Published in final edited form as:

Gene. 2016 September 30; 590(2): 234–243. doi:10.1016/j.gene.2016.05.009.

Endocrine disrupting chemical, bisphenol-A, induces breast cancer associated gene HOXB9 expression *in vitro* and *in vivo*

Paromita Deb¹, Arunoday Bhan¹, Imran Hussain¹, Khairul I. Ansari¹, Samara A. Bobzean², Tej K Pandita³, Linda I Perrotti², and Subhrangsu S. Mandal^{1,*}

¹Epigenetics Research Laboratory, Department of Chemistry and Biochemistry, The University of Texas at Arlington, Arlington, Texas 76019

²Department of Psychology, The University of Texas at Arlington, Arlington, Texas 76019

³Department of Radiation Oncology, The Houston Methodist Research Institute, Houston TX 77030

Abstract

HOXB9 is a homeobox-containing gene that plays a key role 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 the endocrine disrupting chemical bisphenol-A (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) in the HOXB9 promoter are required for BPA-induced expression. Estrogen-receptors (ERs) and ER-co-regulators such as MLL-histone methylase (MLL3), 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, like estradiol, increases HOXB9 expression in breast cells both *in vitro* and *in vivo* through a mechanism that involves increased recruitment of transcription and chromatin modification factors.

Graphical Abstract

*Corresponding author. smandal@uta.edu; Fax: 817-272-3808.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

HOXB9; gene expression; estrogen signaling; bisphenol-A; endocrine disruption; epigenetics

Introduction

The members of the homeobox (HOX) containing gene family represent a subset of evolutionarily conserved transcription factors that play crucial roles in embryonic development (Yu et al., 1995; Lappin et al., 2006). Expression of HOX genes regulate patterning of the anterior-to-posterior axis from the level of the hindbrain to the end of the spine (Hueber and Lohmann, 2008). However, HOX genes are also expressed in various normal adult tissues (Takahashi et al., 2004; Mallo and Alonso, 2013). HOXB9, one of the 39 HOX genes, plays key roles in skeletal and mammary gland development (Chen and Capecchi, 1999). HOXB9 is a direct transcriptional target of WNT/TCF4 (Nguyen et al., 2009) and is involved in cell proliferation, cell-cycle progression, differentiation, embryonic segmentation, limb patterning and angiogenesis (Maeda et al., 2005; Hatzis et al., 2008; Nguyen et al., 2009; Hayashida et al., 2010; Tomioka et al., 2010; Chiba et al., 2012). Recently it was also shown that HOXB9 expression confers resistance to ionizing radiation indicating its potential roles in the DNA damage response and in maintenance of genomic integrity (Chiba et al., 2012).

Increasing evidence suggests that altered HOXB9 expression is associated with a variety of cancers, including breast, head and neck, lung, hepatocellular, colon carcinomas and gliomas (Fang et al., 2014; Darda et al., 2015; Sha et al., 2015; Zhan et al., 2015). HOXB9 overexpression in breast tumors induces breast cancer metastasis by altering the tumor microenvironment, and promotes disease progression (Hayashida et al., 2010). In fact, HOXB9 expression is an important prognostic factor in breast cancer (Seki et al., 2012). 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 MCF7 breast cancer cells (Ansari et al., 2011b; Shrestha et al.,

2012). We also 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 (TGF β) etc. and induces three dimensional (3D) tumor colony formation (Shrestha et al., 2012). 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 (Shrestha et al., 2012).

Since HOXB9 is found to be overexpressed in breast cancers, aids 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 cancer. 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 (Schonfelder et al., 2002a; Fernandez and Russo, 2010; Abdel-Rahman et al., 2012; Fucic et al., 2012; Singh and Li, 2012; Sengupta et al., 2013). 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*. Notably, BPA is major environmental and health concern due to its ubiquitous presence in the environment and its chronic human exposure (Murray et al., 2007). BPA is commonly used for manufacturing polycarbonate plastics and epoxy resins, in the inner lining of the food and beverage containers, dental sealants and others (Brotons et al., 1995; Olea et al., 1996; Biles et al., 1997; Fernandez and Russo, 2010). BPA has been detected in varying concentrations in maternal and fetal plasma, placental tissue and in human urine samples (Ikezuki et al., 2002; Schonfelder et al., 2002b; Calafat et al., 2005). Numerous studies indicate that low levels of BPA exposure imposes human health risks. Exposure to BPA induces developmental changes in the female reproductive tract and alters mammary gland development *in vivo* (Murray et al., 2007). Importantly, fetal exposure to low doses of BPA has been linked with long-lasting effects in the mouse mammary gland that are manifested during postnatal stages. These include increased number of estrogen-receptor and progesterone receptor-positive cells at puberty and increased lateral branching, and development of ductal hyperplasias at postnatal days (in rats) (Murray et al., 2007) (Munoz-de-Toro et al., 2005). The increased number of intraductal hyperplasia in the mammary gland is considered to be linked with precursors of carcinomas both in rodents and humans (Ernst et al., 2004). Thus, chronic exposure to BPA, even at low levels, is a major health concern to human. Here, our results demonstrate that HOXB9, which is a critical player in mammary gland development and breast cancer, is upregulated upon exposure to BPA in breast cancer cells *in vitro* and *in vivo*.

Experimental procedure

Cell culture and treatment with estradiol (E2) and BPA

Estrogen receptor (ER) positive MCF7 breast carcinoma cells were purchased from the American Type Cell Culture Collection (ATCC) and cultured as described previously (Bhan et al., 2013; Bhan et al., 2014a; Bhan et al., 2014b; Hussain et al., 2015). Treatment of MCF7 cells with E2 and BPA was carried out (Bhan et al., 2013; Bhan et al., 2014a; Bhan et al., 2014b; Darda et al., 2015; Hussain et al., 2015) by growing the cells in phenol red-free

DMEM-F12 media containing 10 % charcoal stripped FBS for at least three generations. Cells (60–70 % confluent) were then grown in 60 mm cell culture plates and treated with varying concentrations of E2 (0 – 100 nM) and BPA (0 – 1000 nM) for 6 h. The control (treated with diluent DMSO) and treated MCF7 cells were harvested for RNA/protein extraction as well as for ChIP assays as needed (Ansari et al., 2011b; Ansari et al., 2012a; Shrestha et al., 2012; Ansari et al., 2013b; Bhan et al., 2014b; Hussain et al., 2015).

