MIXED LINEAGE LEUKEMIA HISTONE METHYLASES IN HORMONAL REGULATION OF HOX GENES AND THEIR DISRUPTION BY ENDOCRINE DISRUPTING CHEMICALS

by

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ABSTRACT

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The University of Texas at Arlington, 2012

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Homeobox (HOX) genes are highly conserved family of genes that play vital roles during cell differentiation and embryogenesis. Of the 39 HOX genes present in human, HOXC6 is a critical players in mammary gland development and milk production. I demonstrated that HOXC6 is transcriptionally regulated by estrogen (E2) in JAR cells. The HOXC6 promoter contains two putative estrogen response elements (EREs). Promoter analysis using luciferase-based reporter assay demonstrated that both EREs are responsive to E2, $ERE1_{1/2}$ being more responsive than $ERE2_{1/2}$. Estrogen receptors alpha and beta (ERα and ERβ) bind to these EREs in an E2-dependent manner and antisense-mediated knockdown of ERs suppressed the E2-dependent activation of HOXC6 expression. Similarly, knockdown of mixed lineage leukemia (MLL) histone methylases, MLL2 and MLL3, decreased E2-mediated activation of HOXC6. However, depletion of MLL1 or MLL4 showed no significant effect. MLL2 and MLL3 were bound to the HOXC6 EREs in an E2-dependent manner. In contrast, MLL1 and MLL4 that were bound to the HOXC6 promoter in the absence of E2, decreased upon exposure to E2. MLL2 and MLL3

play key roles in histone H3K4-trimethylation and recruitment of general transcription factors and RNA polymerase II (RNAPII) in the HOXC6 promoter during E2-dependent transactivation. Nuclear receptor corepressors, N-CoR and SAFB1, were bound to the HOXC6 promoter in absence of E2 and that binding were decreased upon E2 treatment indicating their critical role in suppressing HOXC6 gene expression under non-activated condition.

Furthermore, I examined the expression of HOXC6 in breast cancer tissue and its gene expression status in MCF7 cells in presence of endocrine disrupting chemicals such as bisphenol-A (BPA) and diethylstilbestrol (DES). My studies demonstrated that HOXC6 is differentially over-expressed in breast cancer tissue. Similar to E2, HOXC6 expression is also transcriptionally induced by BPA and DES. MLL histone methylases MLL2 and MLL3 coordinate with ERs, bind to the HOXC6 promoter upon exposure to BPA and DES, increase histone H3K4-trimethylation and recruitment of RNAPII to the HOXC6 promoter and transcriptionally activates HOXC6 expression.

In another study, roles of E2 and E2-like compounds (BPA and DES) in regulation of another HOX gene HOXA5 has been studied. HOXA5 expression is important for the development of lung, gastro-intestinal tract and vertebrae. My studies demonstrated that HOXA5 is over-expressed in breast cancer patient tissue and ER-positive breast cancer cells. HOXA5 expression is critical for cell viability as shown by flow cytometry, TUNEL and growth assay. Similar to E2, HOXA5 expression is also transcriptionally induced by BPA and DES. Promoter analysis confirms three $ERE_{1/2}$ sites but only $ERE1_{1/2}$ and $ERE2_{1/2}$ were found induced by E2, BPA and DES using luciferase-based reporter assay. MLL histone methylases MLL2 and MLL3 coordinate with ERs, bind to the HOXA5 promoter upon exposure to E2, BPA and DES, increase histone H3K4-trimethylation, histone acetylation and recruitment of RNAPII to the HOXA5 promoter and transcriptionally activate HOXA5 gene expression.

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ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
CGBP	CpG dinucleotide binding protein
ChIP	Chromatin immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle's medium
Dpy30	Dpy30 domain containing protein
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
ERE	Estrogen response element
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HMT	Histone methyl-transferase
HOX	Homeobox
IPTG	Isopropyl-D-1-thiogalactopyranoside
LB	Lysogeny broth medium
MLL	Mixed lineage leukemia
MMLV	Moloney murine leukemia virus
NR	Nuclear receptor
PBS	Phosphate buffered saline
PMSF	Phenylmethanesulfonylfluoride
PR	Progesterone receptor
RA	Retinoic acid
RAR	Retinoic acid receptor
Rbbp5	Retinoblastoma binding protein 5
RNAPII	RNA polymerase II
RT-PCR	Reverse transcriptase polymerase chain reaction
SAM	S-adenosyl methionine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SET	Su(var) 3-9, enhancer of zeste and trithorax
SRC-1	Steroid receptor coactivator-1
Tris	Tris (hydroxymethyl) aminomethane
Wdr5	WD40 repeats containing protein 5

CHAPTER 1

MIXED LINEAGE LEUKEMIA HISTONE METHYLASES IN GENE EXPRESSION, HORMONE SIGNALING AND ENDOCRINE DISRUPTION

1.1 Introduction

In eukaryotes, DNA is associated with histones and is present in the form of chromatin [1]. Nucleosome is the repeating unit of chromatin that contains four histone proteins (H2A, H2B, H3 and H4) in duplicates to form a histone octamer that is wrapped around with DNA [2]. The basic N-termini of histones are subjected to a number of post-translational modifications, including lysine acetylation, lysine and arginine methylation, serine phosphorylation, and ubiquitination [3,4]. These post-translational modifications have roles in gene expression, epigenetic regulation, and disease [1,5,6]. While histone acetylation is generally associated with transcriptional activation, histone methylation has roles in both gene activation as well as silencing [1].

Mixed lineage leukemias (MLLs) are histone H3 lysine-4 (H3K4)-specific methyl-transferases (HMTs) that are involved in gene activation [1,7]. Notably, MLL plays an important functional role in maintaining the expression of homeobox (HOX) genes during embryo development [7,8]. MLLs are shown to interact with nuclear hormone receptors (such as estrogen receptors (ERs)) and modulate the estrogen (17β-estradiol or E2)-dependent gene activation [8,9]. MLLs are well-known as the master regulator of HOX genes [1,7,8]. Several HOX genes have been shown to be transcriptionally regulated by E2 and MLL histone methylases play a major role(s) in this process [9]. MLLs are also found to be linked with endocrine disruption of multiple HOX genes (unpublished data).

1.2 MLLs are H3K4-specific histone methyl-transferases

Methylation of histone H3 at lysine 4 (H3K4) is an epigenetic mark and often associated with transcription activation [1,8,10]. H3K4-methylation marks can be recognized by specific proteins resulting in the recruitment of downstream effectors of transcription [1,8]. In yeast, H3K4-methylation is carried out by an enzyme called Set1 that plays vital role(s) in transcriptional regulation [1]. This enzyme has been diverged and become more complex in mammals. In mammals, there are six different Set1 homologs such as Set1A, Set1B, MLL1, MLL2, MLL3, and MLL4 [1,11]. Each of these enzymes possesses H3K4-specific histone HMT activity [12,13]. Each of them contains a conserved SET domain that is responsible for their HMT activity [14,15]. MLLs transfer the methyl group from S-adenosyl methionine (SAM) to the ε-nitrogen on lysine. SAM is the methyl group donor which is converted to S-adenosyl homocysteine (SAH) (Figure 1.1) [16]. The function of H3K4-methylation is well studied and characterized in yeast [17]. In yeast, Set1 interacts physically with RNA polymerase II (RNAPII) during transcription and involves histone H2B monoubiquitination, Paf1 complex and other transcription factors leading to transcriptional regulation [18,19].

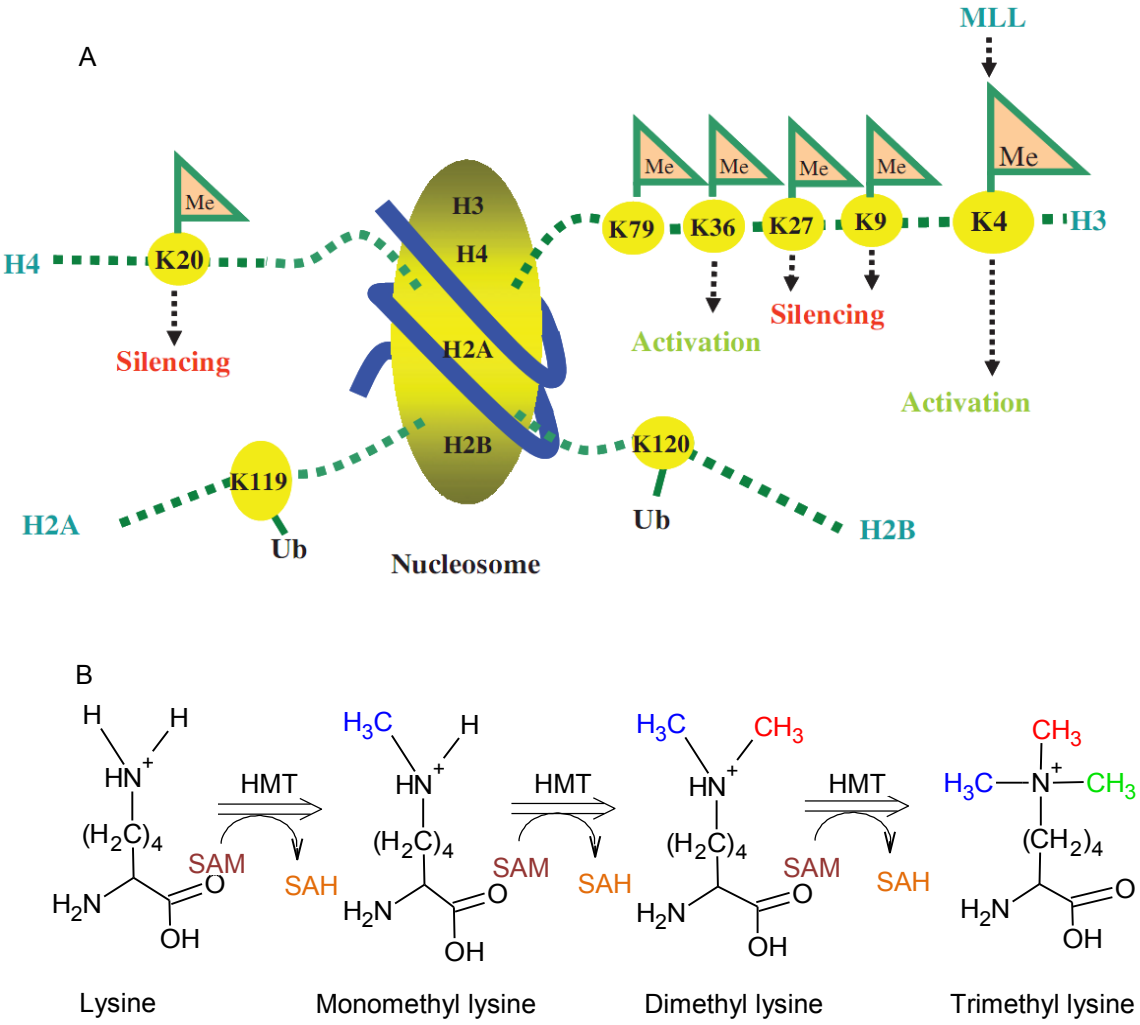


Figure 1.1 Methylation of lysine residue on histone H3. (A) Methylation of Histone H3 at lysine-4 (H3K4) by MLLs and Set1 is linked with gene activation. (B) Structure of lysine, and mono-, di- and tri-methyl lysine. HMTs add methyl group on ε-amino group of lysine. SAM is the methyl group donor which is converted to SAH [16].

For the optimal and proper HMT activity, the physical interactions among all the subunits are required [2]. Although, WD40 repeat containing protein (Wdr5) has been shown to be important for the stable interaction with the SET domain but the presence and interaction of Ash2 and retinoblastoma binding protein 5 (Rbbp5) are essential for the full HMT activity [12]. Wdr5 alone is insufficient for optimal HMT activity. However, Ash2 and Rbbp5 are unable to

stably interact with MLL1 even though these proteins are capable of stimulating MLL1 HMT activity [20].

MLLs are well-known for H3K4-trimethylation and regulate HOX genes [1,7]. They act as transcription factors and bind to the promoter of target genes involved in cell differentiation, cell cycle, angiogenesis and cardiovascular diseases and also interact with other HOX cofactors such as Pbx1 and Meis1 to influence the transcription of target genes [1,8,21,22]. Some of these target genes such as renin have already been studied in detail [23]. Although the role of MLL is well studied in regulating genes under non-hormonal environment but there is not much known about the roles of MLL family of HMTs in hormone signaling and associated diseases. Furthermore, studies on the roles of various coactivator and corepressor proteins need to be done in detail that will bring meaningful insight into the underlying complex biological pathway.

1.3 MLLs exist as complex of proteins

Considering the importance in epigenetic regulation, development and diseases, many scientists have isolated different MLL family of HMTs from human cells and characterized their multi-subunit complex protein and their function [2]. These studies demonstrated that these HMTs are present in distinct multi-protein complexes within the cells and each of these complexes shares several common subunits which includes Ash2, Dpy30 (dosage compensation protein), Rbbp5 and Wdr5 along with several unique components [1]. For example, MLL1-4 is shown to interact with ERs [9,39]. Biochemical techniques have revealed that MLL family of HMTs have some common proteins such as Ash2, Rbbp5, Wdr5, Dpy30, and Cgbp that are human homolog of yeast Set1 complex components Bre2, Swd1, Swd3, Sdc1, and Spp1 respectively [1]. Using affinity purification method, Ansari *et al.* (2008) have shown that CpG binding protein Cgbp interacts with three different HMTs such as MLL1, MLL2 and hSet1 and regulates MLL target HOX gene expression. Cgbp is a well-known CXXC zinc finger domain containing protein that binds to unmethylated CpG islands and plays critical role(s) in gene expression [2]. Immunoprecipitation experiment demonstrated that Flag-Cgbp-IP pulled down MLL1, MLL2 and

hSet1 HMTs along with their associated components. The recruitment of Cgbp into the promoter of MLL target gene HOXA7 further confirms its involvement with MLL. These observations were further supported by the immuno-colocalization experiments performed on Flag-Cgbp stable cells with anti-Flag, anti-MLL1, anti-MLL2 and anti-hSet1 antibodies wherein Cgbp colocalized perfectly with MLL1, MLL2 and partially with hSet1. Furthermore, the functional significance of Cgbp was evaluated when decreased MLL1 recruitment and low level of H3K4-trimethylation was observed in the promoter of HOXA7 upon knockdown of Cgbp. In another study, Cho *et al*. in 2007 discovered Dpy30 as a common interacting subunits of MLL1, MLL2, MLL3, MLL4, Set1A, and Set1B complexes [24]. One of the vital partner of MLL1 core complex Wdr5 has been shown to interact with MLL1, MLL2, MLL3, MLL4, Set1A, and Set1B [1,12]. Dou *et al.* have shown that Ash2/Rbbp5 heterodimer has a weak intrinsic H3K4 HMT activity [25]. Although, these protein complexes have been isolated and characterized, the detail functions of MLLs and their protein subunits in epigenetics, gene expression, and pathogenesis is still not very clear.

1.4 MLLs contain several functional domains

MLLs contain multiple functional domains such as SET, post-SET, high mobility group (HMG), AT-hooks, a cysteine rich CXXC motif, plant homeodomain (PHD), bromodomain, transcription activation domain (TAD), FYRN, FYRC, and LXXLL (NR-boxes) domains etc (Figure 1.2) [17,26]. The conserved SET domain (130 amino acid long) is responsible for their HMT activities in coordination with post-SET domain and other protein subunits. In addition, these MLLs contain several protein-protein and protein-DNA interaction domains (Figure 1.2) [14,15,26]. For example, MLL1-4 contain several DNA binding AT-hooks that make these proteins an important regulator of target gene. They bind to the AT-rich region in DNA. HMG domains are involved in binding DNA with low sequence specificity. PHD, RING, FYRN, and FYRC domains are involved in protein-protein interactions. Coiled coil domain performs homo-oligomerization. RNA recognition motif is present in Set1A and Set1B but absent in MLLs. MLL1 and 4 contain

CXXC zinc finger motif that may be involved in target DNA binding and gene regulation. Each of the MLLs (MLL1-5) contain multiple PHD domains**.** The PHD domains present in MLLs have been implicated in interactions with histone deacetylases, polycomb group proteins, and with methylated lysines in histone tails [1,26,27]. The FYRN and FYRC are two domains that are present in MLL^N and MLL^C regions of MLL1 [28]. These including SET domain are involved in forming hetero-dimers between MLL^N and MLL^C terminal fragments produced upon proteolysis of MLL1 by taspase **[28].** Taspase cleaves MLL1 into separate N-terminal (320 kDa) and C-terminal (180 kDa) part. LXXLL domains present in MLLs is significant for nuclear receptor-mediated gene activation and hormone signaling [9,29]. These NR-boxes work synergistically with both AF1 and AF2 domains present in nuclear receptors. In fact, MLL1 contains one whereas MLL2, MLL3, and MLL4 contain multiple LXXLL domains that are shown to interact with nuclear receptors and play important roles in gene regulation [9,29,39]. Despite great similarity in domain structure and same enzymatic activity (H3K4-methylation), sequence analysis demonstrates that MLLs have ~30 % sequence homology. Further studies are needed to elucidate the detail and diverse functions of other domains present in MLLs and their importance in epigenetics, gene regulation, and pathogenesis [30].

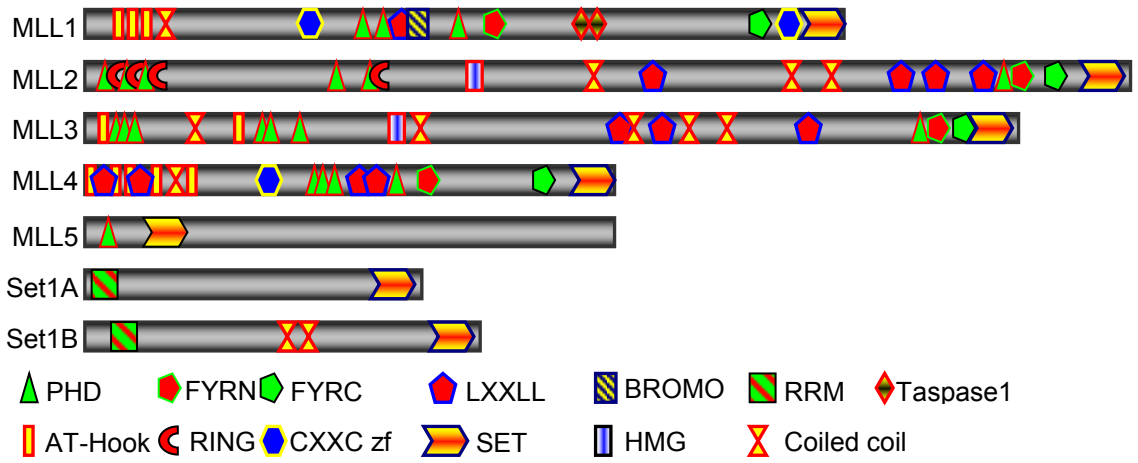


Figure 1.2 Domain structures of Set1A, Set1B, MLL1, MLL2, MLL3, MLL4, and MLL5 [1]. PHD domain (plant homeodomain) is usually involved in protein-protein interactions; FYRN and FYRC domains are involved in hetero-dimmerization between MLL^N and MLL^C terminal fragments. LXXLL domain is involved in interaction with nuclear receptor. BROMO domains are involved in the recognition of acetylated lysine residues in histone tails. RRM is the RNA recognition motif. Taspase1 site is the proteolytic site for the protease taspase1; AT-hook is a DNA binding domain; RING fingers are involved in protein-protein interactions; CXXC-zf is a Zn-finger domain involved in protein-protein interactions; SET domain is responsible for histone lysine methylation. HMG domain is involved in binding DNA with low sequence specificity. Coiled coil domains perform homo-oligomerization.

1.5 MLL histone methyl-transferases in hormone signaling

MLLs are key enzymes that methylate histones and play critical role in gene activation [31]. In humans, there are several MLLs such as MLL1, MLL2, MLL3, MLL4, Set1A, and Set1B. Each has histone H3-lysine 4 (H3K4)-specific HMT activity [2,12,13,32]. In general, MLLs are critical regulator of HOX genes [17,33]. MLLs are often rearranged and misregulated in various types of cancer [1,15,34].

MLLs contain multiple functional domains (Figure 1.2). One of the important domains, LXXLL domain present in coactivators such as MLLs interacts with nuclear receptor (NR) and plays crucial role in hormone-dependent gene activation [1,9,29,35]. Notably, ERα and ERβ belong to the large family of NR that are responsible for sensing the presence of estrogen [36]. ER-mediated gene expression and signaling is a complex process and may follow diverse mechanisms. In a classical mechanism, upon binding to estrogen, ERs undergo structural

changes that are recognized by a helical LXXLL motif present in the transcriptional coactivators and functionally convert ERs to transcriptional activator. Activated ERs, along with various coregulators, are targeted to the EREs present in the promoter of estrogen-sensitive genes leading to their transactivation (Figure 1.3) [36,38].

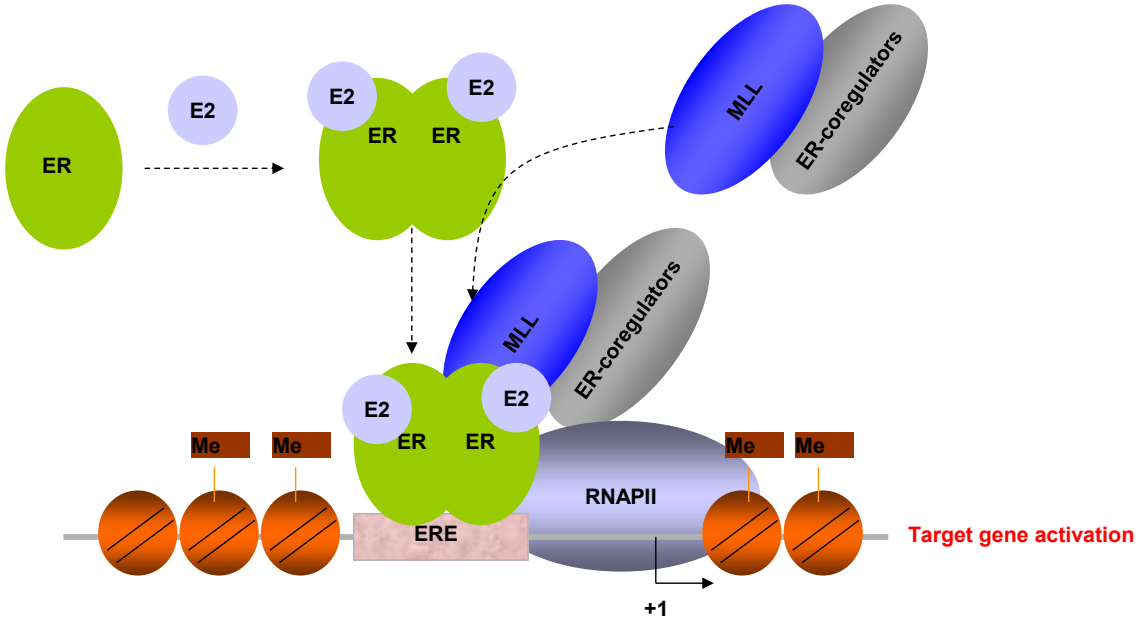


Figure 1.3 ERE-dependent mechanism of gene regulation with the involvement of MLL**.** Once estrogen (ligand) enters into the cell, it binds to ER and activates it. The activated ER (along with activators and coactivators) are recruited to the EREs present in the promoter of estrogen-responsive gene leading to its transcriptional activation. This figure is adopted from "Histone methylases MLL1 and MLL3 coordinate with estrogen receptors in estrogen-mediated HOXB9 expression" by Ansari *et al.* [23].

MLL2, MLL3 and MLL4 contain five, three and four NR-boxes respectively and MLL1 contains only one [9]. MLL2 interacts with ERα through its NR-boxes and acts as coactivator for E2-dependent activation of estrogen-responsive genes, cathepsin D and pS2 [9,29]. MLL2, MLL3, and MLL4 which are components of activating signal coregulator-2, ASC2, complexes (ASCOM complexes), act as coactivator in regulating several hormone-responsive genes [24,29,37]. Recently, we also found that MLL1, MLL2, MLL3 and MLL4 coordinate with ERs and play critical role in E2-mediated activation of several HOX genes [23,54,55]. Little is known about the

involvement of specific MLL in E2-mediated gene activation and appear to depend on the specific gene and cellular environments.

During transcriptional activation of E2-responsive genes, ERs associate with distinct subset of cofactors depending on the target gene, binding affinities and relative abundance of these factors in the cells [39]. These coactivators and corepressors usually exist as multi-protein complexes, possess multiple enzymatic activities, and (in a simplified view) bridge ERs, to either chromatin components such as histone, to components of the basal transcription machinery, or to both (Figure 1.4) [40]. Intense research has identified a large number of cofactors including the three members of the steroid receptor coactivator-1 (SRC-1) family (SRC-1, SRC-2/GRIP1/TIF2, and SRC-3/AIB1/ACTR/pCID/RAC3/TRAM1), CREB binding protein (CBP (cAMP response element binding protein) /p300), p/CAF, and thyroid hormone receptor protein (TRAP)/vitamin D3 receptors (VDR)-interacting proteins (DRIP), ASCOM complexes which consist of some of the MLLs and many others [9,37,40,41]. In addition, menin is also a component of MLL1/MLL2 complexes and has been shown to act as coregulators for ERα and regulates estrogen-responsive genes [29].

1.5.1 Role of ASCOM complex

ASCOM complexes are transcriptional coactivators that are involved in different types of NR-mediated gene activation (such as retinoic acid receptors (RAR), thyroid receptors (TR), liver X receptors (LXR) and ER) [42]. ASC2 binds not only to transcription factors TBP (TATA binding protein) and TFIIA (transcription factor IIA) but also to transcription coactivators SRC-1 and CBP/p300 [41,43]. ASC2 contains two NR-boxes through which it interacts with various NRs either alone or in conjunction with SRC-1 and p300 and regulates NR-mediated gene activation [29,37]. One of the key features of the ASCOM complex is that it exists as massive steady state complex containing a unique interacting protein, ASC2, along with other components and specific MLL histone methylases (MLL2, or 3 or 4) [42,44]. Depending on the target genes and cellular environment, specific MLL-containing ASCOM complexes might be involved in gene

activation. Notably, MLL2 exists as a multi-protein complex containing Ash2, Wdr5, Rbbp5, Cgbp and Dpy30 which has H3K4-specific HMT activity [13,32]. In addition, MLL2 is also shown to interact with ASCOM complex which plays critical role(s) in activated gene expression [29].

1.5.2 Role of menin as a coactivator

Menin, the product of multiple endocrine neoplasia type 1 (MEN1) gene, is a tumor suppressor [29]. Menin is shown to interact with various nuclear proteins (including HDAC1/2, NF-kB, JunD etc) and plays critical role in transcriptional regulation [45]. However, role of menin in MEN1 syndrome is still remain unclear. Recently, menin is shown to be associated with MLL1/MLL2 HMT complexes. Like ASC2, menin also contains two NR-boxes and acts as coactivator for E2-responsive gene pS2 [29].

1.5.3 Roles of other ER coregulators such as CREB binding proteins (CBP/p300), p/CAF and SRC-1 family of ER coregulators

1.5.3.1 CBP/p300

CBP and its close homolog p300 are involved in large numbers of ligand-dependent hormone signaling events [46]. CBP and p300 harbor HAT activity and have been implicated in ER-mediated gene regulation [47]. Furthermore, CBP/p300 is shown to interact with ASCOM complexes [41,43].

1.5.3.2 p/CAF

p/CAF is homolog of yeast histone acetyl-transferase protein and p/CAF complex resembles the yeast chromatin remodeling protein complex GCN5/SAGA [48]. p/CAF interacts with CBP and members of SRC-1 family of coactivators possibly links with RNAPII core machinery during transcription and participates in NR-mediated gene activation [49].

1.5.3.3 SRC-1 family of coactivator

SRC-1 family of protein is well known to be involved in NR-mediated gene activation [41,50]. Based on sequence homology, they are grouped in three families (SRC-1, SRC-2 and

SRC-3). Several members of the SRC family of coactivators contain multiple LXXLL signature motifs and therefore participate by interaction with NRs [51].

