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Transcriptional activation of EGFR by HOXB5 and its role in breast cancer cell invasion

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ABSTRACT

HOX genes are transcription factors that play important roles in body patterning and many cellular processes during embryonic, fetal, and adult development. Given their important function in normal tissues, it is reasonable to assume that abnormal expression of HOX genes in adults could lead to serious diseases such as cancer. Our previous study reported HOXB5 to be significantly up-regulated in breast cancer, and its expression was found to be associated with tumor cell proliferation and invasion. Furthermore, the epidermal growth factor receptor (EGFR), a cellular tyrosine kinase that plays an important role in breast cancer progression, was found significantly up-regulated by HOXB5 in ER-positive breast cancer cells. In the present study, we demonstrated that HOXB5 regulates EGFR expression at the transcriptional level by directly binding to its promoter region and promotes phosphorylation of EGFR as well as its downstream effectors. Patients with ER-positive breast cancer, having high co-expression of HOXB5 and EGFR, had poor prognosis than those with low expression. Knockdown studies validated a key role played by EGFR in the HOXB5-induced invasion of breast cancer cells. These results suggest that targeting EGFR could be an effective strategy to treat breast cancer in patients with high HOXB5 expression.

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1. Introduction

HOX genes are highly conserved homeobox genes that encode transcriptional factors required for normal development and differentiation [1,2]. They are not only expressed during embryonic development, but also continue to be expressed in the adult, and regulate various cellular pathways such as cell proliferation, cell adhesion, migration, and apoptosis [3]. Recently, deregulated HOX gene expression was observed in different cancers, including breast cancer, and has been implicated in tumorigenesis, cancer progression, and metastasis [4,5].

HOXB5 is involved in a variety of developmental processes including lung, gut, and neural crest development [6–8]; its expression is also found to be associated with several types of cancer [9–13]. Additionally, our previous studies have demonstrated that HOXB5 contributes to cell proliferation, invasion, and tamoxifen

Abbreviations: HOX, homeobox; EGFR, epidermal growth factor receptor; ChIP, Chromatin immunoprecipitation; DMFS, distant metastasis free survival; MEK, Mitogen-activated protein kinase kinase.

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resistance of breast cancer cells, and that high expression of HOXB5 is prognostic of poor breast cancer survival [14,15]. We have shown earlier that epidermal growth factor receptor (EGFR) gene expression is regulated by either overexpression or knockdown of HOXB5 in ER-positive breast cancer cells [15]. However, the mechanism of HOXB5-mediated regulation of EGFR expression and the relevance of its contribution to the invasive phenotypes of HOXB5-overexpressing cells has not been elucidated yet.

In this study, we explored the role of HOXB5 in the regulation of EGFR expression and signaling activation in HOXB5-overexpressing MCF7 cells. Our results show that EGFR is a direct target of transcriptional regulation by HOXB5 and the resulting HOXB5-mediated EGFR activation plays an important role in enhancing the invasive phenotype of the breast cancer cells.

2. Methods

2.1. Cell lines and cell culture

MCF7 and HEK293T cells were cultured in Dulbecco's modified eagle's medium (DMEM; Welgene Inc., Daegu, Korea) supplemented with 10% of fetal bovine serum (FBS, Welgene) and 1× antibiotic-antimycotic solution (Welgene) at 37 °C in an

