AN INVESTIGATION INTO THE ROLE OF HUMAN MESODERM INDUCTION-EARLY RESPONSE 1a (hMI-ER1a) IN REGULATING GROWTH OF HUMAN NORMAL AND BREAST CARCINOMA CELLS

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By

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ABSTRACT

Mesoderm induction early response 1 (*mi-er1*) was first isolated as a novel fibroblast growth factor (FGF)-inducible immediate-early gene activated during mesoderm induction in *Xenopus* embryos (Paterno *et al.*, 1997). The human orthologue of *mi-er1* shares 91% similarity to the *Xenopus* sequence at the amino acid level. Human *mi-er1* was shown to be highly expressed in breast carcinoma cell lines and breast tumours while remaining barely detectable in normal breast cell lines and breast tissue (Paterno *et al.,* 1998). In addition, hMI-ER1 was found to interact with estrogen receptor α (Savicky *et al.*, unpublished data) whose dysregulated expression contributes to breast tumour development. These data suggest that the expression of human MI-ER1 is associated with the neoplastic state in breast cancer.

The alternate use of a facultative intron at the 3' end of *hmi-er1* gives rise to two major protein isoforms, hMI-ER1α and hMI-ER1β. Transcripts encoding the β isoform are predominant in almost all tissues. Interestingly, the potent nuclear hormone receptor interaction motif (LXXLL; L represents leucine and X represents any amino acid) is present only in the C-terminus of hMI-ER1α and *hmi-er1α* mRNA is observed mainly in endocrine tissues (Paterno *et al.*, 2002). Sequence analysis also revealed that the only *bona fide* nuclear localization signal (NLS) is located in the C-terminus of β isoforms (Paterno *et al.*, 2002), suggesting distinct cellular functions of hMI-ER1α and β isoforms. With all the conserved, functional protein domains, hMI-ER1 was found to interact with various transcriptional and growth regulatory proteins. Such interactions will be important for modulating cell growth in normal and neoplastic cells.

In this study, we investigated the functional role of hMI-ER1α in regulating growth of normal and breast cancer cells. Transient overexpression of hMI-ER1α suppressed growth of normal cell lines, but has no significant effect on three breast carcinoma cell lines. In contrast, blocking hMI-ER1α expression by antisense strategy resulted in growth-inhibitory effects on breast cancer cell lines. And the preliminary data from Hoechst staining suggests that the growth-suppressive function of antisense hMI-ER1α in breast carcinoma cell lines is not due to induction of apoptosis.

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List of Abbreviations

aa	amino acid
Ada2	adaptor coactivator 2
AS	antisense
ATP	adenosine triphosphate
BSA	bovine serum albumin
Caspases	cysteine aspartate-specific proteases
CDKs	cyclin-dependent kinases
CDKIs	cyclin-dependent kinase inhibitors
CIP	CDK inhibitor protein
CMV	cytomegalovirus
DAB	3,3' diaminobenzidine
DBD	DNA binding domain
DCIS	ductal carcinoma in situ
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagles's medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide phosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ELM2	EGL-27 and MTA1 homology domain 2
ER	estrogen receptor
ERE	estrogen response element
FCS	fetal calf serum
FGF	fibroblast growth factor
FGFRs	fibroblast growth factor receptors
g	grams
HAT	histone acetyl transferase
HBAC	human β-actin
HDAC	histone deacetylase
hmi-er1	human mesoderm induction-early response 1 DNA or RNA
hMI-ER1	human mesoderm induction-early response 1 protein
HRP	horseradish peroxidase

Hsp40	heat shock protein 40
IAP	inhibitor of apoptosis
IDC	infiltrating ductal carcinoma
ILC	infiltrating lobular carcinoma
KIP	kinase inhibitor protein
LB	Luria-Bertani
M	molar
M-MLV	Moloney murine leukemia virus
mRNA	messenger RNA
NCoR	nuclear receptor co-repressor
NLS	nuclear localization signal
NPC	nuclear pore complexes
OD	optical density
PAS	polyadenylation signals
PBS	phosphate buffered saline
PI	protease inhibitor
PMSF	phenylmethylsulfonyl fluoride
Rb	retinoblastoma protein
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription-polymerase chain reaction
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH3	src homology domain 3
SID	Sin3A interaction domain
STS	staurosporine
Swi3	switching-defective protein 3
TBE	Tris-borate/EDTA electrophoresis buffer
TBSt	tris buffered saline-tween
TFIII	transcription factor for RNA polymerase III
TLCK	N^{α}-*p*-tosyl-L-lysine chloromethyl ketone
UTR	un-translated region
UV	ultra violet

Chapter 1: Introduction

1.1 Principles of Cell Proliferation

Life depends on the ability of cells to store, retrieve, and translate the genetic instructions required to make a living organism. This hereditary information is stored in the form of double-stranded deoxyribonucleic acid (DNA) polymers made from a sequence of nucleotides (adenine, guanine, cytosine, and thymine) (Alberts *et al.*, 2002). Information expressed by DNA provides cells an instruction manual for synthesizing other molecules when needed. The two key classes of molecules are ribonucleic acids (RNAs) and proteins. The biological information stored in DNA flows primarily from DNA to RNA to protein. This process occurs in two sequential steps: transcription and translation. First, DNA serves as a template for the synthesis of RNA; it is known as transcription. Later, RNA molecules are translated by the complex protein-synthesis machinery, giving rise to proteins. The segment of DNA sequence encoding a single protein is defined as a gene. In eukaryotes, DNA is folded into compact chromosomes and enclosed in the nucleus (Alberts *et al.*, 2002). To form a functional chromosome, DNA must be able to replicate, and replicated copies must be separated and passed on to daughter cells at cell division. This orderly procession of DNA replication and cell division is collectively known as the cell cycle (Schafer, 1998).

Accurately distributing the properly replicated DNA into two daughter cells is the fundamental requirement for normal cell proliferation. However, DNA lesions can occur under many conditions. For example, free radicals generated as byproducts of normal

metabolic processes and environmental factors such as UV-radiation both threaten the integrity of DNA (Coultas and Strasser, 2000). Cells respond to DNA damage either by undergoing cell cycle arrest or apoptosis (programmed cell death). In multicellular organisms, a balance between cell proliferation and cell death is the key to normal homeostasis. Cell proliferation is a complex, genetically regulated process. Many genes involved in the control of cell proliferation are also important factors in triggering cell death. An imbalance between positive and negative signals determining decisions between life or death leads to diseases linked with aberrant cell growth or unwanted apoptosis (reviewed in Vermeulin *et al.*, 2003). Therefore, it is important to note that apoptosis and cell proliferation are thoughtfully coupled.

1.2 An Overview of the Cell Cycle

The multiplication of a single cell involves two essential processes: (i) DNA replication; and (ii) segregating the replicated DNA into two daughter cells by cell division. This process of duplication and division is known as the cell cycle (reviewed in Schafer *et al.*, 1998). The most fundamental purpose of the cell cycle is to pass on genetic information to the next generation of cells. The eukaryotic cell cycle is divided into four sequential phases: G_1, S, G_2 and M phases (Figure 1.1). In the two *gap phases* (G_1 and G_2), cells take time to ensure that environmental conditions are favourable and preparations are completed before committing to DNA synthesis and mitosis. The length of G_1 phase is the most variable; it depends on external conditions and extracellular signals from other cells. DNA replication occurs during S phase (S for *synthesis*). After S phase, chromosome segregation and cell division occur in M phase (M for *mitosis*)

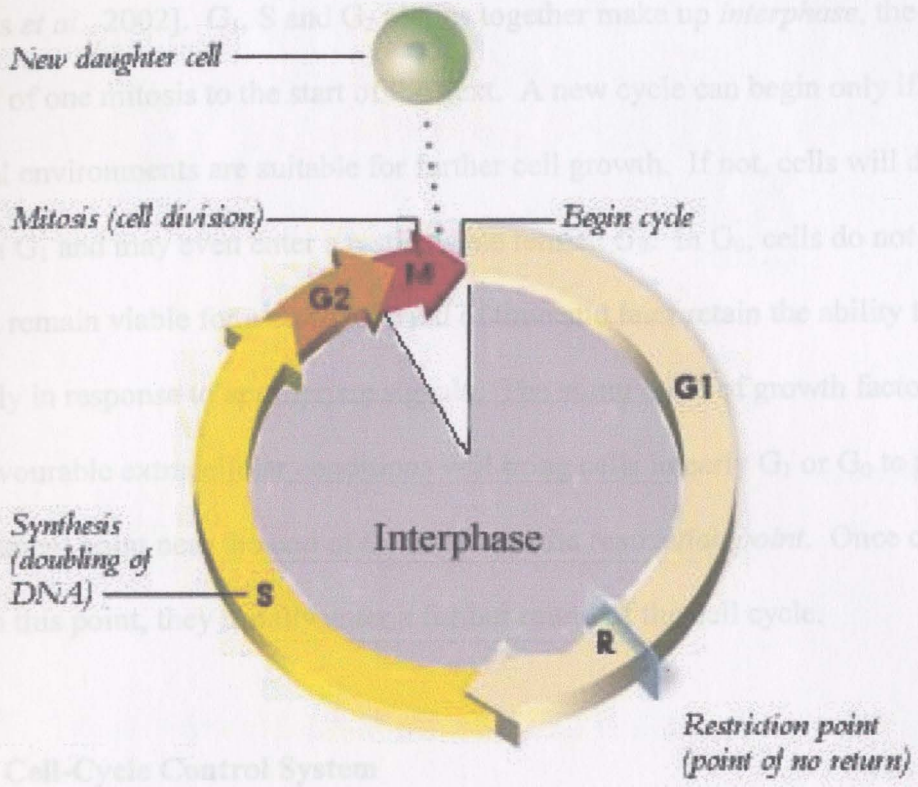


Figure 1.1 The phases of the cell cycle

The cell cycle can be divided into four main stages: (1) the *M phase*, which consists of nuclear and cytoplasmic divisions; (2) the *G_1 phase*, which is the gap between M phase and S phase; (3) the *S phase*, in which DNA replication occurs; (4) the *G_2 phase*, which is the gap between S phase and M phase. G_1, S, and G_2 together are called *interphase.* The restriction point near the end of G_1 is where cells commit to entering the cell cycle (Alberts *et al.*, 2002). This diagram is modified from:

http://www.geocities.com/CollegePark/Lab/1580/cycle.html

[Alberts *et al.*, 2002]. G_1, S and G_2 phases together make up *interphase*, the period from the end of one mitosis to the start of the next. A new cycle can begin only if internal and external environments are suitable for further cell growth. If not, cells will delay progress through G_1 and may even enter a resting state termed G_0. In G_0, cells do not proliferate but can remain viable for a certain period of time and later retain the ability to replicate normally in response to appropriate signals. The stimulation of growth factors together with favourable extracellular conditions will bring cells in early G_1 or G_0 to pass a commitment point near the end of G_1 known as the *restriction point*. Once cells progress through this point, they usually enter a further round of the cell cycle.

1.3 Cell-Cycle Control System

An elaborate network of regulatory proteins involved in governing the events of the cell cycle is known as the cell-cycle control system. If some errors happen during the course of the cell cycle, the control system will delay entry into the next phase. These delays not only provide time for the malfunction to be repaired, but also prevent genomic instability and survival of damaged cells. There are several points in the cell cycle, called checkpoints, at which the cycle can be arrested if basic criteria directing proper cell cycle progression are not satisfied (Stein *et al.*, 1998). As shown in the Figure 1.2, the core of the cell-cycle control system consists of two distinct families of proteins: the cyclins and the cyclin-dependent kinases (CDKs). Cyclins undergo a cycle of synthesis and degradation in each cell cycle and function as regulatory subunits of CDKs. The CDKs regulate the cell cycle events by phosphorylating a broad spectrum of intracellular proteins (Murray, 2004). There is another group of proteins, the cyclin-dependent kinase

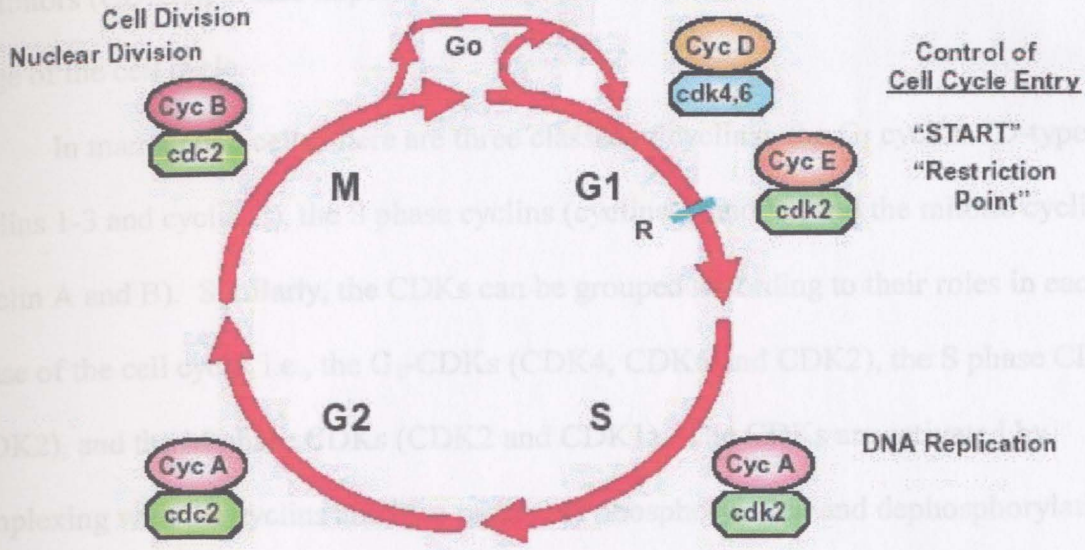


Figure 1.2 Expression of the cyclins and CDKs at different stages of the cell cycle

At the core of the cell cycle is the cyclin-dependent kinase (CDK) family. The activity of CDKs is dependent on its association with a regulatory subunit known as cyclins. In mammalian cells, different cyclin-CDK complexes are active and required at different phases of the cell cycle. CDK4 and CDK6 are active in early G_1 by complexing with cyclin D to regulate restriction point progression. Cyclin E-CDK2 activated at the end of G_1 is important for initiating DNA replication. Once cells enter S phase, cyclin A-CDK2 is required to continue DNA replication. Cyclin A later binds to a key cell-cycle regulator, Cdc2, through G_2 and M phases. The entry into mitosis is promoted by cyclinB-cdc2 complex, which also plays a role in nucleus disassembling and cell division (reviewed in Ivanchuk and Rutka, 2004). This diagram is reproduced from:

http://cpmcnet.columbia.edu/dept/eukaryotic/cobriniknotes2.pdf

inhibitors (CDKIs), is also important for signal transduction and coordination of each stage of the cell cycle.

In mammalian cells, there are three classes of cyclins: the G_1 cyclins (D-type cyclins 1-3 and cyclin E), the S phase cyclins (cyclins A and E), and the mitotic cyclins (cyclin A and B). Similarly, the CDKs can be grouped according to their roles in each phase of the cell cycle, i.e., the G_1-CDKs (CDK4, CDK6 and CDK2), the S phase CDK (CDK2), and the M phase CDKs (CDK2 and CDK1). The CDKs are activated by complexing with the cyclins and by a pattern of phosphorylation and dephosphorylation at specific residues on the kinases. When activated, CDKs are responsible for the phosphorylation of effector molecules such as the retinoblastoma (Rb) protein, a key regulator of G_1 progression (Ivanchuk and Rutka, 2004). In order to maintain cell cycle integrity, cyclin-CDK complexes are inactivated by protein degradation (proteolysis) at certain stages. In addition to the degradation of various cyclins when necessary, CDKIs also have a central role in regulating cycin-CDK activity. There are a variety of CDKI proteins, which function as the "brake" in the system. Such inhibitory proteins are classified into two major groups: the INK4 (specific inhibitor of CDK4) family and the CDK inhibitor protein (CIP)/ kinase inhibitor protein (KIP) family (Pavletich, 1999).

Each of the cyclin-CDK complexes, together with the CDKIs, are responsible for controlling different stages of cell cycle by preventing progression if DNA is damaged. Dysregulation of this cell-cycle control system contributes to uncontrolled cell growth and carcinogenesis.

1.4 Programmed Cell Death (Apoptosis)

Cell viability and death are mutually exclusive partners. Cell cycle is an integral component of viability, and some cell cycle regulators also influence programmed cell death (Sherwood *et al.*, 1994). The balance between positive and negative signals determining the decision between life and death is critical for normal cell growth. If cells are no longer needed, they commit suicide by activating an intracellular death program commonly called apoptosis. Apoptosis is critical and necessary in a variety of cellular events; for example, elimination of selected cells during embryogenesis, in the course of normal tissue turnover and in the developing vertebrate nervous system (White, 1996). It is also involved in many pathological conditions including cancer, cardiovascular and neurodegenerative diseases (Carson and Ribeiro, 1993).

Apoptotic cells show a characteristic morphology (Figure 1.3). The distinct morphological changes including chromatin condensation and margination, cell shrinkage, membrane blebbing and ultimate cellular fragmentation was first described by Kerr *et al.* (1972). In the last decade, the deliberate intracellular apoptotic mechanism is a focus of research. It has become apparent that apoptosis is not a series of clearly defined pathways, but rather, a multitude of highly regulated and interconnected cascades. A number of genes and proteins, some acting within the cells themselves and others acting extrinsically, have now been identified which regulate apoptosis.

1.4.1 Caspases

Caspases (cysteine aspartate-specific proteases), which are a family of intracellular proteins, function as the central proteolytic system of apoptosis. They

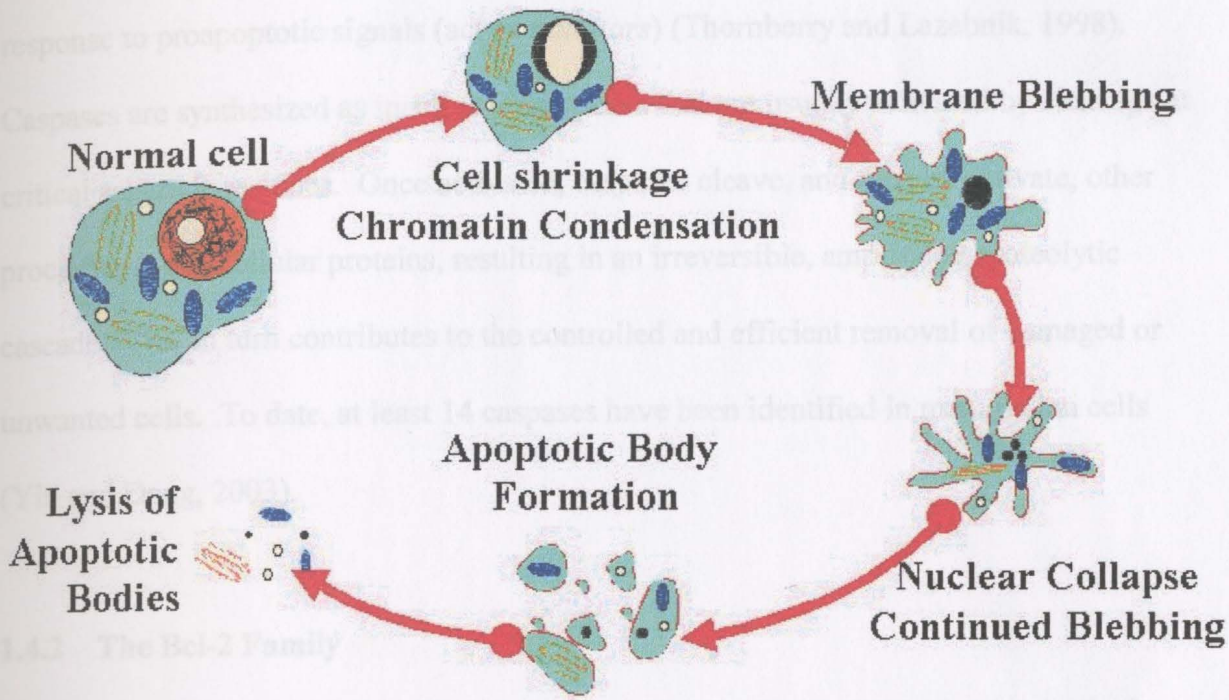


Figure 1.3 Morphological changes of a cell dying through apoptosis

Apoptotic cells undergo a series of morphological changes. The process starts with chromatin condensation and cell shrinkage, followed by membrane blebbing and nucleus destruction. Later, apoptotic bodies are lysed and eliminated by macrophages. Reproduced from:

http://www-micro.msb.le.ac.uk/3035/kalmakoff/baculo/baculohostinteract.html

participate in both cell disassembly (act as *effectors*) and in initiating this disassembly in response to proapoptotic signals (act as *initiators*) (Thornberry and Lazebnik, 1998). Caspases are synthesized as inactive procaspases that are usually activated by cleavage at critical aspartate residues. Once activated, caspases cleave, and thereby activate, other procaspases and cellular proteins, resulting in an irreversible, amplifying proteolytic cascade. This in turn contributes to the controlled and efficient removal of damaged or unwanted cells. To date, at least 14 caspases have been identified in mammalian cells (Yin and Dong, 2003).

