Investigations into estrogen regulation of HOXB2 and its relation with ERα in breast cancer

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By

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DECLARATION

The thesis titled **"Investigations into estrogen regulation of HOXB2 and its relation with ERα in breast cancer"** represents my original research work carried out in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, under the supervision of Dr. Anil Mukund Limaye and Dr. Sachin Kumar.

Sincere efforts have been made to acknowledge contributions from other investigators that helped in conceptualizing and executing the research work. Those who have provided suggestions and technical help have been duly acknowledged. All the research articles and resources used have been cited in the reference section.

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CERTIFICATE

This is to certify that the work described in the thesis titled, **"Investigations into estrogen regulation of HOXB2 and its relation with ERα in breast cancer"** submitted by Ajay Kumar (Roll No. 126106033) to the Indian Institute of Technology Guwahati, India, for the award of the degree of Doctor of Philosophy is an authentic record of the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India.

This thesis or any part thereof has not been submitted elsewhere to award any other degree or diploma.

25th June 2021

Dr. Anil Mukund Limaye
Thesis Supervisor

Dr. Sachin Kumar
Co-supervisor

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List of abbreviations

AbdB	Abdominal B
AF	Activation function
Ang-2	Angiogenin 2
ANGPTL2	Angiopoietin Like 2
ANOVA	Analysis of variance
Antp	Antennapedia
AP	Activator protein
bFGF	basic fibroblast growth factors
BRCA	Breast Invasive Carcinoma
BSA	Bovine serum albumin
bx	Bithorax
bxd	Bithoraxoids
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
CDH17	Cadherin 17
cDNA	Complimentary Deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
ChIP-Seq	Chromatin immunoprecipitation sequencing
CI	Confidence interval
CRC	Colorectal cancer cells
CREBP	cAMP-response element-binding protein
csFBS	Charcoal stripped FBS
CSTA	Cystatin A
CycA	Cyclophilin A
D	Dexamethasone
DCIS	Ductal carcinoma in-situ
DMFS	Distant metastasis-free survival
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DPBS	Dulbecco's phosphate-buffered saline
E1	Estrone
E2	17β-Estradiol
E3	Estriol

ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMSA	Electrophoretic mobility shift assay
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ENC TF	Encode transcription factor
ER	Estrogen receptor
ERBB2	Receptor tyrosine-protein kinase
ERE	Estrogen response elements
ERK	Extracellular-signal-regulated kinase
EtBr	Ethidium bromide
EtOH	Ethanol
FBS	Fetal bovine serum
GPER1	G-protein coupled estrogen receptor 1
HB-EGF	Heparin-binding EGF-like growth factor
HCl	Hydrochloride
HEPES	4-(-2hydroxyethyl)-piperazineethanesulfonic acid
HER2	Human epidermal growth factor receptor 2
HOX	Homeobox
HR	Hazard ratio
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IARC	International Agency for Research on Cancer
IDC	Invasive ductal carcinoma
IgG	Immunoglobulin G
IGFBP1	Insulin-like growth factor-binding protein 1
IHC	Immunohistochemistry
ILC	Invasive lobular carcinoma
IL8	Interleukin 8
IP	Immunoprecipitation
kb	Kilobase
kDa	Kilo Dalton
KM Plotter	Kaplan-Meier plotter
LBD	Ligand binding domain
LCIS	Lobular carcinoma in situ

MAPK	Mitogen-activated protein kinases
MEIS1	Meis homeobox-1
mL	Milliliter
mRNA	Messenger ribonucleic acid
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
NaCl	Sodium chloride
N-CAM	Neural cell adhesion molecule
NCI	National Cancer Institute
NHGRI	National Human Genome Research Institute
nER	Nuclear estrogen receptors
NOS	Nitric oxide synthase
OS	Overall survival
OSCC	Oral squamous cell carcinoma
P4	Progesterone
PAGE	Polyacrylamide gel electrophoresis
PAPC	Paraxial protocadherin
PBS	Phosphate-buffered saline
PBST	PBS containing 0.05% Tween 20
PBX	Pbx homeobox-1
PCDH8	Protocadherin-8
PCR	Polymerase chain reaction
PI3	Phosphatidylinositol 3
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PKA	protein kinase A
PPS	Post progression survival
PPT	Propylpyrazoletriol
PR	Progesterone receptor
pS2/TFF-1	Trefoil factor-1
qRT-PCR	Quantitative reverse transcription PCR
RAR	Retinoid acid receptor
RET	Rearranged during transfection
RFS	Relapse free survival
RNA	Ribonucleic acid
RNase	Ribonuclease
RPKM	Reads per kilobases million
RNA-seq.	RNA Sequencing
RPMI-1640	Roswell Park Memorial Institute-1640 medium

RT-PCR	Reverse transcription-polymerase chain reaction
Scr	Scrambled
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SERDs	Selective estrogen receptors down-regulators
SERMs	Selective estrogen receptor modulators
SHBG	Sex hormone-binding globulin
siRNA	Short interfering ribonucleic acid
SP1	Specificity protein 1
SRA	Sequence Read Archival
T	Testosterone propionate
TAM	4-hydroxytamoxifen
TBST	Tris-buffered saline containing 0.05% Tween 20
TCGA	The Cancer Genome Atlas
Tm	Melting temperature
TGF	Transforming growth factor
TSS	Transcription start site
UCSC	University of California, Santa Cruz
VDR	Vitamin D receptor
VDRE	Vitamin D response element
VEGF	Vascular endothelial growth factor
ZO-1	Zonula occludens-1
ng	Nanogram
nm	Nanomolar
µm	Micrometer
µM	Micromolar
µl	Microliter

CHAPTER 1

Introduction

1.1. Introduction

Homeobox (HOX) genes are a family of evolutionarily conserved transcription factors. These genes are widely known for their role in various developmental processes. They play a crucial role in determining cell identity, body patterning, and regional specification along the anterior-posterior axis during embryonic development (McGinnis and Krumlauf, 1992; Favier and Dolle, 1997).

Dysregulation in the expression of developmental genes is observed in a variety of cancers. Many studies have generated considerable interest in the possible roles of HOX transcription factors in the etiology of solid tumors, as deduced from their aberrant expression in malignant tissues (De Vita et al., 1993; Makiyama et al., 2005). HOX proteins stringently control genes that play an essential role in the cell cycle, differentiation, migration, angiogenesis, and apoptosis. Therefore, deregulation in HOX gene expression could affect various genes having tumor-promoting or tumor-suppressing functions and, ultimately, cell growth, invasiveness, and survival (Bromleigh and Freedman, 2000; Carè et al., 2001; Chu et al., 2004; Svingen and Tonissen, 2006; Lee et al., 2015; Mustafa et al., 2015). Overt HOX gene expression is observed in cancers of lungs (Inamura et al., 2007), oral (Xavier et al., 2014), pancreas (Kuo et al., 2019), prostate (Huang et al., 2014), bladder (Liu et al., 2019), endometrium (Zhang et al., 2014) and ovary (Lu et al., 2016).

HOX genes are aberrantly expressed in breast cancer (Caré et al., 1998; Cantile et al., 2003; Gendronneau et al., 2010; Hur et al., 2014). Their overt expression is linked with breast tumor development and progression (Caré et al., 1998; Gendronneau et al., 2010). Breast cancer is one of the most common endocrine-related cancer among women. Estrogen is an essential sex hormone that plays a pivotal role in the development of the female reproductive system. However, abnormal estrogen signaling is associated with breast cancer development and progression (Secreto and Rinaldi, 1998). Several investigations show that estrogen regulates various HOX genes in cell lines and tissues (Taylor et al., 1998; Pathiraja et al., 2014; Hussain et al., 2015). These HOX genes are proposed to have growth-promoting or growth-inhibiting functions (Pathiraja et al., 2014; Hussain et al., 2015).

The majority of the breast tumors express ERα, and when ligand-bound, it induces the proliferation of breast cancer cells (Clarke et al., 1997). This forms the basis of the utility of tamoxifen, a selective estrogen receptor modulator (SERM) for the treatment of ERα positive breast tumors (Cleator et al., 2009). Yang and co-workers showed an increased expression of HOXB cluster genes in the tamoxifen-resistant MCF-7 cell line. They found an epigenetic basis behind the increased expression of these genes (Yang et al., 2018). HOXB13, an estrogen-suppressed gene, reduces ERα mRNA and protein expression in breast cancer cells and is associated with tamoxifen resistance (Shah et al., 2013). Similarly, some other estrogen-regulated HOX genes (Daftary and Taylor, 2006) are also linked with endocrine resistance (Jin et al., 2012; Jin and Sukumar, 2016), suggesting a possible connection between estrogen-ERα signaling axis and HOX genes.

HOXB2, a HOXB cluster gene, is overtly expressed in breast cancer, where malignant breast tumors express it at higher levels than normal breast tissues (Cantile et al., 2003; Hur et al., 2014). Using an in-vivo screen for tumor growth regulators, Boimel and co-workers found that HOXB2 had a negative effect on breast tumor growth. They overexpressed or knockdown HOXB2 to study its effect on breast tumor formation. On the one hand, HOXB2 overexpression reduced breast tumor growth, whereas, on the other hand, HOXB2 knockdown increased breast tumor formation. Their findings proposed a tumor suppressor function for this gene (Boimel et al., 2011).

Investigations in our laboratory to characterize early estrogen-regulated genes in MCF-7 breast cancer cells using expression microarray analysis showed that estrogen represses HOXB2 mRNA by -4.8 fold (Manjegowda et al., 2015). Given that estrogen exerts mitogenic effects on breast epithelial cells (Lippman et al., 1976), this observation, although in a single cell line, may support a potential tumor suppressor role for HOXB2 (Boimel et al., 2011). The literature on the role of HOXB2 in breast cancer is minimal. Therefore, further attention is needed to investigate the estrogen regulation of HOXB2 and its role in breast cancer.

1.2. Aim and scope of the present work

Recent investigations show that aberrant HOX gene expression may help in cancer prognosis (Lu et al., 2016; Eoh et al., 2017a). Two independent studies show that malignant breast tumors express high levels of HOXB2 compared to normal breast tissues (Cantile et al., 2003; Hur et al., 2014). Neve and co-workers showed high HOXB2 expression in ER-positive breast cancer cells than ER-negative cells (Neve et al., 2006). Since HOXB2 is suggested to be a tumor suppressor, the significance of its high expression in breast tumors and ER-positive breast cancer cells is not known. Further, HOXB2 expression data is variable in different tumor types. Colorectal (Li et al., 2019), glioma (Tian et al., 2002), bladder (Liu et al., 2019), Wilms tumor (Jing et al., 2020), and leukemia (Lindblad et al., 2015) show high levels of HOXB2 expression. High HOXB2 expression in pancreatic (Segara et al., 2005), lungs (Inamura et al., 2007), and acute myeloid leukemia (Lindblad et al., 2015) are correlated with poor prognosis. However, the effect of HOXB2 on survival and its association with the molecular markers of breast cancer is still unclear. The present study seeks to address changes in the mRNA expression of HOXB2 in primary breast tumors using data from The Cancer Genome Atlas (TCGA) database (Koboldt et al., 2012) and its effect on the survival of breast cancer patients using the online tool, Kaplan-Meier Plotter.

HOXB13 modulates the ERα expression in breast cancer cells and is also associated with tamoxifen resistance (Ma et al., 2004; Wang et al., 2007; Sgroi et al., 2013; Shah et al., 2013; Liu et al., 2018). HOXB2 is a tumor suppressor of breast cancer and is also downregulated by estrogen. Both HOXB2 and ERα are transcription factors and may influence each other. It is not known that manipulation in the expression of HOXB2 may affect ERα and its downstream target genes.

Mechanisms of estrogen regulation have been extensively studied (Sanchez et al., 2002). However, in context to HOX genes, much of our understanding deals with estrogen-induced HOX genes. Estrogen suppression of HOX gene expression is poorly understood. Despite the estrogen-dependent nature of breast tumors, estrogen regulation of HOXB2 has not yet been addressed. The present study explores the mechanism of estrogen-mediated regulation of HOXB2 and presents insights into the link between HOXB2 and ERα using breast cancer cell lines as a model system.

1.3. The objectives of the present investigation are

1. To analyze HOXB2 expression in primary breast tumors by mining of the TCGA data.
2. To investigate the molecular mechanism of estrogen-mediated suppression of HOXB2 in breast cancer cells.
3. To study the functional link between HOXB2 and ERα in breast cancer cells.

CHAPTER 2

Review of literature

2.1. Breast cancer

Breast cancer is one of the most common malignancies that originate from the mammary glands and accounts for the majority of cancer-related deaths among women (Jemal et al., 2011). Mammary epithelial cells depend on hormones for their proliferation and differentiation. Estrogen is the steroid hormone that includes estrone (E1), estradiol (E2), and estriol (E3). E2 is the most bioactive form of estrogen that plays a wide range of functions, such as the development of reproductive functions, regulation of the cardiovascular system, central nervous system, and skeletal system. It also contributes to breast cancer initiation and development (Gruber et al., 2002).

Breast cancer can be categorized into two types, namely, ductal and lobular carcinoma. A ductal carcinoma arises in the linings of milk ducts, whereas; lobular carcinoma starts in lobules (milk glands) of the breast. Based on the pathological condition, breast cancer can be divided into three main categories: invasive, non-invasive, and metastatic breast cancer. Invasive breast cancer includes invasive or infiltrating breast cancer, invasive ductal carcinoma (IDC), and invasive lobular carcinoma (ILC). Ductal carcinoma in situ (DCIS) is non-invasive breast cancer, whereas metastatic breast cancer includes stage IV or advanced breast cancers (Feng et al., 2018). Breast cancer can be immunohistochemically (IHC) divided into three different classes; hormone receptor-positive [estrogen receptor (ER), progesterone receptor (PR)], human epidermal receptor (HER) 2 / neu positive, and triple-negative (ER, PR and HER2/neu negative) (Kittaneh et al., 2013). Further, genome-wide expression profiling and hierarchical clustering categorized breast cancer into different subtypes. These subtypes include luminal A, luminal B, luminal C, HER2-enriched, basal-like, claudin-low, and normal breast-like. This subtype differs in its response to any therapy and overall survival, focusing on the need for a precise and personalized treatment regimen (Kittaneh et al., 2013).

Kamangar and co-workers have reported that out of ten diagnosed cases for different cancers, one will be breast cancer (Kamangar et al., 2006). The International Agency for Cancer Research (IARC) estimated 2 million new breast cancer cases in 2018 (Bray et al., 2018). In India, breast cancer contributes to 27% of all cancer cases in women (Ferlay et al., 2018).

Various risk factors are associated with developing breast cancer, such as; age, family history, reproductive or menstrual history, long term postmenopausal hormone therapy, body weight, smoking, alcohol consumption, and genetic alterations in genes like BRCA-1 and BRCA-2 (Paul and Paul, 2014; Kamińska et al., 2015).

Several treatments are available for breast cancer management. This treatment profile depends on various factors like age, stage of the disease, and the extent of metastasis (Kamińska et al., 2015). Some general treatment options like surgery, radiation therapy, and chemotherapy, are routinely used to treat any type of cancer. Endocrine therapy is the specialized treatment used to manage hormone receptor (ER, PR, and HER2) positive breast cancer. It allows administering aromatase inhibitors, selective estrogen receptor down modulators (SERMs), and estrogen receptor down regulators (Reinert and Barrios, 2016; Tong et al., 2018). Tamoxifen, herceptin, and aromatase inhibitors (letrozole and exemestane) are used to treat ER-positive and HER-2 overexpressing tumors (Fan et al., 2015). Chemotherapy is the standard treatment for poorly differentiated tumors lacking HER2, ER, and PR. Hence, better therapeutic strategies with minimal side effects are needed that can improve the survival of breast cancer patients (Henry and Hayes, 2008).

2.2. Role of estrogen and estrogen receptors in breast cancer

2.2.1. Estrogen

Estrogens are steroid hormones that are primarily synthesized by the ovary and testis. It plays a critical role in the growth, differentiation, and development of sexual and reproductive functions. Uterus, ovary, vagina, and mammary glands in females are the estrogen's primary target organ, whereas, in males, testes, prostate, and epididymis are the main target organs. Estrogen relays its signal through ligand-activated transcription factors known as ER (Kuiper et al., 1997).

2.2.2. Estrogen receptors

ER is expressed in the uterus, oviduct, mammary gland, and various other tissues. It includes nuclear and non-nuclear ERs. The nuclear ERs are comprised of ERα and ERβ. ERα was the first nuclear ER to be discovered and used to diagnose and treat breast cancer patients. The human ERα cDNA was first cloned in 1986, and its chromosomal location was identified

two years later (Greene et al., 1986; Ponglikitmongkol et al., 1988). On the other hand, ERβ was characterized in 1990 (Kuiper et al., 1996). ESR1 and ESR2 genes code for ERα and ERβ, respectively. These genes are located on chromosomes 6q24-27 and 14q22-24 and encode 66 and 60 kDa proteins, respectively (Yaşar et al., 2017).

Both ERα and ERβ are divided into six different domains (Figure 2.1). It includes the N-terminal domain (A/B region), the most conserved and variable domain in sequence and length. This is followed by a DNA binding domain (C, DBD) that aids in DNA recognition and receptor binding. Next to the DBD is the hinge region (D). It helps in the nuclear localization of the receptor and assists interaction with the heat shock proteins. Followed by the hinge region, the next domain is the ligand-binding domain (E, LBD). It contains a hormone-binding region, a dimerization interface (homo or heterodimer), and one protein-protein interaction site known as activation function-2 (F, AF-2). In comparison, the AF-1 is located at the N-terminus. Both AF-1 and AF-2 help in regulating the ER transcriptional activity (Nilsson et al., 2001). The structure of human ERs is provided in figure 2.1.

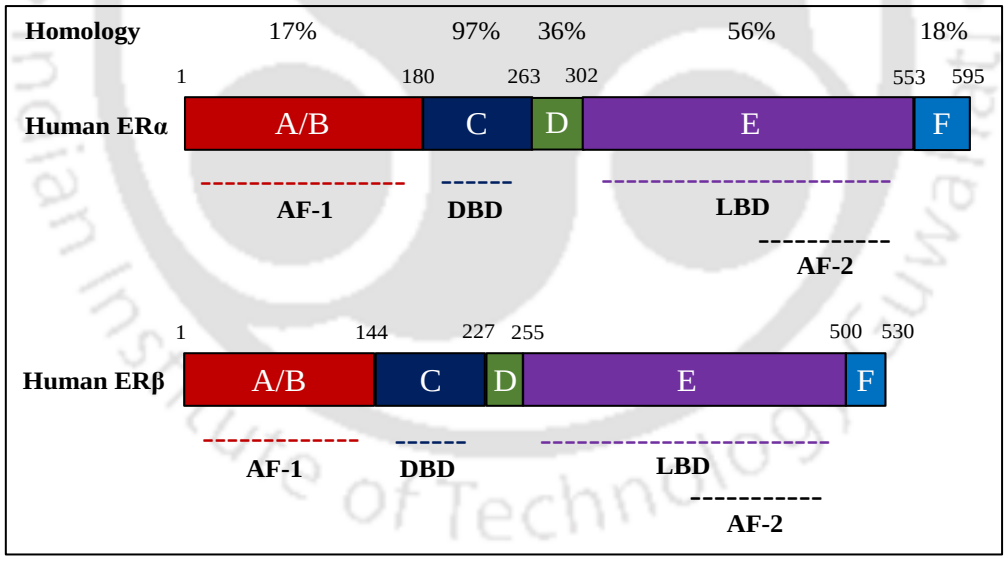


Figure 2.1. Schematic representation of human ERα and ERβ structural regions. Human ERs are comprised of six different domains (A-F). ERα and ERβ are comprised of 595 and 530 amino acids, respectively. AF-1 and AF-2 represent activation functions 1 and 2, DBD and LBD represent DNA and ligand-binding domain.

2.2.3. Estrogen receptor signaling

2.2.3.1. Genomic pathway

The ligand-dependent estrogen signaling events regulate the expression of genes containing an estrogen response element (ERE). This ERE is present at different distances from the transcription start site (TSS) or within a gene. This ERE consists of 5'-GGTCAnnnTGACC-3' DNA palindrome, where n denotes a random nucleotide. Before interacting with the E2, ERs are cloistered by the multimeric chaperone machinery associated with heat shock proteins (hsp70, hsp90) in the cytoplasm. The binding of E2 to ERs causes a conformational change to the receptors. As a result, the receptor dissociates from molecular chaperons, followed by dimerization and translocation into the nucleus. The ligand-activated receptor recognizes the ERE located in the regulatory sequence of their downstream target genes. This DNA-protein complex recruits other cofactors (co-activators or co-repressors) and regulates gene expression (Klinge, 2001).

The ligand-bound ERs can also regulate the expression of genes that do not contain an ERE. Here the E2 bound ER interacts with other transcription factors such as activator protein (AP-1) and stimulatory protein (SP-1) (Safe and Kim, 2008). This constitutes the ERE-independent mode of estrogen signaling. The AP-1 transcription factor is comprised of Fos, Jun, and activating transcription factors. These transcription factors, along with ligand-bound ER, form protein-protein complexes. These complexes further recognize and bind to the AP-1 and SP-1 sites and regulate the expression of estrogen-responsive genes such as cyclin D (Edeuilh, 1999), cathepsin D (Xing and Archer, 1998), and uteroglobin (Duffy, 2006). ER binding to DNA is not needed; therefore, ER lacking a DBD can also activate this pathway (Safe and Kim, 2008).

In addition to the ligand-dependent estrogen-signaling pathway, ER can also be activated in a ligand-independent manner. Here various growth factors such as; epidermal growth factor (EGF) and insulin growth factor (IGF) binds to their cognate receptors. This binding activates a cascade of signaling events such as mitogen-activated protein kinase (MAPK). The activated kinases phosphorylate the ER at serine-118 through MAPK signaling. Then the phosphorylated ER regulates the expression of estrogen-responsive genes. All these

signaling events require the interaction of ER and the chromatin complex and are hence known as genomic actions of estrogen (Yaşar et al., 2017).

2.2.3.2. Non-genomic pathway

Estrogen can trigger the rapid biological responses that occur within a scale of seconds to minutes. This cellular response of estrogen is known as the non-genomic pathway. Some of these effects are known to be mediated by cell surface receptors such as; membrane-bound ERs, G-protein coupled estrogen receptor 1 (GPER1), and sex hormone-binding globulin (SHBG) receptor (Fortunati et al., 1999). The activation of these receptors causes various downstream changes such as production and activation of adenylate and guanylate cyclase, MAPK pathway, nitric oxide synthase (NOS), membrane tyrosine kinase activation, and phosphatidylinositol 3,4,5-triphosphate (PIP_3) synthesis and intracellular mobilization of calcium (Hall et al., 2001; Chetana Revankar et al., 2005).

2.3. HOX transcription factors

HOX genes are a superfamily of transcription factors that specify regional differences during embryonic segmentation along the anterior-posterior axis. These genes are conserved throughout evolution and expressed in a highly coordinated manner in the developing embryo. They are expressed in almost all tissues and organs throughout life (Lappin et al., 2006).

HOX genes were first identified by Morgan and Bridges in the early 1900s when they observed a striking mutation in the fruit fly, *Drosophila melanogaster.* This mutation results in phenotypic changes that lead to body structures forming in an inappropriate place, such as antennae to legs. The phenotypic changes in the body structure due to this mutation gave Morgan, and Bridges reasons to think about some genes responsible for maintaining the correct spatial management of fruit flies body structure (Bridges, 1921; Bridges and Dobzhansky, 1933).

HOX genes were discovered due to two striking mutations known as antennapedia (transformation of antennae into legs) and bithorax mutation (transformation of haltere into wings). These are due to a mutation in '*antp'* and *'bx'* or '*bxd'* genes for antennapedia and bithorax mutation, respectively. This change of one body structure into another is known as

homeotic transformation (Lewis, 1978; Garberl et al., 1983; Sato, 1988). These homoeotic transformations lead to the concept that HOX genes could be the master regulator of the tissue-specific identity. The term "homeotic gene" means that the HOX gene controls the segmentation of the fruit fly. Further, during embryogenesis, HOX proteins provocate the cells to select a particular development path (Favier and Dolle, 1997; Taylor, 2002).

HOX genes are evolutionarily conserved. They are present in almost all organisms, ranging from lower organisms like hydra to higher organisms, including plants and animals (Lappin et al., 2006). Their earliest expression is observed during gastrulation in the developing embryo. The chromosomal organization of HOX genes is from the 3' to 5' direction. The majority of mammalian 3' HOX genes are derivative of the 3' drosophila HOM-C (labial) gene. This helps the formation of the anterior structures of the body. Similarly, most mammalian 5' HOX genes are derivative of the 5' drosophila HOM-C, AbdB gene (Abdominal B). These genes facilitate the formation of the posterior structure of the developing embryo. This characteristic feature of temporal and spatial gene expression is known as temporal co-linearity (Kmita and Duboule, 2003; Lappin et al., 2006).

Vertebrates HOX genes consist of 39 HOX transcription factors. There are 4 HOX clusters, namely HOXA, HOXB, HOXC, and HOXD. These HOX clusters are present on chromosomes -7p15- (HOXA), -17q21.2- (HOXB), -12q13- (HOXC),-and -2q31- (HOXD), respectively (Lappin et al., 2006). Based on the sequence homology, every HOX cluster has 9 to 11 HOX members. Some of the HOX genes are missing from human paralog groups. Hence, they do not contain all the HOX genes (Nam and Nei, 2005; Duboule, 2007).

Mammalian HOX genes contain two exons separated by an intron that differs from 200 base pairs (bp) to many kilobases (kb). The second exon in every HOX gene has a highly conserved sequence of 183 bp. This nucleotide sequence codes for 61 amino acid long sequence, referred to as homeodomain. Homeodomain is composed of three alpha-helices and has a helix-turn-helix DNA binding motif. Homeodomain is the signature sequence of all the HOX genes and is conserved across the species (Gehring, 1987; Favier and Dolle, 1997). The third helix of the homeodomain confers the DNA binding property of HOX genes. The homeodomain binds to a short sequence (ATTA/ATAA/TTAT/TAAT/TAATTA), signifying a very low functional specificity (Desplan et al., 1988; Garcia et al., 2020).

2.4. Cofactors and their effect on HOX-DNA binding specificity

Most HOX proteins bind to a short DNA sequence of four base pairs, compromising their specificity to a particular DNA sequence (Garcia et al., 2020). Other DNA binding proteins, such as cofactors, help HOX proteins to interact with the specific DNA sequences and enhance their binding activity and specificity. These include TALE (three amino acid loop extension) homeodomain proteins, MEIS, and PBS classes of homeodomain proteins. These proteins bind cooperatively to the specific DNA sequence and HOX proteins (Moens and Selleri, 2006; Richard S et al., 2009).

