Biochemical and Biophysical Research Communications 440 (2013) 652–657

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Homeobox A7 stimulates breast cancer cell proliferation by up-regulating estrogen receptor-alpha

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ARTICLE INFO

Article history:
Received 24 September 2013
Available online 4 October 2013

Keywords:
HOX
Estrogen receptor
Breast cancer

ABSTRACT

Breast cancer is the most common hormone-dependent malignancy in women. Homeobox (HOX) transcription factors regulate many cellular functions, including cell migration, proliferation and differentiation. The aberrant expression of HOX genes has been reported to be associated with human reproductive cancers. Estradiol (E2) and its nuclear receptors, estrogen receptor (ER)-alpha and ER-beta, are known to play critical roles in the regulation of breast cancer cell growth. However, an understanding of the potential relationship between HOXA7 and ER in breast cancer cells is limited. In this study, our results demonstrate that knockdown of HOXA7 in MCF7 cells significantly decreased cell proliferation and ERα expression. In addition, HOXA7 knockdown attenuated E2-induced cell proliferation as well as progesterone receptor (PR) expression. The stimulatory effects of E2 on cell proliferation and PR expression were abolished by co-treatment with ICI 182780, a selective ERα antagonist. In contrast, overexpression of HOXA7 significantly stimulated cell proliferation and ERα expression. Moreover, E2-induced cell proliferation, as well as PR expression, was enhanced by the overexpression of HOXA7. Neither knockdown nor overexpression of HOXA7 affected the ER-beta levels. Our results demonstrate a novel mechanistic role for HOXA7 in modulating breast cancer cell proliferation via regulation of ERα expression. This finding contributes to our understanding of the role HOXA7 plays in regulating the proliferation of ER-positive cancer cells.

1. Introduction

Breast cancer is the most common malignancy in women. As is true for other hormone-dependent cancers, estrogen exposure is the most well-established risk factor for human breast cancer. Other established factors associated with the risk of breast cancer are reproductive in nature and are connected to sex steroid exposure, including nulliparity, early age at menarche, older age at menopause and older age of first full-term pregnancy [1].

Estrogen exhibits its growth-promoting effects by binding to the estrogen receptor (ER), which is a member of the steroid/nuclear receptor superfamily and functions as a ligand-activated transcription factor that can regulate gene expression [2]. Two ERs, ERα and ERβ, have been identified in mammals. ERα and ERβ both function as signal transducers and transcription factors to modulate the expression of target genes [3]. In addition, ER has been identified as a transcriptional regulator by interacting with other signals that are triggered by different stimuli. ER transcriptional activity can be directly regulated by estrogenic or anti-estrogenic molecules. Growth factor-induced signaling pathways can also indirectly affect the transcriptional activity of ER [4,5]. Indeed, even in the absence of estrogen, many non-estrogen signaling pathways can also activate ERα [6]. The homeobox (HOX) genes are transcriptional regulators that are also reported to be involved in mediating ER activation and regulating ER-positive breast cancer cells [6].

Homeobox genes were first identified in the fruit fly *Drosophila melanogaster* [7]. The HOX family is an important group of developmental transcriptional regulators that is defined by a highly conserved homeodomain and is encoded by homeobox genes. In mammalian species, there are at least 39 HOX genes that are located on different chromosomes. These genes are numbered from 1 to 13 and are organized into four clusters labeled A, B, C and D [8]. In humans, 39 HOX genes are expressed, and the four HOX gene clusters are conserved with regard to their collinear expression along the anterior–posterior axis [9]. The HOX family

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http://dx.doi.org/10.1016/j.bbrc.2013.09.121

is critical for various aspects of differentiation and morphogenesis both in the embryo and in adult tissues. Recently, an increasing number of studies have indicated that HOX genes play important roles in oncogenesis [9,10]. Many cancers exhibit altered HOX gene expression, particularly leukemia, breast, prostate and lung carcinomas [11–14]. Considering that E2 and its nuclear receptors play critical roles in the regulation of breast cancer growth, the relationship between HOX genes and ER in breast cells is not well understood.

