HOXB7 Promotes Malignant Progression by Activating the TGFβ Signaling Pathway

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Abstract

Overexpression of HOXB7 in breast cancer cells induces an epithelial–mesenchymal transition and promotes tumor progression and lung metastasis. However, the underlying mechanisms for HOXB7-induced aggressive phenotypes in breast cancer remain largely unknown. Here, we report that phosphorylation of SMAD3 was detected in a higher percentage in primary mammary tumor tissues from double-transgenic MMTV-Hoxb7/Her2 mice than tumors from single-transgenic Her2/neu mice, suggesting activation of TGFβ/SMAD3 signaling by HOXB7 in breast tumor tissues. As predicted, TGFβ2 was high in four MMTV-Hoxb7/Her2 transgenic mouse tumor cell lines and two breast cancer cell lines transfected with HOXB7, whereas TGFβ2 was low in HOXB7-depleted cells. HOXB7 directly bound to and activated the TGFβ2 promoter in luciferase and chromatin immunoprecipitation assays. Increased migration and invasion as a result of HOXB7 overexpression in breast cancer cells were reversed by knockdown of TGFβ2 or pharmacologic inhibition of TGFβ signaling. Furthermore, knockdown of TGFβ2 in HOXB7-overexpressing MDA-MB-231 breast cancer cells dramatically inhibited metastasis to the lung. Interestingly, HOXB7 overexpression also induced tumor-associated macrophage (TAM) recruitment and acquisition of an M2 tumor-promoting phenotype. TGFβ2 mediated HOXB7-induced activation of macrophages, suggesting that TAMs may contribute to HOXB7-promoted tumor metastasis. Providing clinical relevance to these findings, by real-time PCR analysis, there was a strong correlation between HOXB7 and TGFβ2 expression in primary breast carcinomas. Taken together, our results suggest that HOXB7 promotes tumor progression in a cell-autonomous and non–cell-autonomous manner through activation of the TGFβ signaling pathway.

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Introduction

The HOX family of homeobox-containing genes encodes transcription factors that are highly conserved from Drosophila to Homo sapiens (1–3). The homeobox, a characteristic feature of this family of genes, is an 180-bp DNA sequence encoding a trihelical 60 amino acid homeodomain (3, 4). It is usually located at a terminal or subterminal position of the corresponding homeoprotein and is responsible for recognizing and binding sequence-specific DNA motifs (ATT/TAAT; refs. 5, 6). HOX genes have been identified as master transcriptional regulators controlling the coordinated expression of genes involved in development and differentiation (7). Recently, a growing body of literature has emerged on the involvement of HOX genes in the pathogenesis of cancers (8). Recently, a few lines of evidence were presented to suggest that HOXB7 also plays a role in tumorigenesis. First, HOXB7 was found to be frequently overexpressed in melanoma, ovarian, and breast cancer cell lines as well as primary tumor cells (9–11). Second, overexpression of HOXB7 in the breast cancer cell line SKBR3 increased proliferation and angiogenesis by upregulating basic fibroblast growth factor (bFGF; refs. 9, 12, 13). In addition, overexpression of HOXB7 in breast cancer cells induced epithelial–mesenchymal transition (EMT) and rendered breast cancer cells resistant to tamoxifen treatment through activation of the EGFR pathway (14, 15).

To study the role of Hoxb7 in breast tumorigenesis, our lab generated an MMTV-Hoxb7 FVB/N transgenic mouse model where expression of HOXB7 is regulated by the mouse mammary tumor virus (MMTV) promoter (16). Although overexpression of HOXB7 alone was not sufficient to cause tumour formation, in crosses of MMTV-Hoxb7 mice with MMTV-Her2/neu transgenic mice, it dramatically impacted oncogene Her2/neu-induced tumorigenesis. In double-transgenic mice, overexpression of HOXB7 delayed tumor onset and lowered tumor multiplicity (16), but promoted tumor progression and metastasis. This contrasting phenotype was intriguing and reminiscent of the dual role of TGFβ in breast cancer. Siegel and colleagues used transgenic...
mouse models to demonstrate that TGFβ signaling suppressed Her2/neu-induced mammary tumor growth while promoting subsequent lung metastasis (17). This led us to hypothesize that HOXB7 may directly or indirectly regulate TGFβ signaling.

In line with this hypothesis, we have now demonstrated that overexpression of HOXB7 induces the expression of TGFβ2 in both mouse and human breast cancer cell lines, leading to increased cell motility and invasiveness, and recruitment and activation of macrophages. Expression of HOXB7 and TGFβ2 is strongly correlated in primary breast cancer tissues and is associated with advanced stages of tumor progression. Overall, our results suggest that HOXB7 may be a potential therapeutic target in invasive and metastatic breast cancer.

