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ROLE OF FIBROBLAST GROWTH FACTORS AND THEIR RECEPTORS IN PROSTATE CANCER

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To my family

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ABSTRACT

Prostate cancer (PCa) is the most common non-cutaneous malignant disease among males in the developed countries. Radical prostatectomy (RP) is an effective therapy for most PCa patients with localized or locally invaded tumors but in some cases the cancer recurs after RP. PCa is a heterogeneous disease, which is regulated by many factors, such as androgen receptor (AR), estrogen receptors α and β (ERα and ERβ), fibroblast growth factors (FGFs) and their receptors (FGFRs). In this study, the role of ERβ, FGF8, FGF13 and FGFRL1 was investigated in PCa. Previous studies have suggested that ERβ is protective against PCa whereas FGF8 has been shown to induce PCa in transgenic mice. FGF13 and FGFRL1 are poorly understood members of the FGF and FGFR families, respectively.

Transgenic mouse models were used to investigate the ability of inactivated ERβ to facilitate FGF8-induced prostate tumorigenesis. Human PCa tissue microarrays (TMAs) were used to study the expression pattern of FGF13 and FGFRL1 in PCa and the results were correlated to corresponding patient data. The targets and biological functions of FGF13 and FGFRL1 were characterized using experimental in vivo and in vitro models.

The results show that deficiency of ERβ, which had been expected to have tumor suppressing capacity, seemed to influence epithelial differentiation but did not affect FGF8-induced prostate tumorigenesis. Analysis of the TMAs showed increased expression of FGF13 in PCa. The level of cytoplasmic FGF13 was associated with the PCa biochemical recurrence (BCR), demonstrated by increasing serum PSA value, and was able to act as an independent prognostic biomarker for PCa patients after RP. Expression of FGFRL1, the most recently identified FGFR, was also elevated in PCa. Cytoplasmic and nuclear FGFRL1 was associated with high Gleason score and Ki67 level whereas the opposite was true for the cell membrane FGFRL1. Silencing of FGFRL1 in PC-3M cells led to a strongly decreased growth rate of these cells as xenografts in nude mice and the experiments with PCa cell lines showed that FGFRL1 is able to modulate the FGF2- and FGF8-induced signaling pathways. The next generation sequencing (NGS) experiments with FGFRL1-silenced PC-3M cells revealed candidates for FGFRL1 target genes.

In summary, these studies provide new data on the FGF/FGFR signaling pathways in normal and malignant prostate and suggest a potential role for FGF13 and FGFRL1 as novel prognostic markers for PCa patients.

Keywords: FGF8, FGF13, FGFRL1, ERβ, prostate cancer, prognostic marker

Lan Yu

FIBROBLASTIKASVUTEKIJÖITTEN JA NIITTEN RESEPTORIEN OSUUS ETURAUHASEN SYÖVÄN KASVUNSÄÄTELYSSÄ

Solubiologia ja anatomia, Biolääketieteen laitos, Turun yliopisto, Turku. Turun molekyylilääketieteen tohtoriohjelma (TuDMM)

TIIVISTELMÄ

Prostatasyöpä on yleisin miesten pahanlaatuinen sairaus kehittyneissä maissa. Radikaali prostatektomia (RP) on tehokas hoitomuoto paikallisen tai paikallisesti levinneen syövän hoitoon mutta silti muutamilla potilailla syöpä uusiutuu RP:n jälkeen. Prostatasyöpä on heterogeeninen kasvain, jonka syntyä ja etenemistä säätelevät monet tekijät, kuten androgeenireseptori, estrogeenireseptorit α ja β (ERα ja ERβ) ja fibroblastikasvutekijät (FGF) sekä niiden reseptorit (FGFR). Tässä väitöskirjatutkimuksessa selvitettiin, miten ERβ ja FGF8, joiden tiedetään indusoivan prostatasyöpää siirtogeenisissä hiirissä ja huonosti tunnetut FGF/FGFR-reitin molekyylit FGF13 ja FGFRL1, osallistuvat prostatasyövän kasvun säätelyyn tutkimalla kliinisiä syöpänäytteitä sekä käyttämällä kokeellisia malleja.

Työssä tutkittiin geenimuunneltujen hiirimallien avulla, pystyykö ERβ, jonka on esitetty toimivan tuumorisuppressorina, suojaamaan prostataa FGF8:n indusoimalta syövältä. FGF13:n ja FGFRL1:n ilmentymistä kliinisissä prostatasyöpänäytteissä tutkittiin monikudosleikkeitten avulla ja tuloksia verrattiin vastaaviin potilastietoihin. FGF13:n ja FGFRL1:n biologisia ominaisuuksia ja vaikutuskohteita selvitettiin kokeellisten in vivo ja in vitro -mallien avulla.

Tulokset osoittavat, että ERβ näyttää säätelevän prostataepiteelin erilaistumista mutta sen inaktivoituminen poistogeenisessä, FGF8:aa ilmentävässä hiirimallissa ei lisännyt tai jouduttanut tuumoreitten muodostumista, kuten oli hypotetisoitu kirjallisuudessa esitetyn ERβ:n tuumorisuppressoriaktiivisuuden perusteella. Kliinisten monikudos-leikkeitten analysoiminen osoitti, että FGF13 on lisääntynyt prostatasyövässä. Erityisesti korkea sytoplasminen FGF13-taso liittyi prostatasyövän uusiutumisriskiin (biochemical recurrence, BCR), joka todettiin seerumin kohonnneen PSA-tason perusteella, ja se toimi RP:n jälkeen itsenäisenä syövän uusiutumista ennustavana prognostisena markkerina. FGFRL1 oli myös lisääntynyt prostatasyövässä, missä sytoplasminen ja tumaan sijoittunut FGFRL1 korreloivat korkeaan Gleason-luokkaan ja proliferaatioaktiivisuuteen (Ki67-antigeenin värjäytyminen), kun taas solukalvolle sijoittuvan FGFRL1:n määrän suhteen tilanne oli päinvastainen. FGFRL1:n inaktivoituminen PC-3M–prostatasyöpäsoluissa johti voimakkaasti estyneeseen tuumorikasvuun atyymisissä hiirissä. Prostatasyöpäsoluilla tehdyt in vitro -kokeet osoittivat, että FGFRL1 pystyy säätelemään FGF2- ja FGF8-välitteisiä signalointireittejä ja RNA-sekvensointikokeitten perusteella tunnistettujen kohdegeenien ilmentymistä.

Yhteenvetona voi todeta tämän tutkimuksen tuottaneen uutta tietoa FGF/FGFR-reitin merkityksestä prostatasyövän kasvussa ja etenemisessä. On mahdollista, että FGF13:n ja FGFRL1:n määrittämistä voidaan tulevaisuudessa käyttää hyväksi arvioitaessa prostatasyöpäpotilaitten ennustetta ja vastetta radikaaliin prostatektomiaan.

Avainsanat: FGF8, FGF13, FGFRL1, ERβ, prostatasyöpä, ennusteellinen tekijä

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ABBREVIATIONS

AdjPr	Adjacent prostate	IP_3	Inositol triphosphate
AP	Anterior prostate	LNCaP	Prostate cancer cell line
ARVs	AR splice variants	MAPs	Microtubule-associated proteins
BCR	Biochemical recurrence	NCI-H660	Prostate cancer cell line
BERKO	Estrogen Receptor Beta Knockout	NLS	Nuclear location sequence
BPH	Benign prostatic hyperplasia	PC-3	Prostate cancer cell line
cDNA	Complementary DNA	PC-3M	Prostate cancer cell line
DAG	Diacylglycerol	PCa	Prostate cancer
DLP	Dorsolateral prostate	PCR	Polymerase chain reaction
ER	Estrogen receptor	PIN	Prostatic intraepithelial neoplasia
ERK1/2	Extracellular signal-regulated protein kinases	PIP_2	Phosphatidylinositol 4, 5-bisphosphate
ERα	Estrogen receptor alpha	PNT1a	Immortalized prostate epithelia cell line
ERβ	Estrogen receptor beta	PSA	Prostate-specific antigen
FGF	Fibroblast growth factor	qRT-PCR	Quantitative RT-PCR
FGFR	Fibroblast growth factor receptor	RP	Radical prostatectomy
FHF	FGF homologous factor	SH2	Scr homology 2
GAB1	GRB2 associated binding protein 1	TMA	Tissue microarray
GRB2	Growth factor receptor bound protein 2	VCaP	Prostate cancer cell line
GS	Gleason score	VGSCs/ NaVs	Voltage-gated sodium channels
HGPIN	High grade prostatic intraepithelial neoplasia	VP	Ventral prostate
IHC	Immunohistochemistry		

LIST OF ORIGINAL PUBLICATIONS

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

I. Elo T, Yu L, Valve E, Mäkelä S, Härkönen P. Deficiency of ERβ and prostate tumorigenesis in FGF8b transgenic mice. Endocr Relat Cancer. 2014, 21(4): 677-690.

II. Yu L, Toriseva M, Tuomala M, Seikkula H, Elo T, Tuomela J, Kallajoki M, Mirtti T, Taimen P, Boström P, , Alanen K, Nurmi M, Nees M, Härkönen P. High expression of fibroblast growth factor 13 in prostate cancer is associated with a shortened time to biochemical recurrence after radical prostatectomy. Int J Cancer. 2016, 139(1):140-52

III. Yu L, Toriseva M, Erickson A, Seikkula H, Nurmi M, Taimen P, Boström P, Mirtti T, Alanen K, Kallajoki M, Tuomela J, Nees M, Härkönen P. Increased FGFRL1 expression is associated with prostate cancer progression. Manuscript, 2016

1 INTRODUCTION

Prostate cancer (PCa) is the most common noncutaneous cancer and the second leading cause of cancer related death in men in developed countries. Most prostate cancer patients are today diagnosed with localized disease due to the wide use of prostate specific antigen (PSA) testing. Radical prostatectomy (RP) and radiation therapy are curative treatment options of localized or locally advanced prostate cancer. Radical prostatectomy (RP) is a curative treatment for most patients diagnosed with localized prostate cancer, but still in the follow-up more than 20% of patients show increased serum PSA levels suggesting biochemical or clinical recurrence. Over 20% of the patients with increased postoperative PSA values, called biochemical recurrence (BCR) suffer from metastatic disease or encounter PCa specific death. Also 15-20% of the patients with newly diagnosed prostate cancer have metastatic disease with no possibilities for curative treatment. Nowadays, the clinical parameters, such as the prostate specific antigen (PSA) value, the Gleason score, and pathological stage (pTNM) and nomograms based on these parameters, are used to predict the patient's outcome after RP. However, because of the heterogeneous characteristics of PCa, exclusive application of these parameters is not always sufficient to predict the prognosis. Therefore, novel biomarkers for recurrence of PCa after radical RP are essential for patient counseling and adjuvant therapy application. Meanwhile, further understanding of the mechanisms of human prostate tumorigenesis and PCa progression at the molecular level is critical for searching novel targeted therapies for the disease.

As a hormone related cancer, androgens and estrogens are suggested to be involved in regulation of prostate tumorigenesis and tumor growth via androgen receptor (AR) and estrogen receptor (ER) α and β. Androgens and AR play a critical role in prostate development and differentiation, as well as in PCa initiation and progression (Barfeld et al. 2014, Cooper, Page 2014). ERα is considered to be an oncogenic factor which promotes cell proliferation, inflammation and malignancy, whereas ERβ has been proposed to serve as an anti-carcinogenic, pro-apoptotic and anti-inflammatory factor (Ellem, Risbridger 2009, Chang, Prins 1999, Leav et al. 2001). Hyperplasia and decreased differentiation of epithelial cells in prostate have been reported in ERβ knockout (BERKO) mice.

The fibroblast growth factors (FGF) and their receptor (FGFR) signaling pathways drive crucial biological processes, including angiogenesis, wound healing, and cell proliferation, survival and migration. Therefore, deregulated FGFs and FGFRs play critical roles in cancer initiation and progression. In PCa, elevated expression of FGF1, FGF2, FGF6, FGF8, FGF9, FGF17, FGF19, and FGF23, as well as FGFR1 and FGFR4 have been identified (Cronauer et al. 2003, Grose, Dickson 2005, Nagamatsu et al. 2015, Yang et al. 2013). FGF8, as a mitogenic growth factor, is involved in prostate tumorigenesis and PCa angiogenesis and bone metastasis (Tuomela et al. 2010, Valta et al. 2008, Elo et al. 2010). Our query on cBioPortal database indicated that FGF13 and FGFRL1 are also altered at the mRNA level in PCa, 31% and 17%, respectively (MSKCC, Cancer Cell 2010) (Cerami et al. 2012, Gao et al. 2013). Until now, there are no studies concerning FGF13 and FGFRL1 in prostate cancer and generally only a few studies on FGF13 and FGFRL1 in cancer.

FGF13, also known as FHF2, belongs to a FGF subfamily comprising fibroblast growth factor homologous factors (FHFs) which are expressed at the highest level in the nervous system (Goldfarb 2005). An association of FGF13 with cancers has not been studied much. Increased FGF13 mRNA level is observed in paratracheal lymph node metastasis of lung adenocarcinoma compared to primary tumors (Chen et al. 2014). The elevated level of FGF13 expression has been found to be associated with poor prognosis of pancreatic and cervical cancers (Missiaglia et al. 2010, Okada et al. 2013).

FGFRL1 is the most recently discovered member of the FGFR family. It is able to bind FGFs but cannot signal via the canonical FGF/FGFR pathway because of lacking functional intracellular tyrosine kinase domain. The function and expression of FGFRL1 in malignant diseases are conflicting. FGFRL1 shows aberrant expression in ovarian tumors with big variations (Schild, Trueb 2005), whereas it displays decreased expression in bladder cancer (Martino et al. 2013). However, increased FGFRL1 expression is found in esophageal squamous cell carcinoma (ESCC) and it is positively related to poorly differentiated carcinoma type and occurrence of lymph node metastasis (Tsuchiya et al. 2011, Shimada et al. 2014).

In this thesis work, the expression and clinical significance of FGF13 and FGFRL1 in human PCa were investigated. Biological functions of FGF13 and FGFRL1 were studied with both *in vivo* and *in vitro* experiments. The role of

ERβ in FGF8b-induced prostate tumorigenesis was studied using cross-bred FGF8b transgenic and ERβ knockout mice.

2 REVIEW OF THE LITERATURE

2.1 Prostate cancer

In human, the prostate is a branched ductal gland that is located at the base of bladder and surrounds the urethra. The prostate produces and secretes proteins to seminal fluid. Prostate cancer (PCa) is one of the most diagnosed cancers in men worldwide. Advanced age, family history and race are known risk factors of PCa. PCa is a very heterogeneous disease, which can be indolent for a long time but which can also behave aggressively. For localized PCa, active surveillance, radical prostatectomy (RP) and radiation therapy with external beam or intraprostatic brachytherapy are the common therapeutic options. Androgen deprivation therapy (ADT) is used in metastatic prostate cancer or as neoadjuvant and adjuvant treatment combined to radiation therapy in high risk localized or locally advanced PCa. In PCa patients progressed to castration resistant prostate cancer (CRPC), also chemotherapy with docetaxel or new androgen pathway modulators abiraterone or enzalutamide are used (Damber, Aus 2008).

2.1.1 Epidemiology and etiology of prostate cancer

Prostate cancer is globally the second most common malignant disease in men and the incidence is increasing (Engholm et al. 2010). In 2012, PCa accounted for 15% of diagnosed cancer in men with an estimated 1.1 million new cases worldwidely (Chen et al. 2014). In Europe, the estimated numbers of new PCa cases and deaths in 2012 were 417,000 and 92,000, respectively (Ferlay et al. 2013). Western Europe showed the highest number in both incidence and mortality (178,000 and 28,000) (Ferlay et al. 2014). In Finland, PCa almost takes up one third of all cancers diagnosed in men and 14% of cancer related death, although the mortality have decreased 3% per year since 2000, (Engholm et al. 2010, Engholm et al. 2016). Programmes for screening of PSA, early detection and subsequent treatment may have contributed to the reduced mortality. The high incidence of PCa in developed countries is largely due to a wide adoption of prostate-specific antigen screening and subsequent biopsies (Ferlay et al. 2010).

The factors that cause PCa are not well known but some risk factors, such as increasing age, family history, and ethnic origin have been identified

(Grönberg 2003, Heidenreich et al. 2011). About 85% of PCa patients are diagnosed after the age of 65 years and less than 0.1% of diagnosed patients are younger than 50 years (Grönberg 2003). The risk to be diagnosed with PCa is at least doubled if one first-degree relative bears the disease (Eeles et al. 2014). The suggested high-penetrance susceptibility genes are summarized as HPC20, HPC2, HPC1, PCaP, CAPB, HPCX, RNASEL, MSRL, CHEK2, CAPZB, BRCA2 (Bratt 2002, Demichelis, Stanford 2015). Several other genetic regions and risk SNPs have been identified in genome-wide association studies showing wide genetic heterogeneity in prostate cancer (Eeles et al. 2014). Moreover, recent study indicated that the germline copy number variation is also associated with PCa susceptibility (Laitinen et al. 2016). Both incidence and mortality are relative high in African descent and low in Asia (Ferlay et al. 2014). In addition to other established risk factors, AR in the first place, chronic intraprostatic inflammation and hormones are the most discussed factors that are plausible to be involved in PCa (De Marzo et al. 2007, Diakos et al. 2014, Härkönen, Mäkelä 2004). Moreover, ionizing radiation, smoking, diet, weight and physical activity have also been linked to PCa (Cuzick et al. 2014, Discacciati, Wolk 2014, Gong et al. 2006, Nair Shalliker et al. 2012, Patel, Stephenson 2011).

