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FUNCTIONAL STUDY OF ONCOGENIC TRANSCRIPTION FACTOR *ERG* AND ITS SIGNALING IN PROSTATE CANCER

by

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To my family

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Functional Study of Oncogenic Transcription Factor ERG and its Signaling in Prostate Cancer

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ABSTRACT

TMPRSS2–ERG is the most frequent type of genomic rearrangement present in prostate tumors, in which the 5- prime region of the TMPRSS2 gene is fused to the ERG oncogene. TMPRSS2, containing androgen response elements (AREs), is regulated by androgens in the prostate. The truncated TMPRSS2-ERG fusion transcript is overexpressed in half of the prostate cancer patients. The formation of TMPRSS2-ERG transcript is an early event in prostate carcinogenesis and previous *in vivo* and *in vitro* studies have shown ectopic ERG expression to be associated with increased cell invasion. However, the molecular function of ERG and its role in cell signaling is poorly understood. In this study, genomic rearrangement of ERG with TMPRSS2 was studied by using comparative genomic hybridization (CGH) in prostate cancer samples. The biological processes associated with the ERG oncogene expression in prostate epithelial cells were studied, and the results were compared with findings observed in clinical prostate tumor samples. The gene expression data indicated that increased WNT signaling and loss of cell adhesion were a characteristic of TMPRSS2-ERG fusion positive prostate tumor samples. Up- regulation of WNT pathway genes were present in ERG positive prostate tumors, with frizzled receptor 4 (FZD4) presenting with the highest association with ERG overexpression, as verified by quantitative reverse transcription-PCR, immunostaining, and immunoblotting in TMPRSS2-ERG positive VCaP prostate cancer cells. Furthermore, ERG and FZD4 silencing increased cell adhesion by inducing active β1-integrin and E-cadherin expression in VCaP cells. Furthermore, we found a novel inhibitor, 4-(chloromethyl) benzoyl chloride which inhibited the WNT signaling and induced similar phenotypic effects as observed after ERG or FZD4 down regulation in VCaP cells. In conclusion, this work deepens our understanding on the complex oncogenic mechanisms of ERG in prostate cancer that may help in developing drugs against TMPRSS2-ERG positive tumors.

Keywords: β1-integrin, CGH array, *ERG*, *FZD4*, prostate cancer, VCaP, WNT signaling.

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ERG onkogeeni ja sen signalointi eturauhassyövässä
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ABSTRAKTI

TMPRSS2-ERG fuusiogeeni on yleisin eturauhassyövissä kuvatuista genomisista uudelleenjärjestymisistä. Fuusiogeeni muodostuu TMPRSS2-geenin 5´-alueen ja ERG-onkogeenin yhdistymisestä. TMPRSS2 geeni ilmentyy androgeenien vaikutuksesta eturauhasessa. TMPRSS2-ERG geenifuusio löytyy noin puolelta eturauhassyöpäpotilailta ja se on aikaisessa vaiheessa tapahtuva syövän syntyä edesauttava muutos. Aiemmat soluviljelmillä ja eläinmalleilla tehdyt tutkimukset ovat osoittaneet, että ERG-onkogeenin ektooppinen ilmentyminen lisää solujen invaasiota. Kuitenkin ymmärryksemme ERG-geenin toiminnan vaikutuksesta soluissa toimivien signalointireittien säätelyssä on vielä puutteellista. Tässä työssä tutkimme ERG:n ja TMPRSS2:n välistä genomista uudelleenjärjestymistä eturauhassyöpänäytteissä käyttämällä vertailevaa genomista hybridisaatiota (CGH). ERG onkogeenin toimintaan liittyviä biologisia prosesseja tutkimme viljellyissä eturauhasen epiteelisoluissa ja vertasimme tuloksia kliinisissä eturauhassyöpänäytteissä näkyviin muutoksiin. Tulokset geenienekspressioanalyyeista osoittivat WNT-signaloinnin aktivaation ja vähentyneen soluadheesion olevan ERG-positiivisille syöpänäytteille tyypillisiä piirteitä. Osoitimme kvantitatiivisella käänteistranskriptaasi-PCR:llä ja immuno-värjäyksin WNT signalointiin osallistuvien geenituotteiden, erityisesti frizzled-4 (FZD4) reseptorin, lisääntyneen ERG-positiivisissa syöpänäytteissä. ERG- ja FZD4-geenien hiljentäminen lisäsi syöpäsolujen tarttumiskykyä aktivoimalla β1-integriinin ja E-kadheriinin ekspressiota VCaP-soluissa. Löysimme uuden inhibiittorin, 4-klorometyyli-bentsoyylikloridin, joka vähensi WNT-signalointia ja aiheutti samankaltaisia muutoksia kuin ERG:n tai FZD4:n ilmentymisen esto VCaP-soluissa. Tämä työ lisää ymmärrystämme ERG-onkogeenin toiminnasta eturauhassyövässä ja saattaa edesauttaa TMPRSS2-ERG-positiivisiin eturauhassyöpiin kohdistuvaa lääkekehitystä.

Avainsanat: β1-integriinin, CGH, eturauhassyöpä, ERG, FZD4, VCaP, WNT signalointi.

CONTENTS

ABBREVIATIONS

Official symbol	Official name
4-CMBC	4-(chloromethyl) benzoyl chloride
AML	Acute myelogenous leukemia
AR	Androgen receptor
ARE	Androgen responsive element
ATCC	American type culture collection
BSA	Bovine serum albumin
CDH1	E-cadherin
CDS	Coding sequence
CGH	Comparative genomic hybridization
COL	Collagen
COPA	Cancer outlier profile analysis
CRPC	Castration resistant prostate cancer
CTB	Celltiter-blue
CTG	Celltiter-glo
DAPI	4',6-diamidino-2-phenylindole
EMT	Epithelial-to-mesenchymal transition
ERG	V-ets erythroblastosis virus E26 oncogene homolog (avian)
ESR	Estrogen receptor
ETS	E-twenty six
ETV1/4/5	ETS variant 1/4/5
FN	Fibronectin
FZD4	Frizzled receptor 4
GFP	Green fluorescencent protein
GSEA	Gene set enrichment analysis
GTI	Gene tissue index
HDAC1	Histone deacetylase 1
HRP	Horseradish peroxidase
IP	Immuniprecipitation
LAM	Laminin
MET	Mesothelial-to-epithelial transition

PCa	Prostate cancer
PI	Propidium iodide
RACE	Rapid amplification of cDNA ends
RT-PCR	Reverse transcriptase PCR
siRNA	Small interfering RNA
SNAI1/2	Snail homolog 1 /2(Drosophila)
TCF/LEF	T-cell factor/lymphoid enhancer factor
TFF3	Trefoil factor 3
TLR4	Toll-like receptor-4
TMPRSS2	Transmembrane protease, serine 2
VDR	Vitamin D receptor
VIM	Vimentin

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are mentioned in the text by I-III. The original communications have been reproduced with the permission of the copyright holders. Unpublished data is also included.

I Iljin K, Wolf M, Edgren H, **Gupta S**, Kilpinen S, Skotheim RI, Peltola M, Smit F, Verhaegh G, Schalken J, Nees M, Kallioniemi O: TMPRSS2 fusions with oncogenic ETS factors in prostate cancer involve unbalanced genomic rearrangements and are associated with HDAC1 and epigenetic reprogramming. Cancer Res 2006, 66(21):10242-10246.

II **Gupta S**, Iljin K, Sara H, Mpindi JP, Mirtti T, Vainio P, Rantala J, Alanen K, Nees M, Kallioniemi O: FZD4 as a mediator of ERG oncogene-induced WNT signaling and epithelial-to-mesenchymal transition in human prostate cancer cells. Cancer Res 2010, 70(17):6735-6745.

III **Gupta S**, Iljin K, Mpindi JP, Arjonen A, Ivaska J, Kallioniemi O. Oncogenic transcription factor ERG regulates adhesion and β1-Integrin traffic in prostate cancer. Submitted.

