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HOXC6 is transcriptionally regulated via coordination of MLL histone methylase and estrogen receptor under estrogen environment

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Abstract

Homeobox containing gene HOXC6 is a critical player in mammary gland development, milk production and is overexpressed in breast and prostate cancer. We demonstrated that HOXC6 is transcriptionally regulated by estrogen (E2). HOXC6 promoter contains two putative estrogen-response elements (EREs), termed as $ERE1_{1/2}$ and $ERE2_{1/2}$. Promoter analysis using luciferase based reporter assay demonstrated that both EREs are responsive to E2, $ERE1_{1/2}$ being more responsive than $ERE2_{1/2}$. Estrogen receptors, ERα and ERβ, bind to these EREs in an E2-dependent manner and antisense-mediated knockdown of ERs suppressed the E2-dependent activation of HOXC6 expression. Similarly, knockdown of histone methylases, MLL2 and MLL3, decreased E2-mediated activation of HOXC6. However, depletion of MLL1 or MLL4 showed no significant effect. MLL2 and MLL3 were bound to the HOXC6 EREs in an E2-dependent manner. In contrast, MLL1 and MLL4 that were bound to the HOXC6 promoter in the absence of E2, decreased upon exposure to E2. MLL2 and MLL3 play key roles in histone H3K4-trimethylation and recruitment of general transcription factors and RNAP II in the HOXC6 promoter during E2-dependent transactivation. Nuclear receptor corepressors N-CoR and SAFB1 were bound in the HOXC6 promoter in absence of E2 and that binding were decreased upon E2-treatment indicating their critical roles in suppressing HOXC6 gene expression under non-activated condition. Knockdown of either ERα or ERβ abolished E2-dependent recruitment of MLL2 and MLL3 into the HOXC6 promoter demonstrating key roles of ERs in recruitment of these MLLs into HOXC6 promoter. Overall, our studies demonstrated that HOXC6 is an estrogen-responsive gene and histone methylases MLL2 and MLL3, in coordination with ERα and ERβ, transcriptionally regulate HOXC6 in an E2-dependent manner.

Introduction

Homeobox (HOX) genes are group of evolutionarily conserved genes that play critical roles in embryonic development.[1,2] HOX genes also continue to be expressed at varying levels throughout postnatal life. There are 39 different HOX genes in human that are clustered in four different groups HOXA, B, C, and D and expression of each HOX gene is tightly regulated.[3] Recent studies demonstrate that HOX genes are associated with various

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oncogenic transformations.[4-9] In particular, HOXC6, a critical player in mammary gland development and milk production, is expressed in osteosarcomas, medulloblastomas, as well as carcinomas of the breast, lung, and prostate.[10-16] HOXC6 regulates expression of BMP7 (bone morphogenic protein 7), FGFR2 (fibroblast growth factor receptor 2), IGFBP3 (insulin-like growth factor binding protein 3) and PDGFRA (platelet-derived growth factor receptor α) in prostate cells and influences the Notch and Wnt signaling pathways *in vivo*.[13,15] HOXC6 regulates various genes including CD44 that are important for prostate branching morphogenesis and bone metastasis of prostate cancer. Although, HOXC6 is critical in so many hormonally regulated processes and diseases, the mechanism by which it may be regulated is mostly unknown.

In general, mixed lineage leukemia (MLL) family of proteins are well known as master regulators of HOX genes.[17-19] MLLs are evolutionarily conserved trithorax family of proteins that play critical roles during development.[20] MLL1 is also well known to be rearranged in leukemia.[19-21] Biochemical studies demonstrate that MLLs are human histone H3 lysine-4 (H3K4) specific methyl-transferases (HMTs) that are key players in gene activation and epigenetics.[17-19,21-40] There are several MLLs in human such as MLL1, MLL2, MLL3, MLL4, SET1A, and SET1B and each exists as multi-protein complexes with several common subunits such as ASH2, WDR5, RBBP5, CGBP, and DPY30.[22,24,41,42] Although, different MLLs and SET1 possess similar enzymatic activities (H3K4-methylation) and are all critical players in gene activation, multiplicity of MLLs suggests their distinct roles beyond histone methylation. Recently, we and others showed that MLLs play key roles in cell cycle regulation, stress response, and HOX gene regulation.[28,38,43-49] Knockdown of MLL1 results in cell cycle arrest in G2/M phase.[34] Beyond their roles in histone H3K4-methylation, several MLLs are found to interact with nuclear hormone receptors (including estrogen receptors), nuclear receptor coregulatory complexes and play critical roles in regulation of hormone responsive genes.[35,50-52]

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

Materials and Methods

Cell culture, estrogen treatment and antisense experiments

Human choriocarcinoma placenta cells (JAR, ATCC) were grown and maintained in RPMI 1640 supplemented with 10 % FBS, 2 mM L-glutamine and penicillin/streptomycin (100 unit and 0.1 mg/mL respectively).[52,53] Human breast cancer cells (MCF7) and ER negative adenocarcinoma breast cell (MDA-MB-231) were maintained in DMEM supplemented with 10 % FBS, 2 mM L-glutamine and penicillin/streptomycin (100 unit and 0.1 mg/mL respectively). For the estrogen treatment, cells were grown and maintained (for at least 3 rounds) in phenol red free DMEM-F-12 media (Sigma) supplemented with 10 % charcoal stripped FBS, 2 mM L-glutamine and penicillin/streptomycin (100 unit and 0.1 mg/mL, respectively). Cells were grown up to 70 % confluency, treated with varying concentrations (0 - 1000 nM) of 17β-estradiol (E2) and incubated for 8 h (or varying time points for temporal studies) and then harvested for RNA and protein extraction.

For antisense experiments, JAR cells were grown up to 60 % confluency (60 mm plate) and transfected with different antisense oligonucleotides (commercially synthesized from IDT) in FBS free media using ifect transfection reagent (MoleculA) and following manufacturer's

instruction. In brief, a cocktail of antisense and ifect transfection reagent was made in 300 μL DMEM-F-12, applied to cells in presence of 1.7 mL supplement free medium, and incubated for 7 h. Then 2 mL media containing all supplements and 20 % charcoal stripped FBS were added and incubated for additional 48 h. Depending on the need, antisense treated cells may have been exposed to 100 nM E2.

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

RNA/protein extracts, RT-PCR and western blot

Cells were harvested and collected by centrifugation at 500 g. The RNA and protein were extracted as described previously.[17,45] For the reverse transcriptase-PCR (RT-PCR), reverse transcription reactions were performed in a total volume of 25 μL containing 500 ng of RNA, 2.4 μM of oligo dT (Promega), 100 units of MMLV reverse transcriptase, 1 × first strand buffer (Promega), 100 μM each of dATP, dGTP, dCTP and dTTP (Invitrogen), 1 mM dithiothreitol (DTT), and 20 units of RNaseOut (Invitrogen). The cDNA was diluted to 100 μL. PCR reactions were performed in a 10 μL reaction volume containing 5 μL diluted cDNA and gene specific primer pairs (Table 1). Protein extracts were analyzed by western blotting using antibodies against MLL1, MLL2, MLL3, MLL4, ERα, ERβ, and β-actin. Western blots were developed using alkaline phosphatase method.

Chromatin Immuno-precipitation (ChIP) experiment

ChIP assays were performed by using JAR cells and EZ Chip™ chromatin immuno-precipitation kit (Upstate) as described previously.[17,34,45] In brief, JAR cells were treated with 100 nM E2 for varying time points, fixed in 4% formaldehyde, lysed in lysis buffer and sonicated to shear the chromatins. The fragmented chromatin was pre-cleaned with protein-G agarose beads and subjected to immuno-precipitation with antibodies specific to ERα, ERβ, MLL1, MLL2, MLL3, MLL4, RNAPII, histone H3, H3K4-trimethyl, H3K9-dimethyl, N-CoR, SAFB1, TBP, TAF250 or β-actin overnight. Immuno-precipitated chromatins were washed and de-proteinized to obtain purified DNA fragments that were used as templates in PCR amplifications using various primers corresponding to different EREs of HOXC6 promoter (Table 1).

