THE ROLE OF INTEGRIN-LINKED KINASE IN TUMOUR ANGIogenesis AND INFLAMMATION

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF COMBINED DOCTOR OF PHILOSOPHY AND DOCTOR OF MEDICINE in

THE FACULTY OF GRADUATE STUDIES

(Biochemistry and Molecular Biology)

THE UNIVERSITY OF BRITISH COLUMBIA
April 2006

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Abstract

Integrins act as transducers of extracellular matrix-mediated cell signaling. A critical
signaling component downstream of integrin engagement is integrin-linked kinase (ILK),
a serine/threonine protein kinase and adaptor protein, that interacts with the cytoplasmic
domains of β1 and β3 integrins. ILK couples integrins and receptor tyrosine kinases to
the cytoskeleton, thus mediating multiple downstream signaling events that regulate cell
adhesion, survival, proliferation, migration and differentiation. Inappropriate ILK
activity as a result of deregulated upstream mechanisms or altered transcription, results in
epithelial cell transformation that recapitulates malignant cancer cell behaviour.

I provide novel data demonstrating that ILK plays an indispensable role in
regulating downstream targets that induce nitric oxide synthase (iNOS) expression and
nitric oxide (NO) production in murine macrophages, and cyclooxygenase-2 (COX-2), a
pro-inflammatory macromolecule, expression in both murine and human macrophages.
Cells treated with a highly specific small molecule ILK inhibitor or transfected with a
dominant-negative mutant of ILK showed decreased ILK activity resulting in inhibition
of lipopolysaccharide (LPS) stimulated iNOS and NO production and reduction in
COX-2 expression in a protein kinase B (Akt/PKB)-dependent manner. I also propose a
novel model for induction of NF-κB transcription, where phosphorylation of IkB at the
serine 32 position directly or indirectly by ILK or Akt/PKB targets IkB for degradation;
consequently NF-κB translocates to the nucleus to initiate transcription of pro­
inflammatory molecules. In addition, ILK and Akt/PKB are stimulated in a PI3-kinase
dependent manner.

I provide evidence for a new paradigm involving ILK in the improper production
of vascular endothelial growth factor (VEGF) in normoxic conditions, shedding light on
the role of ILK in tumor angiogenesis. Mechanistically, I show that activated ILK
stimulates the expression of hypoxia-inducible factor-1α (HIF-1α) transcription factor,
the major inducer of VEGF expression, through the phosphorylation of mammalian target
of rapamycin in an Akt/PKB dependent manner in prostate cancer cells. In a positive
feedback process, exposure of endothelial cells to VEGF stimulates a transient increase in
ILK kinase activity in a PI-3 kinase-dependent manner. Inhibition of ILK activity using
small interfering RNA or exposure to the ILK inhibitor uncouples endothelial cell
migration and proliferation in response to VEGF.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine trisphosphate</td>
</tr>
<tr>
<td>BM</td>
<td>Basement Membrane</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony-stimulating factor-1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DOC</td>
<td>Sodium deoxycholate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescent</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fl/fl</td>
<td>floxed/floxed</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
</tr>
<tr>
<td>INOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>MEOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal Goat Serum</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PI 3-K</td>
<td>Phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl Fluoride</td>
</tr>
<tr>
<td>PTEN</td>
<td>Protein with tensin homology</td>
</tr>
<tr>
<td>SAGE</td>
<td>Serial analysis of gene expression</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SiRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue Microarray</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
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Acknowledgements

I would like to take this opportunity to thank my supervisor Dr. Shoukat Dedhar for challenging me to reach far and guiding me through out my graduate career. I have had an ideal environment to carry out my research, having adequate time, appropriate pace and space, using sophisticated cell and molecular biology techniques working in the Dedhar lab, the MD/PhD program and the University of British Columbia Graduate Studies Program, as well, I have gained some experience in different research areas. I would also like to thank my supervisory and advisory committee, Dr. Alice Mui, Dr. David Huntsman, Dr. Aly Karsan, Dr. Anthony Chow and Dr. Lynn Raymond.

A special thanks to the past and present members of the Dedhar Lab, Julia Mills, Tim McPhee, Arusha Oloumi, Sarah Attwell, Armelle Troussard, Paul MacDonald, Virginia Gray, Nasrin Mawji, Severine Hennequart-Cruet, Nolan Filipenko, Sujata Persad and Larissa Ivanova. I would also like to thank the faculty, staff and future colleagues/fellow students in the Faculty of Medicine, in particular, Eric Tam, Gayle Pelman, Aruna Somasiri the past and present UBC MD/PhD students Cheng-Han Lee, Paul Yong, Ryan Hung, Jimmy Lee, Michael Rauh, Claire Sheldon, Suvendrini Lena, Amy Webber and Aaron Joe, and the Medical Class of 2006.

Finally, I would like to thank my partner Eric Tam, parents, sister and brother-in-law, my close and extended family for their support in this fantastic journey called graduate school.

This work was supported by a MD/PhD studentship from the Canadian Institutes of Health Research of Canada and the Canadian Cancer Society.
1 INTRODUCTION

Tumors are a heterogeneous mass consisting of extracellular matrix (ECM), cancer cells and normal cells, such as fibroblasts and infiltrating macrophages. Critical to the study of cancer is to understand cancer cell physiology in the context of cell-ECM and cell-cell interactions (Bissell et al., 1982; Bissell et al., 2005; Bissell and Radisky, 2001). Integrins are the key mediators of cell interactions with the extracellular environment (Hynes, 1987; Hynes, 2002). Intigrin-linked kinase (ILK) is a unique adaptor and kinase first discovered to bind to the cytoplasmic domain of integrins (Hannigan et al., 1996). In cancer, deregulated ILK activity is involved in several processes such as anchorage and growth signal independence, evasion of programmed cell death (apoptosis), sustained angiogenesis, and tissue invasion and metastasis (Hannigan et al., 2005) alterations in cell physiology which promotes formation of tumors and the manifestation of disease (Hanahan and Weinberg, 2000). Tumor growth can also be characterized in three phases: initiation, promotion and progression (Hanahan and Weinberg, 2000). Promotion of tumor growth relies on growth signals secreted by inflammatory cells in a paracrine manner to promote the proliferation of malignant cells (Balkwill and Mantovani, 2001). For tumors to progress, they require an adequate blood supply (Folkman, 1976; Folkman and Cotran, 1976). In this introductory chapter, I will review integrins, the extracellular matrix, and ILK. Furthermore, I will focus on the cellular and molecular mechanisms mediated by ILK in cancer progression, blood vessel formation and inflammation.
1.1 Extracellular Matrix

During normal embryonic development and in adult life, signaling between extracellular and intracellular components needs to be precisely coordinated and integrated in a spatial-temporal specific manner. The proper regulation of differentiation signals of cell growth and death must be critically balanced to prevent the manifestation of disease such as cancer. Organs are organized into a framework of cells, stroma and extracellular matrix (ECM), a visible network of secreted proteins, glycoproteins and polysaccharides that include collagen, fibronectin, vitronectin and laminin (Boudreau and Jones, 1999). This surrounds all the connective tissues, and underlines all the epithelial and the endothelial sheets of cells (Bissell et al., 2005; Bissell and Radisky, 2001). This provides not only a physical scaffold for the support of tissues and organs, but also serves as the basin for storage, release and presentation of growth factors, and a direct mediator of cell signaling (Bissell et al., 2005). Through interactions with specific transmembrane receptors, the ECM components have profound control over cell survival, proliferation, morphogenesis, migration, polarity and differentiation in a temporal and spatial-specific manner (Bissell et al., 2005). Many transmembrane receptors are responsible for interacting with the ECM, including heparan sulphate proteoglycans, syndecans, hyaluronan binding molecules, and integrins (Zamir and Geiger, 2001). Integrins, a family of cell surface receptors, are the primary mediators of mechanical and chemical signals involved in regulating cytoplasmic kinase activities, growth factor receptors, ion channels, and actin cytoskeleton organization during cell survival, proliferation, differentiation and migration (Hynes, 1987). Indeed, integrins are dynamic, bidirectional interactions that link the intracellular environment to macromolecular
extracellular assemblies. They regulate many intracellular signaling pathways through integrin-mediated cell adhesion and integrin clustering (outside-in signaling). These pathways are vital in regulating such diverse processes as embryonic development, cell survival, cell cycle progression, growth, differentiation, motility, and gene expression (Boudreau and Jones, 1999; Clark et al., 1998; Dedhar and Hannigan, 1996; Giancotti and Mainiero, 1994; Hynes, 1987). As well, integrins may transform from inactive to active state in response to intracellular signals (inside-out signaling). This regulation can then modulate the adhesive affinity of integrins to the ECM, and can activate ECM-degrading enzymes (Brakebusch et al., 2002). In addition to ECM and cytoplasmic proteins, integrins also associate with several membrane proteins, including IAP (Integrin Associated Protein), caveolin, and the tetraspanins (TM4) (Schwartz and Ginsberg, 2002). It is thought that these proteins function to associate integrins with intracellular signaling proteins and pathways to elicit specific responses to extracellular signals.

1.2 Integrins

Integrins can be found in all metazoan species and are composed of non-covalently linked, single span α- and β- subunits, each consisting of a long extracellular domain (up to 1104 residues and 78 residues respectively) and a short intracellular domain (Whittaker and Hynes, 2002). In mammals, 18 α- and 8 β-subunits have been identified (Figure 1.2) (Hynes, 1987; Hynes, 2002) and are known to assemble into 24 α,β functional heterodimeric receptors (see figure 1.1). Integrins are so critical to life that defects in integrin expression and function are responsible for many human diseases, including cancer and autoimmune disease. Targeted knockout studies in various organisms and in tissue culture have underscored the essential roles integrins play in development (Hynes,
Integrins are highly selective, specific heterodimers that only bind a specific set of substrates. Many of the integrins, such as α5β1, αvβ1 and αvβ3 integrins, recognize peptide sequences of ECM components that include the tripeptide arg-gly-asp (RGD), a sequence shared by several ECM proteins, such as fibronectin and vitronectin (Ruoslahti and Pierschbacher, 1987).

Although integrins are capable of binding various ECM components to influence cell behaviour, the cytoplasmic tail itself does not have any known intrinsic catalytic activity, unlike receptor tyrosine kinases; therefore, integrins are dependent on intracellular proteins to relay messages. Likewise, intracellular protein signals are capable of manipulating the avidity and affinity of the extracellular integrin domain for particular ligands. The RGD sequence interacts with integrins to activate several tyrosine kinases and protein kinase signal transduction pathways (Miyamoto et al., 1996). For example α5β1 and αvβ3 integrin engagement results in the up-regulation of Bcl-2 and cell survival (Ruoslahti and Pierschbacher, 1987; Ruoslahti et al., 1987; Zheng et al., 1995).
1.2.1 Molecular Mechanisms in the Regulation of Integrin Function

The cell surface conformation of integrins is critical to their function. Integrins can be found in three states, the inactive (bent form), primed (extended form) and active state (ligand-bound form) (Legate et al., 2006). The majority of the integrins are in the inactive conformation, characterized by bent extracellular domains that hide the ECM-binding pocket. This conformation is stabilized by interactions between integrin transmembrane domains, membrane-proximal extracellular domains and a salt bridge between the α- and β- integrin cytoplasmic domains (Legate et al., 2006). The present model suggests that phosphatidylinositol phosphate kinase type-1γ (PIPK1γ) and talin, a cytoskeletal protein, complex to interact with the cytoplasmic tail of the β integrins (Critchley, 2004). This causes the α- and β- cytoplasmic tails to dissociate, inducing the
integrin extracellular domains to extend and unmask the ligand-binding site to adopt the primed conformation. This conformation allows the integrin to recognize and bind to specific ECM molecules. The separated integrin cytoplasmic domains and talin form a base for the recruitment of other proteins to form focal adhesions. The addition of cytoplasmic and signaling proteins including talin, α-actinin, vinculin and filamin, adaptors such as paxillin, Rack-1, ICAP-1 and β3-endonexin and kinases such as Src tyrosine kinase and focal adhesion kinase (FAK) (Miyamoto et al., 1996), to the base occurs in a hierarchical and sequential manner that leads to the maturation of focal adhesions, clustering of active, ligand-bound integrins and the assembly of a multiprotein complex that is capable of linking integrins to the actin cytoskeleton and coordination of signaling pathways (Zaidel-Bar et al., 2003). These plaques also facilitate cross-talk between integrins and growth factor receptors, which will be further discussed in the next section. This section focuses on the pathways immediately associated with the formation of the focal adhesions.

1.2.2 Focal adhesions

Focal adhesions are sites on the cell where integrins and proteoglycan-mediated adhesions link to the actin cytoskeleton. Focal adhesions are composed of scaffolding molecules, GTPases, and enzymes including kinases, phosphatases, proteases, and lipases. There are several different focal adhesion structures that are defined by their subcellular location, size and composition. Focal complexes are small focal adhesions at the periphery of the spreading or migrating cells. They are regulated by cytoskeletal proteins such as Rac and Cdc42 (Nobes and Hall, 1995; Nobes et al., 1995) and precede
the larger focal adhesions that are regulated by Rho activity (Chrzanowska-Wodnicka and Burridge, 1996; Ridley and Hall, 1992). Focal adhesions are structures found at the cell periphery and center. They are associated with the ends of stress fibers in cells cultured on two-dimensional rigid surfaces. Fibrillar adhesions are elongations of focal adhesions that specifically contains α5β1 integrin and tensin (Pankov et al., 2000). The formation and maturation of the focal adhesion is a hierarchical sequence of integrin clustering and protein aggregation, resulting in a focal platform for signal transduction initiation. Intensive efforts have been made to dissect and distinguish the components of these structures and elucidate their specific roles. A key component of the focal adhesion is the focal adhesion kinase, FAK (Hauck et al., 2002), which mediates signal transduction downstream from integrin molecules that have engaged the matrix. FAK can directly or indirectly interact with the cytoplasmic tail of in the β integrins. Upon integrin engagement, FAK becomes activated through autophosphorylation, the activated PI-3 kinase which produces secondary phospholipid messengers which, in turn, activates ILK and its downstream signaling components such as Akt/PKB to promote cell proliferation. FAK is a pivotal tyrosine kinase, which also recruits Grb and SOS, and initiates the Ras/Raf cascade that diverges to promote proliferation and survival.

Of particular interest is the formation of the Integrin-linked kinase/PINCH/Parvin (IPP) multiprotein complex that forms a platform at the focal adhesion and recruits more signaling and structural proteins (Legate et al., 2006).

Prior to going into more details about the IPP complex, it is worthwhile to briefly discuss another focal adhesion protein. It appears that FAK is upstream of ILK, but new evidence demonstrates that ILK may regulate FAK (Cohen and Guan, 2005).
1.3 Integrin-linked kinase (ILK)

Integrins do not have an intrinsic catalytic activity but are complex mechanochemical sensors and signal transducers of extracellular and intracellular signals. They also have important roles in bringing together the associated proteins required to modulate a myriad of intracellular activities. In fact, it is these associated proteins that define and initiate signals to modulate cell function and transcription, and if not tightly regulated, will lead to profound changes in a cell that confer a selective advantage to progressing to a disease state such as cancer. In cancer research, studies have focused on identifying and elucidating the protein mediators associated with integrins, and the dissection and investigation of the physiological integrin-mediated and growth factor receptor-mediated signals involved in orchestrating the network of pathways that are linked to functional activities in the cell. Therefore, further investigation of integrin-associated proteins will provide more insight into their roles in modulating cell function. To further investigate the multiple integrin-mediated pathways, the Dedhar lab focused their efforts on the study of integrin-associated proteins, specifically the proteins that are or would be associated with the cytoplasmic tail of the β-integrin, in the hope of finding a downstream therapeutic target for the control of cancer progression. This led to the discovery of Integrin-linked kinase (ILK).

1.3.1 ILK Structure and Function

Integrin linked kinase (ILK) was identified 10 years ago in a yeast-2 hybrid screen as a protein capable of interacting with the cytoplasmic tails of β1 and β3-integrins (Hannigan et al., 1996). Since the discovery of ILK, there has been a large accumulation of
evidence, both *in vitro* and *in vivo*, from a number of laboratories consistently demonstrating that ILK is an important adaptor protein in regulating actin polymerization and an essential serine-threonine kinase capable of initiating downstream signaling cascades critical for microenvironment-responsive cell signals, normal development and cancer progression (Hannigan et al., 2005).

Integrin linked kinase consists of 452 amino acid residues and is arranged into three conserved functional domains (see figure 1.2). The N-terminal domain contains four ankyrin repeats, which mediate protein-protein interactions with LIM domain-containing adapter proteins, PINCH-1, PINCH-2 and Paxillin (Legate et al., 2006). The ankyrin repeat has been likened to a cupped hand structure in which the palm and the fingers are represented by the α-helix bundle and the β-hairpins, respectively (Sedgwick and Smerdon, 1999; Wu, 1999), and facilitates the localization of ILK to focal adhesions, sites where growth factor receptor and integrin-mediated pathways can be bridged to allow cross-talk. Immediately following the ankyrin repeats is a pleckstrin homology motif domain, which can bind phosphoinositol-3,4,5-trisphosphate (PIP3), a secondary messenger and PI-3 kinase product, to augment the kinase activity of ILK. The largest domain is the kinase catalytic domain located at the C-terminus. This domain mediates binding to the cytoplasmic tail of β1 and β3 integrins and has serine-threonine kinase activity (Figure 1.4). It is predicted to fold into a bi-lobated structure that is characteristic of other kinase domains (Dedhar et al., 1999; Wu, 1999). A sequence analysis of the ILK kinase domain reveals divergence at three generally highly conserved subdomains. In the highly conserved GXGXXG sequence of subdomain I, 2 of the 3 critical glycine residues are missing in ILK (NENHSG). As well, variance in subdomains VIB (HRDL) and VII
(DFG) also continues to fuel the debate of whether ILK is a protein kinase, however, the ILK sequence does not show any variance in the crucial subdomain VIII APE sequence, nor in the critical lysine residue between subdomain I and II which is necessary for the binding of ATP. Furthermore, other proteins lacking all three glycine residues in subdomain I have been identified as true protein kinases. To date, ILK has repeatedly been shown to phosphorylate a peptide representing the β1 integrin cytoplasmic domain (Hannigan et al., 1996), Akt/PKB at serine 473, and GSK-3 on serine 21/9 in vitro and in cells (Delcommenne et al., 1998; Hannigan et al., 1996; Persad et al., 2000; Persad et al., 2001a). Other physiologically important substrates which ILK can phosphorylate in vitro are myosin light chain, the adaptor protein β-Parvin/Affixin (Deng et al., 2002; Deng et al., 2001; Paralkar et al., 1992; Yamaji et al., 2001), PHI-1, KEPI, CPI-17, GSK3β, Myosin phosphatase target subunit 1 and αNAC (reviewed in (Hannigan et al., 2005). The C-terminus of ILK is also critical as it serves to bind to proteins associated with actin cytoskeleton rearrangement such as α-Parvin/CH-ILKBP/actopaxin, β-Parvin/affixin, γ-Parvin and Paxillin (Nikolopoulos and Turner, 2000; Nikolopoulos and Turner, 2001; Nikolopoulos and Turner, 2002), to facilitate diverse signaling pathways.
Figure 1.2  Schematic diagram of integrin-linked kinase demonstrating the multiple domains and highlighting the conserved sequence. (from (Hannigan et al., 2005))

1.3.2 ILK Chromosomal Localization and Animal Models

The ILK gene is located on human chromosome 11p15.5 – p15.4. (Hannigan et al., 1997) and is highly evolutionary conserved with homologues found in *C. elegans* and *drosophila*. In the mouse, ILK is located in the 7E1 and 9E1 – 3 region of chromosome 11 and shares 99% identity with the human gene (Hannigan et al., 1996; Li et al., 1997; Mackinnon et al., 2002; Nikolopoulos and Turner, 2001; Zervas et al., 2001). The phenotypes of both *Drosophila* and *C. Elegans* ILK mutants highlight the importance of ILK in integrin-mediated adhesion. In the *C. Elegans* pat-4 mutant, embryonic muscle cells were able to form integrin foci, but were unable to recruit vinculin, UNC-89, actin and myosin filaments, proteins normally found in focal adhesions (Mackinnon et al. 2002). In *Drosophila*, ILK mutants phenotype was embryonic lethal and embryos had
defects in muscle attachment secondary to the detachment of actin filaments, similar to the integrin mutants. As well, clones of cells lacking ILK in the adult wing formed wing blisters as the result of a lack of proper cell adherence (Zervas et al. 2001). ILK deficient mice die at the peri-implantation stage due to a failure in epiblast polarization as a result of abnormal F-actin accumulation at the sites of integrin attachment to the basement membrane and cavitate (Sakai et al., 2003). Mice with chondrocyte-specific disruption of ILK develop chondrodysplasia, characterized by a disorganized growth plate, dwarfism and early death as the result of decreased proliferation, adhesion, spreading and focal adhesion formation (Grashoff et al., 2003; Terpstra et al., 2003). The dependent PKB/Akt and GSK-3 phosphorylation, and thus the role of ILK as a kinase and signal effector continued to be debatable at that time. Overexpression of ILK in the mammary epithelium under control of the mouse mammary tumor virus promoter (MMTV) caused the formation of benign hyperplasia and mammary tumors ranging from papillary adenocarcinoma to spindle cell tumors (White et al., 2001). Moreover, phosphorylation of Akt/PKB at serine 473, and GSK-3 on 9/21 was observed. This emphasizes the role of ILK as a kinase signaling molecule. Furthermore, recent tissue-specific ILK knock-out models demonstrate the importance of ILK in embryonic development (Sakai et al., 2003) and organogenesis, specifically cortex and cerebellar development, (Mills et al., 2003; Mills et al., 2006; Niewmierzycka et al., 2005), and function, specifically in diapedesis (leukocyte migration), and blood vessel formation (Cho et al., 2005; Friedrich et al., 2004; Friedrich et al., 2002; Li et al., 2003a; Liu et al., 2005).
1.3.3 Integrin-linked kinase Interactions

Since its discovery, other groups have also shown that ILK binds directly to the cytoplasmic tails of β1 and β3 integrins (Pasquet et al., 2002; Plante et al., 2005; Yamaji et al., 2001). ILK also binds paxillin, through a binding site in its kinase domain, linking ILK to F-actin though interactions with α-parvin/CHILK-BP/actopaxin and the actin-binding adaptor molecule vinculin (Nikolopoulos and Turner, 2000; Turner et al., 1999; Wood et al., 1994). MIG2/kindlin-2 binds to the cytoplasmic tail of β1 and β3 integrins, as well as ILK, and associates with migfilin to create a bridge associating the ILK/Pinch/Parvin (IPP) (discussed in the following section) complex with filamin, F-actin and other integrins (Feng and Walsh, 2004; Tu et al., 2003) (see figure 1.3). These interactions appear to enhance clustering of integrins and provide another link between ILK and the actin cytoskeleton.

