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LIV-1 Promotes Prostate Cancer Epithelial-to-Mesenchymal Transition and Metastasis Through HB-EGF Shedding and EGFR-mediated ERK Signaling

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LIV-1 PROMOTES PROSTATE CANCER EPITHELIAL-TO-MESENCHYMAL TRANSITION AND METASTASIS THROUGH HB-EGF SHEDDING AND EGFR-MEDIATED ERK SIGNALING

by

HUI-WEN LUE

Under the Direction of Dr. Leland W.K. Chung

ABSTRACT

LIV-1, a zinc transporter, is an effector molecule downstream from soluble growth factors. This protein has been shown to promote epithelial-to-mesenchymal transition (EMT) in human pancreatic, breast, and prostate cancer cells. Despite the implication of LIV-1 in cancer growth and metastasis, there has been no study to determine the role of LIV-1 in prostate cancer progression. Moreover, there is no clear delineation of the molecular mechanism underlying LIV-1 function in cancer cells. In this study, we found increased LIV-1 expression in a progressive manner in benign, PIN, primary and bone metastatic human prostate cancer. We characterized the mechanism by which LIV-1 drives prostate cancer EMT in an androgen-refractory human prostate cancer cell (ARCaP) bone metastasis model. LIV-1, when overexpressed in ARCaP_E cells (deriva-

tive cells of ARCaP with epithelial phenotype), promoted EMT irreversibly. LIV-1 overexpressed ARCaP_E cells had elevated levels of HB-EGF and matrix metalloproteinase (MMP) 2 and MMP 9 proteolytic enzyme activities, without affecting intracellular zinc concentration. The activation of MMPs resulted in the shedding of heparin binding-epidermal growth factor (HB-EGF) from ARCaP_E cells, eliciting constitutive epidermal growth factor receptor (EGFR) phosphorylation and its downstream extracellular signal regulated kinase (ERK) signaling. Further investigation of the HB-EGF promoter revealed that both Stat3 and AP-1 controlled HB-EGF promoter activity. Ectopic LIV-1 overexpression induced AP-1 and Stat3 activation. Blockade of both Stat3 and AP-1 by specific inhibitors or dominant negative expression vectors diminished the HB-EGF promoter activity induced by LIV-1 overexpression. These results suggest that LIV-1 is involved in prostate cancer progression as an intracellular target of growth factor receptor signaling which promotes EMT and cancer metastasis. LIV-1 could be an attractive therapeutic target for the eradication of pre-existing human prostate cancer and bone and soft tissue metastases.

INDEX WORDS: LIV-1, EMT, Prostate cancer progression, EGFR, HB-EGF, ERK, AP-1, Stat3

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by

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Georgia State University

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TION AND METASTASIS THROUGH HB-EGF SHEDDING AND EGFR-MEDIATED ERK
SIGNALING

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1 INTRODUCTION

1.1 *Prostate cancer*

Prostate cancer is the most commonly diagnosed cancer and second leading cause of cancer-related death in men in the United States. The American Cancer Society estimated that more than 217,730 new cases of prostate cancer were diagnosed and over 32,050 men died of the disease in 2010 (Bubendorf et al., 2000; Keller et al., 2001). Roughly 1 man in 6 will be diagnosed with prostate cancer during his life time and 1 in 36 will die of prostate cancer. If prostate cancer is detected at an early stage, curative treatment by radical prostatectomy or radiotherapy is possible (Bagshaw et al., 1994; Zincke et al., 1994). However, once prostate cancer metastasizes the mortality rate is extremely high.

The prostate is a gland which belongs to the male reproductive system and produces fluid for semen. The main cell types of the prostate epithelium are the basal, secretory glandular and neuroendocrine cells. The glandular cells secrete PSA and prostatic acid phosphatase into the glandular lumina and are the major cell type in normal and hyperplastic epithelium. The secretory glandular cells have high AR expression and thus are androgen-dependent for their growth. In contrast, basal cells express low or undetectable AR and locate on the basement membrane. Neuroendocrine cells also locate on the basement membrane and both basal and neuroendocrine cells are androgen insensitive. Almost all prostate cancers develop from secretory epithelial cells of the prostate gland and often grow slowly within the gland. It is believed that high grade prostatic intraepithelial neoplasia (HG PIN) is a precursor of prostate cancer. PIN, first described in 1969 (McNeal, 1969), is a neoplastic proliferation of prostatic epithelial cells. Over time, these cancer

cells begin uncontrolled proliferation to form a tumor. Eventually, the tumor cells may gain migratory and invasive ability and invade surrounding tissue, circulate in the bloodstream and lymphatic system, and finally colonize at metastatic sites.

PIN glands characteristically contain basal cells around their periphery (Figure 1.1). The presence of basal cells is an indicator differentiating PIN from prostatic adenocarcinoma in which the basal cells are absent. Normal prostate, PIN, and prostatic carcinoma can be easily distinguished using specific basal cell marker (p63) and the prostate cancer marker Alpha-methylacyl-CoA racemase (AMACR), since AMACR is expressed at a much higher level in adenocarcinoma than in non-neoplastic prostatic glands. Typically, normal prostate only shows basal cell staining, whereas prostate carcinoma only shows AMACR marker staining. However, HGPIN exhibits both markers, which is different from normal prostate or adenocarcinoma (Zynger and Yang, 2009).

Early prostate cancer usually has no symptoms and is usually diagnosed by a PSA test. If the prostate cancer is confined within the gland, radical prostatectomy is potentially curative. However, prostate cancers that have spread outside the gland are typically treated with hormone therapy. Hormone therapy, also called androgen deprivation therapy, aims to reduce levels of testosterone and dihydrotestosterone. Castration induces apoptosis of the majority of prostate cancer cells and causes tumors to shrink or grow more slowly. Most prostate cancers will have an initial favorable response to hormone therapy, but over time prostate cancer cells adapt to the low androgen environment and start to grow again. As the cancer progresses, prostate cancer cells gradually become androgen-independent and stop responding to hormone therapy. Chemotherapy is given if prostate cancer has already metastasized and hormone therapy fails. However, patients with metastatic prostate cancer are not curable and usually die within 1-2 years. A fuller

understanding of the mechanisms underlying cancer metastasis is vital for developing new therapeutic drugs.

1.1.1 Tumorigenic signaling pathways in prostate cancer

Androgen signaling. Androgen and androgen receptor (AR) both play critical roles in normal prostate development as well as prostate cancer(Suzuki et al., 2003). For example, transgenic mice engineering express high levels of the AR in the prostate tend to develop PIN(Stanbrough et al., 2001). AR is a ligand-activated transcription factor which belongs to a steroid hormone receptor family. AR controls the expression of numerous mitotic gene products, such as PSA, c-fos, Drg-1 and caveolin-1, which are important for the normal and neoplastic development of the prostate. In vitro studies showed that AR activation leads to stimulation of the survival signals and metastatic potential in LNCaP cells treated with androgen (Li et al., 2001; Topping et al., 2003). As cancer progresses, prostate cancer changes from androgen-dependent to androgen-independent. Many mechanisms have been proposed and changes of AR signaling are believed to play a crucial role. In androgen-independent prostate tumors, the aberrant AR activation may be due to AR amplification, AR mutation, ligand-independent receptor activation, and an increase of co-activator expression or decrease of co-repressor expression. In fact, AR amplification has been found in 20-30% of hormone refractory patients(Koivisto et al., 1998). Increase of AP expression allows cancer cells to survive in a low or depleted androgen environment. Over 80 mutations of AR have been identified, and most of them are mutated in the transactivation domain or ligand-binding domain, thus causing gain-of-function mutations(Gottlieb et al., 2004). In a transgenic model, AR wild type and AR mutants were specifically introduced into mice

prostate(Han et al., 2005). Only AR mutants caused development of prostate cancer, but not wild type AR, addressing the importance of AR mutations. In addition, AR activity could be activated in the absence of androgen by several growth factor cascades, including EGF, IGF-1, KGF, IL-6 and PKA pathway (Culig et al., 1994; Grossmann et al., 2001; Ueda et al., 2002). These factors are ligands for receptor tyrosine kinases and activation of these pathways may stimulate AR activation and promote growth of cancer cells in a low androgen environments. Furthermore, an increase of coactivator expression is another mechanism which causes AR activation(Gregory et al., 2001). In vitro studies showed that overexpression of AR coactivator enhance AR activity to low levels of androgen. In clinical specimens, AR coactivators- transcriptional factor 2, steroid receptor coactivator 1, and nuclear receptor coactivator amplified in breast cancer 1-have been shown to enhance expressions along with increases of AR expression in androgen-independent prostate cancer. Thus increases of coactivator expressions enhanced AR responses similar to AR mutation.

Wnt/ β -catenin signaling cascades. The aberrant activation of the canonical Wnt/ β -catenin signaling pathway also contributes prostate cancer progression. In the absence of Wnt signaling, free cytoplasmic β -catenin is quickly turned over by a destruction complex consisting of the adenomatous polyposis coli protein (APC), axin, glycogen synthase kinase 3-beta (GSK3 β) and casein kinase Iepsilon(CKI)17, 18. CKI and GSK3 β phosphorylate N-terminal serine/threonine residues of β -catenin(Amit et al., 2002; Liu et al., 2002). The phosphorylated β -catenin is targeted by an E3 ubiquitin ligase called β -TrCP (beta-transducin repeat-contain protein) and then degraded. Binding of Wnt molecules to the Frizzled-LRP5-LRP6 receptor complex leads to the inhibition of this degradation complex. Therefore, free cytoplasmic β -catenin is stabilized, accumulates in the cytosol and translocates to the nucleus. Nuclear β -catenin acts as a transcriptional

activator in a complex with the LEF/TCF DNA binding proteins (Behrens et al., 1996; Korinek et al., 1997; van Noort and Clevers, 2002). TCF/LEF molecules bind to promoter regions of target genes in a sequence-specific manner by recognizing the consensus sequence motif T/A T/A CAAAG₂₄. In the absence of nuclear β -catenin, TCF/LEF usually binds to members of the groucho/TLE proteins which are transcriptional repressors, thus causing inhibition of transcription of target genes. When β -catenin enters the nucleus, it displaces groucho/TLE proteins and binds to LEF/TCF to increase transcription of target genes including regulators of cell cycle, cell proliferation and metastasis (Daniels and Weis, 2005; Gavert and Ben-Ze'ev, 2007)(Figure 1.2). Several Wnt ligands have been reported to express at significant levels in prostatic stromal cells, androgen-dependent and independent cell lines and tumor tissues (Chen et al., 2004; Zhu et al., 2004). Moreover, high levels of Wnt-1 and β -catenin were detected in 77% of patients with lymph node metastasis and 85% in skeletal metastasis, suggesting the significant of this pathway.

Hedgehog signaling cascades. Abnormal hedgehog signaling has also found to cause cancer. The expression of hedgehog signaling components were found to be up-regulated in prostate cancer cells compared to normal prostate tissue. Increases of sonic hedgehog ligand, SHH, lead to the activation of the GLI-1 transcription factor which controls tumorigenic genes of cyclin D1 and c-Myc, resulting in sustaining growth of prostate cancer cells (Fan et al., 2004; Karhadkar et al., 2004; Sanchez et al., 2004).

Cytokine signaling cascades. The up-regulation of several cytokines in the serum of prostate cancer patients seems to be associated with the development of more malignant types of prostate cancer. For instance, higher expression of IL-6 appears in serum and tissues from patients with high grade prostate cancer, and is associated with poor patient outcome (Culig et al., 2005; Hobisch et al., 2001). In addition, Il-6 has been reported to mediate ligand-independent

activation of AR in androgen-independent prostate cancer cells, suggesting a role in promoting androgen-independent progression of prostate cancer (Yang et al., 2003). TGF- β has also been found to have high levels in serum of patients with advanced prostate cancer. TGF- β has dual functions in prostate. TGF- β inhibits the growth of normal prostate epithelial cells, but can promote EMT and metastasis in advanced prostate cancers (Bhowmick et al., 2004). Thus, targeting of these tumorigenic signaling pathways may provide a good therapeutic benefit.

1.1.2 Tumor microenvironment

The interactions between epithelial cells and their microenvironment are crucial in normal prostate development and adult function. Disregulation of stromal-epithelial interactions has been suggested to contribute to malignant progression and tumorigenesis (Hayward et al., 1996; Hayward et al., 1998; Hayward et al., 1997). In particular, the interaction of tumor cells with platelets, lymphocytes, fibroblasts, and macrophages was proved to be involved in tumor progression. The interaction between tumor cells and host cells is reciprocal. First, tumor cells may secrete some soluble factors, such as TGF- β and PDGF, to induce stromal fibroblasts to undergo myofibroblast transition, a process shared by wound healing and tumorigenesis. Tuxhorn et al. provided evidence that prostate cancer epithelium induced fibroblast-to-myofibroblast transition with an increase of α -smooth muscle actin, vimentin, and calponin expressions, which are characteristic of the myofibroblast phenotype, in the surrounding stroma (Tuxhorn et al., 2002). Evidence shows that myofibroblasts are also crucial for cancer cell progression. Chung et al. demonstrated that co-inoculation of prostate cancer cells and normal stromal fibroblasts from the fetal urogenital sinus inhibits the growth of cancer cells. In contrast, prostate cancer cells inoculated

with cancer-associated myofibroblasts show enhanced growth and metastatic potential, suggesting a role for myofibroblasts in cancer progression (Chung et al., 1989). In addition, cancer-associated stroma may release cytokines or neuroendocrine factors, such as HGF, VEGF, IGF-1 and IL-6, which may stimulate cancer cell invasiveness, angiogenesis, and tissue remodeling. Cat et al. showed that myofibroblasts can be induced by tumor cell-derived TGF- β , with an increased release of HGF, VEGF, and IL-6 (Cat et al., 2006), which have been shown to be involved in EMT and androgen-independent progression. In vivo studies also demonstrated that tumor growth and metastasis is significantly reduced in fibroblast-deficient mice. In summary, cancer cells not only produce some factors which favor their survival and growth, but also secrete factors which may promote host stromal cells produce more effectors which in turn act as tumor stimulators to enhance the invasiveness of tumor cells. Thus, a better understanding of stromal-epithelial interactions would be expected to reveal novel therapeutic options.

1.2 EMT

1.2.1 The concept of EMT

Epithelial-to-mesenchymal transition (EMT) is a phenomenon by which epithelial cells acquire migratory and invasive potential during physiological and pathological processes such as embryonic development, wound healing, and cancer progression. Epithelial cells and mesenchymal cells are distinguished by their unique visual appearance. Epithelial cells are adherent cells and they attach laterally to each other to form a sheet of cells called an epithelium. Cell-to-cell junctions and adherent proteins hold neighboring epithelial cells tightly together and restrain epithelial cells from moving away from the epithelium. A typical epithelium is one cell

thick and is polarized along an apical-basal axis where the basal surface interacts with basal membrane. In addition, the intracellular cytoskeleton network maintains the cell structure and provides rigidity and polarization. In contrast, mesenchymal cells are non-polarized and have a diffuse network; therefore they are irregular in shape and less rigid, accounting for the increased migratory ability.

The concept of “Epithelial-Mesenchymal Transformation” was first described by Elizabeth Hay in 1995 using the chick primitive streak as a model (Hay, 1995). Hay proposed that epithelial cells can undergo dramatic changes and transform into mesenchymal cells during embryonic development. Since it is clear that EMT is a reversible process and mesenchymal cells can revert back to epithelial phenotype, termed “Mesenchymal-Epithelial Transition” (MET), the term of “Epithelial-Mesenchymal Transformation” has been replaced with “Epithelial-Mesenchymal Transition”.

Turning epithelial cells into mesenchymal cells requires profound changes in epithelial cell organization (Kalluri, 2009; Kalluri and Weinberg, 2009). First, epithelial cells need to disassemble the cell-cell junctions and lose their apical-basal polarity. Cell surface proteins like E-cadherin which mediate epithelial cell to epithelial cell connection are replaced by N-cadherin which provides weaker adhesive properties, allowing cells to adopt the mesenchymal phenotype. In addition, cytoskeletal networks are reorganized and the cytokeratin intermediate filaments are replaced by vimentin. These changes convert the cell from a cuboidal to a spindle shape and are crucial for cells to leave the epithelium and begin to migrate individually. Second, in order to migrate and invade into the extracellular matrix, cells need to express proteases that degrade extracellular matrix simultaneously. Thus, upon undergoing EMT, cells lose epithelial cellular polarity, detach from their primary site and acquire migratory and invasive properties, allowing

them to migrate through the extracellular matrix (Rorth, 2009). Once arriving at their destination, these mesenchymal cells may undergo the reverse process of MET and establish themselves as one of many possible cell types. In summary, the characteristics of EMT are a loss of epithelial marker expressions, particularly E-cadherin, and an increase in mesenchymal marker expressions, such as N-cadherin, vimentin and fibronectin.

1.2.2 EMT during embryonic development

EMT happens in many biological and pathological events such as embryonic development, normal tissue repair and wound healing, and cancer progression. Primary EMT takes place during the implantation of the embryo, gastrulation, and organ development. During embryonic implantation, the trophoectoderm cells undergo EMT to invade into the endometrium and anchor in the placenta (Pijnenborg et al., 1980).

Gastrulation is a process by which the initial epithelial layer-epiblast forms a three germ layer, the ectoderm, the endoderm, and mesoderm. During gastrulation, the first EMT is the breakdown of the basement membrane underlying the epiblast. Cells in the primitive streak undergo EMT, resulting in the ingression of these cells within the primitive streak. The ingressing cells then either undergo MET to form the endoderm or remain mesenchymal to form the mesoderm.

Another example of primary EMT happens during neural crest formation. Epithelial cells of the neuroectoderm undergo EMT and generate a group of migratory neural crest cells (Tucker, 2004). These migratory neural crest cells disperse throughout the embryo and give rise to differ-

ent cell types including the neurons of the peripheral nervous system, pigment cells, and the cells of the adrenal medulla.

1.2.3 EMT during tissue regeneration and fibrosis

Inflammatory cells and fibroblasts mediate the release of inflammatory agents as well as components of extracellular matrix including collagens, fibronectins, elastin, and tenacins. Under pathological conditions, these stromal cells release inflammatory signals to stimulate normal epithelial cells undergoing EMT. Such EMT is found to be associated with progressive fibrotic diseases of the kidney, liver, heart, lung, and intestine (Kim et al., 2006; Potenta et al., 2008; Zeisberg et al., 2007a; Zeisberg et al., 2007b). Many studies used fibroblast-specific protein 1 (FSP1), α -SMA, and collagen I as mesenchymal markers for the EMT that occurs during fibrosis (Okada et al., 1997; Strutz et al., 1995). The expression of these markers was found to be correlated with the prognosis and extent of fibrosis. Such cells express FSP1 mesenchymal marker and α -SMA, but these cells still display epithelial morphology as well as E-cadherin. The behavior of these cells indicates that under inflammatory stimuli, epithelial cells can have different degrees of EMT, termed “partial EMT”. These cells then leave the epithelial layer and eventually accumulate in the tissue with a loss of all the epithelial markers and gain of a fully fibroblastic phenotype (Okada et al., 1996).

1.2.4 EMT during cancer progression

Most cancer deaths are due to metastatic tumors instead of the primary tumor. Metastasis is the spread of cancer cells which leave the site of primary tumors and disseminate to distant

sites. The process of metastasis in epithelial cancer consists of multiple steps. First, cancer cells lose cell-cell contact, become motile and gain the ability to invade to surrounding tissue. Once the cancer cells escape the basement membrane, they intravasate into local blood vessels, circulate through the blood stream, extravasate from the blood vessel and finally colonize at the secondary site (Chambers et al., 2002; Woodhouse et al., 1997). Metastasis will not happen if any of these steps fail (Figure 1.3).

Accumulated evidence indicates that EMT is associated with cancer progression (Thiery, 2002). EMT phenomena have been observed in many cancers, including breast, pancreatic, ovarian, colon, lung, esophageal and prostate. The characteristics of oncogenic EMT include disassembly of tight junctions and adherent junctions, loss of apical-basal polarity, cytoskeleton rearrangement, and a gain of mesenchymal phenotype with increased migratory and invasive properties. The process of EMT during cancer progression and metastasis closely parallels developmental EMT. Numerous EMT inducers in cancer cell lines have been identified including Transforming Growth Factor- β (TGF- β), Wnt, Snail/Slug, Twist and Six1, and these abnormally expressed EMT inducers are also critical during developmental EMT. Extensive mouse studies and cell line experiments have demonstrated that cancer cells can undergo EMT and acquire migratory and invasive properties when treated with EMT inducers. Blocking specific regulators could revert the mesenchymal phenotype as well as suppress invasive ability. Moreover, cells exhibiting EMT properties are often seen at the invasive front of primary tumors where EMT is likely to be induced by exposure to cytokines or extracellular stimuli. These cells are considered to be the ones that eventually invade the surrounding stroma and spread to distant sites. Thus, EMT is thought to be a critical mechanism for the metastatic spread of cancer cells. In addition, clinical studies have also suggested that EMT inducers in cancer correlate with poor clinical out-

come and tumor malignancy. However, phenotype changes in EMT are much more difficult to observe *in vivo*, since only a subset of tumor cells may undergo EMT at any one time. The main argument for the lack of a role of EMT in cancer is that from a histopathological point of view, metastatic tumors seem no different from the primary tumors. Therefore, the significance of EMT in cancer metastasis is still being debated (Christiansen and Rajasekaran, 2006; Garber, 2008; Thompson et al., 2005).

Recently, circulating tumor cells isolated from patients with progressive metastatic solid tumors, with a focus on men with castration-resistant prostate cancer (CRPC) and women with metastatic breast cancer (BC), were found to coexpress both epithelial and mesenchymal markers, suggesting that EMT processes indeed exist in clinical CTC (Armstrong et al.). In addition, EMT cells and non-EMT cells both are required for metastasis (Tsuji et al., 2009). When only EMT cells or only non-EMT cells were subcutaneously injected, no metastasis was observed. EMT cells could be found in the blood stream, but failed to colonize a secondary site. Metastatic tumor was observed only when both EMT and non-EMT cells were co-injected. Moreover, metastasis was observed when non-EMT cells were *i.c* injected into mouse tail vein, but not EMT cells. These findings suggest that EMT cells are responsible for invasion into the circulation, and only non-EMT cells are able to colonize at secondary sites. EMT cells and non-EMT cells need to cooperate in order to metastasize. Thus, EMT is now considered to play a critical role in the invasive steps of the metastatic cascade, causing invasive metastatic spread of tumors.

1.2.5 Molecular mechanism of EMT

E-cadherin regulation. One of the most important characteristics of EMT is the loss of cell-cell adhesion with down regulation of epithelial cadherin (E-cadherin). E-cadherin, a calcium-dependent transmembrane protein, is the most important adhesion molecule in epithelial cells. The intracellular part of E-cadherin is linked to the actin cytoskeleton through interaction with β -catenin. The extracellular part of E-cadherin binds to the binding sites of other E-cadherin on adjacent cells. Cadherin-cadherin binding is strong and serves as a major cell-cell adhesion force. The cadherin-catenin complex is essential for cell architectural integrity. Disruption of either of the complex components causes significant changes in cellular behavior and often results in tumorigenesis. Cadherin-mediated cell-cell adhesion is highly dynamic, enabling the reorganization and dispersal of cells. E-cadherin has been extensively studied in human epithelial cancers and loss of E-cadherin expression results in tumor progression, metastasis, and poor prognosis in various human cancers (Chan et al., 2003; Dorudi et al., 1993; Gould Rothberg and Bracken, 2006; Kowalski et al., 2003).

Transcription factors. Multiple mechanisms of E-cadherin loss have been demonstrated, including transcriptional, genetic or epigenetic changes that cause a functional loss of E-cadherin. The loss of E-cadherin expression at the transcriptional level was first identified in several cancer cell lines as well as human cancers, including prostate, breast, colorectal, lung, pancreatic, and thyroid cancers (Strumane et al., 2004; Van Aken et al., 2001). Later, the E-box response elements in the proximal E-cadherin promoter which determine epithelium-specific expression and sites of repressor binding were identified (Behrens et al., 1991; Rodrigo et al., 1999). Importantly, several developmental important transcription factors that induce EMT also repress E-cadherin during tumor progression. These transcription factors includes the snail family of zinc finger proteins (Snail, Slug), zinc finger proteins (ZEB1, ZEB2), and bHLH protein (Twist), which can

directly bind to E-cadherin promoter to repress E-cadherin expression (Batlle et al., 2000; Cano et al., 2000; Comijn et al., 2001; Eger et al., 2005). Among them, Snail is one of the key regulators of EMT. Overexpression of Snail reduces not only E-cadherin and other adhesion molecules but also induces mesenchymal markers to promote EMT and mesenchymal phenotype (Cano et al., 2000). In addition, Snail expression could be up-regulated by many known oncogenic signaling pathways, such as transforming growth factor- β (TGF- β) receptor pathway, epidermal growth factor (EGF) receptor pathway, and fibroblast growth factor (FGF) pathway. Furthermore, abnormal expressions of these transcriptional repressors have been found in many human cancers. During EMT, these transcriptional repressors not only suppress E-cadherin expression but also repress other adhesion molecules and induce mesenchymal phenotype (Aigner et al., 2007; De Craene et al., 2005; Vandewalle et al., 2005).

Genetic and epigenetic control. Besides transcriptional control, E-cadherin can be regulated by genetic or epigenetic mechanisms. Mutations of E-cadherin are found in gastric cancer (Becker et al., 1994; Brooks-Wilson et al., 2004) and lobular breast cancer (Sarrio et al., 2003). E-cadherin promoter polymorphism is potentially a good marker for the risk of bladder cancer recurrence (Lin et al., 2006). In addition, hypermethylation of the E-cadherin promoter region is found in various human cancers, resulting in loss of e-cadherin expression (Graff et al., 1995; Yoshiura et al., 1995). Furthermore, the extent of methylation of the E-cadherin region during cancer progression is unstable and heterogeneous, suggesting that methylation may induce EMT through downregulation of E-cadherin expression to promote metastatic progression (Graff et al., 1995).

microRNAs. Recently, small non-coding RNAs of 20-22-nucleotides (microRNAs) that inhibit gene expression at the post-transcriptional level have been identified to play a role in the

regulation of EMT. The miR-141, miR-200b, and miR-205 families are critical controls in ZEB1 and ZEB2 expression, leading to EMT regulation (Gregory et al., 2008; Park et al., 2008). Selective knockdown of miR-141, miR-200b, and miR-205 family miRNAs was sufficient to suppress E-cadherin expression and induce EMT in MDCK3 and HCT116 cells. Additionally, overexpression of these miRNAs results in E-Cadherin re-expression and MET in mesenchymal cells. The mechanisms of microRNA involvement in EMT need to be elucidated in more detail.

1.2.6 Extracellular signals and intracellular networks regulating EMT

Interaction between cancer cells and the tumor microenvironment profoundly influences the behavior of cancer cells. The tumor microenvironment is composed of ECM, cancer-associated fibroblast, myofibroblast, and immune cells. EMT is observed particularly at the invasive front, suggesting that the microenvironment plays a critical role in regulating EMT (Le et al., 2008). EMT can be induced by growth factors, cytokines, or ECM proteins secreted by the microenvironment. For example, conditioned media from cancer-associated fibroblasts induce EMT in breast cancer cells (Lebret et al., 2007). In addition, TGF- β signaling by stromal myofibroblasts can induce secretion of hepatocyte growth factor which promotes cancer cell proliferation and invasion (Lewis et al., 2004).

EMT regulators. Cell signaling pathways are critical inducers of EMT through transcriptional or post-transcriptional induction of several EMT transcription factors, including Snail, Slug, twist, ZEB1, and ZEB2. A variety of extracellular signals have been shown to induce EMT in cancers. TGF- β , EGF family members, FGF, HGF, and IGF have all been shown to trigger EMT in an autocrine or paracrine manner (Huber et al., 2005). One of the most potent and best

studied EMT inducers is TGF- β . The TGF- β family of cytokines binds to transmembrane receptor serine/threonine kinases which in turn activate cytoplasmic Smads (Massague et al., 2005). Activated Smads translocate to the nucleus and activate e-cadherin repressors of the Snail family. TGF- β has dual roles. TGF- β inhibits the growth of normal epithelial cells, but can promote cancer progression at certain stages through its ability to induce EMT (Shi and Massague, 2003; Siegel and Massague, 2003). In vitro studies showed that TGF- β induced EMT in many types of cancer cells with a loss of cell-cell adhesion and cell polarity, and gain of mesenchymal phenotype (Ozdamar et al., 2005; Peinado et al., 2003; Zavadil and Bottinger, 2005; Zhao et al., 2008). In early stage breast cancer, TGF- β is a tumor suppressor through its growth inhibitory effect (Reynisdottir et al., 1995). In contrast, TGF- β promotes metastasis in later stages of breast cancer, at least in part through its ability to induce EMT (Muraoka-Cook et al., 2006; Muraoka et al., 2002). In vivo, TGF- β also enhanced tumor aggressiveness in a mouse model (Muraoka-Cook et al., 2006) and blockade of TGF- β reduced metastasis and primary tumor growth in a mouse model (Siegel et al., 2003). In addition, clinical studies also support a positive correlation between expression of TGF- β ligands and poor prognosis (Ghellal et al., 2000; Mu et al., 2008).

Wnts, a large family of cysteine-rich, secreted lipid-modified signaling proteins, are potent regulators of cell proliferation and differentiation (Willert et al., 2003). The Wnt/ β -catenin pathway is implicated in EMT during development and cancer (Logan and Nusse, 2004; Reya and Clevers, 2005). Loss of function in Wnt signaling leads to developmental abnormalities, whereas constitutively active Wnt signaling causes tumorigenesis (Polakis, 2007; Taipale and Beachy, 2001). In vitro studies showed that activation of the Wnt pathway induces EMT by upregulating Snail expression in numerous cancer cell lines (Gilles et al., 2003; Kim et al., 2002).

Furthermore, active Wnt signaling also clinically correlates with poor outcome in breast cancer patients (Logullo et al., ; Prasad et al., 2009).

1.3 LIV-1

1.3.1 LIV-1 belongs to LIV-1 subfamily of ZIP transporters

Zinc is an essential metal for all cells and plays an important role in a variety of physiological and biochemical processes, including gene expression, growth, metabolism, development, and differentiation (Vallee and Falchuk, 1993). Zinc deficiency is associated with diverse disorders, such as impaired immunity, retarded growth, brain development disorders, delayed wound healing, retarded skeletal development, and development of osteoporosis (Andrews and Gallagher-Allred, 1999; Eberle et al., 1999; Hall et al., 1999; Nishi, 1996; Rink and Gabriel, 2000). Therefore zinc homeostasis needs to be tightly controlled. Zinc cannot passively diffuse across cell membranes because of its charges, so two families of mammalian zinc transporters are required to transport zinc across cell membranes: ZnT (Zinc transporter) proteins and the Zip (Zrt and Irt-like) proteins. They have opposite functions in cellular zinc transportation. ZnT transporters are responsible for transporting zinc out of cells to reduce intracellular zinc, whereas ZIP transporters promote zinc uptake to increase intracellular zinc. Normal prostate gland accumulates high levels of citrate and zinc compared to other tissues. Zinc accumulation by the prostate epithelial cells is achieved through the ZIP family of zinc uptake transporters. The ZIP family contains four subfamilies (Guerinot, 2000). Subfamily I is mainly fungal and plant sequences, while subfamily II consists of mammalian, nematode and insect genes. The *gufA* subfamily is related to the *gufA* gene of *Myxococcus Xanthus* which has unknown function, and the LIV-1

subfamily. 15 members of ZIP family proteins have been identified, but only a few of them have been functionally characterized. Among them, hZIP1 has been proposed to be the major zinc transporter for many tissues (Gaither and Eide, 2001; Guerinot, 2000). In addition, hZIP1 has been demonstrated to have constitutive expression in normal prostate cells and function in the uptake and accumulation of zinc in prostate cells (Franklin et al., 2003). Clinical and experimental studies showed that hZIP1 gene expression is down regulated and zinc is depleted in malignant prostate compared to normal prostate (Franklin et al., 2005a). Furthermore, overexpression of hZIP results in decrease of malignancy of prostate cancer cells *in vitro* and *in vivo* (Golovine et al., 2008; Huang et al., 2006a).

LIV-1, which was originally identified as an estrogen-regulated gene in metastatic breast cancer, has been characterized as a new subfamily of ZIP zinc transporters termed LZT (el-Tanani and Green, 1995). Based on a computer analysis of secondary structure, LIV-1 contains 6-8 transmembrane domains, a long extracellular N terminus, a short extracellular C terminus, and a consensus sequence for a catalytic zinc-binding site of metalloproteases (HEXPHE) with a molecular mass of 84 kDa (Taylor et al., 2003; Taylor and Nicholson, 2003) (figure 1.4). The catalytic zinc is required for the proteolytic metalloprotease activity (Massova et al., 1998). Of interest, no other zinc transporters contain this potential metalloprotease motif, suggesting that LIV-1 may have different function other than transporting zinc. In addition, by using V5-tag LIV-1 expression vector, LIV-1 has been shown to locate on the plasma membrane, especially concentrating on lamellipodiae. Moreover, LIV-1 has been demonstrated to be able to transport zinc in human neuroblastoma cells (Chowanadisai et al., 2008).

1.3.2 LIV-1 is associated with EMT and cancer progression

LIV-1 was first identified in the breast cancer cell line ZR-75-1 as an estrogen-regulated gene (el-Tanani and Green, 1995), and is predominately expressed in hormonal controlled tissues with high levels in breast, prostate, pituitary gland and brain (Taylor et al., 2003). In clinical samples, LIV-1 expression correlates with ER α in breast tumor biopsies (Dressman et al., 2001; Tozlu et al., 2006) and is associated with the spread of breast cancer to the regional lymph nodes (Manning et al., 1994), suggesting a role in metastasis.

EMT has been implicated in the progression of many solid tumors, including prostate cancer (Whitbread et al., 2006; Xu et al., 2006; Zhau et al., 2008), and is considered a key molecular event in cancer progression (Thiery, 2003). LIV-1 was reported to be a downstream target of STAT3 and essential for the nuclear localization of Snail in zebrafish gastrula organizing cells for their migration (Yamashita et al., 2004). LIV-1 cooperates with Snail by binding to E-cadherin promoter and repressing its transcription (Batlle et al., 2000). LIV-1 mRNA was recently shown to be higher in cervical cancer in situ than in normal tissues (Zhao et al., 2007a). RNAi mediated suppression of LIV-1 in HeLa cells significantly inhibited their proliferation, colony formation, migratory, and invasive ability (Zhao et al., 2007b). LIV-1 has also been reported to be elevated in clinical pancreatic carcinoma and induced EMT in pancreatic cancer cells (Unno et al., 2009). However, LIV-1 expression was also reported to correlate with E-cadherin expression (Shen et al., 2009) and to be associated with better outcome in breast cancer patients (Kasper et al., 2005). Although results are conflicting, LIV-1 is still thought to be an obligatory co-factor regulating EMT-associated genes. Since the potential diagnostic and prognostic value of LIV-1 in human prostate cancer has not been investigated, the purpose of this study

is to investigate the function of LIV-1 in cancer progression and evaluate the therapeutic potential of LIV-1.

1.4 EGFR (Epidermal growth factor receptor) signaling in cancer

1.4.1 Receptor activation

Epidermal growth factor receptor (EGFR; ErbB1), a 170kDA membrane protein, belongs to the ErbB family of receptor tyrosine kinases (RTKs), which includes HER2 (ErbB2), ErbB3, and ErbB4. These ErbB receptors share a common structure with an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic tyrosine kinase domain. Under unstimulated conditions, the ErbB receptors are present as a monomer. Upon binding of receptor-specific ligands, the receptors undergo dimerization and cause transactivation of the intracellular tyrosine kinase domain in which specific residues are phosphorylated. Subsequently, intracellular signal proteins are recruited by the phosphorylated residues, resulting in the activation of intracellular pathways, including the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3 kinase (PI-3K) pathways, which ultimately modulate gene transcription (Olayioye et al., 2000; Yarden and Sliwkowski, 2001).

Another mechanism to cause ErbB receptor activation is known as receptor transactivation. For example, GPCR agonists, such as endothelin-1, bombesin, and thrombin, could cause receptor activation through stimulating metalloproteinases which in turn cleave EGF-like ligand precursors, leading to phosphorylation of receptors (Carpenter, 2000; Gschwind et al., 2002; Gschwind et al., 2001; Prenzel et al., 1999). In addition, cytokine has been shown to indirectly activate ErbB receptors through janus tyrosine kinase 2 (Jak2) (Yamauchi et al., 1997). Moreo-

ver, wnt signaling has also been shown to be able to induce receptor activation through metallo-proteinase-mediated cleavage of EGF-like ligands (Civenni et al., 2003).

1.4.2 EGFR function in normal development and in cancer

EGFR functions are ubiquitously expressed in multiple cellular processes including embryogenesis, differentiation, survival, proliferation and tumor progression. The importance of EGFR in developmental processes is supported by knockout mouse experiments. EGFR knockout mice are embryonic lethal at day 10.5-13.5 p.c. In addition, null mutation of EGFR causes developmental defects in the skin, lung, pancreas, GI tract and central nervous system (Miettinen et al., 1995; Sibilio et al., 1998; Sibilio and Wagner, 1995; Threadgill et al., 1995). EGFR is important not only because it has essential roles in normal physiological processes during development, but also because it is involved in numerous types of human cancers. The ErbB receptors were found to be dysregulated in several malignant tumors including lung, breast, colon, squamous cell cancer of the head and neck, and prostate cancer (Mendelsohn, 2002; Salomon et al., 1995). Cancer patients whose tumors have increased expression of EGFR or ErbB2 tend to have a more malignant tumors associated with a poor outcome (Allred et al., 1992; Hynes and Stern, 1994; Nicholson et al., 2001; Salomon et al., 1995; Sjogren et al., 1998; Slamon et al., 1987). Aberrant receptor signaling is due to receptor overexpression (Hirsch et al., 2003), receptor mutation causing ligand-independent activation (Moscatello et al., 1995), or autocrine activation by ligand overexpression (Prenzel et al., 1999). Different studies have shown that overexpression of EGFR or ErbB-2 could induce both in vitro and in vivo transformations. It is thought that high levels of the receptor promote spontaneous dimerization, thus leading to constitutive ErbB acti-

vation. Many genetic alterations of ErbB receptors have been demonstrated in human cancers. Among those mutations, the EGFRvIII mutant receptor mutated in the extracellular domain has been found to occur in breast, ovarian, lung, and glioblastoma cancer and is associated with ligand independent EGFR activity (Kuan et al., 2001; Pedersen et al., 2001). Moreover, EGFR agonists, such as EGF, TGF- α , HB-EGF, and amphiregulin, are confirmed by a number of studies to show overexpression of these proteins in a variety of solid tumors (Normanno et al., 2005a; Salomon et al., 1995). For example, TGF- α is frequently coexpressed with EGFR in prostate cancer (Seth et al., 1999), breast cancer (Umekita et al., 2000), lung, ovary, non-small cell lung cancer (Hsieh et al., 2000), and gastrointestinal stromal tumors (Cai et al., 1999). All these alterations contribute to constitutive active ErbB signaling that leads to cancer development.

The ErbB receptors are able to activate different intracellular signaling pathways, including the ras/raf/MAPK, PI3K/Akt, and STAT pathways (Figure 1.5). Activation of these signaling proteins has been shown to regulate cellular functions involved in cancer development and progression (Normanno et al., 2006). It is well known that activated MAPK promotes cell migration. Clinical studies demonstrated that tumors with high levels of active MAPK have a particularly poor prognosis (Feldkamp et al., 1999). Activation of the MAP kinase pathway is associated with increasing prostate cancer Gleason score and tumor stage (Gioeli et al., 1999). Overexpression of Ras enhances androgen hypersensitivity in LNCaP cells (Bakin et al., 2003b). In contrast, overexpression of dominant negative Ras converted androgen-independent cells back to androgen-dependent status in C4-2 prostate cancer cells (Bakin et al., 2003a). The PI3K/Akt pathway is very important in mediating cell survival, since activated PI3K may inhibit proapoptotic molecules resulting in cell survival (Datta et al., 1997). An increase of PI3K has been found in prostate cancer and expression of Akt was much higher in prostate cancer tissue compared to normal

prostate tissue or PIN (Liao et al., 2003). In addition, PTEN, a negative regulator of PI3K signaling, has been identified to frequently mutate and be inactivated in prostate cancers (Li et al., 1997). Stat3 has also been implicated in cell survival. Inhibition of Stat3 in vivo results in a decrease of anti-apoptotic Bcl-X_L and increased cell death (Grandis et al., 2000). Together, numerous data provide strong evidence that dysregulated ErbB signaling plays a crucial role in cancer development and metastasis.