Real Time RT-PCR

For gene expression analysis RNA was extracted from cells by using RNAGEM tissue plus RNA extraction kit (ZyGEM). The reverse transcription reactions were performed with 1 μg total RNA by using MMLV reverse transcriptase as mentioned above and the cDNA was diluted to 50μL 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. These results were analyzed using the CFX Manager. The real time PCR analysis of the ChIP DNA fragments were done with primers specific to $ERE1_{1/2}$ and $ERE2_{1/2}$ regions of HOXC6 promoter. Each PCR reaction was done in triplicates.

Dual luciferase reporter assay

HOXC6 promoter spanning $ERE1_{1/2}$-$ERE2_{1/2}$ regions (-1107 to +208 nt), and $ERE1_{1/2}$ (alone, -184- to +208 nt), and $ERE2_{1/2}$ (alone -1107 to -697 nt) were cloned and inserted upstream of the promoter of firefly luciferase gene in pGL3-promoter vector (Promega)

(primers are listed in Table 1). JAR cells (4×10^5 in 6 well plate) were co-transfected with 1500 ng of these ERE containing luciferase reporter construct along with 150 ng of a reporter plasmid containing renilla luciferase (pRLTk, Promega) as an internal transfection control using FuGENE6 transfection reagent. Control transfections were done using pGL3 promoter vector without any ERE insertion or with a luciferase construct-containing segment of HOXC6 promoter containing no ERE (non specific control, non-ERE). At 24 h post transfection, cells were treated with 100 nM E2 and incubated for additional 8 h and then subjected to luciferase assay using Dual luciferase reporter assay kit (Promega) as instructed. Firefly luciferase activities were assayed and normalized to those of renilla luciferase. Each treatment was done in four replicates and the experiment was repeated at least twice.

Statistical analysis

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

Results

HOXC6 gene is transcriptionally regulated by estrogen

To examine if HOXC6 is transcriptionally regulated by estrogen, we treated JAR cells (a human placental choriocarcinoma origin) with varying concentrations of E2 and analyzed its impact on HOXC6 expression. Notably, JAR cell is a placental choriocarcinoma cell line and placenta is known to produce various steroid hormones that are circulated to fetus as well as the mother.[54] JAR cells have been previously used for steroid hormone related studies.[55] Our analysis showed that JAR cells express both ERα and ERβ (data not shown). We isolated RNA from the E2-treated and control (not treated with E2) cells, reverse transcribed into cDNA and analyzed by PCR using primers specific to HOXC6. The cDNA was also analyzed by real-time PCR for quantification. β-actin was used as control. Interestingly, we observed that HOXC6 expression was increased upon treatment with E2 in a concentration dependent manner (Fig. 1A). HOXC6 expression was about 4 fold higher in 100 nM E2-treated JAR cells in comparison to control (compare lane 1 with 5, Fig. 1A). Temporal studies demonstrated that transcriptional activation of HOXC6 was increased with the increase in incubation time with maxima at ~8 h and then decreased gradually (likely due to squelching) (Fig. 1B). We also analyzed the E2-dependent expression of HOXC6 in additional ER-positive breast cancer cell line MCF7 and an ER-negative breast cancer cell line MDA-MB-231. Our results showed that HOXC6 is also transcriptionally activated by E2 in a concentration dependent manner in MCF7, but not in ER-negative MDA-MB-231 cells (Supplementary figure S1). The stimulation of HOXC6 in two independent steroidogenic cell lines but not in the ER-negative cell suggested that it is an E2-responsive gene. As JAR cells showed more robust response to E2, we performed all mechanistic studies in JAR cells.

HOXC6 promoter contains estrogen response elements (EREs)

Estrogen-responsive genes are regulated via diverse mechanisms involving estrogen receptors (ER) and various ER-coregulators.[56] Commonly, upon binding to estrogen, ERs

get activated and then targeted to specific DNA sequence elements called estrogen response elements (EREs) present in the promoter of estrogen-responsive genes leading to their transcriptional activation.[57] As HOXC6 showed E2-dependent stimulation, we examined its promoter sequence (up to -3000 nt) for the presence of any consensus EREs (GGTCAnnnTGACC). We found that HOXC6 promoter contains two $ERE_{1/2}$ sites (GGTCA) located at –125 nt and –1143 nt regions located upstream of the transcriptional start site (Fig. 2A). Analysis of the neighboring sequences around these $ERE_{1/2}$ sites revealed that $ERE_{1/2}$ at -125 nt region has GGTCAnnTGACT sequence which has one base pair difference in the palindrome compared to the consensus full ERE (GGTCAnnnTGACC). Furthermore, the palindromic sequences are also separated by two nucleotides instead of three nucleotide separation in a typical full ERE. This analysis suggested that the ERE-sequence located at -125 nt region might be an imperfect ERE (termed as $ERE1_{1/2}$, Fig. 2A). The second $ERE_{1/2}$ site located at -1143 nt regions has no similarity to a consensus full ERE (termed as $ERE2_{1/2}$, Fig. 2A).

To examine the potential involvement of HOXC6 promoter EREs in estrogen-response, we cloned the promoter region containing $ERE1_{1/2}$ and $ERE2_{1/2}$ and also each ERE separately in a luciferase based reporter construct, pGL3 (clones 1-3, Fig. 2A). A non-ERE sequence from the HOXC6 promoter was cloned as negative control (clone 4, Fig. 2A). We transfected each ERE-pGL3 constructs into JAR cells separately, then exposed to E2 (100 nM for 8 h) and then analyzed the luciferase induction using a commercial luciferase detection kit. We also cotransfected a renilla luciferase construct and analyzed the renilla expression as an internal transfection control that was used for normalization of luciferase expression from ERE-pGL3 constructs in the absence and presence of E2. Our analysis showed that transfection with control plasmid (empty pGL3) or with non-ERE plasmid (nonERE-pGL3) followed by treatment with E2, did not have any significant effect on luciferase induction (Fig. 2B). However, transfection with $ERE1_{1/2}$-$ERE2_{1/2}$-pGL3 (clone 1), $ERE1_{1/2}$-pGL3 (clone 2), or $ERE2_{1/2}$-pGL3 (clone 3) constructs followed by exposure to E2, increased the luciferase induction by about 7.5, 5.6 and 2.3 fold respectively compared to the control (Fig. 2B). The highest E2-response (luciferase activity) was observed for the construct that contain both EREs together. The higher E2-response of the $ERE1_{1/2}$-pGL3 than $ERE2_{1/2}$-pGL3 is likely due to higher homology of the $ERE1_{1/2}$ with a consensus full ERE than just ERE-half site present in $ERE2_{1/2}$ region. Point mutations in $ERE1_{1/2}$ (GGTCA to AATCA) keeping $ERE2_{1/2}$ intact (in clone 1) significantly decreased the luciferase activity (from 7.5 fold to 2.6 fold), while mutation in $ERE2_{1/2}$ keeping $ERE1_{1/2}$ intact showed relatively less impact on luciferase induction (from 7.5 to 5.1 fold). Mutation for both EREs simultaneously (TGACC to TGAAA) abolished the E2-dependent luciferase induction almost to basal level (Fig 2B). These observations suggest that $ERE1_{1/2}$ is major regulator in E2-dependent regulation of HOXC6, though both EREs appear to have interdependent roles.

Estrogen receptors (ERs) are essential for E2-mediated activation of HOXC6

As ERs are key players in transcriptional regulation of estrogen-sensitive genes[56], we examined roles of ERs in E2-mediated activation of HOXC6. We knocked down ERα and ERβ separately using specific antisense oligonucleotides (Table 1) and then exposed the ER-knocked down cells to E2. A scramble antisense (with no homology to ERs) was used as negative control. The knockdown efficiency of ERα and ERβ by respective antisense was analyzed at protein levels using western blot and as expected, application of either ERα or ERβ antisense (9 μg) knocked down respective ER (lane 3 for ERα knockdown and lane 4 for ERβ knockdown, Fig. 3A). RNA from ER-knocked down and E2-treated cells was reverse transcribed and cDNA was PCR-amplified using primers specific to β-actin (control), ERs and HOXC6. Our results demonstrated that HOXC6 expression was increased

as expected upon exposure to E2 (lane 2, Fig. 3B, real-time quantification in panel C). Application of scramble antisense did not have any significant effects on E2-mediated activation of HOXC6 (lane 3, Figs. 3B and C). Interestingly, upon depletion of either ERα or ERβ, the E2-dependent activation of HOXC6 was suppressed (compare lanes 4 and 5 with lanes 2-3, Figs. 3B-C). Combined knockdown of ERα and ERβ, further suppressed E2-dependent HOXC6 expression (lane 6, Figs. 3B-C). These results demonstrated that both ERα and ERβ play important roles in E2-dependent HOXC6 expression.