Auto and cross regulatory interactions between HOX proteins and their cofactors help these genes regulate their downstream targets. The specificity of HOX proteins for their target DNA sequence also depends on various cofactors (Kobrossy et al., 2006). HOX proteins interact and bind with CREB binding protein (CBP) and affect histone acetyltransferase activity. This suggests the alternate possibility of gene regulation by HOX proteins without directly binding to the DNA (Shen et al., 2002).

2.5. HOX genes and mammary gland development

Mammary glands are specialized structures that help in postnatal care by providing immune protection and nutrition (Vorbach et al., 2006). Epithelial-mesenchymal transition (EMT) plays a crucial role in the growth and development of the mammary gland. The process starts with mammary lines, followed by ectoderm thickening in the developing embryo and stretching between the fore and hind limbs (Robinson, 2012). HOX genes are actively involved in the mammary gland pre and postnatal development (Schep et al., 2016). HoxC6 is expressed in the mammary gland throughout birth, puberty, and adulthood, and its expression is reduced during pregnancy (Friedmann et al., 1994). The paralogous Hox genes such as HoxA9, HoxB9, and HoxD9 are required to differentiate mammary epithelium (Chen and Capecchi, 1999).

2.6. Downstream targets of HOX genes

HOX genes are widely known for their role in development. These genes are known as the master regulator genes that interact with many molecules inside the cell (Svingen and

Tonissen, 2006). Jones and co-workers identified the first target for mammalian HOX genes. HoxB9 regulates the expression of neural cell adhesion molecules (N-CAM), which mediates cell adhesion in the neuronal cells (Jones et al., 1992). Subsequently, various other targets for HOX genes were identified. Some of these HOX targets include p53 (Raman et al., 2000a), PR (Raman et al., 2000b), pleiotrophin (Chen et al., 2005), IGFBP1 (Foucher et al., 2002), BRCA (Gilbert et al., 2010), β3-integrin (Daftary et al., 2002), p21 (Bromleigh and Freedman, 2000), and VEGF (Caré et al., 1996). These genes are involved in critical cellular processes like cell cycle, differentiation, cell adhesion, invasion, angiogenesis, and apoptosis (Svingen and Tonissen, 2006). In breast cancer, HOXA5 exerts its tumor suppressor role by negatively regulating the expression of CDH1 and CD24 (Teo et al., 2016), and HOXB7 controls the expression of EGFR and MYC (Jin et al., 2012). This shows that HOX genes play a critical role in maintaining cellular homeostasis. Deregulation in the expression of HOX genes affects homeostasis during tumor progression and development (Bhatlekar et al., 2014).

2.7. Regulation of HOX genes

2.7.1 Estrogen

Estrogen regulates the expression of many HOX genes in steroid-responsive cells and tissues. The binding of estrogen to the ER results in transcriptional regulation of HOX genes through the estrogen response element (ERE) present in the HOX gene promoter. A detailed list of estrogen-regulated genes is given in Table 2.1.

Table 2.1. List of estrogen-regulated HOX genes

HOX gene	Model System (Cell lines, Tissue)	Nature of Modulation	Methodology	Reference
HOXA10	Endometrial stromal cells and Ishikawa cells	Increase in mRNA expression	Northern blotting	Taylor et al., 1998
HOXA10	Ishikawa cells	Transcriptional activation	Luciferase assay and EMSA	Eda Akbas et al., 2004
	Ishikawa and MCF-7 cells	Transcriptional activation	Luciferase assay and EMSA	Martin et al., 2007

Table 2.1 Continued

	Murine uterus	Repression	RNase protection assay and Northern blotting	Ma et al., 1998
HOXA11	Endometrial stromal cells and Ishikawa cells	Increase in mRNA expression	Northern blotting	Taylor et al., 1999
HOXB9	JAR cells and MCF7 cells	Transcriptional activation	Luciferase and ChIP assay	Khairul I et al, 2013
	MCF-7 cells	Transcriptional activation	Luciferase and ChIP assay	Deb et al., 2016
HOXB13	MCF7 and T47D cells	Reduction in mRNA expression	qRT-PCR	Wang et al., 2007
HOXC4	Mouse spleen, lymph nodes and Peyer's patch B cells, human *2E2 B cell* line	Transcriptional activation	Luciferase, EMSA, and ChIP assay	Mai et al., 2010
	MCF-7 cells	Increase in mRNA expression	Microarray experiment	Frasor et al., 2003
HOXC5	MCF-7 cells	Increase in mRNA expression	Microarray experiment	Frasor et al., 2003
HOXC6	MCF-7 cells	Increase in mRNA expression	Microarray experiment	Frasor et al., 2003
	JAR cells, MCF-7 cells, and Mammary tissue	Transcriptional activation	Luciferase and ChIP assay, ERα knockdown	Ansari et al., 2011; Hussain et al., 2015
HOXC10	JAR cell line	Transcriptional activation	Luciferase and ChIP assay, ERα knockdown	Ansari et al., 2012
HOXC13	JAR cell line	Transcriptional activation	ChIP assay, ERα knockdown	Ansari et al., 2009

2.7.2 Retinoic acid (RA)

Retinoids are signaling molecules derived from vitamin-A, which plays a vital role in the development of vertebrates. Under physiological conditions, the metabolism of vitamin A

results in the synthesis of retinoic acid in the embryonic tissue. Retinoic acid can regulate HOX gene expression in embryonic cell lines and tissue (Marshall et al., 1996; Simeone et al., 1991). HOX genes are differentially regulated by retinoic acid in the human embryonic carcinoma cell line, NT2/D1. Expression of 24 HOX genes was observed within 24 h of treatment with 10^{-5} M of RA, whereas 14 HOX genes remain inactive at the end of the experiments, suggesting a significant role of RA in the HOX gene regulation (Simeone et al., 1991).

2.7.3 Vitamin D

Vitamin D plays a crucial role in calcium absorption and bone metabolism. It also helps in the development of the reproductive tract. Its metabolism produces 1, 25-dihydroxycholecalciferol, an active metabolite that binds to vitamin D receptors (VDR) and regulates gene expression. VDR binds to vitamin D response elements (VDREs) located in its target genes' regulatory sequences. 1, 25- dihydroxycholecalciferol regulates HOXA10 expression in hematopoietic and endometrial cells by binding to VDRE (Du et al., 2005).

2.8. HOX genes and cancer

During morphogenesis, the tissues and organs acquire a shape that is necessary for their function. The cells attain positional information to ensure that the uncommitted cells differentiate into specific tissue types suitable for their embryo location. This process is well regulated to maintain a balance between cell proliferation and differentiation, which is necessary for fetal development; however, this balance is often compromised in cancer conditions. The involvement of HOX genes in embryogenesis and their differential expression in neoplastic conditions leads to the idea that 'oncology recapitulates ontogeny'(Grier et al., 2005).

HOX gene expression may be gained or lost in different cancers, which credits these genes as tumor promoters or tumor suppressors (Kanai et al., 2010). HOX transcription factors regulate various genes involved in the cell cycle, cell proliferation, cell differentiation, and apoptosis. Therefore, abnormal expression of HOX genes also affects their downstream targets and, eventually, the crucial cellular processes leading to tumor progression (Svingen

and Tonissen, 2006). HOX genes are overtly expressed in a variety of cancers. A detailed list of HOX genes involved in various cancers is provided in Table 2.2.

Table 2.2. HOX genes and their expression in various cancers

HOX gene	Model System (Cell lines, Tissue)	Nature of Expression	Methodology	Reference
HOXA1	Cervical carcinoma cells (CCC)	Expressed only in CCC, not in normal cervical tissue	RT-PCR	Hung et al., 2003
	Breast cancer tissue	Low expression in cancerous tissue	qRT-PCR	Makiyama et al., 2005
	Breast cancer tissue	Hypermethylation/Low expression	Association analysis	Pilato et al., 2013
HOXA2	Breast cancer tissue	Low expression in cancerous tissue	qRT-PCR	Makiyama et al., 2005
HOXA3	Breast cancer tissue	Low expression in cancerous tissue	qRT-PCR	Makiyama et al., 2005
HOXA4	Epithelial ovarian cancer cells	Overexpression	RT-PCR, Western Blotting	Ota et al., 2009
HOXA5	Breast cancer tissue	Low expression in cancerous tissue	qRT-PCR	Makiyama et al., 2005
	Squamous cell carcinoma tissue	High expression in squamous cell carcinoma tissue	qRT-PCR	Abe et al., 2006
HOXA6	Oral squamous cell carcinoma (OSCC) cell lines	Hypermethylation/Low expression	Methylation-qPCR array	Xavier et al., 2014
HOXA7	OSCC cell lines	Hypermethylation/Low expression	Methylation-qPCR array	Xavier et al., 2014
HOXA9	OSCC cell lines	Hypermethylation/Low expression	Methylation-qPCR array	Xavier et al., 2014
	Colorectal cancer cells (CRC) and tissue	High expression in colorectal tumors	qRT-PCR	Watanabe et al., 2018

Table 2.2 Continued

HOXA10	squamous cell carcinoma tissue	High expression in squamous cell carcinoma tissue	qRT-PCR	Abe et al., 2006
HOXA11	OSCC cell lines	Hypermethylation/Low expression	Methylation-qPCR array	Xavier et al., 2014
HOXA13	Breast tumors	High expression	RT-PCR	Hur et al., 2014
HOXB1	H1944 lung cancer cells	Low expression	RT-PCR and Western blotting	Cui et al., 2020
HOXB3	Primary prostate cancer tissue	High expression	RT-PCR and Western blotting	Chen et al., 2012
HOXB4	Cervical carcinoma cells (CCC)	Expressed only in CCC, not in normal cervical tissue	RT-PCR	Hung et al., 2003
	Breast tumors	High expression	RT-PCR	Hur et al., 2014
HOXB5	Breast tumors	High expression	RT-PCR	Hur et al., 2014
HOXB6	OSCC cell lines	Hypermethylation/Low expression	Methylation-qPCR array	Xavier et al., 2014
HOXB7	Gastric cancer and normal mucosa tissues	High expression in cancerous tissues	RT-PCR and Western blotting	Tu et al., 2015
HOXB8 and HOXB9	Colorectal and hepatocellular carcinomas tissue	High expression in cancerous tissues	qRT-PCR	Kanai et al., 2010
HOXB13	Breast cancer tissue	Hypermethylation/Low expression	Association analysis	Pilato et al., 2013
HOXC4, HOXC5, HOXC6, HOXC8	Prostate cancer cell lines and lymph node metastases	High expression	qRT-PCR	Miller et al., 2003
HOXC5	Cervical carcinoma cells (CCC)	Expressed only in CCC, not in normal cervical tissue	RT-PCR	Hung et al., 2003

Table 2.2 Continued

HOXC6	Prostate cancer cells, Primary, metastasized, and castration-resistant prostate cancer	High expression	qRT-PCR	Hamid et al., 2015
HOXC8	Cervical cancer cell lines and tissue	High expression	qRT-PCR	Huang et al., 2018
HOXC9	Neuroblastoma cells and tissue	High expression	Association analysis	Kocak et al., 2013
HOXC10	Cervical carcinoma cells (CCC)	Expressed only in CCC, not in normal cervical tissue	RT-PCR	Hung et al., 2003
HOXC11	Breast cancer tissue	Low expression in cancerous tissue	qRT-PCR	Makiyama et al., 2005
HOXC13	Esophageal squamous cell carcinoma (ESCC) tissue and cell lines	High expression	RT-PCR and Western blotting	Luo et al., 2018
HOXD1	Colorectal and hepatocellular carcinomas tissue	High expression in cancerous tissues	qRT-PCR	Kanai et al., 2010
HOXD3	Breast cancer tissue	Low expression in cancerous tissue	qRT-PCR	Makiyama et al., 2005
HOXD4	Colorectal and hepatocellular carcinomas tissue	High expression in cancerous tissues	qRT-PCR	Kanai et al., 2010
HOXD8	CRC tissue and cell lines	Downregulated	RT-PCR and Western blotting	Mansour and Senga, 2017
HOXD9 and HOXD10	Breast cancer tissue	Low expression in cancerous tissue	qRT-PCR	Makiyama et al., 2005
HOXD11	Esophageal squamous carcinoma (ESC) and paraneoplastic tissues	High expression	RT-PCR immunohistochemical staining	Liu et al., 2016
HOXD12	Colon cancer tissue	High expression	qRT-PCR	Lu et al., 2020

Table 2.2 Continued

HOXD13	Different tumor types	High or low expression in tumor tissue	qRT-PCR	Cantile et al., 2009

* HOXB2 has been excluded from this table, and the details of its involvement in various cancers is provided in separate sections, table 2.3.

2.9. HOX genes and breast cancer

Breast cancer is one of the most frequently diagnosed cancer among women worldwide. Metastasis of breast tumors accounts for breast cancer-related mortality (Olivia et al., 2012). Breast cancer is initiated due to the changes in the architecture of breast tissue and interaction between cells and the extracellular environment. The expression of genes that control these cellular processes is also affected during breast tumor progression (Bissell et al., 1999).

HOX genes are the master regulators of embryonic development. Many investigations have shown that HOX genes are either over or under-expressed in breast cancer. Deregulated HOX gene expression is associated with tumor growth and progression (Brotto et al., 2020).

Hur and co-workers and Cantile and co-workers analyzed HOX gene expression in normal breast tissue and breast tumors. They showed that most of the HOX genes were differentially expressed across normal breast tissue and breast tumors, suggesting their involvement in breast cancer development (Cantile et al., 2003; Hur et al., 2014).

Aberrant expression of HOX genes is associated with breast cancer; many of these genes have tumor-promoting or inhibiting properties, are associated with EMT, and affect a wide range of functional properties of cells (Garcia et al., 2020). A study profiling the expression pattern of HOX genes in normal breast tissues and human invasive ductal breast cancer tissues shows differential expression of several HOX genes. Expression of HOXA cluster genes, including HOXA1, HOXA2, HOXA3, HOXA5, and HOXA9, was significantly less in breast tumors compared to normal breast tissues (Makiyama et al., 2005). Similar patterns were observed for HOXD cluster genes, including HOXD3, HOXD4, HOXD8, HOXD9, and HOXD10. In contrast, HOXC11 expression was more in breast tumors as compared to normal ones. These results suggest that these HOX genes may be regulated by a

common upstream element in adult breast tissue, dysfunction of which occurs in breast cancer (Makiyama et al., 2005).

ER-positive breast tumors express high HOXB5 compared to ER-negative ones. The survival analysis shows that HOXB5 is associated with poor distant metastasis-free survival (DMFS) of ER-positive breast cancer patients. *In-vitro* studies show that forced expression of HOXB5 in ER-positive MCF-7 breast cancer cells causes EMT with more proliferation and invasion. HOXB5 overexpression or knockdown modulated the expression of Erb-B2 receptor tyrosine kinase (ERBB2), ret proto-oncogene (RET), and epidermal growth factor receptor (EGFR); however, no difference was observed in estrogen receptor 1 (ESR1) expression. It is suggested that HOXB5 may be a positive modulator of ER-positive breast cancer (Lee et al., 2015).

HOXB13 expresses at high levels in primary breast tumors (Cantile et al., 2003; Hur et al., 2014). HOXB13 suppresses the expression of ERα at the level of transcription. Low ERα expression may attribute to tamoxifen resistance in ER-positive breast cancer (Shah et al., 2013).

Promoter hypermethylation is associated with the pathogenesis of breast cancer. HOXA5 is hypermethylated in breast tumors, which is associated with loss of ERα expression. ERα predicts the response to endocrine therapy. Therefore, the low expression of ERα due to HOXA5 promoter methylation may alter the response of ERα-positive breast tumors to endocrine treatment (Bagadi et al., 2008). HOXA9 (Sun et al., 2013) and HOXA10 (Chu et al., 2004) are potential tumor suppressors of breast cancer. They are hypermethylated in breast cancer. The combination of HOXA9 and HOXA10 promoter methylation is correlated with the prognosis of breast cancer patients (Park et al., 2017).

HOX transcription factors can transcriptionally activate or suppress the expression of their downstream target genes. Raman and co-workers show that HOXA5 positively regulates p53 expression in MCF-7 breast cancer cells. They show that HOXA5 mediated regulation of p53 induces apoptosis via p53 dependent pathway in MCF-7 cells (Raman et al., 2000a).

HOXA9 is underexpressed in breast cancer cell lines and breast tumors. Low expression of HOXA9 is associated with aggressive disease features in ER/PR-negative breast

tumors, including late-stage disease, high-grade tumors, distant metastasis, and reduced survival. Besides, low HOXA9 expression is associated with migration, invasion, and metastasis of breast cancer cells. HOXA9 regulates BRCA1 expression in MDA-MB-231 breast cancer cells. Further, overexpression of HOXA9 increases BRCA1 expression, which promotes the differentiation of breast tumors and inhibits tumor progression (Gilbert et al., 2010).

Care and co-workers show that HOXB7 overexpressing SkBr3 breast cancer cells express basic fibroblast growth factors (bFGF), which shows enhanced cell proliferation and low growth factor dependence (Caré et al., 1998). High HOXB9 expression in breast cancer cells induces the expression of various angiogenic factors such as vascular endothelial growth factor (VEGF), bFGF, transforming growth factor-beta (TGF-β), angiopoietin-like 2 (ANGPTL2), Interleukin-8 (IL-8), and ErbB. Increased expression of these growth factors triggers their respective pathways and increases proliferation, differentiation, cell-cell adhesion, vascular permeability, and cells acquire mesenchymal phenotype. In the *in-vivo* conditions, HOXB9 stimulates the formation of a well-vascularized tumor that invades and metastasizes to the lung. Hence, aberrant expression of HOXB9 causes breast tumor growth and lung metastasis by inducing various growth factors that modulate the tumor-stromal environment (Hayashida et al., 2010).

2.10. HOXB2

HOXB2 is located on the HOXB homeobox cluster present on chromosome 17, which encodes a nuclear protein with a homeobox DNA-binding domain. HOXB2 proteins function as a sequence-specific transcription factor and is involved in development. HOXB2 plays an essential role in patterning the anterior-posterior and dorsal-ventral axis. HOXB2 coordinates with HOXA2 to produce rhombomere margins along the dorsal-ventral axis at the r2/r3 regions during hindbrain development (Davenne et al., 1999). HOXB2 is also expressed in the second branchial arch and specifies the skeletal structures, such as the hyoid, stapes, and stylohyoid ligament. HOXB2 homozygotic mutants developed facial muscle paralysis and atrophy. They failed to form the motor components of the VIIth (facial) nerve, suggesting that loss of HOXB2 proteins is directly associated with the facial defects (Barrow and Capecchi, 1996).

2.11. HOXB2 and cancer

HOX genes were initially studied to understand their role in forming the body axis during embryonic development. Recent investigations have shown that these genes are either upregulated or downregulated in different tumors, suggesting their potential role in cancer development. HOXB2 is also aberrantly expressed in various cancers and affects crucial cellular processes like cell proliferation, migration, and apoptosis (Brotto et al., 2020). A detailed list of cancers where HOXB2 is aberrantly expressed is provided in Table 2.3.

Table 2.3. HOXB2 and its expression in various cancers

HOXB2-Cancer Type	Model System (Cell lines, Tissue)	Nature of Modulation	Methodology	Reference
Cervical cancer	Cervical carcinoma cells (CCC)	Expressed only in CCC, not in normal cervical tissue	RT-PCR	Hung et al., 2003
Oral cancer	OSCC	High expression in OSCC than normal mucosa	RT-PCR	Destro et al., 2010
Bladder cancer	Bladder tumor samples	Methylation/low expression	Pyrosequencing	Marsit et al., 2010
	Bladder cancer cell lines	High expression	RT-PCR and Western blotting	Liu et al., 2019
Ovarian cancer	Ovarian cancer tissue and cell lines	High expression	qRT-PCR and Western blotting	Yu and Pan, 2020
Colorectal cancer	CRC cell lines	Overexpression	qRT-PCR and Western blotting	Li et al., 2019
Lung cancer	Lung cancer HOP-62 cell line	Suppression	siRNA-mediated knockdown	Inamura et al., 2008
	Lung adenocarcinoma samples	High expression	qRT-PCR	Inamura et al., 2007
Pancreatic cancer	Pancreatic cancer tissue	High expression	Immunostaining	Segara et al., 2005
Acute myeloid leukemia	AML patients with FLT3-ITD mutation	High expression	Gene expression data analysis	Lindblad et al., 2015

2.12. HOXB2 and breast cancer

Compared to other cancers, HOXB2 is less studied in the context of breast cancer. Two independent studies by Cantile and co-workers and Hur and co-workers show high HOXB2 expression in breast tumors than normal breast tissue (Cantile et al., 2003; Hur et al., 2014). In a microarray study, Neve and co-workers show high HOXB2 expression in ER-positive breast tumors and cell lines compared to ER-negative ones (Neve et al., 2006).

Boimel and co-workers used an in-vivo screen to identify genes that promote or inhibit human breast tumor growth. They used a knockdown or overexpression approach to explore the influence of HOXB2 on breast cancer growth. HOXB2 ORF or HOXB2 shRNA expressing breast cancer cells were injected into the mouse mammary fat pad. HOXB2 overexpression reduced the percentage of cells in the mitotic cell division phase, whereas HOXB2 shRNA expressing tumors increased it. Further, they did not observe any change in the *in-vitro* cultured cell growth. Their findings conclude that changes in breast tumor growth after HOXB2 overexpression or knockdown may depend on the tumor microenvironment. They proposed HOXB2 as a negative growth regulator of breast cancer (Boimel et al., 2011).

HOX genes are essential in the development and are also involved in various cancers. The literature on the role of HOXB2 in breast cancer is meager. Although HOXB2 has growth inhibitory functions and breast tumors express high levels of HOXB2, its role in breast cancer is not clear. With a handful of evidence showing the involvement of HOXB2 in breast cancer, underlying molecular mechanisms have not yet been elucidated. Therefore, it requires a detailed study to investigate the mechanism of HOXB2 regulation and its significance in breast cancer.

CHAPTER 3

Materials and methods

3.1. Plasticware, chemicals, and reagents

All cell culture plasticware was purchased from Greiner Bio-one (Frickenhausen, Germany), Eppendorf (Hamburg, Germany), or Tarsons (Kolkata, India). Fetal bovine serum (FBS) and charcoal-stripped FBS (csFBS) were from Gibco (NY, USA). Dulbecco's phosphate-buffered saline (DPBS), trypsin-EDTA, and antibiotics were purchased from HiMedia (Mumbai, India). ERα and HOXB2 specific siRNAs (Cat No. 4392420 and Cat No. AM16708), scrambled siRNA (Cat No. AM4611), Lipofectamine RNAimax, and PowerUp™ SYBR® Green PCR master mix was from Thermo Scientific (PA, USA). Protein G plus-Agarose beads were purchased from Merck Millipore (Burlington, USA). Polyclonal histone H3 antibody (Cat No. BB-AB0055) and normal rabbit IgG antibodies (Cat No. AB0001) were from BioBharati LifeScience Pvt. Ltd. (Kolkata, India). Polyclonal ERα (Cat No. sc-543) and monoclonal PCDH8 (Cat No. sc-81817) antibodies were from Santa Cruz Biotechnology (CA, USA), whereas polyclonal HOXB2 (Cat No. PA5-39210) and monoclonal β-actin (Cat No. AM4302) antibodies were from Thermo Scientific (PA, USA). EMT antibody sampler kit (9782T) was purchased from Cell Signaling Technology (Massachusetts, USA). All other chemicals and buffers were purchased from Merck (Mumbai, India), Sigma-Aldrich (MO, USA), or SRL (Mumbai, India).

3.2. Cell lines

MCF-7 and T47D breast cancer cells were purchased from the National Center for Cell Science, Pune, India.

3.3. Cell culture and treatments

MCF-7 cells were routinely cultured in Dulbecco's Modified Eagle's medium (DMEM). T47D were cultured in Roswell Park Memorial Institute -1640 medium (RPMI-1640). Media were supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin, and 100 µg/mL streptomycin (hereafter referred to as M1 medium) and maintained under the humidified environment with 5% CO_2 at 37°C. In experiments that involved treatment with various estrogenic ligands, MCF-7 or T47D cells were treated with phenol red-free DMEM-F12 or RPMI-1640, respectively, supplemented with 10% csFBS, 100U/mL penicillin, and 100 µg/mL streptomycin (hereafter referred to as M2 medium). Table 3.1 shows a list of

various ligands used in this study. In experiments involving stimulation of cells with E2 for more than 48 h, the cells were treated with fresh M2 medium containing E2 after every 48 h.

Table 3.1. List of ligands used in the study

S. No.	Ligands	Catalogue No.	Company	Solvent	Abbreviation
1	17β-estradiol	E8875	Sigma	Ethanol	E2
2	Propylpyrazoletriol	H6036	Sigma	Ethanol	PPT
3	Testosterone propionate	86541	Sigma	Ethanol	T
4	Progesterone	P8783	Sigma	Ethanol	P4
5	Dexamethasone	D4902	Sigma	Ethanol	D

3.4. Subculturing and seeding

For routine cell culture, the cells were grown in M1 medium until 80-90 % confluence. The cells were then washed with DPBS and treated with trypsin-EDTA. Once the cells got detached from the surface, 1 ml of medium was added to inhibit the trypsin activity. Then, 200 µl of the cell suspension was seeded into T25 flasks containing fresh M1 medium. In cell culture experiments, cells were counted by mixing equal volume (20 µl) of cell suspension and trypan blue dye. The mixture was loaded in the hemocytometer, and live cells were counted by the trypan blue dye exclusion method. Equal numbers of cells were seeded in cell culture dishes depending upon the doubling time, surface area, and treatment duration.

3.5. Design of experiments and treatment protocols

3.5.1. Time course experiment to study the effect of E2 on gene expression

2×10^5 MCF-7 or T47D cells were seeded in 35 mm dishes and grown in M1 medium until 60-70% confluent. Cells were washed with DPBS and fed with the M2 medium. After 24 h, the spent M2 medium was removed. The cells were then treated with ethanol (vehicle) or 10 nM E2 in M2 medium for indicated periods.

3.5.2. Dose-dependent effect of E2 on gene expression

2×10^5 MCF-7 or T47D cells were seeded in 35 mm dishes and grown in M1 medium for 24 h. The cells were washed with DPBS and fed with the M2 medium. After 24 h, the cells were treated with varying concentrations of E2 (0.1 to 100 nM) or ethanol (vehicle) in the M2 medium for a period of 72 h.

3.5.3. Effect of various hormones on gene expression

2×10^5 MCF-7 cells were seeded in 35 mm dishes and grown for 48 h in M1 medium. Cells were then washed with DPBS and fed with M2 medium for 24 h. Then the cells were treated with 10 nM of various steroid hormones such as E2, PPT, testosterone propionate, progesterone, and dexamethasone for 72 h. Ethanol-treated cells served as control.

