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The regulation of LIV-1 mRNA in MDA-MB-231 human breast cancer cells and its association with E-cadherin (CDH1)

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**The regulation of LIV-1 mRNA in
MDA-MB-231 human breast cancer cells and
its association with E-cadherin (CDH1)**

Leelyn Chong

B.S., University of Connecticut, 2010

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

Approval Page

Master of Science Thesis

The regulation of LIV-1 mRNA in MDA-MB-231 human breast cancer cells and its association with E-cadherin (CDH1)

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List of Abbreviations

Adh1	Alcohol Dehydrogenase
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BRCA	Breast Cancer Associated
CDH1	E-cadherin
CDF	Cation Diffusion Facilitator
CES	Cell Death Specification
DMEM	Dulbecco's Minimal Essential Media
DNA	Deoxyribonucleic Acid
DTPA	Diethylenetriaminepentaacetate
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
HepG2	Hepatoblastoma
HRT	Hormone Replacement Therapy
IGF-1	Insulin like Growth Factor-1
IGF-1R	Insulin like Growth Factor-1 Receptor
IL	Interleukin
JAK	Janus Kinase
LPS	Lipopolysaccharides
MAPK	Mitogen-Activated Protein Kinase
MCF	Microcentrifuge
MRE	Metal Response Element
MT	Metallothionein
MTF-1	Metal Responsive Transcription Factor
MTA	Metastasis Associated Protein
NO	Nitric Oxide
RDA	Recommended Daily Allowances
RNA	Ribonucleic Acid
RNAi	RNA Interference
ROS	Reactive Oxidative Species
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
shRNA	Short Hairpin RNA

SOD-----Superoxide Dismutase
STAT-----Signal Transducer and Activator of Transcription
T-----Thionein
TE-----Trypsin-EDTA
TMD-----Transmembrane Domain
TPEN----- N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine
ZIP-----Zrt- and Irt-like Proteins
Zn-----Zinc
ZnT-----Zinc Transporters

Abstract

LIV-1, the estrogen-regulated gene encoding a member of the ZIP family of zinc transporters (SLC39A6), has been implicated in both growth and metastasis of estrogen-receptor (ER) positive (+) and negative (-) breast cancer cells. In ER⁺ breast cancer cells, LIV-1 expression is induced by both estrogen and insulin. In embryonic cells, LIV-1 has also been negatively associated with the expression of the cell adhesion protein, E-cadherin (CDH1), thought to play a role in metastasis. In the present study, we investigated the regulation of LIV-1 and its relationship to CDH1 in MDA-MB-231 ER⁻ breast cancer cells. Cells were treated with insulin and EGF for 24 hours and the mRNA concentrations of LIV-1 and CDH1 were analyzed by RT-PCR. EGF treatment induced LIV-1 mRNA by about 2-fold whereas insulin had minor effects. The mRNA expression of CDH1 paralleled that of LIV-1 with these treatments. MDA-MB-231 cells were also transfected with an shRNA construct designed to knock down LIV-1 expression. This procedure reduced LIV-1 mRNA concentrations by approximately 40% and expression of CDH1 was reduced to a similar extent. This positive association between LIV-1 and CDH1 expression in MDA-MB-231 cells suggests that LIV-1 may be a regulator of CDH1 in ER⁻ cells.

To further understand the linkage between LIV-1 and CDH1, expression of SNAI1, thought to be a transcriptional repressor of CDH1, was measured. However, neither EGF nor LIV-1 knockdown altered the expression of SNAI1 suggesting that this factor does not mediate influences of LIV-1 on CDH1 in these cells. We next investigated the expression of another Snail member, SNAI2, to

explain the observed effects. EGF tended to decrease SNAI2 expression and LIV-1 knockdown increased its expression. Thus SNAI2 may be a modulator of a more aggressive and hormonally independent type of breast cancer cells. We also used ^{65}Zn to characterize the role of LIV-1 in zinc uptake. Knockdown of LIV-1 reduced ^{65}Zn uptake in parallel to the reduction in LIV-1 expression, demonstrating the important role of this transporter for zinc homeostasis in MDA-MB-231 cells. Overall, our current findings suggest that LIV-1 is regulated by the EGFR signaling pathway in ER⁻ breast cancer cells. SNAI2 is inversely regulated by LIV-1 to repress CDH1, increasing the potential for metastasis.

Chapter One: Introduction

Zinc is an important nutrient needed by all organisms to maintain life. Zinc is necessary for cell growth and cell division. A wide variety of enzymes and also transcription factors require zinc (1-2). Because it is so essential for function, cellular zinc homeostasis is tightly regulated. The influx and efflux of zinc are controlled by specific zinc transporters (1-3). There are two gene families of mammalian zinc transporters and they have opposite roles in cellular zinc homeostasis. The ZnT family [solute linked carrier 30 (SLC30A)] of zinc transporters is responsible for transporting zinc out of cells or into intracellular organelles from the cytoplasm. The ZIP (Zrt- and Irt-like proteins) family [solute linked carrier 39 (SLC39A)] is responsible for transporting zinc into the cytoplasm from the extracellular space and intracellular organelles (1-3). The tissue specific distribution and regulation of these transporters has become a major focus for zinc research.

Disturbances in cellular zinc concentration have recently been associated with breast cancer. In fact, recent epidemiological studies have demonstrated a relationship between high zinc concentrations in breast tumors and tumor development (4). Aberrant zinc status was also found to have a significant role in cellular dysfunction, especially in cancer progression and growth (5). Furthermore, studies have shown that a member of the ZIP zinc influx transporter family, LIV-1, may play a role in the growth or metastasis of breast cancer cells (5). LIV-1 was recognized as an estrogen-regulated gene and was suggested as a

potential marker of hormonally responsive tumors. The gene has been discovered to be expressed in not only ER⁺ breast cancer cells (MCF-7) but also in ER⁻ breast cancer cells (MDA-MB-231). These latter cells grow rapidly and are thought to represent a less differentiated more invasive type of breast cancer. MDA-MB-231 cells are also responsive to hormonal treatments such as epidermal growth factor and insulin (5-10).

The induction of a zinc influx transporter by a trophic hormone is consistent with zinc's role in growth. Taylor et al. (5) (11) have suggested that LIV-1 may down-regulate the expression of the cell adhesion protein, CDH1, by activating expression of a zinc finger protein known as SNAI1. SNAI1 in turn is thought to repress CDH1. Thus, elevation of LIV-1 would repress CDH1 and promote metastasis of breast cancer cells. Hence, while LIV-1 was originally thought of as an indicator of a beneficial prognosis because it indicated hormonal responsiveness, downregulation of LIV-1 may be therapeutically useful, because it may slow growth by reducing zinc availability and reduce metastasis by increasing CDH1.

In this study, MDA-MB-231 cells are used for the investigation of the regulation of LIV-1 and its relationship to CDH1 as well as for the characterization of LIV-1's role in this type of carcinoma. Studying the relationship between LIV-1 and CDH1 will give us greater insights into the role(s) that LIV-1 plays in the metastasis and zinc homeostasis of ER⁻ cells.

Chapter Two: Review of Literature

2.1. Zinc:

Zinc is the 25th most abundant element on earth. It comprises about 0.004% of the earth's crust (1). Despite its average abundance, zinc is vital for all organisms because this trace element is used by thousands of proteins (2). Zinc has the ability to form a number of stable yet flexible complexes with macromolecules (1-3). Approximately 300 enzymes were discovered to contain zinc (8). Additionally, proteins known as zinc fingers were found about 15 years ago. These proteins contain zinc binding motifs and are involved in a variety of cellular functions such as DNA or RNA binding and protein-protein interactions (9-10). More than a thousand zinc fingers have also now been identified. Indeed, the success of genetic message transmission depends on zinc's properties (11-12).

Besides its involvement in transcription, zinc also plays an important role in life processes such as the immune system, nervous system, and reproduction at the physiological level (6). At the cellular level, it is involved in functions such as apoptosis, cell signaling, and cell proliferation (13-15). In order for cells to proliferate and undergo mitosis, they need zinc. Zinc enables cells to proceed beyond the S-phase of the cell cycle (16). Zinc is also needed to act on different signaling cascades. For example, the activation of p70S6k, a mitogenic signal transduction molecule, depends on the presence of zinc (17-18). Therefore, these vital cellular processes may be inhibited when zinc is deficient.

Since zinc has the ability to regulate the cell cycle and cell signaling, zinc may be able to function as a regulator in cancer (19). Zinc has been found to both induce and inhibit the important cellular process of removing damaged/abnormal cells known as apoptosis (13) (20) (21). Furthermore, irregular zinc status can cause immune and neurological disorders (22). These disorders could be related to zinc status because caspase-3 is activated to degrade protein when there is a low intracellular zinc concentration (22). Zinc status also alters the expression of an important tumor suppressor gene known as p53 (23). This gene needs zinc for its DNA-binding activity. Interestingly, p53 expression is elevated by both zinc deficiency and zinc supplementation (23-24).

The importance of zinc in cellular processes requires a properly regulated mechanism to maintain zinc homeostasis. Zinc homeostasis in the body is disrupted when there are changes in zinc absorption and excretion in the gastrointestinal tract. However, the disturbance can quickly be restored by zinc redistribution in the tissues and cells (25). The redistribution process depends on zinc transporters. Because it is a highly charged hydrophilic species, passive diffusion cannot be used as a means of transport (26). Therefore, the plausible mechanism of maintaining homeostatic zinc regulation in cells is by zinc influx and zinc efflux transporters. The zinc efflux transporters belong to the ZnT family [solute linked carrier 30 (SLC30A)]. They are responsible for moving zinc out of cells or into intracellular organelles from the cytoplasm (1). The ZIP (Zrt- and Irt-like proteins) family [solute linked carrier 39 (SLC39A)] are responsible for importing zinc into the cytoplasm from the extracellular space and intracellular

organelles (27). An abnormality in zinc transporter expression is a problem because it may lead to the uncontrolled growth of cells. In humans, fourteen members of the SLC39A family have been identified and nine out of the fourteen members belong in the LIV-1 subfamily of ZIP transporters (28-29).

Zinc availability and trafficking in cells are not only influenced by zinc transporters but also by metallothioneins (MT). MT are ubiquitous metal binding proteins that sequester zinc and release it during the events of signal transduction (25). The action of MT on zinc concentration is performed through the MT/thionein (T) coupling system. In the cluster structure of MT, there are cysteine sulfur ligands whose affinity for zinc is reduced in the oxidized state (25).

The dysregulation of zinc transporters could have dramatic consequences on zinc regulation. In fact, an interruption in the zinc regulatory process may lead to the initiation or progression of breast cancer. There is a range of evidence that supports this idea. Cells cannot proliferate without zinc (4) (30) and thus zinc plays an important role in tumor growth (32-35). In 2001, Paski and Xu demonstrated that the cell proliferating effects of growth factors are accompanied by an increase in the concentrations of labile zinc (33). Metals themselves have even been showed to exert estrogen-like activity in MCF-7 breast cancer cells (34). The exact molecular mechanisms of zinc transport in regulating and impacting cancers remain unclear. Hence it is important to understand the molecular mechanisms involved in cellular zinc homeostasis (34).

2.2. Physiological Roles of Zinc:

Zinc plays a crucial role in biological systems. Because of zinc's unique chemical characteristics and involvement with numerous proteins, a number of cellular, physiological, or metabolic processes would cease functioning without zinc. Zinc is the second most abundant micronutrient in the body. Its importance in nutrition, clinical medicine, and public health was not recognized until 1961 (35-36). That year, Prasad *et al.* (35-36) found zinc was responsible for a syndrome of adolescent dwarfism in Middle Eastern countries. The investigations on the significance of zinc expanded after the discovery that altered zinc metabolism led to the symptoms of acrodermatitis enteropathica, an autosomal recessively inherited disease in humans (37). Nowadays, we have come to know that zinc plays an essential role in physiological processes such as growth and development, normal thyroid, pancreatic and parathyroid function (38), immune function (39), bone mineralization, sense of taste and olfaction (40), central nervous system function (41), retinal function (aid in vitamin A metabolism), insulin synthesis and action, and lipid metabolism. Several pathological conditions have been associated with the interrupted regulation of zinc.

2.3. Zinc Homeostasis:

In multi-cellular organisms, most of the zinc is found in the intracellular compartments (42). Approximately 30–40% of the intracellular zinc is in the nucleus and 50% of it is found in the cytoplasm, organelles, and specialized vesicles. The remainder of zinc (~10%) is present in the cell membrane (42). Due to the numerous roles that zinc plays in physiological processes, cellular zinc

homeostasis must be regulated tightly. In the cell, a very low concentration of zinc exists in the form of labile free zinc pools and most of the zinc in cells is bound to proteins. The precise concentration of free labile zinc in cells is still not established (22) (25). Cells are able to use free labile zinc for various reactions but bound zinc is not readily available to conduct cellular signaling although that bound to MT is released under some circumstances (43) (44).

