoting for NIHT3 cells (Arese *et al.,* 1999). However, a recent study showed that the cytoplasmic 18 kDa form of FGF2 bound to FGFR1 can interact with intracellular trafficking protein CEP57, which leads to disruption of centriole duplication and to mitotic instability in the prostate cancer cells (Cuevas *et al.,* 2013). This provides a new mechanism by which intracellular FGF2 could promote prostate tumorigenesis. The role of FGF8 in prostate tumorigenesis will be discussed in detail in chapter 2.3.6.3.1.

Interestingly, an increased expression of an endocrine FGF, FGF19, was recently shown in primary and metastatic prostate cancer tissue specimens and in prostate cancer cell lines. The ability of FGF19 to promote growth, invasion and colony formation of prostate cancer cells was also demonstrated (Feng *et al.,* 2013). FGF7 and FGF9 are expressed in prostate cancer but as they are expressed also in the normal prostate, their role in tumorigenesis is unclear. The expression of FGF9 (Giri *et al.,* 1999b), FGF7 (Ropiquet *et al.,* 1999) and FGF17 (Polnaszek *et al.,* 2004) is upregulated in BPH specimens, and these GFs may be more important in benign than malignant growth of prostate. Expression of FGF10 is not upregulated in human prostate cancer. However, in a mouse prostate regeneration system that uses tissue recombinant technique, an increased stromal expression of FGF10 causes a multifocal prostate cancer and upregulation of AR expression in the prostate epithelium (Memarzadeh *et al.,* 2007).

#### *2.3.5.2 FGFRs in prostate tumorigenesis*

Studies have provided evidence that deregulated FGFR expression associates with prostate tumorigenesis. Increased expression/activation of FGFR1 and FGFR4 are likely to be important in prostate cancer (Figure 6). Increased expression or activation of FGFRs is present in many cancer types and can result from genomic alterations such as mutations, translocations or amplifications in the genes coding for FGFRs. In contrast to other cancer types, including breast, ovarian and endometrial cancer, such genomic alterations in FGFRs are extremely rare in prostate cancer, and the mechanisms underlying the deregulation of FGFRs in prostate cancer remain mostly unknown (Turner & Grose, 2010).

It is notable that normal luminal epithelial cells of the prostate do not express FGFR1, but it is expressed in normal basal and stromal cells (Giri *et al.,* 1999a, Hamaguchi *et al.,* 1995). However, Giri *et al.* (1999a) reported expression of FGFR1 in the cancer cells of moderately and poorly differentiated prostate cancers. More recent studies showed significantly increased expression of FGFR1 in prostate cancers regardless of the disease stage or grade (Murphy *et al.,* 2009, Sahadevan *et al.,* 2007). Furthermore, besides prostate cancer, Valve *et al.* (2001) found expression of FGFR1 already in premalignant PIN-lesions. Due to lack of antibodies specific for FGFR1IIIb and -IIIc isoforms, the above mentioned studies could not define which of the FGFR1 isoforms were expressed in prostate cancer specimens. However, Valve *et al.(*2001) showed the presence of FGFR1IIIc-isoform mRNA in the prostate cancer samples. Consistent with the results from human studies, FGFR1IIIc is expressed in poorly differentiated prostate cancers in the TRAMP-model (Huss *et al.,* 2003). In addition, FGFR1IIIb is expressed at high levels in the tumor vasculature of TRAMP mice. According to Dunning rat prostate carcinoma model and *in vitro* and *in vivo* experiments with TRAMP-derived cell lines, FGFR1 is capable to accelerate the tumorigenicity of prostate epithelial cells (Feng *et al.,* 1997, Freeman *et al.,* 2003a). Two prostate-targeted FGFR1 TG mouse models, one using constitutively active FGFR1 (caFGFR1) (Wang *et al.,* 2004) and another using a chemically inducible FGFR1 (iFGFR1) (Freeman *et al.,* 2003b) demonstrate that FGFR1 activation results in the development of PIN lesions.

Moreover, when the activation time of FGFR1 in the iFGFR1TG mice was prolonged to 42 weeks, the mice developed prostatic adenocarcinoma and other prostatic malignancies (Acevedo *et al.,* 2007). Development of mixed epithelial and stromal malignancies in the iFGFR1 TG prostates in association with changes in the expression of EMT-associated genes was considered as a sign of EMT (Acevedo *et al.,* 2007). Interestingly, a microarray analysis of LuCaP xenografts revealed that upregulation of FGFR1 mRNA in general was associated with prostate cancer progression to androgen independent stage and the result was further confirmed with the immunohistochemical (IHC) analysis on the human prostate cancer specimens (Devilard *et al.,* 2006). In line with this, a recent gene profiling experiment using archival prostate cancer specimens found that increased expression of FGFR1 (isoform not defined) associated with the transition of hormone naïve prostate cancer to CRPC (Armstrong, 2011). Activation of FGFR1 can drive prostate tumorigenesis by several

different mechanisms. These mechanisms include disrupting the normal epithelial-stromal cross-talk, promoting angiogenesis, contributing to development of androgen independency and by inducing EMT (Acevedo *et al.,* 2009). Recently, the requirement of FGFR1-signaling in the prostate cancer metastasis of the TRAMP-model was shown by studying the TRAMP mice with conditional, tissue specific KO of FGFR1 (Yang *et al.,* 2013). Taken together, there is convincing evidence that FGFR1 is important in prostate tumorigenesis and it has roles in initiating and in disease progression.

According to the *in vitro* and *in vivo* studies, in contrast to FGFR1, FGFR2 has tumorigenesis suppressing and homeostasis maintaining functions. The expression of FGFR2 is downregulated in the prostate epithelial cells of the Dunning rat prostate cancer model during malignant transformation (Feng *et al.,* 1997). In the same model, there is a switch in the FGFR2 expression from the IIIb to IIIc isoform as the cancer progresses to androgen independent phase (Yan *et al.,* 1993). In addition, restoration of FGFR2IIIb expression in Dunning rats and human derived prostate cancer cells (Feng *et al.,* 1997, Yasumoto *et al.,* 2004) and the expression of inducible FGFR2 in TRAMP-mice-derived prostate cancer cells suppresses proliferation and tumorigenity of the cancer cells. Expression of inducible FGFR2 in the mouse prostate does not result in hyperplasia or tumorigenesis (Freeman *et al.,* 2003b). Mice with conditional KO of FGFR2 fail to develop normal prostates (Lin *et al.,* 2007). Furthermore, suppression of FGFR2IIIb signaling in mouse prostate epithelium by using dominant negative FGFR2IIIb caused the disruption of homeostasis and hyperplasia with increased frequency of neuroendocrine cells (Foster *et al.,* 2002). Moreover, suppression of FGFR2 in the caFGFR1-TG mice accelerated the formation of PIN lesions (Jin *et al.,* 2003). Observations in the human prostate cancer specimens are controversial. Some studies show that in prostate cancer, the level of FGFR2 is increased (Giri *et al.,* 1999a, Valve *et al.,* 2001) whereas other studies show the opposite (Naimi *et al.,* 2002). Sahadevan *et al.* (2007) reported a similar change in the FGFR2 isoform expression in human prostate cancer samples as was previously found in the Dunning rat model, whereas in other studies such a switch in FGFR2 isoform expression was only found in a subgroup of prostate cancers (Kwabi-Addo *et al.,* 2001), or not at all (Naimi *et al.,* 2002). However, even if there was an expressional switch from FGFR2IIIb to FGFR2IIIc isoform in the prostate cancer, it is unlikely that this gives any advantage to tumor cells, because FGFR2 may be more of a tumor suppressior than tumor promotor. One possibility is that increased expression of FGFR2IIIc in the cancer cells is a manifestation of an ongoing EMT, because this isoform is normally expressed in the stroma.

Expression of FGFR3 does not seem have a central role in human prostate cancer. Different studies have reported variable levels of FGFR3 and its isoforms in benign and malignant prostate epithelium, but no changes in the expression levels between normal prostate and prostate cancer exist (Gowardhan *et al.,* 2005, Ittman & Mansukhani, 1997, Kwabi-Addo *et al.,* 2004, Valve *et al.,* 2001). Mutations in FGFR3 are associated with some cancer types such as bladder, colon and cervix cancers and multiple myelomas. In prostate cancer, FGFR3 mutations associate with a subset of

low grade prostate tumors as well as with the presence of concurrent tumors in bladder or skin (Hernandez *et al.,* 2009).

Increasing evidence implicates FGFR4 in prostate tumorigenesis. Wang *et al.* (2004) detected increased levels of FGFR4 in PIN lesions and prostatic adenocarcinomas. Subsequent studies show increased levels of FGFR4 associated with advanced prostate cancer and with adverse survival of the prostate cancer patients (Gowardhan *et al.,* 2005, Murphy *et al.,* 2009, Sahadevan *et al.,* 2007). Drafael *et al.* (2010) demonstrated that activation of FGFR4 inhibits proapoptotic NFkB-signaling. Moreover, a common Arg388Gly polymorphism, in the transmembrane domain of FGFR4, associates with the risk of various cancers including prostate cancer in several population based studies (Liwei *et al.,* 2011, Ma *et al.,* 2008, Wang *et al.,* 2004, Xu *et al.,* 2011, Xu *et al.,* 2011). The Arg388-variant of FGFR4, which is associated with increased prostate cancer risk, is more stable and sustains phosphorylation for an extended time than the Gly388-variant of FGFR4 (Wang *et al.,* 2008a). In addition, Arg388-variant of FGFR4 induces increased motility and invasion of immortalized prostate epithelial cells (Wang *et al.,* 2004) and upregulates the expression of genes for uPAR and Ehm2, which can promote invasion and metastasis (Wang *et al.,* 2004, Wang *et al.,* 2006). Furthermore, the Arg388-variant of FGFR4 induces increased activation of ERK and serum response factor (SRF) and AP1 and upregulates expression of several prostate cancer-related genes (Yu *et al.,* 2011b). Silencing the Arg388-variant reduces prostate cancer cell invasiveness and metastasis *in vitro* and *in vivo* (Yu *et al.,* 2011b). Interestingly, recent studies on 3-D prostate cancer cell cultures and xenograft models show that the Arg388-variant of FGFR4 increases membrane-type 1 matrix metalloproteinase (MT-MPP1) activation, which enhances ECM degradation and cancer cell invasion and induces EMT in cancer cells, whereas the Gly388-variant of FGFR4 had the opposite, cancer growth suppressing effects (Sugiyama *et al.,* 2010a, Sugiyama *et al.,* 2010b). Altogether FGFR4 expression is important in prostate cancer progression and its inhibition in the Arg-388-variant bearing patients is a potential target for inhibiting cancer invasion.

#### *2.3.5.3 Negative regulators of FGF signaling in prostate tumorigenesis*

Deregulation of endogenous inhibitors of FGF signaling is also associated with prostate cancer. Expression of Sprouty1, Sprouty2 and Sprouty4 is downregulated in human prostate cancer (Kwabi-Addo *et al.,* 2004, McKie *et al.,* 2005). A recent study, which analyzed the prostate phenotypes of mice with inactivated Spry1 and Spry2 (Spry-KO) in the prostate epithelium and prostate phenotype of Pten-KO mice crossed with Spry-KO mice and with Spry2 overexpressing mice, concluded that Sprouty genes function to suppress prostate tumorigenesis *in vivo* (Schutzman & Martin, 2012). According to several studies hSEF is downregulated in advanced prostate cancer (Darby *et al.,* 2006, Murphy *et al.,* 2009, Zisman-Rozen *et al.,* 2007) and reduction in the expression of hSEF associates with decreased survival in prostate cancer patients (Murphy *et al.,* 2009). hSEF is able to reduce cancer cell proliferation *in vitro* and repress cancer progression *in vivo* in a xenograft model (Darby *et al.,* 2009).

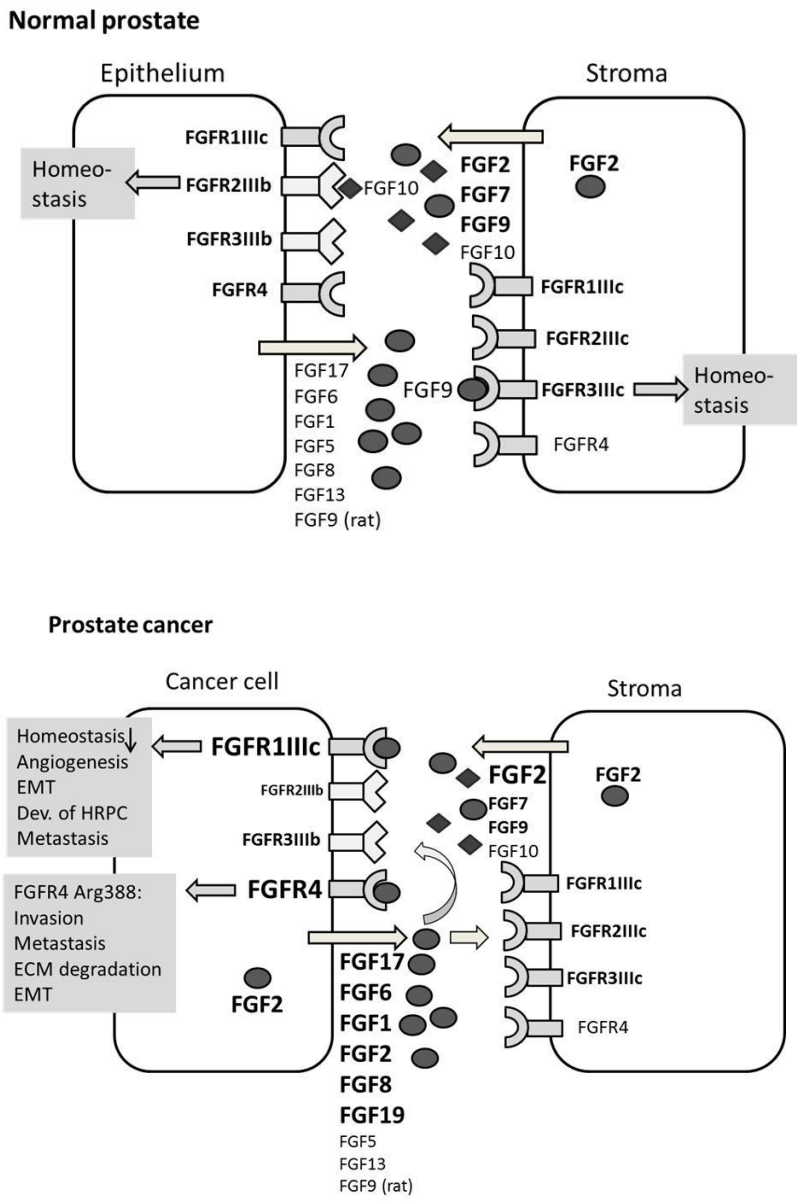


**Figure 6.** FGF-signaling in the normal prostate (top) and in prostate tumorigenesis (bottom). In the normal human prostate, FGFs are secreted by the epithelium and stroma at low levels, except for FGF2, FGF7 and FGF9, which are present in significant quantities. FGF signaling is important in homeostasis-maintaining reciprocal communication between the epithelium and stroma. In the experimental rodent models, homeostasis is mediated by stromal FGF7/FGF10 via epithelial FGFR2IIIB and by epithelial FGF9 via stromal FGFR3IIIC (Jin *et al.,* 2004, Lu *et al.,* 1999, Schmitt *et al.,* 1996). In prostate cancer, this reciprocal communication becomes disturbed, as several FGFs (FGF17, -6, -1,-2,-8 and FGF19) and FGFR1IIIc and FGFR4 are expressed at elevated levels (Gowardhan *et al.,* 2005, Kwabi-Addo *et al.,* 2004, Murphy *et al.,* 2009, Sahadevan *et al.,* 2007) enabling both autocrine and paracrine activation of FGFRs in the epithelium and stroma. Tumorigenesis promoting functions of FGFR1 and FGFR4 occur in prostate cancer (Acevedo *et al.,* 2007, Feng *et al.,* 2013, Freeman *et al.,* 2003b, Sugiyama *et al.,* 2010a, Sugiyama *et al.,* 2010b, Wang *et al.,* 2004, Wang *et al.,* 2004, Yu *et al.,* 2011b).

