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Histone methylase MLL1 and MLL3 coordinate with estrogen receptors in estrogen-mediated HOXB9 expression

Khairul I. Ansari, **Bishakha Shrestha**, **Imran Hussain**, **Sahba Kasiri**, and **Subhrangsu S. Mandal***
Department of Chemistry and Biochemistry, The University of Texas at Arlington, Arlington, Texas 76019

Abstract

Homeobox gene HOXB9 is a critical player in development of mammary gland and sternum and in regulation of Renin which is closely linked with blood pressure control. Our studies demonstrated that HOXB9 gene is transcriptionally regulated by estrogen (E2). HOXB9 promoter contains several estrogen-response elements (ERE). Reporter assay based experiments demonstrated that HOXB9 promoter EREs are estrogen-responsive. Estrogen receptors ERα and ERβ are essential for E2-mediated transcriptional activation of HOXB9. Chromatin immuno-precipitation assay demonstrated that ERs bind to HOXB9 EREs as a function of E2. Similarly, histone methylases MLL1 and MLL3 also bind to HOXB9 EREs and play critical role in E2-mediated transcriptional activation of HOXB9. Overall, our studies demonstrated that HOXB9 is an E2-responsive gene and ERs coordinate with MLL1 and MLL3 in E2-mediated transcriptional regulation of HOXB9.

Post-translational modification of histone plays critical roles in gene expression and silencing (1, 2). Mixed lineage leukemias (MLLs) are highly evolutionarily conserved human histone methylating enzymes that specially methylate histone H3 at lysine 4 (H3K4) and regulate gene activation (3–15). Set1 is the sole MLL homologue and H3K4-specific histone methylase present in yeast (16). In higher eukaryotes, H3K4-specific HMTs are diverged with increased structural and functional complexity. In human, there are at least eight H3K4-specific HMTs that include MLL1, MLL2, MLL3, MLL4, MLL5, hSet1A, hSet1B, and ASH1 (17). The high conservation and multiplicity of MLLs suggest that they have crucial and distinct functions in the cell, though their detailed mechanisms of action are largely unknown (17–19). Each possesses a SET domain that is responsible for their histone H3K4-specific methylation activity and is linked with gene activation. MLL1 is often rearranged in human leukemias (20). During rearrangement MLL1 gets fused with various fusion proteins (called MLL-fusion partners) in frame leading to generation of chimeric proteins and oncogenic transformation. MLLs are also well known as master regulators of HOX genes that play key roles in embryonic development (3, 21). Mll1 knockout (mice) is embryonic lethal. Mll1 mutant mice also exhibit hematopoietic abnormalities and is associated with decreased expression of a number of Hox genes (Hoxa7, a9, a10, a4) in the Mll1 mutant fetal liver (22, 23). Biochemical studies demonstrated that MLLs (MLL1-4 and

*Corresponding author. Department of Chemistry and Biochemistry, The University of Texas at Arlington, 700 Planetarium Place, Room: 349CPB, Arlington, Texas 76019; Tel: 817-272-3804; Fax: 817-272-3808; smandalf@uta.edu.

Supporting Information. Table 1: Nucleotide sequences of primers and antisense oligonucleotides used in the manuscript; Figure S1: The plot of the actual values of luciferase levels for different ERE-PGL3 constructs in the absence and presence of E2, as discussed in figure 2; Figure S2: Western blot analysis of proteins samples obtained from MLL-antisense treated JAR cells showing the respective MLL-knockdown in protein levels. Figure S3: RT-PCR analysis showing the effect of knockdown of MLL1 and MLL3 on expression of ERα and ERβ in JAR cells. Supplemental materials may be accessed free of charge online at http://pubs.acs.org.

Set1A-B) exist as multiprotein complexes with several common core protein subunits including Ash2, Wdr5, Rbbp5, Cgbp and Dpy30 (4, 17, 18). MLL core proteins are involved in their recruitment in specific promoter and in regulation of di- and trimethylation states of histone H3K4 (2, 6, 17). In addition to core subunits MLLs also associate with distinct proteins and that might be linked with their distinct functions in cells (6, 17). Recent studies demonstrated, in addition to their general roles in gene activation, MLLs (MLL1-4) participate in hormone mediated gene activation and signaling (24–27). MLLs interact with nuclear hormone receptors and act as novel coregulators of hormone dependent gene activation (24–27).

HOX genes are evolutionarily conserved homeobox containing genes that play critical roles during embryonic development (28, 29). There are 39 HOX genes in human that are arranged in four different clusters (HOXA-D) and each plays unique function during development. Recent studies demonstrate that several HOX genes are associated in various types of diseases including cardiovascular diseases and cancer (30, 31). In particular relevance to our study, HOXB9 is a critical player in skeletal and mammary gland development (32). HOXB9 is involved in regulation of Renin, an aspartyl protease that cleaves angiotensinogen into angiotensin I (33). Angiotensin I is further cleaved into an octapeptide angiotensin II with the help of angiotensinogen converting enzyme and ultimately influences blood pressure, fluid homeostasis and electrolyte balance (33). HOXB9 along with PBX (another homeodomain containing protein) bind to the proximal promoter of Renin gene regulating its expression (33). Independent studies showed that Angiotensinogen and Renin gene expression are stimulated by estrogen (34).

As HOXB9 is expressed in hormone sensitive mammary gland development and is linked with control of steroid hormone regulated Renin-Angiotensinogen system, we examined if HOXB9 is also transcriptionally regulated by steroid hormone such as estrogen. Although, it is well recognized that MLLs are master players in HOX gene regulation, little is known about their roles in hormonal regulation of HOX genes. Our studies demonstrated that HOXB9 is an estrogen-responsive gene. Estrogen-receptors and MLL histone methylases play key roles in estrogen-dependent activation of HOXB9.

Experimental section

Cell culture, estrogen treatment and antisense–mediated knockdown experiments

In general, JAR (human choriocarcinoma placenta cells, ATCC) and MCF7 (human breast cancer cells, ATCC) were grown and maintained in RPMI-1640 and DMEM media respectively, supplemented with 10 % FBS, 2 mM L-glutamine and penicillin/streptomycin (100 unit and 0.1 mg/mL respectively) in presence of 5% CO_2 at 37 °C (35–37). For the estrogen treatment, cells were initially grown in phenol red-free media containing charcoal stripped FBS for three rounds and then treated with varying concentrations of E2 followed by incubation for varying time periods as needed.

For antisense-mediated knockdown experiments, JAR cells were grown up to 60 % confluency and transfected with varying amounts (0.6 to 1.8 μg/ml) of different antisense oligonucleotides (custom synthesized from IDT-DNA) in FBS-free media using ifect transfection reagent (MoleculA) and following manufacturer's instruction. In brief, antisense and ifect transfection reagents were mixed in 300 μL DMEM-F-12 was applied to cells (60 mm plate) in presence of 1.7 mL supplement free medium. The cells were incubated for 7 h followed by addition of 2 mL media containing all supplements and 20 % charcoal stripped FBS. Cells were then incubated for additional 48 h. Depending on need, antisense-treated cells may have been exposed to 100 nM E2 and incubated for additional 6 h and then harvested for RNA/protein extraction.

