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LIM KINASE I MODULATES EXPRESSION OF MATRIX METALLOPROTEINASES AND ASSOCIATES WITH $\gamma$-TUBULIN: DUAL ROLE IN INVASION AND MITOTIC PROCESSES

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Molecular Biology and Microbiology in the Burnett College of Biomedical Sciences at the University of Central Florida Orlando, Florida

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ABSTRACT

LIM kinase 1 (LIMK1) is a unique dual specificity serine/threonine kinase containing two N-terminal LIM domains in tandem, a PDZ domain and a C-terminal catalytic domain. LIMK1 is involved in modulation of actin cytoskeleton through inactivating phosphorylation of the ADF (actin depolymerization factor) family protein cofilin. Recent studies have shown that LIMK1 is upregulated in breast and prostate cancer cells and tissues, promotes metastasis in animals and induces acquisition of an invasive phenotype when ectopically expressed in benign prostate epithelial (BPH) cells. Furthermore, overexpression of LIMK1 was associated with altered sub cellular localization of the membrane type 1 matrix metalloprotease (MT1-MMP). Matrix metalloproteases (MMPs) are a family of zinc dependant proteolytic enzymes that hydrolyze extra cellular matrix and cell surface molecules. A number of MMPs including MMP-2, MMP-9 and their activator MT1-MMP are over expressed in a variety of cancers including prostate cancer. The abundant expression of these enzymes contributes to changes in the tumor microenvironment, which facilitate degradation of the surrounding collagen matrix and migration of cells through the matrix defects. In this study, we show that MMPs are involved in LIMK1 induced invasion of otherwise non-invasive BPH cells. We also show that (a) the kinase activity of LIMK is not essential for the invasive behavior of the cells and (b) the absence of LIM domains significantly retards cell invasion. We have established transfected sub lines of BPH cells stably expressing 1) constitutively active LIMK1 (BPHL^CA), 2) kinase dead LIMK1 (BPHL^KD) and 3) only the kinase domain of LIMK1 (BPHL^K) for our study. In vitro invasion assays revealed that LIMK1 induced invasion was inhibited by the MMP specific inhibitor, GM6001, and that cells expressing kinase-dead LIMK1 were equally invasive. Furthermore,
BPH cells expressing LIMK1 mutants expressed higher amounts of MMP-2 and MMP-9. Substrate zymography revealed increased concentration of secreted MMP-2 and MMP-9 in the media of BPHL<sup>CA</sup> and BPHL<sup>K</sup> cells respectively compared to BPH<sup>V</sup> (vector control) cells. Quantitative RT-PCR also showed a ~10 fold increase in the steady state concentration of MMP-2 in BPHL<sup>CA</sup> cells compared to the control BPHL<sup>V</sup> cells. Expression of active LIMK1 stimulated cell-surface expression of MT1-MMP in BPHL<sup>CA</sup> cells as determined by flow cytometry. A modest increase in expression of MT1-MMP was noted in BPHL<sup>KD</sup> cells compared to BPHL<sup>K</sup> and BPHL<sup>V</sup> cells. Immunofluorescence analysis indicated differential localization of MT1-MMP and LIMK1 in BPH cells expressing different mutants of LIMK1. Co-localization of LIMK1 and MT1-MMP in the plasma membrane and in the perinuclear region was also evident in these cells. Furthermore, here we provide evidence that suggests a functional role for phosphorylated (activated) LIMK1/2 (p-LIMK1/2) during mitosis through its association with γ-tubulin. Immunofluorescence analysis showed distinct co-localization of γ -tubulin and p-LIMK1/2 in the centrosomes during mitosis from early prophase to the beginning of telophase. No association was seen in the interphase or in late telophase. Phospho-LIMK1/2 was co-precipitated in immunoprecipitates of γ -tubulin using an anti- γ -tubulin antibody suggesting a physical association between these proteins in a complex. This finding reveals a novel role of LIMK1 in the mitotic process.

In summary, our data suggests that MMPs are involved in LIMK1 induced invasion of prostate epithelial cells, and that this effect is mediated through altered expression and activation of specific MMPs. Furthermore, LIMK1 induced invasion is dependant on the presence of LIM domains more than the kinase activity. Finally, we show that phosphorylated LIMK1 and
LIMK2 are involved in the mitotic process in a stage specific manner through its association with the centrosomal protein $\gamma$-tubulin. Because LIMK1 promotes invasion in vitro, regulates expression of MMPs, and is involved in mitotic processes, it is an attractive drug target for prostate cancer therapy.
Dedicated to my family and friends.

Your support, confidence and love carried me.
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1. INTRODUCTION

1.1 Facts about Prostate Cancer

Prostate cancer is the second most deadly cancer in men after lung cancer in the United States and the sixth leading cause of death of men overall [Ward-Smith, 2006]. In fact, one in every six men will be diagnosed with the disease at some point in their life (Figure 1). Prevalence rates of prostate cancer remain significantly higher in African Americans compared to white men and Hispanics [Theodorescu and Pollack, 2003]. Furthermore, mortality rates for African Americans are at least doubled compared to men of other racial and ethnic groups. In addition, family history is one of the strongest risk factors for prostate cancer [Paiss et al., 2006]. The 5-year relative prostate cancer survival rate for men with localized prostate cancer is 100%; however men with advanced stage of the disease have only a 33% chance of survival. Prostate cancer is mainly diagnosed in men over age 65, nonetheless younger populations are also susceptible to the disease. It affects all racial/ethnic groups and is a major health concern.

1.2 Stages of the Disease

The prostate rests in the pelvis (Figure 2) and is made up of branching glands with ducts that are lined with secretory epithelial cells and basal cells [McNeal, 1988]. Prostate cancer is a disease in which the epithelial cells that line the prostate gland mutate and begin to proliferate without regulation. It is divided into six stages [Savage et al., 1997]. Stage 1 (T1) cancer is localized only in the prostate. Stage 2 (T2) cancer is still localized in the prostate gland, but is larger in size. Stage 3 (T3) cancer is no longer confined to the prostate gland, but is larger in size.
Figure 1 Prostate Cancer Distribution
Figure 2 Anatomy of the Prostate

The prostate gland is located below the bladder and surrounds the upper portion of the urethra.

Oregon Health and Science University health center. medicalcenter.osu.edu/
Stage 3 (T3) cancer is no longer confined to the prostate but has spread to nearby seminal vesicles. Stage 4 (T4) cancer is detected in tissues far from the prostate, including lymph nodes (Stage N+) or even bone (Stage M+). Symptoms when present, may include, frequent and painful urination, blood or semen in urine, erectile dysfunction and pain or stiffness in lower back, hips or thighs. There are several tests available for the screening and diagnosis of prostate cancer; these include PSA test, digital rectal exam, MRI scan, ultrasound, CT scan and biopsy. Of these, PSA test combined with digital rectal exam are routinely performed. The PSA test measures the concentration of PSA (prostate specific antigen) in blood; which increases in men who have prostate cancer. In the digital rectal exam, the physician indirectly assesses the prostate tissue for irregularity and roughness. It is recommended that men over age 50 have PSA test and DRE done yearly [Rinnab et al., 2005].

1.3 Treatments

The treatment options for prostate cancer are dictated by the stage of the cancer. In some cases of “early staged” cancer, treatment is withheld until symptoms appear or change. Treatments for stage 1 and 2 prostate cancer include radiation therapy, cryotherapy, prostatectomy and transurethral resection of the prostate (TURP). The goal of these treatments is to destroy cancer cells before they spread to the rest of the body. Hormone therapy (also called anti-androgen treatment) is the treatment option of choice for stage 3, stage 4 and stage N+ prostate cancer. This option is effective in slowing the rate of cancer growth by inhibiting hormones necessary for growth, development, differentiation and function of the prostate [Abrahamsson, 1999]. Decursin has been recently identified as a novel therapeutic agent having potent anti-androgen activities [Guo et al., 2007]. Hormonal therapy also slows the spreading of cancerous cells to
other parts of the body. In the most severe form of prostate cancer, M+, where cancerous cells become hormone resistant, there is currently no cure. Nonetheless, administration of chemotherapeutic agents such as docetaxel combined with a bisphosphonate can be effective in extending life and reducing pain [Westendorf and Hoeppner, 2007]. However, these powerful toxic drugs eliminate both cancer cells and healthy cells. Herein lies one of the major issues that challenges prostate cancer treatment; the absence of reliable markers [Pu et al., 2005]. Thus, the need to identify molecular targets specifically involved in the growth and spread of cancer cells is urgent.

1.4 Molecular Biology of Prostate Cancer

Cancer is a group of more than 200 diseases, thus the causes of cancer are complex and in many cases unknown. The disease is initiated by mutations caused by carcinogenic agents that exert their effect by either direct interaction with DNA or by indirect epigenetic events that modulate gene expression without directly reacting with the base sequence of DNA [Ruddon, 1995]. Carcinogenic agents are generally classified as either chemical, biological, genetic, or radiation [Pitot and Dragan, 1991]. They modify DNA sequences that code for proteins that either increases the malignancy of a tumor cell or increases the chance that a normal cell develops into a tumor cell [Osborne et al., 2004]. These mutated DNA sequences termed oncogenes, may manifest as transcription factors, growth factors, regulatory GTPases, cytoplasmic serine/threonine kinases, or cytoplasmic tyrosine kinases [Robert G. McKinnell, 2006]. In contrast, tumor suppressor genes code for proteins that have a repressive effect on regulation of the cell cycle or promote apoptosis.
1.4.1 Cell Cycle Regulation in Prostate Cancer
Cancerous cells are capable of going through unlimited number of cell divisions without any signs of senesence. In mammalian cells, cell cycle progression is tightly regulated through the activity of cyclin dependant kinases (CDKs) [Sherr, 1996]. CDKs are stage specific kinases that allow transition of cells from one stage to the next. Alteration of the activity and expression level of CDKs results in proliferation of cancer cells [Hashimoto et al., 2006]. The cell cycle is also controlled by checkpoints that detect errors in DNA replication and chromosome segregation. Concomittant with excess cell proliferation, cancerous cells lose sensitivity to signals for adherence, differentiation or death [Collins et al., 1997]. Cyclin D1 and its binding partners (CDK4 and CDK6) are important regulators of G1 to S phase progression. In prostate cancer, Cyclin D1 positively influences tumor growth by aberrant regulation of androgen receptor [Burd et al., 2006]. Similarly, Cdc6, a cell cycle regulatory gene involved in formation of pre-replication complex is down regulated in prostate cancer [Robles et al., 2002].

1.4.2 Growth Factors Common to Prostate Cancer
The ability of cancer cells to promote cell proliferation, inhibit apoptosis and induce angiogenesis is a reflection of the upregulation of certain oncogenic signaling pathways [McCarty, 2004]. Biochemically, misregulation of growth factors and their respective signaling pathways is associated with prostate cancer progression [Reynolds and Kyprianou, 2006]. Specifically, EGF/EGFR signaling pathway is over active in advanced prostate cancer [Culig et al., 1994]. Inhibition of EGFR tyrosine kinase caused apoptotic death of metastatic prostate cancer cell lines LNCap, DU145, and PC3 [Mimeault et al., 2007a]. EGF treatment also increased androgen dependant AR transactivation in the recurrent prostate cancer cell line CWR-
RI [Gregory et al., 2004]. Administration of the antiandrogen bicalutamide combined with the antiestrogen tamoxifen reduces the synthesis of IGF-I in prostate cancer cells [Boccardo et al., 2006]. In addition, recent studies showed that in DU145 prostate cancer cells, IGF-I induced EGFR transactivation leading to mitogenic activity [Zhou et al., 2006]. TGF-β suppresses growth in normal cells, however in aggressive tumor cells it actively participates in angiogenesis and extracellular matrix deposition [Pinkas and Teicher, 2006].

1.4.3 Malfunctioning Genes in Prostate Tumors

Tumor Suppressor Genes

The p53 protein is the most commonly mutated tumor suppressor gene associated with human cancers [Harvey Lodish, 2000]. In normal cells, p53 causes arrest in G1/G2 phase in response to DNA damaged caused by γ-irradiation. Cells lacking functional p53 are allowed to continue cycling in the presence of damaged DNA. Mutations in p53 mainly occur in late stage prostate cancer in association with androgen independence and metastasis, but also occur in approximately 30% of early prostate cancer [SD Downing MSc, 2000]. Furthermore, restoration of wild type p53 by electroporation into PC3 cell xenografts markedly suppressed tumor growth compared to xenografts transfected with mutant p53 [Mikata et al., 2002].

