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The Relationship between LIV-1 and E-cadherin (CDH1) in Prostate Cancer Cells

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The Relationship between LIV-1 and E-cadherin (CDH1) in Prostate Cancer Cells

Lei Cao

B.S., University of Connecticut, 2012

A Thesis submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Connecticut, 2012

Approval Page

Master of Science Thesis

The Relationship between LIV-1 and E-cadherin (CDH1) in Prostate Cancer Cells

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Table of Content

Contents

Acknowledgements iii

Table of Content iv

List of Figures vii

List of Tables viii

List of Abbreviations ix

Abstract xi

Chapter 1: Literature Review 1

1.1 Zinc in human physiology 1

1.2 Zinc in cancer 2

1.3 zinc transporters 5

1.4 Zinc transporters in cancer 6

1.4.1 Zinc transporters and breast cancer 7

1.4.2 Zinc transporters in prostate cancer 8

1.5 The zinc importer LIV-1 and cancer 9

1.6 EGF and EGFR 11

1.7 EMT and E-cadherin (CDH1) 12

1.8 Snail and Slug 14

1.9 MMP-9 14

Chapter 2: Hypotheses 17

Chapter 3: Materials and Methods 18

3.1 Cell Culture and Subculture: 18

3.2 EGF treatment 18

3.2 Transfection 19

3.3 Cell Proliferation Assay 20

3.4 RNA extraction 20

3.5 RNA concentration measurements 21

3.6 Two-step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) 22

3.7 PCR Product Gel Electrophoresis 25

3.8 Protein Extraction 25

3.9 Protein Concentration Measurement 26

3. 10 Western Blot 26

3.11 MMP Proteolytic Assay 28

3.12 Effects of DTPA on zinc distribution 30

3.13 Statistical Analyses 30

Chapter 4: Results 31

4.1 The Effects of EGF on LIV-1 and CDH1 Expression in DU145 and LNCaP cells 31

4.2 Knockdown of LIV-1 with shRNA in DU145 cells 31

4.3 The Effects of Knockdown on Snail, Slug and MMP-9 mRNA Expression in DU145 cells 35

4.4 Effects of EGF on DU145 and LNCaP cell proliferation 35

4.5 Effects of LIV-1 knockdown on DU145 cell proliferation 35

4.6 Effect of LIV-1 knockdown on proteolytic activity of MMP-9 38

4.7 Effect of DTPA on zinc distribution in DU145 cells 38

Chapter 5: Discussion 40

References 47

List of Figures

Figure 1: Effects of EGF Treatment on LIV-1 and CDH1 mRNA and protein in DU145 cells

Figure 2: Effects of EGF Treatment on LIV-1 and CDH1 mRNA and protein in LNCaP cells

Figures 3: Effects of shRNA Transfection on LIV-1 and CDH1 Expression in DU145 cells

Figure 4: Effects of LIV-1 knockdown on Snail, Slug and MMP-9 mRNA expression in DU145 cells

Figure 5: Effects of EGF treatment on DU145 and LNCaP cell growth

Figure 6: Effects of LIV-1 knockdown on DU145 cell growth

Figure 7: The effect of LIV-1 knockdown on MMP-9 activity in DU145 cells

Figure 8: The effect of 50uM DTPA in 65Zinc efflux in DU145 cells

Figure 9: Schematic illustrating the relationship between LIV-1 and CDH1 in current study

List of Tables

Table 1: Specific Primer Sequences

List of Abbreviations

ATP-- Adenosine-5'-triphosphate

BPH--- Benign prostatic hyperplasia

CDH1---E-cadherin

DNA---Deoxyribonucleic Acid

DTPA---Diethylenetriaminepentaacetate

ECM ---Extracellular matrix

EGF--Epidermal Growth Factor

EGFR---Epidermal Growth Factor Receptor

EMT--Epithelial-Mesencyhmal Transition

FBS---Fetal Bovine Serum

RPMI--Roswell Park Memorial Institute medium

IL---Interleukin

MMP---Matrix Metalloproteases

JAK---Janus Kinase

RNA--Ribonucleic Acid

RT-PCR--------------------------Reverse Transcriptase Polymerase Chain Reaction

SDS-PAG------------Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

shRNA--Short Hairpin RNA

STAT------------------------------Signal Transducer and Activator of Transcription

TE---Trypsin-EDTA

ZIP---Zrt- and Irt-like Proteins

Zn---Zinc

ZnT--Zinc Transporters

Abstract

The zinc importer LIV-1, also known as ZIP6, is widely distributed, mainly in hormonally controlled tissues such as breast, prostate, kidney, and pituitary. Attention has focused on its role in breast cancer, especially its regulation by estrogen and epidermal growth factor (EGF) and its link to the Epithelial–Mesenchymal Transition (EMT) marker, E-cadherin (CDH1). EMT is important for tumor progression and metastasis and therefore a potential target for cancer therapy. CDH1 expression is under complex control, including by two transcriptional repressors, Snail and Slug. Similar to CDH1, MMP-9 is also a cancer progression marker. Elevated MMP-9 expression has been linked to increased metastasis and tumor stage.

In the present study, we investigated the relationship between LIV-1 and CDH1 in prostate cancer cell lines, LNCaP and DU145. Cells were treated with or without 10ng/ml EGF for 24 hours, and the mRNA and protein expressions of LIV-1 and CDH1 were analyzed by two-step RT-PCR and western blot, respectively. In both DU145 cells and LNCaP cells, EGF induced LIV-1 protein expression by about 20% and decreased CDH1 mRNA and protein by approximately 40%. However, no significant change in LIV-1 mRNA was seen with EGF treatment, indicating a post-transcriptional mechanism. EGF also promoted proliferation in the two cell lines.

LIV-1 shRNA transfection was used to understand whether LIV-1 knockdown would influence CDH1. LIV-1shRNA vectors decreased the LIV-1 mRNA and protein expression in DU145 cells by around 40%. The effects of LIV-1 knockdown on CDH1, Snail, Slug and MMP-9 mRNA expression were also measured. LIV-1

knockdown increased CDH1 mRNA (80%), while it decreased the expression of Snail mRNA (60%) and MMP-9 mRNA (40%) significantly. There was no significant change observed in Slug mRNA expression. It was also found that LIV-1 knockdown inhibited cell proliferation of DU145 cells, suggesting that LIV-1 may contribute to the EGF-stimulated cell proliferation.

In summary, this study established an inverse relationship between LIV-1 expression and CDH1 in prostate cancer cells. LIV-1 could be a potential biomarker and a therapeutic target in prostate cancer progression and metastasis study.

Chapter 1: Literature Review

1.1 Zinc in human physiology

Zinc is present universally in body tissues and fluids. The total body zinc content has been estimated to be 30 mmol (2 g). Zinc in skeletal muscle accounts for approximately 60% of the total body zinc content and bone mass for approximately 30%. The concentration of zinc in lean body mass is approximately 0.46mmol/g (30 mg/g). Plasma zinc has a rapid turnover rate and it represents only about 0.1% of total body zinc content. This level is under tight homeostatic control. High concentrations of zinc are found in the choroid of the eye as well as in prostatic fluids. [1]

Zinc is necessary for normal biochemical functions in association with proteins, such as catalytic, structural and regulatory. [2] In terms of catalytic effects, zinc is required by more than 50 enzymes. Removal of zinc from zinc metalloenzymes can cause activities to decrease and adding back zinc can restore enzyme activities. The structural role of zinc was established by the discovery of the zinc finger motif, which is contained in all kinds of proteins, such as those participating in cellular differentiation or proliferation, signal transduction, cellular adhesion, or transcription. Zinc is also involved in maintaining structures of some enzymes, for instance, CuZn superoxide dismutase, where copper is at the active site and zinc maintains the enzymatic structure. Another essential zinc function is to regulate gene expression. More than 2000 transcription factors need zinc to maintain their structural integrity and ability to bind to DNA. [2]

Zinc is essential for various physiological processes, such as cell proliferation, reproduction, immune function and defense against free radicals. [3] The symptoms of zinc deficiency include impaired growth, loss of hair, thickening and hyperkeratinization of the epidermis, and testicular atrophy in humans. Severe zinc deficiency can affect various organs, such as the gastrointestinal, central nervous, immune, skeletal and reproductive systems. [4] Chronic diseases, such as gastrointestinal disorders, renal disease, sickle cell anemia, some cancers, pancreatic insufficiency and autoimmune arthritis, have been shown to cause suboptimal zinc status in humans. [4]

Zinc deficiency increases lipid peroxidation in mitochondrial and microsomal membranes which makes zinc beneficial to the integrity of the subcellular organelles. Since zinc has protective efficiency in regulating the activities of antioxidant enzymes, thyroid hormones, and liver marker enzymes, it is also essential to normal cell physiological functions. [5]

1.2 Zinc in cancer

The role of zinc in cancer has received increasing attention. The link between zinc deficiency and cancer progression has been established in human, animal and cell culture studies. [3] It was found as early as 1976 that primary osteosarcoma patients had elevated serum zinc, while patients with metastases had depressed zinc levels. [6]

Zinc is essential to various enzymes and transcription factors that regulate key cellular functions. [7] Insufficient accessibility to intracellular zinc could result in a

decrease of activities of those zinc-dependent proteins involved in the maintenance of DNA integrity and may contribute to the development of cancer. [7] For instance, zinc is required for site-specific DNA binding and proper transcriptional activation of the tumor suppressor protein p53. Both insufficient zinc and excess zinc could cause p53 to miss-fold and result in functional loss. [7] Zinc deficiency has also been shown to upregulate expression of another tumor suppressor protein nuclear factor κB (NF-κB) in rat glioma C6 cells. [5] It has been suggested that a decrease in cellular zinc alone causes DNA damage and impairs DNA damage response mechanisms, resulting in a loss of DNA integrity and the potential for increased cancer risk. [5] There are many reports of abnormal zinc levels in serum and malignant tissues of patients with various types of cancer. In breast cancer, for instance, tissue zinc concentrations were increased greatly, however, in kidney carcinoma, concentrations were decreased markedly. [8] Similar to kidney, zinc content in malignant prostate tissues are significantly lower than the levels found in normal prostate and benign prostate hyperplasia. [9]

The reduced ability of malignant cells to accumulate zinc is one of the most important factors in the development and progression of prostate malignancy. [10] The peripheral zone glandular secretory epithelium in the prostate accumulates extraordinarily high levels of zinc, three to ten times higher than that of other soft tissues. This is special because mammalian cells generally need to avoid the accumulation of high zinc, especially mobile reactive zinc which is toxic. However in the peripheral zone of the prostate, an especially high mitochondria zinc level is

essential to inhibit m-aconitase activity which can prevent the oxidation of citrate and lead to the accumulation and secretion of citrate. [10] The suppressed citrate oxidation caused by inhibition of m-aconitase is lethal in other mammalian cells, since it eliminates the coupled energy production that normally occurs from Krebs cycle oxidation. [10] Without citrate oxidation, 14 Adenosine-5'-triphosphate (ATP) are produced from one glucose, compared with 38 ATP when citrate oxidation exists. The malignant prostate cells become energy-efficient cells in the absence of high zinc concentration, in contrast to the energy-inefficient, citrate-producing cells. Additional energy required for the malignant cell to perform its potential malignant activities is produced without the inhibition of zinc on m-aconitase activity. [10]

Findings of an association between zinc and prostate cancer risk have been inconsistent. One study of vitamin and mineral supplement use found that zinc supplements had a protective function for prostate cancer. [11] In a population-based case-control study, little association was found between dietary zinc intake and prostate cancer. [12] However, in one follow-up study, it was found that chronic zinc oversupply could increase the risk of advanced prostate cancer. [13] Similarly, another case-control study done in Italy found a direct association between high zinc intake and prostate cancer risk, particularly for advanced cancers. [14] Different methods of acquiring dietary zinc intake data could affect the results but it is still not clear if zinc supplements can reduce or elevate prostate cancer risk.

