***HOXC10* is overexpressed in breast cancer and transcriptionally regulated by estrogen via involvement of histone methylases MLL3 and MLL4**

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Abstract

HOXC10 is a critical player in the development of spinal cord, formation of neurons, and associated with human leukemia. We found that *HOXC10* is overexpressed in breast cancer and transcriptionally regulated by estrogen (17 β -estradiol, E₂). The *HOXC10* promoter contains several estrogen response elements (ERE1–7, half-sites). A luciferase-based reporter assay showed that ERE1 and ERE6 of *HOXC10* promoter are E₂ responsive. ER α and ER β play critical roles in E₂-mediated activation of *HOXC10*. Knockdown of ER α and ER β downregulated E₂-induced *HOXC10* expression. ER α and ER β bind to ERE1 and ERE6 regions in an E₂-dependent manner. Additionally, knockdown of histone methylases *MLL3* and *MLL4* (but not *MLL1* and *MLL2*) diminished E₂-induced expression of *HOXC10*. *MLL3* and *MLL4* were bound to the ERE1 and ERE6 regions of *HOXC10* promoter in an E₂-dependent manner. Overall, we demonstrated that *HOXC10* is overexpressed in breast cancer, and it is an E₂-responsive gene. Histone methylases *MLL3* and *MLL4*, along with ERs, regulate *HOXC10* gene expression in the presence of E₂.

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Introduction

Homeobox (*HOX*) genes are evolutionarily conserved genes that play critical roles in cell differentiation and embryonic development (Lawrence *et al.* 1996, Lappin *et al.* 2006). *HOX* genes are also essential in differentiation of adult tissues (Daftary & Taylor 2006). In general, *HOX* genes encode transcription factors that bind to promoters of various target genes through their homeodomain controlling their expression. *HOX* genes are associated in specifying primary and secondary body axis of embryos in animals (Gehring 1993, Huang *et al.* 2007). In humans, there are 39 *HOX* genes arranged in four clusters (*HOX A–D*) located in four different chromosomes. Based on sequence similarities and location within the cluster, *HOX* genes are classified into 13 paralogous groups (Alexander *et al.* 2009). *Hox* genes located in the 3'-end of the cluster are expressed in the anterior (head) region of the embryo, while genes located in the 5'-end of the cluster are expressed in the exterior (tail) region. The colinearity of expression boundaries and position is highly conserved from flies to mammals (Alexander *et al.* 2009). *HOX* proteins are also associated with oncogenic transformation of hematopoietic cells (van Oostveen *et al.* 1999, Fu *et al.* 2003).

Hoxa10, *Hoxc10*, and *Hoxd10* are three paralogous genes that are expressed in the lumbar spinal cord and have distinct expression patterns (Choe *et al.* 2006).

Inactivation of these genes either in combination or alone affects motor neuron patterning (Choe *et al.* 2006). *Hoxc10* and paralogous genes have also been implicated in endometrial differentiation (Akbas *et al.* 2004). Gene expression analysis in preinvasive and invasive cervical cancer cells demonstrated that *HOXC10* expression is elevated in invasive cells and linked with the invasiveness of human papillomavirus-immortalized keratinocytes and cervical cancer cells (Lopez *et al.* 2006, Zhai *et al.* 2007). Knockdown of *HOXC10* expression reduces the invasiveness of cervical cancer cells, indicating its key role in cervical cancer progression (Zhai *et al.* 2007). Although *HOXC10* is a critical gene in the spinal cord and neuronal development and in cervical cancer progression, the mechanism of *HOXC10* gene expression still remains elusive. Recently, several hormones (such as estrogen, progesterone, and retinoic acids) have been shown to regulate different *HOX* genes and allow generation of structural and functional diversity in embryonic and adult tissues (Ma *et al.* 1998, Lane *et al.* 2004, Daftary & Taylor 2006, Huang *et al.* 2007, Taylor 2008).

Mixed lineage leukemias (MLLs) are well known as master regulators of *HOX* genes (Hess 2004, Guenther *et al.* 2005). MLLs are evolutionarily conserved family of histone methyltransferases (HMTs) that specifically methylate histone H3 at lysine 4 (H3K4) and regulate gene activation (Hanson *et al.* 1999, Yokoyama *et al.* 2004, Bannister & Kouzarides 2005, Glaser *et al.* 2006,

Sims & Reinberg 2006, Bhaumik *et al.* 2007, Issaeva *et al.* 2007, Agger *et al.* 2008, Ansari *et al.* 2009*a,b,c*). MLLs are also associated with various oncogenic transformations including myeloid and lymphoid leukemia (So & Cleary 2004, Meyer *et al.* 2006). In humans, there are several proteins of MLL family such as MLL1, MLL2, MLL3, MLL4, etc. They exist as distinct multiprotein complexes inside cells with several common subunits including ASH2, WDR5, RBBP5, CGBP, and DPY30 (Lee & Skalnik 2005, Crawford & Hess 2006, Dou *et al.* 2006, Ansari *et al.* 2008, Trievel & Shilatifard 2009). Recently, we have demonstrated that MLL1 and H3K4 trimethylation have distinct dynamics during cell cycle progression and play critical roles in gene activation (Ansari *et al.* 2008, 2009*a,b,c*, Mishra *et al.* 2009). Knockdown of MLL1 results in cell cycle arrest at G2/M phase, suggesting its critical role in cell cycle progression (Takeda *et al.* 2006, Ansari *et al.* 2009*a,b,c*). In particular relevance to our study, sequence analysis demonstrated that MLLs (MLL1–4) possess one or more LXXLL domains (also called NR-box) (Dreijerink *et al.* 2006, Mo *et al.* 2006, Ansari *et al.* 2009*a,b,c*, Lee *et al.* 2009). Proteins containing NR-boxes are well known to interact with nuclear hormone receptors and play key roles in hormone signaling (Nilsson *et al.* 2001, Lee *et al.* 2006, 2009). Indeed, MLL2 interacts with estrogen receptors (ERs) and regulates estrogen-dependent activation of cathepsin D (Mo *et al.* 2006). Similarly, MLL3 and MLL4 play essential roles in nuclear receptor-mediated gene activation including liver X-receptor (LXR; Lee *et al.* 2006, 2009).

Herein, in an effort to understand the mechanism of *HOX* gene regulation by MLLs, we found that *HOXC10* is overexpressed in breast cancer and is transcriptionally regulated by estrogen. Mechanistic studies demonstrated that histone methylases MLL3 and MLL4, in coordination with ERs, play critical roles in estrogen-mediated activation of *HOXC10*.

