# INTEGRIN-LINKED KINASE IS AN ESSENTIAL MEDIATOR OF ERG-INDUCED EPITHELIAL-TO-MESENCHYMAL TRANSITION IN PROSTATE CANCER MODELS

by

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B.Sc., Universidade Federal do Rio Grande do Norte, 2006

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Genetics)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

March 2011

# Abstract

Approximately 50% of human prostate cancers carry a gene fusion involving the 5' untranslated region of *TMPRSS2*, an androgen-regulated gene, and the protein-coding sequences of *ERG*, which encodes an ETS transcription factor. Exogenous expression of ERG in human prostatic epithelial cell lines (PrECs) promotes phenotypic changes associated with epithelial-to-mesenchymal transition (EMT), a process implicated in the invasion and metastasis of carcinomas. To gain insight into the biological mechanism by which ERG promotes EMT, I used two immortalized PrECs stably infected with a lentiviral vector expressing a Flag epitope-tagged ERG3 (fERG-PrECs). qRT-PCR and Western blotting show that integrin-linked kinase (ILK) mRNA and protein levels are increased in fERG-PrECs. The mesenchymal markers and downstream effectors of ILK, LEF-1 and Snail, are also upregulated in fERG-PrECs. Depletion of ILK expression by siRNA or inhibition of its activity with a highly selective small molecule inhibitor, QLT-0267, results in a substantial decrease in ERG-mediated upregulation of Snail and LEF-1. Furthermore, I show that inhibition of ILK activity impairs the *in vitro* invasive properties and suppresses the anchorage-independent growth of fERG-PrECs. In conclusion, I have provided novel insights into critical pathways by which aberrant ERG expression may promote prostate cancer progression. In particular, I presented evidence to support the hypothesis that ERG-mediated oncogenesis in prostate cancer involves activation of ILK signaling, leading to key cancer-promoting phenotypic effects, such as EMT.

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# Acknowledgments

I owe my deepest gratitude to my supervisor, Dr. Shoukat Dedhar, who has made available his support in a number of ways. A few words are not enough to express my appreciation for the opportunity he has given me to pursue my graduate studies under his supervision. I have had an ideal environment to carry out my research, working in the Dedhar laboratory at the BC Cancer Research Centre. I am greatly thankful to the members of my advisory committee, Dr. Poul Sorensen and in special, Dr. Michael Cox, for the opportunity to work in collaboration in an exciting research project, as well as for very valuable and insightful discussions. I would like to express my gratitude to Dr. Calvin Roskelley for being part of my thesis examination committee. I am also grateful to my former supervisor, Dr. Anthony Griffiths for the precious opportunity of exposure to the genetics research field during my undergraduate studies. Many thanks to the past and present members of the Dedhar laboratory, especially Jamie Lim, Paul McDonald, Arusha Oloumi, Andrew Fielding, Yuanmei Lou, Isabel Serrano, and Mykola Maydan. I extend a special appreciation to the staff and faculty in the Genetics Graduate Program, in particular, Dr. Hugh Brock.

I am thankful to my parents, brothers and sisters without whose love and support I would not have been able to start or finish my studies. Finally, I am forever grateful to Gilles Hennenfent, for his love and patience in happy and tough times during this journey.

*This work was supported by the National Cancer Institute of Canada with funds from the Terry Fox Foundation and by a University Graduate Fellowship (UGF) from UBC.*

*To my father, who will live*
*forever in my heart*

# Chapter 1. Introduction

Human cancer is a multigene disorder in which several somatic mutations in cancer predisposition genes are required in a stepwise manner (Vogelstein and Kinzler 1993). A major breakthrough in prostate cancer research was the identification of gene fusions between the androgen-responsive gene *TMPRSS2* (transmembrane protease serine 2), and the ETS transcription factor *ERG* (ETS-related gene) (Tomlins et al 2005). *TMPRSS2-ERG* is found in roughly 50% of all prostate cancers and results in high level expression of the ERG gene (Clark et al 2007, Darnel et al 2009, Furusato et al 2008, Hermans et al 2006, Kumar-Sinha et al 2008, Saramaki et al 2008, Yoshimoto et al 2008). Several studies have linked ERG activation to invasive properties of prostate cancer, although the precise mechanisms and pathways of ERG-mediated oncogenesis remain poorly understood.

The acquisition of invasiveness by epithelial cells requires dramatic molecular and biological changes, such as, loss of cadherin-mediated cell-cell adhesion, remodeling of cell-matrix adhesion sites, and basement membrane breakdown. These events together are termed epithelial-to-mesenchymal transition (EMT) (Savagner 2001). EMT, is both a fundamental process in embryonic development and is implicated in the invasion and metastasis of carcinomas (Kalluri and Weinberg 2009, Thiery 2003). A protein that has been shown to play a central role in EMT is integrin-linked-kinase (ILK), an intracellular adaptor and serine/threonine kinase. ILK interacts with several proteins that are involved in cytoskeletal dynamics and cell-signaling cascades

(McDonald *et al.*, 2008). Through these interactions ILK can regulate not only EMT, but many other cellular processes implicated in tumourigenesis (McDonald *et al.*, 2008). In this introductory chapter, I will review ERG expression in prostate cancer, the EMT process, and ILK. Furthermore, I will focus on the cellular and molecular mechanisms mediated by ILK in cancer progression.

## 1.1 Prostate gland

The prostate is a compound tubualveolar gland of the male reproductive system that surrounds the urethra in the pelvic cavity, and is located in front of the rectum. A healthy human prostate is approximately three centimeters long and weighs about twenty grams (Thibodeau and Patton, 2007). Its main function is to produce and secrete an alkaline viscid fluid that constitutes about 30% of the seminal fluid volume. Constituents of prostatic fluid include enzymes such as hyaluronidase and prostate-specific antigen (PSA). Prostatic fluid plays an important role in sperm activation, viability, and motility. In addition, the prostate participates in the ejaculation mechanism, allowing the emission of the semen (Thibodeau and Patton, 2007).

The prostate gland is dependent on steroid hormones – androgens – for its development and maturation. Androgens regulate genes that promote cell survival through binding with the androgen receptor (AR), a ligand responsive transcription factor. Testosterone is the principal androgen in circulation, and when it enters prostate cells it is usually converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase. Binding to DHT, activates the AR which subsequently dimerizes, translocates to the nucleus, and regulates transcription through interaction with androgen-responsive elements of specific genes (Heinlein and Chang 2004).

## 1.2 Prostate cancer overview

Prostate cancer is the most common malignancy to afflict men in western societies (Jemal et al 2010). The lifetime risk of being diagnosed with prostate cancer is approximately 1 in 6. Globally, the highest incidence of prostate cancer is found in the United States, Canada, and Scandinavia, and the lowest in China and other parts of Asia (Grönberg 2003). In 2010 alone, an estimated 24,600 Canadian men were diagnosed with prostate cancer, and more than 4,000 were expected to die of the disease (source: Canadian Cancer Society[1]).

The vast majority (95%) of prostate cancers are classified as adenocarcinomas. An adenocarcinoma is a malignant cancer that starts in the cells lining the ducts and tubes of a glandular organ. As with other cancers, prostate tumourigenesis develops in the background of diverse genetic and environmental factors (Abate-Shen and Shen 2000). Multiple complex molecular events characterize prostate cancer initiation, unregulated growth, invasion, and metastasis. Distinct sets of genes and proteins dictate progression from a precursor lesion, known as prostatic intraepithelial neoplasia (PIN), to localized disease, and finally to metastatic disease (Lapointe et al 2007).

At present, the only widely accepted screening tools for prostate cancer are digital rectal examination (DRE) and PSA testing. PSA is a glycoprotein produced in the ducts of the prostate, which can be measured in serum. Elevations of serum PSA indicate an abnormality in the prostate, whether benign enlargement, inflammation, or

[1] Canadian Cancer Society's Steering Committee: Canadian Cancer Statistics 2010. Toronto: Canadian Cancer Society, 2010.

prostate cancer. Despite the ability of PSA screening to find early-stage prostate cancer, controversy exists over its ability to impact cancer-relative survival. Autopsy studies have shown that a great proportion of men without a clinical diagnosis of prostate cancer have asymptomatic disease – which means that many men die with, rather than of, prostate cancer. Hence, the advent of widespread PSA screening has the positive effect of identifying patients with life-threatening prostate cancer at a time when they are more curable and the negative effect of identifying many patients with non–life-threatening cancer who are susceptible to overtreatment (Klotz 2007, Strope and Andriole 2010).

When detected in early stages, treatment is usually effective and typically consists of surgery or radiation to remove or destroy the cancerous cells that are still confined within the prostate capsule. Unfortunately, current methods of treating advanced disease often prove to be insufficient in the long-term (Michaelson et al 2008). Huggins and Hodges received the 1967 Nobel Prize for their discovery that surgical or medical castration could produce a striking regression of advanced prostate cancer and alleviate disease-related symptoms (Huggins and Hodges 1941a, Huggins and Hodges 1941b). Since then, hormonal suppression therapy (also called androgen deprivation therapy [ADT]) has become widely accepted as the mainstay of therapy for the treatment of advanced prostate cancer. Although ADT is initially successful, most men eventually fail this therapy by developing androgen-independent disease, also known as castration resistant prostate cancer (CRPC), a lethal form of this disease that progresses and metastasizes. Treatment options at this point in disease progression

are mainly palliative, with only docetaxel-based chemotherapy demonstrating a modest survival benefit (Petrylak et al 2004, Tannock et al 2004).

Recent research has therefore sought to identify new molecular pathways by which investigators can distinguish indolent prostate cancers from those that go on to pursue a more aggressive clinical course, as well as to discover new targets for the treatment of advanced disease.

## 1.3 ERG expression in prostate cancer

Cancer can be defined as a genetic disease, resulting as a consequence of multiple events associated with initiation, promotion and metastatic growth. These events are characterized by mutations in genes that promote cancer (i.e. oncogenes) or that prevent cancer (i.e. tumor suppressor genes). Mutations include insertion, deletion, or substitution of single nucleotides, and chromosomal gain, losses, or rearrangements (Hanahan and Weinberg 2000).

Recurrent mutations often disrupt genes that play important roles in cancer development and can be exploited for diagnosis, disease subclassification, prognosis and therapy. Many causal cancer genes have been identified through the analysis of recurrent chromosomal rearrangements and resulting gene fusions, which characterize leukemias, lymphomas, and sarcomas (Mitelman et al 2007). In contrast, recurrent rearrangements had not been identified in common epithelial tumors until 2005, when

gene fusions involving members of the E twenty-six (ETS) family of transcription factors were reported in prostate cancer (Tomlins et al 2005).

### 1.3.1 The ETS gene family

Transcription factors are proteins that regulate gene expression via binding to specific DNA sequences present in target genes. ETS is an important family of transcription factors that contains a conserved 80-amino-acid ETS DNA-binding domain that binds the core DNA sequence 5'-GGA(A/T)-3' (Seth and Watson 2005). The human genome has 28 *ETS* genes which are subdivided in 10 subfamilies (Hollenhorst et al 2004, Maroulakou and Bowe 2000, Seth and Watson 2005).

The first member of the ETS gene family, v-Ets oncogene, was identified as a part of the gag-myb-Ets fusion oncogene of the avian retrovirus E26, which is responsible for leukemic transformation of chicken erythroblasts and myeloblasts (Leprince et al 1983, Nunn et al 1983). Since then, a number of genes related to the v-Ets oncogene have been identified in a variety of cell types from *Drosophila* to human (Macleod et al 1992). The ETS gene family is divided into subgroups on the basis of amino acid sequence similarity and overall structure. Of the genes known to be involved in prostate cancer, ETS-related gene, *ERG,* belongs to the 'ERG subgroup' of this family, which contains a PNT (pointed) protein interaction domain. The normal *ERG* gene has at least nine splice forms with multiple transcriptional initiation sites and alternative exons encoding at least five different proteins (Duterque-Coquillaud et al

1993, Owczarek 2004, Rao et al 1987, Wang 2008). The ETS variant 1,4 and 5 genes, *ETV1*, *ETV4* and *ETV5,* belong to the 'PEA3 subgroup' (Laudet et al 1999).

ETS proteins control the expression of genes that are critical for several biological processes, including cellular proliferation, differentiation, development, transformation, and apoptosis (Seth and Watson 2005). Aberrant expression of ETS factors has also been implicated in a range of malignancies, such as, Ewing's sarcoma, myeloid leukemia, and cervical carcinomas (Seth and Watson 2005, Turner and Watson 2008). Recently, ETS transcription factors have emerged as important elements in prostate tumourigenesis (Tomlins et al 2005).

### 1.3.2 ETS gene fusions in prostate cancer

The first clue that ETS genes could be involved in prostate cancer was the report of high level expression of *ERG* in about half of all prostate tumors by Petrovics and colleagues in 2005 (Petrovics 2005). In the same year, applying the Cancer Outlier Profile Analysis (COPA) - which was developed to analyze DNA microarray data for outlier genes (those markedly overexpressed in a subset of cases) - to the Oncomine database, Tomlins *et al* identified strong outlier profiles for *ERG* and *ETV1* in prostate cancer. In further analysis of the joint expression profiles of *ERG* and *ETV1* across several prostate cancer data sets, they found that *ERG* and *ETV1* invariably showed mutually exclusive outlier profiles, suggesting a redundant role in prostate cancer (Tomlins et al 2005). Characterizing cases with *ERG* or *ETV1* outlier expression led to the identification of fusions of the 5' untranslated region of the prostate-specific

androgen-induced transmembrane protease serine 2 gene, *TMPRSS2*, to the respective ETS gene. The fusion of *TMPRSS2* with *ERG* or *ETV1* only occurred in cases with overexpression of the respective ETS gene, and fusions were not detectable in benign prostate tissues (Tomlins et al 2005).

After the initial report of ETS fusions in prostate cancer, subsequent studies have identified novel 5' and 3' fusion partners. Screening additional microarray data sets for ETS gene outlier expression led to the identification of fusions involving *ETV4, ETV5* and *ELK4* (Helgeson 2008, Rickman et al 2009, Tomlins 2006). In addition to *TMPRSS2*, studies have characterized at least 12 additional 5' partners involved in ETS fusions (Attard et al 2008b, Han et al 2008, Hermans 2008a, Hermans 2008b, Tomlins 2007). These novel 5' fusion partners appear to contribute mostly to *ETV1*, *ETV4*, *ETV5* and *ELK4* gene fusions. While additional ETS genes involved in rearrangements or fusions are likely to be rare, it is probable that either a few prominent or many rare 5' partners remain to be discovered.

### 1.3.3 *TMPRSS2-ERG* gene fusions

The most common ETS gene fusion in prostate cancer involves the transcription factor *ERG*, and the androgen regulated gene *TMPRSS2*. *TMPRSS2–ERG* is present in 40-80% of prostate tumours, and is now defined as the most common genetic alteration in human prostate cancer (Clark et al 2007, Hermans et al 2006, Kumar-Sinha et al 2008, Tomlins et al 2009). Both genes are located ~3Mbp apart on chromosome 21, and the fusion can occur either through interchromosomal insertion or through deletion of the

intervening region on chromosome 21 (Clark and Cooper 2009, Tomlins et al 2009). In either type of fusion, the androgen responsive promoter of the abundantly expressed *TMPRSS2* becomes fused to the *ERG* coding sequences, leading to overexpression of full-length or N-truncated ERG protein (Fig. 1.1) (Perner et al 2006).

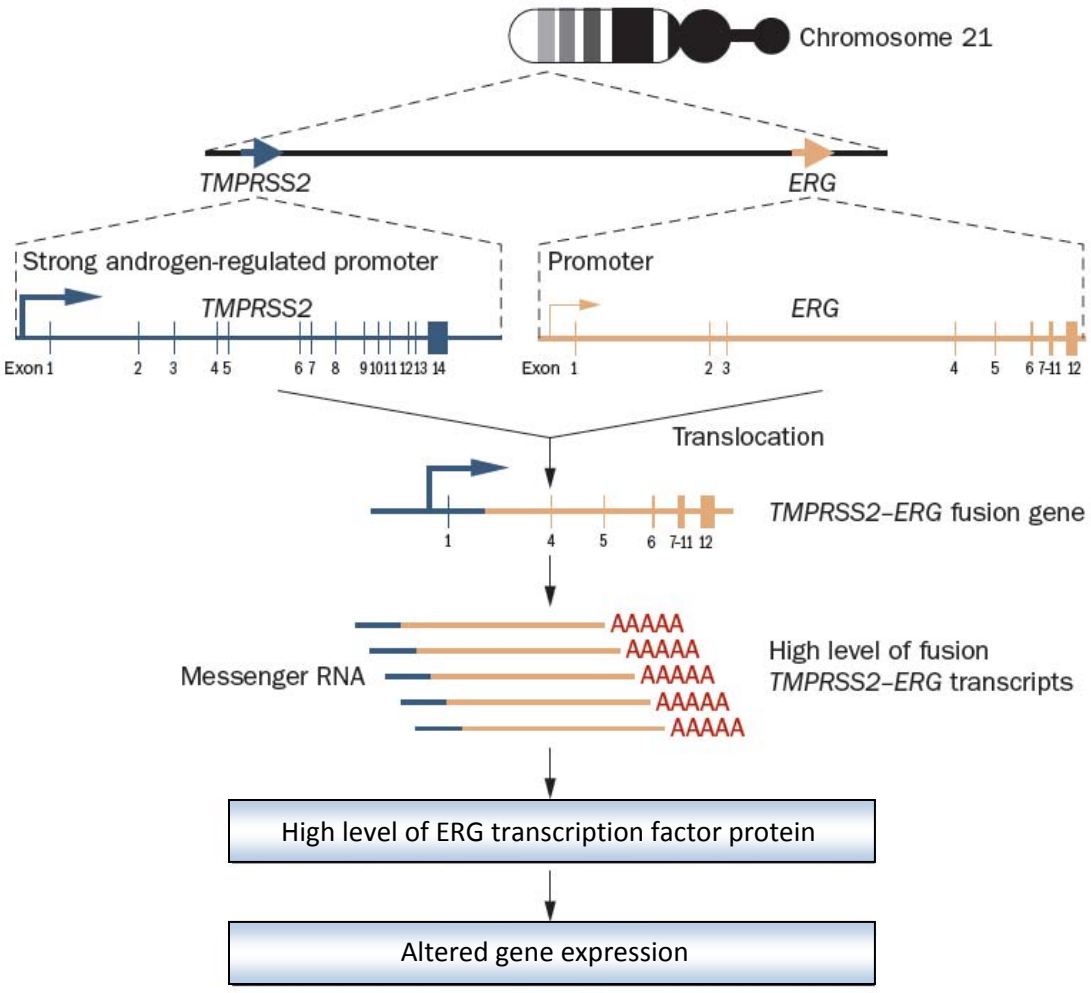



**Figure 1.1 Schematic diagram of formation and consequences of *TMPRSS2-ERG* gene fusion (adapted with permission from Clark and Cooper 2009).**

Much diversity is observed in the transcripts derived from this gene fusion (Fig. 1.3). Multiple *TMPRSS2–ERG* splice variant transcripts have been found both within individual cancers and in comparisons of different cancer samples. Many variants are predicted to be noncoding and are probably nonfunctional, but two variants encode full length ERG protein, eight encode N-terminal truncated (NTT) *ERG* sequences, and one encodes a TMPRSS2–ERG fusion protein. The most commonly reported TMPRSS2–ERG transcripts are T1/E4 (*TMPRSS2* exon 1 fused to *ERG* exon 4) and T1/E5, which encode the truncated proteins III and IV, respectively (Clark et al 2007). These proteins are equivalent to those encoded by two normal ERG splice forms. Proteins III and IV retain both the PNT protein interaction domain and the ETS-binding domain, which are essential for normal transcription factor function (Fig. 1.2).

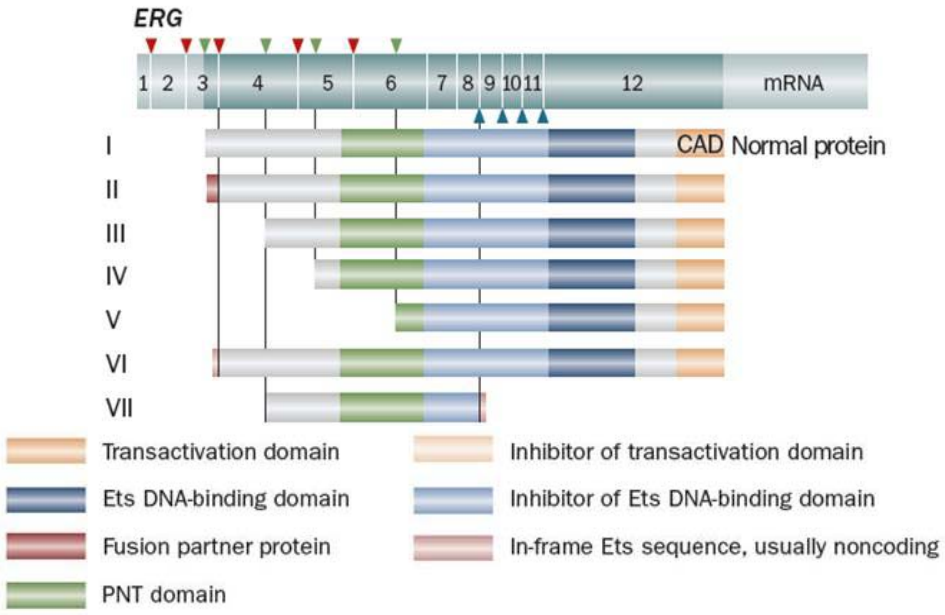



**Figure 1.2 Schematic diagram of transcripts and encoded proteins for the wild-type ERG and fusion transcripts detected in prostate cancer (adapted with permission from Clark and Cooper 2009).**

### 1.3.4 ERG biology and pathology

Developmentally, ERG expression is tightly restricted to specific mesodermal lineages, particularly during the early phases of differentiation of cells in the hematopoietic, chondrocytic and endothelial lineages. In addition, ERG is expressed in mesenchyma of the developing genital ridge and subsequently during renal and urogenital tract differentiation. Later in development, its expression is highest in the capillaries (Maroulakou and Bowe 2000). ERG has been implicated as an important regulator of gene expression involved in chondrogenesis and angiogenesis (Iwamoto 2000, McLaughlin et al 2001), and as a critical modulator of endothelial cell survival and differentiation (Birdsey et al 2008). In addition, *ERG* is believed to have a role in self-renewal-associated proliferation (Loughran 2008).