3.5.4. Effect of E2 and PPT on gene expression

2×10^5 MCF-7 or T47D cells were seeded in 35 mm dishes and grown for 48 h in M1 medium. Cells were then washed with DPBS and fed with M2 medium for 24 h. After that, the cells were treated with 10 nM E2, 10 nM PPT or ethanol (vehicle) for 48 h.

3.5.6. Effect of ERα or HOXB2 knockdown on gene expression

2×10^5 MCF-7 or T47D cells were seeded in 6-well plates and cultured in M1 medium for 24 h. The cells were transfected with either ERα or HOXB2 specific siRNA or scrambled (Scr) siRNA (control) for 24 h using Lipofectamine RNAiMax transfection reagent, as per manufacturer's instructions. After 24 h, cells were washed with DPBS and incubated for 24 h in M2 medium. Then the cells were treated with ethanol or 10 nM E2 for 48 h in M2 medium.

3.5.7. Validation of treatment protocol

All treatment protocols were validated by examining the expression of known estrogen-induced genes called trefoil factor 1 (also referred to as pS2) (Berry et al., 1989) and progesterone receptor (PR) (Ing and Belen Tornesi, 1997).

3.5.8 Experimental replicates

All cell culture experiments had at least three biological replicates. For quantitative analysis of gene expression, mRNA or protein expression in control cells was set to 1, and those observed for treated cells were expressed relative to control.

3.6. MTT assay

After the termination of the experiment, the spent medium was aspirated, and 100 µL of MTT reagent (0.5 mg/ml) was added to the cells and incubated for 3 h. Excess of MTT reagent was removed, and DMSO was added to dissolve the formazan crystal. Then the absorbance was measured at 570 nm and 690 nm (reference wavelength). The difference between the absorbance values was considered as a measure of cell viability.

3.7. Gene expression analysis

3.7.1. Primers

For routine RT-PCR and qRT-PCR, primers were designed manually based on the following criteria; length 18-23 bp, GC content, 50-60 %, and melting temperature (Tm) between 55 to 65 °C. The Tm was calculated using formula 4 X (G+C) +2 X (A+T). Forward and reverse primers were designed so that each of them mapped in two separate exons separated by one intervening intron. Details of the primers used in the present study are provided in Table S (Appendix 1).

3.7.2. RNA Isolation

Total RNA was isolated using TRIzol (Invitrogen Corporation, Grand Island, NY, USA) or a similar reagent prepared in-house. Briefly, after the termination of the experiment, cells were washed with DPBS, and TRIzol was added to the cells. The lysed cells were transferred to a fresh tube, and 0.2 ml chloroform was added per 1 ml of TRIzol followed by vigorous shaking for 15-30 sec. Samples were then incubated for 2-3 min at room temperature and centrifuged at 12000 rpm for 15 min at 4° C. The aqueous phase containing RNA was transferred to a fresh tube, and the chloroform extraction step was repeated. An equal volume of isopropanol was added to precipitate the RNA and incubated for 10 min at room

temperature. The samples were centrifuged at 14000 rpm for 10 min at 4°C, and the supernatant was discarded. The RNA pellet was then washed with 75 % ethanol, followed by centrifugation at 14000 rpm for 5 min at 4° C. The RNA pellet was air-dried, dissolved in the appropriate volume of nuclease-free water, and stored at -80° C until use.

3.7.3. DNAse I digestion of RNA and cDNA synthesis

The quality of isolated RNA was examined by agarose gel electrophoresis and quantified using a Bio spectrophotometer (Eppendorf, Hamburg, Germany). 30 µg of total RNA was treated with DNase I (Promega, USA) and column purified with PureLink® RNA Mini Kit (Thermofisher Scientific, USA) as per manufacturer's instructions. To confirm the removal of genomic DNA, 20 ng of purified RNA was taken for PCR using primers shown in (Table S, appendix I). 2 µg of purified RNA was reverse transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystems, USA) according to the manufacturer's instructions.

3.7.4. RT-PCR and quantitative real-time RT-PCR (qRT-PCR)

For RT-PCR analysis, cDNA was diluted 5 times, and 1 µl was used as a template in PCR reactions using gene-specific primers (Appendix I). PCR products were resolved in 2% agarose gel containing Ethidium bromide (EtBr), and images were captured (Gel Doc™ EZ gel imaging system). Images were background corrected, and integrated densities of bands were determined by Image J software. Expression in treated groups relative to control was calculated after normalization against cyclophilin A (CycA).

Quantitative real-time RT-PCR was carried out in the Applied Biosystems® 7500 real-time PCR system (Life Technologies, Gaithersburg, MD, USA) or Agilent AriaMx Real-time PCR System (Agilent, CA, US). PowerUp™ SYBR® Green PCR master mix (Thermo Scientific, PA, USA) was used to analyze gene expression. The relative gene expression was analyzed by the comparative ΔΔCt method (Livak and Schmittgen, 2001) using CycA as an internal control. A melt curve was included in all real-time PCR reactions to check the specificity of the amplicon. A no-template control was also included for each primer set.

3.8. Western Blotting

3.8.1. Isolation of proteins

After the termination of experiments, cells were washed with DPBS and lysed with 1.5 X Laemmli sample buffer. Following lysis, samples were heated at 95°C for 10 min and centrifuged at 14000 rpm for 10 min, and the supernatant was stored at -20°C until use. In the case of TRIzol lysates, the phenolic phase was used for protein extraction. Total protein was precipitated using isopropanol, and the pellet was washed 3 times with 0.3 M guanidinium hydrochloride prepared in 95% ethanol. The pellets were solubilized in 1% SDS solution, followed by heating at 60°C for 30-40 min. The samples were then centrifuged at 14000 rpm for 10 min at 4° C, and the supernatant containing the proteins were collected and stored at -20° C until use.

3.8.2. Electrophoresis, blotting, and detection

Protein samples were resolved by 8 or 10% SDS-PAGE and transferred into nitrocellulose membranes (HiMedia, Mumbai, India) using a semi-dry transfer method. Following the transfer, blots were stained with Ponceau S dye, and images were captured in the ChemiDoc XRS+ system (Bio-Rad Laboratories, Hercules, CA, USA). To remove the Ponceau S stain, blots were washed 2-3 times with Tris-buffered saline containing 0.05% v/v Tween 20 (TBST). The membranes were then blocked with 1% (w/v) gelatin prepared in Tris-buffered saline containing 0.05% v/v Tween 20 (TBST) for 2 h at room temperature. The blots were probed with primary antibodies for 2 h at room temperature. The blots were then washed 5 times with 1×TBST followed by incubation with HRP conjugated secondary antibody for 1 h at room temperature. After 5 washes with 1×TBST, the blots were developed by enhanced chemiluminescence reagent (Bio-Rad Laboratories, Hercules, CA, USA), and images were captured in ChemiDoc XRS+ system (Bio-Rad Laboratories, Hercules, CA, USA)

3.9. Analysis of TCGA-BRCA data

The Cancer Genome Atlas (TCGA) is a cancer genomics project initiated by the National Cancer Institute (NCI) in collaboration with the National Human Genome Research Institute (NHGRI). This project contains information on 20,000 primary cancers across 33

cancer types (Koboldt et al., 2012). Breast cancer (BRCA) transcriptome RNA seq data (Illumina HiSeq 2000 RNA Sequencing platform) from the TCGA-BRCA dataset was used for the analysis. RNA-Seq data were in terms of $\log_2$(RPKM+1). The dataset was downloaded and accessed through the UCSC Xena browser (https://xenabrowser.net/) in 2017.

3.9.1. Analysis of HOXB2 expression in normal breast tissues and primary breast tumors

RNA-seq data from TCGA-BRCA were divided into normal and primary breast tumors. HOXB2 mRNA expression in 113 normal breast tissues and 1095 primary breast tumors was analyzed by Welch's two-sample *t*-test. The distribution of HOXB2 mRNA expression was represented as box plots.

3.9.2. Analysis of the association between HOXB2 expression and histopathological parameters

The mRNA expression levels of HOXB2 in normal breast tissues and breast tumors were extracted from the TCGA-BRCA dataset. Further, HOXB2 expression was accessed in primary breast tumors that express various hormonal receptors such as ERα, PR, or HER2. HOXB2 mRNA expression in various molecular subtypes of breast cancer that is Luminal A, Luminal B, HER2, Basal, and normal subtypes and in various grades of breast tumors, was also analyzed. The distribution of HOXB2 mRNA expression levels in these categories was visualized as boxplots. Finally, a correlation analysis was done between HOXB2 mRNA with ERα, PR, or HER2 expression in normal breast tissues and tumor samples.

3.9.3. Survival analysis

The effect of HOXB2 mRNA expression on survival was analyzed using KM plotter online tool (www.kmplot.com) (Győrffy et al., 2010). Tumor samples were segregated into two groups (HOXB2-high and HOXB2-low) using the median expression value as its threshold. Based on KM Plotter guidelines, the probe "205453_at" for HOXB2 was used for survival analysis. The relationship between HOXB2 expression and the overall survival (OS), relapse-free survival (RFS), distant-metastasis-free survival (DMFS), and post-progression survival (PPS) was analyzed. We further performed a Kaplan-Meier survival analysis of breast tumors that expressed HOXB2 and were either ERα-positive or negative. The results obtained

from the analysis are graphically depicted as Kaplan-Meier plots by using the default settings.

3.10. Analysis of HOXB2 upstream region by MatInspector and JASPAR

The upstream region of HOXB2 was extracted from the UCSC genome browser. This sequence was analyzed by MatInspector (Quandt et al., 1995) and JASPAR (Sandelin et al., 2004) for the presence of ERα binding sites.

3.11. Chip-Seq Analysis

HOXB2 locus was searched for ERα occupancy by analyzing Chip-seq data retrieved from Sequence Read Archive (SRA). For MCF-7 cells, Chip-Seq data (SRA accession ID: ERP000380) was used for the study, as described earlier (John Mary et al., 2020). A subset of files corresponding to MCF-7 cells was examined in Galaxy web-based platform (Afgan et al., 2018), and the reads were mapped to reference the human genome (hg19). The results were visualized in the UCSC genome browser to locate the specific binding of ERα in the HOXB2 or TFF1 locus. For T47D cells, the "ENC TF Binding" super track in the UCSC genome browser was searched for ERα occupancy.

3.12. Chromatin immunoprecipitation (ChIP) Assay

3.12.1. Cross-linking

MCF-7 and T47D cells were treated with vehicle (ethanol) or 10 nM E2 for 24 h. Cells were fixed with 0.75% (v/v) formaldehyde for 10 min at room temperature. The reaction was stopped by adding 125 mM glycine. Cells were washed three times with ice-cold DPBS, scrapped off, and transferred into a 15 ml tube. Then, the cells were pelleted by centrifugation at 1000 rpm for 5 min.

3.12.2. Cell lysis and sonication

The pellet obtained after centrifugation was lysed in a buffer containing 50 mM HEPES (pH 7.5), 140 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and 1×Protease inhibitor cocktail (ChIP lysis buffer). Lysates were then sonicated to shear the DNA at an amplitude of 30% for 30 cycles, each with a 10-sec pulse on and 25-sec pulse off. After that, sheared chromatin was centrifuged at 14000 rpm for

10 min, and the supernatants containing chromatin were stored in -80^{o} C until use. The beads were pelleted, and the precleared chromatin in the supernatants was collected.

3.12.3 Immunoprecipitation (IP)

50 µg of sonicated chromatin in ChIP lysis buffer was precleared by incubating with Protein G plus-Agarose beads, which were precoated with bovine serum albumin (BSA) and herring sperm DNA. The beads were pelleted, and the precleared chromatin in the supernatants was collected. 5% of the chromatin samples were kept aside as input, and the remaining was incubated either with ERα antibody or normal rabbit IgG antibody for 2 h. 20µl of precoated protein G plus-Agarose beads were added to each sample and incubated for 2 h. The beads were then pelleted by centrifugation and washed extensively with a series of wash buffers, as described earlier (Shivaswamy and Iyer, 2007).

3.12.4. Elution, reverse Cross-linking, DNA purification, and PCR amplification

Immunoprecipitated chromatin was eluted by incubating the beads in 100µl elution buffer (50 mM Tris-HCl pH 8.0, 1% SDS, 10 mM EDTA) at 65° C for 30 min. The eluted immunoprecipitated chromatin was then centrifuged at 14000 rpm for 2 min, and the supernatant was transferred to a fresh tube. 100 µl elution buffer was then again added to the beads, and the elution step was repeated. After that, the elutes were pooled and reverse cross-linked by heating overnight at 65° C. An equal volume of Tris EDTA (TE) buffer and Proteinase K (100 µg/ml) was added to the eluted chromatin and incubated for 3 h at 37° C (Shivaswamy and Iyer, 2007). Eluted chromatin was column purified and used in PCR reactions with primers to amplify specific regions of interest in the HOXB2 or TFF-1 locus (Table S1, Appendix 1). TFF-1 locus, known to be occupied by ERα, was used as a positive control (Shang et al., 2000) to validate the experimental protocol.

3.13. Statistical analysis

Analyzed results of all the quantitative data are represented as mean ± SD. All the experiments were performed at least three times, and the number of replicates is also mentioned in the respective figure legends. The digital images were quantified using Image J software. The association of HOXB2 mRNA concerning various histopathological parameters

was tested utilizing the chi-square test. Data were analyzed by Welch two-sample t-test or Mann–Whitney U test for two-group data or one-way ANOVA followed by Tukey's HSD or Kruskal-Wallis test, followed by Dunn's test for multiple comparisons. The correlation between HOXB2 mRNA with molecular markers of breast cancer was analyzed by Pearson's correlation test. For all the tests, data were analyzed in the R-statistical package and graphed using MS Excel. In all the statistical tests, $P < 0.05$ was considered as significant (significance codes: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

CHAPTER 4

HOXB2 expression in primary breast tumors

4.1. Introduction

HOX genes play a pivotal role in development. Their expression is increased or decreased in different cancers (Brotto et al., 2020). Aberrations in the expression of HOX genes influence the survival of cancer patients (Lu et al., 2016; Eoh et al., 2017). HOX genes can affect the prognosis of various cancers such as epithelial ovarian cancer (Lu et al., 2016; Eoh et al., 2017), cervical cancer (Eoh et al., 2017), pancreatic cancer (Segara et al., 2005; Kuo et al., 2019) lung cancer (Inamura et al., 2008), oral cancer (Destro et al., 2010; Aquino et al., 2015), and prostate cancer (Zhou et al., 2019).

HOX genes also affect the prognosis and survival of breast cancer patients. HOXD3 is a poor prognostic indicator of breast cancer (Shaoqiang et al., 2013). High HOXB5 expression in ER-positive breast tumors is associated with poor DMFS of ER-positive breast cancer patients (Lee et al., 2015). Xia and co-workers showed that low HOXA11 expression is associated with short overall breast cancer survival (Xia et al., 2017).

HOXB2 expression has been studied in a variety of cancers. It is aberrantly expressed in different cancers, such as bladder cancer (Liu et al., 2019), lung cancer (Inamura et al., 2008), and colorectal cancer (Li et al., 2019). HOXB2 expression also affects the prognostic outcome of cancer patients. Segara and co-workers show that ectopic expression of HOXB2 is associated with non-resectable tumors in pancreatic cancer. Lack of HOXB2 expression is associated with a survival advantage for patients undergoing operative resection (Segara, 2005). In lung adenocarcinoma, high HOXB2 expression is associated with poor prognosis in p-stage I patients (Inamura et al., 2007). Lindblad and colleagues proposed that HOXB2 is a tumor suppressor of FLT3-ITD directed AML (Lindblad et al., 2015).

In the context of breast cancer, HOXB2 is less studied. Two independent studies showed high HOXB2 expression in breast tumors than the normal breast tissue (Cantile et al., 2003; Hur et al., 2014). A microarray study shows that ER-positive breast tumors express high levels of HOXB2 compared to ER-negative ones (Neve et al., 2006). Using an in-vivo screen for tumor growth regulators, Boimel and co-workers injected HOXB2 overexpressing breast cancer cells in the mouse mammary fat pad. They observed a reduction in breast tumor growth

in HOXB2 overexpressing breast tumors compared to vector control. Based on their findings, they proposed a tumor suppressor role for HOXB2 (Boimel et al., 2011).

Prognosis is crucial in the management of any cancer (Simon and Altman, 1994; Cortesi et al., 2010). Therapeutic decisions for breast cancer management are based on the status of ER, PR, and HER2 expression (Dai et al., 2016). These molecular markers predict breast tumor's response to endocrine therapy (Mary Cianfrocca, 2004).

The literature on the role of HOXB2 in breast cancer is limited. It neither explains the reason for high HOXB2 expression in breast tumors nor provides information on the prognostic importance of HOXB2. Therefore, in this study, we aimed to explore the prognostic potential of HOXB2 in primary breast tumors and its association with histopathological parameters by mining the TCGA-BRCA data.

4.2. Results

4.2.1. Effect of HOXB2 on the survival of breast cancer patients

Kaplan-Meier survival analysis was performed to ascertain the prognostic potential of HOXB2. Based on the median HOXB2 mRNA expression, tumor samples were divided into HOXB2-high and HOXB2-low. The survival analysis was done by taking all the tumor samples regardless of their subtypes. The results showed that high HOXB2 expression was associated with prolonged OS, RFS, and PPS but not DMFS. The details of the analysis, including a 95% confidence interval (CI) and *p*-values, are shown in figure 4.1

4.2.2. Effect of HOXB2 on the survival of ER-positive and ER-negative breast cancer patients

Survival analysis was performed to explore the role of HOXB2 on the survival of ER-positive and ER-negative breast cancer patients. The analysis showed that high HOXB2 expression in ER-positive breast tumors is associated with prolonged OS and PPS. Whereas no significant difference was observed for RFS and DMFS. HOXB2 did not affect the survival of ER-negative breast cancer patients. The details of the analysis, including a 95% CI and *p*-values, are shown in figure 4.2.

4.2.3 HOXB2 expression in normal and primary tumor samples

HOXB2 mRNA expression data (log_2RPKM+1) within the TCGA-BRCA dataset was extracted through the UCSC Xena browser (https://xenabrowser.net/). HOXB2 mRNA expression was analyzed in normal and primary breast tumors. The mean HOXB2 mRNA expression in normal breast tissues (8.17±0.57) was significantly lower than in primary breast tumors (8.60±1.89). The distribution of HOXB2 expression is represented as box plots (Figure 4.3).

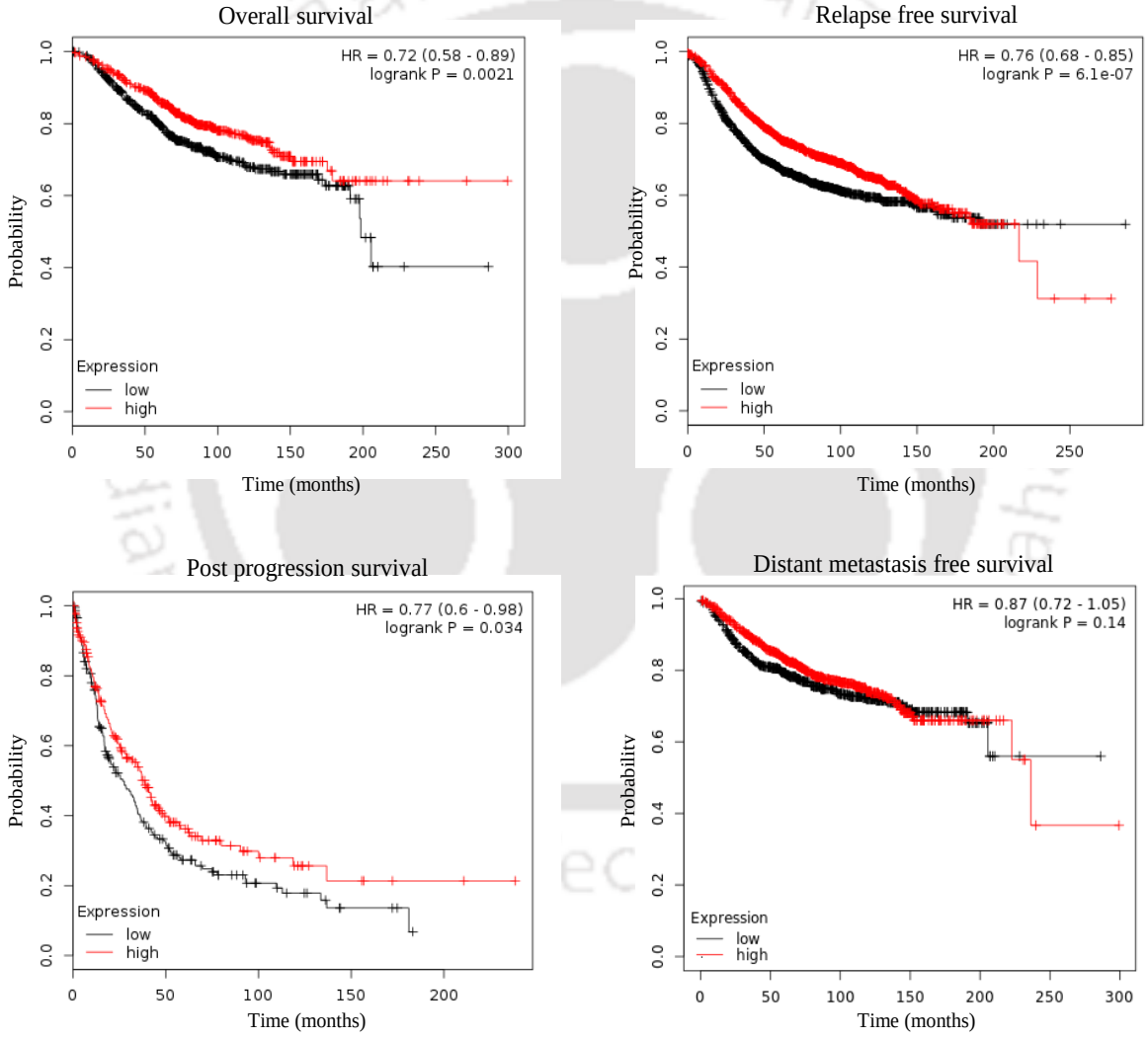


Figure 4.1. Kaplan-Meier survival analysis of breast cancer patients stratified by HOXB2 expression. In-silico survival analysis was performed using the Kaplan-Meier plotter (www.kmplot.com) online tool. The probe set for HOXB2, "205453"_at was used for the survival analysis. Breast tumors were segregated into HOXB2-high and HOXB2-low using the median expression value as the threshold, and survival data were analyzed for OS (n = 1408), RFS (n = 3951), PPS (n = 414), and DMFS (n = 1803).

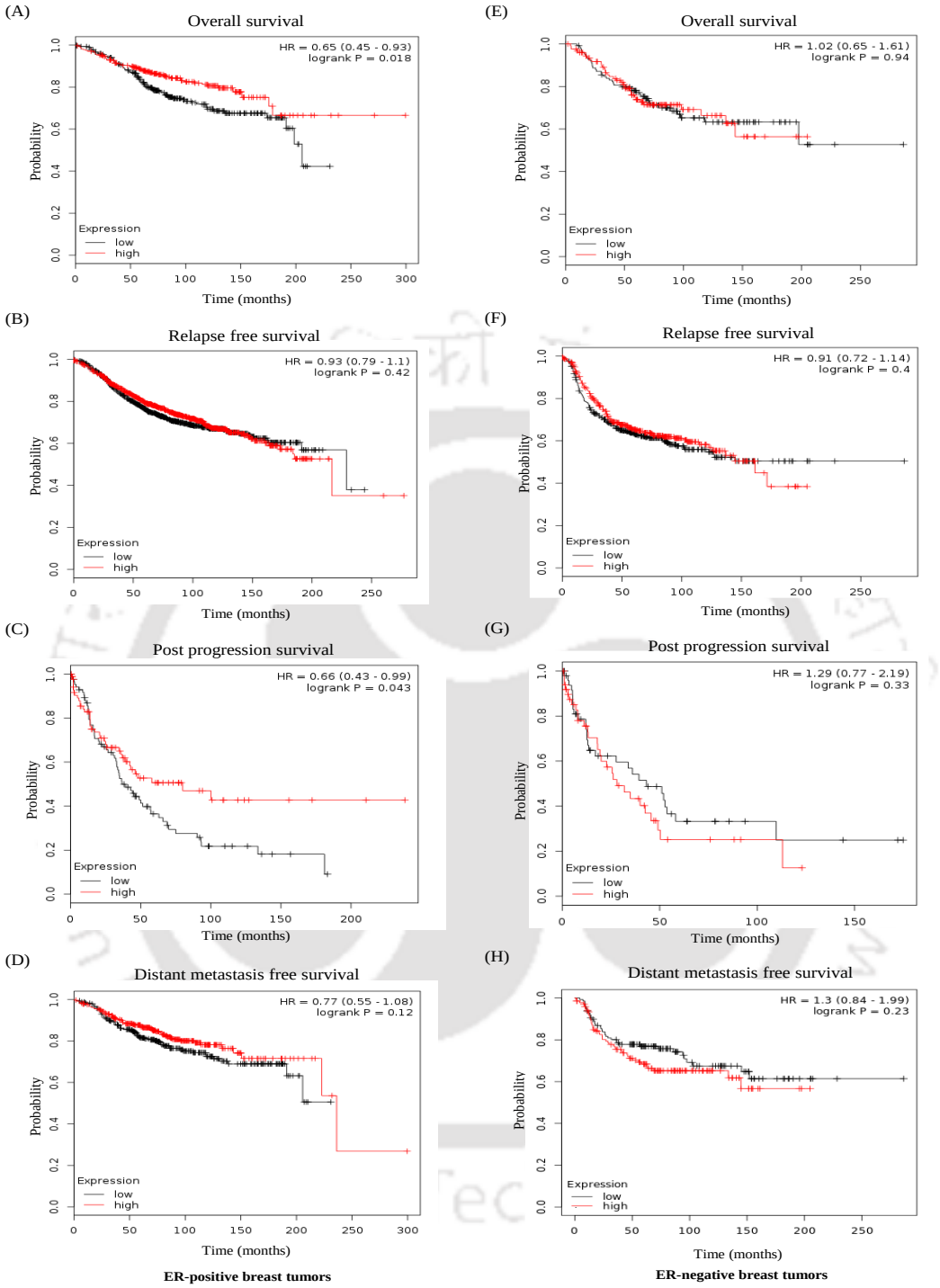


Figure 4.2. Kaplan-Meier survival analysis stratified by HOXB2 expression in ER-positive and ER-negative breast cancer patients. In-silico Survival analysis was performed using the Kaplan-Meier plotter (www.kmplot.com) online tool. The probe set for HOXB2, "205453"_at was used for the survival analysis. Breast tumors were segregated into HOXB2-high and HOXB2-low using the median expression value as threshold and ER-positive and ER-negative using the ER status. The survival analysis relating to HOXB2 expression in ER-positive, A (n = 548), B (n = 2061), C (n = 173), and D (n = 664) and ER-negative, E (n = 251), F (n = 801), G (n = 100), and H (n = 275) breast cancer patients is represented in Kaplan-Meier plots using the default settings.