Once zinc is inside the cells, it can be in four possible forms (45). Under physiological conditions, most intracellular zinc is bound tightly to metalloproteins as a structural or catalytic component (46). In yeasts, about 20% of the zinc is distributed in major zinc metalloenzymes Cu/Zn superoxide dismutase (SOD) and alcohol dehydrogenase (Adh1) (47-50). Secondly, zinc could be bound to MT (50). Upon receiving various stimuli such as the activation of the nitric oxide (NO) pathway, zinc could be released from the Zn-MT complex to supply zinc to proteins or reactions. Zn-MT complexes were demonstrated to not only release zinc in the cytoplasm but also to translocate, for example to the nucleus, in response to cellular signaling events. Thus, they could provide a targeted zinc pool to specific organelles. Moreover, Zn-MT complexes could also serve as buffer of zinc. The third possible fate for intracellular zinc is getting distributed among cell organelles such as endosomes, lysosomes, golgi complexes, endoplasmic reticulum and specialized organelles like synaptic vesicles and secretory granules (51-53). The biological function of this pool of zinc is not clear but it is believed to be involved in detoxification and zinc storage.

The fourth form of zinc consists of the very low concentration of free labile intracellular zinc discussed above (54).

A wide range of intracellular zinc concentrations between 10^{-5} to 10^{-12} M have been reported by different literature (55-59). These huge consistencies are thought to be due to the different approaches used to measure zinc. There could be contamination contributed by the bound or organellar zinc or zinc released from protein sites due to oxidation or proteolysis. These findings indicated that there is a requirement for homeostasis maintenance and cellular signaling events between zinc transport, sequestration, and binding with metalloproteins. Therefore, labile or stored zinc must be coordinately balanced when cells are subject to fluctuations in environmental conditions, intracellular metabolism, and or regulatory responses. Cells require a fine mechanism to transport, distribute, and accumulate zinc within the cell in response to changes.

The discovery of the first zinc transporter for maintaining zinc homeostasis has led to the findings of a number of zinc transporters. Although the exact mechanism of zinc movement by each transporter is not completely understood, the fact that cells efficiently use zinc transporters to influx and efflux zinc to maintain zinc homeostasis has been firmly established. The next section discusses the two families of zinc transporters and their role in regulating zinc homeostasis.

2.4. Zinc Transporters:

A well-regulated mechanism in regulating zinc homeostasis is crucial for maintaining numerous cellular processes. As a small and hydrophilic divalent

cation, zinc cannot permeate biological membranes through passive diffusion (12). Therefore, the transport of zinc requires a separate mechanism. In fact, zinc trafficking has been found to 1) be time and temperature dependent, 2) be sensitive to changes in pH, and 3) involve both saturable and non-saturable components (12) (60-62). These findings suggested that the movement of zinc involves transporters. In fact, intracellular zinc homeostasis is maintained by different finely coordinated proteins that are involved in uptake, excretion and intracellular storage/trafficking (63). Even though zinc transporters are known to play important roles, more information needs to be obtained to understand the distribution and regulation of these transporters. To date, 24 transporters involved in zinc regulation have been discovered. There are 14 members in the ZIP (Zrt and Irt-like proteins) or Solute Linked Carrier 39 (SLC39) gene family and there are 10 transporters belonging to the ZnT (Zinc Transporters) or CDF (Cation Diffusion Facilitator) or SLC30 family (63-64).

2.5. The Family of ZnT (SLC30) Proteins:

The SLC30 family of genes consists of Cation Diffusion Facilitators (65). They are commonly referred to as “ZnT” and they are responsible for transporting zinc from the cytoplasm to the extracellular space or into the lumen of intracellular organelles. Therefore, ZnT transport proteins decrease the cytoplasmic zinc levels. They are located both on cell plasma membranes and intracellular organelle membranes to facilitate the efflux of zinc. Even though these transporters are called cation diffusion facilitator (CDF), they are thus far known only to transport the metal zinc. Some members in this family have also

been demonstrated to confer zinc resistance for both cell survival and intracellular zinc homeostasis maintenance (65).

The CDF family is divided into three subfamilies: I, II, and III. Subfamily I is found mainly in prokaryotes whereas mammalian zinc transporters are assigned to subfamilies II and III (66). However, subfamilies II and III are also found in prokaryotic hosts. Currently there are ten identified members of CDF in mammals. Most ZnT proteins have six (I-VI) transmembrane domains (TMDs) and amino and carboxyl termini on the cytoplasmic side of the membrane. These proteins also have a histidine rich loop that has a variable length between domains IV and V. ZnT-5 is the only CDF member that has 12 TMDs (66).

2.6. The Family of ZIP (SLC39) Proteins:

The ZIP (Zrt/Irt like proteins) family of protein are named after Zrt1 and Zrt2 proteins in *Saccharomyces cerevisiae* (yeast) and Irt1 proteins in *Arabidopsis thaliana* (groundnut) (67-69). Furthermore, these proteins were the first members of the ZIP family to be identified. In *Saccharomyces cerevisiae*, Zrt proteins were discovered to be zinc uptake transporters and Irt was identified as the major iron uptake transporter in *Arabidopsis thaliana*. Therefore, the proteins in the SLC39 gene family are uptake channels and perform the opposite action to ZnT family members. More specifically, the ZIP family proteins transport zinc from the extracellular space or intracellular organelles into the cytoplasm to increase the cytoplasmic zinc levels. Factors of pH and temperature affect the uptake of zinc. Most of the ZIP family members contain eight TMDs and have similar topologies with the amino and carboxyl termini of the protein located on the extra-

cytoplasmic face of the membrane. The majority of the transporters in SLC39 family have a long loop between TMDs 3 and 4. Between the regions of TMDs 3 and 4, a histidine rich sequence is observed to have an ability to bind metal. Hence, this domain is reported to have a function in regulating and transporting zinc (70-72).

2.7. Zinc and Cancer:

Cancer is caused by the dysregulation of cell multiplication. Without intervention, the dysregulation would ultimately lead to uncontrolled growth. Mutations in genes such as proto-oncogenes (Ras) or tumor suppressor gene (p53) that regulate the cell cycle would lead to uncontrolled cell proliferation and thus cause cancer. Zinc plays an important role in cancer because it is needed in the functioning of these cell cycle regulating proteins. Furthermore, zinc has been reported to be involved in the DNA repair mechanism as well as serving as an antioxidant. Having the capability to regulate cell cycle and act on different signaling cascades, zinc is thought to have a function in cancer (73).

In terms of the relationship between zinc and cell growth, zinc dependent transcription factors are required for the replication and transcription of DNA to regulate the cell cycle (74). There are also enzymes involved in cell growth that require zinc. In addition to its role in cell growth, zinc plays a role in apoptosis. Animal as well as *in vitro* studies have demonstrated that zinc deficiency induces apoptosis (74). It is most likely that zinc acts on signaling pathways that are responsible for causing apoptosis. Both *in vitro* and *in vivo* studies have indicated that zinc supplementation reduced apoptosis (75).

As an antioxidant, zinc is involved in many parts of the oxidant defense system. Zinc plays a part in intracellular and extracellular SOD. Moreover, it can regulate MTs, which can scavenge redox-active metals. Moreover, zinc can compete with metals such as copper or iron for membrane binding sites (76). If zinc were absent, these redox active metals could catalyze oxidation reactions which would result in oxidation of proteins, lipids, and DNA. Growing cell cultures in zinc deficient media led to a high concentration of oxidants and zinc deficient rats were found to have increased DNA damage and oxidative injury (77). Hence without zinc, reactive oxidative species (ROS) can elevate and increase oxidative stress. Increase in oxidative stress would lead to DNA damage in cells as well as failure to activate DNA repair mechanisms. The accumulation of these effects would lead to the development of cancer. Given these diverse functions, zinc may be useful for preventing cancer (78).

2.8. The Role of Zinc in Cell Growth:

Transcription factors that interact with DNA and RNA are dependent on zinc to function since they contain zinc-finger motifs. Besides zinc finger transcription factors, DNA polymerases, RNA polymerases, t-RNA synthetase, and alkaline phosphatases also require zinc to perform in cells (54). Furthermore, cell proliferation does not take place in the absence of zinc. Therefore, highly proliferating systems such as the immune system, reproductive system, and the skin would be severely affected without zinc (2-3).

Moreover, there are enzymes involved in cell growth that require zinc, for example, aspartate transcarbamylase. This enzyme is important as it catalyzes the

first step in DNA synthesis. Without zinc, synthesis of DNA would get interrupted. Another zinc requiring enzyme is thymidine kinase; which is involved in the synthesis of collagenous connective tissue (79). Zinc is also important to matrix metalloproteinases such as collagenases and stromelysins. Lastly, zinc metalloenzymes, gelatinase A and gelatinase B, that have been implicated in wound healing and extracellular matrix remodeling are also zinc dependent (80). Hence, many important biological processes would be severely affected when zinc is not available.

2.9. The Effects of Zinc on DNA Repair Mechanisms:

2.9.1. Zinc and the p53 Gene

Zinc is required for DNA repair mechanisms. Many of the base and nucleotide excision repair proteins are dependent on zinc as they are either zinc-finger or zinc-associated proteins (76). The tumor suppressor gene, p53, is a zinc finger protein that is involved in apoptosis, DNA repair and cell proliferation/differentiation (81). It is also able to induce arrest at the G1 phase of the cell cycle to allow DNA repair before the next phase (82). This gene is constitutively expressed in a latent form in most cells and tissues. The gene is activated when exposed to various forms of genotoxic and non-genotoxic stress. The activated form of p53 coordinates several anti-proliferative pathways to permanently delete cells containing damaged DNA from the pool of actively proliferating cells. As a transcription factor, p53, binds DNA through a structurally complex domain stabilized by a zinc atom. The architecture of this

domain can be disrupted by zinc chelation and changed to a phenotype identical to that of many mutant forms of p53 (83).

When stress occurs, p53 gets induced by post-translational modifications that stabilize the protein. Conformational changes also occur to increase the affinity of p53 for specific DNA sequences. Studies have demonstrated that p53's expression is elevated by both zinc deficiency and zinc supplementation (83). The active form of p53 not only regulates the transcription of target genes but interacts with heterologous factors to mediate negative regulation of cell-cycle progression and apoptosis induction. p53-transactivated genes such as WAF-1 (a cyclin kinase inhibitor acting in both G1 and G2 phases), GADD45 (a growth arrest and DNA damage response factor involved in a G2/M checkpoint), and 14-3-3s (a signal transduction factor that inhibits G2/M progression) are all involved in controlling cell cycle arrest (83).

In 50% of cancer, p53 was reported to be mutated (83). Hence, DNA repair would be severely compromised. Most of the p53 gene mutations in cancer are missense mutations. These mutations are scattered in the region of the gene that encodes the sequence-specific DNA-binding domain (residues 102-296), underlying the importance of this domain in the suppressive activity of p53 (13-18). *In vitro* studies showed that metal chelators induced wild-type p53 to lose its bound zinc and oxidized several important cysteines that reside in the DNA-binding domain (13). Based on these observations, zinc plays an important structural role in the p53 protein. As aforementioned, there is an increase in oxidative stress in the absence of zinc and thus leads to increased susceptibility to

DNA damage and inability to activate repair mechanisms (77). A cumulative effect of these factors could be cancer development.

2.9.2. Zinc and p21 Gene

Zinc status also affects p21, a cell cycle progression regulatory gene. When DNA damage occurs, p21 gets over-expressed and leads to growth arrest at the G1 and G2 phases or S phase (84). Mechanisms such as transcriptional regulation, epigenetic silencing, mRNA stability, and ubiquitin-dependent and independent protein degradation all modulate the cellular concentrations of p21 (84). Both p53-dependent and independent pathways are involved in the transcriptional modulation of p21 expression. Two conserved p53-binding sites are located in the p21 promoter. At least one of these two sites is needed for p53 responsiveness after DNA damage (84). As for the p53-independent activation of p21 transcription, it requires a variety of transcription factors such as Sp1, Sp3, Ap2, STATs, C/EBP α , C/EBP β , and the bHLH proteins, BETA2 and MyoD, to be induced by a number of different signaling pathways (84).

Interestingly, p21 plays a role in the stabilization of the assembly of cyclin D1-cdk4/cdk6. The integrity of cyclin D1-cdk4/cdk6 is essential for G1/S transition. p21 changes it into active complexes that modulate cell cycle positively (19). p21 as an assembly factor for cyclin D1-cdk4/cdk6 is peculiar since it functions completely opposite of its established role as a cdk inhibitor. The formation of cyclin D1-cdk4/cdk6 complex is negatively impacted when p21 is repressed and results in impaired cell cycle progression. In fact, it has been reported that in endothelial cells, p21 repression mediated by the Notch pathway

decreased cyclin D1-cdk4 formation and nuclear targeting. The reduction of cyclin D1-cdk led to reduced retinoblastoma protein phosphorylation, as well as depressed S phase entry and cell cycle progression (84). Another finding has revealed that Sp1 repression of p21 transcription mediated by Sp1-binding sites in the proximal p21 promoter in smooth muscle cells decreased cyclin D1-cdk4/cdk6-p21 complex formation and subsequently inhibited cell growth (19). Therefore, p21 repression may lead to depressed cell cycle progression and cell growth.