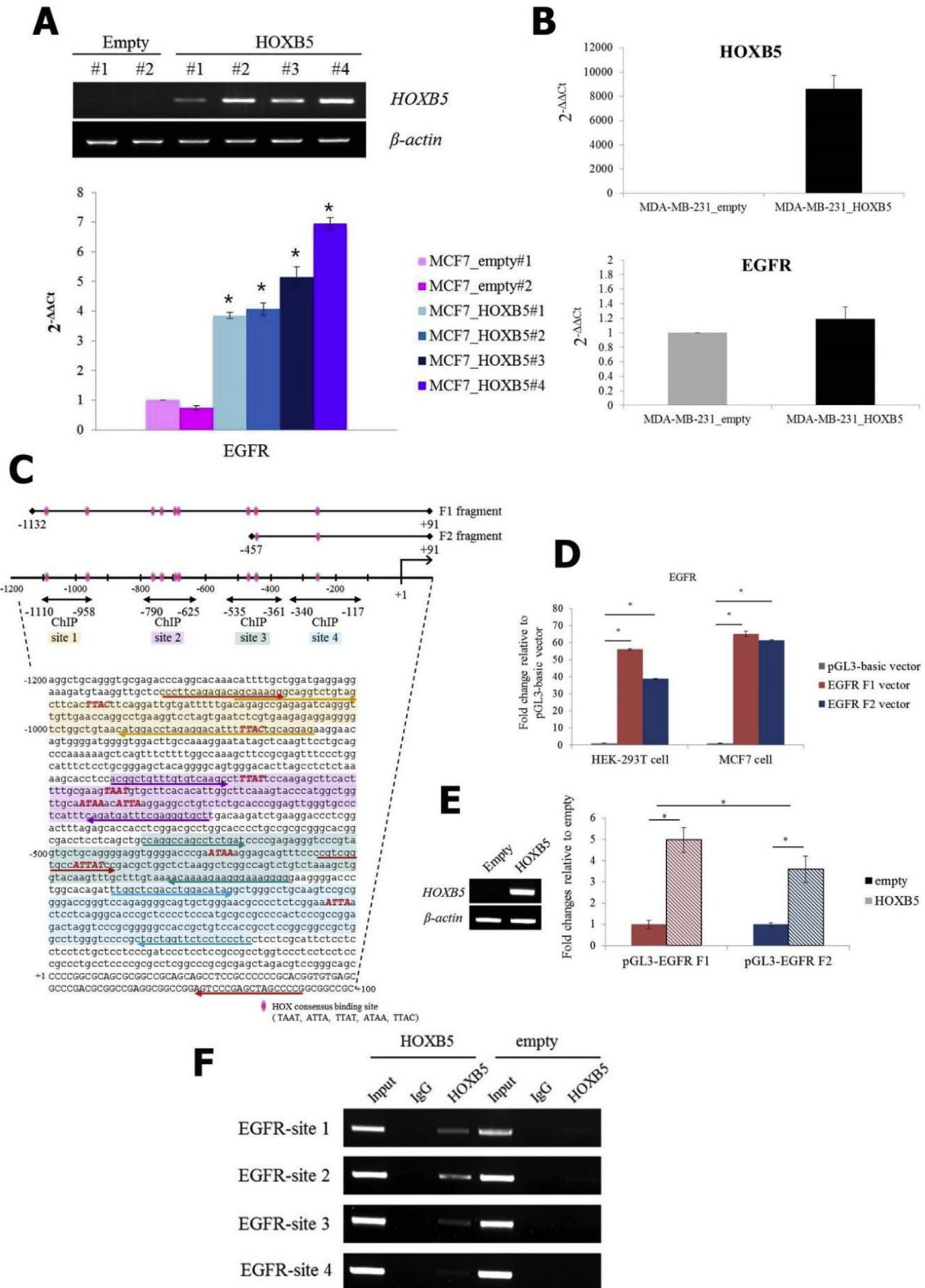


Fig. 1. Transcriptional regulation of EGFR by HOXB5. (A) HOXB5 and EGFR expression levels were examined in MCF7 stable cells expressing HOXB5 (MCF7_HOXB5#1, #2, #3, and #4) and MCF7 control cells harboring empty vector (MCF7_empty#1 and #2). The fold change shown in the graph was quantitated by real-time PCR. The data are represented as mean ± SEM. **p* < 0.001 vs. MCF7_empty#1. (B) The mean value of HOXB5 and EGFR expression level from three independent MDA-MB-231 stable cells expressing HOXB5 (MDA-MB-231_HOXB5#1, #2, and #3). MDA-MB-231 cells harboring empty vector were used as controls. (C) Schematic diagram and sequence analysis of the 1.1 kb upstream sequence of EGFR. Putative HOX core consensus binding elements (TAAT/ATTA/TTAT/ATAA/TTAC) are marked with pink lines and written in red upper case italicized letters in the sequence. The transcription start site (TSS) is written in upper case bold. The primers for cloning F1 and F2 fragments are indicated by red arrows. The promoter regions for the validation of ChIP-PCR (site 1 to 4) are colored orange, purple, green, and blue, respectively. The forward and reverse primers for each site are marked by arrows of same color. (D) Dual luciferase reporter assay for EGFR F1, F2, and control vector in HEK-293T and MCF7 cells. (E) Effect of HOXB5 overexpression on the promoter activity of EGFR F1 and F2 fragments. pGL3-EGFR