2.1.2 Histopathology of prostate cancer

Histologically, the prostate is composed of glands and stroma. The gland is constituted by luminal epithelia, basal cells and neuroendocrine cells; and stroma comprises smooth muscle fibers, collagenous tissue, nerve fibers and capillaries. PCa can be grouped to acinar adenocarcinoma, which contributes to the vast majority of PCa, and rare non-acinar carcinoma, such as bascal cell carcinoma, squamous and adenosquamous carcinoma. Normal prostatic acini have two cell layers: (1) tall columnar secreting epithelia, which is androgen-dependent and limited in proliferation, and (2) basal layer which maintains proliferation capacity and is independent of androgens. Neuroendocrine cells are dispersed in the basal layer (Fig. 1). High grade prostatic intraepithelial neoplasia (HGPIN) lesions are perceived as precursors of PCa. Characteristics of HGPIN are crowding proliferative luminal epithelia with four architectural patterns (tufting, micropapillary, cribriform and flat), enlarged nuclei with prominent nucleoli and disrupted basal cell layer (Bostwick, Cheng 2012). Prostatic adenocarcinoma has several variants named according to their specific features, such as foamy gland carcinoma, small cell carcinoma, ductal

adenocarcinoma, colloid (mucinous) carcinoma, and pseudohyperplastic adenocarcinoma. The architecture of typical PCa shows small glands of back-to-back or fused pattern with little or no stroma between them. The cytologic features show enlarged prominent nuclei and nucleoli and the absence of basal cell layer is an important characteristic of PCa (Figure 1).

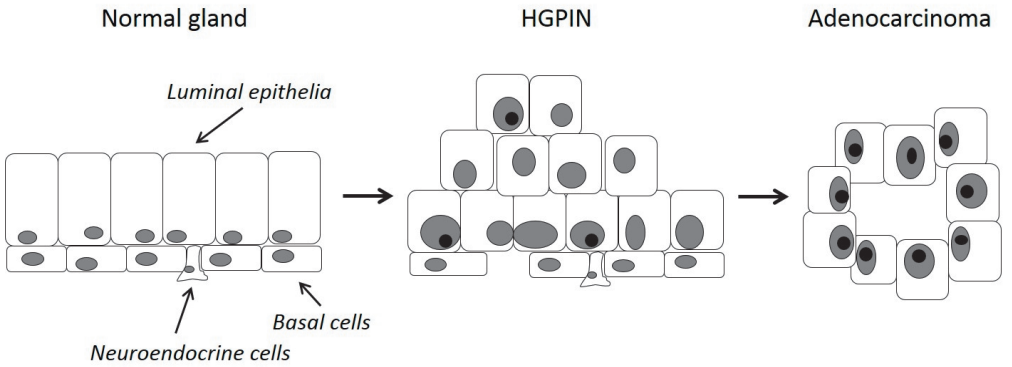

Figure 1. Prostate cancer development from normal epithelium to HGPIN and adenocarcinoma.

2.1.3 Pathologic parameters and biomarkers of prostate cancer

PCa is a heterogeneous malignancy. It is typically an indolent disease but it can also be aggressive. PCa could be controlled for decades by early detection and radical treatment with prostatectomy or radiation therapy but still BCR occurs in up to 30% of operated patients. Efficient prognostic factors for identifying patients at high risk of clinical progression will give information for patient counseling and for selecting proper intervention treatment. In the clinic, some parameters are being used to characterize the disease, such as clinical and pathological TNM classification (cTNM and pTNM), positive surgical margin (PSM), Gleason score (GS), pre-operative PSA, perineural invasion (PNI), vascular invasion, and tumor volume. Based on these parameters, several models have been established by various combinations to predict outcome of PCa patient after RP (HAN et al. 2003, Kattan, Wheeler & Scardino 1999, Partin et al. 1995, Stephenson et al. 2005, Suardi et al. 2008). However, these models are not perfect due to the extremely heterogeneous nature of PCa and limitations of discrimination, calibration, validation and

different study populations. Addition of effective biomarkers would improve the predictive accuracy to some extent.

2.1.3.1 TNM system

The TNM system, extent of the primary tumor (T category), evaluation of regional lymph node metastasis (N category) and distant metastasis (M0-1), is used for classifying the anatomic severity of tumor. The pathological TNM classification (pTNM) is based on the microscopical diagnosis of specimens from radical prostatectomy while the clinical TNM classification (cTNM) is based on the pretreatment data, like digital rectal examination (DRE), transrectal ultrasonography (TRUS) or magnetic resonance imaging (MRI). (Table 1)

Table 1. Pathological TNM classification. (Summarized from (Sobin, Fleming 1997, Cheng et al. 2012)

	pTNM	cTNM
T0	-	No evidence of tumor
T1	-	Inapparent tumor neither palpable nor visible by imaging
T2	Tumor confined in prostate	
T3	Tumor extent breaks through the prostate capsule	
T4	Tumor invades to adjacent organs other than seminal vesicles such as rectum, levator muscle and /or pelvic wall	
NX	Regional lymph node metastasis is not observed	
N0	Regional lymph node metastasis is not observed	
N1-N3	Increasing degree of metastases to regional lymph node(s)	
M0	No distant metastasis	
M1	Distant metastasis is observed at: a. non-regional lymph nodes; b. bone; c. other with or without bone disease)	

2.1.3.2 Surgical margin

Surgical margin is defined as: 'tumor extending to the inked surface of the prostatectomy specimen which the surgeon has cut across' (Epstein et al. 2005b). However, the margin cannot be precisely determined since the prostate is not a dissociative organ but surrounded by multiple structures and the prostate is not always excised exactly along the prostate capsule. The prognostic value of the surgical margin has been reported in several studies. In general, positive surgical margin (PSM) is deemed to negatively relate to the outcome (Adamis, Varkarakis 2014). However, the independence of application of PSM to predict the disease progression is still controversial. Estham et al. indicated that PSM could be used as a predictor factor for BCR while Vis et al. support a conservative opinion saying that PSM is not associated with BCR (Eastham et al. 2007, Vis, Schröder & van der Kwast 2006). Moreover, the site, number and extent of the PSM are studied and suggested to be considered as risk factors (Pettus et al. 2004, Sofer et al. 2002).

2.1.3.3 Gleason score

The strongest predictor for PCa is currently the GS. PCa is classified by five Gleason patterns 1-5, from well differentiated to poorly differentiated based on the architectural features of PCa tumor. The patterns 1 and 2 are assigned as low-grade patterns while 3-5 are assigned as high-grade patterns. Radical prostatectomy specimen is evaluated and assessed as primary and secondary grades by the percentage of cancer area and as tertiary grade if a small component of high-grade pattern is observed in addition to the primary and secondary grades. The GS is the sum of primary and secondary pattern numbers if there are only two patterns. The GS of 1+1=2 should not be diagnosed as PCa nowadays and it is referred as adenosis. Low grade pattern could be ignored if its area is less than 5% of the tumor focus. For example, if we count tumor area as 100% and Gleason pattern 3 represents 99% and Gleason pattern 2 represents 1% of it, the case would be diagnosed as GS 3+3=6. If a tertiary grade pattern exists, the GS is the sum of primary pattern number and tertiary grade pattern number, or GS is recorded as the sum of primary and secondary pattern numbers with a comment of tertiary pattern alternatively (Epstein et al. 2005a, Epstein 2010). These two evaluation methods are still under debate. Discussion of whether PCa of Gleason 6

should be called cancer at all is also ongoing (Loeb, Montorsi & Catto 2015). Currently, the new grading system based on the 5-year BCR-free progression has been accepted by the 2016 World Health Organization (WHO). This grading system consists of five groups: grade group 1 (GS ≤6), grade group 2 (GS 3 +4 = 7), grade group 3 (GS 4 +3 =7), grade group 4 (GS 4 + 4= 8), and grade group 5 (GS 9–10) (Matoso, Epstein 2016).

2.1.3.4 Serum biomarkers

Prostate specific antigen (PSA), also called kallikrein-related peptidase 3 (KLK3), is one of the most widely used serum markers for screening and monitoring the disease progression. Biologically, PSA is secreted to seminal plasma by prostate epithelial cells. The elevation of serum PSA could be caused by leakage of PSA from tumor cells due to disappeared glandular structures in PCa as well as by physical trauma and benign prostate disease (Ulmert et al. 2009). A cut-off value of 4ng/ml PSA value is proposed as an indicator for further examination of detecting tumor, like prostate biopsy, but tumor may present even if serum PSA is less than 4ng/ml (Stephan, Ralla & Jung 2014, Wilt, Thompson 2006). In many clinical laboratories age-specific cut-off values are used for PSA evaluation. Serum PSA value over 20ng/ml is considered as a high-risk factor for PCa (Goldberg, Baniel & Yossepowitch 2013). Several programs to screen PSA for detecting PCa have been used but the benefits are still controversial. The European Randomized Study of screening for Prostate Cancer (ERSPC) by 182,000 men indicates that PSA-based screening reduces the rate of death (Schröder et al. 2009) and the decrease remains significant when the follow-up time is extended to 13 years (Schröder et al. 2014, Pound et al. 1999) but it is associated with substantial overdiagnosis. Study from Prostate, Lung, Colorectal, and Ovarian (PLCO) by 76,693 men suggests that there is no significance between PSA annual screening and usual care groups after 7-10 years of follow-up (Andriole et al. 2009). Moreover, several studies have demonstrated that pre-operative PSA values could serve as an independent marker for predicting BCR (HAN et al. 2003, Stephenson et al. 2005). Besides pre-operative PSA, PSA velocity and PSA doubling time are also studied and suggested to be used as prognostic factors (Crook, Ots 2013, Dijkstra, Mulders & Schalken 2014).

Altogether, PSA is still an irreplaceable factor in clinical practice, although the biochemical progression (BCR), indicated by increased serum PSA value, may not always be consistent with clinical progression (Pound et al. 1999).

2.1.3.5 Molecular biomarkers

Clinically based parameters described above have been used widely to predict the outcome of PCa patients after RP but their benefit is limited when estimating the prognosis of individual patients. The application of molecular biomarkers is a promising approach to overcome the limitation of existing markers. Extensive studies of the molecular mechanisms of PCa growth have provided several suggestions for potential biomarkers for predicting BCR, such as non-coding RNA PCA3, apoptosis-related Bcl-2 family, cell-cycle related p27, cellular adhesion-related E-cadherin, cytokine interleukin-6 (IL-6), angiogenesis-related VEGF(R) family, and transforming growth factor-β1 (TGFβ1) (Lopergolo, Zaffaroni 2009, Miyake, Fujisawa 2013). As a potential genetic markers, TMPRSS:ERG fusion gene appears in approximately 50% of PCa cases and was suggested to be related to an increased risk of BCR but the status has not been proven (Boström et al. 2015). In addition, epigenetic biomarkers, such as DNA hypo- and hyper-methylation, deregulation of microRNAs, histone modification and histone variants on specific genes would also provide information of prognosis (Valdés-Mora, Clark 2014). Copy number variarion (CNV) and genetic information from nucleic acids in periphera blood and circulating tumor cells may have a prognostic role in PCa patients (Boström et al. 2015). Although the molecular biomarkers have shown evidence for prognostic values and some of them, like IL-6 and TGFβ1, have increased the predictive accuracy of existing markers, the standardization of the methods and additional validation studies are requires before clinical application.

2.2 Androgen receptor and prostate cancer

Androgen signaling plays an important role in the male sexual organ development as well as in the development of prostate and PCa.

Androgen receptor belongs to nuclear receptors and consists of an N-terminal transactivation domain (NTD), a DNA-binding domain (DBD), a C-

terminal ligand binding domain (LBD), and a hinge region (H) (Claessens et al. 2008). NTD contains an activation function 1 (AF1) domain that acts as a ligand-independent transcriptional activation domain. AF2 is located at LBD and regulates transcription in a ligand-dependent way. The androgenic ligands, dihydrotestosterone (DHT) and/or testosterone (T), bind to the LBD of AR and induce its nuclear translocation and therefore regulate target gene expression (Lamont, Tindall 2010). AR amplification and ovexpression and AR mutations also play central role in PCa progression (Waltering, Urbanucci & Visakorpi 2012). Recently, several AR splice variants (ARVs) have been reported to play a role in PCa progression (Maughan, Antonarakis 2015). ARVs share the similar NTD and DBD as full length AR (AR-FL) but lacks LBD (Dehm et al. 2008, Zhang et al. 2013). Studies with ectopic AR-Vs expression demonstrate that ARVs are able to regulate transcription of the genes like PSA and TMPRSS2, which are regulated by canonical AR signaling in the absence of androgens, (Sun et al. 2010). Moreover, AR-Vs could also induce nuclear translocation of AR-FL (Sun et al. 2010). It has been reported that increased expression of AR-Vs is correlated with CRPC and BCR (Zhang et al. 2013, Maughan, Antonarakis 2015).

At least 22 known AR splice variants have been reported in the literature (Maughan, Antonarakis 2015). AR-V7 (AR3) is one of the major splice variants of which transcriptional activity is independent of androgens or antiandrogens (Guo et al. 2009). However, AR-V7 is repressed by androgens and is likely to have an acute response to castration therapy (Watson et al. 2010).

2.3 Estrogen receptor and prostate cancer

2.3.1 Estrogen receptor in normal prostate

Estrogens belong to sex steroid hormone family including estrone (E1), estradiol (E2) and estriol (E3). In men, estrogens are mainly converted from testosterone by aromatase enzyme expressed in the testicles, the adipose tissue, the adrenal glands, and even the prostate. Estrogens can execute functions by (1) directly binding to estrogen receptors (ERα and ERβ) and (2) negatively regulating androgen level through hypothalamus-pituitary-gonadal axis (Bonkhoff, Berges 2009). In addition, estrogens can also affect prostate by increasing prolactin (PRL) (Härkönen, Mäkelä 2004). In DNA-binding and ligand-binding domains, ERα and ERβ share 97% and 56% amino acid

homology, respectively (Katzenellenbogen et al. 2001). However, the dissimilar structures in N-terminal or C-terminal ends contribute to their different behavior despite binding to the physiological ligand with similar affinities (Kuiper et al. 1997). In normal prostate, ERα is restricted to stromal cells and ERβ is mainly expressed in luminal epithelia and basal cells (Fixemer, Remberger & Bonkhoff 2003, Leav et al. 2001, Bonkhoff et al. 1999).

2.3.2 Estrogen receptor β in prostate cancer

Studies on estrogen receptors suggest that ERα and ERβ play opposing roles in PCa. ERα is considered to be an oncogenic factor which promotes cell proliferation, inflammation and malignancy, whereas ERβ serves as an anti-carcinogenic, pro-apoptotic and anti-inflammatory factor (Ellem, Risbridger 2009, Chang, Prins 1999, Leav et al. 2001). ERα-knockout mice do not develop prostate cancer after testosterone and/or estrogen treatment, whereas mice lacking ERβ develop prostate cancer after the addition of sex hormones, similarly to wildtype mice (Ricke et al. 2008). Fixermer and colleagues suggested that ERβ is retained in untreated primary and metastatic PCa but absent or decreased in a proportion of HGPIN and recurrent PCa (Fixemer, Remberger & Bonkhoff 2003). The phenotypes of ERβ knockout (BERKO) mice produced in different laboratories are controversial. Some suggest that lacking of ERβ impaired ventral prostate epithelia differentiation and increased proliferation (Imamov et al. 2004, Weihua et al. 2001) while others refer that inactivation of ERβ does not lead to prostate epithelia hyperplasia (Dupont et al. 2000). In addition, ERβ is able to block epithelial-to-mesenchymal transition and to regulate cell cycle process (Christoforou, Christopoulos & Koutsilieris 2014).

2.4 Fibroblast growth factors and their receptors

Fibroblast growth factors and their receptors execute a broad spectrum of biological functions by activating the FGF/FGFR signaling pathways.

2.4.1 FGFs

The fibroblast growth factor (FGF) family is constituted of eighteen secreted and four intracellular polypeptide growth factors which are divided to 8 subfamilies (Table 2). FGFs are expressed and secreted in virtually all tissues. They serve important roles in preimplantation, organogenesis, development, and wound healing. Disorders of FGFs expression have been documented to play an important role in different types of cancer.

To date, 22 FGFs have been identified in both human and mouse, they share 13-71% amino acid homology with the different molecular mass (17 to 34 kDa) (Ornitz, Itoh 2001).

The fibroblast growth factors 3-8, 10, 17-19/15, and 21-23, which contain N-terminal signal peptides, are secreted via the endoplasmic-reticulum-Golgi (ER/Golgi) pathway. The FGF9 subfamily, including FGF9, FGF16, FGF20, has a non-cleaved amino-terminal hydrophobic sequence required for their secretion (Miyake et al. 1998, Ohmachi et al. 2000). In contrast, FGF1 and FGF2 do not have a secretory signal sequence and cannot be secreted by the ER/Golgi pathway. However, they have been found on the cell surface and in the extracellular environment. It is assumed that FGF1 and FGF2 are released by died/injured cells. A recent study suggests a mechanism of an unconventional secretion pathway which is phosphoinositide-dependent (Steringer, Müller & Nickel 2015). Like FGF1 and FGF2, members of FGF11 subfamily do not bear any secretory signal sequence, either. However, unlike FGF1 and FGF2, there is no evidence for either secretion or release of FGF11-14. The FGFs 11-14 are therefore considered as intracellular FGFs (Goldfarb 2005, Schoorlemmer, Goldfarb 2001).

The nuclear localization of FGF1 and FGF2 has been demonstrated in several studies (Antoine et al. 1997, Arnaud et al. 1999, Imamura et al. 1994). The role of nuclearly localized FGFs is still unclear but FGF1 has been suggested to be involved in DNA synthesis but not in FGF-induced cell proliferation (Wiedłocha et al. 1994). Nuclear translocation of FGF2 is demonstrated to promote cancer cell invasion (Coleman et al. 2014b).