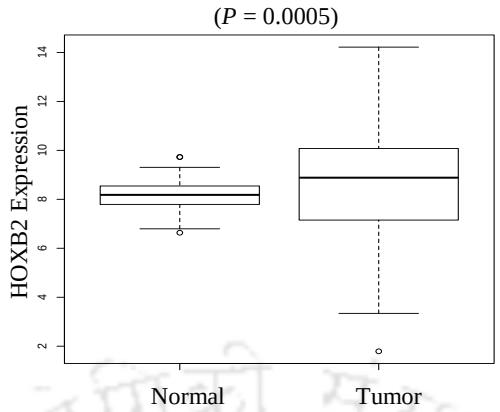


Figure 4.3. HOXB2 mRNA expression in normal breast tissues and breast tumors. Box plot showing HOXB2 mRNA expression in normal breast tissue (n=113) versus primary breast tumors (n=1095). The difference between the mean (log_2RPKM+1) of the two groups was analyzed by Mann–Whitney U test. The p-value is indicated above the box-plot.

4.2.4. Association of HOXB2 expression with histopathological parameters

HOXB2 expression was analyzed in the breast tumors classified based on the immunohistochemistry (IHC) data. Mean HOXB2 expression in ERα-positive tumors (8.84±1.78) was significantly high (p<0.00001) than in ERα-negative tumors (7.40 ± 1.93). Similarly, HOXB2 expression in PR-positive tumors (8.89 ± 1.75) was significantly high (p<0.00001) compared to PR-negative tumors (7.76 ± 2.00). However, no significant difference was observed between HER2-positive tumors (8.81± 1.90) and HER2-negative tumors (8.46 ± 1.93). The distribution of HOXB2 mRNA expression levels is represented as boxplots (Figure 4.4).

Further, the primary tumors were segregated into two groups, namely HOXB2-high and HOXB2-low, based on the HOXB2 RNA median value as the cutoff. Then, the association of HOXB2 with histopathological parameters was analyzed by chi-square test. The results show that HOXB2 expression was significantly associated with age, ERα, PR, and molecular subtypes of breast cancer. However, no significant association was observed with the HER2 status and tumor stage (Table 4.1). HOXB2-high tumors were frequently associated with ERα-positive or PR-positive status. HOXB2-low tumors were related to ERα-negative or PR-negative status. Except for Luminal A and Luminal B, HOXB2-high tumors were relatively less frequent in the rest of the four subtypes. Luminal-B subtype had the highest representation of HOXB2-high cases (61.98%), followed by Luminal-A (57.01%), HER2-enriched

(56.72%), Normal-like (34.78%), and Basal-like (6.38%). However, no significant association was observed between HOXB2 expression and tumor stage (Table 4.1).

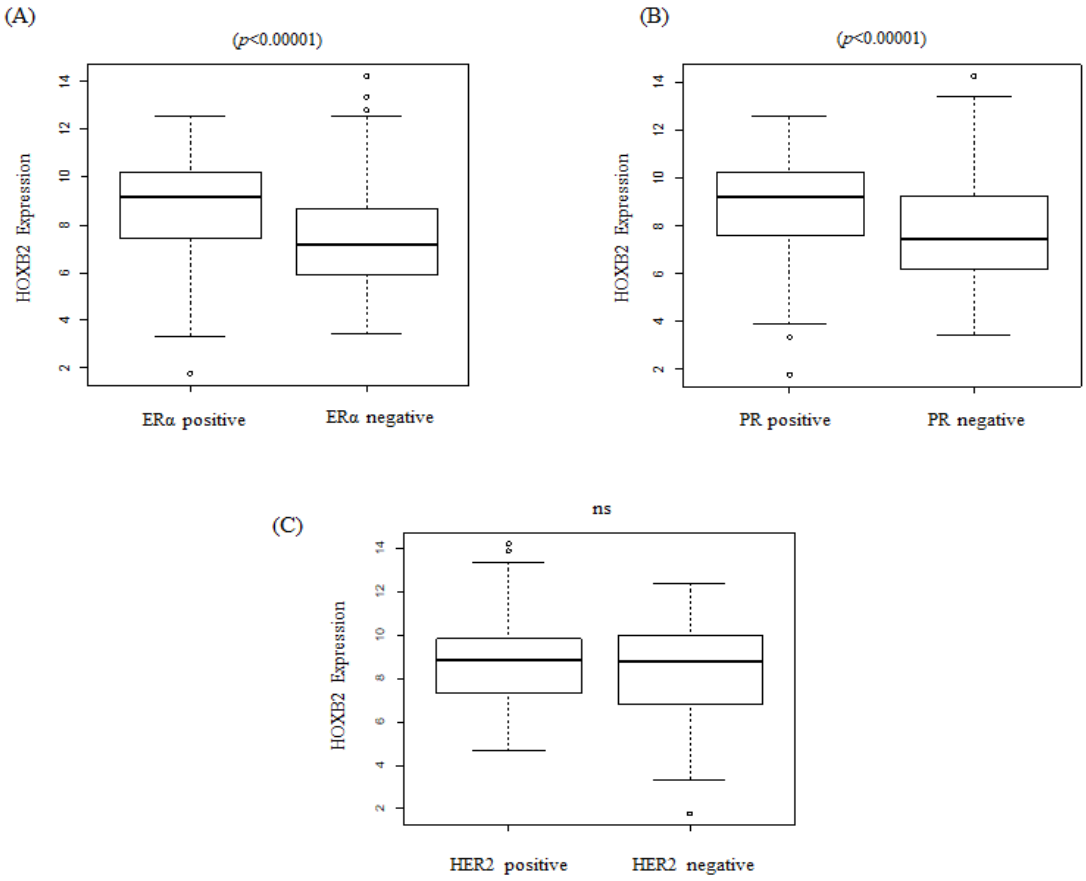


Figure 4.4. HOXB2 mRNA expression in primary breast tumors. Box plots showing the distribution of HOXB2 mRNA expression in ERα-positive (n=603) versus ERα-negative tumors (n=179), (A), PR-positive (n=523) versus PR- negative (n=256), (B) and HER2-positive (n=115) and HER2-negative (n=653), (C). The difference between the mean (log_2RPKM+1) of the two groups was analyzed by Mann–Whitney U test. The *p*-value is indicated above the box-plot. The ns denotes a non-significant value.

4.2.5. HOXB2 expression in molecular subtypes and different stages of breast tumors

HOXB2 mRNA expression in the various molecular subtypes of primary breast tumors was analyzed. The mean HOXB2 expression in the Basal tumor subtype (6.60 ± 1.44) was low when it was compared with Luminal A (8.90 ± 1.96), Luminal B (8.93 ± 1.94), and HER2 (9.10 ± 1.44) subtypes. The mean HOXB2 expression in normal breast tissue was significantly low compared with Luminal A ($p < 0.001$), Luminal B ($p < 0.001$) and HER2 ($p < 0.01$) tumor subtypes. Whereas HOXB2 expression in basal breast tumors was significantly low ($p < 0.00001$) compared to normal breast tissue. The data were analyzed by Kruskal Wallis test,

followed by multiple comparisons using Dunn's test. The distribution of HOXB2 expression in the groups is represented as box plots (Figure 4.5A). The adjusted *p*-values for the pairwise comparison are provided in Table 4.2. Further, HOXB2 expression was analyzed in various stages of breast cancer. No significant difference in the mean HOXB2 expression was observed across different stages (Figure 4.5B).

Table 4.1. Association of HOXB2 expression with various clinical characteristics

	HOXB2-high	**HOXB2-low**	***p***
Age			
Mean ± SD	59.7±13.2	57.2±13.0	**M: 0.0024**
Median	59	57	
Range	28-90	26-90	
ERα			
ERα-positive	327 (57.17)	245 (42.83)	**<0.0001**
ERα-negative	37 (22.29)	129 (77.71)	
PR			
PR-positive	307 (58.81)	215 (41.19)	**<0.0001**
PR-negative	74 (29.01)	181 (70.98)	
HER2			
HER2-positive	58 (50.88)	56 (49.12)	0.62
HER2-negative	314 (48.16)	338 (51.84)	
Molecular type			
Normal-like	8 (34.78)	15 (65.22)	**<0.0001**
Luminal A	240 (57.01)	181 (42.99)	
Luminal B	119 (61.98)	73 (38.02)	
Basal-like	9 (6.38)	132 (93.62)	
HER2-enriched	38 (56.72)	29 (43.28)	
Tumor Stage			
Stage I	95 (52.20)	87 (47.80)	0.09
Stage II	290 (47.54)	320 (52.46)	
Stage III	139 (56.97)	105 (43.03)	
Stage IV	8 (42.11)	11 (57.89)	
Stage X	6 (42.86)	8 (57.14)	

Numbers within the brackets indicate % of HOXB2-high or HOXB2-low in various categories. The *p*-values (*p*) are from the chi-squared test; M indicates the *p*-value from the Mann–Whitney U test. In all the tests, $p < 0.05$ was considered significant.

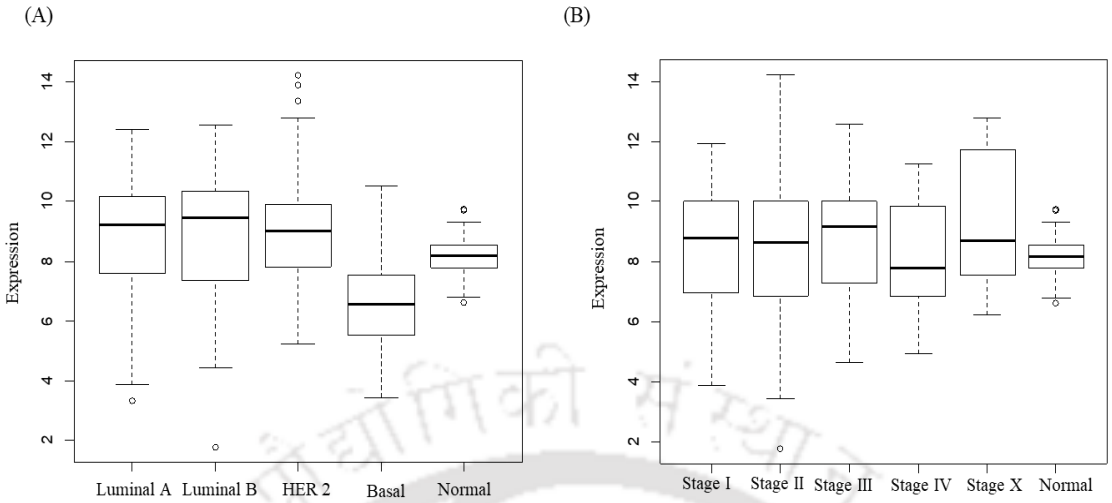


Figure 4.5. HOXB2 expression in various molecular subtypes and various stages of breast cancer. Box plots showing the distribution of HOXB2 mRNA expression in the indicated subtypes (A) and across different stages of breast cancer (B). The data were analyzed Kruskal-Wallis test, followed by multiple comparisons using Dunn's test.

Table 4.2. Analysis of HOXB2 expression in molecular subtypes of breast tumor.

Comparison	Z-value	P.unadjusted	P.adjusted
Basal - Her2	-8.28709752	1.160455e-16	1.160455e-15
Basal - Luminal.A	-12.68575440	7.092762e-37	7.092762e-36
Her2 - Luminal.A	-0.03551466	9.716694e-01	1.000000e+00
Basal - Luminal.B	-11.38607213	4.906109e-30	4.906109e-29
Her2 - Luminal.B	-0.23354033	8.153419e-01	1.000000e+00
Luminal.A-Luminal.B	-0.32688529	7.437546e-01	1.000000e+00
Basal - Normal	-5.72622795	1.026882e-08	1.026882e-07
Her2 - Normal	3.28597594	1.016297e-03	1.016297e-02
Luminal.A - Normal	4.82635601	1.390538e-06	1.390538e-05
Luminal.B - Normal	4.55279367	5.293819e-06	5.293819e-05

HOXB2 expression in various breast tumor subtypes was analyzed by the Kruskal-Wallis test, followed by multiple comparisons using Dunn's test.

4.2.5. Correlation of HOXB2 mRNA with the molecular markers of breast cancer

A correlation of HOXB2 mRNA expression with ERα, PR, or HER2, was done in normal and

tumor samples. There were 113 normal and 1095 primary breast tumor samples. HOXB2 mRNA expression in the normal breast tissue was positively correlated, significantly with ERα (r = 0.25, p = 0.008). However, no significant correlation was observed with that of PR (r = 0.07, p = 0.48) and HER2 (r = -0.05, p = 0.62). In tumor samples, HOXB2 mRNA was positively, significantly correlated with that of ERα (r = 0.36, p < 0.00001), PR (r = 0.29, p < 0.00001) or HER2 (r = 0.14, p < 0.00001), respectively. The correlation analysis is provided in Table 4.3.

Table 4.3. Correlation of HOXB2 expression with ERα, PR, and HER2.

	Normal	***p*-value**	**Tumor**	***p*-value**
ERα	0.24762	**0.008**	0.35557	**<0.0001**
PR	0.06619	0.48	0.28962	**<0.0001**
HER2	-0.04679	0.62	0.14363	**<0.0001**

Correlation analysis between HOXB2 and ERα, PR, or HER2 was done by Pearson's correlation test (n = 113 for each group). Pearson's correlation coefficients are shown in the table above. The statistically significant values are represented in bold.

4.3. Discussion

The literature presents a diverse role of HOXB2 in various cancers. It has tumor-promoting functions in bladder cancer (Liu et al., 2019), lung cancer (Inamura et al., 2008), and colorectal cancer (Li et al., 2019). However, in breast cancer, overexpression of HOXB2 was reported as resulting in a reduction of breast tumor growth (Boimel et al., 2011). Conversely, by others, breast tumors were reported to express higher levels of HOXB2 than normal breast tissue (Cantile et al., 2003; Hur et al., 2014). There is a lack of clarity in the information of expression of HOXB2 and on the correlation of HOXB2 over/under-expression on the survival of breast cancer patients. Therefore, we assessed HOXB2 expression by analyzing databases of primary breast tumors and determined the prognostic potential of its expression in breast cancer.

As in previous studies (Cantile et al., 2003; Hur et al., 2014), our TCGA-BRCA data analysis shows high HOXB2 expression in breast tumors compared to normal breast tissue. Investigations show that high HOXB2 expression predicts an adverse prognosis in lung cancer (Inamura et al., 2007) and pancreatic cancer (Segara, 2005). Therefore, we hypothesized that

high HOXB2 expression in breast tumors might also negatively affect the prognostic outcome of breast cancer patients. To address this, we performed the Kaplan-Meier survival analysis. Analyzing a database containing 7576 breast cancer samples, our results show that high HOXB2 expression is associated with prolonged OS, RFS, and PPS of breast cancer patients in all tumor subtypes. This suggests that when overexpressed in breast cancers, HOXB2 may have a tumor/growth-suppressive role that may prevent breast cancer cells from becoming aggressive and progressing to metastasis. This is in line with findings by Boimel and co-workers who showed in xenograft assays that overexpression of HOXB2 reduces the percentage of cells in mitosis and reduces tumor growth (Boimel et al., 2011). Therefore, the high expression of HOXB2 in breast tumors might affect the pathways involved in cell division.

HOXB5 affects the survival of ER-positive breast cancer patients. Its high expression in ER-positive breast tumors is associated with poor DMFS of ER-positive breast cancer patients (Lee et al., 2015). Neve and co-workers showed high HOXB2 expression in ER-positive breast tumors compared to ER-negative ones (Neve et al., 2006). Our in-silico survival analysis of TCGA breast cancer databases shows that HOXB2 expression is associated with prolonged OS and PPS in patients with ER-positive breast cancer, implying the clinical significance of HOXB2 in ER-positive breast cancer. The selection of a therapeutic regimen for breast tumors depends on the levels of ER, PR, and HER2 expression (Dai et al., 2016). HOXB13 expression is high in ER-negative breast cancer tissue compared to ER-positive ones. Further, HOXB13 expression is negatively correlated with ER or PR expression and positively with HER2 expression in ER-positive tumors (Wang et al., 2007). High HOXB2 expression is observed in ER-positive compared to ER-negative breast tumors and cell lines (Neve et al., 2006). We examined HOXB2 expression in ERα, PR, or HER2-positive or negative breast tumors, and HOXB2 expression in ERα or PR-positive tumors was significantly higher compared to ERα or PR-negative breast tumors. Correlation analysis shows that HOXB2 and ERα are positively correlated in normal and breast tumor samples. Further, HOXB2 expression is high in luminal A and luminal B breast cancer subtypes compared to the others. A similar pattern of expression was observed for ERα (John Mary et al., 2017). Therefore, the direct relationship between HOXB2 and ERα is also apparent from their expression levels in molecular subtypes of breast tumors. Association analysis of

HOXB2 expression with histopathological parameters shows that HOXB2 is significantly associated with ERα, PR, and molecular subtypes of breast cancer. Shah and co-workers showed that HOXB13 negatively regulates ERα in breast cancer cells (Shah et al., 2013), and HOXB13 tumors develop tamoxifen resistance (Ma et al., 2004). A positive correlation between HOXB2 and ERα suggests the possibility that HOXB2 may also be involved in tamoxifen resistance.

In summary, the present study indicates that HOXB2 may be a prognostic indicator of breast cancer. HOXB2 is positively correlated with ERα in normal breast tissue and breast tumors. This study provides evidence to examine a functional link between HOXB2 and ERα and to investigate the estrogen-mediated regulation of HOXB2.

CHAPTER 5

Molecular mechanism of estrogen-mediated suppression of HOXB2

The results presented and discussed in this chapter are published in (Kumar et al., 2021), Gene.

5.1. Introduction

HOX transcription factors are the master regulators of embryonic development that specify positional identity along the anterior-posterior axis (Favier and Dolle, 1997; Grier et al., 2005). Their target genes are involved in various cellular processes, such as proliferation, adhesion, and differentiation during limb or organ development and tissue homeostasis. A variety of solid tumors, including those of the breast, show aberrant HOX gene expression (Cillo, 1994; Chen and Sukumar, 2003; Bhatlekar et al., 2014). An altered HOX gene expression network disrupts tissue homeostasis during tumor development and progression (Bhatlekar et al., 2014).

Many Hox genes are expressed in the mouse mammary gland. They are developmentally or estrogen-regulated, have roles in mammary gland development, or are aberrantly expressed in the neoplastic mouse mammary tissues (Friedmann et al., 1994; Lewis, 2000; Chen and Sukumar, 2003). In humans, as many as 17 HOX genes are expressed in normal breast tissues, and several are misexpressed in breast tumors (Cantile et al., 2003). Breast cancer cell lines also differentially express HOX genes (Hur et al., 2014). A forced expression or knockdown of HOX genes affects proliferation rates and other cellular properties associated with malignancy (Raman et al., 2000; Zhang et al., 2003; Gilbert et al., 2010; Hayashida et al., 2010; Lee et al., 2015). Taken together, *in vitro, in vivo,* and clinical data suggest that altered expression of HOX genes and the consequent disruption of their target gene networks are mechanistically linked to tumor development and progression.

HOXB2 is a member of the HOXB gene cluster. A variety of solid tumors show altered expression of HOXB genes (Bhatlekar et al., 2014). In relation to cancer, HOXB2 is the least studied member. Few studies on HOXB2 in relation to cancers, including those of the breast, are reported (Segara et al., 2005; Kanai et al., 2010; Boimel et al., 2011; Hur et al., 2014; Nagata et al., 2017). In an *in vivo* screen to identify promoters or suppressors of breast tumors, Boimel and co-workers found that HOXB2 expression had a negative impact on tumor formation (Boimel et al., 2011). They proposed a tumor suppressor role for this gene. On the other hand, two independent studies showed that malignant breast tissues express higher levels of HOXB2 than normal (Cantile et al., 2003; Hur et al., 2014).

Estrogen promotes the growth and development of the breast, and induces its differentiated functions. Aberrant estrogen signaling can cause breast cancer (Henderson et al., 1986). Estrogen induces genomic effects in target cells via two estrogen receptors (ERs), namely ERα and ERβ. ERα, a ligand-dependent transcription factor, is expressed in a majority of breast tumors (Anderson et al., 2002). Upon estrogen binding, ERα modulates the expression of target genes by recognizing estrogen response elements (EREs) in their promoters (Klein-Hitpass et al., 1988) and activates pathways involved in cell survival and proliferation (Osborne et al., 2001). This forms the basis for the use of tamoxifen, a selective estrogen receptor modulator (SERM), or fulvestrant, a selective estrogen receptor downregulator (SERD) for treatment of ERα positive breast tumors (Cleator et al., 2009). However, despite initial success with the use of SERMs, tumors often relapse due to endocrine resistance. Interestingly, several Hox genes, such as HOXA1, HOXA10, HOXB7, and HOXB13, are estrogen-regulated (Daftary and Taylor, 2006), or associated with endocrine resistance (Jin et al., 2012; Shah et al., 2013), suggesting a possible interaction between estrogen-ERα pathway and Hox genes.

The relation between estrogen signaling and HOXB2 expression is unknown. In an attempt to identify estrogen-regulated genes in breast cancer cells, a microarray analysis of ER-positive breast cancer cells treated with E2 was performed in our laboratory (Manjegowda et al., 2015). HOXB2 was amongst the estrogen suppressed genes. This observation motivated us to investigate, mechanistically, the relationship between HOXB2 and estrogen signaling, given the importance of estrogen target genes in breast cancer, and the suggested tumor suppressor function of HOXB2 (Boimel et al., 2011). Here, we show that estrogen downmodulates the expression of HOXB2 in human breast cancer cell lines. Estrogen-mediated suppression of HOXB2 depends on ERα and is associated with increased ERα occupancy in the 5' upstream region of HOXB2.

5.2 Results

5.2.1. Effect of E2 on HOXB2 mRNA expression

In a dose-response experiment, MCF-7 or T47D breast cancer cells were treated with varying concentrations of E2 (0.1 to 100 nM) for a period of 72 h. All concentrations of E2

significantly reduced HOXB2 mRNA expression compared to ethanol-treated control (Figure 5.1 A, B). At each concentration of E2, the extent of reduction was lesser in T47D compared to MCF-7. In a time-course experiment, MCF-7 or T47D cells were treated with ethanol or 10 nM E2 for a period of 0 to 96 h. qRT-PCR results showed a significant reduction of HOXB2 mRNA from 6 h and 12 h onwards in MCF-7 and T47D cells, respectively (Figure 5.1 C, D). At each time-point, the extent of reduction in T47D was lesser than that in MCF-7.

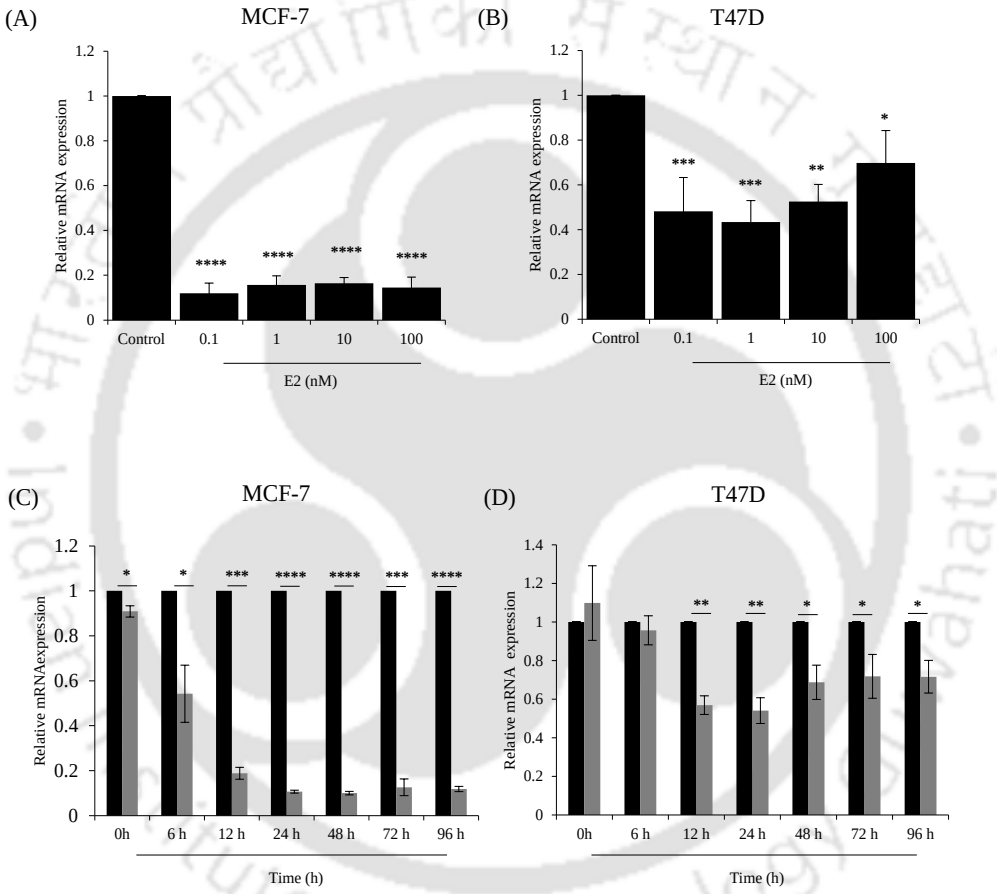


Figure 5.1. Regulation of HOXB2 mRNA by E2. In dose-response experiments, MCF-7 (A) and T47D cells (B) were treated with indicated concentrations of E2 for 72 h. HOXB2 mRNA expression was analyzed by qRT-PCR. Cyc A served as the internal (normalizing) control. The normalized HOXB2 expression in control samples was set to 1, and those in treated samples were expressed relative to control. Bars represent mean relative expression ± sd (n=3). The data were analyzed by ANOVA, followed by Tukey's HSD. In time-course experiments, MCF-7 (C) and T47D (D) cells were treated with 10 nM E2 for the indicated period. HOXB2 mRNA expression was analyzed by qRT-PCR. Cyc A served as the internal (normalizing) control. For each time point, the normalized HOXB2 expression in control samples (black bars) was set to 1, and those in treated samples were expressed relative to control (grey bars). Bars represent mean relative expression ± sd (n=3). The data for each time point were analyzed by Welch's two-sample *t*-test. Asterisks (**p* <0.05, ***p* <0.01, ****p* <0.001, *****p* <0.0001) denote significant results relative to control.