atmosphere of 5% CO₂. Generation of HOXB5-overexpressing cell lines was performed as described previously [15].

2.2. RNA isolation and RT-PCR

Total RNA was isolated from the cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was generated using ImProm-II™ Reverse Transcriptase (Promega, Madison, WI, USA). PCR was performed in replicates using Taq polymerase (Bioneer, Daejeon, Korea). Quantitative PCR was performed using SYBR green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) on an ABI7300 real-time PCR system (Applied Biosystems). The PCR primers used in this study are listed in Table S1.

2.3. Dual luciferase assay

Dual luciferase assay was performed as described previously [16]. Genomic DNA fragments of the EGFR promoter region (EGFR F1 and EGFR F2; Fig. 1) were amplified using Pfu polymerase (Sol-Gent, Daejeon, Korea) and cloned into the pGL3-Basic vector (Promega) using *KpnI* and *SmaI* sites. Restriction enzyme sites were incorporated into the primers (Table S1). Control pGL3-Basic vector or the pGL3-EGFR constructs were transfected into HEK293T or MCF7 cells with the Renilla luciferase vector (pRL Renilla luciferase control) using Attractene transfection reagent (Qiagen). Promoter activity was measured using the Dual-Luciferase Reporter Assay System (Promega) after 48 h of transfection and normalized using Renilla luciferase activity. Effect of HOXB5 on the promoter activity was measured in MCF7 cells after the co-transfection of Renilla luciferase vector and pGL3-EGFR vector with either control pcDNA3 or pcDNA3-HOXB5. Assays were conducted in triplicate for each experiment. Similar results were obtained from three independent experiments.

2.4. Chromatin immunoprecipitation (ChIP) analysis

ChIP was performed with chromatin prepared from MCF7 cells. The cells were fixed in 37% formaldehyde for 15 min, and then quenched with glycine to a final concentration of 0.125 M for 10 min. Sonication was performed using Sonics Vibra-Cell™ to obtain approximately 500–1000 bp DNA fragments. Antibodies against HOXB5 (sc-81099; Santa Cruz) were used along with IgG (sc-2025; Santa Cruz). DNA, purified from immunoprecipitated chromatin and from direct input, was used for PCR. The primer sequences for ChIP-PCR are shown in Table S1.

2.5. Western blotting

Western blot analysis was performed as previously described [15]. Antibodies against EGFR (#4267; Cell Signaling Technology, Inc. MA, USA), phosphor-EGFR (Tyr1068; #3777; Cell Signaling), Src (#2110; Cell Signaling), phosphor-Src (Tyr416; #6943; Cell Signaling), MEK1/2 (#4694; Cell Signaling), phosphor-MEK1/2 (Ser217-221; #9154; Cell Signaling), ERK1/2 (#9102; Cell Signaling), phosphor-ERK1/2 (T202/T204; #9101, Cell Signaling), Akt1 (#2967; Cell Signaling), phosphor-Akt (Ser473; #4058; Cell Signaling), phosphor-Akt (Thr308; #9275; Cell Signaling), and β -actin (ab6276; Abcam, Cambridge, UK) were used to detect each

protein.

2.6. Effect of EGFR knockdown on tumor cell viability and invasion

Cell viability assay was performed by using the Cell Counting Kit-8 (CCK-8) following the manufacturer's instruction (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Invasion assay was performed using Matrigel™ (BD) as described previously [15], in HOXB5-overexpressing and control MCF7 cells. For knockdown study, the cells were transfected with 50 nM EGFR siRNA (#L-003114-00-0005; Dharmacon, Waltham, MA, USA) or non-specific siRNA (#D-001810-10-05; Dharmacon) using G-Fectin reagent (Genolution, Seoul, Korea).