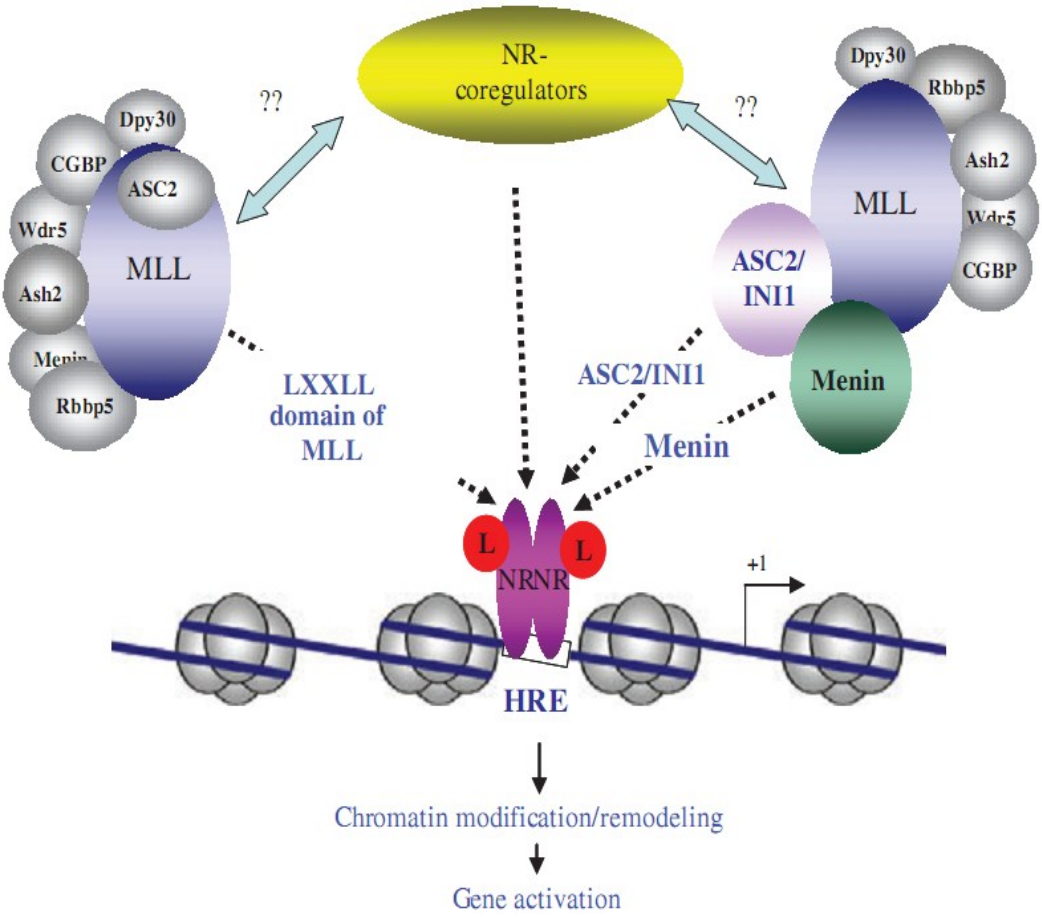


Figure 1.4 MLLs are coregulators for NR-mediated gene activation. During hormone-mediated gene regulation NRs interact with various coregulators. Proteins containing LXXLL domains interact with NRs and act as a coregulator. MLLs (MLL1-4) contain one or more LXXLL domains and may interact directly with NRs. Alternatively, MLLs might interact with NRs via different MLL interacting proteins such as ASC2, Menin, INI1 that contain multiple LXXLL domains. In addition, there are various other NR coregulators. Functional interplay between MLLs and other NR coactivators (CBP/p300, p/CAF, SRC-1 family, etc) needs further investigation [7].

1.6 MLLs are key regulators of HOX genes

MLLs are key enzymes that methylate histone H3 at lysine-4 (H3K4) and regulate gene activation [10,12,17,24,52]. MLLs are well-known as master regulator of HOX genes [26,53]. MLLs are also associated with various oncogenic transformations [15]. Although, the importance of MLLs in

HOX gene expression is well recognized, their roles in hormone-dependent HOX gene regulation are mostly unexplored.

Recent studies indicate that MLLs, in coordination with ERs, act as coactivator for the estrogen-mediated gene activation [23,54,55]. MLL2 in particular is shown to interact with ERα and regulates estrogen-responsive genes such as pS2 and cathepsin D [9]. Recently, menin, an interacting partner of MLL1 and MLL2 has been shown to associate with ERα and recruits H3K4 methylases in estrogen-responsive genes [29]. Studies in our laboratory have already demonstrated that MLLs are involved in the transactivation of HOXC6, HOXB9, HOXC13 and HOXC10 genes under estrogen environment [9,23,54,55,] . What interesting in these findings is that each HOX gene has different MLL requirements under E2-induced transactivation and some are required for basal transcription. For example, in an independent study, we found that histone methylases MLL2 and MLL3 bind to the promoter of HOXC6 in an estrogen-dependent manner and knockdown of MLL2 and MLL3 diminished the estrogen-dependent transactivation of HOXC6 [54]. These results suggest that both these MLLs are essential for the estrogen-dependent transactivation of HOXC6 while MLL1 and MLL4 are required for basal transcription. In another study, MLL1 and MLL3 are required for the estrogen-mediated expression of another HOX gene HOXB9 [23]. Recent studies also shows that MLLs also participate in endocrine disruption of several HOX genes such as HOXA5, HOXB5, HOXC5 and HOXC6 (unpublished data).

1.6.1 HOX genes and their functions

HOX genes are evolutionarily conserved genes that play critical roles in cell differentiation and tissue patterning during development [33,53]. HOX genes are one of two sub-groups of homeobox genes which produce transcription factors containing highly conserved homeodomains [23,33]. There are 39 HOX genes in human, which are categorized in four clusters (A-D) based on their location in different chromosomes (7, 17, 12, and 2) respectively in human and each has unique functions during development (Figure 1.5) [33]. Although, HOX genes

expression is crucial in embryonic development, they are also expressed in different adult tissues such as bone marrow stem and reproductive cells etc [1,7,54].

Recent studies from our laboratory demonstrated that MLLs as well as HOX genes (HOXA5, A7 and A10) play critical role in cell cycle regulation [1,2]. There is change in RNAPII, H3K4-methylation as well as MLL1 levels on HOX gene promoters as a function of cell cycle as demonstrated by ChIP experiments and corresponds to differential gene expression during cell cycle. Depletion of MLL1 results in cell cycle arrest at G2/M phase and ultimately apoptosis [7]. Malfunctions of HOX genes are associated with various types of developmental defects, autism and cancer [1,7]. HOX gene over-expression is associated with different types of cancer. HOXA5, anti-angiogenic gene, is involved in lung, vertebrae, spinal cord and gastro-intestinal tract development [58]. HOXC9 and C10 are associated with cervical carcinomas [23,54]. HOXC13 is involved hair follicle development and alopecia, and in keratin gene regulation [55]. HOXB9 is a central player in skeletal and mammary gland development, and is involved in renin-angiotensin system, angiogenesis and cancer [23,59].

Increasing amount of evidence suggests that hormones play critical role in regulation of HOX genes expression [56]. In the normal mammary gland, the expression of HOX genes is coordinately regulated by hormone and extracellular matrix (ECM) and other unknown factors in a spatial and temporal manner in both stromal and epithelial cells. Animals with misexpressed HOX genes displayed different extents of defects in ductal proliferation, side branching, and alveoli formation implying that these genes are important for normal mammary gland development. Ovariectomy in mice affects the expression of HoxC6 and other Hox genes ultimately affecting mammary gland development and milk production [54,56]. For example, retinoic acids markedly affect HOX gene expression and produce homeotic transformation [60]. Expression of HoxA10 in uterus fluctuates over different stages of pregnancy by estrogen and progesterone [57]. Although, multiple *in vivo* studies indicate that HOX genes are influenced by endocrines during development and most of HOX genes are over-expressed in breast

carcinomas, the molecular mechanism by which these HOX genes are expressed and regulated by steroid hormones are mostly unknown and is the subject of my study.

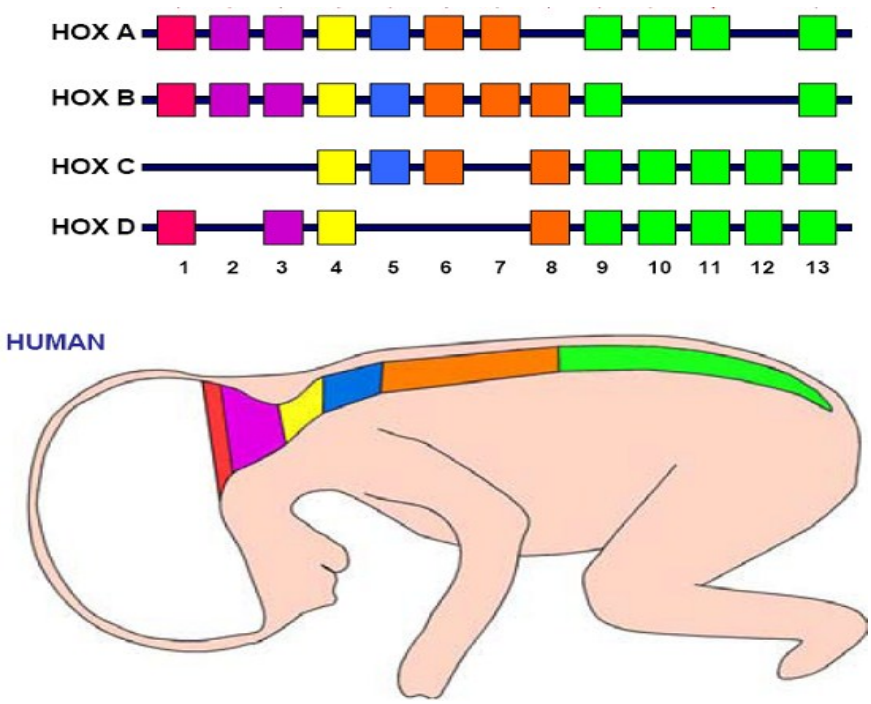


Figure 1.5 Schematic view of classification of HOX genes and their expression pattern in mammalian embryo. Those near 3′-end of chromosome are more involved in anterior sections and near 5′-end HOX genes are more responsible for posterior body section development [33].

1.6.2 HOX genes and their roles in cancer

A growing body of evidence suggests that HOX gene expression is altered in breast cancer and various other types of tumorigenesis. HOXA5 which regulates p53 and progesterone receptor is lost in 60 % of breast carcinomas and over-expression of HOXA5 induces apoptosis in breast cancer cells [58]. HOXC6 regulates expression of various genes (eg BMP7, FGFR2, PDGFRA and CD44) that are key players in prostate cells, prostate cancer and metastasis (Figure 2.1) [58]. HOXC6 is often over-expressed in prostate cancer, breast cancer, esophageal cancer and neuroblastomas [54,58,59]. In the human breast cancer cell line MCF7, many HOX gene transcripts, including HOXA1, HOXA4, HOXB6, HOXC6, HOXB7, and

HOXA10 were detected [60]. In primary breast carcinomas, HOXC6, HOXB3, and HOXB4 were reported to be detectable immunocytochemically in over 90% of neoplastic cells [59]. HOXB7 is over-expressed in more than 80% of breast carcinomas and breast cancer cell lines [61]. HOXA4, B7, C13, D3 and D13 are upregulated while HOXB3 is downregulated in most breast cancer lines and primary breast tumors compared to normal breast cells [62]. Defects in HOXA13 result in hand-foot-genital syndrome that is associated with the duplication of genital tract. HOXB13, that is a key player in cellular homeostasis, is over-expressed in prostate and ovarian cancer [63]. However, this gene is suppressed in various ER-positive breast cancer cells [63,64]. Recent studies suggest that HOXB13 expression is for critical breast cancer invasiveness and the ratio of HOXB13-IL17BR is related with tumor aggressiveness and recurrence of breast cancer in tamoxifen-treated breast cancer patients [65]. Although, HOX genes are key players in various hormonally influenced organogenesis and tumorigenesis, the mechanism by which they are expressed and regulated is mostly unknown.

1.7 Estrogen, bisphenol-A and diethylstilbestrol and their impact on gene regulation

Steroid hormones have important roles in cell development and differentiation. 17β-estradiol (E2), produced mainly in ovaries and is present in the physiological range of 10^{-6} – 10^{-10} M, is principle and most potent estrogen in both males and females but lower levels of estrogens, estrone (E1) and estriol (E3), are also present [66]. The biological effects of estrogens are mediated through ERα and ERβ, which are members of a large superfamily of nuclear receptors [66,67]. ERα is found to be more active than ERβ in regulation of estrogen-responsive genes and generally more tightly bound to the EREs [67]. These receptors act as ligand-activated transcription factors. The classical mechanism of ER action involves estrogen binding to receptors in the nucleus, after which the receptors dimerize and bind to specific response elements known as EREs located in the promoters of target genes [66]. Hormone binding also induces a conformational change within the ligand binding domain of the receptors and this conformational change allows coregulatory proteins to be recruited [68]. However, evidence for

signaling pathways that deviate from this classical model has emerged and it is now established that ERs can regulate gene expression by a number of distinct mechanisms.

ERs can regulate gene expression without binding directly to DNA by modulating the function of other classes of transcription factors through protein-protein interactions in the nucleus [69]. The interaction of ERα with the activator protein 1 (AP-1) transcription factor complex is a typical example of such ERE-independent genomic actions. Also ERs are involved in protein-protein interaction with specificity protein 1 (Sp1) to regulate gene expression in an E2 environment. A number of estrogen-responsive genes that lack full EREs contain ERE half-sites or binding sites for the orphan nuclear hormone receptor SF-1 [SF-1 response elements (SFREs)] that serve as direct ER binding sites [69]. ERα, but not ERβ, is able to bind to SFREs [70]. In addition, estrogen induces rapid generation of a number of signaling cascades including cyclic AMP, inositol-1,4,5-trisphosphate (IP3) and phospholipase C. Some of these cascades involve several cell signaling lipids as second messengers. During E2-mediated gene activation, depending on the cell requirement various coactivator and corepressor proteins coordinate with ERs to carry out E2-mediated gene regulation. Although mechanism is not investigated, selected HOX genes (such as HOXA9, A10 and A11) are reported to be over-expressed upon exposure to estrogen especially during clinical treatment [71].

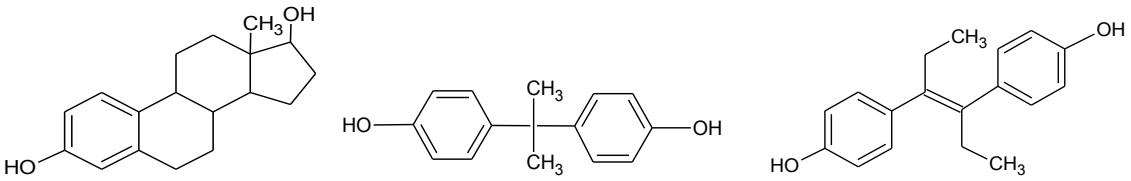
Endocrine disruptors are chemicals that interfere with hormone system in animals including humans. These disruptors can cause cancer, birth defects, and other developmental disorders. Specifically, they are known to cause learning disabilities, severe attention deficit disorder, cognitive and brain development problems, deformations of the body (including limbs); sex organ development problems, feminizing of males or masculine effects on females etc [72,73]. Any system in the body controlled by hormones can be derailed by hormone disruptors. The critical period of development and differentiation for most organisms is between the transitions from a fertilized egg into a fully developed infant. As the cells begin to grow and differentiate, critical balance of hormones are required. Endocrine disruptors are substances that interfere

with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for development, behavior, fertility, and maintenance of homeostasis (normal cell metabolism) [72]. They are sometimes also referred to as hormonally active agents, endocrine disrupting chemicals or endocrine disrupting compounds (EDCs) [73,74].

The estrogen-mimic bisphenol-A (BPA) is used as a model agent for endocrine disruption. BPA is used in the manufacture of polycarbonate plastics and epoxy resins from which food and beverage containers and dental materials are made. Perinatal exposure to environmentally relevant BPA doses result in morphological and functional alterations of the male and female genital tract and mammary glands that may predispose the tissue to earlier onset of disease, reduced fertility, mammary and prostate cancer [75].

Diethylstilbestrol (DES), a powerful synthetic estrogen introduced in 1938 was prescribed to prevent pregnancy complications, but was later shown to have a series of adverse side effects in the offspring, including cancer of the vagina. Recent evidence indicates that *in utero* exposure to DES is also linked to breast cancer in women over the age of 40 years. This evidence provides support to the hypothesis that *in utero* exposures may affect breast cancer risk in adult life [76]. The endocrine disruptors or E2-mimics have structural similarity with ERs that allow them to disrupt E2-ER-ERE complex at the target gene promoter aiding in misregulation of target genes (Figure 1.6). BPA has already been reported to exert its effect via binding to ERs. BPA has also been demonstrated to be inhibiting the aromatase activity by 50% while DES inhibits the aromatase activity by 75%. BPA is suspected to disturb estrogen functions in the brain tissues, since BPA has been shown to reach the brain of both mother and fetus within 1 h after injection to mother rat [77]. BPA and DES have also been demonstrated to induce synaptic plasticity, i.e., long term depression (LTD) enhancement in different histological divisions of the hippocampus, which is a similar effect to that of estradiol [78]. BPA might be activating ERα as predicted from the results of LTD and spinogenesis. However, the binding affinity of BPA to water soluble ERα has been reported to be much lower (approximately

1/2000) than that of 17β-estradiol [79]. The ligand binding affinity of BPA to ERα has been shown to be 1/100–1/1000 of that of 17β-estradiol [80]. All these evidences strongly suggest that both BPA and DES are the potent endocrine disruptors and possess the potential to dysregulate the E2-mediated gene expression via binding to estrogen receptors.



A B C

Figure 1.6 Structures of 17β-estradiol (A), bisphenol-A (B), and diethylstilbestrol (C).

In summary, HOX genes are known to be involved in differentiation and development. Several HOX genes are also misregulated in different cancer cells [1,33]. Estrogen and ERs are also major players in embryogenesis, development and are associated with different types of cancer including breast, prostate and other types of cancer [35,81]. Although, both HOX genes and estrogen receptors are well-known to be associated with cancer and are potential biomarkers, little is known about their inter-dependent functions.

The regulation of gene expression by estrogen is a highly complex and tissue-specific multifactorial processes involving both genomic and non-genomic actions that converge at certain response elements located in the promoters of genes [81]. The final gene response, however, could depend on a number of conditions such as the various combinations of transcription factors bound to a specific gene promoter, the cellular localization of ERs, levels of chromatin modifiers, the levels of different coregulatory proteins, signal transduction elements as well as the nature of intra- and extra-cellular stimuli. These variables are highly cell-specific. In this way E2 evokes multiple gene responses in different types of target cells [81]. Furthermore, MLLs are well-known regulator of HOX genes under non-hormonal environment [15]. However, almost nothing is known about the roles of MLL in regulating HOX genes in hormonal

environment. Our studies demonstrated the novel regulatory mechanism of HOX genes under the environment of estrogen and estrogen-mimic compounds involving MLLs. However, further studies are needed to unravel the detailed mechanism. Our findings indicate that MLL histone methylases can be used as one of the therapeutic targets in hormone-sensitive cancer cells. As HOX genes, MLLs and ERs are all critical player in different types of cancer, these mechanistic studies will advance our understanding about the molecular mechanism of cancer cell biology and will open up new avenues for novel cancer therapy.

CHAPTER 2

HOXC6 IS TRANSCRIPTIONALLY REGULATED VIA COORDINATION OF MLL HISTONE METHYLASE AND ESTROGEN RECEPTOR IN AN ESTROGEN ENVIRONMENT

2.1 Introduction

Homeobox (HOX) genes are group of evolutionarily conserved genes that play critical role in embryonic development [33,82]. HOX genes also continue to be expressed at varying levels throughout post-natal life. There are 39 different HOX genes in human that are clustered in four different groups HOXA, B, C, and D and expression of each HOX gene is tightly regulated [83]. Recent studies demonstrate that HOX genes are associated with various oncogenic transformations [22,62,84]. In particular, HOXC6, a critical player in mammary gland development and milk production, is expressed in osteosarcomas, medulloblastomas, as well as carcinomas of the breast, lung, and prostate [58,59]. HOXC6 regulates expression of BMP7 (bone morphogenic protein 7), FGFR2 (fibroblast growth factor receptor 2), IGFBP3 (insulin-like growth factor binding protein 3) and PDGFRA (platelet-derived growth factor receptor α) in prostate cells and influences the notch and wnt signaling pathways *in vivo* (Figure 2.1) [58]. HOXC6 regulates various genes including CD44 that are important for prostate branching morphogenesis and bone metastasis of prostate cancer. Although, HOXC6 is critical in so many hormonally regulated processes and diseases, the mechanism by which it may be regulated is mostly unknown.

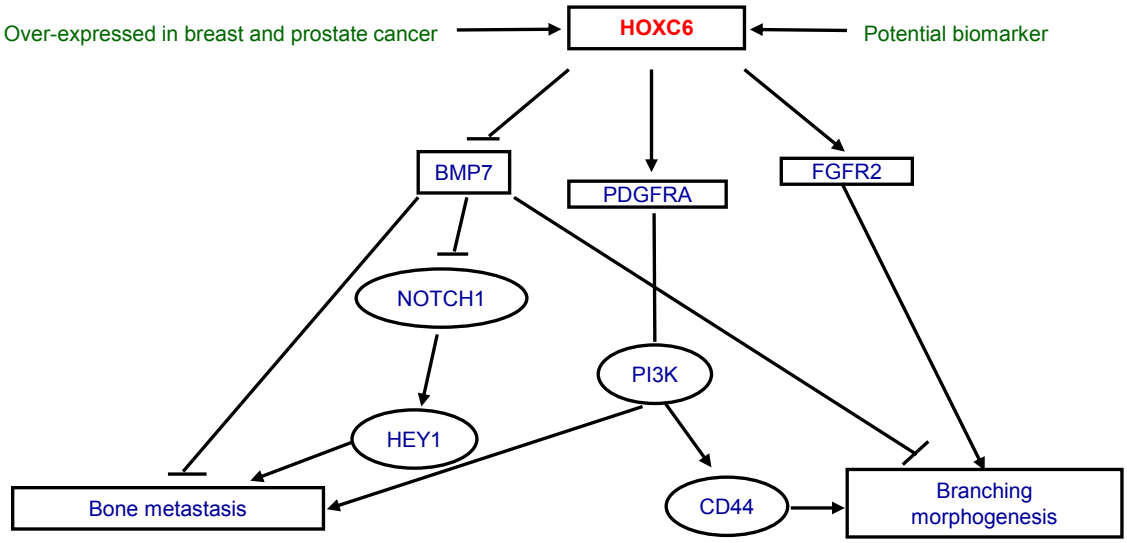


Figure 2.1 Role of HOXC6 in prostate cancer depicting distinct downstream target genes linked with cell signaling components. It regulates BMP7, FGFR2, IGFBP3, and PDGFRA in prostate cells and influences the notch and wnt signaling pathways [58].

In general, mixed lineage leukemia (MLL) family of proteins is well-known as master regulators of HOX genes [2,15,17]. MLLs are evolutionarily conserved trithorax family of proteins that play critical roles during development [85]. MLL1 is also well known to be rearranged in leukemia [15,85,86]. Biochemical studies demonstrate that MLLs are human histone H3 lysine-4 (H3K4)-specific methyl-transferases (HMTs) that are key players in gene activation and epigenetics [1,2,7,8,9,13,15,17.] There are several MLLs in human such as MLL1, MLL2, MLL3, MLL4, SET1A, and SET1B and each exists as multi-protein complexes with several common subunits such as Ash2, Wdr5, Rbbp5, Cgbp, and Dpy30 [1,12,14,88]. Although, different MLLs and Set1 possess similar enzymatic activities (H3K4-methylation) and all are critical players in gene activation, multiplicity of MLLs suggests their distinct roles beyond histone methylation. Recently, we and others showed that MLLs play key roles in cell cycle regulation, stress response, and HOX gene regulation [28,28,34,89]. Knockdown of MLL1 results in cell cycle arrest at G2/M phase [87]. Beyond their roles in histone H3K4-methylation, several MLLs are found to interact with nuclear hormone receptors (including estrogen receptors), nuclear receptor coregulatory complexes and play critical roles in regulation of hormone responsive genes [9,29,55].

As HOXC6 expression is associated with various steroid hormone regulated developmental processes and is over-expressed in various hormonally influenced carcinomas, I examined if it is transcriptionally regulated by steroid hormone. My studies demonstrated that HOXC6 is an estrogen-responsive gene and histone methylases MLL2 and MLL3, along with estrogen receptors (ERs), play critical role in 17β estradiol (E2)-induced HOXC6 expression.

2.2 Materials and methods

2.2.1 Cell culture, estrogen treatment and antisense-mediated knockdown experiment

Human choriocarcinoma placenta cells (JAR, ATCC) were grown and maintained in RPMI 1640 supplemented with 10 % FBS, 2 mM L-glutamine and penicillin/streptomycin (100 unit and 0.1 mg/mL respectively) [55,89]. Human breast cancer cells (MCF7) and ER negative adenocarcinoma breast cell (MDAMB231) were maintained in DMEM supplemented with 10 % FBS, 2 mM L-glutamine and penicillin/streptomycin (100 unit and 0.1 mg/mL respectively). For the estrogen treatment, cells were grown and maintained (for at least 3 rounds) in phenol red free DMEM-F-12 media (Sigma) supplemented with 10 % charcoal stripped FBS, 2 mM L-glutamine and penicillin/streptomycin (100 unit and 0.1 mg/mL respectively). Cells were grown up to 70 % confluency, treated with varying concentrations (0-1000 nM) of 17β-estradiol (E2) and incubated for 8 h (or varying time points for temporal studies) and then harvested for RNA and protein extraction.

For antisense experiments, JAR cells were grown up to 60 % confluency in 60 mm plate and transfected with different antisense oligonucleotides (commercially synthesized from IDT) in FBS free media using ifect transfection reagent (MoleculA) and following manufacturer's instruction. In brief, a cocktail of antisense and ifect transfection reagents was made in 300 µL DMEM-F-12, applied to cells in presence of 1.7 mL supplement free medium, and incubated for 7 h. Then 2 mL media containing all supplements and 20 % charcoal stripped FBS were added and incubated for additional 48 h. Depending on the need, antisense treated cells may have been exposed to 100 nM E2.

Antibodies were purchased from commercial sources as follows: MLL1 (Abgent, AP6182a); MLL2 (Abgent, AP6183a); MLL3 (Abgent, AP6184a); MLL4 (Sigma, AV33704); ERα(D-12) (Santa Cruz, sc-8005); ERβ(H-150) (Santa Cruz, sc-8974); H3K4-trimethyl (Upstate, 07-473; H3 (Upstate, 07-499); H3K9-dimethyl (upstate, 07-441); RNAPII (abcam, 8WG16); TBP (abcam, ab28175); TAF250 (Upstate, 05-500); N-CoR(C-20)(sc-1609); SAFB1 (Upstate, 05-588); β-actin (Sigma, A2066).

2.2.2 RNA/protein extraction, RT-PCR and western blot

Cells were harvested and collected by centrifugation at 1500 rpm. The RNA and protein were extracted as described previously [2,89]. For the reverse transcriptase-PCR (RT-PCR), reverse transcription reactions were performed in a total volume of 25 μL containing 500 ng of RNA, 2.4 μM of oligo dT (Promega), 100 units of MMLV reverse transcriptase, 1X first strand buffer (Promega), 100 μM each of dATP, dGTP, dCTP and dTTP (Invitrogen), 1 mM dithiothreitol (DTT), and 20 units of RNaseOut (Invitrogen). The cDNA was diluted to 100 μL. PCR reactions were performed in a 10 μL reaction volume containing 5 μL diluted cDNA and gene specific primer pairs (Table 2.1). Protein extracts were analyzed by western blotting using antibodies against MLL1, MLL2, MLL3, MLL4, ERα, ERβ, and β-actin. Western blots were developed using alkaline phosphatase method.

2.2.3 Chromatin immunoprecipitation (ChIP) experiment

ChIP assays were performed by using JAR cells and EZ Chip™ chromatin immunoprecipitation kit (Upstate) as described previously [2,87,89]. In brief, JAR cells were treated with 100 nM E2 for varying time points, fixed in 4% formaldehyde, lysed in lysis buffer and sonicated to shear the chromatins. The fragmented chromatin was pre-cleaned with protein-G agarose beads and subjected to immunoprecipitation with antibodies specific to ERα, ERβ, MLL1, MLL2, MLL3, MLL4, RNAPII, histone H3, H3K4-trimethyl, H3K9-dimethyl, N-CoR, SAFB1, TBP, TAF250 or β-actin overnight. Immunoprecipitated chromatins were washed and de-proteinized to obtain purified DNA fragments that were used as templates in PCR

amplifications using various primers corresponding to different EREs of HOXC6 promoter (Table 2.1).