### 2.3.6 Fibroblast growth factor 8 (FGF-8)

FGF8 was initially cloned from Shionogi mouse mammary tumor-derived androgen-sensitive SC3 cell line and nominated as androgen-induced growth factor (AIGF) (Tanaka *et al.,* 1992). FGF8 subfamily consists of FGF8, FGF17 and FGF18, which share similar gene structure and receptor binding specificities and overlapping expression patterns during the embryonic development (Ornitz & Itoh, 2001).

#### *2.3.6.1 Structure of FGF8*

The gene coding for human FGF8 locates in human chromosome 10q24 (Payson *et al.,* 1996) and it consists of three exons, the first of which further divides into four subexons (1A-1D) that can be alternatively spliced to generate four different FGF8 human isoforms (a, b, e and f) and eight mouse isoforms (a-h) (Gemel *et al.,* 1996, MacArthur *et al.,* 1995b) (Figure 7). The different isoforms of FGF8 are similar in their C-terminal domains but differ in their N-termini. At the amino acid level the human and mouse FGF8 isoforms a and b are 100 percent identical (Ghosh *et al.,* 1996). In fact, the a and b isoforms of FGF8 are highly conserved, whereas isoforms e, f, g and h are present only in the placental mammals (Sunmonu *et al.,* 2011). The biological significance of different FGF8 isoforms is mostly unknown (MacArthur *et al.,* 1995a) but they differ in their transforming potential (MacArthur *et al.,* 1995a), in their ability to activate FGFRs (Blunt *et al.,* 1997, MacArthur *et al.,* 1995b), and in their ability to induce angiogenesis (Mattila *et al.,* 2001). Of the FGF8 isoforms, the FGF8b-isoform has the highest potential to transform NIHT-cells *in vitro* (MacArthur *et al.,* 1995a) and it has stronger angiogenic potential than FGF8a and FGF8e (Mattila *et al.,* 2001). Based on studies in mouse and chick embryos, FGF8b and FGF8a have different roles in the early development of mid- and hindbrain (MHB) (Sunmonu *et al.,* 2011). The results of structural studies that have analyzed the binding of FGF8a and FGF8b to FGFR2IIIc suggest that the functional differences of the isoforms are explained by their different affinity to FGFRs, and that the increased binding affinity of FGF8b could affect both the intensity and the quality of downstream signaling (Olsen *et al.,* 2006).

#### *2.3.6.2 Expression and function of FGF-8 in normal tissues*

Members of the FGF8 subfamily are expressed in the epithelial tissues. They activate the mesenchymally/stromally expressed IIIc-isoforms of FGFR1-3 and FGFR4, therefore mediating the epithelial to mesenchymal communication, important in several phases of embryologic development (MacArthur *et al.,* 1995b, Zhang *et al.,* 2006). During embryologic development, FGF8 is expressed at several sites that instruct growth and patterning, such as the tail bud, the apical ectodermal ridge (AER) of the limb bud, the primitive streak and the midbrain-hindbrain junction. FGF8 is also expressed in the developing branchial arches, hypothalamus and otic vesicles (Crossley & Martin, 1995, Crossley *et al.,* 2001, Ohuchi *et al.,* 1994).

FGF8 plays important roles in several embryonic tissues. In fact, homozygous FGF8-KO mice die prenatally at embryonic day E9.5 because they fail to undergo

gastrulation, the process in which the three germ layers are formed (Sun *et al.,* 1999). Before and during gastrulation, FGF8 is required for the formation and migration of the mesoderm (Sun *et al.,* 1999). Moreover, in the primitive streak, expression of FGF8 is necessary for development of left-right asymmetry of the internal organs (Meyers & Martin, 1999). Development of mice with hypomorphic FGF8 mutations and conditional site-specific KO mice has enabled studying the function of FGF8 in the development after the embryonic day E9.5 (Meyers *et al.,* 1998). These experimental mouse models show that expression of FGF8 is required for normal development of brain (Storm *et al.,* 2003), kidney (Perantoni *et al.,* 2005), limb (Lewandoski *et al.,* 2000), inner ear (Ladher *et al.,* 2005), cardiovascular and craniofacial structures (Frank *et al.,* 2002, Trumpp *et al.,* 1999), the nasal cavity (Kawauchi *et al.,* 2005) and the male reproductive system (Kitagaki *et al.,* 2011). Interestingly, the phenotype of mice with hypomorphic mutation of FGF8 resemble the phenotype of human 22q11 deletion syndrome (DiGeorge syndrome) including anomalies in the craniofacial and cardiovascular structures and hypoplasia and aplasia of thymus and parathyroids (Frank *et al.,* 2002), although the human FGF8 gene does not locate in the chromosome 22. In humans, mutations in the FGF8 gene are associated with congenital cleft lip and/or palate (Riley *et al.,* 2007) and with gonadotropin releasing hormone deficiency (Falardeau *et al.,* 2008).

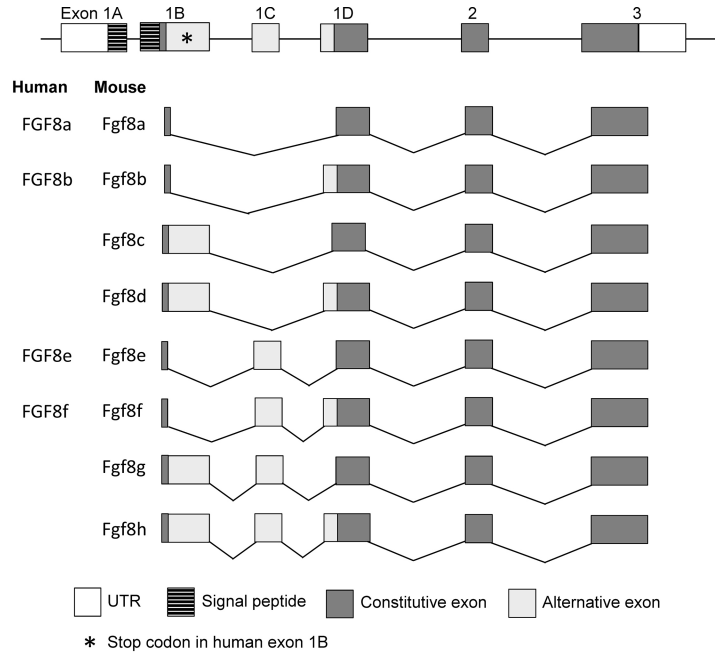


**Figure 7.** The structure of FGF8 gene and FGF8 isoforms in human and mouse. (Modified from Sunmonu *et al.,* 2011).

In human adult tissues, the expression of FGF8 is considered to be generally low, but no comprehensive analysis of FGF8 expression exists. FGF8 is expressed in the adult human breast, kidney, prostate, testis, cerebral neurons, skin, bowel and

endometrium (Dorkin *et al.,* 1999a, Ghosh *et al.,* 1996, Marsh *et al.,* 1999, Zammit *et al.,* 2002). Expression of FGF8 is upregulated in the lactating breast suggesting that FGF8 might be involved in the regulation of lactating cells (Zammit *et al.,* 2002). Furthermore, FGF8 is expressed in blood leucocytes and in the bone marrow specimens of healthy donors, suggesting that FGF8 might have a role in controlling hematopoiesis (Nezu *et al.,* 2005). In the adult mouse, FGF8 mRNA is expressed in the ovaries and testis (Fon Tacer *et al.,* 2010). In addition, low levels of FGF8 mRNA are expressed in the epididymis, eye and in some parts of the brain (Fon Tacer *et al.,* 2010). Altogether, the restricted expression pattern of FGF8 in adult tissues suggests that FGF8 may have functions in the adult tissues, especially in the hormonally regulated reproductive organs.

#### *2.3.6.3 Activation of FGF-8 in hormonal cancer*

Expression of FGF8 is upregulated in human hormonal cancers originating from hormone regulated tissues (Mattila & Harkonen, 2007) such as in breast (Marsh *et al.,* 1999), prostate (Dorkin *et al.,* 1999a, Leung *et al.,* 1996, Tanaka *et al.,* 1998, Valve *et al.,* 2001) and ovarian cancers (Valve *et al.,* 2000) and in testicular germ cell tumors (Suzuki *et al.,* 2001). Accordingly, FGF8 is expressed in several cancer cell lines derived from human prostate, breast and ovarian carcinomas (Mattila & Harkonen, 2007). Only few other human cancer types such as esophageal (Tanaka *et al.,* 2001) and nasopharyngeal cancers (Lui *et al.,* 2011) over-express FGF8, and interestingly, the growth of esophageal cancer seems to be androgen regulated (Tanaka *et al.,* 2001). In prostate and ovarian cancers the expression of FGF8 correlates with disease progression or differentiation status, respectively (Dorkin *et al.,* 1999a, Valve *et al.,* 2000, Valve *et al.,* 2001). Importantly, hormonal cancers express FGFRs that can bind FGF8 (Mattila & Harkonen, 2007, Valve *et al.,* 2000, Valve *et al.,* 2001).

Tumor-promoting functions of FGF8 in hormonal cancers have been demonstrated. FGF8b induces morphological changes and increases anchorage-independent growth and invasiveness of breast cancer cells *in vitro* and elevates tumor growth and angiogenesis *in vivo* (Mattila *et al.,* 2001). In LNCaP prostate cancer cells, FGF8b induces increased growth rate and invasiveness *in vitro* and *in vivo* (Song *et al.,* 2002). In addition, in co-culture experiments of LNCaP cells and stromal cells, FGF8b secreted by the LNCaP cells stimulated the growth of the stromal cells and decreased the inhibitory effect of stromal cells on epithelial cell growth (Song *et al.,* 2000). Moreover, overexpression of FGF8 under the MMTV-promoter in TG mice induced development of neoplasias in the mammary and salivary glands and stromal hyperplasia in the ovarios (Daphna-Iken *et al.,* 1998).

FGF8 was initially found as an androgen-induced growth factor from the Shionogi mouse mammary carcinomas derived SC3 cells (Tanaka *et al.,* 1992) and similar androgen-dependent expression of FGF8 is observed in S115 cells, which derive from the same tumors (Minesita & Yamaguchi, 1965). Androgen regulation of FGF8 expression is still under debate, because similar androgen-dependent expression does not exist in other mouse or human tissues or cell lines. In fact, there is evidence

that the androgen regulation of FGF8 in the S115 cells is not an inherent feature of the FGF8 gene but caused by genomic rearrangements in the tumor such as proviral integration (Valve *et al.,* 1998) or genomic translocation (Erdreich-Epstein *et al.,* 2006). However, androgen increases the expression of FGF8 in the human prostate cancer cell line LNCaP (Gnanapragasam *et al.,* 2002), the breast cancer cell line MDA-MB-231 (Payson *et al.,* 1996) and esophageal cancer cell line KSE1 (Tanaka *et al.,* 2001). In the LNCaP cells, Gnanapragasam *et al.* (2002) showed putative androgen responsive elements (AREs) in the FGF8 promoter area. Recently, the expression of AR and FGF8 mRNA were shown to be downregulated by prenatal anti-androgen treatment in the genital tubercles of hypospadiac rats, suggesting androgen regulation of FGF8 expression (Liu *et al.,* 2012). Furthermore, in both breast and prostate cancer, the expression of FGF8 correlates with the expression of AR (Tanaka *et al.,* 2001, Wang *et al.,* 1999). Moreover, in breast cancer, the expression of FGF8 correlates with the expression of PSA, a known androgen-regulated factor (Tanaka *et al.,* 2002). In prostate cancer, the expression of FGF8 persists in androgen-independent disease (Dorkin *et al.,* 1999a). Altogether, there is strong evidence that androgen has a role in the regulation of FGF8 expression, but the exact molecular mechanisms by which androgen controls the transcription of FGF8 gene in normal tissues and during hormonal tumorigenesis should be studied further.

In breast and prostate cancer cell lines, expression of FGF8 is regulated also by other hormones acting via nuclear receptors such as estradiol, glucocorticoids (Yamanishi *et al.,* 1995), retinoid acid (Brondani & Hamy, 2000, Brondani *et al.,* 2002) and vitamin D3 (Kawata *et al.,* 2006). According to studies by Brondani and Hamy (Brondani & Hamy, 2000), treatment of LNCaP cells with retinoid acid induced an expression switch from the most mitogenic FGF8b-isoform to less mitogenic FGF8a-isoform. This is interesting concerning the regulation of differentiation both during the embryonic development and in tumorigenesis. Moreover, FGF8 is downregulated by TGFβ in SC3 breast cancer cells (Takayashiki *et al.,* 2005) and upregulated by nuclear factor kappa b (NfKB) in prostate cancer cells (Armstrong *et al.,* 2006).

Taken together, *in vitro* and *in vivo* studies suggest that FGF8 has a functional role in hormonal cancers. More studies are needed to illuminate the role of FGF8 in different phases of tumor progression and to understand the regulation of FGF8 gene expression.

#### *2.3.6.3.1 FGF-8 in prostate tumorigenesis*

FGF8 is one of the most studied members of the FGF-family in the prostate cancer. In normal prostate the expression of FGF8 is low or undetectable (Dorkin *et al.,* 1999a). Several studies have shown increased levels of FGF8 in human prostate cancer specimens (Dorkin *et al.,* 1999a, Leung *et al.,* 1996, Murphy *et al.,* 2009, Tanaka *et al.,* 1998, Valve *et al.,* 2001) and some studies have also demonstrated that increased expression of FGF8, or more specifically FGF8b, correlates with advanced stage and grade of the prostate cancer (Dorkin *et al.,* 1999a, Gnanapragasam *et al.,* 2003,

Murphy *et al.,* 2009) and with decreased patient survival (Dorkin *et al.,* 1999a, Gnanapragasam *et al.,* 2003).

