ANTICANCER RESEARCH *41*: 3409-3417 (2021)
doi:10.21873/anticanres.15128

HOXB5 Confers Tamoxifen Resistance in Breast Cancer Cells and Promotes Tumor Aggression and Progression

CLARA YURI KIM, YU CHEON KIM, JI HOON OH and MYOUNG HEE KIM

Department of Anatomy, Embryology Laboratory, Brain Korea 21 project for Medical Science, Yonsei University College of Medicine, Seoul, Republic of Korea

Abstract. *Background/Aim: ER-positive breast cancer patients commonly undergo endocrine therapy with drugs such as tamoxifen. Despite tamoxifen being a highly effective drug, long-term treatment results in resistance in one-third of the patients. Although many explanations for the development of tamoxifen resistance have been put forward, a clearly defined underlying mechanism is still lacking. Materials and Methods: The expression level of HOXB5 was evaluated between MCF7 breast cancer cells and tamoxifen-resistant MCF7 (TAMR) cells by RT-PCR. Then, the effect of HOXB5 on invasion and migration abilities as well as on cancer stemness were investigated through 3D culture and spheroid formation assay. Results: In this study, we provide evidence that HOXB5 is up-regulated in TAMR cells. EGFR is concurrently overexpressed, and the EGFR signaling cascade is activated, resulting in migratory and invasive phenotypes in TAMR cells compared to MCF7 cells. However, HOXB5 knockdown in TAMR cells resulted in the de-activation of the EGFR signaling pathway, less aggressive phenotypes and restoration of sensitivity to tamoxifen treatment. More interestingly, TAMR cells expressed higher levels of stem cell markers, and as a result, their enhanced stemness allowed for a better formation of spheroids than MCF7 cells. When HOXB5 was overexpressed in MCF7 cells, they were able to form a larger number of spheroids as in TAMR cells. Conclusion: HOXB5 is one of the key factors involved in tumor aggression and progression in tamoxifen-resistant breast cancer cells.*

Correspondence to: Myoung Hee Kim, Department of Anatomy, Yonsei University College of Medicine, 50-1, Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea. Tel: +82 222281647, Fax: +82 23650700, e-mail: mhkim1@yuhs.ac and Ji Hoon Oh, Department of Anatomy, Yonsei University College of Medicine, 50-1, Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea. Tel: +82 222281657, Fax: +82 23650700, e-mail: rednovember@yuhs.ac

Key Words: Breast cancer, endocrine-resistance, HOXB5, EGFR, cancer stem cell.

The homeobox (*HOX*) genes are an evolutionarily conserved family of 39 genes organized into 4 different clusters on different chromosomal loci. *HOX* genes play key roles during embryonic development and regulate various cellular and physiological processes (1). Lately, the roles of numerous *HOX* genes in several types of cancer have also been investigated (2).

The HOXB gene family is involved in the progression of various cancers such as breast, gastric, lung and pancreatic cancer (3-7). We have previously reported that HOXB5 promotes cell proliferation and invasion when overexpressed in MCF7 cells (3). Conversely, the knockdown of HOXB5 and its neighboring genes (HOXB4 and HOXB6) in tamoxifen-resistant MCF7 (TAMR) cells resulted in sensitivity to tamoxifen (8). To further elucidate the underlying mechanisms and role of HOXB5, which was highly overexpressed in TAMR cells, it is crucial to identify the molecular pathways it is involved in.

Epidermal growth factor receptor (EGFR) and its associated signaling pathway have received much attention in relation to cancer. Numerous works have demonstrated that EGFR contributes to tumorigenesis by modulating diverse cellular processes such as cell growth, cell cycle, differentiation, invasion, migration and survival of cancer cells (9-11). Moreover, the dysregulation of EGFR and the associated signaling pathway has been described in the acquired resistance to drugs in breast cancer (12, 13).

Here, we investigated the function of up-regulated HOXB5 in TAMR cells. In the presence of HOXB5, EGFR expression as well as the signaling cascade was stimulated. As a result, TAMR cells were migratory, invasive and highly proliferative even with tamoxifen treatment. Nevertheless, when HOXB5 was depleted from TAMR cells, migration and invasion abilities, as well as cell viability were markedly reduced. Moreover, we reveal that HOXB5 is associated with stemness and contributes to the establishment of the more aggressive cancer stem cells from TAMR cells.