The level of p21 can be altered by zinc status in certain cell types. In fact, reductions of zinc status may promote apoptosis in certain cell types and depress G1/S cell cycle progression in human hepatoblastoma (HepG2) cells. However, more studies need to be conducted to further validate the effects of zinc on p21 (84).

2.10. Zinc and Clinical Cases of Cancer:

It is well established that zinc plays an essential role in cell growth and cell cycle. Since these two processes are reliant on zinc, cancer could occur in cases of zinc deficiency. Epidemiological studies have emphasized this importance by demonstrating that there is an association between tissue zinc levels and incidence of cancer (85). Although there has been a connection made between zinc level and other types of cancers, prostate and breast cancers are the two most defined cancers that are related to zinc status.

2.10.1. Prostate Cancer

The prostate gland contains a very high concentration of zinc (85) (86). The cells that contain a very high level of zinc and also citrate are identified as peripheral glandular secretory epithelial cells. The high concentration of zinc in these cells is required to prevent the oxidation of citrate by m-aconitase which in turn is secreted in these cells. In cases of malignant prostate cancer cells, a decrease in both zinc and citrate levels is found. Therefore, these two characteristics are now considered as very reliable markers for malignancy in these tissues (85).

Besides synthesizing, storing, and secreting high concentrations of citrate, peripheral secretory epithelial cells also contain high concentrations of mitochondrial and cellular zinc. Zinc is present in high level so that it can be readily available to act as an inhibitor on m-aconitase which reduces the terminal oxidation of the citrate in these cells and thereby produces less energy. Because cancer cells have increased energy requirements, the concentrations of zinc are decreased and thus activate m-aconitase. The activation in turn triggers the oxidation of citrate to produce energy (85). Besides having a metabolic effect, zinc could also have an apoptotic effect in these cells. It is believed that reduced apoptosis along with increased energy availability allows for the proliferation of malignant cells. Although the exact mechanism for the accumulation of zinc in prostate cells is not fully known, recent studies have shown that up-regulation of the zinc influx transporter, ZIP1, in prostate cells increases zinc accumulation whereas downregulation decreases zinc concentration in prostate cancer cells (85).

2.10.2. Breast Cancer

Breast cancer is one of the leading causes of death among women. The disease makes up 18% of all cancer deaths (87). It is estimated that 50% who are diagnosed with breast cancer will die from it (87). Approximately 30-40% of breast cancers do not possess estrogen receptors (88). Breast cancer cells that are ER⁻ are known to be more invasive than those that exhibit ER⁺ status (89-90). It has been suggested that ER⁺ breast cancer cells that are less invasive rely on estrogen receptors to enhance their growth. Therefore, breast cancers that do not have estrogen receptors may need a separate mechanism to support their motility and development. There is evidence suggesting that ER⁻ breast cancer cells may depend on epidermal growth factor receptor (EGFR) for their growth and viability (90).

The etiology of breast cancer is multi-factorial and is still not completely understood. However, hormones and growth factors have been linked to breast cancer. Zinc status has also been linked with the disease (88). Furthermore, zinc concentrations were found to be higher in biopsies removed from mastectomies or lumpectomies than in normal breast tissues (87). In fact, the effects of hormones and growth factors may play a role in inducing zinc transporters that mediate breast tumor growth (88, 90).

With the discovery of LIV-1 as a zinc importer, the association of zinc with breast cancer becomes even more compelling. LIV-1 is an estrogen receptor regulated gene that is associated with estrogen receptor positive breast cancer. It is now used as a marker of luminal A type breast cancer (91). However, the exact

relationship between zinc, LIV-1, and breast cancer is still not completely understood. Therefore, the connection between growth hormones, dysregulated zinc homeostasis, and zinc transporter function in breast cancer needs to be investigated to develop proper prevention and diagnosis for the disease.

2.11. Zinc Influx Transporter: LIV-1 (Zip 6):

LIV-1 or Zip 6 was identified as an estrogen regulated gene in MCF-7 and ZR-75 breast cancer cells (13). This transporter belongs to the LIV-1 subfamily of ZIP transporters. The LIV-1 subfamily of ZIP transporters is also termed as LZT (LIV-1 subfamily of ZIP zinc Transporters). LZT is characterized by eight TMDs that reside in the plasma membrane (4). It also contains a long extracellular N terminus, a short extracellular C terminus, and a consensus sequence for the catalytic zinc-binding site of metalloproteases. Furthermore, the potential metalloprotease motif (HEXPHEXGD) in LZT sequences is unusual because it consists of proline and glutamic acid which are not seen in any other metalloprotease motifs (7-8). LIV-1 is one of the LZT that needs to be studied because it is an estrogen-regulated gene and its expression in breast tumors is associated with ER status (6). In addition, LIV-1 is linked with the metastatic spread of the cancers in breast to the regional lymph nodes (7). Since the discovery, LIV-1 has been considered as a reliable marker for ER⁺ cancers (92) (93). However, the role of LIV-1 in ER⁻ cells remains to be addressed.

LIV-1 has also been demonstrated to play an important role in host cell immune response through the regulation of cellular zinc homeostasis (94). TPEN-induced zinc deficiency or LPS increased the surface expression of MHC class II

molecules. Interestingly, the addition of zinc or over expression of LIV-1 eliminated the response (94).

El-Tanani et al. examined the role of insulin and its interaction with estradiol in controlling the expression of LIV-1 and pS2 mRNA (7). Like LIV-1, pS2 is an estrogen-induced gene expressed in MCF-7 cells. Both LIV-1 and pS2 mRNA levels increased when MCF-7 cells were treated with insulin and estradiol but each by a distinct mechanism. These separate mechanisms both involved stimulation of gene transcription. Estradiol bound to its intranuclear receptors which caused a conformational change in the receptor protein that permitted it to bind tightly to specific DNA sequences. The activated estradiol hormone receptor could then interact with the pre-initiation complex to stimulate transcription of genes in the vicinity of the hormone response element (7). Insulin, on the other hand, and other signal molecules with plasma membrane receptors, regulated gene transcription by indirect means. Insulin induction relied on the continual synthesis of protein and its regulation involved the phosphorylation/dephosphorylation of specific transcription factors. These results demonstrated that both estradiol and insulin could stimulate the transcription of these estrogen-inducible genes (LIV-1 and pS2) by separate mechanisms (7). However, the signaling effects of both insulin and estradiol could be blocked by a pure antiestrogen of ICI 164384.

El-Tanani and his team also examined LIV-1 and pS2 responses to growth factor stimulation of MCF-7 cells (7). They found that in the absence of estradiol, LIV-1 and pS2 were increased by EGF and insulin-like growth factor-1 (IGF-1) at the transcriptional level. It was found that the induction by growth factors but not

by estradiol relied on the synthesis of protein. However, both mechanisms required the presence of ER (8).

Taylor et al. noted that LIV-1 is closely related to the ZIP transporters (6-7). They identified LZT in this zinc-binding site motif and thus they predicted that LIV-1 could act as a zinc-influx transporter. To confirm their prediction, they used Chinese hamster ovary cells to express the protein. They found that LIV-1 was localized to the plasma membrane lamellipodiae, similar to the membrane-type metalloproteases, which correlated with metastasis. To further confirm their prediction of LIV-1 as a LZT protein, they demonstrated that LIV-1 regulated zinc uptake of the cells. The structure of LIV-1 along with their observation in breast tumor demonstrated that LIV-1 indeed behaved as a zinc-influx transporter. Their discovery suggested that the combination of LIV-1's zinc-uptake and potential metalloprotease activities could play a role in breast cancer progression (32).

Kasper et al. determined if LIV-1 expression levels could serve as a prognostic marker for breast cancer (6). They looked at LIV-1 mRNA and protein levels in tumors from 111 human breast cancer patients by in situ hybridization and immunohistochemistry. They found that LIV-1 mRNA quantity when combined with a positive ER status made a better marker of a good clinical outcome in breast cancer patients than progesterone receptor status. They also discovered that LIV-1 protein was negatively correlated with tumor size, grade, and stage. The high level of expression in LIV-1 protein apparently was associated with a longer relapse free and overall survival in breast cancer patients

with invasive ductal carcinoma. Their data suggested that LIV-1 could have the potential in serving as a prognostic marker for breast cancer patients (6).

Despite the possibility of LIV-1 serving as a positive therapeutic marker for breast cancer cells, some studies have indicated a high expression level of LIV-1 in ER⁺ tumors is associated with spread to the lymph nodes. The increased level of LIV-1 in cells may be the result of an interruption in its regulation and this disturbance may have contributed to the uncontrolled growth of cells. Therefore, the role of LIV-1 in breast cancer and tumors still remain unclear and more investigations need to be conducted to make a better assessment (2) (7) (11).

2.12. Epidermal Growth Factor and Epidermal Growth Factor Receptor:

An over expression of EGFR in breast cancer is an indication of a more aggressive and invasive type of tumor (95). MCF-7 cells, which are ER⁺ and considered to be less invasive than MDA-MB-231 cells, transform to estrogen independent and to a more cancerous phenotype when EGFR is over expressed (95). In fact, the expression of the EGFR is twice as likely in breast cancers which are missing both the ER and the progesterone receptors (95).

EGFR is a type I transmembrane protein that consists of 1186 amino acid residues. Its N-terminus extends into the extracellular space (96). Its structure is unique in the sense that it can trap and respond to a number of ligands. All EGFR members have the consensus sequence of Z_nCX₇CX₂₋₃GXCX₁₀₋₁₃CSCS₃YXGXRCX₄LX_N (96). The effects that each EGFR exhibits depend on the location of the EGFR. The receptor is known to crosstalk with other signaling pathways to exhibit its effects (8). Taylor et al. has proposed that activation of

EGFR may be able to induce LIV-1 through a STAT3 and MAPK modulated signaling pathway, as illustrated below (8):

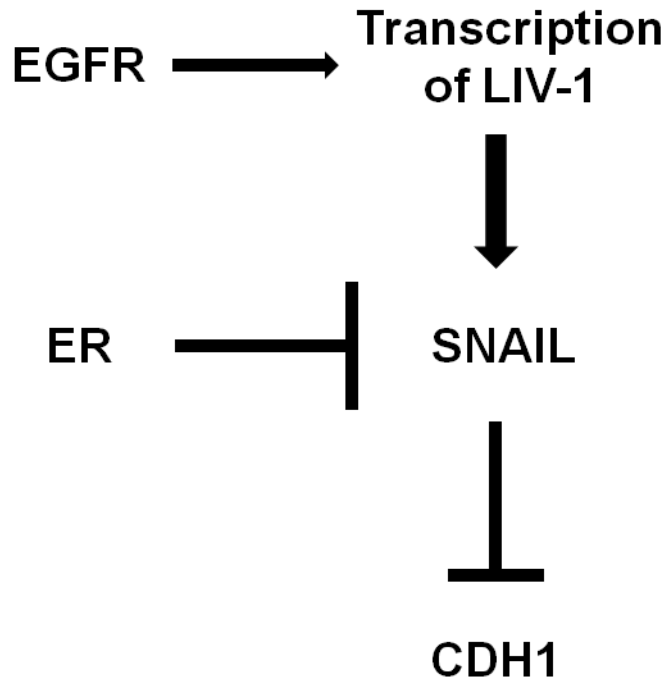


Figure 1: Proposed Regulation of LIV-1 by Epidermal Growth Factor and LIV-1's Effects on SNAIL and CDH1 based on Taylor KM, Hiscox S, Nicholson RI. *Zinc transporter LIV-1: a link between cellular development and cancer progression.* Trends in Endocrinology Metabolism. 2004. **15:**461-3.

Both MAPK and STAT3 are downstream targets of EGFR activation. The over-expression of EGFR in ER⁺ cells could lead to a cancerous phenotype that is independent of estrogen effects (97).

2.13. Insulin:

Insulin is an anabolic hormone that produces significant effects on a number of tissues. The insulin receptor, also known as cluster of differentiation 220 (CD220) (39), consists of 2 alpha and 2 beta subunits encoded by the same

gene (39). The receptor also shares homology with that for IGF-1 (98). Because its structure is homologous to IGF-1, insulin is able to bind and activate the ubiquitous IGF-1 receptors (IGF-1R) at high concentration. In fact, crosstalk is exhibited between insulin and IGF-1 signaling pathways.

Besides binding to IGF-1R, insulin can also express its activity through the binding to its own receptors. Its binding results in the activation of a tyrosine kinase, which triggers the autophosphorylation and subsequent activation of docking sites for downstream proteins such as insulin receptor substrate protein (98). The activation of insulin receptor substrate protein further generates other downstream signaling effects (98).

In relation to breast cancer, elevated concentrations of insulin and diabetes are risk factors for breast cancer development in postmenopausal women (99). Once insulin binds to its corresponded receptor, it can induce proliferative effects in breast cancer cells. The invasive and estrogen receptor negative breast cancer cells, MDA-MB-231, contain a high concentration of insulin receptors (99). These findings indicated that insulin could be a potential target for lowering breast cancer risk.