FGF8 and especially its isoforms a, b and e, are expressed already in the premalignant PIN lesions (Valve *et al.,* 2001), suggesting its involvement in the initial steps of human prostate cancer. On the other hand, FGF8 is also expressed in HRPC (Dorkin *et al.,* 1999a) and in the bone metastasis of prostate cancer (Valta *et al.,* 2008). There is evidence that FGF8 can upregulate the expression of FGF17, which is over-expressed in prostate cancer (Heer *et al.,* 2004) and in prostate cancer bone metastasis (Valta *et al.,* 2008). Co-localized expression of FGF8 and FGF2 and FGF1 in prostate cancer tissues exists (Dorkin *et al.,* 1999b). Importantly, prostate cancers express increased amount of FGFRs (FGFR1IIIc, FGFR4 and possibly FGFR2IIIc) that can be activated by FGF8 (Kwabi-Addo *et al.,* 2004). Furthermore, the expression of FGF8 in prostate cancer specimens also correlates with the expression of VEGF and combined expression of these factors associates with reduced patient survival (West *et al.,* 2001). This is interesting in terms of tumor angiogenesis, a process that is essential for tumor growth, in which VEGFs and FGFs are known to co-operate.

In line with clinical studies, FGF8 is expressed in human prostate cancer cell lines originating from both primary tumor (ALVA-31cell line) and variable metastatic sites (LNCaP, DU145 and PC-3 cell lines) (Ghosh *et al.,* 1996, Tanaka *et al.,* 1995). As pointed out in the previous chapter of this thesis, *in vitro* and *in vivo* studies implicate several functional roles for FGF8 in prostate tumorigenesis. In experiments with prostate cancer cell lines LNCaP and PC-3, overexpression of FGF8b induces increased growth rate and invasiveness *in vitro* and *in vivo* (Song *et al.,* 2000, Valta *et al.,* 2009). Accordingly, inhibition of FGF8 in DU145 cells by antisense constructs significantly reduced their growth rate and *in vivo* tumorigenity in nude mice (Rudra-Ganguly *et al.,* 1998). In xenograft models in which FGF8b overexpressing PC-3 cells were injected subcutaneuosly or orthotopically to mice, FGF8b induced greater tumor growth and angiogenesis compared to control (Tuomela *et al.,* 2010, Valta *et al.,* 2009).

The angiogenic property of FGF8 has been demonstrated using S115 breast cancer cells (Mattila *et al.,* 2001). Gene expression studies show that in S115 cells FGF8 can downregulate the expression of thrombosbondin 1 (TSP1), a known negative regulator of angiogenesis (Mattila *et al.,* 2006). TG mice with prostate-targeted over-expression of FGF8b develop PIN lesions implicating that FGF8 has a role in initiation of prostate cancer (Song *et al.,* 2002). In another genetically modified mouse model, over-expression FGF8 acted in synergy with the heterozygous KO of PTEN-tumor suppressor in the prostate and these mice developed late-onset prostate metastatic adenocarcinomas (Zhong *et al.,* 2006). Furthermore, there is evidence that FGF8 can function in the differentiation of osteoclasts and osteoblasts (Mattila M. M., Thesis 2006, Valta *et al.,* 2006) and an *in vivo* model of intratibial PC-3 tumors implicates a role for FGF8 in prostate cancer bone metastasis (Valta *et al.,* 2008).

In conclusion, FGF8 expression is upregulated in human prostate cancer and it has potential functional roles in several phases of cancer progression, such as in cancer initiation, tumor growth, angiogenesis and development of bone metastasis.

### 2.3.7 Targeting FGF signaling in the treatment of prostate tumorigenesis

As enhanced FGF signaling is involved in several stages of prostate cancer development and progression, FGF signaling is a potential target for drug development. FGF signaling can be inhibited by several strategies - by small-molecule tyrosine kinase inhibitors (TKIs), monoclonal antibodies and FGF-ligand traps.

Small molecule TKIs target the ATP-binding site of the tyrosine kinase domain of the RTKs. Several FGFR inhibitors such as dovotinib, vargatel, lenvantinib masantinib and brivanib are currently in clinical trials for multiple cancer types, including breast, ovarian, and prostate cancers (Brooks *et al.,* 2012, Daniele *et al.,* 2012, Dieci *et al.,* 2013, Gallick *et al.,* 2012). However, these TKIs are not specific for FGFRs but also inhibit PDGFRs and/or VEGFRs. Targeting several RTKs similarly can be more effective, but also more likely to cause side effects. Inhibitors more specific to FGFRs, such as SU5402, PD173074, AZD4547, AZ8010, FIIN-1, BGJ398 and LY2874455, are currently being developed (Brooks *et al.,* 2012, Dieci *et al.,* 2013, Liang *et al.,* 2012, Wesche *et al.,* 2011). Some of these are in clinical trials for treatment of cancers of other organs than prostate. However, it is notable that besides FGFRs, some of the more specific FGFR TKIs also inhibit VEGFRs at higher doses. The effects of AZ8010, which inhibits all FGFRs, were tested by *in vitro* and *in vivo* experiments using immortalized PNT1a prostate epithelial cells and VCaP prostate cancer cells (Feng *et al.,* 2012). These studies showed significant inhibition of cancer cell proliferation and angiogenesis and increased apoptosis.

Monoclonal antibodies can target either FGF ligands or FGFRs, thereby inhibiting ligand binding, receptor dimerization or enhancing removal of the tumor cells by the immune system. The benefit of the monoclonal antibodies lies in their specificity to target a specific FGF or FGFR isoform. This strategy of inhibiting RTK signaling has been successfully used in several cancers with dysregulated RTK signaling (Lemmon & Schlessinger, 2010). Preclinical and clinical trials are ongoing for FGF/FGFR monoclonal antibodies for anticancer treatment (Brooks *et al.,* 2012, Liang *et al.,* 2012). *In vitro* and *in vivo* data on the effectiveness of monoclonal antibody against FGF8 have been promising in experiments with breast and prostate cancer cells (Maruyama-Takahashi *et al.,* 2008, Shimada *et al.,* 2005).

Ligand traps function by sequestering FGFs and hence preventing their binding to FGFRs. For example, A FGF-trap FP-1039 is a fusion protein, which contains the extracellular domain of FGFR1IIIc and therefore it binds the ligands of this receptor. This FGF-trap is in clinical trials for treatment of advanced or recurrent endometrial cancers with specific FGFR2 mutations (Brooks *et al.,* 2012, Liang *et al.,* 2012).

Disadvantages of the above described therapeutical strategies targeting FGF signaling are that the responses are often low or moderate at least partly due to

resistance to therapy. Combinations of several inhibitors or combination with chemo- or radiotherapy may be required to achieve better responses (Dieci *et al.,* 2013). The research on targeting FGF-signaling in prostate cancer compared to many other cancer types is still at an early stage, and only few clinical trials are reported to be ongoing. The possibility to affect stromal-epithelial interaction by interfering with FGF signaling holds great potential in combating prostate cancer and future studies will show whether relevant clinical applications can be developed.

## 2.4 MOUSE MODELS OF PROSTATE CANCER

In contrast to humans, naturally occurring prostate cancer is extremely rare in mice. Therefore hormonal or genetic manipulation is required to induce tumorigenesis when modeling prostate cancer in mice. An ideal mouse model of prostate cancer should mimic the progression of the human disease, pose similar molecular changes and have similar response to therapeutic agents. Different kinds of mouse models have been used to model prostate cancer with the most commonly used being xenograft and GEM models (reviewed in detail in chapter 2.4.2). In xenograft models, immunodeficient mice are used as recipients for human tumor tissue, cancer cell lines or primary cell cultures. A limitation of this strategy is that it is likely that the deficiency of immune system of the recipient mice affects tumor progression, which impedes the interpretation of the results. However, xenograft systems are important in studying how different cell types interact and comparing tumorigenicity and metastatic capacities of different cancer cell lines (Jeet *et al.,* 2010, Valkenburg & Williams, 2011).

### 2.4.1 Techniques for generating GEMs

The most common basic strategies to study the function of the gene of interest in GEM models are to overexpress the gene or to delete the gene product. These strategies are applied by generating TG or KO mice.

To produce TG mice, the cDNA of gene of interest is linked to a promoter, which directs the expression of the gene to specific tissues or cell types or allows universal expression if desired. The transgene construct is then introduced by microinjection into the male pronuclei of fertilized oocytes, which are subsequently transferred to uterus of pseudopregnant female mice. The mice carrying the transgene in their genome can be identified by PCR techniques. A problem associated with the microinjection technique is that the integration of the transgene construct into the genome is random and cannot be controlled. Usually a concatemeric insertion, in which multiple copies of the TG construct integrate in tandem to simple genomic loci in the right orientation, is desirable. Sometimes, gene insertions to a genome can cause a disruption of another gene (Belizario *et al.,* 2012, Houdebine, 2007).

The production of conventional KO mice is obtained by the use of homologous recombination, which enables the insertion of the targeting construct to the gene of interest. Homologous recombination is achieved by attaching DNA sequences homologous to the endogenous gene to flank the mutation in the targeting construct.

The targeting construct usually contains a neomycin cassette, which serves two roles - it disrupts the exon in which it is inserted and it can be used for selection of clones positive for the targeting construct with antibiotics. In addition, the targeting construct usually contains a negative selection marker, which is excised from the construct when homologous recombination occurs. The targeting construct is transfected by electroporation to mouse embryonic stem cells (ES cells) of which the clones with targeted insertion of the construct can then be selected by the selection based on resistance to antibiotics. Subsequently, ES-cells positive for the targeted mutation are injected to the inner cell mass of mouse blastocysts which are then transferred to uteri of pseudopregnant mice. The mice born are chimeric and heterozygous for the KO, but mice homozygotes for the KO can be obtained by breeding the chimeras (Belizario *et al.,* 2012, Houdebine, 2007). One problem associated with conditional KO mice is that insertion of the KO-construct leads to a universal abrogation of the gene product in all tissues and can cause embryonic lethality or other severe phenotypes that impede studying the effects in the tissues of interest. To avoid this problem, conditional KO mice, which rely on site-specific DNA recombinase technology, have been developed (Chan *et al.,* 2007). With these technologies, it is possible to obtain site- or temporally specific loss of function of a gene. The most common strategies used to generate conditional KO mice are the Cre-LoxP (Kuhn *et al.,* 1995) and the FLP-FRT systems (Dymecki, 1996). In both of these techniques, two separate genetic modifications are needed. In principal, the generation of conditional KO mice by the Cre-LoxP system involves targeting the LoxP sites to flank an exon of a gene of interest and breeding the mice with this modification with TG mice expressing Cre-recombinase under the control of a tissue specific or drug-inducible promoter. The deletion of the LoxP-targeted exon will occur only when the Cre-recombinase is activated.

In the future, novel technologies such as zinc finger nuclease (ZNF)-based genetic modification, which provides a highly efficient and fast method to establish KO-organisms of several species (Geurts *et al.,* 2009) without requirement for ES clone selection, may become a popular method to generate KO mice and rats.

### 2.4.2 GEM models for prostate tumorigenesis

Several kinds of GEMs, which model prostate tumorigenesis, exist. Most of these models have used promoters derived from prostate specific genes such as rat probasin (PB, LPB or $ARR_2PB$), rat C3 prostate steroid binding protein, human PSA, mouse mammary tumor virus (MMTV) or mouse cryptin-2 gene to target transgene expression, or more recently for generating a tissue specific KO mice (Jeet *et al.,* 2010).

The first generation of these GEM models was based on the transgenic expression of simian virus 40 (SV40) oncogenes, more precisely large and/or small t antigens (Tag and tag, respectively), in the mouse prostate (Table 2). Expression of Tag and tag promotes tumorigenesis by inhibiting tumor suppressors such as p53, retinoblastoma (rb) and protein phosphatase 2. The most extensively studied models, based on prostate targeted SV40 oncogene expression, are the TRAMP and the LPB-

Tag TG mouse (LADY) models. The TRAMP model uses the rat probasin promoter (PB) to direct the expression of tag and Tag. The TRAMP mice develop HGPIN at 12 weeks of age and poorly differentiated adenocarcinoma at 18-30 weeks of age which commonly metastasize to lymph nodes and lung (Gingrich *et al.,* 1996, Greenberg *et al.,* 1995). Importantly, TRAMP tumors exhibit androgen depletion independent growth upon castration and develop mutations in the AR gene (Gingrich *et al.,* 1997), mimicking the progression of human prostate cancer. The LADY model uses a different version of the probasin promoter (LB) to direct the expression of SV40 Tag. Progression of prostate tumorigenesis in LADY mice resembles that reported in the TRAMP mice, but the tumors tend to have a less aggressive phenotype (Kasper *et al.,* 1998, Masumori *et al.,* 2001). The high rates of tumor incidence and metastases and the development of androgen independence make the TRAMP and the LADY models clinically significant and they have been used for studying the late stages of prostate cancer as well as for evaluation of new cancer therapies and potential chemopreventive agents. Disadvantages of the SV40 based TG models are the neuroendocrine (NE) phenotype of the prostate tumors presenting a rare subtype of human prostate cancer, and the rarity of bone metastases. Other limitations reside in the androgen dependence of the used promoters and uncertain relevance of viral oncogenes in human prostate tumorigenesis (Jeet *et al.,* 2010).

The second generation of GEM models is based on the overexpression or deletion of single or multiple genes found to be altered in the human prostate cancer (Table 2). These models provide the opportunity to study the effects of deregulated genes controlling the differentiation, growth and apoptosis in the prostate. Target genes of such GEMs include transcription factors, GFs and GFRs, tumor suppressors, regulators of cell cycle and apoptosis and signaling pathways (Jeet *et al.,* 2010). Amplification of the gene of a transcription factor, c-myc, is frequently observed in human PIN and primary prostate cancer. $ARR_2$PB-c-Myc TG mice were the first non-SV40-based TG mice that developed prostatic adenocarcinoma (Ellwood-Yen *et al.,* 2003). The tumors in these mice also develop partial castration resistance. As the activation of AR is known to be critical in prostate cancer, a mouse model with elevated AR expression in the prostate has been generated. The $ARR_2$PB-AR TG mice developed focal HGPIN lesions in the prostate in the age of over one year (Stanbrough *et al.,* 2001). Several TG mouse models based on the overexpression of GFs - such as FGFs, TGFβ, IGF1, EGFRs or their receptors in the prostate have been established. These GF based GEMs develop PIN lesions and adenocarcinoma. In general, tumor progression in the GEM models based on GFs has been slow but these models have been important in studying the early stages of prostate cancer (Jeet *et al.,* 2010, Valkenburg & Williams, 2011). Multiple tumor suppressor genes, such as p53, retinoblastoma (Rb), Nkx3.1 and Phosphatase and tensin homologue in chromosome 10 (Pten) have also been targeted in the KO mouse models to study the effect on the prostate phenotype (Jeet *et al.,* 2010, Valkenburg & Williams, 2011). Many of these KO-mouse models develop PIN lesions and some such as the Pten-KO mice, develop adenocarcinoma (Wang *et al.,* 2003). Pten has an antagonistic effect on the PI3K-pathway and its deletion leads to constitutive activation of downstream targets such as Akt. Prostate-specific deletion of Pten results in the development of PIN in six weeks

and adenocarcinoma after nine weeks. Importantly, the tumors are androgen resistant and have gene expression profile resembling that of human cancers (Wang *et al.,* 2003). The only true disadvantage of the Pten-KO model is the lack of bone metastasis.