Materials and Methods

Cell lines and culture. MCF7 and MCF7-TAMR cells were used. Both cells were cultured in Dulbecco's modified Eagle's medium (WelGENE Inc., Daegu, Republic of Korea). The medium was

Table I. *List of primers used for PCR and ChIP-PCR.*

Primers	Forward primer (5'-3')	Reverse primer (5'-3')
h-HOXB1	CTTATGGGAACGAGCAGACC	CTGACACCTTCGCTGTCTTG
h-HOXB2	TTCACCAGTACGCTCTGTGC	AAAGATAACCGAGTGCCCAAT
h-HOXB3	CAGAAGTCCAGCATTGCTCA	CATGACAGGAAACACAATGTCC
h-HOXB4	CATTCACTGAGGGCCAGAAT	CCAGCTCCCAGAACTCAACT
h-HOXB5	CCAATTTCACCGAAATAGACG	CGGTCATATCATGGCTGATG
h-HOXB6	TCTACCGCGAGAAAGAGTCG	GGAGGAACTGTTGCACGAAT
h-HOXB7	CGAGTTCCTTCAACATGCAC	GTTTGCGGTCAGTTCCTGAG
h-HOXB8	CGTGGATCTCCTTCCCTTCT	GAATTACGGCGTGAATAGGC
h-HOXB9	GGGAGCTGCTCAAACAGG	GGAGGGGTTGGTTTGATCC
h-HOXB13	GGAAAAGGCCAAAGAGTGTG	GGAAGGCAGAAAGTGACCTG
h-OCT4	CTGATCTGCTGCAGTGTGG	CCTTCCCACCTGCACAGAT
h-NANOG	CCTTCCTCCATGGATCTGCT	TGAGGTTCAGGATGTTGGAGAG
h-SOX2	ACATGAACGGCTGGAGCA	TGCTGCGAGTAGGACATGC
β-Actin	CATGTTTGAGACCTTCAACACCCC	GCCATCTCCTGCTCGAAGTCTAG

supplemented with heat-inactivated 10% FBS (WelGENE Inc.) and penicillin-streptomycin (WelGENE Inc.). Cells were maintained at 37˚C in a 5% CO_2 incubator. To establish an *in vitro* model for acquired tamoxifen-resistance (MCF7-TAMR), MCF7 cells were cultured under the same conditions with long-term exposure to 1 μM of 4-hydroxytamoxifen (Sigma, St. Louis, MO, USA). Stable HOXB5 knockdown and overexpressing cells were established as previously described (4). Briefly, a set of pLKO.1 lentiviral vectors containing seven different shRNA targeting HOXB5 purchased from Thermo Fisher Scientific (Rockford, IL, USA) was used for transduction in TAMR cells. Stable cell lines were obtained using puromycin selection at a concentration of 0.5 μg/ml and the protein levels were confirmed using Western blot. For HOXB5 overexpression studies, a full-length cDNA of the HOXB5 gene was cloned into the EcoRI-XbaI site of the pcDNA3-HA-tagged expression vector. To establish stable cell lines, G418 was added for 2~3 weeks at a concentration of 300 μg/ml.

Total RNA isolation and RT-qPCR. Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized with 1 μg of total RNA using ImProm-II™ Reverse Transcriptase (Promega, WI, USA). PCR amplification was performed under the following conditions: initial denaturation for 5 min at 94˚C, followed by 27-35 cycles of 94˚C for 40 s (depending on target gene), 58˚C for 20 s and 72˚C for 30 s. For quantitative PCR, a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and Power SYBR Green PR Master Mix (Applied Biosystems) were used. All PCR reactions were performed in triplicate, and β-Actin and GAPDH were used as internal controls. PCR primers are listed in Table I.

siRNA and transfection. Knockdown studies were performed by transfecting MCF7-TAMR cells for 48 h with 20 nM siHOXB5 using G-fectin (Genolution, Seoul, Republic of Korea) following the manufacturer's protocol. The SMARTpool ON-TARGETplus HOXB5 siRNA (Cat. L-017532-02-0005) was purchased from GE Dharmacon (Lafayette, CO, USA).

Cell proliferation assay. Relative cell proliferation was measured using the Cell Counting Kit-8 (Dojindo Molecular Technologies Inc., Kumamoto, Japan) following the manufacturer's protocol. Briefly, 7.5×10^3 cells/well were plated and grown on 96-well plates, stained with 10 μl of WST-8, and incubated for 3 hours at 37˚C in a 5% CO_2 incubator for three consecutive days. The plate was then analyzed using a Softmax Pro microplate reader (Molecular Devices, San Jose, CA, USA) at an absorbance of 450 nm.

Western blot analysis. Western blot analysis was performed as previously described with minor modifications (3). Each cell line was treated under the appropriate conditions and lysed in RIPA buffer, after which their protein contents were determined using Pierce BCA Protein Assay Kit (Thermo Scientific). Each sample was loaded onto 8% SDS polyacrylamide gels, and then electrotransferred to PVDF transfer membranes (Bio-Rad, Hercules, CA, USA). Immunoreactive bands were detected using suitable primary antibodies. Anti-EGFR (#4267; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phospho-EGFR (Tyr1068; #3777; Cell Signaling), anti-SRC (#2110; Cell Signaling), anti-phospho-SRC (Tyr416; #6943; Cell Signaling), anti-MEK1/2 (#4694; Cell Signaling), anti-phospho-MEK1/2 (Ser217-221; #9154; Cell Signaling), anti-ERK1/2 (#9102; Cell Signaling), anti-phospho-ERK1/2 (T202/T204; #9101, Cell Signaling) and anti-β-Actin (ab6276; Abcam, Cambridge, UK) antibodies were used to detect each protein.