2.2.4 Real-time PCR

For gene expression analysis RNA was extracted from cells by using RNAGEM tissue plus RNA extraction kit (ZyGEM). The reverse transcription reactions were performed with 1 µg total RNA by using MMLV reverse transcriptase as mentioned above and the cDNA was diluted to 50 µL final volume. The cDNA was amplified using SsoFast EvaGreen supermix (Bio-Rad) and primers as described in Table 2.1, using CFX96 real-time PCR detection system. These results were analyzed using the CFX manager. The real-time PCR analysis of the ChIP DNA fragments were done with primers specific to $ERE1_{1/2}$ and $ERE2_{1/2}$ regions of HOXC6 promoter. Each PCR reaction was done in triplicates.

2.2.5 Dual luciferase reporter assay

HOXC6 promoter spanning $ERE1_{1/2}$-$ERE2_{1/2}$ regions (-1107 to +208 nt), $ERE1_{1/2}$ (alone, -184 to +208 nt), and $ERE2_{1/2}$ (alone -1107 to -697 nt) were cloned and inserted upstream of the promoter of fire fly luciferase gene in pGL3 promoter vector (Promega) (primers are listed in Table 2.1). JAR cells (~ 4 x 10^5 cells in 6 well plate) were co-transfected with 1500 ng of these ERE-containing luciferase reporter construct along with 150 ng of a reporter plasmid containing renilla luciferase (pRLTk, Promega) as an internal transfection control using FuGENE6 transfection reagent. Control transfections were done using pGL3 promoter vector without any ERE insertion or with a luciferase construct-containing segment of HOXC6 promoter having no ERE (non-specific control, non-ERE). At 24 h post transfection, cells were treated with 100 nM E2 and incubated for additional 8 h and then subjected to luciferase assay using dual luciferase reporter assay kit (Promega) as instructed. Firefly luciferase activities were assayed and normalized to those of renilla luciferase. Each treatment was done in four replicates and the experiment was repeated at least twice.

2.2.6 Statistical analysis

Each experiment was done in 2-3 replicates and then cells were pooled (and treated as one sample), subjected to RNA extraction, RT-PCR and ChIP analysis and each experiment was repeated at least thrice (n = 3). For luciferase assay each treatment was done in four replicates and the experiment was repeated at least twice. The real-time PCR analysis of each sample was done in three replicate reactions and repeated so in all three independent experiments (n = 3). Normally distributed data were analyzed by ANOVA and non-normally distributed data were analyzed using student-*t* tests (SPSS) to determine the level of significance between individual treatments. The treatments were considered significantly different at $P < 0.05$.

Table 2.1 Primers used for cloning, RT-PCR, ChIP, and antisense experiments

Primers	Forward primer (5′- 3′)	Reverse primer (5′ - 3′)
PCR primers		
β-actin	AGAGCTACGAGCTGCCTGAC	GTACTTGCGCTCAGGAGGAG
HOXC6	CAGACCCTGGAACTGGAGAA	CTTCCCGCTTTTCCTCTTTT
HOXC6-ERE1$_{1/2}$	TTTTTCCCCCTTCCTGACAT	GCCTTTACCTGGTCGGTCTA
HOXC6-ERE2$_{1/2}$	AGCCTCATAGCTCAGGTCCA	CCAGAAAGAGAAGGCTGGTG
HOXC6-Non-ERE	TATGAGGGGAGCTGAGCAAT	CCCTCGCACACAGATACACA
MLL1	GAGGACCCCGGATTAAACAT	GGAGCAAGAGGTTCAGCATC
MLL2	AGGAGCTGCAGAAGAAGCAG	CAGCCAAACTGGGAGAAGAG
MLL3	CATATGCACGACCCTTGTTG	ACTGCTGGATGTGGGGTAAG
MLL4	CCCTCCTACCTCAGTCGTCA	CAGCGGCTACAATCTCTTCC
ERα	AGCACCCTGAAGTCTCTGGA	GATGTGGGAGAGGATGAGGA
ERβ	AAGAAGATTCCCGGCTTTGT	TCTACGCATTTCCCCTCATC
Cloning primers		
HOXC6-ERE1$_{1/2}$	CCACCAAACCAGTTCCCTTA*	ATCATAGGCGGTGGAATTGA*
HOXC6-ERE2$_{1/2}$	AGCCTCATAGCTCAGGTCCA*	CTCCTTCTCAGGACCCCTCT*
HOXC6-Non-ERE	TATGAGGGGAGCTGAGCAAT*	CCCTCGCACACAGATACACA*
Antisenses		
MLL1 antisense	TGCCAGTCGTTCCTCTCCAC*	
MLL2 antisense	ACTCTGCCACTTCCCGCTCA**	
MLL3 antisense	CCATCTGTTCCTTCCACTCCC**	
MLL4 antisense	CCTTCTCTTCTCCCTCCTTGT**	
ERα antisense	CATGGTCATGGTCAG**	
ERβ antisense	GAATGTCATAGCTGA**	
Scramble antisense	CGTTTGTCCCTCCAGCATCT**	

*Flanked by appropriate restriction sites; **Phosphorothioate antisense oligonucleotide.

Note: These antisense oligonucleotides were designed by Dr. Subhrangsu S. Mandal and Dr. Khairul I. Ansari and kindly provided by them.

2.3 Results

2.3.1 HOXC6 gene is transcriptionally regulated by estrogen

To examine if HOXC6 is transcriptionally regulated by estrogen, I treated JAR cells with varying concentrations of 17β-estradiol (E2) and analyzed its impact on HOXC6 expression. Notably, JAR cell is a placental choriocarcinoma cell line and placenta is known to produce various steroid hormones that are circulated to fetus as well as the mother [91]. JAR cells have been previously used for steroid hormone related studies [92]. My analysis showed that JAR cells express both ERα and ERβ (data not shown). I isolated RNA from the E2-treated and control (not treated with E2) cells, reverse transcribed into cDNA and analyzed by PCR using primers specific to HOXC6. The cDNA was also analyzed by real-time PCR for quantification. β-actin was used as control. Interestingly, I observed that HOXC6 expression was increased upon treatment with E2 in a concentration-dependent manner (Figure 2.2A). HOXC6 expression was about 4-fold higher in 100 nM E2-treated JAR cells in comparison to control (compare lane 1 with 5, Figure 2.2A). Temporal studies demonstrated that transcriptional activation of HOXC6 was increased with the increase in incubation time with maxima at ~8 h and then decreased gradually (likely due to squelching) (Figure 2.2B). I also analyzed the E2-dependent expression of HOXC6 in additional ER-positive breast cancer cell line MCF7 and an ER negative breast cancer cell line MDAMB231. My results showed that HOXC6 is also transcriptionally activated by E2 in a concentration-dependent manner in MCF7 but not in ER negative MDAMB231 cells (Figure 2.2C and 2.2D). The stimulation of HOXC6 in two independent steroidogenic cell lines but not in the ER negative cell suggested that it is an E2-responsive gene. As JAR cells showed more robust response to E2, we performed all mechanistic studies in JAR cells.

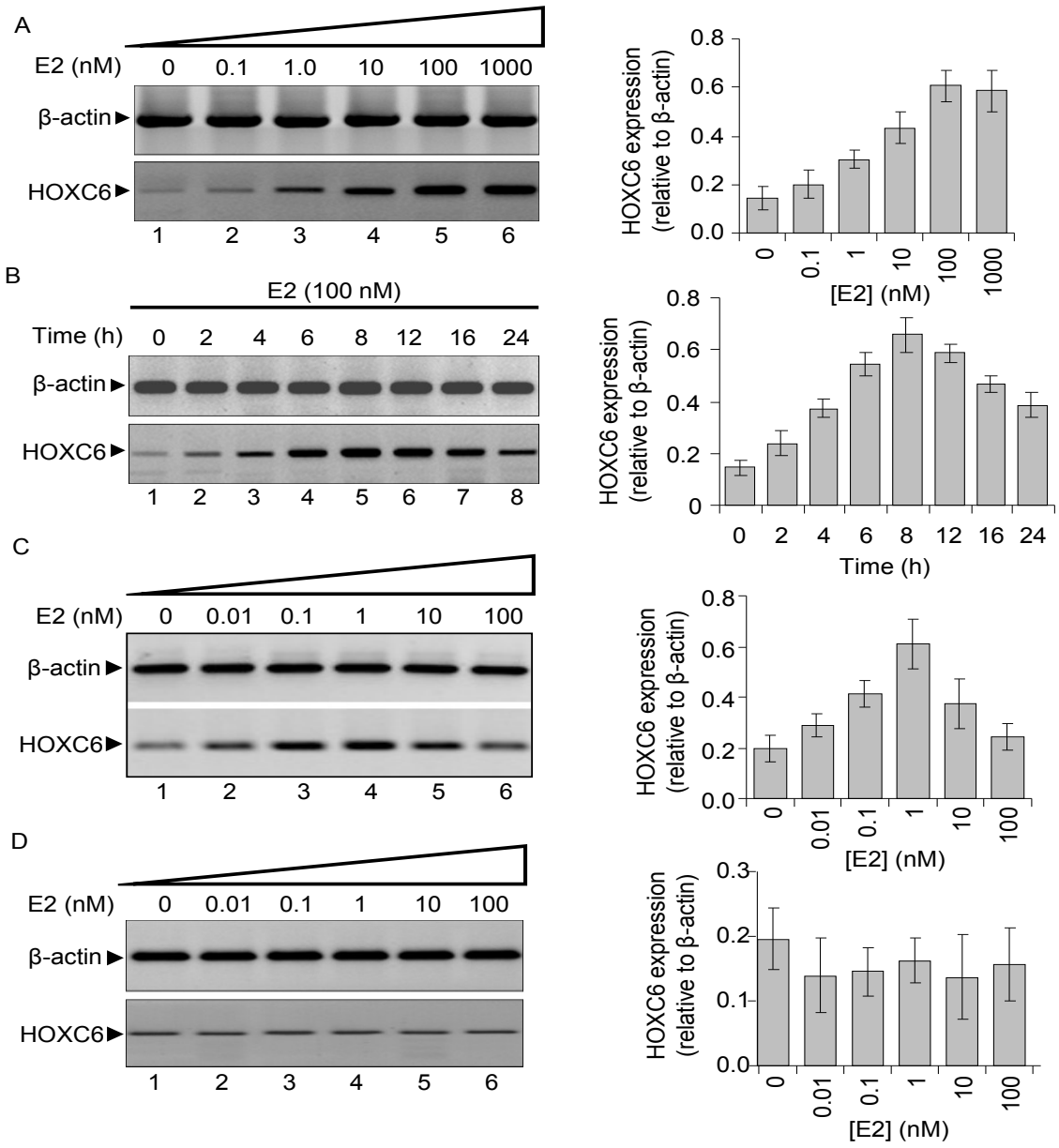


Figure 2.2 Effect of estrogen on HOXC6 gene expression in different cell lines. (A) JAR cells were treated with varying concentrations of E2. RNA from the control and E2-treated cells was isolated, converted to cDNA and analyzed by RT-PCR using primers specific to HOXC6. β-actin was used as a loading control. (B) JAR cells were treated with 100 nM E2 for varying time periods (0-24 h) and reverse transcribed PCR products were analyzed in agarose gel. (C) MCF7 cells were treated with varying concentrations (0-100 nM) of E2. The total RNA was analyzed by PCR using primers specific to HOXC6. (D) MDAMB231 cells was treated with 0-100 nM of E2 and total RNA was analyzed by regular RT-PCR. Expression of HOXC6 (relative to β-actin) was analyzed by real-time PCR as shown on the right of each respective panel. Each experiment was repeated at least thrice. Bars indicate standard errors ($p<0.05$) [54].

2.3.2 HOXC6 promoter contains estrogen response elements (EREs)

Estrogen-responsive genes are regulated via diverse mechanisms involving estrogen receptors (ER) and various ER coregulators [39]. Commonly, upon binding to estrogen, ERs get activated and then targeted to specific DNA sequence elements called estrogen response elements (EREs) present in the promoter of estrogen-responsive genes leading to their transcriptional activation [93]. As HOXC6 showed E2-dependent stimulation, I examined its promoter sequence (up to -3000 nt) for the presence of any consensus EREs (GGTCAnnnTGACC). I found that HOXC6 promoter contains two $ERE_{1/2}$ sites (GGTCA) located at 125 nt and 1143 nt regions upstream of the transcriptional start site (Figure 2.3A). Analysis of the neighboring sequences around these $ERE_{1/2}$ sites revealed that $ERE_{1/2}$ at -125 nt region has GGTCAnnTGACT sequence which has one base pair difference in the palindrome compared to the consensus full ERE (GGTCAnnnTGACC). Furthermore, the palindromic sequences are also separated by two nucleotides instead of three nucleotide separation in a typical full ERE. This analysis suggested that the ERE sequence located at -125 nt region might be an imperfect ERE (termed as $ERE1_{1/2}$, Figure 2.3A). The second $ERE_{1/2}$ site located at -1143 nt regions has no similarity to a consensus full ERE (termed as $ERE2_{1/2}$, Figure 2.3A).

To examine the potential involvement of HOXC6 promoter EREs in estrogen response, I cloned the promoter region containing $ERE1_{1/2}$ and $ERE2_{1/2}$ and also each ERE separately in a luciferase-based reporter construct, pGL3 (clones 1-3, Figure 2.3A). A non-ERE sequence from the HOXC6 promoter was cloned as negative control (clone 4, Figure 2.3A). I transfected each ERE-pGL3 constructs into JAR cells separately, exposed to E2 (100 nM for 8 h) and then analyzed the luciferase induction using a commercial luciferase detection kit. I also co-transfected a renilla luciferase construct and analyzed the renilla expression as an internal transfection control that was used for normalization of luciferase expression from ERE-pGL3 constructs in the absence and presence of E2. My analysis showed that transfection with control plasmid (empty pGL3) or with non-ERE plasmid (non-ERE-pGL3) followed by treatment

with E2 did not have any significant effect on luciferase induction (Figure 2.3B). However, transfection with $ERE1_{1/2}$-$ERE2_{1/2}$-pGL3 (clone 1), $ERE1_{1/2}$-pGL3 (clone 2), or $ERE2_{1/2}$-pGL3 (clone 3) constructs followed by exposure to E2 increased the luciferase induction by about ~7.5, ~5.6 and ~2.3 fold respectively compared to the control (Figure 2.3B). The highest E2 response (luciferase activity) was observed for the construct that contain both EREs together. The higher E2 response of the $ERE1_{1/2}$-pGL3 than $ERE2_{1/2}$-pGL3 is likely due to higher homology of the $ERE1_{1/2}$ with a consensus full ERE than just ERE half site present in $ERE2_{1/2}$ region. Point mutations in $ERE1_{1/2}$ (GGTCA to AATCA) keeping $ERE2_{1/2}$ intact (in clone 1) significantly decreased the luciferase activity (from ~7.5 fold to ~2.6 fold), while mutation in $ERE2_{1/2}$ keeping $ERE1_{1/2}$ intact showed relatively less impact on luciferase induction (from ~7.5 to ~5.1 fold). Mutation for both EREs simultaneously (TGACC to TGAAA) abolished the E2-dependent luciferase induction almost to basal level (Figure 2.3B). These observations suggest that $ERE1_{1/2}$ is major regulator in E2-dependent regulation of HOXC6 though both EREs appear to have interdependent roles.

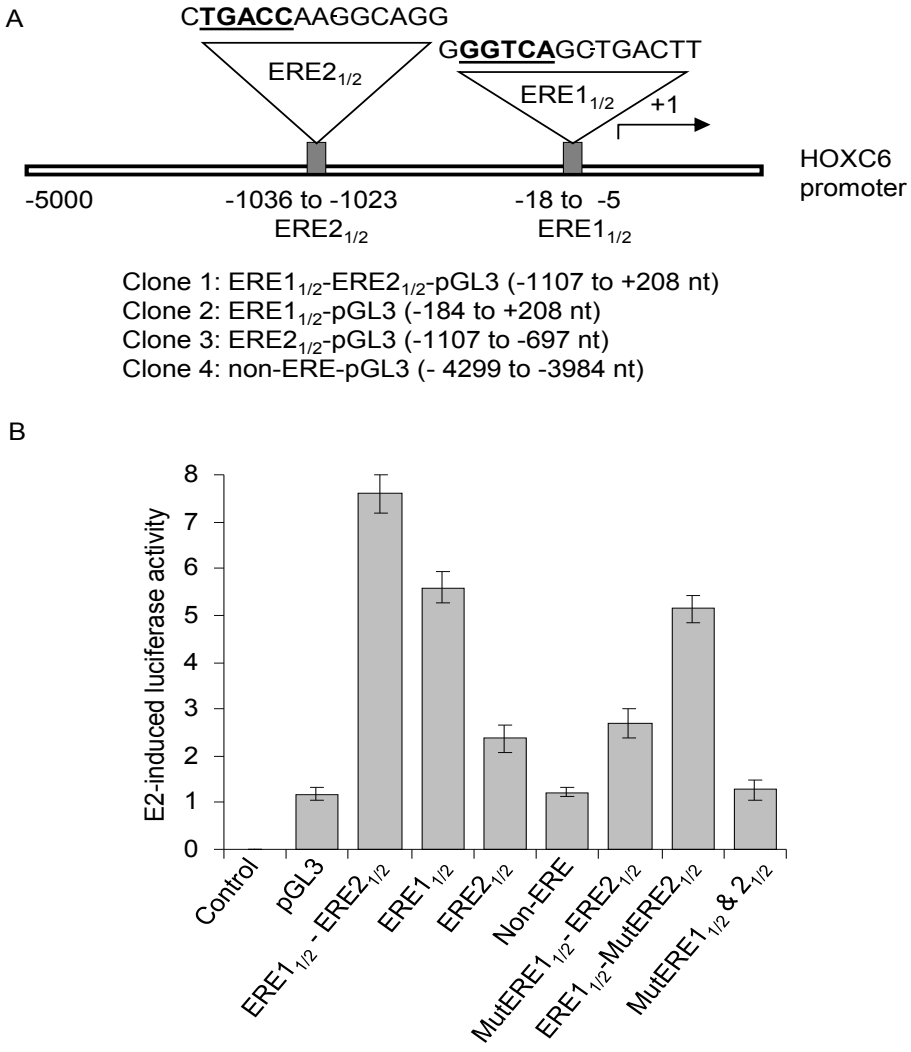


Figure 2.3 HOXC6 promoter EREs and their estrogen response. (A) HOX gene promoter EREs (termed as $ERE1_{1/2}$ and $ERE2_{1/2}$, locations and the neighboring sequences are shown). HOXC6 promoter regions spanning $ERE1_{1/2}$ to $ERE2_{1/2}$, $ERE1_{1/2}$ (alone), $ERE2_{1/2}$ (alone) and a non-ERE regions were cloned (clones 1-4) into a luciferase-based reporter construct, pGL3, used for transfection and reporter assay. In the mutant pGL3 constructs, clone 1 used for mutation of either $ERE1_{1/2}$ or $ERE2_{1/2}$ alone or both $ERE1_{1/2}$ and $ERE2_{1/2}$ simultaneously. For mutations, the GG of $ERE1_{1/2}$ and CC of $ERE2_{1/2}$ were mutated to AA. (B) Luciferase-based reporter assay. $ERE1_{1/2}$-pGL3 and $ERE2_{1/2}$-pGL3 constructs were transfected into JAR cells for 24 h. Control cells were treated with empty pGL3 vector and non-ERE-pGL3. A renilla luciferase construct was also co-transfected along with ERE-pGL3 constructs as internal transfection control. Cells were then treated with 100 nM E2 and subjected to luciferase assay by using Dual-Glo Luciferase Assay System (Promega). The luciferase activities (normalized to renilla activity) in presence of E2 over untreated controls were plotted. The experiment with four replicate treatments was repeated at least twice. Bars indicate standard errors [54].

2.3.3 Estrogen receptors play critical role in E2-mediated transcriptional activation of HOXC6

As ERs are key players in transcriptional regulation of estrogen-sensitive genes [39], I examined roles of ERs in E2-mediated activation of HOXC6. I knocked down ERα and ERβ separately using specific antisense oligonucleotides (Table 2.1) and then exposed the ER-knocked down cells to E2. A scramble antisense (with no homology to ERs) was used as negative control. The knockdown efficiency of ERα and ERβ by respective antisense was analyzed at protein levels using western blot and as expected application of either ERα or ERβ antisense (2.25 μg/ml) knocked down respective ER (lane 3 for ERα knockdown and lane 4 for ERβ knockdown, Figure 2.4A). RNA from ER knocked down and E2-treated cells was reverse transcribed and cDNA was PCR-amplified using primers specific to β-actin (control), ERs and HOXC6. Our results demonstrated that HOXC6 expression was increased as expected upon exposure to E2 (lane 2, Figure 2.4B, real-time quantification in panel C). Application of scramble antisense did not have any significant effects on E2-mediated activation of HOXC6 (lane 3, Figures 2.4B and C). Interestingly, upon depletion of either ERα or ERβ, the E2-dependent activation of HOXC6 was suppressed (compare lanes 4 and 5 with lanes 2 and 3, Figures 2.4B and C). Combined knockdown of ERα and ERβ, further suppressed E2-dependent HOXC6 expression (lane 6, Figures 2.4B and C). These results demonstrated that both ERα and ERβ play important roles in E2-dependent HOXC6 expression.

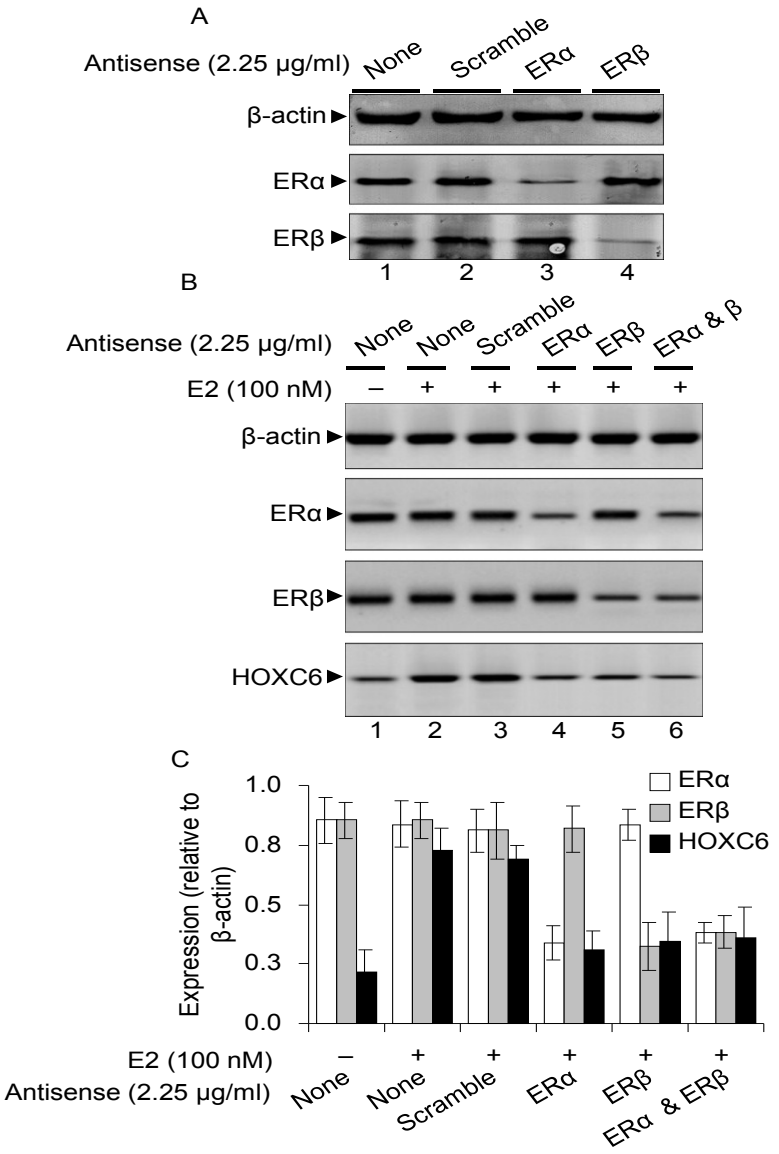


Figure 2.4 Effects of depletion of ERα and ERβ on E2-induced expression of HOXC6. (A) Assessment of ERα and ERβ antisense-mediated knockdown of respective ERs. JAR cells were transfected with ERα, ERβ or scramble antisense (2.25 μg/ml each) for 48 h and proteins were analyzed by western blot using ERα, ERβ, and β-actin antibodies. (B and C) Effects of ERα and ERβ knockdown on E2-mediated activation of HOXC6. JAR cells were transfected with ERα, ERβ or scramble antisense (2.25 μg/ml each) for 48 h separately and treated with E2 (100 nM) for additional 8 h. RNA was isolated and subjected to reverse transcriptase PCR analysis by using primers specific to HOXC6, ERα, and β-actin (loading control). PCR products were analyzed in agarose gel and quantified using real-time PCR (panel C). Each experiment was repeated at least thrice (n = 3). Bars indicate standard errors. Notably, ER knockdown experiments were carried out by Dr. Khairul I. Ansari and these figures are kindly provided by him.

2.3.4 MLLs are essential for regulation of HOXC6 under estrogen environment

As MLL histone methylases are key regulators of HOX genes and several MLLs are implicated in estrogen signaling via their interaction with ERs, I examined if MLLs are involved in E2-dependent activation of HOXC6 expression. I knocked down MLL1, MLL2, MLL3, and MLL4, independently by using specific antisense oligonucleotides (Table 2.1), then exposed the cells to E2 (100 nM for 8 h) and analyzed their impacts on HOXC6 expression using RT-PCR. The application of MLL antisenses resulted in specific knockdown of respective MLLs both at mRNA (compare lane 3 with lanes 1 and 2, Figures 2.5A-D for MLL1 to MLL4, respectively) and protein levels (data not shown). A scramble antisense (with no homology to MLLs) was used as negative control. As seen in figure 2.5A, the application of MLL1-antisense specifically knocked down MLL1 but not β-actin (control) (compare lanes 1 and 2 with lane 3, Figure 2.5A). MLL1 knockdown has no significant effect on E2-mediated activation of HOXC6 (compare lanes 2 and 3, Figure 2.5A). Interestingly, the application of MLL2 antisense not only knocked down MLL2 but also suppressed E2-induced expression of HOXC6 (compare lane 3 with lanes 1 and 2, Figure 2.5B, real-time quantification is in the bottom panel). Similar to MLL2, knockdown of MLL3 also resulted in suppression of E2-mediated activation of HOXC6 (Figure 2.5C, real-time quantification in the bottom panel). However, similar to MLL1, knockdown of MLL4 did not show any significant effect on E2-dependent HOXC6 expression (compare lane 3 with lanes 1 and 2, Figure 2.5D). These observations demonstrated that MLL2 and MLL3 play critical roles in E2-mediated activation of HOXC6.