RNA extraction, Reverse transcription and Real Time PCR

The cells were harvested by centrifugation at 500g. The total RNA was extracted by using RNAGEM tissue plus RNA extraction kit (ZyGEM) following manufacturer's instruction. The reverse transcription reactions were performed with 1 μg of total RNA in a 25 μL reaction cocktail containing 2.4 μM of oligo dT (Promega), 100 units of MMLV reverse transcriptase, 1 x 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 final volume. For semi-quantitative PCR the cDNA was PCR amplified by using Taq DNA Polymerase (Genscript) and primers as described in supplementary Table 1. For real-time PCR analysis, the cDNA was amplified using SsoFast EvaGreen supermix (Bio-Rad) using CFX96 real-time PCR detection system. The real-time PCR results were analyzed using the CFX Manager software. The experiments were repeated at least twice with three replicates each time.

Protein extraction and western blotting

Whole cell protein extracts from E2 and/or antisense-treated and control cells were prepared in whole cell extract buffer (50 mm Tris/HCI, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.05% NP-40, 0.2 mM phenylmethanesulfonyl fluoride, 1× protease inhibitors) as described previously (3, 37). The protein extract was analyzed by western blotting using antibodies specific to MLL1 (Upstate), MLL2 (Bethyl laboratory), MLL3 (Abgent), MLL4 (Sigma), ERα (Santa Cruz), ERβ (Santa Cruz), and β-actin (Sigma). Western blots were developed using alkaline phosphatase method.

Chromatin Immuno-precipitation (ChIP) experiment

ChIP assays were performed by using JAR cells and EZ Chip^{TM} chromatin immuno-precipitation kit (Upstate) as described previously (3, 12, 37). Briefly, 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 chromatin (~150–450 bp in length). The fragmented chromatin was pre-cleared with protein-G agarose beads and subjected to immuno-precipitation with antibodies specific to ERα, ERβ, MLL1, MLL2, MLL3, and MLL4, RNA polymerase II (abcam, 8WG16 clone), and H3K4-trimethyl (upstate). Immuno-precipitated chromatin was washed and de-proteinized to obtain purified DNA fragments that were used as templates in semi quantitative and/or real-time PCR amplifications with various primers corresponding to different EREs of HOXB9 promoter (supplementary Table 1).

Luciferase reporter assay

Four $ERE_{1/2}$ along with their flanking regions (350–400 nt in total) were cloned and inserted upstream of luciferase gene in pGL3-promoter vector (Promega) (primers are listed in supplementary Table 1). JAR cells were transfected with these ERE-containing luciferase reporter constructs using FuGENE6 transfection reagent. Control transfections were done using empty pGL3 promoter vector without any ERE insertion or with a luciferase construct-containing segment of HOXB9 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 6 h and then subjected to luciferase assay using ONE-Glo Luciferase Assay System (Promega) as instructed and detected using a micro plate reader (Flowstar-Omega). Each treatment was done in four replicates and the experiment was repeated at least twice (n = 8).

Results

Effects of estrogen (E2) on HOXB9 gene expression

As HOXB9 is linked with various hormonally regulated physiological processes including mammary gland development, Renin gene expression and cardiovascular function, we examined if its expression is influenced by steroid hormones such as estrogen (32–34). To examine steroid hormone-mediated regulation of HOXB9, we treated two different steroidogenic human cell lines, JAR (Placental choriocarcinoma origin (27, 38) and MCF7 (human breast cancer), with 17β-estradiol (E2) and analyzed its impact on HOXB9 expression. Initially cells were grown in phenol-red free media containing charcoal stripped fetal bovine serum and then treated with varying concentrations of E2 for 8 h. RNA was isolated from these E2-treated cells, reverse transcribed into cDNA and analyzed by regular PCR as well as real-time PCR using primers specific to HOXB9 (Fig. 1A-B). Beta-actin was used as loading control. Real-time PCR quantification of HOXB9 expression (relative to β-actin) as a function of E2-treatment are shown in the respective bottom panels of figures 1A and B. Interestingly, in JAR cells, HOXB9 expression was induced upon treatment with E2 in a dose dependent manner (Fig. 1A). HOXB9 expression was increased by ~4 fold in 10–100 nM E2-treatment in comparison to control (compare lane 1 with 4 and 5, Fig. 1A). Treatment with E2 also induced HOXB9 expression in MCF7 cells, though the level of induction was less in comparison to JAR cells (Fig. 1B). The highest expression of HOXB9 was observed at 1 nM of E2 in MCF7 cells (Fig. 1B). Time-dependent studies (in JAR cells) demonstrated that E2-induced transcriptional activation of HOXB9 reached to maxima at about 4–8 h post E2-treatment (Fig. 1C, real-time PCR quantification is shown in right panel). The induction of HOXB9 expression in two independent steroidogenic cell lines demonstrated that HOXB9 is an estrogen-responsive gene.

HOXB9 promoter contains potential estrogen-response elements

In order to examine mechanism by which HOXB9 may be transcriptionally activated upon exposure to E2, we analyzed its promoter sequence for presence of any putative estrogen-response elements (EREs). These analyses revealed that there are four $ERE_{1/2}$ sites (GGTCA or TGACC, termed as ERE1- ERE4) present within first 3000 nt upstream of HOXB9 transcription start site (Fig. 2A). Analysis of the neighboring sequences of $ERE_{1/2}$ sites did not show presence of any typical consensus full ERE (GGTCAnnnTGACC) (39, 40). However, ERE4 (TGTCCnnnGGTCA) appears to have very close homology (with one nucleotide difference) with a consensus full ERE (Fig. 2A). These analyses suggested that ERE4 might be a potential imperfect full ERE while others (ERE1-3) are just $ERE_{1/2}$ sites (Fig. 2A).