The expression pattern of FGFs varies, from ubiquitous, like FGF2, to highly restricted to a specific cell type and stage, like FGF4. Likewise, FGFs execute biological functions in a spatially and temporally specific manner. FGFs sustain cell proliferation in most of the cases, but specific cell types have diverse responses to FGFs. For example, FGF signaling can cause a cell cycle arrest and induce differentiation in chondrocytes (Sahni et al. 1999). In

osteoblasts, activation of FGF signaling leads to increased proliferation in immature osteoblasts whereas differentiation (Valta et al., 2006) or apoptosis is induced in primed osteoblasts (Mansukhani et al. 2000, Valta et al. 2006).

Table 2. The FGF subfamilies and FGF binding specificities to FGFRs. (Modified from Ornitz, Itoh 2015)

Subfamily	FGF	Secretion	Co-factor	Activated FGFR
FGF1 subfamily	FGF1 (aFGF)	Paracrine	Heparin/ Heparan sulfate proteoglycan (HSPG)	FGFR1-4
	FGF2 (bFGF)			FGFR1c, 3c > 2c, 1b, 4
FGF4 subfamily	FGF4, FGF5,FGF6			FGFR1c, 2c > 3c, 4
FGF7 subfamily	FGF3, FGF7(KGF), FGF10, FGF22			FGFR2b > 1b FGFR1b
FGF8 subfamily	FGF8, FGF17, FGF18			FGFR3c > 4 > 2c > 1c >>3b
FGF9 subfamily	FGF9, FGF16, FGF20			FGFR3c > 2c > 1c, 3b >> 4
FGF19 subfamily	FGF19/15*, FGF21, FG23	Endocrine	α/β Klotho	FGFR1c, 2c, 3c, 4
FGF11 subfamily	FGF11, FGF12, FGF13, FGF14	Intracrine	-	NO

* FGF15 and FGF19 are orthologues in vertebrates, and termed FGF15 in rodents and FGF19 in other vertebrates respectively.

2.4.2 FGF receptors

The FGF receptor family belongs to receptor tyrosine kinases (RTKs) and play important roles in development and homoeostasis of organisms (Lin, Wang 2010). Accumulating evidence shows that mutations and aberrant

expression of FGFRs are involved in initiation and progression of cancers, such as bladder and prostate cancer (Turner, Grose 2010).

2.4.2.1 Structure

The canonical FGFR family consists of four members, FGFR1-4, which are comprised of an extracellular domain, a single-pass transmembrane segmentation, and an intracellular domain. The intracellular domain encompasses a docking-protein-binding site, tyrosine kinase domains and a C-terminal tail (Lin, Wang 2010). Fibroblast growth factor receptor like 1 (FGFRL1), also termed as FGFR5, is the most recently identified FGFR family member and it has similar structural components except for the intracellular domain which lacks the tyrosine kinase part (Trueb et al. 2003).

The extracellular domain of FGFRs is composed of three immunoglobulin-like domains (Ig-like D I - D III) and a liner (acid box) between D I and D II. D I and acid box are supposed to act as an autoinhibitor while D II and D III are the FGF binding sites (Jorgen, Kaisa & Ellen 2011, Olsen et al. 2004). Alternative splicing in D III generates two isoforms named b and c which determine the specificity of binding to FGFs (Eswarakumar, Lax & Schlessinger 2005). Expression of the two isoforms is tissue specific with FGFR IIIb restricted to the epithelial lineages and FGFR IIIc restricted to the mesenchymal lineages (Ornitz et al. 1996). The alternative splicing occurs in case of FGFR1-3 but not FGFR4 and FGFRL1.

In mouse, disruption of FGFR1 and FGFR2 leads to early embryonic lethality and disruption of FGFR3 leads to bone overgrowth (Eswarakumar, Lax & Schlessinger 2005). Mouse with FGFR4 deletoin is viable but shows disordered cholesterol metabolism and bile acid synthesis (Yu et al. 2000).

2.4.2.2 The FGF/FGFR signaling pathways

Canonically, FGFs activate FGFR signaling by binding to corresponding receptors together with heparin sulfate proteoglycans (HSPGs). The formation of ternary complexes of FGF/heparin/FGFR induces transphosphorylation of the intracellular tyrosine kinase and activates intracellular signal transduction cascades. The FGFR substrate 2α (FRS2α), as a major substrate for FGFR kinase, binds to the juxtamembrane region of the intracellular domain via phosphotyrosine binding (PTB) domain constitutively. Activation of FGFR phosphorylates FRS2α and makes it serve as a docking site for adaptor proteins,

such as growth factor receptor bound protein 2 (GRB2) and GRB2 associated binding protein 1 (GAB1). Upon FGFR activation, the adaptor protein GRB2 recruits SOS and activates the RAS GTPase, and thereby activates the MAPK (mitogen-activated protein kinase) signaling pathway, which leads to activation of the downstream signaling pathways, including the extracellular signal-regulated protein kinases (ERK1/2), p38 and JNK. The GRB2 can also recruite GAB1 and activate the PI3K (phosphoinositide 3-kinase)/AKT signaling pathway. The intracellular phosphotyrosines of FGFR could also serve as docking sites for SH2 (Scr homology 2) domain of PLC γ (phospholipase C γ). The phosphorylated PLCγ hydrolyses PIP_2 to IP3 and DAG, which increases intracellular calcium ions (Ca^{2+}) and activates protein kinase C, respectively. Activated FGFRs also induce phosphorylation and nuclear translocation of STAT1, STAT3, and STAT5, along with activation of their downstream target genes (Figure 2).

The activated downstream cascade of MAPK, PI3K/Akt, PLCγ, and STATs pathways regulates gene transcription, cell proliferation, differentiation, survival, and migration sequentially.

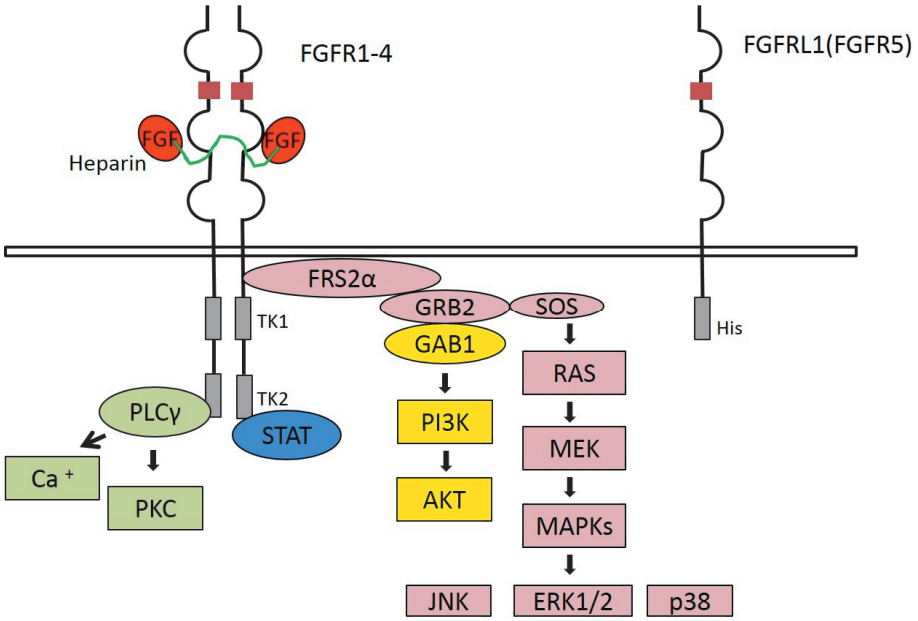

Figure 2. A simplified model for the FGF/FGFR signaling pathways (Modified from Jorgen, Kaisa & Ellen 2011)

Several factors have been documented to regulate FGFR signaling. Sprouty (SPRY) family, which has four isoforms SPRY1-4, is a negative regulator of

RTKs. SPRY specifically inhibits FGF-induced RAS-MAKP signaling by competing with FGR2α binding to GRB2-SOS complexes (Mason et al. 2006, Thisse, Thisse 2005). CBL, an E3 ubiquitin, ubiquitinates phosphorylated FRS2α and induces endocytosis and FGFRs degradation (Wong et al. 2002, Jean et al. 2010). SEF (similar expression to FGF) is a transmembrane protein and regulated by FGFs (Tsang et al. 2002). SEF spatial negatively regulates FGFR signaling by binding to activated MEK and inhibits dissociation of the phosphorylated ERK from the MEK-ERK complex, and therefore blocks nuclear translocation of activated ERK (Torii et al. 2004). DUSP6 is a negative feedback factor that inhibits the FGFR pathway by directly dephosphorylating MAKP residues (Li et al. 2007). MKP3 inhibits MAPK cascade by dephosphorylating ERK2 (Kim, Rice & Denu 2003). FGFRL1 is initially described as a decoy receptor which sequesters FGFs away from the receptors and therefore attenuates FGFR signaling (Steinberg et al. 2010b). Nevertheless, subsequent studies demonstrate that FGFRL1 may also have other functions than as a decoy only. The role of FGFRL1 will be introduced in the chapter 2.4.5.

Besides the signaling pathway, FGFs along with FGFRs are able to traffic to cytoplasm and then translocate to nuclei (Bryant, Stow 2005, Coleman et al. 2014a). The role of FGFRs in nuclei is not very clear, but translocation of FGFR1 into nuclei may influence cell differentiation, proliferation and cell movement (Stachowiak, Maher & Stachowiak 2007, Chioni, Grose 2012, Coleman et al. 2014b).

2.4.2.3 FGFs and FGFRs in the prostate

In the prostate, androgens and FGF/FGFR signaling mediate mesenchymal-epithelial interaction and therefore play important roles in prostate development, homeostasis as well as tumorigenesis.

Studies with human normal prostate tissues and prostate epithelial and stromal cells show that FGF1 mRNA level is barely detectable in normal prostate. In addition, FGF2 is mainly expressed in stroma and less in epithelia (Ittman, Mansukhani 1997). FGF2 has been shown to be mitogenic to prostate stromal cells but to a lesser extent to epithelial cells (Giri, Ropiquet & Ittmann 1999a). FGF7 is the major FGFs produced in normal prostate and is exclusively expressed in the stroma (Ittman, Mansukhani 1997, Kwabi Addo, Ozen & Ittmann 2004). FGF10 is expressed at a very low level in normal prostate compared to FGF7 (Ropiquet et al. 2000a). Both FGF7 and FGF10

show the ability to act as a paracrine factor and stimulate the growth of prostatic epithelial cells but not stromal cells (Ittman, Mansukhani 1997, Thomson, Cunha 1999, Yan et al. 1992). Moreover, FGF7 and FGF10 are demonstrated to regulate prostate ductal-branching morphogenesis (Thomson, Cunha 1999, Sugimura et al. 1996). Androgens have been shown to regulate the mRNA expression of FGF7 and FGF10, but the results are conflicting in the *in vivo* and *in vitro* studies (Thomson, Cunha 1999, Yan et al. 1992, Lu et al. 1999). FGF9 is expressed exclusively in prostatic stromal cells and the expression level is equal to FGF2 but much higher than that of FGF7 (Kwabi Addo, Ozen & Ittmann 2004, Giri, Ropiquet & Ittmann 1999a). FGF9, which has high affinity to FGFR3 IIIc, is a more potent mitogenic factor for epithelium than FGF7 and for stromal cells than FGF2 (Giri, Ropiquet & Ittmann 1999a). Nevertheless, a study with rat prostate shows that FGF9 is mainly secreted from epithelial cells and that it binds directionally to stromal FGFR3 (Jin et al. 2004).

FGFR3 is expressed at the highest level in normal prostate in both epithelium and stroma where FGFR3 IIIb and IIIc RNAs are found at similar level. FGFR2 is expressed in stroma and it exhibits higher expression level than FGFR1. FGFR2 IIIb is mainly found in epithelia while FGFR2 IIIc is localized in stroma (Ittman, Mansukhani 1997). This is consistent with the rule that FGFRs in epithelia are activated by FGFs, which is expressed and secreted from stroma. Therefore, the FGFR2 IIIb, which is activated by FGF7, is expressed in epithelia but not in stroma. FGFR1 is expressed more exclusively in basal epithelia but not in luminal epithelia and FGFR1 IIIc is the predominant isoform (Hamaguchi et al. 1995). FGFR4 expression is less present in normal prostate (Kwabi Addo, Ozen & Ittmann 2004). (Figure 3)

2.4.2.4 FGFs and FGFRs in prostate cancer

Fibroblast growth factors and their receptors (FGFs/FGFRs) are involved in a broad spectrum of functions, including cell proliferation, migration, and wound healing. Therefore, dysregulated and constitutively activated FGF/FGFR pathways have been documented to be involved in initiation and progression of different types of cancers, including PCa. (Figure 3)

Directional and reciprocal interactions of FGFs and FGFRs are important for prostate homeostasis. The aberrant expression and activation of FGF/FGFRs in epithelia and/or stroma of the prostate could lead to

morphogenetic disorders and carcinogenesis (Cronauer et al. 2003, Grose, Dickson 2005).

FGF1 is a potent mitogen and a universal FGFR ligand that could bind to all the receptors independent of alternatively spliced isoform of D3 (Olsen et al. 2004). FGF1 is expressed at a very low level in normal prostate (Ittman, Mansukhani 1997) but the expression is increased in most prostate tumors and is associated with the GS (Dorkin et al. 1999a).

Increased FGF2 in PCa tissues is demonstrated by ELISA, western blot and IHC, and of note, the overexpressed FGF2 is only present in stromal and endothelial cells in PCa (Giri, Ropiquet & Ittmann 1999b). Moreover, PCa patients have increased FGF2 in serum (Cronauer et al. 1997). These indicate that FGF2 may act as a paracrine and/or endocrine factor in PCa. It has been demonstrated that elevated FGF2 plays an important role in PCa progression (Polnaszek et al. 2003).

Both FGF1 and FGF2 have been shown to promote rat PCa cell proliferation (Shain et al. 1996). Moreover, FGF2 is an angiogenic factor and thus contributing to tumor angiogenesis (Basilico, Moscatelli 1992). Studies with human PCa cell lines show that FGF2 is expressed in the metastatic PCa cell lines PC3 and DU145, although it is absent in localized PCa (Nakamoto et al. 1992, Cronauer et al. 1997).

FGF6 shows weak immune-staining in the basal cells in normal prostate but is markedly increased in the basal cells in HGPIN and prostate cancer cell (Ropiquet et al. 2000b).

FGF8 is barely detectable in normal prostate but its elevated expression in PCa has been demonstrated in several studies (Leung et al. 1996, Dorkin et al. 1999b). Increased FGF8 is also associated with advanced GS and the TNM stage (Dorkin et al. 1999b). Detailed introduction of FGF8 is in the chapter 2.4.3.

FGF17 belongs to the FGF8 subfamily and could be induced by FGF8. It also has a similar expression pattern as FGF8 during embryogenesis. Overexpressed FGF17 in localized PCa has been related to a poor outcome and a risk to metastasis PCa (Heer et al. 2004).

FGF19 and FGF23, which function like endocrine factors, seem also be involved in PCa progression.

Overexpression of FGFR1 in prostate tumors is correlated with poorly differentiated PCa (Giri, Ropiquet & Ittmann 1999b). The inducible FGFR1 (iFGFR1) prostate mouse model indicated that FGFR1 promotes PCa progression by inducing epithelial-to-mesenchymal transition (EMT) (Acevedo et al. 2007). A study by Armstrong et al. with clinical samples suggests that FGFR1 is associated with the transition of hormone-naive to castrate-resistant PCa (CRPC) (Armstrong et al. 2011). One more recent study shows that FGFR1 is an essential factor for PCa progression and metastasis (Yang et al. 2013). It is worth noting that FGF1, FGF2, FGF6, FGF8, FGF19 and FGF23, which all are increased in PCa, are able to bind FGFR1. This strongly supports the pivotal roles of FGFR1 in PCa.

In contrast to FGFR1, the expression and role of FGFR2 in PCa are conflicting in different studies. To date, the studies trend to agree that the switch of FGFR2 IIIb to FGFR2 IIIc is associated with PCa progression (Kwabi Addo et al. 2001, Sahadevan et al. 2007). It has been shown that overexpressed FGFR2 IIIb in PCa cell lines suppresses proliferation (Yasumoto et al. 2004). The role of FGFR2 in PCa needs to be investigated further.

So far, the role of FGFR3 in PCa is unknown. There is no evident difference in FGFR3 expression between benign prostate and PCa (Sahadevan et al. 2007). The role of mutational activation of FGFR3 has been discussed but the studies are limited and conflicting (Koufou et al. 2010, Hernández et al. 2009).

FGFR4 expression is reported to be elevated in PCa (Sahadevan et al. 2007, Gowardhan et al. 2005). Of note, the most discussed issue of FGFR4 in PCa is common polymorphism of FGFR-4 in at amino acid 388, the FGFR-4 Arg(388) polymorphism is present in most PCa patients and it has been indicated to involved in PCa initiation and progression (Wang, Stockton & Ittmann 2004, Wang et al. 2008).

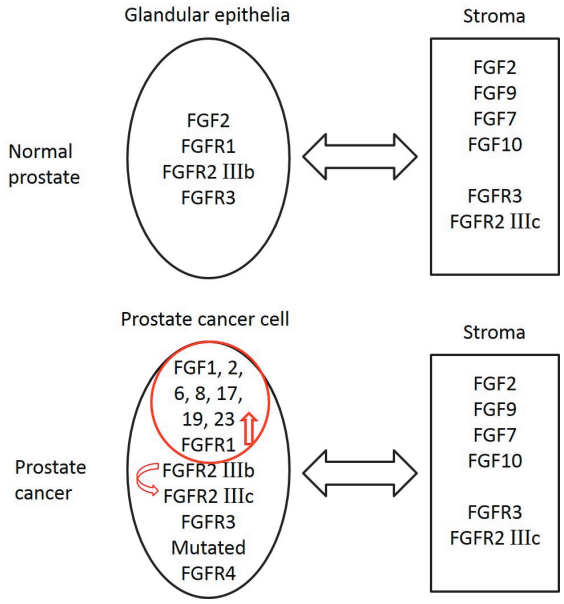

Figure 3. Expression of the members of the FGF and FGFR families in the epithelial and stromal cells in normal prostate (upper panel) and cancerous and stromal cells in PCa (lower panel)

2.4.3 The FGF8 subfamily with an emphasis on FGF8b

The FGF8 superfamily consists of FGF8, FGF17 and FGF18. They are expressed in the epithelial tissues and therefore mediating the epithelial to mesenchymal communication.