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

Total cellular RNA and protein were isolated as described previously (Ansari et al., 2008; Ansari et al., 2011b; Bhan et al., 2013; Bhan et al., 2014a; Bhan et al., 2014b; Hussain et al., 2015). The isolated RNA pellets were air-dried, dissolved in DEPC treated water containing 0.5 mM EDTA and quantified using a Nanodrop spectrophotometer. Synthesis of cDNA was carried out and each cDNA product was diluted to 100 μ L, and 5 μ L of the diluted cDNA was subjected to RT-qPCR or regular RT-PCR amplification using specific primer pairs described in Table 1. Regular RT-PCR reactions were carried out for 33 cycles (30 s at 94 °C for denaturation, 30 s at 60 °C for annealing, 45 s at 72 °C for elongation) and the PCR products analyzed by 1.5 % – 2 % agarose gel electrophoresis. For the RT-qPCR reactions, 5 μ L of diluted cDNA were mixed with 5 μ L of Sso EvaGreen supermix (Bio-Rad) and 2 μ M of each primer and final volume was made up to 12 μ L. PCR reactions were carried out in CFX96 real-time detection system (Bio-Rad) for 40 cycles (5 s at 95 °C for denaturation and 10 s at 60 °C for both annealing and elongation). Data analyses were performed using CFX manager software (Bio-Rad). Each experiment was repeated three times with three replicates each time.

Chromatin Immunoprecipitation assay (ChIP assay)

For ChIP analyses, MCF7 cells were treated with 1 nM E2 or 100 nM BPA for 6 h as described previously by us (Ansari et al., 2008; Ansari et al., 2011b; Bhan et al., 2013; Bhan et al., 2014a; Darda et al., 2015; Hussain et al., 2015). In brief, 6 h post E2 or BPA treatment, cells were crosslinked with 1% formaldehyde for 10 min at 37 °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 pre-cleared with protein-G agarose beads and subjected to immunoprecipitation using antibodies specific to ER α (Santa Cruz; 2Q418), ER β (Santa Cruz; SC-8974), H3K4-trimethyl (EMD-Millipore; 07–473), RNAPII (Abcam; ab5408), MLL3 (Abgent; AP6184a), CBP (Santa Cruz, sc-369), p300 (Santa Cruz; sc-585), N-CoR (Santa Cruz; sc-1609), histone-acetylation (EMD-Millipore; 06–599) and β -actin (Sigma, A2066). Immunoprecipitated chromatin was washed, de-crosslinked and deproteinized at 65 °C in presence of 5 M NaCl, followed by incubation with proteinase K (Sigma) at 45 °C for 1 h. Purified ChIP DNA was PCR amplified using primers spanning ERE4 present in the HOXB9 promoter (Table 1). The analysis of ChIP DNA was carried out via regular PCR and qPCR using appropriate primers (Table 1).

Dual luciferase reporter assay

For ERE luciferase assays, HOXB9 promoter DNA containing the estrogen response elements ERE1 (−6 to −393nt), ERE2 (−999 to −1316 nt), ERE3 (−1174 to −1492 nt), and ERE4 (−1430 to −1858 nt) regions were inserted upstream of the luciferase gene in the pGL3-promoter vector (Promega) (Table 1). MCF7 cells were co-transfected with 1500 ng of construct DNA and 150 ng of a reporter plasmid containing renilla luciferase gene (pRLTk, Promega), as an internal transfection control, using FuGENE6 transfection reagent. MCF7 cells transfected with empty pGL3 served as an experimental control. Twenty-four hours post transfection, the cells were treated with 100 nM BPA for 6 h and then subjected to luciferase assay, using Dual luciferase reporter assay kit (Promega) as instructed by the manufacturer's protocol. Luciferase activities were normalized to the renilla and plotted. Each treatment was performed in four parallel replicates and the experiment was repeated at least twice (Ansari et al., 2009a; Ansari et al., 2009b; Ansari et al., 2011a; Ansari et al., 2011b; Ansari et al., 2012a; Ansari et al., 2012b; Bhan et al., 2013; Bhan et al., 2014a; Bhan et al., 2014b).

Animal studies

Subjects—Ninety day old, experimentally naïve, adult, female Sprague Dawley rats (n=12) were triple housed and maintained 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. All animals were maintained and cared for in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The University of Texas at Arlington's Animal Care and Use Committee approved all experimental procedures and protocols. Notably, all the animals were maintained under equivalent caging, bedding, food, and water bottles conditions to normalize the effects of any exogenous estrogen source on our results. **Ovariectomy:** Female rats were anesthetized with 2–3% isoflurane-oxygen vapor mixture and ovariectomized (OVX) as previously described by us (Bhan et al., 2014a) and others (MacLusky et al., 2005; Betancourt et al., 2010; Eilam-Stock et al., 2012). The animals were allowed to recover for 4–5 days post-surgery, daily vaginal lavage testing was performed for 8 consecutive days to confirm cessation of estrous cycling, thus verifying the completion of the OVX procedure. All OVXs performed were confirmed complete and thus, no animals were eliminated on the basis of an incomplete procedure. **Acute treatments with E2 and BPA:** 10 mg of BPA was dissolved in 1 mL of ethanol to create a stock solution that was stored at −4 °C. E2 was 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) or E2 (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 (MacLusky et al., 2005; Betancourt et al., 2010; Eilam-Stock et al., 2012).

RNA extraction from the mammary glands—RNA extraction was carried out using ZyGEM kit (Hamilton, New Zealand) according to the manufacturer's protocol (Ansari et al., 2013b). The RNA was reverse transcribed and subjected to RT-qPCR and regular RT-PCR using rat specific primers (Table 1).

Results

HOXB9 is overexpressed in breast and other types of cancer

To investigate the association of HOXB9 with different types of cancer, we first analyzed the cancer genome databases using the cbiportal (<http://www.cbiportal.org/public-portal/>) data analysis tool, (Cerami et al., 2012; Gao et al., 2013). This analysis found that the HOXB9 gene is amplified, mutated or deleted in various cancers but primarily in breast, cervical, ovarian, uterine and prostate (Figure 1). 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 (Ansari et al., 2011b; Seki et al., 2012). The overexpression of HOXB9 in breast, prostate, uterine and ovarian tissues suggests potential regulation by steroid hormones and a potential target for mis-regulation by endocrine disrupting chemicals.

