CHARACTERIZATION OF HUMAN MESODERM INDUCTION EARLY RESPONSE 1 (hMIER1) AS A COREGULATOR OF THE ESTROGEN RECEPTORS, ERα AND ERβ

HEATHER FIFIELD

Characterization of Human Mesoderm Induction Early Response 1 (hMIER1) as a coregulator of the Estrogen Receptors, ERα and ERβ

By Heather Fifield

A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the

requirements for the degree

of Masters of Science

Division of Biomedical Sciences

Faculty of Medicine

Memorial University of Newfoundland

October 2008

Newfoundland and Labrador

St. John's

Abstract

The human mesoderm induction early response gene 1 (*hmier1*) is a growth factor induced gene encoding a novel transcription factor. There are two major isoforms of hMIER1, hMIER1α and hMIER1β, which differ in their C-termini (Paterno *et al.*, 2002). In particular, hMIER1α, but not hMIER1β, contains a conserved motif important for interaction with nuclear hormone receptors, the LXXLL motif. Further analysis revealed that hMIER1 was differentially expressed in normal human breast compared to breast carcinoma cell lines and tissues, implying it may have a role in the neoplastic state (Paterno et al., 1998; Paterno et al., 2002).

In this study, the role of hMIER1 as a coregulator of the estrogen receptor (ER) was investigated. The physiological effects of estrogen are mediated by two receptors: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). It has previously been shown that both ERα and ERβ interact with hMIER1α and hMIER1β *in vitro* (Fifield, Honours dissertation). Electrophoretic mobility shift assays (EMSAs) were performed to determine if hMIER1α or hMIER1β interaction with ERα or ERβ affected the ability of the ER to bind a consensus ERE in the absence and presence of specific ligands. These studies revealed that hMIER1α inhibited DNA binding of both ER subtypes in the absence or presence of ligand. In contrast, hMIER1β inhibited the DNA binding of the ERβ in presence and absence of ligand, but has little effect on the DNA binding of ERα.

Further studies were performed to determine if the *in vitro* interaction and effect on DNA binding would be translated into functional effects *in vivo*. Experiments in HEK 293 cells showed that in the absence of ligand, hMIER1α and hMIER1β enhanced ERE-

driven transcription with the ERα, but not ERβ. In the presence of ligand, such as estrogen or the ERα-specific agonist, propylpryazole-triol (PPT), neither hMIER1α nor hMIER1β significantly affected ERE-driven transcription with ERα In the presence of diarylpropionitrile (DPN), an ERβ specific agonist, neither hMIER1α nor hMIER1β affected ERE-driven transcription with ERβ .

Overall these results imply that hMIER1α and hMIER1β are involved in ligand independent activation of ERα, but have no functional effect on the ERβ. This ER subtype selectivity, along with activation of ER signalling in the absence of estrogen or other ligands, warrant further investigation of the role that hMIER1 plays in estrogen receptor signalling.

Acknowledgments

I would like to take this opportunity to thank my supervisor, Dr. Laura Gillespie. I could not have asked for a better teacher and cannot thank her enough for her constant support, encouragement and patience. I would also like the thank the members of my committee, Dr. Gary Paterno and Dr. Ken Kao for their insight and advice.

I would like to thank everyone in the Terry Fox Labs for both their technical and moral support. I would especially like to thank Corrine who has been there for me and helped me through every step of the way. I am also thankful to the staff and students, both past and present, for their help, support and unforgettable friendship. I am a better person for having met all of you and I cannot imagine a better place to have faced this challenge.

I would like to express my appreciation to my family and friends for their love and encouragement. Thank you for listening to me, for inspiring me and for reminding me not to sweat the small stuff.

Lastly I would like to thank my fiancée, Jay, for your unbelievable patience and support. Your faith in me has inspired work harder and to accomplish so much more than I ever believed possible. Thank you!

Table of Contents

List of tables and figures

List of Abbreviations

µg	microgram
µl	microlitre
°C	degrees Celsius
ADA2	adaptor coactivator 2
AF1	activation function 1 domain
AF2	activation function 2 domain
AP1	activator protein-1
βgal	β galactosidase
CAT	Choramphenicol acetyltransferase
CBP	CREB-binding protein
CS	calf serum
cpm	counts per minute
β-M-EtOH	beta mercaptoethanol
BSA	bovine serum albumin
DBD	DNA binding domain
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPN	diarylpropionitrile
DTT	dithiothreitol
E2	17-β-estradiol (estrogen)
EDTA	ethyldiamine tetraacetic acid
EGF	epidermal growth factor
Egl-27	gene first identified in *Caenorhabditis elegans*
ELM2 domain	Egl-27 and MTA-1 like domain
ER	estrogen receptor
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERα/β	estrogen receptor alpha/beta heterodimer
ERE	estrogen response element
FBS	fetal bovine serum
FGF	fibroblast growth factor
g	gram
GST	glutathione S-transferase
GST–α	GST hMIER1α fusion protein

GST-β	GST hMIER1β fusion protein
HAT	histone acetyl transferase
HDAC	histone deacetylase
HEK 293	transformed human embryonic kidney cells
hmier1	human mesoderm induction early response gene 1 DNA/RNA
hMIER1	human mesoderm induction early response 1 protein
HRE	hormone response element
hsp90	heat shock protein 90
IGF-1	insulin like growth factor 1
IPTG	isopropyl-b-thiogalactopyranoside
kDa	kilodalton
LB	luria bertani medium
luc	luciferase
LXXLL motif	nuclear receptor box (L=amino acid leucine; X= any amino acid)
M	molar
MAPK	mitogen activated protein kinase
ml	millilitre
MPP	methyl-pieridinopyrazole
mRNA	messenger ribonucleic acid
MTA	metastasis associated protein
NCOR	nuclear receptor corepressor
NF-κB	nuclear transcription factor kappa-B
NLS	nuclear localization signal
NP-40	nonidet P-40
NuRD	nucleosome remodelling complex
OD	optical density
ONPG	ortho-nitrophenyl-β-galactoside
PBS	phosphate buffered saline
PFR DMEM	phenol red free Dulbecco's modified Eagle's medium
PI	protease inhibitors
PKA	protein kinase A
PMSF	phenylmethylsulfonyl fluoride
PPT	propylpryazole-triol
RLU	relative luciferase units
RNA	ribonucleic acid
^{35}S	radioactive sulphur

SANT domain	domain first identified in SWI3, ADA1, NCOR, TFIIIB
SDS	sodium dodecyl sulfate
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERMs	selective estrogen receptor modulators
SH3	SRC homology 3
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
SRC	steroid receptor coactivator
SSB	SDS sample buffer
SWI3	type of SWI/SNF chromatin remodelling complex
TBE	tris borate/ EDTA electrophoresis buffer
TCA	tricholoroacetic acid
TEMED	tetramethyethylenediamine
TFIIIB	transcription factor for RNA polymerase III subunit B
TGF-β	transforming growth factor beta
tk	Thymidine kinase
TnT	transcription-translation reaction
xmier1	*Xenopus* mesoderm induction early response 1 gene DNA/RNA
XMIER1	*Xenopus* mesoderm induction early response 1 protein

1. Introduction

1.1 General Introduction

1.1.1 DNA and Cell Proliferation

Deoxyribonucleic acid (DNA) is a complex macromolecule that serves as a carrier of genetic information in all cells. The information carried in DNA is essential for organization, function and behaviour of most living cells. Genes are distinct functional units within the DNA that are the basis of hereditary characteristics. Genes provide the method through which DNA conveys information within the cell, as well as between generations.

Normal cells are capable of replicating their DNA and passing it on to daughter cells in a series of events known as the cell cycle. A key event in cell division is the replication of DNA, which occurs during S phase. In order for the information stored in DNA to be successfully passed through generations, DNA must be replicated accurately and any damage to the DNA must be detected and repaired. The cell machinery is capable of recognizing alterations in the DNA and arresting the cell cycle at various checkpoints during G1, S, G2, or M phase to repair the DNA. Failure to recognize and repair DNA mutations can result in abnormal cell growth or development leading to many life threatening diseases. For example, cancer is often the result of mutations in cell cycle control genes. This disrupts normal cell growth control mechanisms and while impaired cell proliferation control is not the only contributing factor in cancer, it is a fundamental underlying feature.

1.1.2 DNA Packaging

The amount of DNA that a cell must accommodate is extremely large, even in organisms that have relatively small genomes. DNA must be efficiently packaged but still be accessible to the cell machinery for replication and information signalling. In eukaryotic cells, DNA packaging is quite complex. In a non-dividing cell, the DNA is packaged with proteins as chromatin fibres dispersed throughout the nucleus. The proteins which play an important role in chromatin structure are known as histones. These proteins have a strong positive charge allowing them to bind to negatively charged DNA. Histones impose a repeating structural organization upon DNA that causes the chromatin to resemble 'beads on a string'. As the cell gets ready to divide, the chromatin fibres condense and fold to form compact structures known as chromosomes.

The regulation of chromatin and its structure controls important nuclear processes such as DNA transcription, replication, repair, mitosis and apoptosis (Hodawadekar and Marmorstein, 2007). Chromatin structure regulates the availability of DNA to cellular processing and is the basis for the differential expression of genes (reviewed in Gal-Yam *et al.*, 2007). For example, DNA methylation in the mammalian genome is the addition of a methyl group to cytosine residues followed by guanine residues, known as CpG dinucleotides. Regions of the DNA with high cytosine-guanine dinucleoutide content are called CpG islands. These usually occur in the 5' regulatory region of genes; in fact many promoters are embedded in the CpG islands. These regions, unlike the CpG dinucleotides, are usually unmethylated and methylation often results in long term gene repression.

Another type of DNA processing is histone modification, histones normally undergo many post translational modifications that result in changes in chromatin structure, gene expression, and DNA repair. In order for genes to be actively expressing information, they must be available to the cell machinery. In the compacted form of chromatin this is difficult, requiring changes in histone structure. One mechanism of altering histone structure is through acetylation, which is the addition of an acetyl group to specific lysine residues within the N-terminal tail of histone molecules (Hodawadekar and Marmorstein, 2007). Histone acetyltransferases (HATs) are enzymes that function to add acetyl groups to histones, and this acetylation facilitates the access of transcriptional machinery to the gene promoters by loosening the packing of nucleosomes. Histone deacetylase (HDAC) proteins catalyze the removal of acetyl groups from histone proteins, resulting in a chromatin structure that is unavailable to the transcriptional machinery and susequent repression of gene expression.

1.1.3 Transcription

The flow of information in cells proceeds from DNA to ribonucleic acid (RNA) to protein. The genes encoded in DNA work as a template for the synthesis of RNA in a process called transcription (Becker *et al.*, 2003). Like DNA, RNA is made up of nucleotide subunits; RNA copied from DNA that encodes for protein is known as messenger RNA or mRNA. In eukaryotic cells, there are three enzymes that are involved in transcription, RNA polymerase I, II, and III. RNA polymerase II are the enzymes that transcribe the genes that code for mRNA to be translated into protein. RNA polymerase

II binds and locally unwinds the DNA double helix to allow the initiation of mRNA synthesis. This enzyme is responsible for the four stages of transcription; binding, initiation, elongation and termination.

RNA polymerase binds to a specific region of the gene known as the promoter; this site determines where RNA synthesis starts and which DNA strand is to serve as the template. Binding of the RNA polymerase to DNA requires the participation of additional proteins known as transcription factors. Transcription factors that are required for an RNA polymerase molecule to bind its promoter sequence and initiate transcription, regardless of the specific gene, are known as general transcription factors. Many genes have short DNA sequences outside the core promoter to which other, more specific, transcription factors bind and affect transcription. These are known as regulatory transcription factors and upon binding, their specific DNA elements can recruit coactivator or corepressor proteins to control the gene expression in a cell in response to the environmental and physiological conditions.

1.1.4 Cell proliferation and Cancer

The development, differentiation, and growth of cells in a multicellular organism must be under tight control to ensure the needs of the organism are met. Cancer is an example of what happens when cell proliferation continues unabated, and often results from errors in cell cycle and transcriptional control mechanisms.

DNA mutations occur spontaneously, but can also be the result of mutagens in the environment. If these mutations are not repaired, accumulation of multiple lesions can

cause a cell to become cancerous. Proto-oncogenes are normal cellular genes that code for proteins that regulate cell growth and differentiation. If mutated, these regulatory genes have the potential to become oncogenes, which in turn may promote tumour growth. Most oncogenes code for components or regulators of growth factor signalling pathways such as growth factors, receptors, or transcription factors.

1.2 Nuclear Hormone Receptors

1.2.1 Structure

Nuclear hormone receptors are a family of receptors which are targets for lipid soluble molecules such as steroid hormones (reviewed in Singh and Kumar, 2005). These receptors, unlike membrane bound receptors, are intracellular and capable of directly affecting their target genes. The activation of these genes results in regulation of many physiological functions, including growth and development. Dysregulation of the receptors and their target genes leads to the development of many types of cancer and other serious diseases such as diabetes.

Nuclear hormone receptors share a common structure that consists of three independent functional regions (Figure 1) (reviewed in Nilsson *et al.*, 2001). The first is the A/B domain at the N-terminus, which encodes the ligand independent activation function domain (AF-1). This region of the receptor is involved in protein-protein interactions as well as transcriptional activation of target gene expression. Adjacent to the A/B domain is the DNA binding or C domain, this is important for binding of the receptor to specific DNA sequences called hormone response elements (HRE's) and

contains the first of two interfaces important in receptor dimerization. The last functional domain is the ligand binding or E domain. This domain contains a receptor dimerization interface, it mediates ligand binding, nuclear translocation and transactivation of target gene sequences. This region also contains a second activation function domain (AF-2) which is the ligand-dependant transcriptional activation domain. The F domain at the carboxy terminus of the ER is a variable region, whose specific function is currently unknown (reviewed in Hanstein *et al.*, 2004) Linking the C domain to the E/F domain is the D region or hinge region. The D region is poorly characterized and not well conserved between different nuclear hormone receptors. It has been shown to be associated with molecular chaperone proteins such as heat shock protein 90 (hsp90) (reviewed in Pettersson & Gustafsson, 2001).

1.2.2 Model of Steroid Nuclear Receptor Action

Steroid hormone receptors are a subset of the nuclear hormone receptor family that interact with small hydrophobic ligands capable of regulating growth, differentiation, and homeostasis in eukaryotic cells (reviewed in Hanstein *et al.*, 2004). This class of nuclear hormone receptors are transcription factors whose activity is regulated by ligand binding. Using the estrogen receptor (ER) as an example, it is possible to examine how these receptors can function through several specific mechanisms; namely the ligand-dependent classical or non-classical pathways, and the ligand-independent pathway.

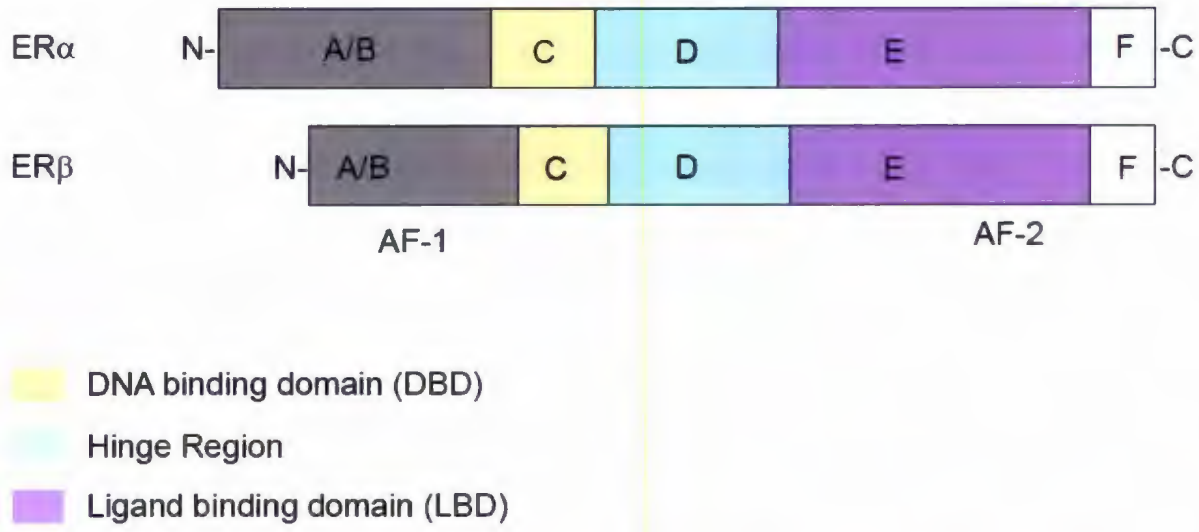

Figure 1: Domain structure of Steroid Nuclear Hormone Receptors ERα and ERβ

Schematic representation of the ERα and ERβ. At the N terminus is the A/B domain which contains an acidic activation domain important for ligand-independent transactivation (AF-1). Adjacent to the A/B domain is the DNA binding domain or C domain. This portion of the receptor is essential for recognition of specific estrogen response elements (EREs). The C domain is linked to the ligand binding domain, or E domain, by a hinge region or D domain. The E domain contains a second activation domain responsible for ligand-dependent transcriptional activation (AF-2). The F domain is a variable region.