2.4.3.1 The structure and biological functions

FGF8 is originally identified in the androgen-dependent mouse mammary tumor SC-3 cell line (Tanaka et al. 1992). FGF8 gene locates on human chromosome 10q24 and has six exons (Payson et al. 1996). Alternatively, splices of exon 1 generate four different FGF8 isoforms in human (a, b, e, and f) (Gemel et al. 1996) and eight isoforms in mouse (a-h) (Tanaka et al. 1992). The FGF8 isoforms mainly differ in amino terminal ends and the isoforms a and b are highly conserved (Sunmonu, Li & Li 2011). FGF8b is identical in human and mouse (Ghosh et al. 1996). FGF8 is an androgen-induced growth factor (AIGF) and acts by binding to its receptor, and therefore, activates the signaling pathways, such as Ras-MAPK, PI3K-AKT, and PLCγ pathway. Of

the isoforms, FGF8b has the highest ability to activate FGFRs, especially FGFR2IIIc, FGFR3IIIc and FGFR4, whereas FGF8a has the least potential (Blunt et al. 1997). None of the FGF8 isoforms is able to active the b splice form of FGFR1-3.

FGF8 is expressed in a spatially and temporally restricted pattern and is essential for the development. FGF8 knockout mice die prenatally (E9.5) due to the failure of gastrulation (Sun et al. 1999). Studies with hypomorphic FGF8 mutation mice suggest that FGF8 is required for the differentiation and organogenesis of the brain, kidney, limb, inner ear, cardiovascular and craniofacial structures, and the reproductive track (Storm, Rubenstein & Martin 2003, Lewandoski, Sun & Martin 2000, Ladher et al. 2005, Frank et al. 2002, Kitagaki et al. 2011).

2.4.3.2 The role of FGF8b in prostate cancer

In adult, FGF8 is expressed at very low level and is mainly found in sex hormone target tissues, including prostate (Mattila, Härkönen 2007). Overexpression of FGF8 in PCa was first reported by Leung et al. (Leung et al. 1996) and later confirmed by more studies (Tanaka et al. 1998, Valve et al. 2001). Moreover, FGF8 is also frequently expressed in PCa bone metastatic sites (Valta et al. 2008). FGF8 expression is found to correlate with high GS and advanced tumor stage (Valve et al. 2001). Among the isoforms, FGF8b has the highest potential to transform NIH3-cells (MacArthur et al. 1995) and is the one that has been indicated to relate with late- and high-stage of the disease with decreased survival. FGF8b is also elevated in CRPC (Dorkin et al. 1999b). Studies of transgenic mice with prostate targeted overexpression of FGF8b show that FGF8b is involved in prostatic pre-malignant lesion and prostatic intraepithelial neoplasia (PIN) which indicate that FGF8b is involved in PCa initiation (Song et al. 2002, Elo et al. 2010).

Along with the tissue sample studies, FGF8 is expressed in PCa cell lines, including primary tumor cell line ALVA-31 and metastatic PCa cell lines (LNCaP, DU145, and PC-3) (Tanaka et al. 1995, Ghosh et al. 1996). *In vitro* and *in vivo* studies indicate that FGF8b is a mitogenic factor which accelerates prostate tumor growth (Song et al. 2000, Valta et al. 2009). Studies with both prostate and breast cancer cells imply that FGF8b also has an angiogenetic role by regulating thrombospondin 1 (TSP1), which is a known negative regulator of angiogenesis (Mattila et al. 2001, Valta et al. 2009, Tuomela et al. 2010).

FGF8b also functions along with deficient PTEN in a synergic manner to induce PCa (Zhong et al. 2006). Of note, in addition to its mitogenic and angiogenic role, FGF8 is able to induce expression of Sprouty, which is a negative regulator of Ras-MAPK pathway (Chambers et al. 2000).

2.4.4 FHFs subfamily with the emphasis on FGF13

FHFs members were found through a combination of random cDNA sequencing by virtue of their sequence similarity to FGFs. The FHF family contains four members: FGF11, FGF12, FGF13 and FGF14 (also known as FHF3, FHF1, FHF2 and FHF4, respectively) (Smallwood et al. 1996). In human, FGF11, FGF12, FGF13, and FGF14 are located on chromosomes 17, 3, X, and 13, respectively. The four FHFs bear 58-71% amino acid identity with each other and are defined as a highly conserved branch of FGF family. The homologous segment of FHFs and canonical FGFs is a pseudo threefold-symmetry structure called β-trefoil (Olsen et al. 2003). The non-homologous substitutions of a few amino acids of FHFs make them different from other FGFs in a notable way that they are not capable of activating FGF receptors.

In adult mouse, all the FGF11-14 are expressed in the brain, Fgf13 being at the highest level. Fgf12, Fgf13 and Fgf14 are also detected in the eye; and Fgf12 and Fgf14 in testis; and Fgf13 in heart (Smallwood et al. 1996). (Table 3)

Table 3. The expression patterns of FGF11-14 in adult mouse.

	high level	low level
FGF12 (FHF1)	olfactory bulb	cerebellum, deep cerebellar nuclei, cortex, midbrain
FGF13 (FHF2)	hippocampus	multiple brain
FGF11 (FHF3)	Purkinje cell layer	olfactory bulb, hippocampus, cerebellum
FGF14 (FHF4)	granular layer of cerebellum	hippocampus, olfactory bulb

Alternative promoter usage and alternative splicing at 5'-exon of FHFs generate at least 10 FHF isoforms in human. Based on the sequence, the isoforms are named as A and B. Isoform A bears the identified bipartite NLS which is conserved among the FGF11-14, therefore the isoform A of FGF11-14 is verified to localize mainly in the nuclei. FGF12 and FGF14 have isoforms A and B, respectively. FGF13 displays the most diverse splicing which results in 5 isoforms, 1S and 1U refers to isoform A and B, respectively. Table 4 shows the sequence and localization of FGF11-14 isoforms.

Table 4. The amino acid sequences of the alternative amino termini for the FHF isoforms in human and mouse.

Exons	Sequence	Species	Subcellular localization
FGF12			
1A	MAAAIASSLIROKROARESNSDRVSASKRRSSPSKDGRSLCERHVLGVFSKVRFCSGRKRPV RRRP/EPQL…	Human	Nuclear
	----------/EPQL…	Mouse	Absent in nucleoli
1B	MESK/EPQL…	Human	Cytosolic nuclear
	----/EPQL…	Mouse	
FGF13			
1S(A)	MAAAIASSLIRQKRQARAREKSNACKCVSSPSKGKTSCDKNKLNVFSRVKLFGSKKRRRRRP /EPQL…	Human	Nuclear nucleoli
	----------/EPQL…	Mouse	
1U(B)	MALLRKSYS/EPQL…	Human	Cytosolic nuclear
	---------/EPQL…	Mouse	
1V	MSGKVTKPKEEKDASK/EPQL…	Human	Cytosolic nuclear
	----------/EPQL…	Mouse	
1Y	MLRQDSIQSAELKKKESPFRAKCHEIFCCPLKQVHHKENTEPE/EPQL…	Human	Cytosolic nuclear
	----------P----------/EPQL…	Mouse	
1Y + 1V	MSGKVTKPKEEKDASK/VLDDAPPGTQEYIMLRQDSIQSAELKKKESPFRAKCHEIFCCOLK QVHHKENTEPE/EPQL…	Human	
	----------/----------P- ----------/EPQL…	Mouse	Cytosolic nuclear
FGF11			
1A	MAALASSLIRQKREVREPGGSRPVSAQRRVCPRGTKSLCQKQLLILLSKVRLCGGRPARPDR GP/EPQL…	Human	ND
	----------T-Q-- -/EPQL…	Mouse	
1B	MSLS/EPQL…	Mouse	ND
FGF14			
1A	MAAAIASGLIRQKRQAREQHWDRPSASRRRSSPSKNRGLCNGNLVDIFSKVRIFGLKKRRLR RQ/DPQL…	Human	Nuclear
	----------F----------/DPQL…	Mouse	Absent in nucleoli
1B	MVKPVPLFRRTDFKLLLCNHKGLFFLRVSKLLGCFSPKSMWFLWNIFSKGTHMLQCLCGKSL KKNKNPT/DPQL…		ND
	----------/DPQL…	Mouse	Cytosolic

Modified from (Munoz-Sanjuan, Smallwood & Nathans 2000, Goetz et al. 2009). Immunofluorescence was used to identify the subcellular localization

by tranfecting isofom sequences to cells. A slash indicates the junction between the exons. ND, not determined.

2.4.4.1 Fibroblast growth factor 13

Human *FGF13* locates on chromosome Xq26 where a variety of X-linked mental retardation syndromes (XLMSs), including Börjeson-Forssman-Lehmann syndrome (BFLS), have been mapped (Gecz et al. 1999, Smallwood et al. 1996). FGF13 lacks the classical signal sequence but contains nuclear localization sequence (NLS) as FGF1 and FGF2. This suggests that FGF13 could be released from the cells similar to FGF1 and FGF2 (Smallwood et al. 1996). But untill now, there is no evidence showing that FGF13 is a secreted protein.

The exon 2-5 of *FGF13* is conserved among all FGF13 isoforms. The alternative splicing of the first exon generated at least 5 different isoforms (1S, 1U, 1V, 1Y and 1V+Y) in human and mouse. Nevertheless, the truncated isoform FGF13 1B31 which has the alternative splicing at exon 3 has also been reported (Gecz et al. 1999). FGF13 1S and 1U coded by transcript variant 1 and 6 are the most studied isoforms and known as isoforms A and B, respectively. In transfected HEK 293 cells, FGF13 A isoform localizes to the nucleus in a consistent pattern and other isoforms localize to both cytoplasm and nucleus due to the lacking NLS (Munoz-Sanjuan, Smallwood & Nathans 2000). Although FGF13 is unable to bind or activate any known FGFRs, it is able to bind heparin by using a similar mechanism as other FGFs (Olsen et al. 2003). FGF13 A isoform binds to heparin more tightly than FGF13 1V and FGF13 1V+1Y (Munoz-Sanjuan, Smallwood & Nathans 2000). This suggests that the interaction of FGF13 isoforms with the extracellular matrix differ from each other.

2.4.4.2 FGF13 in embryonic development

During mouse embryogenesis (E9.5-E16.5), overall expression of Fgf13 rises first and then falls, the peak appears at E12.5-14.5 (Hartung et al. 1997). A study of Fgf13 isoforms by hybridization shows that Fgf13 A, 1V and 1Y account for the most of the signal and the distribution varies from each other (Munoz-Sanjuan, Smallwood & Nathans 2000). The distribution of Fgf13 isoforms is overlapping but distinct from each other. (Table 5)

Table 5. The distribution of the Fgf13 isoforms in mice

Methods	In situ hybridization			RNase protection
	E12.5	E18.5	Adult mouse	Adult mouse
Fgf13 A	central nervous system, liver, vertebral bodies	brain, spinal cord, enteric ganglia, heart	cortex, hippocampus, midbrain nuclei	brain, eye, spleen, testis
Fgf13 B	Spinal cord	undetectable	-	heart
Fgf13 1V	central nervous system, heart, limbs	brain, spinal cord, retinal, heart, enteric ganglia, tongue, condensing cartilage in the limbs, connective tissue around the vertebrae and ribs	cerebellum, thalamus, olfactory bulb, amygdala, facial nucleus, medial geniculate nuclei, basal brainstem nuclei	brain, heart, eye
Fgf13 1Y				-

Summarized on the basis of the data from (Munoz-Sanjuan, Smallwood & Nathans 2000, Liu, Yang & Dudley 2014).

FGF13 deficient mice generated by in utero electroporation show a defect in neuronal polarization and migration. Of note, the FGF13B, but not FGF13A, is able to reverse the migratory deficiency (Wu et al. 2012). The Emx1-Cre/Fgf13$^{F/Y}$ (cerebral-cortex-specific knockout) and Ella-Cre/Fgf13$^{F/Y}$ mice show that the neocortical laminar formation is delayed and the hippocampal formation is abnormal (Wu et al. 2012). The Ella-Cre/Fgf13$^{F/Y}$ mice do not show motor defect but display an impaired memory and learning ability and probable mood disorders (Wu et al. 2012).

In adult human tissues, FGF13 transcript is detectable in most of the organs, including the prostate and peripheral lymphocyte (Hartung et al. 1997), with highest expression in brain and skeletal muscle (Gecz et al. 1999).

2.4.4.3 Cellular interactions of FGF13

It was demonstrated that FHFs could bind to heparin by using a similar mechanism as other FGFs. The binding affinity of FHFs to heparin is equal to FGF7 and FGF10 but much lower compared to FGF1 and FGF2. However, recombinant FHFs could not activate any of the known functional FGF receptors. Moreover, FHFs could not antagonize the effects of FGF1 with FGFRs. Olsen and Garbi found two residues, Val95 and Arg52, which were fully conserved and unique in FHFs. They are expected to prevent FHF binding to FGF receptors. At the same time, the N terminus and the β8-β9 loop may also make the FHF conformation incompatible with FGFRs (Olsen et al. 2003). This suggests that FGF13 may execute their function by a pathway other than FGFRs. Instead of activating FGFRs, FGF13 is indicated to bind microtubules, voltage gated sodium channels and Islet-Brain-2.

2.4.4.3.1 Binding sites of FGF13 with IB2

The mitogen-actived protein kinases (MAPKs) include the ERK1/2, c-Jun NH2 terminal kinases (JNK), and the p38 MAPKs. Islet-Brain-2 (IB2), known as JNK interacting protein 2 (JIP2), is mainly expressed in brain and pancreatic islet cells (Negri et al. 2000). Two JIP proteins, JIP1 and JIP2 (known as IB1 and IB2), are scaffold proteins which regulate the JNK pathway by assembling components of the MAPK cascade. Both FGF12 and FGF13 are found to bind IB2, but not IB1, with high specificity. In contrast, FGF1 cannot bind to IB2 (Schoorlemmer, Goldfarb 2001). Moreover, the present of FGF12 and FGF13 can facilitate IB2 binding to p38δ in a dose dependent manner (Schoorlemmer, Goldfarb 2001). Therefore, it is suggested that FGF13 support p38δ activation. The p38 MAPK pathway is associated with extracellular stimuli and is abbarrently activated in PCa (Uzgare, Kaplan & Greenberg 2003, Royuela et al. 2002). The p38α and p38δ are the two of the four p38 isoforms that could be detected in prostate at protein level (Frank, Miranti 2013). It is suggested that p38δ may play a role like oncogenic in PCa (Frank, Miranti 2013). Moreover, p38δ potentionally has overlapping functions with p38α but distinct characteristics have already been observed, for instance, formation of complex with ERK1/2 and phosphorylation of microtubule-associated protein such as Tau and Stathmin (Efimova, Broome & Eckert 2003, Goedert et al. 1997, Parker et al. 1998).

2.4.4.3.2 Binding sites of FGF13 with ion channels

Voltage gated sodium channels (VGSCs), also called Na_Vs, comprise 9 α and 4 β subunits, which are marked as Nav1.1-1.9 based on the α-subunit. VGSCs are classically expressed at excitable cells, like neurons (Nav1.1, Nav1.2, Nav1.3, Nav1.6, Nav1.7, Nav1.8, and Nav1.9), skeletal muscle (Nav1.4) and cardiac muscle (Nav1.5); where their mechanisms are well understood. Besides the excitable tissues, Na_Vs are also found to be functionally involved in immune cells, fibroblasts, as well as cancers cells, such as breast cancer, cervix cancer, colon cancer, lung cancer, prostate cancer, lymphoma and so on (Brackenbury 2012, Fraser et al. 2014). In non-excited cells, Na_Vs can regulate cell proliferation (Bennett et al. 2013), differentiation (Chopra et al. 2010), endocytosis, secretion, and motility (Black, Liu & Waxman 2009). The VGSCs α subunits are referred to cell migration and invasion while β subunits are considered to mediate cellular adhesion and process extension (Brackenbury 2012). However, the mechanisms of Na_Vs in cancer cells are poorly known. Regulation of pH, secretion, gene expression and intracellular Ca^+ are considered as the potential mechanisms of Na_Vs involved in cancer progression (Brackenbury 2012, Diss et al. 2005). Epidermal growth factor (EGF), nerve growth factor (NGF) and steroid hormones, like β-estradiol and dihydrotestosterone (DHT) are documented to regulate the Na_V expression (Brackenbury 2012).

The total Na_V expression is reported to be increased in PCa tissues compared to normal prostate tissues (Abdul, Hoosein 2002, Diss et al. 2005) and Na_Vs are involved in metastatic process in PCa (Yildirim et al. 2012). Concerning the isotopes, the mRNAs of all Na_Vs except $Na_V1.8$ are detectable in normal prostate tissues. In BHP, NaV1.2 and $Na_V1.5$ are the predominant VGSCs while $Na_V1.8$ and 1.9 are not detectable. In the PCa cell lines (PC3 and LNCaP), both $Na_V1.6$ and $Na_V1.7$ exhibit much higher expression than other Na_Vs. They are also markedly increased (6-27-fold) when compared to normal prostate and BHP (Shan et al. 2014, Suy et al. 2012). Extended study of Na_V protein expression in PCa cells lines (CWR22Rv-1, LNCaP, C4-2, C4-2B, DU145, PC3 and PC3M) shows that $Na_V1.5$, $Na_V1.6$, $Na_V1.8$ could be detected in all 7 PCa cell lines while $Na_V1.3$ and $Na_V1.4$ are undetectable; $Na_V1.1$, $Na_V1.2$ and $Na_V1.9$ expression levels are higher in DU145, PC3 and PC-3M cell lines and $Na_V1.7$ is absent from PC-3M and CWR22Rv-1 cell lines (Suy et al. 2012). In PCa tissue, $Na_V1.7$ is the predominant isotype (Diss

et al. 2005, Diss et al. 2001). Moreover, it has been shown that $Na_V1.8$ expression is related to pathological stage and the GS in PCa (Suy et al. 2012).

