



HOXA5 Expression Is Elevated in Breast Cancer and Is Transcriptionally Regulated by Estradiol

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HOXA5 is a homeobox-containing gene associated with the development of the lung, gastrointestinal tract, and vertebrae. Here, we investigate potential roles and the gene regulatory mechanism in HOXA5 in breast cancer cells. Our studies demonstrate that HOXA5 expression is elevated in breast cancer tissues and in estrogen receptor (ER)-positive breast cancer cells. HOXA5 expression is critical for breast cancer cell viability. Biochemical studies show that estradiol (E2) regulates HOXA5 gene expression in cultured breast cancer cells *in vitro*. HOXA5 expression is also upregulated *in vivo* in the mammary tissues of ovariectomized female rats. E2-induced HOXA5 expression is coordinated by ERs. Knockdown of either ER α or ER β downregulated E2-induced HOXA5 expression. Additionally, ER co-regulators, including CBP/p300 (histone acetylases) and MLL-histone methylases (MLL2, MLL3), histone acetylation-, and H3K4 trimethylation levels are enriched at the HOXA5 promoter in present E2. In summary, our studies demonstrate that HOXA5 is overexpressed in breast cancer and is transcriptionally regulated via estradiol in breast cancer cells.

Keywords: HOXA5, breast cancer, estradiol, gene expression, estrogen receptors, chromatin

INTRODUCTION

HOX genes are a highly conserved family of homeobox-containing genes that play pivotal roles in cell differentiation, development, and embryogenesis (Krumlauf, 1994; Zakany and Duboule, 2007). The collinear expression of HOX genes are critical in anterior–posterior patterning during embryogenesis (Akam, 1987; Mlodzik et al., 1988; Duboule and Dolle, 1989; McGinnis and Krumlauf, 1992; Mallo et al., 2010). In humans, there are 39 HOX genes that are classified as HOXA-D clusters (Acampora et al., 1989; Garcia-Fernandez, 2005; Holland et al., 2007; Mallo and Alonso, 2013), and they primarily function as transcription factors (Svingen and Tonissen, 2006; Plowright et al., 2009; Ladam and Sagerstrom, 2014; Zheng et al., 2015; Morgan et al., 2016). They bind to their target gene promoters via their homeodomain and control gene expression

(Hanson et al., 1999; Shah and Sukumar, 2010; Boube et al., 2014; Taniguchi, 2014). Though HOX genes were originally described to exhibit collinear expression, especially during embryogenesis, recent studies demonstrate that each HOX gene may be independently regulated and expressed in various adult tissues (Taylor et al., 1998; Neville et al., 2002; Takahashi et al., 2004; Dunwell and Holland, 2016; Rux and Wellik, 2017). Additionally, HOX gene expressions are misregulated in a variety of cancers (Grier et al., 2005; Shah and Sukumar, 2010; Gray et al., 2011; Bhatlekar et al., 2014). HOX genes are associated with cancer cell proliferation, angiogenesis, and tumor growth (Hayashida et al., 2010; Ansari et al., 2011a,b; Shrestha et al., 2012; Hussain et al., 2015; Deb et al., 2016). HOX gene expression is increasingly recognized as a potential biomarker and target of therapeutic intervention. For example, HOXB9, a key player in mammary gland development, is upregulated in breast cancer, regulates growth and angiogenic factors, and is a potential biomarker (Ansari et al., 2011b; Shrestha et al., 2012). Similarly, another HOX gene, such as HOXC6, implicated in mammary gland development and breast cancer, regulates tumor growth factors and induces 3-D-colony formation (Ansari et al., 2011a; Hussain et al., 2015). HOXB7 overexpression is linked with cell proliferation, neoplastic transformation, and tumorigenesis (Wu et al., 2006; Nguyen Kovochich et al., 2013). Spinal cord associated HOXA10 expression is upregulated in breast cancer and is regulated by estradiol (Chu et al., 2004). Thus, HOX genes are emerging as major players in gene regulation and cancer; however, their detailed functions and gene regulatory mechanism remains elusive.

Homeobox-containing gene HOXA5 is a critical player in the development of lung, gastrointestinal tract, spleen, kidney, and vertebrae (Larochelle et al., 1999; Jeannotte et al., 2016). It regulates the expression of various proteins in conjunction with other paralogs during the ontogeny of normal development (Larochelle et al., 1999; Jeannotte et al., 2016). Studies also demonstrate that HOXA5 expression plays a critical role in development of the murine central nervous system (Joksimovic et al., 2005). In addition to its critical role in development, HOXA5 expression is dysregulated in breast epithelium and is linked to breast cancer biogenesis (Raman et al., 2000a,b). Here, we investigate if HOXA5 expression is associated with breast cancer and study its transcriptional regulatory mechanism in breast cancer cells. We demonstrate that HOXA5 expression is elevated in breast cancer, and its expression is regulated by estradiol (E2).

EXPERIMENTAL SECTION

Cell Culture, Antisense Oligonucleotide (ASO)-Transfection, and E2 Treatments

CCL228 (colon cancer), H358 (lung cancer), HeLa (cervical cancer), JAR (choriocarcinoma placenta), MCF10 (normal breast epithelial), MCF7 (ER-positive breast cancer), T47D (ER-positive breast cancer), and MDA-MB-231 (ER-negative breast cancer) cells (from ATCC) were cultured in DMEM that was

supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin/streptomycin (100 units and 0.1 mg/mL, respectively) (Wang et al., 2010; Ansari et al., 2011a, 2012a; Shrestha et al., 2012; Kasiri et al., 2013; Bhan et al., 2014a; Hussain et al., 2015; Deb et al., 2016).

For the estrogen treatment, MCF7 cells were cultured in phenol red-free DMEM-F-12 supplemented with 10% charcoal-stripped FBS for at least 3 generations. Cells were then treated with 17 β -E2 for 6 h and then subjected to RNA and protein extraction.

All the ASOs were synthesized commercially from IDT-DNA. MCF7 cells (grown in phenol red-free DMEM-F-12 supplemented with 10% charcoal-stripped FBS) were transfected with ASOs using iFECT transfection reagent (KD Medicals, Inc.) with antisense oligonucleotides for 48 h (Ansari et al., 2011a; Kasiri et al., 2013). ASO-transfected cells were then treated with 1 nM E2 for 4 h followed by RNA and protein extraction for further analysis.

Immunohistological Analysis of Breast Cancer Tissue Microarray

Breast cancer tissue microarrays (US Biomax Inc) were immunostained with DAB staining using HOXA5 antibody as described by us previously (REF). Microarray slides were initially deparaffinized by immersing in xylene (twice, 10 min each), then sequentially in 100, 95, and 70% ethanol (5 min each) and then incubated in 0.1 M sodium citrate buffer (at 95°C, 15 min) for antigen retrieval. For the immunostaining, the slide was immersed in 3% H₂O₂ (15 min), washed with PBS (x2), blocked with buffer containing donkey serum, and then incubated with HOXA5 antibody (overnight). Slides were washed thrice (PBS) and then incubated (1.5 h) with donkey secondary antibody (biotinylated). Slides were then washed and incubated with avidin-biotin complexes (1.5 h), again washed with PBS, followed by washes with 0.1 M Tris-HCl (pH 7.4, twice). The slide was subjected to peroxidase labeling (incubated with a DAB substrate, Vector Laboratories), dehydrated (immersed sequentially in 70, 95, and 100% ethanol), cleaned (incubated in citrisolv agent), and mounted using DPX mounting solution. Slides were analyzed under a microscope (Nikon Eclipse TE2000-U, Japan).

