**Research Paper**

**HOXA10 Regulates p53 Expression and Matrigel Invasion in Human Breast Cancer Cells**

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**ABSTRACT**

HOX genes regulate cell differentiation during embryonic development. Here we demonstrate HOXA10 expression in both benign and malignant adult human breast tissue and in MCF-7, but not BT20 breast cancer cells. We have previously shown that HOXA10 mediates uterine differentiation in response to estrogens. The mechanism of action of estradiol and other estrogen receptor modulators on breast cancer cell growth is still poorly understood. MCF-7 cells, which are ER (+) and express HOXA10, were used to assay the effect of estradiol and tamoxifen on HOXA10 expression. Semi-quantitative RT-PCR and northern analysis revealed that treatment with either estradiol or tamoxifen increased HOXA10 mRNA expression. BT20 cells, which are ER (-) and do not endogenously express HOXA10, were used to assay the effect of increased HOXA10 expression on p53 expression and on the invasive phenotype. Constitutively expressing HOXA10 in BT20 cells increased p53 protein expression. Increased HOXA10 also reduced invasiveness through matrigel. The mechanism by which estrogen and other estrogen receptor modulators influence both normal breast development as well as breast cancer may involve the regulation of developmental control genes such as HOXA10; HOXA10 in turn regulates expression of key downstream genes such as p53 and regulates tumor cell functional phenotype.

**INTRODUCTION**

Breast cancer is one of the most common malignancies in the Western world. Established factors associated with the risk of breast cancer include reproductive factors associated with sex steroid exposure; increased risk is seen with nulliparity, early age at menarche, older age at first full-term pregnancy, and older age at menopause. Recently, the Women’s Health Initiative investigators reported a 26% increase in invasive breast cancer cases in the trial arm administered an estrogen and a progestin compared to the placebo arm after 5 years of follow-up. The estrogen receptor (ER) is also an important therapeutic target for the treatment and prevention of breast cancer. Selective estrogen receptor modulators (SERMs), including tamoxifen and raloxifene, are compounds that bind ER and exert tissue-specific effects. Tamoxifen was the first SERM approved by the F.D.A. for the treatment and prevention of breast cancer; it has been shown to reduce the incidence of estrogen receptor-positive breast cancer. It is therefore of interest to investigate the molecular mechanisms by which these agents influence breast cancer.

ER target genes are likely to be essential to the signal transduction pathways that regulate breast cancer cells. We have previously demonstrated that at least three HOX genes are regulated by estrogens. HOX genes are transcription factors that act during normal embryogenesis in the patterning and development of the embryonic body plan. They are essential regulators of tissue and cell differentiation in most organ systems. Alteration in these developmental mechanisms have a demonstrated role in carcinogenesis. In particular, HOX genes have a well-characterized role in hematologic malignancies. Several HOX genes are expressed in the normal and neoplastic mouse mammary gland.

As transcription factors, HOX genes are known to regulate the expression of other genes, including the p53 gene, a tumor suppressor gene which protects cells against malignant transformation. Cells that develop mutations in p53 demonstrate a diminished apoptotic potential and DNA repair, which contribute to tumor growth and metastasis. There are consensus HOX binding sites in the p53 promoter. Raman et al. demonstrated a potential role for HOX5 in human breast tumors; transient transfection of HOX5 activated the p53 promoter. Conversely, expression of HOX5 in epithelial cancer cells expressing wild-type p53 led to increased p53 and apoptotic cell death. Both breast cancer cell lines and human tumors displayed a coordinate loss of p53 and HOX5 mRNA and protein expression.
While HOXA5 has a well documented role in breast cancer, it is not known to be steroid-responsive; we have previously demonstrated that estrogen acts as a regulator of HOXA10 gene expression in the adult human uterus.5-7 We therefore hypothesized that HOXA10 would mediate some of the effects of sex steroid hormones on the human breast. Here we demonstrate that HOXA10 is expressed in normal human breast and in breast cancer, and that HOXA10 expression is regulated by estrogen and tamoxifen in breast cancer cells. HOXA10 in turn regulates p53 expression in breast cancer cells and also modulates the ability of these cells to invade.

