HOXB7, a Homeodomain Protein, Is Overexpressed in Breast Cancer and Confers Epithelial-Mesenchymal Transition

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Abstract

Epithelial-mesenchymal transition (EMT) is increasingly recognized as a mechanism whereby cells in primary noninvasive tumors acquire properties essential for migration and invasion. Microarray analyses of microdissected epithelial cells from bone metastasis revealed a HOXB7 overexpression that was 3-fold higher than in primary breast carcinomas and 18-fold higher compared with normal breast. This led us to investigate the role of HOXB7 in neoplastic transformation of breast cells. Expression of HOXB7 in both MCF10A and Madin-Darby canine kidney (MDCK) epithelial cells resulted in the acquisition of both phenotypic and molecular attributes typical of EMT. Loss of epithelial proteins, claudin 1 and claudin 7, mislocalization of claudin 4 and E-cadherin, and the expression of mesenchymal proteins, vimentin and α-smooth muscle actin, were observed. MDCK cells expressing HOXB7 exhibited properties of migration and invasion. Unlike MDCK vector–transfected cells, MDCK-HOXB7 cells formed highly vascularized tumors in mice. MDCK-HOXB7 cells overexpressed basic fibroblast growth factor (bFGF), had more active forms of both Ras and RhoA proteins, and displayed higher levels of phosphorylation of p44 and p42 mitogen-activated protein kinase (MAPK; extracellular signal–regulated kinases 1 and 2). Effects initiated by HOXB7 were reversed by specific inhibitors of FGF receptor and the Ras-MAPK pathways. These data provide support for a function for HOXB7 in promoting tumor invasion through activation of Ras/Rho pathway by up-regulating bFGF, a known transcriptional target of HOXB7. Reversal of these effects by HOXB7-specific siRNA further suggested that these effects were mediated by HOXB7. Thus, HOXB7 overexpression caused EMT in epithelial cells, accompanied by acquisition of aggressive properties of tumorigenicity, migration, and invasion. (Cancer Res 2006; 66(19): 9527-34)

Introduction

HOX genes, a subset of the homeobox gene family, are well conserved at the genomic level during evolution. In addition to their roles as master transcriptional factors critical in the regulation of embryonic development, their stringently regulated expression patterns in various tissues and organs in adulthood indicate fundamental roles in maintaining homeostasis. A growing body of literature has emerged in the last decade on the involvement of HOX genes in the pathogenesis of cancer. Many studies have reported aberrant expression of a number of HOX genes in cancer. Some examples are HOXA7 and HOXD13 in lung cancer (1), HOXC4 and HOXC8 in prostate cancer (2), HOXB7 in ovarian cancer (3), and HOXA10 in endometrial cancer (4). Focusing on breast cancer, HOXA5 expression was lost in 60% of breast cancers (5). Expression of HOXA5 in MCF7 cells induced p53-dependent apoptosis, whereas in HS578T cells, which harbor mutant p53, cell death was mediated by the caspase pathway (6). HOXD10 was reported to be progressively reduced in epithelial cells as malignancy increases, and restored expression of HOXD10 in a highly invasive breast cancer cell line, MDA-MB-231, could significantly impair migration (7). The ratio of HOXB13 versus interleukin-17β receptor was documented to be an accurate biomarker for prediction of tumor recurrence in the setting of adjuvant tamoxifen monotherapy, and overexpression of HOXB13 could increase MCF10A motility and invasion in vitro (8).

Epithelial-mesenchymal transition (EMT), initially recognized as an essential step for embryogenesis in the early 1980s (9), is now considered a major mechanism for the conversion of early-stage tumors to invasive malignancies (4, 10–12). During passage through EMT, epithelial cells lose epithelial adherens and tight junction proteins, consequently lose polarity and cell-cell contacts, and undergo a dramatic remodeling of the cytoskeleton to facilitate cell motility and invasion (13). Transcriptional factors like Snail (10) and Twist (14, 15) were unveiled as key regulators in induction of EMT in breast cancer and other cancers and act by suppressing the expression of epithelial specific adhesion molecule, E-cadherin. E-cadherin expression is irreversibly lost in invasive lobular breast cancer (16). Besides these transcriptional factors, growth factors like hepatocyte growth factor (HGF; ref. 17), transforming growth factor (TGF)-β (18), and epidermal growth factor (EGF; ref. 19), as well as matrix metalloproteinase (MMP)-3 (20), also induce EMT in various cell lines. These studies also suggested that Snail was located at the hub of these growth factor signaling pathways leading to EMT because the activated receptor tyrosine kinases could up-regulate the expression of Snail by activating the Ras-mitogen-activated protein kinase (MAPK) pathway (21). In this study, we identified HOXB7 as one of the promising candidate genes, which was overexpressed at increasingly higher levels from normal epithelial cells to primary metastatic breast tumors to bone metastatic lesions by microarray analysis of purified epithelial cells. HOXB7 was reported to be involved in tissue remodeling of the normal mammary gland (22) and was associated with the development of breast cancer (23, 24). cDNA-based comparative genomic hybridization revealed that HOXB7 was located in a novel amplicon at 17q21.3, and this amplification correlated with poor prognosis in a panel of 186 breast cancer cases (25). Overexpression of HOXB7 in SKBR3 breast cancer cells was found to directly or indirectly regulate the expression of many angiogenic and growth factors, including basic fibroblast growth factor (bFGF), vascular

endothelial growth factor (VEGF), interleukin 8, Ang1, Ang2, and MMP9, and resulted in the formation of well-vascularized tumors when grown as xenografts in nude mice (23, 24). In this article, we provide evidence that HOXB7 is overexpressed in primary breast carcinomas and distant metastasis to various organs. In cell culture models, we show the ability of HOXB7 to confer the biological and molecular characteristics of EMT to epithelial cells.