1. INTRODUCTION

The TMPRSS2 gene is fused to ERG (an oncogenic ETS transcription factor) in more than half of the tumors occuring in patients with localized prostate cancer (Tomlins et al., 2005; Demichelis et al., 2007). The 5-prime region of the TMPRSS2 gene has androgen response elements (AREs), and is controlled by androgens in the prostate, and therefore, TMPRSS2 is expressed in both normal prostate and prostate tumor samples (Tomlins et al., 2005). TMPRSS2–ERG gene fusion positive prostate cancer patients were reported to have poor prognosis (Demichelis et al., 2007). The morphological characteristics of TMPRSS2-ERG positive prostate tumors are large nucleoli, intraductal spread of cancer cells as well as embedded acini (Zong et al., 2009). *In vivo* and *in vitro* studies of TMPRSS2-ERG in the prostate have confirmed its role in cell invasion and cell signaling, e.g. in the PI3K pathway activation (Tomlins et al., 2008; Zong et al., 2009). TMPRSS2-ERG overexpression in a mouse model led to the development of prostate intraepithelial neoplasia but not to invasive adenocarcinoma (Klezovitch et al., 2008; Zong et al., 2009). However, the molecular mechanism of the formation of TMPRSS2-ERG gene fusion and its signaling in prostate tumors is still unclear.

The main aim of this thesis is to explore the genetic mechanisms leading to gene fusions in advanced prostate tumors, and the molecular mechanisms of ERG-initiated oncogenesis in prostate cancer. *In silico*, *in vitro,* and *in vivo* studies were performed to identify the potential downstream pathways linked with ERG activation in prostate cancer. Cell adhesion mechanisms induced by TMPRSS2-ERG in VCaP prostate cancer cells were determined. Furthermore, a novel compound was identified that inhibited WNT signaling and induced cell-cell and cell-matrix adhesion in TMPRRS2-ERG positive VCaP cells. In conclusion, this work will help us to understand the complex regulatory pathways, like WNT signaling and cell adhesion, involved in TMPRSS2-ERG positive prostate carcinogenesis.

2. REVIEW OF THE LITERATURE

2.1. Prostate Cancer

Prostate cancer is one of the most common cancers, and also one of the most common causes of cancer-related death in men (http://www.cancer.org, Rosenberg et al., 2010). It is more common in Western countries than in Asia (Hsing et al., 2000; Whittemore et al., 1995). Due to the multifocal and heterogeneous nature of prostate cancer, it is quite difficult to study its progression (Scardino, 2000). The prostate gland is an integral part of the male reproductive system, and it is located under the urinary bladder (Dixon *et al*. 1999) and controlled by androgens. Among other substances, prostate produces prostate-specific antigens and prostatic acid phosphatase involved in e.g. sperm liquidification (Rao AR et al., 2008; Balk et al., 2003; Ross et al., 1999; Mooradian et al., 1987; Ostrowski and Kuciel, 1994).

The steroidal hormones, testosterone and its active metabolite dihydrotestosterone (DHT), are important for prostate growth and differentiation, and bind strongly to androgen receptors (AR, a member of nuclear receptor superfamily) (Roy et al., 1999; He et al., 1999; Heinlein and Chang, 2002a; Heinlein and Chang, 2002b; Marker et al., 2003; Tindall and Rittmaster, 2008). AR plays an important role in prostate specific gene regulation, and in modulation of gene expression by interacting with the regulatory regions (androgen response elements) of its target genes (Riegman et al., 1991), and the activation of various signaling pathways may eventually lead to development of hormone refractory prostate cancer (Culig et al., 1994; Seaton *et al.*, 2008; Malinowska *et al.*, 2009).

The majority of prostate cancer cases are adenocarcinomas, arising from prostate epithelial cells in the peripheral zone rather than in the transition zone (Myers, 2000; Haggman et al., 1997; Shin et al., 2000; McNeal and Haillot, 2001; McNeal and Bostwick, 1986; Scardino, 2000). Typically, many isolated malignant zones can be observed within the same prostate gland (Sakr et al., 1994; Dickinson, 2010). Age, diet, family history, and ethnic background are known risk factors in prostate cancer development (Pienta and Esper, 1993). Among these, age is the most important risk factor, as prostate cancer incidence increases dramatically with age (Hankey et al., 1999). Inflammation may also play a role in prostate cancer progression (Nelson et al., 2004). Prostatic intraepithelial neoplasia (PIN) is a premalignant proliferation (lesion with increased cells) arising within ducts and acini of the prostate gland. The PIN lesion can be categorized to either low-grade or high-grade PIN (Bostwick and Qian, 2004; Joniau et al., 2005). High-grade PIN (HGPIN) is more invasive, has epithelial crowding and stratification, and has larger nuclei than the low-grade PIN. HGPIN may

progress into cancer (Bostwick and Brawer 1987; Kovi et al., 1988; Montironi et al., 2007) and eventually result in mortality. There are a number of genetic alterations frequently detected in PIN, including chromosomal amplifications at 7p, 7q and 8q, inactivations of chromosomal regions (8p, 13q, 16q, etc.), mutations in tumor suppressor genes (such as NK3 homeobox 1 (NKX3-1), Phosphatase and Tensin Homolog (PTEN), and overexpression of oncogenic proteins, such as c-Myc and Bcl-2 (Qian et al., 1995; Qian et al., 1997). There are also several gene products that can be used for diagnostic and therapeutic purposes for prostate cancer, such as Fatty Acid Synthase, Glycoprotein A80, Alpha Methyl Acyl CoA Racemase (AMACR) and Glutathione S-Transferase pi 1 (GSTP1) (Joniau et al., 2005; Swinnen et al., 2002; Coogan et al., 2003; Rubin et al., 2002; Ananthanarayanan et al., 2005; Jeronimo et al., 2002).

2.2. ETS Transcription Factors

In humans, nearly 30 ETS family members have been identified so far. A common feature to ETS transcription factors is a conserved DNA-binding domain, and a subclass of them also has sterile alpha motif (SAM) and pointed domain (PNT) (Seth and Watson, 2005; Oikawa and Yamada, 2003). ETS family members can act as transcriptional activators, repressors or both (Gutierrez-Hartmann et al., 2007; Verger and Duterque-Coquillaud, 2002; Sharrocks, 2001; Oikawa, 2004). The ETS transcription factors are associated with different type of cancers and are involved in different biological processes, such as cellular differentiation, cell cycle, motility and proliferation (Bassuk and Leiden, 1997; Oikawa and Yamada, 2003; Hollenhorst et al., 2010; Look, 1995). For instance, ETS2 transcription factor may physically interact with Fos-Jun, ERG or ETS1 by forming heterotrimers (Basuyaux et al., 1997). The EWS-Fli-1 fusion gene is known to induce c-Myc oncogene expression in the Ewing carcinoma cells (Dauphinot et al., 2001). *TEL–JAK2* gene fusion activates Stat1 and Stat5, and promotes T-cell lymphoma development (Carron et al., 2000). ETS1 gene is overexpressed in multiple cancer types, and is involved in cancer invasion and metastasis via degradation of the extracellular matrix (ECM) and is associated with poor prognosis (Behrens et al., 2001; Davidson et al., 2001). *ERG* is located on chromosome 21 and it has been described as an oncogene in multiple cancer types (Reddy et al., 1987; Owczarek et al., 2004). In human myeloid leukemia cells, the *ERG* gene is fused with the *TLS/FUS* gene on chromosome 16 (Kong et al., 1997) and *ERG* is fused with *TMPRRS2* gene in more than half of the prostate tumors (Tomlins et al., 2005).

2.3. Chromosomal Rearrangements and TMPRSS2-ERG in Prostate Cancer

Chromosomal rearrangements, deletions, translocations, inversions and gene amplifications, play an important role in cancer development (Chen et al., 2005; Gajecka et al., 2008; Sheen et al., 2007; Vissers et al., 2007). Chromosomal rearrangement can lead to cancer due to the deletion or disruption of a tumor suppressor gene or due to an over-expression of an oncogene (Tanaka et al., 2006). Accordingly chromosomal rearrangement can transform a proto-oncogene into a cancer-causing gene (oncogene) leading to cancer progression (Lonnig and Saedler, 2002).

TMPRSS2 (transmembrane protease, serine 2), located on chromosome 21, is highly expressed in normal prostate epithelial cells as well as prostate cancer cells, and is controlled by androgens and estrogens (Paoloni-Giacobino et al., 1997; Setlur et al., 2008). The 5-prime untranslated region of *TMPRSS2* is frequently fused to the ERG oncogene in prostate tumors. This has been confirmed by several studies (Attard et al., 2008; Attard et al., 2010; Dehm, 2008; Helgeson et al., 2008; Hermans et al., 2009; Hu et al., 2008; Jhavar et al., 2008; King et al., 2009; Klezovitch et al., 2008; Mao et al., 2008; Mehra et al., 2008; Mosquera et al., 2008; Perner and Rubin, 2008; Saramaki et al., 2008; Yu et al., 2010), initially discovered by using a bioinformatics approach based on outlier gene expression analysis followed by RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE) and sequencing (Tomlins et al., 2005). Fusion of *TMPRSS2* and *ERG* resulted in androgen mediated ERG overexpression in prostate cancer (Tomlins et al., 2005) (Fig. 1).