FGF11-14 are documented to bind to the intracellular C-terminal domain (CTD) of the Na_V α subunit and are assumed to constitute auxiliary subunits for Na_Vs (Goetz et al. 2009). So far, FGF12 (FHF1) has been found to bind to $Na_V1.5$ and $Na_V1.9$ (Liu et al. 2003, Liu, Dib-Hajj & Waxman 2001), FGF13 (FHF2) binds to NaV1.5, $Na_V1.6$ and $Na_V1.9$ (Wittmack et al. 2004, Goldfarb 2005), and FGF14 (FHF4) is demonstrated to interact with $Na_V1.1$, $Na_V1.5$ and hippocampal sodium channels (Lou et al. 2005). Although convincing evidence has shown that FGF11-14 could regulate Na_Vs, the mechanism underlying is far from clear because of the inconsistent observations between different isoforms and different systems.

Both isoforms of FGF13 A and B are found to be colocalized with $Na_V1.6$ and exhibit different effects on it (Rush et al. 2006). FGF13B also emerges in the complexes of $Na_V1.9$ (Rush et al. 2006, Wittmack et al. 2004, Goldfarb 2005).

2.4.4.3.3 *FGF13 is a microtubule stabilization protein*

Microtubules are highly dynamic polymers which are essential for cytoskeleton structure. Dynamics of microtubules contribute to the cell mitosis, movement and endocrine signaling pathways. In human, eight α- and seven β-tubulin isotypes have been identified for microtubules structures. The aberrant expression and post-translation of isotypes are verified in cancers (Parker, Kavallaris & McCarroll 2014). In PCa, overexpression of class IIIβ-tubulin is associated with advanced PCa and taxanes resistance, which is the only chemotherapy class to show a survival benefit in metastatic CRPC (Ploussard et al. 2010, Tsourlakis et al. 2014). Microtubule-associated proteins (MAPs), including MAP1-4, Tau, etc., are a family that regulates microtubule dynamic by binding to and stabilizing them. Various roles of MAPs have been reported in malignancies and their resistance to microtubule-targeting agents. For instance, MAP2 is assumed as a diagnostic marker in oral squamous cell carcinoma, but it has also been suggested to inhibit cell proliferation and melanoma metastasis (Chen et al. 2004). Tau, as one of the most studied MAPs, is inversely associated with the sensitivity of breast cancer cells to paclitaxel due to its ability of stabilizing microtubules (Rouzier et al. 2005). By presenting the FGF13 ability of direct interaction and

polymerizing microtubules, Wu et al. assume that FGF13 works as a microtubule stabilization protein in brain development (Wu et al. 2012).

2.4.4.4 FGF13 and cancers

A query on the cBioPortal database indicated that *FGF13* gene alterations were found at a high frequency (31%) in PCa (Cerami et al. 2012, Gao et al. 2013). Study of FGF13 in pancreatic cancer showed that FGF13 expression is increased in metastatic tumor compared with primary tumor and its expression is related to aggressive tumor and proliferation marker Ki67. Moreover, FGF13 is positively associated with reduced progression-free survival of pancreatic cancer (Missiaglia et al. 2010). High FGF13 expression is found in cisplatin-resistant Hela cells and FGF13 transcript variant 2/3/5 is indicated to play a pivotal role in acquisition of platinum-drug resistantance (Okada et al. 2013). High FGF13 expression is also shown to positively correlate with poor prognosis of cervical cancer (Okada et al. 2013). Additionally, a decreased FGF13 mRNA level was observed by comparison with lung adenocarcinoma cells from primary cancer to metastatic paratracheal lymph nodes (Chen et al. 2014).

2.4.5 Fibroblast growth factor receptor like 1 (FGFRL1)

Fibroblast growth factor receptor like 1 (FGFRL1) is the fifth member of FGF receptor family, which was identified from the human cartilage (Wiedemann, Trueb 2000). *FGFRL1* comprises seven exons and is located on the short arm of chromosome 4 p16 in a close proximity to *FGFR3* in human.

2.4.5.1 Structure of FGFRL1

FGFRL1 has 504 residues with the molecular mass of 55 kDa (Rieckmann et al. 2009). The human FGFRL1 contains four glycosylation sites for carbohydrate attachment. Therefore, the difference in carbohydrates attached results in 10 kDa difference in FGFRL1 protein molecular mass (Rieckmann, Kotevic & Trueb 2008).

FGFRL1 is a transmembrane-spanning receptor, which contains three Ig-like extracellular domains and an intracellular domain. Its extracellular domain shares up to 40% amino acid similarity to FGFR1-4 (Rieckmann et al. 2009). It has therefore been shown that FGFRL1 is able to bind some of the

FGFs (Steinberg et al. 2010b) and heparin (Rieckmann, Kotevic & Trueb 2008). The FGFRL1 binding ability varies between FGFs showing strong binding affinity to FGF3, FGF4, FGF8, FGF10 and FGF22; intermediate affinity to FGF2, FGF5, FGF17 and FGF23. FGFRL1 is unable to bind FGF1, FGF6, FGF7, FGF9, FGF12, FGF16, FGF19, FGF20 and FGF21. However, FGFRL1 exhibits higher binding affinity to heparin than the conventional receptors (Steinberg et al. 2010b).

Steinberg et al. demonstrated that the extracellular domain of FGFRL1 could be shed from the cell membrane, and the cleavage site is supposed to be in the membrane-proximal region (Gly17-Ala18) (Steinberg et al. 2010b). But the proteases, like broad spectrum metalloproteases inhibitor, leupeptin, and pepstatin, have no effect on the shedding event. However, the shedding of FGFRL1 is not a universal rule that happens on all the cell types (Steinberg et al. 2010b). In contrast to the extracellular domain, the intracellular domain of FGFRL1 does not show similarity to any of the conventional FGFRs and any of the other protein sequences (Steinberg et al. 2010b). The intracellular domain of FGFRL1 contains only 100 residues without any protein tyrosine kinase and therefore cannot signal via transautophosphorylation. Instead, this intracellular domain contains a dileucine, two tyrosine-based motifs in tandem form, and a histidine-rich C-terminal tail. Dileucine and tyrosine-based motif are known to medicate endocytosis and transmembrane protein trafficking (Bonifacino, Traub 2003); and the histidine-rich tail is documented to interact with zinc and nickel ions (Zhuang et al. 2009). It is assumed that the intracellular domain is necessary for FGFRL1 turnover by guiding FGFRL1 to endosomes and lysosomes (Rieckmann et al. 2009).

Riechman et al. report that FGFRL1 could form constitutive homophilic dimers regardless of existent of FGFs or heparin (Rieckmann, Kotevic & Trueb 2008). Both extracellular and intracellular domains of FGFRL1 are needed for homophilic dimer formation. The study does not find evidence of heterophilic formation between FGFRL1 and FGFR3/FGFR4 which have similar tissue distribution with FGFRL1.

Alternative splicing of FGFRL1 is found from murine lymph node stromal cells which lacks first Ig-like domain (Sleeman et al. 2001). The expression pattern and relative study of this splicing variant in human has not been reported yet.

2.4.5.2 Expression of FGFRL1 in normal tissue

The gene of FGFRL1 is expressed in all vertebrates but the expression level is relatively lower than that of conventional FGFRs. Study of Fgfrl1 RNA expression in mouse embryos shows that Fgfrl1 expression is very low in early mouse embryos stage; but it steadily increases with prominent expression between embryonic days (E)15.5-18.5 (Trueb, Taeschler 2006). In late mouse embryos (E 16.5-17.5), Fgfrl1 is found primarily in the developing vertebral bodies and cartilage but is expressed at a very low levels in inner organs, brain and the spinal cord (Trueb, Taeschler 2006, Trueb et al. 2003). In newborn mice, Fgfrl1 is found in most of the tissues with high expression in the cartilaginous structures (Trueb, Taeschler 2006). In human, FGFRL1 mRNA is expressed at a high level in pancreas, thyroid and adrenal gland, kidney, skeletal muscle and heart (Kim et al. 2001). Immunohistochemical stainings confirmed that FGFRL1 expression in pancreas and skeletal muscle as well as bladder is higher than in liver and spleen (di Martino et al. 2013). In contrast, FGFR1L expression is negative in lung, stomach, esophagus, and smooth muscle (di Martino et al. 2013, Kim et al. 2001, Sleeman et al. 2001).

Homozygous Fgfrl1 knockout mice died after birth because of the dysplastic diaphragm muscle. The diaphragms of Fgfrl1 knockout mice are 40% thinner than the control mice, but the remaining diaphragm muscles are well differentiated and innervated (Baertschi, Zhuang & Trueb 2007). The diaphragm deficient phenotype is not related to gender (Catela et al. 2009). Other skeletal muscles do not show any defects. Another striking phenotype of Fgfrl1 knockout mice is the severe hypogenesis of both metanephric kidneys (Gerber et al. 2009). In these mutant mice, lackof Fgfrl1 causes defects in ureteric branching and nephrogenic mesenchymal differentiation. Moreover, the significantly declined markers of the nephrogenesis, such as *Pax8*, *Lhx1*, *Wnt4* and *Fgf8*, are also observed in Fgfrl1 deficient mice (Gerber et al. 2012). Catela et al. report another Fgfrl1 knock-out mouse model, in which homozygous Fgfrl1 $^{-/-}$ mice also died around birth because of the agenesis of the diaphragm. In contrast to the other models, this study also reports the hypoplastic skeletal system, such as shortened axial and malformed vertebrae, and congenital heart defects that could cause transient fetal anemia (Catela et al. 2009). Heterozygous Fgfrl1 $^{+/-}$ mice do not show any discernible abnormalities in all the established models described above. However, mice with targeted distribution of the intracellular domain of Fgfrl1

are vital and only exhibit a reduced number of glomeruli but not show similar phenotypes as described above (Bluteau et al. 2014).

Subcellular expression of wild type FGFRL1 is found mainly in vesicular structures and Golgi complex, whereas deletion or mutation of the tandem tyrosine-based motif or histidine-rich region retain FGFRL1 in the plasma membrane (Bonifacino, Traub 2003).

2.4.5.3 Biological functions of FGFRL1

Studies of transgenic mouse models have shown that FGFRL1 plays an important role in specific muscle and kidney development (Baertschi, Zhuang & Trueb 2007, Gerber et al. 2009). The zebrafish model and *in vitro* studies have also been developed to explore the potential functions of FGFRL1. Nonetheless, there is still no consensus of FGFRL1 function.

FGFRL1 is assumed to exert negative effects on FGF signaling because it binds to FGFs but lacks the tyrosine kinase domain for signaling. The first reported negative effect of FGFRL1 is the inhibition of cell proliferation (Trueb et al. 2003). This study shows that overexpressed FGFRL1 reduced the proliferation rate in MG-63 cells but decreased FGFRL1 does not affect cell proliferation. In addition to the anti-proliferative effect, overexpressed FGFRL1 has also been found to induce cell apoptosis in HEK293 cells (Steinberg et al. 2010a). Moreover, injection of FGFRL1 mRNA to *Xenopus* embryos reproduced the XFD-phenotype which was generated by injection of domain-negative FGFR (XFD) and displayed gastrulation development defects. The effect of overexpressed FGFRL1 on *Xenopus* embryos could be revised by coinjection of FGFR1 mRNA. The interference with FGF signaling is also supported by the evidence of interaction between FGFRL1 and Spred1, a negative regulator of FGF (Zhuang, Villiger & Trueb 2011).

Nevertheless, the negative regulation of FGF signaling is challenged by the evidence of showing genes that are regulated by Fgfrl1. When kidney gene expression profile of Fgfrl1 deficient mice are compared with control mice, more than 50 genes which involved in Fgf, Wnt, Bmp, Notch, and Six/Eya/Dach signaling pathways are downregulated. Interestingly, *Fgf8*, as one of the favourite ligands for Fgfrl1 and an essential factor for kidney development, is greatly reduced in *Fgfrl1* deficient mice compared to normal control (Gerber et al. 2012). This suggests that Fgfrl1 might play a positive role in FGF signaling, at least in kidney development. Another example is the

study of FGFRL1 on pancreatic cells. FGFRL1 is highly expressed on the plasma membrane and insulin secretory granules of human beta-cells. The study shows that the intracellular domain of FGFRL1 could activate phosphorylation of ERK1/2 in a ligand independent manner and interact with a phosphatase SHP-1 in beta-cells (Silva et al. 2013). The results suggest that insulin processing might be regulated, at least partly, via FGFRL1 signaling. These studies imply that FGFRL1 might act not only as a negative regulator of FGF signaling pathway.

In addition to the role in FGF/FGFR signaling, FGFRL1 is also involved in cell adhesion. FGFRL1 is found to be accumulated at the cell-cell contact surface and is therefore presumed to play a role in cell contacts. There is evidence that culture surfaces coated with recombinant FGFRL1 are able to accelerate cell adhesion (Rieckmann, Kotevic & Trueb 2008). The adhesion promoting effect is mediated by heparin sulfate glycosaminoglycans and could be abolished by heparin preincubation in a dose-dependent manner or by mutation of the heparin-binding site. In contrast to the adhesion induced by fibronectin, the cells attached to recombined FGFRL1 are reluctant to spread (Rieckmann, Kotevic & Trueb 2008).

Cell-cell fusion is an important process during development. FGFRL1 is the first reported mammalian protein that is able to induce cell fusion (Steinberg et al. 2010a). It is reported that Fgfrl1 is sharply upregulated when C2C12 myogenic cells start to fuse to multinucleated syncytia (Catela et al. 2009). Hence, FGFRL1 might play an important role during cell-cell fusion process, at least in differentiation of myoblasts to myotubes. The cell-cell fusion mechanism induced by FGFRL1 is poorly understood but the Ig-like domain D3 and transmembrane segments are needed in this process (Steinberg et al. 2010a).

2.4.5.4 FGFRL1 in human disease

The first FGFRL1 mutation was reported by Bonifacino et al. in Antley-Bixler syndrome (Bonifacino, Traub 2003). The mutation was identified in the intracellular domain of FGFRL1 and the disease presents with craniosynostosis, radio-ulnar synostosis and genital anomalies. Genital disorder could be explained by the mutation of P450 oxidoreductase and the mutation of FGFRL1 offers causes of bony phenotype.

Wolf–Hirschhorn syndrome (WHS) is a development disorder which is caused by deletion of the short arm of chromosome 4 which contains *FGFRL1* genes. WHS is characterized by craniofacial dysgenesis, congenital heart defects, short stature and mental retardation (Battaglia et al. 2000, De Keersmaecker et al. 2002). In the Fgfrl1 null mouse model introduced by Catela et al., Fgfrl1 deficient mice have been shown to display several features overlapping those of WHS patients, such as skeletal malformation, short stature and heart defects (Catela et al. 2009). Of note, FGFRL1 is expressed in the primordia of bones and cartilaginous tissues. FGFRL1 has thus been shown to be a candidate gene of WHS and Fgfrl1 null mice could be used as a model to study WHS.

Deletion of chromosome 4p is also a common event in bladder cancer. Loss of heterozygosity in 4p16.3 including FGFRL1 has been shown to happen in 9% of the bladder tumors (di Martino et al. 2013). This implies FGFRL1 as a putative deletion target in bladder cancer but there is no evidence to support it until now. This study also exhibits downregulation of FGFRL1 expression in bladder cancer compared to normal bladder tissue but the decrease is not related to 4p16.3.

The FGF/FGFR signaling has been documented to play important roles in embryogenesis, development as well as carcinogenesis. As a potential regulator of the FGF/FGFR signaling pathway, Schild et al. showed high *FGFRL1* RNA expression level in several bone-cartilage and muscle related sarcoma cell lines (MG63, SW1353 and A204) whereas the level was low in others (HT1080, SK-LMS-1, SK-UT-1HeLa) (Schild, Trueb 2005). In the same study, alterations of FGFRL1 mRNA expression were also reported in a profiling array of ovarial tumor samples.

FGFRL1 has also been studied in esophageal squamous cell carcinoma (ESCC) (Shimada et al. 2014, Tsuchiya et al. 2011). Tsuchiya et al. show that a relatively high level of FGFRL1 mRNA is found in 20% of well differentiated ESCC but in 68% of poorly differentiated tumors. Moreover, the same study demonstrates that FGFRL1 is able to stimulate cell growth by preventing cell cycle arrest at G_1/G_0 (Tsuchiya et al. 2011). The later study by Shimada et al. shows that FGFRL1 staining is both on the plasma membrane and in the cytoplasma in ESCC, but mainly in cytoplasm. Overall and cause-specific survival analysis showed that positive FGFRL1 group displays worse prognosis than negative group. This study also showed that FGFRL1 is

associated with lymph node metastasis in ESCC, although it failed to be an independent prognostic factor (Shimada et al. 2014).

3 AIMS OF THE STUDY

The aim of the thesis study was to investigate the role of the selected members of the FGF/FGFR pathways and estrogen receptor β in initiation, growth and progression of PCa.

The specific aims were:

1. To study the effect of ERβ deficiency on FGF8b-induced prostate tumorigenesis by establishing FGF8b-TG-BERKO$_{FVB}$ gene-modified mouse models.

2. To evaluate the expression pattern of FGF13 utilizing human PCa tissue microarrays and to explore the prognostic value of FGF13 in estimation of PCa patient outcome by associating the FGF13 expression results with the related clinical data.