1.4.3 EGFR in angiogenesis

Angiogenesis is essential for tumor growth and metastasis. It has been demonstrated that the ErbB receptor/ligand network regulates the process of neovascularization, in which endothelial cells proliferate and undergo differentiation. On the one hand, endothelial cells themselves express ErbBs. On the other hand, EGFR signaling regulates the production of proangiogenic factors, the most potent being VEGF, in different tumor cells. For example, upon EGF treatment, glioma cells upregulate the secretion of VEGF (Goldman et al., 1993). Conditioned media from EGF-stimulated glioma cells induced tube formation of human umbilical vein endothelial cells (HUVECs), and this effect was blocked by an anti-VEGF antibody. In addition, EGFR activation was shown to regulate VEGF promoter activity in glioblastoma cells through the MAPK and PI3K pathways, which were independent of the hypoxia-induced HIF-1 pathway, a potent inducer of VEGF (Maity et al., 2000). In prostate cancer cells, EGFR was also shown to regulate expressions of angiogenic factors. In particular, EGF significantly increased the secretion of VEGF in prostate cancer cells, and treatment with the EGFR tyrosine kinase inhibitor gefitinib sup-

pressed EGF-induced VEGF expression both in prostate cancer cells cultured in vitro or implanted in the flank of nude mice (Bianco et al., 2004; Sini et al., 2005).

ErbB ligands also have a direct effect on endothelial cells. Using an orthotopic nude mice model, TGF- α -expressing tumor cells directly induced EGFR expression in endothelial cells (Baker et al., 2002). Baker et al. demonstrated that only tumor-associated endothelial cells obtained from EGF/TGF- α positive cancers express functional EGFR, which can be activated upon stimulation with either EGF or TGF- α (Baker et al., 2002). In addition, stimulation of HUVECs with EGF or HB-EGF resulted in an increase of EGFR phosphorylation and ERK activation (Sini et al., 2005). Taken together, evidence supports the idea that tumor cells may induce expression and activation of the EGFR pathways in endothelial cells to promote tumor-associated angiogenesis. Thus, a better understanding the role of the EGFR/ligand network in endothelial cell/tumor cell interactions will provide strategies to inhibit angiogenesis which in turn could block tumor proliferation, survival and metastasis.

1.4.4 EGFR in bone metastasis

The most common metastatic site of prostate cancer is the bone (Li et al., 2006). Nearly 80% of advanced prostate cancer cases result in bone metastasis and generate severe pain and disability (Landis et al., 1999). Bone metastasis results from dysregulation of the bone formation and bone resorption processes. The cells responsible for bone remodeling are osteoblasts, which secrete new bone, and osteoclasts, which dissolve bone matrix. Two factors necessary and sufficient for osteoclast formation and activation are macrophage colony stimulating factor (M-CSF), which promotes proliferation and differentiation of pre-osteoclast cells, and RANKL, which ac-

tivates pre-osteoclasts and is secreted by osteoblasts and bone marrow stroma cells (Boyle et al., 2003). Cancer cells are able to synthesize many growth factors and cytokines which can lead to the activation of osteoclasts (Roodman, 2001). EGFR signaling involvement in the pathogenesis of bone metastasis was demonstrated in a clinical trial of the EGFR inhibitor gefitinib in breast cancer patients (von Minckwitz et al., 2005). A significant improvement in bone pain was observed in patients treated with gefitinib. Evidence showed that both EGF and TGF- α are able to stimulate bone turnover and osteoclastogenesis in different systems (Guise et al., 1993; Ibbotson et al., 1983; Ibbotson et al., 1985; Zhu et al., 2007). EGFR inhibitor treatment inhibits M-CSF and RANKL production in bone marrow stromal cells and thus inhibits osteoclast formation (Normanno et al., 2005b). These data suggests that EGFR signaling regulates the ability of bone marrow stroma cells to induce osteoclastogenesis.

Anti-EGFR inhibitors also have an effect on prostate cancer cells. For example, gefitinib treatment reduced the ability of conditioned media from prostate cancer cells to induce RANKL expression in osteoblasts (Angelucci et al., 2006). In addition, EGFR signaling activates the expression of proteases which play an important role in metastasis. For instance, the urokinase-type plasminogen-activator (uPAR) and matrix metalloproteinases (MMPs) are necessary for the invasive ability of tumor cells and metastasis (Guise and Mundy, 1998; Nemeth et al., 2002). Gefitinib treatment inhibited the expressions of uPAR and MMP-9 in prostate cancer cells, and reduced the metastatic potential of these cancer cells (Angelucci et al., 2006).

1.4.5 EGFR as a therapeutic target

Cancer patients whose tumors show dysregulated EGFR or ErbB-2 tend to have a more aggressive disease and a poor clinical outcome. Because of the importance of ErbB receptors, a huge effort has been made to develop therapies that target ErbBs. Two current successful approaches have been developed: anti-EGFR monoclonal antibodies and EGFR-specific tyrosine kinase inhibitors (TKIs). Anti-EGFR MAbs, such as cetuximab and panitumumab, bind to the extracellular domain of EGFR, thus preventing the binding of EGFR ligands and activating the receptors (Sato et al., 1983). In contrast, TKIs, such as gefitinib and erlotinib, prevent the binding of adenosine triphosphate to the intracellular tyrosine kinase domain, thus inhibiting intracellular tyrosine kinase activity and subsequent signaling (Lichtner et al., 2001).

Both MAbs and TKIs result in decreases in the MAPK, PI3K/Akt, and Jak/Stat pathways. However, anti-EGFR MAbs also cause downregulation of EGFR expression. Cetuximab is a chimeric anti-EGFR MAb which is approved for treating patients with metastatic colorectal cancer refractory to irinotecan-based chemotherapy. Cetuximab has higher affinity toward EGFR compared to TGF- α or EGF and induces EGFR internalization and antibody-dependent cell-mediated cytotoxicity. In preclinical studies in nude mice bearing irinotecan-resistant colorectal tumors, cetuximab exerted strong antitumor activity (Prewett et al., 2002). However, cetuximab only showed a 10-20% response rate in several clinical trials (Cunningham et al., 2004; Saltz et al., 2004). TKIs are low molecular weight synthetic molecules which block the intracellular kinase activity of EGFR. For example, Gefitinib was approved for the treatment of patients with locally advanced or metastatic non-small cell lung cancer after failure of both docetaxel and platinum-based chemotherapies. In phase II clinical trials, Gefitinib treatment showed a significant response rate in patients with advanced non-small cell lung cancer (Fukuoka et al., 2003). In subsequent phase III trials, Gefitinib treatment did not improve overall survival or delay tumor pro-

gression (Giaccone et al., 2004; Herbst et al., 2004). However, Gefitinib did show a significant response rate in a particular population of patients with specific EGFR mutation (Inoue et al., 2009; Inoue et al., 2006). Gefitinib treatment showed a 50-70% responsive rate with great progression-free survival in this group of patients and gefitinib was approved in Europe for treating patients with advanced or metastatic non-small cell lung cancer carrying EGFR mutation.

Anti-EGFR therapy has shown significant responses in some cancers, but no therapeutic response resulted in the majority of cancer patients. In fact, patients initially responsive to anti-EGFR treatment develop drug resistance over time. Potential mechanisms of resistance to EGFR-targeted therapies have been proposed. For example, constitutive activation of downstream signaling of EGFR, such as PI3K mutations and K-ras mutations, has been demonstrated as one of the possible causes (Prenen et al., 2009; Sartore-Bianchi et al., 2009). In addition, activation of an alternative receptor or EGFR mutation are also possible mechanisms for the development of resistance to anti-EGFR therapies. Thus, a better understanding of the molecular mechanisms of resistance to EGFR-targeted therapies will further increase our understanding of EGFR signaling and ultimately improve treatment strategies.

1.5 The Ras/Raf/MEK/ERK cascade in cancer progression

The ERK cascade is activated by a large number of extracellular stimuli. Activation of this cascade controls a variety of cellular processes, including proliferation, differentiation, development, cell survival, migration, apoptosis, and oncogenic transformation (Seger and Krebs, 1995; Torii et al., 2004; Viala and Pouyssegur, 2004; Yoon and Seger, 2006). Signaling is usually initiated by activation of a small G protein, Ras, which transmits the signal further by recruit-

ing Raf kinase to the plasma membrane (Wellbrock et al., 2004) (Figure 1.6). Once Raf is activated, Raf transmits signaling by phosphorylating MEK and activates MEK (Ahn et al., 1991). Upon activation, MEKs act as dual specific kinases and phosphorylate the Tyr and Thr residues of ERKs, leading to ERKs activation (Seger et al., 1992). Once phosphorylated by MEKs, ERKs become a potent protein kinase and are able to phosphorylate a large number of downstream targets (Yoon and Seger, 2006). In particular, ERKs phosphorylate and activate a series of transcription factors, such as c-fos (Murphy et al., 2002), c-jun (Morton et al., 2003), p53 (Milne et al., 1994), Elk1 (Gille et al., 1992), and Ets1/2 (Yang et al., 1996), which play critical roles in the initiation and regulation of proliferation and oncogenic transformation. The inactivation of ERKs is mainly mediated by protein Ser/Thr phosphatases or MAPK phosphatases (Sun et al., 1993). MAPK phosphatases inactivate MAPKs by simultaneously removing phosphates from both Tyr and Thr residues, thus terminating the signaling cascade.

1.5.1 ERK1/2 is necessary for cell proliferation

ERK1 and ERK2 were originally identified as mitogen-stimulated phosphoproteins of 41-45 kDa (Cooper et al., 1984; Nakamura et al., 1983). The direct involvement of ERK1/2 in the mitogenic response was shown by the inhibition of ERKs. Overexpression of dominant negative ERK1 or ERK1 siRNA inhibited fibroblast cell proliferation (Pages et al., 1993). In addition, treatment with MEK1/2 inhibitors which prevent ERKs from activation was shown to reduce the proliferation of various cell types, including fibroblasts, T lymphocytes, smooth muscle cells, hepatocytes and epithelial cell lines (Brunet et al., 1994; DeSilva et al., 1998; Dudley et al., 1995; Karpova et al., 1997; Sebolt-Leopold et al., 1999; Seufferlein et al., 1996; Talarmin et al., 1999;

Williams et al., 1998). In contrast, overexpression of constitutive active MEK1 led to increases in cell proliferation (Brunet et al., 1994; Cowley et al., 1994). The role of ERK1/ERK2 in the regulation of cell proliferation was further demonstrated by knockout mice experiments. Analysis of *Erk1*^{-/-} mice revealed that ERK1 is not required for embryonic development and does not affect cell proliferation rate. However, *Erk2*^{-/-} mice showed a defect in tropoblast development, resulting in embryonic lethality.

1.5.2 The ERK pathway in cancer

The ERK pathway is the best studied of the mammalian MAPK pathways, and dysregulation of ERK signaling has been found in approximately one-third of all human cancers. Besides a basic role in cell proliferation, ERK signaling is involved in many other aspects of tumorigenesis, so the signals which activate ERK are of particular interest. ERK signaling could be activated by a variety of extracellular stimuli. Most cancer-associated lesions show constitutive activation of ERK signaling due to overexpression of receptor tyrosine kinases, activating mutations in receptor tyrosine kinases, sustained autocrine or paracrine production of ligands, Ras mutations and Raf mutations.

Aberrant overexpression or mutation of receptor tyrosine kinases can cause activation of Ras leading to upregulated MAPK signaling (Lynch et al., 2004; Stephens et al., 2004). In particular, EGFR has been found to be overexpressed or mutationally activated in many human cancers (Grandis and Sok, 2004). In addition, increased EGFR ligands, such as TGF- α , HB-EGF, and amphiregulin, could cause constitutive activation of EGFR, which in turn stimulates ERK signaling (Gangarosa et al., 1997; McCarthy et al., 1995). Increased expression of EGFR ligands

has been observed in a variety of Ras- or Raf-transformed cell types. Moreover, approximately 30% of human cancers showed aberrant Ras expressions resulting from amplification of ras oncogene or activating mutations (Flotho et al., 1999; Stirewalt et al., 2001). Recently, it was shown that B-raf is frequently mutated in certain cancer types, especially melanoma (27-70%), papillary thyroid cancer (36-53%), colorectal cancer (5-22%) and ovarian cancer (30%) (Davies et al., 2002; Garnett and Marais, 2004; Libra et al., 2005). The most common B-Raf mutation is B-Raf (V600E) in which nucleotide 600 changes from valine to glutamic acid (Garnett and Marais, 2004). This B-Raf mutation accounts for 90% of the B-Raf mutations found in melanoma and thyroid cancer and causes constitutive activation of B-Raf, which leads to downstream MEK and ERK activation.

1.5.3 ERK in cell migration and invasion

Cancer cells need to gain migratory and invasive ability to successfully metastasize to distant sites. Previous studies indicated that ERK can phosphorylate and activate myosin light-chain kinase leading to increased phosphorylation of the myosin light chain and enhanced cell motility (Klemke et al., 1997). Many growth factors have been demonstrated to enhance cell migration by activation of receptor tyrosine kinase involving the Ras/MAPK signal pathway (Hartmann et al., 1994; Klemke et al., 1994). Another important target of ERK involvement in cell migration is Rho signaling. ERK may promote a more malignant phenotype by dysregulating Rho signaling. For example, ERK upregulates the AP-1 transcription factor which controls Rho signaling to promote cell migration (Vial et al., 2003).

Gaining the ability to invade the surrounding basement membrane is another key event during cancer progression. MAPKs have been shown to be able to regulate the expression of proteases responsible for basement membrane degradation. In response to extracellular stimuli, phospho-ERK activates some transcription factors, including AP-1. The promoters of many proteases such as MMP-1, MMP-3, MMP-7, MMP-9, MMP-11, MMP-13, MMP-19, and uPA contain AP-1 response elements. Because of its ability to degrade ECM, all these proteases have been implicated in cancer progression (Brogley et al., 1999; Gum et al., 1996; Kondapaka et al., 1997; Mazumdar et al., 2001; Overall and Lopez-Otin, 2002; Reddy et al., 1999; Ree et al., 1998). Furthermore, angiogenesis has been linked with the ERK pathway. In a mouse model, oncogenic Ras expression caused an increase of VEGF expression, which promotes angiogenesis and tumor maintenance (Chin et al., 1999). ERK also was found to directly activate HIF-1 α and Sp1, which were shown to induce VEGF expression (Richard et al., 1999). Because of the importance of ERK signaling, the ERK pathway has been intensely studied. A better understanding of this signaling process will be the key to novel strategies and new therapies.

1.6 Signal transducer and activator of transcription 3 (Stat3)

STATs are a family of cytoplasmic transcription factors which transmit signals to the nucleus where STATs bind to specific DNA promoter sequences and regulate gene transcription. These transcription factors are activated by a series of extracellular signals such as cytokine, growth factors, and hormones that bind to specific cell-surface receptors. Upon stimulation, STATs become activated typically through tyrosine phosphorylation by Janus kinases (JAKs) or growth factor receptor tyrosine kinases such as EGFR and PDGF. STATs have also been demon-

strated to be activated through non-receptor tyrosine kinases, such as v-src, c-src, and abl. Once activated, phosphorylated STATs form homodimer or heterodimer and go into the nucleus to regulate gene expression.

1.6.1 The mechanisms of STAT activation

Cytokines. STAT proteins are involved in signaling by many cytokines, such as IL-6 family cytokines. For example, IL-6 first binds to and activates its receptors, causing receptor aggregation. IL-6 receptors, which lack intrinsic tyrosine kinase activity, recruit JAKs to the receptors (Ihle, 1996). JAKs not only phosphorylate themselves to become activated, but also phosphorylate the C-terminal of the receptors. The receptor phosphotyrosines serve as a docking site for recruitment of STATs (Seidel et al., 1995). STATs in turn are phosphorylated by JAKs and then phosphor-STATs form either homo- or heterodimers to translocate to the nucleus, where they bind to the promoters of target genes and induce gene expression.

Growth factors. STATs can also be activated by numerous growth factors, including EGF and PDGF. Activated EGFR has been shown to be able to directly phosphorylate STAT proteins. In most cases, c-Src is involved in STAT activation by EGF and PDGF receptors (Chaturvedi et al., 1998; Chaturvedi et al., 1997; Olayioye et al., 1999; Wang et al., 2000). Inhibition of c-Src abrogates EGF or PDGF induced Stat3 phosphorylation, indicating that c-Src is required for EGF or PDGF-mediated Stat3 activation. In addition, EGFR-mediated growth of transformed epithelial cells is dependent on the activation of Stat3. Inhibition of Stat3 by siRNA or dominant negative Stat3 suppressed EGF-induced cell growth (Grandis et al., 1998). It has been shown that

constitutive active Stat3 correlates with EGFR signaling in breast cancer cells, and inhibition of Stat3 resulted in apoptosis of breast cancer cells (Garcia et al., 2001; Garcia et al., 1997).

1.6.2 Stat3 in cancer development and progression

The STAT family consists of seven different members: STAT1, 2, 3, 4, 5A, 5B, and 6. Aberrant STAT signaling, in particular Stat3 and Stat5, results in the development and progression of human cancers such as prostate, breast, pancreas, brain, lung, and head and neck cancer (Bowman et al., 2000; Catlett-Falcone et al., 1999; Coffey et al., 2000; Garcia et al., 2001; Lin et al., 2000; Song and Grandis, 2000), by either preventing apoptosis, inducing cell proliferation, or both (Herrington et al., 2000). Stat3^{-/-} knockout mice resulted in embryonic lethality, suggesting its role in early embryonic development. Tissue-specific gene deletions have shown that Stat3 plays a critical role in the regulation of epithelial cell apoptosis, skin remodeling, macrophage inactivation, and cell growth. Constitutively activated STAT3 has been demonstrated to be able to induce transformation, and is associated with tumor development and progression (Bowman et al., 2000). Tumorigenesis is a complex process involving a balance of several events, including escape from contact-mediated growth arrest, inhibition of apoptosis, angiogenesis, increased proliferation and enhanced migratory and invasive potential (Hahn and Weinberg, 2002). Studies have shown that Stat3 could regulate this process through upregulation of anti-apoptosis proteins (Bcl-x, Mcl-1), cell cycle regulator (cyclins D1/D2, c-Myc), and angiogenic factors (VEGF) both in vitro and in vivo (Sinibaldi et al., 2000; Yu and Jove, 2004).

The persistent activation of Stat3 is frequently observed in prostate cancer as well as other solid tumors. Activated Stat3 is found in both primary human prostate cancer samples and prostate cancer cell lines (Dhir et al., 2002; Mora et al., 2002; Ni et al., 2002). Stat3 activation depends mainly on deregulation of the tyrosine receptors or associated JAKs. The inhibition of Stat3 reduced proliferation and induced apoptosis (Gao et al., 2005). Studies of constitutively active Stat3 have shown that overexpression of constitutively active Stat3 leads to anchorage-independent growth as well as androgen-independent growth in prostatic epithelial cell lines (DeMiguel et al., 2002; Huang et al., 2005). Because of its important role in tumorigenesis, Stat3 has been identified as a new anticancer target. Studies in cell culture and animal models established that Stat3 is a promising therapeutic target in a variety of human cancers (Bowman et al., 2000; Turkson and Jove, 2000). Inhibition of Stat3 signaling has been demonstrated repeatedly to cause growth inhibition and apoptosis in tumor cells harboring constitutive active Stat3 (Cattlett-Falcone et al., 1999; Lin et al., 2000).

1.7 Matrix metalloproteinase 2/9 (MMP2/9)

1.7.1 MMP2/9 in cancer progression

The MMPs are a family of highly conserved zinc-dependent endopeptidases, which are able to degrade most components of the basement membrane and extracellular matrix. MMPs, which are produced by both cancer cells and stromal cells, play a central role in cancer progression by proteolyzing ECM and allowing tumor cells to escape from the primary site and invade the vascular and lymphatic systems (Chambers et al., 2002; Mareel and Leroy, 2003). Enhanced levels of MMPs are now considered to be characteristic of most malignant tumors, and in some

carcinomas specific MMPs have been shown to be significant prognostic indicators (Liabakk et al., 1996; Murray et al., 1998; Murray et al., 1996; Sier et al., 1996; Talvensaaari-Mattila et al., 1998). Angiogenesis, the formation of new blood vessels, requires proliferation of endothelial cells from preexisting blood vessels, breakdown of extracellular matrix and migration of endothelial cells. Because ECM proteolysis is a required process for angiogenesis, MMPs also play a critical role during angiogenesis. For example, tumor angiogenesis and tumor growth in MMP-2-deficient mice is highly reduced (Itoh et al., 1998). In addition, MMPs was shown to be involved in regulating tumor growth in both primary and secondary tumors. For example, MMPs were shown to degrade insulin-like growth factor binding proteins. The degradation led to the release of more IGFs, thus contributing to the growth-regulatory functions of MMPs (Fowlkes et al., 1995).

MMPs have also been implicated in EMT, a hallmark of cancer metastasis. Activation of growth factors and cleavage of adhesion molecules are the potential mechanisms underlying MMP-induced EMT. Among all the MMPs, MMP9 was shown to induce cell migration and invasion in both physiological and pathological processes (Freije et al., 2003; Fridman et al., 2003; Himelstein et al., 1994). Secretion of MMP-9 can be triggered by a variety of factors, such as cytokines and growth factors. For example, EGF stimulates MMP-9 secretion in breast cancer cells (Kondapaka et al., 1997). TGF- β , a potent EMT inducer, also activates MMP-9 in breast and prostate cancer cells (Samuel et al., 1992; Sehgal and Thompson, 1999; Welch et al., 1990). In addition, overexpression of Snail, a master regulator of EMT, also induced MMP-9 transcription expression in MDCK cells, suggesting a role in regulating EMT (Jorda et al., 2005; Kondapaka et al., 1997).

In prostate cancers, the gelatinases (MMP-2 and MMP-9) have been found to be specifically associated with prostate cancer metastasis. Increased levels of MMP-2 and MMP-9 in the serum and urine have been correlated with metastasis in prostate cancer patients (Gohji et al., 1998; Moses et al., 1998; Zhang et al., 2004). Secretion of MMP-2 and MMP-9 induce tumor angiogenesis in prostate cancer cells (Stearns et al., 1999; Wood et al., 1997). In addition, increased expression of MMP-2 and MMP-9 along with decreased E-cadherin expression at biopsy could predict advanced prostate cancer at radical prostatectomy (Kuniyasu et al., 2000; Kuniyasu et al., 2003). Moreover, studies showed that the expression of activated MMP-2 was undetectable in normal prostate, benign PIN, and prostate cancer with low Gleason score. Activated MMP-2 was detected in prostate cancers with higher Gleason score and in lymph node metastases (Stearns and Stearns, 1996).

1.7.2 The regulation of MMP2/9

Transcriptional regulation. MMP-2 and MMP-9 are similar enzymes, but have different regulation, glycosylation, proenzyme activation and substrate specificity. MMP-2 is a 72-kDa nonglycosylated protein, whereas MMP-9 is a 92-kDa glycosylated protein (Kotra et al., 2002). MMP-9 is known to be inducible and under the control of growth factors, chemokines and other stimulatory signals (Hipps et al., 1991). There are two important binding sites within MMP-9 promoter: the Activating Protein-1 (AP-1) site and the Polyoma Enhancer Activator (PEA3) site. Cytokines and growth factors that activate MMP-9 expression act through a MAPK pathway, such as ERK and JNK. The inducers include EGF, PDGF, HGF, bFGF, TGF- α , TNF- α , IL-1 β , and IFN- γ . The AP-1 site is located approximately 70 bp upstream of the transcriptional activa-

tion site and is required for the transcriptional activation of the promoter (Benbow and Brinckerhoff, 1997; Crawford and Matrisian, 1996). In addition, the PEA3 site binds members of the Ets family transcription factors. The PEA3 site was found to be necessary for basal transcription and transactivation by cytokines and growth factors. It has been demonstrated that the ETS and AP-1 binding sites cooperate to enhance transcription, suggesting a synergistic effect between these two sites (Crawford and Matrisian, 1996).

Proenzyme activation. The gelatinases are synthesized as inactive proenzymes, and need to be activated for fully catalytic activity. Therefore, the activation of proMMP-2 or proMMP-9 is another step in the regulation of MMP activity. The MMPs become catalytically active when the propeptide domain is cleaved. The membrane-type1 MMP (MT1-MMP) is the key molecule involved in proMMP-2 activation. MT1-MMP and MMP-2 have been shown to be co-localized to the plasma membrane of tumor cells (Sato and Seiki, 1996). The activation of proMMP-2 by MT1-MMP has been observed in many tumor types, such as lung, brain, and stomach (Nomura et al., 1995; Sato and Seiki, 1996; Strongin et al., 1993; Tokuraku et al., 1995; Yamamoto et al., 1996). Thus, MT1-MMP activates the proMMP-2 and active MMP-2 in turn activates MMP-9.

1.8 Heparin-binding epidermal growth factor-like growth factor (HB-EGF)

1.8.1 HB-EGF belongs to EGF-like growth factor family

HB-EGF was first identified as a heparin binding molecule secreted by macrophages (Higashiyama et al., 1991). It is a 208 amino acid protein of 20-22 kDa in size. Under physiological conditions, the N-terminal of HB-EGF, which has more basic amino acids, can interact with negatively charged heparin sulfate proteoglycans both on the cell surface and in the extracellular ma-

trix. HB-EGF is a member of the EGF family of growth factors. The EGF family of growth factors is composed of four groups. The first group includes EGF, TGF α , and amphiregulin which specifically bind to EGFR (ErbB1). The second group includes HB-EGF, betacellulin and epiregulin which bind to both EGFR and ErbB4. The other two groups include neuregulins 1-4 (Higashiyama et al., 2008). These EGF family ligands exert their function through binding to their cognate receptors and activating them.

HB-EGF gene expression has been detected in many tissues, including the heart, lung, brain and skeletal muscle (Abraham et al., 1993). HB-EGF is a potent mitogen and chemoattractant for numerous different cell types and is involved in many biological processes, such as skin wound healing (Shirakata et al., 2005), angiogenesis (Ongusaha et al., 2004), development (Iwamoto and Mekada, 2006; Iwamoto et al.), and cell migration and invasion (Rahman et al.). Recently, more and more studies have shown that HB-EGF plays a critical role in cancer growth and cancer progression (Higashiyama et al., 2008; Ongusaha et al., 2004; Wang et al., 2007a; Yagi et al., 2008).

1.8.2 Ectodomain shedding of HB-EGF and EGFR transactivation

HB-EGF is initially synthesized as a membrane-anchored precursor protein-proHB-EGF with a short cytoplasmic tail (Massague and Pandiella, 1993). The membrane-anchored HB-EGF needs to be cleaved by metalloproteases in order to release the mature soluble form of HB-EGF in a process termed "ectodomain shedding" (Goishi et al., 1995). Regulation of proHB-EGF processing is very critical since it controls ligand availability and receptor activation. This step is also crucial for cancer development and cancer progression because many proteases which

cleave proHB-EGF are dysregulated and EGFRs are often constitutively activated in cancers (Hynes and Lane, 2005; Mosesson and Yarden, 2004; Murphy, 2008).

Ectodomain shedding of HB-EGF can be stimulated by various stimuli, such as GPCR ligands (Prenzel et al., 1999), growth factors (Murphy, 2008), cytokines and cellular stress caused by reactive oxygen and osmotic shock (Kim et al., 2005; Krieg et al., 2004; Tanida et al., 2004). These shedding stimuli cause release of soluble mature HB-EGF, leading to EGFR trans-activation and subsequent intracellular signaling activation. Most proteases implicated in ectodomain shedding belong to the disintegrin and metalloproteases (ADAMs) family or the matrix metalloproteases (MMPs) family and have been associated with different types of tumors (Higashiyama, 2004; Higashiyama and Nanba, 2005). For example, ADAM17, one of the major proteases responsible for ectodomain shedding, is found to be upregulated in a range of tumor types (Sunnarborg et al., 2002; Yin and Yu, 2009). In addition, MMP2 and MMP9, well documented proteases which are upregulated in various tumors and involved in cancer metastasis, have also been shown to be involved in the ectodomain shedding of HB-EGF (Lucchesi et al., 2004; Roudabush et al., 2000).

The ectodomain shedding of pro-HB-EGF produces two fragments: soluble extracellular HB-EGF and a c-terminal intracellular fragment. Soluble extracellular HB-EGF exerts its function in an autocrine or paracrine manner through binding to EGFRs and transactivating them. Mice expressing non-cleavable HB-EGF showed severe developmental abnormalities, suggesting that soluble HB-EGF mediates major functions (Yamazaki et al., 2003). Recently, studies showed that the HB-EGF c-terminal fragment also has functionality. The HB-EGF c-terminal fragment was shown to be able to enter the nucleus to promote S-phase entry by exporting PLZF and Bcl6 which are both transcriptional cell cycle repressors (Nanba et al., 2003).

1.8.3 Expression of HB-EGF is high in human cancer

HB-EGF expression is increased in a lot of different cancer types including bladder (Kramer et al., 2007), breast (Ito et al., 2001c), colon (Ito et al., 2001a), liver (Ito et al., 2001d), ovarian (Tanaka et al., 2005), pancreatic (Ito et al., 2001b) and prostate cancers (Freeman et al., 1998), compared with normal tissues. Tumors isolated from ovarian cancer patients showed high levels of HB-EGF as did ascetic fluid acquired from ovarian cancer patients compared to normal samples or patients with benign tumors. Indeed, HB-EGF expression was one or two orders of magnitude higher than other EGF family members in ovarian cancer patients (Miyamoto et al., 2004). In bladder cancer, HB-EGF was also shown to express ten to hundred fold higher than other EGFR ligands (Thogersen et al., 2001). These results indicate that HB-EGF might be the major ligand responsible for EGFR activation, at least in ovarian and bladder cancers.

1.8.4 HB-EGF promoter regulation

The HB-EGF gene is expressed in a variety of cells and tissues and its expression has been shown to be induced by a wide range of signals, such as growth factors, tumor necrosis factor α , phorbol ester, thrombin and angiotensin II. HB-EGF gene is highly expressed in smooth muscle cells and MyoD, AP-1 and Ets-2 have been implicated in stimulating HB-EGF mRNA expression through binding to its promoter region during myogenesis, oxidative stress and oncogenic raf (Chen et al., 1995; McCarthy et al., 1997; Sakai et al., 2001). In addition, regulatory macrophages upregulate HB-EGF mRNA transcription through Sp-1 transcription factors (Ed-

wards et al., 2009). Knockdown of Sp-1 abrogates HB-EGF expression by regulatory macrophages.

1.8.5 HB-EGF is involved in the malignant phenotype of tumors

Since HB-EGF expression is higher in tumors, HB-EGF has been studied for its potential role in cancers. It is well demonstrated that HB-EGF can promote cell proliferation and might increase the proliferative potential of tumor cells. Overexpression of HB-EGF in normal rat kidney cells or chicken embryo fibroblasts induces oncogenic transformation and anchorage-independent growth (Fu et al., 1999; Harding et al., 1999). In addition, blocking HB-EGF by anti-HB-EGF antibodies reduced proliferation in human glioblastoma cells (Mishima et al., 1998). Overexpression of HB-EGF in human ovarian cancer cells enhanced tumor formation, and knockdown of HB-EGF negated tumor formation completely (Miyamoto et al., 2004).

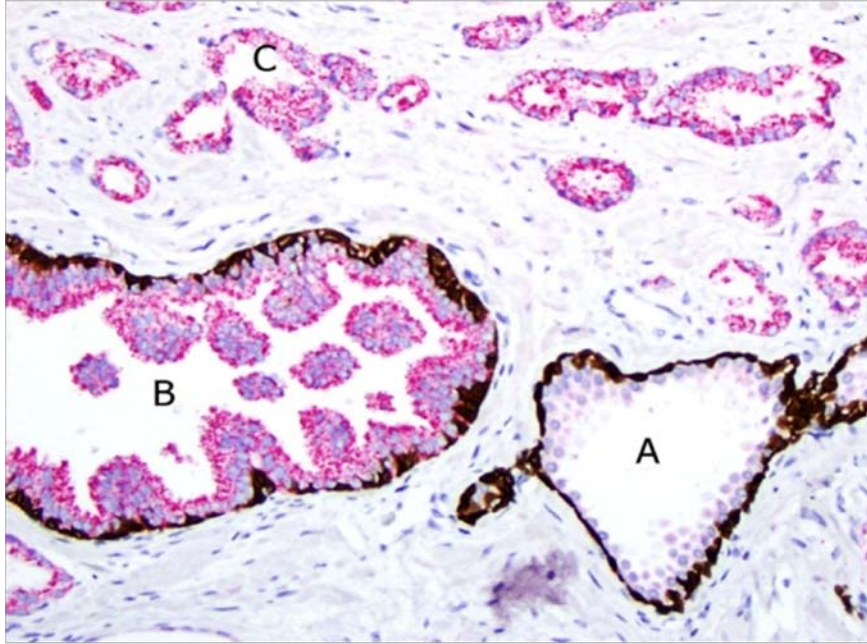
Soluble HB-EGF has been shown to promote cell migration. Expression of soluble HB-EGF, but not non-cleavable HB-EGF enhanced MDCK cell motility (Singh et al., 2004). HB-EGF has also been shown to promote prostate cancer cell migration and invasion (Madarame et al., 2003). Recently, studies demonstrated that HB-EGF is involved in EMT in gastric and ovarian cancer cells (Yagi et al., 2008; Yin et al.). Treatment with recombinant HB-EGF protein in ovarian cancer cells decreased E-cadherin expression but increased Snail expression (Yagi et al., 2008). In addition, overexpression of non-cleavable HB-EGF increased E-cadherin expression

and decreased cell motility in pancreatic cancer cells (Wang et al., 2007a). In summary, HB-EGF is involved in cancer cell proliferation, migration and invasion.

1.8.6 HB-EGF as a therapeutic target

Since HB-EGF is involved in tumor development, migration and invasion, many attempts have been made to target HB-EGF as an anti-cancer treatment. CRM197 is a non-toxic mutant of diphtheria toxin used as an antitumor drug in clinical trials (Buzzi, 1982; Uchida et al., 1971). CRM197 can bind to both soluble HB-EGF and proHB-EGF and then prevent HB-EGF binding to EGFRs to stimulate a mitogenic effect (Mitamura et al., 1995). In ovarian cancers, HB-EGF is the predominantly expressed EGF ligand. Ovarian cancer tumor growth in xenografted mice was significantly suppressed by CRM197 (Miyamoto et al., 2004). In addition, CRM197 has shown enhanced bioactivity in combination with conventional chemotherapeutic agents (Sanui et al., ; Yagi et al., 2009).

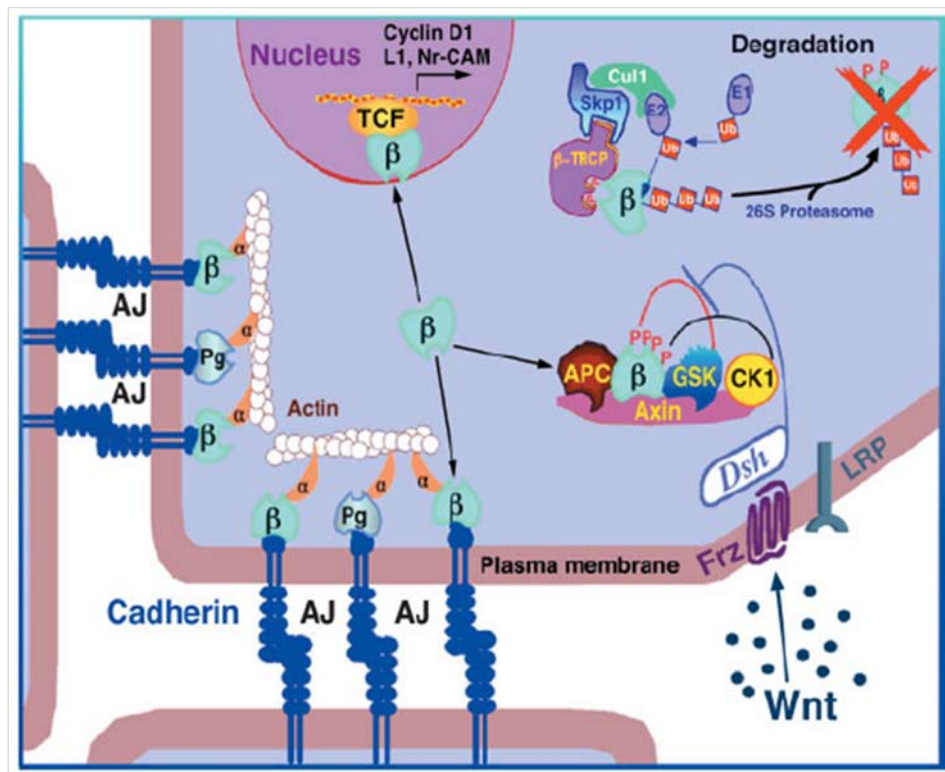
Another strategy is to inhibit HB-EGF ectodomain shedding. Several small molecule inhibitors targeting metalloproteases have been developed to inhibit proHB-EGF shedding. Although these inhibitors do not specifically inhibit HB-EGF alone, but inhibit all ErbB ligands, they did show tumor growth suppression and induction of apoptosis. Therefore, targeting HB-EGF may be worth trying as a strategy for anticancer treatment.



Debra L. Zynger and Ximing Yang, 2000

Figure 1.1

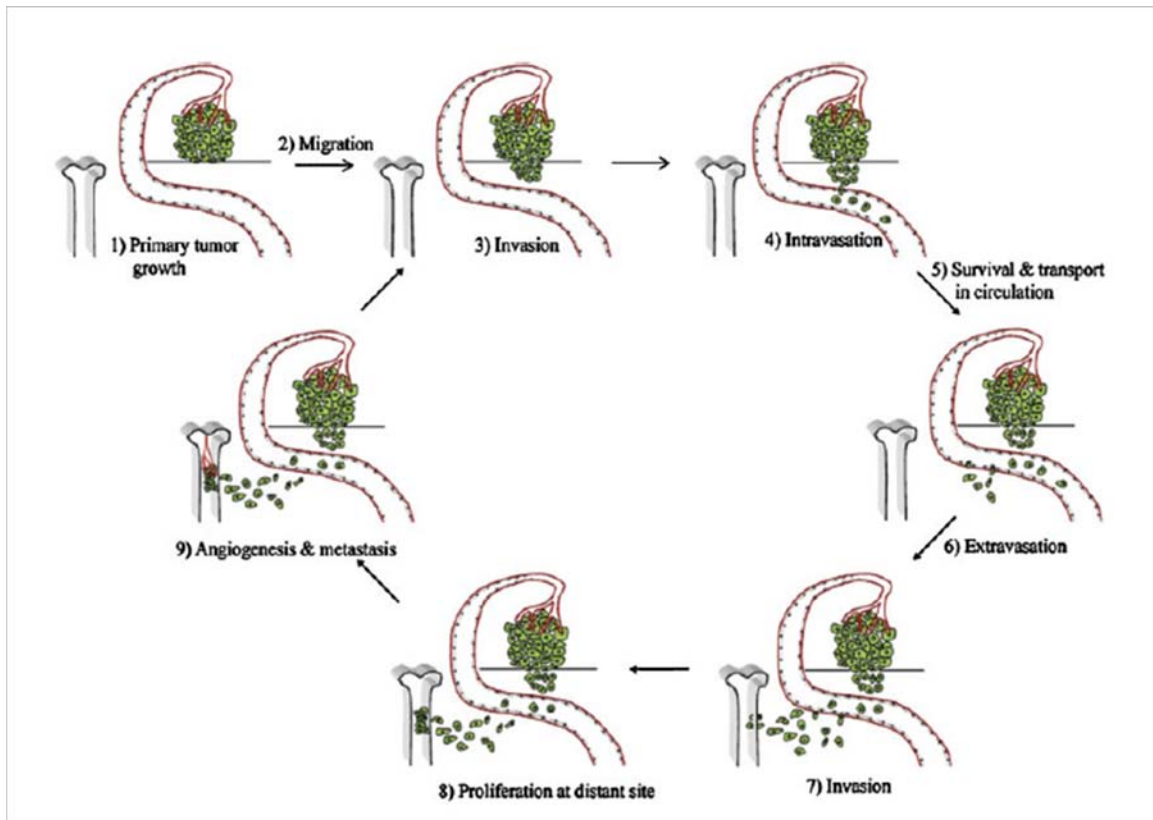
Triple antibody staining (AMACR, p63 and HMWCK). **A.** Benign gland with basal cell staining (brown) and minimal AMACR staining (red). **B.** HGPIN gland with both basal cell staining (brown) and strong AMACR staining (red) in neoplastic cells. **C.** Adenocarcinoma with no basal cell staining but strong AMACR staining (red only).