1.3 zinc transporters

There are two families of zinc transporters in humans. One is the ZnT (SLC30) family of transporters with 10 members, which carry zinc from the cytoplasm into the organelles or outside to the extracellular space. The other is the ZIP (SLC39) family with 14 members, which are responsible for taking zinc from the extracellular space or organellar lumen into the cytoplasm. [15]

Some of the zinc transporters have wide tissue distributions, such as ZnT1 and ZIP1. But many others have restricted tissue expressions. For instance, ZnT5 mRNA is highly expressed in human endocrine pancreas, ovary, prostate, and testis tissues. Meanwhile, the liver, brain, and small intestine have the highest levels of ZnT6 mRNA over other tissues or organs. In addition, mRNA and protein concentrations of ZnT6 differed within a tissue, which suggested some undefined processing step. [16]

Several ZnT and ZIP family members are major factors in regulation of zinc homeostasis, such as ZnT1, ZIP4, and ZIP5 in intestinal zinc transport, ZIP10 and ZnT1 in renal zinc reabsorption, and ZIP5, ZnT2, and ZnT1 in pancreatic release of endogenous zinc. Many of them are also involved in other physiological functions, for example, ZnT2 in lactation, ZIP14 in the hypozincemia of inflammation, ZIP6, ZIP7, and ZIP10 in metastatic breast cancer, and ZnT8 in insulin processing and as an autoantigen in diabetes. [15]

The ZnT4 Zn transporter, expressed in breast epithelial cells, is responsible for the inherited zinc deficiency observed in lm (lethal milk) mice, showing that ZnT4 is required to supply zinc in breast milk. In addition, human breast-fed infants studies

have also revealed a potential role of ZnT2 in the transport of zinc to milk.

The mRNA and protein of ZIP4 were regulated in response to zinc availability and mutations in ZIP4 cause acrodermatitis enteropathica, a rare recessive-lethal human genetic disorder. [17] A number of studies have found that the expression levels of zinc transporters in human tumors correlate with their malignancy, suggesting that alteration of intracellular zinc homeostasis can contribute to the severity of cancer. These findings indicate that regulation of zinc homeostasis by zinc transporters plays important roles, disruption of which may lead to disease states. [4]

1.4 Zinc transporters in cancer

Many zinc transporters, such as ZIP4, ZIP6, ZIP7, and ZIP10, have been shown to be aberrantly expressed in various cancers. For instance, the expression of ZIP4 is activated in pancreatic and hepatocellular cancers. [18-21] In a nude mouse model with orthotopic xenografts, silencing of ZIP4 by RNA interference in pancreatic cancer cell lines reduced incidence of tumor metastasis, downsized the tumor grade, and significantly increased their survival rate. [18] Knockdown of ZIP4 in mouse Hepa cells significantly activated apoptosis and modestly slowed progression from G0/G1 to S phase when cells were released from hydroxyurea block into zinc-deficient medium. It was shown that knockdown of ZIP4 in Hepa cells depressed their migration; moreover ZIP4 overexpression in Hepa cells and MCF-7 cells enhanced migration. [19] In another study, treatment with physiological concentrations of zinc increased the abundance of ZnT-1 mRNA in a rat insulinoma

and two human ductal adenocarcinoma cell lines but not in normal human islet cells. [22] However, the more significant correlations were found between zinc transporters and breast and prostate cancers.

1.4.1 Zinc transporters and breast cancer

In the United States, breast cancer is the most common malignant tumor expected to occur in women, accounting for 28% of incident cases. It is also the second leading cause of cancer death in women, following lung and bronchus cancer. [23] ZIP6, ZIP7, and ZIP10 are all suspected to be involved in metastatic breast cancer. [20, 21, 24, 25]

ZIP7 expression can be enhanced by exogenous zinc in a tamoxifen-resistant breast cancer model (TamR cells). Knockdown of ZIP7 with small interfering RNA terminated the activation of epithelial growth factor receptor (EGFR)/IGF-I receptor/Src signaling. [25] In cancer, the abnormal balance between cell death and survival makes cancer cells survive under condition that would normally induce apoptosis. EGF provides a strong cell survival signal and offers antiapoptotic ability to promote cancer cell survival in inappropriate environments. [26]

The zinc transporter ZIP10 plays an essential role in the migratory activity of highly metastatic breast cancer as well. Screening of clinical samples for ZIP10 mRNA expression indicated that ZIP10 was significantly correlated with the metastasis of breast cancer to the lymph nodes. In addition, the expression of ZIP10 mRNA was higher in the invasive and metastatic breast cancer cell lines compared

with less metastatic ones. In *in vitro* cell migration assays, the depletion of ZIP10 and intracellular zinc inhibited the migratory activity of invasive and metastatic breast cancer cell lines MDA-MB-231 and MDA-MB-435S. [24]

The zinc transporter LIV-1, also known as ZIP6, is estrogen regulated and present in greater concentration in estrogen receptor–positive breast cancers as well as in tumors that spread to the lymph nodes. [27] LIV-1 has been associated with signal transducer and activator of transcription 3 (STAT3), a molecule linked with breast cancer progression. This association was first observed in zebrafish embryos where LIV-1 was shown to be the downstream target of STAT3 and essential for the nuclear localization of another transcription factor, Snail, which causes loss of cell adhesion by reducing adherence proteins. [21] Further evidence for an involvement of LIV-1 with Snail was provided by the observation that LIV-1 siRNA reduced HeLa cell invasion via a Snail pathway. [20]

1.4.2 Zinc transporters in prostate cancer

In the United States, prostate cancer is the most common malignant tumor expected to occur in men, accounting for 28% of incident cases. It is also the second leading cause of cancer death in men, after lung and bronchus cancer. [23]

Growth of human prostate cancer cells, LNCaP and PC-3, was inhibited by zinc treatment in a dose-dependent manner. [28] Up-regulated gene expression of metallothioneins (MTs) and ZnT1 in both cell lines were observed after zinc treatment. Since BPH (Benign prostatic hyperplasia) accumulates much more zinc

than prostate malignant tissue, the overall balance of zinc is decreased in the prostate cancer cells. It was not surprising that compared with BPH, prostate cancer tissues expressed significantly lower levels of ZnT1 gene. [28] ZnT4 expression also reduced in malignant prostate tissue compared to normal and benign prostate tissue. [29]

Similarly, mRNA and protein expressions of the zinc uptake transporter, ZIP1, are significantly down-regulated in prostate adenocarcinomatous tissue compared with normal prostate tissue. These changes occur early in malignancy and are maintained through its progression in the peripheral zone. [30] However, overexpressed ZIP1 in PC-3 prostate cancer cells results in significant inhibition of NF-kB activity and induced secretion of NF-kB-controlled tumorigenic cytokines, such as IL-6 and IL-8. NF-kB-regulated genes have an established role in malignant transformation, metastatic progression of prostate cancer, and resistance to therapeutic regimens. Moreover, ZIP1 overexpression induces suppression of prostate tumor growth in a xenograft model. [31] It has been shown that both ZIP2 and ZIP3 are down regulated in malignant cells in situ as demonstrated for ZIP1. [32] In contrast to the ZIP1 localization at the basolateral membrane, ZIP2 and ZIP3 transporter proteins were localized predominantly at the apical cell membrane. It was proposed that ZIP2 and ZIP3 appeared to be associated with the re-uptake of zinc from prostatic fluid. [32]

1.5 The zinc importer LIV-1 and cancer

The zinc importer LIV-1 is estrogen-regulated and expressed universally, mainly in hormonally controlled tissues such as breast, prostate, placenta, kidney, pituitary

and corpus callosum. [27] One investigation in human breast cancer patients found that LIV-1 mRNA and protein expression levels are weakly correlated, indicating posttranscriptional regulations. [33] In contrast to the usual finding that LIV-1 is found in increased amounts in estrogen receptor–positive breast cancer as well as in tumors that spread to the lymph nodes[27], another study found that LIV-1 mRNA had a trend to lower expression in tumors with lymph node metastasis, although this was not significant at the 5% level. [33] Meanwhile high LIV-1 protein expression seems to be associated with a longer relapse free and overall survival in breast cancer patients with invasive ductal carcinoma. A negative correlation of LIV-1 protein but not mRNA levels with tumor size, grade and stage reflected an association of LIV-1 protein expression with less aggressive tumors. [33]

LIV-1 has been identified as a gene whose expression is stimulated by estrogen treatment in MCF-7 and ZR-75 breast cancer cells. [27] An investigation of LIV-1 expression in clinical breast-tumour populations revealed its significant correlation with estrogen receptor status. [27] LIV-1 mRNA correlation with the estrogen receptor and its regulation by the receptor have been reported in several studies. [27, 33]

LIV-1 has been shown to contain a novel potential metalloprotease motif similar to that in the MMPs (matrix metalloproteases), which have an important and well-documented role in metastasis. [34] Recently, it was found that overexpression of LIV-1 in prostate cancer ARCaPE cells resulted in elevated MMP-2 and MMP-9 proteolytic enzyme activities. [35]

In zebrafish gastrula organizer, LIV-1 was shown to control epithelial-mesenchymal transition (EMT) by being a downstream target of the transcription factor STAT3, which has a proven role in the development of cancer. LIV-1 was essential for the nuclear localization of the zinc finger transcription factor Snail, a master regulator of EMT. [21] The significant association between LIV-1, Snail and CDH1 suggested a link between the LIV-1 and EMT in breast cancer.