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

To understand the potential impacts of estrogenic EDCs on HOXB9 gene regulation, we treated ER-positive MCF7 breast cancer cells with varying concentration of a well-known estrogenic EDC, bisphenol-A (BPA) and analyzed its impacts on HOXB9 gene expression. Additionally, we also treated MCF7 cells with estradiol (E2) as a positive control. Total cellular RNA was isolated, reverse transcribed and the levels of HOXB9 mRNA measured by regular RT-PCR and RT-qPCR, using primers specific to HOXB9 (Table 1) and GAPDH expression as a control. Both BPA and E2-treatment induced HOXB9 mRNA expression in a dose dependent manner (Figures 2A–D). Notably, HOXB9 expression increased by about 6.6 fold at 1 nM E2 and 9.4 fold at 100 nM BPA, respectively. Since HOXB9 is optimally induced in breast cancer cells by 1 nM E2 and 100 nM BPA, we used these E2 and BPA concentrations for future biochemical experiments. Time-dependent analysis showed that HOXB9 induction in MCF7 cells was similarly optimal at 6 h post-treatment with either 1 nM E2 or 100 nM BPA (data not shown). Additionally, in order to understand the impacts of BPA exposure in the presence of E2, we treated MCF7 cells with varying concentrations of BPA in the presence and absence of E2 (1 nM) and analyzed their impacts on HOXB9 expression. As seen in figure 2 (panels E–F), low concentrations of BPA has nominal impacts on E2-induced HOXB9 expression. However at higher concentrations (100 nM) of BPA, the level of E2-induced HOXB9 is decreased in comparison to E2 alone and this might be likely due to the competition between BPA and E2 for same binding sites on ER. 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 evaluate the impact of BPA on HOXB9 expression *in vivo*, we treated ovariectomized (OVX) Sprague Dawley rats with BPA, as well as E2, and analyzed the impact on HOXB9 mRNA expression in mammary glands. Female Sprague Dawley rats were subjected to ovariectomy, to minimize the impacts of endogenous esrogens and then were subcutaneously

injected twice with E2 (5 µg) and BPA (25 µg) 24 h and 4 h before sacrifice. These doses of E2 and BPA treatment were previously used and shown to affect gene expression and impact behavioral traits and other neural functions (Bhan et al., 2014a; Bhan et al., 2014b; Bobzean et al., 2014). Control OVX rats were treated with the diluent peanut oil (vehicle). Total RNA from mammary glands of control and E2/BPA treated rats was isolated and analyzed by qPCR and regular RT-PCR. Analysis showed that E2 and BPA treatments increased HOXB9 mRNA levels, in the rat mammary glands by ~4 and ~5 fold, respectively, (Figures 2E–F). These observations suggested that HOXB9 expression is transcriptionally regulated by E2 in the mammary glands and it is also up-regulated upon exposure to BPA, in vivo.

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 HOXB9 expression. The HOXB9 promoter contains several putative estrogen-response elements (EREs) (Ansari et al., 2011b). 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 (Nilsson and Gustafsson, 2000; Nilsson and Gustafsson, 2002; Lee et al., 2006; Mo et al., 2006). ERs aid in the recruitment of various ER-co-regulators and epigenetic modifiers to the gene promoters that facilitate histone modification and chromatin remodeling leading to target gene activation (Nilsson et al., 2001; Nilsson and Gustafsson, 2002; Barkhem et al., 2004; Chen et al., 2004). Promoter analysis revealed that there were three ERE half sites (GGTCA) located at –208 nt, –1201 nt and –1395 nt upstream of the HOXB9 transcriptional start site (TSS) (Figure 3A). There is also an imperfect ERE (TGTCCnnnGGTCA) located at –1600 nt (Figure 3A) that is 1 nt different from the consensus full ERE (GGTCAnnnTGACC) and ERE-half sites are flipped within the palindrome. Given the sequence homology to the consensus ERE, we examined the BPA (and E2) responsiveness of each ERE (ERE1-ERE4) site using a luciferase based reporter assay (Ansari et al., 2011b). Initially, we cloned each ERE along with ~150 nt of flanking sequences on both sides into a luciferase expression construct, pGL3 (Figure 3A) (Ansari et al., 2011b). A promoter region without an 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) or 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 E2 or BPA treatment of ERE4-pGL3 (which is the imperfect ERE) transfected MCF7 cells resulted in a ~3 and ~4 fold increase in luciferase activity whereas none of the other ERE-GL3 construct displayed any responsiveness to E2 or BPA. Therefore, the ERE4 element, which is an imperfect full ERE, is likely the E2/BPA responsive ERE and may also be associated with BPA-induced HOXB9 expression.

Estrogen-receptors (ERs) and ER co-regulators are enriched at the HOXB9 promoter upon treatment with BPA

As ERs are key players in transcriptional up-regulation of estrogen-responsive genes (Nilsson and Gustafsson, 2000; Nilsson and Gustafsson, 2002), we examined if ERα participates in BPA-induced HOXB9 expression. We examined if BPA and E2 treatment