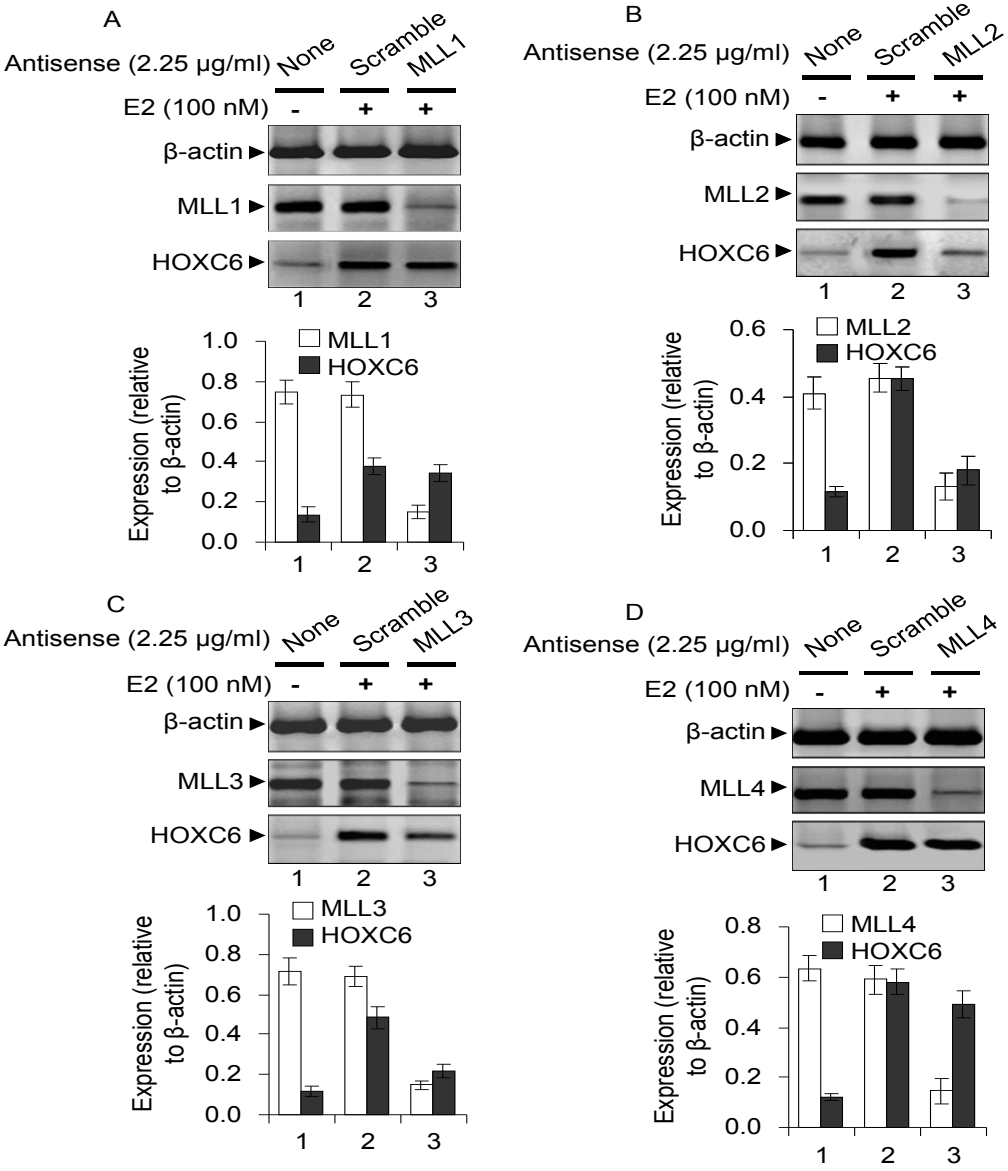


Figure 2.5 Effects of depletion of MLL1, MLL2, MLL3, and MLL4 on E2-induced expression of HOXC6. JAR cells were transfected with 2.25 μg/ml of MLL1 (A), MLL2 (B), MLL3 (C), and MLL4 (D) specific phosphorothioate antisenses separately. Control cells were treated with a phosphorothioate scramble antisense. The antisense-treated cells were incubated for 48 h followed by treatment with 100 nM E2 for 8 h. RNA was isolated from treated and control cells and subjected to reverse transcriptase-PCR by using primers specific to HOXC6 along with MLL1, MLL2, MLL3, and MLL4. β-actin was used as loading control. The PCR products were analyzed by agarose gel and quantified. Real-time PCR quantification of the cDNA relative to β-actin showing the relative levels of respective MLL and HOXC6 expression are shown in the respective bottom panel. Notably, MLL knockdown experiments were carried out by Dr. Khairul I. Ansari and these figures are kindly provided by him.

2.3.5 ERs and MLLs bind to HOXC6 promoter in an E2-dependent manner

As HOXC6 promoter contains two $ERE_{1/2}$ sites and ERs are involved in E2-dependent stimulation of HOXC6, I analyzed the *in vivo* bindings of ERα and ERβ to the HOXC6 promoter EREs in the absence and presence of E2 using chromatin immunoprecipitation (ChIP) assay. In brief, JAR cells were treated with E2 (100 nM for 8 h), fixed with formaldehyde, sonicated to shear the chromatin and then subjected to ChIP with antibodies against ERα, ERβ and β-actin (control). The immunoprecipitated DNA fragments were PCR-amplified using primers spanning $ERE1_{1/2}$ and $ERE2_{1/2}$ regions of HOXC6 promoter (Figures 2.6A and B). The real-time PCR quantifications of the ChIP DNA fragments are shown in figure 2.6C. A promoter segment (-4299 to -3984 nt) containing no ERE site was used as control (non-ERE). As seen in figures 2.6B and C, no significant binding of β-actin was observed in $ERE1_{1/2}$, $ERE2_{1/2}$ and non-ERE regions irrespective of E2. However, the binding of ERα and ERβ were enhanced in both $ERE1_{1/2}$ and $ERE2_{1/2}$ in an E2-dependent manner (compare lane 1 with 2, and 3 with 4, Figures 2.6B and C). No significant binding of ERs was observed in non-ERE region (lane 5 and 6, Figure 2.6B). These observations suggested that ERα and ERβ are both associated with E2-mediated activation of HOXC6 via binding to ERE regions.

Next, I examined the E2-dependent binding of different MLLs (MLL1-4) in the HOXC6 promoter using ChIP assay with antibodies specific to different MLLs. These analyses demonstrated that binding of MLL2 and MLL3 was increased in both $ERE1_{1/2}$ and $ERE2_{1/2}$ in presence of E2 (compare lanes 1 with 2 for binding in $ERE1_{1/2}$, and 3 with 4 for binding in $ERE2_{1/2}$ regions, Figures 2.6D, real-time PCR analysis of the ChIP DNA samples is shown in figure 2.6E). E2-dependent binding of MLL2 and MLL3 are more robust in $ERE1_{1/2}$ region in comparison to the $ERE2_{1/2}$ (compare lanes 1 and 2 with 3 and 4, Figures 2.6D and E). In contrast to MLL2 and MLL3, binding of MLL1 and MLL4 were not enhanced in presence of E2, instead decreased level of binding of MLL1 (to $ERE2_{1/2}$) and MLL4 (to $ERE1_{1/2}$) were observed

in presence of E2 (Figures 2.6D and E). These results demonstrated further that, in addition to ERs, MLL2 and MLL3 play critical role in E2-dependent activation of HOXC6.

To further confirm the E2-dependent binding of ERs and MLLs to HOXC6 promoter, I analyzed their binding pattern in a time-dependent manner using ChIP assay with ERs and MLLs antibodies. ChIP DNA samples were PCR-amplified using real-time PCR and plotted (Figure 2.7). In agreement with above studies, I observed that bindings of ERα and ERβ were enhanced in both $ERE1_{1/2}$ and $ERE2_{1/2}$ regions in presence of E2 in a temporal manner (Figures 2.7A and B). ERα and ERβ enrichments were observed as early as 15 min and increased with time reaching a saturation within 2-3 h, (Figures 2.7A and B). Recruitment of MLL2 and MLL3 were also enhanced in both $ERE1_{1/2}$ and $ERE2_{1/2}$ regions in presence of E2, though kinetics of their recruitment to different EREs was different (Figures 2.7C and D). In the $ERE1_{1/2}$ region, the enhanced recruitment of MLL3 was observed as early as 15 min after E2 treatment and then reached to saturation within 2 h and this kinetics appeared to be very similar to recruitment of ERα and ERβ in the $ERE1_{1/2}$ region (Figure 2.7C). However, the E2-dependent binding of MLL2 to the $ERE1_{1/2}$ was delayed to 4 h post E2 treatment and then increased and reached to saturation at around 6-8 h (Figure 2.7C). However, in the $ERE2_{1/2}$ region, similar to the kinetics of recruitment of ERs, E2-dependent recruitment of MLL2 and MLL3 was initially increased and then reached to saturation within 2-3 h (Figure 2.7D). Interestingly however, in agreement with my observation in figure 2.5, significant amount of constitutive binding of MLL4 to $ERE1_{1/2}$ and MLL1 to $ERE2_{1/2}$ regions were observed and these bindings were gradually decreased in a time-dependent manner in presence of E2 (Figures 2.7C and D). No binding of MLL1 to the $ERE1_{1/2}$ and MLL4 to the $ERE2_{1/2}$ were observed irrespective of presence of E2 (Figures 2.7C and D). These studies further suggested that MLL2 and MLL3 along with ERα and ERβ play key roles in E2-dependent activation of HOXC6, while MLL1 and MLL4 might be involved in basal transcription of HOXC6.

As E2 treatment enhanced recruitment of MLL histone methylases onto HOXC6 EREs, I analyzed if H3K4-trimethylation level at the HOXC6 promoter is also enhanced upon exposure to E2. I observed that H3K4-trimethylation level and RNA polymerase II (RNAPII) recruitment were increased in the ERE regions of HOXC6 promoter in a time-dependent manner as a function of E2, while the net level of histone H3 at the HOXC6 promoter region remained almost constant (Figures 2.7E and F).

It is known that general transcription factor TFIID interacts with trimethylated histone H3K4 and facilitates the pre-initiation complex (PIC) assembly at the gene promoter [94]. I examined if components of TFIID are also concomitantly recruited in the HOXC6 promoter with increase in H3K4-trimethylation and RNAPII in presence of E2. My ChIP analysis showed that along with enrichment in H3K4-trimethylation and RNAPII level, there is increased recruitment of TBP (TATA binding protein, component of TFIID) and TAF250 (TBP-associated factor 250) in the HOXC6 promoter EREs in presence of E2 (lanes 2 and 3, Figure 2.8A and B). Knockdown of either MLL2 or MLL3 decreased the E2-dependent enrichment of TBP, TAF250, H3K4-trimethylation and RNAPII (lanes 4 and 5, Figure 2.8A and B), indicating critical roles of MLL2 and MLL3 histone methylases in PIC assembly at the HOXC6 promoter during E2-mediated gene activation.

To understand if any corepressors were involved in maintaining transcriptionally repressed state of HOXC6 in absence of E2, I examined the binding of N-CoR (nuclear receptor corepressor) and SAFB1 (scaffold attachment factor B1), the two well-known nuclear receptor corepressors, using ChIP assay [95]. Interestingly, we found that both N-CoR and SAFB1 were bound to the HOXC6 promoter (in both EREs) in the absence of E2 and their binding was gradually decreased upon exposure to E2 (Figures 2.8C and D, quantification in the respective bottom panels) indicating their critical role in suppressing HOXC6 gene expression under non-activated condition. Bindings of both N-CoR and SAFB1 were decreased upon treatment with E2 in a time dependent manner while the binding of ERα was increased.

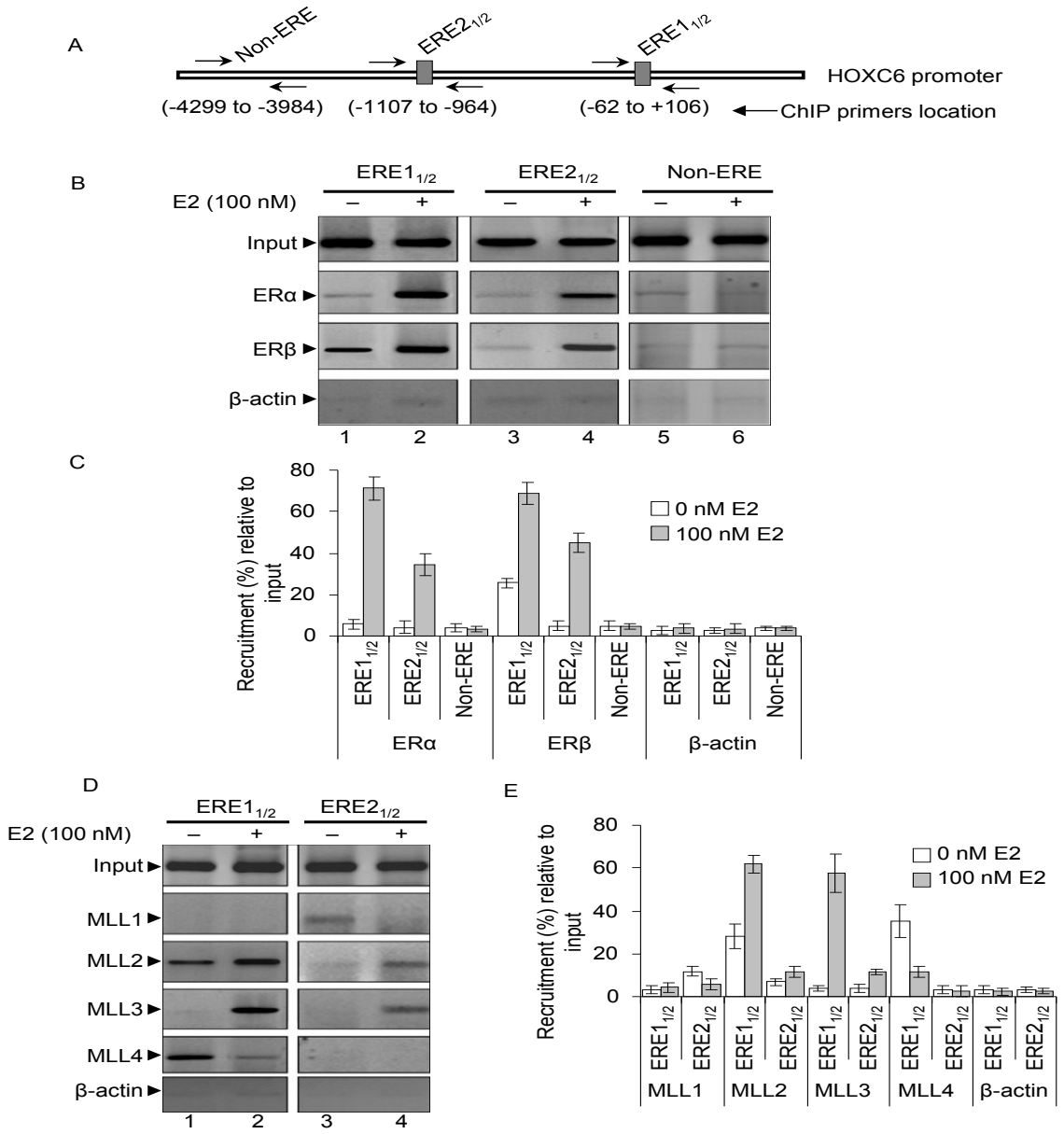


Figure 2.6 E2-dependent recruitment of ERs and MLLs in the ERE regions of HOXC6 promoter. (A) Scheme showing positions of ChIP PCR primers. (B and C) Recruitment of ERs: JAR cells were treated with 100 nM E2 for 8 h and subjected to ChIP assay using antibodies specific to ERα and ERβ. The immunoprecipitated DNA fragments were PCR-amplified using primers specific to $ERE1_{1/2}$ and $ERE2_{1/2}$ of HOXC6 promoter. Primers specific to a promoter sequence containing no ERE (Non-ERE) was used as negative control. ChIP DNA fragments were analyzed by real-time PCR and shown in the figure C. (D and E) Recruitment of MLLs (MLL1-MLL4): JAR cells were treated same way as explained in figure 2.6 (B and C) using antibodies specific to MLL1, MLL2, MLL3 and MLL4. Each experiment was repeated at least thrice. Bars indicate standard errors [54].

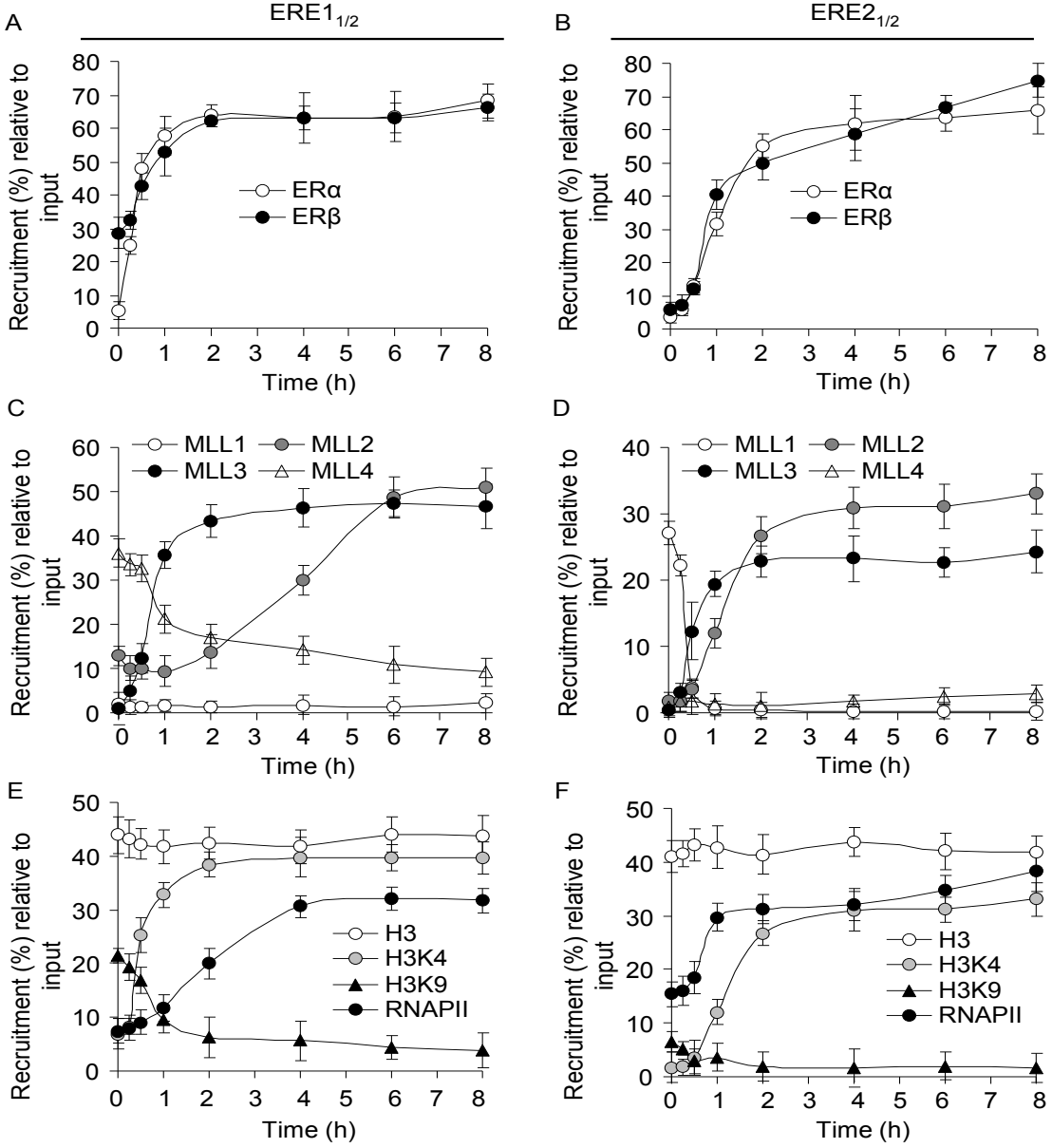


Figure 2.7 Dynamics of recruitment of ERs and MLLs onto HOXC6 promoter: Cells were treated with 100 nM E2 for varying time periods (0 - 8 h) and then subjected to ChIP assay using antibodies specific to ERα, ERβ, MLL1, MLL2, MLL3, MLL4, H3K4-trimethyl and RNAPII. Immunoprecipitated DNA fragments were PCR-amplified using primers specific to $ERE1_{1/2}$ and $ERE2_{1/2}$ of HOXC6 promoter respectively, quantified and plotted. (A and B) Recruitment of ERα and ERβ in the $ERE1_{1/2}$ and $ERE2_{1/2}$. (C and D) Recruitment of MLL1-4 in the $ERE1_{1/2}$ and $ERE2_{1/2.}$ (E and F) Recruitment of RNAPII and level of histone H3 (control), H3K4-trimethylation and H3K9-dimethylation. Each experiment was repeated at least thrice. Bars indicate standard errors [54].

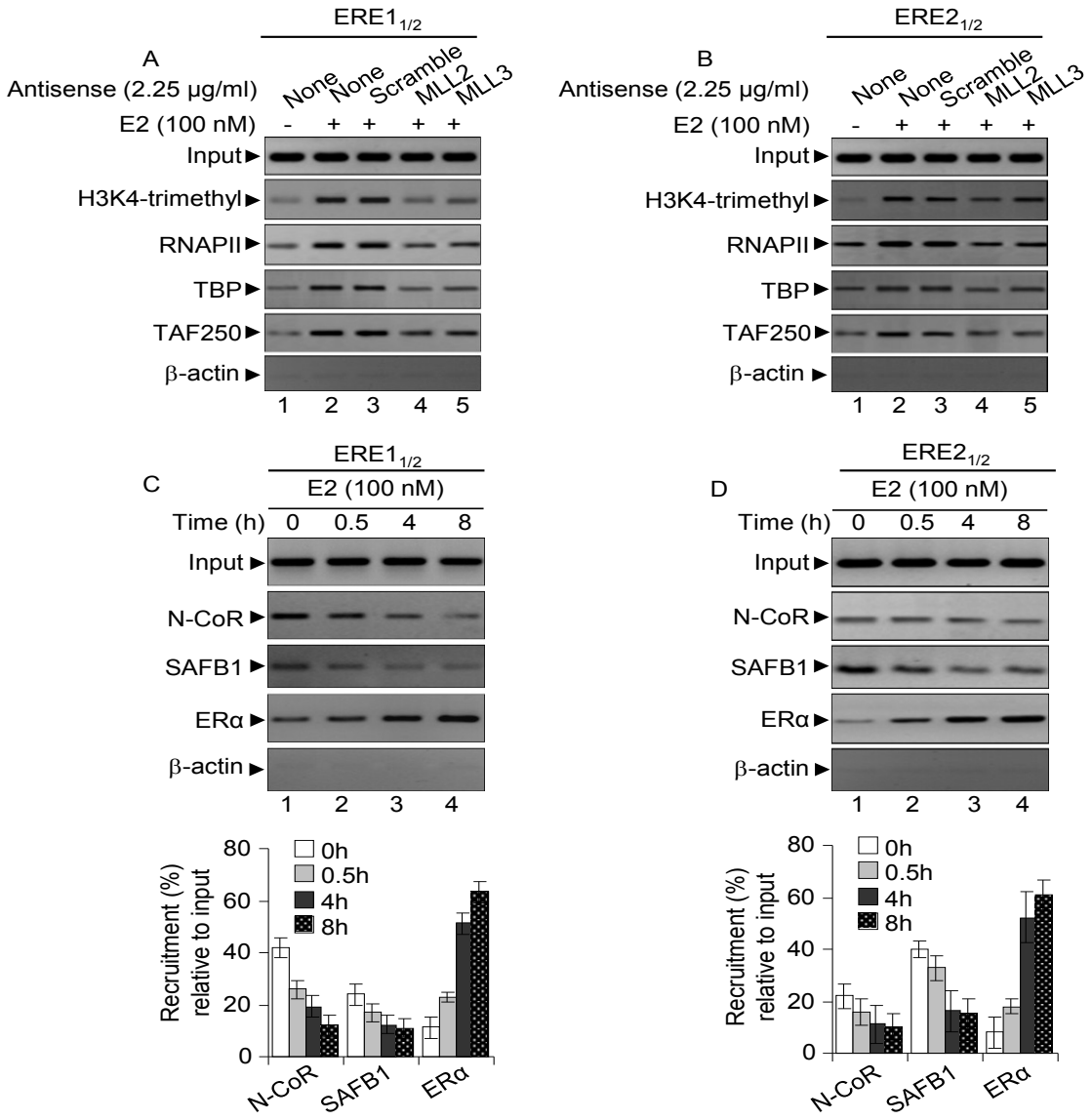


Figure 2.8 Roles of MLL2 and MLL3 in E2-dependent assembly of general transcription factors and RNAPII in the HOXC6 promoter and E2-dependent recruitment of N-CoR and SAFB1 in ERE regions of HOXC6 promoter in absence and presence of E2. (A and B) JAR cells were transfected with MLL2, MLL3 or scramble antisenses for 48 h and then treated with 100 nM E2 for additional 8 h and subjected to ChIP assay by using antibodies specific to H3K4-trimethyl, RNAPII, TBP, TAF250. The immunoprecipitated DNA fragments were PCR-amplified using primers specific to $ERE1_{1/2}$ and $ERE2_{1/2}$ regions of HOXC6 promoter. β-actin are used as negative control IgG. (C and D) JAR cells were treated with 100 nM E2 for varying time periods and subjected to ChIP assay using antibodies specific to N-CoR and SAFB1. Antibodies specific to ERα and β-actin are used as positive and negative control IgG. The real-time PCR quantification of the recruitment level is shown below the respective panels. Each experiment was repeated at least thrice. Bars indicate standard errors [54].

2.3.6 MLL2 and MLL3 are recruited to the HOXC6 promoter in an ER-dependent manner

ERs are well known to bind directly to EREs of estrogen-responsive genes via their own DNA binding domain. Notably, MLLs (MLL1-4) also have several DNA binding domains and these DNA binding domains may facilitate their direct binding with the promoter [1]. Alternatively, MLLs may be recruited to the promoter via interactions with ERs or other associated proteins. Notably, MLL2 and MLL3 have multiple LXXLL domains (NR-boxes) and are previously reported to interact with ERα in presence of estrogen [9,29]. I examined if MLL2 and MLL3 that are involved in E2-mediated activation of HOXC6 bind to HOXC6 EREs directly or their bindings are dependent on ERs. To examine this, I knocked down ERα and ERβ separately, then exposed the cells to E2 (100 nM for 8 h) and analyzed the status of MLL2 and MLL3 recruitment to $ERE1_{1/2}$ and $ERE2_{1/2}$ regions of HOXC6 promoter in the absence and presence of E2 (Figure 2.9). Our results demonstrated that binding of MLL2 and MLL3 were increased in both $ERE1_{1/2}$ and $ERE2_{1/2}$ regions in presence of E2 (lanes 1, 2 and 5, 6, Figure 2.9). However, knockdown of either ERα or ERβ, decreased (or even abolished) the recruitment of MLL2 and MLL3 onto both the EREs (compare lanes 3 and 4 with 2 and lanes 7 and 8 with 6, Figure 2.9). These results demonstrated that binding of both MLL2 and MLL3 to the HOXC6 promoter (in presence of E2) is dependent upon ERα and ERβ.

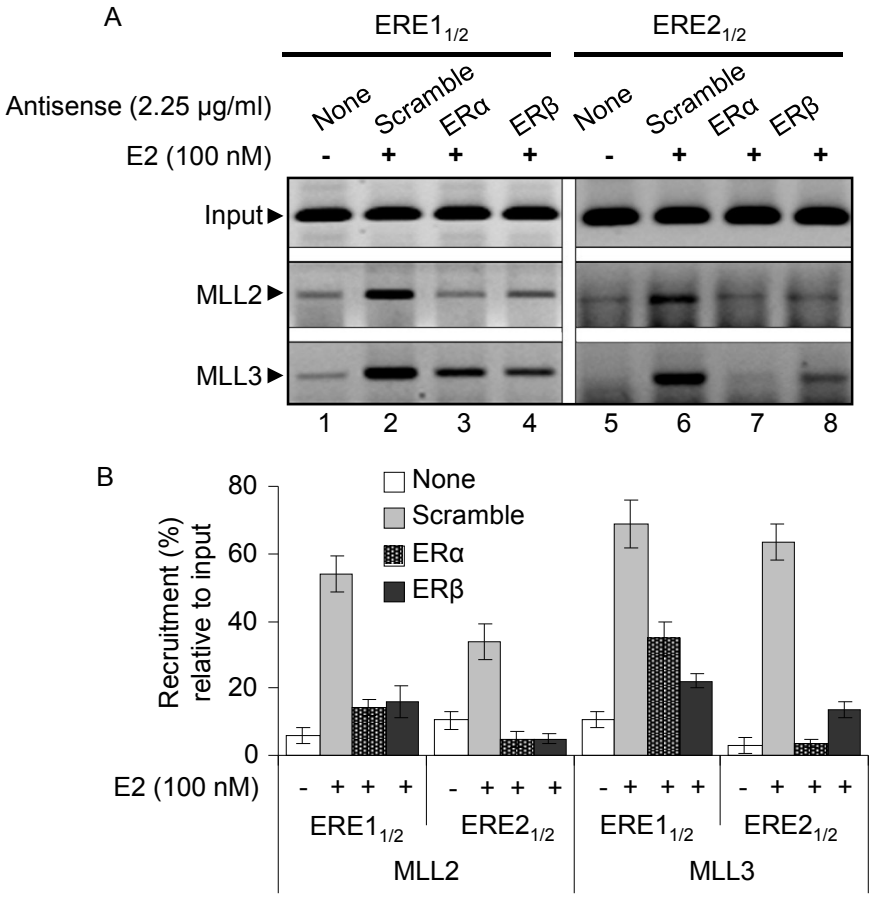


Figure 2.9 Roles of ERα and ERβ on E2-dependent recruitment of MLL2 and MLL3. JAR cells were transfected with ERα and ERβ antisense for 48 h followed by exposure to E2 (100 nM for additional 8 h). Cells were harvested and subjected to ChIP assay using anti-MLL2 and anti-MLL3 antibodies. The immunoprecipitated DNA fragments were PCR-amplified using primers specific to $ERE1_{1/2}$ and $ERE2_{1/2}$ regions of HOXC6 promoter and subjected to real-time PCR quantification and plotted in panel B [54].