Our previous studies have demonstrated that HOXA4, A7 and A10 are overexpressed in high malignant serous ovarian cancer compared with serous tumors that have low malignant potential or are benign [15]. Of these genes, HOXA7 is one of the most consistently overexpressed HOX genes in ovarian cancers [15]. It has also been reported that HOXA7 is expressed in ovarian tumors exhibiting mullerian-like features, and this expression is correlated with the generation of anti-HOXA7 antibodies by patients [16]. The ectopic expression of HOXA7 can enhance the epithelial phenotype of immortalized ovarian surface epithelial cells [16]. More recently, we also found that HOXA7 is overexpressed in granulosa tumor cells, which is where HOXA7 increases cellular proliferation via up-regulation of epidermal growth factor receptor (EGFR) expression [17]. However, the regulatory role of HOXA7 in other reproductive tumors, especially in human breast cancer cells, remains unclear.

In the present study, we used gain- and loss-of-function approaches to examine the role of HOXA7 in the regulation of cell proliferation in MCF7 breast cancer cells. We demonstrate that the knockdown of HOXA7 results in both decreased cell proliferation and decreased ERα expression. Consequently, cell proliferation and ERα expression were both increased by HOXA7 overexpression. ERβ levels were not affected by this HOXA7 modulation. Our results indicate that HOXA7 stimulates MCF7 cell proliferation by up-regulating ERα expression, and that a link exists between HOXA7 expression and the regulation of proliferation and ER expression in human breast cancer cells.

2. Materials and methods

2.1. Cell culture and treatment

The human breast cancer cell line, MCF7, was obtained from American Type Culture Collection (Manassas, VA). Cells were grown in a phenol red-free 1/1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (F12) (DMEM/F12; Sigma, St. Louis, MO) supplemented with 10% charcoal/dextran-treated fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in the air. E2, ICI 182780 or both were added to the cultures as concentrated stock solutions in pure ethanol (0.1% of the total volume) to a final concentration of 10 nM. The untreated cells were exposed to an equivalent volume of ethanol.

2.2. Antibodies and reagents

The monoclonal HOXA7 antibody (sc-28600), polyclonal estrogen receptor-α antibody (sc-542), polyclonal estrogen receptor-β antibody (sc-6822), polyclonal progesterone receptor antibody (sc-538) and polyclonal actin antibody (sc-1615) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories (Hercules, CA). Horseradish peroxidase-conjugated donkey anti-goat IgG was obtained from Santa Cruz Biotechnology. E2 and ICI 182780 were purchased from Sigma.

2.3. Small interfering RNA (siRNA) transfection

To knock down endogenous HOXA7, cells were transfected with 50 nM ON-TARGET*plus* SMART*pool* siRNA targeting human HOXA7 (Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX

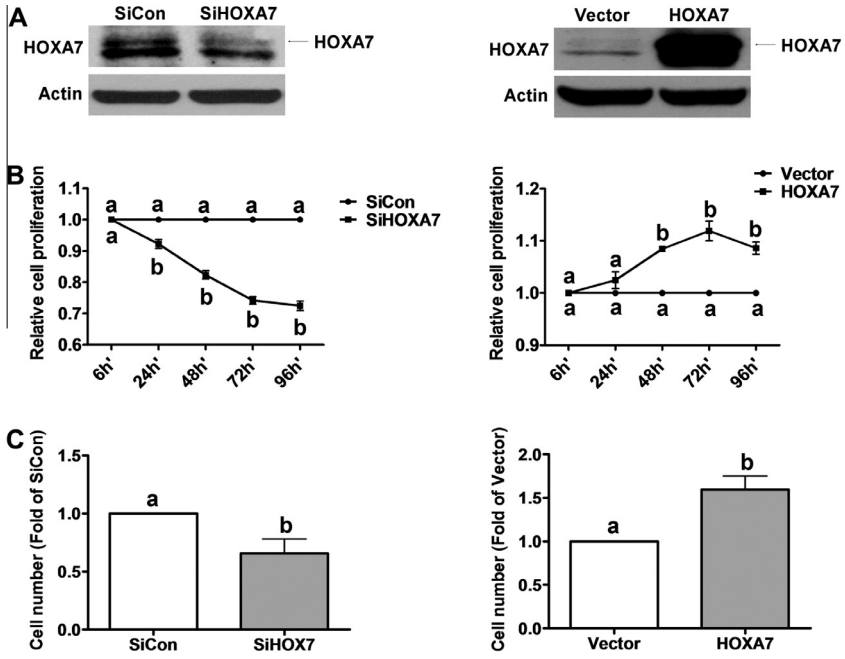

Fig. 1. HOXA7 regulates cell proliferation in MCF7 cells. (A) The cells were transfected with control siRNA (siCon) or HOXA7 siRNA (SiHOXA7) to knockdown endogenous HOXA7. The cells were transfected with the control vector (Vector) or HOXA7 plasmid (HOXA7) to overexpress HOXA7. The efficiencies of HOXA7 knockdown and overexpression were examined by Western blotting. The arrow indicates the HOXA7 band. (B) Cells were transfected with HOXA7 siRNA and overexpressed HOXA7, and cell proliferation was examined by an MTT assay. (C) Cells were transfected with HOXA7 siRNA and overexpressed HOXA7, and cell proliferation was examined by trypan blue exclusion assay after 72 h in culture. The results are expressed as the mean ± SEM of at least three independent experiments. Values without a common letter are significantly different ($p < 0.05$).

(Invitrogen, Life Technologies). The siCONTROL NON-TARGETING *pool* siRNA (Dharmacon) was used as the transfection control.

2.4. Plasmid constructs and transfection

Full-length HOXA7 (917 bp) was amplified by RT-PCR using the Phusion RT-PCR Kit (New England BioLabs, Ipswich, MA) and the following primers (5′–3′): TCA TTC CTC CTC GTC C and ATG AGT TCT TCG TAT TAT GAA CG. The gel-purified PCR product was cloned into the pcDNA3.1 (+) vector using the pCR-Blunt II-TOPO Expression Kit (Invitrogen) and was confirmed by DNA sequencing (Child & Family Research Institute DNA Sequencing Core Facility, Vancouver, BC). pcDNA3.1/CAT was used as a control vector. The cells were cultured in growth medium, as described above, to achieve approximately 80–90% confluence at the time of transfection. Transient transfections were carried out using Lipofectamine 2000 Reagent (Invitrogen) following the manufacturer's protocol. After transfection, cells were processed directly for mRNA/protein extraction, treated with E2 for various periods of time, or reseeded onto a 96-well plate 36 h after transfection for further cell viability analysis.

2.5. MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) assay was used to determine cell proliferation. Cells were seeded at 1×10^4 cells/well in 96-well plates. MTT was added to a final concentration of 0.5 mg/mL. The cells were then incubated for 4 h, and the medium was removed. DMSO was added to each well, and absorbances were measured at 490 nm using a microplate reader.