Prior to the identification of ERG overexpression in prostate cancer, aberrant expression of this gene had been described in acute myeloid leukemia, Ewing's sarcoma and primitive neuroectodermal tumours (Hsu et al 2004, Oikawa and Yamada 2003). Translocations involving ERG and EWS are causative lesions in Ewing's sarcomas and some primitive neuroectodermal tumors (Giovannini et al 1994). Several translocation variants for the EWS-ERG have been found, all of which involve fusion of EWS with carboxy-terminal ERG sequences that include the signature ETS-binding domain. The resulting chimeric proteins are described as affecting the intrinsic RNA splicing function of EWS (Yang et al 2000), and as altering gene expression profiles via ERG-mediated transcriptional activity (Prasad et al 1994). Likewise, ERG translocations with TLS/FUS are associated with transformation in certain acute myeloid leukemias

(Ichikawa et al 1994, Kong et al 1997). In this context, the chimeric fusion proteins include the amino-terminal SAM/PNT domain and the ETS-binding domain, and are believed to mediate oncogenic transformation by ERG transcriptional activation.

In regards to prostate cancer, recognition that approximately 20% of high-grade PIN harbor *TMPRSS2-ERG* fusions suggests that these translocations occur early in disease development (Cerveira et al 2006, Perner et al 2006). Although, attempts by several independent groups to investigate the role of this gene fusion in prostate cancer initiation have produced conflicting results (reviewed in (Clark and Cooper 2009, Tomlins et al 2009). Two *TMPRSS2-ERG* transgenic mouse studies have demonstrated that the expression of the fusion transcript causes increased prostatic dysplasia and perhaps prostatic intraepithelial neoplasia (PIN), but not cancer (Klezovitch 2008, Tomlins 2008). These results suggested that ETS fusions might influence malignant progression in the context of other genetic lesions. Indeed, subsequent studies have shown that combined expression of ERG with additional genetic alterations associated with prostate cancer can lead to disease progression. These studies have demonstrated that ERG overexpression collaborates with alterations in PI3K signaling, such as PTEN knockdown or Akt upregulation (Carver et al 2009, Zong et al 2009), and AR hyperactivation (Zong et al 2009). Importantly, studies show that in human prostate cancer, PTEN loss and ERG genetic rearrangements are concomitant events and predict poorer prognosis (Carver et al 2008, King 2009, Yoshimoto et al 2008).

Several *in vitro* and clinical biopsy-based studies support a role for *TMPRSS2-ERG* fusions in mediating the transition to an invasive phenotype (reviewed in Tomlins

*et al.*, 2009). It has been shown that overexpression of truncated *ERG* or different *TMPRSS2-ERG* isoforms in primary or immortalized benign prostatic epithelial cells increases cell migration and invasion (reviewed in Tomlins *et al.*, 2009). Similarly, knockdown of *ERG* or *TMPRSS2-ERG* in the VCaP cell line, which harbors the *TMPRSS2-ERG* gene fusion, decreases invasion (reviewed in Tomlins *et al.*, 2009). Furthermore, the presence of ERG-associated gene fusions in prostate cancer specimens has been reported to be associated with more aggressive disease (Attard et al 2008a, Demichelis 2007, Mehra 2008, Nam et al 2007, Perner 2006, Rajput et al 2007, Wang et al 2006, Yoshimoto et al 2008). Although it is clear that ERG possess oncogenic properties, the precise molecular events and signaling mechanisms contributing to ERG-mediated prostate cancer progression still remain to be understood.

## 1.4 Epithelial-to-mesenchymal transition

Metastasis remains one of the most life-threatening pathological events, responsible for 90% of cancer death in humans (Sakamoto and Kyprianou 2010). The metastatic progression is a complex, multistep process that entails local invasion, followed by dissemination of the malignant cells, and finally re-establishment at distant sites resulting in metastasis formation.

Epithelial tissues represent the origins of most solid tumors, including prostate cancer. The organization of the epithelial cell layers in normal tissues is incompatible with the motility and the invasiveness displayed by malignant carcinoma cells. In order

to acquire motility and invasiveness, carcinoma cells must shed many of their epithelial phenotypes, detach from epithelial sheets, and undergo a drastic alteration – the epithelial-to-mesenchymal transition (EMT) (Savagner 2001).

EMT is a cellular mechanism long recognized as a central feature of embryonic development (Kalluri and Weinberg 2009). Several developmental milestones, including gastrulation, neural crest formation and heart morphogenesis, rely on the plastic transition between epithelium and mesenchyme. In addition, EMT is also involved in tissue regeneration and organ fibrosis. Accumulating evidence suggests a critical role for EMT in cancer progression. An essential difference between the embryonic and tumourigenic processes is that the tumourigenic processes involve genetically abnormal cells that progressively lose their responsiveness to normal growth-regulatory signals and possess the ability to evolve. Such evolution derives from the genetic and epigenetic instability that is inherent in most neoplastic cell types (Polyak and Weinberg 2009).

### 1.4.1 Induction and mechanisms of EMT

EMT is a highly dynamic, multistep process requiring the accomplishment of some key events. These occur in a coordinated way and include loss of intercellular cohesion, disruption of the tumour basement membrane and underlying extracellular matrix (ECM), activation and rearrangement of the cytoskeleton resulting in increased motility, and finally migration into the interstitium of the stromal compartment (Kalluri and

Weinberg 2009). The following subsections will describe the most common molecular and structural features associated with EMT.

**Intercellular interactions and cell dissociation**

Normal epithelia are multicellular structures comprised of polarized, closely associated cells that reside on the top of a basement membrane and are largely immobile. Instead, mesenchymal tissue consists of non-polarized loosely associated cells living more individually within the extracellular matrix (ECM) and endowed with high ability for movement. While the main type of adhesion system in epithelia is E-cadherin mediated cell-cell interaction, which stabilizes the whole multicellular architecture, the adhesion system in mesenchymal cells comprises dynamic integrin-mediated cell matrix interactions that allow the motility of single elements (Kalluri and Weinberg 2009).

The key event occurring in both normal and pathological EMTs is the downregulation of E-cadherin (Conacci-Sorrell et al 2002). This transmembrane glycoprotein provides a physical link among adjacent cells and is crucial for the establishment and maintenance of polarity and integrity of epithelia. Its extracellular domain interacts with the E-cadherin molecule on adjacent cells, and its intracellular domain is associated with a multiprotein complex comprising α-, β-, γ- and p120-catenin. β-catenin binds tightly to the cytoplasmic domain of E-cadherin and through α-catenin to the actin microfilament network of the cytoskeleton, thereby connecting cell-cell adhesion with the intracellular machinery involved in cell shape and migration regulation (Savagner 2001). The binding of E-cadherin with catenins and actin

cytoskeleton is essential for the formation of strong cell-cell adhesion, and any event that perturbs this complex leads to reorganization of the actin cytoskeleton and destabilization of cell-cell contacts. By linking together the cells, E-cadherin based junctional complexes keep epithelial cells in a stationary, non-motile state and, hence, disruption of cell-cell adhesion is of major importance in tumour invasion, a process in which motility is critical. Indeed, E-cadherin acts as a tumor suppressor against invasion and metastasis, and its function is abolished during the malignant progression of most carcinomas by a variety of mechanisms, which I will discuss later in this chapter (Hirohashi 1998, Mareel and Leroy 2003).

During EMT, expression of a typical fibroblastic marker – N-cadherin – is often acquired in place of E-cadherin (Maeda et al 2005). Nevertheless, in contrast to E-cadherin down-regulation, up-regulation of N-cadherin is not always associated with EMT, suggesting that its role could be associated to a subset of tumours. Moreover, proteins localized in tight junctions like claudins, connexins, occludins and zonula occludens have also been found to be involved in EMT (Kalluri and Weinberg 2009).

**Cell-matrix interactions, cell motility and invasion**

Disruption of cell-cell contacts is necessary but not sufficient to activate cell motility. To reach a particular location, cells must also modify their relationship with the ECM, migrate through it and proceed to its proteolysis. Attachment to the ECM is mainly performed via transmembrane receptors of the integrin family which allow the communication between the ECM and the internal actin cytoskeleton, as well as cell

contraction and movement (Ridley et al 2003). Integrin expression and role in migration is cell-type- and differentiation-stage-specific, as well as dependent on the ECM constitution (Hynes 2002). Several integrins have been implicated in EMT. Binding to ECM leads to integrin molecule clustering on the membrane, which enhances the interaction of their cytoplasmic tail with cellular factors. This results in the formation of large multi-protein plataforms that link the ECM to the actin cytoskeleton and appear as a point of attachment of the cell to the external surface, called focal adhesions (Hynes 2002). A protein that plays a key role in focal adhesions and that has been largely implicated in EMT is integrin-linked kinase (ILK). ILK is an intracellular adaptor and kinase that transduces signals derived from integrin-matrix interactions and growth-factor-receptor stimulation. The specific roles of ILK in EMT will be discussed later in this chapter.

The first step to migration includes a front-rear polarization affecting surface receptors, vesicule trafficking, Golgi apparatus localization and microtubules organization, controlled in part by the Rho family small guanosine triphosphate (GTP)-binding proteins (Rho-GTPases). These proteins are also implicated in the actin polymerization leading to the formation of actin-based cell protusions in the leading edge of the cell. The two major structures are called lamellipodia (large structure; actin-filament meshwork) and philopodia (spike-like structure; radially oriented actin filaments). These protusions are implicated in the attachment to the ECM and are necessary to the migratory mechanism since they serve as traction sites. Behind the leading edge, filamentous actin forms contractile stress fibers responsible for the

contraction of the cell body and retraction of the trailing edge (Adams 2001, Clark et al 1998). Members of the Rho family of GTPases play a pivotal role in transmitting signals from growth factor and cell adhesion receptors to effector proteins of actin cytoskeleton remodeling. Growth factor-mediated stimulation of receptor tyrosine kinases and their downstream mediators Ras and PI3K, as well as ECM-generated signals, are all able to modulate the activity of Rho family of GTPases. RhoA, Rac1, and Cdc42 are the best studied members of this family, and their critical role in cell migration and invasion has been repeatedly shown (Narumiya et al 2009, Sahai and Marshall 2002, Vega and Ridley 2008). With regard to cell migration RhoA induces actin stress fiber formation and regulates cytoskeletal configurations affecting cell-cell or cell-matrix adhesion. Conversely, Rac1 is involved in lamellipodia and membrane ruffle formation, Cdc42 excites filopodia formation (Ridley 2006).

In addition to actin microfilaments, intermediate filaments (IFs) and microtubules are major components of the cytoskeleton and play an important role in mesenchymal migration. IFs are related to cell physiological function, show high molecular diversities and are expressed in tissue-specific programs. For instance, keratins define epithelial tissues whereas Vimentin defines mesenchymal origin. Indeed, Vimentin is often upregulated during EMT and its expression is associated with acquisition of a migratory phenotype and invasion (Omary et al 2004).

Once motility is acquired, to proceed with degradation of the ECM, the cell sets up specific mechanisms that concentrate protease activity in the pericellular environment, mainly by membrane-anchored proteases called the transmembrane

matrix metalloproteinases (MMPs) and the endogenous proteolytic urokinase-type plasminogen activator (uPA) system. The most studied mechanism in ECM degradation during invasion is the activation of MMPs that can be directly linked to the plasma membrane by specific interactions with integrins or other cell surface receptors (Polette et al 2004). Several MMPs have been found to be upregulated in EMT (Przybylo and Radisky 2007).

**Resistance to anoikis**

Anoikis, a Greek word meaning 'homelessness', is apoptosis induced by loss of cell adhesion or inappropriate cell adhesion (Frisch and Francis 1994). Cancer cells, rather than normal cells, are usually not sensitive to anoikis, and many have developed anchorage independence, meaning that they do not require adhesion to ECM to proliferate and survive (Bissell and Radisky 2001). Indeed, identification of anchorage-independent growth with a 'soft agar colony formation assay' which measures proliferation in a semisolid culture medium, is a characteristic of malignancy and/or EMT *in vitro* (Gupta and Massagué 2006, Roberts et al 2006). Importantly, resistance to anoikis has been shown to promote metastasis, since tumour cells can enter and disseminate into the bloodstream. In agreement with a higher metastatic potential, cells that have undergone EMT display resistance to anoikis (Onder 2008).

### 1.4.2 Extracellular signals and pathways regulating EMT

Since EMT is a highly complex process requiring extensive changes in adhesion, cell shape and gene expression, its regulation is expected to be similarly complex. Indeed, various mechanisms leading to specific gene repression and activation, transduction signaling pathways and a multitude of mediator molecules seem to cooperate in controlling EMT. The molecular analysis of EMT-associated events has revealed extensive cross-talking among individual pathways leading to very complex biochemical circuits.

The tumour microenvironment composed of ECM, cells and soluble factors, plays an important role in EMT induction and further in metastasis. Indeed, interaction of tumour cells with their local microenvironment can induce the autocrine and/or the paracrine secretion of growth factors, cytokines and ECM proteins further leading to EMT (Moustakas and Heldin 2007, Thiery and Sleeman 2006). During the past years, a great number of growth factors and signaling pathways have been associated with EMT induction, such as the epidermal growth factor (EGF) via the Janus-activated kinase (JAK) pathway (Lo et al 2007), the fibroblast growth factor (FGF) via the ERK/MAPK (Boyer and Thiery 1993) and the hepatocyte growth factor (HGF) (Grotegut et al 2006). The most extensively studied effect is that of the transforming growth factor β1 (TGF-β1) acting via the Smad proteins or the ERK and PI3K signaling pathways (Moustakas and Heldin 2007, Rahimi and Leof 2007). In addition, WNT, Hedgehog, Notch, and nuclear factor-κB (NF-κB) signaling pathways have been found to be important for EMT induction (Huber et al 2005, Moustakas and Heldin 2007).

EMT can be programmed by pleiotropically acting transcription factors that are normally involved in various steps of early embryogenesis. Signals released by the stromal microenvironment of a cancer cell, operating together with genetic and epigenetic alterations of the cancer cell genome, are often responsible for inducing expression of these transcription factors. As detailed earlier, E-cadherin is a very important molecule in cancer progression and EMT induction. In a variety of human cancers, E-cadherin loss is linked to poor prognosis, tumour progression and metastasis, underlying that its regulation is a key step in tumour spreading. Among the high number of factors and mechanisms specifically implicated in E-cadherin regulation, repressors of gene expression have strongly been associated with EMT and tumour progression (Peinado et al 2007). The first repressors to be identified were the zinc finger proteins Snail (Batlle et al 2000, Cano et al 2000) and Slug (Bolos et al 2003) and the Smad-interacting proteins ZEB1 and ZEB2 (Comijn et al 2001), all capable of binding the E-boxes on E-cadherin promoter. Other repressors include the basic helix–loop–helix transcription factors E12/E47 (TCF3) (Pérez-Moreno et al 2001) and Twist (Yang et al 2004). These proteins actively repress transcription by recruiting co-repressors (Postigo and Dean 1999, Postigo et al 2003) and known repressor complexes (Herranz et al 2008, Peinado et al 2004) but also by influencing the activity of other E-cadherin repressors (Guaita et al 2002). In parallel, hypermethylation of E-cadherin promoter leading to silencing has emerged as another important mechanism for the downregulation of this protein during EMT and in many carcinomas (Strathdee 2002).

In addition, the integrity of the cell adhesion system mediated by E-cadherin can be regulated by post-translational mechanisms like tyrosine phosphorylation of catenins (Lilien and Balsamo 2005). In response to particular signaling pathways catenins are phosphorylated and subsequently released from the complex containing E-cadherin (Nelson and Nusse 2004). When β-catenin is released in the cytosol, it is phosphorylated in a complex containing the APC protein, axin and GSK3β, responsible for targeting β-catenin to degradation through the ubiquitin–proteasome system (Peifer and Polakis 2000). During transduction of WNT-related signals, GSK3β phosphorylation inhibits this process (Papkoff et al 1996) and allows β-catenin to accumulate in the cytoplasm and translocate to the nucleus, where it functions as a cofactor for members of the LEF/TCF family of transcription factors which further activate the transcription of important genes (Behrens et al 1996, Huber et al 1996b). Altered expression of these genes, like c-MYC has been implicated in carcinogenesis and EMT (He et al 1998). In human cancer, mutations in proteins implicated in the WNT signaling and/or the β-catenin degradation are mainly responsible for the accumulation of β-catenin to the cytoplasm and further translocation to the nucleus.

### 1.4.2 Mesenchymal-to-epithelial transition (MET)

Studies suggest that primary tumours displaying a gene expression signature characteristic of EMT are more likely to be associated with eventual distant metastasis and shorter periods of distant metastasis-free survival (Bloushtain-Qimron 2008, Liu et al 2007, Sheridan et al 2006, Shipitsin et al 2007). An apparent paradox comes from

repeated observations that the EMT-derived migratory cancer cells typically establish secondary colonies at distant sites that resemble, at the histopathological level, the primary tumour from which they arose, accordingly, they no longer exhibit the mesenchymal phenotypes ascribed to metastasizing carcinoma cells. Reconciling this behaviour with the proposed role of EMT as a facilitator of metastatic dissemination requires the additional notion that metastasizing cancer cells must shed their mesenchymal phenotype via a mesenchymal-to-epithelial transition (MET) during the course of secondary tumor formation. METs – the reversal of EMTs – are hypothesized to occur following dissemination and the subsequent formation of distant metastasis (Polyak and Weinberg 2009).

Cancer cells may undergo METs owing to influences originating in their microenvironment. For example, this was demonstrated by upregulation of E-cadherin expression and acquisition of differentiated epithelial cell features when prostate cancer cells were co-cultured with normal hepatocytes. This return to an epithelial state involved, among other things, the formation of cell-cell interactions between normal hepatocytes and the cancer cells, ostensibly mediated by homotypical E-cadherin bridges formed between them (Yates et al 2007). In support of the MET hypothesis, dynamic expression of E-cadherin in cancer progression has been documented (Tsuji et al 2009).

## 1.5 Integrin linked-kinase

The ECM is a complex structural entity surrounding and supporting cells that are found within tissues and organs. The ECM binds to substrate-adhesion molecules on the surface of cells and influences various intracellular signaling pathways that regulate survival, proliferation, polarity and differentiation. An important family of adhesion molecules that bind to the ECM are the integrins. Integrins are heterodimeric transmembrane molecules that consist of α and β subunits, and they are composed of large extracellular domains and relatively small cytoplasmic domains (Legate et al 2006).

In the search for intracellular molecules that bind the cytoplasmic regions of integrins, in 1996 Integrin-linked kinase (ILK) was discovered in the Dedhar laboratory, through a yeast-two hybrid screen as an β1-integrin subunit cytoplasmic domain interactor (Hannigan et al 1996). To date, genetic studies in cultured mammalian cells, *Caenorhadbditis elegans, Drosophila melanogaster, Xenopus,* and murine models have demonstrated that ILK plays a central role in transducing many of the biochemical signals that are initiated by cell-ECM interactions (Hannigan et al 2005). ILK is implicated in the regulation of several fundamental processes, such as growth, proliferation, survival, differentiation, migration, invasion and angiogenesis (reviewed in Hannigan et al 2005). Evidence now points to ILK as having critical, multifunctional roles in the normal development and function of several tissues. Dysregulation of ILK has been implicated in several diseases, including tumourigenesis.

### 1.5.1 ILK structure and function

The gene encoding ILK is mapped to the distal tip of human chromosome 11, at band 11p15.4 – 15.5 and is highly evolutionary conserved with homologues found in *C. elegans* and *Drosophila* (Hannigan et al 1997). ILK consists of 452 amino acid residues and has three conserved functional domains. The first domain consists of four amino-terminal ankyrin repeats (Fig. 1.3). These mediate protein interaction with the LIM domain-containing adaptor protein PINCH (particularly interesting new cysteine-histidine protein) and with ILKAP (ILK-associated protein) (reviewed in Legate et al 2006), which are critical for the ILK localization to focal adhesion sites and regulation of its function. The central domain is a pleckstrin homology-like motif domain, which can bind phosphoinositol-3,4,5-trisphosphate (PIP3), a secondary messenger and PI3 kinase product. The largest domain is a kinase catalytic domain located at the C-terminus. This domain mediates binding to the cytoplasmic tail of of $\beta$1 and $\beta$3 integrins, and has been shown to have serine-threonine kinase activity. Nevertheless, the ability of ILK to function as a protein kinase has been questioned because of its atypical kinase domain. The kinase domain of ILK is atypical in that it lacks the highly conserved Asp-Phe-Gly (DFG) and His-Arg-Asp (HRD) motifs, but contains the invariant Lys residue involved in ATP binding, as well as the invariant Ala-Pro-Glu (APE) motif (reviewed in Hannigan et al. 2005). This has resulted in the classification of ILK as a pseudokinase (Boudeau et al 2006, Wickstrom et al 2010). However, in a recent study, Maydan et al have shown that ILK is indeed an authentic protein kinase capable of phosphorylating protein and peptide substrates with comparable kinetics to those of other protein kinases. This study

revealed that ILK is a $Mn^{2+}$-dependent kinase. Analysis of the kinase activity of wildtype ILK and of the K220A mutation (which is unable to co-ordinate ATP) has determined that ILK is significantly more active in the presence of $Mn^{2+}$ (Maydan et al 2010). To date, ILK has repeatedly been shown to phosphorylate integrin β1 cytoplasmic domain (Hannigan et al 1996), Akt serine 473, GSK3β on serine 9/21 (Delcommenne 1998, Maydan et al 2010, Persad 2000, Persad et al 2001a, Troussard et al 2006), 20-kDa regulatory light chains of myosin (LC20) (Maydan et al 2010), the ILK-binding protein, β-parvin (Deng et al 2001, Deng et al 2002), the myosin targeting subunit of myosin light chain phosphatase, MYPT1, protein phosphatase inhibitors, PHI-1, KEPI and CPI-17 and α-NAC (reviewed in Hannigan et al. 2005). The C-terminus of ILK also mediates interactions with proteins associated with actin cytoskeleton rearrangement such as α-Parvin, β-Parvin and Paxillin (reviewed in Hannigan et al. 2005).

