HOXB7 Is an ERα Cofactor in the Activation of HER2 and Multiple ER Target Genes Leading to Endocrine Resistance

Kideok Jin¹, Sunju Park¹, Wei Wen Teo¹, Preethi Korangath¹, Sean Soonweng Cho¹, Takahiro Yoshida¹, Balázs Győrffy², Chirayu Pankaj Goswami³, Harikrishna Nakshatri³, Leigh-Ann Cruz¹, Weiqiang Zhou⁴, Hongkai Ji⁴, Ying Su⁵, Muhammad Ekram⁵, Zhengsheng Wu⁶, Tao Zhu⁶, Kornelia Polyak⁶, and Saraswati Sukumar¹
ABSTRACT

Why breast cancers become resistant to tamoxifen despite continued expression of the estrogen receptor-α (ERα) and what factors are responsible for high HER2 expression in these tumors remains an enigma. HOXB7 chromatin immunoprecipitation analysis followed by validation showed that HOXB7 physically interacts with ERα, and that the HOXB7–ERα complexes enhance transcription of many ERα target genes, including HER2. Investigating strategies for controlling HOXB7, our studies revealed that MYC, stabilized via phosphorylation mediated by EGFR-HER2 signaling, inhibits transcription of miR-196a, a HOXB7 repressor. This leads to increased expression of HOXB7, ER target genes, and HER2. Repressing MYC using small-molecule inhibitors reverses these events and causes regression of breast cancer xenografts. The MYC-HOXB7–HER2 signaling pathway is eminently targetable in endocrine-resistant breast cancer.

SIGNIFICANCE: HOXB7 acts as an ERα cofactor regulating a myriad of ERα target genes, including HER2, in tamoxifen-resistant breast cancer. HOXB7 expression is controlled by MYC via transcriptional regulation of the HOXB7 repressor miR-196a; consequently, antagonists of MYC cause reversal of selective ER modulator resistance both in vitro and in vivo. Cancer Discov; 5(9): 944–59. © 2015 AACR.

INTRODUCTION

In order to antagonize estrogen receptor-α (ERα) function in ER-positive breast cancer, various endocrine therapies have been used, such as selective ER modulation via SERMs, ligand deprivation using aromatase inhibitors, and ER downregulation with fulvestrant (1–4). Despite the worldwide use of tamoxifen as adjuvant treatment for postmenopausal women with ER-positive breast cancer, cancer recurs in about one third of tamoxifen-treated patients (5). Thus far, two major pathways of endocrine resistance, through ER itself or receptor tyrosine kinases (RTK), have played pivotal roles in endocrine therapy. In acquired tamoxifen resistance, ER expression is maintained at detectable levels in the majority of tumors, and ER continues to promote tumor proliferation (6). With regard to RTKs, upregulation of ERBB/HER family members, such as EGFR and HER2, was observed in endocrine-resistant tumors, but the mechanism underlying increased expression of ERBB/HERs is not clear (6–8).

Several studies on HOX genes, including HOXB7, HOXB13, HOXC10, and HOXC11, and a cofactor for the homeobox gene, PBX1, have been reported in endocrine-resistant breast cancer (9–13). We recently reported that HOXB7 overexpression confers tamoxifen resistance through upregulation of EGFR signaling (9). Here, we have provided evidence for the function of HOXB7 as an ER cofactor in the activation of ER target genes and HER2. Furthermore, we have identified the upstream regulators of HOXB7 that are amenable to therapeutic targeting directed at overcoming endocrine resistance in breast cancer.