1.4.2 The Bcl-2 Family

The Bcl-2 family of proteins constitutes a crucial intracellular checkpoint in the intrinsic cell death pathway to determine whether a cell should live or die. They are essential mediators of cell survival and apoptosis. The proto-oncogene *Bcl-2* (for B-cell leukemia 2) was first identified as a gene activated by chromosome translocation in human lymphoma (Tsujimoto *et al.*, 1985;Cleary and Sklar, 1985;Bakhshi *et al.*, 1985). Members of the Bcl-2 family that are very similar in sequence and structure generally fall into two functional classes with respect to their effects on cell survival. One class including *Bcl-2* itself and other members (such as *Bcl-xl* and *Bcl-w*) inhibits apoptosis. The other class (such as *Bax*, *Bok* and *Bak*) promotes procaspase activation and cell death (Heath, 2001). Both anti- and proapoptotic family members have been demonstrated to be essential for the completion of apoptotic programs (Lindsten *et al.*, 2000;Wei *et al.*, 2001).

1.4.3 The IAP Family

The inhibitor of apoptosis (IAP) family is another important intracellular apoptosis regulator. They function as endogenous caspases inhibitors. The IAPs regulate the activity of both initiator and effector caspases in two ways: they bind to targeted procaspases to prevent their activation, and they bind to active caspases to inhibit their activity (Alberts *et al.*, 2002). Controlled expression of the IAPs has been shown to influence cell death in a variety of contexts, including in hyper-proliferative disorders, such as cancer (Miller, 1999).

1.5 Introduction to Cancer

In normal cell growth, there is a tightly controlled balance between growth-promoting and growth-restraining signals such that proliferation occurs only when required. At the most basic level, cancer is a multifaceted disease where cellular proliferation is no longer under normal control mechanisms (reviewed in Garrett, 2001). Cancer cells acquire accumulative mutations of proto-oncogenes, tumour-suppressor genes, and other genes that regulate, directly or indirectly, cell proliferation, thus growing in an uncontrolled fashion (Hahn and Weinberg, 2002). A number of characteristics which differentiates tumour cells from normal cells are: (1) Self-sufficiency in growth signals, (2) insensitivity to antigrowth signals, (3) evading apoptosis, (4) limitless replicative potential, (5) induced vascularization for receiving oxygen and nutrients (angiogenesis), (6) migrating from the site of origin to a distant part of the body (metastasis), and (7) genome instability (Hanahan and Weinberg, 2000).

Classification of cancer according to the site of origin describes the type of tissue from which the cancer cells arise. Cancers originating in epithelial cells are termed *carcinomas*; those arising from connective tissue or muscle cells are termed *sarcomas*. Besides these two broad categories, there are many other types of cancer, such as *leukemias*, originating in tissues that produce blood cells; *melanoma*, derived from a pigment cell in the skin, and cancers derived from cells of the nervous system (Alberts *et al.*, 2002).

1.6 Cancer-Critical Genes

Cancer is a genetic disease of somatic cells: it involves dynamic alterations in the genome (Knudson, 2002). Many genes that are mutated in human cancer have been identified in the past three decades. These cancer-critical genes, meaning all genes whose mutation contributes to the development of cancer, are grouped into two broad classes. The first class describes genes with a gain-of-function mutation lead to cancer development, known as *proto-oncogenes*; their mutant, overactive forms are known as *oncogenes*. Genes of the second class, for which a loss-of-function mutation results in the removal of the functional brake on cell growth, are called *tumour suppressor genes* (Alberts *et al.*, 2002).

1.6.1 Proto-oncogenes and Oncogenes

Genetically dominant proto-oncogenes with mutation of a single allele can have growth-promoting effects on cells and prompt them to enter a tumourigenic state. Once proto-oncogenes mutate to become oncogenes they retain their functionality, but no

longer respond to normal regulatory signals. Oncogenes were first identified in retroviruses capable of transforming cells in culture and inducing tumours in animals (Bishop *et al.*, 1985). The types of genetic alterations that can transform proto-oncogenes into oncogenes fall into three basic categories, as shown in Figure 1.4: A change of a single nucleotide base pair in coding sequence (point mutations), a larger-scale alteration such as a partial deletion, or chromosomal translocation that results in the production of a hyperactive fusion protein. Alternatively, the cancer-critical genes may be overexpressed because of DNA amplification caused by errors in DNA replication (Alberts *et al.*, 2002).

Oncogenes and proto-oncogenes represent a diversity of functions, sequences and cellular locations. The majority of oncogenes fall into four familial functional classes: growth factors, receptors, signal transducers and transcription factors (Heath, 2001). There is no single consistent activation mechanism of any one oncogene. Whatever the mechanism for oncogene activation, the end result is to produce a protein which can cause abnormal cell growth.

1.6.2 Tumour suppressor genes

Tumour suppressor genes are defined as genes involved in the control of abnormal cell proliferation and whose activation can suppress tumourigenicity (Macdonald and Ford, 1997). Early efforts to identify these genes were guided by Knudson's hypothesis of biallelic gene inactivation; both copies (alleles) of recessive tumour suppressor genes must be removed or inactivated before an effect is seen (Knudson, Jr., 1971;Comings, 1973). Over the past 15 years, many tumour suppressor genes have been identified and their three classical properties are defined. First, tumour suppressor genes are recessive,

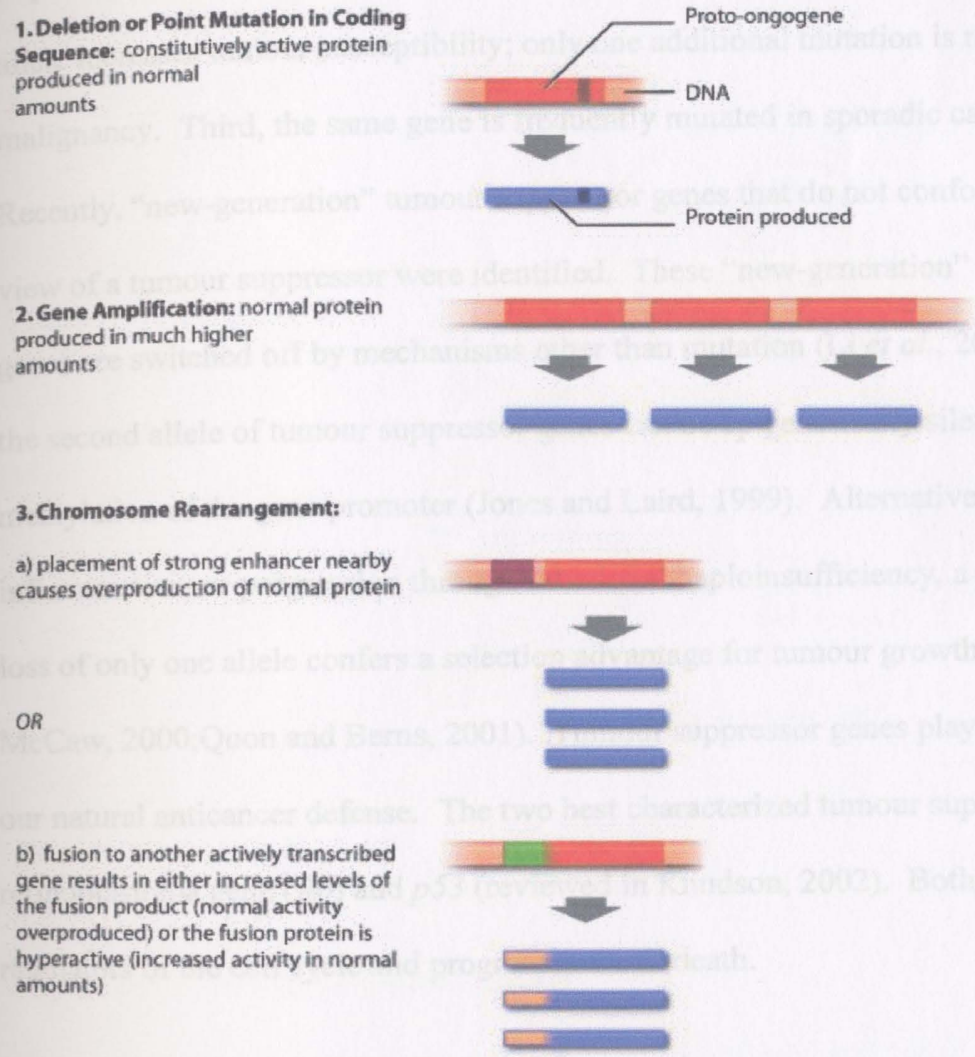


Figure 1.4 Mechanisms of oncogene activation

Proto-oncogenes can be converted to oncogenes in three ways: Deletion or point mutation in coding sequence, gene amplification or chromosomal rearrangement. Adapted from Weinberg (1998).

requiring "two-hit" inactivation of both alleles. Second, inheritance of a single mutant allele increases tumour susceptibility; only one additional mutation is needed to produce malignancy. Third, the same gene is frequently mutated in sporadic cancers (Sherr, 2004). Recently, "new-generation" tumour suppressor genes that do not conform to the classical view of a tumour suppressor were identified. These "new-generation" tumour suppressor genes are switched off by mechanisms other than mutation (Li *et al.*, 2002). For example, the second allele of tumour suppressor genes can be epigenetically silenced by methylation of the gene promoter (Jones and Laird, 1999). Alternatively, they may influence tumour progression through functional haploinsufficiency, a process in which loss of only one allele confers a selection advantage for tumour growth (Cook and McCaw, 2000;Quon and Berns, 2001). Tumour suppressor genes play a crucial role in our natural anticancer defense. The two best characterized tumour suppressor genes are retinoblastoma gene (*Rb*) and *p53* (reviewed in Knudson, 2002). Both are important regulators of the cell cycle and programmed cell death.

1.7 Development of Breast Cancer

Breast cancer is one of the most common cancers in women in the developed countries of the world. In 2004, the most frequently diagnosed cancers continue to be breast cancer for women in Canada; an estimated 21,200 women will be diagnosed with breast cancer and 5,200 will die of it (Canadian Cancer Society, 2004).

The main function of the human breast is to produce life-sustaining milk for the young. As shown in Figure 1.5, the female breast is made up mainly of lobules (milk-producing glands), ducts (milk passages connecting the lobules to the nipple), and stroma

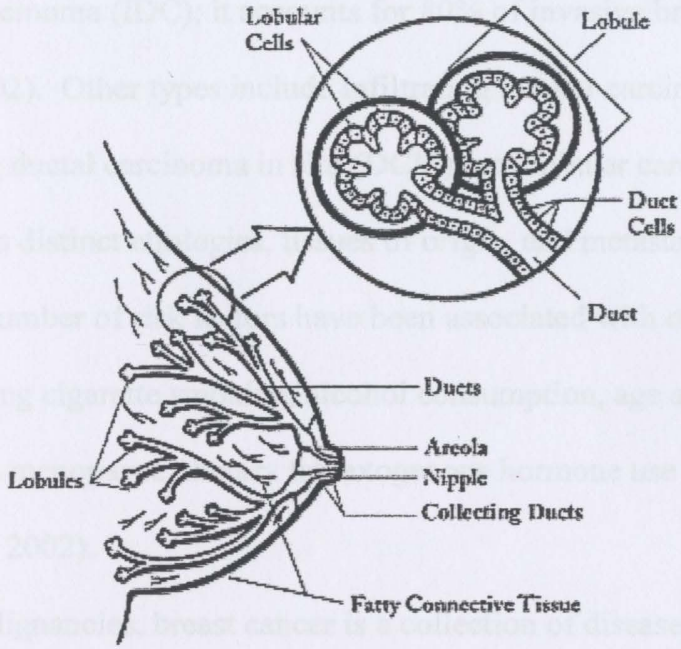


Figure 1.5 Normal Breast Structure

The human breast is made of the secretory glandular tissue and surrounding fatty tissue. The glandular tissue comprises between 15 to 20 lobes with ducts and lobules surrounded by connective tissue. The nipple is surrounded by a pigmented area known as the areola (Donegan and Spratt, 2002). Reproduced from American Cancer Society: http://www.cancer.org

(fatty tissue surrounding the glands and ducts) [Donegan and Spratt, 2002]. Most breast cancers begin in the ducts (ductal). And the most common type of breast cancer is infiltrating ductal carcinoma (IDC); it accounts for 80% of invasive breast cancers (Galmarini *et al.*, 2002). Other types include infiltrating lobular carcinoma (ILC), mucinous carcinoma, ductal carcinoma in situ (DCIS), and lobular carcinoma in situ (LCIS), etc. Each has distinct etiologies, tissues of origin, and metastatic behaviours (Greifzu, 2004). A number of risk factors have been associated with development of breast cancer, including cigarette smoking, alcohol consumption, age at menarche, age at first childbirth, age at menopause, dietary fat, exogenous hormone use and family history (Donegan and Spratt, 2002).

Like most malignancies, breast cancer is a collection of diseases. It is the result of a complex and heterogeneous combination of genetic alterations that promote development of tumour cells. The great majority of breast cancer is due to acquired somatic mutations; however, hereditary cases account for 5-10% of all breast cancer. Several genes that contribute to the occurrence of hereditary breast cancer have been identified. Among these breast cancer susceptibility genes, *BRCA1* and *BRCA2* are responsible for the majority of hereditary breast cancer (reviewed in Yang and Lippman, 1999). Many studies indicate that both *BRCA1* and *BRCA2* are involved in transcriptional regulation, cell-cycle control and DNA damage repair pathways (Wong *et al.*, 1997;Scully *et al.*, 1997;Anderson *et al.*, 1998;Yang and Lippman, 1999). Interestingly, mutations of *BRCA1* and *BRCA2* rarely appear in sporadic breast cancer (reviewed in Yang and Lippman, 1999). It is possible that they function through different mechanisms such as differential splicing or altered cellular location (Thakur *et al.*, 1997).

1.8 Role of the Estrogen Receptor in Breast Cancer

The ovarian steroid hormones, estrogen and progesterone, are necessary for normal mammary growth and development. These steroid hormones regulate the expression of numerous growth factors that mediate growth and differentiation signals. Estrogen has long been implicated in the pathogenesis and progression of breast cancer (Allegra *et al.*, 1979). The action of estrogen is mediated by the estrogen receptors (ERs) that are members of the steroid/thyroid hormone nuclear receptor superfamily, including receptors for steroid and thyroid hormones, vitamin D and retinoic acid (Mangelsdorf *et al.*, 1995;Tenbaum and Baniahmad, 1997). As a class, these receptors are transcription factors whose activity is regulated by ligand binding.

The ER is located predominantly in the nucleus (King and Greene, 1984). Two estrogen receptors have been identified, ERα and ERβ. Increased ERα expression may be one of the very earliest changes occurring in the process of breast cancer development (Khan *et al.*, 1994;Lawson *et al.*, 1999). ERα is therefore considered as a biomarker of breast cancer hormone sensitivity and of differentiation which predicts disease-free survival (Leclercq, 2002). The exact role of ERβ in the growth of breast cancer is still unclear. However, some studies suggest that ERβ might interact with and negatively modulate the actions of ERα (Hall and McDonnell, 1999).

Steroid hormones such as estradiol and progesterone are lipophilic and they enter cells by diffusing through the cell membrane. Once in the cell, estradiol binds the ER with high affinity and specificity. This ligand binding induces a conformational change leading to dimerization of receptors, which allows the receptor-hormone complex to bind to its specific DNA target, the estrogen responsive element (ERE), followed by

recruitment of co-activators (or co-repressors) as well as other transcription factors (Hanstein *et al.*, 2004). The EREs are DNA sequences located in the promotor region upstream of the transcriptional start site of many genes. Upon ERE binding the liganded receptor activates transcription of target genes (Jordan, 1998). ERs also regulate transcription of a variety of genes at alternative response elements, such as AP-1 site, that binds the Jun/Fos transcription factor but not ER (Kushner *et al.*, 2000).

About 35% of patients with ER-positive breast tumours fail to respond to hormonal therapy implying that ER is likely mutated in these tumours. Several mutations and ER splice variants have been identified in neoplastic breast tissue, some of which function as dominant negatives; they are transcriptional inactive and also render co-expressed wild-type receptors transcriptional inactive. In addition, some ER variants/mutants are constitutively active in the absence of ligands, and others alter the agonist/antagonist activity of selective ER modulators (Osborne *et al.*, 2001). Expression of the ER variants/mutants observed in breast tumours may possibly explain disease progression and lack of responsiveness to endocrine therapy (Murphy *et al.*, 1997). However, further research is needed to support this theory.

Alteration in ER expression at the early stages of breast tumour development implies that dysregulation of ER expression is an important factor contributing to tumourigenesis. Therefore, the measurement of ER protein expression in the routine screening of breast tumours may provide some significant clinical correlations.

1.9 Immediate-Early Genes

Extracellular signals are transduced into the nucleus through a variety of signaling pathways to induce changes in patterns of gene expression. The very first genes to be activated along these ordered signaling cascades are termed as *immediate-early genes*. Therefore, the characteristic of immediate-early genes is that their expression can be rapidly and transiently induced upon stimulation of cells. Their expression is generally low in non-stimulated cells; however, it is rapidly activated within minutes to a few hours once stimulated and does not require *de novo* protein synthesis (Caputto and Guido, 2000). Immediate-early genes usually encode proteins, such as transcription factors, that play an important part in transducing extracellular messengers into long-term changes in cellular phenotype. After translation, these inducible transcription factors re-enter the nucleus and regulate the expression of "later response" genes (Walton *et al.*, 1999). Most prominent among the immediate-early genes are *fos* and *jun* gene families (Sheng and Greenberg, 1990;Morgan and Curran, 1991).

Because immediate-early genes encode proteins that act as pleiotropic regulators of a large variety of cellular and developmental processes, their complex interactions together with additional modulating signals result in fundamental cellular decisions of whether to proliferate or die (Kelly and Siebenlist, 1995). The dysregulated expression of these immediate-early gene products may lead to tumour development since signal pathways specifying cell growth and division become over-stimulated and uncontrolled.

1.10 Human Mesoderm Induction Early Response 1 (*hmi-er1*) Gene

A few years ago, investigations into the role of fibroblast growth factors (FGFs) and their receptors (FGFRs) in regulating cell differentiation and growth during development of *Xenopus* embryos led to the isolation of a novel FGF-regulated immediate-early gene, which was later named mesoderm induction early response 1 (*mi-er1*) (Paterno *et al.*, 1997). Expression levels of *mi-er1* were increased during mesoderm induction by FGF in *Xenopus*. A human orthologue of *Xenopus mi-er1* was later cloned and characterized. It was found that human and *Xenopus* MI-ER1 share 91% similarity at amino acid level (Paterno *et al.*, 1998b). Sequence analysis revealed that *hmi-er1* is a single copy gene located at 1p31.2 and is 63 kb in size. As shown in Figure 1.6, *hmi-er1*, which consists of 17 exons including one skipped exon (exon 3A) and a facultative intron (intron 15), gives rise to 12 distinct transcripts (Paterno *et al.*, 2002).

Alternate promoter usage and splicing at distinct 5' ends that arise from transcripts including either exon 1A or 1B and alternate use of exon 3A, give rise to mRNAs encoding three N-termini domains: N1, N2 and N3 (Figure 1.6A). N1 and N2 have the same translational start (ML-), but N1 has an additional sequence encoding a 25 amino acid (aa) cysteine-rich domain. N3 with the translational start (MAE-) results from transcription from a different promoter. The alternate inclusion of intron 15 and together with alternate use of three polyadenylation signals (PASi-iii) produce four distinct 3' ends: *a, bi, bii* and *biii* (Figure 1.6B). These four distinct 3' ends encode two specific C-terminal domains: α and β. The β C-terminus represents a longer sequence (102 aa) than α C-terminus (23 aa) and arises from inclusion of facultative intron 15. The three distinct N-terminal domains, a common internal region, and together with the two possible C-

Figure 1.6 Structure and N-termini sequences of the human *mi-er1* gene

(A) The sequences of the three distinct N-termini with two possible translational starts (ML- and MAE-) are shown; N1 includes sequence from exon 3A. (B) Schematic illustrating the structure of the *hmi-er1* gene and transcripts. The three distinct 5' ends (N1, N2 and N3), the central common coding region, and the four alternate 3' ends (*a*, *bi*, *bii*, and *biii*) of *hmi-er1* transcripts are demonstrated. Adapted from Paterno *et al.*, 2002.