GEM models with multiple (two to five) genetic modifications have been generated to define the effect of combined mutations to prostate tumorigenesis (Table 2). Generally GEM models with more than one genetic modification tend to have more aggressive and rapidly developing prostate tumors compared to GEMs with only a single modification (Jeet *et al.,* 2010, Valkenburg & Williams, 2011). As the TMPRSS2-ERG-fusion gene is a frequent genomic change in human prostate cancer (Tomlins *et al.,* 2005), a TG-mouse model expressing the TMPRSS2-ERG fusion gene has recently been generated (Tomlins *et al.,* 2008). These mice develop PIN lesions at 12-14 weeks of age, but the progression to prostate cancer has not been observed.

GEM models have provided information about molecules and mechanisms important in the initiation and progression of prostate cancer. However, no GEM model generated so far is able to mimic all the stages of human prostate tumorigenesis. The rarity of bone metastasis in GEM models poses a challenge for prostate cancer research. In the future, the development of GEMs will probably be focused on generation of models with multiple genetic modifications and in utilizing conditionally inducible KO technology as well as other novel technologies. Developing new non-androgen dependent prostate-specific promoters would also be important to enable studying the androgen dependence during cancer progression (Jeet *et al.,* 2010, Valkenburg & Williams, 2011).

**Table 2.** Examples of published GEM models with prostate phenotype.

| Category | Mouse model | Gene and modification | Prostate phenotype | References |
|---|---|---|---|---|
| SV40 based | **TRAMP** ($ARR_2PB$-tag/ Tag) | **SV40 Tag and tag,** expression | Metastatic NE carcinoma, (LN, Lu, Bn, Kid), reactive stroma | (Gingrich *et al.,* 1996, Greenberg *et al.,* 1995) |
| | **LADY** (LPB-Tag ) | **SV40 Tag,** expression | Metastatic NE carcinoma (LN, Li, Lu) | (Masumori *et al.,* 2001) |
| SHRs | **PB-AR** | **AR,** overexpression | HGPIN | (Stanbrough *et al.,* 2001) |
| GF signaling | **$ARR_2PB$-FGF8b** | **FGF8b,** overexpression | HGPIN, stromal hyperplasia | (Song *et al.,* 2002) |
| | **$ARR_2PB$-iFGFR1** | **FGFR1,** inducible activation | Adenocarcinoma, sarcomatoid carcinoma, sarcoma, reactive stroma | (Acevedo *et al.,* 2007) |
| | **BK5-IGF1** | **IGF1,** expression in basal cells | Adenocarcinoma | (DiGiovanni *et al.,* 2000) |
| | **FSP1-Cre; $TGF\beta R2^{flox}$** | **TGFβR2,** KO in stromal fibroblasts | HGPIN and stromal hyperplasia | (Bhowmick *et al.,* 2004) |
| Tumor suppressors | **PB-p53 mutant** | **p53,** expression of mutant form | mPIN | (Elgavish *et al.,* 2004) |
| | **PSA-Cre; $Nkx3.1^{+/flox}$** | **Nkx3.1**, conditional KO | mPIN | (Abdulkadir *et al.,* 2002) |
| | **PB-Cre4; $Pten^{loxp/loxp}$** | **Pten,** conditional KO | Metastatic adenocarcinoma (LN, Lu), reactive stroma | (Wang *et al.,* 2003) |
| Transcription Factors | **$ARR_2PB$-myc-PAI** | **Myc,** overexpression | Local adenocarcinoma | (Ellwood-Yen *et al.,* 2003) |
| Composite TG mice | **$Nkx3.1^{-/-}$ x $Pten^{-/-}$** | **Nkx3.1 and Pten,** KO | Metastatic adenocarcinoma (LN) | (Abate-Shen *et al.,* 2003) |
| | **$Pten^{-/-}$ x p53 $^{-/-}$** | **Pten and p53**, KO | Invasive tumors, sarcomatoid carcinoma | (Martin *et al.,* 2011) |
| | **$Pten^{+/-}$ x $ARR_2PB$-FGF8b** | **Pten,** KO; **FGF8b,** overexpression | Metastatic adenocarcinoma (LN), Mucinous adenocarcinoma | (Zhong *et al.,* 2006) |
| | **LADY, x PB-hepsin** | **SV40 Tag** and **hepsin**, expression | Metastatic NE carcinoma (Bn) | (Klezovitch *et al.,* 2004) |

$ARR_2PB$, improved probasin promoter; PB, probasin; LPB, long probasin; SHR, steroid hormone receptor; BK5, bovine keratine 5 ;NE, neuroendocrine; LN, lymph node; Lu, lung; Li, liver; Bn, bone; Kid, kidney.

# 3. AIMS OF THE PRESENT STUDY

Previous studies have demonstrated dysregulation of FGF/FGFR signaling and increased expression of FGF8b in human prostate cancer. Other studies have suggested ERβ protection against prostate tumorigenesis and reported hyperplasia, inflammation and incomplete differentiation of the epithelial cells in the prostates of BERKO mice.

The aim of this study was to generate and investigate GEM models to study the function of FGF8b and ERβ in the prostate with the specific interest on prostate tumorigenesis. The effects of FGF8b expression were studied also in the other reproductive organs.

The specific aims were:

1. To establish a TG mouse model with prostate-targeted FGF8b overexpression to determine if FGF8b plays a role in prostate tumorigenesis.

2. To study the histological and molecular effects of FGF8b overexpression in the epididymis and testis of the FGF8b-TG mice.

3. To determine the effect of ERβ deficiency on FGF8b-induced pathological changes in the mouse prostate by crossing FGF8b-TG mice with $BERKO_{FVB}$ mice.

# 4. MATERIALS AND METHODS

## 4.1 MICE

### 4.1.1 Generation of FGF8b-TG mice (I)

FGF8b transgene construct was generated by ligating the 530-bp long DNA fragment containing the probasin ($ARR_2PB$) promoter (a gift from Dr. R.J. Matusik) in front of 648 bp long human FGF8b cDNA (a gift from Dr. P. Roy-Burman) and Bgh polyA sequence using recombinant DNA techniques. Functional *in vitro* testing of the FGF8b transgene construct in the promotorless pGEM7Zf(+) vector was performed in the PC-3 cells co-transfected with an AR-expression vector. The ability of the construct to produce FGF8b mRNA and protein were checked by Northern and Western blots. To produce TG mice in the FVB/N background, the transgene construct was purified by agarose gel by a Quick-Pick electroelution Capsule kit (Qiagen, Valencia, CA) and Elutip DEAE columns (Schlaicher & Shüell, Dassel, Germany) and thereafter microinjected into the pronuclei of fertilized FVB/N mouse oocytes.

For genotyping of the offspring, total DNA was extracted from tail or ear biopsies using DNeasy columns (Qiagen, Valencia, CA). The presence of the FGF8b transgene insertion in the genome was tested by using PCR with primers F: 5'-TACAACTGCCAACTGGGATG-3' and R: 5'-GGCGGGTAGTTGAGGAACTC-3' specific for the sequence $ARR_2PB$ promoter and the hFGF8b gene, respectively. Genotyping-PCR resulted in a 762 bp product from DNA samples from mice positive for the transgene and no product from WT mice. Mice positive for the transgene were used as founder mice and mated with WT FVB/N mice to establish four FGF8b-TG mouse lines (nominated as L2–L5), which were used for further expressional and phenotypic analysis. For genomic stability, FGF8b-TG mice were kept heterozygous for the transgene.

### 4.1.2 Generation of FGF8b-TG-BERKO$_{FVB}$ mice (III)

BERKO-mice in the C57BL/6J strain were produced by Krege *et al.*(1998) by inserting a NEO-cassette in the exon three of the ERβ gene. These BERKO-mice were crossbred with FVB/N mice for 7 generations. To produce FGF8b-TG-$BERKO_{FVB}$ mice, breeding in two generations was performed. First, female FGF8b-TG mice from FGF8b-TG lines L2 and L4 were mated with male BERKO ($ER\beta^{-/-}$) mice, which gained mice heterozygotes for $BERKO_{FVB}$ (hz $BERKO_{FVB}$, $ER\beta^{+/-}$) of which half were positive for the FGF8b-transgene. In the next generation, FGF8b-TG; hz$BERKO_{FVB}$ female mice were bred with $BERKO_{FVB}$ and hz$BERKO_{FVB}$ male mice to obtain offspring with FGF8b-TG-$BERKO_{FVB}$, $BERKO_{FVB}$, WT, FGF8b-TG-hz$BERKO_{FVB}$ and hz$BERKO_{FVB}$ genotypes. Mice with FGF8b-TG and WT genotypes were also obtained by mating FGF8b-TG with WT mice. Mice were genotyped from DNA extracted from ear biopsies by performing two PCRs, the FGF8b-genotyping PCR (presented in the section 4.1) and the $BERKO_{FVB}$-genotyping PCR with the protocol

and primers by Windahl *et al.* (1999). Studies were performed from male mice with genotypes of $BERKO_{FVB}$, FGF8b-TG, FGF8b-TG-$BERKO_{FVB}$ and WT and they were kept until the age of 12-15 months.

### 4.1.3 Animal experimentation (I-III)

All the animal experiments were approved by the Finnish Animal Ethics Committee, and the institutional policies of the University of Turku (Turku, Finland) on animal experimentation fulfill the requirements defined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were kept under controlled environmental conditions (12 h light / 12 h darkness, temperature 21 ± 3°C) and standard pelleted chow and tap water were offered *ad libitum*.

For collecting the tissue samples, the mice were killed using carbon dioxide and cervical dislocation. After killing the mice, their body weights were measured and tissues of interest for RNA analyses were quickly dissected and frozen in liquid nitrogen. For RNA analyses, the prostate lobes (VP, DLP and AP) and the epididymal segments (IS, caput, corpus and cauda) were microdissected separately. In some cases, the IS was prepared along with the rest of the caput epididymides (caput + IS). For histological preparations, prostate blocks, epididymides and testes were dissected, weighed and fixed overnight in 10% neutral buffered formalin or in 4% PFA.

To induce the FGF8b transgene expression prepubertially by androgens, 14-day-old FGF8b-TG (n = 13) and WT (n = 7) male mice were treated for a period of two-weeks with a daily subcutaneous injection of T propionate (7 µg/g of body weight) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) dissolved in maize oil (+ 1% ethanol). Control animals were treated with corresponding amounts of the solvent.

## 4.2 ANALYSIS OF RNA (I-III)

### 4.2.1 Extraction of RNA

Total RNA from tissues of interest was extracted using Trizol (Invitrogen, Life Technologies, Inc., Carslbad, CA) or TRIsure (Bioline, London, UK) reagent, according to the manufacturers' instructions. For quantitative RT-PCR (qRT-PCR) and microarray analyses, the RNA was subsequently purified and DNAse-treated in RNeasy columns (Qiagen, Valencia, CA).

### 4.2.2 Reverse-transcriptase PCR (RT-PCR) (I, III)

The expression of *Ffg8b* and *Erβ* mRNAs in mouse tissues was studied by RT-PCRs. First, 1 µg of total RNA was used for cDNA synthesis in a 20 µl reaction containing random hexanucleotides and AMV reverse transcriptase enzyme (Finnzymes, Espoo, Finland). PCRs with 35 amplification cycles were performed in the Eppendorf Mastercycler. Successful cDNA synthesis was verified by beta-actin PCR. The sequences of the primers used in *Fgf8b* RT-PCR were: F 5'-

GGACACCTTTGGAAGCAGAG-3' and R 5'-CAACAGATGGCTGGCAACTA-3' and in β-actin RT-PCR: F 5'-TTGTAACCAACTG GGACGATATGG-3' and R 5'-GATCTTGATCTTCATGGTGCTAGG-3'. The primers used in *Erβ* RT-PCR were described earlier (Krege *et al.,* 1998).

### 4.2.3 Quantitative RT-PCR (I-III)

The cDNAs were synthetized from 1 µg of total RNA in the similar way as for normal RT-PCR. Next, 1/20 dilution of the cDNA was made and 10 µl of the diluted cDNA was subsequently used per each quantitative RT-PCR (qRT-PCR) reaction. In qRT-PCR cDNAs were amplified using using the SYBR® Green chemistry-based Quantitect SYBR green® reagent for two-step RT-PCR (Qiagen) or Dynamo™ F-410 kit (Finnzymes, Espoo, Finland) with amplification conditions recommended by the manufacturer's handbook. Reactions were performed in duplicate in a volume of 20 µl in DNA Engine Opticon (MJ Research Inc., Waltham, MA) or in CFX96™ machine (Bio-Rad Laboratories, Hercules, CA). Primer sequences and annealing temperatures are given in Table 3. The PCR efficiency for each primer pair was determined using qRT-PCR analysis of a dilution series. The relative expression levels for genes of interest were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) using beta-actin as an internal control for data normalization. Results are given as mean ± standard deviations (SD) and represent the relative expression values compared with those obtained in WT caput epididymidis or testis, respectively, which were set to 1 in arbitrary units.