2.6. RNA extraction and RT-qPCR

At the end of the treatment period, the medium was removed from the culture plates, and RNA was extracted using TRIzol (Invitrogen). The RNA concentration was measured based on the absorbance at 260 nm. The isolated RNA was reverse transcribed into

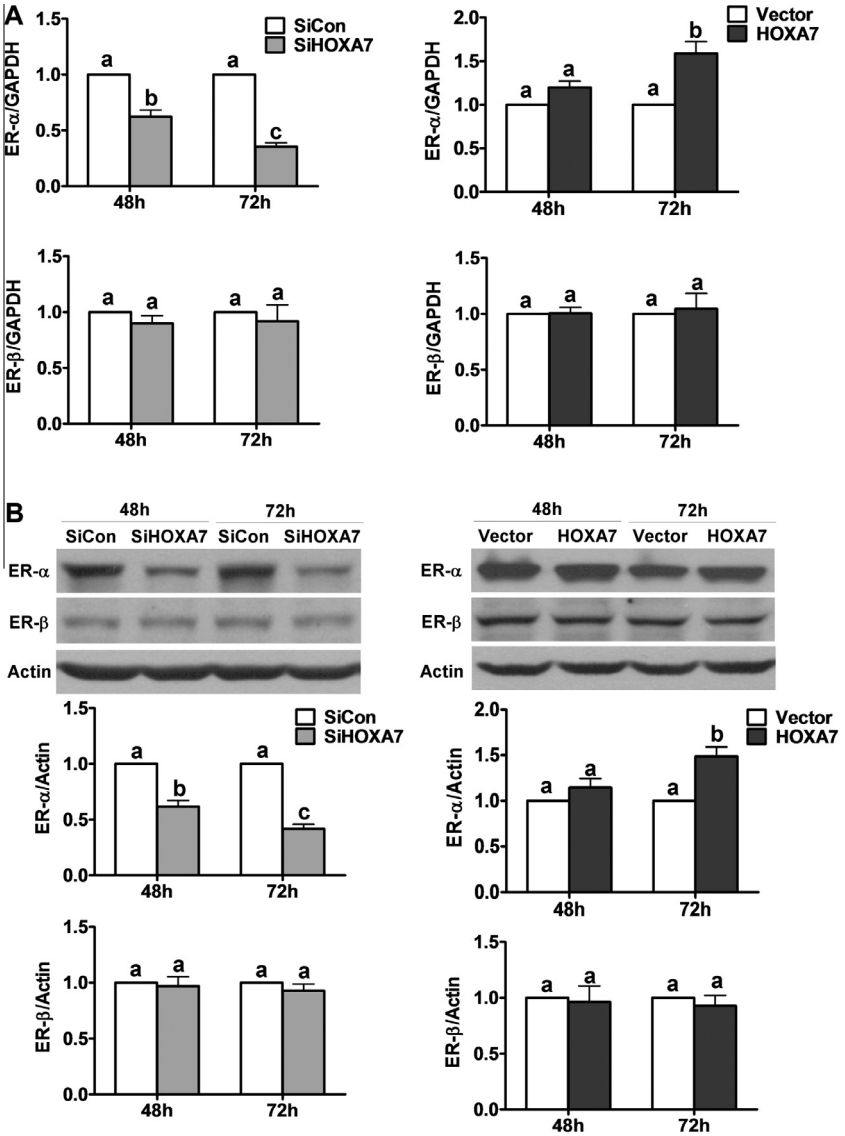

Fig. 2. HOXA7 regulates ERα but not ERβ expression in MCF7 cells. Cells were transfected with control siRNA (siCon) or HOXA7 siRNA (SiHOXA7) to knockdown endogenous HOXA7. The cells were transfected with the control vector (Vector) or HOXA7 plasmid (HOXA7) to overexpress HOXA7. mRNA (A) and protein (B) levels of ERα and ERβ were examined by RT-qPCR and by Western blotting, respectively. The results are expressed as the mean ± SEM of at least three independent experiments. Values without a common letter are significantly different ($p < 0.05$).