1.6 EGF and EGFR

Cancer cells have a characteristic ability to survive under conditions that would normally induce apoptosis. Epidermal growth factor (EGF) provides a strong cell survival signal and cancer cells promote their survival in inappropriate environments through this type of survival signaling. [36]

The EGF receptor (EGFR) is a transmembrane protein that regulates the intracellular effects of ligands such as EGF and transforming growth factor-α (TGF-α). [37] With ligand binding to the EGFR extracellular domains, the proportion of dimerized receptor increases and the enzymatic activity of its intracellular tyrosine kinase domain is induced dramatically. The EGFR exerts its function in the cellular environment directly or indirectly through its tyrosine kinase activity. [37] Increased expressions of the ligands and/or receptors, as well as ligand-independent receptor activation, have been found in many epithelial cancers, especially gliomas and breast, pancreas, and liver carcinoma. Human carcinomas frequently express high EGFR and this has been associated with a more invasive clinical behavior. Moreover, activation

of high levels of EGFR in nonmalignant cell lines can lead to a transformed phenotype. [38]

Multiple signalling pathways can be activated by EGFR, such as the PI3K/AKT, RAS/ERK and JAK/STAT pathways. [26] STAT3 and AKT activation correlated markedly with EGFR status in malignant astrocytic gliomas. [39] A significantly positive correlation between nuclear STAT3 and EGFR expression in breast cancers was also reported. [36] STAT3 activation has been shown to play a role in oncogenesis and activated STAT proteins are found in human cancer. STAT proteins constitute a family of transcription factors that are activated by cytokine and non-cytokine receptors. Activation of STAT causes tyrosine phosphorylation, dimerization and translocation from the cytoplasm to the nucleus. [36]

1.7 EMT and E-cadherin (CDH1)

EMT is a rapid and mostly reversible change of cell phenotype. [40] During this process, epithelial cells loosen their cell–cell adhesion structures such as adherens junctions and desmosomes, modulate their polarity and rearrange their cytoskeleton. As a result, they become isolated, motile and resistant to apoptosis. [40] EMT has been recognized as a critical phase of embryonic development in animal species. [41] Such EMT-like processes are also evoked in tumor progression and metastasis. [40] Many in vitro studies show that various carcinoma cell lines undergo EMT or partial EMT. EMT leads to the dissemination of single carcinoma cells from the sites of the

primary tumors. More generally, EMT might be involved in the dedifferentiation process that causes malignant carcinoma. [41]

Several signal-transduction pathways for EMT have been identified based on research conducted in embryonic model systems and in normal and transformed cell lines, including the activation of several receptor tyrosine kinases and transforming growth factor-β receptors. [42] Transcriptional repressors of CDH1 are induced in these pathways, leading to the loss of the epithelial phenotype. Declining expression of CDH1 mRNA and protein are hallmarks of EMT, both in embryonic development and in cancer progression. [42] Down-regulation of CDH1 is associated with cell–cell dissociation and invasion in pancreas, prostate and mammary gland mouse cancer models. [40] CDH1 is required for the maintenance of stable junctions and regarded as one of the hallmarks of the epithelial phenotype. In epithelial cells, early contacts are mediated by CDH1 molecules that cluster into small junctional complexes, which then expand to establish stable adherens junctions and promote the formation of desmosomes. Loss of CDH1 expression seems to be strongly involved in EMT since there is a direct link between lack of CDH1 production and loss of the epithelial phenotype. [41]

Another cadherin, N-cadherin, by contrast, is produced in some carcinoma cells that have lost CDH1 and, in these cells, N-cadherin appears as a weak intercellular adhesion system. [41]

Specific transcription factors, in particular Snail (Snail1), Slug (Snail2), Twist, SIP1/Zeb and E47, negatively regulate CDH1 expression. These factors participate in

most physiological EMT situations, and their overexpression in epithelial cells usually induces EMT. [40]

1.8 Snail and Slug

During tumor progression, various mechanisms can inactivate or silence CDH1, such as somatic mutations, promoter hypermethylation, histone deacetylation, and, importantly, transcriptional repression. Several EMT-inducing developmental regulators repress CDH1 transcription by binding with specific E-box domains in the proximal CDH1 promoter. [42] Most prominent are the Snail-related zinc-finger transcription factors Snail and Slug, the repressors ZEB-1 and ZEB-2. [42]

Endogenous Snail protein is present in invasive mouse and human carcinoma cell lines and tumors in which CDH1 expression has been lost. Epithelial cells that overexpressed Snail showed a fibroblastoid phenotype and acquired tumorigenic and invasive properties. [43] In pancreatic cancer, Snail and Slug are expressed, but not in normal tissue. [44]. It was also found Slug and Snail correlated negatively with CDH1 in transformed breast cell lines. [45] Inconsistent correlations between Snail expression and tumor progression in human breast cancer have been seen in another study where reduced Snail expression corresponded with higher tumor grades. [46]

1.9 MMP-9

Controlled degradation of extracellular matrix (ECM) is critical for the growth, invasion, and metastasis of malignant tumors, and for tumor-induced angiogenesis.

[47] Because of their capacity to degrade ECM, resulting in migration of endothelial cells, matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9 are known to play a role in angiogenesis, tumor growth and metastasis. [48] Both MMP-2, also known as gelatinase A, and MMP-9, also known as gelatinase B, are cancer-associated, secreted, zinc-dependent endopeptidases, belonging to the gelatinase subgroup of MMP. [49] 72-kDa MMP-2 is expressed in various cell types, such as osteoblasts, monocytes, and different types of transformed cells. 92-kDa MMP-9 is produced by, for instance, normal alveolar macrophages, osteoclasts, invading trophoblasts, and several types of transformed cells. [49]

Besides extracellular matrix, MMP-2 and MMP-9 cleave many different targets, such as cytokines, growth factors and cytokine/growth factor receptors which in turn regulate key signaling pathways in cell growth, migration, invasion, inflammation and angiogenesis. [49] Expression of MMP-9 is induced at the transcriptional level, by growth factors and cytokines, oncogenes, hormones, and contact to the ECM. For instance, inhibition of the tumor repressor protein NF-κB reduced the expression of MMP-9 in vascular smooth muscle cells. [50] It was found in the MDCK epithelial cell line that MMP-9 transcription is also activated in response to Snail overexpression. [51]

Elevated levels of MMP-2 and/or MMP-9 have been reported in many kinds of cancers, such as breast, brain, ovarian, pancreas, colorectal, bladder, prostate and lung cancers. Since they are overexpressed in a variety of malignant tumors and their

expression and activity are often associated with tumor aggressiveness and a poor prognosis, they are regarded as cancer biomarkers. [49]

MMPs have been shown to play a role in the processes of tumor angiogenesis, tumor growth and metastasis, albeit that these processes are intermingled with each other. [48] Elevated MMP-9 expression has been linked to increased metastasis and tumor stage in a number of studies, e.g. malignant versus benign breast tumors and advanced versus benign ovarian tumors. In several invasive cell lines, MMP-9 expression was increased compared to non-invasive counterpart. [48] Also MMP-9 overexpression increased the incidence of metastatic diseases in immunocomprized mice. [48]

The relation between MMP-9 and CDH1 has been investigated but is still not clear. One study in epithelial ovarian cancer cells showed that MMP-9 was involved in EGF-dependent down-regulation of CDH1 and recombinant MMP-9 or transient expression of MMP-9 was sufficient to reduce CDH1 levels in the absence of EGF. [52] Incubation of epithelial ovarian carcinoma cells with exogenous MMP-9 catalyzed CDH1 ectodomain shedding, suggesting posttranslational modification of CDH1 function via MMP-9. [53] The soluble CDH1 fragment thus released inhibits CDH1 functions in a paracrine way. [54] However, some other studies indicated that CDH1 might be the upstream regulator of MMP-9. In invasive bronchial BZR tumor cells, expression of CDH1 resulted in decreased MMP-9 both at the mRNA and at the protein levels. [55]

Chapter 2: Hypotheses

Hypothesis I:

EGF treatment will induce LIV-1 expression and decrease CDH1 expression in prostate cancer cell lines, DU145 and LNCaP.

Hypothesis II:

EGF treatment will stimulate growth of these cells.

Hypothesis III:

Knockdown of LIV-1 will increase CDH1 expression in DU145 cells. CDH1 repressors, Snail, Slug and MMP-9 may play essential roles in this process.

Chapter 3: Materials and Methods

3.1 Cell Culture and Subculture:

Androgen-independent human prostate cancer cells (DU145) and androgen-responsive human prostate cancer cells (LNCaP) were obtained from the American Type Cell Culture Collection (ATCC, Rockville, MD) and grown as recommended in 5% CO_2 at 37˚C and 95% humidity. The cells were maintained in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% fetal bovine serum. T-75 flasks were used to maintain DU145 and LNCaP cells in 15 ml of media. When the cells reached confluency, the media was removed and the cell monolayer was rinsed with 5 mL of Phosphate Buffered Saline (PBS). To detach the cells, 3 ml of 1XTE (0.05% Trypsin / 0.53 mM EDTA) was added to the cell layer and incubated for 5 minutes. Once the cells were observed to be detached, 7 ml of media was added to the cell suspension. This 10 ml of cell suspension was next aliquoted to two T-75 flasks. Fresh media (10 ml) was added to each flask after the division.

3.2 EGF treatment

DU145 and LNCaP cells were grown in T-25 flasks until they reached an approximately 60% confluency. Cells were incubated in serum-free RPMI for 24hrs. Serum-free RPMI with or without 10ng/ml of EGF (Sigma, St. Louis, MO) was used for another 24 hours and then RNA and proteins were extracted. Three flasks were assigned to each treatment group---non-EGF as control, and 10 ng/ml EGF.