**Table 3.** Primers and annealing temperatures used in qRT-PCRs.

| Gene symbol | Forward primer 5'-3' | Reverse primer 5'-3' | Ann. °C |
|---|---|---|---|
| ***Ar*** | GTCTCCGGAAATGTTATGAA | AAGCTGCCTCTCTCCAAG | 58 |
| ***Apod*** | ACGGAAACATCGAAGTGCTAA | TGGCTTCACCCTTTACTTGG | 58 |
| ***Actb*** | CGTGGGCCGCCCTAGGCACCA | TGGCCTTAGGGTTCAGGGGG | 60 |
| ***Clu*** | TGCTGATCTGGGACAATGG | CTCCCTTGAGTGGACAGTTCT | 60 |
| ***Ctgf*** | TGACCTGGAGGAAAACATTAAGA | AGCCCTGTATGTCTTCACACTG | 60 |
| ***Crisp4*** | TGCCTTTGTTCCTGTTGTGA | TGTCACAGTACCTCGCCAAGATTC | 55 |
| ***Dusp6*** | CGAGACCCCAATAGTGC | AATGGCCTCAGGGAAA | 55 |
| ***Egr1*** | CCTATGAGCACCTGACCACA | TCGTTTGGCTGGGATAACTC | 58 |
| ***Esr1*** | CCG TGTGCAATGACTATG CC | GTGCTTCAACATTCTC | 58 |
| ***Etv5*** | AAGGGGTGGGTCCTGCCCCG | CGGTGGTAGCTGGGGCGACTA | 60 |
| ***FGF8b*** | CCAAGCCCAGGTAACTGTTCA | GGTGCGGCTGTAGAGTTGGTA | 60 |
| ***Fgfr1b*** | GACAGTGAGCCACGCAGACT | GCATTCGGGAATTAATAGCTCG | 60 |
| ***Fgfr1c*** | TCCAGAACGGTCAACCATG | ACCGACAAGGAAATGGAGGT | 60 |
| ***Fgfr2b*** | GGATCAAGCACGTGGAAAAG | ACTGGT TGGCCTGCCCTATA | 60 |
| ***Fgfr2c*** | GGTCCTGAAGGCCGCCGGTGTTA | TCTCTCTCACAGGCGCTGG | 60 |
| ***Fgfr3b*** | AAGGCCTTCTCAGCCACG | GAATGTGGAGGCAGACGC | 60 |
| ***Fgfr3c*** | CCAGCCACGCAGAGTGATG | AGGCGCTAACACCACCGA | 60 |
| ***Fgfr4*** | CGCCAGCCTGTCACTATACAAA | CCAGAGGACCTCGACTCCAA | 60 |
| ***Foxq1*** | CAGGTCGGTGCCTGAGAC | CGCTTATGCTATCGGTCTGG | 60 |
| ***Il17a*** | TCATCCCTCAAAGCTCAGCG | TTCATTGCGGTGGAGAGTCC | 58 |
| ***Il6*** | CCGGAGAGGAGACTTCACAG | CAGAATTGCCATTGCACAAC | 60 |
| ***Lcn2*** | CCATCTATGAGCTACAAGAGAACAAT | TCTGATCCAGTAGCGACAGC | 60 |
| ***Lcn5*** | GTTTTTAGGCTTCTGGTATGA | CTGATATTCTGGTGACCTTGTA | 55 |
| ***Muc1*** | GTGCCAGTGCCGCCGAAAGA | TGCCGAAACCTCCTCATAGGGGC | 60 |
| ***Muc2*** | GCCAGATCCCGAAACCAC | TGTAGGAGTCTCGGCAGTCA | 60 |
| ***Rpl19*** | TCGTTGCCGGAAAAACAC | CCGAGCATTGGCAGTACC | 60 |
| ***Spp1*** | CCCGGTGAAAGTGACTGATT | TTCTTCAGAGGACACAGCATTC | 60 |
| ***Svs2*** | ACAGTCAGCTGTGTTTGTACAATATG | GCCTTTCTGACCAAGCATAAA | 60 |
| ***Sprr1A*** | CCTGAAGACCTGATCACCAGA | AGGCAATGGGACTCATAAG | 60 |
| ***Sprr2A*** | CTGCTCCGGAGAACCTGAT | ACAAGGCTCAGGGCACTTC | 60 |
| ***Shh*** | TCCACTGTTCTGTGAAAGCAG | GGGACGTAAGTCCTTCACCA | 60 |
| ***Tgfb1*** | CAACAATTCCTGGCGTTACCTTGG | GAAAGCCCTGTATTCCGTCTCCTT | 60 |
| ***Tgm4*** | TGCAGAGAGAGGTAGCAGGAC | TCTCTCCACATTCACAGCGTA | 60 |
| ***Tnf*** | CCCCAAAGGGATGAGAAGTT | CACTTGGTGGTTTGCTACGA | 60 |
| ***GI 38086179**** | CCTGGAGGACAGACAGCATC | TGGCAGGAATGTGTACAGATAG | 60 |

*Unknown gene

### 4.2.4 *In situ* hybridization (I)

*In situ* hybridizations for Fgf8b were carried out for formalin fixed, paraffin embedded 5 µm tissue sections. To synthesize riboprobes for Fgf8b, human Fgf8b cDNA derived from pcDNA 3.1 (-) expression vector (a gift from Dr. P. Roy-Burman) was used as a template to amplify the sequence by PCR. Sequences of the PCR primers used which contained the binding sites of T7 and T3 RNA polymerases, respectively (indicated with bold letters) were: F: 5'-CAGAGATGCA**TAATACGACTCACTATAGGGAG A**CCAAGCCCAGGTAACTGTTCAGTCC-3' and R: 5'-CCAAGCCTTC**ATTAACC CTCACTAAAGGGAGA**TTCCCCTTCTTGTTCATGCAGATGT -3'. Purification of the PCR product and synthesis of the digoxigenin-labeled riboprobes, as well as pretreatment of sample slides, the hybridization procedure and the subsequent treatments were carried out as described previously by Jiborn *et al.* (2004). Hybridization was performed overnight at 55°C with probe concentrations of 100 ng/ml.

### 4.2.5 Microarray analysis (I)

To study the gene expression in the ventral prostates (VPs) of three-month-old FGF8b-TG and WT mice Illumina Sentrix® Mouse-6 oligomicroarray BeadChips (Illumina, San Diego, CA) containing 47,769 oligos were used. The microarray hybridization protocol as well as quality control of RNA and were performed at the Finnish DNA microarray center (Turku, Finland). Gene Spring 7.2 software (Agilent Technologies, Santa Clara, CA) was used for analysis of the microarray data and Ingenuity Pathways analysis software (IPA, Ingenuity Systems, http://www.ingenuity.com) for mining the data and constructing functional regulatory networks. Microarray data verification concerning 13 selected genes was carried out by means of a probe-based qRT-PCR method (ProbeLibrary, Roche Diagnostics GmbH, Roche Applied Science, Basel, Switzerland) at the Turku Center of Biotechnology (Turku, Finland). An online assay design program (Probe Finder; http://www.universalprobelibrary.com) was used to design suitable primer pairs (Table 3). L19 was used as a housekeeping gene for normalization of the expression levels.

## 4.3 ANALYSIS OF PROTEINS (I-III)

### 4.3.1 Immuhistochemical and histochemical stainings (I-III)

Paraffin-embedded 5-µm sections on Superfrost slides were used for histochemical stainings immunostainings, with the exception of CD68 staining which was performed using frozen sections. Tissue sections were first deparaffinized, rehydrated in decreasing ethanol series and washed in PBS. Then, depending on the antigen of interest, the antigen retrieval was performed with either 10 mM citrate buffer, pH 6, in a microwave oven, with citric acid buffer pH 6, in a microwave oven or in a pressure cooker or with proteinase K or Ficin at 37˚C (Table 4). In some cases antigen retrieval was not necessary. Endogenous peroxidases were blocked by 20 min incubation in 2%

$H_2O_2$ in PBS. To minimize the nonspecific binding of IgG, blocking with normal serum for 1 h was performed prior to primary antibody incubation in most staining protocols. Incubation with primary antibodies was performed overnight in +4˚C. Biotinylated secondary antibodies, avidin-biotinylated peroxidase complex (Vectastain ABC kit, PK-4000, Vector Inc. Burlingame, CA) and 3,3'-diaminobenzidine (DAB substrate kit SK-4100, Vector Inc.) were used for visualization of the antibody binding. Mayer's hematoxylin was used for counterstaining and the slides were washed, dehydrated, treated with xylene and mounted with Mountex. The primary antibodies used and antigen retrievals performed are specified in the Table 4. Histochemical stainings for PAS (periodic acid Schiff) and Massons Trichrome staining for visualizing of collagen were performed by standard protocols.

**Table 4.** Primary antibodies and antigen retrieval methods used in the IHC stainings.

| ***Antibody against*** | ***Origin, (clone)*** | ***Manufacturer (catalogue #)*** | ***Antigen retrieval*** | Dilution |
|---|---|---|---|---|
| AR | Rabbit PAb, (N-20) | Santa Cruz biotechnology, (SC-816) | CA, pc | 1:100 |
| CD3 (T-lymphocytes) | Rabbit PAb | Abcam, (ab5690) | Citr., mw | 1:250 |
| CD45R/B220 (B-lymphocytes) | Rat MAb, (RA3-6B2) | BD Pharmingen, (550286) | Citr., mw | 1:50 |
| CD68 (macrophages) | Rat MAb, (FA-11) | AbD serotec, (MCA1957) | None | 1:200 |
| Cytokeratins | Rabbit PAb | DAKO, (Z0622) | Prot. K, 37 °c | 1:1000 |
| FGF8b | Goat PAb | R&D systems, (AF-423-NA) | Citr., mw | 1:100 |
| Laminin | Rabbit PAb | Sigma, (L9393) | Ficin, 37 °c | 1:50 |
| p63 | Mouse MAb (4A4) | BD Pharmingen, (559951) | CA, mw | 1:500 |
| SMA (Smooth muscle actin) | Mouse MAb (1A4) | NeoMarkers (MS-113-P) | None | 1:200 |
| SPP1 (Osteopontin) | Goat PAb | R&D systems, (AF808) | Citr., mw | 1:100 |

PAb, Polyclonal antibody; MAb, Monoclonal antibody; CA, 10 mM Citric acid buffer, pH 6; pc, pressure cooker; Citr., 10 mM Citrate buffer, pH 6; mw, microwave oven; Prot. K, Proteinase K.

## 4.4 ANALYSIS OF HISTOLOGICAL CHANGES (I-III)

### 4.4.1 Histological analysis of the prostate (I, III)

Histological analysis for the hematoxylin eosin (HE)-stained prostate sections was done in a blinded manner by T.E. with the assistance of S.M, using the Bar Harbor classification system as a quide line (Shappell et al 2004). Histological sections from several levels of the prostates were evaluated using Olympus BX60 stereomicroscope. In study I, the results of the analysis were confirmed by a clinical pathologist (P.K.). In

study I, prostate sections of altogether 72 FGF8b-TG mice from lines L2, L4 and L5 and of 43 littermate WT mice were analysed. In study III, prostate sections of altogether 52 mice (8 WT, 12 $BERKO_{FVB}$, 21 FGF8b-TG and 11 FGF8b-TG-$BERKO_{FVB}$) of the age of 10.5-14 months were included in the analysis.

In order to study the association between the epithelial and stromal changes in the prostate and between the epithelial changes and inflammation presented by lymphocytes and macrophages in the stroma,TG mice (age 9-20 months, n = 49) were divided into four groups according to the most advanced histological change in their prostate epithelium (normal, hypercellular, mPIN and carcinoma). A numeric score (from 1 to 4) was given to each sample according to the most advanced histological change present in the prostate stroma as follows: 1, normal stroma; 2, hypercellular stroma; 3, atypical stroma; and 4, malignant stroma (I). Evaluation of the degree of prostatic inflammation (scores from 0 to 3) was based on the average number of inflammatory cell aggregates in the prostate stroma in x40 microscopic field as follows: 0, no signs of inflammation; 1, mild inflammation presented by a 1–3 aggregates of inflammatory cells; 2, moderate inflammation with 4–7 aggregates of inflammatory cells and 3, pronounced inflammation with >7 large aggregates of inflammatory cells (I, III).

### 4.4.2 Evaluation of androgen receptor staining (I, III)

Staining grade of androgen receptor (AR) was defined from sections stained by AR IHC by T.E. by estimating the percentage of positively stained nuclei in the VP epithelium and stroma. In cases of normal histology or stromal proliferation, average percentage of AR-positive cells was defined by evaluating at least five representative microscopic fields using x40 objective. For mPIN lesions, the proportion of AR-positive cells was estimated for each mPIN containing acinus and the average proportion per acinus was calculated. For carcinoma, stromal atypia and sarcoma, total areas containing the respective changes were evaluated.

### 4.4.3 Histological analysis of the epididymis and testis (II)

Histological analysis of the epididymis and testis was performed by T.E. with the assistance of P.K., P.S. (epididymis) and J.T. (testis) in a blinded manner from hematoxylin–eosin stained samples of 3- to 20-month-old FGF8b-TG (epididymis, n = 29; testis, n = 26) and WT mice (epididymis, n = 20; testis, n = 20). The samples were collected from generations $F_0$-$F_3$ of the FGF8b-TG lines L2, L4 and L5.

## 4.5 ANALYSIS OF SERUM TESTOSTERONE (II)

Measurement of serum T levels was performed by a standard RIA method as previously described (Huhtaniemi *et al.,* 1985). Samples analysed were from 3- to 5-month-old WT (n = 7) and L4 FGF8b-TG (n = 4) mice, and from 7- to 16-month-old WT (n = 12) and FGF8b-TG mice of lines L2 (n = 7), L4 (n = 8) and L5 (n = 2).

## 4.6 STATISTICAL ANALYSIS (I,II,III)

Statistical analyses were performed using SPSS 11.0 software for Windows (SPSS, Chicago, IL) and by GraphPad Prism 6 (GraphpadSoftware, Inc.). Differences in the frequencies of the histological changes between groups were tested by the $chi^2$-test. For qRT-PCR results, the normal distribution of the data was tested using the Shapiro–Wilk *W*-test. Then, the two-sample *t*-test, the Mann–Whitney *U*-test or analysis of variance (One-way ANOVA) or Kruskall-Wallis test were applied to test for the differences between groups. In analysis containing multiple comparisons, Bonferroni corrections or Dunn's multiple comparison tests for *p*-values were applied. Logistic regression analyses (LRAs) were applied to study which histological changes (inserted as binary categorial variables, present or absent) could predict the presence of mPIN lesions in FGF8b-TG mouse prostates. The predictive ability of each factor was studied separately and adjusted for age**.** Values of $p<0.05$ were considered statistically significant.

# 5. RESULTS

## 5.1 CHARACTERIZATION OF THE FGF8B-TG MICE (I, II)

### 5.1.1 FGF8b is expressed in the reproductive organs of the FGF8b-TG mice

Analyses of multiple mouse tissues by RT-PCR indicated that FGF-8b was expressed in all the prostate lobes (VP, DLP and AP), the ductus deferens, the urethra, the epididymides and the seminal vesicles of FGF8b TG mice with the highest expression in the VP. According to the qRT-PCR data, FGF-8b mRNA levels in the VPs were hundred to several thousand times higher than in the WT mice depending on the FGF8b-TG mouse line and the expression levels remained high in the VPs of old (12- to 16-month-old) FGF8b-TG mice and persisted at a high level independent of the generation ($F_1$-$F_5$) of the FGF8b-TG mice. Based on the macroscopic phenotype of urogenital organs and expression level of the transgene, FGF8b TG lines L2, L4 and L5 were selected for further analysis of prostate and epididymis. Analyses of epididymal segments (caput, corpus and cauda) and testis by qRT-PCR showed that the expression of FGF8b was significantly upregulated in all the epididymal segments of the FGF8b-TG mice. In the FGF8b-TG mouse lines L4 and L5, the expression was highest in the caput epididymidis, and in the TG mouse line L2 in the cauda epididymidis. Moreover, it was discovered, that in the L4 mouse line, the epididymal expression of FGF8b mRNA was especially high in the $F_1$ generation, whereas in the subsequent generations ($F_2$-$F_5$), the FGF8b mRNA level was still overexpressed, but at a much lower level. In the testes of FGF8bTG mice, the FGF8b mRNA level was upregulated in the L2 mice and in the $F_1$ generation of L4 mice, whereas no upregulation of FGF8b was found in the testes of L4 mice in the $F_2$-$F_5$ generations or in the L5 mice. Low levels of endogenous FGF8b mRNA were found in the caput and corpus epididymis and in the testis of WT mice.