PTEN, was the first phosphatase identified as a tumor suppressor gene. It has the ability to remove phosphate groups attached to serine, threonine and tyrosine residues. In prostate cancer, PTEN is inactivated by a combination of mechanisms including hemizygous deletion, homozygous deletion, point mutations and promoter methylation [Isaacs and Kainu, 2001]. Wu et al demonstrated that in prostate cancer cells with inactivated PTEN, the AKT/phosphoinositide-3 kinase pathway was constitutively activated due to increased
accumulation of PTEN substrate PIP3 [Wu et al., 1998]. Activation of this pathway resulted in suppression of apoptosis and increased cell survival. Mutations in PTEN are usually seen in advanced prostate cancer and may play a role in acquisition of metastatic potential.

Oncogenes

Proto-oncogenes are normal genes that regulate cell growth and differentiation and become tumor inducing upon activation. Activation can be accomplished by mutations that cause a change in protein structure resulting in increased protein activity or concentration. In prostate cancer, the c-myc oncogene has an important role in regulation of cellular proliferation, differentiation and apoptosis [Grandori et al., 2000]. Overexpression and amplification of c-myc gene is more prevalent in recurrent tumors and metastatic lesions than primary tumors [Buttyan et al., 1987]. Recent studies demonstrated that down regulation of the c-myc gene resulted in growth inhibition and apoptosis in prostate cancer cell lines [Napoli et al., 2006].

The Rho GTPases belong to the Ras superfamily of small GTP binding proteins that act as molecular switches involved in cell motility and invasion, cytoskeleton organization and signal transduction [Yao et al., 2006]. RhoC GTPase is over expressed in aggressive cancers and is required for prostate cancer cell invasion [Gioeli, 2005].

The best characterized members of the Rho subfamily, RhoA, Rac1 and Cdc42 play important roles in cellular proliferation, transformation and cell cycle progression [Adnane et al., 1998]. Ghosh et al have shown that RhoA participates in signaling pathways controlling prostate epithelial cell proliferation in a cell line derived from transgenic mice with prostate adenocarcinoma [Ghosh et al., 2002]. In addition, high levels of Rac1 activity has been shown to
suppress the expression of cyclin dependant kinase inhibitor p21 and promote uncontrolled cell
growth in prostate cancer cell lines [Knight-Krajewski et al., 2004].

Aurora kinases, another family of serine/threonine kinases involved in cell cycle
progression from G2 phase to mitosis. Aurora kinase A is involved in coordination of
microtubule organizing center and Aurora kinase B functions in attachment of mitotic spindle to
the centromere. All three members (Aurora-A/B/C) of this gene family were reported to be over
expressed in many human cancers [Katayama et al., 2003]. Deregulation of Aurora kinase A/B
induces aberrant mitosis leading to chromosomal instability and centrosomal irregularity [Lee et
al., 2006]. Absence of regulated cell division eventually induces aneuploidy, which is a driving
force in malignant progression [Ewart-Toland et al., 2005]. Recent studies show that Aurora
kinase B expression in prostate tumors positively correlates with increased Gleason score
[Chieffi et al., 2006]. Over expression of Aurora kinase A is noted in most high grade prostatic
intraepithelial neoplasia lesions [Buschhorn et al., 2005]

1.4.4 Role of Androgen
The growth, development, differentiation and maintenance of the prostate gland relies on the
production of androgens, mainly in the form of testosterone [Lindzey et al., 1994]. Androgen
secretion from leydig cells is stimulated by leutinizing hormone which is released in response to
binding of gonadotropin releasing hormone to gonadotropes in the anterior pituitary [Grossmann
et al., 2001]. Androgens exert their effect through binding to androgen receptor (AR). AR is a
member of the steroid receptor superfamily and contains an N-terminal transcription activation
domain, a central DNA binding domain, and a C-terminal steroid binding domain [MacLean et
al., 1997]. Once testosterone enters the cell, 5α-reductase converts it to dihydrotestosterone
which has an higher affinity for AR [Denmeade et al., 1996]. Upon ligand binding, AR becomes activated and binds promoter sequences (androgen response elements) that drive the transcription of androgen responsive genes.

Initially, like the normal prostate, cancerous prostate relies on androgens for growth. Thus, blocking the action of androgens causes tumor regression. However many tumors eventually begin to regrow independent of androgens. [Bhuiyan et al., 2006]. There are several possible mechanisms by which prostate cancer cells become androgen independent. Mutations in AR may allow it to bind ligands non-specifically and thus activate AR [Koivisto et al., 1998]. Similarly, mutations in hormone binding not only causes increased sensitivity to other hormones but also may cause AR to be responsive to antiandrogens [Akimoto, 1998]. In addition, androgen refractory prostate cancer cells may bypass AR signaling as a result of constitutive action of regulatory molecules downstream of AR [Bonkhoff, 1998]. Comprehensive understanding of the molecular mechanisms by which prostate cancer cells become androgen independent is necessary in order to develop effective therapies for advanced prostate cancer.

1.4.5 Roles of Actin Cytoskeleton and Microtubules

Cell migration is characterized by changes in the actin cytoskeleton and is a critical component of tumor cell invasion and metastasis. Altered distribution and activity of microtubules also contribute to the cells’ ability to migrate and interact with neighboring cells. Reorganization of the actin cytoskeleton is the primary mechanism for cell migration and is subject to microtubule dependant activities [Yamazaki et al., 2005]. Recent studies indicate that microtubule influence on actin cytoskeleton is mediated through substrate contact formation [Rodionov et al., 1993]. Rho family of small GTPases (RhoA, Rac1,Cdc42) regulate the dynamics of actin cytoskeleton
associated with cell shape and behavior [Zhuge and Xu, 2001]. These GTP binding proteins are active when GTP bound and inactive when GDP bound. This activation status is regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEF). Rho activation induces assembly of stress fibers and focal adhesions, GTP bound Rac induces peripheral actin accumulation and membrane ruffling and Cdc42 induces filipodia [Edwards et al., 1999]. The highly invasive prostate cancer cell line PC3 display 3-fold higher expression of RhoA compared to minimally invasive PC3 cells [Hodge et al., 2003]. Activated Rac1 and Cdc42 facilitate pRb hyperphosphorylation which induces E2F responsive gene transcription thereby allowing entry in S phase [Gjoerup et al., 1998]. An increase in these actin remodeling events are characteristic of metastatic tumor cells in various types of cancer [Wittekind and Neid, 2005]. More recent studies show that the presence of lamellipodia (membrane ruffling) positively correlates with cell motility and polarity in prostate cancer cell lines [Strock et al., 2006]. In addition, EGFR signaling causes activation of Rac1 and subsequent membrane ruffling and lamellipodia formation [Malliri et al., 1998]. LIMK1 is a downstream effector kinase of Rac1 and Cdc42 that is directly involved in actin reorganization. Furthermore, increased activity of LIMK1 enhances cell spreading, cell migration and lamellipodia formation Misra et al., 2005].

### 1.5 Angiogenesis and Metastasis

Solid tumors eventually stimulate outgrowth of capillaries by which new blood vessels are formed; a process known as angiogenesis. These blood vessels penetrate the tumor growth supplying nutrients and oxygen and removing waste products [Varner, 2006]. This process is actually initiated by release of growth factors by tumor cells that signal the surrounding normal
host tissue to make proteins that stimulate growth of new blood vessels [Montesano et al., 1993]. This event marks the beginning of the transition of the tumor to a malignant state. Tumor cells metastasize by invading blood vessels or lymphatic channels where they are free to seed out onto tissue surfaces and develop secondary tumors. This is the most deadly aspect of cancer. In this process a tumor cell has to detach from the primary tumor and from the extracellular matrix (ECM). Many cell adhesion proteins such as E-cadherin are missing or compromised in tumor cells [Sato et al., 1999]. This helps the cell to escape adhesion to other cells and allows it to move more freely. During metastasis, tumor cells degrade the basement membrane in order to disperse into the circulatory system [Yee and Shiu, 1986]. Penetration of the basement membrane is accomplished through the action of degradative enzymes that may be secreted by the tumor cells themselves. The steps involved in initiation, angiogenesis and metastasis are illustrated in Figure 3.

1.5.1 Matrix Metalloproteinases

Matrix metalloproteinase’s are a family of at least 26 enzymes, 15 of which contain zinc in their active site [Visse and Nagase, 2003]. There are two classes of MMPs, secreted proteins and membrane bound proteins. Soluble MMPs are secreted into the extracellular space upon activation. Similarly, membrane bound MMPs become inserted into the plasma membrane when activated. Nonetheless, most MMPs are characterized by a regulatory pro-domain, a catalytic domain, a linker region and a hemopoxin domain [Hideaki Nagase, 2004].(Figure 4). MMPs are primarily involved in the turnover of the ECM. They also have roles in embryonic development, tissue remodeling, wound healing and angiogenesis [Giambernardi et al., 1998].
Figure 3 Stages in the Development of a Tumor. Outline of the process of tumor initiation and acquisition of metastasis

Nelson, et al., 2000
Figure 4 Structure of Matrix metalloproteinases

72kD Pro-MMP-2, 92kD, Pro-MMP-9, 66kD Pro-MT1-MMP. Upon activation, the signal peptide and pro domain becomes cleaved exposing the active site of the enzyme. Signal peptide (purple), propeptide/regulatory prodomain (gray), hemopoxin domain (red), fibronectin domain (blue). Catalytic domains are represented by double arrows.
Membrane-type MMPs are anchored to the membrane by a transmembrane domain or by GPI anchored proteins. Activity of MMPs is regulated by a family of four endogenous inhibitors TIMP 1-4 [Blavier et al., 2006]. Extracellular matrix metalloproteinase inducer (EMMPRIN) plays a role in activation of MMPs and correlates with tumor progression [Ueda et al., 2007]. This family of proteolytic enzymes are collectively capable of degrading all components of the ECM and are therefore major implicators of tumor cell invasion in a variety of cancers [Karadag et al., 2004].

1.5.2 Membrane Type I Matrix Metalloproteinase

Membrane type 1 matrix metalloprotease also called MMP-14, is anchored to the plasma membrane through its transmembrane domain such that the catalytic domain is exposed on the surface of the cell [Nagakawa et al., 2000]. Activation of MMP-14 requires removal of the pro-peptide by a member of the proprotein convertase family, furin, resulting in a 57kDa active enzyme [Yana and Weiss, 2000]. Once cleaved, MMP-14 is inserted into the plasma membrane where TIMP-2 binds to its N terminus and it associates with a neighboring MMP-14 protein to activate matrix metalloproteinase 2. Substrates for MMP-14 include, collagens I-III, fibronectin, laminin-1, vitronectin, dermatan sulfate proteoglycan, proMMP-2 and proMMP13. The known steps of the activation mechanism of MT1-MMP are outlined in figure 5a.

Expression of MT1-MMP is strongly associated with tumor progression and metastasis in almost all cancers [Hofmann et al., 2000]. Sounni et al showed that over expression of MT1-MMP promotes tumor growth and angiogenesis in breast cancer cells [Sounni et al., 2002]. Elevated levels of MT1-MMP were shown to facilitate tumor progression and increase the growth of
Figure 5 Activation mechanisms of MMPs

A: Pro-MMP-2 and Pro-MT1-MMP activation mechanism. Upon cleavage by furin convertases inside the cell, the pro-peptide regulatory domain of MT1-MMP is cleaved. Next the MT1-MMP is inserted into the plasma membrane with the catalytic domain exposed to the extra cellular space. Activation of pro-MMP-2 by MT1-MMP is assisted by binding to a TIMP-2/MT1-MMP complex, which presents pro-MMP-2 to an adjacent active MT1-MMP. B: Activation of Pro-MMP-9. Tissue inhibitor of matrix metalloprotease 1 (TIMP-1) binds to and inhibits pro-MMP-9. In the absence of TIMP-1, MMP-3 readily activates pro-MMP-9. Formation of the ternary complex TIMP-1/MMP-3/pro-MMP-9 causes the interaction between pro-MMP 9 and TIMP1 to be weakened. ProMMP 9 partially dissociates from the complex and can be readily activated by free MMP-3.
malignant melanoma in vivo[Iida et al., 2004]. With its catalytic domain exposed to the ECM environment, MT1-MMP is especially important in pericellular proteolysis. In prostate cancer, MT1-MMP has been shown to correlate with tumor stage and promote metastasis of prostate cancer cells to the lymph node and lung [Cao et al., 2005]. MMP-14 degrades several components of the ECM; this degradative activity is enhanced by activation of latent MMP-2 and MMP-9. Hence MT1-MMP is the most important proteinase involved in cancer cell migration, invasion, and metastasis.