Three-dimensional (3D) in vitro cell culture. 3D culture was performed as previously described (14). Briefly, an eight-well chamber slide was pre-coated with 70 μl of Matrigel™ (BD Biosciences, San Jose, CA, USA) and incubated at 37˚C for at least 1 h for solidification. Afterwards, 5×10^3 cells diluted in 2% Matrigel-containing media were plated onto the eight-well chamber slide. Culture media was changed every 3 days, supplemented with 2% Matrigel. On the 14th day of culture, cells were stained with anti-phalloidin (Invitrogen) and fluorochrome 4', 6-diamidino-2-phenylindole (DAPI). Images were acquired using a Zeiss LSM 700 confocal microscope and were analyzed using Zen 2011 software (Carl Zeiss, Oberkochen, Germany). 3D images were created from z-stack scans accumulated by incremental stepping through the 3D sample using a focal drive.

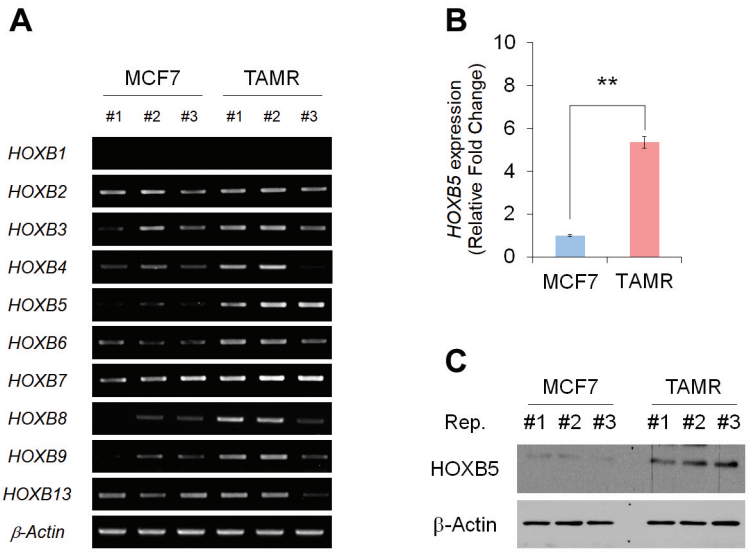

Figure 1. *HOXB5 is up-regulated in tamoxifen-resistant MCF7 cells. (A) The middle HOXB cluster is up-regulated in TAMR cells. (B) HOXB5 mRNA expression levels in MCF7 and TAMR cells from three different passages were determined by RT-qPCR. (C) HOXB5 protein expression levels in MCF7 and TAMR cells from three different passages were determined by western blot. β-Actin was used as an internal control. All experiments were performed in triplicate.*

Spheroid formation assay. Spheroid culture was performed as previously described with minor improvements (15). 1×10^4 cells/ml were counted and re-suspended carefully using a 25G syringe needle to obtain a single-cell suspension. The cells were pelleted, washed with cold PBS, and syringe-filtered again to ensure a single-cell suspension. Cells were plated onto Ultra-Low attachment 6-well plates (Corning, Corning, NY, USA) with 2 ml DMEM/F12 media (WelGENE Inc.) supplemented with 1% PSA, 2% B27, 10 ng/ml FGFb, 20 ng/ml EGF, 5 μg/ml insulin and 4 μg/ml heparin. Cells were maintained at 37˚C in a 5% CO_2 incubator. The number of spheroids with a diameter greater than 50 μm was regularly counted, and when there were approximately 100 spheroids, they were collected by gentle centrifugation, dissociated, and then passaged for the assessment of self-renewal.

Statistical analysis. Data are expressed as the mean±SD. Statistical differences were determined using Student's *t*-test for pairwise comparisons. *p*-Values of <0.05 were considered significant.

Results

HOXB5 is up-regulated in tamoxifen-resistant MCF7 cells

To investigate the underlying mechanism regarding HOXB5 up-regulation in tamoxifen resistance, we first conducted RT-qPCR for the entire *HOXB* genes with ER+ breast cancer cells (MCF7) and tamoxifen-resistant breast cancer cells (TAMR) from three different passages. The mRNA expression of *HOXB4*, *HOXB5*, *HOXB6*, *HOXB8* and *HOXB9* were noticeably up-regulated in TAMR cells (Figure 1A and 1B). *HOX* genes are known to function as clusters, and may have coinciding cellular functions, which still needs to be elucidated. However, since the expression of *HOXB5* was the most up-regulated, and based on our preceding findings, we focused on *HOXB5* for our study. Moreover, by performing western blot, the overexpression of HOXB5 protein level in TAMR cells was further confirmed (Figure 1C).