Materials and Methods

Purification of epithelial cells, RNA amplification, and labeling for microarray. Epithelial cells were isolated from freshly resected mammoplasty tissue (normal breast tissue; $n = 2$) by immunopurification. Malignant epithelial cells from frozen breast tissue specimens, primary invasive ductal carcinomas with lymph node metastasis ($n = 2$), and from bone metastases ($n = 3$) were purified by laser capture microdissection. Laser capture microdissection was carried out using a PixCell II LCM system (Arcturus Engineering, Mountain View, CA) as per instructions of the manufacturer. Total RNA from purified epithelial cells was extracted with RNeasy Mini kit (Qiagen, Inc., Valencia, CA) including a DNase treatment step. RNA (~ 10 ng total RNA) from each sample was amplified with the RiboAmp RNA Amplification kit (Arcturus Engineering). The amplified RNA was labeled with the ENZO BioArray HighYield RNA transcript labeling kit (Affymetrix, Santa Clara, CA). To obtain appropriate concentrations for hybridization, three bone metastasis samples were pooled. Biotin-labeled RNA samples (12 μg RNA of normal, invasive ductal carcinoma, and pooled bone metastases) were then fragmented and hybridized to the GeneChip Human Genome U133A 2.0 Array (Affymetrix).

Reverse transcription-PCR, quantitative PCR, and statistical analysis. Immortalized cell lines derived from normal human mammary epithelial cells, MCF10A, normal Madin-Darby canine kidney (MDCK) breast cancer cell lines (American Type Culture Collection, Manassas, VA), normal organoid, and tumor RNA were extracted by Trizol method, and all cDNAs were prepared with 1 μg of RNA in SuperScript II (Invitrogen, Carlsbad, CA) reactions according to the instructions of the manufacturer. Reverse transcription-PCR (RT-PCR) amplifications of HOXB7, bFGF, and internal control gene 36B4 were done with the following primer pairs: HOXB7, forward 5′-AGAGTAACTTCCGGATCTA-3′ and reverse 5′-TCGGCT-TCAGCCCTGTCTT-3′; bFGF, forward 5′-TCAAAGGAGTGTGTGCTAACCG-3′ and reverse 5′-CTGCCCAGTTCGTTTCAGTG-3′; and 36B4, forward 5′-GA-TTGGCTACCCAACTGTTGCA-3′ and reverse 5′-CAGGGGCAGCAGCCACA-AAGGC-3′.

Quantitative real-time PCR was done and analyzed essentially as described (12) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization. Primer sequences of HOXB7 for Q-PCR were as follows: forward 5′-AGAGTAACTTCCGGATCTA-3′ and reverse 5′-CAGGTAGCGAT-TGTAGTG-3′ with the TaqMan probe FAM-ACCCCTGGATGCGAAGCTCA-TAMRA (Applied Biosystems, Foster City, CA). Two-tailed Student's t test was used for analysis of the real-time PCR results.

Plasmids, siRNA, and transfection. The expression vector for Flag-tagged HOXB7 was a kind gift from Dr. Judith Gasson (University of California at Los Angeles, Los Angeles, CA). Wild-type HOXB7 was amplified with primers B7-F (5′-ATGAGTTCATTGTATTATGCGAATG-3′) and B7-R (5′-ACTCTTCCTCTTCCTCCTCTGCTTCAG-3′) and subcloned into pcDNA3.1-V5-His-Topo vectors (Invitrogen). Flag-tagged HOXB7 and wild-type HOXB7 plasmids were transfected into MCF10A or MDCK cells with Genejammer (Stratagene, La Jolla, CA) and cells were selected in 800 μg/mL G418–containing medium to establish stable clones. Two pairs of HOXB7 siRNA oligos [SA1, CAUUCUGUGUGUAUCUAAA (sense) and UUUAGAUA-CACACAGAAUG (antisense); and SA2, GGACUCUCCUUCUGUAAUA (sense) and UAUUACAGAAUUAGAGUCC (antisense)] were synthesized and transfected into MDA-MB-435 cells with Lipofectamine 2000 (Invitrogen).

Western blot, immunofluorescence assays, and antibodies. Western blot and immunofluorescence were done as described (13). Antibodies to Flag (Stratagene), HOXB7, claudin 1, claudin 4, claudin 7 (Invitrogen), E-cadherin, vimentin, β-actin (BD Biosciences, Franklin Lakes, NJ),

α-smooth muscle actin (Sigma, St. Louis, MO), Ras (Santa Cruz Biotechnology, Santa Cruz, CA), MAPK, and phospho-MAPK (Cell Signaling, Danvers, MA) were used. Alexa Fluor 488–labeled phalloidin (Invitrogen) was used to detect F-actin.

Wound healing assay. For the wound healing assay, cell cultures at varying confluence were serum starved for 8 hours. After scratching the monolayer, cells were washed with PBS, cultured in 10% fetal bovine serum (FBS)-DMEM, and photographed under $10\times$ objective lens every 3 hours.

Matrigel invasion assay. Into the Biocoat Matrigel invasion chambers (BD Biosciences) were seeded 4×10^4 cells in 2% FBS medium, and in the lower wells 10% FBS was added as the chemoattractant. After 36 hours of incubation, the filters were stained with crystal violet, and the number of cells that had penetrated through the filter was counted under $\times 20$ magnification (10 randomly selected high-power fields). For inhibition of invasion assays, the FGF receptor inhibitor Su5402 (40 μmol/L), the MAPK/extracellular signal–regulated kinase kinase (MEK) inhibitor U0126 (10 μmol/L), and the Raf inhibitor Bay43-9006 (4 μmol/L; Calbiochem, San Diego, CA) were added to the medium in the top chamber at the final concentrations indicated.

Ras and RhoA activation assay. Cell lysates were incubated with glutathione S-transferase (GST)-Raf-1 Ras binding domain or GST-Rhotekin RhoA binding domain agarose beads (Upstate, Charlottesville, VA). GTP-bound Ras or RhoA that precipitated with the beads were detected by SDS-PAGE and immunoblotting with anti-Ras antibody (Santa Cruz Biotechnology) or anti-RhoA antibody (Upstate).

Xenograft analysis. A suspension of 1×10^6 MDCK-vec or MDCK-B7 cells in 100 μL of Matrigel (BD Biosciences) was injected into the mammary fat pad (one on either side) of 10 female Swiss nu/nu mice. Tumors were removed 8 weeks later and subjected to H&E and immunohistochemical staining for histopathologic examination.