2.7. Statistical analysis

For all experiments, a minimum of three independent biological replicates were analyzed for quantitation. Data are expressed as the mean value with standard error. Statistical differences were determined by Student's *t*-test. A *p*-value of <0.05 was considered statistically significant. The online Kaplan-Meier plotter (<http://kmplot.com>) was used to analyze the clinical significance of HOXB5 and EGFR expression in patients with breast cancer.

3. Results

3.1. Transcriptional regulation of EGFR by HOXB5 in MCF7 breast cancer cells

Our previous studies showed that EGFR is one of genes regulated by HOXB5 in breast cancer cells [15]. To investigate the molecular mechanism underlying HOXB5-mediated EGFR regulation, we first monitored mRNA expression level of EGFR in four independent clones of MCF7 cell lines stably expressing HOXB5. Compared to the levels in two MCF7 cell lines harboring empty vector, EGFR expression in HOXB5-overexpressing MCF7 clones was significantly high (Fig. 1A). In contrast, overexpression of HOXB5 in ER-negative MDA-MB-231 cells had no effect on EGFR expression (Fig. 1B). We then examined the effect of HOXB5 on the promoter activity of EGFR by performing luciferase reporter assay. Two different-sized genomic fragments, EGFR-F1 spanning –1132 to +91 bp and EGFR-F2 spanning –457 to +91 bp, contained a series of putative HOX-binding sites and had strong promoter activity both in HEK-293T and MCF7 cells (Fig. 1C and D). The ability of the larger fragment, EGFR-F1, to drive expression of the reporter was significantly greater than that of the shorter fragment, EGFR-F2 (Fig. 1D), indicating that the performance increased proportionally with the number of HOX-binding sites. Importantly, the transcriptional activity of the reporter, driven by EGFR-F1 or EGFR-F2, was clearly induced by exogenous HOXB5 expression (Fig. 1E). The direct binding of HOXB5 to the promoter region of EGFR was further confirmed by ChIP analysis using MCF7 cells (stably overexpressing HOXB5) and control MCF7 cells (harboring an empty vector). HOXB5 binding was observed in sites 1 to 4, with site 2 showing the strongest binding and site 4 the weakest, probably depending on the number of HOX-binding sites (Fig. 1F). This data demonstrates that binding of HOXB5 to the EGFR promoter correlates with the ability of HOXB5 to regulate EGFR gene expression.

promoter constructs (pGL3-EGFR-F1 and F2) were co-transfected with effector plasmid containing HOXB5 or empty vector. HOXB5 mRNA expression was confirmed by RT-PCR. Luciferase activity was measured after 48 h of transfection and normalized to that of Renilla luciferase, used as an internal control. The data are represented as mean \pm SEM. **p* < 0.001 vs. empty control. (F) ChIP-PCR results show HOXB5 binding to EGFR upstream regions. Chromatin, isolated from MCF7 cells overexpressing HOXB5 or empty vector, was precipitated with HOXB5 antibody or control IgG. ChIP-ed DNA was amplified with specific primer sets shown in (C). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Activation of EGFR downstream signaling pathway in HOXB5-overexpressing cells

EGFR expression is known to be associated with aggressive phenotypes of breast cancer through the activation of downstream signaling pathways that regulate various cellular processes, such as cell proliferation, differentiation, and adhesion [17]. Here, we examined HOXB5-mediated activation of EGFR and its effect on downstream signaling pathways using two clones of MCF7 cells, stably expressing HOXB5, and two clones of control cells (Fig. 2). Consistent with the findings at the mRNA level, the total protein and phosphorylation levels of EGFR were found to be up-regulated in HOXB5-overexpressing cells. Activated EGFR enhanced Src and MEK/ERK signaling, but did not affect Akt phosphorylation. Our data imply that HOXB5 overexpression induces EGFR activation and potentially affects the downstream Ras/Raf/MEK/ERK pathway rather than the Ras/PI3K/PTEN/Akt pathway.