Materials and Methods

Primary tissue samples and cell culture

Human breast cancer tissue samples were obtained through the South Carolina Tissue Bank with approval from the Institutional Review Board at the University of South Carolina (Columbia, SC). Tissue samples were randomly collected from patients who were diagnosed with invasive breast ductal carcinoma between 2003 and 2007. Their clinicopathologic characteristics are summarized in Supplementary Table S1. Adjacent normal tissues that were at least 2 mm away from the tumor margins and confirmed to be free of tumor deposits were used as normal control in this study. The isolation of carcinoma cells from tumors developing in MMTV-Her2/neu transgenic mice and establishment of the primary HER2 tumor cell line, H605, were described previously (18). All human breast cancer cell lines were obtained from ATCC, and with the exception of H605, were maintained in DMEM/F12 containing 5% horse serum, g/ml hydrocortisone, 100 ng/mL insulin, 0.5 µg/mL hydrocortisone, 100 ng/mL cholera toxin, and 20 ng/mL EGF.

Plasmids and stable cell lines

To generate luciferase reporter plasmids containing the TGFβ2 promoter sequences, DNA fragments containing different lengths of the TGFβ2 promoter sequence (nucleotide from −1015 to +68) were amplified using genomic DNA. The PCR products were cloned into pGL3-promoter vector. The plasmids for TGFβ2-183 and TGFβ2-25 were generated by cutting plasmid TGFβ2-1083 with Kpn I and Sac I, respectively. To construct HOXB7-overexpressing plasmids, mouse HOXB7 cDNA was inserted into pcDNA3, and human HOXB7 cDNA was inserted into pMSCV-IRES-GFP. To generate HOXB7 knockdown and TGFβ2 knockdown plasmids, shRNA for human HOXB7 and TGFβ2 were inserted into pSHH1-puro. The sequences for HOXB7 knockdown and TGFβ2 knockdown are ATCTTGATCTGTCTTTCCG and ATCTTGATCTGTCTTTCCG, respectively.

To generate HOXB7-overexpressing or knockdown stable cell lines, 293T cells were cotransfected with retroviral constructs of pMSCV-HOXB7 or pSHH1-HOXB7 along with the respective package system plasmids for 2 days. The supernatants were collected to infect different human breast cancer cell lines for 2 days, and then cells were collected for RT-PCR and Western blot analysis. To generate HOXB7-overexpressing MDA-MB-231 (MDA-B7), plasmid pB-HOXB7-eGFP and the empty vector were transfected into MDA-MB-231 cells. The GFP-positive cells were flow sorted and used in the experiments. The HOXB7-overexpressing MDA-MB-231 cells were further transfected with pSHH1-TGFβ2 to generate the TGFβ2 knockdown cell line (MDA-B7-β2KD). HOXB7-overexpressing H605 cell line was established by transfection of Her2 transgenic mouse mammary tumor cells. H605 with pcDNA3-HOXB7 or empty vector plasmids followed by G418 selection for establishing stable clones.

Animal experiments

All procedures were conducted in accordance with NIH regulations, and approved by the Institutional Animal Care and Use Committee of the University of South Carolina. In the tail-vein injection experiments, a total of 1 × 10⁶ MDA-MB-231 cells (MDA-con), MDA-MB-231/HOXB7 (MDA-B7) per animal were suspended in sterile PBS and injected into the tail vein of six NOD-SCID mice, 6 to 8 weeks of age. At 60 days after injection, the mice were killed and the lungs were photographed under fluorescent microscope, and then fixed in 10% formalin. Hematoxylin and eosin (H&E) staining of the entire paraffin-embedded lung tissue was performed. The metastatic foci were counted under ×200 magnification (ten randomly selected high-power fields). In the orthotopic transplantation experiments, 1 × 10⁶ H605 cells (mammary tumor cell line from MMTV-Her2 transgenic mice) per site were injected into the mammary fat pad of 6- to 8-week-old female FVB/N mice. At 30 days after injection, the tumors were collected and processed for FACS assay and immunofluorescence staining analysis.

Immunohistochemistry and immunofluorescent assay

Immunohistochemical (IHC) staining of formaldehyde-fixed, paraffin-embedded primary or xenograft tumor tissues was carried out as previously reported (14), using the human p-SMAD3 antibody at 1:100 dilution. Stained slices were scored according to intensity of staining and percentage of tumor cells staining positive for p-SMAD3 [0%, IHC level 0; 1%–30%, IHC level 1++; 31%–70%, IHC level 2++; and >70%, IHC level 3+].

For immunofluorescence staining of macrophages, fresh tumor tissues were embedded in OCT gel and kept at −80°C. Frozen tissue sections (10-µm slices) were fixed in 4% paraformaldehyde. Immunostaining was performed using FITC-conjugated anti-F4/80 (dilution, 1:200) and PE-conjugated anti-CD206 (dilution, 1:200). The sections were then counterstained with DAPI.