Gavert N. et al, 2007

Figure 1.2

Wnt/β-catenin signaling in cancer.



Khamis et al, 2011

Figure 1.3
Steps of metastasis.

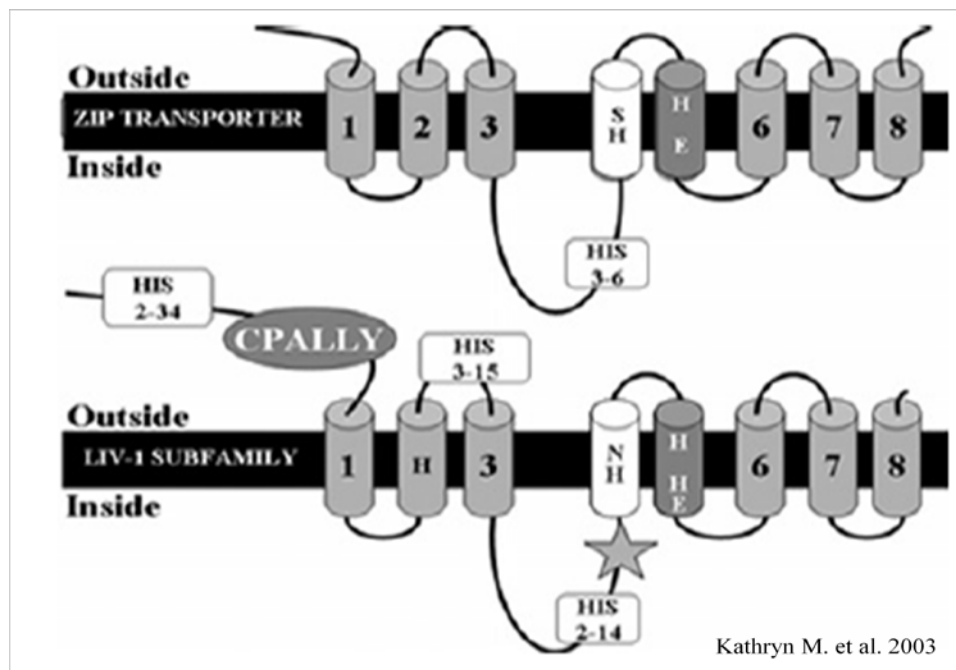
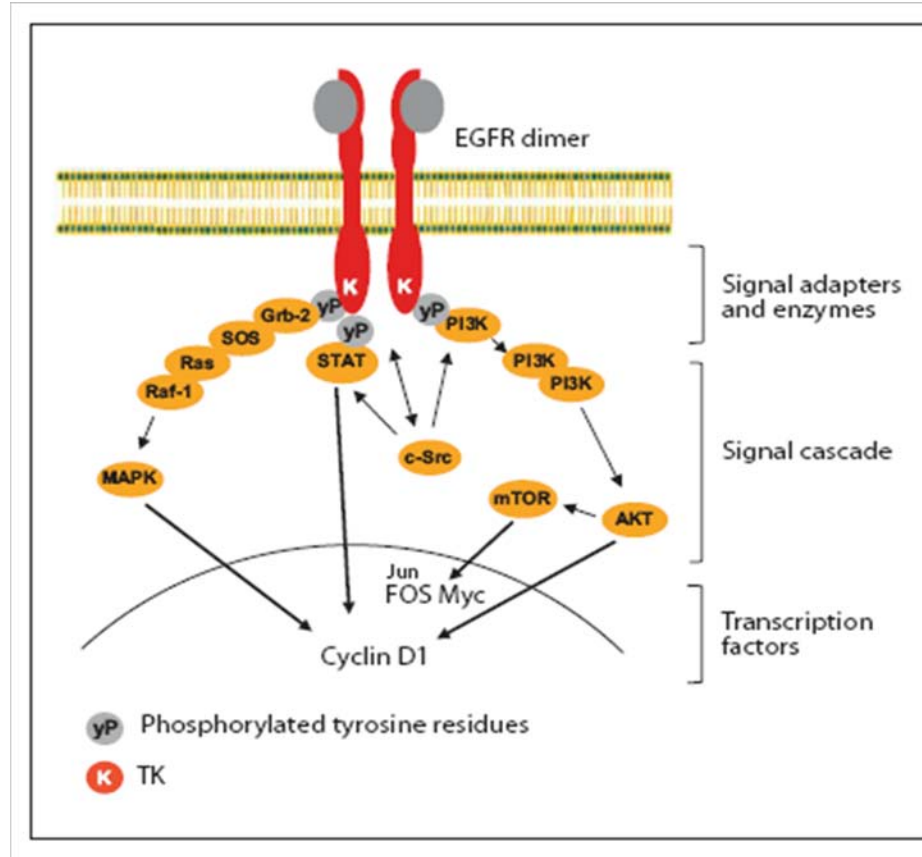


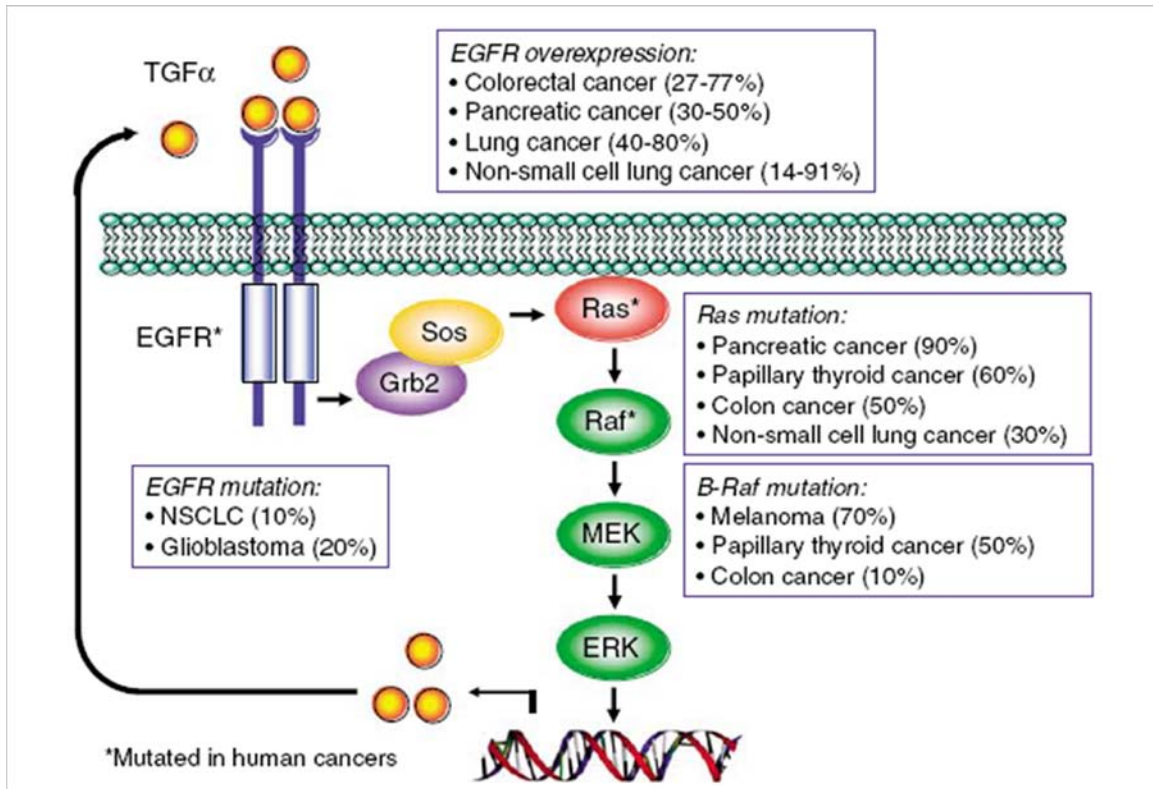
Figure 1.4

Secondary structure analysis of LIV-1.



Georg L. et al. 2009

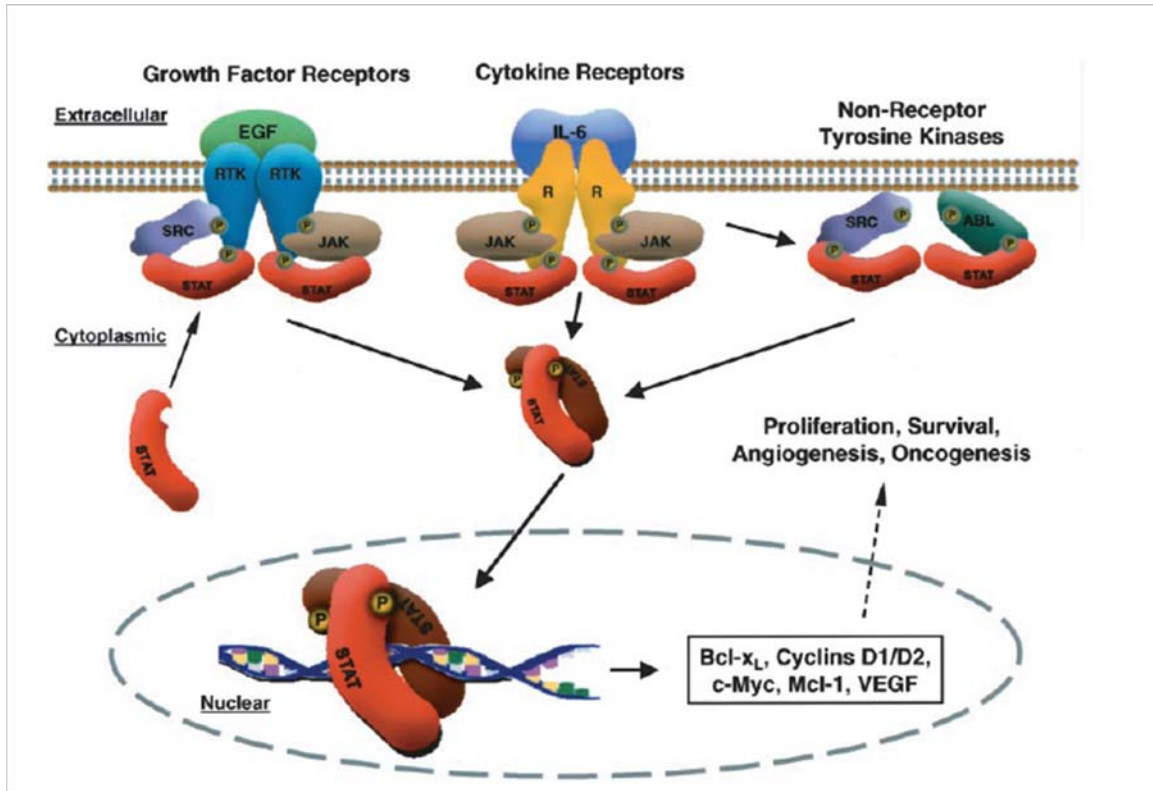
Figure 1.5
EGFR signaling pathways.



Roberts PJ et al. 2007

Figure 1.6

Ras/Raf/MEK/ERK signaling in tumors.



Ralf B. et. al. 2002

Figure 1.7

Multiple ways lead to activate Stat3.

2 LIV-1 PROMOTES HUMAN PROSTATE CANCER EPITHELIAL-TO-MESENCHYMAL TRANSITION AND SKELETAL AND SOFT TISSUE METASTASES

2.1 Abstract

Metal transporter LIV-1 is an effector molecule downstream from soluble growth factors, promoting epithelial to mesenchymal transition (EMT). We examined the role of LIV-1 in promoting prostate cancer progression and metastasis using the human prostate cancer ARCaP EMT model. Upon overexpressing LIV-1 in ARCaP_E cells (ARCaP cells expressing epithelial phenotype), we observed a permanent transition of ARCaP_E to mesenchymal phenotype, with mesenchymal stromal morphology and expression of stromal markers N-cadherin and Snail, but loss of epithelial E-cadherin. Importantly, LIV-1-overexpressing ARCaP_E cells exhibited increased migratory and invasive potential *in vitro* and enhanced subcutaneous tumor formation *in vivo*, while intracardiac inoculation resulted in marked bone and soft tissue metastasis in athymic mice. In addition, we found that LIV-1 level in clinical human prostate cancer specimens is abnormally elevated, with the highest expression seen in bone metastatic tumor. Our study supports the concept that LIV-1 is an important downstream effector to soluble growth factors, promoting EMT and prostate cancer growth and metastasis. LIV-1 can be considered as a novel target for future therapeutic development.

2.2 Introduction

Epithelial to mesenchymal transition (EMT) is a common feature of cancer where tumor cells acquire increased growth, survival, migratory, invasive and metastatic behaviors as the disease progresses. This is probably due to a reawakening of the biologic functions of embryonic tissues during development and normal tissue repair upon wound healing (Acloque et al., 2009; Dvorak, 1986; Micalizzi and Ford, 2009). Through EMT, embryonic cells gain invasive and migratory potential for organogenesis and cytodifferentiation. Recapitulating these embryonic processes, pathologic EMT is known to promote fibrosis, inflammation, and cancer cell migration, invasion and metastasis (Acloque et al., 2009; Thiery et al., 2009). EMT in solid tumors including prostate cancer is considered as a key molecular event in cancer progression (Whitbread et al., 2006; Xu et al., 2006; Zhau et al., 2008).

At the cellular level, EMT precedes the loss of epithelial cellular polarity, causing detachment of epithelial cells from their primary site and increasing their migratory and invasive properties. These changes ultimately accelerate the dissemination of cancer cells to distant organs. In both normal development (*e.g.* metazoan gastrulation and neural crest development) and neoplastic progression, epithelial cells reach secondary sites and re-establish cell polarity, with increased cell adhesion and interaction with resident cells. This phase of the process is indicative of a reversal of EMT, or mesenchymal to epithelial transition (MET) (Duband et al., 1995; Hay and Zuk, 1995). Due to the promotion of interaction with resident cells, cancer cell MET may lead to cancer-associated bone turnover and tumor angiogenesis, with devastating effects in cancer-bearing hosts (Baum et al., 2008; Kim and Salgia, 2009). The cancer-host interaction enhances regenerating ability (or stemness) of cancer cells thus promoting tumor growth, survival, and leading to potential secondary cancer metastases (Hugo et al., 2007; Spaderna et al.,

2007; Thompson and Williams, 2008). Delineating the molecular mechanism of EMT and MET is critical to developing effective therapies for prostate cancer progression and bone metastasis.

LIV-1 is a candidate regulator of EMT and MET processes. As the prototype of the LIV-1 subfamily of ZIP metal transporters (Taylor, 2000; Taylor et al., 2003), it represents a group of proteins that are similar to ZIP transporters in secondary structure and in their ability to transport metal ions. Originally identified in the breast cancer cell line ZR-75-1 as an estrogen-regulated gene (Manning et al., 1988), LIV-1 has been associated with ER α status in breast tumor biopsies (Dressman et al., 2001; Tozlu et al., 2006), and with the spread of breast cancer to the regional lymph nodes (Manning et al., 1994). In cervical cancer, expression of LIV-1 was shown to be higher in tumor cells than in normal tissues (Zhao et al., 2007a). RNAi-mediated suppression of LIV-1 in HeLa cells significantly inhibited cell proliferation and colony formation, leading to reduced migratory and invasive ability (Zhao et al., 2007b). In zebrafish, LIV-1 was reported to be essential for the nuclear localization of Snail, a master transcription factor stimulating EMT, causing migration of the gastrula organizing cells (Yamashita et al., 2004). Additionally, LIV-1 was shown to cooperate with Snail by binding to promoter of the epithelial marker E-cadherin gene to repress its transcription (Cano et al., 2000). It appears that LIV-1 is an obligatory co-factor regulating EMT-associated genes.

Potential biologic functions and the diagnostic and prognostic values of LIV-1 in human prostate cancer have not been investigated. Since zinc plays important roles in the maintenance of prostate epithelial cell homeostasis (Bataineh et al., 2002), and Snail is an master transcription factor controlling EMT in prostate cancer cells (Barrallo-Gimeno and Nieto, 2005; Batlle et al., 2000; Odero-Marrah et al., 2008), LIV-1 may be an active participant in the promotion of EMT during prostate cancer progression and bone metastasis. In this study, we determined the level of

LIV-1 in human prostate cancer cell lines and clinical tissue specimens to define the relationship between LIV-1 expression and human prostate cancer progression and metastasis. The ARCaP human prostate cancer progression cell model was used to evaluate the role of LIV-1 in the induction of EMT. This study found that LIV-1 promotes prostate cancer cell EMT, leading to both bone and soft tissue metastases. In addition, the results from this study demonstrate that molecular suppression of LIV-1 could be a therapeutic strategy to inhibit prostate tumor cell EMT and prostate cancer bone metastasis.

2.3 *Material and Methods*

2.3.1 Ethics statement.

All animal work had been conducted according to relevant national and international guidelines, and was approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University School of Medicine (Permit number 254-2008).

2.3.2 Cell lines and cell culture.

Human prostate cancer ARCaP_E and ARCaP_M cells were established in our laboratory (Xu et al., 2006). The cells were cultured in T-medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA). Human embryonic kidney HEK293 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM (Invitrogen) supplemented with 10% FBS. RPMI-1640 was pur-

chased from Invitrogen (Carlsbad, CA). All the culture media were supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). Cell cultures were maintained at 37°C in a humidified atmosphere supplemented with 5% CO₂.

2.3.3 Antibodies and reagents.

Polyclonal rabbit antibody against LIV-1 was generated in our laboratory. Rabbits were immunized by standard immunization protocol with conjugated peptide KLH-CPDHDS DSSGKDPRNS, corresponding to residues 146-161 of the LIV-1 protein (GenBank accession number NM_012319). Blood was taken 2 weeks after the fourth boost and IgG were purified and tested for specific immune reactivity. Polyclonal antibody to E-cadherin (E-cad), and monoclonal antibody to vimentin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to N-cadherin (N-cad) was from BD Transduction Laboratories (San Diego, CA). Monoclonal antibody to β -actin was from Sigma-Aldrich (St. Louis, MO).

Growth factors of IGF-1 and TGF- β 1 were purchased from Diagnostic Systems Laboratories (Webster, TX). EGF was from R&D Systems (Minneapolis, MN).

2.3.4 Transfection.

Full-length coding region for human LIV-1 cDNA was cloned and confirmed by DNA sequencing. The cDNA was then cloned downstream from a cytomegalovirus early promoter in the mammalian expression vector pcDNA3.1/V5-His (Invitrogen). HEK293 and ARCaP_E cells were seeded at 3×10^5 cells per well in 6-well plates 24 hours before transfection. The cells were transfected with 4 μ g of the LIV-1 expression construct using 8 μ l Lipofectamine 2000 (Invitrogen). To isolate clones stably overexpressing the LIV-1, transfected ARCaP_E cells were treated with G418 (600 μ g/ml) 2 days after the transfection. Four individual clones overexpressing LIV-1 protein (LIV#8, #12, #14 and #17) and two clones transfected with control vector (con1 and con2) were used for the studies.

2.3.5 siRNA knockdown.

LIV-1 siRNA was purchased from Invitrogen. ARCaP_M cells were seeded at 3×10^5 cells per well in 6-well plates for 24 hrs. The cells were transfected with 2.5 μ l of 20 μ M LIV-1 siRNA or equal amount of universal control siRNA, using 8 μ l Lipofectamine 2000 per well. Cells were harvested and assayed 48 hours after transfection.

2.3.6 RNA extraction.

The RNeasy Mini kit (Qiagen, Valencia, CA) was used to extract total RNA from cells. 350 μ l of buffer RLT with 3.5 μ l of β -mercaptoethanol was added to cells grown in a 6-well plate. 350 μ l of 70% ethanol was added to each sample, mixed well and transferred to an RNeasy spin column. Each sample was centrifuged for 30 seconds at 10,000 rpm. Each column was washed with 700 μ l of RW1, and centrifuged for 30 seconds at 10,000 rpm. Then each column was washed with 500 μ l of RPE twice, and centrifuged for 30 seconds at 10,000 rpm. Next, each column was centrifuged for additional 30 seconds at 10,000 rpm and added 30 μ l of RNase-free water to elute RNA. RNA samples were stored at -80°C until use.

2.3.7 Semiquantitative expression analysis with reverse transcription-polymerase chain reaction (RT-PCR).

Total RNA was isolated from cultured cells using the RNeasy Mini kit (Qiagen, Valencia, CA). From each sample, equal amount of RNA (2 μ g) was used in first-strand cDNA synthesis reaction with the Superscript First-Strand cDNA Synthesis kit (Invitrogen). Basically, 2 μ g of total RNA is combined with 1 μ l of 50 ng random hexamer or 50 μ M oligo dT primer and DEPC-treated water to 6 μ l, incubate for 5 minutes at 65°C, and place on ice for additional 5 minutes. Next, 2 μ l of 10 RT buffer, 4 μ l of 25 mM MgCl₂, 2 μ l of 0.1M DTT, 1 μ l of RNaseOUT, and 1 μ l of SuperScript III RT was added to each sample. The samples are incubated at 25°C for 10 minutes, followed by 50°C for 50 minutes, 85° C for 5 minutes, and chilled on ice. Equal volume of cDNA (3 μ l) from each reaction was used for PCR analysis using gene-specific oligonucleotide primer pairs: 5'-GCAATGGCGAGGAAGTTATCT-3' and 5'-CTATTGTCTCTAGAAAGTGAG-3' for LIV-1; 5'-TGCCCAGAAAATGAAAAAGG-3' and

5'-GTGTATGTGGCAATGCGTTC-3' for E-cad; ; 5'-CCATCACTCGGCTTAATGGT-3' and 5'-GATGATGATGCAGAGCAGGA-3' for N-cad; 5'-CGAAAGGCCTTCAACTGCAAAT-3' and 5'-ACTGGTACTTCTTGACATCTG-3' for Snail; and 5'-TTAGCACCCCTGGCCAAGG-3' and 5'-CTTACTCCTTGGAGGCCATG-3' for GAPDH. The reactions were initiated with a 4-minute incubation at 94°C, followed by 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. The reaction was completed with a 7-minute extension at 70°C for 7 minutes. PCR products were visualized after electrophoresis through a 1.2% agarose gel and stained by ethidium bromide (0.5 µg/ml).

2.3.8 Western blotting.

Cells at 80% confluence were lysed in a whole-cell lysis buffer as previously reported (Wang et al., 2007b). The lysates were incubated on ice for 30 minutes and centrifuged at 10,000 rpm at 4°C for 10 minutes. From each sample, 35 µg protein in the supernatant was resolved by SDS-PAGE and blotted onto a nitrocellulose membrane (BioRad, Hercules, CA), which was blocked in 5% skim milk in PBST (137 mM NaCl, 12 mM phosphate, 2.7 mM KCl, and 0.1% Tween 20) at room temperature for 20 minutes; and incubated with primary antibody at 4°C overnight. The membranes were then washed three times in PBST, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. After five washings in PBST, specific signal was detected by incubating the membrane with ECL reagent (Amersham-Pharmacia Biotech, Piscataway, NJ). Exposure time might vary depending on the intensity of signal. After exposure, the membrane was stripped by stripping buffer (Pierce) and reprobed with different primary antibodies.

2.3.9 Scratch wound healing assay.

ARCaP_M cells transfected transiently with LIV-1 siRNA or universal siRNA control were used to determine migratory behavior. Gently and slowly scratch the cells with a 10 μ l pipette tip across the center of the well 48 hours after transfection. After scratching, gently wash the well twice with medium to remove the detached cells. Replenish the well with fresh medium. Photos were taken after scratch at 0 and 48 hrs later. Multiple views of each well were documented, and three independent experiments were performed.

2.3.10 Trans-well migration and invasion assays.

To perform a trans-well migration assay, 2.5×10^4 cells in the top chamber of 24-well trans-well plates of 8 μ m pore size (BD Biosciences) were incubated for 16 hours in complete medium that was added to the bottom chamber. Cells were then fixed with formalin and stained with 0.5% crystal violet. The non-migrated cells inside the chamber were removed by swabbing. Crystal violet of the migrating cells was solubilized into the Sorenson's buffer (0.1 M sodium citrate and 50% ethanol, pH 4.2) and was measured for absorbance at OD₅₉₀. Invasion assay was performed using BD BioCoat Matrigel invasion chambers (BD Biosciences; 8- μ m pore size). The same procedures described above were used, except the filters were pre-coated with 100 μ l Matrigel at a 1 : 4 dilution in RPMI-1640.

2.3.11 Assessment of tumorigenic and metastatic potentials.

The functional roles of LIV-1 in prostate tumor formation and metastasis were assessed as we have reported previously (Thalmann et al., 2000). To assess local tumor growth, 4-weeks old athymic male mice (Ncr-nu/nu, National Cancer Institute, Frederick, MD) were inoculated subcutaneously with ARCaP_E cells (1×10^6 in 50 μ l PBS) stably transduced with LIV-1. Tumor dimension was measured with a caliper at days 23, 32, 43, and 50 after injection, and tumor volume was calculated as length \times width \times height \times 0.5236 (Janik et al., 1975). To assess cancer metastases, athymic male mice were inoculated intracardiacally with ARCaP_E cells (2×10^6 in 100 μ l PBS) stably transduced with LIV-1 to the left ventricles. Animals were observed for 4 months for development of metastatic lesions. Bone metastases were recorded by X-ray radiography and soft tissue metastases was confirmed by histopathology.

2.3.12 Immunohistochemistry (IHC) of tissue microarray (TMA).

The normal and diseased prostate tissues analyzed were from: 1) One custom-made TMA with normal prostate tissues from 4 healthy men; matched cancer, benign, and PIN tissues from 12 prostate cancer cases; matched benign and cancer tissues from 11 cases; matched PIN and cancer from 1 case; benign prostatic hyperplasia (BPH) from 2 cases; and prostate cancer bony metastasis from 3 prostate cancer cases. 2) One custom-made TMA consists 47 bone metastasis tissues from 11 prostate cancer patients; and 3) Four TMAs each containing 66 cases of prostate cancer and benign prostate disorders (US Biomax, Rockville, MD).

The IHC protocol for evaluating gene expression has been reported (Wang et al., 2007b). Briefly, specimens were deparaffinized, rehydrated and subjected to antigen retrieval. After endogenous peroxidase block, the specimens were incubated with primary antibody at 4°C overnight, followed by a 30-minute incubation with DakoCytomation EnVision+ HRP reagent. Signals were detected by adding diaminobenzidine as chromogen and counterstained by hematoxylin. Pre-immunization rabbit serum was served as negative controls. IHC staining was scored by two investigators independently based on four staining intensities from 0 to +++ as previously reported (Wang et al., 2007b).

2.3.13 Statistical analysis.

To analyze the potential association of LIV-1 protein expression and prostate cancer progression from normal/benign, prostatic intraepithelial neoplasia (PIN), localized primary cancer, to bone metastasis, LIV-1 expression level was divided into two categories: staining intensity of high (3) vs. medium to null (2 to 0, respectively). Kruskal Wallis non-parametric test was used to determine the equality of population medians among prostate cancer progressions of normal/benign, PIN, primary cancer, and bone metastasis. This test is equivalent to the parametric ANOVA test used when there are more than two groups were compared. Mann-Whitney non-parametric test was applied to determine the equality of population medians between two cancer progressions, 1) bone metastasis vs. localized cancer; and 2) bone metastasis vs. benign, PIN, and primary localized cancer. This test is equivalent to the parametric t-test used when there are only two groups compared. Logistic regression was used to model the relationship between binary Gleason scores which were divided into binary variables of well differentiated ($GI \leq 6$) vs. moderate to poorly differentiated ($GI \geq 7$) prostate cancer. SAS and Minitab were used in this analysis.

2.4 Results

The human prostate cancer ARCaP cells established in our laboratory (Xu et al., 2006) can be readily promoted to undergo EMT in response to soluble factors and matrix proteins present in the milieu of tumor microenvironment (Graham et al., 2008; Jossion et al., ; Odero-Marah et al., 2008; Zhau et al., 2008). To elucidate the molecular mechanism regulating EMT, the epithelial ARCaP_E was analyzed for differential gene expression, in response to soluble factors, in comparison to its ARCaP_M counterpart, which displayed a mesenchymal stromal phenotype. LIV-1 was one of the differentially expressed genes identified (Zhau et al., 2008). In the current study, we investigated the role of LIV-1 in regulating EMT in ARCaP cells to assess the possible mechanism of LIV-1 action in the promotion of prostate cancer bone and soft tissue metastases.

2.4.1 LIV-1 was involved in promoting EMT in ARCaP cell model.

We have previously reported that ARCaP_E cells underwent EMT when treated with soluble factors including IGF-1, EGF, TGF- β 1 and β -2 microglobulin (β -2M) (Graham et al., 2008; Jossion et al., ; Odero-Marah et al., 2008; Zhau et al., 2008). In the present study, when ARCaP_E cells were treated with either TGF- β 1 or IGF-1, an induction of LIV-1 expression was detected by both RT-PCR and Western blotting analyses (Figure 2.1). When different concentrations of IGF-1 were added to the induction medium, the responsiveness of LIV-1 expression was found to be dose-dependent (Figure 2.1). IGF-1-induced LIV-1 expression in ARCaP_E cells occurred concomitantly with a switch of cell morphology and gene expression toward mesenchymal phenotype, *i.e.*, the loss of tightly adhesive polarized epithelial morphology to become loosely dis-

persed fibroblastic cells with increased expression of N-cad and vimentin but decreased expression of E-cad, a hallmark retained by polarized epithelial cells (Figure 2.2). An activated LIV-1 expression seemed to occur concurrently with the transition of ARCaP_E to ARCaP_M, an ARCaP mesenchymal variant (Xu et al., 2006).

To define the role of LIV-1 in mediating EMT, we transiently reduced LIV-1 level in the mesenchymal-like ARCaP_M cells by siRNA knockdown. ARCaP_M cells treated with specific LIV-1 siRNA showed markedly reduced LIV-1 transcripts (Figure 2.3). Importantly, the treated cells showed decreased expression of mesenchymal markers N-cad and Snail, but increased expression of the E-cad gene in both RT-PCR and Western blotting analyses (Figure 2.3). In addition, ARCaP_M cells treated with specific LIV-1 siRNA exhibited much reduced migratory and invasive ability in scratch wound-healing (Figure 2.4) and transwell invasion assays (Figure 2.5). These results suggested that LIV-1 expression is associated with EMT and decreased LIV-1 expression lead to mesenchymal to epithelial transition (MET), a reversal of the EMT. The presence of LIV-1 appeared to be required for the maintenance of a mesenchymal phenotype.

We next examined whether an elevated LIV-1 in the epithelial-like ARCaP_E cells would be sufficient to initiate EMT, assessed by molecular analyses. Following transient transfection with a LIV-1 expression construct, ARCaP_E cells were examined by both RT-PCR and western blotting assays for the expression of EMT-associated markers. The transfected ARCaP_E cells displayed markedly increased LIV-1 expression (Figure 2.6), accompanied by increased N-cad and Snail but a decreased E-cad expression. These expressional changes were in agreement with those seen in growth factor-elicited EMT (Figure 2.2). Results from LIV-1 siRNA knockdown and LIV-1 overexpression studies in respective ARCaP_M and ARCaP_E cells suggested that LIV-1 could be a key regulator of EMT in human prostate cancer cells.

2.4.2 Production and characterization of polyclonal antibodies to human LIV-1.

To evaluate if LIV-1 expression is associated with clinical progression of human prostate cancer, we raised polyclonal antibodies by immunizing rabbits with a KLH-conjugated LIV-1 peptide. Specificity of LIV-1 antibodies was confirmed by Western blotting of the whole-cell extracts from cells overexpressing exogenous LIV-1. From the HEK293 cells transiently transfected with LIV-1, we observed a single immune-reactive LIV-1 protein, at 110 kDa (Figure 2.7). Since the calculated molecular weight of LIV-1 protein is 90 kDa (Taylor et al., 2003), the differential 20 kDa between the detected and the predicted sizes was likely attributed to N-linked glycosylation of the LIV-1 protein, as previously reported (Taylor et al., 2003). Importantly, the signal detected by the LIV-1 antibodies was abolished when the antibodies were pre-adsorbed with the LIV-1 peptide used in immunization. In addition, increased signal intensity was detected in ARCaP_E cells transiently transfected with the LIV-1 expression construct, while a reduction of the signal was seen in ARCaP_M cells treated with a transient LIV-1 knockdown vector in both Western blotting and IHC assays (Figure 2.7&2.8). These results indicated that the LIV-1 antibodies produced could detect specifically LIV-1 protein, which was modified in the cell lines tested.

2.4.3 Stable LIV-1 overexpression induced EMT in ARCaP_E cells.

Following transient knockdown of LIV-1 in ARCaP_M cells, an expected reversal of the mesenchymal fibroblastic cell shape to epithelial morphology was observed. These morphologic switches were readily detectable by gene expression changes (Figure 2.3). In contrast, however,

transiently overexpressing LIV-1 in ARCaP_E cells did not bring forth conspicuous mesenchymal fibroblastic morphology, despite of concerted expressional changes indicative of EMT (Figure 2.6). We suspected that the lack of morphologic changes may be attributable to the nature of the transient transfection. Accordingly, stable ARCaP_E clones were established to evaluate whether LIV-1 is a critical regulator associated with morphologic as well as expressional and behaviorally transition from an epithelial to a mesenchymal phenotype.

We isolated 4 ARCaP_E clones (LIV#8, 12, 14 and 17) stably expressing high levels of LIV-1 protein, as detected by Western blotting (Figure 2.9). Two control clones (con1 and con2) were also isolated from transfection with the control vector. The ones overexpressing LIV-1 showed typical EMT-like expressional changes, with decreased E-cad expression but increased N-cad and Snail expressions (Figure 2.9). Significantly, all the clones showed markedly changed cellular morphology: instead of the small cell size with cobblestone-like shape with tightly arranged intercellular contact typical of the epithelial cell-like ARCaP_E, all the four clones adapted remarkably altered morphology displayed a loss of intercellular contact, exhibiting typical spindle-shaped mesenchymal cell morphology (Figure 2.10). The morphologic transition was permanent and irreversible, persisting after more than 30 passages in continuous culture, while the two vector-transfected clones remained epithelial cell-like. It seems that stable LIV-1 overexpression could bring forth both morphologic and biochemical EMT transition. LIV-1 is thus a potent promoter of EMT in ARCaP_E cells.

The effects of LIV-1 on behavioral changes were assessed for its promotion of cell migration and invasion as assessed by Boyden chamber assays. While the control neo transfected ARCaP_E clones showed similar migration and invasion capabilities closely mimic those of the parental ARCaP_E cells, repeated assays revealed LIV-1 overexpression conferred significantly

increased migratory capability (Figure 2.11) and invasive potential to penetrate extracellular matrices (Figures 2.12). Taken together, these data support the notion that increased LIV-1 levels promote prostate cancer cells to gain motility and invasive behaviors.

2.4.4 LIV-1 overexpression promoted in vivo prostate tumor formation and distant metastases.

We examined the role of LIV-1 stably expressed in ARCAP_E cells in modulating their subsequent tumorigenic and metastatic behaviors in mice. We compared local and distant metastatic growth of ARCaP_E tumors by the use of subcutaneous and intracardiac tumor cell inoculation protocols as described previously (Thalmann et al., 2000; Xu et al., 2006).

Following subcutaneous implantation, LIV-1 overexpressing clones induced similar incidence of tumor formation as the vector-transfected controls, each group having 6 tumors from a total of 8 inoculations. Nonetheless, LIV-1-overexpressing clones formed significantly larger tumors than the control clones when the tumors were measured at 43 and 50 days after inoculation (Figure 2.13). Due to the large tumor burden in the LIV-1 transfected experimental group, these studies were terminated at 50 days. At this time, the average tumor size of LIV-1-overexpressing clones was 3 - 5 times larger than that of the control clones, with no evidence of distant metastases.

We then used intracardiac inoculation to evaluate the metastatic fate of LIV-1 overexpressing ARCaP_E cells. Four months after intracardiac administration, the mice inoculated with LIV-1 overexpressing ARCaP_E clones presented with significantly elevated incidence of tumors at multiple organ sites, including the bone and soft tissues of lymph nodes, adrenal glands, and

the lung, compared to vector-transfected controls (Table 1&Figure 2.14). Among the seven animals inoculated with LIV-1-overexpressing ARCaP_E clone 8, two were found to have multiple bone metastases, both in tibial, mandibular, and spinal bones (Figure 2.15A), while another four mice were found to harbor soft tissue tumors of the adrenal glands and the lung (Figure 2.15B). All the tumors were confirmed by histopathologic analysis. In a parallel study, five of the nine animals inoculated with LIV-1-overexpressing ARCaP_E clone 14 were found to bear bone metastasis. In sharp contrast, intracardiac inoculation of vector-transfected controls did not produce any detectable metastases in bone or soft tissues (Table 1). This series of assays demonstrated that by increasing migratory and invasive behaviors of prostate cancer cells, LIV-1 promoted metastatic growth of prostate cancer cells to the bone and the soft tissues.

2.4.5 Enhanced LIV-1 expression in clinical prostate cancer specimens.

In order to examine the correlation of LIV-1 expression and cancer malignancy, we first assess LIV-1 expression in different prostate cancer cell lines. We choose isogenic LNCaP series (LNCaP, C4-2 and C4-2B) and ARCaP series (ARCaP_E, ARCaP_M and ARCaP_{M2}) cell lines. Both series are from low potential of metastasis to high potential of metastasis, with C4-2, C4-2B, ARCaP_M and ARCaP_{M2} specifically metastasize to bone. We found that LIV-1 expression correlated with the metastatic potential of these isogenic cell models. LIV-1 expression is higher in the bone metastatic cell lines than those of the non- or low-metastatic cells such as LNCaP and ARCaP_E (Figure 2.16).

Based on the findings in ARCaP cells where LIV-1 was shown to induce EMT and prostate cancer local growth and distant metastases and LIV-1 expression in different cancer cell

lines, we seek to demonstrate the clinical relevance of these findings by performing a series of IHC assays. A LIV-1 polyclonal antibody established by our laboratory was used to detect LIV-1 status in clinical specimens on two custom-made TMAs of normal, benign/PIN, localized and bone metastatic prostate cancers and four commercial TMAs of benign and prostate cancer specimens. These TMAs represented a total of 344 prostate specimens including bone metastasis specimens from 14 patients. Results from Kruskal Wallis non-parametric test showed remarkable positive correlation of LIV-1 expression with disease progression from normal, benign, PIN, primary to bone metastasis ($P < 0.001$). Figure 2.17 showed the representative IHC images of increased LIV-1 expression in human prostate specimens from benign to bone metastasis. Significant differences were also found when LIV-1 expression was subjected to Mann-Whitney non-parametric test between either bone metastasis and localized cancer ($p=0.002$); or bone metastasis and normal/ benign, PIN, primary cancer and metastasis ($p=0.001$) (Figure 2.18). There was, however, no positive correlation between LIV-1 expression in well-differentiated ($GI \leq 6$) and moderate to poorly-differentiated ($GI \geq 7$) prostate cancers by Logistic regression test.