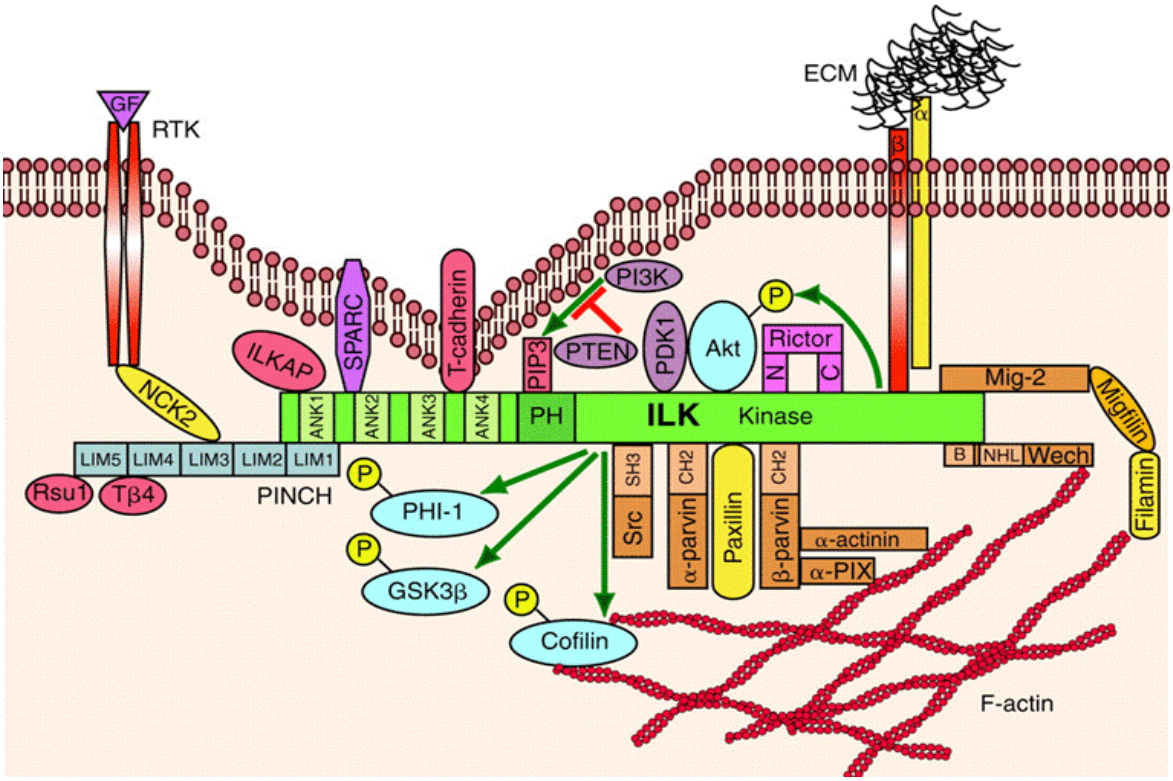



**Figure 1.3 Schematic diagram of ILK demonstrating the multiple domains and highlighting the multiprotein complex in focal adhesions (reproduced with permission from McDonald et al. 2008).**

Recently, proteomic approaches to identify ILK binding partners have revealed a novel role for ILK in mitotic spindle organization. Using SILAC (stable isotope labeling with amino acids in cell culture)-based mass spectrometry, proteins which specifically interact with ILK were identified (Dobreva et al 2008). In addition to its known focal adhesion, actin associated binding partners, several components of the microtubule cytoskeleton, particularly those localized to centrosomes, were found. These included α and β tubulin, RuvB-like protein 1 (RUVBL1) and colonic and hepatic tumor

overexpressing gene (ch-TOG) (Fig. 1.4). Several of these novel interactions were confirmed endogenously (Dobreva et al 2008) and biochemical analyses of purified mitotic spindles revealed ILK to be present in two different pools in mitotic cells (Fielding et al 2008). The first was a soluble fraction where it co-fractionated with PINCH and α-Parvin, known focal adhesion binding partners. The second fraction was the purified mitotic spindles, where ILK co-fractionated with α-tubulin and two known centrosomal proteins which were also identified as ILK binding partners, RUVBL1 and ch-TOG. Immunofluorescence analysis confirmed that ILK localized to the centrosome in interphase and dividing cells, where it co-localized with RUVBL1 and ch-TOG at the minus ends of microtubules. Co-immunoprecipitation experiments revealed that ILK was present in two separate protein complexes within the cell (Dobreva et al 2008), a PINCH/Parvin containing complex and a tubulin/RUVBL1 complex, accounting for ILK's focal adhesion and centrosome localizations respectively. The finding that ILK was present in a tubulin containing complex at centrosomes was interesting as several reports had suggested that ILK may play a role in mitosis.

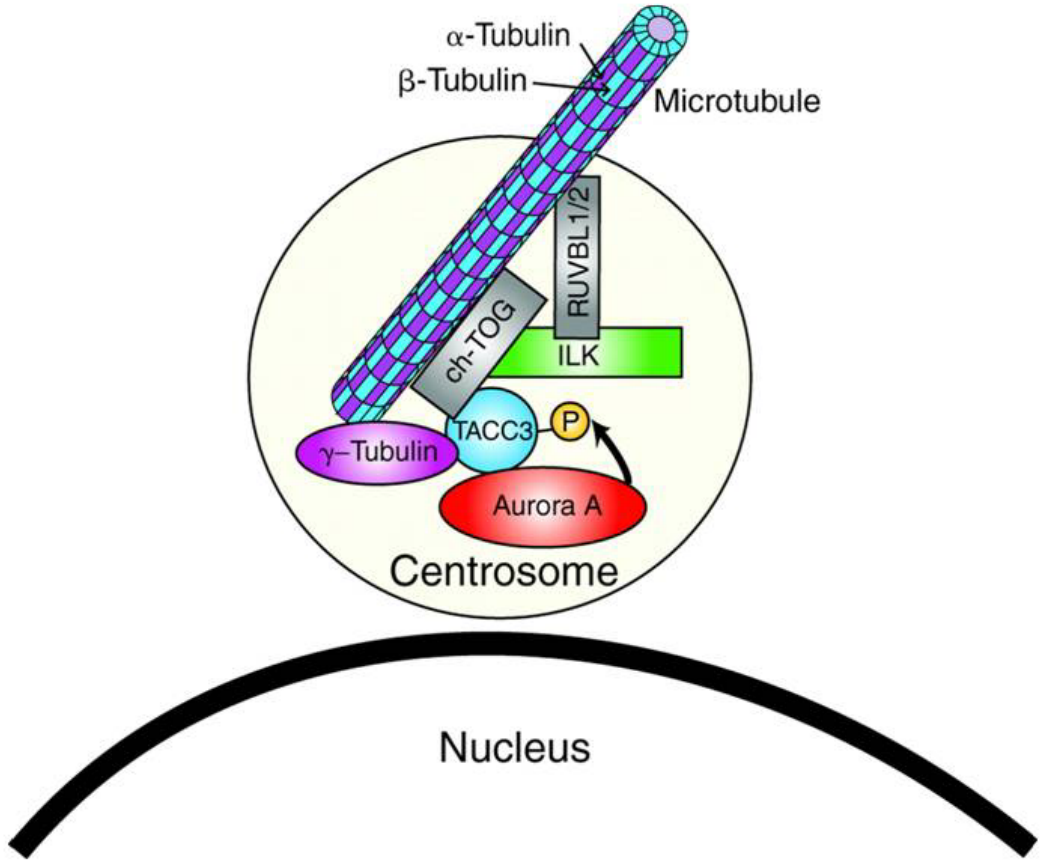



**Figure 1.4 Schematic diagram of ILK and centrosomal binding partners (reproduced with permission from McDonald et al. 2008).**

### 1.5.2 Regulation of ILK

The kinase activity of ILK is stimulated by integrin engagement and soluble mediators, including growth factors and chemokines, and is regulated in a PI3K-dependent manner (Delcommenne 1998, Imanishi et al 2007, Li et al 2003a, Rosano et al 2006, Xie et al 2004). ILK contains a consensus sequence for phosphoinositide-binding PH domains, which suggests a mechanism for the PI3K-dependent activation of ILK, through direct

interaction with lipid products of PI3K. Indeed, the direct and specific binding of ILK to phosphatidylinositol 3,4,5-trisphosphate (PIP3) was recently validated through a proteomics strategy (Pasquali et al 2007). PIP3 has been shown to specifically stimulates the activity of ILK *in vitro*, and in addition, membrane targeted constitutively active PI3K activates ILK *in vivo* (Delcommenne 1998). Conversely, the tumor suppressor PTEN, which acts as an antagonist of PI3K signaling, dephosphorylates PIP3 to phosphatidylinositol 4,5-diphosphate (PIP2), resulting in the inhibition of ILK activity. Mutational loss or inactivation of PTEN is commonly seen in many tumors, including prostate cancer. PTEN-null prostate carcinoma cells have constitutively increased levels of ILK activity (Persad 2000, Persad 2001, Persad et al 2001b). The catalytic activity of ILK is also negatively regulated by the ILK associated protein (ILKAP), a serine/threonine phosphatase. Decrease in ILKAP expression and an increase in ILK activity correlate with poor patient outcome in melanoma (Dai et al 2003). Another protein phosphatase, stomach-cancer-associated tyrosine phosphatase 1 (SAP-1), induces apoptosis by inhibiting ILK kinase activity and Akt activation. Although this phosphatase is thought to inhibit ILK activity indirectly by disrupting focal adhesions (Takada 2002). In addition, ILK expression has been shown to be upregulated by hypoxia (Abboud et al 2007, Lee et al 2006).

### 1.5.3 ILK expression in cancer

ILK is ubiquitously expressed in human tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (McDonald et al 2008). Significant defects in normal development and tissue homeostasis occur when ILK is deleted (Hannigan et al 2005, McDonald et al 2008). In contrast, constitutive activation or overexpression of ILK either in cell culture or in transgenic mouse models results in cancer development and progression (reviewed in Hannigan et al 2005).

ILK expression is elevated in a range of malignancies including: prostate cancer (Graff et al 2001, Schmitz et al 2007); melanoma (Dai et al 2003); ovarian cancer (Ahmed et al 2003); breast cancer (reviewed in Hannigan et al 2005); colon cancer (Bravou et al 2003, Marotta et al 2001); gastric cancer (Ito et al 2003); Ewing's sarcoma (Chung et al 1998); primitive neuroectodermal tumors (Chung et al 1998); anaplastic thyroid (Younes et al 2005); pancreatic cancer (Sawai et al 2006); non-small-cell lung cancer (Takanami 2005); mesothelioma (Watzka et al 2008); medulloblastoma (Chung et al 1998). The increase in ILK expression predicts poor patient survival in several types of cancers (Dai et al 2003, Graff et al 2001, Takanami 2005, Yau et al 2005).

In regards to prostate cancer, it has been reported that ILK expression increases dramatically with increasing tumor grade, indicating that ILK is upregulated with prostate tumor progression (Graff et al 2001). In this study Graff and collaborators have shown that ILK is uniformly expressed specifically in poorly differentiated prostate cancers and is directly related to key biological parameters associated with progression, such as, disproportionate increase in proliferation over apoptosis. Besides, they found that

patients whose tumors had the highest ILK expression were the least likely to survive for 5 years, suggesting that ILK overexpression has implications for prognosis (Graff et al 2001). Another study shows that ILK is upregulated in prostate adenocarcinoma cells when compared with benign epithelial cells. This study concluded that ILK can be used as an internal control marker to monitor specific prostate adenocarcinoma cell isolation following laser microdissection (Kieffer et al 2005).

Despite being upregulated in many malignancies, the mechanisms surrounding the increase in ILK expression are not well delineated, and might vary among tumors.

### 1.5.4 ILK-activated signaling pathways in cancer

ILK can influence a range of cellular functions and phenotypes, depending on cellular and tissue contexts. In normal cells ILK is likely to be transiently activated. However, in cancer cells in which ILK is overexpressed, or constitutively activated, many signaling pathways can be affected. ILK has been implicated in the regulation of several downstream targets that promote cell proliferation, survival, migration, and invasion (Hannigan et al 2005, McDonald et al 2008). The signaling pathways activated by ILK and their biological consequences are shown in Fig. 1.5, and most of them will be described in the next subsections.

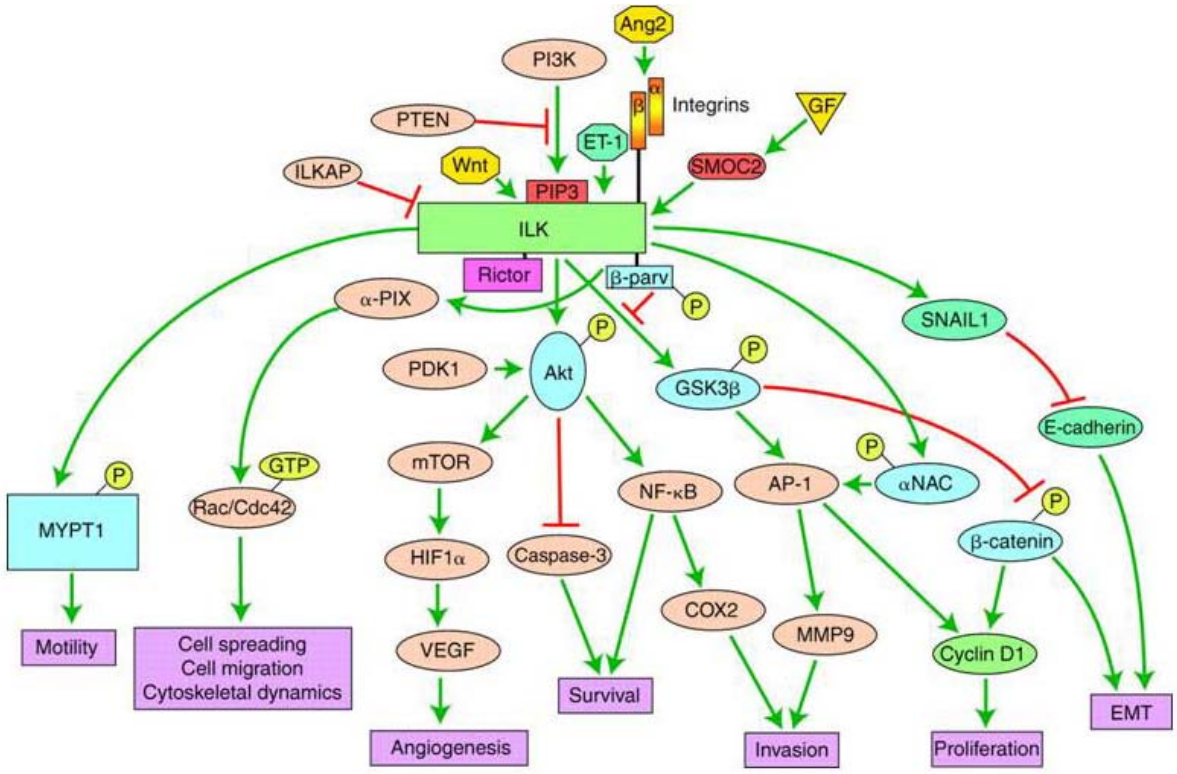



**Figure 1.5 Schematic diagram of ILK-dependent pathways and phenotypes (reproduced with permission from McDonald et al. 2008).**

### 1.5.5 Role of ILK in anchorage-independent growth and cell-cycle progression

Growth and survival of non-transformed epithelial cells require adhesion to the ECM, as cells kept in suspension arrest in G1. Loss of adhesion causes reduced expression of G1 cyclins, leading to G1 arrest and induction of apoptosis. Overexpression of ILK increases the expression of several key components of the cell cycle machinery, including cyclin A, cyclin D1, and CDK4 proteins, leading to stimulation of anchorage-independent cell-cycle progression (Radeva et al 1997).

Several lines of evidence demonstrate that the inhibition of GSK3β as a result of ILK-mediated phosphorylation leads to stimulation of cyclin D1 expression. The inhibition of GSK3β leads to activation of CREB (cyclin-AMP-responsive-element-binding protein) transcription factor or β-catenin/LEF transcription factor, resulting in the stimulation of cyclin D1 expression, and the subsequent promotion of transition from G1 to S phase (D'Amico et al 2000, Persad and Dedhar 2003, Troussard et al 1999). Furthermore, PTEN inhibition of ILK-GSK3β signaling suppresses the nuclear translocation of β-catenin and stimulation of cyclin D1 expression. In addition, the negative regulator of ILK activity, ILKAP, decreases GSK3β phosphorylation and nuclear translocation of β-catenin (Leung-Hagesteijn et al 2001). Moreover, the inhibition of GSK3β prevents the phosphorylation of c-Jun, an AP-1 transcription factor component, allowing AP-1 to bind DNA, thus stimulating the expression of cyclin D1. Alternatively, the direct phosphorylation of Jun transcriptional co-activator α-NAC by ILK also promotes the activation of AP-1 (Quelo et al 2004).

### 1.5.6 Role of ILK in cell survival and anoikis

Cell-ECM adhesion is a key factor for cellular homeostasis. Disruption of such interaction has adverse effects on cell survival, leading to a specific type of apoptosis known as anoikis (Frisch and Francis 1994). Resistance to anoikis is a crucial step during tumourigenesis and in particular during the metastatic spreading of cancer cells.

ILK is a key effector of integrin function regulating ECM-dependent cell adhesion (Grashoff et al 2004, Hannigan et al 2005), and as a result, it plays a major role in signal transduction leading to anoikis protection (Attwell et al 2000). The promotion of cell survival by ILK is primary due to the ability of ILK to promote the phosphorylation and activation of Akt on serine 473. Upon phosphorylation, Akt stimulates downstream anti-apoptotic pathways, such as activation of NF-κB and inactivation of pro-apoptotic proteins as BAD (Nicholson and Anderson 2002). Inhibition of ILK activity or expression suppresses Akt phosphorylation in many types of cancer cells (Attwell et al 2000, Makino et al 2004, Obara et al 2004, Persad et al 2000, Tan et al 2004, Troussard et al 2006). However, inhibition of ILK in normal cells, such as breast, mouse embryo fibroblasts and chondrocytes, does not affect Akt phosphorylation and activation (Grashoff et al 2003, Sakai et al 2003, Troussard et al 2006). Therefore, the role of ILK in the regulation of Akt serine 473 phosphorylation is likely to be cell-type and context dependent.

### 1.5.7 Role of ILK in EMT

EMT is of critical importance to the oncogenic and invasive properties of tumors cells derived from epithelial origins. The overexpression of ILK in epithelial cells results in a morphological transformation into a fibroblastic state accompanied by a down-regulation of E-cadherin, increased production of fibronectin (Wu et al 1998), nuclear translocation of β-catenin (Novak 1998), downregulation of the epithelial markers Cytokeratin18 and MUC1, and the upregulation of the mesenchymal markers LEF-1, Snail and Vimentin

(Barbera et al 2004, Bravou et al 2003, Guaita et al 2002, Novak et al 1998, Persad et al 2001b, Somasiri et al 2001, White et al 2001, Wu et al 1998, Xie et al 2004, Xie et al 1998). As described earlier in this chapter, these characteristics are reminiscent of EMT.

The most prominent hallmark of ILK-induced EMT is the loss of E-cadherin protein and mRNA expression, indicating that increasing ILK expression in epithelial cells, beyond a certain threshold, results in the repression of transcription of the gene encoding E-cadherin (Li et al 2003b, Novak et al 1998, Somasiri et al 2001, Wu et al 1998). The upregulation of the transcription factor Snail, a zinc finger protein that represses E-cadherin by binding the E-boxes present in the E-cadherin promoter (Thiery 2003), is thought to be the main mechanism that regulates E-cadherin expression (Batlle et al 2000). The inhibition of ILK in SW480 colon carcinoma cell line has been shown to result in the downregulation of the activity of the Snail promoter, concomitant with upregulation of the activity of the E-cadherin promoter (Tan et al 2001). Analysis of the Snail promoter identified a 65 base pair region in the 5' promoter of Snail, termed the Snail ILK responsive element (SIRE) (Tan et al 2001). A subsequent study identified Poly-ADP-ribose polymerase (PARP-1) as a bona fide SIRE-binding protein (McPhee et al 2008). PARP-1 binds the SIRE in an ILK-dependent manner. Inhibition of PARP-1 expression in PC3 cells results in a concomitant decrease in Snail levels, leading to re-expression of E-cadherin (McPhee et al 2008).

In addition to Snail, Zeb-1 is another transcriptional repressor that has been shown to be induced by ILK overexpression (Guaita et al 2002). Guaita and colleagues

have demonstrated that Zeb-1 plays a role in the maintenance of E-cadherin transcription, and in the induction of the mesenchymal phenotypes in ILK-mediated EMT (Guaita et al 2002). This study shows that ILK indirectly regulates the transcription of Zeb-1 via Snail (Guaita et al 2002).