METHODS

Cell Culture and Treatment. Breast cancer cell lines MCF7 and BT20 were obtained from the American Type Culture Collection (Rockville, MD) and maintained at 37°C, 5% CO2. MCF7 cells were cultured in a 1/1 mixture of Dulbecco's modified Eagle's medium (DEM) and Ham's F12 (F12) (DMEM/F12) containing 10% calf serum.22 BT20 cells were cultured in RPMI 1640 medium supplemented with 10% calf serum. Before treatment, cells were grown in serum free, phenol red free DMEM/HAM F-12 (Sigma Chemical Co.; St. Louis, MO) for 48 h in order to eliminate steroids. Estradiol, tamoxifen, or both were added to the cultures as concentrated stock solutions in pure ethanol (0.1% of the total volume) to final concentrations of 10−8 M. Untreated cells were exposed to an equivalent amount of stock solutions in pure ethanol (0.1% of the total volume) to final concentrations of 10−8 M. Untreated cells were exposed to an equivalent amount of ethanol. Hormone-treated cells were incubated for 24 h.

Tissue Specimens. Informed consent was obtained from individual subjects for all procedures. The study was approved by the institutional review board of Yale University. Normal and malignant breast tissue was obtained from 8 consecutive patients diagnosed with breast cancer and operated upon at Yale New Haven Hospital. During mastectomy, samples from nonneoplastic breast cancer tissue were taken, as well as intact normal breast tissue from an area within a few centimeters of the tumor. Each sample was split into two parts; one was analyzed by a pathologist for clinical diagnosis, including exclusion of the presence of infiltrated tumor cells in normal appearing breast tissues. The other part of the sample was snap-frozen in dry ice and stored at −80°C until RNA was extracted.

RNA Extraction and Northern Analysis. One hundred to 200 mg of tissue were homogenized in Trizol reagent. MCF7 cells were treated with hormones for 24 h. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 20 μg of RNA were loaded per lane in a 1% agarose/0.66M formaldehyde gel and, after partial hydrolysis with NaOH, transferred onto nylon membranes and immobilized by UV irradiation. Northern blot hybridizations were performed as described previously in using vitro transcription with [α-32P]-dUTP.15 A 103 bp fragment of the 3' untranslated region of human HOXA10 was used as the template for generating the riboprobes. [32P] labeled probe/mRNA hybrids were visualized by autoradiography. Equal loading of samples was verified by stripping the membrane and reprobing with a probe to G3PDH. Quantification was performed using laser densitometry (Molecular Dynamics; Sunnyvale, CA).

Reverse Transcriptase-polymerase Chain Reaction (RT-PCR). mRNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase and poly(dT) as a primer. After 2-μg aliquots of poly(A)+ RNA were reverse transcribed, 1/25 of the resulting cDNA was used for each amplification. Following initiation under hot start conditions, PCR reactions were continued for 22 cycles on a PE 480 thermal cycler (PE Biosystems, Foster City; California, USA) using 92°C for denaturation, 55°C for annealing, and 72°C for extension (1 min/step). The following primers were used: HOXA10 forward, 5'-GCCCTTCAGGACAGCAGCAAG-3' and HOXA10 reverse, 5'-AGGTTGACGCTTGAGCAATCTTCA-3'; β-actin forward, 5'-GGTGGGGCGCCCCAGGACCA-3' and β-actin reverse, 5'-CTCCTTAAATCGGACAGATTCA-3'. Amplified products were separated on a 1% agarose gel and visualized under UV light. The HOXA10 primers selected yielded a 211 bp reaction product from mRNA and a 1389 bp reaction product from DNA, which allowed control for possible DNA contamination.

Transient Transfections. Transient transfection of cultured BT20 cells was carried out using LipofectAMINE (Life Technologies Inc.). Cells were plated at a density of 1 x 104/well in a 24-well culture plate and transfected the next day. Typically 10 ng of construct DNA were transfected with 4 μl of LipofectAMINE. The medium was replaced after 5 h. Transfection efficiency had been previously tested with a luciferase reporter vector and measurement of luciferase activity (Dual-Luciferase Assay system, Promega; Madison, WI). Transfections were performed in triplicate. Two different sets of transfection experiments were performed. Cultured BT20 cells were transiently transfected with either a HOXA10 expression vector comprised of HOXA10 cDNA cloned into pcDNA3.1 or the empty vector pcDNA3.1. BT20 Tet-On cells were transiently transfected with the empty vector pTRE2 or a HOXA10 expression vector where HOXA10 cDNA was cloned into the pTRE2 response plasmid.