5.2.2. Effect of E2 on HOXB2 protein expression

E2 reduced HOXB2 mRNA expression in MCF-7 and T47D cells. Therefore, we next checked the effect of E2 on HOXB2 protein expression. In a time-course experiment, MCF-7 or T47D cells were treated with 10 nM E2 for a period of 24 to 72 h. The western blotting results show that E2 reduced HOXB2 protein expression in both the cell lines for all the time points. The suppression in HOXB2 protein expression increased after 48 or 72 h of E2 treatment (Figure 5.2).

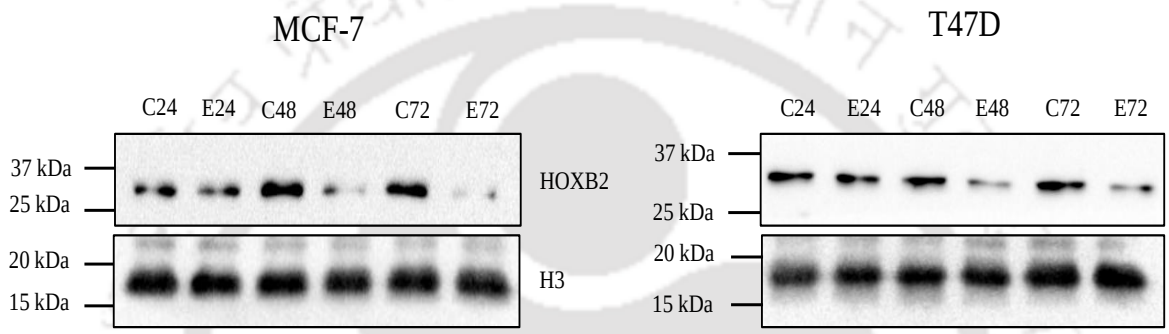


Figure 5.2. Effect of E2 on HOXB2 protein expression. MCF-7 or T47D cells were treated with 10 nM E2 for the indicated time points. The total protein was isolated and subjected to western blotting. H3 was used as an internal control.

5.2.3. Regulation of HOXB2 by steroid hormones and PPT

In MCF-7 cells, E2 reduced HOXB2 mRNA expression within 6 h. Therefore, we hypothesized that ERα might regulate its expression. We tested the effect of propylpyrazoletriol (PPT), an ERα agonist, on HOXB2 mRNA expression. In addition, we also studied the regulation of HOXB2 by various other steroid hormones. MCF-7 cells were treated with 10 nM PPT or a similar concentration of various other hormones for 72 h. Their effect on HOXB2 mRNA was accessed by semi-quantitative RT-PCR. The result shows that E2 and PPT significantly reduced HOXB2 mRNA. However, no significant difference was observed with other steroid hormones (Figure 5.3).

5.2.4. E2 suppresses HOXB2 expression in MCF-7, and T47D cells via ERα

PPT reduced HOXB2 mRNA expression in MCF-7 cells. Further, to check its effect on HOXB2 mRNA and protein expression in MCF-7 and T47D cells, we treated both the cell lines with PPT. It significantly reduced HOXB2 mRNA in MCF-7 (Fig 5.4A) and T47D cells

(Figure 5.4C). Analysis of HOXB2 protein by western blotting yielded similar results (Figure 5.4B and D).

We further studied the effect of ERα knockdown on E2-mediated suppression of HOXB2. Breast cancer cells, pre-treated with scrambled or ERα-specific siRNA, were stimulated with E2 or vehicle. The ERα specific siRNA abrogated ERα protein expression to levels beyond detection (Figure 5.5C and D). As expected, E2 treatment significantly downmodulated the expression of HOXB2 mRNA in both the cell lines transfected with scrambled siRNA (Figure 5.5A and B, bars 1 and 2). However, in both the cell lines transfected with ERα specific siRNA, no significant downmodulation of HOXB2 mRNA was observed (Figure 5.5A and B, bars 3 and 4). Interestingly, ERα specific siRNA, on its own, significantly induced HOXB2 mRNA in MCF-7 cells (Fig 5.5A, bars 1 and 3) but not in T47D cells (Fig 5.5B, bars 1 and 3).

The pattern of HOXB2 protein expression in both the cell lines was similar to the mRNA. In both the cell lines transfected with scrambled siRNA, E2 downmodulated HOXB2 protein. When analyzed by ANOVA followed by multiple comparison tests using Tukey's HSD, the downmodulation of HOXB2 protein in T47D cells was significant (Fig 5.5D, bars/lanes 1 and 2), but not in MCF-7 cells (Fig 5.5C, bars/lanes 1 and 2). However, when analysed by Welch's two-sample *t*-test, the downmodulation was significant in MCF-7 cells ($p < 0.0003$). E2 treatment did not modulate HOXB2 expression in both the cell lines transfected with ERα specific siRNA (Fig 5.5C and 4D, bars/lanes 3 and 4). ERα-specific siRNA alone significantly induced expression of HOXB2 protein in MCF-7 cells (Fig 5.5C, bars/lanes 1 and 3) but significantly down-modulated HOXB2 protein in T47D cells (Fig 5.5C, bars/lanes 1 and 3).

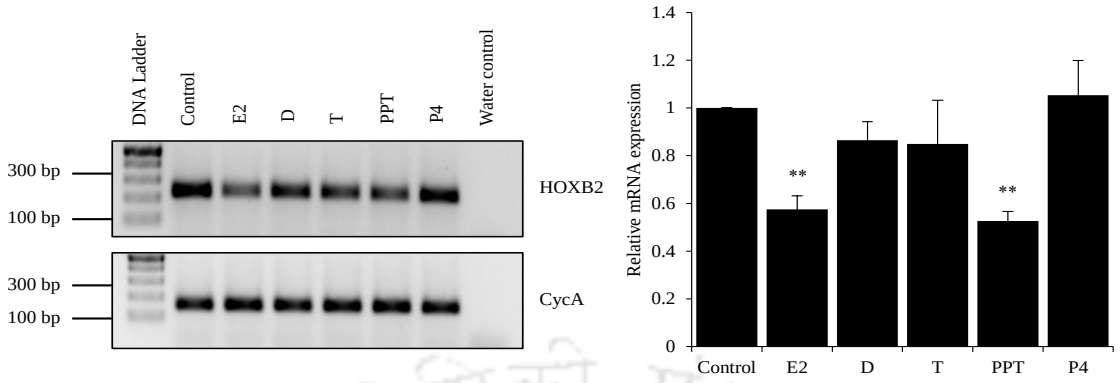


Figure 5.3. Effect of various steroid hormones and PPT on HOXB2 mRNA expression. MCF-7 cells were treated with various hormones; 10 nM E2, 10 nM dexamethasone (D), 10 nM progesterone (P4), 10 nM testosterone propionate (T), and 10 nM PPT (ERα-specific agonist) for 72 h. HOXB2 mRNA expression was analyzed by semi-quantitative RT-PCR. CycA served as the internal control. The bar graph on the right is the quantitative representation of the data shown on the left. Bars represent mean relative expression ± sd (n=3). The data were analyzed by ANOVA, followed by Tukey's HSD. Asterisks (*p <0.05, ** p <0.01, ***p <0.001, ****p <0.0001) denote significant result relative to control.

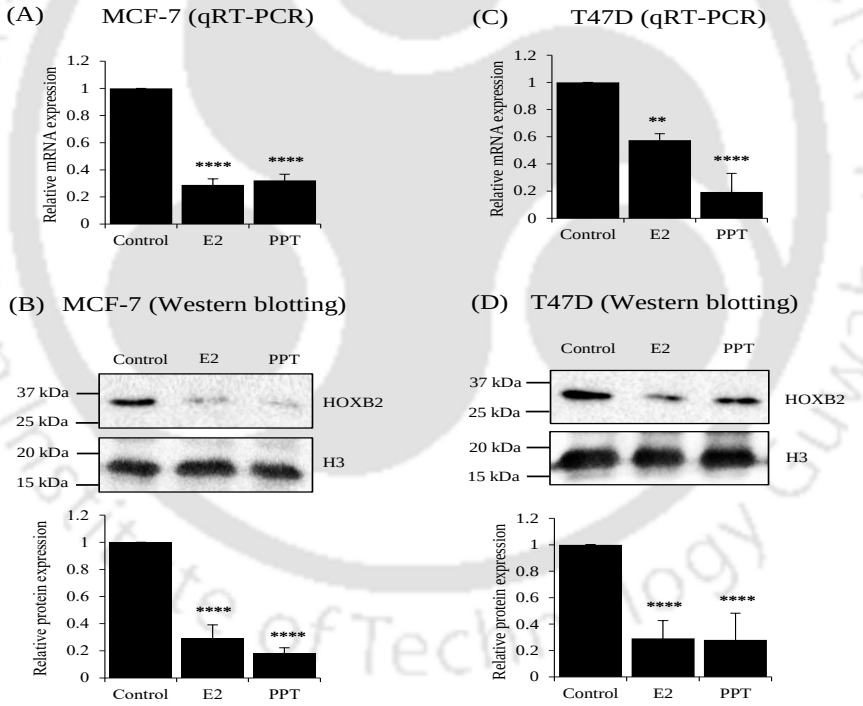


Figure 5.4. Effect of PPT on HOXB2 mRNA and protein expression. MCF-7 (A, B) and T47D cells (C, D) were treated with 10 nM E2 or 10 nM PPT for 48 h. Total RNA or protein was extracted and subjected to qRT-PCR (A, C) or western blotting analysis (B, D). Cyc A and histone H3 served as normalizing control in qRT-PCR and western blots, respectively. The western blots in B and D are representative of 3-4 independent experiments, and the bar graphs shown below are quantitative representations. The normalized HOXB2 expression in control samples was set to 1, and those in treated samples were expressed relative to control. Bars represent mean relative expression ± sd (n=3 for A, B, and C; n=4 for D). The data were analyzed by ANOVA, followed by Tukey's HSD. Asterisks (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001) denote significant result relative to control.

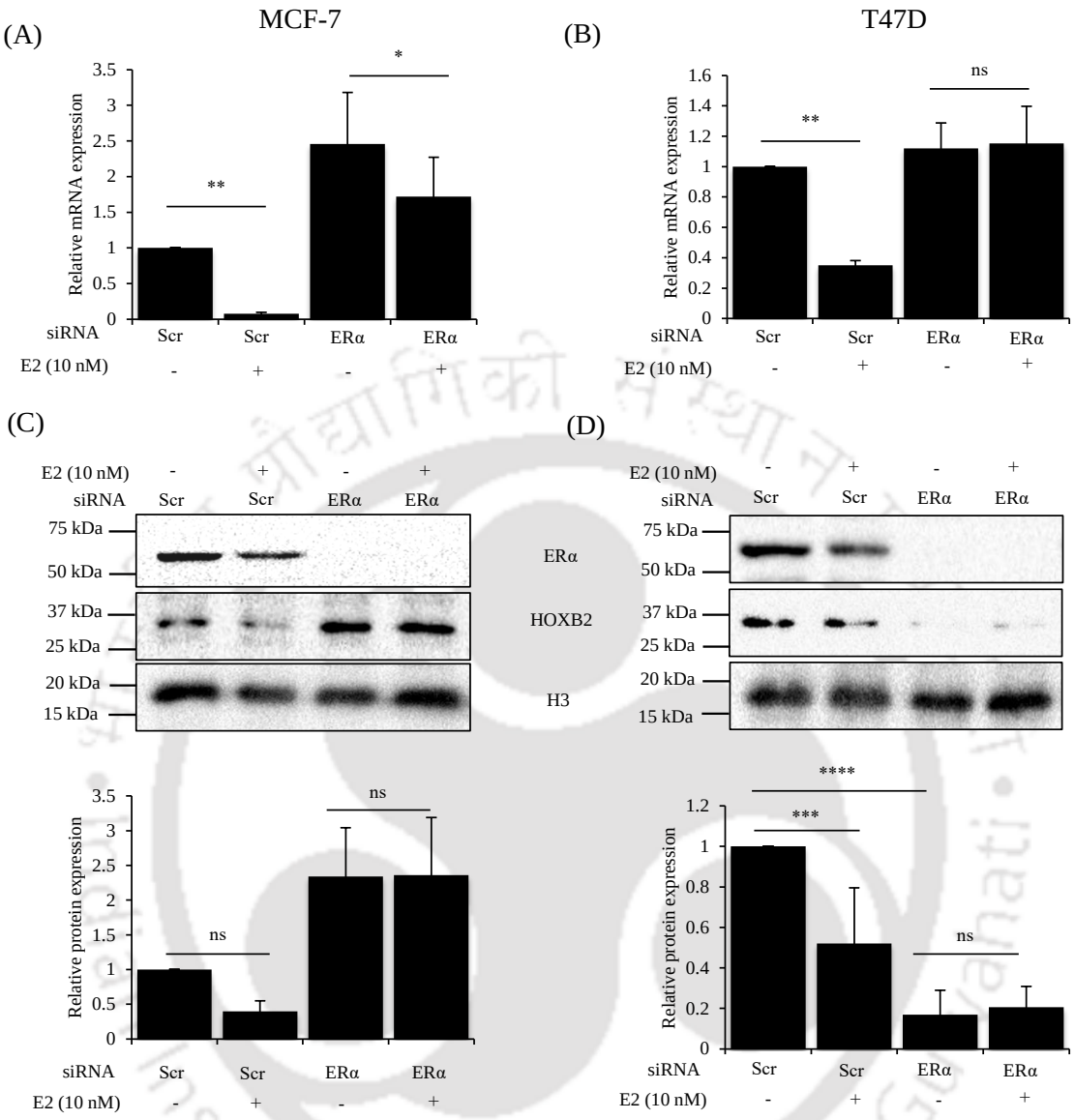


Fig. 5.5. Effect of ERα knockdown on E2 mediated suppression of HOXB2. MCF-7 (A, C) and T47D cells (B, D) were transfected with scrambled siRNA (Scr) or ERα specific siRNA and incubated for 24 h followed by treatment with vehicle or 10 nM E2 for the 48 h. Total RNA or protein was isolated for analyzing HOXB2 expression using qRT-PCR (A, B) or western blotting (C, D). The western blots in C and D are representative of 5 independent experiments, and the bar graphs shown below are quantitative representations. HOXB2 expression in Scr + vehicle-treated (control) cells was set to 1, and those in other treatment groups were expressed relative to control. Bars represent mean relative expression ± sd (n=6 for A, n=3 for B, and n=5 for C and D). The data were analyzed by ANOVA, followed by Tukey's HSD. Asterisks (*p <0.05, **p <0.01, ***p <0.001, **** p<0.0001) denote significant results relative to control.

5.2.5. HOXB2 upstream region has EREs that binds to ERα

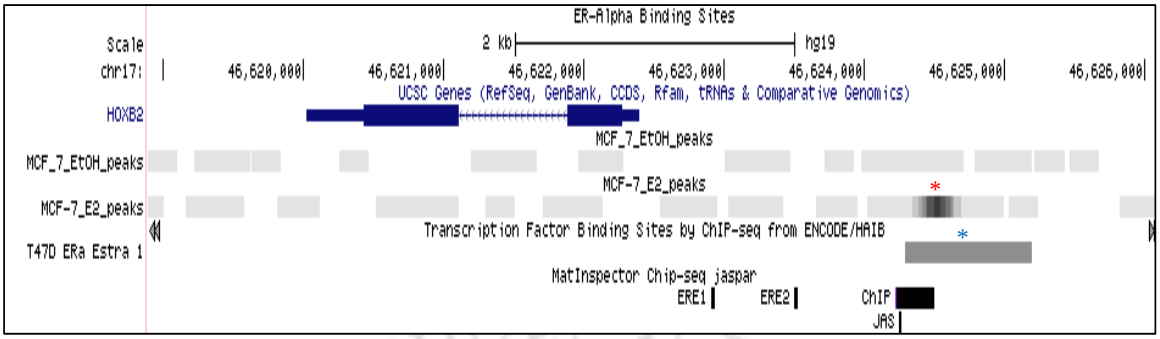
The dependence of HOXB2 expression on ERα prompted us to examine the promoter and intronic regions of HOXB2 for estrogen response elements (EREs). Approximately 7 kb

genomic sequence of human chromosome 17, which harbors the HOXB2 gene, was analyzed by Matinspector. Two EREs (referred to as ERE1 and ERE2) were predicted in the upstream region of HOXB2 mRNA (Fig. 5.6A). Sequencing data of fragmented chromatin samples immunoprecipitated by anti-ERα antibody from MCF-7 cells treated with ethanol or E2 are freely available in the Sequence Read Archive (SRA accession ID: ERP000380). The data were analyzed using Galaxy and visualized in the UCSC Genome Browser. Figure 5.6A shows enriched ERα occupancy in the 5' upstream region of HOXB2 mRNA (indicated by a red asterisk) in the E2 treated samples. Data from a similar experiment in T47D cells also revealed ERα occupancy in the same region (Figure 5.6A, indicated by a blue asterisk). Matinspector did not predict any EREs in this region. However, analysis of the sequence using JASPAR revealed an ERE downstream of the ERα binding site (referred to as JAS, Fig.5.6A).

5.2.6. Estrogen treatment of MCF-7 and T47D cells increases ERα occupancy in the HOXB2 upstream region.

We carried out ChIP experiments to independently assess ERα binding in the HOXB2 locus. MCF-7 and T47D cells were treated with ethanol or 10 nM E2 for 24 h, and fixed chromatin fragments were immunoprecipitated with normal rabbit IgG or anti-ERα antibodies. Immunoprecipitated DNA samples were subjected to PCR with primer pairs designed to amplify regions that harbored ERE1, ERE2, or a region representing the ERα binding site shown by the Chip-Seq data (Fig. 5.6A, indicated by a black rectangle, ChIP (hereafter referred to as ERE3). This region encompasses the ERE predicted by JASPAR (JAS, Fig 5.6A). Primers that amplify known sites of ERα occupancy in the pS2 locus were also used as a positive control. In both the cell lines, none of the immunoprecipitated DNA samples yielded any PCR products with primers that were designed to amplify ERE1. In both the cell lines, 10 nM E2 treatment resulted in enrichment of ERE3 in ERα immunoprecipitated samples. ERE2 was not detected in any of the immunoprecipitated DNA samples from T47D cells. However, in MCF-7 cells, 10 nM E2 treatment appeared to enrich ERE2 in three out of four replicates in ERα immunoprecipitated samples. These results strongly indicate that 10 nM E2 enhanced ERα binding in the HOXB2 upstream region in both the cell lines (Figure 5.6B).

(A)



(B)

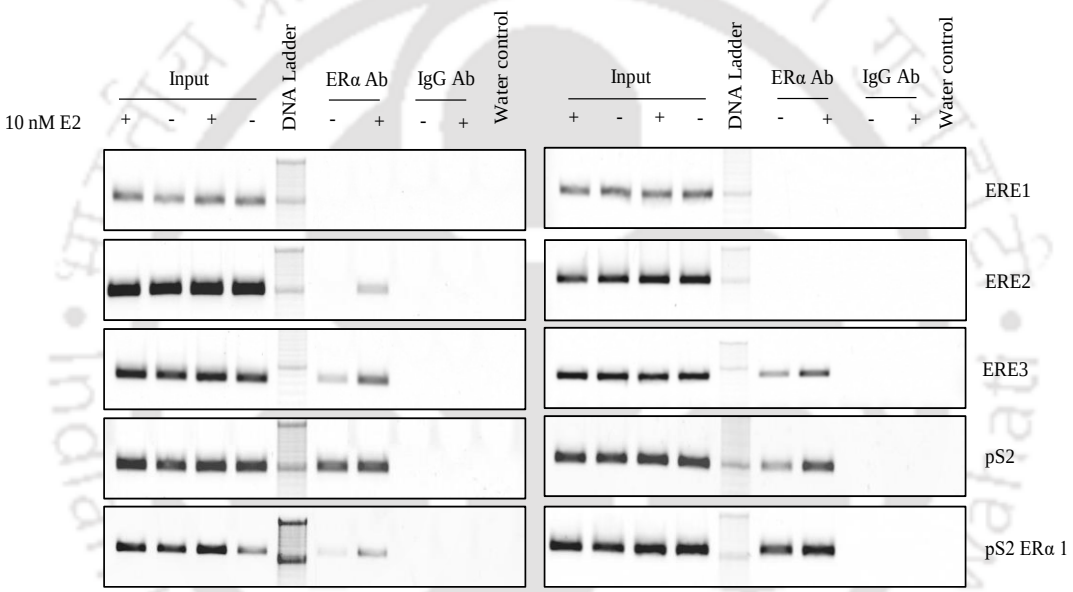


Figure. 5.6. Estrogen-induced binding of ERα in the upstream region of HOXB2. A. Schematic showing the location of EREs in the HOXB2 locus. ERE1 and ERE2 are those revealed by Matinspector. Results of ChIP-seq. analysis are shown below the HOXB2 mRNA (blue), which has a reverse orientation relative to Chr. 17 sequence. The red and blue asterisks mark the regions of enhanced ERα binding in MCF-7 and T47D cells, respectively. B. ChIP analysis of MCF-7 and T47D cells with or without estrogen treatment. Sonicated chromatin samples were immunoprecipitated with ERα-specific or normal IgG antibody (negative control) as described in materials and methods. Immunoprecipitated DNA was subjected to PCR using primers that will amplify the region shown as a black rectangle.

5.3. Discussion

The present study conclusively demonstrates the estrogen regulation of HOXB2 in human breast cancer cell lines. We show that estrogen directly suppresses HOXB2 in MCF-7 and T47D breast cancer cell lines via a mechanism that involves ERα binding to the ERE located upstream of the HOXB2 gene. The finding reinforces the increasingly appreciated role

of estrogen in the regulation of HOX genes and aligns with the proposed tumor suppressor role of HOXB2.

The estrogen-ERα signaling axis is central to estrogen-induced proliferation and differentiated functions of the breast epithelial cells. It is also involved in breast tumorigenesis and metastasis (Saha Roy and Vadlamudi, 2012). Given the importance of estrogen in the etiology of breast cancer, its target genes and their expression networks that regulate proliferation and cellular phenotype are particularly relevant. These include hormones, cytokines, growth factors and their receptors, and transcription factors and their co-regulators (Frasor et al., 2003). Development and organogenesis, on the one hand, and tumor initiation and progression on the other co-opt similar processes and molecular pathways. The idea that tumorigenesis results from the deregulation of developmental genes is widely accepted (Abate-Shen, 2002; Samuel and Naora, 2005; Bhatlekar et al., 2014). HOX genes encode transcription factors, which not only play a vital role as master regulators in development but are also instrumental in positional identity and maintenance of differentiated state during adulthood. Thus, aberrant expression of HOX genes in tumors, including those of the breast, is a result of deregulation. Interestingly, several HOX genes are regulated by estrogen in breast cancer cell lines. This study uncovers HOXB2 as a new player in the link between estrogen signaling and the HOX gene expression network.

Estrogen downmodulated HOXB2 expression in both MCF-7 and T47D cells. The dependence on ERα and its binding to the 5' upstream region upon estrogen stimulation was observed in both the cell lines. However, the effects of ERα knockdown alone in the two cell lines were different. In MCF-7 cells, ERα knockdown alone increased both mRNA and protein levels of HOXB2. In T47D cells, ERα knockdown had no effect on mRNA but significantly decreased HOXB2 protein level. Thus, in the absence of estrogen stimulation, the perturbation in ERα protein expression appears to have differential effects in the two cell lines. One possible explanation is the existence of additional cell-type-specific ligand-independent pathways of ERα-mediated regulation of HOXB2. The disparate observations are, however, independently in line with two other aspects of the ERα-HOXB2 relationship. On the one hand, the effect of ERα knockdown observed in MCF-7 is consistent with the role of ERα in

HOXB2 suppression. On the other hand, the T47D cells behave similarly to ER-negative breast tumors that express lower levels of HOXB2.

Estrogen regulates several HOX genes, and there is evidence for the involvement of ERα, modulation of promoter activity, and interaction of ERα to the upstream regulatory region. Clearly, a majority of these estrogen-regulated HOX genes are estrogen-induced. It is striking that so far, HOXB13 and HoxA10 are the only estrogen-suppressed HOX genes reported in cell culture and animal models, respectively. It is not clear whether HOXB13 and HoxA10 are regulated at the level of transcription. Our ChIP experiments clearly demonstrate the binding of ERα to the upstream region of HOXB2 following estrogen treatment. This indicates a mechanism of transcriptional suppression of HOXB2 in MCF-7 and T47D cells, a point of departure from other estrogen-regulated HOX genes.

HOX genes regulated by estrogen are proposed to be oncogenes, tumor suppressors, or tumor modulators. For example, HOXA10 is an estrogen-induced gene in the uterus, breast, and MCF-7 breast cancer cells (Chu et al., 2004; Eda Akbas et al., 2004; Martin et al., 2007). Forced expression of HOXA10 in ER-negative BT20 breast cancer cell line induces p53 expression and leads to a reduction in invasiveness. On this basis, HOXA10 is described as a tumor suppressor (Chu et al., 2004). HOXB2 expression data in other cancers and its correlation with survival are reported. In pancreatic cancer, HOXB2 expression is associated with non resectable tumors. Lack of HOXB2 expression is associated with tumor resectability, which in turn provides a significant survival advantage in patients undergoing operative resection (Segara, 2005). In lung adenocarcinoma, increased HOXB2 expression in p-stage I patients is associated with poor prognosis (Inamura et al., 2007). HOXB2 appears to have a tumor suppressor role in AML that is driven by the mutant receptor tyrosine kinase FLT3 (Lindblad et al., 2015). HOXB2 is also expressed in HPV+ cervical tumors (Gonzalez-herrera and Velazquez-velazquez, 2015). Aberrant expression of HOXB2 in breast tumors is known. However, neither its role in breast tumor development and progression nor its prognostic importance is clearly understood. Our findings from chapter 4 show that HOXB2 is a good prognostic indicator of breast cancer and is associated with better survival of breast cancer patients.

The present study is significant in that it helps in the reconciliation of divergent data on HOXB2 expression in breast cancer and perceptions about its role. Boimel and co-workers reported that knockdown of HOXB2 expression in breast cancer cell line led to increased tumor growth when injected into mammary fat pads. They described it as a tumor suppressor. Consistent with this, high-grade breast tumors show significantly lower HOXB2 expression (Boimel et al., 2011). Independently, two research groups reported higher expression of HOXB2 in malignant breast tissues compared to normal (Cantile et al., 2003; Hur et al., 2014), consistent with the pro-tumorigenic role of HOXB2. Hur and co-workers suggested that HOXB2 is a tumor suppressor and interpreted the higher expression of HOXB2 in malignant breast tissues as a counteracting response (Hur et al., 2014). Estrogen promotes the proliferation of ER-positive and estrogen-responsive breast cancer cells (Laidlaw et al., 1995). Hence, estrogen-mediated suppression of HOXB2, on the one hand, provides supporting evidence for its tumor suppressor function. On the other hand, higher expression of HOXB2 in malignant tumors could be interpreted as a loss of estrogenic control of HOXB2. This interpretation could be relevant in the context of endocrine resistance. Interestingly, perturbation of HOXB2 expression does not affect the proliferation of breast cancer cells *in vitro* (Boimel et al., 2011). Therefore, it is likely that HOXB2 suppression is not essential for the estrogen-mediated proliferation of breast cancer cells in culture.