results in recruitment of ER to the HOXB9 promoter, especially the ERE regions, using a chromatin immunoprecipitation (ChIP) assay (Ansari et al., 2011b). Along with ER, ER co-regulators also play key roles in E2-induced gene regulation (Nilsson et al., 2001; Nilsson and Gustafsson, 2002; Barkhem et al., 2004). Therefore, along with ER α , we also examined selected ER-co-regulators (MLL3, CBP/p300 and N-CoR) for enrichment at the HOXB9 promoter upon treatment with BPA or E2 (Dreijerink et al., 2006; Lee et al., 2006; Mo et al., 2006). Notably, MLLs are well-recognized histone methyl-transferases that specifically introduce histone H3 lysine-4 (H3K4) methylations and regulate gene activation (Barkhem et al., 2004; Ernst et al., 2004; Hess, 2004; Guenther et al., 2005; Ansari et al., 2009b; Ansari et al., 2009c; Lee et al., 2009; Ansari and Mandal, 2010; Mandal et al., 2010; Ansari et al., 2011a; Ansari et al., 2011b; Bhan et al., 2013). MLLs are recently implicated in estradiol/BPA induced gene activation (Ansari et al., 2009b; Ansari et al., 2011a; Ansari et al., 2011b; Bhan et al., 2013; Bhan et al., 2014a; Bhan et al., 2014b). In particular, our previous studies indicated involvement of MLL3 in HOXB9 gene regulation (Ansari et al., 2011b) 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 (Bulyanko and O'Malley, 2011; Ansari et al., 2013a). N-CoR is a repressive NR-coregulator that maintains the repressive state of gene in the absence of activated or liganded ERs (Shibata et al., 1997; Oesterreich et al., 2000; Jiang et al., 2006). 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 4) because it was the ERE regulating E2/BPA-treatment response in the luciferase analysis (Figure 3B). 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 ER α , 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 the HOXB9 promoter ERE4 region (also in other ERE, see Figure S1) (Figures 4B–C). ChIP analysis showed that ER α , MLL3, CBP and p300 were enriched in the ERE4 region in the presence of either BPA or E2 (Figures 4B–C). Beyond ERE4, we also found some amount of enrichment of ER α , MLL3, CBP and p300 in the ERE3 region while no significant binding were observed in the ERE1, ERE2 and the non-ERE regions (Figure S1). No significant binding of β -actin was observed at the HOXB9 promoter (used as a non-specificity control, Figures 4B–C). These observations demonstrate that, along with ERs, ER-coregulators such as histone methylase MLL3 and histone acetylases, CBP and p300, bind to HOXB9 promoter ERE (primarily ERE4) in a BPA-dependent manner and regulate BPA induced HOXB9 expression. We also found that the occupancy levels of nuclear receptor co-repressor (N-CoR) were 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 (Strahl and Allis, 2000; Martin et al., 2003; Bannister and Kouzarides, 2004; Hess, 2004; Guenther et al., 2005; Crawford and Hess, 2006). Since MLL3, CBP/p300 were 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) 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 4B–C). 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 suggests its potential involvement in the maintenance of the basal level HOXB9 transcription, especially in the absence of any stimuli such as E2 or BPA.

Discussion

HOXB9 is a critical player in skeletal and mammary gland development (Chen and Capecchi, 1999). Mice homozygous for HOXB9 mutation show developmental defects and a significant decline in newborn survival (Chen and Capecchi, 1997); it also leads to abnormal breast epithelium branching and lobulo-alveolar development, eventually leading to the inability of the mother to nurse pups (Chen and Capecchi, 1999). 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 (Pan et al., 2001). Recent studies demonstrate that HOXB9 is overexpressed in 42% of human breast tumors (Hayashida et al., 2010). 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 (Hayashida et al., 2010; Shrestha et al., 2012). Studies from our laboratory demonstrated that HOXB9, through its 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), TGF β -1 (tumor growth factor β -1) control 3D tumor colony formation (Shrestha et al., 2012). Furthermore, we demonstrated that HOXB9 expression is transcriptionally induced by E2, in cultured breast cancer cells (Ansari et al., 2011b). 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 publicly available cancer genome database, using Cbioportal data analysis software (Cerami et al., 2012; Gao et al., 2013). This analysis demonstrated that HOXB9 gene is amplified, mutated and deleted in variety of cancer cells (Figure 1). 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 mRNA expression is not only 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 coordinate with the estradiol-dependent gene activation and signaling (Brzozowski et al., 1997; Feng et al., 1998; Paige et al., 1999; Ansari et al., 2011b). These co-regulators usually possess enzymatic activity, modify chromatin, and bridge ERs with transcription machinery (Chen et al., 2004; Mo et al., 2006). 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), etc (Mangelsdorf et al., 1995; Lee et al., 2001; Dreijerink et al., 2006; Lee et al., 2009). Sequence analysis demonstrated that HOXB9 promoter contains multiple ERE1/2 sites (ERE1-3) and a potential imperfect full ERE (ERE4) (Nilsson and Gustafsson, 2002). 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 ER α 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 (Strahl and Allis, 2000; Martin et al., 2003; Bannister and Kouzarides, 2004; Hess, 2004; Guenther et al., 2005; Crawford and Hess, 2006). Studies from our laboratory and others have shown that MLLs functionally coordinate with ERs during estrogen-dependent gene activation (Ansari et al., 2009b; Ansari et al., 2011a; Ansari et al., 2013a; Ansari et al., 2013b; Bhan et al., 2013; Bhan et al., 2014a; Bhan et al., 2014b; Hussain et al., 2015). 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 (Ansari et al., 2011b). 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 increased the epigenetic marks of histone H3K4-trimethylation, and histone acetylation, and increased RNA polymerase II enrichment at the HOXB9 promoter region (ERE4). We also observed that NCoR, a nuclear receptor co-repressor, is localized at the HOXB9 promoter in the absence of E2 or BPA-treatment and is delocalized following 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 as well as 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 5). 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 classes 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 (Richter et al., 2007). Chronic and/or acute exposure to EDCs results in harmful health effects including birth defects, diabetes, cancers, reproductive problems, early puberty, and obesity (Fernandez and Russo, 2010; Abdel-Rahman et al., 2012; Fucic et al., 2012; Singh and Li, 2012; Sengupta et al., 2013). 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 (Schonfelder et al., 2002a). BPA is commonly found in plastics, metallic storage containers, and other routinely used consumables (Fernandez and Russo, 2010). 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 (Fernandez and Russo, 2010). The impacts of BPA exposure on human health have been extensively studied. Exposure to BPA leads to developmental and reproductive anomalies (Schonfelder et al., 2002a). Perinatal exposure to BPA alters mammary gland development *in vivo* (Schonfelder et al., 2002a). BPA exposure has also been shown to alter the epigenetic states at the cellular level and that may be transmitted through multiple passages post BPA exposure. In particular, a study by Weng et al, showed that low-dose BPA treatment to breast epithelial cells (progenitor cells) induced epigenetic changes that were transmitted to their differentiated progeny through various epigenetic mechanisms even when the cells were not exposed to BPA at the differentiation stages (Weng et al., 2010). Low-dose BPA exposure to breast epithelial progenitor cells induced nuclear internalization of ER α and affected ER-signaling pathways and ER-target genes expression. Upon exposure to BPA, the expression of lysosomal-associated membrane protein 3 (LAMP3) became epigenetically silenced via promoter CpG-island methylation, in breast epithelial cells (Weng et al., 2010). Similarly, an independent study by Qin et al examined the effect of BPA on cellular proliferation and senescence in human mammary epithelial cells (HMEC) (Qin et al., 2012). These studies demonstrated that BPA exposure at an early passage stage increased the proliferation and the sphere size of HMEC up to passage 16. Briefly, early exposure to BPA induced epigenetic alterations in the form of epigenetic memory and that were transmitted for several generations (passages) even in the absence of BPA. The results of BPA-induced epigenetic alterations include increased protein levels of p16 and cyclin E, which are known to induce cellular senescence and promote proliferation, respectively. Furthermore, DNA methylation levels of genes related to development of most or all tumor types, such as BRCA1, CCNA1, CDKN2A (p16), and others, were increased in BPA-exposed HMEC. These observations suggest that BPA has the capability to modulate cell growth in breast epithelial cells via

modulation of the epigenetic states and these may be heritable and may contribute to abnormal mammary development and in mammary carcinogenesis (Qin et al., 2012). 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank all the Mandal lab members for helpful discussions. Research in Mandal laboratory is supported by grant from NIH (1R15 ES019129-01) and Pandita laboratory is supported by RO1 CA129537, RO1GM109768 and RO1CA154320.