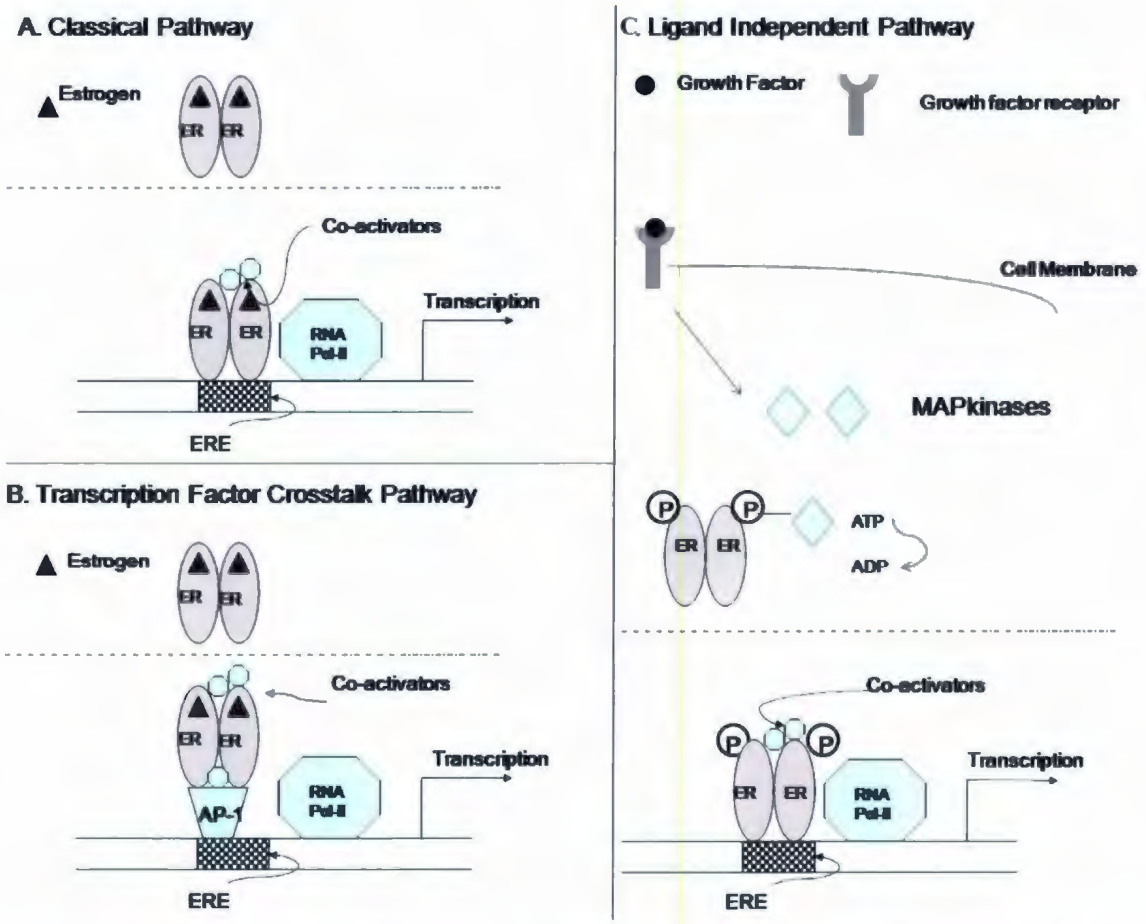

Figure 2: Ligand dependent classical (A), non-classical (B) and ligand independent (C) models of estrogen receptor activation.

A. Classical model of estrogen receptor activation begins with binding of ligand (E2). The receptor then forms homo or heterodimers and translocates to the nucleus of the cell, where it binds to its specific hormone response element and activates transcription through recruitment of coactivator complexes.

B. Non-Classical model of estrogen activation also begins with ligand binding, dimerization and translocation to the nucleus. In the non-classical model the ER does not bind DNA directly but is recruited to another DNA bound transcription factor such as Sp1 or AP-1. The receptor then enhances transcription through association with coactivator complexes.

C. Ligand independent model of estrogen receptor activation occurs in the absence of a typical ligand such as E2. The pathway is usually activated by the binding of a peptide growth factor to its membrane associated receptor. This results in a signalling cascade that results phosphorylation of the ER by specific kinases. The phosphorylated form of the ER is activated, binds the hormone response element and enhances transcription through the association with coactivator complexes.

[Adapted from Nilsson et al., 2001, and Heldring et al., 2007]

activation without ER directly binding DNA. ER also interacts with the fos/jun transcription factor complex on AP1 sites to stimulate gene expression (reviewed in Kushner *et al.*, 2000). Agonist bound ERα enhances AP1 activity via interaction with the p160 family of coactivators whereas antagonist bound ERβ also enhances AP1-dependent transcription by sequestering corepressors away from the AP1 complex. C. Ligand-independent model. The fact that ER activity was observed in the absence of estrogen challenged the view of ER as a ligand-dependent transcription factor. In the ligand-independent model, peptide growth factors, protein kinase A (PKA) activating factors, and cyclins can induce ER mediated activity in the absence of ER-specific ligands (reviewed in Pettersson and Gustafsson, 2001). The N-terminus of the ER contains several conserved serine residues that are targets for phosphorylation. In the absence of ligand, growth factor signalling leads to the activation of kinases that phosphorylate and activate the ER or its associated coregulators (reviewed in Heldring *et al.*, 2007). For example, epidermal growth factor (EGF) is a peptide growth factor that can mimic the effects of estrogen through the ER. EGF binds its membrane-bound receptor/tyrosine kinase, which in turn activates a mitogen-activated protein kinase (MAPK) signalling cascade. MAPKs phosphorylates the ER in the AF-1 domain resulting in an activated ER that can then proceed through the classical or non classical models of activation.

1.3 The Estrogen Receptor

1.3.1 Estrogen Receptor: ERα and ERβ

It was discovered, based on the binding of 17-β-estradiol (E2) in the uterus, that the biological effects of the steroid hormone estrogen were mediated by a nuclear hormone receptor labelled the estrogen receptor (ER). This receptor was believed to be the single mediator of the physiological effects of estrogen until 1996, when a novel estrogen receptor was discovered in the rat prostate (Kuiper *et al.*, 1996). To distinguish the two receptors, the original estrogen receptor was re-named ERα and the new second receptor was designated ERβ. ERα and ERβ are not splice variants from the same gene but rather distinct products of different genes on separate chromosomes (reviewed in Matthews and Gustafsson, 2003). The ER isoforms belong to the family of nuclear hormone receptors and thus contain highly conserved structure. The DNA binding domains of the two receptors are almost identical, displaying 97% sequence similarity (reviewed in Pettersson and Gustafsson, 2001). The ligand binding domain is the least well conserved between the two isoforms with an overall amino acid identity of 55%, however specific regions involved in ligand binding and the AF-2 display a higher sequence similarity. The main difference between the receptor subtypes is in the affinity for various selective estrogen receptor modulator compounds (SERMs) and the transcriptional response elicited by these compounds.

ERα and ERβ are both capable of binding estrogen and have similar binding specificities. Both receptor subtypes produce genomic and non-genomic effects in response to estrogen (reviewed in Singh and Kumar, 2005). The non-genomic effects are

rapid responses within the cytoplasm that are mainly due to the activation of various protein kinase cascades. However, this study is focused on the different effects of the receptor subtypes at the genomic level.

Recently, there has been evidence that the presence of ERβ in cells that also express ERα results in a distinct profile of gene expression compared to cells expressing only ERα or ERβ (Chang *et al.*, 2006; Williams *et al.*, 2007). In the absence of estrogen stimulation, ERβ regulate genes that are normally regulated by ERα with estrogen (Chang *et al.*, 2006). In the presence of estrogen, ERβ also stimulates the expression of a unique subset of genes that are not regulated by ERα alone. Many of the genes modulated by ERβ are those involved in cell cycle progression and apoptotic mechanisms. Recent studies have shown that ERβ inhibits the expression of ERα-regulated genes and is also capable of opposing ERα-E2 induced proliferation (Williams *et al.*, 2007). This provides evidence that ERβ may have a protective effect from the proliferative response elicited by the ERα in the presence of estrogen. The relative levels of ERα and ERβ in normal and abnormal breast tissue is an important issue in determining how the cells will respond to different endocrine therapies.

When ERα and ERβ are co-expressed in a cell, they do not merely function as separate homodimers at the ERE, it has been shown that ERα and ERβ are capable of forming heterodimers (denoted ERα/β in this text) and these heterodimers are capable of binding EREs (Cowely *et al.*, 1997; Pace *et al.*, 1997). Upon DNA binding, the ERα/β heterodimer is also capable of recruiting ER cofactors and stimulating ERE-driven transcription. As homodimers, the ER subtypes clearly exhibit differential effects in the

response to estrogen and the effects on transcription from a consensus ERE (reviewed in Nilsson *et al.*, 2001). The function of the heterodimer, in normal and abnormal cell proliferation is currently unknown.

1.3.2 Coregulators of the Estrogen Receptor

The ER is not solely responsible for mediating the effects of estrogen; upon DNA binding the ER recruits other proteins known as coregulators to form large multiprotein complexes. It is through the interaction with coregulators that chromatin remodelling, histone modification, transcription initiation elongation, splicing or terminal degradation occurs (reviewed in Moggs and Orphanides, 2001). Coregulators are critical for proper function of the ER and its effects on development, physiology and reproduction. These proteins are classified based on their effects on the transcriptional activity of ER, as coactivators, corepressors or cointegrators. It is important to note that these proteins do not function exclusively of one another, coactivators and corepressors may be found in the same large multifunctional protein complex despite their opposing action (reviewed in Pettersson and Gustafsson, 2001).

Well characterized examples of ER coactivators are the members of the p160 or steroid receptor coactivator, SRC, family. These cofactors contain three of the LXXLL motifs typically found in ER cofactors, where L is leucine and X is any amino acid (Ding *et al.*, 1998). SRC-1 mediates functional interactions between the AF-1 and AF-2 domain resulting in AF domain synergy. The SRC-3 member of this family exhibits ER subtype selectivity, with a higher affinity and activation with ERα over ERβ (Suen *et al.*, 1998).

SRC proteins also mediate interactions with cointegrator proteins such as p300 family members.

p300 and Creb-binding protein (CBP) have been characterized as a coactivators of many factors such as nuclear receptors, p53, and nuclear factor NF-κB (reviewed in Petersson and Gustafsson, 2001). These coactivators possess intrinsic HAT activity, and are capable of acting synergistically with ligand-bound ER to enhance transcription. CBP interacts with both SRC-1 and the ER and the ER/SRC-1/CBP complex forms a stable ternary structure for coactivation of the ER (Hanstien *et al.*, 1996).

There are a variety of proteins that repress transcriptional activity of the ER. The most extensively studied corepressors include nuclear receptor corepressor (NCOR), and silencing mediator of retinoid and thyroid receptors (SMRT) (reviewed in Hall and McDonnell, 2005). These corepressors interact with ER and other nuclear receptors through a specific domain known as the CoRNR box, which contains sequences similar to that of the LXXLL domain found in nuclear receptor coactivators. NCOR and SMRT have both been found to associate with ERα in the presence of an antagonist such as tamoxifen (Smith et al., 1997; Lavinsky et al., 1998). In contrast, ERβ has been found to bind NCOR and SMRT in the presence of agonists, but not antagonists, via the LXXLL-like motif box (Webb et al., 2003). Differences in cofactor recruitment may be the basis for alternative function of the two ER isoforms.

1.3.3 Estrogen Receptor in Breast Development

Estrogen plays a key role in development and morphogenesis of reproductive tissues (reviewed in Pettersson and Gustafsson, 2001). The two subtypes of ER mediate the effect of estrogen on its target tissues and play distinct non-redundant roles in normal development and maintenance of tissues in the reproductive, immune, skeletal, cardiovascular, and central nervous systems. ERα and ERβ bind estrogen with relatively similar affinity, but differ in the response elicited at the promoter region of estrogen responsive genes. The proliferative effects of estrogen on many systems in the body appear to be the result of a fine balance between ERα and ERβ signalling. Extensive studies have been conducted on the role that the ER subtypes and their relative levels play in breast development and growth.

Studies conducted in mice have provided much insight into the role of ERα and ERβ in the breast. In the adult mouse mammary gland, ERβ is expressed in over 60% of the ductal epithelial cells while ERα is only expressed in approximately 20% of the cells in the same gland. Also, ERβ, but not ERα, is expressed in the stroma of the adult gland (reviewed in Heldring *et al.*, 2007). It is interesting to note that in a developing 2 week old mouse embryo ERα and ERβ are both strongly expressed in the ductal epithelium and stroma. ERα -/- knockout mice demonstrate limited mammary growth even in the presence of estrogen (Kenney et al., 2003). When an ERα -/- mammary fat pad of a 3 week old mouse is transplanted into the mammary fat pad of a wild type 3 week old mouse, ductal growth fails to occur around the ERα -/- implant, suggesting that it is secreting growth inhibitory substances. Since ERβ is still highly expressed in the 3 week

old mouse this suggests that ERβ stimulates secretion of a growth repressor such as transforming growth factor β (TGF-β) (reviewed in Heldring et al., 2007). ERβ -/- knockout studies suggest that it also has an important role in growth regulation and differentiation of the mammary epithelium. In fact, the ERβ -/- knockout mice show incomplete differentiation of mammary epithelium and as the mice age the gland continues to proliferate abnormally and fills with large cysts. This implies that loss of ERβ also leads to loss of mammary gland growth repression mechanisms. Other murine studies imply that ERβ may also play a role in the organization and adhesion of epithelial cells and, therefore, affect differentiated tissue morphology (Forster *et al.*, 2002).

1.3.3 Selective Estrogen Receptor Modulators

Compounds known as selective estrogen receptor modulators (SERMs) demonstrate remarkable differences in effectiveness and tissue selective action in response to estrogens. SERMs are tissue selective, functioning as agonists in some tissues while simultaneously acting as antagonists in other tissues. The discovery of ERβ led to a possible mechanism by which SERMs may produce tissue selective responses (reviewed in Katzenellenbogen and Katzenellenbogen, 2000). As previously discussed, ERα and ERβ are highly conserved and the majority of the differences between the two subtypes is in the ligand binding domain. This difference presents the possibility for some compounds such as steroids, phytoestrogens or androgen-derived diols to have affinity and potency preference for either ERα or ERβ. The response at any given target gene may vary based on the ER subtype or nature of its ligand (McDonnell, 1999). As a result

ERα and ERβ specific agonists and antagonists have been developed. In the case of ERα, it has been discovered that a compound propypryazole-triol (PPT) (Figure 3) has over 400-fold higher affinity for and is approximately 1000-fold more potent with ERα than with ERβ as an agonist (Stauffer *et al.*, 2000). Similarly, methyl-pieridinopyrazole (MPP) is an antagonistic pyrazole compound that demonstrates approximately a 200-fold binding affinity for ERα (Sun *et al.*, 2002). ERβ subtype specific ligands have also been discovered, for example the non-steroidal estrogen diarylpropionitrile (DPN) is an ERβ specific agonist with approximately 30-fold higher affinity for ERβ over ERα (Sun *et al.*,2003). ERβ-specific antagonists include compounds such as R,R-terahydrohrysene (Meyers *et al.*, 2001) and mifipristone (Escande et al., 2006). Further understanding and development of ER subtype specific ligands and their pharmacology is an important tool to discovering the exact function of each ER subtype and the role they play in estrogen-related diseases.

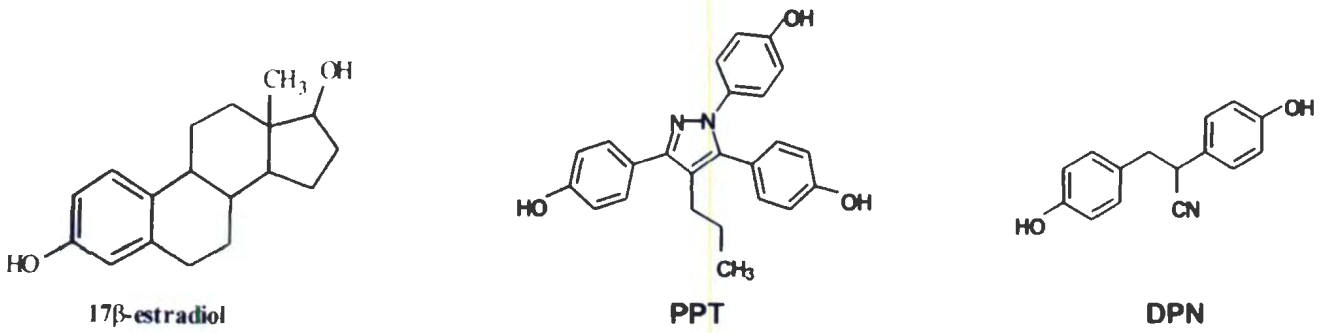

Figure 3: Structure of estrogen receptor selective ligands Estradiol, PPT and DPN. Estradiol is a natural estrogen that has similar binding affinity with both ERα and ERβ. Propypryazole-triol (PPT) is a synthesized ERα specific ligand, which has over 400 fold higher affinity for ERα over ERβ. Diarylpropionitrile (DPN) is a synthesized ERβ specific agonist that has a 30 fold higher affinity for the ERβ subtype.

[Reproduced from Harrington et al., 2003; Marino et al., 2005]

1.3.4 Estrogen Receptor in Cancer

Due to the ability of ERα and ERβ to differentially regulate estrogen-mediated growth and development of the breast, much focus has been placed on characterizing the mechanisms by which the ER subtypes affect breast cancer. Estrogen controls cell growth and differentiation through the ER in normal tissues and hormone responsive tumours through its ability to interact with specific ERE elements in estrogen-responsive genes and subsequently regulate transcription. For example, the ability to induce the expression of c-myc and cyclin D is responsible for much of the estrogen-regulated progression through the cell cycle (Doisneau-Sixou *et al.*, 2003).

Overexpression or hyperactivity of ER can result in an increase in cell cycle progression and enhanced tumour growth. However, the exact mechanism through which the ER functions in the proliferation of breast cells in response to estrogen is not fully understood. In normal murine mammary gland, proliferating ductal epithelial cells do not express ERα, indicating that E2 mediates its effects indirectly (reviewed in Petersson and Gustafsson, 2001).Yet in ERα expressing breast cancers, the effect of estrogen appears to occur directly through the ERα as it is blocked via treatment with the anti-estrogen tamoxifen which targets ERα. It is proposed that ERα may mediate breast growth indirectly by increasing growth factor secretion, but this does not explain why normal ERα-expressing mammary cells do not proliferate in response to estrogen but do so in breast cancer. This suggests that ERβ might play an important role in growth factor regulation and a fine balance of ERα and ERβ is necessary to maintain normal mammary growth.