Materials and methods

Cell culture, estrogen treatment, and antisense experiment

Human choriocarcinoma placenta cells (JAR), human adenocarcinoma mammary (MCF7), ER-negative human adenocarcinoma mammary (MDA-MB-231), ductal carcinoma mammary (T47D), hepatocellular carcinoma (HEPG2), cervical cancer (HeLa), colorectal adenocarcinoma (SW480), chronic myelogenous leukemia (K562), bronchoalveolar carcinoma (H358), normal breast epithelial (MCF10), normal lung fibroblast (HFL1), and normal placenta (Hs 798.PI) cells were obtained from ATCC (Manassas, VA, USA) and grown in Phenol Red-free DMEM-F-12 (or RPMI as

needed, Sigma), supplemented with 10% charcoal-stripped fetal bovine serum, 2 mM L-glutamine, and penicillin/streptomycin (100 units and 0.1 mg/ml). For estrogen treatment experiments, cells were grown in 10 ml culture dish to 70% confluency and treated with different concentrations (0–1000 nM) of estrogen (17 β -estradiol, E₂) in the absence or presence of tamoxifen and incubated for 8 h (or varying time points for temporal studies) and then harvested for RNA and proteins extraction.

For the antisense experiments, JAR cells were grown up to 60% confluency in 60 mm culture plate and transfected with varying amounts (3–9 μ g) of different antisenses in FBS-free media as described by us previously (Ansari *et al.* 2008, 2009*a,b,c*; Table 1). In brief, a cocktail of antisense and infect transfection reagents (MolecularA) was made in 300 μ l culture media (without supplements) as instructed by the manufacturer. Cells were washed twice with culture media (without supplements) and 1.7 ml media (without supplements) was added to the plate. The antisense-transfection reagent cocktail was applied to the cells, incubated for 7 h before addition of 2 ml culture media with all supplements and 20% charcoal-stripped FBS. Cells were then incubated for an additional 48 h.

Preparation of RNA and protein extract

The RNA and proteins were extracted using similar procedure as described by us previously (Ansari *et al.* 2008, 2009*a,b,c*). In brief, the cell pellets were resuspended in diethyl pyrocarbonate (DEPC)-treated buffer A (20 mM Tris-HCl, pH 7.9; 1.5 mM MgCl₂; 10 mM KCl and 0.5 mM dithiothreitol (DTT); and 0.2 mM phenylmethylsulphonyl fluoride), incubated on ice for 10 min, and centrifuged at 3500 *g* for 5 min. The supernatant containing the cytoplasmic extracts was subjected to phenol-chloroform extraction followed by LiCl precipitation of cytoplasmic mRNA by incubating for 1 h at –80 °C. The RNA was washed with DEPC-treated 70% EtOH, air dried, and resuspended in DEPC-treated water.

Reverse transcription-PCR and western blotting

Reverse transcription (RT) reactions were performed in a total volume of 25 μ l containing 500 ng RNA, 2.4 μ M oligo-dT (Promega), 100 units MMLV reverse transcriptase, 1 $\times$ first-strand buffer (Promega), 100 μ M each of dATP, dGTP, dCTP, and dTTP (Invitrogen), 1 mM DTT, and 20 units RNaseOut (Invitrogen). The cDNA was diluted to 100 μ l. PCR was performed in a 10 μ l reaction volume containing 5 μ l diluted cDNA and gene-specific primer pairs (Table 1). For western blot analysis, an equivalent amount of cell protein extracts (25 μ g) was

Table 1 Primers used for RT-PCR and ChIP experiments

Primers	Forward primer (5'–3')	Reverse primer (5'–3')
(A) PCR primer		
GAPDH	CAATGACCCCTTCATTGACC	GACAAGCTTCCCGTTCTCAG
<i>HOXC10</i> -ORF	ACCACAGGAAATTGGCTGAC	GATCCGATTCTCTCGGTTCA
MLL1	GAGGACCCCGGATTAAACAT	GGAGCAAGAGGTTTACGATC
MLL2	GTGCAGCAGAAGATGGTGAA	GCACAATGCTGTCAGGAGAA
MLL3	AAGCAAACGGACTCAGAGGA	ACAAGCCATAGGAGGTGGTG
MLL4	GTCTATGCGCAGTGAGACA	AGTCTGCATCCCGTATTTG
<i>HOXC10</i> -ERE1	ATGGCGCTGTTACTCCACTC	CCAATGGGATTTGAAAATGG
<i>HOXC10</i> -ERE2	TGACCCCTCCCTGGCCCA	CTCTCCCGCTCCTCTTTT
<i>HOXC10</i> -ERE3	TGGCTTCCTCTTTCCCTAC	ACTGGGAATCTCCAGCCTCT
<i>HOXC10</i> -ERE4	CTGAGATGGTGACCCAGGAG	AAAGGAGGTTCCGAATGGAC
<i>HOXC10</i> -ERE5	CGCAGGTTCCCTCTTTCTTT	CCGTTTCTGCTGCTTTACTT
<i>HOXC10</i> -ERE6	CGTGCGCTCTGTAAGGAC	GAGCCTCGGTTCAGTTTGAG
<i>HOXC10</i> -ERE7	CAAGTCTGGGGCCTAAACAG	CCGAAAACTGGAAGGAAAT
<i>HOXC10</i> -non-ERE	TCTTGTGAGGAGTGGAGT	CAAGGGAGAGGACAGAGTCG
ERE1-pGL3	CCCTTCTCCCCAGGAGTTAC ^a	AGGGGAGGGAGGAAAAAGA ^a
ERE2/3-pGL3	TGGCTTCCTCTTTCCCTAC ^a	CTTACCTTGGGGATGGGAGT ^a
ERE4/5-pGL3	CGCAGGTTCCCTCTTTCTTT ^a	AAAGGAGGTTCCGAATGGAC ^a
ERE6-pGL3	CCTAGGCTCTGTCCAAAGGA ^a	CCCCGCTAAAACAGGGAATA ^a
ERE7-pGL3	TCAAACCTGGTGGGAAAGAC ^a	GTTTCTAAGGAGGCCAAGC ^a
Non-ERE-pGL3	GAGAAACCAGGTGCTTCTG ^a	TGTGCATACGACCTGACAAA ^a
(B) Antisense oligonucleotide		
ER α antisense	CATGGTCATGGTCAG ^b	
ER β antisense	GAATGTCATAGCTGA ^b	
MLL1 antisense	TGCCAGTCGTTCTCTCCAC ^b	
MLL2 antisense	ACTCTGCCACTTCCCGCTCA ^b	
MLL3 antisense	CCATCTGTTCTTCCACTCCC ^b	
MLL4 antisense	CCTTCTCTTCTCCCTTGT ^b	
Scramble antisense	CGTTTGCCCTCCAGCATCT ^b	