Results

HOXB7 is overexpressed in primary breast carcinoma and metastasis. To identify genes involved in breast cancer progression, oligonucleotide array analysis was done on total RNA isolated from immunobead purified epithelial cells from two normal mammoplasty samples, epithelial cells microdissected from two lymph node–positive primary breast tumors, and three bone metastasis samples. Whereas validation of differentially expressed genes is an ongoing study in our laboratory, our interest was piqued by the 3- and 18-fold overexpression of HOXB7 in the primary and metastatic lesions, respectively, compared with normal mammoplasty samples (Fig. 1A). Because we have previously identified loss of HOXA5 in breast carcinoma (5) and showed its involvement in both p53-dependent and p53-independent apoptotic pathways (5, 6), and accumulating evidence points to a role for HOXB7 in breast cancer, we chose to focus our efforts on this gene.

To validate the microarray data, HOXB7 mRNA was examined by RT-PCR and was found to be expressed in 6 of 10 breast cancer cell lines at higher levels compared with both finite life span and immortalized human mammary epithelial cells (Fig. 1B). These data were further extended and confirmed on 31 primary breast carcinoma samples and 19 metastatic breast lesions by quantitative RT-PCR. HOXB7 mRNA was expressed at higher levels in primary breast carcinomas ($P < 0.0001$) and distant metastasis ($P = 0.0005$), compared with nine purified normal mammary epithelial organoid samples (Fig. 1C). Thus, the results of the microarray analysis were validated in large sample sets of both primary and metastatic breast cancer.

HOXB7 was reported to be present in a novel amplicon on chromosome 17; further fluorescence *in situ* hybridization (FISH) analysis of 346 tumors showed gene amplification in 10.2% of primary breast cancers, which correlated to poor prognosis (25).
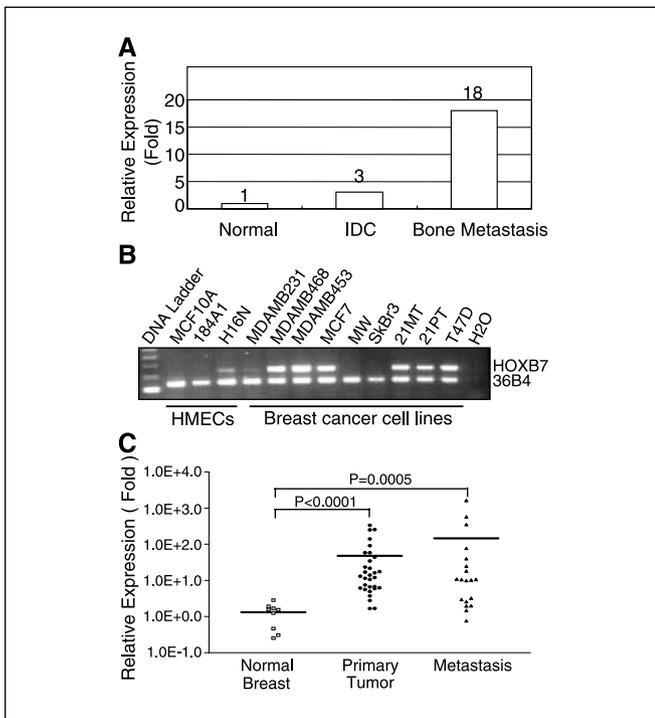
Figure 1. HOXB7 mRNA levels in normal breast, primary breast carcinomas, and distant metastases. *A,* relative expression levels of HOXB7 mRNA in purified epithelial cells from human mammary epithelial cells (*Normal*), lymph node–positive invasive ductal carcinoma (*IDC*), and bone metastasis by microarray analysis. *B,* RT-PCR analysis of HOXB7 in normal human mammary epithelial cells (*HMECs*) and breast cancer cell lines. *36B4,* a ribosomal protein mRNA that served as an internal loading control. *C,* the relative levels of HOXB7 mRNA as measured by quantitative real-time RT-PCR in nine purified mammary epithelial organoids from normal reduction mammoplasty samples, 31 primary breast carcinomas, and 19 breast metastasis to various organs. The two-tailed Student *t* test was applied for statistical analysis.

We determined if the overexpression of HOXB7 seen in primary tumors can be traced to gene amplification by the use of real-time PCR on tumor DNA for HOXB7 and the internal control gene GAPDH. Our analysis of DNA from the same tumor panel showed that <10% tumors had more than two copies of HOXB7 (data not shown). Thus, gene amplification seems to be the underlying mechanism accounting for only a small percentage of tumors that overexpress HOXB7 mRNA.

HOXB7, a novel factor that induces EMT. To determine whether HOXB7 protein expression plays a contributory role in tumor progression, we stably transfected FLAG-tagged HOXB7 (FB7) in MCF10A cells, an immortalized normal mammary epithelial cell line with undetectable HOXB7 expression. Intriguingly, both pooled clones and 70% (16 of 23) of the G418-selected stable clones of MCF10A-FB7 appeared spindle shaped and fibroblastic in monolayer culture, whereas HOXB7-vector control cells, like MCF10A parental cells, maintained their cobblestone-like phenotype (Fig. 2*A*). This morphologic change implied that the MCF10A-FB7 cells have undergone trans-differentiation from epithelial cells to mesenchymal cells. Consistent with this observation, rearrangement of cytoskeleton as a signature of the transition was observed by phalloidin staining. In contrast with control cells exhibiting a peripheral F-actin staining with slim central stress fibers, MCF10A-FB7 cells showed a decrease in marginal F-actin but contained much thicker central stress fibers (Fig. 3*B-1*).

MDCK cells grow as tight islands of cobblestone-shaped cells and have been widely used as a prototypic model to study EMT (26, 27). To determine if the phenotypic change induced in MCF10A cells by HOXB7 is effective in other cell lines, stable clones of MDCK cells expressing HOXB7 were generated. In comparison with the parental and control MDCK cells, >80% clones of MDCK-B7 showed the spindle shape morphology (Fig. 2*A*). All subsequent experiments were conducted with pools of the MDCK clones. Thus, phenotypic changes typical of EMT were elicited on expression of HOXB7 in both MCF10A and MDCK epithelial cells.