In human breast cancer, the evaluation of the role of the ER in pre-invasive mammary tumours is of particular interest (Roger *et al.*, 2001). When cases of proliferating benign breast disease, non proliferating benign breast disease, and carcinoma *in situ* are compared, the percentage of ERβ expressing cells was high in normal mammary glands and non-proliferating benign breast disease, but decreased significantly in proliferating benign breast disease and carcinoma *in situ*. The ERα showed an inverse expression when compared to ERβ. As well, in a normal resting mammary gland, ERβ is present in both luminal and myoepithelial cells whereas ERα is only found in some luminal epithelial cells. ERα shows a very different distribution and proportion which suggests a potentially protective effect of ERβ against the mitogenic activity of estrogen. Some laboratories have reported a correlation between the presence of ERβ and lower tumour stage and grade, as well as a predictive factor in the response to tamoxifen therapy in breast cancer (reviewed in Heldring *et al.*, 2007; Hopp *et al.*, 2004). However, these studies are widely debated and the potential of ERβ as predicative factor or diagnostic marker in breast cancer is not clearly defined.

1.4 MIER1

1.4.1 MIER1: Structure

Mesoderm Induction Early Response 1 gene or *mier1* was initially discovered in *Xenopus laevis* as a novel immediate early gene target of fibroblast growth factors (FGF) (Paterno *et al.*, 1997). FGFs are important for both embryonic and adult development. FGF plays an important role in cells derived from the middle embryonic layer of early embryos known as the mesoderm (Becker *et al.*, 2003). The receptors for FGF are

receptor tyrosine kinases that upon phosphorylation activate a cascade of events that leads to cell proliferation and differentiation. This signal transduction is essential for proper development of many cell types such as muscle, cartilage, bone and blood. The first genes to be transcribed in response to such growth factor signalling are known as immediate early or early response genes. Many early response genes are transcription factors that are capable of enhancing or repressing the transcription of other genes.

Early experiments with *Xenopus mier1* (*xmier1*) showed that FGF treatment resulted in *xmier1* levels that were 3-4 fold higher than in non FGF treated controls (Paterno et al., 1997). To confirm that *xmier1* was an early response gene and that the expression was not dependent upon *de novo* protein synthesis, the expression was measured in the presence of a protein synthesis inhibitor, cycloheximide. xMIER expression was unaffected by the presence of cyclohximide, confirming that it is an early response gene. Further analysis of *xmier1* revealed that its product, xMIER1, had the potential for nuclear localization and contained stretches of acidic amino acids characteristic of acidic activation domains found in transcription factors (Paterno *et al.*, 1997; Post *et al.*, 2001). Upon further examination, it was determined that xMIER1 was actually targeted to the nucleus and that the N-terminus functioned as a potent activator of transcription. Combined, these results support *xmier1* as an early response gene that codes for a novel transcription factor xMIER1. Since its discovery many studies have been conducted to further characterize the structure and function of *mier1*.

A human orthologue of *xmier1*, known as *hmier1* displaying 91% sequence similarity was also discovered and characterized (Paterno *et al.*, 1998; Paterno *et al.*,

2002). The structure of *hmier1* and its splice variants is shown in Figure 4. *hmier1* is a single copy gene consisting of 17 exons spanning 63 kb. Exon 3A functions as a skipped exon and the last intron (intron 15) functions as a facultative intron. As a result of this, the single copy of *hmier1* ultimately gives rise to 6 predicted protein isoforms with common (exons 4-15) internal regions and varying N-terminal and C-terminal domains (α and β). The variation in the C-terminal domain of the protein arises from alternative splicing of intron 15 at the 3' end of the gene. This, in turn, encodes two distinct C-terminal domains, α and β. The α C-terminus results from the removal of intron 15, which encodes for a 23 amino acid C-domain. The β C-terminus arises from the inclusion of intron 15, which encodes a larger 102 amino acid C-terminal domain. The alternative C termini suggest alternative function and give rise to two dominant forms of hMIER1, hMIER1α and hMIER1β.

The internal conserved region of hMIER1 was found, upon comparison to other known proteins, to contain several conserved functional motifs as seen in Figure 5. This common region contains a highly acidic region, a proline rich motif, an ELM2 domain, and a signature SANT domain. The α and β isoforms also contain motifs specific to their differential splicing, a LXXLL motif and a nuclear localization signal (NLS), respectively. These domains provide indications as to the possible function of this novel protein.

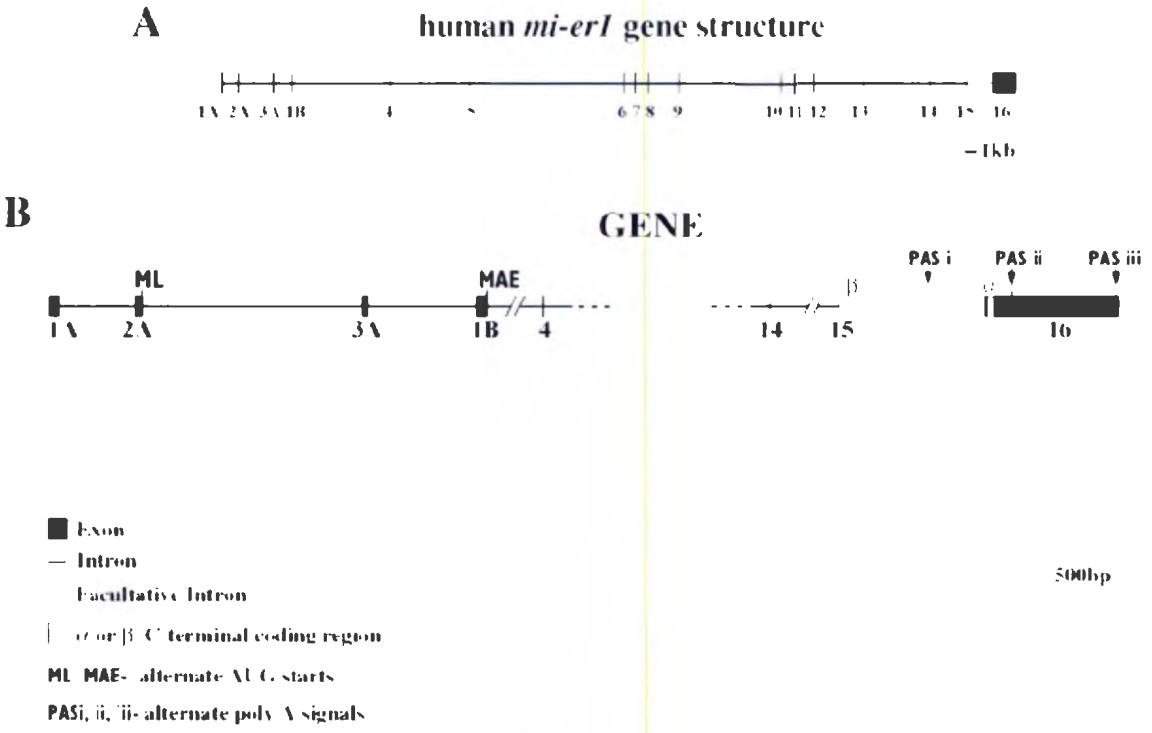

Figure 4: Structure of *hmier1* gene and splice variants

A schematic of *hmier1* gene where the exons are shown in black, the introns as horizontal lines, the facultative intron is shown in grey and the white area represents the position of α and β c-terminal coding regions. A. Organization of the *hmier1* gene is shown with numbers below denoting the exons. B. An enlargement of the gene structure shown in A, demonstrating usage of promoter 2A or 1B to produce distinct 5' ends. Also shown is the variation of the 3' end generated from alternative splicing, alternative promoter usage and alternative polyadenlyation signals (PAS).

[Reproduced from Paterno et al., 2002]

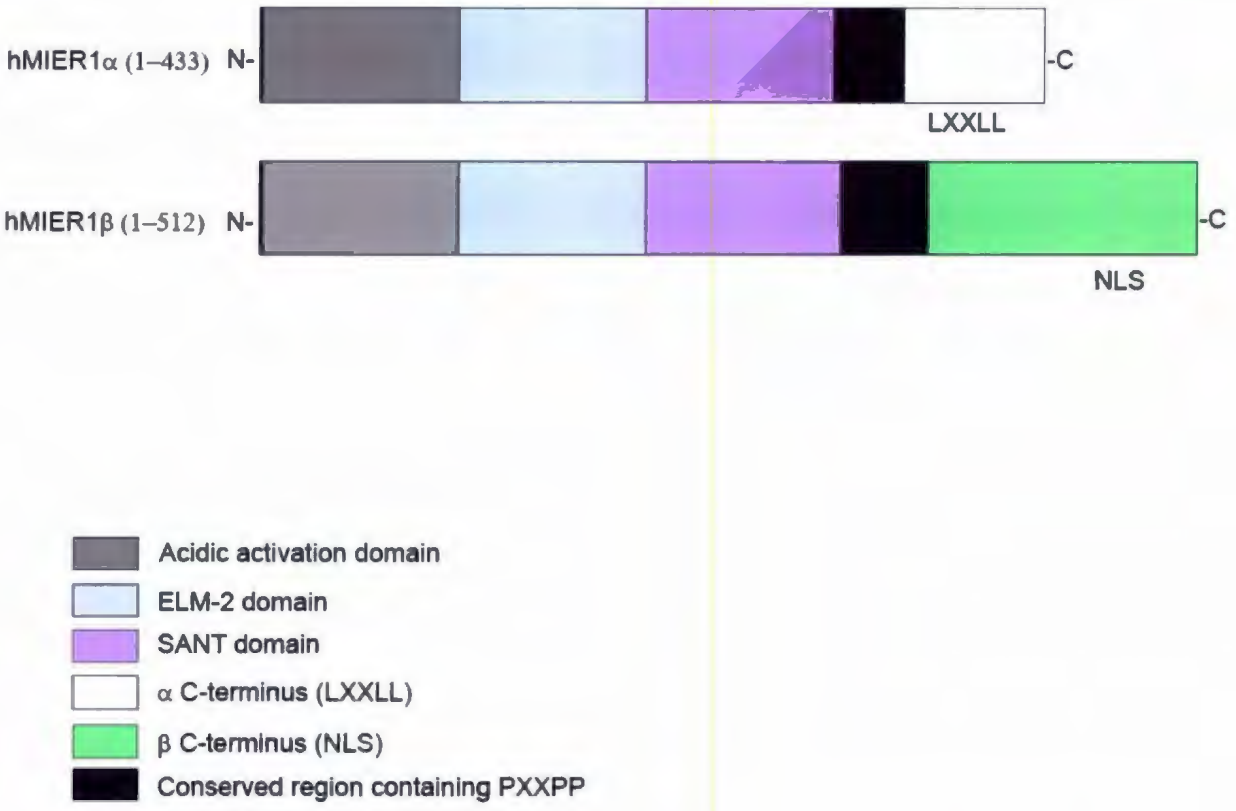

Figure 5: Structure of hMIER1 isoforms

Schematic illustration of hMIER1α (433 amino acids) and hMIER1β (512 amino acids). Both isoforms contain several conserved motifs; at the N terminus there are four regions rich in acidic amino acids or an acidic activation domain, an ELM2 domain, a SANT domain and C terminal to the SANT domain is the PXXPP motif. hMIER1α and hMIER1β are distinct at their C termini, hMIER1α contains a LXXLL motif in this region whereas hMIER1β contains a nuclear localization signal (NLS).

1.4.2 MIER1 Functional Domains

I. Acidic Activation Domain

The N terminus of xMIER1 and hMIER1 contains four stretches of highly acidic amino acids. These stretches correspond to a well characterized motif called the acidic activation domain that can be found in many transcription factors (reviewed in Struhl, 1987). Acidic activation domains are particularly associated with transcriptional activation as they aid in the recruitment of transcriptional machinery (Melcher, 2000). Constructs of the xMIER1 protein revealed that while full length xMIER1 did not activate transcription, a construct containing the four acidic stretches of the N terminal alone was capable of stimulating transcription (Paterno *et al.*, 1997). The deletion construct containing only the first three of the acidic stretches was a more potent transactivator that stimulated transcription 80 fold. This suggests that MIER1 has the potential to function in activating transcription. Since hMIER1 and xMIER1 share 91% sequence homology this suggests that the hMIER1 acidic activation domain may also play a role in activation of transcription in human cells.

II The ELM2 domain

The ELM2 domain is also found in both forms of the hMIER1 protein, and gets its name from the EGL-27 and MTA1 homology domain 2. This domain was originally discovered in *Caenorhabditis elegans* protein EGL-27 and the human metastasis-associated (MTA1) protein (Solari *et al.*, 1999). The EGL-27 protein was found to be very similar to MTA1 with conserved functional domains ELM1 and ELM2. EGL-27 is

important in developmental patterning of *C. elegans*, and MTA is a protein known to be part of a complex involved in transcriptional repression through the recruitment of HDAC (Wang *et al.*, 2006). In proteins containing ELM2 domains, it has been found that this domain plays an important role in recruitment of HDAC activity (Ding *et al.*, 2003). In assays using a G5tkCAT reporter plasmid, which contains chloramphenicol acetyltransfease (CAT) linked to 5 GAL4 DNA binding sites and the thymidine kinase (tk) promoter, hMIER1α and hMIER1β both exhibit the ability to repress transcription. Further analysis showed the ELM2 domain of hMIER1 is essential for the binding of HDAC1 and transcriptional repression.

III The SANT domain

The SANT domain was originally found in proteins SWI3, ADA2, N-CoR, and TFIIIB from which the name is derived (reviewed in Aasland *et al.*, 1996). SWI3 and ADA2 are components of either the SWI-SNF or ADA transcriptional activation complexes, N-CoR is a corepressor that regulates nuclear hormone receptors, and TFIIIB is the B subunit of the RNA polymerase III initiation complex. The function of the SANT domain is not fully understood; however it is highly related to the DNA binding domain of the MYB protein, containing 3 alpha helices in a helix-turn-helix motif.

Recent studies have revealed that despite the similarity to the MYB DNA binding domain, the SANT domain contains hydrophobic residues in its recognition helix that are predicted to be incompatible with DNA binding (reviewed in Boyer *et al.*, 2004). It has been implied, therefore, that the SANT domain may play an essential role in regulation of

chromatin accessibility through protein-protein interactions. Additionally the SANT domain is essential for both HAT and HDAC activity. For example, in yeast the Ada2 is a conserved subunit in the Gcn5 containing HAT complex. Minor deletion in the SANT domain shows reduced ability for binding histone tails, and results in Ada2 complexes that are inactive in HAT assays. In the case of nuclear corepressors such as NCOR and SMRT, the SANT domain is involved in the HDAC activity. These corepressors both contain two closely spaced SANT domains and are known to recruit HDAC to the target genes through interaction with unliganded receptors. NCOR and SMRT alone with the HDAC3 subunit is sufficient for active HDAC, and deletion of the amino SANT domain disrupts the binding of HDAC3 to SMRT emphasizing the role of SANT in protein-protein interactions. The mechanism by which this domain is responsible for activation of the catalytic unit of the HDAC is unclear.

In the case of hMIER1, there is no HAT or HDAC activity associated with the SANT domain. The SANT domain is required for association of hMIER1 with the chromatin of its own promoter via Sp1. The SANT domain, which does not bind the DNA directly, physically interacts with Sp1 and through interaction hMIER1 represses Sp1 driven transcription. As a result, the SANT domain of hMIER1 appears to repress transcription from its own promoter by a HDAC independent mechanism.

IV The Proline Rich Region

Another highly conserved motif found in hMIER1 is a proline rich region denoted as PXXPP, where P represents the amino acid proline and X represents any other amino acid. In other proteins, the binding of SRC homology 3 (SH3) domains has been shown

to be mediated by this proline rich motif (reviewed in Li, 2005). SH3 domains are a group of well characterized small interaction modules ubiquitously expressed in eukaryotes that mediate protein-protein interaction. These domains play important roles in mediation of many biological processes such as assembly of large multi-protein complexes, subcellular localization of components in signalling pathways, and regulation of enzymes via intramolecular interactions.

The proline rich region in hMIER1 may function as a recognition site for this family which provides numerous avenues by which it may play a role in cell signalling and development. For example, overexpression of xMIER1 in Xenopus development results in embryos with abnormalities in both anterior and posterior structures (Teplitsky *et al.*, 2000). Further studies revealed that only Proline 365 located in the consensus SH3 binding motif was required for this effect on embryonic development. This demonstrates that xMIER1 developmental effects are mediated by the proline rich region. Since xMIER1 and hMIER1 are highly conserved it also implies that hMIER1α may also have a functional SH3 binding motif within its proline rich region. Further investigation of this domain in combination with the evidence from the previously discussed conserved regions may demonstrate a role for hMIER1 in regulation of cell growth via protein-protein interactions.

V The LXXLL motif

In the hMIER1α C terminus, there is a conserved motif known as the LXXLL motif, where L is leucine and X is any amino acid. LXXLL motifs are found in many proteins that interact with nuclear hormone receptors and are required for the binding of

transcriptional regulators to the ligand binding domain in nuclear receptors (Heery *et al.*, 1997). Mutation of the hydrophobic residues around specific helices in the ligand binding domain disrupts ligand-dependent interaction of the LXXLL motif with the ER and its corresponding cofactors. Mutation of these residues also abolishes ligand-independent (AF-2) function. The ability of nuclear receptor cofactors, such as SRC-1, to bind in the presence of ligand is dependent upon an intact LXXLL motif.

VI. Nuclear Localization Signal

Studies with xMIER1 identified a functional nuclear localization signal in the C-terminal domain (Post *et al.*, 2001). Analysis of the human isoforms of MIER1 revealed that the hMIER1β C terminus displays a high degree of similarity to that of the xMIER1 Paterno *et al.*,2002). Transfection assays with NIH 3T3 cells revealed that hMIER1β was targeted exclusively to the nucleus while hMIER1α remained cytoplasmic. This implies that alternative splicing of hMIER1α and hMIER1β may provide functionally distinct roles within the cell. The lack of an NLS in hMIER1α does not rule out the possibility of transport into the nucleus through binding to other proteins, such as to nuclear hormone receptors.