1.5.3 Matrix Metalloproteinase-2/9
Matrix metalloprotease-2 and matrix metalloprotease-9 also termed gelatinase A and gelatinase B respectively, are members of the matrixin family that are secreted into the extracellular space. Like most MMPs, gelatinases are characterized by a signal peptide, a propeptide, and a catalytic domain. Gelatinases have an additional fibronectin type II domain and gelatinase B has a unique collagen like domain (Figure 4). Gelatins, collagen IV and V, aggrecan, elastin, and vitronectin are substrates common to both gelatinases [Kherif et al., 1998]. Collagen I, VII, X, and XI, tenascin C and β-amyloid protein precursor are substrates unique to Gelatinase A. Likewise, collagen XIV is an additional MMP-9 substrate. However, recent studies have identified Ym1 and S100 proteins as substrates for MMP-2, MMP-9 or both [Greenlee et al., 2006]. These novel substrates have key roles to play in the response to parasitic infections [Hung et al., 2002] and inflammation [Eue et al., 2002]. Although MMP-2 and MMP-9 have been implicated in tumor cell invasion in a variety of cancers, their role in prostate cancer is poorly defined. Activation of
MMP-2 occurs at the plasma membrane and is mediated by MMP-14 and TIMP-2 [Hernandez-Barrantes et al., 2000]. Active MMP-3 cleaves and activates proMMP-9, however a number of proteolytic enzymes are capable of activating MMP-9 in vitro [Ramos-DeSimone et al., 1999]. The matrixin family of ECM degrading enzymes are capable of increasing their proteolytic effect by activating each other and substrate overlap. The known steps of MMP-2, MMP-9 and MMP-14 activation are depicted in (Figure 5b).

Enhanced expression of MMPs has been correlated with increasing malignancy [Golubkov et al., 2005]. Nagakawa et al have shown that there is increased expression of MMP-14 in androgen independent and invasive prostatic cancer cell lines [Nagakawa et al., 2000]. In addition, MMP2 expression has been shown to be involved in the process of prostate cancer invasion and metastasis [Liao et al., 2003]. Further, increased activity of MMP-9 in prostate cancer cells has been correlated with tumor cell metastasis [Wilson et al., 2004].

1.6 LIM Kinase 1

LIM kinases and testes specific protein kinase (TESK) are subfamilies of LISK kinases, each with two family members; LIMK1/2 and TESK1/2 respectively. LISK kinases are morphogenesis kinases that stabilize the structure of actin cytoskeleton through phosphorylation and inactivation of cofilin [Manning et al., 2002]. LIMK1 is a dual specificity serine/threonine and tyrosine kinase that has two zinc finger motif LIM domains, a PDZ domain, and a kinase domain (Figure 6). Within the PDZ domain there are two nuclear export signal and within the kinase domain there is a nuclear localization signal [Yang and Mizuno, 1999]. LIM kinases are predominantly expressed in the brain and neural tissue [Yang et al., 2004]. There is substantial
Figure 6 Structure of LIMK1

LIMK1 contains four domains, two consecutive LIM domains, a PDZ domain and a kinase domain. Within the PDZ domain two nuclear export signals are present. Within the kinase domain there is a nuclear localization signal.
evidence identifying LIMK1 gene deletion as a cause for development of cognitive disorder observed in patients with Williams Syndrome [Hoogenraad et al., 2004].

1.6.1 Regulation and Function of LIMK 1

LIMK1 activity influences actin reorganization by regulating the activation status of actin depolymerization factor cofilin. Upon phosphorylation by LIMK1, cofilin is unable to induce globular actin (G-actin) formation. In eukaryotic cells, cofilin binds to filamentous actin (F-actin) causing a conformational change in the actin subunits resulting in severing of F-actin to G-actin. Cofilin bound globular actin then associates with ADFprofilin, which causes cofilin/G-actin dissociation and promotes actin polymerization [Moriyama et al., 1996]. This cycle of actin polymerization and depolymerization is continuous. LIMK1 inactivates cofilin by phosphorylation on serine 3 residue [Toshima et al., 2001]. Thus inactive cofilin cannot sever actin polymers and there is an accumulation of filamentous actin in the cell. Further, increased activity of LIMK1 causes actin remodeling events that may induce formation of lamellipodia, filopodia and stress fibers. Activity of LIMK1 is regulated through the Rho subfamily of small GTPases [Lou et al., 2001]. Stimulation of EGF receptor signaling pathway has been shown to activate Rho GTPases [Nogami et al., 2003]. GTP bound Rac 1, a member of this subfamily, binds to PAK (p-21 activated kinase) homodimers at the N-terminus regulatory domain [Parrini et al., 2002]. Binding of Rac 1 or its family member Cdc42, causes release of PAK’s auto inhibitory domain from the kinase domain, resulting in cross phosphorylation and activation of PAK1/PAK4 which can, in turn, activate LIMK1 [Stofega et al., 2004]. Activation of LIMK1 by Rac1 and Cdc42 mediated by PAK causes the formation of lamellipodia and filopodia
respectively. Inactive LIMK1 is maintained in “closed” conformation with LIM domains associated with the kinase domain (Figure 7). Upon phosphorylation at T508, LIM domains of LIMK1 dissociate from the C-terminal and the kinase domain is available for substrate phosphorylation. LIMK1 inactivates cofilin. As a result cofilin is unable to bind to and induce severing of filamentous actin. Panel C: Dephosphorylation of cofilin by slingshot restores it’s activity. Cofilin can then bind to F-actin causing dissociation of actin subunits and formation of G-actin. Binding of ADF-profilin frees cofilin from G-actin.

In its active conformation the N-terminus LIM domains of LIMK1 are accessible. Active LIMK1 functions to inhibit the actin depolymerizing factor cofilin as mentioned above. LIMK1 can also be activated by a third Rho subfamily member RhoA. In this pathway, RhoA stimulates Rho-associated serine/threonine kinase (ROCK) which in turn phosphorylates and activates LIMK1 [Lin et al., 2003]. ROCK mediated activation of LIMK1 induces stress fiber formation. In addition, bone morphogenic protein type II receptor (BMPR-II) negatively regulates LIMK1 through binding of the tail domain [Foletta et al., 2003]. The signaling mechanism underlying cytoskeletal changes directed by LIMK1 is depicted (Figure 7). In addition, recent studies show that LIMK forms homodimer; an interaction promoted by Hsp90 resulting in transphosphorylation and increased stability [Li et al., 2006].

1.6.2 Role of LIMK 1 in Cancer Progression

In order for tumor cells to become metastatic they must detach from neighboring cells and degrade the extracellular matrix. Both of these events require reorganization of the actin cytoskeleton. Through the activity of LIMK1 there is reorganization of the actin cytoskeleton that coordinates the formation of lamellipodia and filipodia; structures which enhances cell
Figure 7 Activation and function of LIMK1

Accumulation of F-actin gives protrusive force to the leading edge of a cell; it may also facilitate formation of...
Panel A: GTP bound Cdc42 and Rac1 bind to PAK1 homodimers resulting in dissociation and transphosphorylation of PAK1. Active PAK1 phosphorylates LIMK1 at T508, causing a conformational change in LIMK1 where kinase domain is available for substrate binding and LIM domains accessible for interacting partners. Panel B: Phosphorylation of cofilin at Ser3 by LIMK1 inactivates cofilin. As a result cofilin is unable to bind to and induce severing of filamentous actin. Panel C: Dephosphorylation of cofilin by slingshot restores it’s activity. Cofilin can then bind to F-actin causing dissociation of actin subunits and formation of G-actin. Binding of ADF-profilin frees cofilin from G-actin.
migration by projecting the leading edge of the cell [Small et al., 1999]. Davila et al have shown that partial reduction in LIMK1 abolished invasiveness of the metastatic prostate cancer cell line PC3 [Davila et al., 2003]. In addition, ectopic expression of LIMK1 promoted acquisition of invasiveness in benign prostate epithelial cells. Introduction of a dominant negative mutant of LIMK1 in breast cancer cells decreased motility and metastatic lesions in animals [Yoshioka et al., 2003]. In fact, LIMK1 has been implicated in breast tumor cell growth, angiogenesis and invasion [Bagheri-Yarmand et al., 2006]. Taken together, current findings implicate LIMK 1 as a putative oncogene in prostate cancer.

1.6.3 LIMK1 in Cell Cycle Regulation

LIMK1/2 regulate actin cytoskeletal reorganization through phosphorylation of cofilin. Because actin reorganization is a key event in mitosis and cytokinesis, LIMK1 has been implicated in cell cycle progression. Reduction of LIMK1 alters cell proliferation in prostate cancer cells by arresting cells in G2/M. In addition, during prometaphase and metaphase LIMK1 becomes hyperphosphorylated and then returns to basal level as cells enter telophase [Amano et al., 2002]. This phenomenon is evident in both normal breast cells (HUMEC) and metastatic breast cancer cells (MDAMB231), Figure 8. Sumi et al have also shown that LIMK1 activity is low during interphase and peaks during mitosis [Sumi et al., 2002]. Moreover LIMK1 has been shown to interact with 14-3-3ζ and p57Kip2, proteins involved in cell cycle check point control and regulation of CDKs, respectively [Yokoo et al., 2003], [Peng et al., 1997].
Figure 8 Gamma tubulin co-localizes with phospho-LIMK1 to the centrosome during mitosis

Panel A: Metastatic breast cancer cells stained with p-LIMK1 (red, arrow) and γ-tubulin (green, arrow) showed co-localization to centrosome (merged image). Panel B: During interphase p-LIMK1 staining was abolished. γ-tubulin remained localized to the centrosome (green, arrow). Panel C: Co-localization of γ-tubulin and p-LIMK1 to the centrosome is also evident in normal breast epithelial cells (HUMEC).
1.7 Research Done in this Laboratory

Studies from our laboratory showed that ectopic expression of LIM kinase 1 is required for prostate epithelial cell invasion. Over expression of LIMK1 was also observed in prostate tumors and prostate cancer cell lines. Our previous works showed that reduced expression of LIMK1 resulted in G2/M arrest in metastatic prostate cancer cell lines. In addition, ectopic expression of LIMK1 promoted invasiveness in benign prostate epithelial cells. Our studies also revealed that the level of phosphorylated cofilin remained unchanged upon reduction of LIMK1 expression. Immunofluorescence analysis showed localization of MT1-MMP to lamellipodia structures and perinuclear region of PC3 cells.

However, antisense RNA mediated reduction of LIMK1 in these cells reduced the accumulation of MT1-MMP at the cell periphery.

**HYPOTHESIS:** We speculate that LIMK1 induced invasion in prostate epithelial cells is possibly occurring independent of cofilin inactivation and may involve members of MMP family of ECM degrading enzymes.

1.8 Aims of Research

In this study, we attempted to elucidate the roles of LIM domains and the kinase domain of LIMK1 in the invasion process and determine whether matrix metalloproteinaese are involved in LIMK1 induced invasion of BPH cells. We have also attempted to determine any possible association between LIMK1 and γ-tubulin as LIMK1 is localized to the centrosomes during early phases of mitosis (unpublished observation).
**Experimental Design:**

Cloning of constitutively active LIMK1, kinase dead LIMK1 and only the kinase domain of LIMK1 in a mammalian expression vector

Generation of stable cell lines and selection of clones that express maximum amounts of LIMK1 by western blot analysis

Invitro invasion assays of transfected cells expressing different domains of LIMK1

Flow cytometric analysis to study the cell surface expression and activation of MT1-MMP in transfected sublines

Gelatinase zymography was used to evaluate secretion of soluble MMP-2 and MMP-9 by transfected cell lines

Invitro invasion assays utilizing inhibitors against MMPs to confirm their roles in LIMK1 mediated invasion

Immunoflourescence staining to visualize subcellular distribution of LIMK1 and it’s co-localization with MT1-MMP

Immunoprecipitation reactions with γ-tubulin were done in order to substantiate a role of LIMK1 in mitotic processes
2: MATERIALS AND METHODS

2.1 Cell Culture

Benign prostate hyperplasia (BPH) cells were used as the model cell line to study the function of LIMK1, as these cells express low levels of the LIMK1. BPH cells are immortalized luminal epithelial cells originally obtained from a 68 year old male diagnosed with BPH [Hayward et al., 1995]. BPH cells were maintained in high glucose containing DMEM (pH 7.1) (Invitrogen), supplemented with 10% heat inactivated fetal bovine serum 1% antibiotic/antimycotic and 1% glutamine at 37°C humidified incubator with 5% CO₂ and 95% air. Routinely, cells were subcultured by trypsinization. To detach the cells, the monolayer was washed with 1X PBS (Table 1), incubated with 5mL/100mm dish of 1X trypsin (Table 1) at room temperature for 5 minutes. Next, the trypsin was poured off and the dish was incubated at 37°C for approximately 10 minutes. Detached cells were resuspended in complete medium and plated at 1:5 split. Confluency is usually attained within 48 hours.