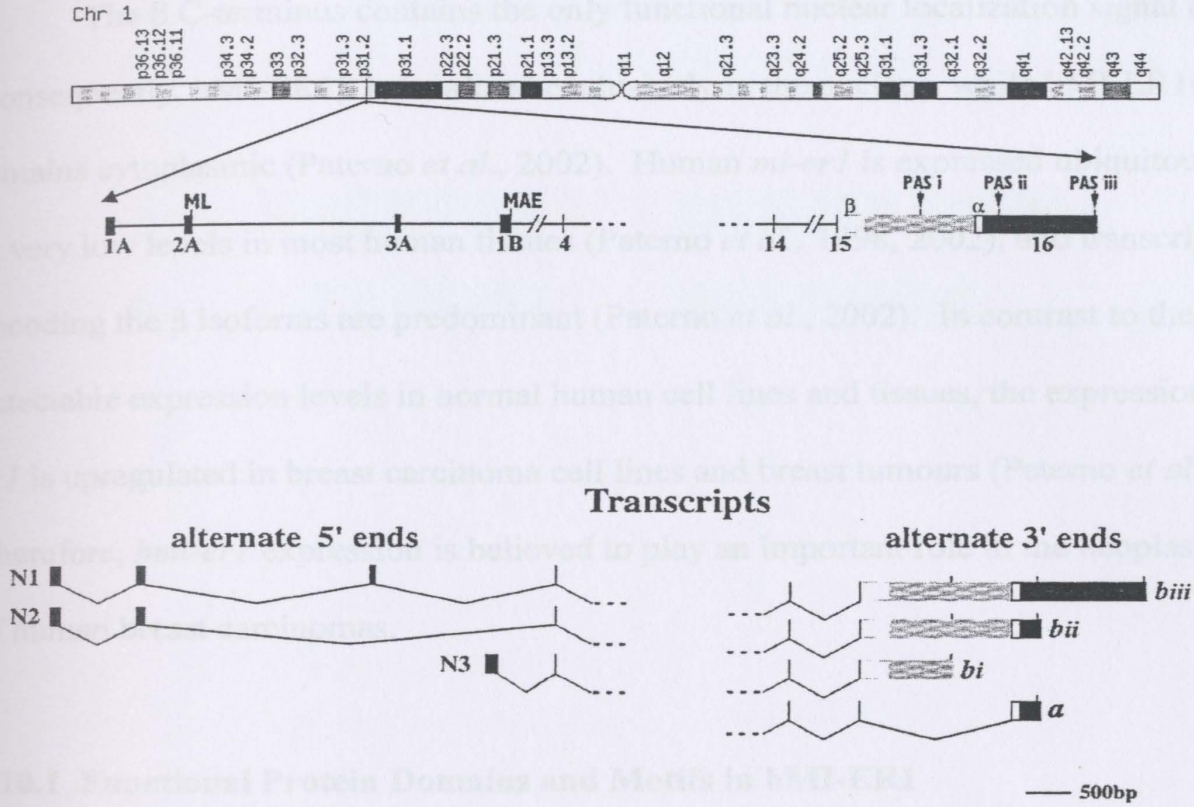
A. N-termini:

N1: **ML**KMCIRCLCLIGLQTVCGLFSCQITQ-

N2: **ML-**

N3: **MAE-**

B.


Chr 1
p36.13
p36.12
p36.11
p34.3
p34.2
p33
p32.3
p31.3
p31.2
p31.1
p22.3
p22.2
p21.3
p21.1
p13.3
p13.2
q11
q12
q21.3
q23.3
q24.2
q25.2
q25.3
q31.1
q31.3
q32.1
q32.2
q41
q42.13
q42.2
q43
q44
ML
MAE
PAS i
PAS ii
PAS iii
β
α
1A
2A
3A
1B
4
14
15
16
Transcripts
alternate 5' ends
alternate 3' ends
N1
N2
N3
biii
bii
bi
a
500bp
Exon
Intron
Facultative Intron
α or β C-terminal coding region
ML, MAE- alternate AUG starts
PASi, ii, iii- alternate poly A signals
N1, N2, N3- alternate N-termini

termini result in the production of six hMI-ER1 protein isoforms: N1α, N1β, N2α, N2β, N3α, and N3β (Paterno *et al.*, 2002b).

The β C-terminus contains the only functional nuclear localization signal (NLS). Consequently, hMI-ER1β is localized exclusively in the nucleus, while hMI-ER1α remains cytoplasmic (Paterno *et al.*, 2002). Human *mi-er1* is expressed ubiquitously, but at very low levels in most human tissues (Paterno *et al.,* 1998, 2002), and transcripts encoding the β isoforms are predominant (Paterno *et al.*, 2002). In contrast to the barely detectable expression levels in normal human cell lines and tissues, the expression of *hmi-er1* is upregulated in breast carcinoma cell lines and breast tumours (Paterno *et al.*, 1998). Therefore, *hmi-er1* expression is believed to play an important role in the neoplastic state of human breast carcinomas.

1.10.1 Functional Protein Domains and Motifs in hMI-ER1

The structure of hMI-ER1 was closely examined and compared to other known proteins using various computer programs (MOTIF: http://www.Motif.genome.ad.jp; PSORT: http://www.psort.nibb.ac.jp, DART: http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi and BLAST search). The overall results revealed a number of putative functional protein domains and motifs in hMI-ER1, as shown in Figure 1.7. Studying each of these functional domains and motifs will provide indications as to the possible cellular functions of *hmi-er1*.

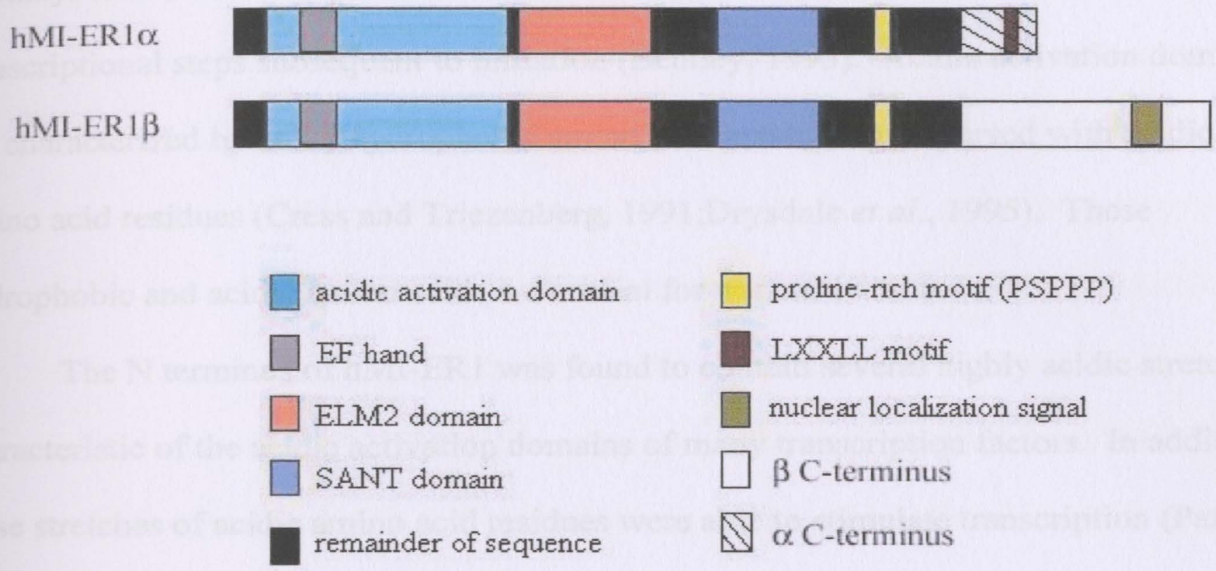


Figure 1.7 hMI-ER1 functional protein domains and motifs

Schematic illustrating the functional protein domains and motifs that are common and unique to the hMI-ER1α and β isoforms.

(A) Acidic activation domains

Acidic activation domains were first described in yeast transcriptional activators in 1987 (Ma and Ptashne, 1987). It was later found that these activation domains not only play a role in recruitment of the transcriptional machinery by protein-protein interactions (Abmayr *et al.*, 1988;Choy and Green, 1993;Struhl, 1998), but also stimulate transcriptional steps subsequent to initiation (Bentley, 1995). Acidic activation domains are characterized by bulky hydrophobic amino acid residues interspersed with acidic amino acid residues (Cress and Triezenberg, 1991;Drysdale *et al.*, 1995). These hydrophobic and acidic amino acids are crucial for transactivation.

The N terminus of hMI-ER1 was found to contain several highly acidic stretches, characteristic of the acidic activation domains of many transcription factors. In addition, these stretches of acidic amino acid residues were able to stimulate transcription (Paterno *et al.*, 1997). Therefore, hMI-ER1 functions as a transcription factor.

(B) EF-hand motif

The EF-hand motif is a highly conserved calcium-binding motif found in a large number of intracellular proteins. It was first described in the structure analysis of carp muscle calcium-binding parvalbumin (Kretsinger and Nockolds, 1973). The EF-hand motif is a helix-loop-helix structure with a Ca^{2+} ion bound to the interhelical loop region. In many calcium-binding proteins, Ca^{2+} binding induces a conformational change in the EF-hand motif and in turn regulates a vast number of target proteins (Yang *et al.*, 2002). Therefore, the EF-hand-containing proteins employ this helix-loop-helix motif to carry

out their diverse biological functions, thus controlling many cellular processes in addition to free calcium levels in the cell (Falke *et al.*, 1994;Kretsinger, 1997).

(C) ELM2 domain

The EGL-27 and MTA1 homology domain 2 (ELM2) was first described in *egl-27*, a *C. elegans* gene that regulates the activity of transcription factors involved in embryonic patterning (Solari *et al.*, 1999) through its interaction with HOX proteins (Ch'ng and Kenyon, 1999). This highly conserved ELM2 domain is found in a number of transcription factors. The role of ELM2 domain in transcriptional regulation was first reported by Ding *et al.*(2003); the ELM2 domain within hMI-ER1 functions as a transcriptional repressor by recruiting histone deacetylase 1 (HDAC1) (Ding *et al.*, 2003). HDACs are involved primarily in transcription repression by tightly compacting chromatin structure and preventing accessibility of transcription factors (de Ruijter *et al.*, 2003).

(D) SANT domain

A SANT domain was identified in hMI-ER1, downstream of the ELM2 domain. The SANT domain was initially found in Swi3 (switching-defective protein 3), Ada2 (adaptor 2), NCoR (nuclear receptor co-repressor), and TFIIIB (transcription factor); all of these proteins are involved in transcriptional regulation. Further sequence analysis revealed its homology to the DNA binding domain (DBD) of *c-myb* (Aasland *et al.*, 1996). Like Myb DBDs, the SANT domain also consists of three α-helices arranged in a helix-turn-helix motif; each α helix contains a highly conserved, bulky aromatic residue playing

a key role in helix packing (Ogata *et al.*, 1994;Aasland *et al.*, 1996b;Tahirov *et al.*, 2001). Interestingly, many ELM2 domain-containing proteins also contain SANT domains (Sloaris *et al.*, 1999). The SANT domain seems to play an essential role in chromatin-remodeling complexes. For example, a SANT domain is found within ATP-dependent remodeling complexes (SWI/SNF, RSC, etc.), as well as histone acetyltransferase (Ada2) (Aasland et al., 1996) and deacetylase (coREST, NCoR, etc.) complexes (Aasland *et al.*, 1996a;Humphrey *et al.*, 2001). Several recent reports suggest that SANT domains may function as histone-binding modules (Boyer *et al.*, 2002;Grune *et al.*, 2003). This supports the idea that SANT domain-containing proteins, like hMI-ER1, act as critical transcriptional regulators through interaction with chromatin-remodeling complexes. In hMI-ER1, Ding *et al.*, (2004) showed that the SANT domain is important for binding Sp1 and regulating its function.

(E) Proline-rich motif

Both hMI-ER1α and β isoforms contain a motif, PXXP (P represents proline and X represents any amino acid) that conforms to the consensus for SH3 (Src homology 3) binding domains (Cohen *et al.*, 1995). The SH3 domain, which is a small conserved sequence of about 60 amino acid residues, is critical for the assembly of many intracellular signaling complexes and pathways (Zarrinpar *et al.*, 2003). By binding with proline-rich motifs, these proline recognition domains target proteins involved in cell growth (Rozakis-Adcock *et al.*, 1993;Buday and Downward, 1993) and other key cellular processes (McPherson, 1999). Site-directed mutagenesis of the proline-rich domain of

Xenopus MI-ER1 showed that proline 365 is a critical residue for embryonic development and mesoderm induction (Teplitsky *et al.*, 2003).

(F) LXXLL motif

Sequence analysis of hMI-ER1 revealed a core LXXLL motif (L denotes leucine and X denotes any amino acid) is present in the C-terminus of α isoform, but not in the β isoform (Paterno *et al.*, 2002). This motif is required for ligand-dependent binding of many transcriptional co-activators, such as SRC1 and CBP/p300, to nuclear receptors (Torchia *et al.*, 1997;Heery *et al.*, 1997a;Heery *et al.*, 2001b). Transcriptional co-activators interact with the conserved inducible activation domain (AF-2) of nuclear receptors via LXXLL motif (Heery *et al.*, 1997b;Nolte *et al.*, 1998;McInerney *et al.*, 1998;Leers *et al.*, 1998). Further structural analysis showed that LXXLL motifs form a amphipathic α helix with the conserved hydrophobic leucine residues aligned on one side of the helix (Darimont *et al.*, 1998). This leucine-rich motif also has very strong similarity to Sin3A interaction domain (SID) contained in the family of MAD transcriptional repressors (Brubaker *et al.*, 2000). The number and sequence of LXXLL motifs varies considerably among the co-activators and is likely to influence the selectivity and affinity of co-activators for different nuclear receptors. The LXXLL motifs showing the strongest interaction with nuclear receptors tend to have a hydrophobic amino acid residue at the -1 position relative to the core (Paige *et al.*, 1999;Chang *et al.*, 1999;Heery *et al.*, 2001a). hMI-ER1α does have a hydrophobic amino acid at position -1, suggesting its interaction with nuclear receptors. This has been

confirmed by Savicky *et al.* (unpublished data) who showed that hMI-ER1 interacts with the ERα.

(G) Nuclear localization signals

All passive and active transport into and out of the nucleus takes place through the nuclear pore complexes (NPC), structures that penetrate the nuclear envelope (Nigg, 1997;Wente, 2000). The NPC has a channel of 9 nm (Goldberg and Allen, 1992) so that larger molecules can not enter the nucleus unless they carry a specific nuclear targeting signal or nuclear localization signals (NLS). There are at least three different classes of NLS; one class comprises a single short stretch of basic amino acids (Kalderon *et al.*, 1984); another class consists of two stretches of basic amino acids separated by a spacer of 10-12 amino acids (Robbins *et al.*, 1991), and the third class has polar/charged residues interspersed with non-polar residues (Chan and Jans, 2002). NLS are sufficient and necessary for nuclear import of the proteins carrying them. Sequence analysis showed that hMI-ER1 contains several predicted NLS; however, the only functional NLS is located in the C-terminus of the β isoform. As predicted, hMI-ER1β is targeted exclusively to the nucleus, while hMI-ER1α is cytoplasmic (Paterno *et al.*, 2002). hMI-ER1 disappears from the nucleus and remains in the cytoplasm in human breast cancer tissue, as compared to normal breast samples (Paterno *et al.*, unpublished data), suggesting hMI-ER1 may have a functional role in breast cancer cells.

1.11 Project Goals

The LXXLL motif in hMI-ER1α indicates its possible interaction with nuclear hormone receptors. This was later confirmed by showing that hMI-ER1α but not β affects ERα activity *in vivo* (Savicky *et al.*, unpublished data). And it has long been appreciated that the abnormal activity of estrogen receptor is strongly associated with the development and progression of human breast cancer. It would be interesting to understand the significance of hMI-ER1α in human breast cancer with respect to its interaction with nuclear hormone receptors. Therefore, my work focused on the impact of the hMI-ER1α isoform on cell growth, using hMI-ER1β as the control for transfection rate. The purpose of this study was to investigate the functional role of hMI-ER1α in regulating growth of normal and breast cancer cells. In addition, the possible mechanism underlying the effect of hMI-ER1α on cell growth was also studied.

Objective 1: Investigation of the role of hMI-ER1α in growth regulation in normal cells

Human embryonic kidney cell line (HEK 293) and normal breast cell line (Hs574) were transfected with either sense or antisense hMI-ER1α. The colony formation assay was then conducted in order to determine the effect of hMI-ER1α expression on normal cell growth.

Objective 2: Investigation of the role of hMI-ER1α in regulating growth of breast carcinoma cells

Two ER-negative human breast carcinoma cell lines (MDA-MB-231 and MDA-MB-468) and one ER-positive breast cancer cell line (BT-474) were transfected with either sense or antisense hMI-ER1α. Those transfected cells were then examined for their colony-forming ability in order to determine the effect of hMI-ER1α expression on growth of breast cancer cells.

Objective 3: Analysis of the possible mechanism whereby hMI-ER1α regulates growth of normal and breast cancer cells

The inhibitory effect of antisense hMI-ER1α expression on growth of breast cancer cells was examined in order to understand whether it is due to induction of programmed cell death (apoptosis). Two breast carcinoma cell lines (MDA-MB-231 and MDA-MB-468) were transfected with antisense hMI-ER1α, followed by Hoechst staining to study apoptotic cells.

Chapter 2: Materials and Methods

2.1 Cell Culture

Human embryonic kidney 293 cells (HEK 293), normal human mammary gland cells (Hs574), and three human breast carcinoma cell lines (BT-474, MDA-MB-231 and MDA-MB-468) were obtained from the American Tissue Culture Collection (ATCC). HEK 293, Hs574 and BT-474 cells were maintained at 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and 100U/ml antibiotics (penicillin/ streptomycin) [Invitrogen]. MDA-MB-231 and MDA-MB-468 were cultured in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% FCS and 100U/ml antibiotics. They were maintained at 37°C in a humidified atmosphere without CO_2.

2.2 Plasmids and Constructs

A. CS3+MT and CS3+MT-hMI-ER1α/β (myc-hMI-ER1α/β)

CS3+MT vectors having the Myc epitope tag (a kind gift from Dr David Turner, University of Michigan) were engineered to contain full-length *hmi-er1α* (N3α) or *hmi-er1β* (N3β) (Ding *et al.*, 2003) as follows: the entire coding sequence of either hMI-ER1α or hMI-ER1β [accession numbers AY124187 and AF515447, respectively] was amplified using specific primers incorporating 5' and 3' *Bam*HI sites. The PCR fragments were then inserted into the *Bgl*II site of CS3+MT plasmid.

B. pcDNA3.1, pcDNA$_{3.1}$-hMI-ER1α and antisense pcDNA$_{3.1}$-hMI-ER1α

The pcDNA$_{3.1}$ mammalian expression vector (Invitrogen) was engineered to contain full-length *hmi-er1α* cDNA (N3α) in sense and antisense orientations as described in Paterno *et al.* (2002). All constructs were verified by sequencing the junctions.

2.3 Transient Transfections

HEK 293 and Hs574 cells were seeded on 6-well plates at 5 x 10^5 cells per well and grown overnight (approximately 18h) in the 37°C incubator. The three human breast carcinoma cell lines, BT-474, MDA-MB-231 and MDA-MB-468 cells, were seeded at 4 x 10^5 cells per well since they were bigger cells and grew faster. Cells were then transfected with 1.5 μg of the empty vector (CS3+MT or pcDNA$_{3.1}$), sense (myc-hMI-ER1α/β or pcDNA$_{3.1}$-hMI-ER1α) or antisense (AS-pcDNA$_{3.1}$-hMI-ER1α) plasmid DNA by Lipofectamine Plus reagent (Life Technologies, Inc.) according to the supplier's protocol. Briefly, 1.5 μg of plasmid DNA were incubated with 6 μl Lipofectamine and 6 μl Plus reagent in the total of 200 μl serum-free medium at room temperature for 30 min. The mixture was added to cells previously seeded in 6-well plates containing 800 μl of serum-free medium and incubated at 37°C for 4h, following which serum-free medium was replaced by the regular medium of each cell line.

2.4 RNA Isolation

5 x 10^5 cells were used for preparation of total RNA. At 24h after transfection, total cellular RNA from five human cell lines (HEK 293, Hs574, BT-474, MDA-MB-231 and

MDA-MB-468) that were transfected with 1.5 μg of the empty vector $pcDNA_{3.1}$ or antisense $pcDNA_{3.1}$-hMI-ER1α, was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA extracted from each cell line was then treated with RQ1 RNase-free DNase (1 U/μl) [Promega] to digest chromosomal DNA and eliminate DNA contamination. This was followed by incubating the mixture at 37°C for 20 min and two subsequent washings with phenol/choloroform/isoamyl alcohol (Invitrogen) to remove the enzyme. After final extraction with diethylpyrocarbonate (DEPC) [Sigma] treated H_2O-saturated chloroform (Fisher), the purified RNA was precipitated overnight with 1/10 the volume of 3M sodium acetate (pH 5.2) and 2.5X the volume of 100% ethanol (Fisher). The final RNA samples were then resuspended in 35 μl DEPC-treated H_2O and stored at -70°C until required. The integrity of the RNA samples was checked on a 1% agarose gel. RNA was quantified by measuring ultraviolet (UV) absorbance at 260 nm of each sample with a spectrophotometer. 1 ml of DEPC-treated H_2O was used as a blank to calibrate the machine. 4 μl of each RNA sample in the total of 1 ml DEPC-treated H_2O (dilution: 1 in 250) was then assessed.

