CHARACTERIZATION OF HUMAN MESODERM
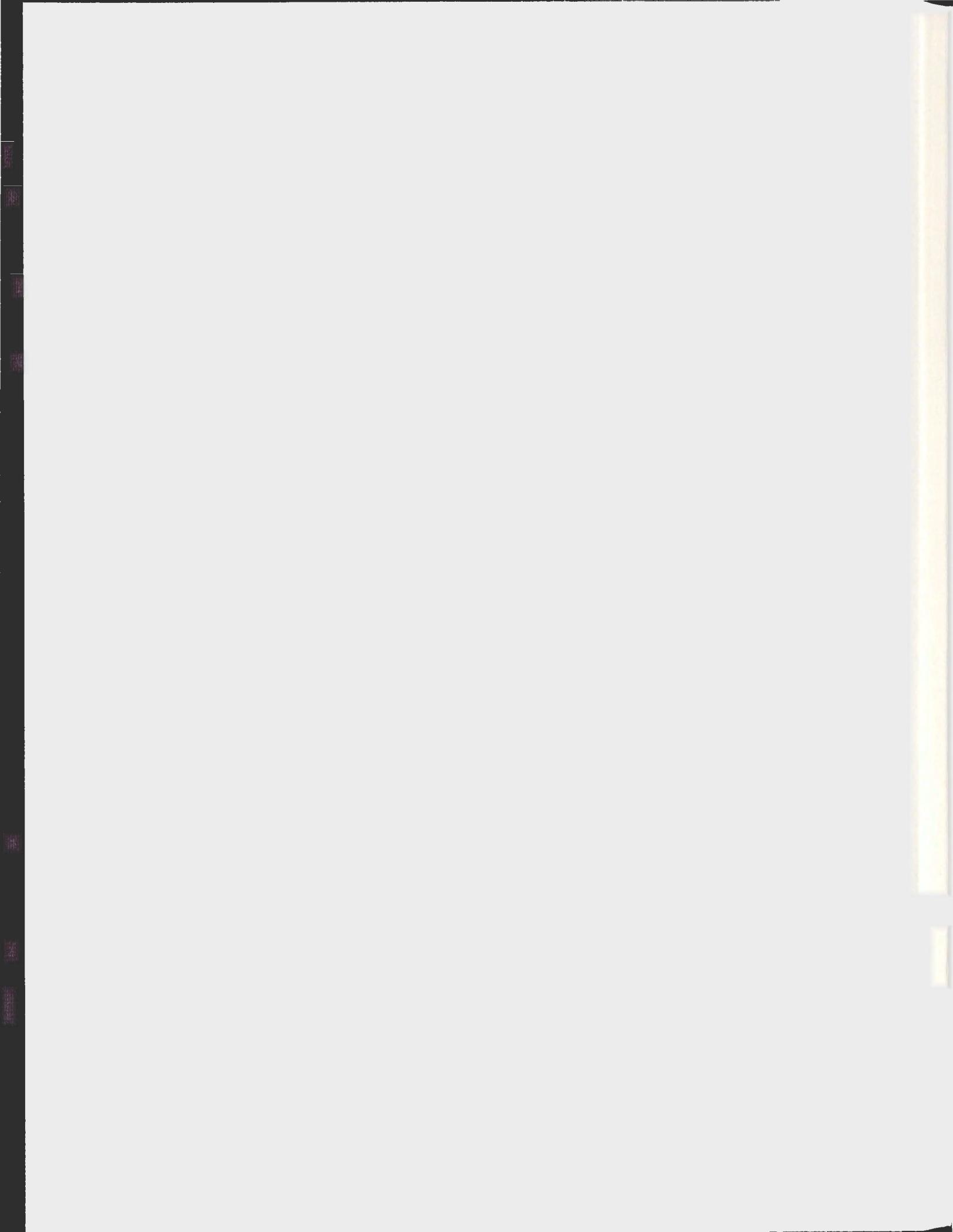# INDUCTION-EARLY RESPONSE 1 (hMI-ER1)
AS A NUCLEAR HORMONE RECEPTOR COFACTOR

MARIANNE SAVICKY

CHARACTERIZATION OF HUMAN MESODERM INDUCTION-EARLY RESPONSE 1 (hMI-ER1) AS A NUCLEAR HORMONE RECEPTOR COFACTOR

By Marianne Savicky

A thesis submitted to the
School of Graduate Studies
in partial fulfillment of the
requirements for the degree of
Masters of Science

Division of Basic Medical Sciences

Faculty of Medicine

Memorial University

May 2004

St. John's

Newfoundland

ABSTRACT

Fibroblast growth factors (FGFs) are a family of proteins whose signaling in cells play important roles in cell growth and development, cell differentiation, and angiogenesis [Powers, et al., 2000]. Investigations into the role of FGF in cell growth and differentiation in *Xenopus laevis* embryos led to the identification of a novel immediate-early gene, or initial target, of FGF signal transduction, which was later named mesoderm induction early response 1 (*mi-er1*) [Paterno, et al., 1997]. A human orthologue of *xmi-er1, hmi-er1,* was also cloned in our laboratory [Paterno, et al., 1998; Paterno, et al., 2002]. hMI-ER1 was found to be consistently expressed at low levels in normal human tissues, however, breast carcinoma cell lines and tumour tissue samples displayed elevated levels, indicating that hMI-ER1 may have a role in the neoplastic state [Paterno, et al., 1998; Paterno, et al., 2002].

Examination of the protein structure of hMI-ER1 revealed the presence of a number of protein interacting motifs, namely an acidic activation domain, ELM2 and SANT domains. Each of these motifs are present in both hMI-ER1α and hMI-ER1β isoforms. Notably, the hMI-ER1α isoform also contains an LXXLL motif (L represents the amino acid leucine and X can be any amino acid), a domain not found in hMI-ER1β but commonly found in nuclear hormone receptor interacting proteins. Therefore, my hypothesis is that hMI-ER1α is involved in protein-protein interactions involving nuclear hormone receptors. The finding that hMI-ER1 recruits HDAC (histone deacetylase) activity through the ELM2 domain [Ding, et al., 2002], indicates that hMI-ER1 is

involved in transcriptional regulation, and will therefore, also likely play a role in the regulation of transcription by nuclear hormone receptors.

The studies described in this thesis revealed that both hMI-ER1α and hMI-ER1β interact with a number of nuclear hormone receptors (ERα, RARα, RARβ, RARγ, RXRα, and RXRγ) *in vitro* and with ERα (estrogen receptor alpha) *in vivo*. Further characterization of the *in vitro* interaction between hMI-ER1 and ERα demonstrated that the region important for interaction is located between amino acids 325-357 of hMI-ER1.

In vivo studies confirmed the interaction between hMI-ER1α or hMI-ER1β and ERα in the absence of the ER ligand, estrogen (E$_2$). Interaction in the presence of E$_2$ appeared to depend on the antibody used for immunoprecipitation.

Studies into the functional consequences of the interaction show that hMI-ER1β enhances estrogen response element (ERE)-driven transcription while hMI-ER1α has a negative, or negligible, effect on ERE-driven transcription. Overall, these results indicate that both hMI-ER1α and hMI-ER1β interact with the estrogen receptor alpha and do indeed play a role in the functioning of the estrogen receptor in cells.

ACKNOWLEDGMENTS

Table of Contents

2. Materials and Methods

3. Results

List of Tables and Figures

List of Abbreviations

μg	microgram
μl	microlitre
aa	amino acid
AD	activation domain
ADA2	adaptor coactivator 2
AF1	activation function 1 domain
AF2	activation function 2 domain
AIB3	member of SRC-1 family of coactivators/corepressors
AP1	activator protein-1
AR	androgen receptor
bcl-2	anti-apoptotic gene originally recognized as a proto-oncogene translocation in most human follicular B cell lymphomas
β-gal	β galactosidase
BSA	bovine serum albumin
CAT	chloramphenicol acetyl transferase
CBP	CREB-binding protein
ChIP	chromatin immunoprecipitation
CIA	coactivator independent of AF-2 function
CoREST	corepressor to the REST (RE1 silencing transcription factor) transcription factor
DAD	deacetylase activation domain
DBD	DNA binding domain
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
E_2	17-β-estradiol (estrogen)
EDTA	ethyldiamine tetraacetic acid
Egl-27	gene first identified in *Caenorhabditis elegans*
ELM2	EGL27 and MTA1 homology 2
ER	estrogen receptor
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERE	estrogen response element
FCS	fetal calf serum
FGF	fibroblast growth factor
g	gram
GAL4DBD	pM plasmid containing GAL4 DNA binding domain
GR	glucocorticoid receptor
GREB1	genes regulated by estrogen in breast cancer 1
GST	glutathione S-transferase

GST-hMI-ER1	glutathione S-transferase tagged hMI-ER1
HAT	histone acetyl transferase
HDAC	histone deacetylase
HER2 (Erb2)	human epidermal growth factor receptor 2
hmi-er1	human mesoderm induction early response 1 gene DNA/RNA
hMI-ER1	human mesoderm induction early response 1 protein
HRE	hormone response element
HRP	horseradish peroxidase
Hsp40	heat shock protein 40
IκB	inhibitor of NFκB protein
IL-1	interleukin 1
IL-6	interleukin 6
IP	immunoprecipitation
ISW1	imitation switch gene 1 (member of SWI2/SNF2 family)
kDa	kilodalton
LBD	ligand binding domain
LCoR	ligand dependent corepressor
LXXLL motif	NR [nuclear receptor] box (L = amino acid leucine; X= any amino acid)
luc	luciferase
M	molar
MAPK	mitogen activated protein kinase
Mi2	member of NuRD histone deacetylase complex
MICoA	MTA1-interacting coactivator
ml	millitre
MLS	membrane localization signal
mM	millimolar
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MTA	metastasis associated protein (isoforms: MTA1, MTA2, MTA3, MTA1s)
NCOR	nuclear receptor corepressor
NFκB	nuclear transcription factor kappa-B
NLS	nuclear localization signal
NSD1	NR-(nuclear receptor) binding SET-domain containing protein
NuRD	nucleosome remodeling and histone deacetylation
nt	nucleotide
°C	degrees Celcius
PBS	phosphate buffered saline
pmoles	picomoles
PRF DMEM	phenol red free DMEM (Dulbecco's modified Eagle's medium)

PCAF	p300/CREB binding protein associated protein
PR	progesterone receptor
pRb	retinoblastoma protein
pS2	estrogen-responsive gene
PTα	prothymosin alpha
RA	retinoic acid
RAR	retinoic acid receptor
RARα	retinoic acid receptor alpha
RARβ	retinoic acid receptor beta
RARγ	retinoic acid receptor gamma
REA	repressor of estrogen activity
RIP140	receptor interacting protein 140
RLU	relative luciferase units
RNA	ribonucleic acid
rpm	revolutions per minute
RXR	retinoid X receptor
RXRα	retinoid X receptor alpha
RXRγ	retinoid X receptor gamma
SANT	domain first identified in SWI3, ADA1, NCoR, TFIIIB
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SERMs	selective estrogen receptors modulators
SH3	src homology domain 3
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
Sp1	promoter specific transcription factor 1
SRC-1	steroid receptor coactivator 1
SRC-3	steroid receptor coactivator 3
SWI2/SNF2	family of DNA-stimulated ATPases (*switch* and *sucrose nonfermenting*)
SWI3	type of SWI/SNF chromatin remodeling complex
TAF	TBP associated factors
TAF-Iβ	template activating factor Iβ
TBP	TATA-binding protein
TFII	transcription factors for RNA polymerase II
TFIII	transcription factors for RNA polymerase III
TFTC	TBP-free TAF containing complex
TGFα	transforming growth factor alpha
Tk	thymidine kinase
TNF	tumour necrosis factor
TR	thyroid hormone receptor
TRABID	TRAF binding domain

TRAF	TNF-receptor associated factor
TRAP	thyroid hormone receptor-associated protein
WB	Western blot (immunoblot)
Xbra	*Xenopus* homologue of the *Brachyury* gene
xmi-er1	*Xenopus* mesoderm induction early-response 1 gene DNA/RNA
XMI-ER1	*Xenopus* mesoderm induction early-response 1 protein

1. Introduction:

1.1 General Introduction

1.1.1 Cell Proliferation

DNA (deoxyribonucleic acid), a molecule found in the nucleus of eukaryotic cells, encodes genetic information that determines the structure, function and behaviour of a cell. DNA is organized into chromosomes and each chromosome contains a number of genes, each of which carry the code for specific proteins. Normal cells have the ability to replicate their DNA and produce daughter cells, when necessary. However, alterations or mutations in the structure of DNA can occur in a number of ways, including through environmental agents such as ultraviolet light, radiation, and genotoxic chemicals, or through products of cellular metabolism, such as byproducts of oxidative respiration or lipid peroxidation [reviewed in Hoeijmaker, et al., 2001]. Lesions in the DNA can block transcription, the process of copying the information from DNA and producing messenger ribonucleic acid (mRNA) for protein production, and interfere with DNA replication. The cell's machinery can recognize alterations in the DNA and arrest the cell at specific checkpoints in G1, S, G2 or M phases of the cell cycle in order to allow DNA repair, before the cell converts these alterations into permanent mutations [reviewed in Zhou, et al., 2000]. A cell can become cancerous through the accumulation of multiple DNA lesions, especially mutations in oncogenes and tumour suppressor genes. Oncogenes are genes that, when inappropriately expressed, can release the cell from the normal

1

constraints of cell growth, and change the cell into a cancer cell. Tumour suppressor genes code for proteins which negatively regulate the cell cycle, however, inactivation of such genes can lead to rapid cell proliferation.

1.1.2 Transcription

The transcription of information, into mRNA, contained within a gene is followed by a process called translation, where proteins are synthesized according to the mRNA sequence. Eukaryotic cells have sophisticated cellular machinery to control and regulate the process of gene transcription, including general transcriptional machinery, sequence-specific transcriptional regulators, mediators and cis-acting regulatory elements within the DNA itself. Genes are transcribed by RNA polymerase. RNA polymerase I will transcribe genes that encode ribosomal RNA; RNA polymerase II is the central protein responsible for mRNA synthesis and RNA polymerase III will transcribe genes that encode transfer RNA and small nuclear RNA; RNA polymerase II will assemble with general transcription factors, forming what is referred to as basal transcriptional machinery, at the promoters of genes (sequences of DNA located upstream of a gene) [reviewed in Woychik, et al., 2002]. General transcription factors for RNA polymerase II include: TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH, all of which assist with promoter melting and unwinding of the DNA. The basal transcriptional machinery also requires the presence of sequence or gene-specific transcriptional activators, which specify the gene to be transcribed, and a modulator complex, called Mediator, which will transduce

2

regulatory information between gene-specific transcription factors and the core RNA polymerase II machinery [Myers, et al., 2000]. Sequence-specific regulators will bind to their corresponding DNA cis-acting regulatory elements, such as TATA box sequences and GC boxes, which are part of gene promoters, and to enhancers. The number, orientation and location of multiple cis-acting regulatory elements will determine the efficiency and specificity of the transcriptional activity of a promoter [Alberts, et al., 2002]. Enhancers can be located a significant distance from promoters and can function in either orientation. The basal transcriptional machinery can also associate with sequence-specific transcriptional repressors, rather than activators, which will inhibit transcription.

1.1.3 Chromatin Structure

DNA found within the nucleus of a cell is wrapped around histone proteins H2A, H2B, H3 and H4, each of which is present in duplicate. The octamer of histones and the DNA wrapped around it is called a nucleosome, and the mass of nucleosomes and DNA found in a nucleus is called chromatin. The structure of chromatin helps to condense the long sequence of nucleotides into the small space within a cell. Transcription is regulated, in part, by this chromatin structure, as DNA in this conformation is only partially accessible to transcriptional machinery. Post-translational modification of histone proteins through processes such as acetylation and methylation, strongly influence the architecture of chromatin. Multi-protein complexes that bind to promoters

3

and enhancers, and that are responsible for communication between activators and repressors at sites of transcription initiation, are required to modify this higher-order chromatin structure [reviewed in Narlikar, et al., 2002]. Major classes of chromatin-modifying complexes include: ATP-dependent remodeling complexes, histone acetyl transferase (HAT) or histone deacetylase (HDAC) complexes and DNA methyltrasferases.

A. **ATP-dependent remodeling complexes**: these complexes utilize ATP-hydrolysis to increase the accessibility of nucleosomal DNA, a fundamental requirement for transcription. ATP-dependent remodeling complexes can be divided into three categories, depending on their catalytic ATPase subunit: i)SWI2/SNF2 family, ii) ISWI family, and iii) Mi-2 family [Narlikar, et al., 2002].

B. **HATs and HDACs**: Addition of acetyl groups to lysine residues in –terminal regions of histone proteins, by an enzyme called HAT, removes the positive charge on the lysine residue thereby decreasing the affinity between histone proteins and DNA. The result is a destabilization of chromatin structure which causes the chromatin structure to open thereby permitting better access of basal transcriptional machinery to the DNA. HDAC enzymes remove the acetyl groups from histone proteins thereby stabilizing chromatin structure and inhibiting transcription.

C. **DNA methylation**: Another fundamental determinant of chromatin structure is DNA methylation, the addition of methyl groups to cytosine nucleotides, mediated by enzymes called DNA methytransferases [Bestor, et al., 1988]. DNA methylation of cytosine, in the

4

context of CpG dinucleotides, will inactivate genes, while hypomethylation of gene

promoters will affect DNA-protein interactions and chromatin condensation and allow for

transcriptional activation [Geimann, et al., 2002; Keshet, et al., 1986; Richardson, 2003].

In mammals, patterns of methylation are established early in embryogenesis and are

stably inherited during normal cell growth [Martienssen, et al., 1995]. Disruption or

deregulation of this heritable pattern of DNA methylation, such as hypomethylation of

oncogenes or hypermethylation of CpG islands of tumour suppressor genes, is

consistently observed early in human cancers [Laird, et al., 1994; Baylin, et al., 1991].

1.1.4 Development and Cancer

Both development and cancer are processes involving shifts between cell

proliferation and differentiation. Research has suggested that deregulation of certain

developmental genes may result in, or contribute to, oncogenesis. For example, a number

of cancers, including leukemia, colon, skin, prostate and breast cancers, exhibit

expression or alterations in homeobox genes [Bijl, et al., 1997; Lawrence, et al., 1996;

Vider, et al., 1997; Care, et al., 1996; Ford, 1998]. Homeobox genes are regulatory genes

encoding transcription factors that regulate various aspects of morphogenesis and cell

differentiation during normal embryonic development [Nunes, et al., 2003].

Overexpression of the homeobox gene, HSIX1, has been observed in 44% of primary and

90% of metastatic breast lesions, indicating that HSIX1 may play a role in the progression

of breast cancer [Ford 1998]. Another example of developmental genes that have been

linked to cancer are FGFs. These genes play a significant role in gastrulation and

5

organogenesis, particularly with the nervous system, the lungs and limbs [Powers 2000].

FGFs are known to stimulate growth, survival and/or differentiation of various

mesenchyme-derived cells and have also been implicated in malignant tumours [Auguste,

et al., 2001]. For example, FGF receptor genes, FGFR1 and FGFR4, have been found to

be amplified in 20% and 30% of breast cancer cases, respectively [Dickson, C., et al.,

2000].

1.2 *Nuclear Hormone Receptors*

1.2.1 *Structure*

Nuclear hormone receptors are commonly referred to as ligand-inducible

transcription factors. They include steroid hormone receptors (androgen receptor [AR],

estrogen receptor [ER], glucocorticoid receptor [GR], progesterone receptor [PR],

mineralocorticoid receptor [MR]) and non-steroid receptors (vitamin D receptor [VDR],

retinoic acid receptor [RAR], retinoid X receptor [RXR], thyroid hormone receptor [TR])

that regulate transcription through vitamin and endocrine signaling. These receptors

possess the same basic structure (Figure 1) with an A/B region at the N–terminus which

contains an activation function-1 (AF-1) domain [steroid receptors] and is responsible for

ligand-independent transcriptional activation. This region is also involved in protein-

protein interactions. Adjacent to this A/B domain is the C region or the DNA binding

6

domain (DBD). This is the region of the receptor responsible for recognizing specific

sequences in the promoters of genes which they regulate, called hormone response

elements (HREs). C-terminal to the D region, or hinge region, is the E region which

contains the ligand binding domain (LBD) responsible for ligand-dependent

transcriptional activation as well as homo/heterodimerization, nuclear translocation and

interaction with heat shock proteins. This region also contains the activation function 2

(AF-2) motif. A number of nuclear receptor coregulators interact with the AF-2 region.
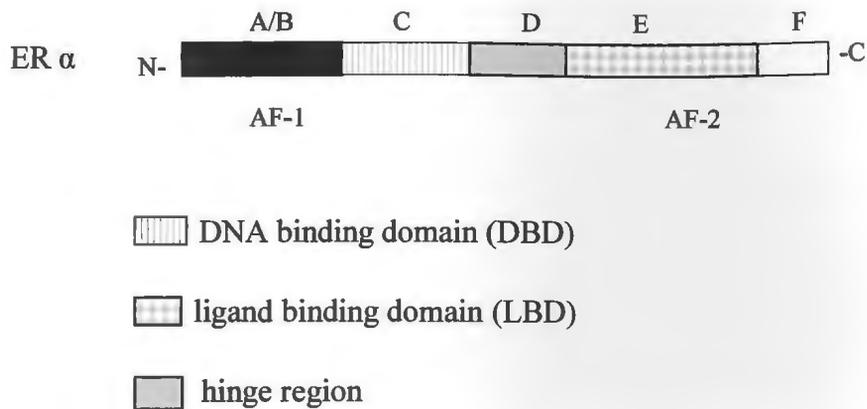
Figure 1: Domain structure of ERα

Schematic representation of ERα. The A/B domain, at the N-terminus, is responsible for ligand-independent transcriptional activation (AF-1). The DNA binding domain (DBD), or C domain, follows and confers the ability of the receptor to recognize estrogen response elements (EREs) and thereby activate or repress genes. The D domain is the hinge region between the DBD and the E domain, or ligand binding domain (LDB). This domain contains the AF-2 (activation function 2) motif responsible for ligand-dependent transcriptional activation. The F domain is a variable region.

1.2.2 Models for ER Function

Steroid hormone receptors are responsible for various biological functions important during growth, differentiation, inflammation, pregnancy and homeostasis. Studies examining the role of the estrogen receptor in such processes has led to the realization that it can function through classical (genomic) and non-classical (non-genomic) methods.

A. Classical Model: The classical model for nuclear hormone receptor action (Figure 2A) begins with ligand binding, which induces conformational changes in the receptor. Steroid hormone receptors will form homodimers or heterodimers whereas non-steroid receptors will heterodimerize with retinoid X receptor (RXR). Dimers will then undergo nuclear translocation where they will next bind directly to HREs, which are target sequences located in the regulatory sequences present in the flanking regions of target genes. At this point receptors will recruit coactivator complexes to activate transcription. If receptors are not bound by agonists or are bound by an antagonist, they will associate with corepressor complexes, whose members usually possess HDAC activity, in order to inhibit transcriptional activation.

Nuclear hormone receptors require multiple protein-protein interactions in order

to mediate transcription. Protein complexes involved in this transcriptional regulation include members of the basal transcriptional machinery as well as coactivator or corepressor molecules. Coactivators are commonly associated with HAT activity whereas corepressors are associated with HDAC activity.

Figure 2: Examples of classical (A) and non-classical (B) modes of action of the estrogen receptor.

A. Classical mode of ER action involves binding of the ligand (estradiol: E_2), receptor dimerization, nuclear translocation, binding to hormone response elements (HREs), and activation of transcription through association with coactivator complexes that include coactivators such as CBP/p300 (CREB binding protein), P/CAF (p300, CBP associated protein), and SRC-1 (steroid receptor coactivator 1).

B.i. NFκB complexes are kept in an inactive state in the cytoplasm by the association of inhibitory proteins (IκB). Exposure of cells to inducers, such as IL-1 (interleukin-1), will result in the degradation of the IκB proteins, allowing for the NFκB dimers to move into the nucleus where they activate transcription. ER has been found to associate with the *c-rel* subunit of the NFκB complex, which prevents the complex from binding to, and activating, the appropriate genes.

B.ii. ERs have been found to enhance Sp1-DNA association, and thereby enhance transcription.

B.iii. FOS and JUN proteins are transcription factors that will heterodimerize and form complexes that regulate transcription by associating with AP1 sites in the promoters of certain genes. ER will associate with FOS/JUN proteins and ultimately enhance transcription.