3.2 Transfection

pLKO.1-puro vector plasmids (Sigma, St. Louis, MO) with short hairpin RNA (shRNA) specifically targeting the LIV-1 gene were utilized for the transfection process. pLKO.1-puro non-target shRNA control vector plasmids were used as control. The pLKO.1-puro shRNA plasmids contained a puromycin selection marker for stable transfection. The whole plasmid with shRNA targeting LIV-1 was transfected into DU145 cells with Fugene HD Transfection reagent (Roche Applied Science, Indianapolis, USA). DU145 cells were plated in T-25 flasks with the confluency around 80%. Fugene HD transfection reagent, opti-MEM (diluent for DNA) and LIV-1shRNA plasmids were taken out, and adjusted to room temperature. Plasmids (2 ug) were diluted into 100 uL opti-MEM. 9uL of Fugene HD transfection reagent was added to the plasmids-opti-MEM mixture to make the ratio between transfection reagent and plasmids 9:2. The transfection complex was mixed and incubated for 15 to 40 minutes then added to the prepared DU145 cells. After 3 days incubation, media were replaced with normal growth media. Ten ug/ml puromycin (Fisher Scientific, Pittsburgh, PA) was added to select the cells with plasmids before the RNA and proteins were extracted.

A titrating test was used to determine the concentration of puromycin for transfection selection. Untransfected DU145 cells (2×10^5) were plated in each well of a 6-well plate containing 3 ml of the growth media with increasing concentrations of puromycin (0, 5.0, 7.5, 10.0 and 12.5 μg/ml). Fresh selective media were replaced after 2 days. After two additional days, the percentages of surviving cells were

counted. Ten and 12.5 ug/ml puromycin were able to kill 100% of the cells, so the concentration of puromycin used for selection was determined to be 10 ug/ml.

3.3 Cell Proliferation Assay

Cell numbers were routinely quantified with a hematocytometer. Cells were detached with trypsin. A 50 ul cell suspension, 400 ul PBS and 50 ul trypan blue (Sigma, St. Louis, MO) were mixed in a microcentrifuge tube and left at room temperature for 5 minutes. A small amount of the trypan blue-cell suspension was transferred to a chamber on the hematocytometer. After counting the cells, about 5 x 10^4 cells were inoculated in each well of a 96-well plate and returned to the incubator.

Before the cell proliferation was tested, 120 ul of a mixture of fresh medium and CellTiter-Blue reagent (Promega Corporation, Madison, WI) in a ratio of 100:20 were used to replace the media in each well. After incubation at 37 °C for 1 hour, the 96-well plate was transferred to a micro-spectrophotometer and the florescence values were read using an excitation wavelength at 560 nm and emission at 590 nm.

3.4 RNA extraction

Total cellular RNA was extracted from DU145 and LNCaP cells grown in T-25 flasks using the Trizol reagent (Invitrogen, Carlsbad, CA). The media were decanted off and cells were rinsed with 3 ml of PBS. 1XTE (1.5 ml) was next added to detach the cells. The detached cells were poured into labeled 15 ml polypropylene tubes and centrifuged at 1000 rpm for 3 minutes. After the cell pellets were obtained, 500 µl of

Trizol was added to each tube to dissolve the cell pellets. The cell pellets were vortexed and transferred to micro-centrifuge tubes and 250 ul chloroform was added. The tubes were vortexed and incubated on ice for 3 minutes and then were further centrifuged at 4°C in a micro-centrifuge (Eppendorf, New York, NY) at 10,000 X g for 21 minutes to separate the aqueous and the organic phases. The dissolved RNA in the aqueous phase was carefully pipetted out into a fresh micro-centrifuge tube. To precipitate the RNA from the aqueous phase, 500 µl isopropanol (Fisher Scientific, Pittsburgh, PA) was added and mixed. After incubation overnight at -20°C, the precipitated RNA was obtained through centrifuging at 10,000 rpm at 4°C for 15 minutes. To get rid of the contaminating salts and proteins, the pellets were then washed with 500 µl 75% ethanol, vortexed, and centrifuged at 10,000 rpm for 5 minutes. The pellets were air dried for 10 minutes to evaporate the alcohol. The dry RNA pellets were then dissolved in Diethylpyrocarbonate (DEPC)-treated water (Fisher Scientific, Pittsburgh, PA). They were stored at -80°C if they were not to be processed immediately for experiments.

3.5 RNA concentration measurements

The concentrations of RNA in each sample were estimated using a NanoDrop 1000 Spectrophotometer. (NanoDrop products, Wilmington, DE) Sample concentration in ng/ul was based on absorbance at 260 nm and the selected analysis constant 40 ng-cm/ul. The ratio of absorbance at 260 and 280 nm was used to assess the purity of RNA. A ratio close to 2.0 was accepted as appropriate for RNA.

3.6 Two-step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Reverse transcription and PCR were performed sequentially in two separate reaction tubes using the Qiagen Two-Step RT-PCR kit (Qiagen, Valencia, CA). RNA (4ug) was incubated for 5 minutes at 65°C then placed immediately on ice. Oligo-dT (1 ul), 4 ul of 5x LongRange RT Buffer, 2 ul of dNTP Mix, 1 ul of LongRange Reverse Transcriptase and 0.2 ul of LongRange RNase inhibitor were added to the denatured RNA from each sample. RNase-free water was added to make the final volume 20 ul. For synthesis of complementary DNA (cDNA), the mixes were incubated for 90 minutes at 42°C and then heated at 85°C for 5 minutes to inactivate the enzyme.

Long Range PCR Buffer (5ul of 10x), 2.5ul of dNTP mix, 2ul of forward primer solutions, 2ul of reverse primer solutions, 10 ul of Q-Solution, 0.4 ul of LongRange PCR Enzyme Mix and 26.1 ul of RNase-free water were thawed and used to make the PCR mix, following the manufacturer's protocol. Template cDNA (2 µl) were added to each tube containing the reaction mix. For a simplified hot start, the tubes were placed immediately into a thermal cycler that was preheated to 93°C for 3 min and the cycling program started with 93˚C for 15 seconds, 62˚C for 30 seconds, and 68˚C for 1 minute.

Primers for MMP-9 were designed using PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest/). Forward and reverse primers were selected with the following criteria: optimal product size (~500), higher

GC/AT ratio, and minimal probability of primer dimerization. The NCBI database (http://www.ncbi.nlm.nih.gov/) was used to gather the complete sequence information of each gene and to predict the PCR product sizes. Sequences of primers for LIV-1, CDH-1, Snail, Slug and 18S Primers were obtained from previous published literature. [56] The primers were ordered from Integrated DNA Technologies (IDT, San Diego, CA). Before usage, a final primer concentration of 10 μM was prepared in DEPC treated water. The primers used and their expected product sizes are shown in Table 1.

Table 1: Specific Primer Sequences

Gene	Sequence	Product size	Cycle numbers
LIV-1	Forward: 5'-GGTGATGGCCTGCACAATTTC-3' Reverse: 5'-TTAACGGTCATGCCAGCCTTTAGTA-3'	161	27
CDH1	Forward: 5'-GTCATTGAGCCTGGCATTT-3' Reverse: 5'-GCTTGAACTGCCGAAAAATC-3'	462	35
SNAI1	Forward: 5'-CCAGAGTCAGCCCTTAGTTC-3' Reverse: 5'-AGGAGAGAGTCCAGAGGATG-3'	284	30
SNAI2	Forward: 5'-TCGGACCCACACATTACC-3' Reverse: 5'-CTGGAGCAGAGGTTGTTAGC-3'	282	30
MMP-9	Forward: 5'-TACCACCTCGAACTTTGACAGCGA-3' Reverse: 5'-ATCGCCAGTACTTCCCATCCTTGA-3'	504	30
18S	Forward: 5'-GCTTAATTTGACTCAACACGGGA-3' Reverse: 5'-AGCTATCAATCTGTCAATCCT-3'	84	25

In order to obtain an appropriate detection before reaching the plateau of the PCR reaction, each pair of primers was tested for its optimal cycle number. The thermo cycler was set to run until the cycle number of 35. At the end of 25, 27, 30 and 32 cycles, 5µl of sample was pipetted out into a 0.2 ml thin-wall PCR tube. The samples were next separated on 1% agarose gel.

3.7 PCR Product Gel Electrophoresis

The amplified samples from two-step RT-PCR were separated by electrophoresis through 1% agarose gels (0.5 g of agarose (Invitrogen, Carlsbad, CA)), in 50 ml TBE buffer (Fisher Scientific, Pittsburgh, PA) containing 5 µl ethidium bromide (10 mg/ml). PCR amplified product (5 µl), 1 µl of 10 X loading dye, and 4 µl of distilled water were mixed and loaded carefully into each lane of the 1% agarose gel. 100bp DNA ladder (New England Biolabs, MA, USA) was added to one of the lanes and run alongside the samples to validate the PCR product sizes. The gel was run at 120 volts for 30 minutes. After the run was completed, the gel was visualized under UV light using a ChemiDoc System (BioRad, Hercules, CA) and the bands quantified using Quantity One Software (BioRad, Hercules, CA).

3.8 Protein Extraction

Cells grown in T-25 flasks were rinsed with 2 ml of PBS and detached with 2 ml of 1XTE. The cell suspensions were transferred to 15 ml polypropylene tubes and centrifuged at 1000 rpm for 3 minutes. The supernatant was removed and 50 µl of

RIPA lysis buffer (Teknova, Hollister, CA) containing 30 μl/ml protease inhibitor cocktail (Sigma, St. Louis, MO) was added to the pellets. The resuspended pellets were transferred to pre-cooled microcentrifuge tubes after vortexing briefly. The tubes were incubated with constant agitation on a platform shaker at 4˚C for 30 minutes. Afterwards, the tubes were centrifuged at 10,000 rpm for 10 minutes at 4˚C to pellet cell debris. The protein supernatants were transferred into pre-cooled tubes and stored at -80˚C.

3.9 Protein Concentration Measurement

The BCA protein assay (Thermo Fisher Scientific, IL) was used for the measurement of protein concentrations. Standards were prepared with albumin and absorbance was measured at 562 nm. Protein concentrations were calculated with a standard curve after absorbance was measured. RIPA buffer was used to make a five times dilution of the sample solution.

3. 10 Western Blot

A 10% polyacrylamide resolving gel made with 1.5 M TrisHCl was cast and milli-Q water was used to remove bubbles. After the gel polymerized for 1 hour, the top layer of milli-Q water was removed before a 5% polyacrylamide stacking gel was added and allowed to polymerize for 30 minutes.