1.5 Purpose of this Study

The structure of hMIER1 implies that it has a potential role as a transcription factor. Its acidic activation domain, ELM2, SANT and proline rich region also demonstrate that hMIER1 is capable of protein-protein interaction. An LXXLL motif in hMIER1α indicates that this protein can also interact with nuclear receptors such as the

ER. Previous studies in our lab have characterized hMIER1 as a coregulator of the human ERα (Savicky *et al.*, unpublished). The purpose of this study was to further characterize the interaction of hMIER1 with ERα and to investigate the interaction of hMIER1 with the novel subtype ERβ. DNA binding assays were performed to determine if the interaction of hMIER1 with the ER subtypes affects the ability of ER to bind its consensus ERE. In order to provide functional insight, the effect of hMIER1 on ERE-driven transcription in response to ERα and ERβ mediated signalling was investigated.

Given the fact that hMIER1α has previously been shown to interact strongly with both ER subtypes *in vitro*, and given the presence of an LXXLL domain, it is hypothesized that the hMIER1α subtype will interact with ER at its consensus ERE and have an effect on ER driven transcription. hMIER1β also interacts with both ER subtypes *in vitro*, and due to differential structure hMIER1β may differ from hMIER1α in both physical and functional effects on the ER at its consensus ERE. Analysis of hMIER1 interaction with the ERα and ERβ may provide insight for the role of this novel transcription factor in estrogen signalling and estrogen related diseases, such as breast cancer.

Objective 1: *To determine the effect of hMIER1 on the binding of ERα and ERβ to a consensus ERE in the absence and presence of ligand.*

Some transcriptional regulators affect transcription by enhancing or repressing the ability of the ER to bind DNA. Since hMIER1 has previously been shown to interact with both ERα and ERβ *in vitro,* electrophoretic mobility shift assays (EMSAs) were performed to investigate whether hMIER1 affected DNA binding of *in vitro* synthesized

ERα and ERβ.

Objective 2: *To characterize the role of hMIER1 in ERE-driven transcription in the absence of ligand*

The structure of hMIER1 reveals characteristics of both a transcriptional activator and repressor. The functional effect of hMIER1 cannot be determined *in vitro,* so hMIER1α, hMIER1β, ERα, and ERβ proteins were co-expressed in HEK 293 cells. Since ERα and ERβ are activated differently based on whether a ligand is present, the effect of hMIER1 was first determined in the absence of ligand. Assays in which ERE was linked to a reporter gene were performed to determine the ligand-independent effect of hMIER1 interaction on ERα and ERβ driven transcription.

Objective 3: *To characterize the role of hMIER1 in ERE-driven transcription in the presence of estrogen, DPN and PPT.*

In the presence of specific ligands ERα and ERβ respond differently based on differences in the ligand binding domain. Estrogen transactivates both of the receptor subtypes similarly, whereas specific SERMs such as PPT are specific for one ER subtype. The hMIER1α, hMIER1β, ERα and ERβ proteins were co-expressed in HEK 293 cells which were treated with specific ligands. Assays using an ERE linked to a reporter gene were used to determine the ligand-dependent effects of hMIER1 on ER driven transcription.

2. Materials and Methods

2.1 Materials

2.1.1 Plasmids and Constructs

I. pCS3+MT; CS3+MT-*hmier1α/β*

pCS3+MT is a mammalian expression vector with 6 N-terminal repeats encoding the amino acid residues MEQKLISEEDLNE of the c-myc protein (gift from David Turner, University of Michigan). The expression vectors containing full length *hmier1α* or *hmier1β* (accession numbers AY124187 and AD51 5447, respectively) were engineered in our laboratory by Z. Ding.

II. pGEX-4T-1; GST-*hmier1α*, GST-*hmier1β*

pGEX-4T-1 is a Glutathione S-transferase gene fusion vector for the expression, detection and purification of GST fusion proteins in bacteria (Pharmacia, Biotech). GST-*hmier1α/β* constructs were engineered in our laboratory by Z.Ding. The appropriate isoform was cloned into the pGEX-4T-1 vector.

III pCMX, pCMX-*mER α/β*

The mouse ERα was a gift from Dr. Christine Pratt (University of Ottawa; accession number NP_031982) and the mouse ERβ was a gift from Dr. John White (accession number U81451). Partial sequences were obtained to verify the identity of the received plasmids.

IV. 3XERE-TATAluc

The 3XERE-TATAluc plasmid contains three copies of the vitellogenin estrogen response element (ERE) sequence (ggtcacagtgacc) preceding the luciferase gene in a mammalian expression vector pGl2-TATA. This plasmid was purchased from Addgene, Inc.

V. pRSV βgal

The pRSV βgal plasmid is a mammalian expression vector containing the β-galactosidase gene (βgal) downstream of a rous sarcoma virus (RSV) promoter (Promega). This construct is commonly used for monitoring the transfection efficiency in mammalian cells.

2.1.2 Plasmid preparation and purification

Prior to preparation of the plasmids XL Blue chemically competent cells (Stratagene Inc.) were transformed by adding approximately 1 µg of DNA to 100 µl of cells. The reaction was gently mixed with a pipette tip and incubated on ice for 30 minutes. The reaction was then heat shocked at 42°C for 40 seconds and placed back on ice for 1-2 minutes. To the reaction was added 250µl of Luria broth (LB) medium (5 g peptone, 2.5 g yeast extract, 5 g NaCl, 500 ml dH_2O; autoclaved) was added and the culture was shaken at 37 °C for 60 minutes. After incubation 100 µl of the culture was plated on a LB ampicillin (5 g peptone 2.5 g yeast, 5 g NaCl, 7.5 g Agar, 500 ml dH_2O; autoclaved; 50 µg/ml ampicillin) and incubated at 37°C overnight. The following day, one colony from each plate was selected and allowed to grow in a 150 ml LB medium with 50 µg/ml ampicillin culture, shaking at 37 °C overnight. The following morning the

cultures were centrifuged at 3000xg to pellet the bacteria. At this point DNA purification was performed using the Nucleobond PC500 EF plasmid maxi prep kit (Clontech Laboratories Inc.) was carried out as per the manufacturer's instructions.

2.2 GST Fusion Protein Production

To produce GST fusion protein, approximately 10 ng of the pGEX-4T1 plasmid was added to 100 µl of BL21 Codon Plus RP chemically competent cells (Stratagene). The reaction was gently mixed with a pipette tip and incubated on ice for 30 minutes. The reaction was then heat shocked at 42°C for 40 seconds and placed back on ice for 1-2 minutes. Then, 250 µl of LB medium was added and the culture was shaken at 37 °C for 60 minutes. After incubation 100 µl of the culture was plated on a LB ampicillin plate and incubated at 37°C overnight. The following day, one colony from each plate was selected and allowed to grow in a 5 ml LB medium with 50 µg/ml ampicillin culture, shaking at 37 °C overnight. The next morning, 1 ml of the culture was used to inoculate 250 ml of LB medium with 50 µg/ml ampicillin and allowed to grow at 37 °C, shaking, for approximately 3.5 hours. The optical density (OD) of the culture was measured at this time at 600 nm in a spectrophotometer (Beckman Du-64). Provided the culture had reached an OD of 0.6-0.8, 25 µl of IPTG (isopropyl-b-D-thiogalactopyranoside; Invitrogen) was added to induce protein production. The culture was then allowed to grow with shaking at 37 °C for an additional 3.5 hours. The culture was poured into a 250 ml Nalgene polypropylene bottle and centrifuged at 4000 rpm in a Sorval centrifuge for 15 minutes. All of the supernatant was drained and the pellet was re-suspended in 5 ml of ice cold 1X PBS. The re-suspended pellet was placed in a 50 ml falcon tube and 25 µl

of 0.2 M phenylmethylsulfonyl fluoride (PMSF, Sigma) was added. Cells were then lysed by sonication on ice for 2 minutes in 30 second bursts with 10 second rest periods using a Sonic Dismembrator (Model 500, Fisher-Scientific). Following sonication, 500 ul of 10% Triton X-100 (Sigma) was added and the mixture was transferred to a 30 ml Corex glass tube. The sample was balanced to within 0.01 g and centrifuged at 4000xg in a Sorval RC-SB centrifuge for 15 minutes. The supernatant was aliquoted into clean labelled 1.7 ml tubes and placed at -80°C for storage. GST fusion protein expression levels and purity were checked by SDS polyacrylamide gel electrophoresis (SDS PAGE).

2.3 *In vitro* Coupled Transcription-Translation

In vitro translations were performed using the TnT® T7 Coupled Reticulocyte Lysate System (Promega). Each 50 µl reaction mixture contained: 25 µl TnT® Rabbit Reticulocyte Lysate, 2 µl TnT® Reaction Buffer, 1 µl amino acid mixture minus methionine, 1 µl of Ribonuclease Inhibitor (RNA guard, Amersham Biosciences), 1 µl of T7 polymerase, 5 µl of ^{35}S methionine. The appropriate volume of DEPC water was added to make up a final volume of 50 µl per reaction. A master mix of these components was made up to the appropriate volume and distributed equally to all samples. In order to perform both labelled and unlabeled reactions, the master mix was made up as described above and separated into two separate mixtures before the addition of ^{35}S. The radiolabel was added to the labelled reaction mix, while an appropriate amount of amino acid mixture minus leucine was added to the unlabeled mixture. Both master mixtures were aliquoted for each sample, and 1 µg of the appropriate DNA was added to each reaction All samples were incubated together for 90 minutes at 30 °C. The samples were

consequently analyzed using SDS PAGE to determine if the proteins produced were the correct full length product, as is determined by the predicted molecular weight for that protein. Tricholoroacetic acid (TCA) precipitation was performed to determine percent incorporation of radioactive label.

2.3.1 Determination of Incorporation of Radioactive label

After completion of the *in vitro* transcription translation reaction, TCA precipitation assays were conducted to determine the success of radioactive incorporation. For each reaction, 2 µl of the translation product was bleached in 98 µl of 1 N NaOH, 2% H_2O_2 in dH_2O), vortexed briefly and incubated at 37°C for 10 minutes. Following the incubation 900 µl of ice cold 25% TCA/2% casamino acids (Merck) was added to precipitate the translation product. This mixture was incubated on ice for 30 minutes. The precipitation product was then collected on Whatman filter paper (Fisher) under vacuum as follows: 1 ml of 5% TCA was filtered under vacuum; next 1 ml of sample and 1 ml of 5% TCA (used to wash the sample tube) were filtered under vacuum, followed by 2 washes of 3 ml of 5% TCA. Finally, 3 ml of 100% acetone was added to dry the filter paper. To determine ^{35}S incorporation, the filter was placed in Biodegradable Counting Scintillant (Amersham) and analyzed in a Beckman S3801 Scintillation Counter.

2.4 Cell Culture

Human embryonic kidney cells (HEK 293) were obtained from the American Tissue Culture Collection (ATCC). The cells were cultured in Dulbecco's Modified

Eagle's medium (DMEM, Invitrogen), with the addition of 7.5% Calf Serum (CS; Invitrogen) and 2.5% Fetal Bovine Serum (FBS; Invitrogen). The cells were cultured in a 37°C incubator with 5% CO_2

2.5 Transfections

Approximately 18 hours prior to the transfection procedure, HEK 293 cells were seeded at a density of 5.0×10^5 cells/well in 6 well plates (Corning Inc.). Cells were seeded in phenol red free (PRF) DMEM media with 10% charcoal stripped FBS. Approximately 1 hour prior to transfection, the supplemented media was aspirated and PRF media minus serum was added. Cells were transfected with the following plasmids in co-transfecion experiments: 3xERE-TATA luc and βgal along with either pCS3+MT, pCS3+MT-*hmier1*α, or pCS3+MT-*hmier1*β, and *ER*α or *ER*β. Cells were transfected with a total amount of 1.9µg of plasmid DNA with 6µl of Plus reagent (Invitrogen) according to the manufacturer's protocol. Briefly, the 1.9 µg of DNA was mixed with 6 µl of Plus reagent in the appropriate amount of PRF medium (minus serum) and incubated at room temperature for 15 minutes. The mixture was then added to 6 µl of Lipofectamine reagent (Invitrogen) diluted in the appropriate amount of PFR medium (minus serum) and incubated for an additional 15 minutes at room temperature. Additional PRF (minus serum) media was added to the samples to make up the final volume and 1 ml of the mixture was added to the cells in each well. The cells were incubated for 4 hours at 37°C, following which the PFR medium (minus serum) was replaced with PFR medium plus 10% charcoal stripped FBS and incubated at 37°C for an additional 48 hours.

2.6 Reporter Assays

2.6.1 Luciferase Reporter Assay

HEK 293 cells were seeded in 6 well plates in PRF DMEM approximately 18 hours prior to transfections. For the ERα or ERβ homodimer experiments, cells were transiently transfected with 0.5 µg of 3xERE-TATAluc reporter plasmid, 0.2 µg of βgal reporter plasmid, 0.4 µg of either ERα, ERβ or CS3+MT, and 0.8 µg of either CS3+MT, pCS3+MT*hmier1α* or pCS3+MT*hmier1β*. Approximately 24 hours following transfection, cells were treated for an additional 24 hours with either 10^{-8} M 17 β-estradiol (Estrogen) (Sigma-Aldrich), 10^{-8} M PPT (Sigma-Aldrich), 10^{-8} M DPN (Tocris) or an equivalent volume of vehicle as appropriate to each experiment. HEK 293 cells were harvested 48 hours after transfections as follows; the PFR DMEM was removed by aspiration, each well was washed with 1 ml 1xPBS which was also aspirated, then 400 µl of 1x Cell Lysis Buffer (Promega) was added to each well. Each well was scraped gently and cells were incubated with lysis buffer for 20 minutes on ice. The lysate was then transferred into clean labeled 1.7 ml centrifuge tubes and spun at 12000xg for 15-20 seconds at room temperature. The supernatant was collected into new 1.7ml tubes. The lysate was either assayed immediately or stored at -80°C until further use. Luciferase assay substrate was removed from -80°C and allowed to thaw for a minimum of 30 minutes before use. Luciferase assays were performed by mixing 10 µl of cell lysate with 50 µl of luciferase assay substrate for 10 seconds. The sample was then quantified using a Monolight 2010 luminometer (Analytical Luminescence Laboratory). The relative luciferase units were recorded for each sample and later normalized according to

transfection efficacy (described below). Each experiment was performed three times in triplicate and statistical analysis using students t-test, $p<0.01$.

2.6.2 β-galactosidase Reporter Assay

Beta-galactosidase (βgal) reporter assays were performed in order to correct the RLU according to the transfection efficiency of each sample. Thawed samples were vortexed briefly to ensure thorough mixture of the lysate. 200 µl of βgal buffer [Z buffer (16.1 g/L Na_2HPO_4, 5.5 g/L NaH_2PO_4, 0.75 g/L KCl, and 0.246 g/L $MgSO_4$), 4 g/L ortho-nitrophenyl-β-galactoside (ONPG), and 0.27% β-metcaptoethanol (β-M-EtOH)] was added to labelled 1.7 ml tubes. At timed intervals 10 µl of cell lysate was added to the appropriate tube and votexed for 1-2 seconds. A negative control sample containing cell lysis buffer instead of cell lysate was included for each assay. The samples were allowed to incubate at room temperature for 2 minutes, at which point a yellow color began to develop, then 200 µl of a stop buffer (1 M Tris pH 11) was added at timed intervals in the same sequence as the lysate. Once all reactions were stopped, the 200 µl of each sample was loaded onto a 96 well plate. The plate was then read in a microplate spectrophotometer at a wavelength of 415 nm. The value for the sample containing cell lysis buffer alone was subtracted from all cell lysate samples to provide specific values.

2.7 Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSAs) were performed using a radio-labelled vitellogenin consensus ERE (5' tcg agc aaa gtc agg tca cag tga cct gat caa t 3'). Five picomoles (pmol) of the double stranded DNA was incubated in a 25 µl reaction

containing 25μCi γ-^{32}P ATP and 10 units T4 polynucleotide kinase (Invitrogen) in 1X forward reaction buffer (Invitrogen) at 37°C for 30 minutes. This T4 kinase enzyme transfers the γ-phosphate of ATP to the 5' end of the DNA, labelling the DNA with the ^{32}P. The DNA was then purified using an ethanol/sodium acetate overnight precipitation at -20 °C. The precipitation reaction contained 2.5μl (1/10th the original volume) of sodium acetate, 62.5 μl 100% EtOH (2.5x the original volume) and 2ul of tRNA (10mg/ml). After 18-24 hours, labelled probe was centrifuged at 12000xg for 20 minutes, the pellet was washed with 70% EtOH to remove excess free ^{32}P, and centrifuged again at 12000xg for an additional 15 minutes. The pellet was re-suspended in 40 μl if dH_2O and 1 μl of this solution added to 3 ml Biodegradable Counting Scintillant (Amersham) was analyzed in a Beckman S3801 Scintillation counter to determine ^{32}P incorporation. Based on this, the probe was diluted to100,000 cpm/μl in dH_2O. For example if 1 μl of the re-suspended probe was 300,000 cpm this means there was a total of approximately 1,200,000 cpm in total. The probe would be suspended in a final volume of 120 μl of dH_2O, which requires adding an additional 80 μl of dH_2O to the sample.

For the EMSA reaction, a master mix was prepared such that each 20 μl reaction contained a final concentration of 2 μg/μl Poly(dI-dC), 5% glycerol, 85 g/ml bovine serum albumin (BSA), 50 mM HEPES, 4 mM Tris pH7.6, 1 mM dithiothreitol (DTT), 50 mM KCl, 5 mM $MgCl_2$, and 10 μM $ZnSO_4$. A 100,000 cpm aliquot of labelled probe was incubated in this reaction mixture for 20 minutes at room temperature with 200 ng of GST, GST-hMIER1α, or GST-hMIER1β and the unlabeled equivalent of 100,000cpm ERα, ERβ or ERα/β TnT as measured by labelled TnTs of each protein performed in

tandem. The TnT samples were treated with either 10^{-8} M estrogen, PPT, DMSO or an equivalent volume of vehicle for 30 minutes prior to being added to the reaction.