Treatment of macrophages with tumor cells conditioned media

To obtain conditioned media, stable cell lines MDA-B7, MDA-B7-β2KD, and the control MDA-con were seeded in a 10-cm dish at 5 × 10⁵ cells per dish and cultured till 90% confluence. The media was then replaced with serum-free DMEM. After 24 hours, the supernatants were collected and filtered through a 0.22-µm filter. To obtain mouse macrophages, mice were injected intraperitoneally with 3 mL of 3% thioglycollate in sterile PBS. Three days later, mice were euthanized and the peritoneal macrophages were harvested by lavaging the peritoneal cavity with 2 × 10 mL of PBS. Cells were suspended with DMEM media containing 10% FBS, and plated in 6-well plates. After 1 hour, the nonadherent cells were removed by PBS, and the adhered macrophages were further cultured in serum-free DMEM for 24 hours, followed by treatment with control...
DMEM or indicated tumor cell conditioned media for another 24 hours.

Other methods
Quantitative real-time RT-PCR analysis, chromatin immunoprecipitation (ChIP) assay, Western blot analysis, wound-healing assay, Matrigel invasion assay, and FACS assay were performed using standard protocol (14, 16, 19). See Supplementary Methods for more details.

Statistical analyses
The statistical analyses were conducted with R and GraphPad software packages (GraphPad). The association between the level of p-SMAD3 and overexpression of HOXB7 in mammary tumors was assessed with the Fisher exact test using R software. A Student t test or ANOVA test was used for comparison of quantitative data. The linear correlations between HOXB7 and TGFβ ligand expression in primary breast cancer tissues were evaluated with Pearson correlation coefficient analysis. Values of \( P < 0.05 \) were considered significant.

Results
Activation of TGFβ signaling in HOXB7-overexpressing mammary tumors
The dual role of HOXB7 in Her2/neu-induced tumorigenesis led us to hypothesize that HOXB7 may activate TGFβ signaling, which is known to inhibit tumor onset and promote tumor progression in several types of cancers (16, 17). TGFβ has been shown to signal most commonly through the SMAD family signaling pathway (20). The activation of the transmembrane TGFβ receptor leads to the phosphorylation and activation of SMAD3, which then forms a complex with SMAD4 to regulate the transcription of target genes by binding to DNA at specific SMAD-binding elements (20). To assess the involvement of the TGFβ signaling in both mammary tumor formation and subsequent metastasis, we examined the phosphorylation status of SMAD3 in primary tumors arising in MMTV-Her2/neu and MMTV-Hoxb7/neu transgenic mice. IHC staining analysis of SMAD3 phosphorylation revealed a striking difference between these two groups of tumors (Fig. 1A). The majority of primary mammary tumors from MMTV-Her2/neu mice showed undetectable (level 0) or weakly detectable (level 1+) staining signal and about 15% of tumors display moderate (level 2+) or strong (level 3+) p-SMAD3 staining. In contrast, all of the tumors from MMTV-Hoxb7/neu double-transgenic mice were positive for p-SMAD3 staining. MMTV-Hoxb7/neu mammary tumors (54.6%) showed strong staining (level 3+). Overall, overexpression of HOXB7 in mammary tumors was strongly associated with increased phosphorylation of SMAD3 (\( P = 0.048 \)).

Our previous studies have shown that overexpression of HOXB7 promotes lung metastasis (16). We therefore examined whether there is a correlation between the phosphorylation level of SMAD3 and the number of metastatic foci in the lung. As shown in Fig. 1B, tumors with higher phosphorylation level of SMAD3 were more likely to metastasize to lung. The number of metastasis foci was significantly higher in tumors with p-SMAD3 staining at the IHC level 3+ than in p-SMAD3negative tumors (\( P = 0.036 \)). Together, these findings suggested that HOXB7 may promote tumor progression and metastasis via enhancement of the TGFβ–SMAD3 pathway.

HOXB7 upregulates TGFβ2 expression in mammary tumors and breast cancer cells
Given that HOXB7 is a classical transcription factor, we performed RT-PCR analysis to screen the TGFβ signaling pathway genes in primary tumor cells with, and without HOXB7 overexpression. Semiquantitative RT-PCR analysis demonstrated an increase in TGFβ2 mRNA in all of the HOXB7-overexpressing primary mouse tumors compared with Her2/neu tumors. In contrast, no significant changes in the expression of TGFβ1 and TGFβ3 were observed (Fig. 1C). We next determined whether increased TGFβ2 mRNA in HOXB7-overexpressing primary tumors was reflected in increased TGFβ2 protein. TGFβ2 mRNA encodes a precursor protein of approximately 40 kDa, which is cleaved to an approximately 12 kDa active secreted protein. We found increased production of pro-TGFβ2 in HOXB7-overexpressing tumor cells in comparison with control cells (Fig. 1D). TGFβ signaling activation is associated with the activation (via phosphorylation) of SMAD3; indeed, we found increased phosphorylation of SMAD3 in HOXB7-overexpressing Her2 tumor cells compared with Her2 tumor cells (Fig. 1D).