[adapted from Nilsson, et al., 2001, and Robyr, et al., 2000]

11

A. Classical Mode of Action
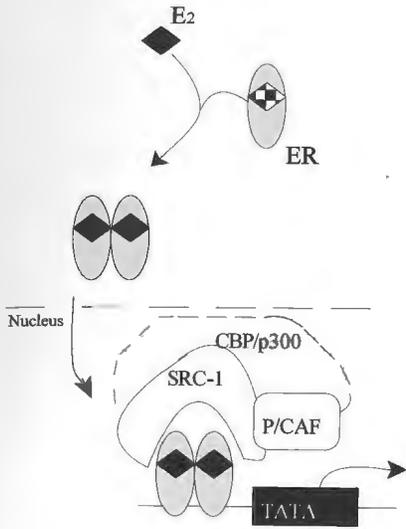
B.i. ER and NFκB
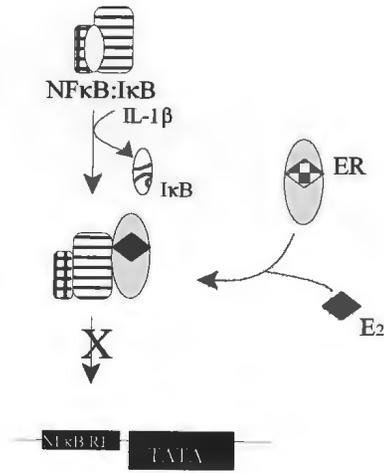
B.ii. ER and Sp1 transcription factors
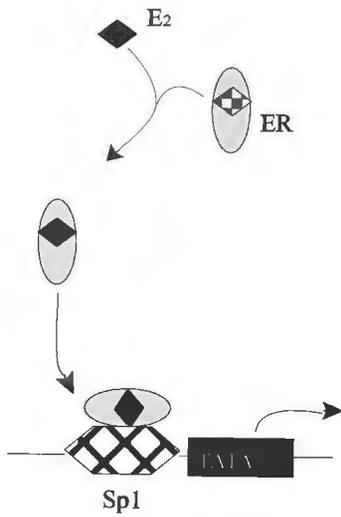
B.iii. ER and AP1 transcription factors
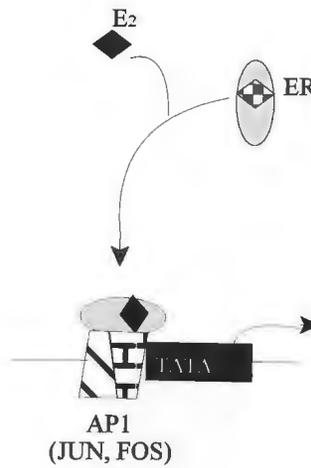
B. Non-classical Models of ER Action:

ERs have also been found to have non-genomic effects, which are often immediate and transient, via signaling pathways more commonly associated with growth factors of cell surface receptors. ERs can also regulate transcription through response sites that bind transcription factors including Rel/NFκB, Sp1, and AP1, likely through protein-protein interactions since ER does not bind to these sites directly [Nilsson, et al., 2001]. Ligand-independent activation of the ER can also occur through posttranslational modifications, such as phosphorylation and acetylation, as well as through interaction with cyclin D1.

i) Rel/NFκB is a family of transcriptional regulators, whose activity is controlled by subcellular localization. They exist in inactive states in the cytoplasm where they can be associated with inhibitory IκB proteins. Treatment of cells with various inducers (such as lipopolysaccharides, TNF or IL-1) results in degradation of the IκB through phosphorylation and ubiquitination. This releases the Rel/NFκB dimer allowing nuclear translocation where they can activate appropriate genes. ERα interacts with the *c-rel* subunit of the NFκB complex. This interaction prevents NFκB from binding to and stimulating expression from the interleukin-6 (IL-6) promoter (Figure 2B). Therefore, ER can inhibit expression of the cytokine IL-6 through a protein-protein interaction [Galien, 1997].

12

ii) Both ERα and β physically interact with the Sp1 transcription factor to enhance Sp1-DNA binding (Figure 2C). For example, both ERα and β can activate transcription of the RARα1 gene by the formation of ER-Sp1 complexes on the Sp1 sites of the RAR1 promoter [Sun, et al., 1998; Zou, et al., 1999]. Estradiol (E_2) also induces c-fos protooncogene expression in MCF-7 cells, which is dependent on the formation of a transcriptionally active ER/Sp1 complex [Duan, et al., 1998].

iii) ERα and β also interact with the FOS/JUN proteins of the AP1 complex (Figure 2D). E_2 bound ERα enhances AP1 activity by interacting with the p160 family of coactivators whereas ERβ bound to anti-estrogens, such as tamoxifen, enhances AP1 activity by sequestering corepressors away from the AP1 transcriptional complex [Philips, et al., 1993; Webb, 1999; Peach, et al., 1997]. In the absence of E_2, ER can also be activated by phosphorylation and thereby enhance transcription through AP1 sites.

iv) Overexpression of epidermal growth factor receptors (ErbB2 or HER2) has been identified in a number of cancer cells. Overexpression of these receptors can activate a number of signal transduction pathways in the cell, including MAP kinase (MAPK). MAPK hyperactivity has been shown to phosphorylate serine-118 in ERα, resulting in ligand-independent receptor activation [Bunone, et al., 1996; Kato, et al., 1995]. Overexpression of HER2 can also induce MTA1 expression. MTA1 decreases estrogen

13

response element (ERE)-driven transcription by interacting with ER and allowing for the development of E_2-independent growth [Mazumdar, et al., 2000].

v) Direct acetylation of the hinge region of ERα by p300 has been shown to regulate transactivation and to suppress ligand sensitivity of the ER [Wang, et al., 2001]. TAF-Iβ (template-activating factor-Iβ) will interact with the DNA-binding domain and C-terminus of ERα and represses p300-mediated acetylation of histone proteins and of ERα, decreasing ERα-mediated transcription [Loven, et al., 2003].

vi) Cyclin D1, which is overexpressed in a significant number of human cancers, is able to act as a bridging factor between unliganded ER and SRC-1 (steroid receptor coactivator 1) and thereby recruit coactivators with HAT activity to ER in the absence of ligand [Zwijsen, et al., 1998]. For example, Cyclin D1 can trigger activation of ER by interacting with a protein containing HAT activity, P/CAF (p300/CREB-binding protein-associated protein), and thus facilitate the interaction between ER and P/CAF [McMahon, et al., 1999]. This interaction can stimulate the transcriptional function of ER in the absence of its ligand, estrogen (E_2).

1.3 *Nuclear Hormone Receptors and Cancer*

Approximately two thirds of breast cancer patients have endocrine-responsive

disease, and therefore, countless studies have been undertaken in order to more fully

examine the role of nuclear hormone receptors in breast cancer and to determine which

hormone therapies could play a critical role in the management of the disease. Down-

regulation of RARβ and overexpression of RXR have both been associated with

progression of carcinogenesis in breast cancer [Xu, et al., 1997; Lawrence, et al., 1998].

ERα mRNA was shown to be up-regulated during carcinogenesis of breast tumours

[Iwao, et al., 2000] while ERβ expression was shown to be down-regulated in

proliferative preinvasive mammary tumours [Roger, et al.,2001].

Both estrogens and progestins effect cell growth and/or differentiation in

mammary epithelial cells [Vienonen, et al., 2003]. Through interaction with the ER,

estrogen (E_2) can control cell growth and differentiation in normal tissues and in

hormone-responsive tumours by inducing expression of both immediate early genes as

well as hormone responsive genes important for cell growth [Perillo, et al., 2000]. For

example, estrogens have been found to increase expression of PR in breast cancer cells

[Nardulli, 1988] and to induce both c-Myc and cyclin D1, the induction of either is

sufficient to affect cell cycle progression. Overexpression of cyclin D1 has been found in

many breast cancers [Zwijsen, 1998] and activation of either c-Myc or cyclin D1 will lead

to early activation of cyclin E-Cdk2, phosphorylation of pRB and release of E2F. This

15

signaling cascade will ultimately result in DNA synthesis, and cell cycle progression from

G1 to S phase [Doisneau-Sioux, et al., 2003]. E_2 has also been found to increase RAR

mRNA levels in breast cancer cells [Roman, et al., 1993] as well as *bcl-2* transcription in

MCF-7 cells [Perillo, et al., 2000]. Other target genes for ERα in hormone-responsive

breast tumours have been identified, including: pS2, TGF-α, cathepsin D, and GREB1

[Schurr, et al., 2001; Masiakowski, et al., 1982; Bates, et al., 1988; Cavailles, et al., 1991;

Ghosh, et al., 2000].

Overexpression or hyperactivation of the ER can, thus, drive cells though the cell

cycle, lead to the activation of numerous genes and ultimately enhance growth and

progression of breast cancers. This has lead to a great deal of interest in the exploration of

ways to functionally inactivate ER by suppressing ER-mediated gene-transcription and

cell proliferation. These approaches have involved the use of anti-estrogens, dominant-

negative ERs and corepressors.

Eventually, many breast cancers will develop hormone-independence and

simultaneous amplification of growth factor receptors which results in the development

of a more invasive and aggressive cancer. Hormone independence can also arise due to

loss of ER expression by tumours (rare), mutations in ER, alterations in the binding of

anti-estrogens in breast cancer cells or abnormal interactions between ER and

16

coregulators. ER can interact with corepressors such as SMRT and NCoR in the presence

of antagonists such as tamoxifen and RU486, however, these become agonists when the

ligand-independent activation function (AF-1) of the ER is activated by the MAPK

pathway. This will result in the release of corepressors and recruitment of coactivators

[Lavinsky, et al., 1998]. This pathway may be one explanation for the development of

tamoxifen-resistance in some breast cancer patients.

Gene amplification and overexpression of a number of ER coactivators such as

SRC-3, AIB3, TRAP220 have been identified in breast and ovarian cancer [Anzick, et al.,

1997; Lee, et al., 1999; Zhu, et al., 1999]. Changes in expression patterns of corepressors

or the ratio between coactivators and corepressors in breast cancer cells may contribute to

the development of chemotherapy-resistance in breast cancer treatment [Lavinski, et al.,

1998]. The expression of *hmi-er1* (see below) was found to be negligible in fifty normal

human tissues tested, however, *hmi-er1* was consistently expressed in nine breast

carcinoma-derived cell lines and eight breast tumour tissues samples tested, indicating

that hMI-ER1 may indeed be associated with human breast carcinoma [Paterno, et al.,

1998]. At present, a definitive link between cofactor levels or activity and cancer has yet

to be established, however, evidence does point toward a relationship between cofactors

and carcinogenesis. Therefore, a clearer understanding of the mechanism of action of

17

estrogen, as well as cross-talk with other signal transduction pathways, is crucial for the

development of more effective breast cancer therapies.

1.4 *hMI-ER1*

1.4.1 *MIER-1 Structure*

A number of years ago, investigations into the role of fibroblast growth factor

(FGF) in cell growth and differentiation in *Xenopus laevis* embryos led to the

identification of a novel immediate-early gene, or initial target, of FGF signal

transduction, which was later named mesoderm induction early response 1 (*mi-er1*)

[Paterno, et al., 1997]. FGF increased expression levels of *Xenopus mi-er1* (*xmi-er1*) in

embryo explants during mesoderm induction [Paterno, et al., 1997]. During early embryo

development, *xmi-er1* mRNA levels are evenly expressed during early cleavage (stages 2,

6, and 7), slightly increased at blastula stage and decreased by 6-fold during gastrula,

neurula and tailbud stages (stages 12, 17 and 22), and undetectable in subsequent

developmental stages [Paterno, et al., 1997].

A human orthologue of *xmi-er1, hmi-er1,* was also cloned in our laboratory

[Paterno, et al., 1998; Paterno, et al., 2002]. A number of studies have since been

conducted in order to characterize its structure and function. *hmi-er1* is a single copy, 63

18

kilobase gene, that ultimately gives rise to 6 protein isoforms with a common internal

region and varying – (N1, N2, and N3) and C-termini (α and β): N1α (457 aa), N1β (536

aa), N2α (432 aa), N2β (511 aa), N3α (433 aa) and N3β (512 aa) (Figure 3). *hmi-er1*

consists of 17 exons, with exons 4 to15 encoding the sequence for the common internal

region, exon 3A acting as a skipped exon which gives rise to one of the alternate

N–termini (N1), and intron 15 functioning as a facultative intron and giving rise to one of

two alternate C-termini (α and β). Close analysis of the hMI-ER1β-isoform and XMI-

ER1 proteins revealed that they display 91% sequence similarity [Paterno, et al., 1998].

Two alternate promoters were identified for *hmi-er1*. Use of the first promoter,

and inclusion of exon 3A, will result in an ML- amino acid N–terminal sequence and a 25

amino acid insert (N1) whereas use of the first promoter alone results in an ML- amino

acid N–terminal sequence (N2). The second promoter gives rise to an MAE- N–terminal

amino acid sequence (N3). Two distinct C-termini arise due to alternate use of the

facultative intron 15. The α C-terminus arises from removal of the intron to result in a

rarer, 23 amino acid, sequence whereas the β C-terminus, which arises from inclusion of

intron 15, encompasses 102 amino acids. Different amino acid sequences for α and β

termini suggest alternate functions.

19

A. **N- termini**

N1: MLKMCIRCLCLIGLQTVCGLFSCQITQ-

N2: ML-

N3: MAE-

B.

N3α (1-433)

N3β (1-512)

ELM-2 domain (aa 180-284)   SANT domain (aa 288-331)

acidic activation domain

α C-terminus
(aa 411-433)

β C-terminus
(aa 411-512)
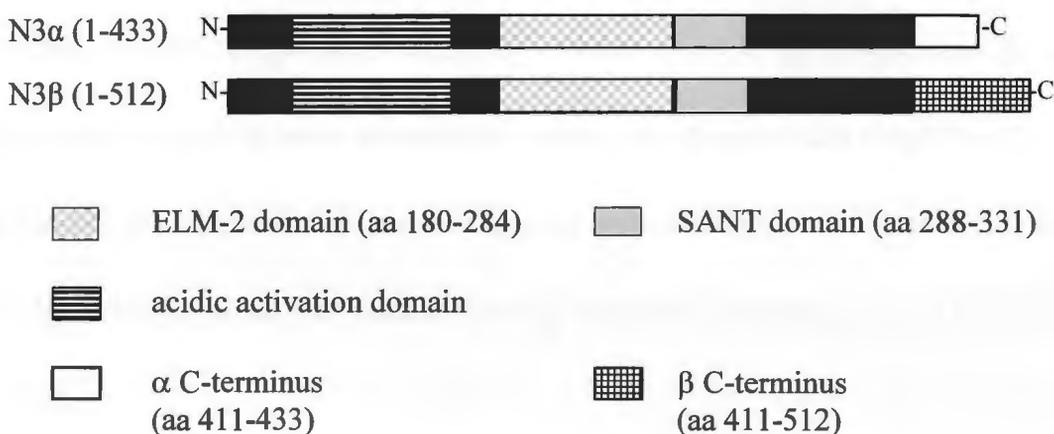
Figure 3: Structure of hMI-ER1

A. The amino acid sequences of the three alternate N-termini are shown. The first promoter and inclusion of exon 3A will give rise to an ML- amino acid start sequence (N1), however, the first promoter alone will result in an ML- amino acid N–terminal sequence (N2). The second promoter gives rise to an MAE- N–terminal amino acid sequence (N3).

B. Schematic illustrating two isoforms of hMI-ER1: N3α (433 aa) and N3β (512 aa). Each of the six protein isoforms contain the common internal region (410 aa) that includes the acidic activation domain, ELM-2 and SANT domains as well as one of two alternate C-termini and one of three alternate N-termini : N1α (457 aa), N1β (536 aa), N2α (432 aa), N2β (511 aa), N3α (433 aa) and N3β (512 aa). The number of amino acids present in each isoform is indicated in brackets on the left.

20

1.4.2 Functional Protein Motifs

Studies examining the role of *xmi-er1* in embryonic development gave the first

indications as to possible functions for this novel gene *in vivo*. Recently, a study found

that overexpression of *xmi-er1* in *Xenopus laevis* embryos lead to truncations of the

anterioposterior axis and dramatically reduced the percentage of explants induced to form

mesoderm by FGF-2 [Teplitsky, et al., 2003]. Whole mount *in situ* hybridization assays

for *brachyury (Xbra)*, an early mesodermal marker, also demonstrated a significant

reduction of *Xbra* expression in *xmi-er1* injected embryos. This study indicates that XMI-

ER1 negatively regulates FGF, thereby limiting mesoderm induction *in vivo* [Teplitsky, et

al., 2003].

Close examination of the protein structure of both XMI-ER1 and hMI-ER1, and

comparison to other known proteins, led to the identification of a number of putative

functional protein motifs, each of which provide indications as to possible function of this

novel protein.

A. **ELM2 Domain**: This domain derives its name from EGL-27 and MTA1 homology 2.

It is a domain commonly found in proteins, such as XMI-ER1 and in the common region

of hMI-ER1, with roles in transcriptional regulation, a number of which also include a

21

SANT domain. The ELM2 domain was recently found to recruit histone deacetylase 1 (HDAC1) activity to hMI-ER1 [Ding, et al., 2003]. HDACs catalyze the removal of acetyl groups from histone proteins, resulting in closing of the chromatin structure and repression of transcription. In order to examine the role of hMI-ER1α and hMI-ER1β in transcription HeLa cells were transfected with a reporter plasmid, G5tkCAT [containing CAT (chloramphenicol acetyltransferase) and five Gal4 DNA binding sites], and either GAL4DBD, GAL4DBD-MIER1α, or GAL4DBD-MIER1β constructs. GAL4DBD is a pM plasmid containing a nuclear localization signal (NLS) and the GAL4 DNA binding domain alone or fused to *hmi-er1α* or *hmi-er1β*. Cell extracts from cells transfected with G5tkCAT and GAL4DBD will display a basal level CAT expression, which is a measure of transcriptional activation. If hMI-ER1α or hMI-ER1β affect transcription, the level of CAT expression would either be increased or decreased in cells transfected with G5tkCAT and GAL4DBD-MIER1α or GAL4DBD-MIER1β. This study found that both hMI-ER1α and hMI-ER1β physically associate with a functional HDAC1 protein *in vivo*, and repress transcription of the G5tkCAT reporter plasmid in a dose dependent manner [Ding, et al., 2003], indicating that hMI-ER1 has a definite role in the regulation of transcription.

B. **SANT Domain**: A single SANT domain was identified in the common region of hMI-

ER1, downstream of the ELM2 domain. This domain, which is similar to the DNA-binding domain of Myb-related proteins, was identified in transcription factors SWI3, ADA2, NCoR and TFIIIB, for which the SANT domain was named [Aasland, 1996]. Proteins with one or more copies of the SANT domain are also commonly involved in protein-protein interactions including those involved in regulating nuclear hormone activity (ex: NCoR, SMRT), chromatin remodeling (ex: MTA-1 and MTA-2) and developmental events (Egl-27, CoREST) [You, 2001]. SANT domains can be found in proteins in HAT and HDAC complexes. SANT containing proteins, SMRT (silencing mediator for retinoid and thyroid receptors) and NCoR (nuclear receptor co-repressor) interact with multiple HDACs and mediate repression for a number of transcription factors, including unliganded nuclear hormone receptors. The N–terminal SANT domain (SANT1) of both SMRT and NCoR interact with, and activate, HDAC3 via a deacetylase activation domain (DAD) [Guenther, 2001]. The second SANT domain (SANT2) of SMRT, located within a histone-interaction domain (HID), inhibits acetylation of histone proteins by HATs [Yu, 2003]. The SANT domain of Ada-2, however, is essential for HAT activity of Gcn5p-containing HAT complexes [Boyer, 2002)].

C. **LXXLL motif (or NR-box)**: This motif, where L represents leucine and X can represent any amino acid, was identified in the α C-terminus of hMI-ER1 and is

23

commonly found in nuclear hormone receptor coactivators (SRC-1, CBP, MICoA) and

corepressors (NCoR, SMRT, DAX-1, RIP140, LCoR) [Heery, et al., 1997]. The LXXLL

domain of MICoA (MTA1-interacting coactivator), for example, will bind to the AF-2

(activation function-2) motif in the estrogen receptor (ER). Interaction between MICoA

and ER will stimulate ER-transactivation function in one of two ways: MICoA will act as

a coactivator itself, associating with ER and its estrogen response element (ERE), or

interact with other coactivators [Mishra, et al., 2003]. MTA1 can bind to the LXXLL

motif in MICoA to repress MICoA-mediated stimulation of ERE-driven transcription

[Mishra, et al., 2003]. The LXXLL domain of MICoA is responsible for its interaction

with ER and MTA1 and the transactivation function of MICoA, as well as for its

movement to the nucleus [Mishra, et al., 2003].

Both DAX-1 and RIP140 (receptor interacting protein 140) inhibit transcriptional

activation of a number of nuclear hormone receptors. DAX-1 inhibits the transcriptional

activation of liganded ERα and ERβ by binding directly to the receptors via an N -

terminal repeat domain, containing multiple LXXLL motifs [Zhang, et al., 2000]. The

single LXXLL motif of LCoR (ligand-dependent corepressor) is responsible for

recruitment of LCoR to agonist-bound nuclear receptors [Fernandes, et al., 2003].

Some LXXLL-containing proteins have both coactivator and corepressor function,

such as CIA (coactivator independent of AF-2 function) [Sauve, et al., 2001]). Other

LXXLL-containing proteins will interact with nuclear hormone receptors and affect their

function, but will not interact through this motif. REA (repressor of estrogen activity),

for example, will bind to ligand-bound ER and suppress ER-activated gene transcription,

however, the LXXLL motif in REA is not required for interaction with ER [Martini, et

al., 2003].

An 8-amino acid sequence spanning from position -2 to +6, relative to the first

leucine residue of the LXXLL motif, influences the affinity and selectivity of coregulators

for nuclear hormone receptors. A hydrophobic residue at position -1 and a

nonhydrophobic residue at +2 conveys high affinity of these proteins for steroid and

retinoid receptors [Heery, et al., 2001]. This is indeed the case with hMI-ER1α, with a

hydrophobic isoleucine at position -1 and a hydrophilic glutamine at position +2.

D. **Acidic Activation Domain**: There is much evidence to support a role for acidic

activation domains, areas rich in acidic amino acid residues, in the recruitment of

transcriptional machinery by protein-protein interactions [Melcher, 2000]. Four regions

rich in acidic amino acid residues have been identified in the common, --terminal region

of hMI-ER1. Transient transfection assays were completed with NIH 3T3 cells using

constructs containing various regions of *xmi-er1* fused to the GAL4DNA binding domain

25

and a CAT reporter plasmid [Paterno, et al., 1997]. The first 98 amino acids of the

N–terminus of XMI-ER1 stimulated transcription 80-fold whereas full length XMI-ER1

did not activate transcription, indicating that the –terminus of XMI-ER1 contains a

transcriptional activation domain [Paterno, et al., 1997]. XMI-ER1 could, therefore,

function as a transcriptional activator.

E. **Proline Rich Motif**: SH3 domains are small protein motifs, of about 60 amino acids in

length, commonly found in signaling proteins and enzymes which recognize proline-rich

motifs as their interacting partners [Vidal, et al., 2001]. A proline rich motif (PXXP; P

represents proline, X represents any amino acid motif) was identified in the common

region of hMI-ER1 and in XMI-ER1, located C-terminal to the SANT domain. Studies

investigating the importance of a number of functional domains of XMI-ER1

demonstrated that proline 365, within the proline rich motif, mediates the activity of

XMI-ER1 in Xenopus embryo development, ultimately affecting the extent of mesoderm

formation during embryonic development [Teplitsky, et al., 2003].

1.4.3 Subcellular Localization of hMI-ER1

Immunohistochemical studies of *Xenopus laevis* embryos during various stages of

development revealed that XMI-ER1 is found exclusively in the cytoplasm during early

26

developmental stages but progressively accumulates in the nuclei of marginal zone cells

of stage 8 blastulae, and is located exclusively in nuclei in the animal hemisphere by late

blastula stage (stage 9) [Luchman, et al., 1999]. Transfection assays with NIH 3T3 cells

revealed that XMI-ER1 translocated exclusively to the nuclei [Paterno, et al., 1997]. Four

putative nuclear localization signals (NLS; NLS1, NLS2, NLS3 and NLS4) were

identified in XMI-ER1, however, there is only one functional NLS (NLS4) located in the

C-terminal domain [Post, et al., 2001]. Further analysis of hMI-ER1α and β revealed

that hMI-ER1β is targeted exclusively to the nucleus whereas hMI-ER1α remained

cytoplasmic [Paterno, et al., 2002]. These results were expected as hMI-ER1α has no

functional NLS, however, they do not rule out the possibility that hMI-ER1α could be

transported into the nucleus through interaction with other proteins, such as HDAC or

nuclear hormone receptors.

1.5 Purpose of this Study The presence of an acidic activation domain, ELM2 and

SANT domains as well as proline rich motif, indicates that hMI-ER1 is likely involved in

protein-protein interactions and an LXXLL motif in hMI-ER1α indicates that this protein

likely interacts with nuclear hormone receptors. Evidence also indicates that hMI-ER1

plays a role in the regulation of transcription. The purpose of this study was to determine

if hMI-ER1α interacts with nuclear hormone receptors, through the LXXLL motif, and

thereby affect the functioning of nuclear receptors as transcription factors.

Objective 1: *Identification of hMI-ER1 interacting nuclear hormone receptors.* A number of GST-pull down assays were completed with GST-hMI-ER1α or GST-hMI-ER1β and *in vitro* translated ^{35}S-labeled nuclear hormone receptors in order to obtain preliminary evidence of an interaction between the receptor(s) and hMI-ER1.

Objective 2: *Identification of the region of hMI-ER1 responsible for interaction with ERα.* Previous data indicated that hMI-ER1 levels were increased in breast tumour cells and tissues [Paterno, et al., 1998], therefore, further studies focused on characterizing the interaction between hMI-ER1 and ERα, a nuclear hormone receptor with significant implications in breast cancer. GST-hMI-ER1 deletion constructs were used in a number of GST-pull down assays in order to determine the region of hMI-ER1 responsible for interaction with ERα.

Objective 3: *Characterization of the in vivo interaction between hMI-ER1 and ERα.* A human estrogen receptor positive breast carcinoma cell line (MCF-7) and a human embryonic kidney cell line (HEK 293) were used for assays which helped to further characterize the interaction of hMI-ER1 and ERα within cells.

Objective 4: *Characterization of the role of hMI-ER1 in ERE-driven transcription.* The recruitment of HDAC1 activity to hMI-ER1 through the ELM2 domain indicates that

28

hMI-ER1 is involved transcriptional regulation. Assays were therefore completed to determine if the interaction between ERα and hMI-ER1 would influence ERE-driven transcription.

