BMB Reports - Manuscript Submission

Manuscript Draft

Manuscript Number: BMB-18-020

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Article Type: Article

Keywords: Breast cancer; tamoxifen resistance; HOX genes; histone modification

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Up-regulation of HOXB cluster genes are epigenetically regulated in tamoxifen-resistant MCF7 breast cancer cells

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Running Title: HOXB cluster genes in tamoxifen resistance

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ABSTRACT

Tamoxifen (TAM) is commonly used to treat estrogen receptor (ER)-positive breast cancer. Despite the remarkable benefits, resistance against TAM presents a serious therapeutic challenge. Since several HOX transcription factors have been proposed as strong candidates in the development of resistance against TAM therapy in breast cancer, we generated an *in vitro* model of acquired TAM resistance using ER-positive MCF7 breast cancer cells (MCF7-TAMR), and analyzed the expression pattern and the epigenetic states of *HOX* genes. HOXB cluster genes were uniquely up-regulated in MCF7-TAMR cells. Survival analysis of *in slico* data showed the correlation of high expression of *HOXB* genes with a poor response to TAM in ER-positive breast cancer patients treated with TAM. Gain- and loss-of-function experiments showed that the overexpression of multi *HOXB* genes in MCF7 renders cancer cells more resistant to TAM, whereas the knockdown restores TAM sensitivity. Furthermore, the activation of *HOXB* genes in MCF7-TAMR was associated with histone modifications, particularly the gain of H3K9ac. These findings imply that the activation of *HOXB* genes are involved in the development of TAM resistance, and could serve a target for developing new strategies to prevent or reverse TAM resistance.

Key words: Breast cancer, tamoxifen resistance, HOX genes, histone modification

INTRODUCTION

Estrogen receptor (ER)-positive breast patients constitute almost 70% of breast cancer cases (1) and are likely to benefit from endocrine therapies such as tamoxifen (TAM) and aromatase inhibitors (AI). These drugs either work by competitively binding to ER or by preventing the systemic conversion of testosterone to estrogens (2, 3). Even though endocrine therapy has been proven to be relatively safe and has significant therapeutic effects, one-third of women treated with TAM for 5 years will have a relapse of the disease within 15 years (4). For decades, on the strength of extensive studies regarding molecular mechanisms that are related with endocrine therapy resistance, several important factors, such as ER gene (ESR1) mutations, epigenetic aberrations, or crosstalk between ER and growth factor signaling, have now come to our attention (5, 6). However, the investigation and discovery of novel biomarkers are still strongly required for predicting responses to TAM resistance and for developing personalized treatment strategies.

HOX are highly conserved transcription factors that have crucial roles in development, and now it becomes evident that many HOX genes are associated with cancer (7-9). Many previous studies have demonstrated abnormal HOX expression patterns in breast cancer tissues and culture cells, and furthermore, their roles in tumorigenesis and metastasis of breast cancer (10-14). In addition, many HOX genes, such as HOXB5, HOXB7, HOXB13, HOXC10, HOXC11, and non-coding RNAs in the HOX clusters were found to be associated with breast cancer endocrine resistance via repression of ER expression or activation of several receptor tyrosine kinase pathways (15-19). However, the expression patterns and the functional characterization of the

whole HOX cluster genes in TAM resistant breast cancer cells have not been investigated.

Here, we generated an *in vitro* TAM resistance model using ER-positive MCF7 breast cancer cells (MCF7-TAMR), and the expression patterns of *HOX* genes as well as their epigenetic status were analyzed. The correlation of *HOX* gene expression in breast cancer patients and its impact on survival have been further examined using publicly available datasets of human breast cancers. In addition, we investigated the functional impact of *HOX* gene expression on TAM sensitization and resistance by doing gain-of-function and loss-of-function experiments.

RESULTS

HOXB genes are up-regulated in MCF7-TAMR cells

We generated an *in vitro* TAM resistant MCF7 cell line (MCF7-TAMR) and confirmed the resistance to TAM in a concentration-dependent manner (Fig. 1A). MCF7 and MCF7-TAMR showed the same cell proliferation rates in normal medium, however, MCF7-TAMR cells showed increased TAM resistance time-dependently up to day 4 (Fig. 1B). To investigate *HOX* gene expression alterations in TAM resistant cells, we analyzed 39 *HOX* gene expression levels in the parental MCF7 and -TAMR cells, and found that the entire *HOXB* cluster genes (*HOXB2-B13*) were significantly up-regulated in MCF7-TAMR cells (Fig. 1C). However, only minor changes were detected in *HOXA*, *HOXC* and *HOXD* cluster genes (Fig. 1C and Supplementary Fig. 1). Representatively, the up-regulation of HOXB5 and HOXB6 expression in MCF7-TAMR cells was also confirmed at the protein level by immunocytochemistry and Western blotting (Fig. 1D and E). To investigate whether the up-regulation of any *HOXB* gene is linked to other nearby *HOXB* gene expression, we analyzed publicly available datasets related to human cancer. The finding that each *HOXB* gene is co-expressed with other *HOXB* genes in human breast cancer patients (Supplementary Table 1) suggests that each *HOXB* gene expression is highly correlated with nearby *HOXB* genes.

Up-regulation of *HOXB* midcluster genes is associated with poor clinical outcome in ER-positive breast cancer patients

To assess the survival length of breast cancer patients depending on their *HOXB* expression levels, DMFS curves were plotted and compared using the Kaplan-Meier method and the log-rank test. There was no significant difference in the DMFS curves between the *HOXB*-high and -low groups in all patients (Fig. 2A). However, when analyzed only with ER-positive patients treated with TAM for therapy, DMFS was significantly lower in the *HOXB*-high groups (Fig. 2B). Among *HOXB* genes, *HOXB5*, *B6* and *B7* genes, in particular, showed a significant difference in the DMFS between high- and low expression groups (Fig. 2C-E). Due to the lack of dataset, the impact of *HOXB4* expression on breast cancer survival was not determined. Multigene prognosis tests also confirmed that the high expression of midcluster *HOXB* genes (*HOXB5-B7*) is associated with the poor survival of ER-positive breast cancer patients treated with TAM, compared with the anterior (*HOXB1-B3*) or posterior *HOXB* genes (*HOXB8-B13*) (Fig. 2F-H). In contrast, there were no significant differences in the DMFS curves between the expression-high and -low groups for *HOXA* and *HOXC* cluster genes in both all patients and ER-positive patients group treated with TAM (Supplementary Fig. 2A-D). In case of *HOXD* cluster genes, high expression was associated with poor patient prognosis, but the expression levels were not induced in MCF7-TAMR cells (Supplementary Fig. 1, and 2E and F).