2.14. E-cadherin (CDH1):

E-cadherin (CDH1) is a transmembrane glycoprotein that makes up the main component of adherent junctions (the actin-linked cell-cell junction of epithelial cells). It mediates calcium-dependent intercellular adhesion and is involved specifically in epithelial cell-to-cell adhesion (100). It is essential for intercellular adhesion, cell polarity, cell signaling, maintenance of cellular

differentiation, and tissue morphology for normal epithelial cells (100). The altered expression or function of CDH1 often occurs during embryogenesis and carcinogenesis. The diminished expression of CDH1 results in the promotion of tumor invasion, malignant transformation, and metastasis *in vitro* and *in vivo* (100). In fact, CDH1 expression is inversely correlated with tumor stage. In other words, tumors in the most aggressive form have the lowest expression in CDH1. Furthermore, the suppression of CDH1 increases the resistance of breast cancer cells to chemotherapeutic agents (100).

The functional loss of CDH1 in an epithelial cell has been considered a hallmark of epithelial-mesenchymal transition (EMT) (101). When the transition occurs in breast cancer cells, their intercellular cohesion and polarity is lost (101). This increases the cells' motility and proteolytic activity. In fact, the loss of CDH1 induces breast cancer cells to remodel their cytoskeleton and allows them to undergo a process similar to embryogenesis (101). Moreover, the transformation enables invasive carcinomas to travel to lymphatic or vascular channels and metastasize to regional nodes and distant sites (101).

The down regulation of CDH1 may arise through transcriptional repression (102). For instance, it has been suggested that CDH1 expression may be reduced by a zinc-finger containing transcriptional repressor known as Snail (SNAI1). SNAI1 has been implicated by Taylor et al. in the regulation of the phenomenon of EMT (102). SNAI1 inhibits the expression of CDH1 by binding the E-box DNA-binding sequences found within the CDH1 promoter (102). The

transfection of SNAI1 into epithelial cells changes them to a more fibroblastic phenotype (102).

Yamashita et al. have indicated that there is a significant association between EGFR, LIV-1, SNAI1, and CDH1 in breast cancer (103). In ER⁻ tumors, the expression of SNAI1 was demonstrated to be higher than in ER⁺ tumors. The increased expression of SNAI1 suppresses the expression of CDH1; suggesting ER⁻ breast cancer could thus exhibit an invasive phenotype (103). SNAI1 alone does not suppress CDH1. Interestingly, SNAI1 cooperates with LIV-1 to reduce the transcription of CDH1. The potential significance of LIV-1 and SNAI1 in regulating CDH1 in breast cancer was established through the nuclear localization of SNAI1 as a Snail-green fluorescent protein (GFP) conjugate in zebrafish gastrula organizer cells. The nuclear localization of SNAI1 in zebrafish failed to occur in the absence of LIV-1 (103). The opposite effect was observed when the embryos had LIV-1 expressed. Failing to localize to the nucleus without LIV-1, SNAI1 negative cells were able to express CDH1 (103). Therefore, LIV-1 is needed for the nuclear localization of SNAI1 in organizer cells of zebrafish. Due to the association between LIV-1 and SNAI1, a link between LIV-1, SNAI1, and CDH1 was established in zebrafish. Based on this finding, Taylor proposed that a similar relationship could exist between LIV-1, SNAI1, and CDH1 in human cancer cells through the activation of EGFR (104-105). Because this is still not fully understood, the link between EGFR, LIV-1, SNAI1, and CDH1 needs to be further investigated. Studying their relationship could help us discover novel therapeutic targets for improving breast cancer prognosis.

2.15. Snai1 and Snai2 Genes:

The product of *Drosophila* Snail gene represents the founding member of a superfamily of transcriptional regulators (106). The members of this family have been associated with mesoderm and neural crest formation as well as in the pathological progression of epithelial tumors. In mammals, the best-characterized members of the Snail superfamily are Snail1 and Snail2. These two transcription factors are also known as Snail and Slug.

The Snail superfamily is characterized by a common protein organization. All the Snail members have a highly conserved region at the carboxyl terminus of the protein. In this region, a range of four to six zinc fingers of the C₂H₂ type are found (106). The zinc-finger domains in Snail and Slug proteins serve to mediate sequence-specific interactions with DNA. Therefore, this highly conserved zinc-finger region recognize similar target sequences and bind specifically to consensus binding sites that contain the core sequence of 5'-CAGGTG/5'-CACCTG (106). The core sequence resembles the E-box (CANNTG) motif and thus serves as the binding targets for basic helix-loop-helix (bHLH) transcription factors. Under some circumstances, the Snail family proteins may compete with bHLH proteins for the core sequence (107). The amino termini of the Snail superfamily members are less well conserved but the SNAG domain at the extreme amino termini have been evolutionarily conserved in most vertebrate members. The Slug genes are differentiated from other members of the superfamily by the aforementioned conserved sequence motif near the zinc fingers.

The members of the Snail superfamily function as transcriptional repressors (107). The function of transcriptional repression is associated with various conserved protein domains. The SNAG domain in vertebrates is crucial to the transcriptional repression function of Snail family members. In both *Drosophila melanogaster* (107) and mice, the Snail gene is essential for normal development. Homozygous null embryos in both species fail to produce mesoderm, resulting in a failure to gastrulate. The failure of mesodermal specification in Snail mutant embryos led to the characterization of Snail as a master regulator of epithelial to mesenchymal transitions. Slug, on the other hand, is not embryonic lethal in mice. Mice that are homozygous null in Slug can still be viable and fertile (107).

Although Slug is not needed for normal development in mice, it has been implicated in EMT during development in other vertebrates. Slug plays an important role in embryogenesis of chick and *Xenopus* by inducing neural crest specification (107). Overall, Snail and Slug promote changes in cell fate during development and thus lead to the production of migratory mesenchymal cells.

Since a major conserved function of Snail proteins is to control cell motility, there is a possibility that Snail can regulate cancer progression. In fact, some members of the Snail superfamily have been implicated in cell survival. For example, the protein of *Caenorhabditis elegans* CES-1 (for cell death specification) can block programmed cell death of the NSM sister neurons during embryogenesis (108). In vertebrates, Slug has been found in certain leukemias to be aberrantly upregulated by the E2A-HLF oncoprotein. Slug's aberrant

upregulation could lead to increased cell survival (108). Further analysis of Slug during hematopoiesis has further revealed a non-pathological role for Slug in promoting cell survival in hematopoietic progenitor cells (108). Aberrant expression of Snail or Slug has been proposed as a key determinant of pathological epithelial to mesenchymal transitions during tumor progression (108). Therefore, phenotypic alterations can be induced by these transcription factors in epithelial breast cancer cells, including loss of cell-cell contacts and acquisition of invasive growth.

Chapter Three: Hypotheses and Rationale

Hypothesis I:

The expression of LIV-1 in breast cancer cells is inversely related to the expression of CDH1 in MDA-MB-231 cells.

Hypothesis II:

The uptake of ^{65}Zn in cells is parallel with the expression of LIV-1.

Rationale:

LIV-1 is a zinc importer and its elevated expression may inhibit CDH1 expression because it stimulates the expression of a zinc finger transcription factor, SNAI1 or SNAI2, which in turn suppresses the expression of CDH1.

Chapter Four: Materials and Methods

4.1. Cell Culture:

Estrogen receptor negative human breast cancer cells (MDA-MB-231) were obtained from American Type Cell Culture Collection (ATCC, Rockville, MD) and grown as recommended in 5% CO₂ at 37°C and 95% humidity. The cells were maintained in Dulbecco's Modified Eagles Media (DMEM) supplemented with 50 mL/L fetal bovine serum, 50,000 IU/L penicillin, 50 mg/L streptomycin, and 2 X 10⁻⁶ M of Fungizone.

4.2. Procedure for Subculturing Cells:

T-75 flasks were used to maintain MDA-MB-231 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 1XTE (0.05% Trypsin / 0.53 mM EDTA). To detach the cells, 1XTE (3 ml) was added to the cell layer and incubated for a period of 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 divided equally to two T-75 flasks. Fresh media (10 ml) was added to each flask after the division.

4.3. Hormonal Treatments:

MDA-MB-231 cells were grown in T-25 flasks until they reached an approximately 60-75% confluency. Cells were treated with or without EGF (10ng/ml) and insulin (6µg/ml) for 24 hours and then RNA and proteins were extracted. Four flasks were assigned to each treatment group---control, EGF, and insulin.

4.4. Transfection Process:

The short hairpin RNA (shRNA) lentiviral plasmid (pLKO.1-puro) was utilized for the transfection process. Two shRNA sequences that specifically targeted the LIV-1 gene were designed and synthesized by Sigma (St. Louis, MO). The shRNA plasmid pLKO.1-puro contained a puromycin selection marker for stable transfection. The whole plasmid with shRNA targeting LIV-1 was transfected into MDA-MB-231 cells with Fugene HD Transfection reagent, using standard protocols supplied by the manufacturer (Roche Applied Science, Indianapolis, USA).

Control and negative control cells were also used. The controls were cells without shRNA insertion. The negative control cells contained non-targeting shRNA that activated the RNAi (RNA interference) pathway but had no matches in the human genome. Transfected cells were then selected using 4 μ l of puromycin (final concentration in media: 0.8 μ M) every 48 hours for two weeks. After the selection period, the RNA and protein were extracted to assess LIV-1 expression.

4.5. RNA Extraction:

Total cellular RNA was extracted from MDA-MB-231 cells using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. The media was decanted off and cells were washed with 3 ml of 1XTE. 1.5 ml of 1XTE was next added to detach the cells. The detached cells were poured into labeled 5 ml polypropylene tubes (Fisher Scientific, Pittsburgh, PA) and centrifuged at 500 X g (1000 rpm) in a Beckman J6-B centrifuge. After

the cell pellet was obtained, 500µl of Trizol was added to dissolve the cell pellets. The dissolved cell pellets were vortexed and transferred to a micro-centrifuge tube. To acquire the aqueous and the organic phase separately, 250µl of chloroform was added, the tubes were vortexed and incubated at room temperature for 3 minutes. The tubes were further centrifuged at 4⁰C in a micro-centrifuge (Eppendorf, New York, NY) at 10,000 X g for 21 minutes. The dissolved RNA in the aqueous phase was carefully pipetted out into a fresh micro-centrifuge tube. To precipitate the RNA out of the aqueous phase, 500 µl of isopropanol (Fisher Scientific, Pittsburgh, PA) was added and incubated overnight at 4⁰C.

The precipitated RNA was obtained using centrifugation at 10,000 rpm at 4⁰C for 15 minutes. The pellet was next washed with 500 µl of 70% ethanol in DEPC water, vortexed, and centrifuged at 10,000 rpm for 5 minutes. To evaporate the alcohol, the pellet was air dried for 10 minutes or until all traces of alcohol disappeared. The dry RNA pellet was then dissolved in 50 µl of DEPC treated water. They were placed at -80 ⁰C if they were not to be processed immediately for experiments.

4.6. RNA Concentrations Estimation:

The concentrations of RNA in each sample were estimated using a Beckman Coulter DU800 spectrophotometer (Fullerton, CA). To prepare for the estimation, 1 µl of the sample was added to 999 µl autoclaved Tris-EDTA buffer (10mM Tris pH 7.5, 1mM EDTA) in a sterile microfuge tube. The samples were

mixed and absorbance was measured in disposable cuvettes at 260nm, 280nm, and 320nm.

After obtaining the absorbance values, the RNA concentration was calculated using the following formula:

$$\text{RNA conc. } (\mu\text{g}/\mu\text{l}) = [A_{260} - A_{320} \times 1000 \text{ (dilution factor)}] / 40$$

The $A_{260\text{nm}}/A_{280\text{nm}}$ ratio was used to determine the purity of RNA after subtracting the absorbance at 320 as a correction for background. Any ratio observed in the range of 2.0 is indicative of RNA. Any ratio obtained below 1.9 indicated a contamination with either DNA or protein and these samples were excluded for RNA quantification. The RNA samples were diluted with RNAase free water to a final concentration of 1 $\mu\text{g}/\mu\text{l}$ and stored at -80°C .

4.7. Assessment of RNA Quality:

To check for RNA quality, aliquots of RNA were electrophoresed through an agarose gel containing formaldehyde. The presence and appropriate ratio of 2:1 (28S:18S) rRNA bands were confirmed.

4.8. RNA Expression Analysis:

RNA was quantified using a semi-quantitative reverse transcriptase PCR reaction using gene specific primers.