To further determine whether HOXB7 can activate TGFβ2 expression in human breast cancer cells, human HOXB7-expressing plasmids were transiently transduced into two breast cancer cell lines (MDA-MB-231 and SKBR3) with low levels of HOXB7 expression (14). Semiquantitative RT-PCR and Western blot analysis were performed to examine the expression of HOXB7 and TGFβ2. Consistent with the results obtained in mouse tumor cells, overexpression of HOXB7 in two human breast cancer cell lines, MDA-MB-231 and SKBR3, increased TGFβ2 expression at both mRNA and protein levels (Fig. 1E and 1F), and induced an autocrine TGFβ signaling as evidenced by increased phosphorylation of SMAD3 in HOXB7-overexpressing cells compared with control cells (Fig. 1E). Conversely, HOXB7 knockdown in two HOXB7-expressing breast cancer cell lines (MDA-MB-468 and T47D) suppressed TGFβ2 expression at both mRNA and protein levels (Fig. 1G and H), with concomitant decrease of phosphorylation of SMAD3 (Fig. 1H). These data indicated that HOXB7 can upregulate TGFβ2 expression in breast cancer cells.

HOXB7 directly binds to the TGFβ2 promoter and upregulates its transcription activity
All of the HOX proteins bind to the consensus binding site containing the ATTA/TAAAT motif in in vitro assays (8, 21). The in vivo binding affinity and specificity is determined by the adjacent sequences and/or binding cofactors (21). Searching through the upstream sequence of TGFβ2 promoter allowed us to identify 10 potential HOXB7-binding sites within the proximal 1.5 Kb of TGFβ2 5’ flanking region (relative to the transcription start site). To investigate whether HOXB7 binds to the TGFβ2 promoter directly (by interacting with the cis elements) or indirectly (by influencing another protein that interacts with the cis elements) in vivo, we first performed the ChIP assay. Cell lysates were sonicated to generate approximately 200-bp chromatin fragments before immunoprecipitation. Chromatin that coimmunoprecipitated with anti-HOXB7 (or control IgG) antibody was amplified by real-time PCR using primers flanking the proximal (−126 to −354 bp) or
Overexpression of HOXB7 increases TGFβ2 expression and phosphorylation level of SMAD3 in mouse primary MMTV-HER2/HoxB7 bitransgenic mammary tumors and human breast cancer cells. A, overexpression of HOXB7 is associated with increased phosphorylation level of SMAD3 in the mouse mammary tumors. Representative IHC images of primary mouse mammary tumors stained with antibody against p-SMAD3 are presented. IHC 0, negative; IHC 1, 1%–10% weak positive; IHC 2, 11%–30% weak positive; IHC 3, >30% strong positive. *Fisher exact test with R software. B, correlation between p-SMAD3 level in primary tumors and the number of metastatic foci in lung. C, RT-PCR analysis of TGFβ2, TGFβ3, and HOXB7 mRNA expression in primary MMTV-neu and MMTV-Hoxb7/neu transgenic mouse mammary tumor tissues. D, Western blot analysis of HOXB7, TGFβ2, SMAD3, and p-SMAD3 levels in primary mammary tumors described above. E and G, RT-PCR analysis of TGFβ1, TGFβ2, TGFβ3, and HOXB7 mRNA expressions in primary MMTV-Hoxb7-overexpressing (E) and HOXB7 knockdown (G) human breast cancer cells. Different breast cancer cells were transduced with HOXB7-overexpressing retroviruses or HOXB7 knockdown lentiviruses for 48 hours before harvesting for RT-PCR analysis of mRNA expression. F and H, Western blot analysis of HOXB7, TGFβ2, SMAD3, and p-SMAD3 levels in transiently HOXB7-overexpressing (F) and HOXB7 knockdown (H) human breast cancer cells described above. V, vector; B7, HOXB7; Scr., Scrambled shRNA; shB7, HOXB7 shRNA.

