EPIGENETIC MECHANISM OF REGULATION OF HOX GENES AND NEUROTRANSMITTERS VIA HORMONES AND LNCRNA

by

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ABSTRACT

EPIGENETIC MECHANISM OF REGULATION OF HOX GENES AND NEUROTRANSMITTERS VIA HORMONES AND LNCRNA

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Endocrine disrupting chemicals (EDCs) are classes of environmental contaminants whose exposure even at very low concentrations interfere with normal endocrine signaling and induce many adverse health effects including increased risk of birth defects, diabetes, obesity, abnormal reproduction, and cancer. EDC exposure alters the epigenetic programming of cells resulting in altered gene expression and regulation and cell signaling. These EDCs include bisphenol-A (BPA), diethylstilbesterol (DES), polychlorinated biphenyl (PCB), phthalates, phytoestrogens, methoxychlor, fungicides, insecticide, herbicide, and some heavy metals. As a part of my work, I have investigated epigenetic impacts of EDCs on HOX (homeobox containing genes) genes, which are critical players in embryonic development. They determine segmental identity along the anterio-posterior (AP) body axis. HOX genes are also expressed in adults for functional differentiation. Notably, deregulation of HOX gene expression contributes to severe human diseases including cancer. The knowledge on the regulatory mechanisms that drive HOX expression is limited. Studies demonstrate that HOX gene expressions are influenced by variety of hormones that include estradiol, progesterone, testosterone, retinoic acid, vitamin D and others. By regulating HOX genes, hormonal signals utilize a conserved

mechanism that allows generation of structural and functional diversity in both developing and adult tissues. In my research, I have investigated the mechanism of endocrine regulation (by estradiol) and disruption (by EDCs) of a particular HOX gene, HOXB9, *in vitro* and *in vivo*. Notably, HOXB9 is a homeobox-containing gene that plays key roles in mammary gland development and is associated with breast and other types of cancer. Here, we demonstrate that HOXB9 expression is transcriptionally regulated by estradiol (E2), *in vitro* and *in vivo*. We also demonstrate that endocrine disrupting chemical BPA induces HOXB9 expression in cultured human breast cancer cells (MCF7) as well as *in vivo* in the mammary glands of ovariectomized (OVX) rats. Luciferase assay showed that estrogen-response-elements (EREs) at the HOXB9 promoter are induced by BPA. Estrogen-receptors (ERs) and ER-coregulators such as mixed lineage leukemia 3 (MLL3) histone methylase, histone acetylases, CBP/p300, bind to the HOXB9 promoter EREs in the presence of BPA, modify chromatin (histone methylation and acetylation) and lead to gene activation. In summary, our results demonstrate that BPA exposure alters the epigenetic programming of the HOXB9 promoter leading to its endocrine disruption *in vitro* and *in vivo*.

Hypoxia signaling plays a critical role in tumor growth, angiogenesis, metastatic cancer, and aging. Under hypoxic condition, hypoxia-inducible factors (HIFs) are stabilized and they coordinate the process of hypoxia-induced gene expression and cell signaling pathways and leads to increased tumor cell proliferation, angiogenesis and growth. Recent studies indicate that non-coding RNAs which are closely associated with cancer are abnormally expressed under hypoxia. Here, we have investigated the transcriptional regulation of a cancer associated long non-coding RNA (lncRNA), homeobox transcript antisense RNA (HOTAIR) under hypoxic conditions. Our studies demonstrate that HOTAIR expression is upregulated under hypoxia in colon cancer and several other types of cancer cells. HOTAIR transcription is regulated by HIF1α which binds to the hypoxia response elements (HRE) present in the

HOTAIR promoter under hypoxia. HIF1α knockdown results in decreased HOTAIR expression under hypoxia. Along with HIF1α, histone acetyltransferase, p300, histone methyl-transferase, MLL1, and RNA polymerase II are enriched at the HOTAIR promoter in a hypoxia dependent manner. The levels of H3K4-trimethylation and histone acetylation are also enriched at the HOTAIR promoter under hypoxic conditions. Overall, our studies demonstrate that HOTAIR expression is induced under hypoxic environment via coordination of HIFs and other transcription activators which may contribute to its roles in tumorigenesis.

Long noncoding RNAs are abundant in the mammalian transcriptome and many of them are specifically expressed in the brain. HOTAIR, a long intergenic noncoding RNA (lincRNA), is known to be overexpressed in several diseases. However, the role of HOTAIR in neurons/neurological disorders remains unclear. Neurotransmitters and neurotrophic factors are critical to neuronal signaling, growth and neuroprotection. Brain derived nerotrophic factor (BDNF) is a critical neurotrophic factor that plays key roles in neuronal growth, maintenance and protection and abnormal levels of BDNF in brain causes neurological disorders. Here we have investigated the mechanism of BDNF expression, especially, mediated by lncRNA HOTAIR. Our studies have demonstrated that HOTAIR is a key regulator of BDNF. Knockdown of HOTAIR resulted in induction of BDNF expression in hippocampal (HT22) cells. Furthermore, enzymatic inhibition of a HOTAIR-interacting partner, EZH2, also resulted in BDNF induction. Chromatin immunoprecipitation analysis demonstrated that levels of EZH2 (HOTAIR-interacting histone trimethylase) and LSD1 (HOTAIR-interacting histone demethylase) were reduced at the BDNF promoter under EZH2-inhibition condition. The level of repressive histone modification marks such as H3K27-trimethylation (which is introduced by EZH2) is decreased at the BDNF promoter. Taken together, these observations indicate that HOTAIR along with with interacting partners such as EZH2 are involved in repression of BDNF expression.

Beyond lncRNA, estrogen is also known to modulate neural plasticity, cognition and neuroprotection. Indeed, our studies demonstrate that estradiol (E2) treatment induces the expression of BDNF mRNA and protein levels in HT22 cells *in vitro*. Estrogen receptor ERα/ERβ and ER-coregulators (CBP/p300) and MLL3 were enriched at the BDNF promoter in the presence of E2. Additionally, we showed that E2 induced-BDNF expression is mediated by the displacement of silencing factors, EZH2 and LSD1 at BDNF promoter and subsequent recruitment of active transcription machinery. These results reveal the function and mechanisms of lncRNA, HOTAIR to enforce silent chromatin state at BDNF promoter which in turn is rescued upon treatment with positive regulators such as E2.

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HOX	Homeobox genes
E2	17β-estradiol
ERs	Estrogen receptor
ERE	Estrogen response element
AP1	Activation protein-1
SP1	Specific-protein 1
NCOR	Nuclear receptor co-repressor
HDAC	Histone deacetylase
HAT	Histone acetylase
CBP	CREB-binding protein
PRC2	Polycomb repressive complex 2
BDNF	Brain-derived neurotrophic factor
OXT	Oxytocin
BPA	Bisphenol-A
ChIP	Chromatin Immunoprecipitation
DEPC	Diethyl pyrocarbonate
DES	Diethylstilbestrol
DMEM	Dulbecco's modified eagle's medium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
HOTAIR	HOX antisense intergenic RNA
LncRNA	Long non-coding RNA
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
HIF	Hypoxia inducible factors
VHL	Von-Hippel Lindau tumor suppressor gene
HRE	Hypoxia Response Element/Hormone response element
FIH-1	Factor inhibiting HIF-1
PHD	Proline hydroxylase
RNAPII	RNA polymerase II
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SRC-1	Steroid Receptor Coactivator-1
SUZ12	Suppressor of Zeste 12 Homolog
RbAp46/48	Retinoblastoma Associated Protein 46/48
EED	Embryonic Ectoderm Development

EZH2	Enhancer of Zeste homolog 2
LSD1	Lysine-Specific Demethylase-1
OVX	Ovariectomized
snRNAs	Small nuclear RNAs
snoRNAs	Small nucleolar RNAs
tRNAs	Transfer RNAs
TSSRNAs	Transcription start-site-associated RNAs
PASRs	Promoter-associated small RNAs
PROMPTS	Promoter upstream transcripts
AP-axis	Anterio-posterior axis
RXR	Retinoid X receptor
SMRT	Silencing mediator of retinoic acid
VDR	Vitamin D receptor
VDRE.	Vitamin D response element
NGF	Nerve growth factor
NT3/4	Neurotrophin 3/4
FGF-2	Fibroblast growth factor
TGF	Transforming Growth Factor
VEGF	Vascular endothelial growth factors
EPO	Erythropoietin
CNTF	Ciliary neurotrophic factor
LIF	Leukemia inhibitory factor
rRNA	Ribosomal RNA
tRNA	Transfer RNA
piRNA	Piwi-interacting RNA
eRNA	Enhancer RNA
UCA1	Urothelial Carcinoma Associated 1
ERR	Estrogen-related receptors
AS-RNA	Antisense RNA
REST	RE1-Silencing Transcription factor
ERK	Extracellular signal-regulated kinase
PAH	Polycyclic aromatic hydrocarbons
PCB	Polychlorinated biphenyl
PBB	Polybrominated biphenyls
HPG	Hypothalamus pituitary gonadal
HOTAIR	Homeobox transcript antisense RNA

CHAPTER 1

ENDOCRINE REGULATION OF HOX GENES AND NEUROTRANSMITTERS

1.1 Introduction

The endocrine system is the collection of glands that produces hormones which regulate diverse cellular and physiological functions including metabolism, growth, development, reproduction, sleep, mood and others. Hormones are molecules that are produced in one cellular location in an organism, and whose effects are seen in another tissue or cell type. Hormones can be peptides, proteins or steroids. The protein hormones do not enter the cell, but bind to receptors in the cell membrane and mediate gene expression through intermediate molecules. Steroids homrones do enter inside the cell and interact with steroid receptors and trigger downstream signaling and gene expression. Secretion of hormones is regulated by a system of sensing elements possessing the means to detect the need for both increased and decreased secretion. The particular sensing network, feedback elements, and network of control responses are unique for each hormone. Hormonal pathways maintain homeostasis and adjustments in secretion usually result in changes that will help maintain the status quo.

1.2 Homeobox (HOX) genes

The family of HOX gene consists of 4 to 48 members per genome depending on the animal. HOX genes are involved in patterning of AP axis of the body in nearly all metazoans.[1] The development of embryo is under the control of the HOX gene family that results in different segmental identities in developing embryos. It is well known that clustered organization of HOX genes determine positional identities of different segments in the embryo. Different structural segments in embryos are arranged in

a collinear fashion in accordance with the relative position of the genes within the cluster.[2] HOX genes guide development in higher animals following many principles. 1) Spatial collinearity: 5′-HOX genes are linked to more posterior and caudal structures,[3] while 3′-HOX gene expression are necessary for normal oesophagus development.[4] 2) Posterior prevalence**:** 5'-located HOX genes will have a more dominant phenotype than 3'-located HOX genes.[5] 3) Temporal collinearity**:** 3' located HOX genes in the cluster are expressed earlier than 5' located HOX genes.[6] Overall, HOX gene expression critically guides the developmental processes.

1.2.1. Key features of HOX genes

HOX genes code for transcription factors that contain a characteristic homeodomain through which they recognize the promoters of target genes and regulate their expressions.[7] The homeodomain is a highly conserved DNA-binding domain (homeobox) that is made up of 62-amino acid motif and hence the name homeobox genes or HOX genes.[2] They are known as HOM genes in non-human vertebrate whereas HOX genes in humans.[2] Mutation of HOX genes in *Drosophila melanogaster* results in irreversible homeotic transformations of specific segments along AP axis.[8,9] In the fly, Drosophila melanogaster, HOM complex (HOM-C) is made up of 2 separate clusters encoding Antennapedia (Antp) and bithorax complex (BX-C).[10,11,9,12,13,14] The genes arranged on these clusters are successively expressed along the AP axis of the developing embryo and contribute to the development. The group of genes that belong to these clusters include labial (lab), proboscipedia (pb), deformed (dfd), sex combs reduced (scr), antennapedia (anp), ultrabithorax (ubx), and abdominal-A (abd-A) (Figure 1).[14] Most vertebrates have 39 homeobox containing genes arranged in four clusters: A, B, C and D.[15] These genes are located on chromosomes 6, 11, 15, and 2, respectively, in mice and 7, 17, 12, and 2, respectively, in humans.[2,14] HOX gene family members have been subdivided into 13 groups/subfamilies according to

the chromosomal order of alignment which shows a great potential for functional redundancy and similarities.[2] Such highly related group of genes is known as paralogs. Paralogous HOX genes are shown in the form of schematic representations in Figure 1.

1.2.2. Functional significance of HOX genes in development and cancer

HOX gene expression is critical for embryonic development. They are expressed in a cluster and the genes expressed in the anterior end of the cluster overlap with the posterior domain of the adjacent gene on the chromosome and the posterior gene expression dictates the positional identity.[16] The HOX genes are also expressed in the "secondary axes" of the embryo, the limbs and developing genitalia, as well as in many other developing tissues.[17,18] HOX genes are critical for the development of hindbrain region of the vertebrate nervous system,[19,20] the reproductive tract and other organs.[21,22,23,24,25,26] The continuous expression of HOX genes in reproductive tract in adults accounts for various changes in the endometrial receptivity during pregnancy.

Beyond their crucial roles in embryonic development, increasing eveidences suggest that HOX genes expression is deregulated in various forms of cancers.[27] Aberrant expression of HOX genes deregulate cancer signaling pathways contributing to tumor growth.[27] HOX genes may be associated with transcriptional activation and repression of target genes.[28] HOX gene expression may favor oncogenic or tumor suppression functions. Our laboratory and many others have shown that HOX gene expression in tumors is altered *via* DNA methylation and histone modification.[29,30,31,32,33] HOX genes deregulation has been linked to a variety of cancers including prostate, breast, oesophageal, lung, colon, neuroblastoma, thyroid, ovarian, bladder, kidney, and leukemia.[34,27,35,36,37,38,39,40] For example, several HOX genes encoding HOXA6, HOXA13, HOXB2, HOXB4, HOXB5, HOXB6, HOXB7, HOXB8, HOXB9, HOXC5, HOXC9, HOXC13, HOXD1, and HOXD8 are aberrantly expressed in breast cancer.[39] HOXC4, HOXC5, HOXC6, and HOXC8 genes are overexpressed in malignant prostate cancer

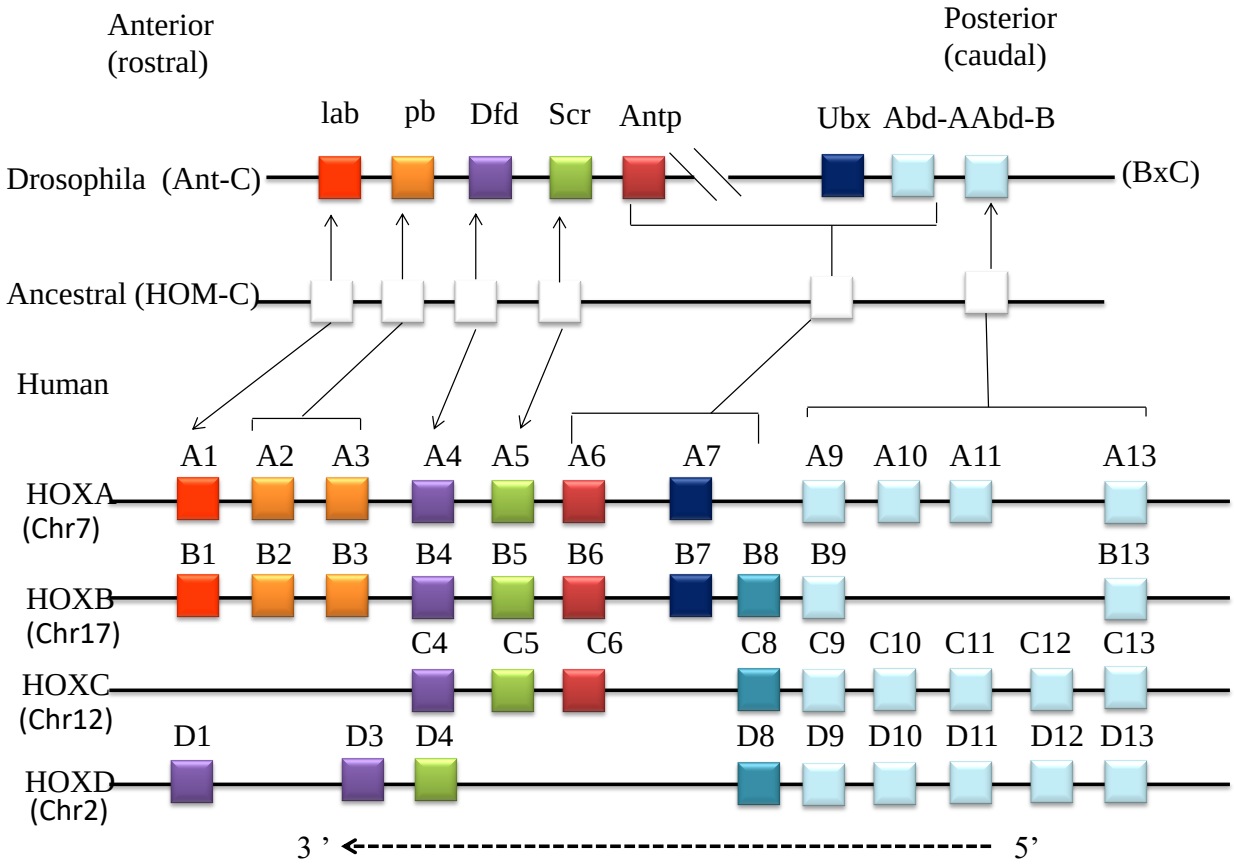


Figure 1. Genomic organization and colinear expression patterns of Drosophila HOM genes and human HOX genes.[43] Although more 3’ HOX genes are expressed in the rostral and anterior regions of the body axis, the more 5’ HOX genes are expressed caudally and in more posterior regions. For each HOX domain of expression, the colored fields represent the characteristically well-defined anteriormost limits of expression. Posteriorly in each domain the HOX expression levels fade so that the boundaries of each expression domain overlap in more caudal regions.

cell lines and lymph node metastases.[27] HOXA9, HOXA13, HOXB13, HOXD13, and HOXC11 genes are critical for embryological development of the prostate gland, whereas HOXC4, HOXC5, HOXC8, HOXB13, and HOXA9 are aberrantly expressed during prostate carcinogenesis.[37] Studies from the Mandal laboratory have shown that HOXB9 regulates expression of growth and angiogenic factors, facilitates tumor growth *in vitro*, and is overexpressed in breast cancer tissue.[35,36] However, I have specifically investigated the estradiol-mediated transcriptional regulation of HOXB9 and its potential endocrine disruption by EDCs in breast cancer cells and *in vivo*. Notably, due to their tissue specific expression patterns, HOX genes are also being explored as potential diagnostic biomarkers and targets for novel cancer therapy.

1.2.3. Endocrine regulation of HOX genes and the mechanism

The process of organogenesis and functional differentiation in the reproductive tract has long been linked with sex steroids.[40] Since HOX genes are related to segmental identity, understanding the sex steroid-mediated regulation of HOX genes is important. Several studies have shown that sex steroids are important regulators of differential HOX gene expression leading to the functional downstream effects such as maturation of endometrium.[41] Alteration of HOX gene expression leads to reproductive tract anomalies and reproductive failure.[41] HOX gene expression in reproductive tracts and associated cancer is highly influenced by steroid hormones. For example, HOXA5 expression in the ovary vary at different stages of the estrous cycle and gestation and this suggests that HOXA5 is regulated by ovarian steroid homrones such as estradiol.[41] HOXB7 is involved in a variety of developmental processes, including hematopoietic differentiation and lymphoid and mammary gland development.[42] HOXB7 expression is significantly elevated in both the primary cancer and distant metastasis.[42] HOXB7 expression is also significantly reduced with estradiol stimulation. These findings support that HOXB7

expression is regulated by estrogen and this action is dependent on the presence of a functional ERα.[42] Our laboratory has also demonstrated that HOXC6, HOXC10, and HOXC13 are transcriptionally regulated by estrogen.[29,30] Overall, it is evident that HOX gene expressions are transcriptionally regulated by steroid hormones and this is important for deveolment of reproductive organs, reproduction and also in endocrine disorders including breast, prostate, and ovarian cancers.

Steroid hormones (such as estradiol or E2) mediated gene expression may follow diverse mechanisms: genomic and non-genomic pathways. The genomic mechanisms may also follow two different pathways. 1) Direct mode: E2-liganded estrogen-receptors (ER) bind directly to an estrogen response element (ERE) present in the target gene promoters. ER-binding allows the recruitment of coactivator proteins and components of the RNA polymerase II machineries resulting in transcription activation. 2) Indirect mode: Liganded ER gets recruited to the target gene promoters via interaction with other DNA-bound transcription factors such as activation protein-1 (AP1), specific-protein 1 (SP1) or nuclear factor Kβ (NF-Kβ) and coorindate the process of transcription activation (Figure 2).[40] In the non-genomic signaling pathway, E2 activates membrane associated forms of ER and triggers intracellular signal transduction pathways that results in rapid ER-signaling and tissue responses.[40] My studies are directed towards the investigation of the epigenetic mechanism of expression of HOXB9 by estradiol and estrogenic EDC like BPA.

1.2.4. Endocrine disruptors: Mechanism of action and impacts on health

Endocrine disrupting chemicals (EDCs) are those chemicals that mimic natural hormones in the body which interfere with normal hormone signaling pathways resulting in adverse physiological and developmental impacts.[44] Adverse health impacts include birth defects, diabetes, cancers, endometrial

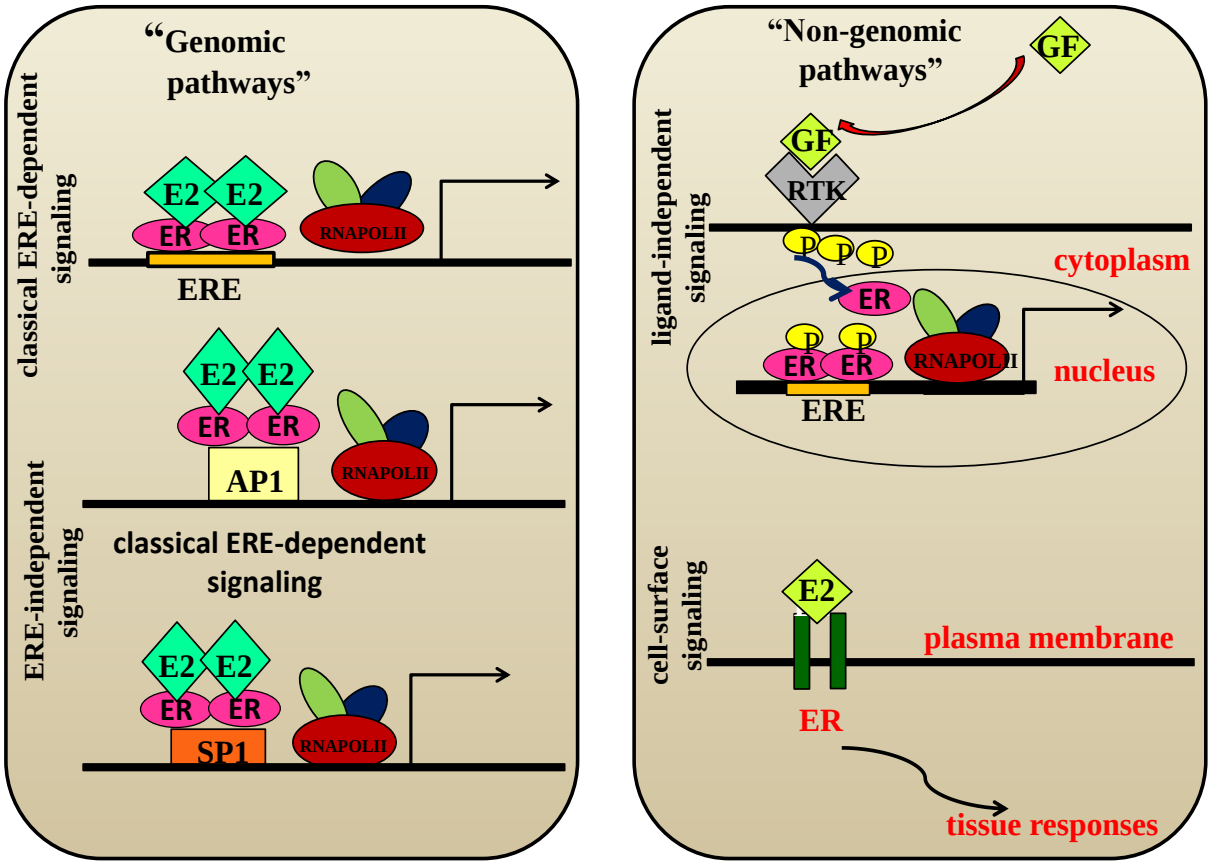


Figure 2. The multifaceted mechanisms of estradiol and estrogen receptor signaling.[43]

1) Genomic ER pathways which include: a) classical ERE-dependent pathways, E2-ER complexes bind to EREs in target promoters leading to an up- or downregulation of gene transcription and subsequent tissue responses; b) ERE-independent pathway, E2-ER complexes alter transcription of genes containing alternative response elements such as AP-1 through association with other DNA-bound transcription factors (Fos/Jun), which tether the activated ER to DNA, resulting in an up-regulation of gene expression whereas several genes containing GC-rich promoter sequences are activated via an ER-SP1 complex; 2) Non-genomic pathways: a) ligand-independent pathways, growth factors (GF) or cyclic adenosine monophosphate (not shown) activate intracellular kinase pathways, leading to phosphorylation (P) and activation of ER at ERE-containing promoters in a ligand-independent manner; b) Cell-surface (nongenomic) signaling, E2 activates a putative membrane-associated binding site, possibly a form of ER linked to intracellular signal transduction pathways that generate rapid tissue responses.

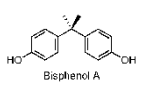
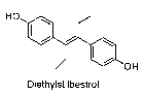
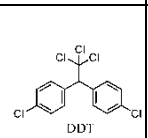
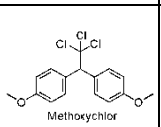
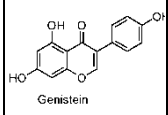
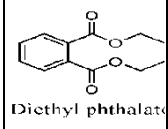
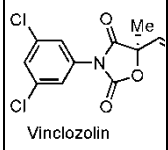
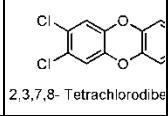
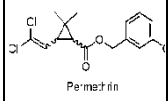
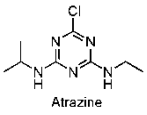
hyperplasia, secondary sexual developmental changes, fetal neurobehavioral alterations, obesity, early puberty, abnormal reproduction, autism, cancer and increased risk of cardiovascular, immunological and neurological disorders. EDC exposure may come from a variety of sources including commonly used medicine, herbicides, pesticides, biocides, heat stabilizers, chemical catalysts, plastic contaminants, pharmaceuticals, petroleum by-products, dietary components and many others.[44] EDCs may have synthetic or natural origin that include xenoestrogens,[45] environmental hormones,[46] hormonally active agents, and other environmental agents. Examples of well know EDCs include BPA, DES, pesticides like methoxychlor, herbicides like atrazine, polycyclic aromatic hydrocarbons (PAH), pesticides, PCBs, polybrominated biphenyls (PBBs), dioxins, some drugs (e.g., antiepileptic drugs), plasticizers (phthalates), dichlorodiphenyltrichloroethane (DDT), fungicides (vinclozolin), and heavy metals (Table 1).[47]

1.2.5. Mechanism of action of EDC

1.2.5.1. Interference in nuclear receptor mediated cell signaling

EDC mimics hormones and therefore, their actions are likely mediated via involvement of diverse types of receptors- nuclear hormone receptors (NR), non-nuclear steroid hormone receptors (*e.g.*, membrane ERs), nonsteroid receptors (*e.g.*, neurotransmitter receptors), orphan receptors, and other enzymatic pathways involved in biosynthesis and metabolism.[47] In general, during NR-signaling, signaling molecules (ligands) bind to their cognate NRs and activate them and induce specific cell signaling pathways that exert a wide variety of different cellular and metabolic responses including NR target gene expression.[48] In a classical mechanism of genomic mode of hormone signaling, upon binding to the ligand, receptors undergo conformational change and get activated (Figure 3A and 3B). Activated NRs migrate into the nucleus and bind to the target gene promoters. Along with NRs, a variety of NR-coregulators (coactivators and corepressors) are also recruited to the promoter.

Table 1. Examples of well-known EDCs, their structures, sources, and potential health impacts

EDC	Structure	Source	Health Impacts	References
Bisphenol A		Foods, plastics, and dental sealants	Obesity, early puberty. miscarriage	*32,49,50*
Diethylstilbesterol		Pharmaceuticals, feed additive for cattle, poultry, and sheep	Clear cell adenocarcinoma (CCA), Reproductive tract structural pregnancy complications, infertility	*51,52,53*
DDT		Paints, plastics, rubber products, dyes, carbonless copy paper	Premature birth, decrease in semen quality, congenital hypothyroidism, shorter menstrual cycles, breast cancer	*54,55,56*
Methoxychlor		Pesticide	Loss of body weights, prolonged estrous cycles, reduced fertility, cystic ovary, reduced sperm counts, and altered reproductive organ weights	*57,58,59*
Genistein		Soy-containing foods and dietary supplements	Reproductive health of developing fetus, brain effects include alteration of social and sexual behavior, modulates adult hypothalamic–pituitary–gonadal (HPG) axis function	*60,61,62*
Pthalate		Insecticide, textile and carpet, wood preservative	Neurodegeneration, DNA damage, decreased heart cell membrane fluidity, increased cholesterol content, protein and lipid oxidation in heart cells	*63,64,65*
Vinclozolin		A fungicide used to control various plant diseases	Reduced sperm count and motility, hypercholesterolemia, kidney and prostate disease, abnormal immune system function, cancer	*66 67 68*
TCDD		Detergents, plastic clothes, soaps, shampoos, nail polishes	Affects the reproductive system	*69,70,71*
Permethrin		Insecticide used in agriculture	DNA damage, decreased heart cell membrane fluidity, increased cholesterol content, and protein and lipid oxidation in heart cells.	*72,73,74*
Atrazine		Herbicede that controls crops (corn) and is used on turfs and lawns,	Feminization of gonads in males, abortion, impaired mammary and neural development, cancer, immune function suppression	*75,76,77*

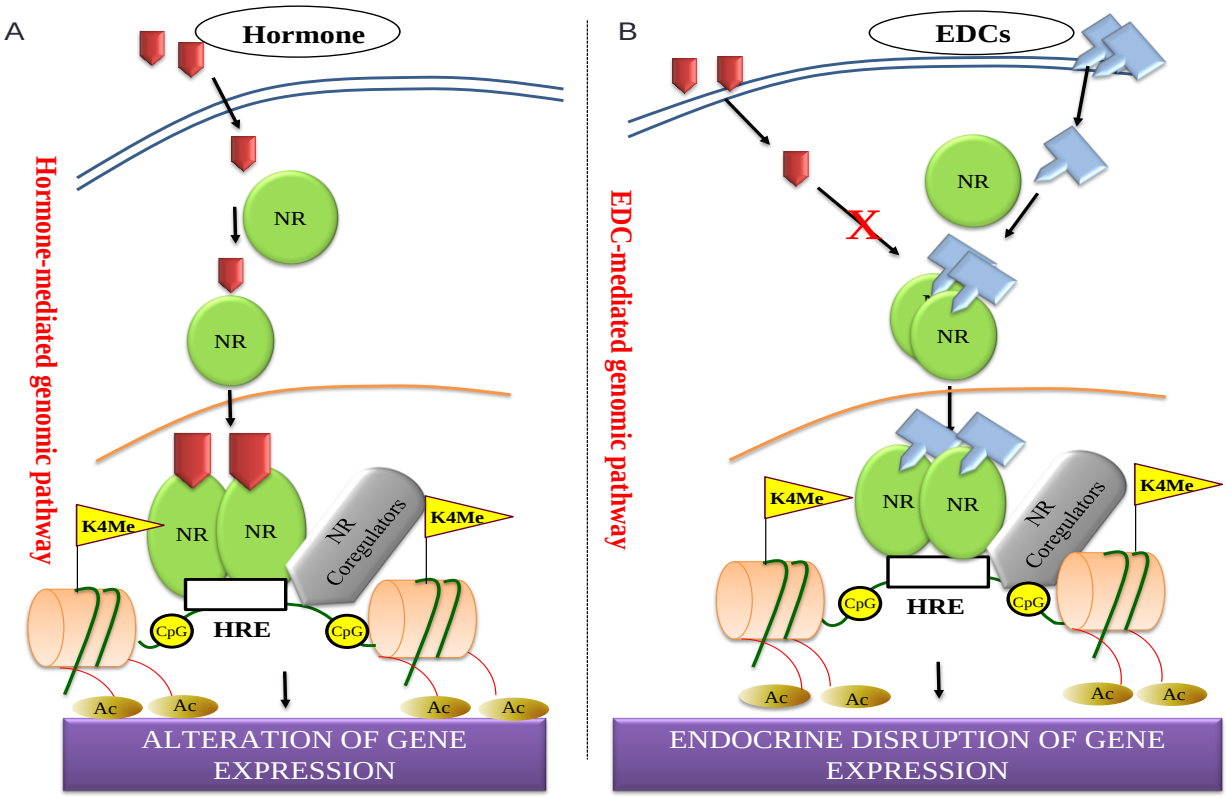


Figure 3. The classical mechanisms of hormonal and EDC-mediated genomic signaling pathway.[87]

A) In the absence of natural hormone, NR is located in the cytoplasm in a complex with chaperones. Upon hormone-binding, NR dimerizes, translocates to the nucleus binds to hormone response element (HREs) present at the promoter of NR-target genes. NR-co-regulators are recruited that remodel the promter and induces transcription of target genes. B) In the 'genomic pathway' of EDC signaling, EDCs, which mimic the action of natural hormones, compete with them for binding to the cognate NRs and initiate NR-signaling with the recruitment of coregulators and thus alter the transcription of target genes.