2. Materials and Methods:

2.1 Plasmids and Constructs

A.CS3MT, CS3MT-*hmi-er1* α/β

Expression vectors were engineered, by Z. Ding in our laboratory, to contain full length *hmi-er1*α or *hmi-er1*β fused to the Myc epitope tag of CS3+MT plasmid (gift from David Turner, University of Michigan). The entire coding sequence of either hMI-ER1α or hMI-ER1β [accession numbers AY124187 and AF515447, respectively] was amplified by PCR with the use of specific primers incorporating 5' and 3' BamHI sites (Table 1). The PCR fragments were inserted into the *Bgl*II site of the CS3+MT plasmid [Ding, et al., 2003]. PCR primers used for preparing *hmi-er1* constructs are listed in Table 1.

B. GST-*hmi-er1*α/β

GST-*hmi-er1* fusion constructs were produced by Z. Ding, obtained by subcloning the appropriate cDNA isoform into the pGEX-4T-1 vector (Pharmacia, Biotech). A number of hMI-ER1 deletion mutants were generated by amplifying fragments encoding the appropriate amino acid residues of hMI-ER1α/β, using the primer pairs listed in Table 2. PCR products were cloned into pCR3.1 and *EcoRI* fragments were then inserted into the complementary sites of the pGEX-4T-1 plasmid.

30

C. pcDNA3-*ERα*

The pcDNA3 vector, containing the complete coding sequence of estrogen receptor α [accession number NM_000125], was a kind gift from Dr.Christine Pratt, University of Ottawa. A partial nucleotide sequence was determined and the remaining sequence was determined by Toronto's Hospital for Sick Children, DNA Sequencing Facility.

D. RARs, RXRs

pCMX-hRARα, pRC/RSV-hRARβ, pGEM-mRARγ, pSG5-mRXRα, and pSG5-mRXRγ, were kind gifts from Dr. Christine Pratt, University of Ottawa [accession numbers NM_000964, NM_000965, M34475, M84817, and M84819, respectively]. Partial sequences were determined in order to verify clones received.

E. pS2, ΔpS2, vERETKLuc

pS2-ERELuc, pS2ΔERELuc, and vERE(3) TKLuc were kind gifts from Dr. Vincent Giguere, McGill University.

pS2-ERELuc contains ~1050 bp pS2 promoter [Berry, et al., 1989] preceding the luciferase reporter of pGL3 (Promega)[Tremblay, et al., 1997]. In the pS2ΔERELuc construct, the ERE sequence was replaced by sequences encoding *Eco*RI-*Eco*RV sites, generated by PCR mutagenesis using the ExSite kit from Stratagene as described by the manufacturer [Tremblay, et al., 1997]. vERE(3) TKLuc was constructed by ligating three copies of the vitA2-ERE

31

oligonucleotide (5'-TCGACAAAGTCAGGTCACAG-TGACCTGATCAAG-3')

into *Sal*1-*Bam*HI-digested TKLuc vector [Tremblay, et al., 1997].

F. pGL3-Basic and pGL3-Control

Control vectors were obtained from Promega Corporation. pGL3-Basic lacks

eukaryotic promoter and enhancer sequences whereas pGL3-Control vector

contains SV40 promoter and enhancer sequences allowing for strong expression

of luciferase in many mammalian cell types.

2.2 *In vitro Transcription-Translation and TCA Precipitation*

▸ Coupled transcription-translation reactions (TₙT; Promega) were performed as per

manufacturer's instructions. Briefly, 10 µl DEPC water and the following reaction

components, provided by the manufacturer, were assembled in a 1.5ml centrifuge tube: 2

µl TₙT reaction buffer, 25 µl reticulocyte lysate, 1 µl T7/SP6/T3 polymerase, 1 µl amino

acid mixture (minus methionine). 1 µl of ribonuclease inhibitor (RNA Guard (27-0815-

01); Amersham Biosciences) and 5 µl [^{35}S] methionine were then added to the master mix

and finally, 1 µg of plasmid DNA template was added to the master mix. Reactions were

incubated for 90 minutes at 30 °C.

▸ TCA (tricholoroacetic acid) precipitation assays were conducted in order to determine

the efficiency of the *in vitro* transcription/translation reaction. Briefly, 98 µl of a 1N

NaOH/2%H$_2$O$_2$ solution was mixed with 2 µl of the TₙT. The reaction was incubated at

37 °C for 10 minutes. 900 µl of ice cold 25% TCA/2% casein amino acid solution was then added and the reactions were incubated for 30 minutes on ice. Precipitate was then collected on Fisher filter paper and counted in a Beckman S3801 Spectrophotometer.

2.3 *GST Pull Down Assays*

2.3.1 *Glutathione Sepharose 4B Beads*

Glutathione Sepharose 4B beads (Pharmacia Biotech) were prepared as per manufacturer's instructions. Briefly, beads were gently resuspended by shaking. 1.33ml of the 75% slurry was placed into a 15ml falcon tube. The beads were centrifuged at 500 x g for 5 minutes. Supernatant was removed and 10 ml of ice cold 1X PBS was added to the tube. GST beads and PBS were then mixed by inversion. The beads were centrifuged again at 500 x g for 5 minutes. Supernatant was removed and 1 ml of 1X PBS was added to result in a 50 % slurry. Beads were kept for up to a month at 4 °C.

2.3.2 *Fusion Protein Production*

10 ng of a pGEX-4T1-1plasmid was added to 100 µl of BL21Codon Plus RP competent E. coli [Strategene]. The reaction mixture was kept on ice for 30 minutes then placed in a water bath at 42°C for 45 seconds. 900 µl of Luria broth (LB) medium [5g peptone, 2.5g yeast extract, 5g NaCl, 500mL dH$_2$O; autoclaved] was added and the culture was shaken for 1 hour at 37°C. Samples were then quickly centrifuged (~10 - 20 seconds), after which most of the supernatant was removed, leaving approximately 100 µl. The pellet was resuspended in the remaining liquid. Using aseptic technique, all of the resuspended

33

pellet was added to an agar-ampicillin plate. Agar plates were prepared by mixing 5g

peptone, 2.5g yeast extract, 5g NaCl, and 500mL dH$_2$O. The agar solution was

autoclaved, and when liquid had cooled, ampicillin was added for a final concentration of

50 μg/ml. This solution was poured into petri dishes (enough to cover the bottom of

plate). The agar-ampicillin plate was inverted and incubated overnight at 37 °C. The next

morning, the plate was placed into a refrigerator at 4 °C. In the afternoon, 5ml LB

medium, with 50μg/ml ampicillin, was inoculated with one colony from the agar-

ampicillin plate. This culture was shaken overnight at 37 °C. The next morning, 250 ml

LB medium, with 50 μg/ml ampicillin, was inoculated with 2ml of the overnight culture

and shaken at 37 °C. After approximately 2.5 - 3 hours, the OD of the culture was

verified. Briefly, 1ml of LB medium was added to one cuvette for calibration and 1ml of

the inoculated culture was added to another cuvette. OD was read in a spectrophotometer

at a wavelength of 595nm. The inoculated culture was grown until the OD reading was

between 0.6 - 0.8. At that point, 25 μl of 1M IPTG (isopropyl-β-D-

thiogalactopyranoside) was added to the culture, which was subsequently shaken at 37 °C

for another 3 hours. The inoculated culture was poured into a 250ml Nalgene

polypropylene bottle and centrifuged in a Sorval centrifuge at 4000 rpm for 10 minutes.

All the liquid was drained off and the pelleted cells were resuspend in 5ml of ice cold 1X

PBS. The resuspended pellet was placed into a new falcon tube where 25 μl of 0.2M

PMSF and 50 μl 100x protease inhibitor (PI) [10 mg aprotinin, 10 mg leupeptin, 50 mg

nor-P-Tosyl-L-Lysine Chloromethyl Ketone, 10 ml water] was added. Cells were then

lysed by 1 minute of mild sonication. At this point, 500 μl of 10% Triton X-100 was added to the tube which was then centrifuged at 16,000 x g for 15 - 20 minutes. The soluble fraction (supernatant) was removed and placed into a new tube and stored at -70 °C. GST fusion protein level and purity were determined by SDS-PAGE.

2.3.3 GST pull down assays

ERα, RARα, RARβ, RARγ, RXRα, or RXRγ were labeled with [^{35}S] methionine through *in vitro* transcription/-translation reactions.

For each GST pull down reaction, 50 μl of a 50% slurry of GST-Sepharose beads were washed two times with GST buffer [20mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40, 1x PI (protease inhibitor), 0.2% bovine serum albumin (BSA)]. Beads were then resuspended in GST buffer (minus BSA) and rotated at 4 °C for 1 hour with indicated GST-hMI-ER1 fusion protein. GST-bound beads were subsequently washed five times with 500 μl of GST buffer, resuspended in GST buffer (minus BSA) and rotated 2 hours with [^{35}S] methionine-labeled *in vitro* translated ER α, RARα, RARβ, RARγ, RXRα, or RXRγ (100,000 cpm) at

4 °C. Beads were then washed three times with 500 μl of GST buffer (minus BSA), twice with 500 μl of GST buffer (minus BSA and NP40) and, finally, twice with 500μl of 150 mM NaCl. Beads were resuspended in 30 μl of 2x SDS loading buffer [5ml 0.5 M Tris pH 6.8, 5 ml 20% SDS, 2.5ml β-mercaptoethanol, 5ml glycerol, 5ml dH$_2$O], boiled 4 minutes and loaded on 8% SDS-polyacrylamide gel. Samples were analyzed by

autoradiography. Input lanes contained 1/10 volume of the indicated TNT used for each reaction.

▸ For serial dilutions (Figures 4 - 9), 0.25 μg , 0.5 μg, 1 μg or 2 μg of GST-MIER1β fusion protein was used for the pull down assays, as indicated.

▸ For GST-pull down assays completed with retinoic acid (RA) [ligand for RAR α and RARβ] or 17β-estradiol (E_2) [ligand for ERα] 1 μg of GST-MIER1α or GST-MIER1β were used in each pull down assay [Figures 10 - 12]. 0.5μl of 1 mM RA or 1μl of 5 mM E_2, or an equal volume of vehicle (DMSO or ethanol, respectively), was added directly to the GST buffer in order to obtain a final concentration of 10^{-6} M. After GST beads and GST-MIER1 fusion proteins were incubated together for one hour (as described above), beads were resuspended in GST-buffer with added ligand [without BSA] and finally 100,000 cpm of [^{35}S] methionine-labeled *in vitro* translated ERα, RARα, or RARβ was added to the reaction.

▸ For GST-pull down assays completed with pre-incubation of ligand and receptor [Figure 13],

1μl of 5 mM E_2 (final concentration of 10^{-6} M E_2), or an equal volume of ethanol (vehicle), was pre-incubated for 15 minutes with [^{35}S] methionine-labeled *in vitro* translated ERα [100,000 cpm] and GST buffer [without BSA] before being added to the GST-bound beads.

▸ GST-pull down assays with various GST-hMI-ER1 deletion constructs [Figures 15 and 16] were completed with 0.1 pmoles of GST-MI-ER1α, GST-MI-ER1β, GST-Δ4, GST-

36

Δ5, GST-Δ9, GST-Δ10, GST-Δ11, GST-Δ13, GST-Δ16, GST-Δ17, GST-Δ18, or GST-Δ19 in 500μl.

2.4 Cell Culture

Human cell lines, MCF-7 (human breast carcinoma) and HEK 293 (transformed human embryonic kidney), were obtained from the American Tissue Culture Collection. MCF-7 cells were cultured at 10% CO_2 and 293 cells were cultured at 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM)[Hyclone] containing 10% fetal calf serum (FCS) [Invitrogen].

2.5 Transfections: MCF-7 Cells

3.0×10^5 cells/well of MCF-7 cells were seeded in 6-well plates 18 h prior to transfection in phenol red free (PRF) DMEM with 10% charcoal stripped FCS (HyClone). All transfections were performed in duplicate in 6-well plates with 4 μl Lipofectamine Plus per well, according to the manufacturer's instructions (Life Technologies, Inc.), with the indicated amounts of DNA. Complexes were left on cells for 4 hours after transfection at which point medium with lipofectamine was aspirated from the cells and 2ml of fresh PRF DMEM was added to each well. Cells were grown in PRF DMEM with charcoal stripped FCS, for approximately 48 hours after transfection and exposed to either 10^{-9} M 17β-estradiol (E_2) [Sigma], or an equal volume of ethanol (vehicle), for 6 hours prior to harvesting. 10^{-9} M is considered to be an E_2-saturating condition for MCF-7 cells but also a physiologically relevant concentration, with the physiological range of E_2 between 10^{-12}

to 10^{-9} M [Hayashi, et al., 2003].

For *in vivo* **immunoprecipitation (IP)** assays with **MCF-7 cells**, cells were transfected with 1 μg of pCS3+MT, pCS3+MT-*hmi-er1*α, or pCS3+MT-*hmi-er1*β per well of a 6-well plate. 48 hours after transfection, and after a 6 hour incubation with 10^{-9} M E_2 or an equivalent volume of ethanol, each well was washed with 2ml 1X PBS. In order to consolidate two wells for one IP, a 500 μl of a 1X Triton Solution [10 ml 1M Tris, pH 7.5, 10ml 100% Triton, 20 ml 0.5M EDTA, 1 ml 2% sodium-azide and 59 ml dH_2O; 50 μl 0.2M PMSF and 100μl 100X PI were added to buffer immediately before IP] was added to each well. Cells were scraped and plates were placed on ice for 20 minutes. Lysate was pulled through a syringe ~ 15 - 20 times and lysate from two wells was transferred to one 1.5ml tube. Insoluble material was removed by centrifugation at 16,000 x g, 4 °C, 5 minutes. Supernatant was removed and placed in a new 1.5ml tube at which point a 1:25 dilution of anti-Myc (9E10, Developmental Hybridoma Bank) antibody was added for IP. Tubes were rotated overnight at 4 °C.

After an overnight rotation, 50 μl of a 50% slurry of Protein G beads was then added to the tubes and rotated 1 hour at 4 °C. Finally, bound beads were washed three times with 1X Triton Solution [with PMSF and PI added just before washes], and twice with 150 mM NaCl. Beads were resuspended in 30 μl of 2x SDS loading buffer, boiled 4 minutes and loaded on 8% SDS-polyacrylamide gel. ERα TNT lanes contained 2μl (between 100,000 to150,000 cpm) of [^{35}S] methionine-labeled *in vitro* translated ERα and 30 μl SDS loading buffer. Gels were run for approximately 1.5 hours at 30 mA in order to

38

allow full separation of protein components. SDS gels were shaken and washed three times in Transfer buffer [200ml 5X stock buffer (60.54g Tris, 288.4g glycine, 3L dH$_2$O), 200 ml methanol, 600 ml dH$_2$0] for 15 minutes to help remove SDS for efficient protein transfer. Proteins were then transferred to a Hybond-ECL nitrocellulose membrane (Amersham) for 2 hours at 60 V. The blot was then shaken in 5% skim milk powder/1X TBS-T [20mM Tris pH7.6, 137 mM NaCl, 0.1% Tween-20, dH$_2$O], at room temperature for 2 hours. **Western blot** analysis for **IP samples** was performed with a 1:800 dilution of a rabbit polyclonal anti-ERα (Santa-Cruz sc-543) antibody in 1X TBS-T. Blots were agitated in this solution overnight at 4 °C, washed 4-5 times in 1 hour at room temperature in 1X TBS-T, and agitated for 1 hour at room temperature in a 1:2500 dilution of donkey anti-rabbit conjugated to horseradish peroxidase (HRP) secondary antibody in 1X TBS-T. Finally, blots were washed 4-5 times in 1 hour, shaking in 1X TBS-T, at room temperature and analyzed with chemiluminescent detection using ECL Western Blotting System and Hyperfilm ECL from Amersham Technologies.

For **Western Blot** analysis of pure **protein extracts**, in order to verify protein expression, transfected cells were washed with 2ml 1XPBS. Then 200 µl of 2XSDS loading buffer was added per well. The plate was placed on ice for 5 minutes then the lysate was pulled through a syringe 10 times. The lysate was stored at -20 °C until needed. For loading on 8% polyacrylamide gel, 35µl of straight protein lysate was added to a new Eppendorf tube along with 5 µl 2X SDS loading buffer (plus bromophenol blue dye). Samples were boiled and loaded directly on an 8% polyacrylamide gel and run as above. After proteins

were run on the SDS gel, the gel was washed 3-4 times, shaking, in 1X transfer buffer for 15 minutes and then transferred onto a nitrocellulose membrane, as above. The membrane was then placed in 5% skim milk powder/1X TBT, shaking at room temperature for 2 hours. Western blot analysis was performed with a 1:1000 dilution of a rabbit polyclonal anti-ERα (Santa-Cruz sc-543) antibody in 1X TBS-T or a 1:200 dilution of anti-Myc antibody in 5% skim milk powder/1X TBS-T. Blots were shaken overnight at 4 °C and then washed 4-5 times in 1 hour at room temperature in 1X TBS-T. Blots were then shaken 1 hour at room temperature in a 1:2500 dilution of donkey anti-rabbit HRP secondary antibody in 1X TBS-T or 1:3000 dilution of sheep anti-mouse HRP in 5% skim milk powder/1X TBS-T. Finally, blots were washed 4-5 times in 1 hour, shaking in 1X TBS-T, at room temperature. Samples were analyzed with chemiluminescent detection using ECL Western Blotting System and Hyperfilm ECL from Amersham Technologies.

2.6 *Transfections: HEK 293 Cells*

For *in vivo* **immunoprecipitation (IP)** assays with **HEK 293** cells, 6.0×10^5 cells/well HEK 293 cells were seeded in 6-well plates 18 h prior to transfection in phenol red free (PRF) DMEM with 10% charcoal stripped FCS (HyClone). Cells were transfected with 0.4 μg of pcDNA3.1- *ERα* and 0.4 μg of either pCS3+MT, pCS3+MT-*hmi-er1α* or pCS3+MT-*hmi-er1β*. All transfections were performed in triplicate in 6-well plates with 4 μl Lipofectamine Plus per well, according to the manufacturer's instructions.

Complexes were left on cells for 4 hours after transfection at which point medium with lipofectamine was aspirated off of cells and 2ml of fresh PRF DMEM was added to each well. Cells were grown in PRF DMEM with charcoal stripped FCS, for approximately 48 hours after transfection and exposed to either 10^{-9} M E_2, or an equal volume of ethanol, for 6 hours prior to harvesting. Each well was then washed with 2ml 1X PBS. In order to consolidate three wells for one IP, 333 μl of a different lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl 10% glycerol, 1% Triton, 40 mM sodium pyrophosphate ($Na_4P_2O_7$ ·$10H_2O$), 50 mM NaF, 1 mM orthovanadate; 1M PMSF and 1X PI were added to buffer immediately before IP] was used rather than 1X Triton Solution. Cells were scraped and allowed to sit on ice for 20 minutes. Lysate was pulled through a syringe ~15 - 20 times and lysate from three wells was transferred to one 1.5ml tube. Insoluble material was removed by centrifugation at 16,000 x g, 4 °C, 5 minutes. Supernatant was removed and placed in a new 1.5ml tube at which point the a 1:100 dilution of a rabbit polyclonal anti-ERα antibody was added for IP. Tubes were rotated overnight at 4 °C.

After an overnight rotation, 50 μl of a 50% slurry of Protein A beads was then added to the tubes and rotated 1 hour at 4 °C. Finally, bound beads were washed three times with the lysis buffer above [with PMSF and PI added just before washes], and twice with 150 mM NaCl. Beads were resuspended in 30 μl of 2x SDS loading buffer, boiled 4 minutes and loaded on 8% SDS-polyacrylamide gel. ERα TNT lanes contained 2μl (between 100,000 to150,000 cpm) of [^{35}S] methionine-labeled *in vitro* translated ERα and 30 μl

41

SDS loading buffer. Gels were run for approximately 1.5 hours at 30 mA in order to allow full separation of protein components. After proteins were run on the SDS gel, the gel was washed 3-4 times, shaking, in 1X transfer buffer for 15 minutes. Proteins were then transferred to a Hybond-ECL nitrocellulose membrane (Amersham) for 2 hours at 60 V.. The blot was then shaken in 5% skim milk powder/1X TBT-T [20mM Tris pH7.6, 137 mM NaCl, 0.1% Tween-20, dH_2O], at room temperature for 2 hours.

Western blot analysis for **IP samples** was performed with a 1:200 dilution of an anti-Myc antibody in 5% skim milk powder/1X TBS-T. Blots were shaken overnight at 4 °C and then washed 4-5 times in 1 hour at room temperature in 1X TBS-T. Blots were then shaken 1 hour at room temperature in a 1:3000 dilution of a sheep anti-mouse conjugated to HRP secondary antibody in 5% skim milk powder/1X TBS-T. Finally, blots were washed 4-5 times in 1X TBS-T, shaking for 1 hour, at room temperature. Samples were analyzed with chemiluminescent detection using ECL Plus Western Blotting System and Hyperfilm ECL from Amersham Technologies. **Western Blot** analysis of pure **protein extracts**, were completed as above.

2.7 *Non-Transfected MCF-7 Cells: Immunoprecipitation and Immunoblotting*

MCF-7 cells were seeded at 1.5×10^6 MCF-7 cells/100mm plate, in phenol red free (PRF) DMEM with 10% charcoal stripped FCS. Two 100 mm plates were used for each IP. Cells were grown for 48 hours in phenol red free (PRF) DMEM with 10% charcoal stripped FCS and exposed to either 10^{-9} M estradiol (E_2), or an equal volume of ethanol (vehicle), for 6 hours prior to harvesting with 1X Triton Solution [1M PMSF and 1X PI; added to buffer immediately before IP]. Plates were washed with 10 ml 1X PBS then 500 μl of 1X Triton solution was added to each plate. Cells were scraped and allowed to sit on ice for 20 minutes. Lysate was pulled through a syringe ~ 15 - 20 times and lystate from two 100mm plates was transferred to a 1.5ml tube. Insoluble material was removed by centrifugation at 16,000 x g, 4 °C, 5 minutes. Supernatant was removed and placed in a new 1.5ml tube at which point a 1:100 dilution of anti-pan hMI-ER1 antibody or 1:50 dilution of anti-hMI-ER1α or β- specific antibodies [produced in our laboratory, (Paterno, et al., 2002)] was added. Anti-hMI-ER1 antiserum was prepared by immunizing rabbits, as in Ryan and Gillespie (1994), with the following synthetic peptides: i. pan hMI-ER1: 16 - CSDDHEFGPSTDMLVHD - 32; ii. α-specific: 413 - CQMLLPVHFSAISSR - 426; iii. β-specific: 466 - CDFDEKSERPAKRRRV - 480. Tubes were rotated overnight at 4 °C. 50 μl of a 50% slurry of Protein A beads was then added to tubes and rotated 1 hour at 4 °C. Bound beads were washed three times with 1X Triton Solution, and twice with 150 mM NaCl. Beads were resuspended in 30 μl of 2x SDS loading buffer, boiled 4

minutes and loaded on 8% SDS-polyacrylamide gel. ERα TNT lanes contained 2μl (between 100,000 to150,000 cpm) of [^{35}S] methionine-labeled *in vitro* translated ERα and 30 μl SDS loading buffer. After proteins were run on the SDS gel, the gel was washed 3-4 times, shaking, in 1X transfer buffer for 15 minutes, then proteins were transferred to a nitrocellulose membrane, as above. The blot was then shaken in 5% skim milk powder/1X TBT-T [20mM Tris pH7.6, 137 mM NaCl, 0.1% Tween-20, dH$_2$O], at room temperature for 2 hours. **Western blot** analysis for **IP samples** was performed with a 1:800 dilution of a mouse monoclonal anti-ERα (Santa Cruz; sc-8005) antibody in 1X TBS-T. Blots were shaken overnight at 4 °C and then washed 4-5 times in 1 hour at room temperature in 1X TBS-T. Blots were then shaken 1 hour at room temperature in a 1:3000 dilution of sheep anti-mouse conjugated to HRP secondary antibody in 1X TBS-T. Finally, blots were washed in 1X TBS-T, shaking, for 1 hour at room temperature. Samples were analyzed with chemiluminescent detection using ECL Western Blotting System and Hyperfilm ECL from Amersham Technologies.

Western Blot analysis of pure **protein extracts**, were completed as above except for verification of endogenous hMI-ER1, the nitrocellulose membrane was shaken in 5% skim milk powder/ 1XTBS-T for two hours and then for 2 hours in a 1:2500 dilution of anti-pan hMI-ER1 antibody in 0.5% skim milk powder/1X TBS-T. The membrane was washed 4 to 5 times, shaking 1 hour in 0.5% skim milk powder/1X TBS-T, then shaken in 1:2500 dilution of donkey anti-rabbit HRP secondary antibody in 0.5% skim milk powder/1X TBS-T. Finally, membranes were washed 1 hour, shaking, in 1X TBS-T.

2.8 *Reporter Assays*

2.8.1 *Luciferase Assays*

For luciferase assays, 3.0×10^5 MCF-7 cells/well were seeded in 6-well plates in PRF

DMEM ~18 hours prior to transfection. For the first set of luciferase assays, cells were

transiently transfected with 0.5 μg of vERE(3)TKLuc reporter plasmid and 0.5 μg of

either pCS3+MT, pCS3+MT-*hmi-er1α*, or pCS3+MT-*hmi-er1β*. The second set of

luciferase assays were completed with 0.8 μg of pS2-ERELuc, or 0.8 μg pS2ΔERELuc

reporter plasmids and 0.8 μg of either pCS3+MT, pCS3+MT-*hmi-er1α* or pCS3+MT-

hmi-er1β . Cells were treated with 10^{-9} M E_2 [Sigma], or an equivalent volume of ethanol,

for 48 hours after transfection. MCF-7 cells were collected by trypsinization as follows:

medium was aspirated, cells were washed with 2ml 1X PBS per well. PBS was aspirated

off cells and 200 μl of trypsin (Gibco) was added per well. Tyrpsin was left on cells for

approximately 6 minutes at which point 1 ml of DMEM (plus serum) was added to the

well. Medium was pipetted up and down to break up cell clusters and the 1ml was then

placed into an Eppendorf tube. Tubes with trypsinzed cells were then centrifuged at 600 x

g for 8 minutes. Supernatant was aspirated and the pelleted cells were stored at -70 °C.