RESULTS

HOXB7 Promotes ER Target Gene Expression

It is known that the cross-talk between ER and other transcription factors can promote ER target gene expression (14–16). We had previously observed that estrogen supplementation of mice caused an increase in the size of xenografts of ER-positive MCF7 cells overexpressing HOXB7, but not of vector control MCF7 cells (9). This finding motivated the hypothesis that HOXB7 may participate in the ER signaling pathway as an ER coactivator. Microarray expression analysis was performed with MCF7–HOXB7 cells and MCF7 vector cells. A mean rank gene set enrichment test (17) using moderated t statistics (Supplementary Methods) showed a significant number of genes induced by HOXB7-overlapping genes in the same direction altered in MCF7 cells in response to estrogen (18). Specifically, we observed a family-wise error rate (FWER) of < 0.001 (Fig. 1A; Supplementary Fig. S1A, S1B; Supplementary Table S1). This finding is also corroborated by a Gene Ontology (GO) analysis (Supplementary Fig. S1C). A large-scale cross-sample gene expression correlation analysis based on the ChIP-PED dataset (n = 13,182; ref. 19; which uses the combined strength of ChIP/ChIP and Chip-Seq data along with the large amounts of publicly available gene expression data to discover...
Figure 1. HOXB7 interacts with ERα and enhances expression of ER target genes. **A**, heatmap representing relative expression of ER target genes (identified in ref. 18) in MCF7–HOXB7 cells compared with MCF7 vector cells determined by microarray analysis. **B**, density curves for cross-sample (n = 13,162) gene expression correlations between HOXB7 and ER target genes versus randomly selected genes. Correlation between HOXB7 and ER target genes is significantly (P < 10^{-15}) different from correlation between HOXB7 and random genes. **C**, real-time RT-qPCR analysis of ER target genes in stable HOXB7-overexpressing MCF7 cells. Immunoblot analysis of HOXB7 and ER target genes in D, MCF7–HOXB7 and E, MCF7-TMR1 and H12 (MCF7–EGFR/HER2) cells compared with vector control cells. F, RT-qPCR analysis of ER target genes in HOXB7-depleted MCF7–TMR1 cells using siRNAs. Immunoblot analysis of HOXB7 and ER target genes in HOXB7-depleted TMR1 G and BT474 H cells compared with vector control cells. I, RT-qPCR analysis of ER target genes in MCF7–HOXB7 stable cells incubated in estrogen-deprived medium (5% charcoal-stripped serum in phenol red-free DMEM) for 48 hours before treatment with vehicle (veh). 10 nmol/L E2 and 1 μmol/L tamoxifen (TAM) for 24 hours. Co-IP analysis performed in J, TMR1 cells using anti-ER or HOXB7 antibody and Western blot analysis using anti-HOXB7 or ER antibody, or K, Co-IP analysis in MCF7–FLAG-tagged-HOXB7 cells using anti-ER antibody and Western blot analysis using anti-FLAG antibody. L, Co-IP analysis performed with anti-FLAG antibodies in MCF7 cells transfected with FLAG-tagged full-length and deletion mutants of HOXB7 constructs. WT, full-length HOXB7; N1, N-terminal deletion 1 (1–14); N2, N-terminal deletion 2 (38–79); WM, W129F and M130I; ΔH3, deletion of Helix domain 3 of homeodomain (183–192); ΔGlu, deletion of glutamic acid tail; TMR, tamoxifen-resistant MCF7 cells. Mean ± SD for three independent replicates. *, P < 0.001.
new biologic contexts with potential transcription factor (TF) regulatory activities) allowed us to confirm that HOXB7 expression significantly correlated with ER target genes (Fig. 1B; Supplementary Fig. S1D–S1K; Supplementary Table S2). Real-time qPCR and immunoblot analysis showed that HOXB7 enhances endogenous ER target gene expression, resulting in overexpressing McCF7–HOXB7 cells (two clones: McCF7–B7-1 and McCF7–B7-2; Fig. 1C and D), T47D–HOXB7 and ERIN–HOXB7 (MCF10A-ER; Supplementary Fig. S2A–S2C), tamoxifen-resistant McCF7 cells (TMR), and McCF7 cells overexpressing both EGFR and HER2 (H12; Fig. 1E). Conversely, siRNA-mediated depletion of HOXB7 expression in cells with high endogenous HOXB7 expression, TMR (Fig. 1F and G) and BT-474 cells (Fig. 1H), resulted in reduced expression of ER target genes. Interestingly, we observed that when cells were treated with estrogen (E2), the mRNA expression of the ER target genes was not significantly altered in ER-binding regions of the CA12 gene in McCF7–HOXB7 cells, ER coactivators were strikingly enriched at levels higher than the HOXB7 coactivators. In contrast, the recruitment of NCOR, an ER corepressor, was decreased at the ER-binding sites (Supplementary Fig. S3G). Following knockdown of HOXB7 expression, recruitment of both ER and HOXB7 coactivators was significantly reduced, whereas NCOR recruitment was increased (Supplementary Fig. S3H). When tamoxifen binds to ER in tamoxifen-sensitive cells, it induces a conformational change in ER and recruits corepressors to inhibit ER target gene transcription. However, when tamoxifen binds to ER in tamoxifen-resistant cells, coactivators are recruited to ER-binding sites instead, resulting in select ER target gene transcription. However, the detailed mechanism remains unclear (26). To shed light on this question, we investigated whether HOXB7 functions as a major recruiter of ER coactivators in tamoxifen-resistant cells. The same ChIP assays were performed as in Supplementary Fig. S3G and S3H, now, after tamoxifen treatment. As predicted, in the presence of tamoxifen, recruitment of coactivators at the ER-binding site in the CA12 gene locus was higher in McCF7–HOXB7 cells than in vector controls. Depletion of HOXB7, on the other hand, resulted in enrichment of corepressors at the site (Fig. 2C and D). These events were also confirmed by HOXB7 ChIP analysis to ER-binding sites at a second ER target gene, MYC (Supplementary Fig. S3I–S3L). In addition, we found that the recruitment of ER coactivators or repressors to ER-binding sites in CA12 or MYC loci was regulated by HOXB7 expression in a dose-dependent manner (Supplementary Fig. S4A and S4B). To investigate the detailed mechanism of how the ER–HOXB7 complex promotes CA12 transcription, we created CA12–luciferase constructs, where we subcloned the genomic 1.7-kb CA12-promoter sequence, including the ER-binding sites and two putative HOXB7-binding sites, into the pGL3 promoter vector (Supplementary Fig. S4C and S4D). We found that overexpression of HOXB7 enhanced luciferase activity, and HOXB7 depletion (using shRNA) resulted in decreased luciferase activity for the CA12-WT and CA12-HOXB7-2A constructs, but not for the CA12-HOXB7-1A construct. These data suggested that HOXB7 was recruited to the HOXB7-1 site in the CA12 gene locus (Fig. 2E and F), and that the ER–HOXB7 complex is critical for activating CA12 transcription (Supplementary Fig. S4E and S4F). To further confirm these findings in a second ER target gene, we created MYC–luciferase constructs containing an ER enhancer region (27) tagged to three different putative HOXB7-binding regions in MYC. These regions were MYC-B7-1, MYC-B7-2, and MYC-B7-3, which were selected through analysis of DNase-seq

Identification of Novel HOXB7-Binding Sites in ER Target Genes

Given the robust upregulation of ER target genes by HOXB7, we explored the role of HOXB7 in regulating the interaction of ER with chromatin at the promoters of ER target genes. ER-binding sites are frequently located further upstream of the genes, such as in enhancer regions (20–23). Using ER-binding sites identified by ER-ChIP analysis in published data profiles (24), present in the proximal promoter or intron regions in ER target gene loci (Supplementary Fig. S3A), we performed HOXB7 chromatin immunoprecipitation (ChIP) analysis of known ER-binding regions in the ER target genes loci, RARA, GREB1, MYC, XBPI, FOS, CCND1, CXCL12, NRIP1, and CA12. ER ChIP analysis was also performed as a positive control. As predicted, following estrogen or tamoxifen treatment, ER was recruited to its binding sites. Interestingly, HOXB7 was also recruited significantly to the ER-binding site. In addition, the occupancy of HOXB7 to the site was enhanced with tamoxifen treatment in ER target gene loci (Fig. 2A and Supplementary Fig. S3B and S3C). A Re-ChIP assay confirmed concurrent presence of both HOXB7 and ER proteins at the same ER-binding region within the target genes (Fig. 2B and Supplementary Fig. S3D–S3F). To investigate the mechanism by which the HOXB7–ER complex may enhance CA12 gene transcription, ChIP assays were performed with pioneer factors FOXA1 (24) and PBX1 (25), ER cofactors (AIB1, SRC-1, CBP, p300, NCOR, and PAX2), and HOXB7 cofactors (PBX2 and MELIS1) to measure their occupancy at the ER-binding site within the CA12 gene in McCF7–HOXB7 cells compared with McCF7 vector cells. Although the recruitment of ER itself and pioneer factors was not significantly altered in ER-binding regions of the CA12 gene in McCF7–HOXB7 cells, ER coactivators were strikingly enriched at levels higher than the HOXB7 coactivators. In contrast, the recruitment of NCOR, an ER corepressor, was decreased at the ER-binding sites (Supplementary Fig. S3G). Following knockdown of HOXB7 expression, recruitment of both ER and HOXB7 coactivators was significantly reduced, whereas NCOR recruitment was increased (Supplementary Fig. S3H). When tamoxifen binds to ER in tamoxifen-sensitive cells, it induces a conformational change in ER and recruits corepressors to inhibit ER target gene transcription. However, when tamoxifen binds to ER in tamoxifen-resistant cells, coactivators are recruited to ER-binding sites instead, resulting in select ER target gene transcription. However, the detailed mechanism remains unclear (26). To shed light on this question, we investigated whether HOXB7 functions as a major recruiter of ER coactivators in tamoxifen-resistant cells. The same ChIP assays were performed as in Supplementary Fig. S3G and S3H, now, after tamoxifen treatment. As predicted, in the presence of tamoxifen, recruitment of coactivators at the ER-binding site in the CA12 gene locus was higher in McCF7–HOXB7 cells than in vector controls. Depletion of HOXB7, on the other hand, resulted in enrichment of corepressors at the site (Fig. 2C and D). These events were also confirmed by HOXB7 ChIP analysis to ER-binding sites at a second ER target gene, MYC (Supplementary Fig. S3I–S3L). In addition, we found that the recruitment of ER coactivators or repressors to ER-binding sites in CA12 or MYC loci was regulated by HOXB7 expression in a dose-dependent manner (Supplementary Fig. S4A and S4B). To investigate the detailed mechanism of how the ER–HOXB7 complex promotes CA12 transcription, we created CA12–luciferase constructs, where we subcloned the genomic 1.7-kb CA12-promoter sequence, including the ER-binding sites and two putative HOXB7-binding sites, into the pGL3 promoter vector (Supplementary Fig. S4C and S4D). We found that overexpression of HOXB7 enhanced luciferase activity, and HOXB7 depletion (using shRNA) resulted in decreased luciferase activity for the CA12-WT and CA12-HOXB7-2A constructs, but not for the CA12-HOXB7-1A construct. These data suggested that HOXB7 was recruited to the HOXB7-1 site in the CA12 gene locus (Fig. 2E and F), and that the ER–HOXB7 complex is critical for activating CA12 transcription (Supplementary Fig. S4E and S4F). To further confirm these findings in a second ER target gene, we created MYC–luciferase constructs containing an ER enhancer region (27) tagged to three different putative HOXB7-binding regions in MYC. These regions were MYC-B7-1, MYC-B7-2, and MYC-B7-3, which were selected through analysis of DNase-seq
data detailed in Methods (Supplementary Fig. S4G). We found that overexpression of HOXB7 enhanced luciferase activity, and HOXB7 depletion (using shRNA) resulted in decreased luciferase activity for constructs containing MYC-B7-1 or MYC-B7-2 but not MYC-B7-3 (Supplementary Fig. S4H and S4I). These results suggested that HOXB7 was recruited to the binding sites 1 and 2 in the MYC gene. Furthermore, we verified the formation of a chromatin loop between the ER-binding site and HOXB7-binding sites by using the chromosome conformation capture (3C) assay for the MYC gene in MCF7–HOXB7 cells after treatment with estrogen and tamoxifen (Supplementary Fig. S4J–S4L). This finding confirmed the occurrence of dynamic long-range chromatin interaction (~65 kb) between ER and HOXB7 bound to their cognate sites, in order to promote MYC transcription. Collectively, these results suggest that, when overexpressed HOXB7 binds to tamoxifen-bound ER, the HOXB7–ER complex tethers coactivators, resulting in ER target gene transcription in tamoxifen-resistant cells. Both HOXB7 and ER cooperate to upregulate CA12 and MYC expression, and HOXB7 augments ER genomic functions as an important coactivator (Fig. 2G and Supplementary Fig. S4M and S4N).