Overexpression of midcluster *HOXB* gene induce TAM resistance in MCF7

Previous studies have shown that several HOXB genes, such as HOXB5, HOXB7, and HOXB13, play a role in TAM resistance individually (16, 17, 19). However, there is no evidence whether the overexpression of multiple HOXB genes produces additive or synergistic effects. To explore whether the combinatorial overexpression of multiple

HOXB genes can induce higher TAM resistance compared with that of a single *HOXB* gene, we performed cell proliferation assay in the presence of TAM after 24 hrs post transfection. Midcluster *HOXB* genes (*HOXB5*, *B6* and *B7)* were used in this experiment because of their potential roles in TAM resistance. The overexpression of each *HOXB* gene after transfection was confirmed by RT-PCR (Fig. 3A and C). The co-expression of *HOXB5*, *HOXB6* and *HOXB7* significantly increased cell proliferation rate in the presence of TAM, compared with a single *HOXB* gene (Fig. 3B). The proliferation activities of MCF7 cells transfected with any combination of two *HOXB* genes (*B5/B6*, *B6/B7* and *B5/B7*) were slightly higher than the values of control cells, and furthermore, the overexpression of three midcluster *HOXB* genes (*HOXB5-B7*) led to the highest cell proliferation in the presence of TAM (Fig. 3D). For loss-of-function studies, *HOXB4*, instead of *HOXB7*, was included as siRNA targets together with *HOXB5* and *B6* because the up-regulation of *HOXB4* in TAMR cells (Fig. 1C) was considered much more relevant than the expression changes of *HOXB7*. In a series of knockdown experiments, reduced mRNA expression of *HOXB4*, *B5*, and *B6* was confirmed (Fig. 3E, G, and I). Individual sets of MCF7-TAMR cells transfected with siRNA for a single gene showed slightly reduced cell proliferation activities in the presence of TAM, but the effect was more pronounced when three *HOXB* genes were silenced simultaneously (Fig. 3F). The same patterns were observed when at least two genes (*HOXB4/B6* or *HOXB5/B6*) were silenced in either single or combination (Fig. 3H and J).

Activation of *HOXB* gene expression in MCF7-TAMR cells is epigenetically regulated

The expression of HOX genes is tightly regulated by epigenetic mechanisms during normal development and cancer (20, 21). Since the *HOXB* cluster genes were generally upregulated as a whole in MCF7-TAMR cells, we expected that different epigenetic states can be generated in the *HOXB* locus during the transition to acquired TAM resistance. To test whether histone modifications at the *HOXB* locus serve as markers for gene expression differences in MCF7 and MCF7-TAMR cells, we performed ChIP analysis. Based on various sources of ChIP-Seq data in MCF7 cells deposited into the UCSC database, we determined the amplicon sites for the promoter region of each *HOXB* gene (Fig. 4A). ChIP-qPCR results revealed that increased H3K9ac at the proximal promoter region of each *HOXB* gene was associated with decreased H3K27me3, as the transcript levels increased in MCF7-TAMR cells (Fig. 4B).

DISCUSSION

In this study, we have shown that the *HOXB* cluster genes are activated as a whole in TAM resistant MCF7 breast cancer cells. The results of survival analysis indicate that high expression of *HOXB*, especially midcluster *HOXB*, associates with poor survival in patients with ER-positive breast cancer who are treated with TAM therapy. Our functional studies with overexpression and knockdown experiments clearly confirm that the midcluster *HOXB* genes contribute to TAM resistance, and the activation of *HOXB* gene expression is mediated by epigenetic mechanisms.

HOX genes play diverse functions in adult tissues as well as during embryogenesis under endocrine control. Therefore, endocrine-HOX signaling has important clinical and molecular implications for physiology and human pathology (22). In human endometrium, HOX genes are dynamically expressed under the control of steroid hormones, and the decreased HOXA10 expression was considered as a possible mechanism of progesterone resistance in endometriosis (23). There is accumulating evidence supporting the contribution of HOX genes in endocrine therapy resistant breast cancer (15). Although several HOX genes, such as HOXB7 and HOXB13, in TAM resistance have been well characterized (17, 19), cooperative and/or synergistic actions of clustered genes in TAM resistance have not been explored. Of note, the driving forces, which induce dysregulated gene expression in cancer, include gene copy number variations, epigenetic regulation, and coordinated actions of transcription factors. In this study, we minded The Cancer Genome Atlas (TCGA) breast cancer data to elucidate the association between copy number amplification and HOX gene expression and found that there is no positive correlation between the expression of HOXB mRNA and copy

number in breast cancer patient samples (Supplementary Fig. 3), suggesting a rare functional relevance of HOXB amplification. In support of this, the copy number assay for each HOXB5 and HOXB6 gene locus in cell lines demonstrated no HOX amplification in MCF7-TAMR cells compared to MCF7 (Supplementary Fig. 4). These data strongly suggest that the possibility that the increase in copy number may have contributed to the increase in the expression level can be excluded. Thus, our argument that the HOXB genes are up-regulated epigenetically in MCF7-TAMR cells seems more persuasive. Moreover, our findings support that consecutive *HOXB* genes have similar biological functions in mediating TAM resistance.

Yet more studies need to be required to explain the causal mechanism, there seem to be several possibilities on how they work together. Many HOX proteins sharing high homology are likely to share common molecular targets, probably involving in common signaling pathways. Moreover, non-coding RNAs such as miRNAs and long non-coding RNAs (lncRNAs) located in HOX cluster could participate in regulating coordinated multi-gene expression during development of TAM resistance. Several studies have shown that miRNAs are associated with drug resistance development and prediction of outcome and therapeutic response in breast cancer (24, 25). MiR-196a and miR-10a, which are located in the *HOXB* loci, in particular, have been identified as players in mediating endocrine resistance (15, 26). Although the potential *cis*- or *trans*-actions of these non-coding RNAs in multi-gene regulation, particularly in breast cancer cells resistant to TAM, remain to be characterized, this possibility must be taken into consideration.

Meanwhile, several studies have reported that epigenetic alterations are associated with drug resistance in breast cancer (27, 28). In this work, we showed the up-regulation

of *HOXB* gene expressions in TAM resistant breast cancer cells, with accompanying changes in histone modification along the whole cluster. Consistent with these findings, a previous study of barrett esophagus (BE) showed that the activation of three consecutive midcluster *HOXB* genes (*HOXB5-B7*) are epigenetically correlated with H3K27me and AcH3 levels and chromatin compaction (29). In many human diseases including cancer, the high-order chromatin structure has been reported to be altered (30). Particularly, *HoxBlinc* (hoxb locus-associated long intergenic non-coding RNA) located between *hoxb4* and *-b5*, has been known to regulate *hoxb* gene transcription by modulating local chromatin alterations during murine embryonic stem cell differentiation (31). Considering the role of *HoxBlinc* RNA during embryogenesis in recruiting Set1/MLL complexes containing methyltransferase activity for H3K4 and its effect on 3D chromatin architecture, we would expect that a large variety of lncRNAs can participate in modulating chromatin structure and gene expression in cancer cells.