The human caucasian breast cancer cells MDA-MB-231 were also maintained in high glucose containing DMEM (pH 7.1) (Gibco), supplemented with 10% non-heat inactivated serum, 1% antibiotic/antimycotic and 1% penicillin streptomycin. To detach the cells, the monolayer was washed with 1X PBS (Table 1), incubated with 2mL/100mm dish of 1X trypsin (Table 1) at 37°C for 2 minutes. Detached cells were resuspended in complete medium and plated at 1:5 split. Confluency is usually attained within 48 hours. Both BPH and MDA-MB-231 cells were harvested by resuspending trypsinized
<table>
<thead>
<tr>
<th>Name of Buffer</th>
<th>Application</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis Buffer</td>
<td>Protein extraction</td>
<td>50mM Tris pH 8.0, 120mM NaCl, 2.5mM EDTA, PMSF, 1mM Na3Vo4, 1%NP-40, 10ug/mL leupeptin/aprotinin, 50mM NaF, 40mM β-glycerophosphate</td>
</tr>
<tr>
<td>Transfer Buffer</td>
<td>Western blot</td>
<td>192mM glycine, 25mM Tris base, 20% methanol</td>
</tr>
<tr>
<td>Running Buffer</td>
<td>SDS-PAGE</td>
<td>192mM glycine, 25mM Tris base, 0.1% SDS</td>
</tr>
<tr>
<td>Blocking Buffer</td>
<td>Western blot</td>
<td>5% milk, 137mM NaCl, 0.1% Tween, 20mM Tris pH 7.6</td>
</tr>
<tr>
<td>Tris buffered saline</td>
<td>Western blot</td>
<td>137mM NaCl, 20mM Tris pH 7.6</td>
</tr>
<tr>
<td>Tris buffered saline-</td>
<td>Western blot</td>
<td>137mM NaCl, 0.1% Tween, 20mM Tris pH 7.6</td>
</tr>
<tr>
<td>Renaturing Buffer</td>
<td>Zymography</td>
<td>2.5% Triton X-100 in distilled water</td>
</tr>
<tr>
<td>Developing Buffer</td>
<td>Zymography</td>
<td>250mM Tris pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol</td>
</tr>
<tr>
<td>Staining Solution</td>
<td>Zymography</td>
<td>50mM Tris pH 7.8, 0.2M NaCl, 5mM CaCl, 0.02% NP-40</td>
</tr>
<tr>
<td>Destaining Solution</td>
<td>Zymography</td>
<td>0.5% w/v Coomassie blue R-250, 5% v/v methanol, 10% v/v acetic acid</td>
</tr>
<tr>
<td>1X PBS</td>
<td>Cell Culture</td>
<td>50mM NaPO4, 300mM NaCl, 10% glycerol</td>
</tr>
<tr>
<td>1X Trypsin</td>
<td>Cell Culture</td>
<td>50mM NaPO4, 300mM NaCl, 10% glycerol, 5mM imidazole</td>
</tr>
<tr>
<td>10X MOPS buffer</td>
<td>RNA Gel preparation</td>
<td>50mM NaPO4, 300mM NaCl, 10% glycerol, 150mM imidazole</td>
</tr>
<tr>
<td>Kinase Assay buffer</td>
<td>Kinase Assay</td>
<td>50mM Hepes, 150mM NaCl, 5mM MgCl, 10mM NaF, 1mM Na3VO4, 5mM MnCl</td>
</tr>
<tr>
<td>RIPA buffer</td>
<td>Immunoprecipitation</td>
<td>1% NP-40, 50mM Tris pH 7.5, 2mM EDTA, 150mM NaCl</td>
</tr>
</tbody>
</table>
cells in 1XPBS and precipitating at 500xg at 4°C for 10 minutes. Cell pellets were washed with 5mL of 1XPBS and centrifuged again at 500xg at 4°C for 10 minutes. Pellets were stored at -4°C until used for protein extraction.

2.2 Preparation of LIMK1 Constructs

A DNA fragment containing full-length coding sequence of LIMK1 (Figure 9) originally cloned in pIND vector (Invitrogen) was used to amplify a 878bp fragment of LIMK1 ORF flanking the stop codon using primers shown in table 2. The reverse primer was designed to obtain a mutated stop codon and a XbaI restriction site. The PCR product was cloned in pGEM-T Easy vector (Promega). In parallel, the 3X flag epitope of the p3XFLAG-CMV vector (Sigma E4901) was amplified using primers containing XbaI and ClaI sites and cloned in pGEM-T Easy vector. Next, LIMK1 fragment (878bp) was cloned in pGEMT Easy containing 3X Flag at the 3’ end. Cloning was done by digesting both plasmids with SSTI and XbaI. Since SSTI restriction site is present in the multiple cloning site of pGEM-T Easy, the digested product was used as an insert for pGEM-T Easy plasmid containing 3X flag epitope. DNA sequence of the LIMK1 fragment with mutated stop codon and 3X flag tag was confirmed by sequence analysis (this construct was already prepared in the lab). Next, the LIMK1 fragment was sub-cloned into pIND vector containing the full length LIMK1 sequence replacing the original 878bp sequence. This cloning generated full-length LIMK1 coding sequence with 3X flag tag at the 3’ end. Full length LIMK1 with 3X flag fusion protein (Figure 10) was subsequently cloned into Sal I and Cla I site in pRev-TRE mammalian expression vector driven by CMV promoter (Clontech).
Figure 9 LIMK1 coding sequence

LIM1 domain (pink), LIM2 domain (green), PDZ domain (yellow), Kinase domain (teal).

Mutated stop codon (red) and extra bases (gray). Flag sequence and Cla I site (dark yellow, turquoise). Primer binding site for amplification of kinase domain only (*,****). Primer binding site for kinase dead mutant amplification (**,****). Primer binding site for constitutively active mutant (***,****).
Table 2 Primers used for generate LIMK1-3XFLAG fusion protein

A. PCR primers used for the generation of 878bp LIMK1 fragment with mutated stop codon.  B. Primers used to amplify 3XFLAG epitope .

<table>
<thead>
<tr>
<th>Template</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIMK1-pIND</td>
<td>RC147 GCTATGATGAGAAGGTGGATGTGT</td>
<td>RC258 CCTCTAGACTCGTCACTACTTTGTC</td>
</tr>
<tr>
<td>p3XFLAG-CMV</td>
<td>RC257 CAGATATCCAGCGACTACAAAGAC</td>
<td>RC258 CCTCTAGACTCGTCACTACTTTGTC</td>
</tr>
</tbody>
</table>
Figure 10 LIMK1 ORF amino acid sequence

Amino acid sequence of LIMK1 ORF. Pink: LIM domain 1. Yellow: LIM domain 2. Blue: PDZ domain. Red: Kinase domain. Black residues are linker sequences. To generate constitutively active LIMK1, T<sup>508</sup> (green) was removed and two glutamic acid residues inserted. To generate kinase dead LIMK1 an aspartic acid residue (green) within the kinase domain was replaced with alanine. Amino acid sequences for 3X Flag epitope are in bold (purple).
2.2.1 Generation of LIMK1 Mutants

Constitutively active LIMK1 (BPHL<sup>CA</sup>) and kinase dead (BPHL<sup>KD</sup>) constructs were generated using Stratagene’s Quick Change Site Directed Mutagenesis kit (Figure 11). Procedures were followed according to the manufacturers protocol. Briefly, using wild type LIMK1 as a template, two mutagenic primers (Table 3) were used for amplification with PCR conditions mentioned in table 4. The PCR product was digested with Dpn1 and used for transformed of XL1-Blue Super competent cells. Antibiotic resistant colonies were picked and DNA extracted using the boiling mini prep method. The construct containing the kinase domain only was generated by PCR amplification using primers flanking the kinase domain. The forward primer was designed to introduce an initiation codon. Then the amplified product was cloned into Sal I and Cla I sites in the pRev-TRE vector. Cloning of the LIMK1 fragments were initially verified by restriction digestion. Finally, DNA sequences of all constructs were confirmed by sequence analysis.

2.3 Generation of Stable Cell Lines

Plasmid DNA containing LIMK1 fragments or the empty vector DNA were purified using Qiagen spin mini prep DNA purification kit. The day before transfection BPH cells (5X10<sup>5</sup>) were seeded into six well dishes in complete growth media. Five to seven micrograms of cDNA were used for transfection using Lipofectamine Plus 2000. Next day, media were replaced with warm OptiMEM (800uL) and cells were incubated for two hours at 37°C. Plasmid DNA was warmed to room temperature and mixed with lipofectamine diluted in OptiMEM (100uL). Lipofectamine (12uL) Invitrogen.)
Figure 11 LIMK1 Constructs

To generate constitutively active LIMK1, threonine 508 was removed and two glutamic acid residues inserted. To generate kinase dead LIMK1 an aspartic acid 460 was mutated to alanine. Both mutations are within the kinase domain of LIMK1.
Table 3 Primers used for generation of LIMK1 mutants

<table>
<thead>
<tr>
<th>Construct</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>RC271 GTGCACATGCCTCACGATAACACT</td>
<td>RC269 ATCGATTCACTACTTTGTCAT</td>
</tr>
<tr>
<td>KD</td>
<td>RC300 CATCATCCACCGAGCCCTCAACTCCAC</td>
<td>RC301 GTGGGAGTTGAGGGCTCGGGATGATG</td>
</tr>
<tr>
<td>CA</td>
<td>RC294 CAAGAAGCGCTACGAAGAGGTGGTGGCAAC</td>
<td>RC295 GTTGCCCACCACCTCTCGTACGCTTTTG</td>
</tr>
</tbody>
</table>

Table 4 PCR Conditions for LIMK1 Mutagenesis

To achieve multiple amino acid insertions and deletions, 18 cycles of amplification was necessary.

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>18</td>
<td>95°C</td>
<td>30s</td>
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<tr>
<td></td>
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<td>1min</td>
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<tr>
<td></td>
<td>68°C</td>
<td>2min</td>
</tr>
</tbody>
</table>
DNA mixed with OptiMEM (100uL) was added to the mixture and incubated at room temperature for 30 minutes. Next the DNA mixture was added to BPH cells containing 800uL of OptiMEM. Transfected cells were incubated at 37° in a humidified CO₂ incubator for 3 hours. Next, one mL of complete growth media supplemented with 20% FBS was added to the cells and incubation continued for 24hrs.

After 48-72hrs post transfection, cells were treated with hygromycin (8ul/ml-Invitrogen). Antibiotic resistant cells were cloned by incubating them with trypsin soaked cloning disc for 7-10 minutes at room temperature and transferring the disc into 24 well dish (1 disc/well). Cells were maintained in complete growth media containing hygromycin and screened for transgene expression as described below.

2.4 RNA Extraction

Total RNA was extracted from transfected cells using Promega total RNA extraction kit. Approximately 1.5X10⁶ cells were harvested and processed for RNA extraction according to the manufacturers protocol. Briefly, cell pellets were homogenized in denaturing solution by vortexing vigourously. Then phenol chloroform was added to the suspension and samples were incubated on ice for 15 minutes. Samples were then centrifuged for 20 minutes at 10000xg at 4°C. After centrifugation, the aqueous layer was recovered and an equal volume of isopropanol was added. Samples were then incubated at -20°C for 18 hours to precipitate the RNA. The next day 1mL of ice cold 70% ethanol was added to the solution and then centrifuged at 10000xg for 10 minutes at 4°C. The resulting pellet was air dried for approximately 20 minutes and then resuspended in nuclease free water. Total RNA concentration of each sample was determined in a spectrophotometer at 260nm wavelength. Integrity of the RNA samples were determined by
formaldehyde (3%) agarose gel (1%) electrophoresis in 1XMOPS buffer (Table 1). RNA samples were prepared for analysis by adding 15μL of RNA sample loading buffer (Sigma 4268) to 5μL of RNA sample (5-7μg) and boiling as 65°C for 15 minutes.

2.5 Reverse Transription PCR

Copy DNAs of the transgenes from LIMK1 expressing cell lines were amplified using stratagene one step RT-PCR kit, according to the manufacturers protocol. Each reaction contained total RNA sample (1μg), RNase free water, RT-PCR buffer, primers (20uM, Table 5), and dNTPs (0.4mM each, provided with kit). Then samples were denatured at 65 °C for 15 minutes and then cooled to 37°C for 3 minutes in thermocycler. Next, reverse transcriptase and DNA polymerase were added to the reaction and samples were incubated for the specified time (Table 6) for reverse transcription.