**HOXB7 Enhances HER2 Expression**

Upregulation of HER2 leads to poor prognosis in ER-positive breast cancer (28). As a consequence, the nature of the cross-talk between HER2 and ER has been studied for

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**Figure 2.** The ER-HOXB7 complex directly enhances transcriptional activity of ER target genes. A, HOXB7 ChIP assay of known ER-binding sites in ER target genes was performed in MCF7–HOXB7 cells incubated in estrogen-depleted medium (5% charcoal-stripped serum in phenol red-free DMEM) for 48 hours before treatment with vehicle, 10 nmol/L E2, and 1 μmol/L tamoxifen (TAM) for 45 minutes. B, ER-re-ChIP analysis following HOXB7 ChIP was performed as in A. ChIP analysis of each factor to the ER-binding site in the CA12 gene locus in (C) MCF7–HOXB7 cells compared with MCF7 vector cells and in (D) HOXB7-depleted TMR cells treated with tamoxifen. RNA Polymerase II ChIP analysis was performed for the CA12 promoter region as a positive control for CA12 transcription. E, luciferase assay was performed in MCF7 cells transiently cotransfected with HOXB7 or vector control plasmid along with wild-type (WT) CA12 promoter–luciferase constructs, or those with deleted HOXB7 binding sites—HOXB7-1Δ or HOXB7-2Δ. F, same assay was performed as in E in HOXB7-depleted TMR cells. G, an activation model of CA12 transcription through interaction between the HOXB7–ER complex and coactivators in tamoxifen-resistant cells. Mean ± SD for at least three independent replicates. *P < 0.001. RLU, relative luminescence units.
MYC Fine-Tunes HOXB7–ERα-Mediated Control of Target Genes

RESEARCH ARTICLE

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more than a decade (29), with a recent study concluding that ER and its cofactors directly regulate HER2 transcription (24). Our previous studies had also shown that HER2 expression is upregulated in HOXB7-overexpressing breast cancer (30). ChIP-PED analysis (19) showed a strong correlation \( r = 0.4670; P < 10^{-15} \) between HOXB7 and HER2 expression (Supplementary Fig. S5A). In the absence of evidence of direct transcriptional regulation, and based on the finding that HOXB7–ER interactions occur at many ER target gene promoters, we hypothesized that HOXB7 can regulate HER2 transcriptional activity via direct binding to its enhancer region along with ER, in a manner similar to that observed in the regulation of other ER target genes. Western blot analysis showed that overexpression of HOXB7 in MCF7 cells resulted in enhanced HER2 expression and phosphorylation at the Y1248 residue in the kinase domain, and activation of downstream effectors of HER2 signaling, such AKT, p44/42 MAPK, S6K, and 4EBP1 (Fig. 3A). Similar overexpression of EGFR and HER2, but not HER3 and HER4, was observed in TMR1 and TMR2 cells with endogenous high HOXB7 expression (Supplementary Fig. S5B), and similar HER signaling responses were seen in TMR and H12 cells (Supplementary Fig. S5C) and in two additional ER-positive breast cancer cells, T47D–HOXB7 and ERIN–HOXB7 cells (Supplementary Fig. S5D).

To define the precise contribution of HOXB7 in HER2 overexpression and tamoxifen sensitivity, we used tamoxifen-resistant TMR and BT474 cells. Stable depletion of HOXB7 in both cell lines using \( \text{HOXB7} \) shRNAs decreased HER2 expression and the downstream effectors of the HER2 signaling pathway (Fig. 3B). Knockdown of
HOXB7 in MCF7-TMR (Supplementary Fig. S5E) and in tamoxifen-resistant BT474 cells (Fig. 3C and Supplementary Fig. S5F) restored tamoxifen sensitivity. TMR and BT474 cells are dependent on HER2 for growth. Treatment of TMR and BT474 cells with the anti-HER2 antibody trastuzumab (31) or the dual EGFR/HER2 inhibitor lapatinib (Fig. 3C and Supplementary Fig. S5G; ref. 32) resulted in enhanced cell death, and this effect was even more significant upon depletion of HOXB7 (Fig. 3C and Supplementary Fig. S5H–S5J). Xenografts of MCF7-TMR-shHOXB7 regained sensitivity to tamoxifen and showed significant tumor regression (Fig. 3D; \( P < 0.001 \)). Growth of MCF7–HOXB7 cells, which also overexpress HER2, in culture (Fig. 3E) and as xenografts (IHC; Supplementary Fig. S5B), remained unaffected by tamoxifen alone, but was suppressed by the HER2 inhibitor trastuzumab. The addition of tamoxifen to trastuzumab caused significantly greater inhibition of tumor growth (Fig. 3F). These results suggest that depletion of HOXB7 may be a potential strategy for reversing tamoxifen resistance in breast cancer showing concurrent overexpression of HER2.