HOXB5 enhances migratory and invasive abilities in TAMR cells. To explore any aggressive phenotypes associated with HOXB5 in TAMR cells, we examined migratory and invasive abilities by performing three-dimensional (3D) *in vitro* cell culture using matrigel, which closely mimics the *in vivo* microenvironment. The 3D *in vitro* culture demonstrated that TAMR cells were more able to create cancer cell aggregates and spread out across the matrigel, with a typical "starburst" pattern, displaying highly migratory and invasive characteristics compared to MCF7 cells (Figure 2). Next, to prove that these phenotypes are due to the overexpressed HOXB5, we performed 3D culture using MCF7: HOXB5 overexpressing cells and TAMR with shRNA down-regulated HOXB5 cells along with corresponding MCF7 vector control and TAMR shRNA control cells. MCF7 cells overexpressing HOXB5 showed widespread aggregates, similar to that of wildtype TAMR cells. On the contrary, TAMR cells with stably knocked down HOXB5 formed very tight and spherical 3D structures embedded in the matrigel (Figure 3). As a result, HOXB5 seems to be a crucial factor in modulating migratory and invasive characteristics of breast cancer cells.

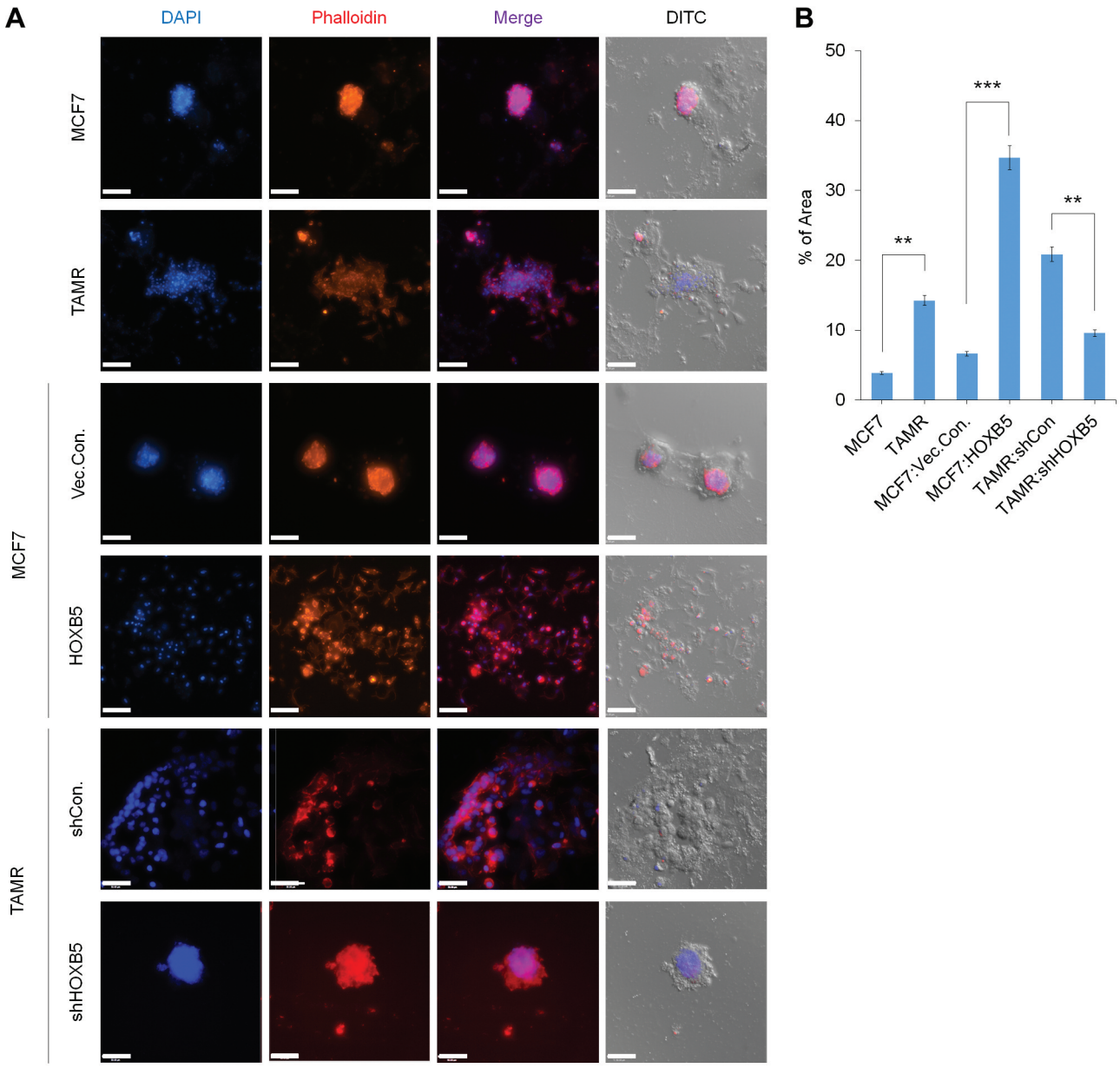

Figure 2. *HOXB5 enhances migratory and invasive characteristics. (A) Confocal microscopy images of MCF7, TAMR, MCF7: vector control, MCF7: HOXB5, TAMR shRNA control, TAMR shHOXB5 cells in 3D culture system at day 14. Cells were stained with DAPI (blue) and phalloidin (red). DAPI was used to stain the DNA in the nucleus, and phalloidin was used to stain the F-actin filaments to visualize cell shape. Differential interference contrast (DITC) images are shown merged with DAPI and phalloidin staining to show the entire 3D colony morphology. The migratory and invasive features were observed in the cross-section images of cells. The scale bar in each panel represents 50 μm. (B) Quantification of the phalloidin signal of the ICC assay.*