4.9. Primer Design, Selection, and Preparation:

Sequences of primers for LIV-1, CDH-1, and GAPDH were obtained from previous published literature (54-55). Primers for SNAI1 and SNAI2 were

designed using Primer 3 software (<http://frodo.wi.mit.edu/>). Left and right gene specific primers were selected with the following criteria: optimal product size (<400), 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. Primers were ordered from Integrated DNA Technologies (IDT, San Diego, CA). Before usage, a final primer concentration of 10 μ M was prepared in DEPC treated water. The primers used and their expected product size are shown in **Table 1**:

Gene	Sequence	Product size
LIV-1	Forward: 5'-GGTGATGGCCTGCACAATTTC-3' Reverse: 5'-TTAACGGTCATGCCAGCCTTTAGTA-3'	161
CDH1	Forward: 5'-GTCATTGAGCCTGGCATTTC-3' Reverse: 5'-GCTTGAACTGCCGAAAAATC-3'	462
SNAI1	Forward: 5'-CCAGAGTCAGCCCTTAGTTC-3' Reverse: 5'-AGGAGAGAGTCCAGAGGATG-3'	284
SNAI2	Forward: 5'-TCGGACCCACACATTACC-3' Reverse: 5'-CTGGAGCAGAGGTTGTTAGC-3'	282
GAPDH	Forward: 5'-CATGCCAGTGAGCTTCCCGTT-3' Reverse: 5'-GTGGAGTCTACTGGCGTCTTC-3'	412

Table 1: Specific Primer Sequences

4.10. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):

Reverse transcription polymerase chain reaction was performed using 2 µg of total RNA. The RNA was transcribed into cDNA in a 50 µl reaction volume using the Qiagen One-Step RT-PCR kit (Qiagen, Valencia, CA). A master mix was prepared with 1X RT-PCR Q-buffer, 5X RT buffer (TrisCl, KCl, (NH₄)₂SO₄, 12.5 mM MgCl₂, DTT; pH 8.7), 400 µmol/L dNTP, and 0.6 µmol/L primers for target genes. Aliquots of the master mix were added to individual 0.2 ml PCR tubes containing 2 µg of RNA, 2 µl of RT-PCR enzyme mix, and appropriate volume of RNase-free water. Reverse transcription and polymerase chain reaction were carried out using a GeneAmp 9700 (Applied Biosystems) thermocycler. Reverse transcription was performed for 30 minutes at 50°C followed by a 1 minute incubation at 95°C for HotStart Taq DNA polymerase activation and inactivation of the reverse transcriptase enzyme. Polymerase chain reaction amplification was carried out for 34 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute.

4.11. Optimization of the Cycle Number:

In order to obtain a sensitive detection before the plateau of the reaction, each primer was tested for its optimal cycle number. The thermocycler was set to run until the cycle number of 40. At the end of 25, 30, and 35 cycles, 5µl of sample was pipetted out and placed into a 0.2 ml PCR tube. At the end of the 40th cycle, the tubes were put back into the thermocycler to complete the extension. The samples were next separated on 1% agarose gel.

4.12. Gel Electrophoresis:

The amplified samples from RT-PCR were separated on horizontal 1% agarose gels [0.5 g of Agarose (Fisher Scientific, Pittsburgh, PA), in 50 ml TBE buffer [(0.89 M Trizma Base, 0.89 M Boric acid and 25 mM EDTA; pH 8.3) containing 25 µl ethidium bromide (10 mg/ml)]. The samples were prepared by using 5 µl of amplified DNA, 1 µl of 10 X loading dye, and 14 µl of distilled water. The prepared samples were then loaded carefully into each lane of the agarose gel. A 1 kb plus DNA ladder (Invitrogen; 3 µl of ladder, 6 µl of distilled water and 1 µl of 10 X loading dye) was added to one of the lanes and run alongside with the samples to validate the product size of the DNA. The gel was run at 120 volts for 50 minutes. After the run was completed, the gel was visualized under UV light using ChemiDoc System (BioRad, Hercules, CA) and the signals quantified using Quantity One Software (BioRad).

4.13. Generation of LIV-1 Antibody:

Rabbit anti-LIV-1 antibody was raised against a synthetic peptide sequence that corresponded to the human gene of LIV-1, QNIGIDKIKRVHIIHHDHC. Using the NCBI database for protein sequence analysis, the sequence was predicted to be an extracellular region. Antibodies were prepared and affinity purified by New England Peptide (Gardner, MA).

4.14. Extraction of Protein:

Treated cells were rinsed with ice cold 1X PBS and detached with 1XTE. The cell suspensions were centrifuged at 1000 X G for 5 minutes. The supernatant was removed and 200µl of RIPA lysis buffer (Teknova, Hollister, CA) containing

10 µl/ml RIPA buffer of protease inhibitor cocktail (Sigma) was added to the pellets. The resuspended pellets were transferred to pre-cooled labeled microcentrifuge tubes after vortexing briefly. The tubes were incubated with constant agitation on a platform shaker at 4°C for 50 minutes. After the incubation, the tubes were centrifuged at 10,000 X g for 10 minutes at 4°C to pellet cell debris. The protein supernatants were transferred into pre-cooled tubes and stored at -80°C.

4.15. Estimation of Protein Concentration:

The Bio-Rad DC assay kit (Bio-Rad Laboratories, Hercules, CA) was used for the estimation of protein concentrations. Standards were prepared with bovine serum albumin and absorbance was measured at 750 nm. Protein concentrations were calculated using a standard curve after absorbance was measured.

4.16. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis:

A 12% polyacrylamide resolving gel made with 1.5 M TrisHCl was cast and isobutanol (300 µl) was used to remove bubbles on top of the resolving gel. After the gel polymerized for 1.5-2.0 hours, the top layer of isobutanol was rinsed out with milli-Q water before a 4% stacking gel was added and allowed to polymerize for 1 hour.

Equal amounts of protein (30 µg) and 3.3 µL of 6X loading dye were used for each sample. The samples were incubated in a hot water bath at 95°C for 10 minutes to denature the protein. The heated samples were loaded in the wells of stacking gel alongside a Precision plus Kaleidoscope Ladder (5 µl) protein standard (Bio-Rad Laboratories, Hercules, CA). The gel was electrophoresed in

running buffer (1X Tris/Glycine: 25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3) for 2 hours at 120 volts at room temperature.

4.17. Transfer to Nitrocellulose Membrane:

The gel was removed from the glass plates and the stacking gel was cut off. An appropriate 'sandwich' was then made such that the sponges, filter papers, gel, and nitrocellulose membrane were carefully placed on the negative side of the transfer cassette. The transfer cassette was loaded in a cartridge and placed in transfer buffer (200 mL of methanol, 720 mL of Milli-Q water, and 80 mL of 10X Tris/Glycine stock solution: 25 mM Tris, 192 mM glycine, 10% methanol). The transfer was carried out for 2 hours at 100 volts at room temperature using the Mini Transblot Electrophoretic Cell with a stir bar (Bio-Rad Laboratories, Hercules, CA).

4.18. Western Blot Antibody Probing:

After transfer, the membrane was removed from the transfer cassette and was blocked with 10 mL of a 5% non-fat dry milk solution in a tray for one hour at 4 C on a platform shaker. The blocking buffer (0.5% nonfat dry milk in TBS/T [0.1% Tween-20 in Tris-buffered saline]) was poured off and the membrane was incubated with primary antibody overnight on the shaker at 4 °C. The dilution of LIV-1 antibody used was 1:500, and for anti-actin was 1:8000. Both primary antibodies were diluted in blocking buffer. The next 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 secondary antibody was added (1:2000) and incubated for 1 hour on the platform shaker.

The secondary antibody solution was poured off and the membrane was washed twice (5 minutes/wash) with TBS/T prior to developing.

4.19. Western Blot Antibody Detection:

ECL reagents (luminal/enhancer chemiluminescent substrate, GE Healthcare (Chalfont St. Giles, UK)) were mixed together. The solution was poured onto the washed membrane and incubated for 5 minutes with periodic shaking. The membrane was removed with a tweezers and was dragged with the protein side down along a piece of Kimwipe before being inserted between sheet protectors. The membrane was exposed using the ChemiDoc System (BioRad, Hercules, CA) and quantified using Quantity One Software (BioRad).

4.20. Cellular Uptake of ^{65}Zn :

Radioactive ^{65}Zn (ZnCl_2 ; 62.5 MBq/mg) was purchased from Perkin Elmer (Shelton, CT). To study the effects of EGF and LIV-1 knockdown on the uptake of ^{65}Zn from media, equal numbers of MDA-MB-231 and LIV-1 knockdown cells were plated in a 24-well plate for overnight attachment. The MDA-MB-231 cells were then treated with EGF (10 μM) for 24 hours whereas the transfected cells were provided with fresh media. After the treatment period was over, the media was removed and the cells were incubated with 30,000 cpm of ^{65}Zn in fresh medium for varying times. The radioactive medium was removed and the cells were washed with PBS. Cells were detached with 1XTE and the ^{65}Zn content of media, PBS wash, and cells were quantified by gamma spectroscopy (Cobra II System, Packard, Meriden, CT).

4.21. Confirmation of ⁶⁵Zn Uptake Experiment Effects with Protein Estimation:

For each ⁶⁵Zn uptake experiment, an additional 24-well plate was prepared alongside to confirm that the effects on ⁶⁵Zn uptake were not due to cell number differences. The cells were treated as described above for the ⁶⁵Zn uptake experiment. After the treatment period was over, they were extracted and protein content measured as described before.

4.22. Statistical Analyses:

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

Chapter Five: Results

5.1. Knockdown of LIV-1 in MDA-MB-231 cells:

In order to achieve the objectives in our study, we utilized an shRNA approach to knockdown LIV-1's expression in MDA-MB-231 cell line. We used shRNA and a lentiviral plasmid pLKO.1-puro containing sequences that specifically target LIV-1. The expression of LIV-1 mRNA and protein was decreased by 40%. Transfection of LIV-1 shRNA into MDA-MB-231 cells did not influence the mRNA and protein expression of the housekeeping genes, GAPDH and β -actin. The expression of LIV-1 was also not altered in cells transfected with the non-target shRNA (Figures 2a, 2b, 2c, and 2d).

Figure 2a:

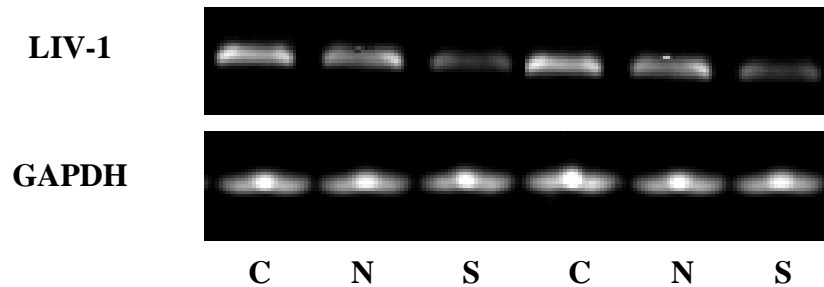


Figure 2b:

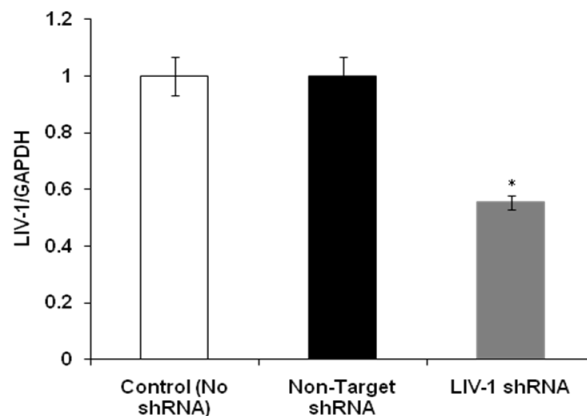


Figure 2c:

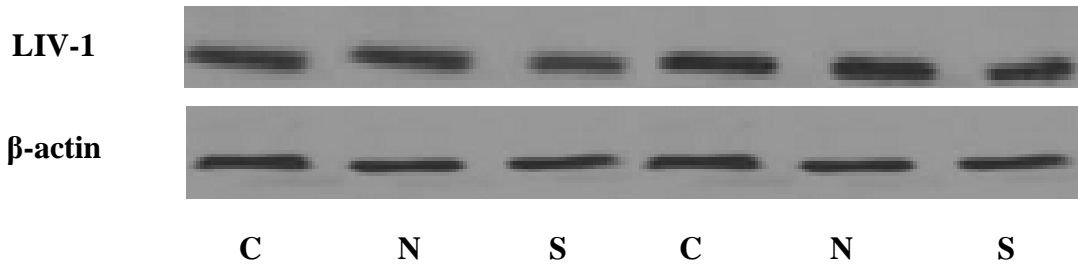
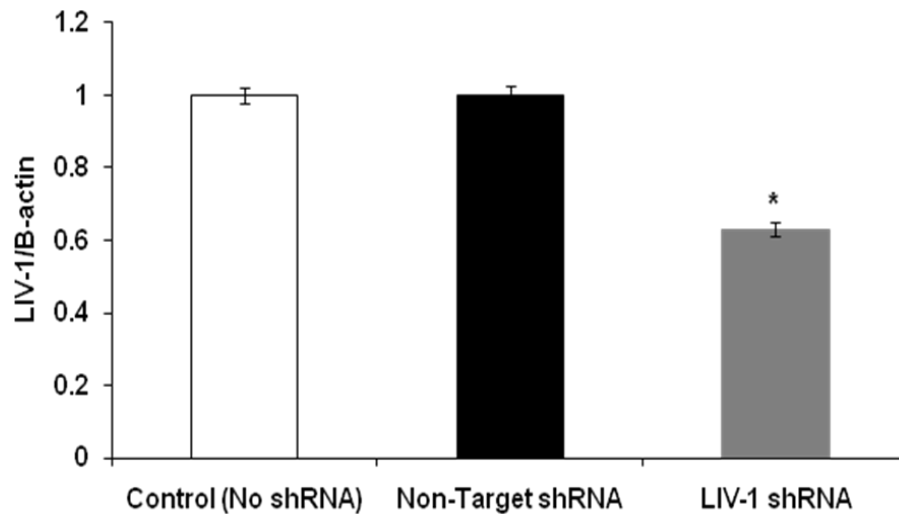