Irrespective of their sequence homology with consensus full ERE, we examined the estrogen-responsiveness of each $ERE_{1/2}$ sites using a luciferase based reporter assay (41). We cloned each ERE along with ~150 nt flanking sequences on both side into a luciferase expression construct pGL3 (Fig. 2A). A promoter region containing no ERE (non-ERE) sequences was also cloned as negative control. Each ERE-pGL3 construct was transfected into JAR and MCF7 cells separately, and then cells were exposed to E2 for 6 h and subjected to luciferase analysis using a commercial luciferase detection kit (Fig 2B-C). Our results demonstrated that transfection with empty pGL3 construct did affect luciferase activity upon E2-treatment in both JAR and MCF7 cell lines (Figs. 2B and C and figure S1). However transfection with ERE1-pGL3, ERE3-pGL3 and ERE4-pGL3 followed by E2-exposure increased the luciferase activity by 2.1, 2.9, and 2.6 fold respectively in JAR cells (Fig. 2B). Transfection with either ERE2-pGL3 or non-ERE-pGL3 did not have any significant effect on luciferase activity (Fig. 2B). In contrast to JAR cells, E2-dependent luciferase induction was observed only for ERE4-pGL3 in MCF7 cells (Fig. 2C). No E2-

response was observed for ERE1-, ERE2- and ERE3-pGL3 in this cell line (Fig. 2C). These results suggested that ERE4 which is an imperfect full ERE, is likely an E2-responsive ERE and may be associated with E2-mediated activation of HOXB9. As ERE1 and ERE3 also showed E2-responses in JAR cells, they may also be involved in E2-mediated transcriptional regulation of HOXB9 in this cell line. Nevertheless, as E2-induced expression of HOXB9 was more robust in JAR cells in comparison to MCF7 cells, and ERE1, ERE3 and ERE4 were E2-responsive in JAR cells, we investigated the mechanism of E2-dependent HOXB9 expression in JAR cells.

Estrogen receptors play critical roles in E2-mediated transcriptional activation of HOXB9

As HOXB9 is an E2-responsive gene and its promoter contains multiple estrogen-response elements, we hypothesized that estrogen-receptors (ERs) are involved in E2-mediated transcriptional regulation of HOXB9 (42). To examine the roles of ERs, we initially knocked down ERα and ERβ separately in JAR cells using specific antisense oligonucleotides (supplementary Table 1) and then treated with E2 (100 nM for 6 h). RNA and protein from ER knocked down and E2-treated cells were analyzed for the expression of ERs as well as HOXB9. A scramble antisense (with no homology to ERs) was used as negative control. Our results demonstrated that ERα and ERβ were knocked down effectively and specifically by respective antisenses (Figs. 3A-B and data not shown). As seen in figure 3A, treatment with E2 increased the expression of HOXB9 (lane 2, Fig 3A). Interestingly, upon knockdown of ERα, E2-dependent activation of HOXB9 was decreased (lane 4, Fig. 3A, real-time PCR data in right panel). Similarly, knockdown of ERβ also suppressed E2-dependent induction of HOXB9 (lanes 2 and 4, Fig. 3B, real-time PCR data in the right panel). We also performed the combined knockdown of ERα and ERβ in JAR cells and found that E2-dependent activation of HOXB9 was suppressed almost to the basal level which is more than that observed under individual knockdown conditions (lane 5, Fig. 3B). These results demonstrated that both ERα and ERβ play critical roles in E2-mediated transcriptional activation of HOXB9.

ERα and ERβ bind to HOXB9 promoter in presence of E2

As both ERα and ERβ played essential roles in HOXB9 gene expression, we examined their E2-dependent binding in HOXB9 promoter using chromatin immuno-precipitation (ChIP) assay (12). JAR cells were treated with E2 (100 nM for 6 h), fixed with formaldehyde, sonicated to shear the chromatin and then subjected to immuno-precipitation with ERα and ERβ specific antibodies. ChIP experiment was performed in parallel with β-actin antibody as non-specific antibody control. The immuno-precipitated DNA fragments were PCR-amplified using primers spanning different ERE regions (ERE1-ERE4) of HOXB9 promoter (Fig. 4A). The real-time PCR quantifications of the ChIP DNA fragments are shown in figure 4B. A promoter segment (−660 to −791 nt) containing no ERE site was used as negative control (non-ERE). ChIP analysis demonstrated that β-actin was not bound to any EREs both in absence and presence of E2 (Fig. 4A). However, the binding of ERα was increased in ERE1, ERE3, and ERE4 regions in presence of E2-treatment (Figs. 4A-B). Similarly, increased binding of ERβ was observed in all ERE regions (ERE1- ERE4, but not in non-ERE regions). These observations demonstrated that ERα and ERβ were bound to HOXB9 EREs especially ERE1, ERE3 and ERE4 regions upon treatment with E2. Some amounts of constitutive binding of ERs was observed in the ERE regions prior to addition of E2 and this might be associated with basal transcription of the gene in absence of external stimuli. Notably, the same set of EREs (ERE1, ERE3 and ERE4) showed E2-dependent luciferase response in reporter assay in JAR cells (see Fig. 1) further indicating the involvement of these EREs in E2-dependent transcriptional regulation of HOXB9.

To further confirm and also to examine the detail of ER binding to HOXB9 promoter, we examined the binding of ERs in different EREs in a time-dependent manner. These analyses demonstrated that ERα was bound to ERE4 as early as 15 minutes post E2-treatment and continued to increase up to 8 h (Fig. 4C, right panel). The binding of ERβ to ERE4 was slightly delayed to about 2 h post E2-treatment and increased upon longer time incubation (Fig. 4C right panel). In ERE1 and ERE3 regions, the binding of ERα and ERβ were observed at ~30 min after E2-treatment and increased with time (Fig. 4C). Although, at this point, it is not clear about the significance of differential binding kinetics of ERs to different ERE regions, these studies demonstrated that both ERα and ERβ were bound to ERE1, ERE3 and ERE4 regions as a function of E2.

Roles of MLLs in E2-mediated regulation of HOXB9

ER-mediated gene activation requires various ER-coregulators (43). Recent studies showed that MLL histone methylases interact with ERs in an E2-dependent manner and act as ER-coregulators in E2-dependent transcriptional activation of estrogen responsive genes (24, 26, 27, 44). To examine if MLLs are associated with E2-dependent activation of HOXB9, we knocked down different MLLs (MLL1, MLL2, MLL3, and MLL4) separately by using specific antisense oligonucleotides (supplementary Table 1), then exposed the MLL-knocked down cells to E2 and analyzed its impact on E2-dependent activation of HOXB9. MLL-specific knockdowns were confirmed by analyzing their respective gene expression both at RNA (compare lane 3 with lane 1, Figs. 5A-D for MLL1 to MLL4, respectively) and protein levels (supplementary Fig S2). A scramble antisense with no homology to MLLs was used as negative control. Our results demonstrated that application of MLL1-specific antisense specifically knocked down MLL1 but not MLL2 or β-actin, and the knockdown of MLL1 resulted in decrease of E2-dependent activation of HOXB9 (Fig. 5A). Knockdown of MLL3 also significantly affected the E2-dependent expression of HOXB9 (Fig. 5C). We also performed combined knockdown of MLL1 and MLL3 (Figs. 5E-F). Our analysis demonstrated that combined knockdown of MLL1 and MLL3 suppressed E2-induced HOXB9 expression almost to basal level and the level of suppression was more than corresponding independent knockdowns indicating further about the critical roles of MLL1 and MLL3 in E2-induced HOXB9 expression (Figs. 5E-F). In contrast, knockdown of MLL2 did not affect E2-induced HOXB9 expression, whereas MLL4-knockdown showed a modest suppression (Figs. 5B-D). These results demonstrated that MLL3 and MLL1 play major roles in E2-mediated activation of HOXB9. Notably, knockdown of either MLL1 or MLL3 did not have any significant effect on the expression of ERα and ERβ, suggesting direct roles of these MLLs in E2-induced HOXB9 expression (supplementary figure S3).