The accepted paradigm of EMT dictates that cells lose markers typical of epithelial cells, such as adhesion molecules (E-cadherin) at the adherens junctions and tight junction proteins (claudins and ZO-1) at the apical junctions (13). Numerous lines of evidence have shown that loss of these proteins impairs cell-cell adhesion and cell-cell communication and facilitates dissemination of metastatic cells (28, 29). Changes in expression of these proteins were examined by Western blot and/or by immunofluorescence analyses. By Western blot analysis, expression of tight junction proteins, claudin 1 and claudin 7, was undetectable in MCF10A-FB7 pooled clones and single clones, and significantly decreased in MDCK-B7 cells (Fig. 3*A*). Curiously, unlike MCF10A-FB7 cells, expression levels of E-cadherin and claudin 4 were not dramatically changed in MDCK-B7 cells (Fig. 3*A*). However, by immunofluorescence staining, in contrast to the MDCK control cells where both E-cadherin and claudin-4 proteins had clear membrane peripheral staining pattern, in MDCK-B7 cells their distributions were mainly diffusely cytoplasmic (Fig. 3*B, 2* and *5*).

During EMT, loss of epithelial markers is usually accompanied by the expression of markers typical of mesenchymal cells. As shown in Fig. 3*B*, *de novo* α-smooth muscle actin expression was observed in MCF10A-FB7 cells, and vimentin expression level was dramatically higher in MDCK-B7 cells (Fig. 3*A*). In MDCK control and
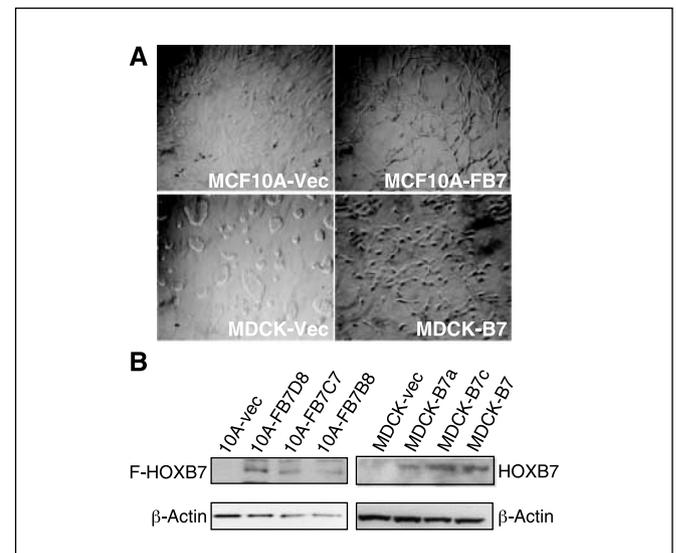
Figure 2. Overexpression of HOXB7 in both MCF10A and MDCK cells induces EMT. *A,* phase-contrast photomicrographs of HOXB7 transfectants and control cell lines growing at low density on tissue culture plastic. Bar, 10 μm. *B,* Western blot analysis of Flag tagged HOXB7 (*left*) and HOXB7 (*right*) expression in MCF10A vector control (*MCF10A-vec*), four different clones of MCF10A-Flag-HOXB7 cell lines (*10A-FB7D8, 10A-FB7C7,* and *10A-FB7B8*), MDCK vector control (*MDCK-vec*), and two different stable clones of MDCK-HOXB7 cell lines (*MDCK-B7a* and *MDCK-B7c*) and MDCK-HOXB7 pooled clones (*MDCK-B7*).
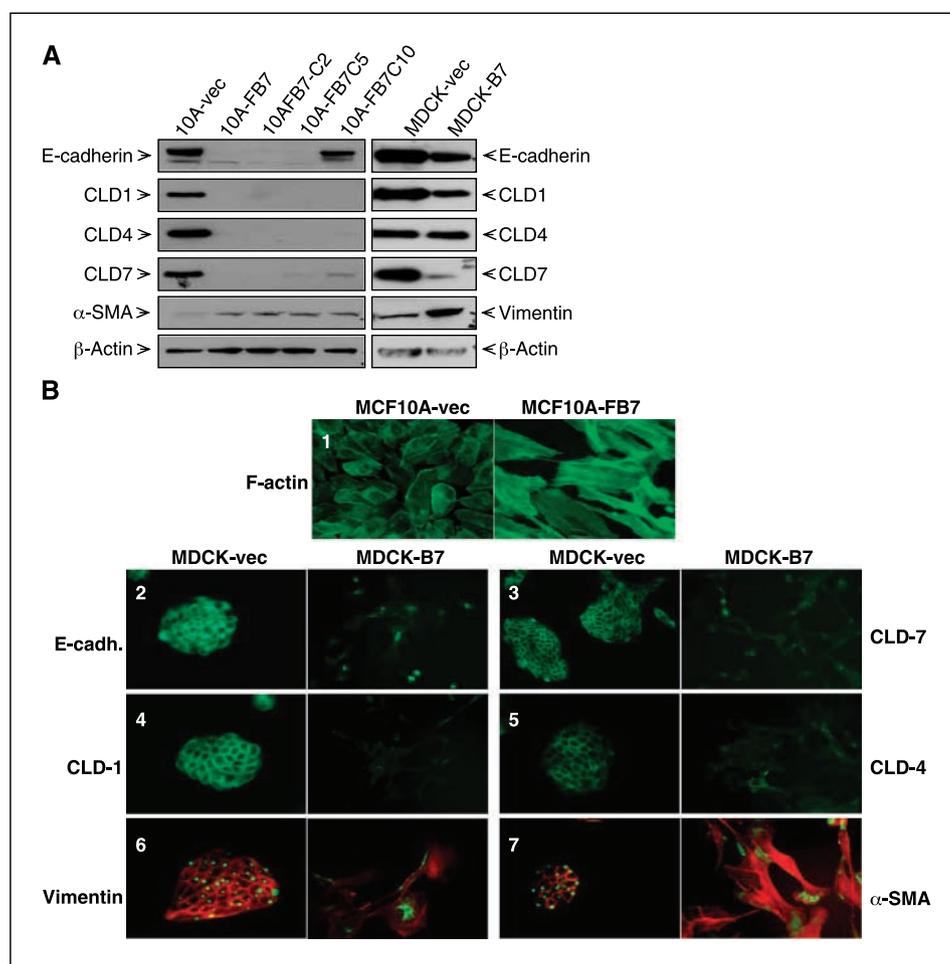
Figure 3. Cells with high expression of HOXB7 express mesenchymal markers and show loss of epithelial markers. *A,* Western blot analysis of E-cadherin, claudin 1 (*CLD1*), claudin 4 (*CLD4*), claudin 7 (*CLD7*), α-smooth muscle actin (α-*SMA*), and vimentin in MCF10A-vec, pooled clones of MCF10A-FB7 (MCF10A-FB7), and three separate clones (*10A-FB7-C2, 10A-FB7-C5,* and *10A-FB7-C10*), MDCK-vec and pooled clones of MDCK-B7 cells. *B,* MCF10A-vec and MCF10-FB7 cells were fixed and stained for F-actin with Alexa Fluor 488-phalloidin. MDCK-vec and MDCK-B7 cells were fixed and processed for immunofluorescence with antibodies recognizing E-cadherin (*2*), claudin 7 (*3*), claudin 1 (*4*), claudin 4 (*5*), vimentin (*6*), and α-smooth muscle actin (*green; 7*). *6* and *7,* the same cells were costained with Alexa Fluor 555-phalloidin (*red*) and vimentin or α-smooth muscle actin (*green*).