2.4 Discussion

HOX genes are critical players in the development and diseases and therefore understanding their roles and regulation is important [83]. Vast bodies of literature exist that address the functions of different HOX genes during embryonic development in various types of organism. Increasing amounts of evidence suggest that beyond their critical roles in development, various HOX genes are misregulated and over-expressed in variety of disease including breast and prostate cancer [83]. HOX genes are potential target for novel biomarker

development and targeted gene therapy [22,62,84]. In spite of their roles in development and disease, little is known about the mechanism by which these HOX genes may be expressed and regulated in different types of tissues or in cancer cells. Increasing amounts of studies indicate that HOX genes (especially HOXA cluster genes) are potentially regulated by steroid hormones and may be misregulated upon exposure to endocrine disruptors [57,96]. In my studies I focused towards understanding the regulatory mechanism of HOXC6 especially in presence of steroid hormone estrogen. HOXC6 is expressed in various steroidogenic tissues [58,59,60]. HoxC6 homozygous mutant female mice showed the absence of epithelial cells in thoracic mammary gland [59,62]. In mammary glands of ovariectomized animals, HoxC6 transcript levels are substantially elevated compared to glands from intact virgin mice indicating a link between ovarian hormones and HoxC6 repression [58,59,62]. HOXC6 expression is associated with osteosarcomas, medulloblastomas, breast and prostate carcinomas [58,59,62]. My studies demonstrated that HOXC6 gene is transcriptionally activated upon exposure to E2 in human breast cancer (MCF7) and placental choriocarcinoma cell lines (JAR).

I have also investigated the molecular mechanism by which E2 regulates HOXC6 gene expression. Sequence analysis revealed that HOXC6 promoter contains two putative EREs, within first 3000 nt upstream of transcription start site. $ERE1_{1/2}$ which is located at -5 nt, is a nearly complete full ERE, whereas $ERE2_{1/2}$ (at -1023 nt) region is a ERE half-site. Luciferase-based reporter analysis demonstrated that both $ERE1_{1/2}$ and $ERE2_{1/2}$ are responsive to E2, $ERE1_{1/2}$ being more responsive than $ERE2_{1/2}$. Mutation of $ERE1_{1/2}$ resulted in significant loss in E2-dependent luciferase induction, in comparison to mutation in the $ERE2_{1/2}$ (Figure 2.3). $ERE1_{1/2}$ and $ERE2_{1/2}$ appeared to have interdependent E2 response in luciferase induction suggesting their potential coordination during E2-mediated gene activation. The enhanced E2 response of $ERE1_{1/2}$ over $ERE2_{1/2}$ further suggests that the $ERE1_{1/2}$ is potentially an imperfect full ERE. Notably, genes with imperfect EREs are well-known to be regulated by estrogen and estrogen receptors[57,97]. Though, it is obvious that ERE-pGL3 are artificial constructs and do not

represent a native chromatin environment of HOXC6 promoter present in the cell nucleus, the induction of luciferase activity upon E2 expsoure suggested that $ERE1_{1/2}$ and $ERE2_{1/2}$ sequences of HOXC6 promoter are responsive to estrogen.

Antisense-mediated knockdown experiments demonstrated that both ERα and ERβ are involved in E2-mediated activation of HOXC6. Generally, ERs bind to the EREs of estrogen-responsive genes as a function of estrogen. Depending on the target gene and cell types, ERα and ERβ, can form homo- and/or heterodimers prior to binding to the response elements on the target gene promoters. ChIP analysis demonstrated that both ERα and ERβ bind to the $ERE1_{1/2}$ as well as $ERE2_{1/2}$ (Figure 2.6) although binding to the $ERE1_{1/2}$ is slightly more efficient than $ERE2_{1/2}$. Some amount of constitutive binding of ERβ is also observed in the $ERE1_{1/2}$ region in the absence of E2, which may have implication in regulation of HOXC6 under basal environment (Figure 2.6A). Temporal studies (Figure 2.7) also demonstrated that binding of ERs to $ERE1_{1/2}$ takes places at earlier time points than $ERE2_{1/2}$. The higher and faster response of $ERE1_{1/2}$ than $ERE2_{1/2}$ towards ERs binding is likely due to the difference in imperfect full ERE ($ERE1_{1/2}$) versus ERE-half site ($ERE2_{1/2}$). Importantly, in the transient transfection-based luciferase assay (Figure 2.3), I also observed higher sensitivity of $ERE1_{1/2}$ towards E2 exposure than $ERE2_{1/2.}$

During E2-mediated gene regulation, in addition to ER, various other activators and coactivators (commonly known as ER coregulators) participate in the process and bind to the promoter of estrogen-sensitive genes leading to their activation [93]. Diverse arrays of ER coregulators have been discovered and many of them contain enzymatic activities (such as acetyl-transferase activity) that presumably modify chromatin, lead to structural changes and chromatin remodeling resulting in transcription activation [39,98]. Recent studies demonstrated that histone methylases MLL2, MLL3, and MLL4, act as co-activators for ERs in regulation of E2-responsive genes [9,29,37,55]. Notably proteins containing LXXLL (NR-box) domains are known to interact with nuclear hormone receptors (NRs) and play critical roles in ligand-dependent gene

activation [1]. Sequence analysis of the MLLs showed that MLL1 contains only one LXXLL domain that remains buried in its globular domain [1,7]. Whereas MLL2, MLL3, and MLL4 contain multiple NR boxes indicating their more facile interaction with ERs [1,7]. My studies (Figure 2.5) demonstrated that, antisense-mediated knockdown of MLL2 and MLL3 resulted in downregulation of E2-dependent activation of HOXC6. However, knockdown of MLL1 and MLL4 had no significant effect in this process. ChIP analysis (Figures 2.6 and 2.7) demonstrated that, MLL2 and MLL3 were bound to the $ERE1_{1/2}$ and $ERE2_{1/2}$ regions of the HOXC6 promoter in an E2-dependent manner. These results demonstrated that MLL2 and MLL3 play critical roles in E2-mediated activation of HOXC6. In contrast to MLL2 and MLL3, I observed binding of some amount of MLL1 (to $ERE2_{1/2}$) and MLL4 (in the $ERE1_{1/2}$) even in the absence of E2 (Figures 2.6 and 2.7) and these bindings of MLL1 and MLL4 were decreased upon addition of E2. Thus, these observations suggest that, upon transcriptional activation by E2, there may be an exchange of basal transcription factors (such as MLL1 and MLL4 in this case) with the factors that are associated with activated transcription (such as MLL2 and MLL3). These observations further indicate that MLL1 and MLL4 may be involved in E2-independent basal transcriptional regulation and maintenance of HOXC6 expression, while MLL2 and MLL3 are critical for E2-dependent transcription activation of HOXC6.

ERs are well-known for binding to the EREs of estrogen-responsive genes via their DNA binding domains [39]. However, the recruitment of MLL2 and MLL3 to the HOXC6 promoter could have different options. Both MLL2 and MLL3 contain DNA binding domains in their N-terminus which may lead to their direct binding to the promoter, though it may not depend on estrogen. Otherwise, these MLLs may interact with ERs via their NR-boxes leading to their recruitment onto the ERE regions via ERs. My results demonstrated that independent knockdown of both ERα and ERβ resulted in decreased binding of MLL2 and MLL3 into the EREs of HOXC6 suggesting ER-dependent mode of binding of these MLLs (Figure 2.10). Notably, both ERα and ERβ are known to regulate ER-responsive genes either independently or in combination [39]. JAR

cells do express both ERα and ERβ, and both ERs are involved in E2-mediated activation of HOXC6. So it is likely that MLL2 and MLL3 interact with ERα and ERβ and bind to EREs that facilitate the recruitment of MLL2 and MLL3 into the HOXC6 promoter leading to HOXC6 transactivation.

What could be the potential roles of MLL2 and MLL3 in E2-mediated HOXC6 activation? MLL2 and MLL3 are both histone H3K4-specific methyl-transferases and H3K4-trimethylation is critical for transcription activation. Analysis (Figures 2.7E and 2.7F) of the H3K4-trimethylation status in HOXC6 promoter demonstrated that similar to MLL2 and MLL3, the level of H3K4-trimethylation is increased in the HOXC6 promoter upon exposure to E2. This finding suggests that MLL2 and MLL3 may be acting as the histone H3K4-trimethylases that help in promoter opening (via recruitment of other chromatin remodelers) and recruitment of general transcription factors (GTFs) including RNAPII, leading to transcription activation. Indeed our results (Figure 2.8A and 2.8B) demonstrated that along with enrichment of H3K4-trimethylation and RNAPII recruitment, binding of TFIID components such as TBP and TAF250 were increased upon treatment with E2 and these bindings were decreased upon knockdown of either MLL2 or MLL3 indicating key roles of MLL2 and MLL3 in E2-dependent histone H3K4-trimethylation, recruitment of GTFs, RNAPII and assembly of transcription pre-initiation complexes.

Furthermore, I observed that histone methylases MLL2 and MLL3 are actively exchanged with MLL1 and MLL4 upon treatment with E2 and ER binding causing the transition from basal to activated transcription state of HOXC6. The obvious question is “Does HOXC6 remain repressed in the absence of E2?” To address this I analyzed the binding of two well-known nuclear receptor corepressors N-CoR and SAFB1 in the absence and presence of E2. Notably, SAFB1 and N-CoR function as ER corepressors, they can directly interact as well as with ER, and repress transcription[35,95]. Our studies demonstrated that indeed N-CoR and SAFB1 were bound to both $ERE1_{1/2}$ and $ERE2_{1/2}$ in the absence of E2 (Figure 2.8C and 2.8D). Bindings of both N-CoR and SAFB1 were decreased upon treatment with E2 in a time dependent manner

while the binding of ERα was increased. These observations suggested that HOXC6 transcription was originally repressed by corepressors in the absence of E2 and this repression was revived in the presence of E2 which is mediated via binding of ERs and various ER coactivators including MLL2 and MLL3. Constitutive binding of ERs (as observed in Figure 2.6A) may be responsible for the recruitment of N-CoR and SAFB1 corepressors in the HOXC6 promoter in absence of E2. The detailed mechanism of transcriptional repression, functional interactions of N-CoR, SAFB1 with the HOXC6 promoter and different histone modification states and their coordination with MLL1 and MLL4 still needs to be investigated. In addition, I also cannot explain why there is an exchange of MLL1 and MLL4 with MLL2 and MLL3 upon E2 expsoure, even though MLL1 and MLL4 could have done the histone methylation job, interaction with ERs, and promoter opening. It may be hypothesized that MLL2 and MLL3, in addition to their histone methylation activities, specifically interact with and recruit various other ER coregulators that are specific to HOXC6 gene expression and regulation.

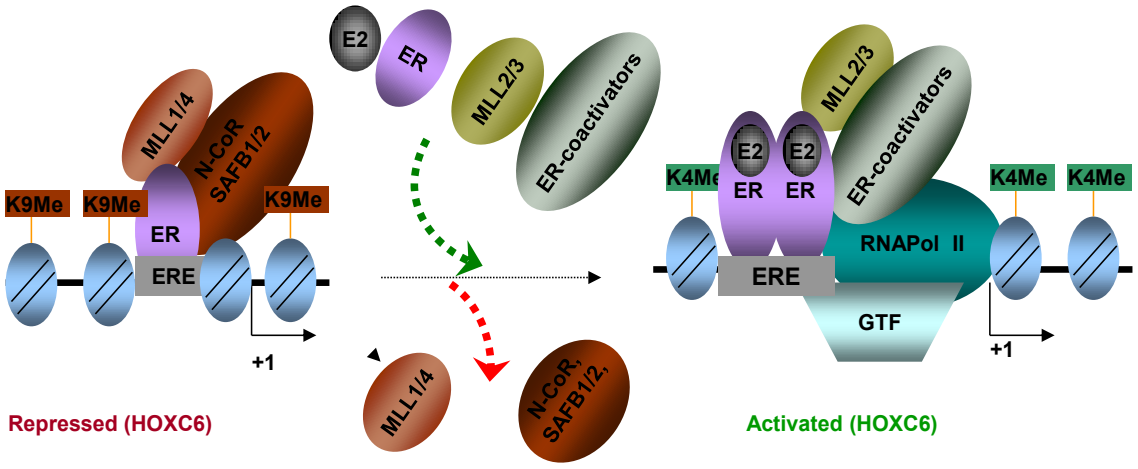


Figure 2.10 Graphical model of HOXC6 gene regulation by MLLs in an estrogen environment. HOXC6 is transcriptionally regulated by E2 and histone methylases MLL2 and MLL3, along with ERs, play critical roles in E2-mediated activation of HOXC6. MLL1 and MLL4, and NR corepressors (N-CoR, SAFB1) are bound to the HOXC6 promoter in the absence of E2 and are replaced with MLL2 and MLL3 during E2-mediated gene activation [54].

Notably, HOXC6 is expressed in various steroidogenic tissues and over-expressed in hormone-sensitive breast and prostate cancers indicating critical roles of steroid hormone in

transcriptional regulation of HOXC6 [58,59,62]. In contrast, increased expression of HoxC6 in mammary glands of ovariectomized female mice indicates potential negative regulation of this gene by ovarian hormone [84,99]. These observations suggest that HOXC6 expression could both be positively and negatively regulated by steroid hormones and which is likely dependent on tissue type. My studies demonstrated that HOXC6 is transcriptionally activated by estrogen in MCF7 as well as JAR cells. Overall my studies demonstrated that histone methylases MLL2 and MLL3 coordinate with ERα and ERβ, bind to the HOXC6 promoter in an estrogen-dependent manner and transcriptionally regulate HOXC6 gene expression.

CHAPTER 3

BREAST CANCER ASSOCIATED GENE HOXA5 IS REQUIRED FOR CELL GROWTH, VIABILITY AND IT IS TRANSCRIPTIONALLY REGULATED BY ESTRADIOL AND ESTROGENIC ENDOCRINE DISRUPTING CHEMICALS BISPHENOL-A AND DIETHYLSTILBESTROL

3.1 Introduction

Homeobox-containing genes (HOX genes) are highly conserved family of genes that play vital roles during cell differentiation and embryogenesis [1,7,8]. HOX genes expression controls the anterio-posterior patterning during embryogenesis [1,7,33]. In human there are 39 HOX genes that are classified in four different groups (HOXA-D) based on the sequence similarity and chromosomal locations [1,33]. HOX proteins primarily act as transcription factors [23,33]. They bind to their target gene promoters via homeodomain and regulate target gene expression [1,23]. Though HOX genes were originally described to exhibit collinear expression especially during embryogenesis, recent studies demonstrate that each HOX gene appears to be independently regulated and expressed in adult tissues. It is increasingly being recognized that HOX genes are dysregulated in a variety of cancers and closely associated with oncogenesis [1,7,60]. HOX genes expression is critical for cancer cell proliferation, angiogenesis and tumor growth [1,7,8]. For example, expression of HOXA1, HOXA4, HOXB3, HOXB4, HOXB6, HOXC6, HOXB7, and HOXA10 were detected in a human breast cancer cell line and also in primary breast carcinomas [60]. HOXB7 over-expression is linked with cell proliferation, neoplastic transformation and tumorigenesis [65]. Studies also demonstrated that HOXA5 expression is lost in 60 % human breast cancer [58]. This loss occurs via promoter hypermethylation in breast cancer cell lines and carcinomas. In this study, I demonstrate that HOXA5 is differentially over-expressed in breast cancer tissues and in estrogen receptor positive breast cancer cells.

Though HOX genes appear to play critical roles in oncogenesis and tumor cell proliferations, their detailed mechanism of regulation remains unclear. Steroid hormones are known to influence the expression of hormone-responsive genes during hormonally regulated organogenesis and disease [7,8,9]. For example, HOXC6 which is a critical player in mammary gland development, is over-expressed in breast and prostate cancer, is transcriptionally regulated by estradiol [54]. HOXC10, a crucial player in spinal cord development, is transcriptionally regulated by estrogen and is over-expressed in breast cancer cells and tissues [7].

In addition to natural hormones, various environmental chemicals interfere with endocrine signaling and long term exposure to endocrine disrupting chemicals has been linked with abnormal reproduction, developmental defects as well as carcinogenesis [91]. Agents with estrogen-like activity such as bisphenol-A (BPA), diethylstilbestrol (DES) and methoxychlor are shown to induce organ-specific developmental changes in the female reproductive tract [97,100]. Notably, BPA is commonly found in plastics, metallic storage containers and various other routinely used consumables and DES is an orally active synthetic nonsteroidal estrogen drug that has been used extensively for estrogen replacement therapy and induction of fertility among others [101]. The endocrine disruptors or E2-mimics such as BPA and DES etc have structural similarity with ERs that allow them to disrupt E2-ER-ERE complex at the target gene promoter aiding in misregulation of target genes. BPA, DES and many other endocrine disrupting chemicals (EDCs) are major environmental and health concern, though knowledge is limited in terms of identifying potential EDCs, assessing their risk, management, and understanding their mechanism of action.

My studies demonstrated that HOXA5 is over-expressed in breast cancer tissue and estrogen-receptor positive breast cancer cells. HOXA5 expression is critical for cell viability. Furthermore, we also demonstrate that HOXA5 is transcriptionally regulated by estrogen and dysregulated by endocrine disrupting chemicals such as BPA and DES.

3.2 Materials and methods

3.2.1 Cell culture condition, E2, BPA and DES treatment

CCL228 (colon cancer), H358 (bronchioalveolar carcinoma), HeLa (cervical cancer), JAR (choriocarcinoma placenta), MCF10 (normal breast epithelial) , MCF7 (ER-positive adenocarcinoma mammary), T47D (ER-positive ductal carcinoma mammary), and MDAMB231 (ER-negative adenocarcinoma mammary) cells were purchased from ATCC, grown and maintained in DMEM containing 10 % FBS, 2 mM L-glutamine and 1% Penicillin/Streptomycin (100 unit and 0.1 mg/mL respectively) as described by us previously [2,55,87,89].

For the estrogen, BPA and DES (all purchased from Sigma) treatment, MCF7 cells were grown and maintained for one round in phenol red free DMEM-F-12 media supplemented with 10 % charcoal stripped FBS, 2 mM L-glutamine and penicillin/streptomycin (100 unit and 0.1 mg/mL respectively). Cells were grown up to 70 % confluency in phenol red free DMEM-F-12 media supplemented with 10 % charcoal stripped FBS, treated with varying concentrations (0-10 nM) of 17β-estradiol (E2), 0-1000 nM each of BPA and DES, and incubated for 6 h (or varying time points for temporal studies) and then harvested for RNA extraction.

3.2.2 Antisense experiment

For treatment of MCF7 cells with antisense oligonucleotides, cells were grown up to 60% confluency and transfected with 1.75 µg/ml of different antisense oligonucleotides (commercially synthesized from IDT-DNA)) in FBS free media using iFECT transfection reagent (KD Medicals Inc) by following manufacturer's instruction. Briefly, cocktails of antisense oligonucleotide and transfection reagents were made in the presence of 300 µL of DMEM-F-12 media (without supplements) by incubating for 30 min as instructed by the manufacturer. Cells were washed twice with supplement free culture media and finally submerged in 1.7 mL of media (without supplements). The antisense oligonucleotide／transfection reagent cocktail was applied to the cells and incubated for 24 h before the addition of 2 mL of culture media with all

supplements and 20% extra activated charcoal-stripped fetal bovine serum. The cells were then incubated for an additional 24 h before being treated with 1 nM E2 for 4 h.

HOXA5 knockdown experiment was performed in MCF7 cells exactly as mentioned above except heat-inactivated fetal bovine serum and normal red DMEM was used and last 24 h incubation is not followed by E2 treatment.

3.2.3 Immunohistological analysis of breast cancer tissue microarray

The breast cancer tissue microarray slides containing 6 different cases (duplicates of each) of breast cancer and their corresponding adjacent normal tissue were purchased from US Biomax Inc and subjected to immunohistological staining. Briefly, the paraffin embedded tissue microarray slide was immersed twice in xylene (10 min) and then sequentially immersed in 100%, 95% and 70% ethanol (5 min each) to deparaffinize the tissue. Antigen retrieval was done by incubating the slide in 0.1 M sodium citrate buffer at 95 ^{0}C for 15 min following supplier's instruction. For immunohistological staining, the tissue microarray slide was then incubated with 3% H_2O_2 for 15 min, washed with PBS thrice, and blocked with blocking buffer containing donkey serum. The slide was then incubated with HOXA5 antibody overnight, washed thrice in PBS, and then incubated with biotinylated donkey secondary antibody for 1.5 h. The slide was washed thrice with PBS, incubated with avidin–biotin complexes for 1.5 h, washed twice with PBS and then twice with 0.1 M Tris-HCl (pH 7.4). Slide was incubated with a DAB substrate (Vector laboratories) for peroxidase labeling. The tissue microarray slide was dehydrated with sequential immersion under 70 %, 95 %, and 100 % ethanol and then cleaned by sequential incubation in citrisolv clearing agent (Fisherband). Tissue sections were finally mounted with DPX mounting solution, photographed and examined under microscope (Nikon Eclipse TE2000-U, Japan).

3.2.4 RNA extraction, reverse transcription and real-time PCR

For RNA extraction, cells were harvested by centrifugation at 1500 rpm for 5 min, the cell pellets were resuspended in diethyl pyrocarbonate (DEPC) treated buffer A (20 mM Tris-

HCl, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl and 0.5 mM DTT; 0.5 mM EDTA), incubated on ice for 10 min, centrifuged at 3500 rpm for 5 min. The supernatant containing the cytoplasmic extracts was subjected to phenol-chloroform extraction followed by ethanol precipitation of cytoplasmic RNA by incubating for 1 h at - 80 ºC. Reverse transcription reactions were performed in a total volume of 25 µL of cDNA containing 500 ng of RNA, 2.4 µM of oligo dT (Promega), 100 units of MMLV reverse transcriptase, 1X first strand buffer (Promega), 100 µM each of dATP, dGTP, dCTP and dTTP (Invitrogen), 1 mM dithiothreitol and 20 units of RNaseOut (Invitrogen). The cDNA was diluted to 100 µL. PCR was performed in a 10 µL reaction volume containing 5 µL diluted cDNA and gene specific primer pairs (Table 3.1). For the real-time PCR amplification, 5 µL of this cDNA was mixed with 5 µL of real-time PCR mix (SsoFast EvaGreen supermix, Bio-Rad) containing respective PCR primers (100 µM each), subjected to PCR amplification (and detection) using CFX96 real-time PCR detection system. The results were analyzed using the CFX manager software and plotted. The real-time PCR analysis of the ChIP DNA fragments was done in the similar manner with primers specific to ERE1, ERE2, and ERE3 regions of HOXA5 promoter (Table 3.1). The experiments were repeated at least twice with three replicates each time (n=3).

3.2.5 Protein extraction and western blotting

Cells were harvested by centrifugation at 1500 rpm for 5 min, resuspended in buffer A for 10 min on ice, centrifuged at 3500 rpm for 5 min. The supernatant was considered as cytoplasmic extract. The nuclear pellet was resuspended in buffer C (20 mM Tris-HCl pH 7.9; 1.5 mM $MgCl_2$; 0.42 M NaCl; 0.5 mM DTT; 0.2 mM PMSF and 0.5 mM EDTA) for 20 min on ice, centrifuged at 13000 rpm for 10 min. The supernatant containing the nuclear protein was separated. Nuclear extract was analyzed by western blotting using anti-HOXA5 antibody (Life Span Biosciences Inc) and nitrocellulose membrane was developed by alkaline phosphatase method.

3.2.6 Dual luciferase reporter assay

Three HOXA5 independent EREs along with their flanking regions (200-350 bp) were cloned and inserted upstream of the promoter of a fire fly luciferase gene in pGL3 promoter vector (Promega) (primers are listed in Table 3.1). MCF7 cells (~4 x 10^5 in 6 well plate) were co-transfected with 1500 ng of these ERE-containing luciferase reporter construct along with 150 ng of a reporter plasmid containing *renilla* luciferase (Promega) as an internal transfection control using lipofectamine transfection reagent (Invitrogen). Control transfection was done using empty pGL3 promoter vector without any ERE insertion. No transfection control was also done in parallel. At 30 h post-transfection, cells were treated with 1 nM E2, 10 nM BPA, 100 nM DES and incubated for an additional 8 h. Total protein was extracted and then subjected to luciferase assay using Dual-Glo Luciferase Assay System (Promega) as instructed and detected using a microplate reader (Flowstar-Omega). Each treatment was done in four replicates and the experiment was repeated at least twice (n = 2).

3.2.7 Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed on MCF7 cells using EZ Chip chromatin immunoprecipitation kit (Upstate) as described previously by us [2,55,89]. In brief, MCF7 cells were treated with 1 nM E2, 10 nM BPA, and 100 nM DES for 4 h, fixed in 4% formaldehyde for 15 min at room temperature, quenched the excess formaldehyde with 125 mM glycine, washed twice with cold PBS containing protease inhibitor (5 $\mu L.mL^{-1}$) and PMSF (5 $\mu L.mL^{-1}$). The cells were then lysed in SDS lysis buffer and sonicated to shear the chromatin to ~150-450 bp in length. The sonicated chromatin was pre-cleaned using protein-G agarose (Millipore) beads and subjected to immunoprecipitation using antibodies specific to ERα (Santa Cruz), ERβ (Santa Cruz), H3K4-trimethyl (Upstate), RNAPII (Abcam), MLL2 (Bethyl laboratory), MLL3 (Abgent), TAF250 (Upstate), N-CoR (Santa Cruz), CBP (Santa Cruz), and H4 acetylation (Upstate). The immunoprecipitated chromatin was deproteinized by incubating at 65 ^oC in presence of NaCl, followed by incubation in presence of proteinase K (Sigma). The digested protein is removed by

phenol-chloroform treatment and obtained purified DNA fragments following enthanol precipitation overnight at -80ºC. The purified DNA was then PCR-amplified using promoter-specific primers (Table 3.1).

3.2.8 Terminal dUTP nicked end labeling (TUNEL) assay

TUNEL assay was performed on MCF7 cells using apo alert DNA fragmentation assay kit (Clontech) as per manufacturer's instruction. MCF7 cells were grown up to 60 % confluency and transfected with 1.75 µg/ml each of HOXA5 and scramble (no homology with HOXA5 gene) antisense oligonucleotide in FBS free media using iFECT transfection reagent followed by the addition of 2 mL of culture media with all supplements and 20% extra activated charcoal-stripped fetal bovine serum.. Cells were incubated with antisense for 48 h and then subjected to terminal nicked end labeling using fluorescent dUTP. DAPI and propidium iodide staining were performed to visualize nucleus of all and dead cells respectively.

3.2.9 Fluorescence activated cell sorting (FACS) analysis

MCF7 cells were grown to 60% confluency and transfected with 1.75 µg/ml of HOXA5 and scramble antisense oligonucleotides separately using iFECT transfection reagent by following supplier's instruction. The antisense oligonucleotide／transfection reagent cocktail was applied to the cells and incubated for 24 h before the addition of 2 mL of culture media with all supplements and 20% extra activated charcoal-stripped fetal bovine serum. The cells were then incubated for an additional 24 h. Control and antisense treated cells were harvested upon trypsinization with all dead and live cells, washed twice with cold PBS, centrifuged and fixed in 70% ethanol overnight at 4 ºC, washed twice with PBS, treated with 1 µg/ml RNase solution and stained with propidium iodide (final concentration of 0.5 µg/ml). The cells were analyzed by flow cytometry, using a Fusing Beckman Coulter (Fullerton, CA, USA) Cytomics FC500 flow cytometry analyzer. This assay tells about the involvement of any gene in cell cycle regulation and maintenance.