In summary, estrogen suppresses HOXB2 expression in breast cancer cells directly in an ERα dependent manner. This work is an initial step in understanding the role of HOXB2 in breast cancer and presents a new player in the estrogen-regulated and HOX gene expression network. Since HOXB2 is a transcription factor, further studies are aimed at addressing how perturbation of HOXB2 expression leads to the alteration of estrogen-regulated gene expression. This aspect has been addressed in chapter 6 of the thesis.

CHAPTER 6

Functional link between HOXB2 and ERα in breast cancer

6.1. Introduction

Two important leads have emerged from the previous chapters. The TCGA data shows that HOXB2 and ERα are positively correlated with each other (chapter 4), and ERα knockdown affects the expression of HOXB2 in MCF-7 and T47D cell lines (chapter 5). In chapter 4, we have shown that ERα regulates HOXB2, but whether modulation of HOXB2 will affect ERα has not been addressed. Therefore, this chapter begins with the hypothesis that modulation of HOXB2 expression may affect ERα. This hypothesis is supported by the two studies, which show that some of the HOX genes regulate ERα expression in breast cancer cells (Shah et al., 2013; Zhang et al., 2013).

The results presented in the last chapter show that ERα is upstream of HOXB2. ERα binds directly to ERE in the HOXB2 locus and suppresses its transcription, and the effect is observed at both mRNA and protein levels (chapter 5). Since HOXB2 is a transcription factor, it can modulate the expression of other genes. It is envisaged that there will be a subset of estrogen-regulated genes, which will require HOXB2 as an intermediate effector. Therefore, this chapter addresses whether the presence/absence of HOXB2 will affect the expression of E2 regulated genes.

Various estrogenic or anti-estrogenic molecules regulate ERα in different cells and tissues (Osborne et al., 1980; Clarke et al., 1994). HOX genes regulate the expression of their downstream targets by binding to a short DNA sequence of four base pairs (ATTA/ATAA/TTAT/TAAT) (Desplan et al., 1988; Garcia et al., 2020). Using gel retardation assay, Guazzi, and co-workers reported that HOXB2 interacts with ACTTACTT sequence in the upstream of the Otx2 gene in the embryonal carcinoma cells (Guazzi et al., 1998). Zhang and co-workers have shown that HOXA7 overexpression induces ERα expression and cell proliferation in MCF-7 breast cancer cells (Zhang et al., 2013). Shah and coworkers have demonstrated that HOXB13 reduces ERα expression in MCF-7 and T47D cells. It suppresses ERα expression at the level of transcription (Shah et al., 2013).

In this chapter, using HOXB2 siRNA, we studied the effect of HOXB2 modulation on the expression of ERα and some of its target genes. We also checked the effect of HOXB2 on cell proliferation and the expression of various EMT markers.

6.2 Results

6.2.1. Validation of HOXB2 knockdown

Breast cancer cells were transfected with scrambled siRNA or HOXB2 specific siRNA, followed by treatment with E2 or vehicle. HOXB2 knockdown was confirmed by qRT-PCR (Figure 6.1A and B, bar 3) and western blotting (Figure 6.1C and D, lane 3) for MCF-7 and T47D cells, respectively. As expected, E2 reduces HOXB2 mRNA (Figure 6.1A and B, bar 2) and protein expression (Figure 6.1C and D, lane 2) in both the cell lines transfected with scrambled siRNA. However, in HOXB2 siRNA transfected cells, E2 treatment further reduces the HOXB2 mRNA (Figure 6.1A and B, bar 4) and protein expression (Figure 6.1C and D, lane 4). The reduction in HOXB2 mRNA and protein expression following HOXB2 siRNA treatment confirmed the HOXB2 knockdown.

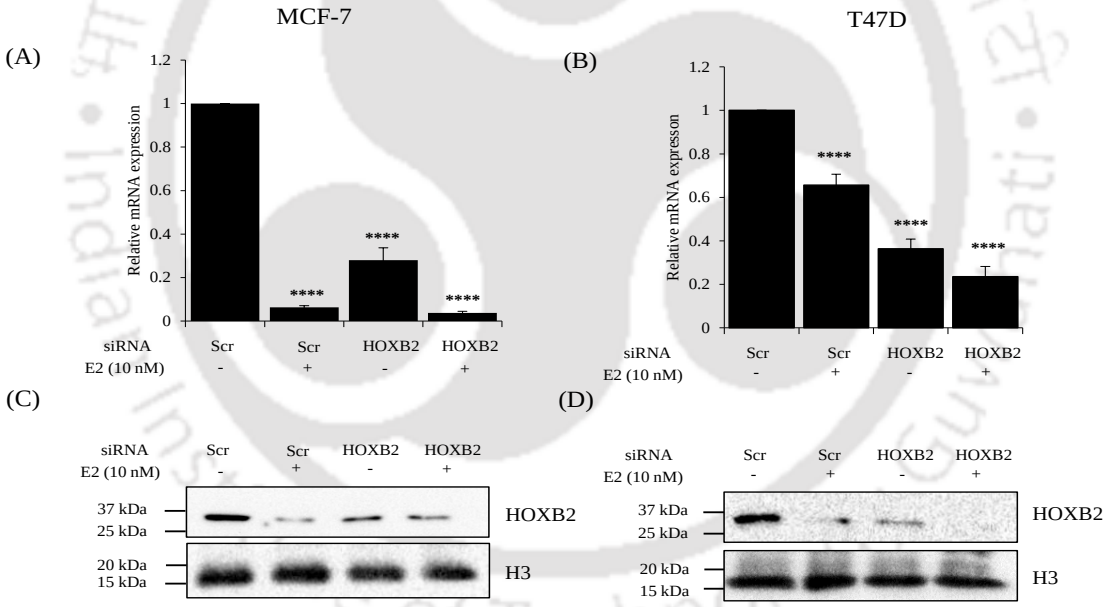


Figure 6.1. Confirmation of HOXB2 knockdown. MCF-7 and T47D cells were transfected with scrambled (Scr) or HOXB2 specific siRNA and incubated for 24 h. The cells were treated with vehicle or 10 nM E2 for the next 48 h. HOXB2 knockdown was confirmed by qRT-PCR (A and B) and Western blotting (C and D). Bars represent mean relative expression ± sd (n=7 for A and n=4 for B). The data were analyzed by ANOVA, followed by Tukey's HSD). Asterisks (*p <0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001) denotes significant results.

6.2.2. HOXB2 modulates ERα and PR protein expression in MCF-7 and T47D cells

To obtain insights into the function of HOXB2 in breast cancer cells, we tested the effect of HOXB2 knockdown on ERα. The protein samples used to test HOXB2 knockdown

(Figure 6.1C and D) were also included in this western blotting experiment, along with three other biological replicates. In both the cell lines transfected with scrambled siRNA, E2 treatment reduced ERα protein expression (Figure 6.2A and B), which is an expected observation. Interestingly, MCF-7 cells transfected with HOXB2 siRNA alone significantly reduced ERα protein expression, the extent of reduction being comparable to that after E2 treatment alone (Figure 6.2A lane and bars 2 and 3). Whereas, in T47D cells, ERα protein expression increased after HOXB2 knockdown (Figure 6.2B, lane and bar 3). We further checked the PR protein expression in these protein samples. HOXB2 knockdown alone had no significant effect on the PR protein expression. However, it caused a significant difference in fold change after E2 treatment between scrambled siRNA and HOXB2 siRNA treated cells (Fig. 6.2A and B).

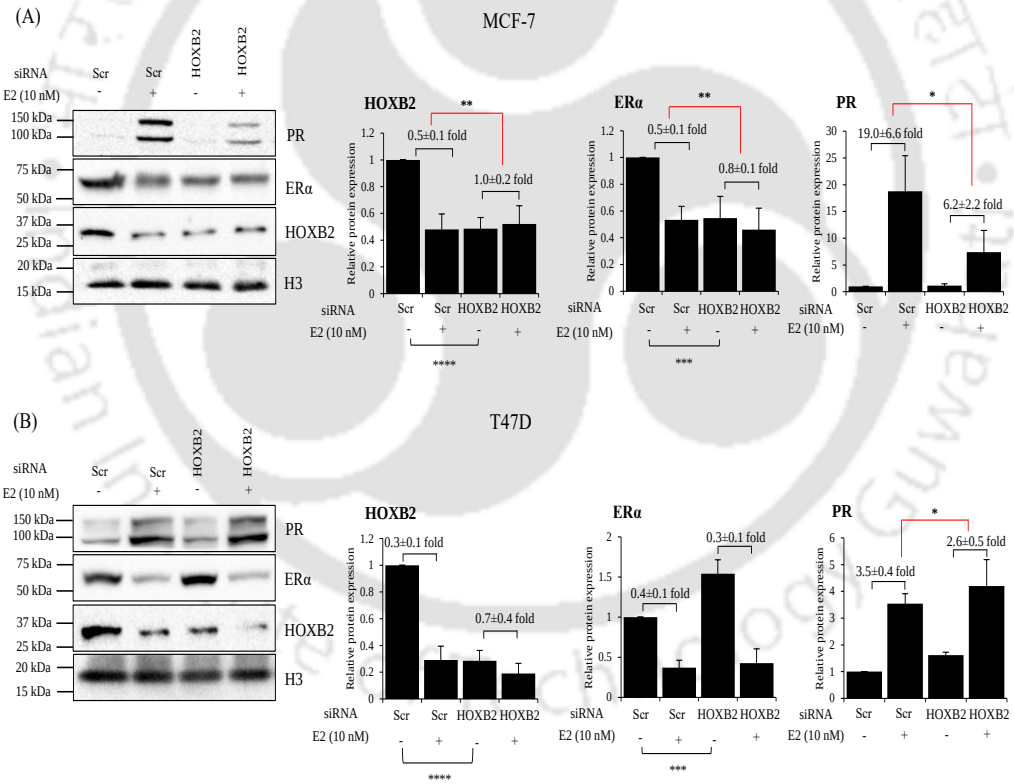


Figure 6.2. Effect of HOXB2 knockdown on ERα and PR protein expression. MCF-7 (A) and T47D (B) cells were transfected with scrambled (Scr) or HOXB2 specific siRNA and incubated for 24 h. Then, the cells were treated with vehicle or 10 nM E2 for 48 h. Effect of HOXB2 knockdown on HOXB2, ERα, and PR protein expression was accessed by western blotting. Bar Graphs on the right are the quantitative representations for the blots shown on the left. The data were normalized against histone. Bars represent mean relative expression ± sd (n=4). Data were analyzed by Welch two-sample *t*-tests (represented by asterisks above the bar graph) and ANOVA and Tukey's HSD (represented by asterisks below the bar graphs) relative to control (scrambled siRNA + vehicle-treated). Asterisks (*p <0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001) denotes significant results.

6.2.3. HOXB2 modulates the expression of ERα target genes in MCF-7 cells.

HOXB2 knockdown reduces ERα protein expression in MCF-7 cells. Therefore, we tested the influence of HOXB2 knockdown on some of the known ERα target genes such as; PR (Ing and Belen Tornesi, 1997) and pS2 (Berry et al., 1989), E2 induced genes, and CSTA, which is an E2 suppressed gene (John Mary et al., 2020). We also selected some of the E2 suppressed genes from our microarray data (Manjegowda et al., 2015), namely PCDH8 and GRAMD4. As expected, E2 induced mRNA expression of pS2 and PR and reduced CSTA, PCDH8, and GRAMD4 expression (Figure 6.3A, bar 2). HOXB2 knockdown caused a significant difference in E2-mediated fold change between scrambled siRNA and HOXB2 siRNA transfected cells for all the genes except ERα and CSTA (Figure 6.3A). Interestingly, HOXB2 knockdown alone significantly increased the mRNA expression of CSTA, PCDH8, and GRAMD4 (Figure 6.3A, bar 3). In line with PCDH8 mRNA, E2 treatment suppressed, and HOXB2 knockdown increased its protein expression (Figure 6.3B, lanes 2 and 3).

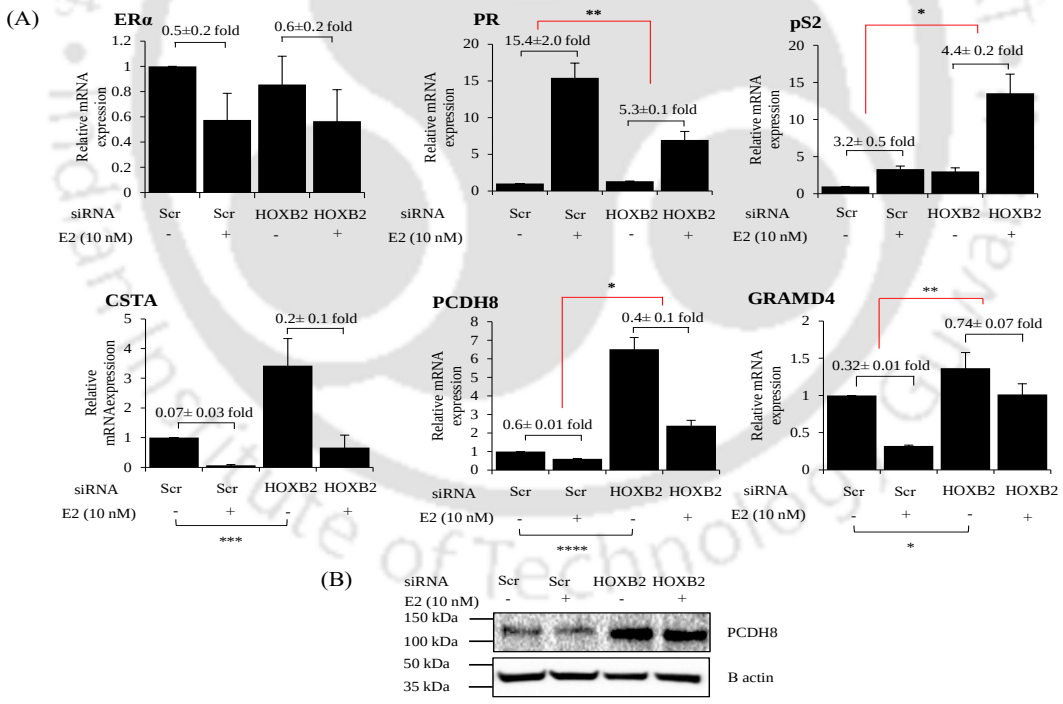


Figure 6.3. Effect of HOXB2 knockdown on ERα target genes in MCF-7 cells. MCF-7 cells were transfected with scrambled (Scr) or HOXB2 specific siRNA for 24 h. After transfection, cells were treated with vehicle or 10 nM E2 for 48 h. Effect of HOXB2 knockdown on gene expression was accessed by qRT-PCR using CycA as normalizing control (n=3), (A), and PCDH8 protein expression was accessed by western blotting (B). Data were analyzed by Welch two-sample t-test (represented by asterisks above the bar graph) and ANOVA and Tukey's HSD (denoted by asterisks below the bar graphs) relative to control (scrambled siRNA + vehicle-treated). Asterisks (*p <0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001) denotes significant results.

6.2.4. HOXB2 modulates the expression of ERα target genes in T47D cells.

HOXB2 knockdown increases ERα protein expression in T47D cells. Therefore, we tested the effect of HOXB2 knockdown on the mRNA expression of the genes shown in figure 6.3. qRT-PCR results show that E2 caused a significant difference in fold change after HOXB2 knockdown between scrambled siRNA and HOXB2 siRNA transfected cells for pS2, PR, and GRAMD4 (Figure 6.4). Like MCF-7 cells, E2 suppressed mRNA expression of PCDH8 and GRAMD4 (Figure 6.4, bar 2), and HOXB2 knockdown increased their expression (Figure 6.4, bar 3).

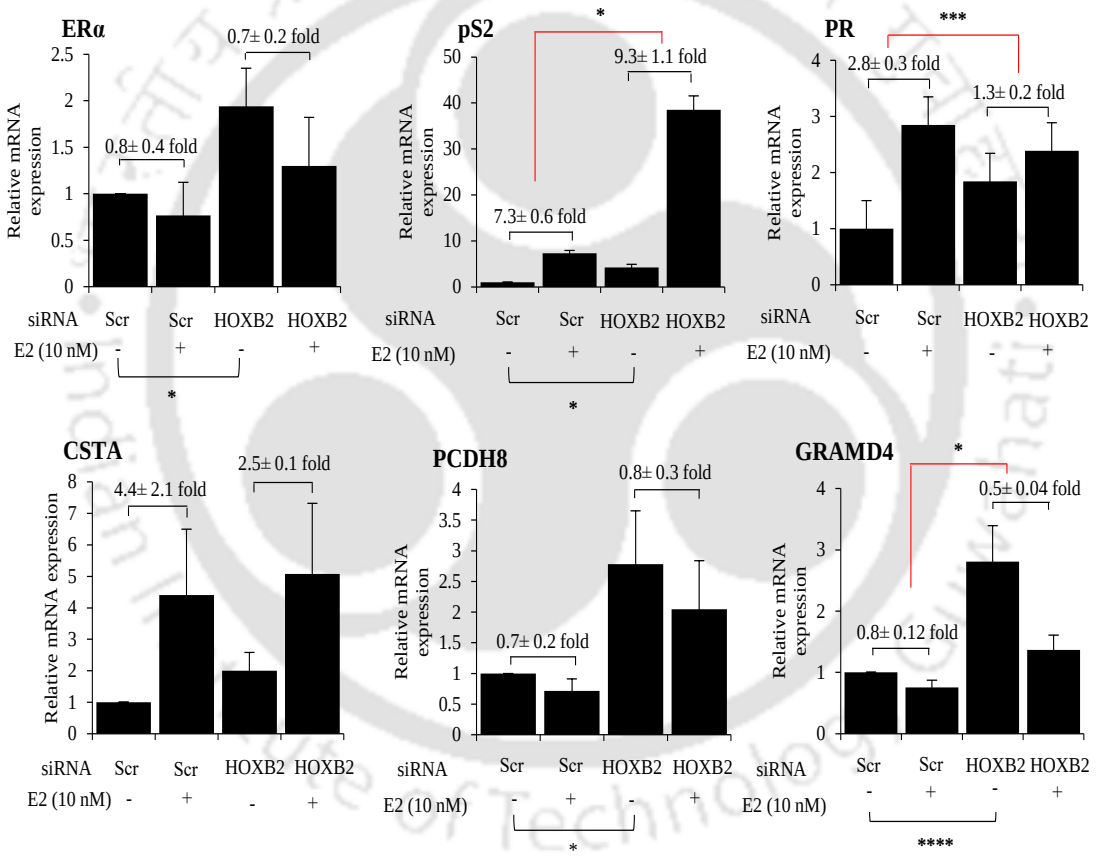


Figure 6.4. Effect of HOXB2 knockdown on ERα target genes in T47D cells. T47D cells were transfected with scrambled (Scr) or HOXB2 specific siRNA for 24 h. After transfection, cells were treated with vehicle or 10 nM E2 for 48 h. Effect of HOXB2 knockdown on ERα regulated genes was accessed by qRT-PCR using CycA as normalizing control (n=4). Data were analyzed by Welch two-sample *t*-test (represented by asterisks above the bar graph) and ANOVA and Tukey's HSD (represented by asterisks below the bar graphs) relative to control (scrambled siRNA + vehicle-treated). Asterisks (**p* <0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001) denotes significant results.

6.2.5. ERα and its target genes contain the putative HOXB2 binding sites

Our results show that HOXB2 modulates the expression of ERα, PR, pS2, CSTA, PCDH8, and GRAMD4 in MCF-7 and T47D cells. HOXB2 binds to the "ACTTACTT" sequence in the upstream region of the Otx2 gene (Guazzi et al., 1998). Therefore, we hypothesized that the HOXB2 modulated genes might also contain HOXB2 binding sites. To test this, the DNA sequence of intronic and upstream regions of these genes was extracted from the UCSC genome browser and searched for the "ACTTACTT" sequence. The region containing this particular sequence was visualized in the UCSC genome browser. The intronic region of ERα, PR, and CSTA contains a full HOXB2 binding site, whereas PCDH8 upstream contains an "ACTTACT" binding sequence with a difference in the 8th nucleotide (Figure 6.5). Further, no HOXB2 binding sites were observed on pS2 and GRAMD4.

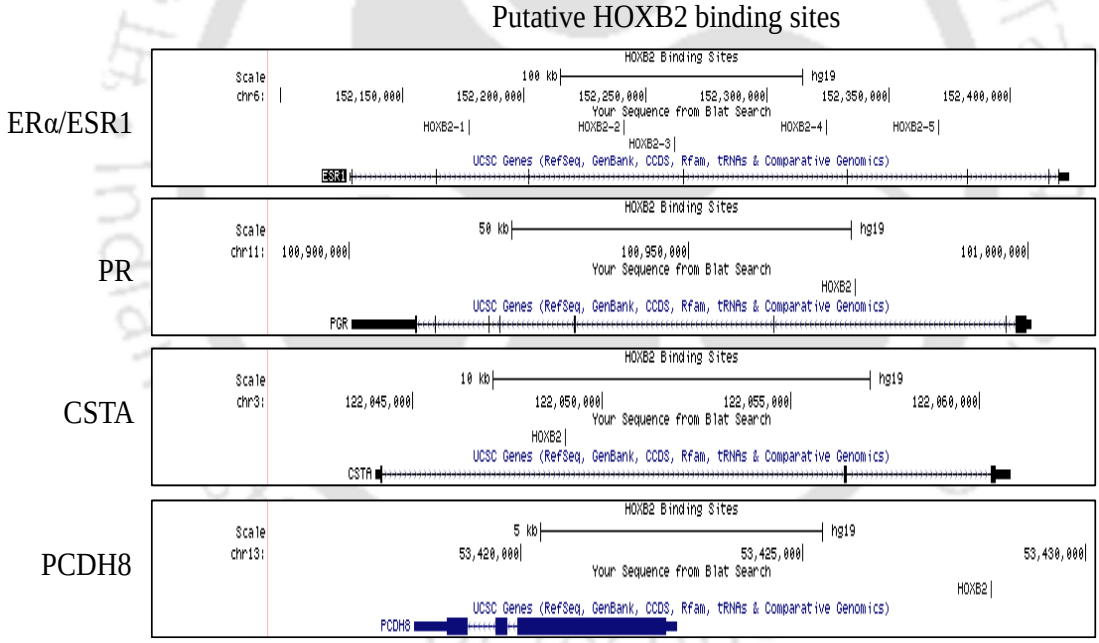


Figure 6.5. Demonstration of putative HOXB2 binding sites on ERα and its target genes. A schematic showing potential HOXB2 binding sites on ERα, PR, CSTA, and PCDH8. A minimum of 20 bp sequences can be located in the genome using the UCSC genome browser. The HOXB2 binding sequence consists of 8 bp. Therefore a total of 20 bp harboring the HOXB2 binding sites was searched in the UCSC genome browse. A vertical black line above the UCSC gene represented as HOXB2 is the putative HOXB2 binding site location.

6.2.6. Effect of HOXB2 knockdown on the cell proliferation

MTT assay was performed to investigate the effect of HOXB2 knockdown on the proliferation of MCF-7 and T47D cells. The results show more proliferation in E2 treated

MCF-7 and T47D cells (Figure 6.6, A and B, bar 2), an expected observation. HOXB2 knockdown had no significant effect on cell proliferation (Figure 6.6, A and B, bar 3). Furthermore, the HOXB2 knockdown did not alter the estrogen-induced proliferation of both the cell lines (Figure 6.6, A and B, bars 2 and 4).

6.2.7. HOXB2 knockdown modulates the expression of epithelial and mesenchymal markers.

Western blotting results show that HOXB2 reduces ERα expression in MCF-7 cells. ERα knockdown modulates the expression of EMT markers and induces EMT (Bouris et al., 2015). Therefore, to explore the role of HOXB2 in EMT, we checked the expression of EMT markers in HOXB2 siRNA transfected MCF-7 cells. Western blotting results showed that HOXB2 knockdown reduces the expression of E-cadherin and Zo-1 (epithelial markers) and induces snail and slug expression (mesenchymal markers) (Figure 6.7).

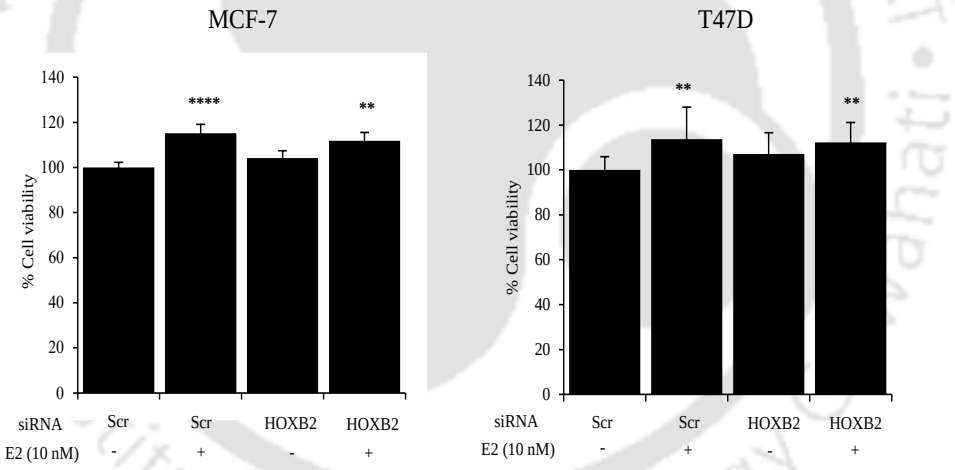


Figure 6.6. Effect of HOXB2 knockdown on cell proliferation. MCF-7 (A) and T47D cells (B) were seeded into 96-well plates, and cells were allowed to grow for 48 h. Then the cells were transfected with scrambled (Scr) or HOXB2 specific siRNA for 24 h. After transfection, cells were treated with vehicle or 10 nM E2 for 48 h. After the treatment, the cell viability was measured by MTT assay. The number of viable cells in Scr + vehicle-treated (control) cells was set to 100, and those in other treatment groups were expressed relative to control. Bars represent mean relative expression ± sd (n=9 for A and n=7 for B). Data were analyzed by ANOVA and Tukey's HSD relative to control (scrambled siRNA + vehicle-treated). Asterisks (*p <0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001) denotes significant results.