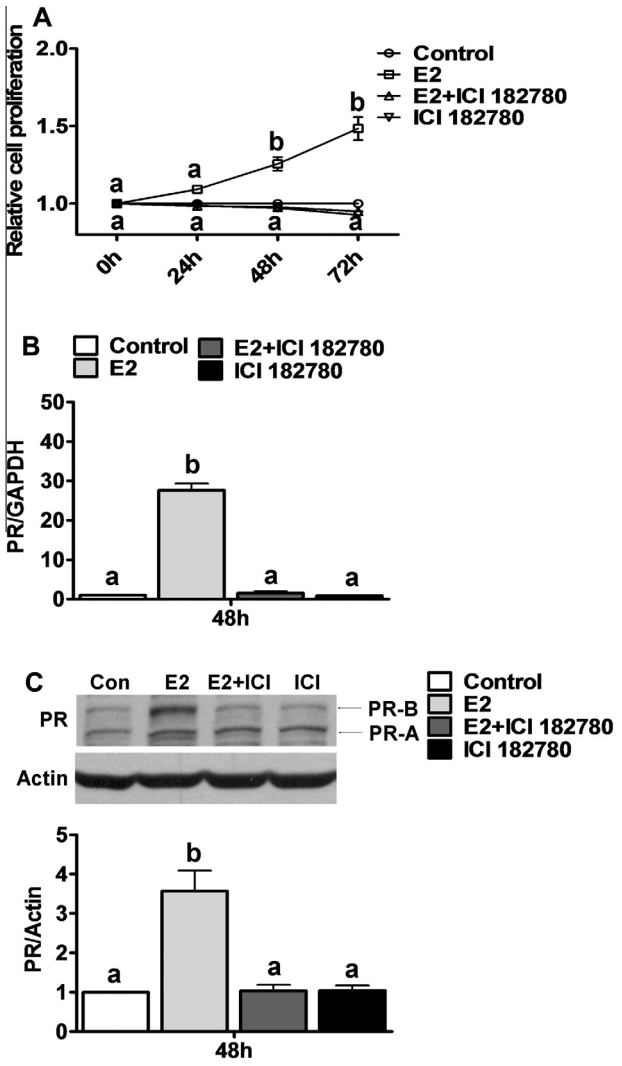

Fig. 3. E2 induces MCF7 cell proliferation and PR expression. (A) Cells were left untreated (Control) or treated with E2 (10 nM) and/or ICI 182780 (10 nM) for 24–72 h. Cell proliferation was examined by an MTT assay. (B and C) Cells were left untreated (Control) or treated with E2 (10 nM) and/or ICI 182780 (10 nM) for 48 h. The mRNA (B) and protein (C) levels of PR were examined by RT-qPCR and Western blotting, respectively. The results are expressed as the mean ± SEM of at least three independent experiments. Values without a common letter are significantly different ($p < 0.05$).

first-strand cDNA using M-MLV reverse transcriptase (Promega BioSciences, San Luis Obispo, CA, USA). The primers used for SYBR Green RT-qPCR were designed using Primer Express Software v2.0 (PerkinElmer Applied Biosystems, Foster City, CA, USA). The following primers were used: ERα: sense, 5′-TGG AGA TCT TCG ACA TGC TG-3′ and antisense, 5′-TCC AGA GAC TTC AGG GTG CT-3′; ERβ: sense, 5′-TGG AGT CTG GTC GTG TGA AG-3′ and antisense, 5′-CTT CAC CAT TCC CAC TTC GT-3′; PR: sense, 5′-CGC GCT CTA CCC TGC ACT C-3′ and antisense, 5′-TGA ATC CGG CCT CAG GTA GTT-3′; and GAPDH: sense, 5′-ATG GAA ATC CCA TCA CCA TCT T-3′ and antisense, 5′-CGC CCC ACT TGA TTT TGG-3′. RT-qPCR was performed using the Applied Biosystems 7300 Real-Time PCR System equipped with a 96-well optical reaction plate. The specificity of each assay was validated by melting curve analysis and agarose gel electrophoresis of the PCR products. All of the RT-qPCR experiments were run in triplicate, and the mean values were used to determine the mRNA levels. Water and mRNA without RT were used as negative controls. Relative quantification of the mRNA levels was performed using the comparative Ct method with GAPDH as the reference gene and the formula $2^{-\Delta\Delta Ct}$.