HOXB5 increases spheroid formation in breast cancer cells. More aggressive cancer cells usually display more mesenchymal and stem cell-like characteristics, and in fact, have a larger population of cancer stem cells (CSCs) with abilities to self-renew and differentiate (16-18). Additionally, drug-resistant tumors are enriched with CSCs, and this subset of population aids survival and promotes cancer growth despite drug treatment by establishing higher invasiveness. To determine if TAMR cells display more stem-like properties compared to MCF7 cells, we screened for any differential gene expression in stem cell markers (*OCT4*, *NANOG*, and *SOX2*) by RT-PCR analysis. All three

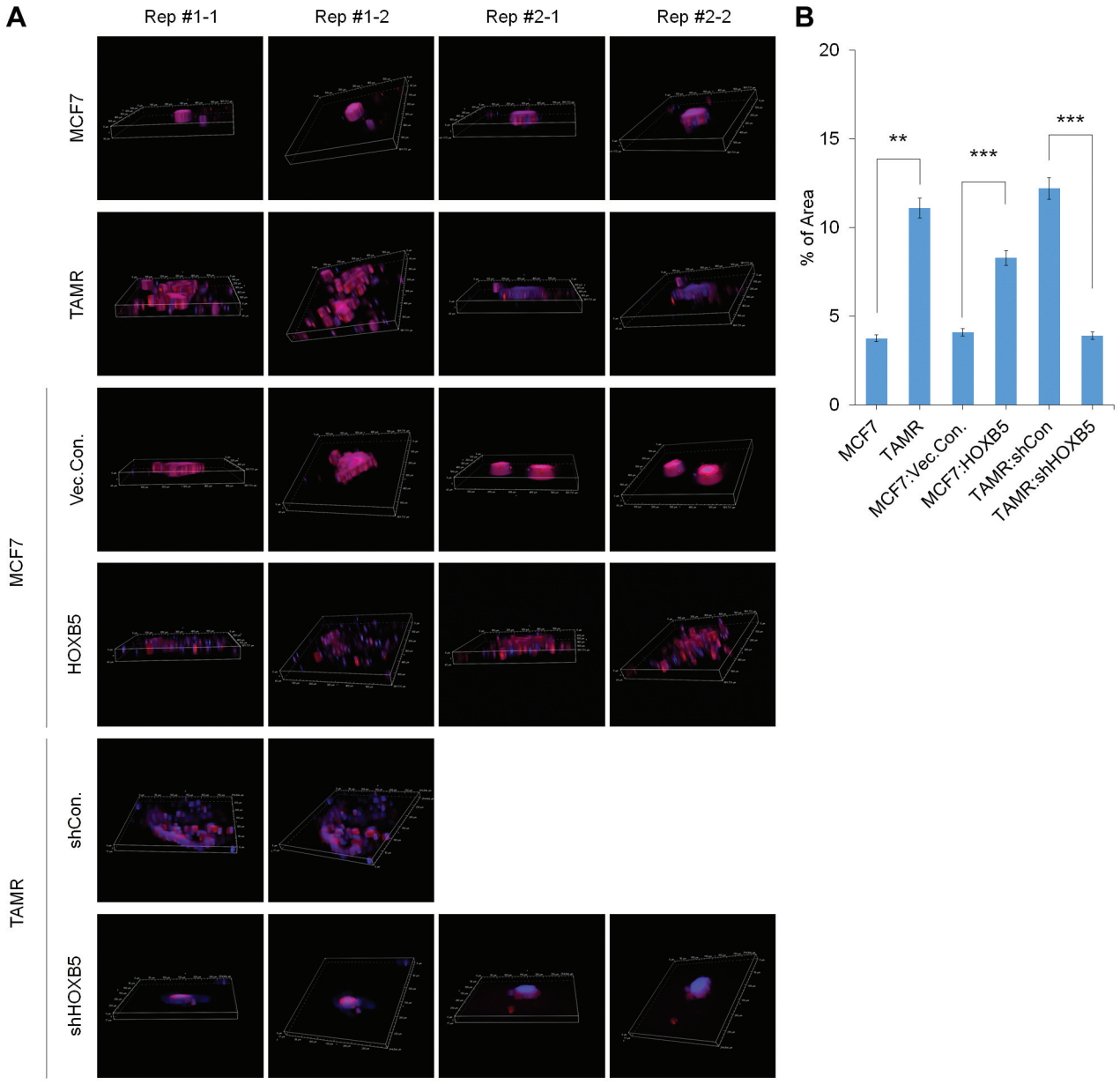

Figure 3. *(A) Confocal microscopy images of MCF7, TAMR, MCF7: vector control, MCF7: HOXB5, TAMR shRNA control, TAMR shHOXB5 cells in 3D culture system at day 14. Cells were stained with DAPI (blue) and phalloidin (red). DAPI was used to stain the DNA in the nucleus, and phalloidin was used to stain the F-actin filaments to visualize cell shape. The migratory and invasive features were observed in total colony structures at different angles. The scale bar in each panel represents 50 μm. (B) Quantification of the phalloidin signal of the ICC assay.*

genes were dramatically up-regulated in TAMR cells (Figure 4A), which allowed us to infer that TAMR cells are more stem cell-like, hence their aggressive phenotype. To better understand the phenomena underlying up-regulated HOXB5 and stem cell markers, we overexpressed HOXB5 in MCF7 cells, and knocked down HOXB5 from TAMR cells, then performed the same RT-PCR. Results showed that overexpression of HOXB5 in MCF7 cells increased gene expression of *OCT4* and *NANOG* compared to parent and vector control cells. In the meantime, HOXB5-depleted TAMR cells expressed significantly lower levels of *OCT4* and *NANOG* than parental and siRNA control cells. Interestingly, *SOX2* mRNA levels were unchanged in both knockdown and overexpression cell lines (Figure 4B).