3.3. Contribution of EGFR to cancer cell invasion in HOXB5-overexpressing cells

EGFR is known to promote migration and invasion of cancer cells [17–19]. Since HOXB5 was also shown to have a role in invasion [15], we confirmed and extended these findings to examine the contribution of EGFR to the invasion of HOXB5-overexpressing breast cancer cells. The mRNA expression of HOXB5 and EGFR were much higher in the MCF7 cells stably overexpressing HOXB5, compared to that in the control cells, and the transfection of EGFR siRNA in both cells specifically suppressed EGFR expression (Fig. 3A). Invasion assays showed that the numbers of invaded cells

increased significantly in HOXB5-overexpressing MCF7 cells compared to that in the control cells harboring empty vector (Fig. 3B). In the EGFR-siRNA knockdown cells, the numbers of cells invaded was significantly decreased, even below the basal levels seen in vector control cells (Fig. 3B). Unlike the effect on invasion, EGFR silencing in HOXB5 overexpressing cells did not affect cell viability (Fig. 3C). These results suggest that the invasive properties seen in HOXB5-overexpressing cells were largely dependent on EGFR up-regulation and its downstream signaling activities.

3.4. Clinical significance of HOXB5 and EGFR in breast cancer

We had previously reported that high expression of HOXB5 is linked to poor prognosis in breast cancer [15]. In this study, the Kaplan-Meier method was employed to examine the prognostic value of combined HOXB5/EGFR expression. Importantly, we found that HOXB5 and EGFR are more likely to be associated with poor clinical outcome in ER-positive breast cancers when both genes are overexpressed simultaneously, as compared to when HOXB5 or EGFR is overexpressed alone (Fig. 4A–B). In the ER-negative patient group, co-expression of HOXB5 and EGFR appear to have a poorer prognosis, but the number of patients was relatively small and did not show a significant difference (Fig. 4C–D). In addition, based on our results, it is likely that EGFR overexpression in ER-negative breast cancer is independent of HOXB5. Collectively, these results indicate that HOXB5-induced up-regulation of EGFR in ER-positive breast cancer may contribute to accelerate tumor progression, due to which high expression of both HOXB5 and EGFR has a negative synergistic effect on patient outcome.

4. Discussion

In this study, we have demonstrated that HOXB5, in breast cancer cells, regulates EGFR at the transcriptional level by binding to the EGFR promoter region. Activation of EGFR then leads to the propagation of downstream signal that promotes cancer development. EGFR is the key player that mediates the effect of HOXB5 on cancer cell invasion.

In recent years, deregulated HOX gene expression has been observed in different human tumors [4,5]. Several groups, including our own, have identified many HOX genes with altered expression, especially in breast cancer [14,20–22]. HOXB5 is one of the genes up-regulated in breast cancer and associated with its progression [14,15]. Similar to HOXB5, HOXB7 overexpression has also been reported to promote breast tumorigenesis and render MCF7 cells resistant to tamoxifen through the activation of EGFR pathway [23]. These observations suggest functional redundancy among the HOX genes.

EGFR overexpression in breast cancer is known to be associated with large tumor size, poor differentiation, and poor clinical outcome, and is, therefore, one of the first identified targets of antitumor agents [17,24,25]. However, it remains difficult to define the group of patients who might benefit from a specific drug. Therefore, development of specific biomarkers for categorizing patients according to their molecular characterization is absolutely necessary to proceed towards personalized medicine. According to our studies, overexpression of HOXB5 and the resulting EGFR up-regulation appears to play an important role in ER-positive breast cancer rather than ER-negative breast cancer. Our previous study showing HOX gene expression pattern in different breast cancer cell lines indicated that HOXB5 is predominantly expressed in ER-positive breast cancer cell lines [14]. As shown in this study, the expression of EGFR was regulated by HOXB5 overexpression or silencing in ER-positive cells such as MCF7 and T47D [15], but not in ER-negative MDA-MB-231 cells. The prognosis of HOXB5 and EGFR