Coactivators and coregulators may include variety of chromatin modifying enzymes including histone acetyl transferases, histone deacetylases, histone methylases and demethylases, DNA methylase and demethylases. Upon recruitment of these NR-coregulators, the histone and DNA in the promoter gets modified and remodel the chromatin leading to gene activation/silencing. EDCs being structurally similar to the natural ligands of NRs, compete with natural substrates and bind to NRs.

Similar to estrogen, upon binding to EDC, NRs also get activated and recruited to NR-target gene promoters along with NR-coregulators, modify and remodel chromatins, alter the epigenetic states of the chromatin and ultimately result in abnormal NR-target gene expression. Precisely, EDCs may directly compete and bind to NR ligand binding domains or may also interact via allosteric mechanism. EDCs may displace α-helices forming the ligand-binding domain (LBD) of nuclear receptors differently,[78] and this decides its ability to act as agonist/antagonist. EDCs can affect steroid hormone signaling via interfering with other pathways such as targeted degradation of NRs,[79,80] and also via activation of non-genomic pathways through membrane ERs (Figure 4).

1.2.6. Bisphenol-A (BPA) and its health impacts

The prototypical endocrine disrupter, BPA is an estrogenic chemical produced in large quantities for use primarily in the production of polycarbonate and epoxy resins.[81,82] Humans are widely exposed to BPA, a common component of plastics used to contain food products and in dental composite resins. BPA is a purported endocrine disruptor, first recognized as such, when it leached from polycarbonate laboratory flasks and confounded an estrogenicity assay.[83,84] The potential hazards that may stem from exposure to this xenoestrogen and its mechanism of action have not been fully characterized. BPA is known to be much less potent than estradiol with regard to the activation of ER. Various coregulatory factors may differentially alter BPA and E2 activities.[85] Homeodomain- containing proteins are critically

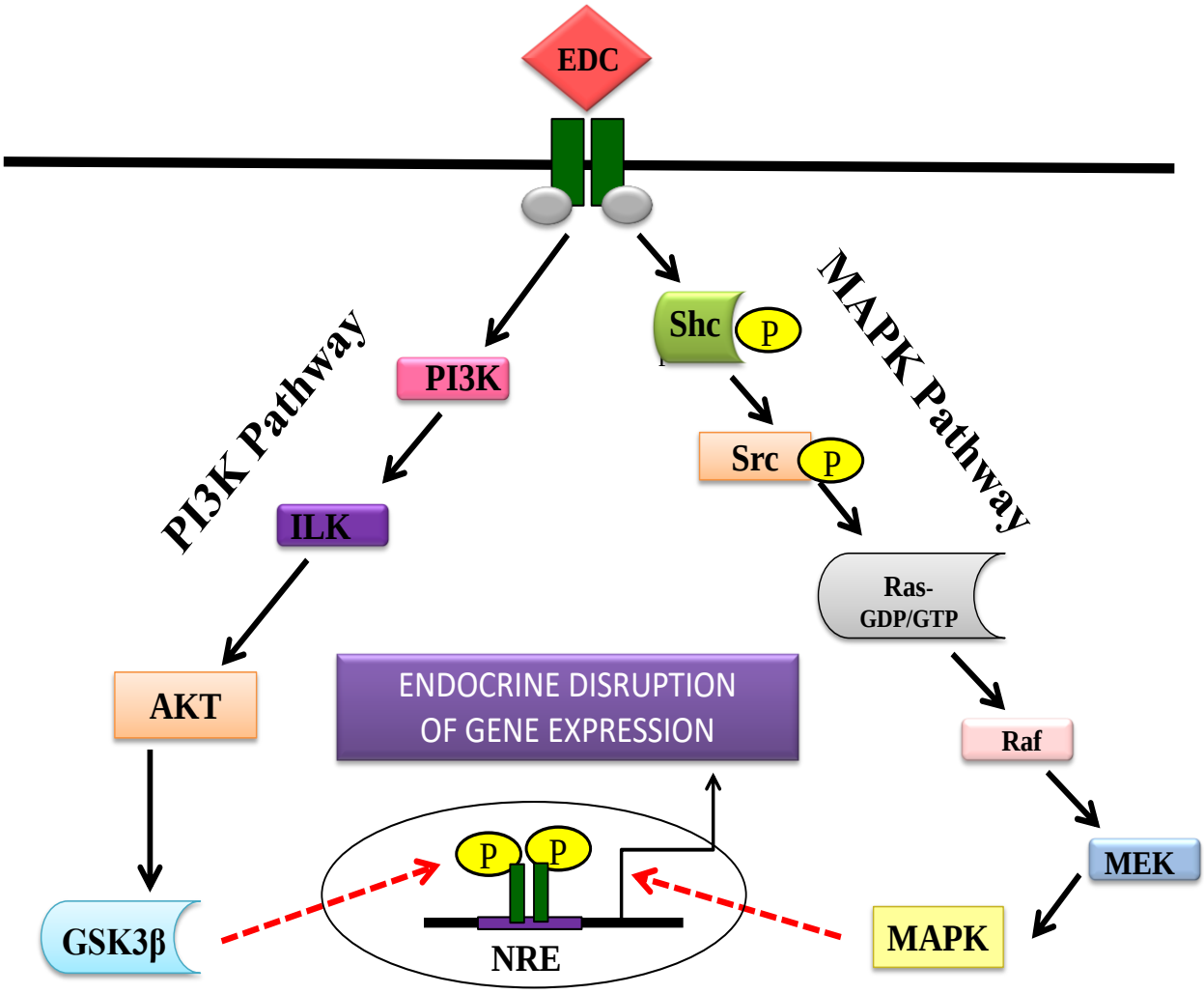


Figure 4. The 'non-genomic pathway' of EDC action may occur through membrane ERs located in the cytoplasmic membrane. [87]

Activation of membrane ERs by EDCs leads to rapid downstream cellular signaling. This induces subsequent stimulation of 2 pathways- a) ERK/MAPK pathway via activation of Shc/Src/Ras/ERK protein cascade or the PI3K/AKT pathway by alteration of PI3K/ILK/AKT/GSK3β cascade expression which in turn may affect the transcription of target genes in the nucleus. The resulting changes cause irregular cellular response which produce adverse effects in humans.

important transcription factors that regulate the expression of multiple genes involved in development and differentiation.[86] Steroidal hormones are closely associated with embryonic growth. BPA is a well-known estrogenic endocrine disruptor. Once BPA enters in the body, it has potential to bind ERs and interfere with normal estrogen-signaling processes contributing towards various human diseases including reproductive and developmental defects and metabolic disorders.[88,89]

1.2.7. Endocrine Disruption of HOX Gene Expression

Alteration of HOX gene expression has been demonstrated to lead to reproductive tract anomalies and reproductive failure.[90] Aberrant regulation of HOX genes likely underlies the mechanism of disruption of natural steroid signaling via certain xeno steroids known as endocrine disrupting chemicals such as BPA or DES. The general population is exposed to a number of hormonally active compounds on a daily basis. More specifically, exposure to estrogenic EDCs during critical stages of differentiation can interfere with the hormonal signaling necessary for normal development and can also result in persistently altered gene expression. Since HOX genes are critical for development and their expression is regulated by estrogen, hence, disturbances in the normal endocrine regulation of HOX genes are purported mechanisms of estrogenic endocrine disruption. The dysregulated HOX gene expression resulting from exposure to EDCs further elucidates the critical role of hormonal regulation of the homeobox genes.

BPA exposure has been shown to alter the HOXC6 and HOXB9 genes.[32,33] Exposure to DES also has been shown to impact expression of HOXC6 and HOXB9 genes.[33] As HOX genes are potential targets of endocrine hormones and hence endocrine disruption, thus, understanding the mechanism and epigenetic impacts of EDCs on HOX gene expression is important and is the subject of my research (chapter 2).

1.3. Neurotransmitters and neurotrophic factors and their functional significance

The study of adult neurogenesis continues to gain momentum as it generates the hope that people with neurological disorders might someday benefit from its study and utilization. Neurogenesis or generation of new neurons continue to occur in adult mammals, including humans, predominantly in the anterior subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the CNS on each side of the brain.[91] Such neurogenesis can be increased with therapeutic agents such as neurotrophic factors and neurotransmitter drugs. In rodents, new immature neurons, called neuroblasts, migrate through a distinct rostral migratory stream to the olfactory bulb. There, some of the neuroblasts mature and integrate with the neuronal circuitry, whereas others die. These new neurons migrate over a very short distance into the neighboring granule cell layer where some become functioning neurons. The functional significance of the new neurons is becoming clearer and seems to include olfactory function and certain forms of memory.[92,93]

1.3.1. Neurotrophic factors

Proteins that bind to transmembrane receptors have been divided into categories such as neurotrophic factors, cytokines, growth factors etc. Well-known examples of neurotrophic factors include brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophins (NT), vascular endothelial growth factors (VEGFs), the transforming growth factor (TGF). Depending on the cell type or context, many of these proteins can exert a variety of effects on cells, ranging from proliferation to maturation and trophic support. Among the neurotrophins, BDNF is known for its survival-promoting effects on new neuroblasts through the tyrosine receptor kinase β (Trkβ) receptor.[94] BDNF and NGF can also act through the common p75 receptor found in the SVZ.[95] NGF also enhances survival of new hippocampal neurons, most likely by increasing cholinergic tone.[96] NT-4 is also known to signal through Trkβ but does not affect ischemia-increased neurogenesis.[97] NT-3 promotes

hippocampal neurogenesis and the related long-term potentiation (LTP) and spatial memory.[98] Basic fibroblast growth factor or FGF-2 is well-known for its mitotic effects in hippocampal neurogenesis, LTP and memory consolidation, but not spatial memory.[99] TGF family plays an important role in neurogenesis. Additionally, infusion of glial cell-line derived neurotrophic factor (GDNF) into the post-ischemic striatum promotes proliferation, recruitment of neuroblasts into the striatum and survival of new neurons.[100] The angiogenic response to injury is associated with increased levels of various vascular growth factors including vascular endothelial growth factors (VEGFs) and angiopoietin-1 (Ang-1). These proteins directly or indirectly promote survival of newly arrived neuroblasts.[101] These studies reemphasize the complexity of the significance of neurogenesis to cognitive function and serves as a reminder of the translational barriers to humans.

1.3.2. Neurotransmitters

Several neurotransmitter systems such as serotonin (5-HT) regulate adult CNS neurogenesis. Neurogenesis can be increased with serotonin reuptake inhibitors such as the antidepressant fluoxetine (Prozac).[102] Dopamine is also known for its effects on neurogenesis. For example, D2 dopamine agonists can increase neurogenesis in mice and in mouse Parkinson models.[103,104] Another monoamine, noradrenaline, probably only plays a role in adult hippocampal neurogenesis. Glutamate also plays a role in neurogenesis. Oxytocin, a neuropeptide acts to protect the hippocampus from the damaging effects of elevated glucocorticoids by promoting neuronal growth.[105] Targeting specific receptor combinations most likely will provide a refined method of directing genesis of appropriate types of neurons and their functional integration.

1.3.3. Neurological disorders and risks

Structural, biochemical or electrical abnormalities in the brain, spinal cord or other nerves can result in a range of neurological disorders including Huntington's disease, muscular dystrophy,

Parkinson's disease and Alzheimer's disease, epilepsy, brain tumors, meningitis etc. Examples of symptoms include paralysis, muscle weakness, poor coordination, loss of sensation, seizures, confusion, pain and altered levels of consciousness. There are many recognized neurological disorders, some relatively common, but many rare. Neurological disorders are very complex, and while the causes are semi-known, research is perpetually turning up new contributing factors to the causes of neurological disorders. Certain neurological conditions are caused by deregulation of the cell signaling pathway controlling the expression of neurotrophic factors/ neurotransmitters. In conditions such as dystonia, Huntington's disease, and muscular dystrophy, the recognition of such genes as risk factors has considerable prognostic value. Other neurological conditions result from the interplay of one or more target genes and environmental toxins. Examples include sporadic Alzheimer's disease, epilepsy, multiple sclerosis, and Parkinson's disease. Due to the lack of accuracy (due to inadequate sensitivity and/or specificity), therapies to target such susceptibility genes are limited. However, such susceptibility genes such as neurotrophic factors or neurotransmitters are of great interest because they may provide insights into the mechanisms that lead to these complex disorders.

In my study, I have investigated the mechanism of transcriptional regulation of a critical neurotropic factor BDNF using *in vitro* and *in vivo* animal models. In particular, we have investigated the roles of steroid hormone estradiol and a noncoding RNA in transcriptiona regulation of BDNF.

1.4. Noncoding RNA and their roles in gene regulation

Human genome, "the blueprint of life" is huge in size containing more than 3 billion base pairs. Sequencing and analysis revealed that human genome contains about 21000 protein coding genes which occupy less than 2% of the whole human genome.[106,107] The rest of the human genome were considered to be mostly non-functional "Junk DNA".[108 109] Recently, Encyclopedia of DNA elements (ENOCDE)

project was launched by National Human Genome Research Institute (NHGRI) to investigate the functional relevance of human genome and gain insights into the roles of these non-protein-coding regions present in the human genome and understand in greater detail the transcriptional landscape of the human genome.[110] Interestingly, this project revealed that ~ 80% of the human genome consists of DNA elements that are functional but do not code for proteins.[111,112,113] These DNA elements either code for non-coding RNAs (ncRNAs) that are mostly not translated into proteins, or regulatory DNAs elements that are interaction sites for various proteins and transcription factors that in turn regulate transcription.[114,115] The discovery of ncRNA from the so-called "Junk DNA" region of the genome made the junk DNA as the "Gold Mine" for biomedical researcher and for the discovery of the mechanism of disease pathogenesis, potential biomarkers and even treatment of disease with genomic medicine. Many of these ncRNAs are processed, released into the blood stream and even excreted in the urine. Though detailed functions of most ncRNA are yet to be discovered, they appear to play crucial roles in chromosome dynamics, gene regulation, mRNA processing, splicing, translation, cell differentiation and development and many others. Misregulation by ncRNA has been found to be increasingly associated with variety of human diseases including cancer, cardiovascular diseases, neurological and immunological disorders.

The ncRNAs have been classified into three subgroups on the basis of their size.[114,116] NcRNAs that are ~ 20-50 nucleotides (nt) in length are called as short ncRNAs.[117] Intermediate/medium ncRNAs are 50-200 nt long and long ncRNAs (lncRNA) are >200 nt long.[118,119,120,121,122] Examples of short ncRNAs include microRNA (miRNA), small interfering RNA (siRNA), piwi-interacting RNA (piRNA), and transcription initiating RNA (tiRNA).[122] Small nuclear RNAs (snRNAs), small nucleolar RNA (snoRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), transcription start site associated RNA (TSSaRNA), promoter associated small RNAs (PASRs) and promoter upstream transcripts (PROMPTs)

are examples of medium sized ncRNAs.[114,123,124,125] Examples of lncRNA are long-intergenic non-coding RNA (lincRNA), transcribed ultra-conserved RNAs (T-UCRs) and various other long non-coding RNAs.[114,123,124,125]

1.4.1. Long- noncoding RNA

LncRNAs have been shown to control every level of the multi-level regulated gene expression pathway.[126] For example, they have long been implicated in post-transcriptional gene regulation through controlling processes like protein synthesis, RNA maturation and transport, and very recently, in transcriptional gene silencing through regulating the chromatin structure.[127,128] Structurally different RNAs engage in diverse mechanisms that lead to different regulatory outcomes. Some of the lncRNAs converge on chromatin structure to silence multiple genes located on the overlapping and non-overlapping sides. They interact with DNA and/or chromatin-modifying proteins and recruit them to their target regions. The exact physical association between these lncRNAs and chromatin modifiers and/or gene promoter chromatin remains to be elucidated.

1.4.2. LncRNA HOTAIR and its function in chromatin dyamics and gene sielncing

HOTAIR is a 2.2 kb long lncRNA that is transcribed from antisense strand of HOXC gene cluster present in chromosome 12.[129] Like protein coding genes, HOTAIR is transcribed by RNA polymerase II, spliced, polyadenylated and 5'-capped.[88,114,130,131] HOTAIR was originally discovered based on tiling microarray analysis by Rinn *et al.* in 2007 and they showed that HOTAIR is located at the HOXC locus in chromosome 12, flanked by HOXC12 and HOXC11.[129] Strand specific reverse transcriptase PCR analysis confirmed that HOTAIR is transcribed in an antisense manner with respect to HOXC genes.[129] HOTAIR was the first lncRNA to be discovered to regulate gene expression *in trans* fashion. HOTAIR leads to transcriptional repression of HOXD locus genes present in chromosome

2, including HOXD8, HOXD9, HOXD10 and HOXD11.[129] However, it does not affect the transcription of HOXC cluster genes present in chromosome 12, where HOTAIR is actually coded.[129] HOTAIR knockout mice studies have shown that there is a de-repression of numerous genes, resulting in homeotic transformation of the spine, loss of vertebral boundary specification during development and malformation of metacarpal–carpal bones.[132]

HOTAIR is a scaffold lncRNA that bridges two complexes, polycomb repressive complex 2 (PCR2) and the LSD1/REST/CoREST-complex, both of which are associated with gene silencing.[133,134] Each complex contains a number of subunits, one of which is an enzyme enhancer of zeste homologue 2 (EZH2), which confers methyl transferase activity to PRC2.[135,136] This enzyme is capable of introducing mono-, di- and tri-methyl groups at the H3K27, to yield H3K27me, H3K27me2, and H3K27me3, methyl marks associated with heterochromatin.[137,138,139] Lysine specific demethylase 1 (LSD1) confers demethylase activity to the RE1-silencing transcription factor (REST/CoREST) complex.[133,140] This enzyme demethylates di- and mono- methylated H3K4 to yield demethylated H3K4, a methyl mark associated with an active state of chromatin.[141,142,143,144] In my studies (chapter 4), I have investigated the contribution of HOTAIR/PRC2/LSD1 in BDNF gene silencing and neuroprotection.

1.4.3. LncRNAs and neural development

Multiple studies have implicated non-coding RNAs in brain development and function. Dynamic expression of lncRNAs has been observed in human-induced-pluripotent stem cells (iPSCs),[145] and human embryonic stem (ES) cells during neuronal differentiation.[146] Neurogenesis-associated lncRNAs were found to associate directly with SUZ12 (a component of the polycomb repressive complex 2, PRC2), REST and SOX2 (a pluripotency-associated TF) suggesting that lncRNAs may act as guides for these proteins. Importantly, knockdown of these lncRNAs by RNAi has been shown to result in impaired neuronal differentiation, suggesting that lncRNAs are critical regulators of neurogenesis.[146]

The importance of lncRNAs in the brain is exemplified by their involvement in the maintenance of pluripotency, neuroectodermal differentiation, neuronal-glial cell fate determination, neuron-specific relaxation of epigenetic imprinting, repression of neural genes in non-neural cells, brain tissue patterning and synaptogenesis.[147] The involvement of lncRNAs in neurodegenerative, neurodevelopmental and neuropsychiatric disorders, and in brain cancers further underlines their importance in CNS development and function. LncRNAs may either themselves drive or mediate the disease pathophysiology (as in the case of *ATXN8OS* and *FMR4*), or they may regulate the expression of disease-associated genes (as in the case of *BACE1-AS*). The detailed functions of lncRNA in different neurological disorders are yet to be revealed. I have hypothesized and hence, explored that lncRNAs may also be involved in expression of BDNF gene in the hippocampus (Chapter 4).[147]

1.4.4. LncRNAs and hypoxia signaling

Both excessive and insufficient levels of oxygen are detrimental to cell biology, and so cells have developed tightly coordinated homeostatic mechanisms to respond to altered oxygen concentration. Chief amongst these is the regulation of gene expression by the hypoxia-inducible factors (HIFs).[148] HIFs are heterodimers containing a regulated HIF-α subunit (HIF1α, HIF2α or HIF3α) and a constitutive β-subunit (HIF1β also called aryl hydrocarbon nuclear translocator, ARNT).[149] HIF1 and HIF2 regulate distinct but overlapping transcriptional profiles comprising many hundreds of protein-coding genes that act to restore oxygen levels by reducing oxygen demand and increasing oxygen delivery. Lack of oxygen or tissue hypoxia is a key feature of many of the major causes of myocardial and cerebral ischemia, and cancer.[150,151] There are multiple lines of evidence linking tumor hypoxia and the consequent HIF activation with an aggressive phenotype in cancer.[152]

Hypoxia regulated expression of protein coding genes have been well investigated and many of these signaling pathways are major targets for cancer therapy. However, the significance of the impacts

of hypoxic signaling on noncoding RNA expression has mostly remained unexplored. As ncRNAs, especially lncRNAs are abundant in cells and major players in cancer, it is important to understand their roles and regulation under tumor microenvironment, such as hypoxia. In my study (chapter 3), I have investigated the transcriptional regulatory mechanism of lncRNA HOTAIR under hypoxia. Notably, many lncRNA expressions are found to be altered under hypoxia. These include lncRNA LET, H19, and p21.[153,154,155] While these lncRNAs could be induced either directly or indirectly by HIF (or indeed in some cases by non-HIF-mediated mechanisms), there is good evidence that a number of them are direct transcriptional targets of HIF.[156]

1.5. Conclusion

Understanding the mechanism of gene function and regulation is critical for the development of novel and effective therapy. Gene expression and regulation may be affected by a variety of factors that include homones, genders, nutrients, and environmental factors such as EDCs. HOX genes are critical players in embryonic development and are misregulated in a variety of human diseases including cancer. In my studies, I have investigated the epigenetic mechanism of transcriptioinal regulation of a homeobox gene, HOXB9 (a critical player in mammary gland development and breast cancer), in the presence of steroid hormone, estradiol. I have also investigated the impact and the mechanism of action of EDC (such as BPA and DES) on HOXB9 expression (Chapter 2). In addition to protein coding genes, I have also investigated the transcriptional regulatory mechanism of an lncRNA, HOTAIR, under hypoxic condition which is a critical driver of tumor growth (Chapter 3). Neurotrophic factors play crucial roles in neuronal growth, maintenance, and repair. They influence growth and maintenance of variety of neurons including dorsal root ganglion neurons, hippocampal, and cortical neurons. On the other hand, expression of neurotrophic factors is reduced in neurological disorders like Alzheimer's,

Parkinson's, and Huntington's diseases and low levels of neurotrphins contribute to neuro-degeneration, affect cognitive abilities, and causes dementia.[157,158,159] Neurotrophin levels may also be affected by a variety of factors that include steroid hormones, physical exercise, electrical simulation, and others. The mechanisms by which expression of neurotrophins is regulated in healthy brain and misregulated in neurological disorders are not clear. Chapter 4 deals with the analysis of the mechanism of BDNF gene regulation and its impacts on neuroprotection. Overall, my studies are aimed towards understanding the mechanism of transcriptional regulation of protein coding genes, neurotransmitters and non-coding RNAs critlcal to human health and diseases. These studies will have long-term impacts in understanding the mechanism of pathogenesis and in development of novel targets for cancer and neurological disorders.

CHAPTER 2

ENDOCRINE DISRUPTING CHEMICAL, BISPHENOL-A, INDUCES BREAST CANCER ASSOCIATED GENE HOXB9 EXPRESSION *IN VITRO* AND *IN VIVO*

2.1 Introduction

The members of homeobox (HOX) containing gene family represent a subset of evolutionarily conserved transcription factors that play crucial roles in embryonic development.[160,161] HOX genes are expressed not only in embryos but also in various normal adult tissues.[162,163] Expression of HOX genes regulate patterning of the AP axis from the level of the hindbrain to the end of the spine.[164] The HOX gene family comprises of 39 HOX gene members arranged in four different clusters (HOXA-D).[114,160,161,162,163,165] HOXB9, a HOX gene located in chromosome 17, plays key roles in skeletal and mammary gland development.[166] HOXB9 is a direct transcriptional target of WNT/TCF4.[167] HOXB9 is involved in cell proliferation, cell-cycle progression, differentiation, embryonic segmentation, limb patterning and angiogenesis.[167,168,169,170,171,172] HOXB9 acts as a transcription factor and regulates expression of renin, an aspartyl protease that cleaves angiotensinogen into angiotensin I,[173] and hence it is involved in controlling blood pressure, fluid homeostasis, and electrolyte balance.[173] Recently it was also shown that HOXB9 expression confers resistance to ionizing radiation indicating its potential roles in DNA damage response and in maintenance of genomic integrity.[169]

Increasing evidences suggest that HOXB9 expression alterations are associated with variety of cancers, including in breast, head and neck, lung, hepatocellular, colon carcinomas and gliomas.[174,175,176,177] HOXB9 overexpression in breast tumors induces breast cancer metastasis by altering the tumor microenvironment, and promotes disease progression.[168] Infact, HOXB9 expression is

an important prognostic factor in breast cancer which promotes invasiveness, metastatic ability, and tumor neovascularization.[178] The overexpression of HOXB9 in breast cancer and its critical roles in mammary gland development indicates its potential regulation by endocrine hormones such as estradiol. Indeed recent studies from our laboratory demonstrated that HOXB9 is overexpressed in breast cancer tissues, in ER-positive breast cancer cells and it is transcriptionally regulated by estradiol in cultured breast cancer cells (MCF7).[30,35] We found that HOXB9 regulates the expression of growth and angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor β (TGFβ) etc and induces three dimensional (3D) tumor colony formations.[35] The homeodomain of HOXB9 is essential for the transcriptional regulation of tumor growth factors and in 3D colony formation, suggesting the importance of the HOXB9 homeodomain in tumorigenesis.[35]

Since HOXB9 is found to be overexpressed in breast cancers, aides in tumorigenesis and is transcriptionally regulated by E2, we hypothesized that its transcription may also be affected upon exposure to estrogenic endocrine disrupting chemicals (EDCs) and that may contribute towards HOXB9 associated diseases including breast cancers. Notably, endocrine disruptors are a class of compounds that alters the functioning of the endocrine system and leads to adverse health effects and is a major health concern.[179,180,181,182,183,184] In the present study, we analyzed the impact of a well-known estrogenic EDC, bisphenol-A (BPA) [and also estradiol (E2)] in transcriptional regulation of HOXB9 gene *in vitro* and *in vivo*. Our results demonstrate that the HOXB9 gene expression is upregulated upon exposure to BPA in breast cancer cells *in vitro* and also in mammary glands *in vivo*.

2.2. Experimental procedure

2.2.1. Cell culture and treatment with estradiol (E2) and BPA

Cell lines:

The estrogen receptor (ER) positive breast carcinoma cells, MCF7 cells were purchased from the American Type Cell Culture Collection (ATCC) and were cultured as described previously by us.[33,88,89,185]

Materials used for culturing mammalian cells:

Reagents used for mammalian cell culture include: DMEM Media (Dulbecco's Modified Eagle's Medium, Sigma), phenol-red-free media such as DMEM-F12 Media (Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham, Sigma), heat inactivated fetal bovine serum (Sigma) (FBS), 2 mM L-glutamine, 100 units/mL of penicillin and 0.1 mg streptomycin/mL (Sigma), 1X PBS (Phosphate buffer saline), trypsin-EDTA solution, charcoal stripped heat inactivated fetal bovine serum/dextran coated charcoal (Sigma) for stripping of serum, 17β-estradiol (E2) and Bisphenol A (BPA) (Sigma).

Recipes:

Cell growth media (500 mL) used to culture all the cell lines was made using 440 mL DMEM supplemented with 50 mL FBS, 2 mM L-glutamine (5 mL), 100 units/mL penicillin and 0.1 mg streptomycin/mL (5 mL).

Growth media for estrogen-treatments was particalularly made using 440 mL DMEM-F12 supplemented with 50 mL charcoal stripped FBS, 2 mM L-glutamine (5 mL), 100 units/mL penicillin and 0.1 mg streptomycin/mL (5 mL).

1X PBS solution was prepared using 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4. Adjust pH to 7.4. The solution was sterilized by autoclaving.

Procedures for cell culture:

Thawing and culturing of MCF7 cells:

1. MCF7 cells were grown in 100 x 20 mm tissue culture dishes under normal tissue culture conditions. Cells were thawed and grown in a humidified cell culture incubator (at 37 °C with 5% CO_2) for at least three generations using regular cell growth media containing DMEM supplemented with 50 mL FBS, 2 mM L-glutamine, 100 units/mL penicillin and 0.1 mg streptomycin/mL.

2. It's important to have healthy growing cells for the E2 treatment experiments. Therefore, before passaging of the cells, the cell morphology and confluency was repeatedly checked under a light microscope.

3. The cells were passaged when the confluency reached 80-85%.

Passaging of cells:

1. Cells were washed 2 times with 5 ml of 1X PBS (pH 7.4), 1 mL of trypsin/EDTA solution was added to trypsinize for 3 min at 37 °C, followed by addition of 5 mL of media. Cells were pipetted up and down to resuspend the cell pellet in the media. 2 mL of resuspended cells was added into three 100 x 20 mm plates.
2. 8 mL of extra cell culture containing media was added to each tissue culture dish and made upto a volume of 10 mL. It was made sure that the media covered the entire area of the dish. The cells were transferred to a humidified incubator and maintained at 37 °C with 5% CO_2.

Charcoal stripping of heat inactivated fetal bovine serum:

1. 2 g dextran coated activated charcoal (Sigma) was added per 100 mL heat-inactivated FBS, into sterile falcon tube.
2. Tubes were placed on a shaker in a 4 °C cold room and stirred for overnight (12 h). Although it is difficult to maintain sterile conditions during charcoal stripping, care was taken to avoid contamination and to keep the serum at 4 °C.
3. Tubes were centrifuged for additional 30 min at 13,000 rpm at 4 °C.
4. Gently serum was poured off into new sterile centrifuge tube and centrifuged for another 30 min at 13,000 rpm at 4 °C.
5. Gently serum was poured off into new 15 mL falcon tubes and divided into 10 mL aliquots in sterile falcon tube and stored at -80 °C.
6. Prior to use, a tube of charcoal-stripped medium was thawed and used immediately to supplement complete medium.
7. The medium was filter sterilized using a 0.22 μm filter and collected in a sterile bottle and stored at 4 °C.

Estrogen and Bisphenol-A treatment of cells:

1. For E2 and BPA treatment, MCF7 cells were grown and maintained for at least 3 generations in phenol-red free DMEM-F12 media supplemented with 10% charcoal stripped FBS, 2 mM L-glutamine and 100 units/mL penicillin and 0.1 mg/mL streptomycin (Hormonal starvation).
2. The cells were allowed to reach a confluency of about 80–90% confluency (3^{rd} generation).
3. The cells were then passaged into 60 mm cell culture plates and incubated until the confluency reached 50–60%, followed by treatments with varying concentrations of E2/BPA.

4. Prior to the E2/BPA treatment, E2/BPA (stock solution: 100 mM in ethanol) was diluted (using DMEM -F12 media) freshly to yield concentration of 10000 μM (stock solution) and then they were serially diluted to yield concentrations such as 1000 μM, 100 μM, 10 μM, 1 μM, 0.1 μM, 0.01 μM and 0.001 μM.

5. To achieve the final concentrations of 1000 nM, 100 nM, 10 nM, 1 nM, 0.1 nM, 0.01 nM and 0.001 nM (i.e. 1000 times dilution), 10 μL of appropriate μM solutions was added to 10 mL of the treatment media.

6. Control cells were treated with equally diluted ethanol.

7. Cells were incubated for 4 h (or varying time periods as needed).

Harvesting of MCF7 cells for RNA and protein extraction:

1. The growth medium was removed and disposed off appropriately.

2. Cells were washed twice with 10 mL of cold 1X PBS.

3. 1mL of 1X PBS (pH 7.4) was added and the cells were scraped in a 1.5 mL microcentrifuge tube.

4. The cells were pelleted at 1500 rpm for 10 min at 4 °C.

5. The PBS was decanted by pipetting it off and the resulting cell pellet was stored at -80 °C before proceeding to the analysis.

2.2.2. RNA extraction cDNA synthesis, RT-PCR and RT-qPCR

Materials:

$MgCl_2$, KCl, dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), Tris-HCl, ethylenediaminetetraacetic acid (EDTA), phenol–chloroform–isoamyl alcohol mixture (125: 24: 1, pH 4.5, Sigma) and ethanol (100%) were used for real-time PCR reactions.

Recipes:

RNA extraction buffer:

20 mM Tris-HCl, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl and 0.5 mM dithiothreitol (DTT); diethyl pyrocarbonate (DEPC) treated ultra-pure, nuclease-free water (Sigma) was used for making up the volume.

RNA extraction protocol:

1. The cell pellet was resuspended in RNA extraction buffer, incubated for 10 min on ice and then centrifuged at 3500 rpm for 5 min at 4 °C.
2. The supernatant was transferred to a new tube and subjected to phenol-chloroform extraction followed by ethanol precipitation.
3. The RNA pellets were air dried to remove the residual ethanol and dissolved in DEPC treated water containing 1 mM EDTA
4. RNA concentrations were quantified using nanodrop spectrophotometer.
5. The RNA extracts were analyzed by RT-qPCR.

Phenol-chloroform extraction and ethanol precipitation for purification of RNA:

1. 1 volume sample (from which RNA to be purified) was taken and 1 volume of phenol: chloroform: isoamyl alcohol (125: 24: 1, pH 4.5). It was vortexed for 1 min and centrifuged at 10000 rpm for 10 min at 4 °C.