For antibody supershift assays, the EMSA reaction was performed as described above, however after the 20 minute incubation, the supershift reactions were incubated for an additional 30 minutes at room temperature with 2 ug of anti ERα (HC-20, Santa Cruz Biotechnology Inc.) or anti-ERβ (Y19, Santa Cruz Biotechnology Inc.) polyclonal antiserum. The samples were then resolved by non-denaturing electrophoresis on 5% polyacrylamide gels made in TBE buffer. Each gel was allowed to run at 60V for 1 hour and 30 minutes in a 0.5x TBE buffer. The bands were fixed in a solution containing 20% methanol and 6% acetic acid, and dried by vacuum for 1 hour. The results were analyzed by autoradiography on Kodak biomax MS film.

3. Results

3.1 The effect of hMIER1 on the binding of ER subtypes to a consensus ERE.

Previous work has shown that both ERα and ERβ interact with hMIER1 *in vitro*, but the functional effects of this interaction are largely unknown. In this study, electrophoretic mobility shift assays (EMSAs) were performed to determine if hMIER1 interaction with the ER subtypes causes any disruption of ER binding to its consensus ERE. Also, because hMIER1α contains an LXXLL motif not found in hMIER1β, it was investigated whether hMIER1α and hMIER1β would differ in their interaction with the ER at its consensus ERE in the presence and absence of different ligands.

3.1.1 In Vitro synthesized ERα and ERβ specifically bind a consensus ERE

Preliminary EMSAs were performed using the *in vitro* synthesized ERα and ERβ proteins to verify the formation of the appropriate homodimers. The dimers are identified based on the relative size of the bands as has been described in (Vanacker et al., 1999; Cowely et al, 1997). The ERα protein is approximately 66 kDa, which is slightly larger than the ERβ at 55 kDa. Therefore, the ERα homodimer-ERE complex is expected to migrate more slowly in the gel than the ERβ homodimer-ERE complex; the ER subtypes are indicated by arrows in Figure 3.1A.

The ER homodimers were incubated with a ^{32}P-labelled ERE in the reaction buffer for 20 minutes at room temperature. The specific ERE-ER complexes were identified by competition with a 40 fold excess of unlabelled ERE (Figure 3.1A, lanes 3 and 5). To confirm that each specific band contained the expected ER subtype, parallel

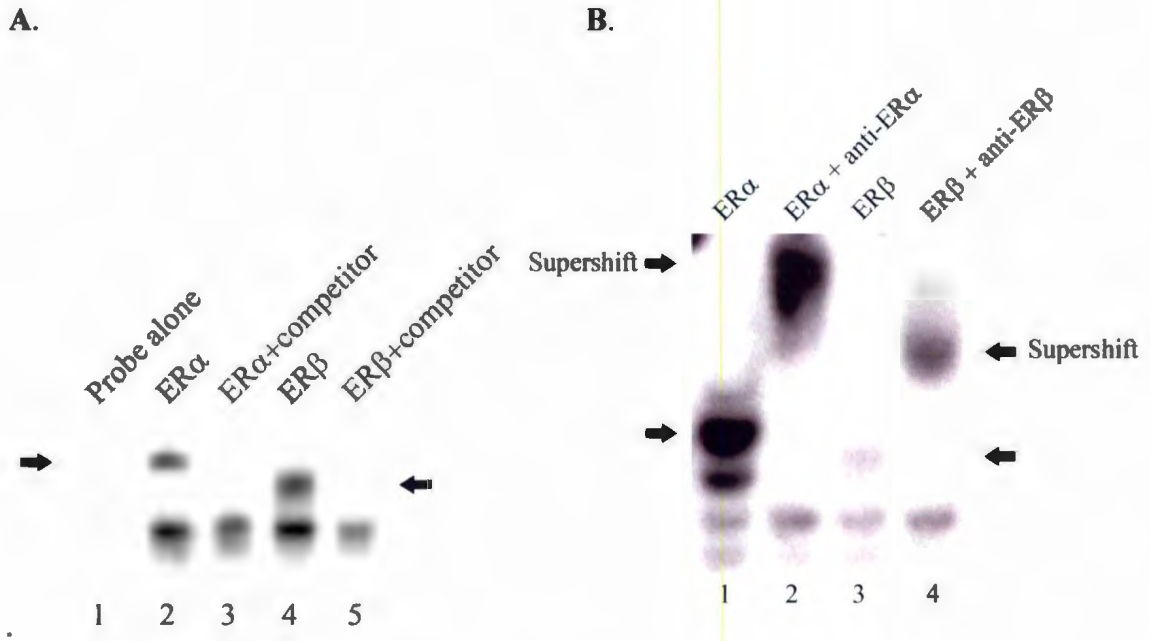

Figure 3.1: ERα and ERβ specifically bind the consensus ERE.

A. An EMSA performed using ^{32}P-labelled ERE probe showing the probe alone (lane 1) and specific ER subtype binding in the absence (lanes 2 & 4) and presence (lanes 3 & 5) of excess unlabelled ERE probe. **B.** The ERα homodimer (lane 1) was supershifted using an anti-ERα antibody (lane 2) (Santa Cruz, HC-20X). The ERβ homodimer (lane 3) was supershifted using an anti-ERβ antibody (lane 4) (Santa Cruz Y-19X)

reactions were performed and incubated with anti-ERα- or ERβ-specific antibodies. Specific DNA-protein complexes containing ER will migrate through the polyacrylamide gel slower, a phenomenon known as a supershift. The supershifted ERα and ERβ complexes are indicated in Figure 3.1B, lanes 2 and 4.

3.1.2 The effect of hMIER1 on ERα ER-binding in the absence and presence of ligand.

In order to investigate the effects hMIER1α and hMIER1β have on the DNA binding ability of ERα, additional EMSAs were performed in the presence and absence of GST-hMIER1α and hMIER1β. EMSAs were attempted using *in vitro* synthesized hMIER1 protein; however the transcription-translation (TnT) reaction did not show an effect on ER-ERE binding. Since using TnTs of hMIER1 and ER in a 1:1 ratio would not determine if excess hMIER1 would disrupt ER-ERE interaction, another method of protein production had to be used to obtain more concentrated protein. Production of hMIER1 in the pGEX vector yielded large amounts of GST-hMIER1 fusion protein. The proteins could subsequently be purified using glutathione sepharose beads to determine the concentration of each GST-hMIER1 fusion protein and a fixed amount of 200ng could be consistently added to each reaction.

To examine at the effects of hMIER1 in the presence of estrogen, the ERα *in vitro* synthesized protein was treated with either 10^{-8}M estrogen or an equivalent volume of vehicle for 30 minutes prior to incubation with GST alone, GST-hMIER1α, or GST-hMIER1β and a ^{32}P labelled ERE probe at room temperature. Incubation with GST alone acted as a control to show that the GST portion of the proteins would not disrupt the ability of ERα to bind its consensus ERE.

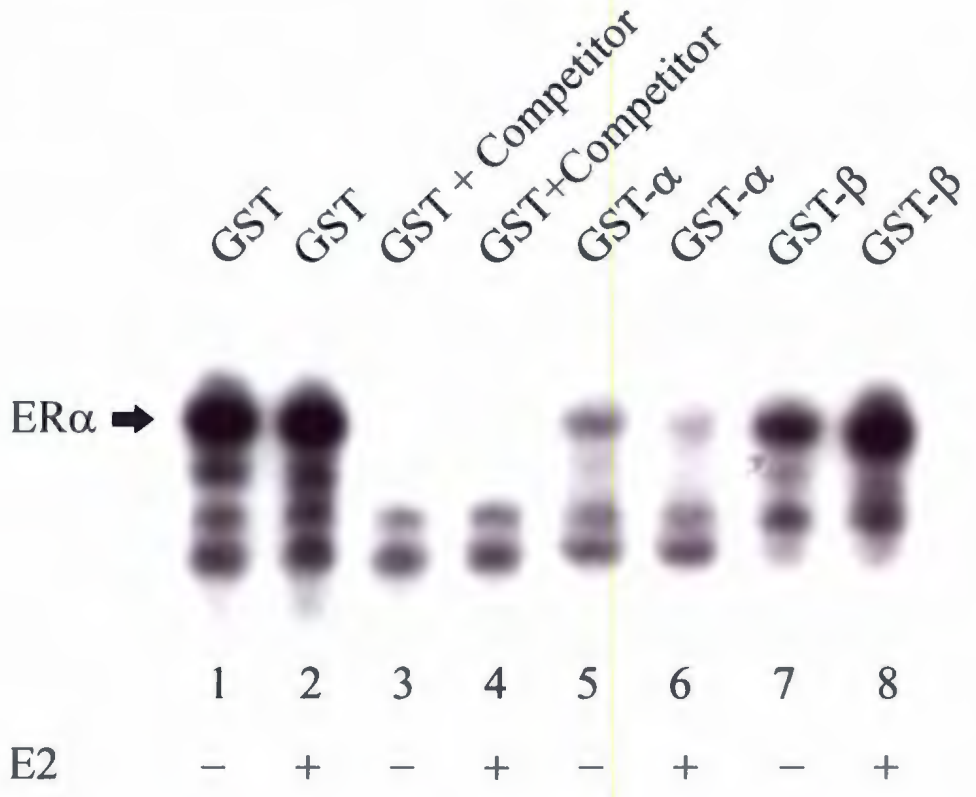

Figure 3.2: GST fused hMIER1α (GST-α) interferes with the ability of ERα to bind its consensus ERE in the presence of estrogen.

Electrophoretic mobility shift assays (EMSAs) were used to investigate whether hMIER1α or hMIER1β would affect the binding of ERα to the ERE in the absence and presence of estrogen. *In vitro* translated ERα was incubated with GST alone (lanes 1&2), GST-hMIER1α (lanes 5&6), or GST-hMIER1β (lanes 7&8). Unlabelled ERE probe was used as cold competitor to ensure the specificity of the ERα band (lanes 3&4). The protein-DNA complexes were resolved through non-denaturing PAGE, and visualized by autoradiography. The position of ERα is indicated. Results were obtained from three independent experiments and shown is a representative autoradiograph.

As shown in Figure 3.2, ERα with GST alone shows no difference in DNA binding in the absence or presence of estrogen (lanes 1 and 2, respectively) and is successfully competed out with unlabelled ERE probe (lanes 3 and 4). hMIER1α disrupts the ERα homodimer DNA binding either in the absence or presence of ligand (lanes 5 and 6). When compared to the control, there is little difference in the DNA binding of ERα with hMIER1β in the absence and presence of estrogen (lanes 7 and 8).

The effects of hMIER1α, and hMIER1β on the binding of ERα to the ERE in the presence of an ERα specific ligand, PPT, are shown in Figure 3.3. PPT has a 410 fold binding affinity preference for the ERα subtype and activates transcription through ERα only (Stauffer et al., 2000). While the ligand binding domains of the ER subtypes have only 55% amino acid identity, the specific residues that are involved with ligand binding are nearly identical. Studies indicate that the selectivity of PPT lies in its differential preference for a specific leucine residue (Leu 384) in the ERα ligand binding domain where ERβ contains a methonine (Met 336).

hMIER1α disrupts the DNA binding of ERα and this effect does not change in the presence of PPT (lanes 5 and 6). In contrast, hMIER1β does not appear to inhibit the ability of ERα to bind its consensus ERE (lanes 7 and 8).

Overall these results show that hMIER1α, but not hMIER1β, disrupts the ability of ERα to bind its consensus ERE either in the presence or absence of ligand.

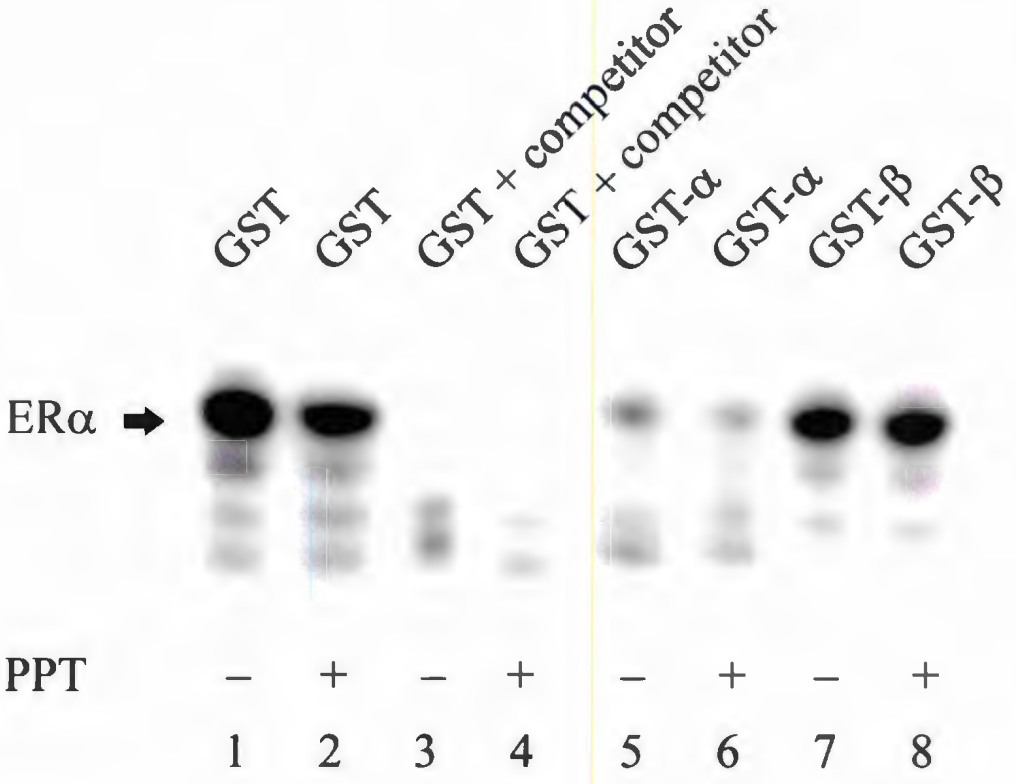

Figure 3.3: GST-hMIER1α (GST-α) interferes with the ability of ERα to bind its consensus ERE in the presence of an ERα specific agonist, PPT.

Electrophoretic mobility shift assays (EMSAs) were used to investigate whether hMIER1α or hMIER1 β would affect the binding of ERα to the ERE in the absence and presence of PPT. *In vitro* translated ERα was incubated with GST alone (lanes 1&2), GST-hMIER1α (lanes 5&6), or GST-hMIER1β (lanes 7&8). Unlabelled ERE probe was used as cold competitor to ensure the specificity of the ERα band (lanes 3&4). The protein-DNA complexes were resolved through non-denaturing PAGE, and visualized by autoradiography. The position of ERα is indicated. Results were obtained from three independent experiments and shown is a representative autoradiograph.

3.1.3 The effect of hMIER1 on ERE binding of ERβ in the absence and presence of ligand.

EMSAs were also performed with *in vitro* translated ERβ protein in the presence or absence of GST-hMIER1α, and hMIER1β (Figure 3.4). To determine the effect of hMIER1α and hMIER1β on ERβ DNA binding in the presence of estrogen, the *in vitro* translated ERβ was treated with 10^{-8} M estrogen or an equivalent volume of vehicle. The protein was then incubated with GST, GST-hMIER1α, or GST-hMIER1β. Incubation with GST alone acted as a control to show that the GST portion of the proteins would not disrupt the ability of ERβ to bind its consensus ERE. To ensure the ERβ band was specific, it was competed with unlabelled probe in the presence and absence of estrogen (Figure 3.4, lanes 3 and 4).

As shown in Figure 3.4, both hMIER1α (lanes 5 and 6) and hMIER1β (lanes 7 and 8) appear to disrupt the ability of ERβ to bind DNA in the presence and absence of estrogen. In the lanes treated with hMIER1α or hMIER1β, it is interesting to note that an additional larger complex appears. The antibodies available for hMIER1 did not function in a supershift, so it is not certain whether hMIER1 interacts with ERβ and supershifts the complex or if truly disrupts the DNA binding. In the latter, this larger complex may be a result of non-specific binding.