2.6 Preparation of Crude Protein Extracts

Fresh or frozen cell pellets were resuspended in protein extraction buffer (Table 1) with inhibitors and then subjected to freezing and thawing for 6-7 cycles in a dry ice ethanol bath. First suspended cells were frozen for approximately 1 min, thawed at 37°C, and then vortexed vigourously for 20-30 seconds. To ensure complete lysis, lysates were observed under the microscope. Then lysates were centrifuged at 500xg for 15 minutes at 4°C. The supernatants were transferred to clean microfuge tubes and protein concentrations were measured using Bradford assay (BioRad). A standard curve was generated using BSA (New England Biolabs) to quantify the protein concentration.
Table 5 Primers used in RT-PCR

Description of primers used in reverse transcriptase PCR. Amplification using Flag specific primer (RC269) confirmed expression of LIMK1 transgene.

<table>
<thead>
<tr>
<th>LIMK1 ORF</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position 872, PDZ domain</td>
<td>RC145, TGGACGAGATTGACCTGCTGATTC</td>
<td>RC269, ATCGATTCACTACTTTGTCAT</td>
</tr>
<tr>
<td>Position 1247, kinase domain</td>
<td>RC130, CAGGTGAGGGTGATGGTGATGAAGG</td>
<td>RC269, ATCGATTCACTACTTTGTCAT</td>
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</table>
Table 6  RT-PCR Amplification Summary

Description of PCR conditions used in reverse transcriptase PCR.

<table>
<thead>
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<th>Cycle</th>
<th>Temperature</th>
<th>Time</th>
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<td></td>
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<td>4min</td>
</tr>
<tr>
<td>1</td>
<td>68°C</td>
<td>10min</td>
</tr>
</tbody>
</table>
2.7 Western Blot Analysis

Fifty micrograms of total proteins in crude cell lysates were denatured in 1X sample buffer (Table 1), boiled for 5 mins and separated by SDS-PAGE. Separated proteins in the gels were transferred to a polvinylidene fluoride membrane (Pall Corporation) at room temperature for 18hrs at 20v in transfer buffer (Table 1). Membranes were stained with India ink (1:1000) for 15 minutes at room temperature to visualize lanes. Next, membranes were incubated in blocking buffer (5% milk TBS-T) for 90 minutes at room temperature. Proteins in the membrane were detected by reacting with specific primary and secondary antibodies (Table 7) diluted in blocking buffer (Table 1) for specified the time (Table 7). Membranes were washed extensively in washing buffer (Table 1) and positive signals detected by chemiluminescence using a west femto chemiluminescence HRP substrate detection kit from Pierce.

2.8 In vitro Invasion Assay

Treatment of Cells Prior to Seeding

To sensitize cells for treatment with epidermal growth factor, 60% confluent dishes were washed with 1XPBS (Table 1) and incubated with phenol red free DMEM complete media supplemented with *10% charcoal stripped fetal bovine serum (CSFBS) for 48 hours. Then the monolayer was washed with 1XPBS and incubated with serum free phenol red free DMEM media for 24hrs. (2.5 grams of charcoal was combined with .25 grams of dextran and q.s. to 1L in 1M tris and stored at 4° o/n. The next day, the charcoal was autoclaved and combined with serum(1:1) ratio and stirred for 1hr at 45°. To remove charcoal the serum was centrifuged)
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Application</th>
<th>Concentration</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flag (Sigma)</td>
<td>Western blot</td>
<td>1:2000</td>
<td>1hr @ RT</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Western blot</td>
<td>1:500,000</td>
<td>1hr @RT</td>
</tr>
<tr>
<td>LIMK1 (Transduction laboratories)</td>
<td>Western blot</td>
<td>1:100</td>
<td>O/N @ 4°C</td>
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<td>Western blot</td>
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<tr>
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<td>1hr @ RT</td>
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<td>Immunofluorescence</td>
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<td>Immunofluorescence</td>
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<td>Immunoprecipitation</td>
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<td>4hrs @ 4°C</td>
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<tr>
<td>MT1-MMP (Chemicon)</td>
<td>Flow cytometry</td>
<td>5ug/million cells</td>
<td>3hrs @ 4°C</td>
</tr>
</tbody>
</table>
Invasion Assay

For the invasion assay, ECM fluorometric kit (ECM 554) from Chemicon was used. Experiment was carried out according to the manufacturers protocol with some modifications. Cells were seeded at a density of 1.25X10^5 in serum free media in matrigel coated inserts that had been rehydrated for 30 minutes. In addition, EGF was added to the cell suspension at a final concentration of 10ng/mL. For inhibition studies, the matrix metalloproteinase inhibitor GM6001 was added at a final concentration of 25uM. EGF (100nM) was added to the lower chamber as the chemoattractant. Cells were incubated at 37°C in a CO_2 incubator for 48hrs. At the end of incubation, media was removed from each insert and discarded. Inserts were transferred to clean wells (24 well dish) containing 225uL of cell detachment buffer (provided with the kit) and incubated at 37°C in a CO_2 incubator for 1 hour. During this incubation, samples were gently rocked back and forth every 15 minutes to facilitate cell detachment. Also, cells that had completely traversed the matrigel membrane to the bottom wells were counted. Next, 75uL of lysis buffer/dye solution (provided with the kit) was added to each well and incubated at room temperature for 15 minutes. Lysis buffer/dye solution with no cells was used as a blank. After incubation, 200uL from each sample was transferred to a clean 96 well plate for fluorescence measurement in a Wallac Victor 2 at 480/520 excitation/emission settings.

2.9 Gelatin Zymography

Transfected cells (2.5X10^5) cells were seeded into six well dishes and incubated for 24hrs. Next day, media was replaced with phenol red free DMEM supplemented with 10% charcoal stripped FBS and cells were incubated for at 37°C in a CO_2 incubator for 48hrs. Cells were then serum starved for 24hr at 37°C in a CO_2 incubator. Next, cells were treated with EGF at a final
concentration of 10ng/mL for 24hrs at 37°C in a CO₂ incubator. After incubation, the conditioned media was harvested and centrifuged at 500xg for 5minutes at 4°C. Supernatant was transferred to a clean microfuge tube and stored at -20° C. For analysis, 15uL of each sample was incubated with 5uL of non-reducing loading buffer (Table 1) at room temperature for 15-20 minutes. Samples were then separated on a 10% SDS gel co-polymerized with 1mg/mL gelatin. Gelatin was dissolved at 60°C for 20 minutes. Gels were run at 125v until the dye front ran off the gel. For better separation, gels were run for an additional 10 minutes. Next, gels were incubated in renaturing buffer (Table 1) for 30 minutes. Gels were washed twice and then incubated in developing buffer (Table 1) for 30 minutes. At this point, the developing buffer was decanted, fresh developing buffer was added and gels were incubated at 37°C for 24hrs. Next day, gels were stained in staining buffer (Table 1) until they became uniformly blue. To see gelatinic bands, gels were destained (Table 1) as needed. Once bands appeared, gels were immediately dried for 3hrs. Bands were quantitated by densitometric analysis using Gene snap software.

2.10 Quantitative Real-Time PCR

Total RNA extracts were obtained as described in section 2.3. For cDNA synthesis, BioRad iScript kit was used according to manufacturer’s protocol. Briefly, RNase free water and 5X reaction mix (provided with kit) were added to total RNA samples (1ug). Next, samples were denatured at 65°C for 15min, cooled to 37° C for 3 minutes in a thermocycler. Reverse transcription was carried out at 42°C in thermocycler for 90min. Real-time PCR was performed using a kit (Biorad SYBR green). This kit provided a 2X reaction mix to which primers,
cNDA (6uL), and nuclease free water was added to a final volume of 25uL. A RT-PCR reaction without reverse transcriptase was used as a control. Primer sequences and PCR cycles are indicated in tables 8 and 9, respectively. Reactions were carried out in BioRad iCycler thermocycler.

*Calculation of Gene Expression*

Quantification of the target gene was calculated using \( \Delta Ct \) method and GAPDH as a reference gene.

To calculate relative expression, the target gene expression was normalized for each sample:

\[
2^{(C_{t}(\text{GAPDH})-C_{t}(\text{target gene}))} = \text{Expression}
\]

Fold change expression was calculated as a ratio of expression of target gene in normal cell line to expression of target gene in test cell line: Normal expression = \( \Delta Ct \) normal cells/\( \Delta Ct \) normal cells; Tumor expression = \( \Delta Ct \) tumor cells/ \( \Delta Ct \) normal cells.

**2.11 Flow Cytometry**

*Treatment of Cells Prior to Seeding*

To sensitize cells for treatment with epidermal growth factor, 60% confluent cells were incubated with phenol red free DMEM complete media supplemented with 10% charcoal stripped fetal bovine serum for 48 hours. Next, cell monolayers were washed with 1XPBS (Table 1) and incubated with serum free and phenol red free DMEM complete media for 24hrs. At this point, EGF (10ng/mL) was added to each dish and cells were incubated for 24 hours.
Table 8  Quantitative PCR amplification summary

Data was collected after each cycle of amplification.

<table>
<thead>
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<th>Cycle</th>
<th>Temperature</th>
<th>Time</th>
</tr>
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<tr>
<td>40</td>
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<td>30s</td>
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<tr>
<td></td>
<td>56°C</td>
<td>30s</td>
</tr>
</tbody>
</table>
Table 9  Primers used in Real Time PCR

Description of target sequences amplified in quantitative PCR.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Length</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>85bp</td>
<td>ATGGCCTTCCGTGTCCTAC</td>
<td>TGATGTCATCATACTTGGCAGG</td>
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<tr>
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<td>295bp</td>
<td>GTCTCCTGCTCCCTCCCT</td>
<td>CGAACATTGCGCTTGGATCTCA</td>
</tr>
<tr>
<td>MMP-2</td>
<td>501bp</td>
<td>GCAAGTTTCCGTCCGCTTCC</td>
<td>CAGTACCAGTGTCAGTATCAGC</td>
</tr>
</tbody>
</table>
MT1-MMP Surface Staining

EGF treated cells were harvested by incubating with 3ml/00mm dish of cell stripper (Cell Gro) for 15-20 minutes. Two ml of PBS was added to each dish and cells were collected. Five X $10^5$ cells were aliquoted into 5mL falcon tubes and centrifuged at 500xg for 10minutes. Cells were resuspended in 1XPBS with 3%FBS containing MT1-MMP antibody (Chemicon) at a concentration of 5ug/million cells. Cells were incubated at 4°C with end over end rocking for three hours. Next, cells were washed in PBS with 3% FBS and centrifuged at 500xg for 5 minutes. This step was repeated twice. Samples were incubated with the secondary antibody conjugated with Alexa 488 (molecular probes) at a concentration of 1:800 for 30min at 4°C with end over end rocking. Cells were washed next in 1XPBS with 3%FBS and centrifuged at 500xg twice and resuspended in the fixation buffer (2% paraformaldehyde in 1XPBS). To prevent clumping, pluronic solution (Sigma) was added to the cell suspension at a concentration of 100uL/mL. Prior to use, fixation buffer was autoclaved and filter sterilized. Samples were stored at 4°C until acquisition in a flow cytometer (FACS Calibur/BD Biosciences).

2.12 Dual label Immunofluorescence Analysis

Preparation of Polylysine Coated coated Coverslips

Coverslips were coated with 1% Poly-L-lysine diluted in sterile borate buffer (2mM EDTA, 89mM Tris/boric acid). First, coverslips (12mm diameter, Fisher) were sterilized in 85% ethanol and allowed to air dry. Next, 200uL of 1Xpoly-L-lysine was added to each coverslip and incubated for 10mins at room temperature. Poly-L-lysine was removed at the end of incubation leaving behind a thin layer. Coverslips were allowed to air dry for approximately 30 minutes.
Treatment of Cells Prior to Staining

Typically, 2X10^4 cells were seeded on poly-L-lysine coated 12mm diameter coverslips (Fisher) in 24 well dishes and incubated overnight at 37°C in a CO2 incubator. The next day or once cells adhered, media was changed to phenol red free DMEM containing 10% charcoal stripped FBS and incubation continued for 48hrs at 37°C in a CO2 incubator. Next, cells were treated with serum free media and incubated for another 24hrs. After 24hrs, cells were treated with EGF (10ng/mL) in phenol red free DMEM media for 30 minutes at 37°C in a CO2 incubator.