In conclusion, we have shown the simultaneous activation of *HOXB* genes in TAM resistant breast cancer cells and demonstrated the functional roles of midcluster *HOXB* genes in sensitizing and desensitizing TAM effect. These findings not only provide insight into the cumulative effect of *HOXB* gene expression in TAM resistance, but may also help develop new therapeutic approaches to resensitize resistant tumors by finding factors that control the whole clustered *HOXB* genes all together.

MATERIALS AND METHODS

Cell culture and establishment of MCF7-TAMR cells

MCF7 cells were provided by Drs. Kyung Tae Kim (National Cancer Center, Korea) and were karyotyped in the Korean Cell line Bank (Seoul, Korea). MCF7 cells were cultured as described previously (14). To generate an *in vitro* model of acquired TAM resistance, MCF7 breast cancer cells were grown in the same condition and exposed to 0.1 μM 4-hydroxytamoxifen (4-OH-TAM) (Sigma, St. Louis, MO, USA) for at least six months.

Cell proliferation assay

Cell proliferation assay was carried out by using the Cell Counting Kit-8 (CCK-8) following the manufacturer's instructions (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, cells were plated in 96-well plates ($9x10^3$ cells per well) and stained with 5 μl of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] for 3.5 hr at 37°C on designated days. Using ELISA reader (Softmax Pro) the absorbance was measured at 450 nm.

Total RNA isolation and RT-PCR

Total RNA isolation, RT-PCR, and qPCR were performed as described (16). All reactions were performed in triplicates, and β-actin was used as an internal control. Primer sequences used for amplification of HOX genes were previously presented (14).

Immunocytochemistry

For immunocytochemistry, cells were fixed and blocked as described previously (16). After blocking, cells were incubated overnight with primary antibodies to HOXB5 (Abcam, ab260791) and HOXB6 (Sigma, HPA042063). The cells were then washed three times in 500 µl of blocking solution. Secondary antibodies (Alexa Fluor® 594-conjugated anti-Rabbit) were added to the cell and incubated for 1.5 ~ 3 hr at room temperature. Then, DAPI (final conc. 5 µg/ml) was added and incubated for 20 min. The cells were then washed three times in 500 µl of blocking solution. Fluorescent images were taken using Olympus IX 70 fluorescent microscope.

Western blotting

MCF7 and MCF7-TAMR cells were treated under the appropriate conditions and lysed. After determining protein concentration, equal amounts of proteins were loaded and blotted as described previously [16]. Anti-HOXB5 (Abcam, ab109375), anti-HOXB6 (Sigma, HPA042063), and anti-ACTIN-B (Abcam, ab6276) antibodies were used to detect the each protein.

Plasmids, siRNA and transfection

A full-length cDNA of HOXB5, -B6 and -B7 gene was cloned into a pcDNA3-HA-tagged expression vector. Single or multiple plasmid DNAs were transfected into MCF7 cells by using Attractene transfection reagent (Qiagen, Hilden, Germany). After 24 hrs, the cells were used for RNA isolation or prepared for cell proliferation assay. For knockdown studies, MCF7-TAMR cells were transfected with 15 nM siRNA by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. siRNAs were purchased from GE Dharmacon (Waltham, MA, USA); ON-TARGETplus Non-targeting Pool (Cat. # D-001810-10-05 5 nmol), SMARTpool: ON-

TARGETplus HOXB5 siRNA (cat. L-017532-02-0005 5 nmol) and Santa Cruz (CA, USA); HOXB4 siRNA(h) (sc-38692), HOXB6 siRNA(h) (sc-38694). Single or pools of individual siRNAs of different genes were transfected into MCF7 cells.

Chromatin Immunoprecipitation (ChIP) analysis

ChIP was done using the Abcam X-ChIP protocol (Abcam, Cambridge, UK), with minor modifications: anti-H3K9ac antibody (ab12179), anti-Histone H3 (tri methyl K27) antibody (ab6002), and normal mouse IgG (Santa Cruz, sc-2025) were used. MCF7 and MCF7-TAMR cells were fixed in 37% formaldehyde for 15 min, and quenched by incubation with 2.5 M Glycine for 10 min. Chromatin was fragmented (about 500-1,000 bp) by sonication using Sonics Vibra Cell™ with the following conditions: Time-8 min, Pulse-10 sec Interval-10 sec. The sonicated chromatin fragment was incubated with appropriate antibodies and salmon sperm DNA/protein coated A/G agarose beads (Santa Cruz, CA, USA) overnight at 4°C with gentle shaking. The following day, the immunoprecipitated DNA was purified and used for PCR with appropriate primers (Supplementary Table 2).

In silico analysis

Survival analysis was done using publicly available database (Kaplan-Meier plotter, http://kmplot.com/) that is capable of assessing the effects of 54,675 genes on survival using 10,461 breast cancer samples. Gene expression data and relapse free and overall survival information are downloaded from GEO (Affymetrix microarrays only), EGA and TCGA. The database is handled by a PostgreSQL server, which integrates gene expression and clinical data simultaneously. To analyze the prognostic value of HOX

genes, the patient samples were categorized into low- and high-expression groups using the median as the auto select best cutoff. Distant metastasis-free survival (DMFS) time was calculated from the operation date to the first distant metastasis date. In addition, cBioPortal (http://www.cbioportal.org/) dataset was utilized to search for co-expression pattern among HOX genes in patients with breast cancer. Gene expression was analyzed with the dataset for Breast Invasive Carcinoma (TCGA, Cell 2015; 817 samples).

Statistical analysis

In Kaplan–Meier method, survival curves of two groups were compared using a log-rank test. The *in vitro* results are expressed as the mean ± SEM and analyzed with the paired t-test. A statistical significance was determined by two-sided statistical tests and *p*-value. Differences were considered significant at $*p < 0.05$, $**p<0.01$, and $***p<0.001$

ACKNOWLEDGEMENTS

We thank Clara Yuri Kim for editing the manuscript. This research was supported by the National Research Foundation (NRF) funded by the Korean Government (MSIP, NRF-2014R1A1A2056986, NRF-2016R1D1A1B03930822, and NRF-2016R1A2B2011821).