To evaluate whether the HOXB7–ER complex could directly regulate HER2 transcription, we performed ChIP assays with HOXB7, ER, and their cofactors to the previously described ER-binding site in the enhancer present in the first exon of the HER2 gene (24). Consistent with previous studies (24, 33), and our prior results with other ER target genes, we found that HOXB7, ER, and their cofactors were recruited to the ER-binding site within the HER2 gene (Supplementary Fig. S6A–S6C). Together, these results supported our hypothesis that the HOXB7–ER complex regulates HER2 transcriptional activity in tamoxifen-resistant breast cancer (Supplementary Fig. S6D).

To investigate the clinical relevance of HOXB7-mediated regulation of HER2 expression, we performed real-time qPCR analysis of HOXB7 and HER2 from 48 breast cancer cell lines that were divided into three groups according to their relative HER2 gene expression levels. We found that high HOXB7 expression correlated significantly (\( P < 0.0001 \)) with high expression of HER2 (Supplementary Fig. S6E). Furthermore, upon analysis of The Cancer Genome Atlas (TCGA) dataset, HOXB7 mRNA levels were found to be significantly increased (\( P < 0.0001 \)) in HER2-positive tumors (Supplementary Fig. S6F).

**miR-196a Regulates HOXB7 Expression in Tamoxifen Resistance**

In malignant melanoma cells, miR-196a binds directly to the HOXB7 3′-UTR and inhibits HOXB7 expression (34). miRNA array analysis showed that miR-196a expression is downregulated in endocrine-resistant breast cancer cells (35). From these observations, we posited that the upregulation of HOXB7 in MCF7-TMR cells was likely caused by reduced miR-196a expression. Consistent with this notion, we found that the miR-196a expression level was lower in TMR cells than in the parental MCF7 cells, as well as in BT474 cells, in comparison with other ER-positive tamoxifen-sensitive cells, such as T47D, CAMA1, and ZR75-1, where HOXB7 expression was relatively low (Fig. 4A). Overexpression of miR-196a inhibited HOXB7 expression, leading to the decrease of ER target gene and HER2 expression in TMR cells, as shown by RT-qPCR (Fig. 4B) and Western blot analysis for prototypic molecules (Fig. 4C). Enforced miR-196a expression resensitized TMR-miR-196a cells to tamoxifen, as shown by colony formation (Fig. 4D) and MTT assays (Fig. 4E), and caused regression of stably transfected BT474-miR-196a tumors upon treatment with tamoxifen (Fig. 4F). Introducing the miR-196a inhibitor (36) into MCF7 cells increased ER target gene expression (Fig. 4G). Using HOXB7–3′-UTR luciferase constructs (WT and mutant MT-A10 bp miR-196a target sequence), we confirmed the inhibitory effect of miR-196a on HOXB7 transcription in MCF7–miR-196a (Supplementary Fig. S7A), H12 (MCF7–EGFR–HER2; Supplementary Fig. S7B), TMR (Supplementary Fig. S7C), and MCF7–miR-196a inhibitor–expressing cells (Supplementary Fig. S7D). We also confirmed that ER transcriptional activity is not modulated by miR-196a by conducting ERE-luciferase assays in TMR cells and in MCF7 cells. ERE-luciferase activity was not significantly altered in both miR-196a–overexpressing TMR and miR-196a inhibitor overexpressing MCF7 cells (Supplementary Fig. S7E). These findings suggest that downregulation of miR-196a induced HOXB7 overexpression in TMR cells, and conversely, reconstituting miR-196a expression in cells should elicit therapeutic effects. Also, as demonstrated by others (37), the success in restoring miRNA expression with nanoparticle preparations to achieve therapeutic advantage supports the rationale of this approach.

**Stability of MYC Controls miR-196a–HOXB7 Signaling**

The relationship between miRNAs and their target genes has been studied in depth; however, the regulation of miRNA expression itself remains largely unclear (38). So, we considered alternate approaches to resolving this problem. It was established early on that EGFR–HER2 signaling regulates MYC stability (39–41). Recent reports suggest that MYC activity may be a key pathway in endocrine resistance (42) and that MYC has the ability to repress miRNA expression (43). These studies led us to propose that a result of MYC-mediated repression of miR-196a expression would be a subsequent upregulation of HOXB7 expression. To determine how MYC functions in tamoxifen resistance, we first examined MYC protein stability following treatment with cycloheximide. We found that MYC protein was more stable in TMR (Fig. 5A and B), H12 (MCF7–EGFR–HER2), and MCF7–HOXB7 cells (Supplementary Fig. S8A–S8D) when compared with control cells. MAPK and AKT signaling pathways phosphorylate the MYC protein at the serine 62 residue, which stabilizes MYC protein (41, 44). Interestingly, we found hyperphosphorylation of MYC at serine (Ser62), but not at threonine (Thr58), occurring through EGFR–HER2 signaling in MCF7–HOXB7, TMR, and H12 cells (Fig. 5C and Supplementary Fig. S8E and S8F). MYC phosphorylation levels are responsive to HOXB7 levels; stable depletion of HOXB7 in TMR or BT474 cells resulted in significantly lower levels of MYC phosphorylation at Ser62 (Supplementary Fig. S8G). As predicted, depletion of MYC in TMR cells by MYC shRNAs resulted in decreased HOXB7, HER2, and ER target gene expression as measured by Western blot analysis (Fig. 5D); here, a higher level of miR-196a gene expression was also observed (Supplementary Fig. S8H). Similarly, inhibiting MYC using the MYC–MAX dimerization inhibitor 1058F4 (45) also resulted in higher levels of miR-196a.
expression in TMR cells (Supplementary Fig. S8I). In addition, depletion of MYC or overexpression of a dominant-negative MYC (S62A) in MCF7 cells caused a dramatic decrease in 3′-UTR- HOXB7 luciferase activity (Supplementary Fig. S8J and S8K). This allowed us to conclude that depletion of MYC decreased HOXB7 expression, in all probability, via upregulation of miR-196a. Thus far, the results support our hypothesis that MYC inhibits miR-196a expression, which results in decreased HOXB7 expression. However, MYC does not directly upregulate HOXB7 and HER2 function as shown by lack of change in HOXB7 promoter-luciferase and HER2 promoter-luciferase assays in scrambled or shMYC-transfected cells (Supplementary Fig. S8L and S8M). miR-196a was identified as a putative MYC target gene through global mapping of MYC-binding sites (46). We performed ChIP analysis to investigate if MYC can bind directly to putative binding sites in the miR-196a gene locus using both TMR–shMYC cells and TMR cells treated with MYC inhibitors. We found that MYC binds directly to the MYC-binding site (chr17:46707861–46708471) and recruits corepressors to the miR-196a gene locus to inhibit miR-196a transcription (Fig. 5E and Supplementary Fig. S8N–S8P).