RNA and Protein Extraction, RT-PCR, and Western Blots

RNA extraction: cells were resuspended in DEPC-treated buffer A (20 mM Tris-HCl, pH 7.9; 1.5 mM MgCl₂; 10 mM KCl and 0.5 mM DTT; 0.5 mM EDTA), incubated on ice (10 min), and then centrifuged. The supernatant was subjected to phenol-chloroform extraction. RNA was precipitated using ethanol. The precipitated RNA was dissolved in DEPC-treated water, quantified, and then reverse-transcribed into cDNA as described by us previously (500 ng RNA, 2.4 μ M of oligo-dT, 100 units MMLV reverse transcriptase, 1X first strand buffer, 100 μ M dNTPs each, 1 mM DTT, and 20 units of RNaseOut in 25 μ L total reaction volume). The cDNA was diluted to 100 μ L and then analyzed using regular PCR and qPCR (PCR primer in **Table 1**). For the qPCR, 5 μ L cDNA was mixed with 5 μ L of SsoFast EvaGreen supermix (Biorad) and PCR primers (100 μ M

TABLE 1 | Primers used for cloning, RT-PCR, ChIP, and antisense experiments.

Primers	Forward primers (5'-3')	Reverse primers (5'-3')
PCR PRIMERS		
GAPDH	CAATGACCCCTTCATTGACC	GACAAGCTTCCC GTTCTCAG
HOXA5	ACTCATTGCGGTGCTAT	TTGTAGCCGTAGCCGTACCT
HOXA5-ERE1	CGAGTCCGGCTGAACGGCGG	TAGGCACCCAAATATGGGGTA
HOXA5-ERE2	TTATTTCTCCAATTGGCTAAA	CCGGCGAGGATGCAGAGGAT
ER α	AGCACCCCTGAAGTCTCTGGA	GATGTGGGAGAGGATGAGGA
ER β	AAGAAGATTCCCGGCTTTGT	TCTACGCATTCCCCTCATC
ANTISENSES		
HOXA5	GTCCCTGAATTGCTCGCTCA**	
ER α antisense	TCCACCTTTCATCATTCCC**	
ER β antisense	GCCACACTTCACCATCCCCA**	
Scramble antisense	CGTTTGTCCCTCCAGCATCT**	

**Phosphorothioate antisense oligonucleotides.

each), and then PCR-amplified in a CFX96 qPCR system. Protein extracts: Whole cell protein extracts were made by suspending the cells in whole cell extraction buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA; 0.05% NP-40; 0.2 mM PMSF; 0.5 mM DTT; and 1 \times protease inhibitor cocktail). Proteins were quantified and analyzed by Western blotting (alkaline phosphatase method).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP analyses were performed using similar procedures described by us previously (Wang et al., 2010; Ansari et al., 2011a, 2012a; Shrestha et al., 2012; Kasiri et al., 2013; Bhan et al., 2014a; Hussain et al., 2015; Deb et al., 2016). Briefly, MCF7 (treated with different conditions) cells were fixed in 4% formaldehyde (15 min), followed by quenching with 125 mM glycine and washed with PBS (twice). They were lysed (SDS lysis buffer), and the chromatin was sheared by sonication (~150–450 bp in length, checked by agarose gel). The sonicated chromatin (precleared using protein-G agarose beads) were immunoprecipitated with antibodies against ER α , ER β , H3K4-trimethyl, RNAPII, MLL1, MLL2, MLL3, CBP, p300, H4 acetylation, and β -actin, independently. The immunoprecipitated DNA fragments were purified and analyzed by PCR using promoter-specific primers shown in Table 1.

Flow Cytometry Analysis

MCF7 cells transfected with HOXA5 and scramble ASOs (48 h) were harvested and centrifuged (1,000 \times g for 5 min at 4°C). Cells were washed with PBS (cold, twice), fixed (70% ethanol/PBS, 1 h, 4°C), and stored overnight at -20°C. Cells were stained with propidium iodide (PI) by resuspending in PI solution (0.5 μ g/mL, 2 h) and then analyzed by FACS (Beckman Coulter, Fullerton, CA, USA) (31).

Animal Studies

Adult, 90-days-old, experimentally naïve, female Sprague-Dawley rats ($n = 12$) (purchased commercially) and maintained in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals as described by us previously (Betancourt et al., 2010; Bhan et al., 2014a,b; Hussain et al., 2015). Rats were anesthetized (2%–3% isoflurane-oxygen vapor mixture), ovariectomized (OVX), and allowed to recover (for 4–5 days post-surgery) as described previously (Betancourt et al., 2010; Bhan et al., 2014a,b; Hussain et al., 2015). Vaginal lavage testing was performed daily for 8 consecutive days to confirm cessation of estrous cycling (Inagaki et al., 2012). Rats were given subcutaneous injections of E2 (5 μ g/kg, dissolved in peanut oil, 5 μ g/mL stock) ($n = 4$) 24 and 4 h prior to sacrifice (via rapid decapitation). Mammary gland tissue was collected, flash frozen on dry ice, and stored at -80°C. RNA and proteins were extracted from mammary tissues and analyzed by RT-PCR and Western blotting (Betancourt et al., 2010; Bhan et al., 2014a,b; Hussain et al., 2015).

Statistical Analyses

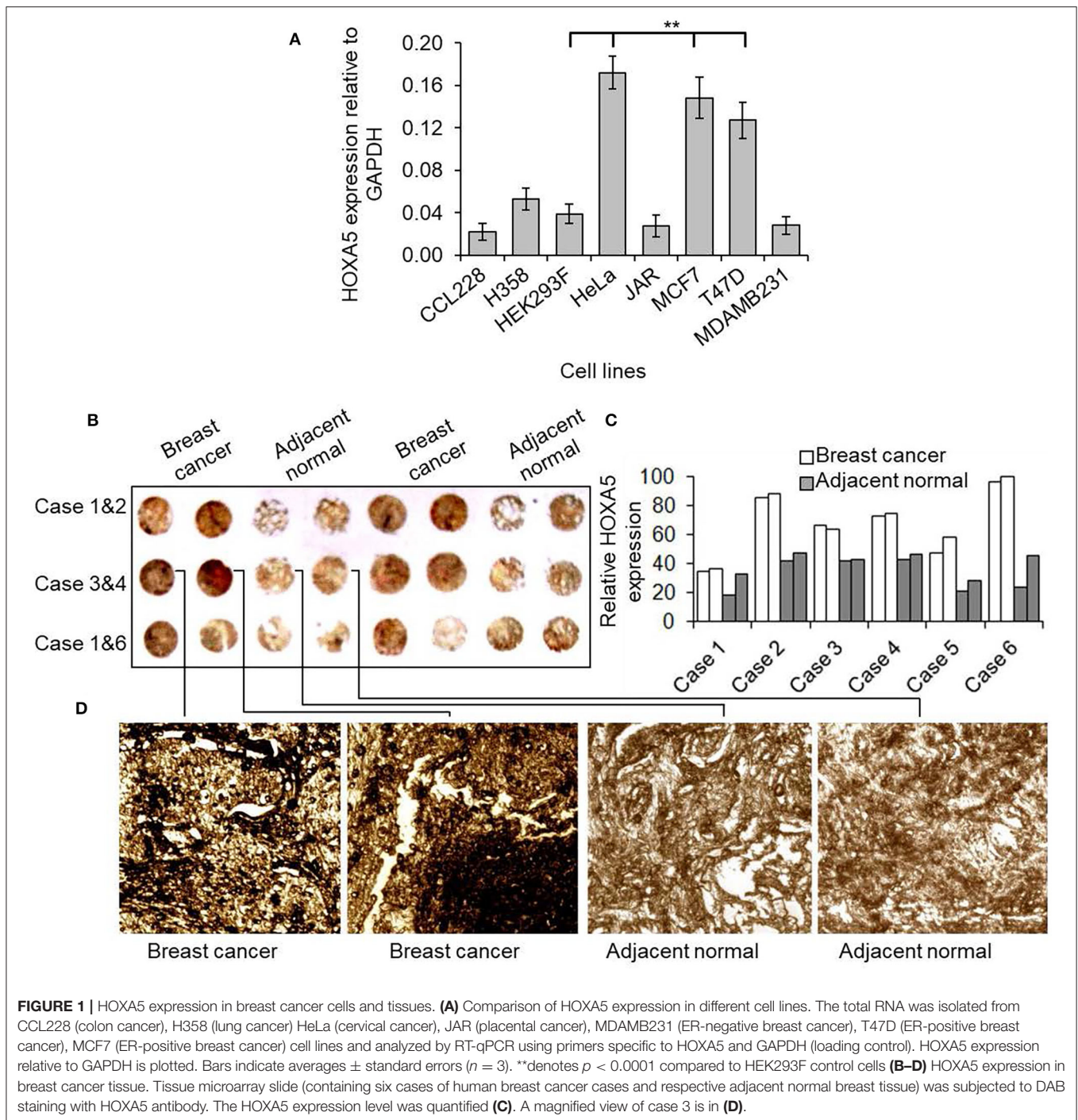
Experiments were repeated at least thrice ($n = 3$). The RT-qPCR experiments were performed in three parallel replicates and repeated at least thrice. The data presented in the figures is expressed as averages \pm standard error. Statistical significance between various groups (control vs. treated) were determined using one- or two-way analysis of variance (ANOVA) followed by multiple comparisons with Dunnett correction (GraphPad Prism). Other statistical evaluations were performed using the Student t -test. Data was considered statistically significant when the p -value was <0.05 .