^aFlanked by appropriate restriction sites.^bAll phosphorothioate.

electrophoresed through SDS–PAGE (10 and 15% respectively) and transferred to nitrocellulose membrane. The membrane containing the protein extracts was probed with anti-MLL1 (Bethyl Laboratory, Montgomery, TX, USA), anti-MLL2 (LifeSpan BioSciences Inc., Seattle, WA, USA), anti-MLL3 (Abgent, San Diego, CA, USA), anti-MLL4 (Sigma), anti-ER α (Santa Cruz, Biotechnologies, Santa Cruz, CA, USA), anti-ER β (Santa Cruz), and anti- β -actin (Sigma) antibodies. Western blots were developed using the alkaline phosphatase method.

Immunohistological analysis of breast cancer tissue microarray

The breast cancer tissue microarray slide containing six different cases (duplicates of each) of breast cancer and their corresponding adjacent normal tissue were purchased from US Biomax, Inc. (Rockville, MD, USA) and subjected to immunohistological staining. For staining, the paraffin-embedded tissue microarray slide was immersed twice in xylene (for 10 min) and then sequentially immersed in 100, 95, and 70% ethanol (5 min each) to deparaffinize the tissue. Antigen retrieval was done by incubating the slide in 0.01 M sodium citrate buffer at 95 °C for 15 min following the supplier's

instruction. For immunohistological staining, the tissue microarray slide was incubated with 3% H₂O₂ for 15 min, washed with PBS thrice, and then blocked with blocking buffer containing donkey serum. The slide was then incubated with *HOXC10* antibody overnight, washed three times in PBS, and then incubated with biotinylated donkey secondary antibody for 1.5 h. The slide was washed thrice with PBS, incubated with avidin–biotin complex (ABC; Vector Laboratories, Burlingame, CA, USA) for 1.5 h, washed twice with PBS, and then washed twice with 0.1 M Tris–HCl (pH 7.4). The slide was incubated with diaminobenzidine (DAB) substrate (Vector Laboratories) for peroxidase labeling. The tissue microarray slide was dehydrated with sequential immersion under 70, 95, and 100% ethanol and then cleaned by sequentially incubation (1, 5, and 10 min) in CitriSolv clearing agent (Fisherbrand, Pittsburgh, PA, USA). Tissue sections were finally mounted with distrene-plasticizer-xylene mounting solution (Sigma), photographed, and examined under a microscope (Nikon Eclipse TE2000-U, Japan).

Dual-luciferase reporter assay

The *HOXC10* full promoter, full promoter with estrogen response element (ERE)-specific mutation,

and independent ERE regions along with their flanking regions (350–400 bp) were cloned and inserted upstream of the promoter of a firefly luciferase gene in pGL3 promoter vector (Promega) (primers are listed in Table 1). JAR cells (4×10^5 in a six-well plate) were cotransfected with 1500 ng of these ERE containing luciferase reporter construct along with 150 ng of a reporter plasmid containing renilla luciferase (pRLTk; Promega) as an internal transfection control using FuGENE6 transfection reagent. Control transfections were done using pGL3 promoter vector without any ERE insertion or with a luciferase construct-containing segment of *HOXC10* promoter containing no ERE (nonspecific control, non-ERE). At 24 h after transfection, cells were treated with 100 nM E_2 and incubated for an additional 8 h and then subjected to luciferase assay using a dual-luciferase reporter assay kit (Promega) as instructed. Firefly luciferase activities were assayed and normalized to those of renilla luciferase. Each treatment was performed in four replicates and the experiment was repeated at least twice.

Chromatin immunoprecipitation experiment

Chromatin immunoprecipitation (ChIP) assay was performed using an EZ Chip Chromatin immunoprecipitation kit (Upstate, Billerica, MA, USA) as described previously (Ansari *et al.* 2008, Mishra *et al.* 2009). In brief, estrogen-treated and control cells (JAR) were fixed in 4% formaldehyde, lysed, and sonicated to shear the chromatin. The fragmented chromatin was precleaned with protein-G agarose and subjected to immunoprecipitation with antibodies specific to ER α , ER β , MLL1, MLL2, MLL3, MLL4, H3K4 Tri-Met, and RNAPII (overnight). Immunoprecipitated chromatin was washed and de-proteinized to obtain purified DNA fragments that were PCR amplified using primers specific to different ERE regions of *HOXC10* promoter (Table 1).

Real-time PCR analysis

For gene expression analysis, total RNA was extracted from different cells by using RNAGEM tissue plus the RNA extraction kit (ZyGEM, Hamilton, New Zealand). The RT reactions were performed with 1 μ g total RNA by using MMLV reverse transcriptase as mentioned above and the cDNA was diluted to 50 μ l final volume. The cDNA was amplified using SsoFast EvaGreen supermix (Bio-Rad) and primers as described in Table 1, using CFX96 real-time PCR detection system. The results were analyzed using the CFX Manager. The real-time PCR analysis of the ChIP DNA fragments was performed with primer specific to different ERE regions of *HOXC10* promoter. Each PCR was performed in triplicate.

Statistical analysis

Each experiment was done in two to three replicates and then cells were pulled (and treated as one sample), subjected to RNA extraction, RT-PCR, and ChIP analysis, and each experiment was repeated at least thrice ($n=3$). For luciferase assay, each treatment was performed in four replicates and the experiment was repeated at least twice ($n=2$). The real-time PCR analysis of such samples was performed in three parallel replicate reactions and each experiment was repeated at least thrice ($n=3$). Normally distributed data were analyzed by ANOVA and non-normally distributed data were analyzed using Student's *t*-tests (SPSS, Chicago, IL, USA) to determine the level of significance between individual treatments. The treatments were considered significantly different at $P<0.05$.