3. To evaluate the expression level and cellular distribution of FGFRL1 in human PCa and the relation of the results to the clinical parameters and to study the mechanisms of FGFRL1 action at both *in vitro* and *in vivo* levels.

4 MATERIALS AND METHODS

4.1 Human prostate cancer samples and clinical data (II, III)

Tissue samples were collected at Turku University Hospital (Turku, Finland). Specimens from estimated benign and tumor areas were collected from patients who underwent RP. Each piece was cut into two pieces for frozen samples and formalin fixed paraffin-embedded (FFPE) samples. Frozen samples were later used for mRNA analysis and FFPE samples were used for histology and TMA construction. Hematoxylin and eosin (H&E) staining from all FFPE samples were reviewed by experienced pathologists. Histological features, percentage of carcinoma and benign areas, and inflammation scores were recorded. The GS grading was performed according to the World Health Organization's classification system for PCa (Epstein et al. 2005a, Epstein 2010). Interpretable clinical data of studied patients was collected via Turku Prostate Cancer Consortium. BCR was defined as at least 2 consecutive postoperative measurements with serum PSA level ≥ 0.2ng/ml, and the date of the first PSA value ≥ 0.2 ng/ml was assigned as the date for BCR. GS ≥ 4+3, PSA ≥ 10ng/ml, pTNM ≥ T3a or PSM were considered as intermediated to high risk factors. The endpoint of follow-up was defined by the months from RP until BCR or the date of last follow-up. The current study is approved by the ethical committee of the Hospital District of Southwest Finland (ETMK 130/180/2008, ETMK: 3/180/2013) and is conducted in compliance with the current revision of the Declaration of Helsinki guiding physicians and medical research involving human subjects. Patient information connected to tissue material was kept anonym and thus protected according to the ethical requirements.

4.2 Construction and evaluation of tissue microarray (II, III)

Three TMA sets were designed to evaluate FGF13 and FGFRL1 protein expression in PCa. A manual tissue-arraying instrument (Beecher Instruments, Sun Prairie, WI) was used to constructe the arrays. The most representative areas were selected and marked on H&E stained slides which indicated the donor site. For each FFPE samples, duplicate tissue cores (φ1.5mm) were punched from representative areas and then arrayed into the recipient blank paraffin block.

The TMA stainings were assessed using IHC score system McCarty et al., 1985), evaluated by two independent observers. Staining intensity (Int) was classified into 4 levels: 0 (negative), weakly positive (Int1), moderately positive (Int2) and strongly positive (Int3). The extent of the area with positive staining in the whole annotated area (%Pos) was scored as well. Thus, IHC score = Int1 * %Pos + Int2 * %Pos + Int3 * %Pos giving the range from 0 to 300.

4.3 Animal experiments

4.3.1 Generation of FGF8b-TG-BERKO$_{FVB}$ mice (I)

Generation of FGF8-TG mice was described before (Elo et al. 2010). BERKO mice in strain C57B/6J (Krege et al. 1998) were crossbred with FVB/N mice for seven generations first and then crossbred with FGF8-TG mice to generate FGF8b-TG-BERKO$_{FVB}$ mice.

4.3.2 Subcutaneous cancer cells inoculation (III)

Five weeks old nude mice (Harlan Winkelman GmbH, Borchen, Germany) were used for the subcutaneous inoculation. The mice (1st experiment: N = 12, repeated experiment: N = 20) were randomized to two groups and the cells were implanted subcutaneously ($1x10^6$ cells in 100 µl). Mice were housed under controlled conditions (12h light/12h darkness, temperature 21 ± 3 ℃) and fed with standard chow food and tap water ad libitum. Tumor growth and body weight were measured every five days. Mice were kept for 45 days after inoculation.

4.4 Cell culture (II, III)

All cell cultures were maintained in a humidified incubator at 37°C and 5% CO_2 (Table 1). PNT1a, LNCaP, DU145, PC3, NCl-H660, and VCaP cell lines were obtained from American Type Culture Collection (ATCC) and PC-3M cell line was obtained from Caliper Life Sciences. Establishment of PSK-1 cell line with neuroendocrine characteristics has been published (Kim et al. 2000). Cell line authentication was performed by IdentiCell Laboratories (Department of Molecular Medicine at Aarhus University Hospital Skejby, Århus, Denmark). The shRNA and plasmid transfection was performed using Lipofectamin 2000 (Thermo Fisher). Single cell clones were selected by application of puromycin.

Table 1. Cell lines and culture conditions used

Cell lines	Culture condition
PNT1a, LNCaP, VCaP, PSK-1	RPMI containing 10% fetal bovine serum (FBS, Gibco), GlutaMAX (2 mM, Gibco) and 1% penicillin/streptomycin
PC3, PC-3M, DU145,	DMEM containing 10% FBS and 1% penicillin/streptomycin
NCI-H660	RPMI containing 5% FBS, insulin (10 µg/ml), transferrin (5.5 µg/ml), sodium selenite (40 nM), hydrocortisone (10 nM), β-estradiol (10 nM), EGF (10 ng/ml), Glutamax (2mM) and 1% penicillin/streptomycin

4.5 FGFRL1 gene silencing experiments (III)

FGFRL1 short hairpin RNA (shRNA) plasmid was purchased from Santa Cruz. The donor FGFRL1 plasmid was obtained from Addgene (Addgene 23600). Sequence containing amino acid residues 1- 504 of FGFRL1 was inserted into the Ecor I/BamH I site of the expression vector pEGFP-C2 for green fluorescent protein.

4.6 RNA analysis (I-III)

Total RNA from the cells and tissues of interest was isolated using RNeasy Mini kit (GIAGEN) (II and III) or TRIzol reagent (Invirtrogen) (I) according to the manufactures' instruction. Extracted RNA was reversed to cDNA for further qRT-PCR analysis or for RNA sequencing. Primers used in the thesis were listed in Table 2.

Table 2. Primers for qRT-PCR

Gene	Sequence	Size (kb)	Ann. °C	Used in
Actin	F: CGTGGGCCGCCCTAGGCACCA R: TGGCCTTAGGGTTCAGGGGG	242	60	I
Ar	F: GTCTCCGGAAATGTTATGAA R: AAGCTGCCTCTCTCCAAG	293	58	I
Era	F: CCGTGTGCAATGACTATGCC R:GTGCTTCAACATTCTCCCTCCTC	245	58	I
FGF13	F: GTTACCAAGCTATACAGCCGAC R:ACAGGGATGAGGTTAAACAGAGT	113	60	II

FGF13 v2/3/5	F: CCCTTTCGTGCTAAGTGTCA R: CTTAAGCTGAGGCTCTTCCG	93	60	II
FGF13v1	F: GACCAG CTG CGA CAA AAA CAA R: TGA GGC TCT GGT CTT CTT CTG C	96	60	II
FGF13v4	F: CCTAAGCACTCTCCCAAGTCC R: TATACCCTTAAGCTGAGGCTCCTT	149	60	II
FGF13v6	F: GTCGTATTCAGAGCCTCAGCT R: GATAGCCACCACTCGCAGAC	166	60	II
FGFRL1	F: CCATGTGGACCAAGGATGGC R: CTAATGTCATCCAGCACGACG	181	60	III
Il17	F: TCATCCCTCAAAGCTCAGCG R: TTCATTGCGGTGGAGAGTCC	167	58	I
Il6	F: CCGGAGAGGAGACTTCACAG R: CAGAATTGCCATTGCACAAC	134	60	I
Muc1	F: GTGCCAGTGCCGCCGAAAGA R: TGCCGAAACCTCCTCATAGGGGC	154	60	I
Muc2	F: GCCAGATCCCGAAACCAC R: TGTAGGAGTCTCGGCAGTCA	127	60	I
TBP	F: GAATATCCCAAGCGGTTT R: ACTTCACATCACAGCTCCCC	223	60	II, III
Tgfb	F: CAACAATTCCTGGCGTTACCTTGG R: GAAAGCCCTGTATTCCGTCTCCTT	128	60	I
Tnfa	F: CCCCAAAGGGATGAGAAGTT R: CACTTGGTGGTTTGCTACGA	132	60	I

4.7 Protein analysis

4.7.1 Immunohistochemistry (I-III)

TMA sections (3 μm) were used for immunohistochemistry (IHC) performed using a Lab Vision autostainer (Thermo Scientific). 5 μm sections from FFPE blocks of animal experiment were cut and used for IHC. Specific protocols have been reported in Yu et al., 2016 (paper II) and Yu et al. manuscript (paper III).

4.7.2 Western blot (II-III)

Total cell protein lysates were prepared in sample buffer (65 mM Tris-HCI pH 6.8, 10% glycerol, 2% SDS, 0.005% bromophenol blue, and 5% 2β-mercaptoethanol). Cytoplasmic and nuclear fragments were isolated by using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology). Conditioned media were collected and concentrated from PC-3M, LNCaP and

VCaP cells, which were cultured in serum free medium for 48 hours. Protein concentration was measured using a standard BCA assay. Equal amounts of protein were applied on gels for protein examination performed by standard protocols. Signal was detected by the LI-COR system (LI-COR Inc).

4.7.3 Immunofluorescence (II)

Adherent cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for immunofluorescence (IF) staining. Normal goat serum (30%) was used for blocking of unspecific staining followed by incubation with a primary antibody overnight (4°C). Primary antibodies were detected using corresponding fluorochrome-conjugated secondary anti-antibodies for 1 h (RT). Samples were mounted with ProLong gold antifade reagent (Invitrogen).

4.8 Primary and secondary antibodies (I-III)

Primary and secondary antibodies used in the thesis were listed in Table 3 and 4.

Table 3. Primary antibodies

Antigen	Supplier	Dilution	Appli-cation	Used in
a tubulin	Abcan; ab4074	1:50,000	WB	II ,III
a tubulin	Abcam; ab7291	1: 200	IF	II
AKT	Cell signaling technology; 9272	1:5000	WB	III
Ar	Santa cruz; sc-816, N20	1:100	IHC	I
CD20	Ventana; L26, 760-2531	ready to use	IHC	II
CD3	Ventana; 2GV6, 790-4341	ready to use	IHC	II
CD34	Santa cruz; sc-18917	1:50	IHC	III
CD68	DAKO; clone PG-M1	1:100	IHC	II
CDK2	Santa cruz; sc-163	1:1000	WB	III
CDK4	Santa cruz; sc-23896	1:1000	WB	III
CDK6	Santa cruz; sc-7181	1:1000	WB	III
cyclin D1	Abcam; ab16663	1:500	WB	III
cyclin E	Santa cruz; sc-481	1:1000	WB	III
FGF13	Atlas antibodies; HPA002809	1:50	IHC, WB, IF	II
FGFRL1(FGFR5)	Abcam; ab95940	1:1000	WB	III

FRS2-α	Santa cruz; sc-8318, H-91	1:1000	WB	III
Ki67	DAKO; monoclonal clone MIB-1	1:200	IHC	II ,III
p44/42 MAPK (Erk1/2)	Cell signaling technology 9102	1:3000	WB	III
p63	BD Pharmingen; 559951	1:500	IHC	I
phospho-AKT (ser 473)	Santa cruz; sc-33437	1:5000	WB	III
phospho-FRS2-α (Y196)	Cell signaling technology; 3864s	1:1000	WB	III
Phospho-Histone H3 (PHH3)	Cell signaling technology; 9701	1:200	IHC	III
phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204)	Cell signaling technology 9101	1:2000	WB	III
SMA	NeoMarkers; MS-113-P	1:100	IHC	I
SP1	Abcam; ab77441	1:1000	WB	II

Table 4. Secondary antibodies

Antigen	Supplier	Dilution	Applica-tion	Used in
Goat anti-rabbit AlexaFlour 488	Abcam; 150077	1:500	IF	II
Goat anti-guinea pig AlexaFlour 647	Abcam; 150187	1:500	IF	II
Goat anti-rabbit	Vector; BA-1000	1:200	IHC	III
IRDye® 680RD Donkey anti-mouse IgG	LI-COR; 926-68072	1:25,000	WB	II , III
IRDye® 800CW Donkey anti-rabbit IgG	LI-COR; 926-32213	1:25,000	WB	II , III
Poly-HRP-anti Ms/Rb/Rt	ImmunoLogic;	Ready to use	IHC	II
Rabbit anti-rat	DAKO; E0468	1:200	IHC	II

4.9 Microscopy (I-III)

TMA stainings were scanned by Pannoramic scanner (3DHISTECH, Hungary) and digital images were obtained for analysis. IF sections were imaged using Olympus BX60 and Axiovert-200M microscopes, equipped with Yokogawa CSU22 spinning disc confocal unit and Plan-Neofluar 63x NA oil objective (Carl Zeiss Microimaging GmbH, Oberkochen, Germany).

4.10 Next generation sequencing (NGS) (III)

Total RNA was extracted from PC-3M control-knockdown (ctrl-KD) and PC-3M FGFRL1-knockdown (FGFRL1-KD) cells. RNA quality control, library preparation, and high-throughput sequencing were performed by The Finnish Microarray and Sequencing Centre (FMSC). All samples were sequenced with Illumina HiSeq 2500 instrument using TruSeq v3 sequencing chemistry (Kinghorn Centre for Clinical Genomics (KCCG), Sydney, NSW, AU). The data was analyzed by comparing gene expression profiles of PC-3M ctrl-KD clones to PC-3M FGFRL1-KD clones using Chipster software v3.6 (CSC - IT Center for Science Ltd., Espoo, Finland).

4.11 Statistical analysis (I-III)

The statistical analysis was performed using SPSS software, version 22 (SPSS Inc.) and GraphPad Prism 6 (Graphpad Softerware, Inc.). Shapiro-Wilk W-test, One-way ANOVA, Mann Whitney U test, Kruskal-Wallis, Wilcoxon matched-pairs signed rank test, Spearmen correlation test, Fisher's exact test and Pearson chi square test (χ^2 test), Kaplan-Meier and log-rank test, Cox proportional hazards model were used to analyze the data based on data's requiremend. Every test was rated by *p* value (2-tailed). Difference was considered statistically significant when *p* value was less than 0.05.

5 RESULTS

5.1 Characterization of transgenic (TG) mouse models: FGF8b-TG, BERKO$_{FVB}$ and FGF8b-TG-BERKO$_{FVB}$ (I)

This study was aimed to explore the effect of deficient ERβ in FGF8b induced pathological changes in the mouse prostate.

5.1.1 FGF8b-TG, BERKO$_{FVB}$ and FGF8b-TG-BERKO$_{FVB}$ mice were viable

In our previous studies, it has been verified that FGF8b was highly expressed in all three prostate lobes (ventral prostate/VP, dorsolateral prostate/DLP and anterior prostate/AP) of FGF8b-TG mice (Elo et al. 2010). Analysis by RT-PCR and sequencing indicated that ERβ mRNA in BERKO$_{FVB}$ mice was shorter than the wild type because of the NEO-cassette contained early stop codons. Therefore, the shorter ERβ mRNA in BERKO$_{FVB}$ mice is not translated to functional, full length ERβ proteins. Offspring of all genotypes were viable and had a normal macroscopic phenotype.

5.1.2 FGF8b-TG-BERKO$_{FVB}$ mice present increased mucinous metaplasia and inflammation in the prostate

Prostate histology was evaluated from HE-stained sections of 10-14-month old mice. Previous study of FGF8b-TG mice had shown that the prostate of FGF8b-TG mice was bigger in size and often swollen. Epithelial and stromal hypercellularity with atypic cells, mouse PIN (mPIN), and inflammation was clearly observed in FGF8b-TG mice (Elo et al. 2010). Prostatic malignant lesions, like adenocarcinoma, sarcoma and carcinosarcoma were also presented in FGF8b-TG mice (Elo et al. 2010). Histological evaluation of the prostate in BERKO$_{FVB}$ mice showed that the frequency of focal epithelial hypercellularity and stromal inflammation tended to be increased compared to WT mice.

The histological changes in the prostates of FGF8b-TG-BERKO$_{FVB}$ mice were largely similar to those FGF8b-TG mice but the epithelial and stromal hyperplasia was less extensive in prostate of FGF8b-TG-BERKO$_{FVB}$ mice than in FGF8b-TG mice. However, in prostate epithelium of FGF8b-TG-BERKO$_{FVB}$ mice, the focal mucinous metaplasia represented by Goblet-like

cells with PAS positive staining was significantly more frequent than in FGF8b-TG mice. In addition, compared to FGF8b-TG mice, the frequency of inflammation in the stroma represented by T-cells, B-cells, and macrophages showed a step-wise increasing from WT, $BERKO_{FVB}$ to FGF8b-TG and FGF8b-TG-$BERKO_{FVB}$ mice (I, Fig.1).

5.1.3 Comparision of the prostate of FGF8b-TG and FGF8b-TG-$BERKO_{FVB}$ mice

Imamov *et al* has previously reported an increased p63-positive cells number in the prostate epithelium of BERKO mice (Imamov et al. 2004). In the present study, there was an increase in the percentage of p63-positive cells in hypercellular foci of $BERKO_{FVB}$ mice (I, Fig. 2B). There was a trend of decreased number of p63-positive cells in epithelia of FGF8b-TG mice, especially in mPIN lesions (I, Fig. 2C). The frequency of p63-positive cells in FGF8b-TG-$BERKO_{FVB}$ mice differed in different area (I, Fig. 2D). However, there was no difference in the overall frequency of 63-postive cells among the four groups when larger areas were included. (I, Fig. 2E).