We then investigated the effect of HOXB7 overexpression on activity of the TGFβ2 promoter. For these studies, a series of reporter gene constructs based on the potential binding sites were generated, representing truncations of the TGFβ2 5′ flanking region (Fig. 2D, left). These reporter constructs (or empty reporter vector) were cotransfected into MCF7 cells with a plasmid overexpressing HOXB7 (or empty vector). HOXB7 overexpression significantly increased reporter gene expression from all, except the T-25 construct (Fig. 2D, right). The significant increase of the reporter activity from the T-183 demonstrates that the other nine upstream binding sites may only contribute minimally to the induction of TGFβ2 by HOXB7 (Fig. 2D, right), which is consistent with the results of ChIP assays. Overall, these results suggested that HOXB7 directly binds to, and activates the TGFβ2 promoter in breast cancer cells.
TGFβ2 induction is critical for HOXB7-mediated cell migration and invasion

Our previous study had shown that overexpression of HOXB7 promotes cell migration and invasion (14). We next determined whether TGFβ2 mediates the effect of HOXB7 on cell migration and invasion in breast cancer cells. We first utilized the TGFβ2-specific shRNA to knock down TGFβ2 expression in HOXB7-overexpressing (MDA-B7) and control (MDA-con) MDA-MB-231 cells. Western blot analysis showed that TGFβ2 shRNAs achieve a knockdown of TGFβ2 expression in HOXB7-overexpressing MDA-MB-231 cells by 50% to 60% (Fig. 3A). Using these TGFβ2 knockdown cells, we performed cell invasion assay and found that cell invasion in HOXB7-overexpressing MDA-MB-231 cells was significantly greater than in vector cells (Fig. 3B and C). The invasive response was decreased significantly in HOXB7-overexpressing cells after transfection with TGFβ2 shRNA (Fig. 3C). Although transfection of TGFβ2 shRNA also decreased invasion of control cells, the percentage of decrease was significantly smaller, compared to HOXB7-overexpressing cells (45.5 ± 11.1% for control cells vs. 80.1 ± 6.2% for HOXB7-overexpressing cells; Fig. 3C). We also performed wound-healing assays under the same conditions. The transfection of TGFβ2 shRNA into vector cells slightly slowed down migration, whereas a knockdown of TGFβ2 expression in HOXB7-overexpressing cells dramatically inhibited cell migration (Fig. 3D). To further confirm that the TGFβ2–SMAD3 signaling pathway is critical for HOXB7-mediated cell migration and invasion, we treated HOXB7-overexpressing or control cells with TGFβ receptor I inhibitor SB431542. Western blots of cell lysates were probed with antibodies to total and p-SMAD3 to confirm the blockage of the TGFβ signaling pathway. As expected, the phosphorylation level of SMAD3 was decreased by SB431542 treatment of HOXB7-overexpressing MDA-MB-231 cells (Supplementary Fig. S1A). More importantly, the inhibition of TGFβ signaling significantly attenuated cell migration and invasion abilities in HOXB7-overexpressing cells, but only slightly reduced cell migration and invasion abilities in vector cells (Supplementary Fig. S1B–S1D). This is consistent with our hypothesis that autocrine TGFβ2 production induced by HOXB7 in breast cancer cells stimulates cell migration and invasion through activation of the classical TGFβ2–SMAD3 signaling pathway.

Knockdown of TGFβ2 expression impairs HOXB7-induced tumor metastasis in vivo

To further investigate whether upregulation of TGFβ2 is critical for HOXB7-induced metastasis in vivo, we utilized shRNA to stably knockdown TGFβ2 expression in HOXB7-overexpressing MDA-MB-231 cells (Fig. 4A). HOXB7 overexpression had no effect on cellular morphology and growth rate of these MDA-MB-231 cells (Supplementary Fig. S2B). To determine lung colonization ability of these cells, the stable cell lines (MDA-B7 and MDA-B7-β2KD) and control MDA-MB-231 (MDA-con) cells were injected through the tail vein into NOD-SCID mice. As showed in (Fig. 4B), MDA-B7 cells formed more lung metastases than MDA-B7-β2 and MDA-con cells after 2 months. The number of metastatic foci in the lungs of mice injected with MDA-B7 cells
was almost 4-fold higher than in mice injected with MDA-con cells (11.7 ± 2.2 vs. 3.0 ± 0.6; P = 0.0037). More importantly, knocking down TGFβ2 dramatically reduced the number of metastatic foci in the lung (11.7 ± 2.2 vs. 2.5 ± 0.7; P = 0.0028; Fig. 4C). Therefore, TGFβ2 plays a pivotal role in HOXB7-promoted metastasis of MDA-MB-231 cells to the lung.