Immunostaining

Cells were washed in 0.1M phosphate buffer (3X), and fixed in freshly prepared 4% paraformaldehyde in 0.1M phosphate buffer for 10mins. Cells were washed in 0.1M phosphate buffer (3X) and permeabilized by incubating in 0.2% triton X100 for 10 minutes at room temperature. Next, cells were washed with 0.2% tritonX100 in 0.1M phosphate buffer. At this point, coverslips were removed from the culture dish and placed cell side up onto filter paper covered with parafilm. Cells were subjected to blocking at room temperature in 10% goat serum, 0.2% triton X100 in 0.1M phosphate buffer for 1.5 hour. Primary and secondary antibodies (Table 7) were diluted in blocking buffer and applied (75uL) to coverslips. Primary antibodies were combined and applied to coverslips for 1hr. Secondary antibodies were centrifuged at 12000xg for 3 minutes prior to use. Coverslips were washed extensively with 0.1M phosphate buffer (six times for 5 minutes each). Cells were postfixed for 5 minutes and then stained with phalloidin (Company 1:40) for 15 minutes at room temperature. Coverslips were washed with 0.1M phosphate buffer (3X) and then mounted with biomedia gel mount.
(BioMeda). Mounted cells were stored in the dark for 18hrs. Flourescent cells were visualized in a confocal microscope (Carl Ziess) at 60X oil objective. Images were also deconvoluted.

2.13 Preparation of Nuclear and Cytoplasmic Extracts

Nuclear extracts of MDA-MB-231 and BPH-LIMK were prepared using NE-PER kit (Pierce). Briefly, ice cold cytoplasmic extraction reagent I was added to cell pellet and vortexed vigorously for 15 seconds and incubated on ice for 10 minutes. Then cytoplasmic reagent II was added to each suspension, vortexed for 5 seconds and incubated on ice for 1 minute. To collect cytoplasmic extracts, samples were centrifuged at 16000g for 5 minutes. To obtain the nuclear fraction, nuclear extraction buffer was added to the remaining pellet, vortexed for 15 seconds then incubated on ice for 10 minutes. The cycle of 15 second vortex and 10 minutes on ice was repeated until 40 minutes elapsed. Sample were then spun down at 16000g for 10 minutes. Protein concentrations were determined using Bradford assay (BioRad). Reagent dye (BioRad) and BSA (New England Biolabs) were used in preparation of standards.

2.14 Immunoprecipitation

Cell lysates were prepared as described above in RIPA buffer (Table 1). Protein concentrations were determined using Bradford assays. Typically, 1ug antibody per 100ug of protein was incubated at 4°C for 4 hours. Next, 30uL of protein A/G sephrose beads (Santa Cruz Biotechnology) was added to the tube and incubated for 18 hours at 4°C with end over end rocking. Next day, samples were centrifuged at 100xg for 2 minutes at 4°C. Supernatants were removed and saved for analysis. Beads were washed three times 5 minutes each in 500uL of RIPA buffer and centrifuged at 200xg for 5 minutes. To separate protein complexes and beads,
16uL of distilled water and 5uL of 4X sample buffer were added to the beads, boiled for 5 minutes and separated by SDS-PAGE. Immunoprecipitated proteins were detected by western blotting as described above.

2.14.1 Calf Intestinal Alkaline Phosphatase Treatment

Nuclear extracts (500ug) resuspended in modified RIPA buffer (1% nonidet P-40, 50mM Tris pH 8.5, 2mM EDTA, 150mM NaCL) were dephosphorylated by calf intestinal alkaline phosphatase (CIAP/New England Biolab). Extracts were diluted in 1XCIAP buffer (550uL) and treated with CIAP (5uL at 10U/uL) buffer. Extracts were incubated at 37°C for 30 minutes and used for immunoprecipitation as described in the previous section.
3. RESULTS

3.1 Expression Analysis of LIM kinase 1 Transgene in Transfected BPH cells

To confirm that transfected cells were expressing LIMK1 transgene, total RNA extracts containing mRNA of BPHLCA, BPHLK and BPHLK cells were used for RT-PCR. RNA concentration was determined by spectrophotometric analysis. RNA integrity was detected on a formaldehyde (3%) agarose gel (1%) which showed intact 18S and 28S ribosomal RNA bands (Figure 12). One microgram of each RNA sample was used for reverse transcriptase PCR reaction. BPH cells expressing transgenes of LIMK1 (BPHLCA, BPHLK), were amplified using upstream PDZ domain primers and downstream Flag specific primers (Figure 12a). Each sample yielded the expected band size of 1.3 kb as a result of LIMK1 transgene amplification. For BHPLK mRNA an upstream kinase domain primer and downstream flag specific primer were used which yielded a 1000bp cDNA fragment (Figure 13b).

Because phosphorylation at T508 activates LIMK1, we wanted to study the effect of expression of active LIMK1 or kinase dead LIMK1. In addition, a cell line expressing only the kinase domain of LIMK1 was generated in order to see whether LIM domains are essential for the effect of LIMK1. BPH cells expressing constitutively active (BPHLCA), kinase dead (BPHLKD) and kinase domain only (BPHLK), LIMK1 were generated by stable transfection as mentioned before. BPHLCA was generated by replacing T508, where it is phosphorylated with two glutamic acid residues. This mutation mimics phosphorylation by providing positive charge and altering structural conformation (Figure 7).
Figure 12 Electrophoresis analysis of total RNA showing intact ribosomal RNA

Figure 13 Reverse transcription PCR

A: Amplicons of BPHL<sup>CA</sup> and BPHL<sup>KD</sup> Panel B: Amplicons of BPHL<sup>K</sup>. After RT-PCR, cDNAs were analyzed by gel electrophoresis.
In addition, this mutant of LIMK remains in the open conformation $\text{BPHL}^{\text{KD}}$, was generated by removal of an aspartic acid residue from the activation loop and replacing it with an alanine residue. Mutation at this position has been shown to inhibit it’s kinase activity. [Rosso et al., 2004].

To identify clones that had the highest expression level of the transgene, western blot analysis was done using anti-Flag monoclonal antibody, (Figure 14). The housekeeping gene GAPDH was used as a loading control. Total protein was extracted from each cell line expressing LIM kinase 1 mutants. These include $\text{BPHL}^{\text{CA}}$ (constitutively active), $\text{BPHL}^{\text{KD}}$ (kinase dead), $\text{BPHL}^{\text{K}}$ (kinase domain only) and $\text{BPHL}^{\text{V}}$ (vector only). Immunoblot analysis showed that all LIM kinase 1 mutants expressed Flag tags (Figure 14). Protein extracts were also analyzed for LIMK1 expression, which showed that BPHL sublines expressing different domains of LIMK1, express higher level of LIMK1 relative to untransfected BPH cells, and cells transformed with the vector (Figure 15).
Figure 14  Flag expression confirms integration of LIMK1 mutants in BPH cell chromosome. Lane 1 vector, Lane 2 constitutively active LIMK1, Lane 3 kinase dead mutant, Lane 4 kinase domain only mutant. Fifty microgram of protein was loaded and blot probed with anti Flag 1:2000 followed by anti-mouse-HRP 1:3X10^4 antibodies.
Figure 15 Expression analysis of LIMK1. BPH cells transfected with LIMK1 mutants
Lane 1: untransfected parental cell line, Lane 2: vector only cell line BPHV, Lane 3: constitutively active mutant BPHCA. Lane 4: kinase dead mutant, BPHKD. Lane 5: kinase domain only mutant. Lane 6: PC3-prostate cancer cells which overexpress LIMK. Fifty microgram of total protein loaded in each lane and blot probed with antiLIMK1 antibody at 1:100 dilution.
3.2 Catalytically Inactive LIMK1 is Invasive

Previously, our lab has shown that a reduction in LIMK1 expression results in loss of invasiveness in PC3 cells. Also, ectopic expression of LIMK1 in BPH cells resulted in acquisition of highly invasive phenotype. In this study, we wanted to determine which domains of LIMK1 is essential for the invasive property of the protein. We used a fluorometric invasion assay using BPH cells expressing LIMK1 mutants (BPHL$^{CA}$, BPHL$^{KD}$, BPHL$^{K}$) or the vector (BPH$^{V}$). In order to stimulate the EGF signaling pathway, transfected cell lines were treated with EGF (10ng/mL) for 24hrs prior to the assay. EGF signaling pathway stimulates Rho GTPases activity in which LIMK1 is a downstream effector kinase. In addition, we used EGF (100nM) as the chemoattractant for invasion assays of BPHL cell lines. Cells that invaded through the matrigel were quantitated by fluorescence reading (Figure 16). Cells that traversed through the matrigel and accumulated in the bottom chamber were counted (Figure 17). The raw fluorescence data showed that the cells expressing BPHL$^{KD}$ had the number of invaded cells comparable to that of BPHL$^{CA}$. Transfected cell lines expressing BPHL$^{K}$ and BPH$^{V}$ had comparable RFU values and were approximately equal to the RFU values of wells without any cells. The cell count data indicated that BPHL$^{CA}$ cells had the highest percentage of invaded cells compared to the control cells BPH$^{V}$. For the BPHL$^{KD}$, cell line the percentage of invaded cells was similar to that of BPHL$^{CA}$. BPHL$^{K}$ cells showed a reduced number of invaded cells.
Figure 16 Invitro Invasion assays of EGF treated transfected BPH cells.

With EGF as an chemoattractant. Graphical representation of population of cells that penetrated matrigel coated membranes. Values are mean +/- SE of three experiments. * $P=0.820$ ** $P=0.045$ and *** $P=0.003$ when compared to control (BPHV) cells.
Figure 17 Percent of invasion of BPH-LIMK1 cell lines

Relative abundance of the invaded BPH cells BPH^K, BPH^KD, BPH^CA, compared to BPH^V cells.

Values are mean +/- SE of three experiments. * P=0.009, ** P=0.009, *** P=0.002 when compared to control (BPH^V) cells.
3.3 LIMK 1 Induced Invasion is Mediated by Matrix metalloproteinases

To determine whether MMPs are involved in LIMK1 mediated invasion, we repeated the above assay using GM6001, an inhibitor of matrix metalloproteinase -2 and -9 [Nelson et al., 2000]. The broad spectrum matrix metalloproteinase inhibitor is GM6001 is a synthetic polypeptide that competively inhibits matrix metalloproteinases by occupying the substrate binding sites [Boghaert et al., 1994]. Xue et al have shown that GM6001 specifically inhibits MMP-2 and MMP-9 in cultured human keratinocytes as observed in in migration assays [Xue et al., 2004]. For invasion assays, cells were seeded with or without GM6001 (25μM) added to the media and the assay was continued as mentioned previously. Invaded cells were quantitated by fluorescence measurement and cell counting as described above. RFU values showed that there was a considerable decrease in invasion of BPHL cell lines expressing constitutively active or kinase dead LIMK1 upon treatment with of MMP inhibitor GM6001 (Figure 18). BPHL cell lines expressing constitutively active or kinase dead LIMK1 were equally inhibited. There was no change in the invasiveness of BPHL^K cells in fluorometric assays upon addition of GM6001. However, the number of BPHL^K cells that traversed the matrigel membrane were decreased in the presence of GM6001 (Figure 19). The cell count values the showed a greater inhibition of invasion in BPHL^CA and BPHL^K upon treatment with GM6001. This result supports the hypothesis that LIMK1 induced invasion is in part mediated by LIM domains.
Figure 18 Inhibition of MMPs decreased invasion of BPH cell lines

Cell lines expressing different domains of LIMK1 were inhibited. Graphical representation of population of cells that penetrated matrigel coated membranes. Values are mean +/- SE of three experiments. MMPs inhibitor (GM6001) substantially reduced the ability of BPHLCA and BPHLKD to invade matrigel coated membranes. BPH cells expressing only the kinase domain of LIMK1 (BPHLK) were not inhibited by GM6001.
Figure 19 MMP inhibitor GM6001 reduced the invasiveness of BPH cells expressing LIMK1 Transgenes with intact LIM domains. Graphical representation of population of cells that penetrated matrigel coated membranes. Percentage of cells that traversed through the matrigel membrane and accumulated in the lower chamber. Values are mean +/- SE of three experiments.
3.3.1 LIMK1 Expression Altered the Concentration of Secreted pro-MMP2 and pro-MMP9 and increased mRNA Concentration of MMP2

Substrate Gel Analysis

To analyze any alteration in the expression of MMPs in BPH cells expressing LIMK 1 mutants, we used gelatinase zymography. Matrix metalloproteinase -2 and -9 are soluble MMPs which are secreted into the extracellular environment. Therefore, conditioned media from each mutant of LIMK1 was harvested and analyzed on 10% SDS gel co-polymrized with denatured collagen (gelatin). Our results showed a graded amount of secreted latent MMP2 (72kD) with the maximum, intermediate and least secretion observed in BPHL CA, BPHL KD, and BPHL K cells respectively (Figure 20). BPHL K cells showed the highest level of latent MMP-9 (92kDa), (Figure 21). No secretion of latent MMP9 was observed in the media of BPHL CA, BPHL KD and BPHV cells.