In summary, our study demonstrated that abnormally enhanced LIV-1 confers the ability for prostate cancer cells to migrate and invade *in vitro* and metastasize to the bone and soft tissues in experimental mouse model. ARCaP cell model of EMT allows us to gain additional insight linking LIV-1, a zinc transporter, to cancer cell growth and survival through the induction of EMT. In addition, LIV-1 expression was found to be correlated with cancer progression in clinical specimens. Thus, LIV-1 could be a new therapeutic target for prostate cancer progression and metastasis.

Table 1. LIV-1 promotes prostate cancer cell metastasis.*

Clones	Incidence of metastasis	Sites of metastasis
con1	0/6	N/A
con2	0/6	N/A
LIV#8	6/7 (86%)	2, adrenal gland
		2, lung
		1, leg bone
		1, jaw and spine
LIV#14	5/9 (56%)	2, leg bone
		1, iliac bone
		1, femur
		1, zygomatic and spine

* LIV-1-overexpression clones (LIV#8 and LIV#14) and vector-transfected clones (con1 and con2) were intracardially inoculated to athymic mice. Incidence and sites of metastasis were followed up to 4 months by necropsy and histopathologic confirmation.

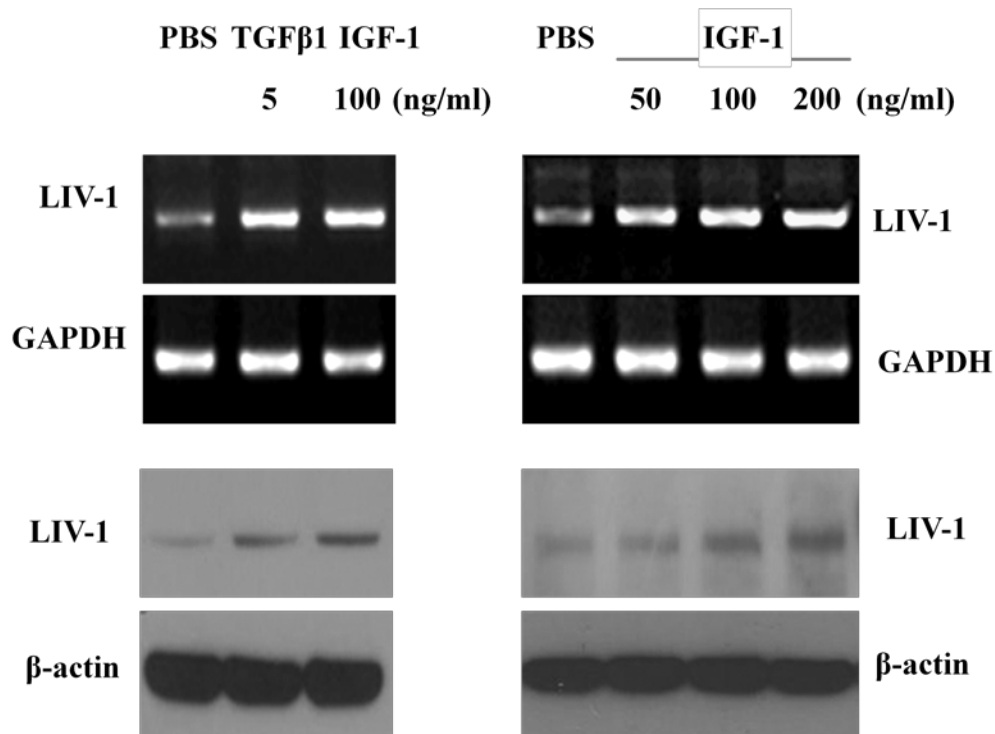


Figure 2.1

LIV-1 is a mediator in ARCaP_E cell EMT. The role of LIV-1 was assessed by its changed expression during EMT. **A**, ARCaP_E cells were treated for 48 hours with growth factors to induce EMT. RT-PCR and Western blotting were used to show increased LIV-1 expression (left panel), and dose responsiveness of the expression (right panel).

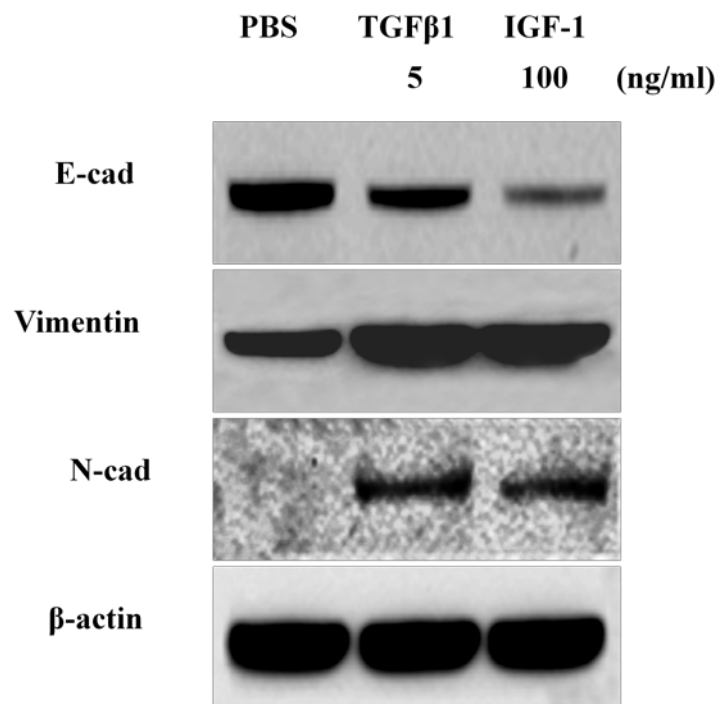


Figure 2.2

Treatment of growth factors induced EMT. Western blotting was used to confirm EMT-like expressional changes in the treated ARCaP_E cells.

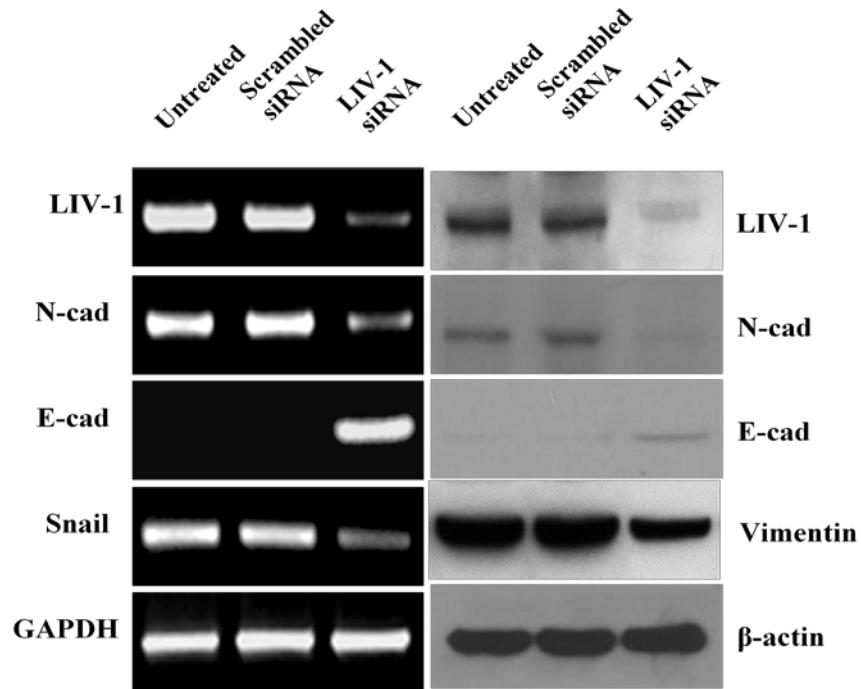


Figure 2.3

Knockdown of LIV-1 induced MET. The mesenchymal cell-like ARCaP_M cells were subjected to siRNA knockdown for LIV-1 expression for 48 hours. RT-PCR and Western blotting were used to detect expressional changes reflecting reverse of EMT in the treated cells.

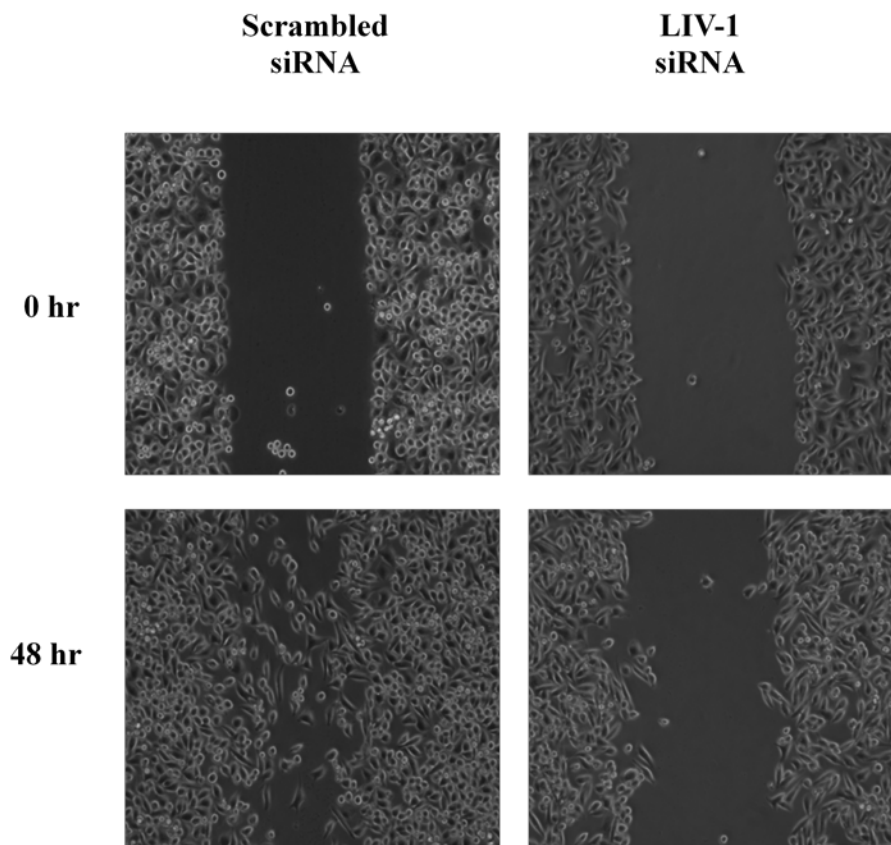


Figure 2.4

Knockdown of LIV-1 reduced migratory capability. Scratch wound healing was used to determine the migratory and invasive behavior in the siRNA treated ARCaP_M cells.

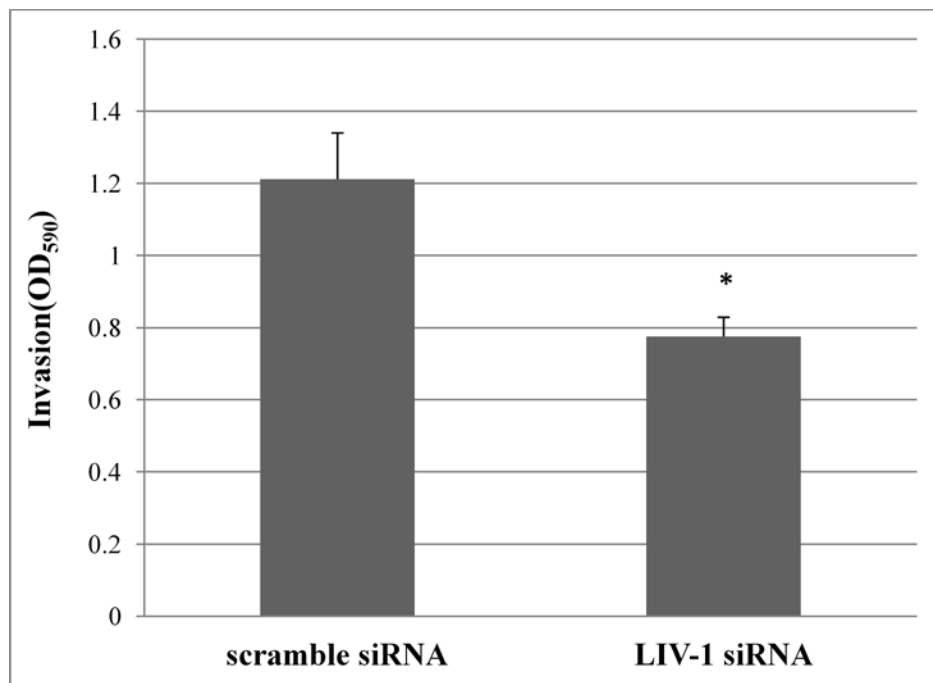


Figure 2.5

Knockdown of LIV-1 suppressed invasive ability. Transwell invasion assays were used to determine the migratory and invasive behavior in the siRNA treated ARCaP_M cells. * indicates statistical significance compared to the con1 control clone ($P < 0.05$).

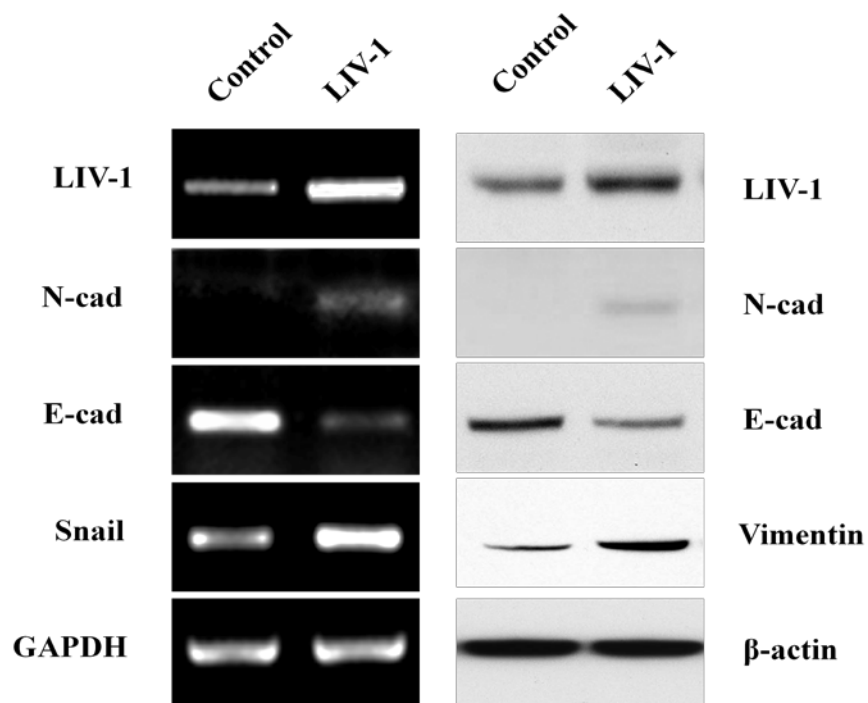


Figure 2.6

Transient overexpression of LIV-1 induced EMT. ARCaP_E cells were transfected with LIV-1 expression construct. RT-PCR and western blotting were performed 48 hours after the transfection to detect expressional changes reflecting EMT-like events. GAPDH served as an internal control for RT-PCR reactions, and β -actin was used as a loading control in Western blotting.

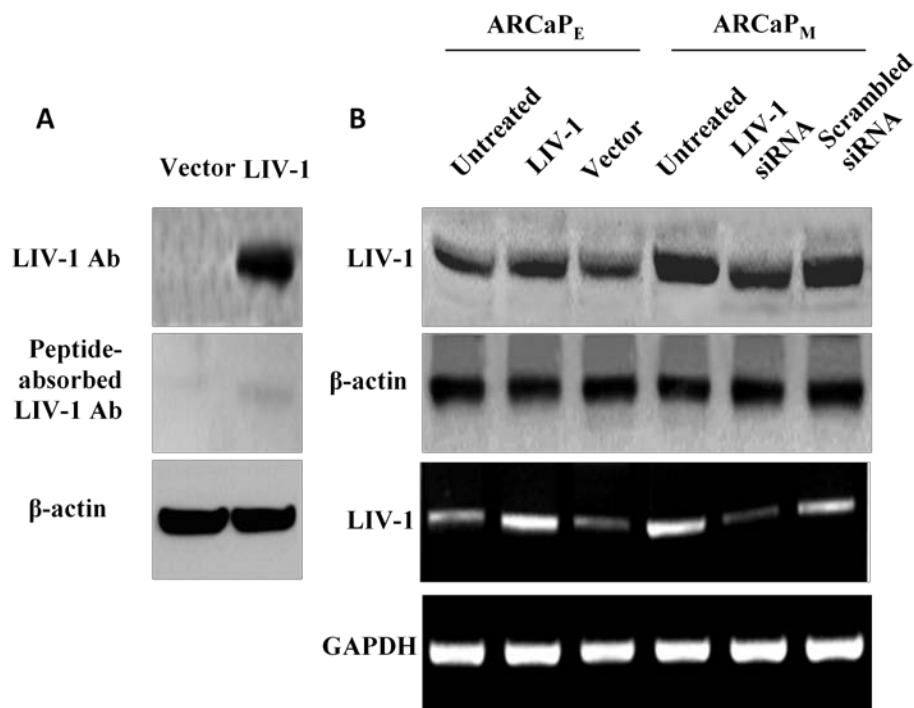


Figure 2.7

The produced antibodies to LIV-1 were subjected to validation for specificity. A, HEK293 cells transiently transfected with the LIV-1 expression construct were subjected to Western blotting analysis with the antibodies to LIV-1 (upper panel). Antibody specificity was determined by pre-absorbing the antibody with the immunizing peptide (middle panel). B, ARCaP_E cells were transiently transfected with the LIV-1 expression construct to overexpress LIV-1 and ARCaP_M cells with the specific siRNA to suppress LIV-1 expression. In the upper 2 panels, Western blotting was performed 48 hours later with the antibodies to LIV-1. In the lower 2 panels, these cells were examined by RT-PCR to confirm the LIV-1 expression. β -actin was used as control in Western blotting and GAPDH was used as control for RT-PCR analysis.

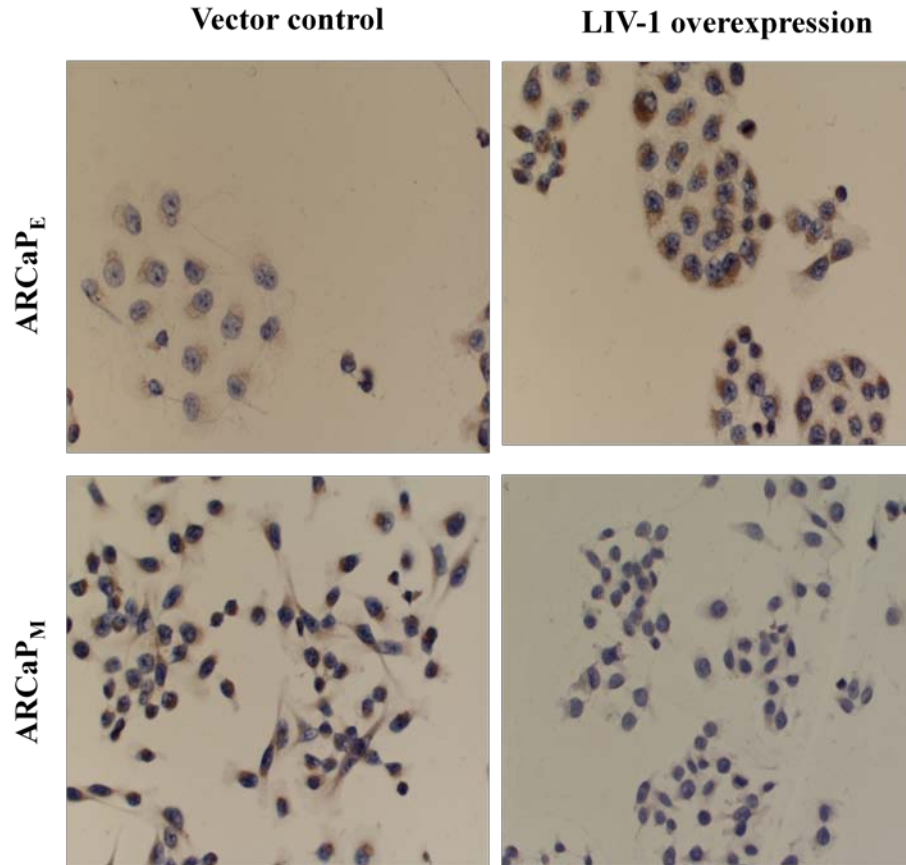


Figure 2.8

Specificity of LIV-1 antibody was confirmed by IHC. IHC was conducted to further confirm LIV-1 Ab specificity in ARCaP_E cells transfected with the LIV-1 expression construct and ARCaP_M cells transfected with the specific siRNA.

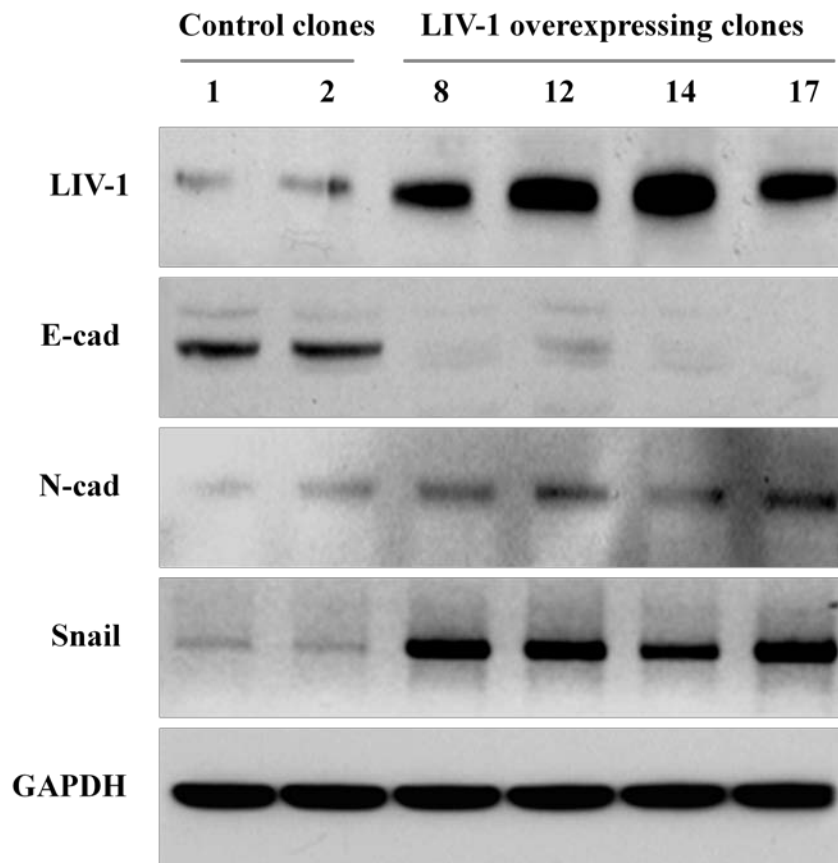


Figure 2.9

LIV-1 overexpression induced EMT. ARCaP_E clones overexpressing LIV-1 displayed EMT-like changes in gene expression. All four LIV-1 overexpressing ARCaP_E clones showed EMT-like expressional changes as detected by Western blotting, while the two vector control clones (1 and 2) retained an epithelial cell-like expression profile.

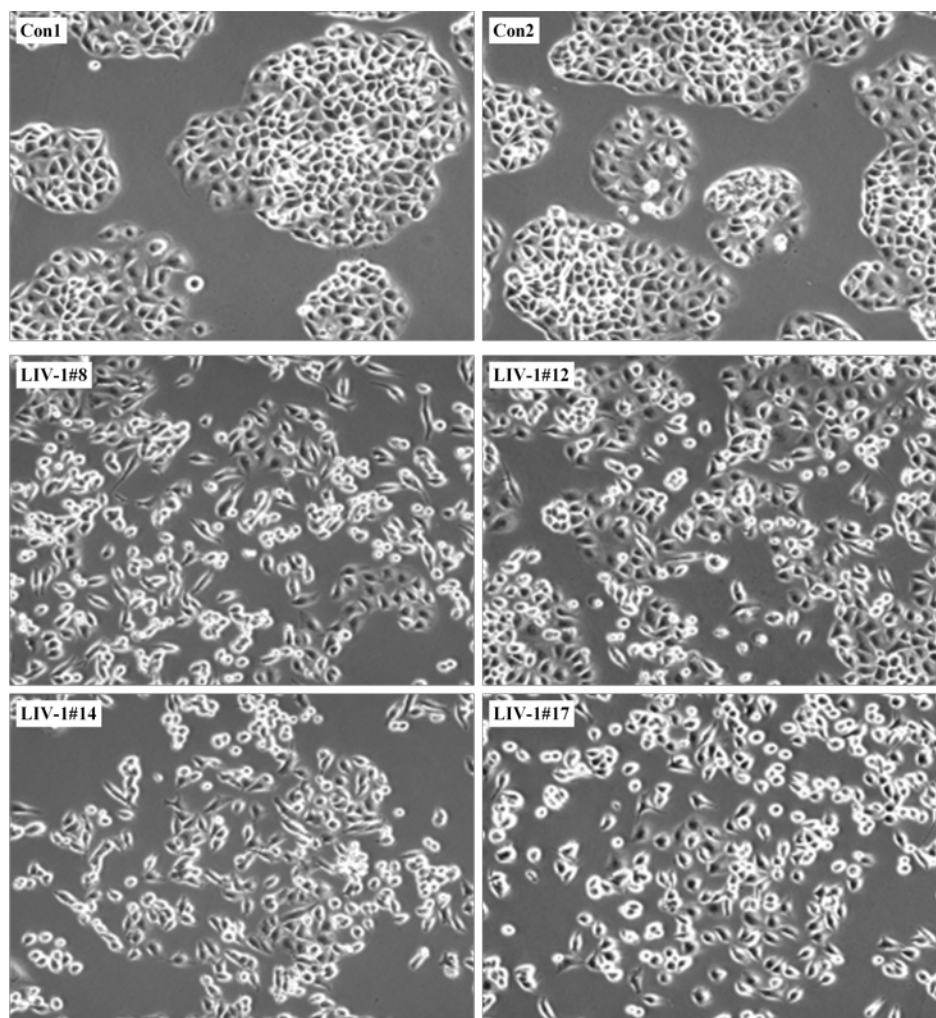


Figure 2.10

ARCaP_E clones overexpressing LIV-1 displayed EMT-like changes in cellular morphology. Cellular morphology of the LIV-1 overexpressing cells showed marked changes from the control clones (200 ×).

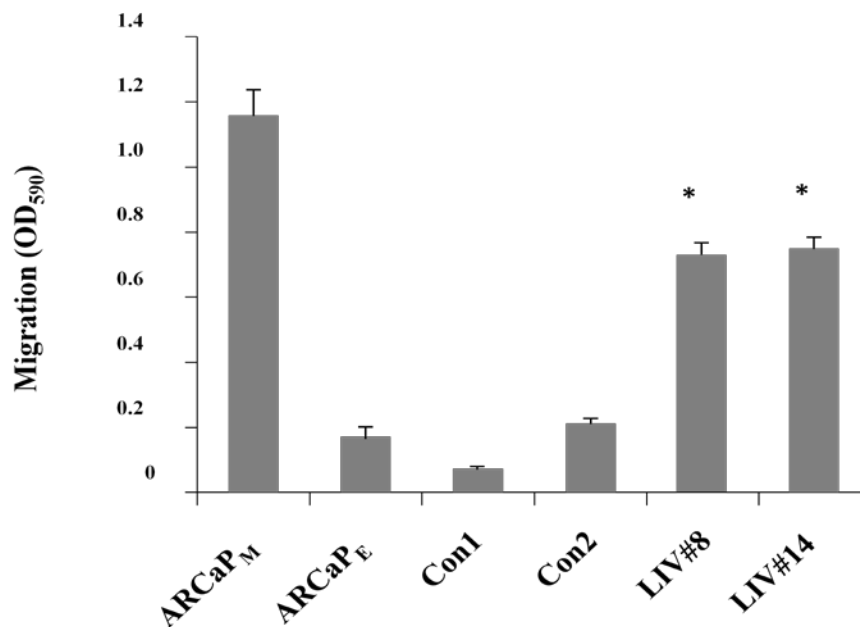


Figure 2.11

Overexpression of LIV-1 exhibited increased migratory ability. LIV-1 overexpressing cells (LIV#8 and LIV#14) were compared with vector control clones 1 and 2 and parental ARCaP_E and ARCaP_M cells for altered migratory capability in transwell assays. Each result is the mean \pm standard deviation of a triplicate assay. * indicates statistical significance compared to the con1 control clone ($P < 0.05$).

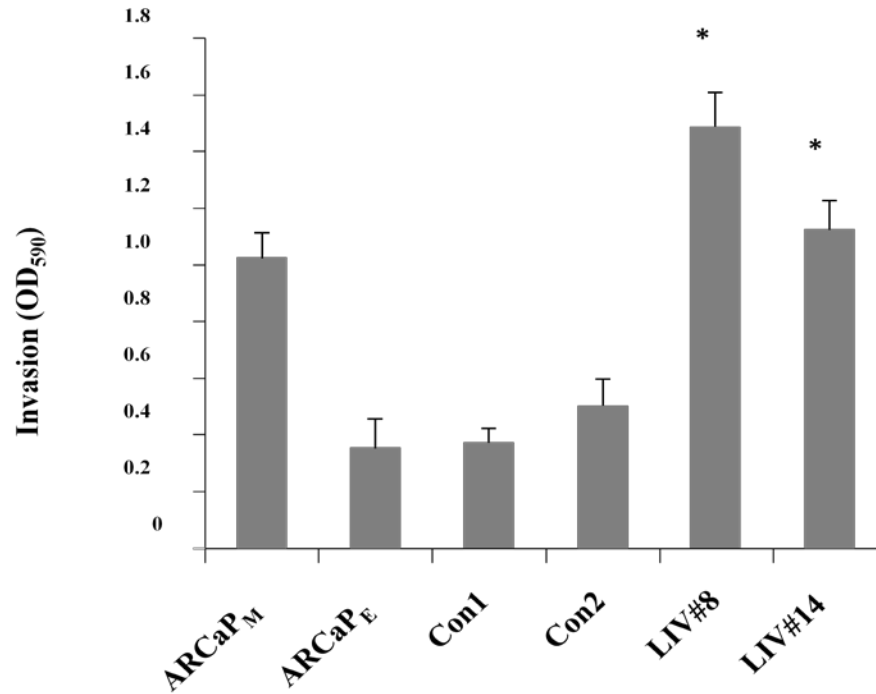


Figure 2.12

Overexpression of LIV-1 exhibited enhanced invasive ability. LIV-1 overexpressing cells (LIV#8 and LIV#14) were compared with vector control clones 1 and 2 and parental ARCaP_E and ARCaP_M cells for invasiveness in transwell invasion assays. Each result is the mean \pm standard deviation of a triplicate assay. * indicates statistical significance compared to the con1 control clone ($P < 0.05$).

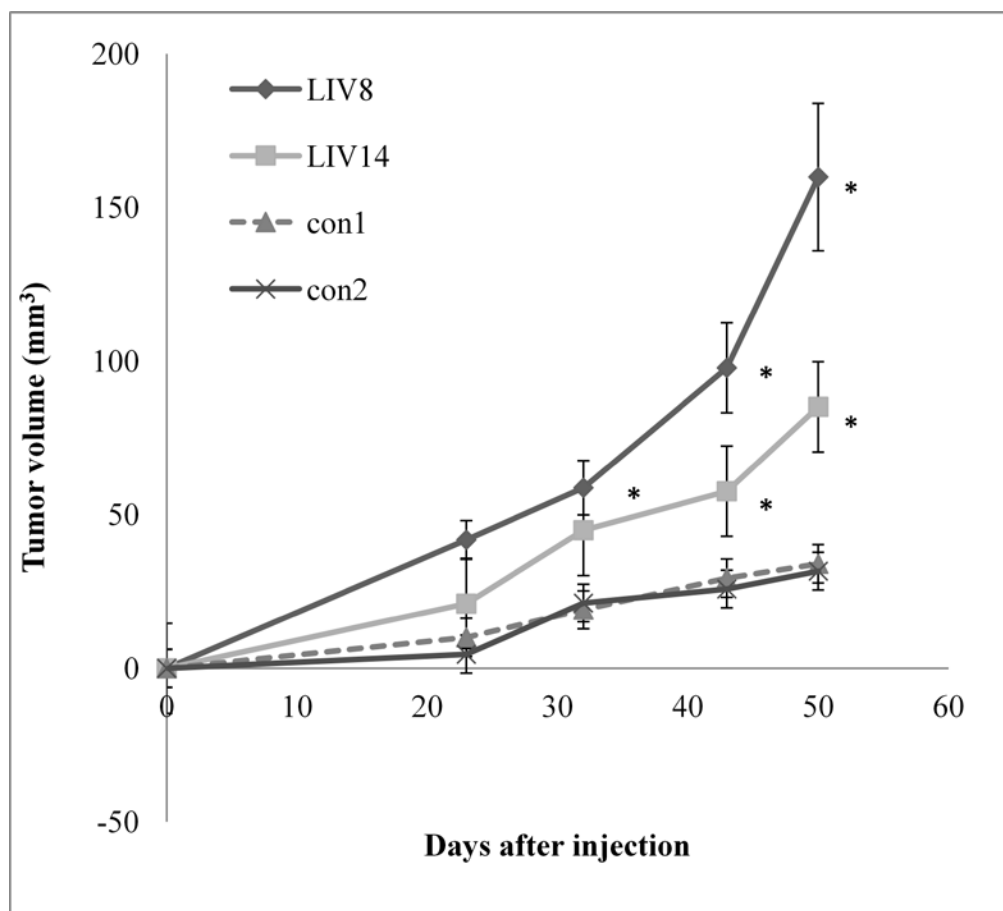


Figure 2.13

LIV-1 overexpression promoted subcutaneous tumor growth. Upon subcutaneous inoculation, the two ARCaP_E clones overexpressing LIV-1 (LIV8 and LIV14) were compared with vector control clones (con1 and con2) for tumor formation in athymic mice. Growth of the tumors at day 23, 32, 43, and 50 is shown. Each result represents the mean \pm standard deviation of six tumors. An asterisk indicates statistical significance compared to the con1 control clone ($P < 0.05$).

i. Bone metastasis



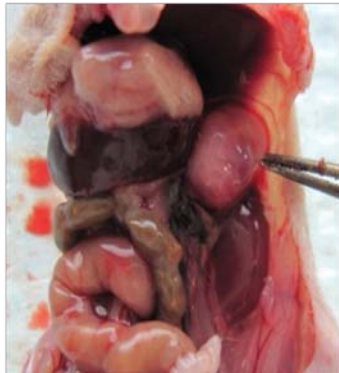
ii. Lung metastasis



iii. Jaw metastasis



iv. Adrenal gland metastasis



v. Right front leg metastasis



Figure 2.14

LIV-1 overexpression promoted cancer bone and soft tissue metastasis. Upon intracardiac inoculation, ARCaP_E clones overexpressing LIV-1 caused metastatic tumor formation in athymic mice (see Table 1). Representative results depict the metastatic tumors.

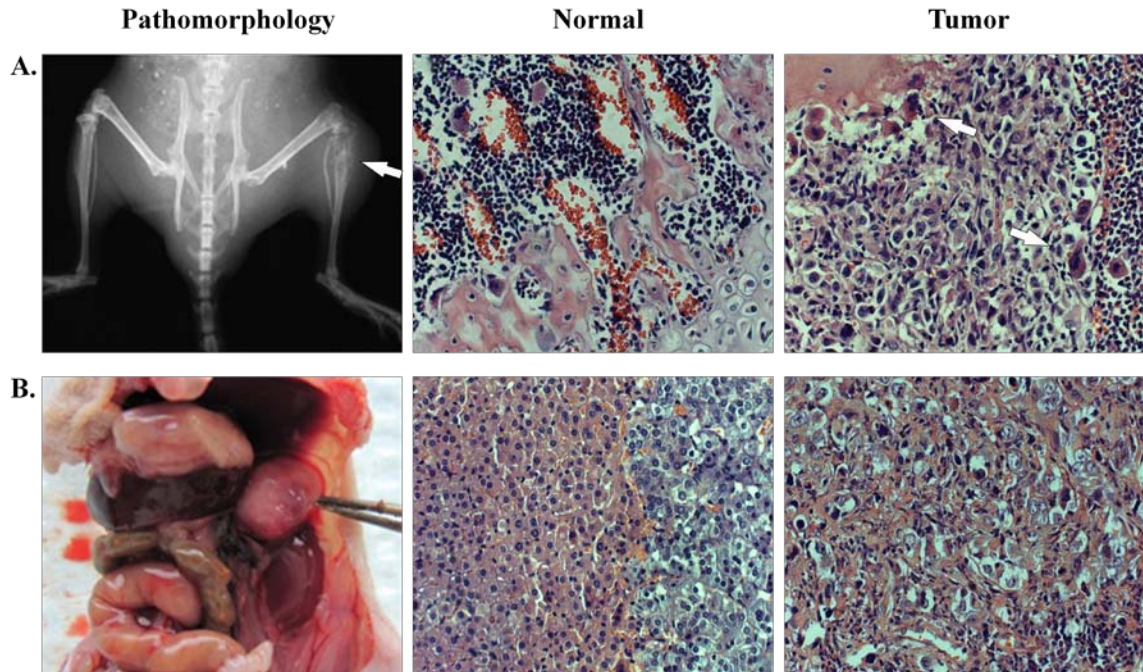


Figure 2.15

Histopathologic confirmation of bone and soft tissue metastasis. A, a tibial tumor (arrow) was identified by X-ray (Pathomorphology). The tumor was subjected to H&E staining for histopathologic confirmation. Compared to the tibia of the opposite leg (Normal), the tibial tumor (Tumor) showed histopathology typical of a metastatic bone lesion. B, an adrenal gland metastasis (held by forceps) destroyed the gland (Pathomorphology). Compared to the unaffected gland (Normal), tumor cells were found in every part of the affected gland (Tumor) (250 ×).

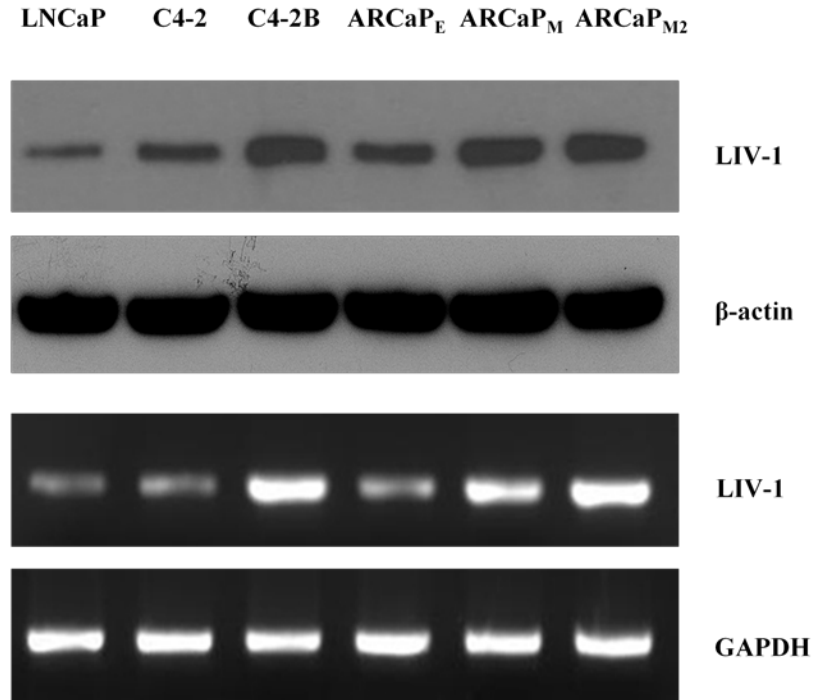


Figure 2.16

LIV-1 expression is associated with human prostate cancer progression. RT-PCR and Western blotting were used to determine LIV-1 expression in different prostate cancer cell lines. β -actin was used as control in Western blotting and GAPDH was used as control for RT-PCR analysis.