RESULTS

HOXA5 Is Elevated in Breast Cancer

In an effort to understand the potential role of HOXA5 in breast cancer, initially we examined HOXA5 expression in various cancer cells and breast cancer tissues. Briefly, RNA from different cell lines was analyzed by RT-qPCR. These analyses demonstrated that HOXA5 expression is elevated in MCF7 and T47D (ER-positive breast cancer cells) in comparison to MDA-MB-231 (ER-negative breast cancer cells) (Figure 1A). For the tissue expression analysis, a breast cancer tissue microarray (commercial) was analyzed for HOXA5 expression level. Briefly, the tissue microarray containing the breast cancer tissue and surrounding normal tissue was immunostained using a HOXA5 antibody and analyzed under a microscope. Interestingly, these analyses show that HOXA5 expression is elevated in most breast cancer tissues relative to their normal counterparts (Figures 1B–D).

To investigate further, we also examined HOXA5 expression in different types of breast cancer patients using a preexisting cancer gene expression database using cBioPortal online data analysis tool (<https://www.cbioportal.org>) (Figure 2). The data provided by cBioPortal includes mutations, deletions, and copy-number amplifications (Cerami et al., 2012; Gao et al., 2013). These analyses demonstrate that HOXA5 expression is



significantly amplified in various breast cancer tissues, such as in MBC project, BRCA (INSERM 2016), breast (METABRIC), and invasive breast carcinoma (TCGA PanCan), further suggesting potential association of HOXA5 in breast cancer. Taken together, these observations demonstrate that HOXA5 is overexpressed at least in some subset of breast cancer and may contribute to breast cancer growth. The overexpression of HOXA5 in the ER-positive breast cancer cells suggests its potential regulation by E2.

HOXA5 Knockdown Suppressed the Breast Cancer Cell Viability

As HOXA5 expression is upregulated in breast cancer, we knocked it down in MCF7 cells and analyzed its impacts on viability. HOXA5 antisense or scramble-ASO were transfected into MCF7 cells separately (48 h) followed by analysis of HOXA5 expression levels using the RNA (RT-qPCR) and protein levels (Western blots). Cells were also visualized under a fluorescence

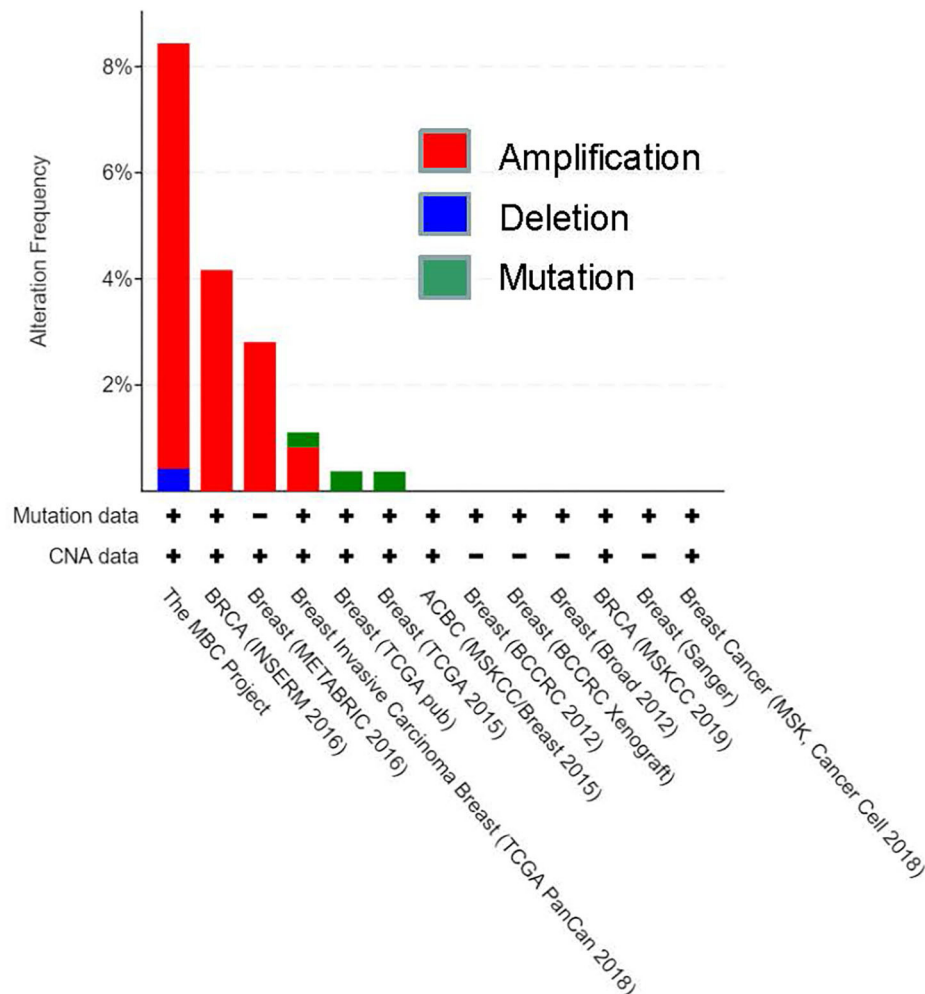


FIGURE 2 | CBioportal analysis of breast cancer database for HOXA5 expression. HOXA5 expression in different types of breast cancer patients using a preexisting cancer gene expression database using a cBioPortal online data analysis tool (<https://www.cbioportal.org>). The data provided by cBioPortal includes mutations, deletions, and copy-number amplifications for HOXA5; CAN, copy number alteration.