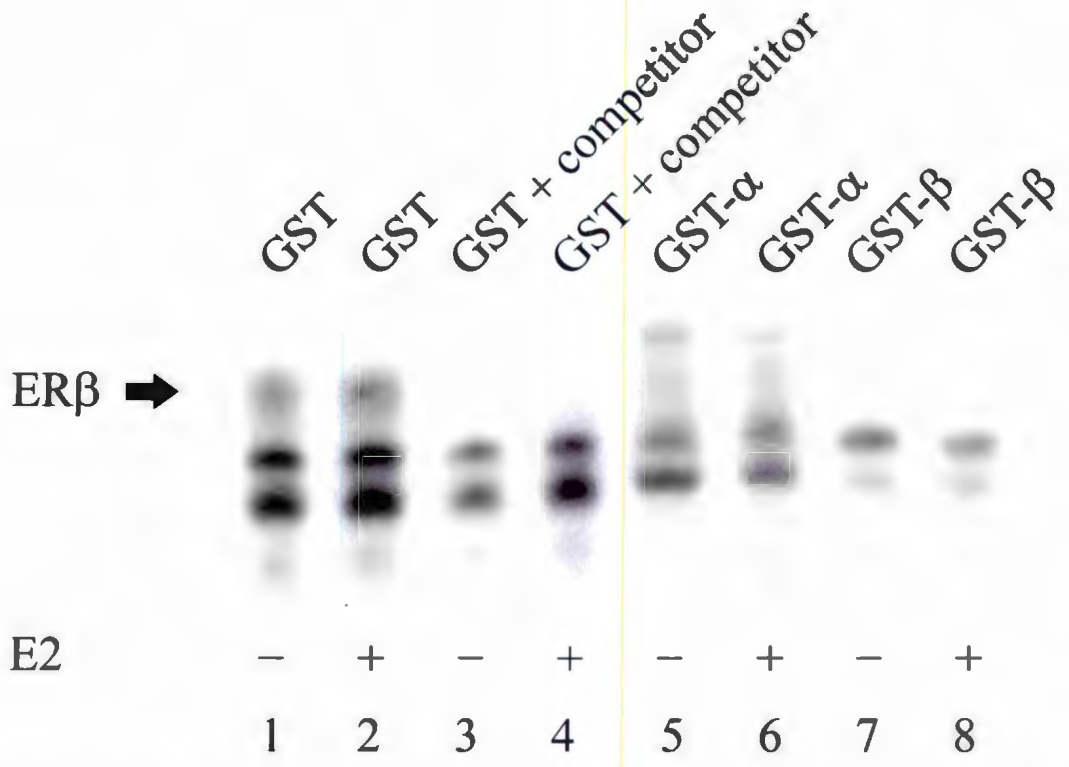

Figure 3.4: GST-hMIER1α (GST-α) and GST-hMIER1β (GST-β) interfere with the ability of ERβ to bind its consensus ERE in the presence of estrogen

Electrophoretic mobility shift assays (EMSAs) were used to investigate whether hMIER1α or hMIER1β would affect the binding of ERβ to the ERE in the absence and presence of estrogen. *In vitro* translated ERβ was incubated with GST alone (lanes 1&2), GST-hMIER1α (lanes 5&6), or GST-hMIER1β (lanes 7&8). Unlabelled ERE probe was used as cold competitor to ensure the specificity of the ERβ band (lanes 3&4). The protein-DNA complexes were resolved through non-denaturing PAGE, and visualized by autoradiography. The position of ERβ is indicated. Results were obtained from three independent experiments and shown is a representative autoradiograph.

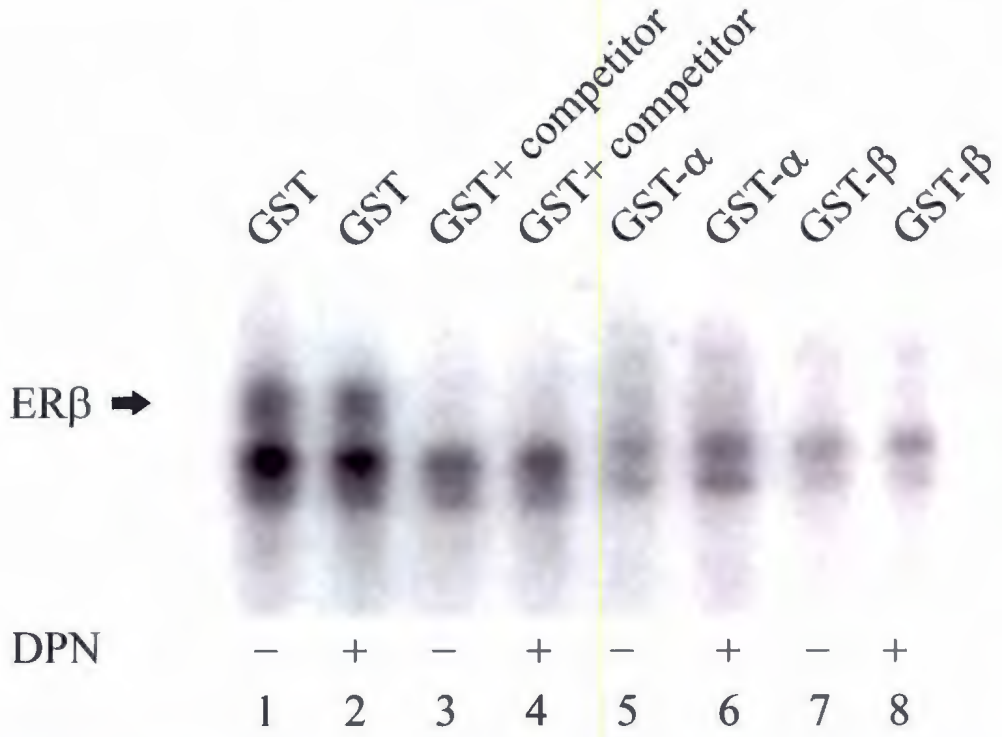

Figure 3.5: GST-hMIER1α (GST-α) and GST-hMIER1β (GST-β) interfere with the ability of ERβ to bind its consensus ERE in the presence of an ERβ specific agonist, DPN.

Electrophoretic mobility shift assays (EMSAs) were used to investigate whether hMIER1α or hMIER1β would affect the binding of ERβ to the ERE in the absence and presence of DPN. *In vitro* translated ERβ was incubated with GST alone (lanes 1&2), GST-hMIER1α (lanes 5&6), or GST-hMIER1β (lanes 7&8). Unlabelled ERE probe was used as cold competitor to ensure the specificity of the ERβ band (lanes 3&4). The protein-DNA complexes were resolved through non-denaturing PAGE, and visualized by autoradiography. The position of ERβ is indicated. Results were obtained from three independent experiments and shown is a representative autoradiograph

The effect of hMIER1α and hMIER1β on ERβ DNA binding was also examined in the presence of an ERβ specific agonist, DPN. The basis for the selectivity of DPN for ERβ appears to be a result of the differences between specific amino acid residues in the ERα and ERβ ligand binding domain. The N-terminal region of the ERβ ligand binding domain through helix 3 and a critical methoinine (Met336) are responsible for the specificity of DPN (Sun et al., 2003). The ERβ protein was incubated with 10^{-8} M DPN or an equivalent volume of ethanol prior to incubation with GST, GST-hMIER1α, or GST-hMIER1β. The ability of ERβ to bind DNA was inhibited by hMIER1α and hMIER1β in both the absence and presence of DPN (Figure 3.5, lanes 5-8).

These results demonstrate that hMIER1α and hMIER1β disrupted the ability of ERβ to bind its consensus ERE, regardless of the presence of ligand. This effect is likely mediated by a region common to both hMIER1α and hMIER1β as both isoforms have the same effect. However, with DPN there is no supershift complex similar to that seen with hMIER1 in the presence of E2 (Figure 3.4, lanes 5-8).

3.2 Effect of hMIER1 on ERE-Driven Transcription in HEK 293 cells.

It has previously been shown that hMIER1α and hMIER1β interact with ERα *in vitro* (Savicky, Masters dissertation). Through GST pulldown assays, it has also been shown the hMIER1α and hMIER1β interact with ERβ in *vitro* (Fifield, Honours dissertation). Given that ERα and ERβ interact with hMIER1 *in vitro*, and that the EMSAs showed that hMIER1α and hMIER1β are capable of affecting the DNA binding of the ER *in vitro*, it was important to determine the functional effect of this interaction on ERE-driven transcription by the ER subtypes. Due to the fact that ERα and ERβ can

be activated in the absence of a ligand and that the cofactor recruitment may differ from that of ligand-dependent activation, assays were performed both in the absence and presence of ligand. Luciferase assays were performed using a 3xERE-TATA-luciferase reporter construct, which contains 3 repeats of an ERE, a TATA box, and a luciferase gene downstream of the ERE. Estrogen receptors recognize and interact with the promoter region of genes that carry one or more copies of a consensus ERE sequence (5'GGTCAnnnTGACC- 3') or variants of this sequence.

Transcription of the ERE results in the production of the enzyme luciferase, that upon substrate binding will produce a light reaction that can be quantified as relative luciferase units (RLU). To determine the effect of hMIER1, two controls were in place, the first control contained only the 3xERE-TATA-Luc to determine if there was any endogenous ER driven expression. The second control contained only the 3xERE-TATA-Luc and the ER subtype in question. Since the CS3+MT vector used in these experiments contains 6 N-terminal repeats encoding the c-myc protein, this control ensured that any effects seen with hMIER1α or hMIER1β were not due to the myc tag itself. The ability of hMIER1α and hMIER1β to regulate transcription can be analyzed by comparing the level of luciferase activity relative to that of ERα with the empty CS3+MT vector. A plasmid containing the reporter β-galactosidase was also transfected into the cells for each assay as a measure of transfection efficiency. After raw luciferase readings were obtained for each lysate, βgal assays were also performed on the samples to determine the transfection efficacy. The raw luciferase data was normalized using the βgal values to account for variability in transfection rates between samples.

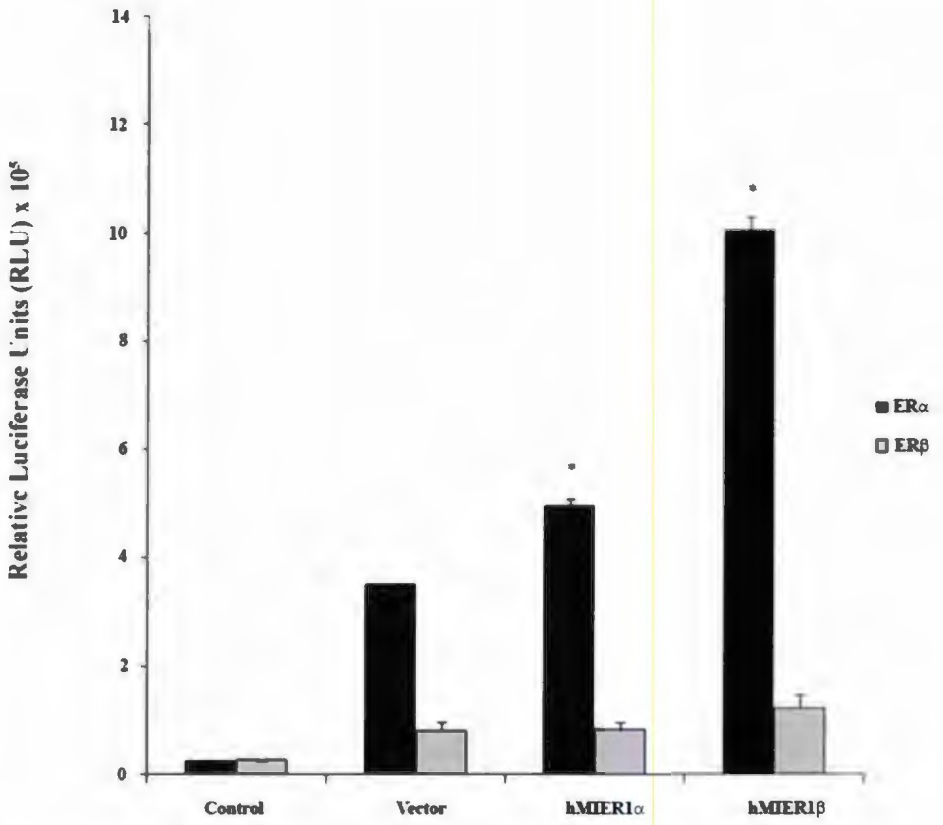

Figure 3.6: hMIER1α and hMIER1β enhance ERα ERE-driven transcription in the absence of ligand.

HEK 293 cells were transfected with 0.5 μg of the 3xERE-TATA-Luc reported plasmid and 0.4 μg of ERα or ERβ and 0.8 μg of myc tagged empty vector, hMIER1α, or hMIER1β. The control samples were transfected with the 3xERE-TATA-luc and empty vector to control for activation by endogenous ERs. Cells were cultured in PRF DMEM in the absence of a ligand, and harvested 48 hours after transfection. The relative luciferase units (RLU) were determined and values normalized to transfection efficiency. Average values and standard error of 7 independent experiments are shown. The Myc empty vector of was compared to hMIER1-α and hMIER1-β, and significance evaluated by a standard t-test.

* represents statistical significance $p<0.05$

3.2.1 The effect of hMIER1 on ERE-driven transcription with ERα and ERβ in the absence of ligand.

When compared to the control vector, both hMIER1α (denoted myc-α) and hMIER1β (myc-β) significantly enhance ERα-activated transcription at the ERE in the absence of ligand (Figure 3.6). hMIER1β appears to be a more potent activator of ERα transcription than hMIER1α at the ERE. With the ERβ, neither hMIER1α nor hMIER1β significantly affect transcription. The relative levels of activation with ERβ reflect the well established fact that ERβ typically is a weaker transcriptional activator than ERα in most cell systems (Pettersson and Gustafusson, 2001). While the ER subtypes are highly conserved in most regions, these results imply that hMIER1α and hMIER1β enhance transcription through a region of the ER specific to ERα, as it has no effect on ERβ transcription.

3.2.2 The effect of hMIER1 on ERE-driven transcription with ERα in the presence of ligand.

To investigate the effects of hMIER1 in the presence of a ligand, luciferase assays were performed as described in section 3.1 with the ERα in the presence of 10^{-8} M estrogen or PPT. The effects of hMIER1α and hMIER1β were examined in comparison to the Myc-tagged empty vector control. A graph of the averages with standard error of three independent experiments is shown in Figure 3.7.

It was found that hMIER1α and hMIER1β have no significant effect on ERα driven transcription at the ERE in the presence of estrogen.

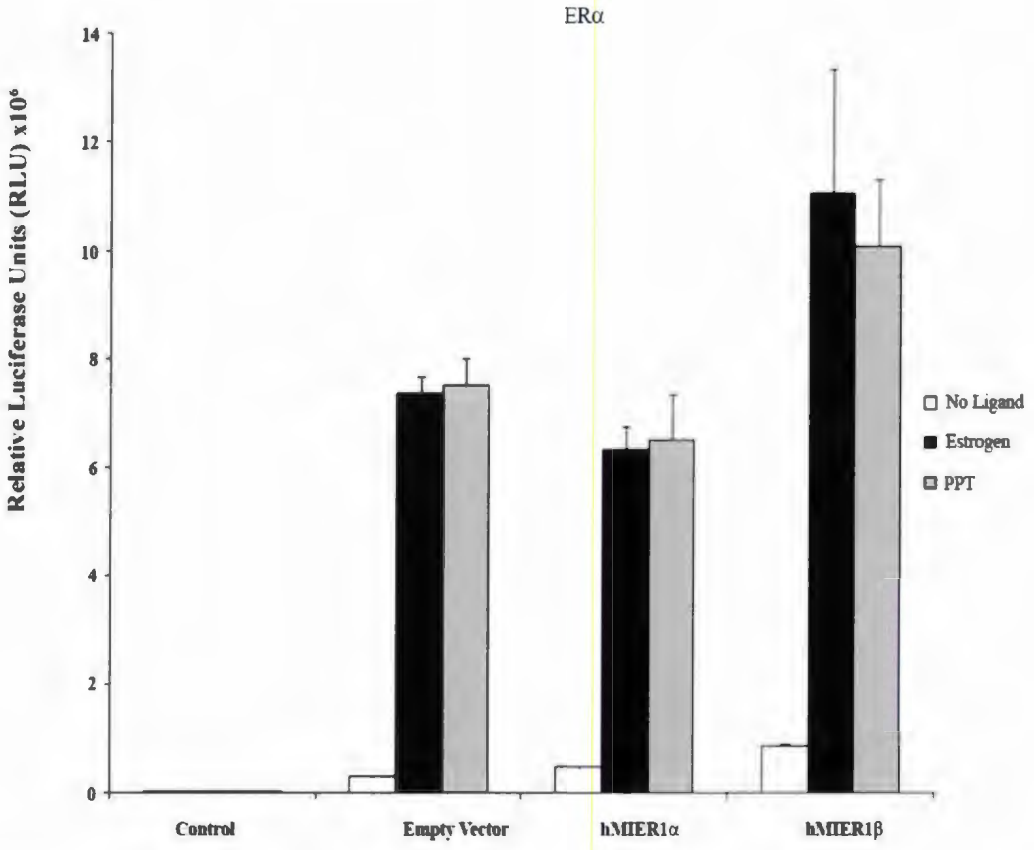

Figure 3.7: hMIER1α and hMIER1β do not significantly affect ERE-driven transcription with ERα in the presence of ligand.

HEK 293 cells were transfected with 0.5 μg of the 3xERE-TATA-Luc reported plasmid and 0.4 μg of ERα, and 0.8 μg of myc tagged empty vector, hMIER1α, or hMIER1β. The control samples were transfected with the 3xERE-TATA-luc and empty vector to control for activation by endogenous ERs. Cells were cultured in PRF DMEM and treated with 10^{-8}M estrogen, PPT or vehicle for 24 hours prior to harvesting. The relative luciferase units (RLU) were determined and values normalized to transfection efficiency. The averages and standard errors of 3 independent experiments are shown. The Myc empty vector was compared to hMIER1α and hMIER1β for each ligand and significance evaluated by a standard t-test.

To investigate the effect of hMIER1 on ERE-driven transcription in the presence of an ERα specific ligand, luciferase assays were also performed in the presence 10^{-8}M PPT. In the presence of PPT, hMIER1α and hMIER1β have no significant effect on ERα driven transcription. These results indicate that hMIER1α and hMIER1β do not affect ERα driven transcription in the presence of ligand.

3.2.3 The effect of hMIER1 on ERE-driven transcription with ERβ in the presence of ligand.

Luciferase assays were performed as described in section 3.1 with the ERβ in the presence of 10^{-8}M estrogen or DPN. The effects of hMIER1α and hMIER1β were examined in comparison to the Myc-tagged empty vector control. A graph of the means with standard error of three independent experiments is shown in Figure 3.8.

In the presence of estrogen, hMIER1α and hMIER1β have no effect on ERβ driven transcription at the ERE.

In the presence of DPN, hMIER1α and hMIER1β have no effect on ERβ driven transcription. These results indicate that hMIER1α and hMIER1β have no effect on ERβ driven transcription in the presence of ligand.