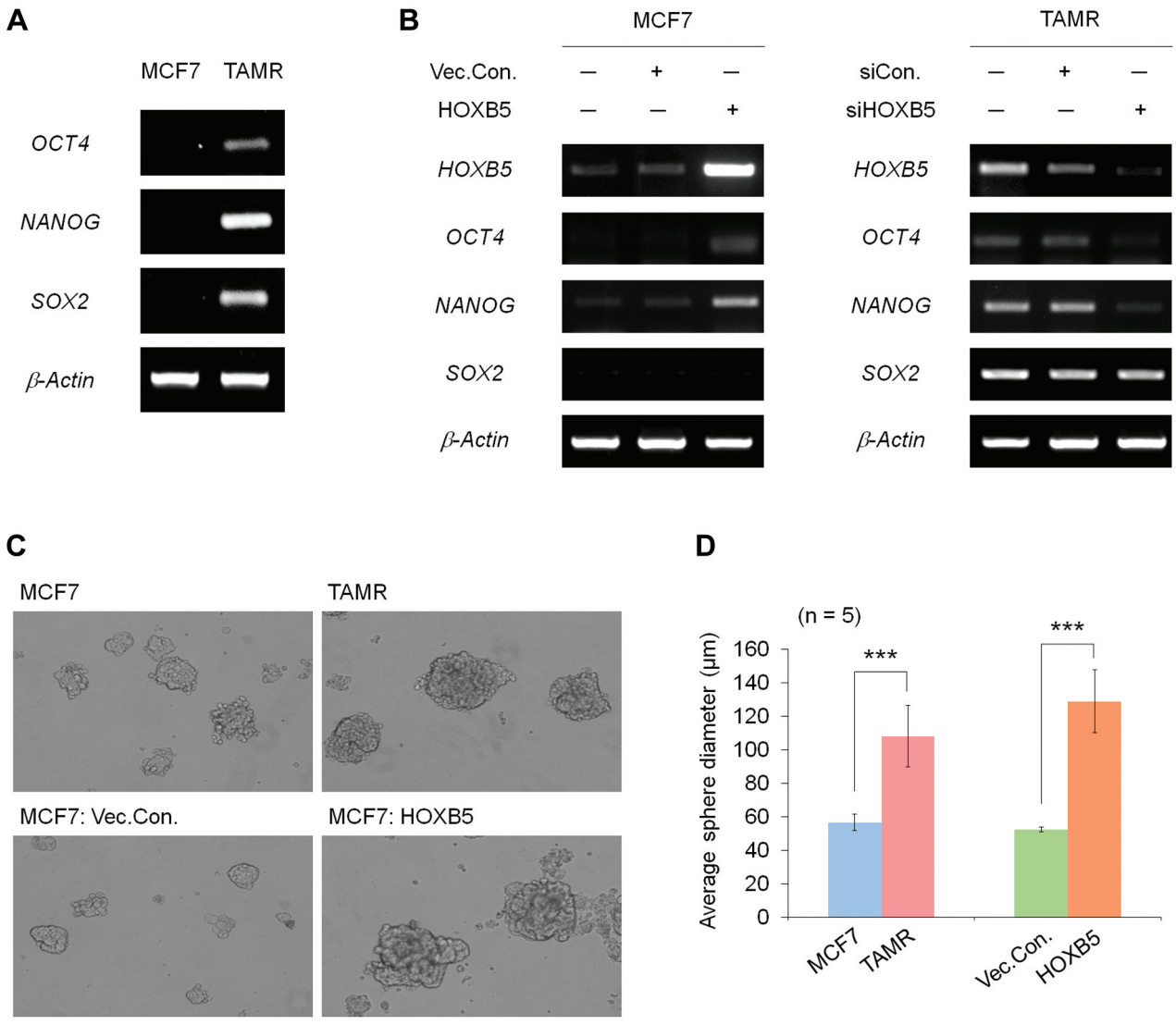

Figure 4. *Stemness and spheroid formation ability increases with HOXB5 expression. (A) mRNA expression levels of OCT4, NANOG, and SOX2 in MCF7 and TAMR cells were determined by RT-PCR. (B) Overexpression of HOXB5 in MCF7 cells up-regulated the expression of stem cell markers, and knockdown of HOXB5 in TAMR cells reduced expression of stem cell markers compared with controls. (C) Growth of spheroids under floating culture in spheroid medium. Spheroid formation was analyzed after 2 weeks. MCF7, TAMR, MCF7: vector control, and MCF7: HOXB5 cells were used. (D) Quantification of average sphere diameter. Data represented as mean±SD; 5 independent spheroid formation assays were performed (n=5). p-Values were determined using Student's t-test (***p<0.001).*

Since the gene expressions of stem cell markers were up-regulated in TAMR cells, we performed spheroid formation assay to further analyze stem cell properties brought upon by HOXB5. Spheroid formation assay allows for the determination of self-renewal and spontaneous aggregation abilities of CSCs. The spheroid formation assay revealed that the size of spheroids from parent TAMR cells were qualitatively higher by ~2-fold, compared to parent MCF7 cells (Figure 4C and 4D). To confirm that this enhanced spheroid formation ability is due to the up-regulated HOXB5 in TAMR cells, we stably overexpressed HOXB5 in MCF7 cells. When MCF7 vector control and MCF7 HOXB5-overexpressing cells were used to perform spheroid formation assay, the latter were significantly more enriched with spheroids with a much bigger in size, further clarifying the role of HOXB5 in cancer stemness (Figure 4C and 4D).

HOXB5 activates the EGFR signaling cascade in tamoxifen-resistant MCF7 cells. EGFR is one of the factors that has been well-studied in association with breast cancer aggressiveness,