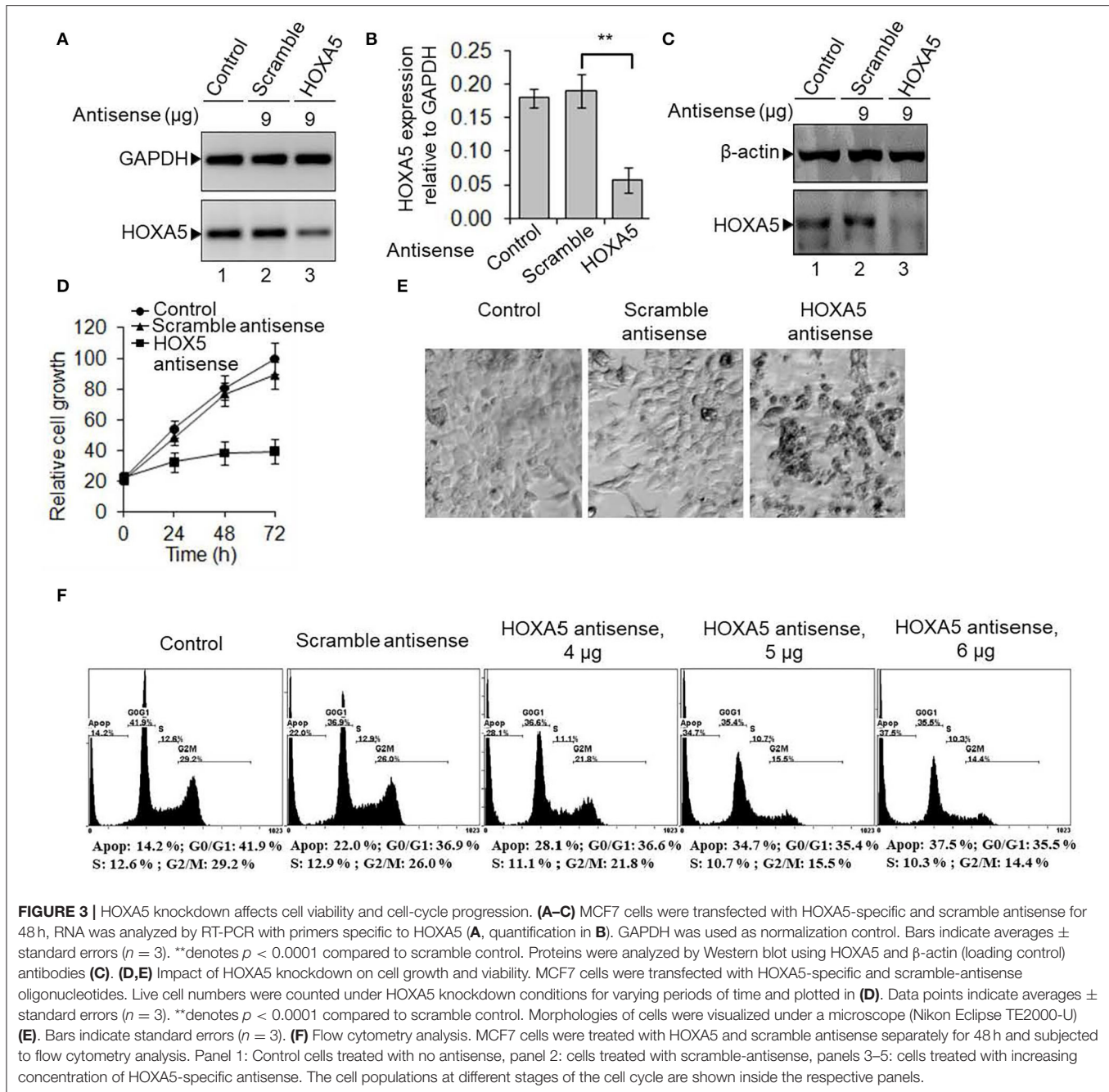
microscope or analyzed by flow cytometry. Our analyses show that HOXA5 expression is decreased upon transfection with HOXA5 ASO (Figures 3A–C), and this results in decrease in growth of MCF7 cells compared to controls (Figure 3D). Microscopic analysis shows that, upon HOXA5 knockdown, cells appear unhealthy, rounded up, and dead (Figure 3E).

To examine if HOXA5 may regulate cell-cycle progression, we knocked it down in MCF7 cells using HOXA5 ASO and analyzed it by flow cytometry. Interestingly, upon HOXA5-ASO transfection, G0/G1 and S phase cell populations were decreased with an increase in apoptotic cells compared to controls (Figure 3F). At 4 μ g of HOXA5 ASO treatment, the cell population at the S (decreased from 12.9 to 11.1%) and G2/M phases (reduced from 26 to 21.8%) were reduced although about 28% of cells were apoptotic, and these affected were further pronounced at higher HOXA5 ASO concentrations (Figure 3F), suggesting the critical role of HOXA5 in regulation of cell-cycle progression.

E@ Regulates HOXA5 Gene Expression *in vitro* and *in vivo*

As HOXA5 expression is elevated in breast cancer cells and tissues, we investigated its potential regulatory mechanism by E2 in MCF7 cells. Briefly, MCF7 cells were treated with 17 β -E2. RNA was isolated and analyzed by RT-PCR for the expression of HOXA5. An antiestrogen, tamoxifen, was also used in conjunction with E2 to explore the specificity and to understand the E2-mediated regulatory mechanism of HOXA5. Interestingly, E2 treatment induced HOXA5 expression in a concentration-dependent manner (Figures 4A,B). HOXA5 expression was increased by 4.5-fold upon exposure to 1 nM of E2. Notably, treatment with the antiestrogen tamoxifen suppressed E2-induced HOXA5 expression (Figures 4A,B). These observations suggest potential regulation of HOXA5 via E2 and ERs.

To further examine the physiological significance of HOXA5 and its endocrine regulation *in vivo*, we analyzed its expression in

