**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 (17β-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. ERα and ERβ play critical roles in E2-mediated activation of HOXC10. Knockdown of ERα and ERβ downregulated E2-induced HOXC10 expression. ERα and ERβ 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, Zhai et al. 2007). Knockdown of MLLs 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.
Sims & Reinberg 2006, Bhaumik et al. 2007, Issaeva et al. 2007, Agger et al. 2008, Ansari et al. 2009a,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, CBP, 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, 2009a,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. 2009a,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. 2009a,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 (17β-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 µg) of different antisenses in FBS-free media as described by us previously (Ansari et al. 2008, 2009a,b,c, Table 1). In brief, a cocktail of antisense and infect transfection reagents (MoleculA) was made in 300 µl 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, 2009a,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 cytoplasmatic 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 µl containing 500 ng RNA, 2.4 µM oligo-dT (Promega), 100 units MMLV reverse transcriptase, 1× first-strand buffer (Promega), 100 µM each of dATP, dGTP, dCTP, and dTTP (Invitrogen), 1 mM DTT, and 20 units RNaseOut (Invitrogen). The cDNA was diluted to 100 µl. PCR was performed in a 10 µl reaction volume containing 5 µl diluted cDNA and gene-specific primer pairs (Table 1). For western blot analysis, an equivalent amount of cell protein extracts (25 µg) was
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-ERα (Santa Cruz, Biotechnologies, Santa Cruz, CA, USA), anti-ERβ (Santa Cruz), and anti-β-actin (Sigma) antibodies. Western blots were developed using the alkaline phosphatase method.

**Table 1** Primers used for RT-PCR and ChIP experiments

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
</tr>
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<tbody>
<tr>
<td>(A) PCR primer</td>
<td>CAATGACCCCTTCAATTGACC</td>
<td>GACAAGCTTCCGTTCGCCAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACCAGGGGAAATGGGCTGAC</td>
<td>GATCCTATTCCCTCTGGTCA</td>
</tr>
<tr>
<td>HOXC10-ORF</td>
<td>GAGGACCCCGAGTATAAACC</td>
<td>GGAGCAGAGGGTCGAGTCA</td>
</tr>
<tr>
<td>MLL1</td>
<td>GTGACAGCAGAAGATGGTGA</td>
<td>GACAAATGGTTCCAGAGAA</td>
</tr>
<tr>
<td>MLL2</td>
<td>AAGCAACGCCATTGCAGGAG</td>
<td>ACAAGCCATAGGAGTGGT</td>
</tr>
<tr>
<td>MLL3</td>
<td>GTCTAGCGCGACTGGAGGAC</td>
<td>AGTCTGGATCCGTTT</td>
</tr>
<tr>
<td>MLL4</td>
<td>ATGGCCTGTTACTCCACTC</td>
<td>CCAATGGGATTTGAGAG</td>
</tr>
<tr>
<td>HOXC10-ERE1</td>
<td>TGACCTCCCTCTCAGCCA</td>
<td>CTCCTTCCCTTCCTTT</td>
</tr>
<tr>
<td>HOXC10-ERE2</td>
<td>TGGCTTCCCTCTCCTTCT</td>
<td>ACTGGCAATTCACAGG</td>
</tr>
<tr>
<td>HOXC10-ERE3</td>
<td>CGGATGTGAGCCAGCAGGAG</td>
<td>CCAAGGAGGAGAGGAAG</td>
</tr>
<tr>
<td>HOXC10-ERE4</td>
<td>CCAATGGACCTCCCAAGGAG</td>
<td>CCGGAAAAGTGGAAGGA</td>
</tr>
<tr>
<td>HOXC10-ERE5</td>
<td>TCTTGTAGGAGGTGAA</td>
<td>CAAAGGAGGAGGAGAG</td>
</tr>
<tr>
<td>ERE1-pGL3</td>
<td>CCCTTCTCCCAAGAGATTAC</td>
<td>AGGGGAGGGAGGAAAAAG</td>
</tr>
<tr>
<td>ERE2-pGL3</td>
<td>TGCTTCTCTCTCTCTCTCAT</td>
<td>CTTACCTGGGATGGGAGT</td>
</tr>
<tr>
<td>ERE4/5-pGL3</td>
<td>CCAGGCTTCCTTCTCTTCTT</td>
<td>AAAGGAGGAGGAGGAG</td>
</tr>
<tr>
<td>ERE6-pGL3</td>
<td>CCAAGGCTCCTCTGCAAGGAG</td>
<td>CCCCCTCAAACAGAGGAA</td>
</tr>
<tr>
<td>ERE7-pGL3</td>
<td>TCAAACTGGGTGAGGAAAAGA</td>
<td>GTTCTTAAGGGCGCCCAG</td>
</tr>
<tr>
<td>Non-ERE-pGL3</td>
<td>GAGAAAACAGGAGGTTTCTTG</td>
<td>TGTCGTAACACAGACAC</td>
</tr>
<tr>
<td>(B) Antisense oligonucleotide</td>
<td>CATGGTGATCTTCCAG</td>
<td>GAATTGCATAGTCTAG</td>
</tr>
<tr>
<td>ERα antisense</td>
<td>GAACTGCATAGTCTAG</td>
<td>GAATTGCATAGTCTAG</td>
</tr>
<tr>
<td>MLL1 antisense</td>
<td>TGGCAATGTTCCCTCTCCAC</td>
<td>CTTAAGGCGGAGGAAAG</td>
</tr>
<tr>
<td>MLL2 antisense</td>
<td>ACTCTGGCAATCCTCCCTCA</td>
<td>TTACCTGGGATGGGAGT</td>
</tr>
<tr>
<td>MLL3 antisense</td>
<td>CACTGGTCTTCCCTCCCTT</td>
<td>AAAGGAGGAGGAGGAG</td>
</tr>
<tr>
<td>MLL4 antisense</td>
<td>CTCCTTCTCTCCCTCTGT</td>
<td>CCCCCTCAAACAGAGGAA</td>
</tr>
<tr>
<td>Scramble antisense</td>
<td>GTTTGTGCTCCTCCAGACTT</td>
<td>TGTCGTAACACAGACAC</td>
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*Flanked by appropriate restriction sites.
All phosphorothioate.