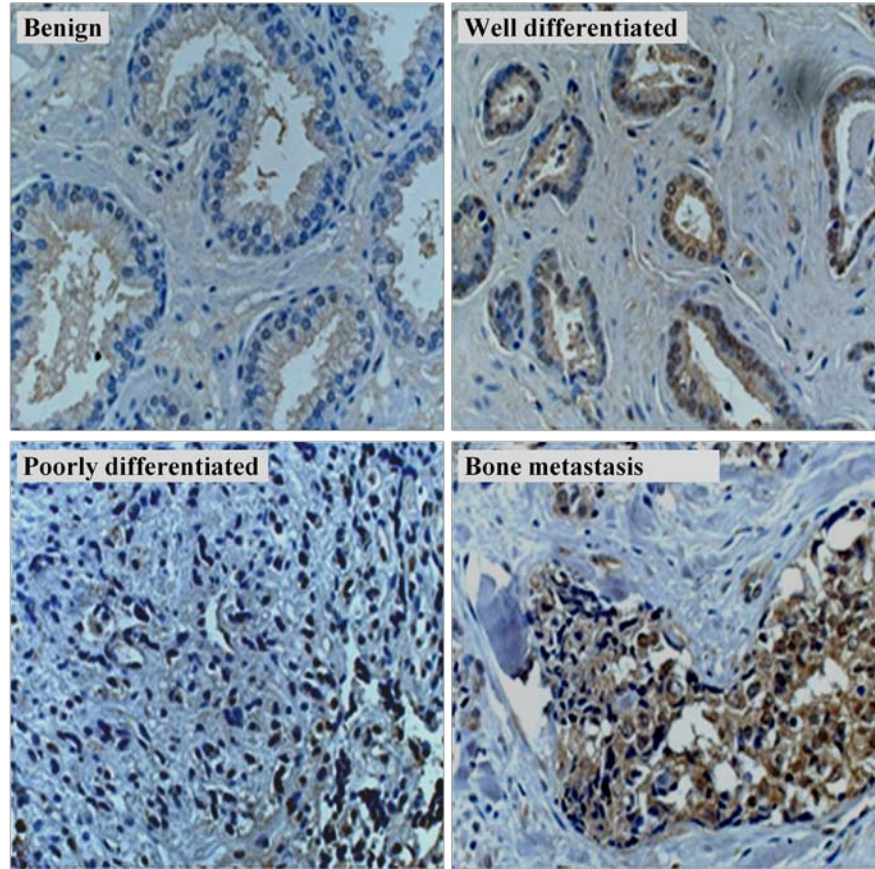


Figure 2.17

LIV-1 expression was correlated with prostate cancer progression. Representative IHC images show increased LIV-1 expression in human prostate specimens from benign to bone metastasis (125 ×).

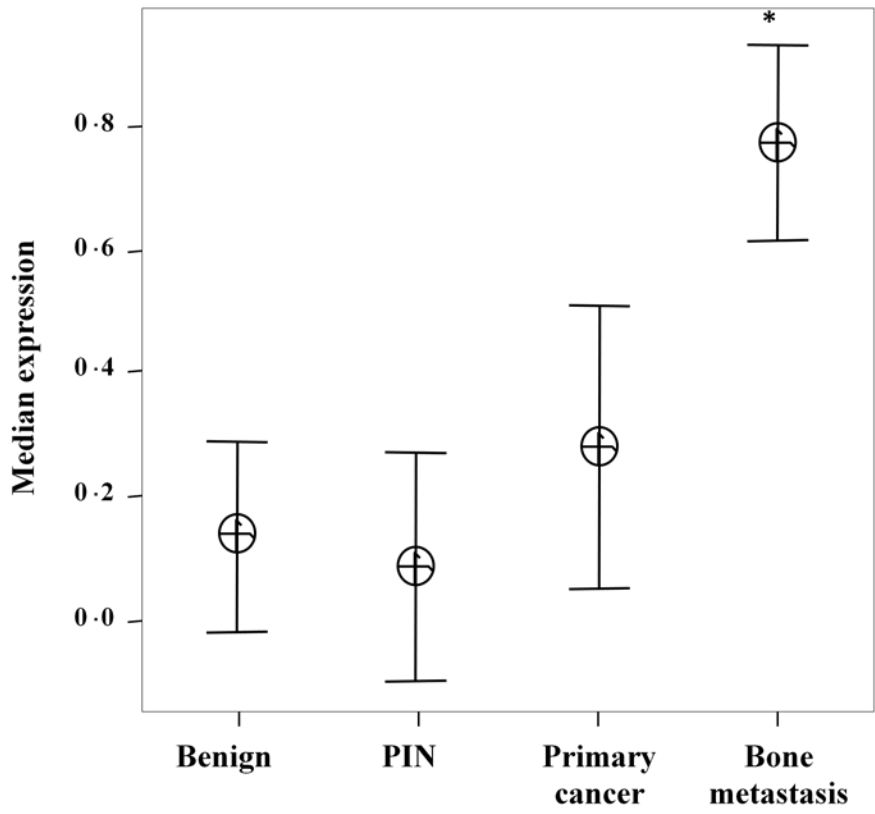


Figure 2.18

Statistical analysis of LIV-1 expression correlated with prostate cancer progression. Interval plot of LIV-1 expression is shown versus prostate cancer progression from normal/benign, PIN, primary cancer to bone metastasis. The data was shown with 95% confidence interval (n = number of cases analyzed). The median expression for LIV-1 in bone was significantly greater than those in normal/benign, PIN, and primary cancer ($P < 0.001$) and that those in primary cancer only ($P = 0.002$) as analyzed by Mann-Whitney test.

3 LIV-1 PROMOTES HUMAN PROSTATE CANCER EMT AND METASTASIS THROUGH HB-EGF SHEDDING AND CONSTITUTIVE EGFR-MEDIATED ERK SIGNALING

3.1 Abstract

LIV-1, a zinc transporter, is an effector molecule downstream from soluble growth factors. This protein has been shown to promote epithelial-to-mesenchymal transition (EMT) in human pancreatic, breast, and prostate cancer cells. Despite the implication of LIV-1 in cancer growth and metastasis, there has been no study to determine the role of LIV-1 in prostate cancer progression. Moreover, there was no clear delineation of the molecular mechanism underlying LIV-1 function in cancer cells. In the present communication, we found increased LIV-1 expression in benign, PIN, primary and bone metastatic human prostate cancer. We characterized the mechanism by which LIV-1 drives human prostate cancer EMT in an ARCaP prostate cancer bone metastasis model. LIV-1, when overexpressed in ARCaP_E cells, promoted EMT irreversibly. LIV-1 overexpressed ARCaP_E cells had elevated levels of HB-EGF and MMP 2 and MMP 9 proteolytic enzyme activities, without affecting intracellular zinc concentration. The activation of MMPs resulted in the shedding of HB-EGF from ARCaP_E cells that elicited constitutive EGFR phosphorylation and its downstream ERK signaling. These results suggest that LIV-1 is involved in prostate cancer progression as an intracellular target of growth factor receptor signaling which promoted EMT and cancer metastasis. LIV-1 could be an attractive therapeutic target for the eradication of pre-existing human prostate cancer and bone and soft tissue metastases.

3.2 *Introduction*

LIV-1, a cell surface protein and a candidate mediator of the growth factor-elicited signaling molecule, has been associated with several important biologic processes by serving as a transporter for zinc and other ions (Chowanadisai et al., 2008; el-Tanani and Green, 1996, 1997; Taylor et al., 2004; Taylor et al., 2003). As a prototype of the LIV-1 subfamily of ZIP metal transporters (Taylor, 2000; Taylor et al., 2003), LIV-1 shares secondary structure with ZIP transporters and may have the ability to transport metal ions. LIV-1 was shown to be a mediator downstream from signal transducer and activator of transcription 3 (STAT3) and Snail, cooperating with Snail in the repression of epithelial marker E-cad gene transcription (Cano et al., 2000). LIV-1 was also shown to be an interacting partner for the estrogen receptor (ER) in hormone-sensitive tissues (El-Tanani and Green, 1997; McClelland et al., 1998). In ER-positive ZR-75-1 breast cancer cell line, LIV-1 transcription is induced by estrogens (Manning et al., 1988). In breast tumors, LIV-1 expression is associated with ER status (Dressman et al., 2001), and is positively correlated with the spread of cancer to regional lymph nodes (Manning et al., 1994). In cervical cancer, expression of LIV-1 was shown to be higher in tumor than normal tissues; RNAi-mediated suppression of LIV-1 significantly inhibited cell proliferation, colony formation, and reduced migratory and invasive ability of the HeLa cells (Zhao et al., 2007a). LIV-1 has also been reported to be elevated in clinical pancreatic carcinoma and induced EMT in pancreatic cancer cells (Unno et al., 2009). In zebrafish, LIV-1 is essential for the nuclear localization of Snail, a master transcription factor promoting epithelial to mesenchymal transition (EMT), causing migration of gastrula organizing cells (Yamashita et al., 2004). LIV-1 thus is an obligatory co-factor regulating EMT-associated genes (Unno et al., 2009; Yamashita et al., 2004; Zhao et al., 2007b).

Epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases and EGFR signaling has been well documented that dysregulation of EGFR signaling is involved in tumorigenesis and cancer metastasis (El-Rayes and LoRusso, 2004; Hynes and MacDonald, 2009). Activation of EGFR is controlled by binding of their ligands, which are members of the EGF family of growth factors. Ligand binding to EGFR induce receptor dimerization and intrinsic kinase domain activation, which in turn lead to the activation of subsequent downstream signaling events, such as mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/AKT, and signal transducers and activators of transcription (STAT) signaling pathways (Fischer et al., 2003; Lurje and Lenz, 2009) which are important in regulating cell proliferation, migration and tumor progression. Constitutive activation of EGFR is often found in several malignant cancers. Mechanisms involving in the dysregulation of EGFR include receptor overexpression, receptor mutation or coexpression of ligands and the receptor and all these result in constitutively active EGFR signaling which will lead to malignant transformation and tumor growth (Hardy et al.).

Heparin-binding epidermal growth factor (HB-EGF) is a member of the EGF family of growth factors and is involved in many biological processes including cell adhesion, invasion and also cancer progression (Miyamoto et al., 2006). HB-EGF is synthesized as a membrane-anchored protein and is cleaved by zinc metalloproteases at the cell surface to release the mature soluble form of HB-EGF (Huang et al., 2006b). The soluble form of HB-EGF then binds to EGFR to cause EGFR transactivation. This proteolytic process is known as ectodomain shedding. Multiple zinc-binding metalloproteases of the matrix metalloprotease (MMP) and a disintegrin and metalloprotease (ADAM) families are reported to be involved in this ectodomain shedding process (Higashiyama et al., 2008; Shah and Catt, 2004). MMP2 and MMP9 are of particularly

interest because of their role in the regulation of HB-EGF shedding (Roudabush et al., 2000; Song et al., 2007) and also in early cancer development, invasion and metastasis (Mook et al., 2004; Shen et al., 2009).

The potential diagnostic and prognostic values of LIV-1 in human prostate cancer have not been investigated. Since zinc plays important roles in the maintenance of prostate epithelial cell homeostasis (Bataineh et al., 2002), and Snail is a master transcription factor controlling prostate cancer cell EMT (Barrallo-Gimeno and Nieto, 2005; Batlle et al., 2000; Odero-Marah et al., 2008), LIV-1 may be an active participant in the promotion of EMT during prostate cancer progression and bone metastasis. In this study, we determined the level of LIV-1 in human prostate cancer cell lines and clinical tissue specimens to define the relationship between LIV-1 and prostate cancer progression and metastasis. The ARCaP human prostate cancer progression cell model was used to evaluate the role of LIV-1. Our study found that LIV-1 overexpression promotes prostate cancer cell EMT and facilitates its metastasis to bone and soft tissues. Further mechanistic investigation revealed that LIV-1 overexpression could upregulate HB-EGF and MMP2 and MMP9 expression. The latter could enzymatically cleave membrane-bound HB-EGF, to produce soluble HB-EGF that constitutively activated EGFR via increased EGFR phosphorylation and its downstream ERK signaling. This is the first finding that LIV-1 overexpression could activate EGFR signaling through upregulation of HB-EGF and MMP2/ MMP9 to promote cancer cell invasiveness. Last, the results from this study demonstrate that molecular suppression of LIV-1 could be a therapeutic strategy to inhibit prostate tumor cell EMT and prostate cancer bone metastasis.

3.3 *Material and Methods*

3.3.1 Cell lines and cell culture.

Human prostate cancer ARCaPE and ARCaPM cells were established in our laboratory (21). The cells were cultured in T-medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA). Human embryonic kidney HEK293 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM (Invitrogen) supplemented with 10% FBS. RPMI-1640 was purchased from Invitrogen (Carlsbad, CA). All the culture media were supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). Cell cultures were maintained at 37°C in a humidified atmosphere supplemented with 5% CO₂.

3.3.2 Antibodies and reagents.

Polyclonal rabbit antibody against LIV-1 was generated in our laboratory. Rabbits were immunized by standard immunization protocol with conjugated peptide KLH-CPDHSDSSGKDPRNS, corresponding to residues 146-161 of the LIV-1 protein (GenBank accession number NM_012319). Blood was taken 2 weeks after the fourth boost and IgG were purified and tested for specific immune reactivity. Polyclonal antibody to phospho-EGFR antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to EGFR was from BD Transduction Laboratories (San Diego, CA). Antibodies to p44/42 MAP kinase and the phosphorylated isoforms were from Cell Signaling Technology (Danvers, MA). Monoclonal antibody to β-actin was from Sigma-Aldrich (St. Louis, MO).

Growth factors of EGF was from R&D Systems (Minneapolis, MN). Tyrphostin AG1478, U0126 and MMP2/9 inhibitor III were obtained respectively from Alomone labs (Jerusalem, Israel), Cell Signaling Technology (Danvers, MA), and Calbiochem (Darmstadt, Germany).

3.3.3 Transfection.

Full-length coding region for human LIV-1 cDNA was cloned and confirmed by DNA sequencing. The cDNA was then cloned downstream from a cytomegalovirus early promoter in the mammalian expression vector pcDNA3.1/V5-His (Invitrogen). HEK293 and ARCaPE cells were seeded at 3×10^5 cells per well in 6-well plates 24 hours before transfection. The cells were transfected with 4 μg of the LIV-1 expression construct using 8 μl Lipofectamine 2000 (Invitrogen). To isolate clones stably overexpressing the LIV-1, transfected ARCaPE cells were treated with G418 (600 $\mu\text{g}/\text{ml}$) 2 days after the transfection. Four individual clones overexpressing LIV-1 protein (LIV#8, #12, #14 and #17) and two clones transfected with control vector (con1 and con2) were used for the studies.

3.3.4 Semiquantitative expression analysis with RT-PCR.

Total RNA was isolated from cultured cells using the RNeasy Mini kit (Qiagen, Valencia, CA). From each sample, equal amount of RNA (2 μg) was used in first-strand cDNA synthesis reaction with the Superscript First-Strand cDNA Synthesis kit (Invitrogen). Equal volume of cDNA (3 μl) from each reaction was used for PCR analysis using gene-specific oligonucleotide

primer pairs: 5'-GCAATGGCGAGGAAGTTATCT-3' and 5'-CTATTGTCTCTAGAAAGTGAG-3' for LIV-1; 5'-TGCCCAGAAAATGAAAAAGG-3' and 5'-GTGTATGTGGCAATGCGTTC-3' for E-cad; ; 5'-CCATCACTCGGCTTAATGGT-3' and 5'-GATGATGATGCAGAGCAGGA-3' for N-cad; 5'-CGAAAGGCCTTCAACTGCAAAT-3' and 5'-ACTGGTACTTCTTGACATCTG-3' for Snail; and 5'-TTAGCACCCCCTGGCCAAGG-3' and 5'-CTTACTCCTTGGAGGCCATG-3' for GAPDH. The reactions were initiated with a 4-minute incubation at 94°C, followed by 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. The reaction was completed with a 7-minute extension at 70°C for 7 minutes. PCR products were visualized after electrophoresis through a 1.2% agarose gel and stained by ethidium bromide (0.5 µg/ml).

3.3.5 Western blotting.

Cells at 80% confluence were lysed in a whole-cell lysis buffer as previously reported (22). The lysates were incubated on ice for 30 minutes and centrifuged at 10,000 rpm at 4°C for 10 minutes. From each sample, 35 µg protein in the supernatant was resolved by SDS-PAGE and blotted onto a nitrocellulose membrane (BioRad, Hercules, CA), which was blocked in 5% skim milk in PBST (137 mM NaCl, 12 mM phosphate, 2.7 mM KCl, and 0.1% Tween 20) at room temperature for 20 minutes; and incubated with primary antibody at 4°C overnight. The membranes were then washed three times in PBST, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. After five washings in PBST, specific signal was detected by incubating the membrane with ECL reagent (Amersham-Pharmacia Biotech, Piscataway, NJ).

3.3.6 Measuring intracellular zinc concentration.

Two methods were used to determine intracellular zinc concentration. To prepare samples for assaying total intracellular zinc by the inductively coupled plasma mass spectrometry (ICP-MS), cultured cells at 80% confluence were trypsinized and washed in PBS, and were then incubated in 300 μ l of 70% nitric acid at 37°C for 2 hours. The cells were then placed in 2% nitric acid and subjected to ICP-MS with a Varian instrument. A standard curve was generated with serial dilutions of zinc instrument standards. To prepare samples for assaying intracellular labile zinc by fluorometric method, cells were seeded at 3×10^5 cells per well in 6-well plates the day before measurement. The cells were loaded with 2 μ M of FluoZIN-3 AM (Invitrogen) for 1 hour in Opti-MEM containing 0.02% Pluronic F127 (Invitrogen). After washing in PBS, the loaded cells were incubated in indicator-free Opti-MEM for 30 minutes. Fluorescence of the FluoZin-3 was measured using a PE Victor3 V plate reader.

3.3.7 Trans-well migration and invasion assays.

To perform a trans-well migration assay, 2.5×10^4 cells in the top chamber of 24-well trans-well plates of 8 μ m pore size (BD Biosciences) were incubated for 16 hours in complete medium that was added to the bottom chamber. Cells were then fixed with formalin and stained with 0.5% crystal violet. The non-migrated cells inside the chamber were removed by swabbing. Crystal violet of the migrating cells was solubilized into the Sorenson's buffer (0.1 M sodium citrate and 50% ethanol, pH 4.2) and was measured for absorbance at OD590. Invasion assay was performed using BD BioCoat Matrigel invasion chambers (BD Biosciences; 8- μ m pore size).

The same procedures described above were used, except the filters were pre-coated with 100 μ l Matrigel at a 1 : 4 dilution in RPMI-1640.

3.3.8 Gelatin zymography.

All gelatin zymography reagents were purchased from Invitrogen. Cells were cultured in serum-free RPMI1640 medium for 24 hours and conditioned medium was collected. Protein in the medium was concentrated with AmiconUltracel 30 KDa filter (Millipore, Billerica, MA). Equal amount of protein (10 μ g/sample) was mixed with 2 \times Novex Tris-Glycine SDS sample buffer, and fractionated on a 10% gelatin gel under non-reducing conditions. The gel was then incubated at 37°C in renaturing buffer for 30 minutes and in developing buffer for 30 minutes. Finally, the gel was stained in SimplyBlue Safestain, and bands representing gelatinase activity of MMP2 and MMP9 were quantified.

3.3.9 Enzyme-linked immunosorbent assay (ELISA) for HB-EGF.

Cells were cultured in serum-free RPMI1640 medium for 24 hours. Conditioned medium was collected and analyzed for HB-EGF concentration with the Human HB-EGF DuoSet ELISA kit (R&D Systems), following manufacturer's recommended protocol. Basically, a 96-well plate was coated with the HBEGF capture antibody and incubated overnight at room temperature. The plate was then washed three times with wash buffer and blocked by adding reagent diluents for at least 1 hour at room temperature. The plate was washed twice with wash buffer and added with HBEGF condition medium, incubating 2 hours at room temperature. The plate was then washed

three times with wash buffer and added with the HBEGF detection antibody, incubating 2 hours at room temperature. The plate again was washed three times with wash buffer and added with Streptavidin-HRP, incubating 20 minutes at room temperature. Repeat the wash steps and then substrate was added to each well for 20 minutes at room temperature. Next, stop solution was added to each well and optical density at OD₄₅₀ was measured by using a plate reader (subtraction of OD₅₄₀). The results were plotted. Each sample was assayed in triplicate from three independent experiments.

3.4 Results

3.4.1 LIV-1 overexpression activates EGFR and downstream ERK signaling.

Our previous study showed that LIV-1 induced EMT *in vitro* and promoted prostate cancer metastasis *in vivo*. We next explored the mechanism by which LIV-1 promoted prostate cancer EMT, progression and metastasis. We examined the phosphorylation status of AKT, p38, JNK, Smad, NF- κ B, β -catenin and ERK because these regulatory proteins were shown to be altered by a soluble growth factor, β 2-M, which also promoted EMT and LIV-1 expression (Graham et al., 2008; Jossion et al., ; Zhau et al., 2008). In this study, we observed that ERK signaling was significantly activated (Figure 3.1). ERK signaling has been well studied that p-ERK is involved in cell proliferation, migration, invasion as well as metastasis. Since ERK was frequently activated by growth factor receptor signaling, we examined specifically the phosphorylation status of IGF-1R, TGF- β receptor and EGFR proteins. This series of analyses revealed that there was a specifically increased EGFR phosphorylation in the LIV-1-overexpressing cells (Figure 3.1). To elucidate the relationship between EGFR and ERK activation, we used the specific in-

hibitor AG1478 to block EGFR activation. After AG1478 treated the cells for 2 hours, whole cell lysates was collected to further do Western blotting analysis. Inhibition of EGFR phosphorylation led to a simultaneous reduction in ERK phosphorylation (Figure 3.2), suggesting that EGFR activation is responsible for downstream ERK phosphorylation.

3.4.2 Block of EGFR and ERK signaling reduce migratory and invasive abilities of the LIV-1 overexpressing cells.

We examined next whether EGFR-induced ERK activation were responsible for the increased metastatic behaviors of LIV-1 overexpressing ARCaP_E cells in transwell migration and invasion assays. Blocking EGFR activation by AG1478 was accompanied by reduced migratory and invasive capabilities (Figure 3.3). In support of these results, ERK inhibitor U0126 treatment also reduced both the migratory and invasive capabilities (Figure 3.4), similar to those observed following AG1478 treatment. These results, taken together, demonstrated that EGFR and downstream ERK activation is the major signaling pathway stimulating migratory and invasive behavior of the LIV-1 overexpressing cells.

3.4.3 LIV-1 overexpression did not enhance intracellular zinc concentration of LIV-1 overexpressing cells.

Since LIV-1 is a member of the ZIP metal transporters that may regulate intracellular zinc ion homeostasis (Chowanadisai et al., 2008; Lopez and Kelleher, ; Taylor et al., 2003), we evaluated whether the increased EGFR constitutive phosphorylation in LIV-1 overexpressing

clones was due to changes in intracellular zinc concentration. Intracellular labile Zn was assessed with Zn-specific fluorophores and LIV-1-overexpressing cells showed very slightly increase of labile Zn when compare to control cells (Figure 3.5). Total intracellular zinc concentration measured by ICP-MS did not show big differences between ARCaPE-LIV-1 and ARCaPE-neo cells (Figure 3.6). As a summary, compared to the control clones, there were no statistically significant differences in both the total zinc determined by ICP-MS analysis and the labile fraction measured with fluorometric assay method (Figure 3.5&3.6).

3.4.4 LIV-1 activated EGFR signaling by increasing HB-EGF, MMP2 and MMP9 expression.

We then tested the hypothesis that as a result of activation of EGFR in LIV-1 overexpressing cells, a positive feedback consisting of an autocrine/paracrine loop of growth factors may be elicited to account for increased tumorigenicity and metastatic potential. We examined whether the increased EGFR phosphorylation was a result of increased production of cognate ligands such as EGF and HB-EGF proteins (Fischer et al., 2003; Lurje and Lenz, 2009).

Western blotting revealed that LIV-1 overexpressing ARCaPE clones and the control clones had similar levels of EGFR protein, and upon ligand treatment both clones could be drastically phosphorylated, which was abolished by specific inhibitor AG1478 (Figure 3.7). Nonetheless, LIV-1 overexpressing LIV-1 cells showed a constitutive EGFR phosphorylation in the absence of exogenous ligand, and this phosphorylation could be abolished by AG1478 treatment (Figure 3.7). To investigate the cause of the constitutive EGFR phosphorylation, we found while both of these cell types produced low or undetectable levels of EGF as detected by RT-PCR, sig-

nificant increased levels of HB-EGF were expressed by LIV-1 overexpressing clones compared to the control clones (Figure 3.8). These results indicated that HB-EGF may be a constitutive inducer for EGFR signaling via increased EGFR phosphorylation.

3.4.5 Increase of MMP2 and MMP9 activity caused an increase of soluble HB-EGF, which in turn activates EGFR signaling.

HB-EGF is synthesized as a membrane-anchored form which needs to be released from the plasma membrane by matrix metalloproteases (MMPs) in order to bind EGFR (Miyamoto et al., 2006; Roudabush et al., 2000; Song et al., 2007). Interestingly, there was significantly higher MMP2 and MMP9 expression in the LIV-1-overexpressing clones (Figure 3.8), while gelatin zymography demonstrated that MMP2 and MMP9 enzymatic activities were also enhanced (Figure 3.9). Importantly, treatment with MMP2/9 inhibitor III led to a sensitive reduction of soluble HB-EGF in the culture media as determined by ELISA (Figure 3.10), suggesting that these proteolytic enzymes were involved in HB-EGF shedding. In other experiments, MMP2/9 inhibitor III treatment caused a time-dependent loss of phosphorylation in both EGFR and the downstream ERK proteins (Figure 3.11), confirming that the proteolytic enzymes acted upstream of EGFR-elicited MAPK signaling. Consequently, MMP2/9 inhibition significantly reduced the migratory and invasive ability of LIV-1-overexpressing cells in transwell assays (Figure 3.12). It seemed likely that the function of LIV-1 was to stimulate the expression of MMP2, MMP9 and HB-EGF proteins, which in turn activated EGFR and downstream ERK signaling, leading to EMT that facilitated local tumor growth and its distant metastases to bone and soft tissues (Figure 3.13).

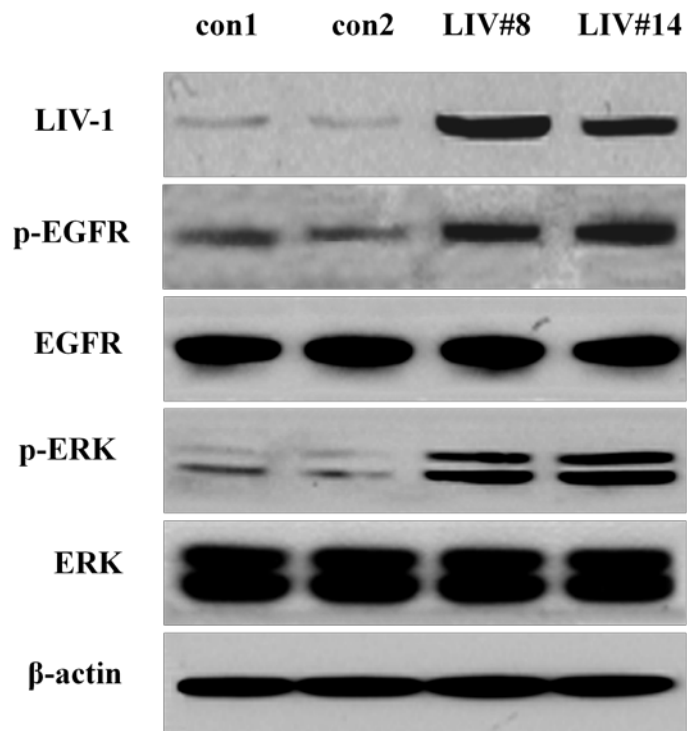


Figure 3.1

LIV-1 overexpressing cells (LIV#8 and LIV#14) showed increased phosphorylated EGFR (p-EGFR) and ERK (p-ERK).

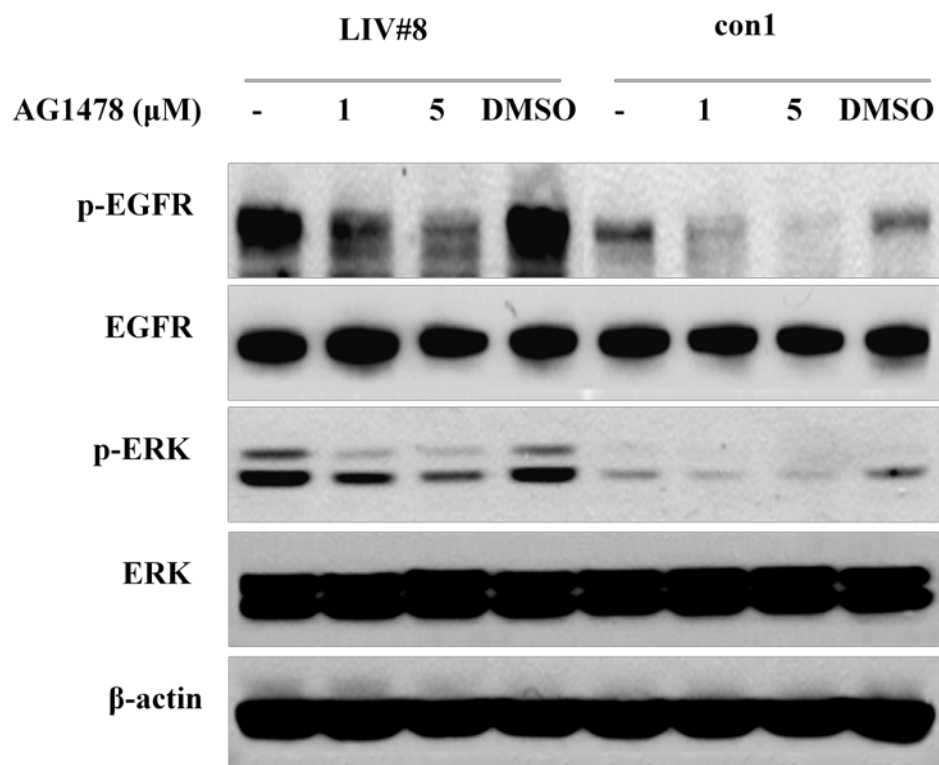


Figure 3.2

EGFR inhibitor (AG1478) treatment reduced phosphorylated EGFR and ERK in LIV-1 overexpressing cells.

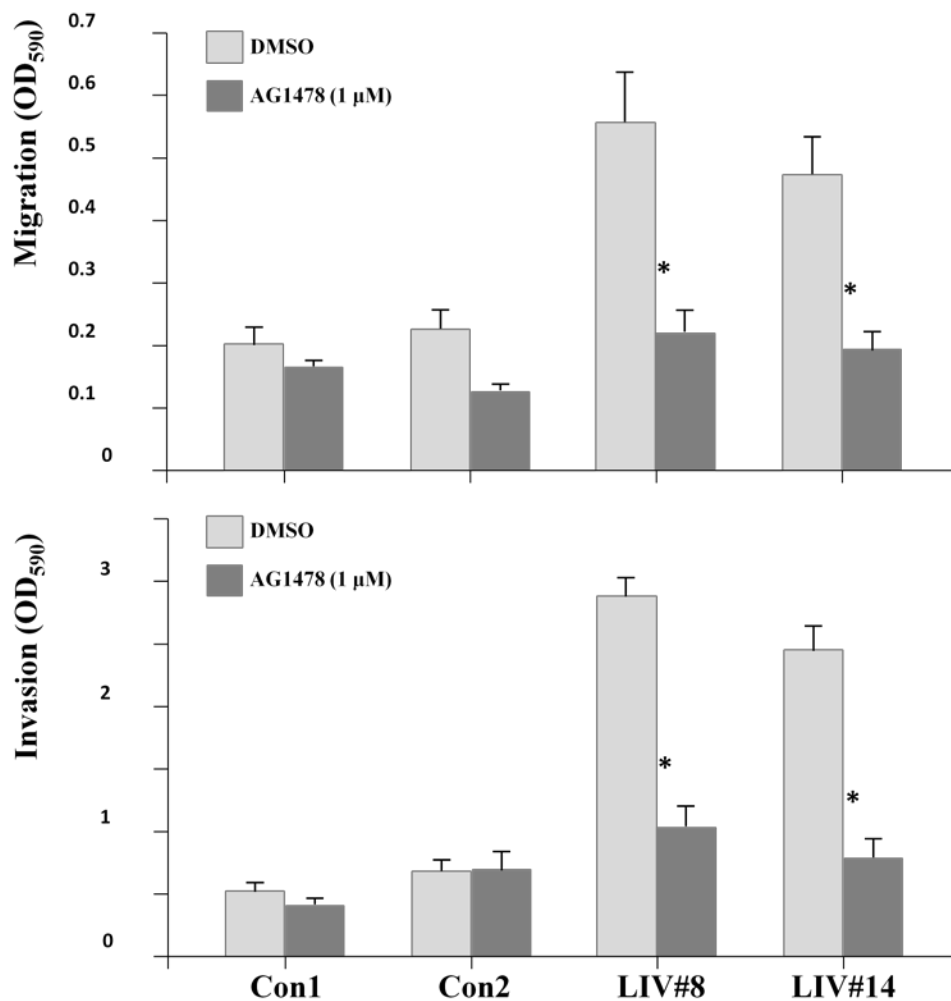


Figure 3.3

Inhibition of EGFR suppressed migratory ability and invasive ability of LIV-1 overexpressing clones in transwell assays 24 hours after the treatment. * indicates statistical significance compared to the control of the same group ($P < 0.05$).

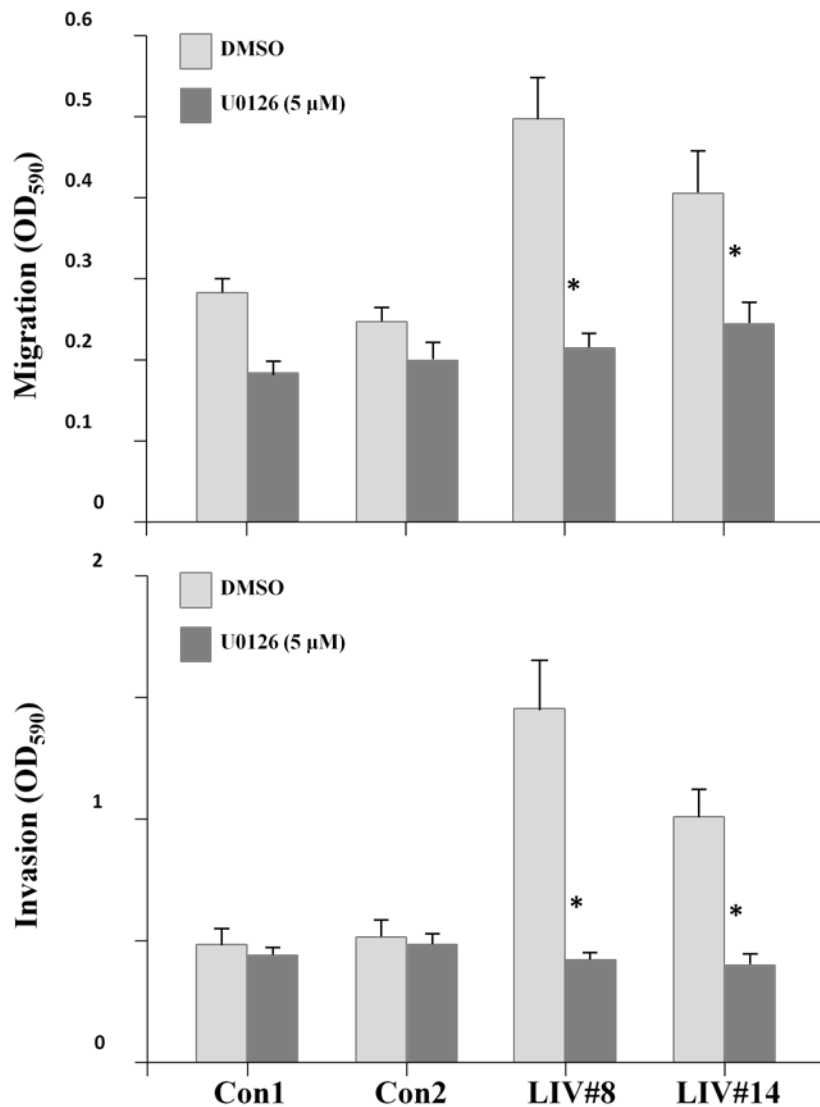


Figure 3.4

Inhibition of ERK signaling resulted in similar suppression of cellular motility to the EGFR inhibition. * indicates statistical significance compared to the control of the same group ($P < 0.05$).

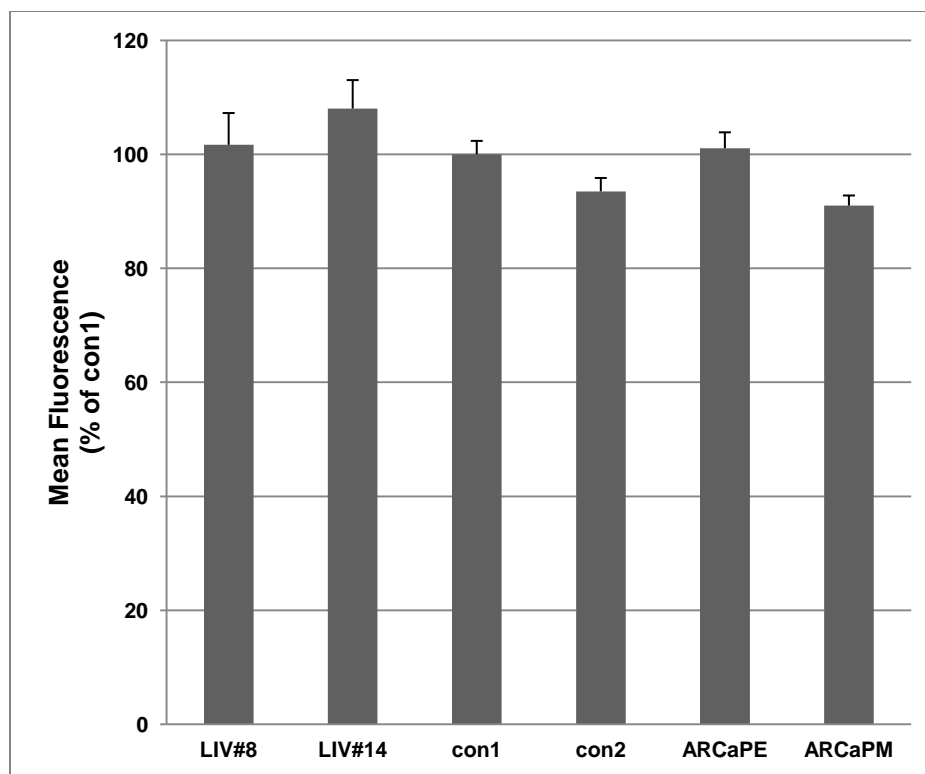


Figure 3.5

Intracellular labile Zn was assessed with Zn-specific fluorophores and measured by a fluorescence plate reader.