MLLs are essential in regulation of HOXC6 under estrogen environment

As MLL histone methylases are key regulators of HOX genes and several MLLs are implicated in estrogen-signaling via their interaction with ERs, we examined if MLLs are involved in E2-dependent activation of HOXC6 expression. We knocked down MLL1, MLL2, MLL3, and MLL4, independently by using specific antisense oligonucleotides (Table 1), then exposed the cells to E2 (100 nM for 8 h) and analyzed their impacts on HOXC6 expression using RT-PCR. The application of MLL-antisenses resulted in specific knockdown of respective MLLs both at mRNA (compare lanes 3 with lane 1, Figs. 4A-D for MLL1 to MLL4, respectively) and protein levels (data not shown). A scramble antisense (with no homology to MLLs) was used as negative control. As seen in figure 4A, the application of MLL1-antisense specifically knocked down MLL1 but not β-actin (control) (compare lanes 1 and 3, Fig. 4A). MLL1-knockdown has no significant effect on E2-mediated activation of HOXC6 (compare lane 2 and 3, Fig. 4A). Interestingly, the application of MLL2-antisense not only knocked down MLL2 but also suppressed E2-induced expression of HOXC6 (compare lane 3 with lanes 1 and 2, Fig. 4B, real-time quantification is in the bottom panel). Similar to MLL2, knockdown of MLL3 also resulted in suppression of E2-mediated activation of HOXC6 (Fig. 4C, real-time quantification in the bottom panel). However, similar to MLL1, knockdown of MLL4 did not show any significant effect on E2-dependent HOXC6 expression (compare lane 3 with lanes 1 and 2, Fig. 4D). These observations demonstrated that MLL2 and MLL3 play critical roles in E2-mediated activation of HOXC6.

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

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

As MLL2 and MLL3 were found to be critical in E2-mediated activation of HOXC6, we examined the E2-dependent binding of different MLLs (MLL1-4) in the HOXC6 promoter using ChIP assay with antibodies specific to different MLLs. These analysis demonstrated that binding of MLL2 and MLL3 was increased in both $ERE1_{1/2}$ and $ERE2_{1/2}$ in presence of E2 (compare lanes 1 with 2 for binding in $ERE1_{1/2}$, and 3 with 4 for binding in $ERE2_{1/2}$

regions, Figs. 5D, real-time PCR analysis of the ChIP DNA samples is shown in panel 5E). E2-dependent binding of MLL2 and MLL3 are more robust in the $ERE1_{1/2}$ region in comparison to the $ERE2_{1/2}$ (compare lanes 1 and 2 with 3 and 4, Figs. 5D-E). In contrast to MLL2 and MLL3, binding of MLL1 and MLL4 were not enhanced in presence of E2, instead decreased level of binding of MLL1 (to $ERE2_{1/2}$) and MLL4 (to $ERE1_{1/2}$) were observed in presence of E2 (Figs. 5D-E). These results demonstrated further that, in addition to ERs, MLL2 and MLL3 play critical role in E2-dependent activation of HOXC6.

To further confirm the E2-dependent binding of ERs and MLLs to HOXC6 promoter, we analyzed their binding pattern in a time-dependent manner using ChIP assay with ER and MLL antibodies. ChIP DNA samples were PCR-amplified using real-time PCR and plotted (Fig. 6, agarose gel analysis of the PCR products are shown in the supplementary figure S2). In agreement with our above studies, we observed that binding of ERα and ERβ was enhanced in both $ERE1_{1/2}$ and $ERE2_{1/2}$ regions in presence of E2 in a temporal manner (Figs. 6A-B). ERα and ERβ enrichments were observed as early as 15 min and increased with time reaching saturation within 2-3 hrs, (Figs. 6A-B). Recruitment of MLL2 and MLL3 were also enhanced in both $ERE1_{1/2}$ and $ERE2_{1/2}$ regions in presence of E2, though kinetics of their recruitment to different EREs was different (Figs. 6C-D). In the $ERE1_{1/2}$ region, the enhanced recruitment of MLL3 was observed as early as 15 min after E2 treatment and then reached to saturation within 2 hr and this kinetics appeared to be very similar to recruitment of ERα and ERβ in the $ERE1_{1/2}$ region (Fig. 6C). However, the E2-dependent binding of MLL2 to the $ERE1_{1/2}$ was delayed to 4 hr post E2-treatment and then increased and reached to saturation at around 6-8 hr (Fig. 6C). However, in the $ERE2_{1/2}$ region, similar to the kinetics of recruitment of ERs, E2-dependent recruitment of MLL2 and MLL3 was initially increased and then reached to saturation within 2-3 hr (Fig. 6D). Interestingly however, in agreement with our observation in figure 5, significant amount of constitutive binding of MLL4 to $ERE1_{1/2}$ and MLL1 to $ERE2_{1/2}$ regions were observed and these bindings were gradually decreased in a time dependent manner in presence of E2 (Figs. 6C and D). No binding of MLL1 to the $ERE1_{1/2}$ and MLL4 to the $ERE2_{1/2}$ were observed irrespective of presence of E2 (Figs. 6C and D). These studies further suggested that MLL2 and MLL3 along with ERα and ERβ, play key roles in E2-dependent activation of HOXC6, while MLL1 and MLL4 might be involved in basal transcription of HOXC6.

As E2-treatment enhanced recruitment of MLL histone methylases onto HOXC6 EREs, we analyzed if H3K4-trimethylation level at the HOXC6 promoter is also enhanced upon exposure to E2. We observed that H3K4-trimethylation level and RNA polymerase II (RNAPII) recruitment were increased in the ERE regions of HOXC6 promoter in a time-dependent manner as a function of E2, while the net level of histone H3 at the HOXC6 promoter region remained almost constant (Figs. 6E-F). Notably, we also observed the presence of relatively low amount of H3K9-dimethylation marks present in HOXC6 EREs and these marks decreased upon treatment with E2.

It is known that general transcription factor TFIID interacts with trimethylated histone H3K4 and facilitates the pre-initiation complex (PIC) assembly at the gene promoter.[58] We examined if components of TFIID are also concomitantly recruited in the HOXC6 promoter with increase in H3K4-trimethylation and RNAPII in presence of E2. Our ChIP analysis showed that along with enrichment in H3K4-trimethylation and RNAPII level, there is increased recruitment of TBP (TATA binding protein, component of TFIID) and TAF250 (TBP-associated factor 250) in the HOXC6 promoter EREs in presence of E2 (lane 2, Fig. 7A-B). Knockdown of either MLL2 or MLL3 decreased the E2-dependent enrichment of TBP, TAF250, H3K4-trimethylation and RNAPII (lanes 4-5, Figs. 7A-B), indicating critical roles of MLL2 and MLL3 histone methylases in PIC assembly at the HOXC6 promoter during E2-mediated gene activation.

To understand if any corepressor is involved in maintaining transcriptionally repressed state of HOXC6 in absence of E2, we examined the binding of N-CoR (nuclear receptor corepressor) and SAFB1 (scaffold attachment factors B1), the two well known nuclear receptor corepressor[59-61] using ChIP assay. Interestingly, we found that both N-CoR and SAFB1 were bound to the HOXC6 promoter (in both EREs) in the absence of E2 and their binding was gradually decreased upon exposure to E2 (Figs. 7C-D, quantification in the respective bottom panels).