Protein Extraction and Western Analysis. After transient transfection, cells were further incubated for 48 h prior to protein extraction. To harvest protein, cells were washed once with PBS, harvested using a cell scraper into 100 μl of passive lysis buffer (50 mM Tris pH 7.5, 5 mM MgCl2, 5 mM EGTA, 10% glycerol, 0.25% TritonX-100, containing a 1:100 dilution of a freshly added protease inhibitor cocktail (Sigma P8340; St. Louis, MO)), passed through a 22G 1/2” needle, and incubated on ice for 30 min. The cell lysate was centrifuged at 10,000 xg for 10 min at 4°C and the supernatant was transferred to a new tube. Samples were quantified using the Bio-Rad protein assay kit. 20 microgram of cell lysate were separated on a 7.5% SDS-PAGE gel and transferred to nitrocellulose. After blocking in PBS containing 5% milk and 0.1% Tween-20 for 1 h, membrane was incubated with monoclonal p53 antibodies at 1:400 (Neomarkers) or monoclonal β-actin antibodies at 1:20,000 (Sigma A5441; St. Louis, MO) for 1 h at room temperature. The membrane was then washed 3x5 min with wash buffer (1x PBS/0.1% Tween). The blot was incubated in a 1:3000 dilution of HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The membrane was washed 3x5 min with wash buffer and bands were visualized using chemiluminescence (ECL, Amersham Biosciences; Piscataway, NJ).

Cell Invasion Assays. BT20 cells stably transfected with the regulator plasmid, BT20-TetOn cells, (CLONTECH; Palo Alto, CA) were transiently transfected with either pTRE2 empty vector or the expression vector pTRE2/HOXA10, in which HOXA10 cDNA was cloned into pTRE2 downstream of the tet response element. Doxycycline was used to induce the expression of the gene downstream of the tet-response element (TRE). The two BT20/TetOn cells (pTRE2 and pTRE2/HOXA10) were evaluated in a chemoinvasion assay as described23 and modified by us. Briefly, cells were loaded onto the upper compartments of Boyden chambers (105 cells/chamber) and incubated for 48 h. Cells that invaded the Matrigel barrier toward DME/F12 media containing 10% Nu Serum (Becton Dickinson) were counted on the undersides of filters after fixation and staining with crystal violet. The membranes were mounted on slides for evaluation under light microscopy. Each experiment was performed in quadruplicate. Two different sets of transfection experiments were performed.

RESULTS

We first established that HOXA10 is expressed in adult human breast and differentially expressed in two breast cancer cell lines. After total RNA extraction, eight pairs of normal and neoplastic breast tissue yielded sufficient RNA to perform northern analysis and RT-PCR. HOXA10 gene expression was detected in both the normal and tumor samples (Fig. 1A). Two breast cancer cell lines were used in our experiments. MCF-7 cells are well-differentiated breast cancer cells that are known to be estrogen receptor positive. BT20 cells are poorly differentiated breast cancer cells that express estrogen receptor negative. By RT-PCR, we amplified HOXA10 from MCF-7 cells but not from BT20 cells (Fig. 1B).
RNA isolation and RT-PCR using specific oligonucleotides that specifically amplify to HOXA10. Beta-actin was amplified as a control. Both tumor and normal breast tissue expressed HOXA10. (B) Expression of HOXA10 in MCF-7 but not BT20 cells. RT-PCR detected expression of HOXA10 in the MCF-7 breast cancer cell line but not in the BT20 breast cancer cell line. Each experiment was repeated three times.

To determine whether HOXA10 expression could be altered by sex steroid hormones, we treated MCF-7 cells, which express HOXA10 as well as estrogen receptor, with estradiol and/or tamoxifen in concentrations that mimic physiologic and/or therapeutic conditions. Northern analysis demonstrated increased HOXA10 expression after treatment with estradiol as well as tamoxifen. Treatment with both estradiol and tamoxifen induced a higher level of HOXA10 expression than with either hormone alone (Fig. 2). Densitometry determined that HOXA10 mRNA increased approximately two-fold after treatment with either estradiol or tamoxifen, and was increased 2.5 fold with concomitant treatment. Semi-quantitative RT-PCR confirmed that estradiol and tamoxifen regulate the expression of HOXA10 (data not shown).