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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FIGURE LEGENDS

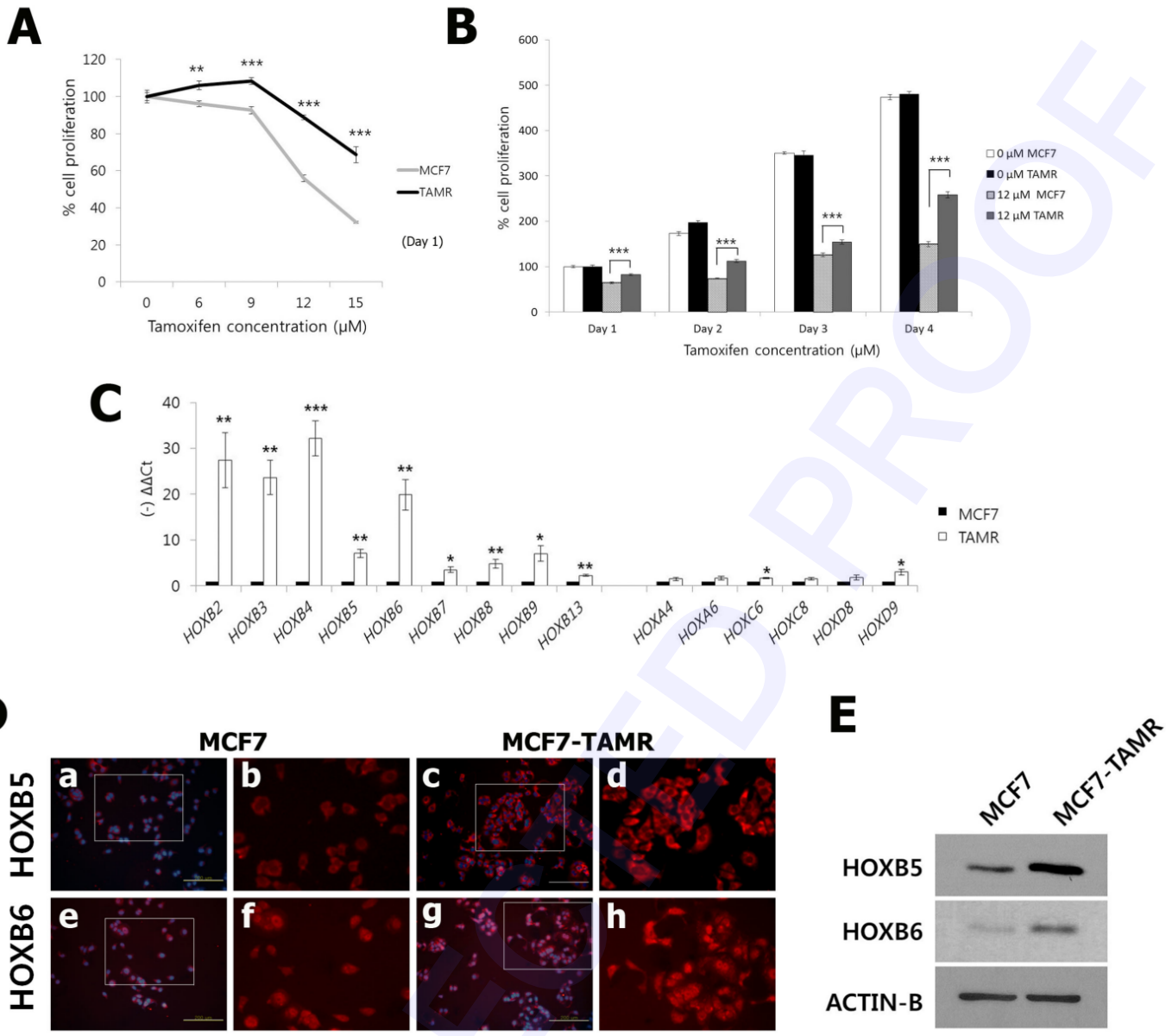
Figure 1. **Up-regulation of *HOXB* genes in MCF7-TAMR cells.** (A) Cell viability test with MCF7 and MCF7-TAMR cells at Day 1 after treatment with indicated concentration of TAM. (B) Cell viability was measured from day 1 to day 4 after treatment with 0 and 12 μM TAM. (C) Real-time PCR analysis of whole *HOXB* genes, *HOXA4*, *HOXA6*, *HOXC6*, *HOXC8*, *HOXD8*, and *HOXD9* in MCF7 and MCF7-TAMR cells. (A-C) Data are presented as mean ± SEM from at least three independent assays. *p<0.05; **p<0.01; ***p<0.001 *vs*. MCF7, by t-test. (D) Protein expression and (E) Western blotting of HOXB5 (a-d) and HOXB6 (e-h) in MCF7 and MCF7-TAMR cells. The images in a, c, e, and g were overlaid with DAPI counterstain (x200), and boxed regions were magnified in b, d, f, and h, respectively (x400).

Figure 2. Kaplan-Meier analysis of DMFS for the breast cancer patients based on *HOXB* expression. (A-B) Survival rate was compared between the *HOXB*-high and –low expression groups with (A) all patients and with (B) ER-positive patients who received TAM therapy. (C-E) DMFS of ER-positive patients who received TAM monotherapy were stratified by (C) *HOXB5*, (D) *HOXB6*, and (E) *HOXB7* expression level. (F-H) In a multigene analysis, ER-positive breast cancer patients were divided into two groups according to the expression level of (F) anterior *HOXB* (*HOXB1-B3*), (G) middle *HOXB* (*HOXB5-B7*), and (H) posterior *HOXB* (*HOXB8-B13*) and analyzed DMFS.

Figure 3. Effects of midcluster *HOXB* genes expression in TAM sensitivity. (A-B) MCF7 cells were transfected with vector only (MCF7:Vec) and single plasmid DNA (MCF7:HOXB5, MCF7:HOXB6, MCF7:HOXB7), or co-transfected with multiple plasmid DNAs simultaneously (MCF7:HOXB5/6/7): (C-D) co-transfected with 2-3 plasmid DNAs for *HOXB5*, *B6*, and *B7* with different combinations (MCF7:HOXB5/6, MCF7:HOXB6/7, MCF7:HOXB5/7 and MCF7:HOXB5/6/7). (A and C) RT-PCR analysis of *HOXB5*, *B6*, and *B7* in each transfectant. *β-ACTIN* was used as an internal control. (B and D) All transfectants were treated with 10 μM of TAM and measured for cell proliferation rate by CCK-8 assay at day 1. (E-F) MCF7-TAMR cells were transfected with siRNAs for control, *HOXB4*, *HOXB5*, *HOXB6* (TAMR-siCON, TAMR-siHOXB4, TAMR-siHOXB5, TAMR-siHOXB6), or co-transfected simultaneously (TamR-siHOXB4/5/6): (G-H) *HOXB4*, *HOXB6* (TAMR-siCON, TAMR-siHOXB4, TAMR-siHOXB6): (I-J) *HOXB5*, *HOXB6.* (E, G and I) Q-PCR analysis for each HOXB expression. (F, H, and J) CCK-8 assay for cell proliferation rate at day 1. Data are presented as mean ± SEM from at least three independent assays. *p<0.05, **p<0.01, ***p<0.001 *vs*. MCF7:Vec or TAMR-siCON, by t-test. $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 *vs*. MCF7: HOXB5/6/7 or TAMR-siHOXB4/5/6 (in F) or TAMR-siHOXB5/6 (in J), by t-test.