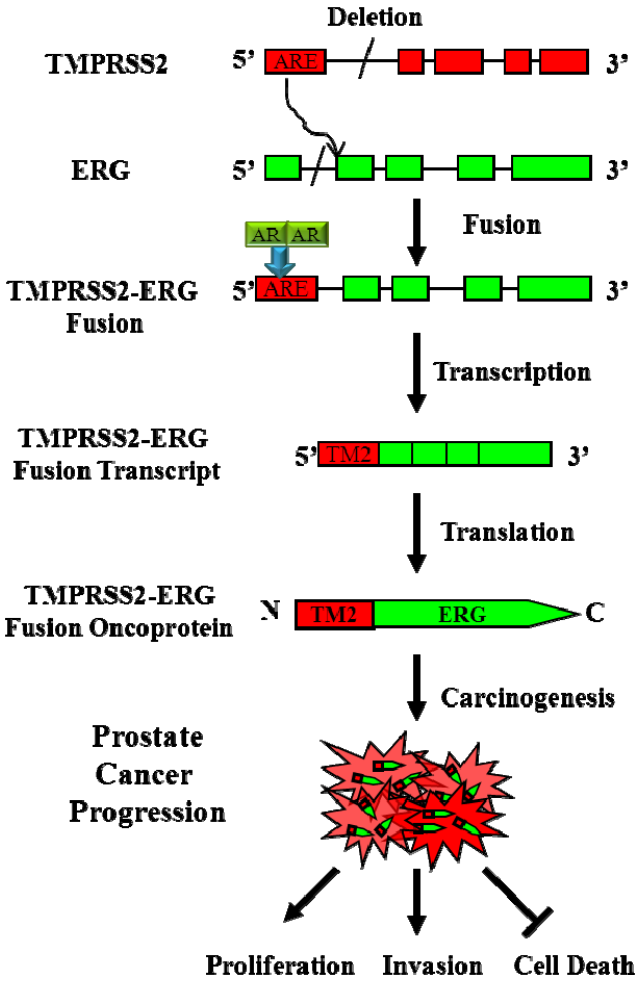

Figure 1: Schematic representation of the *TMPRSS2-ERG* gene fusion model. The strong *TMPRSS2* gene promoter containing androgen response elements (AREs) is fused to the *ERG* oncogene to form the *TMPRSS2–ERG* fusion transcript. The *TMPRSS2-ERG* fusion gene is now controlled by androgen via androgen receptor (AR) and produces high levels of TMPRSS2-ERG mRNA and protein, which is involved in prostate cancer progression. The fusion protein takes part in cell proliferation and invasion in prostate cancer (Tomlins et al., 2005).

2.4. Role of ERG in Prostate Cancer

TMPRSS2-ERG genomic rearrangement occurs early in prostate cancer development, but its prognostic significance may depend on cytogenetic characteristics of the tumor, of the overexpressed *ERG* transcripts, and of the disease stage (Attard et al., 2008; Hermans et al., 2009). Also the phenotypes of ERG positive prostate cancer mouse models varies considerably, which is probably due to differences in hereditary

backgrounds of the mice and different isoforms of ERG in overexpression constructs (Zong et al., 2009).

In vivo overexpression of ERG in a transgenic mouse model resulted in PIN formation and increased integrin β4 expression. ERG overexpression in mouse prostate glands also resulted to the disruption of basal layer by luminal cells which allows contact with the stromal microenvironment (Klezovitch et al., 2004). Results from histological analysis of mice transduced with a lentiviral ERG overexpression construct in prostate epithelial cells indicated that ERG is sufficient to induce neoplastic changes in prostate epithelium but does not produce invasive adenocarcinoma (Klezovitch et al., 2008; Tomlins et al., 2008; Zong et al., 2009). However, both i*n vivo* and *in vitro* studies of ERG overexpression resulted into increased invasion of prostate cancer cells (Tomlins et al., 2008). The urokinase plasminogen activator (PLAU) was upregulated in ERG overexpression study in prostate RWPE cells, and resulted to increased cell invasion (Tomlins et al., 2008). ERG overexpression in the prostate cell model has been connected also with the induction of c-Myc induced neoplastic changes (Sun et al., 2008). Zong et al. (2009) by using immunohistochemistry showed that c-Myc and c-JUN (which is a binding partner of ERG) are overexpressed in ERG-transduced prostate gland as compared with normal glands (Verger et al., 2001). Furthermore, simultaneous overexpression of AR and ERG in prostate epithelial cells by lentiviral systems developed invasive adenocarcinoma, while AR or ERG overexpression alone did not induce prostate cancer (Zong et al., 2009). In addition, in one *in vivo* study the ERG overexpression together with AKT kinase resulted to invasive prostate cancer, but when expressed alone developed only PIN. Zong et al. (2009) also confirmed that ERG overexpression in connection with PTEN knockdown led to the development of prostate adenocarcinoma but PTEN knockdown alone formed only PIN and hyperplasia.

ERG also plays an important role in regulation of phospholipase A2, group VII (PLAG7), vitamin D receptor and prostaglandin signaling in prostate cancer (Vainio et al., 2011; Hendrickson WK et al., 2011; Mohamed et al., 2011). One of the recent studies confirmed also the up regulation of Toll-Like Receptor-4 (TLR4) and NF-kB pathway activation in *TMPRSS2-ERG* positive tumors, which led to prostate cancer development (Wang et al., 2011). Trefoil factor 3 (*TFF3*) which is overexpressed and regulated by ERG, promotes cell invasion in castration-resistant prostate cancer (CRPC) cells (Rickman et al., 2010). Enhancer of Zeste Homolog 2 (EZH2) is also activated by *TMPRSS2-ERG* and mediates the epigenetic silencing in prostate cancer (Yu et al., 2010). The most relevant cancer related pathways linking to ERG oncogenesis is summarized in table1.

Table 1: Studies on the effects of ERG oncogene in biological pathways which are linked in prostate cancer progression.

Pathways	Genes/molecules	Phenotypes	Experimental Models	References
PI3K Pathway	PTEN, and AKT	ERG and AKT overexpression: -Cribriform growth with cell crowding -Embedded acini -Nuclear atypia - Increased invasion	Mouse model	Zong et al., 2009
Arachidonic Acid	PLA2G7	-Up-regulated in prostate cancer -Increased growth and survival -Regulation of oxidative stress	*In silico* (IST) Prostate cancer tissues VCaP cells LNCaP cells	Vainio et al., 2011
Vitamin D Receptor Signaling	VDR	Up-regulated VDR expression: -Low prostate-specific antigen (PSA) -Lower Gleason score -Less advanced tumour stage -Most likely having TMPRSS2:ERG fusion -Reduced risk of lethal prostate cancer	Prostate cancer tissues	Hendrickson WK et al., 2011
Prostaglandin Signaling	HPGD and PGE_2	-HPGD and PEG2 expression is regulated by ERG protein -ERG silencing affects the prostaglandin E2 dependent cell growth and urokinase-type plasminogen activator (uPA) expression -Increased inflammation	VCaP cells	Mohamed et al., 2011
NF-κB Pathway	PAR1, CCL2, FOS, TLR3, and TLR4	-PAR1, CCL2, FOS, TLR3, and TLR4 genes were up regulated in TMPRSS2-ERG positive tumors -Increased NF-κB–mediated transcription -Inhibition of NF-κB pathway reduced cells proliferation in VCaP	Prostate cancerous and normal samples LNCaP cells PC3 cells	Wang et al., 2011

2.5. WNT Signaling in Prostate Cancer

The WNT pathway plays an important role in embryonic development as well as in tissue and stem cell maintenance in adults (Boyer et al., 2010). The WNT pathway is linked also to other signaling pathways, including regulation of cell adhesion and cell-cell contacts mediated via E-cadherin, which binds to β-catenin and sequesters it to the plasma membrane of the cells (Schmalhofer et al., 2009). The loss of E-cadherin has profound effects on β-catenin sub-cellular localization and signaling activity, and is a hallmark of EMT, a process involved in aggressive tumor cell behavior and invasion (Schmalhofer et al., 2009; Cavallaro and Christofori, 2004; Gottardi et al., 2001; Kuphal and Behrens, 2006; Onder et al., 2008).