and the activation of EGFR results in the instigation of the EGFR signaling cascade which leads to numerous cellular phenotypes (19). EGFR signaling pathway has been described to contribute to tumor cell migration and invasion in craniopharyngiomas and oral squamous cell carcinoma (20, 21). In addition, activation of EGFR has been reported to regulate stemness in head and neck squamous cell carcinoma by inducing stem cell marker genes such as *OCT4* and *NANOG* (22). Based on these previous findings, we hypothesized that EGFR may be involved in regulating the aggressive phenotypes associated with TAMR cells, and we sought to investigate whether HOXB5 influences EGFR signaling. To examine HOXB5-mediated activation of the EGFR signaling pathway in TAMR cells, western blot was performed. The total protein levels of EGFR and its downstream factors like SRC, MEK, and ERK1/2 were similar between MCF7 and TAMR cells. Yet, the phosphorylated-protein levels of EGFR/SRC/MEK/ERK1/2 were all up-regulated in TAMR cells compared to MCF7 cells (Figure 5A). These results suggest that the up-regulated HOXB5 induces EGFR and its downstream signaling cascade. To confirm, we knocked down HOXB5 using siRNAs in TAMR cells and performed western blot for the same target proteins. Results revealed that without HOXB5, the EGFR-signaling pathway was deactivated and the phosphorylated-protein forms could not be detected (Figure 5B). Altogether, these results suggest that HOXB5 plays a critical role in regulating EGFR signaling in TAMR cells, and by doing so, leads to breast tumor aggression and progression.

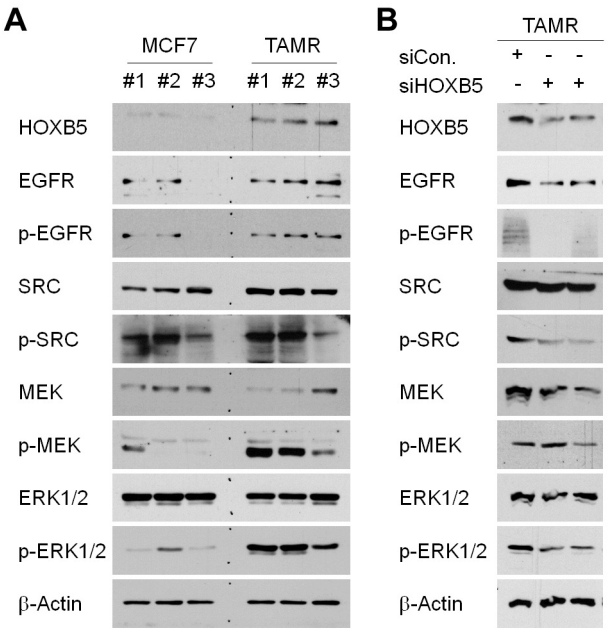

Figure 5. *HOXB5 induces EGFR expression and activates its signaling pathway. (A) EGFR signaling pathway activation was compared between MCF7 and TAMR cells through western blot analyses. (B) The deactivation of EGFR signaling pathway was evaluated in TAMR cells treated with siRNA control and/or siHOXB5 through western blot analyses. β-Actin was used as an internal control. All experiments were performed in triplicate.*