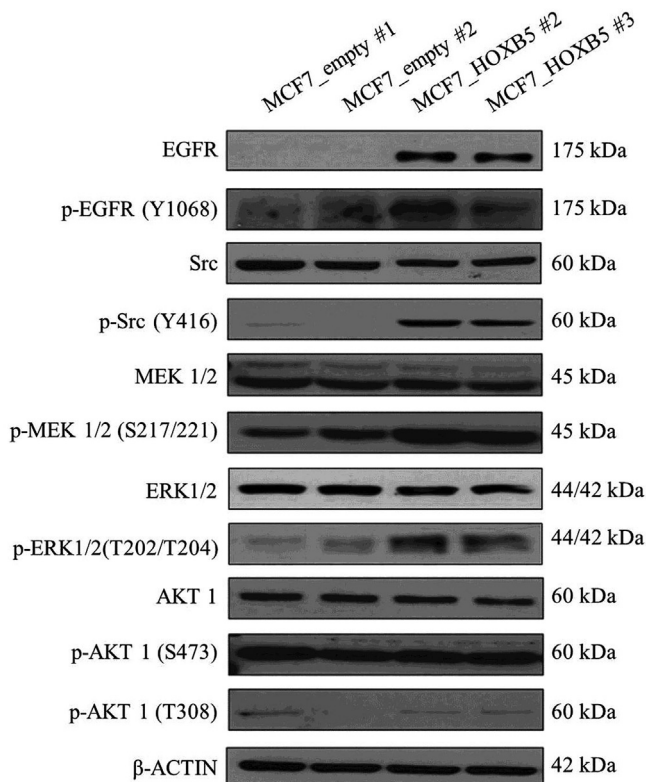


Fig. 2. Western blot analyses of EGFR and downstream signaling pathway activation. Protein extracts, isolated from MCF7 cells overexpressing HOXB5 (MCF7_HOXB5#2 and MCF7_HOXB5#3) and control MCF7 cells harboring empty vector (MCF7_empty#1 and MCF7_empty#2), were used for the assay. Antibodies used were EGFR, p-EGFR, Src, p-Src, MEK1/2, p-MEK1/2, ERK, p-ERK1/2, AKT1, p-AKT1, and beta-actin.