MLL histone methylases bind to HOXB9 promoter in presence of E2

As MLL1 and MLL3 are essential in E2-mediated HOXB9 activation, we examined their bindings to the EREs of HOXB9 promoter in presence of E2. We performed ChIP assays in E2-treated and control cells using antibodies specific to different MLLs and β-actin (as non-specific control) and ChIP DNA fragment were PCR-amplified using primers specific to ERE1, ERE3 and ERE4 (Fig. 6A). The ChIP DNA was also quantified using real-time PCR (Fig. 6B). These analyses demonstrated that binding of MLL1 was increased in ERE3 region in presence of E2 (lane 4, Fig 6A-B). However, binding of MLL3 was significantly increased in all the EREs (ERE1, ERE3 and ERE4) in presence of E2 (Fig. 6A-B). No significant E2-dependent binding were observed for MLL2 and MLL4 (Fig. 6A-B). Time-dependent ChIP analysis demonstrated that MLL3 recruitment was increased in ERE1, ERE3, and ERE4 in presence of E2 and this elevated binding was observed as early as 15–30 min post E2-treatment (Fig. 6C). Similarly, E2-dependent MLL1 binding was also observed in the ERE3 region. These results, in agreement with knockdown experiments

(Fig. 5) further demonstrated that MLL1 and MLL3 are involved in regulation of HOXB9 gene expression under E2 environment.

As E2-treatment enhanced recruitment of MLL1 and MLL3 histone methylases onto HOXB9 EREs, we analyzed if H3K4-trimethylation level was also enhanced upon exposure to E2. ChIP analysis using H3K4-trimethyl specific antibody demonstrated that level of H3K4-trimethylation was increased in ERE1, ERE3 and ERE4 regions of HOXB9 promoter (Figs. 7A-C) in a time-dependent manner as a function of E2. Similar to H3K4-trimethylation, level of RNA polymerase II (RNAPII) recruitment was also increased in HOXB9 EREs as function of E2. These observations suggested that MLL1 and MLL3 that are recruited to HOXB9 promoter in presence of E2, introduced H3K4-trimethylation marks into chromatin and led to the increased recruitment of RNAPII leading to HOXB9 gene activation.

MLL1 and MLL3 are recruited to HOXB9 promoter via ERs

ERs are well known to bind directly to EREs via their own DNA binding domain (42, 45). MLLs (MLL1-4) also have several DNA binding domains which may facilitate their direct binding with the HOXB9 promoter (19). Furthermore, MLLs are shown to interact with ERs and therefore, they may be recruited to HOXB9 EREs via interaction with ERs. To test if MLL1 and MLL3 that are associated with HOXB9 gene activation are recruited via ERs, we examined the recruitment (ChIP assay) of MLL1 and MLL3 under ER-knockdown environment. As shown in figure 8, the E2-dependent binding of MLL3 to ERE1, ERE3 and ERE4, and binding of MLL1 into ERE3, were decreased upon independent knockdown of either ERα or ERβ. These observations demonstrated that E2-dependent binding of both MLL1 and MLL3 are dependent on both ERα and ERβ.

Discussion

HOX genes are critical developmental regulators and therefore their expression are likely to be influenced by various types of hormones (29). For example, retinoic acid (RA) influences HOX gene expression controlling the developmental process (46–48). In case of embryonic stem cells, RA influences HOX gene expression in a collinear fashion (3’-5’ direction) (47, 48). However, in matured tissues, the expression and regulation of different HOX genes appear to be independent and tissue specific (47). Steroid hormones are also implicated in regulation of different HOX genes *in vivo*, though their detailed mechanism of action is mostly unexplored (30). Recent studies demonstrated that HOXA10 is transcriptionally regulated by steroid hormones and their expression is misregulated by endocrine disrupting chemicals (30, 31, 49–51). Our studies demonstrated that HOXB9, a homeobox containing gene that is implicated in regulation of Renin-Angiotensinogen system and cardiovascular function, is transcriptionally regulated by estrogen. Renin-Angiotensinogen system which is closely linked with control of hypertension, blood pressure and plasma volume, is also controlled by steroid hormones (34). Thus, our studies showing the estrogen-mediated regulation of HOXB9, extends the mechanistic implication of estrogen signaling in Renin-Angiotensinogen system and cardiovascular function.

Estrogen is a critical player in female reproduction. In addition, estrogen also plays critical roles in bone strength, controlling blood cholesterol, hypertension and other cardiovascular functions in both male and female. Abnormal estrogen signaling leads to critical human disease including cardiovascular disease and cancer (45, 52–58). Estrogen signaling follows diverse pathways including estrogen-dependent activation of estrogen-responsive genes (42, 45). In general, during estrogen-mediated gene activation, estrogen binds to its cognate receptors (ERα and ERβ) and activates them (43). Activated ERs migrate to nucleus and bind to estrogen-response elements (EREs) present in the promoters of estrogen responsive

genes resulting in their transactivation (43). Sequence analysis demonstrated that HOXB9 promoter contains multiple $ERE_{1/2}$ sites (ERE1-3) and a potential imperfect full ERE (ERE4) (39). Luciferase based reporter assay demonstrated that ERE1, ERE3 and ERE4 are responsive to E2-exposure in JAR cells suggesting their potential involvement in E2-dependent activation of HOXB9. However, in MCF7 cells, only ERE4 showed estrogen response in luciferase assay which further indicate that ERE4 is likely an imperfect full ERE. The difference in luciferase response in two different cell lines may lie in the difference in cell lines. Antisense-mediated knockdown and ChIP analysis demonstrated that both ERα and ERβ are essential for E2-mediated activation of HOXB9. Both ERα and ERβ bind to ERE1, ERE3 and ERE4 of HOXB9 promoter as a function of E2, suggesting the involvement of these EREs in HOXB9 regulation. As seen in figure 3, the independent knockdown of ERα and ERβ affected E2-dependent activation of HOXB9. However, upon combined knockdown of ERα and ERβ, the effects were more severe resulting in complete suppression of E2-dependent HOXB9 activation. These observations suggest that both ERα and ERβ are critical in E2-mediated regulation of HOXB9 and it is possible that they form heterodimer and bind to HOXB9 EREs and modulate transcription.