2.5 Reverse Transcription –Polymerase Chain Reaction (RT-PCR) Analysis

The first strand DNA was synthesized at 37°C for 60 min using 1 μg total RNA in a mixture containing 10 μl DEPC-H_2O, 4 μl 5X first strand buffer, 2 μl 0.1 M dithiothreitol (DTT), 2 μl (200 ng) random primer, 2 μl dNTP mix (2.5 mM each of dATP, dCTP, dGTP, dTTP), 1 μl (200 U/μl) Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) and 1 μl (38660 U/ml) RNAguard RNase inhibitor (Amersham). One-eighth of cDNA synthesized was added to a 50 μl PCR reaction mixture containing

1X PCR buffer, 1.5 mM $MgCl_2$, 200μM each of dATP, dCTP, dGTP and dTTP, 4 μg/ml appropriate forward and reverse primers, and 1U platinum Taq DNA polymerase (Invitrogen). All the primers used are listed in Table 2.1. The PCR reaction was done in a thermal cycler (Mastercycler gradient, Eppendorf) using the following program:

1 cycle:	94°C for 4 min
X cycles:	55°C for 1 min
	72°C for 1 min
	94°C for 1 min
1 cycle:	55°C for 1 min
	72°C for 10 min
	30°C for 1 sec

X represented 23 cycles with forward primer (HER 2) and reverse primer (HER 4) for hMI-ER1α amplification. **X** also represented 26 cycles with forward primer (HER 8) and reverse primer (HER 9) for testing the functional antisense hMI-ER1α construct.

Human β-actin was amplified with forward primer (HBAC-1) and reverse primer (HBAC-3) listed in Table 2.1 for 23 cycles and used as an input control. In all experiments, the PCR products were analyzed in the linear range of amplification, which for *hmi-er1α* is 23 cycles, for testing functional antisense *hmi-er1α* is 26 cycles, and for β-actin is 23 cycles.

Ten microliters of each PCR product was eamined on a 1% agarose (Gibco) gel containing ethidium bromide (0.1% μg/ml) [Bio-Rad] in tris borate/EDTA electrophoresis

(TBE) buffer. The relative intensities of the bands representing PCR products were visualized under UV illumination.

		Reverse primer	Expected PCR product sizes
hMI-ER1α amplification	(HER 2) 5'-CCAAATCGTGTT TGCTGAGC-3'	(HER 4) 5'-CAAGGGCTGAAG GCCTATGG-3'	146 bp
Antisense hMI-ER1α examination	(HER 8) 5'-TCAGTTCAAGAG CCAATGCC-3'	(HER 9) 5'-TAAGTGTTGCAA AGTGGCT-3'	224 bp
Human β-actin	(HBAC-1) 5'-ATCTGGCACCACACCT TCTACAATGAGCTGCG-3'	(HBAC-3) 5'-AGCTCGTAGCTC TTCTCCAGG-3'	

Table 2.1 List of primers used for PCR analysis

	Forward primer	Reverse primer	Expected PCR product sizes
hMI-ER1α amplification	(HER 2) 5'-CCAAATCGTGTT TGCTGAGC-3'	(HER 4) 5'-CAAGGGCTGAAG GCCTATGG-3'	146 bp
Antisense hMI-ER1α examination	(HER 8) 5'-TCAGTTCAAGAG CCAATGCC-3'	(HER 9) 5'-TAAGTGTTGCAA AGTGGCT-3'	224 bp
Human β-actin	(HBAC-1) 5'-ATCTGGCACCACACCT TCTACAATGAGCTGCG-3'	(HBAC-3) 5'-AGCTCGTAGCTC TTCTCCAGG-3'	

2.6 Protein Extraction

Cells transfected with either the empty vector $pcDNA_{3.1}$ or $pcDNA_{3.1}$-hMI-ER1α were washed with ice cold PBS and then treated with 100 µl Deox protein lysis buffer (Table 2.3) mixed with 1X protease inhibitor (PI) [1M aprotinin, 1M leupeptin, and 5M nor-*p*-tosyl-L-lysine chloromethyl ketone (TLCK)] and 1 µM phenylmethylsulfonyl fluoride (PMSF) [Sigma] at 24h post-transfection. After 30 min on ice, the lysates were centrifuged at 4°C for 10 min. And the total soluble protein extracted from each transfected cell line was collected and stored at -70°C until required. The protein concentration was then determined by Bio-Rad protein assay kit (Bio-Rad).

Table 2.2 Components of Deox protein lysis buffer

Working solutions	Stock concentration	ml added/ 100ml
50 mM Tris.HCl (pH 8.0)	1 M	5 ml
150 mM NaCl (Fisher)	5 M	3 ml
0.02% Sodium azide (Fisher)	2%	1 ml
Nonidet P-40 (ICN Biomedicals, Inc.)	100%	1 ml
0.1% SDS (Sigma)	10%	1 ml
0.5% Sodium Deoxycholate (Sigma)	---	0.5 g
distilled water	---	89 ml

2.7 Bio-Rad Protein Assay

The Bio-Rad protein assay is a dye-binding procedure responding to various concentrations of soluble proteins. Dilutions of the protein sample are made with Deox protein lysis buffer, and this lysis buffer is used as a blank. Each sample was made up with dH_2O to the total volume of 800 μl and mixed with 200 μl BioRad reagent. The mixture was then left at room temperature for 15 min. The completed reaction is read on a spectrophotometer at a wavelength of 595 nm. A set of serial dilutions made from a bovine serum albumin (BSA) stock (1.4 μg/μl) was used to create a standard curve, which is set up plotting absorbance against BSA protein concentrations (μg/μl). Using this standard curve, the concentration of each protein sample can then be determined.

2.8 Western blotting

Total cell lysates were prepared as previously described. Equal amounts of denatured proteins (25 μg) extracted from each transfected cell line were resolved on an 8% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAG) and transferred onto Hybond-ECL nitrocellulose membranes (Amersham). Filters were blocked by incubation in 5% skim milk in TBS-T (20 mM Tris pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 1h at room temperature. Blots were probed with a 1:2000 dilution of a rabbit polyclonal anti-hMI-ER1 antibody in 5% skim milk/TBS-T for 3h at room temperature, washed with TBS-T for 1h, and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (dilution: 1:4000) [Amersham] in 5% skim milk/TBS-T for 1h. The blots were washed with TBS-T and developed by enhanced chemiluminescence using the ECL kit and exposed to ECL™-Hyperfilm™ (Amersham).

2.9 Colony Formation Assay

Cells were transfected with 1.5 μg of $pcDNA_{3.1}$ empty vector, $pcDNA_{3.1}$-hMI-ER1α or antisense $pcDNA_{3.1}$-hMI-ER1α using Lipofectamine Plus reagents as previously described. At 24h post-transfection, cells were washed with PBS and transferred to 60 mm cell culture dishes. Cells were selected in 500 μg/ml geneticin (Invitrogen). After 10 to 14 days of selection, the medium was removed and cells were washed with PBS. Cells were rinsed with dH_2O and stained with 2% crystal violet in PBS for 20 min. The number of geneticin-resistant colonies or cells were then scored and photographed.

2.10 Immunocytochemistry

Cells were transfected with 1.5 μg of myc-tagged hMI-ER1α or hMI-ER1β constructs using Lipofectamine Plus reagents as previously described. Twenty-four hours after transfection, transfected and non-transfected control cells were washed with PBS and transferred to 8-chamber slides (Becton Dickinson Labware) with 4 x 10^4 cells/ chamber. Cells were fixed in PBS containing 4% (v/v) paraformaldehyde (Fisher) for 30 min, rinsed twice with PBS, and then permeabilized using 0.2% (v/v) Triton X-100 (Sigma) in PBS for 10 min. Nonspecific staining was blocked by incubating the slides with the blocking buffer (1.5% normal goat serum [Invitrogen] in PBS) for 20 min. After washing, the slides were incubated first with a 1:1000 dilution of the anti-MYC monoclonal antibody, 9E10 (Developmental Hybridoma Bank), in the blocking buffer for 2 hours, then with a biotinylated goat-anti-mouse-conjugate (1:500; 30 min; Santa Cruz) and finally with an avidin-biotin-horseradish peroxidase (HRP) complex (1:25; 30 min; Santa Cruz). Reaction was visualized by 3,3' diaminobenzidine (DAB) staining (Sigma). The

slides were mounted in 10% (v/v) glycerol (Fisher) in PBS after removal of gaskets. The cells with brown-stained nuclei or cytoplasm were considered as positive. A total of 200 cells was counted at magnification of 400X and images were taken with a Coolsnap digital camera.

2.11 Apoptosis Assay

Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the DNA-binding fluorochrome bisbenzimide (Hoechst 33342; Sigma). Cells were first transfected with either $pcDNA_{3.1}$ empty vector or antisense $pcDNA_{3.1}$-hMI-ER1α using Lipofectamine Plus reagents. At 24h post-transfection, cells were transferred to 8-chamber slides with 4×10^4 cells/ chamber. Forty-eight hours later, cells were washed with PBS and fixed in 4% paraformaldehye/PBS for 30 min. After washing with PBS, cells were stained with 0.5 μg/ml Hoechst 33342 in PBS for 10 min at room temperature. Cells treated with 1 μM staurosporine (Sigma) in their corresponding cell medium for 48h were used as positive controls. The stained cells were visualized under an Olympus BX 50 fluorescence microscope using a UV filter in the range of 395-450 nm. Condensed or fragmented nuclei were considered as apoptotic cells.

Chapter 3: Results

3.1 Human *mi-er1* transfection efficiency

Previous studies have shown that *hmi-er1* was highly expressed in tumour cell lines and tumour tissues while remaining undetectable in normal breast cell lines and breast tissues (Paterno *et al.*, 1998a). The expression of *hmi-er1* is believed to be associated with the neoplastic state in human breast carcinoma. Therefore, the role of human *mi-er1* in cell growth regulation was investigated in three human breast carcinoma cell lines (BT-474, MDA-MB-231 and MDA-MB-468) using HEK 293 and Hs574 as non-breast carcinoma cell lines for comparison. It has long been appreciated that estrogen receptor (ER) contributes to the metastatic behaviour and progression of breast cancer (Mercer *et al.*, 1984;Coradini *et al.*, 1984). The hMI-ER1 protein was found to interact with estrogen receptor (Savicky *et al.*, unpublished data). Therefore, the functions of *hmi-er1* were examined in three breast cancer cell lines that differ markedly in their estrogen receptor content. The ER-positive (BT-474) and ER-negative (MDA-MB231 and MDA-MB-468) breast cancer cell lines were used.

Transfection refers to a range of techniques used for introducing genes into cells in such a way that they can be taken up by the nucleus and expressed. Cellular transfections provide powerful experimental tools to understand gene regulation *in vivo* and *in vitro* (Kaiser and Toborek, 2001). Achieving a high transfection efficiency is the key to successful experiments in characterizing the function of the genes of interest. Therefore, it is important to determine the percentage of cells in a culture that express

transfected gene (transfection efficiency). This will not only avoid underestimating or overestimating functions of gene of interest, but also closely study its effects on cell growth.

Transfection rates of *hmi-er1* were first determined in human embryonic kidney cells (HEK 293) and in human breast carcinoma cell lines (BT-474, MDA-MB-231 and MDA-MB-468) by immunocytochemical staining. The very slow-growing normal human breast cell line (Hs574) was not included in this part of experiments because they are very fragile and it is hard to work with. Previous studies have showed that hMI-ER1β is localized exclusively in the nucleus, while hMI-ER1α remained in the cytoplasm of mouse NIH 3T3 fibroblasts (Paterno *et al.*, 2002a). Because the cellular localization of *hmi-er1β* is well-characterized with clear nuclear staining, *hmi-er1β* transfectants were used as positive controls here.

Cells were transiently transfected with plasmids containing myc-tagged *hmi-er1α* or *hmi-er1β* cDNA and grown on 8-chamber slides. After 24h, transfected cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). After several washings, the cells were incubated first with the anti-MYC antibody 9E10. After incubation with a biotinylated secondary antibody and with avidin-biotin-horseradish peroxidase complex, staining was visualized using 3,3' diaminobenzidine (DAB). Non-transfected and myc-tagged empty vector-transfected cells were used as controls. Only cells with stronger staining than controls were counted and considered as successful transfectants. Cells that showed abnormal morphology were excluded from the count.

Efficiency of transfection and levels of hMI-ER1 expression were determined by means of immunocytochemistry (Table 3.1 and Figure 3.1). We showed that the average

transfection efficiency of three breast carcinoma cell lines (BT-474, MDA-MB-231 and MDA-MB-468) is 30-40%, which is the expected rate with the use of the conventional protocol (Yamamoto *et al.*, 1999). We also found that the highest transfection efficiency (77%) was achieved in transfected human embryonic kidney (HEK 293) cells. It is believed that certain cell lines are intrinsically easier to transfect than others, although the exact reason for these differences is still unknown (Nikcevic *et al.*, 2003). Weak nuclear staining was observed in HEK 293 cells that had been transfected with myc-tagged empty vector (Figure 3.1A). HEK 293 cells were first described as human embryonic kidney cells transformed by sheared adenovirus 5 DNA (Graham *et al.*, 1977). It is possible that the small but sufficient amount of c-MYC tag was produced in myc-tagged empty vector-transfected HEK 293 cells. This resulted in the recognition of the tag by antibody 9E10, thus leading to weak nuclear staining in vector-transfected HEK 293 cells. The staining of endogenous MYC in HEK 293 cells is another possible explanation. However, no other supporting reference can be found for these proposed explanations. Therefore, it is still unclear why myc-tagged vector-transfected HEK 293 cells showed weak nuclear staining. As expected, *hmi-er1β*-transfected cells showed predominantly nuclear staining. Interestingly, most positive *hmi-er1α*-transfected breast cancer cells showed staining in the nucleus, while HEK 293 cells were stained both in the nucleus and cytoplasm.

Table 3.1 Total numbers and subcellular localization of positive *hmi-er1*-transfected cells

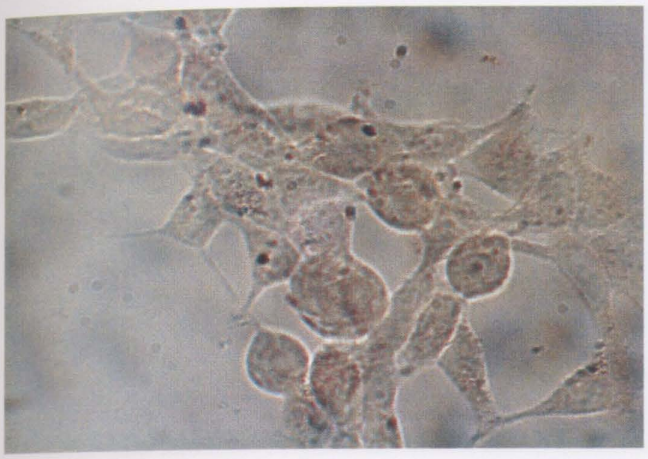
HEK 293				
	Nucleus	Cytoplasm	Unstained	Transfection rate (%)
CS3+MT	84	31	285	--
hmi-er1α	166	144	90	77%
hmi-er1β	262	36	102	75%
BT-474				
CS3+MT	--	--	400	--
hmi-er1α	100	26	274	32%
hmi-er1β	136	8	256	36%
MDA-MB-231				
CS3+MT	--	12	388	--
hmi-er1α	92	30	278	31%
hmi-er1β	128	8	264	34%
MDA-MB-468				
CS3+MT	--	--	400	--
hmi-er1α	102	24	274	32%
hmi-er1β	112	12	276	31%

The first column lists myc-tagged empty vector (CS3+MT), myc-tagged *hmi-er1α* or *him-er1β*-transfected cells. A total of four hundred cells were counted. The data shown are total numbers of two independent experiments. By observing the DAB staining in transfected cells, the subcellular localization of hMI-ER1α and hMI-ER1β was identified.

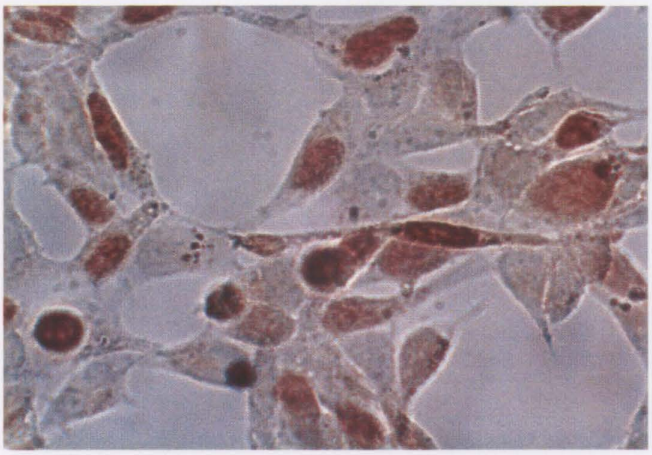
Figure 3.1 Immunocytochemistry of cells transient transfected with hMI-ER1

HEK 293 (A), BT-474 (B), MDA-MB-231 (C) and MDA-MB-468 (D) cells were transfected with 1.5 μg of myc-tagged empty vector (CS3+MT), myc-tagged full-length *hmi-er1α* or *hmi-er1β*, fixed after 48h and stained using the anti-MYC antibody 9E10 as described in "Materials and Methods". DAB stain (brown) indicates that cells were successfully transfected and also revealed the subcellular localization of hMI-ER1α and hMI-ER1β. No staining was detected in non-transfected cells. Cells mock-transfected or transfected with myc-tagged empty vector were used as controls. Representative pictures from two independent experiments were showed.