1.3.4 ILK/PINCH/Parvin (IPP) Complex

Following the priming of the integrin by talin and PIPKIγ but prior to ligand binding, ILK combines with parvin and PINCH in the cytosol to assemble into the IPP complex. ILK is the central component of the IPP complex, binding to PINCH proteins through the N-terminal ankyrin-repeat domain and parvins at the kinase domain. ILK then links the entire IPP complex to the cytoplasmic tails of β1 and β3 integrins. The IPP complex is then immediately recruited to the focal adhesion through interactions with other intracellular proteins, such as paxillin. Simultaneously, other proteins such as vinculin and focal adhesion kinase (FAK) are recruited to the nascent focal complex in a sequential manner (Zaidel-Bar et al., 2003). The stability of the individual IPP
components are dependent on complex formation as shown by RNA interference (Fukuda et al., 2003), as the lack of a complex results in an increased degradation of the IPP components. At least two other factors appear to be required to assemble the IPP complex into focal adhesions; namely, the adaptor molecules paxillin, and MIG/kindlin-2, which both bind directly to the IPP complex through the kinase domain of ILK (Gardner et al., 1996; Mackinnon et al., 2002; Nikolopoulos and Turner, 2000; Nikolopoulos and Turner, 2001; Nikolopoulos and Turner, 2002). The following section will summarize the known binding partners of the IPP complex and how signaling specificity is achieved through differential binding of molecules to PINCH and Parvin isoforms.
Figure 1.3  Schematic diagram of ILK/PINCH/Parvin complex found at the focal adhesions (this figure is taken from (Hannigan et al., 2005)).

1.3.5 Particulary Interesting Novel Cysteine-Histidine rich protein (PINCH) Interactions

Signaling specificity of the IPP complexes depends on the presence of the different PINCH or Parvin isoforms. For example, the ILK-PINCH1 complex transduces integrin-mediated signals that control cell spreading and migration, whereas ILK-PINCH2 complex cannot, therefore it is thought that accessory proteins which differentially bind to PINCH isoforms may be responsible for transducing signals that negatively or positively affect this cell behaviour. Ras-suppressor protein RSU1 is an accessory protein that negatively regulates growth factor induced Jun N-terminal kinase (JNK)
activity through interactions with PINCH1 (Tsuda et al., 1995; Vasaturo et al., 2000), alternatively, thymosin β4 binds to PINCH1 to upregulate ILK activity and positively influence migration and survival of cardiac cells (Bock-Marquette et al., 2004). The PINCH1 interaction with NCK2, an adaptor protein, is necessary for the localization of PINCH1 to the focal adhesion, but the relevance of this is not clear. In addition, studies demonstrate that PINCH1 shuttles between the nucleus and the cytoplasm in Schwann cells and may have a role in gene regulation or signaling between the cytoplasm and nucleus (Campana et al., 2003).

1.3.6 Parvin Interactions

There are three different isoforms of parvins, α-parvin/CHILK-BP/actopaxin, β-parvin/affixin and γ-parvin, all of which have overlapping abilities to bind other partners. The ILK-γ-parvin complex is involved initially in integrin signaling and establishing cell polarity during leukocyte migration (Yoshimi et al., 2006). Alpha-parvin/CHILK-BP/actopaxin is known to bind to F-actin directly and indirectly through paxillin (Attwell et al., 2003; Nikolopoulos and Turner, 2000) and interacts with ILK in a PI3-kinase dependent manner (Attwell et al., 2003). This later study further confirms ILK as a critical kinase and an adaptor necessary for the activation of downstream PKB/Akt and GSK-3 signaling. HIC5 also binds to α-parvin/CHILK-BP/actopaxin and is capable of shuttling to the nucleus where it modulates the expression of several genes (Kim-Kaneyama et al., 2002; Shibanuma et al., 2004). As well, α-parvin/CHILK-BP/actopaxin specifically binds to TESK1, a serine/threonine kinase that phosphorylates and regulates the actin-regulating protein coifin (Edwards et al., 1999). Likewise, studies demonstrate
that β-parvin/affixin binds α-actinin and α-PIX, a guanidine exchange factor, providing a connection between ILK, the IPP complex and actin-regulating GTPases, Rac1 and Cdc42 (Filipenko, 2005 #64; (Rosenberger et al., 2003). Alpha-PIX also regulate the actin dynamics and focal adhesion turnover by binding to PAK1, a Rac1/Cdc42 effector that regulates cytoskeletal dynamics through the LIM kinase-Actin Depolymerizing Factor-Cofilin pathway (Edwards, 1999 #731). In addition, α-PIX also binds to Calpain-4, the protease which cleaves talin an important step in the disassembly of focal adhesions and in cell migration (Rosenberger et al., 2003). As well, β-parvin/affixin has been implicated in membrane repair as it binds to the calcium-dependent membrane-repair protein dysferlin at the sarcolemma of skeletal muscle (Matsuda et al., 2005). This IPP complex forms a platform in which ILK mediated downstream signals and the recruitment of other proteins can occur (see Figure 1.3).

1.3.7 Signals Downstream of IPP Complex

Integrins not only provide the structural bridge between ECM and intracellular actin cytoskeleton, they are also, as previously described, signal transducers by themselves or in collaboration with other growth factor receptors or G-protein coupled receptors. The cross-talk or synergistic interactions between integrins and growth factor receptors mediates and coordinates intracellular signaling cascades downstream of integrin and growth factor receptor engagement, including the Ras/Raf-1/MEK/Erk1/2 pathway, Rho family GTPases, PI-3-Kinase, ribosomal S6 Kinase (RSK), Jun amino-terminal kinase (JNK), FAK and Paxillin, and p130CAS (Comoglio et al., 2003; Giancotti and Tarone, 2003; Lee and Juliano, 2004; Schwartz and Ginsberg, 2002). Proliferation and migration
are examples of cell processes which are dependent on these interactions for the initiation of growth-factor mediated signaling cascades.

Although transmembrane integrin and growth factor receptors are induced to cluster by matrix proteins, intracellular signaling mediated by growth factor receptors, depends on binding of the growth factor. In epithelial cells, proliferation signals from mitogens, such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF), have to collaborate with signals from integrins for progression of the cell cycle (Danen and Yamada, 2001; Pardee, 1989). In endothelial cells, basic FGF/αvβ3 and VEGF/αvβ5 signaling pathways are important for cell survival, but differentially activate Ras-Raf-ERK signaling (Alavi et al., 2003). Hood et al. proposed that these two signals independently account for protection of endothelial cells from distinct mediators of apoptosis in an ERK activated background (Hood and Cheresh, 2002). The αvβ3/bFGF pathway promotes an ERK-independent survival mechanism preventing stress-mediated death based on coupling to the mitochondria, whereas the αvβ5/VEGF pathway prevents integrin receptor-mediated death in an ERK-dependent manner. Together these two signals result in angiogenesis, but the differential activation of these signaling pathways impacts the distinct biological response of bFGF and VEGF. Some integrins are able to cross-talk with more than one type of growth factor receptor, such as αvβ3 integrin, which associates with growth factor receptors and modulates cell cycle progression in response to IGF-1, PDGF, and VEGF (reviewed by (ffrench-Constant and Colognato, 2004). Expression of integrins and growth factor receptors change as the cell responds to the environment. For example, to promote cell survival appropriate intracellular signals are initiated and emphasized. α5β1 integrin and epidermal growth factor receptor
(EGFR) expression is upregulated in normal rat intestinal epithelial cells during serum deprivation to differentially activate the PKB/Akt, but not Erk1/2 (Lee and Juliano, 2004), as cell survival becomes more important than cell proliferation. The interconnected networks of integrin and growth factor signaling are tightly regulated, therefore, any disruptions in either network could lead to a complete shut down of signaling or the propagation of signals that are no longer responsive to the primary stimulus. Both scenarios could result in autonomous growth and transformation.

1.3.8 Regulation of ILK

ILK is regulated by integrin engagement and secondary messenger products of the PI3 kinase. ILK clustering has been observed within adhesion sites in epithelial cells suggesting that ILK binds to primed and activated integrins (Li et al., 1999), and that ILK localization is concomitant with integrin activation (Attwell et al., 2003).

Studied more extensively for its role in cell survival, cell cycle progression and the regulation of transcription factors is the PI3 kinase signaling pathway. The class IA subgroup of the PI3 kinases are composed of a regulatory 85 kDa subunit and a 110 kDa catalytic subunit, and are activated by growth factor receptor tyrosine kinases. Binding of the p85 regulatory subunit to stimulated growth factor receptor tyrosine kinases occurs via interactions with SH2 domain or association with adaptor proteins. This results in the activation of the p110 catalytic subunit to phosphorylate membrane-bound PtdIns-4 (PIP) and PtdIns(4,5)P2 (PIP2), converting them to PIP2 and PtdIns(3,4,5)P3 (PIP3), respectively. The production of PIP2 and PIP3 are tightly regulated, but upon stimulation with growth/survival factors such as PDGF, nerve growth factor (NGF), insulin, or
insulin-like growth factor (IGF), PI3 kinase is activated and phosphoinositides are transiently produced for the duration of the stimulus (Cantley and Neel, 1999). It is thought that the sudden downregulation of this signal is due mostly to the activity of a phosphatase called phosphatase and tensin homolog deleted on chromosome 10, also termed MMAC1 or TEP1 (PTEN), which decreases the levels of PIP2 and PIP3 by dephosphorylating the 3 position (Stambolic et al., 1998). As well, PTEN has been shown to de-phosphorylate proteins such as FAK, although the in vivo significance of this finding requires further investigation (Vivanco and Sawyers, 2002).

ILK-dependent phosphorylation is regulated in a PI3 kinase-dependent manner. Addition of PIP3 or overexpression of PI3 kinase p110 catalytic α-subunit increases ILK-dependent kinase activity in vitro and cell culture studies, respectively (Delcommenne et al., 1998). In cells that lack PTEN, such as prostate cancer cells, ILK is constitutively active (Persad et al., 2000; Persad et al., 2001a; Persad et al., 2001b). The expression of thymosin-β4 in cardiomyocytes has been demonstrated to increase the activity of ILK, as measured by phosphorylation of Akt/PKB on serine 473 residue (Bock-Marquette et al., 2004). Thymosin-β4 binds to LIM domains-4 and -5 of PINCH1, and upregulates ILK activity, positively influencing migration and survival of cardiac myocytes. On the other hand, the catalytic activity of ILK is negatively regulated by the phosphatase ILK-associated protein (ILKAP), a serine/threonine phosphatase, which has been shown to reduce the kinase activity of ILK in vitro and the phosphorylation of GSK-3β in vivo, but not the phosphorylation of Akt/PKB (Kumar et al., 2004; Leung-Hagesteijn et al., 2001). Decrease in ILKAP expression and an increase in ILK activity have been thought to correlate with poor patient outcome with melanoma (Dai et al., 2003). DAB2/DOC2, a
tumour suppressor that is markedly downregulated in ovarian and colorectal carcinoma (Kleeff et al., 2002; Mok et al., 1998), was shown to inhibit proliferation and tumorigenesis, and induce anoikis or apoptosis with the lack of integrin stimulation, with a concomitant downregulation of ILK activity in xenograft ovarian tumors and breast cancer cells, respectively (Mok et al., 1998). Another protein phosphatase, stomach-cancer-associated tyrosine phosphatase-1 (SAP-1), induces apoptosis by inhibiting ILK kinase activity and Akt /PKB activation, though this phosphatase is thought to inhibit ILK activity indirectly by disrupting the focal adhesion (Takada et al., 2002). Work done by Mongroo et al. indicates that β-parvin/affixin inhibits ILK signaling downstream of receptor tyrosine kinases in breast cancer cells (Mongroo et al., 2004). Early studies demonstrate that ILK autophosphorylates in vitro, but this function in vivo requires further investigation (Zervas et al., 2001).

1.3.9 ILK as a Downstream Effector of Signaling Pathways

Molecular and genetic approaches have converged to confirm that ILK serves as a physical link between integrins and cytoskeleton, and is a key effector of signaling pathways during development. However, overexpression of ILK in epithelial cells results in anchorage-independent growth and cell-cycle progression, increased cell survival, epithelial-mesenchymal transformation and nuclear activation of β-catenin, migration, motility and invasion. A brief description of the known primary ILK effectors will be given prior to incorporating them into the context of the cognate pathways. The two main substrates of ILK are Akt/PKB and GSK-3 (see figure 1.4).
There are three isoforms of Akt/PKB: α which is the most abundant, β and γ. All three are composed of an N-terminal PH domain, a central kinase domain, and a C-terminal hydrophobic regulatory domain (Hill and Hemmings, 2002). Akt/PKB is regulated by upstream secondary messengers and secondary activating kinases. PIP2 and PIP3 both bind and localize Akt/PKB to the plasma membrane. This also induces a conformational change to expose the activation loop of Akt/PKB, which is subsequently phosphorylated at the threonine 308 site by 3-phosphoinositide-dependent kinase-1 (PDK-1) (Alessi et al., 1997a; Alessi et al., 1997b). PDK-1 also phosphorylates the
activation loop of other AGC family kinases such as 70 kDa ribosomal S6 kinase (p70^{s6k}), 90 kDa ribosomal S6 kinase (p90^{RSK}), serum and glucocorticoid-induced protein kinase (SGK), and protein kinase C isoforms (Alessi, 2001). For full activation, Akt/PKBα requires subsequent phosphorylation at serine 473 in the C-terminal hydrophobic regulatory domain (Alessi et al., 1997a) by a kinase termed PDK2 or hydrophobic motif kinase (HMK). There are multiple candidates for PDK2/HMK, including ILK, MAPKAP kinase-2, PKC isoforms, DNA-dependent protein kinase, ATM, PDK-1 and Akt/PKB, itself, and now mTOR/rictor (Bayascas and Alessi, 2005).

Akt/PKB is a potent inhibitor of apoptosis, or programmed cell death—an observation that can be partially explained by its regulation of the targets such as BAD, caspase-9, CREB, IKK and the forkhead transcription factors. BAD is a pro-apoptotic member of the Bcl-2 family that initiates an apoptotic cascade by binding to Bcl-XL on the mitochondrial membrane, and opening a channel to cause the release of cytochrome c into the cytosol (Datta et al., 1997). Akt/PKB blocks this cascade by phosphorylating BAD at serine 136, which promotes the association of BAD with 14-3-3, thus sequestering it in the cytosol. In addition, Akt/PKB inactivates the forkhead family of transcription factors by phosphorylation at multiple serine/threonine residues, rendering the transcription factors unable to upregulate a subset of pro-apoptotic factors such as p27 and Fas (Brunet et al., 1999; Burgering and Kops, 2002). Alternatively, CREB transcription factor is activated by Akt/PKB phosphorylation to increase the expression of the anti-apoptotic genes Bcl-2 and Mcl-1 (Du and Montminy, 1998). Akt/PKB also indirectly regulates NF-κB by phosphorylating and activating IKK-α, which, in turn, phosphorylates and targets IκB for degradation, allowing the release and translocation of
NF-κB to the nucleus to upregulate prosurvival factors, such as Bcl-XL, caspase inhibitors, and c-Myb (Barkett and Gilmore, 1999; Lauder et al., 2001). Akt/PKB can phosphorylate MDM2, which then binds to p53 and enhances its degradation, resulting in the inhibition of the p53 mediated cell cycle arrest, apoptosis and DNA (Mayo and Donner, 2001). Akt/PKB also inactivates glycogen synthase kinase -3 (GSK-3) by phosphorylation to promote cell cycle entry, and regulate glucose metabolism. As well, Akt/PKB phosphorylates mTOR to promote growth and translation of proteins (Granville et al., 2006).

GSK-3 was the first substrate of Akt/PKB identified (Cross et al., 1995). GSK-3 regulates both glucose metabolism and cell proliferation. Akt/PKB phosphorylates GSK-3 on serine 9, which results in its inactivation. This then results in the activation of several proteins, including cyclin D1, β-catenin, and the activator protein (AP-1) transcription factor. GSK-3 phosphorylates β-catenin at multiple sites, targeting it for ubiquitination and subsequent degradation. Therefore, when GSK-3 is inactivated (through Wnt signaling), this results in the stabilization and nuclear localization of β-catenin, which then associates with lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factor to promote the expression of cell cycle progression genes such as cyclin D1, c-myc and matrilysin (Hill and Hemmings, 2002). GSK-3 also affects cyclin D1 directly by phosphorylating it at threonine 286, targeting it for ubiquitination and subsequent degradation. Inactivation of GSK-3 by PKB/Akt prevents this phosphorylation, thereby increasing cyclin D1 levels (Diehl et al., 1998). GSK-3 also phosphorylates c-Jun, a component of the AP-1 transcription factor, rendering it inactive.
and unable to promote the expression of a wide variety of genes, including cyclin D1 and VEGF.

1.3.10 Role of ILK in Anchorage-Independent Growth and Cell Cycle-Progression

Proliferation is a tightly regulated complex cellular function. Normal cells pass through a limited number of cell divisions before they undergo cell death. When epithelia are placed on a surface, they proliferate until the surface of the dish is covered by a monolayer of cells just touching each other. Then mitosis ceases. This phenomenon, called contact inhibition results in growth arrest and occurs upon engagement of cadherins, homophilic cell-to-cell receptors, and integrins. Cancer cells are refractory to contact inhibition growth through abnormal expression of proteins that mediate cell cycle progression.

Overexpression of ILK increases the expression of several key components of the cell cycle machinery, including cyclin A, cyclin D1, and CDK4 proteins, and reduced the inhibitory activity of p27kip1 (Radeva et al., 1997). Several lines of evidence demonstrate that the inhibition of GSK3 as a result of ILK-mediated phosphorylation leads to activation of the transcription factor CREB (cyclic-AMP-responsive-element-binding protein) or β-catenin /TCF, 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; Persad et al., 2001b; Troussard et al., 1999). Two other studies further reinforce the role of ILK in promoting cell cycle progression through stimulation of cyclin D1 expression. The inhibition of PTEN suppresses the nuclear translocation of β-catenin, and the presence of ILKAP also decreases GSK3β phosphorylation and nuclear
translocation of β-catenin (Kumar et al., 2004; Leung-Hagesteijn et al., 2001). The regulation of the transcription factor AP-1 involves a GSK-dependent and -independent mechanisms. The inhibition of GSK3 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 AP-1 transcription factor activation (Quelo et al., 2004).