Importantly, during estrogen-mediated gene activation, along with ERs, several ER-coregulators that interact with ERs play essential roles in transactivation of the target gene (42). These coregulators usually possess enzymatic activity, modify chromatin and bridge ERs with transcription machinery (59). Many ER-coactivators have been identified including SRC-1 family of protein, CREB-binding protein (CBP/p300), p/CAF, ASCOM (activating signal cointegrator-2 that also contains MLLs) etc (26, 44, 59–66). Recent studies demonstrated that MLL family of histone methylases, interact with ERs in an estrogen-dependent manner and bind to ERE regions of ER-target genes regulating their expression under hormonal environment (24, 26, 27, 44). Our analysis demonstrated that histone methylase MLL1 and MLL3, play critical roles in HOXB9 gene regulation under estrogen environment. Knockdown of MLL1 and MLL3 resulted in suppression of E2-dependent activation of HOXB9. MLL2 and MLL4 have little or no impact on E2-induced HOXB9 expression. ChIP analysis demonstrated that like ERs, recruitment of MLL3 increased in all the E2-responsive HOXB9 ERE regions (ERE1, ERE3 and ERE4) in presence of E2. MLL1 was also bound to ERE3 in an E2-dependent manner. These results further supported that MLL1 and MLL3 are important players in E2-mediated transcriptional regulation of HOXB9. Notably, like MLL3, MLL2 and MLL4 have several NR-boxes (nuclear receptor boxes) and hence are capable of interacting with ERs, and are also implicated in E2-mediated activation of different estrogen responsive genes (24, 26, 27, 44). The involvement of MLL1 and MLL3 but not MLL2 and MLL4 in E2-induced HOXB9 expression suggested that different estrogen-responsive genes require different MLL-coregulators and MLL3 and MLL1 are specific for HOXB9 regulation.

It is well known that MLLs are chromatin modifying enzymes that methylate histone H3 at lysine 4 (H3K4) and play critical roles in gene activation (7, 18, 21, 67–69). As MLL1 and MLL3 act as a coactivators of ER and is essential for E2-induced HOXB9 expression, recruitment of MLL1 and MLL3 in different ERE regions of HOXB9 may result in histone H3K4-methylation and facilitates recruitment of RNA polymerase II (RNAPII) and enhanced transcription. Indeed, ChIP analysis showed that level of H3K4-trimethylation and recruitment of RNAPII was increased in a time-dependent manner in ERE1, ERE3 and ERE4 regions of HOXB9 promoter under E2-treatment. This result indicated that MLLs plays a critical role in histone methylation and HOXB9 gene activation. Though under normal condition the recruitments of MLLs in the promoter of their target gene are mediated either by their own DNA binding domains or via interacting partners, recruitment of MLL1 and MLL3 under E2 environment may be mediated by ERs. Our analysis of E2-dependent recruitment of MLL1 and MLL3 in ERE1, ERE3 and ERE4 of HOXB9 in ER depleted cells

indicated that recruitment of MLL1 and MLL3 are indeed dependent on by both ERα and ERβ. Notably, E2-dependent interaction between MLLs and nuclear receptors (including ER) are well recognized. For example, histone methylase MLL2 has been shown to directly interact with ERα, via its LXXLL domains and regulate E2-dependent activation of cathepsin D (26). Similarly, MLL-interacting protein, menin that also has LXXLL domains, interacts with ERα and regulates E2-dependent activation TFF1 (pS2) (24). MLL3 and MLL4 complexes are shown to interact with multiple nuclear receptors including ER, RAR and LXR in ligand dependent manner and regulate ligand dependent gene activation (10, 24, 26, 44, 70, 71). Our studies further demonstrated that MLL1 and MLL3 functionally interact with ERs and regulate E2-induced HOXB9 expression. Similar to HOXB9, HOXC13 is also transcriptionally activated by estrogen via coordination of MLLs and ERs (27). However, the mechanism of transcriptional regulation and coregulators requirement between these HOX genes appear to be different. MLL1 and MLL3 are primary ER-coregulators in HOXB9 regulation, while MLL1-4 participate in E2-induced HOXC13 expression. Furthermore, there is no apparent conservation in organization of the promoter elements between HOXB9 and HOXC13 genes indicating their distinct mechanism of transcriptional regulation. The cell type as well as tissue specific distinct mechanism of HOXB9 and HOXC13 gene expression under retinoic acid exposure was also observed previously (47). As MLLs are acting as coactivators of ERs, it is likely that different HOX genes are controlled by different MLL coactivators and in case of HOXB9 they are primarily MLL1 and MLL3. Analyzing the roles of E2 and other steroid hormones in regulation of all 39 HOX genes and elucidating their mechanism is critical in detailed understanding of the steroid hormone mediated regulation of HOX genes.

Overall, our studies demonstrated that HOXB9 is an E2-responsive gene. As estrogen and ERs are closely associated with cardiovascular disease, HOXB9 is closely linked with regulation of Renin-Angiotensinogen system and regulation of blood pressure, plasma volume, electrolyte balance, and our studies revealed a novel epigenetic mechanism by which MLL-histone methylases coordinate with ERs to regulate HOXB9 gene expression. Our studies also implicated potential involvement of MLL histone methylases in regulation of cardiovascular functions such as blood pressure, fluid homeostasis and electrolyte balance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HOX	Homeobox
ER	estrogen receptor
NR	Nuclear receptor
ERE	estrogen response elements
MLL	Mixed lineage leukemia
RNAP II	RNA polymerase II

H3K4	histone H3 lysine 4
HMT	histone methyl-transferase
RT-PCR	Reverse transcriptase polymerase chain reaction
ChIP	Chromatin immunoprecipation
DMEM	Dulbecco's modified Eagle medium