pooled MDCK-HOXB7 cells, although fluorescence signals were observed in both control and HOXB7 transfectants, their expression patterns were completely different. In MDCK control cells, both α-smooth muscle actin and vimentin were localized in a concentrated and polarized pattern. However, in MDCK-B7 cells, α-smooth muscle actin was mainly distributed along the lamellipodia and a network of vimentin intermediate filaments was also clearly visible (Fig. 3*B, 6* and *7*). Thus, changes in morphology and molecular markers in both MCF10A and MDCK cells stably expressing HOXB7 were consistent with EMT.

HOXB7 can promote migration and invasion. The essential contribution of EMT to carcinoma progression is that dissociated epithelial cells acquire migration and invasive ability and are able to actively pass through the basement membrane and traverse to distant organs. To test whether HOXB7-overexpressing cells acquire greater migration and invasive ability, two assays were done: the wound healing and the Matrigel invasion assays. The wound healing assay was conducted at different confluence levels of both MDCK-vec and pooled MDCK-B7 cells. Figure 4*A* shows representative photomicrographs taken 0, 6, 9, and 15 hours after the cell surface was scratched for the wound healing assay. No motility was observed in MDCK-vec cells during the entire observation period, whereas the pooled MDCK-B7 cells started to fill the wound as early as 6 hours after scratching. Pooled MDCK-B7 cells also showed a significantly greater invasive potential than MDCK-vec cells in the Matrigel invasion assay (Fig. 4*B* and *C*). Moreover,

lamellipodia-like structures, an important signature of cell migration, were observed in the majority of pooled MDCK-B7 cells that penetrated and traversed the Matrigel (Fig. 4*B*).

To determine if HOXB7 is critical to promoting invasion of breast epithelial cells, HOXB7 siRNA oligonucleotides, SA1 and SA2, were transiently cotransfected into MDA-MB-435 breast cancer cells that naturally express HOXB7 and exhibit strong invasive ability *in vitro*. By Western blot analysis, cotransfection of both siRNAs, SA1 and SA2, resulted in the knockdown of HOXB7 expression in MDA-MB-435 cells by 80% to 90% (Fig. 5*C*). The siRNAs were relatively specific to HOXB7 because no reduction was observed in the expression of two other homeobox genes tested, HOXA5 and HOXD3 (data not shown). The siRNA-transfected MDA-MB-435 cells were then tested for their invasive ability by the Matrigel invasion chamber assay; knockdown of endogenous expression of HOXB7 with specific siRNAs markedly decreased the invasive ability of MDA-MB-435 cells (Fig. 5*D*).

Ras-MAPK pathway is involved in HOXB7 induced EMT. Because it involves scattering of epithelial cells accompanied by a change in morphology to facilitate these movements, EMT is a reflection of the plasticity of differentiated epithelial cells. Multiple signal transduction pathways have been identified to be involved in the induction of EMT. Local expression of growth factors, TGF-β, HGF, EGF/TGF-α, and FGF-2, have been shown to assist EMT by binding to their cognate receptors on epithelial cells and by initiating signal transduction cascades (13). The Ras-Raf-MAPK