To prepare cell lysate, 100 μl of 1X Reporter Lysis Buffer (E3971, Promega) was added

to the pelleted cells. The Eppendorf tube was vortexed in order to resuspend pelleted

cells. Cells and lysis buffer were incubated on ice for a minimum of 30 minutes. During

this time, luciferase assay substrate (Promega) was removed from -70 °C and allowed to

come to room temperature. After the 30 minute incubation, the lysed cells were re-vortexed and spun at 16,000 x g for two minutes at 4°C. Supernatant was removed and placed into new tubes. Luciferase assays were performed by mixing 20 µl of the cell lysate with 100 µl of luciferase assay substrate and by using a Monolight 2010 Luminometer (Analytical Luminescence Laboratory). The relative luciferase units (RLU) were normalized to the amount of cellular protein (assayed as outlined below) in each sample and plotted, in order to account for possible variations between experimental samples. A control vector, such as β-galactosidase, is sometimes used in such experiments to normalize for transfection efficiency. Such a control was not used in this case, however, due to the fact that hMI-ER1 is known to interact with transcriptional regulatory components such as HDACs and CBP/p300. Endogenous or transfected hMI-ER1 could, therefore, interfere with the transcription, translation and/or activity of β-galactosidase, rendering it an inefficient measure for transfection efficiency. Each experiment was repeated two to three times, as indicated.

2.8.2 Biorad Assays

Biorad assays were completed in order to determine the amount of protein present in each cell lysate. To calibrate the spectrophotometer, 4 µl of 1X lysis buffer was mixed with 796 µl dH$_2$O and 200 µl Biorad reagent. For samples of BSA, five different amounts of BSA were used (1.4, 2.8, 5.6, 8.4 or 11.2 µg) along with 4 µl of 1X lysis buffer for each sample. dH$_2$O was added to bring this volume up to 800 µl, at which point 200 µl of

Biorad reagent was added to each reaction. For each sample, 4 µl of cell lysate was mixed with 796 µl of dH$_2$O and 200 µl of Biorad reagent. All reactions were left at room temperature for a minimum of 10 minutes and all readings were completed in a spectrophotometer at a wavelength of 595nm.

3. Results:

3.1 *In Vitro Interaction of hMI-ER1 With Various Nuclear Hormone Receptors*

In order to investigate whether hMI-ER1α or hMI-ER1β interact with nuclear

hormone receptors, a series of GST-pull down assays were performed using *in vitro*

translated ^{35}S-labeled ERα, RARα, RARβ, RARγ, RXRα, or RXRγ receptors and GST-

hMI-ER1α or GST-hMI-ER1β fusion proteins. Preliminary GST-pull down assays

revealed that both GST-hMI-ER1α and GST-hMI-ER1β interact with each receptor.

Further GST-pull down assays were then completed to determine the amount of GST-

fusion protein required for interaction of GST-hMI-ER1 with each of the receptors. *In*

vitro translated ^{35}S-labeled HDAC was also used as a positive control in a number of the

GST- pull down assays completed.

A series of GST-pull down assays were completed with serial dilutions of GST-

MI-ER1β (0.25, 0.5, 1, or 2 μg) and a constant amount of *in vitro* translated ^{35}S-labeled

receptor (ERα, RARα, RARβ, RARγ, RXRα, or RXRγ) [100,000 cpm]. Each assay was

repeated twice. The *in vitro* translated ^{35}S-labeled receptors did not interact with GST

alone, however, full length hMI-ER1β was found to interact, in a dose dependent manner,

with each of the nuclear hormone receptor listed (Figures 4 - 9, respectively). These

results demonstrated that hMI-ER1β interacts with ERα, RARα, RARβ, RARγ, RXRα,

and RXRγ. For all subsequent GST-pull down assays, 1 μg of full length GST-hMI-

ER1α or GST-hMI-ER1β was used.

48

Figure 4: hMI-ER1β interacts with ERα *in vitro*.

GST-pull down assays were performed by incubating *in vitro* translated ^{35}S-labeled ERα

protein (100,000 cpm) with 0.25 μg, 0.5 μg, 1 μg, or 2 μg of GST alone or GST-MI-

ER1β. An autoradiography of a dried SDS-polyacrylamide gel is shown with the

positions of the marker proteins indicated on the left. The position of ERα (approximately

65.5 kDa) was determined by running an *in vitro* translated ^{35}S-labeled ERα along side
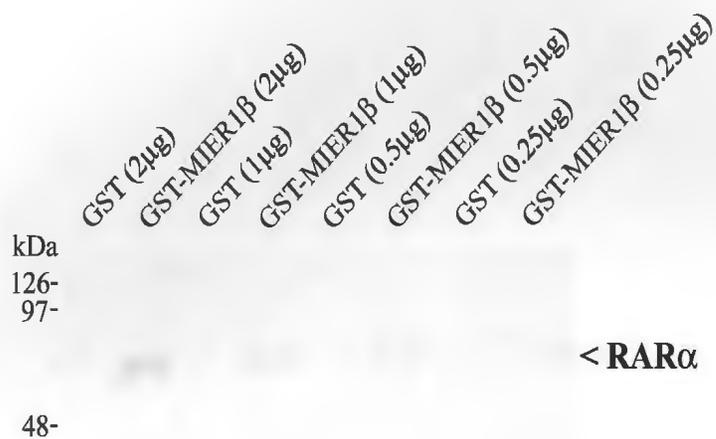
samples.

49

Figure 5: hMI-ER1β interacts with RARα *in vitro*.

GST-pull down assays were performed by incubating *in vitro* translated ^{35}S-labeled

RARα protein (100,000 cpm) with 0.25 μg, 0.5 μg, 1 μg or 2 μg of GST alone or GST-

MI-ER1β. An autoradiography of a dried SDS-polyacrylamide gel is shown with the

positions of the marker proteins indicated on the left. The position of RARα

(approximately 50.1 kDa) was determined by running an *in vitro* translated ^{35}S-labeled
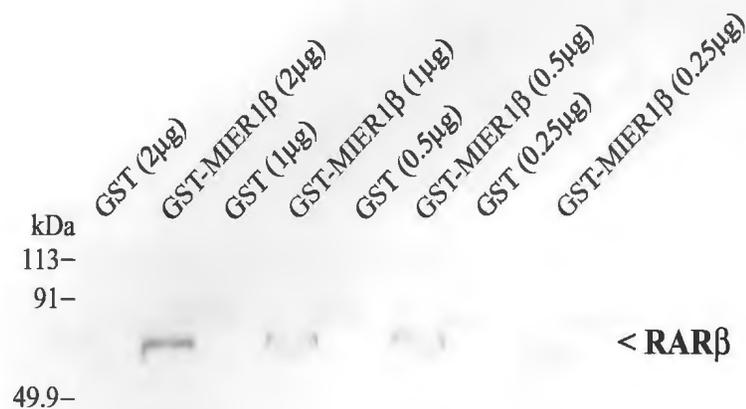
RARα along side samples.

Figure 6: hMI-ER1β interacts with RARβ *in vitro*.

GST-pull down assays were performed by incubating *in vitro* translated ³⁵S-labeled

RARβ protein (100,000 cpm) with 0.25 μg, 0.5 μg , 1 μg or 2 μg of GST alone or GST-

MI-ER1β. An autoradiography of a dried SDS-polyacrylamide gel is shown with the

positions of the marker proteins indicated on the left. The position of RARβ

(approximately 50.0 kDa) was determined by running an *in vitro* translated ³⁵S-labeled

RARβ along side samples.

Figure 7: hMI-ER1β interacts with RARγ *in vitro*.

GST-pull down assays were performed by incubating *in vitro* translated ^{35}S-labeled

RARγ protein (100,000 cpm) with 0.25 μg, 0.5 μg, 1 μg or 2 μg of GST alone or GST-

MI-ER1β. An autoradiography of a dried SDS-polyacrylamide gel is shown with the

positions of the marker proteins indicated on the left. The position of RARγ

(approximately 50.0 kDa) was determined by running an *in vitro* translated ^{35}S-labeled

RARγ along side samples.

Figure 8: hMI-ER1β interacts with RXRα *in vitro*.

GST-pull down assays were performed by incubating *in vitro* translated ^{35}S-labeled

RXRα protein (100,000 cpm) with 0.25 μg, 0.5 μg, 1 μg or 2 μg of GST alone or GST-

MI-ER1β. An autoradiography of a dried SDS-polyacrylamide gel is shown with the

positions of the marker proteins indicated on the left. The position of RXRα

(approximately 51.4 kDa) was determined by running an *in vitro* translated ^{35}S-labeled
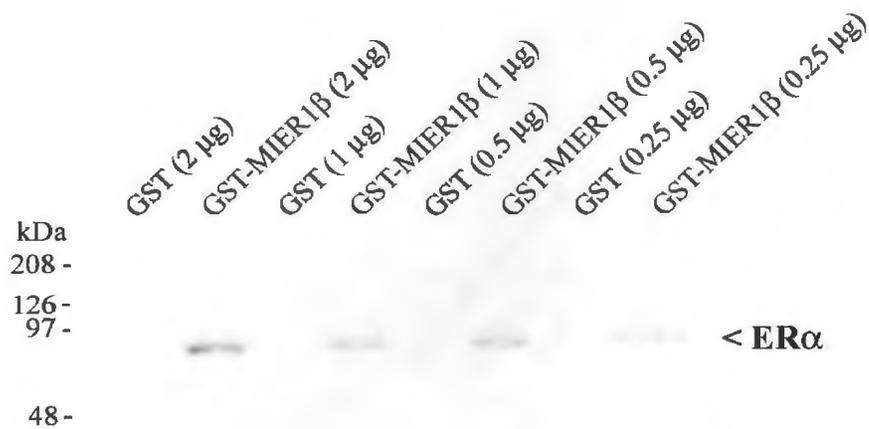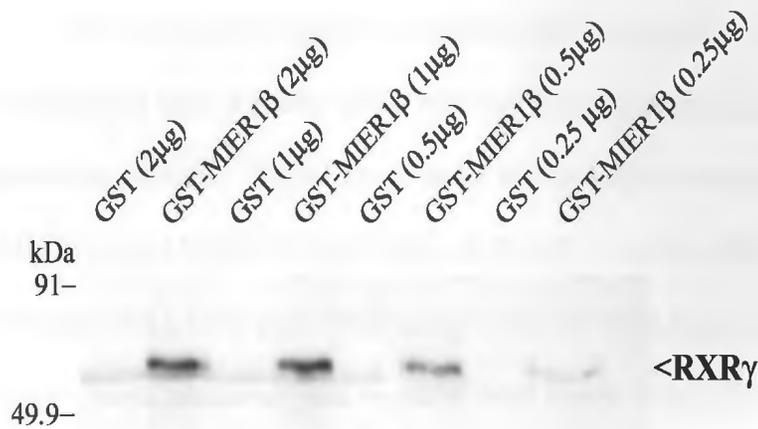
RXRα along side samples.

Figure 9: hMI-ER1β interacts with RXRγ *in vitro*.

GST-pull down assays were performed by incubating *in vitro* translated ^{35}S-labeled

RXRγ protein (100,000 cpm) with 0.25 μg, 0.5 μg, 1 μg or 2 μg of GST alone or GST-

MI-ER1β. An autoradiography of a dried SDS-polyacrylamide gel is shown with the

positions of the marker proteins indicated on the left. The position of RXRγ

(approximately 51.0kDa) was determined by running an *in vitro* translated ^{35}S-labeled

RXRγ along side samples.

3.1.1 *Ligand Independent Interaction of hMI-ER1 and RARα or RARβ In Vitro*

The binding of a ligand to a nuclear hormone receptor will change the receptor conformation and, in many cases, will lead to subsequent changes in nuclear receptor interacting partners. Therefore, in order to investigate whether the interaction between hMI-ER1α or hMI-ER1β and RARα or RARβ would be affected by the presence of ligand (RA), GST-pull down assays with 10^{-6} M RA, or an equal volume of DMSO (vehicle), were conducted and repeated three times. GST, GST-MI-ER1α or GST-MI-ER1β were incubated with GST beads as above. After 5 washes with GST buffer, GST-bound beads were resuspended with GST buffer with RA, or an equal volume of DMSO (vehicle), and 100,000 cpm of *in vitro* translated ^{35}S-labeled receptor. As shown in figures 10 and 11, the presence of RA did not affect the interaction between hMI-ER1α or hMI-ER1β and either of the receptors. These results indicate that the *in vitro* interaction between hMI-ER1α or hMI-ER1β and RARα or RARβ is ligand-independent.

3.1.2 *Ligand Independent Interaction of hMI-ER1 and ERα In Vitro*

To investigate whether the interaction between hMI-ER1α or hMI-ER1β and ERα would be affected by the presence of ligand (E_2), GST-pull down assays were repeated four times with 10^{-6} M E_2, or an equivalent volume of ethanol (vehicle). A representative

55

gel is shown in figure 12. Results from each GST-pull down assay completed demonstrated that both hMI-ER1α and hMI-ER1β interact with ERα *in vitro*, in a ligand-independent manner.

Two GST-pull down assays were also completed where E_2 was pre-incubated with ^{35}S-labeled ERα before being added to the GST-bound beads, in order to determine if the interaction between hMI-ER1α or hMI-ER1β and ERα would be affected by the conformational change induced in ERα upon the binding of ligand. Figure 13 shows that pre-incubation of E_2 and ERα did not affect the *in vitro* interaction between hMI-ER1α or hMI-ER1β and ERα.
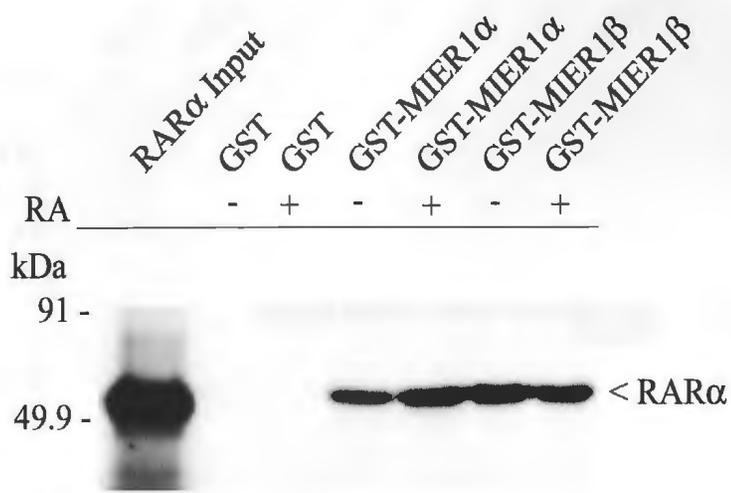
Figure 10: Ligand independent interaction between hMI-ER1α and hMI-ER1β with RARα *in vitro*.

GST-pull down assays were completed with ^{35}S-labeled RARα protein (100,000 cpm) and 1.3×10^{-11} moles of either GST alone, GST-MIER1α, or GST-MI-ER1β and 10^{-6} M retinoic acid (RA)(+) or DMSO (vehicle)(-), and repeated three times. An autoradiography of a dried SDS-polyacrylamide gel is shown with the positions of the marker proteins indicated on the left. The RARα input lane represents 10% of the input of ^{35}S-labeled RARα protein used in each pull down assay.
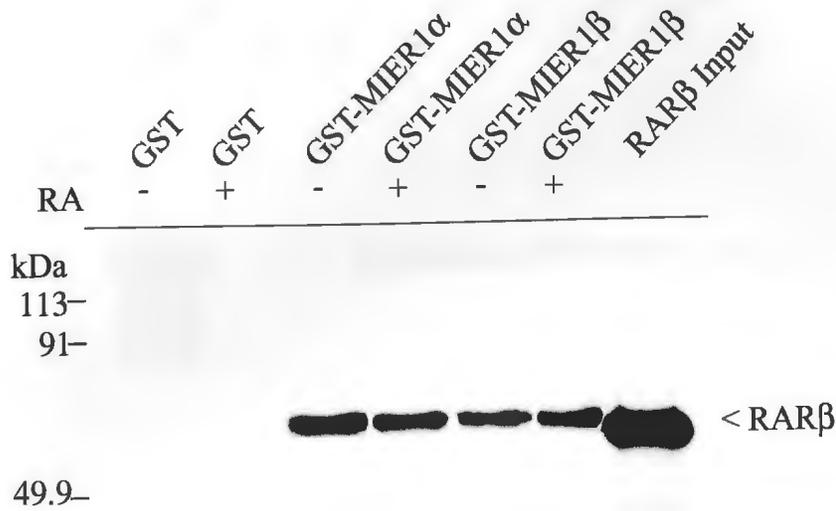
Figure 11: Ligand independent interaction between hMI-ER1α and hMI-ER1β with RARβ *in vitro*.

GST-pull down assays were completed with ^{35}S-labeled RARβ protein (100,000 cpm) and 1.3×10^{-11} moles of either GST alone, GST-MIER1α, or GST-MI-ER1β and 10^{-6} M retinoic acid (RA)(+) or DMSO (vehicle)(-), and repeated three times. An autoradiography of a dried SDS-polyacrylamide gel is shown with the positions of the marker proteins indicated on the left. The RARβ input lane represents 10% of the input of ^{35}S-labeled RARβ protein used in each pull down assay.

58
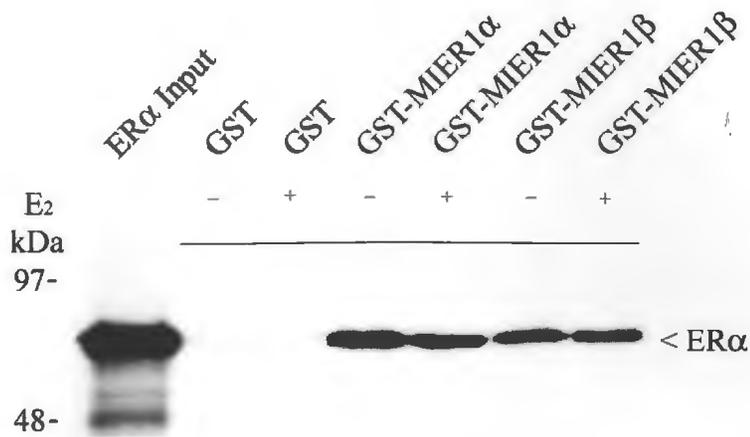
Figure 12: Ligand independent interaction between hMI-ER1α and hMI-ER1β with ERα _in vitro_.

GST-pull down assays were completed with ^{35}S-labeled ERα protein (100,000 cpm) and 1.3 x 10^{-11} moles of either GST alone, GST-MIER1α, or GST-MI-ER1β and 10^{-6} M estradiol (E_2) (+) or ethanol (vehicle)(-), and repeated four times. An autoradiography of a dried SDS-polyacrylamide gel is shown with the positions of the marker proteins indicated on the left. The ERα input lane represents 10% of the input of ^{35}S-labeled ERα protein used in each pull down assay.

59
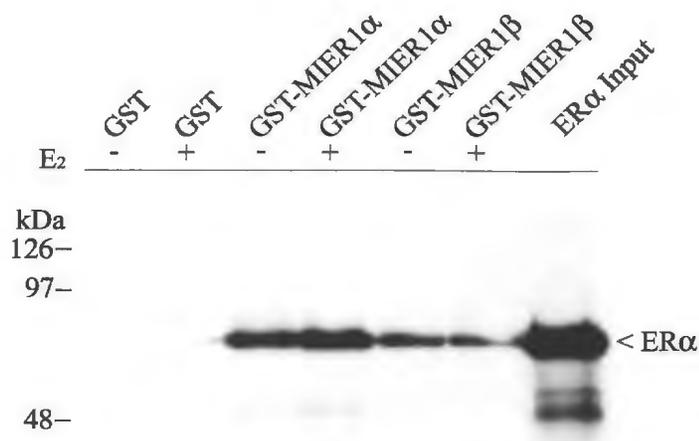
Figure 13: Pre-incubation of E$_2$ and ERα does not alter the interaction between hMI-ER1α or hMI-ER1β with ERα *in vitro*.

GST-pull down assays were completed by incubating 1.3 x 10^{-11} moles of GST alone, GST-MIER1α, or GST-MI-ER1β with GST beads, rotating one hour, at 4°C. ^{35}S-labeled ERα protein (100,000 cpm) and 10^{-6} M estradiol (E$_2$)(+) or ethanol (vehicle)(-) were pre-mixed and incubated together for 15 minutes, before being added to the GST-bound beads. An autoradiography of a dried SDS-polyacrylamide gel is shown with the positions of the marker proteins indicated on the left. The ERα input lane represents 10% of the input of ^{35}S-labeled ERα protein used in each pull down assay.

60

3.1.3 *The Amino Acids of hMI-ER1 Required for Interaction with ERα are Located in the region 325-357*

Previous studies have indicated that hMI-ER1 levels are increased in breast tumour cells and tissues [Paterno, et al., 1998]. Given the importance of ER in breast cancer, subsequent studies for this research focused on characterizing the interaction between hMI-ER1 and ERα. In order to determine which region of hMI-ER1α or β is responsible for the interaction with ERα *in vitro*, GST-hMI-ER1 deletion constructs, expressed and purified from E. coli BL21, were used in a series of GST-pull down assays with *in vitro* translated ^{35}S-labeled ERα. Each GST-pull down assay was repeated four times.

The complete amino acid sequence of hMI-ER1α, shown in figure 14, indicates the locations of the ELM2, SANT and LXXLL domains. The first series of constructs included fusion proteins consisting of the acidic acid domain (aa 1-155), the ELM2 domain (aa 164-283), the acidic activation and ELM2 domains (aa 1-283), the SANT domain and α C-terminus (aa 287-433) or the SANT domain and the β C-terminus (aa 286-512) (Figure 15A). The interaction between these constructs and ERα was compared to the interaction between full length GST-hMI-ER1α or GST-hMI-ER1β and ERα. Figure 15B shows a representative GST-pull down assay. There was no interaction between any of the N–terminal GST-hMI-ER1 constructs (aa 1-155, 164-283 or 1-283) and ERα (Figure 15B). A strong interaction was observed between hMI-ER1 C-terminal

61

constructs (aa 287-433 and 286-512) and ERα (Figure 15B). Results indicate that it is the

C-terminal region of hMI-ER1 that is responsible for the interaction with ERα. These

results also highlight the fact that the interaction between hMI-ER1 and ERα does not

occur through the LXXLL motif, since both hMI-ER1α and β interact with ERα.

To further define the region responsible for the interaction between ERα and hMI-

ER1, GST-pull downs assays were completed with GST-hMI-ER1 deletion constructs

including the SANT domain and extending to the α C-terminus (aa 287-433), the SANT

domain and extending to the end of the common region (aa 287-410), including the

SANT domain and amino acids C-terminal to the SANT domain (aa 287-357), lacking

the last amino acid of the SANT domain (aa 287-330), including the last seven amino

acids of the SANT domain and extending through to the α C-terminus (aa 325-433), and

lacking the SANT domain and including the α C-terminus (aa 355-433) (Figure 16A).

A strong interaction was observed between ERα and GST-hMI-ER1 constructs aa 287-

433, aa 287-410, and aa 287-357, all of which had an intact SANT domain as well as

amino acids downstream of the SANT domain. An interaction was also observed

between ERα and the GST-hMI-ER1 construct aa 325-433. However, deletion of the last

aa of the SANT domain (K), and amino acids flanking the C-terminus of the SANT

domain (aa 287-330 or aa 355-433), completely abolished interaction with ERα (Figure

16B). This deletion analysis revealed that the sequence required for the interaction with

ERα can be found in the region aa 325-357 of hMI-ER1, encompassing the last seven

amino acids of the SANT domain and amino acids flanking the C-terminus of the domain.

```
MAEPSVESSSPGGSATSDDHEFDPSADMLVHDFDDERTLEEEEMM        45

EGETNFSSEIEDLAREGDMPIHELLSLYGYGSTVRLPEEDEEEEE        90

EEEEGEDDEDADNDDNSGCSGENKEENIKDSSGQEDETQSSNDDP       135

SQSVASQDAQEIIRPRRCKYFDTNSEVEEESEEDEDYIPSEDWKK       180

EIMVGSMFQAEIPVGICRYKENEKVYENDDQLLWDPEYLPEDKVI       225

IFLKDASRRTGDEKGVEAIPEGSHIKDNEQALYELVKCNFDTEEA       270

LRRLRFNVKAAREELSV**WTEEECRNFEQGLKAYGKDFHLIQANKV**   315

**RTRSVGECVAFYYMWK**KSERYDFFAQQTRFGKKKYNLHPGVTDYM   360

DRLLDESESAASSRAPSPPPTASNSSNSQSEKEDGTVSTANQNGV       405

SSNGPGIL**QMLL**PVHFSAISSRANAFLK                    433
        ↑
```

Figure 14: Complete amino acid sequence of hMI-ER1α.
The complete amino acid sequence of hMI-ER1α (N3α; aa 1-433) is shown, with
amino acid numbers indicated on the right. The ELM2 domain is underlined, the
SANT domain is indicated in bold and the LXXLL motif is indicated in pink. The
arrow indicates the beginning of the α C-terminus.

63

Figure 15: ERα interacts with the C–terminal regions of hMI-ER1α and hMI-ER1β *in vitro*.

A. Schematic representing GST deletion constructs. The names of each construct, followed by the amino acid numbers present in respective construct, are indicated on the left of each construct. The acidic activation domain, ELM2 and SANT domains as well as alternate α and β C-termini are indicated.