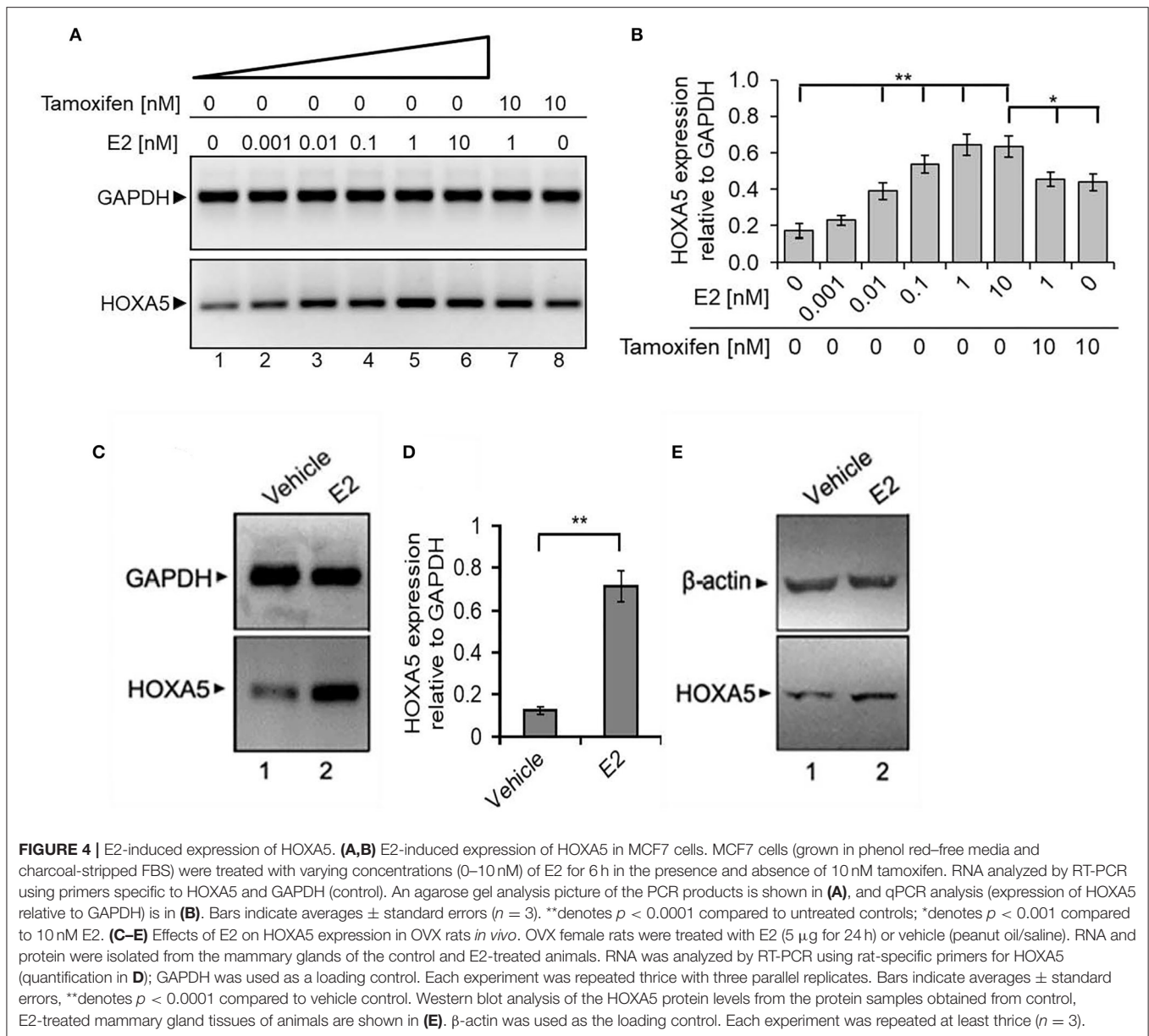


the mammary tissue of OVX female rats treated with E2 (5 μ g/kg) (Betancourt et al., 2010; Bhan et al., 2014a,b; Hussain et al., 2015). The OVX procedure was performed to minimize the effects of endogenous estrogens. Interestingly, RT-qPCR and Western blot analysis demonstrate that HOXA5 expression is elevated in the rat mammary tissues upon treatment with E2 (Figures 4C–E), indicating potential E2-mediated regulation of HOXA5 *in vivo*.

ER Coordinates E2-Induced HOXA5 Expression

ERs are crucial players in estrogen signaling (Nilsson and Gustafsson, 2000, 2002). In general, ERs are activated upon

binding to E2. The activated ERs dimerize and bind to their target gene promoters. Along with ERs, various ER co-regulators and chromatin-modifying enzymes are also recruited and modify the chromatin and induce ER target gene expression (Nilsson et al., 2001; Nilsson and Gustafsson, 2002; Barkhem et al., 2004; Dreijerink et al., 2006; Lee et al., 2006; Mo et al., 2006). To understand the importance of ERs in HOXA5 gene regulation, we analyzed the HOXA5 expression in ER knocked down (using ER α or ER β -specific ASO) and E2-treated MCF7 cells. As seen in Figure 5, ER α or ER β levels were knocked down upon treatment with respective ASO (lanes 5–7, Figure 5A, qPCR data in Figure 5B). Interestingly, the level of E2-induced expression of



HOXA5 was suppressed significantly upon knockdown of ER α or ER β (lanes 5–7, **Figures 5A,B**). Scramble-ASO does not have any major impact on the E2-dependent HOXA5 expression (lanes 2–4, **Figures 5A,B**). The level of E2-induced HOXA5 expression is further suppressed by the combined knockdown of ER α and ER β (lane 7, **Figures 5A,B**).

As ER α and ER β are associated with HOXA5 expression, we examined their binding to the HOXA5 promoter (Ansari et al., 2011b). We performed the ChIP assay using antibodies against ER α , ER β , and β -actin (control), and the immunoprecipitated DNA were analyzed by PCR using primers spanning the estrogen-response element (ERE) regions of the HOXA5 promoter. Notably, promoter sequence analysis shows the presence of two potential EREs at the HOXA5 promoter

upstream of the transcription start site (**Figure 6A**). ChIP analysis shows that levels of ER α and ER β (but not β -actin) were enriched in the ERE1 and ERE2 regions of the HOXA5 promoter in the presence of E2 (**Figure 6B**, qPCR data in **Figure 6C**). Taken together, the antisense-mediated knockdown and ChIP analysis demonstrate the involvement of both ER α and ER β HOXA5 gene expression in the presence of E2.

Along with ERs, ER co-activators are crucial players in ER target gene expression. Therefore, in addition to ERs, we analyzed the enrichment level of several well-known ER co-regulators: CBP/p300 (histone acetylase) and mixed lineage leukemias (MLL, histone methyltransferases) (Ansari et al., 2011b). Additionally, we also analyzed the level of