1.3.11 Role of ILK in Cell Survival and Anoikis

Integrin mediated cell-ECM anchorage promotes cell survival. If cells are prevented from establishing ECM attachment, this often induces a specific form of apoptosis (Kerr, Wyllie, Currie et al. 1972) referred to as anoikis (Frisch and Francis, 1994; Frisch and Screaton, 2001). The mechanisms regulating adhesion dependent cell survival occurs at the level of cytoplasm and in the nucleus. At the cytoplasmic level, it is thought that the underlying mechanism involves the recruitment of the apoptosis initiator, caspase-8, to the plasma membrane by unligated integrins or integrin β subunit cytoplasmic domain, where it becomes activated in a death receptor independent manner. When integrin ligation occurs, the integrin-caspase complex is disrupted and the cells can survive (Frisch and Screaton, 2001). The engagement of integrins induces the clustering of proteins at the focal adhesion to initiate cascades, such as the MAP kinase cascade, PI3 kinase /Akt/PKB pathway to modulate gene expression. This coordination of signals is important in cell migration and the ability for cells to survive without adhesion is advantageous for cancer cell propagation (Frisch and Screaton, 2001).
Molecular and genetic approaches have converged to confirm that ILK is a key effector of integrin function regulating ECM-dependent cell adhesion (Grashoff et al., 2004; Hannigan et al., 2005). The promotion of cell survival by ILK has been studied extensively and compiling evidence demonstrate that this regulation is primarily due to the ability of ILK to promote the phosphorylation and full activation of anti-apoptotic Akt/PKB on serine 473. Upon phosphorylation, Akt/PKB stimulates downstream anti-apoptotic pathways, such as activation of NF-κB, inhibition of forkhead transcription factors (FKHR/AFX/FOX) and inactivation of pro-apoptotic proteins such as BAD (Nicholson and Anderson, 2002). It is interesting to note that both ILK and NF-κB are highly activated in ERBB2-overexpressing breast cancer cells (Makino et al., 2004). Recent studies demonstrate that ILK regulates NF-κB activity in macrophages in an Akt/PKB-dependent manner, thus it is possible that ILK may be involved in the prosurvival functions of NF-κB. It is thought that by an uncharacterized mechanism, ILK might indirectly phosphorylate Akt/PKB indirectly through recruitment of another serine 473 kinase (Hill and Hemmings, 2002) or through α-parvin-mediated targeting of Akt/PKB to lipid rafts (Fukuda et al., 2003). The observation that the lack of PTEN in human cancers, such as prostate, breast and glioblastoma, with concomitant Akt/PKB phosphorylation further validates the critical role ILK plays in cell survival. Exposure of cells to the ILK specific small molecule inhibitor results in a decrease in Akt/PKB phosphorylation and subsequent sensitivity to apoptosis. In addition, Akt/PKB was recently shown to promote survival by transcriptional upregulation of PPARβ, and this control was found to be due, at least in part, to the transcriptional upregulation of ILK (Di-Poi et al., 2005a; Di-Poi et al., 2004; Di-Poi et al., 2005b).
1.3.12 Role of ILK in Epithelial-Mesenchymal Transformation (EMT) and Nuclear Activation of β-catenin

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 and Dedhar, 1999; Novak et al., 1998), downregulation of the epithelial markers cytokeratin18 and MUC1, and the upregulation of the mesenchymal markers LEF1 and vimentin (Barbera et al., 2004; Bravou et al., 2003; Bravou et al., 2006; Guaita et al., 2002; Hannigan et al., 1996; Marotta et al., 2003; Marotta et al., 2001; Novak and Dedhar, 1999; Novak et al., 1998; Persad et al., 2001b; Somasiri et al., 2001; Tan et al., 2001; White et al., 2001; Wu et al., 1998; Xie et al., 2004; Xie et al., 1998). This transformation is reminiscent of epithelial-mesenchymal transformation (EMT), a prominent hallmark of metastatic cancer cells (Bissell and Radisky, 2001; Thiery, 2002). Presently, the mechanisms underlying morphological change can be partially explained by the classical Wnt signaling pathway. In the absence of Wnt signaling, β-catenin is complexed with axin, APC, and GSK3-β and targeted for degradation following constant phosphorylation by GSK3-β (Nelson and Nusse, 2004). In the presence of Wnt signaling, β-catenin is uncoupled from the degradation complex and translocates to the nucleus, where it binds Lef/TCF transcription factor, thus activating genes that direct cell fate, polarity, and proliferation of tumor cells (reviewed in (Cavallaro, 2004; Cavallaro and Christofori, 2004)), such as cyclin D. A primary event that governs EMT is the disruption of the E-cadherin-mediated stable cell-cell interactions (Conacci-Sorrell et al., 2003; Conacci-Sorrell et al., 2002), ultimately leading to the mesenchymal phenotype.
The intracellular domain of E-cadherin interacts with α-, β-, γ- and p120-catenin, to link to the cytoplasmic actin filaments (Cavallaro and Christofori, 2004; Imai et al., 2004) and stabilize cell-cell interactions. The upregulation of the transcription factor SNAIL, a zinc finger protein that represses E-cadherins, by binding to the E-boxes present in E-cadherin promoter (Thiery, 2002), is thought to be the main mechanism that regulates E-cadherin expression (Batlle et al., 2000). The mechanism of regulation of SNAIL expression is still unclear, however decreased GSK-3β activity and the stimulation of the PI3-kinase/Akt/PKB pathway and activation of NF-κB have recently been implicated in regulating its transcription (Bachelder et al., 2005; Grille et al., 2004; Zhou et al., 2004).

To date, the mechanism underlying how ILK regulates SNAIL is unclear but recent studies suggest that ILK may promote the expression of SNAIL repressor through regulation of NF-κB transcription factor activation in an Akt/PKB dependent manner (Barbera et al. 2004; Rosano et al. 2005). Earlier studies propose Zeb-1 as the candidate for a transcriptional repressor of E-cadherin that is upregulated in cells overexpressing ILK, although this activation also appears to be indirect (Guaita et al., 2002). This pattern of the role of ILK in EMT has been validated with histopathology specimens (Bravou et al., 2003; Bravou et al., 2006; Marotta et al., 2003; Marotta et al., 2001). To date the molecular mechanisms of ILK in EMT are unclear and are the focus of future studies. ILK is also a signaling intermediate in transforming growth factor-β (TGF-β)-mediated EMT (Lee et al., 2004; Li et al., 2003a), a crucial event in kidney fibrotic disease and in the progression towards metastasis (Beck et al., 2001). Recent data suggests that PI3K-ILK-Akt pathway is independent of the TGFβ-induced Smad pathway that requires TGFβ-mediated epithelial to mesenchymal transition (Lee et al., 2004).
1.3.13 Role of ILK in Migration, Motility and Invasion

ILK-mediated EMT is accompanied by increased cell migration and invasion (Somasiri et al., 2001). It is well established that the RHO family of GTPases can control cell spreading and motility (Clark et al., 1998). The Rho family of proteins consists of about 20 genes that encode signaling molecules containing a small GTPase domain, and the family is divided into 5 groups: Rho-like, Rnd, CDC42-like, Rac-like and RhoBTB. These proteins regulate numerous cellular activities, including cell growth, differentiation, proliferation and tumor cell invasion. The Rho-like subfamily is necessary for the formation of stress fibers and focal adhesions in cells. Specifically, RhoC has been shown to promote tumor invasion (Clark et al., 1998) and the overexpression and constitutive activation of RhoC upregulate genes that enhance invasion in breast carcinoma cells (Wu et al., 2004). To support this, inhibition of RhoC results in inhibition of melanoma cell spreading (Collisson et al., 2003). The Rac-like subfamily induces membrane-ruffling, lamellipodia generation, and other membrane structures important for cell motility, such as filopodia. The Rac proteins can also stimulate growth transformation, Jun N-terminal kinase (JNK) activation, and promote cell survival.

Filipenko et al. (2005) recently showed a critical role for ILK in the early events of cell attachment and spreading resulting from a dramatic reorganization of the actin cytoskeleton (Filipenko et al., 2005). They demonstrated that ILK is a key player in this cytoskeletal reorganization by activation of the small GTPases, Rac and CDC 42 via the guanine nucleotide exchange activity of α-PIX. The absence of ILK activity and
association with α-PIX results in a delayed or decreased ability of the cell to adhere to extracellular matrix and spread (Li et al., 2003b; Turner et al., 1999).

1.3.14 Role of ILK in Regulating Myosin Light Chain (MLC)

The contraction and relaxation of the smooth muscle is regulated by transient reversible phosphorylation of the myosin light chains by calcium/calmodulin-dependent myosin light-chain kinase (MLCK) and dephosphorylation by myosin light chain phosphatase (MP). Inhibition of MP is thought to be responsible for calcium sensitization of smooth-muscle contraction. ILK promotes contraction by phosphorylating and inactivating the myosin phosphatase target subunit in the absence of calcium. This suggests that ILK may modify the myosin phosphatase (MP) activity (Muranyi et al., 2002), and regulate calcium-sensitization and -independent contraction of smooth muscles by directly phosphorylating myosin light chain or inhibiting MP. ILK is also capable of phosphorylating the myosin light chain phosphatase inhibitor proteins CPI-17 and PH-1, thus inhibiting myosin light chain phosphatase (MLCP) activity bound to myosin. In addition, ILK phosphorylates KEPI, a phosphorylation-dependent type-1 protein phosphatase inhibitor at threonine 73, resulting in an increase in inhibition of the phosphatases PP1C and myosin phosphatase MPH (Erdodi et al., 2003). This presents another possible way ILK may mediate cell migration and morphology, and a novel means by which ILK affects signaling pathways by inhibiting protein phosphatases (Deng et al., 2002). Moreover, Huang et al. showed that ILK activity and localization to focal adhesions is induced in response to the induction of myogenic differentiation, and is required in myogenic differentiation and myotube formation (Huang et al., 2000).
1.3.15 Role of ILK Invasion

Invasion is a process coordinated between malignant and normal cells, the ECM and stroma. Invasion is dependent, in part, on the ECM, ECM-receptors, including integrins and a balance of ECM-degrading enzymes, such as matrix metalloproteinases (MMPs) and their inhibitors, known as tissue inhibitors of metalloproteinases (TIMPs). With the use of genetic cell studies, enzymology and the highly specific small molecule ILK inhibitor Troussard et al (2000) show that ILK stimulates AP-1 activity that results in an increase in MMP-9 (matrix metalloproteinase-9) expression (Troussard et al., 2000) and subsequent ease in invasion into matrigel by intestinal and mammary cells overexpressing ILK. Similarly, this was also shown by inhibiting of ILK activity in glioblastoma cells (Koul et al., 2005). More recently, Zhiyong et al. demonstrate that ILK regulates osteopontin-dependent matrix metalloproteinase-2 (MMP-2) and urokinase-type plasminogen activator (uPA) expression to convey metastatic invasion in murine mammary epithelial cancer cells (Zhiyong et al. 2006).

1.3.16 Role of ILK in Human Disease

To date, ILK has been implicated in multiple cancers and non-cancer diseases and has been shown to be expressed in diseased tumors, specifically cancer and scar contractures (Levinson et al., 2004). ILK expression and possible activity are altered in a variety of human cancers. By immunohistochemistry and immunoblotting, elevated ILK expression has been detected in the following human cancers: prostate (Graff et al., 2001; Kieffer et al., 2005; Levinson et al., 2004; Qi et al., 2005), familial and sporadic colon
(Bravou et al., 2003; Bravou et al., 2006; Marotta et al., 2003; Marotta et al., 2001),
gastric (Ito et al., 2003), ovarian (Ahmed et al., 2004; Ahmed et al., 2003), breast cancer
(reviewed in (Hannigan et al., 2005)), primitive ectodermal tumors and Ewing’s sarcoma
(Chung et al., 1998), pancreatic (Sawai et al., 2006), anaplastic thyroid (Younes et al.,
2005), and melanoma (Dai et al., 2003). In addition, mounting evidence suggests that
the treatments which target constitutively active ILK in PTEN lacking glioblastomas may
be promising (Koul et al., 2005; Morimoto et al., 2000; Nadjar et al., 2005; Xie et al.,
2004).

In melanomas, gastric and colon cancers, increased ILK expression correlates
with a higher-grade of disease and with metastases. Specifically, in human colon cancer,
ILK overexpression results in activation of β-catenin, down-regulation of E-cadherin and
activation of the Akt-FKHR pathway (Bravou et al., 2006). Moreover, ILK activity is
associated with interleukin-1α-induced cancer progression of pancreatic cancer and poor
patient survival (Sawai et al., 2006). In prostate cancer, ILK expression has also been
demonstrated to correlate positively with tumor grade and inversely with patient survival
rates. Interestingly, ILK upregulation has been observed in response to ionizing radiation
of lung cancer cells, hypoxia of hepatocellular carcinoma cells and hyperthermia of
prostate cancer cells (Cordes, 2004; Zhang et al., 2003).

In cancer cells, deregulation of ILK can occur at the transcriptional level.
Although, the mechanisms responsible for the increase in ILK expression are not well
delineated, recent studies demonstrate that ILK transcription is stimulated in
keratinocytes by the nuclear receptor peroxisome-proliferator-activated receptor-β/δ
(PPARβ/δ) (Di-Poi et al., 2005a; Di-Poi et al., 2004). This transcription factor is
regulated by APC, a tumor suppressor, in colon cancer cells and promotes survival of keratinocytes by activating phosphorylating and activating Akt/PKB. Studies by Li et al. suggest that kidney cells stimulated by TGFβ increase ILK expression through the activation of SMAD2 transcription factor (Li et al., 2003a). As well, ILK has been demonstrated to be upregulated in different stages of asbestos-induced carcinogenesis in rats (Sandhu et al., 2000) and the ILK pathway was upregulated in hexachlorobenzene-induced gender-specific rat hepatocarcinogenesis (Plante et al., 2005).

1.4 Angiogenesis

Angiogenesis is the formation of blood vessels from pre-existing blood vessels. This is a major mechanism that underlies physiology, such as wound repair, and pathological neovascularization in adults, rheumatoid arthritis, post myocardial infarction, diabetic retinopathy and tumor progression (Carmeliet, 2005a; Hanahan and Folkman, 1996). Neovascularization is important for embryonic development and also encompasses arteriogenesis, venogenesis and lymphangiogenesis. Vasculogenesis and angiogenesis are coordinated and complex processes involving ECM and vascular endothelial cells and is regulated by various angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), interleukin-8 (IL-8), transforming growth factor-α (TGF-α) (Vinals and Pouyssegur, 2001). These factors promote cell proliferation, chemotactic migration, and capillary/tube-like network formation of vascular endothelial cells in vitro and in vivo. The endothelial-specific mitogen VEGF has been shown to be a key positive regulator of tumor angiogenesis including glioblastoma, colon and breast (reviewed in (Carmeliet, 2005a; Carmeliet,
The interactions between ECM and the cells are required for new blood vessel formation. Integrins cooperate with the VEGF receptors to promote activation of an *in vitro* angiogenic program in vascular endothelial cells.

Cell response to oxygen deprivation drives the expression of many genes important for angiogenesis, red cell production, and glycolysis. These include key vascular and hematopoietic growth factors, such as VEGF, erythropoietin, platelet-derived growth factor (PDGF), and TGF-α (Semenza, 2000). A major VEGF transcription factor is HIF-1α. The transcription of HIF-1α is regulated through the regulation of mammalian target of rapamycin (mTOR), in turn mediating 4E-BP1 (Semenza, 2003).

Briefly, mammalian TOR (mTOR) is a large protein kinase that exists in two distinct complexes within cells. One complex consists of mTOR, GβL and Raptor (regulatory-associated protein of mTOR) (Kim et al., 2002; Kim et al., 2003) and the other complex is comprised of mTOR, GβL and Rictor (Kim et al., 2002; Kim et al., 2003). The first complex is sensitive to rapamycin and regulates cell growth by phosphorylating S6K1. The latter complex is not sensitive to rapamycin and its cellular function is unclear (Sarbassov et al., 2004). Studies demonstrate that upon activation of mTOR by Akt/PKB phosphorylation, mTOR then forms a complex with GβL and raptor, which increases mRNA translation via activation of S6-kinase and inhibition of eIF4E binding protein (4E-BP) (Hay, 2005).

Transcription of HIF subunits is constitutive, however, HIF-1α proteins undergo propyl hydroxylation and are targeted for ubiquitination by the von Hippel-Lindau (VHL) protein and degraded by a proteasome (Ivan and Kaelin, 2001). Under hypoxic or growth factor-induced conditions, unhydroxylated HIF-1α subunits avoid posttranslational
modification, become stabilized and complex with HIF-β subunits to drive transcription of hypoxia-induced promoters (reviewed in (Semenza, 2003). Loss of VHL function results in constitutive HIF stabilization and predisposes to particular tumors, such as renal clear cell carcinomas, cerebellar hemangioblastomas, retinal angiomata and pheochromocytomas, all of which have a major vascular component (Hughson et al., 2003; Semenza, 2003).

Tumor growth and metastasis depends upon angiogenesis, a process by which quiescent vasculature is induced to sprout new capillaries. Avascular tumors cannot expand in size because of a lack of blood supply bringing nutrients and oxygen, and removing waste, but their ability to switch on the production of angiogenic factors explains how they can trigger neovascularization, maintain oxygen-dependent ATP production, expand, and metastasize (Folkman, 1976; Folkman and Cotran, 1976). This cellular activity requires several well orchestrated cell-matrix, cell-cell and signaling pathways that lead to the coordination of physiological cellular activities including proliferation, survival, invasion and migration.

1.4.1 Role of Extracellular Matrix in Angiogenesis

The conformation and abundance of the different components of the ECM regulate and direct cellular processes involved in angiogenesis. Signals from the ECM to the cytoskeleton are transduced through specific integrins. The major integrins responsible for these interactions are α2β1, α1β1 and ανβ3, α5β1, which are collagen and fibrin/fibronectin receptors, respectively (Davis and Senger, 2005).
The ECM components that are encountered by endothelial cells during sprouting angiogenesis, include interstitial fibrin and collagen, which themselves are capable of supporting chemotactic migration alone (Davis and Senger, 2005; Dejana et al., 1985). Endothelial cell proliferation and survival are highly dependent on adhesion to the ECM by integrins (Davis, 2005), which activates the p44/p42 (Erk1/Erk2) mitogen-activated protein kinase (MAPK) signal transduction cascade and suppresses Fas-induced apoptosis (Aoudjit and Vuori, 2001).

Interstitial collagen, the fibrin matrix, and basement membrane laminin differentially regulate various stages of angiogenesis. It has been proposed that laminin-1 rich basement membranes induce persistent integrin-dependent activation of GTPase Rac in endothelial cells which results in the expression of matrix degradative enzymes such as MMPs and ADAMs. Upon degradation of the basement membrane, endothelial cells are exposed to interstitial collagens, which activate signaling pathways that drive cytoskeletal reorganization and sprouting morphogenesis. Cell adhesion to interstitial collagen I results in the activation of both Src and Rho and suppression of protein kinase A (PKA). This promotes the formation of prominent actin stress fibers which mediates endothelial cell retraction and capillary morphogenesis. In addition, collagen I mediated activation of Src disrupts VE-cadherin from cell junctions and promotes disruption of cell-cell contacts, ultimately transforming the cell to have invasion and migration capabilities. During vessel maturation, laminin continues to play an intimate role in directing endothelial cell morphology. In particular, Cdc42 and Rac1 are activated for lumen formation while proliferative pathways such as Ras-MAPK and NF-κB suppressed. (Klein et al., 2002; Mettouchi et al., 2001). The ECM can further regulate angiogenesis
by binding various angiogenic cytokines such as fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) (Brader and Eccles, 2004). This acts as a reservoir for coordinating the spatial and temporal specific release of proangiogenic factors.

1.4.2 Role of Growth Factors and Receptors Associated with Angiogenesis

Numerous inducers of angiogenesis have been identified, including the members of the VEGF family, angiopoietins, transforming growth factor-α and -β (TGF-α and-β), platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNF-α), interleukins, chemokines, and a member of the FGF family. These soluble factors crosstalk with integrin-mediated signaling to promote or inhibit blood vessel formation. This particular section of this chapter will focus on two of the above angiogenesis inducers, FGF and VEGF.

The FGF family consists of at least 22 distinct family members, which have been identified in a variety of organisms (Gospodarowicz, 1974; Ornitz and Marie, 2002) and are involved in the entire program for the acquisition of the angiogenic phenotype (reviewed by (Presta et al., 2005). During embryonic development, FGFs play a critical role in morphogenesis, and in adults, these proteins control the nervous system, tissue repair, wound healing and tumor angiogenesis (Eswarakumar et al., 2005).

Unlike other growth factors, FGFs act in concert with heparin or heparin sulfate proteoglycans (HSPGs) to activate FGF receptors. These receptors are expressed primarily in skeletal tissues and are overexpressed in a variety of human diseases such as lymphoma, prostate and breast cancer. In response to FGF stimulation, a variety of
signaling proteins are phosphorylated including Src homology (Shc) proteins, phospholipase-cy, Signal Transducers and Activators of Transcription 1 (STAT1), Grb2-associated binder -1 (Gab1), and FGFR substrate 2α (FRS2α). Together, these phosphorylation events stimulate intracellular signaling pathways that control cell proliferation, differentiation, migration, survival and morphology (Holgado-Madruga et al., 1996; Holgado-Madruga et al., 1997) (Schaeper et al., 2000) (Maroun et al., 2000) (Kouhara et al., 1997). Pivotal in mediating downstream events is the tyrosine phosphorylation of the docking proteins FRS2α and FRS2β and the subsequent recruitment of Grb to form a FRS2α-Grb platform which is responsible for assembling positive and negative signaling proteins to mediate FGF signal translation (Holgado-Madruga et al., 1996; Holgado-Madruga et al., 1997) (Schaeper et al., 2000) (Maroun et al., 2000). Recruitment of Sos or Gab1 results in the activation of the Ras/MAP kinase signaling pathway and the PI3 kinase/Akt/PKB pathway, respectively (Holgado-Madruga et al 1996, Kouhara et al 1997, Wong et al. 2002). These signals continue until Grb-2-mediated recruitment of Cbl promotes ubiquitination of FGF receptor resulting in a downregulation of the FGF mediated signals (Kaabeche et al., 2004).

An intimate cross-talk exists among basic fibroblastic growth factor (bFGF) and the different members of the VEGF family during angiogenesis, lymphangiogenesis and vasculogenesis (reviewed in (Presta et al., 2005). Studies demonstrate that FGF and VEGF may exert a synergistic effect in different angiogenesis models (Xue and Greisler, 2002), and FGF may require VEGF/VEGFR activation for promoting angiogenesis (Castellon et al., 2002; Jih et al., 2001).
The VEGF family comprises six members, including placental growth factor (PIGF), VEGF-A, VEGF-B, VEGF-C and VEGF-D, that interact differently with three cell surface tyrosine kinase VEGF receptors. VEGF gene expression is up-regulated by a variety of factors such as hypoxia, acidosis and stimulation by growth factors including FGF, TNF, EGF, TGF-β and IL-1. To date, VEGF-A/ VEGF receptor 2 (VEGFR-2) interaction appears to play a major role in blood vessel angiogenesis whereas VEGF-C and -D are mainly involved in lymphangiogenesis through their interaction with VEGFR-3, which is expressed on lymphatic endothelia (reviewed in (Ferrara et al., 2003). VEGFR-2 is the predominant tyrosine kinase receptor in angiogenic signaling and is involved in regulating endothelial cell migration, proliferation, differentiation and survival as well as vessel permeability and dilatation. Upon dimerization, VEGFR-2 undergoes tyrosine phosphorylation at multiple sites that serve as binding sites for adaptor molecules such as Nck, Grb-10, Grb-2, Sck, human cellular protein tyrosine phosphates A (HCTPA) and the regulatory p85 subunit of PI3-kinase (reviewed in (Cebe-Suarez et al., 2006). In particular, phosphorylation of VEGFR at tyrosine 1175 has been implicated in activation of many phospholipase Cγ-1 (PLCγ-1)-dependent signaling pathways (Takahashi et al., 2001). VEGFR-2 is inactivated by internalization into endocytic vesicles and direct dephosphorylation by SHP-1 and SHP-2. VEGFR-2 dependent mitogenesis is thought to occur through the activation of the classical Ras-dependent signaling cascade, and possibly through the S6/Akt/PKB pathway. Interestingly, c-Src and nitric oxide (NO) (Cebe-Suarez et al., 2006) have been implicated as intracellular mediators of VEGF signaling. In addition as well as
components of the ECM such as heparan sulfate (Holmqvist et al., 2004) and fibronectin (Wijelath et al., 2004) can also modulate mitogenic signaling.