B. *In vitro* translated ^{35}S-labeled ERα protein (10,000 cpm) was loaded directly onto the gel (lane 1). For each pull down assay, *in vitro* translated ^{35}S-labeled ERα protein (100,000 cpm) was incubated with GST alone (lane 2), GST-MI-ER1α (lane 3), GST-MI-ER1β (lane 4), or with deletion constructs Δ5, Δ13, Δ4, Δ9, or Δ16 (lanes 5, 6, 7,8, and 9 respectively). An autoradiography of a dried SDS-polyacrylamide gel is shown with the positions of the marker proteins indicated on the left. The ERα input lane represents 10% of the input of ^{35}S-labeled ERα protein used in each pull down assay.

A.

GST

α(1-433) GST

β(1-512) GST

Δ 5 (1-155) GST

Δ13 (164-283) GST

Δ4 (1-283) GST

Δ9 (287-433) GST

Δ 16 (286-512) GST

▤ acidic activation domain   ▨ SANT domain (aa 288-331)

▦ ELM-2 domain (aa 180-284)   ☐ α C-terminus ▦ β C-terminus

B.

KDa
117-
95-

49-

< ERα
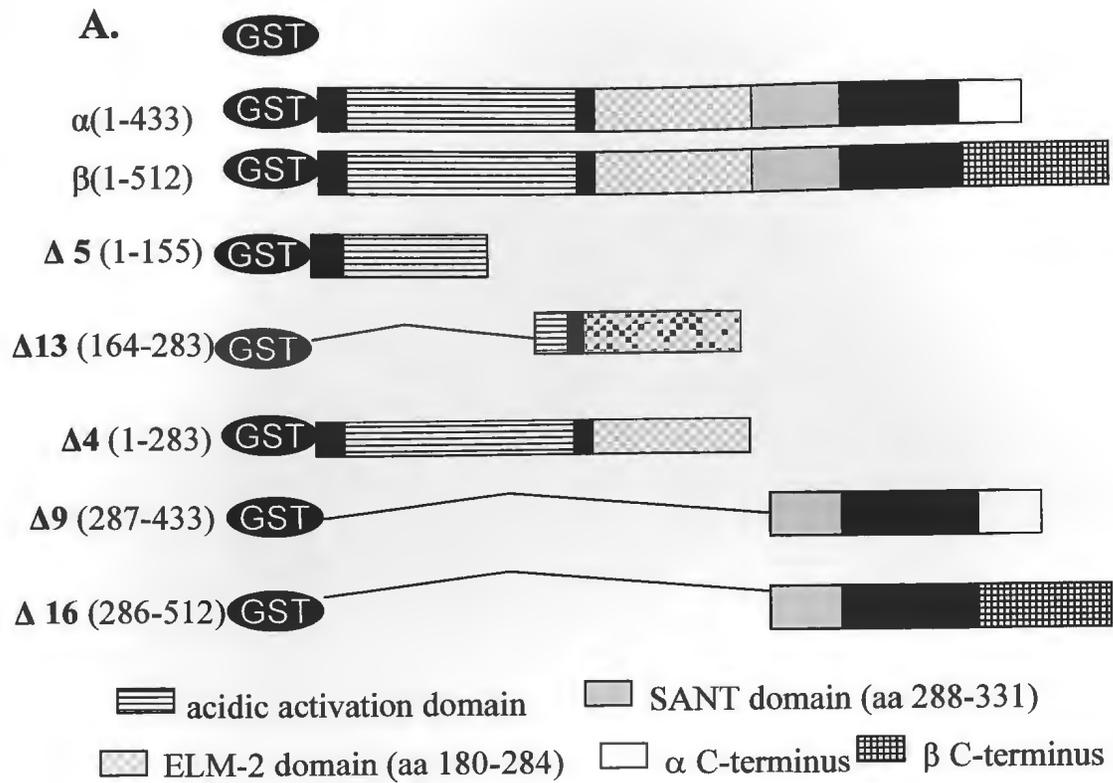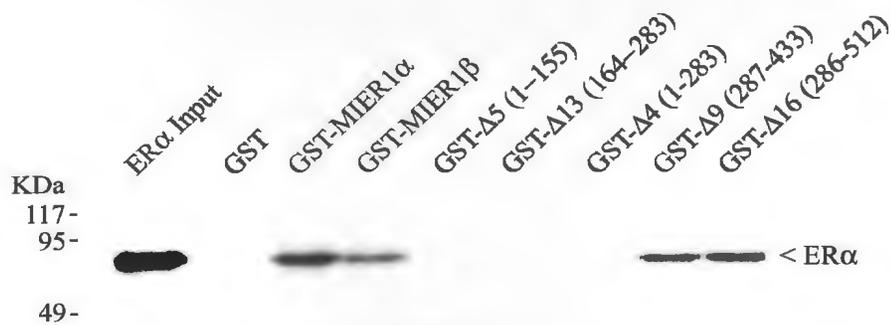
Figure 16: The ERα interaction domain is located within the region of amino acids 325-357 of hMI-ER1 *in vitro*.

A. Schematic representing GST deletion constructs. Amino acid numbers present in respective constructs are given.

B. *In vitro* translated ^{35}S-labeled ERα protein (10,000 cpm) was loaded directly onto the gel (lane 1). GST pull down assays were completed with *in vitro* translated ^{35}S-labeled ERα protein (100,000 cpm) and GST-Δ9, GST-Δ17, GST-Δ18, or GST-Δ19, GST-Δ10, or GST-Δ11 (lanes 2 - 7, respectively). An autoradiography of a dried SDS-polyacrylamide gel is shown with the positions of the marker proteins indicated on the right. The ERα input lane represents 10% of the input of ^{35}S-labeled ERα protein used in each pull down assay.

A.

Δ9 (287-433) GST ——————————————— [SANT | β C-terminus | α]

Δ 17 (287 - 410) GST ——————————————— [SANT | β C-terminus]

Δ 18 (287 - 357) GST ——————————————— [SANT | β]

Δ 19 (287 - 330) GST ——————————————— [SANT]

Δ 10 (325 -433) GST ——————————————— [β C-terminus | α]

Δ 11 (355 - 433) GST ——————————————— [β C-terminus | α]

☐ α C-terminus   ▦ β C- terminus   ▨ SANT domain (288-331)

B.

kDa
113 -

ERα Input | GST-Δ9 (287-433) | GST-Δ17 (287-410) | GST-Δ18 (287-357) | GST-Δ19 (287-330) | GST-Δ10 (325-433) | GST-Δ11 (355-433)

< ERα

49.9 -
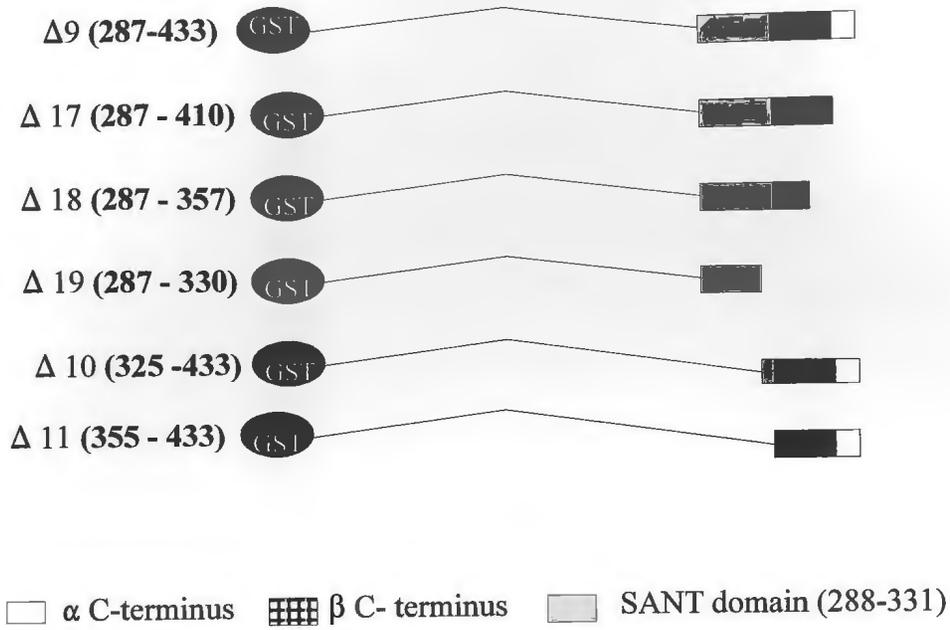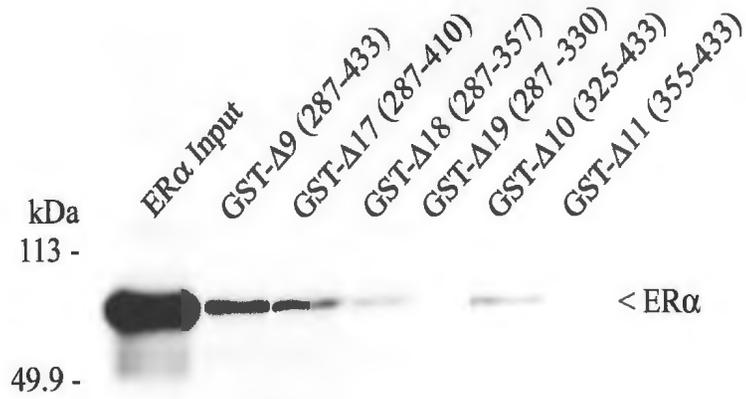
3.2 *In Vivo Interaction of hMI-ER1 and ERα*

3.2.1 *hMI-ER1α and hMI-ER1β interact with ERα, in MCF-7 cells*

To investigate whether the interaction observed between hMI-ER1α or hMI-ER1β and ERα *in vitro* would also occur *in vivo*, a human ER (+) breast cancer cell line (MCF-7), was transiently transfected with pCS3+MT vector (Myc-tagged vector), pCS3+MT-*hmi-er1α*, or pCS3+MT-*hmi-er1β*. Transfection experiments were repeated numerous times under varying conditions (ie: altering concentration of plasmid or antibody, different buffers, varying length of time for E_2 treatment, etc.) in order to optimize cell growth conditions and to examine whether final results would be affected. Cells were subjected to treatment with 10^{-9} M E_2 dissolved in ethanol, or an equal volume of ethanol alone, for 6 hours (repeated four times) or 48 hours (repeated six times) prior to extraction. Results did not vary by changing the exposure time to E_2. A representative blot of a 48 hour E_2-treatment is shown in Figure 17. Expression of Myc-tagged hMI-ER1α and Myc-tagged hMI-ER1β was verified for each experiment by Western blot analysis, of whole cell extracts, with an anti-Myc antibody (Figure 17A) whereas expression of endogenous ERα was verified for each experiment with an anti-ERα antibody (Figure 17B). Cell extracts were also subjected to immunoprecipitation with anti-Myc antibody, followed by Western blot analysis with anti-ERα antibody. Both hMI-ER1α and hMI-ER1β immunoprecipitated with endogenous ERα, in MCF7 cells, but only in the absence of ligand (Figure 17C). Altering the amounts of antibody used,

changing the exposure time to E_2, and co-transfection of both hMI-ER1 and ERα all garnered the same result (data not shown). These studies demonstrated an *in vivo* interaction between hMI-ER1α or β and ERα.

Figure 17: hMI-ER1α and hMI-ER1β interact with ERα in the absence of E$_2$, in MCF-7 cells.

A. MCF-7 cells were grown in PRF DMEM for approximately 18 hours. Cells were transfected with 1 μg of pCS3+MT vector, pCS3+MT-*hmi-er1*α or pCS3+MT-*hmi-er1*β and, after approximately 48 hours, were treated for 6 hours with 10^{-9} M E$_2$, or an equivalent volume of ethanol. Cell lysates were prepared and loaded directly on the gel (lanes 3 - 8). Western blot analysis with anti-Myc antibody followed. ^{35}S-labeled *in vitro* translated Myc-tagged hMI-ER1α or Myc-tagged hMI-ER1β (~100,000 cpm) were loaded directly on the gel (lanes 1 and 2) to indicate the position of ERα in the cell extracts. The position of marker proteins are indicated.

B. Cell lysates of MCF-7 cells transfected with 1 μg of pCS3+MT vector, pCS3+MT-*hmi-er1*α or pCS3+MT-*hmi-er1*β and treated for 6 hours with 10^{-9} M E$_2$, or an equivalent volume of ethanol, were prepared and loaded directly onto the gel, which was followed by Western blot analysis with monoclonal anti-ERα antibody. ERα was expressed but, as expected, ERα was slightly downregulated by the treatment of cells with E$_2$ [Saceda, et al., 1988]. ^{35}S-labeled *in vitro* translated ERα (~100,000 cpm) was loaded directly on the gel (lane1) to indicate the position of ERα in the cell extracts. The position of marker proteins are indicated on the left.

C. Cell lysates from MCF-7 cells, transiently transfected with 1 μg of CS3+MT vector, pCS3+MT-*hmi-er1*α, or pCS3+MT- *hmi-er1*β were prepared and subjected to IP with anti-Myc antibody (1:25); western blot analysis was performed using a polyclonal anti-ERα antibody (1:1000). Cells were treated with 10^{-9} M E$_2$, or an equal volume of ethanol (vehicle), prior to cell lysis. ^{35}S-labeled ERα protein (100,000 cpm, lane 1) and cell lysate from non-transfected MCF-7 cells, treated with 10^{-9} M E$_2$ (lane 2), were added directly to sample buffer and used to indicate the position of ERα in the cell extracts. Protocols were repeated four times. The position of marker proteins are indicated on the left.

68

A.

WB: _____ anti-Myc _____

myc-MI-ER1α TnT
myc-MI-ER1β TnT
vector
vector
myc-MI-ER1α
myc-MI-ER1α
myc-MI-ER1β
myc-MI-ER1β

E₂ − + − + − +

kDa
−117 −

MI-ER1β > − 95 −
MI-ER1α > −49.9−

B.

WB: _____ anti-ERα _____

ERα TnT
vector
vector
myc-MI-ER1α
myc-MI-ER1α
myc- MI-ER1β
myc-MI-ER1β

E₂ − + − + − +

kDa
117−
95− ◄ ERα

C.

IP: ___ anti-Myc _____

WB: _____ anti-ER α _____

ERα TnT
cell lysate
vector
vector
myc-MI-ER1 α
myc-MI-ER1 α
myc-MI-ER1 β
myc-MI-ER1 β

E₂ + − + − + − +

kDa
126 -
97 - ◄ ERα
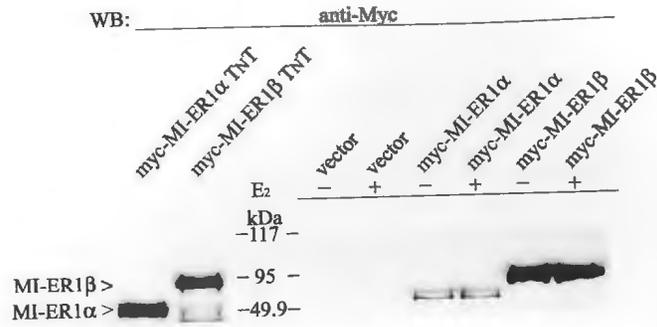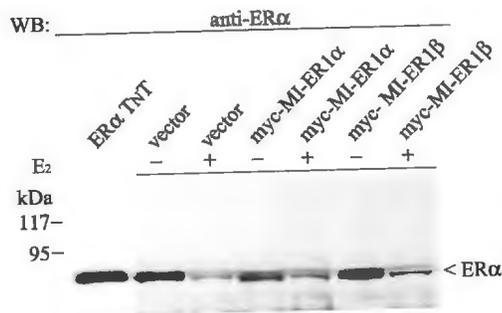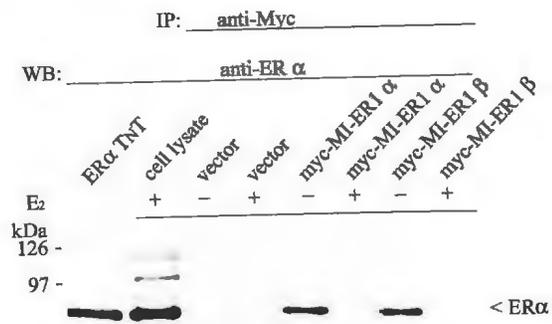
3.2.2 *Interaction Between Endogenous hMI-ER1 and ERα Proteins in MCF-7 Cells*

In order to exclude the possibility that the observed interaction was an artifact of overexpression, the next three sets of experiments were conducted in order to determine if endogenous hMI-ER1α or hMI-ER1β would interact with endogenous ERα in *untransfected* MCF-7 cells. MCF-7 cells were simply grown in phenol red free (PRF) DMEM and subjected to treatment with 10^{-9} M E$_2$, or an equal volume of ethanol, for 6 hours prior to extraction. Cell extracts were either loaded directly (Figure 18, lanes 2 and 3) or subjected to immunoprecipitation with a pan-hMI-ER1 antibody (Figure 18, lanes 4 and 5), pre-immune serum (Figure 18, lanes 6 and 7) or polyclonal anti-ERα antibody (Figure 18, lanes 8 and 9). Western blot analysis with a monoclonal anti-ERα followed. In this case, hMI-ER1α and hMI-ER1β interacted with ERα in a ligand independent manner, unlike earlier *in vivo* interaction analyses but similar to *in vitro* results.

Co-immunoprecipitation was also performed using anti-hMI-ER1α-specific or anti-hMI-ER1β-specific antibodies for immunoprecipitation (Figure 19, lanes 1 - 4). These results were consistent with previous *in vivo* interaction analyses with transfected MCF-7 cells (Figure17C), where hMI-ER1α and hMI-ER1β only immunoprecipitated with endogenous ERα in the absence of ligand.

69

WB: _____ anti-ERα _____

ERα TNT
protein extract
protein extract
IP: anti-pan hMIER1
IP: anti-pan hMIER1
IP: pre-immune serum
IP: pre-immune serum
IP: anti-ERα
IP: anti-ERα

E$_2$ -   +   -   +   -   +   -   +

KDa
95 -

49 -
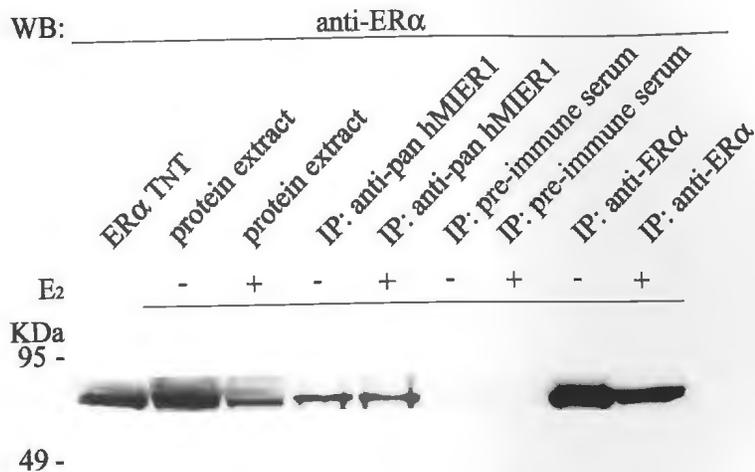
Figure 18: IP of MCF-7 cell lysates with hMI-ER1 antibody shows that endogenous hMI-ER1 interacts *in vivo* with endogenous ERα, in a ligand independent manner. Non-transfected MCF-7 cells were grown in PRF DMEM and charcoal-stripped FCS for ~72 hours and treated with 10^{-9} M E$_2$, or an equal volume of ethanol (vehicle), for 6 hours prior to harvesting, as indicated. Cell lysates were prepared and added directly to sample buffer (lanes 2 and 3) or subjected to IP with anti-hMI-ER1 antibody (1:100; lanes 4 and 5), IP with pre-immune serum (1:100; lanes 6 and 7), or IP with polyclonal anti-ERα (1:100; lanes 8 and 9); western blot analysis was completed with monoclonal anti-ERα (1:800). ^{35}S-labeled ERα protein (100,000 cpm) was used to verify the identity of the bands in the cell extracts (lane 1). The position of marker protein is indicated on the left.

70
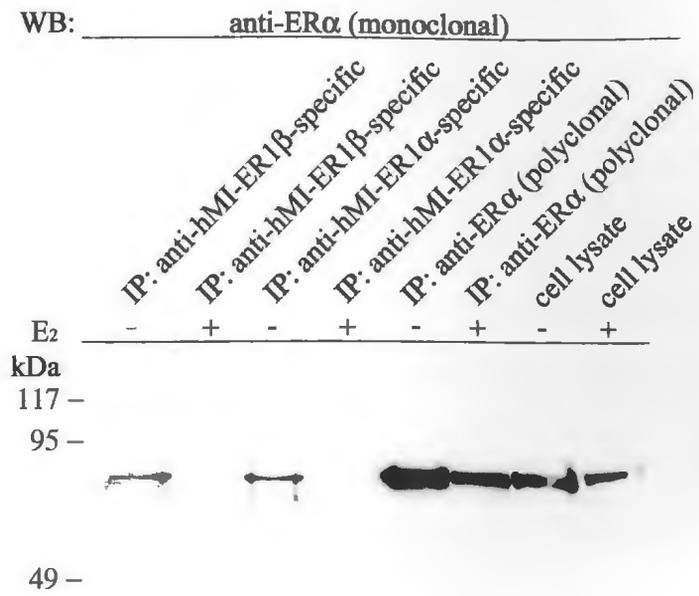
Figure 19: **IP of MCF-7 cell lysates with hMI-ER1α-specific or hMI-ER1β-specific antibody shows that endogenous hMI-ER1α and hMI-ER1β interact *in vivo* with endogenous ERα, in the absence of E_2.**

Non-transfected MCF-7 cells were grown in PRF DMEM and charcoal-stripped FCS for ~72 hours and treated with 10^{-9} M E_2, or an equal volume of ethanol (vehicle), for 6 hours prior to harvesting, as indicated. Cell lysates were prepared and added directly to sample buffer (lanes 7 and 8) or subjected to IP with anti-hMI-ER1β-specific antibody (1:50; lanes 1 and 2), anti-hMI-ER1α-specific antibody (1:50; lanes 3 and 4), or polyclonal anti-ERα (1:100; lanes 5 and 6); western blot analysis was completed with monoclonal anti-ERα (1:800). The position of marker protein is indicated on the left.

3.2.3 *hMI-ER1α and hMI-ER1β interact with ERα in HEK 293 Cells*

Different cell lines have varying expression profiles and can thus behave differently. For example, ERα is found in the cell membrane, cytoplasm and the nucleus of MCF7 cells [Parikh, et al., 1987] and in a study conducted by Paterno, et al., 1998, hMIER1 was expressed in nine breast carcinoma cell lines and eight breast tumour tissue samples, but, was not detectable in normal breast cell lines and breast tissues. To verify if the *in vivo* interaction between hMI-ER1α or hMI-ER1β and ERα would differ in another cell line, *in vivo* studies were repeated, five times, using HEK 293 cells (human embryonic kidney cells). HEK 293 cells were transiently transfected with pcDNA3-*ERα* and either pCS3+MT vector, pCS3+MT-*hmi-er1α*, or pCS3+MT-*hmi-er1β* and subjected to treatment with 10^{-9} M E_2, or an equal volume of ethanol, for 6 or 48 hours prior to extraction (6 hour E_2 treatment shown in Figure 20). Expression of Myc-tagged hMI-ER1α and Myc-tagged hMI-ER1β was verified for each experiment by Western blot analysis with an anti-Myc antibody (Figure 20B) and expression of ERα was verified for each experiment with an anti-ERα antibody (Figure 20C). Cell extracts were subjected to immunoprecipitation with anti-ERα antibody followed by Western blot analysis with anti-Myc antibody (Figure 20A). Both hMI-ER1α and hMI-ER1β interacted with ERα in HEK 293 cells the presence or absence of E_2, however, interaction between hMI-ER1α and ERα appear to be stronger in the absence of E_2.

All of the *in vivo* studies show that hMI-ER1α and hMI-ER1β consistently interact with ERα, in the <u>absence</u> of E_2. There was a difference, however, with the

A.

IP: _____ anti-ERα _____

WB: _____ anti-Myc _____

myc-MI-ER1α TNT   vector   vector   myc-MI-ER1α   myc-MI-ER1α   myc-MI-ER1β   myc-MI-ER1β   myc-MI-ER1β TNT

E_2 −   +   −   +   −   +

kDa
117−
95−

MI-ER1α >

< MI-ER1β

B.

WB: _____ anti-Myc _____

myc-MI-ER1α TNT   myc-MI-ER1β TNT   myc-MI-ER1α   myc-MI-ER1α   myc-MI-ER1β   mcy-MI-ER1β

−   +   −   + E_2

kDa
− 117 −
− 95 −

< MI-ER1β
< MI-ER1α

C.