2. The upper, aqueous phase was transferred to a fresh tube and 1 volume of chloroform: isoamyl alcohol (24: 1) was added to it.
3. It was vortexed for 1 min and centrifuged at 10000 rpm for 10 min at 4 °C.
4. The upper, aqueous phase was transferred to a fresh tube. Any transferred chloroform was removed by centrifugation 10 sec at top spin followed by removal of the bottom phase with a micropipette.
5. 0.1 volume of 3M Sodium Acetate (pH 5.2) and 1 volume of isopropanol or 2.5 volumes of 95% ethanol was added.
6. The above mix was placed in -80 °C for 2 h and centrifuged at 13000 rpm for 25 min at 4 °C.
7. The supernatant was removed and the pellet was washed with 1 mL of 70% ethanol. The pellet was air dried (20 min), then resuspended in TE buffer (made in nuclease-free water), RNA was quantified using nanodrop spectrometer and then stored at -80 °C.

Reverse-transcription-PCR/ cDNA synthesis:

Materials:

Oligo dT (Promega), MMLV reverse transcriptase (Promega), 1X first strand buffer (Promega), dNTPs mix containing dATPs, dCTPs, dGTPs and dTTPs (Promega), DTT (Sigma), RNaseOut (Invitrogen) were used for cDNA synthesis.

Recipes:

RT-2 buffer was made using 100 U of MMLV reverse transcriptase, 1X first strand buffer, 100 µM dNTPs mix, 1 mM DTT and 20 U of RNaseOut. The total volume should be made up to 13 µL per RT-reaction using DEPC treated ultra-pure, nuclease-free water.

Reverse-transcription/ cDNA synthesis protocol:

1. For reverse transcription/cDNA synthesis, 500 ng of RNA (quantified using Nanodrop spectrophotometer) was mixed with 2.4 µM oligo dT (Promega) in a 12 µL total volume and incubated at 70 °C for 10 min in a thermocycler.
2. Then 13 µL of RT-2 buffer was added to the RNA and oligo dT mix (12 µL) and the final volume was made up to 25 µL.
3. This mixture was incubated at 37 °C for 1 h for reverse transcription in a thermocycler.
4. Thus formed, cDNA product was diluted to 100 µL and that was used for regular PCR and real-time PCR (qPCR) analyses.
5. If more than one RNA sample needs to be processed to generate cDNA, one can prepare a master mix containing these components for all the RNA samples at once.

Real-time PCR (qPCR) and RT-PCR:

Materials:

Sso EvaGreen supermix (Bio-Rad), Taq polymerase, 10X Taq buffer solution containing Mg^{2+}, primer pair (forward and reverse) dissolved in TE buffer, dNTPs mix containing dATPs, dCTPs, dGTPs and dTTPs (Promega), 50X TAE buffer, pH 8.0 (Tris-acetate-EDTA), agarose gel were used to perform real-time PCR and RT-PCR .

Recipes:

TE Buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

50X stock TAE buffer (Tris-acetate-EDTA buffer) was made using 242 g Tris Base (MW: 121.1), 57.1 mL glacial acetic acid, 100 mL of 0.5M EDTA. Tris base was mixed using stir bar and dissolved in about 600 mL of deionized water. EDTA and acetic acid were added to bring to a final volume of 1 L with deionized water. It was stored at room temperature.

Real-time PCR (RT-qPCR) protocol:

1. For the qPCR reactions, 3 µL of diluted cDNA or DNA isolated from ChIP chromatin was mixed with 3 µL Sso EvaGreen supermix (Bio-Rad) and 2 µM of primer pair (forward and reverse primers, Table 2, Table 3, Table 4) and final volume was made up to 7 µL.
2. PCR reactions were carried out in CFX96 real-time detection system (Bio-Rad) for 50 cycles.
3. Each cycle contained following steps:
    I. 5 sec at 95 °C for denaturation.
    II. 10 sec at 60 °C for both annealing and elongation, followed by plate read.
4. Data analyses were performed using CFX manager software (Bio-Rad).
5. Each reaction was performed in 3 parallel replicates and reactions were repeated at least three times.

RT-PCR protocol:

1. The RT-PCR master mix was prepared containing 1 µL 10X Taq buffer solution containing Mg^{2+}, 1 µL 0.25 mM dNTP stock, 0.2 µL Forward primer (50 µM), 0.2 µL Reverse primer (50 µM), 0.1 µL Taq polymerase (5 units/µL) and the volume of the reaction mixture was made upto 10 µL per reaction using ultra-pure, nuclease-free water.
2. If more than one cDNA sample needs to be processed for amplification, one can prepare a master mix containing all the components for all the cDNA samples at once.
3. 5 µL of cDNA or ChIP DNA was added to wells of the PCR plates, followed by addition of 5 µL of RT-PCR master mix. Reactions were carried out for 32 cycles. Each cycle contains the following steps:
    a. Initial denaturing: 94 °C for 3 min.
    b. Then 32 cycles of: 94 °C for 30 sec.

i. 60 °C for 45 sec.

ii. 72 °C for 60 sec (about 1 kb/min).

c. Extension: 72 °C for 7 min and at the end of the 33rd cycle, 10 min at 72 °C.

4. PCR products were analyzed in 1.5% agarose gel electrophoresis.
5. cDNA was subjected to PCR using specific primer pairs (Table 2, Table 3, Table 4).
6. Each experiment was repeated thrice (n = 3).
7. GAPDH was used as control. The results were analyzed using the CFX Manager. Expressions of gene relative to GAPDH were plotted.

2.2.3. Chromatin Immunoprecipitation assay (ChIP assay)

Materials:

Antibodies to native protein of interest (or a post-translationally modified form), SDS lysis buffer for ChIP, formaldehyde (37%), glycine (1.375M), ChIP dilution buffer, high salt immune complex wash buffer, low salt immune complex wash buffer, lithium chloride (LiCl) immune complex wash buffer, TE buffer, elution buffer for ChIP, protease inhibitors (Roche), protein A-agarose DNA beads (Millipore), and proteinase K (20 µg/uL) were used to perform ChIP assay.

Antibodies were purchased from commercial sources that are 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), RNA pol II (RNAPII, Abcam, 8WG16), NCoR(C-20, sc-1609), β-actin (Sigma, A2066), CBP (A22, Santa Cruz Biotechnology, Sc369) and p300 (N15, Santa Cruz Biotechnology, Sc584).

Recipes:

1) **SDS Lysis Buffer** was prepared using 1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1.

2) **ChIP Dilution Buffer** was made using 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1 and 167 mM NaCl.
3) **High Salt Immune Complex Wash Buffer** was made using 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl.
4) **Low Salt Immune Complex Wash Buffer** was made using 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl.
5) **Lithium Chloride (LiCl) Immune Complex Wash Buffer** was made using 0.25M Lithium Chloride (LiCl), 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris, pH 8.1.
6) **TE Buffer** was made using 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
7) **Elution buffe**r was made using 200 µL of elution buffer as follows: 10 µL 20% SDS, 20 µL 1M $NaHCO_3$ and 170 µL H_2O.

Cross-Linking of the cells:

1. For E2/BPA treatment, MCF7 cells were grown and maintained for at least 3 generations in phenol-red free DMEM-F12 media supplemented with 10% charcoal stripped FBS, 2 mM L-glutamine and 100 units/mL penicillin and 0.1 mg/mL streptomycin.
2. Once the cells reached ~ 80% confluency (3^{rd} generation), the cells were passaged into 100 x 20 mm cell culture plates and incubated until the confluency reached 50–60% (overnight/12 h), followed by treatments with 0.1 nM E2 for 4 h.
3. To fix the cells, formaldehyde was added directly to cell culture media to a final concentration of 1% (37% formaldehyde) into 10 mL treatment medium.
4. The treated MCF7 cells were incubated on a gentle shaking platform for 15 min at 37 °C.

5. The crosslinking reaction was quenched by adding glycine to a final concentration of 0.125M. It was continued to rock at room temperature for 10 min (1.25M glycine).
6. The media was aspirated and plates were rinsed twice with ice cold 1X PBS containing 0.2 mM PMSF containing 10 µL of 200X protease inhibitor cocktail. Dishes were placed on ice.
7. 1 mL of SDS lysis buffer was added containing 1X Protease Inhibitor Cocktail.
8. Cells were scraped from each dish and transfered into a separate microcentrifuge tube.
9. Cells were pelleted by spinning at 800 rpm at 4 °C for 5 min.

Sonication/Chromatin shearing:

1. MCF7 cell lysate were sonicated on wet ice.
2. MCF7 cells in SDS Lysis Buffer were sheared with 4–5 sets of 15–sec pulses on wet ice using an ultrasonic Processor/Sonicator, 50-watt model equipped with a 2 mm tip and set to 30% of maximum power gave the appropriate length DNA fragments.
3. Cell lysate was kept ice-cold as sonication produces heat that can denature the chromatin.
4. Cell lysate was centrifuged at 10000 rpm at 4 °C for 10 min to remove insoluble material.
5. One 5 µL aliquot was removed for agarose gel analysis of the sheared DNA. To prepare an aliquot for agarose gel analysis, 90 µL H_2O and 4 µL of 5M NaCl were added to the 5 µL samples (unsheared and sheared). Samples were incubated at least for 4-5 hours upto overnight at 65 °C to reverse the DNA-protein crosslinks. The phenol chloroform extraction was performed and the aqueous portion containing the DNA fragments was run on 1% agarose gel.
6. The chromatin was sonicated to an average length between 200–400 bp while keeping samples on ice. The time and number of pulses vary depending on sonicator, cell type and extent of crosslinking.

7. Once appropriate sonication was done (checked by fragmentation analysis in agarose get to 200–400 bp long fragments), sample was centrifuged at 13000 rpm for 10–20 min at 4 °C.
8. The supernatant was transferred into a fresh tube. These contain the sonicated chromatins for ChIP analysis. At this point, chromatin extracts (preferably in 100 µL aliquots) were stored at -80 °C until further use.

Immunoprecipitation (IP) of Cross-linked chromatins:

1. Sonicated chromatin was diluted 10–fold in ChIP dilution buffer (to 1 mL).
2. Samples were centrifuged at high speed in a microcentrifuge for 15 min at 4 °C to pellet the precipitated SDS. The supernatant was transferred to a fresh microcentrifuge tube.
3. The DNA concentration (A_{260}) of the chromatin is measured using nanodrop spectrophotometer. Lysis buffer for ChIP devoid of SDS was used as a blank. The A_{260}/A_{280} ratio should be ~ 1.4–1.6.
4. Aliquot of 100 µg of chromatin per antibody was used.
5. It is important to have an irrelevant antibody such as β-actin as a negative IgG control. Some chromatin was set aside as a no antibody control "input".
6. The chromatin was diluted to a final volume of 300 µL with ChIP dilution buffer supplemented with protease inhibitor cocktail.
7. To pre-clear the chromatin, 50 µL of protein A-agarose bead was added to the chromatin (each vial) and rotated for 2 h at 4 °C.
8. The chromatin samples were centrifuged at 3000 rpm for 5 min at 4 °C.
9. The supernatants were transferred to fresh 1.5 mL microcentrifuge tubes. This was the pre-cleared chromatin extract used for IP.

IP Washes and Elution:

1. Relevant antibodies (antibodies specific to ERα, ERβ, MLL1, MLL2, MLL3, MLL4, H3K4-trimethyl, histone acetylation, NCoR, CBP, p300, β-actin and RNAPII) were added to the pre-cleared chromatin solution and rotated overnight at 4 °C.
2. 50 µL of protein A-agarose beads (washed and equilibrated with 1X PBS) was added to chromatin samples and rotated for 2 h at 4 °C.
3. The samples were centrifuged at 500 rpm for 5 min at 4 °C.
4. The supernatant was carefully pipetted from all samples. Beads that contain the IP should not be removed.
5. 1 mL of high-salt wash buffer (for ChIP) was added to samples containing agarose beads and rotated for 10 min at room temperature.
6. The samples were centrifuged at 500 rpm for 5 min at room temperature.
7. Carefully the supernatant was removed and 1 mL of low-salt wash buffer was added for ChIP. It was rotated for 10 min at room temperature.
8. The samples were centrifuged at 500 rpm for 5 min at 4 °C.
9. The supernatant was removed carefully and 1 mL of LiCl wash buffer was added for ChIP. It was rotated for 10 min at room temperature.
10. The samples were centrifuged at 500 rpm for 5 min at 4 °C.
11. The supernatant was removed and washed twice with 1 mL of TE buffer as above.
12. The beads (containing the immunoprecipitated chromatins) and input samples (sonicated chromatin extract for "input") were resuspended in 50 µL of ChIP elution buffer supplemented with 1 µL of proteinase K (20 µg/µL), and incubated for 2 h at 55 °C.
13. Beads were resuspended by vortexing the samples.

14. Agarose was pelleted by brief centrifugation (13000 rpm for 1 min) and supernatant was collected into new microcentrifuge tubes.
15. The elution steps were repeated for 3 more times and the eluates were combined (total volume = 200 µL).

Reverse crosslinking and isolation of ChIP DNA fragments:

1. 8 µL 5M NaCl was added to samples and incubated at 65 °C for 5 h to reverse the DNA-protein crosslinks.
2. 1 µL of RNase A was added and incubated for additional 30 min at 37 °C.
3. 4 µL of 0.5M EDTA, 8 µL 1M Tris-HCl and 1 µL Proteinase K were added and incubated at 45 °C for 2 h.
4. The samples were centrifuged at 13000 rpm for 5 min at room temperature.
5. Supernatants were transferred to fresh microcentrifuge tubes.
6. They were subjected to phenol: chloroform extraction
7. 1 volume sample and 1 volume of phenol: chloroform: isoamyl alcohol (125: 24: 1, pH 4.5) were taken. They were vortexed for 1 min and centrifuged at 10000 rpm for 10 min at 4 °C.
8. The upper layer aqueous phase was transferred to a fresh tube and 1 volume of chloroform: isoamyl alcohol (24:1) was added. This was vortexed for 1 min and centrifuged at 10000 rpm for 10 min at 4 °C.
9. The upper aqueous phase was transferred to a fresh tube, 0.1 volume of 3M Sodium Acetate (pH 5.2) was added or 2.5 volumes of 95% ethanol.
10. They were mixed and placed at -80 °C for 2 h, and centrifuged at 13000 rpm for 30 min at 4 °C.
11. The supernatant was removed and the pellet was washed with 1 mL of 70% ethanol. The pellet was air dried (20 min).

12. DNA was resuspended in 50 µL of TE buffer, quantified using nanodrop spectrophotometer and analyzed by PCR or real-time PCR (qPCR) using various primers corresponding to different regions on gene promoters (Table 2, Table 3, Table 4).

2.2.4. Dual luciferase reporter assay

Materials:

iFECT transfection reagent (K.D. medicals), DMEM-F12 media (Sigma), pGL3-promoter vector and ERE-pGL3 clones, Dual-glo Luciferase assay kit (Promega) were used to perform dual luciferase reporter assay.

Transfection of MCF7 cells with ERE-pGL3 constructs and treatment with E2/BPA:

1. MCF7 cells were grown for 3 generations in phenol-red-free DMEM (DMEM-F12) containing charcoal-stripped FBS (10% FBS) and appropriate supplements.
2. 2 x 10^5 MCF7 cells were seeded (3^{rd} generation) in 60 mm cell culture plates with 5 mL of DMEM-F12 with 10% charcoal-stripped FBS. The plates were incubated in 5% CO_2 incubator at 37 °C until the confluency reached to about 60%.
3. On the day of transfection, the following solutions were prepared, Solution A: For each transfection, 1.5 µg of ERE-pGL3 and 0.15 µg of reporter plasmid containing renilla luciferase (PRLTK, as an internal transfection control) were diluted into 100 µL of FBS free DMEM-F12 without antibiotics or any supplements. Solution B: For each transfection 6-8 µL of iFECT transfection reagent was diluted into 100 µL of FBS free DMEM-F12 without antibiotics and supplements. Solution A was added to solution B directly.
4. The solution was mixed gently by pipetting and incubating the mixture for 35-45 min at room temperature.

5. The MCF7 cells were washed 3 times with DMEM-F12 without serum, antibiotics and supplements.
6. For each transfection, 0.8 mL of FBS free and antibiotic free DMEM-F12 media were added to each tube containing the ERE-pGL2/iFECT transfection reagent complex. The transfection cocktails were added into the respective plates containing cells and swirled slowly to spread the reagent.
7. The cells were incubated for 24 h at 37 °C in a CO_2 incubator with 5% CO_2.
8. After 24 h, the medium was aspirated and replaced with fresh media containing phenol-red-free DMEM (DMEM-F12), charcoal-stripped FBS (10% FBS) and other supplements.
9. The cells were treated with appropriate concentration of E2/BPA and incubated for 4 h inside the tissue culture incubator.
10. The cells were harvested for luciferase analyses using dual glo luciferase assay kit.

Preparation of MCF7 cell lysate and luciferase assay:

1. 4 h post E2 treatment, growth medium was removed from the treated MCF7 cells.
2. The cells were rinsed in 1X PBS. The final wash was removed as much as possible.
3. A minimal volume of 1X lysis reagent was dispensed into each culture vessel (e.g., 400 µL/60 mm culture dish).
4. Culture dishes were rocked to ensure complete coverage of the cells with lysis buffer.
5. Lysed/attached cells were scraped from the dish, and transferred to a microcentrifuge tube. The tubes were placed on ice.
6. The microcentrifuge tube was vortexed for 10–15 sec, then centrifuged at 13000 rpm for 1 min (at room temperature). The supernatant was transferred to a new tube. This cell lysate was used for luciferase assay.

7. 20 µL of cell lysate was mixed with 100 µL of Luciferase Assay Reagent (LAR), and the firefly luminescence (pGL3 luminescence; with emission filter 590 nm) was measured.
8. Prior to the luciferase assay, LAR: Luciferase assay reagent (LAR) was made by adding Luciferase Assay Buffer (10 mL) to vial of lyophilized luciferase assay substrate (supplied by the manufacturer). The working aliquots of the LAR were stored at -80 °C. Before each use LAR was equilibrated to room temperature.
9. 100 µL Stop & Glo® Reagent was added to the plate and renilla luminescence was measured (with emission filter at 590 nm)
10. Prior to the Renilla luminescence measurement, Stop & Glo® Reagent was prepared by adding 2.1 mL of 50X Stop & Glo® Substrate to 105 mL of Stop & Glo®, vortexed for 10 sec and stored at -20 °C.
11. Firefly luciferase activities were assayed and normalized to those of renilla luciferase
12. The light intensity of the reaction was nearly constant for about 1 min and then decayed slowly, with a half-life of approximately 10 min. Therefore, 5 readings were performed at an interval of every 20 sec.

For ERE luciferase assays, HOXB9 estrogen response elements (EREs) such as ERE1 (-6 to -393 nt), ERE2 (-999 to -1316 nt), ERE3 (-1174 to -1492 nt), and ERE4 (-1430 to -1858 nt) regions were cloned and inserted upstream of luciferase gene in pGL3-promoter vector (Promega) (Table 2).

2.2.5. Animal studies

We exposed ovariectomized (OVX) adult female rats with E2/BPA and analyzed its impacts on gene expression of various genes in different tissues.

Materials: Isoflurane, 17-β-estradiol (E2), Peanut oil were used for animal experiments.

Subjects:

1. 90 days old, experimentally naïve, adult, female, Sprague–Dawley rats were triple housed in a temperature and humidity-controlled environment under a 12 h reversed light/dark cycle with lights on at 7 p.m. and off at 7 a.m.

2. All animals had free access to food and water throughout the study and were maintained and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.[186,187,188]

Ovariectomy:

1. Rats were anesthetized with a 2–3% isoflurane–oxygen vapor mixture and ovariectomized (OVX) using a dorsal approach.[186,187,188]

2. Briefly, both flanks were shaved and swabbed with Betadine, the skin was opened with a 10 mm incision along each rat's midline, just below the ribs, and a 10 mm incision was made through the muscle. The ovary was pulled through the incision and the tissue between the oviduct and uterus were clamped with a hemostat while a ligature was placed just below the hemostat. Next, the ovary was removed with scissors and the hemostats released. This procedure was repeated on the contralateral side. Lastly, the muscle layer was sutured closed and the skin incision closed with 9 mm wound clips.

Vaginal lavage testing:

Following a 4-5 day surgical recovery period, all rats underwent daily vaginal lavage testing daily for 8 consecutive days to confirm cessation of estrous cycling. Vaginal secretion was collected with a plastic pipette filled with 10 μL of 0.9% saline by inserting the tip into the rat vagina, but not

deeply. Unstained material was observed under a light microscope. All OVXs performed were confirmed as complete and thus, no animals were eliminated on the basis of an incomplete procedure. Vaginal lavage testing was performed to confirm cessation of estrous cycling (estrous cycling in female rats is regulated by estrogen).

Estrogen, BPA and DES treatments to OVX animals:

1. Estradiol (17β-Estradiol-3-benzoate (EB; Sigma-Aldrich)) was dissolved in peanut oil to yield final concentrations of 5 μg/mL.

2. Rats were given subcutaneous (s.c.) injections of estradiol (5 μg) and control animals were given peanut oil, 24 and 4 h prior to sacrifice.

3. BPA: 10 mg of BPA was dissolved in 1 mL of ethanol to create a stock solution that was stored at -4 °C. DES and estradiol were dissolved in peanut oil to yield final concentrations of 5 μg/mL. BPA was dissolved in ethanol and brought up to a final concentration of 50 μg/mL with saline. Rats were given subcutaneous injections of either BPA (25 μg/kg), estradiol (5 μg), or DES (5 μg/kg), (n = 4) 24 and 4 h prior to sacrifice. Animals were sacrificed via rapid decapitation and mammary gland tissue was collected from each rat and flash frozen on dry ice and then stored at -80 °C until RNA extraction.[189,190,191]

Tissue preparation

After the respective control and estradiol/bisphenol-A treatments, animals were rapidly decapitated and a midline incision was made along the ventral side of the animal's body using surgical scissors. To access inguinal mammary glands, both skin and mammary tissue were pulled away from the peritoneal wall and dissected from the skin. Upon removal, liver and mammary glands were

immediately removed and placed on dry ice until they could be transferred and stored at -80 °C until commencement of biochemical analyses.[186,187,188]

RNA extraction:

1. Frozen mammary glands were subjected to RNA extraction by ZyGEM kit.
2. A small amount of tissue from the frozen mammary glands (isolated from the control and E2/BPA-treated OVX rats) was homogenized and transferred to a microcentrifuge tube.
3. 10 µL of 10X buffer (Silver) was added to the tube containing homogenized tissue followed by 2 µL of RNA-gem and the volume was made upto 100 µL using DEPC treated ultra-pure, nuclease free H_2O
4. The sample was vortexed for 30 sec and incubated at 75 °C for 10 min.
5. After the 75 °C incubation, 5 µL of DNase buffer and 2 µL of DNase I were added.
6. It was vortexed for 30 sec and incubated at 37 °C for 5 min and then at 75 °C for 5 min.
7. The sample was centrifuged at 10000 rpm for 10 min and the supernatant was collected containing RNA and proteins into a new microcentrifuge tube.
8. RNA was further purified using phenol-chloroform extraction followed by ethanol precipitation and resuspended in TE buffer.
9. RNA was quantified using nanodrop spectrophotometer and then analyzed using RT-qPCR using rat specific primers (Table 2).

Table 2. Nucleotide sequence of primers

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
PCR primers		
hHOXB9	TGGGACGCTTAGCAGCTATT	CGTACTGGCCAGAAGGAAAC
hGAPDH	CAATGACCCCTTCATTGACC	GACAAGCTTCCCGTTCTCAG
rHOXB9	GAAAAAGCGCTGTCCCTACA	TTGAGGAGTCTGGCCACTT
rGAPDH	CTCCCATTCTTCCACCTTTG	TTACTCCTTGGAGGCCATGT
Cloning primers		
HOXB9-ERE1	GTAGCTGGGGCTGAGGTTAC*	ACATTATCCGGGCGCTTG*
HOXB9-ERE2	ACAGGAGGGCTGAAAACTC*	GAACCAGAGCGCCTTTACAT*
HOXB9-ERE3	TGCAGGGCCACAGAATAGAT*	AAGGGTTAAGGCCACTTTCC*
HOXB9-ERE4	ACGAGATGGCTTCATTTGGA*	AGACCCCTTCATTGGAGCTT*
HOXB9-Non-ERE	GGAAGCTCGCAGTCATGTAA*	GAGGGAGGGAGAGCAAGG*
ChIP primers		
HOXB9-ERE4	CCAGAGGAGAACTGGGTCTG	TCTATTCTGTGGCCCTGCAC

*Cloning primers flanked by appropriate restriction sites.

2.3. Results

2.3.1. HOXB9 is overexpressed in breast and other types of cancer

Initially, to investigate the association of HOXB9 with different types of cancer, we analyzed publicly available cancer genome database using cbioportal (http://www.cbioportal.org/public-portal/) data analysis tool.[192,193] This analyses demonstrate that HOXB9 gene is amplified, mutated and deleted in various forms of cancer that includes breast, cervical, ovarian, uterine and prostate (Figure 5). Amplification of HOXB9 gene was observed in 10.3%, 5.4% and 4.1% of the analyzed cases of breast carcinoma. HOXB9 amplification was also observed in 4% of ovarian and 2% of prostate cancer cases. Overexpression of HOXB9 in breast cancer tissues is in agreement with our previous findings and studies from other laboratories.[178,194] The overexpression of HOXB9 in breast, prostate, uterine and ovarian tissues indicate its potential regulation by steroid hormones and it is a potential target for misregulation by endocrine disrupting chemicals.

2.3.2. HOXB9 is induced by BPA in breast cancer cells and in mammary gland tissue of ovariectomized (OVX) rats

To understand the potential impacts of estrogenic EDCs on HOXB9 gene regulation, we treated ER-positive breast cancer cells, MCF7 with varying concentration of a well-known estrogenic EDC, bisphenol-A (BPA) and analyzed its impacts on HOXB9 gene expression. Additionally, we also exposed MCF7 cells with estradiol (E2) as positive control. In brief, cells were grown in phenol-red-free media supplemented with charcoal-treated FBS and were then subjected to treatment with varying concentrations of E2 or BPA, independently. RNA was isolated, reverse transcribed and levels of HOXB9 expressions were analyzed by regular RT-PCR and RT-qPCR, using primers specific to HOXB9 (Table 2). GAPDH expression was analyzed as loading control. This analysis showed that BPA and E2-treatments induced the HOXB9 expression levels in dose dependent manner (Figures 6A-D).

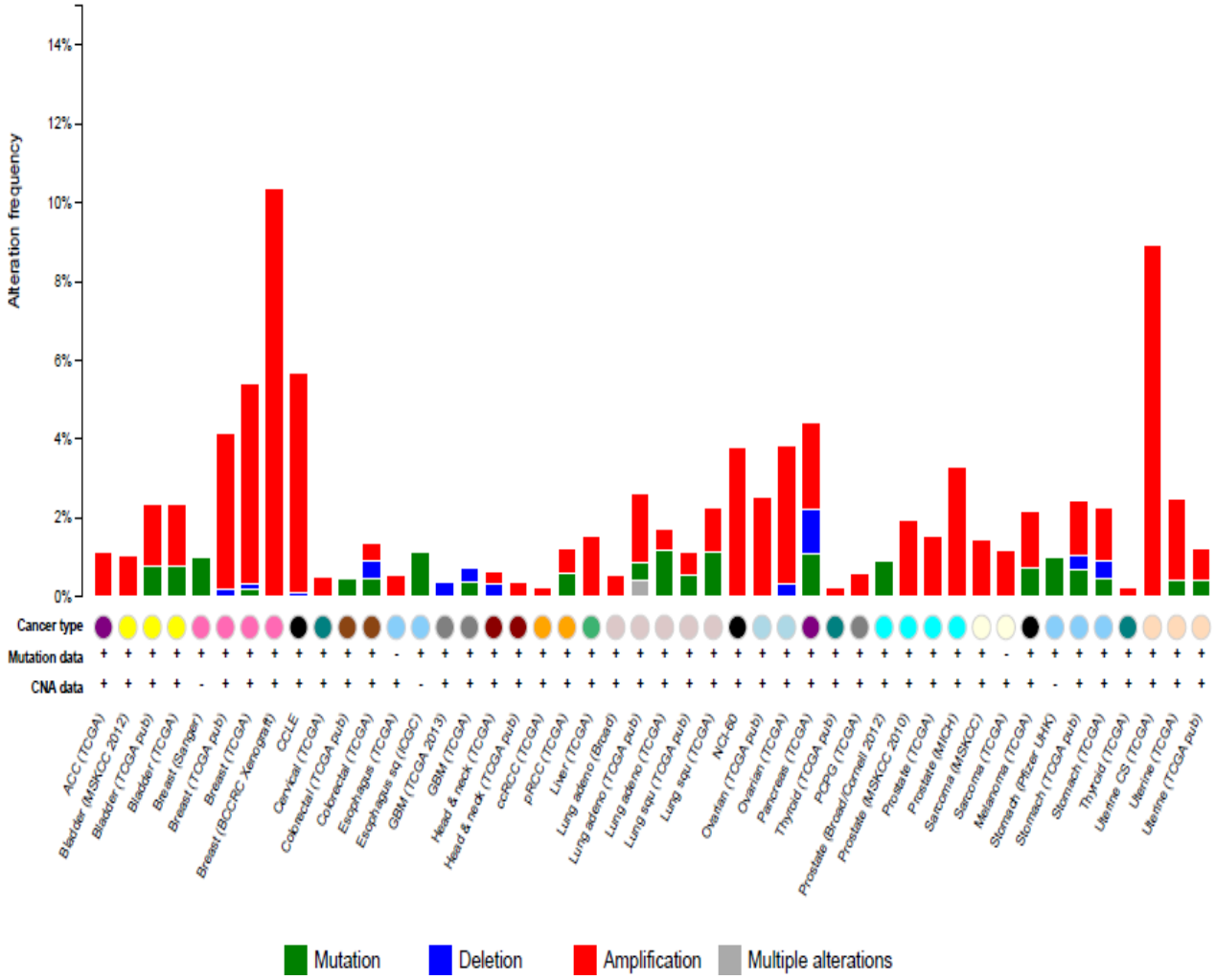


Figure 5. Cross-cancer analysis of copy number alterations and mutations in HOXB9 based on cBioPortal database.[192,193]

Notably, HOXB9 expression was increased by about 6.6 fold at 1 nM E2 and 3.7 fold and 9.4 fold at 10 and 100 nM BPA, respectively. Since HOXB9 is optimally induced in breast cancer cells by 1 nM E2 and 100 nM BPA, we used these concentrations of E2 and BPA for future biochemical experiments. The time-dependent analysis showed that HOXB9 induction in MCF7 cells was optimum at 6 h post-treatment with 1 nM E2 or 100 nM BPA (data not shown). The induction of HOXB9 expression by E2 and BPA in ER-positive breast cancer cells (MCF7) indicate that HOXB9 is transcriptionally regulated by E2 and is potentially misregulated upon exposure to estrogenic EDC like BPA.

To further evaluate the impacts of BPA on HOXB9 expression *in vivo,* we exposed overiectomized (OVX) Sprague Dawley rats with BPA as well as E2, and analyzed their impacts on HOXB9 expression in the mammary glands of OVX rats. In brief, female Sprague Dawley rats were subjected to ovariectomy to minimize the impacts of internal esrogens. We exposed the OVX rats to acute levels of E2 (5 µg/kg) and BPA (25 µg/kg) for 24 h. These doses of E2 and BPA treatment were previously used and shown to affect gene expression and impact behavioral traits and other neural functions.[88,89,195] The control OVX rats were exposed with the diluent peanut oil (vehicle). The mammary glands from control and E2/BPA treated female rats were harvested, RNA was isolated, reverse transcribed and the expression levels of HOXB9 were analyzed using qPCR and regular RT-PCR. These analyses showed that E2 and BPA treatments significantly increased the levels of HOXB9 expression by ~ 4 and ~ 5 folds, respectively, in the rat mammary glands (Figures 6E and 6F). GAPDH was used as the loading control. These

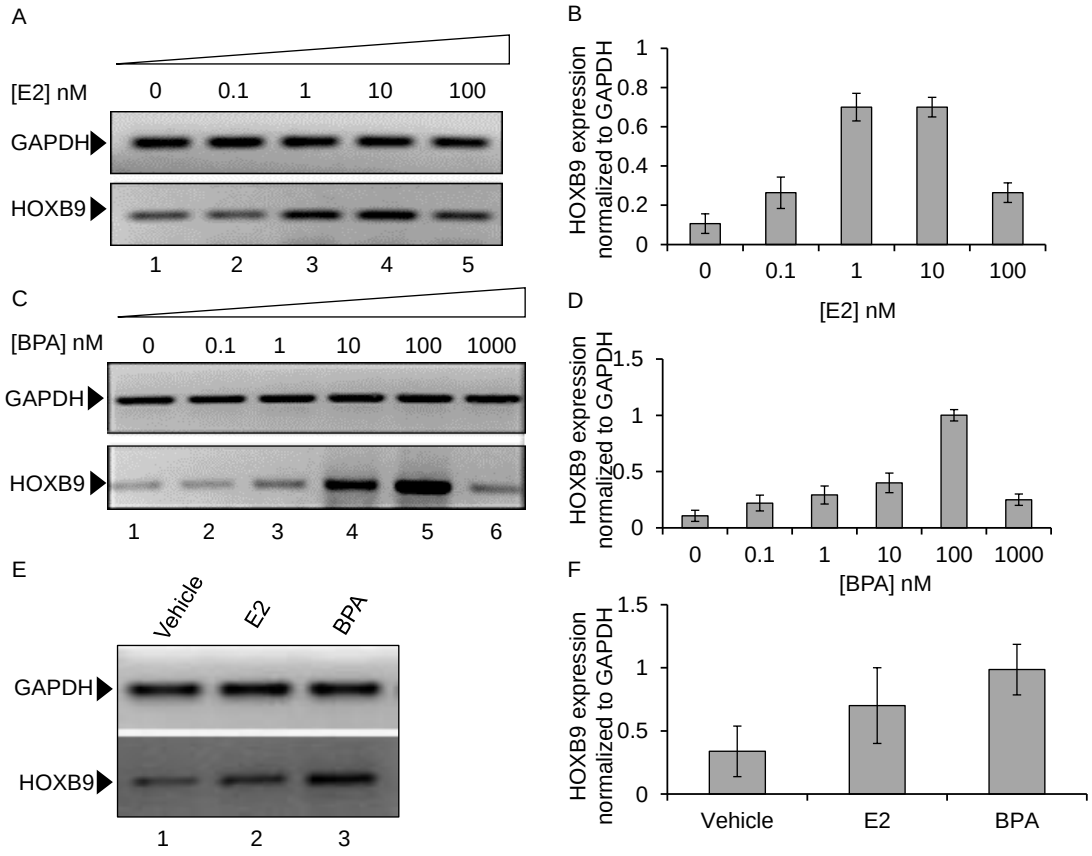


Figure 6. Effects of BPA on HOXB9 expression *in vitro* and *in vivo*.