Results

HOXC10 is overexpressed in breast cancer tissue and placenta choriocarcinoma cells

In an effort to understand the gene regulatory mechanism of *HOXC10*, initially we examined its expression levels in different types of malignant and nonmalignant human cell lines that include T47D (breast cancer), MCF7 (breast cancer), HEPG2 (hepatocellular carcinoma), JAR (placental choriocarcinoma), HeLa (cervical cancer), SW480 (colorectal adenocarcinoma), K562 (chronic myelogenous leukemia), H358 (bronchoalveolar carcinoma), MCF10 (normal breast epithelial), HFL1 (normal lung fibroblast), and Hs798.PI (normal placenta). RNA was isolated from each cell line, reverse transcribed into cDNA, and analyzed by quantitative real-time PCR (qPCR) for the expression of *HOXC10*. These analyses showed that *HOXC10* expression (mRNA level) was relatively higher in breast cancer cells (MCF7 and T47D), placental choriocarcinoma cells (JAR), and H358 (lung cancer) in comparison to several other cell lines including nonmalignant breast (MCF10) and placental cells (Hs 798.PI) (Fig. 1A).

As *HOXC10* expression was higher in breast cancer cells, we further examined its expression in human breast cancer by immunohistological staining of a breast cancer tissue microarray. A tissue microarray containing six cases of breast cancer (in duplicates) along with corresponding adjacent normal tissue was purchased commercially and subjected to DAB staining using *HOXC10* antibody. Immunohistological staining showed that the level of *HOXC10* expression was distinctly higher (as evidenced by more intense DAB staining) in each case of breast cancer tissue compared with its corresponding adjacent breast normal tissue (Fig. 1B; quantification is shown in C; a magnified view

Figure 1 Expression of *HOXC10* in different malignant and nonmalignant human cell lines and human breast cancer tissue. (A) The total RNA was isolated from T47D (breast cancer), MCF7 (breast cancer), HEPG2 (hepatocellular carcinoma), JAR (choriocarcinoma placenta), HeLa (cervical cancer), SW480 (colorectal adenocarcinoma), K562 (chronic myelogenous leukemia), H358 (bronchoalveolar carcinoma), MCF10 (normal breast epithelial cells), HFL1 (normal lung fibroblast HFL1), and Hs798.PI (normal placenta) cell lines, reverse transcribed to cDNA, and analyzed by real-time PCR using primers specific to *HOXC10*. *GAPDH* was used as a loading control. *HOXC10* expression relative to *GAPDH* is plotted. Each experiment was repeated at least thrice ($n=3$). Bars indicate S.E.M. (B–D) Immunohistological analysis of *HOXC10* expression in breast cancer tissue: human breast cancer tissue microarray (six cases of breast cancer along with their matched adjacent normal breast tissue) was obtained from US Biomax and subjected to immunohistological staining (DAB staining) with *HOXC10* antibody. The relative quantification of *HOXC10* expression within the tissue section is presented in panel C. A magnified view of tissue histology showing *HOXC10* expression in case 5 is shown in panel D.

of one set of tissue (case 5) is shown in D). These observations, in combination with cell culture data (Fig. 1A), demonstrated that *HOXC10* is overexpressed in breast cancer and potentially upregulated in placental choriocarcinoma cells.

Notably, MCF7 and T47D are well-known ER-positive breast cancer cell lines and have been widely used for estrogen-related experiments (Jansson *et al.* 2006, Yau & Benz 2008). Similarly, JAR cell is a placental choriocarcinoma cell line and placenta is known to produce various steroid hormones that are circulated to fetus as well as to the mother (Strauss *et al.* 1996). JAR cells are ER-positive cell line and have been previously used for steroid hormone-related studies (Wadsack *et al.* 2003). Thus, overexpression of *HOXC10* in breast carcinoma tissue and also in JAR, MCF7, and T47D cell lines indicated that *HOXC10* is potentially regulated by estrogen.

Effect of estrogen and tamoxifen on *HOXC10* gene expression

To examine whether *HOXC10* expression is regulated by estrogen, we exposed two different steroidogenic ER-positive cell lines such as JAR (Fig. 2) and MCF7 and an ER-negative breast cancer cell MDA-MB-231 (data not shown) with estrogen (E_2) and analyzed its impact on *HOXC10* expression. We treated JAR and MCF7 cells with varying concentrations of E_2 , incubated for 8 h, isolated RNA from the control and E_2 -treated cells, reverse transcribed into cDNA, and subjected to PCR analysis using primer specific to *HOXC10* (Fig. 2 and data not shown). cDNA was also analyzed by real-time PCR for quantification (respective bottom panels). *GAPDH* expression was analyzed as control. Our studies showed that, in JAR cells, the level of *HOXC10* mRNA

Figure 2 Effect of E₂ on *HOXC10* expression. (A) E₂-induced expression of *HOXC10* in JAR cells. The cells were grown in phenol red-free media and treated with varying concentrations of E₂ in the presence and absence of tamoxifen. The total RNA was isolated, reverse transcribed to cDNA, and analyzed by PCR using primers specific to *HOXC10*. *GAPDH* was used as a loading control. Expression of *HOXC10* (relative to *GAPDH*) was examined by real-time PCR (bottom panel). (B) Temporal studies: JAR cells were treated with 100 nM E₂ for varying time periods (0–24 h). RNA was reverse transcribed, analyzed by regular PCR (top panel), and real-time PCR (bottom panel). Each real-time PCR was carried out in three parallel replicates and each experiment was repeated at least thrice ($n=3$). Bars indicate S.E.M.

was increased upon treatment with E₂ in a concentration-dependent manner (Fig. 2A, real-time PCR data in bottom panel). *HOXC10* expression was about fivefold higher in 10 and 100 nM E₂-treated cells in comparison with control (Fig. 2A). Importantly, application of tamoxifen, an estrogen antagonist, inhibited E₂-induced expression of *HOXC10* further demonstrating roles of E₂ in *HOXC10* activation (lanes 7–8, Fig. 2A). Expression of *GAPDH* was unaffected by E₂ (Fig. 2A).

HOXC10 expression is also stimulated by E₂ in MCF7 cells, though the stimulation was observed at relatively lower E₂ concentration (0.1–1 nM) in comparison to JAR cells (data not shown). Application of tamoxifen also inhibited E₂-induced expression of *HOXC10* in MCF7 cells (data not shown). Notably, *HOXC10* expression was not stimulated by E₂ in an ER-negative cell line (MDA-MB-231; data not shown). The stimulation of *HOXC10* expression upon exposure to E₂ in two independent cell lines demonstrated that *HOXC10* gene is transcriptionally regulated by E₂. To determine the optimum time of E₂ exposure, we treated JAR cells with 100 nM E₂ for varying time periods and our results demonstrated that application of E₂ induced *HOXC10* expression as early as 2 h and reached a maximum at ~8 h after E₂ treatment (Fig. 2B, real-time PCR data in the bottom panel). As JAR cells showed a relatively more robust E₂ response toward *HOXC10* expression, we performed all other experiments in JAR cells.