Quantification of mRNA Concentration

To determine whether LIMK1 expression alters the steady state mRNA concentration of matrix metalloprotease -2, quantitative real-time PCR was done using total RNA from the transfected BPHL CA and BPHV cells. The results showed that the mRNA concentration of MMP-2 was~10 fold higher than BPH cells expressing the vector (BPHV) (Figure 22). The concentration of MT1-MMP mRNA was unchanged between LIMK1 over expressing samples and BPHV cells. No difference in the concentration of MT1-MMP mRNA was noted between BPHL CA and BPHV cells. Therefore the upregulation of MT1-MMP observed in cells expressing constitutively active LIMK1 appears to take place at the level of activation level. The house keeping gene, GAPDH was used as a reference.
Figure 20 Gelatin zymography of the secreted Matrix Metalloproteinase 2

(A): Expression of secreted Pro-MMP-2 in transfected BPH cell lines. BPH cells expressing constitutively active LIMK1 (BPHLCA) had the highest concentration of Pro-MMP-2. BPHLKD (kinase dead) and BPHLK cells showed also showed comparable levels of secreted Pro-MMP-2 but substantially lower than BPHLCA cells. BPHV cells showed the least amount of secreted. (B): Densitometric analysis of relative intensity of gelatinoic activity. Values are mean +/- SE of three experiments. * P=0.028, ** P=0.035*** P=0.010 when compared to control (BPHV) cells.
Figure 21 Gelatin zymography of secreted Matrix metalloproteinase 9

(A) BPHL cells expressing only the kinase domain of LIMK1 (BPHLK^), had the maximum amount of secreted Pro-MMP-9. Transfected BPHL^CA, BPHL^KD cells showed significantly lower concentrations of secreted Pro-MMP-9. BPHV cells had the least amount of secreted pro-MMP-9.

(B) Densitometric analysis of the relative intensity of gelatinoic activity. Values are mean +/- SE of three experiments. * P=0.002, ** P=0.018 *** P=0.001 when compared to control (BPHV) cells.
Figure 22 Quantitative Real-time PCR analysis of MMP-2 steady state mRNA

BPH cells expressing constitutively active LIMK1 had 10 fold increase in MMP-2 mRNA concentration relative to vector only control cell line (BPHV). Normalized average ΔCT values for BPHV and BPHLCA were -7 and -3.7, respectively. Values are means +/- SE of three experiments.
3.4 MT1-MMP Expression positively Correlates with the Concentration of active LIMK1

Ectopic Expression of Constitutively Active LIMK1 Increased MT1-MMP Expression

Membrane type I-matrix metalloproteinase is known to activate MMP-2 and possibly MMP-9. Furthermore, because it has potent pericellular ECM degrading activity, MT1-MMP is one of the most important regulators of cell migration and invasion. Upon activation of MT1-MMP through cleavage of the pro-peptide by furin like convertases, it is inserted into the plasma membrane (Figure 5a). To analyze whether there is any change in the cell surface expression of MT1-MMP, an antibody was used that recognizes the catalytic domain of MT1-MMP, the domain exposed to the extra cellular side of the plasma membrane. Flow cytometric analysis indicated that the BPHL<sup>CA</sup> cells expressed the highest amount of MT1-MMP on the surface (Figure 23). BPHL<sup>KD</sup> cells expressing catalytically inactive LIMK1 also showed modest increase in the cell surface expression of MT1-MMP compared to BPH<sup>V</sup> control cell. Expression of MT1-MMP in BPHL<sup>K</sup> and BPH<sup>V</sup> cell lines were comparable but lower than that of BPHL<sup>CA</sup> and BPHL<sup>KD</sup>. Because MT1-MMP is targeted to the plasma membrane in it’s active form, increased surface expression of MT1-MMP could be considered as increased activity of MT1-MMP. Therefore, it could be inferred that increased expression of active LIMK1 increased the activity of MT1-MMP in BPHL<sup>CA</sup> cells. For densitometric analysis, the cells that were emitting fluorescence were gated out from the nonfluorescent cells and calculated as the percentage of the control (BPH<sup>V</sup>) cells (Figure 24).
Figure 23  Flow cytometric analysis of MMP-14 cell surface expression in transfected BPH cell lines.

Two parameter histograms with fluorescence intensities in the X axis and number of cells in the Y axis. A total of 10,000 cells were counted. Black histogram represents unstained cells, red histogram represents population of fluorescent cells within each sample. Data shows a noticeable shift in the red histogram of BPHLCA cells representing high number of cells with fluorescence. The red histogram of BPHKD cells also showed a shift towards the right representing increased number of cells with fluorescence compared to that of BPHV cells. Red histogram of BPHV cells showed minimum shift indicating minimal number of fluorescent cells. A modest shift in the red histogram was noted in BPHLK cells representing fluorescent cells with some degree of MT1-MMP expression.
Figure 24 Relative Expression of MT1-MMP in BPH cells expressing different domains of LIMK1.

Quantitative analysis of fluorescent cells after elimination of cell debris and non-fluorescent cells. Data represents the average +/- SE of three independent experiments. * $P=0.136$, ** $P=0.109$ and *** $P=0.05$. 
Expression of LIMK1 Transgenes Increased Concentration of MT1-MMP

Total protein were extracted from LIMK1 expressing cell lines and analyzed by western blots using anti-MT1-MMP antibody. The results showed a considerable increase in MT1-MMP latent and active protein levels in BPHL<sup>CA</sup> cell line compared to the vector only control cell line BPH<sup>V</sup> (Figure 25). As a positive control PC3 extracts were used as they express high levels of MT1-MMP [Daja et al., 2003]. The level of MT1-MMP observed in BPHLKD was comparable to but less than that of BPHL<sup>CA</sup>. BPHL<sup>K</sup> cells showed the least amount of MT1-MMP. The vector only cell line (BPHV) did not express any MT1-MMP. This data also substantiates a positive correlation between LIMK1 and MT1-MMP protein levels.

MT1-MMP Co-localizes with LIMK1 in the Perinuclear Region

To determine the intracellular localization of MT1-MMP, dual label immunoflorescence using antibodies of MT1-MMP and Flag was performed. A distinct difference in cell morphology was noted in cells expressing active LIMK1, kinase dead LIMK1 and only the kinase domain. Lamellipodia and filopodia extensions were observed in constitutively active (BPHL<sup>CA</sup>) cells (Figure 26). Cellular morphology and localization pattern of MT1-MMP in BPH cells expressing LIMK1 with intact LIM domains (BPHL<sup>CA</sup> and BPHL<sup>KD</sup> cells) were comparable, although more ruffling membranes were noted in BPHL<sup>CA</sup> cells. In BPHL<sup>K</sup> cells, microspikes were observed. Also, BPHL<sup>K</sup> cells were clustered together and showed a more adherent phenotype. Higher concentration of MT1-MMP was also observed in BPHL<sup>CA</sup> cells. In addition, co-localization of MT1-MMP and constitutively active LIMK1 to the perinuclear region was most evident in BPHL<sup>CA</sup> cells. Flag staining observed in was BPH<sup>V</sup> was essentially non-existant compared to BPHL sublines.
Figure 25 Expression of Pro-MT1-MMP and active MT1-MMP in BPH-LIMK cell lines

Western blot analysis of MT1-MMP in BPH crude extracts of expressing LIMK1 mutants.

Metastatic prostate cancer cells (PC3) that express high levels of LIMK1, also showed expression of latent and active MT1-MMP.
Figure 26 MT1-MMP localization studies of BPH cells expressing mutant LIMK1

In BPHL^K cells diffused cytoplasmic and nuclear localization of LIMK1 (green) and appearance of microspikes (arrow) could be seen. Localization of MT1-MMP in these cells was mainly to the perinuclear region. In BPHLKD cells, LIMK1 was localized to the nuclei and to the plasma membrane (arrow). BPHL^KD merged image shows that MT1-MMP is distributed throughout the cytoplasm and co-localized with LIMK1 (arrow). In BPHLCA cells, increased concentration of constitutively active LIMK1 to the plasma membrane was evident. Appearance of lamellipodia also could be seen in these cells. BPHL^CA cells, showed the most intense staining of MT1-MMP and accumulation of LIMK1 to ruffling membranes (merged image, arrows).

MT1-MMP localization was observed in the perinuclear to cytoplasmic region in BPHL^CA and BPHL^KD cell lines. However, in BPHL^KD cells, MT1-MMP staining was more concentrated to the perinuclear region. Further, the results showed modest co-localization between constitutively active LIMK1 and MT1-MMP in the membrane ruffles.
3.5 Phospho-LIMK1/2 Associates with γ-Tubulin

Role of LIMK1 in Mitosis

Disruption of highly orchestrated processes like mitosis and cytokinesis causes chromosomal instability and subsequent tumor progression [Ohbayashi et al., 2007]. In addition, LIMK1 has been shown to coordinate microtubule instability through co-localization with microtubules and complex formation via PDZ domain [Gorovoy et al., 2005]. Induction of microtubule destabilization by treatment with nocodazole resulted in decreased association with LIMK1. Because centrosomes are critically involved in mitotic spindle formation we studied the distribution of phospho-LIMK1/2 in the mitotic cells.

Co-localization studies of p-LIMK1/2 and γ-tubulin

Immunofluorescence analysis of metastatic (MDA-MB-231) and normal (HMEC) breast epithelial cells (HMEC) revealed that phospho-LIMK1 and γ-tubulin co-localize to the centrosome during prophase, metaphase and anaphase (Figure 8). This pattern of localization was also observed in the invasive prostate cancer cell line DU145 (data not shown). This co-localization diminishes at the onset of telophase. Visualization of phospho-LIMK1 and γ-tubulin localization was accomplished using a monoclonal α-gamma tubulin and polyclonal α-phospho-LIMK1 antibodies. To determine optimal dilution of phospho-LIMK1/2 to be used in western blot analysis, both antibodies were titrated using crude nuclear extracts of LNCap and BPH whole cell extracts (Figure 27). Western blot analysis showed that 1:50 was the optimal dilution to use for 50μg of nuclear extracts. Immunoprecipitation with γ-tubulin antibody pulled down
Figure 27  Titration of phospho-LIMK1/2 antibody in BPHL cells

(A) Titration of p-LIMK1.  (B) Titration of and p-LIMK2.  GAPDH was used as a loading control.  Fifty microgram of crude BPH extracts were used.
A
MDA-MB-231 Nuc Extract
IB: pLIMK1

IB: pLIMK1

IB: pLIMK2

IP: Non-specific antibody
IP: γ-Tubulin CIAP treated Nuc Extract

B
BPH-L^FLAG^ Nuc Extract
IP: γ-Tub

IB: pLIMK1

IB: γ-Tubulin

IB: FLAG

C
BPH-L^FLAG^ Nuc Extract
IP: FLAG

IB: pLIMK1

IB: γ Tubulin
Figure 28 Biochemical interactions between phospho-LIMK1 and γ-tubulin

Panel (A) Upper and middle panel: Western blot analysis of p-LIMK1 and p-LIMK1 in crude nuclear extracts and immunoprecipitates with γ-tubulin antibody using MDA-MB-231 cells. Lower panel: Treatment of MDA-MB-231 cells with CIAP or immunoprecipitation with non-specific antibody followed by western blot analysis with anti-phospho-LIMK1. Panel (B): Western blots of p-LIMK1, γ-tubulin and Flag-tagged LIMK1 in crude and γ-tubulin immunoprecipitates of nuclear extracts of BPHLFLAG cells. Panel (C) Western blots of p-LIMK1 and γ-tubulin in Flag immunoprecipitates of nuclear extracts of BPHFLAG cells.
phospho-LIMK1. This interaction between γ-tubulin and p-LIMK1/2 was shown to exist in BPH cells over expressing LIMK1 and MDA-MB-231 breast cancer cells (Figure 28a/b). To confirm that γ-tubulin specifically associated with phospho-LIMK1, nuclear extracts were treated with calf intestinal alkaline phosphatase (CIAP). Treatment with CIAP removes phosphate groups from all phosphorylated protein. Therefore CIAP treated nuclear extracts did not precipitate with γ-tubulin as indicated by post immunoprecipitation immunoblot analysis (Figure 28a). This confirms that γ-tubulin associates with LIMK1 only in phosphorylated form. Immunoprecipitation with an unrelated antibody showed that γ-tubulin was specifically associating with p-LIMK1 (Figure 28a). In addition, immunoprecipitation with Flag antibody also pulled down γ-tubulin and phospho-LIMK1 (Figure 8c). Therefore recombinant LIMK1 expressed in BPH cells is also associating with γ-tubulin.
4. DISCUSSION