Figure 4. Histone modifications in MCF7 and MCF7-TAMR cells. (A) A screenshot of the *HOXB* cluster in UCSC genome browser on Human (GRCh37/hg19) Assembly (http://genome.ucsc.edu/). Amplicon for each *HOXB* gene are marked. ChIP-seq data

for Pol2 (GSM822295), H3K4me3 (GSM945269), H3K27me3 (GSM970218) and H3K27ac (GSM945854) were uploaded as custom tracks in the browser. (B) ChIP-qPCR analysis along the *HOXB* cluster. Immunoprecipitated DNAs and input DNAs were derived using anti-H3K9ac and anti-H3K27me3 antibodies. ChIPed DNAs for IgG were used negative controls. Primers for gene desert regions were used as negative controls (gene desert #1; Chr 16: 62,732,615-62,732,729, gene desert #2; Chr 20: 56,403,369-56,403,521). Data are shown as the percentage of input, after normalization with IgG. *p<0.05, **p<0.01.



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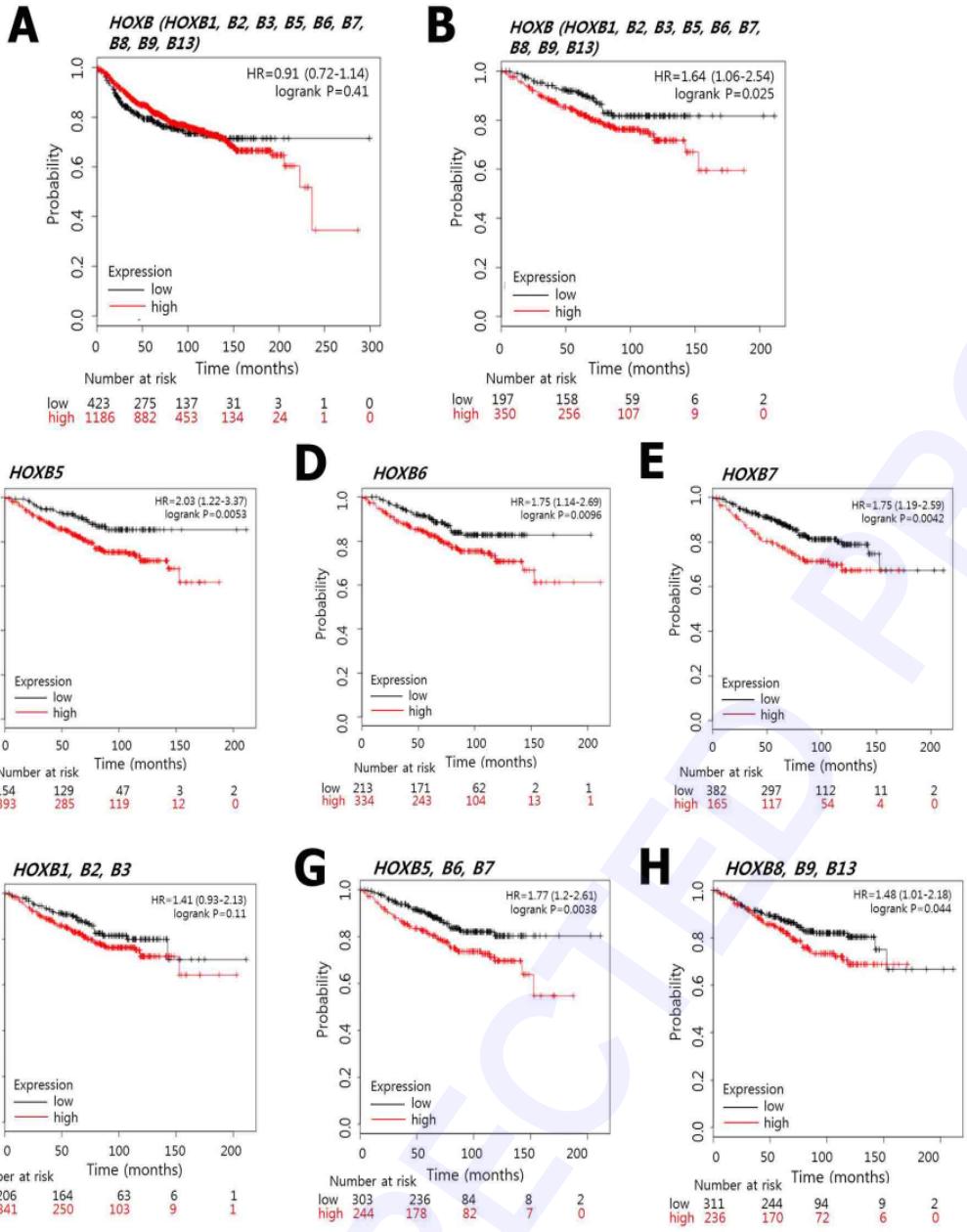

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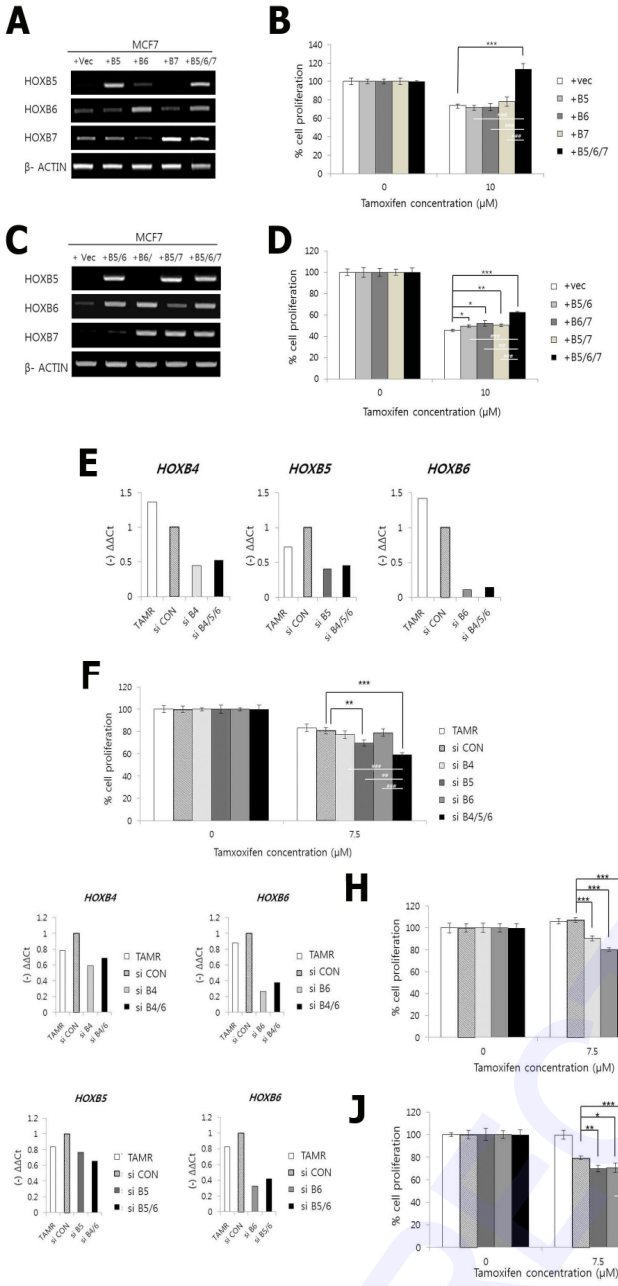