The proportion of AR-positive cells was 90-100% in normal prostate epithelium and 35-50% in normal stroma in all the studied groups. In the hypercellular epitheliums, proportion of AR-positive cells in $BERKO_{FVB}$ mice was similar to normal epithelial cells but the percentage of AR-positive cells was reduced to 50-75 in mPIN lesions of FGF8b-TG and FGF8b-TG-$BERKO_{FVB}$ mice. In FGF8b-TG mice, the percentage of AR-positive cells tended to be decreased in the hypercellular and atypical stroma but increased in the sarcoma-like lesions. Compared to FGF8b-TG group, the percentage of AR-positive cells in hypercellular stroma of FGF8b-TG-$BERKO_{FVB}$ mice displayed decreased, equal or increased variation compared to normal stroma (I, Fig. 3).

Smooth muscle actin (SMA) and Masson Trichrome stainings were used to study the composition of the prostate stroma. In normal prostate, SMA-positive cells surrounded prostate acini in a ring-like structure. In the hypercellular stroma of FGF8b-TG and FGF8b-TG-$BERKO_{FVB}$ mice, SMA-positive staining was occasionally absent from the acini. Compared to FGF8b-TG-$BERKO_{FVB}$ mice, the hypercellular stroma was more extensive and more disorganized in FGF8b-TG mice. The Masson Trichrome stains collagen fibers blue-green and smooth muscle red. In both FGF8b-TG and FGF8b-TG-

BERKO$_{FVB}$ mice, the hyptercellular stroma displayed wider blue-green-staining compared to normal stroma (I, Fig. 3).

5.1.4 Changes in genes expression profiles in the ventral prostate of FGF8b-TG and FGF8b-TG-BERKO$_{FVB}$ mice

Our previous report on the alterations of gene expression in the VPs of *FGF8b*-TG mice indicates that expression of *Spp1* and *Ctgf* were upregulated compared to WT mice (Elo et al. 2010). Similar overexpression of *Spp1* and *Ctfg* in VPs was found in *FGF8b*-TG and *FGF8b*-TG-BERKO$_{FVB}$ mice but there was no significant difference between BERKO and WT mice suggesting that only FGF8b but not ERβ was involved in regulation of these genes. Based on the clue on the altered expression of b and c isoforms of FGFR in the epididymides of *FGF8b*-TG mice (Elo et al. 2012), we explored the FGFR isoform b and c expression in VP in all groups. The results showed an increased *Fgfr1c* mRNA level and decreased *Fgfr2c* level in both VPs of *FGF8b*-TG and *FGF8b*-TG-BERKO$_{FVB}$ mice compared to WT mice. The relative mRNA levels of *Fgfr1b* and *Fgfr2b* in all four groups were similar and expression of *Fgfr3b* and *Fgfr3c* mRNA were undetectable. Expression of *Ar* and *Erα* were analyzed and the statistical significance was only observed in the mRNA level of *Ar* between VPs of BERKO$_{FVB}$ and *FGF8b*-TG mice (I, Fig. 4).

Because of the inflammation and mucinous phenotype were frequently observed in transgenic mice groups, the mRNA expression of pro-inflammatory cytokines, such as tumor necrosis factor α (*Tnf*α), interleukin 6 (*Il6*) and 17 (*Il17*), mucin1 (*Muc*1) and 2 (*Muc*1), and transforming growth factor β1 (*Tgfβ*1) were measured by qRT-PCR. The *Tnf*α mRNA level was significantly increased in *FGF8b*-TG and *FGF8b*-TG-BERKO$_{FVB}$ mouse groups compared with WT mice. However, the rest of the studied genes did not show statistically signifiacant difference between the groups which may partly have been explained by a relatively small number of samples and big variation between individual mice (I, Fig. 5).

5.2 FGF13 expression is increased in human PCa and can be considered an independent prognostic marker for PCa patients after radical prostatectomy (II)

Previous studies demonstrated that FGFs and FGFRs play important roles in PCa. We performed data mining based on genome-wide mRNA expression datasets provided by cBioPortal to identify recurrent alterations of FGF and FGFR expression in PCa. In the data set generated by Memorial Sloan Kettering Cancer Center (MSKCC), we found that FGF13 and FGFRL1 exhibited altered mRNA expression in a high proportion (31% and 17%, respectively) of PCa cases compared to normal prostate (Cerami et al. 2012, Gao et al. 2013). The second part of the thesis was to explore the expression pattern of FGF13 in PCa and evaluate its prognostic value in PCa patients after RP. The TMAs including tissue samples from BPH, adjacent prostate (AdjPr), HGPIN, primary PCa, aggressive PCa (locally invasive and metastatic PCa), and CRPC were stained immunohistocemically for FGF13. The IHC scores were evaluated to estimate the levels of FGF13 protein.

5.2.1 FGF13 expression pattern in human PCa tissues and PCa cell lines

Benign prostate tissues, BPH and AdjPr showed negative or weak cytoplasmic FGF13 staining in both epithelia and stromal cells, and the positive nuclear staining was rare. In malignant cells, FGF13 staining was primarily observed in the cytoplasm and only sporadically observed in the nuclei (II, Fig. 1 a-f). B and T lymphocytes showed strong FGF13 immunoreaction in all prostate samples (II, Supp. Fig. 2).

FGF13 distribution was examined by IF in two PCa cell lines. Along with the IHC results from tissue sections, FGF13 was stained in both cytoplasm and nuclei of PC-3M and LNCaP cells with a heterogeneous pattern. In the cytoplasm, string-like structures were frequently and clearly observed, and FGF13 was generally concentrated around the nucleus and occasionally seemed to be colocalized with the filopodia tips (II, Fig. 4 e-g).

Immunoblotting of total FGF13 protein from prostate cancer cells showed three clear bands which may indicate different isoforms of FGF13. FGF13 could be detected in both cytoplasmic and nuclear fractions of PCa cell lines, this finding was in line with IHC and IF results (II, Fig. 4 c, d).

5.2.2 FGF13 expression is upregulated in human PCa tissues and PCa cell lines

Compared to BPH and AdjPr, both cytoplasmic and nuclear expression of FGF13 was clearly increased in HGPIN and primary PCa. Indeed, when AdjPr and primary PCa samples that were collected from the same patients were paired, the comparison analysis showed a statistically significant increased in the levels of both cytoplasmic and nuclear FGF13 in PCa (II, Fig. 1 g, h). Nevertheless, cytoplasmic and nuclear FGF13 expression in aggressive PCa displayed a decrease compared to primary PCa, and the decrease was more evident in CRPC samples (II, Fig. 1 i, j).

Tissue samples adjacent to samples used for TMAs were used for mRNA analysis. In concordance with TMA studies, FGF13 mRNA level was significantly higher in PCa tissues than benign tissues. Among the transcript variants studied, FGF13v4 showed a clear increase in the PCa group compared to AdjPr (II, Fig. 2).

In vitro studies with PCa cell lines showed that FGF13 mRNA and protein levels were markedly higher in most of PCa cell lines compared to the immortalized prostatic epithelial cell line PNT1a. Relative levels of different variant mRNAs in cell lines were not similar to those of total FGF13 mRNA. In contrast to total FGF13 mRNA levels, PC-3M and LNCaP showed highest FGF13v1 and FGFv4 expression, respectively, while VCaP exhibited highest expression of FGF13v2/3/5 and FGF13v6 (II, Fig. 4 a, b).

5.2.3 FGF13 is an independent indicator for BCR in PCa patient after RP

Analysis of the association of FGF13 expression with clinicopathological parameters, such as age, pre-operative serum PSA, the GS, the PSM, the pTNM staging, and BCR, indicated that cytoplasmic FGF13 staining was significantly related to BCR and nuclear FGF13 staining was related to pre-operative serum PSA (II, Table 1).

The Kaplan-Meier and log-rank tests indicated that high cytoplasmic FGF13 was clearly associated with shortened BCR-free time, and the significance was still obvious in patient stratified as GS $\geqslant$ 4+3, PSM, or pTNM $\geqslant$ T3a groups. Nuclear FGF13 expression was not directly associated with BCR. Interestingly, when nuclear FGF13 was combined with cytoplasmic FGF13,

the group with low cytoplasmic FGF13 and positive nuclear FGF13 showed an impressively long BCR-free time (II, Fig. 3).

The Cox regression models were used to evaluate the predictive value of FGF13 in PCa. Among the clinicopathological parameters, cytoplasmic FGF13 as well as the GS showed significant association with BCR in univariable Cox regression tests and hazard ratio (HR) was 3.276 (95%CI 1.496-7.170) and 1.798 (95%CI 1.235-2.617), respectively. In multivariable Cox regression tests, cytoplasmic FGF13 HR was 3.288 (95%CI 1.488-7.622), and the HR of GS was only half of the cytoplasmic FGF13 showing 1.688 (95%CI 1.128-2.526). Other parameters, such as pre-operative PSA, pTNM staging, PSM, and age did not show significant correlation with BCR in the studied cohort (II, Table 2).

5.2.4 FGF13 localization is overlapping with α-tubulin and voltage-gated sodium channels (Na$_V$s/VGSCs)

It has been reported that FGF13 acts as a microtubule-stabilizing protein and regulates Na$_V$s in neurons. In order to characterize its biological functions, we used co-staining to explore the possible interactions. The staining suggested that there is overlapping between the localizations of FGF13 and α-tubulin or Na$_V$ 1.8 (II, Fig. 4 e-g).

5.3 FGFRL1 expression is increased in PCa and associated with PCa progression (III)

The thrid part of the thesis focused on examining the expression of FGFRL1 in PCa and to explore its possible biological functions and target genes.

5.3.1 FGFRL1 is expressed in benign prostate and PCa, and it shows elevated expression in PCa

Both qRT-PCR and IHC was used to evaluate FGFRL1 expression in human PCa. The results showed that the relative level of total FGFRL1 mRNA was more than two fold higher in PCa than in benign prostate (III, Fig. 3). FGFRL1 IHC staining showed that FGFRL1 was expressed on the plasma membrane, cytoplasm and nuclei in benign prostate but the staining was mainly cytoplasmic in PCa. The IHC scores were used to assess the alterations of

FGFRL1 levels in TMAs. Samples of primary PCa and adjacent non-tumorous prostate tissues, which came from the same patient, were paired and analyzed. Cytoplasmic and nuclear FGFRL1 showed a significant increase while cell membrane associated FGFRL1 exhibited a clear decrease in primary PCa compared to adjacent non-tumorous prostate tissues (III, Fig. 4A-C). To expand the study material, two TMAs of independent aggressive PCa (locally invasive and metastatic PCa) and CRPC cohorts were analysed for comparison. The IHC score for cell membrane FGFRL1 showed highest in benign prostate and declined sequentially in the order of HGPIN > primary PCa > aggressive PCa and CRPC. On the contrary, cytoplasmic FGFRL1 displayed the lowest level in BPH and benign tissues adjacent to PCa. Cytoplasmic FGFRL1 level was increased in HGPIN and primary PCa and showed the highest level in advanced PCa. The highest level of nuclear FGFRL1 was in primary PCa (III, Fig. 4D-F).

5.3.2 FGFRL1 is associated with PCa progression

Clinical data of patients from primary TMA was collected and correlated to the FGFRL1 IHC scores. None of the cytoplasmic, nuclear, or cell membrane associated FGFRL1 scores showed significant correlation to BCR of PCa. The chi square test indicated that FGFRL1 expression was independent from clinical parameters, such as age, PSM and T-stage. Nevertheless, FGFRL1 is associated with the GS, pre-operative PSA, and PSM (III, Table 1). Specific analysis with the Spearman's test showed that decreased membranous FGFRL1 was associated with the high GS and high Ki67 expression. On the contrary, the high GS and Ki67 were correlated to elevated cytoplasmic and nuclear FGFRL1. Nuclear FGFRL1 expression was positively related to pre-operative PSA (III, Table 2).

5.3.3 FGFRL1 may function as a decoy receptor in PCa cells

Because of the lacking intracellular tyrosine kinase domain, FGFRL1 is assumed to act as a decoy receptor in a membrane-anchored or soluble form. Indeed, we were able to detect FGFRL1 in the medium conditioned by PCa cells (III, Fig. 5E). To examine the role of FGFRL1 on FGF/FGFR signaling, FGFRL1 was overexpressed or silenced in PCa cells. The cells were stimulated with FGF8b and FGF2, both of which have high affinity to FGFRL1. FGF8b and FGF2 have also been documented to play an important

role in PCa. FRS2α is a critical mediator of the FGF/FGFR signaling pathway and its phosphorylation demonstrates activation of the intracellular kinase part of FGFRs. The results show that overexpressed FGFRL1 attenuated the phosphorylation of FRS2α while downregulated FGFRL1 exhibited inverse effect. The Ras/Raf/ERK signaling pathway displayed a similar response as FRS2α, showing decreased phosphorylation of ERK1/2 in FGFRL1 overexpressing cells and an opposite response in FGFRL1 silenced cells. Of note, the extent of phosphorylated ERK1/2 was much weaker compared to phosphorylated FRS2α. The AKT signaling pathway did not show clear difference in FGF2 treated groups. However, increased AKT phosphorylation was observed in FGFRL1 silenced cells when treated with FGF8b (III, Fig. 6A-D).

5.3.4 Decreased FGFRL1 reduces in vivo PCa cell growth

FGFRL1 was silenced in PC-3M cells to study its effect on cell growth at both *in vitro* and *in vivo* levels. The *in vitro* proliferation experiments showed that the cells with lower level of FGFRL1 grew slightly faster during 4-day experiments (III, Fig. 7A). However, when the cells were implanted subcutaneously into nude mice, all the control mice generated evident tumors (n=16) while in the FGFRL1-KD groups tumor take was 13 out of 16. Moreover, the xenograft tumors derived from FGFRL1 knockdown cells grew slower and were smaller compared to the control tumors (III, Fig. 7C). There was no difference of the mean body weights of the xenograft bearing mice between the groups (III, Fig. 7C). Immunohistochemical staining of the mitotic marker PHH3 and the endothelial cell marker CD34 were used to assess proliferation activity and angiogenesis. The results showed that there was no difference in proliferation between the two groups (III, Supplementary Fig. 1A). The FGFRL1 knockdown tumors showed lower CD34 staining level compared to control tumors, although the difference is not significant (III, Supplementary Fig. 1B). At the end point of tumor growth, neither ERK1/2 nor AKT pathway showed difference between the groups (III, Supplementary Fig. 1C, D). Cyclins and cyclin-dependent kinases (CDKs) were analyzed by western blotting from the xenograft tumor. Cyclin D1 did not show significant variation between groups. In contrast, expression of cyclin E, CDK2 and CDK4 expression seemed to be downregulated in FGFRL1 knockdown tumors significantly (III, Supplementary Fig. 1E-I).

5.3.5 Altered gene expression profiles between PC-3M ctrl-KD and PC-3M FGFRL1-KD cells

The NGS was used to study the target genes of FGFRL1 in PCa. RNA from control and FGFRL1 knockdown cells was collected and sequenced. Compared to control PC-3M cells, expression of 48 genes was found to be significantly changed in FGFRL1 knockdown cells with cutoff of FDR<0.1 and logFC>1.0 or logFC<-1. The top 15 upregulated and top 15 downregulated genes are listed in Table 3 (III).

6 DISCUSSION

6.1 FGF8b-induced prostate tumorigenesis is not facilitated by inactivation of ERβ

Previous studies indicate that FGF8b is able to induce prostate carcinogenesis and to be involved in PCa progression (Valta et al. 2008, Elo et al. 2010). ERβ has been reported to affect PCa cell proliferation and suggested to be a tumor suppressor (Hurtado et al. 2008, Cheng et al. 2004). Declined ERβ expression has been observed in HGPIN and PCa (Horvath et al. 2001, Leav et al. 2001). However, studies with BERKO mice are conflicting (Prins et al. 2001, Imamov et al. 2004, Dupont et al. 2000). We generated the transgenic mouse model FGF8b-TG-BERKO$_{FVB}$ to study if ERβ inactivation would make these mice more susceptible to prostatic carcinogenesis than FGF8b-TG mice.

Four transgenic mouse groups were studied, including WT, FGF8b-TG, BERKO$_{FVB}$, and FGF8b-TG-BERKO$_{FVB}$ mice. BERKO$_{FVB}$ mice exhibited similar histological changes in prostate as described before, showing epithelial hyperplasia and inflammation in stroma (Weihua et al. 2001). Both FGF8b-TG and FGF8b-TG-BERKO$_{FVB}$ mice showed epithelial and stromal hyperplasia, but the frequency was higher in the FGF8b-TG group. Of note, the mPIN lesion showed similar incidence in FGF8b-TG and FGF8b-TG-BERKO$_{FVB}$ mice but the adenocarcinoma changes observed in FGF8b-TG prostate were absent from FGF8b-TG-BERKO$_{FVB}$ mice. In contrast to the hypothesis, deficient ERβ was not able to enhance the prostate tumorigenesis induced by FGF8b.

We have reported that elevated number of inflammatory cells and abnormal, hypertrophic stroma were found in the stroma of FGF8b-TG mice (Elo et al. 2010). In this study, we showed an increasing frequency of inflammation in four groups with the highest level in FGF8b-TG-BERKO$_{FVB}$ mice. This suggests that deficiency of ERβ enabled recruitment of more inflammatory cells in the prostate overexpressing FGF8b. In other words, ERβ may have an anti-inflammatory role in PCa. This is consistent with published studies (Prins et al. 2001, Mak et al. 2015).

Besides inflammatory changes, significant increase in mucinous metaplasia was observed in FGF8b-TG-BERKO$_{FVB}$ mice compared to FGF8b-TG mice. This suggests that overexpression of FGF8b combined with deficient ERβ has a more evident effect on epithelia differentiation.