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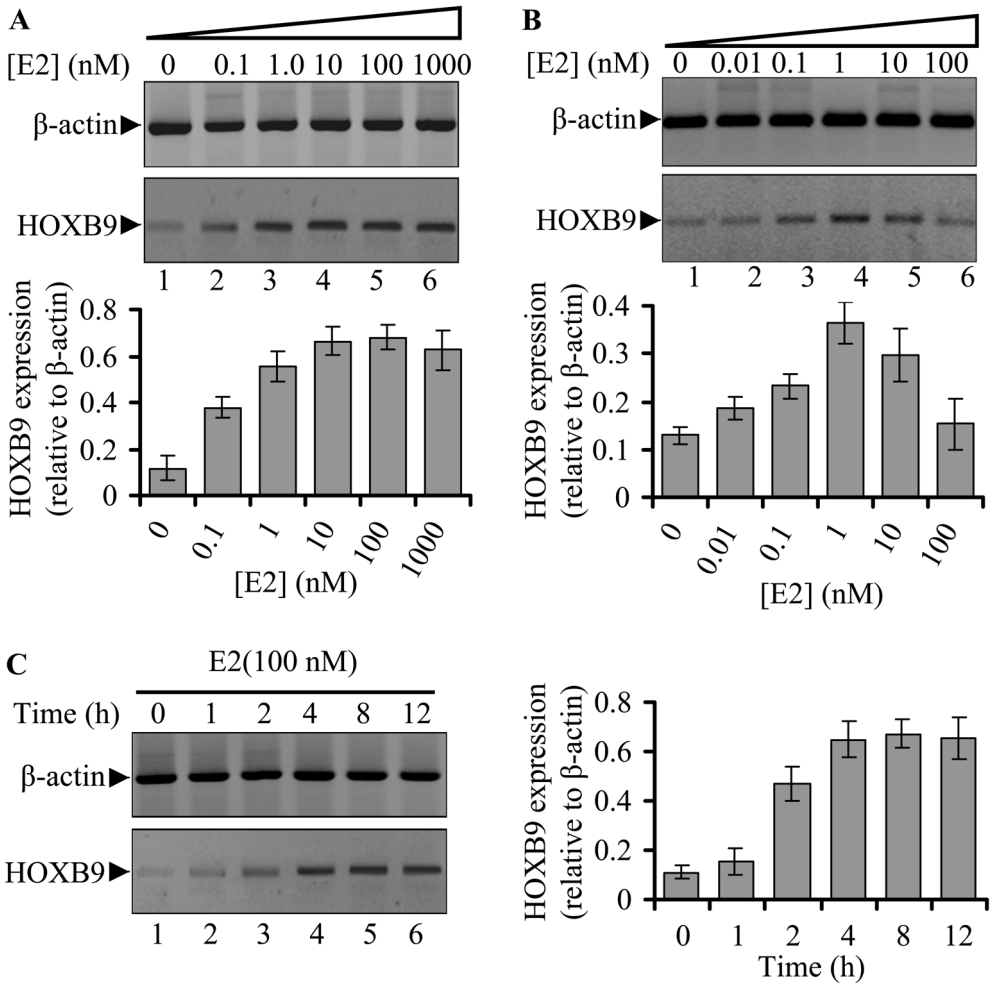

Figure 1.

Effect of E2 on HOXB9 expression. (**A**) JAR cells (grown in phenol-red free media) were treated with varying concentrations of E2. RNA was isolated, reverse transcribed, and subjected to regular PCR (top panel) and real-time PCR (bottom panel) using primers specific to HOXB9. β-actin was used as a loading control. Each experiment was repeated at least thrice. Bars indicate standard errors ($p < 0.05$). (**B**) Effect of E2 on HOXB9 expression in MCF7 cells was analyzed in a similar way as described for JAR cells in figure A. Top panel shows the agarose gel analysis of the PCR products and bottom panel shows the real-time PCR data. (**C**) JAR cells were treated with 100 nM E2 for varying time periods (0 –12 h) and RNA was reverse transcribed and analyzed by regular PCR (left panel) and real-time PCR (right panel) using HOXB9 and β-actin primers. Each experiment was repeated at least thrice ($n = 3$).

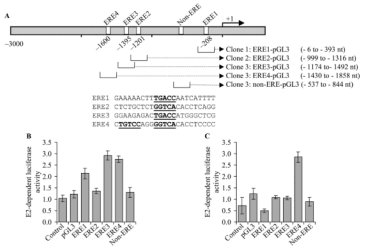

Figure 2.

Analysis of HOXB9 promoter EREs and their E2-response using luciferase based reporter assay. (**A**) HOXB9 promoter (up to -3000 nt) contains three $ERE_{1/2}$ sites named as ERE1 to ERE3 and an imperfect full EREs (ERE4). The promoter segments containing these $ERE_{1/2}$ sites along with ~150 nt on both sides were cloned into luciferase based reporter construct pGL3 and used for transfection. (**B**) E2-response of HOXB9 promoter EREs in JAR cells. The ERE containing pGL3 constructs were transfected into JAR cells for 24 h. Control cells were treated with either none (no construct transfected, control lane), or empty pGL3 vector or non-ERE-pGL3 (non-ERE). Both control as well as plasmid transfected cells were then treated with 100 nM E2 for 6 h and subjected to luciferase assay by using ONE-Glo Luciferase Assay System. The ratio of E2-induced luciferase activities over corresponding E2-untreated samples were plotted. The experiment with four replicate treatments was repeated at least twice. Bars indicate standard errors. (**C**) E2-response of HOXB9 promoter EREs in MCF7 cells. Experiments were performed in MCF7 cells in the same way as described in figure B.

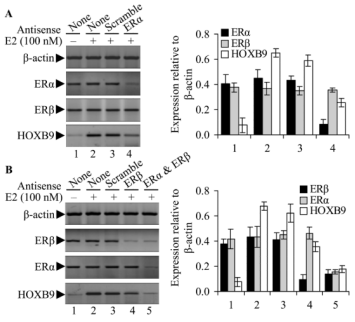

Figure 3.

Roles of ERα and ERβ in E2-induced expression of HOXB9. (**A**) Effect of ERα knockdown: JAR cells were transfected with ERα and a scramble antisense (9 μg each) for 48 h. Antisense-transfected cells were treated with E2 (100 nM for 6 h). RNA was isolated and subjected to reverse transcriptase-PCR analysis by using primers specific to ERα and HOXB9. PCR with ERβ was done to confirm specificity of ERα antisense and β-actin was used quantitative control. PCR products were analyzed in agarose gel. Lane 1: control cells (no E2 control), lane 2: cells were treated with 100 nM E2. Lane 3: cells were initially transfected with 9 μg of scramble antisense followed by exposure to E2. Lane 4: cells were initially transfected with 9 μg of ERα antisense and then treated with E2. Real-time PCR analysis of ERα, ERβ and HOXB9 relative to β-actin is plotted. Each experiment was repeated at least thrice (n = 3). Bars indicate standard errors. (**B**) Effect of ERβ knockdown or a combined knockdown of ERα and ERβ on E2-induced HOXB9 expression. Experiments and analysis were performed in the same way as shown in panel A.

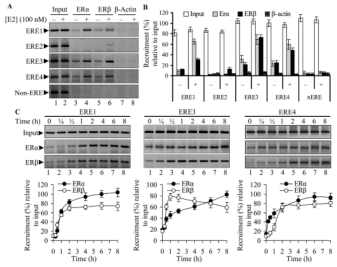

Figure 4.