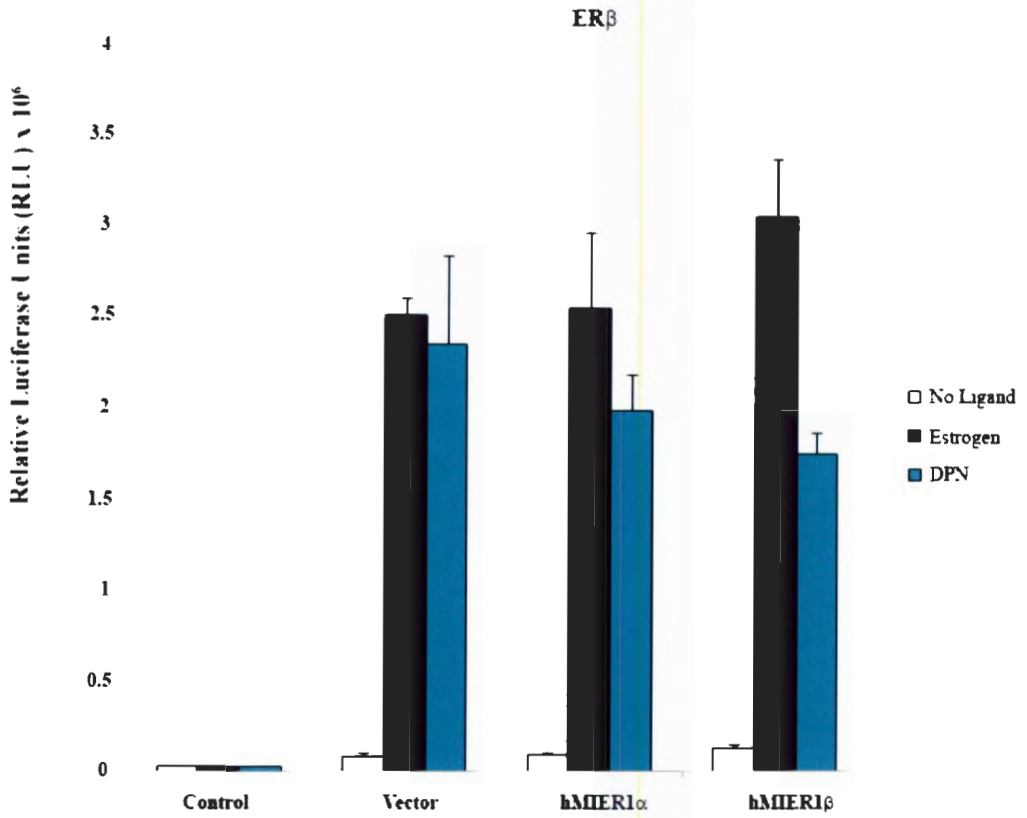

Figure 3.8: hMIER1α and hMIER1β do not significantly affect ERE-driven transcription with ERβ in the presence of ligand.

HEK 293 cells were transfected with 0.5 μg of the 3xERE-TATA-Luc reported plasmid and 0.4μg of ERβ and 0.8 μg of myc tagged empty vector, hMIER1α, or hMIER1β. The control samples were transfected with the 3xERE-TATA-luc and empty vector to control for activation by endogenous ERs. Cells were cultured in PRF DMEM and treated with 10^{-8}M estrogen, DPN or vehicle for 24 hours prior to harvesting. The relative luciferase units (RLU) were determined and values normalized to transfection efficiency. The averages and standard error of 3 independent experiments are shown. The Myc empty vector was compared to hMIER1α and hMIER1β for each ligand and significance evaluated by a standard t-test.

4. Discussion

Nuclear hormone receptors are essential for normal cell growth, differentiation and homeostasis. These proteins play an important role in cell proliferation as well as apoptosis, and excessive activation of their associated signalling pathways is implied in serious diseases such as cancer (reviewed in Singh and Kumar, 2005). Upon hormone binding, the receptors mediate the hormone effects through complex signalling that results in downstream gene expression or repression. However, in the case of hormone receptors such as ER, the pathways become especially complex as the receptor also exhibits hormone-independent signalling. The ER is capable of genomic (nuclear) and non-genomic (membrane and cytoplasmic) signalling which results in a wide range of ER-mediated effects. The genomic effects of ER involve receptor dimerization, nuclear localization, and binding of coregulatory complexes to estrogen response elements. This method of ER signalling is well characterized and may be ligand-dependent or independent. The majority of ligand-independent genomic signalling can be attributed to peptide growth factors such as, epidermal growth factor (EGF) or insulin-like growth factor 1 (IGF-1) (reviewed in Nilsson *et al.*, 2001). The non-genomic effects of ER are a mechanism through which estrogen can rapidly and transiently manifest its activity in a cell (Pietras and Márquez-Gárban, 2007). These effects also account for 1/3 of estrogen-induced genes that lack functional estrogen response elements. In response to their respective ligands, nuclear hormone receptors initiate non genomic signalling from the cell membrane or cytoplasm through interaction with numerous protein-kinase cascades.

The presence of an acidic activation, ELM2, and SANT domains, along with the proline-rich motif imply that hMIER1 is capable of numerous types of protein-protein interactions. This in combination with the LXXLL motif in hMIER1α further suggests that hMIER1 may play a role as a cofactor of nuclear hormone receptors such as ER. Previously hMIER1α has been characterized as an ERα cofactor (McCarthy *et al.*, 2008, in press) and the purpose of this study was to further characterize hMIER1 as an ER cofactor by investigating its interaction with and functional effects on ERα and ERβ.

4.1 *In vitro* interaction of hMIER1 with both ERα and ERβ.

Previous work using GST pull down assays have demonstrated that both hMIER1α and hMIER1β interacted with ERα and ERβ *in vitro* (Fifield, Honours dissertation). These results imply that while the LXXLL motif present in hMIER1α may play a role, it is not fully responsible for the interaction of ER with hMIER1. It is more likely that one of the domains in the common region of hMIER1α and hMIER1β is responsible for the interaction, as ERα and ERβ bind both hMIER1α and hMIER1β.

In a previous study with ERα and hMIER1, it was determined that the SANT domain and the amino acids C-terminal to this domain were critical for ERα interaction (Savicky, Masters dissertation). The SANT domain has been associated with both HAT and HDAC activity in other proteins (Boyer *et al.*,2004), therefore hMIER1 has the potential for both transactivation and transrepression. Future investigation could include determining if this domain is involved with the interaction of hMIER1 with ERβ.

Studies to investigate whether ERβ physically interacted with hMIER1α and hMIER1β *in vivo* were attempted; however, due to lack of efficient ERβ antibodies, the

results were inconclusive. Therefore, future studies would be required to characterize whether or not there is an interaction between endogenous hMIER1 and endogenous ERβ.

4.2 Effect of hMIER1 on ERE-binding of ERα and ERβ

Since previous studies have shown that hMIER1α and hMIER1β interact with the ER *in vitro*, and that hMIER1α also causes repression of ERα driven transcription at a promoter containing a consensus ERE, the effect of hMIER1 on the ability of ER to bind its consensus ERE was studied. Electrophoretic mobility shift assays (EMSAs) were used to determine the effect that hMIER1 might have on ER-DNA binding at a consensus ERE.

The present results showed that hMIER1α inhibited ERα and ERβ DNA binding in both the absence and presence of ligand. hMIER1β also inhibited the DNA binding of ERβ in the absence and presence of ligand, but has no effect on ERα-DNA binding. Taken together, these results implied that the interaction of hMIER1α with the ER subtypes was distinctly different from that of hMIER1β.

It is interesting to note that the non-specific bands seen in lanes 3 and 4 of all EMSA figures, were not competed out with unlabelled ERE, but appeared to be competed out in the presence of GST-hMIER1 proteins. This effect was more prominent in some conditions compared to others, and was always a variable result. The non-specific bands could be caused by proteins in the reticulocyte lysate that non-specifically bound to the ERE, but in the presence of a large protein such as hMIER1, these proteins might simply bind preferentially to hMIER1 instead of the ERE sequence. It is not

believed that this is a significant result, but further studies with controls containing the reticulocyte lysate and hMIER1 could be performed to confirm this.

In the EMSA with ERβ and hMIER1 in the absence or presence of estrogen (Figure 3.4, lanes 5-8) there is an additional band seen that appears to be larger than the ER-ERE complex. This band did not appear in the lanes with ERβ alone, and therefore the specificity cannot be determined by looking at the lanes with excess probe competition (lanes 3 and 4). Unfortunately, the EMSA results did not provide much insight into the mechanism by which hMIER1 may be exerting its functional effects. In a cell, hMIER1 may affect ER driven transcription in more than one way. It may bind the ER directly and sequester it away from its consensus ERE, leading to a decrease in transcription or it may bind the ER on the DNA and act as a transactivator or transrepressor. In the EMSAs, what appears as inhibition of DNA binding may be a result of something entirely different. It is possible that hMIER1 binds the ER and the labelled ERE which results in the supershifted band seen in the ERβ figure. *In vitro* synthesized ERβ has been shown to have a much lower affinity for the ERE and a more diffuse binding pattern than that of ERα and longer exposures were required to get a clear autoradiography picture. Perhaps with different exposures this complex may also show up with ERα. Or, if hMIER1 actually prevents the ER subtypes from binding the ERE, the larger complex may also have been a result of non-specific binding to some component of the reticulocyte lysate product or the GST fusion protein. Further studies were attempted by using hMIER1 antibodies to supershift both the ERα-ERE and ERβ – ERE complexes, but the antibodies available for hMIER1 were not suitable for supershift.

Additional studies could be performed to determine if this complex is present with ERα, and if this complex will be specifically competed with excess unlabelled ERE probe.

It is clear from the EMSAs that ERα and ERβ behave differently in their ability to bind DNA in the presence of hMIER1. The one consistent effect seen across all experiments is the disruption of DNA binding by hMIER1α with both receptor subtypes either in the absence or presence of ligand.

4.3 Effect of hMIER1 on ERE-driven transcription in the absence of ligand

To determine if the EMSA results would reflect the effect of hMIER1 *in vivo* functional assays measuring transcription from a consensus ERE were performed. The results from seven independent experiments in the absence of ligand revealed the hIMER1α and hMIER1β significantly enhanced ERE-driven transcription with the ERα homodimer (Figure 3.6). Neither hMIER1α nor hMIER1β had a significant effect on the ERβ homodimer in the absence of ligand.

The functional difference between the transactivation ablilities of ERα and ERβ appear to be due to differences in the AF-1 domain, the ERα AF-1 domain autonomously initiates transcription when fused to a GAL4 DNA binding domain, but the ERβ AF-1 does not (reviewed in Pettersson and Gustafsson, 2001). This difference in the AF-1 domain, which is responsible for ligand-independent interaction, results in differences in cofactor recruitment and transcriptional activity in the absence of ligand for ERα and ERβ. For example, ERβ, in contrast to ERα, recruits SRC-1 in a ligand-independent manner, and transcriptional activation is dependent upon phosphorylation of critical serine residues in the A/B domain. Perhaps through a similar mechanism hMIER1

appears to be recruited to ERα, but not ERβ, as a coactivator in the absence of ligand. Future studies would include determining which domain of the ER subtypes is responsible for interaction with hMIER1.

Since ERα and ERβ have been shown to form heterodimers, and these heterodimers are capable of recruiting ER cofactors and stimulating ERE-driven transcription, future studies could be performed to investigate the effects of hMIER1α and hMIER1β with the ERα/β heterodimer. Previous studies have shown that with an ERE-TATA similar to the one used in these experiments, the ERα partner dictates the activity of the ERα/β heterodimer in ERE-dependent transcription (Li *et al.*, 2004). Further studies by this group were performed with constructs containing enhancer promoter regions of estrogen responsive genes such as pS2. These studies revealed that when an ERβ fusion receptor homodimer, which does not heterodimerize, is expressed in tandem with a fusion ERα homodimer in HeLa cells, increasing amounts of ERβ resulted in a repression of transcription from the pS2 estrogen responsive promoters. However, when the ERα/β heterodimer was expressed with the ERα fusion homodimer, it augmented the ERE-dependent activity, indicating it worked in synergy with the ERα partner. Based on the results of this previous study, further studies could be performed with hMIER1 and the ER subtypes in the presence of more complex ERE promoters like pS2 in the absence or presence of ligand.

The mechanism by which hMIER1 may enhance ERα transcription in the absence of ligand is unclear. Prior to ligand binding, the ER is usually bound by chaperone complexes and ligand binding results in conformational change to release these

complexes and allow translocation to the nucleus, dimerization and cofactor recruitment (reviewed in Heldring *et al.*, 2007). In the ligand-independent pathway phosphorylation of key residues in the ligand binding domain and the AF-1 domain in the absence of ligand result in coactivator recruitment and conformational change. Perhaps the un-liganded conformation of ERα is more favourable to hMIER1 binding, resulting in ligand-independent recruitment. However, it has already been described that hMIER1 has the potential for many different protein-protein interactions. It is also possible that in the absence of ligand, hMIER1 may sequester corepressors away from the ER and enhance transcription by this mechanism. Further studies could be done to determine the complex in which hMIER1 is present on the ERE and whether hMIER1 itself directly interacts with the ER complex. It would also be of importance to repeat the previously described assays in different cell types to determine if the current results are cell type specific.

4.4 Effect of hMIER1 on ERE-driven transcription in the presence of ligand

To determine the effects of hMIER1 in the ligand-dependent pathway, HEK 293 cells were incubated with estrogen, PPT or DPN 24 hours prior to harvesting. The ER subtypes have similar affinity for estrogen, while PPT is an ERα specific agonist, and DPN is an ERβ specific agonist. The results from these experiments showed the hMIER1α had no ligand-dependent effects on ERα or ERβ.

The ligand binding domain of the ER subtypes is made of 12 α-helices denoted H1-H12. Upon ligand binding, H12 normally forms the AF-2 pocket by folding against H3, H5/6 and H11 (Hall and McDonnell, 2005). The position of H12 is different based on

the structure and function of the ligand bound to the ER. Studies indicate that in antagonist binding H12 occupies the coactivator binding surface, blocking coactivator recruitment, whereas in agonist bound ER, H12 is repositioned to expose a hydrophobic groove to which coactivators can bind (Hall and McDonnell, 2005). In particular, the LXXLL motif in cofactors provides a structural motif that will bind the hydrophobic groove in the ER ligand binding domain with high affinity. Most cofactors with LXXLL motifs are coactivators, but some corepressors have also been found to use this motif to bind the active ER and repress ER transcription. It is possible that the position of the H12 in the ligand binding domain plays some part in the differential binding of hMIER1 in the absence or presence of ligand.

The ligands estrogen, PPT and DPN, all bind to the ER ligand binding domain and use the AF-2 to determine specificity and function. However, these ligands do not bind in the same manner and they do not form identical conformations when bound to the ER. For example, the specificity of PPT and DPN for ERα and ERβ, respectively, is due to differences in a single amino acid residue H6 of the ligand binding pocket (Stauffer *et al.* 1999; Sun *et al.*, 2003). While overall the ligand binding domain is poorly conserved between the receptors at 55% identity, the residues that line the ligand binding pocket and interact with ligand are nearly identical. The conformation change in the ligand bound state of the ER appears to be significant enough to block or suppress the effects of hMIER1α and hMIER1β interaction.

It is also of importance to note that the ligand treatments were for a 24 hour period, perhaps a more standard 48 hour ligand treatment would have produced an effect

with hMIER1 and the ER subtypes. The 24 hour incubation period was chosen due to the fact that previous studies assayed reporter gene activity 24 hours after treatment with PPT and DPN (Sun *et al.*, 2002; Sun *et al.*, 2003; Harrington *et al.* 2003). The estrogen incubation was also 24 hours to provide comparable results, despite the fact that experiments in this laboratory have consistently been performed with a 48 hour estrogen treatment. Further studies should be performed using a time course ligand incubation of 24, 48, and 72 hours to determine the cumulative effects of hMIER1 on ERE-driven transcription in the presence of ligand.

4.5 Conclusions and Future Studies

hMIER1 has been found to be differentially regulated in breast carcinoma tissues and cells lines, implying it may play a role in the normal growth and development of the breast (Paterno et al., 1998). hMIER1 is structurally similar to other well characterized transcriptional regulatory proteins known to be associated with the metastatic state such as the metastasis associated protein, MTA1. Like hMIER1, MTA1 contains an ELM2 domain, a SANT domain, acidic activation domains, and a proline rich region (Toh et al., 1995; Solari et al., 1999; Nicholson et al., 2003). MTA1 or the MTA1 related protein, MTA2, have been found to be involved in chromatin remodelling via the nucleosome remodelling histone deacetylase complex (NuRD). MTA1 has been found to be over expressed in both breast carcinoma tissues and cell lines (Nicholson et al., 2003). Furthermore, MTA1 has been found to be a potent corepressor of the ER via HDAC activity providing a potential mechanism by which it may be involved in the neoplastic

state (Mazumdar et al., 2001). Further investigation into the role that hMIER1 has on estrogen signalling and function may help clarify the role that hMIER1 plays in estrogen related cancers.

In this study the interaction of hMIER1 with the ER and its functional effects was investigated. Characterization of the hMIER1-ER interaction could provide insight into the role that hMIER1 may play in development of normal and abnormal breast tissue. The assays used to characterize the hMIER1-ER interaction in this study gave some conflicting results. In the absence of ligand, hMIER1α and hMIER1β enhance ERα transcription, presumably through the physical interaction represented in previous GST pulldown assays. Yet the interaction of hMIER1α and hMIER1β with the ERβ *in vitro* is not functionally represented *in vivo*, as hMIER1 has no effect on ERβ transcription. The *in vitro* EMSA results imply that, in both the presence and absence of ligand, hMIER1α disrupts ER-ERE DNA binding with both ER subtypes, and hMIER1β also disrupts ERβ DNA binding. These results indicate that hMIER1 may repress transcription by interfering with ER-ERE binding. However, this is not represented *in vivo*, as hMIER1α and hMIER1β enhance transcription in the absence of ligand but have no effect in the presence of ligand.

As previously discussed, hMIER1 could be causing a supershift of the ER band and not actually interfering with the ability of ER to bind its consensus ERE. However, if hMIER1 does not supershift the ER complex and the result seen is actually due to a disruption of DNA binding, it is important to note that *in vitro* DNA binding can also be

very fastidious and slight changes that do not quite match the *in vivo* conditions could cause the ER to be blocked from binding the ERE.