WB: ___ anti-ERα ___

myc-MI-ER1β   myc-MI-ER1β

E_2 −   +

kDa
95−

< ERα

49−
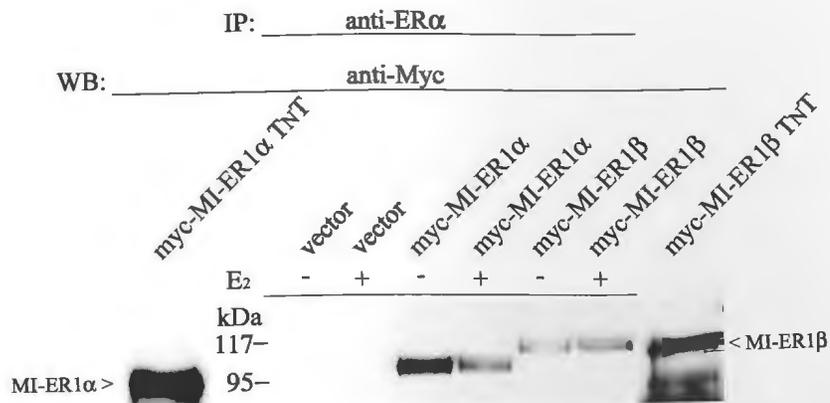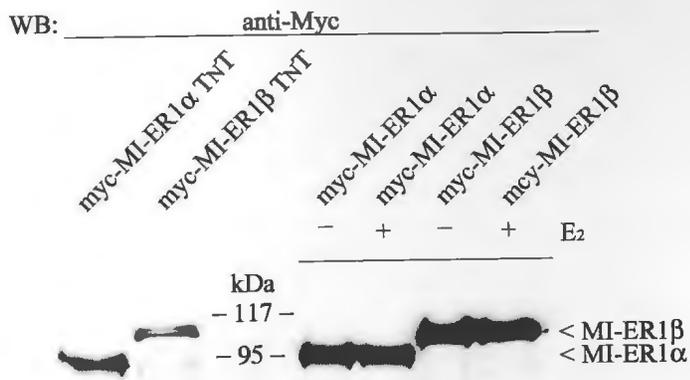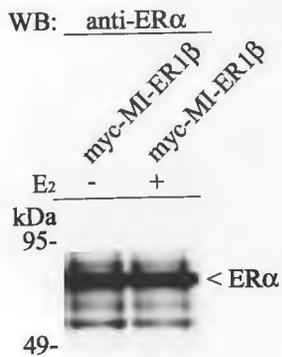
presence of E_2. As summarized in Table 3, no interaction was observed between hMI-ER1α or hMI-ER1β and ERα in the presence of E_2 in MCF-7 cells with protocols using anti-Myc, anti- hMI-ER1α-specific or anti-hMI-ER1β-specific antibodies for immunoprecipitation. An interaction between hMI-ER1α or hMI-ER1β and ERα, in the presence of E_2, was detected in MCF-7 cells when an anti-pan-hMI-ER1 antibody was employed for immunoprecipitation. An interaction between hMI-ER1α or hMI-ER1β and ERα, in the presence of E_2, was also detected in HEK 293 cells when an anti-ERα antibody was used for immunoprecipitation.

Table 3: Summary of *In Vivo* Results

	IP Antibody	WB Antibody	- E_2	+E_2
Transfected **MCF-7** Cells	anti-Myc	anti-ERα	+	-
Non-Transfected **MCF-7** Cells	anti-pan-hMI-ER1	anti-ERα	+	+
Non-Transfected **MCF-7** Cells	anti-hMI-ER1α specific	anti-ERα	+	-
Non-Transfected **MCF-7** Cells	anti-hMI-ER1β specific	anti-ERα	+	-
Transfected **HEK 293** Cells	anti-ERα	anti-Myc	+	+

[Note: "+" indicates an interaction between hMI-ER1 and ERα and "-" indicates no interaction between hMI-ER1 and ERα]

Figure 20: hMI-ER1α and hMI-ER1β interact with ERα, in the presence and absence of E_2, in HEK 293 cells

A. HEK 293 cells were transiently transfected with 0.4 μg of pcDNA3-*ERα* and 0.4 μg of either CS3+MT vector, pCS3+MT-*hmi-er1α*, or pCS3+MT-*hmi-er1β*. Cells were treated with 10^{-9} M E_2, or an equal volume of ethanol (vehicle), 6 hours prior to harvesting. Cell lysates were prepared and subjected to IP with anti-ERα antibody (1:100) (lanes 2-7); western blot analysis was performed using anti-Myc antibody (1:1000). ^{35}S-labeled Myc-tagged hMI-ER1α or Myc-tagged hMI-ER1β protein (100,000 cpm) were used to verify the identity of the bands in the cell extracts (lane 1 and 8, respectively). The position of marker proteins are indicated.

B. In order to verify expression of *hmi-er1α* or *hmi-er1β* in transfected HEK 293 cells, cell lysates of HEK 293 cells transfected with 0.4 μg of pcDNA3-*ERα* and 0.4 μg of either CS3+MT vector, pCS3+MT-*hmi-er1α*, or pCS3+MT-*hmi-er1β* and treated for 6 hours with

10^{-9} M E_2, or an equivalent volume of ethanol, were prepared and loaded directly on the gel (lanes 3 - 6). Western blot analysis with anti-Myc antibody followed. ^{35}S-labeled in vitro translated Myc-tagged hMI-ER1α or Myc-tagged hMI-ER1β (~100,000 cpm) were loaded directly on the gel (lanes 1 and 2, respectively) to verify the bands present in cell extracts. The position of marker proteins are indicated.

C. In order to verify expression of *ERα* in transfected HEK 293 cells, cell lysates of HEK 293 cells transfected with 0.4 μg of pcDNA3-*ERα* and pCS3+MT-*hmi-er1β* and treated for 6 hours with 10^{-9} M E_2, or an equivalent volume of ethanol, were prepared and loaded directly on the gel. Western blot analysis with anti-ERα followed. Expression of ERα in cells transfected with 0.4 μg of pcDNA3-*ERα* and 0.4 μg of either CS3+MT vector or pCS3+MT-*hmi-er1α* were also verified (data not shown). The position of marker proteins are indicated on the left.

74

3.3 *Effect of hMI-ER1 on ERE-Driven Transcription*:

3.3.1 *hMI-ER1β Enhances ERE-driven Transcription in Luciferase Assays with the vERE(3)TkLuc reporter plasmid*

Since both hMI-ER1α and hMI-ER1β were found to interact with ERα *in vitro* and *in vivo*, experiments were performed to determine whether this interaction affects the ability of ERα to bind to EREs and regulate transcription. The first reporter construct used for luciferase assays was vERE(3)TkLuc (Tk: thymidine kinase; Luc: luciferase). The estrogen response element in this construct was that of vitellogenin A2, a known E_2-responsive gene. MCF-7 cells were transfected with vERE(3)TkLuc and either pCS3+MT vector, pCS3+MT-*hmi-er1α*, or pCS3+MT-*hmi-er1β*, and treated with 10^{-9} M E_2, or an equivalent volume of ethanol, for 48 hours prior to harvesting. Cell lysates were collected and relative luciferase units (RLU) were determined and normalized to the amount of cellular protein in each sample. The ability of hMI-ER1α and hMI-ER1β to regulate transcription can be analyzed by comparing the level of luciferase activity in these samples, relative to the Myc-tagged empty vector. In two separate experiments conducted with the vERE(3)TkLuc reporter vector, overexpression of hMI-ER1α did not have an effect on transcription compared to the control (vERE(3)Tkluc + CS3MT), whereas hMI-ER1β enhanced ERE-driven transcription, by approximately 3-fold, in the presence of E_2 (Figure 21).

75

Myc Vector	+	+	-	-	-	-
Myc-hMI-ER1α	-	-	+	+	-	-
Myc-hMI-ER1β	-	-	-	-	+	+
E₂	-	+	-	+	-	+
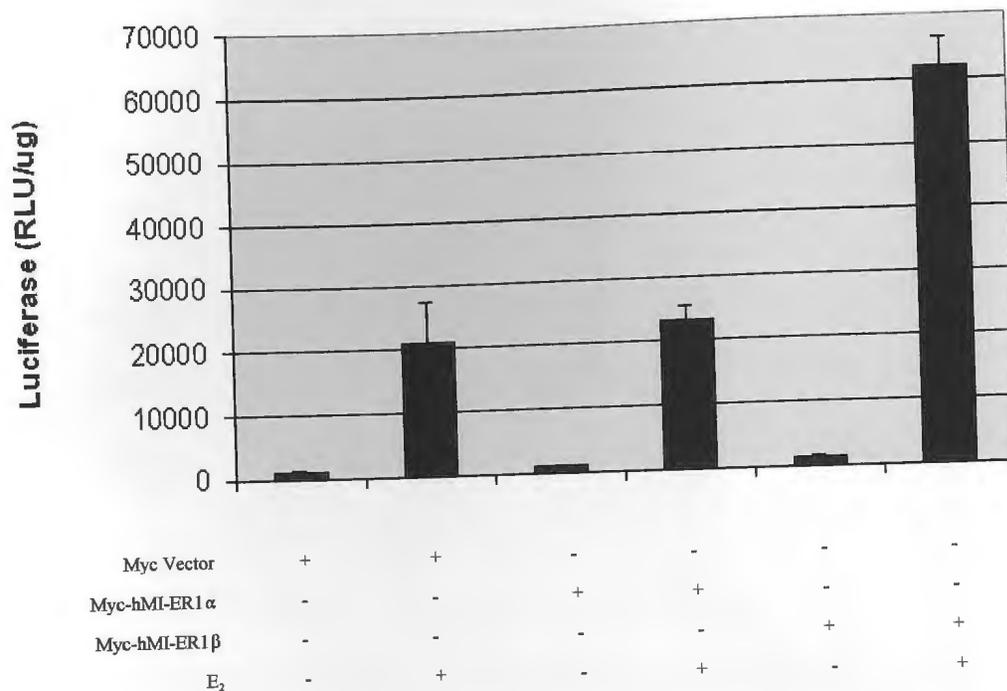
Figure 21: hMI-ER1β enhances ERE-driven transcription in MCF-7 cells transfected with the vERETKLuc reporter construct.

MCF-7 cells were transfected with 0.5 μg of the vERE(3)TKLuc reporter plasmid and 0.5 μg of either CS3+MT vector, pCS3+MT- *hmi-er1α*, or pCS3+MT- *hmi-er1β* and cultured in PRF DMEM with 10^{-9} M estradiol (E₂), or an equal volume of ethanol (vehicle). Cells were harvested approximately 48 hours after transfection and the amount of relative luciferase units (RLU) was determined. Values were normalized to the amount of cellular protein present in each sample. Average values and standard deviations from two independent experiments are shown.

3.3.2 hMI-ER1β Enhances ERE-driven Transcription Whereas hMI-ER1α inhibits ERE-driven Transcription in Luciferase Assays with the pS₂-ERELuc Reporter Plasmid

Thymidine kinase (Tk) belongs to a group of enzymes involved in DNA synthesis and protein synthesis. It had been demonstrated that elements in the Tk promoter contain sequences recognized by various protein complexes, including Sp1 transcription factors [Karlseder, et al., 1996]. Since hMI-ER1 has been found to repress transcription by inhibiting binding of Sp1 proteins to DNA [Ding, et al., unpublished data], luciferase assays were completed with a different reporter construct (pS₂-ERELuc) in order to verify that results obtained with vERE(3)TkLuc were not altered by an interaction between hMI-ER1 and Sp1 sites in the Tk promoter. *pS2*, a member of the trefoil peptide family, is a known ER-responsive gene that is widely used to study estrogen-regulated gene expression. The 5' flanking region of the pS2 gene contains a TATA box, CAAT box and an single imperfect ERE [Jeltsch, et al,. 1987; Nunez, et al., 1989; Kim, et al., 2000]. Estrogen receptors will interact with estrogen responsive genes containing one or more copies of the ERE consensus sequence (5'-GGTCAnnnTGACC'3'), or those that contain variants of this sequence, such as half sites (TGACC). The pS2-ERELuc reporter construct contains 1050 bp of the pS2 promoter [Berry, et al., 1989] preceding the luciferase reporter of pGL3 [Tremblay, et al., 1997]. The pS2ΔERELuc construct, used

77

as a control, contains the same insert in the pGL3 vector except the ERE half site was replaced by sequences encoding *Eco*RI-*Eco*RV sites, generated by PCR mutagenesis [Tremblay, et al., 1997]. Luciferase assays were performed on three separate occasions in MCF-7 cells, as described above, with either pS_2-ERELuc, with an intact ERE, or pS_2-ΔERELuc, with a mutated ERE, and either pCS3+MT vector, pCS3+MT-*hmi-er1α*, or pCS3+MT-*hmi-er1β*. For each luciferase assay conducted, expression of Myc-tagged hMI-ER1α and Myc-tagged hMI-ER1β was verified by Western blot analysis with anti-Myc antibody. Figure 22B shows a representative Western blot.

MCF-7 cells, transfected with the pS_2-ERELuc vector alone, will display a certain level of luciferase activity in the presence of estrogen. In comparing the level of relative luciferase activity of experimental samples (pS_2-ERELuc + hMI-ER1α or pS_2-ERELuc + hMI-ER1β) to the control (pS_2-ERELuc + pCS3+MT vector), it is evident that overexpression of hMI-ER1β enhances ERE-driven transcription almost 2 fold, whereas hMI-ER1α inhibits ERE-driven transcription 1.6 fold (Figure 22A).

Figure 22: ERE-driven transcription is inhibited by hMI-ER1α and enhanced by hMI-ER1β in MCF-7 cells transfected with pS2-ERELuc reporter constructs.

A. MCF-7 cells were transfected with 0.8 µg of either pS2-ERELuc or pS2ΔERELuc reporter plasmids and 0.8 µg of either CS3+MT vector, pCS3+MT-*hmi-er1*α, or pCS3+MT-*hmi-er1*β and cultured in PRF DMEM with 10^{-9} M estradiol (E_2), or an equal volume of ethanol (vehicle). Cells were harvested approximately 48 hours after transfection and the amount of relative luciferase units (RLU) was determined. Values were normalized to the amount of cellular protein present in each sample. Average values and standard deviations from two independent experiments are shown.

B. Expression of Myc-tagged hMI-ER1α and hMI-ER1β protein in each sample was verified by Western blot analysis with anti-Myc antibody. A representative Western blot is shown. *In vitro* translated ^{35}S-labeled Myc-tagged hMI-ER1α or Myc-tagged hMI-ER1β protein (100,000 cpm) were used to verify bands in the cell extracts (lanes 1 and 2, respectively). The position of marker proteins are indicated.

79

A.
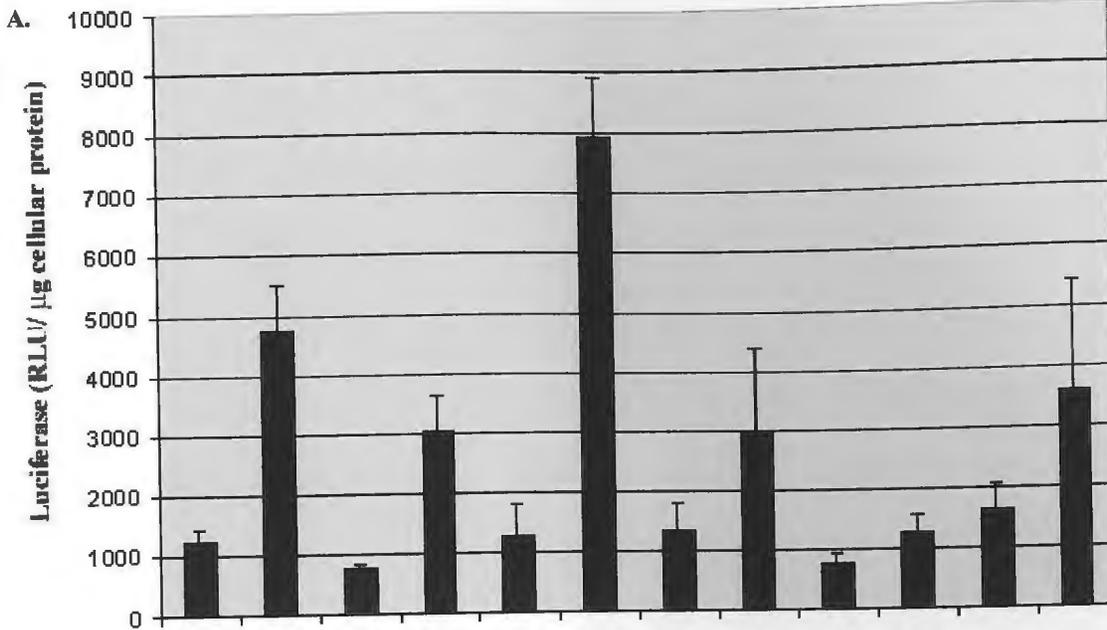
ERE	+	+	+	+	+	+	-	-	-	-	-	-
Myc Vector	+	+	-	-	-	-	+	+	-	-	-	-
Myc-hMI-ER1α	-	-	+	+	-	-	-	-	+	+	-	-
Myc-hMI-ER1β	-	-	-	-	+	+	-	-	-	-	+	+
E₂	-	+	-	+	-	+	-	+	-	+	-	+

B.

WB: anti-Myc
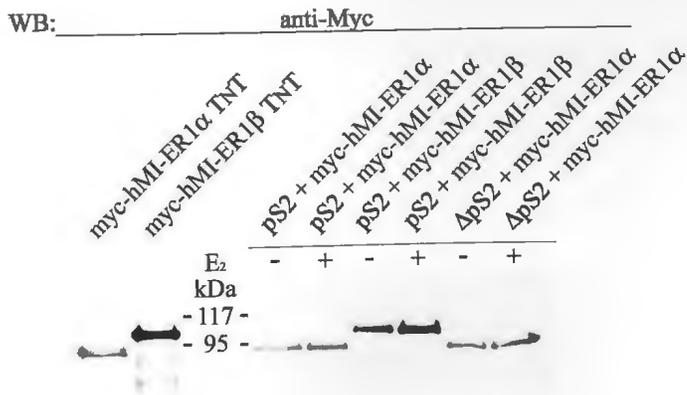
3.4 Results Summary

Results presented in this study confirm the hypothesis that hMI-ER1α and hMI-ER1β interact with a number of nuclear hormone receptors *in vitro* (ERα, RARα, RARβ, RARγ, RXRα, and RXRγ) and with ERα *in vivo*, and indicate that this interaction can affect the transcriptional regulatory function of ERα. The interaction between hMI-ER1 and ERα involves a sequence contained within aa 325-357 of hMI-ER1. Studies presented here highlight the importance of the last amino acid of the SANT domain and amino acids flanking the C-terminus of the SANT domain for this interaction.

The interaction between hMI-ER1α or hMI-ER1β and RARα, RARβ, or ERα are not affected by the presence of ligand *in vitro*. *In vivo* studies confirm that hMI-ER1α and β also interact with ERα in the absence of estrogen, however, results varied with the presence ligand. hMI-ER1α and hMI-ER1β were found to interact with ERα in MCF-7 cells only in the absence of ligand when anti-Myc or anti-hMI-ER1α or β-specific antibodies were used for immunoprecipitaton. However, hMI-ER1α or β interacted with ERα in the presence and absence of estrogen in MCF-7 and HEK 293 cells when either a pan-hMI-ER1 or anti-Myc antibodies were employed for immunoprecipitation, respectively.

Studies examining the possible functional consequences of an interaction between hMI-ER1α or β and ERα indicate that hMI-ER1β enhances ERE-driven transcription.

hMI-ER1α, on the other hand, did not have an effect on ERE-driven transcription with the vERE(3)TkLuc reporter plasmid but had an inhibitory effect on ERE-driven transcription with pS_2-ERELuc reporter plasmids.

4. Discussion:

Nuclear hormone receptors play a critical role in the regulation of cell growth, differentiation and apoptosis and it has become evident that the mechanism of hormone receptor action is a highly complex process. The estrogen receptor (ER), for example, is known to exert both genomic (nuclear) and nongenomic (membrane and cytoplasmic) effects, some of which can be explained by cross-talk with growth factors and cellular kinase pathways. ER genomic activity, which involves ligand binding, receptor dimerization, nuclear translocation and binding of complexes to estrogen-response elements, has been found to be up-regulated by pathways including insulin-like growth factor type- I and epidermal growth factor signaling pathways [Nicholson, et al., 1999]. Through nongenomic effects, nuclear hormone receptors can rapidly and transiently initiate signaling from the cell membrane or cytoplasm, in response to their respective ligands, through interaction with numerous signal transduction cascades [Losel, et al., 2003; Cato, et al., 2002].

Whether exerting genomic or nongenomic effects, the estrogen receptor requires multiple protein-protein interactions, with both basal transcriptional machinery as well as coregulatory molecules, in order to exert their functions in a cell. ER coregulators can themselves be phosphorylated by growth factors, stress-response and cytokine-related pathway kinases, which have been identified as yet another regulatory mechanism for ER signaling [Schiff, et al., 2003]. The presence of an acidic activation domain, ELM2 and SANT domains, as well as a proline rich motif, indicate that hMI-ER1 is likely to be

involved in protein-protein interactions and the presence of an LXXLL motif in hMI-ER1α suggests that one possible group of protein-interacting partners could be nuclear hormone receptors. The purpose of this study was to determine whether hMI-ER1α is indeed a nuclear hormone receptor coregulator and to determine whether it plays a part in regulating the function of the estrogen receptor in the cell.

4.1 *In vitro Interaction of hMI-ER1 with Nuclear Hormone Receptors*:

A series of preliminary GST pull down assays demonstrated that both hMI-ER1α and hMI-ER1β interact *in vitro* with ERα, RARα, RARβ, RARγ, RXRα, and RXRγ (Figures 4 - 9). These results indicated early on that the LXXLL-motif was not responsible for the *in vitro* interaction since hMI-ER1β, which lacks this motif, was also able to interact with the nuclear hormone receptors tested. It is not uncommon for LXXLL-containing cofactors to interact with various nuclear hormone receptors through alternate motifs or through other bridging proteins. REA (repressor for E_2-activity), for example, will interact directly with liganded ER and suppress ER-activated gene transcription by E_2, and although REA contains an LXXLL motif, this motif is not required for interaction with ER [Martini, et al., 2003]. In the case of mADA3, an LXXLL-containing protein, interaction with ERα occurs through a TBP-free-TAF containing complex (TFTC) [Benecke, et al., 2002]. It is the members of the TFTC complex that mediate the interaction with nuclear hormone receptors. ASC-2 is yet another LXXLL-containing protein which does not require this motif for interaction with

83

RXR, RAR, TR, ERα, and GR, or with members of basal transcriptional machinery such as TFIIA, TBP, CBP/p300, and SRC-1 [Lee, et al., 1999].

Since both hMI-ER1α and β were found to interact with the nuclear hormone receptors *in vitro*, it was evident that the domain responsible for this interaction was located in the common region of hMI-ER1. Therefore, hMI-ER1β was used in GST-pull down assays performed to determine whether *in vitro* interaction between hMI-ER1 and ERα, RARα, or RARβ, would be affected by the presence of respective ligands. Results indicated that hMI-ER1β interacts, *in vitro*, with ERα, RARα and RARβ in a ligand independent manner (Figures 10- 13). In future, these assays could be repeated with hMI-ER1α in order to verify that *in vitro* results would be consistent for both hMI-ER1 isoforms.

Previous studies indicated that hMI-ER1 levels were increased in breast tumour cells and tissues, suggesting a role for hMI-ER1 in breast tumourigenesis [Paterno, et al., 1998]. The ER is a nuclear hormone receptor with significant implications in breast cancer, therefore, subsequent studies for the present work focused on characterizing the interaction between hMI-ER1. The next goal was, thus, to determine which region of hMI-ER1 was responsible for interaction with ERα. Another series of GST pull down assays were completed where various GST-hMI-ER1 deletions constructs were tested for interaction with *in vitro* translated ^{35}S-labeled ERα protein. These studies reveal that amino acids within the region of aa 325-357 of hMI-ER1 are required for the interaction with ERα, highlighting the significance of the end of the SANT domain and the amino

84

acids flanking the C-terminus of the currently defined domain for this interaction (Figures 15 and 16). A GST-hMI-ER1 construct containing only aa 325-357 of hMI-ER1, and constructs with N-terminal deletions of this region, should be used for future *in vitro* studies in order to identify the exact amino acids responsible for the interaction between hMI-ER1 and ERα.

It is predicted that, like the ELM2 domain, the SANT domain will turn out to be larger than currently hypothesized. Originally, the ELM2 domain was thought to encompass amino acids 180-239 of hMI-ER1, which had been defined based on sequence conservation in a few available protein sequences [Solari, et al., 1999]. However, a subsequent reexamination of the alignment of hMI-ER1 and other available protein sequences revealed conservation of amino acid sequences C-terminal to the defined ELM2 domain [Ding, et al., 2003]. Data presented in the study by Ding, et al., 2003, illustrated that the functional ELM2 domain extends from amino acids 180 - 284. A BLAST analysis (www.ncbi.nlm.nih.gov/BLAST/) comparing amino acid sequences of SANT domain containing proteins such as hMI-ER1, MTA1, MTA2, and MTA3, also suggests that the region of homology extends downstream of the originally defined SANT domain.

Examining the other SANT domain containing proteins does seem to suggest a domain larger than the predicted 104 amino acids. For example, a region encompassing 179 amino acids of CoREST, a previously identified corepressor for the REST/NRSF transcription factor (RE1 silencing transcription factor/neural restrictive silencing factor),

is responsible for its interaction with HDAC1/2 and its corepressor function [You, et al., 2001]. This 179 amino acid region contains a SANT domain such as that found in other HDAC1/2-interacting proteins including hMI-ER1, NCoR, MTA1 and MTA2. Until recently, only two HDAC1/2 containing complexes had been identified: mSin3 and NuRD-containing complexes [You, et al., 2001].

The exact function of the SANT domain in transcriptional regulators still remains to be elucidated, however, several lines of evidence point to a role in chromatin remodeling since SANT domains can be found in subunits of ATP-dependent chromatin remodeling enzymes, histone acetyltransferases and deacetylases [Boyer, et al., 2002] . The identification of novel HDAC1/2-containing complexes, such as the CoREST-HDAC1/2 complex, leaves room for the possibility that other ELM2/SANT domain-containing proteins may form such complexes and that the ELM2 and SANT domains could play an, as of yet, unexplained but physiologically relevant function. It has been demonstrated that the ELM2 domain of hMI-ER1 will bind to HDAC ½ complexes, and through this interaction, function as a transcriptional repressor [Ding, et al., 2003].