Abbreviations

HOXB9	Homeobox-containing gene B9
E2	17-beta-estradiol
BPA	Bisphenol-A
ER	Estrogen-receptor
MLL	Mixed Lineage Leukemia
CBP	CREB Binding protein
ERE	Estrogen-response element
OVX	Ovariectomized
EDC	Endocrine disrupting chemical

References

- Abdel-Rahman WM, Moustafa YM, Ahmed BO, Mostafa RM. Endocrine Disruptors and Breast Cancer Risk - Time to Consider the Environment. Asian Pacific Journal of Cancer Prevention. 2012; 13:5937–5946.
- Ansari KI, Grant JD, Kasiri S, Woldemariam G, Shrestha B, Mandal SS. Manganese(III)-salens induce tumor selective apoptosis in human cells. Journal of Inorganic Biochemistry. 2009a; 103:818–826. [PubMed: 19297027]

- Ansari KI, Hussain I, Kasiri S, Mandal SS. HOXC10 is overexpressed in breast cancer and transcriptionally regulated by estrogen via involvement of histone methylases MLL3 and MLL4. *J Mol Endocrinol*. 2012a; 48:61–75. [PubMed: 22143955]
- Ansari KI, Hussain I, Shrestha B, Kasiri S, Mandal SS. HOXC6 Is Transcriptionally Regulated via Coordination of MLL Histone Methylase and Estrogen Receptor in an Estrogen Environment. *Journal of Molecular Biology*. 2011a; 411:334–349. [PubMed: 21683083]
- Ansari KI, Kasiri S, Hussain I, Bobzean SA, Perrotti LI, Mandal SS. MLL histone methylases regulate expression of HDLR-SR-B1 in presence of estrogen and control plasma cholesterol in vivo. *Molecular Endocrinology*. 2013a; 27:92–105. [PubMed: 23192982]
- Ansari KI, Kasiri S, Hussain I, Mandal SS. Mixed lineage leukemia histone methylases play critical roles in estrogen-mediated regulation of HOXC13. *Febs Journal*. 2009b; 276:7400–7411. [PubMed: 19922474]
- Ansari KI, Kasiri S, Mandal SS. Histone methylase MLL1 has critical roles in tumor growth and angiogenesis and its knockdown suppresses tumor growth in vivo. *Oncogene*. 2013b; 32:3359–3370. [PubMed: 22926525]
- Ansari KI, Kasiri S, Mishra BP, Mandal SS. Mixed lineage leukaemia-4 regulates cell-cycle progression and cell viability and its depletion suppresses growth of xenografted tumour in vivo. *Br J Cancer*. 2012b; 107:315–24. [PubMed: 22713656]
- Ansari KI, Mandal SS. Mixed lineage leukemia: roles in gene expression, hormone signaling and mRNA processing. *Febs Journal*. 2010; 277:1790–1804. [PubMed: 20236313]
- Ansari KI, Mishra BP, Mandal SS. Human CpG binding protein interacts with MLL1, MLL2 and hSet1 and regulates Hox gene expression. *Biochim Biophys Acta*. 2008; 1779:66–73. [PubMed: 18082152]
- Ansari KI, Mishra BP, Mandal SS. MLL histone methylases in gene expression, hormone signaling and cell cycle. *Front Biosci (Landmark Ed)*. 2009c; 14:3483–95. [PubMed: 19273288]
- Ansari KI, Shrestha B, Hussain I, Kasiri S, Mandal SS. Histone Methylases MLL1 and MLL3 Coordinate with Estrogen Receptors in Estrogen-Mediated HOXB9 Expression. *Biochemistry*. 2011b; 50:3517–3527. [PubMed: 21428455]
- Bannister AJ, Kouzarides T. Histone methylation: Recognizing the methyl mark. *Chromatin and Chromatin Remodeling Enzymes, Pt B*. 2004; 376:269.
- Barkhem T, Nilsson S, Gustafsson JA. Molecular mechanisms, physiological consequences and pharmacological implications of estrogen receptor action. *Am J Pharmacogenomics*. 2004; 4:19–28. [PubMed: 14987119]
- Betancourt AM, Eltoum IA, Desmond RA, Russo J, Lamartiniere CA. In Utero Exposure to Bisphenol A Shifts the Window of Susceptibility for Mammary Carcinogenesis in the Rat. *Environmental Health Perspectives*. 2010; 118:1614–1619. [PubMed: 20675265]
- Bhan A, Hussain I, Ansari KI, Bobzean SA, Perrotti LI, Mandal SS. Bisphenol-A and diethylstilbestrol exposure induces the expression of breast cancer associated long noncoding RNA HOTAIR in vitro and in vivo. *J Steroid Biochem Mol Biol*. 2014a; 141:160–70. [PubMed: 24533973]
- Bhan A, Hussain I, Ansari KI, Bobzean SA, Perrotti LI, Mandal SS. Histone methyltransferase EZH2 is transcriptionally induced by estradiol as well as estrogenic endocrine disruptors bisphenol-A and diethylstilbestrol. *J Mol Biol*. 2014b; 426:3426–41. [PubMed: 25088689]
- Bhan A, Hussain I, Ansari KI, Kasiri S, Bashyal A, Mandal SS. Antisense transcript long noncoding RNA (lncRNA) HOTAIR is transcriptionally induced by estradiol. *Journal of Molecular Biology*. 2013; 425:3707–22. [PubMed: 23375982]
- Biles JE, McNeal TP, Begley TH, Hollifield HC. Determination of bisphenol-A in reusable polycarbonate food-contact plastics and migration to food-simulating liquids. *Journal of Agricultural and Food Chemistry*. 1997; 45:3541–3544.
- Bobzean SAM, Dennis TS, Perrotti LI. Acute estradiol treatment affects the expression of cocaine-induced conditioned place preference in ovariectomized female rats. *Brain Research Bulletin*. 2014; 103:49–53. [PubMed: 24568745]
- Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V, Olea N. Xenoestrogens released from lacquer coatings in food cans. *Environ Health Perspect*. 1995; 103:608–12. [PubMed: 7556016]

- Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, Carlquist M. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*. 1997; 389:753–8. [PubMed: 9338790]
- Bulyanko YA, O'Malley BW. Nuclear Receptor Coactivators: Structural and Functional Biochemistry. *Biochemistry*. 2011; 50:313–328. [PubMed: 21141906]
- Calafat AM, Kuklenyik Z, Reidy JA, Caudill SP, Ekong J, Needham LL. Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. *Environmental Health Perspectives*. 2005; 113:391–395. [PubMed: 15811827]
- Cerami E, Gao JJ, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C, Schultz N. The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data. *Cancer Discovery*. 2012; 2:401–404. [PubMed: 22588877]
- Chen BS, Mandal SS, Hampsey M. High-resolution protein-DNA contacts for the yeast RNA polymerase II general transcription machinery. *Biochemistry*. 2004; 43:12741–9. [PubMed: 15461446]
- Chen F, Capecchi MR. Targeted mutations in *hoxa-9* and *hoxb-9* reveal synergistic interactions. *Dev Biol*. 1997; 181:186–96. [PubMed: 9013929]
- Chen F, Capecchi MR. Paralogous mouse Hox genes, *Hoxa9*, *Hoxb9*, and *Hoxd9*, function together to control development of the mammary gland in response to pregnancy. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96:541–546. [PubMed: 9892669]
- Chiba N, Comaills V, Shiotani B, Takahashi F, Shimada T, Tajima K, Winokur D, Hayashida T, Willers H, Brachtel E, Vivanco MD, Haber DA, Zou L, Maheswaran S. Homeobox B9 induces epithelial-to-mesenchymal transition-associated radioresistance by accelerating DNA damage responses. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109:2760–2765. [PubMed: 21930940]
- Crawford BD, Hess JL. MLL core components give the green light to histone methylation. *Acs Chemical Biology*. 2006; 1:495–498. [PubMed: 17168535]
- Darda L, Hakami F, Morgan R, Murdoch C, Lambert DW, Hunter KD. The role of HOXB9 and miR-196a in head and neck squamous cell carcinoma. *PLoS One*. 2015; 10:e0122285. [PubMed: 25860510]
- Dreijerink KM, Mulder KW, Winkler GS, Hoppener JW, Lips CJ, Timmers HT. Menin links estrogen receptor activation to histone H3K4 trimethylation. *Cancer Res*. 2006; 66:4929–35. [PubMed: 16651450]
- Eilam-Stock T, Serrano P, Frankfurt M, Luine V. Bisphenol-A impairs memory and reduces dendritic spine density in adult male rats. *Behav Neurosci*. 2012; 126:175–85. [PubMed: 22004261]
- Ernst P, Mabon M, Davidson AJ, Zon LI, Korsmeyer SJ. An Mll-dependent Hox program drives hematopoietic progenitor expansion. *Curr Biol*. 2004; 14:2063–9. [PubMed: 15556871]
- Fang L, Xu Y, Zou L. Overexpressed homeobox B9 regulates oncogenic activities by transforming growth factor-beta1 in gliomas. *Biochem Biophys Res Commun*. 2014; 446:272–9. [PubMed: 24582746]
- Feng W, Ribeiro RC, Wagner RL, Nguyen H, Apreletti JW, Fletterick RJ, Baxter JD, Kushner PJ, West BL. Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. *Science*. 1998; 280:1747–9. [PubMed: 9624051]
- Fernandez SV, Russo J. Estrogen and Xenoestrogens in Breast Cancer. *Toxicologic Pathology*. 2010; 38:110–122. [PubMed: 19933552]
- Fucic A, Gamulin M, Ferencic Z, Katic J, von Krauss MK, Bartonova A, Merlo DF. Environmental exposure to xenoestrogens and oestrogen related cancers: reproductive system, breast, lung, kidney, pancreas, and brain. *Environmental Health*. 2012; 11
- Gao JJ, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun YC, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C, Schultz N. Integrative Analysis of Complex Cancer Genomics and Clinical Profiles Using the cBioPortal. *Science Signaling*. 2013; 6
- Guenther MG, Jenner RG, Chevalier B, Nakamura T, Croce CM, Canaani E, Young RA. Global and Hox-specific roles for the MLL1 methyltransferase. *Proc Natl Acad Sci U S A*. 2005; 102:8603–8. [PubMed: 15941828]