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

ERs are well known to bind directly to EREs of estrogen-responsive genes via their own DNA binding domain. Notably, MLLs (MLL1-4) also have several DNA binding domains and these DNA binding domains may facilitate their direct binding with the promoter.[22] Alternatively, MLLs may be recruited to the promoter via interactions with ERs or other associated proteins. Notably, MLL2 and MLL3 have multiple LXXLL domains (NR boxes) and are previously reported to interact with ERα in presence of estrogen.[35,50,51] We examined if MLL2 and MLL3 that are involved in E2-mediated activation of HOXC6 bind to HOXC6 EREs directly or their bindings are dependent on ERs. To examine this, we knocked down ERα and ERβ separately, then exposed the cells to E2 (100 nM for 8 h) and analyzed the status of MLL2 and MLL3 recruitment to $ERE1_{1/2}$ and $ERE2_{1/2}$ regions of HOXC6 promoter in the absence and presence of E2 (Fig. 8). Our results demonstrated that binding of MLL2 and MLL3 were increased in both $ERE1_{1/2}$ and $ERE2_{1/2}$ regions in presence of E2 (lanes 1, 2 and 5, 6, Fig. 8). However, knockdown of either ERα or ERβ, decreased (or even abolished) the recruitment of MLL2 and MLL3 onto both the EREs (compare lanes 3-4 with lane 2 and 7-8 with 6, Fig. 8). These results demonstrated that binding of both MLL2 and MLL3 to the HOXC6 promoter (in presence of E2) is dependent upon ERα and ERβ.

Discussion

HOX genes are critical players in the development and diseases and therefore, understanding their roles and regulation is important.[3] Vast bodies of literature exist that address the functions of different HOX genes during embryonic development in various types of organism. Increasing amounts of evidence suggest that beyond their critical roles in development, various HOX genes are misregulated and overexpressed in variety of disease including breast and prostate cancer.[3] HOX genes are potential target for novel biomarker development and targeted gene therapy.[4-9] In spite of their roles in development and disease, little is known about the mechanism by which these HOX genes may be expressed and regulated in different types of tissues or in cancer cells. Increasing amounts of studies indicate that HOX genes (especially HOXA genes) are potentially regulated by steroid hormones and may be misregulated upon exposure to endocrine disruptors.[62-65] In our studies we focused towards understanding the regulatory mechanism of HOXC6 especially in presence of steroid hormone estrogen. HOXC6 is expressed in various steroidogenic tissues.[10,13,66] HOXC6 homozygous mutant female mice showed the absence of epithelial cells in thoracic mammary gland.[4,12] In mammary glands of ovariectomized animals, HOXC6 transcript levels are substantially elevated compared to glands from intact virgin mice, indicating a link between ovarian hormones and HOXC6 expression.[4,12,15] HOXC6 expression is associated with osteosarcomas, medulloblastomas, breast and prostate carcinomas.[4,10-16] Our studies demonstrated that HOXC6 gene is transcriptionally activated upon exposure to E2 in human breast cancer (MCF7) and placental choriocarcinoma cell lines (JAR).

We have also investigated the molecular mechanism by which E2 regulates HOXC6 gene expression. Sequence analysis revealed that HOXC6 promoter contains two putative EREs,

within first 3000 bp upstream of transcription start site. $ERE1_{1/2}$ which is located at -5 nt, is a nearly complete full ERE, whereas $ERE2_{1/2}$ (at -1023 nt) region is a ERE half-site. Luciferase based reporter analysis demonstrated that both $ERE1_{1/2}$ and $ERE2_{1/2}$ are responsive to E2, $ERE1_{1/2}$ being more responsive than $ERE2_{1/2}$. Mutation of $ERE1_{1/2}$ resulted in significant loss in E2-dependent luciferase induction, in comparison to mutation in the $ERE2_{1/2}$ (Fig 2). $ERE1_{1/2}$ and $ERE2_{1/2}$ appeared to have interdependent E2-response in luciferase induction suggesting their potential coordination during E2-mediated gene activation. The enhanced E2-response of $ERE1_{1/2}$ over $ERE2_{1/2}$ further suggests that the $ERE1_{1/2}$ is potentially an imperfect full ERE. Notably, genes with imperfect EREs are well known to be regulated by estrogen and estrogen receptors.[63,67-69] Though it is obvious that ERE-pGL3 are artificial constructs and do not represent a native chromatin environment of HOXC6 promoter present in the cell nucleus, the induction of luciferase activity upon E2-expsoure suggested that $ERE1_{1/2}$ and $ERE2_{1/2}$ sequences of HOXC6 promoter are responsive to estrogen.

Antisense-mediated knockdown experiments demonstrated that both ERα and ERβ are involved in E2-mediated activation of HOXC6. Generally, ERs bind to the EREs of estrogen-responsive genes as a function of estrogen. Depending on the target gene and cell types, ERα and ERβ, can form homo- and heterodimers prior to binding to the response elements on the target gene promoters. ChIP analysis demonstrated that both ERα and ERβ bind to the $ERE1_{1/2}$ as well as $ERE2_{1/2}$ (Fig. 5) although binding to the $ERE1_{1/2}$ is slightly more efficient than $ERE2_{1/2}$. Some amount of constitutive binding of ERβ is also observed in the $ERE1_{1/2}$ region in the absence of E2, which may have implication in regulation HOXC6 under basal environment (Fig. 5A). Temporal studies (Fig. 6) also demonstrated that binding of ERs to $ERE1_{1/2}$ takes places at earlier time points than $ERE2_{1/2}$. The higher and faster response of $ERE1_{1/2}$ than $ERE2_{1/2}$ towards ERs binding is likely due to the difference in imperfect full ERE ($ERE1_{1/2}$) versus ERE-half site ($ERE2_{1/2}$). Importantly, in the transient transfection based luciferase assay (Fig. 2), we also observed higher sensitivity of $ERE1_{1/2}$ towards E2-exposure than $ERE2_{1/2}$.

During E2-mediated gene regulation, in addition to ER, various other activators and coactivators (commonly known as ER-coregulators) participate in the process and bind to the promoter of estrogen-sensitive genes leading to their activation.[57] Diverse arrays of ER-coregulators have been discovered and many of them contain enzymatic activities (such as acetyl-transferase activity) that presumably modify chromatin, lead to structural changes and chromatin remodeling resulting in transcription activation.[56,70] Recent studies demonstrated that histone methylases MLL2, MLL3, and MLL4, act as co-activators for ERs in regulation of E2-responsive genes.[32,35,50,52] Notably proteins containing LXXLL (NR box) are known to interact with nuclear hormone receptors (NRs) and play critical roles in ligand-dependent gene activation.[22] Sequence analysis of the MLLs showed that MLL1 contains only one LXXLL domain that remains buried in its globular domain.[22,71] Whereas MLL2, MLL3, and MLL4 contain at least four NR-boxes indicating their more facile interaction with ERs.[22,71] Our studies (Fig. 4) demonstrated that, antisense-mediated knockdown of MLL2 and MLL3 resulted in downregulation of E2-dependent activation of HOXC6. However, knockdown of MLL1 and MLL4 had no significant effect in this process. ChIP analysis (Figs. 5-6) demonstrated that, MLL2, and MLL3 were bound to the $ERE1_{1/2}$ and $ERE2_{1/2}$ regions of the HOXC6 promoter in an E2-dependent manner. These results demonstrated that MLL2 and MLL3 play critical roles in E2-mediated activation of HOXC6. In contrast to MLL2 and MLL3, we observed binding of some amount of MLL1 (to $ERE2_{1/2}$) and MLL4 (in the $ERE1_{1/2}$) even in the absence of E2 (Figs. 5-6) and these bindings of MLL1 and MLL4 were decreased upon addition of E2. Thus, these observations suggest that, upon transcription activation by E2, there may be an exchange of basal transcription factors (such as MLL1 and MLL4 in this case) with the factors that are associated with activated

transcription (such as MLL2 and MLL3). These observations further indicate that MLL1 and MLL4 may be involved in E2-independent basal transcriptional regulation and maintenance of HOXC6 expression, while MLL2 and MLL3 are critical for E2-dependent transcription activation of HOXC6.