Discussion

In this work, we show that HOXB5, highly expressed in TAMR cells, results in more migratory and invasive characteristics compared to MCF7 cells. When HOXB5 was depleted from TAMR cells, migration and invasion of cancer cells decreased. Moreover, the presence of HOXB5 increased the stemness of cancer cells. TAMR cells not only expressed higher levels of stem cell markers, but also formed a greater number of spheroids, whereas MCF7 cells were less capable of forming spheroids. Furthermore, when HOXB5 was stably overexpressed in MCF7 cells, these cells were better able to express stem cell markers and generate spheroids similar to those of TAMR cells, demonstrating that HOXB5 is a key factor in establishing a CSC sub-population. We reveal that these phenotypes are induced by elevated HOXB5 and its ability to activate the EGFR signaling pathway.

The infiltration of aggressive tumor cells into neighboring tissues resulting in metastasis is one of the major problems in handling cancers (23-25). Drug-resistant hormone-positive breast cancer cells have been described to be more basal-like, showing similar phenotypes to that of triple-negative breast cancer (TNBC). It has been documented that most TNBC patients overexpress EGFR (26-28), and so EGFR inhibitors have been evaluated for the treatment of breast cancers (29, 30). Yet, the outcomes were not successful, because there was the lack of biomarkers to predict which patients would respond to EGFR inhibitors. TAMR cells, which show more aggressive phenotypes like TNBC cells, also overexpress EGFR. Moreover, the overexpression of HOXB5 has been reported in head and neck squamous cell carcinoma (HNSCC) and hepatocellular carcinoma (HCC). HOXB5 is significantly up-regulated in HNSCC and HCC cells, and upon knockdown, cell migration and invasion were suppressed in line with our study. These studies further corroborate our findings (31, 32). Here, we propose HOXB5, which is together overexpressed with EGFR, as an efficacious biomarker for TNBC patients as well as tamoxifen-resistant breast cancer patients. Yet, further validation will be necessary to establish the direct link between HOXB5, EGFR, and tamoxifen resistance to be applied as a therapeutic target or biomarker in clinic.

Furthermore, CSCs and their role in drug resistance is becoming increasingly more evident (33). This sub-population of tumor cells within the total tumor cells can help evade drug treatment, disseminate for metastasis and

increase the tumor mass. As a result, it is essential to study factors related to CSCs and their development. We demonstrated for the first time that HOXB5 is necessary for the activation of stem cell markers (*OCT4* and *NANOG*) and the formation of spheroids enriched with cancer stem cells. Interestingly, the expression of *SOX2* remained unaffected. Formerly, *OCT4, NANOG,* and *SOX2* were believed to regulate their own transcription by positive-feedback loops through the OCT-SOX enhancers (34-36). In contrast, another study demonstrated that the *SOX2* is dispensable for the activation of OCT-SOX enhancers, and that the expression of *NANOG* was maintained in *SOX2*-null-*OCT4*-rescued cells (37). We further speculate that in our breast cancer system, cells may be less dependent on *SOX2* for stemness, and rely on *OCT4* and *NANOG* to achieve cancer stem cell-like properties.

Our work provides evidence for the first time that HOXB5 is not only involved in tumorigenesis and/or cancer progression but is also a marker of tamoxifen resistance. HOXB5 regulates *EGFR* and its signaling pathway, attaining enhanced migratory and invasive attributes in TAMR cells. Moreover, the presence of HOXB5 induces the expression of stem cell markers *OCT4* and *NANOG*, and allows for a better formation of spheroids. Collectively, our findings show that HOXB5 is a crucial factor that contributes to cancer aggression, and with the appropriate modulation of HOXB5, tamoxifen resistance as well as cancer progression can be combatted.

Conflicts of Interest

The Authors declare that no competing interests exist.

Authors' Contributions

C.Y.K and J.H.O designed the experiments, analyzed data and wrote the manuscript. C.Y.K and Y.C.K performed the *in vitro* studies. M.H.K and J.H.O managed and supervised the study and finalized the manuscript. All authors discussed the results and commented on the manuscript.

Acknowledgements

This research was supported by the grants from Brain Korea 21 Project for Medical Science, Yonsei University and the National Research Foundation (NRF) (No. NRF-2019R1I1A1A01050780 and 2016R1A2B2011821).

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Received May 21, 2021
Revised June 4, 2021
Accepted June 8, 2021