AR has been demonstrated to act as a repressor of E-cadherin expression, e.g. by induction of the SNAI1 transcription factor, a central player in EMT transitions (Hoshino et al., 2009; Liu et al., 2008). Continuous activation of WNT-target genes promotes unrestrained cell growth and cell survival. The WNT pathway and WNT-activated genes such as c-Myc (Sun et al., 2008) and histone deacetylase 1 (HDAC1) have been demonstrated to contribute to cancer progression in a wide range of

epithelial tissues including the prostate, colon, liver, and ovary (Billin et al., 2000). 1) A specific protein–protein interaction has been identified between β-catenin and AR in a prostate cancer cell line model (Schweizer et al., 2008) and 2) β-catenin considerably induces androgen stimulated transcriptional activation. Co-immunoprecipitation confirmed that the two proteins are together in the same complex, and enhanced β-catenin-regulated transcription was reported (Schweizer et al., 2008; Truica et al., 2000). In addition, 3) androgen mediated transcription has been shown to potentiate the transcriptional activities of Wnt/beta-Catenin signaling, which may contribute to the development of androgen insensitivity and hormone-refractory prostate cancer (Schweizer et al., 2008), for which currently no cure exists.

Most of the Wnt proteins bind to cell surface receptors frizzled, and induce dishevelled family protein which results β-catenin translocation and stabilization in the nucleus and results in the activation of Wnt target genes (Chen et al., 2003; Hurlstone and Clevers, 2002; Mao *et al.*, 2001; Sagara et al., 2001; Sheng *et al.*, 1998). *FZD4* is a WNT receptor that is located on chromosome 11 and contains 2 exons (Kirikoshi et al., 1999). *FZD4* is upregulated in high grade PIN and cancer cells compared to benign prostate cells and therefore it could have potential as a diagnostic marker in prostate cancer (Acevedo et al., 2007). The molecular functions of *FZD4* in prostate cancer are still poorly studied, particularly in *TMPRSS2-ERG* fusion positive prostate tumor samples.

3. AIMS OF THE STUDY

Genomic rearrangement leading to the fusion of the androgen responsive gene, *TMPRSS2,* with the oncogenic ETS transcription factor, *ERG,* is detected in nearly half of all human prostate cancers. However, the molecular mechanism of the *TMPRSS2*-ERG fusion in prostate cancer was still unclear. Thus, the first aim of the study was to explore the chromosomal rearrangements leading to *TMPRSS2-ERG* gene fusions in advanced castration resistant prostate tumor samples.

The second aim of the study was to explore the function of the *ERG* oncogene in prostate epithelial cell lines, and to elucidate the potential downstream pathways connected to *ERG* overexpression in clinical prostate tumors.

In vivo studies have shown PIN formation and integrin β4 up regulation in prostates with *ERG* overexpression. However, the role of *ERG* in cell adhesion and invasion was still unclear. Thus, the third aim of the study was to explore the role of *ERG* in modulating cell adhesion in prostate epithelial cells.

Finding a drug, directly targeting an oncogenic transcription factor *ERG* is challenging. Instead, targeting its downstream pathways might provide an alternative way to treat *ERG* positive prostate cancer patients. As such, our fourth aim was to find a small molecule compound that inhibits the growth of *TMPRSS2-ERG* positive prostate cancer cells.

4. MATERIALS AND METHODS

4.1. Tissue Samples and Cell Lines (I, II, III)

Prostate cancer samples were obtained from the Department of Pathology, Turku University Hospital, Finland as freshly frozen samples with subsequent knowledge of the cancer grade. The study methods were approved by the ethical committee of University of Turku, Finland and informed consent of the patients was obtained. The frozen tissue blocks were sectioned (4-6μm) and stored at -80 °C. The immortalized prostate cell lines RWPE1, LnCaP, VCaP, and DuCaP were purchased from the American Type Culture Collection (ATCC) and grown in RPMI medium with appropriate substituents.

4.2. Gene Copy Number and Expression Data (aCGH) (I)

From the clinical samples, RNA was extracted by TRIzol (Invitrogen) and LiCl /urea using standard protocols. Copy number and expression levels for the *ERG*, *TMPRSS2, ETV4,* and *ETV1* loci were obtained by integration of genome-wide data from the same samples. Human genome CGH 44A and 44B oligonucleotide Microarrays were used for array-based CGH. GeneChipscanner (Affymetrix) was used for scanning after the staining. A more detail protocol is described in the materials and methods section of article I.

4.3. *In Silico* Analysis of ERG Target Genes (I)

To identify the prospective ERG target genes, gene expression data from 410 human prostate tissue samples, including normal (178) and tumors (232) were assembled. Then, the patterns of *ERG* co-expressed genes were analyzed by using four different methods. DAVID GO analysis tool (DAVID Bioinformatics) and gene set enrichment analysis (GSEA, MIT and Harvard, USA) were used to elucidate biological processes (as described in the materials and methods section of article I).

4.4. *GFP-ERG* and *GFP-FZD4* Expression Constructs (I, II)

The full length *ERG* cDNA was PCR amplified, using ORF clone from Origene Inc., (# SC108516) (article I materials and methods) as a template. The *GFP-FZD4* expression construct was obtained from Origene (RG217286). FuGENE-6 (Roch) and Lipofectamine 2000 (Invitrogen) transfection reagents (Roche) were used for transfections.

4.5. Western Blotting (II, III)

GFP (1:1 000, rabbit polyclonal, A11122, Molecular Probes), ERG (1:1 000, rabbit polyclonal, SC-353, Santa Cruz), and FZD4 (1:1 000, goat polyclonal, SC-66450, Santa Cruz) antibodies were used for Western blotting. Antibodies against HSPA8 (heat shock protein 70 kDa, 1:1000, rat monoclonal, SPA-815, Stressgen) and β-Actin (1:1,000, mouse-monoclonal, Becton Dickinson) were used as the internal controls in the Western blot analyses (article II, materials and methods).

4.6. siRNA and Lentiviral shRNA Silencing of ERG in VCaP Cells (II, III)

The most efficient siRNAs against *ERG* (SI03089443, Qiagen), *FZD4* (SC-39983, Santa Cruz) as well as a scrambled siRNA (1027310, Qiagen) were transfected using a Lipofectamine 2000 reagent. To make stable VCaP cells with reduced *ERG* mRNA expression, the *ERG* and scrambled shRNA Lentiviral Particles (Mission/Sigma) with a titer of > 10^6 TU/ml were used to infect VCaP cells. Subsequently, stable VCaP clones were selected using puromycin (2μg/ml) (article II, materials and methods).

4.7. Cell Adhesion Analysis (II, III)

The plates were coated with laminin (LAM), fibronectin (FN), or collagen I (COL I) (0.5 μg/ml) overnight, and blocked with 0.1% BSA. *ERG* siRNA, scrambled siRNA, 4-CMBC or DMSO treated VCaP cells were trypsinized and collected and 0.2% soybean trypsin inhibitor was used to inhibit the trypsin effect. Cells were allowed to attach for the indicated time and stained with PI. The attached cells were counted by using Acumen Explorer at 0, 5, 10, 20, and 30 min time points (article II, materials and methods).

4.8. Quantitative Real-Time PCR Analysis (II, III)

We used a standard quantitative real-time PCR analysis protocol which is described in the article II, in the materials and methods section.

4.9. Fluorescence Activated Cell Sorting (FACS) Analysis (II, III)

FACS analysis of VCaP cells with stable ERG knockdown, or scrambled shRNA expression was performed by using antibodies against active β1-integrin (active β1 subunit-containing integrins; ab12G10, anti-mouse, monoclonal, Abcam) and E-cadherin (ab 1416, anti-mouse, monoclonal, Abcam). The cells were incubated in 1:1500 dilutions of the antibodies overnight at +4°C, and Alexa 647 labeled secondary antibodies (1:2000) were used (incubation for 60 min at room temperature). To detect the signal PI was used for cell cycle analysis of 4-CMBC treated VCaP cells. An

Accuri C6 Flow Cytometer was used to measure the mean fluorescence intensity (article II, materials and methods).