ERs are well known for binding to the EREs of estrogen-responsive genes via their DNA binding domains[56] However, the recruitment of MLL2 and MLL3 to the HOXC6 promoter could have different options. Both MLL2 and MLL3 contain DNA binding domains in their N-terminus which may lead to their direct binding to the promoter, though it may not depend on estrogen. Otherwise, these MLLs may interact with ERs via their NR-boxes leading to their recruitment onto the ERE regions via ERs. Our results (Fig. 8) demonstrated that independent knockdown of both ERα and ERβ resulted in decreased binding of MLL2 and MLL3 into the EREs of HOXC6 suggesting their ER-dependent mode of binding of MLLs. Notably, both ERα and ERβ are known to regulate ER-responsive genes either independently or in combination.[56] JAR cells do express both ERα and ERβ, and both ERs are involved in E2-mediated activation of HOXC6. So it is likely that MLL2 and MLL3 interact with ERα and ERβ and bind to EREs that facilitate the recruitment of MLL2 and MLL3 into the HOXC6 promoter leading to HOXC6 transactivation.

What could be the potential roles of MLL2 and MLL3 in E2-mediated HOXC6 activation? MLL2 and MLL3 are both histone H3K4-specific methyl-transferases and H3K4-trimethylation is critical for transcription activation. Analysis (Fig. 6E-F) of the H3K4-trimethylation status in HOXC6 promoter demonstrated that similar to MLL2 and MLL3, the level of H3K4-trimethylation is increased in the HOXC6 promoter upon exposure to E2. This finding suggest that MLL2 and MLL3 may be acting as the histone H3K4-trimethylases that help in promoter opening (via recruitment of other chromatin remodelers) and recruitment of general transcription factors (GTFs) including RNAPII, leading to transcription activation. Indeed our results (Figs. 7A-B) demonstrated that along with enrichment of H3K4-trimethylation and RNAP II recruitment, binding of TFIID components such as TBP and TAF250 were increased upon treatment with E2 and these bindings were decreased upon knockdown of either MLL2 or MLL3 indicating key roles of MLL2 and MLL3 in E2-dependent histone H3K4-trimethylation, recruitment of GTFs, RNAPII and assembly of transcription pre-initiation complexes.

Furthermore, we observed that histone methylases MLL2 and MLL3 are actively exchanged with MLL1 and MLL4 upon treatment with E2 and ER-binding causing the transition from basal to activated transcription state of HOXC6. The obvious question is “Does HOXC6 remain repressed in the absence of E2?” To address this we analyzed the binding of two well known nuclear receptor corepressors N-CoR and SAFB1 in the absence and presence of E2. Notably, SAFB1/2 and N-CoR function as ER corepressor, they directly interact with each other as well as with ER, and repress transcription.[59-61,72] Our studies demonstrated that indeed N-CoR and SAFB1 were bound to both $ERE1_{1/2}$ and $ERE2_{1/2}$ in the absence of E2 (Figs. 7C-D). Binding of both N-CoR and SAFB1 was decreased upon treatment with E2 in a time dependent manner while the binding of ERα was increased. These observations suggested that HOXC6 transcription was originally repressed by co-repressors in the absence of E2 and this repression was relived in the presence of E2 which is mediated via binding of ERs and various ER-coactivators including MLL2 and MLL3. Constitutive binding of ERs (as observed in Fig 5A) may be responsible for the recruitment of N-CoR and SAFB1 corepressors in the HOXC6 promoter in absence of E2. The level of MLL2 and MLL3 binding to HOXC6 EREs in presence of E2 did not seem to be affected significantly by MLL1 or MLL4 knockdown, though the binding of MLL2 (to $ERE1_{1/2}$) is slightly increased in the absence of E2 (see supplementary figure S3). We also examined the level of histone H3K9-methylation in the HOXC6 promoter ERE regions in the absence and

presence of E2. H3K9-methylation is usually associated with transcriptionally repressed chromatin or silenced chromatin.[73] Our ChIP analysis showed that levels of H3K9-dimethylation were relatively low in both $ERE1_{1/2}$ and $ERE2_{1/2}$ regions in the absence of E2, this level was further decreased upon addition of E2. These observations suggested that H3K9-methylation is at least partially responsible for transcriptional repression (basal transcription) of HOXC6 in the absence of E2. The detailed mechanism of transcriptional repression, functional interaction of NCoR, SAFB1 with the HOXC6 promoter and different histone modification states and their coordination with MLL1 and MLL4 still need to be investigated. In addition, we also can not explain why there is an exchange of MLL1 and MLL4 with MLL2 and MLL3 upon E2-expsoure, even though MLL1 and MLL4 could have done the histone methylation job, interaction with ERs, and promoter opening. It may be hypothesized that MLL2 and MLL3, in addition to their histone methylation activities, specifically interact with and recruit various other ER-coregulators that are specific to HOXC6 gene expression and regulation.

Notably, HOXC6 is expressed in various steroidogenic tissues and overexpressed in hormone sensitive breast and prostate cancers indicating critical roles of steroid hormone in transcriptional regulation of HOXC6.[4,10,15] In contrast, increased expression of HOXC6 in mammary glands of ovariectomized female mice indicates potential negative regulation of this gene by ovarian hormone.[5,74] These observations suggest that HOXC6 expression could both be positively and negatively regulated by steroid hormones and which is likely dependent on tissue type. Our studies demonstrated that HOXC6 is transcriptionally activated by estrogen in breast (MCF7) as well as placental choriocarcinoma (JAR) cells and histone methylases MLL2 and MLL3, in coordination with ERα and ERβ, play critical roles in E2-induced HOXC6 expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research highlights

▶ HOXC6 is transcriptionally regulated by estrogen (E2). ▶ Histone methylases MLL2 and MLL3, along with estrogen-receptors, play critical roles in E2-mediated activation of HOXC6. ▶ MLL1 and MLL4, and NR-corepressors (N-CoR, SAFB1), are bound to the HOXC6 promoter in the absence of E2 and are replaced with MLL2 and MLL3 during E2-mediated gene activation.

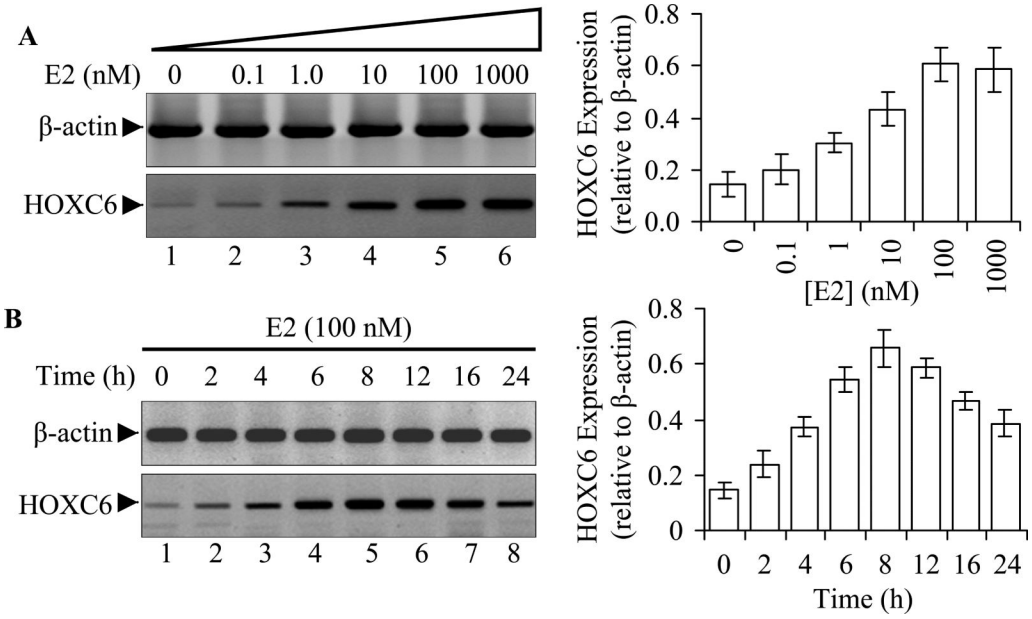

Figure 1.