Also, *in vitro* synthesized proteins may lack important proteins for ER-DNA interaction. The ER-DNA complex is an intricate network of cofactors and stabilizer proteins (reviewed in Petersson and Gustafsson, 2001). As previously discussed, the necessary cointegrator proteins may be endogenously present in a cell system, but absent in the reticulocyte lysate used to synthesize proteins *in vitro*. Studies were attempted using nuclear extracts of breast cancer T47D cells designed with a tetracycline inducible system for hMIER1. However, due to difficulties optimizing the ER subtype expression and hMIER1 induction they could not be completed in this time frame. Future studies should be performed using nuclear extracts specifically expressing the ER subtypes and hMIER1α or hMIER1β to better characterize the effects on DNA in the absence or presence of ligand in a more *in vivo* like system.

Also, the *in vivo* effects of hMIER1 may also be a result of its ability to sequester specific corepressors away from the ER-DNA complex, resulting in enhanced transcription without direct interaction with ER. If hMIER1 does not interact directly with the ER, the *in vitro* assays will be unable to accurately reflect the mechanism by which is exerts its effects on the ER.

Another possible explanation for the discrepancies could involve the distinct C-termini of hMIER1α and hMIER1β which indicate alternate functions and subcellular localizations for the two isoforms. hMIER1α contains an LXXLL motif typical of cofactors of nuclear hormone receptor, in contrast hMIER1β contains a consensus nuclear

localization signal (NLS). It has been found that hMIER1β is targeted to the nucleus in NIH 3T3 cells, whereas hMIER1α, which lacks an NLS, remains cytoplasmic (Paterno *et al.*, 2002).This does not exclude the possibility that hMIER1α is transported to the nucleus through its interaction with other proteins such as nuclear hormone receptors.

Since previous studies show that hMIER1β may already be present in the nucleus, it may be more accessible to the ER. Ligand bound ER subtypes may be in a conformation unfavourable to hMIER1β interaction and hMIER1α translocation. Future studies involving immunocytochemistry and confocal microscopy are necessary to determine the subcellular localization of hMIER1 in both the absence and presence of ligand in different cell types. Also, the determination of whether hMIER1 requires ER for translocation would be of importance.

Taking all the results into consideration, this study successfully confirms that hMIER1 interacts with both forms of the ER *in vitro*, and that it acts as a transactivator in of ERα-ERE driven transcription in the absence of ligand. This study helps characterize both hMIER1α and hMIER1β as novel cofactors of ERα. Given that hMIER1 is differentially expressed in breast cancer tissues and is similar to other known transcription factors involved in breast cancer, such as MTA proteins it is likely that hMIER1 may provide novel insight into estrogen-related cancers. Further studies with hMIER1α and hMIER1β may have significant implications in the development of novel treatments for estrogen related diseases such as breast cancer.

References

Aasland, R.(1996). The SANT domain: a putative DNA-binding domain in the SWI-SNF and ADA complexes, the transcriptional co-repressors N-CoR and TFIIIB. Trends Biochem. Sci. 21, 87-88

Becker,W.M., Kleinsmith,L.J., and Hardin, J. (2003). The world of the cell. San Fransico, CA. Pearson Education Inc.

Boyer, L.A. (2002). Essential role for the SANT domain in the functioning of multiple chromatin remodelling enzymes. Molec. Cell. 10, 935-942

Boyer, L.A., Latek, R.R., and Peterson, C.L. (2004). The SANT domain: a unique histone-tail-binding molecule? Nat. Rev. Mol. Cell Biol. 5 (2), 158-163

Chang, E.C., Frasor, J., Komm, B., and Katzenellenbogen, B.S. (2006). Impact of estrogen receptor β on gene networks regulated by estrogen receptor α in breast cancer cells. Endocrinology 147, 4831-4842

Cheng G., Li, Y., Omoto, Y., Wang, Y., Berg, T., Nord, M., Vihko, P., Warner, M., Piao, Y-S., and Gustafsson, J-A. (2004). Differential regulation of ERα and ERβ in primate mammary gland. J. Clin. Endocr Metab. 90, 435-444

Cowely, S.M., Hoare, S., Mosselman S., and Parker, M.G. (1997). Estrogen receptors α and β form heterodimers on DNA. J. Biol. Chem. 272 (32), 19858-19862

DeNardo,D.G., Cuba, V.L., Kim, H., Wu, K., Lee, A.V., and Brown, P.H. (2007) Estrogen receptor DNA binding is not required for estrogen-induced breast cell growth. Mol. Cell. Endocriol. 277, 13-25

Ding, X.F, Anderson, C.M., Ma, H., Hong, H., Uht, R.M., Kushner, P.J., and Stallcup, M.R. (1998). Nuclear Receptor-binding sites of coactivators glucocorticoid receptor 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities Mol. Endocrinol. 12, 302-313

Ding, Z., Gillespie, L.L, and Paterno, G.D. (2003). Human MI-ER1 alpha and beta function as transcriptional repressors by recruitment of histone deacetylase 1 to their conserved ELM2 Domain. Mol Cell Biol 23,250-258

Ding, Z. Gillespie, L.L, Mercer, F.C., and Paterno, G.D. (2004). The SANT domain of human MI-ER1 interacts with Sp1 to interfere with GC box recognition and repress transcription from its own promoter. J. Biol. Chem. 279, 28009-28016

Doisneau-Sixou, S.F., Sergio, C.M., Carrol, J.S., Hui,R., Musgrove, E.A., and Sutherland, R.L. (2003). Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells. Endocrine-Related Cancer 10, 179-186

Escande, A., Pillon,A., Servant, N., Cravedi, J.P., Larrea, F., Muhn, P., Nicholas, J.C., Cavaillès, V., and Balaguer, P. (2006). Evaluation of ligand selectivity using reporter cell lines stably estrogen receptor alpha or beta. Biochem. Pharmacol. 71, 1459-1469

Fifield, H.A. (2005). Interaction of a transcriptional regulatory factor, hMI-ER1, with the estrogen receptor β. Honors Dissertation.

Förster, C., Mäkelä, S., Wärri, A., Kietz, S., Becker, D., Hultenby, K., Warner, M., and Gustafsson, J-A. (2002). Involvement of estrogen receptor β in terminal differentiation of mammary gland epithelium. Proc. Natl. Acad. Sci. U.S.A. 99,15578-15583

Gal-Yam, E.N., Saito, Y., Egger, G., and Jones. P.A. (2007). Cancer epigenetics: modifications, screening and therapy. Annu. Rev. Med.59, 491-504

Hall, J.M. and McDonnell, D.P. (2005). Coregulators in nuclear estrogen receptor action. Mol. Interventions 5(6), 343-357

Hansein, B., Eckner, R., DiRenzo, J., Halachmi, S., Liu, H., and Searcy, B., (1996). p300 is a component of an estrogen receptor coactivator complex. Proc. Natl. Acad. Sci. USA. 93, 11540-11545

Hanstein, B., Djahansouzi, S., Dall, P., Beckmann, M.W., and Bender, H.G. (2004). Insights into the molecular biology of the estrogen receptor define novel therapeutic targets for breast cancer. Eur. J. Endocrinol. 150, 243-255

Harrington, W.R., Sheng, S., Barnett, D.H., Petz, L.N., Katzenellenbogen, J.A and Katzenellenbogen, B.S. (2003). Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression. Mol. Cell. Endocrinol. 206, 13-22

Heery, D.M., Kalkhoven, E., Hoare,S. and Parker, M.G. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387,733-736

Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M., Ström, A., Treuter, E., Warner, M., and Gustafsson, J.A. (2007). Estrogen receptors: how do they signal and what are their targets. Physiol. Rev. 87, 905-931

Hodawadekar, S.C., and Marmorstein, R. (2007) Chemistry of acetyl transfer by histone modifying enzymes: structure, mechanism and implications for effector design. Oncogene 26, 5528-5540

Hopp, T., Weiss, H., Parra, I., Cui, Y. Osborne, C., and Fuqua,S., (2004). Low levels of estrogen receptor beta predict resistance to tamoxifen therapy in breast cancer. Clinical Cancer Research 10, 7490-7499

Katzenellenbogen, B.S., and Katzenellenbogen, J.K. (2000). Review Estrogen receptor transcription and transactivation estrogen receptor alpha and estrogen receptor beta: regulation by selective estrogen receptor modulators and importance in breast cancer. Breast Cancer Res. 2, 335-344

Kenney, N.J., Bowman, A., Korach, K.S., Barret, J.C., and Salomon D.S., (2003). Effect of exogenous epidermal-like growth factors on mammary gland development and differentiation in the estrogen receptor alpha knockout (ERKO) mouse. Breast Cancer Res. Treat. 79, 161-173

Kim, K., Barhoumi,R., Burghardt, R., and Safe.S. (2005). Analysis of estrogen receptor α-Sp1 interactions in breast cancer cells by fluorescence resonance energy transfer. Mol. Endocriol. 19(4), 843-854

Kuiper, G.G.J.M, Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J-A. (1996). Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. USA 93, 5925-5930

Kushner, P.J., Agard, D.A., Greene, G.L., Scanlan, T.S, Shaiu, A.K., Uht, R.M., and Webb, P. (2000). Estrogen receptor pathways to AP-1. J. Steroid Biochem. Mol. Biol. 74 311-317

Lavinsky, R.M., Jepsen, K., Heinzel, T., Torchia, J., Mullen, T., Schiff, R.,Del-rio, A.L., Ricote, M., Ngo, S., Gemsch, J., Hilsenbeck, S.G., Osborne, C.K., Glass, C.K., Rosenfeld, M.G., and Rose, D.W. (1998) Diverse signalling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. Proc. Natl. Acad. Sci. USA. 95, 2920-2925

Li, X., Huang, J., Yi, P., Bambara, R.A., Hilf, R. and Muyan, M. (2004). Single-chain estrogen eeceptors (ERs) reveal that the ERα/β heterodimer emulates functions of the ERα dimer in genomic estrogen signaling pathways. Mol. Cell. Biol. 24(17), 7681-7694

Li, S.S-C. (2005). Specificity and versatility of SH3 and other proline-recognition domains: structural basis and implications for cellular signal transduction. Biochem. J.390, 641-653

Matthews, J., and Gustafsson, J.A. (2003). Estrogen signalling: a subtle balance between ERα and ERβ. Mol. Interventions 3(5), 281-292

Marino,M., Acconcia, F. and Ascenzi, P. (2005) Estrogen receptor signalling: Bases for drug actions. Curr. Drug Targets Immune Endocr. Metabol. Disord. 5, 305-314

Mazumdar, A., Wang, R.A., Mishra, S.K., Adam, L., Bagheri-Yarmand, R., Mandal, M., Vadlamudi,R.K., and Kumar, R. (2001) Transcriptional repression of oestrogen receptor by metastasis-associated protein 1 corepressor. Nat. Cell. Biol. 3, 30-37

McCarthy, P.L. (2008). Changes in subcellular localisation of MI-ER1 alpha, a novel oestrogen receptor-alpha interacting protein, is associated with breast cancer progression. Br. J. Cancer 99(4) 639-646

McDonnell, D.P. (1999). The molecular pharmacology of SERMs. Trends Endocrinol. Metab. 10, 301-311

Melcher, K. (2000). The strength of acidic activation domains correlates with their affinity for both transcriptional and non-transcriptional proteins. J. Mol. Biol. 301, 1097-1112

Meyers, M.J., Sun, J., Carlson, K., Marriner, G.A., Katzenellenbogen, B.S, and Katenellenbogen, J.A. (2001). Estrogen receptor β potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. J. Med.Chem. 44, 4230-4251

Moggs, J.G. and Orphanides, G. (2001). Estrogen receptors: orchestrators of pleiotropic cellular responses. EMBO reports 2(9), 775-781

Nicolson, G.L., Nawa,A., Toh, Y., Taniguchi, S., Nishimori, K., and Moustafa, A. (2003). Tumor metastasis associated human *MTA1* gene and its MTA1 protein product: role in epithelial cancer cell invasion, proliferation and nuclear regulation. Clin. Exp. Metastasis 20, 19-24

Nilsson, S., Mäkelä, S., Treuter,E., Tujague, M., Tomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., and Gustafsson, J. (2001). Mechanisms of Estrogen Action. Physiol. Rev. 81, 1535-1565

Pace, P., Taylor, J., Suntharalingam, S., Coombes, R.C., and Ali, S. (1997). Human estrogen receptor β binds DNA in a manner similar to and dimerizes with estrogen receptor α. J. Biol. Chem. 272 (41), 25832-25838

Paterno, G.D., Li, Y., Luchmann, H.A., Ryan, P.J., and Gillespie, L.L. (1997). cDNA cloning of a novel , developmentally regulated immediate early gene activated by fibroblast growth factor and encoding a nuclear protein. J. Biol. Chem. 10, 25591-25595

Paterno, G.D, Mercer, C., Chayter, J.L., Yang, X., Robb, J.D., and Gillespie, L.L. (1998). Molecular cloning of human *er1* cDNA and its differential expression in breast tumours and tumor-derived cell lines. Gene 222, 77-82

Paterno, G.D., Ding, Z. Lew, Y., Nash, G.W., Mercer, C.F., and Gillespie, L.L. (2002). Genomic organization of the human mi-er1 gene and characterization of alternatively spliced isoforms: regulated use of a facultative intron determines subcellular localization. Gene 295, 79-85

Pettersson, K., and Gustafsson, J.A. (2001). Role of estrogen receptor beta in estrogen action. Annu. Rev. Physiol. 63, 165-192

Pietras,R.J., and Márquez-Gárban, D.C. (2007). Membrane associated estrogen receptor signalling pathways in human cancers. Clin. Cancer. Res. 13(16) 4672-4676

Post, J., Gillespie, LL. and Paterno, G.D. (2001). Nuclear localization signals in the Xenopus FGF embryonic early reponse 1 protein. FEBS Lett. 502, 41-45

Roger, P., Sahla, M.E., Mäkelä, S., Gustafsson, J-A., Baldet, P., and Rochefort, H. (2001). Decreased expression of estrogen receptor β protein in proliferative preinvasive mammary tumours. Cancer Res. 61, 2537-2541

Safe, S.(2001). Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. Vitam. Horm. 62, 231-252

Savicky, M.W. (2004) Characterization of human mesoderm induction-early response 1 (hMI-ER1) as a nuclear hormone receptor cofactor. Masters of Science, Thesis.

Saville, B., Wormke, M., Wang, F., Nguyen, T., Enmark, E., Kuiper, G., Gustafsson, J.A., and Safe, S. (2000). Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation of GC-rich (Sp1) promoter regions. J. Biol. Chem. 275(8), 5379-5387

Singh, R.R. and Kumar, R. (2005). Steroid hormone receptor signalling in tumorigenesis. J. Cell. Biochem. 96, 490-505.

Solari, F., Bateman, A., and Ahringer J. (1999). The *Caenorhabditis elegans* genes *egl-27* and *egr-1* are similar to MTA1, a member of a chromatin regulatory complex, and are redundantly required for embryonic patterning. Development 126, 2483-2494

Smith, C.L., Nawaz,Z. and O'Malley, B.W. (1997).Coactivator and corepressors regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxtamoxifen. Mol. Endocrinol. 11(6), 657-666

Stauffer, S.R., Coletta, C.J., Tedesco, R., Nishiguchi, G., Carlson, K., Sun, J., Katzenellenbogen, B.S and Katzenellenbogen, J.A. (2000). Pyrazole ligands: structure - affinity /activity relationships and estrogen receptor-α-selective agonists. J. Med. Chem. 43, 4934-4947

Struhl, K. (1987). Promoters, activator proteins, and the mechanism of transcriptional initiation in yeast. Cell 49, 295-297

Suen, C.S., Berrodin, T.J., Mastroeni, R., Cheskis, B.J., Lyttle, C.R. and Frail, D.E. (1998) A transcriptional coactivator, steroid receptor coactivator-3, selectively augments steroid receptor transcriptional activity. J. Biol. Chem. 273(42), 27645-27653

Sun, J., Huang, Y.R., Harrington,W.R., Sheng, S., Katzenellenbogen, J.A, and Katzenellenbogen, B.S. (2002). Antagonists selective for estrogen receptor alpha. Endocrinology 143(3), 941-947

Sun, J., Baudry, J., Katzenellenbogen, J.A, and Katzenellenbogen, B.S. (2003). Molecular basis for the subtype discrimination of the estrogen receptor-β-selective ligand, diarylpropionitrile. Mol. Endocrinol. 17(2), 247-258

Teplitsky Y., Paterno, G.D. and Gillespie, L.L. (2003). Proline^{365} is a critical residue for the activity of XMI-ER1 in *Xenopus* embryonic development. Biochem. Biophys. Res. Commun. 308, 679-683

Toh,Y., Pencil, S.D. and Nicolson, G.L. (1994) A novel candidate metastasis associated gene, *mta1*, differentially expressed in highly metastatic mammary adenocarcinoma cell lines. J. Biol. Chem 269(37), 22958-22963

Vanacker, J.M., Pettersson, K, Gustafsson, J.A, and Laudet,V. (1999). Transcriptional targets shared by estrogen related receptors (ERRs) and estrogen receptor (ER) α, but not by ERβ. The EMBO J 18(15), 4270-4279

Wang, L., Rajan, H., Pitman, J.L.,McKeown, M., and Tsai, C.C. (2006). Histone deacetylase-associating Atrophin proteins are nuclear receptor corepressors. Genes Dev. 20, 525-530

Webb,P., Valentine, C., Nguyen, P., Price, R.H., Marimuthu, A., West, B.L., Baxter, J.D. and Kushner, P.J. (2003). ERβ binds N-CoR in the presence of estrogens via an LXXLL-like motif in the N-CoR C-terminus. Nucl. Recept. 1:4

Williams,C., Edvardsson, K., Lewandowski, S.A., Strom, A. and Gustafsson, J.A. (2007). A genome wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signalling in breast cancer cells. Oncogene 1-14