2.7. Western blotting

Cells were lysed in lysis buffer (Cell Signaling Technology, Danvers, MA), and protein concentrations were determined using a DC protein assay kit with BSA as the standard (Bio–Rad Laboratories). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. After blocking with Tris-buffered saline containing 5% non-fat dry milk for 1 h, membranes were incubated overnight at 4 °C with primary antibodies followed by incubation with HRP-conjugated secondary antibody. Immunoreactive bands were detected with enhanced chemiluminescent substrate (Pierce, Rockford, IL). Membranes were stripped with stripping buffer (62.5 mM Tris, 10 mM Dithiothreitol, 2% SDS, pH 6.7) at 50 °C for 30 min and reprobed with anti-actin as a loading control.

2.8. Statistical analysis

The results are presented as the mean ± SEM of at least three independent experiments. For experiments involving only two groups, the data were analyzed in Excel with a Two-Sample *t*-test assuming unequal variances. Multiple group comparisons were analyzed by one-way ANOVA followed by Tukey's multiple comparison test using PRISM software. Significant differences were defined as $p < 0.05$.

3. Results

3.1. Manipulation of HOXA7 affects MCF7 cell proliferation

To determine the functional role of endogenous HOXA7 in regulating cell proliferation, gain- and loss-of-function approaches were used. Expression of HOXA7 protein was detected in MCF7 cells (Fig. 1A). Transfected cells with HOXA7 siRNA and cells overexpressing HOXA7 significantly down-regulated and up-regulated HOXA7 protein levels, respectively (Fig. 1A). To examine the role of HOXA7 in MCF7 cell proliferation, the cells were transfected with HOXA7 siRNA or overexpressed HOXA7, and cell proliferation was analyzed by an MTT assay. As shown in Fig. 1B, knockdown of HOXA7 decreased MCF7 cell proliferation. Conversely, overexpression of HOXA7 increased cell proliferation. MTT assays have been widely used to measure the mitochondrial metabolic rate to indirectly reflect viable cell numbers. We are aware that the mitochondrial metabolic activity of the cells may be affected by different culture conditions or treatments, which can lead to considerable variation in results reported from MTT assays [18]. Therefore, the effects of HOXA7 on MCF7 cell proliferation were further confirmed by trypan blue exclusion assay. Similar to the results obtained from MTT assay, transfection with HOXA7 siRNA decreased cell number, and overexpression of HOXA7 increased the number of MCF7 cells (Fig. 1C).

3.2. HOXA7 regulates ERα, but not ERβ, in MCF7 cells

To examine whether HOXA7 affects ER expression, the expression levels of ERα and ERβ were examined at both mRNA and protein levels following HOXA7 knockdown or overexpression in MCF7 cells. Knockdown of HOXA7 decreased ERα mRNA levels after a 48 h transfection. Conversely, overexpression of HOXA7 increased ERα mRNA levels after a 48 h transfection (Fig. 2A). Interestingly, the mRNA levels of ERβ were not altered by knockdown and overexpression of HOXA7. Parallel effects of HOXA7 on

ER protein levels were also observed via Western blotting analyses (Fig. 2B).

3.3. E2 increases MCF7 cell proliferation and progesterone receptor expression

Consistent with previous studies [19], treatment with E2 significantly stimulated MCF7 cell proliferation. The stimulatory effects of E2 could be abolished by co-treatment with the selective ERα antagonist, ICI 182780 (Fig. 3A). Because ERα is a transcription factor, it is predicted that loss of ERα will result in the decreased expression of its normal target genes. To test this hypothesis, we examined the progesterone receptor (PR) levels, a known ERα-regulated gene [19]. RT-qPCR and Western blotting results demonstrated that treatment of the MCF7 cells with E2 increased the mRNA and protein levels of PR. The stimulatory effects of E2 could also be abolished by co-treatment with ICI 182780 (Fig. 3B and C).