HOXC10 promoter contains multiple EREs

To understand the mechanism of *HOXC10* gene regulation especially under steroid hormone environment, we analyzed its promoter for the presence of any EREs. We found that *HOXC10* promoter contains multiple (seven within ~3000 nt) putative EREs (ERE_{1/2} sites, GGTCA, or TGACC, Fig. 3A). Notably, during estrogen-mediated gene expression, ERs bind to EREs facilitating recruitment of various transcription factors and ER coregulators leading to gene activation (Nilsson & Gustafsson 2002). The presence of multiple ERE_{1/2} sites in *HOXC10* promoter indicated that *HOXC10* is potentially regulated by estrogen. As *HOXC10* showed E₂ response and its promoter contains several ERE_{1/2} sites, we analyzed the E₂ responsiveness of these ERE_{1/2} sites using a luciferase-based reporter assay. Initially, we cloned each ERE_{1/2} region independently in a luciferase-based reporter construct, pGL3 (Fig. 3A). A non-ERE region was also cloned as a negative control (non-ERE-pGL3, Fig. 3A). Each ERE-pGL3 construct was transfected in JAR cells; cells were then exposed to E₂ and subjected to luciferase activity analysis using a commercial luciferase detection kit. We also cotransfected a renilla luciferase construct (pRLTk) as an internal transfection control, and luciferase activities from the ERE-pGL3 constructs were normalized relative to renilla expression. Our results demonstrated that transfection with either

Figure 3 Characterization of *HOXC10* promoter EREs using luciferase assay. (A) *HOXC10* promoter showing different EREs (ER_{1/2} sites). Each ERE (1–7), a non-ERE region, and a full-length *HOXC10* promoter region were cloned into luciferase-based reporter construct, pGL3. EREs (1–7) were also mutated independently in the full-length promoter pGL3 construct and subjected to luciferase assay. (B) Luciferase assay: each ERE-pGL3 or full-length promoter pGL3 or ERE-mutated pGL3 constructs were transfected into JAR cells for 24 h. Control cells were treated with empty pGL3 vector or non-ERE-pGL3. Cells were also cotransfected with renilla luciferase expression construct as an internal transfection control. Cells were then treated with 100 nM E₂ and subjected to luciferase assay by using Dual-Glo Luciferase Assay System (Promega). The luciferase activities in the presence of E₂ (over untreated controls and normalized against renilla luciferase expression) were plotted. The experiment with four replicate treatments was repeated at least thrice ($n=3$). Bars indicate S.E.M. ($P<0.05$).

control pGL3 (no-ERE) or non-ERE-pGL3 did not induce any significant luciferase activity in the presence of E₂ (Fig. 3B). However, transfection with different ERE(1–7)-pGL3 constructs resulted in E₂-dependent luciferase induction, though the extent of luciferase induction was different for different EREs (Fig. 3B). ERE1-, ERE2/3-, and ERE6-pGL3 showed a more robust response to E₂ (Fig. 3B).

We also cloned the full-length *HOXC10* promoter spanning all the EREs in the pGL3 vector (Fig. 3A) and introduced independent mutation (GGTCA to AATCA or TGACC to TGAAG) to each ERE_{1/2} site and examined their E₂ response using luciferase assay. Our results demonstrated that full-length *HOXC10* promoter is about eightfold more responsive to E₂ exposure compared with empty pGL3, and this E₂ response is higher than the E₂ response of each individual ERE examined separately (Fig. 3B). Mutation of ERE_{1/2} sites (specifically ERE1 and ERE6) resulted in significant loss of E₂-dependent luciferase induction (Fig. 3B). Notably, though the individually cloned ERE_{1/2} sites (ERE2/3, ERE4/5, and ERE7) showed a significant E₂ response, a

point mutation in either of those EREs in a full-length promoter construct did not exhibit a major reduction in E₂-dependent luciferase induction (Fig. 3B). These observations suggested that ERE1 and ERE6 regions of *HOXC10* promoter are potentially involved in transcriptional regulation of *HOXC10* expression in the presence of estrogen.

ERs play critical roles in E₂-mediated activation of *HOXC10*

ERs are key players in estrogen-mediated gene activation (Nilsson *et al.* 2001, Lalmansingh & Uht 2008). ER α and ER β are two major ERs that regulate estrogen-responsive genes (Nilsson *et al.* 2001, Lalmansingh & Uht 2008). To understand the importance of ERs in E₂-induced expression of *HOXC10*, we knocked down both ER α and ER β separately in JAR cells using specific phosphorothioate antisense oligonucleotide (Table 1), exposed the ER knocked down cells to E₂, and analyzed its impact on *HOXC10* expression. A scramble antisense

Figure 4 Effect of ER α and ER β knockdown on E₂-induced expression of *HOXC10*. JAR cells were transfected with ER α - and ER β -specific antisense oligonucleotide for 48 h. A scramble antisense (with no homology with ER α and ER β gene) was used as control. The knocked down cells were treated with E₂ for additional 8 h. RNA from these cells was subjected to RT-PCR analysis by using primer specific to *HOXC10* along with respective ER α and ER β . GAPDH was used as control. (A) Lane 1: control cells; lane 2: cell treated with E₂; lanes 3–4: cells treated with scramble-antisense in the absence and presence of E₂; lanes 5–6: cells treated with ER α and ER β antisense, respectively, followed by exposure to E₂; lane 7: cells transfected with a mixture (1:1) of ER α and ER β antisense, respectively, followed by exposure to E₂. The real-time quantification of the transcript accumulation relative to GAPDH was plotted in bottom panel. (B) Each real-time PCR was carried out in three parallel replicates and each experiment was repeated at least thrice ($n=3$). Bars indicate S.E.M. ($P<0.05$).

(with no homology to ER) was used as a negative control. Our results demonstrated that application of ER α or ER β antisense knocked down the respective mRNA (lanes 5–6, Fig. 4A, real-time PCR data in B). Importantly, upon ER α or ER β knockdown, E₂-induced expression of *HOXC10* was suppressed significantly (lanes 5–6, Fig. 4A and B). Application of scramble antisense did not have any significant effect on E₂-mediated activation of *HOXC10* (lanes 3–4,

Fig. 4A and B). Combined knockdown of ER α and ER β suppressed further (almost to the basal level) the E₂-dependent activation of *HOXC10* (lane 7, Fig. 4A and B). Notably, the basal expression of *HOXC10* may not depend on E₂ and ER. These results demonstrated that both ER α and ER β are critical players in E₂-mediated transcriptional activation of *HOXC10*.