Figure 4. Overexpression of HOXB7 increases cell migration and invasion ability. *A,* wound healing assay with MDCK-vec and pooled MDCK-B7 cells. Microscopic observations were recorded 0, 6, 9, and 15 hours after scratching the cell surface. *B,* the cells were placed in a three-dimensional Matrigel invasion chamber and cells that invaded through Matrigel were fixed, stained with crystal violet, and counted. *Arrow,* a lamellipodia structure. *Inset,* higher magnification. *C,* number of cells that invaded through the Matrigel was counted in 10 fields under 20× objective lens. *Bars,* SD.
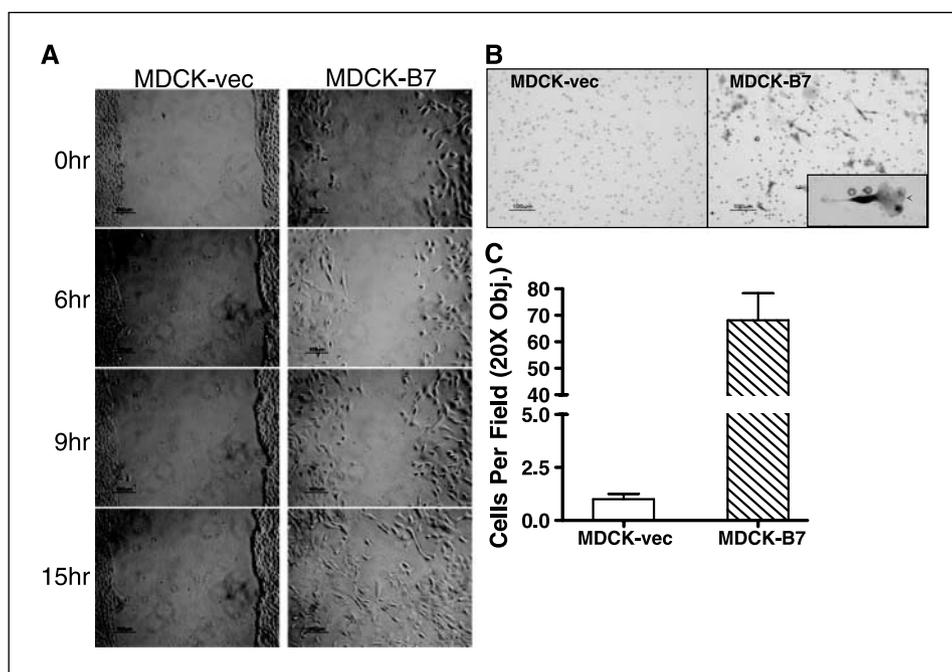
pathway has been shown to be an indispensable link in the chain of signal transduction leading to induction of EMT (18, 30). To test the involvement of this pathway in EMT induced by HOXB7 in MDCK cells, we employed the GST pull-down assay to analyze the active forms of both RhoA and Ras. In contrast to MDCK-vec cells, pooled clones of MDCK-B7 cells had more active forms of both Ras and RhoA proteins (Fig. 5A). Investigating a role for MAPK activation by testing for p44/p42 MAPK, higher levels of activation of MAPK were observed in pooled MDCK-B7 cells compared with the MDCK-vec cells, with no change in the amount of total protein (Fig. 5A). That this activation is attributable to HOXB7 was tested by suppressing endogenous HOXB7 expression in MDA-MB-435 cells using HOXB7 siRNA. Suppression of HOXB7 expression abrogated activation of the Ras-MAPK pathway (Fig. 5C), suggesting a key role for HOXB7 in this phenomenon. Further confirmation was sought that activation of these pathways is associated

with the invasive ability of MDCK-B7 cells. MDCK-vec and pooled MDCK-B7 cells were seeded into Matrigel invasion chambers and treated with the RAF inhibitor Bay43-9006 or the MEK inhibitor U0126. Treatment of the cells with either inhibitor alone or in combination resulted in the complete suppression of the invasive phenotype displayed by the MDCK-B7 cells (Fig. 5B). Similar results were observed in migration assays (data not shown).

bFGF, a wide-spectrum factor functioning in mitogenesis (31), angiogenesis (32), and neurogenesis (33), was previously reported as a directly regulated gene target of HOXB7 (34). bFGF has also been shown to induce EMT in lens cells (35) in response to injury and in kidney cells (36). Furthermore, activation of FGF receptors by autocrine bFGF results in the recruitment and phosphorylation of adaptor protein SHC, which then creates binding sites for the growth factor receptor binding protein-2 adaptor in complex with the Ras-activating nucleotide exchange factor SOS (37). To verify

Figure 5. Involvement of Ras-Raf-MAPK pathway in HOXB7-induced EMT. *A,* activity of Ras and RhoA was analyzed by GST pull-down assay with GST fusion proteins: GST-Raf Ras binding domain and GST-Rhotekin RhoA binding domain, respectively. Active Ras and RhoA, total Ras, and RhoA were determined by Western blot analysis. Phosphorylated MAPK and total MAPK were also determined by Western blot. *B,* inhibition of the activity of RAF and p42/44 MAPK by BAY 43-9006 (*Raf-inh*) and U0126 (*MEK-inh*), respectively, could block invasive ability of pooled MDCK-B7 cells. *C and D,* cotransfection of HOXB7 siRNAs SA1 and SA2 into MDA-MB-435 cells partially inhibited Ras and MAPK activity (*C*) and invasive ability of MDA-MB-435 (*D*) by reducing endogenous HOXB7 expression (*C*).
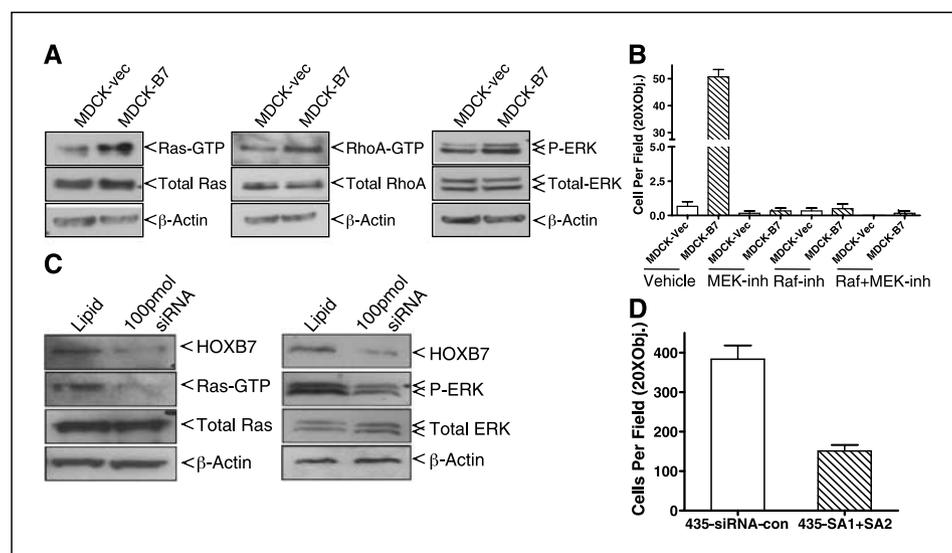
whether this signal transduction pathway is involved in the HOXB7-induced invasive properties in our system, we first tested for bFGF expression by RT-PCR in both pooled MDCK-B7 cells and MDCK-B7 xenograft tumors grown in nude mice. Figure 6*B* shows that bFGF was expressed at higher levels in both MDCK-B7 cells and transplanted tumors compared with MDCK-vector control cells. Treatment of MDCK-B7 cells with the FGF receptor–specific inhibitor Su5402 (Calbiochem) could attenuate the Ras-GTP form (Fig. 6*D*). Further, the FGF receptor–specific inhibitor could inhibit invasion ability of MDCK-B7 cell by ∼80% (Fig. 6*D*). The expression of some other well-known EMT regulators, such as members of the TGF-β pathway and members of the Snail family, was also determined by RT-PCR or Western blot analysis, but no significant change was detected (data not shown). Thus, several lines of evidence point to bFGF as the major mediator of EMT initiated by HOXB7.