The expression of FGF8b was localized to prostate epithelium of FGF8b-TG mice by FGF8b *in situ* hybridization and by FGF8 IHC. In line with mRNA data, FGF8b IHC showed strongest staining in the VP. In the epididymis of FGF8b-TG mice, positive staining for FGF8b was found mainly in the epithelial cells and the ductal lumens but in some cases positive staining was also present in a small proportion of the stromal cells. In approximately half of the FGF8b-TG mice, the developing germ cells in the seminiferous epithelium of the testes showed positive staining for FGF8b. However, there was no stage specificity in the spermatogenesis in the staining pattern. The testes of WT mice showed no staining or a very weak staining for FGF8b which also localized in germ cells.

## 5.1.2 FGF8b-TG mice have alterations in prostate, epidymis and testis

### *5.1.2.1 Prostates of FGF8b-TG mice display progressive epithelial and stromal changes*

The gross phenotype and the body weights of the FGF8b-TG mice were normal. In FGF8b-TG mice older than six months, prostates were bigger in size and weight than in WT mice and in the VPs of FGF8b-TG mice older than 10 months, there were often swellings, hemorrhagic cysts and tumors.

Progressive changes in prostate epithelium and stroma, with frequencies and severity increasing upon aging were found in the histological analysis of the prostates of FGF8b-TG mice from lines L2, L4 and L5. Most changes were located in the VP, whereas some were also found in the DLP and less frequently in the AP. In the prostates of the young (3-6 month old) FGF8b-TG mice, stromal hypercellularity with increased vasculature as well as epithelial hypercellularity were frequently observed. In FGF8b-TG mice older than six months, epithelial hyperplasia, with atypic features, were present. Focal or multifocal mPIN lesions first appeared in a 9 month-old mouse. PIN lesions became more frequent in mice older than 12 months, in which the frequency was nearly 50 percent. Malignant changes, classified as adenocarcinoma, were found in VPs of 8 percent of old FGF8b-TG individuals. Stromal hypercellularity with atypical cells, which in some cases presented a focal phyllodes-type growth pattern, were found in FGF8b-TG mice older than 10 months with the frequency of 32 percent in mice older than 12 months. Based on the histology and destructive growth pattern, some of these stromal atypias were classified as sarcomas or as carcinosarcomas when co-localised with adenocarcinoma. Stromal and epithelial changes and aberrancies often co-existed in the same prostates. Inflammation, presented as aggregates of several types of inflammatory cells, including T and B lymphocytes and macrophages, mainly in the prostatic stroma was also frequent in FGF8b-TG mice older than 6 months (incidence > 50%). The epithelial and stromal changes often co-localized in the same prostates and it was found that the presence of mPIN lesions and malignant changes in FGF-8b-TG prostates was significantly associated with stromal changes and inflammation. Based on the FGF8b IHC, the neoplastic changes including the adenocarcinomas still expressed FGF8b. In contrast to this, sarcomas and carcinosarcomas did not express FGF8b.

### *5.1.2.2 Epididymides of FGF8b-TG mice contain epithelial and stromal alterations*

In the young FGF8b-TG mice, macroscopic abnormalities in the epididymis were infrequent. Upon aging, the frequencies of uni- or bilateral abnormalities of the epididymis became more frequent especially in the FGF8b-TG mouse line L5 but also in the L4 and L2 mouse lines. The abnormalities included increased size, swellings, cysts, hemorrhages and tumor-like morphology of the epididymis and decreased size of the testis. The epididymal abnormalities were most pronounced in the caput epididymides in all the FGF8b-TG mouse lines. The presence and the severity of the epididymal abnormalities seemed to associate with the expression of FGF8b, because

they were more severe in the $F_1$ mice of L4 line than in the subsequent generations of the same mouse line, which had decreased FGF8b mRNA levels.

Histological evaluation of the FGF8b-TG mice epididymides revealed progressive epithelial and stromal hypercellularity and oligospermia. Epithelial hypercellularity were most pronounced in the caput epididymis, but also present in other epididymal segments. Other changes found in the FGF8b-TG epididymis included the dilatation of the epididymal duct, characterized by a remarkably increased luminal space and inflammation characterized by lymphocyte aggregates in the hypercellular stroma. Generally, the histological changes were more frequent and more severe in the L4 and L5 than in the L2 FGF8b-TG mouse line. The epididymal hypercellularities were mild or moderate in the young FGF8b-TG mice but became more frequent and advanced in the older mice, which often had epithelial dysplasias and stromal hyperplasias containing atypic cells. In two macroscopically tumor-like epididymides of old TG mice (> 10 months old) the epididymal stroma was characterized as malignant. Interestingly, in these epididymides, the stroma presented a phyllodes growth pattern, similar that was found in the prostate stroma of some FGF8b-TG mice.

#### *5.1.2.3 Testes of FGF8b-TG mice contain degenerative seminiferous epithelium*

Some FGF8b-TG mice had smaller testis than the WT mice, whereas most of the FGF8b-TG mice had macroscopically normal testis. However, the histological evaluation of the testis revealed changes, such as a degenerative seminiferous epithelium, decreased number of spermatids and less frequently, hyperplasia of the testicular interstitial tissue. Degeneration of the seminiferous epithelium was found already in some 3 month-old FGF8b-TG mice but the frequency was increased upon aging. The degeneration of the seminiferous epithelium was heterogenous along the testicular tubules and did not show any spatial pattern or stage specificity in the spermatogenesis, but seemed to be caused by a general destruction of the developing germ cells in the seminiferous epithelium. There was an association between epidimymal and testicular changes, because changes in the epididymal histology were more frequent in those mice that also had degeneration of seminiferous epithelium in the testis.

#### *5.1.2.4 A subset of the FGF8b-TG male mice is infertile*

Infertility was observed in the FGF8b-TG male of the lines L4 and L5 during mouse breeding. The infertile males had macroscopically abnormal epididymides and in most cases small testis. The number of spermatozoa prepared from the cauda epididymis of the FGF8b-TG mice was dramatically decreased and compared to the WT mice the spermatozoa seemed to be more fragile as their heads and tails were often detached.

### 5.1.3 FGF8b-TG mice have normal serum testosterone levels

The serum T levels of FGF8b-TG mice were compared to those of WT mice of similar age. Both the young and the old FGF8b-TG mice had normal serum T levels. Also, the serum T levels of the infertile TG mice or the TG mice with abnormalities in the

epididymis and testis were similar to the WT mice suggesting that the fertility problems were not caused by insufficient production of T and that the TG expression in the testis did not affect the Leydig cell function.

### 5.1.4 Prostates and epididymides of FGF8b-TG mice display altered staining pattern of epithelial and stromal proteins

The histological changes in the prostate and epididymis were further evaluated by histochemical and IHC staining, which revealed striking similarities in the staining patterns of several markers. The basal lamina, visualized by the laminin staining, was thicker in both the prostates and the epididymides containing hyperplastic stroma in the FGF8b-TG mice. In both prostates and epididymides containing premalignant and malignant changes the basal lamina became progressively more irregular and discontinuous. There was generally a decrease in the proportion of smooth muscle actin (SMA) positive cells in the hypercellular and malignant stroma in both the prostate and the epididymis of the FGF8b-TG mice. SMA-negative stroma was seen also around PIN-lesions in the prostate. Massons Trichrome staining showed that the hypercellular stroma in both the prostates and the epididymis was mainly composed of collagen fibers indicative of fibroblastic cells. Atypical stroma around advanced mPIN lesions and adenocarcinoma in the prostates and in sarcoma-like foci in both prostate and epididymides of FGF8b-TG contained abundantly collagen fibers between expanded masses of malignant cells. Formation of a slightly thickened collagen rich periacinar stroma similar to that observed in the prostates of young adult FGF8b-TG mice could also be induced by T treatment of prepubertal FGF8b-TG mice, which induced the expression of the FGF8b transgene driven by the androgen-induced $ARR_2PB$ promoter. In the hypercellular stroma, the frequency of AR-positive cells was decreased in the prostate, whereas in the hypercellular stroma of the epididymis, it was not clearly changed from the normal. Interestingly, in both the prostates and the epididymides of FGF8b-TG mice, the malignant cells in the stroma with a phyllodes growth pattern were strongly AR positive.

In the prostate, the transformed epithelial cells in the mPIN lesions, but especially in the adenocarcinomas and carcinosarcomas showed decreased staining for cytokeratins indicating dedifferentiation of the transformed epithelia. In line with this, transformed epithelium of the mPIN lesions and adenocarcinomas had also decreased percentage of AR-positive cells. Stromal areas of the TG prostates containing atypical stromal cells and sarcoma-like lesions were negative for cytokeratins. Most of the mPIN lesions and tumor cells in the adenocarcinoma lesions continued to express FGF8b in the prostates of FGF8b-TG mice. In contrast, sarcomatous areas did not show any staining for FGF8b, and tumor cells in carcinosarcoma were not stained either.

### 5.1.5 Expression of *Spp1, Ctgf, Apod* and *Foxq1* is upregulated in the prostates and epididymides of FGF8b-TG-mice

Based on the results of the microarray analysis and further confirmation by qRT-PCR the expression levels of osteopontin (*Spp1*), connective tissue growth factor (*Ctgf*),

apolipoprotein D (*Apod*) and forkhead box 1 (*Foxq1*) were significantly upregulated in the VPs of young FGF8bTG mice. All these genes were also found to be upregulated in the caput epididymides of old FGF8b-TG mice. A significantly upregulated expression of *Spp1* and *Ctgf* was also verified in the VPs of older (12- and 15-month-old) FGF8b-TG mice. Moreover, it was demonstrated by SPP1 IHC that in the FGF8b-TG prostates with stromal proliferation, the stromal cells especially adjacent to the epithelium, were positive for SPP1. Some of the transformed epithelial cells in mPIN and adenocarcinoma lesions also stained positive for SPP1.

The expression of known epididymal FGF target genes, ets (E twenty six) variant 5 (*Etv5*) and *Dusp6*, was downregulated in the caput epididymides of FGF8b-TG mice. The expression of Fgfr1-3 b and c isoforms was also studied by qRT-PCR and it was found that in the caput epididymides with high FGF8b mRNA expression, the expression of *Fgfr1c* was upregulated, and *Fgfr3c* was downregulated compared with the WT mice. No changes in the expression of other *Fgfrs* were found.

## 5.2 CHARACTERIZATION OF THE BERKO$_{FVB}$ AND FGF8B-TG-BERKO$_{FVB}$ MICE (III)

### 5.2.1 FGF-8b TG-BERKO$_{FVB}$ mice (III) are viable and normal

Breeding of FGF8b-TG and BERKO$_{FVB}$ mice in two subsequent generations was successful and the genotypic division of the offspring was as expected. Offspring of all the genotypes, including the FGF8b-TG-BERKO$_{FVB}$ mice, were viable and had a normal macroscopic phenotype.

### 5.2.2 *Erβ1* and *Erβ2* are expressed in the mouse prostate

By RT-PCR of *Erβ* and subsequent cloning and sequencing of the PCR products, we found that the prostates of WT mice expressed mRNA's for the *mErβ1* ("wt isoform") and *mErβ2*. Shorter forms of these *Erβ* mRNAs were found from the prostates of BERKO$_{FVB}$ mice. According to the sequencing results, these shorter Erβ mRNA forms, which are the result of alternative splicing of the NEO-cassette-containing *Erβ* gene, contain early stop codons in exon four as was previously reported by Krege *et al.* (1998) and therefore they are not translated to functional, full length proteins.

### 5.2.3 Prostates of FGF8b-TG-BERKO$_{FVB}$ mice contain increased frequency of mucinous metaplasia and inflammation

The urogenital organs, including the prostate of the BERKO$_{FVB}$ mice, were macroscopically normal. The prostates and less frequently also the epididymides of the FGF8b-TG-BERKO$_{FVB}$ mice were enlargened and abnormal, resembling the phenotype of these organs in the FGF8b-TG mice.

Evaluation of the prostate histology was performed from the prostate samples of 10-14 month old WT, BERKO$_{FVB}$, FGF8b-TG and FGF8b-TG-BERKO$_{FVB}$ mice. For

the most part the prostate histology of $BERKO_{FVB}$ mice was normal, but focal epithelial hyperplasia and inflammation presented by lymphocytes in the stroma tended to be more frequent than in WT mice. As described in our previous study, the prostates of the FGF8b-TG mice contained a high frequency of both epithelial and stromal abnormalities of which some were premalignant or malignant. The histological changes, their frequencies and their presence in different prostatic lobes of the FGF8b-TG-$BERKO_{FVB}$ mice were for the most part similar to those in the prostates of FGF8b-TG mice. However, despite nearly similar frequencies of epithelial and stromal hyperplasia in both mouse groups, epithelial and stromal hyperplasias containing atypic cells seemed to be less frequent and especially the stromal hyperplasias were often less extensive in the prostates of FGF8b-TG-$BERKO_{FVB}$ compared to FGF8b-TG mice. Malignant changes were absent from FGF8b-TG-$BERKO_{FVB}$ prostates. Focal mucinous metaplasia presented by PAS-staining-positive Goblet-like cells in the prostate epithelium, was significantly more frequent in FGF8b-TG-$BERKO_{FVB}$ mice than in FGF8b-TG mice ($p=0,028$). In addition, there was a tendency to increased frequency of inflammation, presented by lymphocytes in the prostate stroma, in FGF8b-TG-$BERKO_{FVB}$ mice compared to FGF8b-TG mice, but inflammation was not more widely spread than in the FGF8b-TG mice.

### 5.2.4 Differential staining patterns of stromal proteins the prostates of FGF8b-TG-$BERKO_{FVB}$ and FGF8b-TG mice

Imamov *et al.* (2004) reported previously that BERKO prostates contain increased numbers of p63- positive basal cells (Imamov *et al.,* 2004). In contrast to this, we did not find differences in the overall frequencies of p63-positive cells in the epithelium among the mouse groups studied (WT; FGF8b-TG; $BERKO_{FVB}$; FGF8b-TG-$BERKO_{FVB}$). However, it seemed that focally, in the hyperplastic foci of the $BERKO_{FVB}$ prostate epithelium, there was an increase in the percentage of the p63-positive cells but this difference was diluted when bigger areas of the prostate were analysed. In the prostates of FGF8b-TG-$BERKO_{FVB}$ mice, there were epithelial foci with increased and decreased number of basal cells, but overall the frequency of p63-positive cells was normal.