Roles of MLL histone methylases in E₂-mediated activation of *HOXC10*

As MLLs are well-known players in *HOX* gene regulation (Guenther *et al.* 2005) and are also involved in nuclear receptor-mediated gene expression (Lee *et al.* 2006, 2009, Mo *et al.* 2006, Ansari & Mandal 2010), we examined whether MLLs are also involved in *HOXC10* gene activation under an E₂ environment. We knocked down different MLLs (MLL1, MLL2, MLL3, and MLL4) separately by using specific phosphorothioate antisense oligonucleotide (Table 1), treated with E₂, and analyzed their effect on E₂-mediated activation of *HOXC10*. Knockdown of each MLL was confirmed both at mRNA (compare lane 4 with lane 1, Fig. 5A–D for MLL1–4 respectively) and at protein levels (data not shown). A scramble antisense (with no homology to MLL1–4) was used in parallel as a negative control. Our results demonstrated that application of MLL1 and MLL2 antisenses efficiently knocked down their respective mRNA (Fig. 5A and B, real-time PCR data in the bottom panels). However, MLL1 and MLL2 knockdown have no significant effect on E₂-induced expression of *HOXC10* (Fig. 5A and B, real-time PCR in bottom panels). In contrast, knockdown of MLL3 or MLL4 suppressed (more than 75%) the E₂-induced expression of *HOXC10* (Fig. 5C and D, real-time PCR data in bottom panels). These results demonstrated that MLL3 and MLL4 are critical players in E₂-dependent activation of *HOXC10*.

Binding of ERs and MLLs into *HOXC10* promoter EREs in the presence of E₂ *in vivo*

As *HOXC10* promoter contains seven ERE_{1/2} sites, and MLLs and ERs are involved in E₂-dependent *HOXC10* activation, we analyzed E₂-dependent binding of ERs and MLLs into these ERE regions *in vivo* using ChIP assay. Initially, JAR cells were treated with E₂, fixed with formaldehyde, and subjected to ChIP assay using antibodies specific to ER α , ER β , and β -actin (as negative control). Immunoprecipitated DNA fragments were PCR amplified using primers specific to ERE1–ERE7 and a non-ERE region of *HOXC10* promoter. ChIP DNA was also analyzed by real-time PCR for relative quantification. As shown in Fig. 6A, no significant binding of β -actin was observed in any EREs irrespective

Figure 5 Effect of knockdown of MLLs (1–4) on E₂-induced expression of *HOXC10*. JAR cells were transfected with different MLL-specific and scramble antisenses (5 µg) for 48 h followed by treatment with 100 nM E₂ for 8 h. RNA was subjected to RT-PCR analysis by using primer specific to *HOXC10*, MLLs (1–4), and *GAPDH* (as control). RT-PCR products were analyzed by agarose gel and quantified by using real-time PCR (bottom panels). (A) Effects of MLL1 knockdown. Lane 1: control cells; lane 2: cells treated with 100 nM E₂; lanes 3–4: cells transfected with scramble and MLL1 antisense, respectively, followed by treatment with E₂. Quantification of transcript accumulation (MLL1 or *HOXC10*) based on real-time PCR analysis is shown in the bottom panel. (B–D) Effects of knockdown of MLL2, MLL3, and MLL4 respectively (using similar experiments as described in A). Real-time PCR quantifications are shown in the respective bottom panels. Each real-time PCR was carried out in three parallel replicates and each experiment was repeated at least thrice ($n=3$). Bars indicate s.e.m. ($P<0.05$).

of E₂ (Fig. 6A). No binding of ERα and ERβ was observed in *HOXC10* promoter region containing no ERE (non-ERE). However, binding of ERα and ERβ was enhanced in the ERE1 region in an E₂-dependent

manner (Fig. 6A, real-time PCR quantifications of ChIP DNA are shown in B). An E₂-dependent increase in ERα binding was also observed in ERE6 region (Fig. 6A and B). Constitutive binding of ERα and ERβ was observed in ERE3, 4, and 7 regions. No binding of ERα and ERβ was observed in ERE2 and ERE5 irrespective of E₂. These results demonstrated that ERE1 and ERE6 are likely involved in E₂-dependent activation of *HOXC10* *in vivo*. As ERE1 and ERE6 were found to be more responsive to E₂ treatment, we performed all other binding analysis on these two ERE regions only.

As MLL3 and MLL4 (but not MLL1 and MLL2) were found to be essential in E₂-dependent activation of *HOXC10*, we examined the binding of these MLLs into E₂-responsive ERE1 and ERE6 regions using the ChIP assay. We observed that binding of MLL3 and MLL4 was enhanced upon addition of E₂ in both ERE1 and ERE6 regions; however, no E₂-dependent recruitment of MLL1 and MLL2 was observed in either of these EREs (Fig. 6C and D, real-time PCR data in bottom panel). These observations further suggested that MLL3 and MLL4 regulate *HOXC10* gene expression in the presence of E₂, likely via interaction with ERE1 and ERE6 regions of *HOXC10* promoter.

Temporal studies demonstrated that ERα and ERβ were bound to ERE1 and ERα in ERE6 regions in an E₂- and time-dependent manner (Fig. 7A). E₂-induced binding of ERα was observed as early as 15 min after E₂ treatment in both ERE1 and ERE6 regions, while binding of ERβ (to ERE1) was delayed (~2 h; Fig. 7A). MLL3 and MLL4 were also bound to the ERE1 and ERE6 regions in an E₂- and time-dependent manner (Fig. 7A). The binding of MLL3 was increased as early as 30 min in both ERE1 and ERE6; however, binding of MLL4 was slightly delayed (Fig. 7A). Similar to MLL3 and MLL4, we also observed the concomitant increase in RNA polymerase II (RNAP II) recruitment and H3K4 trimethylation level in ERE1 and ERE6 regions indicating the involvement of histone H3K4 trimethylation activities in E₂-mediated activation of *HOXC10* (Fig. 7A).

To examine potential roles of MLL3 and MLL4 in E₂-mediated *HOXC10* activation, we examined the level of H3K4 trimethylation and RNAPII recruitment (ChIP assay) at the *HOXC10* promoter after MLL3 and MLL4 knockdowns followed by E₂ treatment. Our results demonstrated that upon knockdown of either MLL3 or MLL4, or their combined knockdown, the level of H3K4 trimethylation and RNAPII recruitment at the ERE1 and ERE6 regions of *HOXC10* promoter was decreased significantly (Fig. 7B and C). These observations indicated that MLL3 and MLL4 mediate histone H3K4 trimethylation and RNAPII recruitment at the *HOXC10* promoter in the presence of E₂ and induce transcription activation.