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 \times 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 (pRL.Tk; 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 E2 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 were preclenched with protein-G agarose and subjected to immunoprecipitation with antibodies specific to ERα, ERß, MLL1, MLL2, MLL3, MLL4, H5K4 Tri-Met, and RNAPII (overnight). Immunoprecipitated chromatin were 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 μg total RNA by using MMLV reverse transcriptase as mentioned above and the cDNA was diluted to 50 μl final volume. The cDNA was amplified using SsoFast EvaGreen supermix (Bio-Rad) and primers as described in Table 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 leukaemia), 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 HOX10 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...
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 (E2) and analyzed its impact on HOXC10 expression. We treated JAR and MCF7 cells with varying concentrations of E2, incubated for 8 h, isolated RNA from the control and E2-treated cells, 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.
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 (ERE1/2 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 ERE1/2 sites in HOXC10 promoter indicated that HOXC10 is potentially regulated by estrogen. As HOXC10 showed E₂ response and its promoter contains several ERE1/2 sites, we analyzed the E₂ responsiveness of these ERE1/2 sites using a luciferase-based reporter assay. Initially, we cloned each ERE1/2 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 (pRL-Tk) 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

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**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.
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 TGAAA) to each ERE1/2 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 ERE1/2 sites (specifically ERE1 and ERE6) resulted in significant loss of E2-dependent luciferase induction (Fig. 3B). Notably, though the individually cloned ERE1/2 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). ERα and ERβ 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 ERα and ERβ 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...
Followed by exposure to E2; lane 7: cells transfected with a mixture of scramble antisense (with no homology with ER) was used as control. The knocked down cells were treated with E2 for an additional 8 h. RNA from these cells was subjected to RT-PCR analysis by using primer specific to HOXC10 along with respective ERα and ERβ. 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 ERα and ERβ antisense, respectively, followed by exposure to E2; lane 7: cells transfected with a mixture (1:1) of ERα and ERβ 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).