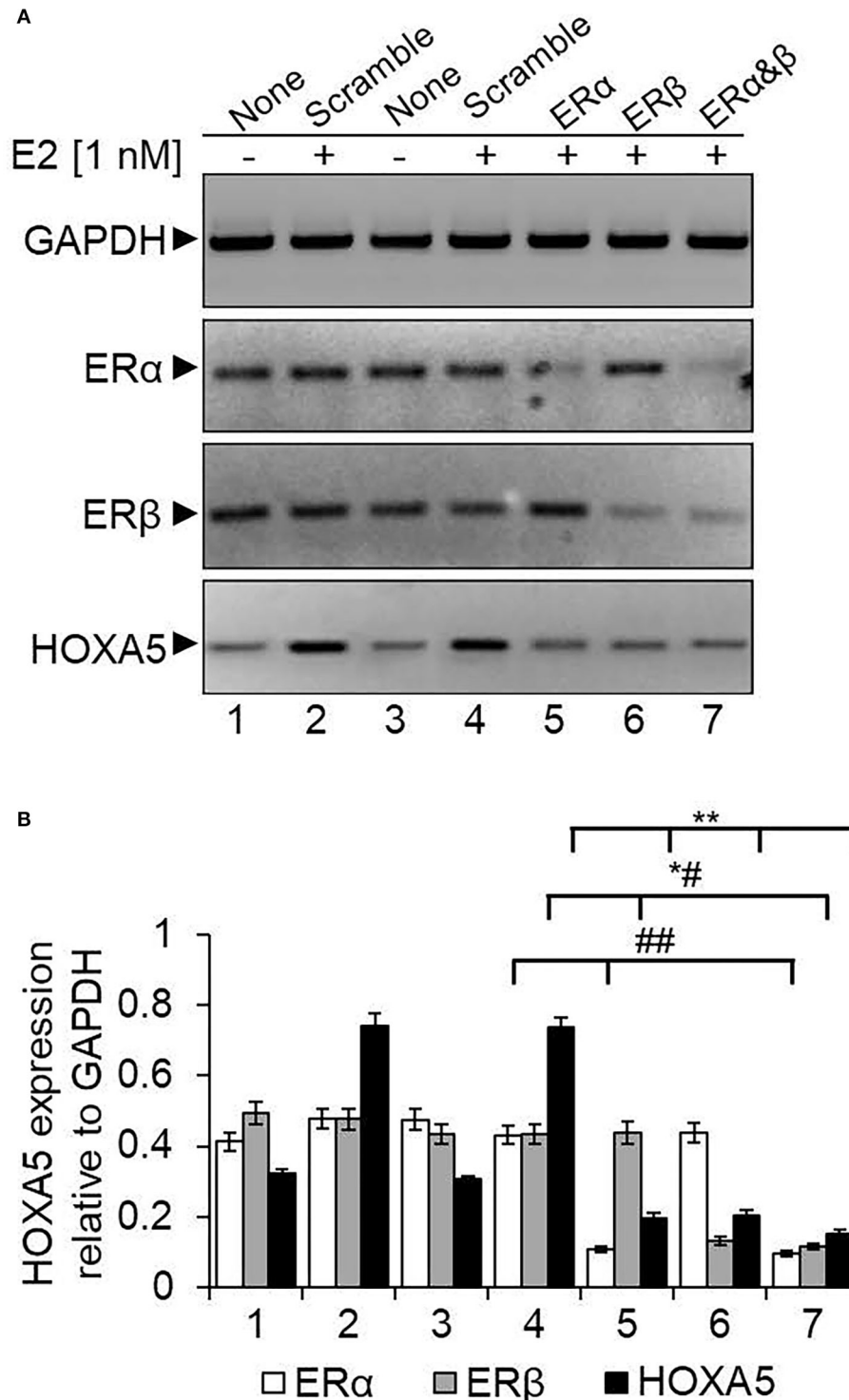


FIGURE 5 | Knockdown of ERs and its impact on E2-induced HOXA5 expression. MCF7 cells were transfected with ERα or ERβ-specific ASOs or a scramble antisense for 48 h, followed by treatment with E2 (1 nM, 6 h). RNA was analyzed by RT-qPCR by using primers specific to HOXA5, ERs, and GAPDH (control) separately **(A)**. Lane 1: control cells (no E2); lane 2: cells treated with E2; lanes 3 and 4: cells treated with scramble antisense in the absence and presence E2; lanes 5 and 6: cells treated with ERα and ERβ antisense, respectively, followed by exposure to E2; lane 7: cells transfected with 1:1 mixture of ERα and ERβ antisenses in the presence of E2. The qPCR analysis data is in **(B)**. qPCR reactions were carried out in three parallel replicates, and each experiment was repeated at least thrice ($n = 3$). Bars indicate averages \pm standard errors. **indicates $p < 0.0001$ compared to E2-treated scramble control (for HOXA5 target); # indicates $p < 0.0001$ compared to E2-treated scramble control (for ERβ target); ## indicates $p < 0.0001$ compared to E2-treated scramble control (for ERα target).

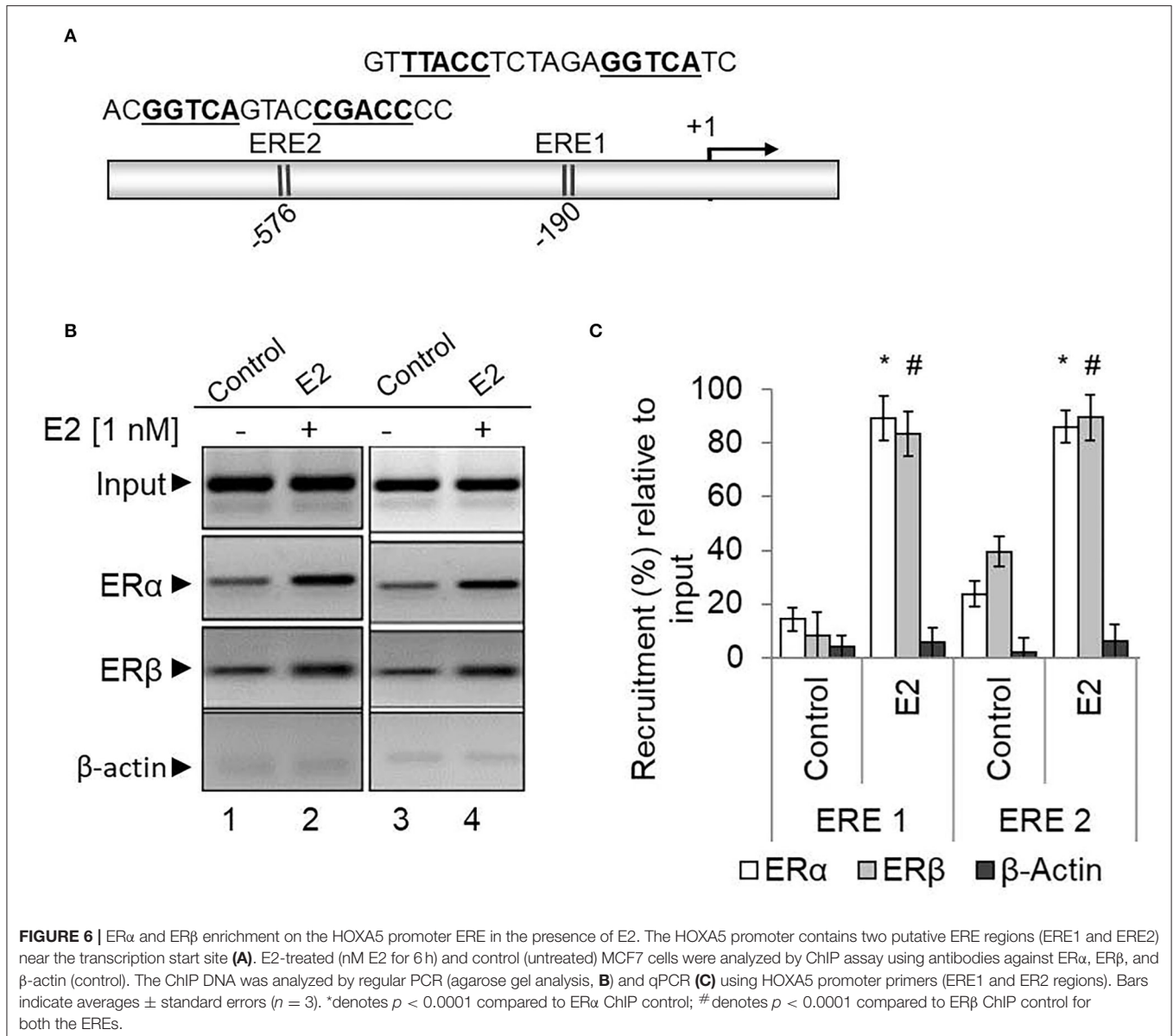
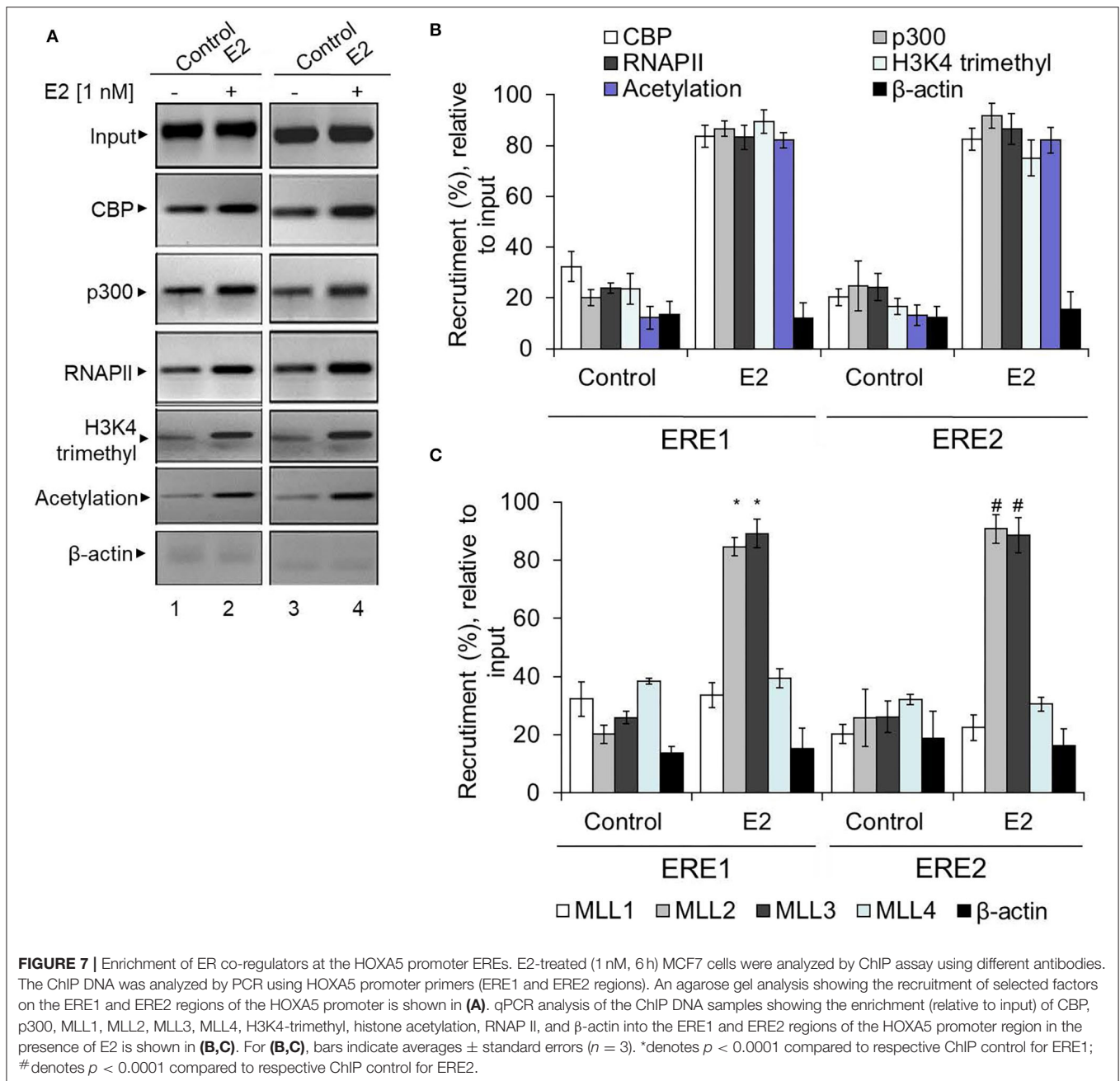