Figure 6 E₂-dependent recruitment of ERs and MLLs onto *HOXC10* EREs. (A and B) JAR cells were treated with 100 nM E₂ for 8 h and analyzed by ChIP assay using ERα and ERβ antibodies. β-Actin antibody was used as control IgG. The ChIP DNA was PCR amplified using primers specific to different ERE regions (ERE1–ERE7) or a non-ERE region of *HOXC10* promoter. Real-time quantification of ChIP DNA samples showing the recruitment of ERα and ERβ (relative to input) into different ERE regions in the presence of E₂ is shown in panel B. (C and D) E₂-dependent binding of MLLs on ERE1 and ERE6 of *HOXC10* promoter. JAR cells were treated with 100 nM E₂ for 8 h and analyzed by ChIP assay using MLL1-, MLL2-, MLL3-, MLL4-, and β-actin (control)-specific antibodies. The immunoprecipitated DNA fragments were PCR amplified using primers specific to ERE1 and ERE6 regions of *HOXC10* promoter. Real-time quantification of the recruitment of MLL1–4 relative to input was plotted in panel D. The experiments were repeated at least twice. Bars indicate S.E.M.

MLL3 and MLL4 are recruited to the *HOXC10* EREs in an ER-dependent manner

ERs have an activation domain and DNA-binding domain and they are well known to bind directly to EREs of estrogen-responsive genes via their DNA-binding domain (Nilsson & Gustafsson 2002). Notably, MLL3 and MLL4 also have DNA-binding domains that may facilitate their direct binding to the ERE sequences. MLL3 and MLL4 also have multiple NR boxes that are responsible for interaction with nuclear receptors (Ansari *et al.* 2009a,b,c). In fact, MLL3 and MLL4 were recently shown to be recruited to *LXR* gene promoter regulating its expression (Lee *et al.* 2009). Herein, to examine the mode of binding of MLL3 and

MLL4 to the *HOXC10* promoter, we knocked down ERα and ERβ separately in JAR cells, then exposed to E₂, and analyzed the status of MLL3 and MLL4 recruitment to ERE1 and ERE6 (Fig. 8, real-time PCR data of ChIP DNA samples is in the bottom panel). As expected, we found that the binding of MLL3 and MLL4 was increased in both ERE1 and ERE6 regions of *HOXC10* promoter in the presence of E₂ and scramble antisense (lanes 1 and 2, Fig. 8). However, knockdown of either ERα or ERβ decreased (or even abolished) the recruitment of MLLs onto both ERE1 and ERE6 regions (compare lanes 3 and 4, with lane 2, Fig. 8). These results suggest that recruitment of MLL3 and MLL4 to the *HOXC10* EREs is dependent on both ERα and ERβ.

Figure 7 (A) Temporal recruitment of ERs, MLLs, RNAPII, and level of H3K4 trimethyl in the ERE1 and ERE6 regions of *HOXC10* promoter. Cells were treated with 100 nM E₂ for varying time periods and analyzed by ChIP assay using ERα, ERβ, MLL3, MLL4, H3K4-trimethyl, and RNAPII antibodies. The ChIP DNA was PCR amplified using primers specific to ERE1 and ERE6 regions of *HOXC10* promoter. (B and C) Effect of MLL3 and MLL4 knockdown on H3K4 trimethylation and RNAPII recruitment. JAR cells were treated with MLL3 and MLL4 antisenses for 48 h followed by E₂ treatment for 8 h. Cells were fixed and subjected to ChIP analysis by using antibodies specific to H3K4 trimethyl and RNAPII. β-Actin antibody was used as control IgG. The immunoprecipitated DNA fragments were PCR amplified using primers specific to ERE1 and ERE6 regions of *HOXC10* promoter. Real-time PCR quantifications are shown in respective bottom panel. Experiments were repeated at least twice ($n=2$) and bars indicate S.E.M.

Discussion

A growing body of evidence suggests that hormones play critical roles in regulation of developmental genes including *HOX* genes (Ma *et al.* 1998, Lim *et al.* 1999, Huang *et al.* 2007). For example, retinoic acids affect *HOX* gene expression and produce homeotic transformation (Huang *et al.* 2007). Although retinoids regulate anterior *HOX* genes, recent data showed that posterior *HOX* genes are regulated by estrogens and progesterones (Lim *et al.* 1999). Neonatal exposure to diethylstilbestrol (DES) downregulates uterine *Hoxa10* expression (Ma *et al.* 1998). *Hoxb13*, a gene that is involved in normal differentiation and secretory

function of the mouse ventral prostate, is suppressed upon exposure to neonatal estrogen (Economides *et al.* 2003). Ovariectomy in mouse affects the expression of *Hoxc6*, which is critical for mammary gland development and milk production (Garcia-Gasca & Spyropoulos 2000). Beyond their developmental roles, expression of several *HOX* genes is associated with breast and prostate cancers that are highly sensitive to steroid hormones (Huang *et al.* 2007).

Hoxc10 is a critical gene that is involved in the development of spinal cord, formation of neurons, and associated with human leukemia (Akbas *et al.* 2004, Choe *et al.* 2006, Lopez *et al.* 2006, Zhai *et al.* 2007). The mechanism by which *HOXC10* expression is regulated is

Figure 8 Effects of ER α and ER β knockdown on E₂-dependent recruitment of MLL3 and MLL4. JAR cells were transfected with ER α , ER β , and scramble antisenses (9 μ g each) for 48 h followed by treatment with 100 nM E₂ for 6 h. Cells were subjected to ChIP assay using MLL3 and MLL4 antibodies and immunoprecipitated DNA fragments were PCR amplified using primers specific to ERE1 and ERE6 regions of *HOXC10* promoter. Real-time quantification of ChIP DNA samples is shown in the bottom panel. Experiments were repeated at least twice and bars indicate s.e.m.