3.4. E2-increased MCF7 cell proliferation and progesterone receptor expression are regulated by the expression of HOXA7

To examine whether HOXA7 can affect E2-induced cell proliferation and PR expression, cells were treated with E2 after knockdown and overexpression of HOXA7. As shown in Fig. 4A, the stimulatory effect of E2 on cell proliferation was attenuated by the knockdown of HOXA7. In contrast, E2-induced cell proliferation was enhanced in cells overexpressing HOXA7. In addition, the E2-induced PR mRNA and protein levels were attenuated after HOXA7 knockdown and were enhanced by the overexpression of HOXA7 (Fig. 4B and C).

4. Discussion

Homeobox genes have been shown to be functionally expressed in the adult organism and to act as transcription factors to regulate aspects of morphogenesis and differentiation. Aberrant spatiotemporal expressions of HOX genes have been found in many disorders of the human reproductive system, such as polycystic ovarian syndrome (PCOS), endometriosis, and granulose cell dysfunction [17,20]. In the current study, we showed that HOXA7 regulated ERα expression, E2-mediated proliferation and PR expression in human ER-positive breast cancer cell line, MCF-7. This finding contributes to the understanding of the proliferative effect of HOXA7 in modulating human breast cancer cell proliferation via the regulation of ERα but not ERβ.

Although the role of estrogen/ER-mediated signaling in human breast cancer progression has been extensively studied [21,22], how the ligand-independent-mediated activation of ER affects the progression of breast cancer remains unclear [6]. Moreover, to date, the transcriptional regulation controlling ERα is not well known [23]. In MCF7 cells, p53 has been shown to increase ERα promoter activity and mRNA levels [24]. Inhibition of STAT5 abolishes the prolactin-induced increase of ERα mRNA levels [25]. In the present study, we found that HOXA7 acted as an upstream regulator of ERα. However, we did not provide evidence that HOXA7 could directly bind to the promoters of ERα. Therefore, it is of interest to study further the direct involvement of HOXA7 in the transcriptional regulation of ERα.

Estrogen is a critical hormone for mammary gland growth and is implicated in the development and progression of breast cancer [26,27]. The regulation of ER is important in cancer development;