Table 3.1 Primers used for cloning, RT-PCR, ChIP, and antisense experiments

Primers	Forward primers (5′ - 3′)	Reverse primers (5′ - 3′)
PCR primers		
GAPDH	CAATGACCCCTTCATTGACC	GACAAGCTTCCCGTTCTCAG
HOXA5	ACTCATTTTGCGGTCGCTAT	TTGTAGCCGTAGCCGTACCT
HOXA5-ERE1	CGAGTCCGGCTGAACGGCGG	TAGGCACCCAAATATGGGGTA
HOXA5-ERE2	TTATTTCTCCAATTGGCTAAA	CCGGCGAGGATGCAGAGGAT
HOXA5-ERE3	CGGACCCTGTGATGAAGATT	CCTGCCGCTCTTAAATGACA
ERα	AGCACCCTGAAGTCTCTGGA	GATGTGGGAGAGGATGAGGA
ERβ	AAGAAGATTCCCGGCTTTGT	TCTACGCATTTCCCCTCATC
Cloning primers		
HOXA5-ERE1	CTCCACCCAACTCCCCTATT*	CGGTCGTTTGTGCGTCTAT*
HOXA5-ERE2	CGCAAAAACAGAGCCGTAAT*	TTCCCTCGCAGTTCCATTAG*
HOXA5-ERE3	CGGACCCTGTGATGAAGATT*	GCAGCTACAATTACGGCTCTG*
Antisenses		
HOXA5	GTCCCTGAATTGCTCGCTCA**	
ERα antisense	TCCCACCTTTCATCATTCCC**	
ERβ antisense	GCCACACTTCACCATTCCCA**	
Scramble antisense	CGTTTGTCCCTCCAGCATCT**	

*Flanked by appropriate restriction sites; **Phosphorothioate antisense oligonucleotides

Note: These antisense oligonucleotides were designed by Dr. Subhrangsu S. Mandal, Dr. Khairul I. Ansari and Bibhu P. Mishra and kindly provided by them.

3.3 Results

3.3.1 HOXA5 is over-expressed in breast cancer cells and tissue

In an effort to understand the mechanism of transcriptional regulation of HOXA5, initially, I examined its expression in various cancer cells and breast cancer tissue. First of all, I examined its expression level in different types of cancer cell lines including ER positive and ER negative breast cancer cell lines. Real-time PCR analysis demonstrated that HOXA5 expression was relatively higher in ER positive breast cancer cells (MCF7 and T47D) in comparison to the ER negative MDAMB231 cells and nonmalignant breast epithelial cells MCF10 (Figure 3.1A). As HOXA5 is over-expressed in breast cancer cells, we further examined its expression in breast cancer tissue using immunohistological staining. A breast cancer tissue microarray slide containing 6 cases of breast cancer (in duplicates) along with corresponding adjacent normal tissue was purchased from commercial source and subjected to DAB staining using HOXA5 antibody. These analyses showed that HOXA5 expression was relatively higher in most breast

cancer tissue examined in comparison to corresponding adjacent breast normal tissues (Figure 3.1B, a magnified view of case 3 is shown in 3.1C). The over-expression of HOXA5 in breast cancer tissue and in ER positive breast cancer cells, and comparatively lesser expression in ER negative cell line suggested its potential regulation by estrogen.

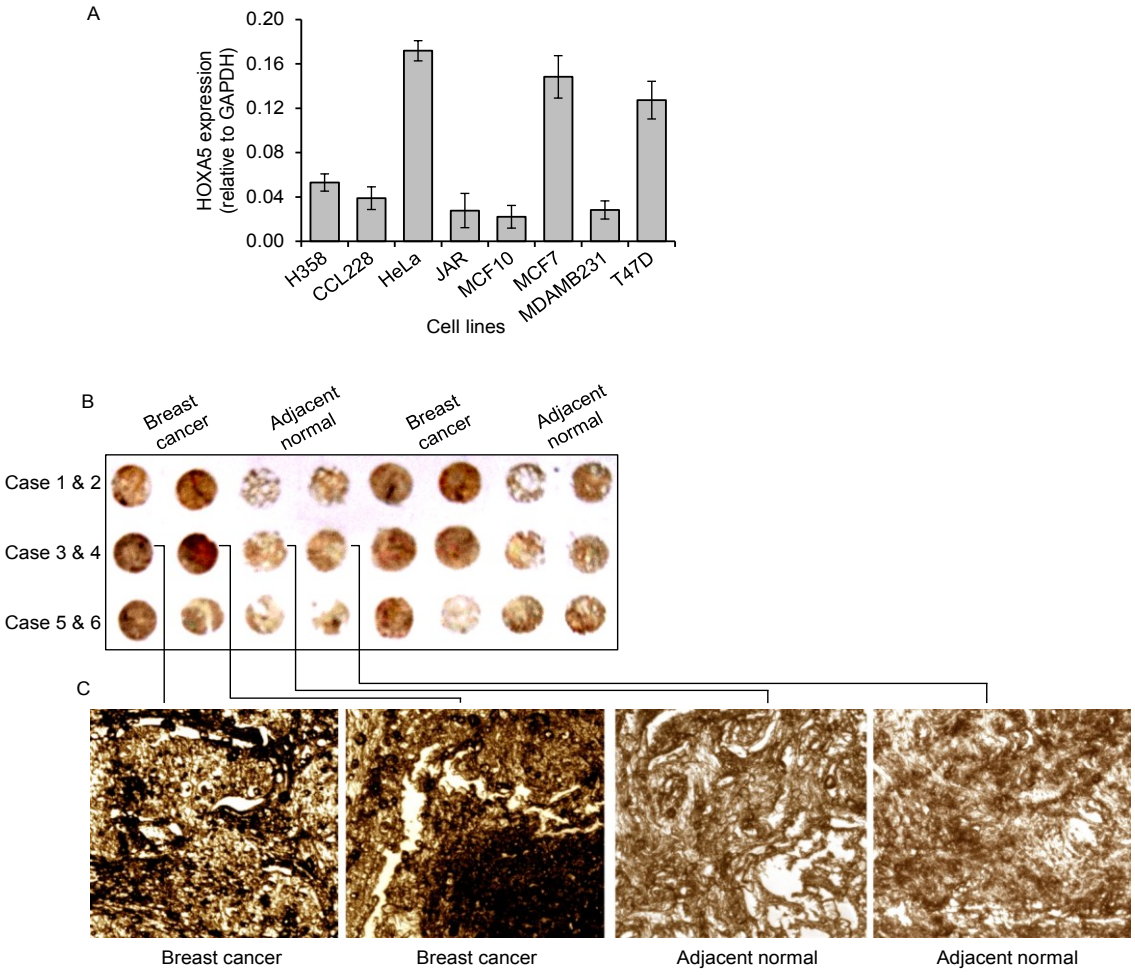


Figure 3.1 Expression of HOXA5 in different malignant and nonmalignant human cell lines and human breast cancer tissue. (A) The total RNA was isolated from CCL228, H358, HeLa, JAR, MDAMB231, T47D, MCF7, MCF10 cell lines, reverse transcribed into cDNA and analyzed by real-time PCR using primers specific to HOXA5. GAPDH was used as a loading control. HOXA5 expression relative to GAPDH is plotted. Each experiment was repeated at least thrice (n = 3). Bars indicate standard errors. (B) Immunohistological analysis of HOXA5 expression in breast cancer tissue: Human breast cancer tissue microarray slide (6 cases of breast cancer along with respective adjacent normal breast tissue) was subjected to immunohistological staining (DAB staining) with HOXA5 antibody. A magnified view of case 3 showing HOXA5 over-expression in breast cancer tissue is also shown. Notably, Dr. Khairul I. Ansari and Arunoday Bhan carried out the immunohistological analysis of HOXA5 expression and figures B and C are kindly provided by them.

3.3.2 HOXA5 plays essential roles in growth and survival of MCF7 cells

To understand the importance of HOXA5 in breast cancer cells, I examined its potential roles in cellular growth and viability. I knocked it down in MCF7 cells using HOXA5-specific antisense and subjected to microscopic analysis, growth and apoptotic assays. My analysis showed that HOXA5 antisense significantly knocked down HOXA5 expression at mRNA level (compare lanes 1 and 2 with lane 3, Figure 3.2A; real-time quantification in figure 3.2B). Interestingly, upon knockdown of HOXA5, the growth of MCF7 cells were severely affected and microscopic analysis demonstrated that cell populations are low in the HOXA5 antisense-treated cells in comparison to the control untreated or scramble antisense-treated MCF7 cells. At longer time point of HOXA5 antisense exposure live cell population was decreased further indicating cell death (Figure 3.2D). Microscopic analysis showed that upon HOXA5 knockdown, cells appeared unhealthy, were rounded up and fragmented into debris (Figure 3.2C).

To examine the nature of cell death, I performed TUNEL and flow cytometry assay. TUNEL assay demonstrated that upon application of HOXA5 antisense cells nuclei were fragmented (green color nuclei indicating dUTP staining) (panel 2, Figure 3.2E). Analysis of the propidium iodide staining showed that most cells colored red are dead cells (panel 3, Figure 3.2E). DAPI staining further showed the nuclear condensation and fragmentation in dead cells (panel 1, Figure 3.2E) demonstrating that knockdown of HOXA5 induced apoptotic cell death. In another study, flow cytometry analysis was done to examine the involvement of HOXA5 in cell cycle. Interestingly as shown in figure 3.2F, upon treatment with the HOXA5 antisense oligonucleotide, the apoptotic cell death increased from 14.2 % (control) to 37.5 % (HOXA5 antisense treated). Notably, application of the scramble antisense oligonucleotide (with no homology to HOXA5) also led to a slight increase in apoptotic cell death (22 %) likely due to the toxic effect of transfection reagent. The HOXA5 antisense-mediated increase in apoptotic cell population indicated that HOXA5 is a critical player for the growth and survival of MCF7 cells.

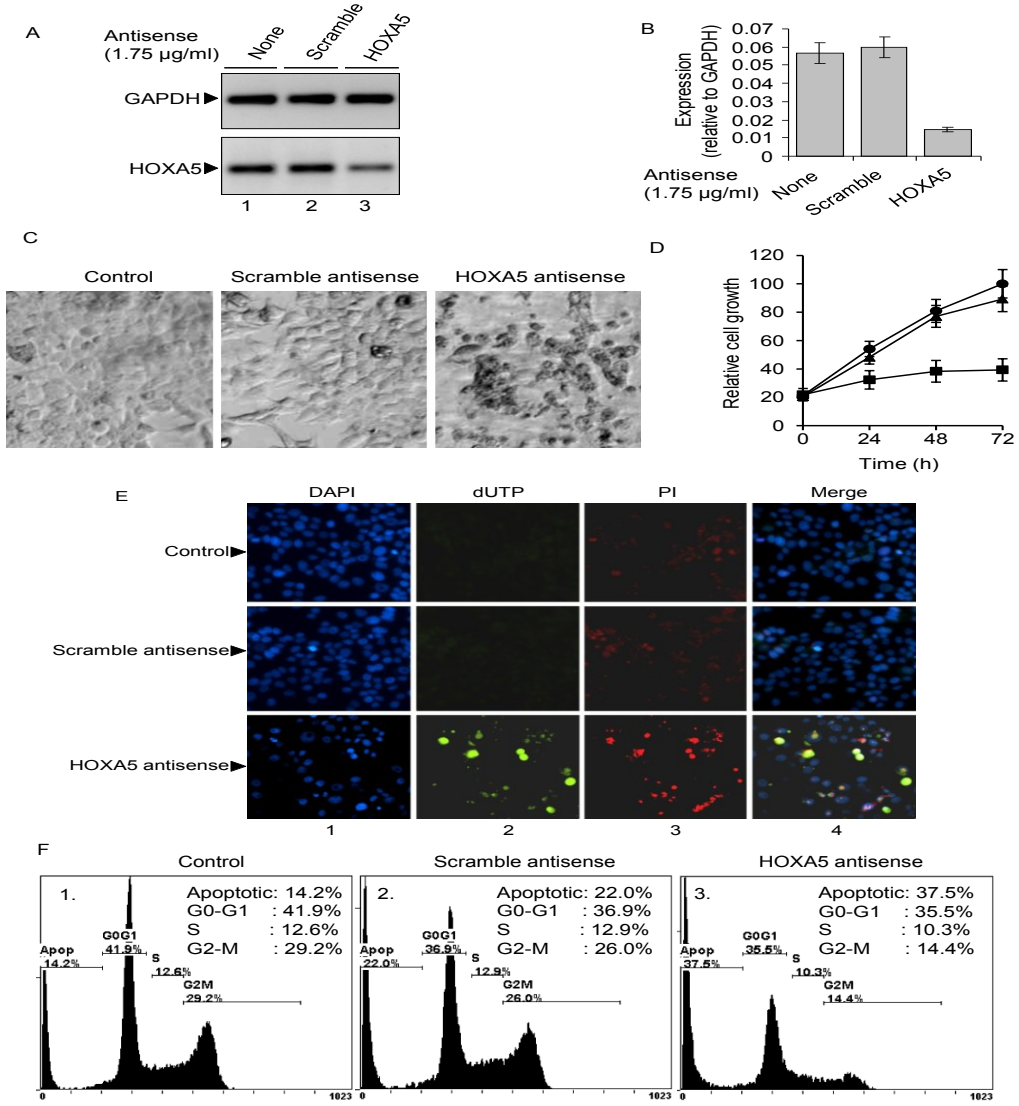


Figure 3.2 Effect of HOXA5 knockdown on cell viability. (A) MCF7 cells were transfected with HOXA5-specific and scramble antisenses for 48 h. The RNA from the control and antisense-treated cells were analyzed by regular RT-PCR with primers specific to HOXA5 (real-time quantification is shown in panel B). GAPDH was used as control. Bars indicate standard errors and experiment was repeated thrice (n = 3). (C) DIC (differential interference contrast) images of MCF7 cells that were treated with HOXA5 and scramble antisenses and visualized under microscope. (D) After HOXA5 knockdown for varying period of time, cell numbers at three regions of DIC picture was counted and relative cell growth versus time was plotted. Experiment was carried out in three parallel replicates and each experiment was repeated at least twice (n = 2). Bars indicate standard errors. (E) TUNEL assay. MCF7 cells were treated with HOXA5 and scramble antisenses and subjected to terminal nicked end labeling using fluorescent dUTP. The result shown here is at 48 h post-treatment of antisense. Green speckles represent apoptotic cells with fragmented nuclei. DAPI and propidium iodide staining were performed to visualize nucleus of all and dead cells respectively. (F) FACS analysis: MCF7 cells were treated with HOXA5 and scramble antisenses separately for 48 h and subjected to flow cytometry analysis.

3.3.3 HOXA5 is an estrogen-responsive gene

As HOXA5 is over-expressed in breast cancer tissue and found to be critical for viability of ER positive MCF7 cells, I examined its potential regulation by estrogen. I exposed MCF7 cells (grown in phenol red free media and charcoal stripped serum) with 17β-estradiol (E2). RNA from the E2-treated and control cells were reverse transcribed and subjected to PCR analysis using HOXA5 open reading frame primers (Table 3.1). The relative expression of HOXA5 was also quantified using real-time PCR (qPCR). GAPDH was used as a loading control. Interestingly, my results demonstrated that HOXA5 expression is induced by E2 in concentration-dependent manner (Figure 3.3A). qPCR analysis showed that HOXA5 was over-expressed by ~3.5-fold at 1 nM in comparison with untreated control and its expression was suppressed with higher concentration of E2 likely due to squelching. HOXA5 expression was maximum at 4 h upon treatment with 1 nM E2 (Figure 3.3B). Notably, the expression of HOXA5 was insensitive to E2, BPA and DES in an ER negative cell line MDAMB231 (Figure 3.3C). Also, the tamoxifen treatment decreased the estrogen-induced HOXA5 expression (compare lane 5 with lane 7, Figure 3.3A). These results demonstrated that HOXA5 is transcriptionally regulated by E2 via involvement of estrogen recoptors in MCF7 cells and this expression is suppressed by tamoxifen.

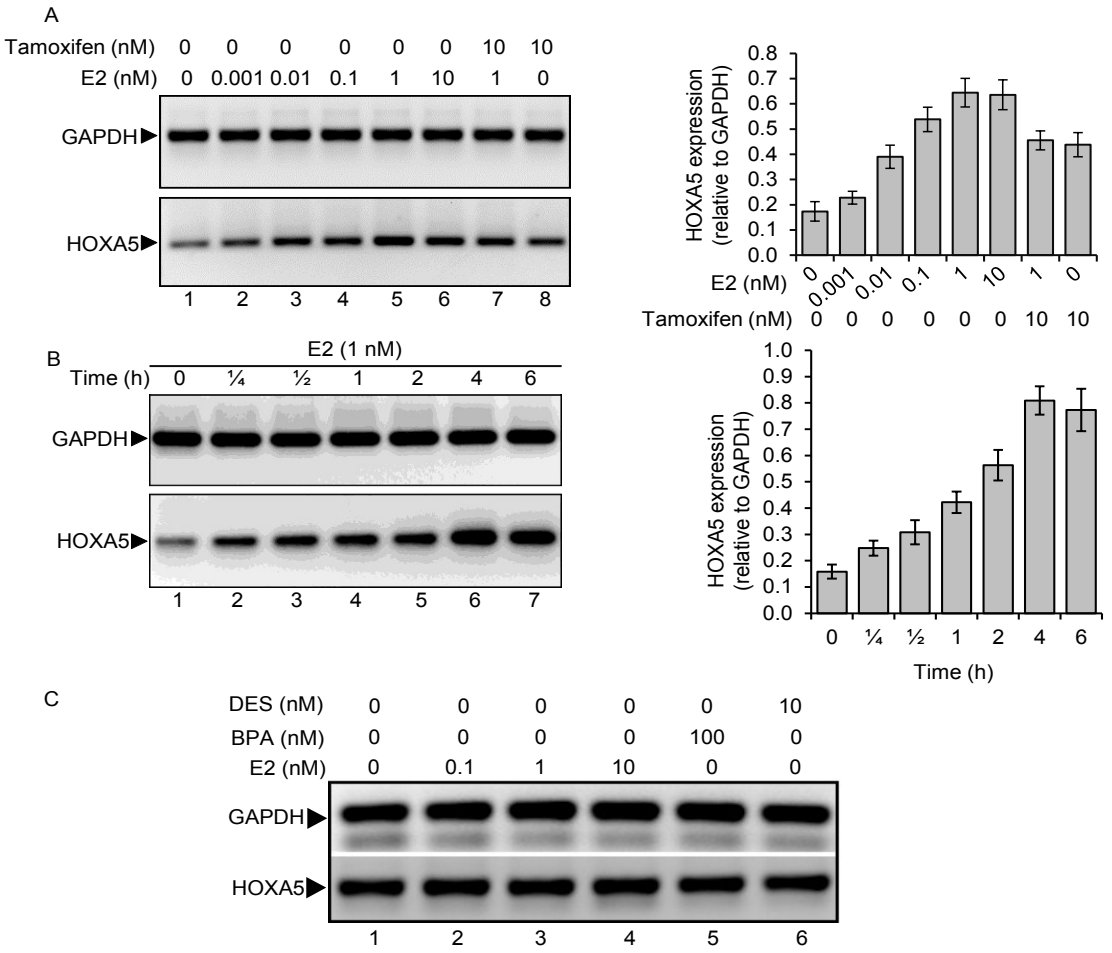


Figure 3.3 Effect of E2 on HOXA5 expression. (A) E2-induced expression of HOXA5 in MCF7 cells. The cells were grown in phenol red free media and treated with varying concentrations (0-10 nM) of E2 in presence and absence of 10 nM tamoxifen. The total RNA was isolated, reverse transcribed to cDNA and analyzed by PCR using primers specific to HOXA5. GAPDH was used as a loading control. Expression of HOXA5 (relative to GAPDH) was examined by real-time PCR (right panel). (B) Temporal studies: MCF7 cells were treated with 1 nM E2 for varying time periods (0 - 6 h). RNA was reverse transcribed, analyzed by regular PCR (left panel), and real-time PCR (right panel). (C) MDAMB231 cells were grown in phenol red free media and treated with varying concentrations (0-10 nM) of E2, 10 nM BPA and 100 nM DES. The total RNA was isolated, reverse transcribed to cDNA and analyzed by PCR using primers specific to HOXA5. Each real-time PCR reaction was carried out in three parallel replicates and each experiment was repeated at least thrice (n = 3). Bars indicate standard errors.

3.3.4 Estrogen receptors play crucial role in E2-induced HOXA5 expression via recognition of promoter EREs

To further examine potential involvement of ERs in HOXA5 expression in MCF7 cells, I knocked down two major estrogen receptors, ERα and ERβ, using respective antisenses (either

seperately or in combination) in MCF7 cells, then treated with E2 and examined its impact on HOXA5 expression using regular RT-PCR and qPCR. My analysis showed that ERα and ERβ application specifically knocked down respective ERs in MCF7 cells (lane 5 for ERα knockdown and lane 6 for ERβ knockdown, Figure 3.4A). Importantly, knockdown of both ERα and ERβ resulted in more reduced E2-dependent HOXA5 expression as shown by qPCR (lane 7, Figure 3.4B). Combined knockdown of ERα and ERβ further reduced the levels of HOXA5 expression indicating crucial roles of both ERα and ERβ in E2-induced HOXA5 expression (Figure 3.4B).

To understand further, I examined the E2-dependent binding of ERs in the promoter of HOXA5 using chromatin immunoprecipitation (ChIP) assay. Notably, analysis of the DNA sequences of HOXA5 promoter demonstrated that HOXA5 promoter contains three potential ERE sites within 1000 nt upstream of the transcription start site (Figure 3.6A). Based on the neighbouring nucleotide sequences, ERE3 is likely a $ERE_{1/2}$ site while ERE1 and ERE2 are pontentially imperfect full EREs. I examined the bindings of ERs especially to these putative ERE sites. Briefly, cells treated with E2 were fixed with formaldehyde, sonicated to shear the chromatin and then subjected to immunoprecipiation using ERα, ERβ and β-actin (control) antibodies. The ChIP DNA were PCR-amplified using primers specific to different ERE regions of HOXA5 promoter. ChIP analysis showed that both ERα and ERβ were enriched in ERE1 and ERE2 region upon treatment with E2, BPA and DES but not on ERE3 (compare lane 1 with lanes 2, 3, and 4 Figure 3.7 A-D). No binding of β-actin was observed both in the absence and presence of E2. Thus, knockdown analysis and binding assay demonstrated that ERα and ERβ play essential roles in E2-induced HOXA5 expression.

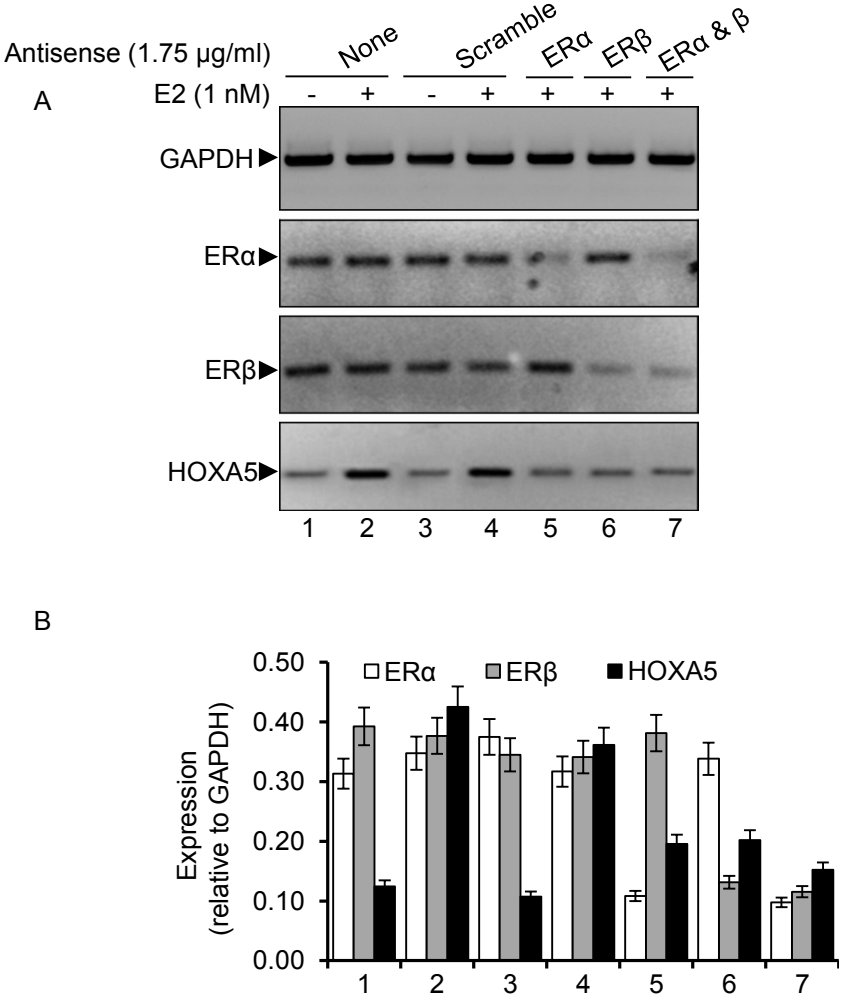


Figure 3.4 Effects of ERα and ERβ knockdown on E2-induced expression of HOXA5. MCF7 cells were transfected with ERα and ERβ-specific antisense oligonucleotides for 48 h. A scramble antisense (with no homology with ERα and ERβ gene) was used as control. The knocked down cells were treated with E2 for additional 4 h. RNA from these cells was subjected to RT-PCR analysis by using primers specific to HOXA5, ERα and ERβ separately. GAPDH was used as control. Lane 1: control cells without E2 treatment; lane 2: cells treated with E2; lanes 3 and 4: cells treated with scramble antisense in the absence and presence E2; lanes 5 and 6: cells treated with ERα and ERβ antisense respectively followed by exposure to E2; lane 7: cells transfected with 1:1 mixture of ERα and ERβ antisenses in the presence of E2. The real-time quantification of the transcript accumulation relative to GAPDH was plotted in (B). Each real-time PCR reaction was carried out in three parallel replicates and each experiment was repeated at least thrice (n = 3). Bars indicate standard errors ($p < 0.05$). Notably, ERs knockdown experiments were carried out by Dr. Khairul I. Ansari and these figures are kindly provided by him.

3.3.5 HOXA5 is transcriptionally induced by bisphenol-A (BPA) and diethylstilbestrol (DES)

Increasing amounts of evidence indicate that environmental chemicals especially endocrine disrupting chemicals may misregulate various critical genes ultimately contributing towards various human disease including cancer. As HOXA5 over-expression is associated with cancer and is transcriptionally induced by E2 in breast cancer cells, I examined the impacts of exposure of various endocrine disrupting chemicals on transcription level of HOXA5 in MCF7 cells. Specifically, I treated MCF7 cells with varying concentrations of BPA and DES that are well-known to interfere with estrogen signaling. Interestingly, I found that treatment with both BPA and DES induced HOXA5 expression in MCF7 signifcantly both in a concentration and time-dependent manner (Figures 3.5A-D). These observations demonstrated that HOXA5, which is an E2-responsive gene, is also activated upon treatment with nanomolar concentrations of estrogen mimics and therefore might be potentially misregulated upon exposure to BPA and DES.