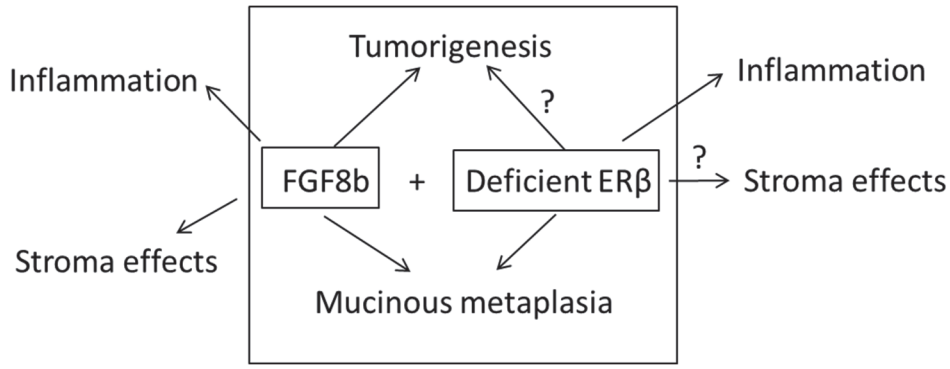

Figure 4. Functions of FGF8b and ERβ in gene-modulated mice ($BERKO_{FVB}$, FGF8b-TG, and FGF8b-TG-$BERKO_{FVB}$)

6.2 Upregulated FGF13 expression is observed in PCa and correlated with BCR

FGF13 expression is increased in metastatic pancreatic cancer and it positively associated with reduced progress-free survival (Missiaglia et al. 2010). Query on cBioPortal database indicated that 31% PCa cases show altered *FGF13* expression (Cerami et al. 2012, Gao et al. 2013) but there are no published reports further analyzing and verifying the data.

6.2.1 FGF13 expression is upregulated in PCa and increased FGF13 is associated with the outcome of PCa patients after RP

In this study, qRT-PCR and TMAs containing different kind of PCa tissues were used to examine FGF13 expression. Elevated expression of FGF13 at both protein and mRNA levels was observed in PCa compared to benign samples. In primary PCa TMA, FGF13 was gradually increased from BPH, adjacent prostate to HGPIN and PCa. The increase of FGF13 in aggressive PCa was also clear compared to non-malignant prostatic tissues but it was at lower level than in primary PCa. Similarly, an increase of FGF13 in CRPC specimens was just slight and non-significant compared to benign prostate. In general, FGF13 expression was increased from non-cancer to HGPIN and primary PCa and then it was relatively decreased upon progression of primary PCa to locally invasive or metastatic PCa and CRPC. This suggests that

FGF13 may be involved in the early stages of PCa, although in our study different cohorts were independent from each other, which makes the comparison of quantitative and relative differences not straightforward.

Overexpressed FGF13 is associated with shorter progression-free survival in pancreatic cancer (Missiaglia et al. 2010) and poor outcome in cervical cancer (Okada et al. 2013). In our study, chi square test showed that cytoplasmic FGF13 was associated with BCR while nuclear FGF13 was associated with the GS. Kaplan-Meier survival and log-rank tests showed that high cytoplasmic FGF13 statistically indicated shortened BCR-free time in PCa patients after RP. The significance was still clear in patient groups of GS $\geq$ 4+3, positive surgical margin (PSM), and pTNM stage $\geq$ T3a. Nuclear FGF13 did not show a significant association with BCR-free survival independently. However, positive nuclear FGF13 along with low cytoplasmic FGF13 exhibited a surprisingly long BCR-free time. This suggests that cytoplasmic and nuclear FGF13 may play opposite roles in PCa. Univariable and multivariable Cox regression analyses indicate that both cytoplasmic FGF13 and the GS could be used as independent prognostic markers for PCa after RP. The cytoplasmic FGF13 prognostic value may be more effective than the GS because the HR was two-fold higher than the GS.

6.2.2 Possible functions of FGF13 in PCa

Studies with PCa cell lines verified the elevation of FGF13 expression in PCa cell lines compared to the immortalized, non-tumorigenic prostate epithelial cell line PNT1a.

Previous studies suggest that FGF13 has at least five isoforms (Munoz-Sanjuan, Smallwood & Nathans 2000). This is in line with our western blot experiment which showed several bands in the predictive region. The isoform FGF13a is mainly located in nuclei while other isoforms are distributed in both cytoplasm and nuclei in a diffused pattern (Munoz-Sanjuan, Smallwood & Nathans 2000). In the present study, the isoforms were detected both in the cytoplasmic and nuclear fractions, which was in agreement with IHC staining results from human PCa tissues. However, we could not identify the location of specific FGF13 isoforms due to the limited availability of specific isoform recognizing antibodies. Nonetheless, in view of the association of BCR-free survival and cytoplasmic/nuclear FGF13, it is possible that differential isoforms play different functions in PCa.

It has been demonstrated that FGF13 acts as a microtubule stabilization protein in neurons (Wu et al. 2012). In our study, IF staining of PCa cells showed that FGF13 staining formed string-like structures. In co-staining experiments these structures seemd to co-localize with α-tubulin staining. Tubulins are actively involved in mitosis, cell division and movement, and endrocine pathway (Mistry, Oh 2013). Altered tubulin stability and isotype expression are involved in cancers and drug resistance (Parker, Kavallaris & McCarroll 2014). In PCa, overexpression of class IIIβ-tubulin is reported to be associated with advanced PCa and docetaxel resistance (Ploussard et al. 2010, Terry et al. 2009, Tsourlakis et al. 2014). According to these results, FGF13 may contribute to PCa via interacting with tubulin but this possibility needs to be explored by detailed further experiments.

As another suggested mechanism, FGF13 is found to regulate Na_Vs in neurons (Goetz et al. 2009, Pablo, Pitt 2014). Interestingly, among the reported Na_V studies in PCa, $Na_V1.8$ shows a similar expression pattern to that of FGF13 (Suy et al. 2012). In our study, co-staining of FGF13 with panNa_V and $Na_V1.8$ suggested overlapping localization of the two enabling a possibility that an interaction of FGF13 and Na_Vs, particular $Na_V1.8$, also in PCa. Abberrant expression and location of Na_Vs have been reported in many cancers, including PCa. Although the exact mechanisms are unclear, it has been suggested that they are involved in cancer cell proliferation, migration, and invasion (Brackenbury 2012, Patel, Brackenbury 2015). However, this putative mechanism of FGF13 interacting with $Na_V1.8$ in PCa also needs to be further elucidated.

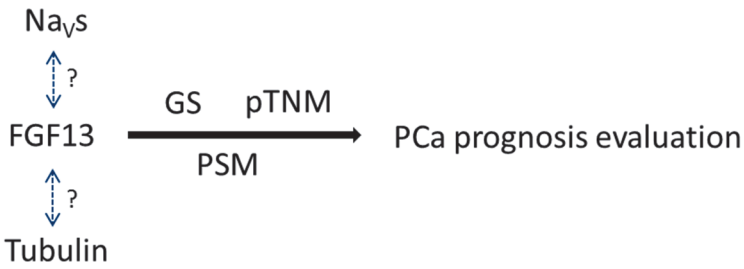

Figure 5. Summary of suggested cellular interactions and clinical significance of FGF13 among established prognostic markers in PCa. (GS, the Gleason Score; PSM, positive surgical margin)

6.3 FGFRL1 may contribute to PCa progression

FGFRL1 plays an important role in the development of diaphragm and metanephric kidney (Baertschi, Zhuang & Trueb 2007, Gerber et al. 2009). Several studies have reported the aberrant expression of FGFRL1 in cancers, such as bladder, ovarian, and colorectal cancer (Schild, Trueb 2005, Donnard et al. 2014, Martino et al. 2013). A TMA study of esophageal squamous cell carcinoma (ESCC) demonstrated that positive FGFRL1 immunoreaction on the cell membrane but mainly in the cytoplasm. Correlation of the TMA analyses with clinicopathological data indicated that FGFRL1 was associated with both lymph node metastasis and tumor growth in the ESCC patients (Shimada et al. 2014).

6.3.1 FGFRL1 expression is upregulated in PCa and associated with the disease progression

In this study, total FGFRL1 mRNA was analyzed by qRT-PCR. Expression of FGFRL1 mRNA was upregulated in primary PCa compared to benign prostate. We then studied human benign prostate and PCa tissues at different stages of the disease to investigate the FGFRL1 expression pattern. Differentiation of FGFRL1 protein staining according to the cellular localization showed that compared to benign prostate adjacent to PCa, the cell membrane-associated FGFRL1 was decreased in HGPIN and even more so in PCa (HGPIN > primary PCa > locally invasive and metastasized PCa and CRPC). In contrast, the proportion of cytoplasmic and nuclear FGFRL1 was clearly increased in primary PCa when it was paired with the adjacent benign prostate tissues which were from the same patient. The step-wise increase in cytoplasmic FGFRL1 was even more evident when aggressive PCa (locally invasive and metastasized PCa) and CRPC were studied, although the fact that the TMAs of aggressive PCa and CRPC represented different patient cohorts and were constructed separately makes the comparison no straightforward. Rieckmann et al. suggests that the intracellular domain of FGFRL1 could act as mediator of endocytosis and transmembrane protein trafficking (Rieckmann et al. 2009). Besides, the extracellular domain of FGFRL1, specifically recognized by the FGFRL1 antibody used in our study, is able to shed from the membrane. Thus, the deregulated distribution of FGFRL1 may be caused by disturbed FGFRL1 trafficking and/or the shedding rate.

Moreover, decreased cell membrane associated FGFRL1 was positively associated with high grade PCa and proliferation marker Ki67. It is possible that the cell membrane FGFRL1 exhibits a protective role, which is gradually lost during PCa progression. Opposite to cell membrane associated FGFRL1, increased cytoplasmic and nuclear FGFRL1 showed positive correlation with the high GS and Ki67 immunopositivity. In addition, increased nuclear FGFRL1 was associated with elevated pre-operative PSA and the correlation was statistically significant. These results suggest that the cytoplasmic and nuclear FGFRL1 could have a biological role and contribute to PCa progression in an active way.

6.3.2 FGFRL1 affects the intracellular FGF/FGFR signaling

Binding of FGFs to FGFRs triggers the dimerization of the receptors and autophosphorylation of the intracellular tyrosine kinase. A central mediator for FGF/FGFRs signaling, FRS2α, is then phosphorylated and initiates downstream signaling, like the Ras/Raf/ERK and PI3K/AKT pathways. The extracellular domain of FGFRL1 is able to bind FGFs at a high affinity but it cannot signal in a canonical way because the intracellular part lacks the tyrosine kinase domain. FGFRL1 is therefore considered to probably function as a decoy receptor. In this study, we used PCa cells in which FGFRL1 was either overexpressed or silenced, and stimulated them with FGF2 or FGF8b, which both are documented to play important roles in PCa. In the cells with decreased FGFRL1, FGF2 and FGF8b evoked phosphorylation of FRS2α more robust than in control cells. Correspondingly, in the cells overexpressing FGFRL1, the response of FRS2α to FGF2 and FGF8b was attenuated. As reported by Steinberg F et al., we also detected FGFRL1 in PCa cultured serum-free medium. Thus, these results together indicate that the cell membrane associated FGFRL1 and/or its soluble form are able to regulate FGF/FGFR signaling negatively at least *in vitro.* The Ras/Raf/ERK signaling pathways showed similar response to FGF stimulation as FRS2α but the response was much weaker. Intriguingly, the PI3K/AKT pathway showed an inverse response in FGF8b treated cells. It is thus possible that FGFRL1 also regulates the FGF/FGFR downstream signaling pathways by other mechanisms than as a decoy receptor.

6.3.3 Effects of reduced FGFRL1 on PCa cells

The Ras/Raf/ERK signaling pathways generally contribute to cell growth while the PI3K/AKT pathway is involved in the maintenance of cell viability. In the *in vitro* cell growth experiments, PC-3M cells in which FGFRL1 was decreased by sh-RNA transfection grew slightly faster than control cells as one would expect if FGFRL1 primarily functions as a dominant negative factor although the effect of FGFRL1 downregulation on *in vitro* proliferation was small. However, the *in vivo* growth of FGFRL1-KD PC-3M cell xenografts was different from that *in vitro*: the FGFRL1 deficient tumors grew markedly slower than controls. This result is in line with that of Trueb et al. (2003) who reported that overexpressed FGFRL1 increased the growth of osteosarcoma MG63 cells. It is possible that the tumor microenvironment, including the tissue oxygenation level and other growth conditions, may influence and determine the functions of FGFRL1. The analyses of the xenograft samples for the proliferation markers at the end point of experiment demonstrated that expression of cyclin E, and cyclin-dependent kinases 2 (CDK2) and CDK4 was decreased but other cyclins, CDKs or the mitosis marker PHH3 did not show marked changes as detected by IHC. Cell cycle is controlled by several cyclins and CDKs. CyclinD-CDK4/6 complexes control G_1 progression and cyclinE-CDK2 complex regulates the G_1-S transition (Stamatakos et al. 2010, Möröy, Geisen 2004). Meanwhile, FGFRL1 is reported to prevent cell cycle arrest in G_1/G_0 phase in the ESCC cells (Tsuchiya et al. 2011). So far, our results demonstrate that FGFRL1 contributes to PCa by regulating tumor growth, possibly affecting cell cycle progression as one of its mechanisms.

The NGS results showed more than 40 markedly changed genes in FGFRL-KD PC-3M cells compared to control knockdown cells. These genes were highly consistent with FGFRL1 associated genes deposited in the public database ciBioPortal (Taylor et al. 2010). Among these genes, at least PIK3C2B, MYO9B, HDAC2, SEPP1, and MAP2K1 are related to PCa (Koutros et al. 2010, Gerstenberger et al. 2015, Makowska et al. 2015, Shahmoradgoli et al. 2013, Amatangelo et al. 2012).

In summary, this study demonstrates that the FGFRL1 expression is upregulated in PCa and it is associated with an advanced stage of the disease. In line with this finding, the growth rate of FGFRL1 deficient PC-3M xenografts was demonstrated to be strongly suppressed. However, the molecular mechanisms of FGFRL1 action on PCa and the xenograft growth need to be further explored in the future experiments (Figure 6).

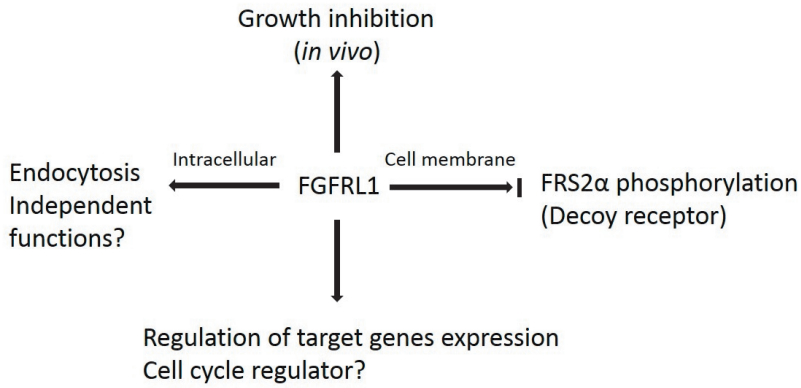

Figure 6. Summary of possible functions of FGFRL1 in PCa.

6.4 Summary of the studies

6.4.1 The possible interactions between the FGF/FGFR pathways and ERβ

The deficiency of ERβ, previously suggested to function as tumor suppressor, could not facilitate FGF8b-induced tumorigenesis in a gene modulated-mouse model. The results demonstrate that ERβ may not affect prostate tumorigenesis, but ERβ did have differentiation promoting and anti-inflammatory effects, which may influence tumor progression. These results provide a new perspective on the role of ERβ in prostate and PCa.

6.4.2 FGFs and FGFRs contribute to PCa

Prostate cancer is a heterogeneous disease that is influenced by hormones and growth factors. There is increasing experimental and clinical evidence showing that FGFs and FGFR, besides AR and ER, are involved in PCa initiation and progression. The results of this thesis study show that besides FGF8, FGF13 and FGFRL1 are also dysregulated in PCa and associated with PCa progression. In human PCa, altered expression of FGF13 and FGFRL1 was associated with PCa progression and prognosis in a statistically significant way. FGF13 and FGFRL1 could thus serve as novel tools for increasing the precision of current evaluation of PCa prognosis. Therefore, validated application of FGF13 and FGFRL1 in PCa could provide information for clinical decision-making.

7 CONCLUSIONS

In the present thesis study, the role of FGF/FGFR pathways in human PCa was evaluated by investigating FGF13 and FGFRL1, which have showed altered expression in a high proportion of PCa but which are poorly understood. In addition, potential tumor suppressing effect of ERβ on FGF8b-induced carcinogenesis was studied in gene-modulated mouse models. Based on these studies, the following conclusions are presented:

1. ERβ is not able to affect FGF8b-induced malignant changes in the prostate of FGF8b transgenic mice, but it obviously promotes differentiation of prostate epithelium and protects it against inflammation, which may be of importance in PCa.

2. FGF13 expression was found to be elevated in PCa. Analysis of the association between FGF13 and the clinical outcome of the patients suggested that FGF13 expression level could predict PCa prognosis independently or in combination with established prognostic criteria, such as TNM, surgical margin and PSA. FGF13 may execute its functions by interacting with tubulins or voltage-gated sodium channels but future experiments are needed to demonstrate and verify the possibility of such protein-protein interactions.

3. FGFRL1 expression is increased in PCa. Elevated cytoplasmic and nuclear FGFRL1, and decreased cell membrane FGFRL1 exhibit a positive correlation with PCa progression. In *in vivo* tumor experiments, the growth of FGFRL1 deficient PC-3M xenografts was strongly suppressed compared to the controls. The *in vitro* experiments suggested that FGFRL1 acts as a decoy receptor in PC-3M cells but the results also provided evidence for independent effects of FGFRL1 on PCa cell function which could be associated with the growth inhibitory effects of FGFRL1 silencing in PC-3M xenografts. Specific alterations of gene expression were identified in the NGS analysis of FGFRL1 deficient cells in comparison to control cells, which provides tools for further functional experiments needed to analyse the mechanisms of FGFRL1 actions in PCa.

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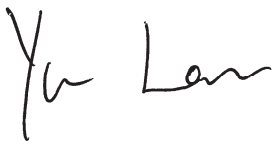

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