TGFβ2 cytokine secreted by HOXB7-overexpressing breast cancer cells directly stimulates peritoneal macrophages to acquire M2 features

It is well documented that tumor-associated macrophages, especially M2 phenotype, promote tumor growth and metastasis by secreting many cytokines, chemokines, and proteases (22–27). It is reported that TGFβ2 can induce macrophages to acquire a M2 phenotype (28). Besides inducing cell migration and invasion via tumor cell–autonomous manner, we wanted to test whether HOXB7 can also promote tumor progression through regulation of tumor microenvironment such as recruitment and activation of macrophages. To examine the effect of HOXB7 on recruitment of macrophages in vivo, we generated a MMTV-Her2 mouse mammary tumor cell line H605 that stably overexpressed HOXB7 for transplantation into immunocompetent syngeneic mice (Supplementary Fig. S3A). HOXB7 overexpression marginally increased the proliferation rate of H605 cells (Supplementary Figs. S3B and S3C; P = 0.058). HOXB7-overexpressing and control H605 cells were injected into mammary fat pad of syngeneic FVB/N mice. The size of tumors derived from HOXB7-overexpressing H605 cells was moderately larger than that in control H605 cells injected mice, but the difference was not statistically significant (Supplementary Fig. S3D; P = 0.059). The recruitment of macrophage phenotype was examined by immunofluorescence staining (Fig. 5A) and FACS analysis (Fig. 5B). Significantly more F4/80 (marker for mouse macrophage) and CD206 (specific marker for M2 phenotype) double-staining positive cells were detected in tumors derived from HOXB7-overexpressing H605 cells than control cells (Fig. 5A). As showed in Fig. 5B and C (top), the average percentage of infiltrating macrophages (F4/80+) in HOXB7-overexpressing tumors was more than 2-fold higher than H605 control tumors (13.1 ± 1.9 vs. 4.8 ± 0.5; P = 0.0031). In addition, the M2 population (F4/80+/CD206+) was also increased by nearly 3-fold (9.8 ± 1.8 vs. 2.8 ± 0.4;
Figure 4.
TGFβ2 is required for HOXB7-induced tumor metastasis in vivo. A, establishment of a stable TGFβ2 knockdown cell line. Western blot analysis was performed to validate the knockdown efficiency. MDA-con, control MDA-MB-231 cells; MDA-B7, HOXB7-overexpressing cells; MDA-B7-β2KD, HOXB7-overexpressing, and TGFβ2 knockdown cells. B, examination of lung metastasis in tail-vein injected animals. MDA-con, MDA-B7, and MDA-B7-β2KD cells were injected via tail vein into NOD/SCID mice (n = 6/group). The mice were sacrificed 2 months later, and the lungs were photographed under fluorescence microscope and then processed for H&E staining. Representative photos are showed. Arrows, metastatic foci. C, quantification of the metastatic foci in lungs described in B. The metastatic foci were counted under ×200 magnification (ten randomly selected high-power fields for each sample; n = 6). * *, P < 0.05.

P = 0.0045; Fig. 5B and C, right). These data strongly suggest that HOXB7 promotes recruitment and induction of macrophages into the M2 phenotype.

We determine whether TGFβ2 is involved in HOXB7-mediated induction of M2 phenotype by using an additional model system. We treated naïve mouse peritoneal macrophages with growth medium conditioned by MDA-B7, MDA-B7-β2, and MDA-con cells for 48 hours. Because the proliferation rates of MDA-B7, MDA-B7-β2, and MDA-con cells are almost identical in tissue culture, this approach enabled us to compare the effects of tumor cell-secreted factors on macrophages. As shown by Western blot analysis in Fig. 5D, expression of typical M2 markers, Arg1 and Ym1, was strongly induced in mouse peritoneal macrophages after stimulation with conditioned medium by MDA-B7 cells than with MDA-con cells, whereas knockdown TGFβ2 expression diminished the upregulation of Ym1 and Arg1 (Fig. 5D). These findings suggest that HOXB7-overexpressing cancer cells stimulate naïve mouse peritoneal macrophages to acquire features of M2 macrophages by secreting cytokines, among which an important constituent is TGFβ2.

HOXB7 and TGFβ2 expression in primary breast cancer is associated with aggressive phenotypes

To explore the clinical relevance of HOXB7 regulation of TGFβ2 expression in breast cancer, we performed quantitative RT-PCR analysis of cDNAs generated from 46 clinically and pathologically annotated cases of breast cancer. Compared with the adjacent normal tissues, primary breast cancers expressed higher levels of HOXB7 (P = 0.03) and TGFβ2 (P = 0.015). TGFβ1 mRNA levels, on the other hand, were comparable between breast carcinomas and adjacent normal tissues (Fig. 6A). After calculating the correlation coefficients between the expression levels of HOXB7 and the TGFβ ligands, we found a significant linear correlation between HOXB7 and TGFβ2 (R = 0.267, P = 0.0002) in primary breast cancer tissues (Fig. 6C). However, we did not observe such an association between the expression of HOXB7 and TGFβ1 (Fig. 6B). These results support our finding that HOXB7 and TGFβ2 are coexpressed in primary breast cancers and our findings in model systems find a close parallel in clinical samples.