FIGURE 6 | ERα and ERβ enrichment on the HOXA5 promoter ERE in the presence of E2. The HOXA5 promoter contains two putative ERE regions (ERE1 and ERE2) near the transcription start site (A). E2-treated (nM E2 for 6 h) and control (untreated) MCF7 cells were analyzed by ChIP assay using antibodies against ERα, ERβ, and β-actin (control). The ChIP DNA was analyzed by regular PCR (agarose gel analysis, B) and qPCR (C) using HOXA5 promoter primers (ERE1 and ERE2 regions). Bars indicate averages ± standard errors (n = 3). *denotes *p* < 0.0001 compared to ERα ChIP control; # denotes *p* < 0.0001 compared to ERβ ChIP control for both the EREs.

histone acetylation and H3K4 trimethylation at the HOXA5 promoter using ChIP in the absence and presence of E2. Notably, promoter histone acetylation and H3K4 trimethylation are post-translational histone modifications associated with gene activation. Interestingly, our ChIP analysis shows that E2 treatment resulted in enrichment of CBP, p300, histone methylases MLL2 and MLL3 (Figures 7A–C). Histone acetylation, H3K4 trimethylation, and RNA polymerase II (RNAPII) levels were also elevated at the HOXA5 promoter with E2 treatments (Figures 7A,B). The E2-dependent enrichment in CBP/p300 acetyltransferase and MLL-histone methylases MLL2 and MLL3 and the histone acetylation and methylation levels, suggest that, along with ERs, these ER co-regulators are associated with E2-dependent HOXA5 gene expression.

DISCUSSION

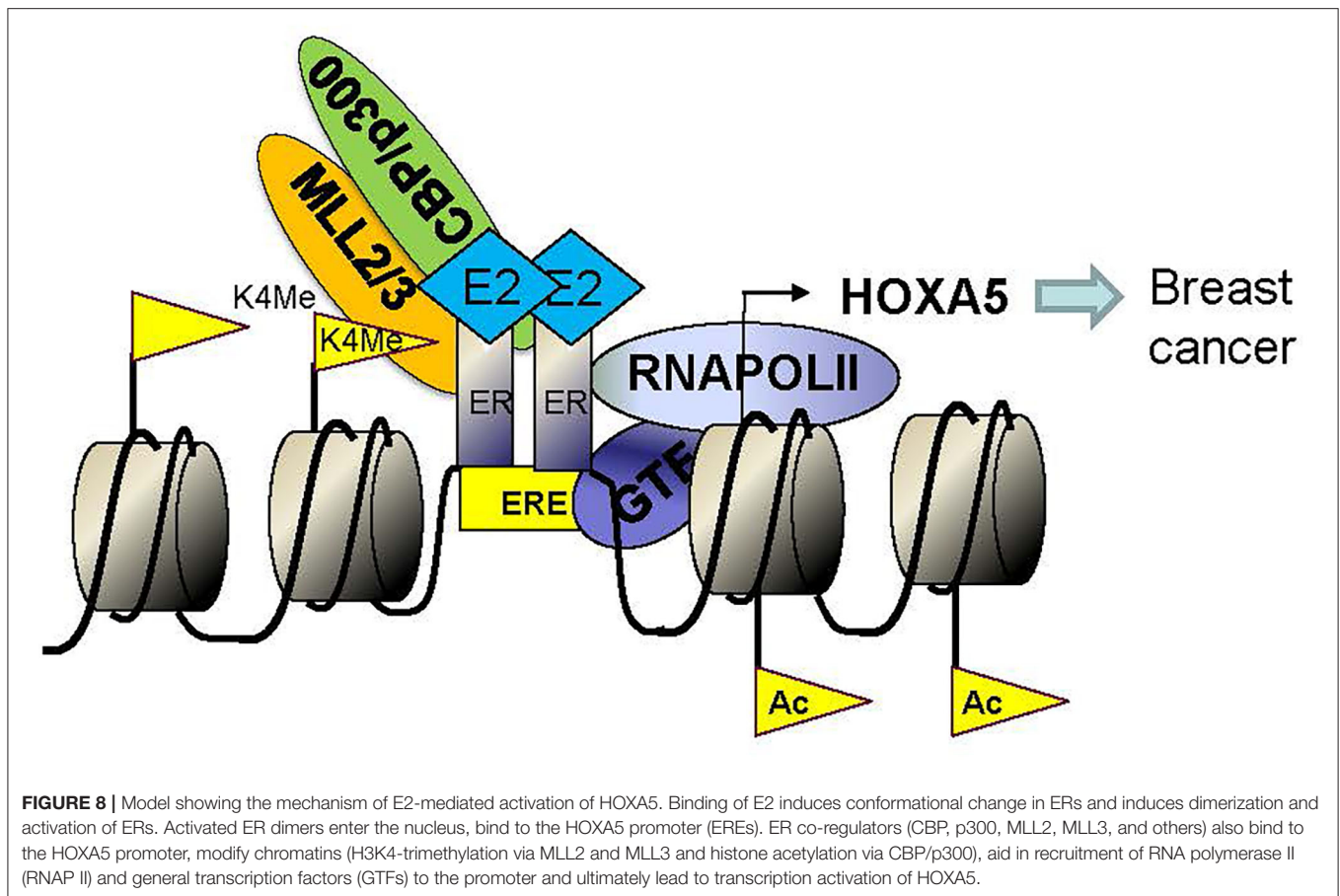
HOX gene expression is well-known to guide cellular differentiation, organogenesis, and development (Krumlauf, 1994; Zakany and Duboule, 2007). Increasing studies demonstrate that hormones, such as estrogen, retinoic acid, etc., influence the expression of developmental genes, including HOX genes. Posterior HOX genes appear to be regulated by estrogens and progesterones, and anterior HOX genes are regulated by retinoids. HOXA5, which acts a transcription factor, plays a critical role in embryonic development. Beyond this, an emerging group of studies shows the dysregulation of HOXA5 in various solid/adult/pediatric/cancers or hematological malignancies and linked with higher pathological grade and poorer disease outcome (Chen et al., 2004; Wang et al.,