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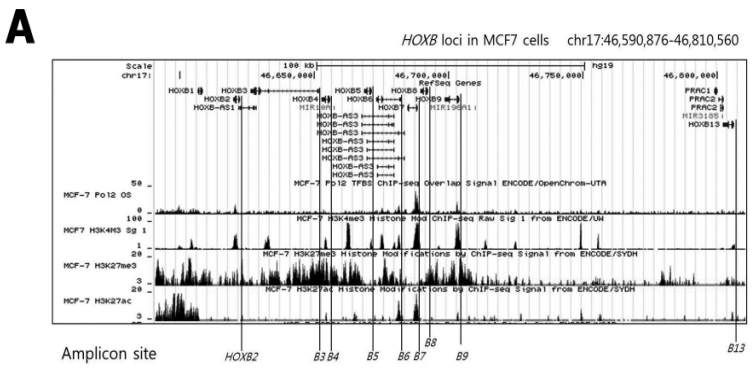

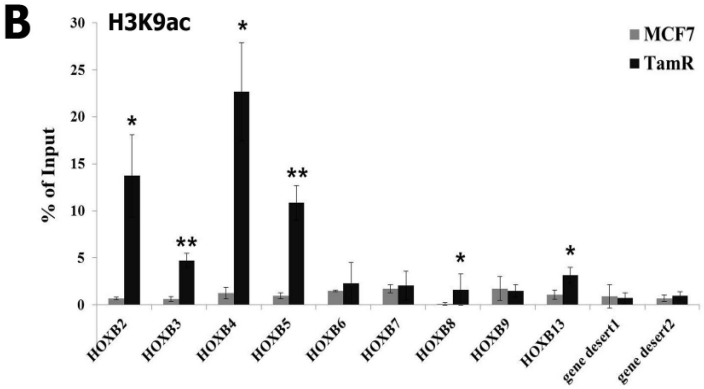

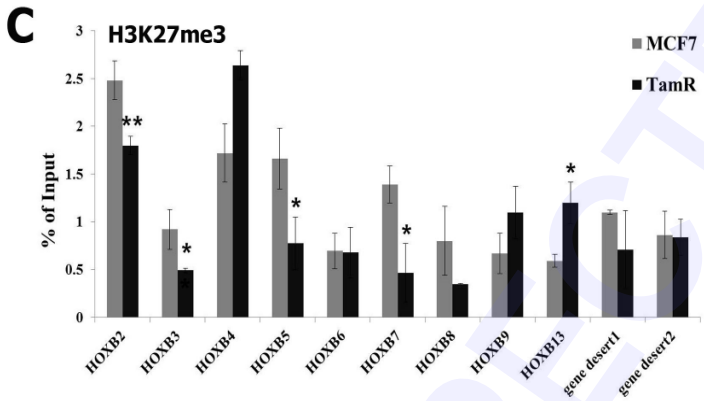

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SUPPLEMENTARY MATAERIALS

Supplementary Table 1. The list of genes with the highest expression correlation with the *HOXB* genes.

Supplementary Table 2. Primers used for ChIP-PCR.

Supplementary Figure 1. mRNA expression levels of *HOXA*, *HOXB*, *HOXC* and *HOXD* genes in MCF7 and MCF7-TAMR cells. *beta-ACTIN* was used as an internal control.

Supplementary Figure 2. Kaplan-Meier analysis of DMFS based on *HOXA*, *HOXC* and *HOXD* expression in the online dataset. Survival was compared between the *HOXA*-high and –low expression groups (A) with all patients (*p* value = 0.34) and (B) with ER-positive patients who received TAM therapy (*p* value = 0.093). Survival was compared between the HOXC-high and –low expression groups (C) with all patients (*p* value = 0.083) and (D) with ER-positive patients who received TAM therapy (*p* value = 0.36). Survival was compared between the *HOXD*-high and –low expression groups (E) with all patients (*p* value = 0.0034) and (F) with ER-positive patients who received TAM therapy (*p* value = 0.042).

Supplementary Figure 3. Correlation of mRNA level and copy number of HOX in TCGA breast cancer samples. (A) HOXB2. (B) HOXB3. (C) HOXB4. (D) HOXB5. (E) HOXB6. (F) HOXB7. (G) HOXB8. (H) HOXB9. (I) COPS5, a reference gene showing positive correlation between mRNA level and copy number.

Supplementary Figure 4. Estimation of copy number for HOXB5 and HOXB6 gene locus using SYBR Green I dye detection method. For qPCR, 7.5 ng of gDNA from MCF and MCF7-TAMR cells was used. Primer sequences are as follow: HOXB5-forward; CAAAGCCAACCTTCTCTCTGTT, HOXB5-reverse; CGTAAATTCTCGCTGATGACC-reverse, HOXB6-forward; GTCTCACTCACTGTTGCACG, HOXB6-reverse; CACTTCCTCCTATTACCCGC; control region-forward; TCATGGGTTGTGAAGACCGA, control region-reverse; GGGGCAGGTCTGTATTCCTT.