Protein solution with 60 μg of protein and loading dye whose volume was half of the protein solution were mixed and incubated at 95℃ for 10 minutes to denature the

protein. The heated samples were loaded in the wells of the stacking gel alongside a Precision plus Kaleidoscope Ladder (5 µl) protein standard (Bio-Rad Laboratories, Hercules, CA). The gel was electrophoresed at room temperature in running buffer (1X Tris/Glycine: 25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3) at 60 volts for 30min then switched to 150 volts for 1 hour.

The gel was removed from the glass plates. Since the molecular weights of LIV-1 and CDH1 vary, 42 kD and 75 kD, respectively, the gel was divided into 2 parts along the 50 kD line and used for detecting the different proteins with different primary antibodies. A piece of PVDF membrane was placed on each half of the gel removing any bubbles between the gel and the membrane. The gel and membrane were sandwiched between sponges and filter paper and placed in the transfer cassette. The gel was placed close to the negative side. The transfer cassette was loaded in a cartridge and placed in transfer buffer (100 mL of methanol, 700 mL of Milli-Q water, and 100 mL of 10X Tris/Glycine (25 mM Tris, 192 mM glycine)). The transfer was carried out for 2 hours at 250 mA at 4°C using the Mini Transblot Electrophoretic Cell with a stir bar (Bio-Rad Laboratories, Hercules, CA).

After transfer, the membrane was removed from the transfer cassette and stained with Ponceau S to ensure the integrity of the protein and the completeness of the transfer. It was then blocked with 20 mL of blocking buffer (0.5% nonfat dry milk in TBS/T [0.1% Tween-20 in Tris-buffered saline]) in a tray for one hour at room temperature on a platform shaker. The blocking buffer was poured off and the membrane was incubated with LIV-1 or CDH-1 primary antibodies (Santa Cruz

Biotechnology, CA, USA) overnight on the shaker at 4 ºC. The dilution of LIV-1 and CDH-1 rabbit polyclonal IgG antibodies used was 1:500. Both primary antibodies were diluted in blocking buffer. The following day, the primary antibody solution was removed and the membrane was washed three times with TBS / T (1% Tween-20) for 5 minutes each wash. After the washes, the goat anti-rabbit IgG-HRP secondary antibody (Santa Cruz Biotechnology, CA, USA) was added (1:1000) and incubated for 1 hour on the platform shaker. The membrane was rinsed with TBS twice before adding the detection reagents.

Western Blotting Luminol Reagents (Santa Cruz Biotechnology, CA, USA) were mixed together. The solution was poured onto the washed membrane and incubated for 5 minutes with periodic shaking. The membrane was removed with tweezers and was dragged with the protein side down along a piece of Kimwipe to remove excess reagents before being inserted between sheet protectors. Light emissions from the membrane were detected using the ChemiDoc System (BioRad, Hercules, CA) and quantified using Quantity One Software (BioRad, Hercules, CA).

The membrane with LIV-1 primary antibodies was then incubated with Western Re-Probe buffer (GBiosciences, MO) for 30 minutes to strip the LIV-1 primary antibodies and then reprobed with β-Actin primary antibodies (Santa Cruz Biotechnology, CA) using the same process as before.

3.11 MMP Proteolytic Assay

Non-target shRNA DU145 cells (5 x 10^4) and LIV-1 shRNA DU145 cells were

plated into each well of a 96-well plate. Six hours after plating, the media were removed and the cells rinsed with PBS. Cells were then incubated with 50 ul of serum-free RPMI. After 20 hours, conditioned media were collected and centrifuged. The supernatants were stored at -80°C before being used. Cell number was assessed using the Cell-Titer Blue assay to make sure the cell growth rate would not influence the MMP assay results.

A 10% polyacrylamide resolving gel embedded with 0.15% gelatin was made and milli-Q water was used to remove bubbles. After the gel polymerized for 1hour, the top layer of milli-Q water was removed and a 5% polyacrylamide stacking gel without gelatin was added and allowed to polymerize for 30 minutes.

Conditioned media (15 ul) and 2 ul of loading dye were mixed and incubated at room temperature for 10 minutes. The samples were loaded in the wells of stacking gel and then electrophoresed in running buffer (1X Tris/Glycine: 25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3) at 120 volts for 90 minutes at room temperature.

After electrophoresis, the gel was rinsed with 1% Triton-100 3 times with 5 minutes each rinse to renature the MMP-9 protein. After incubation with the developing buffer (50 mM Tris, 10 mM $CaCl_2$ and 0.02% NaN_3) for 20 hours at 37°C, the gel was stained with 0.125% Coomassie brilliant blue (BioRad, Hercules, CA) then destained until clear bands could be seen.

3.12 Effects of DTPA on zinc distribution

Radioactive ^{65}Zn ($ZnCl_2$; 62.5 MBq/mg) was purchased from Perkin Elmer (Shelton, CT). DTPA is an extracellular zinc chelator, which has an impact on the zinc distribution between intracellular and extracellular zinc. In primary hepatocytes, DTPA reduced the uptake of ^{65}Zn from the medium and increased efflux from prelabeled cells. However, in GH3 rat anterior pituitary tumor cells, DTPA reduced the uptake of ^{65}Zn from the medium, but promoted the retention of the isotope in the prelabeled cells. [57] The isotope indicated the different effects of DTPA between primary and transformed cells.

To study the effects of DTPA on the efflux of ^{65}Zn in DU145 cells, cells were plated in T-25 flasks and grown to about 75% confluency. The cells were then prelabeled in growth media containing around 4.5 kcpm ^{65}Zn for 48 hours. Afterwards, the cells were provided with normal fresh media with or without 50uM DTPA for another 48 hours. The media were then removed and the cells were rinsed with PBS. Cells were detached with 1XTE. The ^{65}Zn content of media, and cells were quantified by gamma spectroscopy (Cobra II System, Packard, Meriden, CT).

3.13 Statistical Analyses

One way ANOVA with Bonferroni's post hoc test (SPSS version 17.0) was used to analyze data from all experiments. An α-level of $p < 0.05$ was considered statistically significant.

Chapter 4: Results

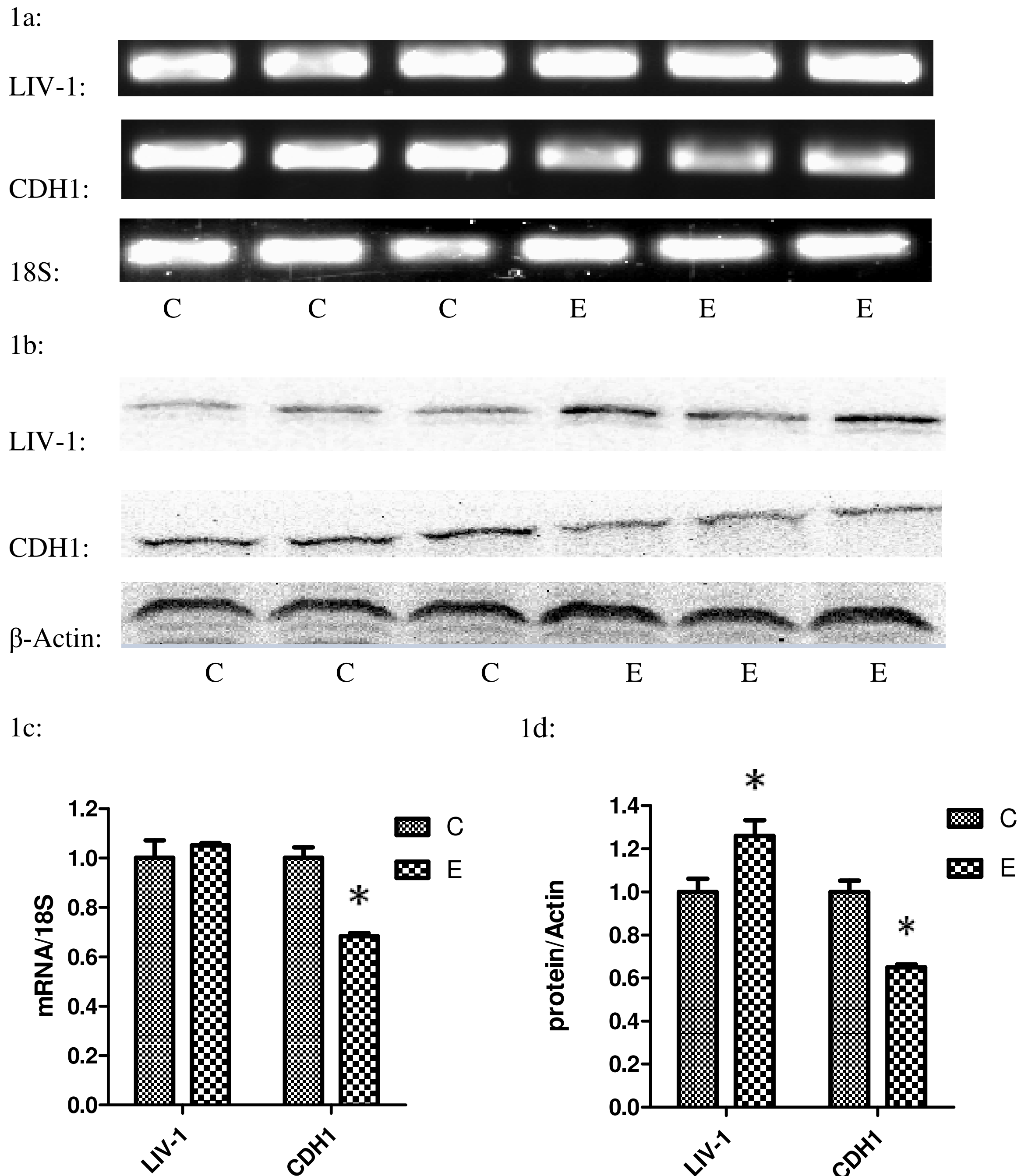
4.1 The Effects of EGF on LIV-1 and CDH1 Expression in DU145 and LNCaP cells

The effects of 24 hours EGF treatment on LIV-1 and CDH1 expressions were investigated in DU145 and LNCaP cells. EGF increased the protein expression of LIV-1 by about 25% and decreased mRNA and protein expressions of CDH1 to approximate 60% in both DU145 (Figure 1) and LNCaP (Figure 2) cells. No change of LIV-1 mRNA was observed in either DU145 or LNCaP cells. Cells treated with serum-free medium without EGF were used to normalize expression.