(A-D) MCF7 cells grown in phenol red free DMEM-F-12 media were treated with varying concentrations of E2 and BPA. RNA from the control and E2 or BPA-treated cells was analyzed by RT-PCR (panels A and C) and RT-qPCR (panels B and D) using HOXB9 specific primers. GAPDH was used as the loading control. Panel A and B are RT-PCR and qPCR analyses of HOXB9 expression upon exposure to varying concentrations of E2, respectively. Panel C and D are RT-PCR and qPCR analyses of HOXB9 expression upon exposure to varying concentrations of BPA, respectively. (E-F) *In vivo* effect of E2 and BPA on HOXB9 expression. Ovariectomized adult female rats were administered with estradiol (5 µg/kg) and BPA (25 µg/kg) for 24 h and 4 h before sacrifice, separately. RNA from the control, E2 and BPA treated mammary glands were analyzed by regular RT-PCR (panel E) and qPCR (panel F). GAPDH was used as a loading control. Each qPCR experiment was repeated for three times with three parallel replicates (n = 3). Bars indicate standard errors ($p \leq 0.05$).

observations suggested that HOXB9 expression is transcriptionally regulated by E2 in the mammary glands and it is upregulated upon exposure to BPA, *in vivo*.

2.3.3. HOXB9 promoter EREs are responsive to BPA

Since HOXB9 is an E2-responsive gene and is induced by BPA, we investigated the potential mechanism of BPA-induced expression of HOXB9. HOXB9 promoter contains several putative estrogen-response elements (EREs).[194] Notably, in the presence of E2, estrogen-receptors (ERs) are activated which in turn bind to EREs present in the promoters of E2-regulated genes and induce transcription.[196,197,198,199] ERs aid in the recruitment of various ER-coregulators and epigenetic modifiers to the gene promoters that facilitate histone modification and chromatin remodeling leading to target gene activation.[199,200,201,202] Promoter analysis revealed that there are three ERE1/2 sites (GGTCA) located at the -208 nt, -1201 nt and -1395 nt regions located upstream of the transcriptional start site (TSS) in the HOXB9 promoters (Figure 7A). There is also an imperfect ERE (GGTCAnnnTGTCC) located at the -1600 nt (Figure 7A) and that is 1 nt different from the consensus full ERE (GGTCAnnnTGACC). Presence of these putative imperfect EREs indicate its potential involvement in E2/BPA induced HOXB9 gene expression.

Irrespective of sequence homology with consensus full ERE, we examined the BPA (and E2) responsiveness of each EREs (ERE1-ERE4) sites using a luciferase based reporter assay.[194] Initially, we cloned each ERE along with ~ 150 nt flanking sequences on both sides into a luciferase expression construct, pGL3 (Figure 7A).[194] A promoter region containing no ERE (non-ERE) sequence was also cloned and used as a negative control. Each ERE-pGL3 construct was transfected into MCF7 cells separately, and the cells were exposed to E2 (1 nM) and BPA (100 nM) for 6 h. An empty pGL3 vector was also transfected as a negative control. The cell extracts from the control and (E2/BPA) treated MCF7 cells were subjected to luciferase analysis using a commercial luciferase detection kit

(normalized to renilla expression). Our results demonstrated that transfection with ERE4-pGL3 (which is the imperfect ERE) construct followed by E2 or BPA treatment induced the luciferase

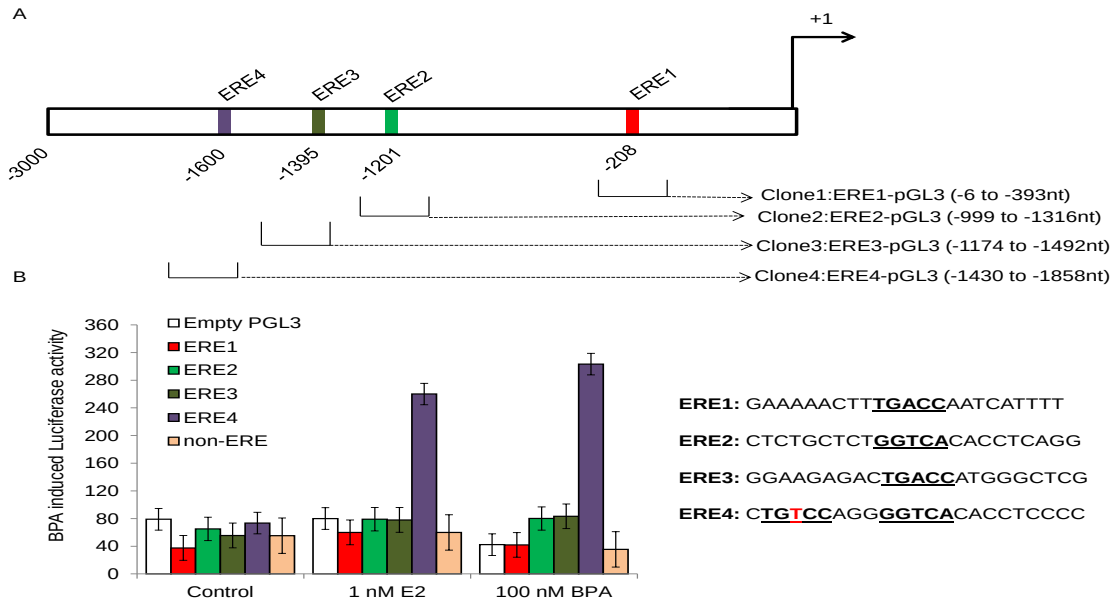


Figure 7. HOXB9 promoter EREs are responsive to BPA treatment.

(A-B) HOXB9 gene promoter EREs, termed as ERE1, ERE2, ERE3 and ERE4 locations and the neighboring sequences are shown in panel A. The promoter regions spanning the EREs were cloned into luciferase based reporter construct, pGL3 and used for luciferase assay (cloned region for each ERE is shown in panel A. (B) Luciferase based reporter assay. ERE containing pGL3 constructs or empty pGL3 (control vector) constructs were co-transfected into MCF7 cells along with renilla luciferase construct (pRLTK; internal transfection control) for 24 h. Cells were then treated with 100 nM BPA and 1 nM E2 for 6 h and then subjected to luciferase assay by using dual-Glo Luciferase Assay kit. The luciferase activities (normalized to renilla activity) were plotted. The experiment was repeated thrice with four parallel replicates (n = 3). Bars indicate standard errors. P values ≤ 0.05 were considered to be significant.

activity by ~ 3 and ~ 4 folds, respectively in MCF7 cells (Figure 7B). Transfection with other ERE-pGL3 constructs or the empty pGL3 vector had no significant impacts on E2/BPA-induced luciferase inductions. These results suggest that ERE4 which is an imperfect full ERE, is likely an E2/BPA responsive ERE and may be also associated with BPA-mediated activation of HOXB9 transcription.

2.3.4. Estrogen-receptors (ERs) and ER-coregulators are enriched at the HOXB9 promoter upon treatment with BPA

As ERs are key players in transcriptional upregulation of estrogen-responsive genes,[198,199] we examined if ERα participates in BPA-induced HOXB9 expression. We examined if BPA and E2 treatment results in recruitment of ERs in HOXB9 promoter regions especially in the ERE regions, using chromatin immunoprecipitation (ChIP) assay.[194] Notably, along with ER, ER-coregulators also play key roles in E2-induced gene regulation.[199,200,201] Therefore, along with ERα, we also examined if selected ER-coregulators (such as MLL3, CBP/p300 and N-CoR) are enriched at the HOXB9 promoter upon treatment with BPA or E2.[196,197,203] Notably, MLLs are well-recognized histone methyl-transferases that specifically introduce histone H3 lysine-4 (H3K4) methylations and regulate gene activation.[30,185,200,204,205,206,207,208,209,210,211,212] MLLs are recently implicated in estradiol/BPA induced gene activation.[30,88,89,185,206,211] In particular, our previous studies indicated involvement of MLL3 in HOXB9 gene regulation,[30] and therefore, here we investigated the potential involvement of MLL3 in BPA-induced HOXB9 regulation. CBP and p300 are well known histone acetyl-transferases that play crucial roles in NR-mediated gene activation.[213,214] N-CoR is a repressive NR-coregulator that maintains the repressive state of gene in the absence of activated or liganded ERs.[215,216,217] Here, we examined the E2/BPA-dependent recruitment of ERs and ER-coregulators (MLL3, CBP/p300 and N-CoR) at the ERE4-region of HOXB9 promoter (Figure 8A). We chose to analyze the ER and ER-coregulators binding in the ERE4-regions because it was the most responsive ERE to E2/BPA-treatment in luciferase

analysis (Figure 8B). In brief, MCF7 cells were treated with E2 (1 nM) and BPA (100 nM) for 6 h, fixed with formaldehyde, sonicated to shear the chromatin and then subjected to immunoprecipitation using ERα, MLL3, CBP, p300 and N-CoR specific antibodies. β-actin antibody was used as a non-specific antibody control. The immunoprecipitated DNA fragments were purified and PCR-amplified using primers spanning ERE4 region of HOXB9 promoter (Figures 8B and 8C). ChIP analysis showed that ERα, MLL3, CBP and p300 were enriched in the ERE4 region in the presence of either BPA or E2 (Figures 8B and 8C). No significant binding of β-actin was observed in the HOXB9 promoter (used as a non-specificity control, Figures 8B and 8C). These observations demonstrate that, along with ERs, ER-coregulators such as histone methylase MLL3 and histone acetylases, CBP and p300, are involved in the BPA induced HOXB9 expression. We also found that the occupancy levels of nuclear receptor co-repressor; N-CoR was significantly reduced at the HOXB9 promoter upon treatment with either E2 or BPA.

MLLs are well known H3K4-specific methyl-transferases and CBP/p300 are histone acetylases.[205,212,218,219,220,221] Since MLL3, CBP and p300 are found to be enriched at the HOXB9 promoter in a BPA/E2-dependent manner, we examined the levels of histone acetylation and histone H3K4-tri-methylation in the HOXB9 promoter in the absence and presence of BPA, using ChIP assay. We also analyzed the recruitment of RNA Polymerase II (RNAP II) on to the HOXB9 promoter in the absence and presence of BPA. Our ChIP analyses demonstrated that the levels of histone H3K4-trimethylation, histone acetylation and the levels of RNA polymerase II were increased at the HOXB9 promoter (ERE4) in the presence of BPA or E2 (Figures 8B and 8C). The enrichment of histone methylases (MLL3) and histone acetylases (CBP/p300) and consequent increase in H3K4-trimethylation and histone acetylation at the HOXB9 promoter in presence of BPA, suggest the potential involvement of these ER-coregulators in the BPA mediated transcriptional activation of HOXB9. The BPA/E2-

induced dissociation of N-CoR from the HOXB9 promoter indicate its potential involvement in the maintenance of the basal transcription levels of HOXB9 especially in the absence of any stimuli such as E2 or BPA.

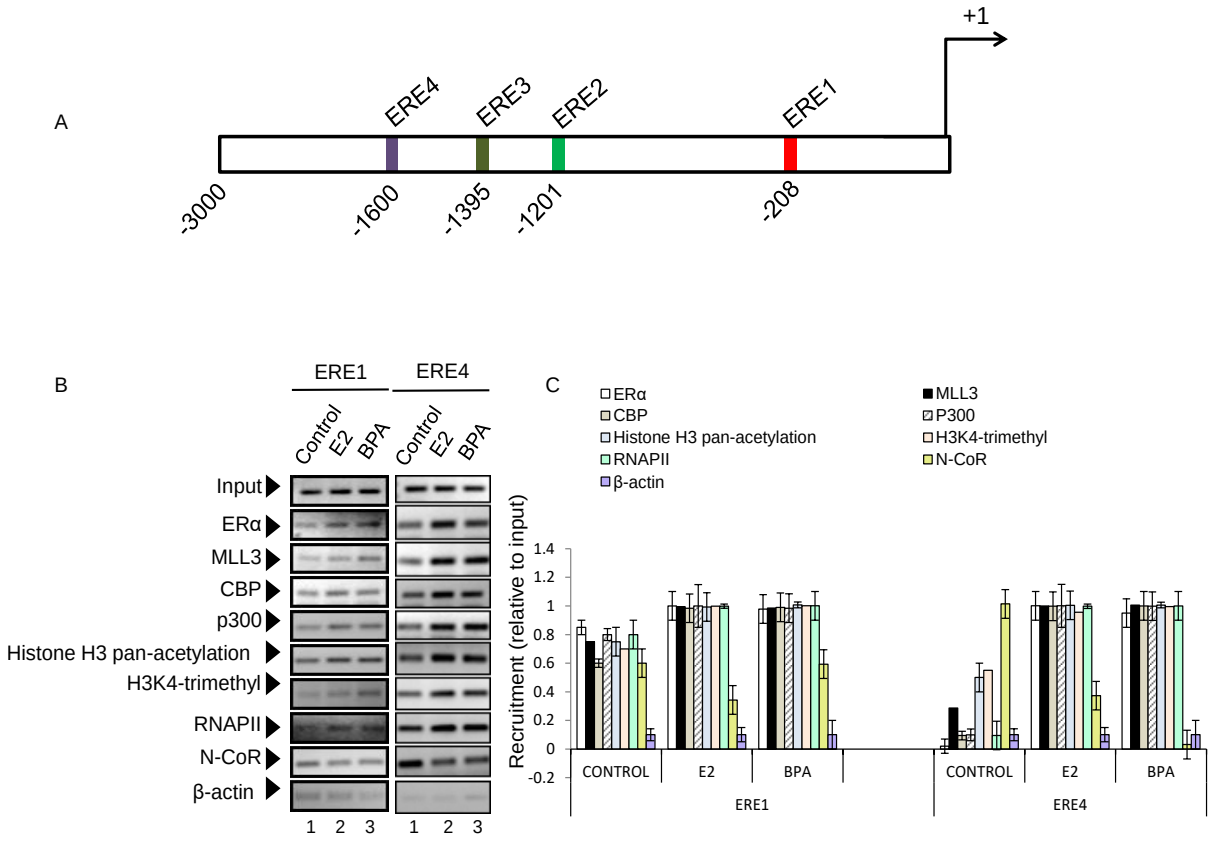


Figure 8. E2 and BPA-dependent recruitment of ER and ER-coregulators at the HOXB9 promoter.

(A-C) MCF7 cells were treated with 1 nM E2 and 100 nM BPA, separately for 6 h and subjected to ChIP assay using antibodies specific to MLL3, CBP, p300, H3K4me3, histone acetyl, RNAP II, N-CoR and β-actin (negative control). ChIP DNA fragments were PCR-amplified and analyzed by qPCR and regular PCR using primers specific to ERE4 region of the HOXB9 promoter. Panel A shows the position of the primer spanning ERE4 region in the HOXB9 promoter. Panels B-C show the ChIP analyses of MLL3, CBP, p300, H3K4-trimethyl marks, histone acetyl marks, RNAP II, N-CoR and β-actin on the ERE4 of the HOXB9 promoter. Each experiment was repeated at least thrice. Bars indicate standard errors. P values ≤ 0.05 were considered to be significant.

2.4. Discussion

HOXB9 is a critical player in skeletal and mammary gland development.[166] Mice homozygous for HOXB9 mutation show developmental defects and a significant decline in newborn survival;[222] it also leads to abnormal breast epithelium branching and lobulo-alveolar development, eventually leading to the inability of the mother to nurse pups.[166] In addition to its critical role in mammary gland and skeletal development, HOXB9 is involved in regulation of renin and hence regulates blood pressure, fluid homeostasis, and electrolyte balance.[173] Recent studies demonstrate that HOXB9 is overexpressed in 42 % of human breast tumors.[168] Furthermore, HOXB9 is a homeodomain containing transcription factor, and is involved in regulation of tumor growth and angiogenic factors and hence plays critical roles in tumorigenesis and metastasis.[35,168] Studies from our laboratory demonstrated that HOXB9, through it homeodomain, binds to the promoters of tumor growth and angiogenic factors like NRG2 (neuregulin-2), VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor), TGFβ-1 (tumor growth factor β-1) and control 3D tumor colony formation.[35] Furthermore, we demonstrated that HOXB9 expression is transcriptionally induced by E2, in cultured breast cancer cells.[30] As HOXB9 is overexpressed in breast cancer, and is involved in mammary gland development and is transcriptionally regulated by estradiol in breast cancer cells, here, we investigated its potential endocrine disruption by estrogenic EDCs such as BPA, *in vitro* and *in vivo*. Additionally, we also analyzed the expression levels of HOXB9 in different types of cancers using publically available cancer genome database, using Cbioportal data analysis software.[192,193] This analysis demonstrated that HOXB9 gene is amplified, mutated and deleted in variety of cancer cells (Figure 5). Specifically, amplification of HOXB9 gene was observed in several analyzed cases of breast, ovarian and prostate cancer. The overexpression of HOXB9 in endocrine regulated tumors such as breast, prostate and ovarian cancers, indicates its potential regulation by endocrine hormones such as estrogens and

androgens and furthermore makes it a potential target of endocrine disruption upon exposure to hormone mimicking EDCs such as BPA. To investigate the potential endocrine disruption of HOXB9, we exposed both cultured breast cancer cells as well as OVX female Sprague Dawley rats with BPA (as well as E2 as control) and analyzed their impacts of HOXB9 expression. Our analysis demonstrated that HOXB9 expression is not induced by E2 but also by BPA both *in vitro* (cultured MCF7 cells) and *in vivo* (mammary glands of OVX rats), indicating its transcriptional regulation by E2 and its potential endocrine disruption upon exposure to estrogenic EDCs like BPA and others. Notably, ERs and ER-coregulators are integral components of estrogen-dependent gene activation and signaling. During estrogen-dependent gene activation, upon binding of the ligand to estrogen-receptors (ERs), ERs undergo conformational changes, and bind to estrogen-response-elements (EREs) present in the promoters of estrogen responsive genes, resulting in their transcriptional activation. Along with ERs, variety of ER-coactivators also coordinates with the estradiol-dependent gene activation and signaling.[194,223,224,225] These coregulators usually possess enzymatic activity, modify chromatin, and bridge ERs with transcription machinery.[196,202] Many ER-coactivators have been identified including SRC-1 family of protein, CREB binding protein (CBP/p300), p/CAF, ASCOM (activating signal cointegrator-2 that also contains MLLs).[203,208,226,227] Sequence analysis demonstrated that HOXB9 promoter contains multiple ERE1/2 sites (ERE1-3) and a potential imperfect full ERE (ERE4).[199] Based on our luciferase based reporter assay, we observed that not only E2 but also BPA response induces HOXB9 promoter activity. ERE4, which is an imperfect full ERE, showed significant induction of luciferase activity in response to both E2 and BPA exposure, indicating its potential involvement in estrogen-mediated gene expression and endocrine disruption by BPA.

ChIP analyses demonstrate that ERα and a variety of ER-coregulators such as histone methylase, MLL3, and histone acetylases, CBP and p300 are enriched at the HOXB9 promoters during E2 as well

as BPA-induced HOXB9 expression. Notably, MLL-family of histone methylases is a well-known family of H3K4-specific methyl-transferases that play key roles in gene activation.[205,212,218,219,220,221] Studies from our laboratory and others have shown that MLLs functionally coordinate with ERs-during estrogen-dependent gene activation.[33,88,89,185,206,211,213,228] MLLs are a novel class of ER-coregulators. Here, our analysis further demonstrated that histone methylase, MLL3 coordinates with the E2 and BPA-induced HOXB9 gene activation. Notably, the involvement of MLL3 in E2-induced HOXB9 activation is in agreement with our previous observation.[194] ER-mediated recruitment of histone methylases and acetylases at the ER-target promoters, are anticipated to modify histone proteins, remodel the chromatin which ultimately contribute to gene activation. Indeed, our ChIP analysis also showed that BPA (as well as E2) treatment altered the epigenetic marks such as, increased histone H3K4-trimethylation, histone acetylation, enrichment of RNA polymerase II at the HOXB9 promoter region (ERE4). We also observed that NCoR, a nuclear receptor corepressor, is pre-occupied at the HOXB9 promoter in the absence of E2 or BPA-treatment and gets delocalized from the HOXB9 promoter upon exposure to either E2 or BPA, indicating the potential role of NCoR in maintenance of basal transcription of HOXB9.

Involvement of ERs and ER-coregulators, changes in histone modifications upon BPA-induced HOXB9 gene activation, indicate that the epigenetic mechanism of transcriptional activation of HOXB9 by BPA is similar to that of estradiol-mediated HOXB9 activation (a model showing the mechanism of BPA induced endocrine disruption of HOXB9 is shown in figure 9). These observations also further indicate that HOXB9 gene expression may be disrupted upon exposure to estrogenic EDCs like BPA and others both *in vitro* and *in vivo*, even in the absence of estradiol, and that may contribute towards abnormal HOXB9 expression and various human pathogenesis including breast cancer. Notably, EDCs are a class of molecules when exposed to our body, they interact with various hormone receptors at very

low concentrations and alter the hormone signaling pathways affecting various hormonally regulated processes including reproduction and development.[229] Chronic and/or acute exposure to EDCs results in harmful health effects including birth defects, diabetes, cancers, reproductive problems, early puberty, and obesity.[179,180,181,182,183] Exposure to EDCs results in alteration in the gene expression profiles. For example, exposure to estrogen mimicking EDCs such as bisphenol-A (BPA) alters uterine HOX gene expression and induces developmental changes in the female reproductive tract.[184] BPA is commonly found in plastics, metallic storage containers, and other routinely used consumables.[182] It is widely used since 1950 as a monomer that is polymerized to manufacture polycarbonate plastic and epoxy resins and found as an environmental contaminant.[182] The impacts of BPA exposure on human health have been extensively studied. Exposure to BPA leads to developmental and reproductive anomalies.[184] Perinatal exposure to BPA alters mammary gland development *in vivo*.[184] Thus, exposure to BPA is of serious health concern. In the present study, we demonstrated that breast cancer (as well as variety of other cancers) associated gene HOXB9, is upregulated upon exposure to BPA both *in vitro* and *in vivo*. Even in the absence of native hormone estradiol, BPA activates estrogen-receptors, that in turn, activates and recruits variety of ER-coregulators at the HOXB9 promoter, alters the promoter histone modification and other epigenetic features resulting in HOXB9 gene induction which is associated with breast and other carcinogenesis. Thus our studies demonstrate that BPA is an estrogenic EDC and therefore its exposure is capable of altering the epigenetic programming in cells causing unwanted gene activation and that may contribute towards increased risk of cancer and other human diseases. In summary, our studies demonstrated a novel epigenetic mechanism of estrogen-mediated regulation and BPA-induced endocrine disruption of a key homeobox containing gene, HOXB9, which is critical for development of reproductive organs and in progression of cancer.

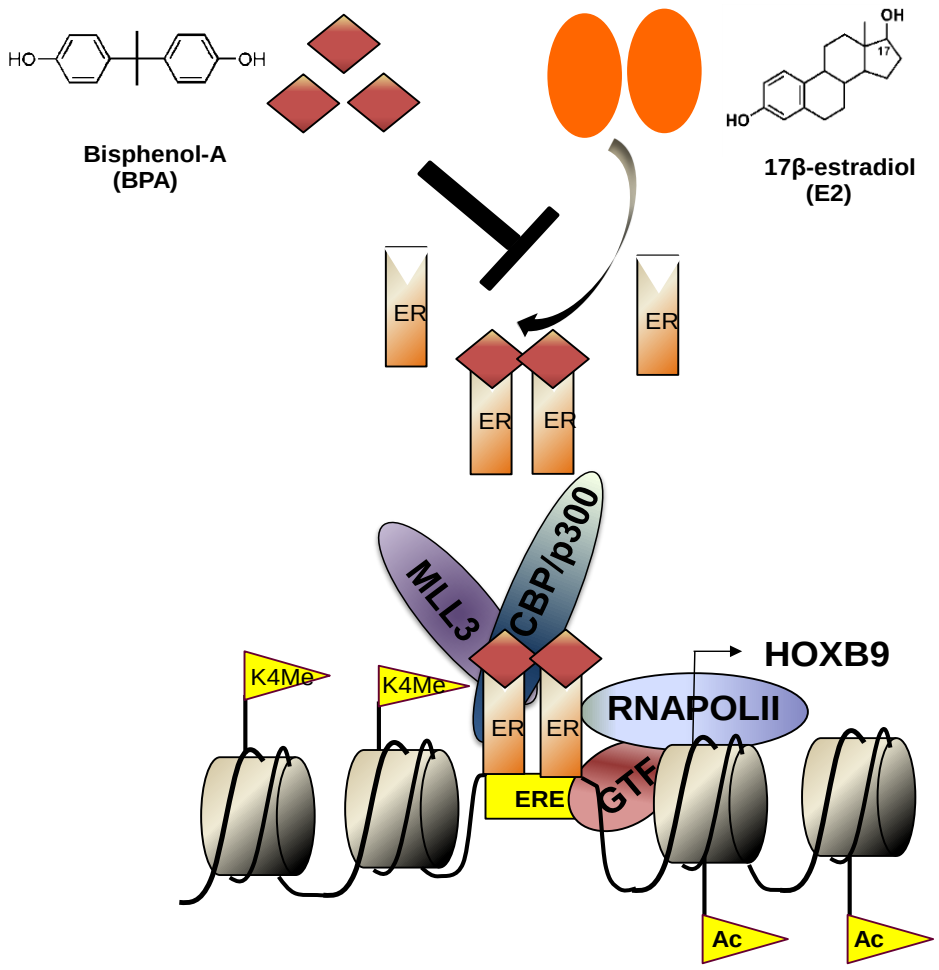


Figure 9. Model showing the roles of ER, MLL and other ER-coregulators in E2 and BPA-mediated upregulation of HOXB9.

During classical E2-stimulated HOXB9 gene expression, ERs dimerizes upon binding to E2, dimerized ERs translocate into the nucleus and bind to the EREs present in the promoter region of HOXB9. ER-coregulators such as MLL3, CBP, p300, and other ER-coregulators are also recruited to the HOXB9 promoter. Promoter histones are methylated via MLL3 and acetylated via the catalytic activity of CBP/p300 and thus allowing access to RNA polymerase II (RNAP II) and other GTFs to the promoter,[202] ultimately leading to transcriptional activation of HOXB9. Exposure of cells to estrogen mimicking endocrine disrupting chemicals (EDCs) such as BPA that in turn competes with endogenous E2 and binds to ERs, leading to activation of ERs and associated ER-target gene activation, in a fashion very similar to E2. Thus, even in the absence of E2, BPA can induce ER-target genes such as HOXB9 leading to their misregulation.

CHAPTER 3

HYPOXIA INDUCES EXPRESSION OF LONG NONCODING RNA HOTAIR

3.1. Introduction

Non-coding RNAs (ncRNAs) are an emerging class of transcripts that are coded by the genome, but are mostly not translated into proteins.[114,230] NcRNAs play key roles in gene expression, cell differentiation, and development. In particular, long non-coding RNAs (lncRNAs) appear to play major roles in the regulation of chromosome dynamics, gene activation, and silencing.[114] Misregulation and mutations in lncRNAs are widely associated with a variety of human diseases including cancer, [130,231] neurological disorders,[147,232,233,234] cardiovascular disease,[89] and diabetes.[35] HOTAIR (HOX Antisense Intergenic RNA) is a recently discovered (2.2 kb long) antisense lncRNA that is associated with gene silencing and chromatin condensation.[235,236,237,238,239] HOTAIR is located on HOXC gene locus in chromosome 12, flanked by HOXC11 and HOXC12.[235] HOTAIR interacts with various chromatin modifying complexes such as PRC2 (polycomb repressive complex 2) and histone demethylases LSD1 (lysine specific demethylase 1).[131,133,240,241] PRC2 is a multiprotein complex containing subunit like EZH2 (a histone H3-lysine 27 (H3K27)-specific histone methylase), SUZ12, EED and RbAp48.[242,243,244,245] EZH2-mediated histone H3K27-methylation is associated with gene silencing.[246,247] Histone demethylase, LSD1 also interacts with transcription repressors such as REST (Repressor element-1 silencing transcription factor) and CoREST complexes.[248,249,250] Moreover, histone H3K4-demethylation is associated with gene silencing as well.[251,252] HOTAIR interacts with PRC2 and LSD1 through its 5'- and 3'-ends, respectively[133] and recruits them into their target gene promoters, that induce H3K27-methylation and H3K4-demethylation which results in suppression in gene expression.[131] Beyond its biochemical roles in gene silencing, HOTAIR appears to be overexpressed in various cancers

including colorectal cancer,[253] hepatocellular carcinoma,[254,255,256] pancreatic cancer,[257] non-small cell lung cancer,[258,259] and others.[260,261,262] HOTAIR expression is linked with tumor metastases and tumor growth. Loss of HOTAIR has been linked with decrease in cancer invasiveness, and HOTAIR overexpression leads to augmentation in cancer invasiveness and tumor growth.[263,264] HOTAIR regulates the expression of various tumor suppressor genes that include homeobox genes (such as HOXD10, HOXD8), protocadherin gamma subfamily A-8 (PCDHGA8), PCDHB5, and others. Overexpression of HOTAIR results in downregulation of tumor suppressor expression leading to tumor cell proliferation. Studies from our laboratory demonstrated that HOTAIR is crucial for cell growth and viability of breast cancer cells and its knockdown induces apoptosis in breast cancer cells.[130] We also demonstrated that HOTAIR is transcriptionally induced by estradiol (E2) and is disrupted by Bisphenol A (BPA) and Diethylstilbesterol (DES) in cultured breast cancer cells and *in vivo*.[88]

Hypoxic (low oxygen environment) tumor microenvironment is a critical driver of tumor cell proliferation, angiogenesis, and metastasis.[265,266,267] Under hypoxic conditions, transcription factors like hypoxia inducible factors (HIFs) are stabilized due to their inhibition of degradation via inhibition of ubiquitination. HIFs control the expression of a large number of genes that play critical roles in various oncogenic pathways such as regulation of proliferation, apoptosis, tumor metabolism, epithelial-to-mesenchymal transition, invasiveness, and pH regulation.[268,269,270,271,272] As HOTAIR is closely associated with tumor growth and metastasis,[131,273,274] we explored if HOTAIR expression is transcriptionally regulated under hypoxic environment and if its expression is regulated by HIFs. Our studies indeed demonstrate that HOTAIR expression is induced upon hypoxia in different tumor cells and this is coordinated via HIFs and the MLL-family of histone methylases.

3.2. Experimental Procedure

3.2.1. Cell culture and hypoxia treatment

Human colon adenocarcinoma cells (CCL228), cervical cancer (HeLa), breast cancer (MCF7), neuroblastoma (SH-SY5Y) were obtained from American Type Cell Culture Collection (ATCC) and cultured in our laboratory as described in Chaper 2.[267,271,29,30,32,33,35,89,275,276] Cells were grown and maintained in Dulbecco's modified Eagle's media (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 0.1 mg streptomycin/mL. All cell lines were maintained in a humidified incubator with 5% CO_2 and 95% air at 37 °C.

For hypoxia treatment, CCL228 cells were grown up to 70–80% confluency in 100 x 20 mm cell culture plates. CCL228 cells were incubated for varying amounts of time (0, 2, 4, and 8 h) in a hypoxia chamber (Stem Cell Technologies) in an atmosphere of hypoxia (low oxygen concentration) by using a combination of gases made up of 0.5% O_2 and 5% CO_2 balanced with N_2 gas (94.5%). Plates were also incubated under normoxic conditions (normal oxygen concentration) in the presence of 21% O_2 and 5% CO_2. At the indicated time points, the cell culture plates were immediately removed. The media was quickly aspirated from each cell culture dish and washed with PBS, and cells were harvested for RNA and protein extraction.

For hypoxia mimetic experiments, CCL228 cells were grown under standard conditions in DMEM. Cells were seeded in 60 x 15 mm plates and treated with varying concentrations of $CoCl_2$ (Sigma-Aldrich, St. Louis, MO) followed by incubation at 37 ˚C in the presence of 5% CO_2 for varying time periods. Control cells were treated with equivalent amounts of DMSO (the vehicle for $CoCl_2$ solution). The cells were harvested for total RNA extraction and subjected to RT-PCR and RT-qPCR analysis.