unknown. Notably, ovarian hormones are known to influence spinal cord physiology (Monks *et al.* 2001). Our studies demonstrated that expression of *HOXC10* is significantly higher in human breast cancer tissue. In agreement with our finding, recent studies showed that along with various other genes, *HOXC10* transcripts were overexpressed in lymph node (+) breast carcinomas (Abba *et al.* 2007). *HOXC10* expression was also higher in different ER-positive breast and placental choriocarcinoma cells, in comparison to various nonmalignant cell lines, indicating potential roles of estrogen in transcriptional regulation of *HOXC10*. Our studies demonstrated that upon exposure to estrogen (E₂), *HOXC10* is transcriptionally activated in both ER-positive breast (MCF7) and placenta choriocarcinoma (JAR) cells. *HOXC10* gene promoter contains several ERE_{1/2} sites and luciferase-based reporter assay demonstrated that *HOXC10* promoter (full promoter) is highly responsive to E₂ exposure. Mutation in the ERE1 and ERE6 regions of *HOXC10* promoter significantly affected its E₂ sensitivity indicating potential involvement of these EREs in E₂-mediated transcriptional activation of *HOXC10*. Notably, the luciferase

reporter assay is based on recombinant plasmid DNA constructs that do not represent native chromatin environment present inside the cell, and therefore, these luciferase-based data may not be directly correlated with E₂ responses of different EREs *in vivo*. Notably, in agreement with the luciferase data, analysis of the binding of ER and ER coregulators (MLL3 and MLL4 in this case) demonstrated that primarily ERE1 and ERE6 of *HOXC10* promoter are most responsive to E₂-dependent binding of ERs and MLLs, and may be involved in E₂-induced regulation of *HOXC10* *in vivo*.

ERs are major players in regulation of estrogen-responsive genes (Nilsson *et al.* 2001). In an ERE-dependent mechanism, in the presence of estrogen, ERs bind to the ERE sequences and mediate gene activation (Nilsson *et al.* 2001). Our results demonstrated that antisense-mediated knockdown of either ER α or ER β abolished E₂-induced activation of *HOXC10*. ChIP analysis demonstrated that both ER α and ER β are recruited to ERE1 and ER α in the ERE6 region of *HOXC10* promoter in an E₂-dependent manner. These observations demonstrated that ERs are involved in E₂-induced expression of *HOXC10*. Notably, as seen in Fig. 6A and B, some amount of constitutive bindings of ER α and ER β was observed in the ERE3, ERE4, and ERE7 region and this binding was not altered by E₂. These observations suggest that these EREs could be associated with basal transcription of *HOXC10*.

Importantly, when activated ERs bind to the promoters, they also recruit various activators and coactivators that lead to structural changes and remodeling of the chromatin leading to transcription activation (Lonard & O'Malley 2007). Recent studies demonstrated that histone methylases MLL2, MLL3, and MLL4 act as a coactivator for ERs in regulation of certain E₂-responsive genes (Mazo *et al.* 1990, Dreijerink *et al.* 2006, Mo *et al.* 2006, Ansari *et al.* 2009a,b,c). Notably, protein containing LXXLL domain (NR box) is known to interact with NRs and play critical roles in ligand-dependent gene activation (Ansari *et al.* 2009a,b,c). Sequence analysis showed that MLL1 contains one LXXLL domain, while MLL2, MLL3, and MLL4 contain at least four NR boxes indicating their potential involvement in E₂-mediated regulation of estrogen-responsive genes (Ansari *et al.* 2009a,b,c). Our studies demonstrated that, similar to ER α and ER β , knockdown of MLL3 and MLL4 resulted in down-regulation of E₂-dependent activation of *HOXC10*.

ChIP analysis demonstrated that MLL3 and MLL4 were bound to ERE1 and ERE6 regions of *HOXC10* promoter in an E₂-dependent manner. In contrast, E₂-dependent binding of MLL1 and MLL2 was not observed, further indicating no significant roles of MLL1 and MLL2 in the process. These observations demonstrated that MLL3 and MLL4 play critical roles in E₂-dependent activation of *HOXC10* and this may be

mediated via binding to ERE1 and ERE6 regions of *HOXC10*. Importantly, knockdown of either MLL3 or MLL4 abolishes the E₂-dependent enrichment of H3K4 trimethylation and recruitment of RNA polymerase II at the *HOXC10* promoter, indicating critical roles of MLL3 and MLL4 in promoter histone H3K4 trimethylation and transcription initiation.

Furthermore, our results demonstrated that knockdown of ERs (ER α or ER β) resulted in decreased binding of MLL3 and MLL4 into the EREs of *HOXC10* gene promoter, indicating critical roles of ER α and ER β in recruiting MLL3 and MLL4 in the *HOXC10* promoter. Notably, in addition to LXXLL domains, MLL3 and MLL4 also contain a DNA-binding domain that may also facilitate their recruitment to their target gene promoters. MLL2 also has NR boxes and has been previously implicated in ER-associated gene activation. However, based on our experiments, MLL2 is not involved in E₂-dependent activation of *HOXC10*. This is likely because different genes may have different coregulator requirements, and based on our data, MLL3 and MLL4 are the ER coregulators for *HOXC10*. Notably, Lee *et al.* (2006), has demonstrated that MLL3 and MLL4 (but not MLL1 and MLL2) are involved in hormone-mediated regulation of *RAR β* . Furthermore, our studies demonstrated that both ER α and ER β are involved in E₂-mediated activation of *HOXC10*. These observations indicate that ER α and ER β may form a heterodimer that recognizes the EREs in the *HOXC10* promoter. Our results also showed that knockdown of either ER α or ER β did not completely abolish the E₂-dependent activation of *HOXC10* expression. This observation suggests that both ER α and ER β can supplement the function of each other while one ER is specifically knocked down. This is likely mediated via formation of a homodimer (instead of a heterodimer) that binds the promoter EREs and helps the recruitment of ER coactivators. Notably, the ERs are well known to form the homo- and heterodimers in recognition of EREs during transcriptional regulation of estrogen-responsive genes (Nilsson & Gustafsson 2002, Lindberg *et al.* 2003).

Overall, our studies demonstrated that *HOXC10* is overexpressed in breast cancer tissue and is transcriptionally activated by estrogen. Histone methylases MLL3 and MLL4 coordinate with ERs and play critical roles in transcriptional regulation of *HOXC10* in the presence of estrogen. Though MLLs are considered major regulators of *HOX* genes, little is known about their roles in regulation of *HOX* genes in a hormonal environment. Our studies revealed novel roles of MLL histone methylases in estrogen-mediated regulation of *HOX* genes. Notably, *Hoxa10*, a *Hoxc10* paralogous gene, has been previously shown to be regulated by estrogen and is critical in endometrial differentiation and uterine development (Taylor *et al.* 1998).

E₂-dependent *Hoxa10* expression is tissue specific and mediated via involvement of ERs and specificity factor Sp1 (Martin *et al.* 2007). Our studies demonstrated that *HOXC10* is also an estrogen-responsive gene and this may be associated with hormone-induced cell differentiation, development, and human diseases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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