4.10. Illumina Array and Bioinformatics Analysis (II, III)

The genome-wide gene expression analysis is explained in detail in article II. Total RNA was isolated from 1) *GFP* or *GFP-ERG* transfected RWPE1 cells, 2) cells transfected with *ERG* siRNA, *ERG* 214shRNA, and 3) 4-CMBC treated VCaP cells in duplicate. RNA was applied for Illumina human Ref-8 v2.0 & 3.0 expression bead chip and MetaCore analytical suite version2.0 (GeneGo) software was used for biological process and network analyses. The raw data of the illumina array analysis was deposited in the GEO database (GSE16671 and GSE23357).

4.11. Confocal Microscopic Imaging (II, III)

For immunostaining and confocal microscopy, 1) scrambled, *ERG* or *FZD4* siRNA treated VCaP cells, as well as 2) stable shRNA or *ERG* shRNA expressing VCaP cells, 3) *GFP*, *GFP-ERG* and *GFP-FZD* overexpressing VCaP cells, as well as 4) 4-CMBC and DMSO treated VCaP cells were grown on coverslips. The primary antibodies recognizing ERG (1:100, rabbit polyclonal, SC-353, Santa Cruz), FZD4 (1:100, goat polyclonal, SC-66450, Santa Cruz), active β1-integrin-12G10 (1:200), total β1-integrin-P5D2 (1:200, anti-mouse, monoclonal, Abcam), E-cadherin (1:200) and Vimentin (1:100, SC6260, anti-mouse, monoclonal, Santa Cruz) were used for immunostaining. Alexa 488, Alexa 555, and Alexa 647 conjugated secondary antibodies (1:500 dilutions in PBST) were used for detection. DAPI (H-1200, Vector laboratories) was used to visualize the nucleus. Microscopy was done by using confocal laser scanning microscope (LSM510, 63X with oil) and images were analyzed by ImageJ software (Collins, 2007) (article II, materials and methods).

4.12. Immunohistochemical Analysis (II)

Five ERG positive, 5 *ERG* negative and 2 normal prostate tissue specimens were used for immunohistochemistry. Six μm thick frozen sections were used for immunohistochemistry. Affinity purified IgG against human ERG (SC-353, Anti rabbit, Santa Cruz, 1:100, also used in WB) and FZD4 (1:100, also used in WB) were diluted with TBS-buffered 1% BSA, and the slides were incubated with the primary antibodies overnight. Biotinylated anti-goat secondary antibodies were used at room temperature for 30 minutes. Olympus BX50 microscope (Olympus Corporation), Nikon ACT-1 (Version 2.62, Nikon Corporation 2000) software and Digital Camera DXM1200 (A.G. Heinze, Inc.) were used for imaging and analysis (article II, materials and methods).

4.13. Cell Titer-Glo (CTG) Assay (II, III)

4-CMBC or DMSO treated VCaP cells were incubated for 72 hrs at 37°C in 5% CO_2 incubator. CellTiter-Glo (CTG) (G7570, Promega) was added into each well and incubated for 30 minutes at room temperature on a shaker. The luminescence signal was then measured using EnVision Multilabel plate reader (PerkinElmer) to analyse the proportion of viable cells per well.

4.14. Reporter Assay (II, III)

TCF/LEF-GFP Reporter (CCS-018G, SABioscience) was used to measure the WNT signaling activity in response to *ERG* or *FZD4* modulation or 4-CMBC treatment in RWPE1 and VCaP cells. All experiments included transfection with a negative control plasmid (*GFP* reporter construct with a minimal promoter) and a positive control (GFP) to quantitate background and confirm the transfection efficiency. To measure WNT activity, 200 ng *TCF-LEF* reporter was used per well. Transfections or compound exposers were done in triplicate and measured 48 and 72h later by the Acumen Explorer ^{TM}. GraphPad Prism4 software was used to plot the results (article II, materials and methods).

4.15. β1-Integrin Internalization Assay (III)

Integrin internalization assay was done as explained previously (Ivaska et al., 2002; Roberts et al., 2001) with a few modifications. β1-integrin internalization was performed in 1) *ERG, FZD4* and scrambled siRNAs transfected VCaP cells, 2) *GFP*, *GFP-ERG* and *GFP*-FZD4 overexpressing cells, and 3) 4-CMBC and DMSO treated VCaP cells at 48 hrs time point. The amount of internalized biotinylated integrin was assayed by immunoprecipitation using anti-β1-integrin (1:1000, P5D2, anti mouse, Abcam), and anti-EGFR (1:1000, LA1, anti mouse, Upstate) antibodies. The HRP-conjugated anti-biotin antibody (1:1000, cat-7075, Cell Signaling) was used as a control to visualize total biotinylated integrin. The total β1-integrin and EGFR were visualized by Western blotting using β1-integrin (1:1000, P4G11, ab78502, anti-mouse, Abcam) and EGFR (1:1000, 2232, anti rabbit, Cell Signaling) antibodies, respectively (article III, materials and methods).

5. RESULTS AND DISCUSSION

5.1. *TMPRSS2* and *ERG* Oncogene Fusion (I)

Genomic rearrangement involving the androgens controlled *TMPRSS2* gene and oncogenic transcription factor *ERG* has been identified in more than half of solid prostate tumors (Tomlins et al., 2005). To visualize the genomic rearrangement of *TMPRSS2-ERG*, we performed a comparative genomic hybridization (aCGH) experiment in prostate cancer samples. The aCGH results showed the presence of the interstitial deletions between *TMPRSS2* and *ERG* loci, resulting to *TMPRSS2-ERG* gene fusions in clinical prostate cancer samples. Accordingly, *ERG* was upregulated in the *TMPRSS2-ERG* gene fusions positive clinical samples. *TMPRSS2* and *ERG* are located in the same chromosomal strand 21 and the closeness of the two genes (~ 2.8 Mb) may facilitate the fusion gene formation in prostate cancer (I, and figure 2 therein). The TMPRSS2-ERG fusion, was the most common genetic alteration we identified by array-CGH in prostate cancer specimens. Two micro deletions at *TMPRSS2* and *ERG* loci were also identified in one of the prostate tumor patient samples, which indicate that more complex genetic rearrangement may cause gene fusion in this prostate cancer patient (I, and figure 2 therein).

5.2. Co-expression and Co-localization of ERG, AR, and HDAC1 (I)

Histone deacetylases is a group of enzymes that catalyze the elimination of acetyl groups from lysine residues in histones and non-histone proteins. Histone deacetylation is a characteristic feature in epigenetic repression of transcription, and is involved in transcriptional regulation of cell cycle progression (Taunton et al., 1996). In our study, *in vivo* co-expression analysis was performed in normal prostate and prostate cancer samples using multiple statistical methods in order to find *ERG*-associated genes and biological processes. *HDAC1* was most consistently among the top *ERG* co-expressed genes in our analysis (I, and figure 3, there in left panel A). Furthermore, *HDAC1* mRNA expression was measured by RT-PCR in *TMPRSS2-ERG* positive and negative prostate cancer samples. Taken together, we found that HDAC1 was overexpressed in hormone-refractory prostate cancers and it is co-expressed with ERG in prostate tumor samples (I, and supplementary figure 2 therein). However, the molecular mechanism of *HDAC1* overexpression in *TMPRSS2-ERG* positive tumors is not well understood. One study has shown evidence that ERG interacts indirectly with HDAC1 via SETDB1 methyl transferase (Yang et al., 2002; Yang et al., 2003).

For pathways analysis, gene set enrichment analysis (GSEA) was performed in 410 human prostate samples, including normal tissues and tumors. As a result, we observed

that the WNT pathway was upregulated, and HDAC target genes and cell death pathways were down regulated in *ERG* positive tumors samples (I, and figure 4 therein). Since HDAC1 was overexpressed and its target genes downregulated in *ERG* positive cancers, we hypothesized that *ERG* positive prostate tumors may benefit from epigenetic therapy by HDAC inhibitors. To validate this hypothesis, we treated *ERG* positive VCaP and DuCaP cells with a 30 nM concentration of a HDAC inhibitor, trichostatin A. According to the hypothesis, both VCaP and DuCaP cells responded strongly to this treatment (I, and supplementary figure S3 therein, Bjorkman et al., 2008). Whereas, ERG negative prostate cancer cells and non-malignant prostate epithelial cells were not that sensitive. Interestingly, Androgen removal further potentiated the growth inhibitory and pro-apoptotic effects of HDAC inhibitors in *TMPRSS2-ERG* positive VCaP cells (Bjorkman et al., 2008).