3.2.2. RNA extraction, cDNA synthesis, RT-PCR, and real-time PCR (qPCR)

For the RNA extraction, similar protocols were followed that were described in Chapter 2.[33,267,271,275] In brief, cells from normoxia and hypoxia environments were harvested, centrifuged at 1800 x g for 5 min at 4 ºC, and then 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 dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] for 10 min on ice. Cells were centrifuged at 8000 x g for 5 min at 4 ºC, and the supernatant was subjected to phenol-chloroform (1: 1 phenol and chloroform saturated with 1X TE) extraction. The aqueous layer was mixed with 2.5 volumes of ethanol, incubated at -80 ºC for 4 h and then centrifuged at 20000 x g for 30 min at 4 ºC. The RNA pellets were air dried, dissolved in DEPC treated water containing 0.5 mM EDTA, and quantified using a nanodrop spectrophotometer.

For cDNA synthesis, 2.4 µM of oligo dT (Promega) was mixed with 500 ng of the RNA extracted in a 12 µL total volume and incubated at 70 °C for 10 min. The mix was then added to a cocktail of 100 units of MMLV reverse transcriptase (Promega), 1X first strand buffer (Promega), 100 µM dNTPs (each), 1 mM DTT, and 20 units of RNaseOut (Invitrogen) and the volume was made up to 25 µL (using DEPC treated water). This mixture was incubated at 37 °C for 1 h for the reverse transcription. Each cDNA product was diluted to 100 µL, and 5 µL of the diluted cDNA was subjected to PCR amplification using specific primer pairs described in Table 3.

In general, PCR reactions were carried out for 31 cycles (30 sec at 94 ºC for denaturation, 30 sec at 60 ºC for annealing, 45 sec at 72 ºC for elongation) and finally PCR products were analyzed in 1.5% agarose gel electrophoresis. For the qPCR reactions, 5 µL of diluted cDNA were mixed with 5 µL Sso EvaGreen supermix (Bio-Rad) and 2 µM of each primer and final volume was made up to 12 µL. PCR reactions were carried out in CFX96 real-time detection system (Bio-Rad) for 40 cycles (5 sec at 95 ºC for denaturation and 10 sec at 60 ºC for both annealing and elongation). Data analysis was performed

using CFX manager software (Bio-Rad). Each experiment was repeated three times with three replicates each time.

3.2.3. Protein extraction and Western blotting

For preparation of whole cell protein extracts, CCL228 cells were incubated in normoxic and hypoxic conditions for indicated time periods. The cells were harvested quickly and resuspended in whole cell extraction buffer [50 mm Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.05% NP-40, 0.2 mM PMSF, 0.5 mM DTT and 1X protease inhibitors cocktail] followed by incubation on ice for 25 min and then centrifuged at 15000 x g for 20 min at 4 °C. The supernatant containing the protein extracts was analyzed by SDS-PAGE and subjected to Western blotting using the alkaline phosphatase method.

3.2.4. Dual-luciferase reporter assay

HOTAIR promoter (+5 to -2050 bases) encompassing the HRE region at (-1131 bases) was cloned into a luciferase-based reporter construct (pGL3; Promega). CCL228 cells were grown up to 50% confluency, in 6-well cell culture plates and co-transfected with 1500 ng of HOTAIR full promoter-pGL3 construct and 150 ng of a reporter plasmid containing renilla luciferase (pRLTk, as an internal transfection control; Promega) using iFECT transfection reagent as previously described. After 24 h of transfection, cells were incubated for indicated amounts of time in normoxic and hypoxic conditions separately and then subjected to luciferase assay using dual-luciferase reporter assay kit (Promega). Luciferase activities were normalized to the renilla expression and plotted. Each treatment was performed in four parallel replicates and the experiment was repeated at least three times.[271]

3.2.5. Chromatin immunoprecipitation assay (ChIP)

ChIP assays were performed using MCF7 cells and EZ Chip™ chromatin immuno-precipitation kit (Upstate) as described in Chapter 2.[33,267,271,275] Briefly, CCL228 cells were incubated for indicated amount of time periods in normoxic (21% O_2, 5% CO_2) and hypoxic conditions (0.5% O_2, 5% CO_2, balanced with N_2 gas, 94.5%), fixed with 1% formaldehyde, and then sonicated to shear the chromatins (~ 300 bp long DNA fragments). The fragmented chromatin was pre-cleaned with protein-G agarose beads and subjected to immunoprecipitation using antibodies specific to HIF1α, HIF2α, MLL, MLL2, MLL3, MLL4, H3K4-trimethyl, acetylated histone, H3K27-trimethyl, p300, RNA polymerase II (RNAPII) and β-actin (control). Immunoprecipitated chromatins were de-proteinized with proteinase K and purified DNA fragments were obtained. The purified immunoprecipitated DNA samples were PCR amplified using various primers corresponding to an HRE and a non-HRE region of HOTAIR promoter (Table 3). Antibodies were purchased from commercial sources that are as follows: MLL1 (Abgent, AP6182a), MLL2 (Abgent, AP6183a), MLL3 (Abgent, AP6184a), MLL4 (Sigma, AV33704), HIF1α (Novus Biologicals, NB100-134), HIF2α (Novus Biologicals, NB100-122), H3K4-trimethyl (Upstate, 07-473), RNA pol II (RNAPII, Abcam, 8WG16), β-actin (Sigma, A2066), H3K27-trimethyl (EMD Millipore, 07-449) and p300 (N15, Santa Cruz Biotechnology, Sc584).

3.2.6. Antisense-mediated knockdown of HIF1α and MLL1

Antisense-mediated knockdown experiment:

Materials: 1X trypsin-EDTA solution (Sigma), DMEM without any supplements, DMEM media with 2X supplements (20% FBS, 2% L-glutamine, 2% penicillin and streptomycin), ERα and ERβ-specific antisense oligonucleotides (custom synthesized from IDT technologies), iFECT (K.D. medicals) were used for antisense mediated knockdown experiments.

Antisense oligonucleotides design

Antisense oligonucleotides interfere with mRNA stability, cause mRNA degradation, and translational arrest and that ultimately reduced the levels of target mRNA in cells. The antisense approach relies on antisense oligonucleotides to efficiently bind to target sequences and depends on antisense oligonucleotides length, sequence content, secondary structure, thermodynamic properties, and target accessibility.[277] The secondary and tertiary structural folding of mRNA/ncRNA molecule may render the RNA inaccessible to an antisense oligonucleotide. Even the sequences that may bioinformatically appear to be accessible may be involved in intra-molecular hydrogen bonding or stacking interactions, which would disrupt hybridization of an antisense oligonucleotide. Therefore predicting the accessible binding sites on a RNA molecule is important, to which the antisense oligonucleotides can hybridize and lead to RNase H mediate degradation. The length of antisense-oligonucleotide may vary from 10-25 nt. Most of our antisense-oligonucleotides were in the range of ~ 22 nt and the *Tm* (melting point) is ~ 42 °C. Phosphorothioate antisense oligonucleotides against HIF1α and MLL1 were designed using IDT DNA antisense design software.

Analyze the efficacy of antisenses in knocking down respective genes such as HIF1α, MLLs etc:

1. 2 x 10^5 CCL228 cells were seeded in 60 mm cell culture plates, grown for 2 generations in DMEM-F12 media with appropriate supplements, with 5 mL of DMEM-F12 with 10% FBS. The plates were incubated in 5% CO_2 incubator at 37 °C until the confluency in the plates reached 55-65%.

2. On the day of transfection, following solutions were prepared, Solution A: For each transfection, 1 µg - 9 µg of antisense oligonucleotides was diluted complementary to the HIF1α and MLL1 mRNA into 100 µL of FBS free DMEM-F12 without antibiotics or any supplements. Solution B: For each transfection, 6-8 µL of iFECT transfection reagent was diluted into 100 µL of FBS free DMEM-F12 without antibiotics and supplements. Solution A was added to solution B.

3. If more than one sample needs to be knocked down, using the same antisense oligonucleotide, one can prepare a master mix containing the components for all the knockdown plates at once.
4. The solution was mixed by pipetting and the mixture was incubated for 35 - 45 min at room temperature in dark.
5. The cells were washed containing plates 3 times with DMEM-F12 without serum, antibiotics and supplements.
6. For each transfection, 0.8 mL of FBS free and antibiotic free DMEM-F12 media was added to each tube containing the antisense/iFECT transfection reagent complex. The diluted complex was gently spread all over plates containing the washed cells and gently swirled for 2 times.
7. The cells were incubated for 24 h at 37 °C in a CO_2 incubator with 5% CO_2.
8. 1 mL of DMEM-F12 media containing 2 times the normal serum and antibiotic concentration was added without removing the transfection reagent.
9. The cells were incubated for an additional 24 - 48 h (varying time periods for the identifying the efficacy of the antisense mediated knockdown).
10. Cells were harvested (by trypsinization) and whole cell protein extracts were made to analyze the levels of HIF1α – knockdown levels using Western blot using HIF1α -specific antibody.
11. The most efficient antisense was used for further studies in understanding the roles of HIF1α/MLLs in hypoxia-induced HOTAIR expression.
12. mRNA was isolated from the harvested cells, reverse-transcribed and analysed by PCR or qPCR using primers specific to HOTAIR and GAPDH (control) (Table 3).

3.2.7. Statistical analysis

Each experiment was performed in two to three replicates and then cells were pooled (and treated as one sample) and subjected to RNA extraction, RT-PCR, and ChIP analysis; each experiment was repeated at least thrice ($n = 3$). For luciferase assay, each treatment was performed in replicates of four and the experiment was repeated at least twice. The real-time PCR analysis of such samples was performed in three replicate reactions and repeated; thus, there are three independent experiments ($n = 3$) in all. Normally distributed data was analyzed using student t-tests (SPSS) to determine the level of significance between individual treatments. The treatments were considered significantly different at $p \leq 0.05$.

Table 3. Nucleotide sequence of antisense and primers

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
PCR primers		
GAPDH	CAATGACCCCTTCATTGACC	GACAAGCTTCCCGTTCTCAG
HOTAIR	GGTAGAAAAAGCAACCACGAAGC	ACATAAACCTCTGTCTGTGAGTGCC
HIF-1α	CCACCTATGACCTGCTTGGT	TATCCAGGCTGTGTCGACTG
HIF-2α	AGCAGCTGGAGAGCAAGAAG	ATGGAAGAGAGAGGGGTGCT
HOXD8	TCAAATGTTTCCGTGGATGA	CGGGAAATTTGTCCTTGTTG
HOXD10	GCTCCTTCACCACCAACATT	GAACCTCAGGGTTCTCAACG
PCDHB5	GCAAAAGACCTGGGTCTTGGG	AACTGCACTGGATTTTCTAGTAAG
PCDHGA8	ATCGCGGTTCCTGGAGCACGT	GTCTGCACGTCCAGGGAGA
ChIP primers		
HOTAIR HRE	CCTCCTGTTAGCTTGTCTACC	CAGGCTGAATTTACAATCCA
HOTAIR non-HRE	GTCACCAAAAACAAAAACAAAAA	GCGAGTCAGAGTTCCCCACTG
Cloning primers		
HOTAIR promoter	GGTACCCTGTGAGGAGACAGCTG CTGGAC[a]	AAGCTTAGGCTGGAACAGATCCCAA ACAAA[a]
Antisense		
HIF-1α	GCCGGCGCCCTCCAT[b]	
MLL1	TGCCAGTCGTTCCTCTCCAC[b]	
Scramble	CGTTTGTCCCTCCAGCATCT[b]	

[a]cloning primers flanked by appropriate restriction sites.
[b]phosphodiester linkages replaced by phosphorothioate linkages.

3.3. Results

3.3.1. HOTAIR expression is upregulated under hypoxic environment

In an attempt to investigate if HOTAIR is transcriptionally regulated under hypoxic conditions, we exposed colon cancer cells (CCL228) to normoxic (21% O_2, 5% CO_2) or hypoxic (0.5% O_2, 5% CO_2, balanced with N_2 gas 94.5%) conditions, separately, for varying time intervals (0 - 24 h). RNA and proteins were isolated from the control and hypoxia-exposed CCL228 cells. RNA was reverse-transcribed and analyzed by RT-PCR and qPCR, and proteins were analyzed by western blot. These analyses show that HIF1α and HIF2α expression (both RNA and protein levels), that are well-known markers for hypoxia, are upregulated under hypoxic conditions in comparison to normoxia (Figures 10 A-C). Interestingly, along with HIFs, the expression of HOTAIR is also induced upon hypoxia treatment in a time-dependent manner. HOTAIR expression level was highest (~ 4.5 fold) at 8 h which started squelching upon increase in time of exposure to hypoxia (Figures 10A and 10B). In order to demonstrate whether this phenomenon was a common feature of tumor cells, we exposed different types of tumor cells to hypoxic conditions and then analyzed the expression of HOTAIR using RT-PCR and RT-qPCR. These analyses also demonstrate that HOTAIR expression is induced significantly in most of the tumor cells including in colon cancer (CCL228, 4.5 fold), cervical cancer (HeLa, 2.2 fold), breast cancer (MCF7, 3.5 fold), neuroblastoma (SH-SY5Y, 1.7 fold) cell lines (Figures 10D and 10E). Here, we have used CCL228 cells for following biochemical experiments.

In order to further verify the hypoxia-induced expression of HOTAIR, we treated CCL228 cells with a widely used hypoxia mimetic compound $CoCl_2$,[235,278,279 260,280,281] and analyzed its impacts on HOTAIR expression. Briefly, CCL228 cells were treated with varying concentrations of $CoCl_2$ for 48 h and the RNA was analyzed for the expression of HOTAIR, HIF1α, and HIF2α using RT-PCR and qPCR. Our analysis shows that $CoCl_2$ treatment leads to an increase in expression of HOTAIR along

with induction of HIF1α and HIF2α in a dose-dependent manner (Figures 11A and 11B). The HOTAIR expression reached to an optimum at ~ 150 μM. Time-dependent studies demonstrated that $CoCl_2$-induced transcriptional activation of HOTAIR reached to maxima at about 48 h post $CoCl_2$-treatment (Figures 11C and 11D). Collectively, based on the hypoxia (low oxygen) treatment or hypoxia mimetic ($CoCl_2$) treatment analysis, our studies demonstrate that HOTAIR expression is transcriptionally activated under hypoxic conditions.

HOTAIR is well-known to regulate several target genes associated with tumor suppression, cancer, and metastasis.[131,231,239] These include tumor suppressor homeobox genes (such as HOXD10, HOXD8), protocadherin gamma subfamily A-8 (PCDHGA8), PCDHB5, and others. HOXD10 functions as a tumor suppressor in variety of cancers that is inactivated through promoter hyper-methylation.[282] HOXD8 acts as a metastasis suppressor and loss of HOXD8 may give the colorectal cancer cells misinformation to recognize liver as an orthotopic organ.[237] PCDHB5 is involved in intercellular adhesion and signaling and is associated with breast carcinogenesis.[238] We analyzed the expression of these HOTAIR target gene expression levels under hypoxia and our analysis revealed that while the level of HOTAIR was upregulated under hypoxia, the expressions of HOTAIR-target genes such as HOXD10, HOXD8, PCDHGA8, and PCDHB5 were significantly repressed (Figures 12A and 12B). Similar to hypoxic conditions, $CoCl_2$ treatment also resulted in downregulation of HOTAIR target genes such as HOXD10, HOXD8, PCDHGA8, and PCDHB5 expression in CCL228 cells while the expression of HOTAIR expression was augmented (Figures 12C and 12D). As HOTAIR is a repressor, its over expression may be linked with the down-regulation of its target genes under hypoxia (Figure 12), though there may also be HOTAIR-independent mechanism of gene expression involved in those target gene expression. Together, these results support our observation that HOTAIR expression is induced under

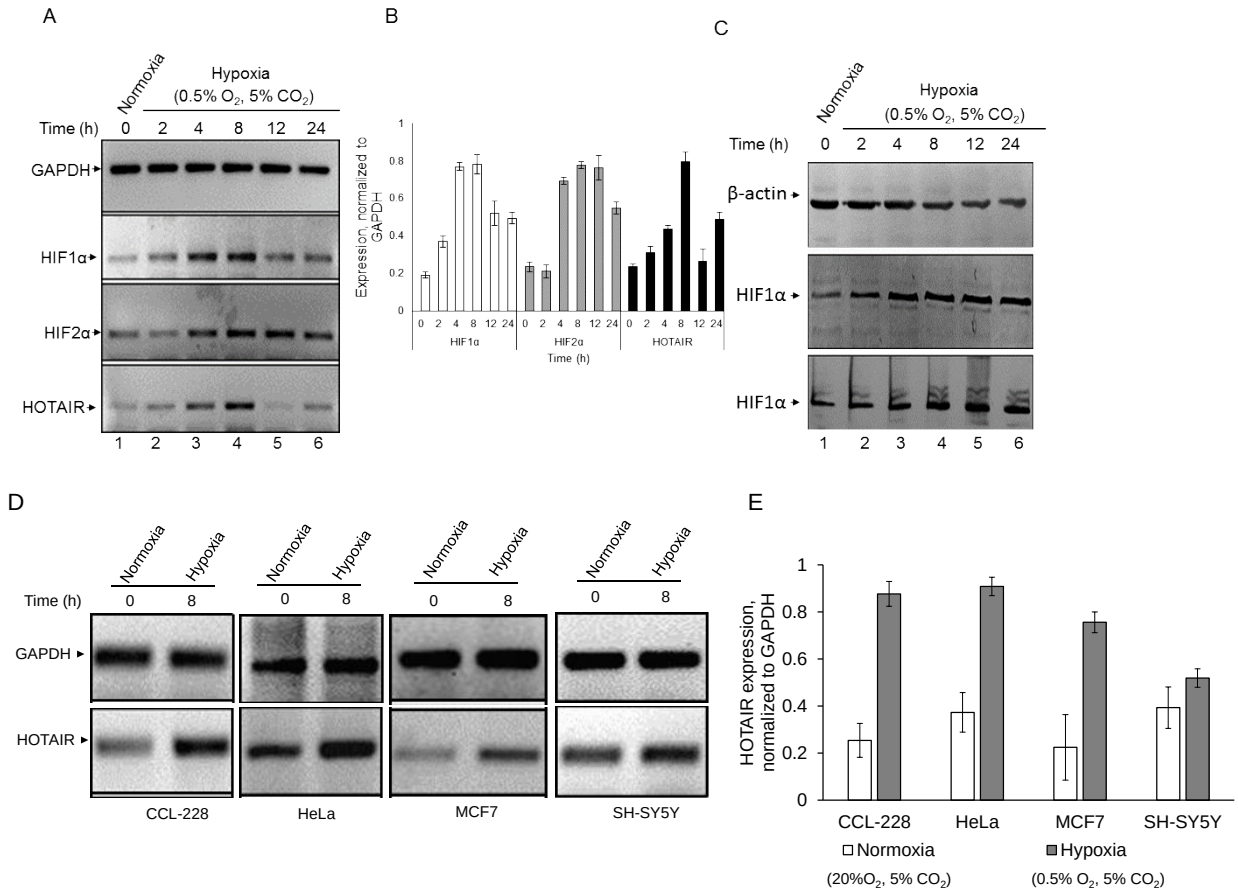


Figure 10. HOTAIR expression is induced by hypoxia in tumor cells.

(A-C**)** CCL228 cells were grown in DMEM media and exposed to hypoxic conditions (0.5% O_2, 5% CO_2, balanced with N_2 gas, 94.5%) for varying time periods (0- 24 h). RNA from the control and hypoxia treated cells were analyzed by regular RT-PCR using primers specific to HOTAIR, HIF1α and HIF2α. GAPDH was used as a loading control (panel A). qPCR analysis of HOTAIR, HIF1α and HIF2α expression, relative to GAPDH is in panel B. The expression of hypoxia induced HIF1α and HIF2α (protein levels) are analyzed by western blot (β-actin as loading control) and is shown in panel C. (D-E): CCL228, HeLa, MCF7 and SH-SY5Y cell lines were cultured under normoxic or hypoxic conditions (8 h). RNA was analyzed by RT-PCR (panel D) and qPCR (panel E) for HOTAIR gene expression. Each experiment was repeated for thrice with three parallel replicates ($n = 3$; $P \leq 0.05$).

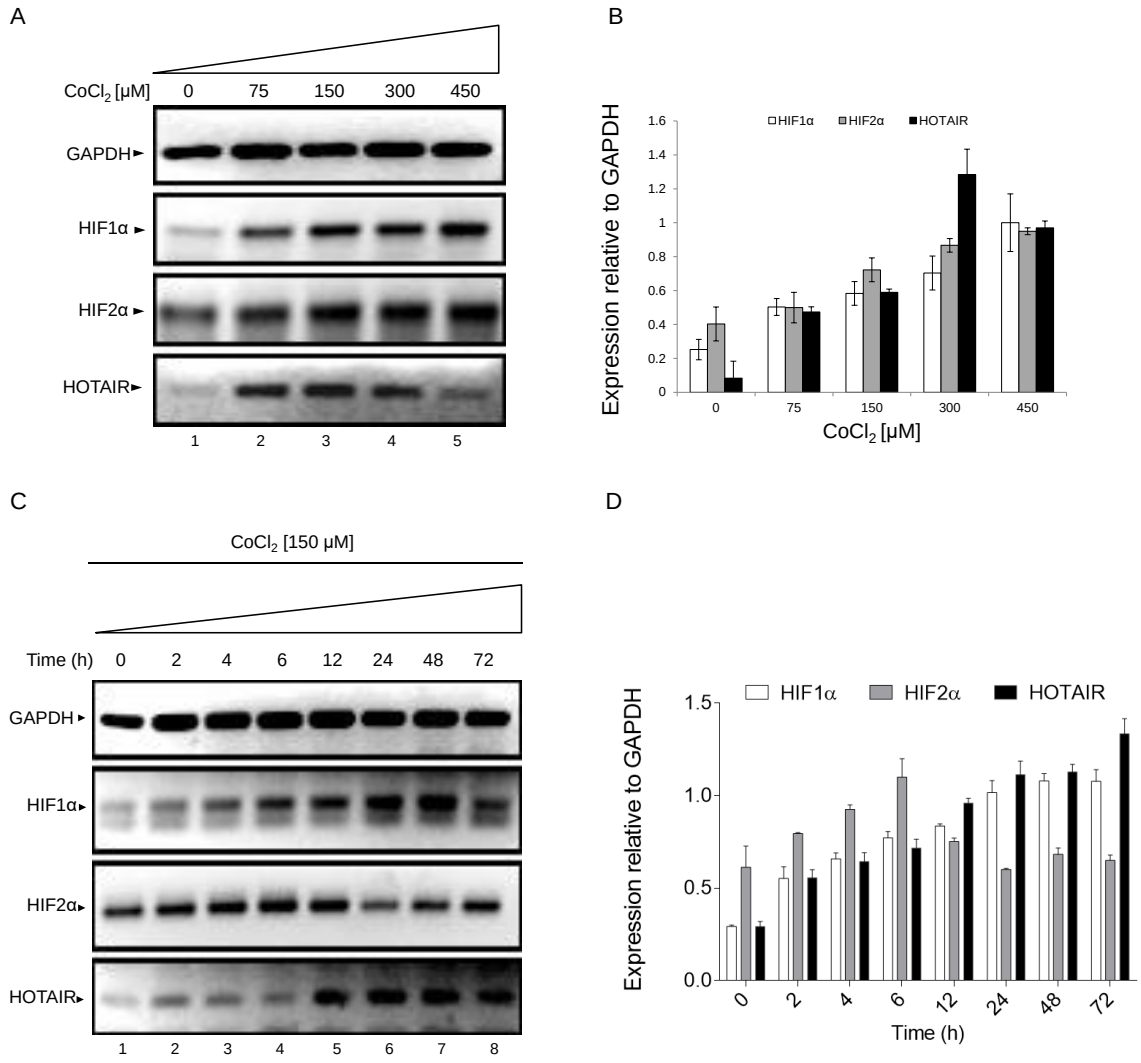


Figure 11. Effects of $CoCl_2$-treatment on HOTAIR expression.

(A-B) CCL228 cells were treated varying concentration of $CoCl_2$ (hypoxia mimetic compound) for 48 h. RNA was reverse-transcribed and analyzed by RT-PCR (panel A) and qPCR (panel B) with primers specific to HOTAIR, HIF1α, HIF2α and GAPDH (control). (C-D) CLL228 cells were treated with 150 µM of $CoCl_2$ for varying time points. RNA was analyzed by RT-PCR (panel C) and qPCR (panel D) for the expression of HOTAIR, HIF1α, HIF2α and GAPDH (control). Each experiment was repeated for thrice with three parallel replicates ($n = 3$; $P \leq 0.05$).

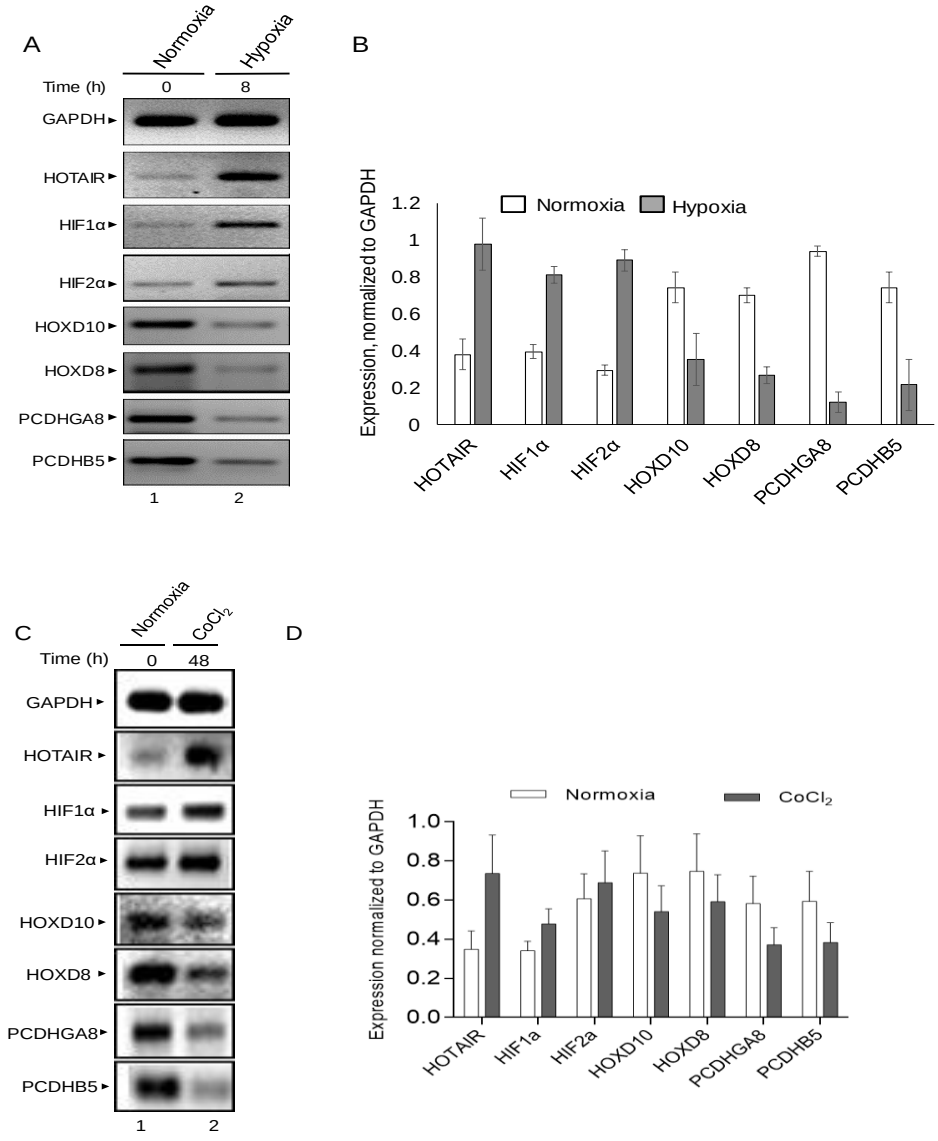


Figure 12. Hypoxia modulates the expression of HOTAIR-regulated target genes.

(A-D) CCL228 cells were cultured under normoxic or hypoxic conditions (8 h). RNA from the control and hypoxia-treated cells was reverse-transcribed and analyzed by regular RT-PCR (panel A) and RT-qPCR (panel B) using primers specific to HOTAIR, HIF1α, HIF2α, HOXD10, HOXD8, PCDHGA8, PCDHB5 and GAPDH (loading control). (C-D) CCL228 cells were treated with 150 μM $CoCl_2$ for 48 h and then harvested for total RNA extraction. RNA was analyzed by RT-PCR (panel C) and qPCR (panel D) using specific primers specific to HOTAIR, HIF1α, HIF2α, HOXD10, HOXD8, PCDHGA8, PCDHB5 and GAPDH (loading control). Each experiment was repeated for thrice with three parallel replicates ($n = 3$; $P \leq 0.05$).

hypoxia and HOTAIR overexpression may lead to its target gene silencing which may contribute to tumorigenesis.

3.3.2. HOTAIR promoter contains functional hypoxia response element

Hypoxia-inducible factors (HIFs) are known to stabilize under hypoxic condition. HIFs are transcription factors and regulate the expression of a variety of target genes under hypoxia and that contribute to the tumor growth and metastasis. Generally, HIFs binds to the hypoxia-response elements (HRE) present in promoters of target genes, recruits variety of activators and co-activators, that induces chromatin modification (histone methylation, acetylation, etc.) and chromatin remodeling, and that ultimately results in target gene activation.[133,240,241,261,262,263,264] To find out if the HOTAIR promoter contains potential hypoxia regulatory elements, we analyzed its promoter sequences and identified a potential HRE (hypoxia response element, ATCGTG) located at 1135 nt position and an HIF ancillary site (HAS; CACAG) located at 1110 nt upstream of the transcription start site (Figure 13A).[31,266,268,283,284,285] The HAS is located 16 nt downstream of the potential HRE and is an imperfect inverted repeat that recruits transcription factor complexes other than HIFs (Figure 13A).[286] The region containing both elements is considered as the typical HRE sequence.[287] The presence of the putative HRE motif at the HOTAIR promoter indicates its potential role in HIF-mediated HOTAIR gene regulation under hypoxic condition.

To functionally characterize the HRE motif, we cloned the HOTAIR promoter containing HRE region (+5 to -2050) into a luciferase based reporter construct, pGL3 (Figure 13A) and tested for its hypoxia responsiveness using luciferase-based reporter assay. Briefly, CCL228 cells were transfected with the HOTAIR promoter- pGL3 construct or with the empty pGL3 vector (with no promoter insert) and then exposed to hypoxia for 8 h. Cells were then harvested and luciferase activity driven by the HOTAIR promoter was measured. Notably, along with HOTAIR promoter-pGL3 constructs, a renilla

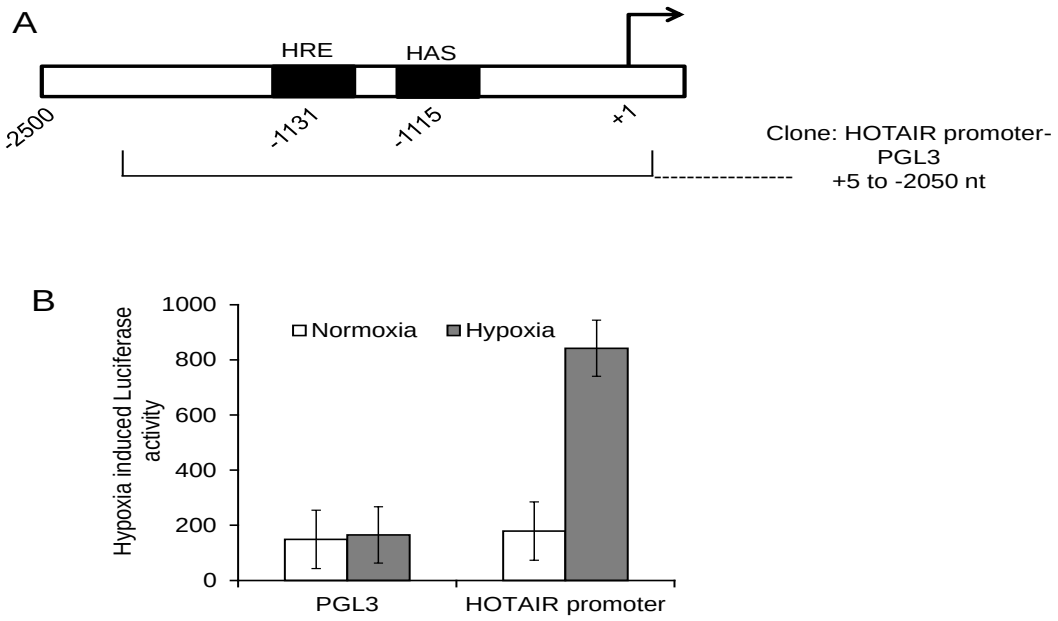


Figure 13. HOTAIR promoter HREs are responsive to hypoxia treatment.