p53 is a well-characterized tumor suppressor gene. We investigated the effect of HOXA10 on p53 expression in breast cancer cells. MCF-7 cells, which endogenously express HOXA10, and BT20 cells, which do not inherently express HOXA10, were transfected with either empty vector pCDNA3.1 as the control or HOXA10 expression vector in which HOXA10 cDNA was cloned into pCDNA3.1. This latter plasmid constitutively expresses HOXA10. Monoclonal p53 antibody was used to detect p53 protein levels by western analysis. MCF-7 cells, which endogenously express HOXA10, did not show a significant change in p53 expression in response to additional HOXA10. BT20 cells, which do not inherently express HOXA10, displayed a marked upregulation of p53 expression in response to HOXA10 treatment (Fig. 3).

To evaluate the physiologic changes affected by these alterations, we investigated the effect of HOXA10 expression on breast cancer cell invasiveness. BT20 cells, which do not inherently express HOXA10, are invasive by nature. In this experiment, we assessed whether the expression of HOXA10 in BT20 cells could alter their invasive potential. Cells transfected with pTRE2/HOXA10 or pTRE empty vector served as the control for baseline BT20 cell invasion and did not differ from one another. Constitutive expression of HOXA10 in BT20 cells transfected with a construct consisting of HOXA10 cDNA cloned into pTRE2 vector resulted in decreased invasion compared to cells not treated with doxycycline (Fig. 4). Each experiment was repeated four times. The BT20 cells invaded at a rate of 44.1 ± 8.0 % and 5.5 ± 0.9% in the control and HOXA10 treated groups, respectively. (P<0.05, students t-test)

**DISCUSSION**

The molecular mechanisms that underlie breast cell differentiation, steroid responsiveness, and tumorigenesis are poorly understood. The aberrant expression of homeobox-containing genes has been reported in a variety of neoplasias. Some members of the homeobox gene family have been reported to be oncogenic and to result in increased expression of HOXA10. One mechanism by which this homeobox gene may regulate both normal cell proliferation, differentiation and invasion.

Previously we have demonstrated that estrogen acts as a regulator of HOXA10 gene expression in the adult uterus. Breast cells are similarly hormonally responsive. Reproductive factors as well as exogenous hormonal exposure, including postmenopausal estrogen replacement therapy, modify breast cancer risk. Here we observed that HOXA10 is expressed in both normal human breast tissue and human breast tumors. In human mammary carcinoma cells, both estrogen and the selective estrogen receptor modulator tamoxifen result in increased expression of HOXA10. One mechanism by which Tamoxifen may effect both normal and neoplastic breast tissue is by altering HOXA10.
HOXA10 is a positive regulator of p53 tumor suppressor gene expression, as expression of HOXA10 upregulated p53 expression and transcriptional activity. Expression of the p53 tumor suppressor gene protects cells against malignant transformation. Consistent with the predicted downstream effect of activated p53 expression, forced expression of HOXA10 resulted in decreased invasiveness and oncogenic potential of human mammary epithelial cancer cells. Homeobox-containing genes have been classified as both oncogene/tumor suppressor genes and as tumor modulators that alter tumor progression. Here we provide evidence that a homeobox-containing protein HOXA10 is a tumor modulator, the enhanced expression of which leads to moderation of the oncogenic potential of human mammary epithelial cancer cells. Similar to the role of HOXA10 in embryonic development, where expression leads to cell differentiation, HOXA10 may induce breast cell differentiation, here promoting p53 induced apoptosis and decreased cell invasion. It is therefore conceivable that agents that lead directly or indirectly to the upregulation of HOXA10, utilized as adjuvant therapy, will increase the efficacy of conventional chemotherapeutic regimes currently utilized to treat mammary carcinoma. This HOXA10 mediated pathway is a potential therapeutic target.

In conclusion, we have demonstrated that regulation of developmental control genes such as HOXA10 has a role in mammary gland differentiation and breast cancer. Estradiol and tamoxifen alter HOXA10 expression in human mammary carcinoma cells that are ER positive. Expression of HOXA10 induces expression of the tumor suppressor gene p53. We also identified HOXA10 as a tumor modulator for human mammary epithelial cells, as constitutive HOXA10 expression results in decreased breast cancer cell invasiveness. Further functional characterization of HOXA10 and other hormonally-regulated homeobox genes in the human mammary epithelial cell will be instrumental in understanding the development and formation of hormone-dependent neoplasia in the mammary gland.

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