4.1 LIM kinase 1 and Matrix metalloproteases

LIM kinase 1 has been identified as a required component for the invasion of prostate epithelial cells [Davila et al., 2003]. To determine the structure-function relationship of LIM kinase 1 mediated invasion, mutant constructs were designed. BPH cells stably over expressing a kinase domain only mutant of LIM kinase 1 (BPHL^K) showed decreased invasiveness when compared to kinase dead mutant (BPHL^KD) and constitutively active mutant (BPHL^CA) of LIM kinase 1. The percent invasion for BPHL^CA and BPHL^KD cell lines were comparable. The kinase domain only of LIMK1 mutant did not promote invasion of BPH cells. Phosphorylation of LIMK1 at T508 allows dissociation of the LIM domains from the kinase domain. When LIM kinase 1 becomes phosphorylated, it’s conformation changes from closed to open, thereby making the LIM domains accessible (Figure 7). Therefore it is reasonable to assume that the highly invasive phenotype seen in BPHL^CA cell line may be due to increased protein:protein interactions involving the LIM and/or the PDZ domain, kinase activity, or both. In fact, current studies showed a correlation between the protein:protein interaction of LIM domain containing proteins PELP1 and FHL2 with prostate cancer progression [Nair et al., 2007]. This data suggest that the LIM domains of LIM kinase 1 may be necessary for the invasiveness of cancerous prostate. This result further implies that LIM kinase 1 is able to induce invasion independent of it’s kinase activity. While there is conflicting evidence as to the role of LIM kinase 1 in promotion or inhibition of tumor cell invasion [Wang et al., 2006], the data presented here supports a pro-invasive role for LIM kinase 1 in the absence of it’s kinase activity in prostate epithelial cells. In addition, little is known about the mechanism by which LIM kinase 1 exerts
it’s effect. However, research from other laboratories showed that the regulation of cofilin by LIMK1 is central to LIM kinase 1 mediated invasiveness [Zebda et al., 2000]. The reason for these opposite findings is not known. However, based on this study, one can speculate that there may be interacting proteins associated with the LIM and PDZ domains, which are in part responsible for invasion, and that the activity of LIM kinase 1 contributes to invasion but is not necessary for invasion.

To understand the mechanism underlying LIM kinase 1 mediated invasion we monitored the effect of inhibition of matrix metalloprotease 2/9 on the ability of BPH-LIMK1 cell lines to invade matrigel coated membrane in vitro. Because MMPs have been shown to be involved in prostate cancer cell invasion [Wilson et al., 2004], we hypothesized that they may also be involved in LIM kinase 1 mediated prostate cancer cell invasion. Inhibition of MMPs resulted in considerable decrease in invasion of BPH cells expressing kinase dead or active LIMK1 through matrigel coated membranes. Matrix metalloprotease inhibition had the greatest effect on BPH cells expressing active LIMK1. There is substantial evidence implicating matrix metalloprotease-2 and matrix metalloprotease-9 as key enzymes in prostate cancer cell invasion, [Vayalil and Katiyar, 2004], [Vijayababu et al., 2006], [Sehgal and Thompson, 1999], [Hong et al., 2006]. In agreement with published reports, we found that MMP-2 and MMP-9 are involved in the invasion of prostate epithelial cells. However, to our knowledge, our study provides novel data that links the expression of LIM kinase 1 activity to the expression of matrix metalloproteases. Gelatinase zymography analysis showed that the BPH^CA cell line produced the highest concentration of secreted latent MMP-2. Conversely, the highest concentration of MMP-9 was seen in BPH^K cell line. Comparable concentration of secreted latent MMP-2 was observed
in BPH\textsuperscript{KD}. In comparison, BPH cells stably expressing kinase dead (BPH\textsuperscript{K}) or the vector (BPH\textsuperscript{V}) had minimal levels of latent MMP-2. These data further confirms that MMPs have an integral role to play in prostate cancer cell invasion. It also showed a positive correlation between LIM kinase 1 induced expression and the secretion of soluble MMP-2. It could be speculated that LIM kinase 1 may have a role in either the expression, regulation, or activation of MMP-2.

Quantitative RT-PCR data showed that LIMK1 is involved in alteration of the mRNA levels of MMP-2. We used BPH cells expressing constitutively active LIMK1 or vector only. The results show a 10 fold increase in the steady state mRNA levels of MMP-2 in BPH cells over expressing LIM kinase 1 relative to the vector control. GAPDH was used as a reference gene. These results showed that LIM kinase 1 expression increased the steady state level of MMP-2 mRNA compared to control BPH cells. Our data on MMP-2 and MMP-9 expression in prostate cancer cells provides further evidence to support current knowledge [Aalinkeel et al., 2004; Pratap et al., 2005; Singh et al., 2004] and also extends the understanding by linking MMP-2 and MMP-9 expression to LIM kinase 1. Our data supports our hypothesis that LIM kinase 1 induced invasion is mediated by MMPs. And further, this correlation is seen at the level of mRNA concentration and protein expression.

Membrane-type I matrix metalloprotease is vital for initiation of ECM degradation [Udayakumar et al., 2004], [Bair et al., 2005], [Golubkov et al., 2005]. MT1-MMP (also called MMP-14) not only directs the degradation of cell surface ECM, but also activates MMP-2, another potent ECM degrading family member [Dong et al., 2005], [Cardillo et al., 2006]. Using flow cytometric analysis we were able to quantitate the level of “processed” MMP-14 at the
plasma membrane in benign prostate epithelial cells. In addition, our results showed that upon treatment with EGF there was a considerable increase in MT1-MMP signal intensity compared to samples not treated with EGF (data not shown). This result complies with current research that identifies EGF signaling as an upregulated pathway in prostate cancer [Mimeault et al., 2007b], [Angelucci et al., 2006]. It is logical to assume that EGF signaling increases the level of active endogenous LIM kinase 1 mediated by Rac 1 while simultaneously inducing MT1-MMP expression [Kheradmand et al., 2002], [Van Meter et al., 2004] [Yeh et al., 2006]. In addition, EGFR signaling increases the activation of both LIM kinase 1 and MT1-MMP in prostate cancer cells. In this study, we show increased expression of MT1-MMP on the cell surface in BPH cells stably expressing constitutively active LIM kinase 1. Cell lines expressing, kinase dead and kinase domain mutants of LIM kinase 1, showed comparable levels of MT1-MMP expression relative to the control. Since MT1-MMP is only inserted into the plasma membrane after cleavage and activation by furin convertases, [Hernandez-Barrantes et al., 2000] we can argue that over expression of LIM kinase 1 correlates with increased expression and increased activity of MT1-MMP.

Immunofluorescence analysis revealed that LIM kinase 1 and MT1-MMP co localize to the perinuclear to cytoplasmic region in BPH. Current research shows that MT1-MMP targeting to the plasma membrane is necessary for migrating cells to become invasive [Strongin, 2006]. Cell lines expressing constitutively active and kinase dead mutants showed distinct formation of lamellipodia and membrane ruffles upon treatment with EGF. However a dramatic effect was seen in cells expressing constitutively active LIM kinase 1. In addition, co-localization of LIM kinase 1 and MT1-MMP in the membrane ruffles was observed in BPHL CA. This data suggest
that LIM kinase 1 and MT1-MMP are both involved in the formation of structures relevant to
tumor cell motility and that these enzymes may be functionally or mechanistically related. We
can also infer that sub cellular localization of MT1-MMP is in part dependant on LIM kinase 1
expression and/or activity. BPH cells expressing only the kinase domain showed substantial
formation of micro spikes; which are consistent with Rho signaling effects [Aznar et al., 2004].
No specific localization was seen in the control cell line expressing the vector. Our localization
studies focused on LIM kinase 1 and MT1-MMP support the hypothesis that LIM kinase 1
induced invasion is mediated by matrix metalloproteases. Moreover, we provide further
evidence in support of the current knowledge that MT1-MMP is involved in the migration of
cancer cells [Lehti et al., 2000]. MT1-MMP and LIM kinase 1 may act synergistically to each
other in the process of tumor cell migration and invasion.

The work presented here is important because it expounds on the key molecules that are
involved in prostate cancer cell progression to invasiveness. Matrix-metalloproteinase’s are
extra cellular matrix degrading enzymes with clear and direct links to malignancy [Nelson et al.,
2000]. Independantly, LIM kinase 1 and matrix metalloproteases expressions have been strongly
implicated with increasing malignancy. Our study shows that there may be cross-talk between
these two proteins in promotion of invasion. This study also provides evidence that LIM kinase 1
mediated invasion can occur independent of it’s kinase activity. The data also indicate that in the
absence of the LIM domains, BPH cells showed considerably decreased invasiveness. This
strongly indicates the LIM domains are critical to the invasiveness of LIM kinase 1. To confirm
this speculation, a mutant of LIM kinase 1 that is both kinase dead and constitutively open can be
studied. Taken together these results elucidate the mechanisms involved in prostate cancer cell
invasion by linking LIM kinase 1 to MMPs. Both of which are independently known to be involved in tumor cell migration and invasion. Identification of molecular drug targets for tumor cell invasion relies on the elucidation of the complex mechanism of metastasis. This data provides evidence that LIM kinase 1 could be a target for anti-metastatic drug that could possibly effect the matrix metalloproteinase’s. In addition, Meng et al have shown that knockout LIM kinase 1 mice display only minor abnormalities; hence LIM kinase 1 represents an ideal target for drug design [2002].

4.2 LIM KINASE 1 and γ-Tubulin

LIM kinase 1 is well established as a potent regulator of the actin cytoskeleton through it’s interaction with coflin [Zebda et al., 2000]. Previous studies have revealed that LIM kinase 1 has a role in the stabilization of microtubules [Gorovoy et al., 2005]. Cell motility, migration and division are processes that rely on the coordination between microtubules and actin cytoskeleton [Rodriguez et al., 2003]. Sumi et al showed that LIM kinase 1 activation and subsequent targeting to the spindle pole is regulated by cell cycle progression in HeLa cells. The data presented here further supports these findings. During interphase, epiflourescence imaging showed that phospho-LIM kinase 1 is not co-localized with γ-tubulin. However upon activation and the onset of mitosis a distinct localization is seen between γ-tubulin and phospho-LIM kinase1 to the spindle poles. This p-LIM kinase 1/γ-tubulin association was abolished during telophase where p-LIM kinase 1 concentrated to the cleavage furrow. This pattern of localization between p-LIM kinase 1 and γ-tubulin was observed in the slightly metastatic prostate cancer cell line DU-145, in the highly invasive breast cancer cell line MDA-MB-231 and in normal benign breast cancer cells, HUMEC. This indicates that this interaction is not
cancer cell specific and that it may represent a novel role for LIM kinase 1 in the regulation of cell division. We propose that inactive LIM kinase 1 is bound to microtubules during interphase and when it becomes phosphorylated it is released from microtubule and targeted to the centrosome where it associates with γ-tubulin. Other studies showed a similar cell cycle dependant activation of Ajuba (a LIM containing protein) and subsequent co-localization with γ-tubulin to the centrosome [Abe et al., 2006].

Immunoprecipitation reactions confirmed that p-LIM kinase 1 associates with γ-tubulin. Nuclear extracts of BPH and MDA-MB-231 immunoprecipitated with γ-tubulin and blotted with p-LIM kinase 1 gave a positive result. Western blot analysis of nuclear extracts immunoprecipitated with a un-related antibody could not be detected by probing with anti-phospho-LIMK1. Similarly, nuclear extracts immunoprecipitated with non-specific antibody and immuno blotted with p-LIM kinase 1 antibody did not yield a signal. This result confirms that p-LIM kinase 1 is specifically associating with γ-tubulin. To prove that p-LIM kinase 1 and not LIM kinase 1 is associating with γ-tubulin, nuclear extracts were treated with calf intestinal alkaline phosphatase (CIAP) to remove phosphate group from all proteins. CIAP treated extracts were processed and showed no band after immunoblotting with p-LIM kinase 1. It is important to mention that p-LIM kinase 2 also associated with γ-tubulin. This result suggest that p-LIM kinase 1 may have an indirect role in mitotic spindle assembly through its interaction with γ-tubulin at the centrosome during prometaphase to anaphase. Therefore over expression of LIMK1 in cancer cells may promote chromosomal instability and spindle defects.
LIST OF REFERENCES


Golubkov VS, Boyd S, Savinov AY, Chekanov AV, Osterman AL, Remacle A, Rozanov DV, 