Using a miR-196a luciferase construct that contained a putative MYC-binding site (chr17:46707861–46708471) and recruits corepressors to the miR-196a gene locus to inhibit miR-196a transcription (Fig. 5E and Supplementary Fig. S8N–S8P). These results suggested that the recruitment of MYC to its putative binding site in the miR-196a promoter contributes to the repression of miR-196a expression in TMR cells.

To determine whether inactivation of MYC can sensitize TMR cells to tamoxifen, we first depleted MYC using shRNA in TMR cells. Decrease in MYC levels significantly attenuated tamoxifen resistance in TMR cells (Supplementary Fig. S8R and S8S). Although targeted drug development against MYC

**Figure 4.** miRNA-196a regulates HOXB7 expression. **A,** real-time RT-qPCR analysis of miR-196a and HOXB7 in TMR parental cells and in four ER-positive breast cancer cell lines. **B,** real-time RT-qPCR analysis; and **C,** immunoblot analysis of ER target genes in TMR cells transiently transfected with miR-196a compared with vector control cells. **D,** crystal violet staining of TMR cells with enforced miR-196a expression in TMR-miR-196a cells compared with vector control cells. **E,** real-time RT-qPCR analysis of ER target genes and HOXB7 expression in MCF7 cells transiently transfected with two different miR-196a inhibitors compared with vector control cells. **F,** tumor growth of BT474 vector cells (same as in Fig. 3D) and BT474–miR-196a cell xenografts (mfp) in NSG mice treated with tamoxifen (pellets). **G,** real-time RT-qPCR analysis of ER target genes and HOXB7 expression in MCF7 cells transiently transfected with two different miR-196a inhibitors compared with vector control cells. **H,** crystal violet staining of TMR cells with enforced miR-196a expression in TMR-miR-196a cells compared with vector control with tamoxifen treatment at different doses. **I,** tumor growth of BT474 vector cells (same as in Fig. 3D) and BT474–miR-196a cell xenografts (mfp) in NSG mice treated with tamoxifen (pellets). **J,** real-time RT-qPCR analysis; and **K,** immunoblot analysis of ER target genes in TMR cells with enforced miR-196a expression in TMR-miR-196a cells compared with vector control with tamoxifen treatment at different doses. **L,** tumor growth of BT474 vector cells (same as in Fig. 3D) and BT474–miR-196a cell xenografts (mfp) in NSG mice treated with tamoxifen (pellets). **M,** real-time RT-qPCR analysis of ER target genes in TMR cells with enforced miR-196a expression in TMR-miR-196a cells compared with vector control with tamoxifen treatment at different doses. **N,** tumor growth of BT474 vector cells (same as in Fig. 3D) and BT474–miR-196a cell xenografts (mfp) in NSG mice treated with tamoxifen (pellets). **O,** real-time RT-qPCR analysis of ER target genes in TMR cells with enforced miR-196a expression in TMR-miR-196a cells compared with vector control with tamoxifen treatment at different doses. **P,** tumor growth of BT474 vector cells (same as in Fig. 3D) and BT474–miR-196a cell xenografts (mfp) in NSG mice treated with tamoxifen (pellets). **Q,** real-time RT-qPCR analysis of ER target genes in TMR cells with enforced miR-196a expression in TMR-miR-196a cells compared with vector control with tamoxifen treatment at different doses.
has been a challenge, we projected that MYC inhibitors could alter tamoxifen resistance via HOXB7 reduction. Using two MYC inhibitors of MYC–MAX dimerization, 10058-F4 and 10074-G5 (47), we observed not only a decrease of HOXB7 expression but also a reduction of HER2 and ER target gene expression (Fig. 5F and Supplementary Fig. S8T and S8U). In addition, combined treatment with 10058-F4 and trastuzumab synergistically reduced cellular viability of BT474 cells (Fig. 5G and Supplementary Fig. S8V) and colony formation (Fig. 5H), effects that could be rescued by exogenous expression of HOXB7 in these cells (Supplementary Fig. S8W).

Furthermore, the drug combination caused significant reduction of BT474 tumor growth in immunodeficient NSG mice compared with single agents (Fig. 5I and Supplementary Fig. S9A–S9C). In addition, fulvestrant reduced cellular viability of TMR and BT474 cells and caused regression of MCF7–HOXB7 xenografts (Supplementary Fig. S9D and S9E). Collectively, the data strongly support the notion that MYC is a critical molecule in regulating HOXB7 expression.

MYC–HOXB7–HER2 Predicts Clinical Outcome in Tamoxifen-Resistant Breast Cancer Patients

In order to examine if the MYC–HOXB7–HER2 pathway can be predictive of endocrine resistance in breast cancer,
we first examined HOXB7 expression in endocrine therapy–treated ER-positive breast cancer patients (n = 1,208) from the Metabric database. We found a statistically significant association between high HOXB7 expression and poor overall survival (OS; HR, 1.37; P = 0.002). In addition, coexpression of HOXB7, HER2, and MYC was significantly prognostic of OS (HR, 2.80; P = 5.5E−06) and more significant than HOXB7 alone. To determine commonality across various datasets, we investigated three independent ER-positive breast cancer patient datasets and found that a combination of HOXB7 and MYC or HOXB7 and HER2 expression was significantly prognostic of OS or relapse-free survival (RFS) in each dataset (Fig. 6A–C and Supplementary Fig. S10A–S10F). To further confirm the results of these analyses, we performed IHC for HOXB7, HER2, and MYC in a tissue microarray containing 72 tumor samples of ER-positive breast cancer from patients treated with tamoxifen monotherapy. We found that a combination of HOXB7 and MYC or HOXB7 and HER2 expression was significantly correlated with OS or RFS (Supplementary Fig. S10G–S10K). These results provide further support to our postulate that the MYC–HOXB7–HER2 signaling pathway is associated with endocrine resistance in patients diagnosed with breast cancer.

**DISCUSSION**

ER-targeted therapy by using a selective ER modulator (SERM), tamoxifen (1, 2, 48), or aromatase inhibitors (AI), such as anastrozole, exemestane, and letrozole (3), or the selective ER downregulator (SERD) fulvestrant (4) is effective in treating breast cancer in postmenopausal women (49). However, long-term endocrine treatment often leads to development of de novo resistance in ERα-positive breast cancer. Many underlying molecular events that confer resistance are known, but a unifying theme is yet to be revealed. Here, we have described findings that support a key role of HOXB7 in endocrine resistance and identify novel potential therapeutic targets among the upstream regulators of HOXB7.