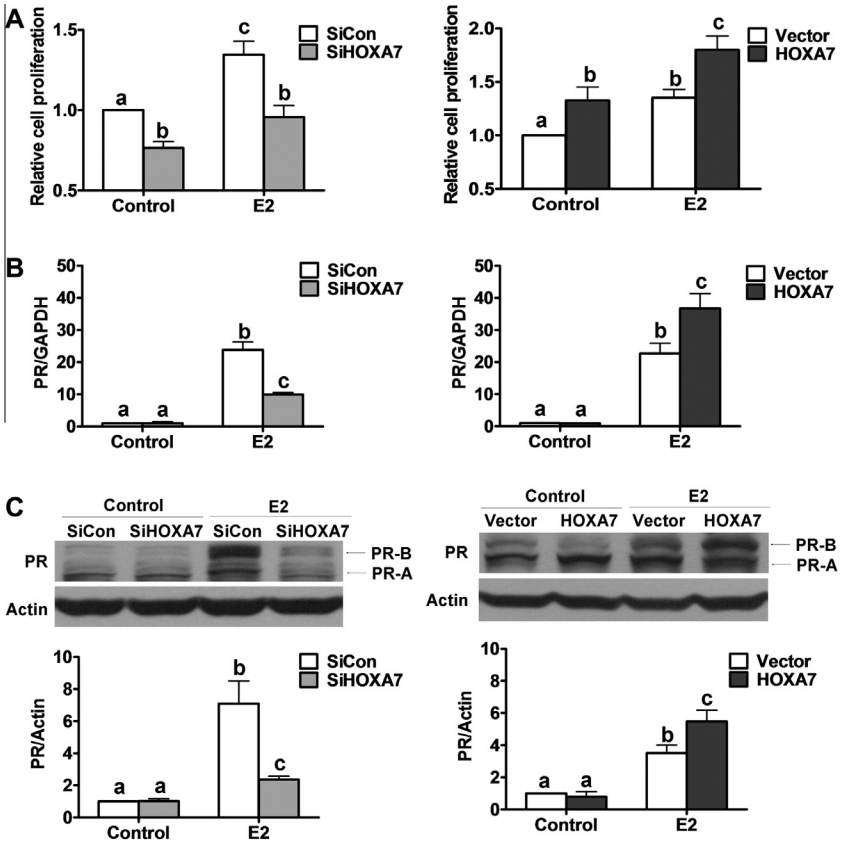

Fig. 4. E2-induced MCF7 cell proliferation and PR expression are affected by the expression levels of HOXA7. Cells were transfected with control siRNA (siCon) or HOXA7 siRNA (SiHOXA7) to knockdown endogenous HOXA7. Cells were transfected with the control vector (Vector) or HOXA7 plasmid (HOXA7) to overexpress HOXA7. (A) After transfection, cells were treated with E2 (10 nM) for 72 h, and cell proliferation was examined by an MTT assay. (B and C) After transfection, cells were treated with E2 (10 nM) for 48 h, and the mRNA (B) and protein (C) levels of PR were examined by RT-qPCR and Western blotting, respectively. The results are expressed as the mean ± SEM of at least three independent experiments. Values without a common letter are significantly different ($p < 0.05$).

however, the ligand-independent regulation of ER remains poorly understood. Previously, it was shown that the homeobox genes, which are transcriptional regulators, are involved in mediating ER activation in ERα-positive breast cancer cells [6,28]. The aberrant expression of HOX genes has been reported in breast cancer. Some members of the HOX gene family are oncogenic and can induce cellular immortalization [29,30]. Recently, it has been reported that HOXA1 stimulates oncogenicity of human breast carcinoma cells and oncogenic transformation through the activation of ERK1/2 signaling [31]. In contrast, other members of the HOX gene family can act as tumor suppressors. In breast cancer, the loss of HOXA5 is accompanied by the loss of p53 expression and is associated with breast cancer development [13]. The down-regulation of HOXA9 is frequently detected in breast cancer, and low levels of HOXA9 are associated with tumor metastasis and patient mortality [32]. HOXA7 hypermethylation and down-regulation of HOXA7 are detected in human breast cancer [33]. However, whether homeobox genes can regulate ER expression in human breast cancer remains unknown. In this study, our results demonstrate a new mechanism for HOX-mediated cancer cell proliferation that may act through the regulation of ERα expression.

Even though HOXA7 regulation of ER expression has not been reported, a few reports have previously demonstrated a potential relationship between steroid hormone receptors and HOX genes. In breast cancer cells, HOXA5 can directly regulate PR expression [34]. It has also been reported that HOX proteins could be required for the ERα-dependent regulation of proliferation-related genes by regulating ERα, but the direct interaction between the HOX protein and ERα may not be required [6]. More recently, it was reported that the estrogen-related receptor α (ERRα) is under the transcriptional control of the homeobox protein prospero-related homeobox1 (Prox1) [35], although the detailed mechanism is still not understood.

In summary, in this study, we demonstrated a novel mechanistic role for HOXA7 in modulating breast cancer cell proliferation via the regulation of ERα but not ERβ. This finding contributes to the understanding of the proliferative effect of HOXA7 in ER-positive cancer cells.

Acknowledgments

We would like to give our thanks to Dr. Qing Huang for technical assistance and instruction. This work was supported by an operating Grant from the Canadian Institutes of Health Research to P.C.K.L. and the National Natural Science Foundation of China (No. 81100468) to H.F.H.

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