Almost 100 percent of the prostate epithelial cells were AR-positive in all mouse groups, but in the mPIN lesions of FGF8b-TG and FGF8b-TG-$BERKO_{FVB}$ mice, there was a focal decline in the percentage of AR-positive cells. In line with our previous results, the percentage of AR-positive cells tended to be decreased in the hypercellular areas of the prostate stroma of FGF8b TG mice whereas in the malignant prostate stroma of FGF8b TG mice there was increased percentage of AR-positive cells. Compared to FGF8b-TG mice, the percentage of AR-positive cells in the hypercellular stroma of FGF8b-TG-$BERKO_{FVB}$ mouse prostates varied more and was either decreased, similar or increased compared to normal stroma. In the hypercellular stroma of prostates of both Fgf8b-TG and Fgf8b-TG-$BERKO_{FVB}$ mice, there were SMA-negative areas surrounding the prostatic acini, but in Fgf8b-TG mice these areas were more extensive. According to Massons trichrome staining, the hypercellular prostate stroma in both the FGF8b-TG and FGF8b-TG-$BERKO_{FVB}$ contained increased amount

of collagen fibers, but in the prostates of FGF8b-TG mice, the collagen-rich areas were generally wider and the collagen fibers were less organized than those in the prostates of FGF8b-TG-BERKO$_{FVB}$ mice. Differences in the staining patterns of hypercellular stroma in the FGF8b-TG and FGF8b-TG-BERKO$_{FVB}$ mice may reflect differences in the cellular composition of the stroma.

### 5.2.5 Changes in the gene expression are similar in the prostate of FGF8b-TG-BERKO$_{FVB}$ and FGF8b-TG mice

Based on the qPCR analysis, expression of FGF8b mRNA was equally high in the VPs of FGF8b-TG-BERKO$_{FVB}$ mice as in the VPs of FGF8b-TG mice. Similar overexpression of *Spp1* and *Ctgf* as previously described by us in the VPs of FGF8b-TG mice was also found in FGF8b-TG-BERKO$_{FVB}$ mice. The expression of *b* and *c* isoforms of *Fgfr1* and *Fgfr2* was studied in VPs of all the mouse groups. Compared to prostates of WT mice, the expression of *Fgfr1c* was found to be upregulated and the expression of *Fgfr2c* downregulated in the VPs of FGF8b-TG and FGF8b-TG-BERKO$_{FVB}$ mice. There were no significant differences in the mRNA levels of *Ar* and *Erα* between the prostates of different mouse groups, despite a tendency to higher level of Ar in the BERKO$_{FVB}$ mice which was actually significantly higher than *Ar* mRNA level of FGF8b-TG mice but did not differ from WT mice. The expression of *Tnfα* was upregulated in the prostates of FGF8b-TG and FGF8b-TG-BERKO$_{FVB}$ mice, but no differences were found in the mRNA levels of other pro-inflammatory cytokines such as *Il6* and *Il17,* or markers associated with cancer progression or mucinous phenotype such as *Tgfβ1*, *Muc1* or *Muc2* among the prostates of different mouse groups.

# 6. DISCUSSION

## 6.1 THE RELEVANCE OF MOUSE MODELS IN STUDYING HUMAN PROSTATE CANCER

In this thesis, GEM models were used to study the effects of a potential oncogene, FGF8b and a potential tumor-suppressor gene ERβ, in the prostate. In addition, the effects of FGF8b overexpression in other male reproductive tissues such as the epididymis and testis were examined. The relevance of using mouse models, including GEMs and xenograft models to study human prostate cancer, can be critizized based on the differential anatomy of the prostate, on the general physiological differences between human and mouse and the fact that naturally occurring prostate cancer is extremely rare in mice (Valkenburg & Williams, 2011). The fact that none of the numerous available mouse models generated to date can mimic all the steps of human prostate tumorigenesis from initiation to development of bone metastasis has also raised questions about the effectiveness of using a mouse as a model animal for prostate cancer (Hensley & Kyprianou, 2012, Ittmann *et al.,* 2013). On an ethical basis, the use of animals to study human diseases can also be generally questioned.

Despite the limits of mice as model organisms in studies of prostatic diseases, there are many facts that support their use. First, despite the morphological and histological differences between human and rodent prostate, there is evidence that the basic molecular mechanism mediating the cell-cell interactions are highly conserved in mammals. Tissue recombinant experiments have proven that rodent UGM can induce growth, ductal morphogenesis and prostate specific differentiation of epithelium derived from adult human prostate or bladder, implicating the similarity of molecular mechanisms between the species and the relevancy of rodent models to study human prostate biology (Cunha, 2008). The mouse and human genomes are also highly similar with the average of 79 percent similarity at the amino acid level (Mouse Genome Sequencing Consortium *et al.,* 2002), which enables studying the function of most human genes in the mouse.

Second, mouse models, and especially GEMs produced to date, have gained valuable information on the function of specific genes in prostate tumorigenesis. Many of the genes studied in prostate-specific GEM models were found initially to be deregulated in the human prostate cancer. By a GEM model, the functional mechanisms involved in prostate tumorigenesis, were explored (Ittmann *et al.,* 2013, Jeet *et al.,* 2010). Third, similar functional studies in the physiological context as in GEM models in the presence of complex stromal microenvironment, functional immune system, vasculature, nerves and endogenous hormones, which are known to contribute to tumorigenesis and cancer progression, are not possible to carry out with other methods currently in use. Another advantage of GEM models over other methods is that with them it is, in theory, possible to study all the stages of prostate cancer progression from initiation to metastasis (Ittmann *et al.,* 2013). Orthotopic xenograft models in which prostate cancer cells are inoculated to nude mouse prostate also enable studying of genetic manipulations in the presence of stromal microenvironment,

but they are impeded by the fact that the immune system of the recipient mice is defective, which can affect tumorigenesis. Xenograft models are limited to the use of available prostate cancer cell lines, which present the advanced stage of prostate cancer and therefore are not suitable for studying earlier stages of tumorigenesis. *In vitro* studies using human prostate cancer cell lines provide a fast method to characterize intracellular mechanisms in prostate cancer cells, but they lack the tumor microenvironment and the physiological context (Hensley & Kyprianou, 2012, Ittmann *et al.,* 2013). *In vitro* studies are limited also by the scarcity of prostate cancer cell lines due to the difficulty in establishing such cell lines. Tissue culture experiments using human prostate tissue are useful in studying the biology and regulation of prostate cancer growth but they also lack the complex physiological context of the body. Human prostate cancer tissue specimens will continue to have a central role in prostate cancer research because they enable the studying of molecular changes and the heterogeneity of the disease at the molecular level. Using new high throughput methods, such as genomic sequencing, will improve analyzing the tissue samples, but *in vivo* models are still required to perform functional studies to understand the biological significance of the changes found and to test new therapeutic methods (Ittmann *et al.,* 2013).

Besides rodents, the use of dogs in prostate cancer research has been implicated, because their prostate anatomy resembles that of human and prostate cancer occurs naturally with high frequency. Dogs might be effective for therapeutic studies, but their use in studying genomic manipulations would be unpractical and expensive compared to rodents (Ittmann *et al.,* 2013, Valkenburg & Williams, 2011).

Altogether, mouse models have provided valuable information about the molecular biology of prostate cancer and broadened the knowledge on the function of specific genes in the prostate. They are central for preclinical therapeutic studies of prostate cancer. As discussed above, mouse models and especially GEMs have certain advantages over other methods used in functional studies on prostate cancer research and the future studies will show which are the optimal *in vivo* models to improve screening and treating of prostate cancer.

## 6.2 EFFECTS OF FGF8b OVEREXPRESSION IN THE PROSTATE

FGF8 is a transforming growth factor, which is expressed at an elevated level in hormonal cancers including prostate cancer (Mattila & Harkonen, 2007) and in premalignant PIN lesions (Valve *et al.,* 2001). This suggests a putative role for FGF8 in the early steps of prostate tumorigenesis. A previous TG mouse model with prostate-targeted overexpression of FGF8b provided further evidence for the involvement of FGF8b in prostate cancer initiation, as mPIN lesions developed in the prostates of these mice (Song *et al.,* 2002).

The results of the present study advance the role that FGF8b plays in prostate tumorigenesis. We showed, for the first time, that overexpression of FGF8b induced neoplastic changes, which were unreported in the study by Song *et al.(*2002) These

included adenocarcinoma, sarcoma and carcinosarcoma in the prostates of old FGF8b-TG mice. Another new finding was the development of progressive stromal changes in the prostate, which preceded the formation of PIN lesions and co-evolved with the epithelial changes upon aging. The hypercellular stroma of FGF8b-TG mice resembled the reactive stroma present in human prostate cancer (Tuxhorn *et al.,* 2002a), because it contained increased amount of collagen and decreased amount of AR-positive smooth muscle cells. The hypercellular stroma also contained inflammation and increased angiogenesis, which are characteristic of the reactive stroma.

Expression of genes previously found to be prostate tumorigenesis promoting factors expressed by the inflammatory cells or the reactive stroma, namely *Spp1* (Castellano *et al.,* 2008, Khodavirdi *et al.,* 2006, Pazolli *et al.,* 2009) and *Ctgf* (Yang *et al.,* 2005) was upregulated in the VPs of FGF8b-TG mice and SPP1 was demonstrated to locate mainly in the hypercellular stroma. As formation of collagen-rich stroma could be induced by a brief androgen treatment, which activated the expression of FGF8b in the prostates of prepubertal FGF8b-TG mice, it was concluded that epithelial FGF8b expression induced stromal growth in a paracrine manner. Stromal alterations and inflammation were associated with epithelial changes and were predictive for the presence of mPIN lesions. Our results support the notion that stromal activation facilitated the development of mPIN lesions and carcinomas in the prostates of FGF8b-TG mice. Interestingly, reactive stroma associated with mPIN lesions and adenocarcinoma is present also in numerous other prostate-targeted GEM models (Ittmann *et al.,* 2013). Reactive stroma is also found in association with human prostate cancer (Ayala *et al.,* 2003, Tuxhorn *et al.,* 2001), but stromal changes tend to be more pronounced in the GEM models (Ittmann *et al.,* 2013). However, a recent study found that the presence of extensive reactive stroma in human prostate cancer associates with aggressive prostate cancer with a lethal outcome (Ayala *et al.,* 2011).

Interestingly, the mixed types of malignant changes found in our FGF8b-TG mice, including adenocarcinomas, sarcomas and carcinosarcomas, closely resemble those earlier observed in the inducible FGFR1-TG (iFGFR1-TG) mice (Acevedo *et al.,* 2007). iFGFR1-TG mice also developed mixed epithelial and stromal malignancies including sarcomatoid carcinomas, which were reported to be a result of EMT and subsequent invasion of the transformed epithelial cells in the stroma. The presence of EMT in our model could not be proven because of insufficient evidence. Epithelial origin of malignant stromal cells was supported by the increased number of AR-positive cells in the sarcoma-like lesions and by the absence of cytokeratin expression in both the malignant epithelium and stroma. On the other hand, expression of FGF8b in the carcinomas, but not in the sarcomas or carcinosarcomas suggested a different origin for epithelial and stromal malignancies. Interestingly, the expression of *Fgfr1c* was found to be upregulated and *Fgfr2c* downregulated in the prostates of old FGF8b-TG mice, suggesting that signaling via FGFR1c might well be involved in the tumorigenesis and explain the similarities with the iFGFR-TG model. Similar upregulation of *Fgfr1c* was also found in the caput epididymides of the FGF8b-TG-mice. This is in line with the previous results of our research group, which have indicated that FGF8b and FGF2 can upregulate the expression of *Fgfr1* in the S115 and in MCF7 breast cancer cells (Ruohola

*et al.,* 1995, Tarkkonen *et al.,* 2012). Upregulation of *Fgfr1* expression by FGF8 occurs also in the neuronal cells (Mott *et al.,* 2010). This method of autoregulation provides a mechanism to enhance tumorigenesis, because according to current knowledge, signaling via FGFR1 and FGFR4 promotes prostate tumorigenesis (Acevedo *et al.,* 2009, Gowardhan *et al.,* 2005, Kwabi-Addo *et al.,* 2004, Murphy *et al.,* 2009, Sahadevan *et al.,* 2007) whereas signaling via FGFR2 has the opposite effect and mainly supports homeostasis (Freeman *et al.,* 2003a, Kwabi-Addo *et al.,* 2004). Signaling via FGFR1 is also supported by upregulation of *Spp1* in the prostates and epididymides of FGF8b-TG mice, because previous studies have shown that expression of *Spp1* can be induced by FGFR1 activation but not by FGFR2 activation (Freeman *et al.,* 2003a). However, it is probable that in our FGF8b-TG model, signaling via other FGF8b-binding FGFRs (FGF2c, FGFR3c and FGF4) expressed in the prostate contributed to the outcome and could partly explain the heterogenous prostate phenotypes observed among FGF8b-TG mice, because as mentioned above, signaling via different FGFRs has different roles in the prostate.

The atypic and malignant lesions in the prostates of FGF8b-TG mice resemble "stromal tumors of uncertain malignant potential" (STUMPs), which are rare stromal lesions in human prostate, some of which can become malignant. The phyllodes growth pattern and AR expression observed in the stromal atypias and malignancies of FGF8b-TG mouse prostates exist in human STUMPs and sarcomas (Hansel *et al.,* 2007). However, sarcomas are rare in the human prostate. Interestingly, sarcomas and sarcomatoid carcinomas associated with adenocarcinomas in the prostate are present in numerous other prostate-targeted GEM models. In many of these models, development of the sarcomatoid carcinomas is believed to be a result of EMT in the transformed epithelial cells (Ittmann *et al.,* 2013). The relevance of stromal malignancies in GEM prostates, in terms of human prostate cancer, remains to be elucidaded.

One limitation of our FGF8b-TG mouse model was that the prostatic changes developed slowly as the mPIN lesions were found, at the earliest, in 10 month-old mice and malignant changes at the earliest, in one year-old mice. In addition, the prostate phenotype was heterogenous and all the mice did not develop any changes. These features complicated studying the phenotype and associated changes in the mice, but they can also be interpreted as advantages, because both the slow progression and the heterogeneity are typical features of human prostate cancer. Therefore, the current model may mimic the tumorigenesis in human prostate better than models with aggressive prostate cancer soon after the puberty.

Taken together, the present results underline the capacity of FGF-8b to induce malignant changes in the prostate and the importance stromal activation in FGF-8b induced prostate tumorigenesis. The results suggest that epithelially expressed FGF8b acts in both autocrine and paracrine manners to induce changes in the prostate epithelium and stroma, which leads to altered crosstalk between the two compartments and subsequently, to gradually developing atypias and malignancies. It is suggested that FGF8 may promote tumorigenesis by activation of stroma also in human prostate cancer.

## 6.3 EFFECTS OF FGF8b OVEREXPRESSION IN THE EPIDIDYMIS AND TESTIS

The FGF8b-TG mice were initially developed to study prostate phenotype. Because the transgene expression and morpholocical changes, associated with infertility, were found also in the epididymis and testis of the FGF8b-TG mice, we wanted to characterize these changes further. Interestingly, the epididymis and the testis are among the few organs that express FGF8 in the adult mouse (Fon Tacer *et al.,* 2010). Actually, besides the FGF8 expressed in the epididymis, the epididymis can be exposed to FGF8 in a lumicrine way, because FGF8 is present in the testis derived RTF (Kirby *et al.,* 2003). FGF8 and its receptors are expressed in both the epididymis and the testis (Cancilla & Risbridger, 1998, Fon Tacer *et al.,* 2010) but its specific function in these organs remains unknown.