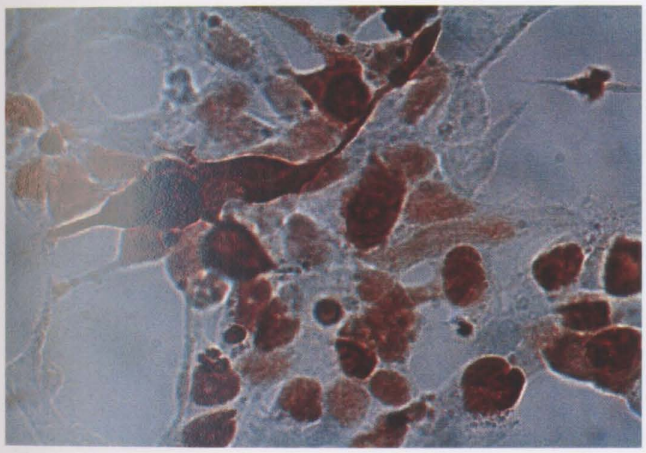
A. HEK 293 cells



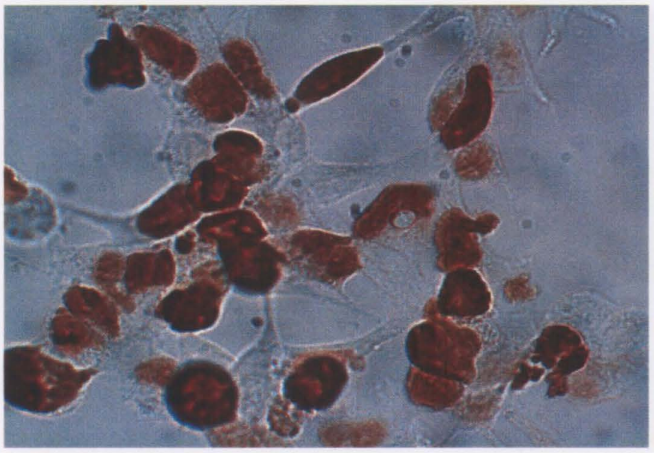
mock



CS3+MT



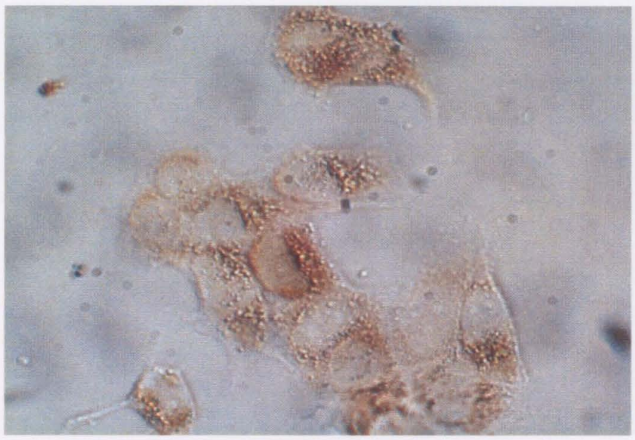
hMI-ER1 α



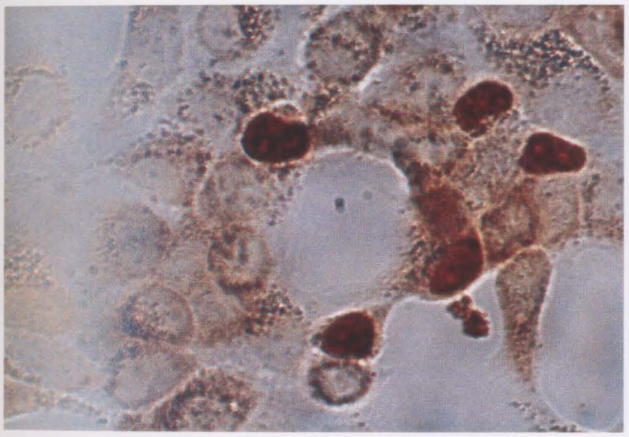
hMI-ER1 β

B. BT-474 cells

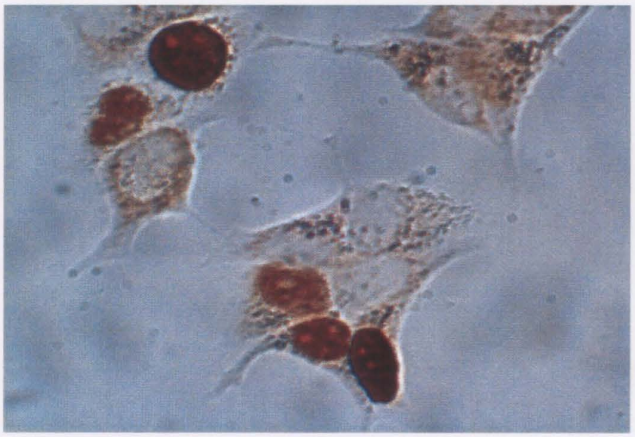
mock



CS3+MT

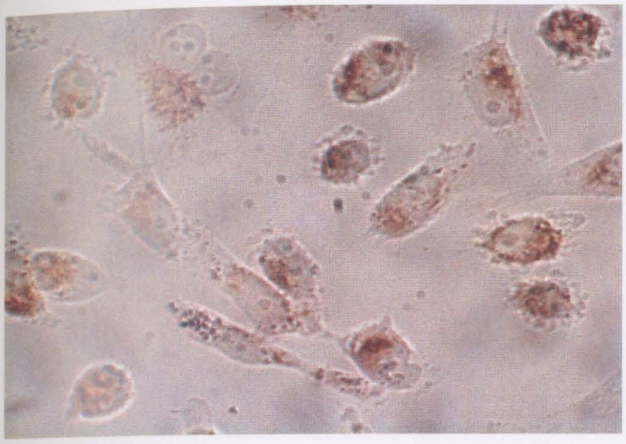


hMI-ER1 α

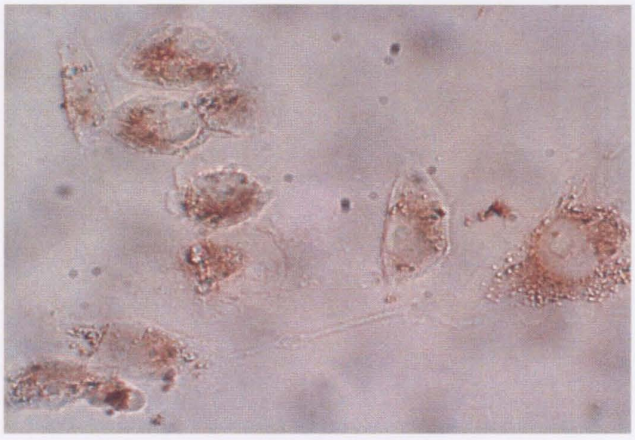


hMI-ER1 β

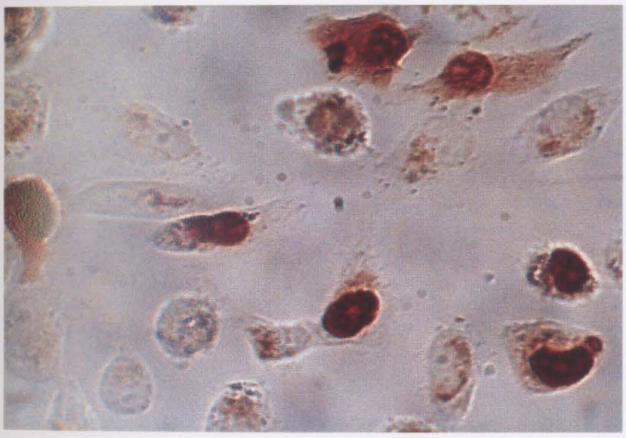
C. MDA-MB-231 cells



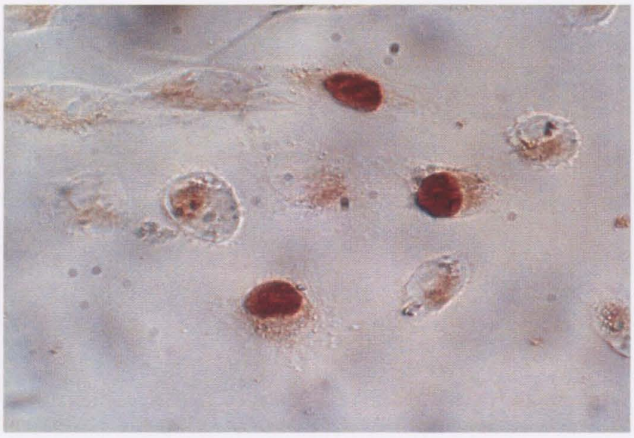
mock



CS3+MT

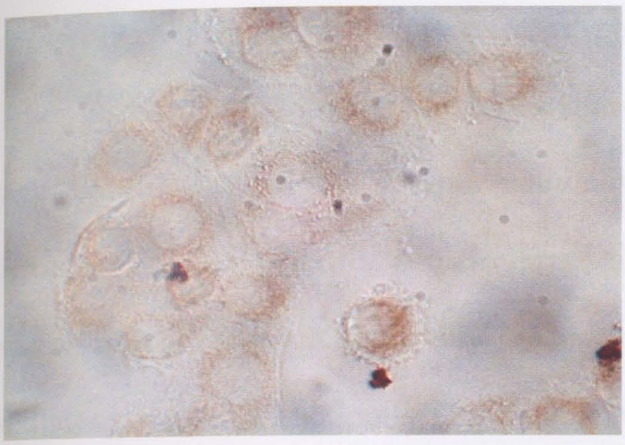


hMI-ER1 α

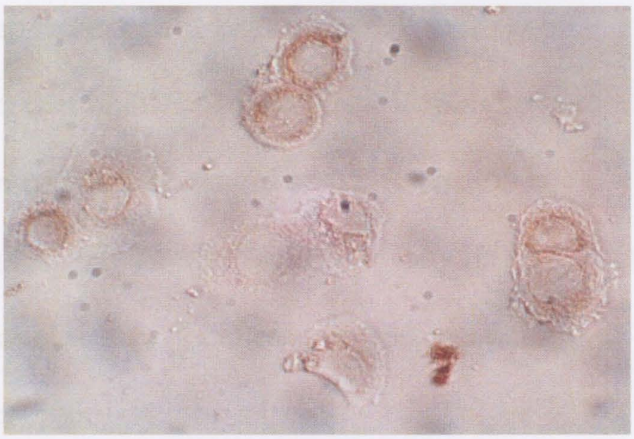


hMI-ER1 β

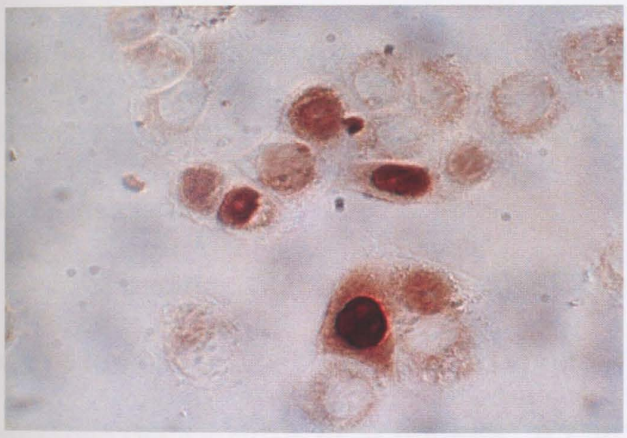
D. MDA-MB-468 cells



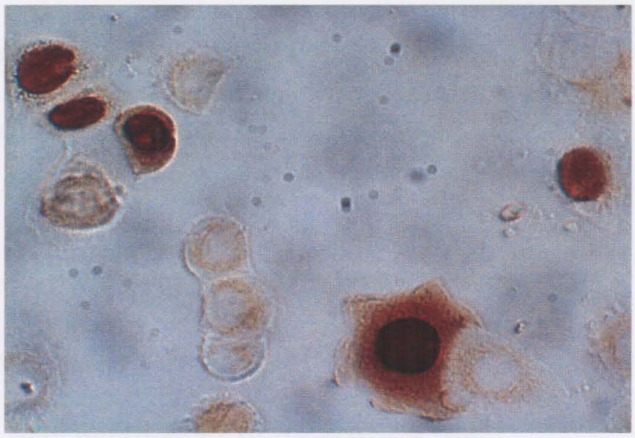
mock



CS3+MT



hMI-ER1 α



hMI-ER1 β

3.2 The overexpression of *hmi-er1α* at the mRNA level in transfected cell lines was evaluated by reverse transcription-polymerase chain reaction (RT-PCR)

Paterno *et al.* have previously suggested that hMI-ER1α and β isoforms may have distinct cellular functions. Further examination of the amino acid sequence of the α isoform revealed a potential protein interacting LXXLL motif (Paterno *et al.*, 2002). This leucine-rich motif (LXXLL; where L denotes leucine and X denotes any amino acid) mediates protein interaction with the nuclear receptors and the assembly of nuclear receptor-co-activator complexes (Heery *et al.*, 1997c). hMI-ER1 is already known to interact with estrogen receptors (Savicky *et al.*, unpublished data) and its expression level is elevated in breast tumour cell lines and tumour tissue, suggesting that the hMI-ER1α isoform is associated with the pathology of human breast cancer. Therefore, my work focuses on the role of hMI-ER1α playing on growth regulation of human breast carcinoma cells.

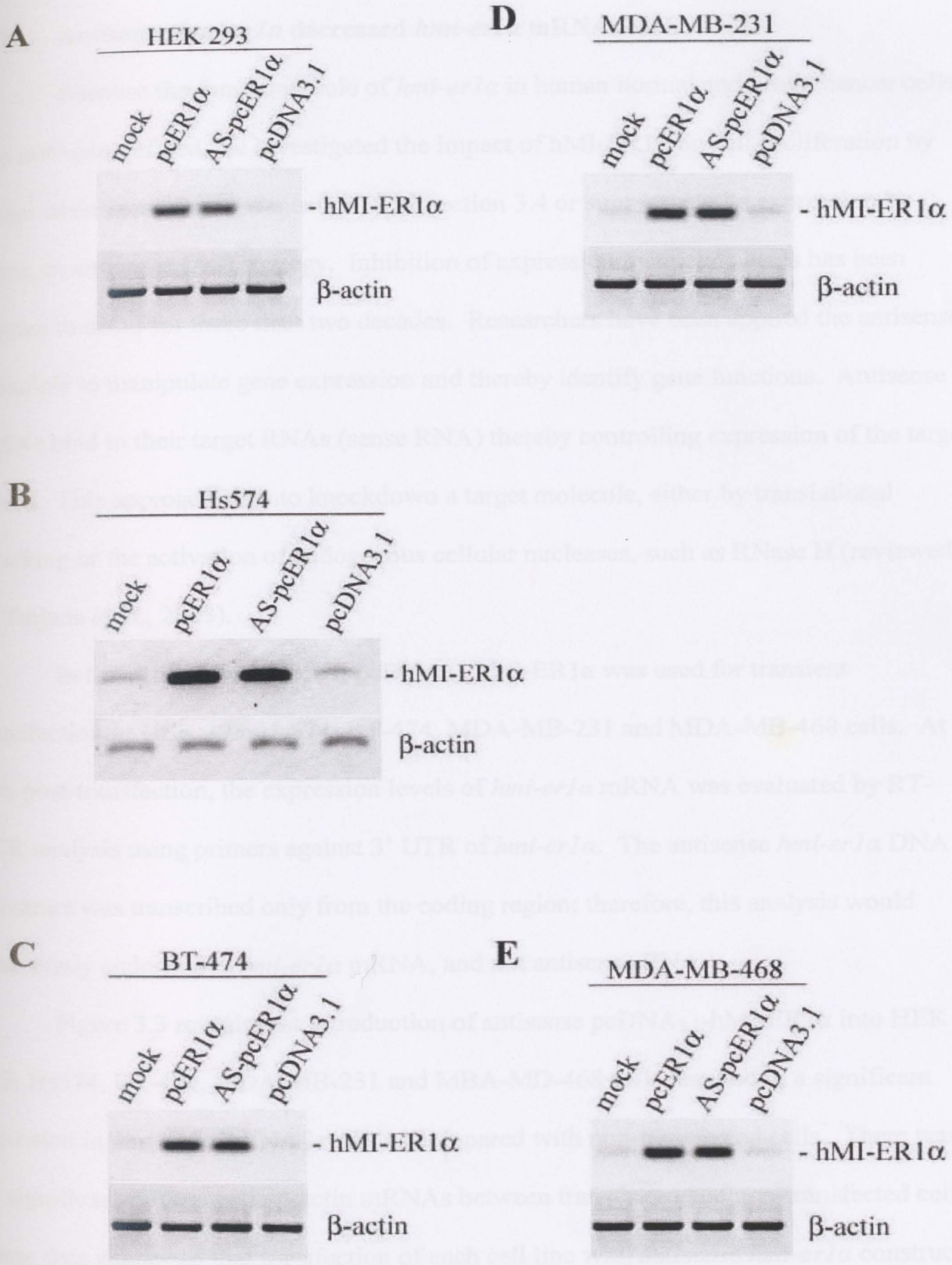
The expression levels of hMI-ER1 were very low in normal human tissues. Its expression can not be detected at the protein level using the available antibodies; thus, we employed the sensitive PCR method. RT-PCR was performed to determine the expression of *hmi-er1α* at the mRNA level in multiple transfected cell lines, including human embryonic kidney cells (HEK 293), normal breast cells (Hs574), and human breast carcinoma cell lines (BT-474, MDA-MB-231 and MDA-MB-468). In addition, RT-PCR was also used to confirm that the antisense construct was effectively downregulating *hmi-er1α* expression. First, the full-length cDNA of *hmi-er1α* was subcloned in sense or antisense orientation under the CMV promoter in $pcDNA_{3.1}$. Each cell line was transiently transfected with $pcDNA_{3.1}$ empty vector, $pcDNA_{3.1}$-hMI-ER1α

(pcER1α), or antisense $pcDNA_{3.1}$-hMI-ER1α (AS-pcER1α). These were transfected at a concentration of 1.5 μg each. Twenty-four hour later, total RNA was extracted from transfected cells using Trizol, and the complementary DNA was prepared by reverse transcription. PCR was then carried out using *hmi-er1α*-specific 5' (HER 2) and 3' (HER 4) primers to confirm the expression of *hmi-er1α* at the mRNA level. These primers recognize the same coding sequence in both endogenous and transfected *hmi-er1α*. Non-transfected cells and cells transfected with $pcDNA_{3.1}$ empty vector served as controls to ensure the entire experiment was properly conducted. The expression of human β-actin was also examined to show integrity and equality of input cDNA in each sample.

As expected, in each cell line the overexpression of *hmi-er1α* was detected in cells transfected with $pcDNA_{3.1}$-hMI-ER1α and those with antisense $pcDNA_{3.1}$-hMI-ER1α (Figure 3.2). This indicated successful cell transfections. Because the set of PCR primers used here could also recognize endogenous *hmi-er1α*, the expression of endogenous *hmi-er1α* mRNA in non-transfected and $pcDNA_{3.1}$ empty vector-transfected cells could be readily detected with an increase of PCR cycle.

Figure 3.2 RT-PCR analysis of *hmi-er1α* expression in human normal and breast carcinoma cell lines

Each cell line was transfected with $pcDNA_{3.1}$ empty vector, $pcDNA_{3.1}$-hMI-ER1α (pcER1α) or antisense $pcDNA_{3.1}$-hMI-ER1α (AS-pcER1α), followed by RNA isolation and reverse transcription as described in "Materials and Methods". cDNAs from each sample were amplified using *hmi-er1α*-specific 5' and 3' primers. The PCR products were loaded and electorphoresed on a 1% agarose gel (*top panels*). The over-expression of *hmi-er1α* was shown in HEK 293 (A), Hs574 (B), BT-474 (C), MDA-MB-231 (D) and MDA-MB-468 (E) cells. The expression of β-actin shown in each bottom panel was used as input control.


A
HEK 293
mock
pcER1α
AS-pcER1α
pcDNA3.1
- hMI-ER1α
β-actin
B
Hs574
mock
pcER1α
AS-pcER1α
pcDNA3.1
- hMI-ER1α
β-actin
C
BT-474
mock
pcER1α
AS-pcER1α
pcDNA3.1
- hMI-ER1α
β-actin
D
MDA-MB-231
mock
pcER1α
AS-pcER1α
pcDNA3.1
- hMI-ER1α
β-actin
E
MDA-MB-468
mock
pcER1α
AS-pcER1α
pcDNA3.1
- hMI-ER1α
β-actin

3.3 Antisense *hmi-er1α* decreased *hmi-er1α* mRNA levels

Because the functional role of *hmi-er1α* in human normal and breast cancer cells has not been defined, we investigated the impact of hMI-ER1α on cell proliferation by either overexpressing it as mentioned in Section 3.4 or suppressing its expression by using an antisense (AS) strategy. Inhibition of expression by nucleic acids has been known to occur for more than two decades. Researchers have been applied the antisense principle to manipulate gene expression and thereby identify gene functions. Antisense RNAs bind to their target RNAs (sense RNA) thereby controlling expression of the target genes. This approach aims to knockdown a target molecule, either by translational blocking or the activation of endogenous cellular nucleases, such as RNase H (reviewed in Tatjana *et al.*, 2003).

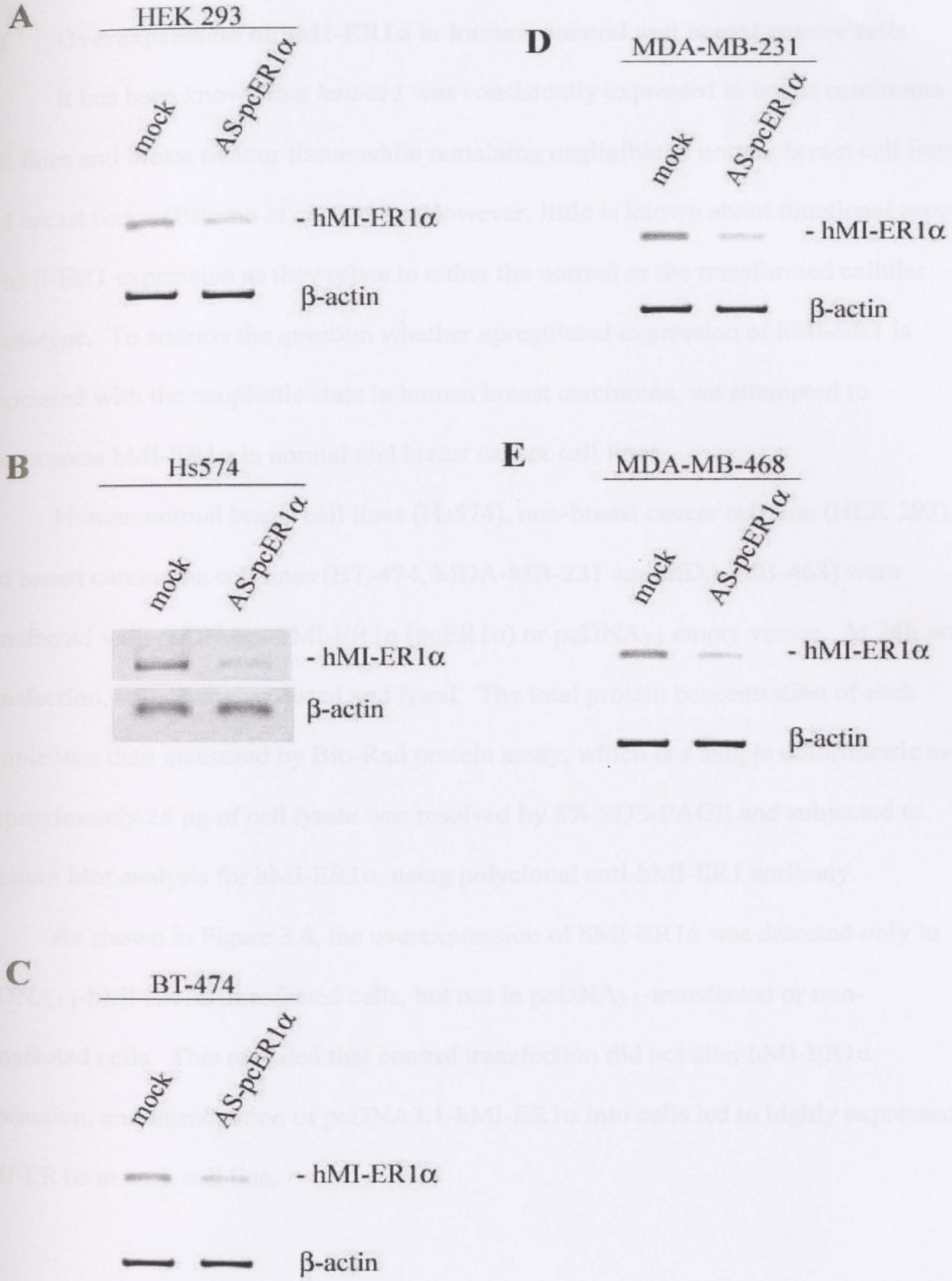
In this study, the antisense $pcDNA_{3.1}$-hMI-ER1α was used for transient transfection of HEK 293, Hs574, BT-474, MDA-MB-231 and MDA-MB-468 cells. At 24h post-transfection, the expression levels of *hmi-er1α* mRNA was evaluated by RT-PCR analysis using primers against 3' UTR of *hmi-er1α*. The antisense *hmi-er1α* DNA construct was transcribed only from the coding region; therefore, this analysis would detect only endogenous *hmi-er1α* mRNA, and not antisense RNA.