Effect of estrogen on HOXC6 gene expression. (**A**) JAR cells (grown in phenol red free media) were treated with varying concentrations of E2. RNA from the control and E2-treated cells was isolated, converted to cDNA and analyzed by PCR using primers specific to HOXC6. β-actin was used as a loading control. The cDNA was also analyzed by real-time PCR and expression of HOXC6 (relative to β-actin) is plotted in the right panel. (**B**) JAR cells were treated with 100 nM E2 for varying time periods (0-24 h) and reverse transcribed-PCR products were analyzed in agarose gel and quantified using real-time PCR (right panel). Each experiment was repeated at least thrice. Bars indicate standard errors ($p<0.05$).

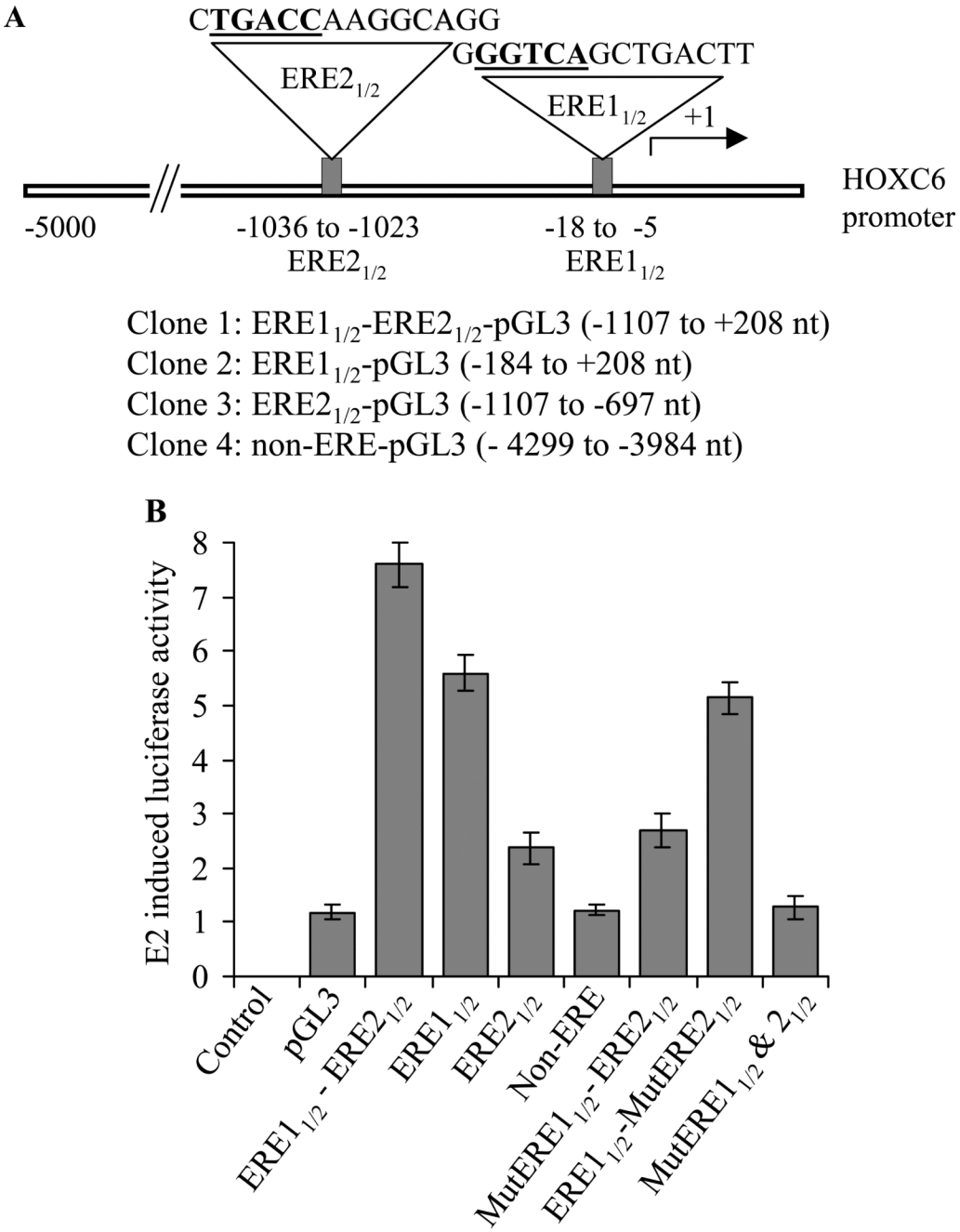

Figure 2.

HOXC6 promoter EREs and their estrogen-response: (**A**) HOX gene promoter EREs (termed as $ERE1_{1/2}$ and $ERE2_{1/2}$, locations and the neighboring sequences are shown). HOXC6 promoter regions spanning $ERE1_{1/2}$ to $ERE2_{1/2}$, $ERE1_{1/2}$ (alone), $ERE2_{1/2}$ (alone) and a non-ERE regions were cloned (clones 1-4) into a luciferase based reporter construct, pGL3, used for transfection and reporter assay. In the mutant pGL3 constructs, clone 1 used for mutation of either $ERE1_{1/2}$ or $ERE2_{1/2}$ alone or both $ERE1_{1/2}$ and $ERE2_{1/2}$ simultaneously. For mutations, the GG of $ERE1_{1/2}$ and CC of $ERE2_{1/2}$ were mutated to AA. (**B**) Luciferase based reporter assay. $ERE1_{1/2}$-pGL3 and $ERE2_{1/2}$-pGL3 constructs were transfected into JAR cells for 24 h. Control cells were treated with empty pGL3 vector and

non-ERE-pGL3. A renilla luciferase construct was also co-transfected along with ERE-pGL3 constructs as 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 (normalized to renilla activity) in presence of E2 over untreated controls were plotted. The experiment with four replicate treatments was repeated at least twice. Bars indicate standard errors.

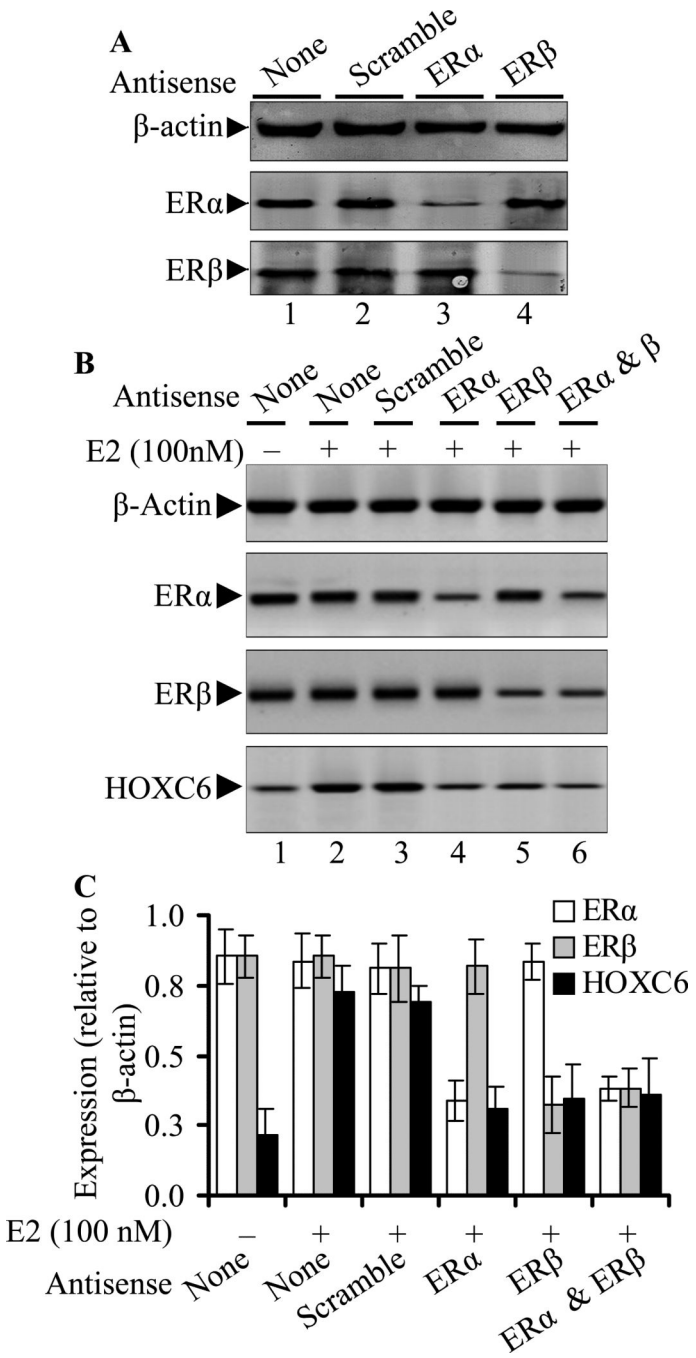

Figure 3.