2019). However, the detailed mechanism by which HOX genes influence differentiation and development and how it is regulated or misregulated in diseases/various carcinomas is still emerging and less understood. Here, we explore any association and function of HOXA5 in breast cancer and investigate its potential mechanism of transcription.

Our studies demonstrate that HOXA5 expression is also augmented in breast cancer tissues in comparison to the corresponding adjacent normal breast tissues. Additionally, *c*-bioportal-based meta-analysis of preexisting gene expression databases in breast cancer patients also show that HOXA5 expression is elevated in breast cancer patients. HOXA5 expression is also found to be elevated in ER-positive breast

cancer cells. Additionally, our studies also demonstrate that HOXA5 expression is important for cell-cycle progression as well as cell viability of breast cancer cells. HOXA5 downregulation results in cell-cycle arrest and apoptosis in breast cancer cells. These observations suggest that HOXA5 expression is elevated in breast cancer at least in some subset of breast cancer and may be critical for breast cancer cell proliferation. As HOXA5 is elevated in ER-positive breast cancer cells and breast cancer tissues, we investigated its gene regulation potential via E2. These studies demonstrate that E2 indeed regulates the transcription of HOXA5 *in vitro*, in ER-positive breast cancer cells (MCF7), and this expression is suppressed upon treatment with an antiestrogen, tamoxifen, suggesting the potential regulation of



HOXA5 via E2 and ERs. Additionally, we also observed that HOXA5 expression is elevated in the mammary tissues of OVX Sprague–Dawley rats, further supporting our observation that HOXA5 expression is regulated by estrogen *in vivo*.

Mechanistic studies demonstrate that E2-dependent HOXA5 expression is coordinated via involvement of ERs and ER co-activators. Knockdown of either ER α or ER β suppressed E2-dependent HOXA5 expression in MCF7 cells. ChIP analysis demonstrates that ERs and ER co-activators, CBP/p300 (acetyltransferases), and MLL2 and MLL3 (histone H3K4-methylases) are enriched at the HOXA5 promoter (ERE regions) in the presence of E2. Along with histone acetyltransferases and histone methylases, the level of histone H3K4 trimethylation and histone acetylation level was elevated at the HOXA5 promoter. Notably, histone acetylation and H3K4 trimethylation are linked to gene activation. The enrichment of histone acetyltransferases (CBP/p300) and histone H3K4 trimethylases (MLL2 and MLL3) and the histone acetylation and H3K4 trimethylations at the HOXA5 promoter in the presence of E2 suggest their important roles in E2-mediated regulation of HOXA5 expression in breast cancer.

Overall, our studies demonstrate that HOXA5 gene expression is regulated by E2, and its expression is upregulated in breast cancers. The E2-mediated regulation of HOXA5 is coordinated via involvement of estrogen receptors, CBP/p300 histone acetyltransferases, and the MLL family of histone

methyltransferases. CBP/p300 and MLL-histone methylases act as ER co-regulators in regulation of HOXA5 expression in breast cancer. A model showing the mechanism of E2-mediated HOXA5 gene activation is shown in **Figure 8**. Notably, multiples lines of evidence support our observations that increased H3K4-trimethylation and histone acetylation contribute to E2-induced HOXA5 expression. For example, Yan et al. demonstrate that inhibition of histone deacetylase HDAC8 (by HDAC inhibitor) increases histone acetylation and HOXA5 expression, which, in turn, induces tumor-suppressor p53 induction (Yan et al., 2013). Another study shows that oxidative stress-mediated repression of HDAC8 induces histone H3 acetylation and HOXA5 expression that controls plasticity of lung cancer stem-like cells (Saijo et al., 2016). Similar to histone acetylation, histone H3K4 trimethylation is well-known to activate HOX gene expression, including HOXA5 expression (Okada et al., 2006; Mishra et al., 2009; Ansari et al., 2012b; Burillo-Sanz et al., 2018). Enhancer elements present around the HOXA cluster regulates HOXA cluster gene spatiotemporal expression, and deletion impairs HOX5 gene activation in embryonic stem cells (Cao et al., 2017). Thus, our results showing the roles of H3K4 trimethylation and histone acetylation are in agreement with previous studies. Although histone acetylation and H3K4-trimethylation result in gene activation, H3K27 trimethylation by polycomb repressive complex (PRC) results in HOXA5 gene silencing and contributes to cell differentiation and development

(Xi et al., 2007). Transcription factor CTCF facilitates the stabilization of PRC2 and H3K27 trimethylation at the HOXA locus and represses HOXA gene (including HOXA5) expression (Xu et al., 2014). Mutation in CTCF results in increased HOXA cluster gene expression. Similarly, ASXL1 mutation facilitates myeloid transformation through inhibition of PRC2-mediated HOXA5 repression (Abdel-Wahab et al., 2012). An independent study reports that HOXA5 expression is repressed in a subset of human breast cancer tissues, and this loss occurs via promoter hypermethylation (Teo et al., 2016). The study shows that HOXA5 loss in tumor cells causes reduced downstream target expression involved in maintaining epithelial integrity, which leads to a subsequent increase in invasion and migration. This suggests the potential presence of multiple modes of action of HOXA5 in different breast cancer subsets. Nevertheless, our studies demonstrate that HOXA5 expression is upregulated, at least in a subset of breast cancer, and is regulated by estrogen *in vitro* and *in vivo*, and therefore, may play critical roles in breast cancer.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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ETHICS STATEMENT

The animal study was reviewed and approved by IACUC, University of Texas at Arlington.

AUTHOR CONTRIBUTIONS

IH did most of the experiments, performed the data analysis, and contributed to the manuscript writing. PD, AC, MO, KA, and AB assisted the different biochemical experiments. SB and LP coordinated the animal experiments. SU, PA, RB, and HD helped with the cancer-related studies. SM designed the experiments, supervised the study, and written the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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