A large number of reports have shown that TGFβ can promote tumor progression at later stages of breast cancer (29, 30). To investigate whether the activation of TGFβ2 expression by HOXB7 contributes to tumor progression, we calculated the correlation coefficient between the expression of HOXB7 and TGFβ2 in subgroups stratified by tumor grade and stage (Table 1). Interestingly, the linear correlation between HOXB7 and TGFβ2 expression is especially pronounced in higher grade tumors, for example, grade 2 tumors (R = 0.485; P = 0.001) and in stage III/IV (R = 0.769; P = 0.01). Furthermore, a significant correlation is only observed in lymph node–positive tumors (R = 0.297; P = 0.007), but not in the lymph node–negative tumors (R = 0.168; P = 0.092). In line with our previous observation that HOXB7 is a prognostic marker in HER2-positive tumors (16), the positive correlation between HOXB7 and TGFβ2 is significant in HER2-positive tumors as well (R = 0.453, P = 0.016). Together, our results support a relationship between HOXB7 and TGFβ2 with effects on breast cancer progression and metastasis.

Discussion

This study contributes to our understanding of the molecular mechanism by which overexpression of HOXB7 in breast cancers
promotes tumor progression. Our data suggested that upregulation of TGFβ2 by HOXB7 is responsible for both tumor cell-autonomous and tumor-stromal interaction mechanisms, leading to an aggressive phenotype and tumor progression to metastasis. As a classical transcriptional factor, HOXB7 may directly or indirectly regulate the transcription of target genes. Among many candidate genes we screened, TGFβ2 expression was selectively upregulated by HOXB7 in both human breast cancer cell lines and mouse primary mammary tumors. Further statistical analysis indicated that there was a positive correlation between expression of HOXB7 and TGFβ2 in the mouse mammary tumors. Although many genes have been shown to be induced or activated as a result of HOXB7 overexpression in cancer cells, only a few genes have been identified as direct targets (directly binding of HOXB7 protein to the promoter of target genes; refs. 9, 12, 13, 21). This is largely due to the ambiguous short binding motif containing ATTA or TAAT sequence, which is present in almost any gene promoter and can be shared by 38 other HOX proteins (21). To demonstrate that TGFβ2 is one of the novel direct targets of HOXB7, we performed quantitative ChIP assay and found that HOXB7 protein directly binds to the two proximal potential binding sites in the TGFβ2 promoter. The direct regulation of TGFβ2 transcription by HOXB7 was corroborated by the observation that HOX cofactor Pbx2 and Meis were recruited to the same target region. Promoter deletion analysis indicated that overexpression of HOXB7 alone was sufficient to activate promoter activity. It is also noteworthy that two other HOX proteins, HOXA10 and HOXB9, can activate TGFβ2 expression as well (31, 32). Although HOXB9-binding sites in TGFβ2 promoter have not been identified (32), HOXA10 binds to two cis elements located at the proximal (−410 to −385) and distal (−1506 to −1478) regions in the TGFβ2 promoter (31), which are different from the main HOXB7-binding site (−75 to −78). Meanwhile, the ATTA/TAAT motif is also present in TGFβ1 promoter region; however, overexpression or knockdown of HOXB7 in breast cancer cells seems to have no effect on its expression and there is no correlation between the expression of HOXB7 and TGFβ1 in the clinical samples either. All of these lines of evidence suggested that HOXB7 can specifically regulate its target gene expression in a context-dependent manner. It remains unknown whether the binding specificity is determined by the adjacent sequences or unknown cofactors.

Another question is whether TGFβ2 is important for the functions of HOXB7 in cancer progression. HOXB7 has been shown to promote cell migration and invasion, and induce EMT and angiogenesis (9, 12–14). A large number of reports indicate that TGFβ can play very similar roles (33, 34). For example, TGFβ expression is higher in invasive breast carcinoma than in ductal

Figure 5.
Overexpression of HOXB7 enhances recruitment and activation of macrophages to acquire M2 phenotypes. A, overexpression of HOXB7 enhances recruitment and activation of macrophages in orthotopic transplantation models of breast cancer. HOXB7-overexpressing and control mouse mammary tumor cell lines were injected into the mammary fat pad of syngeneic FVB/N mice (n = 6/group). One month later, the tumors were harvested for IHC staining using FITC-conjugated anti-F4/80 and PE-conjugated anti-CD206 antibodies. The slices were counterstained with DAPI and photographed. B, the representative pictures of FACS analysis of the infiltrated macrophages in the transplanted tumors. The tumor tissues described in A were digested into single cells and subject to FACS analysis with the FITC-conjugated anti-F4/80 and PE-conjugated anti-CD206 antibodies. C, quantitative analysis of percentage of total and M2 macrophages in the transplanted tumors (n = 5); *, P < 0.05. D, HOXB7 induces peritoneal macrophages to acquire M2 phenotypes. Conditioned media from MDA-con, MDA-B7, and MDA-B7-β2KD and fresh DMEM media were used to treat primary mouse peritoneal macrophages for 24 hours. Expression levels of Arg1 and YM1 were examined by Western blot analysis. Arg1, Arginase, type I.