4.2 *hMI-ER1 Interacts with ERα In Vivo*

4.2.1 *Protein-Protein Interaction:*

In vivo studies demonstrated that when cell extracts from transfected and non-transfected MCF-7 cells were immunoprecipitated with anti-Myc, anti-hMI-ER1α-specific or anti-hMI-ER1β-specific antibodies, followed by immunoblotting with an anti-

86

ERα antibody, both hMI-ER1α and hMI-ER1β interacted with ERα, but only in the

absence of E$_2$ (Figures 17C and 19). However, when non-transfected MCF-7 cells were

immunoprecipitated with anti-pan hMI-ER1 antibody, followed by immunoblotting with

an anti-ERα antibody, a ligand-independent interaction was detected between hMI-ER1α

or hMI-ER1β and ERα (Figure 18). These results could be explained by the fact that the

anti-Myc antibody recognizes the most N-terminal end of Myc-tagged hMI-ER1 and the

anti-hMI-ER1α or β-specific antibodies recognize the most C-terminal sequences of hMI-

ER1 whereas the anti-pan-hMI-ER1 antibody recognizes an amino acid sequence in the

common region of hMI-ER1. Perhaps, in the presence of E$_2$, hMI-ER1 is involved in

protein-complexes where the N- and C-termini are folded in such a way so as to prevent

the antibody from accessing the relevant amino acids and thus an interaction cannot be

detected through immunoprecipitation experiments. It should also be noted that each set

of experiments were performed separately, at different times. In order to exclude the

possibility that results were due to slight differences in experimental procedure, the

experiments could be completed together, at the same time and run on side by side on one

SDS gel.

There was also a difference observed between *transfected* HEK 293 cells and

transfected MCF-7 cells. hMI-ER1α and β were only found to interact with ERα in the

absence of E$_2$ in transfected MCF-7 cells (Figure 17C) however, interacted in the

presence and absence of E$_2$ in HEK 293 cells (Figure 20A). Some critical differences

should be noted between these sets of experiments. Firstly, MCF-7 cells were only

87

transfected with Myc-hMI-ER1α or β and tested for interaction with an *endogenous* ERα, however, HEK 293 cells were *co-transfected* with both Myc-hMI-ER1α or β and ERα. Secondly, immunoprecipitations were completed with anti-Myc antibodies in MCF-7 cells followed by immunoblotting with an anti-ERα antibody whereas immunoprecipitations were completed with anti-ERα in HEK 293 cells followed by immunoblotting with an anti-Myc antibody. In order to investigate whether such differences are critical, results could be verified by reversing antibodies used for immunoprecipitation and western blotting (ex: immunoprecipitation with anti-Myc antibody for HEK 293 cell extracts followed by immunoblotting with an anti-ERα antibody).

The ligand-independent interaction observed between hMI-ER1α or β and ERα in HEK 293 cells immunoprecipitated with anti-ERα could be explained by the fact that hMI-ER1α and β proteins would be denatured when run on an SDS gel, and thus N-termini would be accessible to the anti-Myc antibody for immunoblotting (Figure 23). The N–terminus of hMI-ER1α/β in MCF-7 cell extracts may not have been accessible to the anti-Myc antibody when it was used for immunoprecipitation, prior to the denaturing of the proteins through SDS-PAGE. Co-transfection experiments with both ERα and hMI-ER1α or hMI-ER1β were completed in MCF-7 cells and the same ligand-dependent interaction was observed (data not shown).

Both ERα and β mRNA undergo alternative splicing, ultimately generating transcripts that have duplications or different combinations of exons [Koduri, et al.,

88

E$_2$

Cells transfected with:
ERα and **Myc-hMI-ER1α/β**

MCF-7 cell
extract

HEK 293 cell
extract

hMI-ER1

E$_2$ E$_2$
ERα ERα

Co-activation/Co-
repression complex

hMI-ER1

E$_2$ E$_2$
ERα ERα

Co-activation/Co-
repression complex

IP with **anti-Myc**

IP with **anti-ERα**

hMI-ER1

hMI-ER1

SDS PAGE & WB with anti-
ERα

No proteins were isolated
through IP because the anti-
Myc antibody was unable to
access the N-terminus of Myc-
tagged hMI-ER1

SDS PAGE & WB with anti-Myc

ERα   Linearized proteins
Myc   hMI-ER1
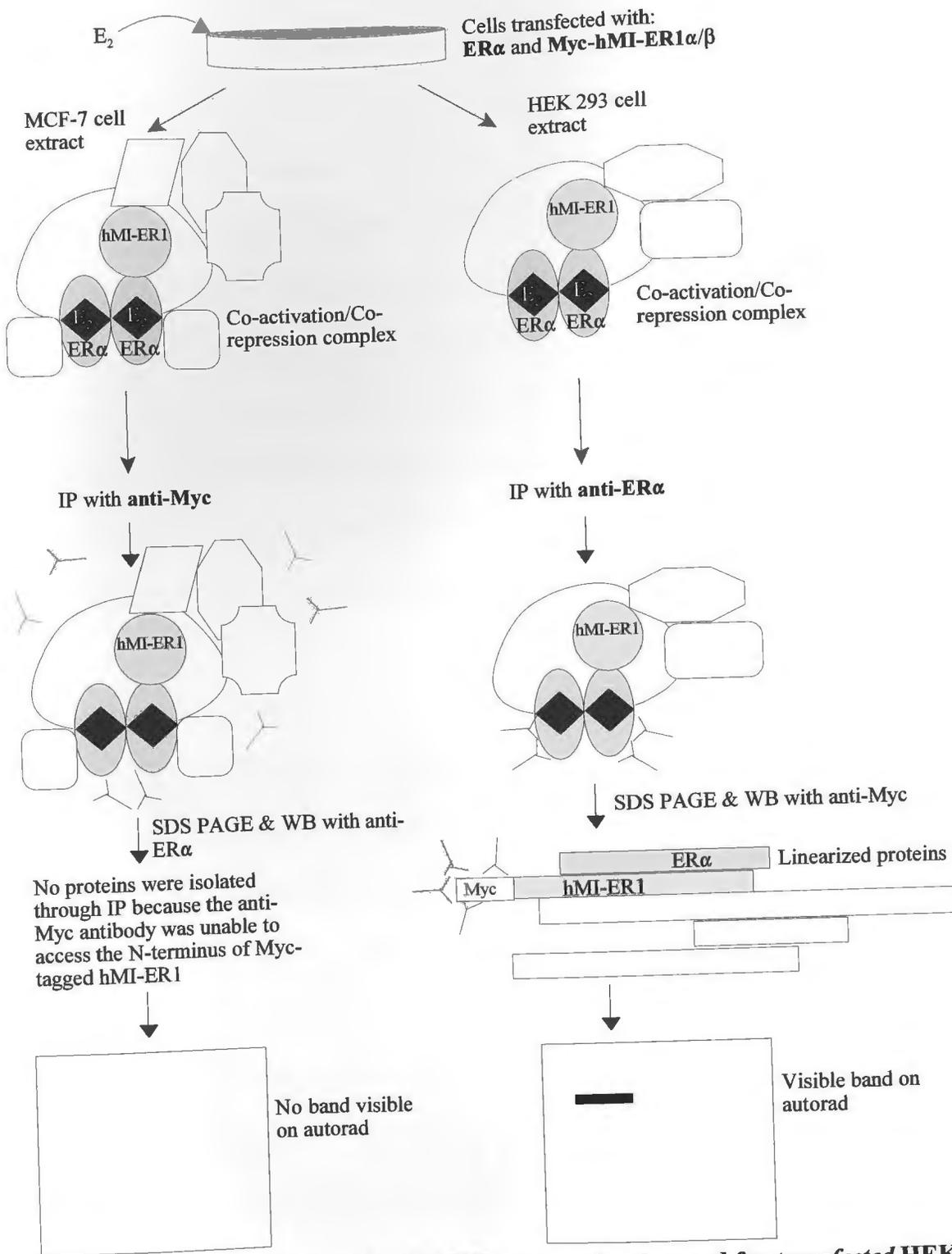
No band visible
on autorad

Visible band on
autorad

**Figure 23: Model to account for the different results observed for *transfected* HEK
293 cells and *transfected* MCF-7 cells**. hMI-ER1 interacting proteins may be cell
context dependent.

89

2001]. A number of studies have shown that these splice variants are present in breast cancer cell lines, including MCF-7 cells. The exact functional role(s) of the splice variants have not been established, although several lines of evidence suggest that some of these variants can be translated into functionally active proteins [Koduri, et al., 2001]. For instance, a 77 kDa ERα protein, with duplications in exons 6 and 7, was found in an MCF-7 cell clone that grows optimally in the absence of E_2 [Pink, et al., 1996]. During the first 6 months of another study, aimed at determining the effects of long term exposure to the anti-estrogen, tamoxifen, a novel population of MCF-7 cells emerged that overexpressed an ERα protein, arising from an mRNA variant lacking exon 3, and ultimately encoding a protein that was missing a 39 amino acid sequence [Fasco, et al., 2003].

In the future it will be important to characterize ERα expression in the MCF-7 cell line used in this study in order to determine if hMI-ER1α and β will also interact with and/or affect the functioning of alternate ERα splice variants It would also be useful to repeat experiments in a number of other breast cancer cell lines in order to verify if the interactions observed between hMI-ER1 and ERα, in non-transfected MCF-7, cells will hold true in other breast cancer cell lines that may express other ERα variants. The heterogeneity of ER isoform profiles in breast cancer cell lines suggests that the expression of alternate ER isoforms may result in variations in response to estrogens and anti-estrogens, altering activation of estrogen-responsive genes and ultimately contributing to tumourigenesis and tumour progression. It is also likely that expression of

splice variants, relative to wild type ER, in a given cell line, may also influence E_2-responsiveness

A number of studies have attempted to determine the cellular factors that influence the observed mechanistic differences between cell lines. Even within a specific cellular classification, such as ER-positive breast cancer cell lines, there is a variability in receptor isoform expression as well variability in coregulator, growth factor and protein kinase expression. Alternate expression profiles exhibited by various cells types could affect the interaction of hMI-ER1 and ERα . For example, hMI-ER1 will interact with transcriptional machinery that enhances transcription (CBP/p300) as well as with that which inhibits transcription (HDAC) . Therefore, the interaction between hMI-ER1 and ERα could vary depending on which hMI-ER1 and/or ERα interaction partners are expressed. The observed differences in interactions between hMI-ER1 and ERα in MCF-7 cells (ligand-dependent interaction) versus HEK 293 cells (ligand-independent interaction) in this study [Figures17c, 19, and 20a], therefore, is not surprising. A separate study confirmed that different breast cancer cell lines have different nuclear hormone receptor and cofactor expression profiles and showed that nuclear receptor regulation is cell line specific [Vienonen, et al., 2003]. For example, E_2 will reduce ERα expression in MCF-7 cells but will induce ERα expression in T-47D and ZR-75-1 cells, each of which are breast carcinoma cell lines [Vienonen, et al., 2003]. Ngwenya, et al., 2003, found that although ZR-75 and MCF-7 cells (both ER-positive breast cancer cell lines) use the same cis-elements and transcription factors to regulate hormone-dependent

91

regulation of E2F-1 gene expression, they do so through different mechanisms. If such significant differences occur in ER-positive breast cancer cell lines, then the differences between breast and kidney cells may be even greater. hMI-ER1 may also have alternate functions, or alternate interaction partners, in normal versus cancer cells. It will, therefore, also be important to repeat experiments conducted in this study in a number of other normal and cancer cell lines in order to determine that interactions observed here are not specific to MCF-7 and HEK 293 cells and in order to further characterize the *in vivo* interaction between hMI-ER1α or hMI-ER1β and ERα in normal and neoplastic states.

4.2.2 Functional Consequences of an Interaction between hMI-ER1 and ERα

In order to determine if the *in vivo* interaction between hMI-ER1α or β and ERα would affect the genomic function of ERα, MCF-7 cells were co-transfected with a reporter construct containing three copies of the vitellogenin A2 (vitA2)-estrogen response element (vERE(3)TkLuc) and with hMI-ER1α or β. Results from two independent experiments revealed that, while hMI-ER1α did not appear to have any effect on ERE-driven transcription, hMI-ER1β enhanced ERE-driven transcription approximately three fold (Figures 21 and 24).

Tk contains several Sp1 sites in its promoter and previous studies have found that hMI-ER1 can function as a transcriptional repressor through interactions at Sp1 sites [Karlseder, et al., 1996; Paterno, et al., unpublished data]. In order to verify that results

92

A. Interaction between hMI-ER1β and ERα in the absence of E₂.

ER α becomes activated by MAPK

ERα releases corepressors and recruits coactivators

ERα/hMI-ER1 containing coactivation complex

HAT

hMI-ER1 β

Transcriptional activation

ERα ERα

ERE

DNA

B. Interaction between hMI-ER1β and ligand-bound ERα.

ERα/hMI-ER1 containing coactivation complex with additional activator proteins

HAT

hMI-ER1 β

Enhanced transcriptional activation + +
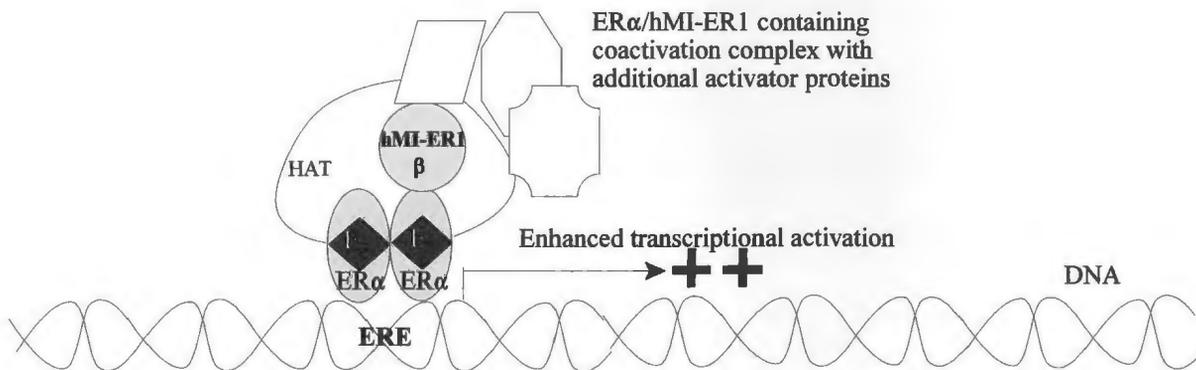
ERα ERα

ERE

DNA
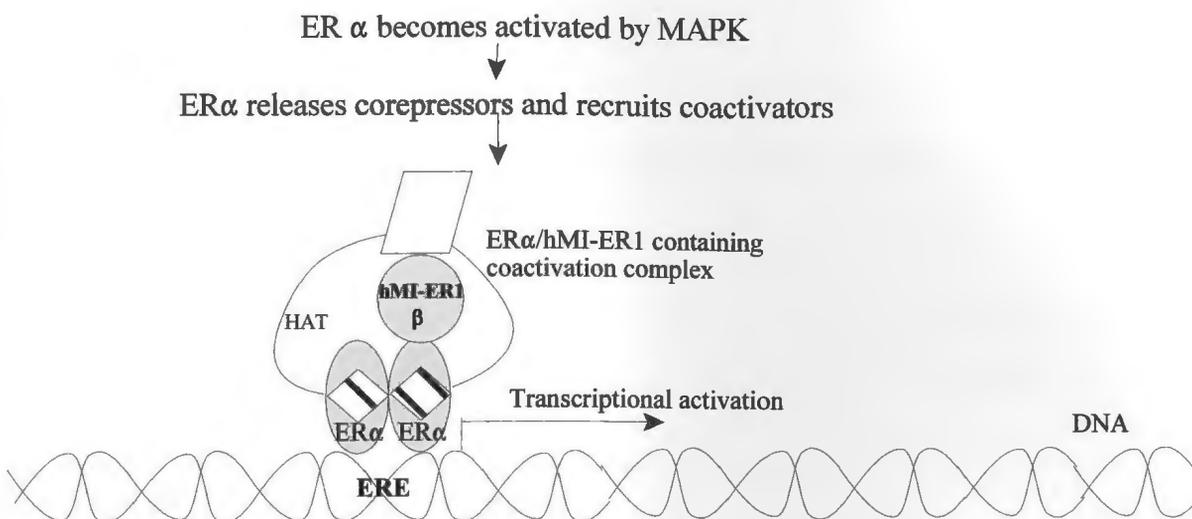
Figure 24: Models demonstrating transactivation of ERE through interaction with hMI-ER1β-containing coactivation complexes.

obtained with the vERE(3)TkLuc reporter construct were not altered by the presence of the Tk promoter, transcriptional reporter assays were performed in MCF-7 cells with one of two luciferase reporter plasmids: pS2-ERELuc (with a functional ERE) and pS2-ΔERELuc (mutated ERE). *pS2* is an estrogen-responsive gene, with a 5' region flanking region containing a TATA box, CAAT box and a single imperfect ERE, that provides a model system for the study of estrogen-regulated genes [Kim, et al., 2000]. Expression of pS2 has been used as a marker of estrogen-responsiveness in ER-positive breast cancer cells, an indicator of disease progression, and a predictor of success of antiestrogen therapy [Nunez, et al., 1989; Schwartz, et al., 1991; Foekens, et al., 1990]. Schwartz, et al., 1991, suggest that patients with ER(+) breast tumours expressing pS2 are more likely to respond to hormone treatment. The results obtained in the present study with pS2-ERELuc and pS2-ΔERELuc showed, once again, that hMI-ER1β enhanced ERE-driven transcription, however, hMI-ER1α repressed ERE-driven transcription (Figure 22A).

The distinct amino acid sequences for α and β C-termini of hMI-ER1 indicated that there could be an alternate function for each isoform. The three isoforms of MTA (MTA-1, MTA-2 and MTA-3), for example, display 60% homology in amino acid sequence, and as such, possess a number of functional similarities and differences [Kumar, et al., 2003]. Both MTA-1 and 2 specifically repress transcription through association with deacetylase activity, whereas MTA-3 does not [Yao, et al., 2003]. Notably, MTA-1 and 2 exist in two biochemically distinct protein complexes and, thus, likely have different functional properties [Yao, et al., 2003]. A shorter, naturally

94

occurring variant of MTA1, MTA1s, has also been identified [Kumar, et al., 2002]. MTA1s is an LXXLL motif-containing protein that has been found to interact with, and sequester, ER in the cytoplasm, enhancing nongenomic functions of ER [Kumar, et al., 2002].

Perhaps hMI-ER1α and hMI-ER1β possess dual, or alternate, activation/repression functions. NRC (nuclear receptor co-regulators), is one such dual-function protein. NRC contains one copy of the LXXLL motif that interacts with nuclear hormone receptors and possesses potent N-terminal (AD1) and C-terminal (AD2) activation domains, however, the C-terminus of the protein, containing the LXXLL-motif, appears to inhibit overall transcriptional activity [Mahajan, et al., 2000]. NSD1, a novel mouse nuclear protein, is another bifunctional coactivator and corepressor that contains a SET domain (motif found in several chromatin-modifying proteins), and separate repression and activation receptor interacting domains (NID-L and NID+L) [Huang, et al., 1998]. NID-L will interact with unliganded LBDs of RAR and TR and allow binding of these receptors with corepressors, whereas NID+L will interact with liganded RAR, TR, RXR and ER and allow for binding of coactivators and transcription factors [Huang, et al., 1998]. hMI-ER1 may also have alternate cell context, ligand and receptor-specific functions. Perhaps, *in vivo* interactions between hMI-ER1α or β and ERα are more highly complex that the *in vitro* results would suggest. hMI-ER1 may interact with ER through more than one motif and/or through novel chromatin remodeling complex(es).

hMI-ER1 has been found to interact with HDAC1 complexes [Ding, et al., 2003],

95

however, early evidence also suggests that hMI-ER1 is also involved in HAT complexes [Blackmore, et al., unpublished data]. In the present study, hMI-ER1α was found to inhibit ERE-driven transcription. Previous studies have also shown that hMI-ER1α and β repress transcription through recruitment of HDAC1 complexes as well as through interactions with Sp1 and interfering with DNA-Sp1 interactions [Ding, et al., 2003; Ding, et al., unpublished data]. Results presented here, however, suggest that hMI-ER1β functions to enhance ERE-driven transcription. Similar results were found with transient transfection assays involving CAT reporter plasmids and Gal4 DBD (DNA-binding domain) plasmids fused to one of various regions of XMI-ER1 [Paterno, et al., 1997]. The N–terminus of XMI-ER1 (aa 1-175) stimulated transcription 80-fold [Paterno, et al., 1997]. Further characterization of this function in hMI-ER1 must be explored in various cell lines, and under different conditions.

hMI-ER1α and β may have alternate functions themselves, or, simply interact with different complexes or interaction partners that control their function. MICoA, for example, can increase E_2-mediated stimulation of ERE activity on its own, however, interaction with coactivator PELP1 and HAT will significantly increase this effect [Mishra, et al., 2003]. Interaction between MICoA and MTA-1 and HDAC2, on the other hand, will suppress MICoA-mediated stimulation of ERE activity [Mishra, et al., 2003].

4.3 *Future Studies*:

A more complete understanding of ER-mediated transcriptional regulation is required for prevention and better treatment of ER-related diseases involving tissues such as breast and bone as well as in the urogenital and cardiovascular systems. It is now evident that hMI-ER1 plays a role in the functioning of ER, however, further studies will be required in order to further characterize the structural and functional implications of this interaction.

1. Examine subcellular localization of hMI-ER1α/β and ERα.

Other than the traditional nuclear ER, biochemical, immunohistological and genetic methods have also established the existence of functional membrane and cytoplasmic ERs [Levin, 2002; Levin, 2001]. Studies conducted in our lab have been identified a functional NLS in the C-terminal domain of XMI-ER1(NLS4) [Post, et al., 2001]. Transfection assays with NIH 3T3 cells revealed that XMI-ER1 translocated exclusively to the nuclei [Paterno, et al., 1997], whereas immunohistochemical studies of *Xenopus laevis* embryos revealed that XMI-ER1 is found exclusively in the cytoplasm during early developmental stages but will progressively accumulate in the nuclei [Luchman, et al, 1999]. Analysis of the β isoform of hMI-ER1 revealed that it is the human orthologue of XMI-ER1. Further analysis of hMI-ER1α and β revealed that hMI-ER1β is targeted exclusively to the nucleus, likely due to a functional NLS, whereas hMI-ER1α, which lacks an NLS, remained cytoplasmic [Paterno, et al., 2002]. These results

97

do not, however, rule out the possibility that hMI-ER1α could be transported into the nucleus through interaction with other proteins, such as HDAC or nuclear hormone receptors. Techniques such as immunohistochemistry or confocal microscopy could be employed in order to determine the pattern of subcellular localization of hMI-ER1α, hMI-ER1β and ERα in the presence and absence of E_2, which would help in determining whether hMI-ER could influence both the genomic and nongenomic actions of ER.

2. Identify the region of ER responsible for interaction with hMI-ER1.

Determining the region of ERα responsible for the interaction with hMI-ER1α and β, will aid in developing a more complete understanding of the nature of the interaction between these proteins. *In vitro* work, such as GST pull down assays could be conducted, as described in Materials and Methods, using with GST-ERα deletion constructs. Co-transfection assays with deletion mutants of ERα and full length hMI-ER1α or β would also help to further understand the nature of *in vivo* interactions.

3. Examine the role of the LXXLL-motif in hMI-ER1α.

Although the LXXLL-motif of hMI-ER1α is not responsible for the interaction with ERα, this motif may be involved in the interaction of hMI-ER1α with other proteins and may influence the functioning of hMI-ER1α as an ERα cofactor, as evidenced from the alternate function of hMI-ER1α and hMI-ER1β in ERE-driven transcription. Site directed mutagenesis of one or more of the leucines in this motif, followed by transfection of the mutated hMI-ER1α, could help to identify a possible role for this motif *in vivo*.

4. Examine the effect of other selective estrogen receptor modulators (SERMs) or

98

ligands on the interaction between ERα and hMI-ER1.

Overexpression of ERα has often been observed in the early stages of breast cancer, therefore, hormone therapies have played a critical role in breast cancer management. Since estrogen plays in role in promoting growth and, thus, progression of breast cancers, anti-estrogens, or selective estrogen receptor modulators (SERMs), are widely used as anti-cancer therapies to modulate the function of ER. The transcriptional, or genomic, activity of ERα and ERβ are influenced by both ligands and by coregulator proteins. Various ER ligands have demonstrated differences in their ability to recruit coregulators, indicating that various ligands can induce different ER conformations [Kraichely, et al., 2000]. Conformational differences will therefore not only affect recruitment of coregulators but will also have functional consequences for the receptor. For example, Schurr, et al., 2001, demonstrated an estrogen-dependent interaction between ERα and the proapoptotic forkhead transcription factor, FKHR, and showed that this interaction increased ERα transactivation through an estrogen response element. The interaction was found to be significantly reduced in the absence of hormone or in the presence of the anti-estrogen, tamoxifen.

ER subtypes also will exert different cellular functions, depending on the nature of the bound ligand. As previously mentioned, ERα and ERβ have been shown to interact with AP1 transcription factors at AP1 sites. In a study conducted in HeLa cells, ERα stimulated an AP1-mediated transcription in the presence of estrogen whereas ERβ demonstrated AP1-mediated activation in the presence of antiestrogens [Paech, et al.,

99

1997]. It would be interesting to determine whether the interaction between hMI-ER1α or hMI-ER1β and ERα would be affected by various SERMs or anti-estrogens.

5. Examine the possibility that hMI-ER1 has other interaction partners.

As evidenced from numerous studies referenced here, the ER requires interaction with multiple proteins in order to regulate its function. As previously mentioned., REA is a potent repressor of ER activity, however, interaction with prothymosin-α (PT-α) will recruit REA away from ER, and thereby allow for proper coactivation of ER [Martini, et al., 2000]. Indeed, preliminary studies have already demonstrated an interaction between hMI-ER1 and HDAC1, HAT, Sp1, retinoblastoma (pRB), heat shock protein 40 (Hsp40), TRABID and core histone proteins [Ding, et al., 2003; Blackmore, et al., unpublished data; Paterno, et al., unpublished data]. Binding of hMI-ER1 to any one of these proteins could competitively affect its binding to another interaction partner, ultimately leading to the formation of multiple protein complexes, or, alternately, hMI-ER1 may form one single complex with all of the above.