Overexpression of HOXB7 in MDCK cells promotes tumor formation and local invasion *in vivo*. All the *in vitro* culture data indicated that the expression of HOXB7 conferred many features of neoplastic transformation to MDCK cells. To test whether MDCK-B7 cells were tumorigenic *in vivo*, MDCK-vec and pooled MDCK-B7 cells were implanted as Matrigel plugs into the mammary fat pads of immunodeficient female nude mice. Tumors formed in nine of the ten sites injected with MDCK-B7 cells (Fig. 6*A*), but no distant metastases were found in tumor-bearing mice. RT-PCR confirmed that HOXB7 expression was retained in the implanted tumors (Fig. 6*B*). In contrast, no palpable tumors were observed in any of

the sites injected with MDCK-vec cells. MDCK-B7 tumors were firmly attached to the surrounding tissues, including the underlying axillary muscle. Histopathologic examination showed that the tumors lacked well-defined capsular margins; tumor cells had infiltrated into the surrounding tissues; and islands of tumor cells dissociated from the main tumor mass were also observed (Fig. 6*C*). Immunohistochemcial examination of tumor sections with a marker of proliferation, Ki67, and of vascular endothelial cells, CD146, revealed that the tumors were proliferative and highly vascularized. Thus, HOXB7-overexpressing MDCK cells were tumorigenic and formed aggressive invasive tumors that were well vascularized.

Discussion

Metastasis accounts for the majority of breast cancer–related mortality, and bone marrow is one the most favored metastatic sites for breast cancer. In this study, we compared gene expression profiles from purified normal and tumor epithelial cells isolated from normal mammoplasty tissues, primary invasive breast tumor, and bone metastasis. HOXB7, a homeobox gene, showed a stepwise increase in expression during tumor progression. EMT was originally recognized as a critical step to metazoan embryogenesis and in defining structures during organ development (38). During the last decade, a number of studies have related EMT to cancer progression and, in parallel, a role for HOX genes in cancer. In this article, we show that HOXB7 conferred EMT to epithelial cells, with a gain of biological features consistent with neoplastic transformation and
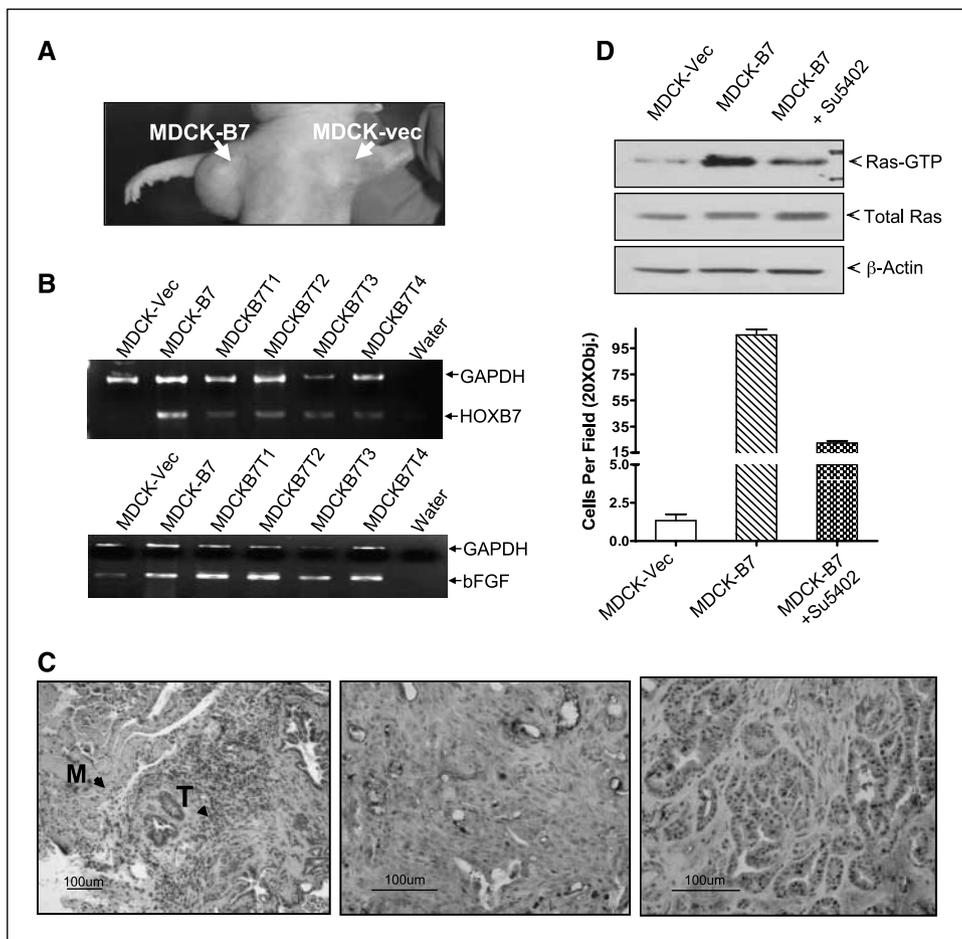
Figure 6. HOXB7 expression in MDCK cells confers tumorigenic potential *in vivo*. *A,* growth of pooled MDCK-B7 and MDCK-vec cells injected into the mammary gland fat pads of Swiss nu/nu mouse. *B,* RT-PCR verification of both HOXB7 and bFGF expressions in HOXB7-MDCK stable cell lines in culture and in xenograft tumors in nude mice. *C,* histopathology of xenograft MDCK-HOXB7 tumors. Tumor sections were stained with H&E (*left*). *Arrows,* invasion into the skeletal muscles (*M*) and tumor cells infiltrating into fat (*T*). Immunohistochemical analysis using monoclonal antibodies to the endothelial cell surface marker CD146 (*middle*) and cell proliferation marker Ki67 (*right*) are shown. Bar, 100 μm. *D,* treatment of MDCK-B7 cells with Su5402, a specific inhibitor of FGF receptor, could attenuate the active form Ras MDCK-B7 cells (*top*) and further reduce its invasive ability (*bottom*).