Interestingly, the histological and the molecular changes found in the epididymides of FGF8b-TG mice closely resembled those earlier described in the prostate of the FGF8b-TG mice. The epididymides of the FGF8-b-TG mice presented stromal and epithelial hypercellularity that progressed upon aging to atypical and malignant changes in the stroma and dysplastic changes in the epithelium. In the similar way as in the prostate, FGF8b was primarily expressed in the epididymal epithelium, but most pronounced phenotype was seen in the stroma. Collagen fibers and inflammatory cells were abundant in the hypercellular stroma of the epididymis of the FGF8b-TG mice in the same way as in the prostates. The smooth muscle layer and basement membrane became irregular in both organs as the stroma became atypic. The malignant changes in the epididymis stroma contained AR-positive cells as was reported in the prostates. As the composition of the stroma and the changes in the gene expression were found to be highly similar in the epididymis and in the prostates of FGF8b-TG mice, it is suggested that the molecular mechanisms induced by FGF8b overexpression in both organs are highly similar.

The epididymis is known as a highly cancer resistant tissue, and epididymal cancers are extremely rare in humans (Ganem *et al.,* 1998) and also difficult to induce in GEM models (Yeung *et al.,* 2012). Therefore it is notable, that malignant changes, namely sarcomas, developed in the epididymis of the FGF8b-TG mice. However, in contrast to the prostate, FGF8b-TG mouse epididymides did not develop adenocarcinomas whereas dysplasias were present in the epithelium of some of the epididymides of old FGF8b-TG mice. This could reflect the high cancer-resistance of the epididymal epithelium. Epididymal cells may have gained resistance to oncogenic stimuli by constitutive expression of angiogenic and oncogenic factors, which are tumor-inducible in other tissues more prone to develop cancer (Yeung *et al.,* 2012). For example, the epithelial cells in the IS of the epididymis are constitutively exposed to growth factors including FGFs present in RTF, but in contrast to most of the other cell types, they do not respond to these factors by proliferating or by differentiating (Cotton *et al.,* 2008). Several other mechanisms such as the anti-tumor mechanisms involved in maintaining spermatozoa in the quiescent stage can potentially contribute to epididymal tumor-resistance, but the issue still remains enigmatic (Yeung *et al.,* 2012). In our TG-model, high level of FGF8b expression was required to induce

morphological and gene expressional changes in the epididymis. In addition, pronounced changes were present in the caput and cauda epididymis, but not in the corpus epididymis, which was the only segment in which FGF8b was endogenously expressed. These results might reflect the resistance of epididymal epithelium to oncogenic FGF8b unless its level is considerably high.

Our data were insufficient to conclude whether the degeneration of the seminiferous epithelium and the hypospermatogenesis in the testis of FGF8b-TG mice was a primary effect of FGF8b overexpression in the seminiferous epithelium or a secondary effect caused by the epididymal changes such as the stromal hypercellularity found in association with degenerative testicular epithelium. FGF8b may have a primary effect on testis because FGF8b-binding FGFRs are expressed in the developing germ cells and in Sertoli cells (Cancilla & Risbridger, 1998). A primary effect of FGF8b is supported by the the histological observation that the degenerative phenotype in the testis did not present a spatial pattern but was sporadic along the tubules. In the case of pressure atrophy caused by an occlusion or a defect in fluid reabsorption, downstream of the testis, a clear spatial pattern in the degeneration of seminiferous epithelium, advancing from the direction of rete testis would be expected, as is the case in the ERKO mice (Zhou *et al.,* 2001). The specific mechanisms underlying the infertility observed in the subset of FGF8b-TG mice also remain unsolved, but it is probable that both the testicular and the epididymal phenotypes of FGF8b-TG mice reduce the fertility by separate mechanisms.

Taken together, the current results show that disruption of normal FGF-signaling by increased expression of FGF8b disturbs the cellular homeostasis in the epididymis and testis and leads to development of hyperplasias, dysplasias and malignancies in the epididymis and to disruption of developing germ cells in the testis, which can cause

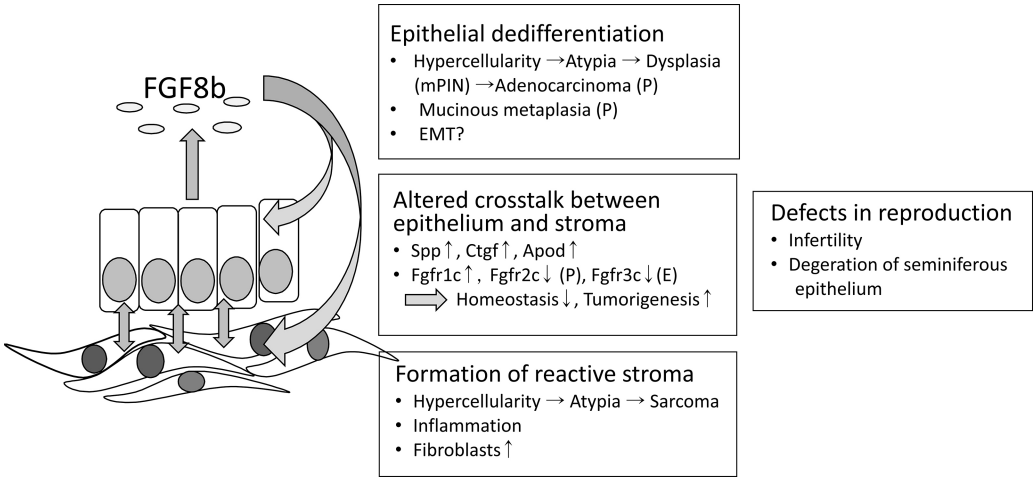


**Figure 8.** Summary of the effects of epithelial FGF8b overexpression in the prostate and epididymis. Epithelial and stromal alterations developed and progressed in parallel fashion but the early appeareance of stromal hypercellularity suggests that FGF8b has direct effects on the stroma. FGF8b may affect the epithelium directly or indirectly via altered crosstalk between the two compartments. P, alteration was present only in the prostate; E, alteration was present only in the epididymis.

infertility. This underlines the importance of controlled FGF-signaling in these organs. The similarity of the changes in the FGF8b-TG mouse epididymis to those in the prostates suggests that the molecular mechanisms underlying the phenotypes are similar in both organs. A summary of the effects of FGF8b in the prostate and epididymis is presented in the figure 8.

## 6.4 COMBINED EFFECT OF FGF8b OVEREXPRESSION AND ERβ DEFICIENCY IN THE PROSTATE

FGF8b-TG-BERKO$_{FVB}$ mouse model was generated based on the results of previous studies, which have shown that FGF8b-TG mice develop prostatic neoplasias including mPIN lesions, adenocarcinomas and sarcomas, whereas BERKO mice develop prostatic hyperplasias and have a defect in the differentiation of prostatic epithelial cells (Imamov *et al.,* 2004, Weihua *et al.,* 2001). In addition, increasing evidence from studies with different kinds of mouse models, human prostate tissue specimens and cell culture experiment implicate that ERβ has anti-tumorigenic and differentiation promoting effects in the prostate (Hartman *et al.,* 2012, Kawashima & Nakatani, 2012). Therefore, it was anticipated that mice with the overexpression of FGF8b and deficiency of ERβ could facilitate and aggravate prostate tumorigenesis, often difficult to induce in GEM models with a single genomic modification.

Histological examination revealed that, in line with the previous studies, prostates of BERKO$_{FVB}$ mice contained epithelial hyperplasia and inflammation in the stroma, but the changes were milder and less frequent than in the BERKO mice of Weihua *et al*.(2001). This is notable, because our BERKO$_{FVB}$ mice originated from the same colony as those of Weihua *et al.* (2001) which were made in the C57BL-strain (Krege *et al.,* 1998), but our mice were crossbred to FVB/N-strain. Moreover, there was a tendency for higher expression of AR and a focally increased number of basal cells in the epithelium of BERKO$_{FVB}$ mice, but in contrast to the previous results (Imamov *et al.* 2004, Weihua *et al*. 2001) these changes were not significant in our BERKO$_{FVB}$ mice. Furthermore, the changes in the prostates of FGF8b-TG mice were for the most part similar to those in the FGF8b-TG mice and in contrast to what was expected, the tumorigenesis was not accelerated compared to FGF8b-TG mice. Notably, the atypic and neoplastic changes especially in the prostate stroma tended to be even less frequent in the FGF8b-TG-BERKO$_{FVB}$ mice than in the FGF8b-TG mice. Therefore, our results suggest that ERβ does not play an important role as a tumor suppressor or protect from the development of atypic/reactive stromal changes in the FGF8b-TG mouse prostates. This is in contrast with the earlier results, which have shown that ERβ agonists can induce apoptosis in the epithelium and stroma of prostate cancer and benign prostatic hyperplasia (McPherson *et al.,* 2010).

Interestingly, mucinous metaplasia was significantly more frequent in the prostate epithelium of FGF8b-TG-BERKO$_{FVB}$ mice than in any other mouse group. This suggests that the lack of ERβ influences the differentiation of the epithelial cells of FGF8b-TG mouse prostates and makes them more prone to adapt the mucin-secreting phenotype. Our previous study (I) had showed an increased frequency of

mucinous metaplasia, associated with mPIN lesions in the prostates of old FGF8b-TG mice. Also, mucinous adenocarcinoma was reported in the PTEN-KO-FGF8b-TG mouse prostates (Zhong *et al.,* 2006). In fact, mucin-secreting Goblet-like cells are found in prostates of several GEM models often in association with adenocarcinoma and this been interpreted as a sign of intestinal differentiation (Ittmann *et al.,* 2013). The significance of these mucin-secreting cells in the prostate is not clear, but in human prostate, benign lesions with mucin secreting cells and mucinous adenocarcinoma, a rare form of human prostate cancer, have been described (Bohman & Osunkoya, 2012).

Inflammation was often present in the prostate stroma of both FGF8b-TG-BERKO$_{FVB}$ and FGF8b-TG mice but was slightly more frequent in the prostates of FGF8b-TG-BERKO$_{FVB}$ mice, suggesting that the proinflammatory effects of the two genomic modifications were additive. Our result is in line with the previous results, which have implicated that the anti-inflammatory functions of estrogens are mediated by ERβ (Harris *et al.,* 2003, Prins & Korach, 2008, Savolainen *et al.,* 2007).

Our results support the role of ERβ as a differentiation promoting and anti-inflammatory factor in the prostate but do not provide evidence for a tumor-suppressive role. Slightly increased frequency of epithelial hyperplasias in BERKO mice compared to WT mice and FGF8-TG-BERKO compared to FGF8b-TG mice, suggests that ERβ may inhibit proliferation. Based on the results found in this study, figure 9 shows a summary of the suggested functions of ERβ. A milder phenotype in the prostates of our BERKO$_{FVB}$ mice compared to that reported earlier (Imamov *et al.* 2004, Weihua *et al.* 2001), may be due to the different genetic backgrounds or possibly by environmental differences, such as differences in the diet or pathogens. ERβ may have differential roles at different phases of prostate development and in different phases of prostate tumorigenesis. Different isoforms of ERβ, of which mERβ1 and mERβ2 were expressed in the prostates of WT mice in the current study, could have opposite functional roles (Lu *et al.,* 2000, Zhao *et al.,* 2005), but the issue remains to be clarified. An inducible, prostate-specific BERKO model would be required to fully analyse the effect of ERβ abrogation in the tumorigenesis and to eliminate the potential influences of the lack of the functional receptor during prostate maturation.

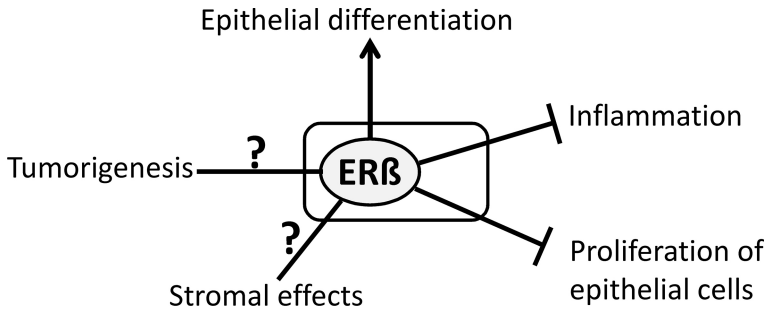


**Figure 9.** Summary of the suggested function of ERβ in the prostate based on the prostate phenotype of BERKO$_{FVB}$ and FGF8b-TG-BERKO$_{FVB}$ mice.

# 7. CONCLUSIONS

In the present study, GEM models were produced to investigate the function of FGF8b and ERβ *in vivo* in the prostate. A special interest was on the effects of FGF8b in prostate tumorigenesis and their possible modulation by inactivation of ERβ. The effects of FGF8b overexpression were also examined in the epididymis and testis. The main conclusions of the study are the following:

1. Epithelial overexpression of FGF8b induces development of progressive alterations in the prostate epithelium and stroma, which progress from mPIN lesions in the epithelium and atypias in the stroma further to neoplasias including adenocarcinomas, sarcomas and carcinosarcomas. The fibrotic stroma in the prostates of FGF8b-TG mice resembles reactive stroma present in the human prostate cancer, and its formation precedes and associates with the development of epithelial neoplasias. Altered crosstalk between the reactive stroma and altered epithelium probably contributes to development of prostatic neoplasias in the FGF8b-TG mice. FGF8b induces upregulation of genes potentially promoting tumorigenesis such as *Spp1, Ctgf, Apod* and *Foxq1* in the prostate. Activation of FGF8b could induce similar mechanisms also in human prostate tumorigenesis.

2. In the epididymis, overexpression of FGF8b induces development of epithelial and stromal hypercellularity, which advance to dysplasias in the epithelium and to atypical cells -containing hyperplasias and sarcomas in the stroma. The similarities in the histological alterations and in the expression of genes such as *Spp1, Ctgf, Apod* and *Fgfr1c* in the epididymis and prostate suggest that the molecular mechanisms induced by FGF8b are highly similar in both organs. It is remarkable that FGF8b can induce stromal malignancies and epithelial dysplasias in the epididymis, which is known for its high resistance to carcinogenesis. Increased FGF8b expression in the epididymis and testis can also cause infertility by directly or indirectly affecting spermatogenesis and/or sperm maturation in the epididymis.

3. Based on the present results, ERβ may have differentiation promoting and anti-inflammatory functions in the prostate. However, deficiency of ERβ does not significantly affect prostate tumorigenesis or at least neoplastic changes induced by FGF8b. Because the published data concerning the role of ERβ in prostate tumorigenesis are conflicting, more optimal GEM models with conditional, inducible prostate-targeted ERβ-ablation would be required to examine its role in detail. The expression and functions of different ERβ-isoforms during prostate tumorigenesis should also be investigated.

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Teresa Elo

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