Figure 2d:



Figures 2: Effects of shRNA Transfection on LIV-1 Expression in MDA-MB-231 cells

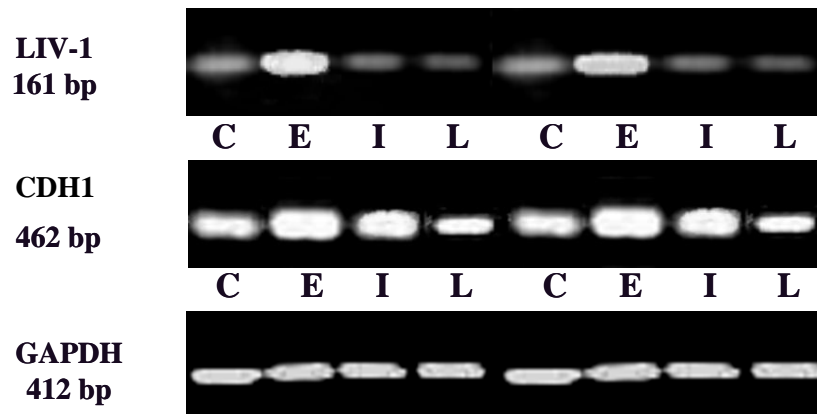
MDA-MB-231 cells were transfected with or without LIV-1 shRNA or non-target shRNA. The cells were then selected for puromycin resistance and allowed to grow for three passages prior to extraction of RNA and protein

(a) LIV-1 and GAPDH mRNA concentrations were measured by RT-PCR with ethidium bromide staining of products separated by agarose gel electrophoresis. (b) Quantified bar graphs of LIV-1 and GAPDH mRNA concentrations. (c) LIV-1 and β -actin proteins were measured by Western blot analysis following SDS-PAGE separation of proteins. (d) Quantified bar graphs of LIV-1 and β -actin proteins.

C = non-transfected cells, N = cells transfected with non-targeting shRNA, S = cells transfected with LIV-1 shRNA. Experiments were repeated four times with three replicates in each group. The asterisk indicate significant difference from the control ($P < 0.05$).

5.2. The Effects of Hormonal Treatments and Knockdown on LIV-1 and CDH1 mRNA Expression:

The effects of EGF and insulin on LIV-1 and CDH1 mRNA expression after 24 hours of treatment were investigated in MDA-MB-231 cells (Figures 3a and 3b). EGF increased the expression of LIV-1 and CDH1 mRNA by approximately two-fold whereas insulin had no significant effect on either mRNA expression. Transfection with LIV-1 shRNA decreased the expressions of both LIV-1 and CDH1 mRNA by approximately 40% (Figure 3c).



LEGEND: C - Control E - EGF I - Insulin L - LIV-1 shRNA

Figure 3a: Effects of Treatments and LIV-1 Knockdown on LIV-1 and CDH1 Expression

Cells were treated as described, RNA extracted and mRNA expression quantified by RT-PCR. Amplified products were separated by agarose gel electrophoresis and visualized with ethidium bromide.

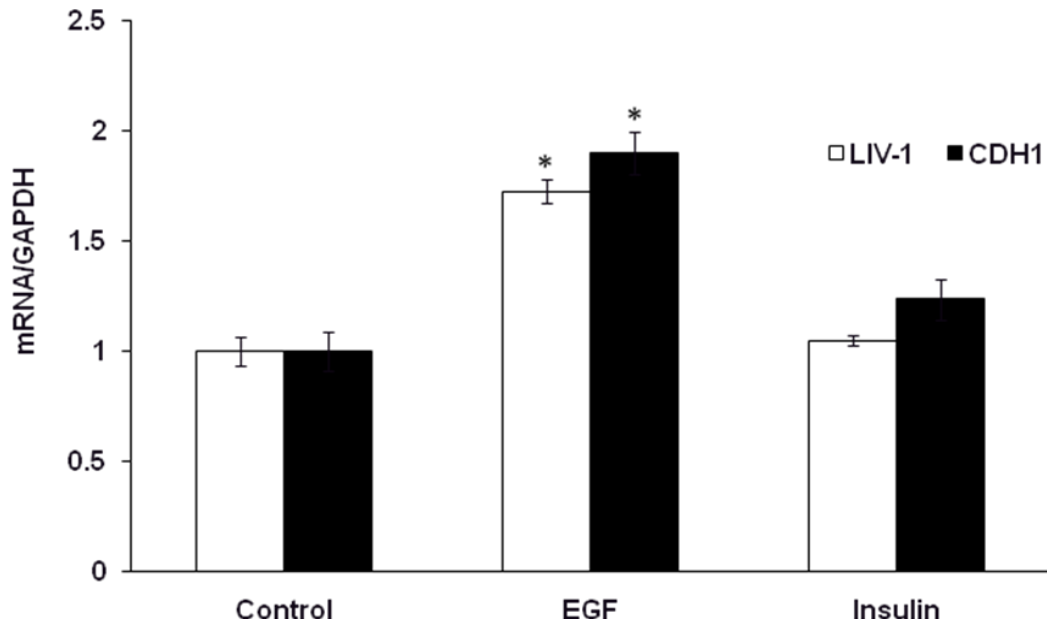


Figure 3b: Quantitative Analysis of Effects of Hormonal Treatments on LIV-1 and CDH1 mRNA

Densitometric analysis of the bands was performed using Quantity One software. The expression of LIV-1 and CDH1 were normalized against GAPDH and expressed quantitatively as a percent of controls. The asterisks indicate significant difference from the control ($P < 0.05$). Bars represent the mean of six observations.

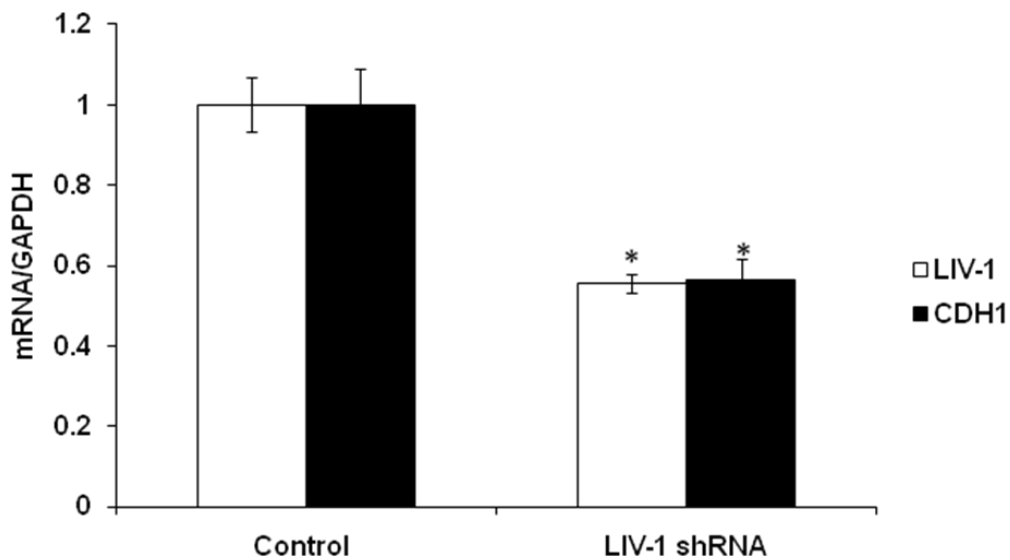


Figure 3c: Quantitative Analysis of Effect of LIV-1 Knockdown on LIV-1 and CDH1 mRNA

Densitometric analysis of the bands was performed using Quantity One software. The expression of LIV-1 and CDH1 were normalized against GAPDH and expressed quantitatively as a percent of controls. The asterisks indicate significant difference from the control ($P < 0.05$). Bars represent the mean of six observations.

LIV-1 Protein Expression:

The effects of EGF, Insulin, and LIV-1 shRNA treatments on the protein expression of LIV-1 were investigated in MDA-MB-231 cells after 24 hours (Figure 4a). The expression of LIV-1 protein did not change significantly with insulin after 24 hours of treatment. However, LIV-1 expression was increased by two-fold with EGF. The expression of LIV-1 protein, however, was diminished by about 40% after knockdown.

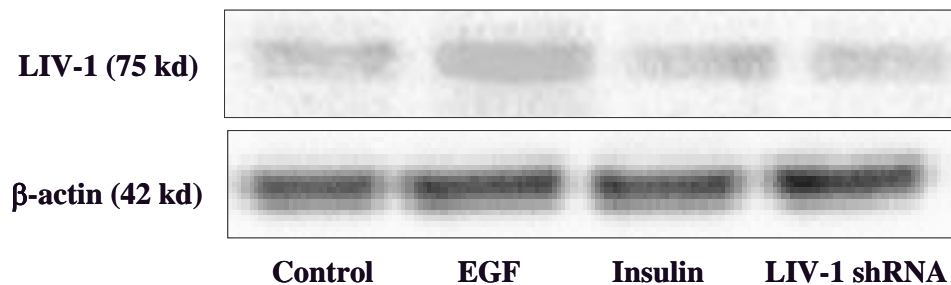


Figure 4a: Effects of Hormonal Treatments and LIV-1 Knockdown on LIV-1 Protein Expression

Cells were treated as described, proteins extracted and protein expression quantified by Western analysis. Proteins were visualized with primary and secondary antibodies and ECL reagents as described in Materials and Methods.

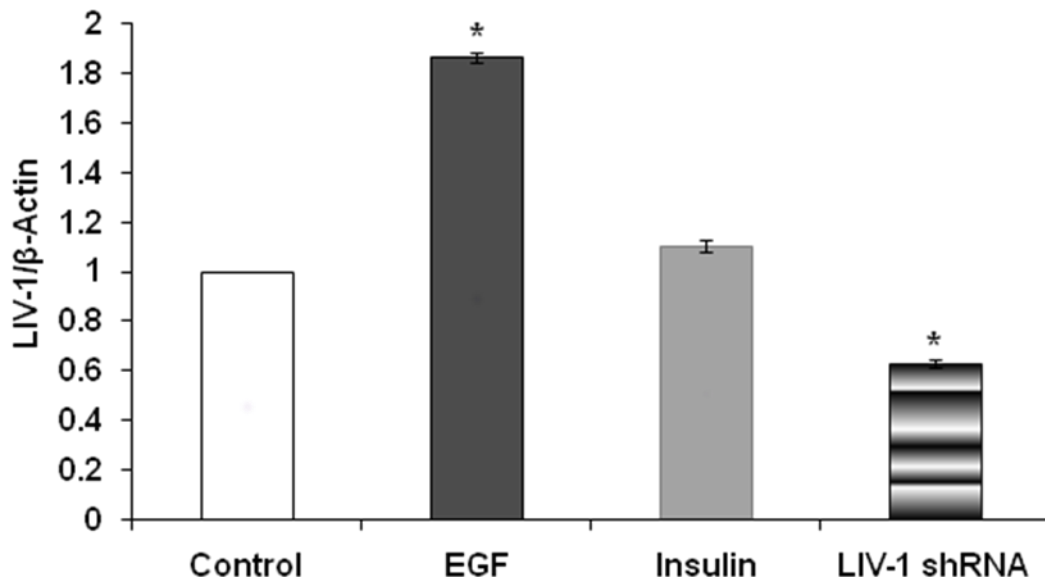


Figure 4b: Quantitative Analysis of LIV-1 Protein following Hormonal Treatments and LIV-1 Knockdown

Densitometric analysis of the bands was performed using Quantity One software. The protein expression of LIV-1 was normalized against β -actin and expressed quantitatively as a percent of controls. The asterisks indicated significant difference from the control. Bars represent the mean of four observations.

5.4. Correlation between LIV-1 mRNA and CDH1 mRNA in MDA-MB-231 cells

The quantitative values of LIV-1 and CDH1 mRNA were plotted against each other to analyze the correlation between the expressions of the two genes. It appeared that the expression of LIV-1 mRNA correlated with CDH1 mRNA expression in a direct proportional manner. Therefore, there was a positive relationship observed between the two genes.