VEGFA-dependent regulation of cytoskeletal organization and cell migration occurs through recruitment of the adaptor protein Shb and activation of PI3 kinase resulting in the regulation of FAK phosphorylation and leading to the recruitment of paxillin, talin or vinculin to the focal adhesions (Kanno et al., 2000). As well, the recruitment of Src, PI-3 kinase and PLCγ-1 also plays a role in cytoskeletal rearrangement (Zeng et al., 2002a; Zeng et al., 2002b). Finally, activation of SAPK/p38 pathways and the small GTPases Rho, Rac and Cdc42 also contributes to actin dynamics and cell contraction in endothelial cell migration.

Cell survival is dependent on VEGFR-2 signaling. Activation of the PI3 kinase/Akt/PKB pathway induces the expression of anti-apoptotic molecules including Bcl-2, A1 and IAP (inhibitor of apoptosis) family of proteins. Unfortunately, this survival signal is dependent on stable adherens junctions that contains a transient tetramer composed of VEGFR-2, PI3-kinase, VE-cadherin and β-catenin (Grazia Lampugnani et al., 2003). Disruption of this complex results in apoptosis.

VEGF mediated signaling is complicated because the VEGF receptors form multiprotein complexes with various coreceptors such as neuropilins, heparan sulphate, integrins and cadherins. These associations allow further coordination of signal amplitude, timing and specificity with extracellular cues from soluble ligands, cell-cell and cell-ECM interactions.
1.4.3 Role of Integrins in Angiogenesis

Specific combinations of integrins and their ligands have been identified on the surface of endothelial cells (Stupack and Cheresh, 2004). These include α5β1 integrin which binds fibronectin (Koivunen et al., 1993; Koivunen et al., 1994), α1β1 and α2β1 integrins which are receptors for laminins and collagens, and the αv integrins (Hynes, 1987): αvβ3 and αvβ5 which recognize RGD sequences in various ECM proteins namely vitronectin, fibronectin, thrombospondins and von Willebrand factor (Eliceiri and Cheresh, 1999; Eliceiri and Cheresh, 2001). In addition, αvβ3 integrins have also been reported to bind to various fragments of collagens, laminins and other matrix proteins (Brooks et al., 1994a; Brooks et al., 1994b).

Fibronectin (FN), a widely distributed ECM component, is expressed in embryonic vascular networks as a component of the basement membrane (Risau and Lemmon 1988; Francis et al., 2002). Mouse knock-out models of α5 or fibronectin produce embryonic lethal phenotypes. Although the studies demonstrate that α5 and fibronectin are not required in endothelial cell development from their angioblast precursors, α5 is critical for tubulogenesis and in its absence, vasculogenesis fails to occur in the yolk sac and embryo (George et al., 1993; Yang et al., 1993). In pathological states, both fibronectin and α5β1 are upregulated by the pro-angiogenic growth factor, bFGF (Kim et al., 2000). This further emphasizes the critical role of α5β1 integrin in coupling fibronectin with intracellular events that are necessary for vasculogenesis and angiogenesis (Koivunen et al., 1993; Koivunen et al., 1994).

Unlike the α5β1 integrins, the α1β1 and α2β1 integrins are less critical for organism development, and instead appear to play a role in vascular remodeling and
pathological angiogenesis. Expressed in endothelial cells, these two integrins are receptors for collagens and laminins and are up-regulated in response to VEGF (Senger et al., 2002). Downregulation of α1β1 and α2β1 activity using blocking antibodies inhibits both VEGF-induced endothelial migration on collagen and angiogenesis (Senger et al., 2002). Similarly, tumor growth and angiogenesis are significantly reduced in α1- and α2-null mice (Chen et al., 2002; Gardner et al., 1996).

There are five integrins that contain a common αv subunit. The αv-null mice have a lethal phenotype and demonstrate extensive blood vessel hemorrhaging. Although αvβ3 and αvβ5 integrins have been shown to be upregulated by angiogenic growth factors, and promote endothelial cell adhesion and migration on vitronectin and other ECM molecules (reviewed in (Stupack and Cheresh, 2004), mice lacking the β subunit (β1, β3, β5, β6 and β8) did not show changes in angiogenesis (Hodivala-Dilke et al., 2003) et al. 1999; Huang et al. 2000). Furthermore, Reynolds et al. (2002) demonstrate that mice lacking αvβ3 and/or αvβ5 integrins undergo enhanced pathological angiogenesis (Reynolds et al., 2002). On the contrary, antagonists, such as antibodies and peptidomimetics, against αvβ3 and αvβ5 integrin activity decreases VEGF-induced angiogenesis and tumor progression. This suggests that the lack of αvβ3 and αvβ5 integrin engagement with the appropriate ECM molecule results in the inhibition of angiogenesis (Stupack and Cheresh, 2004). Clearly, the role of αvβ3 and αvβ5 integrins in angiogenesis requires further study.
1.4.4 Role of Integrin-linked kinase in Vasculogenesis and Angiogenesis

Recent evidence demonstrates the importance of ILK in angiogenesis. The ILK knockout mouse is peri-implantation lethal (Friedrich et al., 2004). Moreover, EC-targeted knockout of ILK in endothelial cells results in embryonic lethality with severe defects in placental and embryonic vascularization (Friedrich et al., 2004) secondary to detachment and apoptosis as the result of the inhibition of cell survival pathways due to the lack of integrin activation or signal transduction through the integrin. Furthermore deletion of ILK in zebrafish using antisense morpholino oligonucleotides results in marked patterning abnormalities of the vasculature and is similarly lethal (Friedrich et al., 2004).

Ex vivo deletion of ILK from purified endothelial cells of adult mice resulted in the downregulation of the active-conformation of β1-integrins concomitant with a reduction in Akt/PKB phosphorylation and caspase 9 activation ultimately leading to apoptosis. Re-introduction of activated Akt/PKB to cells lacking ILK did not completely rescue the cells from apoptosis which suggests that ILK activates other pro-survival signals in addition to Akt/PKB (Friedrich et al., 2004). ILK also regulates VEGF induced vascular morphogenesis in a PI3 kinase-dependent manner (Kanesaki et al., 2005). Kaneko et al. reported that ILK is critical in the chemotactic migration of endothelial cells, as well as cell proliferation due to the decrease in cell survival in the absence of ILK kinase activity (Kaneko et al., 2004). By comparing capillary length, number and relative capillary area per field between VEGF stimulated endothelial cells and cells containing a dominant-negative, kinase deficient ILK, Watanabe et al. demonstrated that ILK is required in mediating capillary network formation and tubulogenesis (Watanabe et al., 2005) in vitro. Cho et al. demonstrated that ILK protects
endothelial cells from anoikis and nutrient-deprived stress induced apoptosis (Cho et al., 2005). Vouret-Craviari et al. proposed that ILK is critical in organizing cell-matrix adhesions that cluster α5β1 integrins on fibronectin and recruit paxillin to the focal adhesion site to induce cytoskeletal changes (Vouret-Craviari et al., 2004). Because of mounting evidence demonstrating the importance of ILK in vasculogenesis, HIF-1α expression has been demonstrated to be regulated by Akt/PKB activity, and ILK is a major regulator of ILK activity, we hypothesized that ILK plays a role as a VEGF mediator and effector of tumor angiogenesis.

1.5 Role of Integrin-linked kinase in Inflammation

Inflammation is a localized protective reaction of tissue to irritation, injury, or infection, characterized by pain, redness, swelling, and sometimes loss of function. It involves the cooperation of many cells and macromolecules. The transcription factor NF-κB is known to be of major importance in the production of pro-inflammatory molecules. NF-κB is widely expressed and important in the regulation of genes involved in mammalian immune and inflammatory responses, apoptosis, and cell proliferation and differentiation (Ghosh and Karin, 2002), and has been associated with cancer and neurodegenerative processes. The NF-κB family is a group of related and inducible transcription factors including RelA/p65, RelB, c-Rel, p50 and p52 (reviewed in (Ghosh and Karin, 2002). In unstimulated cells, NF-κB is sequestered in the cytoplasm and bound to inhibitors of NF-κB (IκB) (Ghosh and Karin, 2002). Upon activation, IκB is degrades and NF-κB translocates to the nucleus to bind DNA. Stimulation of cell with cytokines, bacterial lipopolysaccharides (LPS), phorbo esters, or potent oxidants leads to rapid
phosphorylation and subsequent ubiquitination and proteosome degradation of IκBα (Karin and Ben-Neriah, 2000). The NF-κB heterodimer translocates to the nucleus and binds to a specific κB consensus sequence and associates with transcriptional coactivators to express genes necessary for promoting cell survival and the inflammatory response in a timely and potent manner. There are two pathways, classical and alternate, that lead to the activation of different NF-κB transcription factors and the biological consequences of these pathways. Depending on the stimulus and the specific cell, NF-κB-dependent transcription leads to the expression of proteins involved in inflammation, innate immunity and survival (Karin and Greten, 2005) (Figure 1.9). The classical pathway involves the stimulation of receptors by pro-inflammatory stimuli and genotoxic stress such as pathogenic components and cytokines. This initiates a cascade of events that involve the phosphorylation-dependent degradation of IκBs by IKK-β and γ and the release and nuclear translocation of NF-κB heterodimer (p65 and p50). This is the majority of the heterodimer combinations involved in transcription. The alternate pathway involves stimulation by certain tumor necrosis factor (TNF)-family members and leads to IKK-α dependent phosphorylation of p100, a precursor of p52. The p100 is proteolytically degraded to p52, which complex to RelB to form a p52/RelB heterodimer (reviewed in (Karin and Greten, 2005).

ILK has also been shown to promote cell survival in an Akt/PKB-dependent manner (Hannigan et al., 2005). Based on these observations I hypothesized that ILK is able to activate NF-κB activity through activation of IKK and the degradation of IκB. To test this, I determined if the lack of ILK activity would result in a decrease in NF-κB activity and whether the expression of NF-κB enhanced or induced gene expression of
inducible nitric oxide (iNOS) and cyclooxygenase-2 (COX-2) two genes known to be regulated by NF-κB activity. More recently, Douglas et al (2006) observed nuclear β-catenin in fibroproliferative response to acute lung injury and this was accompanied with increased expression of the cotranscriptional regulator TCF-1, resulting in increase cyclin D expression. This suggests that ILK may regulate proinflammatory processes through this pathway as well. Because mediators of inflammation, cytokines, chemokines, proteases and inhibitors of apoptosis are regulated by NF-κB activity, it has been thought that this nuclear protein might provide the link from inflammation to tumour promotion and progression.

1.6 Summary

Many of the events that occur during the normal progression of vascular development in the embryo are recapitulated during situations of neoangiogenesis in the adult (Carmeliet 2003). Most notably, many tumors promote their own growth and dispersion to form metastases by recruiting host blood vessels to form in the vicinity of the tumor. While there is an abundance of molecular and genetic evidence demonstrating that ILK plays a critical role in vasculogenesis, the role of ILK in tumor angiogenesis remains unclear. We therefore examined whether ILK is involved in the stimulation of VEGF expression in tumor cells, and secondly if ILK is required for VEGF-mediated endothelial cell migration and formation of blood vessels. We found that ILK is involved in both the expression of VEGF in tumor cells, and the cellular mechanisms that promote VEGF-mediated cell activity. Specifically, the present model implicates ILK in the indirect phosphorylation of mTOR by ILK-dependent Akt/PKB, consequently resulting in the
phosphorylation and inactivation of 4E-BP1, a translation inhibitor, by mTOR. In turn, leading to the expression of HIF-1α and ultimately leading to the expression of VEGF. Secondly, we demonstrate that the ILK kinase and adaptor functions are necessary for the VEGF-stimulated endothelial cell invasion, migration and proliferation, however the exact molecular events remain elusive.

ILK is serine/threonine protein kinase and adaptor protein that interacts with the cytoplasmic domain of the β1 and β3 integrin to mediate cytoskeletal reorganization and intracellular signal cascades (Dedhar, 2000; Hannigan, 2005; Zervas, 2001). ILK has been demonstrated to regulate the activity of transcription factors such as β-catenin-TCF/Lef-1, AP-1, and CREB and other kinases such as Akt/PKB through its kinase activity. ILK activity in turn is regulated in a PI3-kinase dependent manner (Delcommenne et al., 1998; Persad et al., 2000). Since the transcription factor, NF-κB has been shown to be activated by Akt/PKB, which is known to lead to activation of iNOS in mice (Xie et al 1994), we wanted to determine whether ILK could also regulate NF-κB activity. We examined the role of ILK in lipopolysaccharide (LPS) stimulated expression of iNOS and NO, a physiologically relevant system for studying the regulation of NF-κB. Since there are species-specific differences between mice and humans, one of these being the COX-2 promoter, we also examined the role of ILK in COX-2 expression in both murine and human macrophages. We found that ILK is an upstream regulator of LPS-mediated phosphorylation of IκB and of NF-κB-dependent expression of iNOS.
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1175 in vascular endothelial growth factor (VEGF) receptor-2 and regulates VEGF-


2 Regulation of tumor angiogenesis by integrin-linked kinase (ILK)\(^1\)

2.1 Summary

Constitutive activation of the PI-3 Kinase pathway is oncogenic and is implicated in the promotion of tumor angiogenesis by stimulating the expression of VEGF. The integrin-linked kinase (ILK) is a PI-3 Kinase-dependent effector of integrin-mediated cell adhesion as well as growth factors, and is an upstream regulator of PKB/Akt. Here we show that ILK is essential for HIF-1\(\alpha\) and VEGF expression in prostate cancer cells, and that it is also essential for VEGF-stimulated endothelial cell migration, tube formation and tumor angiogenesis. Consequently, ILK plays important roles in two key aspects of tumor angiogenesis: VEGF expression by tumor cells, and VEGF stimulated blood vessel formation. Our findings suggest that ILK is a promising therapeutic target for the inhibition of tumor angiogenesis.

We show that Integrin-linked kinase (ILK) stimulates the expression of VEGF by stimulating HIF-1\(\alpha\) protein expression in a PKB/Akt and mTOR/FRAP dependent manner. In human prostate cancer cells, knock-down of ILK expression with siRNA, or inhibition of ILK activity, results in significant inhibition of HIF-1\(\alpha\) and VEGF expression. In endothelial cells, VEGF stimulates ILK activity, and inhibition of ILK expression or activity results in the inhibition of VEGF-mediated endothelial cell migration, capillary formation \textit{in vitro}, and angiogenesis \textit{in vivo}. Inhibition of ILK activity also inhibits prostate tumor angiogenesis and suppresses tumor growth. These


All the work in this chapter was carried out by me except for figures 2.5 B and C.
data demonstrate an important and essential role of ILK in two key aspects of tumor angiogenesis: VEGF expression by tumor cells, and VEGF stimulated blood vessel formation.

2.2 Introduction

Angiogenesis plays a critical role in cancer progression (Hanahan and Weinberg, 2000). Tumor growth and metastasis have been shown to be dependent on angiogenesis, and inhibition of tumor angiogenesis, by selectively inhibiting the growth, survival and migration of endothelial cells, is perceived as an attractive, non-toxic means of regulating tumor progression (Kerbel, 1991).

A variety of proteins have been identified as potential targets of anti-angiogenesis therapy, and despite poor results in clinical trials of some of the anti-angiogenic strategies (Kerbel and Folkman, 2002), the potential of anti-angiogenic therapy continues to be an attractive means of cancer control.

One of the key mediators of angiogenesis is vascular endothelial cell growth factor (VEGF), which can promote the proliferation, survival, and migration of endothelial cells and is essential for blood vessel formation (Ferrara, 2002). VEGF is expressed by activated endothelial cells, but more importantly for tumor angiogenesis, VEGF expression and secretion is stimulated in tumor cells by activation of oncogenes such as Ras (Rak et al., 2000), as well as by the activation of the PI-3 Kinase pathway (Jiang et al., 2001; Fukuda et al., 2002), which has inherent oncogenic properties. The PI-3 Kinase pathway can be constitutively activated via autocrine growth factors, by constitutively activated growth factor receptors such as Erb-B2, by activating mutations...
in PI-3 Kinase or its downstream effector, PKB/Akt, or by the mutational inactivation, or loss of the tumor suppressor, PTEN (Cantley and Neel, 1999). The constitutive upregulation of expression of VEGF by tumor cells is felt to be a major contributor to tumor angiogenesis (Ferrara, 2002).

VEGF expression is regulated at the level of transcription, by a variety of transcription factors which include, AP-1, NF-κB, and hypoxia inducible factor-1α (HIF-1α) (Huang et al., 2000; Ryan et al., 1998; Damert et al., 1997). The major physiological stimulus for VEGF expression is hypoxia, resulting in the transcriptional induction of the VEGF gene by HIF-1α (Forsythe et al., 1996; Carmeliet et al., 1998; Ryan et al., 1998), which is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits (Jiang et al., 1996). The hypoxia-mediated stimulation of HIF-1α expression is regulated by the inhibition of ubiquitin-mediated degradation and consequent stabilization of the HIF-1α subunit under hypoxic conditions. As a result HIF-1α accumulates, dimerizes with HIF-1β, and activates transcription of target genes, including VEGF (reviewed in Harris, 2002). Recently, however, the expression of VEGF via the activation of the PI-3 Kinase pathway has also been shown to be mediated by HIF-1α (Jiang et al., 2001; Fukuda et al., 2002). Signaling via receptor tyrosine kinases induces HIF-1α expression by an independent mechanism involving the stimulation of increased rates of HIF-1α protein synthesis via PI-3 Kinase dependent stimulation of PKB/Akt and mTOR/FRAP which activates the translational regulatory protein eIF-4E binding protein 1 (4E-BP1) and p70 S6 kinase. (Fukuda et al., 2002; Laughner et al., 2001; Gingras et al., 2001; Peterson et al., 1999). These findings indicate that HIF-1α regulates both hypoxia- and growth factor-induced VEGF expression.
One of the components of the PI-3 Kinase pathway, immediately upstream of PKB/Akt is integrin linked-kinase (ILK) (Hannigan et al., 1996; Wu and Dedhar, 2002; Troussard et al., 2003). ILK can interact with the cytoplasmic domain of β integrin subunits and is activated by both integrin activation as well as growth factors (Wu and Dedhar, 2002). ILK is a PI-3 Kinase-dependent kinase (Persad et al., 2001; Delcommenne et al., 1998), and is an upstream regulator of the phosphorylation of PKB/Akt on serine-473 (Troussard et al., 2003; Persad et al., 2001; Persad et al., 2000; Lynch et al., 1999; Delcommenne et al., 1998), one of the two phosphorylation sites required for the full activation of PKB/Akt. Overexpression of ILK induces anchorage independent cell growth and suppression of anoikis, and promotes hyperplasia and tumor formation in vivo (Wu and Dedhar, 2002). ILK activity is also constitutively activated in PTEN-null cancer cells, and the constitutive activation of PKB/Akt in such cells is inhibited upon inhibition of ILK activity (Persad et al., 2000). ILK also promotes cell migration and invasion (Persad and Dedhar, 2003). Because of these oncogenic properties of ILK, we decided to explore the potential role of ILK in promoting tumor angiogenesis. We wanted to determine whether ILK is involved in the stimulation of expression of VEGF in tumor cells, and secondly whether ILK is required for VEGF-mediated endothelial cell migration and formation of blood vessels.

In this paper we report that overexpression of ILK stimulates VEGF expression in a PKB/Akt and HIF-1α-dependent manner, and that inhibition of ILK expression or activity in VEGF expressing prostate cancer cells (DU145 and PC3), results in dramatic inhibition of VEGF expression and secretion via inhibition of PKB/Akt activity and HIF-1α expression. Furthermore, inhibition of ILK activity or expression in VEGF-
stimulated endothelial cells results in the inhibition of endothelial cell migration and blood vessel formation in vitro and in vivo. A small molecule ILK inhibitor suppresses tumour angiogenesis and tumour growth in a PC-3 xenograft tumor model. Our results demonstrate an important and essential role of ILK in two key aspects of tumor angiogenesis: VEGF expression by tumor cells, and VEGF stimulated blood vessel formation, and suggest that ILK may be a promising therapeutic target for the inhibition of tumor angiogenesis.