- Hatzis P, van der Flier LG, van Driel MA, Guryev V, Nielsen F, Denissov S, Nijman IJ, Koster J, Santo EE, Welboren W, Versteeg R, Cuppen E, van de Wetering M, Clevers H, Stunnenberg HG. Genome-wide pattern of TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells. *Molecular and Cellular Biology*. 2008; 28:2732–2744. [PubMed: 18268006]
- Hayashida T, Takahashi F, Chiba N, Brachtel E, Takahashi M, Godin-Heymann N, Gross KW, Vivanco MDM, Wijendran V, Shioda T, Sgroi D, Donahoe PK, Maheswaran S. HOXB9, a gene overexpressed in breast cancer, promotes tumorigenicity and lung metastasis. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:1100–1105. [PubMed: 20080567]
- Hess JL. MLL: a histone methyltransferase disrupted in leukemia. *Trends in Molecular Medicine*. 2004; 10:500–7. [PubMed: 15464450]
- Honma S, Suzuki A, Buchanan DL, Katsu Y, Watanabe H, Iguchi T. Low dose effect of in utero exposure to bisphenol A and diethylstilbestrol on female mouse reproduction. *Reprod Toxicol*. 2002; 16:117–22. [PubMed: 11955942]
- Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenberg JG, vom Saal FS. Exposure to bisphenol A advances puberty. *Nature*. 1999; 401:763–4. [PubMed: 10548101]
- Hueber SD, Lohmann I. Shaping segments: Hox gene function in the genomic age. *Bioessays*. 2008; 30:965–979. [PubMed: 18798525]
- Hussain I, Bhan A, Ansari KI, Deb P, Bobzean SA, Perrotti LI, Mandal SS. Bisphenol-A induces expression of HOXC6, an estrogen-regulated homeobox-containing gene associated with breast cancer. *Biochim Biophys Acta*. 2015; 1849:697–708. [PubMed: 25725483]
- Ikezaki Y, Tsutsumi O, Takai Y, Kamei Y, Taketani Y. Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. *Hum Reprod*. 2002; 17:2839–41. [PubMed: 12407035]
- Jiang SM, Meyer R, Kang KY, Osborne CK, Wong JM, Oesterreich S. Scaffold attachment factor SAFB1 suppresses estrogen receptor alpha-mediated transcription in part via interaction with nuclear receptor corepressor. *Molecular Endocrinology*. 2006; 20:311–320. [PubMed: 16195251]
- Lappin TR, Grier DG, Thompson A, Halliday HL. HOX genes: seductive science, mysterious mechanisms. *Ulster Med J*. 2006; 75:23–31. [PubMed: 16457401]
- Lee JW, Lee YC, Na SY, Jung DJ, Lee SK. Transcriptional coregulators of the nuclear receptor superfamily: coactivators and corepressors. *Cell Mol Life Sci*. 2001; 58:289–97. [PubMed: 11289310]
- Lee S, Kim DH, Goo YH, Lee YC, Lee SK, Lee JW. Crucial Roles for Interactions between MLL3/4 and INI1 in Nuclear Receptor Transactivation. *Molecular Endocrinology*. 2009; 23:610–619. [PubMed: 19221051]
- Lee S, Lee DK, Dou YL, Lee J, Lee B, Kwak E, Kong YY, Lee SK, Roeder RG, Lee JW. Coactivator as a target gene specificity determinant for histone H3 lysine 4 methyltransferases. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103:15392–15397. [PubMed: 17021013]
- MacLusky NJ, Hajszan T, Leranth C. The environmental estrogen bisphenol a inhibits estradiol-induced hippocampal synaptogenesis. *Environmental Health Perspectives*. 2005; 113:675–679. [PubMed: 15929888]
- Maeda K, Hamada J, Takahashi Y, Tada M, Yamamoto Y, Sugihara T, Moriuchi T. Altered expressions of HOX genes in human cutaneous malignant melanoma. *International Journal of Cancer*. 2005; 114:436–441. [PubMed: 15551325]
- Mallo M, Alonso CR. The regulation of Hox gene expression during animal development. *Development*. 2013; 140:3951–3963. [PubMed: 24046316]
- Mandal SS, Ansari KI, Hussain I, Kasiri S, Shrestha B. MLL histone methylases in estrogen-mediated regulation of HOX genes involved in hair follicle development and leukemia. *Faseb Journal*. 2010; 24
- Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. The nuclear receptor superfamily: the second decade. *Cell*. 1995; 83:835–9. [PubMed: 8521507]

- Markey CM, Coombs MA, Sonnenschein C, Soto AM. Mammalian development in a changing environment: exposure to endocrine disruptors reveals the developmental plasticity of steroid-hormone target organs. *Evol Dev*. 2003; 5:67–75. [PubMed: 12492412]
- Martin ME, Milne TA, Bloyer S, Galoian K, Shen W, Gibbs D, Brock HW, Slany R, Hess JL. Dimerization of MLL fusion proteins immortalizes hematopoietic cells. *Cancer Cell*. 2003; 4:197–207. [PubMed: 14522254]
- Mo R, Rao SM, Zhu YJ. Identification of the MLL2 complex as a coactivator for estrogen receptor alpha. *Journal of Biological Chemistry*. 2006; 281:15714–15720. [PubMed: 16603732]
- Munoz-de-Toro M, Markey CM, Wadia PR, Luque EH, Rubin BS, Sonnenschein C, Soto AM. Perinatal exposure to bisphenol-A alters peripubertal mammary gland development in mice. *Endocrinology*. 2005; 146:4138–47. [PubMed: 15919749]
- Murray TJ, Maffini MV, Ucci AA, Sonnenschein C, Soto AM. Induction of mammary gland ductal hyperplasias and carcinoma in situ following fetal bisphenol A exposure. *Reprod Toxicol*. 2007; 23:383–90. [PubMed: 17123778]
- Nguyen DX, Chiang AC, Zhang XHF, Kim JY, Kris MG, Ladanyi M, Gerald WL, Massague J. WNT/TCF Signaling through LEF1 and HOXB9 Mediates Lung Adenocarcinoma Metastasis. *Cell*. 2009; 138:51–62. [PubMed: 19576624]
- Nilsson S, Gustafsson JA. Estrogen receptor transcription and transactivation: Basic aspects of estrogen action. *Breast Cancer Res*. 2000; 2:360–6. [PubMed: 11250729]
- Nilsson S, Gustafsson JA. Estrogen receptor action. *Critical Reviews in Eukaryotic Gene Expression*. 2002; 12:237–257. [PubMed: 12641394]
- Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA. Mechanisms of estrogen action. *Physiological Reviews*. 2001; 81:1535–65. [PubMed: 11581496]
- Oesterreich S, Zhang QP, Hopp T, Fuqua SAW, Michaelis M, Zhao HH, Davie JR, Osborne CK, Lee AV. Tamoxifen-bound estrogen receptor (ER) strongly interacts with the nuclear matrix protein HET/SAF-B, a novel inhibitor of ER-mediated transactivation. *Molecular Endocrinology*. 2000; 14:369–381. [PubMed: 10707955]
- Olea N, Pulgar R, Perez P, OleaSerrano F, Rivas A, NovilloFertrell A, Pedraza V, Soto AM, Sonnenschein C. Estrogenicity of resin-based composites and sealants used in dentistry. *Environmental Health Perspectives*. 1996; 104:298–305. [PubMed: 8919768]
- Paige LA, Christensen DJ, Gron H, Norris JD, Gottlin EB, Padilla KM, Chang CY, Ballas LM, Hamilton PT, McDonnell DP, Fowlkes DM. Estrogen receptor (ER) modulators each induce distinct conformational changes in ER alpha and ER beta. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96:3999–4004. [PubMed: 10097152]
- Pan L, Xie YH, Black TA, Jones CA, Pruitt SC, Gross KW. An Abd-B class HOX center dot PBX recognition sequence is required for expression from the mouse Ren-1(c) gene. *Journal of Biological Chemistry*. 2001; 276:32489–32494. [PubMed: 11432851]
- Qin XY, Fukuda T, Yang LQ, Zaha H, Akanuma H, Zeng Q, Yoshinaga J, Sone H. Effects of bisphenol A exposure on the proliferation and senescence of normal human mammary epithelial cells. *Cancer Biology & Therapy*. 2012; 13:296–306. [PubMed: 22258036]
- Richter CA, Birnbaum LS, Farabollini F, Newbold RR, Rubin BS, Talsness CE, Vandenbergh JG, Walser-Kuntz DR, vom Saal FSV. In vivo effects of bisphenol A in laboratory rodent studies. *Reproductive Toxicology*. 2007; 24:199–224. [PubMed: 17683900]
- Rubin BS, Murray MK, Damassa DA, King JC, Soto AM. Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. *Environ Health Perspect*. 2001; 109:675–80. [PubMed: 11485865]
- Schonfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, Chahoud I. Parent bisphenol A accumulation in the human maternal-fetal-placental unit. *Environmental Health Perspectives*. 2002a; 110:A703–A707. [PubMed: 12417499]
- Schonfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, Chahoud I. Parent bisphenol A accumulation in the human maternal-fetal-placental unit. *Environ Health Perspect*. 2002b; 110:A703–7. [PubMed: 12417499]