4.2 Knockdown of LIV-1 with shRNA in DU145 cells

MISSION® pLKO.1-puro vector plasmids with sequences specifically targeting LIV-1 were used to knockdown LIV-1 expression in DU145 cells. MISSION® pLKO.1-puro non-target shRNA control vectors were used as control. Compared to the non-target shRNA control, LIV-1shRNA vectors decreased the LIV-1 mRNA and protein expression in DU145 cells by around 40% (Figure 3). 18S RNA and β-actin were chosen as the housekeeping gene and protein, respectively and were not affected by LIV-1shRNA. Non-target shRNA transfected cells were used to normalize expression.

Figure 1: Effects of EGF Treatment on LIV-1 and CDH1 mRNA and protein in DU145 cells

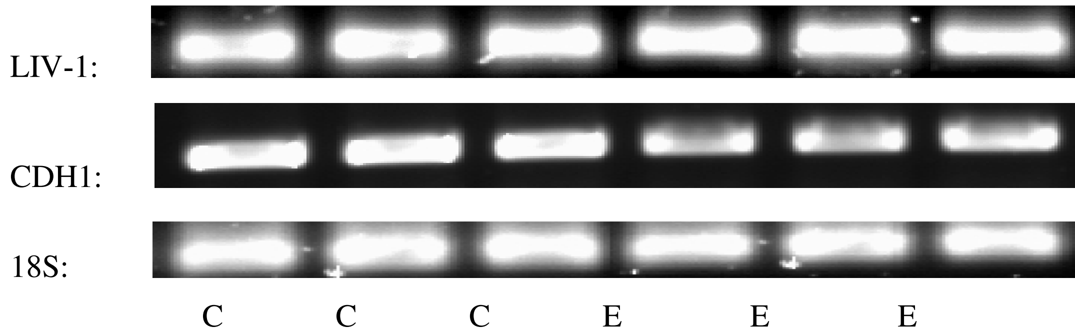


DU145 cells were treated with or without 10ng/ml EGF for 24 hours in serum-free media. **(a)** LIV-1, CDH1 and 18S RNA contents were measured by RT-PCR with ethidium bromide stained agarose gel. **(b)** LIV-1, CDH1 and β-actin proteins were detected by Western blot following SDS-PAGE gel separation of proteins. **(c)** Quantified bar graphs of LIV-1 and CDH1 mRNA concentrations expressed relative to 18S RNA. **(d)** Quantified bar graphs of LIV-1 and CDH1 protein concentrations expressed relative to β-actin.

C = cells treated without EGF used as control, **E =** cells treated with 10ng/ml EGF. The asterisk indicate significant difference from the control ($P<0.05$).

Figure 2: Effects of EGF Treatment on LIV-1 and CDH1 mRNA and protein in LNCaP cells

2a:



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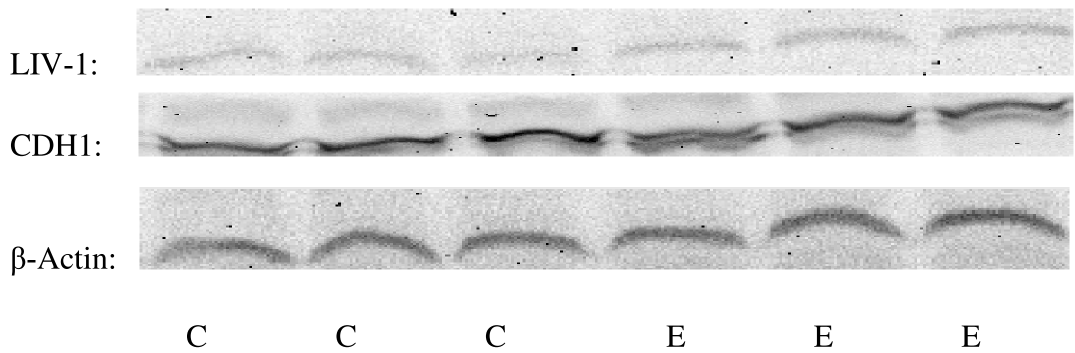
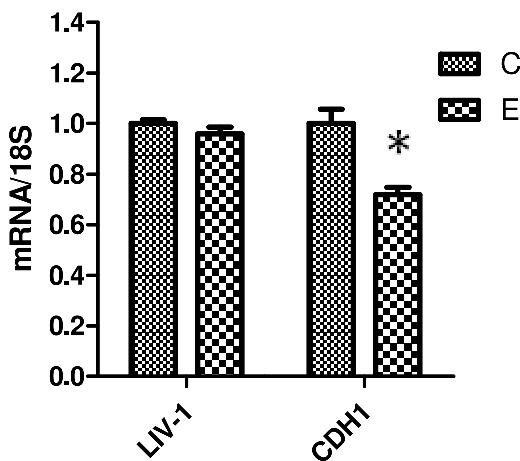
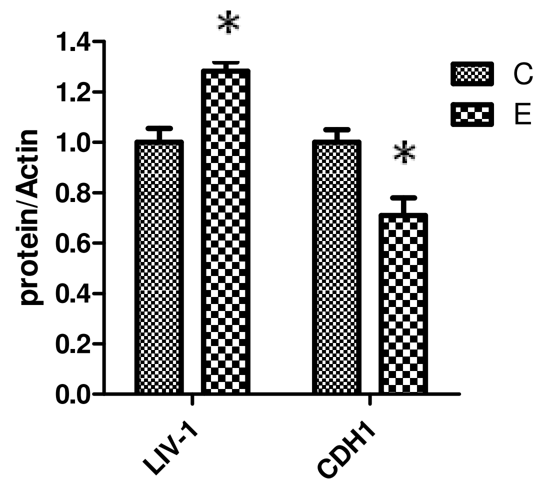


2c:



2d:

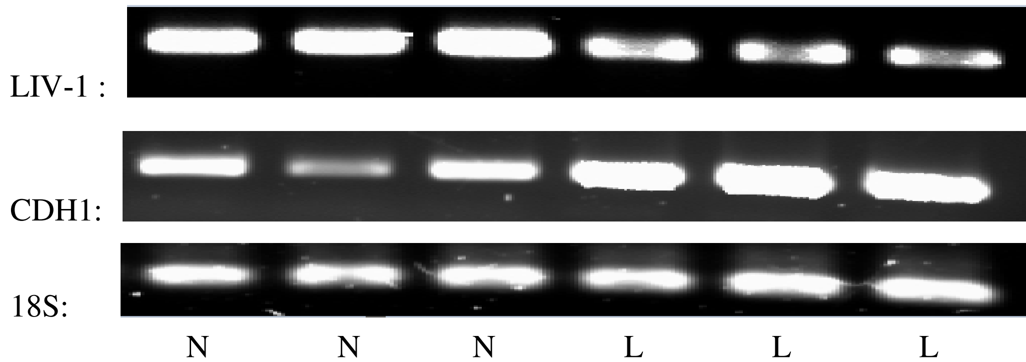


LNCaP cells were treated with or without 10ng/ml for 24 hours EGF in serum-free media. (**a**) LIV-1, CDH1 and 18S RNA contents were measured by RT-PCR with ethidium bromide stained agarose gel. (**b**) LIV-1, CDH1 and β-Actin proteins were detected by Western blot following SDS-PAGE gel separation of proteins. (**c**) Quantified bar graphs of LIV-1 and CDH1 mRNA concentrations expressed relative to 18S RNA. (**d**) Quantified bar graphs of LIV-1 and CDH1 expressed relative to β-Actin.

C = cells treated without EGF used as control, **E =** cells treated with 10ng/ml EGF. The asterisk indicate significant difference from the control ($P<0.05$).

Figures 3: Effects of shRNA Transfection on LIV-1 and CDH1 Expression in DU145 cells

3a:



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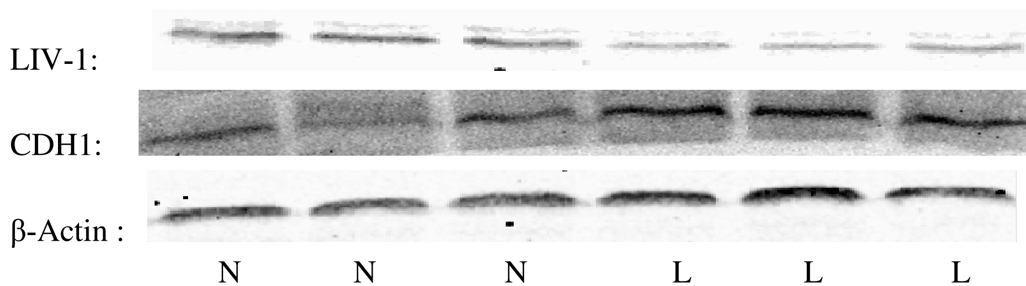

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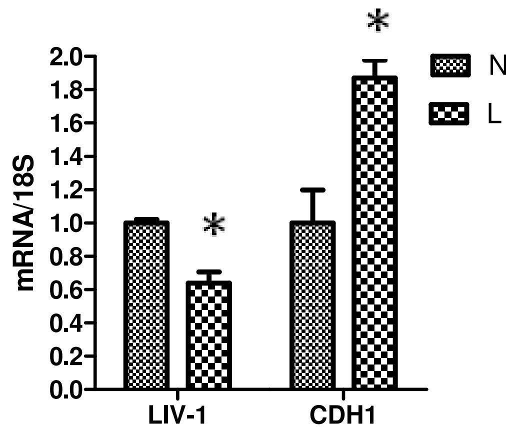

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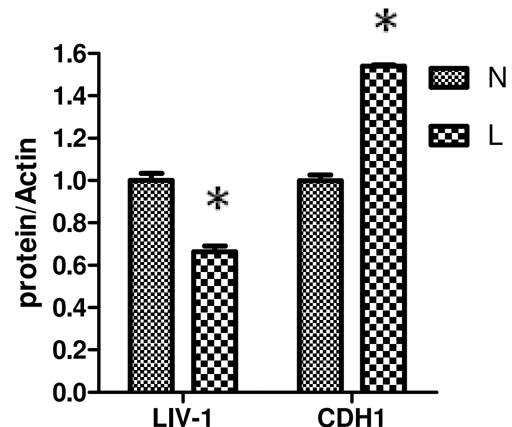


DU145 cells were transfected with LIV-1 shRNA or non-target shRNA. The cells were then selected with puromycin before extraction of RNA and protein.
(a) LIV-1, CDH1 and 18S RNA contents were measured by RT-PCR with ethidium bromide stained agarose gel. **(b)** LIV-1, CDH1 and β-Actin proteins were detected by Western blot following SDS-PAGE gel. **(c)** Quantified bar graphs of LIV-1 and CDH1 mRNA concentrations expressed relative to 18S RNA. **(d)** Quantified bar graphs of LIV-1 and CDH1 expressed relative to β-Actin.
N = cells transfected with non-targeting shRNA, **L =** cells transfected with LIV-1 shRNA. The asterisk indicate significant difference from the control ($P<0.05$).