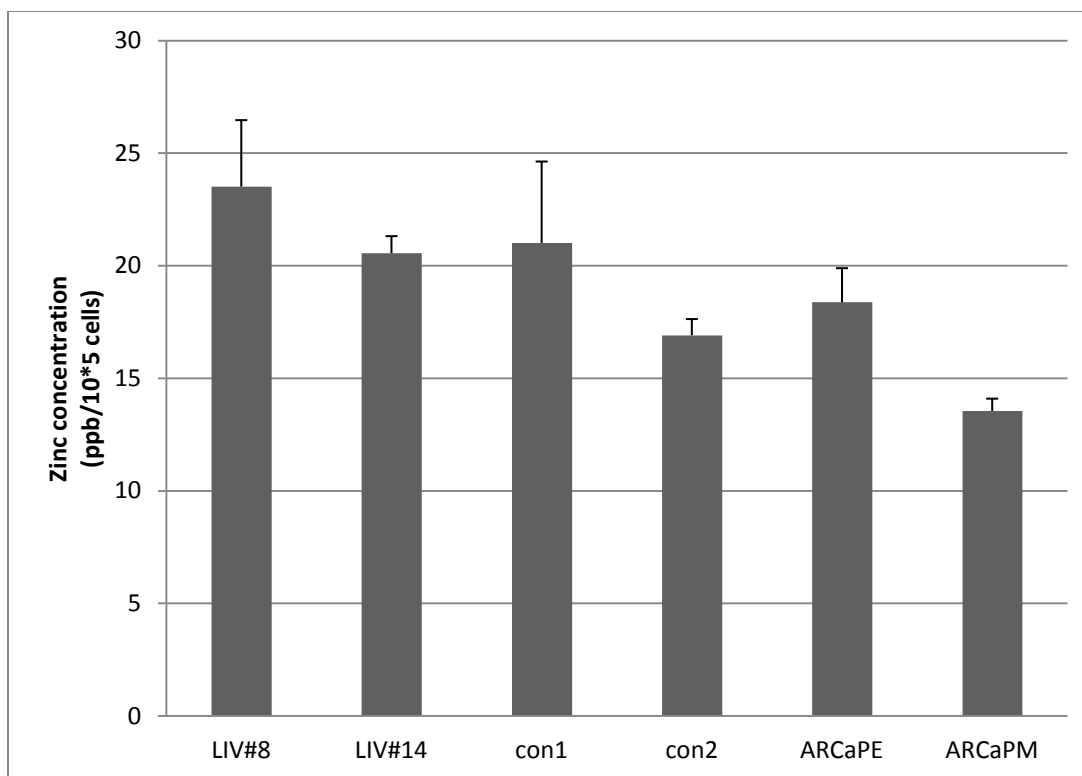


Figure 3.6

Total intracellular zinc concentration was measured by ICP-MS.

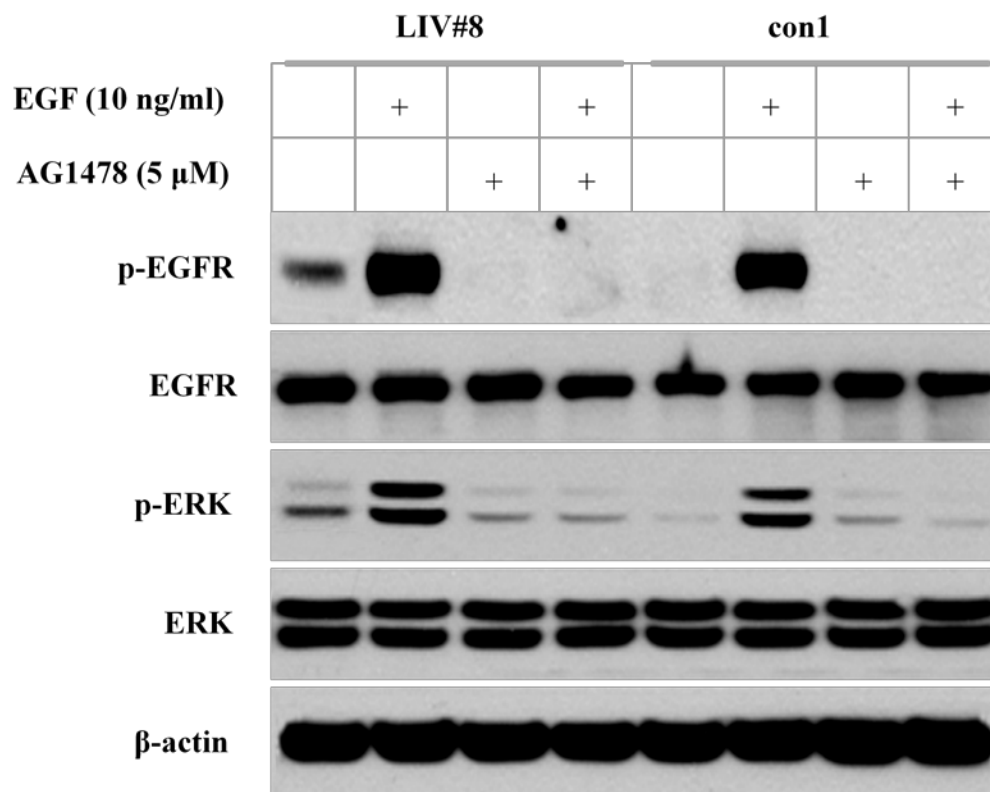


Figure 3.7

Treatment of EGF and EGFR inhibitors in LIV-1 overexpressing cells. LIV-1 overexpressing cells (LIV#8) and control cells (con1) were treated with EGF and AG1478 for 2 hours. Western blotting showed that the EGF-elicited EGFR and ERK phosphorylation was blocked by the AG1478 inhibitor.

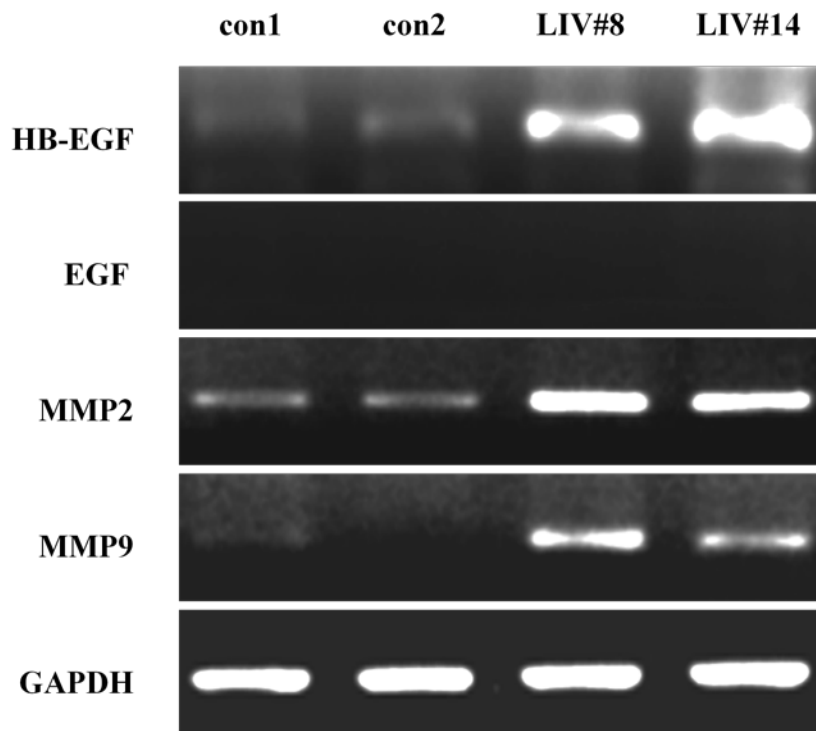


Figure 3.8

RT-PCR showed increased HB-EGF, MMP2 and MMP9 expression in LIV-1 overexpressing cells.

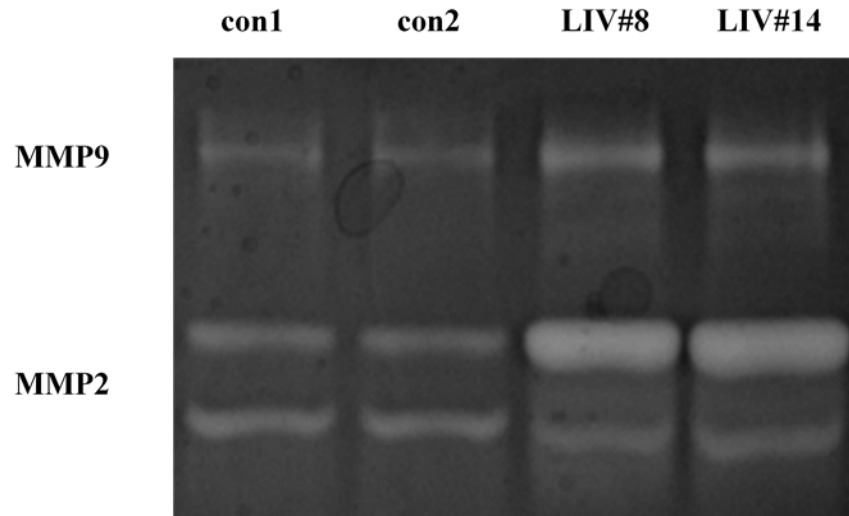


Figure 3.9

MMP2/9 activity was higher in LIV-1 overexpressing cells. LIV-1 overexpressing cells (LIV#8 and LIV#14) were cultured in serum-free medium for 24 hours and the culture media were used to determine the MMP2 and MMP9 enzymatic activity by zymogram assay.

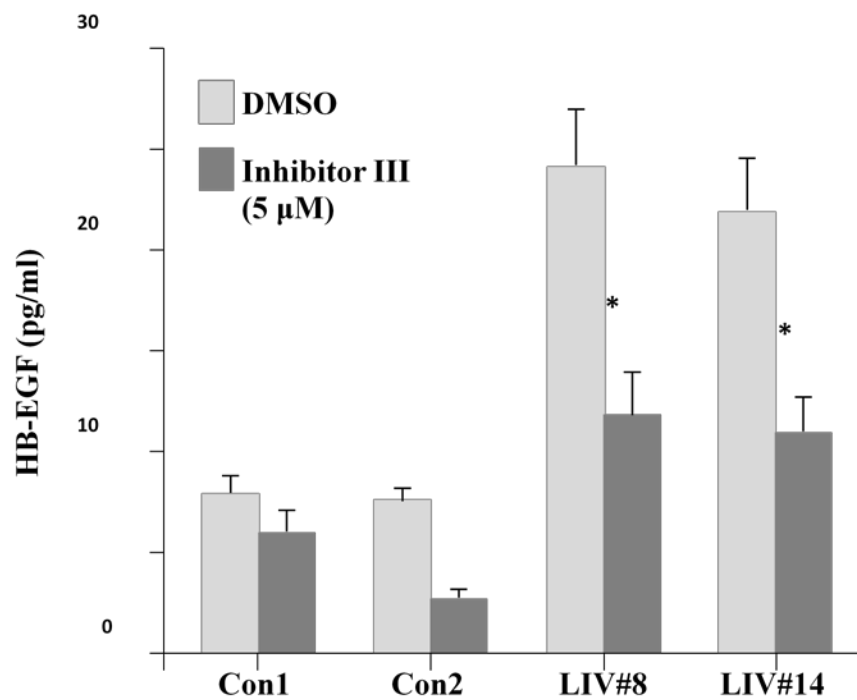


Figure 3.10

The effect of MMP2/9 enzymatic activity on HB-EGF shedding was evaluated by ELISA. LIV-1 overexpressing clones (LIV#8 and LIV#14) secreted more HB-EGF than control clones (con1 and con2), and the secretion was reduced by MMP2/9 inhibition. *indicates statistical significance compared to the control of the group ($P < 0.05$).

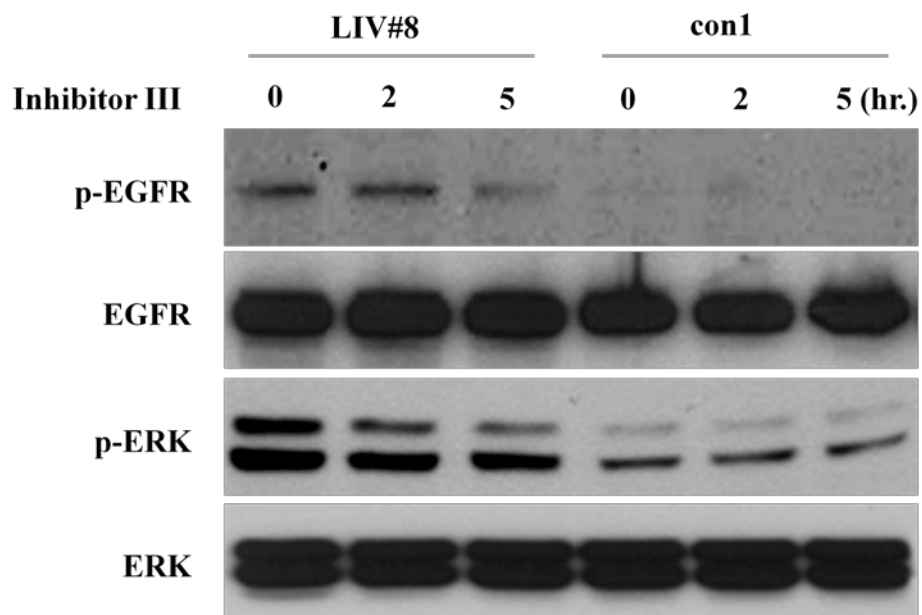


Figure 3.11

Treatment of MMP2/9 inhibitors reduced the levels of phosphor-EGFR and downstream phosphor-ERK. Western blotting showed that inhibition of MMP2/9 activity suppressed EGFR and the downstream ERK phosphorylation.

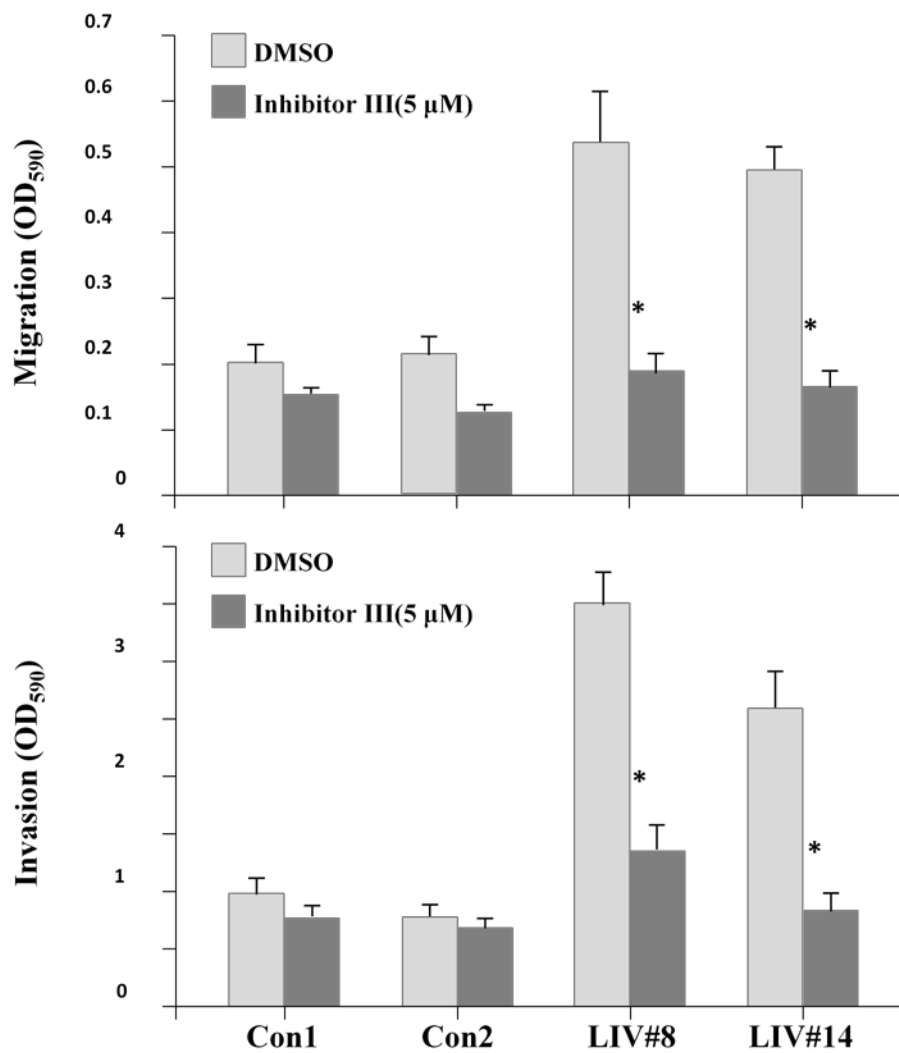


Figure 3.12

Treatment of MMP2/9 inhibitors decreased migratory and invasive ability of LIV-1 overexpressing cells. LIV-1 overexpressing cells were treated with MMP 2/9 inhibitor III for 24 hours in Transwell motility assays. Both migration and invasion of the treated cells were decreased. * indicates statistical significance compared to the control of the group ($P < 0.05$).

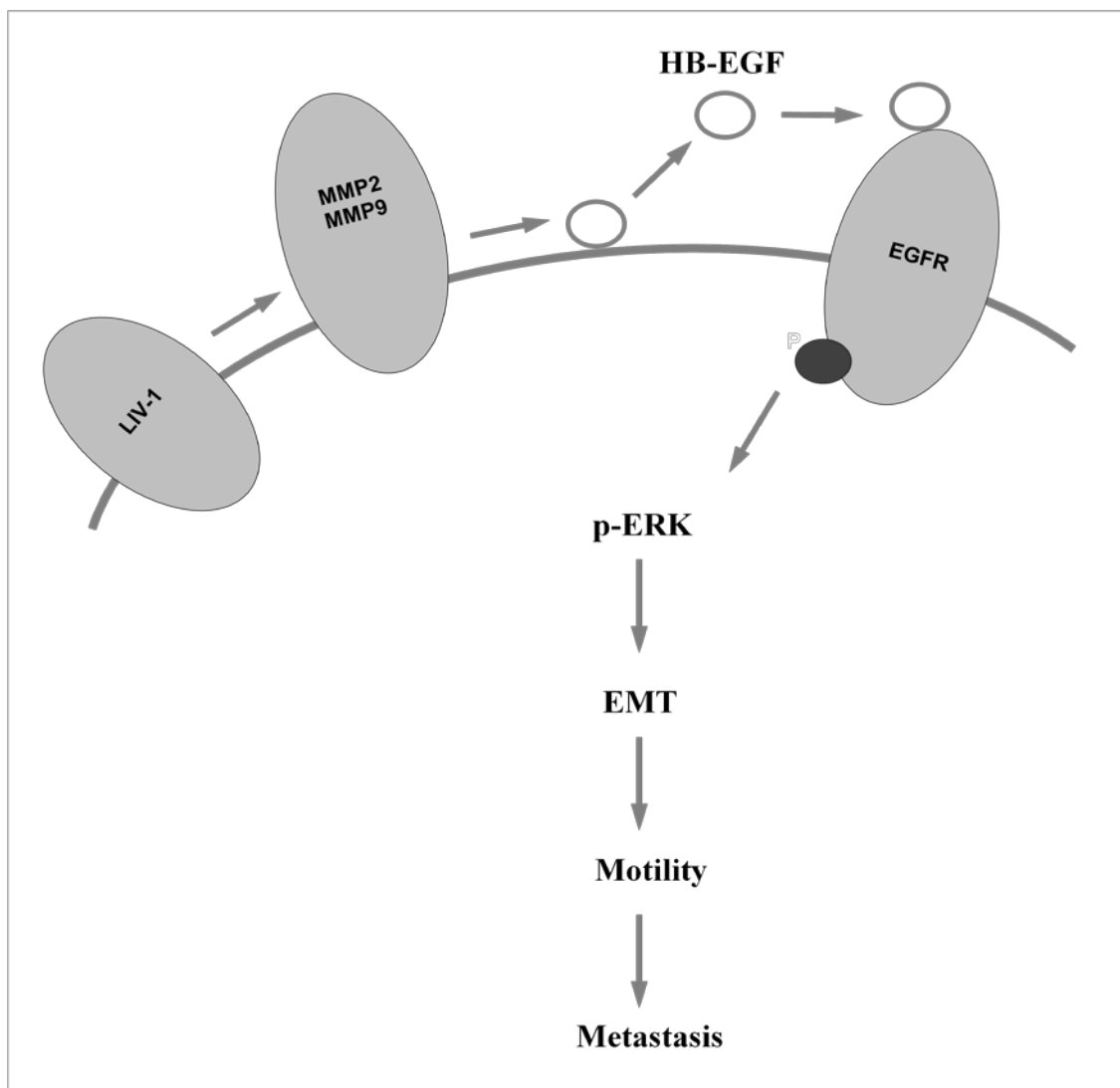


Figure 3.13

Diagram depicts the proposed role of LIV-1 in prostate cancer cell EMT and metastasis.

4 LIV-1 UPREGULATES HB-EGF PROMOTER ACTIVITY THROUGH AP-1 AND STAT3 TRANSCRIPTION FACTORS

4.1 Abstract

LIV-1, a zinc transporter, is an effector molecule downstream from soluble growth factors. LIV-1 overexpression has been shown to induce EMT and cancer progression. LIV-1 overexpression enhanced tumor cells with increased migratory and invasive capability. Constitutive activation of EGFR signaling has been demonstrated to be the major signaling contributing the malignant phenotype of tumor cells. The function of LIV-1 was seemed to stimulate the expression of MMP2, MMP9 and HB-EGF proteins, which in turn activated EGFR and downstream signaling, leading to EMT that facilitated local tumor growth and its distant metastases to bone and soft tissues. Here we found that up-regulation of HB-EGF promoter activity was significantly increased in LIV-1 overexpressing cells and AP-1 and Stat3 were the two major transcription factors controlling HB-EGF promoter activity. Blockade of AP-1 and Stat3 activity diminished the HB-EGF promoter activity. In addition, ChIP experiments showed that both AP-1 and Stat3 bound to HB-EGF promoter *in vivo*, and specific inhibitors of AP-1 and Stat3 inhibited the binding of these transcription factors to the promoter. This study provides a new understanding of the mechanism of LIV-1 driven cancer metastasis and may help to develop a better strategy of anti-tumor therapy.

4.2 Introduction

LIV-1 was first identified in the breast cancer cell line ZR-75-1 as an estrogen-regulated gene (el-Tanani and Green, 1995), and is predominately expressed in hormonal controlled tissues with high levels in breast, prostate, pituitary gland and brain (Taylor et al., 2003). EMT has been implicated in the progression of many solid tumors, including prostate cancer (Whitbread et al., 2006; Xu et al., 2006; Zhau et al., 2008) and is considered as a key molecular event in cancer progression (Thiery, 2003). LIV-1 was reported to induce EMT in zebrafish gastrula organizing cells (Yamashita et al., 2004). LIV-1 mRNA was recently shown to be higher in cervical cancer in situ than in normal tissues (Zhao et al., 2007a). RNAi mediated suppression of LIV-1 in HeLa cells significantly inhibited their proliferation, colony formation, migratory, and invasive ability (Zhao et al., 2007b). Ours and other studies have demonstrated that LIV-1 expression was elevated in clinical prostate and pancreatic carcinoma and induced EMT in prostate and pancreatic cancer cells (Unno et al., 2009). However, LIV-1 expression was also reported to correlate with E-cadherin expression (Shen et al., 2009) and be associated with better outcome of breast cancer patients (Kasper et al., 2005).

HB-EGF is a member of EGF family of growth factors and its expression is found to be increased in a lot of different cancer types. For example, HB-EGF expression was one or two orders of magnitude higher than other EGF family members in ovarian cancer patients (Miyamoto et al., 2004). In bladder cancer, HB-EGF was also been shown to express ten to hundred fold higher than other EGFR ligands (Thogersen et al., 2001), indicating that HB-EGF might be the major ligand which is responsible for EGFR activation at least in ovarian and bladder cancers.

HB-EGF has been shown to promote prostate cancer cell migration and invasion (Madarame et al., 2003). Recently, studies demonstrated that HB-EGF is involved in EMT in

gastric and ovarian cancer cells (Yagi et al., 2008; Yin et al.). Treatment of recombinant HB-EGF protein in ovarian cancer cells decreased E-cadherin expression but increased Snail expression (Yagi et al., 2008). In addition, overexpression of non-cleavable HB-EGF increased E-cadherin expression and decreased cell motility in pancreatic cancer cells (Wang et al., 2007a), suggesting a role of cancer metastasis.

The HB-EGF gene is expressed in a variety of cells and tissues and its expression has been shown to be induced by lots of signals, such as growth factors, tumor necrosis factor α , phorbol ester, thrombin and angiotensin II. HB-EGF gene is highly expressed in smooth muscle cells and MyoD, AP-1 and Ets-2 have been indicated in stimulating HB-EGF mRNA expression through binding to its promoter region during myogenesis, oxidative stress and oncogenic raf (Chen et al., 1995; McCarthy et al., 1997; Sakai et al., 2001). In addition, regulatory macrophages upregulate HB-EGF mRNA transcription through Sp-1 transcription factors (Edwards et al., 2009). Knockdown of Sp-1 abrogates HB-EGF expression by regulatory macrophages.

Previous studies showed that overexpression of LIV-1 induced EMT and cancer metastasis through HB-EGF shedding mediating EGFR activation. Here we demonstrated that HB-EGF promoter activity was significantly increased in LIV-1 overexpressing cells. AP-1 and Stat3 transcription factors were found to be essential for HB-EGF promoter activation in LIV-1 overexpressing cells. Blockade of AP-1 and Stat3 completely suppressed HB-EGF promoter activity. Since AP-1 and Stat3 have been found to play critical roles in cancer metastasis, the purpose of this study is to investigate the molecular mechanism of HB-EGF promoter activity and evaluate the therapeutic potential of HB-EGF induced cancer metastasis.

4.3 *Material and Methods*

4.3.1 Cell lines and cell culture.

Human prostate cancer ARCaP_E and ARCaP_M cells were established in our laboratory (Xu et al., 2006). The cells were cultured in T-medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA). Human embryonic kidney HEK293 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM (Invitrogen) supplemented with 10% FBS. All the culture media were supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). Cell cultures were maintained at 37°C in a humidified atmosphere supplemented with 5% CO₂.

4.3.2 Antibodies and reagents.

Antibody to c-Jun (ChIP grade) was from Abcam (Cambridge, MA). Antibody to phospho-Stat3 (Tyr705) was from Cell Signaling Technology (Danvers, MA). Monoclonal antibody to β-actin was from Sigma-Aldrich (St. Louis, MO). Antibody to RNA Polymerase II and mouse IgG were from Upstate (Billerica, MA). SB203580 and U0126 were obtained from Cell Signaling Technology (Danvers, MA). MMP2/9 inhibitor III, Stat3 inhibitor (WP1066) and AP-1 inhibitor (SR11302) were obtained respectively from Calbiochem (Darmstadt, Germany), Santa cruz biotechnology (Santa Cruz, CA), and Tocris Bioscience (Ellisville, Missouri).

4.3.3 Transfection.

HEK293 and ARCaP_E cells were seeded at 3×10^5 cells per well in 6-well plates 24 hours before transfection. The following morning the cells were transfected with 4 µg of the promoter and expression vector construct using 8 µl Lipofectamine 2000 (Invitrogen). First, 4 µg of the promoter and expression vector construct were diluted in 250µl Opti-Mem reduced serum media (Invitrogen). Then, 250µl of Opti-Mem containing 8 µl of Lipofectamine 2000 was added to 250µl Opti-Mem containing 4 µg of the promoter and expression vector constructs. The complex was incubated for 30 minutes at room temperature and then added directly to the well. The cells were allowed to incubate for 6 hours at 37 °C. The complex was removed and 2 ml of fresh culture media was added to each well. Cells were typically harvested at 24-48 hours post-transfection.

4.3.4 Drug treatment of cells.

Drug treatment of cells allows for the examination of different cellular pathways. Cells were serum starved for approximately 18-24 hours before treatment. As a control, the substance used to dissolve the drug was added to cells alone. Once the treatment was complete the cells were harvested for further analysis.

4.3.5 Dual Luciferase Reporter assay.

Dual Luciferase Reporter assay system (Promega) was used to analyze promoter activity. Cells were transfected with a firefly promoter plasmid and a renilla reporter plasmid as a internal control with or without an expression plasmid. After 24-48 hours post-transfection, the cells were directly lysed using 1X Lysis buffer rocking at room temperature for 15-20 minutes. 20 μ l of the cell lysate was added to a 96 well plate and then 100 μ l of the Luciferase substrate was added to the sample. The samples were read on an EnSpire® Multimode Plate Reader (PerkinElmer) to read the firefly luciferase activity first. Then the Stop-&- Glo reagent was added to the sample and measure again to read the control Renilla activity. The results were a normalization of the Firefly with Renilla luciferase RLU (Firefly/Renilla) from each sample.

4.3.6 Semiquantitative expression analysis with RT-PCR.

Total RNA was isolated from cultured cells using the RNeasy Mini kit (Qiagen, Valencia, CA). From each sample, equal amount of RNA (2 μ g) was used in first-strand cDNA synthesis reaction with the Superscript First-Strand cDNA Synthesis kit (Invitrogen). Equal volume of cDNA (3 μ l) from each reaction was used for PCR analysis using gene-specific oligonucleotide primer pairs: 5'- TGCCCGGCGGAATCTCCTGA -3' and 5'- GATGCAGGAGGGAGCCCGGA -3' for HB-EGF; and 5'-TTAGCACCCCTGGCCAAGG-3' and 5'-CTTACTCCTTGGAGGCCATG-3' for GAPDH. The reactions were initiated with a 4-minute incubation at 94°C, followed by 32 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 1 minute. The reaction was completed with an extension at 68°C for 10 minutes.

PCR products were visualized after electrophoresis through a 1.2% agarose gel and stained by ethidium bromide (0.5 µg/ml).

4.3.7 Chromatin Immunoprecipitation (ChIP).

EZ-ChIP™ kit from Upstate was used to analyze whether a specific transcription factor binds to a known segment of DNA. Cells were seeded in 100 mm culture dishes in culture media so that the following day the confluence would be approximately 90% . Cells were crosslinked by adding Formaldehyde to a final 1% concentration. The cells were incubated for 10 minutes at room temperature. Next, 10X glycine (final concentration 1X) was added to quench unreacted formaldehyde and the cells were incubated for 5 minutes at room temperature. The medium was removed and the cells were washed twice with ice-cold 1x PBS. The cells were harvested in 2 ml PBS containing 1X protease inhibitor cocktail II and centrifuged at 700 g at 4°C for 2-5 minutes. The cells were then lysed in 1 ml SDS Lysis Buffer containing 1X Protease Inhibitor Cocktail II. After Lysis, the samples were sonicated in an ice-water bath with 12 sets of 15-second pulses and 45-second off so that the size of the DNA fragment would be 200-1000 base pairs. The samples were then centrifuged to remove the insoluble materials. 900 µl of Dilution Buffer was added to a 100 µl aliquot of each sample and 60 µl of Protein G Agarose was then added to pre-clear the lysate. Prior immunoprecipitation, a 20 µl pre-cleared lysate was aliquoted as an input control. Next, immunoprecipitation was performed with 1-5 µg of anti-phospho Stat3 or anti-c-Jun antibodies overnight at 4°C with rotation. For positive control, anti-RNA polymerase II was used. For negative control, normal mouse IgG was used. The following day 60 µl of Protein G Agarose beads was added to each sample and incubated for 1 hour at 4°C with rotation. The samples were centrifuged at 1200 rpm for 1 minute and the supernatant was removed. The beads

were then washed with 1 ml each of the cold biffer in the order, Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Immune Complex Wash Buffer, and TE Buffer twice. Following the wash, 100ul of elution buffer (20% SDS, 0.1M NaHCO₃) was added to the beads and incubate for 15 minutes at room temperature to elute the protein/DNA complex. This step was repeated and total of 200 µl eluted sample were collected. The eluted chromatin was then subjected to reverse crosslinking by adding 8 µl of 5M NaCl and incubated at 65°C overnight. The samples was then added 1 µl of RNase A and incubate at 37°C for 30 minutes. Next, 4 µl 0.5 M EDTA, 8 µl 1M Tris-HCl and 1 µl Proteinase K were added to each sample and the samples were incubated at 45°C for 1-2 hours. The DNA was then purified by using Spin Columns and the purified DNA was analyzed with Real-time quantitative PCR.

4.3.8 Real-time Quantitative PCR.

2 µl of purified DNA, 250 nM of the appropriate primers, 1X Fast SYBR Green master mix (Applied Biosystems) and ddH₂O to 20 µl in a MicroAmp fast 96-well Reaction Plate (Applied Biosystems). The plate was covered with a MicroAmp Optical Adhesive Film (Applied Biosystems). The reaction was performed in the Applied Biosystems 7500 Fast Real time system. The primers were listed below: Stat3-F: 5'-AGGGAGGGACAGTGGGATAG-3'; Stat3-R: 5'-CTTCCAACCCATGTGCTTTT-3'; AP-1-F: 5'-AAACAACCTCTCTCCCCAGCA-3'; AP-1-R: 5'-TTTGACTGCCACCTTTTCC-3'; GAPDH-F: 5'-TACTAGCGGTTTTACGGGCG-3'; GAPDH-R: 5'-TCGAACAGGAGGAGCAGAGAGCGA-3'. The reactions were initiated with 15 seconds incubation at 94°C, followed by 40 cycles at 94°C for 3 seconds, 60°C for 30 seconds.

The Ct values were measured and relative Ct method was used to do the following analysis. The results were expressed as a fold difference manner ($2^{-(\Delta\Delta Ct)}$) and plotted in chart form.

4.3.9 Site-directed Mutagenesis.

QuikChange II Site-Directed Mutagenesis Kit (Stratagene) was used to generate the desired mutations. Mutagenesis primers were designed using Stratagene's Primer Design program. For Stat3 Mutant: sense- 5'-acgcgtcacgtccctggccactgaaatacctactgag-3'; antisense- 5'-ctcagtaggtatttcagtgccaggacgtgacgcgt-3'. For AP-1 Mutant1: sense- 5'-atctcttgggtcaggacagagatcccagaagaccctgcaaaag-3'; antisense-5'-cttttcaggggtcttctgggatctctgtcctgacccaagaaat-3'. For AP-1 Mutant2: sense- 5'-agctgggtcctatgggaggagggtccatttttatgttgaggaaaa-3'; antisense- 5'-tttctcaacataaaaaatggacctccctcccataggaccagct-3'. PCR was performed with the HB-EGF promoter construct and the mutagenesis primers according to manufacturer's protocol. After PCR, Dpn I was added to the reaction and incubated for 1 hour at 37°C to remove the unmutated plasmid DNA. Then the mutated plasmid was transformed and the colonies were chosen and sent for sequencing to confirm mutation.

4.4 Results

4.4.1 LIV-1 overexpressing cells up-regulates the transcription of HB-EGF through a phosphor-ERK and phosphor-Stat3 dependent pathway.

Previous studies we have shown that LIV-1 promoted prostate cancer EMT, progression and metastasis. The function of LIV-1 was to stimulate the expression of MMP2, MMP9 and HB-EGF proteins. MMP2 and MMP9 then cleaved membrane-bound HB-EGF to release more soluble form of HB-EGF, which in turn activated EGFR to activate EGFR and downstream p-ERK and p-Stat3 signaling pathways, leading to EMT that facilitated local tumor growth and its distant metastases to bone and soft tissues. In addition, we found that inhibition of MMP2/9 resulted in a sensitive reduction of EGFR activation as well as downstream p-ERK and p-Stat3 signaling (Figure 4.1).

Since LIV-1 overexpressing cells expressed higher HB-EGF mRNA as well as soluble HB-EGF in culture media (Figure 4.2), We then examined whether the increase of HB-EGF mRNA was due to increase in HB-EGF transcription. We used a 1.1kb human HB-EGF promoter following a firefly luciferase reporter and transfected the HB-EGF promoter with a renilla reporter as an internal control into LIV-1 overexpressing cells and control cells. We found that HB-EGF promoter activity showed a 4-5 fold of increase in LIV-1 overexpressing cells comparing to control cells (Figure 4.3). This result suggested that LIV-1 overexpression causes a transcriptional increase of HB-EGF expression. Next, we explored the mechanism controlling HB-EGF transcription. In previous study, we examined the phosphorylation status of AKT, p38, JNK, Smad, NF- κ B, β -catenin and ERK in LIV-1 overexpressing cells because these regulatory proteins were shown to be altered by a soluble growth factor, β 2-M, which also promoted EMT

and LIV-1 expression. We observed that two major signaling pathways were significantly activated which are p-ERK and p-Stat3 (Figure 4.4). To test whether p-ERK and p-Stat3 were involved in the activation of HB-EGF transcription, we performed the promoter assay with different drug treatment. We used U0126 and Stat3 inhibitor to block p-ERK and p-Stat3 activation and we also include a p38 MAPK inhibitor which has been shown to regulate HB-EGF expression. We observed that both U0126 and Stat3 inhibitor suppressed HB-EGF promoter reporter activity, but not SB203580 (Figure 4.5). Additionally, we treated LIV-1 overexpressing cells and control cells with U0126 and Stat3 inhibitor and isolated total mRNA to do a RT-PCR analysis. The result also showed that HB-EGF mRNA was significantly reduced (Figure 4.6). These results suggested that LIV-1 overexpressing cells may up-regulate the transcription of HB-EGF through a phosphor-ERK and phosphor-Stat3 dependent pathway.

4.4.2 Stimulation of the HB-EGF promoter is mediated by AP-1 and phosphor-Stat3 transcription factors.

Since p-ERK and p-Stat3 are involved in the stimulation of HB-EGF promoter activity, we then explored whether AP-1 transcription factor could be the one to regulate HB-EGF promoter activity. AP-1 has been shown to stimulate mouse HB-EGF promoter in mouse smooth muscle cells. In addition, AP-1 is one of the major downstream transcription factor activated by p-ERK and multiple potential AP-1 response elements has been found in HB-EGF promoter sequence by using computer software. We then test whether AP-1 is involved in stimulating HB-EGF promoter activity. To this end, we first used an AP-1 response element-luciferase construct to transfect into LIV-1 overexpressing cells and control cells to see whether AP-1 has been acti-

vated. Clearly, AP-1-luc activity was 6 fold higher in the LIV-1 overexpressing cells comparing to control ones, indicating that AP-1 was activated in LIV-1 overexpressing cells. Furthermore, the increased AP-1-luc activity could be reduced by treating AP-1 inhibitors (Figure 4.7); indicating that AP-1 is activated and AP-1 inhibitors could successfully reduce AP-1 binding to its response element. Next, a similar experiment was carried out with transfecting a HB-EGF promoter reporter and treating with or without AP-1 inhibitors. We found that HB-EGF promoter activity could be reduced to half by treating AP-1 inhibitors, suggesting that AP-1 might be involved in the stimulation of HB-EGF transcription (Figure 4.8). We further confirmed that AP-1 and p-ERK acted in the same pathway. Since both U0126 and AP-1 inhibitor could reduce HB-EGF promoter activity, but no additive effect has been observed with two drugs treating at the same time (Figure 4.9).

Previously we have shown that Stat3 inhibitor suppressed HB-EGF promoter activity, we then used a Stat3 constitutive active expression construct and a dominant negative construct to rule out the toxicity and specificity effect of the drug treatment. HB-EGF promoter reporter was co-transfected with a Stat3 constitutive active expression construct or a dominant negative construct into ARCaP_E cells. Clearly, HB-EGF promoter activity was stimulated by co-transfecting a Stat3 constitutive active expression construct and was a little bit lower by co-transfecting a Stat3 dominant negative expression construct (Figure 4.10). Similar result has been observed when transfecting into HEK293 cells (data not shown). Since both AP-1 and Stat3 inhibitor could reduce HB-EGF promoter activity, we next explored whether HB-EGF promoter activity would be diminished by treating AP-1 and Stat3 inhibitors simultaneously. The result showed that treatment of AP-1 and Stat3 inhibitors completely blocked HB-EGF promoter activity in LIV-1 overexpressing cells (Figure 4.11). Additionally, we treated LIV-1 overexpressing cells and control

cells with U0126, Stat3 inhibitor or both and isolated total mRNA to do a RT-PCR analysis. The result also showed that HB-EGF mRNA was significantly reduced (Figure 4.12). These results indicated that AP-1 and Stat3 are the major transcription factors stimulating HB-EGF transcription.