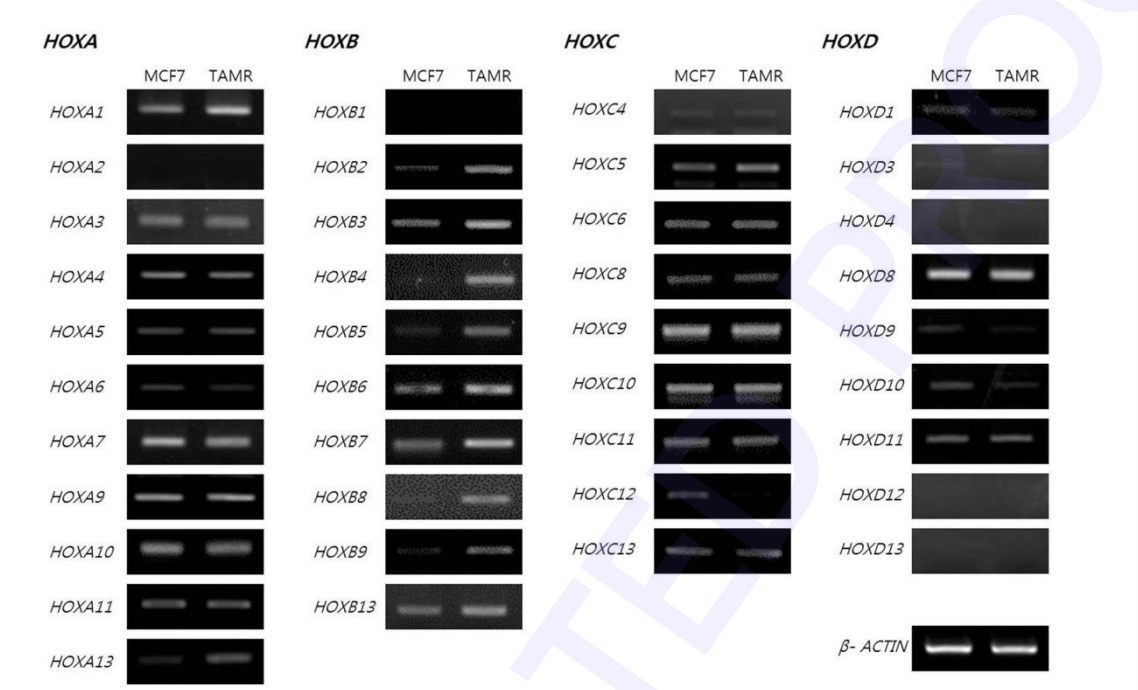
Supplementary Table 1. The list of genes with the highest expression correlation with the *HOXB* genes

HOXB2		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB3	0.69	**0.86**
HOXB4	0.55	0.66
HOXB5	0.39	0.6
HOXB6	0.35	0.6
HOXB1	0.31	0.55
HOXB3		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB4	**0.73**	**0.76**
HOXB2	0.69	**0.86**
HOXB5	0.62	**0.7**
HOXB6	0.6	**0.71**
HOXB8	0.34	0.42
HOXB7	0.33	0.51
HOXB1	0.33	0.54
SKAP1	0.3	0.47
HOXB4		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB3	**0.73**	**0.76**
HOXB5	0.61	**0.71**
HOXB2	0.55	0.66
HOXB6	0.52	**0.71**
HOXB7	0.33	0.52
HOXB5		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB6	**0.79**	**0.89**
HOXB3	0.62	**0.7**
HOXB4	0.61	**0.71**
HOXB8	0.49	0.62
FGF14	0.43	0.34

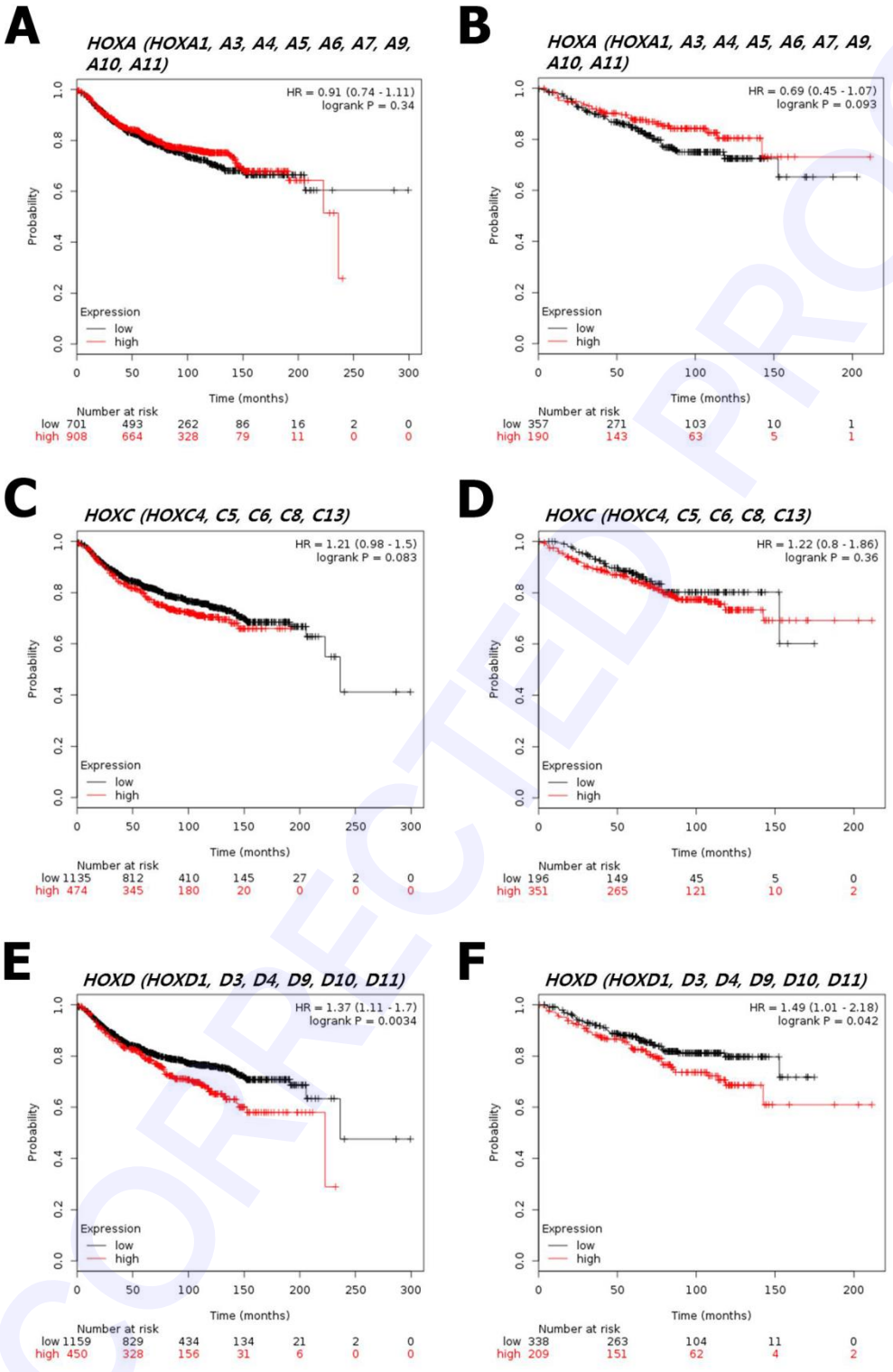
HOXB2	0.39	0.6
HOXB7	0.37	0.63
SKAP1	0.32	0.33
HOXB6		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB5	**0.79**	**0.89**
HOXB7	0.6	**0.75**
HOXB3	0.6	**0.71**
HOXB8	0.57	0.63
HOXB4	0.52	**0.71**
HOXB9	0.37	0.5
HOXB2	0.35	0.6
HOXB7		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB6	0.6	**0.75**
HOXB8	0.42	0.6
PRAC2	0.39	0.33
HOXB9	0.38	0.55
HOXB5	0.37	0.63
HOXB4	0.33	0.52
HOXB3	0.33	0.51
HOXB8		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB6	0.57	0.63
HOXB5	0.49	0.62
HOXB9	0.48	0.59
HOXB7	0.42	0.6
HOXB3	0.34	0.42
HOXB9		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB8	0.48	0.59
HOXB7	0.38	0.55
HOXB6	0.37	0.5