2.3 Materials and Methods

2.3.1 Cell Culture and Transfections

Prostate carcinoma cell lines positive and null for PTEN (DU145 and PC3 respectively) (ATCC) were cultured as suggested by ATCC. PC3 cells were transiently transfected with V5-tagged ILK-dominant negative (ILK-DN:V5), Empty vector, GFP-tagged PTEN (PTEN:GFP), HIF-1α–dominant negative, HIF-1 response element conjugated to GFP reporter (HRE:GFP), and/or Renilla for transfection control using 2 – 3 µg of cDNA with 6 µl of Lipofectin reagent (Life Technologies Inc.), according to the manufacturer’s guidelines. DU145 cells were transfected with 4 µl of Fugene reagent (Roche Molecular Biochemicals). Intestinal epithelial cells (IEC-18) were stably transfected with ILK wild-type (ILK-13 A1a3), ILK dominant-negative (ILK-DN GH31RH) or ILK anti-sense (ILK-14 Antisense) (Hannigan et al., 1996). The parental IEC-18 cells were used as the control. These cells were cultured as previously described (Delcommenne et al., 1998). Human umbilical vein endothelial cells (HUVEC) (ATCC) were cultured as suggested by
ATCC. ILK-13 A1a3 and HUVEC cells were transfected with 6 μl of Lipofectamine 2000 reagent (Life Technologies Inc.), according to the manufacturer’s guidelines.

### 2.3.2 Small Interfering RNA (siRNA)

PC3 cells were transfected with small interfering RNA (siRNA) that specifically targets the ILK gene (ILK-H or ILK-A) or non-silencing control using 6 μl of Lipofectin in OPTI-MEM (GibcoBRL) overnight. The cells were passaged 36 hours after incubation in complete media and harvested 36 to 48 hours later as previously described (Troussard et al., 2003). siRNA duplexes were synthesized by Xeragon Inc., MD. Sequences of the human ILK gene specifically targeting the pH domain (ILK-H) (Troussard et al., 2003) and the integrin binding domain (ILK-A) were chosen. The sequence of the DNA target of ILK-A is 5'-GACGCTCAGCAGACATGTGGA-3'. A non-silencing siRNA (16-base overlap with *Thermotoga maritima*) was used as the control.

### 2.3.3 Chemical Inhibitors

Cells were exposed to the highly specific small molecule inhibitor (KP-392; Kinetek Pharmaceuticals) (Cruet-Hennequart S et al., 2003; Mills J et al., 2003; Tan C et al., 2002; Persad S et al., 2001; Persad S et al., 2001; Tan C. et al., 2001; Persad S et al., 2000) and equivalent amounts of DMSO vehicle. PI-3 Kinase inhibitor LY294002 (Calbiochem) and MEK1 inhibitor PD98059 (Cell Signaling Technology) were dissolved in DMSO as the vehicle.
2.3.4 Western Blotting

Cell lyses and western blotting were carried out as previously described by us (Troussard et al., 2003; Cruet-Hennequart et al., 2002; Tan et al., 2001; Persad et al., 2001). The following antibodies were used in this study: anti-phosphoserine-473 PKB/Akt, anti-PKB/Akt, and anti-phosphoserine-21/9-Glycogen Synthase Kinase-3 (GSK-3) antibodies from New England Biolabs, anti-V5 antibody from Invitrogen, anti-ILK and anti-HIF-1α antibodies from BD Transduction, anti-phosphoserine-2448-mTOR/FRAP and anti-mTOR/FRAP antibodies from Cell Signaling, anti-VEGF antibody from Oncogene Research Products, anti-green fluorescent protein (GFP) antibody from Boehringer Mannheim, and anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase from Jackson Laboratories.

2.3.5 Quantification of VEGF in Conditioned Media

Secreted VEGF was quantified by using an enzyme linked immunoassay (ELISA) kit for human VEGF (Oncogene Research Products) according to the manufacturer’s instructions.

2.3.6 ILK Kinase Assay

HUVECs were starved for 24 hours prior to the experiment. The cells were exposed to increasing amounts of VEGF for 1 or 24 hours, followed by lysis with NP-40 lysis buffer. Equivalent amounts (250 μg) of lysate were immunoprecipitate overnight with 3 μg of mouse monoclonal anti-ILK antibody (Upstate Biotechnologies Institute) at 4°C. The immune complexes were isolated with protein A/G plus agarose beads (Santa Cruz Biotechnology), and washed 3 times with NP-40 lysis buffer and three times with last
wash buffer (50 mM HEPES pH 7, 2 mM MgCl₂, 2 mM MnCl₂, 200 μM Na₃VO₄, protease inhibitors). The kinase assay was performed using 2 μg of GSK-3 fusion protein (New England Biolabs) as a substrate, 200 μM ATP in the reaction buffer (50 mM HEPES pH 7, 2 mM MgCl₂, 2 mM MnCl₂, 200 μM Na₃VO₄, 200 μM NaF) for 30 minutes at 30°C. Phosphorylation of the substrate was detected by Western blot with anti-GSK-3 serine-21/9 antibody.

2.3.7 Invasion and Migration Assay

Invasion and migration was analyzed with a modified Boyden chamber assay (cell culture inserts with a polycarbonate-filter (PVP, 8 μm pore size; Corning Incorporated, NY) covered with growth factor reduced Matrigel (Becton Dickinson, MA)). 200 μl of cell suspension (5x10⁴ cells) were added to the upper wells and allowed to attach for 2 hours at 37°C. Increasing amounts of KP-392 and equivalent amounts of DMSO diluted in 100 μl were added to the upper well for a complete volume of 300 μl. The lower compartment was filled with F-12K medium containing 0 or 20 ng/ml VEGF. Chambers were incubated for 16 hours in a 5% CO₂, 99% humidity and 37°C atmosphere. Cells on the under side of the filter were quantified by staining the cells with crystal violet, followed by counting the number of cells per magnified field of view (6 fields/membrane).

2.3.8 Cell Viability and Proliferation Assay

The metabolic activity of cells was determined in vitro using the colorimetric cell proliferation / tetrazolium salt WST-1 reagent/Electro Coupling Solution (WST-1/ECS) assay kit (Chemicon International, CA) according to manufacturers instructions. The
plate was incubated with the reagent for an additional 30 minutes to 1 hour and quantified by spectrophotometry (OD = 450 nm). All experiments were performed in triplicate.

### 2.3.9 Immunohistochemical Staining.

Tissues were frozen in compound-embedding medium (OCT; Miles Inc. IN), and 10-μm sections were collected on positively charged slides (Wax-It Histology Services Inc., B.C. Canada). Sections were fixed with cold acetone and blocked with 3 % hydrogen peroxidase, followed by 1 % bovine serum albumin and normal rabbit serum. Sections were then incubated with the anti-mouse CD31 antibody (BD Pharmingen) overnight at 4°C, followed by incubation with a horseradish peroxidase conjugated anti-rat secondary antibody (Jackson Laboratories) for 1 hour at room temperature. The tissue was washed 3 times with PBS in between each step. The antibody localization was visualized with NOVARED substrate kit (Vector Laboratories, CA), used as directed by manufacturer and the slides were counterstained with hematoxylin.

### 2.3.10 Endothelial Tube Formation Assay

The endothelial tube formation assay was performed as previously describe with minor modification (Maeshima et al., 2000). A suspension of HUVEC in medium was seeded in triplicate into Matrigel-precoated 24 well plates in the presence of 50 μM KP-392.

### 2.3.11 Endothelial Sprouting Assay

Microcarrier beads coated with denatured collagen (Cytodex3; Sigma) were seeded with HUVECs. Fibrin gels were made by dissolving 2.5 μg/μl bovine fibrinogen (Sigma), 0.05 mg/ml Aprotinin (Sigma) in F-12K medium followed by passing the solution through a 0.22 μm pore size filter to sterilize and mixed with a fraction of HUVEC-
coated bead. This mixture was transferred gently to 96-well plates together with HUVEC-coated beads at a density of 25 beads/well with a wide mouth pipette tip. Clotting was induced by the adding 1.2 units/ml thrombin. After clotting was complete, F-12K medium containing the indicated inhibitors, equivalent amounts of DMSO vehicle, 0 or 20 ng/ml VEGF and 1 % FBS was carefully applied onto the gel. After 3 days of incubation with daily changes of the medium, the number of capillary-like tubes formed/microcarrier bead (sprouts/bead) was counted by microscopy, monitoring at least 150 beads for each treatment. Only sprouts greater than 150 μm in length and comprised of at least 3 endothelial cells were counted.

2.3.12 Chorioallantoic Membrane (CAM) of Chick Embryos Assay

White Leghorn chicken eggs were fertilized and incubated at 37°C under conditions of constant humidity. The developing CAM was separated from the shell by opening a window at the broad end of the egg above the air sac on embryonic day 6. The opening was sealed with Parafilm (American National Can, IL) and the eggs were returned to the incubator. On embryonic day 8, 30 ng/ml VEGF and 50 μM of KP-392 in DMSO or equivalent amounts of DMSO vehicle were loaded onto 2 mm³ gelatin sponges (Pharmacia Upjohn) and placed on the surface of the developing CAM. Eggs were resealed and returned to the incubator for 10 days as previously described (Roskelley et al., 2001).

2.3.13 PC3 xenograft tumor assay

6 to 8 week old male nude mice (Harlan Sprague Dawley, Inc., IN, USA) were allowed to acclimatize for 1 week in the Jack Bell Research Centre animal facility. Procedures
involving animals and their care conform to institutional guidelines (University of British Columbia Animal Care Committee). 100 μl of a PC3 cell suspension (RPMI media (GibcoBRL); 5% FBS; 2X10^7 cells/ml) was injected subcutaneously into the left and right flank regions of the nude mice via a 27-gauge needle under halothane anaesthesia. 10 mice comprised each experimental group. 1 week post inoculation, animals were dosed daily with the ILK inhibitor (KP-307-2) by intraperitoneal injection of 100 mg/kg of ILK inhibitor (at a concentration of 10 mg/ml in 5% Tween80 in saline). Control mice received equivalent volumes of vehicle (5% Tween 80 in saline). 1 tumor was removed 1 week post treatment for angiogenesis marker analysis. The Image-Proplus from Media Cybernetics (Carlsbad, CA) was used to measure the CD31 positive immunostained endothelial lining (red objects) of neovasculature within the tumors. Tumor growth was monitored 3 times a week by measuring the height, length and width of each tumor with a caliper. The tumor volumes were calculated from a formula (axbxc/2) that was derived from the formula for an ellipsoid (πd^3/6). Data were calculated as the percentage of original (day 1) tumor volume and graphed as fractional tumor volume ± SEM. Mice were sacrificed by day 28 after KP-392 or vehicle administration.

2.3.14 Micrographs

All images were generated using the Nikon Eclipse TE 200 microscope and Nikon Cool PIX 950 digital camera.

2.3.15 Plasmid

We would like to thank Dr. Peggy Olive for the HRE:GFP.
2.4 Results

2.4.1 Overexpression of ILK stimulates VEGF expression in a PKB/Akt- and HIF-1α-dependent manner

We have previously demonstrated that overexpression of ILK in IEC-18 rat intestinal epithelial cells results in anchorage-independent cell cycle progression, tumorigenicity in nude mice, activation of PKB/Akt, inhibition of GSK-3, and stimulation of AP-1, NF-κB and β-catenin/LEF transcription factors (reviewed in Wu and Dedhar, 2002). Overexpression of a kinase-deficient mutant of ILK, or ILK anti-sense cDNA, did not result in the stimulation of these pathways or phenotypes. Because activation of other oncogenes such as Ras, or the PI-3 Kinase pathway have been shown to stimulate VEGF expression in tumor cells (Rak et al., 2000), we wanted to determine whether ILK overexpression also resulted in the stimulation of VEGF expression. As shown in Fig. 2.1A, the expression of both isoforms of VEGF is markedly stimulated in the ILK overexpressing clone of IEC-18 cells (ILK-13, A1a3), as compared to control clones expressing the E359K kinase-deficient ILK-dominant negative (ILK-DN, GH31RH), or antisense-ILK (ILK-14) (Novak et al., 1998; Hannigan et al., 1996). In addition the data in Figure 2.1A, also show markedly increased phosphorylation of PKB/Akt on serine-473 in the absence of any changes in PKB/Akt expression. Since one of the major transcriptional regulator of the VEGF gene is hypoxia inducible factor-1α (HIF-1α), we transfected the different IEC-18 clones described above with a HIF-1 response element fused to a green fluorescence protein (GFP) reporter (HRE:GFP) (Ruan and Deen, 2001). As shown in Figure 3.1A, this reporter is only active in the ILK overexpressing clone, suggesting that the stimulation of VEGF expression in these cells is likely mediated by the upregulation or activation of the HIF-1 transcription factor. We were unable to
directly analyze HIF-1α protein expression in these clones because of the lack of availability of suitable anti-rat HIF-1α antibodies. Inhibition of ILK expression in the A1a3 ILK overexpressing cells with ILK siRNA, resulted in the suppression of VEGF expression (Figure 2.1B), showing that ILK is indeed responsible for the stimulation of VEGF expression in these cells. We have previously shown that the ILK overexpressing clones have constitutive high-level expression of cyclin D1 (Radeva et al., 1998), and inhibition of ILK expression by siRNA inhibits cyclin D1 expression (Troussard et al., 2003). As shown in Figure 2.1B, ILK siRNA also results in cyclin D1 expression in the ILK overexpressing cells. These data demonstrate that overexpression of kinase-active ILK results in the stimulation of VEGF expression via the activation of PKB/Akt and the HIF-1 transcription factor.
Figure 2.1 VEGF expression and HIF-1α activity are increased in epithelial cells with a high ILK activity. (A) Immunoblot analysis with the indicated antibodies of NP-40 cell lysates of the indicated cell lines transfected with 3 μg HRE:GFP and pRenilla (transfection efficiency control), and exposed (24 hrs) to 1% FBS media. ILK 13 A1a3 clones overexpress ILK, ILK-DN clones express dominant negative ILK (Hannigan et al., 1996). (B) Immunoblot analysis with the indicated antibodies of the RIPA IEC-18 ILK 13 A1a3 cell lysates 4 days post-transfection with the indicated type and amount of siRNA. ILK-A was ILK specific siRNA and control was non silencing siRNA from *Thermotoga maritima* (Troussard et al., 2003) All figures are a representation of 3 trials.
2.4.2 Inhibition of ILK expression and activation suppresses PKB/Akt and mTOR/FRAP phosphorylation and inhibits HIF-1α and VEGF expression in prostate cancer cells

In order to analyze in more detail the ILK-mediated signaling pathway leading to the stimulation of VEGF, and to assess the relevance of ILK in VEGF expression in cancer cells, we decided to inactivate ILK expression or activity in human prostate cancer cells which express VEGF.

The PI-3 Kinase pathway is constitutively activated in many cancer cells lines. In certain human prostate cancer cell lines (PC3 and LnCAP) the PI-3 Kinase pathway is constitutively activated due to the loss of expression of the tumor suppressor PTEN (Davies et al., 1999; Stambolic et al., 1998). We have previously shown that ILK activity is also constitutively activated in these cells, and that inhibition of ILK activity suppresses PKB/Akt activity in these PTEN-null cells (Persad et al., 2000). VEGF expression has been shown to be constitutively elevated in PC3 cells (Jiang et al., 2001). Because we had found that ILK stimulated VEGF expression (Figure 2.1), we wanted to determine whether inhibition of ILK activity in PC3 cells resulted in the inhibition of VEGF expression. As shown in Figure 2.2A, inhibition of ILK activity by transfection of kinase-deficient, ILK-DN (E359K) (Persad et al., 2000), or wild-type PTEN, resulted in inhibition of VEGF expression at the protein level as determined by Western blotting. In addition, the expression of HIF-1α protein is also substantially inhibited by DN-ILK and PTEN (Figure 2.2A). As expected, DN-ILK also inhibited phosphorylation of PKB/Akt on serine-473 (Figure 2.2A). Kinase-deficient ILK-dominant negative and PTEN also inhibited the activity of the HIF-1 response element (HRE), as shown in Figure 2.2C,
suggesting that the upregulation of VEGF expression in these cells is likely due to the ILK-mediated upregulation of HIF-1α expression.

We have recently utilized double stranded RNA interference (siRNA) to knock down ILK protein expression (Troussard et al., 2003). Furthermore, we have shown that ILK knockdown by siRNA results in significant inhibition of PKB/Akt Serine-473 phosphorylation and activation (Troussard et al., 2003). We therefore exposed PC-3 cells to increasing concentrations of ILK specific siRNA (Troussard et al., 2003). As shown in Figure 2.2B, ILK siRNA resulted in the complete depletion of ILK expression in PC-3 cells. This was associated with a suppression of phosphorylation of PKB/Akt on serine-473. Expression of PKB/Akt was not affected (Figure 2.2B). Furthermore ILK siRNA mediated knockdown of ILK also resulted in significant inhibition of expression of both HIF-1α and VEGF protein (Figure 2.2B). It has been recently shown that PKB/Akt can regulate the expression of HIF-1α protein at the translational level by stimulating the phosphorylation of mTOR/FRAP, which is a regulator of protein synthesis (Gingras et al., 1999; Peterson et al., 1999). We therefore wanted to determine whether the ILK-mediated expression of HIF-1α and VEGF also involved mTOR/FRAP. As shown in Figure 2.2B, siRNA mediated knockdown of ILK resulted in the inhibition of mTOR/FRAP phosphorylation on serine-2448, concomitant with the inhibition of the PKB/Akt phosphorylation. The expression of mTOR/FRAP protein was not affected by the knockdown of ILK. These data suggest that in the PC3 cells, the constitutive activation of ILK drives VEGF expression most likely via HIF-1α through the activation of PKB/Akt and mTOR/FRAP, resulting in increased translation of HIF-1α protein. This is further substantiated by the observation that transfection of a dominant-negative HIF-
1α construct into PC3 cells almost completely inhibits VEGF expression as well HRE activity (Figure 2.2C).
Figure 2.2 VEGF and HIF-1α expression are severely affected by the loss of ILK activity in a PTEN and mTOR/FRAP-dependent manner, in PTEN-null prostate carcinoma cells (PC3). (A) Immunoblot analysis with the indicated antibodies of the RIPA lysate of PC3 cells transfected with 1 – 2 μg of Empty::V5, ILK-DN::V5 or PTEN::GFP vector. (B) Immunoblot analysis with the indicated antibodies of the RIPA lysate of PC3 cells transfected with the indicated type and amount of siRNA. (C) Immunoblot analysis with the indicated antibodies of NP-40 lysate of PC3 cells co-transfected with the indicated constructs and pRenilla after 48 hours. Cells were noted not to be dying by cell viability assay. All figures are a representation of 3 independent trials.
2.4.3 Pharmacological inhibition of ILK activity results in the inhibition of HIF-1α and VEGF expression in prostate cancer cells

We have identified highly selective small molecule inhibitors of ILK activity. These ATP competitive inhibitors have been extensively characterized, and shown to inhibit ILK activity and the activation of all of the downstream effectors of ILK (Cruet-Hennequart et al., 2003; Mills et al., 2003; Tan et al., 2002; Persad et al. 2001; Tan et al., 2001; Persad et al., 2000, Troussard et al., 2000). The inhibitors are equally effective and specific as ILK inhibition by dominant-negative ILK and ILK siRNA (Persad et al., 2001; Troussard et al, 2003). We therefore wanted to determine whether exposure of human prostate cancer cells to the ILK inhibitor would also inhibit the expression of HIF-1α and the expression and synthesis of VEGF. As shown in Figure 3.3A, exposure of both PC3 and DU145 prostate cancer cells to the ILK inhibitor KP-392 (Persad et al., 2001; Persad et al., 2001) resulted in the inhibition of both HIF-1α and VEGF expression in a dose-dependent manner. Despite poor cellular permeability of this inhibitor, resulting in the exposure of cells to relatively high concentrations, it can be seen that there is significant inhibition of both HIF-1α and VEGF expression at 25 μM KP-392, especially in PC3 cells. As well, it can clearly be seen that KP-392 also suppresses the phosphorylation of PKB/Akt on serine-473 in a dose-dependent manner. Again there is no effect on the expression of PKB/Akt. In addition, as shown in Figure 3.3B, not only VEGF cellular expression but also its secretion, as determined by an enzyme-linked immunosorbant assay (ELISA) of the conditioned cell media, is inhibited by the ILK inhibitor KP-392. In contrast to PC3 cells, VEGF expression is not completely inhibited by the ILK inhibitor in DU145 cells, despite substantial inhibition of HIF-1α expression. This suggests cell type differences in the regulation of VEGF expression.
In Figure 3.3C, we demonstrate that HIF-1α expression is stimulated by serum in serum starved PC3 cells, and that inhibition of ILK as well as PI-3 Kinase with the respective pharmacological inhibitors KP-392 and LY294002, inhibits HIF-1α expression.