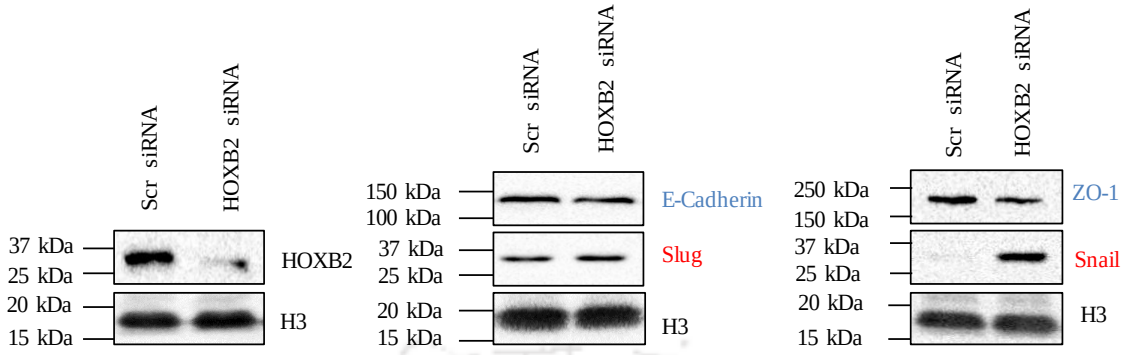


Figure 6.7. Effect of HOXB2 knockdown on the expression of EMT markers. MCF-7 cells were transfected with scrambled or HOXB2 siRNA. Total protein was isolated and subjected to western blotting. Blots were probed with antibodies specific for epithelial (represented in blue color) and mesenchymal markers (represented in red color). H3 was used as the loading control.

6.3. Discussion

The present study is motivated by the hypothesis that HOXB2 may affect ERα and the expression of its target genes. Our results demonstrate that the HOXB2 knockdown modulates ERα expression. As shown in chapter 4, ERα knockdown also affects HOXB2, suggesting both the transcription factors mutually affects each other. The effect of HOXB2 or ERα knockdown was different in both the cell lines. HOXB2 knockdown decreased and increased ERα expression in MCF-7 and T47D cell lines, respectively. In comparison, ERα knockdown increased and decreased HOXB2 expression in MCF-7 and T47D cell lines, respectively (chapter 4). John Marry and co-workers made similar observations, where ERα knockdown increased CSTA expression in MCF-7 and decreased in T47D cell lines (John Mary et al., 2017). The differential effects of HOXB2 knockdown on ERα or vice-versa may depend on the cell context and are probably affected by other molecules expressed in a cell type-specific manner.

Estrogen regulates the expression of PR, pS2, CSTA, and GRAMD4. HOXB2 knockdown also modulates the expression of these genes. These results support the hypothesis that HOXB2 affects ERα, which, in turn, affects the mRNA expression of these genes.

The HOX family consists of 39 members, and most of them recognize a core sequence consist of 4 bp (TAAT/ATTA/ TTAT/ATAA) (Desplan et al., 1988; Garcia et al., 2020). Most of the online tools that locate transcription factor binding sites also rely on this sequence.

Experimental evidence to show the binding of a HOX gene to a particular DNA sequence is limited, as most of them are related to the core HOX binding sequence. Guazzi and co-workers used gel retardation assay to demonstrate that HOXB2 interacts with a sequence of 8 bp, which is ACTTACTT in upstream of the Otx2 gene in the embryonal carcinoma cells (Guazzi et al., 1998). Therefore, to gain insights into the possible role of HOXB2 mediated regulation of ERα, we checked HOXB2 binding sites in the upstream and the intronic region of ERα and all other genes examined in this study. The results showed that ERα intronic region contains five putative HOXB2 binding sequences. Besides, PR, CSTA, and PCDH8 also contain HOXB2 binding sites. This indicates that HOXB2 may likely interact directly with/or via ERα to these loci and affect their expression. However, further experiments are needed to prove whether HOXB2 physically binds to its putative binding sites on these genes.

MTT assay results show that HOXB2 modulation did not alter the proliferation of MCF-7 and T47D cells. Therefore, the question of whether HOXB2 is a tumor suppressor of breast cancer is uncertain. Boimel and co-workers have examined the effect of HOXB2 on cell proliferation. They failed to notice any significant change in cell proliferation in the *in-vitro* conditions. They observed a reduction in breast tumor growth when HOXB2 overexpressing breast cancer cells were injected into the mammary fat pad. They interpreted that the tumor suppressor role of HOXB2 is manifested in the *in-vivo* condition and depends on the tumor microenvironment (Boimel et al., 2011).

Bouris and co-workers have reported that the silencing of ERα in MCF-7 cells leads to various phenotypic changes, modulation in gene and protein expression of various EMT markers. ERα knockdown caused complete loss of epithelial markers, induced expression of mesenchymal markers, and eventually induced the mesenchymal phenotype (Bouris et al., 2015). Our results show that HOXB2 knockdown also reduces ERα expression in MCF-7 cells. Therefore, we hypothesized that modulation in ERα expression upon HOXB2 knockdown might also affect the epithelial-mesenchymal behavior of breast cancer cells. To test our hypothesis, we checked the effect of HOXB2 knockdown on the expression of EMT markers. Western blotting results show that HOXB2 knockdown suppresses the expression of epithelial markers (E-cadherin and ZO-1) and induces mesenchymal markers (Snail and Slug). This suggests that HOXB2 may influence the invasion and metastasis of breast cancer cells.

This reinforces the idea that the tumor suppressor role of HOXB2 will be best manifested in the *in-vivo* condition, a concept that needs to be investigated in mice bearing xenografts of human tumor cell lines silenced for HOXB2. Our finding provides preliminary evidence supporting a need to explore the role of HOXB2 and ERα in EMT.

Taken together, these results show that HOXB2 alters ERα expression in MCF-7 and T47D cells, which eventually modulates the expression of its target genes examined in the study. Putative HOXB2 binding sites on ERα suggest a possible role of HOXB2 in its regulation; however, further experiments are necessary to make a conclusive statement. Our results show that HOXB2 knockdown also alters the expression of EMT markers and provides new insights to explore the connection between HOXB2 and ERα in regulating the EMT. Modulation in the expression of EMT markers suggests that HOXB2 may have a role in cellular behavior, particularly in cell-cell attachment. This is supported by the fact that we find PCDH8, a cell-cell adhesion molecule (Kim et al., 1998), is modulated by HOXB2. Estrogen suppresses HOXB2 and PCDH8, and HOXB2 manipulations also affect PCDH8 and ERα, raising an interesting question of whether ERα can regulate PCDH8. This idea is discussed in the next chapter of this thesis.

CHAPTER 7

Estrogen-mediated regulation of PCDH8

The results presented and discussed in this chapter are published in (Kumar et al., 2019), Gene Reports

7.1. Introduction

The results presented in the previous chapter show that estrogen regulates HOXB2, and manipulations in its expression, also modulate PCDH8. Therefore the present study is based on the hypothesis that estrogen may also regulate PCDH8. PCDH8 is suggested to have a tumor suppressor role in breast cancer (Yu et al., 2008). Its regulation by estrogen is not known. Therefore, investigating the estrogen-mediated regulation of PCDH8 may provide important insights.

Metastasis, a major hurdle in the successful treatment of solid tumors, is the primary cause of cancer-associated mortality (Steeg, 2006). Invasion and metastasis of breast tumors is a complex process that involves extensive remodeling of the extracellular matrix (ECM) and epithelial-mesenchymal transition (EMT) of tumor cells. Altered gene expression in tumor cells during EMT translates into a phenotypic alteration characterized by loss of cell-to-cell contact (adhesion) and migratory property (Vincent-Salomon and Thiery, 2003).

Cadherins are calcium-dependent cell adhesion molecules. They are transmembrane receptors that also enable intercellular contact and signaling via homophilic and heterophilic interactions. Members of the type-I, type-II, and type-III classes of the cadherin superfamily are expressed in the mammary tissue. Several members of these classes show altered expression in breast cancers, have prognostic value and are understood to be tumor suppressors (Andrews et al., 2011). Protocadherins constitute a distinct class of proteins within the cadherin superfamily. In sharp contrast to the other members of the cadherin superfamily, very little is known about the expression, function, and regulation of protocadherins, and their role in malignant transformation of the breast tissue (Andrews et al., 2011).

Protocadherin-8 (PCDH8) is the human homolog of paraxial protocadherin (PAPC), which plays an important role in vertebrate development. PAPC mediates somite organization, negatively influences cell migration and determines cell polarity. In humans, PCDH8 appears to have an analogous role. It prevents migration of breast epithelial cells (Yu et al., 2008). Planas-Silva and Waltz showed that estrogen induces spindle shaped morphology and migratory behaviour in a subset of MCF-7 breast cancer cells with reduced membrane

localization of E-cadherin/β catenin (Planas-Silva and Waltz, 2007). Yu and co-workers have shown that PCDH8 is frequently mutated or silenced in breast tumors, and the consequent loss of PCDH8 activity is associated with increased cell proliferation and migration (Yu et al., 2008). Silencing of PCDH8 by promoter methylation in gastric and bladder cancers is also reported (Lin et al., 2012; Zhang et al., 2012).

In this chapter, we show that estrogen reduces the expression of PCDH8 in MCF-7 and T47D breast cancer cells via a mechanism that depends on ERα expression. ERα knockdown alone appears to induce the level of PCDH8 expression in both the cell lines, which mirrors the negative association between ERα and PCDH8 mRNA expression in breast tumors within the TCGA-BRCA dataset.

7.2. Results

7.2.1. Effect of E2 on PCDH8 mRNA and protein expression

MCF-7 cells were treated with 10 nM E2 for 48 h. Analysis of qRT-PCR and western blotting showed that E2 significantly repressed PCDH8 mRNA and protein expression in MCF-7 cells (Figure 7.1A, B). In a time-course experiment with MCF-7 cells, 10 nM E2 suppressed PCDH8 mRNA (Figure 7.2A). In a dose-response experiment, a range of concentrations of E2 (0.1 to 100 nM) caused MCF-7 cells to express significantly lower levels of PCDH8 mRNA (Figure 7.2B) compared to ethanol-treated control.

7.2.2. E2 suppresses PCDH8 expression via ERα

The genomic effects of estrogen are mediated via ERα. To ascertain whether ERα is involved in estrogen regulation of PCDH8, we studied the effect of PPT on PCDH8 expression. PPT significantly suppressed the levels of PCDH8 mRNA (Figure 7.3A) and protein (Figure 7.3B)

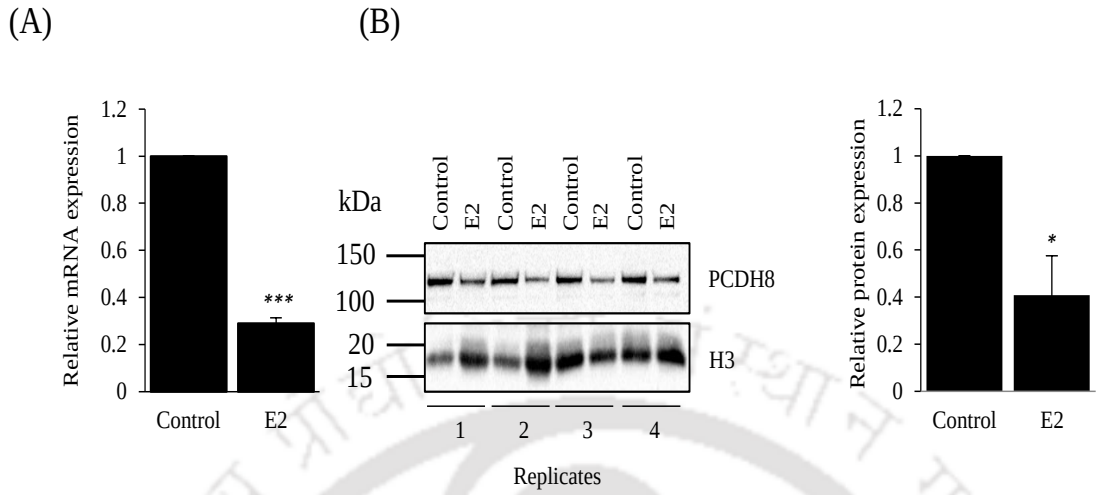


Figure 7.1. E2 reduced PCDH8 mRNA and protein expression in MCF-7 breast cancer cells. MCF-7 cells were treated with vehicle (ethanol) or 10 nM E2 for a period of 48 h. Total RNA was extracted. PCDH8 mRNA expression was analyzed by qRT-PCR using CycA as an internal control (A). PCDH8 protein expression was analyzed by western blotting. PCDH8 band intensities were normalized against Histone H3 bands, which served as an internal control. The bar graph on the right is the quantitative representation of the data shown on the left (B). The data were analyzed by Welch's two-sample *t*-test. Bars represent mean relative expression ± sd (n=3 for A and n=4 for B). Asterisks (*p <0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001) denotes significant results.

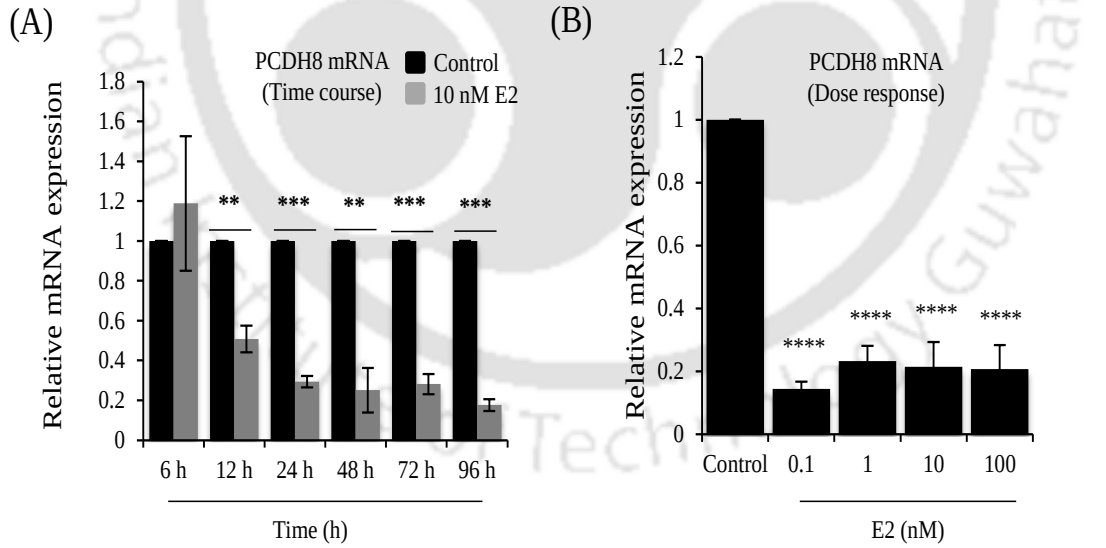


Figure 7.2. E2-mediated suppression of PCDH8 mRNA expression. A time-course study of the suppression of PCDH8 mRNA by 10 nM E2 in MCF-7 cells (n=3). Data were analyzed by Welch's two-sample *t*-test separately for each time point (A). Dose-response study of the effect of indicated concentrations of E2 on PCDH8 mRNA expression in MCF-7 cells (B). The data were analyzed by ANOVA and Tukey's HSD (n=3). Asterisks (*p <0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001) denotes significant results.

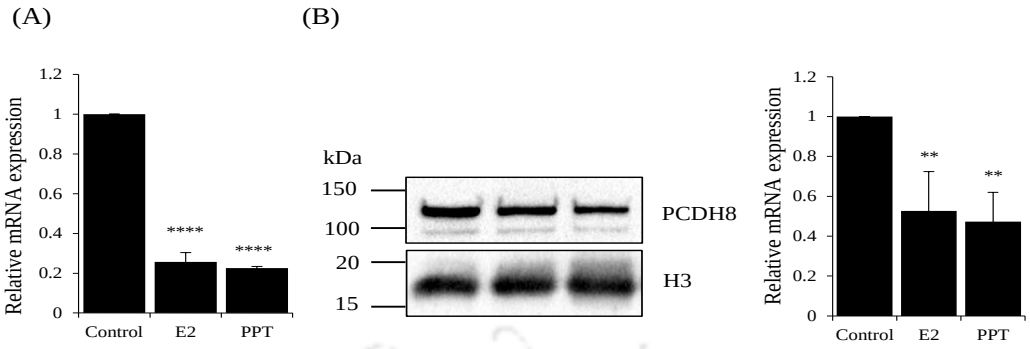


Figure 7.3. Effect of PPT on PCDH8 expression. MCF-7 cells treated with 10 nM E2 or 10 nM PPT for 48 h. Total RNA and protein were isolated and subjected to qRT-PCR (A) and western blot analysis (B). The bar graph on the right is the quantitative representation of the data shown on the left (B). The data were analyzed by ANOVA, followed by Tukey's HSD (n=3). Asterisks (*p <0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001) denotes significant results.

7.2.3. Effect of ERα knockdown on E2 mediated regulation of PCDH8

The role of ERα was also investigated in two ERα positive cell lines, namely MCF-7 and T47D, which were transfected with scrambled siRNA (Scr) or ERα specific siRNA. The ERα specific siRNA caused a reduction in ERα protein to levels beyond detection (Figure 7.4 B). PCDH8 mRNA and protein were suppressed by E2 in both cell lines treated with Scr. In Scr treated MCF-7 cells, the fold change in PCDH8 mRNA expression following E2 treatment was 0.5 ± 0.1 (Figure 7.4A left panel, n=4, P< 0.01, Welch two-sample *t*-test). In Scr treated T47D cells, the fold change in PCDH8 mRNA expression following E2 treatment was 0.4 ± 0.04 (Figure 7.4A, right panel, n=4, P <0.001, Welch two-sample *t*-test). However, in both the cell lines transfected with ERα specific siRNA, there was a significant alteration in the fold change following E2 treatment, indicating that suppression of PCDH8 mRNA expression by estrogen was dependent on ERα. The fold change in PCDH8 mRNA expression by E2 in ERα siRNA transfected MCF-7 cells was 0.8 ± 0.1, which was significantly different than 0.5 ± 0.1in Scr transfected MCF-7 cells (Figure 7.4A, left panel, n = 4, Welch two- sample *t*-test, P< 0.01). Similarly, the fold change in PCDH8 mRNA expression by E2 in ERα siRNA transfected T47D cells was 0.9 ± 0.03, which was significantly different than 0.4 ± 0.04 in Scr transfected T47D cells (Figure 7.4B, right panel, n=4, Welch two-sample *t*-test, P< 0.001). The effect of ERα siRNA on estrogen suppression of PCDH8 protein in both the cell lines was similar to that of PCDH8 mRNA (Figure 7.4B).

7.2.4. Knockdown of ERα increases PCDH8 expression

In MCF-7 cells, ERα knockdown alone caused a significant increase in PCDH8 mRNA by 5.1 ± 0.8 fold (Figure 7.4A, left panel, n=4, ANOVA, and Tukey's HSD, *P*< 0.00001). In T47D cells, ERα knockdown alone caused a significant induction of PCDH8 mRNA by 4.1± 0.98 fold (Figure 7.4A, right panel, ANOVA and Tukey's HSD, **P< 0.01). The pattern of PCDH8 protein expression in these experiments was similar to that of PCDH8 mRNA. In MCF-7 cells, ERα knockdown alone caused a significant increase in PCDH8 protein by 1.7± 0.4 fold (Figure 7.4A, left panel, n=4, ANOVA, and Tukey's HSD, *P*< 0.05). In T47D cells, ERα knockdown alone caused a significant induction of PCDH8 protein by 1.9± 0.5 fold (Figure 7.4B, right panel, ANOVA and Tukey's HSD, **P< 0.01).

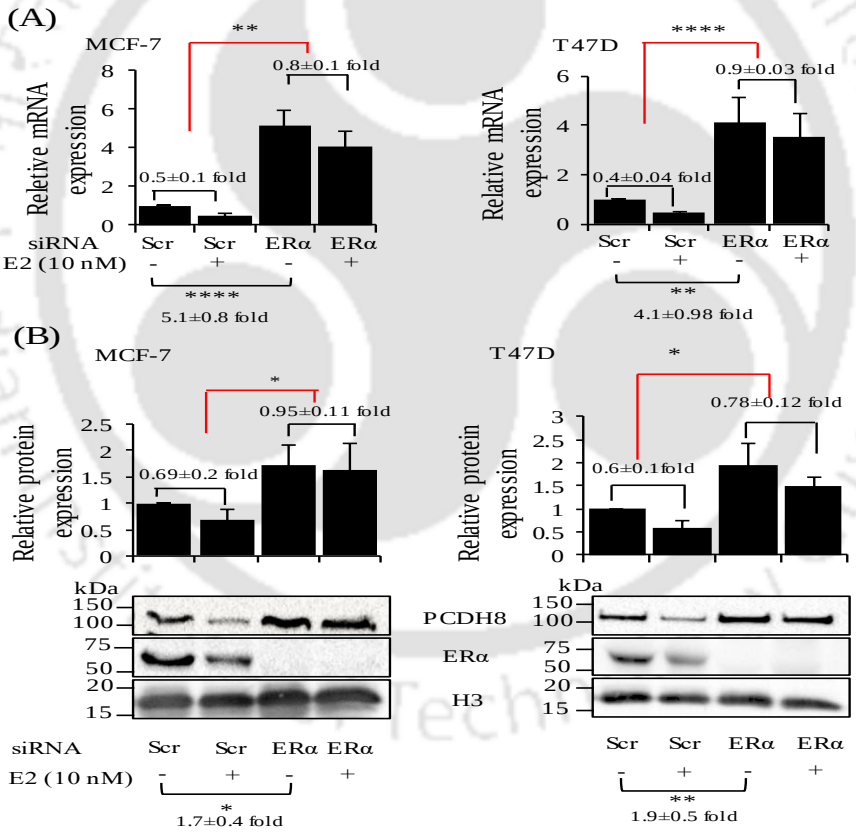


Figure 7.4. Effect of ERα knockdown on estrogen-mediated suppression of PCDH8. MCF-7 and T47D cells were transfected with scrambled (Scr) or ERα siRNA for 24 h. The cells were treated with ethanol or 10 nM E2 for 48 h. Total RNA and protein was isolated, and PCDH8 expression relative to control (scrambled siRNA + vehicle-treated) was determined by qRT-PCR (A) and western blotting (B) using CycA and histone H3 as the internal control. Data were analyzed by ANOVA followed by Tukey's HSD (indicated by asterisks below the bar graphs) and Welch two-sample *t*-tests (indicated by asterisks above the bar graphs). Asterisks (*p <0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001) denotes significant results.

7.2.5. Analysis of PCDH8 upstream by Matinspector and Chip-Seq and ChIP assay

E2 reduced PCDH8 expression within 12 h of treatment. This directed us to believe that ERα may interact with the PCDH8 promoter and hence suppress its mRNA. Therefore, we analyzed the PCDH8 upstream 2kb sequence to locate potential ERα binding sites. Matinspector predicted one putative ERE upstream 2kb of PCDH8. However, Chip did not show any peak in the same locus (Figure 7.5A). We carried out ChIP experiment to demonstrate ERα occupancy on the PCDH8 locus. The increased ERα occupancy in the known ERα binding sites in the pS2 locus validated our ChIP protocol. The immunoprecipitated DNA did not show ERα enrichment in the ERE located upstream of PCDH8. This suggests that E2 mediated regulation of PCDH8 may not be associated with ERα binding on the PCDH8 locus (Figure 7.5B).

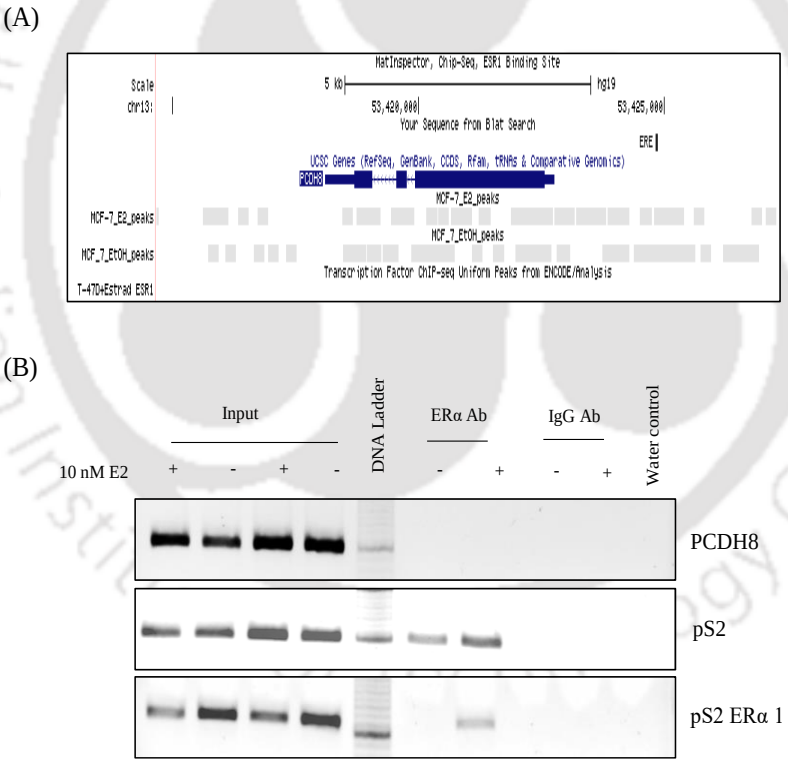


Figure 7.5. Demonstration of a putative ERE and ERα binding in the upstream of PCDH8. Schematic showing the location of ERE upstream of PCDH8 locus, revealed by Matinspector (A). ChIP analysis of MCF-7 cells with or without estrogen treatment. Sonicated chromatin samples were immunoprecipitated with ERα-specific or normal IgG antibody (negative control) described in materials and methods. Immunoprecipitated DNA was subjected to PCR using primers that amplify pS2 and the PCDH8-ERE region (B).

7.2.6. Analysis of PCDH8 expression in breast tumors

We analyzed PCDH8 mRNA expression data from 1215 breast tissue samples within the TCGA-BRCA dataset. We found that the mean PCDH8 mRNA expression in tumors was significantly lower than that in normal breast tissues (Figure 7.6A). The primary tumors expressed significantly higher levels of ERα mRNA compared to normal breast tissues (Figure 7.6B). Based on immunohistochemical data, the primary breast tumors were divided into ERα-positive and ERα-negative groups. ERα-negative tumors expressed significantly higher levels of PCDH8 mRNA than ERα-positive tumors (Fig. 7.6C).