invasiveness. Overexpression of HOXB7 in mammary epithelial cells, MCF10A, and a prototypical model epithelial cell line, MDCK, could induce the conversion of cobblestone-like epithelial morphology to spindle-shape mesenchymal morphology. Consistent with the morphologic change, some hallmark proteins of epithelial cells were lost or reduced during the transition. Intriguingly, in MDCK-B7 cells, instead of a dramatic change in expression levels, the epithelial-specific proteins, E-cadherin and claudin 4, altered their localization from the cell membrane to the cytoplasm. A similar E-cadherin translocation pattern was documented by Bellovin et al. in 2005 (39), where they also showed that cytoplasmic localization of E-cadherin was correlated to EMT and genesis of metastasis of colorectal tumors. These observations suggest that although expression levels of these adhesion and tight junction proteins were not significantly altered, improper subcellular localization could result in protein loss of function and contribute to progression of metastasis. On the other hand, we only observed this phenomenon in MDCK-B7 cells, but not in MCF10A-B7 cells. This implies that the mechanism of EMT induction by HOXB7 could be cell context dependent.

These morphologic and cell-cell contact changes ultimately reflect on cell mobility and invasive ability. It is well known that small GTP binding proteins, such as members of the Ras and Rho families, comprehensively regulate cell migration and invasion. Also well documented is that the Ras-RAF-MAPK pathway plays indispensable role in EMT induced by activation of receptor tyrosine kinase of growth factors like HGF, VEGF, EGF, and bFGF (30). Taking into account published findings that HOXB7 could directly transactivate the expression of bFGF in both melanoma and breast cancer cell lines (23, 34), we investigated bFGF expression and found that it was high in both HOXB7 stably transfected MDCK cells and the xenograft tumors. Further, blocking FGF autocrine signaling cascade with the FGF receptor inhibitor Su5402 could attenuate activation of the Ras-RAF-MAPK pathway and the invasive ability of MDCK-B7 cells. In addition to activation of the Ras pathway, more RhoA-GTP form proteins were observed in HOXB7-transfected cells. RhoA protein is known by its ability to remodel the actin cytoskeleton and form thick stress fibers, which are required for migratory behavior of cells (40). Using MDCK cells, Zondag et al. (27) have shown that a shift in balance between RhoA and RAC activity can control the transition of phenotype from epithelial to mesenchymal. They found that sustained signaling by oncogenic RasV12 permanently down-regulated RAC activity, which led to up-regulation of RhoA activity and EMT. On the other hand, reconstitution of RAC activity by expression of Tiam1 or RACV12 led to down-regulation of Rho activity and restored an epithelial phenotype to mesenchymal, RasV12-transformed cells (27). Although activation of RhoA mediates formation of F-actin stress fibers and enhances cell motility, some studies indicate that excessive activation of RhoA can actually inhibit polarization and motility (41, 42). For example, stimulation of U118 cells with S1P resulted in a 5-fold induction of RhoA activity and inhibition of migration (42). In the case of HOXB7-transformed MDCK cells, higher bFGF expression was observed compared with

vector controls (Fig. 6B). The expression of bFGF could result in constitutively activating Ras signaling through autocrine signaling cascades. Activated Ras signaling pathways could further activate RhoA by ~ 2-fold (Fig. 5A), which could have mediated the formation of contractile stress fiber to facilitate migration and invasion.

This study was initiated from the finding that HOXB7 is an mRNA that showed a stepwise increase in expression during breast cancer progression from primary tumor to distant metastasis. But what is the cause of the abnormally high expression of HOXB7 in breast carcinomas? Kallioniemi's group first reported that over-expression of HOXB7 was related to breast cancer by identification of HOXB7 in a novel amplicon, and that amplification of the gene in 10.2% of primary breast cancers correlated with poor prognosis (25). Our data provide evidence that may mechanistically link the high expression of HOXB7 to poor prognosis of breast cancer.

In our studies, the percentage of tumors displaying HOXB7 expression in comparison with normal tissues was much higher (60% at 5-fold higher than normal) compared with the incidence of gene amplification (10.2%) revealed by FISH analysis (25). Our assessment of HOXB7 gene copy number by real-time PCR in the same tumor panel showed that <10% tumors had more than two copies of HOXB7 (data not shown). This implied that not just genomic amplification but also other transcriptional regulation mechanisms are involved in overexpression of HOXB7 in breast carcinomas. Although homeo-box genes have been known for several decades, the mechanism by which their expression is regulated remains elusive. Periodic expression pattern of HOXB7 during lactation cycles in mice suggests that HOXB7 expression may be under the control of hormones like estrogen, progesterone, and prolactin. In addition, HOXB7 expression could also be controlled by extracellular matrix (22).

Taken together, we show that HOXB7 was highly expressed in both primary metastatic tumors and bone metastasis, and over-expression HOXB7 could induce EMT, which is associated with tumor progression and poor prognosis. Further studies to identify mechanisms regulating HOXB7 expression and HOXB7 down-stream regulated genes would be worthwhile to better understand the pathogenic role of HOXB7 in tumorigenesis and explore the potential of HOXB7 as a therapeutic target.

Acknowledgments

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Correction: HOXB7, a Homeodomain Protein, Is Overexpressed in Breast Cancer and Confers Epithelial–Mesenchymal Transition

In this article (Cancer Res 2006;66:9527–34), which appeared in the October 1, 2006, issue of *Cancer Research* (1), an incorrect Western blot scan was used for β-actin as a loading control in the right panel of Fig. 5C. The corrected panel appears below. The authors regret this error.
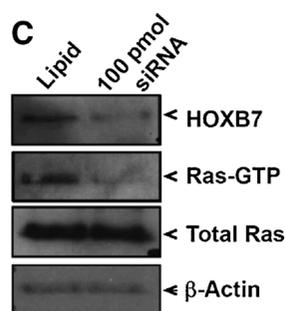

Figure 5.

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HOXB7, a Homeodomain Protein, Is Overexpressed in Breast Cancer and Confers Epithelial-Mesenchymal Transition

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