Effect of depletion of ERα and ERβ on E2 induced expression of HOXC6. **(A)** Assessment of ERα and ERβ antisense-mediated knockdown of respective ERs. JAR cells were transfected with ERα, ERβ or scramble antisense (9 μg each) for 48 h and proteins were analyzed by western blot using ERα, ERβ, and β-actin antibodies. (**B-C**) Effects of ERα and ERβ knockdown on E2-mediated activation of HOXC6. JAR cells were transfected with ERα, ERβ or scramble antisense (9 μg each) for 48 h separately and treated with E2 (100 nM) for additional 8 h. RNA was isolated and subjected to reverse transcriptase-PCR analysis by using primers specific to HOXC6, ERα, and β-actin (loading control). PCR products were analyzed in agarose gel and quantified using real-time PCR (panel C). Lane 1:

control cells, lane 2: cells were exposed to 100 nM E2. Lane 3-5: cells were initially transfected with scramble, ERα, and ERβ antisenses separately followed by exposure to E2. Lane 6: Cells were transfected with a mixture (1:1) of ERα and ERβ antisenses followed by exposure to E2. Real-time quantification of cDNA (showing the relative level of HOXC6 expression) is shown in the bottom panel. Each experiment was repeated at least thrice (n = 3). Bars indicate standard errors.

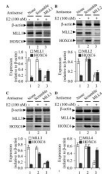

Figure 4.

Effect of depletion of MLL1, MLL2, MLL3, and MLL4 on E2-induced expression of HOXC6. JAR cells were transfected with 5 μg (2×10^6 cells) of MLL1, MLL2, MLL3, and MLL4 specific phosphorothioate antisense oligonucleotides separately. Control cells were treated with a phosphorothioate scramble antisense with no homology with MLL1, MLL2, MLL3, and MLL4 genes. The antisense-treated cells were incubated for 48 h followed by treatment with 100 nM E2 for 8 h. RNA was isolated from treated and control cells and subjected to reverse transcriptase-PCR by using primers specific to HOXC6 along with MLL1, MLL2, MLL3, and MLL4. β-actin was used as control. The PCR products were analyzed by agarose gel and quantified. Real-time PCR quantification of the cDNA showing the relative levels of respective MLL and HOXC6 expression are shown in the respective bottom panel. **(A)** Effects of MLL1 knockdown. (Top) Lane 1: control cells; lane 2: cells that were initially transfected with 5 μg of scramble antisense followed by exposure to E2. Lanes 3: cells were initially transfected with MLL1 antisense and then treated with E2. Expression levels of MLL1 and HOXC6 (relative to actin, average of three replicate experiments, n = 3) were quantified using real-time PCR and plotted in the bottom panel. (**B-D)** These figures show the effects of knockdown of MLL2, MLL3, and MLL4, respectively, in the similar manner as shown for MLL1 in panel A.

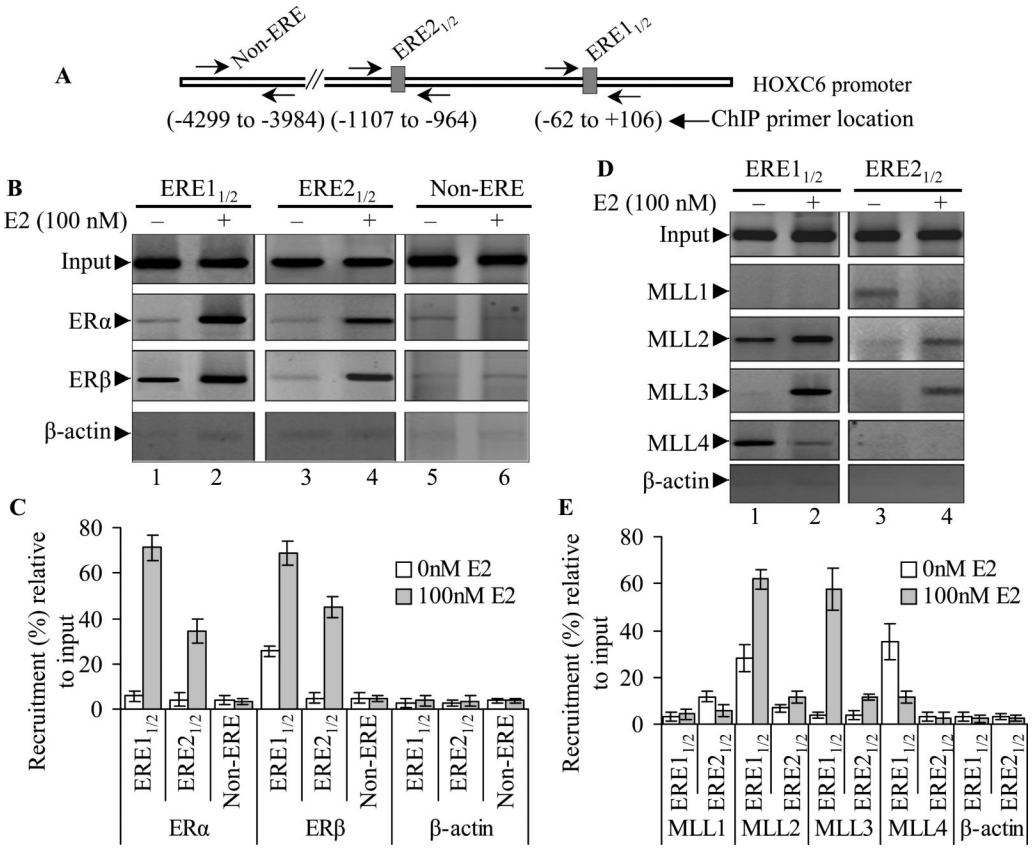

Figure 5.

E2-dependent recruitment of ERs and MLLs in the ERE regions of HOXC6 promoter. (**A**) Scheme showing positions of ChIP PCR primers. (**B-C**) Recruitment of ERs: JAR cells were treated with 100 nM E2 for 8 h and subjected to ChIP assay using antibodies specific to ERα and ERβ. β-actin antibody was used as control IgG. The immuno-precipitated DNA fragments were PCR-amplified using primers specific to $ERE1_{1/2}$ and $ERE2_{1/2}$ of HOXC6 promoter. Primer specific to a promoter sequence containing no ERE (non-ERE) was used as control. ChIP DNA fragments were analyzed by real-time PCR and shown in the panel B. Each experiment was repeated at least thrice. Bars indicate standard errors. (**D-E**) Recruitment of MLLs (MLL1-MLL4): JAR cells were treated with 100 nM E2 for 8 h and subjected to ChIP assay using antibodies specific to MLL1, MLL2, MLL3 and MLL4. ChIP DNA fragments were PCR-amplified using primers specific to $ERE1_{1/2}$ and $ERE2_{1/2}$ of HOXC6 promoter. ChIP DNA fragments were analyzed by real-time PCR and shown in panel D. Each experiment was repeated at least thrice. Bars indicate standard errors.