(A-B) Schematic representation of HOTAIR promoter showing a potential HRE (hypoxia response element) sequence 5'-ATCGTG -3' spanning nucleotides -1141 to -1135 and an HIF ancillary site (HAS) 5'- CACAG -3' spanning nucleotides -1115 to -1110. HOTAIR promoter (spanning from +5 to -2050nt) containing HRE and HAS sequences was cloned into a luciferase-based reporter construct, pGL3, used for transfection into CCL228 cells and subjected to the luciferase assay (panel A) (B) HOTAIR promoter activity under both normoxic and hypoxic conditions were analyzed by luciferase assay. HOTAIR promoter-pGL3 or empty pGL3 (vector control) constructs were co-transfected into CCL228 cells separately for 24 h along with renilla luciferase construct (an internal transfection control) (panel B). Cells were then incubated for normoxic and hypoxic conditions separately and then subjected to luciferase assay using dual-Glo luciferase assay kit. The luciferase activities (normalized to renilla activity) were plotted. The experiment was repeated thrice with four parallel replicate. Bars indicate standard errors ($n = 3$; $P \leq 0.05$). Note: This experiment was performed by Dr. Arunoday Bhan in Dr. Mandal's lab at UT Arlington).[288]

luciferase vector was also co-transfected as an internal transfection control. As seen in Figure 13B, there was a strong transcriptional induction activity (~ 4.25 fold induction by hypoxia) from the plasmid containing the HOTAIR promoter (HOTAIR - pGL3), in comparison to empty pGL3-construct transfection (Figure 13B). These observations indicate that the HOTAIR promoter containing the HRE region is regulated by hypoxic condition.

3.3.3. HIF1α is essential for hypoxia-mediated upregulation of HOTAIR

To understand if HIFs are associated with transcription activation of HOTAIR expression in hypoxic cells, we examined the recruitment of HIFs (HIF1α or HIF2α) to the putative HRE region of the HOTAIR promoter under hypoxia using ChIP assay. In brief, CCL228 cells were exposed to hypoxic and normoxic (control) conditions for 8 h, separately. Cells were fixed with formaldehyde and chromatins were sonicated and immunoprecipitated using antibody against HIF1α, HIF2α, and β-actin (negative control). Immunoprecipitated DNA was analyzed by qPCRs using primers specific to an HRE and a non-HRE region, present in the HOTAIR promoter (Figure 14B and 14C). These analyses showed that there is a significant enrichment of HIF1α (~ 4 fold) in the HRE region of the HOTAIR promoter under hypoxic conditions. No significant enrichment of recruitment of HIF2α or β-actin (negative) was observed in the HRE region. Additionally, there was also no significant enrichment of HIF1α and HIF2α in non-HRE region in the HOTAIR promoter. These observations suggest that HIF1α specifically binds to the HRE region of the HOTAIR promoter under hypoxic conditions to induce HOTAIR gene activation under hypoxia.

To further explore potential roles of HIF1α in HOTAIR expression, we knocked down HIF1α in CCL228 cells and then anlyzed the hypoxia-induced expression of HOTAIR. Briefly, cells were transfected with varying concentrations of HIF1α-specific antisense oligonucleotide or a scramble antisense (control) for 48 h and then exposed to hypoxic conditions for 8 h. RNA and proteins from the

control and treated cells were analyzed by RT-PCR and western blot (Figures 15 A - C). HIF1α-antisense treatment knocked down HIF1α at both RNA and protein levels, without affecting expression of HIF2α (Figures 15 A - C). Interestingly, antisense-mediated knockdown of HIF1α suppressed the hypoxia-induced expression of HOTAIR levels (4 and 8 folds reduction in case of 2 μg and 5 μg antisense treatment respectively, Figure 15 A and 15B). HIF2α (as well as GAPDH and β-actin) was used as specificity control (Figures 15 A - C). The scramble antisense had no signficant impact on HIF1α and HOTAIR expression. These analyses demonstrate that HIF1α plays key roles in hypoxia-induced expression of HOTAIR in CCL228 cells.

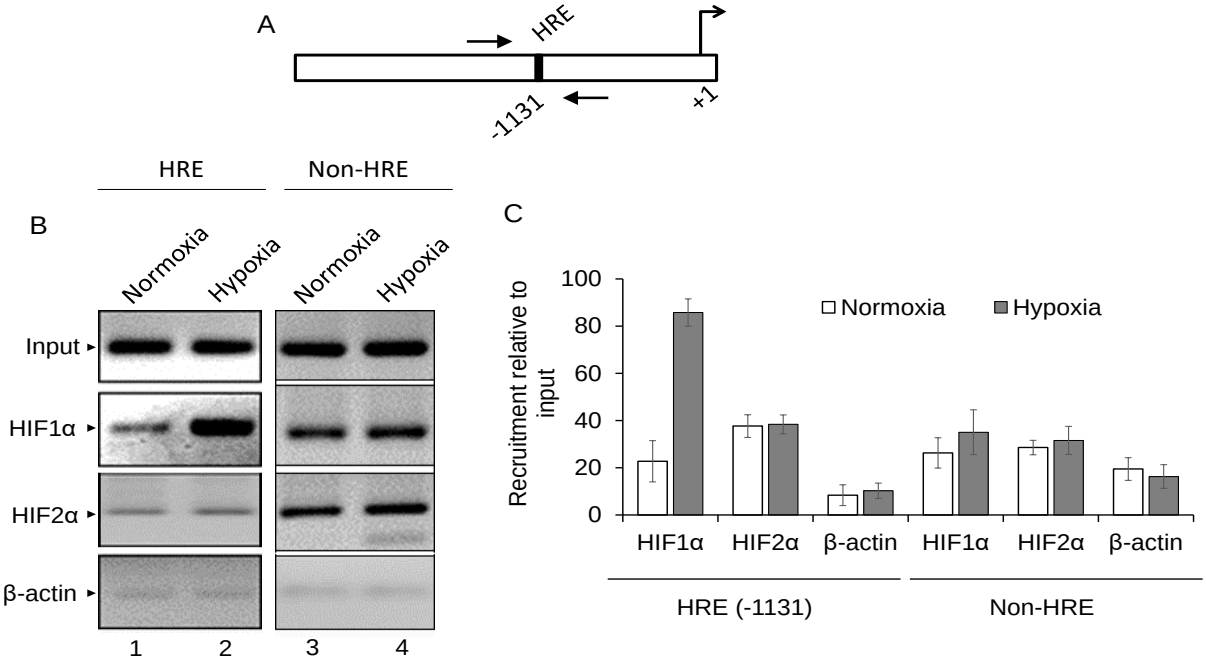


Figure 14. HIF1α binds to hypoxia-responsive elements of the HOTAIR promoter under hypoxic conditions.

(A-B) Schematic representation of the putative binding sites of hypoxia-responsive elements (HREs) in the HOTAIR promoter (panel A). (B-C) CCL228 cells treated with normoxia and hypoxia (8 h) were analyzed by ChIP using antibodies specific to HIF1α, HIF2α and β-actin (control). ChIP DNA fragments were PCR amplified using primers specific to HRE and non-HRE regions in the HOTAIR promoter. Panels B and C shows the regular PCR (agarose gel analysis) and qPCR analysis. The experiment was repeated thrice with four parallel replicate. Bars indicate standard errors ($n = 3$; $P \leq 0.05$).

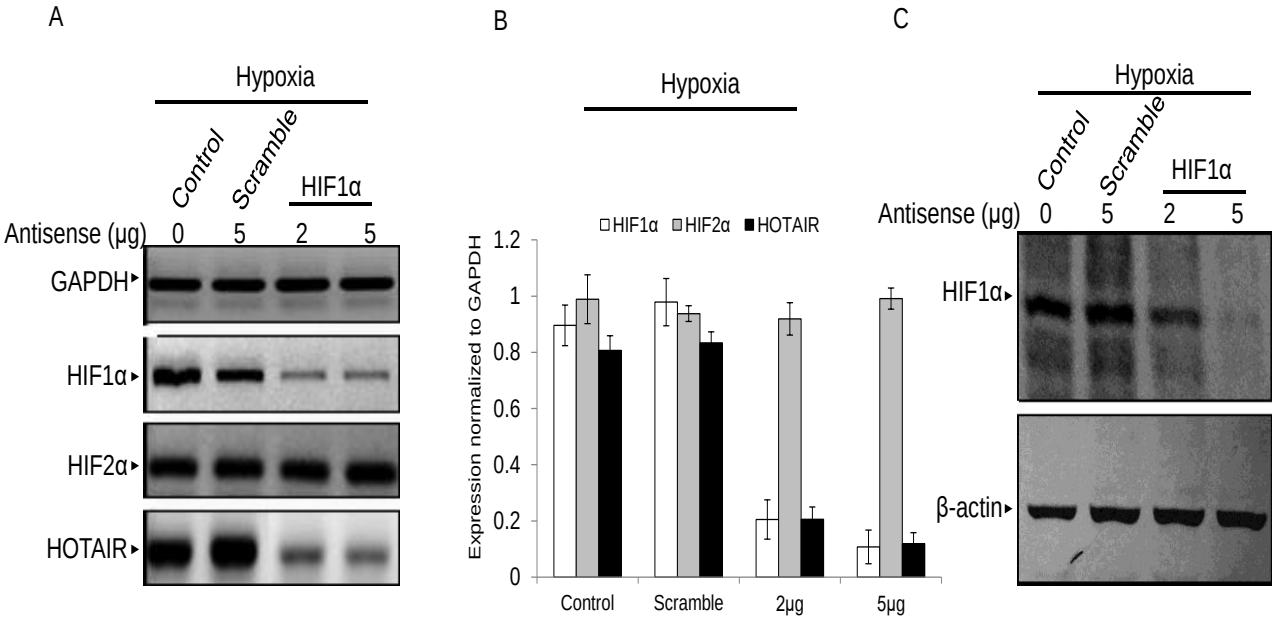


Figure 15. Effect of HIFs on the expression of HOTAIR in CCL228 cells under hypoxia.

(A-B) CCL228 cells were transfected with antisense oligonucleotide specific to HIF1α (2 μg and 5 μg) or scramble antisense (5 μg) separately for 48 h and treated with hypoxia (0.5% O_2) for additional 8 h. RNA was reverse-transcribed and analyzed by regular RT-PCR (panel A) and qPCR (panel B) using primers specific to HIF1α, HIF2α, HOTAIR and GAPDH (control). Bars indicate standard error (n = 3; P ≤ 0.05). (C) HIF1α protein levels were analyzed by Western blotting after HIF1α knockdown upon treatment with normoxic and hypoxic conditions (panel C). β-Actin was used as control.

3.3.4. Histone H3K4-methylases, and histone acetyl-transferases are enriched at the HOTAIR promoter under hypoxia.

HIFs are well known regulators of hypoxia-related genes.[269,270] Along with HIFs, various other transcriptional activators and coactivators are associated with hypoxia associated transcription activation. These include histone acetyl-transferases such as CREB-binding protein (CBP/p300), and other p160 steroid receptor coactivator (SRC) gene family homologs, SRC-1 and SRC-3, TIP60 and TIF-2.[275,289,290,291,292] Notably, along with histone acetylation, histone H3K4-trimethylation is also critical to gene activation. Mixed lineage leukemia (MLL) family of histone H3K4-specific methyl-transferases are well known players in basal gene regulation as well as in gene activation.[31,207,228,242,284,293,294] Recent studies from our laboratory demonstrated that MLL histone methylases are associated with hypoxia signaling, angiogenesis, and tumor growth.[228] In particular, MLL1 is enriched in hypoxic tumor tissue in the region where HIF1α is stablized and enriched.[228,295] MLL1 is also enriched in the basement membranes of blood vessels, indicating potential role of MLLs in hypoxia signaling and angiogenesis.[247,295] Therefore, to explore futher, we examined if MLLs are also invoved in hypoxia-induced activation of HOTAIR. Additionally, histone acetyltransferase, CBP/p300 have also been identified as interaction partners of MLL and p300 is known to be a transcriptional co-activator for HIFs.[289,296,297,298,299] Notably, MLL1 is shown to interact with CBP and this capacity is essential for its ability to activate transcription.[296] Therefore, to explore futher, we examined if MLLs and p300 are also invoved in hypoxia-induced activation of HOTAIR using ChIP assay. In brief, CCL228 cells were exposed to hypoxia (0.5% O_2) or normoxia, for 8 h and subjected to ChIP analysis using MLL1, MLL2, MLL3, MLL4, p300, and β-actin (control). The ChIP analyses showed that histone methylase, MLL1 and histone acetylase p300 are enriched in the HRE region of the HOTAIR promoter in a hypoxia-dependent manner (Figure 16B and 16C). Other MLLs (MLL2, MLL3 and MLL4) and β-

actin were not significantly enriched. These observations indicate that histone methylase, MLL1 and histone acetylase p300 are involved in hypoxia-mediated induction of HOTAIR via binding to its promoter in a hypoxia dependent manner.

H3K4-trimethylation and histone acetylation are key epigenetic modifications associated with transcriptional activation.[287,300,301,302,303] As MLL1 is a H3K4-methylase and p300 is a histone acetyl-transferase[286,304,305,306,307] and both are involved hypoxia-induced HOTAIR expression, we assessed the levels of histone H3K4-trimethylation and histone acetylation at the HOTAIR promoter under normixa and hypoxia using ChIP assay. Interestingly, we observed an elevation in the levels of H3K4 trimethyl and histone acetylation at the HOTAIR promoter (HRE region) under hypoxia while the levels of H3K27-methylation was decreased (Figure 16D and 16E). Notably, H3K27- methylation is critical roles to gene sielncing and a decrease in histone H3K27-methylation is associated with gene activation.[308] These findings suggest that hypoxia-induced H3K4-trimethylation and histone acetylation marks at the HOTAIR promoter which are introduced via associated histone methylases and acetylases respectively coordinate the process of hypoxic induction of HOTAIR expression.

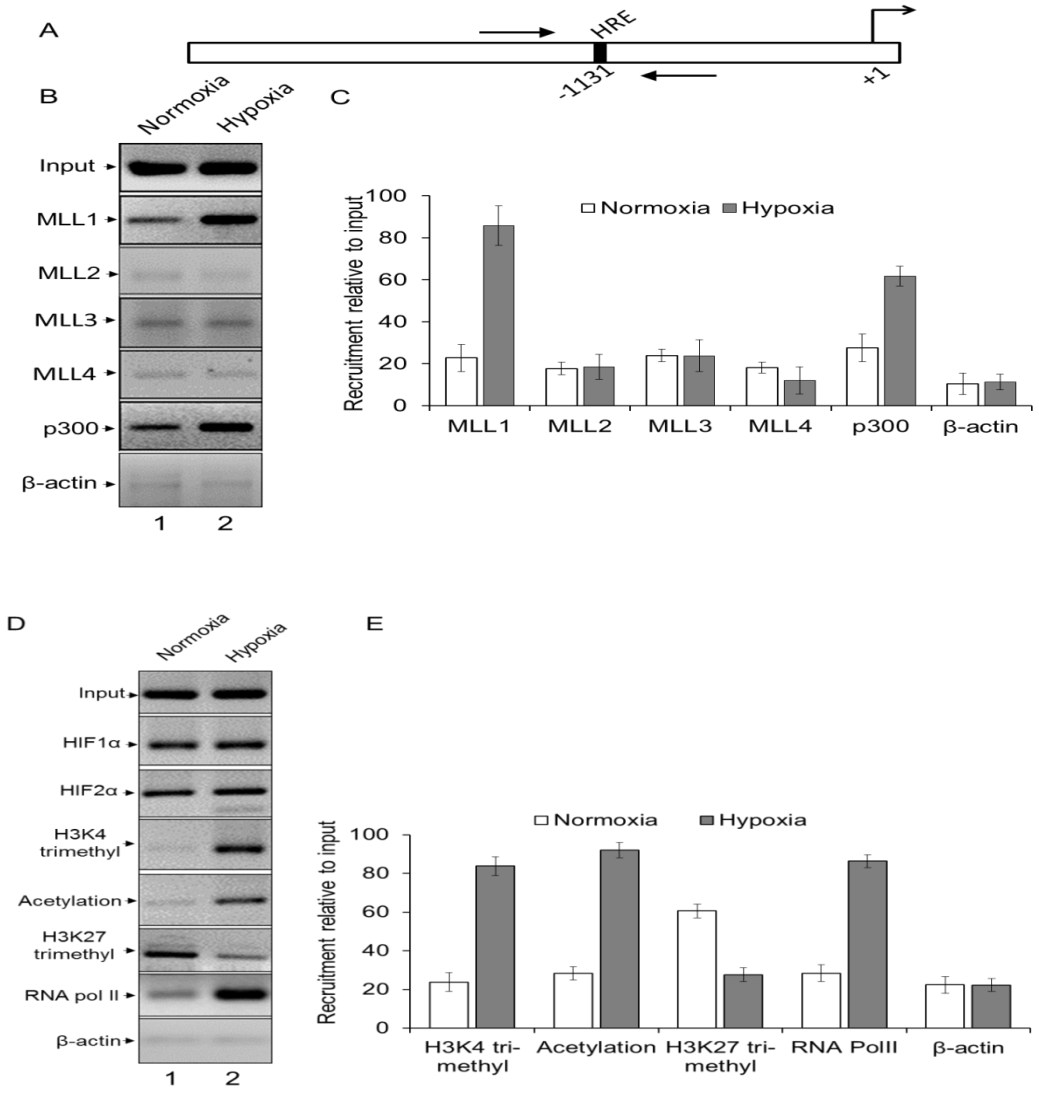


Figure 16. Hypoxia-induced recruitment of MLL-histone methylases and p300 at the promoter of HOTAIR.

(A-E) HOTAIR promoter showing the HRE location (panel A). (B-E) CCL228 cells were treated with normoxia and hypoxia (8 h) conditions separately and subjected to ChIP assay using antibodies specific to MLLs (MLL1–MLL*4*), p300, H3K4-trimethyl, histone acetyl, H3K27-trimethyl, RNAP II and β-actin (control). ChIP DNA fragments were PCR amplified using primers specific to the HRE region in the HOTAIR promoter. Panels B and C shows the agarose gel analysis and qPCR analysis of the ChIP DNA. (D-E) ChIP analysis of H3K4-trimethyl, histone acetyl, H3K27-trimethyl, RNAP II, and β-actin in the HOTAIR-HRE region (D: agarose gel analysis, E: qPCR analysis). Bars indicate standard errors ($n = 3$; $P \leq 0.05$).

3.3.5. MLL1 knockdown down-regulated hypoxia dependent HOTAIR expression

Since MLL1 was found to be recruited to the HOTAIR promoter, we examined if MLL1 is essential for HOTAIR expression in the presence of hypoxia. Therefore, we knocked down MLL1 in CCL228 cells using MLL1 specific antisense oligonucleotides (Table 3). MLL1-knocked-down cells were exposed to hypoxic or normoxic conditions independently for 8 h. RNA and proteins from the control and treated cells were analyzed by qPCR and western blot. These analyses demonstrated that application of MLL1 antisense (MLL1-AS) specifically knocked down MLL1 (Figure 17A). The knockdown of MLL1 decreased hypoxia induced expression of HOTAIR (Figure 17B). MLL1 knockdown shows minimal impact of the basal expression of HOTAIR under normoxia. These observations suggest that histone methylase MLL1 is essential player in hypoxia induced HOTAIR expression.

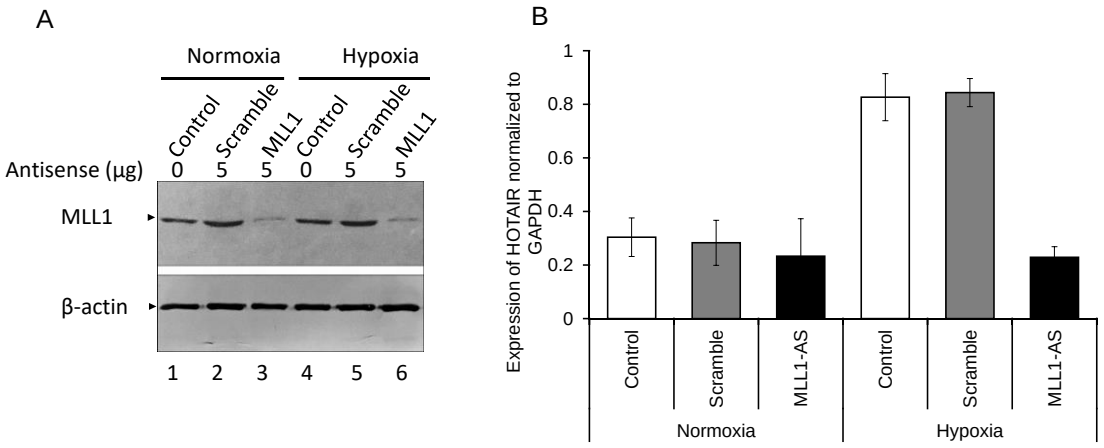


Figure 17. Roles of MLL1 in hypoxia-induced expression of HOTAIR.

(A-B) CCL228 cells were transfected with MLL1 or scramble antisense (5 μg each) separately for 48 h and exposed to hypoxic environment for 8 h. Whole cell proteins were isolated and subjected to western blot using MLL1 and beta-actin (control) antibodies (panel A). RNA was isolated and subjected to qPCR analysis using primers specific to HOTAIR and GAPDH. The levels of HOTAIR expression (relative to GAPDH) under normoxia and hypoxia is plotted in panel B (qPCR). Each experiment was repeated at least thrice (n = 3). Bars indicate standard errors (n = 3; $P \leq 0.05$).

3.4. Discussion

Hypoxic tumor microenvironment is a critical driver of tumor growth and metastasis.[309,310,311,312,313] Hypoxia inducible factors (HIFs) are a family of transcription factors which are stabilized under hypoxic tumor microenvironment due to their inhibition of degradation.[300] Under normoxia, alpha subunits of HIFs are hydroxylated by proline hydroxylase (PHD), which facilitate their polyubiquitination and subsequent degradation.[148,301,314,315] However, under hypoxic conditions, HIFs are not hydroxylated and therefore not degraded. The stabilized HIFs translocate to the nucleus, bind to hypoxia response elements (HREs) and initiate transcription of hypoxia-regulated genes including tumor growth and angiogenic factors.[291,316,317] HIF activation, in turn, leads to increased cell proliferation, angiogenesis, and tumor growth.[228,270,295] Recent studies suggest that, along with protein coding genes, noncoding RNA appears to be closely associated with tumorigenesis. Many ncRNAs especially lncRNA expressions are influenced by hypoxia. Examples of hypoxia-regulated lncRNA include H19, lncRNA-p21, noncoding ultra-conserved transcripts, lnc-RoR, urothelial carcinoma-associated-1 (UCA1) etc which are key players in variety of cancers.[296,297,318,319] Here, we have investigated the transcriptional regulation of an oncogenic lncRNA, HOTAIR which is overexpressed in multiple types of human carcinomas such as colorectal carcinomas, breast carcinomas, etc.[114,123,131,320]

HOTAIR lncRNA gene is located on chromosome 12 within the HOXC locus and regulates the expression of HOXD cluster genes present in the chromosome 2, in a *trans* regulatory fashion.[114,131,235,321] HOTAIR carries out the transcriptional repression of HOXD cluster of genes by interacting and guiding the gene silencing chromatin modifying complexes of PRC2 and LSD1 complexes to the target loci, leading to epigenetic modifications that are in turn associated with the process of transcriptional repression.[236,321] Recent studies from our lab demonstrate that HOTAIR is transcriptionally upregulated by estradiol (E2) and other endocrine disruptors such as bisphenol-A

(BPA) and diethylstilbestrol (DES) *in vitro* and *in vivo*.[88,130] The E2- mediated upregulation of HOTAIR is coordinated via ERs (estrogen receptors) and various ER-coregulators including MLL histone methylases. HOTAIR also regulates various tumor suppressor genes including PCDH10 (Protocadherin-10), PCDHB5 (Protocadherin β-5), JAM2 (Junction adhesion molecule 2) etc and thus is closely associated with the initiation and progression of tumors.[130,239,322,323] Here, our studies demonstrate that HOTAIR is transcriptionally induced upon hypoxic conditions in different types of tumor cells including colon cancer, breast cancer, cervical cancer, and neuroblastoma cells. HOTAIR induction was also observed under hypoxia mimetic condition such as under treatment with $CoCl_2$ (in CCL228 cells). Additionally, we also observed that while HOTAIR expression is induced upon hypoxia, several HOTAIR target genes and tumor suppressors such as HOXD10, HOXD8, PCDHGA8, and PCDHB5 were down-regulated under hypoxic conditions as well as under $CoCl_2$ treatment conditions. This could be due to the fact that HOTAIR recruits PRC2 and LSD1 complexes which introduce H3K27-trimethylation and H3K4-demethylation respectively at the above target gene promoters resulting in their gene silencing.

Normally, many hypoxia regulated gene expression are coordinated via the recruitment of HIF transcription factors which bind to the hypoxia response element (HRE) present in the target gene promoters. Promoter analysis show that HOTAIR promoter contains a putative hypoxia response element (HRE) within close proximity of the transcription start site (within -2000 nt) which indicates potential hypoxic regulation of HOTAIR expression. Luciferase-based reporter assay indeed shows that HOTAIR promoter is functionally activated upon hypoxia. Interestingly, CHIP analysis showed that HIF1α, which is a key regulator of hypoxia driven target gene expression, binds to the HRE region of the HOTAIR promoter. Furthermore, knockdown of HIF1α leads to downregulation of the hypoxia-

induced expression of HOTAIR. These observations demonstrated that hypoxia-induced HOTAIR expression is coordinated via HIF1α.

Notably, along with HIFs, a variety of other factors such as histone acetyl transferases and other coactivators play crucial roles in the hypoxia-dependent gene activation.[32,302,303] Many of these transcription activators and coactivators interact with HIFs, get recruited to the promoters of hypoxia regulated genes and remodel the chromatins leading to gene activation.[282,283,291,307,318,319] ChIP analysis indeed demonstrated histone acetyl-transferase p300 as well as the levels of histone acetylation are enriched at the HOTAIR promoter in a hypoxia-dependent manner. Similar to histone acetylation, histone H3K4-trimethylation is also associated with gene activation. MLLs are well known as human histone H3K4-specific histone methyl-transferases that are integral component of eukaryotic gene activation.[31,207,307] Here, our results demonstrated that along with HIF1α and p300, histone methylase, MLL1 is also enriched at the HOTAIR promoter under hypoxic condition. Histone H3K4-trimethylation and RNAPII levels are also increased at the HOTAIR promoter upon exposure to hypoxia. Knockdown of MLL1 also down regulated the level of hypoxia-induced HOTAIR expression. Notably, involvement of MLL1 in hypoxic tumor microenvironment is previously observed in our laboratory.[247,295] Therefore, the current observation that MLL1 play key roles in regulation of HOTAIR expression under hypoxia further strengthens our findings and hypothesis that MLL1 is a critical player in hypoxia signaling and especially in regulation of hypoxia-driven gene expression and tumorigenesis.

Overall, our studies demonstrated that HOTAIR is a hypoxia responsive gene and its expression is augmented under hypoxia in tumor cells. We demonstrate that along with HIFs, histone methyl-transferase (MLL1), and histone acetyl-transferase (p300) coordinate to induce hypoxia-dependent activation of HOTAIR. Notably, hypoxia-mediated activation of HOTAIR was recently observed in non-small cell lung cancer cells (NSCLC)[306] and thus, our observations about the hypoxia-induced

HOTAIR expression in colon cancer, breast cancer, cervical cancer and neuroblastoma are in agreement with the previous observation in lung cancer cells. Furthermore our observation demonstrate that histone methylase MLL1, which is enriched in hypoxic tumor tissue and associated with angiogenesis,[247,295] coordinates with HIF1α and plays a critical role in regulation of hypoxia-induced expression of HOTAIR. Thus, our studies further strengthen the significance HOTAIR and MLL1 in hypoxia signaling and tumor growth and they are potential targets for novel cancer therapy. A model showing the hypoxia-induced HOTAIR expression, roles of MLL1 and HIFs is shown in figure 18. Notably, HOTAIR is an antisense transcript and an lncRNA. Therefore, our studies also demonstrate that hypoxia can induce antisense transcripts, in a similar fashion as protein coding genes from the sense strands via coordination of HIFs and other transcription factors. As HOTAIR is a key player in a variety of cancers and hypoxia and it plays a major role in tumor growth, our studies showing the hypoxia-induced HOTAIR expression via coordination of HIFs, histone methylases and acetylases shed light on understanding the mechanism of HOTAIR expression in tumor cells, especially under the hypoxic tumor microenvironment.

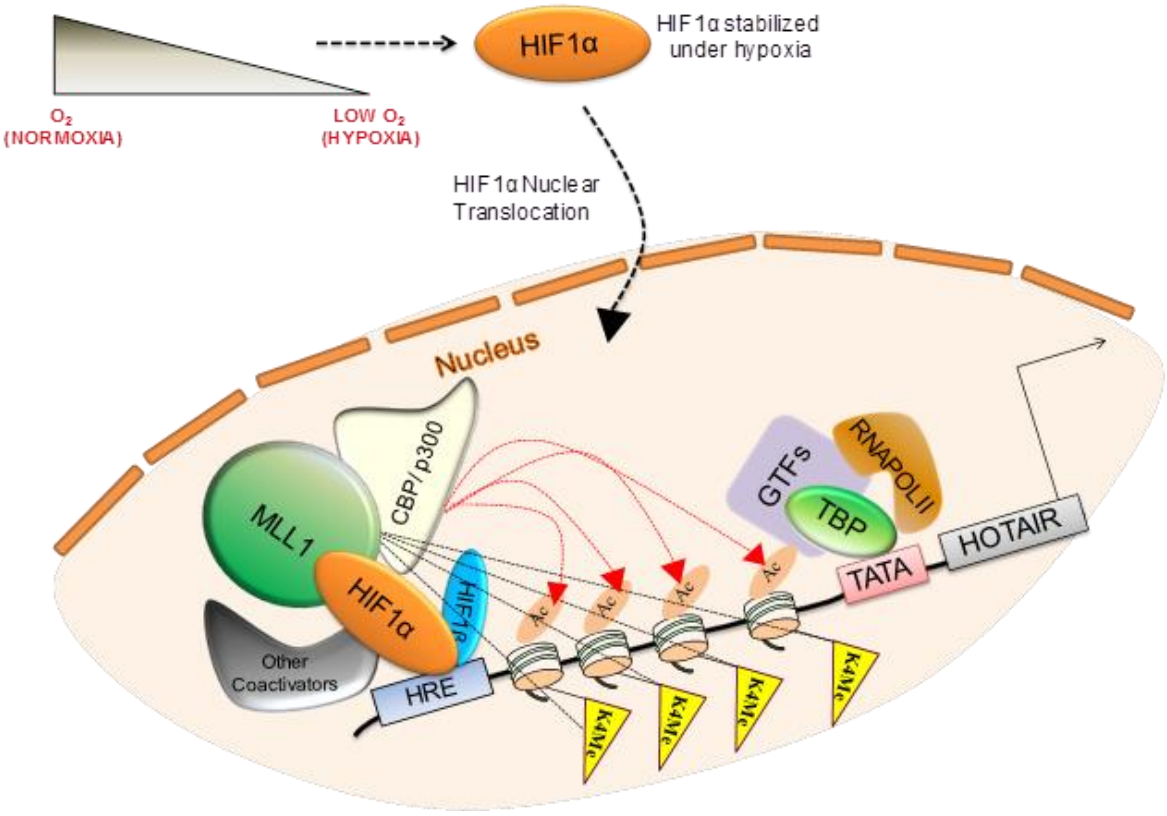


Figure 18. Mechanism of regulation of HOTAIR gene expression under hypoxia.

HIFs are stabilized under hypoxia, translocate to the nucleus, heterodimerise (HIF1α/β) and bind to HRE regions present in the HOTAIR promoter. Along HIFs, histone acetyl-transferase (CBP/p300), histone methylase MLL1 and other coactivators are enriched at the HOTAIR promoter. Promoter histones are methylated at H3K4 via MLL1 and acetylated via HATs such as p300 associated with HIFs, allowing access of RNA Polymerase II and other General Transcription Factors (GTFs) to the HOTAIR promoter, ultimately resulting in HOTAIR gene activation.

CHAPTER 4

TRANSCRIPTIONAL REGULATION OF BRAIN DERIVED NEUROTROPHIC FACTOR (BDNF) VIA LNCRNA HOTAIR AND ESTRADIOL

4.1. Introduction

Brain-derived neurotrophic factor (BDNF) is a critical player in neurogenesis and neuroprotection.[159] BDNF has survival and growth promoting actions on a variety of neurons, including dorsal root ganglion cells, hippocampal, and cortical neurons.[324,325,326] BDNF is a member of the neurotrophin family of growth factors along with nerve growth factor (NGF), neurotrophins-3 (NT-3), NT4/5 and NT-6. BDNF is synthesized in the endoplasmic reticulum (ER) as a 32–35 kDa precursor protein (pro BDNF) that moves through the Golgi apparatus and trans-Golgi network (TGN).[159] In the presence of lipid raft associated sorting receptor carboxy peptidase E (CPE), pro-BDNF is sorted by vesicles and subsequently transported into activity-dependent secretion by post-synaptic dendrites.[327] The terminal domain of pro-BDNF is cleaved by a distinct protein convertase enzyme to form 13 kDa biologically active mature BDNF (mBDNF).[328,329,330] The BDNF gene consists of nine 5' noncoding exons each linked to individual promoter regions, and a 3' coding exon (IX), which codes for the BDNF preprotein amino acid sequence.[331,332] The neuronal activity-dependent regulation of specific promoter regions within the BDNF gene dictates the spatial and temporal expression of specific BDNF transcript isoforms which determine tissue-specific expression of the BDNF transcript.[333,334] Human BDNF structure is closely related to rat and mouse BDNF. Eight distinct mRNAs are transcribed, with transcripts containing exons I–III expressed predominantly in brain and exon IV found in lung and heart. In situ hybridization experiments have revealed that BDNF mRNA is strongly expressed in the brain. The BDNF expression levels are low during fetal development, markedly increase after birth, and then

decrease in adults. There are two types of receptors for neurotrophins, either tropomyosin-kinases (trk) or the p75 receptor.[335] BDNF and NT-4 bind to trkβ, whereas all neurotrophins bind to p75. Full-length trkβ receptors autophosphorylate in response to ligand activation, and this, in turn, activates numerous signal cascades.