TGF-β1 signaling has also been implicated in the regulation of E-cadherin, Snail and EMT (Cui et al 1996, Peinado et al 2003, Welch et al 1990, Wikström et al 1998). Engagement of integrins stimulates the expression and secretion of TGF-β1. The up-regulation of TGF-β1 expression and secretion is shown to be dependent upon the activity of ILK (Ortega-Velazquez et al 2003). In renal tubular epithelial cells, ILK expression is up-regulated in a dose- and time-dependent manner by TGF-β1 signaling in a Smad-dependent manner (Li et al 2003a). EMT has been shown to be mediated by ILK signaling in these cells, since the expression of a dominant negative ILK construct blocks this TGF-β1-initiated EMT (Li et al 2003a). In addition, ectopic expression of wild-type ILK in these cells results in a complete EMT, which phenotypically resembles the one initiated by TGF-β1 (Li et al 2003a).

Furthermore, ILK could also indirectly promote the loss of E-cadherin through the upregulation of LEF-1. Overexpression of LEF-1 has been shown to outcompete E-cadherin for β-catenin binding, thus disrupting cadherin/catenin complexes and resulting in the internalization of E-cadherin by its degradation (Kim et al 2002). Therefore, in addition to its direct effect on repressing the transcription of E-cadherin, ILK could increase the degradation of E-cadherin, which normally has a half life of 5-7 hours, via the upregulation of LEF-1 (Shore and Nelson 1991, Stewart et al 2000).

Besides the loss of E-cadherin, another significant way of regulating epithelial and mesodermal cell fate is through WNT signaling. The downstream effect of the WNT pathway, which is the activation of the β-catenin/LEF(TCF) transcription factor, has been shown to up-regulate several mesenchymal genes (Huber et al 1996a). Overexpression of ILK in two independent epithelial systems, IEC-18 intestinal epithelial cells and scp2 mouse mammary epithelial cells, has been shown to result in the translocation of β-catenin to the nucleus in the absence of a significant alteration in its expression level (Novak et al 1998). In addition, up-regulation of LEF-1 expression has been observed in these ILK overexpressing epithelial cells, resulting in β-catenin/LEF-1 complex formation, and activation of its transcriptional activity. ILK-induced activation of β-catenin/LEF complex formation has been shown to influence the expression of LEF-1/β-catenin-responsive genes such as cyclin D1 (Tan et al 2001). As mentioned earlier in this chapter, ILK has been shown to directly phosphorylate GSK3β (on serine 9 and 21). Stable or transient overexpression of ILK inhibits GSK3β activity, whereas the overexpression of the kinase-deficient form of ILK enhances GSK3β activity (Dedhar 2000, Delcommenne et al 1998, Tan et al 2001, Troussard et al 1999, Troussard et al 2000). The inhibition of GSK3β by ILK ultimately results in the stabilization of β-catenin, and an increase in β-catenin/LEF-1 transcriptional activity (Dedhar 2000, Delcommenne et al 1998, Tan et al 2001). Moreover, in PTEN-null prostate cancer cells where ILK is constitutively active (Persad et al 2000, Persad et al 2001b), high levels of both nuclear β-catenin and cyclin D1 have also been observed. Subsequent transfection of wild-type PTEN, however, has been shown to stimulate phosphorylation and degradation of β-

catenin in these cells through ILK-mediated activation of GSK3β (Persad et al 2001b). ILKAP has been shown to selectively inhibit activation of the LEF-1/TCF transcription factors as the result of inhibition of GSK3β phosphorylation on Ser 9 (Leung-Hagesteijn et al 2001). In another study, investigating the role of ILK in Wnt3a-induced stabilization and activation of β-catenin-mediated transcription, Oloumi and colleagues have shown that inhibition of ILK results in a significant reduction in Wnt3a-stimulated LEF/TCF transcriptional activity (Oloumi et al 2006). This study demonstrated that there is a dramatic reduction in the Wnt3a-induced nuclear accumulation of β-catenin, in both HEK293 and L cells, upon the inhibition of ILK activity. ILK inhibition also appears to reverse the Wnt3a-induced suppression of β-catenin phosphorylation. In addition, it was demonstrated that ILK complexes with APC and GSK3β, suggesting a direct role in regulation of β-catenin turnover. These results revealed an important role for ILK in the regulation of both acute, as well as the prolonged effects of Wnt3a signaling in mammalian cells (Oloumi et al 2006). In addition, recently Oloumi et al have shown cooperation between *Wnt1* and *ILK* transgenes during mammary carcinogenesis (Oloumi et al 2010). A significant acceleration in mammary tumor incidence and growth was observed in the MMTV-Wnt/ILK mice. Besides elevated expression of Wnt/ILK targets, such as β-catenin and cyclin D1, gene-expression profiling identified the activation of the FOXA1 transcription factor. Upregulation of FOXA1, was consistent with the expansion of the enriched luminal progenitor population or $CD29^{lo}CD24^{hi}CD61^{+}$ cells in MMTV-Wnt/ILK tumors (Oloumi et al 2010).

### 1.5.8 Role of ILK in migration, motility and invasion

ILK-mediated EMT is accompanied by enhanced cell migration and invasion (Somasiri et al 2001). ILK has been shown to play a critical role in the early events of cell attachment and spreading, resulting from a dramatic reorganization of the actin cytoskeleton (Filipenko et al 2005). Filipenko and colleagues demonstrated that ILK is a key player in cytoskeleton reorganization by activation of the small GTPases, Rac and Cdc42 via the guanine nucleotide exchange activity of α-PIX (Pak-interactive exchange factor α). The absence of ILK activity and association with α-PIX results in a delayed or decreased ability of the cell to adhere to the ECM and spread (Li et al 2003d, Turner et al 1999). Furthermore, ILK can promote invasion by upregulating and activating the matrix metalloproteinase MMP9, through activation of AP1 transcription factor (Troussard et al 2000). Inhibition of ILK in highly invasive human glioblastoma cells and in ILK-overexpressing mammary epithelial cells resulted in substantial decrease in invasion into Matrigel (Koul et al 2005, Troussard et al 2000).

## 1.6 Previous findings

As mentioned earlier in this chapter, ERG is now implicated in the induction of transformation in prostatic epithelial cells (PrECs) (Carver et al 2009, Hu 2008, Klezovitch et al 2008), although, the underlying mechanisms remain to be fully elucidated. Since ERG translocations lacking *TMPRSS2* exon fusions are associated with a number of malignancies and that transcripts encoding full length ERG are present

in clinical samples of prostate cancer (Hu et al 2008), Dr. Michael Cox laboratory (Vancouver Prostate Centre) hypothesized that the *TMPRSS2* coding sequence found fused to *ERG* were unlikely required for the potential transforming activity of ERG in prostate epithelium. Thus, they chose to assess how aberrant expression of ERG influenced transformation of PrECs. As an approach they engineered an ERG-expression vector including an N-terminal, Flag epitope tagged ERG3 construct (fERG), under transcriptional control of the UbC ubiquitin promoter in a Lenti viral vector system. ERG3 was chosen as it contains all pertinent ORFs described for locus. Three immortalized, but non-tumorigenic human PrEC lines were chosen to test the effect of exogenous expression of ERG on PrECs phenotype. PNT1B and BPH1 are clonal SV40-immortalized normal adult PrEC lines. RWPE1 is a clonal HPV-18-immortalized peripheral zone PrEC line derived from a histologically normal adult prostate. All three cell lines are reported to be Cytokeratin-positive and to display several attributes of apical prostatic epithelial cells. Lenti viral infection and blasticidin resistance were used to create stably Mock- and fERG-infected populations of these three PrEC lines. Following the creation of the stable cell lines, ERG mRNA and protein were readily detected in all three fERG populations. fERG exhibited a predominantly nuclear subcellular localization in PrECs, and further assessing fERG functionality (by examining ERG transcriptional activity), confirmed that fERG is biologically active in PrECs (unpublished observations from Dr. Michael Cox laboratory, Vancouver Prostate Centre).

Assessing the phenotypic consequences of ERG expression revealed that all three fERG-expressing PrEC populations exhibited morphologic transformation. The morphologic changes observed in ERG-transformed PrECs were indicative of acquisition of mesenchymal characteristics associated with aggressive prostate cancer (Thompson et al 2005). Assessing expression of genes typically associated with EMT in Mock- and fERG-PrECs, revealed that Cytokeratin 8 mRNA expression was dramatically decreased in fERG-cells relative to corresponding Mock cells. In contrast, mRNA of the mesenchymal marker Vimentin was increased in all three fERG-transformed PrECs. Immunoblotting confirmed that fERG expression resulted in significant suppression of Cytokeratin 8/18 protein levels and in upregulation of Vimentin. Importantly, in all three fERG-PrEC populations, E-cadherin protein expression was undetected or significantly suppressed. Furthermore, fERG-expressing PrECs acquired anchorage-independent growth *in vitro*, another phenotypic feature associated with EMT. These results demonstrated that ERG expression can profoundly alter the phenotype of immortalized PrECs to one consistent with that observed in aggressive prostate cancer, and that the changes observed are associated with induction of EMT.

Considering the study mentioned above and literature evidence regarding the role of ILK in EMT and its association with aggressive prostate cancer phenotypes, I hypothesized that ILK is a downstream effector of ERG signaling in prostate tumorigenesis. Therefore, the main objective of my studies was to investigate the molecular mechanism by which ERG expression promotes EMT in PrECs, and to

identify downstream effectors of ERG signaling that may play critical roles in prostate cancer progression.

# Chapter 2. Materials and Methods

## 2.1 Cell lines

Human PrEC line, BPH1 ((Hayward *et al.*, 1995), courtesy Dr. S. Hayward, Vanderbilt University), was maintained in DMEM with 10% fetal bovine serum (FBS), while RWPE1 (Bello et al 1997) ATCC) was maintained in GIBCO™ Defined Keratinocyte-Serum-Free Media plus epidermal growth factor and bovine pituitary extract. All media and supplements were from Invitrogen (Burlington, ON, Canada). VCaP cells were obtained from American Type Culture Collection (ATCC) and cultured in modified DMEM containing 1.5 g/L of $NaHCO_3$. Cells were cultured in humidified 5% $CO_2$ environment at 37 ºC. BPH1 and RWPE1 cells stably infected with a Flag epitope-tagged ERG3 (fERG) construct were generated and characterized by Dr. Michael Cox laboratory as following: A Flag epitope tag (MDYKDDDDK) was engineered onto the amino terminus of ERG3 (ERG variant 1, NM_182918) by reverse transcription-polymerase chain reaction (RT-PCR) from HUVEC cell total RNA with forward primer (f):5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGATTAT AAAGATGAT GATGATAAAGCCAGCACTATTAAGGAAGCC-3' and reverse primer (r): 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGTAGTAAGTGCCCAGATGAGAA GCATATGGCTGGTGGG-3'. The resulting attB1-/attB2-flanked Flag-ERG3 cDNA (fERG) PCR product was cloned into pDONR201 Gateway entry vector, sequence-verified and LR cloned into the Lenti viral vector destination plasmid, pDEST/FU(GW)BW (pDEST-Mock: modified from (Lois et al 2002) to contain a

Gateway and blasticidine-resistance cassette), to be under transcriptional control of the UbC ubiquitin promoter to generate pDEST/FU(GW)BW-fERG. Infectious Lenti viral particles were harvested from 0.2 µm filtered conditioned media of HEK293T cells 2 days after co-transfection with pDEST-Mock or -fERG, pR8.9 and pVSVG (Figueiredo et al 2006) and used to create stably Mock- and fERG-infected PrEC populations by infection and selection with blasticidin-containing media (BPH-1: 8 µg/ml, RWPE-1: 5 µg/ml).

**2.2 siRNA transfection**

ILK (AAG ACG CTC AGC AGA CAT GTG GA) or control non-silencing (AAT TCT CCG AAC GTG TCA CGT) siRNAs (QIAGEN, Mississauga, ON, Canada) were introduced into cells by using siLentFect reagent (Bio-Rad Laboratories, Mississauga ON, Canada), according to the manufacturer's instructions. To achieve sufficient ILK knockdown in fERG cells, they were transfected twice (at day 0 and 2) with 100 nM ILK siRNA and harvested on day 3.

**2.3 Protein analysis**

Whole cell extracts were prepared in RIPA lysis buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.5% DOC, 1% NP-40; 0.1% SDS), containing 2 mM $Na_3VO_4$; 1 mM NaF; 2 mM β-glycerolphosphate, and Complete protease inhibitor cocktail (Roche Diagnostics, Laval, QC, Canada). Immunoblot analysis was performed on cell-equivalent lysates subjected to SDS-PAGE and electrophoritic transfer to nitrocellulose membrane (Bio-

Rad Laboratories). Membranes were probed with anti-ERG-1/2/3, anti-Raf-1 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-ILK, anti-Vimentin, anti-E-cadherin, anti-αv integrin (BD Biosciences, Mississauga, ON, Canada); anti-LEF-1, anti-Snail, anti-ERK1/2, anti-$^{Thr202/Tyr204}$ERK1/2, anti-$^{Ser259}$Raf-1, anti-PARP, anti-Akt, anti-$^{Ser473}$Akt, anti-GSK-3β, anti-$^{Ser9}$GSK-3β (Cell Signalling, Danvers, MA); anti-β1 integrin (Chemicon, Temecula, CA) and anti-β-actin (Sigma-Aldrich, Oakville,ON, Canada).

**2.4 mRNA analysis**

Total cellular RNA was prepared using Trizol following manufacturer's instruction and quantified by UV spectroscopy. cDNA was generated from Mock- and fERG-PrEC total RNA by RT-PCR using First-Strand cDNA Synthesis System. Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using Roche Universal Probe Library (UPL) technology (Roche Applied Science, Laval, Quebec, Canada) on an Applied Biosystems (Foster City, CA) qRT-PCR instrument according to the manufacturer's instructions. Briefly, 1 µg of total RNA was used in a 40-µl reaction to make cDNA. Subsequently, 10 µl of qRT-PCR mixture containing 100 nM UPL probe, 200 nM of each primer and TaqMan PCR master mix (Applied Biosystems) was loaded into a 384-well plate. After a preliminary 95°C incubation the samples were read for 40 cycles (95°C: 30 sec, 60°C: 30 sec, 72°C: 60 sec). Samples were analyzed using Applied Biosystems analysis software to determine relative quantity. Results were first normalized to β-actin mRNA levels as a reference loading control and are reported as fold change over the Mock values. All qRT-PCR primers were designed using the

Roche Applied Science online assay design centre[2] and were purchased from Invitrogen.

### 2.5 Anchorage-independent growth and anoikis assay

Anchorage-independent growth was assessed by soft agar colony formation of 5,000 cells cultured for 10 days in 0.35% agar in respective culture media containing 10 μM of QLT-0267 or vehicle (DMSO). Colonies were stained with 0.005% crystal violet and counted and measured microscopically. For evaluation of anoikis, 6-well plates were coated with a solution of polyhydroxyethylmethacrylate (polyHEMA; Sigma), dissolved at 10 mg/ml in 95% ethanol and dried overnight, plates were then washed twice with 1x PBS. Resuspended cells ($5 \times 10^5$) were cultured in serum containing media, with 10 μM of QLT-0267 or vehicle, on polyHEMA coated plates for 48 hours at 37°C and 5% $CO_2$. After cultivation on polyHEMA, cell lysates were prepared and immunoblotted with an antibody against PARP.

### 2.6 Attachment assay

One hundred μl of fibronectin (Sigma; diluted to 10 μg/ml in PBS), laminin (Sigma; diluted to 10 μg/ml in PBS) or vitronectin (Sigma; diluted to 1 μg/ml in PBS) were independently added to the wells of 96-well plates. The plates were incubated for 2 hours at room temperature, and the fluid was then aspirated. Subsequently, potential nonspecific cell adhesion to residual exposed plastic surface was blocked by addition of

[2] http://www.roche-applied-science.com/sis/rtpcr/upl/ezhome.html

200 µl of 10-mg/ml BSA (Sigma) solution to each well with 1h incubation at room temperature. After aspiration of the BSA solution, the plates were washed with PBS. Cells suspensions were then added to the wells (2.5 x $10^4$ per well), and the plates were incubated for 30 min at 37°C and 5% $CO_2$. Unattached cells were removed by aspiration, and adherent cells were washed two times with PBS. Subsequently, 100 µl of media were added to each well, and the residual number of cells per well were quantified by the MTT assay, performed according to manufacturer's protocol (Roche Diagnostics).

**2.7 Three-dimensional (3-D) culture in Matrigel**

3-D on-top culture of cells in Matrigel (BD Biosciences) was carried out in 4-well chamber slides (Nalge Nunc, Rochester, NY) pre-coated evenly with 150 uL overnight-thawed Matrigel (8.4 mg/ml) and placed in a 37°C incubator for 30 min for Matrigel solidification. Single cell suspensions were plated onto Matrigel-coated chamber slides in complete growth medium with 5% Matrigel and allowed to grow for 5 days. Overlay medium containing 5% Matrigel and 10 µM of QLT-0267 or vehicle, was renewed every 2 days.

**2.8 Microscopy**

For immunofluorescence analysis of 2-D monolayer cultures, cells were fixed in −20°C methanol for 10 min. PBS containing 1% BSA was added to cells for 30 min to block nonspecific interactions. Primary antibodies were then added for 2 hours at room

temperature in goat serum/gelatin blocking buffer. Secondary antibodies were added for 1 hour at room temperature also in blocking buffer. Hoechst was added to stain DNA and coverslips were mounted in mounting medium (Vector Laboratories). For immunofluorescence analysis of 3-D cultures on Matrigel we used a protocol described by Lee and colleagues (Lee et al 2007). Cell images were acquired either with a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss Canada Ltd., Toronto,Canada), AxioCam HRc camera and AxioVision3.1 Software, or with a Zeiss Colibri fluorescence microscope, AxioCam MRm camera and AxioVision Rel. 4.7 software. Only brightness/contrast adjustments were made to images using Photoshop CS2 (Adobe).

**2.9 Luciferase reporter gene assay**

Cells were transiently transfected with either pTOPFLASH (TCF Optimal Promoter) or pFOPFLASH (Fake Optimal Promoter) containing multimerized wild-type and mutated TCF/LEF binding sites, respectively, cloned upstream of Firefly luciferase reporter gene. *Renilla reniformis* luciferase (pRenilla) reporter was used as an internal control to allow normalization of transfection efficiencies. Transfections were carried with Lipofectamine 2000 (Invitrogen, Burlington, ON) according to the manufacturer's guidelines. Luciferase activities were measured 48 hours after transfection using the Dual Luciferase Reporter Kit (Promega, Madison, WI). The experiments were done in triplicate transfections and the promoter activities were expressed as the fold activity of TOP over FOP reporters in each condition as relative light units (RLU).

ILK promoter activity was determined as described above, using a 1,006 bp ILK promoter construct (ILK #1) provided by Prof. Nelly Kieffer, Laboratoire Franco-Luxembourgeois de Recherche Biomédicale, Luxembourg (Melchior et al 2002), and two ILK promoter mutants carrying disrupted DNA consensus motifs for Ets transcription factors (1.Ets and 2.Ets) provided by Dr. Ute Reuning, Frauenklinik der Technischen Universität München (Lössner et al 2009).

**2.10 Migration and invasion assays**

Chemotactic migration of tumor cells was analyzed on transwell filters (6.5 mm diameter, 8μM pore size) from Corning Corporation. Equal numbers of the indicated cells were seeded in serum-free medium supplemented with 0.2% fatty-acid free bovine serum albumin into the insert of the culture plate. FBS was added to the lower chamber as a chemoattractant for VCaP cells, and bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF) were used as chemoattractant for RWPE-1 cells. After 24 hours, cells in the upper chamber were removed with a cotton swab and filters were washed twice in PBS. Cells present on the lower surface of the filters were fixed in methanol, stained with either crystal violet or Hoechst and photographed. In a similar manner, the invasiveness of tumor cells was evaluated using transwell filters coated with Matrigel (BD Matrigel Invasion Chamber, BD Biosciences). For ILK inhibition studies, QLT-0267 (10μM) or vehicle control was added to cells for 16 hours before trypsinization and seeding for migration and invasion assays. Each experiment was repeated three times, and results were averaged.

### 2.11 Statistical analysis

The data from each experiment was analyzed from at least three independent biological replicates. Representative immunoblots and micrographs are provided to demonstrate the primary data. Statistical significance of differences in mRNA levels, reporter activity, cell growth, adhesion and invasion were assessed by paired *t* tests using the GraphPad Prism (GraphPad Software, San Diego, CA). A $p$ value $< 0.05$ was considered statistically significant.

# Chapter 3. Results

## 3.1 ILK expression and ILK-regulated pathways involved in EMT are activated in fERG-PrECs

In order to investigate the molecular mechanism involved in ERG-induced EMT, I used two immortalized but non-tumorigenic human prostatic epithelial cell lines (PrECs) – BPH1 and RWPE1 – stably overexpressing full length ERG3 (fERG). These cell lines were previously shown to exhibit downregulation of E-cadherin by immunoblot analysis. As discussed in the introductory chapter, a hallmark of EMT is the downregulation of E-cadherin cell surface expression, which leads to loss of intercellular cohesion. Therefore, in order to verify that the decrease in E-cadherin expression previously observed in fERG-PrECs occurs at the cell membrane level, I first examined the expression of this marker by immunofluorescence staining. As expected, control (Mock) cells which retain the morphology of the parental cell lines, show strong cell-cell contacts based on E-cadherin. In striking contrast, fERG-BPH1 and fERG-RWPE1 cells, which have acquired a mesenchymal associated morphology, display low cell-cell interactions and suppression of E-cadherin at cell-cell junctions (Fig. 3.1).