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This hypothesis is supported by data that TGF-β may also favor invasiveness of breast cancer because both isoforms share similar biologic activities (29, 30). Although most studies analyzed TGF-β1 expression, TGF-β2 may also favor aggressiveness of breast cancer because both isoforms share similar biologic activities (29, 30). This hypothesis is supported by data that TGF-β1 was identified as one of the genes differentially expressed in highly metastatic tumors in a mouse model of spontaneous breast cancer metastasis to bone (37). We therefore proposed that HOXB7 promotes tumor progression through upregulation of TGF-β2 gene in breast carcinomas. Indeed, our data confirmed that chemical inhibition of TGF-β signaling or knockdown of TGF-β2 expression can dramatically decrease HOXB7-mediated migration and invasion abilities. Further in vivo assay demonstrated that HOXB7-promoted metastasis to lung was blocked by TGF-β2 knockdown, indicating that upregulation of TGF-β2 expression at least partially accounts for the function of HOXB7 in promotion of tumor progression in vivo. In line with this finding, the linear correlation between HOXB7 and TGF-β2 expression tends to be more pronounced in later stage and more aggressive breast tumors.

It is well known that macrophages play very important roles in progression of tumors. Tumor cells recruit macrophages via chemokines and in turn activate them to acquire M2 phenotype by secreting cytokines (22, 25, 38–40). M2 macrophages are strongly associated with poor outcomes in patients with a variety of cancers (40). M2 macrophages are believed to promote tumor cell migration, invasion, and intravasation by releasing growth factors such as VEGF and bFGF to induce angiogenesis and by producing proteases such as MMP9 to degrade extracellular matrix (41, 42). Recently, it was reported that TGF-β2 can induce macrophages into M2 phenotype (28), which led us to explore the effect of HOXB7 on activation of macrophages. We found that macrophages were attracted more efficiently into HOXB7-overexpressing tumors than the control tumors, and more importantly, acquired M2 macrophage phenotypes in HOXB7-overexpressing tumors. Further in vitro analysis showed that TGF-β2 secreted by HOXB7-overexpressing breast cancer cells was directly responsible for the induction of M2 macrophage phenotypes. Recruitment and activation of macrophages adds another new mechanism to HOXB7-mediated promotion of tumor progression.

**Table 1** Correlation of HOXB7 and TGF-β2 in primary breast tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>N (%)</th>
<th>R²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>46 (100%)</td>
<td>0.267</td>
<td>0.0002</td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4 (8.6%)</td>
<td>0.023</td>
<td>0.848</td>
</tr>
<tr>
<td>2</td>
<td>19 (41.3%)</td>
<td>0.485</td>
<td>0.001</td>
</tr>
<tr>
<td>3</td>
<td>23 (50.0%)</td>
<td>0.218</td>
<td>0.025</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>9 (19.6%)</td>
<td>0.442</td>
<td>0.051</td>
</tr>
<tr>
<td>II</td>
<td>27 (58.6%)</td>
<td>0.165</td>
<td>0.040</td>
</tr>
<tr>
<td>III and IV</td>
<td>7 (15.2%)</td>
<td>0.769</td>
<td>0.010</td>
</tr>
<tr>
<td>Missing</td>
<td>3 (6.5%)</td>
<td>0.939</td>
<td>0.159</td>
</tr>
<tr>
<td>Lymph node status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>18 (39.1%)</td>
<td>0.168</td>
<td>0.092</td>
</tr>
<tr>
<td>Positive</td>
<td>24 (52.2%)</td>
<td>0.291</td>
<td>0.007</td>
</tr>
<tr>
<td>Missing</td>
<td>4 (8.6%)</td>
<td>0.970</td>
<td>0.015</td>
</tr>
<tr>
<td>HER2 Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>22 (47.8%)</td>
<td>0.098</td>
<td>0.157</td>
</tr>
<tr>
<td>Positive</td>
<td>12 (26.1%)</td>
<td>0.453</td>
<td>0.016</td>
</tr>
<tr>
<td>Missing</td>
<td>12 (26.1%)</td>
<td>0.381</td>
<td>0.032</td>
</tr>
</tbody>
</table>

*Values in bold are statistically significant (P < 0.05).*
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Liu, C. De, D. Fan, S. Sukumar

Writing, review, and/or revision of the manuscript: S. Liu, S. Feng, D. Reisman, D. Fan, S. Sukumar, H. Chen

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Hui, Q. Wang

Study supervision: Q. Wang, H. Chen

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References


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