Several factors regulate the expression of BDNF gene. Significantly, compounds that influence or alter neural development exert a profound effect on the expression of this gene and protein. These include teratogens such as cocaine, alcohol and nicotine,[336,337,338] steroid hormones such as glucocorticoids and estrogen, calcium and other signaling molecules such as cAMP.[339,340,341,342,343] BDNF expression is also influenced by other factors that include light, osmotic stimuli, electrical stimuli, and estrous cycle hormones.[344,345,346,347] Hormonal status can greatly influence the expression of BDNF and/or trkβ expression.[348] Estrogen replacement in young adult, ovariectomized, female rats increases BDNF expression in the olfactory bulb, hippocampus, cortex, amygdala, septum, dorsolateral area of the bed nucleus terminalis and the lateral habenular nucleus.[349,350] The mouse BDNF gene contains a sequence, GGACACAAGTGACC with close homology to the estrogen response element, GGTCAnnnTGACC and estrogen-ligand complexes are capable of binding this sequence and mediate transcriptional regulation.[351] Abnormal levels of BDNF expression causes a variety of neurological disorders.[326] For example, decreased BDNF expression is associated with Alzheimer's disease, Parkinson's disease, and Huntington's disease (HD).[157,352,353,354,355] Increased expression of BDNF is linked with seizure and epilepsy.[356,357] However, investigations into the transcription-regulating mechanisms mediating changes in BDNF gene expression in neurological disorders are lacking, partly because of the complex structure of the BDNF gene. Here, we have investigated the epigenetic mechanisms of BDNF gene regulation. Our study shows that BDNF expression is repressed by a long non-coding RNA HOTAIR (HOX antisense intergenic RNA). HOTAIR that interacts with histone

methylase PRC2 (polycomb repressive complex 2) and histone demethylase LSD1 (lysine specific demethylase 1, that interacts with REST), recruits them to target gene promoters leading to gene silencing.[129,133] We also observed that knockdown of HOTAIR- upregulates BDNF expression in hippocampal neuronal cells (HT22). Alternatively, BDNF expression is also induced by estradiol (E2) which is known to stimulate neuronal repair and regeneration.

4.2. Experimental procedure

4.2.1. Cell culture

HT 22 cells, a subclone of the original clone HT4,[358] are immortalized hippocampal cells that have been characterized previously.[359,360] HT22 cells were cultured in cell culture plates (100 x 20 mm^2) with Dulbecco's modified Eagle's media (DMEM; Sigma–Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma–Aldrich), 2 mM L-glutamine and penicillin–streptomycin (100 units and 0.1 mg/ml, respectively). Cells were cultured and maintained at 37 ^oC in a humidified incubator (5% CO_2 in 95% air).

4.2.2. Treatment with 17β-estradiol

Treatment of HT22 with E2 was carried out as previously described by our laboratory.[88,185,361,362] In brief, HT22 cells were grown in phenol red-free DMEM-F12 media containing 10% charcoal stripped FBS, 2 mM L-glutamine and 100 units/mL penicillin and 0.1 mg/mL streptomycin for at least three generations. Cells were then grown in 60 mm cell culture plates overnight (60-70% confluency), then HT22 cells were treated with varying concentrations of E2 (0 - 1000 nM) for 6 h. The control (treated with diluent DMSO) and treated HT22 cells were harvested for RNA/protein extraction as well as for ChIP assays as needed.[35,194,228,293,363,364]

4.2.3. Treatment with EZH2 Inhibitor

HT22 cells were plated in 6-well plates at an initial seeding density (~ 50%) to ensure cell densities were within linear log phase growth upto day 4 of the time course. Cells were treated with either DMSO or varying concentrations of EPZ005687 (Chemietek).[365] Cells were harvested after 4 days and assayed for RNA/protein extraction.

4.2.4. RNA extraction, cDNA synthesis, RT-PCR and RT-qPCR

For the RNA extraction, similar protocols were followed that have been described in Chapter 3 and 4.[33,267,271,275] In brief, HT22 cells were either treated with antisense-oligonucleotides for 48 h or with E2 for 6 h and harvested, centrifuged at 1800 x g for 5 min at 4 ºC, and then 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 dithiothreitol (DTT), and 0.2 mM PMSF] for 10 min on ice. Cells were centrifuged at 8000 x g for 5 min at 4 ºC, and the supernatant was subjected to phenol-chloroform (1:1 phenol and chloroform saturated with 1X TE) extraction. The aqueous layer was mixed with 2.5 volumes of ethanol, incubated at -80 ºC for 4 h and then centrifuged at 20000 x g for 30 min at 4 ºC. The RNA pellets were air dried, dissolved in DEPC treated water containing 0.5 mM EDTA, and quantified using a nanodrop spectrophotometer.

For cDNA synthesis, 2.4 µM of oligo dT (Promega) was mixed with 500 ng of the RNA extracted in a 12 µL total volume and incubated at 70 °C for 10 min. The mix was then added to a cocktail of 100 units of MMLV reverse transcriptase (Promega), 1X first strand buffer (Promega), 100 µM dNTPs (each), 1 mM DTT, and 20 units of RNaseOut (Invitrogen) and the volume was made up to 25 µL (using DEPC treated water). This mixture was incubated at 37 °C for 1 h for the reverse transcription. Each cDNA product was diluted to 100 µL, and 5 µL of the diluted cDNA was subjected to PCR amplification using specific primer pairs described in Table 4.

In general, PCR reactions were carried out for 31 cycles (30 sec at 94 ºC for denaturation, 30 sec at 60 ºC for annealing, 45 sec at 72 ºC for elongation) and finally PCR products were analyzed in 1.5% agarose gel electrophoresis. For the qPCR reactions, 5 µL of diluted cDNA were mixed with 5 µL Sso EvaGreen supermix (Bio-Rad) and 2 µM of each primer and final volume was made up to 12 µL. PCR reactions were carried out in CFX96 real-time detection system (Bio-Rad) for 40 cycles (5 sec at 95 ºC for denaturation and 10 sec at 60 ºC for both annealing and elongation). Data analysis was performed using CFX manager software (Bio-Rad). Each experiment was repeated three times with three replicates each time.

4.2.5. Whole cell protein/histone extraction and Western blotting

For preparation of whole cell protein extracts, HT22 cells were either treated with antisense-oligonucleotides for 48 h or with E2 for 6 h. The cells were harvested quickly and resuspended in whole cell extraction buffer [50 mm Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.05% NP-40, 0.2 mM PMSF, 0.5 mM DTT and 1X protease inhibitors cocktail] followed by incubation on ice for 25 min and then centrifuged at 15000 x g for 20 min at 4 °C. The supernatant containing the protein extracts was analyzed by SDS-PAGE and subjected to Western blotting using the alkaline phosphatase method.

For histone extraction, HT22 cells were harvested and resuspended in extraction buffer [TEB: PBS containing 0.5% Triton X 100 (v/v), 2 mM phenylmethylsulfonyl fluoride (PMSF)] at a cell density of 10^7 cells/ml (5 mL). Cells were lysed on ice for 10 minutes with gentle stirring or by slow rotation at 4 ^{o}C and centrifuged at 2000 rpm for 10 min at 4 ^{o}C. The supernatant was removed and discarded. The pellet was resuspended in 0.2 N HCl (1.25 mL). The histones were acid extracted over night at 4 ^{o}C by slow rotation. Samples were centrifuged at 2000 rpm for 10 min at 4 ^{o}C. The supernatant containing histones was transferred into a fresh 1.5 ml tube and quantified for western blot analysis.[366]

4.2.6. Chromatin Immunoprecipitation Assay

For ChIP analyses, HT22 cells were either transfected with HOTAIR/scramble antisense oligonucleotide or treated with 10 nM/100 nM E2 for 6 h as described in Chapter 3. ChIP assays were performed using HT22 cells and EZ Chip™ chromatin immuno-precipitation kit (Upstate) as described in Chapter 3 and 4.[33,267,271,275] In brief, post-treatment, cells were crosslinked with 1 % formaldehyde for 10 min at 37 ^{o}C, washed twice in ice-cold PBS containing 1 mM PMSF; and 1 X protease inhibitor cocktail (Sigma-Aldrich) and harvested using SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1). The harvested cells were subjected to sonication to shear the chromatin (~150-450 bp in length). The fragmented chromatin was precleared with protein-G agarose beads and subjected to immunoprecipitation using antibodies specific to EZH2 (Active Motif; 39875), LSD1 (Active Motif; 61607), ERα (Santa cruz; 2Q418), ERβ (Santa Cruz; SC-8974), H3K4-trimethyl (EMD-Millipore; 07–473), RNAPII (Abcam; ab5408), MLL1 (Abgent; AO1146a), MLL2 (Bethyl laboratories; A300-113A), MLL3 (Abgent; AP6184a), MLL4 (Sigma; AV33704), CBP (Santa Cruz,sc-369), p300 (Santa Cruz; sc-585), histone-acetylation (EMD-Millipore; 06–599) and β-actin (Sigma, A2066). Immunoprecipitated chromatin was washed, decrosslinked and deproteinized at 65 °C in presence of 5 M NaCl, followed by incubation with proteinase K (Sigma) at 45 °C for 1 h. The ChIP chromatin was purified. Purified ChIP DNA was PCR amplified using primers spanning the different regions present in the BDNF promoter (Table 4).The analysis of ChIP DNA was carried out via regular PCR and qPCR amplifications.

4.2.7. Antisense-mediated knockdown of HOTAIR

For HOTAIR knockdown experiments, initially, a synthetic oligonucleotide DNA that is complementary to antisense HOTAIR transcript was designed using the IDT-DNA antisense design software and was named as small interfering sense (siSENSE) oligonucleotide. HOTAIR siSENSE sequence was selected based on about 50% GC content and a melting temperature of 40 °C and were

modified synthetically by replacement of regular phosphodiester bonds with phosphorothioate linkages. Approximately, 24 h before transfection, HT22 cells were plated in 2.5 ml complete growth medium per well in a 6-well plate. HT22 cells were grown up to ≥80% confluent prior to transfection confluency and transfected with mouse HOTAIR and scramble sISENSE (no homology to HOTAIR) antisense oligonucleotides independently (Table 4) using TransIT-Oligo Transfection Reagent reagent (Mirus Bio).[367,368] Prior to transfection, a transfection reagent mix was made by adding Opti-MEM Reduced-Serum Medium and warm TransIT-Oligo Reagent in a sterile tube according to manufacturer's instruction. The mixture was pipetted gently to mix completely. 5-7 µL of antisense oligonucleotide (1µg/µl stock) was added in separate tubes for a final concentration of 5-7 µg per reaction mix. The mixture was pipetted again to mix completely and incubated at room temperature for 5-20 min. The TransIT-Oligo Reagent: oligo complexes was then added drop-wise to different areas of the wells and incubated for 48 h and cells were either fixed for chromatin immunoprecipitation or harvested for RNA/protein extraction.

4.2.8. Statistical analysis

Each experiment was performed in two to three replicates and then cells were pooled (and treated as one sample) and subjected to RNA extraction, RT-PCR, and ChIP analysis; each experiment was repeated at least thrice (n = 3). For luciferase assay, each treatment was performed in replicates of four and the experiment was repeated at least twice. The real-time PCR analysis of such samples was performed in three replicate reactions and repeated; thus, there are three independent experiments (n = 3) in all. Normally distributed data was analyzed using student t-tests (SPSS) to determine the level of significance between individual treatments. The treatments were considered significantly different at $p \leq 0.05$.

Table 4. Nucleotide sequence of primers

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
PCR primers		
mGAPDH	ACCCAGAAGACTGTGGATGG	CACATTGGGGGTAGGAACAC
mBDNF	CAGGTGAGAAGAGTGATGACC	ATTCACGCTCTCCAGAGTCCC
mHOXD10	GACATGGGGACCTATGGAATGC	CGGATCTGTCCAACTGTCTACT
mHOTAIR	TCCAGATGGAAGGAACTCCAGACA	ATAGATGTGCGTGGTCAGATCGCT
mHOXD3	GAGACCTGGCACTGGGAATA	TGAGTCCACCCTTGGTCTTC
ChIP primers		
mBDNF-HOT-L	GGTCTGAAATTACAAGCAGATGG	CTGGTGGAACTGTGGGAAG
mBDNF-HOTS	GCCACAGTGTGTTCACTTGC	TGTCAGTTACTGTGCTGTATGTGG
mBDNF-ERE1	TACCGGTTCATCATGTCCAA	CCTAAAGCTGTCTCCGCTGT
Antisense		
mHOTAIR	C*C*T*T*C*C*T*T*C*C*G*C*T*C*T*T *A*C*T*C*T	

*phosphodiester linkages replaced by phosphorothioate linkages.

4.3. Results

4.3.1. HOTAIR knockdown leads to increased BDNF expression

In order to understand the mechanism of BDNF expression, we examined its transcription regulatory mechanism under the basal condition in the absence of any external stimuli. In particular, we examined the function of a long non-coding RNA, HOTAIR and its interacting partner histone methylase EZH2 which are known players in gene silencing. As BDNF expression is known to be expressed in hippocampal neurons, we chose to use a mouse hippocampal neuronal cell line HT22 for our *in vitro* study. Initially, we knocked down HOTAIR in HT22 cells by transfecting a HOTAIR-specific antisense (HOTAIR-AS) followed by analyzing its impacts on BDNF expression.[185] Briefly, HT22 cells were transfected with varying concentrations of the HOTAIR- siSENSE oligonucleotide for 48 h. Cells were also transfected in parallel with a scramble-antisense that has no complementarity to HOTAIR RNA. RNA from the control, siSENSE/ scramble antisense-treated cells were isolated, reverse-transcribed and subjected to PCR-amplification and qPCR using primers specific to HOTAIR, BDNF and GAPDH (non-specific control) (Figure 18, Table 4). Our results showed that application of HOTAIR siSENSE knocked down effectively (~ 70% knockdown) HOTAIR transcript levels in a dose dependent manner, 9 μg siSENSE being the most effective in HOTAIR knockdown (Figure 19A and 19B). Interestingly, HOTAIR knockdown also resulted in increased BDNF mRNA (~ 3 fold). The induction of BDNF expression was also observed at the protein level under HOTAIR knockdown condition (Figure 19C). These observations indicate that lncRNA HOTAIR represses the BDNF transcription. Notably, HOTAIR is known to repress several target genes such as HOXD10 and HOXD3 that are associated neuronal differentiation.[369] Our RT-PCR and qPCR analysis also show that expression of HOXD3 and HOXD10 are augmented under HOTAIR knockdown conditions in HT22

cells. These observations further support that in addition to well known target genes of HOTAIR, BDNF expression is also regulated by HOTAIR in HT22 cells.

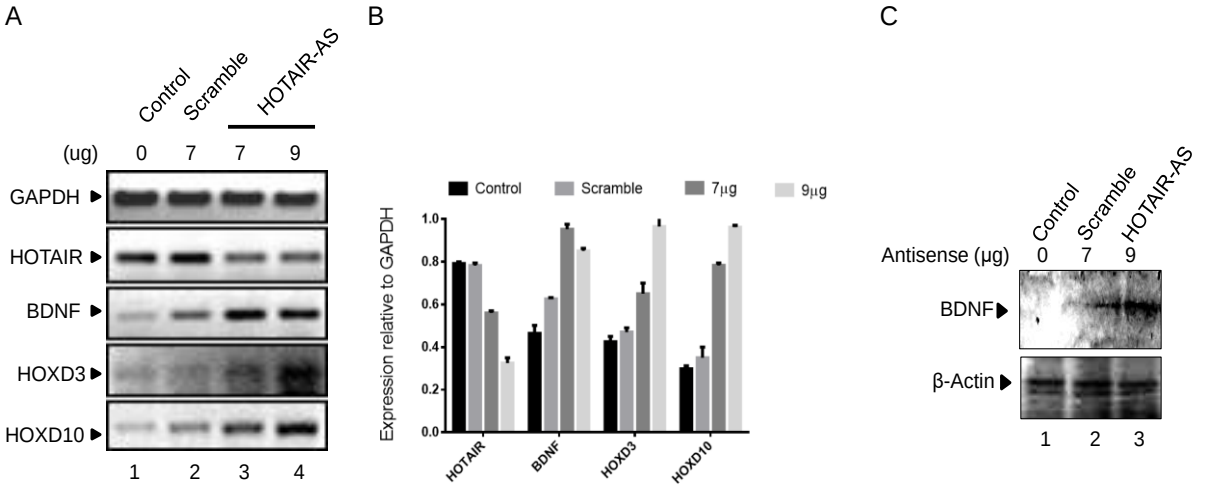


Figure 19. HOTAIR knockdown increases BDNF expression.

(A-C) HT22 cells were transiently transfected with antisense oligonucleotide specific to HOTAIR (7 µg and 9 µg) or scramble antisense (7 µg) separately for 48 h. RNA was reverse-transcribed and analyzed by regular RT-PCR (panel A) and qPCR (panel B) using primers specific to BDNF, HOTAIR, HOXD3 and HOXD10. GAPDH was used as an internal control. Western blot analysis was used to evaluate the expression of BDNF protein levels after HOTAIR knockdown (panel C). β-Actin was used as the internal control. Each experiment was repeated for thrice with three parallel replicates ($n = 3$; $P \leq 0.05$).

4.3.2. Inhibition of EZH2 induces BDNF expression

HOTAIR is a scaffold lncRNA that interacts with gene silencing factors PRC2 and LSD1 and aid their recruitment in target gene promoter inducing transcriptional repression. EZH2, an interacting component of PRC2, is a histone H3K27 specific methyl-transferase that plays key roles in gene silencing. To further understand the roles of HOTAIR in BDNF gene silencing, we inhibited HOTAIR interacting EZH2 using a commercial EZH2 inhibitor and then analyzed its impacts on BDNF and other HOTAIR target gene expression in HT22 cells. Briefly, we treated HT22 cells for 96 h with various concentrations of a potent and selective small-molecule inhibitor of EZH2, EPZ005687.[365] RNA and proteins were isolated from HT22 cells post-treatment with EPZ005687 targeting EZH2. RNA was reverse-transcribed and analyzed by RT-PCR and qPCR using specific primers such as GAPDH (control), BDNF and known HOTAIR target genes such as HOXD3 and HOXD10 (Table 4). These analysis demonstrate that the level of BDNF expression and HOTAIR target genes such as HOXD3 and HOXD10 are increased significantly (~ 5 fold) upon treatment with EZH2-inhbitor in a dose-dependent manner (Figures 20 A-D). Western blot analysis of histone protein from the EZH2-inhbitor treated cell demonstrates that EZH2 inhibitor application reduced the level of histone H3K27-trimethylation (Figure 20E). These observations suggest that similar to HOTAIR, EZH2 also plays key roles in BDNF gene silencing.

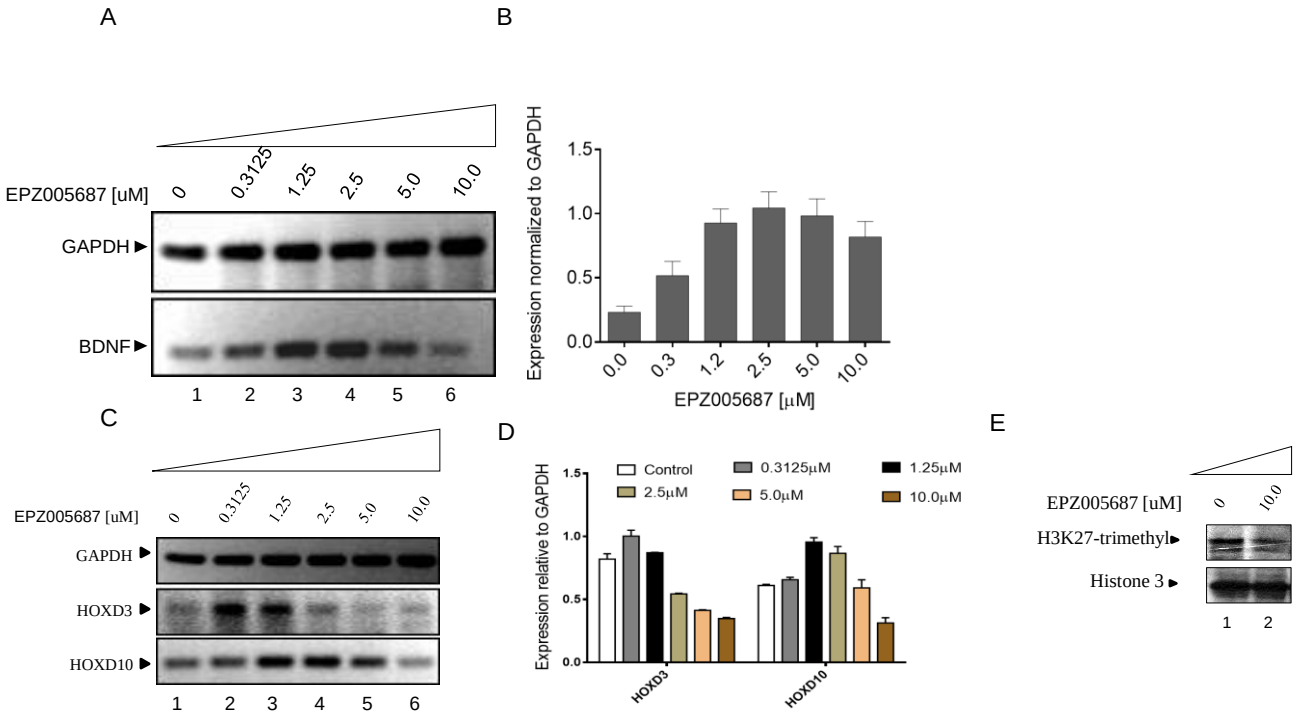


Figure 20. EPZ005687 induces transcriptional activation of BDNF in HT22 cell lines.

(A-E) HT22 cells were treated with varying concentrations of EZH2-inhibitor (EPZ005687) for 96 h. RNA from the control and treated (96 h) cells was reverse transcribed and analyzed by regular PCR and qPCR for expression using primers specific to BDNF, HOXD3, and HOXD10. GAPDH was used as loading control. Panels A and C show the agarose gel analysis wheras Panel B and D shows RT–qPCR analysis of gene expression following 96 h treatment with EPZ005687. Histones proteins were acid-extracted from HT22 cells after treatment with EPZ005687 for 96 h and were examined by Western blotting. HT22 cells show a dose-dependent decrease in H3K27me3 levels with increasing concentrations of EPZ005687 (panel E). Histone was used as a loading control. Each experiment was repeated for thrice with three parallel replicates (n = 3; $P \leq 0.05$).

4.3.3. PRC2 and LSD1 bind to the BDNF promoter in an HOTAIR dependent manner

In order to further understand the mechanism of HOTAIR/PRC2/LSD1-mediated BDNF gene expression, we analyzed the promoter of BDNF for the presence of putative binding sites of PRC2 and LSD1 subunits. Notably, SUZ12 is known to recognize a consensus 9 bp enriched motif of "NNGGANANN" termed as "HOT-S sites" for short whereas LSD1 is known to specifically bind to CG-rich motifs- "NCCNGN" termed as "HOT-L sites" at the promoter of target genes.

We analyzed the BDNF promoter sequences for the presence of potential HOT-S and HOT-L sites. This analysis revealed that along with various other transcription factor binding sites, BDNF promoter contains multiple putative HOT-S and HOT-L sites (Figure 21A). The presence of the multiple HOT-L and HOT-S sites at the BDNF promoter indicates potential roles of PRC2, LSD1 in BDNF gene regulation.

As HOTAIR is a common interacting and guiding partner for PRC2 and LSD1 complexes, we tested if silencing HOTAIR affects the binding of SUZ12 and LSD1 at the HOT-S and HOT-L sites at the BDNF promoter. In brief, we knocked down HOTAIR in HT22 by using a HOTAIR specific antisense (HOTAIR-AS) oligonucleotide and cells were subjected to ChIP assay using antibodies against EZH2, LSD1 and and β-actin (negative control). Immunoprecipitated DNA was analyzed by RT-PCR and qPCR using primers specific to HOT-L (located at 491 bp) and HOT-S site (located at 3394 bp) present in the BDNF promoter. These analysis showed that application of HOTAIR-AS knocked down the HOTAIR RNA level (~ 70%) in HT22 cells. Interestingly, we observed that EZH2 and LSD1 were bound at the BDNF promoter in the normal cells (in the absence of HOTAIR-AS) and their levels were significantly reduced at the BDNF promoter upon HOTAIR-knockdown (Figure 21B and 21C).

Notably, histone H3K4-methylation and histone acetylations are linked with gene activation while H3K27-methylation (which is introcued by EZH2) is linked with gene silencing. As BDNF expression is induced upon HOTAIR knockdown, we also examined the levels of H3K4-and H3K27-methylation and level of RNA polymerase II (RNAP II) enrichments at the BDNF promoter in the absence and presence of HOTAIR knockdowns using ChIP assay. These analsyis demonstrate that along with EZH2, H3K27-methylation levels is decreased at the BDNF promoter upon HOTAIR –knockdown while the levels of H3K4-trimethylation and RNAPII were enriched upon HOTAIR –knockdown (Figure 21B and 21C). These observations indicate that HOTAIR aids in recruitment of EZH2 and LSD1, which induces H3K27-methylation and H3K4-demthylation respectively at the BDNF promoter leading to BDNF repression. However, HOTAIR knockdown derecruits EZH2 and LSD1, which results in increased H3K4-trimethylation and RNAP II recruitment at the BDNF promoter resting in BDNF gene induction.

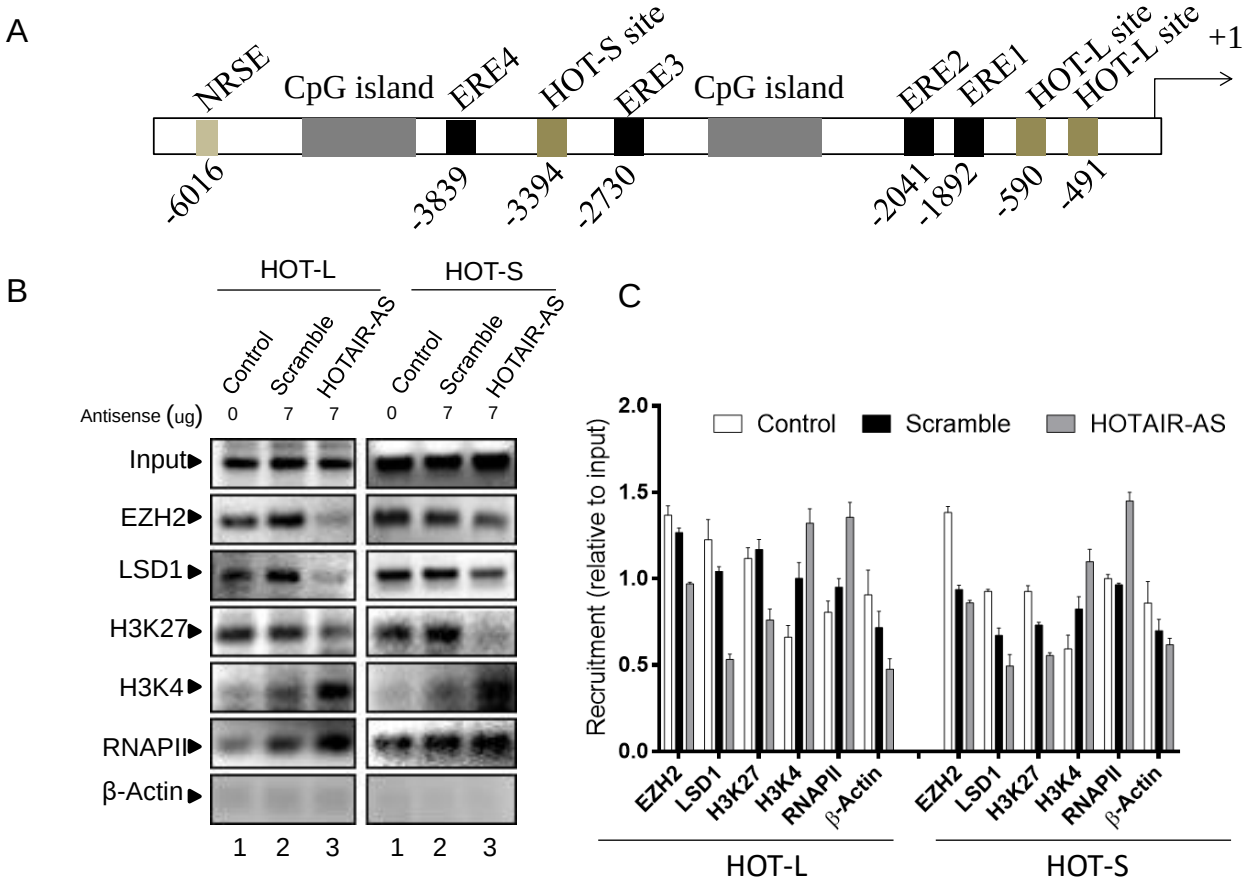


Figure 21. Derecruitment of LSD1 and EZH2 levels and other associated histone marks at the BDNF promoter upon HOTAIR knockdown.

(A-C) Schematic representation of BDNF promoter showing potential PRC/ Suz 12-binding (HOT-S) and LSD1-binding (HOT-L) sequence located at 491 nt and 3394 nt upstream of the transcription start site at the BDNF promoter (panel A) HT22 cells were transfected with antisense oligonucleotide specific to HOTAIR (7 μg) or scramble antisense (7 μg) separately for 48 h, fixed, sonicated and DNA fragments were subjected to ChIP assay using antibodies specific to EZH2, LSD1, H3K4-trimethyl, H3K27-trimethyl, RNAP II and β-actin (control). ChIP DNA fragments were PCR amplified (qPCR) using primers specific to HOT-S and HOT-L regions in the BDNF promoter (panels B and C). Panel B shows agarose gel analysis of EZH2, LSD1, H3K4-trimethyl, histone acetyl, H3K27-trimethyl, RNAP II, and β-actin recruitment in the HOT-S and HOT-L regions. Panel C shows qPCR data analysis. The experiment was repeated thrice. Bars indicate standard errors (n = 3; $P \leq 0.05$).

4.3.4. Estradiol induces BDNF expression in HT22 cells

Neuronal growth and maintenance is well known to be influenced by steroid hormones such as estradiol. Estradiol regulates the process of differentiation and plasticity of hippocampal neurons.[370,371,372,373,374] Studies indicate that the effects of estrogen are linked to the effects of BDNF. For instance, ovariectomy reduces BDNF mRNA and estradiol replacement restores it both *in vitro* and *in vivo*.[339,350,351,375,376,377] Therefore, in addition to investigating BDNF gene regulatory mechanism via lncRNA HOTAIR, we also examined if BDNF expression is transcriptionally regulated by estradiol in HT22 cells and also its mechanism of regulation. Briefly, we treated HT22 cells with various doses (0 to 1000 nM) of estradiol (E2) for 6 h. Cells were then harvested, RNA was isolated, reverse-transcribed and analyzed by qPCR for the expression of BDNF. Our results demonstrated that BDNF expression was stimulated by E2-treatment in a dose-dependent manner which peaked at 10 - 100 nM estrogen exposure (~ 2 folds each) (Figure 22A and 22B). Higher doses (1000 nM) of estradiol resulted in squelching (Figure 22A and 22B). Time course analysis demonstrated that E2-induced BDNF expression increases with time with a maximum at around 6 h, and this induction was decreased at longer incubation time, indicating squelching (Figure 22C and 22D). These results demonstrate that BDNF expression is transcriptionally regulated by estradiol in hippocampal cells.

4.3.5. ERs, MLL-histone methylases, and histone acetylases are enriched at the BDNF promoter upon E2-mediated BDNF gene activation

As BDNF is found to be an E2-responsive gene, we investigated the potential mechanism of E2-induced expression of HOTAIR. Initially, we examined the BDNF promoter for the presence of putative estrogen response elements (EREs) close to the transcription start site. Indeed, we found that BDNF promoter contains a putative ERE which is located at -1892bp upstream of the

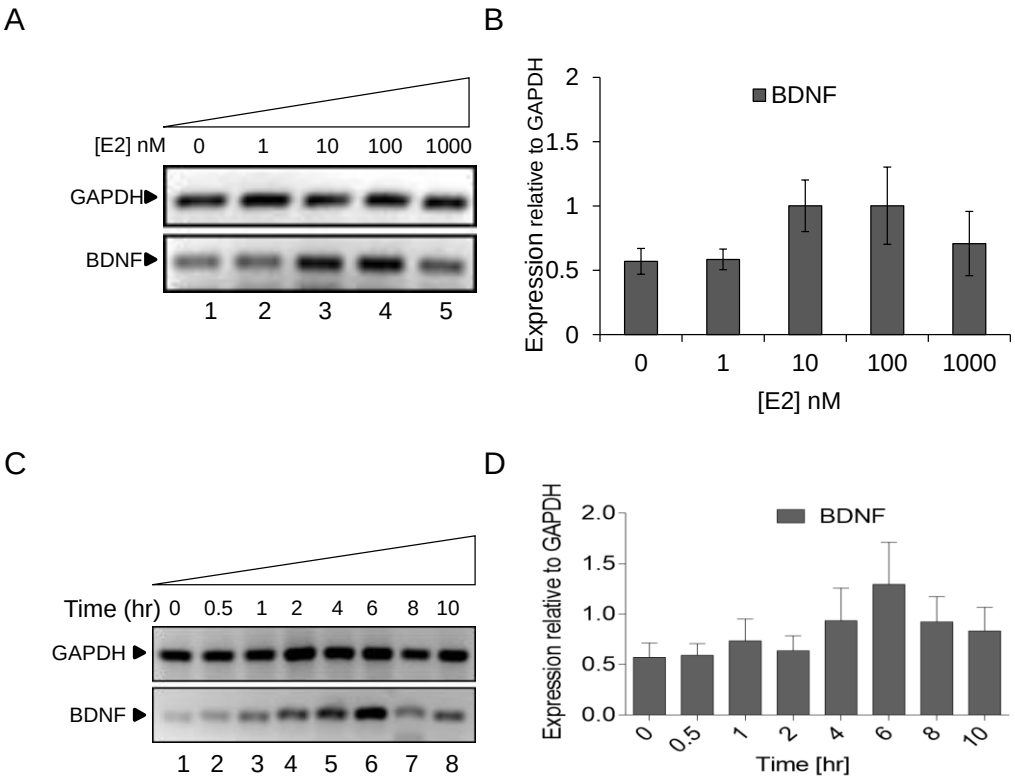


Figure 22. Effects of E2 on BDNF expression *in vitro*.