Next, to understand the putative interactions among ERG, AR, and HDAC1, immunostaining of ERG, AR, and HDAC1 was performed in VCaP cells. The laser scanning microscopic images indicated that ERG was mostly co-localized with AR and HDAC1 within the nucleus (Fig. 2). *In silico* protein-protein and transcription network analyses (pSTIING, http://pstiing.licr.org/) showed that ERG, AR and HDAC1 are in the same protein-protein and transcriptional networks. These preliminary results might suggest a link among ERG, AR and HDAC1 which may cause prostate cancer progression requires further studies.

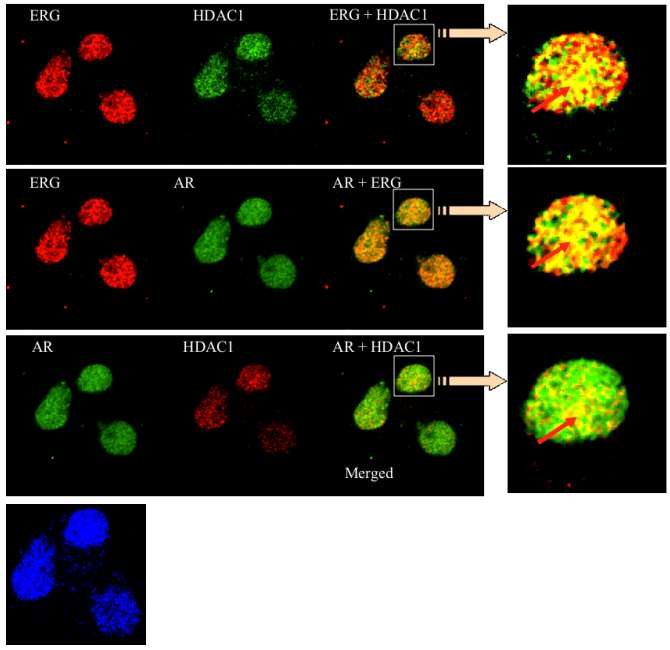

Figure 2: Co-localization study of ERG, AR, and HDAC1 in VCaP prostate cancer cells. AR, HDAC1 and ERG expression was studied in *TMPRSS2-ERG* positive VCaP prostate cancer cells using immunofluorescence staining. Confocal images were analyzed by using the ImageJ program. All the proteins were localized in the nucleus. DAPI staining was used to locate the nucleus. The yellow color in the merged image shows the co-localized proteins.

5.3. *ERG* Regulates Cell-Matrix and Cell–Cell Adhesions (II)

To explore ERG overexpression-related phenotypic changes in prostate cancer cells, knockdown experiments were performed. In particular, we aimed to study tumor cell growth, morphology, invasive properties, and cell motility. Thus, five different *ERG* shRNA or scrambled control shRNA expressing lentiviruses were used to generate stable VCaP cells with puromycin selection. The knockdown of *ERG* was confirmed by quantitative RT-PCR and Western blot analyses. For transient *ERG* knockdown, the silencing capabilities of 10 different siRNAs targeting ERG were tested in VCaP cells. The results indicated that one of the siRNAs was much more effective than the others in reducing *ERG* mRNA and protein levels in VCaP cells (II, and figure 1 therein). VCaP cells stably expressing *ERG*shRNA or transiently transfected with potential *ERG* siRNA formed round cell clusters on culture dish. Our studies indicated that stable knockdown of *ERG* dramatically increased cell adhesion. Therefore, we studied whether these changes were due to ERG silencing induced alterations in the key regulators of cell-cell adhesion. The immunostaining results indicated that *ERG* knockdown upregulated active β1-integrin and E-cadherin expression in VCaP prostate cancer cells (II, and figure 2 therein). To study the cell adhesive properties of the VCaP cells after *ERG* knockdown, we performed adhesion assays using plates coated with different ECM components. The results indicated that *ERG* silencing increased cell-matrix adhesion in response to 10 minute incubation (II, and figure 1 therein) which supports the idea that ERG drives increased invasion in *TMPRSS2-ERG* positive prostate cancer. The loss of E-cadherin is a prerequisite of EMT, a process associated and, /or contributing to aggressive tumor behavior, invasion, and metastasis (Lee et al., 2006). There is relatively little information available concerning the role of E-cadherin loss and prostate cancer. It has been revealed that activated AR is a repressor of E-cadherin. Although this remains as poorly understood mechanisms, AR may promote metastasis also through this process (Liu et al., 2008). Our results showed that ERG downregulates E-cadherin expression which is a central player in EMT transitions, and this may explain the invasive behavior of the *TMPRSS2-ERG* positive cells in prostate cancer.

5.4. Pathway Analysis in *ERG* +/- VCaP Cells (II)

In order to study transcriptional effect of *ERG* modulation, RNA was extracted from *ERG* overexpressed RWPE1 cells, and from the VCaP cells with the silenced ERG with appropriate controls, and the RNA was hybridized in duplicate on Illumina bead arrays. Normalized data from both experiments were compiled and further analyzed in detail, using gene set enrichment analysis (GSEA), various clustering methods and the MetaCore / GeneGo software (www.genego.com). The MetaCore gene ontology and pathway analysis revealed a number of relevant pathways affected by *ERG* overexpression in normal prostate epithelial RWPE1 cells, and after *ERG* knockdown in VCaP prostate cancer cells. The WNT pathway was among the top biological

processes altered in response to *ERG* modulation (activated by ERG overexpression and down-regulated in ERG knockdown). This result is in accordance to *in silico* analysis of co-expression pattern in clinical cancer. The results indicated that the WNT pathway is more strongly activated in the *ERG* positive tumors, as compared with the *ERG* negative tumors or normal tissues samples. In addition, there were several other biological processes or pathways such as Notch-signaling, angiogenesis, stress and immune response, proliferation, and cell cycle that came up as being tied to *ERG* function, in both the overexpression and knockdown analyses.

5.5. *In Silico* Analysis and Clinical Validation of WNT Signature (II)

A more focused and systematic follow up analysis of genes involved in the WNT pathway was performed *in silico,* and identified 226 Wnt pathway genes from the Biocompare database (http://gspd.biocompare.com/) and studied which of these genes were positively associated with the ERG which shows an outlier gene expression in prostate cancer (a subset of prostate cancer samples exhibiting a high expression profile which is different from the rest of the samples) (Kilpinen et al., 2008). The GTI algorithm was used, which is equivalent to the COPA (cancer outlier profile analysis) algorithm (Mpindi et al., 2011; Tomlins et al., 2005). In agreement with our previous study, HDAC1 was one of the genes with the highest association with ERG positivity in the analysis. Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1 (GNAI1) was another gene closely associated with ERG expression in correlation analysis. FZD4 was the third interesting gene among the top WNT pathway genes correlating with ERG. We did not see any change in GNAI1 expression in our ERG modulations experiments. FZD4 was one of the consistent differentially expressed genes in both ERG silenced and overexpressing VCaP and RWPE1 cells, respectively. Top 20 WNT genes with the strongest co-expression with ERG are listed in the article II and supplementary table 6 therein. Additionally, we analyzed the correlations between ERG, WNT pathway genes, and markers for epithelial and mesenchymal differentiation in the IST "*In Silico Transcriptomics*" microarray database (www.genesapiens.org). This analysis consisted of 496 Affymetrix gene expression profiles from clinical samples (including normal prostate and cancer). A significant correlation was confirmed for the expression at WNT pathway genes and the ERG positivity in prostate tumors but not in normal samples (II, and figure 3 therein).

The putative involvement of induced WNT signaling in the epithelial mesenchymal transition was studied by analyzing the co-expression pattern with epithelial and mesenchymal marker genes. As a result, *ERG* expression was found to be associated with increased expression of WNT pathway genes, as well as mesenchymal markers (such as N-cadherin and OB-cadherin), and with decreased expression of epithelial marker genes (such as the basal cytokeratins keratin 5 and keratin 14), and the luminal

keratins- keratin 7 and keratin 18. These results match well with the profiles we observed in the *in vitro* experiments, and strongly suggest the role of *ERG* in regulating WNT signaling, and activating EMT both *in vitro* in cell line model systems and *in vivo* in clinical tumors. In concordance with our previous study (article I) we observed that activation of *ERG* may guide epigenetic reprogramming, increased WNT signaling, and the suppression of cell death pathways, confirming the role of *ERG* with therapeutic significance. We validated the 15 top-ranked ERG co-expressed genes involved in WNT signaling by qRT-PCR in our set of sixteen *ERG*+ (9) and *ERG*- (7) prostate cancer samples. For most of these, we were able to validated significantly higher expression of the WNT pathway components in the *ERG* positive specimens as compared with *ERG* negative tumor samples. As an example, protein kinase D1 that interacts with E-cadherin and is involved in cell invasion and motility, was upregulated in *ERG* positive prostate cancers (Jaggi et al., 2005).