4.4.3 Between -1022 and -682 on the HB-EGF promoter is necessary for promoter activity in LIV-1 overexpressing cells.

Serial deletions of HB-EGF promoter luciferase reporter constructs were generated in order to locate the responsive element. We used PCR to subclone FL, Del1 and Del2 promoter first to Topo TA vector. Next, we used restriction enzyme to cut the fragment and subcloned to pGL3 basic vector (Figure 4.13). Since there were two potential Stat3 binding elements, we want to map the Stat3 binding position. FL, Del1, or Del2 promoter reporter construct was co-transfected with a Stat3 constitutive or dominant negative expression vector into ARCaP_E cells. Only FL promoter reporter construct was able to stimulate by a constitutive Stat3 (Figure 4.14), indicating that responsive element necessary for Stat3 stimulation lies between -1022 to -682. We further transfected FL, Del1, or Del2 promoter reporter construct into LIV-1 overexpressing cells and control cells. Del1 and Del2 promoter construct diminished all the promoter activity comparing to the FL promoter reporter construct (Figure 4.15), suggesting that both AP-1 and Stat3 stimulate HB-EGF promoter activity through this region (-1022 to -682). Furthermore, Del1 and Del2 promoter construct did not show any effect by treating with a Stat3 inhibitor, AP-1 inhibitor or both. HB-EGF FL promoter reporter activity is significantly diminished after treating of both Stat3 inhibitor and AP-1 inhibitor (Figure 4.15). These results indicate that AP-1 and Stat3 medi-

ated HB-EGF promoter activity through this region (-1022 to -682) and are the major transcription factors stimulating HB-EGF promoter activity.

4.4.4 Proximal AP-1 and Stat3 binding elements are required for HB-EGF activity in LIV-1 overexpressing cells.

To investigate the specific binding sites of AP-1 and Stat3 were required for the HB-EGF promoter activity, we used computer analysis to identify consensus AP-1 and Stat3 binding sites in the -1022 to -682 region of HB-EGF promoter sequence. A potential Stat3 binding site located at -945 and two potential AP-1 binding sites locate at -846 and -745 (Figure 4.16). We introduced site-specific mutations into the FL promoter reporter construct at one Stat3 and two AP-1 sites. The mutations we chose were according to published paper which showed successful blocking AP-1 and Stat3 binding and the desired mutations were confirmed by DNA sequencing (Figure 4.17). We then transfected FL wild type, mut stat3, mut AP-1 site1, and mut AP-1 site2 into LIV-1 overexpressing cells and control cells. Mut Stat3 and mut AP-1 site1 promoter reporter constructs showed reduced promoter reporter activity similar to previous results treating by inhibitors (Figure 4.18). However, mut AP-1 site2 still displayed approximately the same activity comparing to the wild type. We also used Stat3 and AP-1 inhibitors to treat the cells transfected with mut stat3 and mut AP-1 site promoter reporter constructs to further confirmed the mutant constructs no longer response to the inhibitors (data not shown). Furthermore, we generated a double mutant which had both mutations of Stat3 and AP-1 site1 and then transfected into LIV-1 overexpressing cells and control cells. Clearly, the double mutant promoter construct almost lost of promoter activity (Figure 4.19). These results suggested that Stat3 (-945) and AP-1(-

846) binding elements are required for the activation of Hb-EGF promoter in LIV-1 overexpressing cells.

4.4.5 AP-1 and phosphor-stat3 bind to HB-EGF promoter in vivo.

The previous results suggested the role of AP-1 and Stat3 in stimulation of HB-EGF promoter activity. To further examine the binding of AP-1 and Stat3 to HB-EGF promoter in vivo, we performed ChIP assays by using c-Jun and phosphor-Stat3 antibodies in LIV-1 overexpressing cells and control cells. Both anti-c-Jun and anti-phospho-Stat3 antibodies did precipitate significant higher amount of the HB-EGF promoter in LIV-1 overexpressing cells comparing to control ones (Figure 4.20 A&B). We also used anti-RNA polymerase II antibody and mouse IgG to immunoprecipitate the GAPDH promoter as a positive and a negative control (Figure 4.20 C). In addition, we treated the Stat3 inhibitor to LIV-1 overexpressing cells and control cells and did the ChIP experiment by using an anti-phospho-Stat3 antibody and anti-c-Jun antibody. The Stat3 inhibitor significantly reduced the amount of HB-EGF precipitated by anti-phospho-Stat3 antibodies (Figure 4.21A). Additionally, the Stat3 inhibitor did not affect HB-EGF promoter precipitated by anti-c-Jun antibodies (Figure 4.21B). The similar ChIP experiments were also carried out by treating AP-1 inhibitors in LIV-1 overexpressing cells and control cells and probing with an anti-c-Jun antibody and anti-phospho-Stat3 antibody. Similarly, the AP-1 inhibitor diminished the amount of HB-EGF precipitated by anti-phospho-c-Jun antibodies (Figure 4.22A) and the AP-1 inhibitor did not affect HB-EGF promoter precipitated by anti-phospho-Stat3 antibodies (Figure 4.22B). We also included a ChIP experiment by using anti-RNA polymerase II as a control to show the specificity of the inhibitors (Figure 4.23).

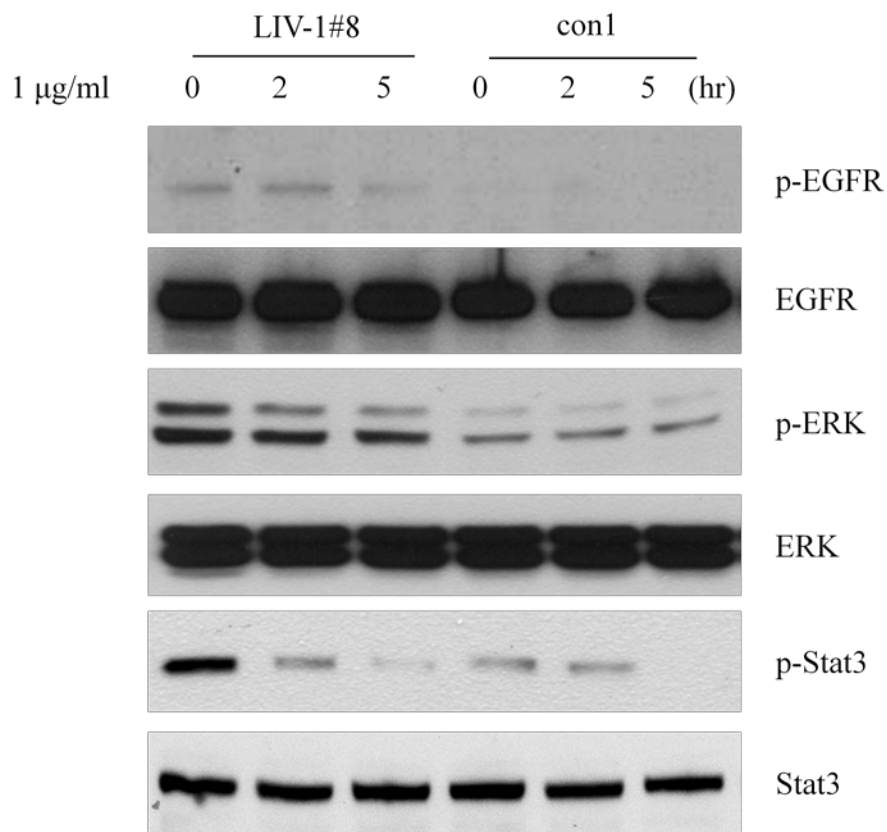


Figure 4.1

MMP 2/9 inhibitors reduced the EGFR and downstream ERK and Stat3 signaling. LIV-1 over-expressing cells and control cells were treated by MMP inhibitor for 0, 2 and 5 hours. Phospho-EGFR activity was decrease as well as p-ERK and p-Stat3.

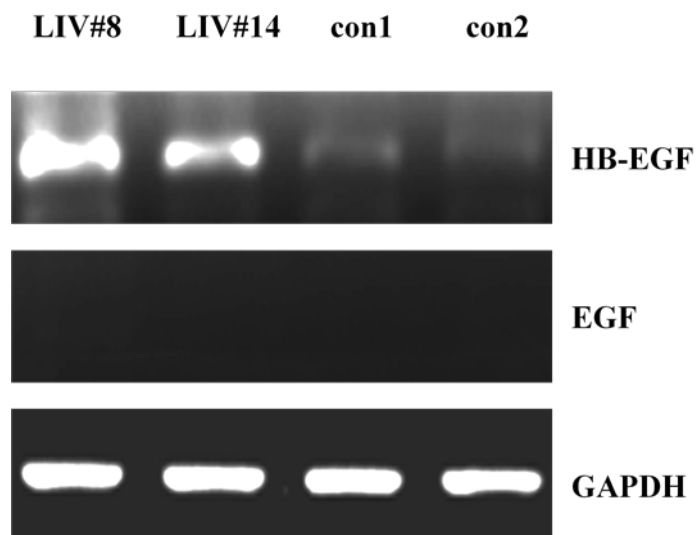


Figure 4.2

HB-EGF mRNA expression was increased in LIV-1 overexpressing cells. RT-PCR showed that LIV-1 overexpressing cells expressed much more HB-EGF transcript but not EGF. GAPDH was used as a control.

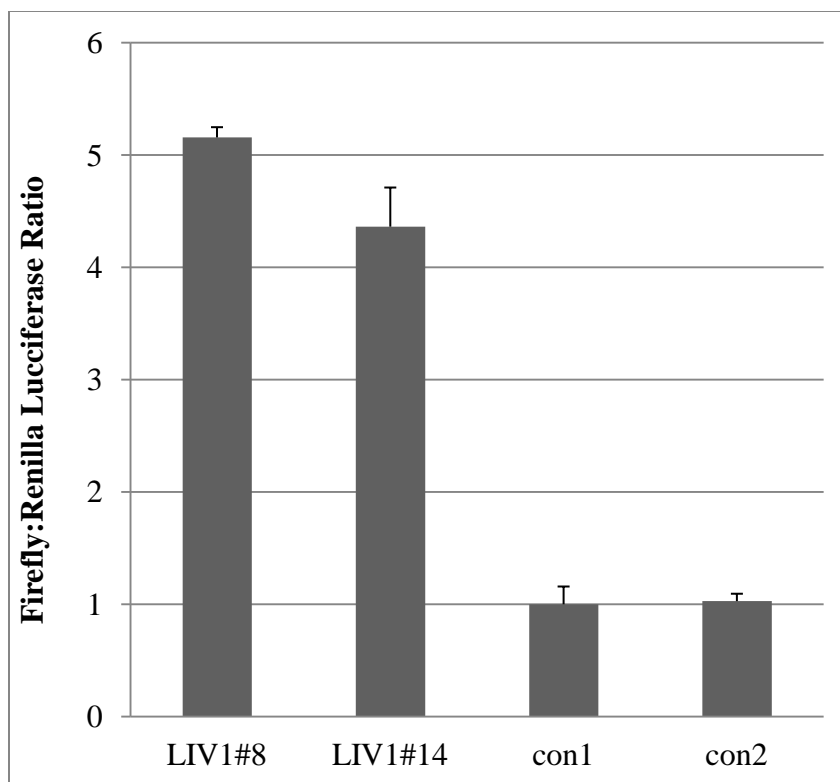


Figure 4.3

HB-EGF promoter activity is significantly increased in LIV-1 overexpressing cells. HB-EGF promoter firefly luciferase reporter was transfected into LIV-1 overexpressing cells and control cells. LIV-1 overexpressing cells showed 4-5 fold of increase in HB-EGF promoter activity. Renilla luciferase reporter was used as a internal control and all the results were normalized with Renilla activity.

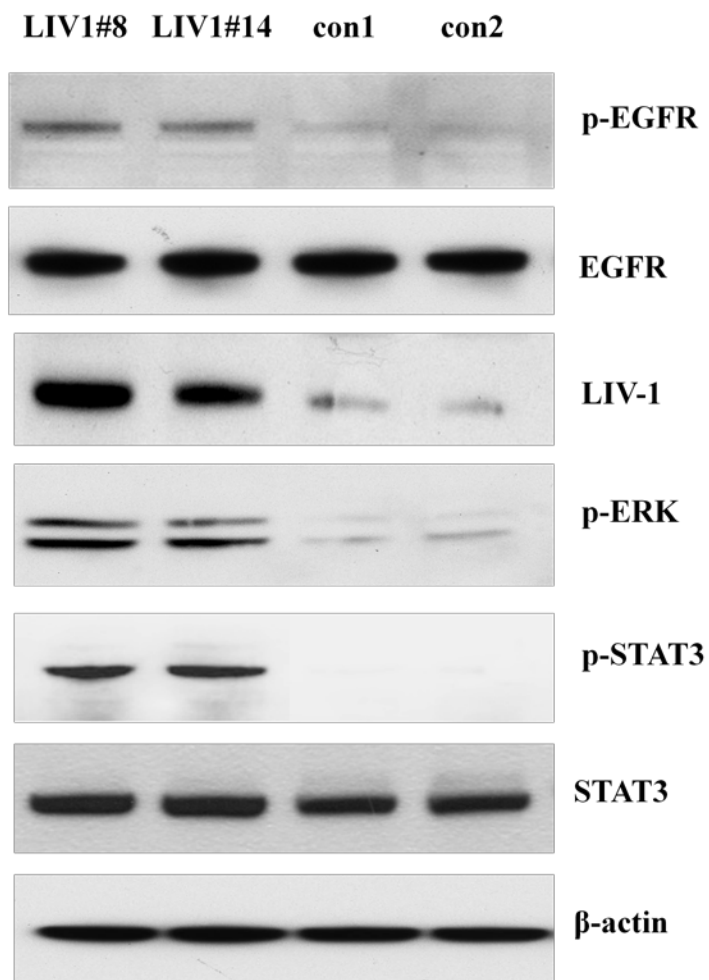


Figure 4.4

The signaling pathways in LIV-1 overexpressing cells. Western blotting showed that LIV-1 overexpressing cells have constitutively activated phospho-EGFR and activated downstream p-ERK and p-Stat3 signaling.

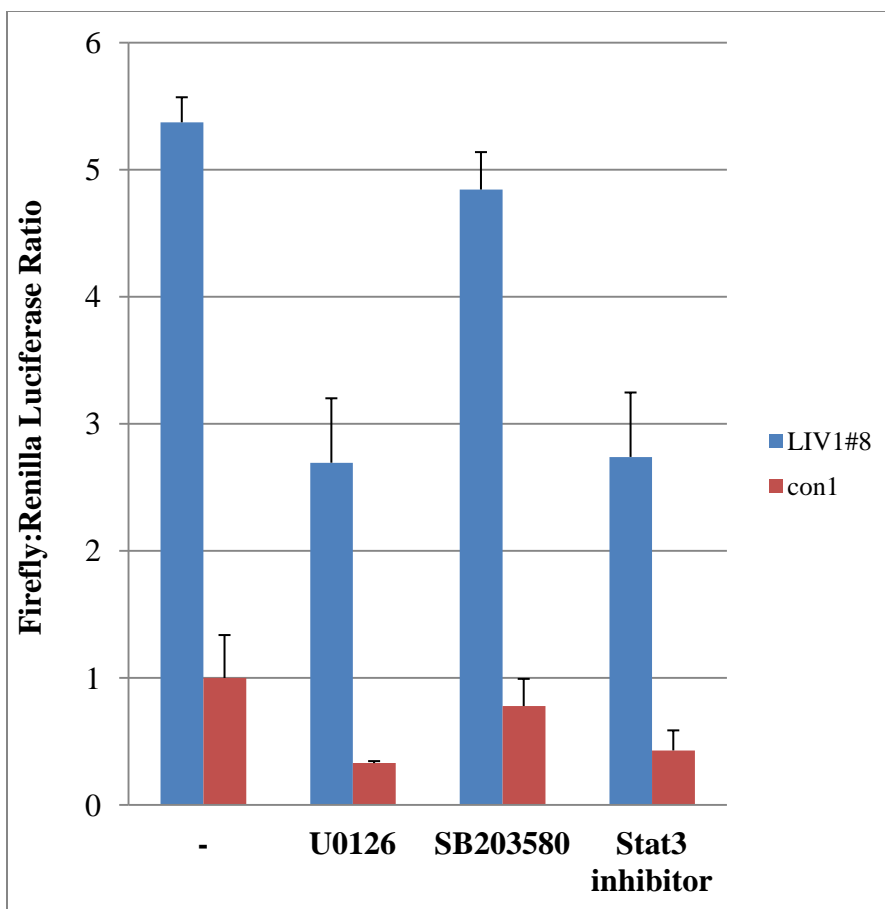


Figure 4.5

Different drugs were used to examine the importance of each signaling pathway. HB-EGF promoter was transfected into LIV1#8 and con1 and then treated with U0126 (10uM), SB203580 (10uM) or stat3 inhibitor (2uM). The result showed that both U0126 and stat3 inhibitor suppressed HB-EGF promoter activity but not SB203580.

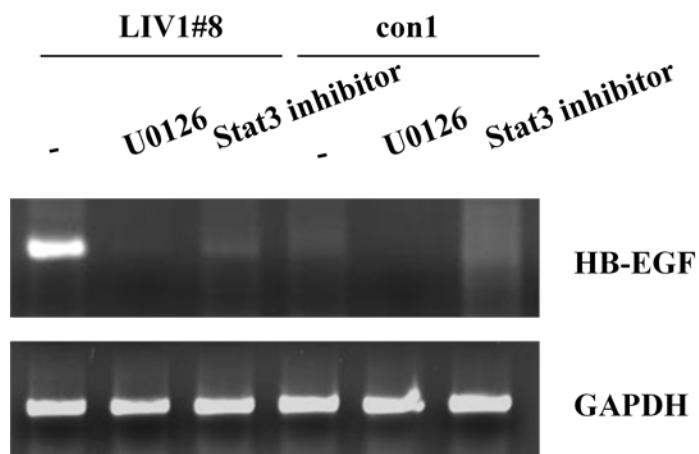


Figure 4.6

Inhibition of ERK and Stat3 signalings suppressed HB-EGF mRNA expression *in vivo*. LIV-1 overexpressing cells and control cells were treated with U0126 (10uM) or stat3 inhibitor (2uM) for 8 hours. RT-PCR showed that both U0126 and stat3 inhibitor suppressed HB-EGF transcript.

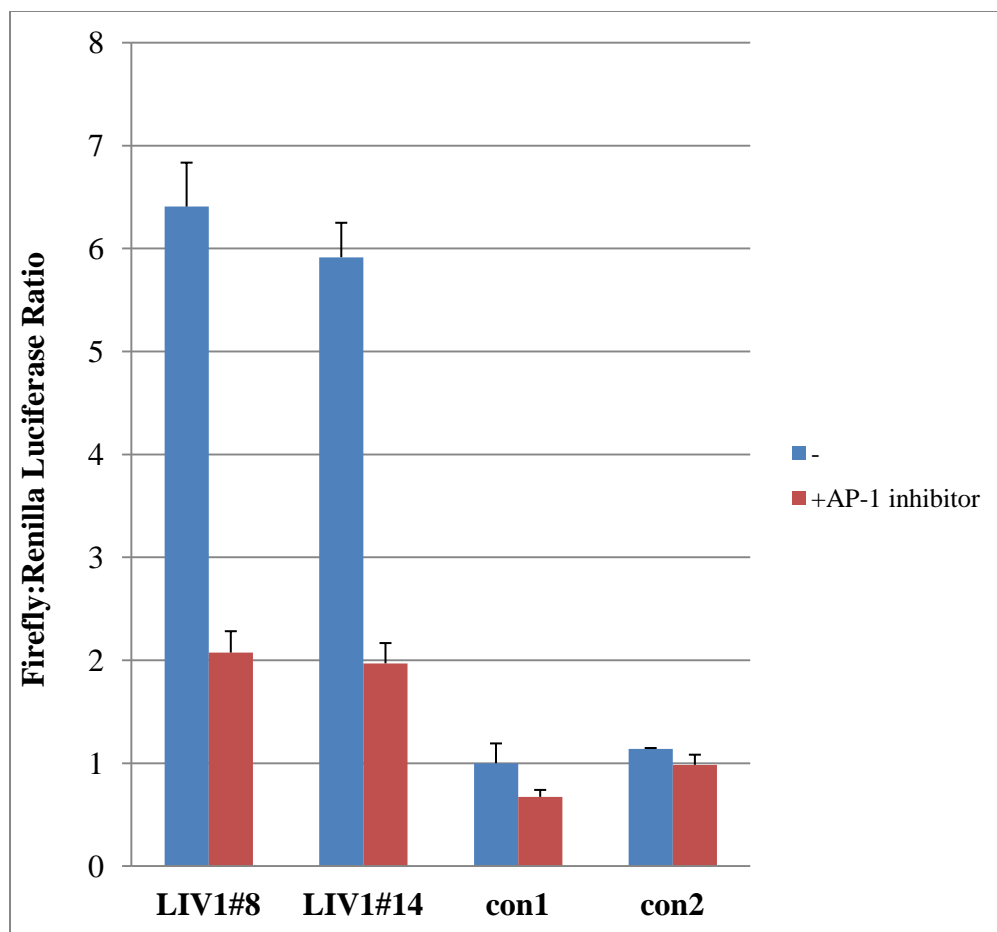


Figure 4.7

AP-1 is one of the major transcription factors downstream of ERK signaling. LIV-1 overexpressing cells and control cells were transfected with AP-1 response element-luc reporter with or without treatment of AP-1 inhibitor. Renilla reporter was an internal control and the results were normalized with Renilla reporter activity.

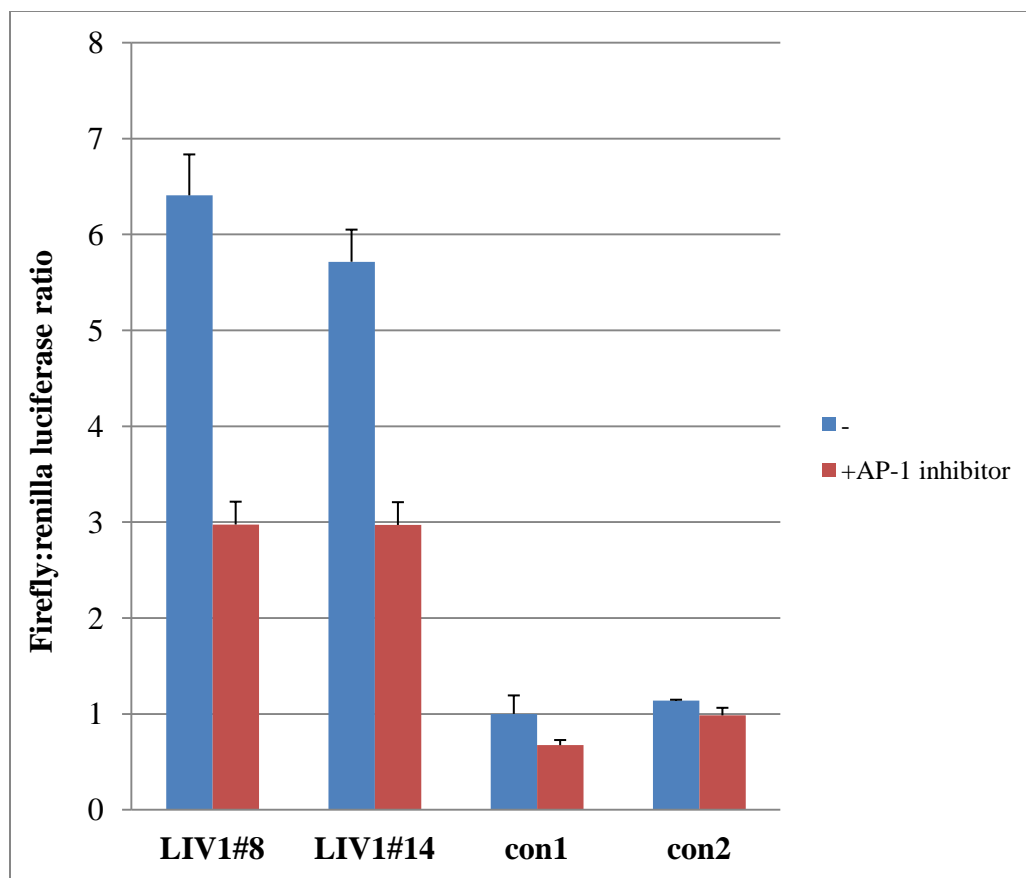


Figure 4.8

Inhibition of AP-1 reduced HB-EGF promoter activity in LIV-1 overexpressing cells. LIV-1 overexpressing cells and control cells were transfected with HB-EGF promoter reporter with or without treatment of AP-1 inhibitor. Renilla reporter was an internal control and the results were normalized with Renilla reporter activity.

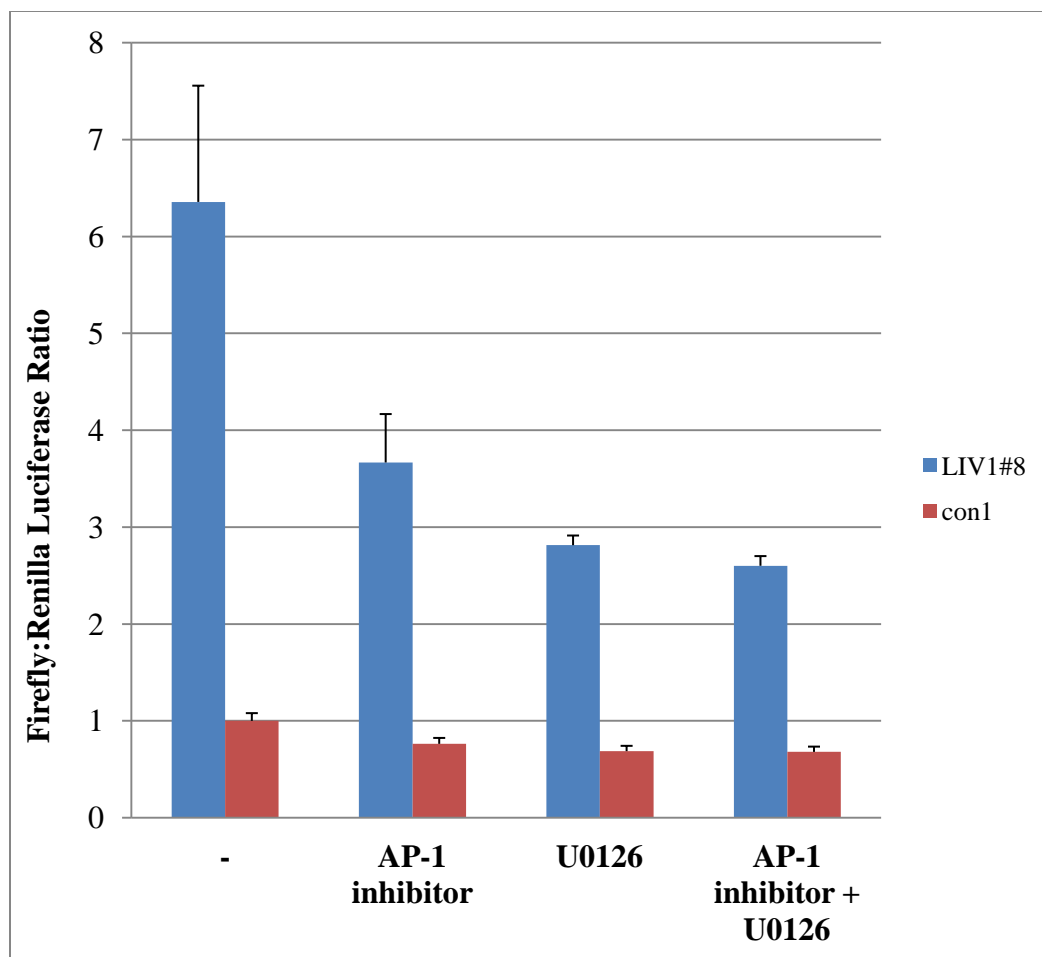


Figure 4.9

ERK and AP-1 acted in the same pathway. LIV-1 overexpressing cells and control cells were transfected with HB-EGF promoter reporter and treated with AP-1 inhibitor, U0126 or both. Renilla reporter was an internal control and the results were normalized with Renilla reporter activity.

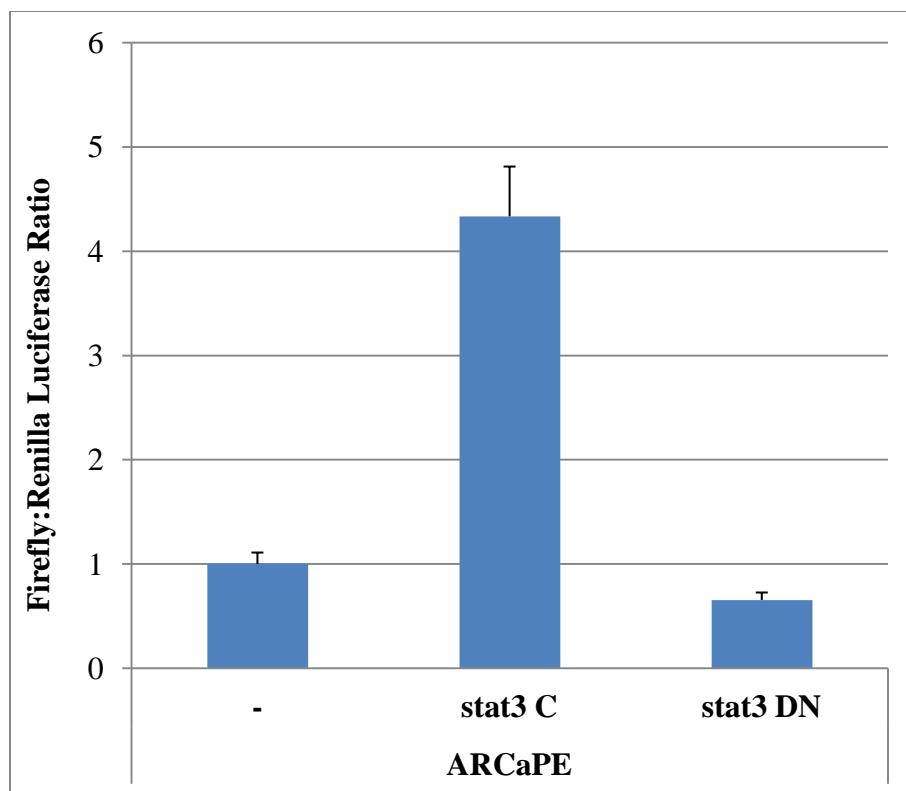


Figure 4.10

Overexpression of constitutive active Stat3 stimulated HB-EGF promoter activity in ARCaP_E cells. ARCaP_E cells were co-transfected with HB-EGF promoter reporter and Stat3 constitutive active or dominant negative expression vector. Renilla reporter was an internal control and the results were normalized with Renilla reporter activity.

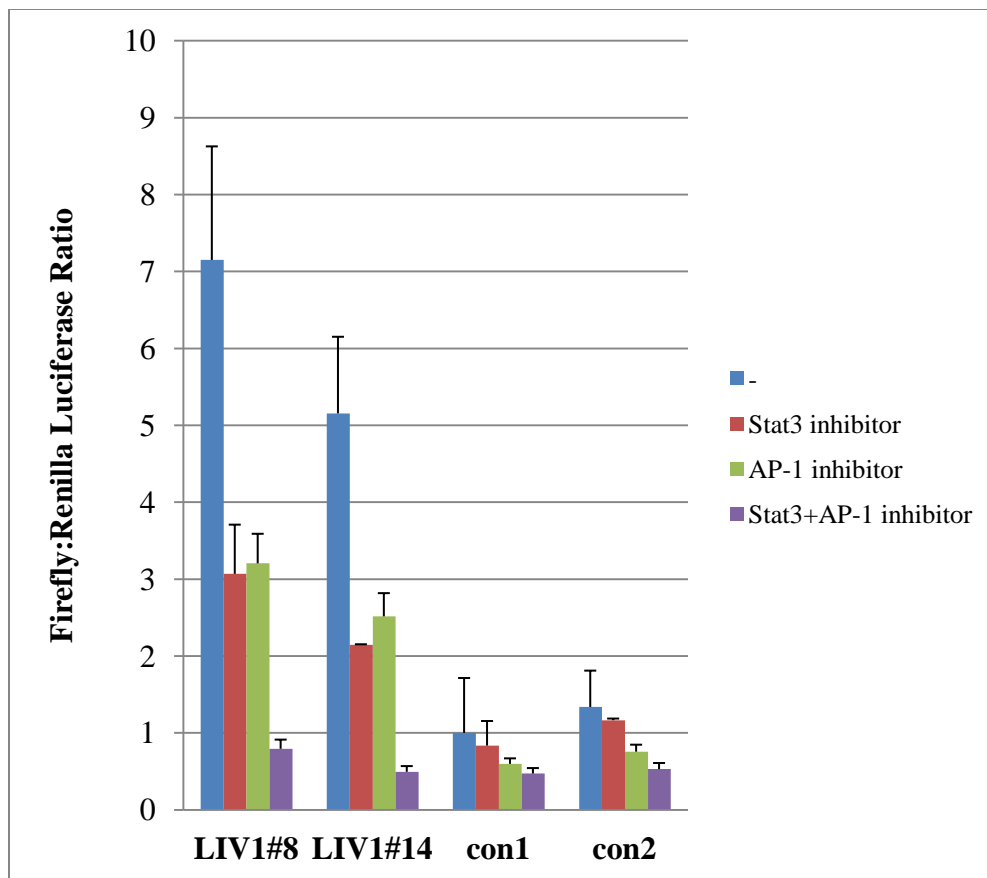


Figure 4.11

Blockade of AP-1 and Stat3 suppressed HB-EGF promoter activity in LIV-1 overexpressing cells. LIV-1 overexpressing cells and control cells were transfected with HB-EGF promoter reporter and treated with Stat3 inhibitor, AP-1 inhibitor, or both. Renilla reporter was an internal control and the results were normalized with Renilla reporter activity.

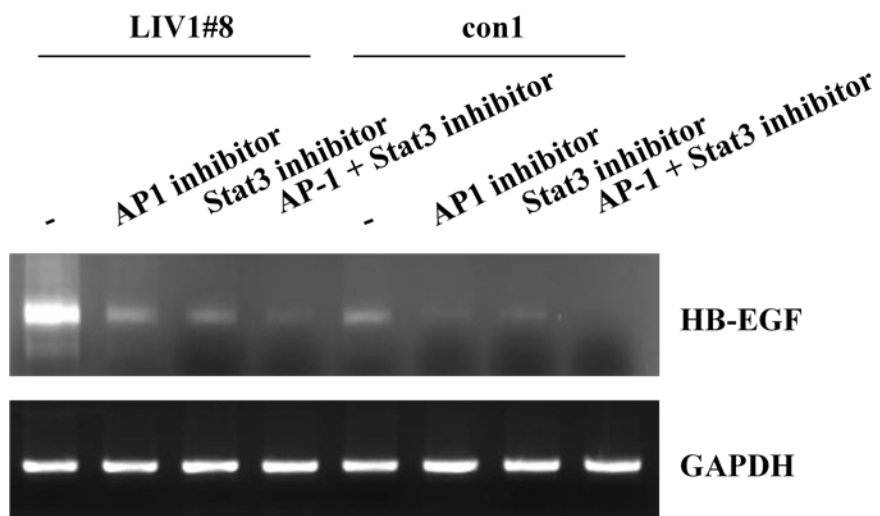


Figure 4.12

Treatment of AP-1 and Stat3 inhibitors inhibited HB-EGF mRNA expression. LIV-1 overexpressing cells and control cells were treated with AP-1(10uM), stat3 inhibitor (2uM) or both for 8 hours. HB-EGF mRNA expression level was determined by RT-PCR.

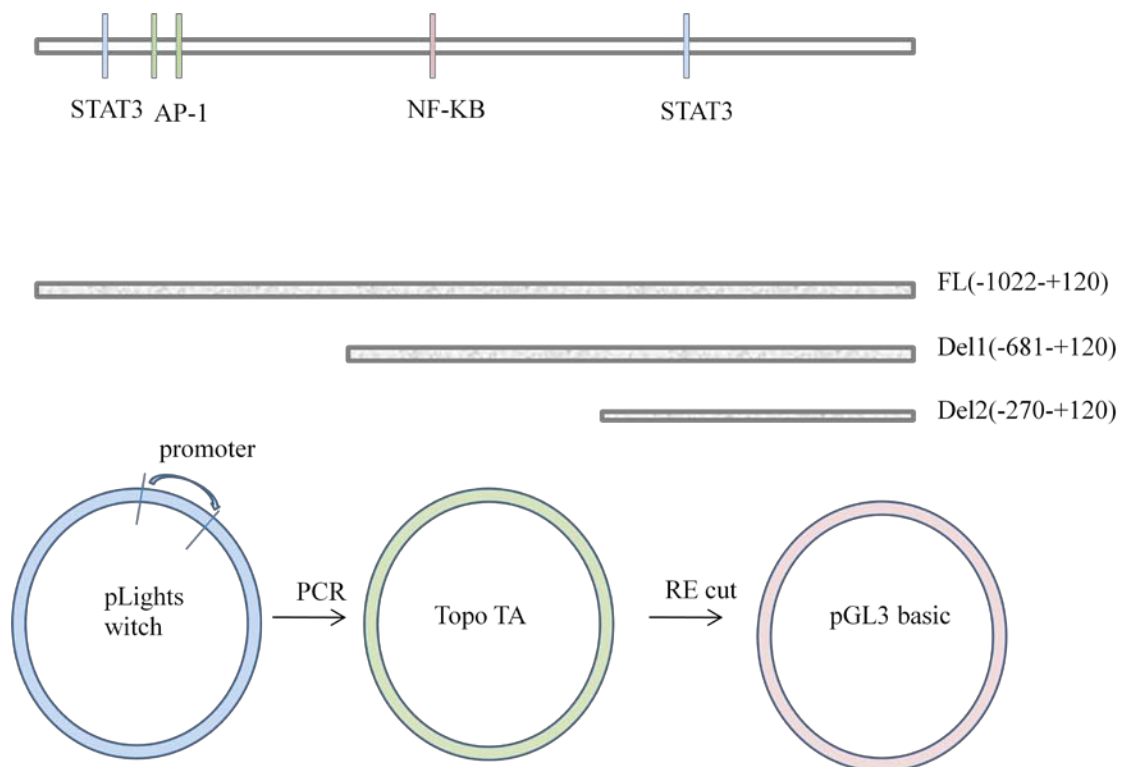


Figure 4.13

HB-EGF promoter deletion constructs.