4.3 The Effects of Knockdown on Snail, Slug and MMP-9 mRNA Expression in DU145 cells

The effects of LIV-1 knockdown on Snail, Slug and MMP-9 mRNA expression were investigated in DU145 cells (Figure 4). There was no change in Slug mRNA expression in LIV-1 knockdown cells, compared with non-target shRNA transfected cells. LIV-1 knockdown, however, decreased the expression of Snail mRNA by approximately 60% whereas it decreased mRNA expression of MMP-9 by 40%.

4.4 Effects of EGF on DU145 and LNCaP cell proliferation

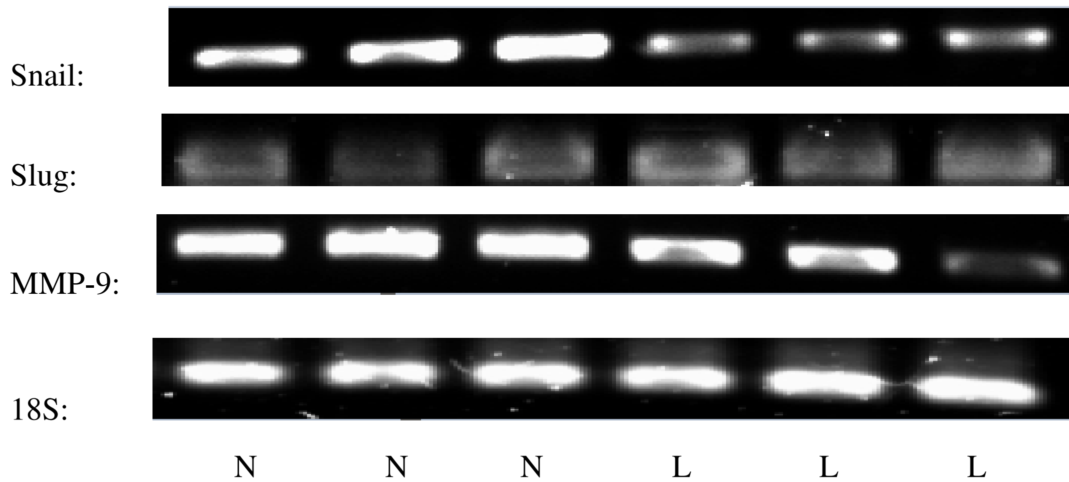
24 hours of 10ng/ml EGF treatment increased the cell growth rates of both DU145 and LNCaP cells, by around 20% and 35%, respectively. The readings for non-EGF treated cells were set up as 100% and the EGF-treated cells were compared to the non-EGF treatment values. (Figure 5)

4.5 Effects of LIV-1 knockdown on DU145 cell proliferation

LIV-1shRNA knockdown in DU145 cells significantly inhibited cell growth to around 85% at 48 hours, compared with non-target shRNA transfected cells. The readings acquired at 6 hours after plating were set up as 100% for both non-target and LIV-1 shRNA transfected DU145 cells. The readings at 24 hours and 48 hours were compared to the 6 hour values. (Figure 6)

Figure 4: Effects of LIV-1 knockdown on Snail, Slug and MMP-9 mRNA expression in DU145 cells

4a:



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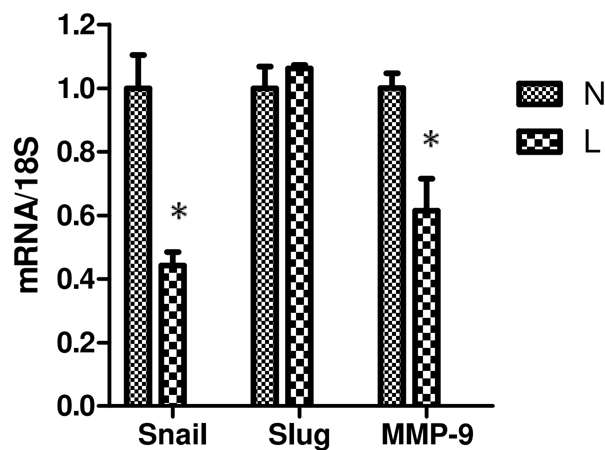
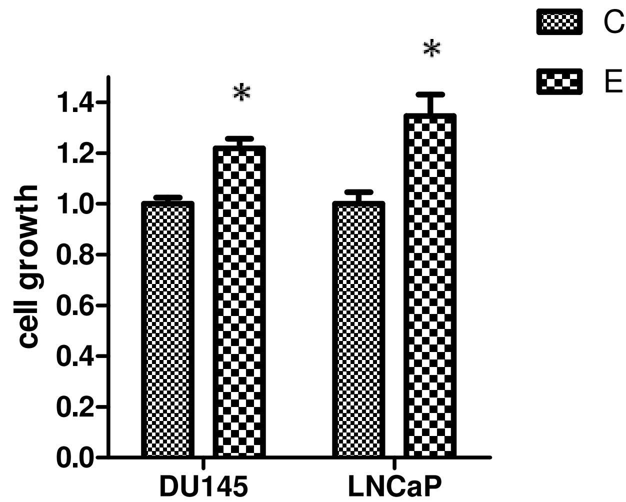


DU145 cells were transfected with LIV-1 shRNA or non-target shRNA. The cells were then selected with puromycin before extraction of RNA. **(a)** Snail, Slug, MMP-9 and 18S RNA contents were measured by RT-PCR with ethidium bromide stained agarose gel. **(b)** Quantified bar graphs of Snail, Slug and MMP-9 mRNA expressed relative to 18S RNA content.

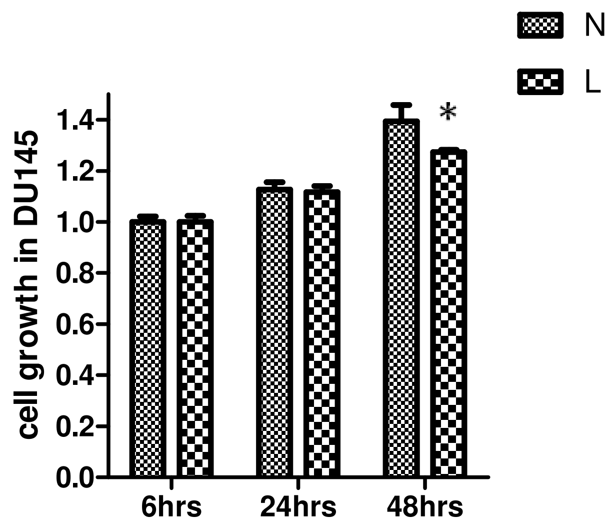
N = cells transfected with non-targeting shRNA, **L =** cells transfected with LIV-1 shRNA. The asterisk indicate significant difference from the control ($P<0.05$).

Figure 5: Effects of EGF treatment on DU145 and LNCaP cell growth



Cells were plated on a 96-well plate and treated with or without 10ng/ml EGF serum-free medium for 24hrs. The cell proliferation was measured by CellTiter-Blue assay. The reader values from non-EGF treated cells were used to normalize.
C = cells treated without EGF used as control, **E** = cells treated with 10ng/ml EGF. The asterisk indicate significant difference from the control (P<0.05).

Figure 6: Effects of LIV-1 knockdown on DU145 cell growth

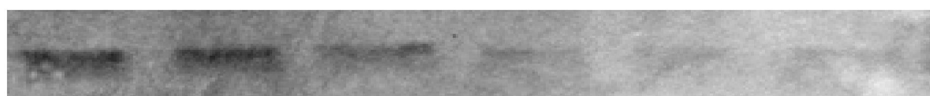


Cells were plated in a 96-well plate. The cell proliferation was detected by CellTiter-Blue assay. The reader values from 6 hours were used to normalize.
N = cells transfected with non-targeting shRNA, **L** = cells transfected with LIV-1 shRNA. The asterisk indicate significant difference from the control (P<0.05).

4.6 Effect of LIV-1 knockdown on proteolytic activity of MMP-9

Conditioned media were collected to investigate the effect of LIV-1 knockdown on MMP-9 proteolytic activity. MMP-9 proteolytic activities were measured by zymography. After electrophoresis, the MMP-9 digests its substrate, gelatin, which was embedded in the SDS-PAGE gel. Staining and destaining reveals the bands where gelatin was digested by MMP-9. DU145 cells transfected with LIV-1 shRNA appeared to have less MMP-9 proteolytic activity compared to those transfected with non-target shRNA. However, the low intensity of the signal in the LIV-1 shRNA knockdown cells precluded the quantification of the data. (Figure 7)

Figure 7: The effect of LIV-1 knockdown on MMP-9 activity in DU145 cells



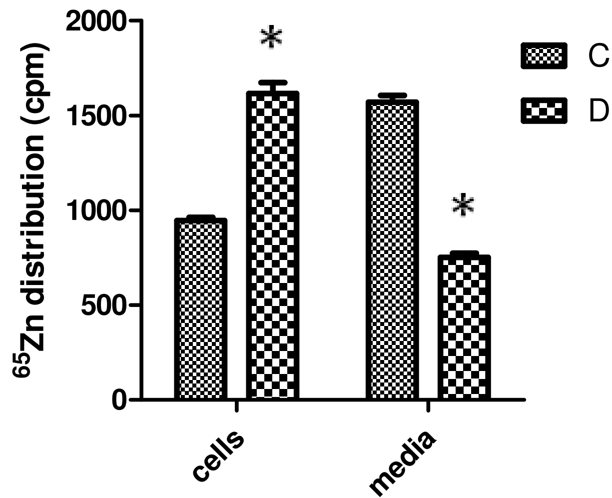
N N N L L L

Cells were plated in a 96-well plate. MMP-9 proteolytic activity were measured by zymography. N = cells transfected with non-targeting shRNA, L = cells transfected with LIV-1 shRNA.
N = cells transfected with non-targeting shRNA, **L =** cells transfected with LIV-1 shRNA.