5.6. Co-expression Study of ERG and FZD4 (II)

The WNT pathway was one of the few systematically altered signaling pathways in the *ERG* modulation experiments, according to bioinformatics analysis by GSEA, MetaCore, and GeneGo pathway analyses. For example, in the *ERG-GFP*-transfected cells, *FZD4* and *DAB2* were upregulated and *DKK1* downregulated. In contrast, *AXIN2*, *CCND1*, *FRAP1*, *LEF1*, and *PPP1CB* were all upregulated in the permanent *ERG*-shRNA expressing VCaP cells, while *FZD4*, *COL4A5*, *MAPK13*, β1-catenin and *UPAR* were downregulated. To summarize, *FZD4*, a WNT receptor, was one of the consistently differentially expressed genes in response to *ERG* modulation experiments *in vitro* in prostate cells, and was, thus, studied in detail in functional experiments (Fig.3). Quantitative RT-PCR validation of *FZD4* mRNA expression showed that *FZD4* was significantly upregulated in all *ERG* positive prostate tumor samples, but was low in ERG negative tumors. *FZD4* was silenced by using siRNA to study its role in cell-cell contacts in VCaP cells. As a result, we observed that *FZD4* siRNA knockdown increases active β1-integrin and E-cadherin expression in VCaP cells similarly as observed in *ERG* silencing (II, and figure5, there in panel A and B). We have also observed that FZD4 silencing downregulates ERG expression in VCaP cells.

E-cadherin mediated cell-cell contact is important for the preservation of embryonic epithelia, and architecture of epithelial tissues (Burdsal et al., 1993; Riethmacher et al., 1995; Wheelock and Jensen, 1992). Fibroblast-like properties, decreased cell-cell contact, and loss of E-cadherin are the common characteristics of EMT, and loss of E-cadherin is one of the key features in cancer invasion (Birchmeier et al., 1991; Hajra et al., 1999). Transcription factors SNAI1/SNAI2 are well studied in loss of E-cadherin expression (Batlle et al., 2000; Bolos et al., 2003). Therefore, we also measured the *SNAI1*/*SNAI2* mRNA expressions in *ERG* modulated prostate cells, and the data

indicated an increased *SNAI1* and *SNAI2* expression, confirming the link between the *ERG* expression and increased WNT signaling and EMT.

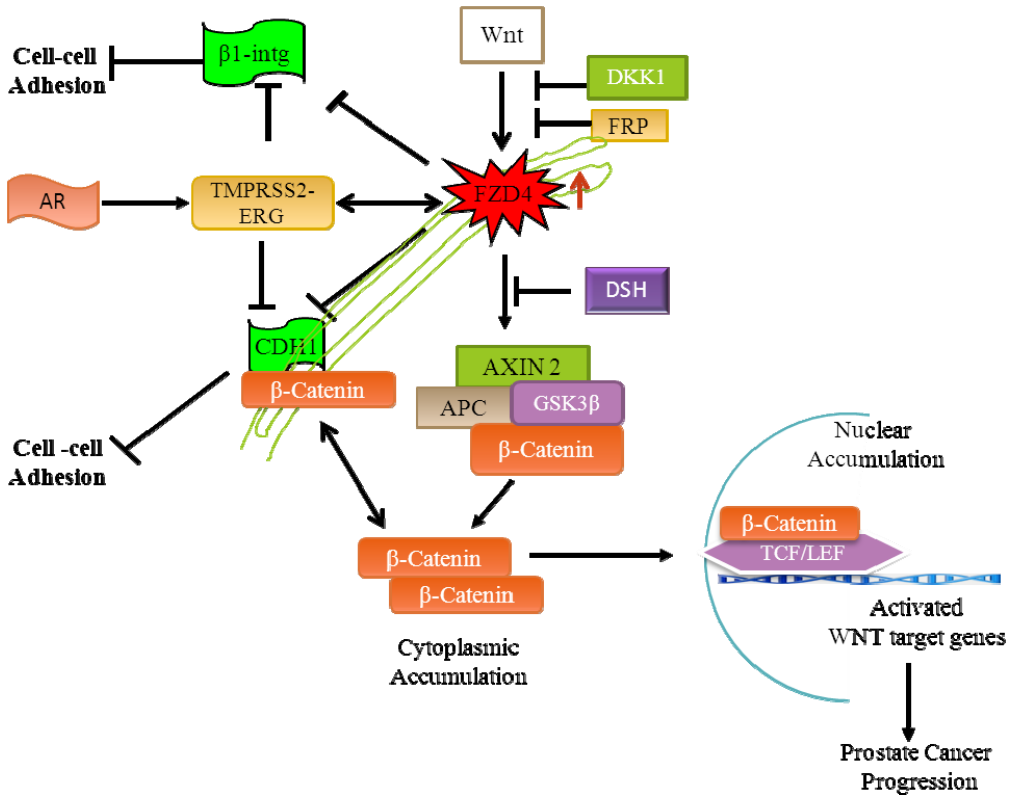

Figure 3. Cell adhesion and WNT signaling in TMPRSS2-ERG positive prostate cancer cells. In the presence of Wnt ligands, Wnt proteins bind to frizzled receptor which activates dishevelled, resulting β-catenin split-up from the APC complex. Next, β-catenin accumulates in the nucleus and mediates transcription of its target genes (Amit et al., 2002; Sakanaka et al., 1999; Willert et al., 1997). In our study, we observed that ERG is co-overexpressed with *FZD4*. *FZD4* was upregulated in our ERG overexpression study in RWPE1 cells and downregulated in *ERG* impaired VCaP cells. FZD4 is the receptor for Wnt family ligands and play important role in Wnt signaling pathway (Malbon, 2004). Overexpression of FZD4 could be one mechanism for the WNT pathway activation in ERG positive prostate cancer samples. There were also other Wnt associated genes, which were differentially expressed in response to ERG modulation. Loss of E-cadherin is one of the common characteristics of cancer invasion (Birchmeier et al., 1991; Hajra et al., 1999). Cell-cell adhesion also depends on the interactions between the cadherins and catenins interactions (Goodwin and Yap, 2004). It has been also observed that E-cadherin binds to β-catenin and prevents nuclear accumulation of β-catenin in colon carcinoma cells (Orsulic et al., 1999). In our study, we observed the downregulation of β-catenin expression in the ERG knockdown VCaP cells. The downregulation of E-cadherin could be one cause for WNT signaling activation in *ERG* positive prostate cancer (figure adapted from http://atlasgeneticsoncology.org/Deep/WNTSignPathID20042.html).

5.7. ERG and FZD4 Induces WNT Signaling (II)

To confirm the induced WNT signaling as a result of *ERG* overexpression, a T-cell factor / lymphoid enhancer factor reporter assay was performed in *ERG* overexpressing RWPE1 cells, and in the *ERG* silenced VCaP cells at different time points. The results indicate a significant increase in WNT activity in response to *ERG* upregulation, and vice versa in in *ERG* impaired VCaP cells. To find out if *FZD4* affects WNT signaling in prostate cancer cells, *TCF/LEF* activity was measured in *FZD4* silenced VCaP cells. The results indicated that *FZD4* siRNA knockdown decreased WNT activity in VCaP cells (II, and figure 6 therein). To confirm our hypothesis that FZD4 mediates the effect of ERG, FZD4 overexpression study was performed in VCaP cells together with ERG knock down using siRNAs. The results confirmed that FZD4 overexpression reversed the effect of ERG knockdown on WNT activity in VCaP cells (II, and figure 6 therein). Finally, we showed ERG-mediated oncogenesis via FZD4 involving WNT signaling activation in prostate cancer.