E2-dependent recruitment of ERα and ERβ in the ERE regions of HOXB9 promoter. (**A**-**B**) JAR cells were treated with 100 nM E2 for 6 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 ERE1-4 of HOXB9 promoter. Primers specific to a promoter sequence containing no ERE (non-ERE) were used as control. Lanes 1, 3, 5 and 7 are no-E2 controls. Lanes 2, 4, 6 and 8, were E2-treated samples. ChIP DNA fragments were analyzed by real-time PCR and shown in panel B. Each experiment was repeated at least thrice. Bars indicate standard errors. (**C**) Dynamics of recruitments of ERα and ERβ onto ERE1, ERE3 and ERE4 of HOXB9 promoter: Cells were treated with 100 nM E2 for varying time periods and then subjected to ChIP assay using antibodies specific to ERα and ERβ. Immuno-precipitated DNA fragments were PCR-amplified using primers specific to ERE1, ERE3 and ERE4 of HOXB9 promoter. Lane 1: control cells (no E2 treatment control). Quantification of recruitment level (% relative to input) is plotted in respective bottom panels. Bars indicate standard errors.

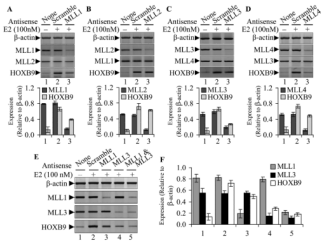

Figure 5.

Effect of knockdown of MLL1, MLL2, MLL3, and MLL4 on E2-induced expression of HOXB9. JAR cells were transfected with 5 μg of MLL1, MLL2, MLL3, and MLL4 specific antisense oligonucleotides separately. Control cells were treated with a scramble antisense with no homology with MLLs. The antisense-treated cells were incubated for 48 h followed by treatment with 100 nM E2 for 6 h. RNA was isolated from treated and control cells and subjected to reverse transcriptase-PCR by using primers specific to HOXB9 along with MLL1, MLL2, MLL3, and MLL4. β-actin was used as control. The PCR products were analyzed by agarose gel. Quantification of transcript accumulation was done by using real-time PCR (shown in respective bottom panel). (A) Effect of MLL1 knockdown. (Top) Lane 1: control cells (no E2-treatment); lane 2: cells that were initially transfected with scramble antisense followed by exposure to E2. Lanes 3: cells were initially transfected with MLL1 antisense and then treated with E2. Real-time PCR analysis of the expression profiles of MLL1 and HOXB9 (relative to β-actin, average of three replicate experiments, n = 3) were quantified and plotted in the bottom panel. MLL2 was used as control to determine target specificity of MLL1 antisense. (B-D) These figures show the effects of knockdown of MLL2 (MLL1 as control), MLL3 (MLL4 as control), and MLL4 (MLL3 as control), respectively, in the similar manner as shown for MLL1 in panel A. (**E-F**) Effect of combined knockdown of MLL1 and MLL3 in E2-induced HOXB9 expression. The knockdown experiments were performed in the same way as shown in panels A-D. For combined knockdown (lane 5), MLL1 and MLL3 antisenses were mixed together in equimolar amounts and then transfected. Real-time PCR analysis of the expression profiles of MLL1, MLL3 and HOXB9 were plotted in panel F.

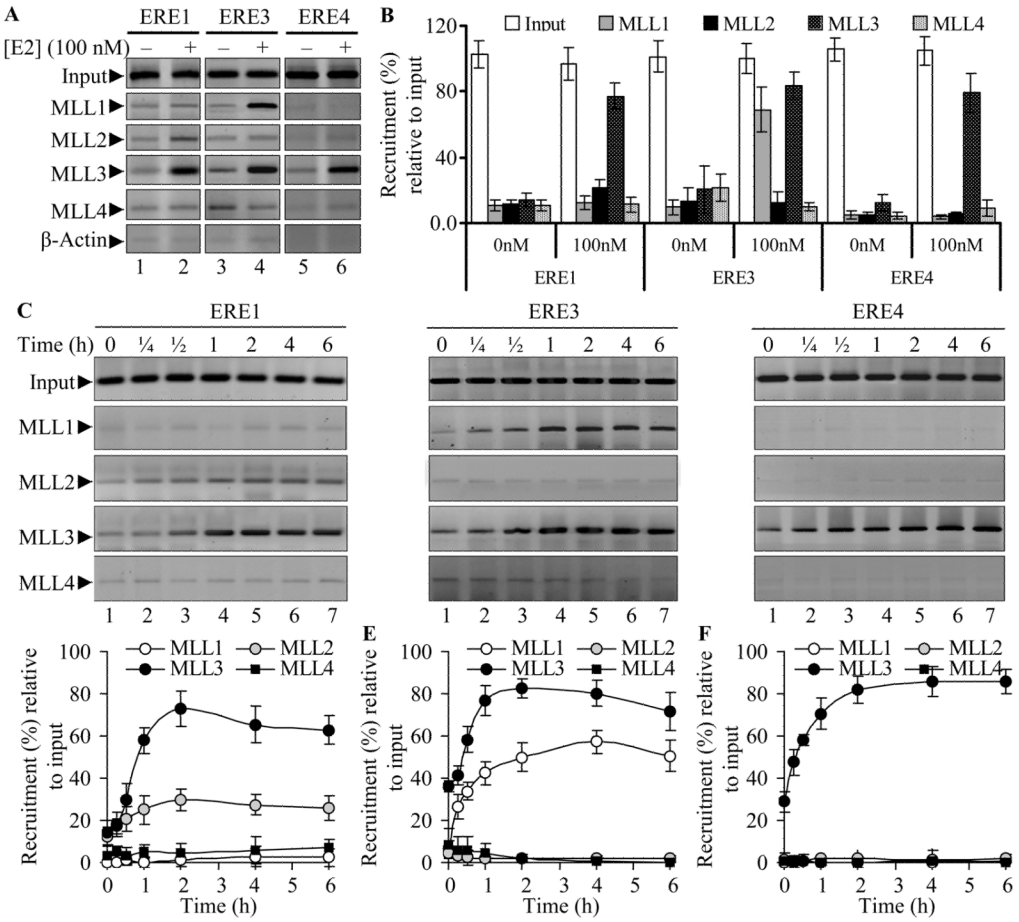

Figure 6.

E2-dependent recruitment of MLLs (MLL1-4) in ERE1, ERE3, and ERE4 of HOXB9 promoter. (A-B) JAR cells were treated with 100 nM E2 for 6 h and subjected to ChIP assay using antibodies specific to MLL1, MLL2, MLL3 and MLL4. β-actin antibody was used as control IgG. The immuno-precipitated DNA fragments were PCR-amplified using primers specific to ERE1, ERE3 and ERE4 of HOXB9 promoter. Lanes 1, 3, and 5 are no-E2 controls. Lanes 2, 4, and 6 were E2-treated samples. ChIP DNA fragments were analyzed by real-time PCR and shown in panel B. Each experiment was repeated at least thrice. Bars indicate standard errors. (C) Dynamics of recruitment of MLL1, MLL2, MLL3 and MLL4 onto HOXB9 promoter: Cells were treated with 100 nM E2 for varying time periods and then subjected to ChIP assay using antibodies specific to MLL1, MLL2, MLL3 and MLL4. Immuno-precipitated DNA fragments were PCR-amplified using primers specific to ERE1, ERE3 and ERE4 of HOXB9 promoter. Lane 1: control cells (no E2 control). Quantifications of recruitment level (% relative to input) are plotted in respective bottom panels. Bars indicate standard errors.