Figure 3.3 reveals that introduction of antisense $pcDNA_{3.1}$-hMI-ER1α into HEK 293, Hs574, BT-474, MDA-MB-231 and MBA-MD-468 cells resulted in a significant reduction in *hmi-er1a* mRNA levels, as compared with non-transfected cells. There was no significant difference in β-actin mRNAs between transfected and non-transfected cells. These data suggested that transfection of each cell line with antisense *hmi-er1α* constructs

specifically suppresses *hmi-er1α* mRNA levels. To find out how many fold is the *hmi-er1α* mRNA being suppressed, densitometric scans should be included in future study.

Figure 3.3 Down-regulation of endogenous hMI-ER1α expression in antisense pcDNA$_{3.1}$-hMI-ER1α-transfected cells

HEK 293 (A), Hs574 (B), BT-474 (C), MDA-MB-231 (D) and MDA-MB-468 (E) cells were transfected with or without antisense pcDNA$_{3.1}$-hMI-ER1α (AS-pcER1α). Total RNA was extracted with Trizol 24h later and used as template for RT-PCRs performed with primers specific for 3' UTR of *hmi-er1α*. PCR products were analyzed on a 1% agarose gel. Antisense pcDNA$_{3.1}$-hMI-ER1α-transfected cells showed a marked reduction in the expression of *hmi-er1α* mRNA (*top panels*). The expression of β-actin was included to confirm that each lane contained the same amount of PCR products (*bottom panels*).


A
HEK 293
mock
AS-pcER1α
- hMI-ER1α
β-actin
B
Hs574
mock
AS-pcER1α
- hMI-ER1α
β-actin
C
BT-474
mock
AS-pcER1α
- hMI-ER1α
β-actin
D
MDA-MB-231
mock
AS-pcER1α
- hMI-ER1α
β-actin
E
MDA-MB-468
mock
AS-pcER1α
- hMI-ER1α
β-actin

3.4 Overexpression of hMI-ER1α in human normal and breast cancer cells

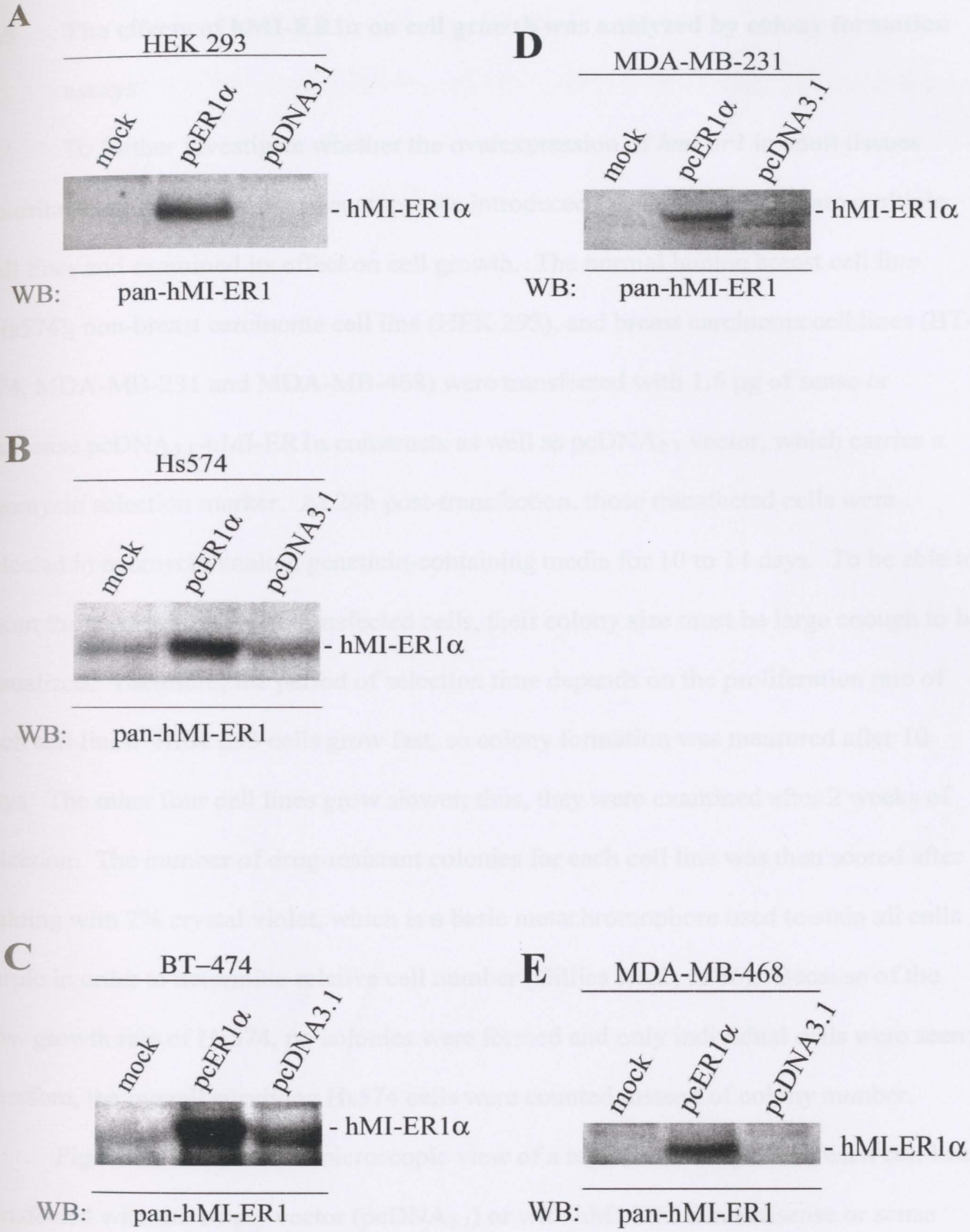
It has been known that *hmi-er1* was consistently expressed in breast carcinoma cell lines and breast tumour tissue while remaining negligible in normal breast cell lines and breast tissue (Paterno *et al.*, 1998). However, little is known about functional aspects of hMI-ER1 expression as they relate to either the normal or the transformed cellular phenotype. To address the question whether upregulated expression of hMI-ER1 is associated with the neoplastic state in human breast carcinoma, we attempted to overexpress hMI-ER1α in normal and breast cancer cell lines.

Human normal breast cell lines (Hs574), non-breast cancer cell line (HEK 293), and breast carcinoma cell lines (BT-474, MDA-MB-231 and MDA-MB-468) were transfected with $pcDNA_{3.1}$-hMI-ER1α (pcER1α) or $pcDNA_{3.1}$ empty vector. At 24h post-transfection, cells were harvested and lysed. The total protein concentration of each sample was then measured by Bio-Rad protein assay, which is a simple colorimetric assay. Approximately 25 μg of cell lysate was resolved by 8% SDS-PAGE and subjected to Western blot analysis for hMI-ER1α, using polyclonal anti-hMI-ER1 antibody.

As shown in Figure 3.4, the overexpression of hMI-ER1α was detected only in $pcDNA_{3.1}$-hMI-ER1α-transfected cells, but not in $pcDNA_{3.1}$-transfected or non-transfected cells. This revealed that control transfection did not alter hMI-ER1α expression, and introduction of pcDNA3.1-hMI-ER1α into cells led to highly expressed hMI-ER1α in each cell line.

Figure 3.4 Western blot analysis of hMI-ER1α expression in multiple cell lines

HEK 293 (A), Hs574 (B), BT-474 (C), MDA-MB-231 (D) and MDA-MB-468 (E) cells transiently transfected with $pcDNA_{3.1}$-hMI-ER1α (pcER1α) or $pcDNA_{3.1}$ control vector, along with non-transfected cells were harvested 24h after transfection. And approximately 25 μg of cell lysate was Western blotted with polyclonal anti-hMI-ER1α antibody, as described in "Material and Method". Only cells transfected with $pcDNA_{3.1}$-hMI-ER1α showed the overexpression of hMI-ER1α.


A
HEK 293
mock
pcER1α
pcDNA3.1
- hMI-ER1α
WB: pan-hMI-ER1
D
MDA-MB-231
mock
pcER1α
pcDNA3.1
- hMI-ER1α
WB: pan-hMI-ER1
B
Hs574
mock
pcER1α
pcDNA3.1
- hMI-ER1α
WB: pan-hMI-ER1
C
BT–474
mock
pcER1α
pcDNA3.1
- hMI-ER1α
WB: pan-hMI-ER1
E
MDA-MB-468
mock
pcER1α
pcDNA3.1
- hMI-ER1α
WB: pan-hMI-ER1

3.5 The effects of hMI-ER1α on cell growth was analyzed by colony formation assays

To further investigate whether the overexpression of *hmi-er1* in adult tissues contributes to the neoplastic phenotype, we introduced *hmi-er1α* isoform into multiple cell lines and examined its effect on cell growth. The normal human breast cell line (Hs574), non-breast carcinoma cell line (HEK 293), and breast carcinoma cell lines (BT-474, MDA-MB-231 and MDA-MB-468) were transfected with 1.5 μg of sense or antisense $pcDNA_{3.1}$-hMI-ER1α constructs as well as $pcDNA_{3.1}$ vector, which carries a neomycin selection marker. At 24h post-transfection, those transfected cells were selected in neomycin analog, geneticin-containing media for 10 to 14 days. To be able to count the colony number of transfected cells, their colony size must be large enough to be visualized. Therefore, the period of selection time depends on the proliferation rate of each cell lines. HEK 293 cells grow fast, so colony formation was measured after 10 days. The other four cell lines grow slower; thus, they were examined after 2 weeks of selection. The number of drug-resistant colonies for each cell line was then scored after staining with 2% crystal violet, which is a basic metachromophore used to stain all cells purple in order to determine relative cell number (Gillies *et al.*, 1986). Because of the slow growth rate of Hs574, no colonies were formed and only individual cells were seen; therefore, the overall surviving Hs574 cells were counted instead of colony number.

Figure 3.5 illustrates a microscopic view of a representative plate of each cell line transfected with the empty vector ($pcDNA_{3.1}$) or with hMI-ER1α in antisense or sense orientations. HEK 293 cells transfected with hMI-ER1α expression plasmids demonstrated a dramatic reduction in the number of colonies suggesting that

overexpression of hMI-ER1α suppresses growth of these cells. The similar anti-growth effect is also seen in human normal breast cell line, Hs574. As shown in Figure 3.5D, the dead Hs574 cells that were fragmented into debris (purple dots) were excluded from the count. BT-474 cells did not grow into big colonies; therefore, the pictures of micro colonies were shown (Figure 3.5E). For each experiment, triplicate plates were prepared and the values represent an average of these independent data. Results from three independent experiments revealed between 70 and 90% growth inhibition of HEK 293 and Hs574 upon overexpression of hMI-ER1α (Figure 3.6A and B). However, very little effects on colony formation (5-10% reduction) were observed in three breast carcinoma cell lines that were transfected with sense hMI-ER1α. In other words, hMI-ER1α-transfected MDA-MB-231, MDA-MB-468 and BT-474 cells maintained their normal growth rate (Figure 3.6C, D and E).

In the case of antisense hMI-ER1α-transfected HEK 293 and Hs574 cells, only a slight reduction in growth rate was observed as compared to the vector controls (Figure 3.6 A and B). Applying the Student's *t-test*, the colony or cell counts for antisense hMI-ER1α-transfected cell lines were compared to that of the control vector transfected cells. Antisense hMI-ER1α has no significant effects on HEK 293 and Hs574 cells ($P = 0.12$ and 0.29 for HEK 293 and Hs574, respectively). Interestingly, a significantly marked reduction in the colony numbers was observed in the breast cancer cells transfected with antisense hMI-ER1α (all P-values < 0.05). Examination of colony formation from three independent experiments showed 40 to 60% decrease in colony-forming efficiency in antisense hMI-ER1α-transfected breast carcinoma cells (Figure 3.6C, D and E). The extent of cell growth inhibition by antisense *hmi-er1α* varied between the three breast

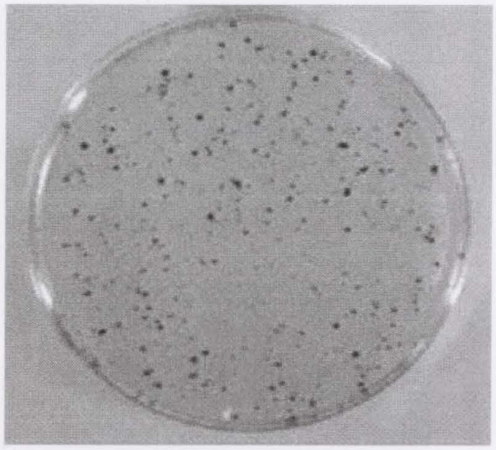
cancer cell lines. This difference may be associated with their content of estrogen receptors (ERs); BT-474 is ER-positive, and both MDA-MB-231 and MDA-MB-468 are ER-negative and highly invasive breast cancer cell lines.

The overall data demonstrate that the impact of hMI-ER1α on growth of human breast carcinoma cells is very different from normal and non-breast cancer cells. The expression of hMI-ER1α caused a noticeable decrease in growth rate of normal breast cells, but it is also essential for growth of breast cancer cells. The hMI-ER1α overexpressing breast cancer cells exhibited a colony formation advantage, while the breast cancer cells with reduced levels of hMI-ER1α were growth suppressed. These findings support an important regulatory role for hMI-ER1α in breast cancer.

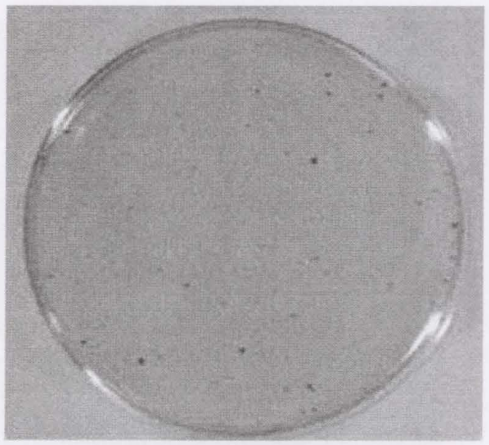
Figure 3.5 Colony formation of various cell lines transfected with either sense or antisense hMI-ER1α expression plasmids

HEK 293 (A), MDA-MB-231 (B), MDA-MB-468 (C), Hs574 (D), and BT-474 (E) were seeded in 6-well plates. After 24h, cells were transfected with empty vector $pcDNA_{3.1}$ (top), $pcDNA_{3.1}$-hMI-ER1α (middle), or antisense $pcDNA_{3.1}$-hMI-ER1α (bottom). At 24h post-transfection, cultures were harvested and equal numbers of cells were placed in 60mm plates with geneticin-containing media. These transfected cells were selected by geneticin for 10 to 14 days. The formed colonies were photographed after staining with 2% crystal violet. The pictures of BT-474 micro colonies were all taken at the same magnification (*Bar*, 1 mm). The pictures of Hs574 surviving cells were taken at 40X magnification (*Bar*, 0.5 mm). Each experiment was carried out three times. Representative plates are shown. Note that hMI-ER1α overexpression resulted in growth suppression in human normal cell lines, but had no significant effects on human breast carcinoma cell lines. Interestingly, a marked reduction in colony-forming efficiency was observed in the antisense hMI-ER1α vs. sense hMI-ER1α-transfected breast cancer cells. Cells transfected with empty vector were used as controls.