5.8. 4-CMBC Inhibits WNT Signaling (III)

4-CMBC is a chemical that is used as a building block in the solid-phase synthesis of Imatinib (Francesco et al., 2007; Sigma Aldrich, 270784). It is well known that Imatinib downregulates WNT signaling in different types of cancer (Rao et al., 2006), but there is no information whether imatinib in *TMPRSS2-ERG* fusion positive prostate tumor samples. We measured the WNT signaling activity using *TCF/LEF-GFP* reporter assay on VCaP cells treated with 4-CMBC at different time points. The results indicated that 4-CMBC treatment for 48 h, at 10 μM concentration, decreased WNT activity in VCaP cells. We observed that 4-CMBC inhibits WNT signaling in *TMPRSS2-ERG* positive VCaP cells. To see the effect of 4-CMBC on *ERG* and *FZD4* expression, we performed qRT-PCR and immunoblotting in 4-CMBC-treated VCaP cells with antibodies against *ERG* and *FZD4* after a 48 h treatment. As a result, we found that 4-CMBC down regulates *ERG* and *FZD4* both at mRNA and protein level significantly (III, and figure 3 there in panel A to D). Finally, based on the above findings, 4-CMBC could be used to inhibit the carcinogenesis in *TMPRSS2-ERG* positive prostate tumors (Fig. 4).

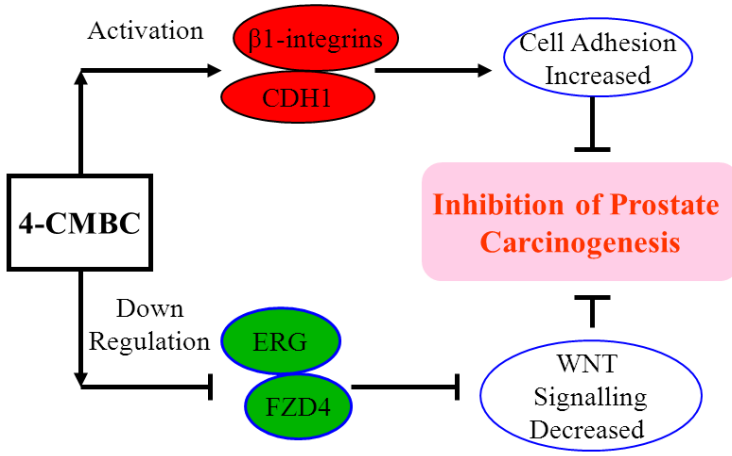

Figure 4. Mechanism of 4-CMBC inhibition of prostate cancer cell growth. 4-CMBC treatment on VCaP cells down regulates ERG and FZD4 at mRNA and protein level expression. *ERG* and *FZD4* activate WNT signaling activation, and thus, 4-CMBC inhibits the WNT signaling in VCaP cells. 4-CMBC exposure activates E-cadherin and β1-integrin, resulting to increased cell-cell and cell-matrix adhesion in VCaP cells. In conclusion, 4-CMBC is a candidate molecule to inhibit prostate cancer progression in *TMPRSS2-ERG* positive tumors.

5.9. Gene Expression Analysis of 4-CMBC-treated VCaP (III)

Genome wide expression analysis was carried out using illumina bead arrays in 4-CMBC-treated VCaP cells at different time points. The MetaCore / GeneGo pathway analysis revealed that cell adhesion and the WNT pathway were the most systematically altered signaling pathways in the 4-CMBC-treated VCaP cells. Furthermore, the genes implicated in the cell adhesion (*CCL2*, *IL8*, *SERPINE2*, PI3K REGclassIA) were downregulated and *PIPKIγ*, vinculin, actins, *RAC1*, Rock, and *ARP1/3* upregulated. The genes involved in WNT signaling such as Wnt, nucleolin, frizzled receptors were downregulated and GSK3beta, vinculin, *MRLC*, *ERK2 (MAPK1/3),* actins and *cRAF1,* upregulated. It could be interesting to study the above mentioned downregulated genes which might be associated with WNT signaling activation and cell adhesion suppression in ERG positive prostate cancer. There were some genes, such as CCL2, and WNT which came across in the 4-CMBC treatment of VCaP cells, that were also observed in our previous *in silico* gene set enrichment analysis of prostate cancer samples (I, and supplementary table 6 therein). Finally, gene set enrichment analysis of differentially expressed genes in 4-CMBC treatment and ERG silencing resulted in similar phenotypes in VCaP cells.

5.10. β1-integrin Internalization in VCaP Cells (III)

We further explored phenotypic changes in VCaP cells in response to 4-CMBC. In our previous study, we had observed that *ERG* silencing in VCaP cells resulted in round

cell clusters that were strongly adherent to cell culture plates. In the present study, we observed that also 4-CMBC-treated VCaP cells formed more rounded structures in comparison to control VCaP cells. Our results indicated that these changes were due to upregulation of active β1-integrin and E-cadherin that are known regulators of cell-cell adhesion. This might explain the dramatically increased cell adhesion properties detected in 4-CMBC-treated VCaP cells. The cell- matrix adhesion in 4-CMBC-treated VCaP cells was studied more specifically by using several extracellular matrix (ECM) proteins that bind β1-integrins. In these studies, the VCaP cells exposed to 4-CMBC were found to bind strongly to laminin, collagen I, and fibronectin extracellular matrix (III, and figure 4 therein).

Integrin heterodimers are repeatedly internalized and rapidly recycled (endo-exocytic cycle) to the cell surface (Bretscher, 1992). Integrins mediate ECM adhesions and regulate key biological processes, such as angiogenesis, differentiation, proliferation, apoptosis, and migration (Hynes, 2002; Naylor et al., 2005; Reddig and Juliano, 2005). Intracellular integrin traffic also regulates cell migration (Caswell and Norman, 2006) and invasion (Caswell and Norman, 2008). To study the role of *ERG* in β1-integrin trafficking, we performed a β1-integrin internalization assay (Ivaska et al., 2002; Roberts et al., 2001) in the VCaP cells. We performed the experiments using *ERG* siRNA or scrambled siRNA, and 4-CMBC and DMSO treated VCaP cells and studied the surface labeled β1-integrin at different time points (0-20 min). The results indicated that *ERG* silencing and 4-CMBC treatment significantly increased β1-integrin internalization (III, and figure 1 and 6 therein). To check the specificity to integrins, EGFR internalization in *ERG* silenced VCaP cells was performed. As a result, we did not observe any significant changes in EGFR internalization (III, and supplementary figure 1). Overall, our results confirm that *ERG* silencing increases cell-cell adhesion due to increased β1-integrin internalization in VCaP cells, which might explain the increased cell invasion and motility in *TMPRSS2-ERG* positive prostate tumors. Finally 4-CMBC, which is linked to mesenchymal to epithelial transition (MET), suggest to provide a new approach to target *ERG* positive prostate tumors.

6. CONCLUSION

In the present study, we showed that *TMPRSS2-ERG* gene fusion overexpression in prostate cancer patients is due to unbalanced genomic alterations. *ERG* is co-expressed with *HDAC1*, and activates several biological processes, such as epigenetic reprogramming and WNT signaling, and suppresses cell death pathways in prostate cancer. Therefore, *ERG* might be used as a potential therapeutic target in prostate cancer treatment. Our study also confirmed that *TMPRSS2-ERG* positive prostate cancer patients could benefit from HDAC inhibitors (epigenetic therapy), and with other combinational approach such as androgen-deprivation therapy.

We found that *ERG* silencing promotes cell adhesion via the regulation of β1-integrin endocytosis, inducing E-cadherin expression, as well as decreasing WNT signaling. Moreover, several other pathways were identified, and could provide novel targets to inhibit *ERG* drive oncogenesis in prostate cancer. This study suggests that it may be possible to target downstream signaling events specifically, such as WNT signaling, for *ERG* positive prostate cancer treatment.

We also observed that *FZD4* is co-overexpressed with *ERG* in prostate cancers. The phenotypic alterations after *FZD4* modulation mimicked those detected by *ERG* modulation in the VCaP prostate cell line model. Similarly to *ERG*, *FZD4* activates WNT signaling in VCaP cells.

Our study also provides a novel mechanism, regulation of β1-integrin endocytosis in *TMPRSS2-ERG* positive cells, by which *ERG* overexpression may increase cell invasion and promote prostate carcinogenesis.

Finally, we identified a compound, 4-CMBC that inhibited WNT activity, *ERG* and *FZD4* expression in *TMPRSS2-ERG* positive VCaP cells, and should, thus, be studied further as a putative compound for the treatment of *TMPRSS2-ERG* positive prostate cancer. Further studies are required to study the growth inhibitory potential compounds in *in vivo*.

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