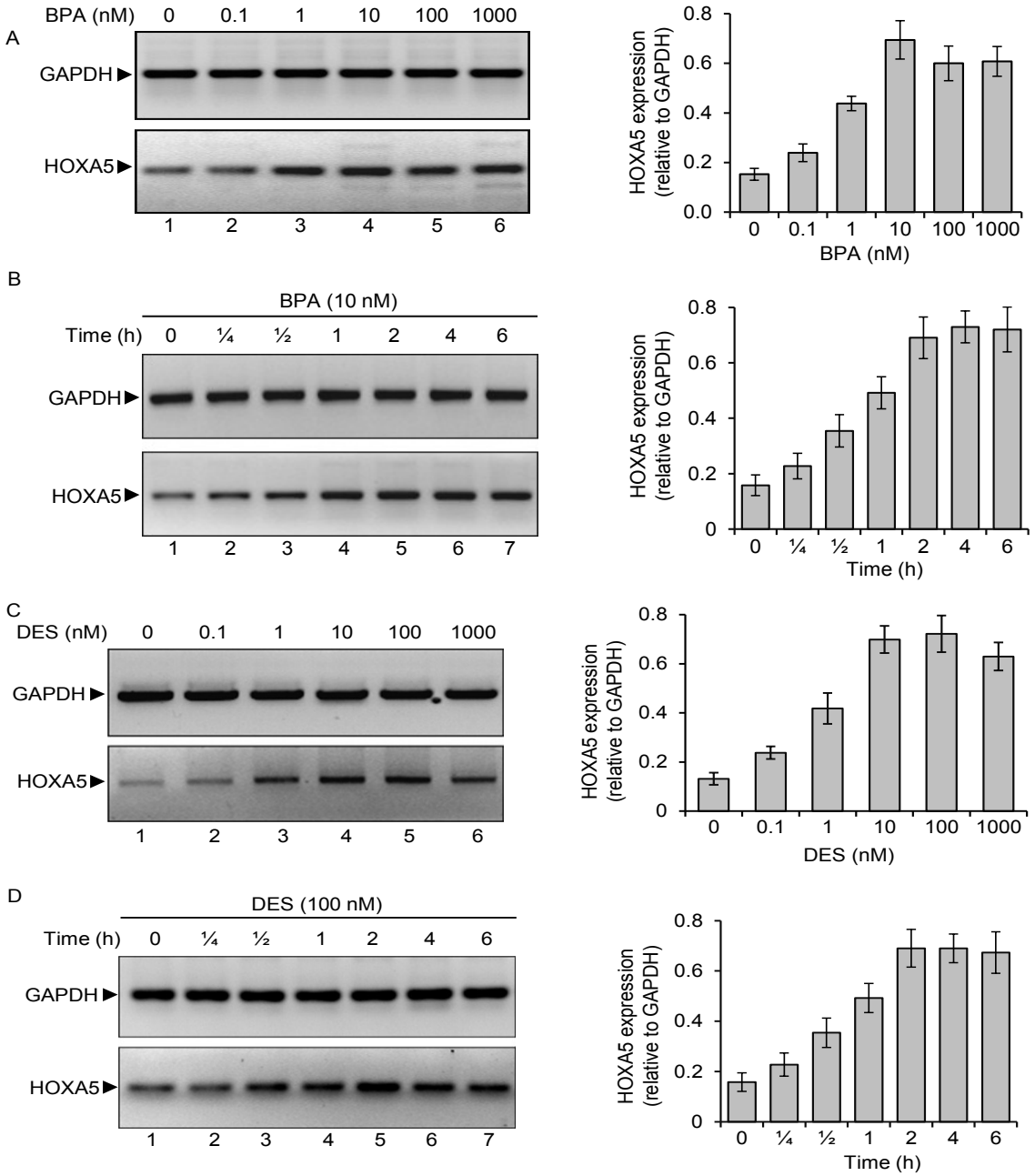


Figure 3.5 Effects of BPA and DES on HOXA5 gene expression. (A and B) MCF7 cells (grown in phenol red free media for one round) were treated with BPA (A) and DES (B) for varying concentrations (0-1000 nM). The total RNA was isolated, converted to cDNA and analyzed by PCR using primers specific to HOXA5. GAPDH was used as a loading control. The cDNA was analyzed by real-time PCR and expression of HOXA5 (relative to GAPDH) is plotted in respective panel below. Each experiment was repeated at least thrice. Bars indicate standard errors ($p<0.05$). (C and D) MCF7 cells were treated with 10 nM BPA (C) and 100 nM DES (D) for varying time periods (0-6 h) and reverse transcribed PCR products were quantified using real-time PCR. Each experiment was repeated at least thrice (n = 3).

3.3.6 HOXA5 promoter EREs are induced by E2, BPA and DES treatments

To examine the potential involvement of HOXA5 promoter EREs, I cloned each individual ERE region in a luciferase-based reporter construct pGL3 and subjected to luciferase assay using a dual luciferase assay kit. Each ERE-pGL3 construct was co-transfected with renilla luciferase construct (supplied by the manufacturer) into MCF7 cells and then treated with either E2 or BPA or DES and subjected to luciferase assay. Luciferase activity of EREs were normalized using renilla expression and plotted (Figure 3.6B). These analyses showed that ERE1 and ERE2 were induced upon treatment with E2, BPA and DES, while ERE3 was relatively insensitive to E2/BPA/DES treatments. These analyses demonstrated that ERE1 and ERE2 regions of HOXA5 promoter particpate in transcriptional induction by E2, BPA and DES, with ERE1 shows more robust response than ERE2.

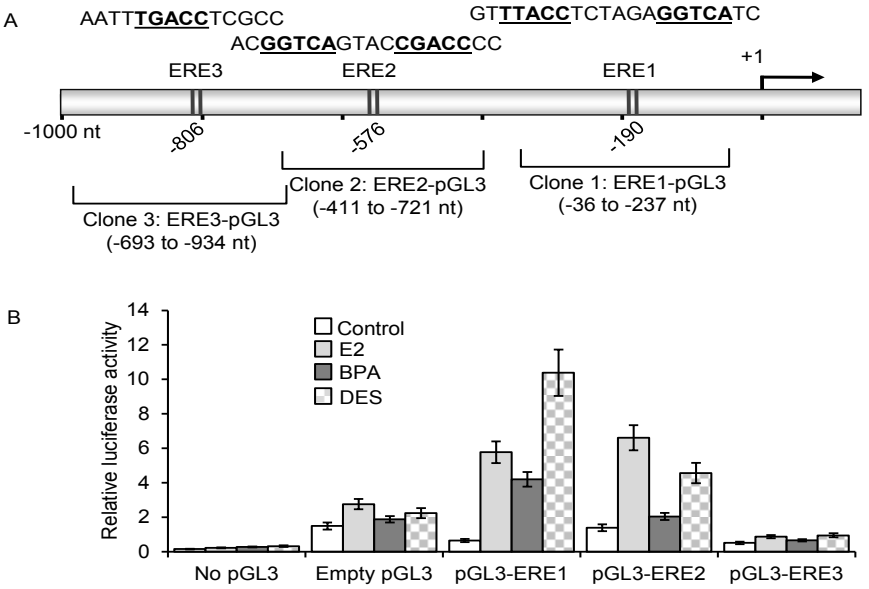


Figure 3.6 Characterization of HOXA5 promoter EREs using dual luciferase reporter assay. (A) HOXA5 promoter showing three EREs within 1000 nt upstream of transcription start site was cloned into luciferase-based reporter construct pGL3. (B) Luciferase assay: ERE-pGL3 construct along with renilla vector was co-transfected into MCF7 cells using lipofectamine transfection reagent. Control cells were treated with empty pGL3 vector and no transfection control was done in parallel. Cells were then treated with 1 nM E2, 10 nM BPA and 100 nM DES and then subjected to luciferase assay. The luciferase activities in presence of E2, BPA and DES (over untreated controls and normalized against renilla luciferase expression) were plotted. The experiment with four replicate treatments was repeated at least thrice (n = 3). Bars indicate standard errors ($p < 0.05$).

3.3.7 ER coactivators are recruited to the HOXA5 promoter upon treatment with E2, BPA and DES

ER coactivators are integral components of estrogen-dependent gene activation. Many ER coactivators have been discovered that includes SRC-1 family, CREB-binding protein, p/CAF, and ASCOM complexes containing MLL histone methyl-transferases. Herein, I examined if any ER coactivators are associated with E2-induced HOXA5 expression. Using ChIP assay, I analyzed the recruitment of CBP, MLL family of HMTs (MLL2 and MLL3) in the HOXA5 promoter in the absence and presence of E2, BPA and DES. Interestingly, I observed that the binding of these factors were enriched in the HOXA5 promoter (Figure 3.7A and 3.7B). Nuclear receptor corepressor N-CoR was bound to the HOXA5 promoter in control and that binding was decreased upon E2/BPA/DES treatment indicating critical role of N-CoR in suppressing HOXA5 gene expression under non-activated condition (Figure 3.7A). Finally I also analyzed the levels of histone acetylation, H3K4-trimethylation and RNA polymerase II into the HOXA5 promoter in the presence of E2, BPA and DES. Figure 3.7A shows that these proteins were recruited to the HOXA5 promoter upon treatment with E2 and E2-mimics. These analyses demonstrated that CBP, MLL2 and MLL3 coordinate with ERs, bind to the HOXA5 promoter upon exposure to E2, BPA and DES, increase histone H3K4-trimethylation, acetylation and recruitment of TAF250 and RNA polymerase II to the HOXA5 promoter and transcriptionally activates HOXA5 gene expression.

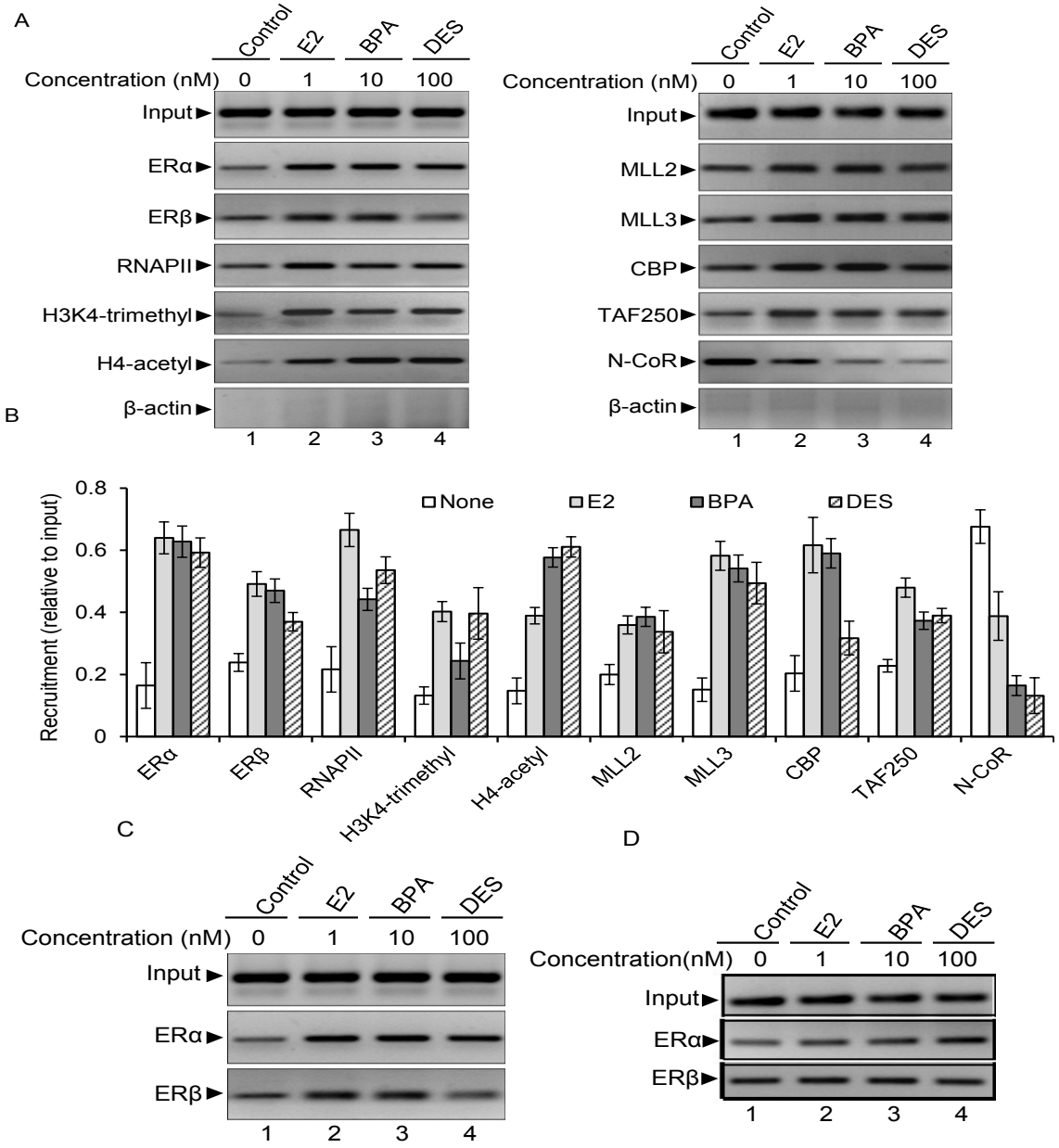


Figure 3.7 E2, BPA, and DES-dependent recruitment of ERα, ERβ, MLL2, MLL3, RNAPII, H4-acetylation, H3K4-trimethylation, CBP, N-CoR, TAF250, and β-actin on HOXA5 promoter ERE. (A) MCF7 cells were treated with 1 nM E2, 10 nM BPA and 100 nM DES for 4 h and analyzed by ChIP assay using antibodies mentioned in the figure. The ChIP DNA was PCR-amplified using primers specific to ERE1 region of HOXA5 promoter. Real-time quantification of ChIP DNA samples showing the recruitment of different factors (relative to input) into ERE1 region of HOXA5 promoter region in presence of E2, BPA and DES is shown in (B). The agarose gel picture of the recruitment of ERα and ERβ on ERE2 region of HOXA5 promoter is shown in panel C and on ERE3 in panel D. The experiments were repeated at least twice (n = 2). Bars indicate standard errors.

3.4 Discussion

In this study, I have examined the roles of HOXA5 in breast cancer cells and its epigenetic mechanism of regulation in presence of estrogen and endocrine disrupting chemicals. My studies demonstrated that HOXA5 is over-expressed not only in ER positive breast cancer cells such as MCF7 and T47D but also in breast cancer tissue in comparison to the corresponding surrounding normal breast tissue. HOXA5 expression is important for cell viability as shown by growth, flow cytometry and TUNEL assay. HOXA5 expression is induced upon exposure to E2 in ER positive breast cancer cells and this is mediated via involvement of ERs, and this expression is suppressed upon tamoxifen treatment. Similar to E2, endocrine disrupting chemicals with estrogenic activities such as BPA and DES also induced the expression of HOXA5 in breast cancer cells. BPA and DES induced the recruitment of ERs and ER coactivators such as histone methylases MLL2 and MLL3. Exposure to BPA and DES also affected the epigenetic states such as increased histone H3K4-trimethylation and histone acetylation levels that ultimately resulted in HOXA5 gene activation. In summary, these studies demonstrated that HOXA5 which is associated with breast cancer is transcriptionally regulated by estrogen and is disrupted upon exposure to endocrine disrupting chemicals such as BPA and DES. My studies provide a direct link with environmental factors in activation of protooncogenes in breast cancer.

Overall, my studies demonstrated that HOXA5 is an E2-responsive gene and it is misregulated by estrogenic endocrine disrupting chemicals. As estrogen and ERs are closely associated with breast cancer cells proliferation and survival [78], and herein HOXA5 is shown to be required for the survival of breast cancer cells and over-expressed in patient breast tissue, it is likely that E2 and ERs are executing their function via regulating HOXA5. Herein, HOXA5 is shown to be regulated by ERs and MLL histone methylases and closely linked with the development of lung, gastro-intestinal tract and vertebrae, and hence implicating estrogen, ERs and MLLs in regulation of these vital organs of the body under hormonal environment [58]. My

studies provide a novel link between estrogen and estrogen-like compounds in regulation of HOXA5 via ERs and MLLs that is implicated in breast cancer survival and growth.

It is well-known that estrogen signaling is a multifactorial complex process involving association of multiple distinct factors at the gene promoter and is highly cell and tissue-specific [1,7,59,93]. Along with ERs, several ER coregulators that interact with ERs play essential roles in transactivation of the target gene. These coregulators usually possess enzymatic activity, modify chromatin and bridge ERs with transcription machinery. Many ER coactivators have been identified till date including SRC-1 family, CREB-binding protein, p/CAF, and ASCOM complexes [66]. Similarly N-CoR, SAFB1 and SMRT are well-known ER corepressors [73]. Further studies are needed regarding the involvement of these coactivators and corepressors to understand more complex association of factors at HOXA5 promoter and hence its involvement in breast cancer.

CHAPTER 4

ENDOCRINE DISRUPTING CHEMICALS BISPHENOL-A AND DIETHYLSTILBESTROL INDUCE THE EXPRESSION OF HOMEOBOX CONTAINING GENE HOXC6 IN BREAST CANCER CELLS

4.1 Introduction

Hormones are important chemical messengers that control various cell signaling and physiological processes crucial to life [102]. Hormones execute their functions at very low concentration through recognition of specific hormone receptors [81,102]. There are different types of hormones such as steroid and peptide hormones. For example, estrogen is a steroid hormone critical for female reproduction and development, progesterone is a female hormone associated with egg formation and androgens are steroid hormones required for male reproduction and development [66,91]. Malfunction in hormonal signaling may cause severe health problems and diseases including abnormal child development, cardiovascular disease, thyroid disease, reproductive disorder, and cancer [66].

Endocrine disrupting chemicals (EDCs) are those that interact with hormone receptors even at very low concentration and interfere with reproduction, development, and other hormonally regulated processes leading to harmful effects like elevated rates of diabetes, cancer, reproductive problem, early puberty, and obesity [96,97]. Recent studies demonstrated that a number of agents with estrogen-like activity induce organ-specific developmental changes in the female reproductive tract [96]. Exposure to bisphenol A (BPA), diethylstilbestrol (DES), and methoxychlor alter uterine HOX gene expression [75,96,97]. BPA is commonly found in plastics, metallic storage containers and various other routinely used consumables [96]. DES is an orally active synthetic nonsteroidal estrogen drug that has been used extensively for estrogen replacement therapy, induction of fertility among others [97]. In addition to these few well-known

EDCs, there are hundreds of chemicals, hormones, and potential EDCs present in our environment originated from routinely used plastics, medicine, drugs, pesticides, fertilizers, growth hormones, and industrial wastes [96]. These known and unknown EDCs have contaminated our drinking water and foods which will have long term impact on our health and environment. In spite of the serious concern and increasing awareness about EDCs, limited knowledge exists in terms of identifying potential EDCs, assessing their risk, management, and understanding their mechanism of action [96]. Herein, I investigated the potential mechanism of endocrine disruption by BPA and DES in HOX gene expression that are crucial players during embryonic developments and human disease.

HOX genes are a family of transcription factors that play crucial roles during embryogenesis and fetal development [1,7,33]. A HOX protein contains highly conserved homeodomain through which it binds to target DNA and regulates gene expression. There are 39 HOX genes in human that are classified into four different clusters HOXA-D [33]. Colinear expression of HOX genes is well recognized for regulating cell differentiation, organogenesis and limb development. Mutation or misregulation of HOX genes exerts developmental defects, autism as well as various other deadly human diseases including cancer [33,54].

In particular relevance to my study, HOXC6 is a well-known player in mammary gland development and milk production [57,59]. HOXC6 is also shown to be over-expressed in breast as well as prostate cancer [38,59]. HOXC6 is a crucial player during prostate branching morphogenesis [58]. My recent study demonstrated that HOXC6 is transcriptionally regulated by estradiol (E2). Importantly, mixed lineage leukemia (MLL) family of histone methyl-transferases (HMTs) coordinate with estrogen receptors (ERs) during E2-mediated transactivation of HOXC6 [54]. Herein, I have investigated the impact of two well-known endocrine disrupting chemicals such as BPA and DES on HOXC6 expression in breast cancer cells. My results demonstrated

that HOXC6 is over-expressed in breast cancer tissues and its transcription is influenced by E2 as well as BPA and DES in breast cancer cells.

4.2 Materials and methods

4.2.1 Cell culture, treatment with estradiol, BPA, and DES

H358 (bronchioalveolar carcinoma), HeLa (cervical cancer), JAR (choriocarcinoma placenta), MCF10 (normal breast epithelial), MCF7 (ER positive adenocarcinoma mammary), T47D (ER positive ductal carcinoma mammary), and MDAMB231 (ER negative adenocarcinoma mammary) cells were purchased from ATCC, grown and maintained in DMEM containing 10 % FBS, 2 mM L-glutamine and 1% Penicillin/Streptomycin (100 unit and 0.1 mg/mL respectively) in presence of 5 % CO_2 in a humidified incubator at 37 ^{o}C as described by us previously [2,55,87,89].

For the estrogen (Sigma), BPA (Sigma) and DES (Sigma) treatment, MCF7 cells were grown and maintained for one round in phenol red free DMEM-F-12 media supplemented with 10 % charcoal stripped FBS, 2 mM L-glutamine and penicillin/streptomycin (100 unit and 0.1 mg/mL respectively). Cells were grown up to 60 % confluency in phenol red free media and charcoal stripped FBS, treated with varying concentrations (0-100 nM) of 17β-estradiol (E2), 0-1000 nM each of BPA and DES for 4 h, and incubated for varying time points (0, ¼ , ½, 1, 2 , 4 and 6 h) before harvesting for temporal study.

4.2.2 Antisense oligonucleotide-mediated knockdown of gene expression

MCF7 cells were grown up to 60 % confluency in 60 mm plates and transfected with 2.25 µg/ml each of ERα, ERβ and a scramble antisense oligonucleotides separately (commercially synthesized from IDT-DNA) in FBS free media using iFECT transfection reagent (KD Medicals Inc) by following manufacturer's instruction. In brief, a cocktail of antisense and iFECT transfection reagent was made in 300 µL DMEM-F-12 media, applied to cells in

presence of 1.7 mL supplement free media and incubated for 24 h. Then 2 mL media containing all supplements and 20 % extra charcoal stripped FBS were added and incubated for another 24 h before treating with 1 nM E2 for additional 4 h.

4.2.3 RNA extraction, reverse transcription and real-time PCR

For RNA extraction cells were harvested by centrifugation at 1500 rpm for 5 min, the cell pellets were resuspended in diethyl pyrocarbonate (DEPC) treated buffer A (20 mM Tris-HCl, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM DTT and 0.5 mM EDTA), incubated on ice for 10 min, centrifuged at 3500 rpm for 5 min. The supernatant containing the cytoplasmic extracts was subjected to phenol-chloroform extraction. This is done in two steps: phenol-chloroform treatment to separate the proteins from the DNA and the chloroform treatment to remove the leftover phenol solution. It is then followed by ethanol precipitation of cytoplasmic RNA by incubating for 1 h at - 80 ºC. The centrifuged RNA was washed with 70% ethanol to remove the impurities. The purified RNA was quantified and used for the regular RT-PCR and real-time PCR reactions.

Reverse transcription reactions were performed in a total volume of 25 µL of cDNA containing 500 ng of RNA, 2.4 µM of oligo dT (Promega), 100 units of MMLV reverse transcriptase, 1X first strand buffer (Promega), 100 µM each of dATP, dGTP, dCTP and dTTP (Invitrogen), 1 mM dithiothreitol and 20 units of RNaseOut (Invitrogen). The cDNA was diluted to 100 µL. PCR was performed in a 10 µL reaction volume containing 5 µL diluted cDNA and 5 µL of reaction mixture including gene specific primer pairs (Table 4.1). For real-time PCR analysis, the cDNA was amplified using SsoFast EvaGreen supermix (Bio-Rad) using CFX96 real-time PCR detection system. The real-time PCR results were analyzed using the CFX manager software. The experiments were repeated at least twice with three replicates each time.

4.2.4 Immunohistological analysis of breast cancer tissue microarray

The breast cancer tissue microarray slide containing 6 different cases (duplicates of each) of breast cancer and their corresponding adjacent normal tissues were purchased from US Biomax Inc and subjected to immunohistological staining. Prior to staining, the paraffin embedded tissue microarray slide was immersed twice in xylene (for 10 min) and then sequentially immersed in 100%, 95% and 70% ethanol (5 min each) to deparaffinize the tissue. Antigen retrieval was done by incubating the slide in 0.01M sodium citrate buffer at 95 ^{0}C for 15 min following supplier's instruction. For immunohistological staining, the tissue microarray slide was incubated in 3% H_2O_2 for 15 min, washed with PBS thrice, and then blocked with blocking buffer containing donkey serum. The slide was then incubated with HOXC6 antibody (produced in rabbit) overnight, washed thrice in PBS, and then incubated with biotinylated donkey secondary antibody for 1.5 h. The slide was washed thrice with PBS, incubated with Avidin-Biotin Complex (ABC, Vector laboratories) for 1.5 h, washed twice with PBS and then twice with 0.1 M tris-HCl (pH 7.4). Slide was incubated with diaminobenzidine (DAB) substrate (Vector Laboratories) for peroxidase labeling. The tissue microarray slide was dehydrated with sequential immersion under 70%, 95%, and 100% ethanol and then cleaned by sequential incubation (1, 5 and 10 min) in citrisolv clearing agent (Fisherband). Tissue sections were finally mounted with DPX mounting solution (Sigma), photographed, and examined under microscope (Nikon Eclipse TE2000-U, Japan).

4.2.5 Dual luciferase reporter assay

The HOXC6 ERE at 125 nt upstream of the transcription start site along with its flanking region (total 396 bp) was cloned and inserted upstream of the promoter of a fire fly luciferase gene in pGL3 promoter vector (Promega) (primers are listed in Table 4.1). MCF7 cells were co-transfected with this ERE-containing luciferase reporter construct along with reporter plasmid containing renilla luciferase (pRLTk, Promega) as an internal transfection control using

lipofectamine transfection reagent (Invitrogen). Control transfection was done using empty pGL3 promoter vector without any ERE insertion. Non-transfection control was also done in parallel. At 30 h post-transfection, cells were treated with 1 nM E2, 10 nM BPA, 100 nM DES and incubated for an additional 8 h. Total protein was extracted and then subjected to luciferase assay using Dual-Glo Luciferase Assay System (Promega) as instructed and detected using a microplate reader (Flowstar-Omega). Each treatment was done in four replicates and the experiment was repeated at least twice (n = 2).

4.2.6 Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed on MCF7 cells using EZ Chip chromatin immunoprecipitation kit (Upstate) as described previously by us [2,55,87]. For ChIP assay, cells were fixed in 4% formaldehyde for 15 min at room temperature, quenched the excess formaldehyde with 125 mM glycine, washed twice with cold PBS containing protease inhibitor (5 $\mu L.mL^{-1}$) and PMSF (5 $\mu L.mL^{-1}$). The cells were then lysed in SDS lysis buffer and sonicated to shear the chromatin to ~150-450 bp in length. The sonicated chromatin was pre-cleaned using protein-G agarose (Millipore) beads and subjected to immunoprecipitation using antibodies specific to ERα (Santacruz), ERβ (Santacruz), H3K4-trimethyl (Upstate), RNAPII (Abcam), MLL2 (Bethyl laboratory), MLL3 (Abgent) and H4 acetylation (Upstate). The immunoprecipitated chromatin trapped in protein-G agarose was washed sequentially with low-salt, high-salt, LiCl, and Tris-EDTA buffer and eluted. The immunoprecipitated chromatin was deproteinized by incubating at 65 ^{o}C in presence of NaCl, followed by incubation in presence of proteinase K (Sigma). The phenol-chloroform was done to remove the proteins and obtain purified DNA fragments after ethanol precipitation. The immunoprecipitated DNA was then PCR-amplified using promoter specific primers (shown in Table 4.1) to analyze the level of recruitment of different proteins.

Table 4.1 Primers used for cloning, RT-PCR, ChIP, and antisense experiments

Primers	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)
PCR primers		
GAPDH	CAATGACCCCTTCATTGACC	GACAAGCTTCCCGTTCTCAG
HOXC6	CAGACCCTGGAACTGGAGAA	CTTCCCGCTTTTCCTCTTTT
HOXC6-ERE	TTTTTCCCCCTTCCTGACAT	GCCTTTACCTGGTCGGTCTA
ERα	AGCACCCTGAAGTCTCTGGA	GATGTGGGAGAGGATGAGGA
ERβ	AAGAAGATTCCCGGCTTTGT	TCTACGCATTTCCCCTCATC
Cloning primer		
HOXC6-ERE	CCACCAAACCAGTTCCCTTA*	ATCATAGGCGGTGGAATTGA*
Antisenses		
ERα antisense	TCCCACCTTTCATCATTCCC**	
ERβ antisense	GCCACACTTCACCATTCCCA**	
Scramble antisense	CGTTTGTCCCTCCAGCATCT**	

*Flanked by appropriate restriction sites; **Phosphorothioate antisense oligonucleotide.

Note: These antisense oligonucleotides were designed by Dr. Subhrangsu S. Mandal and Dr. Khairul I. Ansari and kindly provided by them.