(A-D) HT22 cells (grown in phenol-red free media) were treated with varying concentrations of E2 (Panel A and B). RNA was isolated, reverse transcribed, and subjected to regular PCR (panel A) and real-time PCR (panel B) using primers specific to BDNF. GAPDH was used as the loading control. HT22 were treated with 100 nM E2 for varying time periods (0-10 h), and RNA was reverse transcribed and analyzed by regular PCR (panel C) and real-time PCR (panel D) using BDNF and GAPDH primers. β-actin was used as the loading control. Each experiment was repeated at least thrice (n = 3). Bars indicate standard errors (n = 3; $P \leq 0.05$).

transcription start site. This ERE appear to be an imperfect (GGACACAAGTGACC) ERE with one base pair mismatch in comparison to typical full ERE (GGTCAnnnTGACC). Presence of multiple potential ERE at the BDNF promoter indicates that its potential regulation via estrogen-receptors in presence of estradiol. Hippocampal pyramidal neurons express mRNA for both isoforms of the estrogen receptor, ERα and ERβ in adult rats.[378] HT22 cells also express ERα and ERβ. Therefore, we examined if ERα and ERβ participate in E2-induced expression of BDNF in HT22 cells using chromatin immunoprecipitation (ChIP) assay using antibodies against ERα, ERβ and β-actin (control).[32,33,194] In brief, HT22 cells were exposed to 10 nM and 100 nM E2 for 6 h, separately. Cells were fixed with formaldehyde and chromatins were sonicated and immunoprecipitated using antibodies against ERα, ERβ and β-actin (control). ChIP DNA was analyzed by regular PCR and qPCRs using primers specific to the BDNF promoter ERE region. ChIP analyses demonstrated that both ERα and ERβ are enriched at the ERE region of the BDNF promoter (Figure 23B and 23C).

Notably, along with ERs, ER-coregulators are also integral components of estrogen-mediated gene activation.[198,379] ER-coregulators interact with ERs, bind to the promoter of ER-target genes, modify and remodel chromatins and interact with general transcription machineries and all these contributes to gene activation.[380] Examples of ER-coregulators include SRC1-family of coactivators, CREB-binding protein (CBP/p300), p/CAF, and ASCOM [activating signal cointegrator-2 (ASC2) complexes].[200,226,381,382,383,384,385] CBP/p300 are histone acetylases and out of all the four core histones (H2A, H2B, H3, and H4), acetylation of H3 is particularly important in regulating hippocampal learning and memory,[386] as well as in estrogenic modulation of learning and memory. Also, histone acetylation regulates hippocampal memory and the genes necessary for synaptic plasticity.[387,388] Therefore, along with ERs, we also examined if ER-coregulators (such as CBP/p300) are enriched at the BDNF promoter

upon treatment with E2 using ChIP assay. [196,197,203] ChIP analyses demonstrated significant binding of ER coregulators, CBP and p300 in the BDNF promoter (Figure 24B and 24C).

Studies from our laboratory and others also demonstrate that mixed lineage leukemia (MLL) family of histone methylases act as ER-coregulators during E2-mediated gene activation.[29,30,31,32,33,88,89,130,143,207,212,294,307,389] MLLs are well-recognized histone methyl-transferases that specifically introduce histone H3 lysine-4 (H3K4) methylations that play key roles during gene activation.[31,205,212,218,219,220,221,390] MLLs interact with ERs via their LXXLL domains and participate in E2-mediated gene activation.[31] Recent studies from our laboratory demonstrated that MLLs regulate various E2-responsive genes.[30,185,200,204,205,206,207,208,209,210,211,212] Therefore, we determined if MLLs also participiate in E2-dependent BDNF gene activation. We examined the recruitments of MLLs (MLL1-4) at the BDNF promoter in the absence and presence of E2 in HT22 cells using ChIP assay using antibodies against MLL1-4. ChIP DNA were PCR-amplified using BDNF promoter primers (ERE regions). These analyses demonstrated histone methylase MLL3 is significantly enriched at BDNF promoter in the presence of E2 (Figure 24B and 24C).

Since MLL3 is histone H3K4-methylase and p300 is histone acetyl-transferase and are found to be enriched at the BDNF promoter in an E2-dependent manner, we analyzed the levels of histone acetylation and histone H3K4-trimethylation in the BDNF promoter in the absence and presence of E2, using ChIP assay. As, expected, E2-treatment significantly increased H3K4-trimethylation, histone acetylation and the levels of RNA polymerase II at the ERE1 in the BDNF promoter (Figure 24D and 24E). The enrichment of histone methylases (MLL3) and histone acetylases (CBP/p300) and consequent increase in H3K4-trimethylation and histone acetylation at the BDNF promoter in presence of E2, suggest the potential involvement of these ER-coregulators in the E2 mediated transcriptional activation of BDNF.

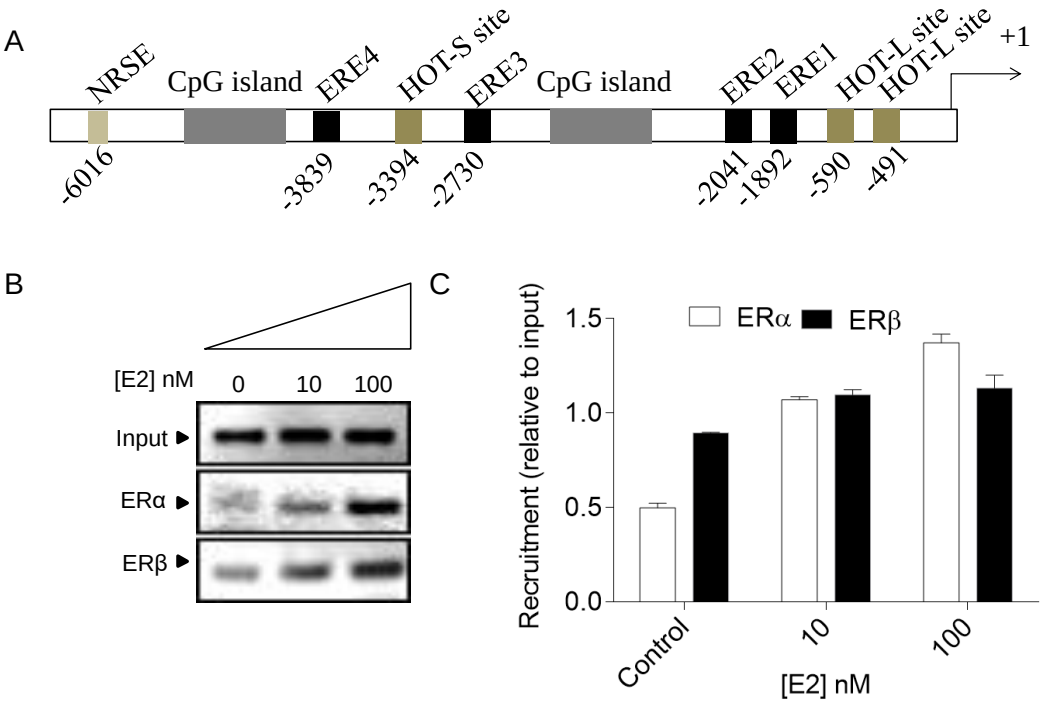


Figure 23. E2 -dependent recruitment of ERs at the BDNF promoter.

(A-C) MCF7 cells were treated with 10 nM E2 and 100 nM E2, separately for 6 h and subjected to ChIP assay using antibodies specific to ERα and ERβ. ChIP DNA fragments were PCR-amplified and analyzed by qPCR and regular PCR using primers specific to ERE1 region of the BDNF promoter. Panel A shows the position of the primer spanning ERE1 region in the BDNF promoter. Panels B-C shows the ChIP analyses of ERα and ERβ on the ERE1 of the BDNF promoter. Each experiment was repeated at least thrice. Bars indicate standard errors. P values ≤ 0.05 were considered to be significant.

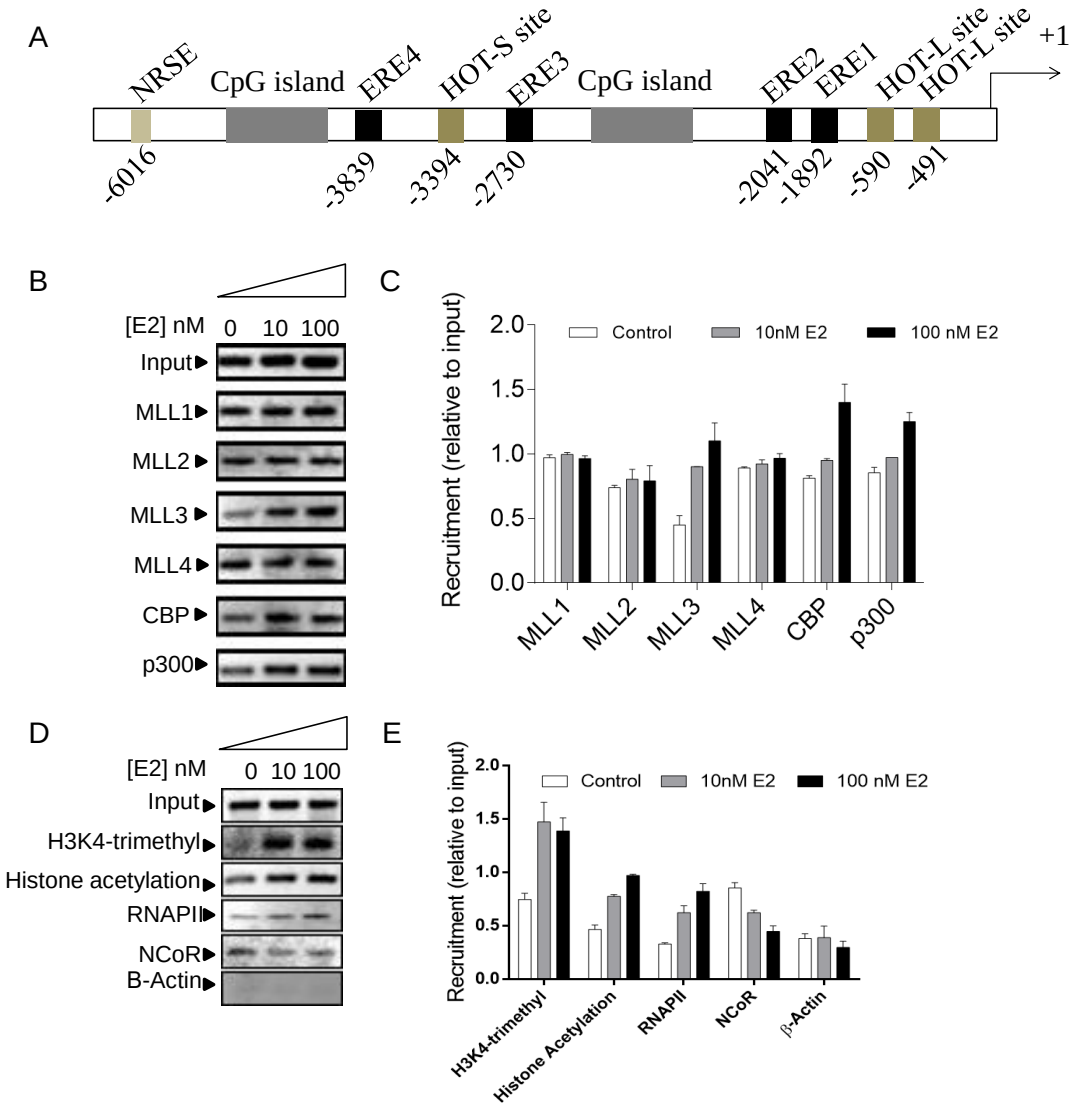


Figure 24. Recruitment of MLL-histone methylase, CBP/ p300 and associated histone marks at the promoter of BDNF upon exposure to E2.

(A-E) HT22 cells were treated with 0nM, 10nM E2 and 100 nM E2 for 6 h and analyzed by ChIP assay using MLL1, MLL2, MLL3, MLL4, CBP, p300, RNAPII, H3K4-trimethyl, histone acetylation, NCoR and β-actin (negative control) antibodies. The ChIP DNA was PCR-amplified using primers specific to ERE1 region of BDNF promoter. The recruitment of MLL3, CBP, p300 in the BDNF promoter are shown in the panel B (RT-PCR) and panel C (qPCR). Panel D-E (qPCR and regular RT-PCR data respectively) shows the levels of RNAPII, N-CoR, H3K4-trimethyl and histone acetylation at the BDNF promoter. Bars indicate standard errors ($p \leq 0.05$).

4.3.6. E2 facilitates the sequestration of silencing machienary by derecruiting EZH2, LSD1 and altering histone methylation and acetylation levels

Our previous observations (as in figure 21B and 21C) showed that in the absence any stimuli (such as E2), BDNF gene is repressed via recruitment of EZH2, LSD1 and HOTAIR. In the presence of E2, BDNF gene expression is induced via recruitment of ERs and ER-coregulators (MLL3, CBP/p300). Does this mean this mean that gene silencing factors (EZH2, LSD1 and HOTAIR) which were bound to promoter in the absence of E2, are dissociated upon treatment with E2 and relieve gene repression? To address this, we further examined the levels of EZH2 and LSD1 binding at HOT-L and HOT-S sites in BDNF promoter upon treatment with E2 using ChIP assay. Briefly, HT22 cells were treated with 10 nM E2 for 6h and and subjected to ChIP assay using antibodies specific to EZH2 and LSD1 and β-actin (negative control). ChIP DNA fragments were PCR amplified using primers specific to the HOT-L (-3394 bp) and HOT-S (-491 bp) sites region in the BDNF promoter. These analysis showed that levels of EZH2 and LSD1 were reduced at both HOT-L and HOT-S sites (2 and 2.5 folds respectively) in the BDNF promoter upon treatment with E2 (Figure 25B and 25C). No significant impacts on β-actin levels were observed at the BDNF promoter upon treatment with E2. Our results suggest that E2 induces a transcriptionally permissive state at the BDNF promoter by derecruiting EZH2 and LSD1 at both HOT-L and HOT-S sites (2 and 2.5 folds respectively) (Figure 25B and 25C).

EZH2 which confers methyl transferase activity to PRC2,[135,136] which introduces mono-, di- and tri-methyl groups at the H3K27 which is associated with heterochromatin.[137,138,139] Lysine specific demethylase 1 (LSD1) confers demethylase activity to the REST/CoREST complex,[133,140] which demethylates di- and mono- methylated H3K4 mark which is associated with an active state of chromatin.[141,142,143,144] Therefore, we investigated the levels of H3K27-trimehyl, H3K4 trimethyl and RNA Polymerase II using ChIP assay in the presence and absence of E2. Our ChIP analysis

demonstrated that E2 treatment significantly reduces H3K27 trimethyl levels whereas increases H3K4 trimethyl levels and also recruits RNA polymerase to the BDNF promoter indicating active gene transcription (Figure 25B and 25C). These data suggest that E2 facilitates the reversal of repression caused by the silencing machinery at the BDNF promoter via abolishing EZH2/LSD1 recruitment, reducing H3K27 trimethyl marks and enhancing H3K4 trimethylation.

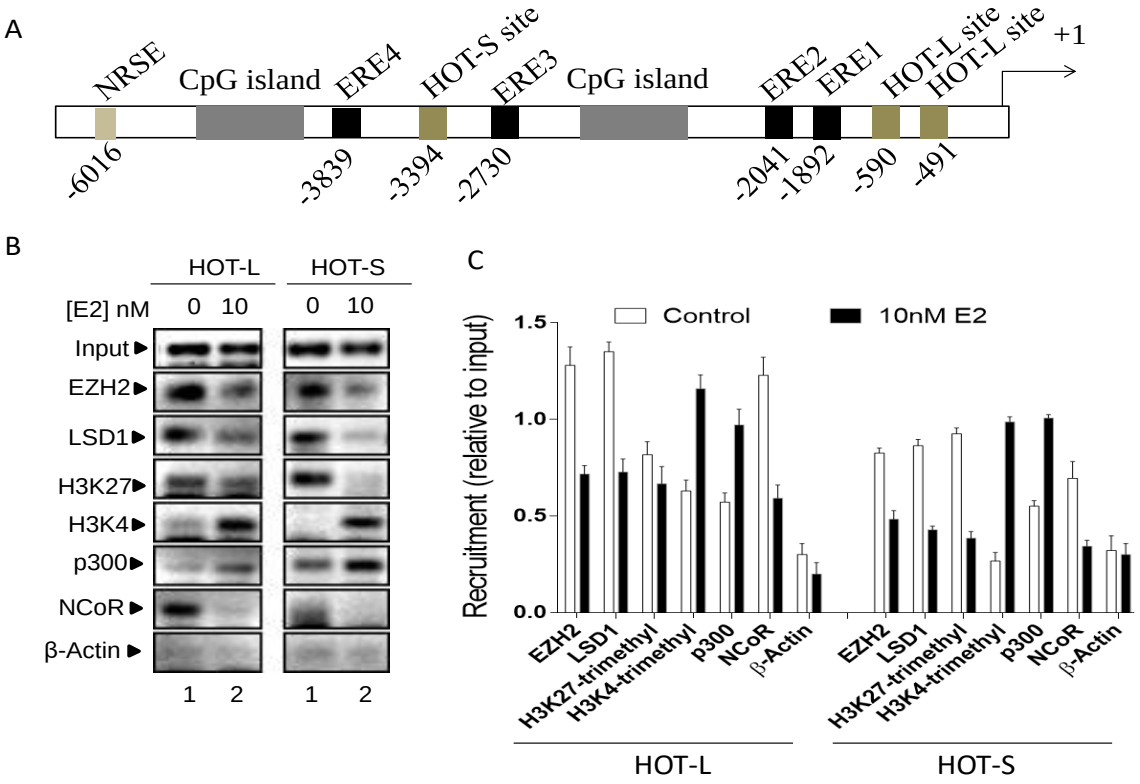


Figure 25. E2 derecruits EZH2 and LSD1 and alters and associated histone marks at the promoter of BDNF.

(A-C) HT22 cells were treated with 0nM and 10nM E2 E2 for 6 h and analyzed by ChIP assay using EZH2, LSD1,H3K27-trimethyl and H3K4-trimethyl,p300, RNAPII, NCoR and β-actin (negative control) antibodies. The ChIP DNA was PCR-amplified using primers specific to HOT-L and HOT-S regions of BDNF promoter. The derecruitment of EZH2 and LSD1 in the BDNF promoter is shown in the panel B (RT-PCR) and panel C (qPCR). The levels of RNAPII, NCoR, H3K4-trimethyl and histone acetylation at the HOT-L and HOT-S sites in the BDNF promoter upon E2 treatment are shown in panels B and C. Bars indicate standard errors ($p \leq 0.05$).

4.4. Discussion

BDNF is a well-known pro-survival factor for the striatal neurons. It is mostly of cortical origin and anterogradely transported to the striatal targets.[391] BDNF levels significantly increase in mice on regimens of dietary restriction,[392] or physical exercise favored by environmental enrichment.[393] A prevailing current theory posits that multiple genetic and environmental factors contribute to the development of most neurological and psychiatric disorders.[394,395] In particular, diverse environmental stressors have been shown to modulate BDNF availability and function in rodents.[396,397,398] Indeed, some risk factors may cause persisting changes in BDNF gene regulation, underlying a lasting epigenetic imprint on the genome, thereby increasing susceptibility to neurological disorders. In this study, we aim to investigate the epigenetic regulation of BDNF in the neuronal cells.

Epigenetic modifications can be mediated by long non-coding RNAs (lncRNAs) which play major roles in regulation of gene transcription, chromatin structure, and mRNA stability during cell development and diseases.[114,399,400] One such lncRNA, HOTAIR, is transcribed from the HOXC locus that acts as a scaffold for histone modification complexes to coordinately interact with PRC2 and lysine-specific demethylase 1 (LSD1).[129,401] HOTAIR carries out the transcriptional repression of HOXD cluster of genes by interacting and guiding the gene silencing chromatin modifying complexes of PRC2 and LSD1 complexes to the target loci, leading to epigenetic modifications that are in turn associated with the process of transcriptional repression.[236,321] Here, we explored the potential roles of HOTAIR in BDNF gene regulations in neuronal cells. Initially to understand the epigenetic mechanism of BDNF gene regulation, we knocked down HOTAIR using an antisense oligonucleotide. We observed that the expression of BDNF mRNA (as well as protein level) is induced upon HOTAIR knockdown in hippocampal neuronal cells (HT22 cell). These observations suggest that HOTAIR plays a significant role in the regulation of BDNF expression. Notably, the expression of several HOTAIR-target genes,

such as HOXD3 and HOXD10,[402,403] were upregulated upon HOTAIR-knockdown, which further confirms the target specificity of HOTAIR-antisense.

To further understand the mechanism of BDNF gene regulation via HOTAIR, we inhibited (small molecule inhibitor) the enzymatic activity of HOTAIR-interacting histone methyl-transferase EZH2 (member of the PRC2-complex). Interstingly, we observed that inhibition of EZH2 also resulted in increased BDNF expression in HTT22 cells. Inhibition of EZH2 indeed reduced the levels of H3K27 trimethyl marks at the BDNF promoter. Additionally, HOTAIR target genes HOXD3 and HOXD10 expression were significantly increased upon inhibition of EZH2. These ovservation demonstrates that EZH2 is also actively involved in transcriptional silencing of BDNF. Analysis of H3K27 trimethylation marks introduced by EZH2 at the promoter of BDNF in HT22 may shed light on how the HOTAIR/EZH2 regulates knockdown-induced BDNF gene activation.

The BDNF gene promoter analysis shows that there are putative binding sites for PRC2 (HOT-S) and LSD1 (HOT-L) in the BDNF promoter.[133] The presence of these sites indicates potential roles of PRC2 and LSD1 in BDNF gene regulation. To further address how HOTAIR regulates BDNF gene expression, we performed ChIP-assay in HT22 cell lines in the presence of HOTAIR knockdown. Results show that HOTAIR silencing decreased the binding of EZH2 and LSD1 within the HOT-S and HOT-L sites at the BDNF promoter in HT22 cells. We also observed significant decrease in enrichment of H3K27 trimethylation marks and increase in H3K4-trimethylation and RNA Polymerase II levels at the BDNF promoter upon HOTAIR-knockdown, suggesting that HOTAIR represses BDNF expression partly by associating with EZH2 and LSD1.

On the other hand, studies in the past have shown that hormonal status can greatly influence the expression of BDNF expression. For instance, estrogen (a potential neuroprotective hormone) replacement in young adult, ovariectomized, female rats increases BDNF expression in the

hippocampus.[339,350,404,405,406,407,408,409,410,411,412] Infact, BDNF-synthesizing neurons co-localize with the estrogen receptors in the forebrain, indicating its role as a biological substrate for the regulation of this gene by estrogen hormone.[413] Hence, we have further analyzed the mechanism of regulation of E2-induced BDNF expression. Our studies demonstrate that treatment with estradiol significantly increased mRNA levels of BDNF in HT22 cells. The sequence corresponding to an ERE-like motif (ERE) in the BDNF promoter differs from the canonical ERE principally with respect to the spacer size between the two pentameric palindrome sequences. Since estrogen action is mainly controlled through ERs and ER-co-regulator molecules, it seems likely some of these may be implicated in E2-mediated BDNF gene activation.

Indeed our ChIP analyses demonstrates that estrogen receptors (ERα, ERβ) and a variety of ER-coregulators such as histone methylase, MLL3, and histone acetylases, CBP and p300 are enriched at the BDNF promoters during E2 -induced BDNF expression. Notably, studies from our laboratory and others have shown that MLLs functionally coordinate with ERs-during estrogen-dependent gene activation.[33,88,89,185,206,211,213,228] Here we demonstrate that MLL3 specially is associated with E2-mediated regulation of BDNF expression in neuronal cells. ER-mediated recruitment of histone methylases and acetylases at the ER-target promoters, are anticipated to modify histone proteins, remodel the chromatin which ultimately contribute to gene activation. Our ChIP analysis showed that E2 treatment resulted in increased histone H3K4-trimethylation, histone acetylation, and enrichment of RNA polymerase II at the BDNF promoter region (ERE). This analysis further demonstrates that BDNF expression is transcriptionally regulated by estradiol in hippocampal cells and this is mediated via coordination of ERs and ER-coregulators like histone methylase MLL3, histone acetylases CBP and p300. These results corroborate with previous findings and demonstrate that estrogen significantly upregulates BDNF expression in hippocampal cells.

So far our studies established that BFNF expression is repressed via coordination HOTAIR/EZH2/LSD1 complexes. Addtionally, we find that BDNF expression is transcriptionally induced by estradiol in neuronal cells. Based on these observations, we hypothesize that BDNF gene is repressed via binding of the repressors, HOTAIR/EZH2/LSD1 complexes at the BDNF promoter and consequent H3K27-methylation. In the presence of inducer stimuli such as E2, the repressor proteins dissociate from the BDNF promoter and that allows the binding of the transcription activation factors, activated receptors (such as ERs), activators/coactivators (ER-coregulators MLL3-histone H3K4-methylase, CBP/p300 histone actylase), consequent alteration in increased H3K4-methylation and increased histone acetylation and chromatin remodeling leading to gene activation. Our ChIP analysis at HOT-S and HOT-L site present in BDNF promoter reveals that the enrichment of EZH2, LSD1 and H3K27-methylation levels are significantly decreased upon treatment with E2, while the levels of E2-associated activation factors such as ERs, MLL3, CBP/p300 and H3K4-trimethy levels were augmented at the BDNF indicating gene activation. Therefore, we propose a model showing positive regulators such as E2 can restore BDNF levels and rescue cells from neuronal damage by displacing silencing machienary from the BDNF promoter and reinforcing active transcription of BDNF gene (Figure 26). This finding may be of particular interest as E2 aids the neuronal regeneration and repair.

Our observations are also in agreement with previous reports which show that loss of the neuronal lncRNAs leads to alteration of neuronal cellular differentiation, and suggests that the lncRNAs play indispensable roles in neurogenesis/ neural cell fate specification, and subsequently brain development.[146] In addition, a similar report describes dramatic changes in expression of lncRNA genes in differentiating neurons, one of which is HOTAIRM1, a cis-acting regulator of the HOXA cluster during myelopoiesis, suggesting a novel role of lncRNAs in neural differentiation.[145,414] Additional evidence suggesting functional roles of lncRNAs in the brain includes a computational analysis of in situ

hybridization data from the Allen Brain Atlas, which identified 849 lncRNAs showing specific expression in the mouse brain.[415] Although lncRNAs have been previously linked to neurogenesis,[416] but little evidence has been presented for functional roles of lncRNAs in the human nervous system. Here, we report that lncRNAs directly regulate the expression of neurotrophic factors. The data presented in this study represent the direct demonstration that lncRNAs are important regulatory units of necessary human nervous system, and imply that deregulation of lncRNA expression may indeed impact the expression of downstream targets like neurotrophic factors thus contributing to neurological disorders. Our observation is in line with a report that showed that there is a strong concordance between the expression pattern of neurotrophins and the estrogen-synthesizing enzyme aromatase in the developing midbrain.[417] These findings may contribute to our understanding of the mechanisms by which steroid hormones influence the differentiation of developing neurons. The relevance of this work may open up a new area of investigation into regulation of BDNF, a neuropeptide that plays crucial roles in neuronal growth, maintenance, and repair. BDNF expression is reduced in Alzheimer's, Parkinson's, and Huntington's diseases and low BDNF level contributes to neuro-degeneration, affects cognitive abilities, and causes dementia.[157,158,159] The mechanisms by which BDNF expression is regulated in healthy brain and misregulated in neurological disorders are still not clear and need further effort to resolve.

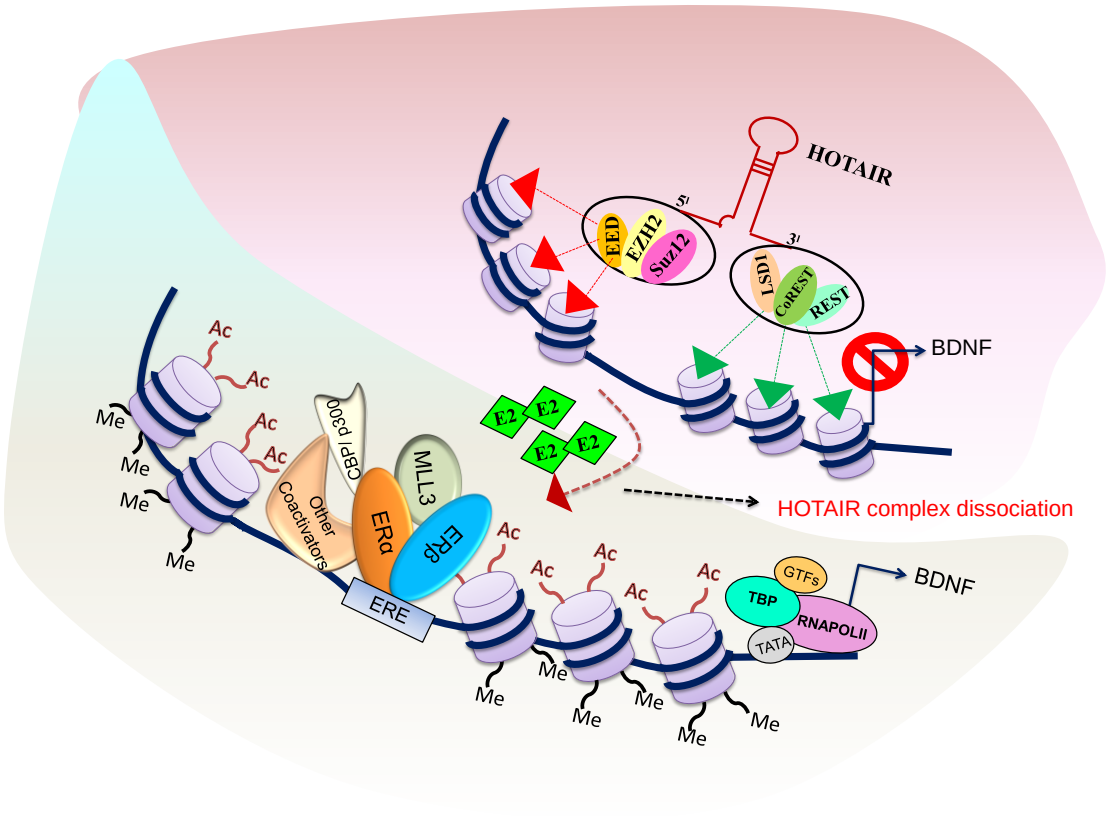


Figure 26. Mechanism of regulation of lncRNA and E2-mediated BDNF gene expression.

BDNF expression is repressed by a long non-coding RNA HOTAIR. HOTAIR interacts with histone methylase PRC2 and histone demethylase LSD1, recruits them to BDNF promoters leading to silencing of BDNF expression. E2, on the other hand, may induce BDNF-gene expression by displacing HOTAIR/EZH2/LSD1 complex at BDNF promoter containing putative cofactor binding sites which in turn may recruit active transcription machienary leading to the induction of BDNF gene.

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BIOGRAPHICAL INFORMATION

The author was born and raised in Kolkata, India. She graduated high school in 2006 from Hariyana Vidya Mandir, Kolkata (India). She received her Bachelor's degree in Chemistry/Zoology/Microbiology in 2009 from St.Joseph's College, Bangalore (India). She completed her Master's degree in Biochemistry in 2011 at University of Calcutta, Kolkata (India). The author moved from Kolkata (India) to Arlington (United States) in August, 2012 and joined Mandal laboratory in Spring, 2013. She worked briefly for Regeneron Pharmaceuticals as a PhD intern from May, 2016 to August, 2016, where she developed bioassays for blood-brain barrier related disorders. Her PhD research seeks to understand the functions of non-coding RNA, HOX genes, neurotransmitters and their transcriptional regulation *in vitro* and *in vivo*. She received her PhD degree in Biochemistry in May, 2017 under the supervision of Dr. Subhrangsu Sekhar Mandal for her dissertation work concerning identification, risk assessment and epigenetic mechanism of target gene regulation by external stimuli such as steroids / endocrine disrupting chemicals. The author wishes to pursue interests in cancer vaccines, molecular epigenetics and mass spectrometry and looks forward to help fight against cancer.