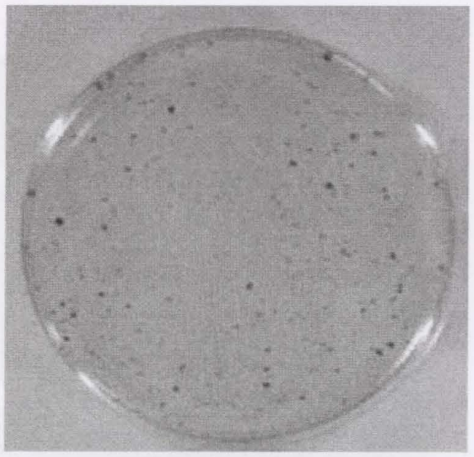
A. HEK 293 cells



pcDNA3.1

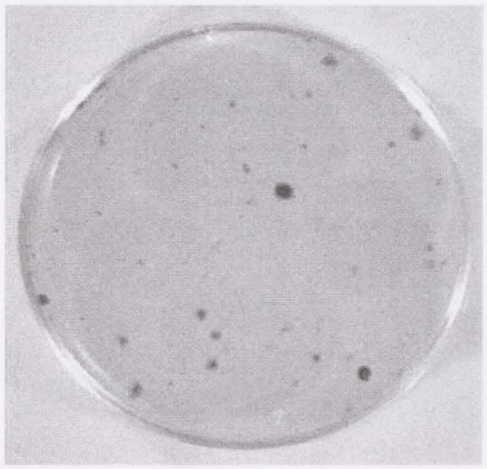


hMI-ER1α

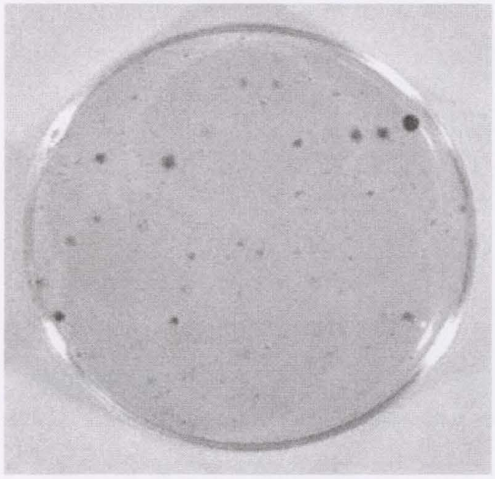


AS-hMI-ER1α

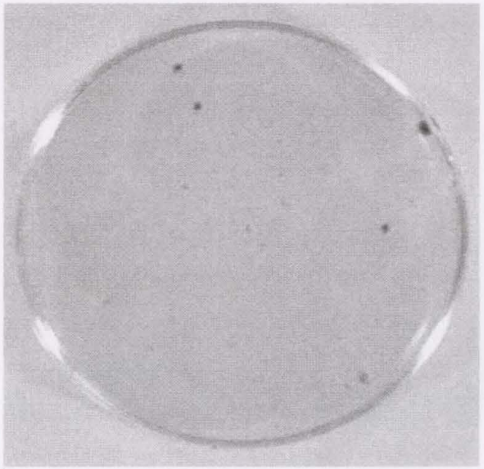
B. MDA-MB-231 cells



pcDNA3.1

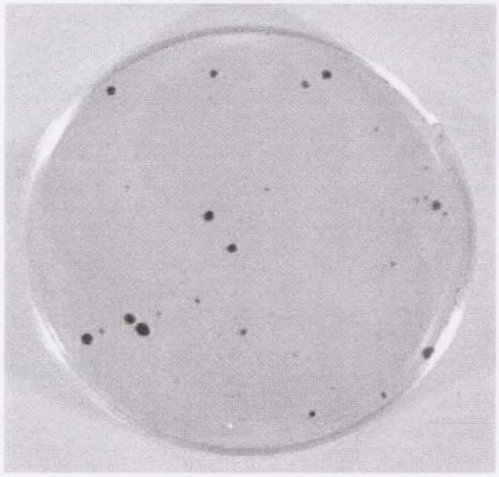


hMI-ER1α

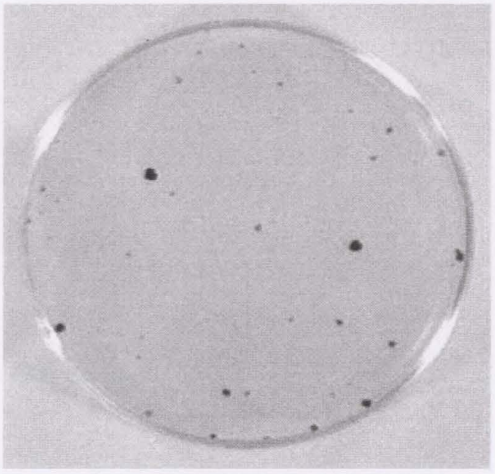


AS-hMI-ER1α

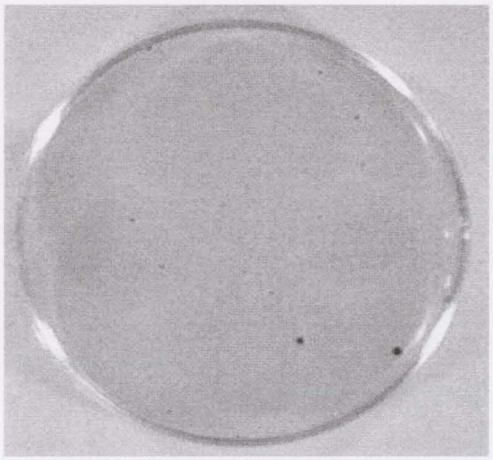
C. MDA-MB-468 cells



pcDNA3.1

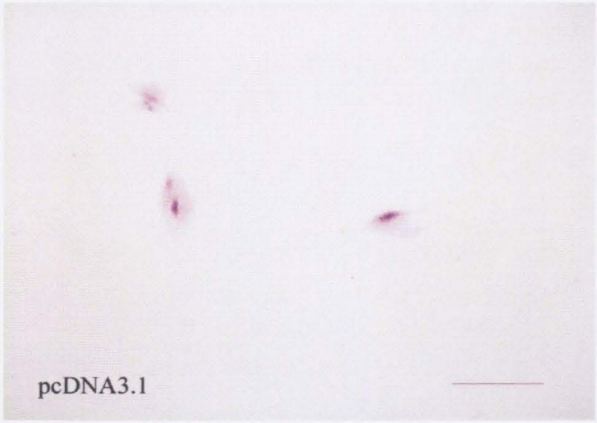


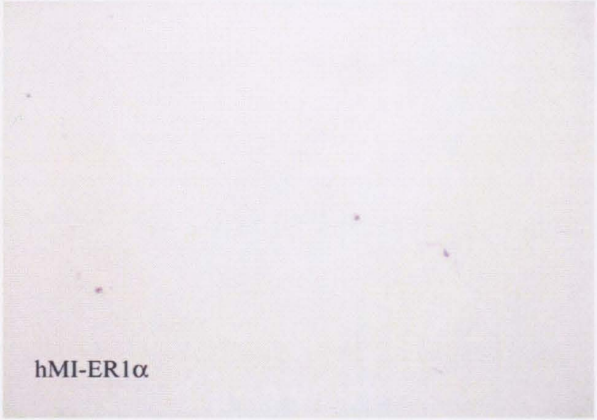
hMI-ER1α

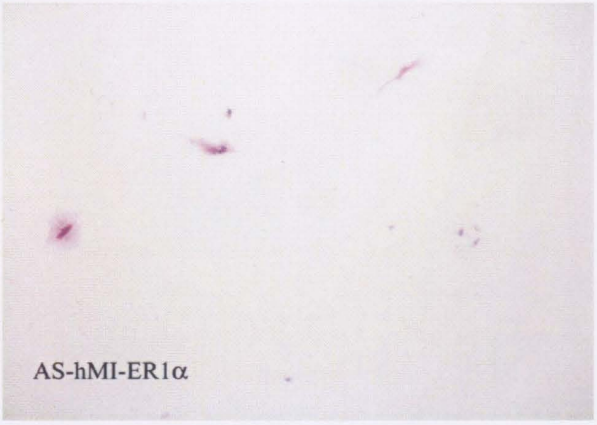


AS-hMI-ER1α

D. Hs574 cells







E. BT-474 cells

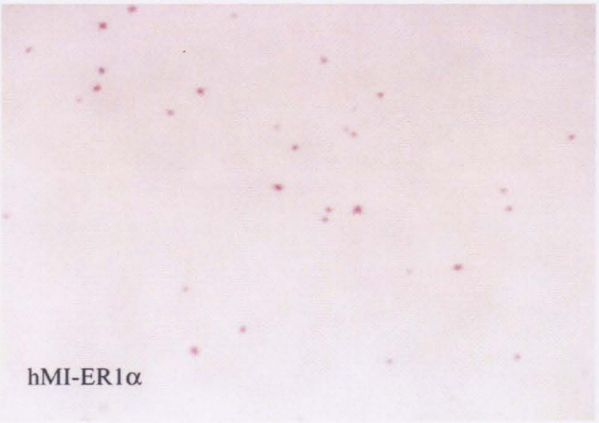
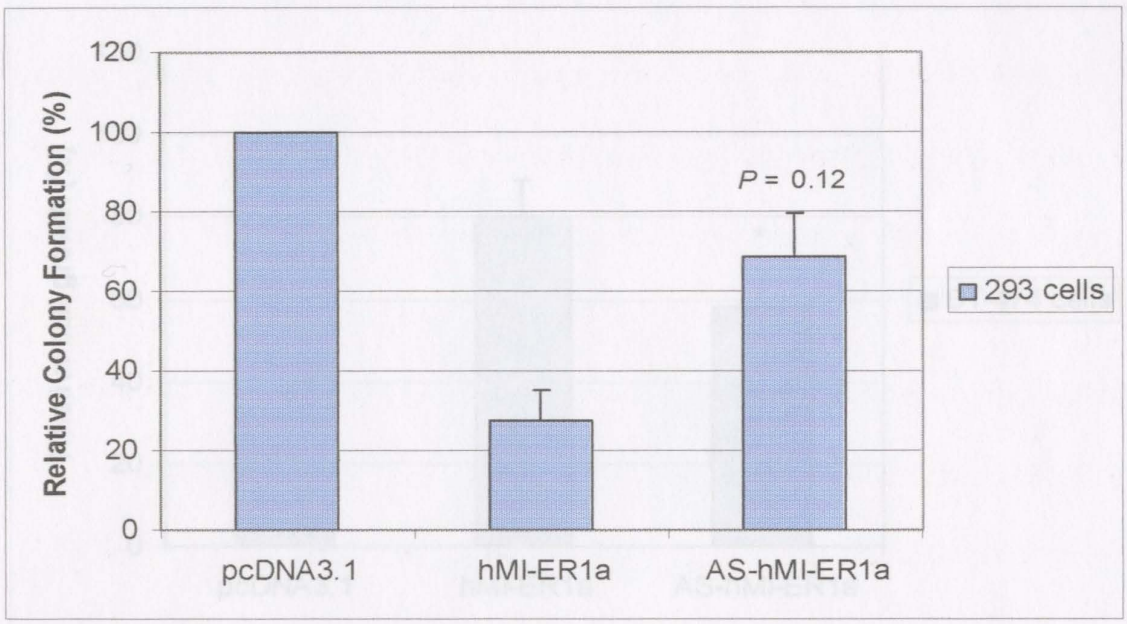


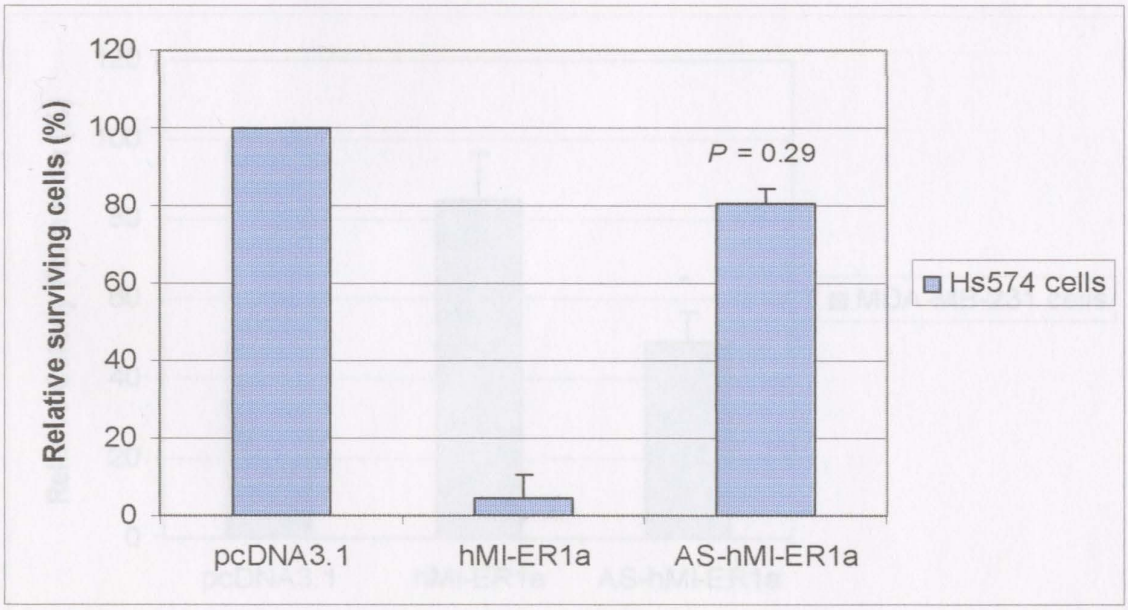
Figure 3.6 Measurement of colony numbers in transfected cell lines

HEK 293, Hs574, BT-474, MDA-MB-231 and MDA-MB-468 cells were transfected with $pcDNA_{3.1}$ control vector, $pcDNA_{3.1}$-hMI-ER1α or AS-$pcDNA_{3.1}$-hMI-ER1α. Transfected cells were selected in the presence of geneticin for 10-14 days. Numbers of drug-resistant colonies or cells were scored after staining with 2% crystal violet. The results represent the average ± SD of three experiments, each performed in triplicate. The overexpression of hMI-ER1α was associated with a marked reduction in the growth rate of two human non-breast carcinoma cell lines, HEK 293 (A) and Hs574 (B), but did not alter the growth rate of three breast cancer cell lines: BT-474 (C), MDA-MB-231 (D) and MDA-MB-468 (E). It is apparent that colony-forming efficiency was significantly reduced by antisense inhibition of hMI-ER1α expression in three breast cancer cell lines. * , Statistical significance is assessed by Student's *t-test* for independent groups (All P-values < 0.05)

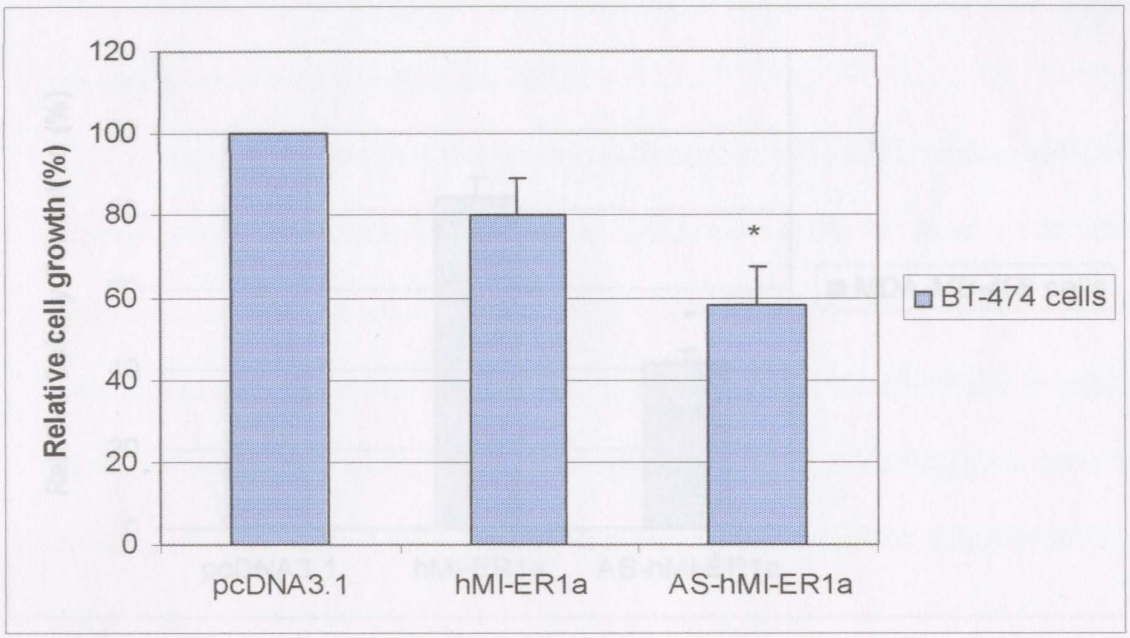
A. HEK 293 cells



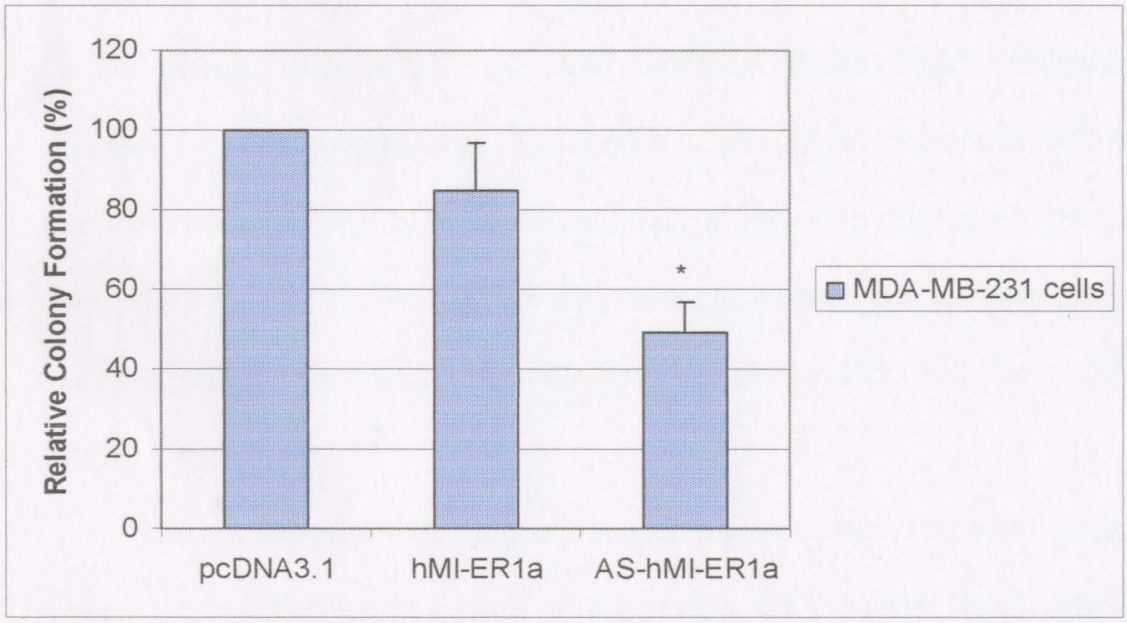
B. Hs574 cells



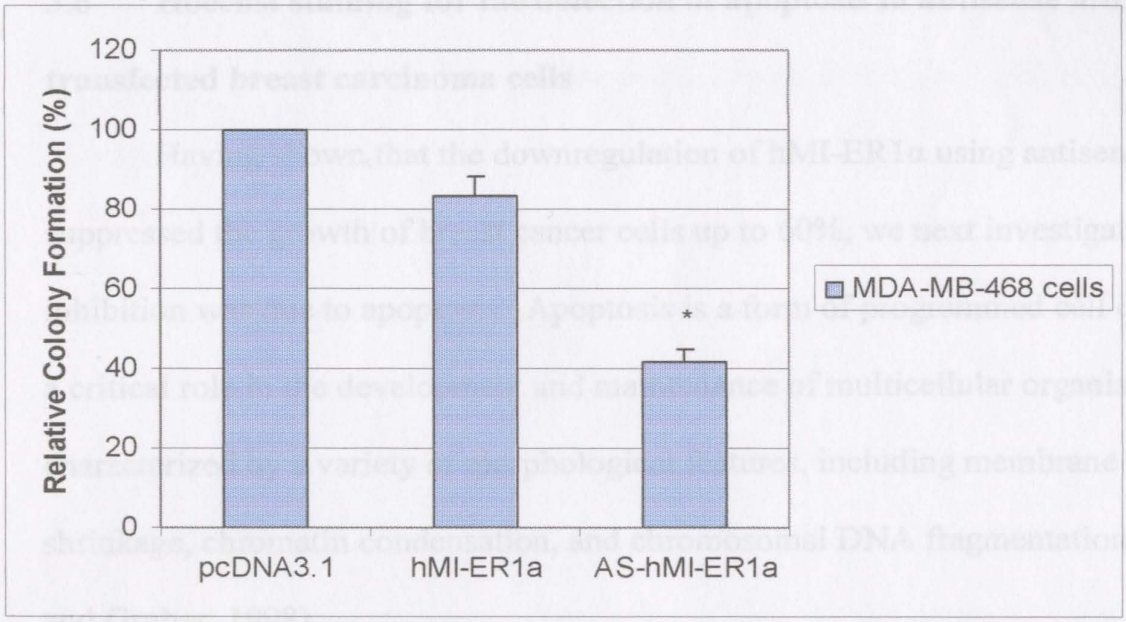
C. BT-474 cells



D. MDA-MB-231 cells



E. MDA-MB-468 cells



3.6 Hoechst staining for the detection of apoptosis in antisense hMI-ER1α-transfected breast carcinoma cells

Having shown that the downregulation of hMI-ER1α using antisense technology suppressed the growth of breast cancer cells up to 60%, we next investigated if this inhibition was due to apoptosis. Apoptosis is a form of programmed cell death that plays a critical role in the development and maintenance of multicellular organisms. It is characterized by a variety of morphological features, including membrane blebbing, cell shrinkage, chromatin condensation, and chromosomal DNA fragmentation, etc. (Sgonc and Gruber, 1998).

HEK 293, MDA-MB-231 and MDA-MB-468 cells were transfected with empty vector $pcDNA_{3.1}$ and antisense hMI-ER1α construct (AS-$pcDNA_{3.1}$-hMI-ER1α). At 24h post-transfection, cells were seeded in 8-chamber slides. After 48h, cells were fixed with 4% paraformaldehyde in PBS and stained with the DNA-binding fluorochrome bis-benzimide dye (Hoechst 33342). Cells were then observed with a fluorescence microscope and photographed. The Hoechst 33342 dye is a popular cell-permeant nuclear stain that emits blue fluorescence when bound to double-stranded DNA. Hoechst dyes preferentially bind to adenine-thymidine (AT) base pair rich regions. It is often used to distinguish condensed pycnotic nuclei in apoptotic cells (Holmquist, 1975;Shapiro, 1981).

HEK 293 cells were used as a negative control here since antisense hMI-ER1α had no profound effect on growth of these cells. On the other hand, staurosporine (STS)-treated MDA-MB-231 and MDA-MB-468 cells were used as positive controls. Staurosporine is a strong protein kinase C inhibitor and a well-studied apoptosis inducer

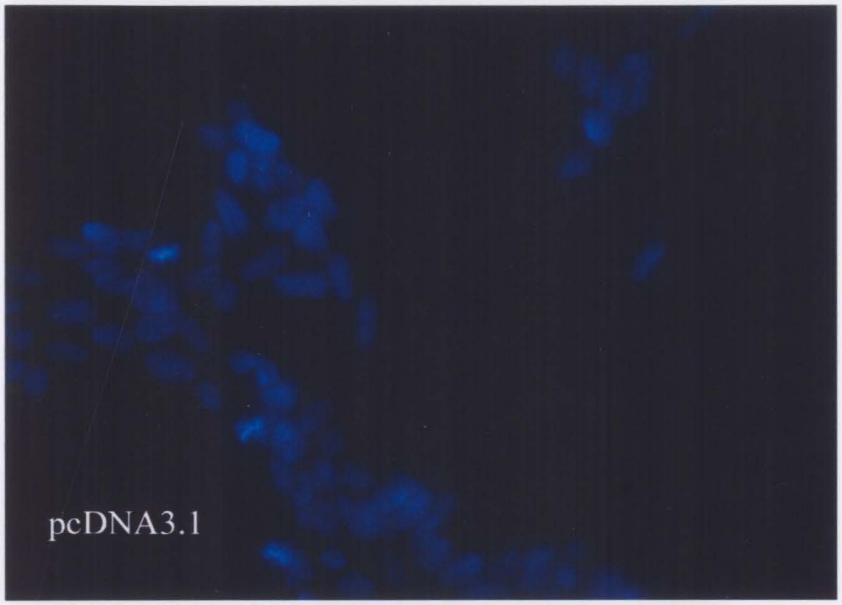
(Tamaoki *et al.*, 1986;Jacobsen *et al.*, 1996;Jiang *et al.*, 2002). Morphological changes in the nuclear chromatin of staurosporine-treated cells undergoing apoptosis were detected by staining with Hoechst.

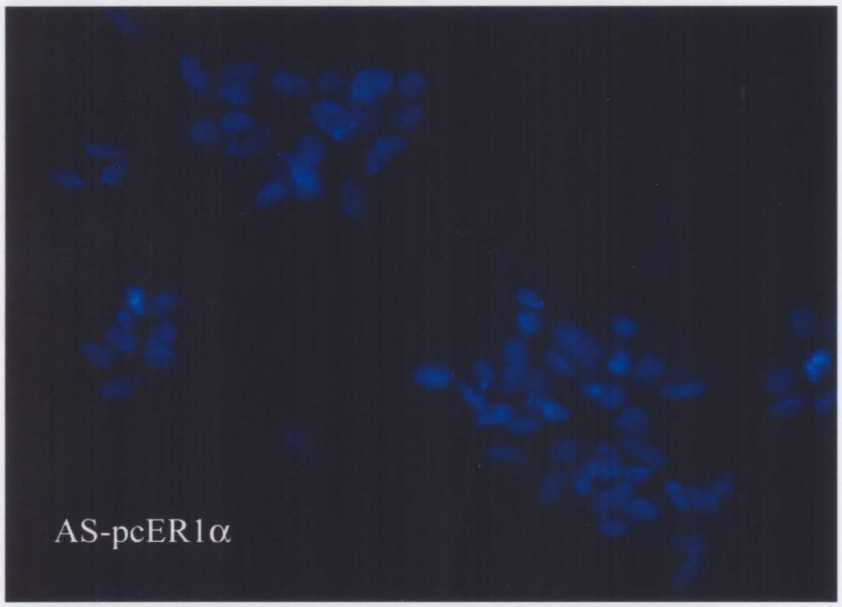
As shown in Figure 3.7, after the treatment with staurosporine, the positive control cells showed significant DNA fragmentation and cell shrinkage that are characteristics of apoptotic cells. In contrast, little or no DNA fragmentation was detected in either empty vector $pcDNA_{3.1}$ or antisense hMI-ER1α-transfected HEK 293, MDA-MB-231 and MDA-MB-468 cells. This preliminary data suggests that the inhibitory effect of antisense hMI-ER1α on growth of breast cancer cells may not be via an apoptotic mechanism. In other words, blocking hMI-ER1α in breast carcinoma cell lines may not induce apoptosis.