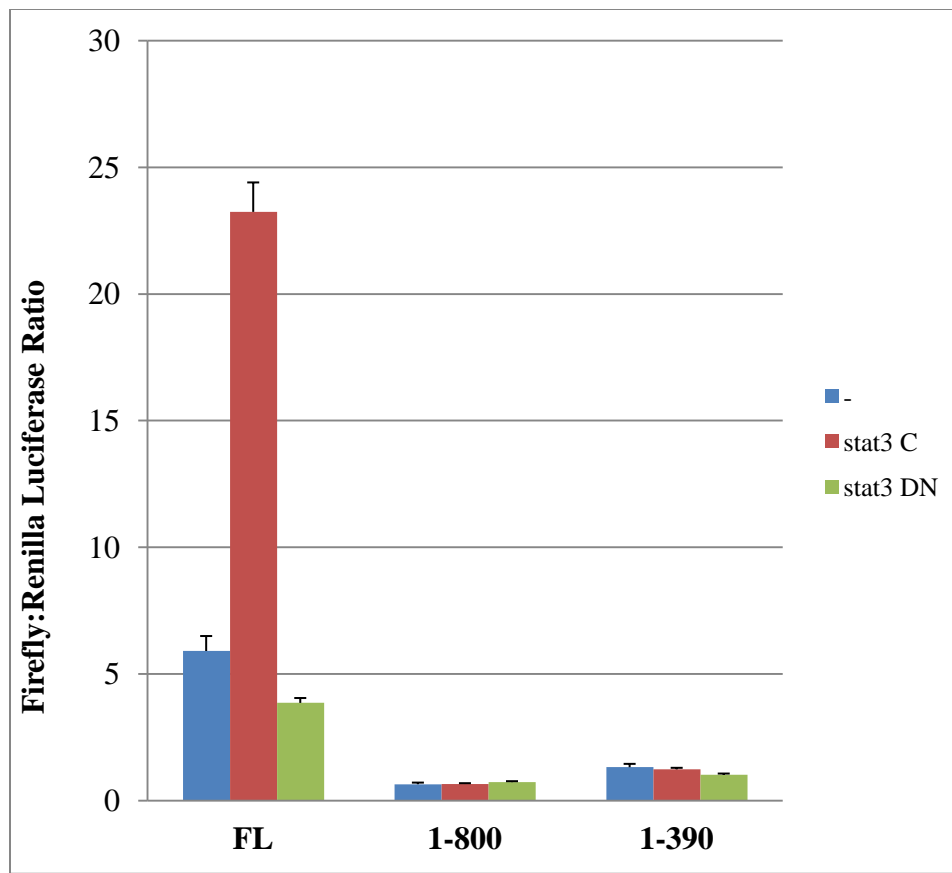


Figure 4.14

Stat3 response elements lied between -1022 to -681. ARCaP_E cells were co-transfected with 3 different length of HB-EGF promoter reporter and Stat3 constitutive active or dominant negative expression vector. Renilla reporter was an internal control and the results were normalized with Renilla reporter activity.

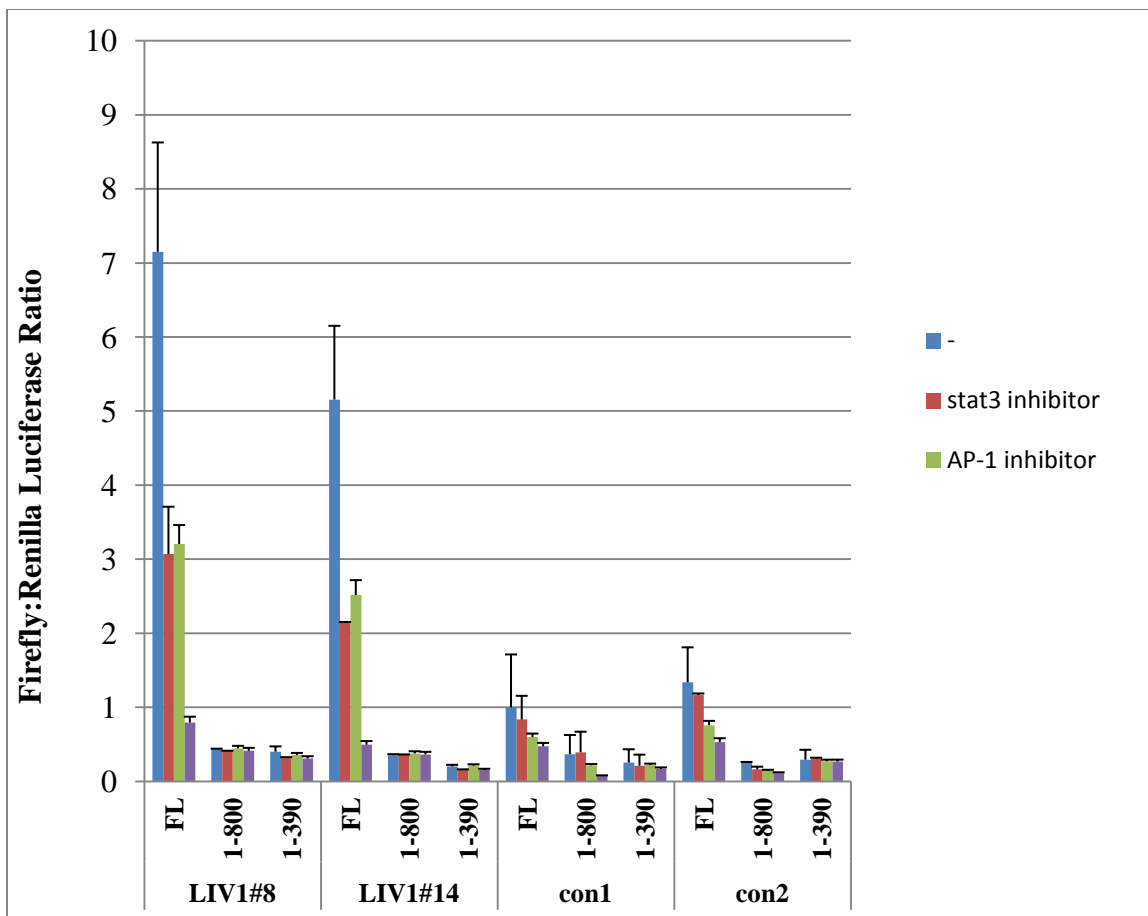


Figure 4.15

Only full length HB-EGF responded to the treatment of Ap-1 and Stat3 inhibitors. LIV-1 over-expressing cells and control cells were transfected with 3 different length of HB-EGF promoter reporter and treated with Stat3 inhibitor, AP-1 inhibitor, or both. Renilla reporter was an internal control and the results were normalized with Renilla reporter activity.



Mut Stat3 (-945): TCCCTGGAA → TCCCTGGCC

Mut AP-1 site1 (-846): AGACCT → CGACCT

Mut AP-1 site2 (-745): AGGCCT → CGGCCT

Figure 4.16

Site-directed mutagenesis of HB-EGF promoter reporter constructs.

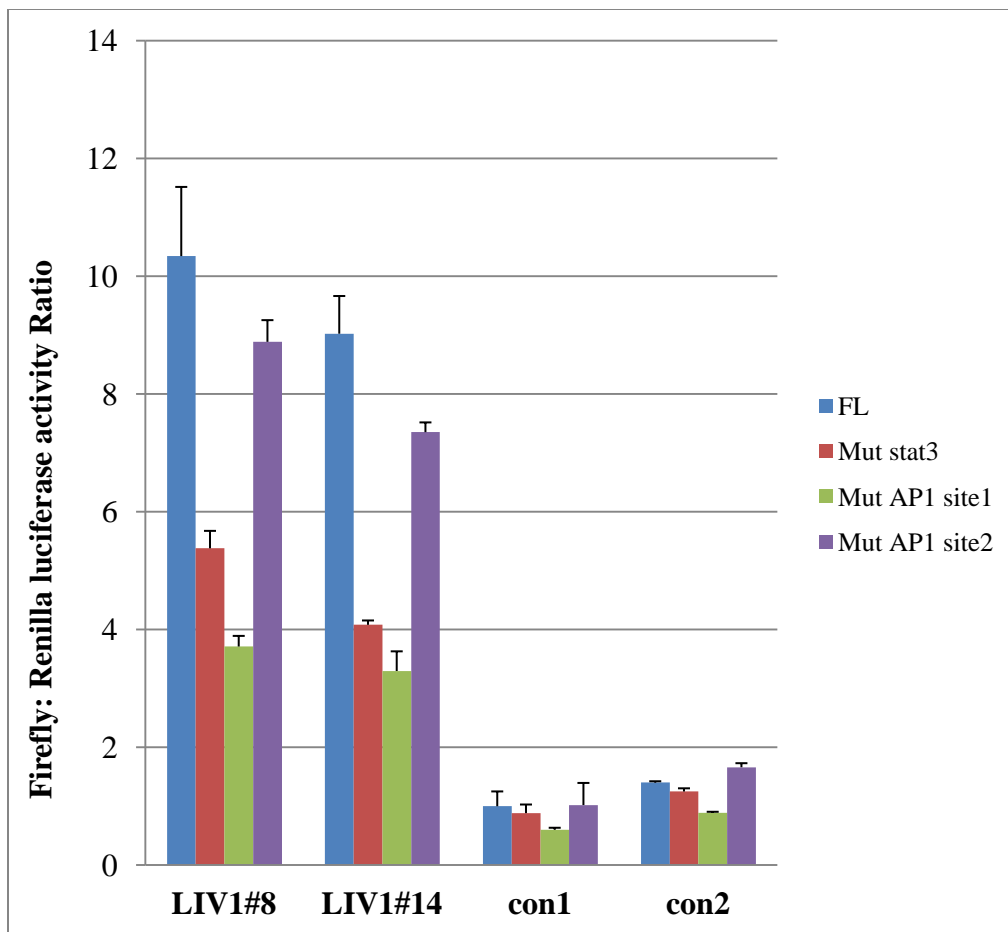


Figure 4.18

Mutation of AP-1 or Stat3 binding sites decreased HB-EGF promoter activity. LIV-1 overexpressing cells and control cells were transfected with 4 different HB-EGF promoter reporter- FL wild type, Stat3 mutant, AP-1 site 1 mutant, or AP-1 site2 mutant. Renilla reporter was an internal control and the results were normalized with Renilla reporter activity.

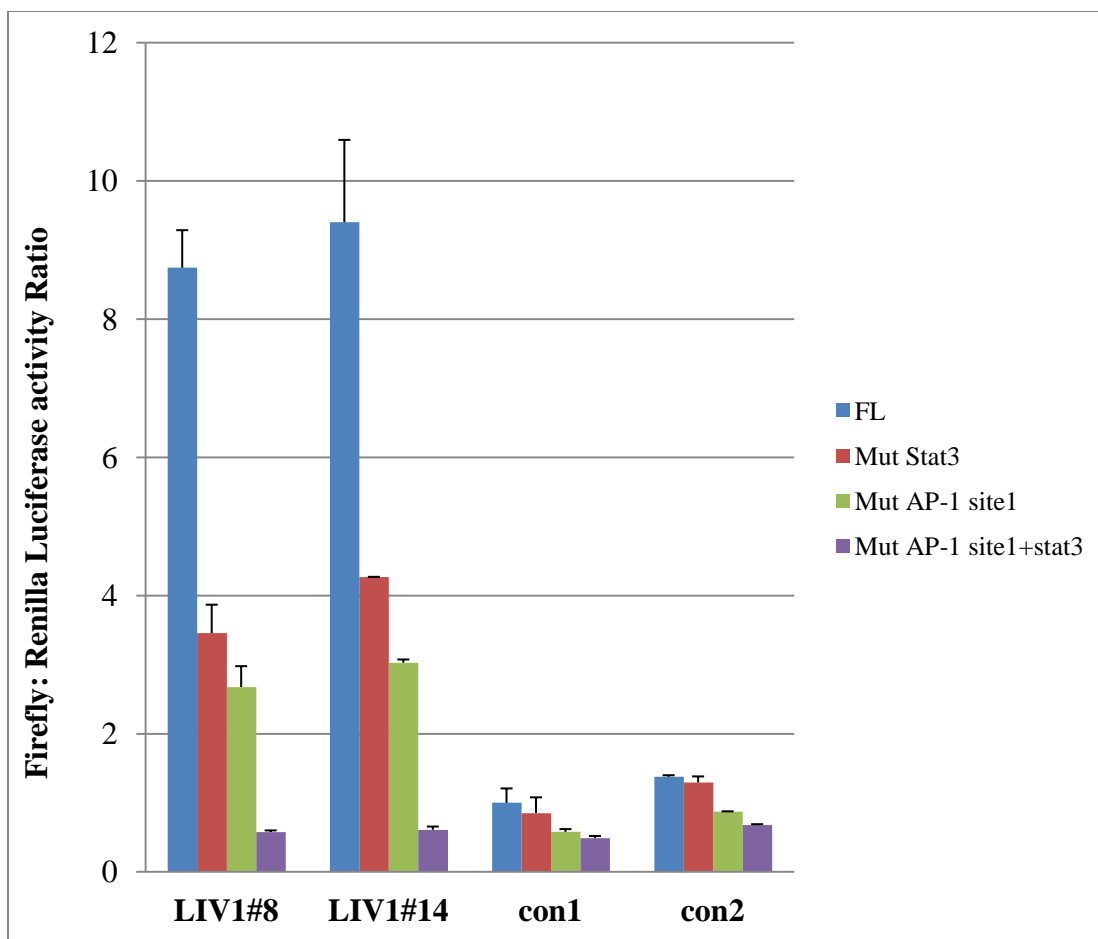


Figure 4.19

Mutation of both AP-1 and Stat3bind sites abrogated HB-EGF promoter activity. LIV-1 overexpressing cells and control cells were transfected with 4 different HB-EGF promoter reporter- FL wild type, Stat3 mutant, AP-1 site 1 mutant or Stat3 and AP-1 site1 double mutant. Renilla reporter was an internal control and the results were normalized with Renilla reporter activity.

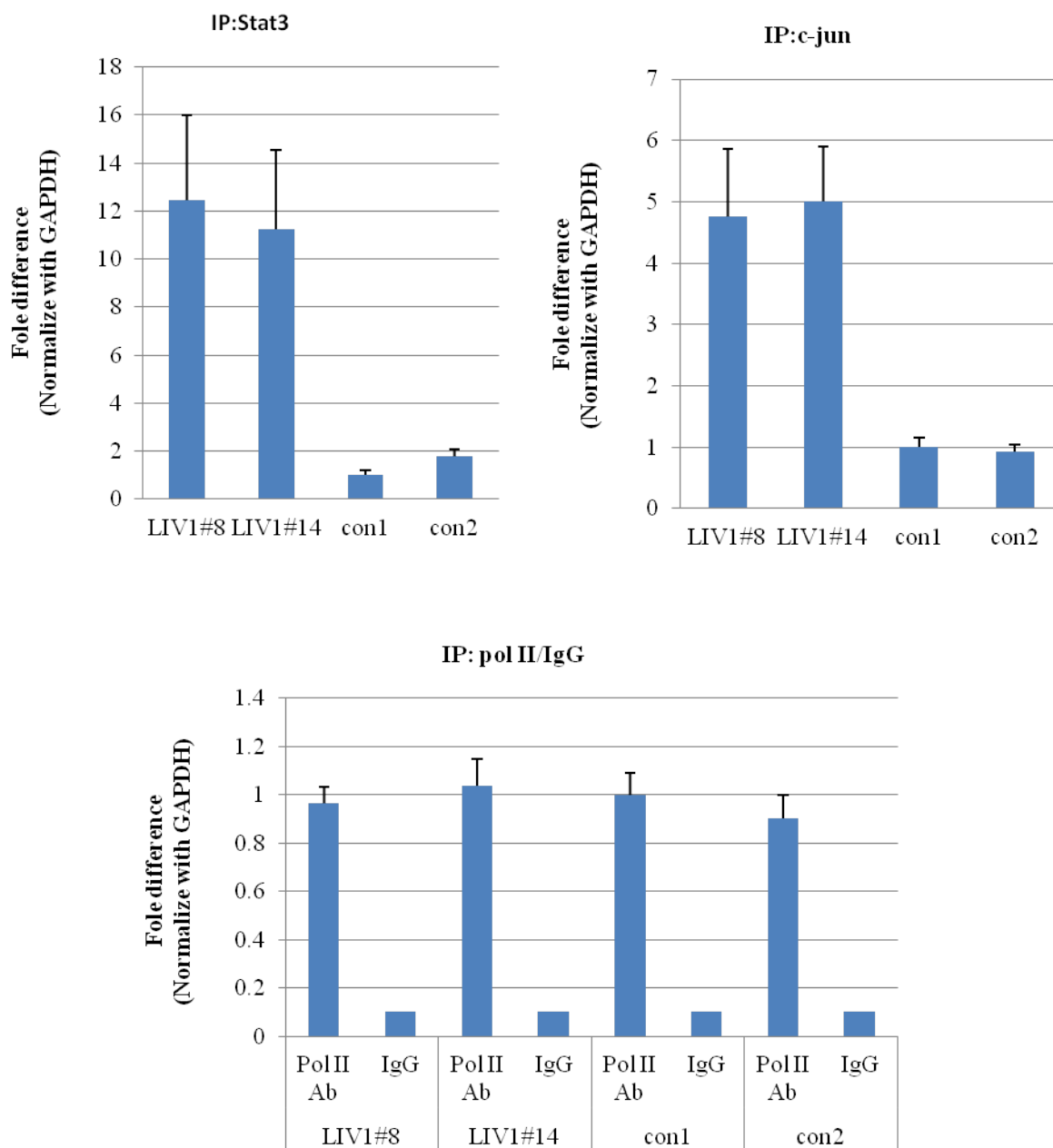


Figure 4.20

Stat3 and c-Jun interact with HB-EGF promoter *in vivo*. (A), Chromatin immunoprecipitations (ChIP) of the HB-EGF promoter by phospho-Stat3 antibody in LIV-1 overexpressing cells and control cells. (B), c-Jun antibody. (C), ChIP of the GAPDH promoter by RNA polymerase II or mouse IgG were used as a positive or negative control. Real-time PCR was performed to determine the quantitative binding of HB-EGF promoter. All the results were normalized with input GAPDH.

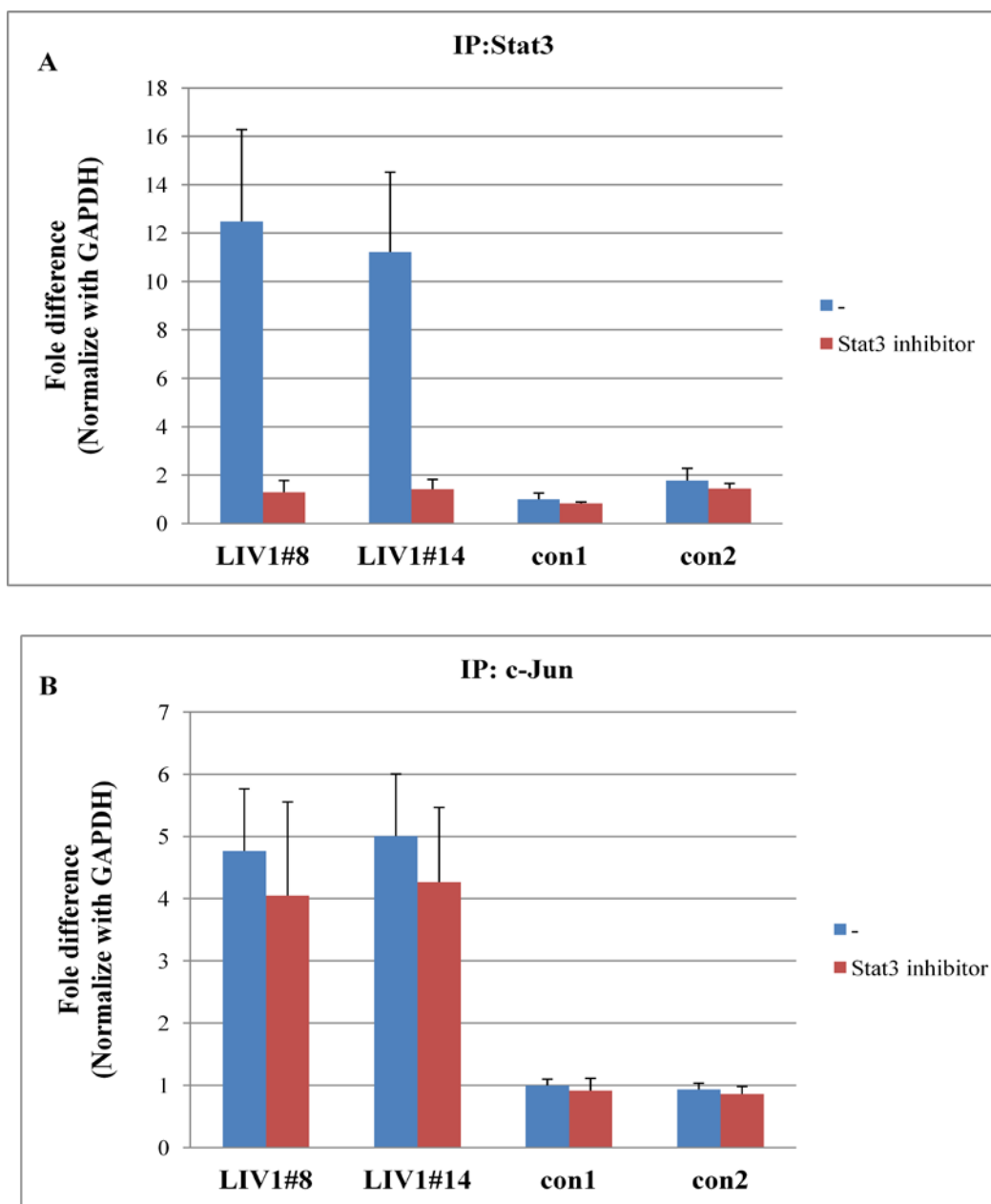


Figure 4.21

Stat3 inhibitor decreases of Stat3 interacting with HB-EGF promoter. (A), Chromatin immunoprecipitations (ChIP) of the HB-EGF promoter by phospho-Stat3 antibody in LIV-1 overexpressing cells and control cells treated with or without Stat3 inhibitor. (B), c-Jun antibody. Real-time PCR was performed to determine the quantitative binding of HB-EGF promoter. All the results were normalized with input GAPDH.

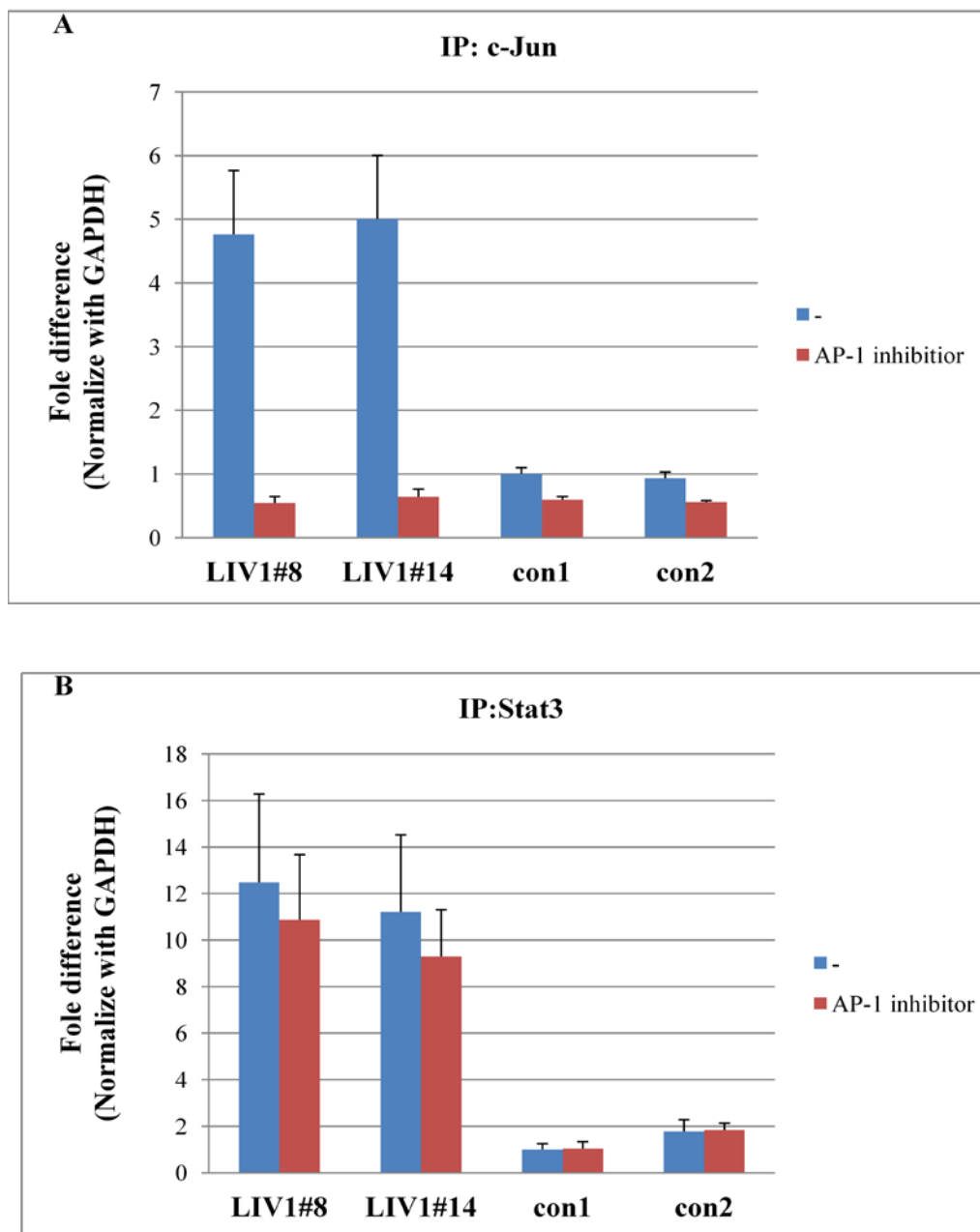


Figure 4.22

AP-1 inhibitor decreases of AP-1 interacting with HB-EGF promoter. (A), Chromatin immunoprecipitations (ChIP) of the HB-EGF promoter by c-Jun antibody in LIV-1 overexpressing cells and control cells treated with or without c-Jun inhibitor. (B), Stat3 antibody. Real-time PCR was performed to determine the quantitative binding of HB-EGF promoter. All the results were normalized with input GAPDH.

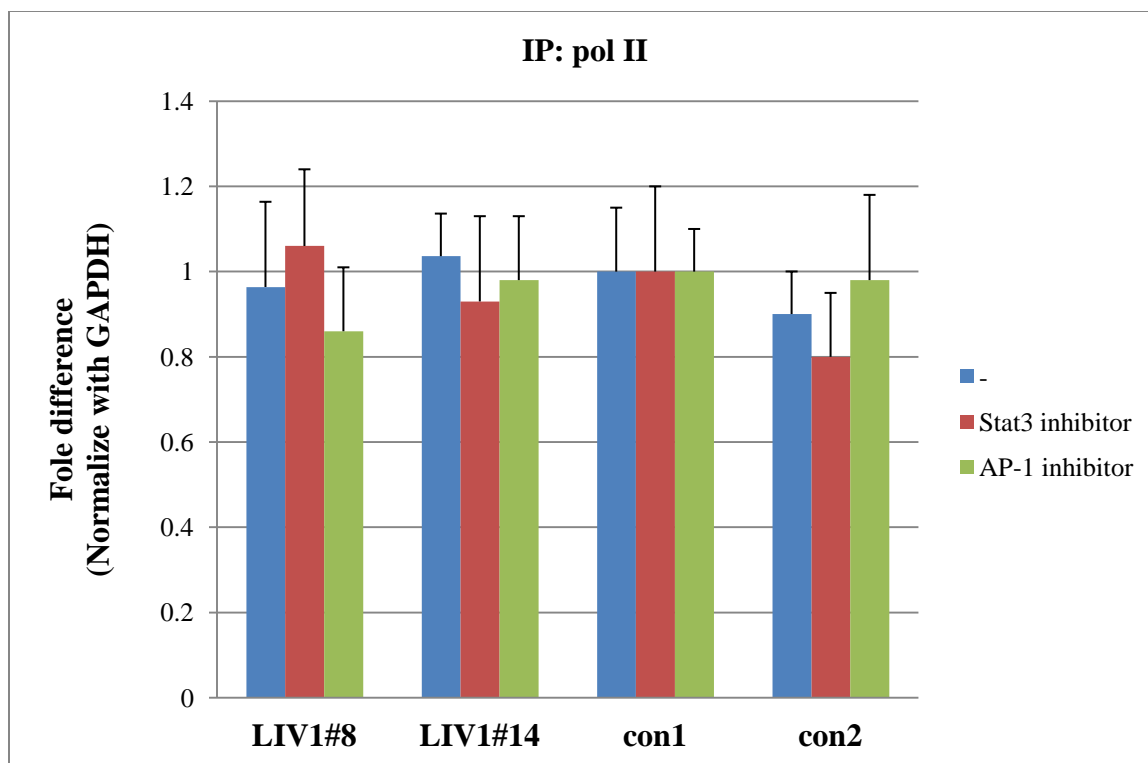


Figure 4.23

ChIP of the GAPDH promoter in LIV-1 overexpressing cells and control cells treated with Stat3 inhibitor or AP-1 inhibitor by RNA polymerase II was used as a positive control. Real-time PCR was performed to determine the quantitative binding of HB-EGF promoter. All the results were normalized with input GAPDH.

5 DISCUSSION

Using the well-characterized ARCaP human prostate cancer progression model, we found that LIV-1 is involved in the promotion of prostate cancer cell EMT, local growth and distant metastases. This conclusion is based on the following evidences, 1) the induction of EMT by IGF-1 or TGF- β 1 in ARCaP_E cells was accompanied by an elevated LIV-1 expression (Figures 2.1 and 2.2); 2) LIV-1 expression was elevated in the isogenic cells expressing mesenchymal phenotype, *i.e.* higher LIV-1 expression was found in ARCaP_M than ARCaP_E cells (Figures 2.3, 2.6 and 2.16); 3) overexpression of LIV-1 in ARCaP_E cells promoted an irreversible EMT of these cells, leading to increased local growth and distant metastases to bone and soft tissues (Figures 2.9-2.15); 4) certain EMT-inducing growth factors, such as β 2-M, could both activate LIV-1 expression and promote bone and soft tissue metastases in prostate, breast, lung and renal cancer cells (Josson et al.); and 5) selected repression of LIV-1 in ARCaP_M cells was accompanied by a reversal of EMT, causing ARCaP_M to adopt a phenotype similar to that of the ARCaP_E cells with decreased migration and invasion (Figures 2.3-2.5). In addition, the function of LIV-1 in the promotion of aggressive cancer behaviors is also supported by the data obtained from the analyses of a large number of clinical prostate cancer specimens (Figures 2.17 and 2.18).

Prostate cancer cells in metastasized tumors are known to display both epithelial and mesenchymal phenotypes in morphology and expression profile, and metastatic prostate tumors are likely comprised of heterogeneous populations of both epithelial and mesenchymal cells. With regard to the mechanism of interconversion between epithelial and mesenchymal phenotypes through EMT and MET, it is interesting to note that an increased LIV-1 expression can be achieved by hormonal induction (El-Tanani and Green, 1997; McClelland et al., 1998) and by growth factor engagement (Figure 2.1). It is conceivable that with induction of LIV-1, cancer

cells can establish metastatic foci through EMT that confers increased migratory and invasive capabilities. Inside the tumor metastasis, LIV-1 expression may be subsided once the inducer withdraws, leading to tumor colonization with cancer cells resuming epithelial morphology and marker expression.

Our study also supports that ARCaP cell EMT is highly reliable and robust as a predictive model for EMT and prostate cancer progression (Zhau et al., 2008). We validated in clinical human prostate cancer specimens that the level of LIV-1 was progressively elevated from normal/benign to PIN in the primary and to metastatic cancer, with the highest expression found in a majority of bone metastases. LIV-1 seems to have a mediatory role, downstream from growth factor signaling in the promotion of prostate cancer EMT under experimental conditions, and likely this role also manifested in clinical progression of prostate cancer cells (Figures 2.17 and 2.18). LIV-1 is thus a candidate marker for prostate cancer progression and metastasis. Suppression of LIV-1 expression and blockade of its role in EMT may be warrant for further clinical investigation as a novel therapeutic approach of cancer growth and metastasis.

By examining large numbers of clinical specimens for both epithelial and mesenchymal stromal markers, it is interesting to notice that tumor cells detected in the bone are predominately epithelial (data not shown). It seems that metastatic tumor is comprised of highly heterogeneous cancer cell population. Alternatively, these results raised the possibility that EMT and MET transitions in metastatic prostate cancer do not necessarily involve a perfect concordance of cell morphology, gene expression and behavioral profiles. Thus, a metastatic tumor may express inherent epithelial genes simultaneously with acquired mesenchymal biomarkers, but the acquired mesenchymal phenotype underscores the increased aggressive and invasive behaviors.

The close correlation between LIV-1 level and cancer progression documented in the clinical specimens is likely a general phenomenon in prostate cancer. Several factors may contribute to abnormally enhanced LIV-1 expression during prostate cancer progression and metastasis. It has been shown that LIV-1 expression is negatively regulated by intracellular zinc concentration (Chowanadisai et al., 2008; Taylor et al., 2003), which provides an auto-regulatory negative feedback (Chowanadisai et al., 2008). Prostate tumor cells are frequently observed with a lowered intracellular zinc pool (Bataineh et al., 2002; Costello et al., 2005; Franklin et al., 2005b), which may induce LIV-1 expression. Additionally, loss of intracellular zinc may prevent cancer cells from apoptotic death, since a lowered zinc level can alter mitochondrial membrane potential to hamper the release of apoptosis-triggering caspases (Truong-Tran et al., 2001). On the other hand, LIV-1 expression may also be stimulated by growth factors in the tumor microenvironment, since treatment with TGF α , TGF- β 1, EGF, IGF-1 and β 2-M all enhanced the LIV-1 level (el-Tanani and Green, 1996, 1997; Zhau et al., 2008). However, no direct evidence shows that LIV-1 overexpression by genomic or epigenomic mechanisms lead to cancer progression. We reported recently that β 2-M-mediated signaling could lead to a decreased intracellular iron which drives EMT and cancer lethality to bone and soft tissues (Josson et al.). Currently it is not clear if LIV-1 is involved in an autoregulatory loop in the regulation of intracellular zinc and iron. In this study, we did not find differences of intracellular total zinc or labile zinc concentrations between LIV-1 overexpressed and neo-control ARCaP_E cells, suggesting that LIV-1 overexpression did not affect intracellular zinc pool in prostate cancer cells. Additional studies should be carried out to determine the role of LIV-1 in determining zinc transport in other prostate cancer cell lines.

Growth factor regulation by LIV-1 is mediated by the activation of Stat3 transcription factor (Yamashita et al., 2004), which orchestrate the nuclear translocation of several important pleiotropic transcription factors. LIV-1 was shown to increase Snail transcription, translation and translocation to cell nucleus in zebrafish organizer (Yamashita et al., 2004). Among many of the common downstream transcription factors responsive to the pleiotropic signals, Snail functions to drive EMT (Huber et al., 2005). By directly repressing E-cadherin transcription, Snail decreases cellular polarity and cell-cell junctional communication, and promotes EMT in not only cancer cells, but also in normal cells as seen in wound healing and renal fibrosis (Battle et al., 2000; Cano et al., 2000; Karreth and Tuveson, 2004; Yang et al., 2004). EMT plays a pivotal role in cell motility during embryonic development (Thiery, 2002), and breast cancer cells undergoing EMT can gain stem cell-like properties with increased ability for self renewal as determined by anchorage-independent growth (Radisky and LaBarge, 2008). The link between EMT and stem cell-like properties could have important implications for the development of novel therapeutic approaches for cancer. In this regard, the relationship between Snail and LIV-1 expression has been reported in breast, cervical and pancreatic cancer progression and lymph node metastases (Manning et al., 1995; Manning et al., 1994; McClelland et al., 1998; Unno et al., 2009; Zhao et al., 2007a). On the other hand, pathophysiologic functions of LIV-1 may be influenced by the background of the cells being studied. It remains unclear why in some breast cancer cell lines, LIV-1 expression has been associated with the suppression of E-cadherin while in other cells LIV-1 knockdown paradoxically increased the invasiveness (Shen et al., 2009).

LIV-1 expression has been reported to be mediated by Stat3 (Yamashita et al., 2004) and our studies showed that LIV-1 could further stimulate Stat3 activation (Figure 4.1). Activation of Stat3 enhanced LIV-1 expression and overexpression of LIV-1 could evoke more Stat3 activa-

tion, suggesting a positive feedback loop exists. Aberrant Stat3 signaling has been frequently observed in different types of human cancers such as prostate, breast, pancreas, brain, lung, and head and neck cancer (Bowman et al., 2000; Catlett-Falcone et al., 1999; Coffey et al., 2000; Garcia et al., 2001; Lin et al., 2000; Song and Grandis, 2000). Constitutively activated Stat3 has been demonstrated to be able to induce transformation, and associated with tumor development and progression (Bowman et al., 2000). Studies have shown that Stat3 functioned in mediating cell proliferation, angiogenesis, and preventing apoptosis (Sinibaldi et al., 2000; Yu and Jove, 2004). Because of its important role in tumorigenesis, Stat3 has been used as a new anticancer target. Studies in cell culture and animal models established that Stat3 is a promising therapeutic target in a variety of human cancers (Bowman et al., 2000; Turkson and Jove, 2000). Inhibition of Stat3 signaling has been demonstrated repeatedly to cause growth inhibition and apoptosis in tumor cells harboring constitutive active Stat3 (Catlett-Falcone et al., 1999; Lin et al., 2000).

Since LIV-1 overexpression was associated with the development of larger prostate tumors (Figures 2.13-2.15) and with accelerated proliferation *in vitro* (data not shown), we carried out mechanistic analyses to elucidate the underlying regulatory pathway. LIV-1 overexpressing clones expressed high levels of HB-EGF (Figures 3.8 and 3.10). At the same time, these clones produced significantly increased MMP2 and MMP9 transcripts (Figure 3.8) as well as their enzymatic activities (Figure 3.9). The enzymatic activities are involved in catalyzing HB-EGF shedding (Figures 3.10). The soluble form of HB-EGF may have interacted with EGFR to cause the constitutive EGFR phosphorylation (Figure 3.11). EGFR phosphorylation will lead to ERK-mediated signaling transduction, which favors cell growth and facilitate cellular motility. In addition, ERK-mediated signaling may promote EMT by downregulating E-cad expression, thus releasing β -catenin from cytoplasmic membrane to enter the nucleus, where β -catenin interacts

with T cell-factor/lymphoid enhancer factor (LEF) transcription factors to promote the growth and survival of cancer cells (Spaderna et al., 2007).

By further examining HB-EGF promoter activity, we found that HB-EGF promoter activity was significantly increased in LIV-1 overexpressing cells (Figure 4.3) and HB-EGF promoter activity was mediated by both AP-1 and Stat3 transcription factors (Figures 4.8 and 4.11). ChIP experiments suggested that AP-1 and Stat3 regulated HB-EGF promoter through directly binding to proximal promoter regions *in vivo* (Figure 4.20). Inhibition AP-1 and Stat3 activity or mutation of AP-1 and Stat3 binding sequences led to completely abrogate HB-EGF promoter activity (Figures 4.15 and 4.18). The HB-EGF expression is increased in a lot of different cancer types (Freeman et al., 1998; Ito et al., 2001a; Ito et al., 2001b; Ito et al., 2001c; Ito et al., 2001d; Kramer et al., 2007; Tanaka et al., 2005). HB-EGF expression was shown to be several to hundred fold higher than other EGFR ligands in ovarian and bladder cancer patients (Miyamoto et al., 2004; Thogersen et al., 2001), indicating the critical role of HB-EGF in mediating EGFR activation at least in ovarian and bladder cancers.

Constitutively active Stat3 has often been found in many tumors, suggesting its important role in tumorigenesis. In addition, Stat3-mediated HB-EGF promoter is not restricted to LIV-1 overexpressing cells, since overexpression of constitutive active form of Stat3 in HEK cells also stimulated HB-EGF promoter activity, suggesting a universal role in regulating HB-EGF promoter. However, more detail studies need to be done to confirm the critical role of Stat3 in regulating HB-EGF gene expression in other types of tumors.

Our results as described herein emphasize a coordinated regulation of LIV-1 expression during the EMT of prostate cancer cells which ultimately gain increased migratory, invasive and metastastic potential. LIV-1 expression increased EGFR-ERK signaling, through the shedding

of HB-EGF from cell surface, by a concomitant induction of MMP2 and MMP9 proteolytic enzymes, which cleave the membrane-bound HB-EGF, and the soluble HB-EGF is responsible for the EGFR phosphorylation and downstream ERK signaling. In addition, both AP-1 and Stat3 controlled HB-EGF gene expression. Both AP-1 and Stat3 are important oncogenic transcription factors found in many tumors. Furthermore, constitutive EGFR activation is a common oncogenic signal in prostate cancer as well as in other malignancies. Our study established a close link for the first time between LIV-1 expression and EGFR-ERK signaling which drives EMT and prostate cancer migration, invasion and metastases. LIV-1 could be a new biomarker and a new therapeutic target for prostate cancer progression and metastasis.

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