Despite the fact that over time many ER-positive breast tumors lose estrogen dependence for growth, nearly 30% retain ER expression and can therefore respond to a different endocrine therapy (6). This implies that activated ER can still serve as a therapeutic target. One of the thought-provoking findings of our previous study was that xenograft growth of ER-positive breast cancer cells overexpressing HOXB7 was more robust upon supplementation of the mice with exogenous estrogen (9), suggesting a cross-talk between HOXB7 and ER. If so, the two transcription factors, acting together, could confer tamoxifen resistance in breast cancer cells. In line with this prediction, we found that a large number of genes induced by HOXB7 were common to those induced by ER. This overlap prompted the hypothesis that HOXB7 may act as an ER cofactor to activate the ER signaling pathway. This hypothesis gained strength through observations of upregulation of ER target genes in conjunction with HOXB7 overexpression and downregulation of ER target genes upon HOXB7 depletion, aided by...
co-IP studies that supported their physical interaction (Fig. 1). ChIP analyses strengthened the finding that the HOXB7–ER complex transcriptionally activates several ER target genes. For the first time, both CA12 and MYC were identified as putative HOXB7 targets (Fig. 2). Our data implicated HOXB7 as a key ER cofactor that promotes ER target gene expression, which could also serve as yet another target in endocrine therapy.

RTKs regulate cellular differentiation and proliferation through transmitting extracellular signals into cells. In endocrine resistance, upregulation of ERBB–HER signaling plays an important role in altering cellular response to tamoxifen (6–8). Thus far, the current strategy for overcoming endocrine-resistant breast cancer is to use (i) an EGFR inhibitor (gefitinib), (ii) a HER2 inhibitor (trastuzumab), (iii) an EGFR/HER2 dual tyrosine kinase inhibitor (lapatinib), or (iv) an mTOR inhibitor (everolimus) combined with tamoxifen or aromatase inhibitors (50–55). Although these inhibitors have shown some clinical benefits for treating tamoxifen or AI-resistant breast cancer, toxicity and development of drug resistance remain major obstacles (56). Furthermore, it highlights the need to debilitate multiple RTK pathways in order to achieve efficient cytotoxic effects. Is it possible to destroy most, if not all, of the RTK-mediated pathways leading to endocrine resistance by targeting just one upstream gene? Our previous data provided evidence that HOXB7 is a direct, upstream regulator of the EGFR signaling pathway, and that long-term exposure to tamoxifen can lead to activation of the HER2 signaling pathway (9). Through a series of studies deciphering the complexes formed at the HER2 enhancer between ER and HOXB7, we were able to provide a common mechanistic basis to the upregulation of HER2 seen in TMR and in HOXB7 overexpressing cells. Functionally, not only did knockdown of HOXB7 correlate with a decrease in HER2 expression, it also restored tamoxifen sensitivity to tamoxifen-resistant cell lines both in vitro and in vivo (Fig. 3). These observations identify HOXB7 as a critical molecule in the control of HER2 expression in tamoxifen resistance.

Searching for clinically feasible strategies for inhibiting HOXB7, we investigated potential upstream regulators of HOXB7 and found miR-196a, a potential negative regulator of HOXB7 expression. We showed that miR-196a binds directly to the HOXB7 3′-UTR, resulting in downregulation of HOXB7 expression. Loss of miR-196a in TMR cells permits overexpression of HOXB7 in TMR breast cancer models both in vitro and in vivo. Through several lines of experimentation, we have provided convincing evidence that HOXB7 expression is regulated by miR-196a (Fig. 4). Although miRNAs constitute a highly attractive target for therapy, the field is in its infancy because there are yet many aspects of targeted delivery that need to be addressed before they become commonly used (57). The first miRNA currently in phase I clinical trials is MRX34, a synthetic miR-34 (a tumor suppressor downstream of p53) incorporated into liposomal nanoparticles, for liver cancer (58), whereas the most advanced is the anti-miR-122, complementary in sequence to miR-122, with a modified locked-nucleic acid structure (Miravirsen), which is directed against the hepatitis C virus (59).

Probing this question further, we investigated the regulation of miR-196a expression, and literature searches identified MYC as its upstream transcriptional regulator (46). This finding was particularly relevant because other studies have also suggested that MYC signaling could be a potential target in endocrine therapy (42, 60, 61). In this study, we show that increased stability of MYC protein, conferred by phosphorylation at serine 62 residue in tamoxifen-resistant cells, results in suppression of miR-196a expression and consequent promotion of HOXB7 expression. In support of this finding, through global mapping in silico of binding sites for MYC and additional ChIP analysis, we confirmed that MYC directly regulates miR-196a expression (Fig. 5). These findings provide a mechanistic explanation of how MYC can contribute to the increase of HOXB7 expression in models of tamoxifen resistance.

In summary, our mechanistic studies present evidence for amplification of a positive feedback loop where the interaction between HOXB7 and ER promotes activation of the HER2 signaling pathway and ER target gene expression, including MYC. Activated HER2 signaling results in phosphorylation and greater stability of MYC, which suppresses miR-196a, permitting HOXB7 expression in tamoxifen-resistant breast cancer (Fig. 7A and B).

Through preclinical testing performed in this study, we propose two potential therapeutic strategies to overcome hormone therapy resistance: First, downregulation of MYC activity by using a MYC inhibitor. Several pharmacologic approaches to inhibit MYC, drugs that interfere with MYC–MAX binding (10058-F4); chromatin modification (JQ1); cell cycle (dinaciclib); SUMOylation (ginkgolic acid); and metabolism (BPTES), have been reported, but MYC-targeted therapeutic strategy has not been clinically successful in treating high–MYC-expressing tumors (62). In this study, a combination of 10058-F4 and trastuzumab reduced tamoxifen-resistant tumor burden by downregulating HER2 expression. Whether other MYC inhibitors have the same effects combined with trastuzumab in tamoxifen-resistant breast cancer deserves attention. Second, enforced miR-196a expression could repress HOXB7 expression. Recently, it was reported that nanoparticle-mediated delivery of a Dicer product (miRNA duplex) or mature miRNA (single-stranded) as plasmid DNA was effective in silencing target gene expression. To carry miRNA, three different nanoparticles are used: (i) an inorganic nanoparticle such as gold (Au), quantum dots, silicon oxide, and iron oxide; (ii) liposomes such as DOTAP and DOTMA; or (iii) polymers such as PEI and PLGA. The advantage of the nanoparticle–miRNA complex is stability, resistance to degradation, and protection from its niche compared with naked miRNA (63). Although miRNAs have multiple targets, we have demonstrated that overexpressed miR-196a resensitizes tamoxifen-resistant cells to tamoxifen by inhibition of HOXB7 expression without much effect on cell proliferation. This implies that increasing miR-196a could be a specific therapeutic intervention for tamoxifen-resistant breast cancer, suggesting that future studies could investigate effects of nanoparticle–miR-196a complexes in HER2–MYC–HOXB7–positive tumors. Finally, our studies showed that destruction of ER using fulvestrant (ICI 182,780; ref. 64) reduced CA12 and MYC luciferase activity in MCF7–HOXB7 cells (Supplementary Fig. S4F). In addition, fulvestrant inhibited growth of TMR cells and caused regression of MCF7–HOXB7 xenografts (Supplementary Fig. S9D and S9E). Thus, fulvestrant, a well-established selective estrogen...
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**Methods**

Additional details are provided in the Supplementary Data.