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

As MLLs are well-known players in Hox gene regulation (Guenter 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 ERE1/2 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 ERα, ERβ, 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 of E2 concentration. Combined knockdown of ERα and ERβ suppressed further (almost to the basal level) the E2-dependent activation of HOXC10 (lanes 3–4, Fig. 4A and B). Combined knockdown of ERα and ERβ 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 ERα and ERβ are critical players in E2-mediated transcriptional activation of HOXC10.
of E$_2$ (Fig. 6A). No binding of ERz and ER$\beta$ was observed in $HOXC10$ promoter region containing no ERE (non-ERE). However, binding of ERz and ER$\beta$ was enhanced in the ERE1 region in an E$_2$-dependent manner (Fig. 6A, real-time PCR quantifications of ChIP DNA are shown in B). An E$_2$-dependent increase in ERz binding was also observed in ERE6 region (Fig. 6A and B). Constitutive binding of ERz and ER$\beta$ was observed in ERE3, 4, and 7 regions. No binding of ERz and ER$\beta$ was observed in ERE2 and ERE5 irrespective of E$_2$. These results demonstrated that ERE1 and ERE6 are likely involved in E$_2$-dependent activation of $HOXC10$ in vivo. As ERE1 and ERE6 were found to be more responsive to E$_2$ 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 E$_2$-dependent activation of $HOXC10$, we examined the binding of these MLLs into E$_2$-responsive ERE1 and ERE6 regions using the ChIP assay. We observed that binding of MLL3 and MLL4 was enhanced upon addition of E$_2$ in both ERE1 and ERE6 regions; however, no E$_2$-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 E$_2$, likely via interaction with ERE1 and ERE6 regions of $HOXC10$ promoter.

Temporal studies demonstrated that ERz and ER$\beta$ were bound to ERE1 and EREz in ERE6 regions in an E$_2$- and time-dependent manner (Fig. 7A). E$_2$-induced binding of ERz was observed as early as 15 min after E$_2$ treatment in both ERE1 and ERE6 regions, while binding of ER$\beta$ (to ERE1) was delayed (~2 h; Fig. 7A). MLL3 and MLL4 were also bound to the ERE1 and ERE6 regions in an E$_2$- 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 E$_2$-mediated activation of $HOXC10$ (Fig. 7A).

To examine potential roles of MLL3 and MLL4 in E$_2$-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 E$_2$ 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 E$_2$ and induce transcription activation.

**Figure 5** Effect of knockdown of MLLs (1–4) on E$_2$-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 E$_2$ 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 panel). (A) Effects of MLL1 knockdown. Lane 1: control cells; lane 2: cells treated with 100 nM E$_2$; lanes 3–4: cells transfected with scramble and MLL1 antisense, respectively, followed by treatment with E$_2$.

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).
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 regions of HOXC10 promoter. 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.
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 Hoxd11, 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 HOX10 expression is regulated is

**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.
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 ERE1/2 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 ERα or ERβ abolished E2-induced activation of HOXC10. ChIP analysis demonstrated that both ERα and ERβ are recruited to ERE1 and ERE6 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 ERα and ERβ was observed in the ERE3, ERE4, and ERE7 region and this binding was not altered by E2. These observations suggest that these ERs 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 ERα and ERβ, 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 (ERα or ERβ) resulted in decreased binding of MLL3 and MLL4 into the EREs of HOXC10 gene promoter, indicating critical roles of ERα and ERβ 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 RARβ. Furthermore, our studies demonstrated that both ERα and ERβ are involved in E2-mediated activation of HOXC10. These observations indicate that ERα and ERβ may form a heterodimer that recognizes the EREs in the HOXC10 promoter. Our results also showed that knockdown of either ERα or ERβ did not completely abolish the E2-dependent activation of HOXC10 expression. This observation suggests that both ERα and ERβ 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 Hox10 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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