- Seki H, Hayashida T, Jinno H, Hirose S, Sakata M, Takahashi M, Maheswaran S, Mukai M, Kitagawa Y. HOXB9 Expression Promoting Tumor Cell Proliferation and Angiogenesis Is Associated with Clinical Outcomes in Breast Cancer Patients. *Annals of Surgical Oncology*. 2012; 19:1831–1840. [PubMed: 22396001]
- Sengupta S, Obiorah I, Maximov PY, Curpan R, Jordan VC. Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells. *British Journal of Pharmacology*. 2013; 169:167–178. [PubMed: 23373633]
- Sha L, Dong L, Lv L, Bai L, Ji X. HOXB9 promotes epithelial-to-mesenchymal transition via transforming growth factor-beta1 pathway in hepatocellular carcinoma cells. *Clin Exp Med*. 2015; 15:55–64. [PubMed: 25081022]
- Shibata H, Spencer TE, Onate SA, Jenster G, Tsai SY, Tsai MJ, O'Malley BW. Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. *Recent Progress in Hormone Research, Proceedings of the 1996 Conference*. 1997; 52:141–165.
- Shrestha B, Ansari KI, Bhan A, Kasiri S, Hussain I, Mandal SS. Homeodomain-containing protein HOXB9 regulates expression of growth and angiogenic factors, facilitates tumor growth in vitro and is overexpressed in breast cancer tissue. *FEBS J*. 2012; 279:3715–26. [PubMed: 22863320]
- Singh S, Li SSL. Epigenetic Effects of Environmental Chemicals Bisphenol A and Phthalates. *International Journal of Molecular Sciences*. 2012; 13:10143–10153. [PubMed: 22949852]
- Strahl BD, Allis CD. The language of covalent histone modifications. *Nature*. 2000; 403:41–45. [PubMed: 10638745]
- Takahashi Y, Hamada J, Murakawa K, Takada M, Tada M, Nogami I, Hayashi N, Nakamori S, Monden M, Miyamoto M, Katoh H, Moriuchi T. Expression profiles of 39 HOX genes in normal human adult organs and anaplastic thyroid cancer cell lines by quantitative real-time RT-PCR system. *Experimental Cell Research*. 2004; 293:144–153. [PubMed: 14729064]
- Tomioka N, Morita K, Kobayashi N, Tada M, Itoh T, Saitoh S, Kondo M, Takahashi N, Kataoka A, Nakanishi K, Takahashi M, Kamiyama T, Ozaki M, Hirano T, Todo S. Array comparative genomic hybridization analysis revealed four genomic prognostic biomarkers for primary gastric cancers. *Cancer Genet Cytogenet*. 2010; 201:6–14. [PubMed: 20633762]
- Weng YI, Hsu PY, Liyanarachchi S, Liu J, Deatherage DE, Huang YW, Zuo T, Rodriguez B, Lin CH, Cheng AL, Huang TH. Epigenetic influences of low-dose bisphenol A in primary human breast epithelial cells. *Toxicol Appl Pharmacol*. 2010; 248:111–21. [PubMed: 20678512]
- Yu BD, Hess JL, Horning SE, Brown GAJ, Korsmeyer SJ. Altered Hox Expression and Segmental Identity in *Mil*-Mutant Mice. *Nature*. 1995; 378:505–508. [PubMed: 7477409]
- Zhan J, Wang P, Niu M, Wang Y, Zhu X, Guo Y, Zhang H. High expression of transcriptional factor HoxB9 predicts poor prognosis in patients with lung adenocarcinoma. *Histopathology*. 2015; 66:955–65. [PubMed: 25324169]

Highlights

- a.** HOXB9 is a homeobox containing gene associated with breast cancer
- b.** HOXB9 expression is induced by estradiol (E2) in vitro and in vivo
- c.** HOXB9 expression is also induced by endocrine disrupting chemical BPA
- d.** ERs coordinate with MLLs during E2/BPA-induced HOXB9 expression
- e.** Results provide mechanistic insight on HOXB9 gene regulation and its endocrine disruption

Figure 1. Cross-cancer analysis of copy number alterations and mutations in HOXB9 based on cBioPortal database (Cerami et al., 2012; Gao et al., 2013)

Figure 2.

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 (panel 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. (Panel E and F are RT-PCR and qPCR analyses of HOXB9 expression upon exposure to varying concentrations of BPA (0 – 100 nM) in the absence and presence of 1 nM E2. *In vivo* effect of E2 and BPA on HOXB9 expression. Ovariectomized adult female rats were administered estradiol (5ug/kg) or BPA (25ug/kg) at 24 h and 4 h before sacrifice. RNA isolated from mammary glands of control, E2 or BPA treated rats were analyzed by RT-PCR (panel G) and qPCR (Panel H). 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 < 0.05).

Figure 3. HOXB9 promoter EREs are responsive to BPA treatment. (A) 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.

Figure 4. 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.

Figure 5.

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 dimerize 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 (Chen et al., 2004), 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.

Table 1

Nucleotide sequence of primers

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

* Cloning primers flanked by appropriate restriction sites