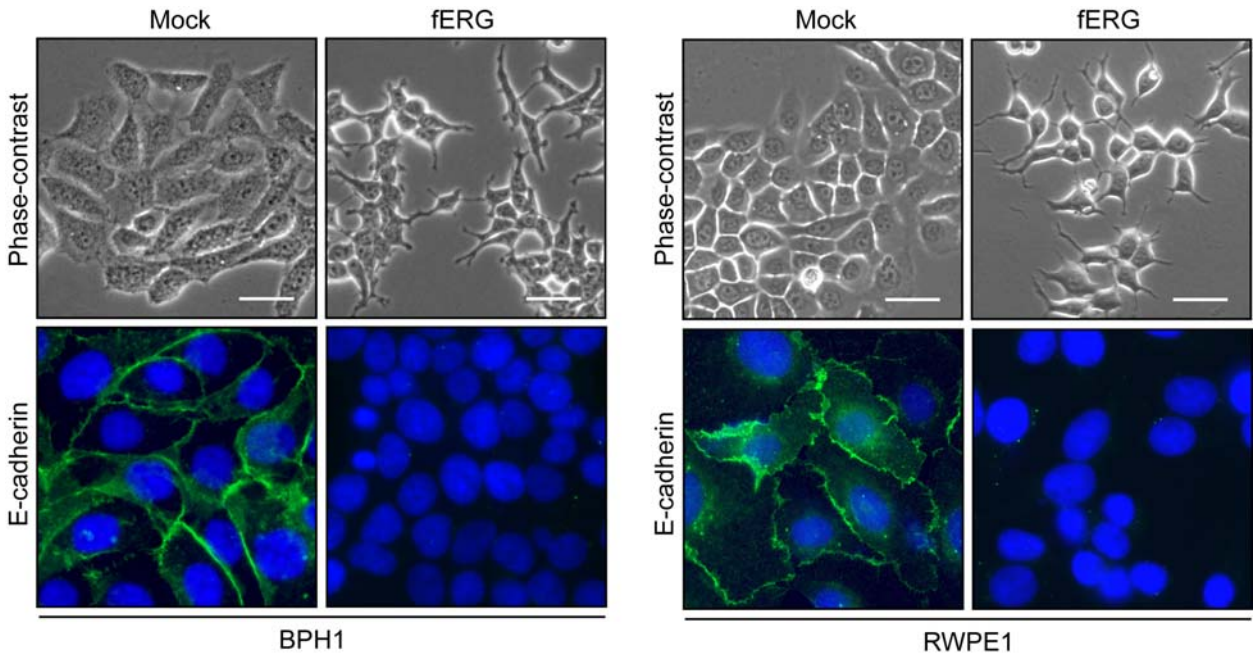



**Figure 3.1 Transformation of fERG-PrECs.** Two immortalized PrECs, BPH1 and RWPE1, were stably infected with a lentiviral vector expressing a Flag epitope-tagged ERG3 (fERG). Control (Mock) cells retain the epithelial morphology of the parental cell lines, and show strong cell-cell contacts based on E-cadherin (green). fERG-BPH1 and fERG-RWPE1 cells exhibit a more undifferentiated morphology with cytoplasmic extensions emanating from the cell surface, and reveal repression of E-cadherin at cell-cell junctions. Hoechst staining (blue) was used to visualize the nuclei. Scale bar, 50 μm.

Following these observations, I proceeded with Western blot analysis of Mock- and fERG-PrECs cell lysates. First, I confirmed the overexpression of ERG in fERG-PrECs and not in the Mock control cells (Fig. 3.2A). In addition, I confirmed the increase in Vimentin expression, and the dramatic decrease in total levels of E-cadherin induced by ERG aberrant expression (Fig. 3.2A). To gain insight into the mechanism involved in the ERG-mediated EMT in PrECs, I examined the expression of ILK, which has been

demonstrated to induce EMT in epithelial cells (Huang et al 2007, Li et al 2003c, Medici and Nawshad 2010, Rosanò et al 2005, Somasiri et al 2001). Western blotting demonstrated that ILK protein levels are markedly increased in fERG- compared to Mock-PrECs (Fig. 3.2A). Following this observation, I assessed the expression of ILK-regulated proteins involved in EMT, and show that fERG-PrECs have a significant increase in the protein expression of transcription factors LEF-1 and Snail (Fig. 3.2A). Subsequently, I assessed phosphorylation levels of primary targets for ILK, GSK-3β and Akt. ILK-mediated phosphorylation of these targets results in Akt activation and GSK-3β auto-inhibition, which can lead to upregulation of both Snail and LEF-1 at the mRNA level. Immunoblotting shows that both fERG expressing cells lines present higher phosphorylation levels in both GSK-3β and Akt, supporting enhanced conventional ILK-mediated signaling in these cells (Fig. 3.2B).

Next, I assessed whether the difference in expression of ILK, Snail, LEF-1 and E-cadherin could also be observed at the mRNA level. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of mRNA expression revealed that the regulation of all these proteins was also affected at the mRNA level (Fig. 3.2C). When compared to the cognate Mock-PrEC populations, ILK expression in the fERG-PrECs is elevated ~2-fold while Snail and LEF-1 expression are elevated at least 10-fold.

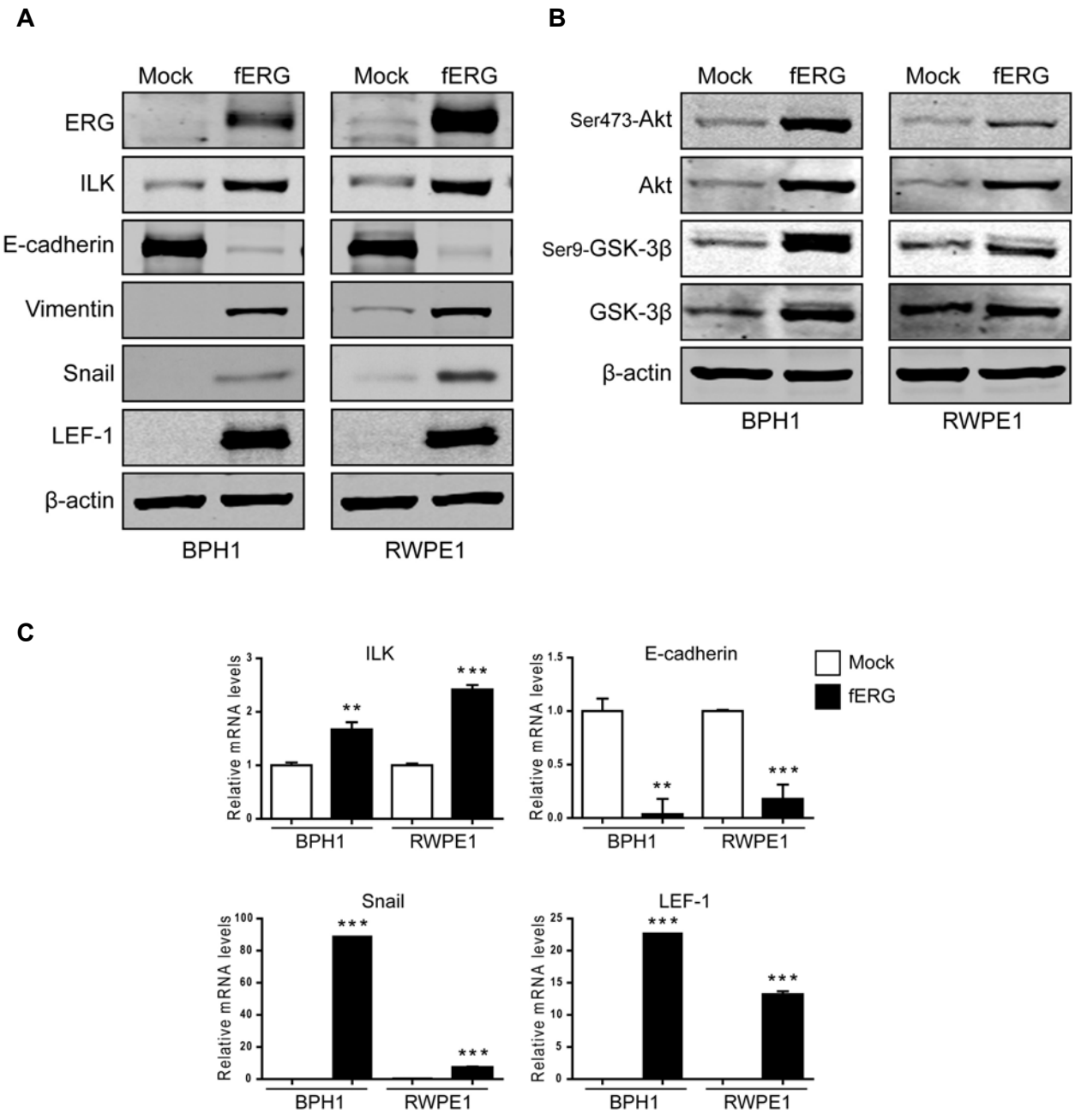



**Figure 3.2 Expression of ILK and ILK-regulated proteins involved in EMT are increased in fERG-PrECs.** (A) Western blot analyses show that ILK protein levels are increased in fERG-PrECs. The epithelial marker E-cadherin is suppressed, and the mesenchymal marker Vimentin is upregulated in these cells. ILK-regulated transcription factors, Snail and LEF-1, are upregulated in fERG-PrECs. (B) The ILK primary targets Akt and GSK-3β are highly phosphorylated in fERG expressing cells. (C) Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of mRNA expression using

Roche UPL technology. Results were first normalized to β-actin mRNA levels as a reference loading control and are reported as fold change over the Mock values (**$p$< 0.001; ***$p$<0.0005).

### 3.3 ILK promoter activity is increased in fERG-PrECs

Next, I investigated whether ERG-dependent elevation of ILK mRNA and protein could be traced back to the level of ILK gene transcription. For this, Mock- and fERG-PrECs were transiently transfected with a luciferase reporter gene vector containing the ILK promoter construct of 1,006 bp length (Melchior et al 2002). After 48 hours, cells were harvested and luciferase activity determined as a measure of ILK promoter activity. fERG-BPH1 cells displayed an up to 2.8-fold enhanced ILK promoter activity when compared to the Mock control cells. fERG-RWPE1 also revealed a statistically significant increase in the ILK promoter activity by 1.7-fold (Fig. 3.3A).

In order to study the involvement of ETS transcription factors in ERG-induced ILK gene transcription, I used two ILK promoter mutants carrying disrupted DNA consensus motifs for ETS transcription: the mutant carrying a disrupted ETS motif at position -462bp (1.Ets) and the mutant carrying a disrupted second ETS motif at position -85bp (2.Ets) (Lössner et al 2009). Disruption of the two DNA consensus motifs for ETS transcription factors containing within the ILK promoter did not lead to a decrease in activity in the ILK promoter of fERG-PrECs. These results suggest that the increase in ILK expression observed in these cells is not being regulated by direct binding of ERG to the ILK promoter (Fig. 3.3B).

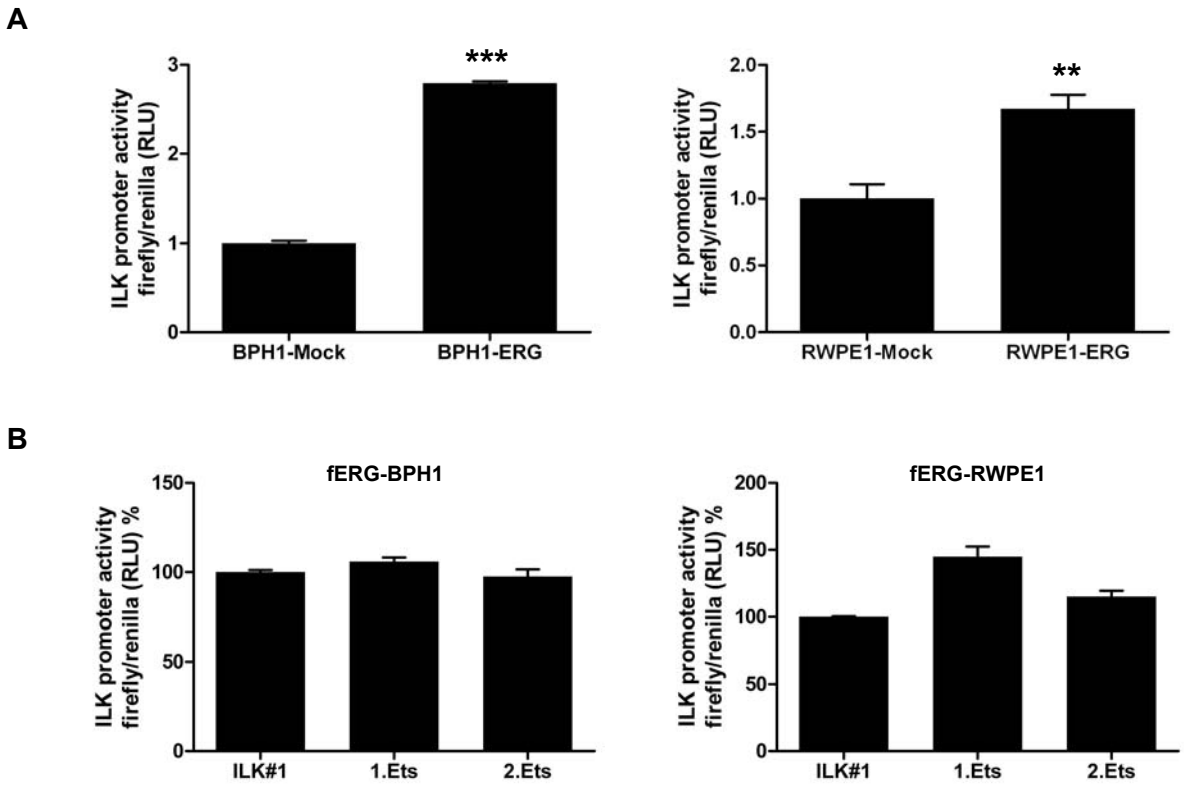



**Figure 3.3 ILK promoter activity is increased in fERG-PrECs but is not inhibited upon disruption of two ETS binding sites contained within the ILK promoter.** (A) Mock- and fERG-PrECs were transiently transfected with the ILK promoter construct ILK #1 (1,006 bp). (B) fERG-PrECs were transiently transfected with ILK promoter mutants carrying two disrupted DNA consensus motifs for ETS transcription factors: the mutant carrying a disrupted ETS motif at position -462 bp (1.Ets) or the mutant carrying a disrupted second ETS motif at position -85 bp (2.Ets). Relative ILK promoter activity was evaluated by calculating the quotient between relative light units (RLU) obtained for firefly and *Renilla* luciferase activity, respectively (mean values of n=3, +- SD, ** $p < 0.002$; *** $p < 0.0001$).

### 3.2 LEF/TCF transcriptional activity is stimulated but β-catenin levels are unchanged in fERG-PrECs

Since ILK overexpression in epithelial cells can lead to nuclear translocation of β-catenin resulting in β-catenin/LEF-1 complex formation and ultimately in activation of LEF/TCF target genes (Novak et al 1998, Tan et al 2001), I next investigated whether a difference in β-catenin pools could be detected in ERG-expressing PrECs. Immunofluorescence staining revealed that there is no noticeable difference in β-catenin localization between Mock- and fERG-PrECs, i.e., there is no translocation of β-catenin into the nucleus of ERG-expressing cells (Fig. 3.4A). However, high levels of LEF-1 are present in the nucleus of fERG-PrECs (Fig. 3.4A).

To assess whether a difference in LEF/TCF transcriptional activity could be detected in these cells, Mock- and fERG-PrECs were transfected with a LEF/TCF response promoter (pTOPFlash) cloned upstream of Firefly luciferase reporter, and with a control reporter *Renilla reniformis* luciferase (pRenilla). Luciferase assays show that there is a significant increase in LEF/TCF activity in fERG-PrECs compared to control cells (Fig. 3.4B). These data suggest that LEF-1 is acting as a transcriptional activator in fERG-PrECs even in the absence of detectable nuclear β-catenin.

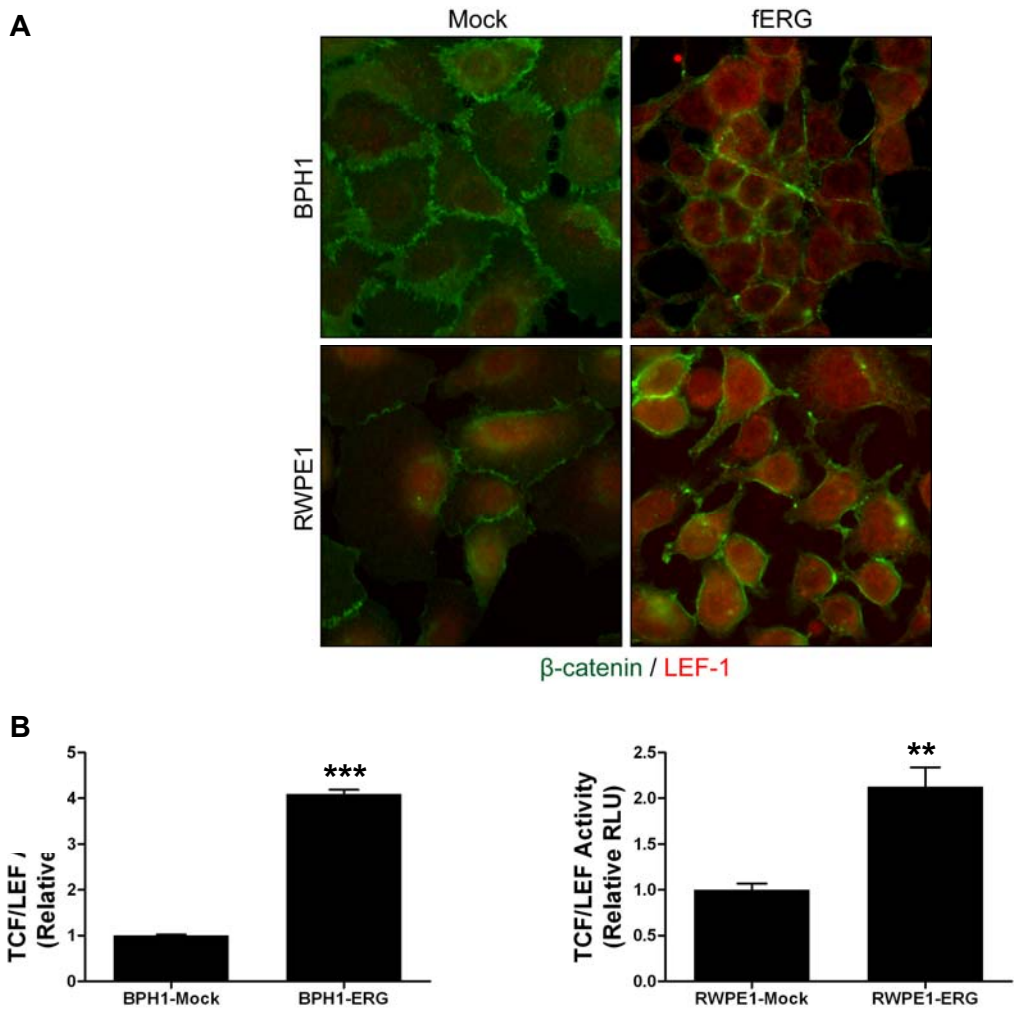



**Figure 3.4 LEF/TCF transcriptional activity is stimulated but β-catenin nuclear levels are unchanged in fERG-PrECs.** (A) Immunofluorescent staining showing β-catenin (green) localized mainly at the membrane of both Mock and fERG cells. LEF-1 (red) levels are increased in fERG cells. (B) Cells were transfected with either a reporter construct bearing copies of an optimal TCF/LEF response promoter (pTOPFlash) or with a reporter construct containing copies of a mutant form of the optimal TCF/LEF-binding site (pFOPFlash), both cloned upstream of Firefly luciferase. The promoter activities were normalized to the control (pRenilla) luciferase activity, and are represented as the fold activity of TOP over FOP reporters in each condition. The luciferase activities were expressed as relative light units (RLU) (**$p$<0.001; ***$p$<0.0001).

### 3.3 Inhibition of ILK decreases Snail and LEF-1 expression in fERG-PrECs

ILK has been previously shown to be involved in the regulation of mesenchymal markers LEF-1 (Novak et al 1998, Tan 2001) and Snail (Barbera et al 2004, McPhee et al 2008, Tan 2001). Therefore, I next sought to examine whether ILK had a direct role in the upregulation of Snail and LEF-1 as a result of ERG aberrant expression. First, I used a potent, second-generation ILK inhibitor, designated QLT-0267. QLT-0267 was developed through the optimization of a lead compound identified in the high-throughput screening of a rationally designed small-molecule library against ILK, and has been shown to highly specific (10-1000 fold selective over other kinases tested under similar conditions) (Younes et al 2005). Here, I show that pharmacological inhibition of ILK by QLT-0267 results in a substantial dose-dependent decrease in ERG-mediated upregulation of Snail and LEF-1 protein levels (Fig. 3.5A). As expected, QLT-0267 does not affect the expression of either ILK or ERG.

To further confirm that the decrease in Snail and LEF-1 protein levels in response to QLT-0267 treatment was ILK-specific, I downregulated ILK expression by siRNA transfection. As shown in Figure 3.5B, targeted siRNA transfection suppressed ILK protein levels by >65% and this correlated with reduction of Snail and LEF-1 protein expression by ~50% in fERG-RWPE1 cells as compared to cells transfected with control siRNA. These results implicate ILK expression and activity in the ERG-mediated EMT by upregulating the expression of Snail and LEF-1 protein levels. The fact that ERG levels do not change upon ILK pharmacological or genetic inhibition, further suggests that ILK a downstream effector in the ERG signaling pathway.