Collectively, the data shown in Figure 3.2 and 3.3 demonstrate that ILK is a critical component of the constitutively activated PI-3 Kinase-PKB/Akt signaling pathway resulting in the stimulation of HIF-1α and VEGF. Inhibition of ILK expression or activity can result in substantial inhibition of the expression of both HIF-1α and VEGF, suggesting that ILK may be an important therapeutic target for the inhibition of expression of the angiogenic factor, VEGF.
Figure 2.3  Inhibition of ILK activity results in decrease of HIF-1α and VEGF expression in a PI-3 Kinase dependent manner in prostate carcinoma cells. (A) Immunoblot analysis with the indicated antibodies of RIPA lysate of PC3 and DU145 cells starved (20 hrs), and then exposed (24 hrs) to the indicated amounts of KP-392, equivalent amounts of DMSO vehicle and 1% FBS. (B) Quantification by ELISA of the level of secreted VEGF protein in conditioned media. The X-axis represents increasing concentrations of KP-392 as shown in the above immunoblot. The first graph represents a single experiment. The second graph is the accumulation of 3 independent trials. (C) Immunoblot analysis of RIPA lysates of DU145 cells cultured under the indicated conditions (24 hrs). The treatment of these cells with KP 392 at the time points shown here caused little to no cell death as assessed by cell viability assay. All figures are a representation of 3 trials.
2.4.4 ILK regulates VEGF-mediated endothelial cell migration and blood vessel formation

VEGF stimulates endothelial cell survival and migration, and promotes the formation of new blood vessels (Ferrara, 2002). Since the activity of ILK is stimulated by various growth factors and chemokines (Wu and Dedhar, 2002; Freidrich et al., 2002), and ILK also promotes cell migration and invasion (Persad and Dedhar, 2003), we wanted to determine whether ILK also played a role in VEGF-mediated endothelial cell migration and vascular morphogenesis. As shown in Figure 2.4A, VEGF stimulates ILK kinase activity in a dose-dependent manner in quiescent human umbilical vein endothelial cells (HUVEC). The stimulation of ILK activity by VEGF is dependent on PI-3Kinase activity since the VEGF stimulation of ILK activity is inhibited in the presence of the PI-3Kinase inhibitor, LY294002. These data support previous studies showing ILK to be a PI-3 Kinase dependent kinase (Delcommenne et al., 1999; Lynch et al., 1998) and demonstrate that VEGF stimulates ILK activity in a PI-3 Kinase dependent manner. We next determined whether ILK was required for the stimulation of VEGF-mediated cell migration of HUVEC cells. As shown in Figure 2.4B, VEGF stimulates the migration of HUVEC cells, and inhibition of ILK activity with the pharmacological ILK inhibitor, KP-392, results in a dose-dependent inhibition of VEGF-mediated HUVEC cell migration. Furthermore, inhibition of ILK expression in HUVEC cells by ILK siRNA also inhibited VEGF stimulated HUVEC cell migration (Figure 2.4C), demonstrating an essential role for ILK in the stimulation of endothelial cell migration by VEGF. We also noted inhibition of Cyclin D1 expression in the ILK siRNA transfected HUVEC cells (Figure 2.4D), indicating that inhibiting ILK may also inhibit VEGF stimulated HUVEC cell proliferation. As shown in Figure 2.4D, HUVEC cell
proliferation in response to VEGF is inhibited in the ILK siRNA transfected cells. Cell viability in the ILK siRNA transfected cells was not significantly altered (data not shown). These data demonstrate an essential role of ILK in VEGF–mediated HUVEC cell migration and proliferation. It has been demonstrated that VEGF promotes its own expression in endothelial cells via a positive autocrine loop involving HIF-1α expression and activity (Stoeltzing et al., 2003; Zhong et al., 2000). It is therefore interesting to note that VEGF stimulated HIF-1α expression in HUVEC cells is inhibited by ILK siRNA (Figure 2.4D), suggesting that ILK is a component of this positive feedback loop.

We next wanted to determine whether ILK is required for VEGF-mediated blood vessel formation. To evaluate this we initially utilized an in vitro endothelial cell sprouting assay. As shown in Figure 2.5A, VEGF significantly stimulated HUVEC capillary sprouting, which was quantified as described in the Experimental Procedures. Both the KP-392 ILK inhibitor and the PI-3 Kinase inhibitor LY294002 inhibited cell sprouting. Exposure of cells to 50 μM KP-392 and 20 μM LY294002 completely inhibited VEGF induced HUVEC sprouting. In contrast, the MEK inhibitor, PD98059, did not have any significant inhibitory effect in this assay. The ILK inhibitor had only minor effects on HUVEC cell viability, and only at very high concentrations (Figure 2.5A). These data demonstrate that PI-3 Kinase and ILK activities are required for VEGF-mediated vascular morphogenesis in vitro.

Another assay that is frequently used for the demonstration of angiogenesis in vitro is the endothelial tube formation assay in which endothelial cells placed on matrigel in the presence of angiogenic factors results in the endothelial cells forming tube like structures morphologically similar to capillaries. This tube formation represents the
contribution of cell survival, migration and proliferation (Folkman and Haudenschild, 1980). As shown in Figure 2.5B, HUVEC cells cultured on matrigel in the presence of VEGF formed tube structures that were completely inhibited by the ILK inhibitor, KP-392. At the same KP-392 concentrations, significant inhibition of HUVEC cell migration and capillary sprout formation was observed (Figure 2.4B and 2.5A).

These three different in vitro assays, demonstrate that the inhibition of ILK activity has a dramatic effect on endothelial cell function in response to VEGF suggesting an essential role of ILK in blood vessel formation.
Figure 2.4 ILK kinase activity is involved in VEGF-stimulated HUVEC activity (A)
ILK kinase activity is stimulated by VEGF in HUVEC. Cells were starved (24 hrs), and
exposed to the indicated amounts of VEGF and LY294002. The ILK kinase assay was
carried out as described in the Experimental Procedures (EP). This is a representation of
3 independent trials. (B) Decrease in ILK activity reduces HUVEC invasion and
migration towards VEGF, and inhibits endothelial cell sprouting in vitro. 2 hours
after HUVEC were seeded on the upper chamber, indicated amounts of KP-392 were
added to this chamber, and the migration assay was performed and analyzed as described
in EP. This graph represents the mean of three experiments ± SD.
Figure 2.4
Figure 2.4  (C) Knockdown of ILK expression reduces HUVEC invasion and migration towards VEGF. Equal number of HUVEC transfected for 3 days with the indicated siRNA (25 nM) were seeded in the upper chamber. The experiment was performed as described in EP. This graph represents the mean of 3 experiments ± SD. (D) Immunoblot analysis with the indicated antibodies of RIPA lysate of transfected HUVEC (25 nM indicated siRNA) that were starved (24 hrs), then exposed (24 hrs) to VEGF (0 or 20 ng/ml) 2 days post-transfection. The graph represents relative HUVEC growth after above conditions, measured by WST
2.4.5 Inhibition of ILK activity inhibits VEGF stimulated angiogenesis in Vivo

We next wanted to determine whether inhibiting ILK activity resulted in the inhibition of VEGF-stimulated angiogenesis in vivo. We utilized a well-established assay for angiogenesis, the chicken chorioallantoic membrane (CAM) assay (Auerbach et al, 1975), to determine the effects of the ILK inhibitor, KP-392. As shown in Figure 2.5C, inhibition of ILK activity had a significant effect on VEGF stimulated blood vessel formation in vivo. In the CAM assay (Figure 2.5C), the incorporation of KP-392, compared to vehicle alone together with VEGF resulted in the complete blockage of growth of blood vessels towards VEGF. It is interesting to note that the blood vessels are not lysed in the presence of KP-392, but rather they fail to grow towards VEGF and seem to be repelled away from VEGF, demonstrating that the inhibition of ILK predominantly inhibits the migration of endothelial cells and blood vessels towards VEGF.
Figure 2.5 KP-392 inhibits angiogenesis in matrigel and in vivo. These are three assays used to assess angiogenesis in vitro and in vivo. (A) VEGF stimulates the early stages of vessel tube formation. HUVEC-coated beads imbedded in fibrin were incubated in the indicated conditions (VEGF (0 or 20 ng/ml); KP-392 (μM)). Micrographs of a typical bead were taken at 72 hours. Number of capillary-like tubes formed per microcarrier bead (sprouts/bead) were counted and analyzed at described in EP. The graph represents the mean of three experiments ± SD. The graph below represents relative cell viability after exposure (24 hrs) to the indicated amounts of KP-392, LY294002 and VEGF, measured by WST-1/ECS assay. Results represent mean absorbance ± SD.
Figure 2.5 (B) This assay shows the formation of endothelial cell-cell interactions in a 3-dimensional culture is disregulated by KP-392. HUVEC suspension in Matrigel were incubated with VEGF and DMSO (Control) or 50 μM KP-392. Micrographs of a typical field were taken to illustrate HUVEC tube formation. (C) Photographs of developing chick chorioallantoic membranes incubated with 30 ng/ml VEGF and DMSO (Control) or 50 μM KP-392. Dashed circle outlines the area covered by the gelatin sponges. Arrows show blood vessels migrating away from the area containing KP-392. All figures are a representation of 3 independent experiments.
2.4.6 Inhibition of tumor angiogenesis and suppression of tumor growth in ILK-inhibitor treated PC-3 xenograft tumor model

The data presented above suggest that inhibition of ILK activity or expression should inhibit tumor angiogenesis and if PC3 tumor growth in vivo is dependent on tumor vascularization then ILK inhibition should also induce tumor growth inhibition. To determine whether inhibition of ILK affected tumor angiogenesis and tumor growth in vivo, we established PC3 tumors in nude mice (Figure 2.6), and treated mice with established tumors with the ILK inhibitor KP-307-2, an analog of KP-392. As shown in Figure 2.6A, there was a statistically significant effect on tumor vascularization as determined by micro-vessel density in anti-CD31 stained KP-307-2 treated and control tumor sections. In addition there was statistically significant tumor growth suppression in the ILK inhibitor treated mice over a 28 day dosing regimen (Figure 2.6B). The inhibitor was well tolerated with no obvious side effects or weight loss (data not shown). These data indicate that ILK is a mediator of prostate tumor angiogenesis, and therefore a target for anti-angiogenic therapy.
Figure 2.6 Inhibition of ILK activity suppresses tumor angiogenesis and tumor growth rate. Nude mice with PC3 flank tumors were treated with daily i.p. injection of 100 mg/kg ILK inhibitor or vehicle for 28 days. (A) 7 days after dosing, 1 tumor from each mouse was harvested and tumor vascular density was analyzed as described in EP. The bar graph shows the means of neovascular densities / field for each group ± SD. Shown are representative photographs of neovasculature in the PC3 tumors. (B) Relative change in tumor volumes in 8 mice in the control group and 10 mice in the treatment group ± SEM. **: p<0.01; *: p<0.05
2.5 Discussion

Angiogenesis is important in cancer progression and is one of the hallmarks of tumor metastasis (Hanahan and Weinberg, 2000). A principal mediator of tumor angiogenesis is VEGF and a major transcriptional activator of the VEGF gene is HIF-1α (Harris 2001). It has been reported that the PI-3 Kinase/Akt signaling pathway mediates angiogenesis and the expression of VEGF in cells, by elevating the levels of HIF-1α protein in cells independent of hypoxic condition (Semenza 2002). This hypoxia-independent stimulation of HIF-1α and VEGF in cancer cells can be mediated by autocrine or chronic stimulation by growth factors such as IGF-1, constitutive activation of PI-3 Kinase, or the constitutive activation of PKB/Akt due to the inactivation of the tumor suppressor, PTEN (Brazil et al., 2002; Galetic et al., 1999). Because ILK is PI-3 Kinase dependent and an upstream target of Akt/PKB, and because an increase in ILK expression is positively correlated with prostate carcinoma grade (Graff et al., 2001), ILK was a likely candidate to be involved in the regulation of VEGF and HIF-1α expression through Akt/PKB activity regulation. In addition, the regulation of HIF-1α translational rate has been shown to be through the regulation of mTOR/FRAP, a downstream target of Akt/PKB (Fukuda et al., 2002).

In this paper we have shown that in human prostate cancer cells, ILK is essential for the regulation of HIF-1α expression and the consequent production of VEGF. Functional inactivation of ILK by exposure to a highly selective chemical inhibitor, or stable or transient transfection of the ILK-dominant negative construct into cell models with high ILK activity, result in a decrease in HIF-1α protein levels and VEGF expression. Furthermore, depletion of ILK protein by siRNA in PC3 cells effectively
decreases Akt/PKB and mTOR/FRAP phosphorylation, HIF-1α levels and VEGF expression. These data suggest that in certain cancer cells, such as prostate carcinoma cells, ILK plays a crucial role in HIF-1α and VEGF expression via activation of PKB/Akt and phosphorylation of mTOR/FRAP (Figure 2.7). VEGF gene transcription can also be stimulated by the transcription factors AP-1 and NF-κB (Harris, 2002). Since ILK has also been shown to regulate the activities of both of these transcription factors (Troussard et al, 2000; Tan et al, 2002), it is possible that in certain cell types ILK could regulate VEGF expression via signaling pathways that activate these other transcription factors. Although in this study we have not ruled out the contribution of AP-1 and NF-κB in the ILK-mediated regulation of VEGF expression in the prostate cancer cell lines examined, the data presented here support a significant role of HIF-1α in the ILK regulation of VEGF expression. This is particularly true for the PC3 cells in which inhibition of ILK expression or activity results in almost complete suppression of both HIF-1α and VEGF expression. Furthermore, in the PTEN-null PC3 cells in which the PI-3 Kinase pathway and ILK are constitutively upregulated, transfection of dominant-negative HIF-1α results in substantial inhibition of VEGF expression. It is interesting to note that ILK mRNA has been shown to be upregulated by hypoxia (Scandurro et al, 2001; Grimshaw et al, 2001), suggesting that ILK may also play a role in hypoxia induced VEGF expression. Thus the role of ILK in hypoxic tumors with constitutive activation of PI-3 Kinase may be quite substantial.
Figure 2.7 Schematic representation of the cell signaling events leading to VEGF production in a prostate carcinoma cell and consequent effects on a neighboring endothelial cell. Shown is our model for the production of VEGF in prostate carcinoma cell and the effects of VEGF on endothelial cell function. Phosphorylation of serine-473 of Akt/PKB by activated ILK in prostate cancer cells results in the full activation of PKB/Akt that promotes the phosphorylation of serine-2448 of mTOR/FRAP. This activates mTOR/FRAP, which increases the levels of HIF-1α protein translation. HIF-1α protein combines with HIF-1β to form an active transcription factor. This heterodimer binds to the VEGF promoter and activates VEGF transcription, expression and secretion. VEGF binds to its receptor and stimulates ILK activity. ILK regulates downstream targets involved in cell survival, proliferation, invasion and migration.
We have also shown here that ILK plays an essential role in VEGF-stimulated endothelial cell-mediated blood vessel formation *in vitro* and *in vivo* (Figure 2.6). Migration and proliferation of human endothelial cells in response to VEGF is inhibited upon inhibition of ILK activity or expression. Furthermore the ability of VEGF stimulated endothelial cells to form capillary-like structures in vitro is also severely inhibited by inhibiting ILK activity. This inhibition appears to be due primarily due to inhibition of cell migration and proliferation, both of which can be regulated by ILK in response to growth factors or engagement of integrins (Wu and Dedhar, 2001; Cruet-Hennequart et al, 2003). The inhibition of angiogenesis *in vivo* in the CAM assay by inhibition of ILK suggests that the primary effect of ILK inhibition is on endothelial cell migration and ability to form vessels, as there did not appear to be any obvious cell lysis in these assays. This agrees with our finding that HUVEC cell survival appears not to be affected as significantly as cell migration and proliferation upon ILK inhibition. Recent evidence from the systemic and targeted knockout of ILK suggests an important role of ILK in cell adhesion and actin accumulation (Sakai et al, 2003), processes crucial for cell morphogenesis and migration, as well as in cell proliferation (Tepstra et al, 2003).

We have also shown here that inhibition of ILK with a highly selective ILK inhibitor results in the statistically significant suppression of tumor angiogenesis as well as tumor growth in a mouse xenograft model of PC3 tumor growth in SCID mice. These data suggest that inhibitors of ILK activity may be considered as angiogenesis inhibitors effective for the suppression of tumor angiogenesis.

The integrins \( \alpha_v \beta_3, \alpha_v \beta_5 \) and \( \alpha_5 \beta_1 \) have also been shown to be crucial regulators of endothelial cell function during angiogenesis (Hood and Cheresh, 2002; Eliceiri and
Furthermore, angiogenesis inhibitors such as endostatin and tumstatin have been shown to function by inhibiting integrin function and signalling (Maeshima et al., 2002). Tumstatin has been shown to inhibit endothelial cell survival by binding to $\alpha_v\beta_3$ and inhibiting $\alpha_v\beta_3$-mediated signalling to PKB/Akt (Maeshima et al., 2002). Since ILK is also regulated by integrins and since ILK is involved in $\alpha_v\beta_3$-regulated cell proliferation (Cruet-Hennequart et al., 2003), as well as in regulating anoikis (Attwell et al., 2000), it is likely that ILK also plays an important role in integrin-mediated endothelial cell function during angiogenesis. We have recently created transgenic mice in which the ILK gene is flanked by Lox-P sites, and have demonstrated that cells from these mice can be used to conditionally knockout ILK expression in cells isolated from these mice (Troussard et al., 2003). The conditional knockout of ILK in endothelial cells will provide further data on the precise mechanism of ILK function in endothelial cells.

The data shown in this paper demonstrate the importance of ILK in the orchestration of tumor angiogenesis by regulating VEGF expression by carcinoma cells, and VEGF stimulated blood vessel formation. ILK occupies a pivotal position in regulating cell adhesion, actin polymerization and signalling. A more detailed understanding of these processes is therefore of importance for therapeutic intervention of tumor angiogenesis.
2.6 References


Persad, S., Attwell, S., Gray, V., Delcommenne, M., Troussard, A., Sanghera, J., and Dedhar, S. (2000). Inhibition of integrin-linked kinase (ILK) suppresses activation of


3 Integrin-linked kinase regulates inducible nitric oxide synthase and cyclooxygenase-2 expression in an NF-κB-dependent manner

3.1 Summary

Nitric oxide (NO) and prostaglandins are produced as a result of the stimulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) respectively in response to cytokines or lipopolysaccharide (LPS). We demonstrate that the activity of integrin-linked kinase (ILK) is stimulated by LPS activation in J774 macrophages. Inhibition of ILK activity by dominant-negative ILK or a highly selective small molecule ILK inhibitor, in epithelial cells or LPS stimulated J774 cells and murine macrophages, resulted in inhibition of iNOS expression and NO synthesis. LPS stimulates the phosphorylation of IκB on serine-32 and promotes its degradation. Inhibition of ILK suppressed this LPS stimulated IκB phosphorylation and degradation. Similarly, ILK inhibition suppressed the LPS stimulated iNOS promoter activity. Mutation of the NF-κB sites in the iNOS promoter abolished LPS and ILK mediated regulation of iNOS promoter activity. Overexpression of ILK stimulated NF-κB activity, and inhibition of ILK or protein kinase B (PKB/Akt) suppressed this activation. We conclude that ILK can regulate NO production in macrophages by regulating iNOS expression through a pathway involving PKB/Akt and NF-κB. Furthermore we also demonstrate that ILK activity is required for LPS stimulated COX-2 expression in murine and human macrophages.


All the work in this chapter was carried out by me.
macrophages. These findings implicate ILK is a potential target for anti-inflammatory applications.

### 3.2 Introductions

Mouse macrophages express an inducible form of nitric oxide synthase (iNOS), which catalyzes the production of nitric oxide (NO) from L-arginine. Macrophage-derived NO is important for host defense, microbial and tumour cell killing (reviewed in MacMicking et al., 1997). Activating stimuli such as lipopolysaccharide (LPS) (Alley et al., 1995) other bacterial cell wall products (Brightbill et al., 1999) and cytokines such as interferon (IFN-gamma) (Gao et al., 1997) all stimulate iNOS expression in induced macrophages. However since excess production of NO results in inappropriate tissue injury and septic shock, iNOS expression is subject to stringent regulatory control.

The mouse iNOS promoter has been extensively studied and consists of two clusters of regulatory elements (Xie et al., 1994; Lowenstein et al., 1993). A proximal region (region I or RI, -48 to -209) functions as the basal promoter containing an octamer element and a NF-κB binding site, which mediates responsiveness to LPS. The distal region (RII, -913 to -1029) functions as an enhancer element and responds to LPS and IFN-gamma through NF-κB, IRF-1, ISRE, and GAS binding sites. The NF-κB sites are essential for LPS-mediated NO production (Xie et al., 1994).

Protein kinase B, (PKB/Akt) has been shown to phosphorylate and activate the IkB kinase (IKK) (Ozes et al., 1999; Romashkova and Makarov, 1999), which in turn phosphorylates IkB. Phosphorylated IkB is targeted for ubiquitin-mediated degradation,
thus releasing active NF-κB and allowing its translocation into the nucleus (Baeuerle and Baltimore, 1996).

Integrin-linked kinase (ILK) is an ankyrin-repeat containing serine/threonine protein kinase that can interact with the cytoplasmic domain of the β1 integrin and regulates integrin dependent functions (Dedhar, 2000; Zervas et al., 2001). It has been demonstrated to regulate the activity of transcription factors such as β-catenin-TCF/LEF-1 (Novak et al., 1998; Tan et al., 2001), AP-1 (Troussard et al., 1999), and CREB (D’Amico et al., 2000). ILK activity is regulated in a PI 3-Kinase dependent manner (Delcommenenne et al., 1998; Persad et al., 2000; Morimoto et al., 2000; Persad et al., 2001) and ILK can regulate the phosphorylation and activation of PKB/Akt (Delcommenenne et al., 1998; Persad et al., 2000; Morimoto et al., 2000; Persad et al., 2001). Since the transcription factor, NF-κB has been shown to be activated by PKB/Akt, which is known to lead to activation of iNOS in mice (Xie et al., 1994), we wanted to determine whether ILK could also regulate NF-κB activity. In order to examine a physiologically relevant system for the regulation of NF-κB by ILK, we examined the role of ILK in lipopolysaccharide (LPS) stimulated expression of iNOS and NO. We found that ILK is an upstream regulator of LPS-mediated phosphorylation of IκB, and of NF-κB-dependent expression of iNOS.