4.7 Effect of DTPA on zinc distribution in DU145 cells

DU145 cells were used to explore the influence of reduced zinc availability on zinc transport. When cells were pre-labeled with ^{65}Zn, more ^{65}Zn was retained in the DTPA-treated cells, indicating significantly decreased efflux of zinc. (Figure 8)

Figure 8: The effect of 50uM DTPA on ^{65}Zinc efflux in DU145 cells



Cells were plated and grown to about 70% confluence. Approximately 43,000 cpm ^{65}Zn in 5ml medium was added to each flask. After 48 hours incubation, media was replaced with unlabeled fresh medium with or without 50 uM DTPA. The cells were incubated for another 48 hrs then media and cell pellets were collected and their radioactivity was measured by gamma spectroscopy.

C = cells treated without DTPA, **D** = cells treated with DTPA. The asterisk indicates significant difference from the control ($P<0.05$).

Chapter 5: Discussion

In this study, the relationship between LIV-1 and CDH-1 expression and the regulation of LIV-1 by EGF were examined in DU145 and LNCaP prostate cancer cell lines. LNCaP and DU145 cells, represent androgen-dependent and androgen-independent cells respectively. EGF has been shown to differentially regulate CDH1 in estrogen receptor positive and estrogen receptor negative breast cancer cell line. [56, 58] EGF treatment increased CDH1 expression in estrogen receptor negative MDA-MB-231 breast cancer cells [56], while it decreased CDH1 in MCF-7 estrogen receptor negative cells. [58]

Zinc is essential for various physiological processes and its concentrations are under tight homeostatic control. [3] The two families of zinc transporters in humans, ZnT and ZIP, are involved in zinc homeostasis. [15] They have also been reported to have other roles in the cells. In fact, many of them are also involved in other physiological functions. For example, ZnT2 functions in lactation, ZIP14 in the hypozincemia of inflammation, ZIP6, ZIP7, and ZIP10 in metastatic breast cancer, and ZnT8 in insulin processing and as an autoantigen in diabetes. [15] Therefore, zinc transporters have been a major focus for scientific research. The peripheral zone glandular secretory epithelium in the prostate accumulates extraordinarily high levels of zinc and one most important factor in the development and progression of prostate malignancy is the lost ability of the malignant cells to accumulate zinc. [10] Several zinc transporters, such as ZIP1, ZIP2 and ZIP3, were down regulated in malignant prostate cancer cells compared to normal prostate cells. [31, 32]

The relationship between the zinc influx transporter, LIV-1, and cancer progression has been investigated in breast cancer and prostate cancer. LIV-1 is expressed widely, mainly in hormonally controlled tissues such as breast, prostate, placenta, kidney, pituitary and corpus callosum. [27] LIV-1 has been identified as a gene whose expression is stimulated by estrogen treatment in MCF-7 and ZR-75 breast cancer cells. [27] In zebrafish gastrula organizer, LIV-1 was shown to control EMT by being a downstream target of the transcription factor STAT3. [21] Loss of CDH1 expression appears to be strongly involved in EMT since there is a direct link between lack of CDH1 production and loss of the epithelial phenotype. [41] Down-regulation of CDH1 is associated with cell–cell dissociation and invasion in various cancer models. [40] A reduced level of CDH1 is regarded as a characteristic of EMT. [40]

One study found that interruption of EGFR signaling cascades results in an inhibition of the growth of both androgen-responsive MDA Pca 2a, MDA Pca 2b and LNCaP cells and androgen-independent DU145 and PC3 prostate cancer cells. This was accompanied by a blockade of the progression from G1 into S phase. [59] EGF treatment could activate the EGFR signaling pathway and stimulate cell growth. In the current study, EGF treatment significantly stimulated cell proliferation rates of both DU145 and LNCaP after 24 hours. Suppressed cell growth by LIV-1 knockdown in DU145 was also found in our study. In HeLa cells, LIV-1 down-regulation also inhibited cell growth. [60] EGF treatment induced LIV-1 and cell growth, but it is

unclear, to which extent, the increased cell growth by EGF is due to the greater LIV-1 protein expression by EGF.

In this study, we investigated whether EGF regulated CDH1 through LIV-1 in prostate cancer cells. EGF treatment did not alter LIV-1 mRNA expression. However, at the protein level, EGF did induce LIV-1. The induction of LIV-1 protein expression but not mRNA expression by EGF indicated post-transcriptional regulation. Effects of EGF on post-transcriptional regulation and protein turnover have been reported in several studies. [61, 62] EGF regulation of EGFR mRNA and protein in human prostate cancer cell lines was found to occur at multiple transcriptional and post-transcriptional levels. EGF regulated EGFR protein turnover by inducing rapid disappearance of EGFR protein in LNCaP and DU145 cells with a half-life of approximate 120 min, compared to approximate 5 h in control cells. [62] Another example is ADAM17, which is a transmembrane metalloprotease involved in the proteolytic release of the extracellular domain of many cell surface molecules, a process known as ectodomain shedding. EGF treatment led to a marked increase in the protein levels of ADAM17, but did not affect the levels of the ADAM17 mRNA. It did not affect the ADAM17 protein synthesis but increased the maturation of the ADAM17 protein and also increased the protein half life. [61] An investigation in human breast cancer patients found that LIV-1 mRNA and protein expression levels are weakly correlated in their tumors, indicating posttranscriptional regulations in vivo. [33] The up-regulation of LIV-1 protein expression with no change in mRNA concentrations in our study suggested posttranscriptional regulation of LIV-1 by EGF

in prostate cancer cells.

Both mRNA and protein levels of CDH1 were measured in the same cells in this study. It was found that EGF repressed the expression of CDH1 at both mRNA and protein levels, raising the possibility that the expression of CDH1 might be down regulated by LIV-1. Knockdown of LIV-1 increased CDH1 expression again demonstrating a negative correlation between LIV-1 and CDH1 expressions.

We found successful knockdown of LIV-1 in DU145 was sufficient to increase the CDH1 amount. CDH1 repressors, Snail and Slug, have been proposed as targets of LIV-1 in human cervical cancer cell line HeLa. [60] Through stimulating Snail or Slug, LIV-1 was proposed to have a negative effect on the expression of CDH1. However, LIV-1 knockdown increased Slug and decreased CDH1 expression in MDA-MB-231 breast cancer cells, while it did not alter Snail expression. [56] In this study, we found decreased mRNA expressions of Snail, but not Slug, in LIV-1 knockdown cells. These results suggested that Snail may mediate the relationship between LIV-1 and CDH1 in prostate cancer cells.

MMP-9 is known to play a role in angiogenesis, tumor growth and metastasis. [48] Overexpressed LIV-1 in prostate cancer cells, ARCaP, through transfection resulted in elevated MMP-9 proteolytic enzyme activity and decreased CDH1 expression. [35] The ability to stimulate MMP-9 proteolytic enzyme activity has been proposed to be another pathway by which LIV-1 promoted EMT transition. However, the relationship between MMP-9 and CDH1 is still not clear. One study in epithelial ovarian cancer cells showed that transient expression of MMP-9 was sufficient to

reduce CDH1 levels. [52] Incubation of epithelial ovarian carcinoma cells with exogenous MMP-9 increased shedding of the CDH1 ectodomain, suggesting posttranslational modification of CDH1 function by MMP-9. [53] However, some other studies have indicated that CDH1 might down regulate MMP-9. In highly invasive bronchial BZR tumor cells, CDH1 expression is absent. Compared with CDH1 negative clones, MMP-9 decreased both at the mRNA and at the protein levels in transfected CDH1-positive clones. [55] Decreased MMP-9 mRNA expression and proteolytic activity were observed in LIV-1 knockdown DU145 cells. It appears that LIV-1 influences the cells migratory and invasive properties through both CDH1 and MMP-9.

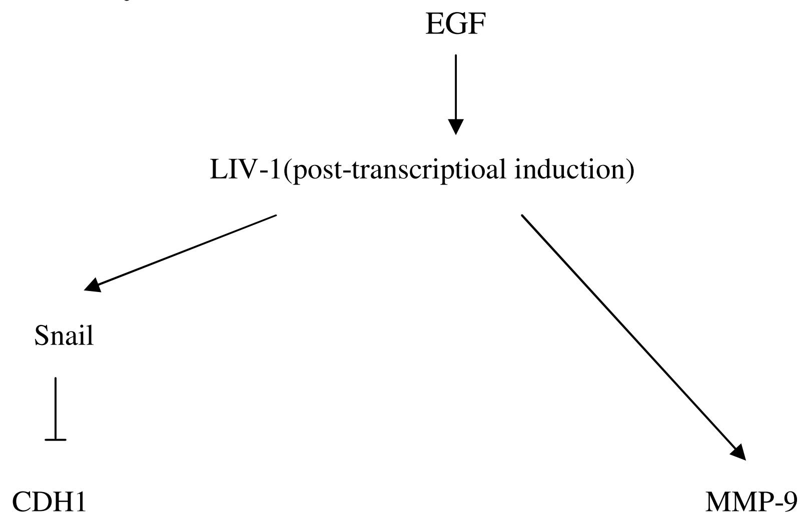
Normal cells and cancer cells responded to DTPA, as an extracellular zinc chelator, in different ways. DTPA promoted efflux of ^{65}Zn from rat primary hepatocytes and pituitary cells, while it increased its retention in rat hepatoma and anterior pituitary tumor cells. [57] More zinc was retained in the DTPA-treated DU145 cells in our study, suggesting that even though prostate cancer cells lose their ability to accumulate high zinc, zinc deprivation still may induce its ability to accumulate zinc, in common with other cancer cell lines.

Post-transcriptional regulation of LIV-1 by EGF was found in this study. More work can be done to look into the precise mode of this regulation, for instance whether EGF increases the translation of LIV-1 or increases protein stability. In addition, it would be useful to show at what time point the LIV-1 and CDH1 expression starts to show alterations in response to EGF. Other growth factors, such

as insulin growth factor may also be able to regulate CDH1 through LIV-1.

In summary, as illustrated in Figure 9, this study showed that LIV-1 was involved in EGF-dependent down-regulation of CDH1 and knockdown of LIV-1 was sufficient to induce CDH1 levels in the absence of EGF by repressing CDH1 repressor Snail in prostate cancer cells. Another tumor metastasis marker MMP-9 was also inhibited with the LIV-1 knockdown, which confirmed the role of LIV-1 in prostate cancer progression.

Figure 9: Schematic illustrating the relationship between LIV-1 and CDH1 in current study



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