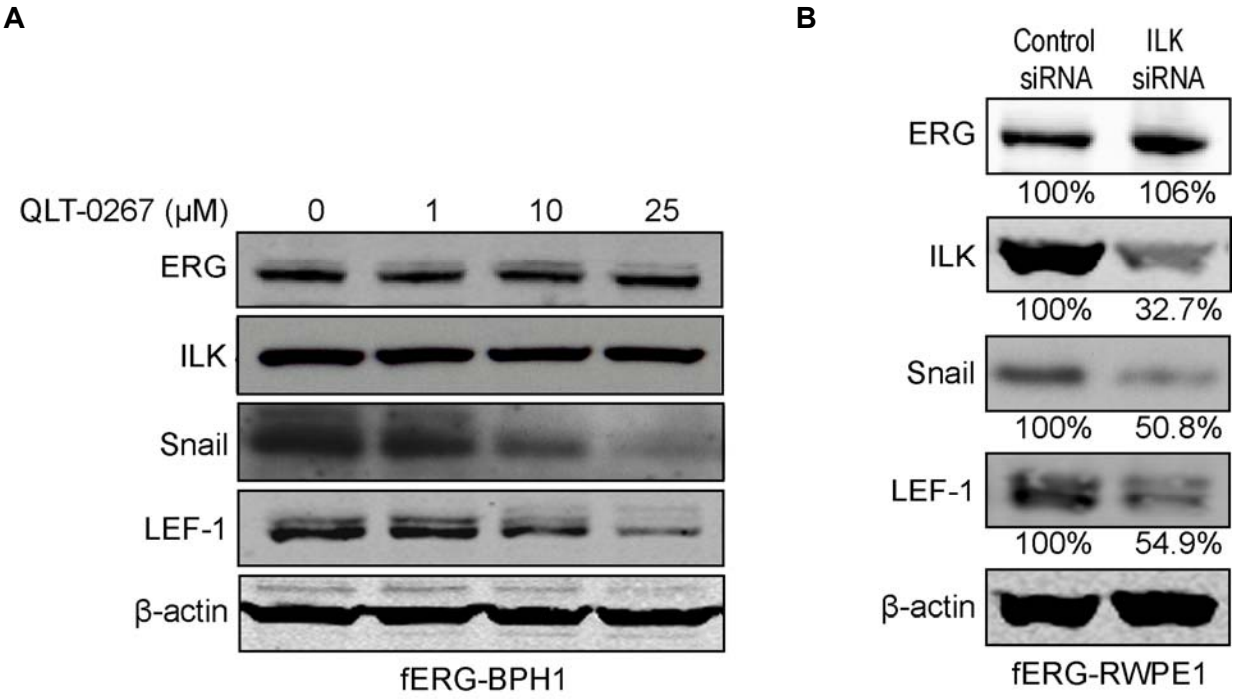



**Figure 3.5 Inhibition of ILK decreases LEF-1 and Snail expression in fERG-PrECs.** (A) Cells were treated for 16 hours with DMSO or increasing concentrations of QLT-0267, a highly selective inhibitor of ILK activity. Western blot analyses show that pharmacological inhibition of ILK results in a dose-dependent decrease in Snail expression and LEF-1 expression. ERG levels are not affected by ILK inhibition. (B) fERG-RWPE1 cells were transfected with 50 nM control or ILK-A siRNA, and reduction in protein expression was confirmed on a Western blot.

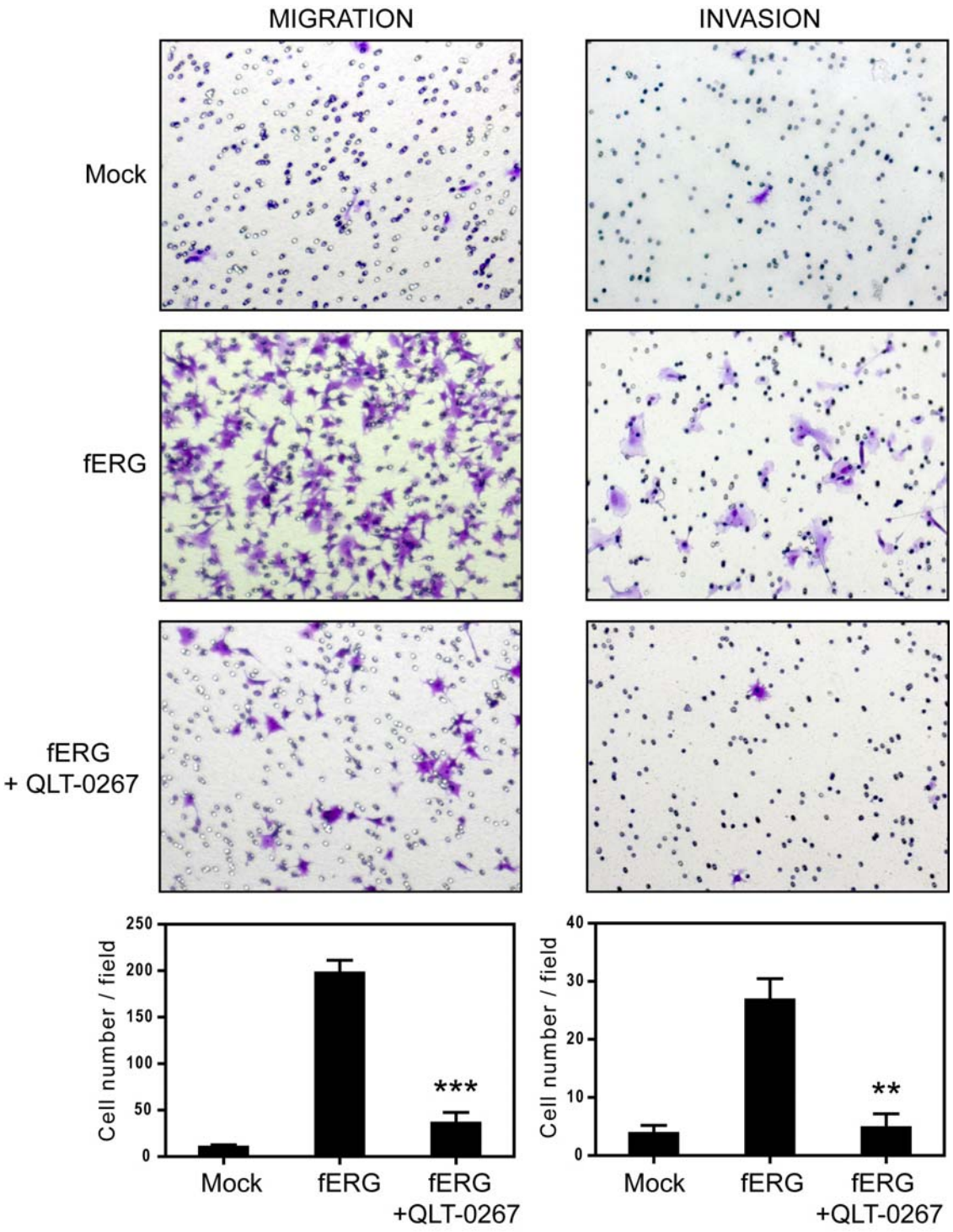
### 3.4 Inhibition of ILK activity impairs in vitro migratory and invasive properties of fERG-PrECs

A phenotypic hallmark of mesenchymal cells is the ability to migrate and invade through extracellular matrices and basement membranes. Therefore, I investigated whether inhibition of ILK could reduce the *in vitro* migratory and invasive properties of fERG-PrECs. To do this, I first used a Boyden Chamber assay which is widely used for studying cell migration and invasion. This assay is based on a chamber of two medium-filled compartments separated by a microporous membrane. Cells are placed in the upper compartment and are allowed to migrate through the pores of the membrane into the lower compartment, in which chemotactic agents are present. After an appropriate incubation time, the membrane between the two compartments is fixed and stained, and the number of cells that have migrated to the lower side of the membrane is determined. In a similar way, invasive capacity can be evaluated using transwell filters coated with Matrigel. Matrigel is a solubilized tissue basement membrane preparation, which contains laminin, collagen type IV, heparan sulfate proteoglycan, entactin, growth factors, and other components. As shown in Figure 3.6A, the data generated using this assays confirmed the highly invasive and migratory capacity of fERG-RWPE1, and shows that pharmacological inhibition of ILK in these cells greatly suppresses cell migration in invasion.

Next, I assessed the invasive capacity of fERG-PrECs using a three-dimensional (3-D) culture of cells in Matrigel, which allows the analysis of EMT in a more 'physiological-like' structure and environment compared with 2-D culture (Moreno-

Bueno et al 2009). The data from this experiment show that 3-D culture of Mock-BPH1 cells in Matrigel results in formation of epithelial spheroid structures, with organized F-actin cytoskeleton and E-cadherin expression at cell surface (Fig. 3.6B), consistent with features of differentiated phenotype of normal prostatic epithelium *in vivo.* In contrast, fERG-BPH1 cells display an aggressive and invasive behavior, forming irregular cellular aggregates with disorganized F-actin cytoskeleton and extensions radiating from the aggregates. Immunofluorescence staining shows absence of E-cadherin at the cell surface of these cells. To evaluate whether inhibition of ILK could reduce the invasive phenotype that fERG-BPH1 cells display in 3-D culture, I exposed these cells to QLT-0267. Treatment of fERG-BPH-1 cells with QLT-0267 dramatically inhibited formation of these disorganized aggregates and resulted in formation of compact spheroid structures resembling structures formed by Mock-BPH-1 cells and exhibiting partial restoration of E-cadherin expression, consistent with a reversion of the EMT phenotype of fERG-expressing PrECs (Fig. 3.6B).

**A**


MIGRATION
INVASION
Mock
fERG
fERG
+ QLT-0267
Cell number / field
250
200
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Mock
fERG
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40
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Mock
fERG
fERG
+QLT-0267

**B**

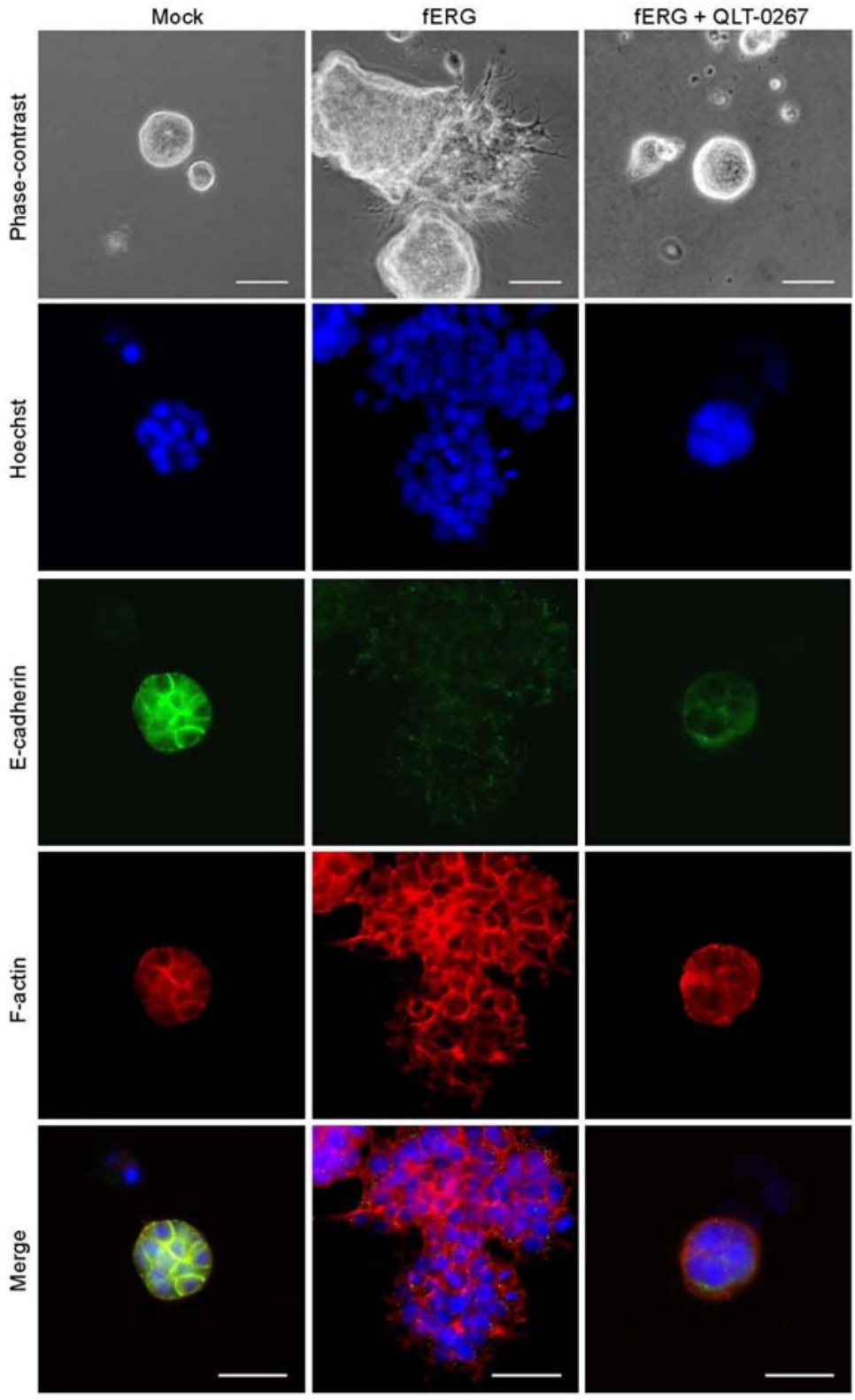
Mock
fERG
fERG + QLT-0267
Phase-contrast
Hoechst
E-cadherin
F-actin
Merge

**Figure 3.6 Pharmacological inhibition of ILK impairs in vitro migratory and invasive properties of fERG-PrECs.** (A) Mock- and fERG-RWPE1 were assayed for migration and invasion using transwell filters (coated with Matrigel for assessment of cell invasion). Photomicrographs of migrated/invaded cells stained by crystal violet are shown. Histogram shows mean number of cells that migrated/invaded per field ± SD (**$p$<0.002, ***$p$<0.0001). (B) Phase-contrast images of 3-D cultures in Matrigel of Mock-BPH1 and fERG-BPH1 reveals the difference in the invasive capacity between these cells. Immunofluorescence staining shows the organized F-actin cytoskeleton and E-cadherin expression at cell surface of Mock-BPH1 cells. In contrast, fERG-BPH1 cells display a disorganized F-actin and absence of E-cadherin at the cell surface. Treatment with QLT-0267 transformed the disorganized aggregates of fERG-BPH1 cells to compact round structures without protrusions. Scale bar, 50 μm.

### 3.5 Inhibition of ILK activity suppresses anchorage-independent growth and induces anoikis in fERG-PrECs

Earlier results with fERG-PrECs demonstrated acquisition of anchorage-independent growth by these cells, another characteristic associated with malignant transformation and EMT. Intriguingly, upregulation of ILK has been previously shown to induce resistance to anoikis and anchorage-independent growth in breast and prostate cancer cells (Attwell et al 2000, Hannigan 1996, Radeva et al 1997). Therefore, in order to examine the role of ILK in fERG-PrECs anchorage-independent growth, I sought to assess the consequence of ILK inhibition on soft agar colony formation. As previously observed, Mock cells are not capable of growing without the support of an ECM, while fERG-BPH1 cells form numerous, large colonies on soft agar. Pharmacological inhibition of ILK by QLT-0267 resulted in a marked suppression of anchorage-independent growth of fERG-BPH1 (Fig. 3.7A).

In order to evaluate whether targeting ILK restored induction of apoptosis due to the loss of substrate adhesion, i.e., anoikis (Frisch and Francis 1994), poly-ADP-ribose polymerase (PARP) cleavage (Kaufmann et al 1993) was assessed in BPH1 cells cultured in suspension for 48 hours. Mock cells grown in suspension readily underwent apoptosis, as evidenced by the almost exclusive detection of cleaved PARP (Fig. 3.7A). In contrast, PARP was predominantly detected in its intact form in fERG-BPH1 cells cultured in suspension, consistent with them being resistant to anoikis (Fig. 3.7A). However, treatment of suspension cultures of fERG-BPH1 cells with QLT-0267 partially restored sensitivity to anoikis as assessed by induction of cleavage of approximately 40% of total PARP (Fig. 3.7A). These results further implicate ILK in ERG-induced prostate tumourigenesis.

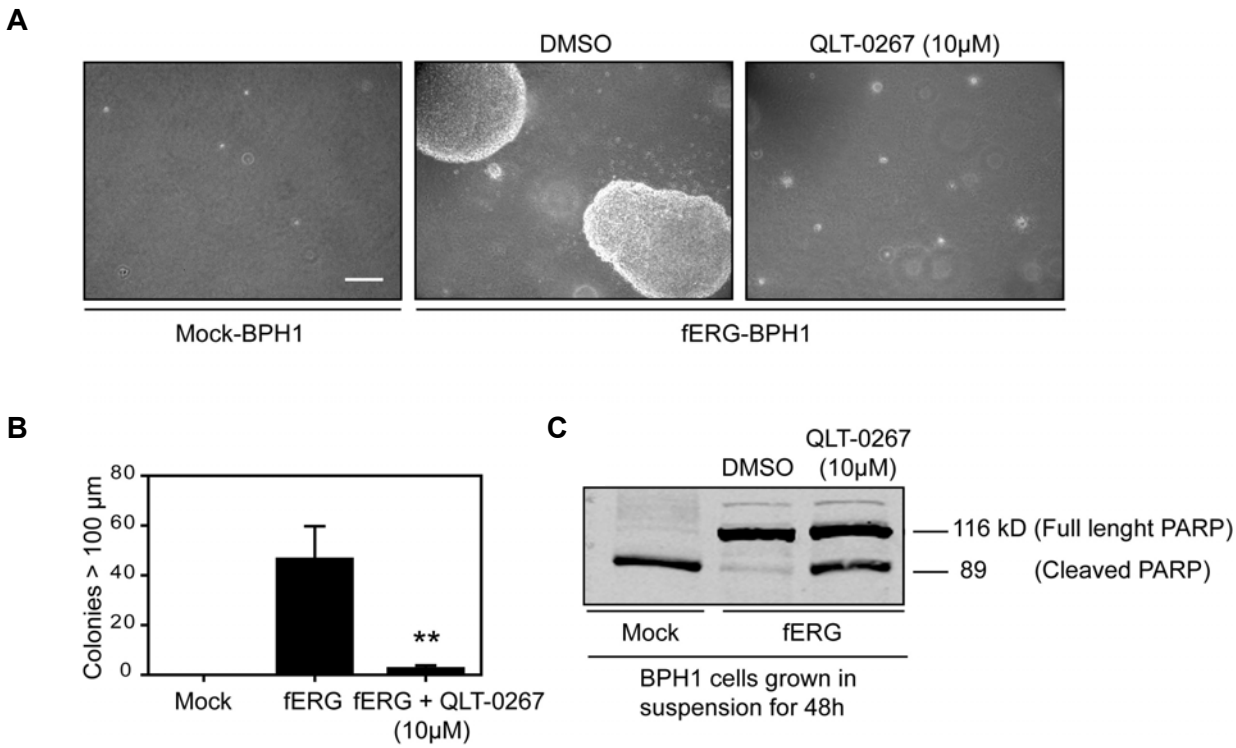


**Figure 3.7 Inhibition of ILK suppresses anchorage-independent growth of fERG-PrECs.** (A) fERG-BPH1 cells were cultured for 14 days in soft agar containing DMSO or 10μM of QLT-0267. The images are representative phase contrast micrographs (10X) of soft agar colonies. Inhibition of ILK kinase activity dramatically suppresses the anchorage-independent growth of fERG-PrECs. Scale bar, 200 μm. (B) Histogram shows mean number of colonies/well > 100 μm ± SD ($p$< 0.005). (C) For evaluation of anoikis, cells were cultured in serum containing media on polyHEMA coated plates for 48 hours. Caspase mediated cleavage of PARP was assessed by Western blotting. Mock cells grown in suspension undergo apoptosis, as evidenced by the detection of fully cleaved PARP. In contrast, ERG expressing cells contain intact PARP, and therefore, are resistant to anoikis. However, inhibition of ILK in these cells by QLT-0267 results in induction of apoptosis.

### 3.6 Cell-ECM interactions are impaired and ERK/MAP kinase signaling is inhibited in fERG-PrECs

During the EMT process, alterations in cell-ECM interactions are known to occur (Kalluri and Weinberg 2009). Therefore, I next investigated whether the binding to ECM substrates was altered in fERG-PrECs. Cell attachment assays using plates coated with fibronectin, laminin and vitronectin, demonstrate a significant reduction in the adhesion of fERG cells to these ECM proteins (Fig. 3.8A). Subsequently, I verified the expression of integrins that can bind to these substrates, and found a dramatic decrease in the protein levels of β1 and αv integrins in fERG-PrECs (Fig. 3.8B). These observations are in agreement with microarray analysis of Mock and fERG-PrECs gene expression profiles performed at Dr. Cox laboratory (Appendix 1), which indicates that transcription of most β and α integrin subunits is downregulated in fERG-PrECs.

Since integrin expression and engagement to the ECM are significantly impaired in fERG-PrECs, I investigated whether ERK/MAP kinase pathway was also affected in these cells. Western blot analysis shows that there is a striking inhibition in the ERK/MAP kinase cascade in fERG-PrECs compared to Mock cells (Fig. 3.8C). The phosphorylation of Raf-1 at Ser-259 causes its inactivation and removal from the membrane, and as a result, ERK/MAP kinase cascade is not activated (Ramos, 2008). Thus, these results suggest that ERG overexpression leads to an inhibition of ERK/MAP kinase signaling, and this may be related to the disruption in cell-ECM interactions observed in fERG-PrECs.