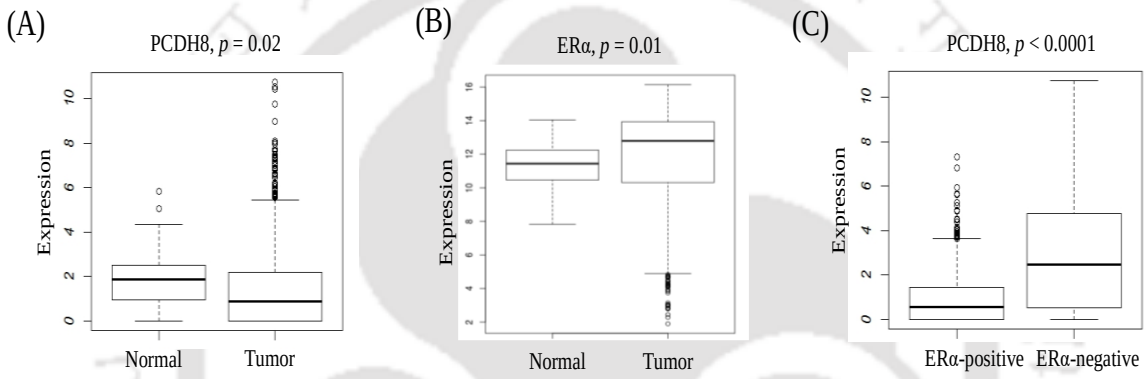


Figure 7.6. Analysis of PCDH8 expression in breast tumors within the TCGA-BRCA dataset. Box plot showing expression of PCDH8 in normal breast tissues (n=113) *versus* breast tumors (n=1102), (A). Box plot showing expression of ERα mRNA in normal breast tissues (n=113) *versus* breast tumors (n=1102), (B). Boxplot showing the distribution of PCDH8 mRNA expression in ERα-positive (n=603) *versus* ERα-negative tumors (n=179), (C). The data were analyzed by Welch's two-sample *t*-tests.

7.3. Discussion

The present study is motivated by two factors; estrogen regulates HOXB2, and modulation in its expression also influences PCDH8. This chapter was driven by the factor to validate the hypothesis that estrogen may also regulate PCDH8, for which the supporting evidence comes from two sources, estrogen-treated cells show low levels of PCDH8, and a microarray study conducted in our lab shows repression in PCDH8 expression in E2 treated MCF-7 cells (Manjegowda et al., 2015).

Estrogen regulation of PCDH8 in breast cancer cell lines, as reported here, is an important insight. Silencing of tumor suppressor genes is well-known. PCDH8 is frequently mutated or silenced in breast, gastric, and bladder cancers (Lin et al., 2012; Yu et al., 2008;

Zhang et al., 2012). Increased cell proliferation and migration upon loss of PCDH8 expression suggest a tumor suppressor role for this protocadherin. Estrogen produces mitogenic responses in breast epithelial cells, and enhanced estrogen signaling causes breast cancer. E2-mediated suppression of PCDH8 expression in MCF-7 and T47D cells is consistent with its tumor suppressor function. The suppression of PCDH8 by estrogen may be one of the key molecular alterations necessary for enhanced proliferation.

Results represented in this chapter show that suppression of PCDH8 expression by estrogen depends on the expression of ERα. Interestingly, the knockdown of ERα expression alone caused a significant increase in PCDH8 expression. This observation mirrors the differential expression of PCDH8 mRNA in ERα positive and ERα negative breast tumors. Consistent with the tumor suppressor role of PCDH8 (Yu et al., 2008), the mean PCDH8 mRNA expression in tumors was lower than that in the normal breast tissues. In view of this observation, the fact that tumors expressed significantly higher levels of ERα than normal breast tissues reiterates the inverse relationship between PCDH8 and ERα.

Loss of cell-cell contact and the ability to migrate is important for the invasion and metastasis of solid tumors. In the context of breast cancer metastasis, loss of cell-cell contact due to loss of E-cadherin expression in tumors lacking ER is known (Fujita et al., 2003). However, ER-positive breast tumors can also metastasize (Weigelt et al., 2005). Molecular alterations leading to loss of cell-cell contact in ER-positive breast tumors is not completely understood. Our results suggest that besides reduced membrane localization of E-cadherin/β catenin (Planas-Silva and Waltz, 2007), estrogen-mediated suppression of PCDH8 could be another molecular event towards spindle-shaped morphology that enables invasion and metastasis of ERα positive tumors

In the present study, we attempted to obtain mechanistic insights into estrogen-mediated suppression of PCDH8. Given that significant suppression of PCDH8 mRNA was observed at 12 h post E2 treatment, it is possible that PCDH8 was regulated directly via ERα at the level of transcription. We examined the PCDH8 locus for potential estrogen response elements (EREs). The Matinspector tool (Quandt et al., 1995) predicted one ERE in the upstream region of PCDH8 mRNA. We also attempted to analyze ERα occupancy in the predicted ERE by chromatin immunoprecipitation (ChIP) assays. However, our results were

not conclusive. Chip-Seq data of chromatin fragments immunoprecipitated with ERα antibody is available for MCF-7 and T47D cells. Analysis of the data using Galaxy (Afgan et al., 2018) followed by visualization in the UCSC Genome Browser (Kent et al., 2002) did not show any peaks of ERα occupancy in the PCDH8 locus. Hence, suppression of PCDH8 by E2 is not associated with a change in ERα occupancy in the PCDH8 locus. Hence, it is likely that estrogen regulates PCDH8 via an indirect mechanism that depends on events downstream of ERα.

The results presented in chapter 6 show that PCDH8 upstream contains a putative HOXB2 binding site (chapter 6, Figure 6.5). Since HOXB2 is a downstream target of ERα (chapter 5), it may mediate the estrogen regulation of PCDH8. Although this chapter validates our hypothesis of estrogen regulating PCDH8, further experiments are necessary to show whether HOXB2 regulates PCDH8 via direct interaction of HOXB2 with PCDH8 promoter or whether, following E2 treatment, HOXB2 engages with PCDH8 promoter through interaction with or upregulation of other factors.

In summary, estrogen causes suppression of PCDH8 expression via ERα in MCF-7 and T47D breast cancer cells. The knockdown of ERα alone induces the expression of PCDH8 in these breast cancer cell lines, which mirrors the differential expression of PCDH8 in ERα-positive and ERα-negative breast tumors of the TCGA-BRCA dataset.

CHAPTER 8

Conclusion

Conclusion

HOX genes are homeodomain-containing transcription factors. They play a pivotal role in the segmentation of developing embryos. HOX genes were initially studied to understand their pattern formation roles and determining the body shape during embryogenesis. Recent investigations have shown that HOX genes are also involved in cancer. These genes are either upregulated or downregulated in different tumors, and their aberrant expression is associated with tumor progression and development (Brotto et al., 2020).

HOXB2 is a member of the HOXB cluster of the homeobox family. It is involved in various developmental processes, such as forming skeletal and facial structures, developing the hindbrain, and erythropoiesis (Barrow and Capecchi, 1996; Davenne et al., 1999). Like other cancers, HOXB2 is also overtly expressed in breast cancer. The literature on the role of HOXB2 in normal breast physiology and breast cancer pathophysiology is meager. On the one hand, HOXB2 expresses at high levels in ER-positive breast tumors (Neve et al., 2006), and on the other hand, HOXB2 is a tumor suppressor of breast cancer (Boimel et al., 2011). Prognostic implications of various tumor-suppressing or tumor-promoting HOX genes are reported (Eoh et al., 2017b). However, the precise role of HOXB2 on the prognosis of breast cancer is not well understood. This study addresses the prognostic utility of HOXB2 in breast cancer.

The Kaplan-Meier survival analysis revealed that high HOXB2 expression was correlated with the prolonged OS, RFS, and PPS of breast cancer patients. Besides, high HOXB2 expression in ER expressing tumors was correlated with prolonged RFS and PPS, indicating that HOXB2 may affect the survival of ER-positive breast cancer patients. TCGA data yielded high HOXB2 expression in primary breast tumors. Similar observations were made by two research groups (Cantile et al., 2003; Hur et al., 2014). HOXB2 is a tumor suppressor, and its high expression correlates with prolonged survival, suggesting that HOXB2 may affect the pathways involved in cell survival. Interestingly, TCGA data analysis revealed high HOXB2 expression in ERα-positive breast tumors than ERα-negative ones. A positive correlation was observed between HOXB2 and ERα in normal breast tissue and primary breast tumors. The direct relationship between them is also reflected from their high

expression in luminal A and luminal B breast cancer subtypes compared to the others. These results provided evidence of a functional link between HOXB2 and ERα and offered a rationale for investigating the estrogen-mediated regulation of HOXB2.

This study unveils the essential role of ERα in estrogen-mediated suppression of HOXB2 expression in breast cancer cells. *In-vitro* experiments showed that estrogen negatively regulates HOXB2 in MCF-7 and T47D breast cancer cell lines. Treatment with PPT and ERα knockdown revealed that ERα is essential for estrogen-mediated suppression of HOXB2. Analysis of ChIP seq data and our ChIP experiments showed that ERα binds directly to the ERE located at the 5' upstream of HOXB2. There was more occupancy of ERα in this particular locus in E2 treated cells. Taken together, this study revealed that estrogen suppresses HOXB2 expression at the transcription level. HOXB2 is a transcription factor and is also a potential tumor suppressor of breast cancer. Therefore, future investigations in our lab will focus on finding global HOXB2 binding sites to explore the significance of HOXB2 in breast cancer.

TCGA data analysis and ERα knockdown experiments provided two important leads. TCGA data analysis revealed that both HOXB2 and ERα are positively correlated. ERα knockdown modulated HOXB2 expression in MCF-7 and T47D breast cancer cell lines. Investigating that modulation in the expression of one transcription factor will affect the other produced interesting results. Not only ERα affects HOXB2 expression, but HOXB2 knockdown also modulated ERα expression in both the cell lines. HOXB2 knockdown also altered the expression of various ERα targets examined in the study. Further, estrogen-mediated regulation of these genes was also affected after HOXB2 knockdown. HOXB2 is a downstream target of ERα and may be an essential mediator of estrogen signaling. HOXB2 knockdown did not alter cell proliferation. Modulation in the expression of EMT markers after ERα knockdown is known (Bouris et al., 2015). Our results showed that HOXB2 knockdown reduces ERα protein expression in MCF-7 cells. Interestingly, HOXB2 knockdown decreased the expression of epithelial markers and increased the expression of mesenchymal markers. These results suggest that HOXB2 may have a role in EMT and can affect breast tumors' invasion and metastasis. Therefore, future investigations may address the role of HOXB2 in EMT mediated by ERα.

HOXB2 knockdown modulates ERα and the expression of various estrogen-regulated genes such as pS2, PR, CSTA, GRAMD4, and PCDH8. Estrogen suppresses HOXB2, and HOXB2 knockdown increased PCDH8 expression. Investigating the estrogen-mediated regulation of PCDH8 revealed that estrogen causes suppression of PCDH8 expression via ERα in MCF-7 and T47D breast cancer cells. This study needs further experiments to explore the role of HOXB2 in PCDH8 regulation.

Taken together, the present study offers novel insights into estrogen regulation of HOXB2 in breast cancer cells. To the best of our knowledge, this is the first study that provides detailed molecular insights into ERα mediated regulation of HOXB2. Survival analysis supports the so purported tumor suppressor function of HOXB2. Further, our results revealed that both HOXB2 and ERα are functionally connected where one's expression affects the other. Here we identified, PCDH8, a novel ERα regulated gene through a search for HOXB2 binding sites in ERα and in a few of its target genes. Our results suggest that HOXB2 may be a mediator in estrogen-mediated gene regulation.

This work provides key valuable insights into the role of HOXB2 in breast cancer and presents HOXB2 as a potential player in the estrogen-ERα and HOX gene expression network. Since HOXB2 is a transcription factor, future investigations should address the mechanism that, how perturbation of HOXB2 expression leads to alteration of estrogen-driven pathophysiology of breast cancer cells. Further identification of genes regulated by HOXB2 using the high throughput technique may unveil the novel signaling pathways that can be potential therapeutic targets.

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APPENDIX

Appendix I-Supplementary Information

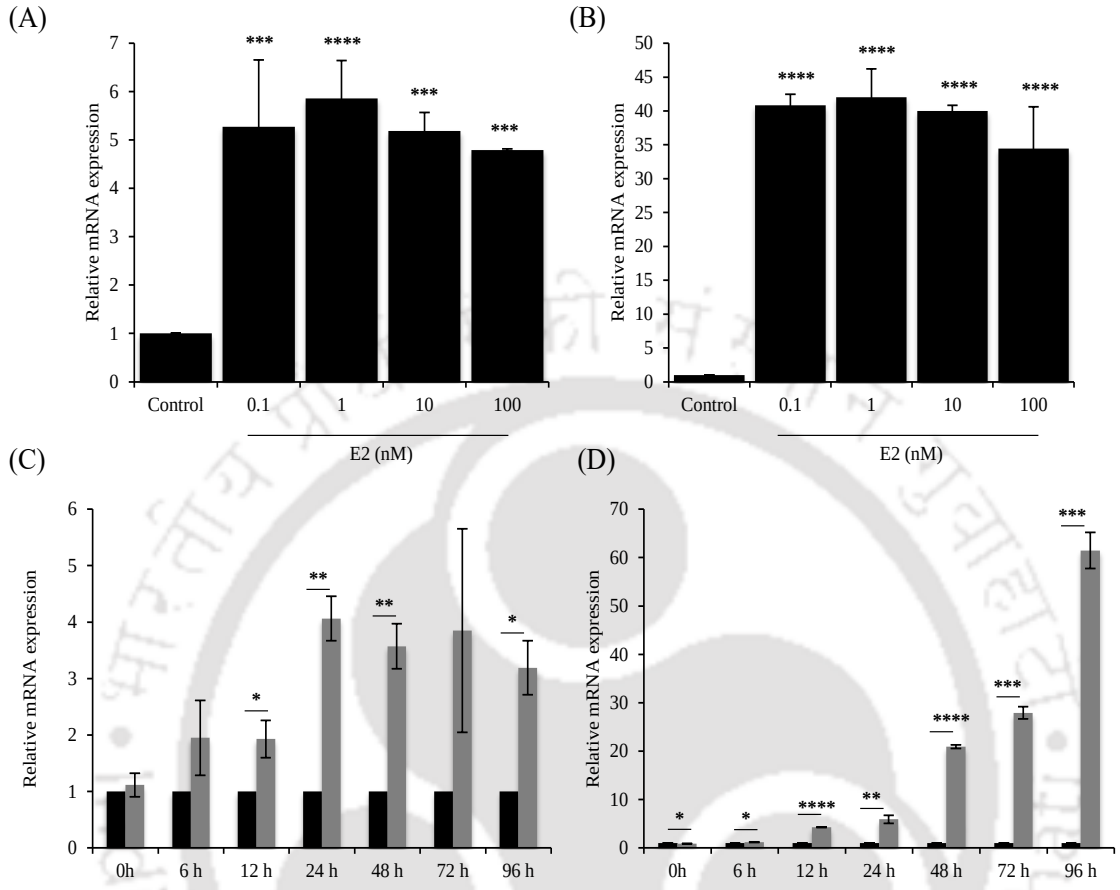
Table S: List of primers used in this study

S. No	Gene Name	Primer sequence (5'----------3')	Amplicon size (bp)	Annealing temperature (°C)	Remarks
1	Cyclophilin A	Forward- GGGCCGCGTCTCCTTTGAGC Reverse- GGCGTGTGAAGTCACCACCC	158	60	Used in qRT-PCR
2	HOXB2	Forward- CCAGGATTCACCTTTCCTTCC Reverse- GCACAGAGCGTACTGGTGAA	156	60	Used in qRT-PCR using MCF-7 cells cDNA
3	HOXB2	Forward- CCAAGAAACCCAGCCAATCC Reverse- TTCGGTGAGGTCCAGCAAGG	242	60	Used in qRT-PCR using T47D cells cDNA
4	TFF1	Forward- AATGGCCACCATGGAGAACA Reverse- ATAGAAGCACCAGGGGACCC	211	60	Used in qRT-PCR
5	PCDH8	Forward- ACAGCGATTCCGACATCAGC Reverse- AGGCAGTGACGTGCTCTTAC	206	60	Used in qRT-PCR

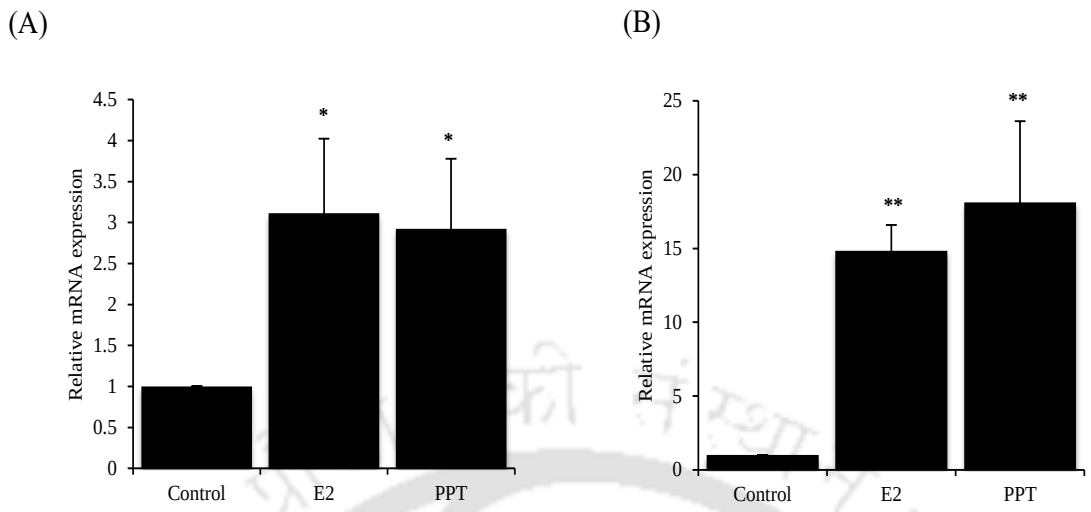
6	GRAMD4	Forward- TGCCAAGCCGGTCACTAACT Reverse- GGCGATGAGGTAATTGAGGG	175	60	Used in qRT-PCR
7	PR	Forward- CGCGCTCTACCCTGCACTC Reverse- TGAATCCGGCCTCAGGTAGTT	121	60	Used in qRT-PCR
8	CSTA	Forward- ATCTGAGGCCAAACCCGCC Reverse- AGCCCGTCAGCTCGTCATC	275	60	Used in qRT-PCR
9	ERα/ESR1	Forward- GCCCTACTACCTGGAGAA Reverse- CCCTTGTCATTGGTACTGG	132	60	Used in qRT-PCR
10	TFF1-A	Forward- TCCCCCTGCAAGGTCACGGT Reverse- TTTGCCTAAGGAGGCCCGGG	350	58	Used in ChIP (+ve control, Shang et al., 2000)
11	TFF1-B	Forward- CATTGCCTCCTCTCTGCTCC Reverse- ACTGTTGTCACGGCCAAGCC	423	58	Used in ChIP (+ve control from Chip Seq. analysis)
12	HOXB2-ERE1	Forward- CATGGAAGCCTGAAAGTGAGG	368	58	Used in ChIP

		Reverse- GCCAGCAGCATCCCCTTC			Harboring HOXB2 ERE1 locus in the genome
13	HOXB2-ERE2	Forward- GCTCAGAGTCTTGTGCCCTC Reverse- AGAACTGAGGTAGCCATTGG	355	58	Used in ChIP Harboring HOXB2 ERE2 locus in the genome
14	HOXB2-ERE3	Forward- GAGGGCTCAGAACAGAGAGA Reverse- ACAGTCTGCCCGAGTTGCCT	279	58	Used in ChIP Harboring HOXB2 ERE3 locus in the genome
15	PCDH8-ERE	Forward- CTACACAGGAGGCTGAGGCA Reverse- GTCACTCTGGGAGACCTGCT	288	58	Used in ChIP Harboring PCDH8-ERE locus in the genome

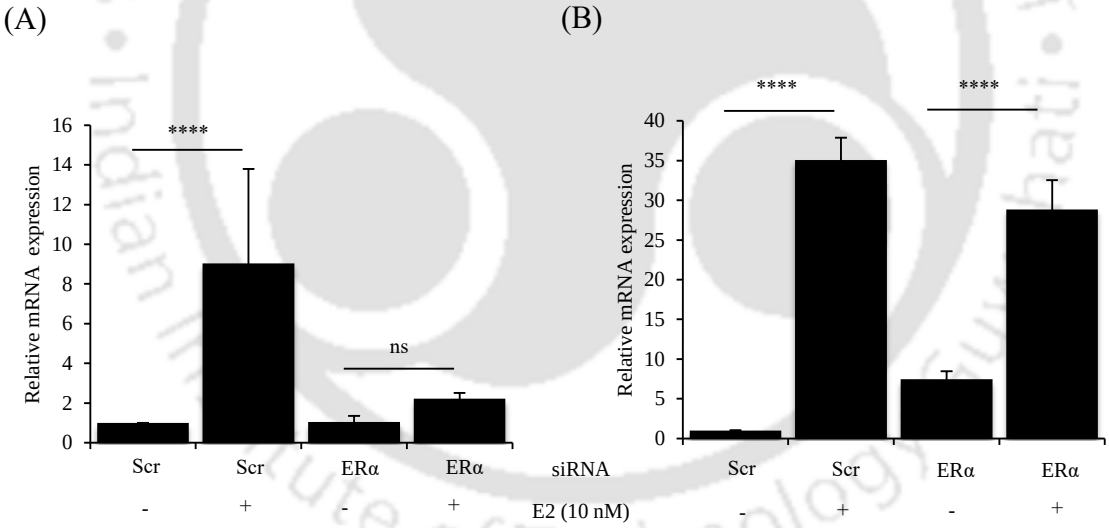
Appendix I-Supplementary Information



Supplementary figure 5.1. Dose and time-dependent effect of E2 on pS2 mRNA expression. MCF-7 (A) and T47D cells (B) were treated with indicated concentrations of E2 for 72 h. After total RNA isolation and cDNA synthesis, gene expression was accessed by qRT-PCR using CycA as a normalizing control. The data were analyzed by ANOVA and Tukey's HSD (n=3). Time course effect of E2 on pS2 mRNA in MCF-7 (C) and T47D (D) cells was analyzed. Both the cell lines were treated with vehicle (black bars) or 10 nM E2 (grey bars) for indicated time periods. Gene expression was analyzed by qRT-PCR using CycA as a normalizing control. Modulation in pS2 mRNA in E2 treated cells was expressed relative to control. Data were analyzed by Welch's two-sample *t*-test separately for each time point. Bars represent mean relative expression ± sd (n=3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.



Supplementary figure 5.2. Modulation in pS2 mRNA by E2 and PPT. MCF-7 (A) and T47D cells (B) were treated with 10 nM E2 or 10 nM PPT for 48 h. Total RNA was extracted, and gene expression was analyzed by qRT-PCR using Cyc A as normalizing control. Bars represent mean relative expression ± sd (n=3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.



Supplementary figure 5.3. Effect of ERα knockdown on pS2 mRNA expression. MCF-7 (A) and T47D cells (B) were transfected with scrambled siRNA (Scr) or ERα specific siRNA and incubated for 24 h. Then, the cells were treated with vehicle or 10 nM E2 for 48 h. The cells were harvested, and total RNA was extracted. The expression of pS2 mRNA was analyzed by qRT-PCR using CycA as an internal control. The expression of pS2 mRNA in Scr + ethanol (control) treated cells was assigned a value of 1, and those obtained for other treatment groups were expressed relative to control. Bars represent mean relative expression ± sd (n=6 for A and n=3 for B). The data were analyzed by ANOVA followed by Tukey's HSD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

Appendix II

List of publications

Publication from the thesis work

Ajay Kumar, Arun Dhillon, Mohan Chowdenahalli Manjegowda, Dixcy Jaba Sheeba John Mary, Neha Singh, Sachin Kumar, Deepak Modi, Anil Mukund Limaye. Estrogen suppresses HOXB2 expression via ERα in breast cancer cells. Gene (2021) 794, 145746. https://doi.org/10.1016/j.gene.2021.145746.

Ajay Kumar, Mohan C. Manjegowda, Dixcy Jaba Sheeba John Mary, Uttariya Pal, Sachin Kumar, and Anil Mukund Limaye Estrogen receptor- α is a determinant of protocadherin-8 expression in breast cancer cells. Gene Reports (2018) 14, 6–11.

Publications outside thesis work

Dixcy Jaba Sheeba John Mary, Girija Sikarwar, **Ajay Kumar**, Anil Mukund Limaye. Interplay of ERα binding and DNA methylation in the intron-2 determines the expression and estrogen regulation of Cystatin A in breast cancer cells. Molecular and Cellular Endocrinology (2020) 504:110701.

Dixcy Jaba Sheeba John Mary, Mohan C Manjegowda, **Ajay Kumar**, Sarbajeet Dutta, Anil Mukund Limaye. The role of cystatin A in breast cancer and its functional link with ERα. Journal of Genetics and Genomics (2017) 44(12):593-597.

Appendix III

List of presentation

Platform presentation

Ajay Kumar, Mohan C Manjegowda, Dixcy Jaba Sheeba John Mary, Sachin Kumar, and Anil M Limaye. Homeobox transcription factor HOXB2 mRNA is an estrogen target in MCF-7 breast cancer cells. Young Scientist presentation at International conference on molecular signaling; Recent trends in biosciences (20^{th}- 22^{nd} November 2015) at North-Eastern Hill University, Shillong.

Poster presentations

Ajay Kumar, Mohan C Manjegowda, Dixcy Jaba Sheeba John Mary, Sachin Kumar, and Anil M. Limaye. Regulation of calcium-dependent cell adhesion protein PCDH8 by estrogen in breast cancer. Poster presentation at 3^{rd} International meet on advanced studies in cell signaling network (18^{th}-20^{th} December 2016) at CSIR-Indian Institute of chemical biology, Kolkata.

Ajay Kumar, Mohan C Manjegowda, Dixcy Jaba Sheeba John Mary JM, Sachin Kumar, and Anil M. Limaye. Estrogen mediated proliferation of ER-alpha positive breast cancer cells is associated with the down-modulation of GRAMD4. Poster presentation at 3^{rd} International conference on perspectives of cell signaling and molecular medicine (8^{th}-10^{th} January 2017) at Bose Institute, Kolkata.

Ajay Kumar, Mohan C Manjegowda, Dixcy Jaba Sheeba John Mary, Sachin Kumar, and Anil M. Limaye. 17β-Estradiol suppresses PCDH8 expression in estrogen receptor-positive breast cancer cells via-ERα. Poster presentation at 86^{th} International conference of the society of biological chemists (16^{th}-19^{th} November 2017) at Jawaharlal Nehru University, New Delhi.

Ajay Kumar, Mohan C Manjegowda, Dixcy Jaba Sheeba John Mary, Sachin Kumar, and Anil M Limaye. Regulation of HOXB2 by estrogen in breast cancer. Poster presentation at National conference on recent developments in Medical Biotechnology and structure-based drug designing (6^{th}-7^{th} December 2015) at the Department of Biosciences and Bioengineering, IIT Guwahati

Appendix IV

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