**Cell Lines**

MCF7, MCF7–HOXB7, MCF7–EGFR, MCF7–HER2, MCF7–EGFR–HER2 (H12), BT474, BT474–HOXB7KO, T47D, T47D–HOXB7, ERIN, and ERIN–HOXB7 cells were used. MCF7, BT474, and T47D cells were purchased from the ATCC (authenticated independently). Cells were cultured for a maximum of 4 weeks before thawing fresh, early passage cells. HOXB7, EGFR, HER2 (H12), BT474, BT474-HOXB7KO, T47D, and T47D–HOXB7 cells were used. MCF7, BT474, and T47D cells were purchased from the ATCC (authenticated using short tandem repeat profile analysis). Stable cell lines were not authenticated independently. Cells were cultured for a maximum of 4 weeks before thawing fresh, early passage cells. HOXB7, EGFR, and HER2 status was confirmed by Western blot analysis and real-time RT-qPCR. All cells were confirmed to be Mycoplasma negative (Hoechst stain and PCR; tested in 2014). For deriving vector control and HOXB7–overexpressing cell lines, the pcDNAs vector or pcDNAs–Flag-HOXB7 was stably transfected into MCF7, T47D, and ERIN cells using Lipofectamine 2000 (Invitrogen, Life Technologies). Cell lines were maintained at 37°C in a 5% CO2 incubator. MCF7 cells and derivatives were cultured in DMEM medium (Mediatech) supplemented with 10% heat-inactivated FBS (Mediatech), 100 IU/mL penicillin, 0.25 μg/mL streptomycin (GIBCO, Life Technologies). BT474 and T47D cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. ERIN (MCF10A cells stably expressing ER) cells (kindly provided by Ben H. Park, Johns Hopkins University) and derivatives were cultured in DMEM/F-12 medium supplemented with 5% horse serum (Invitrogen), 2 mmol/L glutamine, 100 μg/mL streptomycin, 100 IU/mL penicillin, 0.25 μg/mL ampicillin B, 100 μg/mL cholora toxin, 20 ng/mL epidermal growth factor (EMD Millipore), 0.5 μg/mL hydrocortisone (EMD Millipore), and 10 μg/mL insulin. Cell lines were maintained at 37°C in 5% CO2 as described in Supplementary Data.

**Antibodies and Reagents**

The antibodies used in this study were from the following sources: EGFR (1:1,000; Santa Cruz Biotechnology), HER2 (1:1,000; Cell Signaling Technology), HER3 (1:1,000; Cell Signaling Technology), HER4 (1:1,000; Cell Signaling Technology), HOXB7 (1:2,000; Invitrogen and Abcam), ERα (1:1,000; Santa Cruz Biotechnology), MYC (1:1,000; Abcam), phospho-MYC–serine 62 (1:1,000; Abcam), BCL-2 (1:1,000; Cell Signaling Technology), cyclin D1 (1:1,000; Cell Signaling Technology), actin (1:2,000; Sigma-Aldrich), phospho-p44/42 MAPK (1:1,000; Cell Signaling Technology), phospho-AKT–serine and threonine (1:1,000; Cell Signaling Technology), phospho-p70 (1:1,000; Cell Signaling Technology), phospho-MYC–threonine 58 (1:1,000; Applied Biosystems), phospho-p38MAPK (1:1,000; Cell Signaling Technology), phospho-ERK (1:1,000; Cell Signaling Technology), phospho-MAPK (1:1,000; Cell Signaling Technology), phospho-p300 (1:1,000; Cell Signaling Technology), phospho-MYC (1:1,000; Cell Signaling Technology), HDAC (1:1,000; Abcam), p300 (1 μg/mL; Invitrogen), FOXA1 (1 μg/mL; Abcam), PBX-1 (1 μg/mL; Santa Cruz Biotechnology), PBX-2 (1 μg/mL; Abcam), Meis-1 (1 μg/mL; Abcam), PAX-2 (1 μg/mL; Abcam), and RNA polymerase II (1 μg/mL; EMD Millipore). For in vitro assays, 4-hydroxytamoxifen (5 mg), β-estradiol (1 g), 10058-F4, and 10074-G5 (5 mg) were used and purchased from Sigma.
Co-immunoprecipitation

For Co-IP from cell cultures, MCF7 cells were transfected with 3 μg of each plasmid. After 48 hours, cells were washed with cold PBS and harvested in immunoprecipitation buffer [10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM/L EDTA, 0.5% Nonidet P-40, Phospho-stop (Roche Diagnostics), and Complete Protease Inhibitor Mixture (Roche)]. The lysate was then rotated at 4°C for 1 hour, followed by centrifugation at 14,000 rpm for 20 minutes. The supernatants were then combined with 50 μL of protein G Sepharose (GE Healthcare) preincubated with antibodies against FLAG or ER or HOXB7, followed by rotating at 4°C for 2 hours. The protein G Sepharose was pelleted and washed three times using immunoprecipitation buffer. The precipitates were resolved on SDS-PAGE gel and subjected to immunoblot analysis. Immunoblot signals were visualized with chemiluminescence (GE Healthcare). For mapping of the binding region between ER and HOXB7, FLAG-tagged HOXB7 deletion constructs were cotransfected with full-length ER plasmid.

Luciferase Assay

MCF7 or MCF7–TAMR cells were transiently cotransfected with CA12 or 3′-UTR region of the HOXB7–luciferase construct plus pcDNA3.1-empty vector or pcDNA3.1-HOXB7 or HOXB7 shRNAs or miR-196a plus β-galactosidase (β-GAL) plasmid for normalization. Cells were harvested 24 hours after transfection and lysates were assayed sequentially for luciferase and β-galactosidase activity, which were measured by following a protocol (Promega). Assays were conducted in triplicate in a single experiment and then as three independent experiments.

Chromatin Immunoprecipitation

ChIP was carried out according to the manufacturer’s instructions (EMD Millipore) with modifications. Briefly, cells were fixed with 1% formaldehyde for 10 minutes at room temperature. Glycerol quenched samples were lysed in 1 mL of SDS buffer containing protease inhibitors. The lysates were incubated for 10 minutes on ice and sonicated with a Covaris S220 (5% duty cycle, 4 intensity, 200 burst per cycle, 3 cycles of 60 seconds) for 30 minutes on ice. The samples were centrifuged at 10,000 × g at 4°C for 10 minutes and supernatant was taken. With precleared samples, immunoprecipitation was performed by using antibodies and Dynabead-protein G. Eluates were subjected to reverse cross-linking and DNA was recovered by phenol–chloroform–ethanol purification. qPCR was performed using primers described previously (24, 65).

Real-Time RT-qPCR

Total RNA was extracted with TRIzol reagent (Invitrogen), and cDNA was synthesized from total RNA (2 μg) using an M-MLV Reverse Transcriptase (Promega). Aliquots of cDNA were used as templates for a real-time RT-qPCR procedure. Relative quantification of mRNA expression was achieved using real-time PCR (7500 Real-Time PCR system; Applied Biosystems). The Maxima SYBR Green/ROX Master Mix (Fermentas) was used according to the manufacturer’s instructions.