Studies could be completed to further examine these possibilities, including a mammalian two hybrid screen, which detects protein-protein interactions in mammalian cells, and chromatin immunoprecipitation (ChiP) assays, which is a type of gene expression profiling involving the immunoprecipitation of transcriptionally active chromatin. The latter would help to characterize transcription complexes bound to ER-target genes and would involve three steps: 1. *in vivo* formaldehyde cross-linking of

whole cells that freezes protein-protein and protein-DNA interactions, 2. immunoprecipitation of protein-DNA complexes, with antibodies such as anti-hMI-ER1α-specific, anti-hMI-ER1β-specific or anti-ERα, from cell extracts and, 3. use quantitative PCR or Southern Blot to analyze immunoprecipitates. This procedure would help to identify members of hMI-ER1α/ERα and/or hMI-ER1β/ERα protein complexes and to identify genes targeted by such complex(es).

6. Examine other functional consequences of the interaction between hMI-ER1 and ERα, other than just an effect on ERE-driven transcription.

Evidence suggests that estrogen receptors responsible for the nongenomic action of estrogen may be a form of classic ER, or possibly a short translational variant of ERα, found in the cytoplasm and plasma membrane [Li, et al., 2003; Figtree, et al., 2003; Anderson, 1998]. ERα located in the plasma membrane has been found to physically associate with, and activate, insulin-like growth factor receptor following E_2 treatment [Kahlert, et al., 2000]. Estrogen can also activate phosphorylated membrane ER and, through G-proteins c-Src and matrix metalloproteinases, subsequently phosphorylate and activate HER2 [Razandi, et al., 2003]. The interaction between ERα and HER2 prevents tamoxifen-induced apoptosis in HER2 overexpressing breast cancer cells [Chung, et al., 2002]. A number of these interactions lead to the activation of key downstream pathways, such as the MAPK pathway, that will subsequently activate transcriptional activity of ER and other components of the transcriptional machinery, thus linking the genomic and nongenomic actions of ER [Schiff, et al., 2004].

101

It would be of great interest to determine if the interaction between hMI-ER1α or hMI-ER1β and ERα would affect nongenomic functions of ER as opposed to genomic functions, such as ERE-driven transcription. For example, cotransfection experiments with hMI-ER1α/β and either wild type ERα , ERα with a membrane localization signal [MLS] or ERα with an NLS could be completed. Cells would be treated with E_2 or ethanol, and cell extracts could be run on an SDS gel. As discussed previously, ER located in the cell membrane has been found to induce MAPK activation, therefore, immunoblotting could be completed with an antibody specific for phosphorylated MAPK in order to determine if this signal cascade has been affected by the interaction between hMI-ER1α or β and ERα.

7. Examine the interaction between hMI-ER1 and other nuclear hormone receptors.

Finally, it would also be useful to more fully examine the interaction of hMI-ER1α and hMI-ER1β with RARα, RARβ, RARγ, RXRα and RXRγ *in vivo,* by employing procedures described in this study. It would also be useful to examine whether hMI-ER1α and β interact with any other nuclear hormone receptors, such as the glucocorticoid and androgen receptors.

4.4 *Conclusion*:

The purpose of this study was to determine if hMI-ER1α interacts with nuclear hormone receptors, through the LXXLL motif, and in doing so, affect the functioning of

nuclear receptors as transcription factors. Studies have revealed that both hMI-ER1α and hMI-ER1β interact, *in vitro*, with ERα, RAR α, RARβ, RARγ, RXRα and RXRγ and that the *in vitro* interaction between hMI-ER1α or hMI-ER1β and ERα, RAR α, RARβ is ligand independent. *In vitro* studies also indicate that amino acids within the region 325-357 of hMI-ER1 are responsible for the interaction with ERα,.

In vivo studies, completed in MCF-7 cells, confirm an interaction between endogenous hMI-ER1α or hMI-ER1β and endogenous ERα, however, the exact nature of this interaction remains to be elucidated. Functional analysis in MCF-7 cells also revealed that hMI-ER1β enhances ERE-driven transcription whereas hMI-ER1α will have no effect, or inhibit, ERE-driven transcription. This study confirms that hMI-ER1 is indeed involved in ERα actions in the cell, and as such, may have significant implications for the development of treatments for breast cancer.

5. References:

Aasland, R., (1996) The SANT domain: a putative DNA-binding domain in the SWI-SNF and ADA complexes, the transcriptional co-repressor N–CoR and TFIIIB. Trends Biochem Sci, 21(3), 87-88.

Alberts, B., et al., (2002) Molecular Biology of the Cell. New York, Garland Science.

Anderson, R.G., (1998) The caveolae membrane system. Annu Rev Biochem 67, 199-225.

Anzick, S.L., et al., (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 277, 965-968.

Auguste, P, et al., (2001) Inhibition of fibroblast growth factor/fibroblast growth factor receptor activity in glioma cells impedes tumor growth by both angiogenesis-dependent and -independent mechanisms. Cancer Res 61, 1717-1726.

Bates, S.E., et al., (1988) Expression of transforming growth factor alpha and its messenger ribonucleic acid in human breast cancer: its regulation by estrogen and its possible functional significance. Mol Endocrinol 2, 543-555.

Baylin, S.B., et al., (1991) Abnormal patterns of DNA methylation in human neoplasia: potential consequences for tumour progression. Cancer Cells 3 (10), 383-390.

Benecke, A., et al., (2002) ADA-3 containing complexes associate with estrogen receptor alpha. Nucl Acids Res 30 (11), 2508-2514.

Berry, M., et al., (1989) Estrogen-responsive element of the human pS2 gene is an imperfectly palindromic sequence. Proc Natl Acad Sci USA 86, 1218-1222.

Bestor, T.H., (1988) Cloning of a mammalian DNA methyltransferase. Gene 74 (1), 9-12.

Bijl, J.J., et al., (1997) HOXC4, HOXC5 and HOXC6 expression in non-Hodgkin's lymphoma: preferential expression of the HOXC5 gene primary cutaneous anaplastic T-cell and oro-gastrointestinal tract mucosa-associated B-cell lymphomas. Blood 90, 4116-4125.

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

Bunone, G., et al., (1996) Activation of the liganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J 15, 2174-2183.

Care, A., et al., (1996) HOXB7 constitutively activates basic fibroblast growth factor in melanomas. Mol Cell Biol 16, 4842-4851.

Cato, A. C., et al., (2002) Rapid actions of steroid receptors in cellular signaling pathways. Sci STKE, 2002 (138) RE9.

Cavailles, V., et al., (1991) Cathepsin D gene of human MCF-7 cells contains estrogen-responsive sequence in its 5' flanking region. Biochem. Biophys. Res. Commun. 174, 816-824.

Chung, Y.L., et al., (2002) Resistance to tamoxifen-induced apoptosis is associated with direct interaction between HER2/neu and cell membrane estrogen receptor in breast cancer. Int J Cancer 97, 306-312.

Dickson, C., et al., (2000) Tyrosine kinase signaling in breast cancer: Fibroblast growth factors and their receptors. Breast Cancer Res 2, 191-196.

Ding, Z., et al., (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(1), 250-258.

Doisneau-Sioux, S.F., et al., (2003) Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells. Endocrine-Related Cancer 10, 179-186.

Duan, R., et al., (1998) Estrogen-induced *c-fos* protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor SP1 complex formation. Endocrinology 139 (4), 1981-1990.

Fasco, M.J., et al., (2003) Phenotypic changes in MCF-7 cells during prolonged exposure to tamoxifen. Mol Cell Endocrinol 206, 33-47.

Fernandes, I., (2003) Ligand-dependent nuclear receptor corepressor LCoR functions by histone deacetylase-dependent and -independent mechanisms. Molec Cell 11, 139-150.

Figtree, G.A., et al., (2003) Truncated estrogen receptor α 46-kDa isoform in human endothelial cells: relationship to acute activation of nitric oxide synthase. Circulation 107, 120-126.

Foekens, J.A., et al., (1990) Prediction of relapse and survival in breast cancer patients by pS2 protein status. Cancer Res 50, 3832-3837.

Ford, H.L., (1998) Homeobox genes: a link between development, cell cycle and cancer. Dell Biol Int 22, 397-400.

Galien, R., Garcia, T., (1997) Estrogen receptor impairs interleukin-6 expression by preventing protein binding on the NFκB site. Nucleic Acids Research 25 (12), 2424-2429.

Geiman, T.M., (2002) Chromatin remodeling, histone modifications and DNA methylation - how does it all fit together? J Cell Biochem 87 (2), 117125.

Ghosh. M. G., et al., (2000) PDZK1 and GREB1 are estrogen-regulated genes expressed in hormone-responsive breast cancer. Cancer Res. 60, 6367-6375.

Guenther, M.G., et al., (2001) SMRT and N -CoR corepressors are activating cofactors for histone deacetylase 3. Mol Cell Biol, 21(18), 6091-6101.

Hayashi, S.I., et al., (2003) The expression and function of estrogen receptor alpha and beta in human breast cancer and its clinical application. Endo Relat Cancer 10 (2), 193-202.

Heery, D.M., (1997) A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387, 733-736.

Heery, D.M., et al., (2001) Core LXXLL motif sequences in CREB-binding protein, SRC1, and RIP140 define affinity and selectivity for steroid and retinoid receptors. J Biol Chem 276 (9), 6695-6702.

Hoeijmaker, J. H., et al., (2001) Genome maintenance mechanisms for preventing cancer. Nature 411 (6835), 366-374.

Huang, N., et al., (1998) Two distinct nuclear receptor interaction domains in NSD1, a novel SET protein that exhibits characteristics of both corepressors and coactivators. EMBO J 17 (12), 3398-3412.

106

Iwao, K., et al., (2000) Quantitative analysis of estrogen receptor-beta mRNA and its variants in human breast cancers. Intl J Cancer 88, 733-736.

Jeltsch, J. M., et al., (1987) Structure of the human oestrogen-responsive gene pS2. Nucleic Acids Res 15, 1401-1414.

Kahlert, S., et al., (2000) Estrogen receptor α rapidly activates the IGF-1 receptor pathway. J Biol Chem 275, 18447-18453.

Karlseder, J., et al., (1996) Interaction of SP1 with growth- and cell cycle-regulated transcription factor E2F. Mol Cell Biol 16, 1659-1667.

Kato, S., et al., (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270, 1491-1494.

Keshet I., et al., (1986) DNA methylation affects the formation of active chromatin. Cell 44 (4), 535-543.

Kim, J., et al., (2000) Regulation of the estrogen-responsive pS2 gene in MCF-7 human breast cancer cells. J Steroid Biochem Mol Biol 74 (4), 157-168.

Koduri, S., et al., (2001) Quantitation of alternatively spliced estrogen receptor alpha mRNAs as separate gene populations. Steroids 66 (1), 17-23.

Kraichely, D.M., et al., (2000) Conformational changes and coactivator recruitment by novel ligands of estrogen receptor-α and estrogen receptor-β: correlations with biological character and distinct differences among SRC coactivator family members. Endocrinology 141(10), 3534-3545.

Kumar, R., et al., (2002) A naturally occurring MTA1 variant sequesters oestrogen-receptor alpha in the cytoplasm. Nature 418 (6898), 654-657.

Kumar, R., et al., (2003) Emerging roles of MTA family members in human cancers. Semin Oncol 30 (5), Suppl 16, 30-37.

Laird P.W., et al., (1994) DNA methylation and cancer. Hum Mol Genet 3, 1487-1495.

Lawrence, H.J., et al., (1996) The role of HOX homeobox genes in normal and leukemic hematopoiesis. Stem Cells 14, 281-91.

Lawrence, J.A., et al., (1998) A high-risk lesion for invasive breast cancer, ductal carcinoma *in situ*, exhibits frequent overexpression of retinoid A receptor. Cancer Epidemiology Biomarkers Previews 7, 29-35.

Lavinsky R.M., et al., (1998) Diverse signaling pathways modulate nuclear receptor recruitment of NCoR and SMRT complexes. Proc Natl Acad Sci USA 95, 2920-2925.

Lee, S.K., et al., (1999) A nuclear factor, ASC-2, as a cancer-amplified transcriptional coactivator essential for ligand-dependent transactivation by nuclear receptors in vivo. J Biol Chem 274, 34283-34293.

Levin E.R., (2002) Cellular functions of plasma membrane estrogen receptors. Steroids 67, 471-475.

Levin, E.R., (2001) Cell localization, physiology, and nongenomic actions of estrogen receptors. J Appl Physiol 91, 1860-1867.

Li, L., et al., (2003) Plasma membrane localization and function of the estrogen receptor α variant (ER46) in human endothelial cells. Proc Natl Acad Sci USA 100, 4807-4812.

Losel, R.M., et al., (2003) Nongenomic steroid action: controversies, questions and answers. Physiol Rev 83, 965-1016.

Loven, M. A., et al., (2003) A novel estrogen receptor α-associated protein, template-activating factor Iβ, inhibits acetylation and transactivation. Mol Endocrinol 17 (1), 67-78.

Luchman, H.A., et al., (1999) Differential nuclear localization of ER1 protein during embryonic development in *Xenopus laevis*, Mech Dev 80 (1), 111-114.

Mahajan, M.A., et al., (2000) A new family of nuclear receptor coregulators that integrate nuclear receptor signaling through CREB-binding protein. Mol Cell Biol 20 (14), 5048-5063.

Martini, P.G., et al., (2000) Prothymosin alpha selectively enhances estrogen receptor transcriptional activity by interacting with a repressor of estrogen receptor activity. Mol Cell Biol 20 (17) 6224-6232.

Maritini, P.G., et al., (2003) Modulation of estrogen receptor activity by selective coregulators. J Steroid Biochem Mol Biol 85 (2-5), 117-122.

108

Martienssen, R. A., et al., (1995) DNA methylation in eukaryotes. Curr Opin Genet Dev 5 (2), 234-242.

Masiakowski, P., et al., (1982) Cloning of cDNA sequences of hormones regulated genes from MCF7 human breast cancer cell line. Nucleic Acids Res 10, 7895-7903.

Mazumbar, A., et al., (2000) Transcriptional repression of estrogen receptor by metastasis-associated protein 1 corepressor. Nature Cell Biology 3, 30-37.

McMahon, C., et al., (1999) P/CAF associates with cyclin D1 and potentiates its activation of the estrogen receptor. Proc Natl Acad Sci USA 96, 5382-5387.

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, L.C., et al., (2000) Mediator of transcriptional regulation. Annu Rev Biochem 69, 729-749.

Mishra, S.K., et al., (2003) MICoA, a novel metastasis associated protein 1 (MTA1) interacting protein coactivator, regulates estrogen receptor alpha transactivation function. J Biol Chem 278 (21), 19209-19219.

Nardulli, A.M., at al., (1988) Regulation of progesterone receptor messenger ribonucleic acid and protein level in MCF-7 cells by estradiol: analysis of estrogen's effects on progesterone receptor synthesis and degradation. Endocinology 122, 935-944.

Narlikar, G.J., et al., (2002) Cooperation between complexes that regulate chromatin structure and transcription. Cell 108, 475-487.

Ngwenya, S., et al.,(2003) Cell context dependent differences in the induction of E2F-1 gene expression by 17-beta-estradiol in MCF-7 and ZR-75 cells. Endocrinol 144 (5), 1675-1685.

Nicholson, R.I., (1999) Involvement of steroid hormone and growth factor cross-talk in endocrine response in breast cancer. Endocr. Relat. Cancer 6, 373-387.

Nilsson, S., et al., (2001) Mechanisms of Estrogen Action. Physiological Reviews 81 (4), 1535-1565.

109

Nunes, F.D., et al., (2003) Homeobox genes: a molecular link between development and cancer. Patologia 17 (1), 94-98.

Nunez, A.M., et al., (1989) The 5' flanking region of the pS2 gene contains a complex enhancer region responsive to oestrogens, epidermal growth factor, a tumour promoter (TPA), the c-Ha-*ras* oncoprotein and the c-*jun* protein. EMBO J. 8, 823-829.

Paech, K., et al., (1997) Differential ligand activation of estrogen receptors ERalpha and ERbeta at API sites. Science 277 (5331), 1508-1510.

Parikh, I., et al., (1987) Are estrogen receptors cytoplasmic or nuclear? Some immunocytochemical and biochemical studies. J Steroid Biochem 27 (1-3), 185-192.

Paterno, G.D., (1997) cDNA cloning of a novel, developmentally regulated immediate early gene activated by fibroblast growth factor and encoding a nuclear protein. J Biol Chem 272, 25591-25595.

Paterno, G. D., (1998) Molecular cloning of human *er1* cDNA and its differential expression in breast tumours and tumour-derived cell lines. Gene 222, 77-82.

Paterno, G.D., et al., (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, 79-88.

Peach K., et al., (1997) Differential ligand activation of estrogen recptors ER alpha and ER beta at AP1 sites. Science 277, 1508-1510.

Perillo, B., et al., (2000) 17β-estradiol inhibits apoptosis in MCF-7 cells, inducing *bcl-2* expression via two estrogen-responsive elements present in the coding sequence. Mol Cell Biol 20 (8), 2890-2901.

Philips, A., et al., (1993) Estradiol increases and anti-estrogens antagonize the growth factor induced activator protein-1 activity an MFC7 breast cancer cells without affecting c-fos and c-jun synthesis. J Biol Chem 268 (19), 14103-14108.

Pink, J.J., (1996) Models of estrogen receptor regulation by estrogens and anti-estrogens in breast cancer cell lines. Cancer Res 56 (10), 2321-2330.

Poola, I., et al., (2001) Expression of alternatively spliced estrogen receptor alpha mRNAs is increased in breast cancer tissues. J Steroid Biochem Mol Biol 78 (5), 459-469.

Post, J.N. et al., (2001) Nuclear localization signals in the *Xenopus* FGF embryonic early response 1 protein. FEBS Lerr, 502, 41-45.

Powers, C.W., et al., (2000) Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer 7, 165-197.

Razandi, M., et al., (2003) Proximal events in signaling by plasma membrane estrogen receptors. J Biol Chem 278, 2701-2712.

Richardson, B., (2003) DNA methylation and autoimmune disease. Clin Immunol 109 (1), 72-79.

Robyr, D., et al., (2000) Nuclear hormone receptor coregulators in action: diversity for shared tasks. Moled Endo 14 (3), 329 -347.

Roger, P., et al., (2001) Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumours. Cancer Research 61, 2537-2541.

Roman, S.D., et al., (1993) Estradiol induction of retinoic acid receptors in human breast cancer cells. Cancer Res 53 (24), 5940-5945.

Ryan, P.J., et al., (1994) Phosphorylation of phospholipase C gamma 1 and its association with the FGF receptor is developmentally regulated and occurs during mesoderm induction in Xenopus laevis. Dev Biol 166, 101-111.

Saceda, M., et al., (1988) Regulation of the Estrogen Receptor in MCF-7 Cells by Estradiol. Mol Endo 2 (12), 1157 - 1162.

Sauve, F., (2001) CIA, a novel estrogen receptor coactivator with a bifunctional nuclear receptor interacting determinant. Mol and Cell Biol 21(1), 343-353.

Schiff, R., et al., (2003) Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregualtors modulate response. Clin Cancer Res 9, 447S-454S.

Schiff, R., et al., (2004) Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance. Clinical Cancer Res 10, 331s-336s.

Schurr, E. R., et al., (2001) Ligand-dependent interaction of estrogen receptor-α with members of the forkhead transcription factor family. J Biol Chem 276 (36), 33554-33560.

Schwartz, L.H., et al., (1991) pS2 expression and response to hormonal therapy in patients with advanced breast cancer. Cancer Res 51, 624-628.

Solari, F. A., (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.

Sun G., et a., (1998) Estrogen-induced retinoic acid receptor α1 gene expression: role of estrogen receptor-Sp1 complex. Mol Endocrinol 12, 882 - 890.

Teplitsky, Y., et al., (2003) Proline 365 is a critical residue for the activity of xMI-ER1 in *Xenopus* embryonic development. Biochem Biophys Res Commun 308 (4), 679-683.

Tremblay. G.B., et al., (1997) Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Molec Endo 11 (3), 353-365.

Vidal, M., et al., (2001) SH2 and SH3 domains are targets for anti-proliferative agents. Crit Rev Oncol Hematol 40 (2), 175-186.

Vider B. Z., et al., (1997) Human colorectal carcinogenesis is associated with deregulation of homeobox gene expression. Biochem Biophys Res Commun 232. 642-748.

Vienonen, A., et al., (2003) Regulation of nuclear receptors and cofactor expression in breast cancer cell lines. Euro J of Endocrinol 148, 469-479.

Wang, C., et al., (2001) Direct acetylation of the estrogen receptor α hinge region by p300 regulates transactivation and hormone sensitivity. J Biol Chem 276 (21), 18375-18383.

Webb, P., et al., (1999) The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. Mol Endocrinol 13, 1672-1685.

Woychik, N. A., et al., (2002) The RNA polymerase II machinery: structure illuminates function. Cell 108 (4), 453-463.

Yao, Y.L., et al., (2003) The metastasis associated proteins 1 and 2 form distinct protein complexes with histone deacetylase activity. J Biol Chem 278 (43), 42560-42568.

You, A., et al., (2001) CoREST is an integral component of the CoREST-human histone deacetylase complex. PNAS 98 (4), 1454 - 1458.

Yu, J., et al., (2003) A SANT motif in the SMRT corepressor interprets the histone code and promotes histone deacetylation. EMBO J, 22(13), 3403-3410.

Xu, X.C., et al., (1997) Progressive decrease in nuclear retinoic acid receptor beta messenger RNA level during breast carcinogenesis. Cancer Research 57, 4992-4996.

Zhang, H., et al., (2000) DAX-1 functions as an LXXLL-containing corepressor for activated estrogen receptors. J Biol Chem 275 (51) , 39855-39859.

Zhou, B.B., et al., (2000) The DNA damage response: putting checkpoints in perspective. Nature 408 (6811), 433-439.

Zhu, Y.J., et al., (1999) Amplification and overexpression of peroxisome proliferator-activated receptor binding protein (PBP/PPARBP) gene in breast cancer. Proc Natl Acad Sci USA 96, 10848-10853.

Zou, A., et al., (1999) Estrogen receptor beta activates the human retinoic acid receptor alpha-1 promoter in response to tamoxifen and other estrogen receptor antagonists, but not in response to estrogen. Mol Endocrinol 13, 418-430.

Zwijsen, R.M.L., et al., (1998) Ligand-independent recruitment of steroid receptor coactivators to estrogen receptor by cyclin D1. Gene Dev 12, 3488-3498.

6. Appendices

Table 1: PCR primer pairs used for preparing CS3-MT-*hmi-er1* constructs

Construct	Forward primer	Reverse primer
CS3-MT-hmi-er1α aa 1-432	5'-CGGGATCCATATGGCG GAGCCATCTGTTG-3'	5'-CGGGATCCAAAACAAG ACCACAGAAGC-3'
CS3-MT-hmi-er1β aa 1 - 512	5'-CGGGATCCATATGGC GGAGCCATCTGTTG-3'	5'-CGGGATCCTTAGTCATC TGTGTTTTCAAG-3'

[Ding, et al., 2003]

Table 2: PCR primer pairs used for constructing hMI-ER1 and mutating hMI-ER1 plasmids in pGEX-4T1-1 vector

Construct[a]	Forward primer	Reverse primer
hmi-er1α aa 1-433	5'-CACCATGGCGACATCTG TTGAATC-3'	5'-CGGGATCCAAAACAAGAC CACAGAAGC-3'
hmi-er1β aa 1-512	5'-CACCATGGCGACATCTG TTGAATC-3'	5'-TCAATTAGTCATCTGTGTT TTCAAGTTC-3'
aa 1-283 Δ4	5'-CACCATGGCGACATCTG TTGAATC-3'	5'-AATTCCTCTCTAGCTGCTT TTACA-3'
aa 1-129 Δ5	5'-CACCATGGCGACATCTG TTGAATC-3'	5'-CCTTTATTAGGCGGGTGCA GC-3'
aa 287-433 Δ9	5'-CACCATGGTTTGGACAG AGGAAGAGTGTA-3'	5'-CGGGATCCAAAACAAGAC CACAGAAGC-3'
aa 325-433 Δ10	5'-CACCATGGCATTCTATTA CATGTGGAAAAAATCT-3'	5'-CGGGATCCAAAACAAGAC CACAGAAGC-3'
aa 355-433 Δ 11	5'-GTAACGGATTACATGGAT CGTC-3'	5'-CGGGATCCAAAACAAGAC CACAGAAGC-3'
aa 163-283 Δ 13	5'-GAAGAATCTGAAGAAGAT GAAGATT-3'	5'-AATTCCTCTCTAGCTGCTT TTACA-3'
aa 287-512 Δ16	5'-CACCATGGTTTGGACAG AGGAAGAGTGTA-3'	5'-CGGGATCCTTAGTCATCT GTGTTTTCAAG-3'
aa 287-410 Δ17	5'-CACCATGGTTTGGACAG AGGAAGAGTGTA-3'	5'-CTGGTCCATTAGATGACA CTCCA-3'
aa 287-357 Δ18	5'-CACCATGGTTTGGACAG AGGAAGAGTGTA-3'	5'-CCGTTACACCAGGATGAA GATT-3'
aa 287-330 Δ19	5'-CACCATGGTTTGGACAG AGGAAGAGTGTA-3'	5'-CCACATGTAATAGAATGC TACA-3'