Mouse and human macrophages have different iNOS promoters (Zhang et al., 1996). In order to determine a similar role of NF-κB in human macrophages, we analyzed the expression of cyclooxygenase-2 (COX-2), a protein that regulates the production of proinflammatory prostaglandins by catalyzing arachidonic acid into prostaglandins (Smith et al, 2000; Williams et al., 1999). There are two isoforms of
COX, COX-1 and COX-2, which are products of two different genes. COX-1 is constitutively expressed in most tissues and is a housekeeping gene (Funk et al., 1991). COX-2 is not detectable in most normal tissues or resting immune cells, but cytokines, growth factors and endotoxins can induce its expression (Hempel et al., 1994; Riese et al., 1994; Mestre et al., 2001). The role of NF-κB has been demonstrated to be important in mouse and human macrophage/monocytic cells in the induction of COX-2 (Mestre et al., 2001; Lee et al., 2001; Chen et al., 2000; Chen et al., 2001; Allport et al., 2001; Allport et al., 2000; Lukiw et al., 1998). The AP-1 and CREB transcription factors, in addition to NF-κB, have been demonstrated to be important in the regulation of COX-2 expression (Von Knethen et al., 1999; Ogasawara et al., 2001). We demonstrate here that in addition to regulating iNOS gene expression in an NF-κB-dependent manner, ILK activity is also required for LPS-mediated COX-2 expression in murine and human macrophages.

3.3 Materials and Methods

3.3.1 Cell Lines and Cell Culture

Rat intestinal epithelial cells, IEC-18 were obtained from American Type Culture Collections (ATCC). IEC-18 cell clones (ILK-13 Ala3 and A1C3) stably overexpressing wild-type sense ILK cDNA, ILK-14 cells stably expressing antisense ILK cDNA, ILK-KD, kinase-deficient cDNA were all prepared as described previously (Novak et al., 1998; Hannigan et al., 1996). IEC-18 cells and stably transfected cell clones were routinely cultured in α-minimal essential medium (α-MEM) (Gibco BRL) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Gibco BRL), glucose (3.6 mg/ml)
and insulin (10 μg/ml). Stably transfected derivatives were grown in the presence of G418 (80 μg/mL) to maintain selection pressure. Mouse monocyte-macrophage cells J774.1 (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FBS. Primary mouse macrophages were a gift from Dr. Urs Steinbrecher’s lab (University of British Columbia, Vancouver, Canada), and they were isolated as described by Hamilton et al. (38-40). Human monocyte-derived macrophages were a gift from Dr. Anthony Chow’s lab (University of British Columbia, Vancouver, Canada), and they were isolated as described by Sly et al. (Sly et al., 2001; Liu et al., 1994). All cells were grown at 37 °C, in a 99 % humidified atmosphere of 5 % CO₂ in air.

Bone marrow cells were isolated from femurs and plated in tissue culture plates for 2 hours at 37 °C in 100 ng/ml CSF-1 in 10 % FBS/Iscove’s modified Dulbecco’s media supplemented with 30 % conditioned media from cells producing the pZIP-Tex virus (Brunet et al., 1999). The nonadherent cells were then removed and placed in fresh tissue culture plates and allowed to differentiate into macrophages. After several passages over weeks, 100 % of the cells were Mac-1-positive.

### 3.3.2 Transfection

Cells were seeded into 6-well dishes 24 hours prior to transfection such that they would be approximately 60 % confluent on the day of transfection. Transfection of IEC-18 cells were carried out by using Lipofectin (Gibco BRL) according to the manufacturer’s guidelines, and 2 to 3 μg of plasmid per well of a 6-well dish. Transfection of J774 cells was carried out by using LipofectAMINE (Gibco BRL) according to manufacturers instructions, and 2 to 3 μg of plasmid per well as described by Pierce et al., 1996.
Transfections were carried out for 4 to 6 hours in Opti-MEM (Gibco BRL) medium. The transfection medium was then replaced by serum-containing medium for 6 hours prior to the beginning of an experiment.

### 3.3.3 In Vitro Kinase assay

ILK kinase assays were carried out as previously described (Delcommenne et al., 1998; Hannigan et al., 1996). The precipitates were washed and the reactions were carried as described by Hannigan et al., 1996. Myelin basic protein (MBP) was used as a substrate for ILK kinase activity. Phosphorylated MBP was resolved by 15% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE), and visualized by autoradiography. The ILK inhibitor, KP-392, was obtained from Kinetek Pharmaceuticals and was used as previously described (Tan et al., 2001; Persad et al., 2000; Persad et al., 2001; Troussard et al., 2000; Persad et al., 2001). All experiments were done with equivalent amounts of vehicle, DMSO.

### 3.3.4 Western Blot analysis

Cells were harvested in Nonident P-40 lysis buffer and stored at -70°C. Protein concentrations were measured using a BioRad Bradford protein assay kit. Equivalent amounts of protein were resolved in SDS-PAGE, transferred on to PVDF (Immobilion-P Millipore) membranes and probed with antibody. The protein of interest was visualized with enhanced chemiluminescent (ECL) (Amersham) reagents. The following antibodies were used in the experiments: anti-ILK (Upstate Biotechnology Inc), anti-IκB and anti-phosphoserine-32 IκB (New England Biolabs), anti-Akt and anti-phosphoserine-473 Akt
(New England Biolabs), anti-iNOS (New England Biolab), anti-COX-2 (Transduction Labs) and anti-β-Actin (Transduction Labs).

### 3.3.5 Luciferase Assays

Luciferase assays were performed on transiently transfected cells, according to the manufacturer’s instructions (Promega Corp). All assays were normalized for transfection efficiency by measuring a modified luciferase activity (pRenilla; dual luciferase; Promega). Triplicate samples were assayed for each trial of each condition in these experiments.

### 3.3.6 Detection of Nitric Oxide

Lipopolysaccharide (LPS) (Escherichia Coli 055:b5) (Difco (Detroit, MI)) was added at the concentrations indicated or at 500 ng/ml for 24 hour experiments and at 1 µg/ml for 1 hour experiments. Supernatants were removed after incubation times of 24 hours, and nitric oxide concentrations were determined as described by O’Farrell et al (46, 47). Triplicate samples were assayed for each trial of each condition in these experiment.

### 3.3.7 Plasmids

The ILK wild-type and dominant negative plasmids are described in the following articles (Delcommenne et al., 1998; Persad et al., 2000; Persad et al., 2001; Persad et al., 2000). The NF-κB response element promoter conjugated to a luciferase reporter was a kind gift from Dr. Nathan Yoganathan’s lab (Kinetek Pharmaceuticals, Vancouver, Canada). The iNOS promoters were a kind gift from Dr. W.J. Murphy’s lab (University of Kansas Medical Center, Kansas City, USA).
3.3.8 Immunohistochemistry

Human biopsy specimens were fixed in paraformaldehyde and paraffin (Sigma). 5 μm sections were prepared and the sections were placed on Silane (Sigma) coated slides. Conventional deparaffinization and rehydration techniques were employed. The sections were heat-retrieved treated in a pH 5 acetate buffer at 95°C for 10 minutes. They were treated with hydrogen peroxide and Triton X-100 buffer (Sigma), before blocking with 5 % milk. The sections were then incubated with ILK antibody (1/250 dilution) (Upstate Biotechnologies) in 5 % milk overnight at 4°C. The control staining was visualized with strepavidin secondary antibody (Jackson) and DAB (Sigma). Haematoxylin (Sigma) counterstaining was done by soaking the slides in the dye for 2 minutes, then washing with distilled water. Slides were mounted with Permount (Sigma). Micrographs were generated using the Nikon Eclipse TE300 microscope and Nikon D1 digital camera.
3.4 Results

3.4.1 ILK upregulates NF-κB activity

In order to determine if ILK regulates NF-κB activity, reporter assays were performed. These experiments were carried out by transfecting the NF-κB response-element promoter coupled to a luciferase reporter, into IEC-18 intestinal epithelial cells and stably transfected clones overexpressing sense or anti-sense ILK previously characterized (Novak et al., 1998; Delcommenne et al., 1998). Overexpression of wild type ILK, but not kinase-deficient mutant or anti-sense ILK, stimulates NF-κB response-element promoter activity. As shown in Figure 3.1A, NF-κB activity is substantially higher in two independent ILK overexpressing cell lines, relative to the control cell lines. Transfection of kinase deficient-dominant negative ILK cDNA (Figure 3.1B), as well as incubation with highly selective, small molecule inhibitor of ILK (KP-392) (Tan et al., 2001; Persad et al., 2000; Persad et al., 2001; Troussard et al., 2000; Persad et al., 2000) (Figure 2.1D), both inhibit NF-κB response element promoter activity in the ILK overexpressing IEC-18 clone, in a dose-dependent manner. This indicates that the observed stimulation of NF-κB reporter activity in this cell line is ILK-dependent. Furthermore, transfection of the ILK overexpressing cells with a potent dominant-negative PKB/Akt cDNA (PKB-AAA) (Delcommenne et al., 1998; Persad et al., 2000), also resulted in the inhibition of NF-κB activity, suggesting that the ILK induced stimulation of NF-κB in these cells involved PKB/Akt (Figure 3.1C).
Figure 3.1

A

B

C

D
Figure 3.1  (A) ILK upregulates NF-κB activity. IEC-18 cells and stable clones were transfected with the NF-κB response element (pGL3NF-κB response element reporter with a luciferase reporter (□)) or a control plasmid (pGL3Basic, no promoter with a luciferase reporter (■)). 48 hours post-transfection, cells were harvested and assessed for luciferase activity. Samples were normalized with pRenilla and activity is expressed as relative RLU. Data are mean +/- standard deviation of six independent trials. (B) Kinase-dead ILK decreases NF-κB activity in epithelial cells in a dose-dependent manner. ILK wild-type, overexpressing IEC-18 cells (clone A1a3 ILK-13) were co-transfected with increasing amounts of ILK(KD):V5, and the total amount of plasmid was kept constant by supplementing with appropriate amounts of Empty:V5, and 2 μg of pGL3NF-κB response element promoter. After 48 hours, cells were assayed for luciferase activity. All samples were normalized with pRenilla. Data are mean +/- standard deviation of four independent trials. Increased expression of ILK (KD):V5 was monitored by Western blot using anti-V5 antibody. (C) Transfection of dominant-negative PKB/Akt inhibits NF-κB activity in epithelial cells in a dose-dependent manner. ILK wild-type, overexpressing IEC-18 cells (clone A1a3 ILK-13) were co-transfected with increasing amounts of dominant-negative PKB/Akt (AAA):HA and pGL3NF-κB response element promoter. After 48 hours, cells were assayed for luciferase activity. All samples were normalized with pRenilla. Data are mean +/- standard deviation of four independent trials. Increasing PKB/Akt (AAA):HA protein expression was monitored with Western blot using an anti-HA antibody. (D) Pharmacological inhibition of ILK with ILK inhibitor KP-392 decreases NF-κB activity in a dose-dependent manner. ILK-13 IEC-18 cells were transfected with equivalent amounts of either pGL3NF-κB or pGL3Basic and pRenilla. Cells were incubated with complete media for 6 hours post-transfection. Cells were then treated with increasing concentrations of KP-392 for an additional 24 hours. Cells assessed for luciferase activity. All samples were normalized with pRenilla. Data are mean +/- standard deviation of three independent trials.
3.4.2 LPS stimulates NO production in an ILK dependent manner

We next wanted to determine the physiological relevance of the ILK-mediated stimulation of NF-κB. Since LPS is known to stimulate iNOS expression and NO production in murine macrophages in an NF-κB-dependent manner, we examined the role of ILK in this pathway. As shown in Figure 3.2A, LPS stimulates both NO production and NF-κB activity in the J774 macrophage cell line. We next wanted to examine whether LPS had any effect on ILK kinase activity in the cell line. As shown in Figure 3.2B, ILK kinase activity is rapidly and transiently stimulated by LPS, as is the phosphorylation of Akt/PKB on serine-473 and IκB on serine-32. The subsequent degradation of IκB as shown in Figure 2.2B allows release and translocation of NF-κB to translocate to the nucleus. There is no significant change in ILK and Akt/PKB protein levels, within the time course of activation of ILK and Akt/PKB phosphorylation (1 hour).
Figure 3.2  (A) LPS stimulates the production of nitric oxide in J774 cells and upregulates the NF-κB activity. J774 cells were transfected with equivalent amounts of pGL3NF-κB ( ■ ) and pRenilla. After transfection, cells were incubated in complete media for 6 hours. Cells were then stimulated with increasing amounts of LPS in complete media for an additional 24 hours. After 24 hours, nitric oxide production was measured according to the Greiss method and cells were harvested, lysed and assessed for luciferase activity. Data are mean +/- standard deviation of six independent samples. (B) LPS stimulates ILK activity and IκB serine-32 phosphorylation. Cells were exposed to 1 μg/ml of LPS for 60 minutes. ILK kinase activity was determined in J774. AKT serine-473 and IκB serine-32.
3.4.3 Inhibition of ILK suppresses LPS stimulated iNOS expression and NO production in J774 cells and in primary murine macrophages

In order to determine whether the LPS stimulated NO production is ILK-dependent, we exposed J774 cells to LPS and increasing doses of a recently identified, highly selective ILK-inhibitor, KP-392 (Tan et al., 2001; Persad et al., 2000; Troussard et al., 2000; Persad et al., 2001). We observed a parallel dose-dependent inhibition of NO production and iNOS expression (Figure 3.3A). As can be seen in Figure 3.3A, incubation with the KP-392 also inhibits LPS-stimulated ILK activity in these cells in a dose-dependent manner.

To determine if this effect of ILK inhibitor on LPS-stimulated NO production could be demonstrated in primary murine macrophages, murine monocytes were differentiated into macrophages using macrophage-colony stimulating factor (M-CSF) as described in “Materials and Methods”. The macrophages were maintained for 24 hours without M-CSF prior to the experiment. The macrophages were then exposed to LPS in the presence of increasing amounts of ILK inhibitor (KP-392). As shown in Figure 3.3B, the ILK inhibitor decreased LPS-stimulated NO production, in a similar manner to that observed in the J774 cell line after 24 hours.
Figure 3.3
Figure 3.3  (A) Inhibition of ILK kinase activity decreases iNOS expression. J774 cells were incubated with increasing concentrations of KP-392 and 500 ng/ml of LPS for 24 hours in 5% serum. Nitric oxide production was measured according to the Greiss method. Cells were harvested and inducible nitric oxide synthase (iNOS) expression was measured by Western blotting. Parallel experiments of ILK kinase activity was measured as described in materials and methods. Data are mean +/- standard deviation of four independent trials. (B) Inhibition of ILK kinase activity decreases iNOS expression. J774 cell clones were created and incubated with 500ng/ml of LPS. After 24 hours, nitric oxide production was assessed by the Greiss method. Data are mean +/- standard deviation of three independent trials.
Figure 3.3
Figure 3.3 (C) Inhibition of ILK kinase activity decreases iNOS expression. J774 cells were incubated with increasing concentrations of KP-392 and 500 ng/ml of LPS for 24 hours in 5% serum. Nitric oxide production was measured according to the Greiss method. Cells were harvested and inducible nitric oxide synthase (iNOS) expression was measured by Western blotting. Parallel experiments of ILK kinase activity was measured as described in materials and methods. Data are mean +/- standard deviation of four independent trials. (D) Inhibition of LPS-stimulated NO production in primary mouse macrophages by KP-392. Primary mouse macrophages were incubated with 500ng/ml of LPS and increasing amounts of KP-392 as indicated. After 24 hours, nitric oxide production was assessed by the Greiss method. Data are mean +/- standard deviation of three independent trials.
3.4.4 Inhibition of ILK suppresses LPS stimulated NF-κB expression and NF-κB dependent iNOS gene expression

In order to examine the mechanism of the effect of ILK on NO production, we first determined whether inhibition of ILK would also suppress LPS-stimulated NF-κB transcription. J774 cells were co-transfected with increasing amounts of the dominant-negative ILK-KD:V5 plasmid and the NF-κB response element promoter. As can be seen from Figure 3.4A, NF-κB activity is inhibited in a dose-dependent manner by increased expression of the dominant negative ILK. It is well known that macrophages have extremely low transfection efficiency (Mack et al., 1998; Stacey et al., 1993; Pierce et al., 1996; Feng et al., 2000; Mijarovic et al., 1997), therefore, we were unable to detect the levels of ILK-KD expression on a western blot. Therefore, J774 cells were transfected with the NF-κB response element promoter-reporter constructs and reporter activity was measured in LPS stimulated J774 cells exposed to increasing concentrations of KP-392 ILK inhibitor. As shown in Figure 3.4B, inhibition of ILK decreases LPS-stimulated NF-κB response element promoter activity. The mouse iNOS promoter possesses two NF-κB sites, which have been shown to be essential for LPS-mediated NO production. To determine whether LPS-stimulated iNOS expression could also be inhibited by the ILK inhibitor, and whether this ILK dependent iNOS expression was dependent on NF-κB, the J774 cells were transfected with either the full length, wild type iNOS promoter (Xie et al., 1994), or the iNOS promoter with point mutations in the NF-κB sites (Xie et al., 1994). The cells were then stimulated with LPS and treated with increasing concentrations of KP-392 ILK inhibitor. LPS stimulated the full length iNOS promoter but only showed minimal stimulation of iNOS promoter activity containing
point mutations in the NF-κB sites (Figure 3.4C). In addition, the KP-392 ILK inhibitor inhibited the LPS-stimulated iNOS promoter activity in a dose-dependent manner, similar to that observed with the NF-κB response element promoter (Figure 3.4C).
Figure 3.4  (A) Kinase-dead ILK decreases NF-κB activity in J774 macrophage cells in a dose-dependent manner. J774 macrophages cells were co-transfected with increasing amounts of ILK(KD):V5, and the total amount of plasmid was kept constant by supplementing with appropriate amounts of Empty:V5, and 2 μg of pGL3NF-κB response element promoter. Cells were incubated with 500ng/ml of LPS and assayed for luciferase activity 24 hours later. All samples were normalized with pRenilla. Data are mean +/- standard deviation of four independent trials. (B) KP-392 decreases NF-κB activity in a dose-dependent manner. Cells were transfected with equivalent amounts of pGL3NF-κB and pRenilla. After transfection, cells were incubated with 500 ng/ml of LPS and increasing amounts of KP-392 and total mount of drug vehicle was kept constant by the relevant supplementary addition of DMSO for 24 hours, diluted in DMEM with 5% FBS. Cells were harvested and assessed for luciferase activity. Data are mean +/- standard deviation of six independent trials. (C) ILK regulates iNOS transcription in an NF-κB dependent manner. J774 cells were transfected with the indicated plasmids (full-length wild-type murine iNOS promoter or double mutant NF-κB binding sites murine iNOS promoter). Cells were then incubated with 500 ng/ml of LPS and increasing concentrations of KP-392. After 24 hours, cells were harvested and assessed for luciferase activity. Data are mean +/- standard deviation of four independent trials.
3.4.5 Inhibition of ILK suppresses IκB serine 32 phosphorylation and prevents its degradation

To gain further insight into the mechanism of the ILK induced regulation of NF-κB, we examined the effects of inhibition of ILK activity on IκB phosphorylation and degradation. As shown in Figure 3.5, LPS treatment of J774 cells results in a stimulation of phosphorylation of IκB on serine-32, the site for ubiquitin-mediated degradation of IκB (Baeuerle and Baltimore, 1996). As can be seen in Figure 3.5, LPS treatment also leads to degradation of IκB, which correlates with its phosphorylation on serine-32. Exposure of cells to 50 μm KP-392 ILK inhibitor for 1 hour prior to LPS stimulation markedly inhibits IκB phosphorylation, thus stabilizing and preventing its degradation (Figure 3.5), and preventing subsequent NF-κB activation by retaining NF-κB in the cytoplasm.
Figure 3.5 Inhibition of ILK suppresses IκB serine-32 phosphorylation and prevents its degradation. J774 cells were treated with LPS alone or with LPS and KP-392. IκB and IκB serine-32 phosphorylation and protein levels were determined by Western blot analysis in J774 cells exposed to KP-392 (50 μM) for one hour prior to exposure to LPS (1 μg/ml) and KP-392 (50 μM) for the indicated times.
3.4.6 Inhibition of ILK suppresses LPS stimulated COX-2 expression in J774 cells and human macrophages

The regulation of iNOS production differs between human and mouse macrophages, in large part due to the differences between the human and murine iNOS promoters. It is known that the induction of another pro-inflammatory protein, COX-2, by LPS stimulation in mouse macrophages also involves the activation of NF-κB (D'Acquisto et al., 1997; Abate et al., 1998; D'Acquisto et al., 2000; Paik et al., 2000). Recent publications have also identified NF-κB as a major regulator of COX-2 expression in humans (Chen et al., 2000; Macao et al., 2000; Kojima et al., 2000). Therefore, to investigate whether the ILK inhibitor is effective in decreasing inflammatory responses and regulating NF-κB activity in humans, we analysed LPS-stimulated cyclooxygenase-2 (COX-2) expression.

We first determined if ILK was present in human peripheral macrophages. As can be observed in Figure 3.6, ILK expression is readily detectable in human alveolar macrophages. We next incubated J774 cells and human monocyte derived macrophages with LPS and increasing amounts of KP-392. As shown in Figure 3.7A and 3.7B, LPS stimulated COX-2 is inhibited in a dose-dependent manner with increasing amounts of ILK inhibitor, in both mouse and human macrophages.
Figure 3.6 Expression of Integrin Linked kinase in alveolar macrophages. (A) Rabbit IgG control staining on lung biopsy. Human lung biopsy were fixed with paraformaldehyde and imbedded in paraffin. The paraffin blocks were sliced into 5 µm thick sections and stained with haematoxylin and rabbit IgG antibody at the same concentration as the ILK antibody. Staining was carried out as indicated in “Materials and Methods”. (B) ILK staining on lung biopsy. Section from the same block of tissue was stained with ILK (Upstate Biotechnologies) antibody.
Figure 3.7  (A) Inhibition of ILK suppresses COX-2 expression in J774 cells. Cells were incubated with LPS (500 ng/mL) and indicated amounts of KP-392 for 24 hours. COX-2 expression was measured by Western blot.  