Immunoblotting

Cells were rinsed with cold 1× PBS three times and lysed with RIPA buffer. Extracted lysate (40 μg) was vertically electrophoresed on 4% to 12% Bis-Tris NuPage Novex Gel in MOPS SDS running buffer (Invitrogen), and then transferred to Hybond C Extra membrane (GE Healthcare). Membranes were stained with Ponceau stain to confirm protein transfer, then blocked with 4% powdered milk in PBS with 0.2% Tween-20 (PBST) for 1 hour at room temperature. Membranes were probed with primary antibody in 4% milk/PBST overnight, rinsed three times with 1× PBST for 5 minutes, then probed with secondary antibody [either anti-rabbit HRP or anti-mouse HRP (GE Healthcare) at 1:1,000 dilution in 5% milk/PBST for 1 hour]. Membranes were rinsed three times with 1× PBST for 5 minutes, and then treated with ECL Detection Reagent (GE Healthcare) for 1 minute. Membranes were exposed to Hyblot CL autoradiography film to determine protein expression. The quantification of protein level was performed by densitometric scanning and normalizing to intensity of actin.

Cell Viability Assay

Cells were plated at 2.5 × 104 cells per well in 96-well plates, in triplicate, with 200 μL media, with drug treatments at stated concentrations, a combination, or vehicle for 3 days. MTT solution (30 μL; 5 mg/mL in PBS) was added to each well and cells incubated for 2 hours. Media was then aspirated and cells resuspended in 200 μL of DMSO. Absorbance at 560 nm was measured, with background at 670 nm subtracted. Triplicates were averaged for a mean absorbance, and then a percentage calculated of survival of drug-treated cells versus time-matched vehicle-treated cells. Experiments were performed in triplicate.

Microarray Data Analysis

Data were preprocessed using Illumina GenomeStudio, where background-level intensities were subtracted and quantile normalized. Data were exported into R Statistical Software, where probes with P blasts < 0.05 were filtered out and further analysis was performed using the limma package from Bioconductor and custom functions where necessary. An unsupervised clustering heatmap was generated using the 1,000 most variable probes across all samples. Differentially expressed probes, defined as probes with a 2-fold change in expression and an FDR of < 0.05, were converted into Entrez gene identifiers and exported into Cytoscape, where GO analysis was performed using ClueGO with default settings. To understand the relationship between HOXB7 and ER target genes, an established ER target gene set was obtained from Dutertre and colleagues (18) and mapped to their respective probe IDs through Entrez Gene identifiers. Genes that were not represented by the array were discarded and the remaining genes were plotted on a supervised heatmap organized by ER regulation status (up- or downregulated). The limma implementation of mean-rank gene set enrichment (MR-GSE; refs. 17, 66) was used to identify the significance of enrichment of gene sets. MR-GSE uses the average ranks of t statistics, which reduces individual effects of individual genes and gives additional weight to gene sets with a large number of active genes. Such implementation also allows for tests against individual gene sets. Moderated t statistics obtained from limma comparing HOXB7 and vector controls were used as the input into MR-GSE and were tested for enrichment by upregulated or downregulated genes. The gene sets that were tested were the two estradiol response gene sets identified by Dutertre and colleagues (18) and a housekeeping gene set identified by Hsiao and colleagues (66) as a negative control. P values were corrected using Bonferroni correction to obtain FWER (67). The Gene Expression Omnibus (GEO) accession number for the microarray data reported in this article is GSE63607.

Mouse Xenograft Studies

All animal studies were approved by the Institutional Animal Care and Use Committee at Johns Hopkins University (Baltimore, MD). For experiments described in Fig. 3F, 6- to 8-week-old female athymic nude mice were used, and the study was approved by the Johns Hopkins Institutional Animal Care and Use Committee. MCF7–HOXB7 cells were grown to 90% confluence, trypsinized, resuspended in serum-free medium, and mixed 1:1 with Matrigel (BD Biosciences). Estrogen pellets (60-day slow release pellet containing 0.72 mg; Innovative Research of America) were implanted...
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subcutaneously (s.c.) at the nape of the neck two days before 2 × 10^6 cancer cells were injected s.c. with 50 μL of 1:1 PBS/Matrigel (BD Biosciences) in nude or NSG mice. When the tumor size reached about 200 mm^3, 6 mice in each group were treated with either (i) tamoxifen pellet implants, s.c. (60-day slow release pellet containing 5 mg), (ii) 20 mg/kg trastuzumab twice weekly, i.p., or (iii) a combination of tamoxifen and trastuzumab. For Figs. 3D and 4F, tamoxifen pellet was implanted 1 week before 2 × 10^6 BT474-HOXB7KO or BT474-miR-196a or BT474-vector control cells were injected into two mammary fat pads (mfp) of immunodeficient NSG mice with 50 μL of 1:1 PBS/Matrigel (BD Biosciences), using 3 mice per group. For Fig. 5, 2 × 10^4 BT474 cells were injected into the NSG mouse mfp with 50 μL of 1:1 PBS/Matrigel (BD Biosciences). When the tumor size reached about 100 mm^3, 6 mice in each group were treated with either (i) vehicle, (ii) 20 mg/kg trastuzumab twice weekly, i.p., (iii) 30 mg/kg 10058-F4 (Selleckchem), i.p., daily, or (iv) a combination of trastuzumab and 10058-F4. Mice were measured weekly for tumor growth. After 6 to 8 weeks, mice were euthanized and tumors were dissected and fast-frozen, or formalin-fixed and paraffin-embedded and H&E-stained slides were made. Tumor volume was estimated as follows: V = (length × width × height) × 0.5236 mm^3. The 10058-F4 was a kind gift of Selleckchem.

Statistical Analysis

HOXB7 expression levels determined by RT-PCR were dichotomized into low and high groups using the median as cutoff. Kaplan–Meier analysis was performed as described previously (68). The Kaplan–Meier survival plot, hazard ratio, and log-rank P values were calculated and plotted using WinStat 2013 (R. Fitch Software). All statistical tests were two-sided, and differences were considered statistically significant at P < 0.05.

Disclosure of Potential Conflicts of Interest

S. Sukumar is a consultant/advisory board member for CPRIT, Texas; CEPHEID; and CBRCRF. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: K. Jin, S. Park, S. Sukumar


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Korangath, L.-A. Cruz, Y. Su, Z. Wu, T. Zhu, S. Sukumar


Writing, review, and/or revision of the manuscript: K. Jin, S. Park, T. Yoshida, B. Györfi, C.P. Goswami, H. Nakshatri, L.-A. Cruz, S. Sukumar

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Jin, W.W. Teo, K. Polyak, S. Sukumar

Study supervision: T. Zhu, S. Sukumar

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Kideok Jin, Sunju Park, Wei Wen Teo, et al.


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