Figure 3.7 Nuclear staining of cells with Hoechst 33342

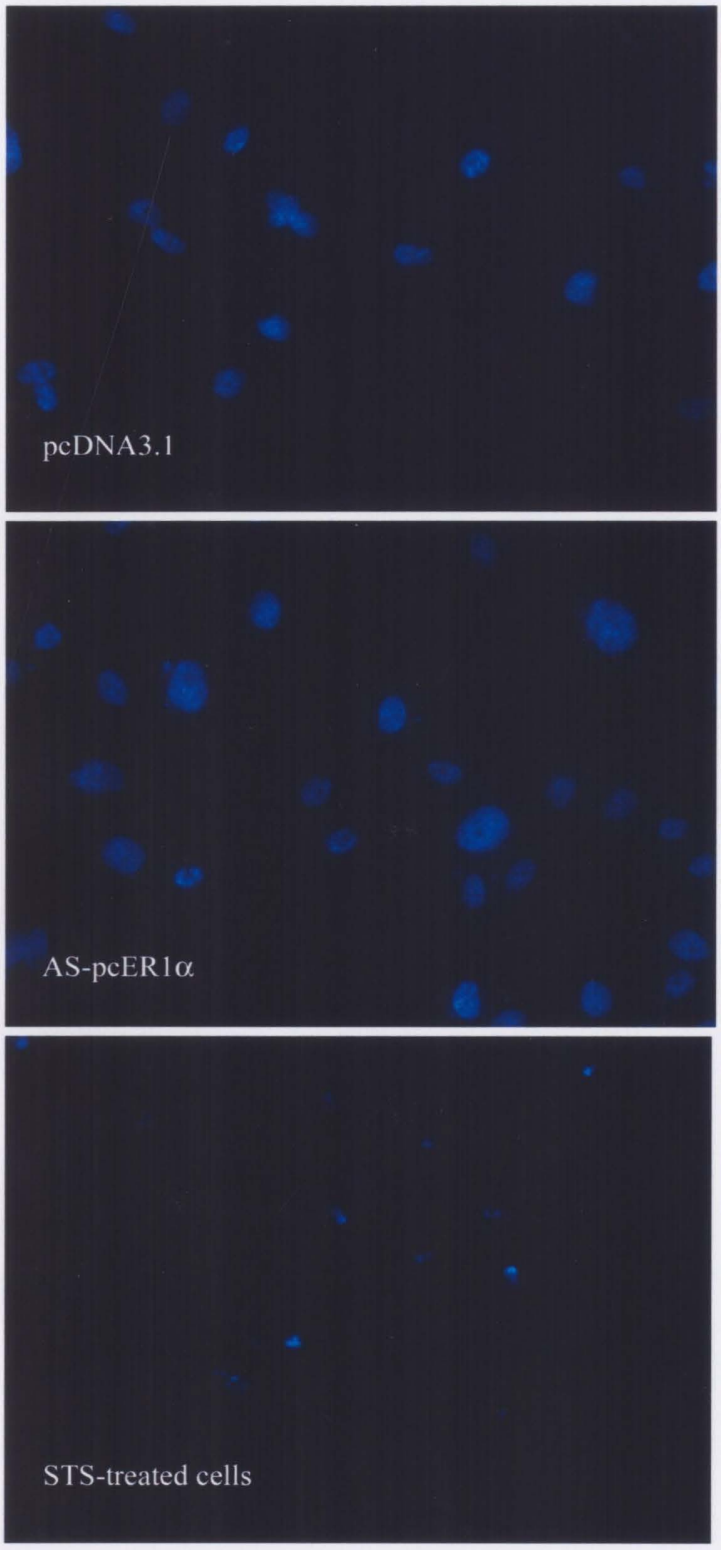
Transfection of HEK 293 (A), MDA-MB-231 (B) and MDA-MB-468 (C) cells with empty vector $pcDNA_{3.1}$ or AS-$pcDNA_{3.1}$- hMI-ER1α (AS-pcER1α) construct, followed by nuclear staining with Hoechst 33342 were viewed and photographed with a fluorescence microscope. All three cell lines transfected with antisense hMI-ER1α revealed no difference in nuclear morphology from that transfected with empty vectors. MDA-231 and MDA-468 cells treated with staurosporine (STS) for 48h were used as positive controls. These control cells with typical punctuated nuclear morphology of apoptotic cells were detected (B and C). Data shown are representative of three independent experiments.

A. HEK 293 cells

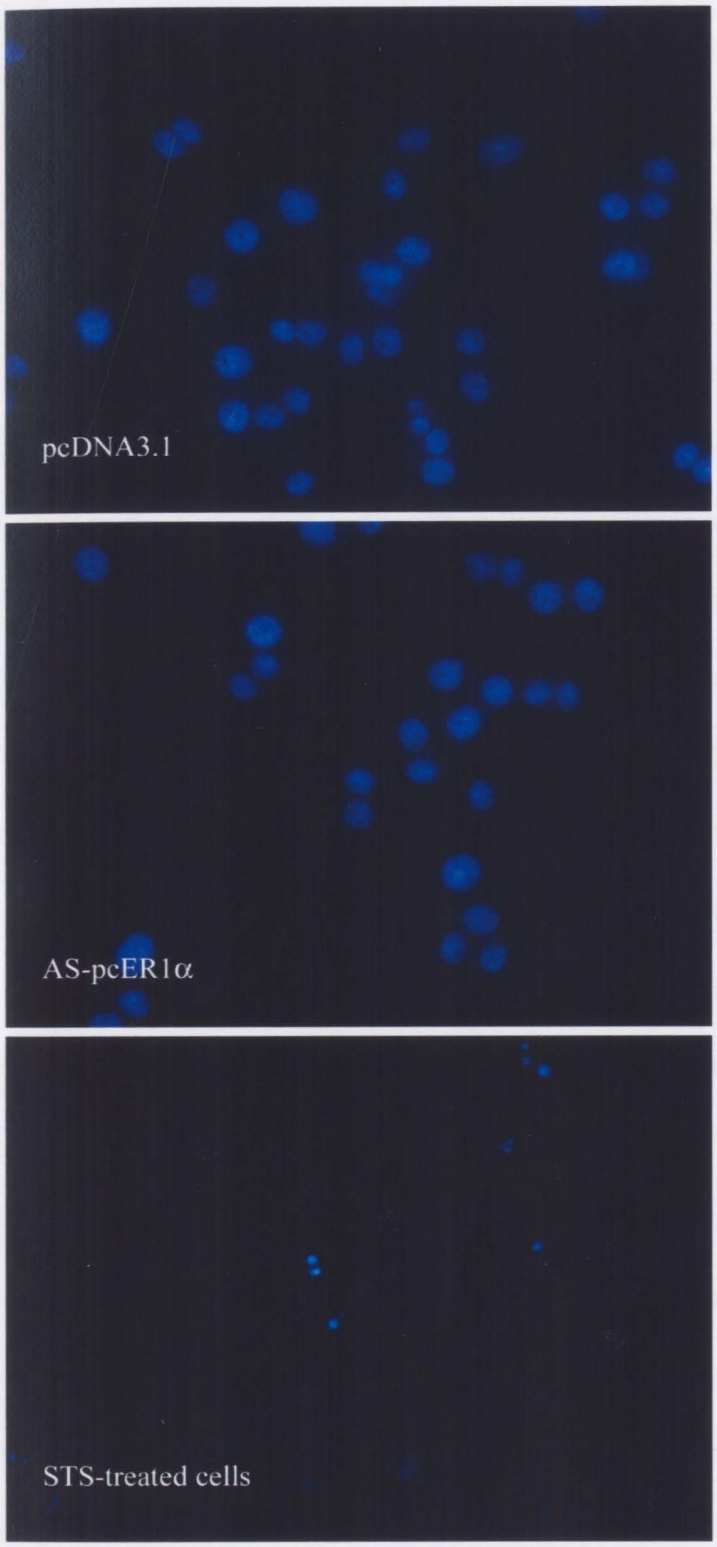




B. MDA-MB-231 cells



C. MDA-MB-468 cells



Chapter 4: Discussion

It is now well accepted that FGF signaling pathway plays an important role in controlling mammary gland development, morphogenesis, and breast cancer progression (Chalbos *et al.*, 1994;Dickson and Lippman, 1995). The recent discovery of a novel FGF-inducible immediate-early gene, *mi-er1*, has revealed a possible "master" regulatory gene involved in a variety of cellular processes. The expression of human *mi-er1* is higher in the testis than in the other tissues (Paterno *et al.,* 1998). In the testis, spermatogenesis occurs as a complex developmental process characterized by a fast rate of cell proliferation that is controlled by an elaborate cascade of transcriptional and regulatory events (Sassone-Corsi, 2002;Lewis *et al.*, 2003). This is consistent with the notion that the hMI-ER1 protein might be involved in normal cellular functions, such as cell proliferation. Paterno *et al.* (1998) have showed that *hmi-er1* is consistently expressed in breast carcinoma cell lines and tumours while being barely detectable in normal breast cell lines and breast tissue. In an attempt to understand its role in regulating growth of human normal and breast cancer cells, several basic cell culture techniques and assays were performed in this study.

In spite of the fact that *hmi-er1* mRNA expression is upregulated in breast tumours (Paterno *et al.*, 1998), the expression levels of endogenous hMI-ER1 protein remain unexplored due to lack of effective antibodies. In this experiment, Western analyses showed no detectable hMI-ER1α protein in breast carcinoma cell lines yet lots in normal human breast cell line, Hs574, (Figure 3.4). The relatively substantial levels of

hMI-ER1α protein in Hs574 cells may be the result of a mixed cell population in this particular cell line since many of available normal breast cell lines are obtained from histologically normal tissue surrounding a breast tumour (Paterno *et al.*, 1998). These observations raise the possibility that a hMI-ER1α mutant protein may be produced in breast cancer cells; therefore, no hMI-ER1α can be detected. This possibility warrants further study.

Previous studies have revealed that the only *bona fide* nuclear localization signal is found in the C-terminus of the hMI-ER1β isoform; hence, hMI-ER1β is located predominantly in the nucleus, while hMI-ER1α is cytoplasmic in transfected NIH 3T3 cells (Paterno *et al.*, 2002). Interestingly, DAB staining of three hMI-ER1α-transfected human breast carcinoma cell lines showed that hMI-ER1α is targeted primarily to the nucleus. Many proteins without an NLS have been found to translocate to the nucleus through interaction with other proteins, including β-catenin (Gan and Khalili, 2004;Townsley *et al.*, 2004) and Smads (Kurisaki *et al.*, 2001;Fink *et al.*, 2003). Therefore, it is possible that hMI-ER1α was transported into the nucleus through interaction with histone deacetylase (HDAC) (Ding *et al.*, 2003), histone acetyltransferase (HAT) (Blackmore *et al.*, unpublished data), nuclear hormone receptors (Savicky *et al.*, unpublished data), or other proteins. Within epithelial cells, diverse appropriately regulated signaling pathways are frequent targets of genetic alteration during progression to carcinoma (reviewed in Hanahan and Weinberg, 2000). A shared property of these pathways is the regulation of transcription factors by tethering within the cytoplasm. Under tightly controlled conditions, paracrine/autocrine signals induce translocation of latent transcription factors from the cytoplasm to the nucleus, resulting in activation of

specific target genes. Genetic alterations found in cancer lead to inappropriate nuclear accumulation of transcription factors. Therefore, nuclear accumulation of hMI-ER1α in breast carcinoma cells may trigger a variety of transcriptional responses and in turn affects cell growth. It is very likely that its altered nuclear functions contribute to tumour progression. Some may argue that nuclear transport of hMI-ER1α in breast cancer cells may be due to cell-type specificity. More studies are needed in order to clear this doubt.

Estrogen receptor (ER) status is an important prognostic biomarker in breast cancer. Loss of expression or function of ER facilitates the metastatic behaviour and progression of breast cancer (Girdler and Brotherick, 2000); thus, ER-negative breast cancer cells are more aggressive and invasive. A recent study has shown that hMI-ER1α interacts with ERα and represses ER element (ERE)-driven transcription in ER-positive MCF-7 breast cancer cells, indicating potential negative regulation between hMI-ER1α and ER (Savicky *et al.*, unpublished data). To determine whether the interaction between hMI-ER1α and ER has an impact on growth of breast cancer cells, sense or antisense *hmi-er1α*-transfected ER-positive and -negative breast carcinoma cell lines were examined. Blocking hMI-ER1α using an antisense strategy inhibited the growth of both ER-positive and –negative cancer cells, but the extent of growth inhibition is greater in ER-negative cells. Therefore, *hmi-er1α* has, at least in part, a direct effect on cellular growth, independent of the estrogen receptor.

Quantitative analysis of colony-forming efficiency by colony-formation assays allows us to investigate the effect of ectopic hMI-ER1α expression on cell proliferation in selected cell lines. Furthermore, it has also provided evidence that hMI-ER1α expression is associated with the change of morphology in human normal breast cells (Figure 3.5B);

it is therefore linked with cell transformation and cell death. In this study, transient overexpression of hMI-ER1α in human normal cell line (Hs574) resulted in a dramatic reduction in the number of colonies indicating that hMI-ER1α can act as a potent growth suppressor in normal cells. In contrast, forced hMI-ER1α expression has no significant effect on growth of three breast carcinoma cell lines.

The functional inactivation of *hmi-er1α,* using antisense technology, led to the reduced colony-forming ability of breast carcinoma cells; however, it appears that antisense constructs did not fully knockout hMI-ER1α expression. It is important to note that the most recognized disadvantage of transient transfection is low efficiency of transfection (30-35%); not all cells are transfected. With a large cell population that is unsuccessfully transfected with antisense constructs, complete knocking down of *hmi-er1* can not be expected. A greater degree of growth inhibitory effect may be seen if hMI-ER1α expression can be completely repressed in cancer cells that are all positively transfected with antisense constructs. This can be achieved by using stable cell lines expressing antisense hMI-ER1α; only successfully transfected cells are selected.

Shortcomings associated with the use of full-length *hmi-er1α* antisense constructs are to increase the likelihood of non-specific inhibition of other genes with sequence homology to the various domains of hMI-ER1α and to induce an interferon response. Together, they in turn alter physiological function of a large number of genes; thus, aberrant cell behavior may result. The problems can be overcome by employing a more effective technology, such as RNA interference (RNAi), to specifically inhibit the expression of *hmi-er1α* in a sequence-specific manner. In addition, use of the lowest effective number of RNAi vectors limits the risk of inducing interferon expression since

small interfering RNAs (siRNAs) are too short to trigger an interferon response (Bridge *et al.*, 2003).

These overall results together with the findings in previous studies using different cancer cells (Ding *et al.*, unpublished data) suggested that hMI-ER1α expression is not only highly regulated, but also plays a specific role in oncogenesis.

To gain some insight into the mechanism underlying the inhibition of cellular proliferation in these antisense *hmi-er1α*-transfected breast carcinoma cells, we employed Hoechst staining for the detection of apoptosis. Programmed cell death is recognized as a critical element in the removal of hazardous, damaged, or unnecessary cells, such as those resulting from DNA damages or during development. Many factors contribute to this process, each demonstrating specificity of function, regulation, and pathway involvement (Ashe and Berry, 2003). Apoptosis can be defined by significant morphological features, including cell shrinkage, chromatin condensation, membrane blebbing and DNA fragmentation (Vermeulen *et al.*, 2003). Therefore, condensed chromatin and nuclear fragmentation can be easily determined by staining cells with DNA specific fluorochromes such as Hoechst dye. This technique is primarily used for qualitative analysis of apoptotic cells.

In our study, downregulation of hMI-ER1α expression did not trigger apoptosis in MDA-MB-231 and MDA-MB-468 cells stained with Hoechst dye. No nuclei fragmentation was detected in these antisense *hmi-er1α*-transfected breast carcinoma cell lines, suggesting that inhibitory effect of antisense *hmi-er1α* on the growth of breast cancer cells was not due to the induction of apoptosis. It is possible that the anti-growth effect of antisense *hmi-er1α* is due to cell cycle arrest since hMI-ER1α has been

demonstrated to interact with retinoblastoma tumour suppressor protein (Rb) (Ding *et al.*, unpublished data). The Rb protein acts as a transcriptional repressor by targeting the E2F transcription factors, whose functions are required for cell cycle entry and DNA synthesis (Harbour and Dean, 2000;Stevaux and Dyson, 2002). Rb proteins are thought to inhibit expression of E2F-regulated genes in several ways, including the recruitment of HDACs, SWI/SNF complexes (Zhang *et al.*, 2000), polycomb group proteins (Dahiya *et al.*, 2001), or methyl transferases (Vandel *et al.*, 2001;Nielsen *et al.*, 2001) that act on the nearby surrounding nucleosome structure. It is clear that Rb plays a pivotal role in making a decision whether a cell should enter or exit the cell cycle. In addition to its role in cell-cycle control, Rb has been implicated in regulating a broad variety of cellular events, including differentiation (Lipinski and Jacks, 1999) and apoptosis (Hickman *et al.*, 2002). It is likely that hMI-ER1 interacts with and presumably modulates the function of Rb, and in turn initiates cell cycle arrest. However, Ding *et al.*, (unpublished data) previously showed that the overexpression of hMI-ER1 also inhibited growth of Rb-inactivated cell lines (C33A and HeLa). Therefore, we can not rule out the possibility that hMI-ER1 regulates cell growth through an Rb-independent mechanism.

A recent study showed that hMI-ER1 represses its own promoter by forming complexes with Sp1 and interfering with the latter's DNA binding activity (Ding *et al.*, 2004). Sp1 is a transcription factor that specially binds to GC-rich sequences and activates a large number of genes, including growth-regulated genes (reviewed in (Black *et al.*, 2001). Many reports suggested a role for Sp1 in the cell cycle. It was shown that Sp1 interacts with cell cycle regulatory proteins such as cyclin D and Rb (Kim *et al.*, 1992;Adnane *et al.*, 1999;Opitz and Rustgi, 2000;Chang *et al.*, 2001), Rb-related protein

p107 (Datta *et al.*, 1995), the transcription factor E2F (Karlseder *et al.*, 1996), p53 (Gualberto and Baldwin, Jr., 1995) and the oncoprotein MDM2 (Johnson-Pais *et al.*, 2001). The requirement of Sp1 for cell cycle progression through G1 phase has been reported (Grinstein *et al.*, 2002). Therefore, the interaction between Sp1 and hMI-ER1 may be involved in regulating the cell cycle and related to positive or negative changes in cell growth.

The sense and antisense hMI-ER1α significantly inhibit growth of human normal and breast carcinoma cells, respectively. Although the preliminary data from Hoechst staining showed that the growth-inhibitory effect of antisense hMI-ER1α on breast cancer cells is not due to induction of apoptosis, this is not necessarily true for the case of sense hMI-ER1α suppressing growth of normal cells. The sense and antisense hMI-ER1α might function through different mechanisms. Therefore, the precise mechanism underlying the anti-growth effect of hMI-ER1α remains unclear. It would be interesting to further investigate whether hMI-ER1α-mediated growth suppression results from the induction of apoptosis or cell cycle arrest.

The expression of hMI-ER1α has been associated with growth inhibition in normal human cells and hMI-ER1α likely has tumour-related functions in breast cancer. These overall results indicate a role for hMI-ER1α in regulating cell growth and suggest that its overexpression in breast carcinoma cells is of functional significance. Therefore, the role of hMI-ER1α in breast cancer development and/or progression is worthy of further study.

Future work would look further into the mechanism underlying the growth-inhibitory activity of hMI-ER1α using sense or antisense hMI-ER1α in the stable-

transfectant HeLa "Tet-ON" system developed in our lab, followed by employing flow cytometry to measure apoptosis and cell cycle progression. Furthermore, repressing hMI-ER1α expression in breast cancer cells using a more effective method known as RNA silencing (RNAi) could prove to be very exciting. The next step would involve investigating whether downregulation of hMI-ER1α by RNAi in breast cancer cells inhibits: (1) cell proliferation, (2) cellular invasion, (3) anchorage-independent growth, and (4) migration using a variety of assays. Moreover, to determine whether subcutaneous injection of hMI-ER1α HeLa Tet-ON cells into nude mice has an impact on tumour growth would be rewarding. Finally, the ultimate approach to understand the function of hMI-ER1 would be to utilize transgenic technology to "knockout" specific hMI-ER1 isoforms in mice. The further analysis of hMI-ER1 isoforms should greatly enhance our understanding the mechanisms of human breast cancer development, progression and endocrine response, and may potentially identify hMI-ER1 as a therapeutic target for breast cancer.

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