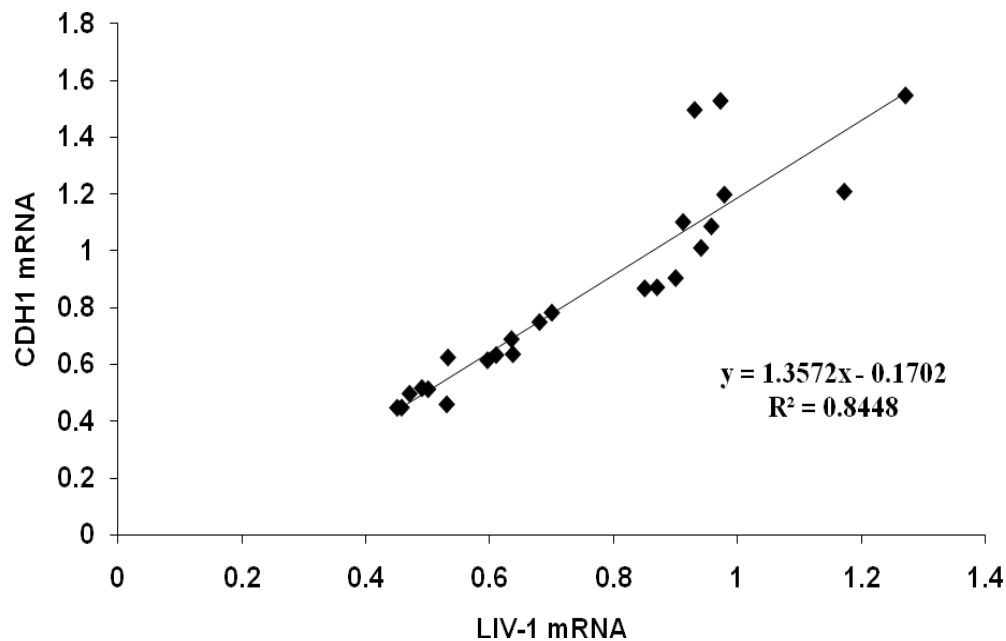


Figure 5: Relationship between LIV-1 and CDH1 mRNA in Hormonal Treatments and LIV-1 Knockdown

The mRNA expressions of LIV-1 and CDH1 were normalized against GAPDH and expressed quantitatively as a percent of controls. These values were used in a scatter plot to determine the relationship between the two genes.

5.5. The Effects of Hormonal Treatments and Knockdown on SNAI1 and SNAI2 mRNA Expression:

The effects of EGF and insulin on SNAI1 and SNAI2 mRNA expression after 24 hours of treatment were investigated in MDA-MB-231 cells (Figures 6a and 6b). Neither EGF nor insulin altered the expression of SNAI1. There was also no change in SNAI1's mRNA expression in LIV-1 knockdown cells. LIV-1 knockdown, however, increased the expression of SNAI2 mRNA by approximately two-fold whereas EGF appeared to decrease mRNA expression of SNAI2 to a minor extent ($p = 0.082$). There was no change observed in SNAI2 mRNA expression with insulin treatment (Figures 6a and 6b).

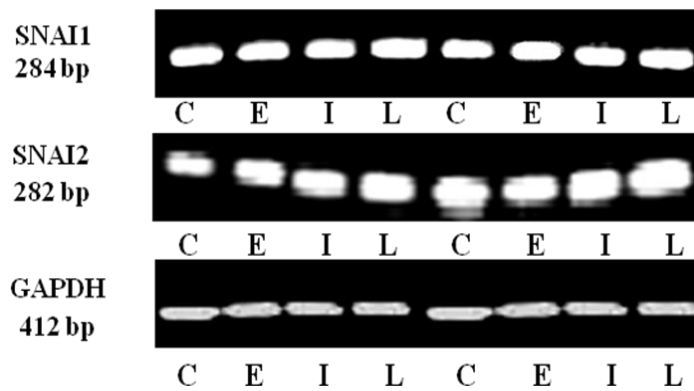


Figure 6a: Effects of Treatments and LIV-1 Knockdown on SNAI1 and SNAI2 Gene Expression

Cells were treated as described, RNA extracted and mRNA expression quantified by RT-PCR. Amplified products were separated by agarose gel electrophoresis and visualized with ethidium bromide.

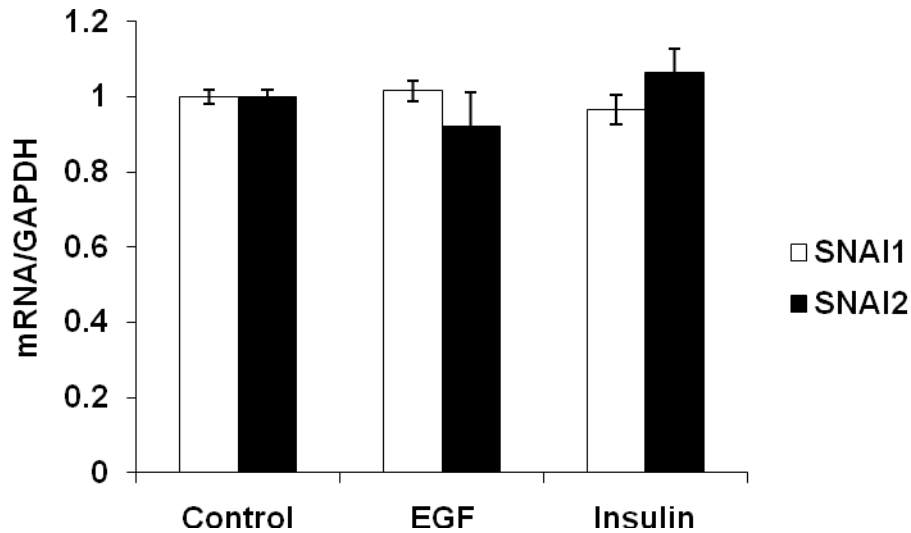


Figure 6b: Quantitative Analysis of Effects of Hormonal Treatments on SNAI1 and SNAI2 mRNA

Densitometric analysis of the bands was performed using Quantity One software. The expression of SNAI1 and SNAI2 were normalized against GAPDH and expressed quantitatively as a percent of controls. The asterisks indicate significant difference from the control ($P < 0.05$). Bars represent the mean of six observations.

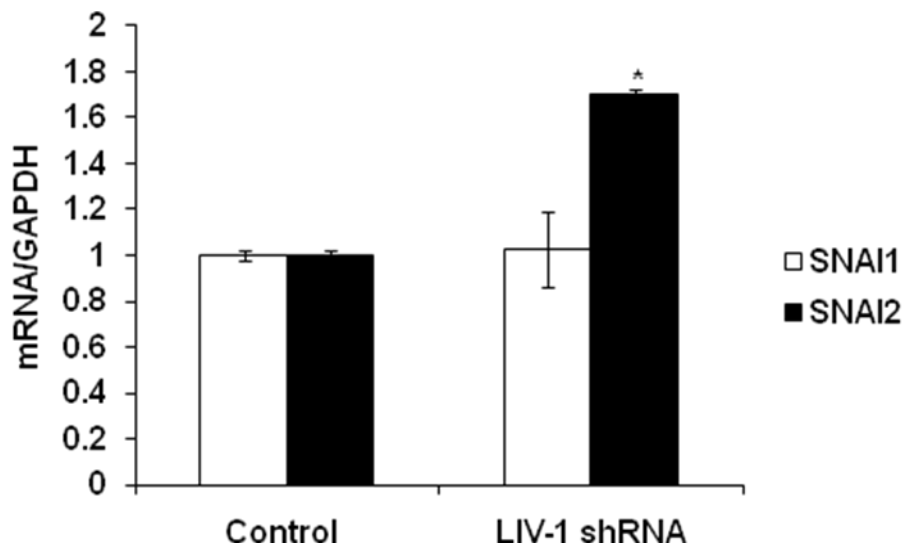


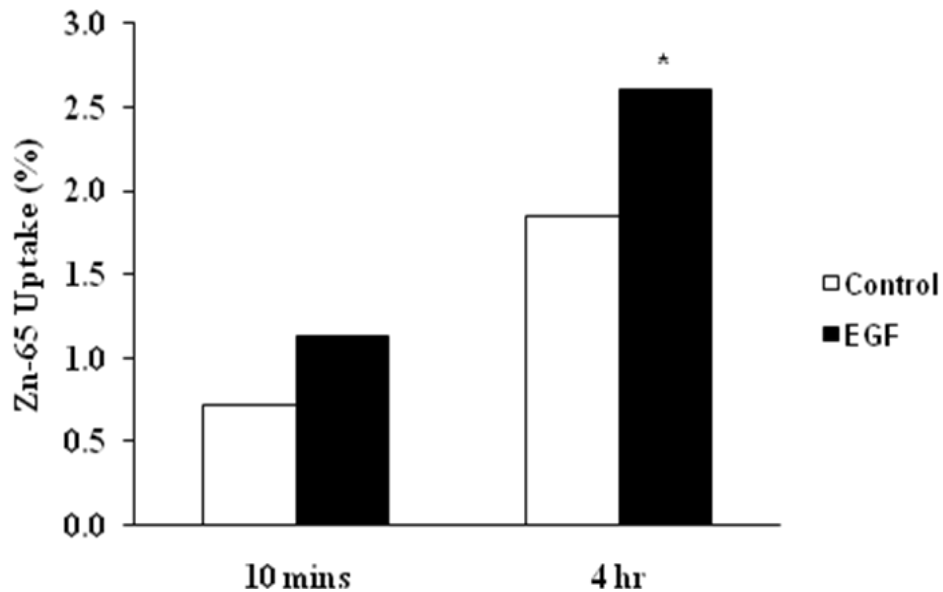
Figure 6c: Quantitative Analysis of Effect of LIV-1 Knockdown on SNAI1 and SNAI2 mRNA

Densitometric analysis of the bands was performed using Quantity One software. The expression of SNAI1 and SNAI2 were normalized against GAPDH and expressed quantitatively as a percent of controls. The asterisks indicate significant difference from the control ($P < 0.05$). Bars represent the mean of six observations.

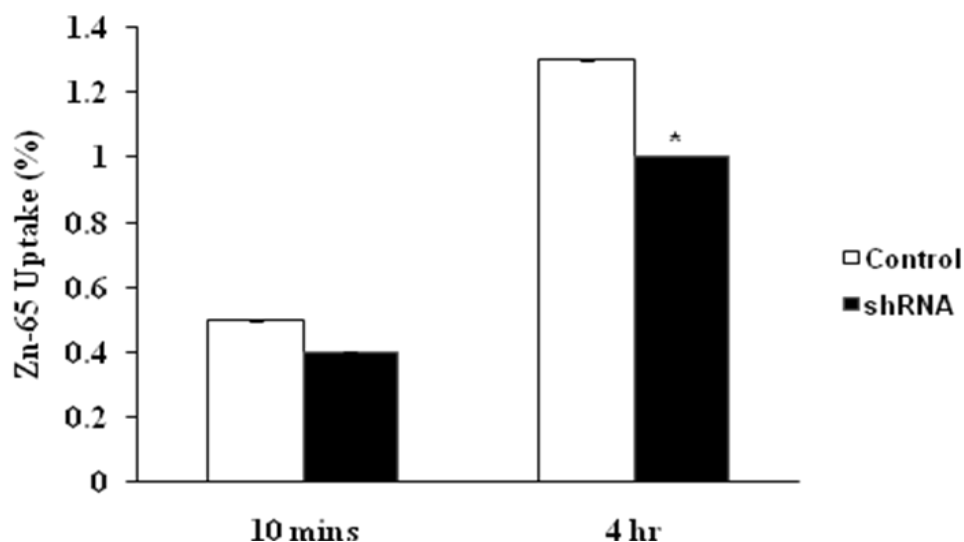
5.6. Effects of EGF and LIV-1 knockdown on ^{65}Zn Uptake in Cells:

MDA-MB-231 and LIV-1 knockdown cells were used for ^{65}Zn uptake experiments to explore the function of LIV-1. Zinc uptake in LIV-1 knockdown cells was significantly decreased whereas it was significantly increased in cells treated with EGF for 4 hours (Figures 7a and 7b). These treatments had no effects on cell number in this time period.

(a)



(b)



Figures 7: Effects of EGF and LIV-1 Knockdown on ^{65}Zn Uptake in MDA-MB-231 cells

MDA-MB-231 cells were treated with or without EGF for 24 hours (7a) or subjected to LIV-1 knockdown (7b) prior to adding ^{65}Zn . After the incubation periods shown, media was collected and cells rinsed and detached or ^{65}Zn measurement. Values are the means \pm S.D. of six experiments, each including at least six replicates of each treatment. Asterisks indicate significant effects with $P < 0.005$.

Chapter Six: Discussion

In this study, the relationship between LIV-1 and CDH-1 expression was examined in ER⁻ MDA-MB-231 breast cancer cells. Breast cancer is one of the leading causes of cancer death in women. Several factors such as gene (BRCA1 and p53) mutations, hormones, and growth factors are associated with breast cancer (8). While many features are involved in the etiology of cancer, diet has clearly been established to significantly impact one's risk of this disease. One dietary factor linked to carcinogenesis is zinc. Zinc is essential for cell growth and cell division (8). It serves as a catalytic cofactor in more than 300 different metalloenzymes and many enzymatic activities in the body are dependent on zinc (3). Moreover, zinc is an important trace element that plays many regulatory and structural roles in cells.