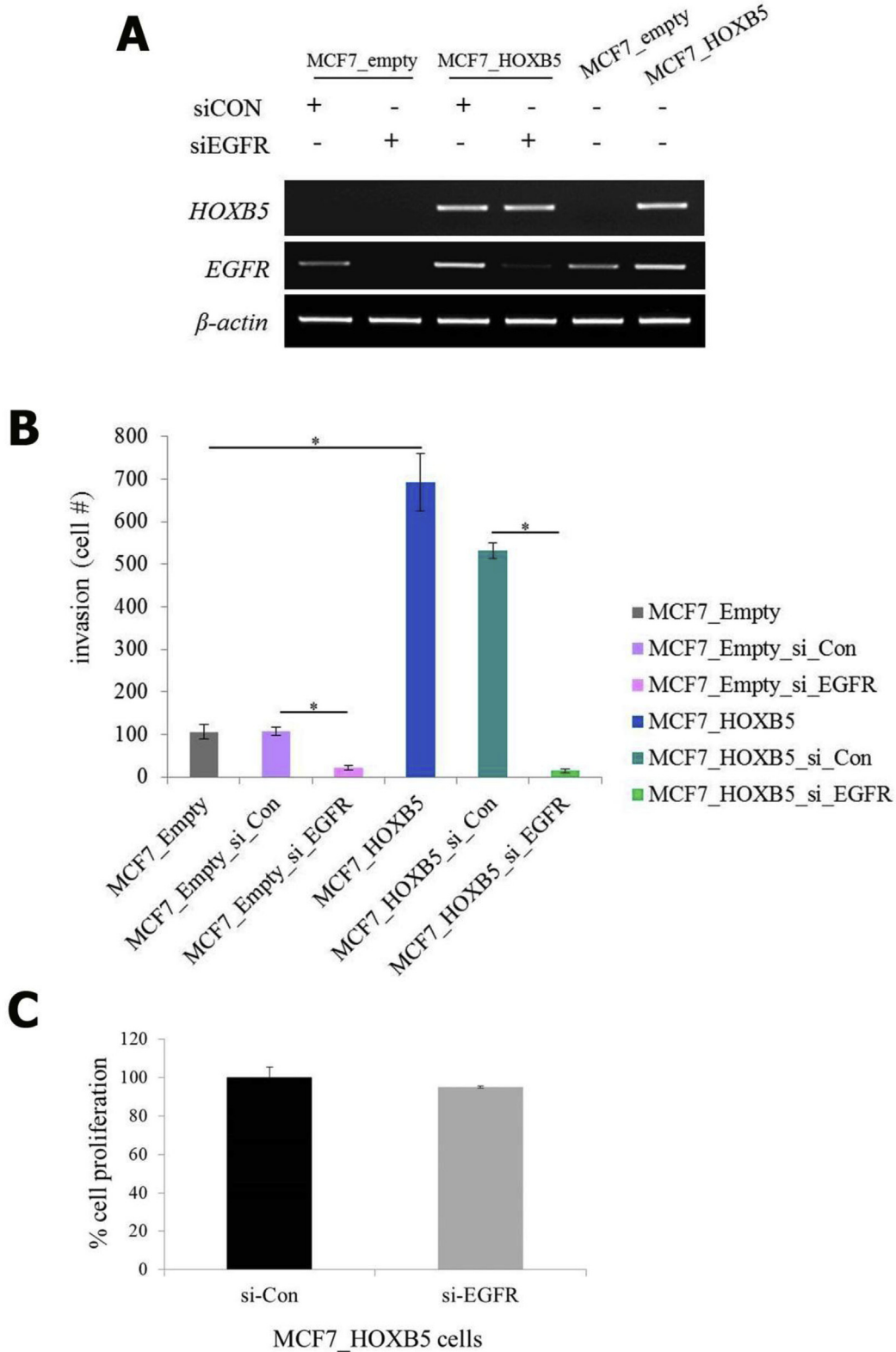


Fig. 3. Contribution of EGFR to the cell invasion and viability of HOXB5-overexpressing breast cancer cells. HOXB5-overexpressing and control MCF7 cells were transfected with either siEGFR or nonspecific siCon and were used for RT-PCR and invasion assay. (A) HOXB5 and EGFR mRNA expression, with or without siRNA knockdown of EGFR; beta-actin used as control. (B) Matrigel invasion assay of MCF7 cells expressing HOXB5 or empty vector, with or without knockdown of EGFR. The number of invading cells was counted after staining with DAPI. (C) Cell viability of HOXB5-overexpressing MCF7 cells, with or without knockdown of EGFR.