4.3 Results

4.3.1 HOXC6 is over-expressed in breast cancer cells and tissue

HOXC6 is known to be associated with mammary gland development and my previous study demonstrated that HOXC6 is transcriptionally regulated by estrogen [54]. Herein, I examined its expression levels in different types of cancer cell lines including ER positive and ER negative breast cancer cell lines. Real-time PCR analysis demonstrated that HOXC6 expression was relatively higher in ER positive breast cancer cells (MCF7 and T47D) in comparison to the ER negative MDAMB231 cells and nonmalignant breast epithelial cells MCF10 (Figure 4.1A).

I further examined the expression of HOXC6 in human breast cancer tissue. I performed immunohistochemical staining (DAB staining) of breast cancer tissue microarray slide that contains 6 cases of breast cancer (in duplicates) along with corresponding adjacent normal tissue using HOXC6 antibody. This analysis demonstrated that HOXC6 is differentially over-expressed in various breast cancer tissue samples in comparison to its corresponding adjacent breast normal tissue (Figure 4.1B, a magnified view of case 6 is shown in figure 4.1C).

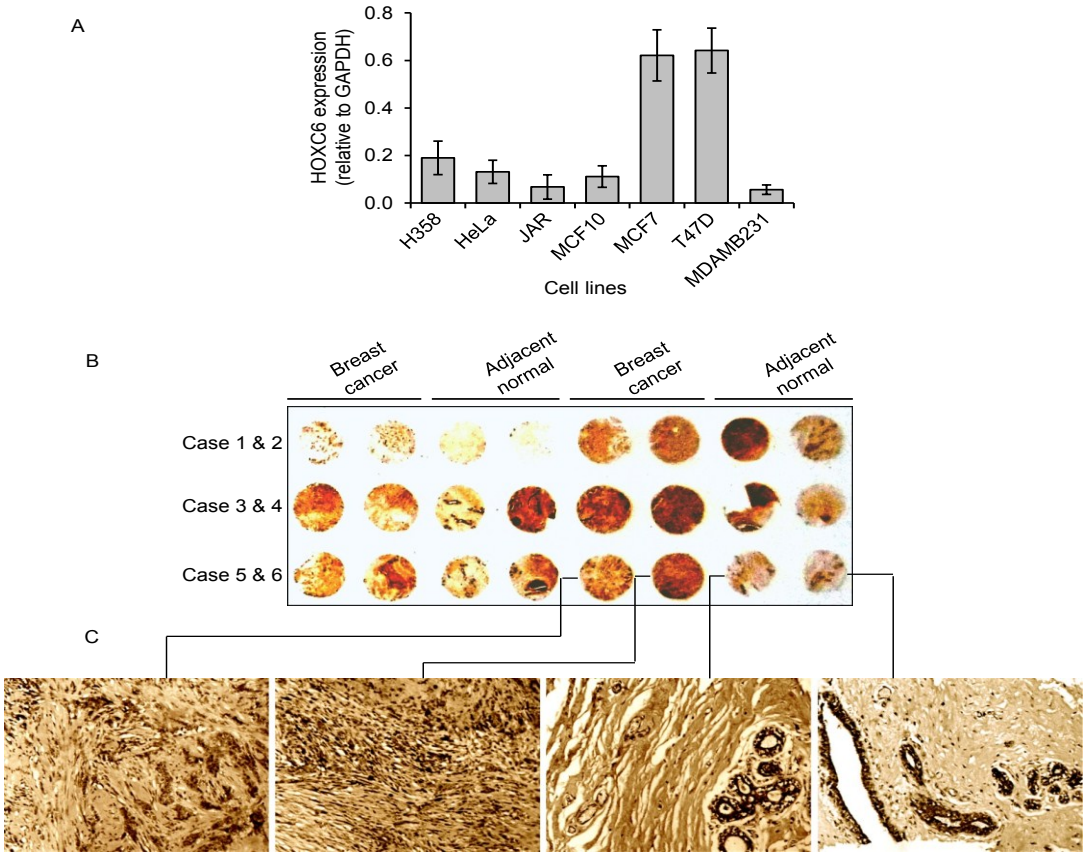


Figure 4.1 HOXC6 expression in different human cell lines and breast cancer tissue. (A) The total RNA was isolated from H358, HeLa, JAR, MCF10, MCF7, T47D, MDAMB231, and reverse transcribed to cDNA and analyzed by real-time PCR using primers specific to HOXC6. HOXC6 expression relative to GAPDH is plotted. Each experiment was repeated at least thrice (n = 3). Bars indicate standard errors. (B) Immunohistological analysis of HOXC6 expression in breast cancer tissue: Human breast cancer tissue microarray (6 cases of breast cancer along with their corresponding adjacent normal breast tissue) was immunostained (DAB staining) with HOXC6 antibody and presented in panel B and the magnified view of case 6 is shown in panel C. Immunohistological analysis of HOXC6 expression was carried out by Dr. Khairul I. Ansari and figures B and C are kindly provided by him.

4.3.2 HOXC6 is transcriptionally activated by E2 in MCF7 cells via ER-dependent pathway

As HOXC6 is over-expressed in ER positive breast cancer cells (MCF7 and T47D) and in breast cancer tissue, I examined its potential regulation by estradiol (E2) in MCF7 cells. I treated the MCF7 cells (grown in phenol red free media and charcoal stripped FBS) with varying concentrations of E2. The RNA from the control and E2-treated cells was reverse transcribed and subjected to PCR analysis using HOXC6 primers. GAPDH was used as a loading control. My analysis demonstrated that HOXC6 transcription is induced upon treatment with E2 in a concentration dependent manner (Figure 4.2A). The highest induction was observed at 1 nM E2. However, HOXC6 expression was relatively insensitive to E2 in ER negative cell line MDAMB231 (Figure 4.2C). Time-dependent analysis demonstrated that E2-induced HOXC6 expression was highest at 4 h post E2 treatment (Figure 4.2B).

As E2-dependent expression was observed in ER positive MCF7 cells and my previous studies demonstrated that ERs are involved in E2-mediated HOXC6 expression in JAR cells [54], I knocked down ERα and ERβ in MCF7 cells, cells were exposed to E2 and analyzed for the HOXC6 expression using RT-PCR. These analyses demonstrated that knockdown of either ERα or ERβ downregulated the E2-dependent induction of HOXC6 in MCF7 cells (Compare lane 2 with lane 3, figure 4.2D and 4.2E), indicating crucial roles of ERs during E2-induced HOXC6 expression.

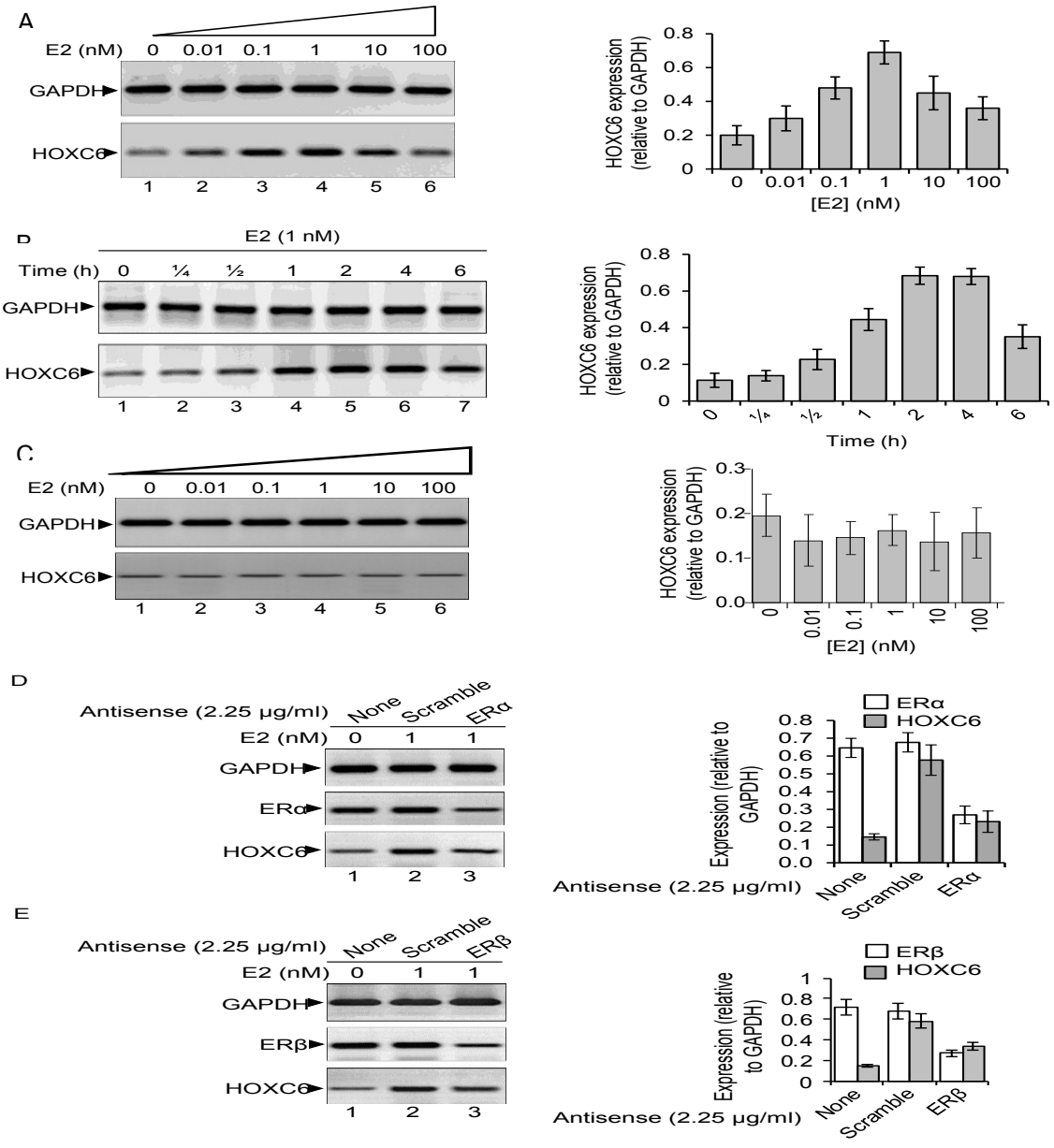


Figure 4.2 Effect of E2 on HOXC6 expression and the roles of ERs. (A) MCF7 cells were treated with varying concentrations of E2. The total RNA was isolated and amplified by RT-PCR and real-time PCR (right panel) using primers specific to HOXC6. Expression of HOXC6 (relative to GAPDH) was examined by real-time PCR (right panel). (B) Temporal studies: MCF7 cells were treated with 1 nM E2 and incubated for varying time periods (0, ¼, ½, 1, 2, 4 and 6 h) post E2 treatment. RNA was reverse transcribed, analyzed by RT-PCR and real-time PCR (right panel). (C) E2-dependent expression of HOXC6 in MDAMB231 by RT-PCR and real-time quantification (right panel). (D and E) MCF7 cells were transfected with ERα and ERβ specific antisenses for 48 h. A scramble antisense was used as negative control. The knocked down cells were treated with E2 for 4 h and analyzed by regular RT-PCR and real-time PCR (right panel). Each experiment was repeated at least thrice (n = 3). Bars indicate standard errors.

4.3.3 HOXC6 is transcriptionally activated by BPA and DES

As HOXC6 is E2-regulated gene in breast cancer cells and is also over-expressed in breast cancer tissue, I examined if this is potentially misregulated by endocrine disrupting chemcials such as BPA and DES. I treated MCF7 cells with varying concentrations of BPA and DES separately and examined their impact on HOXC6 expression using RT-PCR. Interestingly, HOXC6 expression was significantly induced upon exposure to both BPA and DES (Figure 4.3A and 4.3B). BPA-induced expression of HOXC6 was highest at 10 nM concentration while, DES-mediated induction was maximum at 100 nM. These concentrations are relatively higher in comparison to E2. This observation demonstrated that both estrogenic compounds BPA and DES are capable of inducing HOXC6 expression even in the absence of estrogen. Time-dependent analysis demonstrated that both BPA and DES-mediated induction was maximum at 4 h.

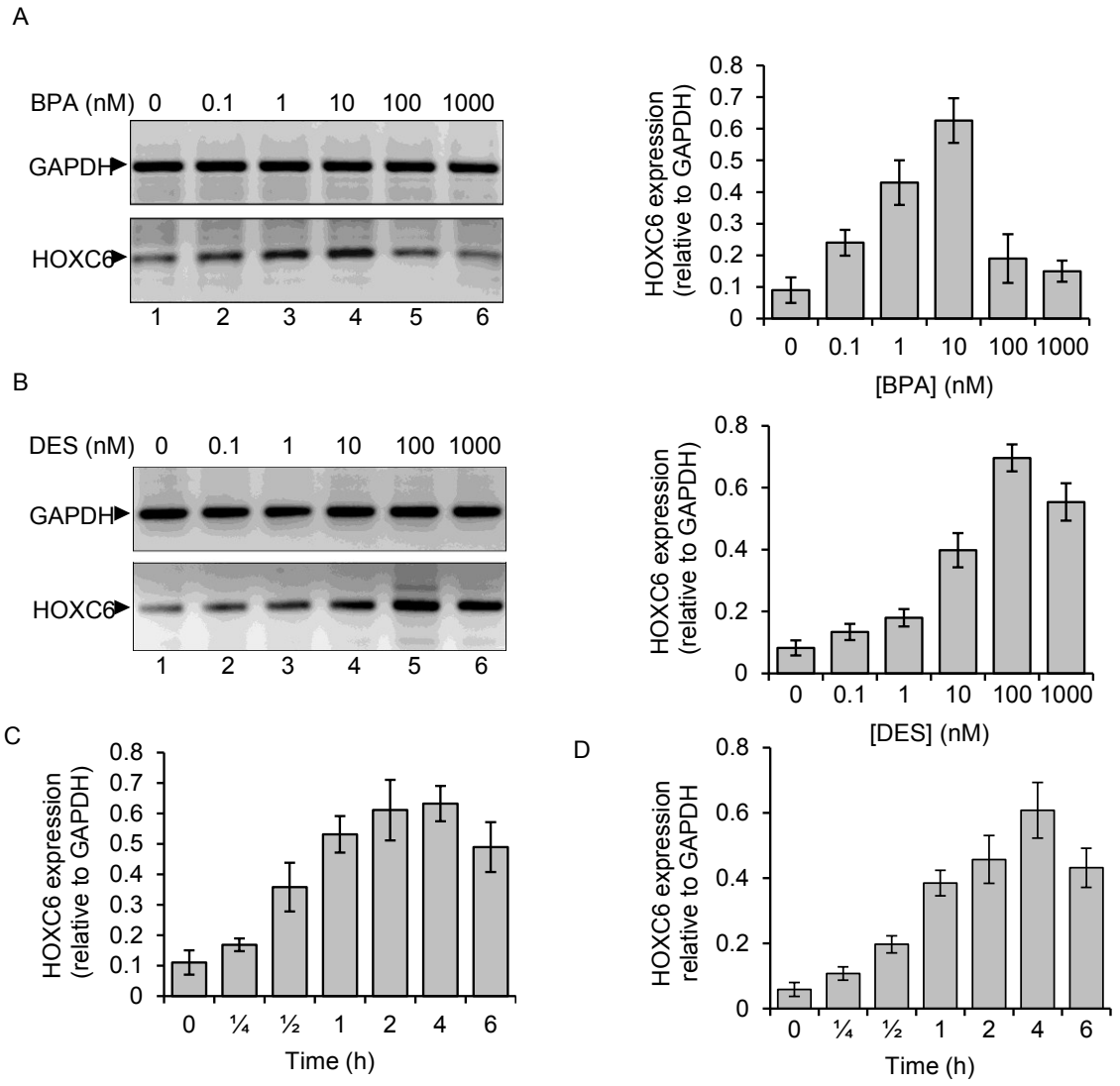


Figure 4.3 Effects of BPA and DES on HOXC6 gene expression. (A and B) MCF7 cells (grown in phenol red free media for one round) were treated with BPA (A) and DES (B) for varying concentrations (0-1000 nM). The RNA was isolated, converted to cDNA and analyzed by RT-PCR using primers specific to HOXC6. GAPDH was used as a loading control. The cDNA was analyzed by real-time PCR and expression of HOXC6 (relative to GAPDH) is plotted in respective right panel. Each experiment was repeated at least thrice. Bars indicate standard errors ($p<0.05$). (C and D) MCF7 cells were treated with 10 nM BPA (C) and 100 nM DES (D) for varying time periods (0-6 h) post E2 treatment. The RNA isolated was converted to cDNA and analyzed by real-time PCR. Each experiment was repeated at least thrice (n = 3).

4.3.4 HOXC6 promoter estrogen response element (ERE) is responsive to E2, BPA and DES

Analysis of HOXC6 promoter demonstrated that it contains an imperfect full ERE located at 125 nt upstream of transcription start site (Figure 4.4A). Recently my study also demonstrated that this ERE participates in E2-mediated HOXC6 activation [54]. Herein I examined if this ERE also participates in BPA and DES-mediated HOXC6 activation using luciferase-based reporter assay. The promoter of HOXC6 containing the ERE region was cloned into luciferase-based reporter construct pGL3. The ERE-pGL3 construct along with reporter vector containing renilla luciferase (transfection control) was tranfected into MCF7 cells followed by expsoure to E2, BPA and DES and the induction of luciferase was determined by using dual luciferase detection kit. Empty pGL3 transfection was used as a negative control. As seen in figure 4.4B, E2-treatment induced the luciferase gene by ~4-fold in ERE-pGL3 transfected MCF7 cells. Interestingly, exposure to BPA and DES also induced luciferase activity by ~2.5 and ~3-fold respectively in comparison to untreated samples (Figure 4.4B). The E2-induced luciferase activity was little higher in comparison to BPA and DES. These analyses demonstrated that similar to E2, BPA and DES also induce HOXC6 expression via involvemnt of HOXC6 promoter ERE.

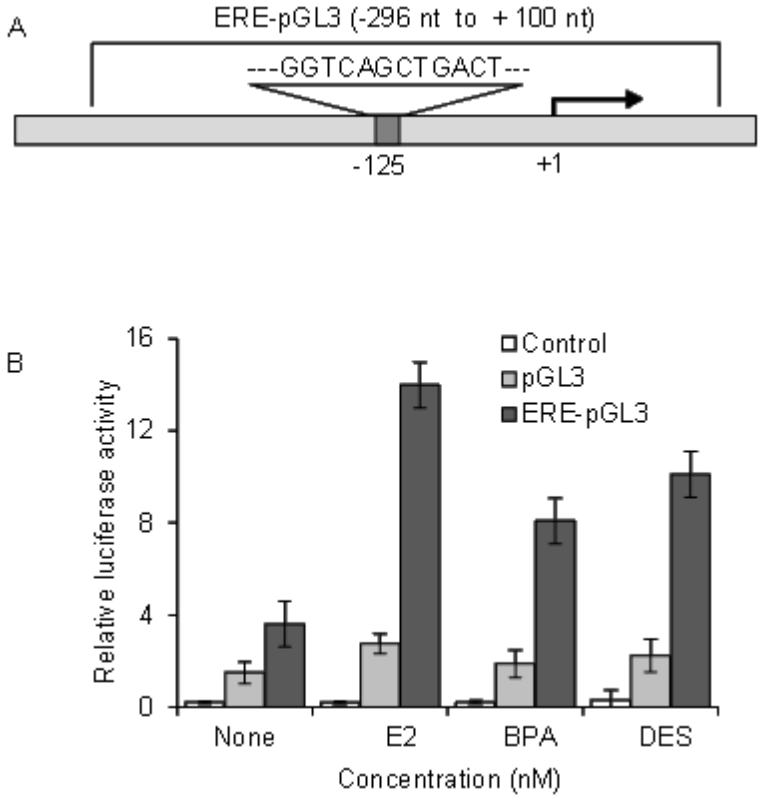


Figure 4.4 Characterization of HOXC6 promoter ERE using luciferase-based reporter assay. (A) HOXC6 promoter showing one ERE was cloned into luciferase reporter construct pGL3. (B) Luciferase assay: ERE-pGL3 construct was transfected into MCF7 cells for 30 h. Control cells were treated with empty pGL3 vector and no transfection control was done in parallel. Cells were also co-transfected with renilla luciferase expression construct as an internal transfection control. Cells were then treated with 1 nM E2, 10 nM BPA and 100 nM DES, and then subjected to luciferase assay by using Dual-Glo Luciferase Assay System (Promega). The luciferase activities in presence of E2, BPA and DES (over untreated controls and normalized against renilla luciferase expression) were plotted. The experiment with four replicate treatments was repeated at least thrice (n = 3). Bars indicate standard errors ($p < 0.05$).

4.3.5 ERs and ER coactivators bind to HOXC6 promoter and modulate histone modification upon exposure to E2, BPA and DES

ER coregulators are well-known to play crucial role during E2-mediated gene activation. In general, in the presence of estrogen, ER coregulators interact with ERs and get recruited to the promoter of target genes leading to gene activation. There are many ER coregulators identified till date. Recent studies demonstrated that MLL family of histone methylases act as ER coregulators during E2-mediated gene activation and signaling. Specifically, histone

methylases MLL2 and MLL3 are shown to bind to the HOXC6 promoter in presence of E2, introduce H3K4-trimethylation and gene activation [54]. Herein, I examined if, similar to E2, treatment with BPA and DES also induces recruitment of ERs and ER coactivators (specifically MLL) and changes the histone methylation status in the HOXC6 promoter. I treated MCF7 cells with E2, BPA and DES separately, control and treated cells were fixed with formaldehyde and subjected to chromatin immunoprecipitation (ChIP) assay. ChIP DNA was analyzed by regular RT-PCR and real-time PCR using HOXC6 promoter primers. Notably, HOXC6 promoter contains an active estrogen response element (an imperfect full ERE) located at 125 nt upstream of the transcription start site and we designed the promoter primers around the ERE region. ChIP analysis demonstrated that, as expected, ERα and ERβ, histone methylases MLL2 and MLL3 were enriched at the HOXC6 promoter upon treatment with E2. Histone H3K4-trimethylation as well as RNA polymerase II recruitment levels were increased upon E2 treatment. Interestingly, treatment with BPA and DES also resulted in increased recruitment of these factors in HOXC6 promoter (Figure 4.5A, real-time quatification is shown in figure 4.5B). These experiments demonstrated that ERs and MLL histone methylases also participate in BPA and DES-dependent HOXC6 activation and misregulation. BPA and DES exposure induced epigenetic changes in HOXC6 promoter affecting its gene expression.

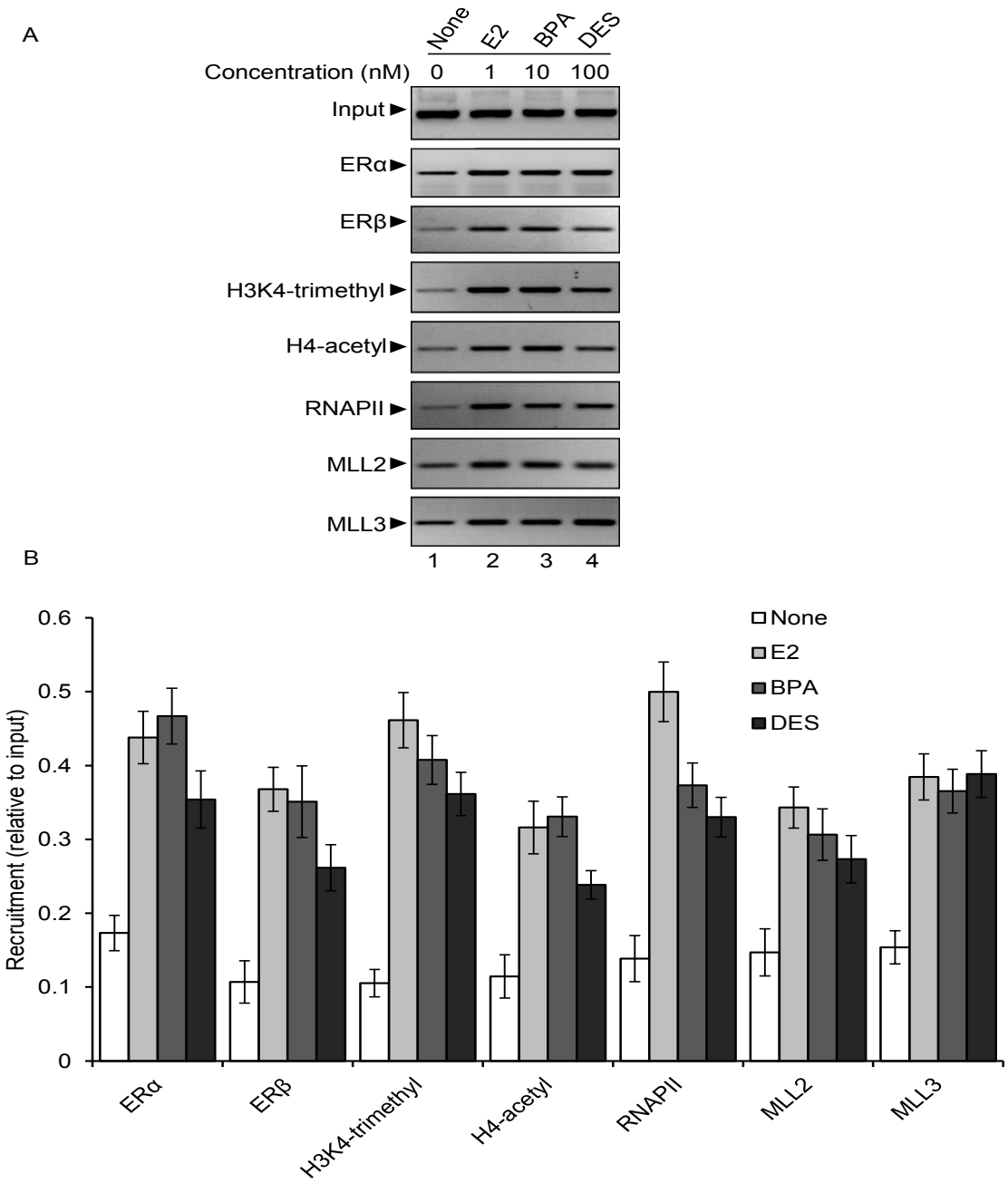


Figure 4.5 E2, BPA and DES-dependent recruitment of ERs, MLL2, MLL3, RNAPII, H4-acetylation and H3K4-trimethylation on HOXC6 ERE. (A and B) MCF7 cells were treated with 1 nM E2, 10 nM BPA and 100 nM DES for 4 h and analyzed by ChIP assay using antibodies indicated in the figure. The ChIP DNA was PCR-amplified using primers specific to ERE region of HOXC6 promoter. Real-time quantification of ChIP DNA samples showing the recruitment of different factors (relative to input) into ERE region in presence of E2, BPA and DES is shown in the panel B. The experiments were repeated at least twice (n = 2). Bars indicate standard errors.

4.4 Discussion

Overall my studies demonstrated that HOXC6 is over-expressed in ER positive breast cancer cells such as MCF7 and T47D. It is also over-expressed in breast cancer tissue in comparison to the corresponding surrounding normal breast tissue. HOXC6 expression is induced upon exposure to E2 in ER positive breast cancer cells and this is mediated via involvement of ERs. Similar to E2, endocrine disrupting chemicals with estrogenic activities such as BPA and DES also induced the expression of HOXC6 in breast cancer cells. BPA and DES induced the recruitment of ERs and ER coactivators such as histone methylases MLL2 and MLL3. Exposure to BPA and DES also affected the epigenetic states such as increased histone H3K4-trimethylation and histone acetylation levels that ultimately resulted in HOXC6 gene activation. In summary, these studies demonstrated that HOXC6 gene which is associated with breast cancer is transcriptionally regulated by estrogen and is disrupted upon exposure to endocrine disrupting chemicals such as BPA and DES. BPA and DES change the epigenetic states of HOXC6 promoter and result in misregulation of its expression. My studies provide a novel epigenetic mechanism by which BPA and DES may be associated with risk of breast cancer in human and provide a direct link with environmental factors in activation of protooncogenes in breast cancer.

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BIOGRAPHICAL INFORMATION

Imran Hussain, a graduate student in the Chemistry and Biochemistry department at the University of Texas at Arlington, is interested in studying epigenetic mechanism of gene regulation under hormonal environment. He completed his Master in Technology degree in biotechnology from Indian Institute of Technology, Kharagpur, India. In his current research, he studied the roles of mixed lineage leukemia histone methylases in hormonal regulation of HOX genes and their disruption by endocrine disrupting chemicals. In future he wants to continue studying the roles of chromatin modifiers in organ development and disease using multicellular organisms as model system.