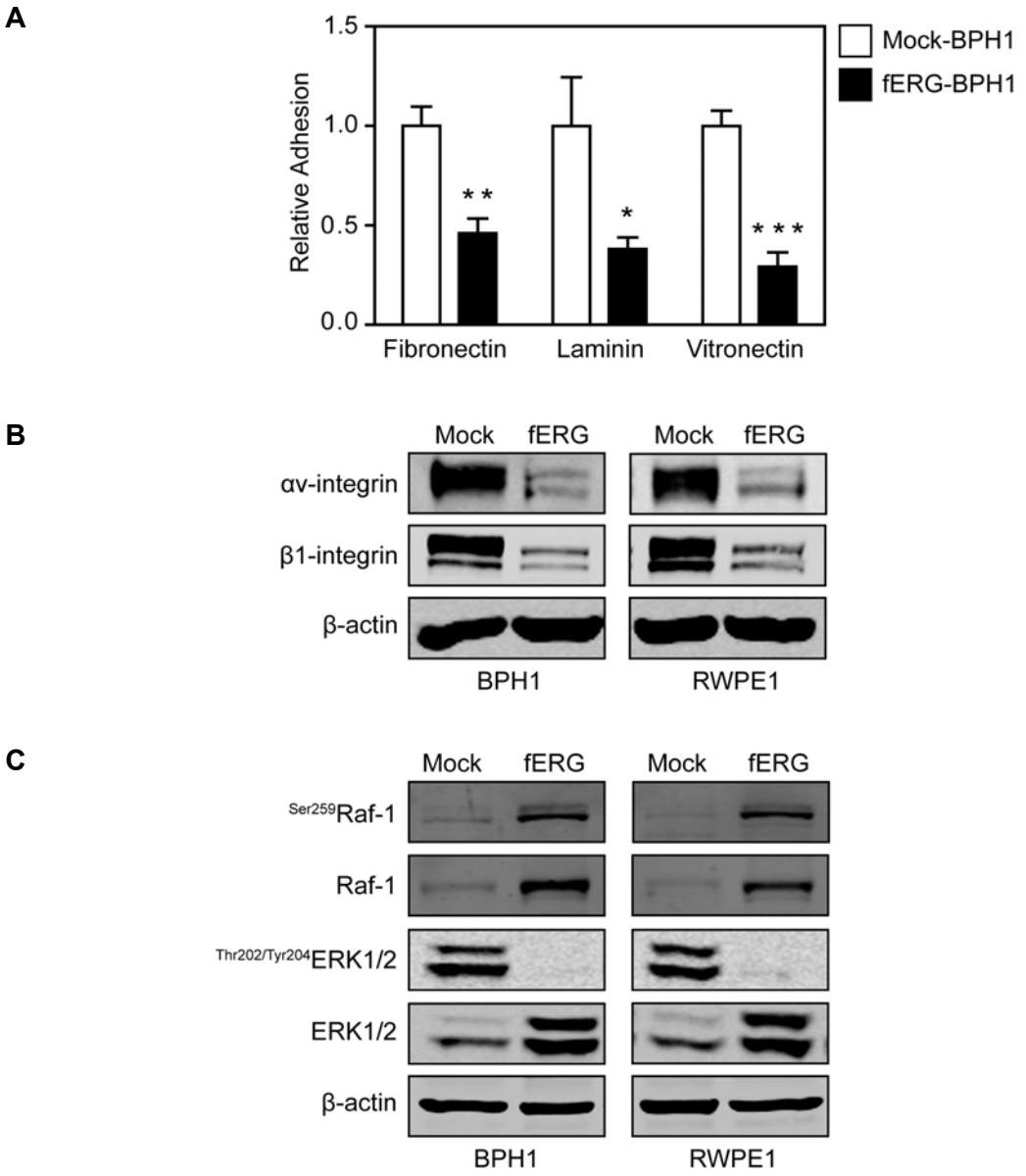


**Figure 3.8 Integrin-mediated cell-ECM interactions are disrupted and ERK/MAP kinase signaling is inhibited in fERG-PrECs.** (A) Attachment assays using plates coated with fibronectin, laminin and vitronectin, demonstrate a significant reduction in the adhesion of fERG cells to these ECM proteins (*$p$<0.02, **$p$<0.002, ***$p$<0.0003). (B) Western blot analyses show a dramatic decrease in β1 and αv integrins in fERG-PrECs. (C) Western blotting shows that there is a striking inhibition in the ERK/MAP kinase cascade in fERG-PrECs compared to Mock-cells. The phosphorylation of Raf-1 at Ser-259 causes its inactivation and removal from the membrane, and as a result, ERK/MAP kinase cascade is not activated.

### 3.7 Upregulation of LEF-1 and downregulation of integrins and ERK/MAP kinase signaling is observed in the metastatic-ERG expressing VCaP cell line

By utilizing human prostatic epithelial cell lines engineered to overexpress ERG, I identified novel downstream effectors of ERG signaling which possibly may play a role in the transition to aggressive prostate cancer phenotypes. In order to investigate whether the same pathways could be dysregulated in a cell line originated from a *TMPRSS2-ERG* positive tumour, I performed experiments using VCaP cells. The VCaP cell line was established from the vertebral bone metastasis of a hormone-refractory prostate tumour, and it harbors the *TMPRSS2-ERG* gene fusion (Korenchuk et al 2001).

As observed in fERG-PrECs, β1 and αv integrins are downregulated and the ERK/MAP kinase signaling is inhibited in VCaP cells (Fig. 3.9B). The mesenchymal marker LEF-1 is also dramatically upregulated in the *TMPRSS2-ERG* fusion-positive cell line. In constrast, downregulation of E-cadherin, Snail and Vimentin are not observed in this cell line (Fig. 3.9A).

Although ILK protein levels in VCaP cells are not as elevated as the levels observed in fERG-PrECs, its expression is clearly higher in VCaP compared to Mock cells. Importantly, the metastatic prostate cancer cell line is still dependent on ILK signaling, given that treatment of these cells with QLT-0267 also leads to inhibition of cell migration and induction of anoikis (Fig. 3.10).

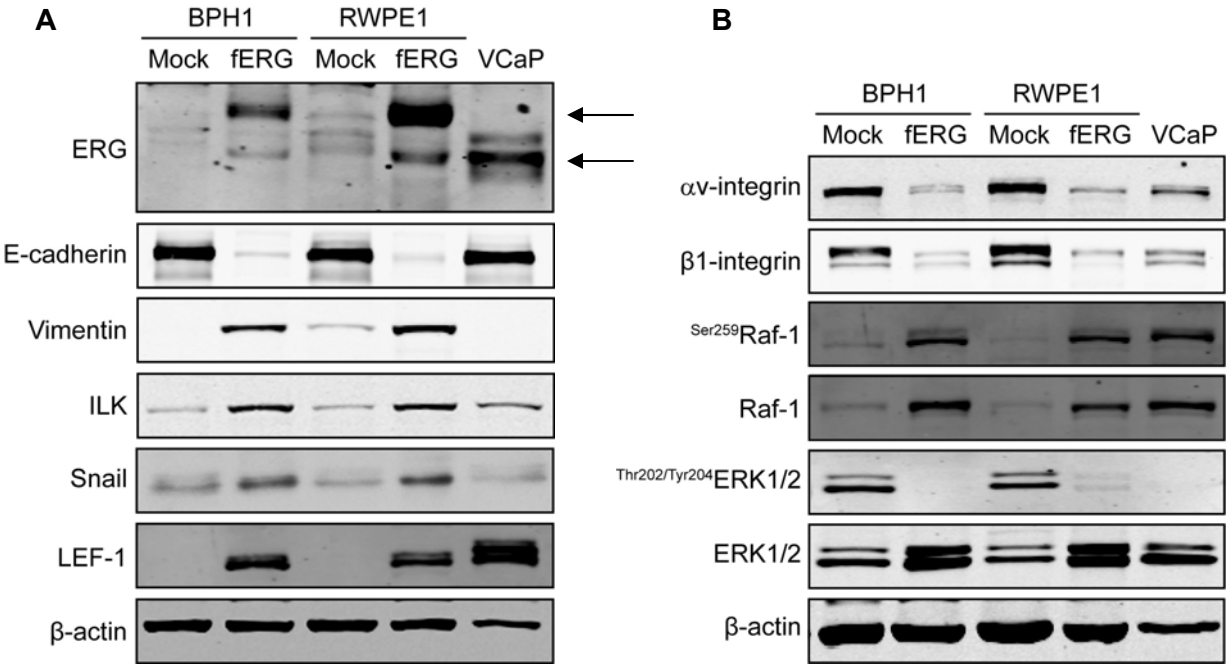


**Figure 3.9 *TMPRSS2-ERG* fusion-positive prostate cancer cell line, VCaP, displays some of the features observed in fERG-PrECs.** (A) Assessment of EMT associated proteins in the VCaP cell line shows that LEF-1 is also significantly upregulated in these cells compared to control PrECs. However, downregulation of E-cadherin, Snail and Vimentin are not observed in these cells. ILK expression is higher in VCaP compared to control cells, but not as elevetated as in fERG. (B) Detection of strong correlation in the inhibition of β1 and αv integrins and ERK/MAP kinase signaling between VCaP and fERG-PrECs.

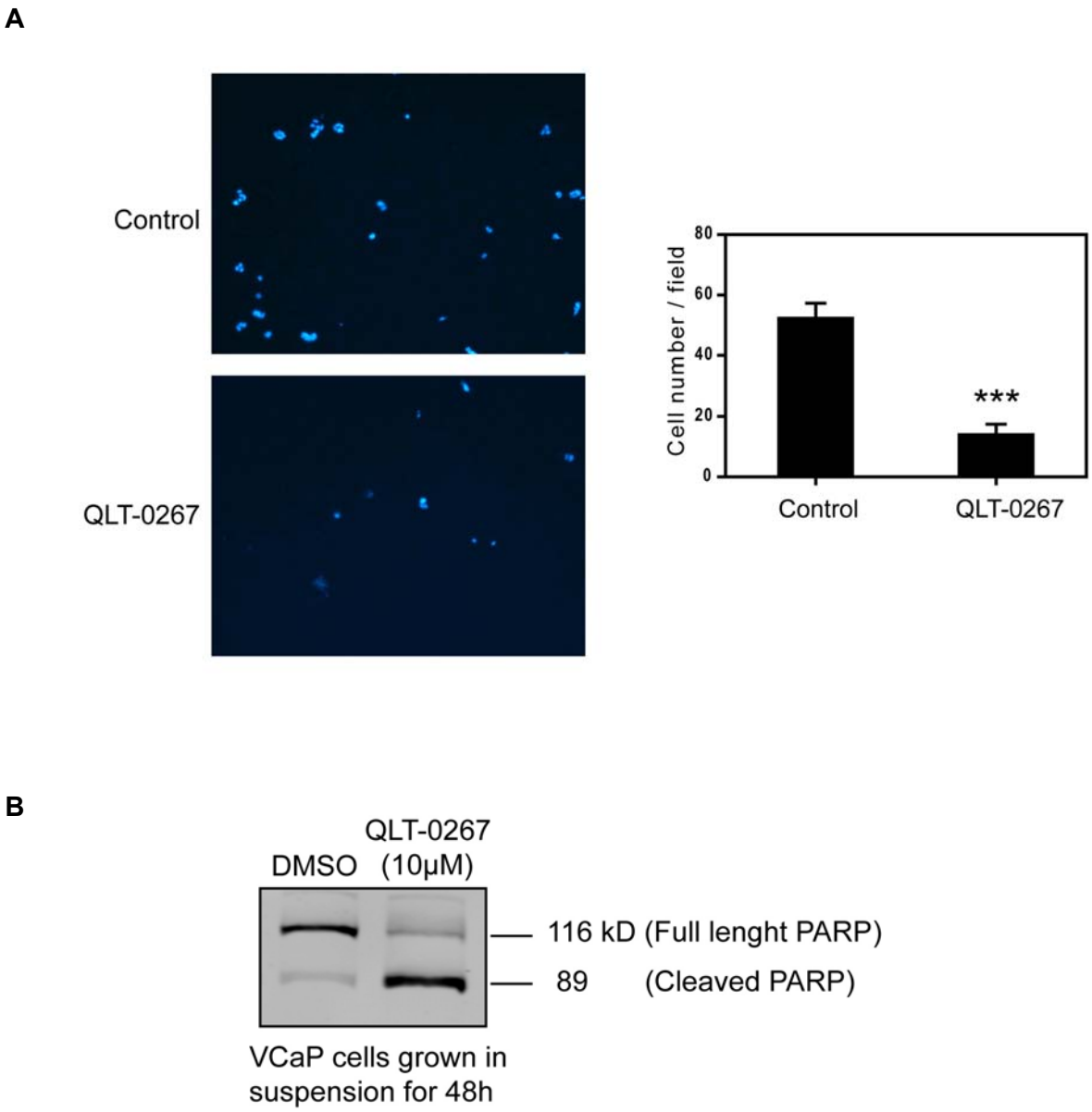


**Figure 3.10 Inhibition of ILK activity impairs migration and induces anoikis in VCaP cells.** (A) VCaP cells were assayed for migration using transwell filters in the absence or presence of the ILK inhibitor (QLT-0267). Photomicrographs of migrated cells stained by Hoechst are shown. Histogram shows mean number of cells that migrated per field ± SD (***$p$<0.0005). (B) For evaluation of anoikis in VCaP cells, caspase mediated cleavage of PARP was assessed by Western blotting.

# Chapter 4. Discussion

Prostate cancer is the most prevalent malignancy in men of the Western world (Jemal et al 2010). Unlike other malignancies, more men die with prostate cancer than from the disease (Albertsen et al 1998). Deciphering the molecular networks that distinguish progressive from nonprogressive disease will lead to identification of biomarkers that can aid in the selection of patients that should be treated, as well as to discovery of new targets for the treatment of metastatic prostate cancers.

Chromosomal rearrangements resulting in the overexpression of the ETS transcription factor, ERG, were recently defined as the most common genetic alterations in human prostate cancer (Kumar-Sinha et al 2008, Tomlins et al 2009). Consistent with its role in Ewing's sarcoma, AML and neuroectodermal tumours, ERG fusions are likely to play a role in prostate tumorigenesis through transcriptional induction or repression of downstream targets. Indeed, several studies have implicated ERG aberrant signaling in prostatic transformation and invasive properties (reviewed in Tomlins et al 2009). Furthermore, the presence of ERG gene fusions in prostate cancer specimens has been reported to be associated with more aggressive disease (Attard et al 2008a, Barry et al 2007, FitzGerald et al 2008, Nam et al 2007, Rajput et al 2007, Yoshimoto et al 2008). However, the molecular pathways by which ERG contributes to aggressive prostate cancer phenotypes are not clearly understood.

Accumulating evidence in recent years indicates that EMT is a critical process not only in development but also in cancer progression. The term EMT refers to a

complex molecular and cellular programme by which epithelial cells shed their differentiation characteristics, including cell-cell adhesion, planar and apical-basal polarity, and lack of motility, and acquire instead mesenchymal features, including motility, invasiveness and a heightened resistance to apoptosis. EMTs have been found to contribute to invasion, metastatic dissemination and acquisition of therapeutic resistance (Polyak and Weinberg 2009). Intriguingly, previous observations from Dr. Cox laboratory revealed that aberrant ERG expression can lead to induction of EMT in human prostatic epithelial cell lines (PrECs). Based on these findings, during my studies I investigated the mechanism underlying ERG-induced EMT in PrECs, and particularly revealed that ILK is a critical mediator in this process.

ILK has been previously implicated in EMT development (Medici and Nawshad 2010, Rosanò et al 2005, Tan et al 2001), and notably, ILK overexpression has also been associated with aggressive prostate cancer phenotypes (Graff et al 2001). Here, I have shown that ILK is upregulated upon ERG exogenous expression in PrECs. The mesenchymal markers Snail and LEF-1, which play major roles in EMT (Barbera et al 2004, Medici and Nawshad 2010, Rosanò et al 2005, Tan et al 2001), are also upregulated in fERG-PrECs, and inhibition of ILK in these cells leads to down-regulation of both mesenchymal markers. Highlighting our findings, recent evidence implicates both Snail and LEF-1 in prostate tumor progression (Heeboll et al 2009, Li et al 2009). Heeboll and co-workers have reported that Snail expression is increased in prostate cancer, and it is associated with invasive properties of prostate cancer cells (Heeboll et al 2009). Li and collaborators have shown that LEF-1 is highly expressed in androgen-

independent prostate cancer, which is a very aggressive form of this malignancy (Li et al 2009). Moreover, this study demonstrates that LEF-1 is a key regulator of AR expression and prostate cancer growth and invasion (Li et al 2009). While the most well-known role of LEF-1 is to transmit WNT signals by binding to β-catenin and recruiting it to target genes for activation, LEF/TCFs have also been shown to cooperate with other factors to regulate transcription independent of β-catenin binding (Arce et al 2006). For example, LEF/TCFs can bind to γ-catenin (plakoglobin), a highly related but distinct catenin, and activate the transcription of c-MYC more effectively than when forming complexes with β-catenin (Kolligs et al 2000).

Activation of EMT is also facilitated by the disruption of cell-ECM adhesions mediated by integrins (Kalluri and Weinberg 2009). Importantly, recent studies have suggested that prostate tumor progression is associated with the suppression of integrin-based cell adhesion (Goel et al 2008, Gorlov et al 2009). Here I show that cell-ECM interactions are disrupted upon ERG expression in PrECs. fERG-PrECs present a significant decrease in adhesion to fibronectin, laminin and vitronectin, concomitantly with a dramatic decrease in the protein levels of β1 and αv integrins. Moreover, microarray analysis of gene expression shows that most β and α integrin subunits are downregulated in the fERG expressing cells (Appendix 1). Consistent with these results, most of the integrins have been reported to be downregulated in prostate cancer (Goel et al 2008, Gorlov et al 2009). For example, β1 integrin has been shown to be downregulated in prostate tumours (Gorlov et al 2009), and transfection-mediated expression of this integrin induces cell adhesion to laminin and prevents tumour growth

(Goel et al 2004). In addition, decrease in β1-integrin expression has been linked to increased invasion in prostate cancer cells (Dedhar et al 1993). However, the mechanisms by which integrin dysregulation occurs still need to be identified. These data suggest that aberrant ERG signaling may be one mechanism that leads to suppression of integrins in prostate cancer. In fact, overexpression of ILK has been shown to inhibit adhesion to integrin susbstrates, while inducing anchorage-independent growth (Hannigan et al 1996). Therefore, the suppression in integrins and consequently in integrin-mediated cell adhesion observed in fERG-PrECs might be mediated through ILK signaling, which should be further explored in the future.

Besides playing a key structural role in cells, integrins are also important initiators and modulators of signal-transduction processes (Hynes 2002, Juliano et al 2004). In fact, integrins are important activators of the ERK/MAP kinase cascade (Ramos 2008). Integrin engagement enhances the efficiency of the cytoplasmic cascade comprising Raf-1, MEK and ERK, and is necessary for trafficking of activated ERK from the cytoplasm to the nucleus. In this study I show that ERK/MAP kinase signaling is greatly inhibited following ERG expression in PrECs, which could be a result of the dramatic suppression in integrin-mediated cell adhesion observed in these cells. Inhibition of ERK/MAP kinase signaling through blocking of ECM-integrin interaction has been shown to keep cells in an undifferentiated state (Hayashi et al 2007, Liu et al 2009, Liu et al 2007). Intriguingly, elevated ERG expression, as a result of the *TMPRSS2-ERG* fusion, has been shown to modulate the growth of prostate cancer cells by abrogating the differentiation of prostate epithelium (Sun et al 2008).

By utilizing human prostatic epithelial cell lines engineered to overexpress ERG, I identified novel downstream effectors of ERG signaling which possibly play a role in the transition to an aggressive prostate cancer phenotype. In order to investigate whether the same pathways could be dysregulated in a cell line originated from a *TMPRSS2-ERG* positive tumour, I performed experiments using the metastatic cell line VCaP. As observed in the ERG expressing PrECs, LEF-1 is dramatically upregulated in VCaP cells. In addition, β1 and αv integrins are downregulated and ERK/MAP kinase signaling is inhibited in these cells. Conversely, downregulation of E-cadherin and upregulation of Snail and Vimentin were not observed in this cell line. This is not very surprising since several lines of evidence suggest that in order to establish colonies at distant sites, metastasizing cancer cells must undergo a reverse conversion called mesenchymal-to-epithelial transition (MET), in which they reacquire at least some of the epithelial features lost during EMT (Polyak and Weinberg 2009). MET is an attractive hypothesis that can explain the histopathological similarity between primary and metastatic tumors. The tendency of disseminated cancer cells to undergo MET likely reflects the local microenvironments that they encounter after extravasation into the parenchyma of a distant organ. Since VCaPs are derived from a bone metastasis of a prostate tumour, it is expected that to be able to successfully proliferate in the bone this cell line had to shed, at least some, of its mesenchymal characteristics. In fact, the observation that these cells express E-cadherin is in accordance with the dynamic expression of E-cadherin observed during the progression of several tumours, including prostate cancer (Tsuji et al 2009). Further supporting these findings, Snail expression has been shown

to be significantly lower in prostate cancer lymph node metastasis than in primary tumours (Heeboll et al 2009). The fact that some of the same pathways dysregulated upon ERG forced expression in PrECs, continue to be dysregulated in the metastatic VCaP cell line, suggests that these pathways may be still playing roles in the late stages of tumor progression. Importantly, inhibition of ILK in VCaPs also led to inhibition of cell migration and induction of anoikis, which suggests that these cells are still dependent on ILK signaling, and therefore targeting ILK might be a valuable option even in late-stage disease.