(B) Inhibition of ILK suppresses COX-2 expression in human peripheral macrophages. Cells were incubated with LPS (500 ng/mL) and indicated amounts of KP-392 for 24 hours. COX-2 expression was measured by Western blot.
3.5 Discussion

The expression of the inducible form of nitric oxide synthase (iNOS), which catalyzes the production of nitric oxide (NO) from L-Arginine, is regulated by the transcription factor NF-κB in murine macrophages in response to LPS and cytokines (MacMicking et al., 1997). Macrophage-derived NO is an important host defense, microbial and tumour cell killing agent (MacMicking et al., 1997), as well as a regulator of pro-inflammatory genes in vivo. The ability to modulate iNOS expression could potentially control chronic and acute inflammatory diseases; therefore, it is important to understand the regulation of iNOS.

In this paper we have provided novel data indicating that the integrin-linked kinase (ILK), which couples integrins and growth factors to downstream signalling pathways (Dedhar, 2000), can regulate iNOS expression and NO production in murine macrophages, and regulate the expression of a pro-inflammatory protein, COX-2, in both murine and human macrophages. ILK is a PI 3 kinase-dependent kinase (Delcommenne et al., 1998; Persad et al., 2000; Morimoto et al., 2000; Wangle et al., 1999) capable of regulating the phosphorylation and activation of PKB/Akt, which has recently been shown to regulate NF-κB activation by activating IkB kinase (Ozes et al., 1999; Romashkova and Makarov, 1999). Here we have shown that ILK can also regulate NF-κB activation in a physiologically relevant system. Our data indicates that ILK activity is rapidly stimulated in response to LPS in murine macrophages, and results in the phosphorylation and degradation of IkB. Importantly, we have shown here that inhibition of ILK using a specific small molecule inhibitor or dominant-negative form of ILK, results in the inhibition of IkB phosphorylation on serine 32 and the prevention of
its degradation. In addition, ILK inhibition suppresses LPS stimulated NF-κB promoter activity as well as iNOS promoter activity and NO production. The inhibition of ILK also inhibits iNOS protein expression.

The precise mechanism involved in the regulation of IκB phosphorylation by ILK is not yet clear and is under investigation. A possible mechanism is that ILK is a critical upstream mediator of NF-κB activation through its capacity to regulate PKB/Akt kinase activity by phosphorylating serine-473 (Persad et al., 2000). In this paper we demonstrated that ILK-stimulated NF-κB activity is inhibited by dominant-negative PKB/Akt. This implicates PKB/Akt in ILK’s regulation of NF-κB.

Our data further suggest that ILK may play a pivotal role in the regulation of NO production by coupling integrin and LPS signalling. It has been shown that NO production is significantly stimulated in the presence of integrin binding RGD peptide (Muller et al., 1997; Attur et al., 2000). Furthermore, ligation of α5β1 integrin with a specific antibody stimulates NO production (Muller et al., 1997; Attur et al., 2000; Schwartz et al., 2001). Since ILK can interact directly with the cytoplasmic domains of integrin β1 and β3 subunits, and can couple integrins to the actin cytoskeleton and downstream signalling components such as Akt/PKB (Dedhar, 2000), it is likely that the integrin-mediated stimulation of NO involves ILK. Thus ILK appears to be an important mediator of NO production by iNOS in macrophages and may play a role in other cell types such as endothelial cells, chondrocytes and osteoblasts.

Since human and mouse iNOS promoters are very different, we have also demonstrated the role of ILK on inflammatory molecule expression in human macrophages, by assessing the expression of COX-2. The LPS-inducible expression of COX-2, also a
proinflammatory enzyme, is regulated by NF-κB in both mice and human promoters (Lee et al., 2001; von Knethen et al., 1999; Muller et al., 1997). As shown by our results, ILK plays a role in regulating the expression of COX-2 in human macrophages. Since ILK has been shown to regulate transcription factors such as AP-1 and CREB (Troussard et al., 1999; D’Amico et al., 2000), it is probable that ILK could regulate the transcription of COX-2 through an NF-κB, AP-1, and/or CREB dependent manner. Investigations are currently underway to determine the identity of the transcription factors involved in ILK regulated transcription activity of the COX-2 promoter.

Macrophages can produce NO and prostaglandins, which are regulated by the expression of iNOS and COX-2 upon stimulation with LPS. Our results show that inhibiting ILK function using either a small molecule (KP-392) or dominant-negative ILK cDNA can result in the inhibition of NF-κB activity as well as NF-κB-dependent expression of both iNOS and COX-2. These results implicate ILK as a novel player in the regulation of NO and prostaglandin production and suggest that the inhibition of ILK may be of therapeutic benefit in controlling destructive inflammatory processes triggered by NO and COX-2.
3.6 REFERENCES


4 Conclusion and Future Directions

4.1 Conclusions

A tumor is a heterogeneous composition of malignant and normal cells, and extracellular matrix (ECM). Within this dynamic microenvironment, growth factors and ECM components interact with each other physically and in a paracrine manner to facilitate and select for malignant cells (Ferrara et al., 1993; Wiseman and Werb, 2002). These malignant cells display increased cell proliferation, survival and migration (Hanahan and Weinberg, 2000), characteristics which are recapitulated in epithelial cells overexpressing ILK. Indeed, molecular and genetic approaches have converged to confirm that ILK is a key effector of integrin and tyrosine kinase receptor cross-talk, as well as an important regulator of cell-cell and cell-ECM mediated intracellular signaling (Delcommenne et al., 1998; Hannigan et al., 2005; Persad et al., 2000; Persad and Dedhar, 2003). Deregulation of ILK activity during these processes may lead to tumorigenesis. In this thesis, I have described three novel roles for ILK in macrophages, cancer cells and endothelial cells. In addition, I have reinforced the kinase function of ILK as a PDK2 candidate in the phosphorylation of Akt/PKB on serine-473.

Angiogenesis is a process involving the coordination of multiple cell signals and mechanisms that result in proliferation, cell survival and migration. These mechanisms have been demonstrated to be PI3-kinase dependent and are critical to blood vessel formation. Hamada et al. demonstrated in an in vivo model that PI3-kinase-Akt/PKB-PTEN pathway in murine endothelial cells is required for normal cardiac and vascular development (Hamada et al., 2005). As well, they demonstrated that the loss of PTEN-mediated control of this pathway contributes both to susceptibility to new tumorigenic
mutations and accelerated tumor growth, secondary to enhanced tumor angiogenesis. Moreover, the loss of PTEN also results in an increased expression of Ang-2, VEGF-A, VEGFR1 and VEGFR2 (Hamada et al., 2005). Likewise, the loss of αvβ3 integrins in mice results in an increase in VEGF receptor 2/Flk1 expression and phosphorylation/activation, amplifying the signals that promote proliferation and survival. ILK has been implicated in several of the processes described above and plays a role in the PI3 kinase cascade (Attwell et al., 2003; Delcommenne et al., 1998; Hannigan et al., 2005; Persad and Dedhar, 2003; Persad et al., 2001b). Therefore, it is possible that ILK is involved in the regulation of VEGFR2 expression in endothelial cells and VEGF-mediated migration, a necessary mechanism in angiogenesis (Attwell et al., 2003; Ferrara, 2005; Ferrara et al., 2003).

Cell migration is documented to be regulated by Rac/Cdc42 (Fryer and Field, 2005). Studies into the mechanism of ILK-mediated Rac activation suggest an important role for the ILK-β-parvin interaction and the activity of the Rac/Cdc42-specific guanine nucleotide exchange factor α-PIX downstream of ILK; linking ILK with the Rac- and Cdc42-mediated actin cytoskeleton reorganization in epithelial cells (Filipenko et al., 2005) in a PI3 kinase dependent manner (Attwell et al., 2003). Together, these new findings imply that upon VEGF stimulation, the role of ILK in endothelial cells may partially involve the initiation of a positive autocrine/paracrine feedback loop by increasing the expression of VEGFR2 and VEGF-A. In addition, ILK may be involved in the reorganization of the cytoskeleton in α-PIX, Rac and Cdc-42 dependent manner in endothelial cells. Moreover ILK may increase the levels of cyclin D, promoting
endothelial cells proliferation, as well as expression of anti-apoptotic molecules in an Akt/PKB dependent-manner upon VEGF stimulation.

For the past 10 years, the identity of the kinase responsible for activation of Akt/PKB has been widely debated. In addition to ILK, several enzymes have been shown to phosphorylate Akt/PKB at serine 473 position, coined as PDK2 or HM activity, including MAPKAP kinase-2, PKC isoforms, DNA-dependent protein kinase, ATM, PDK-1 and Akt/PKB itself (Bayaschas and Alessi, 2005). Recently, Sarbassov and colleagues demonstrate that Rictor/GβL of the mTOR/Rictor/GBL complex is also a possible PDK-2 candidate (Sarbassov et al., 2004). In addition to phosphorylating Akt/PKB directly, data presented in this thesis demonstrate that ILK regulates mTOR through activation of Akt/PKB; though it is not clear whether this is a rapamycin sensitive event. Still it is possible that ILK may directly or indirectly regulate the Rictor/GβL and subsequently Akt/PKB phosphorylation.

In addition, I was able to show that the inhibition of ILK, with the ILK inhibitor, results in a decrease in angiogenesis and growth delay in xenograft prostate tumour size. Although the worked described in this thesis suggests that delayed tumor growth is mostly due to angiogenesis, the observed growth delay could be the result of concurrent decrease in angiogenesis and increased cancer cell death, as long term exposure to the ILK inhibitor results in cell death.

Many heterogeneous tumors, such as prostate and colon cancer, display elevated COX-2 expression as a result of deregulated NF-κB function (Karin and Greten, 2005). Infiltrated within these tumors are special immune cells known as tissue activated macrophages (TAMs) (Lewis and Pollard, 2006), which have been observed to be the
main producers of prostaglandins and COX-2 enzyme (Karin and Greten, 2005). Furthermore, studies have shown that increased prostaglandin levels can stimulate malignant cells to produce VEGF, which initiates and promotes angiogenesis. This is the result of improper HIF-1α transcription factor activation under normoxic conditions (Huang et al., 2005). Thus, ILK may play a key role in at least two critical pathways and cell types within a heterogeneous tumor to promote and sustain the tumorgenic process.

Specifically, I demonstrated that *in vitro* genetic and pharmacological inhibition of ILK suppresses LPS-stimulated, NF-κB mediated expression of iNOS and COX-2, and the subsequent increase in NO and prostaglandin production. NF-κB is the primary mediator of proinflammatory molecules and evidence *in vitro* and *in vivo* suggests that the p65 subunit must interact with acetylated PARP-1 for full activation of NF-κB (Hassa et al., 2003). Recently, it has been shown that acetylation of PARP-1 is mediated by CBP/300 (Hassa et al., 2005), which is phosphorylated and activated by Akt/PKB activity (Du and Montminy, 1998). Since ILK can phosphorylate Akt/PKB (Delcommenne et al., 1998; Hassa et al., 2003; Persad et al., 2001a), it would be interesting to know whether ILK may further control NF-κB activity through the regulation of PARP-1 acetylation. In addition, the phosphorylation of p50, an NF-κB subunit, further activates its transcriptional activity, but the kinase which phosphorplylates the subunit is not well defined (Hou et al., 2003; Li et al., 1994a; Li et al., 1994b). Interestingly, the kinase responsible for this phosphorylation event has not been defined with ILK as a possible candidate kinase for this direct regulation of NF-κB transcriptional activity. In addition to proinflammatory molecules, NF-κB also upregulates prosurvival targets such as Bcl-2, and drug resistance genes such as p-glycoprotein (Karin and Greten, 2005). Thus, over-
activation of ILK may induce the expression of pro-survival targets simultaneously with the expression of COX-2 and iNOS to confer a survival and propagation advantage to diseased cells such as cancer cells.

In summary, I have presented several lines of evidence supporting a key role for ILK in the positive regulation of: Akt/PKB and HIF-1α-dependent expression of VEGF in cancer cells and NF-κB-dependent induction of iNOS and COX-2. Further, my studies indicate that ILK is indispensable for the VEGF-mediated proliferation and migration of endothelial cells, and continue to highlight the importance of ILK as a kinase and adaptor in normal and malignant cell behaviour. Further studies will be required to determine the precise mechanisms by which ILK regulates NF-κB and HIF-1α during processes such as cell survival, proliferation, and migration. In addition, further investigations would be required to understand the mechanisms involved in VEGF-stimulated, ILK-dependent migration and proliferation of endothelial cells. Elucidation of these mechanisms will underscore the importance of ILK activity and ILK signaling pathway as targets for therapeutic intervention in treating cancer.

4.2 Future Directions

ILK is pivotal in mediating and coordinating signal transduction events involving integrins and growth factor receptors during cell migration, survival and proliferation. Deregulation of these tightly controlled cellular processes can result in pathological conditions. The loss of ILK results in poor organogenesis, however, the overexpression or activation of ILK has directly been shown to transform cells to that which resembles a cancer cell (Hannigan et al., 2005; Hannigan et al., 1996; Novak et al., 1998; Radeva et
al., 1997; Somasiri et al., 2001), exhibiting avoidance of apoptosis, increased proliferation, migration and invasion into the surrounding area. Indeed, these phenotypes are the result of specific interactions involving intracellular multiprotein complexes at the membrane, in turn, altering the various cascades of signals to further mediate nuclear complexes and gene transcription. Thus identifying proteins that interact with ILK during different cell activities may assist in further uncovering the role of ILK in development and disease cascades.

ILK activity has been demonstrated to be important in blood vessel sprouting, tubulogenesis and morphogenesis both in vitro and in vivo (Cho et al., 2005; Friedrich et al., 2004; Hannigan et al., 1996; Kaneko et al., 2004; Koul et al., 2005; Vouret-Craviari et al., 2004; Watanabe et al., 2005; Yau et al., 2005). However, little is known about the involvement of integrin and VEGF receptor cross-talk in mediating the negative role of the αvβ3 and αvβ5 (Stupack and Cheresh, 2004), and positive role of α5β1 integrin during angiogenesis. Because ILK can associate with integrins, it is possible that it may be involved in coordinating these intracellular signals. Since the IPP complex can be a positive or negative regulator of downstream events, depending on the different PINCH and Parvin isoforms bound to ILK, uncovering the identity of the proteins associated with the IPP complex would bring insight into the types of proteins and associations required for a specific phenotype. In order to address the dynamic nature of these protein complexes, tandem affinity purification (TAP) tag method (Puig et al., 2001; Rigaut et al., 1999) could be used to purify and identify proteins associating with αvβ3, αvβ5, α5β1 integrins and ILK. This technique has been successfully used to identify over 200 unique multi-subunit complexes in yeast (Gavin et al., 2002).
Since TAP tag method is useful in identifying complexes of proteins, it may also be useful in unraveling the ILK-dependent complexes downstream from ILK activation. The subsequent evaluation of the post-translational modifications of the proteins in the complex will also give insight into how they may be regulated. To further define the importance of each protein interaction, individual or combinations of proteins identified at the complex could be deleted with siRNA, and the cells could be observed for changes in morphology, survival, proliferation and migration. This would also give insight into the downstream events that are dependent on specific upstream protein interactions.

There are an incredible number of kinases and an amazingly complex network of protein phosphorylation pathways. Tools that can down regulate ILK activity, such as siRNA, the Cre-Lox system, and a small molecule ILK inhibitor would help to deconvolute the complexity of ILK signaling and further our understanding into the underlying molecular mechanisms of ILK in cell behaviour, development and disease. Phosphoproteomics (Mumby and Brekken, 2005; Shu et al., 2004) could advance the study of ILK significantly by allowing the study of ILK-dependent protein phosphorylation on a proteome-wide scale.

Small interfering RNA has proven to be an effective means of dramatically decreasing the levels of ILK protein expression (Dykxhoorn et al., 2003; Filipenko et al., 2005; Tan et al., 2004; Troussard et al., 2003). With this tool, future studies could look at how gene transcription and protein phosphorylation profile of cancer cells change upon a decrease of ILK protein expression. As well, the ILK-dependent activation of Akt/PKB can be further evaluated. To answer these questions, cells displaying high ILK activity such as PC3 prostate cancer cells, MDA-MB-231 breast cancer cells, and SW480 colon
cancer cells can be treated with either ILK siRNA, Akt/PKB siRNA or control siRNA. Messenger RNA from treated and control cells at several time points would be used to prepare labeled cDNA to probe commercially available human microarray chips thus creating a temporal transcriptional profile. Transcripts which display a significant variance between control and siRNA treated cells can be further validated by real-time PCR. To further evaluate the cellular phosphorylation profile in the absence of ILK, proteins from siRNA treated and control cells could be differentially labeled using the stable isotope labeling of amino acid in cell culture (SILAC) method (Amanchy et al., 2005; Peri et al., 2003; Peri et al., 2004). Quantification of phosphoprotein levels between the two conditions, and the unequivocal identification of phosphorylated proteins can be accomplished by LC-MS and tandem MS, respectively. Proteins displaying changes in phosphorylation status could be further confirmed by Western blot analysis. These techniques also offer the potential of identifying novel ILK-dependent pathways. In parallel, similar studies could also be done by treating cells with the ILK small molecule inhibitor.

The tumorgenic process that leads a normal cell to a cancerous cell can be divided into three mechanistic phases: initiation, promotion and progression. To date, we have mounting evidence of the critical role of ILK in multiple stages of tumorigenesis stemming from cell culture studies which have been further validated with histopathology studies (Hannigan et al., 2005). Several lines of evidence suggest that the role of ILK in carcinogenesis is at the level of tumor promotion and progression (Hannigan et al., 2005; Troussard et al., 2006). Troussard et al. demonstrated that breast cancer cells versus normal cells have a preferential dependence on ILK for protein kinase B/Akt activation...
and cell survival (Troussard et al., 2006). Bravou et al. observed elevated ILK expression in metastatic lesions of colon cancer (Bravou et al., 2006), while Zhiyong et al. demonstrated that ILK regulates matrix metalloproteinase-2 and urokinase-type plasminogen activator expression to convey metastatic function in murine mammary epithelial cancer cells (Zhiyong et al., 2006). To dissect the role of ILK in various stages of tumorigenesis and angiogenesis, one could start by crossing ILK flox/flox mice with PTEN -/- mice, TRAMP or LADY mice, transgenic mice that spontaneously form prostate cancers (Gingrich et al., 1996; Gingrich and Greenberg, 1996; Greenberg et al., 1995; Greenberg et al., 1994). Progeny mice would be further crossed with tissue-specific promoter Cre-mice (Greenberg et al., 1994; Singh et al., 2002; Young and Dong, 2005), specifically for prostate (probasin promoter Cre) and endothelial cells (Tie2Cre). At various stages of spontaneous tumor promotion and progression, ILK expression can be silenced and the effect on tumorigenesis can be assessed. Hopefully, results from these ILK mouse model would give insight not only on the role of ILK in cancer, but also towards the development of preclinical animal models that would allow better prediction of the outcome and efficacy of anti-ILK therapy on human disease.

An accepted view of cancer is that they are stem cells that fail to differentiate (Harris, 2005). Thus the study of developmental pathways would give insight into the deregulated differentiation signals that may lead to cancer progression. Recently, Mills et al. demonstrated that ILK is associated with the regulation of Sonic Hedgehog (Shh) pathway (Mills et al., 2006), a pathway found to be deregulated in tumors of neurological origin (Wechsler-Reya and Scott, 2001). GSK-3 is a constitutively active serine-threonine kinase thought to be involved in the regulation of several signaling pathways,
including the Wnt, Hedgehog and Notch pathway (Trowbridge et al., 2006). Furthermore, ILK has been demonstrated to regulate GSK-3 through direct phosphorylation and indirectly through activation of Akt/PKB. To further define the role of ILK in these pathways, immunoprecipitation assays of ILK with GSK-3 or Akt/PKB could be performed in the presence and absence of Wnt, Hedgehog and Notch exposure in HEK-293 cells and primitive hematopoietic stem cells to determine direct phosphorylation capabilities. Results from these experiments can provide immediate details of the role of ILK in the regulation of these pathways in a GSK-dependent manner. Fluorescence resonance energy transfer (FRET) technology could also be used to assess transient interactions. Hematopoietic stem cells are a good model because all three pathways have been shown to be critical for cell proliferation, without affecting the mature cells (Trowbridge et al., 2006). These studies would serve to further define the role of ILK in development and provide insight into the function of ILK in cancer.

In summary, I believe that ILK represents an attractive target for treatment of a variety of human diseases and cancers. Though we have evidence of ILK as a potential master regulator of critical biological processes that are relevant to cancer progression such cell-cell, cell-ECM signaling angiogenesis and development, there is still much work to be done. A long line of evidence shows that ILK is critical as a kinase as well as an adaptor in development and cancer progression, however, the proteins that interact with ILK to give signaling complexity and diversity to influence the specificity, strength and timing of intracellular signals are unclear. Another area to be explored would be to further determine the function of specific complex combinations and determine where and when ILK is expressed during tumor progression. The results from these
experiments would give a better understanding of the role of ILK in tumor progression and the events that are the result of deregulated ILK activity. Moreover, understanding how ILK expression and activity are regulated may give insight into the events leading up to ILK deregulation. In turn, ILK could also be used as a biomarker for screening as well as a therapeutic target.

4.3 References


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