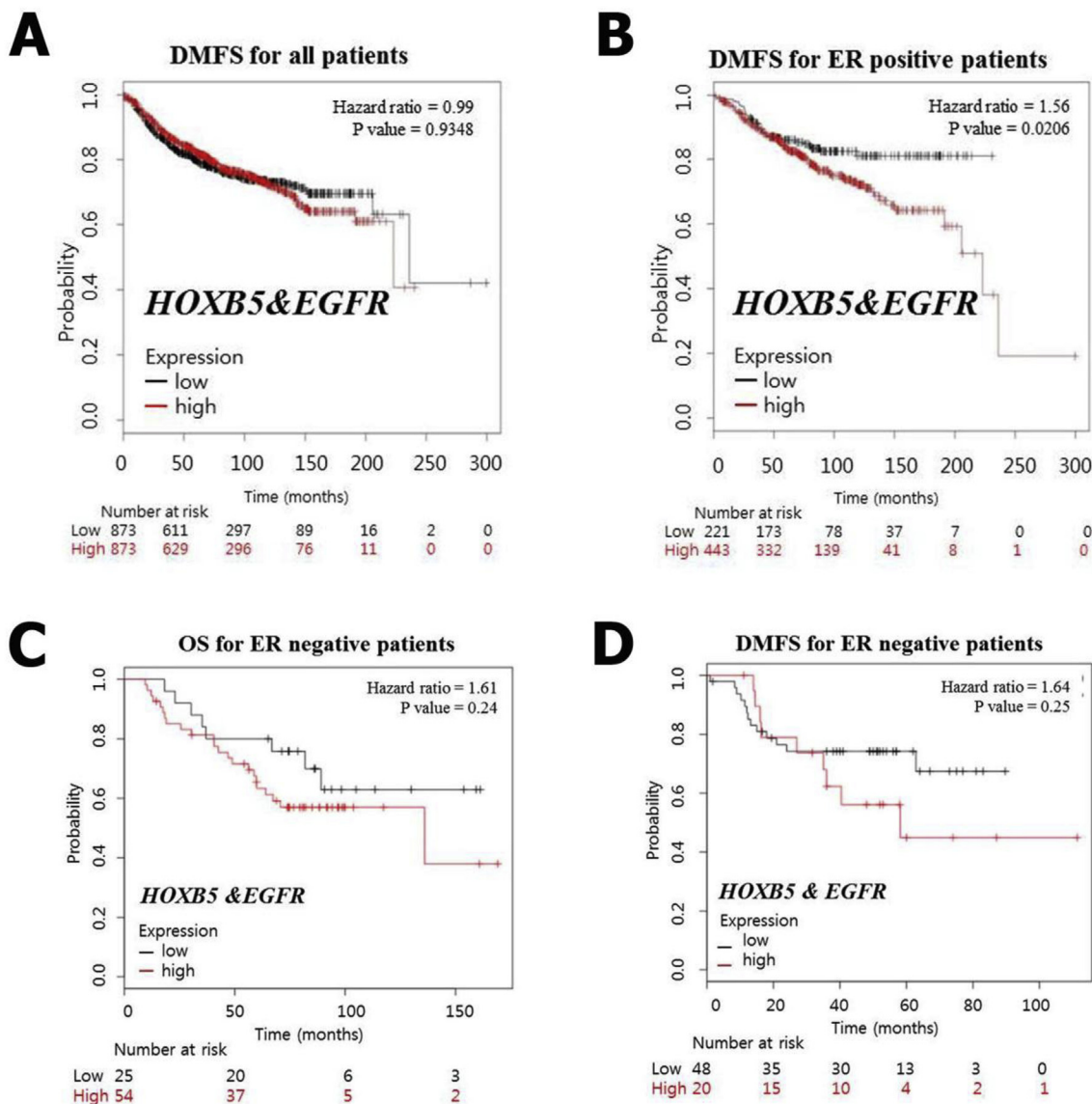


Fig. 4. The prognostic significance of HOXB5 and EGFR expression in breast cancer. (A–B) Kaplan–Meier analysis of distant metastasis-free survival (DMFS) in patients with breast cancer. The red and black lines indicate high and low expression of HOXB5/EGFR in all patients with breast cancer (A) and with ER-positive tumors (B). (C–D) Overall survival (C) and distant metastasis-free survival (D) in ER-negative breast cancers. The red and black lines indicate high and low expression of HOXB5 and EGFR. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

was also significantly worse in ER-positive patients than ER-negative patients, although the impact of the two genes in the ER-negative patient population as a prognostic factor can be concluded with the accumulation of more extensive data. Taken together, our results show a significant role of EGFR in HOXB5-mediated biological pathways in breast cancer cells, thereby suggesting that EGFR-targeted anti-tumor agents may be valuable to ER-positive breast cancer patients with high co-expression of HOXB5 and EGFR.

In conclusion, our data enhance the understanding of the molecular mechanisms underlying HOXB5-mediated pathways in breast cancer, and also provide an evidence for the causal relationship between HOXB5 and EGFR in cancer cell invasion.

Author contributions

J.-Y.L and M.H.K designed and managed the project. J.M.K and

D.S.J performed experiments and analyzed the data. J.-Y.L, J.M.K and M.H.K wrote the paper. All authors discussed the results and commented on the manuscript.

Conflicts of interest statement

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

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.bbrc.2018.08.071>.

Transparency document

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