Collectively, this work provides novel insights into the complex role of ERG in prostate tumourigenesis. Particularly, I show that inhibition of ILK leads to suppression of ERG-mediated oncogenic phenotype by dramatically inhibiting the invasive and anchorage-independent growth properties of ERG-expressing prostate cells. Such results suggest the possibility that ILK may serve as a therapeutic target in ERG-positive prostate cancers. Further supporting a role for ILK as a promising therapeutic target in prostate cancer, recent studies show that *TMPRSS2-ERG* gene fusions and PTEN loss collaborate to promote prostate cancer progression (Carver et al 2009, King et al 2009). Analysis of prostate tissue specimens show that *ERG*-rearranged prostate positive tumours are enriched for *PTEN* loss, and the presence of both aberrations in prostate tumours is associated with poor outcome (Yoshimoto et al 2008). As mentioned earlier, ILK expression in prostate cancer is also correlated with poor prognosis (Graff et al 2001), and ILK has been shown to be negatively regulated by the tumor suppressor PTEN (Morimoto et al 2000, Obara et al 2004, Persad 2000, Persad

et al 2001b). Importantly, inhibition of ILK suppresses activation of Akt and induces apoptosis in PTEN-mutant prostate cancer cells (Persad 2000, Persad et al 2000), and *in vivo*, it delays growth of PTEN-negative tumors (Edwards et al 2005). Thus, targeting ILK in prostate cancer may be very promising since it will potentially affect ERG and PTEN aberrant signaling, which together seem to be pivotal in prostate carcinogenesis.

# Chapter 5. Conclusions and future directions

Approximately half of human prostate cancers harbor translocations of the 5' untranslated region of the androgen-regulated gene, *TMPRSS2,* with the open reading frame of the ETS transcription factor, *ERG*. The result of this genomic rearrangement is aberrant androgen receptor-driven expression of *ERG* (Clark and Cooper 2009, Tomlins et al 2009). Although ERG activation has been linked to invasive properties of prostate cancers, the precise mechanisms and pathways of ERG-mediated oncogenesis remain poorly understood. In this thesis I provided novel insights into critical pathways by which aberrant ERG expression may promote prostate cancer progression. In particular, I presented evidence to support the hypothesis that ERG-mediated oncogenesis in prostate cancer involves activation of ILK signaling, leading to key cancer-promoting phenotypic effects, such as EMT.

During the past few years, EMT has emerged as one of the hot spots of clinical research. Its existence in human tumours can form the basis for explaining characteristics of cancer progression and metastasis, as well as certain cases of drug resistance and relapses after treatment. For example, the expression of ILK has been shown to be responsible for the increased activation of Akt, further leading to EMT and associated drug resistance in hepatocellular carcinoma. Interestingly, inhibition of ILK activity increases mesenchymal sensitivity of these cells to EGFR-targeted therapies in xenografts models (Fuchs et al 2008).

Epidemiologic evidence suggests that prostate cancer carrying ERG fusions predict poor outcome (Attard et al 2008a, Barry et al 2007, FitzGerald et al 2008, Nam et al 2007, Rajput et al 2007, Yoshimoto et al 2008). Here I reveal that ILK is up-regulated upon aberrant expression of ERG in prostatic epithelial cells. These observations are intriguing in light of previous evidence that ILK expression is associated with aggressive prostate cancer phenotypes and correlated with poor prognosis. Analysis of ILK expression in prostate cancer biopsy samples shows that ILK expression levels increase with tumour grade, and that there is an inverse correlation between ILK expression and 5-year patient survival (Graff et al 2001). The constitutively high levels of ILK expression and activity in cancer cells compared with the surrounding normal cells, offers a therapeutic window for downregulating ILK activity in the cancer cells to the level of the normal cells. In addition, it is becoming apparent that, for reasons not fully understood at present, some of the signaling pathways regulating cell survival, proliferation and invasion become more dependent on ILK in cancer cells, which works in favour of ILK as a therapeutic target in cancer (Muranyi et al 2010, Troussard et al 2006). Indeed, I have observed that pharmacological inhibition of ILK suppresses Akt Serine-473 phosphorylation in prostate cancer cells but not in normal prostatic epithelial cells (Appendix 2). In this thesis I have demonstrated that inhibition of ILK leads to a dramatic suppression of ERG-mediated oncogenic phenotype by inhibiting the migratory/invasive properties and anchorage-independent growth ability of ERG-expressing prostate cells. Collectively, the data generated in this study implicates ILK as a downstream effector of ERG signaling in prostate tumourigenesis. Therefore,

the results presented here in concert with previous literature evidence suggest that in exploring specific treatment strategies for ERG-positive prostate cancer patients, it may be an attractive option to target downstream signaling events, such as ILK.

Given the multifunctional role that ILK plays in the regulation of cell signal transduction, further studies will be required to delineate the precise mechanisms by which ILK regulates ERG-induced transformation. The data presented in this thesis suggests that ILK is mediating ERG-induced EMT, at least in part, by inducing the expression of transcription factors, Snail and LEF-1. To dissect the exact role of each of these proteins in ERG-induced tumourigenesis, gain- and loss-of-function experiments should be carried out. For example, siRNA targeting these proteins could be performed in fERG-PrECs, following analyses for gene expression changes of the key epithelial and mesenchymal markers described here, as well as changes in biological endpoints of cell migration, invasion, resistance to anoikis and anchorage-independent-growth. Further investigation will most likely reveal other signaling pathways that are also being regulated by ILK in the context of ERG aberrant expression in prostate epithelial cells. For example, members of the Rho family of GTPases have been shown to play a central role in the regulation of the actin cytoskeleton, and therefore, in cell migration. In fact, ILK has been shown to be a key player in cytoskeleton reorganization by activation of the small GTPases, Rac and Cdc42 via the guanine nucleotide exchange activity of α-PIX (Li et al 2003d, Turner et al 1999) . Therefore, the dramatic suppression in the migratory phenotype of fERG-PrECs observed upon ILK inhibition, might be due to the activation of these small GTPases by ILK.

Despite being upregulated in many malignancies, the mechanisms surrounding the increase in ILK expression are not well recognized. Although the data from the present study reveal that ILK is being regulated at the transcriptional level in fERG-PrECs, the underlying mechanism of how ERG promotes upregulation of ILK remains to be elucidated. ETS proteins control transcription by binding the ETS-binding sites of enhancers or promoters of genes. Therefore, the fact that disruption of the two DNA consensus motifs for ETS transcription factors containing within the ILK promoter region did not lead to a decrease in activity of the ILK promoter in fERG-PrECs, does not rule out the possibility that ERG is binding to other sites (enhancers) outside the promoter region of ILK in these cells. A powerful approach to investigate whether ERG is directly regulating ILK transcription could be through chromatin immunoprecipitation (ChIP) analysis. This method makes use of the ability to crosslink proteins to the DNA, to which they bind in vivo. The ChIP methodology has produced numerous applications in studying the composition and dynamics of transcription factor binding, as well as assessing the interplay between different factors in gene regulation, and therefore could be really useful to reveal the mechanisms surrounding the increase in ILK expression.

In regards to other cancers, ERG overexpression has also been described in acute myeloid leukemia, Ewing's sarcoma and primitive neuroectodermal tumors (Giovannini et al 1994). Intriguingly, high levels of ILK expression have also been documented in these types of cancers (Chung et al 1998) (Muranyi et al 2009, Muranyi et al 2010). Thus, investigating a potential role of downstream ERG signaling in the

regulation of ILK expression in these other malignancies will also be an interesting avenue for future studies.

In addition, in order to further evaluate the role of ILK in ERG-mediated tumourigenesis, experiments *in vivo* must be performed in the near future. For example, studies using murine models carried at Dr. Cox laboratory have demonstrated that fERG-PrECs develop highly mitotic, invasive tumors in renal capsule xenografts. Therefore, it would be very relevant to test the effect of ILK inhibition in these fERG-PrECs xenografts. This could be done by assessing the ability of QLT-0267 to suppress renal capsule fERG-PrECs xenografts growth. It is noteworthy to mention that transgenic mouse models engineered to express prostate-targeted ERG do not develop invasive prostate cancer. This suggests that additional genetic alterations contribute to promote prostate cancer progression in an ERG-positive background. Of note, the PrECs utilized in our study were previously immortalized by viral antigen expression, with SV40 (BPH1) and HPV18 (RWPE1). SV40 and HPV immortalization are predicted on dysregulation of p53 and Rb (Bookstein et al 1993, Cooney et al 1996, Downing et al 2003, Paris et al 2006). Disruption in these tumour suppressor genes have been repeatedly identified in prostate cancer, and therefore could be a possible explanation for the malignant phenotype observed in fERG-PrECs. Supporting the hypothesis that additional genetic alterations are necessary to promote ERG-oncogenic transformation, mouse experiments have shown that combined ERG overexpression and PTEN haploinsufficiency lead to accelerated development of high-grade prostatic PIN and subsequent progression to multifocal, invasive prostate cancer (Carver et al 2009, King

et al 2009). Therefore, it will be extremely valuable to evaluate the role of ILK in mice with PTEN-deficient and ERG-expressing combined backgrounds. The probasin promoter ($ARR_2PB$) has been successfully used to drive prostate-targeted transgene expression in mouse models (Zhang et al 2000), and therefore could be used for future experiments. In fact, at the Vancouver Prostate Centre, prostate specific PTEN deficient mice were generated by crossing PTEN flox/flox mice with an $ARR_2PB$-Cre transgenic line. Thus, as an approach, the vector engineered to overexpress ERG described in our studies could be introduced in the $ARR_2PB$-Cre-PTEN flox/flox mice by Lenti viral transgenesis. The anticipation is that mice with this combined background will form spontaneously invasive carcinoma, in which elevated ILK signaling due to both ERG and PTEN mutations would act cooperatively with ERG expression to induce prostatic transformation. At various stages of spontaneous tumour promotion and progression, ILK expression could then be silenced or its activity inhibited, and the effect on tumorigenesis could be assessed. Hopefully, results from these mouse models will give an insight not only on the role of ILK in ERG-positive tumours, but also towards the development of preclinical animal models that would allow better prediction of the outcome and efficacy of anti-ILK therapy in prostate cancer.

Furthermore, analyses of ERG-positive prostate cancer clinical samples for coexpression of ILK and its downstream effectors, Snail and LEF-1, should be performed. In fact, assessing a cohort of metastatic prostate cancer specimens revealed that in samples where ERG was expressed at high levels E-cadherin expression was significantly suppressed (unpublished observations from Dr. Beatrice

Knudsen laboratory, Fred Hutchinson Cancer Research Center). This observation further supports the hypothesis that ERG aberrant expression leads to the development of aggressive prostate cancer phenotypes through induction of EMT, and therefore, that ILK might play an important role in ERG-positive tumors.

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# Appendix

## Supplementary Material

| | RWPE1 | | BPH1 | | RWPE1 | | BPH1 | |
|---|---|---|---|---|---|---|---|---|
| Name | E/M ratio | p value.adj | E/M ratio | p value.adj | Mock | fERG | Mock | fERG |
| Integrin, alpha 10 | 0.970344 | 0.9898102 | 2.645311 | 0.2298644 | 7.988721 | 7.751805 | 5.363323 | 14.18765 |
| Integrin, alpha 11 | 3.898358 | 0.0050436 | 2.454983 | 0.0469568 | 43.6986 | 170.3528 | 33.3293 | 81.82289 |
| Integrin, alpha 2 | 0.214663 | 0.0134396 | 0.035612 | 0.000022 | 4819.004 | 1034.463 | 10007.15 | 356.3769 |
| Integrin, alpha 2b | 2.16618 | 0.0742322 | 16.18338 | 0.0000042 | 53.30986 | 115.4788 | 18.02326 | 291.6773 |
| Integrin, alpha 3 | 0.062222 | 0.000019 | 0.022768 | 0.0000006 | 17974.29 | 1118.399 | 9614.02 | 218.8914 |
| Integrin, alpha 4 | 2.132616 | 0.0200562 | 5.115753 | 0.0000455 | 29.69473 | 63.32746 | 21.31512 | 109.0429 |
| Integrin, alpha 5 | 0.200399 | 0.0000269 | 1.037336 | 0.9298592 | 2316.571 | 464.2385 | 464.0087 | 481.333 |
| Integrin, alpha 6 | 0.148671 | 0.0018597 | 0.024541 | 0.0000035 | 15640 | 2325.218 | 38514.76 | 945.1757 |
| Integrin, alpha 7 | 14.53479 | 0.0000008 | 21.56763 | 0.0000001 | 481.8672 | 7003.84 | 227.0452 | 4896.825 |
| Integrin, alpha 8 | 3.811043 | 0.000014 | 10.14359 | 0 | 6.945741 | 26.47052 | 7.094994 | 71.9687 |
| Integrin, alpha 9 | 9.324435 | 0.0000027 | 7.431655 | 0.0000061 | 21.73558 | 202.672 | 45.54594 | 338.4817 |
| Integrin, alpha D | 1.782396 | 0.2633472 | 1.106392 | 0.8778362 | 9.786812 | 17.44398 | 7.877956 | 8.71611 |
| Integrin, alpha E | 2.617433 | 0.0162206 | 1.350615 | 0.4738033 | 4720.759 | 12356.27 | 5315.32 | 7178.953 |
| Integrin, alpha L | 4.4547 | 0.0000663 | 0.9829 | 0.9703669 | 7.777069 | 34.64451 | 7.960558 | 7.824436 |
| Integrin, alpha M | 0.670889 | 0.3923457 | 0.627762 | 0.2762505 | 78.69375 | 52.79479 | 41.25266 | 25.89687 |
| Integrin, alpha V | 0.519619 | 0.0593665 | 0.217473 | 0.0001737 | 5956.251 | 3094.982 | 10401.52 | 2262.047 |
| Integrin, alpha X | 1.004095 | 0.9991277 | 0.349826 | 0.3217297 | 5.881712 | 5.905796 | 17.46745 | 6.110573 |
| Integrin, beta 1 | 0.358959 | 0.0003704 | 0.414773 | 0.0011148 | 13027.97 | 4676.504 | 6838.519 | 2836.432 |
| Integrin, beta 2 | 3.13321 | 0.0250095 | 8.116436 | 0.0002938 | 371.4997 | 1163.987 | 13.49232 | 109.5096 |
| Integrin, beta 3 | 0.245868 | 0.0000361 | 1.252373 | 0.4134542 | 33.81041 | 8.312899 | 7.255208 | 9.086226 |
| Integrin, beta 4 | 0.046894 | 0.0001577 | 0.005996 | 0.0000008 | 41518.15 | 1946.938 | 14012.3 | 84.01257 |
| Integrin, beta 5 | 0.564989 | 0.0052285 | 0.508689 | 0.001321 | 6675.344 | 3771.494 | 6677.514 | 3396.776 |
| Integrin, beta 6 | 0.047686 | 0.0000034 | 0.013306 | 0.0000001 | 213.1086 | 10.16225 | 448.5777 | 5.968963 |
| Integrin, beta 7 | 0.388621 | 0.0118965 | 0.271013 | 0.0010682 | 341.5065 | 132.7164 | 297.2669 | 80.56315 |
| Integrin, beta 8 | 0.139685 | 0.0000004 | 0.033876 | 0 | 50.14553 | 7.004603 | 161.0842 | 5.456859 |
| Integrin, beta-like 1 | 0.812555 | 0.7756024 | 0.296144 | 0.0198725 | 12.99386 | 10.55822 | 29.41497 | 8.711056 |

**Table A1. Differential gene expression profile of integrins in fERG- and Mock-PrECs. Results from microarray analysis to profile gene expression in fERG- and Mock-PrECs.** Total RNA was isolated in triplicate and Agilent Low RNA Input Linear Amplification Kit PLUS was used to generate fluorescent cRNA of poly A+ RNA for one-color processing. Cy3-labelled probes were hybridized on Agilent 44K Whole Human Genome 1-color Gene Expression Arrays (G4140-90040). Arrays were analyzed using GeneSpring GX 7.3.1 and normalized using Data Transformation: Set measurements less than 0.01 to 0.01; Per Chip: Normalize to 50th percentile and Per Gene: Normalize to median.

# Appendix

## Supplementary Material

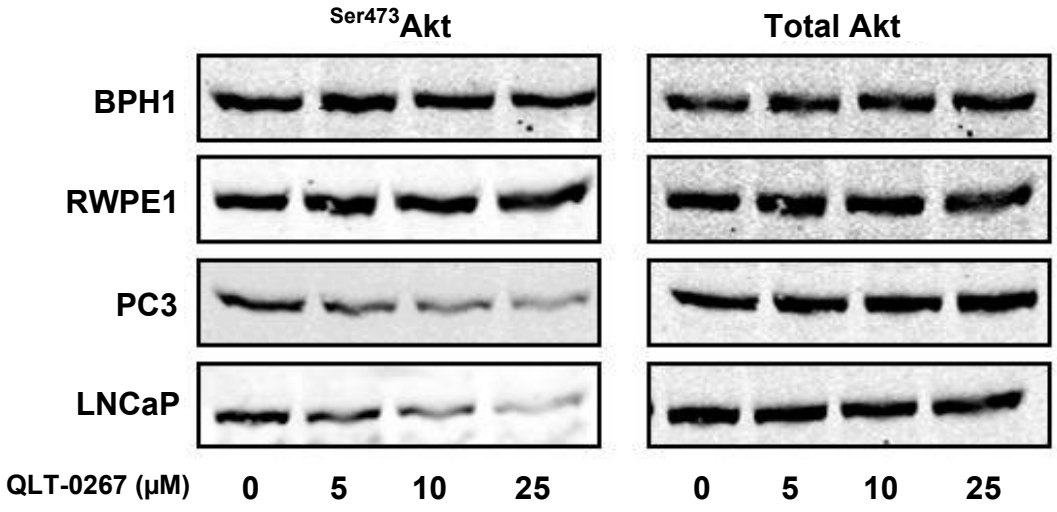


**Figure A1. Pharmacological inhibition of ILK suppresses Akt Serine-473 phosphorylation in prostate cancer cells but not in normal prostate epithelial cells.** Western blot analyses show that treatment of normal immortalized prostate epithelial cells, BPH1 and RWPE1, with the ILK inhibitor QLT-0267 (16 hours incubation) does not lead to inhibition of Akt phosphorylation. In constrast, prostate cancer cells PC3 and LNCaP show a dose dependent decrease in Akt phosphorylation upon treatment with QLT-0267.

# Appendix

## Supplementary Material

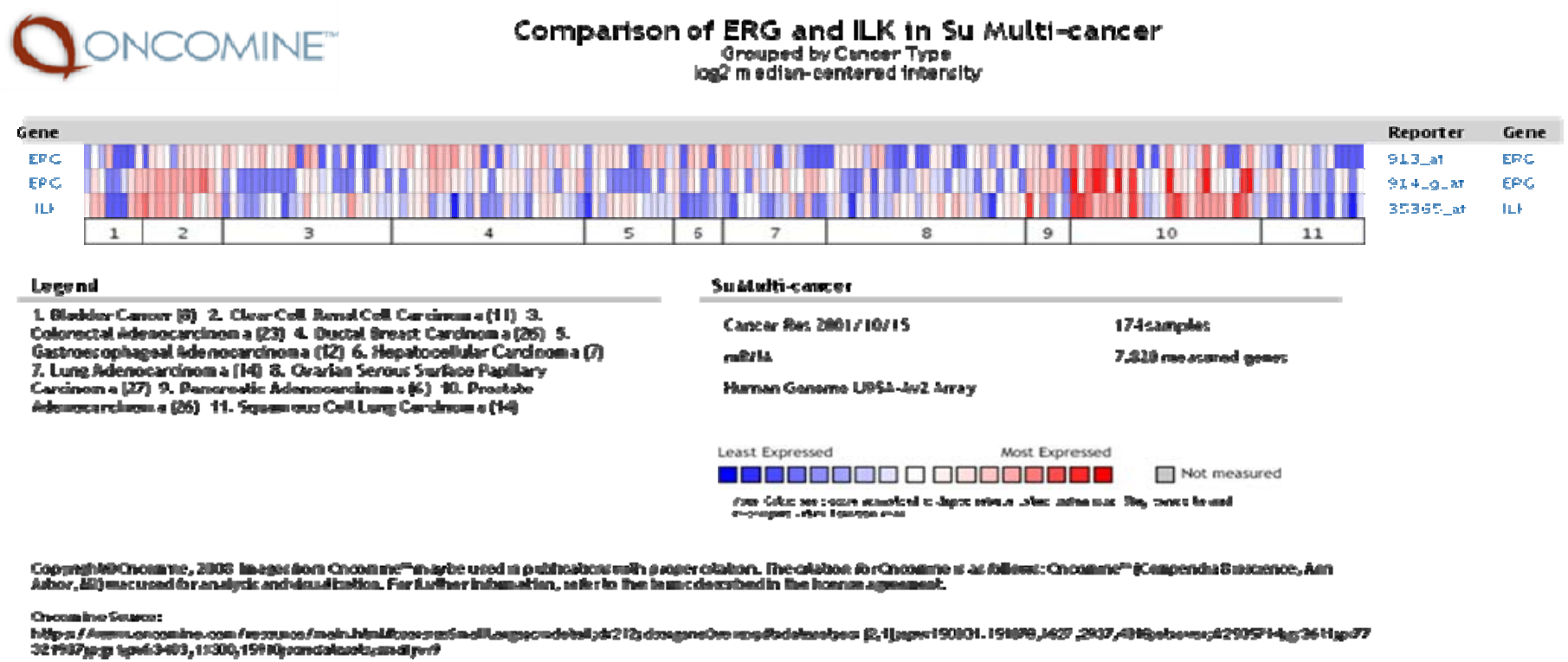


**Figure A2. Comparison of ERG and ILK mRNA expression levels in different types of cancer.** Data generated using Oncomine™ (Compendia Bioscience, Ann Arbor, MI) for analysis and visualization. Number "10" shows the correlation between ERG and ILK expression in prostate cancer.