Supplementary Table 2. Primers used for ChIP-PCR

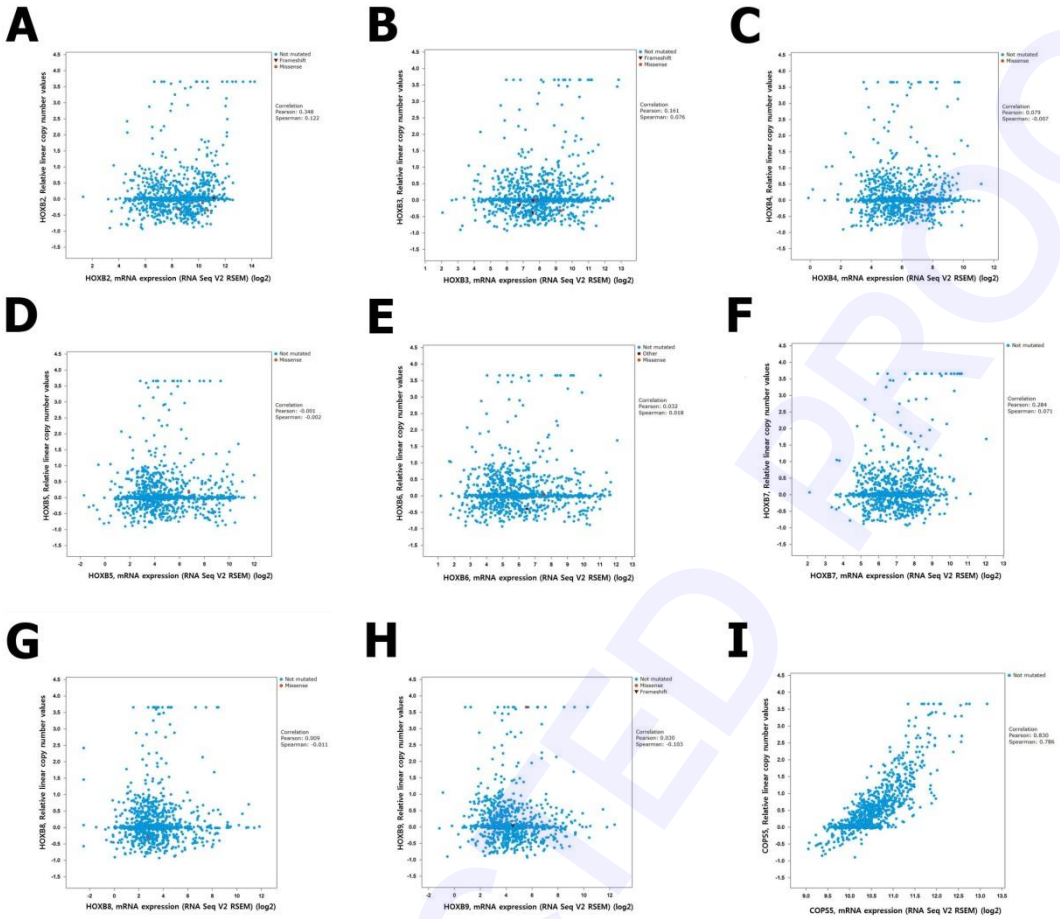
	Forward primer (5’-3’)	Reverse primer (5'-3')
HOXB2	CCTGGTGGAAAACAGAGAGC	CTGCTGGCCACGTAAAGAAG
HOXB3	GGACCTCAGAGACAGAAAGCTA	GTATCGCTTTCCTCTGAGGTG
HOXB4	CTCGGAGGATCACGTGGGCG	TC GACCCCTGACTCGTTTTCCT
HOXB5	CAAAGCCAACCTTCTCTCTGTT	CGTAAATTCTCGCTGATGACC
HOXB6	GTCTCACTCACTGTTGCACG	CACTTCCTCCTATTACCCGC
HOXB7	GTCCCTGCCTACAAATCATCC	CATGTTGAAGGAACTCGGCTC
HOXB8	CACCACTTAAAGAGGTCCTCG	CTCGCTCTGCGTTCTGTC
HOXB9	GATTTATGTCGGAGCTGACGC	GCTAAGCGTCCCAGAAATGG
HOXB13	GGACGTGTAAATGAGACTCTGC	CCTAAAGGCAGAAACTGCG
Gene desert #1	TGGTGGTCTGCCTTCTGCCAGT	TCACGTGGGAGGAAGAAGTAGGGC
Gene desert #2	TCATGGGTTGTGAAGACCGA	GGGGCAGGTCTGTATTCCTT



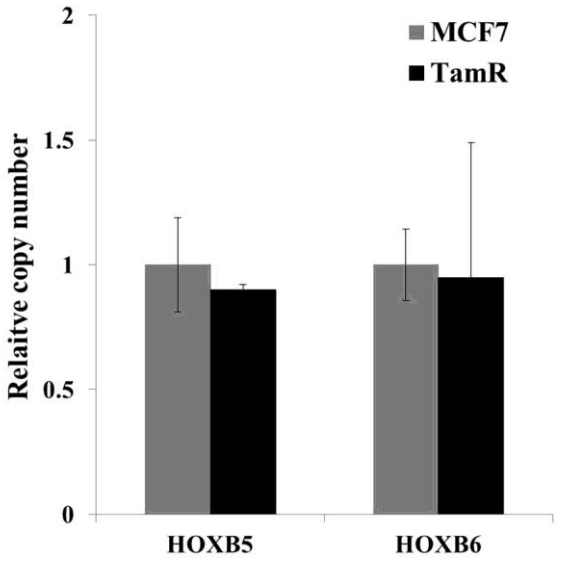
Supple Fig. 1



Supple Fig. 2



Supple Fig. 3



Supple Fig. 4