The zinc level in cells is tightly regulated by zinc transporters that are involved in uptake, excretion, and intracellular storage or trafficking of zinc (2) (13). These proteins include MTs and transmembrane transporters in the ZIP and ZnT family (13). Besides regulating intracellular and extracellular zinc concentration, these two classes of zinc transporters have also been reported to have other roles in the cells. In fact, research has indicated that some members from these two protein families are able to facilitate the transport of other cations. Due to the diverse distribution of these zinc transporters, it is suggested that they serve individual roles in enabling the catalytic, structural, and regulatory roles of zinc (1). Therefore, they have been a major interest in scientific research.

The newly discovered zinc influx transporter, LIV-1, has been suggested to be involved in breast cancer metastasis. Taylor et al. have proposed that LIV-1 may influence breast cancer metastasis due to its association with estrogen receptors and various growth factors that are related to clinical breast cancer (5) (8) (27). The expression of LIV-1 was detected in ER⁺ breast cancer cells such as MCF-7 and studies have established that LIV-1 can be regulated by estrogen in these cells. Thus it became known as an estrogen regulated gene. In fact, the prognosis for breast cancers with LIV-1 present is generally good since anti-estrogen drugs could be utilized to improve patients' survival (29) (30). The prognosis for breast cancer that are absent in LIV-1 is poor. Breast cancer cells that have low or no expression level of LIV-1 are generally negative in ER. They were reported to be more invasive in nature and frequently metastasize. Tumor metastasis has been linked with diminished expression of CDH1. A reduced level of CDH1 is characteristic of EMT. EMT has been implicated in the fundamental steps of embryogenesis as well as tumorigenesis such as invasion and metastasis. The loss of CDH1 has been observed in both of these biological processes. Without CDH1, cells can migrate and invade (101-105).

An appropriate and effective treatment has not been fully established to treat breast cancers that are ER⁻. We have previously detected LIV-1 in MDA-MB-231 ER⁻ cells and to further understand regulation of LIV-1 in these cells, hormonal treatments of EGF and insulin were used. Expression of LIV-1 could be induced two-fold by EGF but insulin had no significant effects on expression. The mRNA level of CDH1 was measured in the same cells. Interestingly, the

expression of CDH1 was increased to a similar extent as LIV-1, suggesting that the expression of CDH1 might be regulated by LIV-1. The knockdown of LIV-1 also decreased CDH1 expression. These observations showed that there was a positive correlation between LIV-1 and CDH1 expression.

The present results contradicted Taylor's hypothesis of an inverse relationship between LIV-1 and CDH1 (5). Taylor proposed that CDH1 could be regulated through two pathways that involved EGFR and ER. The first pathway involved the induction of LIV-1 by EGFR through a STAT3 and Ras/Raf/MAPK modulated signaling pathway. The second pathway consisted of ER activating MTA3, a gene that could suppress the expression of the CDH1 repressor gene known as SNAIL. The EGFR pathway was proposed to have a positive effect on the expression of SNAIL whereas the ER pathway was suggested to have a negative effect. The EGFR pathway was illustrated to explain the invasive nature of cancerous cells whereas the ER pathway described the phenotype of less invasive cells.

Our data indicated that the expression of LIV-1 in MDA-MB-231 breast cancer cells was elevated through the EGFR pathway. However, this was associated with an increased rather than decreased expression of CDH1. The data from our LIV-1 knockdown experiment did not support the role that LIV-1 was proposed to play in EMT. Therefore, we needed to search for an explanation for the relationship we observed in our studies. Interestingly, EMT has been shown to be reversible through the knockdown of SNAIL in recent studies (104). In fact, the phenotype of a more aggressive cell line has been altered after the silencing of

SNAI1 (58). It was demonstrated that an alteration in SNAI1 expression caused the mesenchymal-like phenotype in cancerous cells to reverse to an epithelial-like phenotype (104). The two-fold induction of CDH1 after EGF treatment suggested the possibility of either a phenotype alteration in MDA-MB-231 that reduces SNAI1 or the regulatory mechanism might be different from that proposed by Taylor et al. Therefore, the relationship between LIV-1, SNAI1, and CDH1 was further explored to attain the detailed mechanism about the effect(s) of LIV-1 on breast cancer growth and progression.

Hence, we measured the expression of SNAI1 in our next approach since it would not only help clarify the relationship between LIV-1 and CDH1 but also the importance of LIV-1 with respect to the growth and spread of breast cancer cells. SNAI1 is worthy of investigating because CDH1 was proposed by Taylor as a downstream target of SNAI1, which could repress CDH1 expression at the transcriptional level (91). By analyzing SNAI1, we would further understand the relationship between LIV-1 and SNAI1 as well as SNAI1's role in the development of cancer since an over-expression of SNAI1 is a strong and sufficient inducer of EMT.

We found that there was no change in the mRNA expression of SNAI1 with all treatments, including in LIV-1 knockdown cells. Because our finding did not explain the correlation between LIV-1 and CDH1, we tested another candidate gene that might be involved in the regulation of CDH1. In addition to SNAI1, there is another member of Snail superfamily that can modulate the process of tumor progression in mammals. Hence, we investigated if SNAI2 (Slug) could

contribute to cancer progression and thus changes in CDH1 expression in MDA-MB-231 cells. We observed that there was an increased mRNA expression in SNAI2 in LIV-1 knockdown cells whereas there was no change in cells treated with insulin. EGF, on the other hand, tended to decrease the mRNA expression of SNAI2 ($p=0.082$). These results suggested that SNAI2 may mediate the relationship between LIV-1 and CDH1 in MDA-MB-231, at least partially, and thus influence the cells' migratory and invasive properties.

In addition to having an influence on the expression of SNAI2 and CDH1, LIV-1 was also investigated for its role in zinc homeostasis by ^{65}Zn uptake experiments. As demonstrated, the uptake was decreased when LIV-1 was knocked down in MDA-MB-231 cells but increased when cells were treated with EGF. Therefore, LIV-1 appears to serve as a zinc importer in MDA-MB-231 cells. As a zinc transporter in the ZIP family, LIV-1 expression would be expected to decrease when there is an excess of zinc. This occurs as a means to counteract the accretion of zinc and the cytotoxic effects that can arise from zinc accumulation. The opposite effect of increasing LIV-1 expression level would be more likely if zinc was deprived.

Chapter 7: Summary and Conclusion

7.1. Conclusions:

Cancer is the result of a series of events that include dedifferentiation, uncontrolled cell division, change of cell adhesion properties, and change in invasiveness (107). Breast cancer is one of the top three leading causes of cancer death in women. Several factors such as gene (BRCA1 and P53) mutations, hormones, and growth factors are involved in its progression (24). While many characteristic features are involved in the etiology of cancer, diet has clearly been established to significantly impact one's risk of this disease. One dietary factor linked to carcinogenesis is zinc. In fact, recent epidemiological studies have demonstrated greater zinc concentrations in breast tumors (27) (30). Cellular zinc concentrations are maintained by Zn influx and efflux transporters (ZIP and ZnT respectively).

Studies have shown that a member of the ZIP family, LIV-1, may play a role in the growth or metastasis of breast cancer cells (5) (27) (30). LIV-1 was discovered as an estrogen-regulated gene and was suggested as a potential marker of ER⁺ tumors (5) (27) (30). In addition, some studies have demonstrated that it may down-regulate the expression of the cell adhesion protein, CDH1, thereby promoting metastasis (5). Yet, the regulatory role of LIV-1 on E-cadherin in more invasive breast tumor (ER⁻) is not fully understood. Therefore, we investigated 1) how growth hormones and gene silencing affect LIV-1 expression in invasive tumor cells, 2) how these treatments affect ⁶⁵Zn uptake and 3) the relationship between LIV-1 and CDH1 expression under the same conditions.

We found that elevated expression of LIV-1 is associated with increased expression of CDH1. The suppression of LIV-1 using shRNA resulted in reduced expression of CDH1. An increased uptake of ⁶⁵Zn was observed with EGF treatments but opposite effects with zinc gene silencing. The positive relationship we observed between LIV-1 and CDH1 was contradictory to our hypothesis and thus we looked at two potential genes that could mediate these effects. In different types of carcinoma, an aberrant expression of SNAI1 or/and SNAI2 has been observed and is frequently associated with invasiveness, metastasis, and poor prognosis. Even though we observed no change in SNAI1 expression, SNAI2 expression was altered in a fashion that explained the correlation between LIV-1 and CDH1.

Snail superfamily members have been identified as crucial regulators of cell fate in many biological contexts. Previous literature has demonstrated that loss of cell-cell contact as central to the acquisition of a migratory phenotype (101). In fact, this is a phenotypic alteration promoted by the action of Snail or Slug. The data presented here added the property that Slug is associated with the transcriptional repression of CDH1 in MDA-MB-231 cells; suggesting that the aberrant expression of Snail and Slug in invasive tumors play a role in cell fate. The aberrant expression of Slug could potentially lead to invasive growth of MDA-MB-231 cells and thus affecting the actions of chemotherapeutic agents.

Numerous therapeutic treatments have been applied on patients with metastatic breast cancer. These regimens have demonstrated effectiveness in patients but adverse side effects were also associated with them. ER⁻ tumors are

difficult to treat as they are able to thrive without estrogen and thus are less responsive to endocrine therapy than those that are ER⁺. In addition to being more difficult to treat, this type of tumors is associated with a higher mortality rate. Women with ER⁻ tumors face an 8% to 35% lower five-year survival rate than those whose tumors are ER positive (103).

The aberrant expression of LIV-1 and CDH1 has been previously implicated in various disease stages. Therefore, in order to develop a successful treatment, it was essential to understand the relationship of these two genes. Overall, our study has provided insights into the regulatory mechanism of LIV-1 on CDH1 in ER⁻ breast cancer cells. We also observed the presence of LIV-1 could serve as a prognostic marker for breast cancer patients with invasive tumors since the expression of LIV-1 induced the expression of CDH1 through the repression of SNAI2. Our current findings further indicated that the expression of LIV-1 could be therapeutically beneficial as its expression could affect the expression of both SNAI2 and CDH1. However, the role of SNAI2 in communicating the signal between LIV-1 and CDH1 still requires further clarification.

Although we detected no changes in the mRNA expression of SNAI1, LIV-1 could still have had an effect on the nuclear localization of SNAI1 especially since previous studies have demonstrated that the presence of LIV-1 is essential for the nuclear translocation of Snail family members (105) (107). Moreover, studies in cell lines and transgenic mouse models have demonstrated that SNAI2 regulate EMT in carcinogenesis to a lesser extent than SNAI1 (109).

Our studies, however, indicated that SNAI2 could act as a potential CDH1 repressor in MDA-MB-231 cell line. Thus, SNAI2 is more likely to play a role in the disease progression of this cell line than SNAI1.

Altering the expression of LIV-1 with EGF or shRNA not only influenced the expression of CDH1 but also changed the rate of cellular zinc uptake. The changes detected in CDH1 expression corresponded with those in LIV-1 expression but it is not clear whether changes in intracellular concentrations of zinc played any role in mediating these effects. Alterations in LIV-1 may impact intracellular zinc, which subsequently affects the expression of CDH1. Alternatively, LIV-1 could affect the expression of SNAI2 and then CDH1 through a signal transduction pathway that was independent of zinc. Despite this uncertainty, our discoveries demonstrate that LIV-1 plays a role in the physiology of ER⁺ breast cancer. However, more investigations need to be conducted in order to determine the role of zinc itself in these processes.

7.2. Future Directions:

Our current discovery indicated that 1) reduced LIV-1 expression at both mRNA and protein levels was associated with a similar decrease in zinc uptake and 2) the expression of LIV-1 was parallel to the expression of CDH1 in MDA-MB-231 cell line. Further studies are needed since LIV-1 has been reported to be inversely related to CDH1 in other cell lines. Although we found that SNAI2 could be responsible for the positive relationship that we observed between LIV-1 and CDH1, we still needed to investigate further since an aberrant expression of SNAI1/SNAI2 not only leads to EMT during cancer progression but also causes

dramatic alterations in cell fate and the interaction of cells with their environment. In order to survive under such dramatic changes, cells may need to develop resistance to programmed cell death. In fact, Snail superfamily members have been previously implicated in the protection of cells from programmed cell death during normal development and differentiation (19) (28) (33). Therefore, it would be useful to further understand the role of SNAI2 by investigating how the aberrant expression of SNAI2 protects MDA-MB-231 cells against apoptosis.

It would also be useful to track the phenotypic transition and the proliferation rate of these cells after treating them with EGF and LIV-1 shRNA to better characterize the roles of LIV-1 and SNAI2 in regulating CDH1 and other aspects of cellular behavior. Determining the effects of altered LIV-1 expression on cellular growth and the propensity to metastasize is important because this may help us to identify potential markers for cancer cell behavior as well as to find therapeutic targets for limiting the growth of cancer cells. Therefore, we need to further understand the function of LIV-1 and the regulation of zinc homeostasis in cancer cells as a whole to achieve the objectives.

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