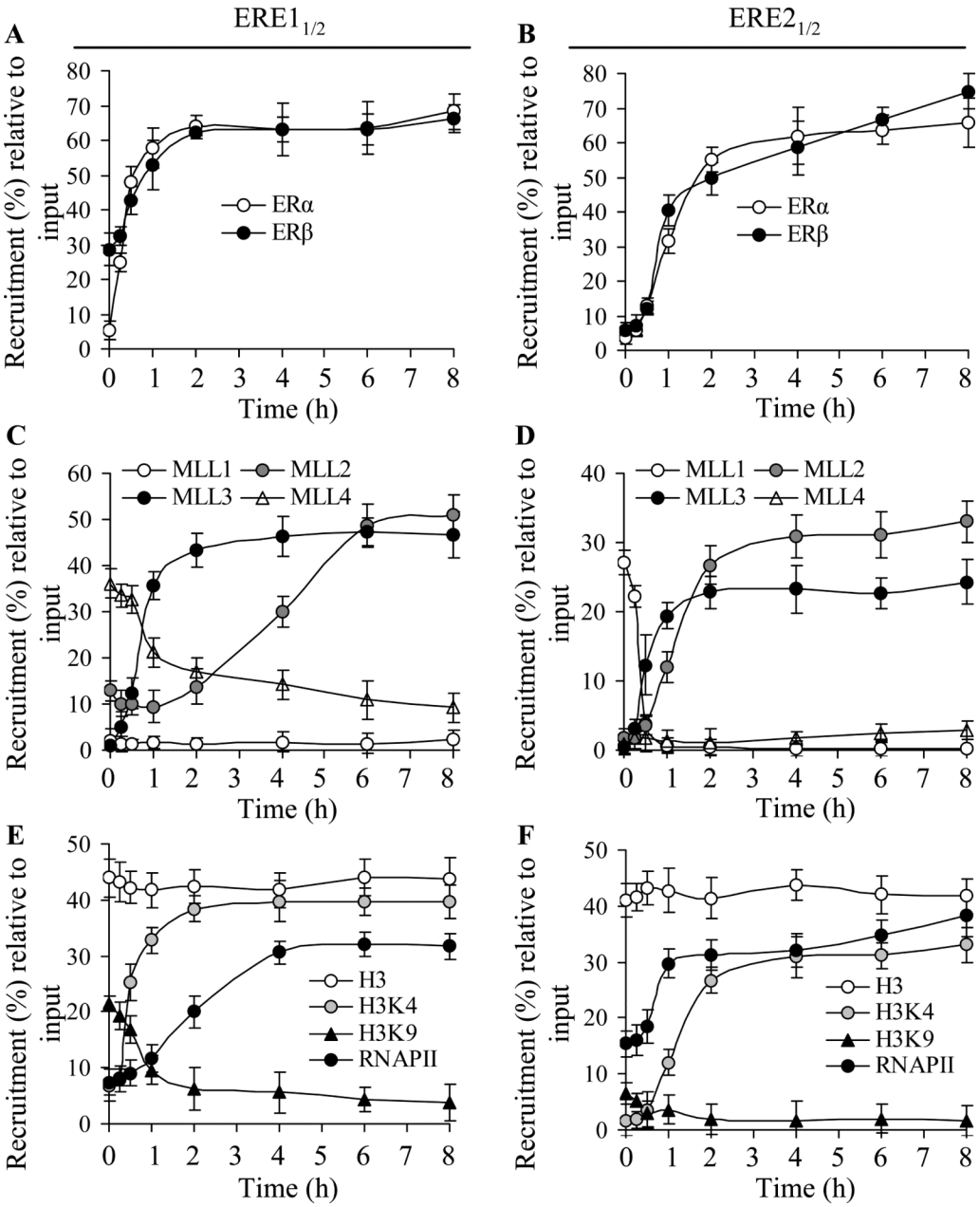

Figure 6.

Dynamics of recruitments of ERs and MLLs onto HOXC6 promoter: Cells were treated with 100 nM E2 for varying time periods (0 - 8 h) and then subjected to ChIP assay using antibodies specific to ERα, ERβ, MLL1, MLL2, MLL3, MLL4, H3k4-trimethyl and RNA polymerase II. Immuno-precipitated DNA fragments were PCR-amplified using primers specific to $ERE1_{1/2}$ and $ERE2_{1/2}$ of HOXC6 promoter respectively, quantified and plotted. (**A-B**) Recruitment of ERα and ERβ in the $ERE1_{1/2}$ and $ERE2_{1/2}$. (**C-D**) Recruitment of MLL1-4 in the $ERE1_{1/2}$ and $ERE2_{1/2}$. (**E-F**) Recruitment of RNA pol II (RNAP II) and level of histone H3 (control), H3K4-trimethylation and H3K9-dimethylation. Each experiment was repeated at least thrice. Bars indicate standard errors.

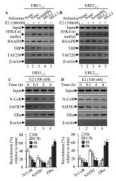

Figure 7.

(A-B) Role of MLL2 and MLL3 in E2-dependent assembly of general transcription factors and RNAP II in the HOXC6 promoter. JAR cells were transfected with MLL2, MLL3 or scramble antisenses for 48h and then treated with 100 nM E2 for additional 8h and subjected to ChIP assay by using antibodies specific to H3K4-tri methyl, RNAPII, TBP, TAF250. β-actin antibody was used as control IgG. The immuno-precipitated DNA fragments were PCR-amplified using primers specific to $ERE1_{1/2}$ and $ERE2_{1/2}$ regions of HOXC6 promoter. (**C-D**) E2-dependent recruitment N-CoR and SAFB1 in ERE regions of HOXC6 promoter in absence and presence of E2. JAR cells were treated with 100 nM E2 for varying time periods (0, 0.5, 4 and 8 h) and subjected to ChIP assay using antibodies specific to N-CoR and SAFB1. Antibodies specific to ERα and β-actin are used as positive and negative control IgG. The ChIP DNA fragments were PCR-amplified using primers specific to $ERE1_{1/2}$ and $ERE2_{1/2}$ of HOXC6 promoter. The real-time PCR quantification of the recruitment level is shown below the respective panels. Each experiment was repeated at least thrice. Bars indicate standard errors.

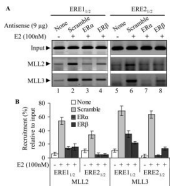

Figure 8.

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

Table 1

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

Primers	Forward primer (5′- 3′)	Reverse primer (5′ - 3′)
PCR primer		
β-actin	AGAGCTACGAGCTGCCTGAC	GTACTTGCGCTCAGGAGGAG
HOXC6	CAGACCCTGGAACTGGAGAA	CTTCCCGCTTTTCCTCTTTT
HOXC6- $ERE1_{1/2}$	TTTTTCCCCCTTCCTGACAT	GCCTTTACCTGGTCGGTCTA
HOXC6- $ERE2_{1/2}$	AGCCTCATAGCTCAGGTCCA	CCAGAAAGAGAAGGCTGGTG
HOXC6-non-ERE	TATGAGGGGAGCTGAGCAAT	CCCTCGCACACAGATACACA
MLL1	GAGGACCCCGGATTAAACAT	GGAGCAAGAGGTTCAGCATC
MLL2	AGGAGCTGCAGAAGAAGCAG	CAGCCAAACTGGGAGAAGAG
MLL3	CATATGCACGACCCTTGTTG	ACTGCTGGATGTGGGGTAAG
MLL4	CCCTCCTACCTCAGTCGTCA	CAGCGGCTACAATCTCTTCC
ERα	AGCACCCTGAAGTCTCTGGA	GATGTGGGAGAGGATGAGGA
ERβ	AAGAAGATTCCCGGCTTTGT	TCTACGCATTTCCCCTCATC
Cloning primer		
HOXC6- $ERE1_{1/2}$	CCACCAAACCAGTTCCCTTA*	ATCATAGGCGGTGGAATTGA*
HOXC6- $ERE2_{1/2}$	AGCCTCATAGCTCAGGTCCA*	CTCCTTCTCAGGACCCCTCT*
HOXC6-Non-ERE	TATGAGGGGAGCTGAGCAAT*	CCCTCGCACACAGATACACA*
Antisense		
MLL1 antisense	TGCCAGTCGTTCCTCTCCAC**	
MLL2 antisense	ACTCTGCCACTTCCCGCTCA**	
MLL3 antisense	CCATCTGTTCCTTCCACTCCC**	
MLL4 antisense	CCTTCTCTTCTCCCTCCTTGT**	
ERα antisense	CATGGTCATGGTCAG**	
ERβ antisense	GAATGTCATAGCTGA**	
Scramble antisense	CGTTTGTCCCTCCAGCATCT**	

*Flanked by appropriate restriction sites

**Phosphorothioate antisense oligonucleotide.