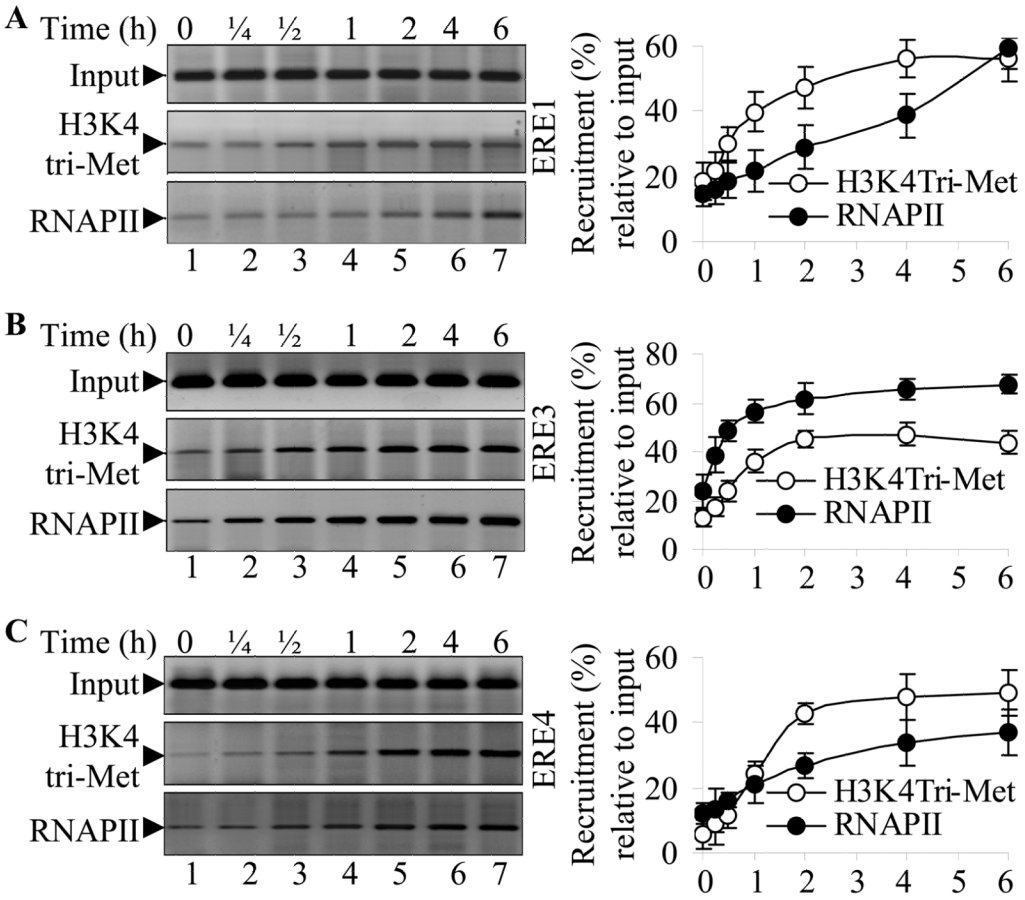

Figure 7.

E2-dependent enrichment of H3K4-trimethylation level and recruitment of RNA polymerase II (RNAP II) in the ERE1, ERE3 and ERE4 of HOXB9 promoter. (**A-C**) JAR cells were treated with 100 nM E2 for varying time periods (0 – 6 h) and then subjected to ChIP assay using antibodies specific to H3K4-trimethylation and RNAPII. Immuno-precipitated DNA fragments were PCR amplified using primers specific to ERE1, ERE3 and ERE4 of HOXB9 promoter respectively. Lane 1: control cells (no E2 control). Lanes 2–7 are E2-treated samples. Quantifications of recruitment level (% relative to input) are plotted in respective right panels. Bars indicate standard errors.

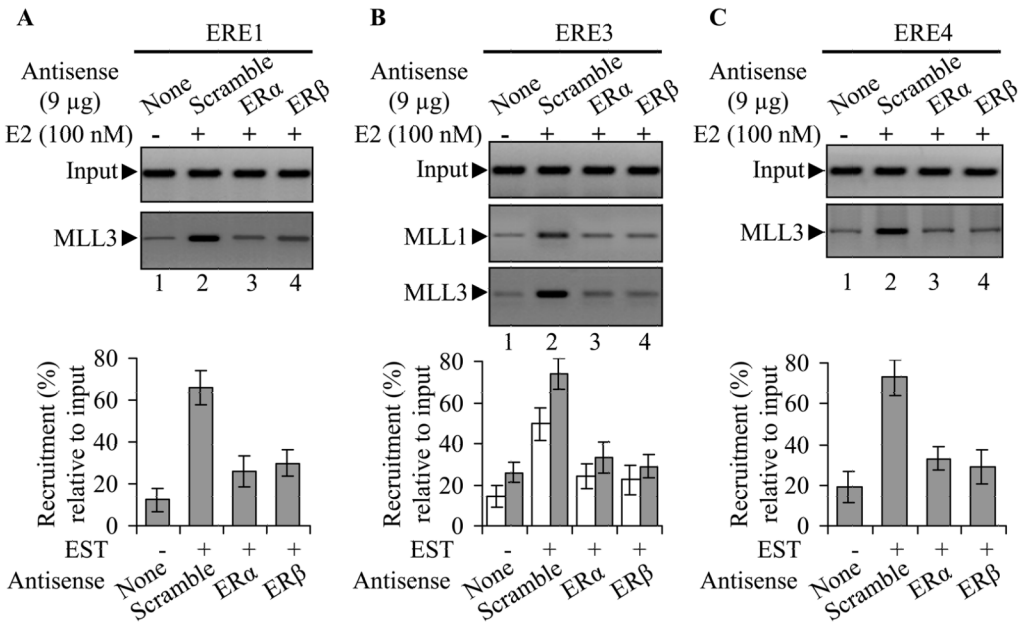

Figure 8.

Effects of ERα and ERβ knockdown on recruitment of MLL1 and MLL3 on ERE1, ERE3 and ERE4 of HOXB9 promoter. JAR cells were transfected with ERα and ERβ antisenses separately for 48 h followed by exposure to E2 (100 nM for additional 6 h). Cells were harvested and subjected to ChIP assay using anti-MLL1 and anti-MLL3 antibodies. The immuno-precipitated DNA fragments were PCR-amplified using primer specific to ERE1, ERE3, and ERE4 regions of HOXB9 promoter. Lane 1: control cells (no E2 control). Lanes 2–4 are E2-treated samples. Real-time quantification of recruitment level (% relative to input) is plotted in respective bottom panels. Bars indicate standard errors.